UNIVERSIDADE D
COIMBRA

Américo José dos Santos Alves

Novel Spiro-lactams as New Antimicrobial Agents

Tese no âmbito do Doutoramento em Química, ramo de Síntese Orgânica, orientada pela Professora Doutora Teresa Margarida Vasconcelos Dias de Pinho e Melo, coorientada pelo Professor Doutor Nuno Eduardo Moura dos Santos da Costa Taveira e pelo Professor Doutor Miguel Prudêncio, e apresentada ao Departamento de Química da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

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Universidade de Coimbra
Departamento de Química – Faculdade de Ciências e Tecnologia

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Coimbra, 2022

Aos meus pais,

“One, remember to look up at the stars and not down at your feet. Two, never give up work. Work gives you meaning and purpose and life is empty without it. Three, if you are lucky enough to find love, remember it is there and don’t throw it away.”

Stephen Hawking

Institutional and Financial Support

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Abstract

The main objective of this PhD thesis was the synthesis of new chiral spiropenicillanates with antimicrobial activity against HIV and *Plasmodium*. This goal was achieved by taking advantage of the reactivity of 6-alkylidenepenicillanates in annulation reactions and in 1,3-dipolar cycloaddition reactions.

Prior bioactivity studies identified a molecule, BSS-730A, with remarkable submicromolar activity against multiple strains of HIV-1 and HIV-2, including multidrug resistant strains, and against both hepatic and erythrocytic stages of *Plasmodium* infection. BSS-730A was selected as lead molecule for the rational structural modulation of the novel spirocyclopentene-β-lactams. This strategy involved the synthesis of twelve novel 6-alkylidenepenicillanates, followed by phosphine-catalyzed [3+2] annulation reactions of the newly synthesized 6-alkylidenepenicillanates with allenoates, which resulted in the synthesis of thirty-eight novel chiral spirocyclopentene-β-lactams. These annulation reactions allowed the structural modulation of position 1', and a simultaneous modulation of both positions 1' and 2'. In this library of novel spirocyclopentene-β-lactams, it was possible to identify six compounds highly active against HIV-1 and HIV-2 in a nanomolar order (*e.g.* IC_{50} HIV-1 < 0.050 μM), with four of them displaying micromolar antiplasmodial activity. Further studies were carried out in order to investigate the interaction between some spiro-β-lactams and BSA.

Concerning the structural modulation of the spirocyclic ring system, two unprecedented synthetic routes were developed. First, we explored the 1,3-dipolar cycloaddition of 6-alkylidenepenicillanates with nitrones, as a route to the synthesis of novel chiral spiroisoxazolidine-β-lactams incorporating the penicillanate nucleus under mild conditions. This process was stereo- and diastereoselective and involved the creation of three new chiral centers. The synthesis of these novel derivatives has resulted in the identification of two new spiro-β-lactams with moderate antiviral activity against HIV-1. The reactivity of 6-alkylidenepenicillanates with nitrile oxides was also explored as a strategy for the synthesis of novel chiral spiroisoxazoline-β-lactams. The major spiro[isoxazoline-4',6-penicillanates] result from the addition of the dipole to the less sterically hindered α-side of the 6-alkylidenepenicillanate and the attack of the dipole's oxygen to the terminal carbon of the exocyclic double bond of the 6-alkylidenepenicillanate. The stereo- and regioselective synthesis of these novel

derivatives was achieved employing three different methodologies: conventional heating, microwave irradiation and continuous flow.

Finally, the synthesis of the first chiral alkylidene-γ-lactams fused to a thiazolidine ring is described. Subsequent *in vitro* evaluation of the anticancer properties of alkylidene-γ-lactams and alkylidene-β-lactams allowed the identification of four compounds with IC_{50} values below 10 μM in A375 human melanoma cells, and three compounds with IC_{50} values below 10 μM in OE19 human esophageal carcinoma cells. Further studies aiming to get insight into the effect of the most promising compounds on cell death mechanism, reactive oxygen species generation as well as the evaluation of their ability to act as MMP-9 inhibitors were also unveiled.

In summary, the work developed during this PhD thesis led to the synthesis of novel families of spiro-β-lactams. This extensive library of new chiral spiro-β-lactams enabled the identification of a set of molecules with remarkable *in vitro* activity against HIV and *Plasmodium*.

Resumo

O trabalho apresentado nesta tese de Doutoramento teve como principal objetivo a síntese de novos espiropenicilanatos quirais com atividade antimicrobiana contra o HIV e o *Plasmodium*. Este objetivo foi alcançado tirando partido da reatividade dos 6-alquilidenopenicilanatos em reações de cicloadição [3+2] formal e em reações de cicloadição 1,3-dipolar.

Estudos anteriores identificaram uma molécula, BSS-730A, com uma notável atividade submicromolar contra variadas estirpes de HIV-1 e HIV-2, incluindo estirpes multirresistentes, e contra ambos os estágios hepáticos e eritrocíticos da infeção por *Plasmodium*. Assim, o BSS-730A foi selecionado como molécula-líder para a modulação estrutural das novas espirociclopenteno-β-lactamas. Esta estratégia consistiu na síntese de doze novos 6-alquilidenopenicilanatos que participaram em reações de cicloadição [3+2] formal com alenoatos catalisadas por fosfina dando origem a trinta e oito novas espirociclopenteno-β-lactamas quirais. Estas reações permitiram a modulação estrutural da posição 1', assim como uma modulação simultânea das posições 1' e 2'. Nesta biblioteca de novas espirociclopenteno-β-lactamas, foi possível identificar seis compostos altamente ativos contra o HIV-1 e o HIV-2 numa escala nanomolar (*e.g.* IC_{50} HIV-1 < 0.050 μM), com quatro destes novos derivados a apresentarem atividade micromolar contra o *Plasmodium*. Foram ainda realizados estudos adicionais com o objetivo de investigar a interação de algumas espiro-β-lactamas com a BSA.

Foram desenvolvidas duas rotas sintéticas sem precedentes com o objetivo de realizar modelações estruturais no anel espirocíclico. Primeiro, foi explorada a cicloadição 1,3-dipolar de 6-alquilidenopenicilanatos com nitronas, como via para a síntese de novas espiro-isoxazolidinas-β-lactamas quirais. Este processo foi estéreo- e diastereoseletivo, e envolveu a criação de três novos centros quirais. A síntese destes novos derivados resultou na identificação de duas novas espiro-β-lactamas com atividade antiviral moderada contra o HIV-1. Em seguida, a reatividade dos 6-alquilidenopenicilanatos com óxidos de nitrilo também foi explorada como estratégia para a síntese de novas espiro-isoxazolinas-β-lactamas quirais. Os produtos maioritários desta cicloadição resultam de uma adição estereosseletiva do dipolo à face com menos impedimento estéreo dos 6-alquilidenopenicilanatos (face-α), e do ataque do oxigénio da espécie dipolar ao carbono terminal da ligação dupla exocíclica. A síntese estéreo- e

regioseletiva destes novos derivados foi conseguida tirando partido de três metodologias experimentais distintas: aquecimento convencional, irradiação por microondas e síntese em fluxo continuo.

Por fim, foi descrita a primeira síntese de alquilideno-γ-lactamas fundidas a um anel de tiazolidina. A subsequente avaliação *in vitro* das propriedades anticancerígenas das alquilideno-γ-lactamas assim como das alquilideno-β-lactamas, permitiu identificar quatro compostos com valores de IC_{50} inferiores a 10 μM em células do melanoma humano (A375), e três compostos com valores de IC_{50} inferiores a 10 μM em células do carcinoma esofágico humano (OE19). Também são descritos estudos adicionais visando compreender o efeito dos compostos mais promissores nos mecanismos de morte celular, geração de espécies reativas de oxigénio, assim como na avaliação da sua habilidade para atuarem como inibidores de MMP-9.

Em suma, o trabalho desenvolvido no âmbito desta tese de Doutoramento levou à síntese de novas famílias de espiro-β-lactamas quirais. Esta biblioteca extensiva de novas espiro-β-lactamas, permitiu a identificação de um conjunto de moléculas com notável atividade *in vitro* contra o HIV e o *Plasmodium*.

Abbreviations, Acronyms and Symbols

τ_0	Average lifetime in the absence of the quencher
[Q]	Concentration of the quencher
^{1}H NMR	Proton nuclear magnetic resonance
^{13}C NMR	Carbon-13 nuclear magnetic resonance
^{19}F NMR	Fluorine-19 nuclear magnetic resonance
2D	Two-dimensional
3D	Three-dimensional
3TC	Lamivudine
6-APA	6-Aminopenicillanic Acid
ABC	Abacavir
AIDS	Acquired Immunodeficiency Syndrome
AnV-FITC	Annexin-V Fluorescein Isothiocyanate
Ar	Aromatic group
ART	Antiretroviral Therapy
ATR	Attenuated Total Reflection
B3LYP	Becke-3-parameter-Lee-Yang-Parr
Bn	Benzyl
Boc	*tert*-Butyloxycarbonyl
br s	Broad singlet
BSA	Bovine serum albumin
CC_{50}	Half maximal Cytotoxic Concentration
CCR5	C-C Chemokine Receptor type 5
CD4	Cluster of Differentiation 4
CI_{95}	Confidence interval of 95%
CMCM	Complete Malaria Culture Medium
COSY	Correlated Spectroscopy
CPE	Constant potential electrolysis
CSA	Camphorsulfonic acid
CXCR4	C-X-C chemokine receptor type 4
Cy	Cyclohexyl
d	Dublet

d4t	Stavudine
DABCO	1,4-Diazabicyclo[2.2.2]octane
DCFH2-DA	2',7'-Dichlorodihydrofluorescein diacetate
DCM	Dichloromethane
dd	Doublet of Doublets
ddd	Doublet of Doublet of Doublets
ddI	Didanosine
DEAE	Diethylethanolamine
DFT	Density Functional Theory
DHE	Dihydroethidium
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DMSO-d_6	Hexadeutero Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DRV	Darunavir
dt	Doublet of triplets
DTG	Dolutegravir
e.g.	For example (from the Latin expression *exempli gratia*)
EI	Entry Inhibitor
Equiv.	Equivalents
ESI	Electrospray
Et	Ethyl
F	Fluorescence intensity in the presence of quencher
F_0	Fluorescence intensity in the absence of quencher
FBS	Fetal Bovine Serum
FTC	Emtricitabine
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human Immunodeficiency Virus
HMBC	Heteronuclear Multi-Bond Correlation
HRMS	High Resolution Mass Spectrometry
HSA	Human serum albumin
HSQC	Heteronuclear Single-Quantum Correlation
IBU	Ibuprofen

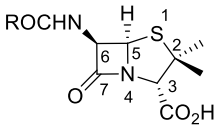
IC_{50}	Half Maximal Inhibitory Concentration
II	Integrase Inhibitor
IUPAC	International Union of Pure and Applied Chemistry
IR	Infrared Spectroscopy
J	Coupling Constant
K_a	Binding constant
K_q	Bimolecular quenching rate constant
K_{SV}	Stern-Volmer quenching constant
LPV	Lopinavir
m	Multiplet
m-CPBA	*m*-Chloroperoxybenzoic acid
M^+	Molecular Ion
Me	Methyl
MMP	Matrix metalloproteinase
MMP-9	Matrix metalloproteinase-9
mp	Melting point
MPI	Maximum percent inhibition
MS	Mass Spectrometry
Ms	Mesyl
MTBE	Methyl *tert*-butyl ether
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Microwave
n.d.	Not Determined
NBS	*N*-Bromosuccinimide
NCS	*N*-Chlorosuccinimide
NHC	*N*-Heterocyclic Carbenes
NMM	*N*-Methylmorpholine
NMO	*N*-Methylmorpholine *N*-oxide
NMR	Nuclear magnetic resonance
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NOE	Nuclear Overhauser Effect
NOESY	Nuclear Overhauser Effect Spectroscopy
NRTI	Nucleoside reverse transcriptase inhibitor

Nu	Nucleophile
ORTEP	Oak Ridge Thermal Ellipsoid Plot
OPTB	*O*-2-[(propan-2-yl)thio]benzyl
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate-buffered saline
Ph	Phenyl
PI	Protease Inhibitor
PIDA	Phenyliodine(III) diacetate
PMB	*p*-Methoxybenzyl
PMP	*p*-Methoxyphenyl
PNB	*p*-Nitrobenzyl
ppm	Parts Per Million
Pr	Propyl
PSDIB	Polymer-supported (diacetoxyiodo)benzene
PTSA	*p*-Toluenesulfonic acid
Py	Pyridine
q	Quartet
RAL	Raltegravir
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute 1640 Medium
RT	Reverse Transcriptase
s	Singlet
SAR	Structure-Activity Relationship
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SCE	Saturated calomel electrode
SQV	Saquinavir
t	Triplet
rt	Room Temperature
TAF	Tenofovir Alafanamida
TBDPS	*tert*-Butyldiphenylsilyl
TBS	*tert*-Butyldimethylsilyl
t-Bu	*tert*-Butyl

$TCID_{50}$	50% Tissue Culture Infectious Dose
TDF	Tenofovir disoproxil fumarate
TEMED	Tetramethylethylenediamine
TFA	Trifluoroacetic Acid
TFT	α,α,α-Trifluorotoluene
THF	Tetrahydrofuran
TLC	Thin Layer Cromatography
TMS	Tetramethylsilane
TOA	Time of addition
TOF	Time of Flight
t^R	Residence time
Ts	Tosyl
TTFA	Thenoyltrifluoroacetone
WAR	Warfarin
ZDV	Zidovudine

Nomenclature

In this PhD thesis, the classical numbering order of β-lactamic compounds was used.[1] This numbering is illustrated in Example 1.



Example 1

The denomination of other organic compounds followed the general IUPAC rules.[2]

[1] Jastrzebski, J. T. B. H.; Koten, G. *Penicillins. In: Comprehensive Heterocyclic Chemistry II* (Eds.: Katritzky, A. R.; Rees, C. W.; Scriven, E. F. V.), Elsevier, Oxford, 1996, vol. 1B, cap. 1.20.

[2] Fernandes, A. C.; Herold, B.; Maia, H.; Rauter,A. P.; Rodrigues, J. A. R. *Guia IUPAC para a Nomenclatura de Compostos Orgânicos*, LIDEL, Lisboa, 2002.

List of Publications Related to the Scientific Topic of the Thesis

Manuscripts published in international journals with peer review:

- *Synthesis of Novel Chiral Spiro-β-Lactams from Nitrile Oxides and 6-(Z)-(Benzoylmethylene)penicillanate: Batch, Microwave-Induced and Continuous Flow Methodologies*, Américo J. S. Alves; João A. D. Silvestre and Teresa M. V. D. Pinho e Melo, *RSC Adv.*, **2022**, *12*, 30879.

- *Unveiling a family of spiro-β-lactams with anti-HIV and anti-plasmodial activity via phosphine-catalyzed [3+2] annulation of 6-alkylidene-penicillanates and allenoates,* Américo J. S. Alves; Nuno G. Alves; Inês Bártolo; Diana Fontinha; Soraia Caetano; Miguel Prudêncio; Nuno Taveira, Teresa M. V. D. Pinho e Melo, *Front. Chem.*, **2022**, 10:1017250.

- *Insights into the Anticancer Activity of Chiral Alkylidene-*β*-Lactams and Alkylidene-γ-Lactams: Synthesis and Biological Investigation*, Américo J. S. Alves, Nuno G. Alves, Mafalda Laranjo, Clara S. B. Gomes, Ana Cristina Gonçalves, Ana Bela Sarmento-Ribeiro, M. Filomena Botelho, Teresa M. V. D. Pinho e Melo, *Bioorg. Med. Chem.*, **2022**, 63, 116738.

- *Synthesis and Structure-Activity Relationships of New Chiral Spiro-*β*-lactams Highly Active Against HIV-1 and Plasmodium,* Nuno G. Alves, Inês Bártolo, Américo J. S. Alves, Diana Fontinha, Denise Francisco, Susana M.M. Lopes, Maria I.L. Soares, Carlos J.V. Simões, Miguel Prudêncio, Nuno Taveira, Teresa M. V. D. Pinho e Melo, *Eur. J. Med. Chem.*, **2021**, 219, 113439.

- *Strategies and methodologies for the construction of spiro-fused γ-lactams: an update*, Américo J. S. Alves, Nuno G. Alves, Maria I. L. Soares, Teresa M. V. D. Pinho e Melo, *Org. Chem. Front.*, **2021**, 8, 3543-3593

- *Recent Advances in the Synthesis of Spiro-β-Lactams and Spiro-δ-Lactams*, Nuno G. Alves, Américo J. S. Alves, Maria I. L. Soares, Teresa M. V. D. Pinho e Melo, *Adv. Synth. Catal.*, **2021**, 363, 2464.

- *Novel spiro-β-lactam BSS-730A displays potent anti-HIV and anti-plasmodial activity*, Inês Bártolo, Bruna S. Santos, Diana Fontinha, Marta Machado, Denise Francisco, Bruno Sepodes, João Rocha, Hélder Mota-Filipe, Rui Pinto, Maria E. Figueira, Helena Barroso, Teresa Nascimento, António P. Alves de Matos, Américo J. S. Alves, Nuno G. Alves, Carlos J. V. Simões, Miguel Prudêncio, Teresa M. V. D. Pinho e Melo, Nuno Taveira, *ACS Infectious Diseases*, **2021**, 7, 421-434.

- *Synthesis of Novel Chiral Spiroisoxazolidine-β-Lactams from 6-Alkylidenepenicillanates: A 1,3-Dipolar Cycloaddition Approach*, Américo J. S. Alves, Teresa M. V. D. Pinho e Melo, *Eur. J. Org. Chem.*, **2020**, 6259-6269.

- *Spiro-Lactams as Novel Antimicrobial Agents*, Américo J. S. Alves, Nuno G. Alves, Cátia C. Caratão, Margarida I. M. Esteves, Diana Fontinha, Inês Bártolo, Maria I. L. Soares, Susana M. M. Lopes, Miguel Prudêncio, Nuno Taveira, Teresa M. V. D. Pinho e Melo, *Curr. Top. Med. Chem.*, **2020**, 20, 140-152.

Submitted manuscripts:

- *High instantaneous inhibitory potential of bictegravir and the new spiro-β-lactam BSS-730A for HIV-2 isolates from RAL- naïve and RAL-failing patients*, Inês Bártolo, Inês Moranguinho, Paloma Gonçalves, Ana Rita Diniz, Pedro Borrego, Francisco Martin, Inês Figueiredo, Perpétua Gomes, Fátima Gonçalves, Américo J. S. Alves, Nuno Alves, Umbelina Caixas, Inês V. Pinto, Isabel Barahona, Teresa M. V. D. Pinho e Melo, Nuno Taveira, **2022,** submitted.

CHAPTER 1

Introduction

Chapter 1 - Introduction

1.1. Lactams

1.1.1. β-Lactams

Azetidin-2-ones, commonly known as β-lactams, are 4-membered cyclic amides, first synthesized by Hermann Staudinger in 1907.[1] The importance of this class of compounds was only recognized after the discovery of Alexander Fleming in 1928, when he unveiled the exceptional potential of these compounds as antibiotics.[2] In fact, the Penicillin G was one of the first molecules to be identified having antibiotic properties and has been recognized as one of the greatest advances in medicine (Figure 1.1). However, the large-scale production and the therapeutic use of the first β-lactam antibiotic only began in the early 1940s, during the World War II.[2b-c, 3] Since then, β-lactamic compounds have been widely used due to their antibacterial and antimicrobial properties, representing a huge contribution to modern medicine. This discovery and the enthusiastic results associated with it, were the starting point for the golden age of natural product antibiotic discovery that reached its peak in the mid-1950s.[4]

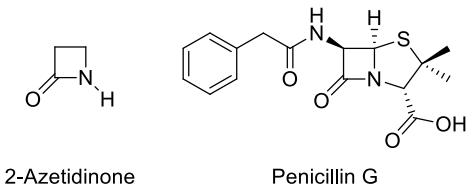


Figure 1.1. Structure of 2-azetidinone and Penicillin G.

Nowadays, the β-lactam ring is a very important moiety in medicinal chemistry and there are a significant number of natural, semi-synthetic and synthetic β-lactam derivatives in pharmacologically active compounds.[1a, 1c] In Figure 1.2, the general structure of different β-lactam antibiotics is represented. It is possible to observe that the β-lactam core may appear in a non-fused system, the monobactams,[5] or fused to 5- or 6-membered cyclic structures (*e.g.* penicillins and cephalosporins).[6] The β-lactam ring is also present in the structure of β-lactamase inhibitors such as clavulanic acid or sulbactam, which are co-administered or as a prodrug in the antibiotic therapy to prevent the inactivation of the β-lactam antibiotic by bacterial β-lactamases.[7] Despite the problems associated to bacterial resistance, β-lactamic antibiotics are still widely

employed due to their high efficacy, broad spectrum of action, low toxicity, and low cost of production.[3b, 8]

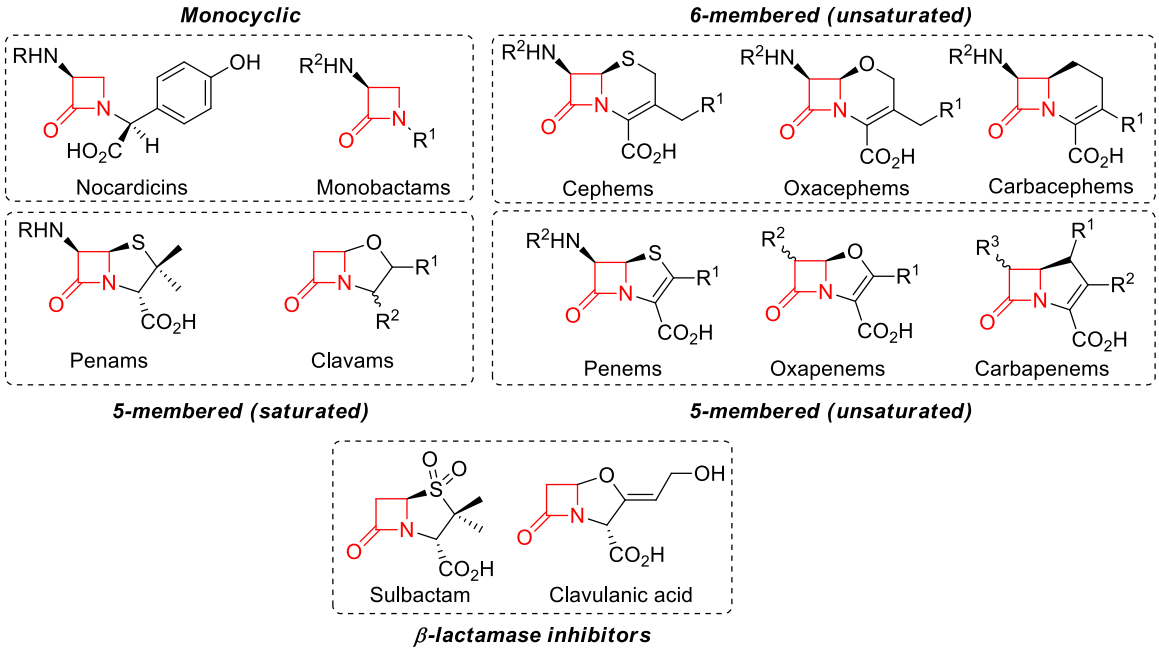


Figure 1.2. Major subfamilies of β-lactams.

Due to the attention that has been given to this structural motif, various other biological activities have been discovered to be associated with β-lactams, such as antioxidant activity,[9] cytotoxic activity,[10] cholesterol absorption inhibition,[11] antiviral activity[12] and antiparasitic activity,[13] among others (Figure 1.3).[14] For all the above-mentioned reasons, it is plausible to affirm that β-lactams are among the most relevant class of compounds in organic and medicinal chemistry.

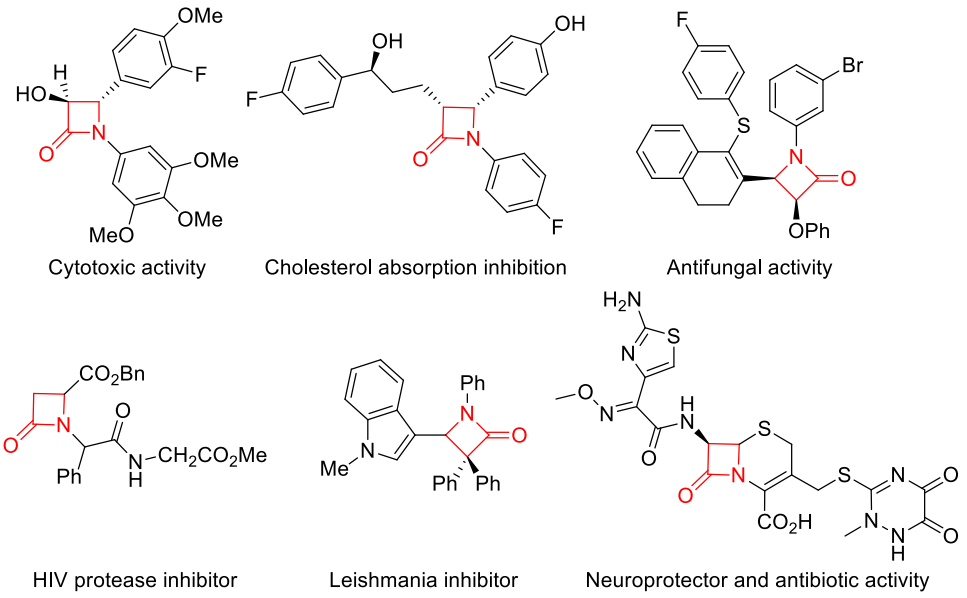


Figure 1.3. Examples of biologically active β-lactams.

The importance of β-lactam compounds led the scientific community to explore a wide range of approaches for the synthesis of the β-lactam ring.[1i, 15] Among the different synthetic approaches that have been described in the literature, the Staudinger ketene-imine cycloaddition is, perhaps, the most famous strategy for the construction of the β-lactam ring. Other approaches such as cyclization reactions, Kinugasa reaction, carbene insertion, carbonylative ring expansion of aziridines were also described in the literature. As expected, this has led to a huge number of review papers that focused on the synthesis and reactivity of either monocyclic or condensed bicyclic β-lactams.[15a-c, 16]

1.1.2. Spiro-β-Lactams

Spirocyclic compounds are molecules containing two rings that share one atom, the quaternary spiro carbon.[17] The tetrahedral nature of the spiro carbon confers rigidity to the molecule leading to a well-defined three-dimensional (3D) structure, allowing an efficient and selective interaction with molecular targets. Moreover, having in mind that important interactions of a compound with a 3D binding site can be achieved more easily using a rigid core (*e.g.* spirocyclic) than a planar one (*e.g.* aromatic systems), the interactions between spirocyclic compounds with enzymes or other biological receptors can also be favored and more specific due to this 3D nature.[18] Consequently, this class of compounds plays a relevant role in medicinal chemistry and it is not surprising that a wide

range of biologically active compounds possess a spirocyclic moiety in its molecular structure.

The spiro-β-lactams, whose general structures are represented in Figure 1.4, are one class of spirocyclic compounds that have attracted the attention of researchers due to its properties.[1f] Spirocyclic-β-lactams are interesting from the biological point of view, due to their structural features and bioactivity. It is known that some spiro-β-lactams can act as antiviral,[19] antifungal,[20] antiplasmodial[19a,c,d, 21] and antibacterial agents[20, 22] (Figure 1.4). Moreover, spiro-β-lactam moiety is also present in cholesterol absorption inhibitors[23] and enzymatic inhibitors[24], and in naturally occurring molecules such as Chartelline A.[25]

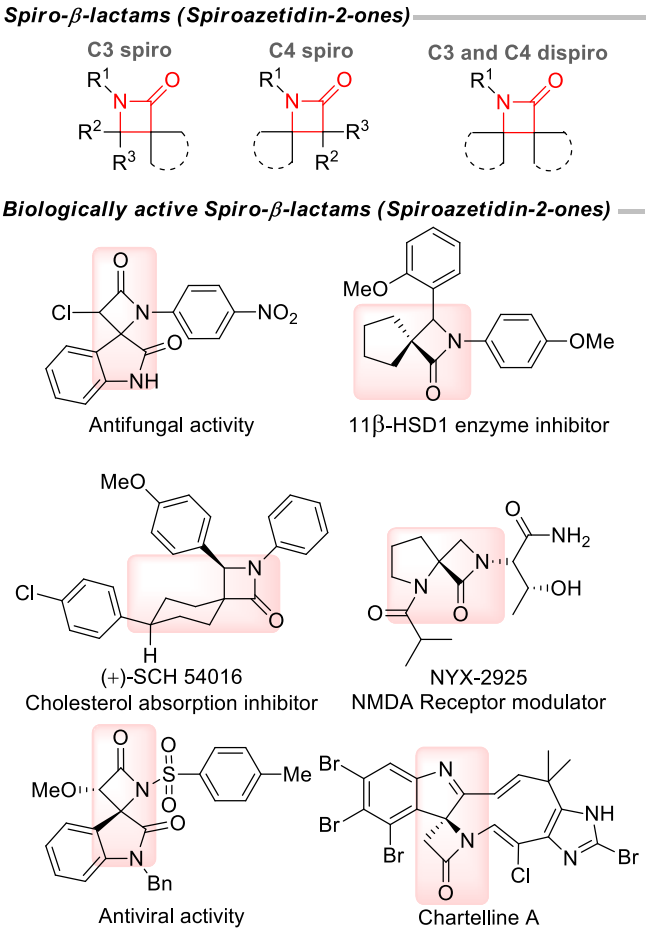
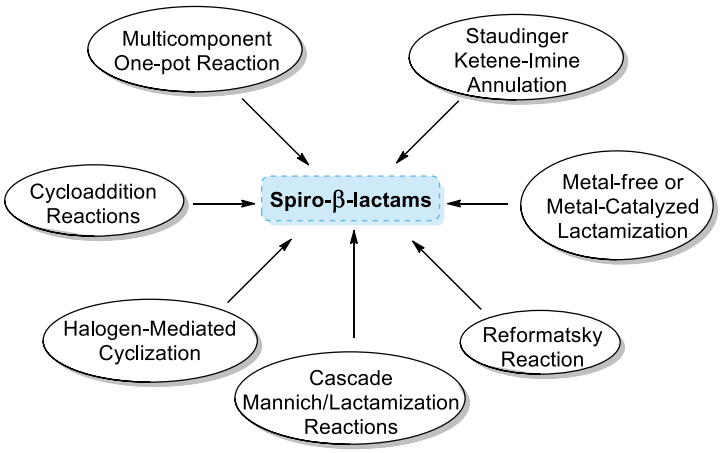


Figure 1.4. General structures of spiro-β-lactams and examples of biologically active derivatives.

Given the above-mentioned facts, there is no doubt that spiro-β-lactams are extremely important in several fields of chemistry and in drug discovery. For this reason,

there is a need to search for alternative methodologies aiming the synthesis of these molecules. As represented in Scheme 1.1, a panoply of synthetic approaches to afford spiro-β-lactams have been described in literature, ranging from the well-known classical Staudinger ketene-imine annulation, lactimization reactions, or 1,3-dipolar cycloaddition reactions to more recent strategies, such as one-pot cascade reactions.[1f, 26] Due to the importance of these spirocyclic compounds, some review papers aimed either on their synthesis or on their biological activity were published in the past few years.[1f, 26a-b]



Scheme 1.1. Synthetic routes to spiro-β-lactams.

1.2. HIV and *Plasmodium*

1.2.1. HIV

Human immunodeficiency virus (HIV) is a retrovirus and the causative agent of acquired immune deficiency syndrome (AIDS). It is believed that HIV infection probably spread from primates to humans by zoonotic transmission over the last century.[27] Nowadays, over 37 million individuals are living with the virus.[27b, 28]

There are two distinct types of HIV (HIV-1 and HIV-2). The first is responsible for approximately 95% of the infections worldwide, while HIV-2 is restricted to West Africa and a couple of European countries with socioeconomic links to West Africa.[27b,d, 29] Despite these two types of HIV share many similarities (*e.g.* intracellular mechanisms of replication and modes of transmission), they lead to distinct immunological and clinical outcomes. Considering that the immunodeficiency occurs slowly in HIV-2

infection when compared to HIV-1, HIV-2 patients have longer disease-free survival. Nevertheless, with the disease progression the mortality risk becomes equivalent.[27a,d, 30]

Regarding virus life cycle (Figure 1.5), HIV enters the cells mediated by the envelope glycoproteins. The first stage of HIV life cycle starts with the binding of the surface proteins to CD4 which is expressed on the surface of T lymphocytes, monocytes, macrophages and dendritic cells. Moreover, to gain access to the host cell, HIV also needs to bind to a co-receptor, usually CCR5 and CXCR4 (natural receptors for α- and β-chemokines).[27a,d, 31] However, there are other paths for HIV entry in cells, namely by endocytosis or cell-to-cell fusion through viral synapses. Once inside the host cell, *ss*RNA is reverse transcribed into HIV DNA, which is then integrated into the host DNA as part of its own genome. After this process, proteins are produced and cleaved, and mature virions are released.[27b, 32] Infection is usually established with a CCR5-tropic strain (R5 isolates), but as the disease progresses and the $CD4^+$ T cell number declines (reduced production and increased destruction), CXCR4-tropic isolates (X4) emerge in most individuals.[27b,d, 31]

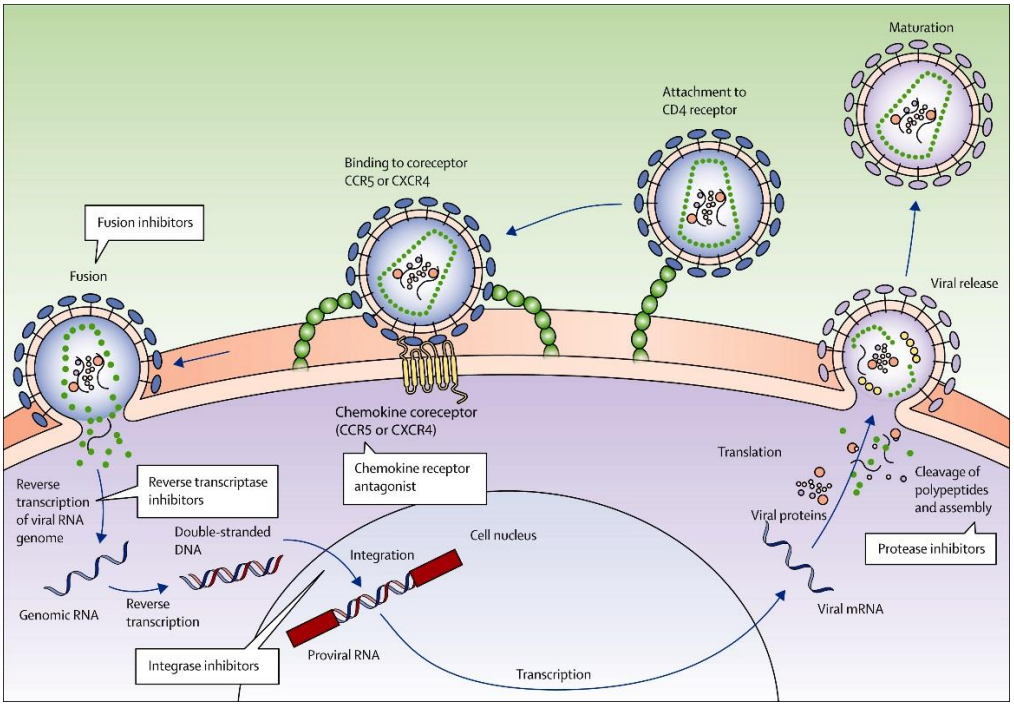


Figure 1.5. HIV life cycle with the sites of action of different classes of antiretroviral drugs (adapted from reference 27d).[27d]

In the past, HIV patients died on average 10 years after the infection. Nowadays, antiretroviral therapy (ART) is highly effective at preventing morbidity and mortality. ART has turned HIV/AIDS into a controlled condition, leading to suppression of viral replication and reducing the plasma HIV viral load to below the limits of detection. These effects lead to a reconstitution of the immune system as verified by an increase in the number of $CD4^+$ T lymphocytes. Since treatment decreases viral load, it reduces the probability of HIV transmission. When used correctly, ART is quite effective, preventing new cells from becoming infected. However, it is important to keep in mind that if the treatment is interrupted, the virus returns and with it a higher risk for developing drug resistance.[27b,d, 32-33]

Notwithstanding the advances in ART, some treatments still fail. The major cause of treatment failure is the development of drug resistance.[29] Thus, in order to maximize viral suppression and avoid drug resistance, all patients newly diagnosed with HIV, should perform drug resistance assays.[34] HIV-1 is characterized by an extreme genetic variability and high evolution rate, which favours the development of antiretroviral resistance. The evolutionary dynamic is the source of a diversified population that can generate drug-resistant variants in response to the ART.[29] Compared to HIV-1, treatment of HIV-2 is more challenging. A problem that new scientific breakthroughs must overcome in the treatment of HIV-2 is that certain antiretroviral drugs, which are effective against HIV-1, do not inhibit HIV-2 propagation. Furthermore, HIV-2 is broadly resistant to some antiretroviral drugs developed to suppress HIV-1.[30]

The different stages of the HIV life cycle present many potential opportunities for therapeutic intervention, however, only a few have been explored. Antiretroviral agents currently approved for clinical use belong to five different classes (Figures 1.6 and 1.7):[27b, 29, 33a-d]

- Nucleos(t)ide reverse transcriptase inhibitors (NRTIs), which act by blocking reverse transcriptase. These inhibitors are analogues of natural nucleos(t)ides, and are merged into HIV DNA, leading to termination of DNA synthesis;
- Non-nucleoside reverse transcriptase inhibitors (NNRTIs), which inhibit reverse transcriptase in a different way from the NRTIs. NNRTIs bind to a

pocket near the active site, causing a conformational change of the enzyme and subsequent inhibition of reverse transcription;

- Entry inhibitors (fusion inhibitors or coreceptor antagonists), that prevent HIV from binding, fusion and entry into the human cell;
- HIV integrase inhibitors (II's), which prevent the HIV genome from being integrated into the host genome;
- Protease inhibitors (PIs), that block the HIV protease, preventing the cleaving of polypeptide chains into functional proteins;

It is noteworthy that current drug regimens do not fully restore health, and their toxicity and the rapid emergence of drug resistant strains calls for the continuous search for new and better antiretroviral drugs.[33d]

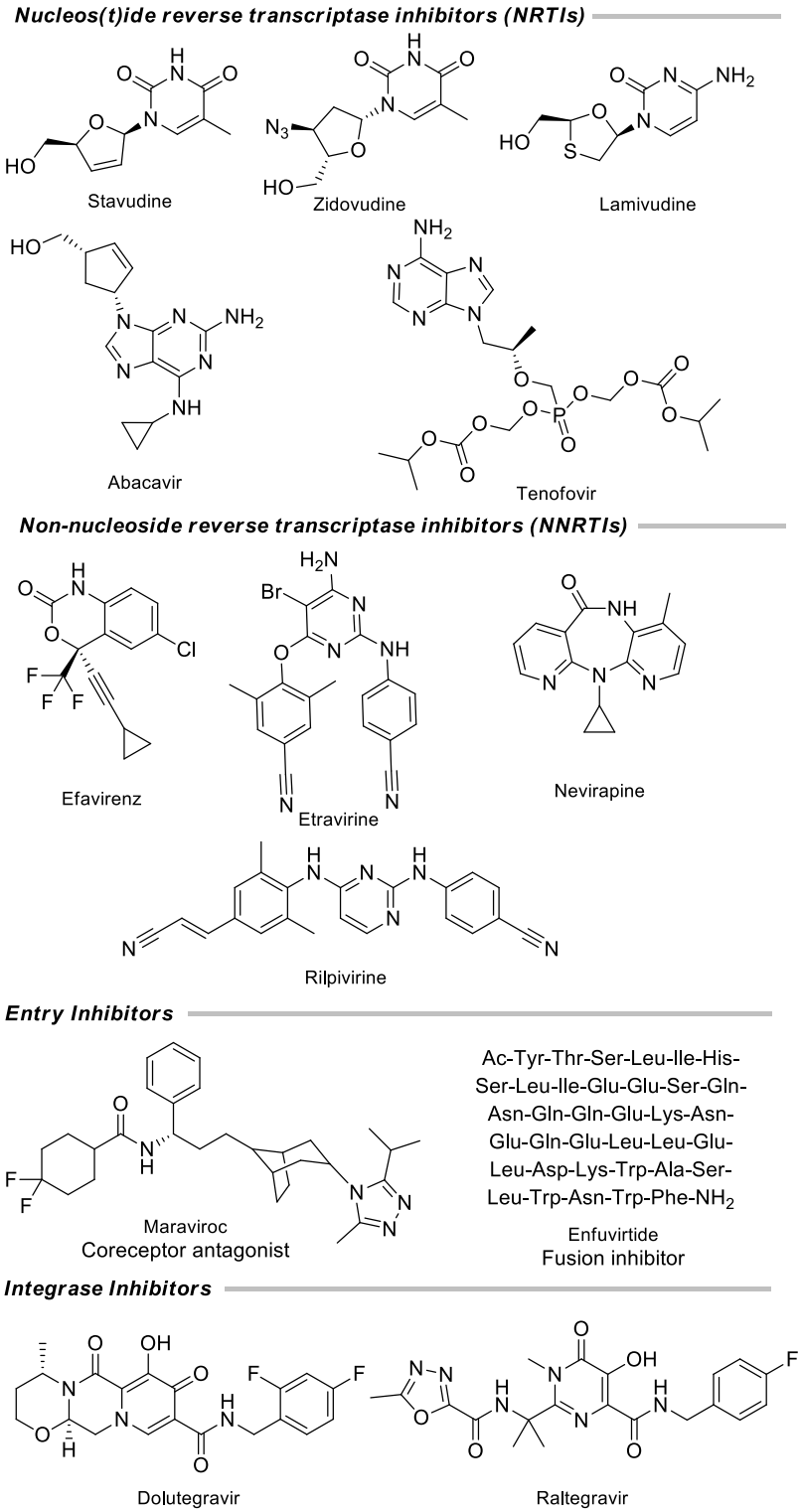


Figure 1.6. Examples of NRTIs, NNRTIs, entry inhibitors and integrase inhibitors.

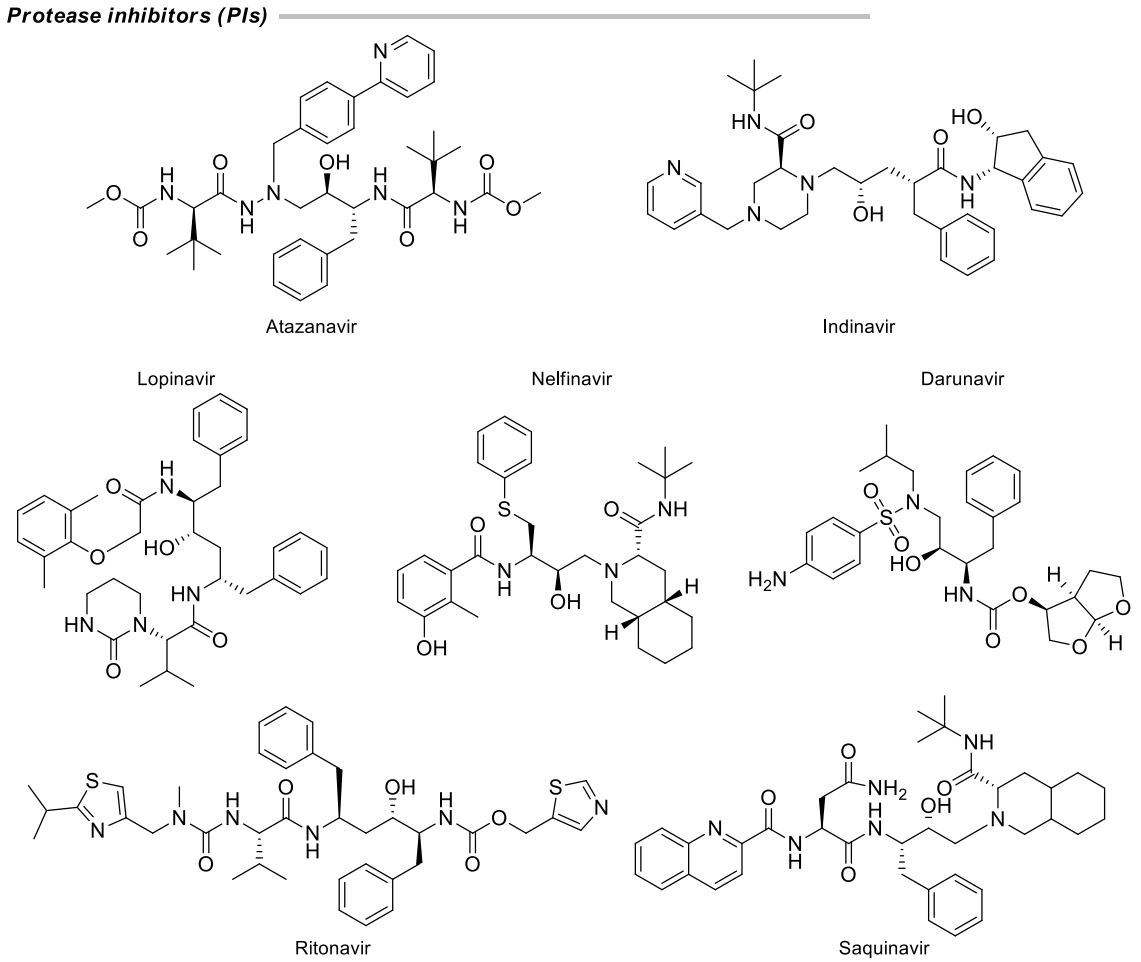


Figure 1.7. Examples of protease inhibitors.

1.2.2. *Plasmodium*

Malaria is a disease that affects humans and other mammalians, caused by protozoan parasites of the *Plasmodium* genus, which includes more than 170 species. However, only five of them are capable of infecting humans: *P. falciparum*, *P. ovale*, *P. vivax*, *P. malariae* and *P. knowlesi*. [35] Malaria remains a major cause of morbidity and mortality, and one of the world's most prevalent tropical diseases.[35a,c, 36] Infection by malaria parasites occurs when an infected female *Anopheles* mosquito bites the mammalian host, releasing *Plasmodium* sporozoites into the host's bloodstream (Figure 1.8).[37]

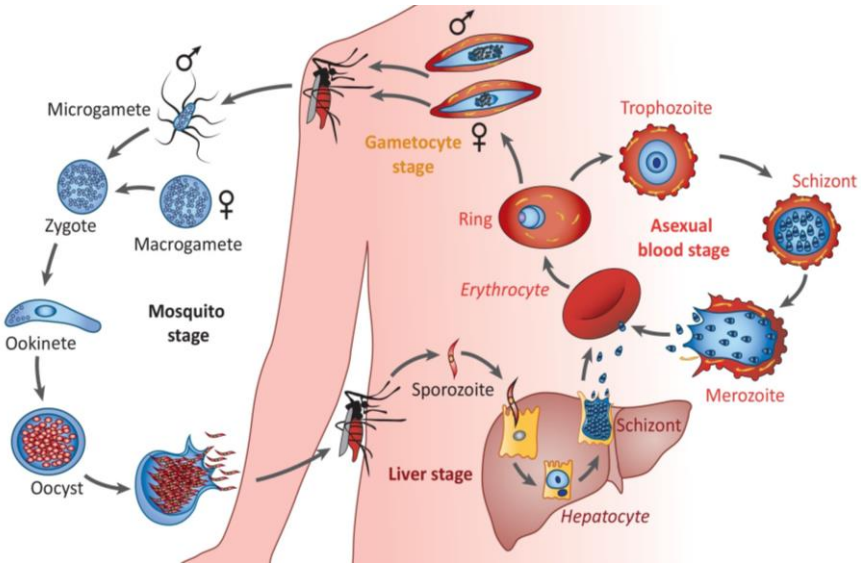


Figure 1.8. Plasmodium life cycle (reproduced from Maier).[38]

The sporozoites injected by the bite of the mosquito use their capacity to move through host cells to reach the blood vessels. Once in the blood, sporozoites migrate to the liver, initiating the liver stage of infection, an essential step in the life cycle of *Plasmodium*. It is noteworthy that sporozoites might also reach the liver via the lymphatic vessels.[35c, 36a, 39] Once in the host's liver, sporozoites infect hepatocytes and develop into exoerythrocytic forms that eventually give rise to merozoites. After few days, parasite-filled vesicles termed merosomes are released into the blood stream, where they finally burst, releasing thousands of merozoites.[35c, 36b, 39-40] This step marks the beginning of the blood stage of infection, responsible for disease symptoms. Each merozoite will invade a red blood cell, starting a cyclic replication cycle that leads to the release of additional merozoites from the infected red blood cell.[35c, 36b, 39-40] In addition, some parasite blood stages differentiate into gametocytes, which can persist in the host bloodstream for weeks. Gametocytes are responsible for the transmission of the malaria parasite from humans to mosquitoes through their ingestion during a subsequent blood meal. This is the starting point of the sexual phase of *Plasmodium* life cycle, which continues with the development of the ingested gametocytes in the mosquito midgut, culminating in the formation of sporozoites that reach the mosquito salivary glands, completing the cycle.[35c, 39b]

Primaquine is the only licensed drug for clinical use against *Plasmodium* liver stages.[35b, 40-41] It is the only drug with demonstrated efficacy against *P. vivax* and *P. ovale* infections. These two *Plasmodium* species can generate hypnozoites, which are dormant parasite forms that can cause relapsing malaria after a period of months or years after the original infection. [35c, 36] However, primaquine presents potentially lethal side effects, specifically hemolytic anemia in people with glucose-6-phosphate dehydrogenase deficiency.[36b] This enzyme deficiency is very common in malarious regions of Africa and Asia.[35b]

Coinfection between HIV and malaria is common, particularly in sub-Saharan Africa, due to the presence of factors that favour transmission, like poverty.[40, 42] Infection with both pathogens is thought to have a synergistic effect. HIV has been shown to increase the risk of development of malaria. On the other hand, some studies reported that malaria leads to a decline in $CD4^+$ T cells, induces HIV-1 replication *in vitro* and *in vivo*, and increases HIV transmission.[40, 42a]

1.3. Spiro-β-Lactams as Antimicrobial Agents

Infectious diseases are a major public health threat due to their high transmission rates and associated mortality.[43] Recently, microbial infections have become a global health problem due to emerging and, in most of cases, resistant strains of viruses, multiresistant bacteria and parasites, increasing the risk of mortality or severe morbidity. As seen by SARS-CoV-2 pandemic, infectious diseases can bring a lot of economic and social problems with a total or partial disruption in economics and in society.[44]

Despite the huge number of bacteria and parasite disease reported cases, the most prominent infectious diseases over the last century were caused by viruses such as HIV, Influenza and Coronaviruses. Having in mind the lack of effective antimicrobial agents there is a need to seek for new alternatives.[45] Through the observation that different viruses use similar pathways to replicate inside a cell, the scientific world took some efforts for the development of broad-spectrum antiviral agents, which are compounds that target more than one virus. Examples of broad-spectrum antiviral agents are represented in Figure 1.9 and include faviparivir, cidofovir, ribavirin and remdesivir.[46]

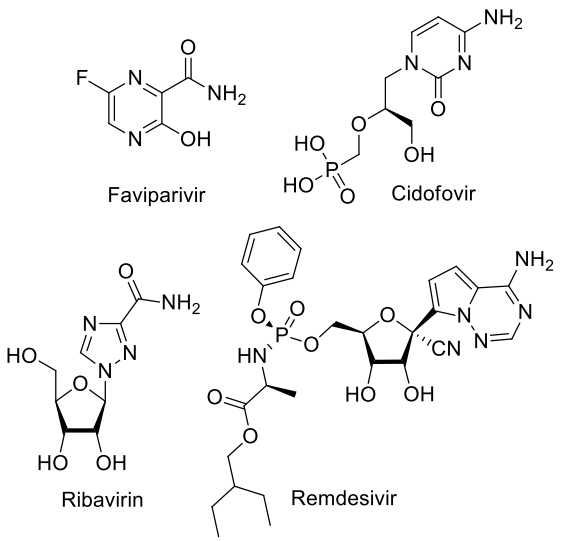


Figure 1.9. Examples of broad-spectrum antiviral agents.

Knowing that some pathogens make use of similar mechanisms to infect hosts opens a window of opportunity to discover novel biological activities of previously approved drugs, a concept called drug repurposing.[47] Notably, drug repurposing allowed the identification of several antiviral agents that demonstrated to be bioactive against other pathogens including bacteria, fungi, or parasites.[46] In Figure 1.10 some examples of known drugs with antimicrobial broad-spectrum activity are represented.

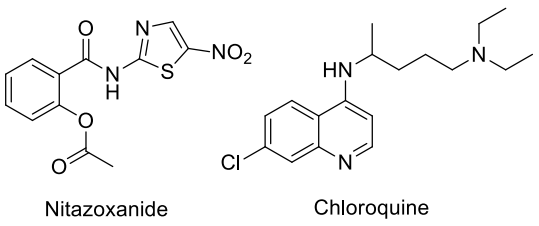


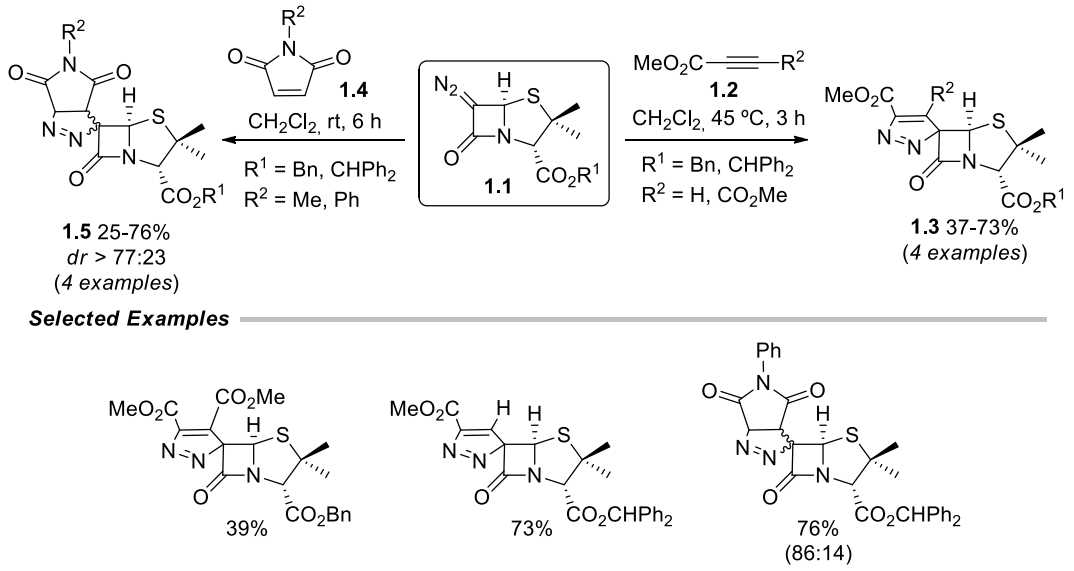
Figure 1.10. Examples of broad-spectrum antimicrobial agents.

Broad-spectrum antiviral agents were commonly developed to target the viral machinery, however, due to rapid mutations leading to drug-resistant virus this strategy may fail after few years.[48] In this context, approaches aiming the synthesis of host-directed antiviral agents have been explored. Drug-enhanced strategies that aim to induce of immune responses, regulation of cytokine storms, and modulation of epigenetic changes are potential interesting approaches to control infections. This host-directed

strategies also reduces the probability of generate drug-resistant strains which is a great advantage. However, there are few approved host-directed agents, with most of them being antiviral agents based on IFNs. Nevertheless, several candidates aiming the inhibition of proteins and enzymes, or even targeting lipid biosynthesis or metabolic pathways, are under preclinical studies.[49]

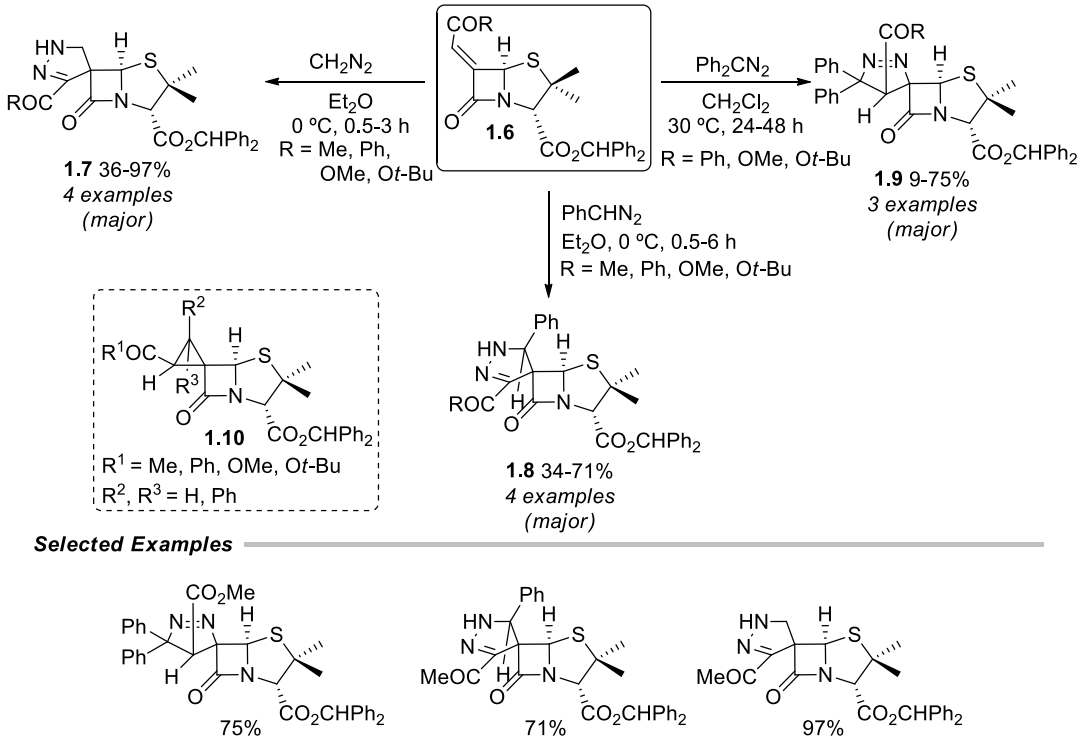
As it has been referred before, spiro-β-lactamic compounds can act as antiviral agents. Given these reports, a multidisciplinary team led by Teresa Pinho e Melo and Nuno Taveira hypothesized that spiro-β-lactams could be developed as HIV inhibitors.[19]

In the last decade, serious efforts were made aiming at the synthesis of novel chiral spiro-β-lactams, by exploring the reactivity of 6-diazopenicillanates and 6-alkylidenepenicillanates, both derived from 6-aminopenicilanic acid (6-APA).[19c-d, 50] The first approach relied on the 1,3-dipolar cycloadditions of 6-diazopenicillanates **1.1** towards electron-deficient alkynes **1.2** for the synthesis of chiral spiro-3*H*-pyrazole-β-lactams **1.3**, which were obtained stereo- and regioselectively, as single products in yields ranging from 37-73% (Scheme 1.2).[50b] The dipolar cycloaddition reaction was also explored with alkenes as dipolarophiles. Carrying out the cycloaddition reaction between diazo derivative **1.1** and acrylonitrile, acrylates, or methyl vinyl ketone, led to the corresponding spiro-2-pyrazoline-β-lactams which were isolated as a diastereosisomeric mixture in moderate to good yields. Similarly, the use of *N*-substituted-maleimides **1.4** as dipolarophiles afforded the expected spiro-1-pyrazoline-β-lactams **1.5**, as an inseparable mixture of diastereoisomers in yields up to 76%. The authors also described the synthesis of spirocyclopropane-β-lactams through the denitrogenation, and subsequent ring contraction, of spiro-1-pyrazoline-β-lactams **1.5** under microwave irradiation.



Scheme 1.2. 1,3-Dipolar cycloaddition reaction of 6-diazopenicillanates **1.1** towards electron-deficient alkenes and alkynes.

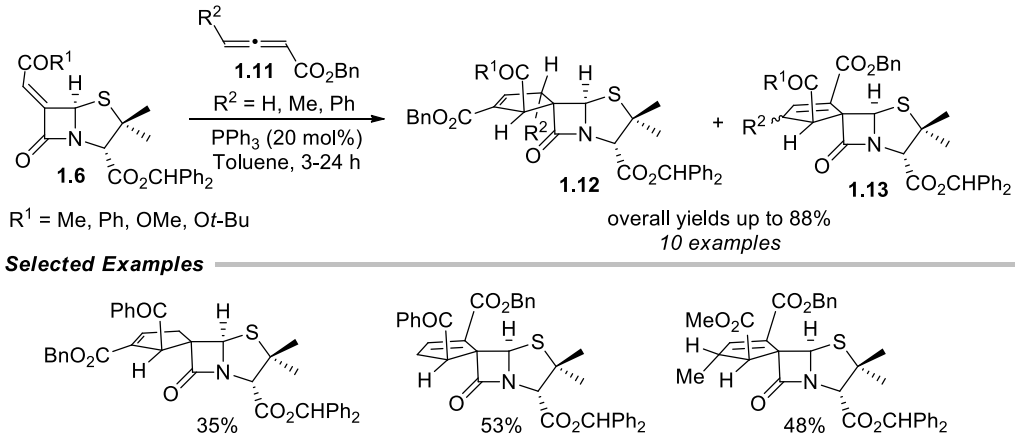
It is known that the synthesis of spiropyrazoline-β-lactams can also be achieved via 1,3-dipolar cycloaddition of 6-alkylidenepenicillanates with diazo compounds.[51] Thus, Pinho e Melo and collaborators have explored that alternative strategy for the synthesis of spiropyrazoline-β-lactams **1.7-1.9** (Scheme 1.3).[50a] The 1,3-dipolar cycloaddition reaction with diazomethane afforded spiro-2-pyrazoline-β-lactams **1.7** as major products in yields up to 97%. The exception is alkylidenepenicillanate **1.6** bearing a *t*-Bu ester as substituent, which cycloaddition reaction with diazomethane led to the corresponding spiro-1-pyrazoline-β-lactam as major product. The same reactivity was observed by carrying out the cycloaddition reactions with phenyldiazomethane as dipole, leading to spiro-2-pyrazoline-β-lactams **1.8** as major adducts. From the 1,3-dipolar cycloaddition reaction of 6-alkylidenepenicillanates **1.6** with diphenyldiazomethane it was possible to obtain spiro-1-pyrazoline-β-lactams **1.9** in yields ranging from 9 to 75%. In addition, the synthesized spiro-1-pyrazoline-β-lactams underwent microwave-induced denitrogenation allowing the synthesis of spirocyclopropane-β-lactams **1.10**.



Selected Examples

Scheme 1.3. 1,3-Dipolar cycloaddition reaction of 6-alkylidenepenicillanates **1.6** towards diazo compounds.

Another approach for the synthesis of spiro-β-lactams is based on phosphine-catalyzed [3+2] annulation of allenoates to 6-alkylidenepenicillanates, leading to chiral spirocyclopentene-β-lactams (Scheme 1.4).[50c] From the reaction of monosubstituted allenoate **1.11a** (R^2 = H) with 6-alkylidenepenicillanates **1.6** in the presence of 20 mol% of triphenylphosphine, regiomeric spiro-β-lactams **1.12** and **1.13** were obtained stereoselectively in overall yields ranging from 41% to 88%. The reactivity of 6-alkylidenepenicillanates **1.6** towards disubstituted allenoates **1.11b**,**c** ($R^2 \neq$ H) was also explored. This allowed the synthesis of the expected cycloadducts in good overall yields (up to 84%). However, it should be noted that carrying the [3+2] annulation reaction between 6-alkylidenepenicillanates **1.6** and allenoate **1.11b** (R^2 = Me) proceeds with high regioselectivity leading exclusively to α-regioisomer **1.13**. In sum, this chemistry allowed the formation of two (R^2 = H) or three ($R^2 \neq$ H) consecutive stereogenic centers, including a quaternary stereocenter in a stereo- and diastereoselective manner.



Scheme 1.4. Phosphine-catalyzed formal [3+2] cycloaddition of allenoates **1.11** with 6-alkylidenepenicillanates **1.6**.

These synthetic approaches that aimed at the synthesis of different classes of spiro-β-lactams allowed the construction of a considerable library of compounds. A library of 26 compounds was selected for the evaluation of their *in vitro* activity against HIV-1 (Figure 1.11; Table 1.1).[19a,c] Among the selected compounds, no significant cytotoxicity was observed in TZM-bl cells, with CC_{50} values ranging from 42.34 μM to 163.76 μM. The exception was compound BSS-972B which showed significant cytotoxicity with a CC_{50} of 9.28 μM. The determination of the maximum inhibition percentage (MPI) was carried out at 10 μg/mL for two HIV-1 strains (SG3.1 and 93AOHDC249) obtained from clinical isolates. The SG3.1 isolate is a X4-tropic strain and 93AOHDC249 isolate is a R5-tropic strain, meaning that the viral nucleoid enters the cell through a CXCR4 or a CCR5 coreceptor, respectively. This study resulted in the identification of three molecules with promising antiviral activity (MPI > 80%).

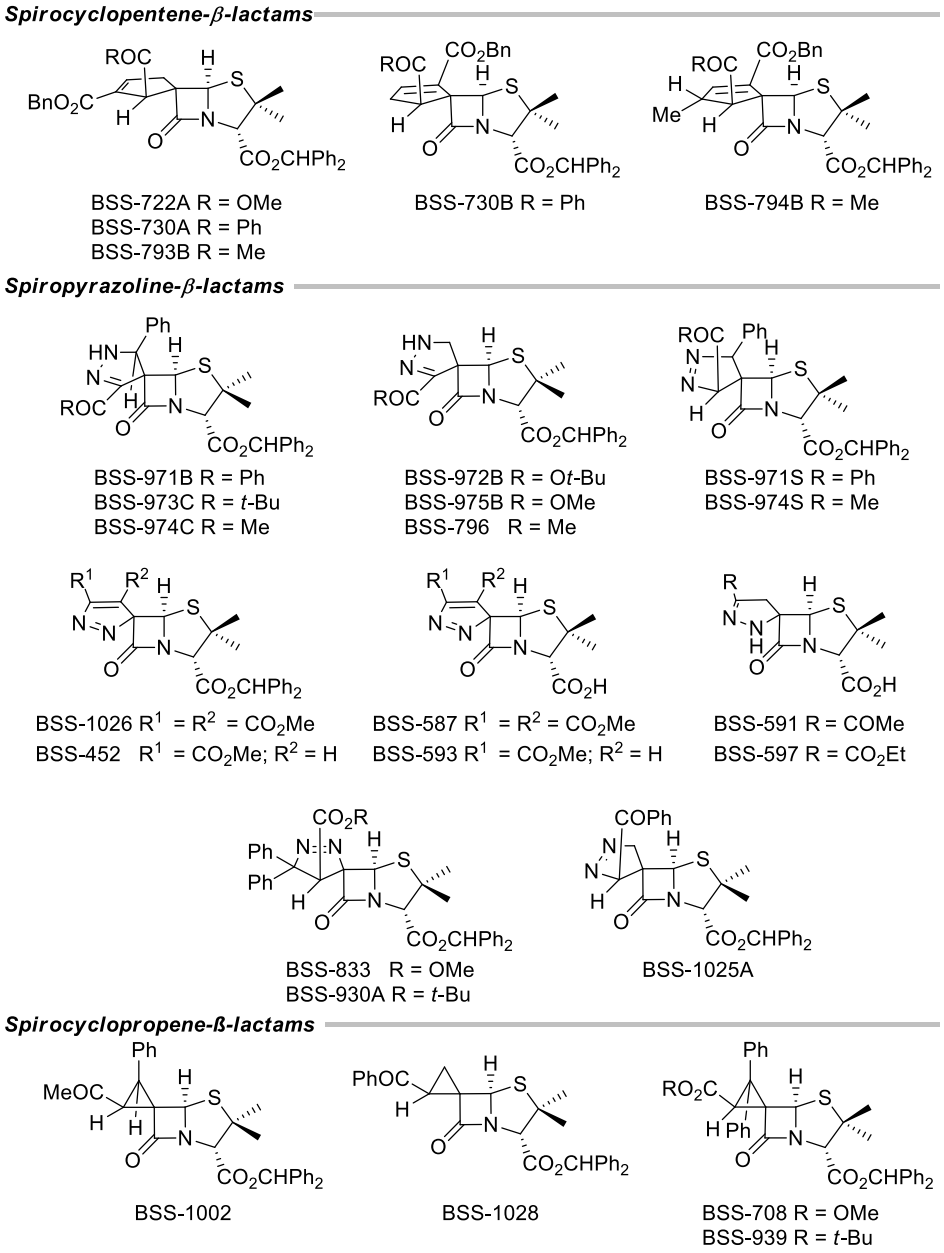


Figure 1.11. Library of spiro-β-lactams evaluated against HIV.

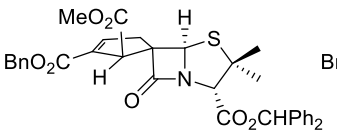
Table 1.1. CC_{50} values and MPI values of anti-HIV-1 *in vitro* screening.

Compound	CC_{50} (µM)	MPI (10 µg/mL) (%) X4-tropism	MPI (10 µg/mL) (%) R5-tropism
BSS-722A	53.74	99	99
BSS-730A	76.84	99	99
BSS-793B	47.69	0	0
BSS-730B	74.45	0	0
BSS-794B	49.02	0	0
BSS-973C	79.80	0	4
BSS-974C	80.79	0	0
BSS-796	98.91	0	1
BSS-971S	81.98	12	65
BSS-974S	91.93	0	0
BSS-591	163.76	n.d.	37
BSS-597	151.79	n.d.	33
BSS-1026	82.01	0	15
BSS-452	104.73	15	0
BSS-587	135.83	n.d.	53
BSS-593	158.14	84	58
BSS-708	83.80	0	0
BSS-833	48.70	0	n.d.
BSS-930A	48.10	0	n.d.
BSS-939	42.34	0	n.d.
BSS-971B	51.14	4	n.d.
BSS-972B	9.28	n.d.	n.d.
BSS-975B	50.10	56	n.d.
BSS-1002	50.25	0	n.d.
BSS-1025A	48.91	0	n.d.
BSS-1028	48.50	79	n.d.

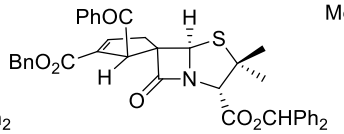
The most promising molecules, BSS-593, BSS-722A, and BSS-730A exhibited potent antiviral activity against HIV-1 isolates with IC_{50} values ranging from 0.004 μM to 0.650 μM (Table 1.2). Compounds having a MPI under 80% were not considered promising enough and their IC_{50} values were not calculated. Nevertheless, these compounds can still constitute a promising starting point for further structural modulations on the search for additional compounds with improved anti-HIV activity.

Particularly interesting was to observe that BSS-722A and BSS-730A also exhibited activity against one HIV-2 strain (03PTHCC19) with IC_{50} values of 0.510 μM and 0.008 μM, respectively.

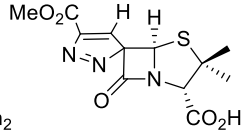
Table 1.2. *In vitro* activity of the most promising spiro-β-lactams against HIV.



BSS-722A



BSS-730A



BSS-593

Compound	CC_{50} (μM)	Virus	Tropism	MPI (10 μg/mL) (%)	IC_{50} (μM)	IC_{90} (μM)	Therapeutic index
BSS-593	158.00	HIV-1	X4	84	0.012	n.d.	13144.76
		HIV-1	R5	58	0.035	n.d.	4553.31
		HIV-2	R5	0	n.d.	n.d.	n.d.
BSS-722A	53.70	HIV-1	X4	97	0.650	1.091	82.64
		HIV-1	R5	99	0.332	0.701	161.80
		HIV-2	R5	90	0.510	1.182	105.29
BSS-730A	76.84	HIV-1	X4	99	0.014	0.025	5584.30
		HIV-1	R5[a]	99	0.026	0.118	2946.32
		HIV-1	R5[b]	94	0.004	0.02	20247.69
		HIV-2	R5	99	0.008	0.064	9605.00

[a] 93AOHDC249 – primary isolate; [b] 93AOHDC250 – primary isolate

The ratio between the CC_{50} and IC_{50} values provide valuable information about the safety of bioactive molecules. This ratio is commonly known as therapeutic index and is described as the ratio between the dosage of a drug that causes a toxic effect and the dosage that causes a therapeutic effect.[52] Consequently, the larger the therapeutic index, the safer the drug is. By the analysis of Table 1.2 it is possible to observe that the assayed spiro-β-lactams showed notable therapeutic index values, up to 20247.69 (for BSS-730A).

To get further inside into BSS-730A's bioactivity, experiments using lead compound BSS-730A were carried out. The antiviral activity of BSS-730A against eight drug-resistant HIV-2 isolates was evaluated. It was observed that this molecule displays high activity with IC_{50} values up to 0.030 μM (Table 1.3). As mentioned in Section 1.2.1., the available drugs for HIV-1 are not highly effective against HIV-2. Consequently, having a molecule showing activity against both HIV-1 and HIV-2, including drug-resistant HIV strains, makes BSS-730A a very promising lead compound for the development of innovative antiviral agents.

Table 1.3. *In vitro* activity of BSS-730A against drug-resistant HIV isolates.

Virus	Strain	Tropism	Susceptibility to antiretroviral drugs	IC_{50} (μM)	IC_{90} (μM)
HIV-2	03PTHCC19	R5	Sensitive	0.008	0.064
	00PTHCC20	X4	resistant to ABC, ZDV, d4T, ddI, LPV	0.018	0.073
	03PTHCC20	X4	resistant to ABC, ZDV, d4T, ddI, LPV	0.019	0.095
	00PTHDECT	R5/X4	resistant to DTG	0.023	0.057
	03PTHDECT	X4	resistant to DTG	0.030	0.082
	03PTHSM9	X4	resistant to SQV, LPV, DRV and TAF	0.016	0.116
	10PTHSJIG	R5	resistant to RAL, DTG, LPV, SQV, DRV and all NRTIs	0.012	0.032
	15PTHSJIG	R5	resistant to RAL, DTG, 3TC and FTC	0.018	0.056
	15PTHCEC	X4	resistant to RAL, DTG, LPV, SQV, DRV, ABC, ddI, TDF, TAF, 3TC, d4T and FTC	0.017	0.051

ABC, abacavir; ZDV, zidovudine; d4T, stavudine; ddI, didanosine; 3TC, lamivudine; FTC, emtricitabine; TDF, tenofovir disoproxil fumarate; TAF, tenovovir alafenamide; LPV, lopinavir SQV, saquinavir; DRV, darunavir; DTG, dolutegravir; RAL, raltegravir; NRTIs, nucleoside reverse transcriptase inhibitors.

In order to investigate the mechanism of action of BSS-730A, some experiments such as time-of-addition assays and structure similarity search were carried out. The time-of-addition (TOA) assays assess for how long the addition of an anti-HIV compound can be postponed within the viral replication cycle before losing its antiviral activity. This methodology may help to unveil the mechanism of action of a new drug by disclosing which step of the HIV cycle was inhibited, through comparison of the new drug's TOA profile with those of reference compounds.[53] The results demonstrated that even after 24 h of delay of BSS-730A addition to the cells, BSS-730A does not shown a significant loss of activity, leading only to a decrease of 10-16% when added 15-24 h after infection (Figure 1.12).

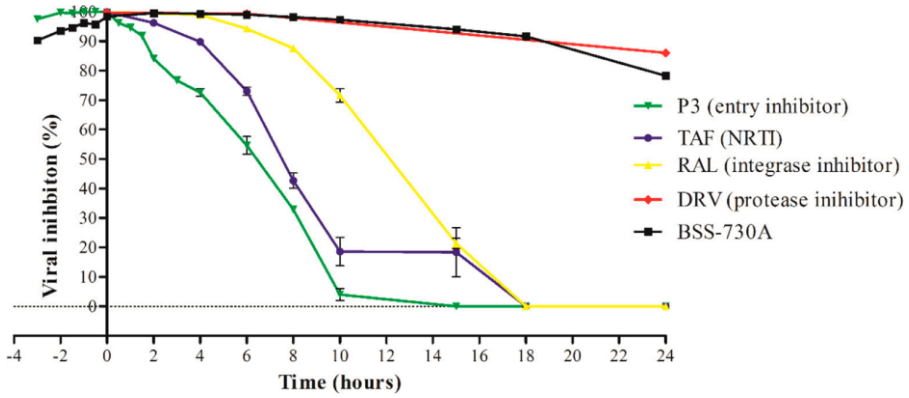


Figure 1.12. Time of addition assay against HIV-1 strain SG3.1.

The structure similarity assays based on extended-connectivity fingerprints, which are molecular 2D structure topological descriptors, revealed similarity between BSS-730A and four molecules with anti-HIV activity. These four molecules are also β-lactams and are reported as HIV protease inhibitors. However, BSS-730A was tested against one HIV-1 recombinant protease and showed no protease inhibitory activity (Table 1.4). Considering the above-mentioned TOA results, together with the lack of protease inhibitory activity, it suggests that BSS-730A acts on the host cells rather than on the virus. Therefore, BSS-730A's mechanism of action may be new and different from those of the currently approved anti-HIV drugs.

Table 1.4. HIV-1 protease inhibition by BSS-730A.

Compound	Concentration (μM)	Control Values Inhibition (%)			Reference Compound	Reference Compound IC_{50} (μM)
		Assay 1	Assay 2	Average		
BSS-730A	10	10.3	5.3	7.8	Pepstatin A	1.2

To further explore the potential of spiro-β-lactams as antimicrobial agents, *in vitro* activity of the three spiro-β-lactams with higher anti-HIV activity (BSS-593, BSS-722A and BSS-730), together with some nonactive derivatives were evaluated against *P. berghei* hepatic infection.[19a,c] Notably, the compounds with the best anti-HIV properties showed interesting activity against *P. berghei* hepatic stage of infection. Nevertheless, two compounds without anti-HIV activity also showed moderate anti-*Plasmodium* activity (BSS-930A and BSS-939: IC_{50} = 3.32 ± 0.23 μM and 2.67 ± 0.22 μM, respectively). It is noteworthy, that these two compounds share a high structural similarity. In fact, BSS-930A is a precursor of BSS-939, the latter being obtained via N_2 extrusion and subsequent ring contraction of the former's 1-pyrazoline ring. The presence of the same pharmacophoric features both in BSS-939 and BSS-930A can be a likely explanation for the similar activities of both compounds.

Remarkably, spiro-β-lactam BSS-730A was identified as the most promising molecule with an IC_{50} value of 0.55 ± 0.14 μM. Excited by these results against the hepatic stage of *Plasmodium* infection, BSS-730A's *in vitro* activity against the erythrocytic stages of infection by the human-infective *P. falciparum* parasite was assessed. Aside from its notable hepatic stage activity, BSS-730A also presents a promising sub-micromolar activity against blood stage of *Plasmodium* infection (IC_{50} = 0.430 μM).[19c]

The outstanding results for BSS-730A's antimicrobial activity against HIV-1, HIV-2, and its dual-stage antiplasmodial activity shows the potential of this molecule as a versatile and broad-spectrum antimicrobial agent. The target and mechanism of action of BSS-730A are still elusive, but its potent activity against two different organisms and the TOA experiments, indicate that BSS-730A targets host cells, which can result in potential activity against other viruses or parasites.

1.4. The Role of the β-Lactam Ring on the Biological Activity

Given the outstanding antimicrobial activity of its initially studied penicillin derived spiro-β-lactams, Pinho e Melo *et al.* conducted structural modulations to gather relevant structure-activity relationships, namely regarding the importance of the β-lactam ring. One of the strategies explored was the synthesis of spiro-γ-lactams analogues, where its four-membered β-lactam ring was replaced by a five-membered ring.[19a]

Spirocyclic compounds containing a γ-lactam ring (spiropyrrolidin-2-ones) have attracted the attention of the scientific community. This structural motif is present in a wide range of natural products, such as elmenol H, annosqualine, spirostaphylotrichin A and spirotaphilotrichin X (Figure 1.13).[54] The spirocyclic γ-lactam motif is also present in non-natural molecules displaying biological activity (*e.g.* the antivomitin drug rolapitant and the anti-malarial compound **1.14**). Furthermore, when compared to the four-membered counterparts, five-membered γ-lactam ring is more stable. In addition, the γ-lactam derivatives are not associated with life-threatening hypersensitivity reactions, which may be a problem in the use of β-lactams.[55]

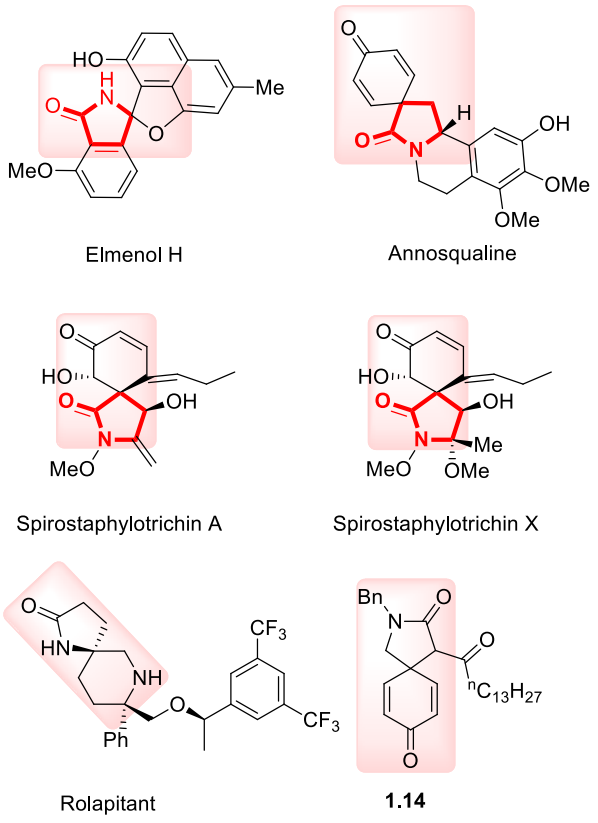
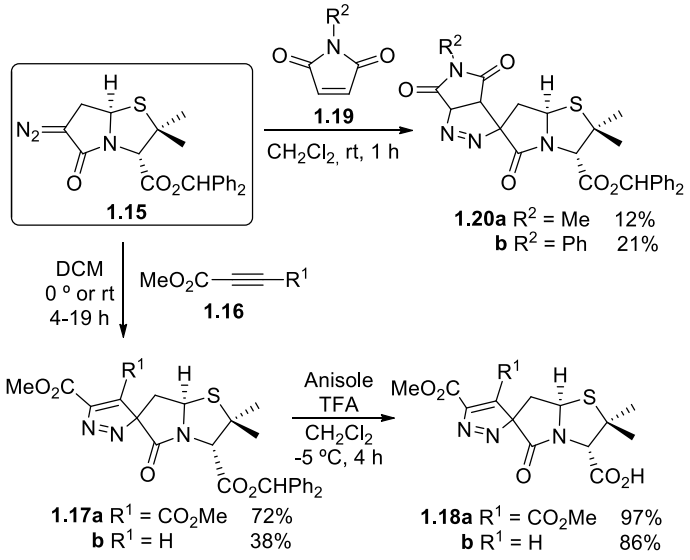


Figure 1.13. Representative natural and/or biologically active spirocyclic γ-lactams.

A synthetic strategy to produce these novel γ-lactams derivatives, analogues of the bioactive molecule BSS-593 was described.[19a] The target spiro-γ-lactamic compounds were synthesized by a similar procedure as described for spiro-β-lactam derivatives, via 1,3-dipolar cycloaddition reaction of a chiral diazo-γ-lactam and electron-deficient dipolarophiles. The synthesis of the chiral diazo-γ-lactam **1.15** derived from *D*-penicillamine will be discussed with further detail in Chapter 5. 1,3-Dipolar cycloaddition reactions of diazo-γ-lactam derivative **1.15**, aiming at the synthesis of γ-lactam analogues of the bioactive molecule BSS-593 were carried out (Scheme 1.5). For this purpose, spiro-γ-lactams **1.17** were synthesized from the cycloaddition reaction of **1.15** with dimethyl acetylenedicarboxylate (**1.16a**) and methyl propiolate (**1.16b**) in 72% and 38% yield, respectively. The cycloaddition of diazo-γ-lactam **1.15** with *N*-substituted maleimides (**1.19**) was also studied, leading to the synthesis of spiro-γ-lactam **1.20a** and **1.20b**, in low yields. Further conversion of the spiro-γ-lactams **1.17** bearing a benzhydryl ester moiety into the corresponding carboxylic acid derivatives **1.18** was also described. The free acid derivatives **1.18** were obtained from the treatment of spiro-γ-lactams **1.17a** and **1.17b** with anisole and TFA in dichloromethane at -5 ºC in excellent yields (97% and 86%, respectively).

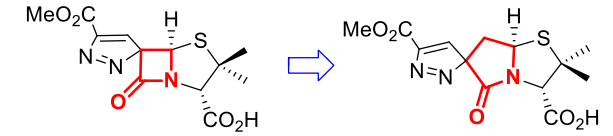


Scheme 1.5. 1,3-Dipolar cycloaddition between a chiral diazo-γ-lactam and electron-deficient dipolarophiles.

After their synthesis, a set of five spiro-γ-lactams derivatives were assayed for their *in vitro* cytotoxicity, and as expected the CC_{50} values did not suggest any relevant cytotoxicity (Table 1.5). However, none of them showed significant anti-HIV activity. The highest MPI values were 30% and 27%, for compounds **1.18a** and **1.18b**, respectively. The antiplasmodial potential of these derivatives was also accessed, however, only compound **1.20a** displayed moderate activity against *P. berghei*, with an IC_{50} value between 1 and 10 μM.

Table 1.5. CC_{50} values and MPI values of spiro-γ-lactams against HIV-1.

Compound	CC_{50} (μM)	MPI (10 μg/mL) (%) X4-tropism
1.17a	30.34	18
1.18a	52.34	30
1.18b	108.60	27
1.20a	46.61	0
1.20b	52.94	0



BSS-593
MPI HIV-1 (10 μg/mL) = 84%
IC_{50} (HIV-1) = 0.012 μM

1.18b
MPI HIV-1 (10 μg/mL) = 27%

Notwithstanding the excellent anti-HIV activity of BSS-593 its γ-lactam counterpart **1.18b** did not share that capability to inhibit the virus. Knowing that the conformational freedom of a 5-membered ring is higher than a 4-membered ring, the authors hypothesized that the lack of antiviral activity could be due to the spiro-γ-lactam pharmacophoric features, stability and/or conformation.

Quantum chemical calculations, at the DFT level of theory, showed that despite the difference on the lactam ring size, both BSS-593 and γ-lactam **1.18b** presented similar minimum energy conformations. On the other hand, molecular superposition of minimum energy conformations and pharmacophoric features mapping of both molecules were also studied. Both studies showed that despite difference between a 4- and 5-membered lactam

ring, both molecules have an almost perfect overlap between pharmacophoric features. This suggest that compound **1.18b** should be capable to reproduce the pharmacophoric 3D disposition from its active BSS-593 analogue, and consequently, to reproduce its interactions with molecular targets. In conclusion, despite those similarities the experimental results clear indicate that the β-lactamic core is a crucial structural feature to ensure activity against both HIV and *Plasmodium*.

1.5. Final Remarks and PhD Project Goals

Spiro-β-lactams are a subclass of β-lactams characterized by having a spiro-fused ring at C3 and/or C4 position of the β-lactam core. As a result of the unique 3D arrangement of spirocyclic compounds, a wide range of biologically active compounds possess a spirocyclic moiety in its molecular structure. Knowing that infectious diseases are a public health problem, the discovery and development of novel alternatives with increased bioactivity and stability, namely broad-spectrum antimicrobial agents, is a priority for the scientific community.

This chapter brings together a brief introduction about β-lactams and its spiro-β-lactam derivatives, and the synthetic efforts carried out since 2012 by Pinho e Melo and collaborators for the synthesis of novel spiro-β-lactams. The present chapter also describes the state of art of spiro-β-lactam antimicrobial activity, which was the trigger for this PhD project. Some key topics which were relevant to set the rationale and goals of this PhD thesis, whose main objective was the synthesis of new spiro-β-lactams with potent antimicrobial activity, have been outlined.

This work was developed after a prior bioactivity study focused on a library of spiro-β-lactams which allowed the identification of spiro-β-lactams displaying a promising dual activity against both HIV and *Plasmodium*. These spiro-β-lactams were considered lead molecules for the rational design of new potentially bioactive chiral spiro-β-lactams.

The purpose of this PhD thesis was achieved through a strategy relying on rational structural modulation of spiro-β-lactam BSS-730A, via phosphane-catalyzed [3+2] annulation of alkylidene-β-lactams with allenoates.

In addition, by taking advantage of the unexplored 1,3-dipolar cycloaddition reaction between alkylidenepenicillanates and nitrones or nitrile oxides, this work also describes the synthesis of two novel classes of spiro-β-lactams (spiroisoxazolidine-β-lactams and spiroisoxazoline-β-lactams, respectively).

The new molecules were assayed for their *in vitro* activity against HIV-1, allowing the gathering of relevant structure-activity relationships. The anti-HIV-2 and anti-*Plasmodium* activity of the most promising compounds was also investigated.

The last chapter of this thesis covers the synthesis of novel alkylidene-γ-lactams, and an *in vitro* study to unveil the bioactivity behaviour of both alkylidene-β-lactams and alkylidene-γ-lactams as anticancer agents.

1.6. References

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CHAPTER 2

Synthesis of Spiro-β-Lactams with Potent Anti-HIV and Anti-Plasmodial Activity *via* Phosphine-Catalyzed [3+2] Annulation of 6-Alkylidenepenicillanates with Allenoates

Abstract

In this chapter, the synthesis of twelve new alkylidenepenicillanates is described. Subsequent phosphine-catalyzed [3+2] annulation reactions of the newly synthesized 6-alkylidenepenicillanates with allenoates resulted in the synthesis of thirty-eight novel spirocyclopentene-β-lactams. Among this library of new compounds, it was possible to identify six compounds highly active against HIV-1 and HIV-2 in a nanomolar order, with four of them displaying micromolar antiplasmodial activity. Further studies comprising the synthesis of spirocyclopentene-β-lactams under continuous flow and their interaction with albumin were also unveiled.

Chapter 2 - Synthesis of Spiro-β-Lactams with Potent Anti-HIV and Anti-Plasmodial Activity via Phosphine-Catalyzed [3+2] Annulation of 6-Alkylidenepenicillanates with Allenoates

2.1. Introduction

Allenes are an attractive class of compounds containing two cumulative C=C bonds, firstly synthesized in 1887 by Burton and von Peachmann.[1] This structural motif presents a high versality, allowing them to participate in a wide range of synthetic transformations, and being important synthons in the synthesis of bioactive compounds.[2] In addition, as represented by the examples in Figure 2.1, the allene moiety is also present in natural or bioactive compounds, such as enzymatic inhibitors, cytotoxic agents, and antiviral agents.[3] Considering the above-mentioned reasons and the wide literature focused on the chemistry of allenes, it is clear that allene-containing compounds are important derivatives in both organic and medicinal chemistry.

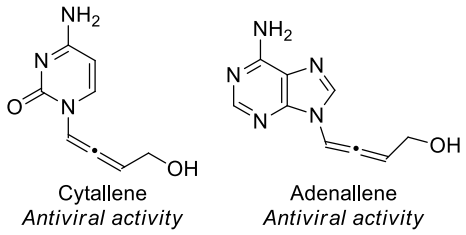
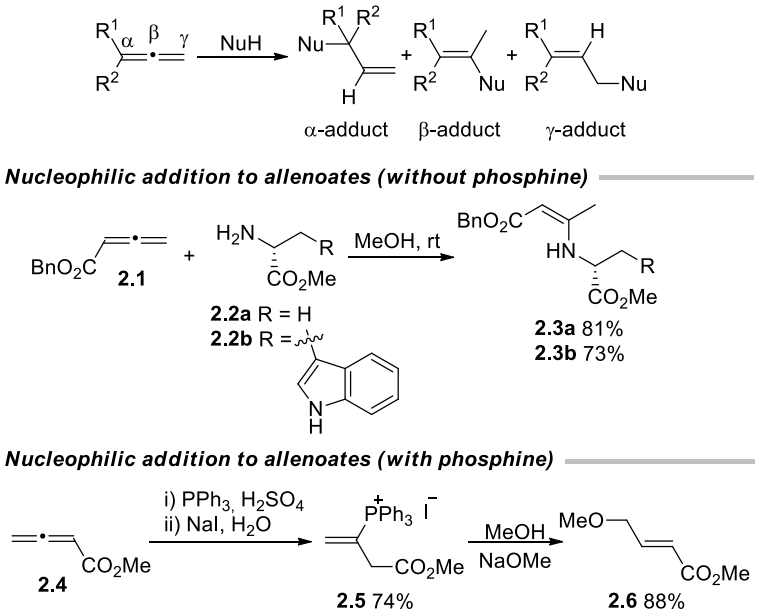


Figure 2.1. Examples of bioactive compounds containing an allene moiety.

As consequence of the two cumulative double bonds some allenes may be unstable, and the nucleophilic addition is highly favored. Moreover, due to the *sp* central carbon, the π-bonds of allenes are orthogonal, consequently, the substituents of α- and γ-carbons are in orthogonal planes, allowing an interesting type of chirality, the axial chirality. Chiral allenes can be used as building blocks for the synthesis of chiral compounds by exploring the transfer of the axial chirality to central chirality.

Allenes can undergo nucleophilic addition reactions in any of its three carbon atoms, depending on their terminal substituents, leading to three possible different products.[4] As outlined in Scheme 2.1, the nucleophilic addition to allenoates, which are allenes containing electron withdrawing groups, occurs in the α,β C=C double bond, leading to β-adducts or Michael adducts.[5] One example is the addition of *D*-alanine or *D*-tryptophan to allene **2.1** leading to the corresponding Michael adducts in 81% and 73% yield, respectively. However, in the presence of phosphines, as reported by Cristau and collaborators, the nucleophilic addition take place in the β,γ C=C double bond, leading to

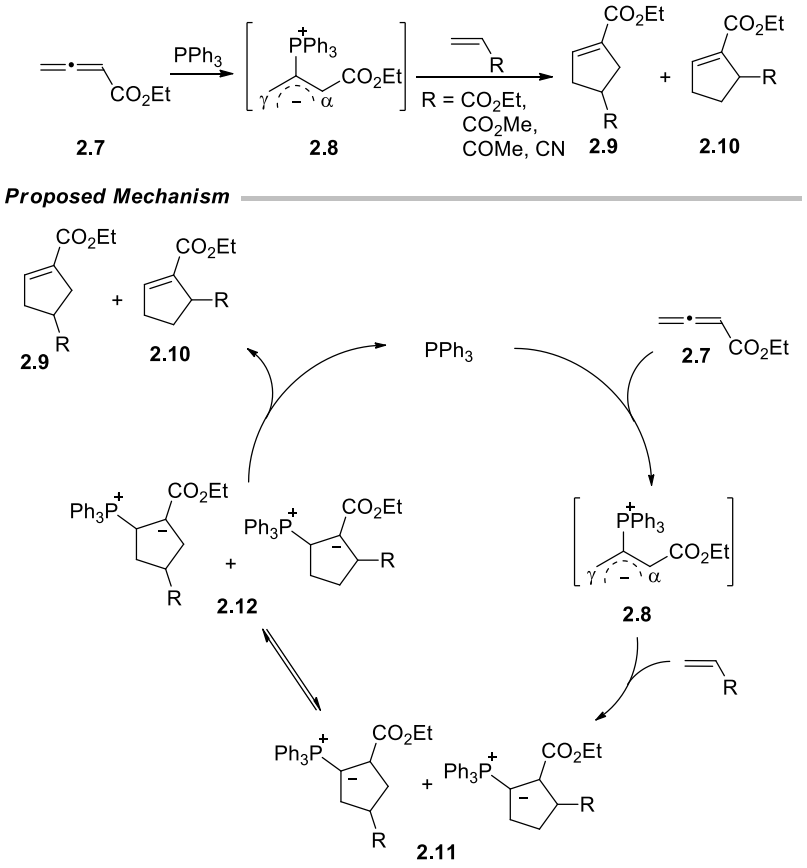
γ-adducts.[5a, 6] As example, treatment of allenoate **2.4** with triphenylphosphine followed by the addition of sodium iodide led to the formation of phosphonium iodide **2.5**. The formation of this salt allows a subsequent nucleophilic addition of the methoxy ion to γ-carbon leading to γ-adduct **2.6** in 88% yield.



Scheme 2.1. Examples of nucleophilic addition to allenes.

This discovery was the starting point for the studies carried out by Lu. Taking advantage of the generation of formal 1,3-dipoles by treatment of allenes with phosphines, Lu and collaborators described for the first time the formal [3+2] cycloaddition reaction of allenoates with electron-poor alkenes.[7] As represented in Scheme 2.2, this approach allowed the synthesis of cyclopentene derivatives **2.9** and **2.10** from the reaction of allenoate **2.7** with acrylates, methyl vinyl ketone or acrylonitrile. This methodology leads to the synthesis of two regioisomers as consequence either of an α- or γ-attack. To better understand the formation of both regioisomers, the proposed mechanism is outlined in Scheme 2.2. The first step comprises the generation of a zwitterionic intermediate **2.8** from the nucleophilic addition of the phosphine to the β-carbon of allenoate. In this intermediate the positive charge is located on the phosphorus atom, while the negative charge is located, essentially on the α- or γ-carbon atoms of the allyl moiety. Consequently, the allyl moiety can act as nucleophile and react with electron-poor alkenes through a nucleophilic attack either from the α- or γ-carbon, leading

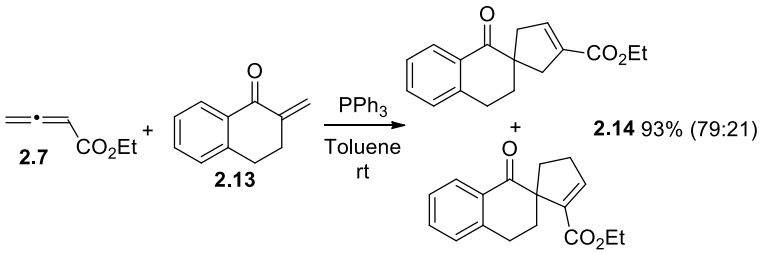
to intermediates **2.11** after a stepwise cycloaddition. Subsequent proton transfer and the elimination of the phosphine afford the expected cycloadducts. Despite the formation of both regioisomers, according to Lu and collaborators, α-adduct **2.9** is the main product of this reaction. This can be explained by a higher stability provided by allenes containing electron-withdrawing groups, making a nucleophilic attack from α-carbon more favorable than by γ-carbon.



Scheme 2.2. Mechanism of phosphine-catalyzed formal [3+2] cycloaddition of allenoates with electron-poor alkenes.

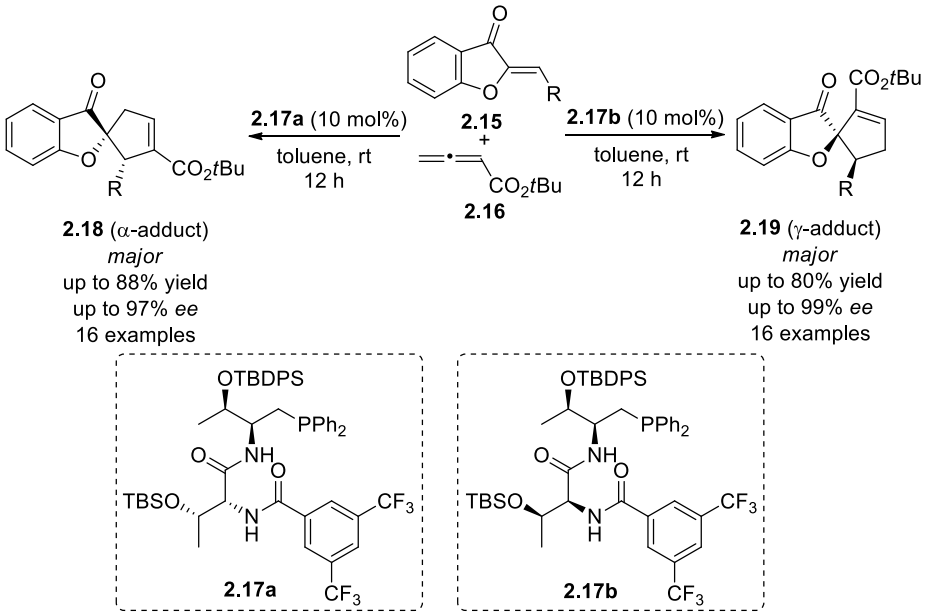
Since these pioneer studies, this strategy has been widely employed for the synthesis of a wide range of carbo- or heterocyclic compounds.[8] It should also be noted the high number of reports containing chiral phosphines or chiral substrates, that allows the enantio- or diastereoselective synthesis of these five-membered adducts, respectively.[9]

Yu and co-workers have described for the first time the formal [3+2] cycloaddition between an allenoate (**2.7**) and an exocyclic carbon-carbon double bond (**2.13**) as an efficient methodology for the synthesis of spirocyclic compounds (Scheme 2.3).[10]



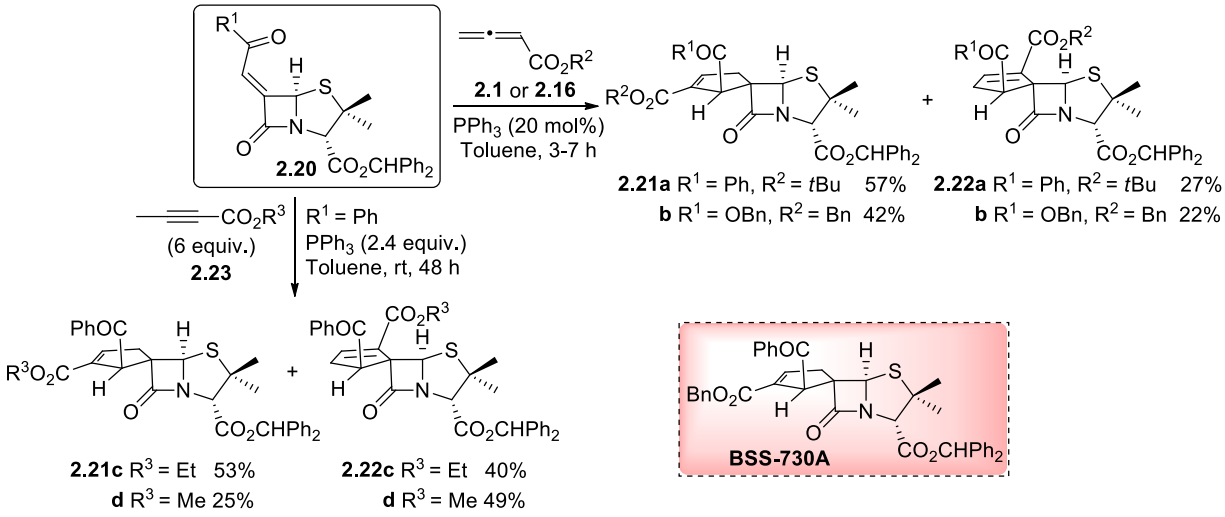
Scheme 2.3. Formal [3+2] cycloaddition between allenoate **2.7** and alkene **2.13**.

After this discovery, the available bibliography contains a panoply of papers reporting the synthesis of spirocyclic compounds applying this methodology.[8b,c, 9c, 11] One representative example of this strategy is the synthesis of spirocyclic benzofuranones carried by Ni *et al.* (Scheme 2.4).[12] Notably, in this work, the authors also have explored a stereo- and regioselective annulation approach, by changing the chirality of the phosphine catalyst. The reaction of aurone **2.15** with allenoate **2.16** catalyzed by a *L-D*-phosphine (**2.17a**) led to spirobenzofuranones **2.18** (α-adducts) as major products, in isolated yields up to 88% with enantiomeric excesses ranging from 91% to 97%. On the other hand, carrying out the reaction under the same conditions but with a *L-L*-phosphine catalyst (**2.17b**) afforded γ-adducts **2.19** in similar yields with enantiomeric excesses up to 99%. Despite the crucial role of the chiral catalysts **2.17** in the asymmetric synthesis of spirobenzofuranones **2.18** and **2.19**, they are also important in the induction of regioselectivity due to their conformation, which favors the formation of a specific transition state leading to specific regioisomers.



Scheme 2.4. Asymmetric synthesis of spirobenzofuranones.

As previously described in Chapter 1 (see Scheme 1.4), the phosphine-catalyzed [3+2] annulation of allenoates with 6-alkylidenepenicillanates was explored by our research group, leading to the discovery of three chiral spirocyclopentene-β-lactams with potent dual antimicrobial activity against both HIV and *Plasmodium*.[13] Recently, aiming the synthesis of novel bioactive molecules, our research group has focused its efforts on the structural modulation of the previously identified lead compound BSS-730A (Scheme 2.5).[14] Novel derivatives **2.21a**,**b** and **2.22a**,**b**, were obtained via phosphine-catalyzed [3+2] annulation between allenoates **2.1** or **2.16** and 6-alkylidenepenicillanates **2.20**. A different approach was explored for the synthesis of derivatives containing short-alkyl chains, due to the volatility of the corresponding allenoates. Hence, the reaction of 2-butynoates **2.23**, as 1,3-dipole precursors, in the phosphane-catalyzed [3+2] annulation reaction with 6-alkylidenepenicillanate **2.20a** was carried out, leading to the expected spirocyclic adducts in good overall yields. Further reactions focused on the elimination of the Michael acceptor system in the cyclopentene ring and the conversion of the benzhydryl ester to the corresponding free acid were also performed.



Scheme 2.5. Synthesis of spirocyclopentene-β-lactams via phosphane-catalyzed [3+2] annulation reactions.

Among these novel derivatives, two of them (**2.21c** and **2.21d**) should be highlighted due to their promising biological activity (Figure 2.2). Both compounds exhibited sub-micromolar IC_{50} values against HIV-1 (0.305 μM and 0.058 μM, respectively), and micromolar IC_{50} values against *Plasmodium berghei* hepatic stage (1.924 μM and 2.289 μM, respectively) and *Plasmodium falciparum* blood stage (0.844 μM and 1.255 μM, respectively). These results emphasize the high potential of this class of spirocyclopentene-β-lactams as therapeutic agents with dual antimicrobial activity.

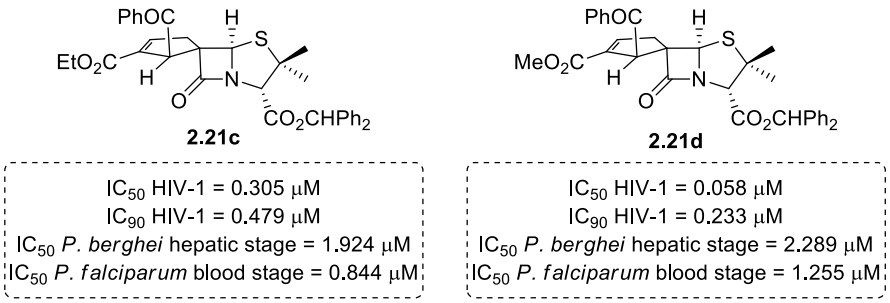


Figure 2.2. Anti-HIV and anti-*Plasmodium* activity of spiro-β-lactams **2.21c** and **2.21d**.

The structural modulations made until now allowed us to draw preliminary structure-activity relationships. Recent studies in our research group also shown that BSS-730A's benzhydryl ester group appear to play a crucial role for the molecule bioactivity, as its removal led to inactive derivatives. On the other hand, small alkyl esters (*e.g.* methyl and ethyl ester) in position 2' of BSS-730A spirocyclopentene ring led to highly bioactive compounds. A similar behaviour was observed when the benzoyl group of BSS-730A was replaced by a methyl ester (BSS-722A) (see Chapter 1, Table 1.2). However, a benzyl group at position 1' led to a total loss of activity. To this point, it is possible to conclude that position 1' and 2' of the cyclopentene ring present in the spirocyclic core of BSS-730A are suitable for structural modulation, without significant loss of biological activity.

The work in the present chapter comprises a step forward on the development of a new class of antimicrobials, by extending the strategy of rational structural modulation of the lead molecule BSS-730A to the synthesis of novel spirocyclopenicillanates. This strategy comprises the individual or cumulative modulation of position 1' (alkylidene moiety) and 2' (allenoate moiety) of BSS-730A spirocyclopentene ring. As consequence of the performed structural modulations it is our goal to gather a significant amount of information regarding the present class of bioactive compounds structure-activity relationship.

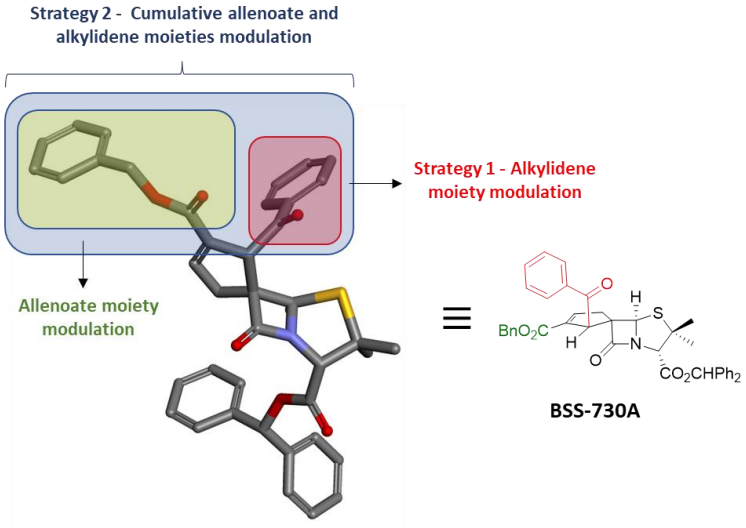
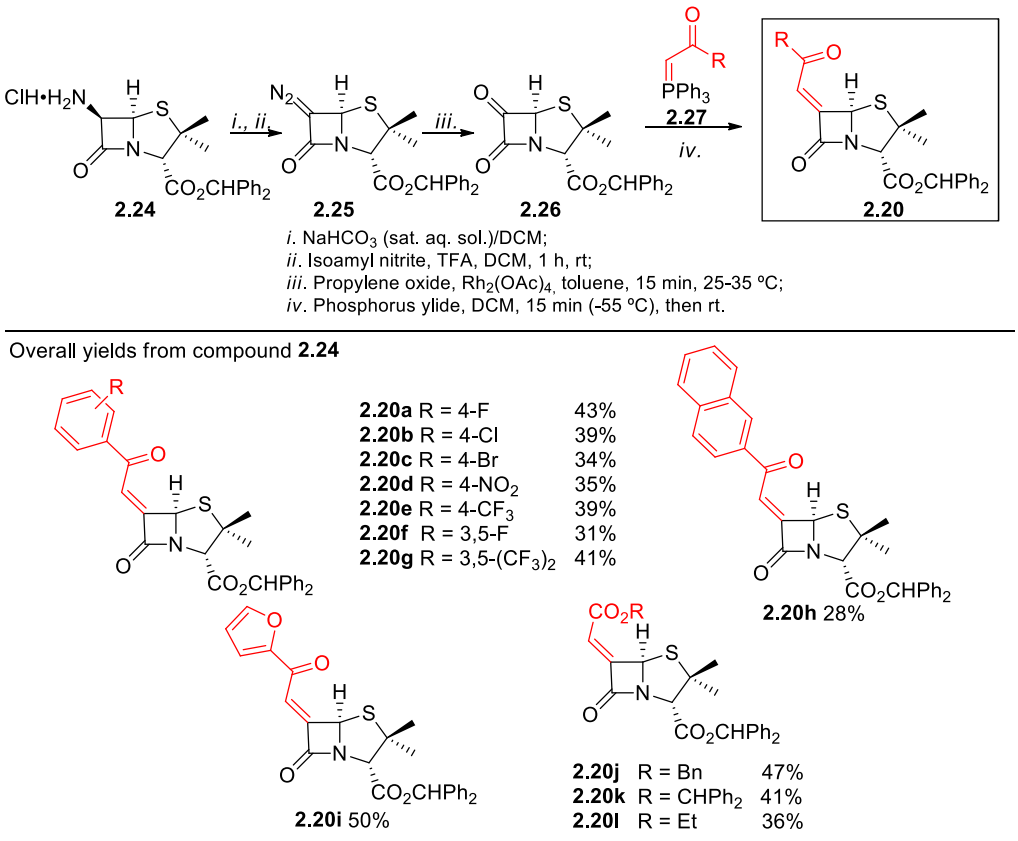


Figure 2.2. Designed structural modulation of lead molecule BSS-730A.

2.2. Synthesis of Spiro-β-Lactams via Phosphine-Catalyzed [3+2] Annulation of 6-Alkylidenepenicillanates and Allenoates

We started the experimental work with the synthesis of 6-alkylidenepenicillanates **2.20** which were the formal dipolarophiles used in all annulation/cycloaddition reactions carried out in the present dissertation. 6-Alkylidenepenicillanates **2.20** were prepared and isolated by a known procedure, having 6-aminopenicillanic acid (6-APA) as starting material, a well-known raw material used for the synthesis of compounds containing the penicillanic core (Scheme 2.6).[13a] This synthetic strategy involves the synthesis of 6-diazopenicillanate **2.25** from benzhydryl 6-β-aminopenicillanate hydrochloride salt **2.24**, obtained via a multistep strategy from 6-APA. A rhodium catalyzed oxidation of 6-diazopenicillanate **2.25** in the presence of propylene oxide led to 6-oxopenicillanate **2.26**. Subsequently, a Wittig reaction of the β-lactam **2.26** with the appropriate phosphorus ylide (**2.27**) afforded the expected 6-alkylidenepenicillanates **2.20** in overall yields ranging from 28% to 50% (starting from 6-β-aminopenicillanate **2.24**).



Scheme 2.6. Synthesis of 6-alkylidenepenicillanates from benzhydryl 6-β-aminopenicillanate hydrochloride.

The molecular structure of alkylidene-β-lactam **2.20h** was determined by single-crystal X-ray diffraction, with its molecular structure represented in Figure 2.3 as an ORTEP3 diagram. Compound **2.20h** crystallized as colourless plates, in the orthorhombic system, $P2_12_12_1$ space group, with one molecule in the symmetric unit. The dihedral angle between the two fused rings in the β-lactam is 48.6(3) º. The absolute structure was determined by a Flack analysis (943 Friedel pairs, η=0.06(8)) that assigns the *S,R* configuration to the chiral centers C3 and C5, respectively. This study unambiguously proves the geometry of carbon-carbon double bond C6-C24 of alkylidene-β-lactam **2.20h** as being the *Z* isomer. The X-ray crystallography studies of compound **2.20h** and the NMR data of all alkylidene-β-lactams, allowed to establish that the Wittig reaction of **2.26** led to the selective synthesis of alkylidene-β-lactams **2.20** with *Z* geometry.

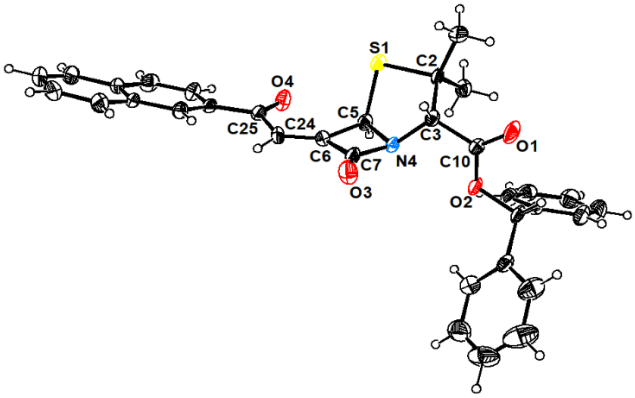
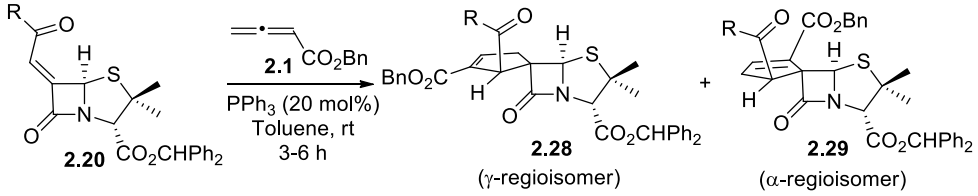


Figure 2.3. ORTEP representation of compound **2.20h**, using 30% level ellipsoids. Selected bond lengths: S1–C2 1.847(5) Å, C2–C3 1.562(7) Å, C3–N4 1.469(6) Å, N4–C7 1.408(7) Å, N4–C5 1.484(6) Å, C5–C6 1.508(8) Å, C6–C7 1.498(8) Å, C7–O3 1.192(7) Å, S1–C5 1.811(6) Å. Selected bond angles: C5–S1–C2 90.3(3)º, C5–N4–C7 93.0(4)º, C7–N4–C3 122.7(5)º, N4–C5–C6 87.3(4)º.

After the synthesis of 6-alkylidenepenicillanates **2.20**, we focused on the exclusive structural modulation at position 1' of the cyclopentene ring of our lead molecule BSS-730A. The synthesis of the new derivatives was achieved by exploring the phosphine-catalysed formal [3+2] cycloaddition reaction of a library of 6-alkylidenepenicillanates **2.20** with benzyl allenoate (**2.1**) (Table 2.1). By carrying out the cycloaddition reactions in the presence of triphenylphosphine at room temperature for a few hours, the expected cycloadducts **2.28** and **2.29** were obtained in excellent overall yields, ranging from 66%

to 97%. The formation of α- (**2.29**) and γ-regioisomers (**2.28**) depend on the initial attack of the dipole, either by the α- or the γ-carbon, to carbon C-6 of the β-lactam. It is noteworthy that due to the inherent chirality of the penicillin-derived alkylidenes and its "butterfly"-like conformation, the approach of the formal 1,3-dipole to the 6-alkylidenepenicillanate occurs through its less hindered α-side, affording the spirocyclopentene-β-lactams in a diastereoselective manner.

Table 2.1. Structural modulation strategy focused on the exclusive modulation of the position 1' of the lead molecule cyclopentene ring.



Entry	**2.20**	R	Products, Isolated yields	
1	**2.20a**	*p*-FC_6H_4	**2.28a**, 47%	**2.29a**, 49%
2	**2.20b**	*p*-ClC_6H_4	**2.28b**, 50%	**2.29b**, 45%
3	**2.20c**	*p*-BrC_6H_4	**2.28c**, 46%	**2.29c**, 39%
4	**2.20d**	*p*-$NO_2C_6H_4$	**2.28d**, 52%	**2.29d**, 29%
5	**2.20e**	*p*-$CF_3C_6H_4$	**2.28e**, 52%	**2.29e**, 35%
6	**2.20f**	3,5-FC_6H_3	**2.28f**, 53%	**2.29f**, 44%
7	**2.20g**	3,5-$(CF_3)_2C_6H_3$	**2.28g:13g** 66% (59:41)[a]	
8	**2.20h**	2-naphthyl	**2.28h**, 42%	**2.29h**, 43%
9	**2.20i**	2-furyl	**2.28i**, 33%	**2.29i**, 55%
10	**2.20j**	$OCHPh_2$	**2.28j: 2.29j** 94% (50:50)[a]	

[a] Ratio determined by ^1H NMR

Unfortunately, spiro-lactams **2.28g**/**2.29g** and **2.28j**/**2.29j** could not be separated by flash chromatography and were obtained as an inseparable mixture of regioisomers, in 59:41 and 50:50 ratios, respectively. Interestingly, when compared to the modulation at the position 2' of the cyclopentene ring, this modulation at the position 1' led to a distinct regioselectivity, with γ-regioisomer being obtained as major product in various

annulation reactions. Given that the modulation of the allenoate substituent does not have a clear impact on the regioselectivity, being α-regioisomers the major products, it is plausible to propose that the introduction of electron-withdrawing substituents at the aromatic ring of the benzoyl group plays a crucial role in the observed regioselectivity leading to γ-regioisomers as major adducts. This trend was more pronounced starting from alkylidenes bearing strong electron-withdrawing substituents at the aromatic ring of the benzoyl group [*e.g.* *p*-NO_2, *p*-CF_3 and 3,5-$(CF_3)_2$], indicating that such aromatic substituents may act as regioselectivity inducers within this specific reactivity context.

Having in mind previously results obtained in our research group from the exclusive modulation at position 2' of the cyclopentene ring, a second modulation was carried out. Thus, a strategy aiming at the simultaneous structural modulation on both positions 1' and 2' of the BSS-730A cyclopentene ring was designed, combining the substituents present at positions 1' and 2' of the derivatives showing higher anti-HIV-1 activity (see Figure 2.4 and Chapter 2.3).[15]

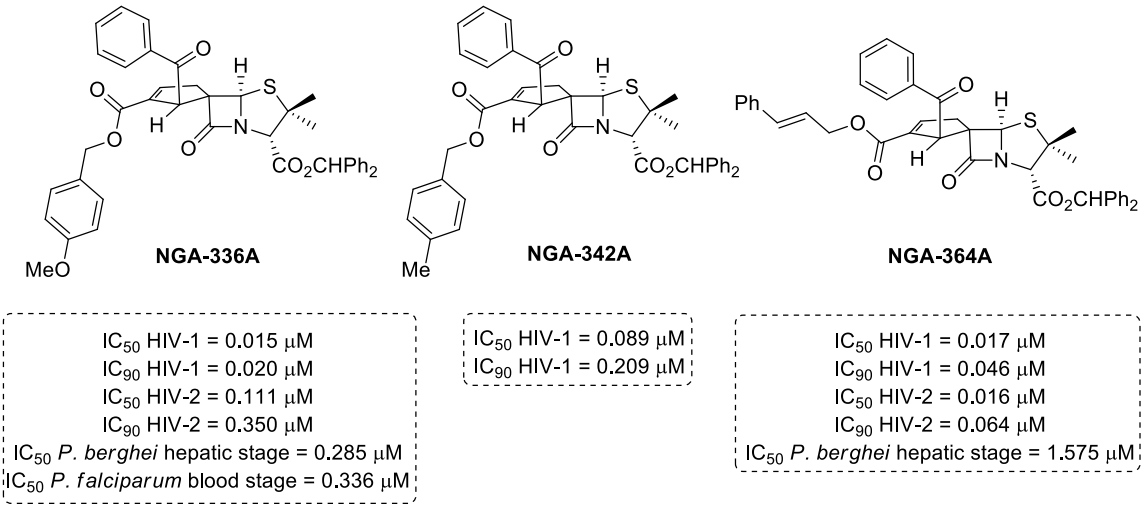
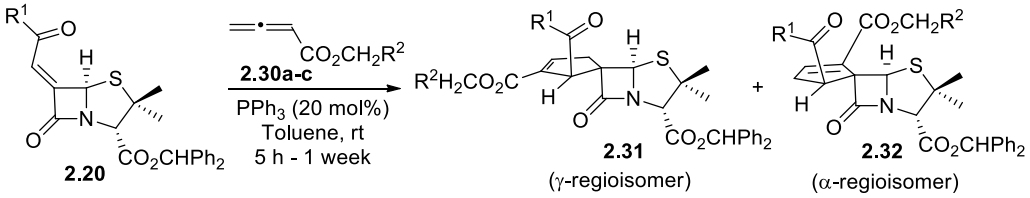


Figure 2.4. Most promising spiro-β-lactams resulting from the structural modulation at position 2' of lead molecule BSS-730A.[15]

The phosphine-catalysed formal [3+2] cycloaddition reactions of (*Z*)-benzoyl-substituted alkylidenepenicillanates **2.20a-c** with allenoates **2.30a-c** were carried out using, as well, 20 mol% of PPh_3 in toluene at room temperature (Table 2.2). However, it should be noted that some annulation reactions were carried out using a mixture of

allenoate with the corresponding 2-butynoate isomer. Nevertheless, this does not affect our reactions because it is known that the treatment of 2-butynoates with triphenylphosphine is an alternative approach to the use of allenoates as 1,3-dipole precursors.[7, 16] These annulation reactions afforded the corresponding spirocyclic adducts **2.31** and **2.32**. Unfortunately, (*E*)-cinnamyl ester derivatives were obtained in low overall yields (up to 33%). On the other hand, moderate to good overall yields were observed for *p*-methoxybenzyl and *p*-methylbenzyl derivatives (up to 78%). It is noteworthy that in comparison with the previously mentioned reactions (Table 2.1), longer reaction times were required up to one week.

Table 2.2. Structural modulation strategy focused on the exclusive modulation of both positions 1' and 2' of the lead molecule cyclopentene ring.



Entry	**2.20**	R^1	**2.30**	R^2	Products, Isolated yields	
1	**2.20a**	*p*-FC_6H_4	**2.30a**[a]	*p*-$MeOC_6H_4$	**2.31a**, 36%	**2.32a**, 39%
2	**2.20a**	*p*-FC_6H_4	**2.30b**	*p*-MeC_6H_4	**2.31b**, 23%	**2.32b**, 32%
3	**2.20a**	*p*-FC_6H_4	**2.30c**[a]	CH=CHPh	**2.31c**, 16%	**2.32c**, 13%
4	**2.20b**	*p*-ClC_6H_4	**2.30a**[a]	*p*-$MeOC_6H_4$	**2.31d**, 42%	**2.32d**, 36%
5	**2.20b**	*p*-ClC_6H_4	**2.30b**	*p*-MeC_6H_4	**2.31e**, 22%	**2.32e**, 24%
6	**2.20b**	*p*-ClC_6H_4	**2.30c**[a]	CH=CHPh	**2.31f**, 16%	**2.32f**, 17%
7	**2.20c**	*p*-BrC_6H_4	**2.30a**[a]	*p*-$MeOC_6H_4$	**2.31g**, 42%	**2.32g**, 36%
8	**2.20c**	*p*-BrC_6H_4	**2.30b**	*p*-MeC_6H_4	**2.31h**, 25%	**2.32h**, 23%
9	**2.20c**	*p*-BrC_6H_4	**2.30c**[a]	CH=CHPh	**2.31i**, 14%	**2.32i**, 14%

[a] Allenoate used in a mixture with the respective 2-butynoate isomer.

2.3. Synthesis of Spiro-β-Lactams Under Continuous-Flow Conditions

Continuous flow chemistry is a term used to describe chemical reactions carried out in a continuous manner.[17] This approach started to be developed in the late 90's, to overcome the existing gap between going from a laboratory scale to an industrial scale.[18] and has been commonly implemented in the past few years due to its advantages. Figure 2.5 represents the five major advantages of flow chemistry which are: 1) Faster reactions; 2) Improved Safety; 3) High reproducibility; 4) Scalability; 5) Facile automation. Continuous flow technology also allows to carry out reactions in a greener and sustainable way, making flow systems extraordinary useful because it is possible to achieve a higher atom economy and reduce the quantities of solvent used.[19] Moreover, by using continuous flow it is possible to reduce the exposure to toxic chemicals and avoid handling with dangerous ones, and, consequently, minimizing the potential of accidents.

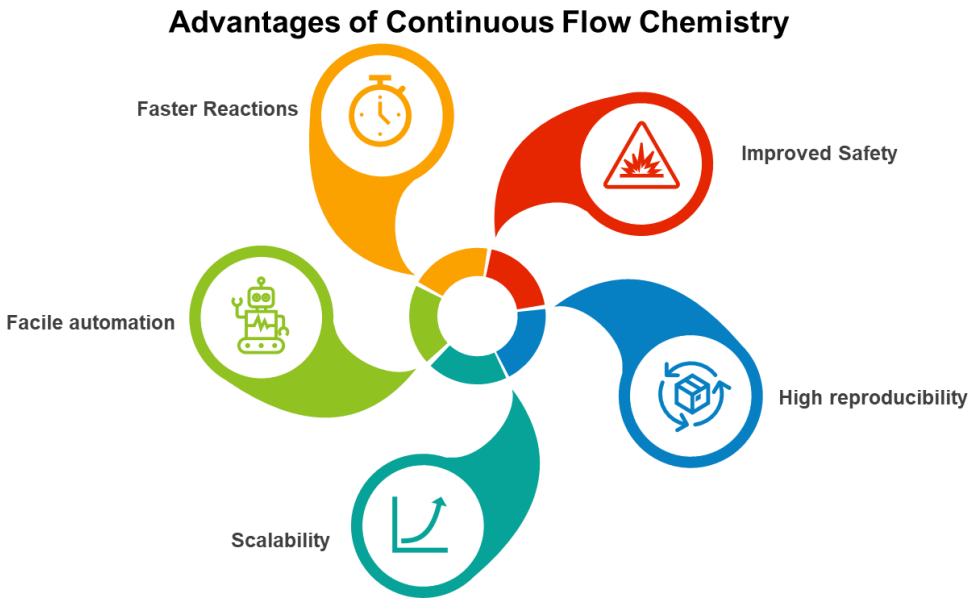


Figure 2.5. Advantages of continuous flow chemistry.

Continuous flow apparatus enables the construction of different systems using the same equipment, by coupling several and distinct reactors (*e.g.* coil reactor, microchips, packed-bed column, among others) in any sequence. This characteristic extends the versality of this methodology allowing to perform multistep reactions.[20] The development of multistep transformations is highly important as it simplifies the synthetic process of a

certain target, and there is no need to isolate or purify intermediates. As consequence, continuous flow chemistry has improved the synthesis of complex molecules such as pharmaceuticals[21] and natural products[22]. In Figure 2.6 it is possible to get an idea about the synthetic rate (per hour) of two active pharmacological ingredients obtained through multistep reaction using continuous flow chemistry. Furthermore, it should be noted that some intermediates can be obtained in kilogram-scale (per hour).

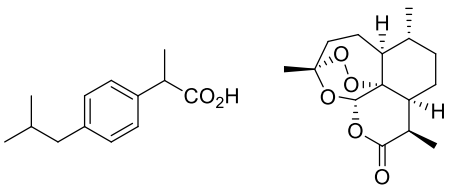


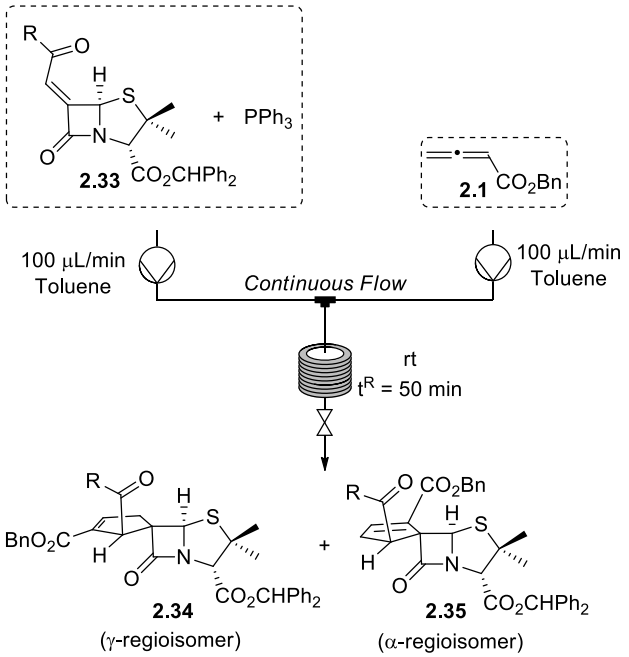
Figure 2.6. Large-scale production of API's under continuous flow.

In this context, in a work developed together with a MSc. student, we put our efforts on the development of a continuous flow approach for the phosphine-catalysed [3+2] annulation of allenoates with alkenes. The reaction conditions were firstly optimized with simple alkenes (*e.g.* methyl vinyl ketone, *N*-substituted maleimides). Then, the [3+2] annulation reaction was extended to more complex alkenes, namely to 6-alkylidenepenicillanates **2.33**, whose reaction with allenoates in the presence of triphenylphosphine was previously described, under batch conditions, leading to spiro-β-lactams with potent antimicrobial properties.[13a]

The formal [3+2] cycloaddition reaction of 6-alkylidenepenicillanates **2.33** with allenoate **2.1** under flow conditions led to the expected spiro-β-lactams in good yields (Table 2.5). The set-up was composed of two inlets, one containing a solution of the appropriate 6-alkylidenepenicillanate **2.33** and 20 mol% of triphenylphosphine, and another one containing a solution of allenoate **2.1**, with both solutions being mixed right before entering in a 10 mL coil reactor. Carrying out the annulation reactions under continuous flow did not lead to isolated yields so high as under batch conditions for the reactions with alkylidenes **2.33b**,**c** [entries 2 and 3 *vs.* batch conditions: **2.34b** (41%), **2.34c** (41%), **2.35c** (27%)], bearing an acetyl and methyl ester, respectively, we should

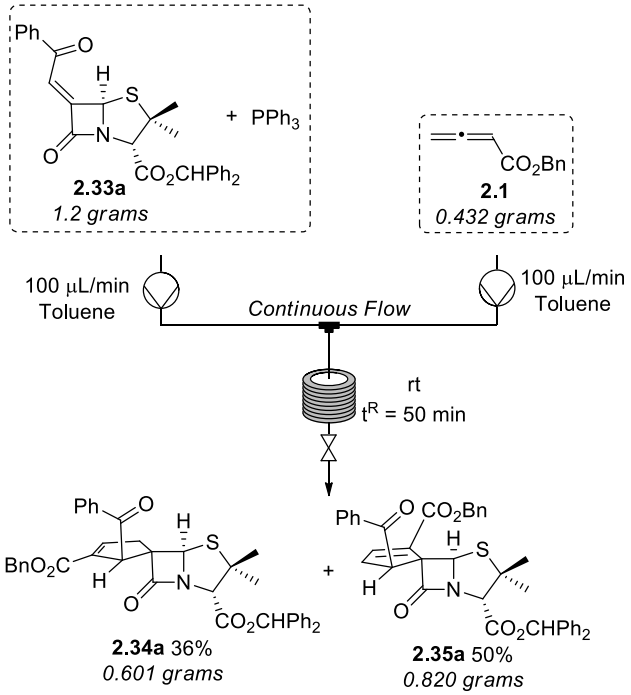
emphasize a similar overall yield obtained for the reaction of alkylidene **2.33a** towards allenoate **2.1** (entry 1 *vs.* batch conditions: 88% overall yield). In fact, the continuous flow conditions led to an excellent result, because it was possible to improve the yield of compound **2.34a** from 35% under batch conditions to 41% under flow conditions. This result is exceptionally important since compound **2.34a** is our lead molecule, BSS-730A. Moreover, it also should be noted that the reactions carried out under batch conditions took 5 hours, while the reactions carried out under flow conditions have a residence time, t^R, of 50 minutes.

Table 2.5. Continuous flow phosphane-catalyzed [3+2] annulation of allenoate **2.1** with 6-alkylidenepenicillanates **2.33**.



Entry	**2.33**	R	Products, Isolated yields	
1	**2.33a**	Ph	**2.34a**, 41%	**2.35a**, 43%
2	**2.33b**	Me	**2.34b**, 31%	**2.35b**, ---
3	**2.33c**	OMe	**2.34c**, 19%	**2.35c**, 34%

To prove the potential of flow chemistry in scale-up reactions, a gram-scale reaction was carried out (Scheme 2.7), wherein 1.2 g of alkylidene **2.33a** reacted with 0.432 g of allenoate **2.1** under the optimized conditions to give 0.601 g of **2.34a** in 36% yield together in **2.35a** in 50% yield (0.820 mg).



Scheme 2.7. Scale-up synthesis of **2.34a** (BSS-730A) under continuous flow.

The potential of the continuous flow approach should be highlighted because it proved to be a very efficient alternative to conventional methodology leading to the expected compounds in competitive yields and in a faster manner, opening avenues for scale-up reactions of biological active spiro-β-lactams.

2.4. Anti-HIV and Anti-*Plasmodium* Activity

2.4.1. Anti-HIV-1 and Anti-HIV-2 Activity

Initially, *in vitro* cytotoxicity assays in TZM-bl cells were performed for all synthesized spiro-β-lactams, a library of twenty-five compounds. None of the studied compounds showed cytotoxicity, with CC_{50} values ranging from 50.65 μM (**2.29c**) to 133.60 μM (**2.31d**) (Table 2.6). These results are in agreement with previous reports on the literature and demonstrate, once again, the low cytotoxic profile of the spirocyclopentene-β-lactams.[13b, 14, 23]

The antiviral activity of the selected twenty-five compounds was evaluated in a single round viral infectivity assay against an HIV-1 CXCR4 tropic isolate. The anti-HIV-1 activity of the two sets of molecules resulting from the two structural modulations will be presented and discussed individually.

The modulation of the position 1' of the lead molecule, afforded sixteen new spirocyclopentene-β-lactams. The antiviral activity of both γ- and α-regioisomers (**2.28** and **2.29**) was accessed. Among these novel compounds, seven γ-regioisomers (**2.28**) showed interesting anti-HIV activity, with MPI values at 25 μg/mL over 75%. The most active molecule of this set was spiro-β-lactam **2.28a** with an $IC_{50\ HIV\text{-}1}$ value of 0.019 μM. It is noteworthy that this molecule has an IC_{50} value comparable to the one presented by BSS-730A ($IC_{50\ HIV\text{-}1}$ = 0.014 μM). However, it was also possible to identify five other molecules (**2.28b-d**, **2.28f** and **2.28i**) with IC_{50} values under 0.200 μM. On the other hand, no anti-HIV activity was observed for α-regioisomers, a behaviour concordant with previously obtained data for similar spirocyclopentenepenicillanates.[13b]

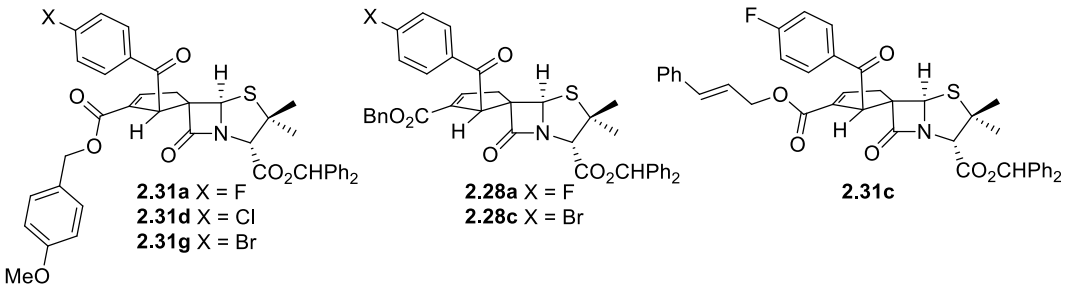
Notably, the last set of compounds (**2.31**) comprising the simultaneous structural modulation on both positions 1' and 2' of the BSS-730A cyclopentene ring led to enthusiastic results. All derivatives showed high inhibition values (MPI at 25μg/mL over 90%) and low $IC_{50\ HIV\text{-}1}$ values. Three molecules (**2.31a**, **2.31c** and **2.31d**) should be highlighted for their IC_{50} values under 0.020 μM. In fact, these molecules have similar or lower IC_{50} values than BSS-730A [**2.31a** ($IC_{50\ HIV\text{-}1}$ = 0.012 μM), **2.31c** ($IC_{50\ HIV\text{-}1}$ = 0.015 μM) and **2.31d** ($IC_{50\ HIV\text{-}1}$ = 0.015 μM)]. To our delight molecules **2.31c** and **2.31d** also showed lower IC_{90} values (0.018 μM and 0.019 μM, respectively) than BSS-730A ($IC_{90\ HIV\text{-}1}$ = 0.025 μM).

Table 2.6. Activity of spiro-β-lactams **2.28**, **2.29** and **2.31** against HIV-1.

Compound	CC_{50} (μM)	MPI (%) (25μg/ml)	IC_{50} (μM)	IC_{90} (μM)	Therapeutic Index (CC_{50}/IC_{50})
BSS-730A	76.84	99	0.014	0.025	5488.57
2.28a	58.14	93	0.019	0.054	3060.00
2.28b	55.99	92	0.063	0.104	888.73
2.28c	55.02	87	0.050	0.116	1100.40
2.28d	53.00	80	0.178	0.413	297.75
2.28e	64.40	76	1.304	1.504	49.39
2.28f	70.14	88	0.132	0.585	531.34
2.28h	73.63	0	n.d.	n.d.	n.d.
2.28i	78.30	87	0.140	0.223	559.29
2.29a	64.77	0	n.d.	n.d.	n.d.
2.29b	63.68	0	n.d.	n.d.	n.d.
2.29c	50.65	0	n.d.	n.d.	n.d.
2.29d	73.79	0	n.d.	n.d.	n.d.
2.29e	54.74	0	n.d.	n.d.	n.d.
2.29f	58.75	0	n.d.	n.d.	n.d.
2.29h	56.17	0	n.d.	n.d.	n.d.
2.29i	61.21	0	n.d.	n.d.	n.d.
2.31a	75.06	100	0.012	0.048	6255.00
2.31b	78.26	95	0.146	0.338	494.93
2.31c	77.61	100	0.015	0.018	5174.00
2.31d	133.60	100	0.015	0.019	8906.67
2.31e	100.10	94	0.211	0.720	474.41
2.31f	87.48	97	0.067	0.198	1305.67
2.31g	100.60	100	0.030	0.099	3353.33
2.31h	72.12	93	0.111	0.297	649.73
2.31i	66.87	95	0.143	0.305	467.62

To get further insight into the antiviral potential of our compounds, the six compounds with better anti-HIV-1 activity ($IC_{50\ HIV\text{-}1}$ < 50 nM) were evaluated against HIV-2 (Table 2.7). All compounds proved to be also very active against HIV-2 with an inhibition of 100% at 25μg/mL. In addition to the observed anti-HIV-1 activity, this set of compounds showed remarkable $IC_{50\ HIV\text{-}2}$ values, ranging from 0.011 to 0.093 μM. Spiro-β-lactams **2.28a** and **2.31a** were the most actives with $IC_{50\ HIV\text{-}2}$ values of 0.011 μM and 0.013 μM, respectively. It should also be highlighted that compound **2.28a** and compound **2.31a** showed lower IC_{90} values (0.055 μM and 0.051 μM, respectively) against HIV-2 virus than lead molecule BSS-730A ($IC_{90\ HIV\text{-}2}$ = 0.064 μM).

Table 2.7. Activity of selected spiro-β-lactams against HIV-2 isolates.



Compound	MPI (%) (25μg/ml)	IC_{50} (nM)	IC_{90} (nM)	Therapeutic Index (CC_{50}/IC_{50})
BSS-730A	99	0.008	0.064	9605.00
2.28a	100	0.011	0.055	5285.45
2.28c	100	0.093	0.159	591.61
2.31a	100	0.013	0.051	5773.85
2.31c	100	0.039	0.103	1990.00
2.31d	100	0.065	0.123	2055.38
2.31g	100	0.087	0.137	1156.32

2.4.2. Anti-*Plasmodium* Activity

2.4.2.1. Hepatic Stage of Infection

The anti-*Plasmodium* activity of the set of six spirocyclopentene-β-lactams with higher anti-HIV-1 activity was also determined. In a preliminary activity screen, the percentage of inhibition of hepatic infection by the rodent *P. berghei* parasite was determined for the entire set of compounds at 1 μM and 10 μM. The results showed that none of these compounds displayed cytotoxicity against the Huh-7 host cells, and that most of them displayed high inhibitory activity at 10 μM. However, compounds **2.28a** and **2.31a** showed inhibition values lower than 50% at 1 μM.

Given these encouraging results, the IC_{50} values of four spirocyclic derivatives against *P. berghei* hepatic infection were determined, with the studied compounds displaying micro to submicromolar activities, ranging from 0.750 μM to 2.593 μM (Table 2.8). Compound **2.31g** had the lowest activity with an $IC_{50\ P.\ berghei}$ of 2.593 μM, followed by compound **2.28d** ($IC_{50\ P.\ berghei}$ = 1.997 μM). Notably, two of the spirocyclopentene-β-lactams screened showed submicromolar activity against the parasite's hepatic stage of infection [**2.28a** ($IC_{50\ P.\ berghei}$ = 0.924 μM) and **2.31a** ($IC_{50\ P.\ berghei}$ = 0.750 μM)]. However, none of them was more potent than the lead molecule BSS-730A ($IC_{50\ P.\ berghei}$ = 0.550 μM).

Table 2.8. Compound activity against *P. berghei* hepatic stage.

Compound	$IC_{50\ P.\ berghei}$ μM
BSS-730A	0.550 ± 0.14
2.28a	0.924 ± 0.027
2.28c	Between 1 and 10
2.31a	0.750 ± 0.107
2.31c	Between 1 and 10
2.31d	1.997 ± 0.458
2.31g	2.593 ± 0.219

2.4.2.2. Blood Stage of Infection

Given their submicromolar activity against hepatic infection by *Plasmodium*, the activity of the two most promising compounds (**2.28a** and **2.31a**) against the erythrocytic stage of *P. falciparum* infection was evaluated (Table 2.9). Both spirocyclopentene-β-lactams showed remarkable activity against this phase of the parasite's life cycle. However, compound **2.28a** showed lower activity ($IC_{50\ P.\ falciparum}$ = 0.560 ± 0.126 μM) than the lead molecule BSS-730A (**3**) ($IC_{50\ P.\ falciparum}$ = 0.430 μM). An opposite behaviour was observed for compound **2.31a** which showed higher activity than BSS-730A against the blood stage of infection with an $IC_{50\ P.\ falciparum}$ value of 0.292 ± 0.062 μM.

Table 2.9. Compound activity against *P. falciparum* blood stage.

Compound	$IC_{50\ P.\ berghei}$ μM
BSS-730A	0.430 ± 0.04
2.28a	0.560 ± 0.126
2.31a	0.292 ± 0.062

2.4.3. Structure-Activity Relationships

2.4.3.1 Anti-HIV-1 and Anti-HIV-2 Activity

The synthesis and antiviral activity determination of twenty-five new BSS-730A's derivatives, allowed to draw structure-activity relationships regarding their ability to act as anti-HIV agents.

Through structural modulation of 6-alkylidenepenicillanates **2.20**, the addition of halogens and other substituents (*e.g. p*-NO_2 and *p*-CF_3) in the aromatic ring of the benzoyl group of BSS-730A was performed. The presence of these substituents has proven to be a successful strategy to obtain new BSS-730A derivatives with potent antiviral activity. Among compounds **2.28** it was not possible to establish a direct correlation between the electron-donating effect of the benzoyl group *para* substituent and the observed anti-HIV-1 activities. However, **2.28a-c** bearing weak withdrawing groups (R = *p*-F, *p*-Cl and *p*-Br) showed higher anti-HIV-1 activity than compounds with strong withdrawing

substituents [R = *p*-NO_2 (**2.28d**) and *p*-CF_3 (**2.28e**)]. Having in mind previous results concerning the modulation of the benzyl ester group, this result reveals that benzoyl group at cyclopentene's 1'position is more tolerant to the introduction of highly electron-withdrawing substituents than the benzyl ester group at cyclopentene's 2'position.

Although being 10 times less active than lead molecule BSS-730A, the furoyl-substituted derivative **2.28i** displays a remarkable submicromolar antiviral activity against HIV-1 (IC_{50} = 0.140 μM). This is an important outcome regarding spirocyclopentene-β-lactams structure-activity analysis, as it shows that cyclopentene position 1' can have its ketone group modulated with the introduction of heteroaromatic rings without causing an abrupt loss of antiviral activity.

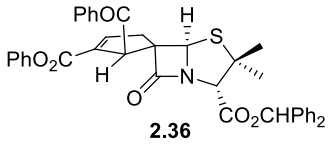
From the simultaneous structural modulation at positions 1' and 2' of the lead molecule, BSS-730A, cyclopentene ring, interesting conclusions can be drawn. It was possible to observe that the simultaneous introduction of *p*-halobenzoyl groups at position 1' together with the introduction of a *p*-methylbenzyl ester group at position 2' (see compounds **2.31b**, **2.31e**, **2.31h**) did not lead to an increase of antiviral activity when compared to the result of the same changes carried out individually (see compounds **NGA-342A**, **2.29a-c**).

On the other hand, the combination of a *p*-halobenzoyl group at cyclopentene's position 1' with a strong electron donating group (R = *p*-OMe) in the aromatic ring of the benzyl ester group at position 2', led to compounds as highly active as the lead molecule BSS-730A (**2.31a**, **2.31d**, **2.31g**) with outstanding IC_{50} values. The result presented by derivative **2.31c**, which contains a *p*-fluorobenzoyl group at position 1' and a cinnamyl ester group at position 2', corroborates the importance of adding conjugation to the phenyl group at position 2' to achieve high anti-HIV activity.

The qualitative structure-activity relationship results presently discussed, show that the presence of aromatic groups with electron-rich substituents in both positions 1' and 2' of the cyclopentene ring, are associated with enhanced anti-HIV-1 inhibition.

With these results, it was also possible to state the key role of a methylene group in the ester substituent on position 2' of the cyclopentene group (CO_2CH_2-R), because it is a common substructure of all the bioactive spirocyclopentene-β-lactams previously and presently identified. It is plausible that the methylene feature may be a critical structural feature for a potential interaction between the spirocyclopentene-β-lactams and its

putative biological target. This structure-activity relationship is also corroborated by the lack of antiviral activity verified for spirocyclopentene-β-lactam **2.36**, which molecular structure differs from lead molecule BSS-730A exclusively by the absence of the previously referred methylene group, since molecule **2.36** has a phenyl ester at position 2' instead of a benzyl ester (Figure 2.7).



2.36

Figure 2.7. Structure of spirocyclopentene-β-lactam **2.36**.

All the six spirocyclopentenepenicillanates evaluated as anti-HIV-2 agents displayed nanomolar activity against HIV-2. Comparing to what was observed for anti-HIV-1 activity, the presence of a *p*-methoxybenzyl ester group at position 2' of the cyclopentene spiro-fused ring does not have a similar impact as observed for anti-HIV-1 activity, leading to a slight decrease of the anti-HIV-2 activity, yet extraordinary, of those compounds (**2.31a**, **2.31d**, **2.31g**).

Additionally, the importance of a fluorine atom to enhance the anti-HIV-2 activity of the synthesized molecules is prominent. Three of the four molecules with higher anti-HIV-2 activity contain a *p*-fluorobenzoyl group at position 1' [**2.28a** ($IC_{50\ HIV\text{-}2}$ = 0.011 μM), **2.31a** ($IC_{50\ HIV\text{-}2}$ = 0.013 μM), **2.31c** ($IC_{50\ HIV\text{-}2}$ = 0.039 μM)]. It should be highlighted that fluorine atoms are well known bioisosteres of hydrogen atoms and its inclusion into a molecule is a commonly used strategy in drug design to improve drug-like features.[24] This rational drug design strategy has already proven valuable on the drug development of other β-lactamic core containing drugs, namely a series of orally active cholesterol absorption inhibitors.[25]

2.4.3.2. Anti-*Plasmodium* Activity

The analysis of the inhibitory activity of spirocyclopentene-β-lactams against *Plasmodium* hepatic infection indicates that the addition of a halogen to the aromatic ring of the benzoyl group at position 1' leads to a decrease in the activity against *Plasmodium* hepatic stages when compared to its counterparts without halogen substituents on that moiety.

On the other hand, recent results demonstrated that the inclusion of a *p*-methoxybenzyl ester group at position 2' of the lead molecule's cyclopentene ring led to a 2-fold increase on its hepatic stage anti-plasmodial activity [NGA-336A ($IC_{50\ P.\ berghei}$ = 0.285 μM) vs BSS-730A ($IC_{50\ P.\ berghei}$ = 0.550 μM). A positive effect of the *p*-methoxybenzyl ester group on the molecules' activity was also observed for *p*-fluorobenzoyl derivatives [**2.28a** ($IC_{50\ P.\ berghei}$ = 0.924 μM) *vs* **2.31a** ($IC_{50\ P.\ berghei}$ = 0.750 μM)], albeit with a decrease in their anti-*Plasmodium* activity.

Few structure-activity relationships can be drawn regarding the spirocyclopentene-β-lactams' anti-plasmodial activity during the blood stage of the parasite's life cycle. This structure-activity analysis focused exclusively on the two compounds assayed, **2.28a** and **2.31a**, as well as on the lead molecule, BSS-730A and on compound NGA-336A. It was observed that the presence of a *p*-MeO substituent on benzyl ester aromatic ring potentiates the anti-plasmodial activity [NGA-336A ($IC_{50\ P.\ falciparum}$ = 0.336 μM)] relative to that of BSS-730A ($IC_{50\ P.\ falciparum}$ = 0.430 μM). This effect is even more pronounced when combined with the presence of a *p*-fluorobenzoyl group at position 1' [**2.31a** ($IC_{50\ P.\ falciparum}$ = 0.292 μM)]. On the other hand, the absence of the *p*-methoxybenzyl ester at position 2' results in a slight decrease of the molecule anti-plasmodial activity, as can be observed for compound **2.28a** which only bears a *p*-fluorobenzoyl group at position 1' of the cyclopentene ring [**2.28a** ($IC_{50\ P.\ falciparum}$ = 0.560 μM)].

Notwithstanding the limited number of results, the qualitative structure-activity relationships identified later for the molecules employed throughout the present dissertation, represent an important achievement for the future rationale design of new spirocyclopentene-β-lactams with potential broad-spectrum antimicrobial activity.

2.5. Interaction Between Spiro-β-Lactams and BSA

Fluorescence spectroscopy is one of the most successful methods applied to study drug-protein interactions due to its high sensitivity.[26] This kind of studies can provide a wide range of information on drug-protein interactions, such as the quenching mechanisms, drug-protein binding affinity, and on the binding site of the protein.[26b, 27]

Plasma proteins play an important role in human physiology and are mainly produced by the liver.[28] They are responsible for some important functions such as: the regulation of blood's osmotic pressure, the clotting process.[29] Plasma proteins also help the immune system to fight some infections and they act as transport proteins, including transport of lipids, vitamins, and drugs.[30]

Despite the huge number of plasma proteins, just two of them are present in sufficient quantity to bind a wide variety of drugs, with such affinity that can cause a significant effect on drug action.[31] One of those proteins is albumin, the most abundant plasma protein playing a vital role in the transport of drug ligands.[30a, 32] Protein binding is important in drug therapy since affects pharmacokinetics and pharmacodynamics, and consequently, can improve or reduce a drug's performance.[31a, 33] This reversible binding can increase the solubility of hydrophobic drugs in plasma which may lead to a more efficient distribution.[33a] Moreover, drug-protein binding can lower their toxicity and prevent metabolic degradation of drugs increasing their half-life.[31b, 32d, 34]

In this work, we carried out fluorescence quenching studies to investigate the interaction between ten spiro-β-lactams with potent antiviral activity and bovine serum albumin (BSA), through quenching of the protein's fluorescence intensity by the ligand (Figure 2.8). BSA was selected for studying the interaction owing to its structural similarity to human serum albumin (HSA).[35]

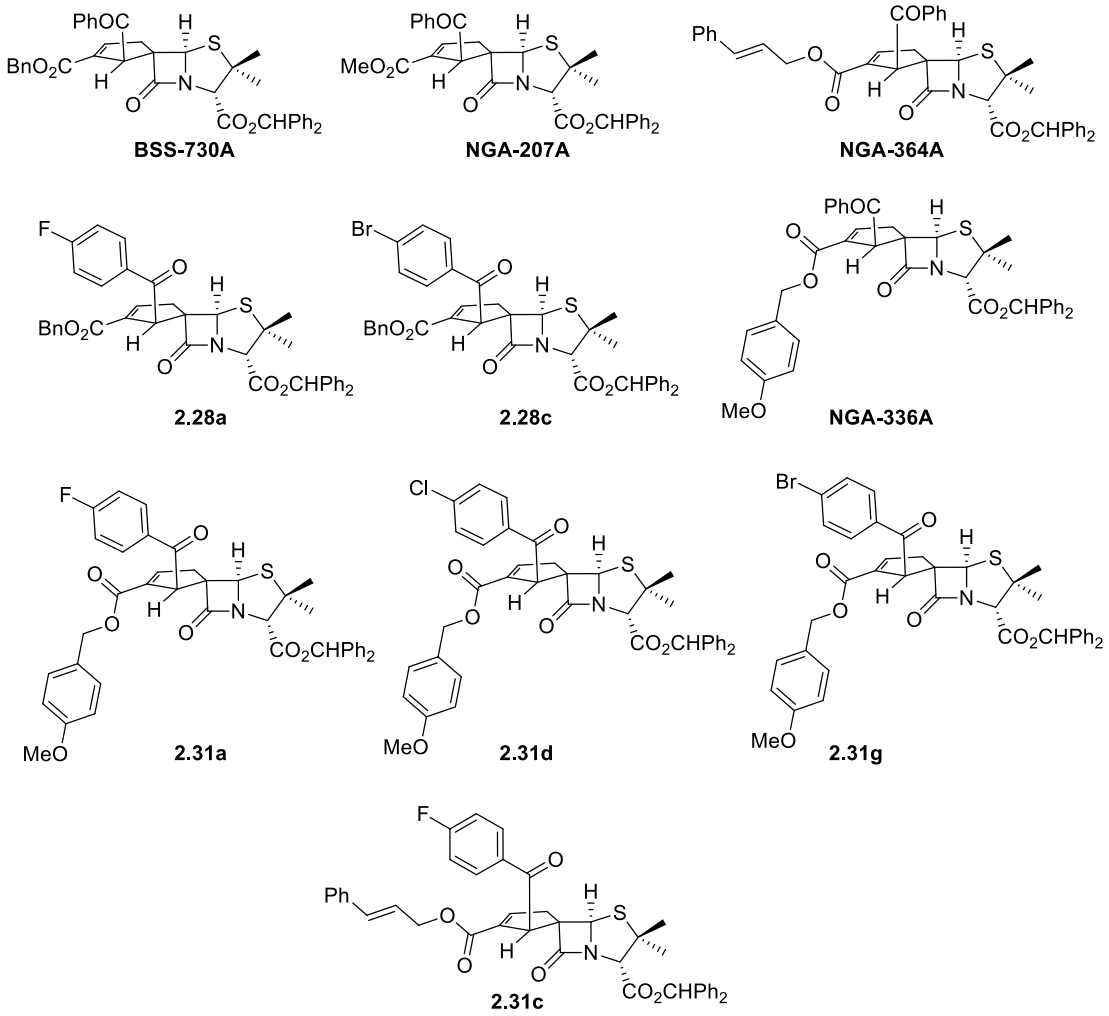


Figure 2.8. Selected spiro-β-lactams for fluorescence spectroscopy studies.

First, the BSA lifetime without the quencher was measured. The average fluorophore's lifetime changed between 6.03 ns at 25ºC to 5.46 ns at 37 ºC. Next, the fluorescence quenching studies were performed. The concentration of BSA was 1.5 μM and the concentrations of spiro-β-lactams varied from 0 to 150 μM. Our aim was to analyse the quenching of BSA fluorescence at 37 ºC, to mimic the corporal temperature, however, in order to verify if the quenching caused by spiro-β-lactams was static or dynamic, we carried out two assays at room temperature.

The quenching of BSA fluorescence at 25 ºC by BSS-730A and NGA-207A showed a correlation with the increase of ligands concentration. The Stern-Volmer plots for the quenching of BSA by the spiro-β-lactams showed a good linearity within the tested concentrations, indicating the existence of only one type of quenching mechanism. The

quenching constants deduced from the slope of the plots, using the Stern-Volmer equation (Equation 1), were 1.14×10^4 $Lmol^{-1}$ and 1.27×10^4 $Lmol^{-1}$ for NGA-207A and BSS-730A, respectively.

$$\frac{F_0}{F} = 1 + K_q \tau_0 [Q] = 1 + K_{SV}[Q]$$

Equation 2.1. where F_0 and F are the fluorescence intensities in the absence and presence of the quencher, K_q is the bimolecular quenching rate constant, τ_0 is the average lifetime of the fluorophore in the absence of the quencher, $[Q]$ is the concentration of the quencher and K_{SV} is the Stern-Volmer quenching constant. The fluorophore's lifetime without quencher is valued 6.03 ns and 5.46 ns at 25 ºC and 37 ºC, respectively.

Further fluorescence quenching studies were carried out for the set of 10 spiro-β-lactams (Figure 2.8) at 37 ºC, showing a similar effect with a decrease on the fluorescence intensity of BSA associated with an increase in the concentration of spiro-β-lactams.

At 37 ºC, the K_{SV} values varied from 9.97×10^3 $Lmol^{-1}$ to 2.41×10^4 $Lmol^{-1}$. For BSS-730A and NGA-207A the values were similar at both temperatures (see Table 2.10), showing a consistent quenching despite the variation of temperature, which agrees with a static quenching. Moreover, the bimolecular quenching constants (K_q) estimated using Equation 2.1 are much greater than the scattering collision quenching constant (2.0×10^{10} $Lmol^{-1}s^{-1}$) suggesting that the quenching is mainly due to the formation of ground state complex and not due to collision/dynamic processes which corroborates the existence of a static quenching mechanism.

The strength of the drug-protein interaction is measured in terms of their binding affinity, which can be expressed by a binding constant (K_a).[26b, 36] The molecules can interact with proteins binding sites independently, and the equilibrium between free and bound molecules is given by Equation 2.2. The K_a values ranged from 7.17×10^2 $Lmol^{-1}$ to 2.27×10^5 $Lmol^{-1}$ (Table 2.10). Moreover, for BSS-730A the binding constant increased with the temperature, indicating an increase of stability of the system at higher temperatures. The values for n were approximately equal to 1 in all the tested spiro-β-lactams, meaning that there is a single binding site in BSA for these ligands, leading to

1:1 adducts. Similar values of K_{SV} and K_a were reported in literature for well-known anti-HIV drugs (*e.g.*, abacavir, efavirenz and emtricitabine).[37]

Table 2.10. Stern-Volmer parameters (K_{SV} and K_q) and binding parameters (K_a and n) for spiro-β-lactams interaction with BSA.

Temperature	Compound	K_{SV} (L.mol^{-1})	K_q (L.M^{-1}.s^{-1})	K_a (L.mol^{-1})	n (BSA:drug)
25 ºC	**BSS-730A**	$1{,}27 \times 10^4$	2.10×10^{12}	4.60×10^3	0.90
	NGA-207A	$1{,}14 \times 10^4$	1.89×10^{12}	7.70×10^3	0.95
37 ºC	**BSS-730A**	$1{,}24 \times 10^4$	2.28×10^{12}	8.06×10^3	0.95
	NGA-207A	$1{,}20 \times 10^4$	2.20×10^{12}	4.27×10^3	0.89
	NGA-336A	$1{,}40 \times 10^4$	2.57×10^{12}	1.45×10^4	1.00
	NGA-364A	$1{,}75 \times 10^4$	3.21×10^{12}	1.41×10^3	0.76
	2.28a	$9{,}97 \times 10^3$	1.83×10^{12}	7.17×10^2	0.74
	2.28c	$2{,}19 \times 10^4$	4.01×10^{12}	2.27×10^5	1.23
	2.31a	$1{,}51 \times 10^4$	2.76×10^{12}	1.84×10^4	1.02
	2.31c	$2{,}09 \times 10^4$	3.82×10^{12}	1.05×10^4	0.94
	2.31d	$1{,}89 \times 10^4$	3.46×10^{12}	3.56×10^4	1.05
	2.31g	$2{,}41 \times 10^4$	4.41×10^{12}	7.96×10^4	1.11

$$\log\left[\frac{(F_0 - F)}{F}\right] = \log K_a + n\ \log[Q]$$

Equation 2.2. where K_a is the binding constant, and n is the number of binding sites. The values of K_a and n for the system were determined from the intercept and slope of log [(F_0–F)/F] vs. log [Q] plot and the results were in Table 1.

Serum albumins have two principal binding sites which appear to be similar both in BSA and in HSA: Sudlow site I and Sudlow site II, located in subdomains IIA and IIIA, respectively (Figure 2.9).[32a, 35, 38] The main differences between both sites are related to the size of the pocket, and to the interactions of the amino acid residues with the ligands. Sudlow site I has been characterized as larger and more flexible than Sudlow site

II. Therefore, due to the lack of flexibility of Sudlow site II, the ligands chirality might have a more prominent influence in the interactions with it.[39] In fact, the interactions between both sites and ligands are somewhat different. While in site I are mainly present hydrophobic interactions, site II comprises a combination of hydrophobic, hydrogen binding and electrostatic interactions. Those binding sites are also associated with specific drugs due to their affinity with a determined binding site. For example, Sudlow site I is also known as warfarin binding site, while Sudlow site II is known as ibuprofen binding site.[30a, 35, 38-39]

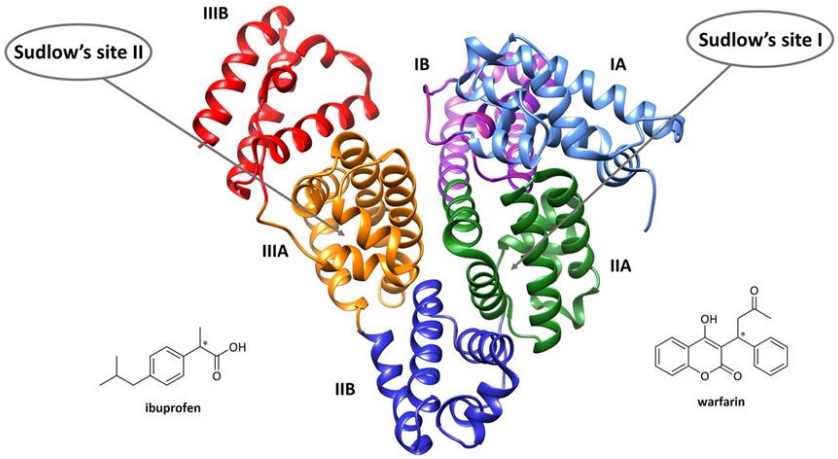


Figure 2.9. A ribbon structure representation of BSA identifying both Sudlow sites I and II and known markers for each site.

Having this in mind, fluorescence studies were carried out for mixtures of BSA with BSS-730A or **2.31a** in the presence and absence of warfarin and ibuprofen. The K_{SV}, K_q and K_a values were determined and are summarized in Table 2.11. The experimental results revealed that both BSS-730A-BSA and **2.31a**-BSA binding in presence of WAR resulted in a reduction in K_{SV} values as compared to BSS-730A-BSA only solutions. In the presence of IBU the Ksv values are similar to the ones without IBU. Therefore, it can be concluded that both BSS-730A and **2.31a** are competing only with WAR to bind at Sudlow site I. These results are in agreement with previous results described by Bocedi *et al.* which stated that anti-HIV agents just compete with WAR on Sudlow site I and were not able of affect Sudlow site II.[40] However, in order to corroborate the experimental results and for a better understanding of the interactions between our spiro-β-lactams and BSA, molecular docking studies should be carried out.

Table 2.11. Stern-Volmer parameters (K_{SV} and K_q) and binding parameters (K_a and n) for spiro-β-lactams interaction with BSA with and without the addition of site markers (WAR and IBU).

Compound	K_{SV} ($L.mol^{-1}$)	K_q ($L.M^{-1}.s^{-1}$)	K_a ($L.mol^{-1}$)	n (BSA:drug)
BSS-730A	$1{,}24 \times 10^4$	2.28×10^{12}	8.06×10^3	0.95
BSS-730A-WAR	1.01×10^4	1.84×10^{12}	1.63×10^3	0.83
BSS-730A-IBU	1.24×10^4	2.26×10^{12}	1.74×10^3	0.81
2.31a	1.51×10^4	2.76×10^{12}	1.84×10^4	1.02
2.31a-WAR	1.34×10^4	2.45×10^{12}	9.70×10^3	0.97
2.31a-IBU	1.38×10^4	2.53×10^{12}	1.95×10^4	1.04
Warfarin (WAR)	9.58×10^4	3.46×10^{12}	6.61×10^4	0.96
Ibuprofen (IBU)	1.17×10^4	3.82×10^{12}	3.04×10^3	0.88

2.6. Conclusions

In this chapter, the synthesis of twelve novel 6-alkylidenepenicillanates and a library of thirty-eight new spirocyclopentene-β-lactams were successfully achieved. The synthetic strategy was conceptualized through rational drug design and structure modulation of a highly promising lead molecule BSS-730A. Previous studies carried out in our research group explored the modulation at position 2' of cyclopentene ring. In this context, our strategy focused on the structural modulation of position 1', and a simultaneous modulation of both positions 1' and 2'.

In order to widen the structurally diversity, the modulated positions were endowed with different functional groups either by replacing the lead molecule substituents with varied aryl esters, cinnamyl esters, or by including substituents, mainly in *para* position, on the terminal aromatic rings of such positions.

All the screened molecules did not show relevant cytotoxicity against TZL-bl cell line, with CC_{50} values ranging from 50.65 μM to 133.60 μM. Among the set of assayed spirocyclopentene-β-lactams sixteen inhibited HIV-1 replication (MPI > 75%), with IC_{50} HIV-1 values as low as 0.012 μM. Among those, the six compounds with higher anti-HIV-1 activity (IC_{50} HIV-1 < 0.050 μM) was also active against HIV-2, with most active compound showing a remarkable IC_{50} HIV-2 value of 0.011 μM. It should be highlighted the presence of a *p*-fluorobenzoyl group at position 1' of the three molecules with higher anti-HIV-2 activity.

The most active spiropenicillanate against *Plasmodium* infectious hepatic stage have an IC_{50} *P. berghei* value of 0.750 μM, while the one which displayed the lower IC_{50} against the parasite blood stage showed an IC_{50} *P. falciparum* value of 0.292 μM.

Both antimicrobial results presented herein, represent an improvement of the activity profile with respect of lead molecule BSS-730A. Considering the high structural similarity between BSS-730A and its new bioactive analogues, it is likely that the new derivatives also share the lead molecule innovative antimicrobial host-based mechanism of action.

As consequence of the diversity of the screened compounds, it was also possible to gather a considerable amount of structure-activity information regarding the spirocyclopentene-β-lactams family of compounds, providing a strong background of

structural and pharmacophoric information for further rational design and synthesis of new potentially bioactive molecules.

The work described in this chapter also comprises fluorescence quenching studies which were carried out in order to investigate the interaction between some spiro-β-lactams and BSA. Similar values and interactions with Sudlow site I as those previously reported for well-known anti-HIV drugs were obtained.

The main conclusions that can be taken from previous work, concerning the development of novel spirocyclopentene-β-lactams as antimicrobial agents, and from this Chapter, which were above-mentioned, are resumed in Figure 2.10.

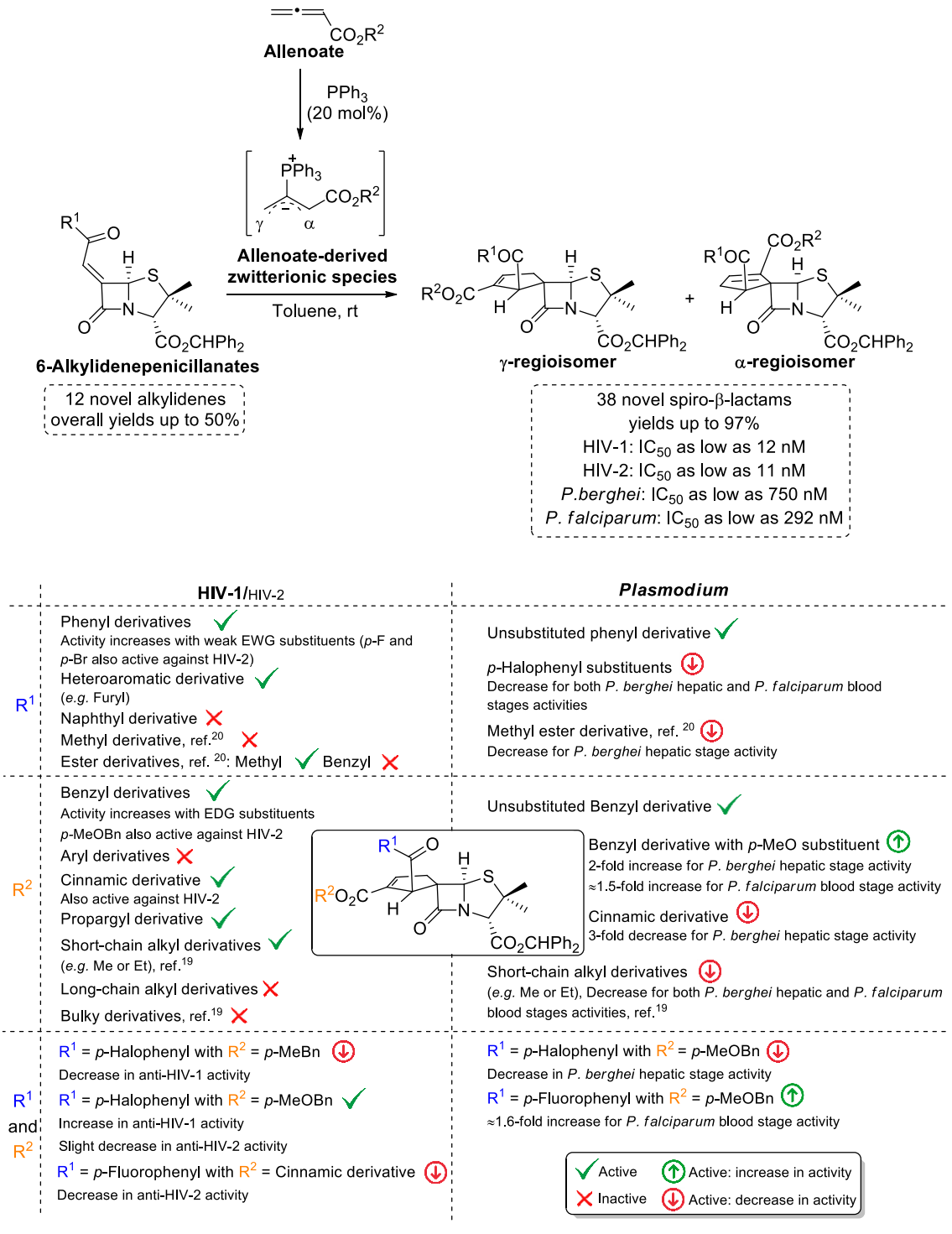


Figure 2.10. Overview of compounds synthesized in Chapter 2 with respective antimicrobial activity, and structure-activity relationships of spirocyclopentene-β-lactams class.

2.7. References

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CHAPTER 3

Synthesis of Novel Chiral Spiro-β-Lactams from 6-Alkylidenepenicillanates and Nitrones

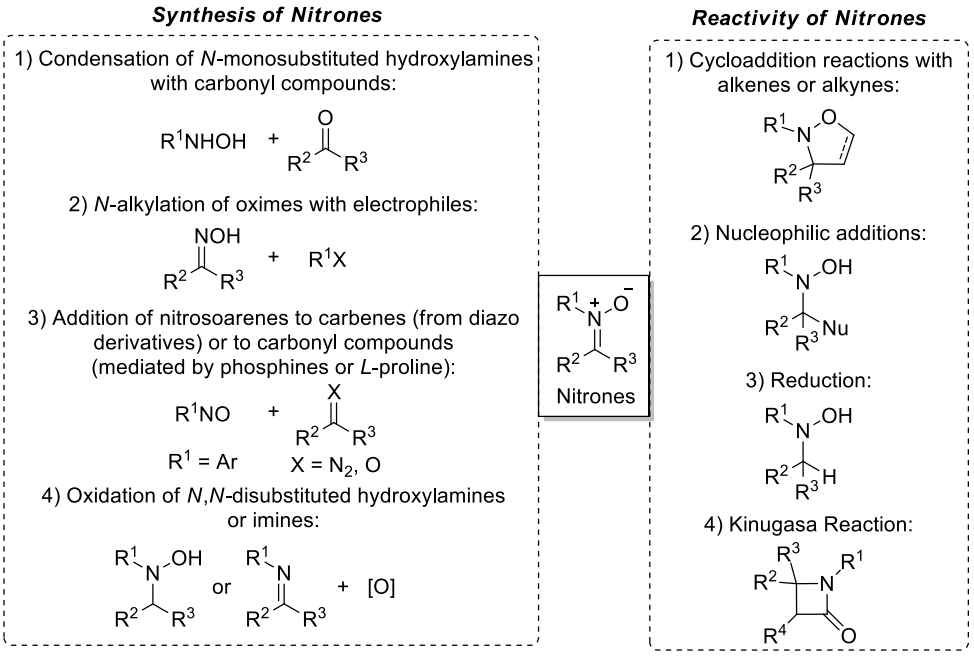
Abstract

This chapter describes the first examples of 1,3-dipolar cycloaddition reactions of 6-alkylidenepenicillanates with nitrones. This strategy allowed the synthesis of novel chiral spiroisoxazolidine-β-lactams incorporating the penicillanate nucleus, in a stereo- and diastereoselective manner with the creation of three new chiral centers. The antiviral activity of these novel derivatives is also reported.

Chapter 3 - Synthesis of Novel Chiral Spiro-β-Lactams from 6-Alkylidenepenicillanates and Nitrones

3.1. Introduction

Nitrones are a class of 1,3-dipoles highly used as building blocks in organic chemistry for the synthesis of nitrogen-containing compounds. The synthesis of nitrones can be achieved through a wide range of approaches from simple reagents under mild conditions. The most common methodologies for the synthesis of these key intermediates are represented in Scheme 3.1 and include: 1) Condensation reactions of *N*-monosubstituted hydroxylamines with carbonyl compounds (aldehydes and ketones); 2) *N*-alkylation of oximes with electrophiles; 3) Addition of nitrosoarenes to carbenes (generated from diazo derivatives) or to carbonyl compounds (mediated by phosphines or *L*-proline); 4) Oxidation reactions of *N*,*N*-disubstituted hydroxylamines or imines.[1]



Scheme 3.1. Overview on the synthesis and reactivity of nitrones.

The versatility of this structural motif allows it to participate in a wide range of transformations, making nitrones chemically rich scaffolds. Scheme 3.1 also presents an overview of the use of nitrones as scaffold in organic chemistry, highlighting some important transformations, namely cycloaddition reactions,[1d-f, 2] nucleophilic additions,[3] among others.[1a-c, 1g, 4] Another application of nitrones as starting material is the reaction

with terminal alkynes leading to β-lactams, an efficient methodology for the construction of the four-membered lactams known as the Kinugasa reaction.[1f, 2b, 5]

The 1,3-dipolar cycloaddition reaction of nitrones with dipolarophiles is one of the most studied reactions with nitrones. In fact, the reaction between nitrones and alkenes is a powerful tool for the synthesis of isoxazolidines, a class of five-membered saturated heterocyclic compounds, containing adjacent oxygen and nitrogen atoms.[6]

The isoxazolidine moiety plays an important role in organic chemistry and can provide access to a wide variety of compounds.[6-7] The most famous example is, perhaps, the reductive N-O bond cleavage leading to 1,3-aminoalcohols which can be achieved under mild conditions. However, isoxazolidines are also relevant scaffolds for the synthesis of oxazines, lactones and lactams. Isoxazolidines also have an outstanding importance in medicinal chemistry due to their biological properties.[2f, 6b] Another interesting features of isoxazolidines is being ribose mimetics, and precursors in the synthesis of nucleoside and nucleotide analogues, which are known for being biologically active against a panoply of pathologies.[8]

Chiral molecules are extremely important, since it is well-known that the 3D nature of a molecule has a high influence on its biological activity. Thus, it should be noted that isoxazolidines have a rich 3D structure, having the potential to have up to three contiguous stereogenic centers, whose can be created in a single step via cycloaddition reactions.

Bringing together all the above-mentioned properties of isoxazolidines and having in mind the advantages of the spirocyclic structural feature, the synthesis of spiroisoxazolidines derivatives have been explored leading to promising biologically active compounds. In fact, the spiroisoxazolidine is an important intermediate in the synthesis of natural occurring compounds and is the core structure of compounds with antibacterial, antitubercular and anticancer properties (Figure 3.1).[6b, 9] With exception of few reports, the synthesis of spiroisoxazolidines is always carried out via 1,3-dipolar cycloaddition of nitrones with alkenes.[6-7]

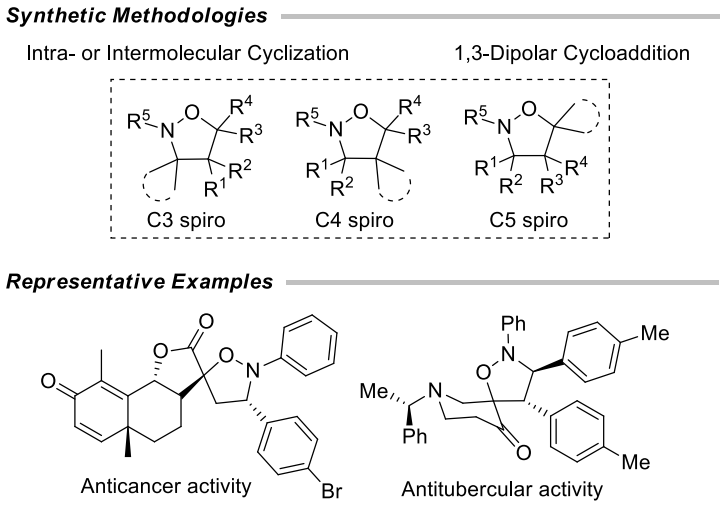
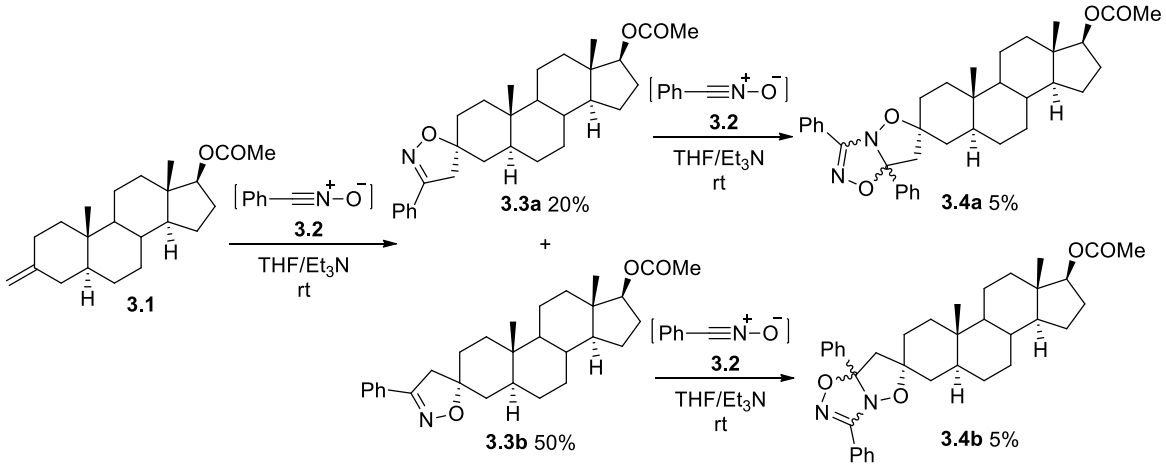


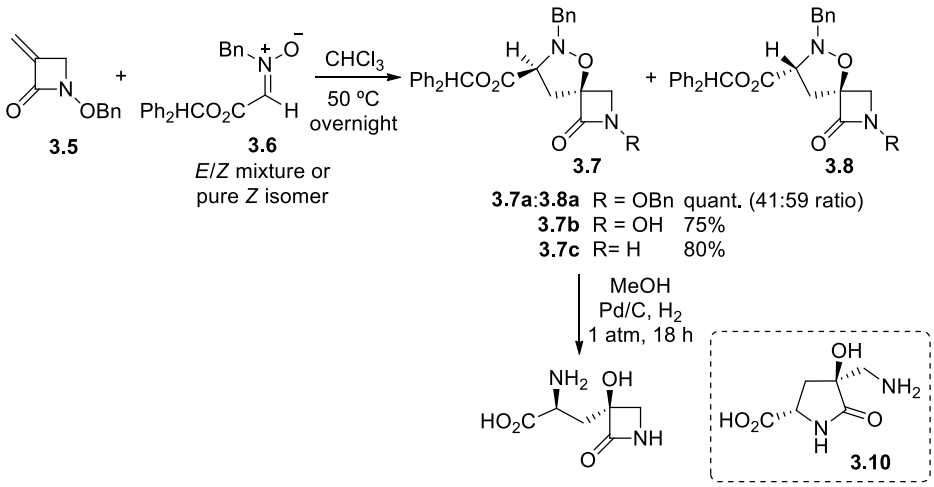
Figure 3.1. General structure of spiroisoxazolidines and representative examples with the corresponding bioactivity.

To the best of our knowledge, the first example describing the synthesis of a spiroisoxazolidine was conducted in 1978 by Colombi et *al.* (Scheme 3.2) through a 1,3-dipolar cycloaddition approach.[10] Interestingly, spiroisoxazolidine **3.4** was not synthesized from a reaction between a nitrone and an alkene, but from two successive reactions between a nitrile oxide and a C=C and C=N double bond. The reaction of steroid **3.1**, bearing an exocyclic double bond, and benzonitrile oxide (**3.2**) afforded the corresponding spiroisoxazolines **3.3** in 70% overall yield. Next, compounds **3.4** containing a spiroisoxazolidine-oxadiazoline moiety were obtained from the treatment of the freshly obtained **3.3** with benzonitrile oxide.



Scheme 3.2. Synthesis of the first spiroisoxazolidine.

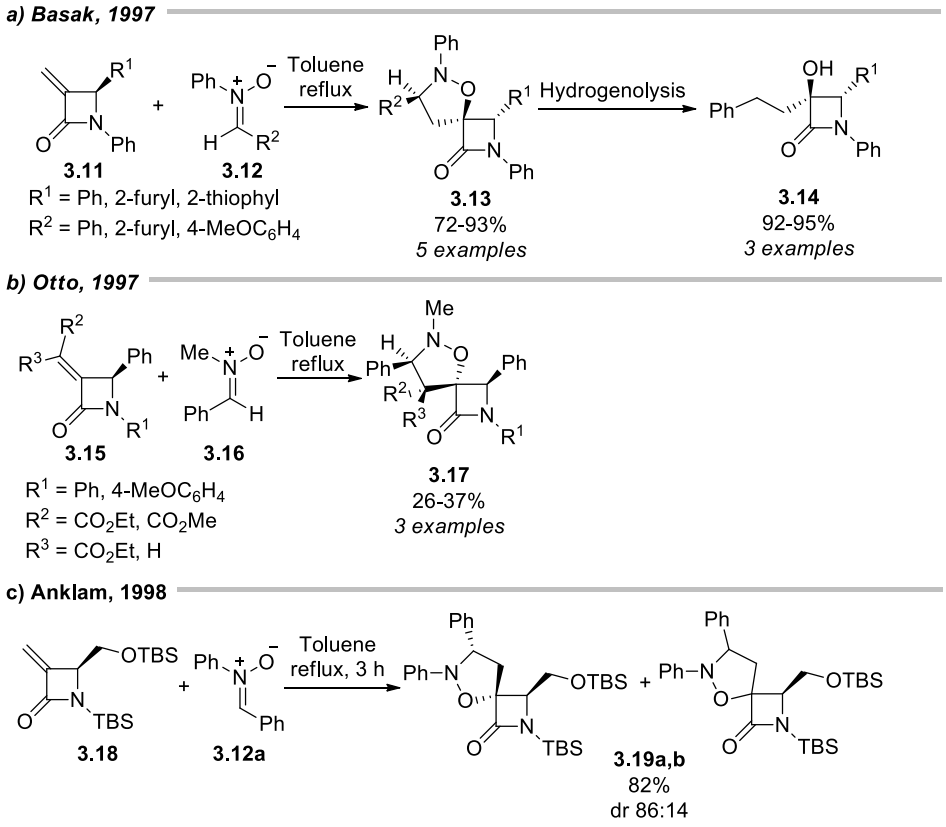
The first example of spiroisoxazolidine-β-lactams was described by Baldwin *et al.* in 1986.[11] Aiming the synthesis of tabtoxinine-β-lactam homologues with a short alkyl chain, the authors decided to explore the 1,3-dipolar cycloaddition reaction of an alkylidene-lactam with a nitrone, by the synthesis of spiroisoxazolidines and further ring-opening reaction leading to the corresponding amino-alcohol (Scheme 3.3). The reaction of alkylidene-lactam **3.5** with nitrone **3.6**, afforded the first spiroisoxazolidine-β-lactams (**3.7** and **3.8**) ever described in a regioselective fashion. Despite of being obtained in quantitative yields, the spirocyclic adducts were obtained as a mixture of stereoisomers **3.7a** and **3.8a**, in a 41:59 ratio, respectively. The authors also proved that the isolated yields are equal if starting from an *E*/*Z* mixture of nitrone **3.6** or from its pure *Z* isomer. Further transformations of compound **3.7a** to the *N*-hydroxy-β-lactam **3.7b** and β-lactam **3.7c** were carried out. Despite the evidence of the synthesis of the desired compound **3.9** by spectroscopic techniques, its tendency to lactamase led to the isolation of compound **3.10**.



Scheme 3.3. Synthesis of spiroisoxazolidine-β-lactams via 1,3-dipolar cycloaddition reactions.

In 1997, Basak *et al.* thought that adding a chiral center at position C-4 of the β-lactam ring could lead to stereoselective reactions (Scheme 3.4a).[12] In fact, the reactions between lactams **3.11** with nitrones **3.12**, proceeded with high regio- and diastereoselectivity, leading to spiroisoxazolidines **3.13** in yields ranging from 72 to 93%. This diastereoselectivity was explained by an approach of the nitrone from the opposite face of the C-4 substituent. Further hydrogenolysis reaction to obtain the respective

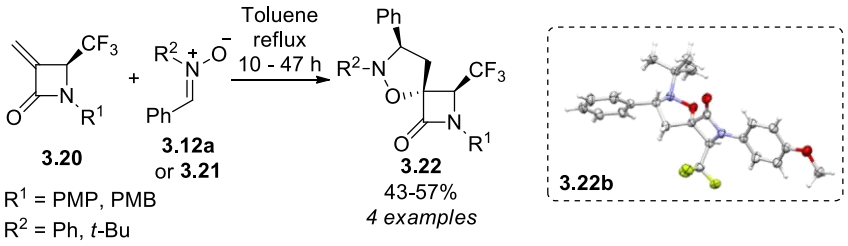
hydroxy-lactams **3.14** was also carried out. In the same year, Otto and co-workers also explored the reactivity of chiral alkylidene-lactams **3.15** with nitrone **3.16**, which led to the synthesis of three novel spiroisoxazolidines **3.17** in yields ranging from 26 to 37% (Scheme 3.4b).[13] In the late 90's other research group have reported the synthesis of a spiroisoxazolidine-β-lactam by studying the reactivity between alkylidene-lactams and 1,3-dipoles (Scheme 3.4c).[14] Thus, from the 1,3-dipolar cycloaddition reaction between the exocyclic double bond of lactam **3.18** and nitrone **3.12a**, under refluxing toluene, spiroisoxazolidines **3.19a** and **3.19b** were obtained in 82% yield with a diastereosisomeric ratio of 86:14.



Scheme 3.4. Synthesis of spiroisoxazolidine-β-lactams via 1,3-dipolar cycloaddition reactions.

D'hooghe and co-workers applied their efforts in the study of alkylidene-lactams reactivity as substrates for Michael additions, electrophilic additions, and cycloadditions. These studies included the 1,3-dipolar cycloaddition reaction of alkylidene-β-lactams with nitrones (Scheme 3.5).[15] Thus, treatment of chiral alkylidene **3.20** with nitrones

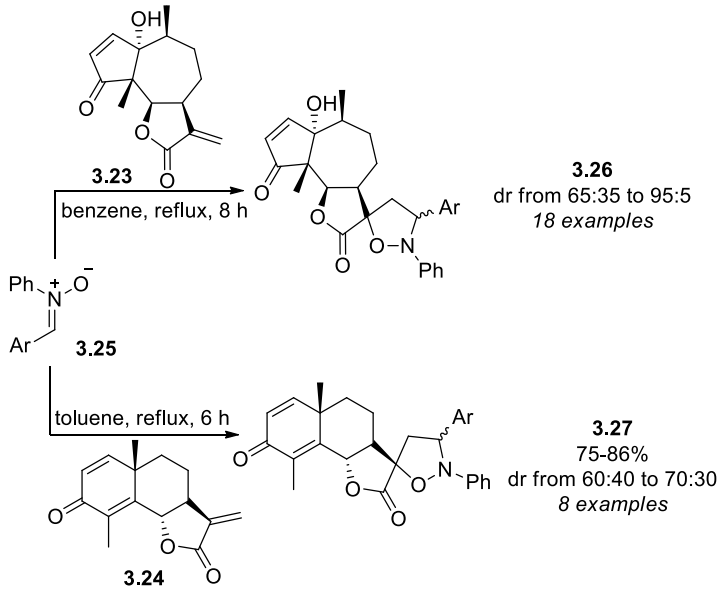
3.12a or **3.21** afforded derivatives **3.22** in moderate yields (43-57%). Additionally, to determine the correct stereochemistry of the spirocyclic adducts, single-crystal X-ray analysis were performed, and the structure of compound **3.22b** (R^1 = PMP; R^2 = *t*-Bu) was unambiguously assigned.



Scheme 3.5. Synthesis of spiroisoxazolidine-β-lactams derived from 3-methylene-4-trifluoromethyl-β-lactams.

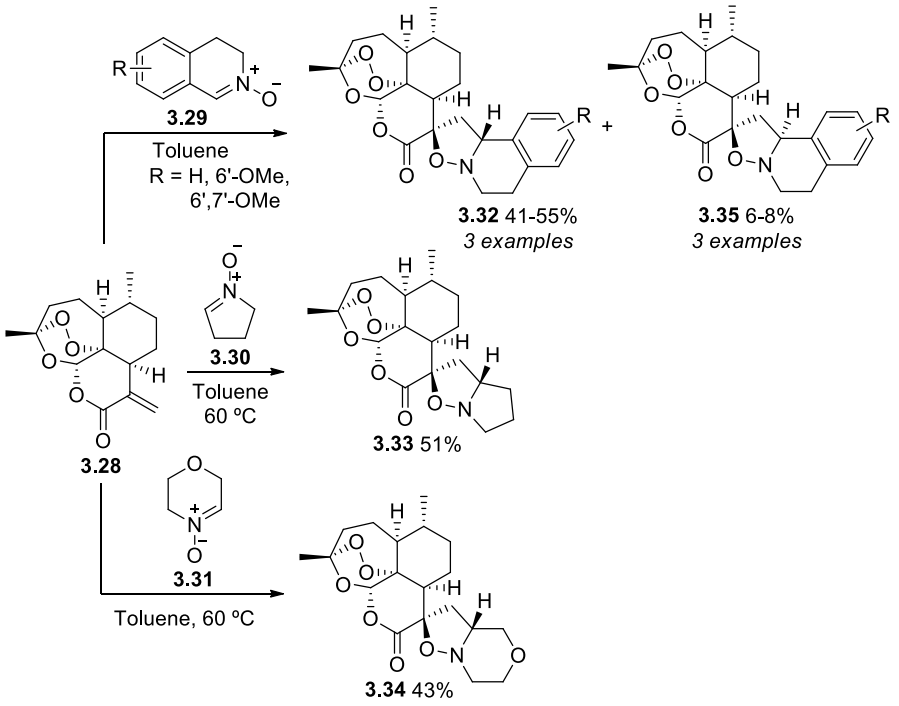
In addition to the above-mentioned examples of 1,3-dipolar cycloaddition reactions between alkylidene-β-lactams as dipolarophiles with nitrones, there are also several reports in the literature using another dipolarophiles for the synthesis of spiro-isoxazolidines, such as: alkylidene-γ-lactams,[16] sesquiterpene lactones with an exocyclic carbon-carbon double bond,[9a,17] chromanones[18] and oxindoles[19].

Thus, since 2000, some papers were published reporting the biological activity of their spirocyclic counterparts. Kumar and co-workers synthesized several spiroisoxazolidine derivatives of two sesquiterpene lactones, parthenin and α-santonin.[9a, 17a] As illustrated in Scheme 3.6, spirocyclic compounds **3.26** and **3.27** were synthesized via a dipolar cycloaddition approach, from the reaction of **3.23** or **3.24** with nitrones **3.25**. The anticancer activity of derivatives **3.26** and **3.27** was studied against several cancer cell lines, showing that these compounds, containing a spirocyclic carbon together with a sesquiterpene and an isoxazolidine core, have very promising properties, with several derivatives displaying IC_{50} values lower than 1 μM.



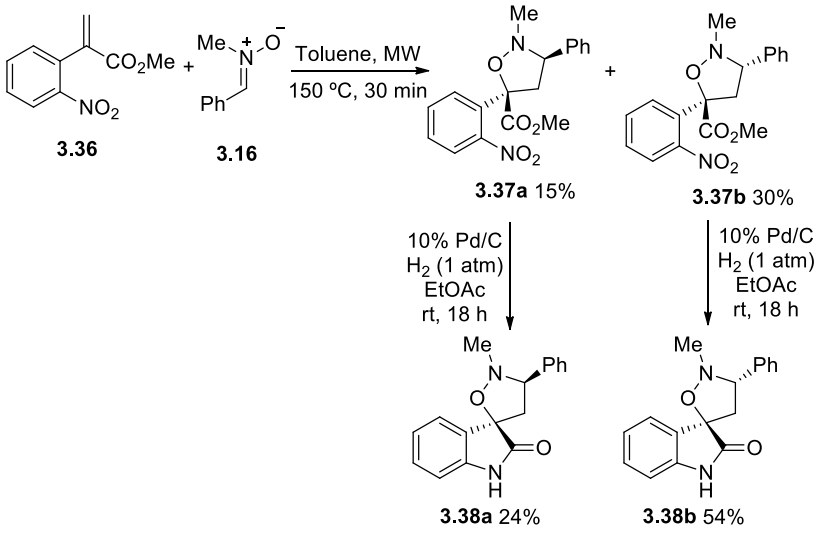
Scheme 3.6. 1,3-Dipolar cycloaddition reaction in the synthesis of spiroisoxazolidine derivatives of two sesquiterpene lactones.

The synthesis and anticancer evaluation of spirocyclic sesquiterpene lactones bearing an isoxazolidine ring was extended to artemisinin derivatives (Scheme 3.7).[17b] Despite the huge importance of artemisinin as antimalarial agent, several artemisinin derivatives also possess anti-cancer properties. The 1,3-dipolar cycloaddition of artemisinin derivative **3.28** with nitrones **3.29**-**3.31** was carried out in toluene, affording the expected cycloadducts **3.32**-**3.34** in moderate yields (41-55%). From the reaction with nitrone **3.29**, it was also possible to obtain diastereoisomers **3.35** as minor products. Further transformations, starting from compound **3.32b** (R = 6'-OMe) were explored, leading to 10-substituted derivatives. This study allowed the identification of four compounds with moderate IC_{50} values, under 10 μM, against three human cancer cell lines.



Scheme 3.7. Synthesis spirocyclic artemisinin derivatives bearing an isoxazolidine ring.

Yong *et al.* have described the synthesis of spiro-oxindoles containing a spiroisoxazolidine moiety (Scheme 3.8).[20] Firstly, isoxazolidines **3.37** were synthesized from the dipolar cycloaddition reaction of **3.36** with nitrone **3.16** under microwave irradiation. Subsequent treatment of adduct **3.37a** with 10% Pd/C in the presence of H_2, afforded spiroisoxazolidine **3.38a** in 24% yield, in a mechanism comprising a reduction as first step, followed by an intramolecular cyclization. Compound **3.38b** was prepared in a similar fashion in 54% yield. An anticancer assay showed that compound **3.38a** has an IC_{50} of 2.6 μM against MCF-7 cell line.



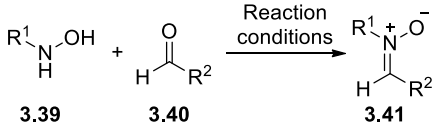
Scheme 3.8. Synthesis of spiro-oxindoles containing a spiroisoxazolidine moiety via intermolecular 1,3-dipolar cycloaddition followed by an intramolecular cyclization.

In this context, we set out to explore the 1,3-dipolar cycloaddition of 6-alkylidenepenicillanates with nitrones, as a strategy to synthesize novel chiral spiroisoxazolidine-β-lactams incorporating the penicillanate nucleus, in a process which allows the generation of three new consecutive stereogenic centers, including a quaternary chiral center.

3.2 Synthesis of Spiroisoxazolidine-β-Penicillanates via 1,3-Dipolar Cycloaddition Reactions

We started the experimental work with the synthesis of five *C*-aryl-*N*-substituted nitrones **3.41** from the condensation reaction between an aldehyde **3.40** and the appropriate hydroxylamine **3.39** by known procedures (Table 3.1). Nitrones **3.41a-c** and **3.41e** were obtained in excellent yields (80-99%) from the corresponding hydroxylamine hydrochloride in the presence of sodium acetate under microwave irradiation.[21] On the other hand, *C*-aryl-*N*-aryl nitrone **3.41d** was obtained in 77% yield, through a methodology described by Hollis *et al.*, from the reaction between *N*-benzylhydroxylamine and benzaldehyde in ethanol at room temperature for 16 hours.[22] Due to the lack of information about the geometry of the synthesized nitrones in the original procedures, their structure was assigned as described elsewhere in the literature and confirmed by NOESY experiments for nitrone **3.41a**. The chemical shift of the C*H* proton and its NOE with the methyl group corroborates the assigned structure as being the *Z*-isomer.[23]

Table 3.1. Synthesis of nitrones **3.41**.



3.39 **3.40** **3.41**

Entry	3.39 R^1	3.40 R^2	Reaction Conditions	Isolated Yields (lit.)
1	Me[a]	Ph	NaOAc, MW, 2 min, 160 ºC	**3.41a.** 89% (90%)[19a]
2	Me[a]	4-$NO_2C_6H_4$	NaOAc, MW, 2 min, 70 ºC	**3.41b.** 80% (86%)[19a]
3	*t*-Bu[a]	Ph	NaOAc, MW, 2 min, 137 ºC	**3.41c.** 99% (86%)[19a]
4	Ph	Ph	EtOH, 16 h, rt	**3.41d.** 77% (95%)[20]
5	Bn[a]	Ph	NaOAc, MW, 5 min, 120 ºC	**3.41e.** 99% (94%)[19b]

[a)] The reaction was carried out with the corresponding hydroxylamine hydrochloride

Next, an initial screening of the previously synthesized *C*-aryl-*N*-substituted nitrones **3.41** with 6-(*Z*)-(benzoylmethylene)penicillanate **3.42** was carried out (Table 3.2). The reaction of **3.41a** with **3.42** was carried out with excess of nitrone (2 equiv.) in toluene at room temperature for 16 hours, and the expected spirocyclic compound **3.43a**

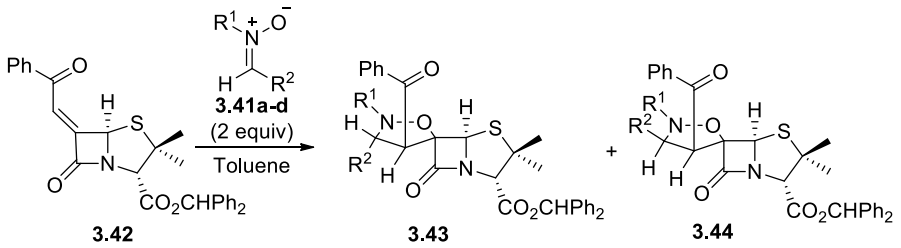
was obtained in 30% yield together with stereoisomer **3.44a** (6% yield). To further optimize the reaction conditions, the temperature was increased to 80 ºC allowing the synthesis of compounds **3.43a/3.44a** in higher overall yield. Notably, an increase in the reaction time to 24 h led to compound **3.43a** in higher yield (70%).

The cycloaddition reaction of a nitrone **3.41b**, bearing a *C*-(*p*-nitro)phenyl group, with 6-alkylidenepenicillanate **3.42** was also explored under the optimized conditions (Table 3.2, entry 4), leading to compound **3.43b** in 56% yield. However, the analysis of ^{1}H NMR spectrum of the crude of reaction between **3.42** and **3.41b** showed that the dipolarophile (**3.42**) was not all consumed. Therefore, the reaction was carried out with longer reaction time, giving after 70 h compound **3.43b** in 64% yield together with the formation of stereoisomer **3.44b** isolated in 10% yield (Table 3.2, entry 5).

In order to access the scope of the reaction we extended the study to the reactivity of 6-(*Z*)-(benzoylmethylene)penicillanate **3.42** towards other nitrones (**3.41c-e**). The reaction of 6-alkylidenepenicillanate **3.42** with nitrone **3.41c** afforded the target spiro-β-lactam **3.43c** in 41% yield. Unfortunately, complex mixtures were obtained from the attempts to carry out the cycloaddition with nitrones **3.41d** and **3.41e**.

The cycloaddition reactions proved to be regioselective, however, the formation of regioisomers **3.45a** (R^1 = Me, R^2 = Ph), **3.45b** (R^1 = Me, R^2 = 4-$NO_2C_6H_4$), and **3.45c** (R^1 = Ph, R^2 = Ph) in trace amounts could be detected in the ^{1}H NMR spectra of spiro-β-lactams **3.44a**, **3.43b** and **3.44c** respectively. This can be explained by an ineffective purification by silica gel flash chromatography due to the same retention factors of both derivatives.

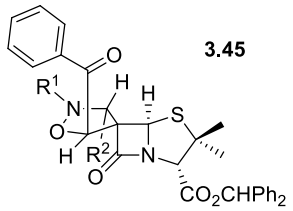
Table 3.2. Screening of the reactivity of a 6-alkylidenepenicillanate towards different nitrones.



Entry	3.41 R^1, R^2	Reaction Conditions	Isolated Yields	
1	**3.41a** Me, Ph	rt, 16 h	**3.43a**, 30%	**3.44a**, 6%[a,b]
2	**3.41a** Me, Ph	80 ºC, 16 h	**3.43a**, 57%	**3.44a**, 23%[a,b]
3	**3.41a** Me, Ph	80 ºC, 24 h	**3.43a**, 70%	**3.44a**, 11%[a,b]
4	**3.41b** Me, 4-$NO_2C_6H_4$	80 ºC, 24 h	**3.43b**, 56%[a,b]	---
5	**3.41b** Me, 4-$NO_2C_6H_4$	80 ºC, 70 h	**3.43b**, 64%[a,b]	**3.44b**, 10%
6	**3.41c** Ph, Ph	80 ºC, 24 h	**3.43c**, 41%[a,b]	---
7	**3.41d** *t*-Bu, Ph	80 ºC, 24 h	Complex Mixture	
8	**3.41e** Bn, Ph	80 ºC, 24 h	Complex Mixture	

[a] Isolated together with the corresponding regioisomer **3.45**

[b] Yield of the major product determined by ^{1}H NMR

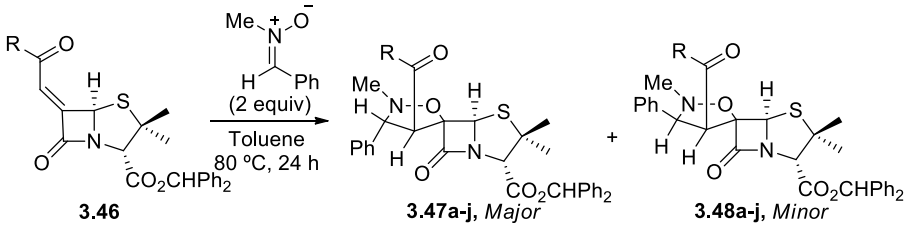


Considering the results obtained regarding the screening with the different nitrones, nitrone **3.41a** was selected to extend 1,3-dipolar cycloaddition reactions to 6-alkylidenepenicillanates bearing different substituted benzoyl groups at the exocyclic carbon-carbon double bond (**3.46a-g**). Additionally, 6-alkylidenepenicillanates where the aryl group was replaced by other aromatic systems such as naphthalene (**3.46h**) and furan (**3.46i**) were also studied (Table 3.3, entries 1-9).

To our delight, spiroisoxazolidine-β-lactams **3.47a-f** and **3.47h,i** were obtained as major products and were isolated as pure stereoisomers in yields ranging from 43% to 69%. In contrast, spiroisoxazolidine-β-lactam **3.47g** was obtained in lower yield (26%)

and could not be isolated in its pure form. From these reaction stereoisomers **3.48a-i** were also obtained as minor products (9-27%). The reactivity of 6-alkylidenepenicillanate **3.46j**, bearing an acetyl group, towards nitrone **3.41a** was also studied (Table 3.3, entry 10). The same stereoselectivity pattern was observed with the synthesis of chiral spiroisoxazolidine-β-lactams **3.47j** and **3.48j** in 30% and 7% yields, respectively.

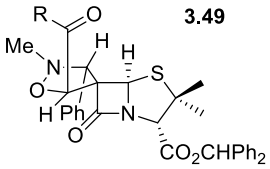
Table 3.3. 1,3-Dipolar cycloaddition of nitrone **3.41a** with alkylidenes **3.46a-j**.



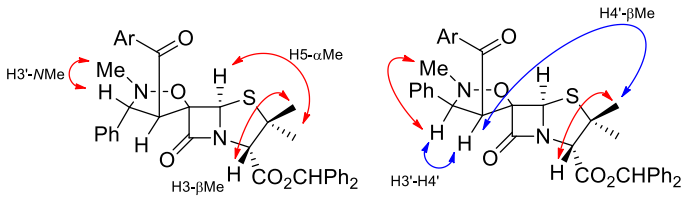
Entry		3.46, R	Products, Isolated Yields	
1	**3.46a**	*p*-FC_6H_4	**3.47a**, 66%	**3.48a**, 13%[a,b]
2	**3.46b**	*p*-ClC_6H_4	**3.47b**, 69%	**3.48b**, 10%
3	**3.46c**	*p*-BrC_6H_4	**3.47c**, 43%	**3.48c**, 12%
4	**3.46d**	*p*-$NO_2C_6H_4$	**3.47d**, 57%	**3.48d**, 27%[a,b]
5	**3.46e**	*p*-$CF_3C_6H_4$	**3.47e**, 53%	**3.48e**, 11%
6	**3.46f**	3,5-$(F)_2C_6H_3$	**3.47f**, 62%	**3.48f**, 14%
7	**3.46g**	3,5-$(CF_3)_2C_6H_3$	**3.47g**, 26%[a,b]	**3.48g**, 9%[a,b]
8	**3.46h**	2-Naphthyl	**3.47h**, 73%	**3.48h**, 10%[a,b]
9	**3.46i**	2-Furyl	**3.47i**, 67%	**3.48i**, 13%[a,b]
10	**3.46j**	Me	**3.47j**, 30%	**3.48j**, 7%

[a)] Isolated together with the corresponding regioisomer **3.49**

[b)] Yield of the major product determined by ^1H NMR



The stereochemistry of the *major* (**3.43**/**3.47**) and *minor* (**3.44**/**3.48**) adducts was determined based on two-dimensional NOE spectra (NOESY) (Figure 3.2 and Figure 3.3). The NOESY spectrum of compound **3.47e** did not show any correlation between isoxazolidine ring protons and penicillanate core protons. However, cross-peaks were observed between protons within the penicillanate core (H3-βMe and H5-αMe) as well as between protons of the isoxazolidine ring (H3'-*N*Me). Some of these correlations were also observed in the NOESY spectrum of compound **3.48e** (shown as red arrows) which further revealed cross-peaks between proton H3' (δ = 4.47 ppm) and proton H4' (δ = 4.78 ppm) confirming their *cis* configuration. The latter also showed correlation with protons of the β-methyl group of the penicillanate core (δ = 1.44 ppm). However, no cross-peaks were observed between protons H5 and any of the methyl groups. The observed correlations were in agreement with the estimated internuclear distances values (see below).



3.47e Ar = *p*-$CF_3C_6H_4$ **3.48e** Ar = *p*-$CF_3C_6H_4$

Figure 3.2. NOESY correlations of compounds **3.47e** and **3.48e**.

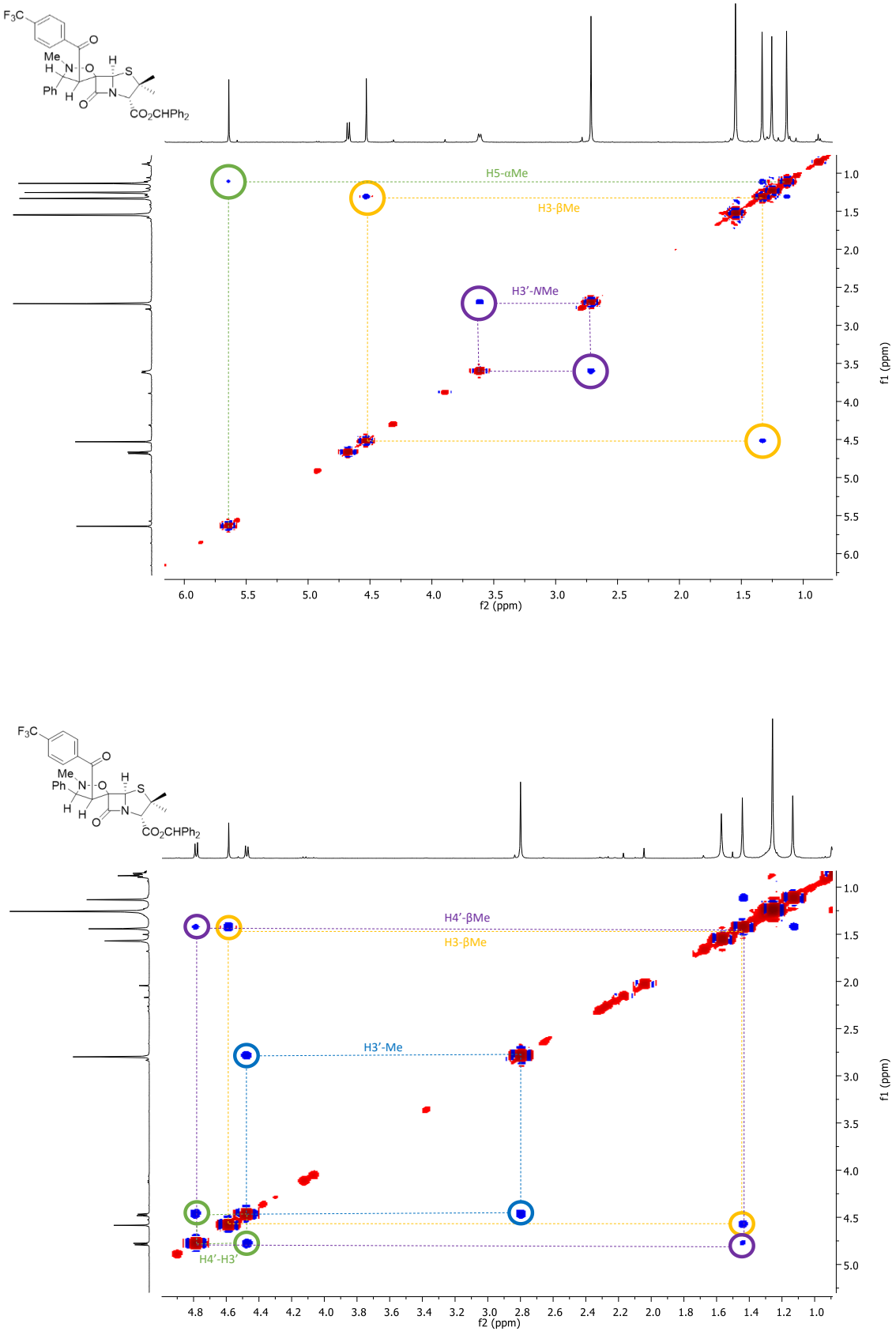
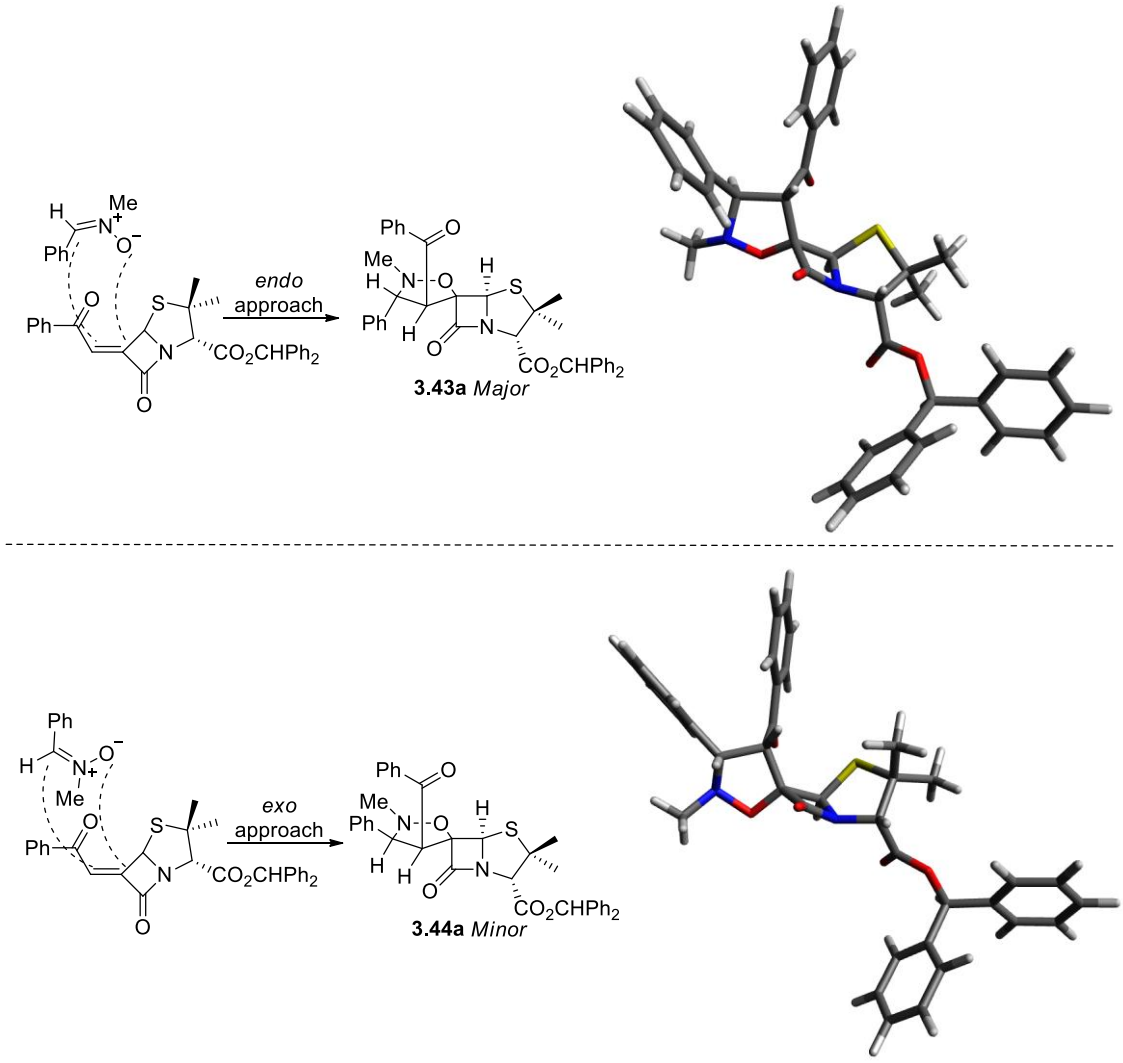


Figure 3.3. NOESY spectra of compounds **3.47e** and **3.48e**.

The bicyclic β-lactam-thiazolidine ring system of the penicillin core exists in a butterfly-like structure. This has been supported by X-ray crystallography studies, namely by the X-ray structure of 6-APA[24] and by the X-ray structure of some spiropyrazolinepenicillanates.[25] Thus, the approach of a given reactant by the convex face (α-side) of the penicillin derivative is usually more favourable. This is in agreement with the stereoselectivity observed in the 1,3-dipolar cycloaddition of 6-alkylidenepenicillanates with nitrones where the major product results from the addition of the dipole to the less sterically hindered α-side of the penicillanates. Furthermore, these spiroisoxazolidine-β-lactams **3.43/3.47** were obtained via an *endo* cycloaddition.

Due to unsuccessful attempts to obtain appropriate crystals for X-ray crystallography studies, quantum chemical calculations, at the DFT level of theory (B3LYP/6-31G(d)), were carried out to determine the 3D structure of spiroisoxazolidine-β-lactams **3.43a** and **3.44a**. The optimized geometries revealed that these novel structures have also a butterfly-like structure with a more open shape in the case of β-lactam **3.43a**. Moreover, the *endo* product (**3.43a**) was estimated to be 7.03 kJ/mol more stable than the *exo* adduct (**3.44a**) which corroborates the experimental results (Scheme 3.9).

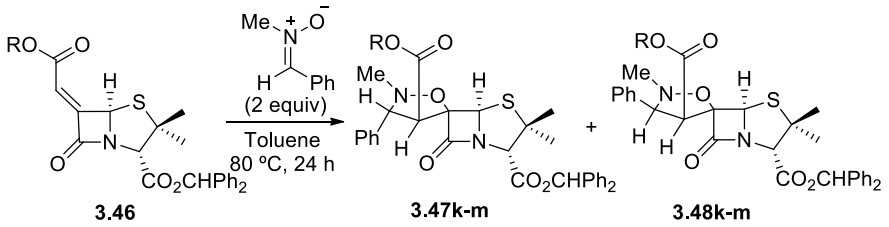
In order to support the NOESY correlations, internuclear distances values were estimated from the optimized structures of derivatives **3.43a** and **3.44a**. The distance between H3'-*N*Me and H3-βMe, which cross-peaks were observed for both spiroisoxazolidine-β-lactams **3.47e** and **3.48e**, are similar in both cases (**3.43a:** 2.376 Å; **3.44a**: 2.387 Å and **3.43a:** 2.416 Å; **3.44a**: 2.576 Å, respectively). Major differences were observed in the distances between H5-αMe and H4'-βMe (**3.43a:** 2.713 Å; **3.44a**: 4.126 Å and **3.43a:** 4.824 Å; **3.44a**: 3.025 Å, respectively) resulting from the more folded structure of the penicillanate core in **3.44a**. Therefore, cross-peaks between H5-αMe were only observed for compound **3.43a** whereas the correlation H4'-βMe was only observed for compound **3.44a**.



Scheme 3.9. *Endo* and *exo*-cycloaddition of alkylidene **3.42** with nitrone **3.41a**, considering the approach of the dipole by the α-face. Optimized geometries (B3LYP/6-31G(d) level) of *endo*-adducts **3.43a** and **3.44a**.

The scope of the reaction was readily extended to 6-alkylidenepenicillanates bearing a carboxylate substituent (**3.46k-m**), as shown by the synthesis of spiro-β-lactams **3.47k-m** and **3.48k-m** (Table 3.4). The major products **3.47k-m** were obtained efficiently (51-80% yield) and resulted also from an *endo* 1,3-dipolar cycloaddition with addition of the nitrone to the α-side of the β-lactams. The stereoisomeric *exo*-cycloadducts **3.48k-m** were isolated as minor products (10 -16% yield). It is noteworthy that the cycloaddition reaction with the most hindered 6-alkylidenepenicillanate **3.46l**, bearing a benzhydryl group, which led to our best result (entry 2), afforded compound **3.47l** in 80% yield and its stereoisomer **3.48l** in 10% yield, corresponding to 90% overall yield.

Table 3.4. 1,3-Dipolar cycloaddition of nitrone **3.41a** with alkylidenes **3.46k-m**.



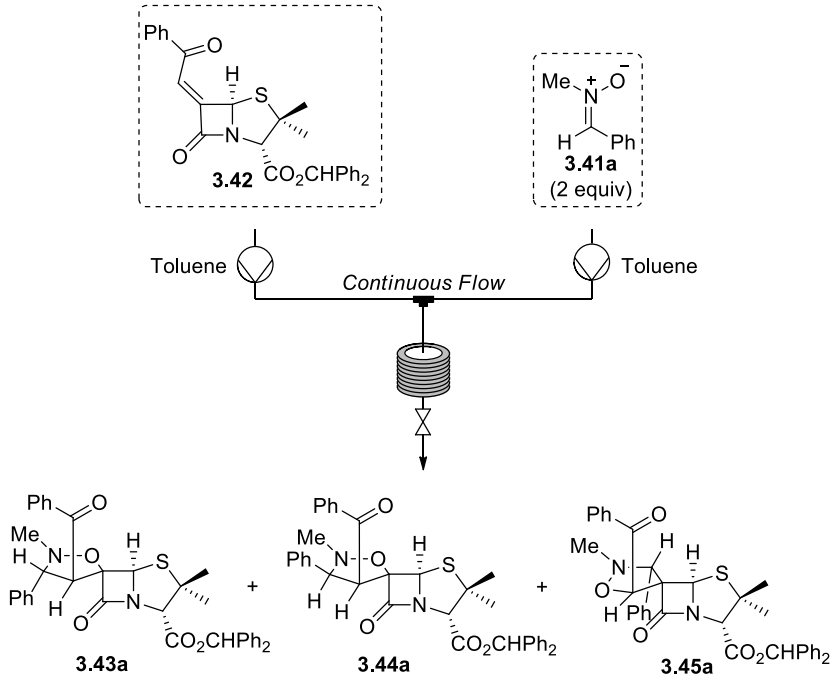
Entry	3.46, R		Products, Isolated Yields	
1	**3.46k**	Bn	**3.47k**, 51%	**3.48k**, 15%[a,b]
2	**3.46l**	$CHPh_2$	**3.47l**, 80%	**3.48l**,10%[a,b]
3	**3.46m**	Me	**3.47m**, 57%	**3.48m**,16%[a,b]

[a)] Isolated together with the corresponding regioisomer **3.49**

[b)] Yield of the major product determined by ^{1}H NMR

The reaction of 6-(*Z*)-(benzoylmethylene)penicillanate **3.42** with nitrone **3.41a** was also explored under continuous flow conditions (Table 3.5). Similarly, the reactions were carried out with an excess of nitrone (2 equiv.) in toluene at 80 ºC (entries 1-4). The first approach for the screening under continuous flow was carried out with a flow rate of 250 μL/min of each solution with a backpressure regulation of 3 bar (entry 1). From this experiment it was possible to achieve a conversion of 79% leading to corresponding spiro-adducts **3.43a**, **3.44a** and **3.45a** in 68:16:16 ratio. By decreasing the flow rate to 100 μL/min it was possible to improve the conversion to 84% (66:16:18 ratio, entry 2). Increasing the pressure to 8 bar led to similar conversion and ratio values (entries 3-4). However, an increase in the pressure (8 bar) together with an increase in the temperature (120 ºC) at a flow rate of 100 μL/min led to our best result with a conversion of 86% together with spiroisoxazolidinepenicillanates **3.43a**, **3.44a** and **3.45a** in 77:12:11 ratio (entry 5). Although it is still necessary to optimize the reaction conditions for the cycloaddition of 6-alkylidenepenicillanates with nitrones, it should be noted that this preliminary study under continuous flow showed a similar reactivity as the observed under batch conditions, leading to derivative **3.43a** as major product. Overall, the results are encouraging and proved to be competitive leading to the desired products in shorter reaction times.

Table 3.5. Continuous flow 1,3-dipolar cycloaddition of 6-alkylidenepenicillanate **3.42** with nitrone **3.41a**.



Entry	Reaction Conditions	t^R	Conversion (ratio)[a]
1	250 µL/min, 80 °C, 3 bar	125 min	79% (68:16:16)
2	100 µL/min, 80 °C, 3 bar	50 min	84% (66:16:18)
3	250 µL/min, 80 °C, 8 bar	125 min	78% (64:18:18)
4	100 µL/min, 80 °C, 8 bar	50 min	80% (68:16:16)
5	100 µL/min, 120 °C, 8 bar	50 min	86% (77:12:11)

[a] **3.43a**:**3.44a**:**3.45a** Ratio determined by ^{1}H NMR

3.3. Anti-HIV Activity

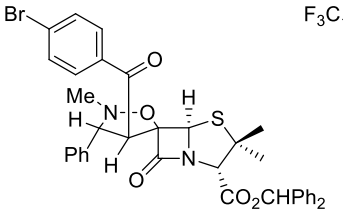
The anti-HIV activity of spiro-β-lactams **3.43a**, **3.47a-f** and **3.47h-m** was evaluated. Initially, *in vitro* cytotoxicity of selected compounds in TZM-bl cells was determined (Table 3.6). Data from cytotoxicity assays showed that from the library of thirteen compounds, none of them displayed relevant toxicity, with CC_{50} values ranging from 63.39 μM to 81.73 μM.

Therefore, the potential of all selected compounds as antiviral agents against HIV-1 was assessed. A first assay was carried out to determine the MPI at 25 μg/mL. Among these, compounds **3.47c** and **3.47e** showed encouraging MPI values (of 80% and 79%, respectively). Since **3.47c** and **3.47e** appeared to be the most promising compounds, with an acceptable MPI their IC_{50} values were determined and found to be 7.18 μM and 9.59 μM, respectively.

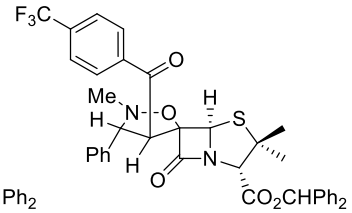
The obtained results showed lower antiviral activity for this set of compounds than for other spiropenicillanate derivatives as previously observed. So far, it is correct to affirm that carbocyclic derivatives in the spirocyclic ring led to outstanding results in contrast with the presence of heterocyclic substituents in the spiro-fused ring. However, it should be noted that the synthesis of spiroisoxazoline-β-lactams allowed the introduction of an oxygen atom in the heterocyclic moiety of our spiropenicillanate derivatives for the first time. Thus, the moderate anti-HIV activity presented by these two biologically active spiroisoxazolidine-β-lactams allows for further modulations, in the search of more active compounds bearing the spiroisoxazoline core.

Table 3.6. Activity of spiro-β-lactams against HIV-1.

Entry	CC_{50} µM	MPI (%) 25µg/mL	IC_{50} µM
3.43a	80.68	0	nd
3.47a	81.73	18	nd
3.47b	77.45	58	nd
3.47c	65.83	80	7.18
3.47d	75.04	0	nd
3.47e	65.95	79	9.59
3.47f	63.39	26	nd
3.47h	67.15	0	nd
3.47i	71.05	0	nd
3.47j	81.47	0	nd
3.47k	76.38	0	nd
3.47l	66.43	0	nd
3.47m	74.44	0	nd



3.47c



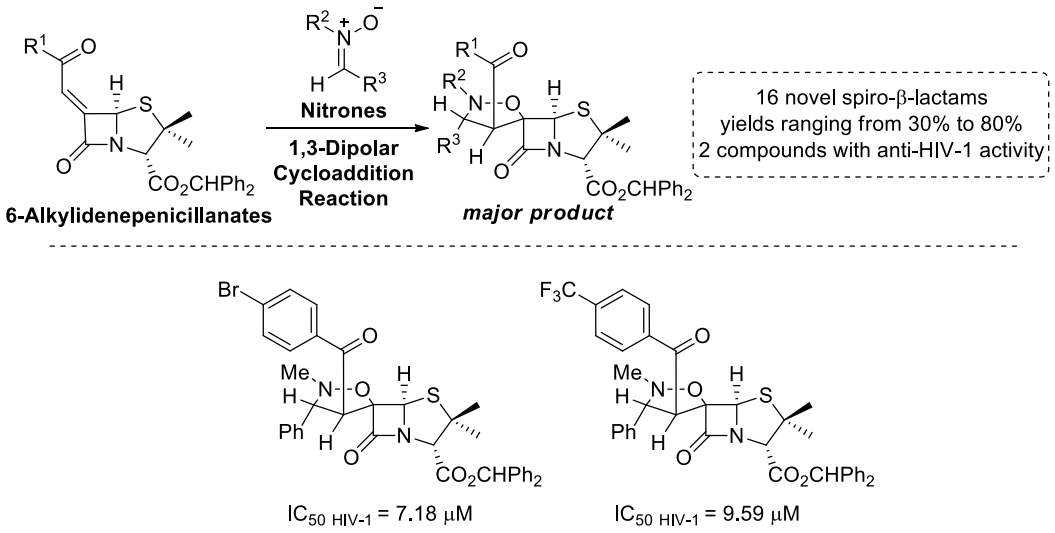
3.47e

3.4. Conclusions

After the successful replacement of substituents in the position 1' and 2' of spirocyclopentene-β-lactam BSS-730A, which resulted in the synthesis of an extensive family of novel spiro-β-lactams with dual activity against HIV (HIV-1 and HIV-2) and *Plasmodium*, our aim was now to extend the modulation to the spirocyclic ring system. The replacement of the cyclopentene ring by an isoxazolidine ring would lead to novel heterocyclic ring spiro-fused penicillanate derivatives.

Aiming at the synthesis of these novel derivatives, the 1,3-dipolar cycloaddition reaction of 6-alkylidenepenicillanates with nitrones was explored. This approach allowed the synthesis of spiroisoxazolidine-penicillanates in a stereo- and diastereoselective manner with the creation of three new chiral centers, with the major product being obtained in isolated yields ranging from 30% to 80% (Scheme 3.10). Furthermore, promising results were also obtained from the preliminary studies of this reactivity under continuous flow.

The synthesis of these novel derivatives has resulted in the identification of two new spiro-β-lactams with moderate antiviral activity against HIV-1, containing a *p*-bromobenzoyl and a *p*-trifluoromethylbenzoyl group at position 4' of the isoxazolidine ring, with IC_{50} values of 7.18 and 9.59 μM, respectively. The low antiviral activity can be explained due to structural differences between the spiro-β-lactam lead molecule BSS-730A and the analogues presented in this chapter, namely in the nature of the spirocyclic ring. However, the identification of these novel bioactive spiro-β-lactams with antiviral activity supports the potential of this class of compounds as highly promising antimicrobial agents and opens an opportunity for further modulations aiming at the synthesis of more active compounds.



Scheme 3.10. Overview of the studied reactivity in Chapter 3 with anti-HIV activity of the most promising compounds.

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CHAPTER 4

Synthesis of Novel Chiral Spiro-β-Lactams from Nitrile Oxides and 6-(*Z*)-(Benzoylmethylene)penicillanate

Abstract

The present chapter describes the first examples of 1,3-dipolar cycloaddition reactions of 6-alkylidenepenicillanates with nitrile oxides. This strategy allowed the synthesis of novel chiral spiroisoxazoline-β-lactams incorporating the penicillanate nucleus, in a stereo- and diastereoselective manner with the creation of two consecutive chiral centers. This chapter also covers the outcomes of these 1,3-dipolar cycloaddition reactions under three different reaction conditions (conventional heating, microwave irradiation and continuous flow).

Chapter 4 - Synthesis of Novel Chiral Spiro-β-Lactams from Nitrile Oxides and 6-(Z)-(Benzoylmethylene)penicillanate

4.1. Introduction

Nitrile oxides, RCNO, are a class of highly reactive 1,3-dipoles, and are usually represented by the mesomeric form illustrated in Figure 4.1. Back in 1894, A. Werner and Buss, have explored the synthesis of nitrile oxides. The treatment of *N*-hydroxybenzimidoyl chloride with sodium carbonate led to the elimination of HCl, and subsequent formation of benzonitrile oxide, which was the first synthesized nitrile oxide.[1]

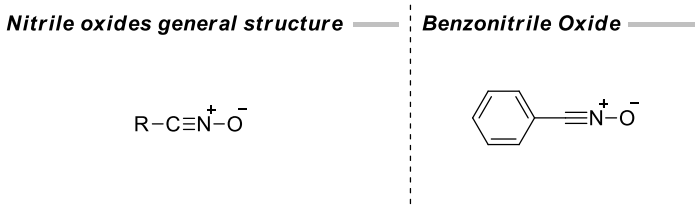
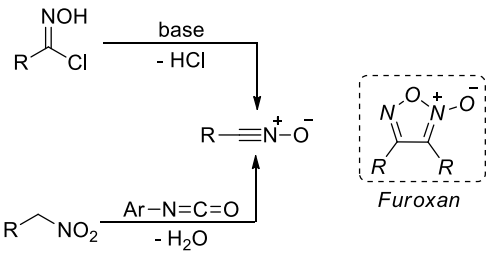


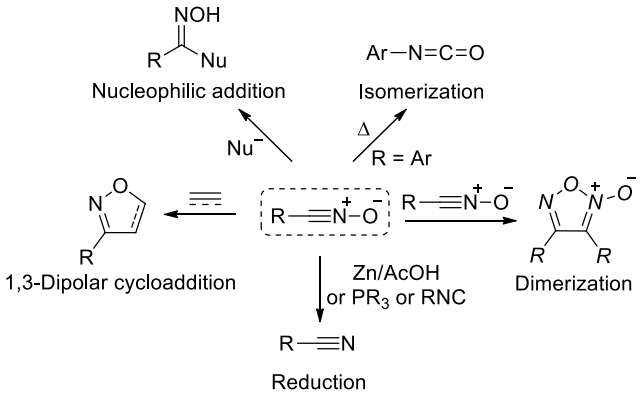
Figure 4.1. Nitrile oxide general structure and benzonitrile oxide structure.

Usually, nitrile oxides can be synthesized through two methods (Scheme 4.1).[1a-c, 2] The first method comprises a dehydrohalogenation of hydroximoyl halides, which are prepared from aldoximes, while the second method, known as Mukaiyama reaction, consists of dehydration of primary nitroalkanes with aryl isocyanates. Despite the stability of some nitrile oxides, it should be noted that most nitrile oxides, in special the aliphatic ones, are extremely unstable and the dimerization reaction is very favored leading to the formation of furoxans.[3] Under the appropriate reaction conditions, aromatic and heterocyclic nitrile oxides can also rearrange to isocyanates. Thus, *in situ* generation of nitrile oxides, firstly applied by Huisgen, is a strategy applied to prevent the above-mentioned side-reaction.



Scheme 4.1. Methods for the synthesis of nitrile oxides.

In fact, Huisgen's pioneering work on the mechanistic studies of 1,3-dipolar cycloaddition reactions of nitrile oxides was the starting point for one of the most well-known and studied transformation of nitrile oxides. The high reactivity of nitrile oxides endows them with a high versatility enabling their participation in a broad range of reactions. Scheme 4.2 outlines an overview concerning nitrile oxides as scaffold in organic chemistry, highlighting some important transformations, namely nucleophilic additions, isomerization to isocyanates, dimerization, and reduction reactions.[1a, b, 2b, 3] Furthermore, nitrile oxides can undergo 1,3-dipolar cycloaddition reaction with alkenes or alkynes as a strategy to afford isoxazolines or isoxazoles, respectively.[1a-c, 2b, c, 3-4]



Scheme 4.2. Overview on the reactivity of nitrile oxides.

Isoxazolines are a class of five-membered unsaturated heterocyclic compounds, containing a nitrogen atom and an adjacent oxygen atom.[5] These compounds can be obtained through 1,3-dipolar cycloaddition reactions,[2a, b, 2d, 4, 6] cyclization reactions of oxime-containing compounds,[7] among others synthetic methodologies.[5, 7a, 8] Isoxazolines are relevant intermediates for the synthesis of aminoalcohols, hydroxyketones and isoxazolidines.[2d, 5a-c, 8b, 9] Moreover, this scaffold has attracted considerable attention among the medicinal chemists, because it is associated to biologically active compounds with anticancer activity, antimicrobial activity, antidiabetic activity, and also insecticidal and parasiticidal activities.[5a-c, 7a, 10]

An important subclass of isoxazolines are the spiroisoxazolines, which motif can be found in natural occurring compounds. Furthermore, spiroisoxazolines have also been widely explored due to the presence of its core structure in biologically active

compounds.[5b, 10a, 11] In fact, compounds with the spiroisoxazoline core have shown anticancer activity,[12] anti-tubercular activity,[13] enzyme or protein inhibition,[12f, 14] anti-*Plasmodium* activity,[15] and antiviral activity.[16] Interestingly, the spiroisoxazoline motif is also present in the structure of the first examples of chiral platinum (II) complexes with ferroelectric properties.[17] Spiroisoxazolines can be synthetized taking advantage from similar reactivities as the ones described for the synthesis of isoxazolines, namely nucleophilic additions and/or cyclization reactions of oximes, or through 1,3-dipolar cycloaddition reactions of nitrile oxides with exocyclic olefins, which is the most studied approach. [4, 5b, 5d, 6b-d, 8b, 10a] The following examples of the synthesis of spiroisoxazolines were carefully chosen, in order to give a better understanding on this thematic, but also to highlight some examples which are relevant for the proposed PhD project.

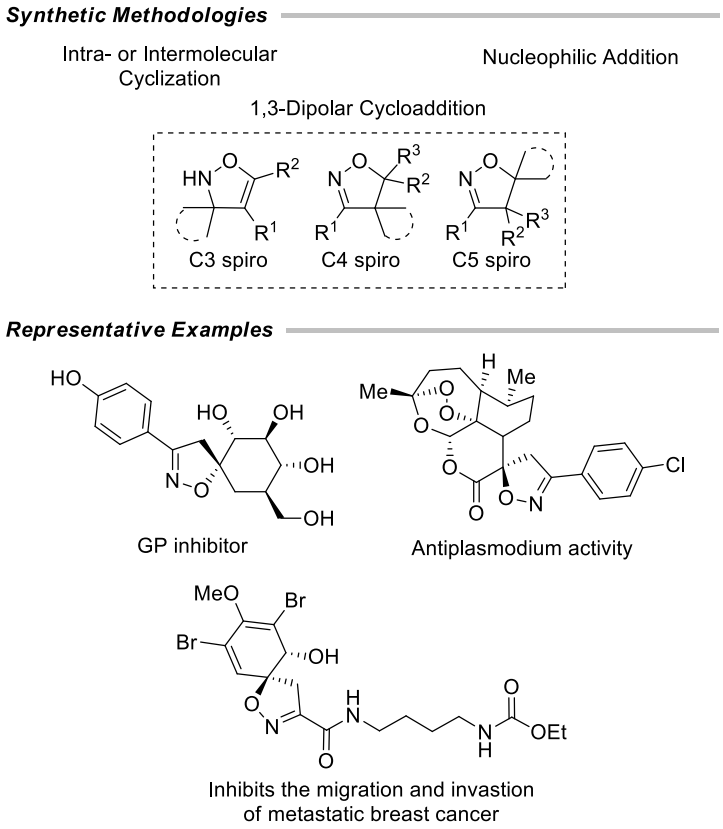
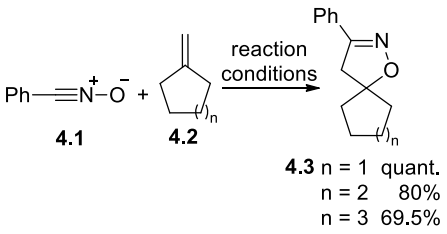


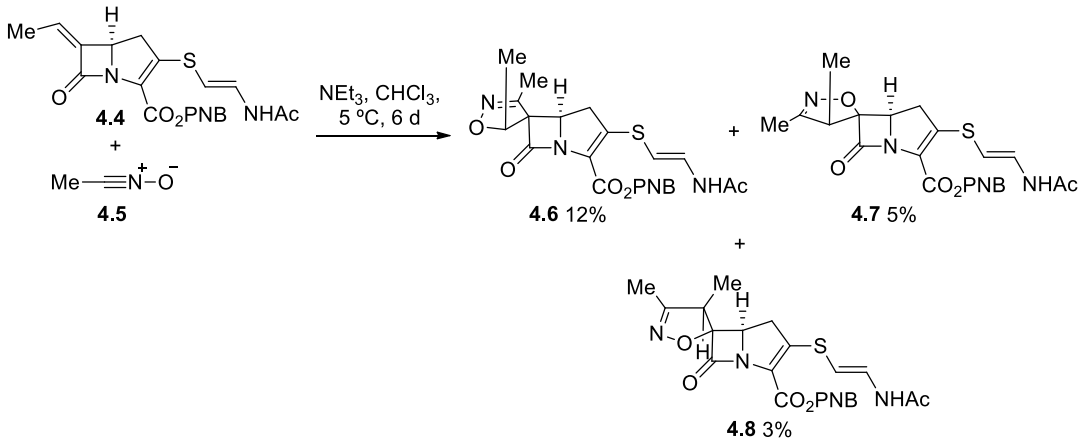
Figure 4.2. General structure of spiroisoxazolines and representative examples with the corresponding bioactivity.

The first examples aiming the synthesis of spiroisoxazolines date back to the 60s, when Barbulesco *et al.* described the reaction of benzonitrile oxide (**4.1**) with methylenecycloalkanes **4.2** (Scheme 4.3).[18] In their study, they have successfully explored the 1,3-dipolar cycloaddition between exocyclic double bond of methylenecycloalkanes **4.2** with benzonitrile oxide (**4.1**), affording the corresponding spiroisoxazolines **4.3** in good yields.



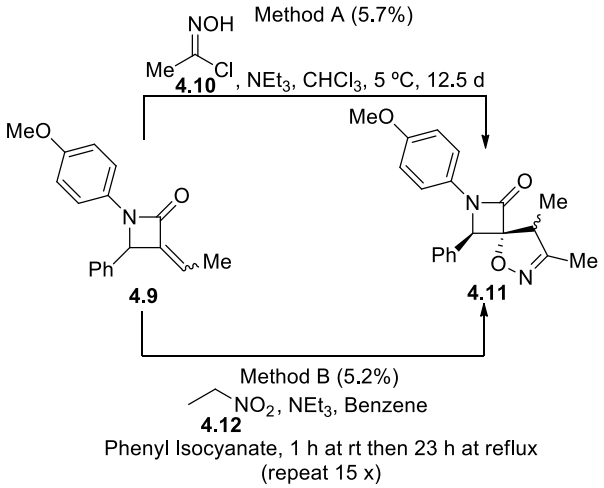
Scheme 4.3. Synthesis of the first spiroisoxazolines.

More than 20 years later, by exploring the reactivity of the methylenic double bond of alkylidene-carbapenem **4.4**, Corbett reported the synthesis of spirocarbapenems **4.6-4.8** containing an isoxazoline moiety (Scheme 4.4).[19] Despite the long reaction time, the reaction between acetonitrile oxide (**4.5**) and alkylidene **4.4** led to compounds **4.6** and **4.7**, in only 12% and 5% yields, respectively. It was also observed the addition of the dipole to the β-face of the carbapenem **4.4** leading to the formation of a third product (**4.8**) in 3% yield.



Scheme 4.4. Synthesis of spirocarbapenems via 1,3-dipolar cycloaddition reaction.

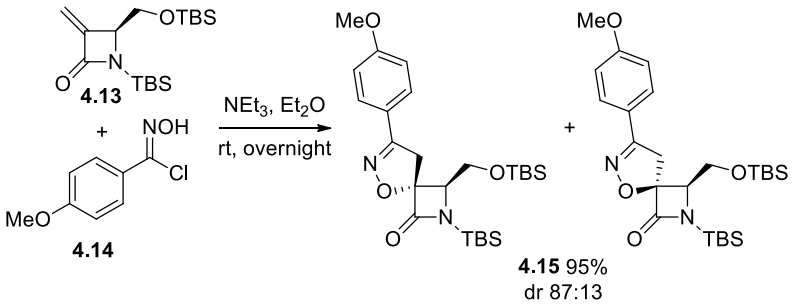
In the late 90s, Otto's and Liebscher's research groups explored the synthesis of spiro-β-lactams via 1,3-dipolar cycloaddition of nitrile oxides to α-alkylidene-β-lactams (Scheme 4.5).[20] Otto and his team described the synthesis of spiroisoxazolidine **4.11** from the cycloaddition reaction of acetonitrile oxide (**4.5**), generated *in situ* from the corresponding hydroximoyl chloride **4.10**, with alkylidene-lactam **4.9** as a (E)/(Z) isomeric mixture.[20a] However, the reaction did not exhibited good functional group tolerance, and consecutive attempts of carrying out the cycloaddition reaction with other dipolarophiles did not lead to the formation of the desired products. To overcome this drawback, the authors explored a different approach (Method B), the Mukaiyama-Hoshino method, which involved the *in situ* generation of the transient nitrile oxide from the treatment of nitroethane with NEt_3. Similarly, carrying out the 1,3-dipolar cycloaddition reaction with Method B only led to the synthesis of compound **4.11** in low yield (5% yield), from alkylidene-lactam **4.9** as starting material, showing that even with a different protocol the reaction did not show good functional group tolerance.



Scheme 4.5. Synthesis of spiroisoxazolines from α-alkylidene-β-lactams.

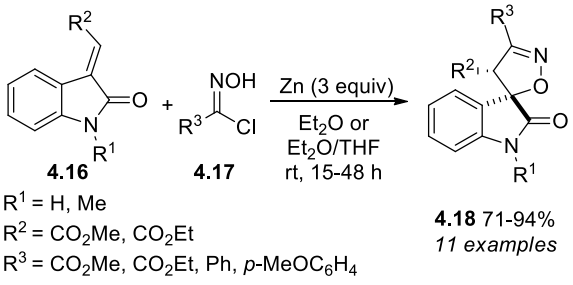
Surprisingly, Liebscher and collaborators faced a similar lack of functional group tolerance while exploring the cycloaddition reaction of 4-methoxybenzonitrile oxide with different alkylidene-lactams. In their work, the exception was the 1,3-dipolar cycloaddition reaction between alkylidene-β-lactam **4.13** and 4-methoxybenzonitrile oxide, generated *in situ* from the dehydrohalogenation of hydroximoyl chloride **4.14**

which afforded spiro-β-lactams **4.15** as a mixture of diastereoisomers (87:13) in 95% yield. (Scheme 4.6).[20b]



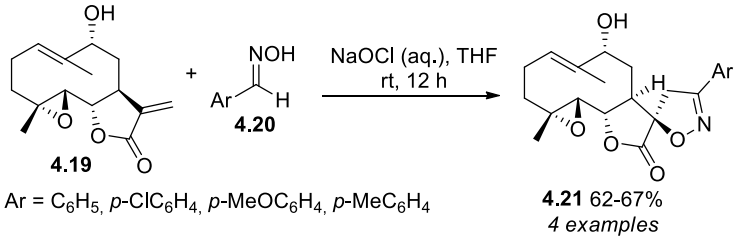
Scheme 4.6. Synthesis of spiro-β-lactams from 4-methoxybenzonitrile oxide.

As above-mentioned, to avoid dimerization, nitrile oxides are often generated *in situ* via dehydrohalogenation of hydroximoyl chlorides. Although triethylamine, sodium hypochlorite solutions or inorganic salt were the most used bases to carry out the dehydrohalogenation step, Ribeiro *et al.* reported, for the first time, the use of zinc as dehydrohalogenating agent to generate transient nitrile oxides (Scheme 4.7).[21] The 1,3-dipolar cycloaddition reaction between indolinones **4.16** with nitrile oxides, generated from the corresponding hydroximoyl chlorides **4.17**, afforded spiroisoxazoline oxindoles **4.18** in high yields (71-94%). Besides being an efficient methodology, carrying out the dehydrohalogenation step with zinc does not require the slow addition of the base, to avoid dimerization reactions.



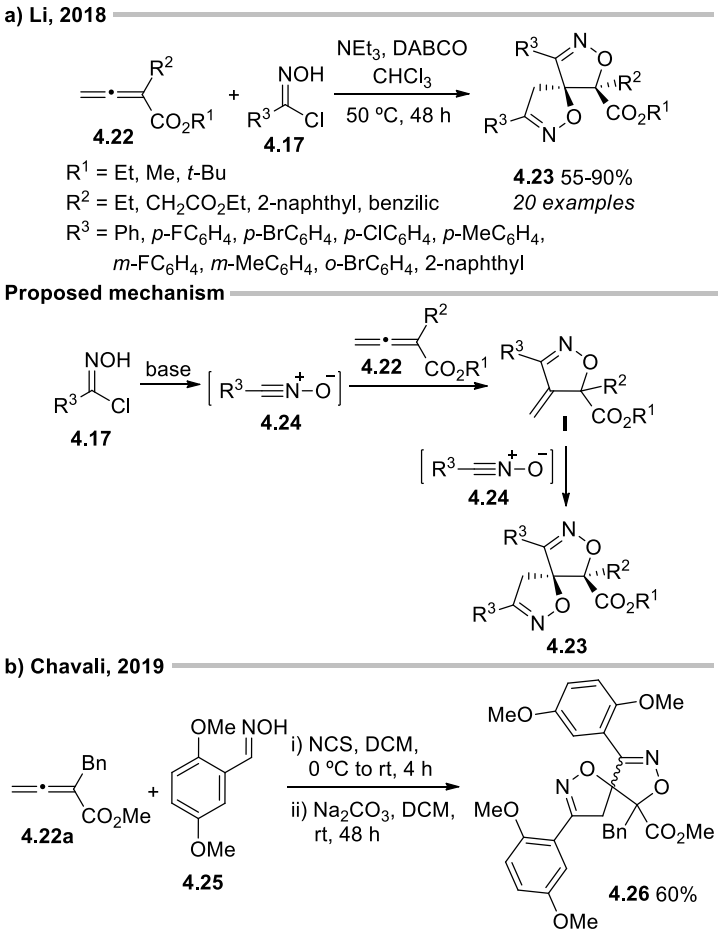
Scheme 4.7. Synthesis of spiroisoxazoline oxindoles via 1,3-dipolar cycloaddition reaction.

Recently, as outlined in Scheme 4.8, Outahar *et al.* described the synthesis of novel spiroisoxazoline derivatives from 9a-hydroxyparthenolide, a compound extracted from the aerial parts of a plant widely used in Moroccan-Algerian traditional medicine (*Anviellea radiata*).[22] Notably, the reaction of **4.19** with aromatic nitrile oxides proceeded in a chemo- and regioselective fashion affording the corresponding spiroisoxazoline derivatives **4.21** in good yields (62-67%).



Scheme 4.8. Synthesis of novel 9a-hydroxyparthenolide-derived spiroisoxazolines.

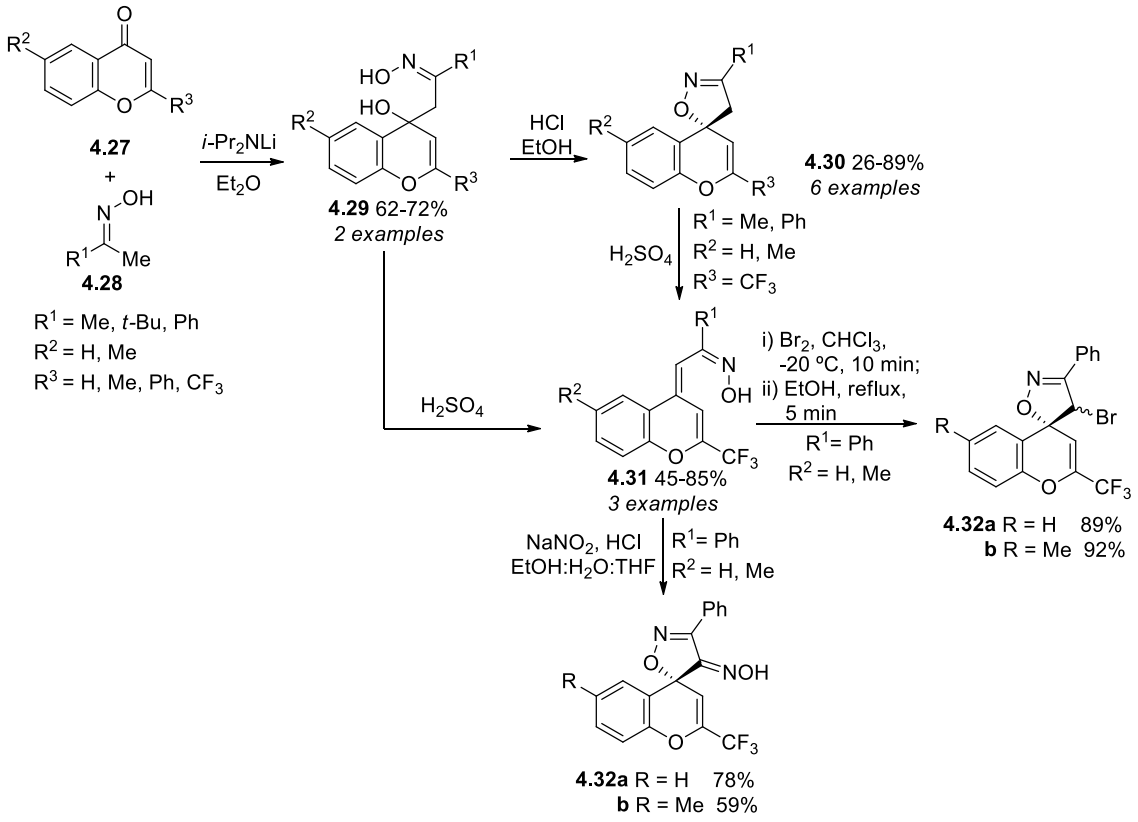
The use of allenoates as dipolarophiles is a common strategy for the synthesis of cycloadducts with an exocyclic double bond. Although there is a panoply of reports where only one carbon-carbon double bond is involved in the cycloaddition reaction, there are a few examples of cycloaddition reactions comprising the reaction of both allenoate's double bonds via sequential cycloadditions in one pot.[23] In 2018, Li and co-workers developed a synthetic approach for the synthesis of spirobiisoxazolines **4.23** via a double 1,3-dipolar cycloaddition of nitrile oxides with allenoates **4.22** (Scheme 4.9a).[24] This synthetic strategy involved a first cycloaddition reaction between nitrile oxides **4.24** and allenoates **4.22**, leading to the synthesis of intermediate **I**, followed by a second 1,3-dipolar cycloaddition between intermediate **I** and a second molecule of the nitrile oxide. The study allowed to build a library of 20 spirobisisoxazolines **4.23**, obtained in moderate to excellent yields (55-90%). The spirobisisoxazoline **4.23** derivd from an alkyl nitrile oxide (R^3 = Et) was obtained in very low yields (< 5%). Control experiments were carried out to elucidate the proposed mechanism, proved that the first cycloaddition reaction occurred in the double bond involving the α- and β-carbon of the allenoate to give intermediate **I**. One year later, the reactivity of nitrile oxide **4.25** towards allene **4.22a** was explored by Chavali and co-workers, where a similar strategy was applied in the synthesis of spirobiisoxazoline **4.26** which was obtained in 60% yield (Scheme 4.9b).[25]



Scheme 4.9. Synthesis of spirobiisoxazolines.

As referred before, the spiroisoxazoline core can be obtained from cyclization reactions. The most common approaches for these cyclizations are acid- or base-catalyzed. Sosnovskikh *et al.* reported an acid-catalyzed cyclization via dehydration of β-Hydroxy oximes **4.29** for the synthesis of spiroisoxazolines (Scheme 4.10).[26] β-hydroxy oximes **4.29** were synthesized from the reaction of 2-trifluoroemethylchromones **4.27** (R^3 = CF_3) with acetophenone *E*-oximes **4.28** in the presence of lithium diisopropylamide. Subsequent acid-mediated dehydration step, with diluted HCl, followed by a O-nucleophilic attack to C-4 of chromone moiety allowed the cyclization into a spirocyclic intermediate leading to spirochromene-isoxazolines **4.30** in moderate yields (26-89%). The exception are aliphatic-containing oximes **4.29** (R^2 = Me, *t*-Bu) which were unstable, and spiroisoxazolines **4.30** were directly obtained without the addition of acid. It should also be noted that, by treating CF_3-containing β-hydroxy oximes **4.29** or spiroisoxazolines **4.30** with H_2SO_4, α,β-unsaturated oximes **4.31** were obtained. The scope of the reaction

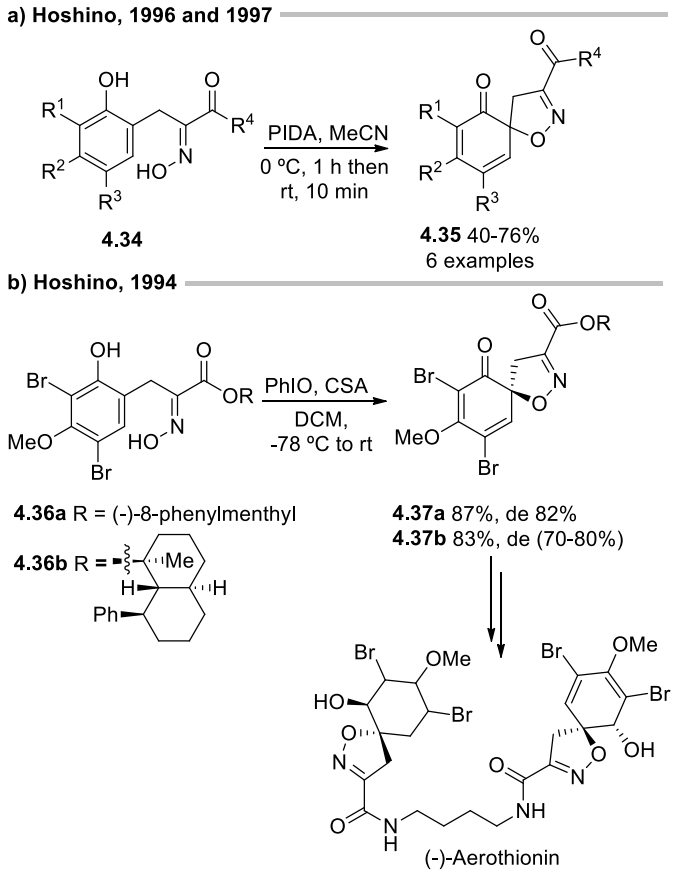
was extended to three other chromones containing different substituents (R^3 = H, Me, Ph) at C-2. 2-Methylchromone and flavone reacted with acetophenone oxime in the presence of lithium diisopropylamide, affording the corresponding methyl and phenyl derivatives in good to excellent yields, while the unsubstituted chromone did not gave the expected product. It should be noted that spiro-adducts **4.30** without the CF_3 moiety at C-2 did not underwent ring-opening reactions to derivatives **4.31** in the presence of concentrated H_2SO_4, showing the potential role of the CF_3 group in these ring-opening reactions. Cyclization reactions of α,β-unsaturated oximes **4.31** were also accomplished, in the presence of HNO_2 or Br_2, leading to compounds **4.32** and **4.33** in good to excellent yields, respectively.



Scheme 4.10. Synthesis of spiroisoxazolines via intramolecular cyclization reactions.

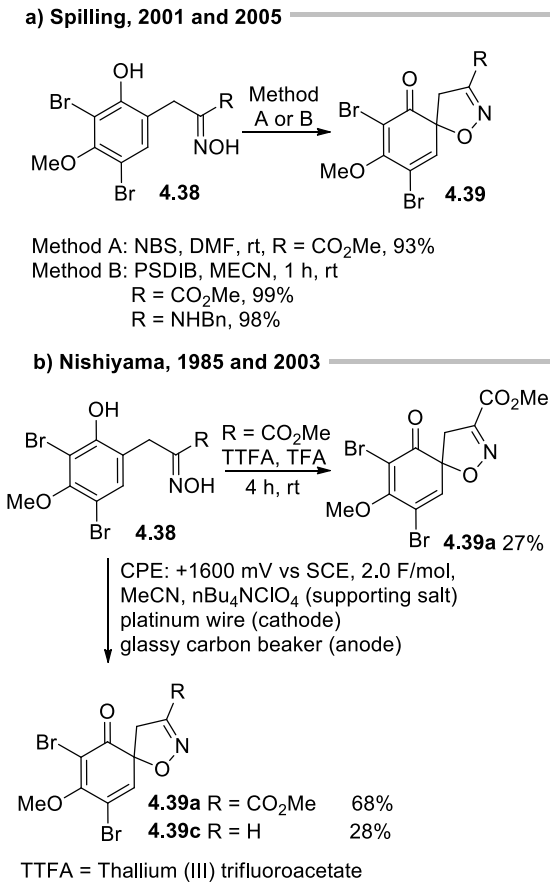
Oxidative cyclizations of phenolic oxime esters are also a well-known approach to obtain spiroisoxazolines. This method has been widely explored and reported by several research groups as a viable route for the synthesis of marine metabolites derivatives. A large amount of scientific advances in the synthesis of natural metabolite

derivatives were reported by Hoshino's, Spilling's and Nishiyama's research groups, which were a driving force for the development of this field. In the last years of the 20th century, Hoshino and co-workers used this synthetic strategy for the synthesis of spiroisoxazolines **4.35** (Scheme 4.11a).[27] Treatment of *o*-phenolic oximes **4.34** with phenyliodonium diacetate (PIDA) under mild conditions afforded the desired spiro-adducts **4.35** in moderate to good yields. The asymmetric version of this methodology was also explored by the same authors (Scheme 4.11b). In this case, the optimized reaction conditions employ a combination of PhIO and camphorsulfonic acid as oxidant. The oxidative cyclization reaction of *o*-phenolic oximes **4.36** containing a chiral auxiliary group proceeds under low temperature, affording chiral spiroisoxazolidines **4.37** in good yields and with good diastereoselectivities. Further transformations were carried out leading to the synthesis of natural metabolite (+)-aerothionin.



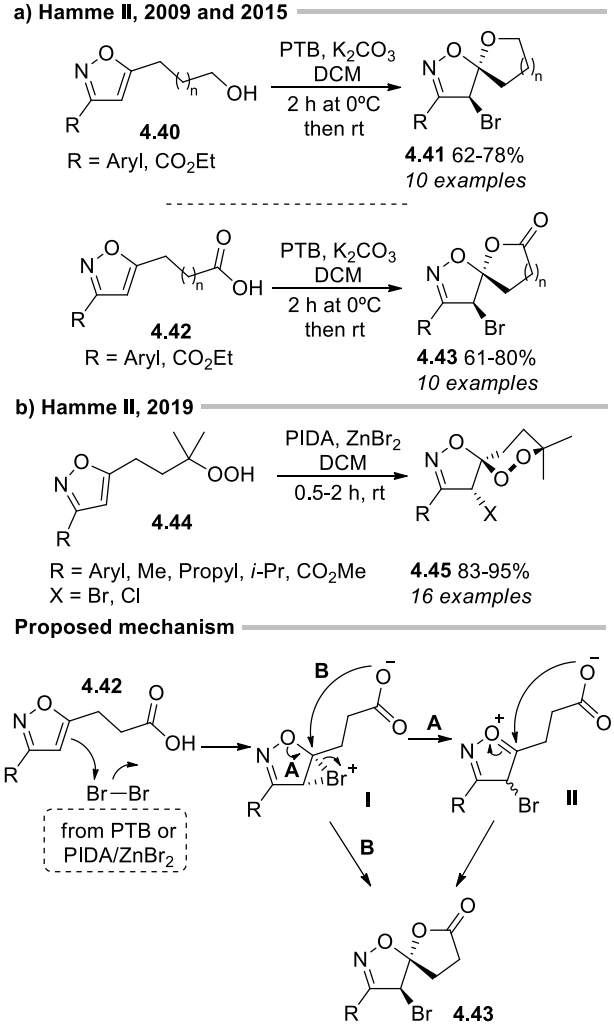
Scheme 4.11. Synthesis of spiroisoxazolines, precursors of marine metabolites derivatives.

The oxidative cyclization reaction of *o*-phenolic oximes can also be achieved by different approaches. Spilling and co-workers described two methods for the synthesis of spiroisoxazolines **4.39** from *o*-phenolic oximes **4.38** (Scheme 4.12a).[28] The first method involves the cyclization of **4.38a** in DMF in the presence of NBS leading to compound **4.39a**. However, attempts to purify compound **4.39a** by column chromatography resulted in its degradation. Therefore, an alternative procedure was developed. Carrying out the reaction of **4.38** in the presence of a polymer-supported oxidant afforded spirocyclic compounds **4.39** in excellent yields, without the need of a chromatographic step. The oxidation of **4.38a** in the presence of thallium(III) trifluoroacetate and trifluoroacetic acid to the corresponding spiroisoxazoline **4.39a** was also achieved in 27% yield (Scheme 4.12b).[29] In 2003, Nishiyama and his co-workers developed an environmental-friendly approach consisting of an anodic oxidation of *o*-phenolic oximes **4.38a** and **4.38c** affording spiroisoxazolines **4.39a** and **4.39c**, in 68% and 28% yields, respectively.[30] It also should be noted that a variety of examples addressing the synthesis of spiroisoxazolines through oxidative cyclizations of *p*-phenolic oximes are known.[11a, 27b, 31]



Scheme 4.12. Oxidative cyclization reaction of *o*-phenolic oximes for the synthesis of spiroisoxazolines.

The oxonium-mediated intramolecular cyclization of halogenated isoxazole intermediates has been also reported as a strategy for the synthesis of spiroisoxazoline ethers, lactones or peroxides (Scheme 4.13).[16, 32] Hamme II and co-workers developed a protocol for the synthesis of spiroisoxazolines **4.41** and **4.43** starting from isoxazoles **4.40** and **4.42**, respectively, in the presence of pyridinium tribromide and potassium carbonate.[32] This approach allowed the synthesis of a wide range of diverse bromo-substituted spiroisoxazolines in good yields. However, the authors faced a drawback with ester-substituted isoxazoles **4.40**, and the spiro-cyclization does not underwent as expected leading to an uncharacterized side product. In order to overcome this unexpected result other bromination agents and reaction conditions were assayed. After some optimization reactions the synthesis of ester-substituted spiroisoxazolines **4.41** was achieved in a system with Br_2/CCl_4 in the absence of light. Spiroisoxazoline peroxides **4.45** were also synthesized in a similar way, through the cyclization of isoxazoles **4.44**.[16b] These reactions were carried out in the presence of PIDA and a bromide source at room temperature, shown good functional group tolerance, allowing the synthesis of alkyl-, aryl- and ester-substituted spiroisoxazolines **4.45** in excellent yields. Further transformations of an ester-substituted derivative were carried out, affording the corresponding carboxylic acid and a library of amine-substituted spiroisoxazolines in good yields. A proposed mechanism for this reactivity is outlined in Scheme 4.13. Under the corresponding reaction conditions the deprotonation of the terminal oxygen atom and the dearomatization of the isoxazole ring via an electrophilic addition of bromine afforded bromonium-ion **I**. Next, two different pathways can be followed. If intermediate **I** follows pathway A, oxonium-ion intermediate **II** was obtained. Herein, both intermediate **I** and **II** underwent intramolecular cyclization leading to the corresponding spiroisoxazolines **4.43**. It is noteworthy, that in both pathways the bromine atom controls the final stereochemistry, where the bromine and the oxygen of the newly formed carbon-oxygen bond have an *anti* relationship. In 2020, the same research group described a similar strategy for the synthesis of fluorine derivatives (Scheme 4.14).[16a]



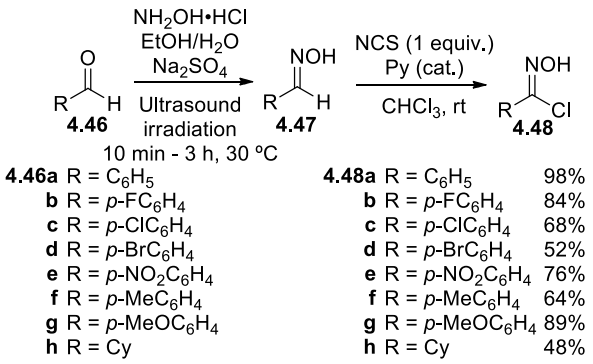
Scheme 4.13. Synthesis of spiroisoxazoline via an oxonium-mediated intramolecular cyclization.

This chapter covers the first examples of chiral spiroisoxazolinepenicillanates, using batch, microwave irradiation and continuous flow methodologies. Aiming to increase the structural diversity of spiropenicillanates derivatives, by adding an isoxazoline ring, spiro-fused to the penicillin core's lactam ring, 1,3-dipolar cycloaddition reactions between a 6-alkylidenepenicillanate with nitrile oxides were carried out.

4.2. Synthesis of Spiroisoxazoline-β-Lactams via 1,3-Dipolar Cycloaddition Reactions

4.2.1. 1,3-Dipolar Cycloaddition Reactions *via* Batch and Microwave-Induced Methodologies

In order to explore the 1,3-dipolar cycloaddition of 6-(*Z*)-(benzoylmethylene)penicillanate to nitriles oxides, we started by synthesizing a library of nitrile oxide precursors, hydroximoyl chlorides **3** (Scheme 4.14). First, the reaction of hydroxylamine hydrochloride with the corresponding aromatic aldehydes **4.46a-g** was carried out in a mixture of ethanol/water with Na_2SO_4 as dehydrating agent under ultrasound irradiation. The condensation reaction led to aldoxime derivatives **4.47a-g**, which were isolated with a simple workup procedure and used without further purification. Next, treatment of aldoximes **4.47** with *N*-chlorosuccinimide (NCS) in the presence of a catalytic amount of pyridine at room temperature afforded aromatic hydroximoyl chlorides **4.48a-g** in good to excellent yields, ranging from 52% to 98%. This synthetic approach also allowed the synthesis of aliphatic hydroximoyl chloride **4.48h** which was obtained in moderate yield (48%).



Scheme 4.14. Synthesis of hydroximoyl chlorides from aldehydes.

Aiming at the synthesis of the novel spiroisoxazoline-β-lactams, the 1,3-dipolar cycloaddition reaction of nitrile oxide **4.49a**, generated *in situ* from benzaldehyde hydroximoyl chloride **4.48a** by the action of potassium carbonate, with 6-(*Z*)-(benzoylmethylene)penicillanate **4.50** was studied for the optimization of both batch and microwave-induced reaction conditions (Table 4.1). The use of an inorganic base ensures

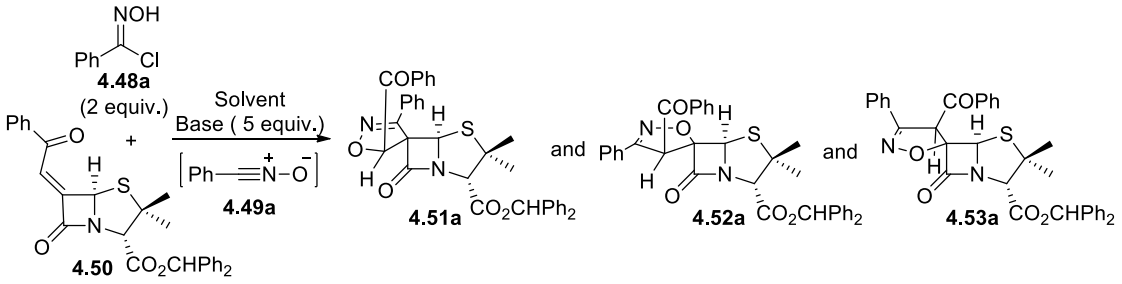
a slow dehydrohalogenation of hydroximoyl chlorides **4.48**, allowing a controlled formation of the corresponding nitrile oxide **4.49** which readily reacts with the desired dipolarophile. The cycloaddition reaction of the transient nitrile oxide **4.49a** with 6-(*Z*)-(benzoylmethylidene)penicillanate **4.50** was carried out in ethyl acetate at room temperature for 7.5 h affording spiroisoxazoline-β-lactam **4.51a** as major product in 60% yield. From this reaction an inseparable mixture of compounds **4.52a** and **4.53a** was also isolated in 32% yield (66:34 ratio) (entry 1). Increasing the reaction time to 24 hours was counterproductive regarding the overall yield but led to spiroisoxazoline-β-lactam **4.51a** as single product isolated in 49% yield (entry 2). However, better results were obtained by increasing the temperature. Carrying out the reaction of **4.49a** with **4.50** under refluxing ethyl acetate the desired products were obtained in excellent overall yield (97%) with a good selectivity for compound **4.51a** which was isolated in 59% yield (entry 3). The same overall yield was obtained by performing the reaction in toluene at 80 ºC (entry 4), with a slight increase in the yield of **4.51a** (entry 3: 59% *vs* entry 4: 65%) and without the formation of **4.53a**.

Additionally, the use of triethylamine for the dehydrohalogenation of hydroximoyl chloride **4.48a** was also explored. Thus, in the presence of triethylamine, the target spiro-β-lactams could also be obtained either in ethyl acetate (98% overall yield, entry 5) or in toluene (48% overall yield, entry 6).

The 1,3-dipolar cycloaddition reaction between nitrile oxide **4.49a** and 6-(*Z*)-(benzoylmethylidene)penicillanate **4.50** was also carried out under microwave irradiation (entries 7-11). It was observed carrying out the reaction in ethyl acetate under microwave irradiation for 30 minutes at 80 ºC afforded compounds **4.51a** and **4.52a** in 32% and 28% yield, respectively (entry 7). The yields were slightly improved using the same conditions for 1 hour, giving the target spiro-β-lactams **4.51a** and **4.52a** in 35% and 34% yield, respectively (entry 8). Changing the reaction solvent to toluene allowed the synthesis of **4.51a** and **4.52a** in 74% overall yield after 30 minutes of microwave irradiation at 80 ºC, with spiroisoxazoline-β-lactam **4.51a** being obtained as major product in 42% yield (entry 9). On the other hand, by increasing the temperature to 120 ºC for 10 min, the reagents were consumed but only a trace amount of **4.51a** was detected (entry 10). Finally, carrying out the microwave-induced reaction in toluene at 80 ºC for 30 min using triethylamine as the dehydrohalogenating agent led to a complex mixture. It should be noted that the cycloaddition reaction between alkylidenepenicillanate **4.50** and nitrile oxide **4.49a** under

microwave irradiation proved to be more selective, with the suppression of the formation of spiro-β-lactam **4.53a**.

Table 4.1. Optimization of the 1,3-dipolar cycloaddition reaction conditions to spiroisoxazoline-β-lactams under batch and microwave-irradiation conditions.



	Entry	Base	Solvent	Reaction Conditions	Isolated Yields (ratio)[a]		Overall yield
Batch	1	K_2CO_3	AcOEt	rt, 7.5 h	**4.51a**. 60%	**4.52a/4.53a**. 32% (66:34)	92%
	2	K_2CO_3	AcOEt	rt, 24 h	**4.51a**. 49%	----	49%
	3	K_2CO_3	AcOEt	reflux, 5 h	**4.51a**. 59%	**4.52a/4.53a**. 38% (84:16)	97%
	4	K_2CO_3	Toluene	80 ºC, 4 h	**4.51a.** 65%	**4.52a**. 32%	97%
	5	NEt_3	AcOEt	rt, 24 h	**4.51a**. 47%	**4.52a/4.53a**. 51% (63:37)	98%
	6	NEt_3	Toluene	80 ºC, 4 h	**4.51a**. 11%	**4.52a/4.53a**. 37% (38:62)	48%
MW	7	K_2CO_3	AcOEt	80 ºC, 30 min	**4.51a**. 32%	**4.52a**. 28%	60%
	8	K_2CO_3	AcOEt	80 ºC, 1 h	**4.51a.** 35%	**4.52a**. 34%	69%
	9	K_2CO_3	Toluene	80 ºC, 30 min	**4.51a**. 42%	**4.52a**. 32%	74%
	10	K_2CO_3	Toluene	120 ºC, 10 min	----	----	----[b]
	11	NEt_3	Toluene	80 ºC, 30 min	Complex mixture		----

[a] Ratio determined by ^{1}H NMR;

[b] Trace amounts of **4.51a**;

The structural assignment of spiroisoxazoline-β-lactams **4.51a** and **4.52a** was supported by one- and two-dimensional NMR spectra (^{1}H NMR, ^{13}C NMR and HSQC; Figures 4.3). As expected, the ^{1}H NMR spectrum of derivatives **4.51a** and **4.52a** show signals corresponding to two methyl groups from the penicillanate core, aromatic protons and to the benzhydryl proton (C*H*Ph$_2$, **4.51a**: 6.98 ppm; **4.52a**: 6.93 ppm). The ^{1}H NMR

and HSQC spectra allowed us to assign the signals corresponding to protons H-3, H-5 of the penicillanate core and to the proton of the isoxazoline ring of both compounds. For the major product, spiro-β-lactam **4.51a**, the following chemical shifts were observed: H-3 at 4.65 ppm, H-5 at 5.86 ppm and isoxazoline proton at 6.17 ppm. A considerable difference was observed in the chemical of the isoxazoline's proton for compound **4.52a** (δ = 5.67 ppm). This difference can be explained by the presence of an oxygen atom in a vicinal position to the proton in the isoxazoline in spiroisoxazoline-β-lactam **4.51a** which promotes a downfield shift. Additionally, from the HSQC spectrum it was possible to confirm the assignment of the spirocyclic carbon of both spiroisoxazoline-β-lactam**s** (**4.51a**: 76.6 ppm; **4.52a**: 102.0 ppm). The difference in the chemical shift of the spirocyclic carbons of these two regioisomers can be explained considering that only in the case of spiro-β-lactam **4.51a** the oxygen atom is attached to the spirocyclic carbon. This NMR data is in agreement with the proposed structures of spiroisoxazoline-β-lactams **4.51a** and **4.52a** resulting from opposite regioselectivity, with **4.51a** and **4.52a** being a spiro[isoxazoline-4',6-penicillanate] (*major*) and a spiro[isoxazoline-5',6-penicillanate], respectively. As previously stated, due to the bicyclic β-lactam-thiazolidine ring system of the penicillin core which exists in a butterfly-like structure, the approach of a given reactant usually occurs by the convex face (α-side) of the penicillin derivative.[33]

This is in agreement with the observed stereoselectivity, with both compounds **4.51a** and **4.52a** resulting from the addition of the dipole to the less sterically hindered α-side of the penicillanates. The stereoselectivity observed in the formation of spiro-β-lactam **4.53a** can be rationalized by an approach of the dipole through the β-side of the penicillanate with the regioselective formation of the cycloadduct where the dipole's oxygen adds to the forthcoming spirocyclic carbon. The ^{13}C chemical shift of the spirocyclic carbons are similar for spiroisoxazoline-β-lactams **4.52a** and **4.53a** as expected for diastereoisomers with the same regiochemistry (^{13}C spirocyclic carbon shift: **4.52a**: 102.2 ppm; **4.53a**: 100.1 ppm).

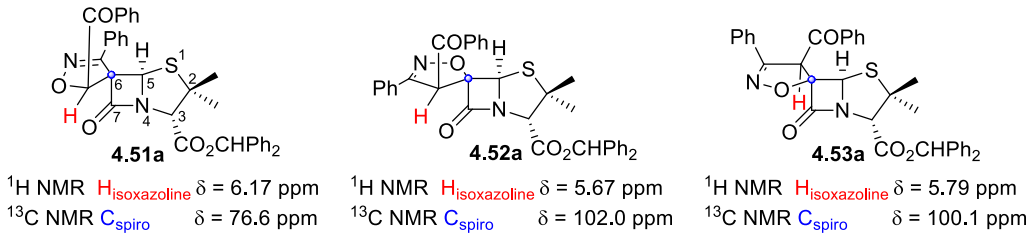


Figure 4.3. Structures of selected spiro-β-lactams highlighting the most important ^{1}H and ^{13}C chemical shifts for the stereochemistry assignment.

The same stereo- and regioselectivity was previously observed by Corbett, while exploring the 1,3-dipolar cycloaddition reaction of nitrile oxides with an alkylidene-carbapenem, aiming at the synthesis of spiroisoxazolinecarbapenems.[19] In this work, it was observed that three products were obtained from the cycloaddition reaction. Two of them were the expected cycloadducts from the addition of the dipole to the α-side of the carbapenem: one results from the attack of the dipole's oxygen to the terminal carbon of the exocyclic double bond of the alkylidene (major product) and the other from the addition of the dipole's oxygen to the forthcoming spirocyclic carbon. It was also observed the formation of a third cycloadduct, a diastereoisomer formed by the addition of the nitrile oxide to the β-side. It is noteworthy that the carbapenem bicyclic system is similar to the penicillanic core in terms of preferred conformation, leading to a more favorable addition of incoming reactants by the α-face.

Although a slight difference was observed in the isolated yields of products of the 1,3-dipolar cycloaddition, by carrying out the reactions in ethyl acetate or in toluene, ethyl acetate was chosen for further studies since it is a "greener" solvent than toluene.[34] Potassium carbonate was selected over triethylamine, as dehydrohalogenating agent, also aiming at developing a more sustainable synthetic methodology.

Then, we extended the reactivity studies of 6-(*Z*)-(benzoylmethylidene)penicillanate (**4.50**) to other nitrile oxides, under batch and microwave irradiation optimized reaction conditions. However, the reactions carried out under conventional heating were monitored by TLC and the reaction time (ranging from 15 minutes to 8 hours) was determined by the total consumption of 6-alkylidenepenicillanate **4.50**.

The 1,3-dipolar cycloaddition reactions with nitrile oxides bearing electron-withdrawing and electron-donating substituents at *para*-position of the phenyl ring were explored (Table 4.2). To our delight, both types of dipole activation led to the expected cycloadducts, with pure chiral spiroisoxazoline-β-lactams **4.51b-f** being obtained as major products, in yields ranging from 49% to 72% under conventional heating and 31-47% under microwave irradiation (entries 1-12).

Notably, the reaction of alkylidenepenicillanate **4.50** with nitrile oxide **4.49b** (R = *p*-FC_6H_4) under conventional heating was highly selective leading to the exclusive formation of a single product, spiroisoxazoline-β-lactams **4 .51b**, in 72% yield (entry 1). On the other hand, performing the same reaction under microwave irradiation (entry 2) led to the synthesis of the same spiro-β-lactam **4.51b** (39% yield) together with spiro-β-lactams **4.52b**/**4.53b** in 32% yield. The 1,3-dipolar cycloaddition reaction with *p*-chloro or *p*-bromo substituted nitrile oxides (**4.49c** and **4.49d**, respectively) led to the corresponding spiroisoxazoline-β-lactams **4.51** (**4.51c** and **4.51d**), as major products, under both methodologies in yields ranging from 41% to 49% (entries 3-6). From these reactions, mixtures of isomeric compounds **4.52c**/**4.53c** and **4.52d**/**4.53d** were also obtained leading to overall yields up to 92% (entries 3-6). The cycloaddition reaction with a nitrile oxide **4.49e**, bearing a *p*-nitrophenyl group, was also explored under the optimized conditions (entries 7 and 8). This reaction allowed the synthesis of spiro-β-lactam **4.51e** as major product in 54% yield under conventional heating (entry 7) as well as under microwave conditions, with compound **4.51e** being obtained in 47% yield (entry 8). In both reactions, mixture of compounds **4.52e**/**4.53e** were also obtained in 32-35% yield (entries 7 and 8). Remarkably, concerning the overall yields, the 1,3-dipolar cycloaddition reaction between **4.49e** and **4.50** was the most efficient under both conventional heating and microwave irradiation (overall yields: 98% and 79%, respectively).

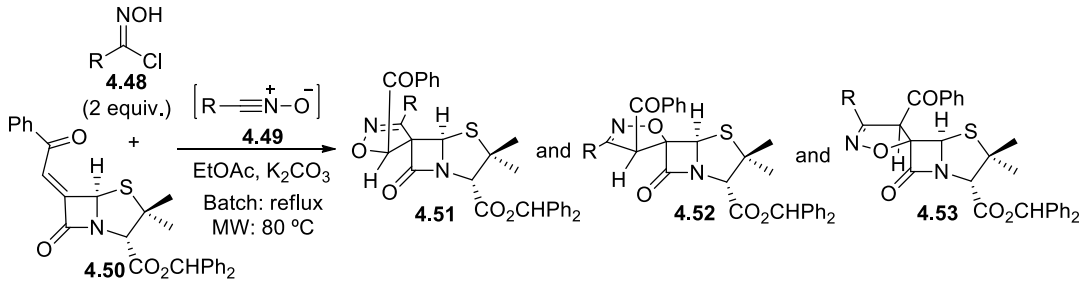
The cycloaddition reaction of 6-alkylidenepenicillanate **4.50** with *p*-methylphenyl derivative **4.49f** also gave the expected major spiroisoxazoline-β-lactam **4.51f** in 50% yield under conventional heating (entry 9), together with spiro-β-lactams **4.52f**/**4.53f** (38% yield). However, under microwave irradiation (entry 10) the overall yield of this cycloaddition reaction was modest leading to compounds **4.51f** and **4.52f**/**4.53f** in 31% and 20% yield, respectively.

The use of a nitrile oxide bearing a stronger electron-donating group (**4.49g**, R = *p*-$MeOC_6H_4$), conducted to an extremely fast 1,3-dipolar cycloaddition under conventional heating (30 minutes) leading to an inseparable mixture of **4.51g**/**4.53g** in 52% yield (entry 11). Under microwave irradiation for 15 minutes the same mixture (**4.51g**/**4.53g**) was isolated in 36% yield, together with pure spiro-β-lactam **4.52g** obtained in 21% yield (entry 12).

The synthesis of spiroisoxazoline-β-lactams derived from an alkyl-substituted nitrile oxide (**4.49h**) were also explored (entries 13 and 14). Under conventional heating, the 1,3-dipolar cycloaddition reaction of dipole **4.49h**, bearing a cyclohexyl moiety, with 6-alkylidenepenicillanate **4.50** led to pure spiroisoxazoline-β-lactam **4.51h** in an exceptional yield of 79%. The formation of compounds **4.52h**/**4.53h** was also observed in 15% yield. Conducting the same reaction under microwave irradiation allowed the synthesis of spiro-β-lactam **4.51h** in 60% yield, together with the mixture of **4.52h/4.53h** isolated in low yield (10%). It should be noted that this cycloaddition was highly selective for the synthesis of spiro-β-lactam **4.51h** and yields were comparable with the ones obtained for the cycloaddition of **4.50** with aryl-substituted nitrile oxides.

Both methodologies led to pure chiral spiroisoxazoline-β-lactams **4.51a-h** (with exception of **4.51g** which is obtained in a mixture with **4.53g**). To our surprise, the reactions under microwave irradiation took longer than expected, and in most cases, there was no substantial differences regarding reaction times using conventional heating or microwave irradiation. In addition, we should highlight the synthesis of spiro-β-lactams **4.51** in higher yields under conventional heating (conventional heating: 49-79% *vs* microwave irradiation: 31-60%) as well as the expected cycloadducts in higher overall yields (conventional heating: 52-98% *vs* microwave irradiation: 51-79%).

Table 4.2. 1,3-Dipolar cycloaddition of 6-(*Z*)-(benzoylmethylidene)penicillanate **4.50** with nitrile oxides **4.49**



Entry	**4.49**	R	Met.	Reaction Time (h)	Isolated Yields (ratio)[a]		Overall Yield
1	**4.49b**	*p*-FC_6H_4	Batch	6	**4.51b.** 72%	**4.52b.** trace amounts	72%
2	**4.49b**	*p*-FC_6H_4	MW	1	**4.51b.** 39%	**4.52b/4.53b.** 32% (84:16)	71%
3	**4.49c**	*p*-ClC_6H_4	Batch	8	**4.51c.** 49%	**4.52c/4.53c.** 38% (71:29)	87%
4	**4.49c**	*p*-ClC_6H_4	MW	1	**4.51c.** 41%	**4.52c/4.53c.** 29% (90:10)	70%
5	**4.49d**	*p*-BrC_6H_4	Batch	2	**4.51d.** 49%	**4.52d/4.53d.** 43% (65:35)	92%
6	**4.49d**	*p*-BrC_6H_4	MW	1	**4.51d.** 44%	**4.52d/4.53d.** 33% (58:42)	77%
7	**4.49e**	*p*-$NO_2C_6H_4$	Batch	2	**4.51e.** 54%	**4.52e/4.53e.** 35% (34:66)	98%
8	**4.49e**	*p*-$NO_2C_6H_4$	MW	1	**4.51e.** 47%	**4.52e/4.53e.** 32% (50:50)	79%
9	**4.49f**	*p*-MeC_6H_4	Batch	5	**4.51f.** 50%	**4.52f/4.53f.** 38% (68:32)	88%
10	**4.49f**	*p*-MeC_6H_4	MW	1	**4.51f.** 31%	**4.52f/4.53f.** 20% (85:15)	51%
11	**4.49g**	*p*-$MeOC_6H_4$	Batch	0.5	**4.51g/4.53g.** 52% (69:31)		52%
12	**4.49g**	*p*-$MeOC_6H_4$	MW	0.25	**4.51g/4.53g.** 36% (69:31)	**4.52g.** 21%	57%
13	**4.49h**	Cy	Batch	6	**4.51h.** 79%	**4.52h/4.53h.** 15% (17:83)	94%
14	**4.49h**	Cy	MW	1	**4.51h.** 60%	**4.52h/4.53h.** 10% (10:90)	70%

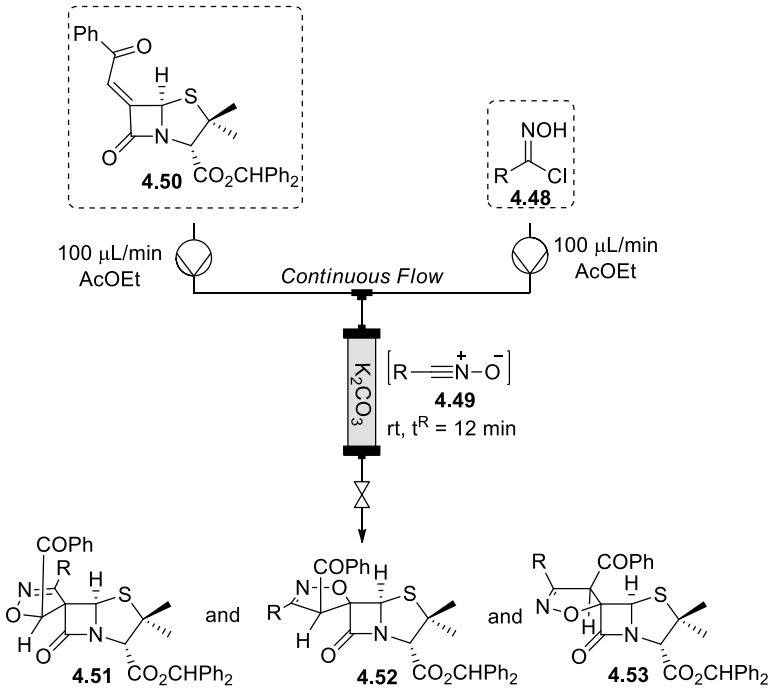
[a] Ratio determined by ^1H NMR

4.2.2. Continuous Flow 1,3-Dipolar Cycloaddition Reactions

Next, in a work developed together with a MSc. student, we have extended our efforts on the development of a continuous flow approach for the 1,3-dipolar cycloaddition reaction of alkylidenepenicillanate **4.50** with nitrile oxides **4.49** (Table 4.3, entries 1-4). The dehydrohalogenation of hydroximoyl chlorides **4.48** for *in situ* generation of the corresponding nitrile oxides **4.49**, relied on the use of a packed-bed reactor filled with potassium carbonate.

The set-up was composed of two inlets, one containing a solution of 6-(*Z*)-(benzoylmethylidene)penicillanate **4.50** and another one containing a solution of the appropriate hydroximoyl chloride **4.48**, with both solutions being mixed right before the entering in the packed-bed reactor. Carrying out the 1,3-dipolar cycloaddition reaction at room temperature with a flow rate of 100 μL/min of each solution led to the expected spiroisoxazoline-β-lactams, in 89% overall yield with the major spiro-β-lactam **4.51a** being obtained in 54% yield (entry 1). The reaction conditions were then optimized. The influence of the residence time, t^R, was investigated by tuning the flow rates of reagents (entry 2). Decreasing the flow rate of each solution to 50 μL/min allowed to increase the overall yield of the reaction to 95%, however, with a slight decrease in the yield of major product **4.51a** to 50%. Unlike to what was observed for reactions carried out in batch or under microwave irradiation, the increase of the temperature to 80 ºC under flow conditions did not lead to better results even with the simultaneous increase of the nitrile oxide equivalents (entries 3 and 4). Considering the above-presented results we selected a flow rate of 100 μL/min together with room temperature and 2 equiv. of the nitrile oxide as the best conditions to continue the study of 1,3-dipolar cycloaddition reactions under continuous flow.

Table 4.3. Optimization of the 1,3-dipolar cycloaddition reaction conditions under continuous flow.



Entry	**4.49**	R	Isolated Yields (ratio)[a]		Overall yields
1	**4.49a**	C_6H_5	**4.51a.** 54%	**4.52a/4.53a.** 35% (33:67)	89%
2	**4.49a**[b]	C_6H_5	**4.51a.** 50%	**4.52a/4.53a.** 45% (45:55)	95%
3	**4.49a**[c]	C_6H_5	**4.51a.** 10%	----	10%
4	**4.49a**[c,d]	C_6H_5	**4.51a.** 22%	----	22%
5	**4.49b**	*p*-FC_6H_4	**4.51b.** 49%	**4.52b/4.53b.** 38% (53:47)	87%
6	**4.49c**	*p*-ClC_6H_4	**4.51c.** 45%	**4.52c/4.53c.** 29% (65:35)	74%
7	**4.49d**	*p*-BrC_6H_4	**4.51d.** 57%	**4.52d/4.53d.** 35% (75:25)	92%
8	**4.49f**	*p*-MeC_6H_4	**4.51f.** 51%	**4.52f/4.53f.** 23% (39:61)	74%
9	**4.49g**	*p*-$MeOC_6H_4$	**4.51g/4.53g.** 54% (51:49)		54%
10	**4.49h**	Cy	**4.51h.** 40%	**4.52h/4.53h.** 3% (39:61)	43%

[a] Ratio determined by ^{1}H NMR
[b] 50 μL/min flow rate, t^R = 24 min
[c] Reaction carried out at 80 ºC
[d] 4 equiv. nitrile oxide

Further 1,3-dipolar cycloaddition reactions of 6-(Z)-(benzoylmethylidene)penicillanate **4.50** with nitrile oxides **4.49b-h** were explored (Table 4.3, entries 5-10). Using *p*-halophenyl-nitrile oxides (R = F, Cl, Br) the expected spiro-β-lactam were obtained in excellent overall yields, ranging from 74% to 92% (entries 5-7). Chiral spiroisoxazoline-β-lactams **4.51b**, **4.51c** and **4.51d** were obtained as major products in 49%, 45% and 57%, respectively. The continuous flow also proved to be a viable approach for the synthesis of spiro-β-lactams using nitrile oxides containing

electron-donating groups at phenyl *para*-position, giving the expected cycloadducts in yields as good as under conventional heating (entries 8 and 9). Unfortunately, among the library of aryl-substituted nitrile oxides used in the present work it was not possible to study the behaviour of nitrile oxide **4.49e** (R = *p*-$NO_2C_6H_4$) under flow conditions due to its partial insolubility in a wide range of solvents.

Finally, the cycloaddition reaction of alkyl-substituted dipole **4.49h** was studied. In this case, the major spiro-β-lactam **4.51h** was obtained in moderate yield (40%) together with a mixture of **4.52h**/**4.53h** (39:61 ratio) in 3% yield. The synthesis of chiral spiroisoxazoline-β-lactams **4.51h**/**4.52h**/**4.53h**, containing a cyclohexane substituent, under continuous flow conditions proved to be less efficient than under conventional heating or microwave irradiation.

The results have shown that the use of continuous flow is an interesting alternative approach to the other methodologies discussed in the present chapter, to carry out 1,3-dipolar cycloaddition reactions leading to chiral spiroisoxazoline-β-lactam. It should also be mentioned that the continuous flow technique allowed the synthesis of the target spiro adducts with a very short reaction time (t^R = 12 minutes) having the great advantage of enabling easy scale-up processes. On the other hand, as outlined in Figure 4.4, continuous flow proved to be the best methodology for the synthesis of the major products (**4.51**) with a production rate ranging from 200 to 400 mg/h of spiro-β-lactams **4.51**, values considerable higher than the ones observed for batch and microwave-induced methodologies.

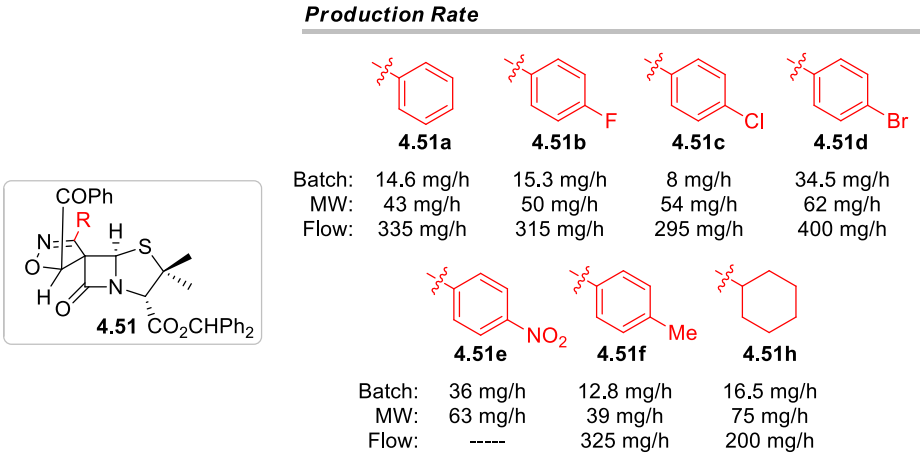
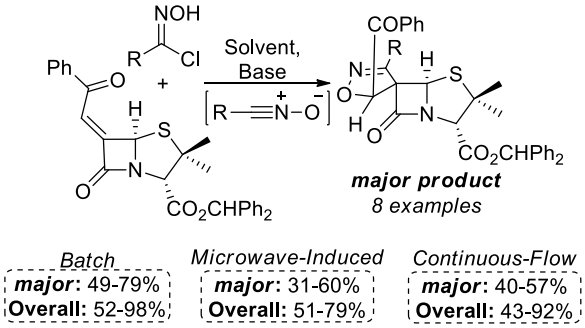


Figure 4.4. Production rates in mg/h for the synthesis of spiro-β-lactams **4.51**.

4.3. Conclusions

In the present chapter a 1,3-dipolar cycloaddition-based approach to a novel family of chiral spiro-β-lactams has been disclosed. 6-(*Z*)-(Benzoylmethylidene)penicillanate reacted with *in situ* generated nitrile oxides to afford novel spiroisoxazoline-β-lactams with the diastereoselective creation of two consecutive stereogenic centers, including a quaternary chiral center. Spiro[isoxazoline-4',6-penicillanates] were firstly synthesized in this work and obtained with stereo- and regioselectivity. The major products results from the addition of the dipole to the less sterically hindered α-side of the 6-alkylidenepenicillanate and the attack of the dipole's oxygen to the terminal carbon of the exocyclic double bond of the alkylidenepenicillanate.

This works also allowed to demonstrate that these spirocyclic adducts can be obtained using three different methodologies: conventional heating, microwave irradiation and continuous flow (Scheme 4.15). Both conventional heating and continuous flow led to the major products in high yields (conventional heating: 49-79%; continuous flow: and 40-57%), affording also the isomeric cycloadducts in high overall yields (conventional heating: 52-98%; continuous flow: 43-92%). The microwave-induced methodology, led to moderate overall yields (51-79%) with the major products being obtained in yields ranging from 31% to 61%. Nevertheless, continuous flow 1,3-dipolar cycloaddition reaction stands out for allowing very short reaction times, and higher production rates than batch or microwave-induced methodologies, with the major products being obtained in up to 400 mg/h.



Scheme 4.15. Overview of the reactivity and methodologies studied in Chapter 4 for the synthesis of chiral spiro-β-lactams.

4.4. References

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CHAPTER 5

Anticancer Activity of Chiral Alkylidene-β-Lactams and Alkylidene-γ-Lactams

Abstract

This chapter describes the synthesis of alkylidene-β-lactams sulfone and sulfoxide derivatives containing the penicillanate core. Herein, is also covered the synthesis of the first chiral bicyclic alkylidene-γ-lactams fused to a thiazolidine ring. The *in vitro* anticancer activity of chiral alkylidene-β-lactams and alkylidene-γ-lactams against four human cancer cell lines was accessed. Further studies aiming to get insight into the effect of the most promising compounds on cell death mechanism, reactive oxygen species generation as well as the evaluation of their ability to act as MMP-9 inhibitors were also unveiled.

Chapter 5 - Anticancer Activity of Chiral Alkylidene-β-Lactams and Alkylidene-γ-Lactams

5.1. Introduction

The first evidence of a hominin tumour dates to 1.98 million years ago, where an osteogenic tumour affected a male *Australpithecus sediba* with a developmental stage equivalent to a human child of approximately 12 years old.[1] According to World Health Organization (WHO) cancer is defined as a group of diseases caused by an unrestrained cell growth that can spread or invade nearby tissues or other parts of the body leading to new tumours. The latter process is called metastasizing.[2] This is triggered by some abnormalities in cell's internal regulatory mechanisms caused by exogenous (*e.g.* radiation, chemicals, lifestyle) and/or endogenous factors (*e.g.* age, genetics and heredity).[3] This leads to some behaviours typical of cancer cells, such as: grow in the absence of signals, ignore signals that induces apoptosis, and trick or hide from the immune system.[4] Figure 5.1 illustrates a representation of normal and cancer cells.

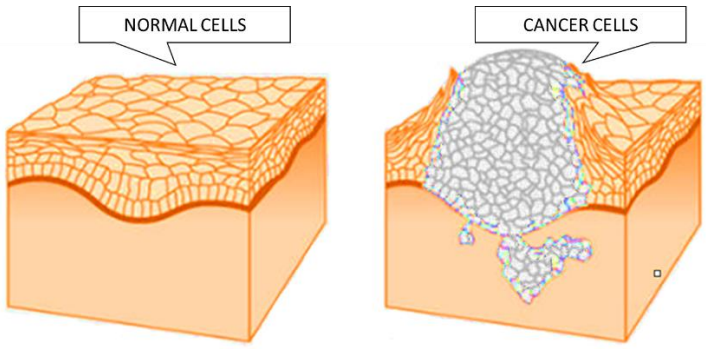


Figure 5.1. Illustration of normal and cancer cells (adapted from NIH).

Nowadays, cancer is a major public health problem and is one of the leading causes of death, being responsible for nearly 10 million deaths annually. The high number of newly diagnosed cancers and its associated mortality is a reflex of population growth and aging, due to human life expectancy that has been increasing at a high rate.[5] Moreover, both cancer and cardiovascular diseases are the leading causes of death between the age of 30 and 70 years. In fact, in high developed countries cancer has surpassed cardiovascular diseases as the prominent cause of premature deaths.[6]

Cancer remains as one of the most difficult diseases to treat, mainly due to the rapid development of drug-resistant cancers (over 90% of cancer patients deaths is due to

drug resistance), as well as the low specificity of some anticancer agents with the associated side effects.[7] Thus, a major challenge of medicinal chemists around the world to overcome some of these obstacles, is the development of more efficient and selective cancer therapies. Chemotherapy is still one of the main methods of cancer treatment, and the pursuit for novel and safer anticancer agents is still a hot topic. This is very evident by looking at the progress that has been made to increase the specificity of cancer treatment, from the discovery and use of nitrogen mustards, as general cytotoxic agents in the 1940s, to Vinca alkaloids in 1960, and more recently to the use of a panoply of inhibitors which target specific receptors and pathways.[8] In parallel, new therapeutic strategies, based on combined anticancer therapies, have also been employed to fight tumour resistance and low drug efficacy. The combination of chemotherapy with other therapies such as immunotherapy, gene therapy, radiotherapy and photodynamic therapy has also been explored to maximize the therapeutic efficiency.[9]

Notwithstanding these alternative strategies to fight cancer, the focus of the work in the present chapter will be the development of novel anticancer agents. Thus, a summary on anticancer agents will be presented to provide a better understanding on this thematic. The literature is very rich in reviews focused on anticancer agents, with several review articles describing the behaviour of a wide range of chemical moieties against cancer.[10]

Examples of famous anticancer agents approved for clinical use are illustrated in Figure 5.2. Among the represented examples it is possible to find a wide diversity of chemical structures, while some of them have simple structures others have more complex ones. They also differ on their source since the selection includes a combination of natural occurring and synthetic compounds.

From the beginning of times to the present day, nature always have inspired scientists to their greatest inventions. It is known that nature provides an entire pool of compounds that might offer an unlimited source of raw materials for drug discovery.[11] The discovery of anticancer agents from natural sources, in the past century, but which are still in use in the 21st century, should motivate and inspire all scientists. The most prominent examples are vincristine, a vinca alkaloid, discovered in 1950s and obtained from *Catharanthus roseus*, and camptothecin first isolated in 1966 from the bark of *Camptotheca acuminata*.[12] Paclitaxel and doxorubicin are other examples of well-known

anticancer agents produced and isolated from natural sources (*Taxus* sp. and *Streptomyces peucetius* var. *caesius*, respectively).[13]

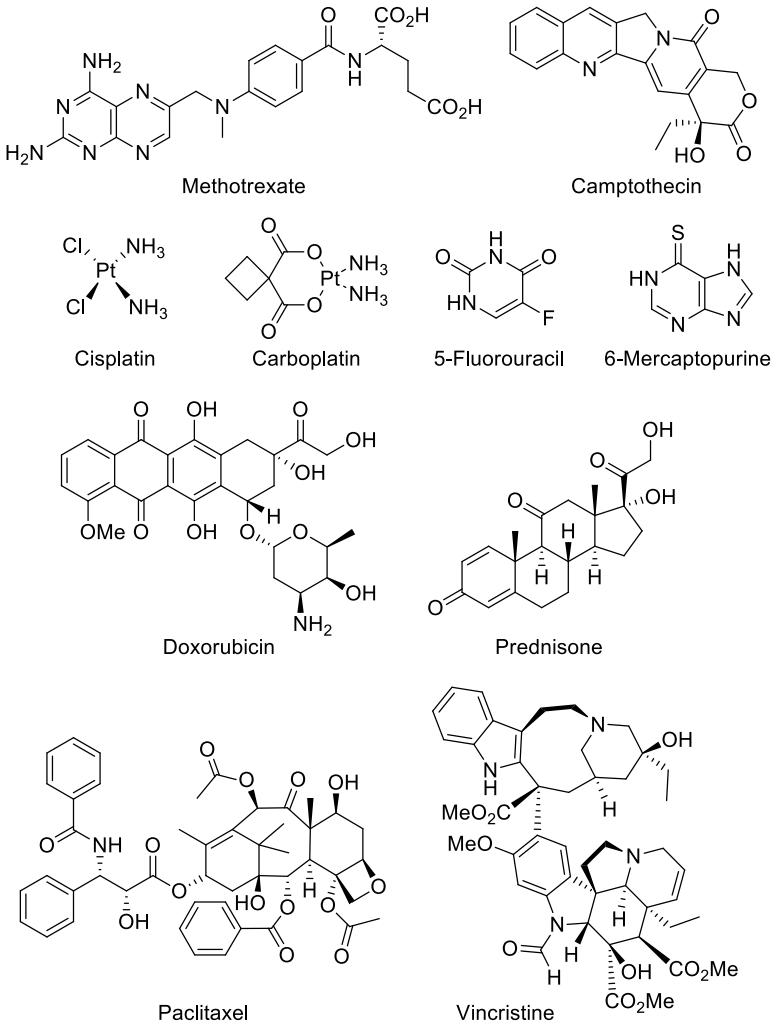


Figure 5.2. Well-known anticancer agents in clinical use.

On the other hand, small modifications of natural compounds can also lead to very active synthetic drugs. Famous examples of those synthetic transformations that can provide potent anticancer agents are 5-fluoruracil, 6-mercaptopurine and methotrexate. Both 5-fluoroacil and 6-mercaptopurine are modified pyrimidine and purine derivatives, two nitrogen-containing bases of nucleic acids.[14] Methotrexate, a folate antagonist, is another example of a synthetic drug with a highly structural similarity with a natural compound (folic acid) having a wide range of biological properties including anticancer activity.[15] Corticosteroids are a well-known class of anti-inflammatory drugs, however

some corticosteroids are also used in the treatment of diverse pathologies. An example of a steroid with that versatility is prednisone, which is also used as an anticancer agent.[16] Platinum-based compounds like cisplatin and carboplatin are another examples of well-succeeded chemotherapeutic agents with its clinical introduction in the mid of 20th century and still widely used these days.[17]

As previously mentioned in this dissertation, it is well stablished that the β-lactam ring is the core structure of a wide range of successful antibiotics. Nevertheless, a wide range of new pharmacologically active β-lactams were discovered displaying a range of other biological activities (*e.g.* antifungal, antiviral, anti-inflammatory, tryptase inhibitors).[18] Additionally, there is also a considerable number of known examples of anticancer agents containing the β-lactam ring in their structure.[19]

Among a wide range of β-lactamic derivatives, alkylidene-β-lactams stood out by being a class of attractive β-lactams that can be used as a scaffold for a wide range of synthetic transformations by exploring the reactivity of the C=C double bond (*e.g.* cycloaddition reactions, olefin metathesis, Michael additions).[20] Furthermore, alkylidene-β-lactams may act as enzymatic inhibitors [*e.g.* human leukocyte elastase, $IC_{50} \geq 0.5$ μM; matrix metalloproteinases (MMPs), $IC_{50} \geq 4$ μM; β-lactamase $IC_{50} \geq 5$ nM], and some derivatives show antioxidant properties.[21] Veinberg *et al.* demonstrated that alkylidene-β-lactams can also exhibit cytotoxic activity against some human and mouse tumour cell lines, namely HT-1080, MG-22A, B16 and Neuro 2A cell lines with IC_{50} values as low as 0.9 μM (Figure 5.3).[22]

The alkylidene-γ-lactam ring is also present in the core of natural and synthetic compounds showing biological properties namely anticancer (IC_{50} values ≥ 20 μM) or analgesic activity (Figure 5.3).[23]

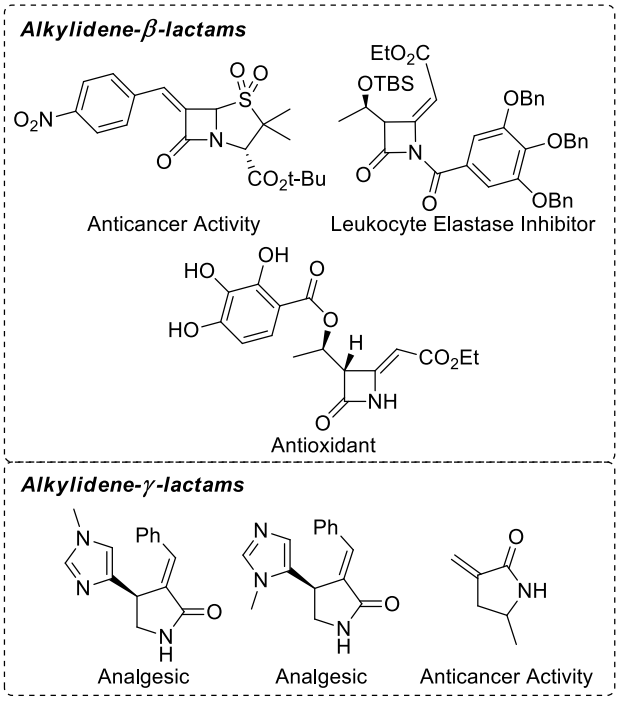


Figure 5.3. Examples of biologically active alkylidene-lactams.

The work presented in this chapter aims to unveil the bioactive profile of alkylidenepenicillanates as anticancer agents. A library of alkylidene-β-lactams and alkylidene-γ-lactams, designed to draw structure–activity relationships (SAR), has been built. The synthesized alkylidene-lactams were assayed for their *in vitro* cytotoxicity against four tumour cell lines, and further studies were carried out with the more promising derivatives in order to get an insight into the induced cell death mechanism.

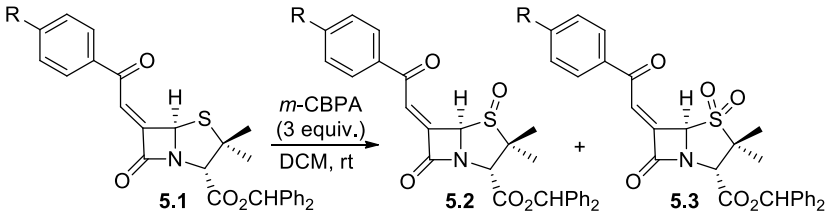
5.2. Anticancer Activity of Chiral Alkylidene-β-Lactams and Alkylidene-γ-Lactams

5.2.1. Synthesis of Chiral Alkylidene-β-Lactams and Alkylidene-γ-Lactams

The experimental work started with the synthesis of a library of eighteen 6-alkylidenepenicillanates (**5.1**, **5.2** and **5.3**). The synthesis of 6-alkylidenepenicillanates **5.1** is described in Chapter 2. Alkylidenes **5.2** and **5.3** were also selected as our target molecules since previous studies on the anticancer activity of alkylidene-penicillanates, have shown that the oxidation to the corresponding sulfoxide and sulfone derivatives can lead to increased biological activity.[22]

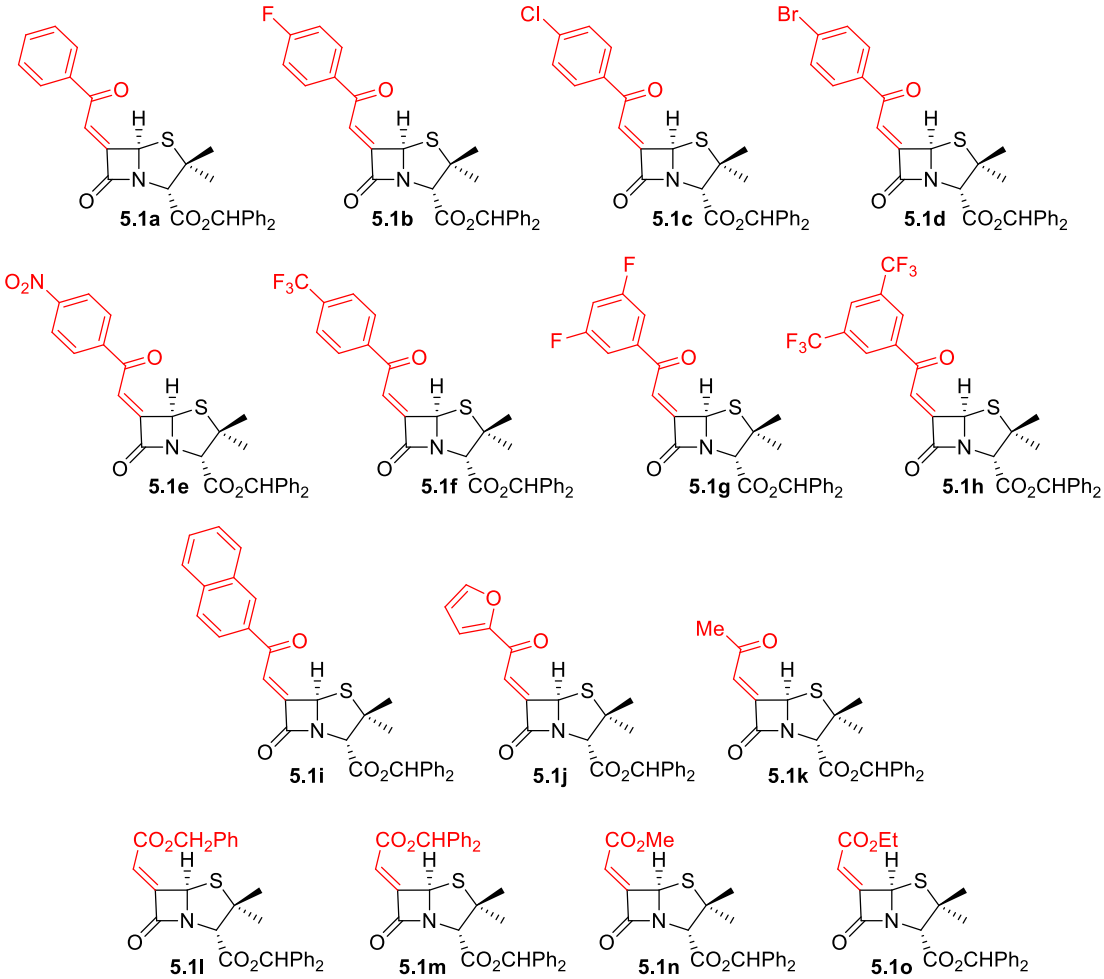
Sulfoxides **5.2** and sulfones **5.3** were prepared from the oxidation of the sulphur atom of 6-alkylidenepenicillanates **5.1a,e** (Table 5.1). Carrying out the oxidation of 6-(*Z*)-(benzoylmethylene)penicillanate (**5.1a**) with 3 equivalents of *m*-CPBA, for 30 minutes, the corresponding sulfoxide **5.2a** was obtained as major product in 75% yield, together with sulfone **5.3a** isolated in 20% yield (entry 1). With the increase of the reaction time to 17 or 24 hours, sulfone **5.3a** becomes the major product, isolated in yields ranging from 56% to 63%, with sulfoxide **5.2a** being isolated in moderate yields (21-29%) (entries 2 and 3). Treatment of 6-alkylidenepenicillanate **5.1e** with *m*-CPBA for 17 hours also afforded the desired oxidation products **5.2b** and **5.3b** in 77% overall yield (29% and 48%, respectively) (entry 4). In this specific case, longer reaction time did not lead to an increase of the isolated yields (entry 5).

Table 5.1. *m*-CPBA oxidation of 6-alkylidenepenicillanates **5.1a,e** leading to sulfoxide and sulfone derivatives.



Entry	**5.1**, R	Reaction time	Products, Isolated yields
1	**5.1a**, 4-H	0.5 h	**5.2a** 75%, **5.3a** 20%
2	**5.1a**, 4-H	17 h	**5.2a** 27%, **5.3a** 56%
3	**5.1a**, 4-H	24 h	**5.2a** 21%, **5.3a** 63%
4	**5.1e**, 4-NO_2	17 h	**5.2b** 29%, **5.3b** 48%
5	**5.1e**, 4-NO_2	24 h	**5.2b** 21%, **5.3b** 37%

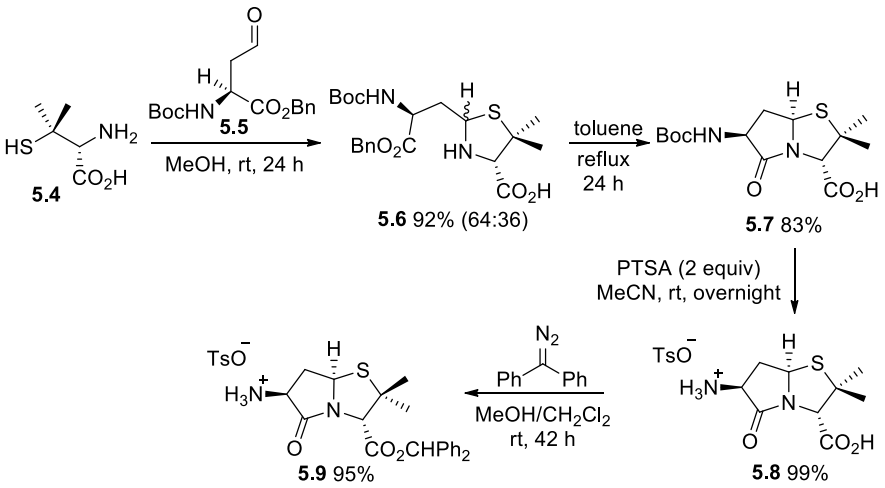
Previously synthesized 6-alkylidenepenicillanates



In order to evaluate if the β-lactam ring is a pharmacophoric feature associated with the anticancer activity, alkylidene-γ-lactams were also synthesized. The target alkylidene-γ-lactams are analogues of three known 6-alkylidenepenicillanates, however, with the replacement of the four-membered β-lactam ring by a five-membered γ-lactam ring.

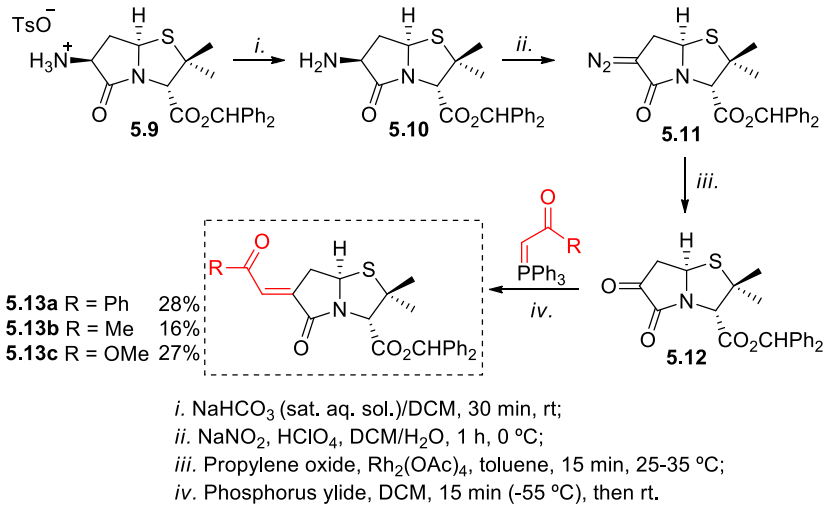
The γ-lactam-thiazolidine bicyclic derivative **5.9** was not commercially available, thus we developed a multistep strategy starting from *D*-penicillamine (**5.4**), an amino acid obtained from the degradation of penicillins, and an L-aspartic acid derived aldehyde **5.5** (Scheme 5.1).[24] *L*-Aspartic acid derived aldehyde **5.5**[25] was synthesized through a method described for the preparation of the *R* enantiomer which involves the initial reduction of an acid to the corresponding alcohol, followed by a Swern oxidation.[26] Thiazolidine **5.6** was obtained (92% yield) from the condensation reaction of D-penicillamine with aldehyde **5.5**, followed by the formation of bicyclic γ-lactam **5.7** (83% yield), through a intramolecular lactamization reaction of thiazolidine **5.6** under refluxing toluene over 24 hours.

A strategy described by Chauvett *et al.* was applied for the *N*-Boc deprotection of compound **5.7**, by its treatment with *p*-toluenesulfonic acid (PTSA), leading to the corresponding tosylate salt **5.8** in quantitative yield.[27] Then, the synthetic methodology involved the reaction of compound **5.8** with diphenyldiazomethane leading to the corresponding γ-lactam benzhydryl ester **5.9** in 95% yield.



Scheme 5.1. Synthesis of the γ-lactam bicyclic core.

The final steps of this newly developed synthetic route aiming at the synthesis of novel chiral alkylidene-γ-lactams are outlined in Scheme 5.2. The neutralization reaction of γ-lactam benzhydryl ester **5.9** with an aqueous solution of $NaHCO_3$ afforded amino-γ-lactam **5.10** in 96% yield. Taking advantage of a protocol described by Sheehan and Commons for β-lactamic systems, diazo-γ lactam **5.11** was obtained in 97% yield by the treatment of amino-γ-lactam **5.10** with sodium nitrite in the presence of $HClO_4$ at 0 ºC. Further conversion of the diazo-γ-lactam **5.11** into the corresponding oxo-γ-lactam **5.12** by the treatment with propylene oxide in the presence of rhodium (II) acetate dimer in toluene was carried out. Finally, the novel chiral alkylidene-γ-lactams **5.13a-c** were obtained via a Wittig reaction of oxo-γ-lactam **5.12** with the appropriate phosphorus ylide in moderate overall yields, ranging from 16% to 28% (from **5.9**).



Scheme 5.2. Synthesis of chiral alkylidene-γ-lactams derived from *D*-penicillamine.

The knowledge about the three-dimensional conformation of alkylidene-γ-lactams **5.13** is important to determine if this class of compounds share structural similarity with their β-lactam analogues, namely the butterfly-like structure of the penicillanate core. Hence, the molecular structure of these interesting chiral alkylidene-γ-lactams, was unambiguously established by single-crystal X-ray diffraction of alkylidene-γ-lactam **5.13c**. Figure 5.4 depicts its ORTEP diagram. The most relevant bond distances (Å) and angles (º) are reported in the figure caption. This derivative crystallized as colourless prisms in the orthorhombic system within the $P2_12_12_1$ space group, showing one molecule

per asymmetric unit. Its molecular structure consists of an oxohexahydropyrrolo[2,1-*b*]thiazole derivative, with a dihedral angle between the two fused rings of 55.7(2) º. The absolute structure was determined by a Flack analysis (803 Friedel pairs, η=0.10(10)) that unambiguously assigns the *S,R* configuration to the chiral centers C3 and C7a, respectively. All distances and angles are within the expected values for similar compounds.[28] This study allowed to unambiguously confirm the geometry of the exocyclic carbon-carbon double bond as being the *E* isomer.

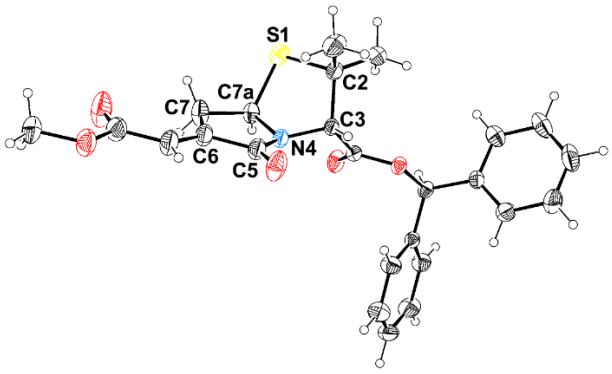


Figure 5.4. ORTEP representation of compound **5.13c**, using 30% level ellipsoids. Selected bond lengths: S1–C2 1.822(5) Å, C2–C3 1.558(6) Å, C3–N4 1.437(5) Å, N4–C7a 1.434(5) Å, N4–C5 1.354(5) Å, C5–C6 1.481(7) Å, C6–C7 1.478(6) Å, C7–C7a 1.520(6) Å, S1–C7a 1.820(5) Å. Selected bond angles: C7a–S1–C2 94.8(2)º, C5–N4–C7a 114.1(4)º, C7a–N4–C3 114.6(3)º, N4–C5–C6 106.7(4)º.

5.2.2. Cell Biology

5.2.2.1. Anticancer activity studies

Initially, *in vitro* studies regarding the anticancer activity of alkylidene-β-lactams **5.1a-h**, **5.2** and **5.3** were carried out against melanoma (A375) and esophageal (OE19) cancer human cell lines. The comparison of the anticancer activity of the tested compounds was made by analysing the corresponding IC_{50} values, calculated from the dose-response curves, which allowed to identify structure-activity relationships. The IC_{50} values against A375 and OE19 cell lines with 48 h of incubation are presented in Table 5.2.

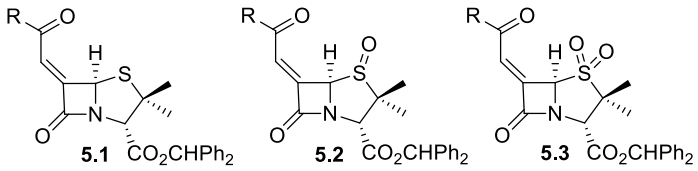
Alkylidene-β-lactam **5.1a** containing an unsubstituted benzoyl moiety showed moderated activity against A375 cell line (IC_{50} = 22.8 μM), and good activity against

OE19 cell line (IC_{50} = 12.8 μM). The introduction of weak electron withdrawing groups (R = F, Cl, Br) at phenyl group *para*-position was also assessed. Comparing with unsubstituted derivative **5.1a**, compound **5.1b** (R = *p*-fluorophenyl) was the only one showing lower IC_{50} values against both cell lines (17.8 μM and 12.1 μM, for A375 and OE19 cell lines, respectively). A decrease in cytotoxicity could be observed for both *p*-CF_3 and *p*-NO_2 phenyl-substituted derivatives. The effect of the introduction of this strong electron withdrawing groups was more pronounced for the nitro derivative (**5.1e**) which showed IC_{50} values of 50.3 μM and 44.1 μM, for A375 and OE19 cell lines, respectively.

The effect of substituents at *meta*-positions of the phenyl group was also evaluated. Interestingly, disubstituted 3,5-difluorophenyl derivative **5.1g** showed IC_{50} values comparable to those obtained for the phenyl (**5.1a**) or *p*-fluorophenyl (**5.1b**) derivatives in A375 cell line, while the 3,5-CF_3 phenyl-substituted derivative (**5.1h**) presented weak activity. Similarly, the newly synthesized sulfoxide **5.2b** and both sulfones **5.3a** and **5.3b** showed poor cytotoxicity against both cell lines. Nevertheless, sulfoxide **5.2a**, derived from alkylidene-β-lactam **5.1a**, proved to be very active against A375 melanoma cell line with an IC_{50} value of 4.5 μM.

Table 5.2. IC_{50} values and respective confidence intervals at 95% (CI_{95}) of 6-(benzoylmethylene)penicillanate derivatives **5.1a-h**, **5.2** and **5.3** in melanoma (A375) and esophageal (OE19) cell lines. Incubation time: 48 h. Values were determined by dose-response sigmoidal fitting. Each experiment was performed in triplicate and repeated in at least two independent experiments ($n \geq 2$).

Compound	R	A375 (48 h)		OE19 (48 h)	
		IC_{50} (μM)	CI_{95}	IC_{50} (μM)	CI_{95}
5.1a	C_6H_5	22.8	[18.1;28.7]	12.8	[10.5;15.6]
5.1b	*p*-FC_6H_4	17.8	[13.6;23.3]	12.1	[10.7;13.6]
5.1c	*p*-ClC_6H_4	48.6	[very wide]	14.6	[10.1;21.1]
5.1d	*p*-BrC_6H_4	29.7	[23.3;38.0]	13.8	[11.3;16.8]
5.1e	*p*-$NO_2C_6H_4$	50.3	[32.8;77.3]	44.1	[29.2;66.8]
5.1f	*p*-$CF_3C_6H_4$	26.7	[19.2;37.2]	15.6	[11.4;22.1]
5.1g	3,5-$F_2C_6H_3$	19.1	[17.7;20.5]	17.6	[13.6;22.8]
5.1h	3,5-$(CF_3)_2C_6H_3$	39.2	[26.3;58.5]	48.5	[very wide]
5.2a	C_6H_5	4.5	[4.2;4.9]	23.7	[15.9;35.3]
5.2b	*p*-$NO_2C_6H_4$	48.2	[27.6;84.2]	92.4	[very wide]
5.3a	C_6H_5	17.5	[12.0;25.4]	53.1	[33.4;84.4]
5.3b	*p*-$NO_2C_6H_4$	76.7	[37.7;156.2]	95.5	[very wide]
Cisplatin	----	8.4	[7.4;9.5]	96.3	[85.8; 108.2]



Anticancer activity studies were extended to the screening of selected 6-(benzoylmethylene)penicillanates with an incubation time of 72 hours (Table 5.3). No significant differences were observed when compared to the 48 h assays, in the case of A375 cell line. However, with 72 h of incubation the cytotoxic effect was higher for OE19 cell line than at 48 h for both tested compounds (**5.1a**, IC_{50} = 8.6 μM; **5.2b**, IC_{50} = 10.7 μM).

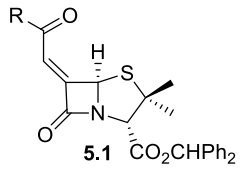
Table 5.3. IC_{50} values and respective confidence intervals at 95% (CI_{95}) of 6-(1-benzoylmethylene)penicillanate derivatives **5.1a,b** and **5.2a** in melanoma (A375) and esophageal (OE19) cell lines. Incubation time: 72 h. Values were determined by dose-response sigmoidal fitting. Each experiment was performed in triplicate and repeated in at least two independent experiments ($n \geq 2$).

Compound	A375 (72 h)		OE19 (72 h)	
	IC_{50} (μM)	CI_{95}	IC_{50} (μM)	CI_{95}
5.1a	22.4	[18.6;26.8]	8.6	[5.4;13.8]
5.1b	19.9	[15.0;26.4]	10.7	[8.1;14.2]
5.2a	39.9	[27.5;57.9]	----	----

To get further details regarding structure-activity relationships of 6-alkylidenepenicillanates as anticancer agents, we studied the activity of 6-alkylidenepenicillanates bearing 2-naphthoyl (**5.1i**), 2-furoyl (**5.1j**) and acetyl (**5.1k**) substituents as well as ester groups (**5.1l-o**) (Table 5.4). It was observed that the replacement of the benzoyl group of **5.1a** by substituents incorporating other aromatic systems, such as naphthoyl and furoyl groups, or the replacement by an acetyl group led to higher IC_{50} values. Nevertheless, interesting results were observed in the study of alkylidene-β-lactams containing an ester moiety at the carbon-carbon double bond. Despite the moderate activity of the methyl ester derivative (**5.1n**) against A375 and OE19 cell lines, both alkylidene-β-lactams **5.1l** and **5.1o** substituted with a benzyl and an ethyl ester, respectively, showed low IC_{50} values against both cell lines (**5.1l**: 7.1 μM and 8.2 μM and **5.1o**: 7.0 μM and 11.2 μM for A375 and OE19 cell lines, respectively). In addition, the 6-alkylidenepenicillanate containing with a benzhydryl ester (**5.1m**) as substituent in the exocyclic carbon-carbon double bond, also showed good activity against the melanoma A375 cell line with an IC_{50} value of 9.1 μM. However, it was somewhat less active than benzyl ester alkylidene-β-lactam **5.1l**, suggesting that the introduction of a second phenyl group in the ester substituent of the exocyclic carbon-carbon double bond impairs its biological activity.

Table 5.4. IC_{50} values and respective CI_{95} of compounds **5.1i-o** in melanoma (A375) and esophageal (OE19) cell lines. Incubation time: 48 h. Values were determined by dose-response sigmoidal fitting. Each experiment was performed in triplicate and repeated in at least two independent experiments ($n \geq 2$).

Compound	R	A375 (48 h)		OE19 (48 h)	
		IC_{50} (μM)	CI_{95}	IC_{50} (μM)	CI_{95}
5.1i	2-naphtyl	36.5	[20.2;65.9]	19.5	[14.2;26.9]
5.1j	2-furyl	26.0	[18.0;37.6]	54.3	[36.3;81.1]
5.1k	Me	63.0	[45.0;88.1]	51.9	[34.0;79.1]
5.1l	OCH_2Ph	7.1	[6.6;7.7]	8.2	[6.7;10.0]
5.1m	$OCHPh_2$	9.1	[6.4;12.8]	21.6	[16.0;29.3]
5.1n	OMe	24.6	[17.6;34.3]	20.2	[14.7;27.6]
5.1o	OEt	7.0	[5.9;8.4]	11.2	[9.8;12.7]
Cisplatin	----	8.4	[7.4;9.5]	96.3	[85.8; 108.2]



5.1

This set of compounds also had their anticancer activity studied with 72 h of incubation time (Table 5.5). Similarly, as previously observed for the early studied 6-alkylidenepenicillanates (see Table 5.3) it only led to better results in OE19 cell line. It is noteworthy to highlight the low IC_{50} values, 4.6 μM and 5.3 μM, observed for both compounds **5.1l** and **5.1o**, respectively, against this cell line.

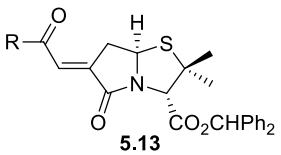
Table 5.5. IC_{50} values and respective CI_{95} of 6-(1-benzoylmethylene)penicillanate derivatives **1l,m** and **1o** in melanoma (A375) and esophageal (OE19) cell lines. Incubation time: 72 h. Values were determined by dose-response sigmoidal fitting. Each experiment was performed in triplicate and repeated in at least two independent experiments (n ≥ 2).

Compound	A375 (72 h)		OE19 (72 h)	
	IC_{50} (μM)	CI_{95}	IC_{50} (μM)	CI_{95}
1l	13.2	[11.8;14.7]	4.6	[4.3;5.0]
1m	19.8	[13.8;28.3]	----	----
1o	14.2	[12.2;16.6]	5.3	[4.9;5.7]

After screening the whole library of alkylidene-β-lactams we carried out *in vitro* anticancer assays to evaluate the potential of the novel alkylidene-γ-lactams. From this study it was possible to conclude that despite the high structural similarity with the β-lactam analogues, as confirmed by the X-ray diffraction study, alkylidene-γ-lactams **5.13** did not show any cytotoxic activity against both A375 and OE19 cell lines, resulting in very high IC_{50} values (Table 5.6). These results confirm the crucial role of the β-lactam nucleus in the anticancer activity.

Table 5.6. IC_{50} values and respective CI_{95} of compounds **5.13** in melanoma (A375) and esophageal (OE19) cell lines. Incubation time: 48 h. Values were determined by dose-response sigmoidal fitting. Each experiment was performed in triplicate and repeated in at least two independent experiments (n ≥ 2).

Compound	R	A375 (48 h)		OE19 (48 h)	
		IC_{50} (μM)	CI_{95}	IC_{50} (μM)	CI_{95}
5.13a	Ph	>100	----	73.3	[42.2;127.3]
5.13b	Me	>100	----	>100	----
5.13c	OMe	>100	----	99.7	[very wide]

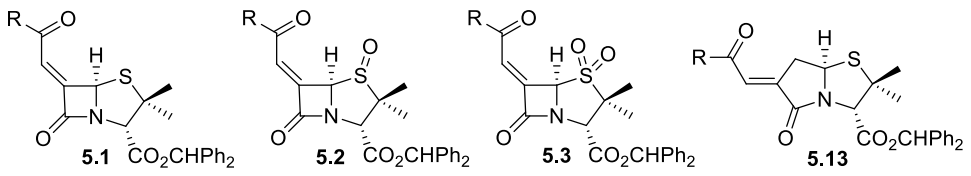


5.13

Next, we completed our anticancer studies extending the assays to HT-1080 (fibrosarcoma cell line) and H1299 (human lung cancer cell line) cell lines, by screening a selection of 6-alkylidenepenicillanates comprising distinct chemical moieties (Table 5.7). Overall, the screening assays indicated a lack of sensibility of these two cell lines against the tested compounds leading to higher IC_{50} values, however, we should highlight the results obtained for compound **5.1l** with IC_{50} values of 11.7 μM and 9.44 μM, against HT-1080 and H1299 cell lines, respectively. It should be noted that this derivative is also one the most active compounds against A375 and OE19 cell lines.

Table 5.7. IC_{50} values and respective CI_{95} of compounds **5.1a**,**b**, **5.1j-m**, **5.1o**, **5.2a**, **5.3a** and **5.13b** in HT-1080 and H1299 cell lines. Incubation time: 48 h. Values were determined by dose-response sigmoidal fitting. Each experiment was performed in triplicate and repeated in at least two independent experiments ($n \geq 2$).

Compound	R	HT-1080 (48 h)		H1299 (48 h)	
		IC_{50} (μM)	CI_{95}	IC_{50} (μM)	CI_{95}
5.1a	C_6H_5	21.1	[18.5;24.0]	39.0	[25.4;59.8]
5.1b	*p*-FC_6H_4	11.2	[9.9;12.6]	25.2	[20.0;31.6]
5.1j	2-furyl	45.5	[35.3;48.8]	61.5	[34.7;109.0]
5.1k	Me	20.8	[18.3;23.5]	24.2	[18.2;32.1]
5.1l	OCH_2Ph	11.7	[10.7;12.9]	9.4	[8.0;11.1]
5.1m	OMe	15.2	[13.3;17.4]	22.4	[17.5;28.8]
5.1o	OEt	25.1	[20.4;30.9]	15.0	[12.1;18.7]
5.2a	C_6H_5	65.5	[36.4;117.8]	92.4	[very wide]
5.3a	C_6H_5	19.5	[16.4;23.3]	50.1	[very wide]
5.13b	Me	>100	----	>100	----
Cisplatin	----	16.3	[14.5;18.3]	69.1	[61.4;77.7]



Carrying out the screening with 72 h of incubation did not improve the observed results, as seen in the previous assays. The exception was compound **5.1o** which showed better activity against HT-1080 cell line after 72 h of incubation (IC_{50} = 19.7 μM) (Table 5.8).

Table S3. IC_{50} values and respective CI_{95} of compounds **5.1b**, **5.1l,m**, and **5.1o** in HT-1080 and H1299 cell lines. Incubation time: 72 h. Values were determined by dose-response sigmoidal fitting. Each experiment was performed in triplicate and repeated in at least two independent experiments (n ≥ 2).

Compound	HT-1080 (72 h)		H1299 (72 h)	
	IC_{50} (μM)	CI_{95}	IC_{50} (μM)	CI_{95}
5.1b	17.8	[14.3;22.1]	----	----
5.1l	12.7	[10.8;14.9]	23.5	[very wide]
5.1m	11.8	[10.3;13.5]	24.9	[very wide]
5.1o	19.7	[15.7;24.7]	27.7	[16.5;46.6]

5.2.2.2. Types of cell death and cell morphology

To get a deeper knowledge about the effect of the compounds on cell viability and the induced mechanism of cell death on A375 cells, flow cytometry studies were performed with four selected alkylidene-β-lactams (**5.1l**, **5.1m**, **5.1o** and **5.2a**) in two concentrations (Figure 5.5). It was possible to conclude that regardless of the studied compound, the treatment with the lower concentration (5 μM) did not lead to significant changes when compared to control cells. On the other hand, when cells were treated with 10 μM, major alterations on the induced mechanism of cell death were visible. Both compounds **5.1l** and **5.1o** induce a significant decreasing in the viable cells (**5.1l**: 56.25%; **5.1o**: 63.00%), an increasing in the late apoptosis/necrosis (**5.1l**: 13.00%; **5.1o**:11.50) and in necrosis (**5.1l**: 24.00%; **5.1o**: 20.75%), comparing to control cells (V: 93.63%; LA/N: 1.83%; N: 1.17%). Despite the lower values observed for apoptosis/necrosis and necrosis, compound **5.1m** presented the same profile (V: 73.50%; LA/N: 7.75%; N: 14.75%). In contrast, compound **5.2a** (V: 89.17%; LA/N: 4.17%; N: 3.00%) did not show major alterations in comparison with control cells, suggesting that this compound is reducing

the metabolic activity of A375 cells without inducing apoptosis and/or necrosis. A plausible rationalization for this observation is that compound **5.2a** may induce the cells to enter in a state of quiescence, where cells stop dividing.[29]

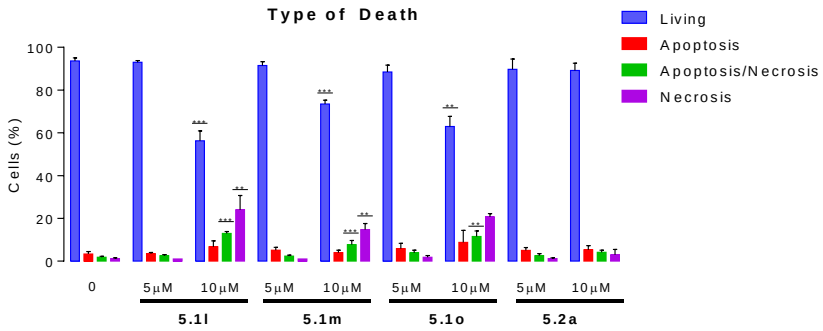


Figure 5.5. Viability and types of cell death induced by compounds **5.1l**, **5.1m**, **5.1o** and **5.2a** after 48 h of incubation in the human melanoma A375 cells, assessed by flow cytometry using dual staining with AV and PI. Results are presented as mean ± SD of viable cells (V), in initial apoptosis (IA), in late apoptosis and/or necrosis (LA/N) and in necrosis (N) of three independent experiments (n=3) performed in duplicate. Statistical significance: $*p < 0.05$; $**p < 0.01$, $***p < 0.001$.

To further complete our studies regarding the induced mechanism of cell death, a morphological evaluation of A375 cells after May-Grünwald-Giemsa staining was carried out. This assay showed that control cells were homogeneous in size with an almost perfect circular shape (Figure 5.6), but after the incubation with alkylidene-β-lactam **5.1l** they lose their perfect shape and it was possible to observe either the formation of several blebs, a typical feature of apoptosis, or the loss of cell integrity.

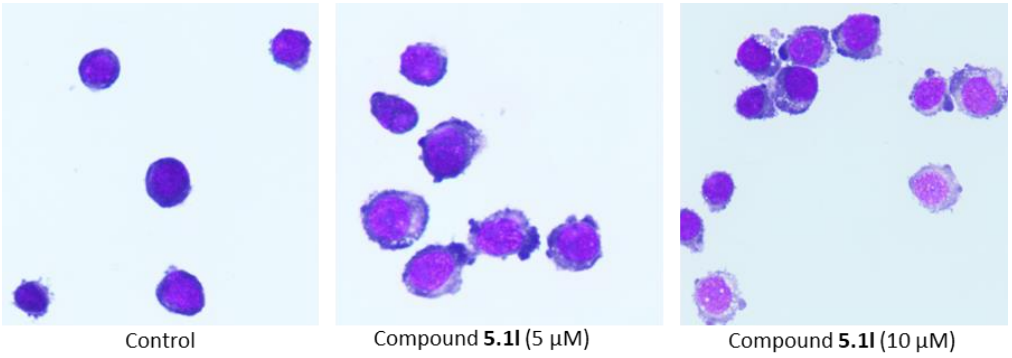


Figure 5.6. Morphological evaluation of A375 cells after May–Grünwald– Giemsa staining. The evaluation was performed in cells not submitted to treatment (control) and submitted to 5 or 10 μM of compound **5.1l** for 48 h. The images were randomly taken and are representative of each condition (50x).

5.2.2.3. Cell Cycle Analysis

The behaviour of alkylidene-β-lactams as anticancer agents was further explored, and cell cycle analysis was conducted in A375 melanoma cell line for β-lactams **5.1l**, **5.1m**, **5.1o** and **5.2a** in two concentrations (5 and 10 μM) (Figure 5.7). Alkylidene-β-lactam **5.1l** at a concentration of 5 μM, decreased the population of cells in the G1 phase (71.25%) with a significant increase of cells in S (19.50%) and G2/M (8.75%) phases, compared to control cells (G1: 83.50%; S:11.33%; G2/M: 5.17%). However, changes were more prominent when cells were treated with 10 μM of **5.1l** (G1 phase: 58.33%; S phase: 29.67%; G2/M phase: 12.00%). Moreover, compound **5.1o** led to similar results at 10 μM, with a decrease of cells population in G1 phase (61.75%) and an increase of cells in S (25.75%) and G2/M (12.50%) phases. Regarding 6-alkylidenepenicillanate **5.1m**, major changes in cell arrest were only noticed when cells were treated with a 10 μM concentration. This leads to a decrease of cells in G1 phase (67.67%) which is linked with a slight increase of the population in S phase (15.33%) and a 2.5-fold increase in the percentage of cells in G2/M phase (13.00%).

Alkylidene-β-lactams **5.1l**, **5.1m** and **5.1o** are inducing cell arrest in S and G2/M phases, so they may act by interfering in the DNA or protein synthesis. Moreover, the presence of a Michael acceptor in the structure of our alkylidene-β-lactams might also strengthen this hypothesis.

Finally, no significant changes were observed in the cell cycle profile for cells treated with compound **5.2a**. This means that compound **5.2a** induces a blockade in the G0/G1

phase, without any effect in the other phases of the cell cycle, corroborating the hypothesis that this compound induces a state of quiescence where cells stop dividing.[29]

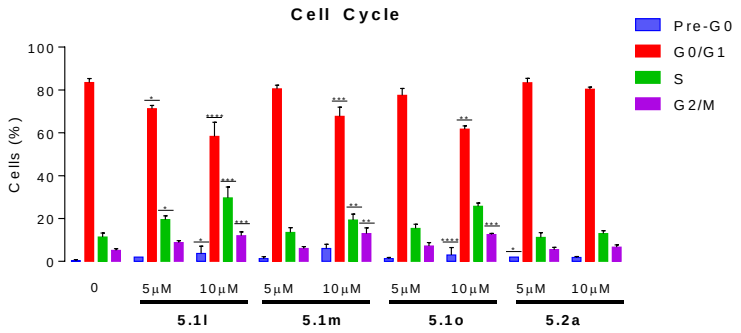


Figure 5.7. Cell cycle analysis after treatment with compounds **5.1l**, **5.1m**, **5.1o** and **5.2a** with 48 h of incubation in the human melanoma A375 cells, assessed by flow cytometry. Results are presented as mean ± SD of three independent experiments (n=3) performed in duplicate. Statistical significance: ∗p < 0.05; ∗∗p < 0.01, ∗∗∗p < 0.001, ∗∗∗∗p < 0.0001.

5.2.2.4. Reactive Oxygen Species

The intracellular reactive oxygen species (ROS) production is highly important because they might be involved in the mechanism of action of anticancer agents. Thus, the presence of intracellular peroxides and superoxide anion was assessed (Figure 5.8). The study was performed in A375 cell line with the selected alkylidene-β-lactams (**5.1l**, **5.1m**, **5.1o** and **5.2a**), in the same concentrations, and with two time points.

From this experiment it was possible to observe a decrease of intracellular peroxides, being more pronounced in the case of compound **5.1l**. A plausible explanation for this decrease could be the activation of antioxidant pathways. Likewise, a reduction in peroxides production was also observed for compounds **5.1o** and **5.1m** (10 μM) with an incubation time of 48 h. On the other hand, with exception of a slight decrease observed for compound **5.1o** (5 μM with 48 h of incubation), no significant changes were observed in the superoxide anion production profile when compared to control values.

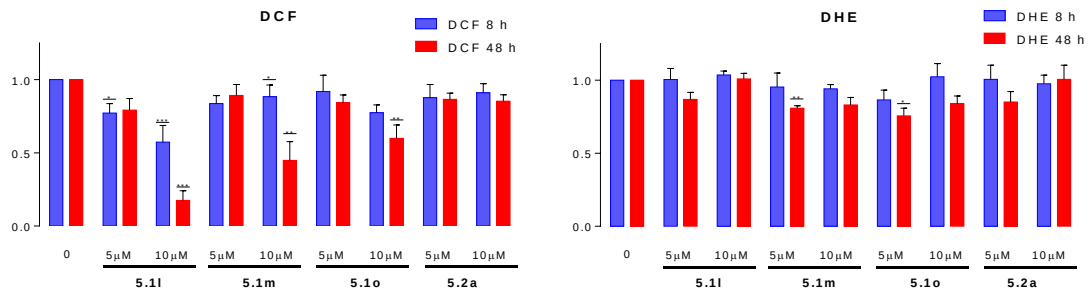


Figure 5.8. Intracellular production of ROS namely peroxides and superoxide anion, in the human melanoma A375 cells after treatment with compounds **5.1l**, **5.1m**, **5.1o** and **5.2a.** The analyses were carried out with 8 h or 48 h of incubation. Results are presented as mean ± SD of two independent experiments (n=2) performed in duplicate. Statistical significance: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

5.2.2.5. Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases responsible for tissue remodeling.[30] MMP-9 is one of the most studied and complex MMPs which belongs to gelatinase family. This MMP is responsible either for inhibiting or stimulating the extracellular matrix degradation process which is essential for tumour invasion or metastasis.[30b, 31] In this context, a set of eleven compounds were screened for their capability to inhibit MMP-9 (Figure 5.9).

The gelatinolytic activity of MMP-9 secreted from fibrosarcoma cell line HT-1080 was evaluated with gelatin zymography. From a library of selected compounds with moderate to good anticancer activity, this study allowed to identify five 6-alkylidenepenicillanates (**5.1a**, **5.1b**, **5.1l**, **5.1m** and **5.1o**) that led to a modest reduction of MMP-9 expression when compared to control values. Interestingly, alkylidene-γ-lactams **5.13a** and **5.13c** proved to be the most efficient MMP-9 inhibitors (61.4±3.90% and 52.9±6.00%, when compared to control, respectively) among the studied compounds. The MMP-9 inhibitory effect observed for alkylidene-γ-lactams, may open the way for further studies, aiming at the development of a new class of alkylidene-γ-lactams-based MMP-9 inhibitors to treat MMP-9 associated diseases such as cardiovascular diseases, lung diseases, arthritis, neurodegenerative diseases, and central nervous system (CNS) disorders.[32]

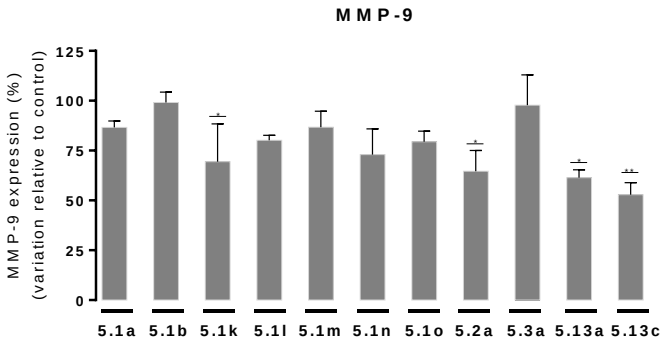


Figure 5.9. MMP-9 expression after treatment with a set of alkylidene-lactams at 10 μM. MMP-9 expression was evaluated by gelatin zymography. Results are presented as mean ± SD of at least three independent experiments (n≥3). Statistical significance: ∗p < 0.05; ∗∗p < 0.01.

5.3. Conclusion

This chapter describes the structural modulation of chiral α-alkylidene-substituted β-lactams and γ-lactams, leading to the synthesis of new chiral alkylidene-β-lactams and alkylidene-γ-lactams. A novel synthetic route for the synthesis of α-alkylidene-γ-lactams from *D*-penicillamine has been described for the first time.

In vitro studies regarding the anticancer activity of 6-aroylmethylene-, 6-acetylmethylene- and 6-alkoxycarbonylmethylene-penicillanates were carried out. Four compounds (**5.1l**, **5.1m**, **5.1o** and **5.2a**) with IC_{50} values below 10 μM in A375 human melanoma cells, and three compounds (**5.1l**, **5.1m**, **5.1o**) with IC_{50} values below 10 μM in OE19 human esophageal carcinoma cells stood out. Remarkably, 6-(*Z*)-(benzoylmethylene)penicillanate sulfoxide derivative showed an IC_{50} value of 4.5 μM against A375 cell line. 6-(*Z*)-(Ethoxycarbonylmethylidene)penicillanate also showed good activity on HT-1080 human fibrosarcoma, H1299 human lung cancer, and particularly against OE19 cell line (4.6 μM). From this work, it was possible to conclude that alkylidene-β-lactams induce cell apoptosis and necrosis and block the cell cycle in S and G2/M phases. The reported results show that the β-lactamic core together with the presence of an ester substituent are important structural features to ensure anticancer activity, a relevant SAR information for the design of further structural modulations aiming at the development of new and more potent drugs against cancer.

On the other hand, it was possible to conclude that alkylidene-γ-lactams are potential MMP-9 inhibitors. This may be the starting point for further studies, aiming at the development of a new class of alkylidene-γ-lactams-based MMP-9 inhibitors with potential biological activity.

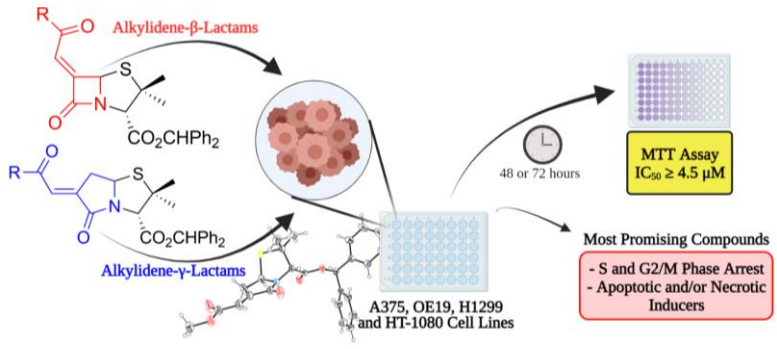


Figure 5.10. Summary of the main results for Chapter 5.

5.4. References

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CHAPTER 6

Experimental Section

Chapter 6 – Experimental Section

6.1. Instrumental

Chromatography

Thin layer chromatography (TLC) analysis was performed on aluminum plates coated with 60 F254 silica. Flash column chromatography was performed with silica gel 60 as the stationary phase.

Continuous Flow

Continuous flow reactions were performed in an E-Series flow chemistry system (Vapourtec), using a 10 mL coil or a packed-bed column as reactor.

Elemental Analysis

Elemental analyses were carried out with an Elemental Vario Micro Cube analyser.

Infrared Spectroscopy

Infrared spectra (IR) were recorded in an Agilent Technologies Cary 630 FTIR spectrometer coupled with a diamond Attenuated Total Reflectance (ATR) sampling accessory.

Mass Spectrometry

High-resolution mass spectra (HRMS) were obtained on a TOF VG Autospect M spectrometer with electrospray ionization (ESI) or on a Orbitrap q-Exactive Focus (Thermo scientific) spectrometer coupled with an HPLC Vanquish (Thermo Scientific) with ESI.

Melting Points

Melting points (mp) were determined in open glass capillaries with a Falc R132467 apparatus and are uncorrected.

Microwaves

Microwave-assisted reactions were performed in a CEM Discover S-Class (CEM Focused Synthesis System), using 10 mL microwave tubes.

Nuclear Magnetic Resonance Spectroscopy

^{1}H NMR, ^{13}C NMR and ^{19}F NMR were obtained in a Bruker Avance III spectrometer, operating at 400 MHz, 100 MHz and 376 MHz, respectively. TMS was the internal standard used. Chemical shifts (δ) and coupling constants (*J*) are indicated in ppm and Hz, respectively. Unless otherwise specified, the solvent was deuterated chloroform ($CDCl_3$).

Optical Rotations

Optical rotations were measured on an Optical Activity AA-5 electrical polarimeter, in a cell of 1 dm path length (l). The solvent used to dissolve the sample is recorded in parentheses.

X-Ray Diffraction

Single-crystal X-ray analysis was carried out at room temperature on a Bruker D8 Quest or on a Bruker APEX-II diffractometer.

6.2 Solvents and Reagents

Ethyl Acetate

Distilled, after being refluxed for 3 hours with potassium carbonate.

Dichloromethane and Chloroform

Distilled after being refluxed for 3 hours with calcium chloride. Chloroform was passed through alumina.

Ethanol and Methanol

Distilled from sodium alkoxide, after being refluxed for 2 hours with magnesium (5 g/L) with iodine scraps (0.5 g/L).

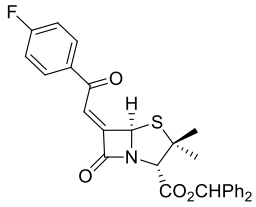
Diethyl Ether, Hexane, Tetrahydrofuran and Toluene

Distilled and stored on 4 Å molecular sieves, after being refluxed with sodium scraps, using benzophenone as an indicator.

Benzhydryl 6-β-aminopenicillanate hydrochloride salt (**2.24**)[1], benzhydryl 6-diazopenicillinate (**2.25**)[2], benzhydryl 6-oxopenicillinate (**2.26**)[3], 6-alkylidenepenicillanates **2.33a-c**[4], **3.42a**,**k**,**n**[4], **4.50**[4] and **5.1a**,**k**,**n**[4], allenoate **2.1**[5], nitrones **3.41a-c**[6], **3.41d**[7] and **3.41e**[6] and Benzyl (2*S*)-2-(*tert*-butoxycarbonylamino)-4-oxobutanoate (**5.5**)[8] were prepared as described in the literature. All other solvents and reagents were purchased from Aldrich, Acros Organics, Alfa Aesar or Fluorochem, and were used without any additional purification.

6.3. Compound Index

6-Alkylidenepenicillanates



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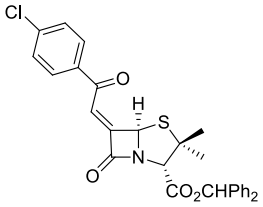

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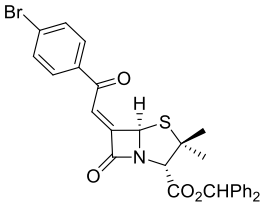

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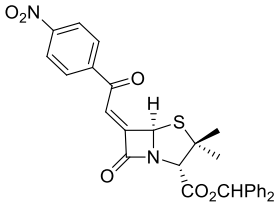

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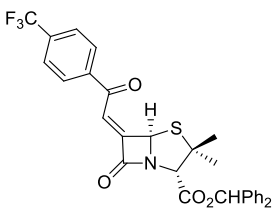

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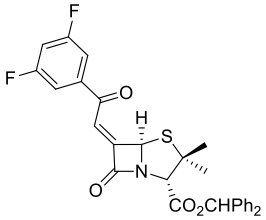

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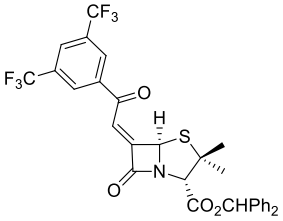

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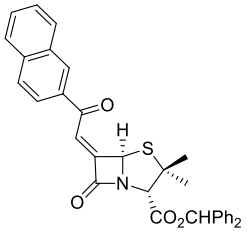

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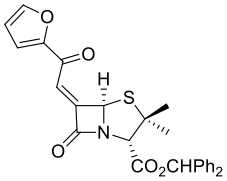

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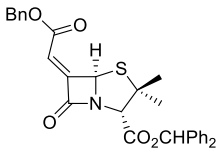

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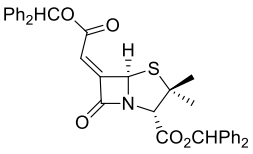

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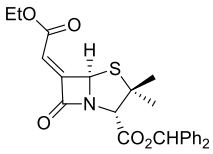

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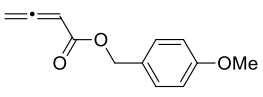
Allenoates



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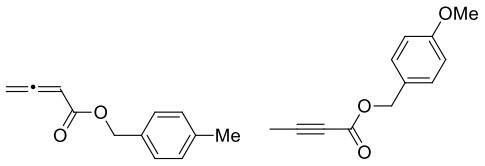
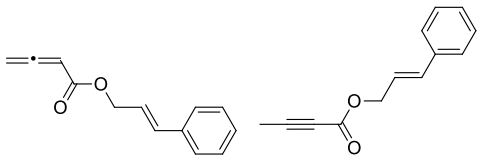


2.30b/**2.30b'**, p. 193



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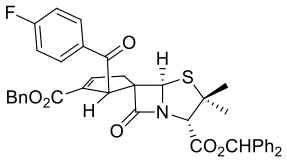
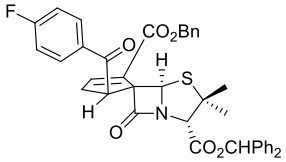
Spirocyclopentene-β-lactams (Part I)

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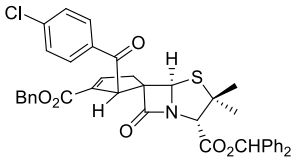
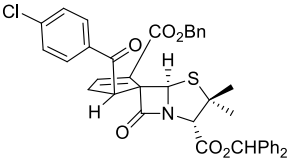
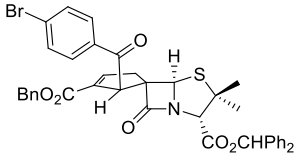
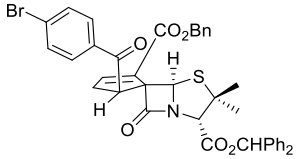
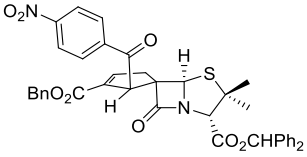
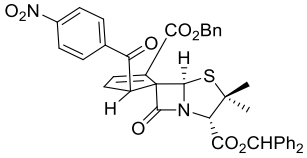



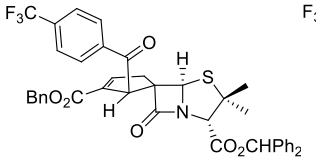
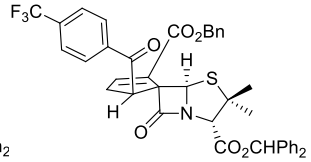
2.28b/**2.29b**, p. 196

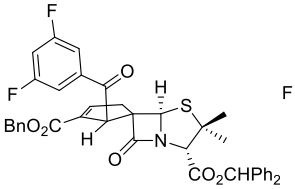
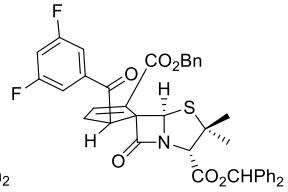
2.28c/**2.29c**, p. 197

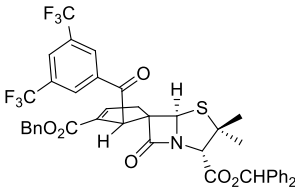
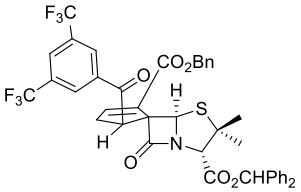
2.28d/**2.29d**, p. 198

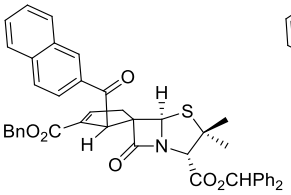
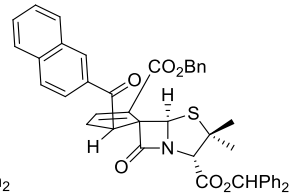
2.28e/**2.29e**, p. 199

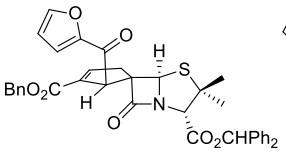
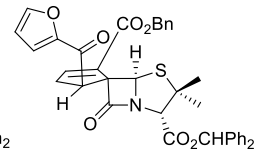
2.28f/**2.29f**, p. 200

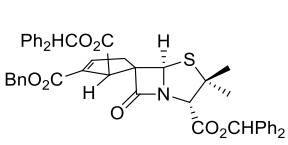
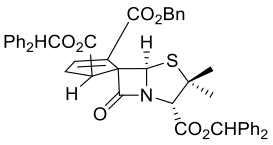
2.28g/**2.29g**, p. 201

2.28h/**2.29h**, p. 202

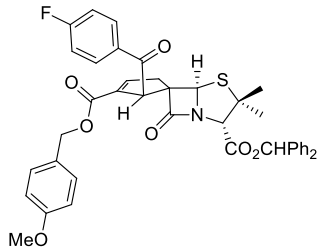



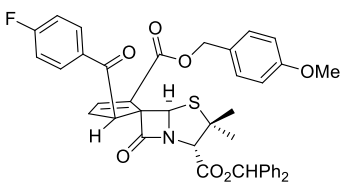
2.28i/**2.29i**, p. 203

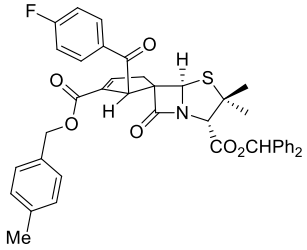
2.28j/**2.29j**, p. 204

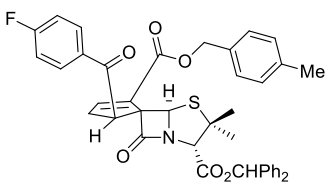
Spirocyclopentene-β-lactams (Part II)



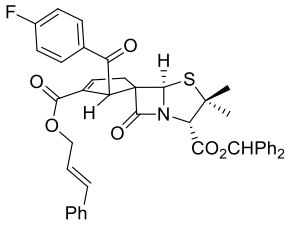


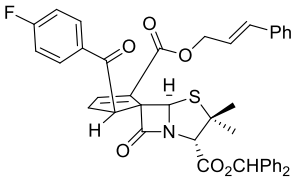
2.31a/**2.32a**, p. 205



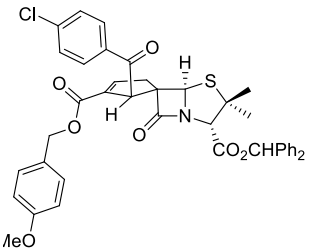


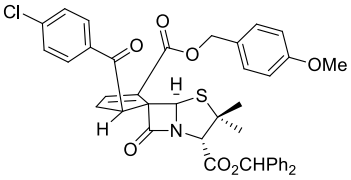
2.31b/**2.32b**, p. 206



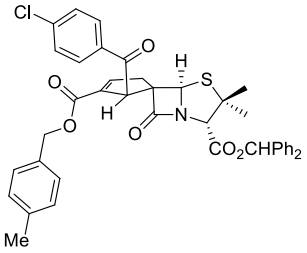


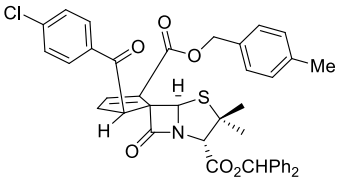
2.31c/**2.32c**, p. 207



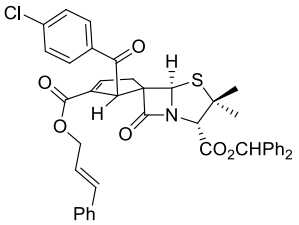


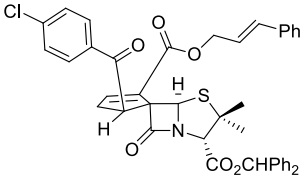
2.31d/**2.32d**, p. 208



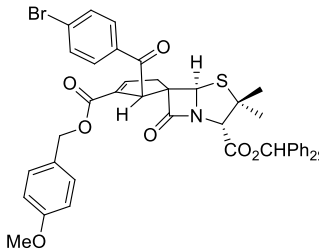


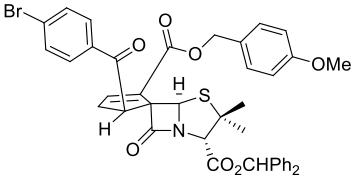
2.31e/**2.32e**, p. 210



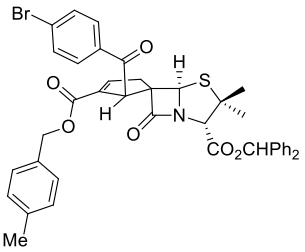


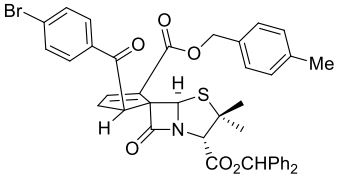
2.31f/**2.32f**, p. 211



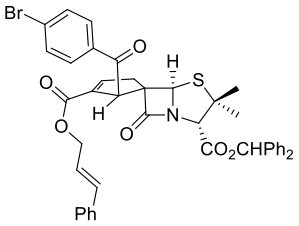


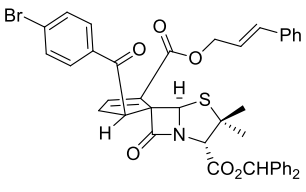
2.31g/**2.32g**, p. 212





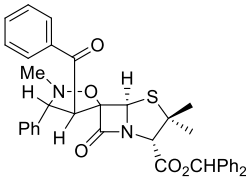
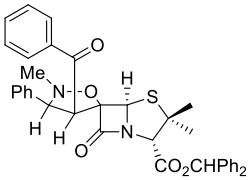
2.31h/**2.32h**, p. 213



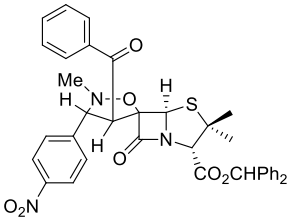
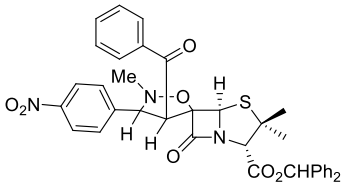


2.31i/**2.32i**, p. 214

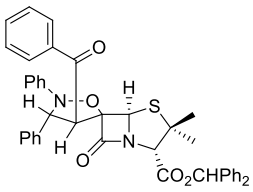
Spiroisoxazolidine-β-lactams (Part I)

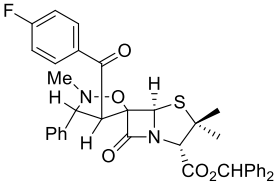
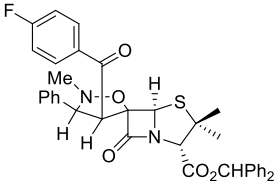
3.43a/**3.44a**, p. 216

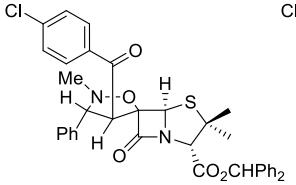
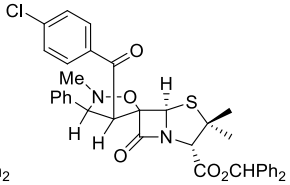
3.43b/**3.44b**, p. 217



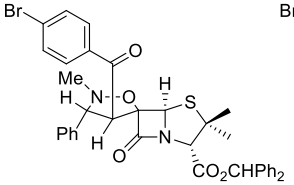
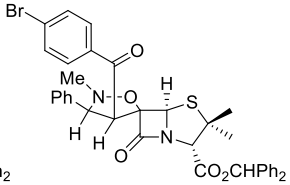
3.43c, p. 218

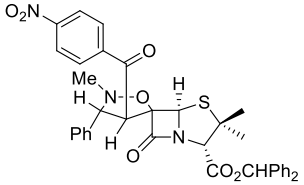
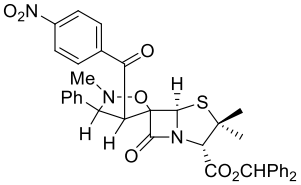
3.47a/**3.48a**, p. 218

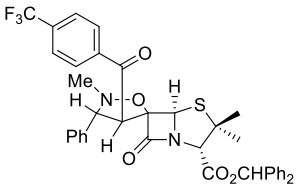
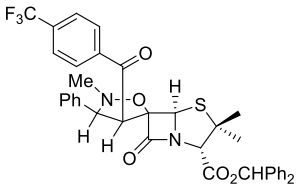
3.47b/**3.48b**, p. 219

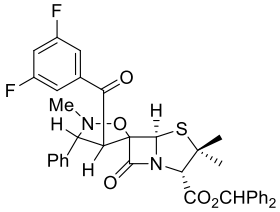
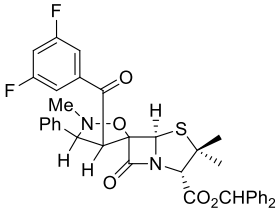
3.47c/**3.48c**, p. 220

3.47d/**3.48d**, p. 221

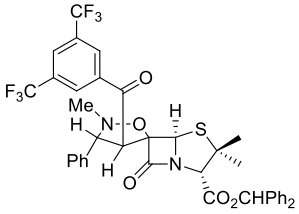
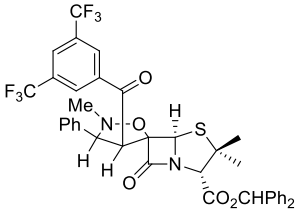



3.47e/**3.48e**, p. 222

3.47f/**3.48f**, p. 223

Spiroisoxazolidine-β-lactams (Part II)

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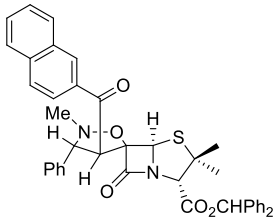
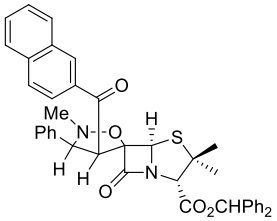

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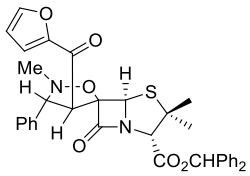
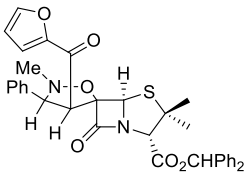
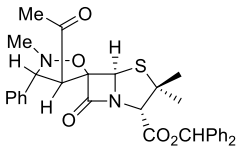
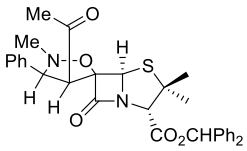
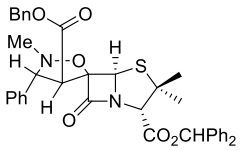
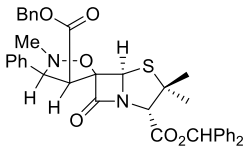



3.47i/**3.48i**, p. 226

3.47j/**3.48j**, p. 227

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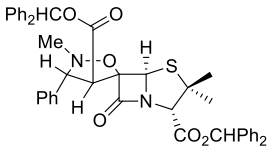
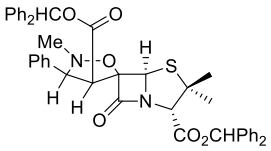
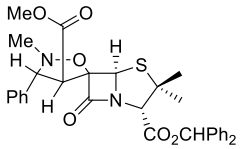
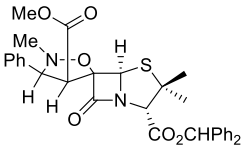
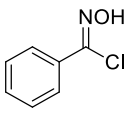



3.47l/**3.48l**, p. 229

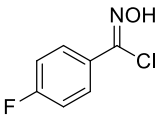



3.47m/**3.48m**, p. 230

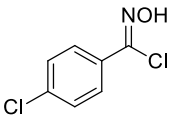
Hydroximoyl Chlorides



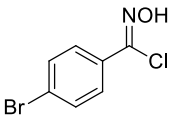
4.48a, p. 231



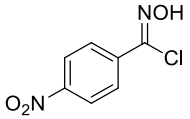
4.48b, p. 231



4.48c, p. 232



4.48d, p. 232



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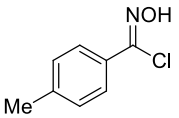
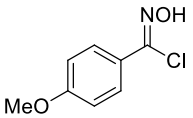
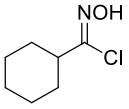


4.48f, p. 233

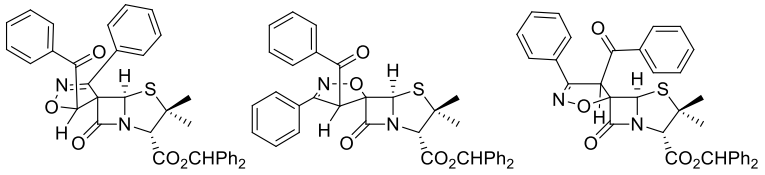


4.48g, p. 233

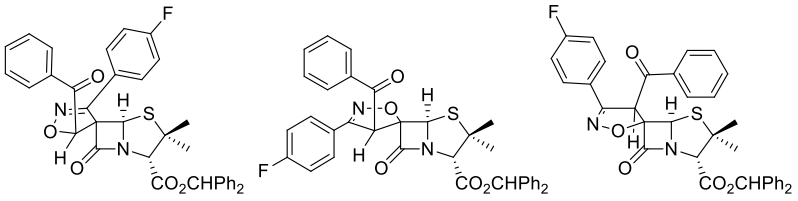


4.48h, p. 234

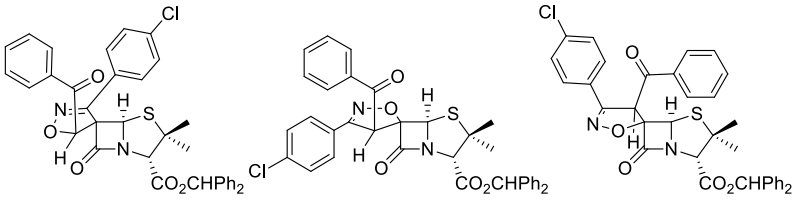
Spiroisoxazoline-β-lactams (Part I)



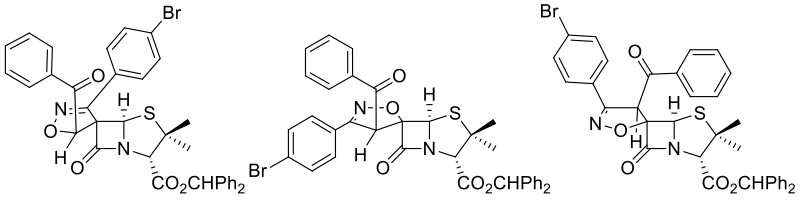
4.51a/**4.52a**/**4.53a**, p. 235



4.51b/**4.52b**/**4.53b**, p. 236



4.51c/**4.52c**/**4.53c**, p. 237



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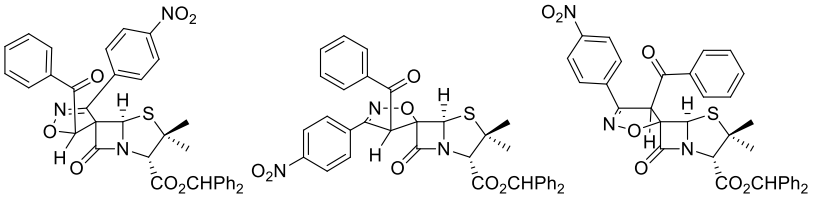

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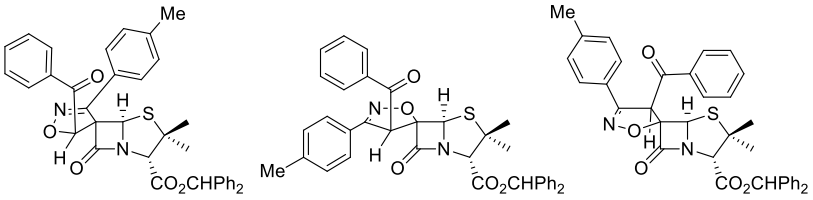
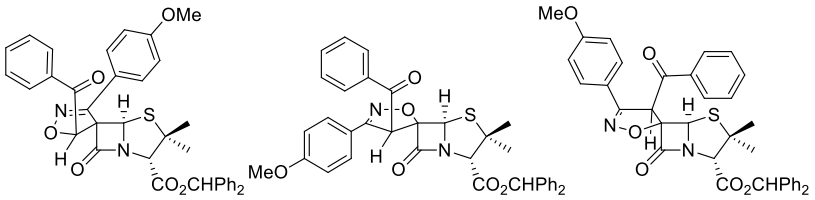
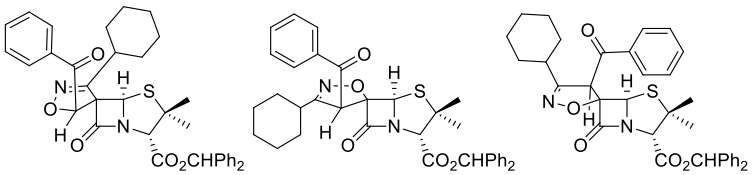


4.51f/**4.52f**/**4.53f**, p. 240

Spiroisoxazoline-β-lactams (Part II)

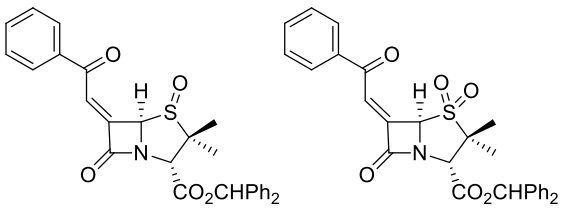


4.51g/**4.52g**/**4.53g**, p. 241

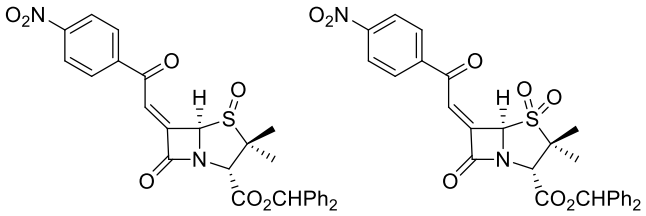


4.51h/**4.52h**/**4.53h**, p. 243

Sulfone and Sulfoxide Alkylidene-β-lactams

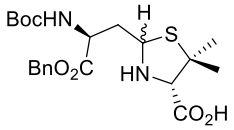


5.2a/**5.3a**, p. 244

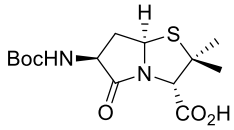


5.2b/**5.3b**, p. 245

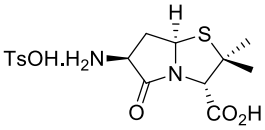
Compounds for the Synthesis of Diazo-γ-Lactam



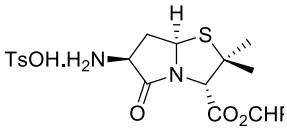
5.6, p. 246



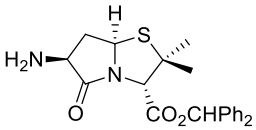
5.7, p. 247



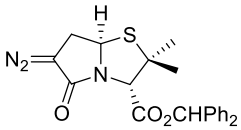
5.8, p. 247



5.9, p. 248

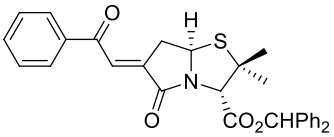


5.10, p. 248

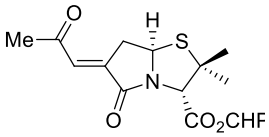


5.11, p. 249

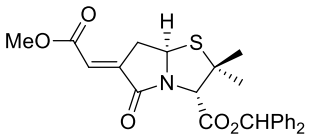
Alkylidene-γ-Lactams



5.13a, p. 250



5.13b, p. 251

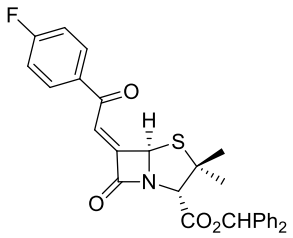


5.13c, p. 251

6.4. Procedures for Chapter 2

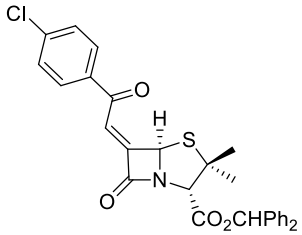
6.4.1. General Procedure for the Synthesis of 6-Alkylidenepenicillanates

A solution of 6-β-aminopenicillanate hydrochloride salt **2.24** (1.00 g, 2.38 mmol) in dichloromethane (75 mL) was washed with a saturated aqueous solution of $NaHCO_3$ (2 × 75 mL), and the aqueous phase was extracted with dichloromethane (2 × 50 mL). The organic extracts were combined, dried and concentrated under reduced pressure to obtain the corresponding 6-β-aminopenicillanate. To a solution of 6-β-aminopenicillanate (2.38 mmol) in ethyl acetate (7 mL), isoamyl nitrite (1.2 equiv., 0.382 mL) was added followed by a catalytic amount of trifluoroacetic acid (two drops). The reaction mixture was stirred at room temperature for 1 h and concentrated under reduced pressure to obtain diazo-lactam **2.25** which was used, without further purification, in the synthesis of 6-oxopenicillinate **2.26**. Rhodium acetate dimer ($4x10^{-3}$ equiv., $9.5x10^{-3}$ mmol) was dissolved in toluene (9 mL) in a two-neck round bottom flask under inert atmosphere. Next, propylene oxide (99 equiv., 235.85 mmol, 16.3 mL) was added dropwise followed by a dropwise addition of a solution of the previously isolated diazo-lactam **2.25** in toluene (9 mL). The reaction was stirred for 15 min and concentrated under reduced pressure to give benzhydryl 6-oxopenicillinate **2.26**. Next, freshly synthesized benzhydryl 6-oxopenicillinate **2.26** was dissolved in dichloromethane (12 mL), the solution was cooled to –55 °C under nitrogen, and a solution of the appropriate phosphorus ylide (2.26 mmol) in dichloromethane (30 mL) was added dropwise. Stirring was continued for 15 min, then the solution was warmed to room temperature and washed with water (20 mL). The products were purified by flash chromatography. The presented yields were calculated from benzhydryl 6-β-aminopenicillanate hydrochloride.



Benzhydryl 6-(*Z*)-(4-fluorobenzoylmethylidene)penicillanate (2.20a)

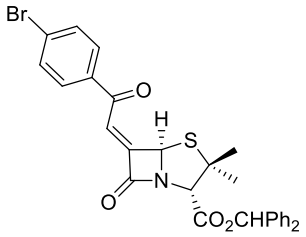
Prepared from benzhydryl 6-oxopenicillanate and the corresponding phosphorus ylide (0.90 g, 2.26 mmol), as described in the general procedure. After purification by flash chromatography (hexane/ethyl acetate, 3:1), compound **2.20a** was obtained as a yellow solid (509 mg, 1.015 mmol, 43%). mp low melting point solid; $[\alpha]_D^{25}$= + 290 (*c* 0.5 in CH_2Cl_2); IR (ATR): ν = 1011, 1154, 1228, 1593, 1636, 1735, 1741, 1770 and 1772 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.28 (s, 3H), 1.58 (s, 3H), 4.68 (s, 1H), 6.15 (s, 1H), 6.97 (s, 1H), 7.19 (t, *J* = 8.6 Hz, 2H), 7.29-7.42 (m, 11H), 8.02 (dd, *J* = 8.8 and 5.3 Hz, 2H) ; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.6, 33.6, 63.9, 69.8, 71.3, 78.6, 115.9, 116.4 (d, *J* = 22 Hz, 2C), 127.2, 127.7, 128.4, 128.5, 128.8, 128.8, 131.6 (d, *J* = 10 Hz, 2C), 133.6 (d, *J* = 3 Hz, 1C), 139.2, 139.3, 156.8, 166.5 (d, *J* = 256 Hz, 1C), 166.9, 167.4, 186.8; ^{19}F NMR (376 MHz, $CDCl_3$): -102.74 (s, 1F) ; Anal. Calcd for $C_{29}H_{24}NO_4SF$: C, 69.45; H, 4.82; N, 2.79; S, 6.39. Found: C, 69.43; H, 5.04; N, 2.76; S, 6.28.



Benzhydryl 6-(*Z*)-(4-chlorobenzoylmethylidene)penicillanate (2.20b)

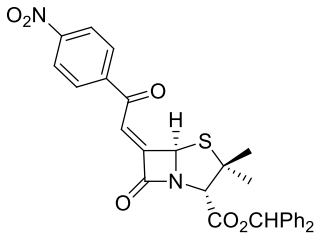
Prepared from 6-oxopenicillanate (2.38 mmol) and the corresponding phosphorus ylide (0.937 g, 2.26 mmol), as described in the general procedure. After purification by flash chromatography (hexane/ethyl acetate, 3:1), compound **2.20b** was obtained as a yellow solid (486 mg, 0.938 mmol, 39%). mp low melting point solid; $[\alpha]_D^{25}$= + 280 (*c* 0.5 in CH_2Cl_2); IR (ATR): ν = 1007, 1233, 1587, 1636, 1735, 1741 and 1772 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.29 (s, 3H), 1.58 (s, 3H), 4.69 (s, 1H), 6.15 (d, *J* = 0.9 Hz, 1H), 6.98 (s, 1H), 7.30-7.42 (m, 11H), 7.48-7.52 (m, 2H), 7.91-7.95 (m, 2H) ; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.6, 33.6, 63.9, 69.8, 71.2, 78.6, 115.8, 127.2, 127.7, 128.4,

128.5, 128.8, 128.8, 129.5, 130.1, 135.4, 139.2, 140.9, 157.0, 166.8, 167.3, 187.2; HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{29}H_{25}ClNO_4S$ $[M+H]^+$ 518.1187; found 518.1188.



Benzhydryl 6-(*Z*)-(4-bromobenzoylmethylidene)penicillanate (2.20c)

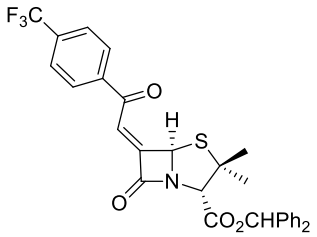
Prepared from 6-oxopenicillanate (2.38 mmol) and the corresponding phosphorus ylide (1.038 g, 2.26 mmol), as described in the general procedure. After purification by flash chromatography (hexane/ethyl acetate, 3:1), compound **2.20c** was obtained as a light-yellow solid (461 mg, 0.82 mmol, 34%). mp 135.4 – 137.2 ºC; $[\alpha]_D^{25}$= + 220 (*c* 0.5 in CH_2Cl_2); IR (ATR): ν = 1005, 1265, 1583, 1631, 1719, 1749 and 1758 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.28 (s, 3H), 1.58 (s, 3H), 4.68 (s, 1H), 6.14 (d, *J* = 0.9 Hz, 1H), 6.97 (s, 1H), 7.30-7.42 (m, 11H), 7.67 (d, *J* = 8.6 Hz, 2H), 7.85 (d, *J* = 8.6 Hz, 2H) ; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.6, 33.6, 64.0, 69.8, 71.3, 78.6, 115.7, 127.3, 127.7, 128.4, 128.5, 128.8, 128.8, 129.8, 130.2, 132.6, 135.8, 139.2, 139.3, 157.1, 166.8, 167.3, 187.4; HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{29}H_{25}BrNO_4S$ $[M+H]^+$ 562.0682; found 562.0693.



Benzhydryl 6-(*Z*)-(4-nitrobenzoylmethylidene)penicillanate (2.20d)

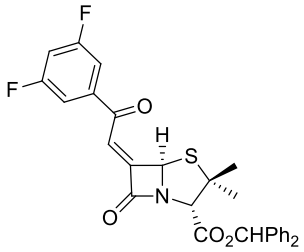
Prepared from 6-oxopenicillanate (2.38 mmol) and the corresponding phosphorus ylide (0.96 g, 2.26 mmol), as described in the general procedure. After purification by flash chromatography (hexane/ethyl acetate, 3:1), compound **2.20d** was obtained as a yellow solid (445 mg, 0.842 mmol, 35%). mp 68.3 – 70.3 ºC; $[\alpha]_D^{25}$= + 270 (*c* 0.5 in CH_2Cl_2); IR (ATR): ν = 1009, 1223, 1341, 1523, 1735, 1741 and 1773 cm^{-1}; ^1H NMR

(400 MHz, $CDCl_3$): δ = 1.29 (s, 3H), 1.59 (s, 3H), 4.71 (s, 1H), 6.15 (d, *J* = 0.9 Hz, 1H), 6.98 (s, 1H), 7.31-7.41 (m, 11H), 8.11-8.16 (m, 2H), 8.35-8.39 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.5, 33.8, 64.1, 69.8, 71.3, 78.7, 115.2, 124.4, 127.2, 127.7, 128.4, 128.6, 128.8, 128.8, 129.8, 139.2, 139.3, 141.3, 150.9, 158.6, 166.7, 166.8, 187.0; Anal. Calcd for $C_{29}H_{24}N_2O_6S$: C, 65.90; H, 4.58; N, 5.30; S, 6.07. Found: C, 66.02; H, 4.83; N, 5.11; S, 6.07.



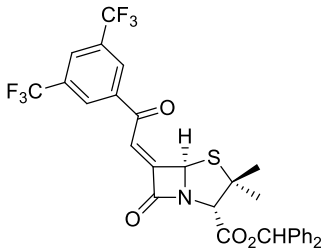
Benzhydryl 6-(*Z*)-(4-trifluoromethylbenzoylmethylidene)penicillanate (2.20e)

Prepared from 6-oxopenicillanate (2.38 mmol) and the corresponding phosphorus ylide (1.013 g, 2.26 mmol), as described in the general procedure. After purification by flash chromatography (hexane/ethyl acetate, 4:1), compound **2.20e** was obtained as a yellow solid (518 mg, 0.94 mmol, 39%). mp low melting point solid. $[\alpha]_D^{25}$ = + 270 (*c* 0.5 in CH_2Cl_2); IR (ATR): ν = 1010, 1168, 1319, 1735, 1741, 1744 and 1773 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.29 (s, 3H), 1.59 (s, 3H), 4.70 (s, 1H), 6.16 (d, *J* = 0.9 Hz, 1H), 6.98 (s, 1H), 7.30-7.40 (m, 11H), 7.79 (d, *J* = 8.3 Hz, 2H), 8.09 (d, *J* = 8.2 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.6, 33.7, 64.0, 69.8, 71.3, 78.7, 115.6, 126.2, 126.3, 127.3, 127.7, 128.4, 128.6, 128.8, 128.8, 129.1, 139.2, 139.3, 139.6, 157.8, 166.8, 187.6; ^{19}F NMR (376 MHz, $CDCl_3$): -63.24 (s, 3F); Anal. Calcd for $C_{30}H_{24}F_3NO_4S$: C, 65.33; H, 4.39; N, 2.54; S, 5.81. Found: C, 65.60; H, 4.39; N, 2.47; S, 5.05.



Benzhydryl 6-(*Z*)-(3,5-difluorobenzoylmethylidene)penicillanate (2.20f)

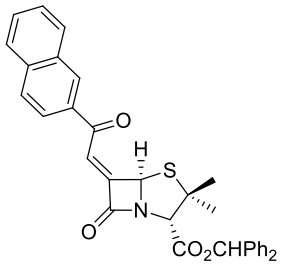
Prepared from 6-oxopenicillanate (2.38 mmol) and the corresponding phosphorus ylide (0.94 g, 2.26 mmol), as described in the general procedure. After purification by flash chromatography (hexane/ethyl acetate, 3:1), compound **2.20f** was obtained as a white solid (386 mg, 0.743 mmol, 31%). mp 150.8 – 152.8 ºC; $[\alpha]_D^{25}$= + 290 (*c* 0.5 in CH_2Cl_2); IR (ATR): ν = 1066, 1121, 1251, 1297, 1593, 1640, 1719 and 1758 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.29 (s, 3H), 1.58 (s, 3H), 4.69 (s, 1H), 6.14 (d, *J* = 1.0 Hz, 1H), 6.97 (s, 1H), 7.09 (tt, *J* = 8.3 and 2.3 Hz, 1H), 7.25 (d, *J* = 1.0 Hz, 1H), 7.27-7.42 (m, 10H), 7.46-7.52 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.6, 33.7, 64.0, 69.8, 71.3, 78.7, 109.6 (t, *J* = 25 Hz, 1C), 111.7 (d, *J* = 19 Hz, 1C), 111.8 (d, *J* = 18 Hz, 1C), 115.1, 127.2, 127.7, 128.4, 128.6, 128.8, 128.8, 139.2, 139.3, 139.8 (t, *J* = 8 Hz, 1C), 158.2, 163.3 (d, *J* = 251 Hz, 1C), 163.4 (d, *J* = 251 Hz, 1C), 166.8, 166.9, 186.0; ^{19}F NMR (376 MHz, $CDCl_3$): -106.90 (s, 2F); Anal. Calcd for $C_{29}H_{23}F_2NO_4S$: C, 67.04; H, 4.46; N, 2.70; S, 6.17. Found: C, 66.93; H, 4.33; N, 2.63; S, 5.61.



Benzhydryl 6-(*Z*)-(3,5-difluoromethylbenzoylmethylidene)penicillanate (2.20g)

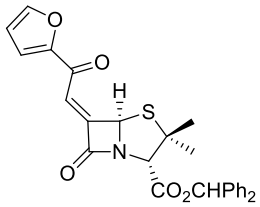
Prepared from 6-oxopenicillanate (2.38 mmol) and the corresponding phosphorus ylide (1.167 g, 2.26 mmol), as described in the general procedure. After purification by flash chromatography (hexane/ethyl acetate, 4:1), compound **2.20g** was obtained as a yellow solid (601 mg, 0.97 mmol, 41%). mp low melting point solid; $[\alpha]_D^{25}$= + 230 (*c* 0.5 in CH_2Cl_2); IR (ATR): ν = 1074, 1132, 1277, 1456, 1647, 1744 and 1773 cm^{-1}; ^1H NMR

(400 MHz, $CDCl_3$): δ = 1.30 (s, 3H), 1.59 (s, 3H), 4.72 (s, 1H), 6.17 (d, *J* = 0.8 Hz, 1H), 6.98 (s, 1H), 7.30-7.41 (m, 11H), 8.13 (s, 1H), 8.42 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.5, 33.9, 64.0, 69.8, 71.3, 78.7, 114.5, 122.9 (q, *J* = 273 Hz, 2C), 124.2, 127.2, 127.7, 128.4, 128.6, 128.7, 128.8, 128.8, 133.0 (q, *J* = 34 Hz, 2C), 138.4, 139.2, 139.3, 159.4, 166.6, 166.7, 185.8; ^{19}F NMR (376 MHz, $CDCl_3$): -63.02 (s, 6F); HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{31}H_{24}F_6NO_4S$ $[M+H]^+$ 620.1325; found 620.1321.



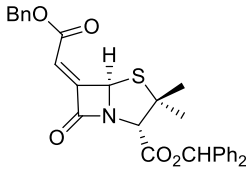
Benzhydryl 6-(*Z*)-(2-naphthoylmethylidene)penicillanate (2.20h)

Prepared from 6-oxopenicillanate (2.38 mmol) and the corresponding phosphorus ylide (0.973 g, 2.26 mmol), as described in the general procedure. After purification by flash chromatography (hexane/ethyl acetate, 3:1), compound **2.20h** was obtained as a yellow solid (355 mg, 0.66 mmol, 28%). mp 100.7 – 102.7 ºC; $[\alpha]_D^{25}$= + 250 (*c* 0.5 in CH_2Cl_2); IR (ATR): ν = 997, 1126, 1252, 1457, 1623, 1719, 1769 and 1771 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.30 (s, 3H), 1.60 (s, 3H), 4.70 (s, 1H), 6.21 (d, *J* = 0.9 Hz, 1H), 6.98 (s, 1H), 7.32-7.40 (m, 10H), 7.57-7.68 (m, 3H), 7.88-8.00 (m, 3H), 8.05 (dd, J = 1.7 and 8.6 Hz, 1H), 8.52 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.6, 33.6, 63.9, 69.9, 71.3, 78.6, 116.4, 123.9, 127.2, 127.3, 127.7, 128.1, 128.3, 128.5, 128.7, 128.8, 129.2, 129.4, 130.0, 131.1, 132.6, 134.6, 136.1, 139.3, 139.4, 156.3, 166.9, 167.7, 188.2; HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{33}H_{28}NO_4S$ $[M+H]^+$ 534.1734; found 534.1734.



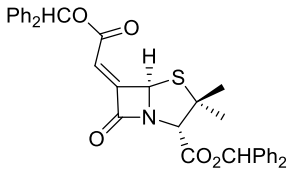
Benzhydryl 6-(*Z*)-(2-furoylmethylidene)penicillanate (2.20i)

Prepared from 6-oxopenicillanate (2.38 mmol) and the corresponding phosphorus ylide (0.837 g, 2.26 mmol), as described in the general procedure. After purification by flash chromatography (hexane/ethyl acetate, 3:1), compound **2.20i** was obtained as a yellow solid (562 mg, 1.18 mmol, 50%). mp low melting point solid; $[\alpha]_D^{25}$ = + 290 (*c* 0.5 in CH_2Cl_2); IR (ATR): ν = 1153, 1251, 1458, 1560, 1566, 1629, 1636, 1735, 1741, 1770 and 1772 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.28 (s, 3H), 1.57 (s, 3H), 4.68 (s, 1H), 6.18 (d, *J* = 1.0 Hz, 1H), 6.62 (dd, *J* = 3.6 and 1.6 Hz, 1H), 6.97 (s, 1H), 7.23 (d, *J* = 1.1 Hz, 1H), 7.31-7.41 (m, 11H), 7.68 (dd, *J* = 1.5 and 0.5 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.5, 33.8, 63.8, 69.8, 71.2, 78.6, 113.3, 116.4, 119.5, 127.2, 127.7, 128.3, 128.5, 128.7, 128.8, 139.3, 139.4, 148.0, 153.4, 156.2, 166.9, 167.4, 176.2; Anal. Calcd for $C_{27}H_{23}NO_5S$: C, 68.48; H, 4.90; N, 2.96; S, 6.77. Found: C, 68.44; H, 5.02; N, 3.09; S, 6.66.



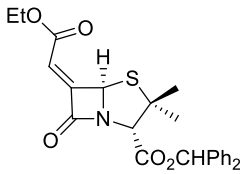
Benzhydryl 6-(*Z*)-(benzyloxycarbonylmethylidene)penicillanate (2.20j)

Prepared from 6-oxopenicillanate (2.38 mmol) and the corresponding phosphorus ylide (0.927 g, 2.26 mmol), as described in the general procedure. After purification by flash chromatography (hexane/ethyl acetate, 4:1), compound **2.20j** was obtained as a colourless oil (576 mg, 0.976 mmol, 41%). $[\alpha]_D^{25}$ = + 270 (*c* 0.5 in CH_2Cl_2); IR (ATR): ν = 1066, 1153, 1170, 1249, 1453, 1496, 1719, 1774 and 2966 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.25 (s, 3H), 1.55 (s, 3H), 4.64 (s, 1H), 5.22 (s, 2H), 6.00 (d, *J* = 1.0 Hz, 1H), 6.33 (d, *J* =1.1 Hz, 1H), 6.94 (s, 1H), 7.32-7.38 (m, 15H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.5, 33.8, 64.1, 67.6, 69.3, 70.9, 77.5, 115.8, 127.2, 127.6, 128.4, 128.5, 128.7, 128.8, 128.8, 128.8, 128.8, 135.1, 139.2, 139.3, 157.1, 163.7, 166.4, 166.8; HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{30}H_{28}NO_5S$ $[M+H]^+$ 514.1683; found 514.1688.



Benzhydryl 6-(*Z*)-(benzhydryloxycarbonylmethylidene)penicillanate (2.20k)

Prepared from 6-oxopenicillanate (2.38 mmol) and the corresponding phosphorus ylide (1.099 g, 2.26 mmol), as described in the general procedure. After purification by flash chromatography (hexane/ethyl acetate, 3:1), compound **2.20k** was obtained as a colourless solid (579 mg, 0.982 mmol, 41%). mp low melting point solid; $[\alpha]_D^{25}$= + 200 (*c* 0.5 in CH_2Cl_2); IR (ATR): ν = 981, 1065, 1170, 1248, 1449, 1496, 1719, 1735 and 1773 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.25 (s, 3H), 1.56 (s, 3H), 4.64 (s, 1H), 6.01 (d, *J* = 1.0 Hz, 1H), 6.40 (d, *J* =1.1 Hz, 1H), 6.94 (s, 1H), 6.97 (s, 1H), 7.30-7.36 (m, 20H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.5, 33.7, 64.3, 69.3, 71.0, 78.6, 78.7, 115.9, 127.2, 127.3, 127.6, 128.4, 128.4, 128.5, 128.8, 128.8, 129.3, 139.3, 139.4, 139.4, 157.1, 163.0, 166.4, 166.8; Anal. Calcd for $C_{36}H_{31}NO_5S$: C, 73.32; H, 5.30; N, 2.38; S, 5.44. Found: C, 73.12; H, 5.67; N, 2.31; S, 4.94.

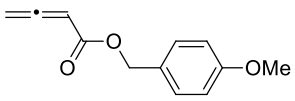


Benzhydryl 6-(*Z*)-(ethoxycarbonylmethylidene)penicillanate (2.20l)

Prepared from 6-oxopenicillanate (2.38 mmol) and the corresponding phosphorus ylide (0.787 g, 2.26 mmol), as described in the general procedure. After purification by flash chromatography (hexane/ethyl acetate, 4:1) compound **2.20l** was obtained as a colourless solid (373 mg, 0.857 mmol, 36%). mp 72.6–74.4 °C; $[\alpha]_D^{25}$= + 280 (c 0.5 in CH_2Cl_2); IR (ATR): ν = 968, 1069, 1171, 1256, 1287, 1497, 1719, 1773 and 2984 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.28 (s, 3H), 1.33 (t, *J* = 7.1 Hz, 3H), 1.57 (s, 3H), 4.20–4.31 (m, 2H), 4.66 (s, 1H), 6.03 (d, *J* = 1.0 Hz, 1H), 6.29 (d, *J* = 1.1 Hz, 1H), 6.96 (s, 1H), 7.29–7.40 (m, 10H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 14.3, 25.6, 33.9, 61.8, 64.0, 69.3, 70.8, 78.6, 116.1, 127.2, 127.7, 128.5, 128.7, 128.8, 139.2, 139.3, 156.6, 163.9, 166.5, 166.9; HRMS (ESI-TOF) m/z: $[M + Na]^+$ Calcd C25H25NNaO5S 474.1346, found 474.1340.

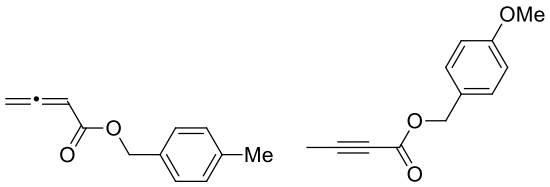
6.4.2. General Procedure for the Synthesis of Monosubstituted Allenoates

The monosubstituted allenoates were obtained through a synthetic procedure, previously reported in the literature.[9] Bromoacetyl bromide (0.252 mL, 2.90 mmol) was added dropwise to a solution of an alcohol (2.90 mmol) and pyridine (0.232 mL, 2.90 mmol) in dichloromethane (10 mL) at 0 ºC, forming a white suspension. The suspension was stirred for 20 min at 0 ºC and then for an additional 30 min at 25 ºC, after which distilled water (15 mL) was added to the reaction mixture. The organic layer was separated, and the aqueous layer was then extracted with dichloromethane (2 x 15 mL). The combined organic layers were washed with brine (15 mL) dried over with $MgSO_4$ and concentrated to give the respective α-bromoacetate as an oil, which was used directly without purification for the next reaction step. The α-bromoacetate obtained in the previous step was added dropwise to a solution of triphenylphosphine (0.754 g, 2.9 mmol) in toluene (20 mL), and was left stirring overnight. The resulting precipitated was filtered, washed sequentially with toluene and hexane, and then dissolved in distilled water (20 mL). NaOH (2 M) was added to keep the pH > 7 and the mixture was left stirring. After 30 min, dichloromethane (20 mL) was added. The organic layer was then separated, washed with brine (15 mL), dried over with $MgSO_4$ and the filtrate was concentrated affording the desired phosphorus ylide, which was used directly without further purification for the next step. The phosphorus ylide was then dissolved in anhydrous dichloromethane (5 mL) in a two-neck round-bottled flask, and NEt_3 (0.405 mL, 2.90 mmol) was added dropwise to the solution. After stirring for 15 min, a previously prepared solution of acetyl chloride (0.212 mL, 2.90 mmol) in anhydrous dichloromethane (5 mL) was added dropwise over 30 min. The reaction mixture was left stirring for 12 h under nitrogen. A precipitate was formed which was filtered and discarded, and the solvent was carefully evaporated under reduced pressure. The desired allenoate was purified by flash chromatography [ethyl acetate/hexane], being obtained as an oil or a low melting point solid.



4-Methoxybenzyl 2,3-butadienoate (2.30a)

Allenoate **2.30a** was obtained as described in the general procedure from the corresponding alcohol (4-methoxybenzyl alcohol) and purified by flash chromatography (hexane/ethyl acetate, 4:1), being obtained as a colourless oil (385 mg, 1.885 mmol, 65%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 3.81 (s, 3H), 5.13 (s, 2H), 5.22 (d, *J* = 6.4 Hz, 2H), 5.66 (t, *J* = 6.4 Hz, 1H), 6.87-6.91 (m, 2H), 7.30-7.33 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 55.4, 66.6, 79.5, 88.1, 114.1, 128.1, 130.2, 159.8, 165.8, 216.1; HRMS (ESI) m/z: Calcd for $C_{12}H_{12}NaO_3$ $[M+Na]^+$ 227.0679; Found 227.0676.

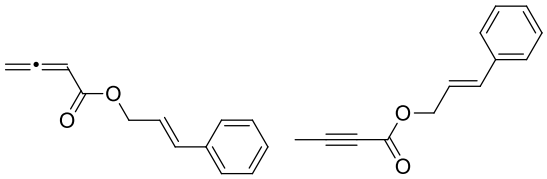


4-Methylbenzyl 2,3-butadienoate (2.30b) and 4-methylbenzyl 2-butinoate (2.30b')

Obtained as described in the general procedure from the corresponding alcohol (4-methylbenzyl alcohol). After flash chromatography (hexane/ethyl acetate, 4:1), a mixture of products **2.30b** and **2.30b'** (1:0.55) was obtained as a colourless oil (300 mg, 1.595 mmol, 55%).

Compound **2.30b**: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.36 (s, 3H), 5.15 (s, 2H), 5.23 (d, *J* = 6.8 Hz, 2H), 5.67 (t, *J* = 6.4 Hz, 1H), 7.16-7.18 (m, 2H), 7.25-7.28 (m, 2H); HRMS (ESI) m/z: Calcd for $C_{12}H_{13}O_2$ $[M+H]^+$ 189.0910; Found 189.0908.

Compound **2.30b'**: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.09 (s, 3H), 2.36 (s, 3H), 5.07 (s, 2H), 7.16-7.18 (m, 2H), 7.25-7.28 (m, 2H); HRMS (ESI) m/z: Calcd for $C_{12}H_{13}O_2$ $[M+H]^+$ 189.0910; Found 189.0908.



(*E*)-Cinnamyl 2,3-butadienoate (2.30c) and (*E*)-cinnamyl 2-butinoate (2.30c')

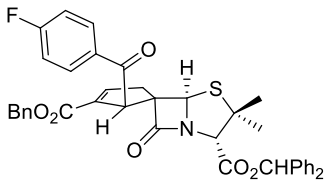
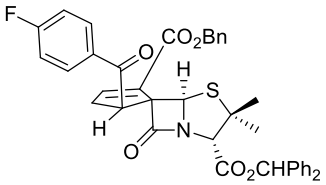
Obtained as described in the general procedure from the corresponding alcohol ((*E*)-cinnamyl alcohol). After flash chromatography (hexane/ethyl acetate, 4:1), a mixture of products **2.30c** and **2.30c'** (1:0.25) was obtained as a yellow oil (383 mg, 1.914 mmol, 66%).

Compound **2.30c**: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.82 (dd, *J* = 6.4 and 1.2 Hz, 2H), 5.25 (d, *J* = 6.4 Hz, 2H), 5.69 (t, *J* = 6.8 Hz, 1H), 6.27-6.34 (m, 1H), 6.67 (d, *J* = 16.0 Hz, 1H), 7.24-7.41 (m, 5H); HRMS (ESI) m/z: Calcd for $C_{13}H_{13}O_2$ $[M+H]^+$ 201.0910; Found 201.0909.

Compound **2.30c'**: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.11 (s, 3H), 4.73 (dd, *J* = 6.4 and 1.2 Hz, 2H), 6.27-6.34 (m, 1H), 6.66 (d, *J* = 16.0 Hz, 1H), 7.24-7.41 (m, 5H); HRMS (ESI) m/z: Calcd for $C_{13}H_{13}O_2$ $[M+H]^+$ 201.0910; Found 201.0909.

6.4.3. General Procedure for the Phosphine-catalyzed [3+2] Annulation of Allenoates with 6-Alkylidenepenicillanates

To a mixture of the appropriate 6-alkylidenepenicillanate (1 equiv.) and PPh_3 (20 mol%) in toluene (2-3 mL), a solution of allene (1 equiv.) in toluene (1-2 mL) was added. The reaction mixture was stirred at room temperature under nitrogen for the time indicated in each case, being monitored through TLC. Upon completion, the solvent was removed under reduced pressure and the crude product was purified by flash chromatography.

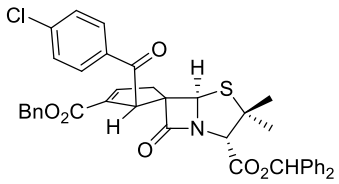
(1'*R*,2'*R*)-Benzhydryl spiro[(2-(4-fluorobenzoyl)-3-benzyloxycarbonylcyclopent-3-ene)-1',6-penicillanate] (2.28a) and (1'*R*,2'*R*)-Benzhydryl spiro[(2-(4-fluorobenzoyl)-5-benzyloxycarbonylcyclopent-4-ene)-1',6-penicillanate] (2.29a)

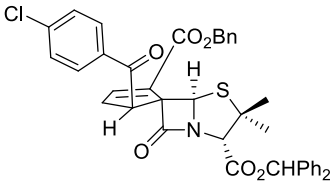
Obtained from allene **2.1** (65 mg, 0.375 mmol) and 6-alkylidenepenicillanate **2.20a** (188 mg, 0.375 mmol) as described in the general procedure (reaction time: 6.5 h). Purification of the crude product by flash chromatography (hexane/ethyl acetate, 4:1), gave, in order of elution, **2.28a** as a colourless solid (119 mg, 0.175 mmol, 47%) and **2.29a** as a colourless solid (125 mg, 0.185 mmol, 49%).

Compound **2.28a**: mp 64.5-66.5 ºC; $[\alpha]_D^{25}$ = + 270 (*c* 0.5 in CH_2Cl_2); IR (ATR): $\tilde{\nu}$ = 1063, 1155, 1201, 1234, 1330, 1456, 1593, 1670, 1718 and 1773 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.11 (s, 3H), 1.50 (s, 3H), 3.11 (dd, *J* = 18.6 and 3.1 Hz, 1H), 3.53 (dt, *J* = 18.6 and 2.3 Hz, 1H), 4.52 (s, 1H), 4.96 (d, *J* = 12.2 Hz, 1H), 5.00 (d, *J* = 12.2 Hz, 1H), 5.10 (d, *J* = 1.2 Hz, 1H), 5.41 (s, 1H), 6.91 (s, 1H), 7.01 (t, *J* = 8.6 Hz, 2H), 7.08 (s, 1H), 7.13-7.18 (m, 2H), 7.28-7.37 (m, 13H), 8.07 (dd, *J* = 9.8 and 5.4, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 26.1, 32.4, 40.8, 52.8, 64.3, 66.9, 69.1, 70.6, 71.1, 78.5, 115.6 (d, *J* = 22 Hz, 2C), 127.2, 127.7, 128.4, 128.5, 128.5, 128.6, 128.7, 128.7, 128.8, 132.1 (d, *J* = 10 Hz, 2C), 133.8 (d, *J* = 3 Hz, 1C), 135.3, 135.7, 139.2, 139.3, 146.2, 162.9, 166.1 (d, *J* = 256 Hz, 1C), 167.0, 176.3, 199.6; ^{19}F NMR (376 MHz, $CDCl_3$): -104.44 (s, 1F); HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd $C_{40}H_{35}FNO_6S$ 676.2164, found 676.2162.

Compound **2.29a**: mp 73.9-75.9 ºC; $[\alpha]_D^{25}$ = + 430 (*c* 0.5 in CH_2Cl_2); IR (ATR): $\tilde{\nu}$ = 977, 1012, 1151, 1176, 1200, 1306, 1374, 1453, 1596, 1679, 1718, 1744 and 1776 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.11 (s, 3H), 1.51 (s, 3H), 2.49 (dd, *J* = 18.6 and 2.5 Hz, 1H), 3.19 (ddd, *J* = 18.6, 9.1 and 2.0 Hz, 1H), 4.50 (d, *J* = 8.4 Hz, 1H), 4.54 (s, 1H), 5.21 (s, 2H), 6.27 (s, 1H), 6.91 (dd, *J* = 2.8 and 2.2 Hz, 1H), 6.94 (s, 1H), 7.16 (t, *J* = 8.6 Hz, 2H), 7.27-7.38 (m, 11H), 7.40-7.48 (m, 4H), 7.96 (dd, *J* = 8.8 and 5.3 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.9, 32.8, 36.0, 49.4, 62.8, 66.6, 69.1, 71.0, 74.0, 78.4, 116.3 (d, *J* = 22 Hz, 2C), 127.5, 127.5, 128.2, 128.2, 128.4, 128.5, 128.7, 128.7, 131.2 (d, *J* = 9 Hz, 2C), 131.6 (d, *J* = 3 Hz, 1C), 134.4, 135.8, 139.6, 144.7, 162.5, 166.1 (d, *J* =

256 Hz, 1C), 166.7, 174.2, 196.9; ^{19}F NMR (376 MHz, $CDCl_3$): -104.18 (s, 1F); HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd $C_{40}H_{35}FNO_6S$ 676.2164, found 676.2156.





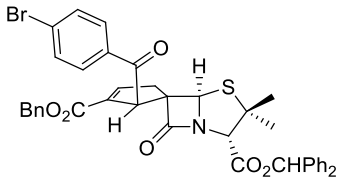
(1'*R*,2'*R*)-Benzhydryl spiro[(2-(4-chlorobenzoyl)-3-benzyloxycarbonylcyclopent-3-ene)-1',6-penicillanate] (2.28b) and (1'*R*,2'*R*)-Benzhydryl spiro[(2-(4-chlorobenzoyl)-5-benzyloxycarbonylcyclopent-4-ene)-1',6-penicillanate] (2.29b)

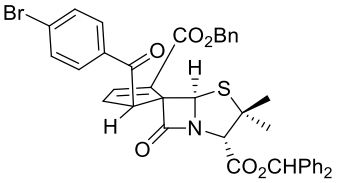
Obtained from allene **2.1** (65 mg, 0.375 mmol) and 6-alkylidenepenicillanate **2.20b** (194 mg, 0.375 mmol) as described in the general procedure (reaction time: 4.5 h). Purification of the crude product by flash chromatography (hexane/ethyl acetate, 4:1), gave, in order of elution, **2.28b** as a colourless solid (130 mg, 0.188 mmol, 50%) and **2.29b** as a colourless solid (116 mg, 0.168 mmol, 45%).

Compound **2.28b**: mp 145.2-146.5 ºC; $[\alpha]_D^{25}$ = + 290 (*c* 0.5 in CH_2Cl_2); IR (ATR): $\tilde{\nu}$ = 1000, 1065, 1177, 1198, 1236, 1328, 1456, 1588, 1668, 1718, 1735 and 1773 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.11 (s, 3H), 1.50 (s, 3H), 3.11 (dd, *J* = 18.6 and 3.1 Hz, 1H), 3.52 (dt, *J* = 18.6 and 2.3 Hz, 1H), 4.52 (s, 1H), 4.95 (d, *J* = 12.2 Hz, 1H), 5.00 (d, *J* = 12.2 Hz, 1H), 5.09 (d, *J* = 1.0 Hz, 1H), 5.40 (s, 1H), 6.91 (s, 1H), 7.08 (s, 1H), 7.14 (dd, *J* = 7.5 and 1.9 Hz, 2H), 7.29-7.35 (m, 14H), 7.97 (d, *J* = 8.7 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 26.1, 32.5, 40.8, 52.8, 64.4, 66.9, 69.1, 70.8, 71.1, 78.5, 127.1, 127.7, 128.4, 128.5, 128.6, 128.7, 128.8, 130.1, 135.2, 135.7, 139.2, 139.3, 140.1, 146.2, 162.9, 165.5, 166.9, 170.3, 176.3, 200.1; HRMS (ESI-TOF) m/z: $[M+NH_4]^+$ Calcd $C_{40}H_{38}ClN_2O_6S$ 709.2134, found 709.2125.

Compound **2.29b**: mp 116.3-118.3 ºC; $[\alpha]_D^{25}$ = + 370 (*c* 0.5 in CH_2Cl_2); IR (ATR): $\tilde{\nu}$ = 977, 1011, 1088, 1176, 1200, 1306, 1453, 1589, 1676, 1718, 1744 and 1774 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.11 (s, 3H), 1.51 (s, 3H), 2.47 (dd, *J* = 18.7 and 2.5 Hz, 1H), 3.18 (ddd, *J* = 18.8, 9.1 and 2.0 Hz, 1H), 4.49 (d, *J* = 8.4 Hz, 1H), 4.54 (s, 1H), 5.21 (s, 2H), 6.26 (s, 1H), 6.90 (t, *J* = 2.5 Hz, 1H), 6.93 (s, 1H), 7.27-7.49 (m, 17H), 7.87 (d, *J* = 8.6 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.9, 32.9, 35.9, 49.4, 62.8, 66.6, 69.1,

71.0, 74.1, 78.4, 127.5, 127.5, 128.2, 128.3, 128.4, 128.5, 128.7, 128.7, 129.4, 130.0, 133.5, 134.4, 135.8, 139.6, 140.3, 144.7, 162.5, 166.7, 174.2, 197.3; HRMS (ESI-TOF) m/z: $[M+NH_4]^+$ Calcd $C_{40}H_{38}ClN_2O_6S$ 709.2134, found 709.2131.





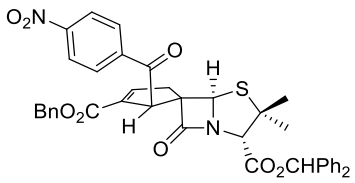
(1'*R*,2'*R*)-Benzhydryl spiro[(2-(4-bromobenzoyl)-3-benzyloxycarbonylcyclopent-3-ene)-1',6-penicillanate] (2.28c) and (1'*R*,2'*R*)-Benzhydryl spiro[(2-(4-bromobenzoyl)-5-benzyloxycarbonylcyclopent-4-ene)-1',6-penicillanate] (2.29c)

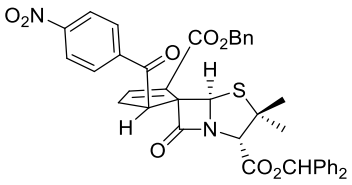
Obtained from allene **2.1** (65 mg, 0.375 mmol) and 6-alkylidenepenicillanate **2.20c** (211 mg, 0.375 mmol) as described in the general procedure (reaction time: 6.5 h). Purification of the crude product by flash chromatography (hexane/ethyl acetate, 4:1), gave, in order of elution, **2.28c** as a colourless solid (129 mg, 0.175 mmol, 47%) and **2.29c** as a colourless solid (109 mg, 0.148 mmol, 39%).

Compound **2.28c**: mp 156.5-158.5 ºC; $[\alpha]_D^{25}$ = + 360 (*c* 0.5 in CH_2Cl_2); IR (ATR): $\tilde{\nu}$ = 998, 1068, 1177, 1201, 1237, 1330, 1456, 1583, 1670, 1718 and 1773 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.11 (s, 3H), 1.50 (s, 3H), 3.11 (dd, *J* = 18.6 and 3.1 Hz, 1H), 3.52 (dt, *J* = 18.6 and 2.2 Hz, 1H), 4.52 (s, 1H), 4.95 (d, *J* = 12.2 Hz, 1H), 5.00 (d, *J* = 12.2 Hz, 1H), 5.08 (d, *J* = 1.1 Hz, 1H), 5.40 (s, 1H), 6.91 (s, 1H), 7.08 (s, 1H), 7.14 (dd, *J* = 7.5 and 1.8 Hz, 2H), 7.27-7.37 (m, 14H), 7.47 (d, *J* = 8.6 Hz, 2H), 7.89 (d, *J* = 8.6 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 26.1, 32.5, 40.8, 52.8, 64.4, 67.0, 69.1, 70.8, 71.1, 77.2, 78.5, 127.1, 127.7, 128.4, 128.6, 128.7, 128.8, 129.0, 130.8, 131.8, 135.2, 135.7, 136.1, 139.2, 139.3, 146.3, 162.9, 166.9, 176.3, 200.3; HRMS (ESI-TOF) m/z: $[M-H]^+$ Calcd $C_{40}H_{33}BrNO_6S$ 734.1217, found 734.1229.

Compound **2.29c**: mp 115.9-117.9 ºC; $[\alpha]_D^{25}$ = + 380 (*c* 0.5 in CH_2Cl_2); IR (ATR): $\tilde{\nu}$ = 976, 1007, 1069, 1176, 1199, 1305, 1374, 1453, 1584, 1676, 1718, 1744 and 1774 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.11 (s, 3H), 1.51 (s, 3H), 2.47 (dd, *J* = 18.5 and 2.7 Hz, 1H), 3.18 (dd, *J* = 18.6 and 9.0 Hz, 1H), 4.48 (d, *J* = 8.9 Hz, 1H), 4.54 (s, 1H), 5.21 (s, 2H), 6.26 (s, 1H), 6.90 (s, 1H), 6.93 (s, 1H), 7.27-7.37 (m, 11H), 7.44 (dd, *J* =

17.0 and 7.3 Hz, 4H), 7.63 (d, *J* = 8.4 Hz, 2H), 7.79 (d, *J* = 8.4 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.9, 32.9, 35.8, 49.4, 62.8, 66.6, 69.1, 71.0, 74.1, 78.4, 127.5, 127.5, 128.2, 128.3, 128.4, 128.5, 128.7, 128.7, 129.0, 130.0, 132.4, 133.9, 134.4, 135.8, 139.6, 144.7, 162.5, 166.7, 174.1, 197.4; HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd $C_{40}H_{35}BrNO_6S$ 736.1363, found 736.1362.





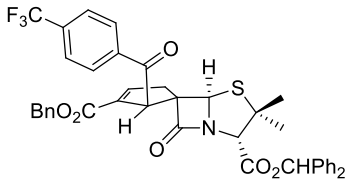
(1'*R*,2'*R*)-Benzhydryl spiro[(2-(4-nitrobenzoyl)-3-benzyloxycarbonylcyclopent-3-ene)-1',6-penicillanate] (2.28d) and (1'*R*,2'*R*)-Benzhydryl spiro[(2-(4-nitrobenzoyl)-5-benzyloxycarbonylcyclopent-4-ene)-1',6-penicillanate] (2.29d)

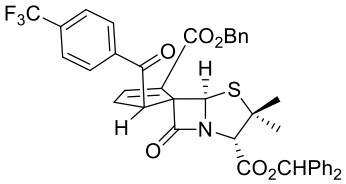
Obtained from allene **2.1** (65 mg, 0.375 mmol) and 6-alkylidenepenicillanate **2.20d** (198 mg, 0.375 mmol) as described in the general procedure (reaction time: 5.5 h). Purification of the crude product by flash chromatography (hexane/ethyl acetate, 3:1), gave, in order of elution, **2.28d** as a colourless oil (138 mg, 0.196 mmol, 52%) and **2.29d** as a colourless solid (77 mg, 0.109 mmol, 29%).

Compound **2.28d**: mp 122.0-123.7 ºC; $[\alpha]_D^{25}$ = + 310 (*c* 0.5 in CH_2Cl_2); IR (ATR): $\tilde{\nu}$ = 845, 950, 1004, 1066, 1179, 1316, 1521, 1675, 1734 and 1759 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.11 (s, 3H), 1.49 (s, 3H), 3.14 (dd, *J* = 18.7 and 3.1 Hz, 1H), 3.52 (dt, *J* = 18.7 and 2.3 Hz, 1H), 4.54 (s, 1H), 4.92 (d, *J* = 12.1 Hz, 1H), 4.99 (d, *J* = 12.1 Hz, 1H), 5.13 (d, *J* = 1.1 Hz, 1H), 5.40 (s, 1H), 6.91 (s, 1H), 7.09-7.13 (m, 3H), 7.26-7.37 (m, 13H), 8.12 (s, 4H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 26.1, 32.8, 40.7, 53.3, 64.8, 67.1, 69.1, 71.1, 71.1, 78.6, 123.6, 127.1, 127.7, 128.4, 128.6, 128.7, 128.7, 128.8, 130.1, 134.9, 135.4, 139.1, 139.2, 141.7, 146.7, 150.4, 162.8, 166.8, 175.8, 200.2; HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd $C_{40}H_{38}N_3O_8S$ 720.2374, found 720.2368.

Compound **2.29d**: mp 122.7-124.7 ºC; $[\alpha]_D^{25}$ = + 330 (*c* 0.5 in CH_2Cl_2); IR (ATR): $\tilde{\nu}$ = 1013, 1154, 1179, 1201, 1313, 1347, 1528, 1686, 1723, 1743 and 1776 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.11 (s, 3H), 1.51 (s, 3H), 2.48 (dd, *J* = 18.5 and 2.5 Hz, 1H), 3.18 (ddd, *J* = 18.6, 9.1 and 2.0 Hz, 1H), 4.53 (d, *J* = 8.5 Hz, 1H), 4.56 (s, 1H), 5.22 (s, 2H), 6.26 (s, 1H), 6.90-6.92 (s, 1H), 6.94 (s, 1H), 7.27-7.39 (m, 11H), 7.41 (d, *J* = 7.1 Hz,

2H), 7.46 (d, *J* = 7.1 Hz, 2H), 8.09 (d, *J* = 8.9 Hz, 2H), 8.34 (d, *J* = 8.9 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.8, 33.1, 35.5, 49.8, 63.2, 66.7, 69.1, 71.0, 74.5, 78.5, 124.3, 127.4, 127.5, 128.3, 128.3, 128.5, 128.5, 128.7, 128.7, 128.7, 129.6, 134.4, 135.7, 139.6, 139.9, 144.3, 150.7, 162.4, 166.6, 173.7, 197.0; HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd $C_{40}H_{38}N_3O_8S$ 720.2374, found 720.2366.





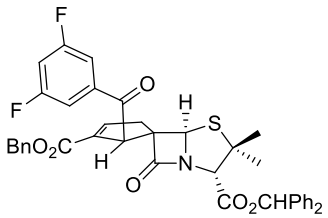
(1'*R*,2'*R*)-Benzhydryl spiro[(2-(4-trifluoromethylbenzoyl)-3-benzyloxycarbonylcyclopent-3-ene)-1',6-penicillanate] (2.28e) and (1'*R*,2'*R*)-Benzhydryl spiro[(2-(4-trifluoromethylbenzoyl)-5-benzyloxycarbonylcyclopent-4-ene)-1',6-penicillanate] (2.29e)

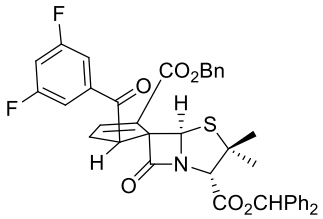
Obtained from allene **2.1** (65 mg, 0.375 mmol) and 6-alkylidenepenicillanate **2.20e** (207 mg, 0.375 mmol) as described in the general procedure (reaction time: 5.5 h). Purification of the crude product by flash chromatography (hexane/ethyl acetate, 4:1), gave, in order of elution, **2.28e** as a colourless solid (143 mg, 0.196 mmol, 52%) and **2.29e** as a colourless solid (96 mg, 0.133 mmol, 35%).

Compound **2.28e**: mp 134.1-135.5 ºC; $[\alpha]_D^{25}$ = + 320 (*c* 0.5 in CH_2Cl_2); IR (ATR): $\tilde{\nu}$ = 1004, 1065, 1135, 1320, 1675, 1735 and 1760 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.12 (s, 3H), 1.50 (s, 3H), 3.13 (dd, *J* = 18.7 and 3.1 Hz, 1H), 3.53 (dt, *J* = 18.6 and 2.3 Hz, 1H), 4.54 (s, 1H), 4.95 (d, *J* = 12.2 Hz, 1H), 4.99 (d, *J* = 12.2 Hz, 1H), 5.14 (d, *J* = 1.1 Hz, 1H), 5.41 (s, 1H), 6.92 (s, 1H), 7.10-7.14 (m, 3H), 7.27-7.35 (m, 12H), 7.60 (d, *J* = 8.3 Hz, 2H), 8.13 (d, *J* = 8.3 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 26.1, 32.7, 40.7, 53.0, 64.6, 67.0, 69.1, 70.1, 71.1, 78.5, 123.8 (d, *J* = 272.8 Hz, 1C), 125.5, 125.5, 127.1, 127.7, 128.4, 128.6, 128.6, 128.6, 128.7, 128.8, 129.5, 134.4, 134.7, 135.1, 135.6, 139.2, 139.3, 140.0, 146.4, 162.8, 166.9, 176.1, 200.5; ^{19}F NMR (376 MHz, $CDCl_3$): δ = -63.03 (s, 3F); HRMS (ESI-TOF) m/z: $[M+NH_4]^+$ Calcd $C_{41}H_{38}F_3N_2O_6S$ 743.2397, found 743.2387.

Compound **2.29e**: mp 141.1-143.1 ºC; $[\alpha]_D^{25}$ = + 330 (*c* 0.5 in CH_2Cl_2); IR (ATR): $\tilde{\nu}$ = 1013, 1067, 1131, 1171, 1319, 1453, 1684, 1719, 1744 and 1776 cm^{-1}; ^{1}H NMR (400

MHz, $CDCl_3$): δ = 1.11 (s, 3H), 1.52 (s, 3H), 2.48 (dd, *J* = 19.0 and 2.7 Hz, 1H), 3.20 (ddd, *J* = 18.6, 9.1 and 2.0 Hz, 1H), 4.54 (d, *J* = 8.6 Hz, 1H), 4.56 (s, 1H), 5.22 (s, 2H), 6.27 (s, 1H), 6.89-6.92 (s, 1H), 6.94 (s, 1H), 7.27-7.38 (m, 11H), 7.42 (d, *J* = 7.2, 2H), 7.46 (d, *J* = 7.2, 2H), 7.76 (d, *J* = 8.3 Hz, 2H), 8.04 (d, *J* = 8.3 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.9, 33.0, 35.6, 49.6, 63.0, 66.7, 69.1, 71.0, 74.2, 78.4, 123.6 (d, *J* = 272.9 Hz, 1C), 126.2, 126.2, 127.5, 127.5, 128.2, 128.3, 128.4, 128.5, 128.7, 128.7, 128.7, 128.9, 134.4, 134.8, 135.1, 135.8, 138.0, 139.6, 144.5, 162.4, 166.7, 173.9, 197.4; ^{19}F NMR (376 MHz, $CDCl_3$): δ = -66.18 (s, 3F); HRMS (ESI-TOF) m/z: $[M+NH_4]^+$ Calcd $C_{41}H_{38}F_3N_2O_6S$ 743.2397, found 743.2386.





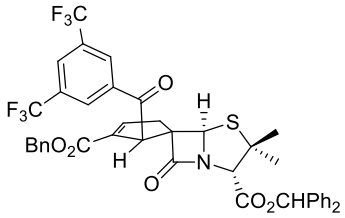
(1'*R*,2'*R*)-Benzhydryl spiro[(2-(3,5-difluorobenzoyl)-3-benzyloxycarbonylcyclopent-3-ene)-1',6-penicillanate] (2.28f) and (1'*R*,2'*R*)-Benzhydryl spiro[(2-(3,5-difluorobenzoyl)-5-benzyloxycarbonylcyclopent-4-ene)-1',6-penicillanate] (2.29f)

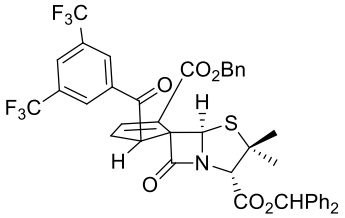
Obtained from allene **2.1** (65 mg, 0.375 mmol) and 6-alkylidenepenicillanate **2.20f** (195 mg, 0.375 mmol) as described in the general procedure (reaction time: 6.5 h). Purification of the crude product by flash chromatography (hexane/ethyl acetate, 4:1), gave, in order of elution, **2.28f** as a colourless solid (138 mg, 0.198 mmol, 53%) and **2.29f** as a colourless solid (116 mg, 0.167 mmol, 44%).

Compound **2.28f**: mp 69.5-71.5 ºC; $[\alpha]_D^{25}$ = + 320 (*c* 0.5 in CH_2Cl_2); IR (ATR): ṽ = 984, 1065, 1120, 1333, 1438, 1593, 1676, 1716, 1741 and 1773 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.13 (s, 3H), 1.52 (s, 3H), 3.12 (dd, *J* = 18.7 and 3.1 Hz, 1H), 3.50 (dt, *J* = 18.7 and 2.3 Hz, 1H), 4.54 (s, 1H), 4.97 (d, *J* = 12.2 Hz, 1H), 4.99 (s, 1H), 5.04 (d, *J* = 12.2 Hz, 1H), 5.39 (s, 1H), 6.92 (s, 1H), 6.93-6.98 (m, 1H), 7.08 (s, 1H), 7.15-7.18 (m, 2H), 7.29-7.35 (m, 13H), 7.55 (dd, *J* = 7.9 and 2.1 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 26.1, 32.8, 40.7, 53.2, 64.6, 66.9, 69.1, 71.0, 71.1, 78.5, 108.8 (t, *J* = 25.4 Hz, 1C), 112.2 (d, *J* = 16.6 Hz, 1C), 112.2 (d, *J* = 26.2 Hz, 1C), 127.1, 127.7, 128.4, 128.6, 128.7, 128.8, 135.1, 135.5, 139.1, 139.3, 140.1, 140.5 (t, *J* = 7.9 Hz, 1C), 146.3, 162.8, 162.8 (d,

J = 250.2 Hz, 1C), 162.8 (d, *J* = 250.0 Hz, 1C), 166.8, 175.9, 199.2; ^{19}F NMR (376 MHz, $CDCl_3$): δ = -108.43 (s, 2F); HRMS (ESI-TOF) m/z: $[M+NH_4]^+$ Calcd $C_{40}H_{37}F_2N_2O_6S$ 711.2335, found 711.2333.

Compound **2.29f**: mp 134.8-136.8 ºC; $[\alpha]_D^{25}$ = + 270 (*c* 0.5 in CH_2Cl_2); IR (ATR): ν̃ = 987, 1119, 1257, 1304, 1333, 1438, 1593, 1686, 1718 and 1769 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.11 (s, 3H), 1.53 (s, 3H), 2.47 (dd, *J* = 19.0 and 2.7 Hz, 1H), 3.19 (ddd, *J* = 18.6, 9.1 and 2.1 Hz, 1H), 4.40 (d, *J* = 8.3 Hz, 1H), 4.56 (s, 1H), 5.21 (s, 2H), 6.25 (s, 1H), 6.90 (t, *J* = 2.5 Hz, 1H), 6.94 (s, 1H), 7.05 (tt, *J* = 8.4 and 2.3 Hz, 1H), 7.27-7.38 (m, 11H), 7.39-7.49 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.9, 33.0, 35.6, 49.7, 63.0, 66.7, 69.1, 71.0, 74.2, 78.4, 109.1 (t, *J* = 25 Hz, 1C), 111.4 (d, *J* = 8 Hz, 1C), 111.6 (d, *J* = 7 Hz, 1C), 127.5, 127.5, 128.2, 128.3, 128.4, 128.5, 128.7, 128.7, 128.7, 134.4, 135.8, 138.2 (t, *J* = 8 Hz, 1C), 139.6, 144.4, 162.4, 163.3 (d, *J* = 252 Hz, 1C), 163.4 (d, *J* = 252 Hz, 1C), 166.6, 173.8, 196.0; ^{19}F NMR (376 MHz, $CDCl_3$): δ = -107.21 (s, 2F); HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd $C_{40}H_{34}F_2NO_6S$ 694.2069, found 694.2067.





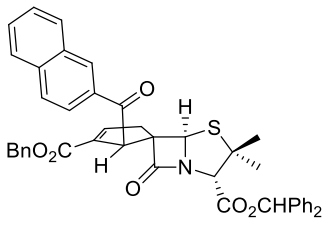
(1'*R*,2'*R*)-Benzhydryl spiro[(2-(3,5-bis(trifluoromethyl)benzoyl)-3-benzyloxycarbonylcyclopent-3-ene)-1',6-penicillanate] (2.28g) and (1'*R*,2'*R*)-Benzhydryl spiro[(2-(3,5-bis(trifluoromethyl)benzoyl)-5-benzyloxycarbonylcyclopent-4-ene)-1',6-penicillanate] (2.29g)

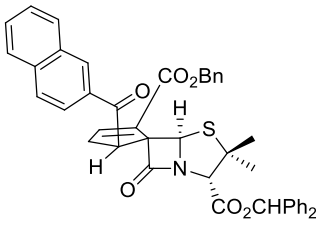
Obtained from allene **2.1** (65 mg, 0.375 mmol) and 6-alkylidenepenicillanate **2.20g** (232 mg, 0.375 mmol) as described in the general procedure (reaction time: 5 h). Purification of the crude product by flash chromatography (hexane/ethyl acetate, 4:1), gave a mixture of **2.28g**/**2.29g** as a yellow oil (197 mg, 0.248 mmol, 66%), in a 59/41 ratio.

Compound **2.28g**: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.12 (s, 3H), 1.51 (s, 3H), 3.15 (dd, *J* = 18.7 and 3.1 Hz, 1H), 3.51 (dt, *J* = 18.7 and 2.2 Hz, 1H), 4.58 (s, 1H), 4.88 (d, *J* = 12.1 Hz, 1H), 5.23 (s, 1H), 5.05 (d, *J* = 12.2 Hz, 1H), 5.41 (s, 1H), 6.91 (s, 1H),

7.06-7.10 (m, 2H), 7.23-7.36 (m, 14H), 7.98 (s, 1H), 8.46 (s, 2H); HRMS (ESI-TOF) m/z: $[M+Cl]^+$ Calcd $C_{42}H_{33}NO_6SF_6Cl$ 828.1627, found 828.1638.

Compound **2.29g**: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.12 (s, 3H), 1.54 (s, 3H), 2.46 (dd, *J* = 19.0 and 3.3 Hz, 1H), 3.22 (ddd, *J* = 18.4, 9.1 and 1.8 Hz, 1H), 4.52 (d, *J* = 8.3 Hz, 1H), 4.58 (s, 1H), 5.12 (s, 2H), 6.26 (s, 1H), 6.94 (s, 1H), 7.06-7.10 (m, 2H), 7.23-7.26 (m, 14H), 8.10 (s, 1H), 8.34 (s, 2H); HRMS (ESI-TOF) m/z: $[M+Cl]^+$ Calcd $C_{42}H_{33}NO_6SF_6Cl$ 828.1627, found 828.1638.





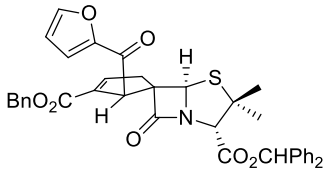
(1'*R*,2'*R*)-Benzhydryl spiro[(2-naphthoyl-3-benzyloxycarbonylcyclopent-3-ene)-1',6-penicillanate] (2.28h) and (1'*R*,2'*R*)-Benzhydryl spiro[(2-naphthoyl-5-benzyloxycarbonylcyclopent-4-ene)-1',6-penicillanate] (2.29h)

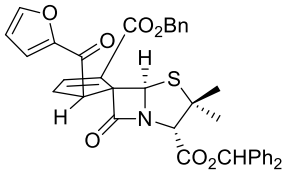
Obtained from allene **2.1** (65 mg, 0.375 mmol) and 6-alkylidenepenicillanate **2.20h** (200 mg, 0.375 mmol) as described in the general procedure (reaction time: 4 h). Purification of the crude product by flash chromatography (hexane/ethyl acetate, 5:1), gave, in order of elution, **2.28h** as a yellow solid (111 mg, 0.158 mmol, 42%) and **2.29h** as a fluffy yellow solid (114 mg, 0.161 mmol, 43%).

Compound **2.28h**: mp 81.5-83.5 ºC; $[\alpha]_D^{25}$ = + 340 (*c* 0.25 in CH_2Cl_2); IR (ATR): $\tilde{\nu}$ = 1175, 1247, 1330, 1457, 1654, 1707, 1718 and 1773 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.07 (s, 3H), 1.49 (s, 3H), 3.15 (dd, *J* = 18.6 and 3.1 Hz, 1H), 3.58 (dt, *J* = 18.6 and 2.2 Hz, 1H), 4.53 (s, 1H), 4.91 (s, 2H), 5.33 (s, 1H), 5.44 (s, 1H), 6.91 (s, 1H), 7.00-7.02 (m, 2H), 7.10-7.16 (m, 4H), 7.28-7.34 (m, 10H), 7.52-7.56 (m, 1H), 7.59-7.63 (m, 1H), 7.80-7.92 (m, 3H), 8.05 (dd, *J* = 8.7 and 1.7 Hz, 1H), 8.66 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 26.1, 32.4, 40.9, 52.9, 64.3, 66.7, 69.1, 70.7, 71.2, 78.4, 124.7, 126.8, 127.1, 127.7, 127.8, 128.2, 128.3, 128.3, 128.4, 128.5, 128.5, 128.8, 128.8, 130.2, 131.8, 132.6, 134.8, 135.2, 136.0, 136.0, 139.2, 139.3, 146.0, 162.9, 167.0, 176.6, 201.0; HRMS (ESI-TOF) m/z: $[M+NH_4]^+$ Calcd $C_{44}H_{41}N_2O_6S$ 725.2680, found 725.2676.

Compound **2.29h**: mp 90.8-92.8 ºC; $[\alpha]_D^{25}$ = + 340 (*c* 0.25 in CH_2Cl_2); IR (ATR): $\tilde{\nu}$ = 984, 1118, 1251, 1323, 1457, 1670, 1718 and 1763 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$):

δ = 1.09 (s, 3H), 1.52 (s, 3H), 2.59 (dd, *J* = 18.8 and 2.5 Hz, 1H), 3.27 (ddd, *J* = 18.6, 9.1 and 2.0 Hz, 1H), 4.56 (s, 1H), 4.72 (d, *J* = 8.5 Hz, 1H), 5.23 (s, 2H), 6.32 (s, 1H), 6.94-6.95 (m, 2H), 7.27-7.40 (m, 11H), 7.41-7.44 (m, 2H), 7.46-7.48 (m, 2H), 7.56-7.65 (m, 2H), 7.88-7.94 (m, 2H), 7.97-8.01 (m, 2H), 8.46 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.9, 32.7, 36.3, 49.6, 62.7, 66.6, 69.2, 71.1, 74.1, 78.4, 124.2, 127.2, 127.5, 127.5, 128.0, 128.2, 128.2, 128.4, 128.5, 128.7, 128.7, 129.0, 129.8, 130.2, 132.5, 132.7, 134.4, 135.9, 139.7, 145.0, 162.6, 166.8, 174.5, 198.4; HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd $C_{44}H_{38}NO_6S$ 708.2414, found 708.2410.





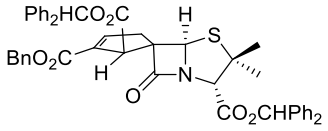
(1'*R*,2'*R*)-Benzhydryl spiro[(2-furoyl-3-benzyloxycarbonylcyclopent-3-ene)-1',6-penicillanate] (2.28i) and (1'*R*,2'*R*)-Benzhydryl spiro[(2-furoyl-5-benzyloxycarbonylcyclopent-4-ene)-1',6-penicillanate] (2.29i)

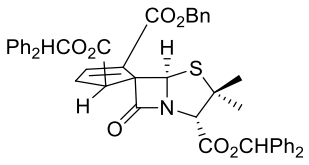
Obtained from allene **2.1** (65 mg, 0.375 mmol) and 6-alkylidenepenicillanate **2.20i** (177 mg, 0.375 mmol) as described in the general procedure (reaction time: 7 h). Purification of the crude product by flash chromatography (hexane/ethyl acetate, 2:1), gave, in order of elution, **2.28i** as a colourless solid (81 mg, 0.12 mmol, 33%) and **2.29i** as a colourless solid (134 mg, 0.21 mmol, 55%).

Compound **2.28i**: mp 76.5-78.5 ºC; $[\alpha]_D^{25}$ = + 360 (*c* 0.25 in CH_2Cl_2); IR (ATR): ῡ = 985, 1177, 1202, 1234, 1292, 1331, 1463, 1560, 1662, 1716 and 1773 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.14 (s, 3H), 1.51 (s, 3H), 3.09 (dd, *J* = 18.6 and 3.1 Hz, 1H), 3.48 (dt, *J* = 18.6 and 2.3 Hz, 1H), 4.53 (s, 1H), 4.92 (d, *J* = 1.0 Hz, 2H), 5.01 (d, *J* = 12.3 Hz, 1H), 5.06 (d, *J* = 12.3 Hz, 1H), 5.41 (s, 1H), 6.47 (dd, *J* = 3.6 and 1.6 Hz, 1H), 6.91 (s, 1H), 7.06 (s, 1H), 7.22 (dd, *J* = 6.7 and 2.8 Hz, 2H), 7.28-7.35 (m, 14H), 7.51 (d, *J* = 1.0, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 26.0, 32.8, 41.0, 53.7, 64.1, 66.8, 69.1, 70.2, 71.1, 78.5, 112.8, 119.3, 127.1, 127.7, 128.3, 128.4, 128.5, 128.6, 128.7, 128.7, 128.8, 135.4, 135.6, 139.2, 139.3, 145.9, 147.0, 153.2, 162.9, 166.9, 176.1, 188.6; HRMS (ESI-TOF) m/z: $[M+NH_4]^+$ Calcd $C_{38}H_{37}N_2O_7S$ 665.2316, found 665.2310.

Compound **2.29i**: mp 112.8-114.8 ºC; $[\alpha]_D^{25}$ = + 370 (*c* 0.5 in CH_2Cl_2); IR (ATR): ῡ = 985, 1055, 1133, 1177, 1254, 1330, 1457, 1654, 1720, 1749 and 1770 cm^{-1}; ^{1}H NMR

(400 MHz, $CDCl_3$): δ = 1.12 (s, 3H), 1.52 (s, 3H), 2.54 (dd, *J* = 18.8 and 2.2 Hz, 1H), 3.16 (ddd, *J* = 18.8, 9.0 and 2.1 Hz, 1H), 4.38 (d, *J* = 8.1 Hz, 1H), 4.55 (s, 1H), 5.20 (s, 2H), 6.25 (s, 1H), 6.57 (dd, *J* = 3.6 and 1.7 Hz, 1H), 6.93 (d, *J* = 4.7 Hz, 2H), 7.23 (d, *J* = 3.6 Hz, 1H), 7.27-7.37 (m, 11H), 7.42 (d, *J* = 7.2 Hz, 2H), 7.46 (d, *J* = 7.2 Hz, 2H), 7.60 (d, *J* = 1.0 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.8, 33.1, 36.1, 49.9, 62.8, 66.5, 69.1, 71.0, 73.6, 78.4, 112.8, 117.8, 127.5, 127.5, 128.2, 128.2, 128.3, 128.4, 128.7, 128.7, 134.2, 135.9, 139.7, 145.1, 146.7, 151.7, 162.5, 166.8, 174.1, 187.8; HRMS (ESI-TOF) m/z: $[M+NH_4]^+$ Calcd $C_{38}H_{37}N_2O_7S$ 665.2316, found 665.2309.



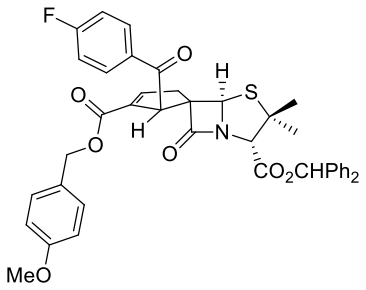


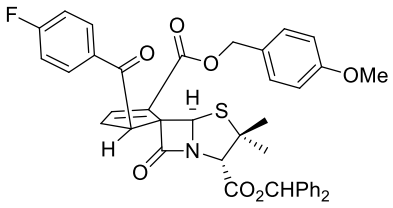
(1'*R*,2'*R*)-Benzhydryl spiro[(2-benzhydryloxycarbonyl-3-benzyloxycarbonylcyclopent-3-ene)-1',6-penicillanate] (2.28j) and (1'*R*,2'*R*)-Benzhydryl spiro[(2-benzhydryloxycarbonyl-5-benzyloxycarbonylcyclopent-4-ene)-1',6-penicillanate] (2.29j)

Obtained from allene **2.1** (65 mg, 0.375 mmol) and 6-alkylidenepenicillanate **2.20j** (221 mg, 0.375 mmol) as described in the general procedure (reaction time: 5 h). Purification of the crude product by flash chromatography (hexane/ethyl acetate, 3:1), gave a mixture of **2.28j**/**2.29j** as a yellow oil (270 mg, 0.353 mmol, 94%) in a 50/50 ratio.

Compound **2.28j**: ^1H NMR (400 MHz, $CDCl_3$): δ = 1.11 (s, 3H), 1.47 (s, 3H), 3.02 (dd, *J* = 18.5 and 3.1 Hz, 1H), 3.30 (dt, *J* = 18.4 and 2.1 Hz, 1H), 4.19 (d, *J* = 0.9 Hz, 1H), 4.49 (s, 1H), 5.06 (d, *J* = 12.4 Hz, 1H), 5.15 (d, *J* = 12.4 Hz, 1H), 5.33 (s, 1H), 6.82 (s, 1H), 6.90 (s, 1H), 6.97-6.99 (m, 1H), 7.24-7.40 (m, 24H), 7.43-4.47 (m, 1H); HRMS (ESI-TOF) m/z: $[M+NH_4]^+$ Calcd $C_{47}H_{45}N_2O_7S$ 781.2942, found 781.2932.

Compound **2.29j**: ^1H NMR (400 MHz, $CDCl_3$): δ = 1.07 (s, 3H), 1.50 (s, 3H), 2.55 (dd, *J* = 18.8 and 2.5 Hz, 1H), 3.10 (ddd, *J* = 18.7, 8.6 and 2.1 Hz, 1H), 3.65 (d, *J* = 7.9 Hz, 1H), 4.52 (s, 1H), 5.19 (d, *J* = 3.7 Hz, 1H), 5.22 (d, *J* = 3.6 Hz, 1H), 6.11 (s, 1H), 6.83 (s, 1H), 6.90 (s, 1H), 6.97-6.99 (m, 1H), 7.24-7.40 (m, 24H), 7.43-4.47 (m, 1H); HRMS (ESI-TOF) m/z: $[M+NH_4]^+$ Calcd $C_{47}H_{45}N_2O_7S$ 781.2942, found 781.2932.





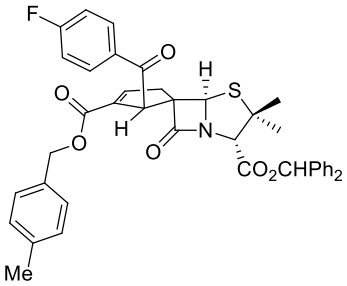
(1'*R*,2'*R*)-Benzhydryl spiro[(2-(4-fluorobenzoyl)-3-(4-methoxybenzyloxycarbonyl)cyclopent-3-ene)-1',6-penicillanate] (2.31a) and (1'*R*,2'*R*)-Benzhydryl spiro[(2-(4-fluorobenzoyl)-5-(4-methoxybenzyloxycarbonyl)cyclopent-4-ene)-1',6-penicillanate] (2.32a)

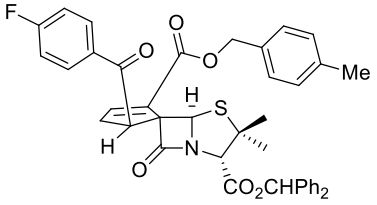
Obtained from allene **2.30a** (51 mg, 0.250 mmol) and 6-alkylidenepenicillanate **2.20a** (125 mg, 0.250 mmol) as described in the general procedure (reaction time: 24 h). Purification of the crude product by flash chromatography (hexane/ethyl acetate, 4:1), gave, in order of elution, **2.31a** as a colourless solid (64 mg, 0.091 mmol, 36%) and **2.32a** as a colourless solid (69 mg, 0.097 mmol, 39%).

Compound **2.31a**: mp 72.9-74.9 ºC; $[\alpha]_D^{25}$ = + 390 (*c* 0.5 in CH_2Cl_2); IR (ATR): $\tilde{\nu}$ = 1001, 1104, 1156, 1176, 1203, 1237, 1330, 1515, 1593, 1670, 1710, 1746 and 1773 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.11 (s, 3H), 1.49 (s, 3H), 3.10 (dd, *J* = 18.6 and 3.1 Hz, 1H), 3.51 (dt, *J* = 18.5 and 2.2 Hz, 1H), 3.81 (s, 3H), 4.51 (s, 1H), 4.88 (d, *J* = 11.9 Hz, 1H), 4.93 (d, *J* = 11.9 Hz, 1H), 5.08 (d, *J* = 1.1 Hz, 1H), 5.40 (s, 1H), 6.80-6.82 (m, 2H), 6.91 (s, 1H), 7.01 (t, *J* = 8.6 Hz, 2H), 7.06-7.09 (m, 3H), 7.29-7.34 (m, 10H), 8.06 (dd, *J* = 5.4 and 8.9 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 26.1, 32.5, 40.8, 52.8, 55.4, 64.3, 66.7, 69.1, 70.7, 71.1, 78.5, 114.0, 115.5 (d, *J* = 21.9 Hz, 2C), 127.1, 127.3, 127.7, 128.3, 128.5, 128.7, 130.5, 132.1 (d, *J* = 9.4 Hz, 2C), 133.9 (d, *J* = 2.7 Hz, 1C), 135.8, 139.2, 139.3, 146.0, 159.9, 163.0, 166.10 (d, *J* = 255.4 Hz, 1C), 167.0, 176.4, 199.6; ^{19}F NMR (376 MHz, $CDCl_3$): δ = -104.56 (s, 1F); HRMS (ESI-TOF) m/z: $[M+NH_4]^+$ Calcd $C_{41}H_{40}FN_2O_7S$ 723.2535, found 723.2531.

Compound **2.32a**: mp 84.6-86.6 ºC; $[\alpha]_D^{25}$ = +350 (*c* 0.5 in CH_2Cl_2); IR (ATR): $\tilde{\nu}$ = 983, 1028, 1122, 1152, 1210, 1237, 1331, 1457, 1514, 1595, 1676, 1712 and 1764 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.11 (s, 3H), 1.51 (s, 3H), 2.47 (dd, *J* = 18.6 and 2.5 Hz, 1H), 3.17 (ddd, *J* = 18.5, 9.1 and 2.0 Hz, 1H), 3.80 (s, 3H), 4.49 (d, *J* = 8.4 Hz, 1H), 4.54 (s, 1H), 5.13 (d, *J* = 12.0 Hz, 1H), 5.17 (d, *J* = 12.0 Hz, 1H), 6.26 (s, 1H), 6.81-6.89 (m, 3H), 6.95 (s, 1H), 7.16 (t, *J* = 8.6 Hz, 1H), 7.27-7.37 (m, 8H), 7.40-7.50 (m, 4H), 7.93-7.98 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.9, 32.8, 35.9, 49.4, 55.4, 62.7,

66.4, 69.1, 71.0, 74.0, 78.4, 114.1, 116.2 (d, *J* = 21.9 Hz, 2C), 127.5, 127.5, 128.0, 128.2, 128.3, 128.7, 128.7, 130.4, 131.2 (d, *J* = 9.4 Hz, 2C), 131.6 (d, *J* = 2.9 Hz, 1C), 134.5, 139.7, 144.5, 159.8, 162.3, 166.1 (d, *J* = 255.6 Hz, 1C), 166.8, 174.3, 196.9; ^{19}F NMR (376 MHz, $CDCl_3$): δ = -104.21 (s, 1F); HRMS (ESI-TOF) m/z: $[M+NH_4]^+$ Calcd $C_{41}H_{40}FN_2O_7S$ 723.2535, found 723.2529.



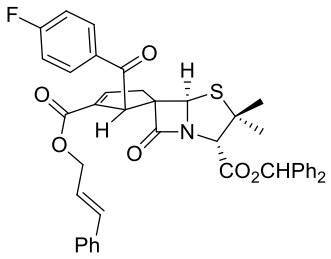


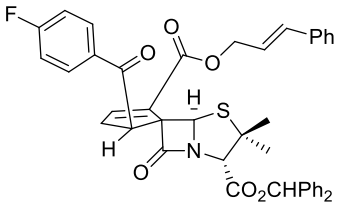
(1'*R*,2'*R*)-Benzhydryl spiro[(2-(4-fluorobenzoyl)-3-(4-methylbenzyloxycarbonyl)cyclopent-3-ene)-1',6-penicillanate] (2.31b) and (1'*R*,2'*R*)-Benzhydryl spiro[(2-(4-fluorobenzoyl)-5-(4-methylbenzyloxycarbonyl)cyclopent-4-ene)-1',6-penicillanate] (2.32b)

Obtained from allene **2.30b** (47 mg, 0.250 mmol) and 6-alkylidenepenicillanate **2.20a** (125 mg, 0.250 mmol) as described in the general procedure (reaction time: 7 d). Purification of the crude product by flash chromatography (hexane/ethyl acetate, 3:1), gave, in order of elution, **2.31b** as a colourless solid (40 mg, 0.058 mmol, 23%) and **2.32b** as a colourless solid (55 mg, 0.079 mmol, 32%).

Compound **2.31b**: mp 71.4-73.4 ºC; $[\alpha]_D^{25}$ = + 310 (*c* 0.5 in CH_2Cl_2); IR (ATR): ῡ = 986, 1000, 1103, 1155, 1201, 1331, 1450, 1508, 1593, 1671, 1711 and 1773 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.11 (s, 3H), 1.50 (s, 3H), 2.35 (s, 3H), 3.10 (dd, *J* = 18.6 and 3.1 Hz, 1H), 3.52 (dt, *J* = 18.5 and 2.2 Hz, 1H), 4.52 (s, 1H), 4.91 (d, *J* = 12.0 Hz, 1H), 4.96 (d, *J* = 12.0 Hz, 1H), 5.09 (d, *J* = 1.1 Hz, 1H), 5.41 (s, 1H), 6.92 (s, 1H), 6.98-7.10 (m, 7H), 7.29-7.35 (m, 10H), 8.06 (dd, *J* = 8.9 and 5.4 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 21.3, 26.1, 32.4, 40.8, 52.8, 64.3, 66.8, 69.1, 70.7, 71.1, 78.5, 115.5 (d, *J* = 21.9 Hz, 2C), 127.1, 127.7, 128.3, 128.5, 128.7, 128.8, 128.8, 129.3, 132.0 (d, *J* = 9.5 Hz, 2C), 132.2, 133.9 (d, *J* = 2.8 Hz, 1C), 135.8, 138.4, 139.2, 139.3, 146.0, 162.9, 166.10 (d, *J* = 255.4 Hz, 1C), 167.0, 176.4, 199.6; ^{19}F NMR (376 MHz, $CDCl_3$): δ = -104.52 (s, 1F); HRMS (ESI-TOF) m/z: $[M+NH_4]^+$ Calcd $C_{41}H_{40}FN_2O_6S$ 707.2586, found 707.2580.

Compound **2.32b**: mp 71.3-73.3 ºC; $[\alpha]_D^{25}$ = + 330 (*c* 0.5 in CH_2Cl_2); IR (ATR): $\tilde{\nu}$ = 981, 1015, 1122, 1210, 1330, 1508, 1595, 1676, 1716 and 1764 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.11 (s, 3H), 1.51 (s, 3H), 2.34 (s, 3H), 2.48 (dd, *J* = 18.6 and 2.5 Hz, 1H), 3.52 (ddd, *J* = 18.6, 9.1 and 2.0 Hz, 1H), 4.50 (d, *J* = 8.5 Hz, 1H), 4.54 (s, 1H), 5.16 (d, *J* = 12.4 Hz, 1H), 5.19 (d, *J* = 12.3 Hz, 1H), 6.28 (s, 1H), 6.88 (t, *J* = 2.5 Hz, 1H), 6.95 (s, 1H), 7.13-7.18 (m, 4H), 7.25-7.36 (m, 8H), 7.42 (d, *J* = 7.1 Hz, 2H), 7.47 (d, *J* = 7.3 Hz, 2H), 7.96 (dd, *J* = 8.8 and 5.3 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 21.4, 25.9, 32.8, 35.9, 49.4, 62.7, 66.5, 69.1, 71.0, 74.0, 78.4, 116.2 (d, *J* = 22.0 Hz, 2C), 127.5, 127.5, 128.2, 128.2, 128.7, 128.7, 129.4, 131.2 (d, *J* = 9.4 Hz, 2C), 131.6 (d, *J* = 2.7 Hz, 1C), 132.8, 134.5, 138.2, 139.7, 144.6, 162.5, 166.10 (d, *J* = 256.0 Hz, 1C), 166.8, 174.3, 196.9; ^{19}F NMR (376 MHz, $CDCl_3$): δ = -104.22 (s, 1F); HRMS (ESI-TOF) m/z: $[M+NH_4]^+$ Calcd $C_{41}H_{40}FN_2O_6S$ 707.2586, found 707.2579.





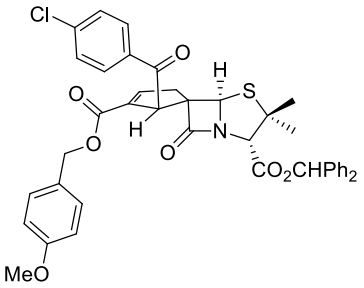
(1'*R*,2'*R*)-Benzhydryl spiro[(2-(4-fluorobenzoyl)-3-cinnamyloxycarbonylcyclopent-3-ene)-1',6-penicillanate] (2.31c) and (1'*R*,2'*R*)-Benzhydryl spiro[(2-(4-fluorobenzoyl)-5-cinnamyloxycarbonylcyclopent-4-ene)-1',6-penicillanate] (2.32c)

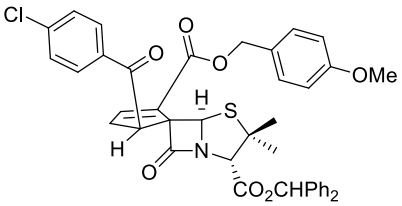
Obtained from allene **2.30c** (50 mg, 0.250 mmol) and 6-alkylidenepenicillanate **2.20a** (125 mg, 0.250 mmol) as described in the general procedure (reaction time: 4 d). Purification of the crude product by flash chromatography (hexane/ethyl acetate, 4:1), gave, in order of elution, **2.31c** as a colourless solid (28 mg, 0.040 mmol, 16%) and **2.32c** as a colourless solid (23 mg, 0.033 mmol, 13%).

Compound **2.31c**: mp 76.5-78.5 ºC; $[\alpha]_D^{25}$ = + 300 (*c* 0.25 in CH_2Cl_2); IR (ATR): $\tilde{\nu}$ = 986, 1060, 1155, 1201, 1235, 1330, 1449, 1593, 1670, 1716 and 1773 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.12 (s, 3H), 1.51 (s, 3H), 3.12 (dd, *J* = 18.6 and 3.1 Hz, 1H), 3.54 (dt, *J* = 2.2 and 18.5 Hz, 1H), 4.53 (s, 1H), 4.58 (ddd, *J* = 12.7, 6.8 and 1.1 Hz, 1H), 4.64 (ddd, *J* = 12.6, 6.6 and 1.1 Hz, 1H), 5.11 (d, *J* = 1.1 Hz, 1H), 5.42 (s, 1H), 6.01 (dt,

J = 15.9 and 6.7 Hz, 1H), 6.50 (d, *J* = 15.8 Hz, 1H), 6.92 (s, 1H), 7.08 (t, *J* = 8.6 Hz, 3H), 7.31-7.35 (m, 15H), 8.13 (dd, *J* = 8.8 and 5.4 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 26.1, 32.5, 40.8, 53.0, 64.3, 65.6, 69.1, 70.7, 71.2, 78.5, 115.6 (d, *J* = 21.9 Hz, 2C), 122.5, 126.8, 127.1, 127.7, 128.3, 128.6, 128.8, 131.2 (d, *J* = 9.5 Hz, 2C), 134.0 (d, *J* = 2.6 Hz, 1C), 134.9, 135.8, 136.1, 139.2, 139.3, 146.0, 162.8, 166.2 (d, *J* = 255.6 Hz, 1C), 167.0, 176.4, 199.6; ^{19}F NMR (376 MHz, $CDCl_3$): δ = -104.34 (s, 1F); HRMS (ESI-TOF) m/z: $[M+NH_4]^+$ Calcd $C_{42}H_{40}FN_2O_6S$ 719.2586, found 719.2581.

Compound **2.32c**: mp 79.3-81.3 ºC; $[\alpha]_D^{25}$ = + 350 (*c* 0.5 in CH_2Cl_2); IR (ATR): ṽ = 982, 1124, 1152, 1211, 1235, 1263, 1331, 1449, 1596, 1677, 1716 and 1764 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.11 (s, 3H), 1.51 (s, 3H), 2.50 (dd, *J* = 18.8 and 2.8 Hz, 1H), 3.20 (ddd, *J* = 18.6, 9.1 and 2.0 Hz, 1H), 4.51 (d, *J* = 8.5 Hz, 1H), 4.55 (s, 1H), 4.83 (dd, *J* = 6.5 and 1.0 Hz, 2H), 6.28 (s, 1H), 6.33 (dt, *J* = 15.9 and 6.5 Hz, 1H), 6.65 (d, *J* = 15.9 Hz, 1H), 6.92-6.93 (m, 2H), 7.16 (t, *J* = 8.6 Hz, 2H), 7.25-7.41 (m, 13H), 7.45 (d, *J* = 7.3 Hz, 2H), 7.97 (dd, *J* = 8.8 and 5.3 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.9, 32.8, 36.0, 49.5, 62.8, 66.6, 69.1, 71.0, 74.0, 78.4, 116.3 (d, *J* = 21.9 Hz, 2C), 123.2, 126.9, 127.4, 127.6, 128.2, 128.2, 128.7, 128.7, 131.2 (d, *J* = 9.3 Hz, 2C), 131.6 (d, *J* = 2.8 Hz, 1C), 134.5, 134.6, 136.4, 139.6, 144.6, 162.5, 166.1 (d, *J* = 256.0 Hz, 1C), 166.8, 174.2, 196.9; ^{19}F NMR (376 MHz, $CDCl_3$): δ = -104.20 (s, 1F); HRMS (ESI-TOF) m/z: $[M+NH_4]^+$ Calcd $C_{42}H_{40}FN_2O_6S$ 719.2586, found 719.2582.





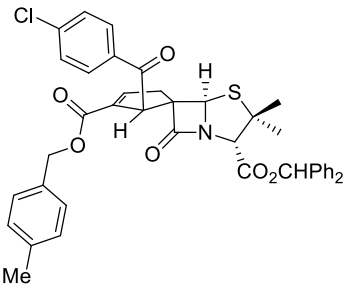
(1'*R*,2'*R*)-Benzhydryl spiro[(2-(4-chlorobenzoyl)-3-(4-methoxybenzyloxycarbonyl)cyclopent-3-ene)-1',6-penicillanate] (2.31d) and (1'*R*,2'*R*)-Benzhydryl spiro[(2-(4-chlorobenzoyl)-5-(4-methoxybenzyloxycarbonyl)cyclopent-4-ene)-1',6-penicillanate] (2.32d)

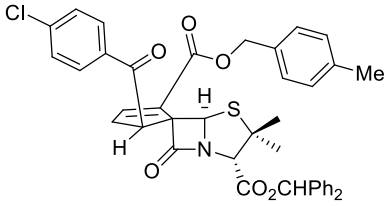
Obtained from allene **2.30a** (51 mg, 0.250 mmol) and 6-alkylidenepenicillanate **2.20b** (130 mg, 0.250 mmol) as described in the general procedure (reaction time: 22.5 h). Purification of the crude product by flash chromatography (hexane/ethyl acetate, 4:1),

gave, in order of elution, **2.31d** as a colourless solid (77 mg, 0.106 mmol, 42%) and **2.32d** as a colourless solid (65 mg, 0.090 mmol, 36%).

Compound **2.31d**: mp 76.1-78.1 ºC; $[\alpha]_D^{25}$ = +260 (*c* 0.5 in CH_2Cl_2); IR (ATR): ṽ = 999, 1092, 1175, 1244, 1331, 1457, 1514, 1587, 1671, 1708, 1742 and 1773 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.11 (s, 3H), 1.49 (s, 3H), 3.10 (dd, *J* = 18.6 and 3.1 Hz, 1H), 3.17 (dt, *J* = 18.6 and 2.3 Hz, 1H), 3.81 (s, 3H), 4.52 (s, 1H), 4.88 (d, *J* = 11.9 Hz, 1H), 4.93 (d, *J* = 11.9 Hz, 1H), 5.07 (d, *J* = 1.0 Hz, 1H), 5.39 (s, 1H), 6.80-6.82 (m, 2H), 6.91 (s, 1H), 7.05-7.07 (m, 3H), 7.26-7.34 (m, 12H), 7.96 (d, *J* = 8.6 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 26.1, 32.5, 40.7, 52.8, 55.4, 64.4, 66.7, 69.1, 70.8, 71.1, 78.5, 114.0, 127.1, 127.3, 127.7, 128.4, 128.5, 128.7, 130.5, 130.7, 135.8, 135.8, 139.2, 139.3, 140.0, 146.1, 159.9, 162.9, 166.9, 176.3, 200.1; HRMS (ESI-TOF) m/z: $[M+NH_4]^+$ Calcd $C_{41}H_{40}ClN_2O_7S$ 739.2239, found 739.2235.

Compound **2.32d**: mp 83.8-85.8 ºC; $[\alpha]_D^{25}$ = + 350 (*c* 0.5 in CH_2Cl_2); IR (ATR): ṽ = 982, 1011, 1090, 1123, 1248, 1330, 1513, 1587, 1676, 1712 and 1763 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.11 (s, 3H), 1.51 (s, 3H), 2.46 (dd, *J* = 18.7 and 2.8 Hz, 1H), 3.17 (ddd, *J* = 18.5, 9.1 and 2.0 Hz, 1H), 3.80 (s, 3H), 4.48 (d, *J* = 8.6 Hz, 1H), 4.54 (s, 1H), 5.13 (d, *J* = 12.0 Hz, 1H), 5.17 (d, *J* = 12.0 Hz, 1H), 6.26 (s, 1H), 6.85-6.87 (m, 3H), 6.95 (s, 1H), 7.27-7.36 (m, 8H), 7.42-7.43 (m, 2H), 7.45-7.48 (m, 3H), 7.87 (d, *J* = 8.6 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.9, 32.8, 35.8, 49.4, 55.4, 62.8, 66.4, 69.1, 71.0, 74.0, 78.4, 114.1, 127.5, 127.5, 128.0, 128.2, 128.3, 128.7, 128.7, 129.4, 129.9, 130.4, 133.5, 134.5, 139.6, 140.2, 144.5, 159.8, 162.5, 166.8, 174.2, 197.2; HRMS (ESI-TOF) m/z: $[M+NH_4]^+$ Calcd $C_{41}H_{40}ClN_2O_7S$ 739.2239, found 739.2236.





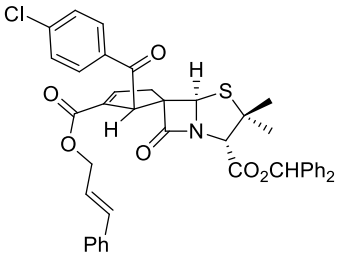
(1'*R*,2'*R*)-Benzhydryl spiro[(2-(4-chlorobenzoyl)-3-(4-methylbenzyloxycarbonyl)cyclopent-3-ene)-1',6-penicillanate] (2.31e) and (1'*R*,2'*R*)-Benzhydryl spiro[(2-(4-chlorobenzoyl)-5-(4-methylbenzyloxycarbonyl)cyclopent-4-ene)-1',6-penicillanate] (2.32e)

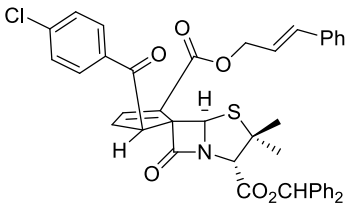
Obtained from allene **2.30b** (37 mg, 0.197 mmol) and 6-alkylidenepenicillanate **2.20b** (102 mg, 0.197 mmol) as described in the general procedure (reaction time: 67 h). Purification of the crude product by flash chromatography (hexane/ethyl acetate, 4:1), gave, in order of elution, **2.31e** as a colourless solid (39 mg, 0.055 mmol, 28%) and **2.32e** as a colourless solid (38 mg, 0.054 mmol, 27%).

Compound **2.31e**: mp 79.5-81.5 ºC; $[\alpha]_D^{25}$ = + 340 (*c* 0.25 in CH_2Cl_2); IR (ATR): $\tilde{\nu}$ = 999, 1092, 1179, 1202, 1237, 1262, 1331, 1457, 1588, 1671, 1713 and 1774 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.11 (s, 3H), 1.49 (s, 3H), 2.35 (s, 3H), 3.10 (dd, *J* = 18.6 and 3.1 Hz, 1H), 3.51 (dt, *J* = 18.5 and 2.2 Hz, 1H), 4.52 (s, 1H), 4.90 (d, *J* = 12.0 Hz, 1H), 4.95 (d, *J* = 12.0 Hz, 1H), 5.07 (s, 1H), 5.39 (s, 1H), 6.91 (s, 1H), 7.02 (d, *J* = 8.0 Hz, 2H), 7.06 (s, 1H), 7.10 (d, *J* = 7.9 Hz, 2H), 7.28-7.34 (m, 12H), 7.95 (d, *J* = 8.7 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 21.4, 26.1, 32.5, 40.7, 52.8, 64.4, 66.9, 69.1, 70.8, 71.1, 78.5, 127.1, 127.7, 128.4, 128.6, 128.7, 128.8, 128.8, 129.3, 130.7, 132.1, 135.7, 135.8, 138.4, 139.2, 139.3, 140.1, 146.1, 162.9, 166.9, 176.3, 200.1; HRMS (ESI-TOF) m/z: $[M+NH_4]^+$ Calcd $C_{41}H_{40}ClN_2O_6S$ 723.2290, found 723.2287.

Compound **2.32e**: mp 81.0-83.0 ºC; $[\alpha]_D^{25}$ = + 360 (*c* 0.25 in CH_2Cl_2); IR (ATR): $\tilde{\nu}$ = 982, 1012, 1091, 1123, 1210, 1261, 1331, 1456, 1588, 1676, 1716 and 1767 cm^{-1}; 1H NMR (400 MHz, CDCl3): δ = 1.11 (s, 3H), 1.51 (s, 3H), 2.34 (s, 3H), 2.46 (dd, *J* = 18.6 and 2.4 Hz, 1H), 3.17 (ddd, *J* = 18.5, 9.1 and 2.0 Hz, 1H), 4.48 (d, *J* = 8.5 Hz, 1H), 4.54 (s, 1H), 5.17 (s, 2H), 6.26 (s, 1H), 6.88 (t, *J* = 2.5, 1H), 6.94 (s, 1H), 7.14 (d, *J* = 7.9 Hz, 2H), 7.25-7.35 (m, 10H), 7.41-7.47 (m, 6H), 7.87 (d, *J* = 8.6 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 21.4, 25.9, 32.8, 35.9, 49.4, 62.8, 66.6, 69.1, 71.0, 74.1, 78.4, 127.5, 127.5, 128.2, 128.3, 128.7, 128.7, 129.4, 129.4, 129.9, 132.8, 133.5, 134.5, 138.2, 139.6,

140.2, 144.5, 162.5, 166.8, 174.2, 197.3; HRMS (ESI-TOF) m/z: $[M+NH_4]^+$ Calcd $C_{41}H_{40}ClN_2O_6S$ 723.2290, found 723.2291.





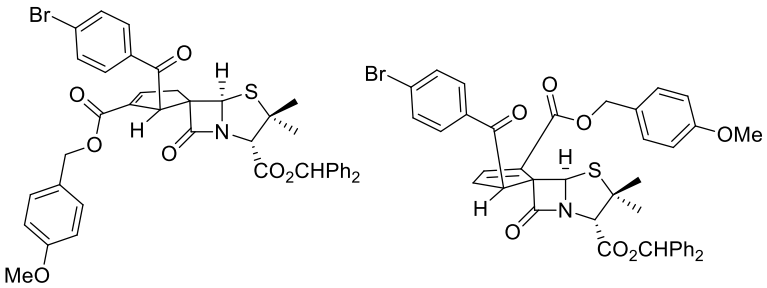
(1'*R*,2'*R*)-Benzhydryl spiro[(2-(4-chlorobenzoyl)-3-cinnamyloxycarbonylcyclopent-3-ene)-1',6-penicillanate] (2.31f) and (1'*R*,2'*R*)-Benzhydryl spiro[(2-(4-chlorobenzoyl)-5-cinnamyloxycarbonylcyclopent-4-ene)-1',6-penicillanate] (2.32f)

Obtained from allene **2.30c** (50 mg, 0.250 mmol) and 6-alkylidenepenicillanate **2.20b** (130 mg, 0.250 mmol) as described in the general procedure (reaction time: 4 d). Purification of the crude product by flash chromatography (hexane/ethyl acetate, 3:1), gave, in order of elution, **2.31f** as a colourless solid (34 mg, 0.047 mmol, 19%) and **2.32f** as a colourless solid (31 mg, 0.043 mmol, 17%).

Compound **2.31f**: mp 81.7-83.7 ºC; $[\alpha]_D^{25}$ = + 280 (*c* 0.25 in CH_2Cl_2); IR (ATR): $\tilde{\nu}$ = 999, 1092, 1176, 1200, 1236, 1261, 1330, 1457, 1588, 1671, 1716 and 1773 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.12 (s, 3H), 1.51 (s, 3H), 3.12 (dd, *J* = 18.6 and 3.1 Hz, 1H), 3.53 (dt, *J* = 18.5 and 2.2 Hz, 1H), 4.53 (s, 1H), 4.57 (ddd, *J* = 12.7, 6.7 and 1.1 Hz, 1H), 4.64 (ddd, *J* = 12.6, 6.7 and 1.1 Hz, 1H), 5.10 (d, *J* = 1.1 Hz, 1H), 5.42 (s, 1H), 6.00 (dt, *J* = 15.8 and 6.7 Hz, 1H), 6.50 (d, *J* = 15.9 Hz, 1H), 6.91 (s, 1H), 7.08 (s, 1H), 7.29-7.38 (m, 15H), 7.39 (d, *J* = 8.6 Hz, 2H), 8.04 (d, *J* = 8.7 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 26.1, 32.6, 40.8, 52.9, 64.4, 65.7, 69.1, 70.8, 71.2, 78.5, 122.5, 126.8, 127.1, 127.7, 128.4, 128.6, 128.8, 128.8, 129.0, 130.8, 135.0, 135.8, 135.9, 136.1 139.2, 139.3, 140.2, 146.0, 162.8, 166.9, 176.3, 200.1; HRMS (ESI-TOF) m/z: $[M+NH_4]^+$ Calcd $C_{42}H_{40}ClN_2O_6S$ 735.2290, found 735.2287.

Compound **2.32f**: mp 85.5-87.5 ºC; $[\alpha]_D^{25}$ = + 320 (*c* 0.25 in CH_2Cl_2); IR (ATR): $\tilde{\nu}$ = 983, 1012, 1091, 1126, 1210, 1261, 1331, 1449, 1492, 1589, 1677, 1716 and 1767 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.10 (s, 3H), 1.51 (s, 3H), 2.49 (dd, *J* = 18.7 and 3.0 Hz, 1H), 3.20 (ddd, *J* = 18.6, 9.1 and 2.0 Hz, 1H), 4.49 (d, *J* = 8.5 Hz, 1H), 4.55 (s,

1H), 4.83 (dd, *J* = 6.5 and 1.0 Hz, 2H), 6.27 (s, 1H), 6.33 (dt, *J* = 15.9 and 6.5 Hz, 1H), 6.65 (d, *J* = 15.9 Hz, 1H), 6.92 (s, 2H), 7.25-7.40 (m, 13H), 7.46 (d, *J* = 7.8 Hz, 4H), 7.88 (d, *J* = 8.6 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.9, 32.9, 35.9, 49.4, 62.8, 65.6, 69.1, 71.1, 74.1, 78.4, 123.2, 126.9, 127.4, 127.6, 128.2, 128.7, 128.7, 129.4, 130.0, 133.6, 134.5, 134.6, 136.3, 139.6, 139.6, 140.3, 144.5, 162.5, 166.8, 174.1, 197.3; HRMS (ESI-TOF) m/z: $[M+NH_4]^+$ Calcd $C_{42}H_{40}ClN_2O_6S$ 735.2290, found 735.2288.

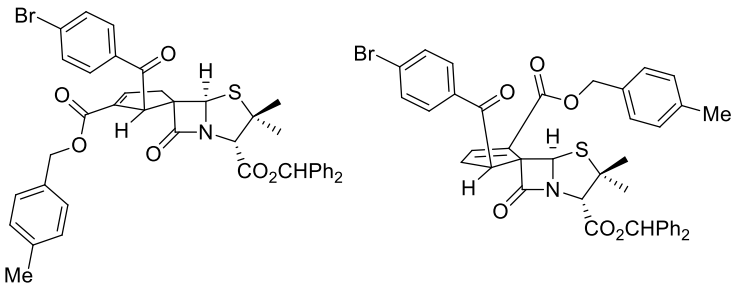


(1'*R*,2'*R*)-Benzhydryl spiro[(2-(4-bromobenzoyl)-3-(4-methoxybenzyloxycarbonyl)cyclopent-3-ene)-1',6-penicillanate] (2.31g) and (1'*R*,2'*R*)-Benzhydryl spiro[(2-(4-bromobenzoyl)-5-(4-methoxybenzyloxycarbonyl)cyclopent-4-ene)-1',6-penicillanate] (2.32g)

Obtained from allene **2.30a** (51 mg, 0.250 mmol) and 6-alkylidenepenicillanate **2.20c** (141 mg, 0.250 mmol) as described in the general procedure (reaction time: 24 h). Purification of the crude product by flash chromatography (hexane/ethyl acetate, 4:1), gave, in order of elution, **2.31g** as a colourless solid (81 mg, 0.105 mmol, 42%) and **2.31g** as a colourless solid (69 mg, 0.090 mmol, 36%).

Compound **2.31g**: mp 82.6-84.6 ºC; $[\alpha]_D^{25}$ = + 320 (*c* 0.25 in CH_2Cl_2); IR (ATR): $\tilde{\nu}$ = 998, 1069, 1175, 1243, 1331, 1458, 1584, 1671, 1708 and 1774 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.11 (s, 3H), 1.49 (s, 3H), 3.10 (dd, *J* = 18.6 and 3.1 Hz, 1H), 3.50 (dt, *J* = 18.6 and 2.3 Hz, 1H), 3.82 (s, 3H), 4.52 (s, 1H), 4.88 (d, *J* = 11.9 Hz, 1H), 4.93 (d, *J* = 11.9 Hz, 1H), 5.06 (d, *J* = 1.1 Hz, 1H), 5.39 (s, 1H), 6.82 (d, *J* = 8.7 Hz, 2H), 6.91 (s, 1H), 7.05-7.07 (m, 3H), 7.29-7.34 (m, 10H), 7.48 (d, *J* = 8.6 Hz, 2H), 7.88 (d, *J* = 8.6 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 26.1, 32.6, 40.7, 52.7, 55.4, 64.4, 66.8, 69.1, 70.8, 71.1, 78.5, 114.0, 127.1, 127.3, 127.7, 128.4, 128.6, 128.8, 128.9, 130.5, 130.8, 131.7, 135.8, 136.2, 139.2, 139.3, 146.1, 159.9, 162.9, 166.9, 176.3, 200.3; HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd $C_{41}H_{37}BrNO_7S$ 766.1469, found 766.1462.

Compound **2.32g**: mp 86.5-88.5 ºC; $[\alpha]_D^{25}$ = + 320 (*c* 0.5 in CH_2Cl_2); IR (ATR): $\tilde{\nu}$ = 983, 1009, 1071, 1123, 1249, 1331, 1514, 1584, 1676, 1715 and 1766 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.11 (s, 3H), 1.51 (s, 3H), 2.45 (dd, *J* = 18.7 and 2.5 Hz, 1H), 3.16 (ddd, *J* = 18.5, 9.1 and 2.0 Hz, 1H), 3.79 (s, 3H), 4.47 (d, *J* = 8.5 Hz, 1H), 4.54 (s, 1H), 5.12 (d, *J* = 12.0 Hz, 1H), 5.17 (d, *J* = 12.0 Hz, 1H), 6.25 (s, 1H), 6.85-6.87 (m, 3H), 6.95 (s, 1H), 7.27-7.36 (m, 8H), 7.41-7.43 (m, 2H), 7.46-7.48 (m, 2H), 7.63 (d, *J* = 8.6 Hz, 1H), 7.79 (d, *J* = 8.6 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.9, 32.8, 35.8, 49.4, 55.4, 62.8, 66.4, 69.1, 71.0, 74.1, 78.4, 114.1, 127.5, 127.5, 128.0, 128.2, 128.3, 128.7, 128.7, 129.0, 130.0, 130.4, 132.4, 134.0, 134.5, 139.6, 144.5, 159.8, 162.5, 166.8, 174.2, 197.4; HRMS (ESI-TOF) m/z: [M+H]+ Calcd $C_{41}H_{37}BrNO_7S$ 766.1469, found 766.1463.



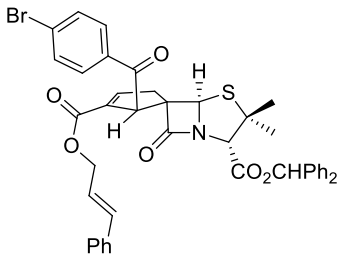
(1'*R*,2'*R*)-Benzhydryl spiro[(2-(4-bromobenzoyl)-3-(4-methylbenzyloxycarbonyl)cyclopent-3-ene)-1',6-penicillanate] (2.31h) and (1'*R*,2'*R*)-Benzhydryl spiro[(2-(4-bromobenzoyl)-5-(4-methylbenzyloxycarbonyl)cyclopent-4-ene)-1',6-penicillanate] (2.32h)

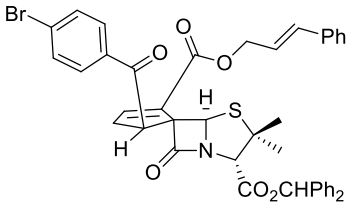
Obtained from allene **2.30b** (47 mg, 0.250 mmol) and 6-alkylidenepenicillanate **2.20c** (141 mg, 0.250 mmol) as described in the general procedure (reaction time: 4 d). Purification of the crude product by flash chromatography (hexane/ethyl acetate, 4:1), gave, in order of elution, **2.31h** as a colourless solid (46 mg, 0.061 mmol, 25%) and **2.32h** as a colourless solid (43 mg, 0.057 mmol, 23%).

Compound **2.31h**: mp 74.9-76.9 ºC; $[\alpha]_D^{25}$ = + 270 (*c* 0.5 in CH_2Cl_2); IR (ATR): $\tilde{\nu}$ = 998, 1069, 1177, 1201, 1237, 1262, 1331, 1458, 1583, 1671, 1712 and 1774 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.11 (s, 3H), 1.49 (s, 3H), 2.36 (s, 3H), 3.10 (dd, *J* = 18.6 and 3.1 Hz, 1H), 3.51 (dt, *J* = 18.6 and 2.3 Hz, 1H), 4.52 (s, 1H), 4.91 (d, *J* = 12.0 Hz, 1H), 4.95 (d, *J* = 12.0 Hz, 1H), 5.07 (d, *J* = 1.1 Hz, 1H), 5.39 (s, 1H), 6.91 (s, 1H), 7.02 (d, *J* = 8.0 Hz, 2H), 7.07 (s, 1H), 7.11 (d, *J* = 7.9 Hz, 2H), 7.29-7.35 (m, 10H), 7.46 (d, *J*

= 8.6 Hz, 2H), 7.88 (d, *J* = 8.6 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 21.4, 26.1, 32.5, 40.7, 52.7, 64.4, 66.9, 69.1, 70.8, 71.1, 78.5, 127.1, 127.7, 128.4, 128.5, 128.7, 128.8, 128.8, 129.0, 129.4, 130.8, 131.7, 132.1, 135.8, 136.1, 138.4, 139.2, 139.3, 146.2, 162.9, 166.9, 176.3, 200.2; HRMS (ESI-TOF) m/z: $[M+NH_4]^+$ Calcd $C_{41}H_{40}BrN_2O_6S$ 767.1785, found 767.1782.

Compound **2.32h**: mp 76.7-78.7 ºC; $[\alpha]_D^{25}$ = + 400 (*c* 0.25 in CH_2Cl_2); IR (ATR): $\tilde{\nu}$ = 982, 1008, 1070, 1121, 1256, 1330, 1449, 1583, 1676, 1716 and 1763 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.11 (s, 3H), 1.51 (s, 3H), 2.34 (s, 3H), 2.46 (dd, *J* = 18.7 and 2.5 Hz, 1H), 3.17 (ddd, *J* = 18.5, 9.1 and 2.0 Hz, 1H), 4.48 (d, *J* = 8.5 Hz, 1H), 4.54 (s, 1H), 5.17 (s, 2H), 6.26 (s, 1H), 6.88 (dd, *J* = 2.9 and 2.2 Hz, 1H), 6.94 (s, 1H), 7.14 (d, *J* = 7.9 Hz, 2H), 7.25-7.35 (m, 10H), 7.42 (d, *J* = 7.1 Hz, 2H), 7.47 (d, *J* = 7.2 Hz, 2H), 7.63 (d, *J* = 8.6 Hz, 2H), 7.79 (d, *J* = 8.6 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 21.4, 25.9, 32.9, 35.8, 49.4, 62.8, 66.6, 69.1, 71.0, 74.1, 78.4, 127.5, 127.5, 128.2, 128.3, 128.7, 128.7, 129.0, 129.4, 130.0, 132.4, 132.8, 134.0, 134.5, 138.2, 139.6, 144.5, 162.5, 166.7, 174.2, 197.4; HRMS (ESI-TOF) m/z: $[M+NH_4]^+$ Calcd $C_{41}H_{40}BrN_2O_6S$ 767.1785, found 767.1777.





(1'*R*,2'*R*)-Benzhydryl spiro[(2-(4-bromobenzoyl)-3-cinnamyloxycarbonylcyclopent-3-ene)-1',6-penicillanate] (2.31i) and (1'*R*,2'*R*)-Benzhydryl spiro[(2-(4-bromobenzoyl)-5-cinnamyloxycarbonylcyclopent-4-ene)-1',6-penicillanate] (2.32i)

Obtained from allene **2.30c** (50 mg, 0.250 mmol) and 6-alkylidenepenicillanate **2.20c** (141 mg, 0.250 mmol) as described in the general procedure (reaction time: 4 d). Purification of the crude product by flash chromatography (hexane/ethyl acetate, 3:1), gave, in order of elution, **2.31i** as a colourless solid (38 mg, 0.050 mmol, 20%) and **2.32i** as a colourless solid (31 mg, 0.040 mmol, 16%).

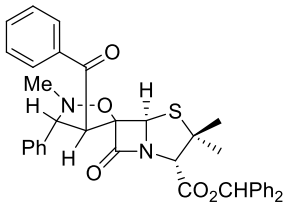
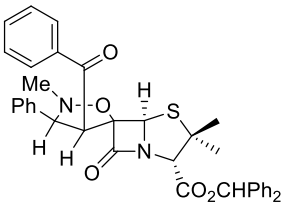
Compound **2.31i**: mp 89.1-91.1 ºC; $[\alpha]_D^{25}$ = + 360 (*c* 0.25 in CH_2Cl_2); IR (ATR): $\tilde{\nu}$ = 998, 1070, 1177, 1201, 1236, 1261, 1330, 1449, 1583, 1670, 1716 and 1773 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.12 (s, 3H), 1.51 (s, 3H), 3.12 (dd, *J* = 18.6 and 3.1 Hz, 1H), 3.53 (dt, *J* = 18.6 and 2.2 Hz, 1H), 4.53 (s, 1H), 4.58 (ddd, *J* = 12.8, 6.9 and 1.1 Hz, 1H), 4.64 (ddd, *J* = 12.6, 6.6 and 1.1 Hz, 1H), 5.10 (d, *J* = 1.0 Hz, 1H), 5.41 (s, 1H), 6.01 (dt, *J* = 15.8 and 6.7 Hz, 1H), 6.50 (d, *J* = 15.9 Hz, 1H), 6.92 (s, 1H), 7.08 (s, 1H), 7.29-7.35 (m, 15H), 7.57 (d, *J* = 8.6 Hz, 2H), 7.96 (d, *J* = 8.7 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 26.1, 32.6, 40.8, 52.9, 64.4, 65.7, 69.1, 70.8, 71.2, 78.5, 122.5, 126.8, 127.1, 127.7, 128.4, 128.6, 128.8, 128.8, 129.0, 130.9, 131.8, 135.0, 135.8, 136.0, 136.3 139.2, 139.3, 146.1, 162.8, 166.9, 176.3, 200.3; HRMS (ESI-TOF) m/z: $[M+NH_4]^+$ Calcd $C_{42}H_{40}BrN_2O_6S$ 779.1785, found 781.1761.

Compound **2.32i**: mp 91.2-93.2 ºC; $[\alpha]_D^{25}$ = + 400 (*c* 0.25 in CH_2Cl_2); IR (ATR): $\tilde{\nu}$ = 983, 1010, 1126, 1209, 1261, 1331, 1449, 1584, 1676, 1718 and 1767 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.10 (s, 3H), 1.51 (s, 3H), 2.49 (dd, *J* = 18.6 and 3.0 Hz, 1H), 3.19 (ddd, *J* = 18.6, 9.0 and 2.0 Hz, 1H), 4.49 (d, *J* = 8.5 Hz, 1H), 4.55 (s, 1H), 4.83 (dd, *J* = 6.5 and 1.0 Hz, 2H), 6.27 (s, 1H), 6.33 (dt, *J* = 15.9 and 6.5 Hz, 1H), 6.65 (d, *J* = 15.9 Hz, 1H), 6.92 (s, 2H), 7.25-7.40 (m, 9H), 7.46 (d, *J* = 7.2 Hz, 4H), 7.46 (d, *J* = 8.6 Hz, 2H), 7.80 (d, *J* = 8.6 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.9, 32.9, 35.8, 49.4, 62.9, 65.6, 69.1, 71.1, 74.1, 78.4, 123.2, 126.9, 127.4, 127.6, 128.2, 128.7, 128.7, 129.0, 130.0, 131.8, 132.4, 134.0, 134.5, 134.7, 136.3, 139.6, 144.5, 162.5, 166.8, 174.1, 197.5; HRMS (ESI-TOF) m/z: $[M+NH_4]^+$ Calcd $C_{42}H_{40}BrN_2O_6S$ 779.1785, found 779.1782.

6.5 Synthesis of Compounds of Chapter 3

6.5.1. General Procedure for the 1,3-Dipolar Cycloaddition of Nitrones with 6-Alkylidenepenicillanates

To a mixture of the appropriate 6-alkylidenepenicillanate **3.42** (1 equiv.) in toluene (2.5 or 5 mL), the corresponding nitrone **3.41** (2 equiv.) was added. The reaction mixture was stirred at 80 ºC for the time indicated in each case, being monitored through TLC. Upon completion, the solvent was removed under reduced pressure and the crude product was purified by flash chromatography.

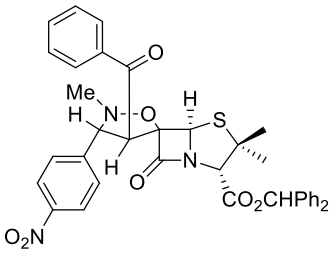
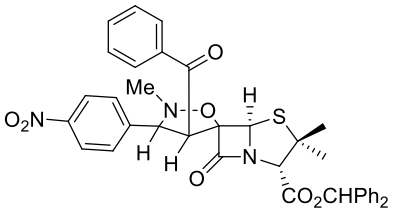



(3'*R*, 4'*S*, 5'*S*)-Benzhydryl spiro[(4-benzoyl-2-methyl-3-phenyl-isoxazolidine)-5',6-penicillanate] (3.43a) and (3'*S*, 4'*S*, 5'*S*)-Benzhydryl spiro[(4-benzoyl-2-methyl-3-phenyl-isoxazolidine)-5',6-penicillanate] (3.44a)

Obtained from *N*-methylphenylnitrone **3.41a** (55.6 mg, 0.412 mmol) and 6-alkylidenepenicillanate **3.42a** (100 mg, 0.412 mmol) as described in the general procedure (reaction time: 24 h). Purification of the crude product by flash chromatography (hexane/ethyl acetate, 5:1 → 3:1), gave, in order of elution, **3.43a** as a colourless solid (90 mg, 0.145 mmol, 70%) and a mixture of **3.44a/3.45a** as a yellow oil (**3.44a**: 11%).

Compound **3.43a**: mp low melting solid; $[\alpha]_D^{25}$= + 280 (*c* 0.5 in CH_2Cl_2); IR (ATR): ν = 981, 1179, 1258, 1449, 1456, 1676, 1741 and 1776 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.13 (s, 3H), 1.33 (s, 3H), 2.71 (s, 3H), 3.61 (d, *J* = 6.9 Hz, 1H), 4.52 (s, 1H), 4.69 (d, *J* = 7.3 Hz, 1H), 5.66 (s, 1H), 6.94 (s, 1H), 7.27-7.37 (m, 15H), 7.44-7.50 (m, 3H), 7.56 (d, *J* = 7.3 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.9, 32.0, 43.3, 60.4, 62.8, 69.3, 71.6, 78.5, 79.5, 96.6, 127.3, 127.6, 128.3, 128.4, 128.7, 128.7, 128.8, 128.9, 129.1, 129.1, 133.9, 137.0, 139.3, 139.4, 166.8, 173.8, 198.9; HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{37}H_{35}N_2O_5S$ $[M+H]^+$ 619.2261; found 619.2253.

Recorded as a mixture of compounds **3.44a/3.45a**: Compound **3.44a**: ^1H NMR (400 MHz, $CDCl_3$): δ = 1.12 (s, 3H), 1.44 (s, 3H), 2.79 (s, 3H), 4.45 (d, *J* = 6.2 Hz, 1H), 4.57 (s, 1H), 4.81 (d, *J* = 6.2 Hz, 1H), 5.79 (s, 1H), 6.93 (s, 1H), 7.00-7.53 (m, 20H); MS (ESI-TOF) m/z: $[M+H]^+$ found 619.23.

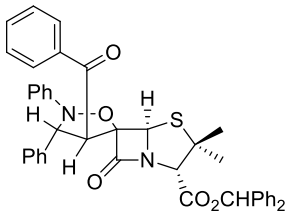



(3'*R*, 4'*S*, 5'*S*)-Benzhydryl spiro[(4-benzoyl-2-methyl-3-(4-nitrophenyl)isoxazolidine)-5',6-penicillanate] (3.43b) and (3'*S*, 4'*S*, 5'*S*)-Benzhydryl spiro[(4-benzoyl-2-methyl-3-(4-nitrophenyl)isoxazolidine)-5',6-penicillanate] (3.44b)

Obtained from *N*-methyl-*C*-4-nitrophenyl nitrone **3.41b** (74 mg, 0.412 mmol) and 6-alkylidenepenicillanate **3.42a** (100 mg, 0.206 mmol) as described in the general procedure (reaction time: 24 h). Purification of the crude product by flash chromatography (hexane/ethyl acetate, 5:1 → 3:1), gave, in order of elution, a mixture of **3.43b**/**3.45b** as a yellow oil (**3.43b**: 64%) and **3.44b** as a yellow oil (14 mg, 0.020 mmol, 10%).

Recorded as a mixture of compounds **3.43b/3.45b**: Compound **3.43b**: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.13 (s, 3H), 1.34 (s, 3H), 2.72 (s, 3H), 3.73 (d, *J* = 6.9 Hz, 1H), 4.53 (s, 1H), 4.63 (d, *J* = 6.9 Hz, 1H), 5.66 (s, 1H), 6.95 (s, 1H), 7.20-7.64 (m, 15H), 7.66 (d, *J* = 8.7 Hz, 2H), 8.20 (d, *J* = 8.8 Hz, 2H); MS (ESI-TOF) m/z: $[M+H]^+$ found 664.21.

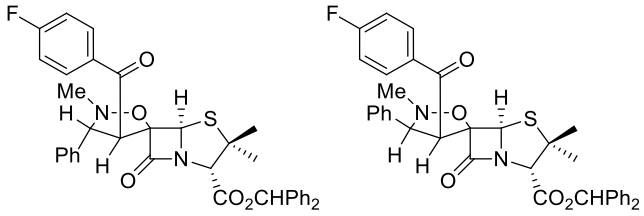
Compound **3.44b**: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.14 (s, 3H), 1.47 (s, 3H), 2.79 (s, 3H), 4.56 (d, *J* = 6.3 Hz, 1H), 4.59 (s, 1H), 4.88 (d, *J* = 6.3 Hz, 1H), 5.78 (s, 1H), 6.94 (s, 1H), 7.17 (t, *J* = 7.8 Hz, 2H), 7.29-7.36 (m, 11H), 7.41 (d, *J* = 8.7 Hz, 2H), 7.45 (d, *J* = 8.5 Hz, 2H), 7.88 (d, *J* = 8.8 Hz, 2H); HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{37}H_{34}N_3O_7S$ $[M+H]^+$ 664.2112; found 664.2111.



(3'*R*, 4'*S*, 5'*S*)-Benzhydryl spiro[(4-benzoyl-2,3-diphenyl-isoxazolidine)-5',6-penicillanate] (3.43c)

Obtained from diphenylnitrone **3.41c** (81 mg, 0.412 mmol) and 6-alkylidenepenicillanate **3.42a** (100 mg, 0.206 mmol) as described in the general procedure (reaction time: 24 h). Purification of the crude product by flash chromatography (hexane/ethyl acetate, 5:1 → 3:1), gave a mixture of **3.43c/3.45c** as a colourless solid **3.43c**: 41%.

Recorded as a mixture of compounds **3.43c/3.45c**: Compound **3.43c:** ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.15 (s, 3H), 1.36 (s, 3H), 4.31 (d, *J* = 6.0 Hz, 1H), 4.56 (s, 1H), 4.74 (d, *J* = 6.0 Hz, 1H), 5.81 (s, 1H), 6.96-6.99 (m, 4H), 7.14-7.18 (m, 3H), 7.31-7.64 (m, 19H); MS (ESI-TOF) m/z: $[M+H]^+$ found 681.24.



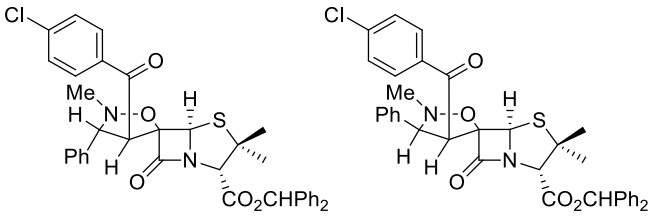
(3'*R*, 4'*S*, 5'*S*)-Benzhydryl spiro[(4-(4-fluorobenzoyl)-2-methyl-3-phenyl-isoxazolidine)-5',6-penicillanate] (3.47a) and (3'*S*, 4'*S*, 5'*S*)-Benzhydryl spiro[(4-(4-fluorobenzoyl)-2-methyl-3-phenyl-isoxazolidine)-5',6-penicillanate] (3.48a)

Obtained from *N*-methylphenylnitrone **3.41a** (55.6 mg, 0.412 mmol) and 6-alkylidenepenicillanate **3.46a** (103 mg, 0.412 mmol) as described in the general procedure (reaction time: 24 h). Purification of the crude product by flash chromatography (hexane/ethyl acetate, 5:1 → 3:1), gave, in order of elution, **3.47a** as a colourless solid (86 mg, 0.135 mmol, 66%) and a mixture of **3.48a/3.49a** as a yellow oil (**3.48a**: 13%).

Compound **3.47a**: mp 139.8-141.5 ºC; $[\alpha]_D^{25}$ = + 240 (*c* 0.25 in CH_2Cl_2); IR (ATR): ν = 849, 1151, 1180, 1194, 1303, 1593, 1676, 1752 and 1774 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.14 (s, 3H), 1.35 (s, 3H), 2.70 (s, 3H), 3.58 (d, *J* = 7.0 Hz, 1H), 4.52 (s, 1H),

4.63 (d, *J* = 7.2 Hz, 1H), 5.63 (s, 1H), 6.94-6.99 (m, 3H), 7.30-7.36 (m, 14H), 7.46 (dd, *J* = 6.6 and 2.9 Hz, 2H), 7.58 (dd, *J* = 8.8 and 5.3 Hz, 2H) ; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 26.0, 32.0, 43.3, 60.4, 62.8, 69.3, 71.5, 78.5, 79.5, 96.5, 116.0 (d, *J* = 22 Hz, 2C), 127.3, 127.6, 128.4, 128.5, 128.6, 128.7, 128.8, 129.2, 131.6 (d, *J* = 10 Hz, 2C), 133.5 (d, *J* = 3 Hz, 1C), 136.9, 139.3, 139.3, 166.3 (d, *J* = 257 Hz, 1C), 166.8, 173.7, 197.3; ^{19}F NMR (376 MHz, $CDCl_3$): -103.59 (s, 1F) ; HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{37}H_{34}FN_2O_5S$ $[M+H]^+$ 637.2167; found 637.2185.

Recorded as a mixture of compounds **3.48a/3.49a**: Compound **3.48a**: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.13 (s, 3H), 1.45 (s, 3H), 2.78 (s, 3H), 4.45 (d, *J* = 6.1 Hz, 1H), 4.57 (s, 1H), 4.73 (d, *J* = 6.2 Hz, 1H), 5.78 (s, 1H), 6.81 (t, *J* = 8.6 Hz, 2H), 6.93 (s, 1H), 7.03-7.50 (m, 17H) ; ^{19}F NMR (376 MHz, $CDCl_3$): -105.09 (s, 1F) ; MS (ESI-TOF) m/z: $[M+H]^+$ found 637.22.



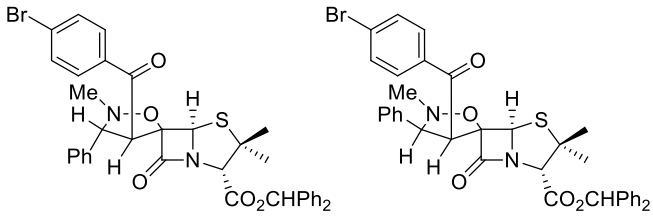
(3'*R*, 4'*S*, 5'*S*)-Benzhydryl spiro[(4-(4-chlorobenzoyl)-2-methyl-3-phenyl-isoxazolidine)-5',6-penicillanate] (3.47b) and (3'*S*, 4'*S*, 5'*S*)-Benzhydryl spiro[(4-(4-chlorobenzoyl)-2-methyl-3-phenyl-isoxazolidine)-5',6-penicillanate] (3.48b)

Obtained from *N*-methylphenylnitrone **3.41a** (55.6 mg, 0.412 mmol) and 6-alkylidenepenicillanate **3.46b** (106.7 mg, 0.412 mmol) as described in the general procedure (reaction time: 24 h). Purification of the crude product by flash chromatography (hexane/ethyl acetate, 5:1 → 3:1), gave, in order of elution, **3.47b** as a colourless solid (93 mg, 0.143 mmol, 69%) and **3.48b** as a yellow oil (13 mg, 0.020 mmol, 10%).

Compound **3.47b**: mp 140.6-142.6 ºC; $[\alpha]_D^{25}$= + 310 (*c* 0.5 in CH_2Cl_2); IR (ATR): ν = 1006, 1089, 1181, 1303, 1584, 1677, 1753 and 1772 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.13 (s, 3H), 1.35 (s, 3H), 2.70 (s, 3H), 3.59 (d, *J* = 7.0 Hz, 1H), 4.52 (s, 1H), 4.62 (d, *J* = 7.2 Hz, 1H), 5.64 (s, 1H), 6.95 (s, 1H), 7.26-7.38 (m, 15H), 7.43-7.51 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 26.0, 32.1, 43.3, 60.5, 62.9, 69.3, 71.5, 78.5, 79.5, 96.6, 127.3, 127.6, 128.4, 128.5, 128.6, 128.7, 128.8, 129.1, 129.3, 130.2, 135.4, 136.8,

139.3, 139.3, 140.6, 166.8, 173.7, 197.8; HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{37}H_{34}ClN_2O_5S$ $[M+H]^+$ 653.1871; found 653.1873.

Compound **3.48b**: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.13 (s, 3H), 1.43 (s, 3H), 2.78 (s, 3H), 4.45 (d, *J* = 6.1 Hz, 1H), 4.57 (s, 1H), 4.73 (d, *J* = 6.2 Hz, 1H), 5.77 (s, 1H), 6.93 (s, 1H), 7.04-7.06 (m, 2H), 7.11-7.13 (m, 2H), 7.18-7.21 (m, 2H), 7.30-7.40 (m, 13H); HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{37}H_{34}ClN_2O_5S$ $[M+H]^+$ 653.1871; found 653.1867.



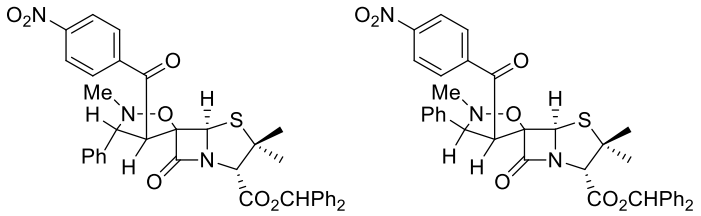
(3'*R*, 4'*S*, 5'*S*)-Benzhydryl spiro[(4-(4-bromobenzoyl)-2-methyl-3-phenyl-isoxazolidine)-5',6-penicillanate] (3.47c) and (3'*S*, 4'*S*, 5'*S*)-Benzhydryl spiro[(4-(4-bromobenzoyl)-2-methyl-3-phenyl-isoxazolidine)-5',6-penicillanate] (3.48c)

Obtained from *N*-methylphenylnitrone **3.41a** (27.8 mg, 0.206 mmol) and 6-alkylidenepenicillanate **3.46c** (57.9 mg, 0.103 mmol) as described in the general procedure (reaction time: 24 h). Purification of the crude product by flash chromatography (hexane/ethyl acetate, 5:1 → 3:1), gave, in order of elution, **3.47c** as a colourless solid (31 mg, 0.044 mmol, 43%) and **3.48c** as a yellow oil (8.7 mg, 0.012 mmol, 12%).

Compound **3.47c**: mp 134.4-136.4 ºC; $[\alpha]_D^{25}$ = + 280 (*c* 0.75 in CH_2Cl_2); IR (ATR): ν = 846, 1004, 1182, 1303, 1577, 1676, 1752 and 1772 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.13 (s, 3H), 1.34 (s, 3H), 2.70 (s, 3H), 3.59 (d, *J* = 7.0 Hz, 1H), 4.52 (s, 1H), 4.62 (d, *J* = 7.2 Hz, 1H), 5.63 (s, 1H), 6.94 (s, 1H), 7.29-7.37 (m, 13H), 7.39-7.47 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 26.0, 32.1, 43.3, 60.5, 62.9, 69.3, 71.5, 78.5, 79.5, 96.6, 127.3, 127.6, 128.4, 128.5, 128.6, 128.7, 128.8, 129.3, 129.4, 130.3, 132.1, 135.8, 136.8, 139.3, 139.3, 166.8, 173.7, 198.0; HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{37}H_{34}BrN_2O_5S$ $[M+H]^+$ 697.1366; found 697.1367.

Compound **3.48c**: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.13 (s, 3H), 1.42 (s, 3H), 2.78 (s, 3H), 4.44 (d, *J* = 6.1 Hz, 1H), 4.57 (s, 1H), 4.72 (d, *J* = 6.2 Hz, 1H), 5.76 (s, 1H),

6.93 (s, 1H), 7.05-7.07 (m, 3H), 7.18-7.20 (m, 2H), 7.27-7.36 (m, 14H); HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{37}H_{34}BrN_2O_5S$ $[M+H]^+$ 697.1366; found 697.1364.

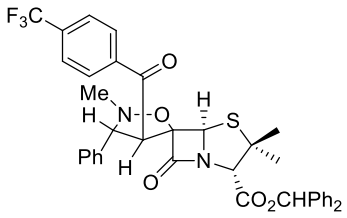
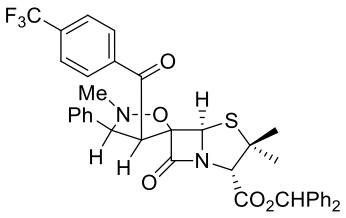


(3'*R*, 4'*S*, 5'*S*)-Benzhydryl spiro[(2-methyl-4-(4-nitrobenzoyl)-3-phenyl-isoxazolidine)-5',6-penicillanate] (3.47d) and (3'*S*, 4'*S*, 5'*S*)-Benzhydryl spiro[(2-methyl-4-(4-nitrobenzoyl)-3-phenyl-isoxazolidine)-5',6-penicillanate] (3.48d)

Obtained from *N*-methylphenylnitrone **3.41a** (27.8 mg, 0.206 mmol) and 6-alkylidenepenicillanate **3.46d** (54.4 mg, 0.103 mmol) as described in the general procedure (reaction time: 24 h). Purification of the crude product by flash chromatography (hexane/ethyl acetate, 5:1 → 3:1), gave, in order of elution, **3.47d** as a colourless solid (39 mg, 0.059 mmol, 57%) and a mixture of **3.48d/3.49d** as a yellow oil (**3.48d**: 27%).

Compound **3.47d**: mp 143.1-144.0 ºC; $[\alpha]_D^{25}$= + 330 (*c* 0.5 in CH_2Cl_2); IR (ATR): ν = 1150, 1179, 1299, 1456, 1669, 1749 and 1773 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.13 (s, 3H), 1.33 (s, 3H), 2.72 (s, 3H), 3.63 (d, *J* = 7.2 Hz, 1H), 4.54 (s, 1H), 4.68 (d, *J* = 7.3 Hz, 1H), 5.63 (s, 1H), 6.95 (s, 1H), 7.30-7.40 (m, 13H), 7.45-7.48 (m, 2H), 7.69 (d, *J* = 8.8 Hz, 2H), 8.14 (d, *J* = 8.9 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.9, 32.3, 43.2, 61.2, 63.3, 69.2, 71.4, 78.6, 79.7, 96.9, 124.0, 127.3, 127.6, 128.4, 128.5, 128.5, 128.8, 128.8, 129.4, 129.5, 129.7, 136.4, 139.2, 139.3, 141.4, 150.7, 166.6, 173.3, 198.1.

Recorded as a mixture of compounds **3.48d/3.49d**: Compound **3.48d**: ^1H NMR (400 MHz, $CDCl_3$): δ = 1.13 (s, 3H), 1.44 (s, 3H), 2.81 (s, 3H), 4.49 (d, *J* = 6.1 Hz, 1H), 4.59 (s, 1H), 4.79 (d, *J* = 6.2 Hz, 1H), 5.77 (s, 1H), 6.94 (s, 1H), 7.04-7.36 (m, 15H), 7.58 (d, *J* = 8.9 Hz, 2H), 7.99 (d, *J* = 8.9 Hz, 2H); MS (ESI-TOF) m/z: $[M+H]^+$ found 664.21.

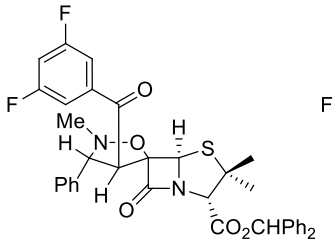
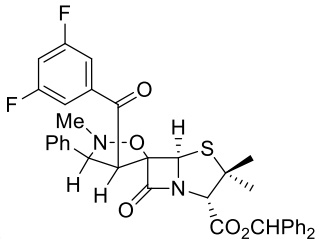



(3'*R*, 4'*S*, 5'*S*)-Benzhydryl spiro[(2-methyl-3-phenyl-4-(4-trifluoromethylbenzoyl)-isoxazolidine)-5',6-penicillanate] (3.47e) and (3'*S*, 4'*S*, 5'*S*)-Benzhydryl spiro[(2-methyl-3-phenyl-4-(4-trifluoromethylbenzoyl)-isoxazolidine)-5',6-penicillanate] (3.48e)

Obtained from *N*-methylphenylnitrone **3.41a** (27.8 mg, 0.206 mmol) and 6-alkylidenepenicillanate **3.46e** (56.9 mg, 0.103 mmol) as described in the general procedure (reaction time: 24 h). Purification of the crude product by flash chromatography (hexane/ethyl acetate, 5:1 → 3:1), gave, in order of elution, **3.47e** as a colourless solid (37,4 mg, 0.054 mmol, 53%) and **3.48e** as a yellow oil (7.7 mg, 0.011 mmol, 11%).

Compound **3.47e:** mp 153.6-155.6 ºC; $[\alpha]_D^{25}$ = + 280 (*c* 0.25 in CH_2Cl_2); IR (ATR): ν = 1066, 1128, 1259, 1321, 1456, 1685, 1748 and 1776 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.13 (s, 3H), 1.33 (s, 3H), 2.71 (s, 3H), 3.62 (d, *J* = 7.0 Hz, 1H), 4.53 (s, 1H), 4.68 (d, *J* = 7.2 Hz, 1H), 5.64 (s, 1H), 6.95 (s, 1H), 7.29-7.38 (m, 13H), 7.45-7.48 (m, 2H), 7.56 (d, *J* = 8.4 Hz, 2H), 7.65 (d, *J* = 8.3 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.9, 32.2, 43.2, 60.9, 63.1, 69.3, 71.4, 78.5, 79.6, 96.8, 123.5 (q, *J* = 273 Hz, 1C), 125.8, 125.9, 127.3, 127.6, 128.4, 128.5, 128.6, 128.8, 128.8, 129.1, 129.3, 129.4, 136.6, 139.2, 139.3, 139.7, 166.7, 173.5, 198.4; ^{19}F NMR (376 MHz, $CDCl_3$): -63.24 (s, 3F); HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{38}H_{34}F_3N_2O_5S$ $[M+H]^+$ 687.2135; found 687.2129.

Compound **3.48e**: ^1H NMR (400 MHz, $CDCl_3$): δ = 1.13 (s, 3H), 1.44 (s, 3H), 2.80 (s, 3H), 4.47 (d, *J* = 6.1 Hz, 1H), 4.58 (s, 1H), 4.78 (d, *J* = 6.2 Hz, 1H), 5.78 (s, 1H), 6.94 (s, 1H), 7.02-7.04 (m, 3H), 7.17-7.20 (m, 2H), 7.30-7.36 (m, 10H), 7.40 (d, *J* = 8.3 Hz, 2H), 7.53 (d, *J* = 8.2 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.6, 33.5, 44.8, 58.3, 62.7, 68.9, 74.2, 76.3, 78.7, 97.6, 123.5 (d, *J* = 273 Hz, 1C), 125.1, 125.2, 127.3, 127.5, 128.0, 128.4, 128.5, 128.8, 128.8, 129.1, 132.6, 139.2, 166.4, 172.5, 195.7; ^{19}F NMR (376 MHz, $CDCl_3$): -63.27 (s, 3F); HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{38}H_{34}F_3N_2O_5S$ $[M+H]^+$ 687.2135; found 687.2162.

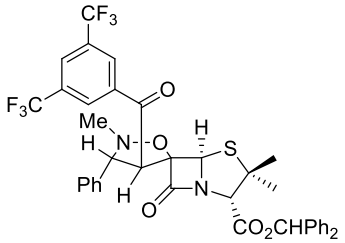
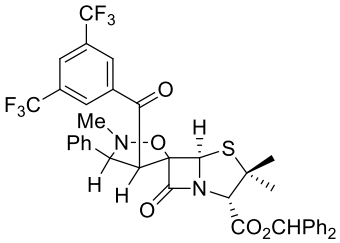



(3'*R*, 4'*S*, 5'*S*)-Benzhydryl spiro[(4-(3,5-difluorobenzoyl)-2-methyl-3-phenyl-isoxazolidine)-5',6-penicillanate] (3.47f) and (3'*S*, 4'*S*, 5'*S*)-Benzhydryl spiro[(4-(3,5-difluorobenzoyl)-2-methyl-3-phenyl-isoxazolidine)-5',6-penicillanate] (3.48f)

Obtained from *N*-methylphenylnitrone **3.41a** (55.6 mg, 0.412 mmol) and 6-alkylidenepenicillanate **3.46f** (107 mg, 0.206 mmol) as described in the general procedure (reaction time: 24 h). Purification of the crude product by flash chromatography (hexane/ethyl acetate, 5:1 → 3:1), gave, in order of elution, **3.47f** as a colourless solid (83.6 mg, 0.128 mmol, 62%) and **3.48f** as a yellow oil (19 mg, 0.029 mmol, 14%).

Compound **3.47f**: mp 140.4-141.4 ºC; $[\alpha]_D^{25}$ = + 260 (*c* 0.5 in CH_2Cl_2); IR (ATR): ν = 977, 1250, 1265, 1438, 1596, 1688, 1720 and 1772 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.15 (s, 3H), 1.37 (s, 3H), 2.71 (s, 3H), 3.58 (d, *J* = 7.1 Hz, 1H), 4.54 (s, 1H), 4.54 (d, *J* = 7.1 Hz, 1H), 5.62 (s, 1H), 6.95 (s, 1H), 6.95 (dt, *J* = 16.5 and 2.3 Hz, 1H), 7.03 (d, *J* = 5.7 Hz, 2H), 7.30-7.40 (m, 13H), 7.45-7.49 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 26.0, 32.3, 43.2, 61.0, 63.2, 69.3, 71.4, 78.5, 79.6, 96.7, 109.2 (t, *J* = 25 Hz, 1C), 111.7 (d, *J* = 19 Hz, 1C), 111.8 (d, *J* = 19 Hz, 1C), 127.3, 127.6, 128.4, 128.5, 128.8, 128.8, 129.4, 129.5, 136.4, 139.2, 139.3, 163.0 (d, *J* = 252 Hz, 1C), 163.1 (d, *J* = 252 Hz, 1C), 166.7, 173.4, 196.9; ^{19}F NMR (376 MHz, $CDCl_3$): -107.47 (s, 2F); HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{37}H_{33}F_2N_2O_5S$ $[M+H]^+$ 655.2073; found 655.2075.

Compound **3.48f**: ^1H NMR (400 MHz, $CDCl_3$): δ = 1.14 (s, 3H), 1.46 (s, 3H), 2.80 (s, 3H), 4.46 (d, *J* = 6.1 Hz, 1H), 4.59 (s, 1H), 4.64 (d, *J* = 6.1 Hz, 1H), 5.76 (s, 1H), 6.76 (tt, *J* = 8.3 and 2.3 Hz, 1H), 6.92-6.94 (m, 3H), 7.08-7.10 (m, 3H), 7.19-7.22 (m, 2H), 7.29-7.36 (m, 10H); ^{19}F NMR (376 MHz, $CDCl_3$): -108.52 (s, 2F); HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{37}H_{33}F_2N_2O_5S$ $[M+H]^+$ 655.2073; found 655.2073.

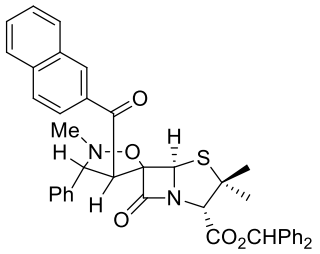
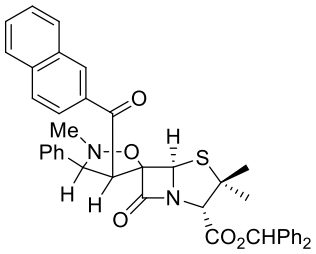



(3'*R*, 4'*S*, 5'*S*)-Benzhydryl spiro[(4-(3,5-bis(trifluoromethyl)benzoyl)-2-methyl-3-phenyl-isoxazolidine)-5',6-penicillanate] (3.47g) and (3'*S*, 4'*S*, 5'*S*)-Benzhydryl spiro[(4-(3,5-bis(trifluoromethyl)benzoyl)-2-methyl-3-phenyl-isoxazolidine)-5',6-penicillanate] (3.48g)

Obtained from *N*-methylphenylnitrone **3.41a** (55.6 mg, 0.412 mmol) and 6-alkylidenepenicillanate **3.46g** (127.6 mg, 0.206 mmol) as described in the general procedure (reaction time: 24 h). Purification of the crude product by flash chromatography (hexane/ethyl acetate, 5:1 → 3:1), gave, in order of elution, **3.47g /3.49g** as a yellow oil (**3.47g**: 26%) and **3.48g /3.49g** as a yellow oil (**3.48g**: 9%).

Recorded as a mixture of compounds **3.47g /3.49g**: Compound **3.47g**: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.16 (s, 3H), 1.38 (s, 3H), 2.72 (s, 3H), 3.55 (d, *J* = 7.3 Hz, 1H), 4.54 (s, 1H), 4.64 (d, *J* = 7.4 Hz, 1H), 5.66 (s, 1H), 6.96 (s, 1H), 7.23-7.47 (m, 15H), 7.90 (s, 1H), 7.98 (s, 1H), 8.06 (s, 1H); ^{19}F NMR (376 MHz, $CDCl_3$): 63.11 (s, 6F); MS (ESI-TOF) m/z: $[M+H]^+$ found 755.20.

Recorded as a mixture of compounds **3.48g /3.49g**: Compound **3.48g**: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.15 (s, 3H), 1.47 (s, 3H), 2.82 (s, 3H), 4.51 (d, *J* = 6.1 Hz, 1H), 4.61 (s, 1H), 4.75 (d, *J* = 6.2 Hz, 1H), 5.78 (s, 1H), 6.95 (s, 1H), 7.00-7.06 (m, 3H), 7.17-7.19 (m, 2H), 7.33-7.37 (m, 10H), 7.79 (s, 3H); ^{19}F NMR (376 MHz, $CDCl_3$): 63.11 (s, 6F); MS (ESI-TOF) m/z: $[M+H]^+$ found 755.20.

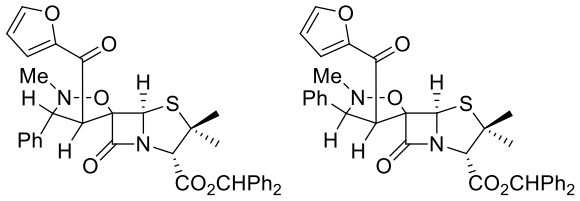



(3'*R*, 4'*S*, 5'*S*)-Benzhydryl spiro[(2-methyl-4-naphtoyl-3-phenyl-isoxazolidine)-5',6-penicillanate] (3.47h) and (3'*S*, 4'*S*, 5'*S*)-Benzhydryl spiro[(2-methyl-4-naphtoyl-3-phenyl-isoxazolidine)-5',6-penicillanate] (3.48h)

Obtained from *N*-methylphenylnitrone **3.41a** (55.6 mg, 0.412 mmol) and 6-alkylidenepenicillanate **3.46h** (110 mg, 0.206 mmol) as described in the general procedure (reaction time: 24 h). Purification of the crude product by flash chromatography (hexane/ethyl acetate, 5:1 → 3:1), gave, in order of elution, **3.47h** as a colourless solid (101 mg, 0.151 mmol, 73%) and a mixture of **3.48h /3.49h** as a yellow oil (**3.48h**: 10%).

Compound **3.47h**: mp 138.3-139.0 ºC; $[\alpha]_D^{25}$= + 300 (*c* 0.5 in CH_2Cl_2); IR (ATR): ν = 1124, 1152, 1178, 1258, 1457, 1670, 1741 and 1773 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.13 (s, 3H), 1.33 (s, 3H), 2.74 (s, 3H), 3.65 (d, *J* = 7.0 Hz, 1H), 4.52 (s, 1H), 4.83 (d, *J* = 7.2 Hz, 1H), 5.71 (s, 1H), 6.95 (s, 1H), 7.30-7.38 (m, 13H), 7.46-7.52 (m, 4H), 7.57 (ddd, *J* = 8.1, 6.4 and 1.7 Hz, 1H), 7.74 (s, 1H). 7.81 (dd, *J* = 8.3 and 5.2 Hz, 2H), 7.86 (dd, *J* = 8.7 and 1.6 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 26.0, 31.8, 43.3, 60.8, 62.7, 69.4, 71.5, 78.4, 79.6, 96.5, 124.1, 127.0, 127.3, 127.6, 127.8, 128.3, 128.4, 128.7, 128.8, 128.8, 129.1, 129.2, 129.9, 131.4, 132.3, 134.3, 135.9, 137.2, 139.3, 139.4, 166.9, 174.0, 198.5; HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{41}H_{37}N_2O_5S$ $[M+H]^+$ 669.2418; found 669.2413.

Recorded as a mixture of compounds **3.48h /3.49h**: Compound **3.48h**: ^1H NMR (400 MHz, $CDCl_3$): δ = 1.10 (s, 3H), 1.42 (s, 3H), 2.81 (s, 3H), 4.52 (d, *J* = 6.7 Hz, 1H), 4.57 (s, 1H), 4.96 (d, *J* = 6.2 Hz, 1H), 5.81 (s, 1H), 6.82-8.11 (m, 32H); MS (ESI-TOF) m/z: $[M+H]^+$ found 669.24.

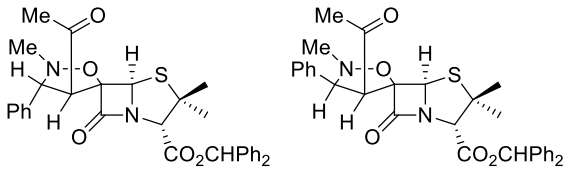


(3'*R*, 4'*S*, 5'*S*)-Benzhydryl spiro[(4-furoyl-2-methyl-3-phenyl-isoxazolidine)-5',6-penicillanate] (3.47i) and (3'*S*, 4'*S*, 5'*S*)-Benzhydryl spiro[(4-furoyl-2-methyl -3-phenyl-isoxazolidine)-5',6-penicillanate] (3.48i)

Obtained from *N*-methylphenylnitrone **3.41a** (55.6 mg, 0.412 mmol) and 6-alkylidenepenicillanate **3.46i** (97,5 mg, 0.206 mmol) as described in the general procedure (reaction time: 24 h). Purification of the crude product by flash chromatography (hexane/ethyl acetate, 4:1 → 3:1), gave, in order of elution, **3.47i** as a colourless solid (84 mg, 0.138 mmol, 67%) and a mixture of **3.48i /3.49i** as a yellow oil (**3.48i**: 13%).

Compound **3.47i**: mp 137.2-138.5 ºC; $[\alpha]_D^{25}$= + 270 (*c* 0.5 in CH_2Cl_2); IR (ATR): ν = 1155, 1179, 1297, 1463, 1660, 1744 and 1786 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.14 (s, 3H), 1.37 (s, 3H), 2.70 (s, 3H), 3.72 (s, 1H), 4.47 (d, *J* = 7.1 Hz, 1H), 4.54 (s, 1H), 5.61 (s, 1H), 6.45 (dd, *J* = 3.5 and 1.6 Hz, 1H), 6.82 (s, 1H), 6.94 (s, 1H), 7.29-7.37 (m, 13H), 7.45 (dd, *J* = 7.3 and 2.1 Hz, 2H), 7.54 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.9, 32.5, 43.2, 60.6, 62.9, 69.2, 71.6, 78.5, 79.3, 96.1, 112.7, 119.3, 127.3, 127.6, 128.3, 128.5, 128.7, 128.7, 129.0, 129.0, 139.3, 139.3, 147.8, 152.9, 166.8, 173.4, 186.4; HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{35}H_{33}N_2O_6S$ $[M+H]^+$ 609.2054; found 609.2068.

Recorded as a mixture of compounds **3.48i /3.49i**: Compound **3.48i**: ^1H NMR (400 MHz, $CDCl_3$): δ = 1.14 (s, 3H), 1.45 (s, 3H), 2.78 (s, 3H), 4.43 (d, *J* = 6.3 Hz, 1H), 4.58 (s, 1H), 4.64 (d, *J* = 5.8 Hz, 1H), 5.77 (s, 1H), 6.22 (d, *J* = 1.9 Hz, 1H), 6.78 (s, 1H), 6.93 (s, 1H), 7.09-7.50 (m, 16H); MS (ESI-TOF) m/z: $[M+H]^+$ found 609.20.

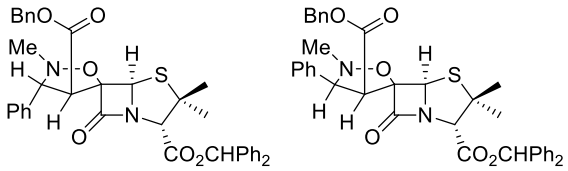


(3'*R*, 4'*S*, 5'*S*)-Benzhydryl spiro[(4-acetyl-2-methyl-3-phenyl-isoxazolidine)-5',6-penicillanate] (3.47j) and (3'*S*, 4'*S*, 5'*S*)-Benzhydryl spiro[(4-acetyl-2-methyl -3-phenyl-isoxazolidine)-5',6-penicillanate] (3.48j)

Obtained from *N*-methylphenylnitrone **3.41a** (55.6 mg, 0.412 mmol) and 6-alkylidenepenicillanate **3.46j** (87.6 mg, 0.206 mmol) as described in the general procedure (reaction time: 24 h). Purification of the crude product by flash chromatography (hexane/ethyl acetate, 4:1 → 3:1), gave, in order of elution, **3.47j** as a colourless solid (34 mg, 0.061 mmol, 30%) and **3.48j** as a yellow oil (7.8 mg, 0.014 mmol, 7%).

Compound **3.47j**: mp 122.8-124.5 ºC; $[\alpha]_D^{25}$= + 250 (*c* 0.5 in CH_2Cl_2); IR (ATR): ν = 968, 978, 1258, 1267, 1718 and 1774 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.21 (s, 3H), 1.53 (s, 3H), 2.13 (s, 3H), 2.65 (s, 3H), 3.49 (d, *J* = 6.9 Hz, 1H), 3.90 (d, *J* = 7.3 Hz, 1H), 4.57 (s, 1H), 5.56 (s, 1H), 6.95 (s, 1H), 7.28-7.44 (m, 13H), 7.52 (dd, *J* = 7.9 and 1.4 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 26.0, 32.7, 33.5, 43.1, 63.0, 65.4, 69.2, 71.2, 78.5, 79.0, 95.9, 127.3, 127.6, 128.4, 128.4, 128.5, 128.7, 128.8, 129.2, 129.3, 137.2, 139.3, 139.3, 166.8, 173.4, 207.0; HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{32}H_{33}N_2O_5S$ $[M+H]^+$ 557.2105; found 557.2108.

Compound **3.48j:** ^1H NMR (400 MHz, $CDCl_3$): δ = 1.20 (s, 3H), 1.52 (s, 3H), 1.58 (s, 3H), 2.77 (s, 3H), 3.91 (d, *J* = 6.2 Hz, 1H), 4.36 (d, *J* = 6.2 Hz, 1H), 4.58 (s, 1H), 5.68 (s, 1H), 6.93 (s, 1H), 7.31-7.36 (m, 15H); HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{32}H_{33}N_2O_5S$ $[M+H]^+$ 557.2105; found 557.2107.

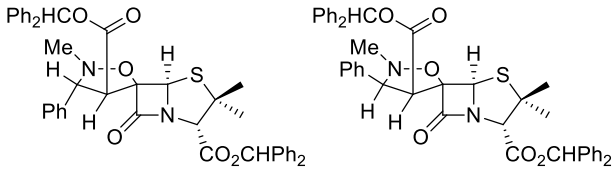


(3'*R*, 4'*S*, 5'*S*)-Benzhydryl spiro[(4-benzyloxycarbonyl-2-methyl-3-phenyl-isoxazolidine)-5',6-penicillanate] (3.47k) and (3'*S*, 4'*S*, 5'*S*)-Benzhydryl spiro[(4-benzyloxycarbonyl-2-methyl -3-phenyl-isoxazolidine)-5',6-penicillanate] (3.48k)

Obtained from *N*-methylphenylnitrone **3.41a** (55.6 mg, 0.412 mmol) and 6-alkylidenepenicillanate **3.46k** (105.8 mg, 0.206 mmol) as described in the general procedure (reaction time: 24 h). Purification of the crude product by flash chromatography (hexane/ethyl acetate, 5:1 → 3:1), gave, in order of elution, **3.47k** as a colourless solid (68.7 mg, 0.106 mmol, 51%) and a mixture of **3.48k /3.49k** as a yellow oil (**3.48k**: 15%).

Compound **3.47k**: mp low melting solid; $[\alpha]_D^{25}$= + 190 (*c* 0.5 in CH_2Cl_2); IR (ATR): ν̃ = 979, 1155, 1256, 1456, 1731, 1735 and 1779 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.18 (s, 3H), 1.40 (s, 3H), 2.65 (s, 3H), 3.66 (t, *J* = 8.4 Hz, 2H), 4.57 (s, 1H), 5.15 (d, *J* = 12.3 Hz, 1H), 5.20 (d, *J* = 12.3 Hz, 1H), 5.54 (s, 1H), 6.95 (s, 1H), 7.29-7.39 (m, 20H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.5, 33.4, 43.2, 62.7, 67.3, 68.8, 71.5, 78.5, 78.9, 95.0, 127.3, 127.6, 128.3, 128.4, 128.5, 128.6, 128.7, 128.7, 128.7, 128.8, 129.0, 129.1, 135.3, 139.3, 139.3, 166.7, 170.8, 172.7; HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{38}H_{37}N_2O_6S$ $[M+H]^+$ 649.2367; found 649.2362.

Recorded as a mixture of compounds **3.48k/3.49k**: Compound **3.48k:** ^1H NMR (400 MHz, $CDCl_3$): δ = 1.21 (s, 3H), 1.49 (s, 3H), 2.79 (s, 3H), 3.40 (s, 2H), 3.70 (d, *J* = 6.3 Hz, 1H), 4.33 (d, *J* = 6.2 Hz, 1H), 4.58 (s, 1H), 5.69 (s, 1H), 6.94 (s, 1H), 7.22-7.43 (m, 20H); MS (ESI-TOF) m/z: $[M+H]^+$ found 649.24.

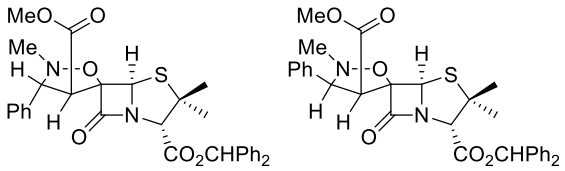


(3'*R*, 4'*S*, 5'*S*)-Benzhydryl spiro[(4-benzhydryloxycarbonyl-2-methyl-3-phenyl-isoxazolidine)-5',6-penicillanate] (3.47l) and (3'*S*, 4'*S*, 5'*S*)-Benzhydryl spiro[(4-benzhydryloxycarbonyl-2-methyl-3-phenyl-isoxazolidine)-5',6-penicillanate] (3.48l)

Obtained from *N*-methylphenylnitrone **3.41a** (55.6 mg, 0.412 mmol) and 6-alkylidenepenicillanate **3.46l** (121 mg, 0.206 mmol) as described in the general procedure (reaction time: 24 h). Purification of the crude product by flash chromatography (hexane/ethyl acetate, 5:1 → 3:1), gave, in order of elution **3.47l** as a colourless solid (119 mg, 0.164 mmol, 80%) and a mixture of **3.48l /3.49l** as a yellow oil (**3.48l:** 10%).

Compound **3.47l**: mp low melting solid; $[\alpha]_D^{25}$= + 190 (*c* 0.5 in CH_2Cl_2); IR (ATR): ν˜ = 953, 978, 1155, 1254, 1449, 1453, 1494, 1735 and 1777 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.08 (s, 3H), 1.20 (s, 3H), 2.66 (s, 3H), 3.70 (t, *J* = 7.7 Hz, 2H), 4.52 (s, 1H), 5.52 (s, 1H), 6.92 (s, 1H), 6.97 (s, 1H), 7.10-7.13 (m, 2H), 7.27-7.36 (m, 23H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.4, 33.1, 43.2, 62.6, 68.8, 71.4, 78.0, 78.5, 79.1, 95.2, 126.9, 127.3, 127.6, 128.0, 128.2, 128.3, 128.5, 128.6, 128.7, 128.8, 129.0, 136.6, 139.3, 139.3, 139.5, 139.5, 166.6, 170.5, 172.9; HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{44}H_{41}N_2O_6S$ $[M+H]^+$ 725.2680; found 725.2683.

Recorded as a mixture of compounds **3.48l /3.49l**: Compound **3.48l**: ^1H NMR (400 MHz, $CDCl_3$): δ = 1.21 (s, 3H), 1.25 (s, 3H), 2.75 (s, 3H), 3.79 (d, *J* = 6.1 Hz, 1H), 4.34 (d, *J* = 6.1 Hz, 1H), 4.49 (s, 1H), 5.58 (s, 1H), 6.71 (s, 1H), 6.92 (s, 1H), 7.08-7.42 (m, 25H); MS (ESI-TOF) m/z: $[M+H]^+$ found 725.27.



(3'*R*, 4'*S*, 5'*S*)-Benzhydryl spiro[(4-methyloxycarbonyl-2-methyl-3-phenyl-isoxazolidine)-5',6-penicillanate] (3.47m) and (3'*S*, 4'*S*, 5'*S*)-Benzhydryl spiro[(4-methyloxycarbonyl-2-methyl-3-phenyl-isoxazolidine)-5',6-penicillanate] (3.48m)

Obtained from *N*-methylphenylnitrone **3.41a** (55.6 mg, 0.412 mmol) and 6-alkylidenepenicillanate **3.46m** (90 mg, 0.206 mmol) as described in the general procedure (reaction time: 24 h). Purification of the crude product by flash chromatography (hexane/ethyl acetate, 4:1 → 3:1), gave, in order of elution, **3.47m** as a colourless solid (67 mg, 0.117 mmol, 57%) and a mixture of **3.48m/3.49m** as a yellow oil (**3.48m**: 16%).

Compound **3.47m**: mp low melting solid; $[\alpha]_D^{25}$= + 220 (*c* 0.5 in CH_2Cl_2); IR (ATR): ν = 980, 1169, 1199, 1255, 1434, 1454, 1495, 1735 and 1779 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.22 (s, 3H), 1.48 (s, 3H), 2.67 (s, 3H), 3.64 (d, *J* = 7.2 Hz, 1H), 3.73 (br s, 4H), 4.59 (s, 1H), 5.54 (s, 1H), 6.95 (s, 1H), 7.30-7.37 (m, 13H), 7.47 (dd, *J* = 7.8 and 1.5 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.6, 33.4, 43.1, 52.6, 62.8, 68.8, 71.5, 78.6, 127.3, 127.6, 128.3, 128.4, 128.5, 128.7, 128.8, 129.0, 129.1, 139.2, 139.3, 166.7, 171.3, 172.7; HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{32}H_{33}N_2O_6S$ $[M+H]^+$ 573.2054; found 573.2062.

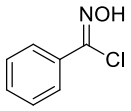
Recorded as a mixture of compounds **3.48m/3.49m**: Compound **3.48m:** ^1H NMR (400 MHz, $CDCl_3$): δ = 1.21 (s, 3H), 1.49 (s, 3H), 2.79 (s, 3H), 3.40 (s, 2H), 3.70 (d, *J* = 6.3 Hz, 1H), 4.33 (d, *J* = 6.2 Hz, 1H), 4.58 (s, 1H), 5.69 (s, 1H), 6.94 (s, 1H), 7.22-7.43 (m, 15H); MS (ESI-TOF) m/z: $[M+H]^+$ found 573.21.

6.6. Synthesis of Compounds of Chapter 4

6.6.1. General Procedure for the Synthesis of Hydroximoyl Chlorides

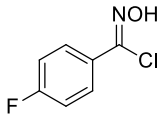
Hydroximoyl chlorides **4.48** were prepared based on previously developed procedures.[10] The appropriate aldehyde (1 equiv.) was dissolved in ethanol (15 mL). A solution of hydroxylamine hydrochloride (1.25 equiv.) in water (3 mL) and anhydrous sodium sulfate (1 equiv.) were added. The reaction mixture was irradiated in the

ultrasonic bath at 30 ºC for the time indicated in each case. The mixture was filtered, and the solvent was evaporated under reduced pressure. The residue was dissolved in CH_2Cl_2, washed with water, and extracted with CH_2Cl_2. The combined organic layers were dried over anhydrous $NaSO_4$, filtered, and the solvents were evaporated affording oximes **4.47** which were used without further purification. Oximes **4.47** were dissolved in chloroform (11 mL) and a drop of pyridine was added. After 5 min, *N*-chlorosuccinimide (1 equiv.) was added stepwise to the stirring reaction mixture. The reaction mixture was stirred for the time indicated in each case, monitored by TLC until completion. The solvent was removed under reduced pressure and the crude product was purified by flash chromatography.



(*Z*)-*N*-Hydroxybenzimidoyl chloride (4.48a)

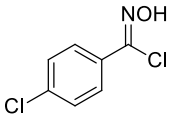
Obtained from benzaldehyde (0.612 mL, 6 mmol) and hydroxylamine hydrochloride (521 mg, 7.5 mmol) as described in the general procedure after 10 minutes of ultrasonic irradiation. The crude mixture reacted overnight with *N*-chlorosuccinimide (799 mg, 6 mmol) as described in the general procedure. Purification by flash chromatography (hexane/ethyl acetate, 3:1) afforded compound **4.48a** as a yellow solid (917 mg, 5.89 mmol, 98%). mp 45.1-47.1 ºC (lit.[11] 47-48 ºC); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.40-7.46 (m, 3H), 7.85 (dd, *J* = 8.1 and 1.5 Hz, 2H), 8.25 (s, 1H). The ^{1}H NMR spectral data are in good agreement with the literature data.[12]



(*Z*)-4-Fluoro-*N*-hydroxybenzimidoyl chloride (4.48b)

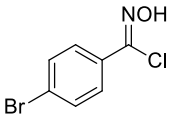
Obtained from 4-fluorobenzaldehyde (0.316 mL, 3 mmol) and hydroxylamine hydrochloride (260 mg, 3.75 mmol) as described in the general procedure after 30 minutes of ultrasonic irradiation. The crude mixture reacted overnight with *N*-chlorosuccinimide (400 mg, 3 mmol) as described in the general procedure. Purification by flash chromatography (hexane/ethyl acetate, 3:1) afforded compound **4.48b** as a colourless

solid (438 mg, 2.52 mmol, 84%). mp 75.1-77.1 ºC (lit.[13] 74.5-75 ºC); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.10 (t, *J* = 8.7 Hz, 2H), 7.84 (dd, *J* = 9.0 and 5.3 Hz, 2H), 7.91 (s, 1H). The ^{1}H NMR spectral data are in good agreement with the literature data.[14]



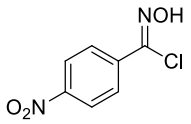
(*Z*)-4-Chloro-*N*-hydroxybenzimidoyl chloride (4.48c)

Obtained from 4-chlorobenzaldehyde (421 mg, 3 mmol) and hydroxylamine hydrochloride (260 mg, 3.75 mmol) as described in the general procedure after 30 minutes of ultrasonic irradiation. The crude mixture reacted overnight with *N*-chlorosuccinimide (400 mg, 6 mmol) as described in the general procedure. Purification by flash chromatography (hexane/ethyl acetate, 3:1) afforded compound **4.48c** as a colourless solid (389 mg, 2.05 mmol, 68%). mp 86.4-88.4 ºC (lit.[11] 88.5-89.5 ºC); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.39 (d, *J* = 8.8 Hz, 2H), 7.79 (d, *J* = 8.8 Hz, 2H), 7.96 (s, 1H). The ^{1}H NMR spectral data are in good agreement with the literature data.[15]



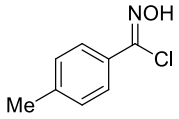
(*Z*)-4-Bromo-*N*-hydroxybenzimidoyl chloride (4.48d)

Obtained from 4-bromobenzaldehyde (555 mg, 3 mmol) and hydroxylamine hydrochloride (260 mg, 3.75 mmol) as described in the general procedure after 30 minutes of ultrasonic irradiation. The crude mixture reacted overnight with *N*-chlorosuccinimide (400 mg, 3 mmol) as described in the general procedure. Purification by flash chromatography (hexane/ethyl acetate, 3:1) afforded compound **4.48d** as a colourless solid (363 mg, 1.55 mmol, 52%). mp 78.3-80.3 ºC (lit.[16] 76-78 ºC); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.55 (d, *J* = 8.7 Hz, 2H), 7.72 (d, *J* = 8.7 Hz, 2H), 7.84 (s, 1H). The ^{1}H NMR spectral data are in good agreement with the literature data.[14]



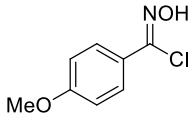
(*Z*)-4-Nitro-*N*-hydroxybenzimidoyl chloride (4.48e)

Obtained from 4-nitrobenzaldehyde (453 mg, 3 mmol) and hydroxylamine hydrochloride (260 mg, 3.75 mmol) as described in the general procedure after 15 minutes of ultrasonic irradiation. The crude mixture reacted overnight with *N*-chlorosuccinimide (400 mg, 3 mmol) as described in the general procedure. Purification by flash chromatography (hexane/ethyl acetate, 3:1) afforded compound **4.48e** as a yellow solid (460 mg, 2.29 mmol, 76%). mp 121.4-123.4 ºC (lit.[11] 124-125 ºC); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.05 (d, *J* = 9.0 Hz, 2H), 8.27 (d, *J* = 9.0 Hz, 2H), 8.32 (s, 1H). The ^{1}H NMR spectral data are in good agreement with the literature data.[14]



(*Z*)-4-Methyl-*N*-hydroxybenzimidoyl chloride (4.48f)

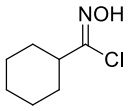
Obtained from 4-methylbenzaldehyde (0.353 mL, 3 mmol) and hydroxylamine hydrochloride (260 mg, 3.75 mmol) as described in the general procedure after 30 minutes of ultrasonic irradiation. The crude mixture reacted overnight with *N*-chlorosuccinimide (400 mg, 3 mmol) as described in the general procedure. Purification by flash chromatography (hexane/ethyl acetate, 3:1) afforded compound **4.48f** as a colourless solid (323 mg, 1.91 mmol, 64%). mp 67.4-69.4 ºC (lit.[17] 71-72 ºC); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.38 (s, 3H), 7.21 (d, *J* = 8.1 Hz, 2H), 7.65 (s, 1H), 7.73 (d, *J* = 8.3 Hz, 2H). The ^{1}H NMR spectral data are in good agreement with the literature data.[15]



(*Z*)-4-Methoxy-*N*-hydroxybenzimidoyl chloride (4.48g)

Obtained from 4-methoxybenzaldehyde (0.364 mL, 3 mmol) and hydroxylamine hydrochloride (0.260 mg, 3.75 mmol) as described in the general procedure after 3 hours of ultrasonic irradiation. The crude mixture reacted overnight with *N*-chlorosuccinimide

(400 mg, 3 mmol) as described in the general procedure. Purification by flash chromatography (hexane/ethyl acetate, 3:1) afforded compound **4.48g** as a colourless solid (498 mg, 2.68 mmol, 89%). mp 85.7-87.7 ºC (lit.[11] 87.5-89 ºC); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 3.85 (s, 3H), 6.91 (d, *J* = 9.0 Hz, 2H), 7.65 (s, 1H), 7.79 (d, *J* = 9.0 Hz, 2H). The ^{1}H NMR spectral data are in good agreement with the literature data.[18]



(*Z*)-*N*-Hydroxycyclohexanecarbimidoyl chloride (4.48h)

Obtained from cyclohexanecarboxaldehyde (0.361 mL, 3 mmol) and hydroxylamine hydrochloride (260 mg, 3.75 mmol) as described in the general procedure after 30 minutes of ultrasonic irradiation. The crude mixture reacted overnight with *N*-chlorosuccinimide (400 mg, 3 mmol) as described in the general procedure. Purification by flash chromatography (hexane/ethyl acetate, 3:1) afforded compound **4.48h** as a colourless oil (235 mg, 1.45 mmol, 48%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.23-1.35 (m, 1H), 1.39-1.50 (m, 2H), 1.66-1.70 (m, 1H), 1.78-1.82 (m, 2H), 1.92-1.96 (m, 2H), 2.46 (tt, *J* = 11.5 and 3.4 Hz, 1H), 8.10 (s, 1H). The ^{1}H NMR spectral data are in good agreement with the literature data.[19]

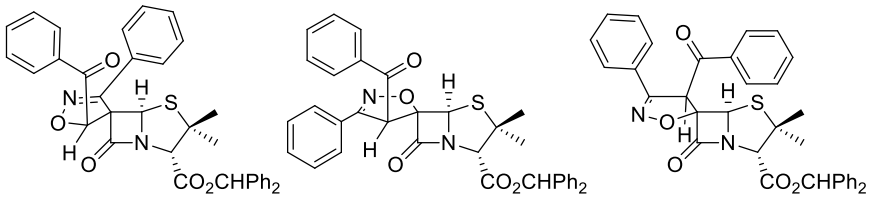
6.6.2. General Procedure for the 1,3-Dipolar Cycloaddition of Nitrile Oxides with 6-(*Z*)-(Benzoylmethylidene)penicillanate

Method A (Batch): A solution of the appropriate hydroximoyl chloride **4.48** (2 equiv.) in ethyl acetate (2 mL) was added to a suspension of potassium carbonate (5 equiv.) and the 6-(*Z*)-(benzoylmethylidene)penicillanate **4.50** (1 equiv.) in ethyl acetate (3 mL). The reaction mixture was stirred under reflux for the time indicated in each case, monitored by TLC until completion and filtered through a pad of Celite, which was washed with ethyl acetate. The solvent was removed under reduced pressure and the crude product was purified by flash chromatography.

Method B (MW irradiation): A suspension of hydroximoyl chloride **4.48** (2 equiv.) with potassium carbonate (5 equiv.) and 6-(*Z*)-(benzoylmethylidene)penicillanate **4.50** (1 equiv.) in ethyl acetate (1 mL) was irradiated in a microwave reactor (CEM

Focused Synthesis System, Discover S-Class) at 80 ºC for the time indicated in each case. Upon completion, the crude mixture was filtered through a pad of Celite, which was washed with ethyl acetate. The solvent was removed under reduced pressure and the crude product was purified by flash chromatography.

Method C (Continuous Flow Chemistry): The flow system was set up according to the scheme in Table 2. A 0.2 M solution of 6-(*Z*)-(benzoylmethylidene)penicillanate **4.50** in ethyl acetate (1 mL) with a 100 μL/min flow rate, was combined at a T-piece with a 0.4 M solution of the corresponding hydroximoyl chloride **4.48** in ethyl acetate (1 mL) with a 100 μL/min flow rate. The combined solution was passed through a packed-bed column filled with K_2CO_3 (≈ 2.1 g, 7 cm), kept at room temperature. The output solution was concentrated under reduced pressure and the crude product was purified by flash chromatography.



(4'*R*,5'*S*)-Benzhydryl spiro[(5-benzoyl-3-phenyl-isoxaxoline)-4',6-penicillanate] (4.51a) and (4'*S*,5'*S*)-Benzhydryl spiro[(4-benzoyl-3-phenyl-isoxaxoline)-5',6-penicillanate] (4.52a) and (4'*S*,5'*R*)-Benzhydryl spiro[(4-benzoyl-3-phenyl-isoxaxoline)-5',6-penicillanate] (4.53a)

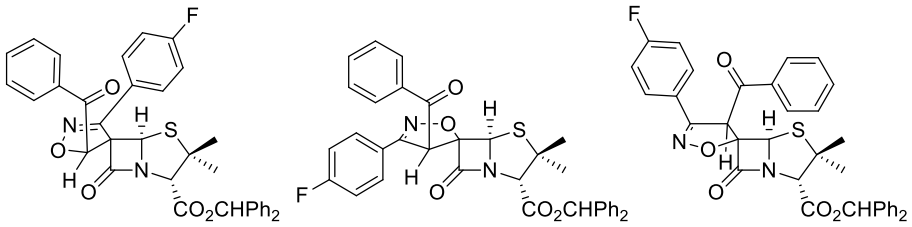
Obtained from hydroximoyl chloride **4.48a** (64 mg, 0.412 mmol) and 6-(*Z*)-(benzoylmethylidene)penicillanate **4.50** (100 mg, 0.206 mmol) as described in the general procedure. The crude product was purified by flash chromatography (hexane/ethyl acetate, 3:1). Method A, gave, in order of elution, **4.51a** as a colourless solid (73 mg, 0.121 mmol, 59%) and a mixture of **4.52a/4.53a** (84:16) as a yellow oil (47 mg, 0.078 mmol, 38%). Method B, gave, in order of elution, **4.51a** as a colourless solid (43 mg, 0.071 mmol, 35%) and **4.52a** as a yellow oil (42 mg, 0.070 mmol, 34%). Method C, gave, in order of elution, **4.51a** as a colourless solid (67 mg, 0.111 mmol, 54%) and a mixture of **4.52a/4.53a** (33:67) as a yellow oil (43 mg, 0.071 mmol, 35%).

Compound **4.51a**: mp 74.0-76.0 ºC; $[\alpha]_D^{25}$ = + 360 (*c* 0.5 in CH_2Cl_2); IR (ATR): ν = 881, 1083, 1156, 1449, 1496, 1586, 1678, 1740 and 1780 cm^{-1}; ^1H NMR (400 MHz,

$CDCl_3$): δ = 1.15 (s, 3H), 1.57 (s, 3H), 4.65 (s, 1H), 5.86 (s, 1H), 6.17 (s, 1H), 6.98 (s, 1H), 7.27-7.35 (m, 12H), 7.43 (t, *J* = 7.5 Hz, 1H), 7.52-7.56 (m, 2H), 7.66 (t, *J* = 7.4 Hz, 1H), 7.81-7.83 (m, 2H), 8.04-8.06 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 26.2, 32.1, 63.7, 68.0, 69.9, 76.6, 78.8, 81.8, 126.7, 127.3, 127.4, 127.5, 127.7, 128.5, 128.6, 128.6, 128.8, 128.9, 129.1, 129.2, 129.4, 131.0, 134.5, 135.0, 139.1, 139.2, 155.6, 166.5, 170.7, 191.6; HRMS (ESI) m/z: Calcd for $C_{36}H_{31}N_2O_5S$ $[M+H]^+$ 603.1948; Found 666.1941.

Compound **4.52a:** mp low melting solid; $[\alpha]_D^{25}$= + 280 (*c* 0.25 in CH_2Cl_2); IR (ATR): ν = 910, 1080, 1176, 1448, 1497, 1595, 1676, 1741 and 1777 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.12 (s, 3H), 1.46 (s, 3H), 4.60 (s, 1H), 5.67 (s, 1H), 5.68 (s, 1H), 6.93 (s, 1H), 7.22-7.24 (m, 2H), 7.30-7.35 (m, 11H), 7.48-7.55 (m, 4H), 7.63 (t, *J* = 7.4 Hz, 1H), 7.95-7.97 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.5, 33.4, 57.9, 63.7, 68.9, 72.9, 77.5, 78.7, 102.2, 127.2, 127.3, 127.5, 127.6, 128.4, 128.6, 128.6, 128.7, 128.8, 128.8, 128.9, 129.0, 129.3, 131.0, 134.5, 137.2, 139.1, 139.1, 156.7, 166.2, 170.3, 194.6; HRMS (ESI) m/z: Calcd for $C_{36}H_{31}N_2O_5S$ $[M+H]^+$ 603.1948; Found 666.1945.

Recorded as a mixture of compounds **4.52a/4.53a** (33:67): Compound **4.53a**: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.30 (s, 3H), 1.33 (s, 3H), 4.46 (s, 1H), 5.56 (s, 1H), 5.79 (s, 1H), 6.93 (s, 1H), 7.22-7.24 (m, 2H), 7.30-7.35 (m, 11H), 7.48-7.55 (m, 4H), 7.63 (t, *J* = 7.4 Hz, 1H), 8.11 (d, *J* = 7.2 Hz, 2H).



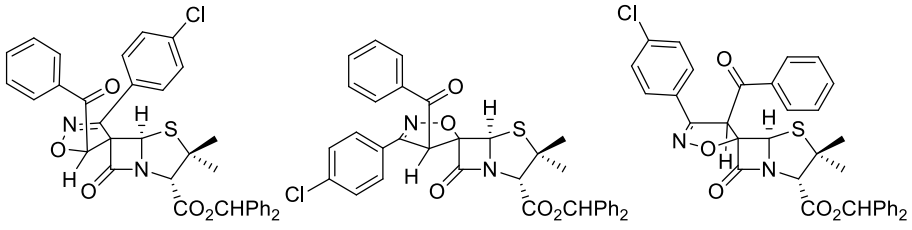
(4'*R*,5'*S*)-Benzhydryl spiro[(5-benzoyl-3-(4-fluorophenyl)-isoxaxoline)-4',6-penicillanate] (4.51b) and (4'*S*,5'*S*)-Benzhydryl spiro[(4-benzoyl-3-(4-fluorophenyl)-isoxaxoline)-5',6-penicillanate] (4.52b) and (4'*S*,5'*R*)-Benzhydryl spiro[(4-benzoyl-3-(4-fluorophenyl)-isoxaxoline)-5',6-penicillanate] (4.53b)

Obtained from hydroximoyl chloride **4.48b** (71 mg, 0.412 mmol) and 6-(*Z*)-(benzoylmethylidene)penicillanate **4.50** (100 mg, 0.206 mmol) as described in the general procedure. The crude product was purified by flash chromatography (hexane/ethyl acetate, 3:1). Method A gave **4.51b** as a colourless solid (92 mg, 0.148 mmol, 72%). Method B, gave, in order of elution, **4.51b** as a colourless solid (50 mg, 0.081 mmol,

39%) and **4.52b/4.53b** (84:16) as a yellow oil (41 mg, 0.066 mmol, 32%). Method C, gave, in order of elution, **4.51b** as a colourless solid (63 mg, 0.101 mmol, 49%) and a mixture of **4.52b/4.53b** (53:47) as a yellow oil (49 mg, 0.079 mmol, 38%).

Compound **4.51b**: mp 167.1-169.1 ºC; $[\alpha]_D^{25}$= + 380 (*c* 0.5 in CH_2Cl_2); IR (ATR): ν = 838, 1081, 1156, 1449, 1509, 1598, 1676, 1736 and 1778 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.17 (s, 3H), 1.57 (s, 3H), 4.65 (s, 1H), 5.84 (s, 1H), 6.17 (s, 1H), 6.90 (t, *J* = 8.7 Hz, 2H), 6.98 (s, 1H), 7.30-7.36 (m, 10H), 7.53-7.56 (m, 2H), 7.66 (t, *J* = 7.4 Hz, 1H), 7.79-7.82 (m, 2H), 8.04-8.06 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 26.1, 32.3, 63.8, 68.0, 69.9, 76.6, 78.9, 81.8, 116.5 (d, *J* = 22 Hz), 123.4 (d, *J* = 3 Hz), 127.2, 127.7, 128.5, 128.7, 128.8, 128.8, 129.0, 129.2, 129.4, 129.4, 129.5, 134.5, 134.9, 139.0 (d, *J* = 2 Hz), 154.6, 164.4 (d, *J* = 252 Hz), 166.5, 170.4, 191.6; ^{19}F NMR (376 MHz, $CDCl_3$): δ = -108.5 (s, 1F); HRMS (ESI) m/z: Calcd for $C_{36}H_{29}N_2O_5FNaS$ $[M+Na]^+$ 643.1673; Found 643.1671.

Recorded as a mixture of compounds **4.52b/4.53b** (53:47): Compound **4.52b**: ^1H NMR (400 MHz, $CDCl_3$): δ = 1.12 (s, 3H), 1.46 (s, 3H), 4.60 (s, 1H), 5.63 (s, 1H), 5.66 (s, 1H), 6.91-6.96 (3H), 7.30-7.35 (m, 10H), 7.47-7.56 (m, 4H), 7.62-7.66 (m, 1H), 7.93-7.95 (m, 2H); Compound **4.53b**: ^1H NMR (400 MHz, $CDCl_3$): δ = 1.30 (s, 3H), 1.34 (s, 3H), 4.46 (s, 1H), 5.56 (s, 1H), 5.74 (s, 1H), 6.91-6.96 (3H), 7.30-7.35 (m, 10H), 7.47-7.56 (m, 4H), 7.62-7.66 (m, 1H), 8.08-8.10 (m, 2H).



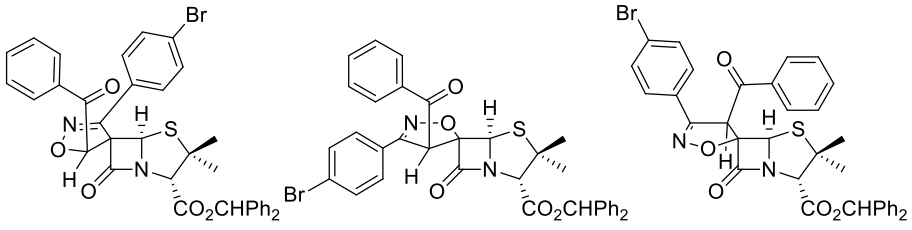
(4'*R*,5'*S*)-Benzhydryl spiro[(5-benzoyl-3-(4-chlorophenyl)-isoxaxoline)-4',6-penicillanate] (4.51c) and (4'*S*,5'*S*)-Benzhydryl spiro[(4-benzoyl-3-(4-chlorophenyl)-isoxaxoline)-5',6-penicillanate] (4.52c) and (4'*S*,5'*R*)-Benzhydryl spiro[(4-benzoyl-3-(4-chlorophenyl)-isoxaxoline)-5',6-penicillanate] (4.53c)

Obtained from hydroximoyl chloride **4.48c** (78 mg, 0.412 mmol) and 6-(*Z*)-(benzoylmethylidene)penicillanate **4.50** (100 mg, 0.206 mmol) as described in the general procedure. The crude product was purified by flash chromatography (hexane/ethyl acetate, 3:1). Method A, gave, in order of elution, **4.51c** as a colourless solid (64 mg,

0.100 mmol, 49%) and a mixture of **4.52c/8c** (71:29) as a yellow oil (50 mg, 0.078 mmol, 38%). Method B, gave, in order of elution, **4.51c** as a colourless solid (54 mg, 0.084 mmol, 41%) and **4.52c/4.53c** (90:10) as a yellow oil (38 mg, 0.21 mmol, 29%). Method C, gave, in order of elution, **4.51c** as a colourless solid (59 mg, 0.093 mmol, 45%) and a mixture of **4.52c/4.53c** (65:35) as a yellow oil (38 mg, 0.060 mmol, 29%).

Compound **4.51c**: mp 198.1-199.4 ºC; $[\alpha]_D^{25}$= + 450 (*c* 0.5 in CH_2Cl_2); IR (ATR): ν = 975, 1086, 1156, 1203, 1450, 1492, 1595, 1667, 1736 and 1779 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.17 (s, 3H), 1.57 (s, 3H), 4.65 (s, 1H), 5.85 (s, 1H), 6.18 (s, 1H), 6.99 (s, 1H), 7.16 (d, *J* = 8.7 Hz, 2H), 7.31-7.36 (m, 10H), 7.53-7.56 (m, 2H), 7.66 (t, *J* = 7.4 Hz, 1H), 7.73-7.77 (m, 2H), 8.03-8.05 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 26.1, 32.8, 63.8, 68.0, 69.9, 76.5, 79.0, 81.9, 125.7, 127.2, 127.7, 128.6, 128.8, 129.0, 129.2, 129.4, 129.6, 134.6, 134.9, 137.2, 139.0, 154.6, 166.4, 170.4, 191.5; HRMS (ESI) m/z: Calcd for $C_{36}H_{29}N_2O_5Cl_2S$ $[M+Cl]^+$ 671.1180; Found 671.1183.

Recorded as a mixture of compounds **4.52c/4.53c** (90:10): Compound **4.52c**: ^1H NMR (400 MHz, $CDCl_3$): δ = 1.12 (s, 3H), 1.46 (s, 3H), 4.60 (s, 1H), 5.64 (s, 1H), 5.67 (s, 1H), 6.93 (s, 1H), 7.21-7.23 (m, 2H), 7.30-7.36 (m, 10H), 7.46-7.53 (m, 4H), 7.62-7.66 (m, 1H), 7.93-7.95 (m, 2H); Compound **4.53c**: ^1H NMR (400 MHz, $CDCl_3$): δ = 1.30 (s, 3H), 1.34 (s, 3H), 4.46 (s, 1H), 5.56 (s, 1H), 5.74 (s, 1H), 6.93 (s, 1H), 7.21-7.23 (m, 2H), 7.30-7.36 (m, 10H), 7.46-7.53 (m, 4H), 7.62-7.66 (m, 1H), 8.07-8.09 (m, 2H).



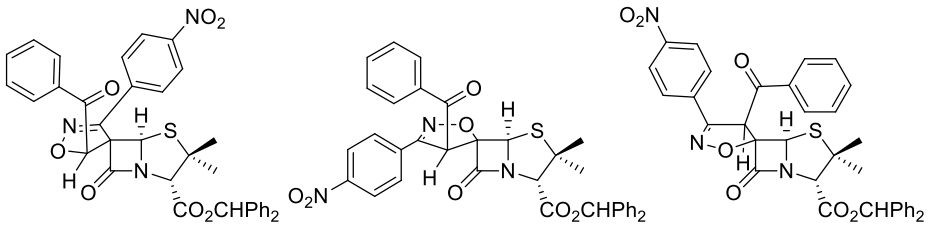
(4'*R*,5'*S*)-Benzhydryl spiro[(5-benzoyl-3-(4-bromophenyl)-isoxaxoline)-4',6-penicillanate] (4.51d) and (4'*S*,5'*S*)-Benzhydryl spiro[(4-benzoyl-3-(4-bromophenyl)-isoxaxoline)-5',6-penicillanate] (4.52d) and (4'*S*,5'*R*)-Benzhydryl spiro[(4-benzoyl-3-(4-bromophenyl)-isoxaxoline)-5',6-penicillanate] (4.53d)

Obtained from hydroximoyl chloride **4.48d** (97 mg, 0.412 mmol) and 6-(*Z*)-(benzoylmethylidene)penicillanate **4.50** (100 mg, 0.206 mmol) as described in the general procedure. The crude product was purified by flash chromatography (hexane/ethyl acetate, 3:1). Method A, gave, in order of elution, **4.51d** as a colourless solid (69 mg,

0.101 mmol, 49%) and a mixture of **4.52d/4.53d** (65:35) as a yellow oil (60 mg, 0.088 mmol, 43%). Method B, gave, in order of elution, **4.51d** as a colourless solid (62 mg, 0.091 mmol, 44%) and **4.52d/4.53d** (58:42) as a yellow oil (47 mg, 0.069 mmol, 33%). Method C, gave, in order of elution, **4.51d** as a colourless solid (80 mg, 0.117 mmol, 57%) and a mixture of **4.52d/4.53d** (75:25) as a yellow oil (49 mg, 0.072 mmol, 35%).

Compound **4.51d**: mp 196.7-198.4 ºC; $[\alpha]_D^{25}$= + 410 (*c* 0.5 in CH_2Cl_2); IR (ATR): ν = 866, 1086, 1155, 1203, 1450, 1492, 1594, 1667, 1736 and 1779 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.18 (s, 3H), 1.57 (s, 3H), 4.65 (s, 1H), 5.86 (s, 1H), 6.18 (s, 1H), 6.90 (t, *J* = 8.7 Hz, 2H), 6.96 (s, 1H), 7.30-7.37 (m, 10H), 7.53-7.56 (m, 2H), 7.65-7.70 (m, 3H), 8.03-8.06 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 26.1, 32.4, 63.8, 68.0, 69.9, 76.5, 79.0, 81.9, 125.7, 126.2, 127.2, 127.7, 128.6, 128.7, 128.8, 129.0, 129.2, 129.4, 132.5, 134.6, 134.9, 139.0, 154.6, 166.4, 170.4, 191.5; HRMS (ESI) m/z: Calcd for $C_{36}H_{30}N_2O_5BrS$ $[M+H]^+$ 681.1053; Found 681.1049.

Recorded as a mixture of compounds **4.52d/4.53d** (58:42): Compound **4.52d**: ^1H NMR (400 MHz, $CDCl_3$): δ = 1.12 (s, 3H), 1.46 (s, 3H), 4.60 (s, 1H), 5.64 (s, 1H), 5.66 (s, 1H), 6.93 (s, 1H), 7.31-7.35 (m, 10H), 7.38-7.40 (m, 3H), 7.47-7.53 (m, 3H), 7.63-7.66 (m, 1H), 7.93-7.95 (m, 2H); Compound **4.53d**: ^1H NMR (400 MHz, $CDCl_3$): δ = 1.30 (s, 3H), 1.34 (s, 3H), 4.46 (s, 1H), 5.56 (s, 1H), 5.73 (s, 1H), 6.94 (s, 1H), 7.31-7.35 (m, 10H), 7.38-7.40 (m, 3H), 7.47-7.53 (m, 3H), 7.63-7.66 (m, 1H), 8.07-8.09 (m, 2H).



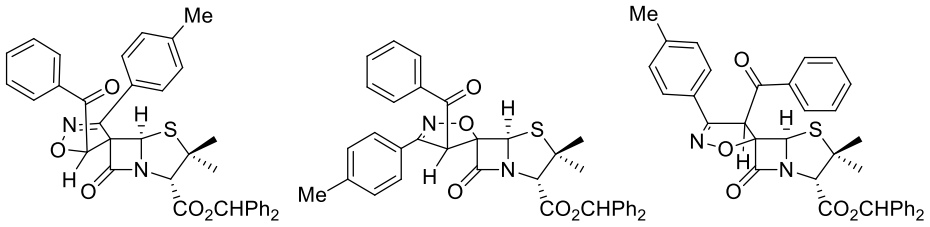
(4'*R*,5'*S*)-Benzhydryl spiro[(5-benzoyl-3-(4-nitrophenyl)-isoxaxoline)-4',6-penicillanate] (4.51e) and (4'*S*,5'*S*)-Benzhydryl spiro[(4-benzoyl-3-(4-nitrophenyl)-isoxaxoline)-5',6-penicillanate] (4.52e) and (4'*S*,5'*R*)-Benzhydryl spiro[(4-benzoyl-3-(4-nitrophenyl)-isoxaxoline)-5',6-penicillanate] (4.53e)

Obtained from hydroximoyl chloride **4.48e** (83 mg, 0.412 mmol) and 6-(*Z*)-(benzoylmethylidene)penicillanate **4.50** (100 mg, 0.206 mmol) as described in the general procedure. The crude product was purified by flash chromatography (hexane/ethyl acetate, 3:1). Method A, gave, in order of elution, **4.51e** as a colourless solid (72 mg,

0.111 mmol, 54%) and a mixture of **4.52e/4.53e** (34:66) as a yellow oil (47 mg, 0.072 mmol, 35%). Method B, gave, in order of elution, **4.51e** as a colourless solid (63 mg, 0.097 mmol, 47%) and **4.52e/4.53e** as a yellow oil (50:50) (43 mg, 0.066 mmol, 32%).

Compound **4.51e**: mp 171.7.-173.7 ºC; $[\alpha]_D^{25}$= + 370 (*c* 0.5 in CH_2Cl_2); IR (ATR): ν = 884, 1082, 1155, 1344, 1449, 1497, 1518, 1597, 1669, 1744 and 1777 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.20 (s, 3H), 1.58 (s, 3H), 4.68 (s, 1H), 5.88 (s, 1H), 6.25 (s, 1H), 7.00 (s, 1H), 7.33-7.38 (m, 10H), 7.54-7.58 (m, 2H), 7.69 (t, *J* = 7.4 Hz, 1H), 7.98-8.06 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 26.1, 32.5, 64.0, 67.9, 69.9, 76.4, 79.2, 82.3, 124.4, 127.1, 127.8, 128.2, 128.6, 128.8, 128.8, 128.9, 128.9, 129.0, 129.3, 129.4, 133.4, 134.8, 138.8, 149.1, 153.8, 166.3, 169.9, 191.4; HRMS (ESI) m/z: Calcd for $C_{36}H_{30}N_3O_7S$ $[M+H]^+$ 648.1799; Found 648.1792.

Recorded as a mixture of compounds **4.52e/4.53e** (50:50): Compound **4.52e**: ^1H NMR (400 MHz, $CDCl_3$): δ = 1.13 (s, 3H), 1.46 (s, 3H), 4.61 (s, 1H), 5.69 (s, 1H), 5.72 (s, 1H), 6.94 (s, 1H), 7.31-7.36 (m, 10H), 7.48-7.56 (m, 2H), 7.76-7.78 (m, 2H), 8.08-8.12 (m, 2H), 8.18-8.20 (m, 2H), 8.33-8.36 (m, 1H); Compound **4.53e**: ^1H NMR (400 MHz, $CDCl_3$): δ = 1.31 (s, 3H), 1.33 (s, 3H), 4.47 (s, 1H), 5.58 (s, 1H), 5.82 (s, 1H), 6.94 (s, 1H), 7.31-7.36 (m, 10H), 7.48-7.56 (m, 2H), 7.69-7.72 (m, 2H), 7.94-7.96 (m, 2H), 8.08-8.12 (m, 2H), 8.33-8.36 (m, 1H).



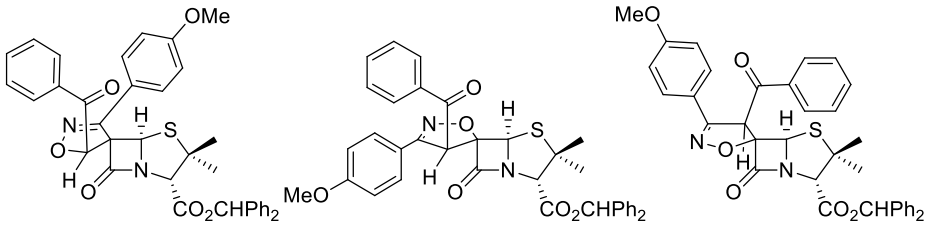
(4'*R*,5'*S*)-Benzhydryl spiro[(5-benzoyl-3-(4-methylphenyl)-isoxaxoline)-4',6-penicillanate] (4.51f) and (4'*S*,5'*S*)-Benzhydryl spiro[(4-benzoyl-3-(4-methylphenyl)-isoxaxoline)-5',6-penicillanate] (4.52f) and (4'*S*,5'*R*)-Benzhydryl spiro[(4-benzoyl-3-(4-methylphenyl)-isoxaxoline)-5',6-penicillanate] (4.53f)

Obtained from hydroximoyl chloride **4.48f** (70 mg, 0.412 mmol) and 6-(*Z*)-(benzoylmethylidene)penicillanate **4.50** (100 mg, 0.206 mmol) as described in the general procedure. The crude product was purified by flash chromatography (hexane/ethyl acetate, 3:1). Method A, gave, in order of elution, **4.51f** as a colourless solid (64 mg, 0.104 mmol, 50%) and a mixture of **4.52f/8f** (68:32) as a yellow oil (48 mg, 0.078 mmol,

38%). Method B, gave, in order of elution, **4.51f** as a colourless solid (39 mg, 0.063 mmol, 31%) and **4.52f/4.53f** (85:15) as a yellow oil (26 mg, 0.042 mmol, 20%). Method C, gave, in order of elution, **4.51f** as a colourless solid (65 mg, 0.105 mmol, 51%) and a mixture of **4.52f/4.53f** (39:61) as a yellow oil (29 mg, 0.047 mmol, 23%).

Compound **4.51f:** mp 88.4-90.4 ºC; $[\alpha]_D^{25}$= + 420 (*c* 0.5 in CH_2Cl_2); IR (ATR): ν = 876, 1156, 1449, 1497, 1596, 1676, 1742 and 1774 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.16 (s, 3H), 1.56 (s, 3H), 2.34 (s, 3H), 4.65 (s, 1H), 5.88 (s, 1H), 6.16 (s, 1H), 6.98 (s, 1H), 7.04 (d, *J* = 8.0 Hz, 2H), 7.26-7.36 (m, 10H), 7.52-7.55 (m, 2H), 7.65 (t, *J* = 7.4 Hz, 1H), 7.72 (d, *J* = 8.2 Hz, 2H), 8.04-8.06 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 21.6, 26.2, 32.1, 63.6, 68.0, 69.8, 76.6, 78.8, 81.6, 124.4, 127.3, 127.3, 127.6, 128.5, 128.5, 128.8, 128.9, 129.1, 129.4, 130.0, 134.4, 135.0, 139.1, 139.2, 141.3, 155.5, 166.6, 170.7, 191.6; HRMS (ESI) m/z: Calcd for $C_{37}H_{33}N_2O_5S$ $[M+H]^+$ 617.2105; Found 617.2098.

Recorded as a mixture of compounds **4.52f/4.53f** (85:15): Compound **4.52f**: ^1H NMR (400 MHz, $CDCl_3$): δ = 1.12 (s, 3H), 1.46 (s, 3H), 2.27 (s, 3H), 4.59 (s, 1H), 5.66 (s, 1H), 5.66 (s, 1H), 6.93 (s, 1H), 7.04 (d, *J* = 8.1 Hz, 2H), 7.30-7.35 (m, 10H), 7.43 (d, *J* = 8.2 Hz, 2H), 7.46-7.52 (m, 2H), 7.62 (t, *J* = 7.4 Hz, 1H), 7.94-7.96 (m, 2H); Compound **4.53f**: ^1H NMR (400 MHz, $CDCl_3$): δ = 1.30 (s, 3H), 1.34 (s, 3H), 2.32 (s, 3H), 4.46 (s, 1H), 5.56 (s, 1H), 5.75 (s, 1H), 6.93 (s, 1H), 7.13 (d, *J* = 8.1 Hz, 2H), 7.30-7.35 (m, 10H), 7.42-7.44 (m, 2H), 7.46-7.52 (m, 2H), 7.56-7.58 (m, 1H), 8.09 (d, *J* = 7.3 Hz, 2H).



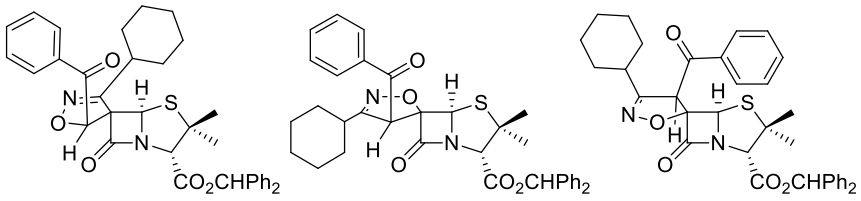
(4'*R*,5'*S*)-Benzhydryl spiro[(5-benzoyl-3-(4-methoxyphenyl)-isoxaxoline)-4',6-penicillanate] (4.51g) and (4'*S*,5'*S*)-Benzhydryl spiro[(4-benzoyl-3-(4-methoxyphenyl)-isoxaxoline)-5',6-penicillanate] (4.52g) and (4'*S*,5'*R*)-Benzhydryl spiro[(4-benzoyl-3-(4-methoxyphenyl)-isoxaxoline)-5',6-penicillanate] (4.53g)

Obtained from hydroximoyl chloride **4.48g** (76 mg, 0.412 mmol) and 6-(*Z*)-(benzoylmethylidene)penicillanate **4.50** (100 mg, 0.206 mmol) as described in the general procedure. The crude product was purified by flash chromatography (hexane/ethyl

acetate, 3:1). The crude product was purified by flash chromatography (hexane/ethyl acetate, 3:1). Method A gave a mixture of **4.51g/4.53g** (69:31) as a yellow oil (68 mg, 0.107 mmol, 52%). Method B, gave, in order of elution, a mixture of **4.51g/4.53g** (69:31) as a yellow oil (47 mg, 0.074 mmol, 36%) and **4.52g** as a colourless solid (28 mg, 0.044 mmol, 21%). Method C gave a mixture of **4.51g/4.53g** (75:25) as a yellow oil (70 mg, 0.111 mmol, 54%).

Recorded as a mixture of compounds **4.51g/4.53g** (69:31): Compound **4.51g**: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.16 (s, 3H), 1.57 (s, 3H), 3.78 (s, 3H), 4.65 (s, 1H), 5.89 (s, 1H), 6.15 (s, 1H), 6.76 (d, *J* = 8.9 Hz, 2H), 6.99 (s, 1H), 7.31-7.36 (m, 10H), 7.51-7.55 (m, 2H), 7.63-7.65 (m, 1H), 7.78-7.80 (m, 2H), 8.03-8.06 (m, 2H); Compound **4.53g**: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.16 (s, 3H), 1.57 (s, 3H), 3.86 (s, 3H), 4.66 (s, 1H), 5.89 (s, 1H), 6.16 (s, 1H), 6.65 (d, *J* = 8.9 Hz, 1H), 6.99 (s, 1H), 7.31-7.36 (m, 10H), 7.51-7.55 (m, 2H), 7.63-7.65 (m, 1H), 7.75 (dd, *J* = 8.9 and 2.2 Hz, 2H), 7.90 (d, *J* = 2.2 Hz, 1H), 8.03-8.06 (m, 2H).

Compound **4.52g**: mp 94.3-96.3 ºC; $[\alpha]_D^{25}$ = + 260 (*c* 0.25 in CH_2Cl_2); IR (ATR): ν = 911, 1717, 1256, 1457, 1516, 1607, 1740 and 1781 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.12 (s, 3H), 1.46 (s, 3H), 3.75 (s, 3H), 4.60 (s, 1H), 5.62 (s, 1H), 5.65 (s, 1H), 6.74 (d, *J* = 8.9 Hz, 2H), 6.92 (s, 1H), 7.30-7.35 (m, 10H), 7.47-7.51 (m, 4H), 7.62 (t, *J* = 7.4 Hz, 1H), 7.94-7.96 (m, 2H);^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.5, 33.4, 55.4, 58.5, 63.7, 68.9, 72.9, 78.7, 101.9, 114.4, 119.7, 127.3, 127.6, 128.4, 128.6, 128.7, 128.8, 128.8, 129.2, 129.3, 134.4, 137.2, 139.1, 139.1, 156.3, 161.7, 166.2, 170.5, 194.6; HRMS (ESI) m/z: Calcd for $C_{37}H_{33}N_2O_6S$ $[M+H]^+$ 633.2054; Found 633.2048.



(4'*R*,5'*S*)-Benzhydryl spiro[(5-benzoyl-3-cyclohexyl-isoxaxoline)-4',6-penicillanate] (4.51h) and (4'*S*,5'*S*)-Benzhydryl spiro[(4-benzoyl-3-cyclohexyl-isoxaxoline)-5',6-penicillanate] (4.52h) and (4'*S*,5'*R*)-Benzhydryl spiro[(4-benzoyl-3-cyclohexyl-isoxaxoline)-5',6-penicillanate] (4.53h)

Obtained from hydroximoyl chloride **4.48h** (67 mg, 0.412 mmol) and 6-(*Z*)-(benzoylmethylidene)penicillanate **4.50** (100 mg, 0.206 mmol) as described in the general procedure. The crude product was purified by flash chromatography (hexane/ethyl acetate, 3:1). The crude product was purified by flash chromatography (hexane/ethyl acetate, 3:1). Method A, gave, in order of elution, **4.51h** as a colourless solid (99 mg, 0.163 mmol, 79%) and a mixture of **4.52h/4.53h** as a colourless oil (13:87) (19 mg, 0.031 mmol, 15%). Method B, gave, in order of elution, **4.51h** as a colourless solid (75 mg, 0.123 mmol, 60%) and a mixture of **4.52h/4.53h** as a colourless oil (10:90) (13 mg, 0.021 mmol, 10%). Method C, gave, in order of elution, **4.51h** as a colourless solid (40 mg, 0.066 mmol, 35%) and a mixture of **4.52h/4.53h** (39:61) as a yellow oil (9 mg, 0.015 mmol, 8%).

Compound **4.51h**: mp 182.7-185.0 ºC; $[\alpha]_D^{25}$ = + 340 (*c* 0.25 in CH_2Cl_2); IR (ATR): ν = 882, 1154, 1176, 1450, 1495, 1597, 1668, 1746, 1773, 2856 and 2390 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.19 (s, 3H), 1.22-1.26 (m, 3H), 1.53 (s, 3H), 1.61-1.79 (m, 5H), 1.97-2.00 (m, 2H), 2.47 (tt, *J* = 11.4 and 3.3 Hz, 1H), 4.59 (s, 1H), 5.84 (s, 1H), 5.94 (s, 1H), 6.97 (s, 1H), 7.32-7.38 (m, 10H), 7.49-7.53 (m, 2H), 7.63 (t, *J* = 7.4 Hz, 1H), 7.99-8.01 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 25.8, 26.0, 26.0, 26.2, 31.4, 31.9, 32.7, 36.1, 63.6, 68.0, 70.0, 78.2, 78.7, 80.0, 127.2, 127.6, 128.5, 128.7, 128.8, 128.9, 129.0, 129.4, 134.3, 135.1, 139.1, 139.3, 160.8, 166.5, 170.7, 192.0; HRMS (ESI) m/z: Calcd for $C_{36}H_{37}N_2O_5S$ $[M+H]^+$ 609.2418; Found 609.2413.

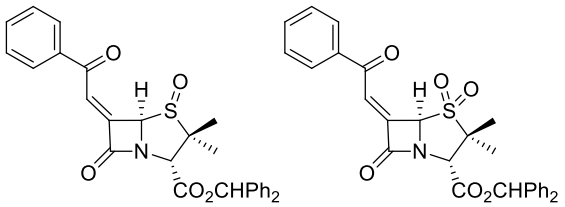
Recorded as a mixture of compounds **4.52h/4.53h** (10:90): Compound **4.52h**: ^1H NMR (400 MHz, $CDCl_3$): δ = 1.10 (s, 3H), 1.38 (s, 3H), 1.40-1.88 (m, 10H), 2.03-2.10 (m, 1H), 4.40 (s, 1H), 5.40 (s, 1H), 5.46 (s, 1H), 6.91 (s, 1H), 7.29-7.36 (m, 10H), 7.53 (t, *J* = 7.8 Hz, 2H), 7.66 (t, *J* = 7.4 Hz, 1H), 7.95-7.97 (m, 2H); Compound **4.53h**: ^1H NMR (400 MHz, $CDCl_3$): δ = 1.10 (s, 3H), 1.38 (s, 3H), 1.40-1.88 (m, 10H), 2.03-2.10

(m, 1H), 4.53 (s, 1H), 5.25 (s, 1H), 5.62 (s, 1H), 6.91 (s, 1H), 7.29-7.36 (m, 10H), 7.46-7.50 (m, 2H), 7.60 (t, *J* = 7.4 Hz, 1H), 8.02 (d, *J* = 7.2 Hz, 2H).

6.7. Synthesis of compounds of Chapter 5

6.7.1. General Procedure for the Oxidation of 6-Alkylidenepenicillanates

A mixture of the 6-alkylidenepenicillanate (0.100 g) and *m*-CPBA (3 equiv.) in dichloromethane (10 mL) was stirred at room temperature for the time indicated in each case (Method A: 0.5 h; Method B: 17 h; Method C: 24 h). Upon completion, the solution was washed twice with a 10% aqueous solution of Na_2SO_4 and the organic layer was extracted with dichloromethane. The combined organic extracts were dried, and the solvent evaporated off. The product was purified by flash chromatography.



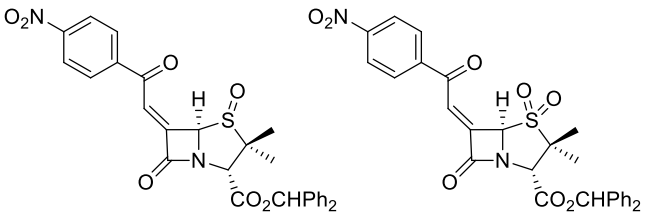
Benzhydryl 1-oxo-6-(*Z*)-(benzoylmethylidene)penicillanate (5.2a) and benzhydryl 1,1-dioxo-6-(*Z*)-(benzoylmethylidene)penicillanate (5.3a)

Prepared from 6-alkylidenepenicillanate **5.1a** (0.206 mmol, 0.100 g), as described in the general procedure. Purification of the crude product by flash chromatography (hexane/ethyl acetate, 2:1), gave, in order of elution, **5.3a** as a colourless solid (Method A: 21 mg, 0.040 mmol, 20%; Method B: 60 mg, 0.116 mmol, 56%; Method C: 67 mg, 0.130 mmol, 63%) and **5.2a** as a colourless solid (Method A: 77 mg, 0.154 mmol, 75%; Method B: 27 mg, 0.053 mmol, 27 %; Method C: 22 mg, 0.044 mmol, 21%).

Compound **5.2a**: mp 71.7–73.7 °C; $[\alpha]_D^{25}$ = + 360 (*c* 0.25 in CH_2Cl_2); IR (ATR): ν = 976, 1107, 1051, 1155, 1232, 1349, 1448, 1595, 1635, 1702, 1747, 1780 and 2972 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.02 (s, 3H), 1.69 (s, 3H), 4.84 (s, 1H), 5.81 (d, *J* = 0.9 Hz, 1H), 7.02 (s, 1H), 7.32-7.41 (m, 10H), 7.53 (t, *J* = 7.7 Hz, 2H), 7.65 (d, *J* = 7.4 Hz, 1H), 7.67 (d, *J* = 1.3 Hz, 1H), 7.99-8.00 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ =

18.3, 19.7, 66.8, 73.8, 78.9, 82.0, 119.8, 127.0, 127.9, 128.7, 128.8, 128.9, 129.0, 129.3, 140.0, 166.1, 167.4, 188.5; HRMS (ESI-TOF) m/z: $[M+Na]^+$ Calcd $C_{29}H_{25}NNaO_5S$ 522.1346, found 522.1340.

Compound **5.3a**: mp 88.5–90.5 °C; $[\alpha]_D^{25}$ = + 270 (*c* 0.5 in CH_2Cl_2); IR (ATR): ν = 976, 1006, 1118, 1168, 1232, 1327, 1149, 1636, 1752, 1787 and 2934 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.21 (s, 3H), 1.61 (s, 3H), 4.61 (s, 1H), 5.60 (d, *J* = 0.9 Hz, 1H), 7.02 (s, 1H), 7.33-7.39 (m, 10H), 7.53 (t, *J* = 7.8 Hz, 2H), 7.66 (t, *J* = 7.4 Hz, 1H), 7.73 (d, *J* = 1.3 Hz, 1H), 8.02-8.04 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 19.3, 19.9, 63.8, 64.6, 71.1, 79.2, 121.7, 126.9, 127.7, 128.4, 128.7, 128.8, 129.0, 129.1, 134.7, 136.1, 138.6, 138.8, 144.0, 166.0, 166.6, 187.1; HRMS (ESI-TOF) m/z: $[M+Na]^+$ Calcd $C_{29}H_{25}NNaO_6S$ 538.1295, found 538.1289.



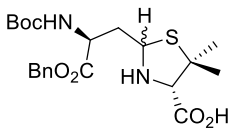
Benzhydryl 1-oxo-6-(*Z*)-(4-nitrobenzoylmethylidene)penicillanate (5.2b) and benzhydryl 1,1-dioxo-6-(*Z*)-(4-nitrobenzoylmethylidene)penicillanate (5.3b)

Prepared from 6-alkylidenepenicillanate **5.1e** (0.189 mmol, 0.100 g), as described in the general procedure. Purification of the crude product by flash chromatography (hexane/ethyl acetate, 2:1), gave, in order of elution, **5.3b** as a yellow solid (Method B: 56 mg, 0.100 mmol, 48%; Method C: 39 mg, 0.070 mmol, 37%) and **5.2b** as a pale-yellow solid (Method B: 32 mg, 0.059 mmol, 29%; Method C: 22 mg, 0.040 mmol, 21 %).

Compound **5.2b**: mp 83.7–85.7 °C; $[\alpha]_D^{25}$ = + 250 (*c* 0.5 in CH_2Cl_2); IR (ATR): ν = 1010, 1067, 1158, 1206, 1341, 1524, 1748, 1782 and 2929 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.03 (s, 3H), 1.70 (s, 3H), 4.85 (s, 1H), 5.81 (d, *J* = 0.7 Hz, 1H), 7.03 (s, 1H), 7.33-7.39 (m, 10H), 7.65 (d, *J* = 1.3 Hz, 1H), 8.16 (d, *J* = 8.9 Hz, 2H), 8.38 (d, *J* = 8.9 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ =18.2, 19.7, 67.0, 74.0, 79.1, 82.0, 118.8, 124.4, 127.0, 127.9, 128.5, 128.8, 128.9, 130.0, 138.9, 139.2, 140.9, 149.1, 151.1, 165.4, 167.2, 187.2; HRMS (ESI-TOF) m/z: $[M+Na]^+$ Calcd $C_{29}H_{24}N_2NaO_7S$ 567.1196, found 567.1191.

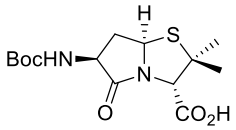
Compound **5.3b**: mp 94.4–96.4 °C; $[\alpha]_D^{25}$ = + 190 (*c* 0.5 in CH_2Cl_2); IR (ATR): ν = 1009, 1118, 1169, 1213, 1323, 1524, 1602, 1647, 1753, 1790 and 2931 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.21 (s, 3H), 1.62 (s, 3H), 4.63 (s, 1H), 5.59 (d, *J* = 0.9 Hz, 1H), 7.02 (s, 1H), 7.33-7.39 (m, 10H), 7.70 (d, *J* = 1.3 Hz, 1H), 8.19 (d, *J* = 8.9 Hz, 2H), 8.39 (d, *J* = 8.9 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ =18.5, 20.0, 64.1, 64.9, 71.2, 79.5, 120.7, 124.5, 127.0, 127.8, 128.6, 128.9, 129.0, 130.2, 138.6, 138.9, 140.3, 146.1, 151.2, 166.0, 166.0, 186.2; HRMS (ESI-TOF) m/z: $[M+Na]^+$ Calcd $C_{29}H_{24}N_2NaO_8S$ 583.1146, found 583.1138.

6.7.2. Procedures for the Synthesis of Diazo-γ-lactam



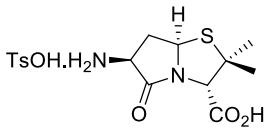
(2*S*,4*R*)- and (2*S*,4*S*)-2-((*S*)-3-(Benzyloxy)-2-((*tert*-butoxycarbonyl)amino)-3-oxopropyl)-5,5-dimethylthiazolidine-4-carboxylic acid (5.6)

To a solution of *D*-penicillamine (73 mg, 0.49 mmol) in methanol (10 mL), aldehyde **5.5** (200 mg, 0.65 mmol) was added. After stirring 18 h at room temperature, the solvent was removed under reduced pressure and petroleum ether was added. The product was filtered and **5.6** was obtained as a colourless solid (199 mg, 0.453 mmol, 92%). The ^1H NMR spectrum showed the presence of two diastereoisomers (ratio 64:36). mp 87.6-89.1 ºC; IR (ATR): ν = 1157, 1366, 1686, 1702, 1734 and 2973 cm^{-1}; *Major isomer*: ^1H NMR (400 MHz, CD_3OD): δ = 1.33 (s, 3H), 1.44 (s, 9H), 1.66 (s, 3H), 2.25-2.37 (m, 2H), 3.64 (s, 1H), 4.21 (dd, *J* = 9.6 and 4.8 Hz), 4.66 (dd, *J* = 8.0 and 5.6Hz), 5.14-5.23 (m, 2H), 7.36-7.39 (m, 5H); HRMS (ESI-TOF) *m/z*: Calcd for $C_{21}H_{31}N_2O_6S$ $[M+H]^+$ 439.18828; found 439.18973.



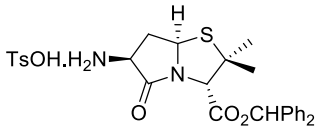
(3*S*,6*S*,7a*R*)-6-((*tert*-Butoxycarbonyl)amino)-2,2-dimethyl-5-oxohexahydropyrrolo[2,1-*b*]thiazole-3-carboxylic acid (5.7)

A solution of thiazolidine **5.7** (3.16 g, 7.21 mmol) in toluene (150 mL) was refluxed over 24 h under N_2 atmosphere. The solvent was removed under reduced pressure and diethyl ether was added. Compound **5.7** precipitates as a colourless solid (1.97 g, 5.96 mmol, 83%). mp decomposes at 225 ºC; $[\alpha]_D^{25}$ = +145 (*c* 1 in MeOH); IR (ATR): ν = 1161, 1279, 1513, 1671, 1708, 2961 and 3399 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.39 (s, 9H), 1.45 (s, 3H), 1.53 (s, 3H), 2.00-2.08 (m, 1H), 2.75-2.81 (m, 1H), 4.31 (s, 1H), 4.49-4.56 (m, 1H), 5.31 (t, *J* = 8 Hz, 1H), 7.30 (d, *J* = 12 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 30.9, 33.4, 36.4, 41.9, 58.7, 62.1, 66.5, 72.4, 83.4, 160.3, 174.6, 176.1; HRMS (ESI-TOF) *m/z*: Calcd for $C_{14}H_{23}N_2O_5S$ $[M+H]^+$ 331.13186, found 331.13222.



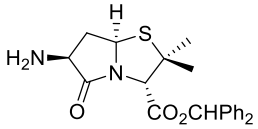
(3*S*,6*S*,7a*R*)-3-Carboxy-2,2-dimethyl-5-oxohexahydropyrrolo[2,1-*b*]thiazol-6-aminium 4-methylbenzenesulfonate (5.8)

To a solution of lactam **5.7** (2.23 g, 6.75 mmol) in acetonitrile (34 mL), *p*-toluenesulfonic acid monohydrate (2.57 g, 13.50 mmol) was added. The reaction mixture was stirred overnight at room temperature. Compound **5.8** precipitates as a colourless solid which was filtered and washed with cold acetonitrile (2.69 g, 6.68 mmol, 99%). mp decomposes at 253 ºC; $[\alpha]_D^{25}$ = +130 (*c* 0.5 in MeOH); IR (ATR): ν = 813, 1006, 1124, 1152, 1224, 1411, 1517, 1617, 1707, 2931 and 3412 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.46 (s, 3H), 1.55 (s, 3H), 2.04 (dd, *J* = 11.6 and 3.4 Hz, 1H), 2.29 (s, 3H), 2.96-3.01 (m, 1H), 4.37 (s, 1H), 4.49 (dd, *J* = 11.4 and 8.0 Hz, 1H), 5.42 (dd, *J* = 8.0 and 5.8 Hz, 1H), 7.11 (d, *J* = 7.8 Hz, 2H), 7.47 (d, *J* = 8.0 Hz, 1H), 8.49 (br s, N*H*$_3$); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 20.8, 25.7, 31.1, 35.6, 52.0, 57.4, 62.2, 67.0, 125.5, 128.1, 137.6, 145.8, 168.2, 168.9; Anal. Calcd for $C_{16}H_{22}N_2O_6S_2$: C, 47.75; H, 5.51; N, 6.96; S, 15.93. Found: C, 47.47; H, 5.28; N, 6.85; S, 15.01.



(3*S*,6*S*,7a*R*)-3-((Benzhydryloxy)carbonyl)-2,2-dimethyl-5-oxohexahydropyrrolo[2,1-*b*]thiazol-6-aminium 4-methylbenzenesulfonate (5.9)

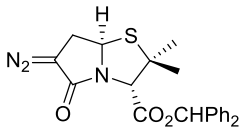
To a stirred solution of compound **5.8** (1 g, 2.49 mmol) in methanol (2.5 mL) a solution of diphenyldiazomethane (483 mg, 2.49 mmol) in dichloromethane (10 mL) was added dropwise. After 24 h at room temperature, an additional portion of diphenyldiazomethane (242 mg, 1.25 mmol) was added and the reaction mixture was stirred for further 20 h. The solvent was removed under reduced pressure and diethyl ether was added. Compound **5.9** precipitates as a beige solid (1.34 g, 2.36 mmol, 95%). mp 117.3-118.7 ºC; $[\alpha]_D^{25}$ = –120 (*c* 0.5 in MeOH); IR (ATR): ν = 812, 1007, 1033, 1123, 1154, 1363, 1420, 1495, 1717 and 2964 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.11 (s, 3H), 1.32 (s, 3H), 2.28 (s, 3H), 2.80-2.86 (m, 1H), 4.25 (br s, 1H), 4.56 (s, 1H), 5.17 (dd, *J* = 6.0 and 7.9 Hz, 1H), 6.87 (s, 1H), 7.05 (d, *J* = 7.8 Hz, 2H), 7.21-7.31 (m, 10H), 7.73 (d, *J* = 8.0 Hz, 1H), 8.37 (br s, N*H*$_3$); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 21.5, 25.7, 30.8, 35.8, 53.1, 58.2, 62.6, 67.2, 78.7, 126.4, 127.0, 127.8, 128.2, 128.5, 128.7, 128.7, 129.1, 139.3, 139.4, 140.5, 141.3, 167.0, 168.5; HRMS (ESI-TOF) *m/z*: Calcd for $C_{22}H_{25}N_2O_6S$ $[M\text{-}TsO]^+$ 397.1580, found 397.1577.



(3*S*,6*S*,7a*R*)-Benzhydryl 6-amino-2,2-dimethyl-5-oxohexahydropyrrolo[2,1-*b*]thiazole-3-carboxylate (5.10)

To a solution of compound **5.9** (300 mg, 0.528 mmol) in dichloromethane (16 mL) a saturated aqueous solution of $NaHCO_3$ (16 mL) was added. The reaction mixture was stirred for 30 min at room temperature. The organic phase was separated off, and the aqueous phase was extracted with dichloromethane. The organic extracts were combined, dried with anhydrous Na_2SO_4 and concentrated under reduced pressure to give compound **5.10** as a yellowish oil (201 mg, 0.507 mmol, 96%); $[\alpha]_D^{25}$ = +128.75 (*c* 4 in CH_2Cl_2); IR (ATR): ν = 732, 976, 1172, 1264, 1399, 1450, 1495, 1705, 1735, 2976 and 3377 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ = 1.24 (s, 3H), 1.52 (s, 3H), 1.73-1.81 (m, 1H), 1.99 (br

s, N*H2*), 2.84-2.90 (m, 1H), 3.80 (dd, *J* = 11.2 and 8.0 Hz, 1H), 4.58 (s, 1H), 5.34 (dd, *J* = 8.0 and 6.0 Hz, 1H), 6.91 (s, 1H), 7.30-7.46 (m, 10H); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 25.4, 30.7, 39.8, 54.9, 57.4, 61.7, 66.9, 77.5, 126.4, 126.9, 127.9, 128.1, 128.3, 128.5, 128.5, 139.8, 139.9, 167.1, 175.2; HRMS (ESI-TOF) *m/z*: Calcd for $C_{22}H_{25}N_2O_3S$ [M+H$^+$] 397.15757, found 397.15804.



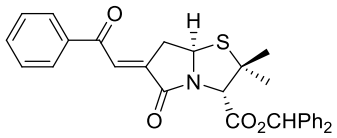
(3*S*,7a*R*)-Benzhydryl 6-diazo-2,2-dimethyl-5-oxohexahydropyrrolo[2,1-*b*]thiazole-3-carboxylate (5.11)

To an ice-cold solution of freshly prepared amino-γ-lactam **5.10** (250 mg, 0.63 mmol) in dichloromethane (40 mL), cold water (40 mL) was added followed by 1M $HClO_4$ (1.3 mL) and $NaNO_2$ (109 mg, 1.58 mmol). The reaction mixture was stirred at 0 ºC for 1 h. The organic phase was separated off, and the aqueous phase was extracted with dichloromethane. The combined organic extracts were washed with cold saturated NaCl, dried (Na_2SO_4) and concentrated under reduced pressure (no heat) to give compound **5.11** as a yellow oil (249 mg, 0.611 mmol, 97%). IR (ATR): ν = 696, 1157, 1262, 1701 and 3286 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.28 (s, 3H), 1.56 (s, 3H), 3.20 (dd, *J* = 14.4 and 2.0 Hz, 1H), 3.54 (dd, *J* = 14.4 and 7.6 Hz, 1H), 4.85 (s, 1H), 5.59 (dd, *J* = 7.6 and 1.6 Hz, 1H), 6.94 (s, 1H), 7.29-7.35 (m, 10H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 26.3, 26.5, 33.1, 52.3, 53.4, 58.8, 64.4, 69.9, 78.3, 126.9, 127.7, 128.1, 128.4, 128.6, 128.6, 139.2, 168.0, 170.7.

6.7.3. General Procedure for the Synthesis of Alkylidene-γ-lactams

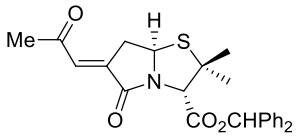
Starting from tosylate salt **5.9** (1.00 g, 1.758 mmol), diazo-γ-lactam **5.11** was prepared following the above-mentioned protocols and used without further purification. Rhodium acetate dimer (4×10^{-3} equiv., 7.0×10^{-3} mmol) was dissolved in toluene (25 mL) in a two-neck round bottom flask under inert atmosphere. Next, propylene oxide (99 equiv., 174 mmol, 13.8 mL) was added dropwise followed by a dropwise addition of a solution of the previously synthesized diazo-γ-lactam **5.11** in toluene (25 mL). The reaction mixture was stirred for 15 min and concentrated under reduced pressure.

Benzhydryl 6-oxopenicillinate **5.12** was used without further purification. Benzhydryl 6-oxopenicillinate **5.12** was dissolved in dichloromethane (10 mL), the solution was cooled to –55 °C under nitrogen, and the appropriate phosphorus ylide (1.67 mmol) in dichloromethane (20 mL) was added dropwise. Stirring was continued for 15 min, then the solution was warmed to room temperature and washed with water (20 mL). The organic layer was separated, dried (Na_2SO_4), and concentrated under reduced pressure. The products were purified by flash chromatography. The presented yields were calculated from tosylate salt **5.9**.



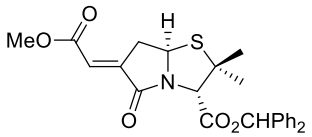
(3*S*,7a,*R*,*E*)-Benzhydryl 2,2-dimethyl-5-oxo-6-(2-oxo-2-phenylethylidene)hexahydropyrrolo[2,1-*b*]thiazole-3-carboxylate (5.13a)

Prepared from 6-oxopenicillanate (1.758 mmol) and the corresponding phosphorus ylide (0.632 g, 1.67 mmol), as described in the general procedure. After purification by flash chromatography (hexane/ethyl acetate, 3:1), compound **5.13a** was obtained as a pale-yellow solid (246 mg, 0.494 mmol, 28%). mp 52.5–54.5 °C; $[\alpha]_D^{25}$ = + 220 (*c* 0.5 in CH_2Cl_2); IR (ATR): ν = 1169, 1254, 1364, 1448, 1496, 1670, 1702, 1738 and 2964 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.32 (s, 3H), 1.59 (s, 3H), 3.37 (dt, *J* = 21.0 and 2.9 Hz, 1H), 3.80 (ddd, *J* = 21.0, 6.9 and 2.8 Hz, 1H), 4.85 (s, 1H), 5.63 (dd, *J* = 6.9 and 2.5 Hz, 1H), 6.99 (s, 1H), 7.34-7.38 (m, 10H), 7.51 (t, *J* = 7.6 Hz, 2H), 7.61 (t, *J* = 7.4 Hz, 1H), 7.79 (t, *J* = 3.0 Hz, 2H), 8.02-8.04 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 26.5, 31.8, 33.6, 59.5, 64.4, 68.6, 78.5, 123.1, 127.0, 127.8, 128.2, 128.4, 128.6, 128.6, 128.8, 133.6, 137.8, 139.2, 147.2, 167.4, 168.3, 190.4; HRMS (ESI-TOF) m/z: $[M+Na]^+$ Calcd $C_{30}H_{27}NNaO_4S$ 520.1553, found 520.1544.



(3*S*,7a*R*,*E*)-benzhydryl 2,2-dimethyl-5-oxo-6-(2-oxopropylidene)hexahydropyrrolo[2,1-*b*]thiazole-3-carboxylate (5.13b)

Prepared from 6-oxopenicillanate (1.758 mmol) and the corresponding phosphorus ylide (0.531 g, 1.67 mmol), as described in the general procedure. After purification by flash chromatography (hexane/ethyl acetate, 3:1), compound **5.13b** was obtained as a colourless solid (121.1 mg, 0.278 mmol, 16%). mp 115.1–117.1 °C; $[\alpha]_D^{25}$ = + 250 (*c* 0.5 in CH_2Cl_2); IR (ATR): ν = 943, 966, 1155, 1169, 1216, 1269, 1357, 1401, 1492, 1637, 1686 and 1756 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.30 (s, 3H), 1.56 (s, 3H), 2.35 (s, 3H), 3.21 (dt, *J* = 21.1 and 2.9 Hz, 1H), 3.65 (ddd, *J* = 21.0, 6.9 and 2.8 Hz, 1H), 4.80 (s, 1H), 5.58 (dd, *J* = 6.9 and 2.6 Hz, 1H), 6.97 (s, 1H), 7.00 (t, *J* = 3.0 Hz, 1H), 7.30-7.36 (m, 10H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 26.6, 31.9, 32.0, 33.4, 59.6, 64.4, 68.7, 78.7, 126.2, 127.1, 127.9, 128.3, 128.6, 128.7, 128.8, 139.3, 145.3, 167.5, 168.4, 198.5; HRMS (ESI-TOF) m/z: $[M+Na]^+$ Calcd $C_{25}H_{25}NNaO_4S$ 458.1397, found 458.1389.



(3*S*,7a*R*,*E*)-Benzhydryl 6-(2-methoxy-2-oxoethylidene)-2,2-dimethyl-5-oxohexahydropyrrolo[2,1-*b*]thiazole-3-carboxylate (5.13c)

Prepared from 6-oxopenicillanate (1.758 mmol) and the corresponding phosphorus ylide (0.558 g, 1.67 mmol), as described in the general procedure. After purification by flash chromatography (hexane/ethyl acetate, 3:1), compound **5.13c** was obtained as a yellow solid (219.3 mg, 0.485 mmol, 27%). mp 165.1–167.1 °C; $[\alpha]_D^{25}$ = + 220 (*c* 0.5 in CH_2Cl_2); IR (ATR): ν = 956, 1155, 1173, 1211, 1259, 1357, 1438, 1458, 1497, 1703, 1706 and 1745 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.30 (s, 3H), 1.56 (s, 3H), 3,21-3,28 (dt, *J* = 20.6 and 3.0 Hz, 1H), 3.63-3.71 (ddd, *J* = 20.6, 7.0 and 2.8 Hz, 1H), 3.79 (s, 3H), 4.81 (s, 1H), 5.59 (dd, *J* = 7.0 and 2.7 Hz, 1H), 6,68 (t, *J* = 3.0 Hz, 1H), 6,97 (s, 1H), 7.28-7.36 (m, 10H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 26.6, 32.0, 33.0, 52.1, 60.0, 64.2, 68.7, 78.7, 121.4, 127.1, 127.9, 128.3, 128.6, 128.7, 128.8, 139.3, 147.6,

166.3, 167.5, 167.6 HRMS (ESI-TOF) m/z: $[M+Na]^+$ Calcd $C_{25}H_{25}NNaO_5S$ 474.1346, found 474.1338.

6.8. Spectroscopic and BSA-binding Studies

A 2 mL solution of 1.5 μM BSA in phosphate buffered saline pH ~ 7.4 was titrated with a 3 mM spiro-β-lactam solution in DMSO. The final protein concentration was 1.5 μM, and the drug concentrations were 0, 1.5, 3, 6, 9, 15, 18, 22.5, 30, 37.5, 45, 52.5, 67.5, 90 and 150 μM. The mixtures were mixed to ensure the formation of a homogeneous solution. Fluorescence spectroscopic studies were performed using a Horiba-Jobin-Yvon Fluorolog 3.22 using a 1 cm quartz cuvette. Fluorescence spectra were corrected for the wavelength response of the system with the appropriate correction files obtained for the instrument. For the spectral determinations, λem was set in the range 270–550 nm succeeding the excitation at λex 280 nm with the excitation and emission slit widths fixed to 2 nm. Emission fluorescence were logged at two different temperatures (298 and 310 K) to monitor the quenching effect for the spiro-β-lactams.

6.9 Biological Evaluation

6.9.1. Anti-HIV Activity

Cell Lines

TZM-bl cells (AIDS Research and Reference Reagent Program, National Institutes of Health, USA) were cultured in complete growth medium that consists of Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin-streptomycin (Gibco/Invitrogen, USA), 1 mM of sodium pyruvate (Gibco/Invitrogen, USA), 2 mM of *L*-glutamine (Gibco/Invitrogen, USA) and 1 mM of non-essential amino acids (Gibco/Invitrogen, USA). Cell cultures were maintained at 37ºC in 5% CO_2.

Cellular Viability Assays

The *in vitro* cytotoxicity of test compounds was evaluated in TZM-bl cells using alamarBlue cell viability reagent (Life Technologies, USA). Cells were cultured in the presence and absence of serial-fold dilutions of the test compounds. Each dilution of each compound was performed in triplicate wells. For each assay we had medium controls (only growth medium), cell controls (cells without test compound) and cytotoxicity controls (a compound that kill cells). The cytotoxicity of each test compound was expressed by the 50% cytotoxic concentration (CC_{50}), which is the concentration of compound causing 50% decrease of cellular viability.

Viruses and Titration

The primary isolates of HIV-1 used in this study were previously described and characterized for co-receptor usage.[20] The HIV-1 SG3.1 subtype B lab-adapted strain was obtained by transfection of HEK293T cells with pSG3.1 plasmid using jetPrime transfection reagent (Polyplus-tranfection SA, Illkirch, France) according to manufacturer's instructions. The 50% tissue culture infectious dose ($TCID_{50}$) of each virus was determined in a single-round viral infectivity assay using a luciferase reporter gene assay in TZM-bl cells[20] and calculated using the statistical method of Reed and Muench.

Antiviral Assays

The antiviral activity of test compounds was determined in a single-round viral infectivity assay using TZM-bl reporter cells, as previously described.[20-21] Briefly, TZM-bl cells were infected with 200 $TCID_{50}$ of HIV-1 or HIV-2 in the presence of serial fold dilutions of the compounds in growth medium, supplemented with DEAE-dextran. After 48 h of infection, luciferase expression was quantified with Pierce Firefly Luc One-Step Glow Assay Kit (ThermoFisher Scientific, Rockford, USA) according to the manufacturer's instructions. For each virus and compound dilution, the assay was set up in triplicate wells. Virus controls, cell controls and inhibitors controls (drugs with a known action in each virus) were used.

Statistical Analysis

Statistical analysis was performed using Prism version 5.01 for Windows (GrahPad Software, San Diego, California USA, www.graphpad.com) with a level of significance of 5%.

6.9.2. Anti-*Plasmodium* Activity

Anti-Plasmodial Activity Against *P. berghei* Hepatic Stage

The inhibition of hepatic stage *Plasmodium* infection by test compounds was assessed by measuring the luminescence intensity of Huh-7 cells infected with a firefly luciferase-expressing *P. berghei* sporozoites (Pb-Luc), as previously described.[22] Briefly, Huh-7 cells, a human hepatoma cell line, were cultured in 1640 RPMI medium supplemented with 10% v/v fetal bovine serum, 1% v/v nonessential amino acids, 1% v/v penicillin/streptomycin, 1% v/v glutamine, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7, and maintained at 37 °C with 5% CO_2.

For infection assays, 1.0×10^4 Huh-7 cells/well were seeded in 96-well plates the day before drug treatment and infection. Serial dilutions of each compound were then prepared in infection medium. On the day of infection, the culture medium was replaced by the appropriate compound concentration and incubated for 1 h at 37 ºC with 5% CO_2. Next, 1.0×10^4 firefly luciferase-expressing *P. berghei* sporozoites, freshly obtained through disruption of salivary glands of infected female *Anopheles stephensi* mosquitoes, were added to each well. An amount of the DMSO solvent equivalent to that present in the highest compound concentration was diluted in infection medium and used as control. Sporozoite addition was followed by centrifugation at 1700×g for 5 min and subsequent incubation for 48 h at 37 ºC with 5% CO_2. The effect of the compounds on the viability of Huh-7 cells was assessed by the Alamar Blue assay (Invitrogen, U.K.) according to the manufacturers protocol, followed by measurement of parasite infection load by a bioluminescence assay (Biotium, USA). All compounds were initially screened at 1 and 10 μM, and the IC_{50} of the most active compounds was determined by evaluating the activity of the drug at seven different concentrations ranging from 0.1 to 10 μM. Nonlinear regression analysis was employed to fit the normalized results of the dose-

response curves, and IC_{50} values were determined using Prism version 5.0 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com).

Anti-Plasmodial Activity Against *P. falciparum* Blood Stage

Ring-stage synchronized cultures of *P. falciparum* strain NF54 at 2.5% hematocrit and at approximately 1% parasitemia were incubated with test compounds or dimethyl sulfoxide (DMSO, vehicle control) in 96-well plates, for 48 h, at 37 °C in a 5% CO_2 and 5% O_2 atmosphere. Stock solutions of chloroquine (positive control) and spiro-β-lactams were prepared in DMSO. Working solutions were prepared from the stock solutions in complete malaria culture medium (CMCM), which consists of RPMI 1640 supplemented with 25 mM HEPES, 2.4 mM l-glutamine, 50 μg/mL gentamicin, 0.5% w/v Albumax, 11 mM glucose, 1.47 mM hypoxanthine, and 37.3 mM $NaHCO_3$. For each measurement, 5 μL of the culture (approximately 800 000 cells) were stained with the DNA-specific dye SYBR green I. After 20 min of incubation in the dark, the samples were analyzed by flow cytometry. Approximately 100 000 events were analyzed in each flow cytometry measurement. Two independent experiments were performed, and all samples were analyzed in triplicate.

6.9.3 Anti-Cancer Activity

Cell Culture

The A375 human melanoma cell line, the HT-1080 human fibrosarcoma cell line and the H1299 human lung cancer cell line were cultured in Dulbecco's Modified Eagle medium (DMEM, Sigma D-5648) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma F7524), 1% Penicillin-Streptomycin (100 U/mL penicillin and 10 μg/mL streptomycin, Gibco 15140-122) and 100 μM sodium pyruvate (Gibco Invitrogen Life Technologies; Gibco 1360). The OE19 esophageal carcinoma cell line was cultured in Roswell Park Memorial Institute 1640 medium (RPMI 1640, Sigma R4130), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma F7524), 1% penicillin-streptomycin (100 U/mL penicillin and 10 mg/mL streptomycin, Gibco 15140-122), and 400 mM sodium pyruvate (Gibco Invitrogen Life Technologies;

Gibco 1360). All cell lines were kept at 37 °C, in a humidified incubator with 95% air and 5% CO_2. For all studies, cells were detached using a solution of 0.25% trypsin-EDTA (Gibco).

Cytotoxicity

The cells were plated in 48-well culture plates at a density of 1×105 cells per well and incubated overnight to allow cell attachment. Stock solutions at 10 mM of all compounds were prepared in dimethylsulphoxide (DMSO, Sigma Aldrich®, EUA). Cells were then incubated in the presence of serial-fold dilutions of the compounds for 48 or 72 h. Each dilution of each compound was performed in triplicate wells. Cell controls (cells without test compound), and vehicle controls (cells with 1% DMSO) were included in each assay. The metabolic activity was determined by MTT colorimetric assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; MTT, Sigma Aldrich®, EUA). Briefly, cell cultures were washed with phosphate saline buffer (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4.2H_2O$, 2.0 mM KH_2PO_4; pH = 7.4) and incubated with 0.5 mg mL^{-1} MTT (Sigma Aldrich®, EUA), pH = 7.4, at 37 °C for 4 h. Then, formazan crystals were dissolved in acid isopropanol (0.04 M 37% hydrochloric acid in isopropanol, Sigma Aldrich®, EUA). Absorbance was measured using an EnSpire Multimode Plate Reader (PerkinElmer). The results allowed to establish dose–response curves and to calculate IC_{50} values, the concentration required to inhibit cell proliferation by 50%.

Types of Cell Death

For types of cell death analysis, the cells were plated in 6-well culture plates at a density of 5×10^5 cells per well and incubated overnight to allow attachment and then were treated with compounds **5.1l**, **5.1m**, **5.1o** and **5.2a** at 5 and 10 μM. Then, cells were collected, centrifuged and washed with PBS prior to incubation with binding buffer, 2.5 μL An-V–FITC and 1 μL propidium iodide (kit Immunotech) for 15 min, at 4 °C in the dark. 400 μL PBS were added and samples were analyzed in a FACSCalibur cytometer (BD Biosciences, EUA). Excitation was set at 488 nm, and the emission filters were set at for An-V–FITC and propidium iodide, respectively.

Cellular Morphology

Morphological characteristics were evaluated by optical microscopy (Nikon Eclipse NI optical microscope, Nikon Instruments, Amsterdam, Netherlands) after staining with May-Grünwald Giemsa medium (Sigma 32856). The photographs were obtained from a Nikon OS-Fi2 camera (Nikon Metrology Inc, Irvine, CA, USA) with magnifications of 50. The representative images of each condition were taken using the NIS-Elements D software (version 4.00).

Cell Cycle Analysis

For analysis of DNA content, 5×10^5 cells were incubated overnight to allow cell attachment and then were treated with compounds **5.1l**, **5.1m**, **5.1o** and **5.2a** at 5 and 10 μM. Cells were collected, centrifuged, and fixed with ice-cold ethanol (70%) for 30 min in the dark. Then, cells were washed with PBS and incubated with PI/RNase solution (Immunostep) for 15 min at rt. Cells were analyzed in the FACSCalibur cytometer with an excitation wavelength of 488 nm, and emission filter at of 585/42.

Production of Reactive Oxygen Species

For analysis of reactive oxygen species, 5×10^5 cells were incubated overnight to allow cell attachment and then were treated with compounds **5.1l**, **5.1m**, **5.1o** and **5.2a** at 5 and 10 μM. Production of intracellular peroxides was determined by incubation of 5 μM of 2′,7′-dichlorodihydrofluorescein diacetate (DCFH2-DA, Invitrogen Life technologies, D399) probe, for 45 min in the dark at 37 °C. After washing, detection was performed with the excitation and emission wavelengths of 485 and 528 nm, respectively. The production of superoxide anion was evaluated using 2 μM dihydroethidium (DHE, Sigma D7008) probe, incubated during 10 min with the cells, at room temperature in the dark. Reading was performed using the excitation and emission wavelengths of 530 and 645 nm, respectively.

Gelatin Zymography

Total protein extracts were prepared on ice using a solution of radioimmunoprecipitation assay (RIPA) buffer, and protein content was determined by

bicinchoninic acid method (Pierce, 23250). Equal amounts of protein were incubated in Gelatin-containing polyacrylamide gels. 7.5% SDS-polyacrylamide separating gels with 4 mg/mL of gelatin (30% bis-acrylamide, 1.5 M tris-HCL pH 8.8, 10% ammonium persulfate, TEMED, 10% SDS and distilled water to reach final volume) and 5% SDS-polyacrylamide staking gels (30% bis-acrylamide, 0.5 M tris-HCL pH 6.8, 10% ammonium persulfate, TEMED, 10% SDS and distilled water to reach final volume) were prepared. The gels were loaded with samples diluted in 5x non-reducing buffer (25% 0.5 M Tris-HCl pH 6.8, 20% glycerol, 4% SDS, and 0.01% bromophenol blue) and the electrophoresis was carried using a constant electric potential of 90 V until satisfactory separation of interest bands. Gels were washed twice on washing buffer (2.5% Triton X-100, 5% 1 M Tris-HCl pH 7.5, 0.25% 2 M $CaCl_2$, 0.001% 0.1 M $ZnCl_2$) for 30 minutes, washed for 10 minutes on incubation buffer (1% Triton X-100, 5% 1 M Tris-HCl pH 7.5, 0.25% 2 M $CaCl_2$, 0.001% 0.1 M $ZnCl_2$) and incubated on incubation buffer for 24 hours at 37 ºC. The gels were stained by using Coomassie Blue staining solution (with 40% methanol and 10% acetic acid) for 0.5-1 h, and destained with a 40% methanol and 10% acetic acid solution. The active form of MMP-9 was visualized on gels. MMP-9 activity was quantified densitometrically with ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis

Statistical analysis was performed using Prism version 5.01 for Windows (GrahPad Software, San Diego, California USA, www.graphpad.com) with a level of significance of 5%. The 50% (IC_{50}) inhibitory concentrations, as well as the dose–response curve slopes, were estimated by plotting the percent metabolic activity (y axis) against the $\log_{10}$ concentration of each compound (x axis) and using the sigmoidal dose–response (variable slope) equation. The normality of the distribution of each quantitative variable was evaluated according to the Shapiro-Wilk test, with the aim of determining the use of parametric or non-parametric tests. The normalized values (superoxide anion, peroxides) of the experimental conditions were compared with the respective normalization value using the Kruskal-Wallis test with the correction of P value for multiple comparisons. The rest of the cellular studies (cell death and cell cycle) and zymography assays were compared with ANOVA test in cases where normal distribution

and homogeneity of variances were verified or with the Kruskal-Wallis test with the correction of P value for multiple comparisons in the otherwise.

6.10. Computational Methodology

Quantum chemical calculations were carried out in order to explore the structure and the preferred conformations of molecules **3.43aa** and **3.44aa**. The structures were optimized at the Density Functional (DFT) level of theory, using the B3LYP hybrid functional[23] and the standard 6-31G(*d*) basis set. All calculations were performed using the GAMESS program package[24] and graphical representations were produced with GaussView. Energy values and Cartesian coordinates are given in Annexes.

6.11. References

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APPENDICES

Appendices

Appendix 1. Copies of ^{1}H, ^{13}C RMN, ^{19}F NMR and Selected 2D NMR Spectra for New Compounds

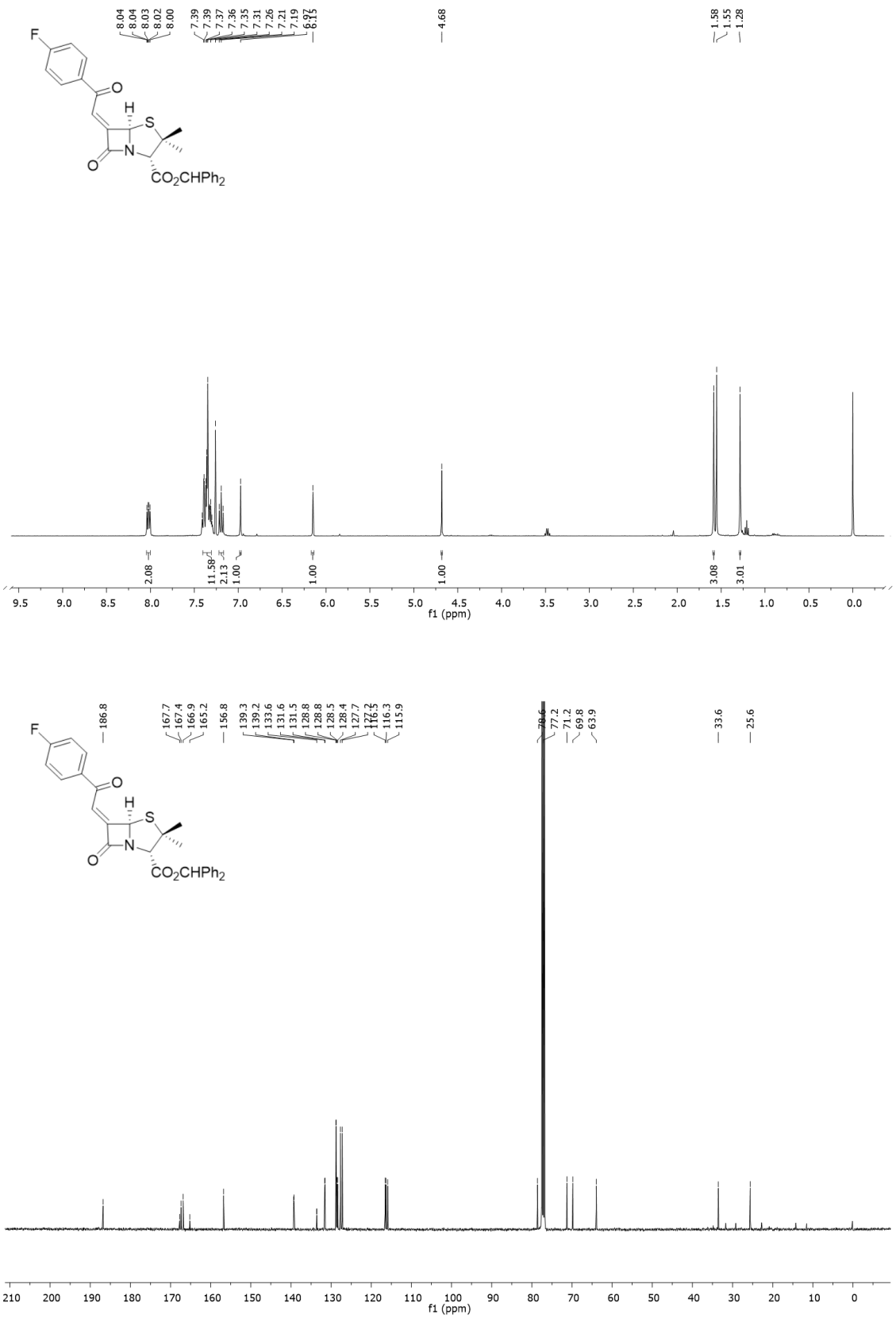


Figure S1: ^{1}H and ^{13}C NMR spectra of compound **2.20a (**$CDCl_3$).

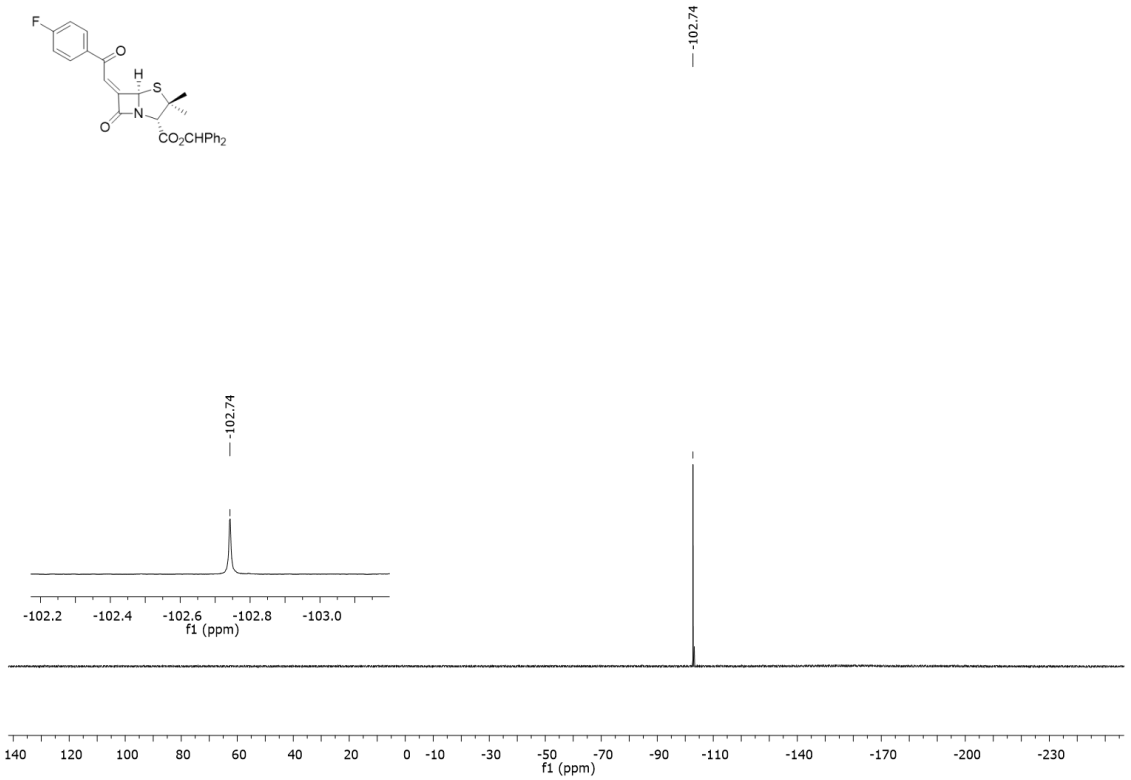


Figure S2: ^{19}F spectrum of compound **2.20a (**$CDCl_3$).

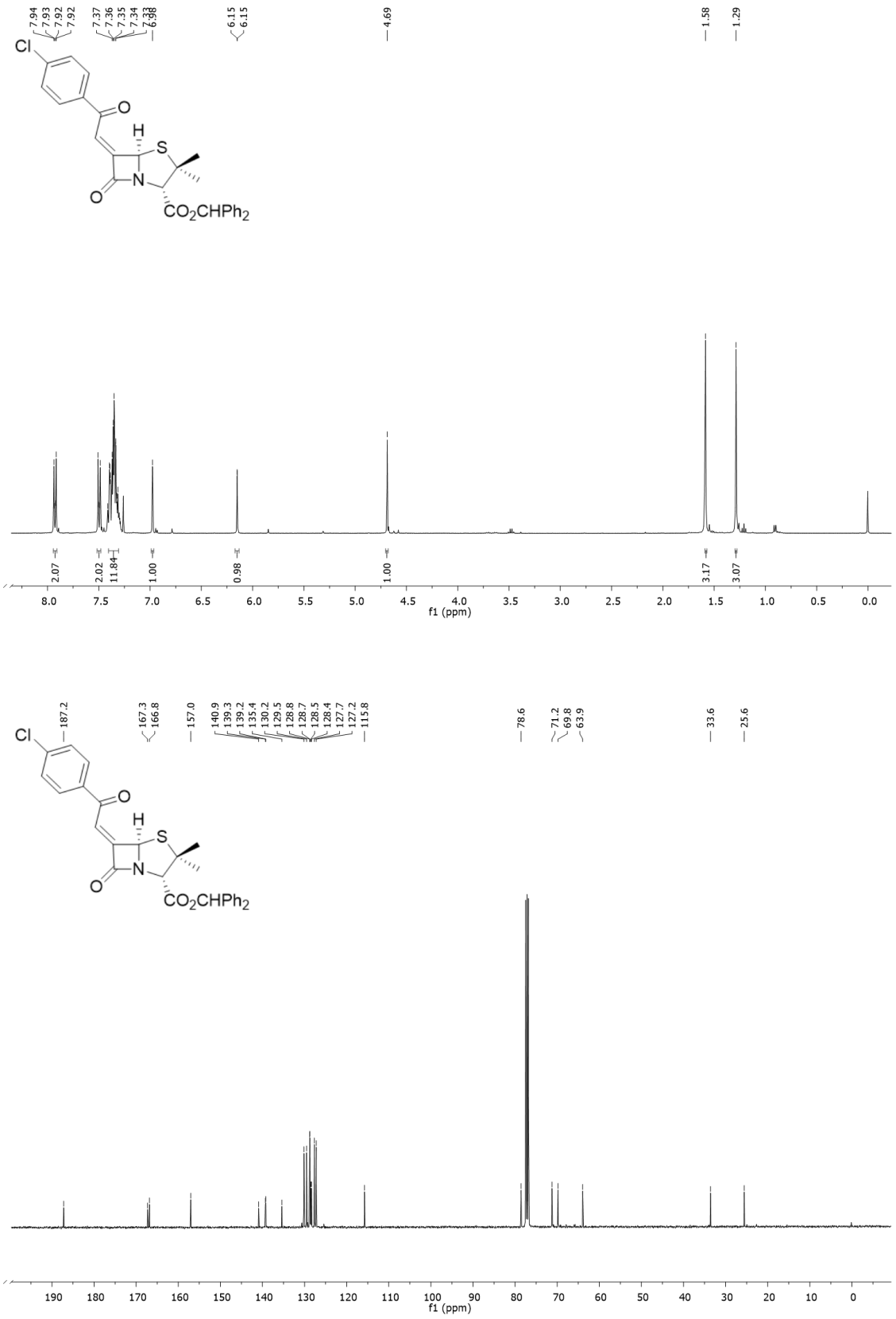


Figure S3: ^{1}H and ^{13}C NMR spectra of compound **2.20b (**$CDCl_3$).

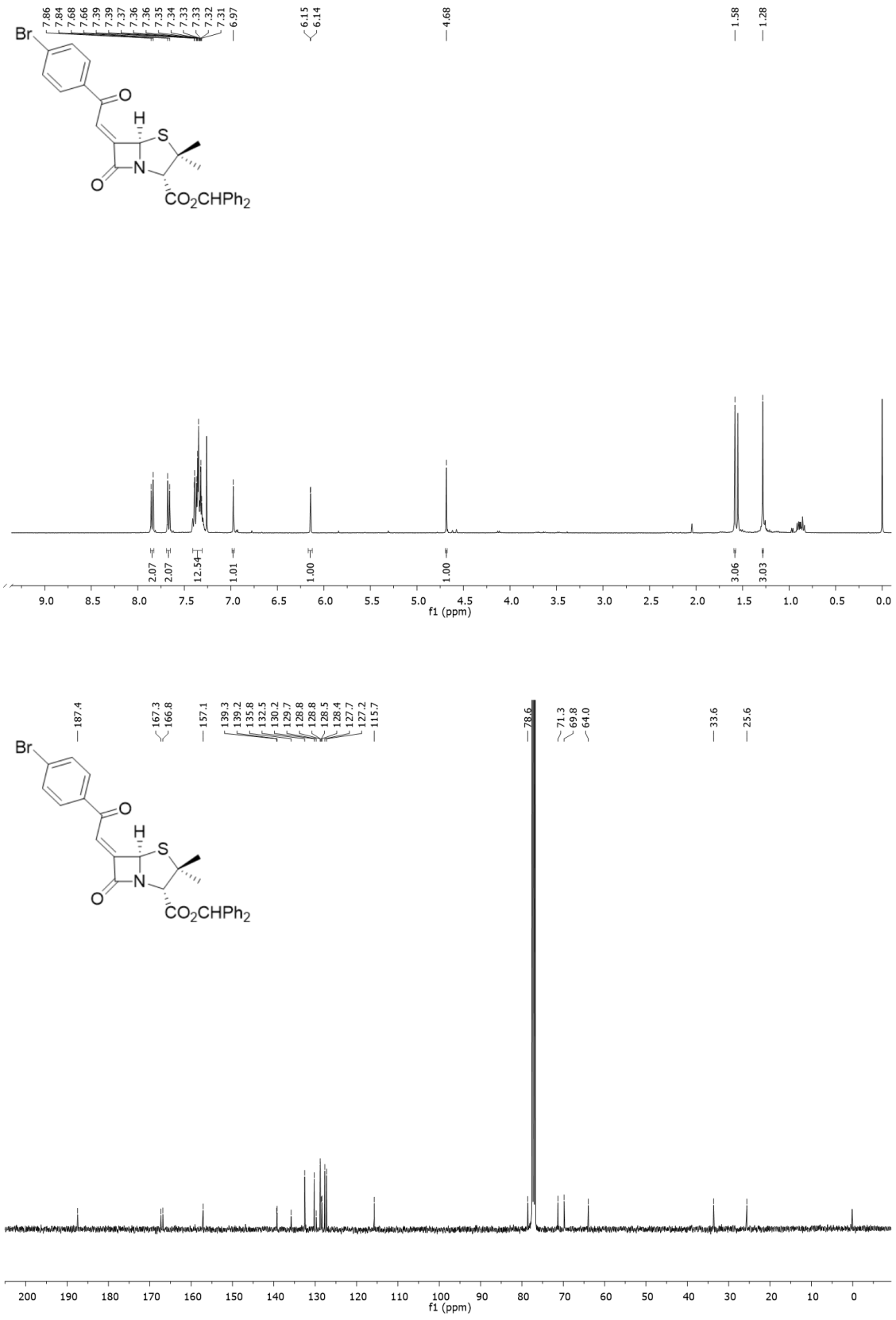


Figure S4: ^{1}H and ^{13}C NMR spectra of compound **2.20c (**$CDCl_3$).

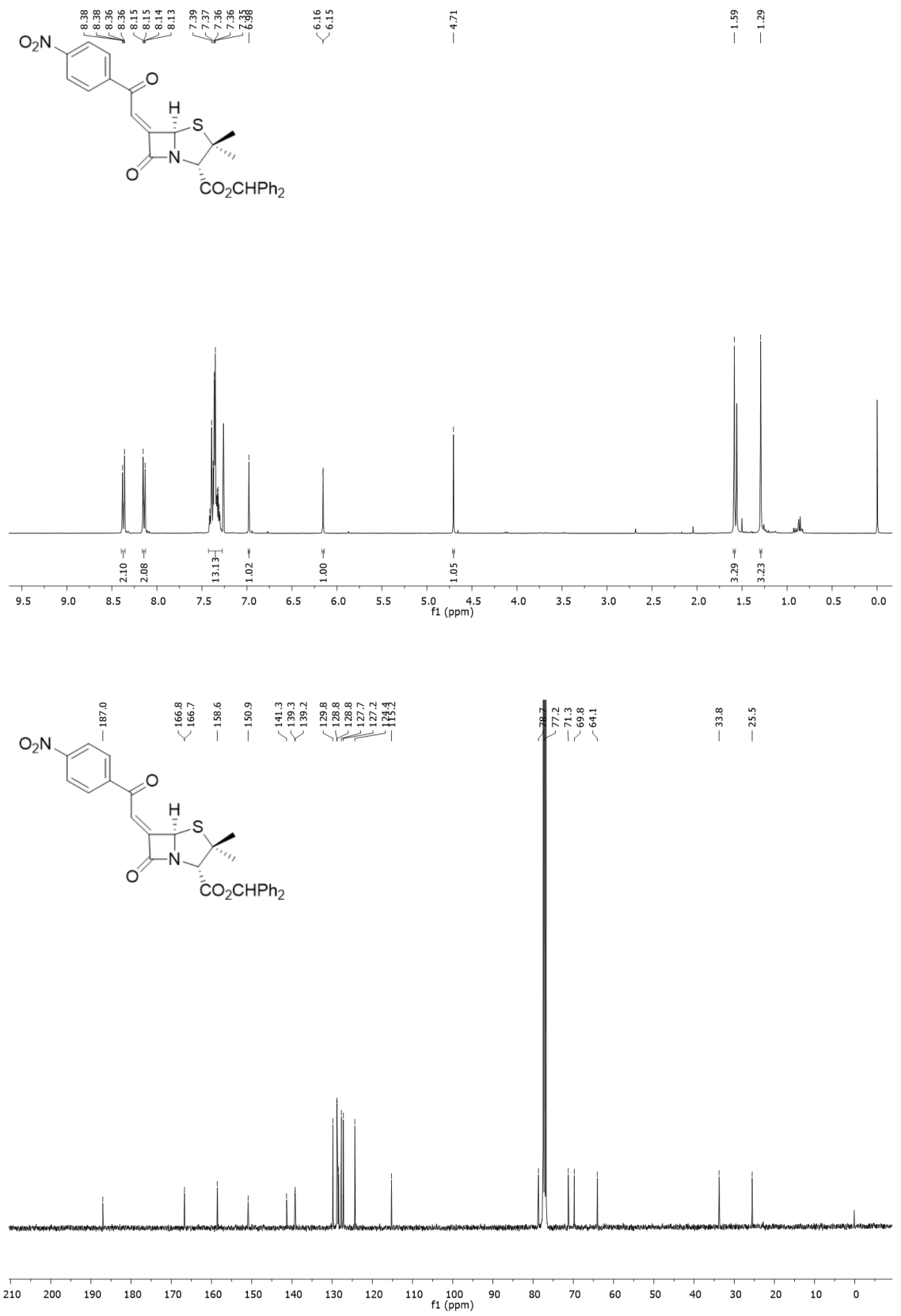


Figure S5: ^{1}H and ^{13}C NMR spectra of compound **2.20d (**$CDCl_3$).

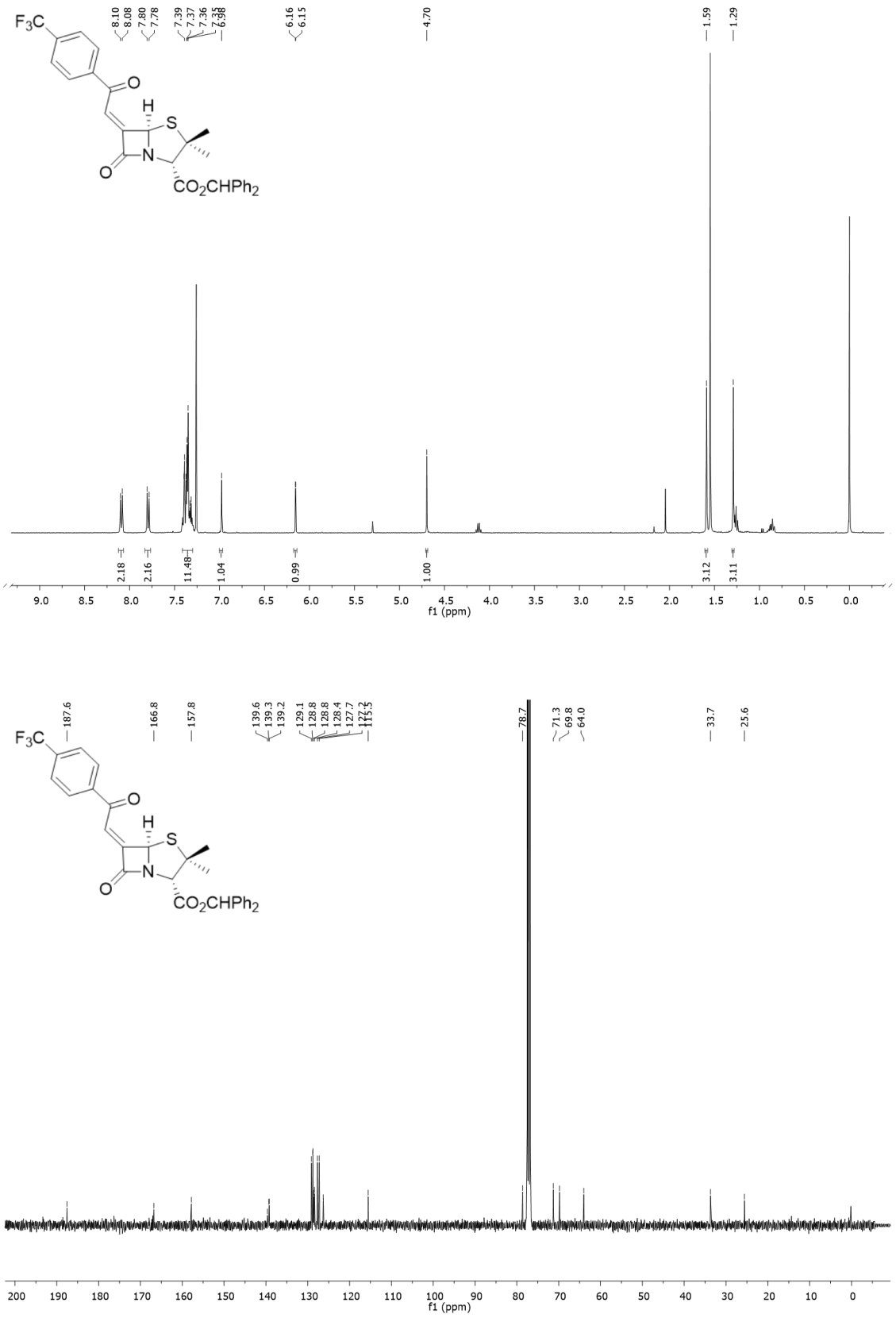


Figure S6: ^{1}H and ^{13}C NMR spectra of compound **2.20e (**$CDCl_3$).

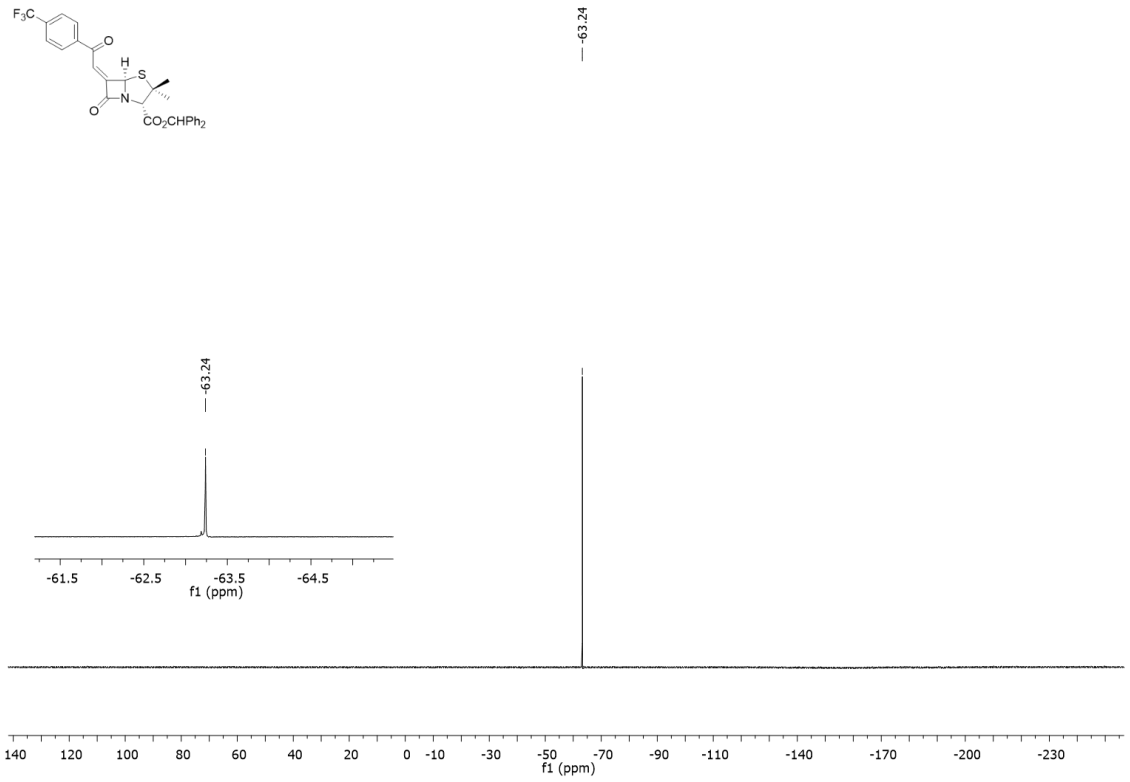


Figure S7: ^{19}F spectrum of compound **2.20e (**$CDCl_3$).

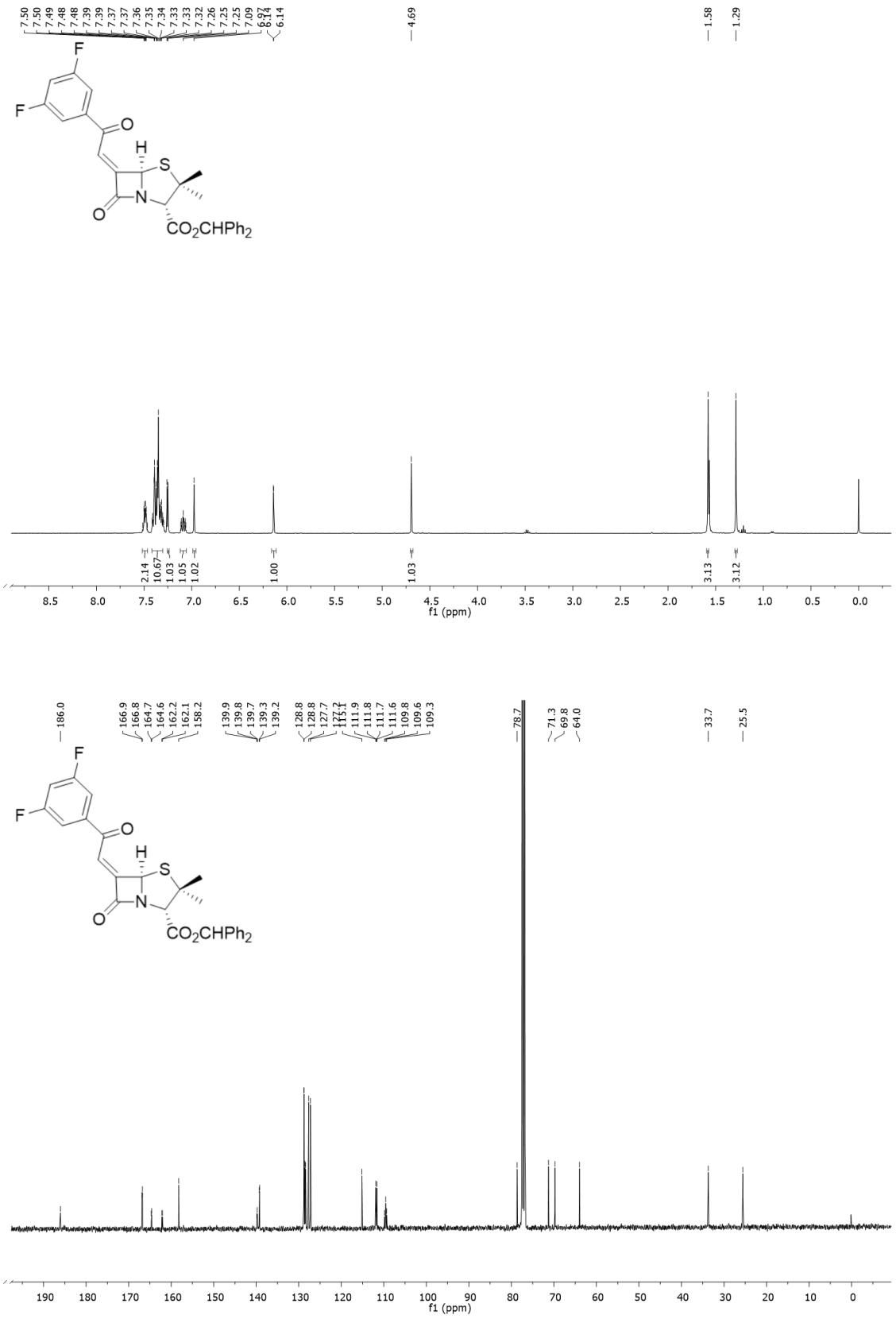


Figure S8: ^{1}H and ^{13}C NMR spectra of compound **2.20f (**$CDCl_3$).

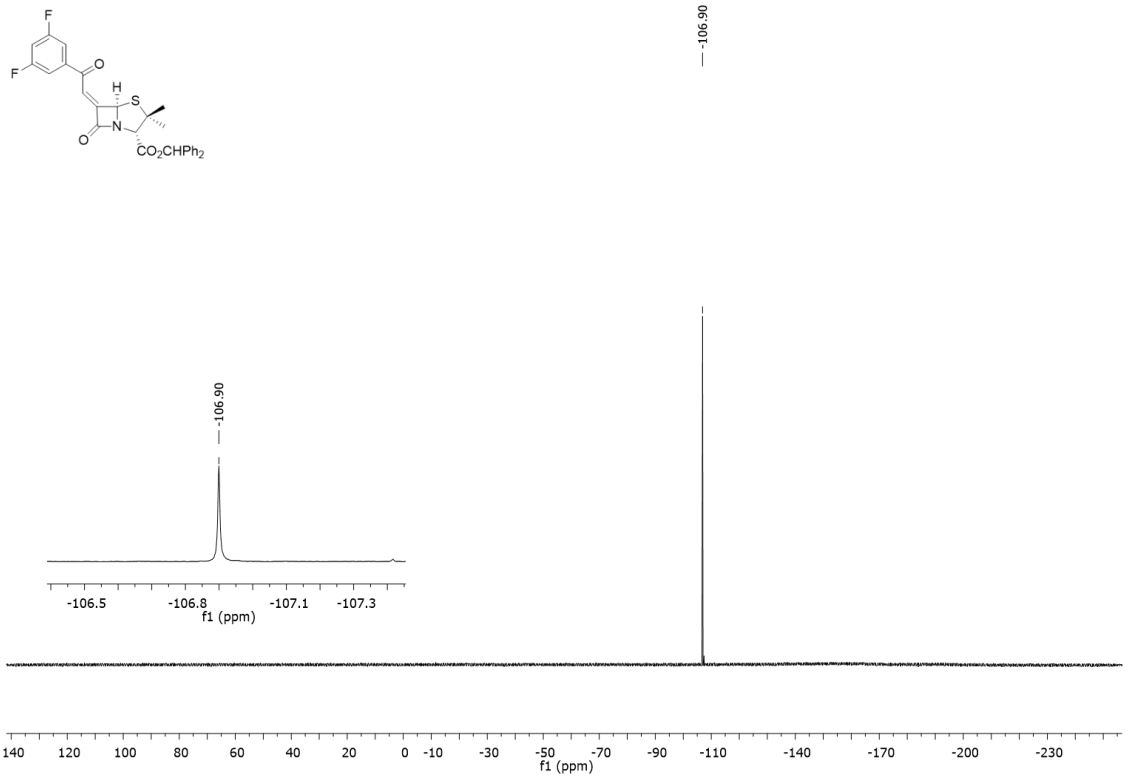


Figure S9: ^{19}F spectrum of compound **2.20f (**$CDCl_3$).

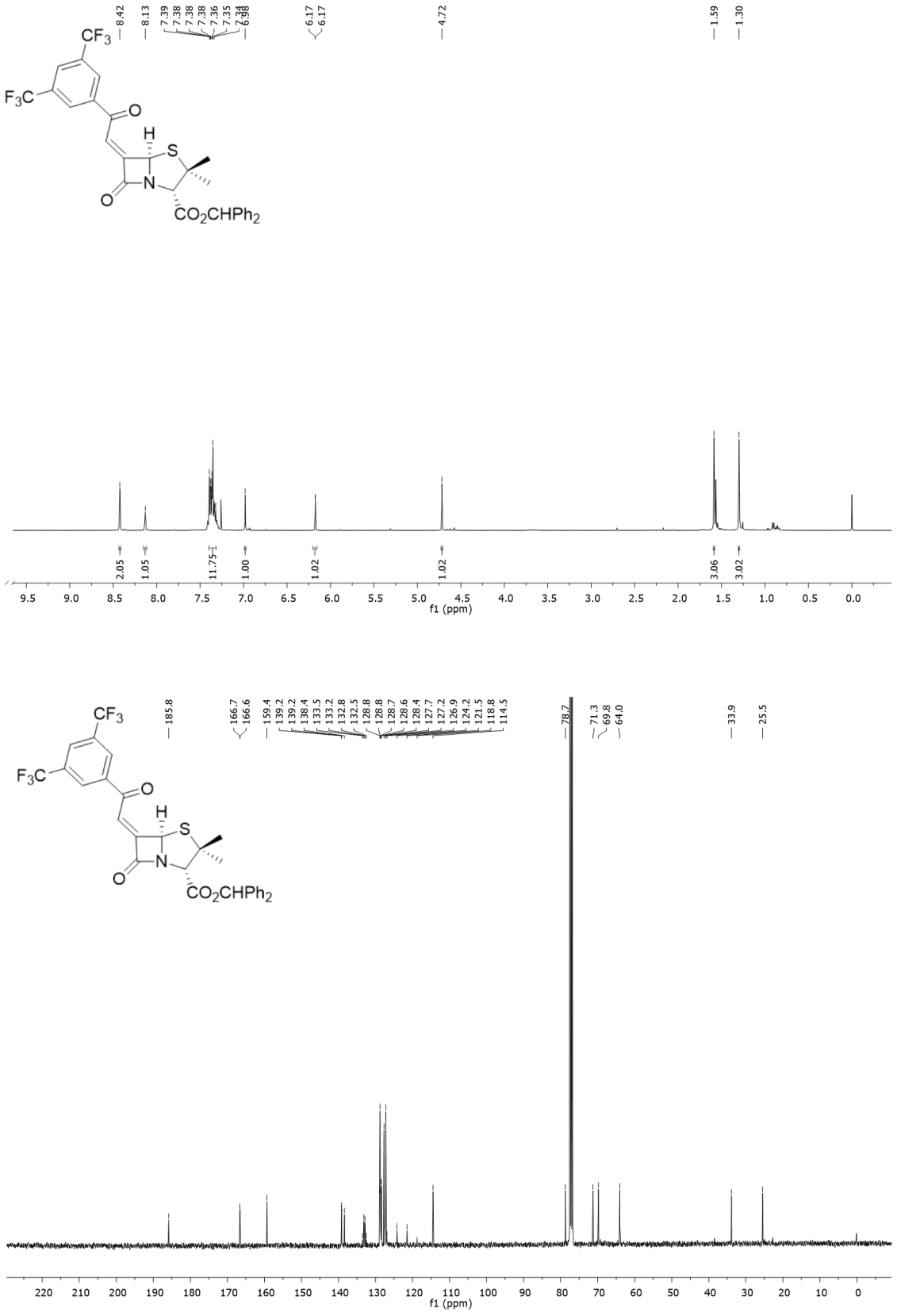


Figure S10: ^{1}H and ^{13}C NMR spectra of compound **2.20g (**$CDCl_3$).

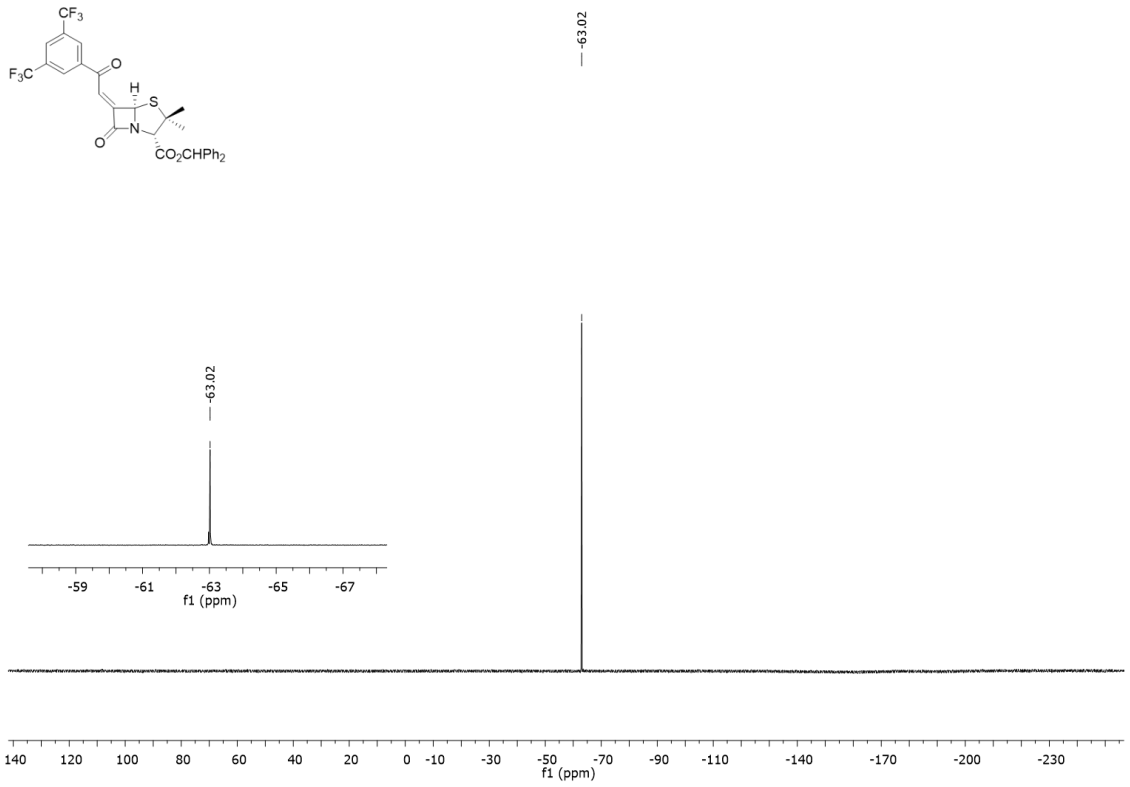


Figure S11: ^{19}F spectrum of compound **2.20g (**$CDCl_3$).

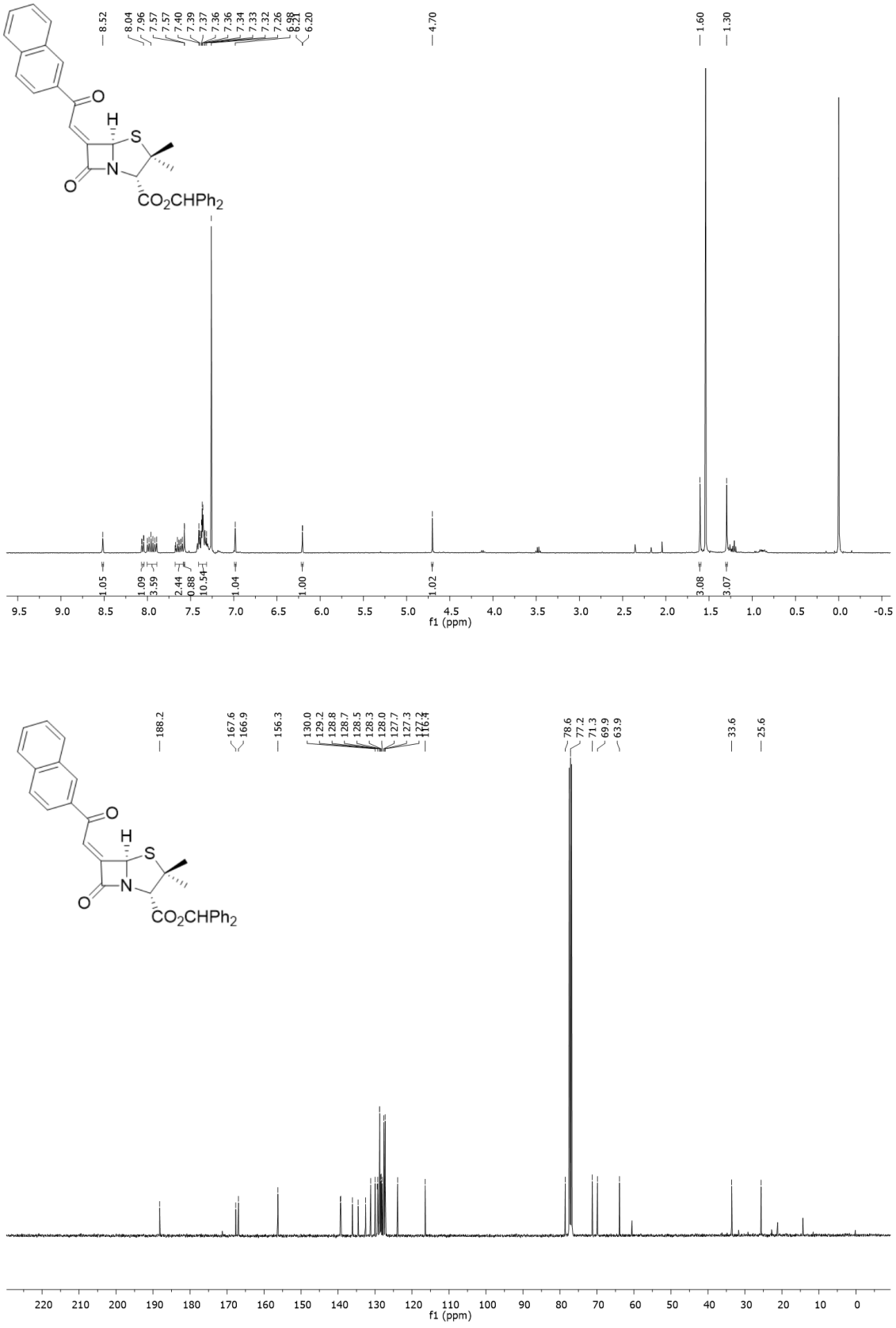


Figure S12: ^{1}H and ^{13}C NMR spectra of compound **2.20h (**$CDCl_3$**)**.

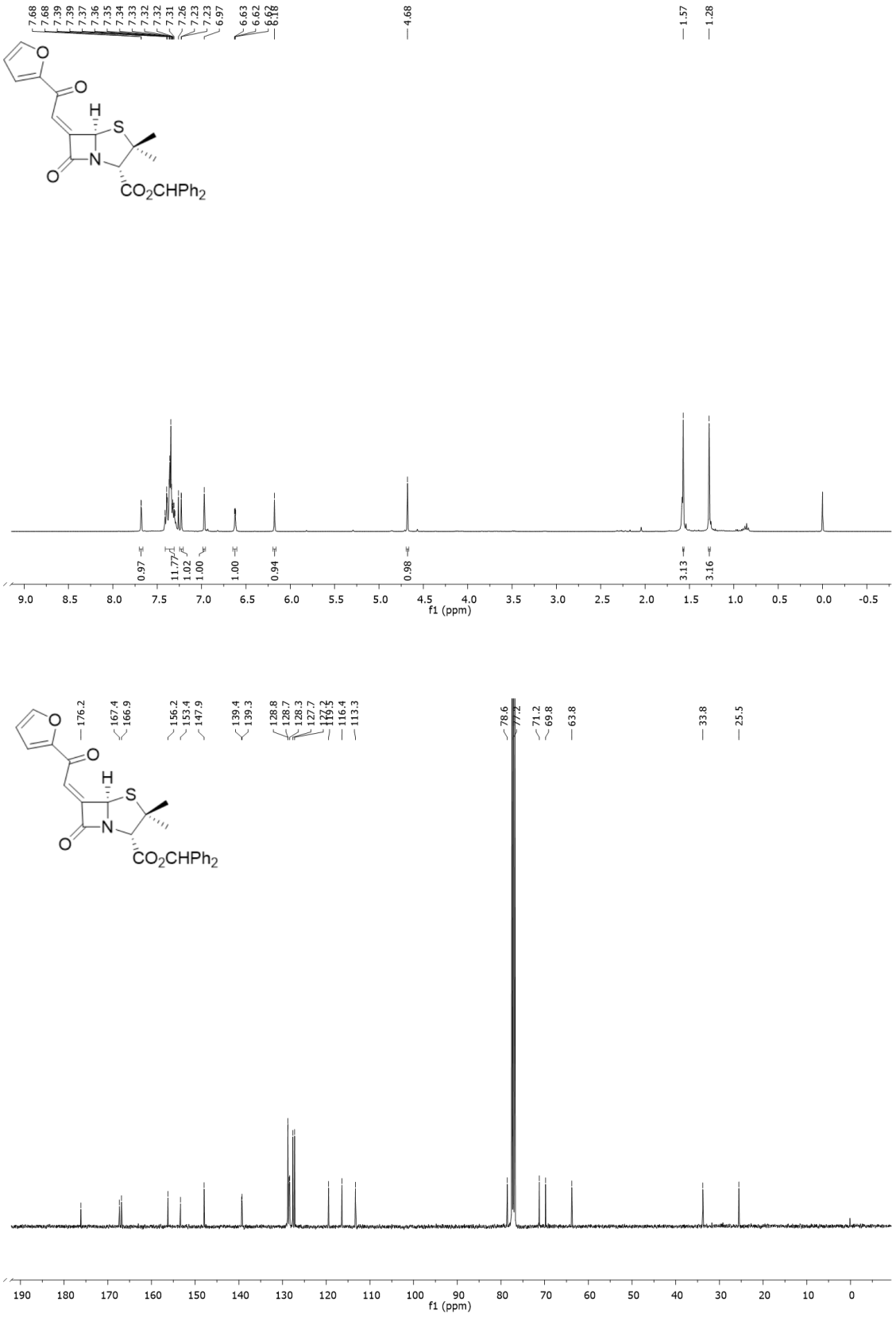


Figure S13: ^{1}H and ^{13}C NMR spectra of compound **2.20i (**$CDCl_3$).

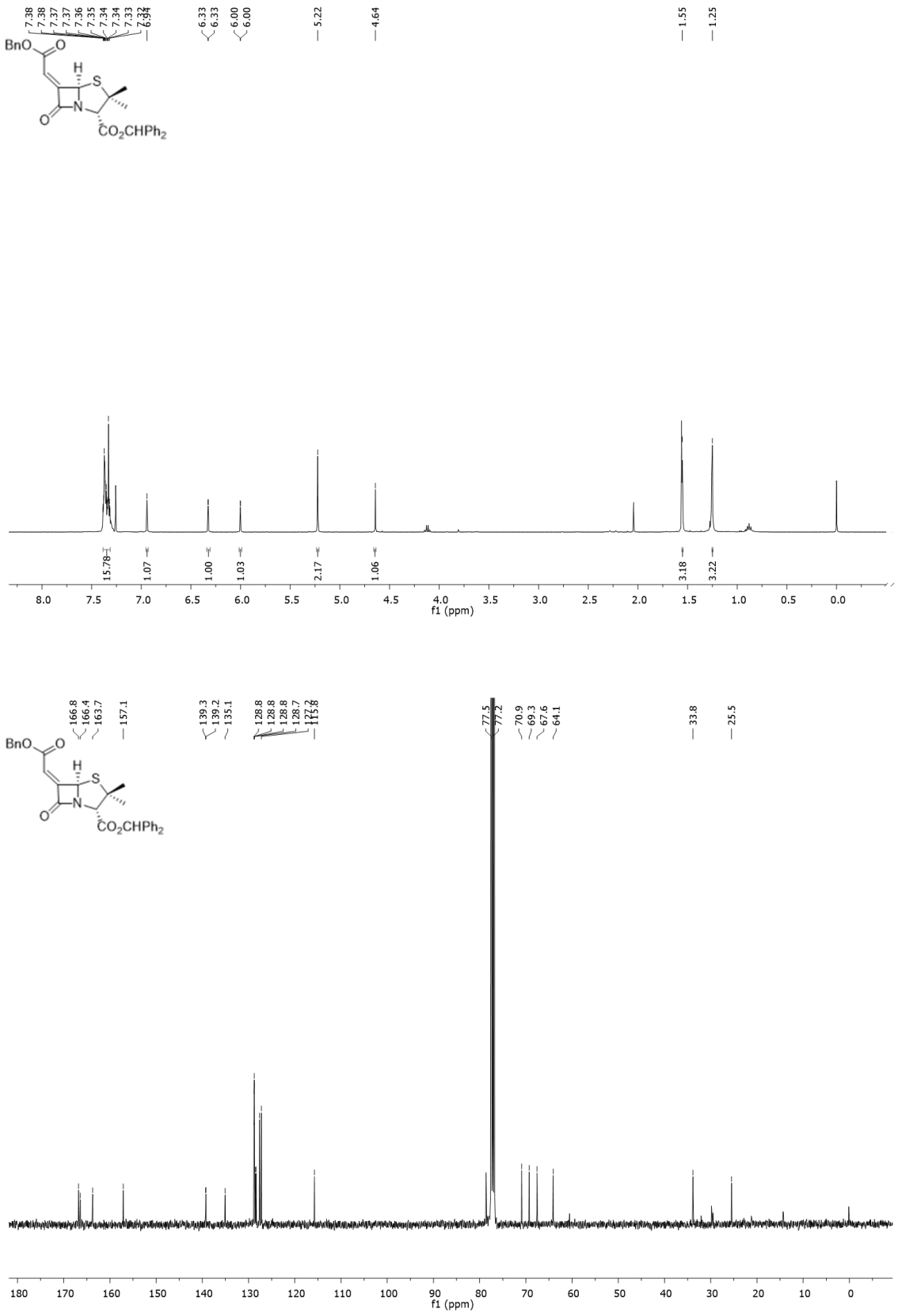


Figure S14: ^{1}H and ^{13}C NMR spectra of compound **2.20j (**$CDCl_3$).

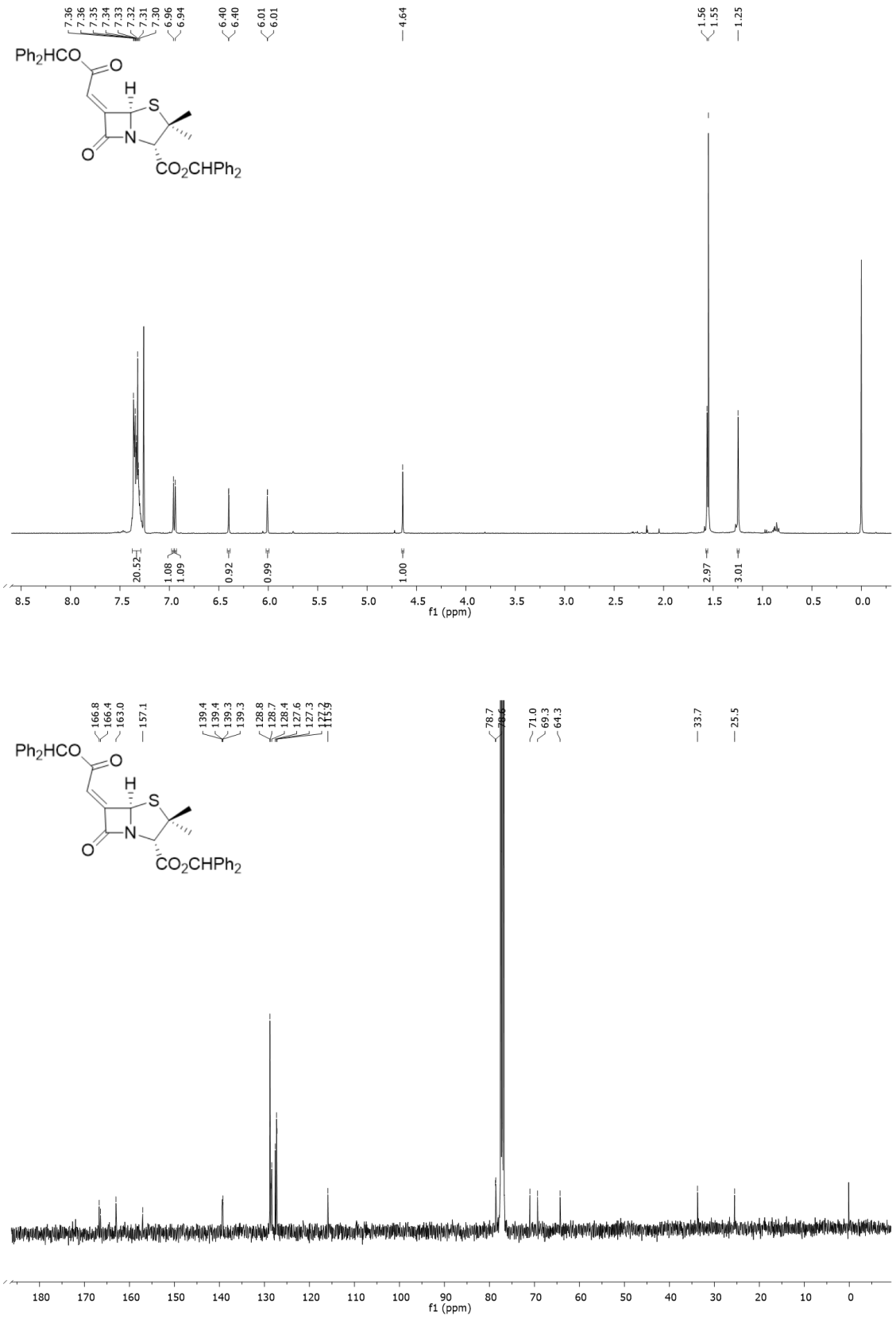


Figure S15: ^{1}H and ^{13}C NMR spectra of compound **2.20k (**$CDCl_3$).

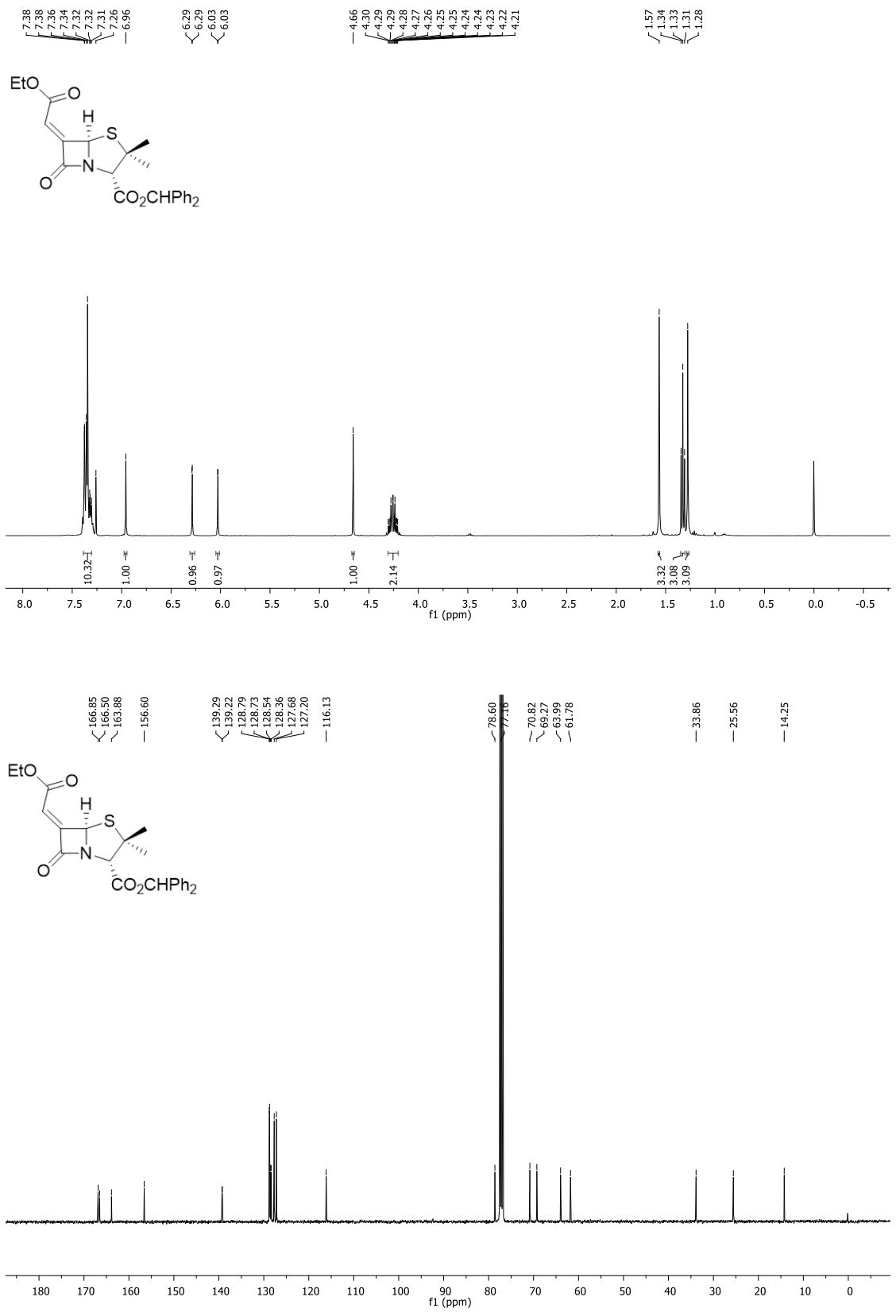


Figure S16: ^{1}H and ^{13}C NMR spectra of compound **2.20l (**$CDCl_3$).

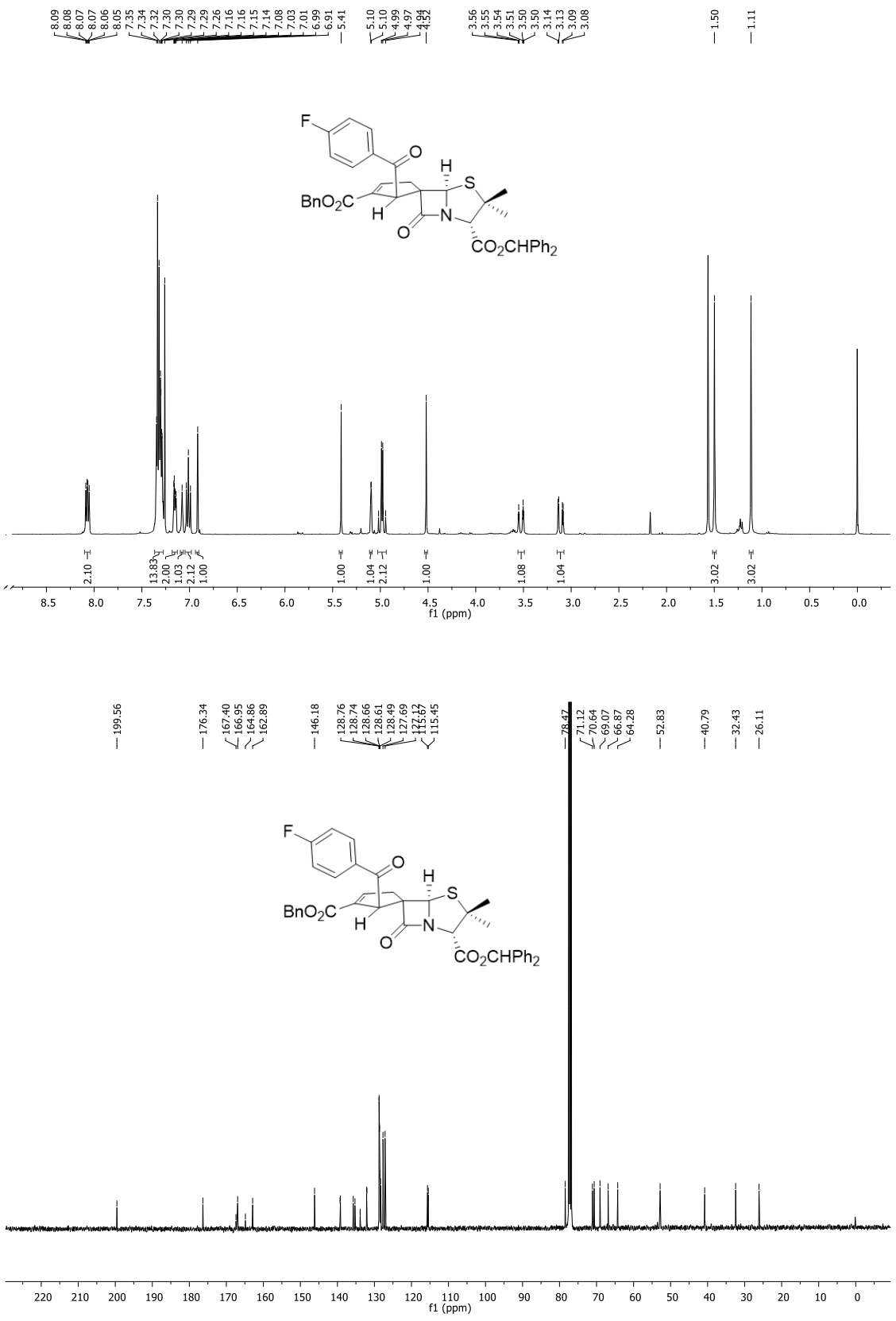


Figure S17: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **2.28a**.

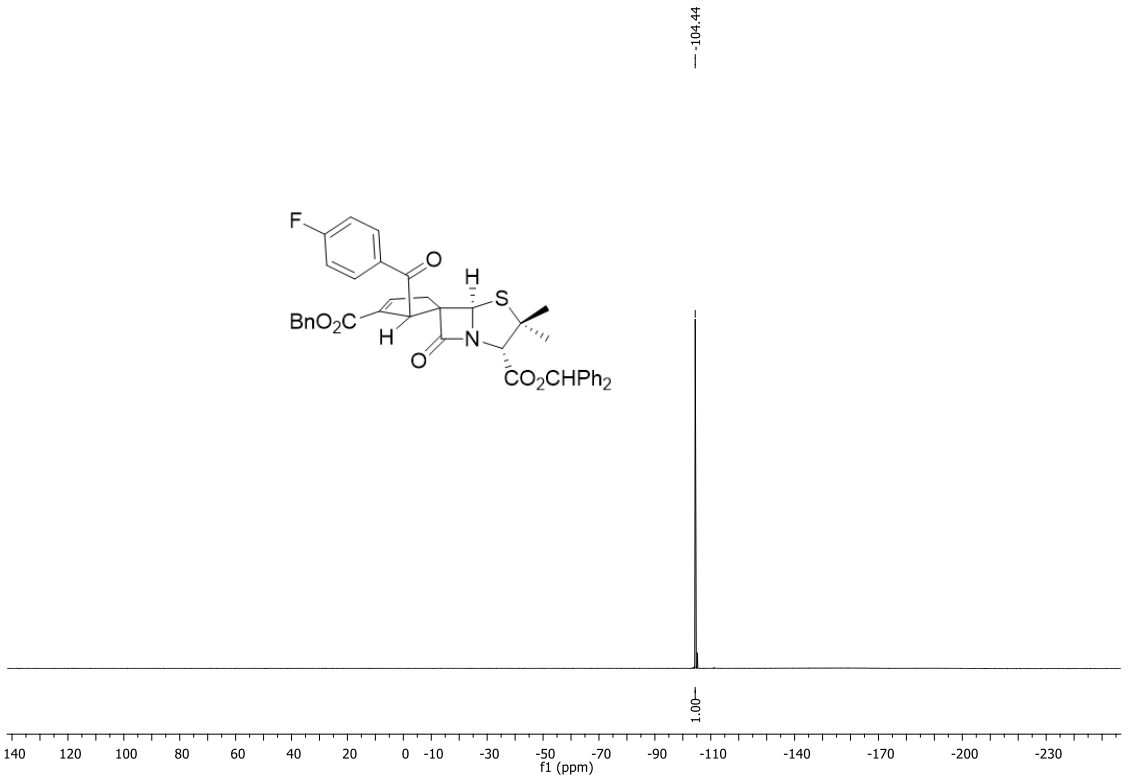


Figure S18: ^{19}F NMR spectrum ($CDCl_3$) of compound **2.28a**.

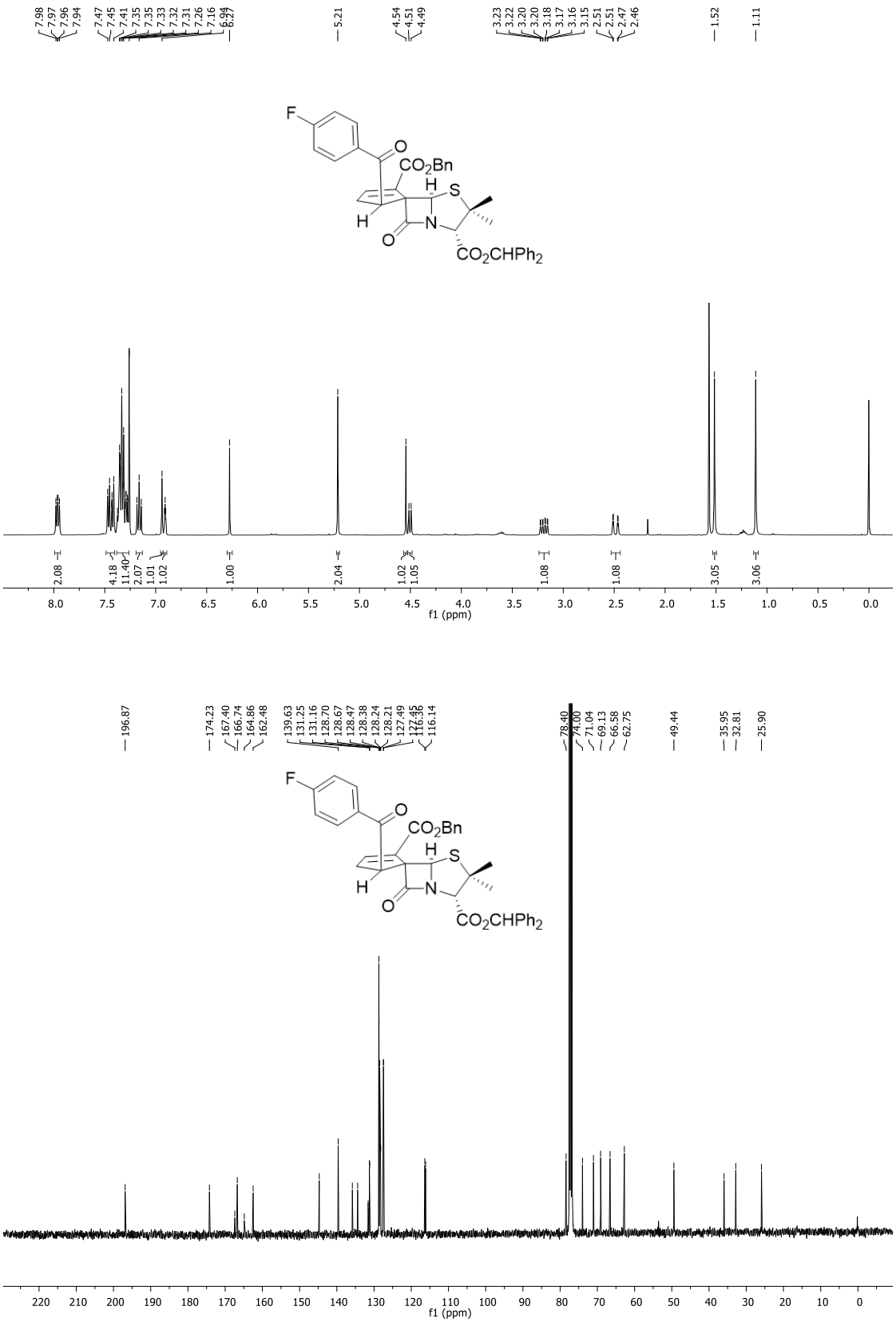


Figure S19: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **2.29a**.

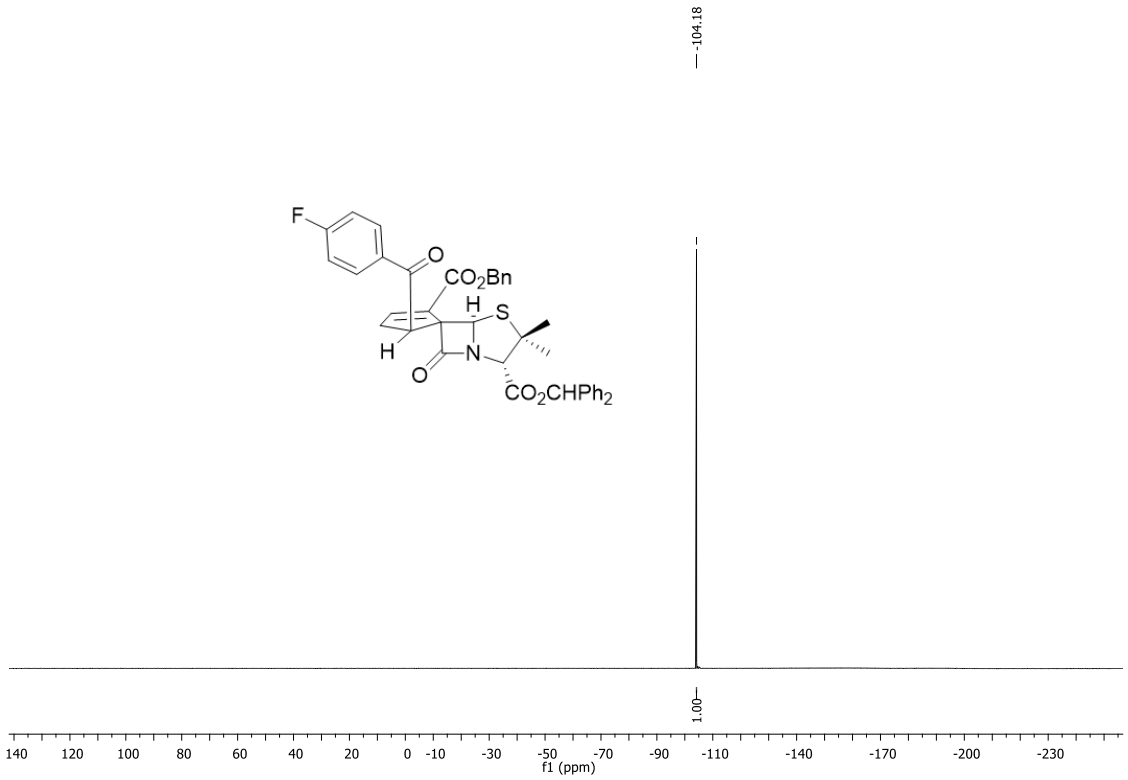


Figure S20: ^{19}F NMR spectrum ($CDCl_3$) of compound **2.29a**.

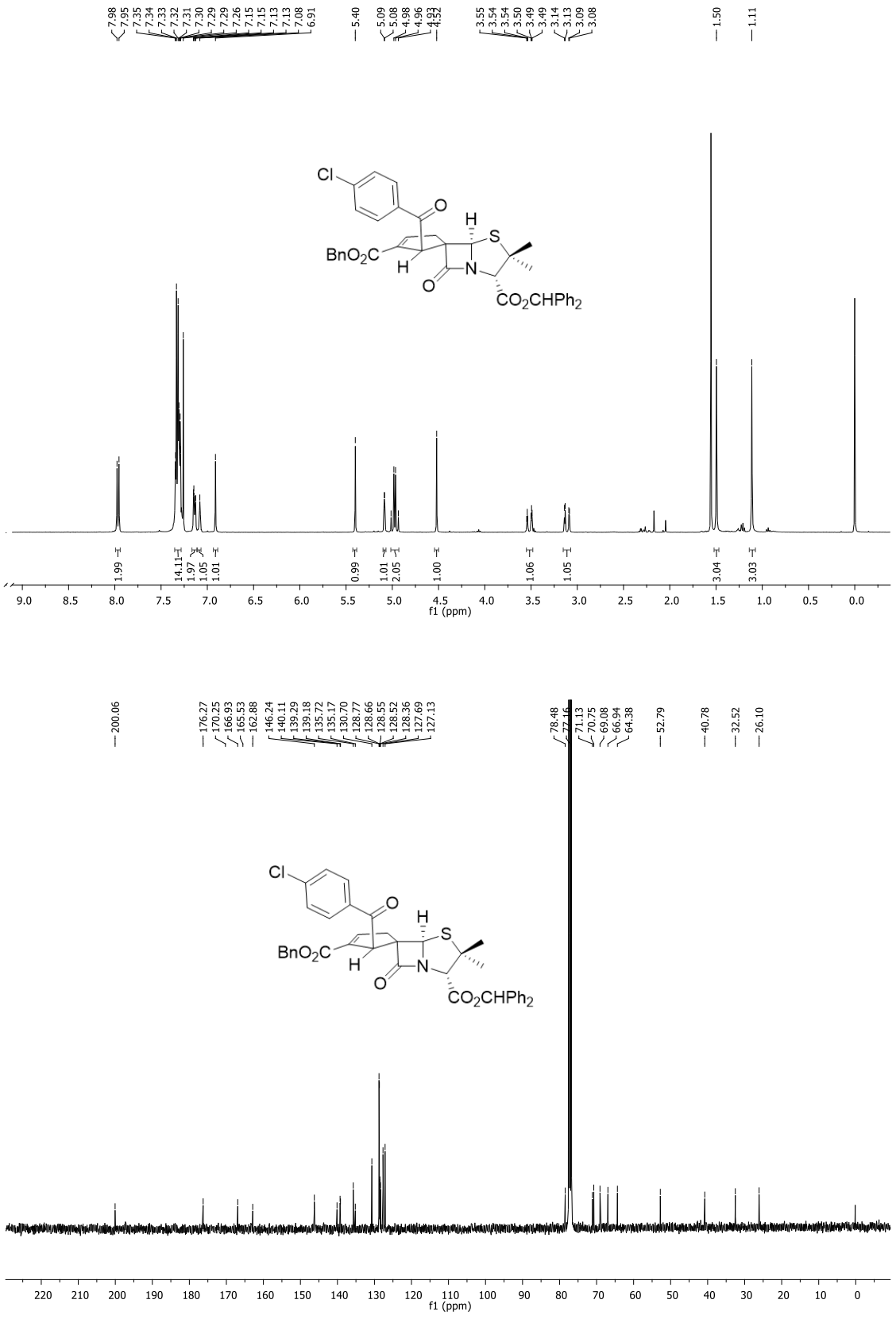


Figure S21: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **2.28b**.

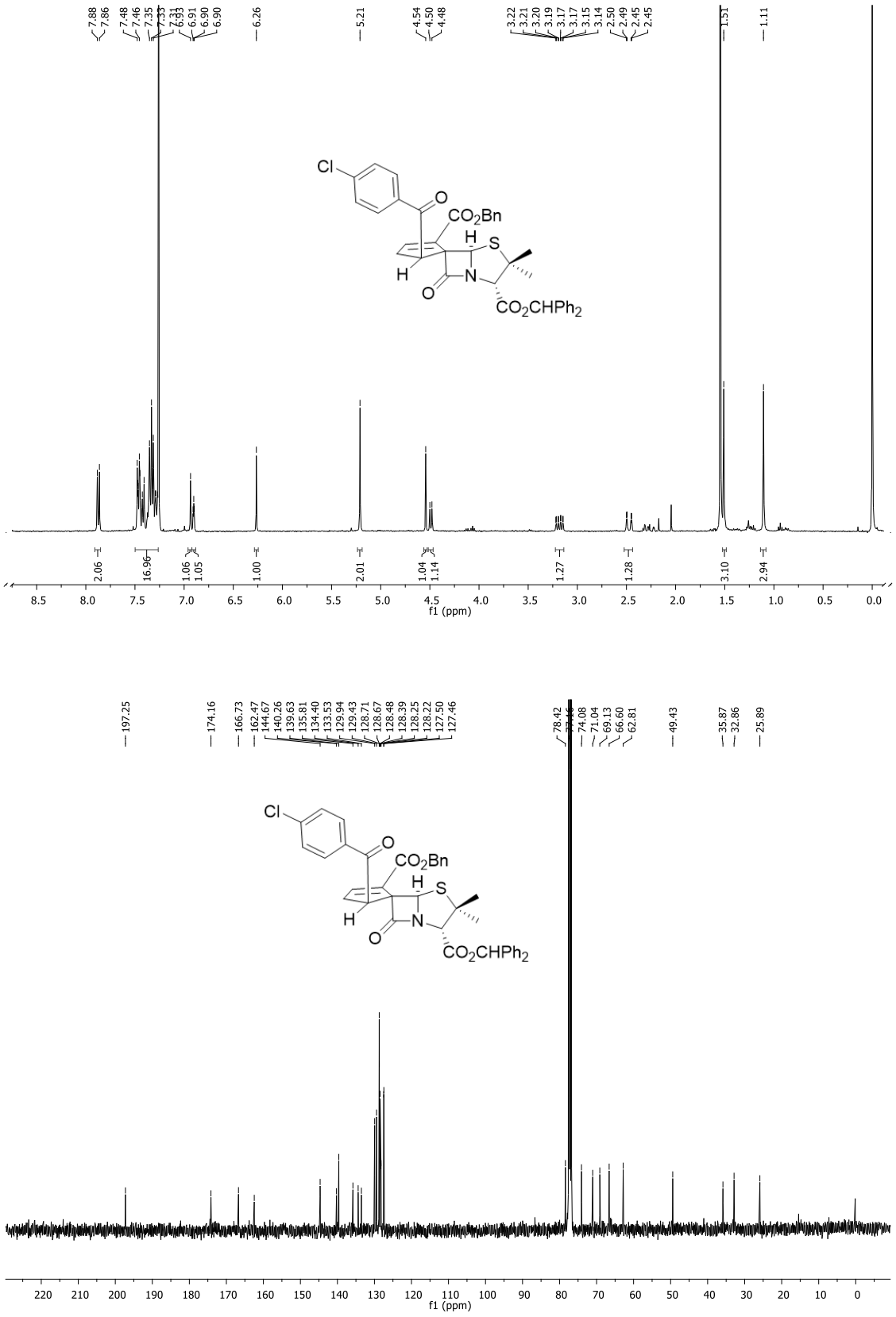


Figure S22: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **2.29b**.

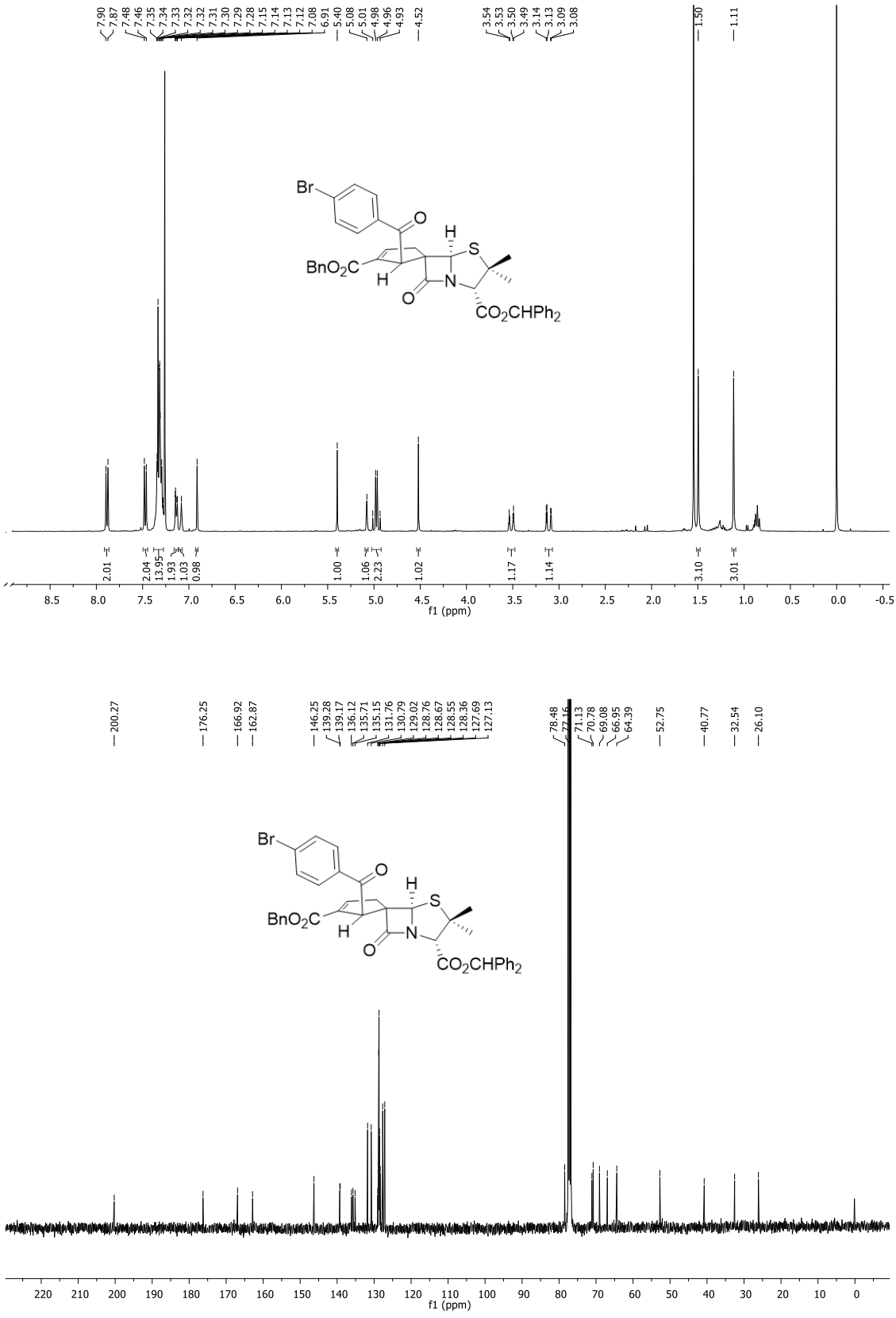


Figure S23: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **2.28c**.

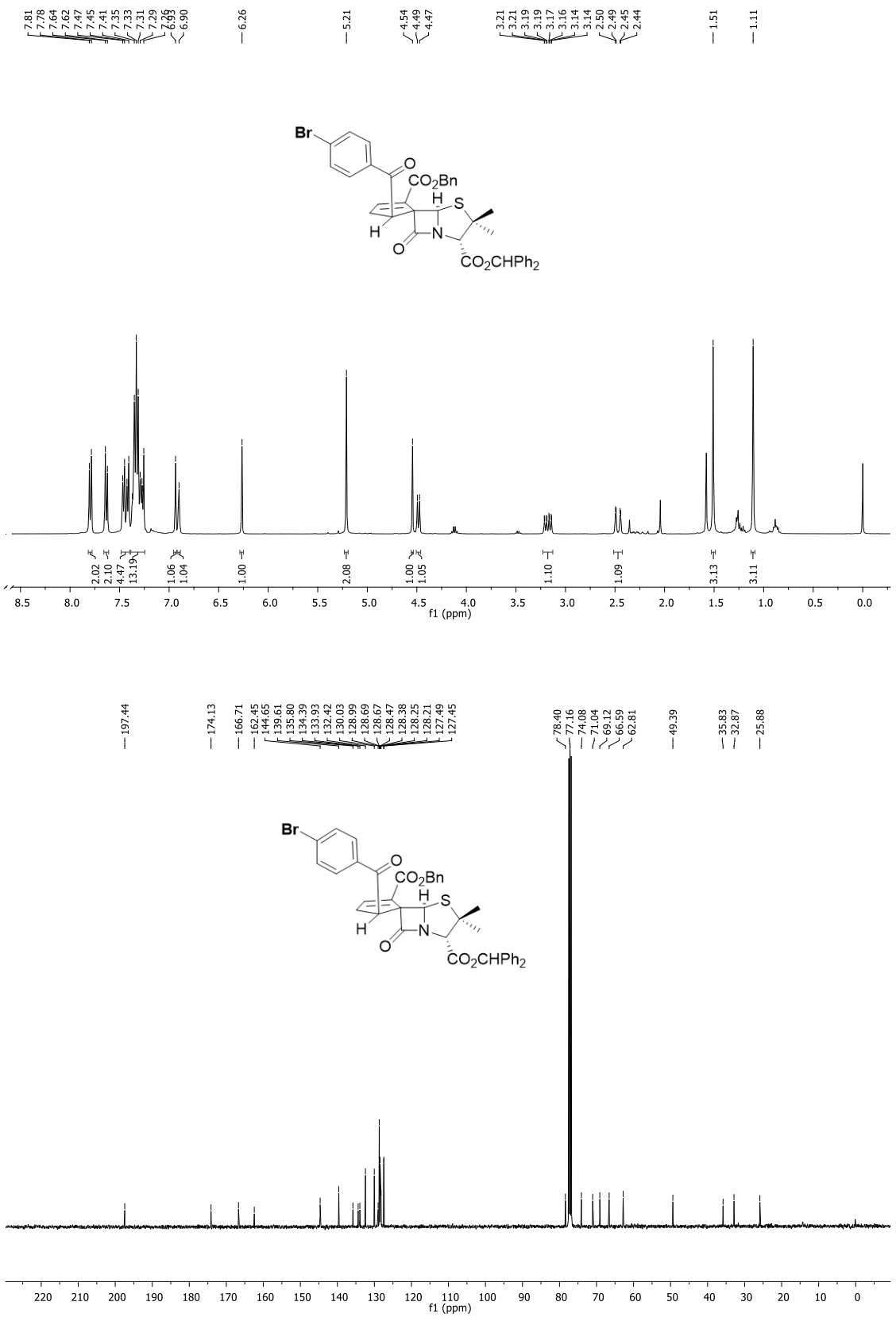


Figure S24: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **2.29c**.

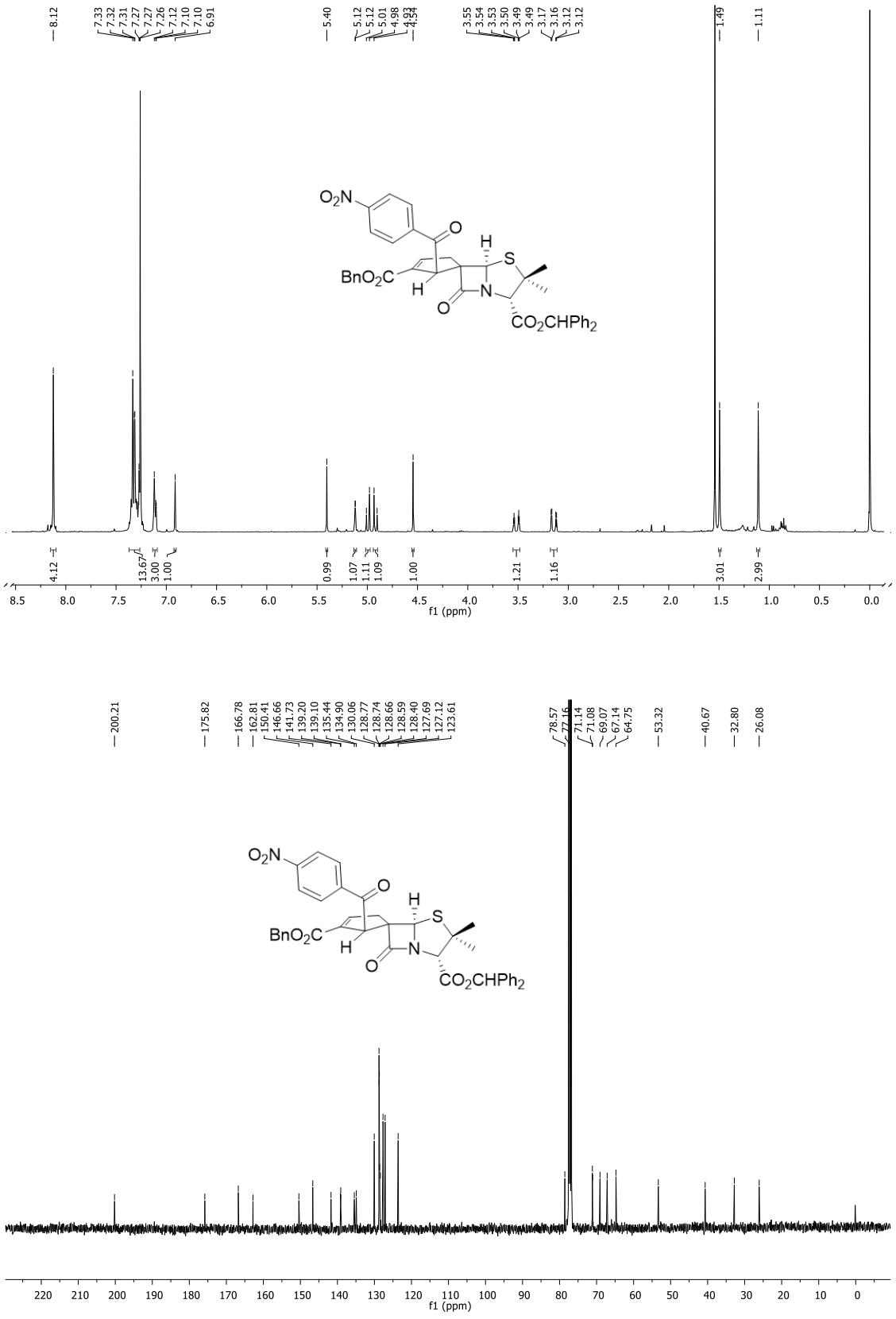


Figure S25: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **2.28d**.

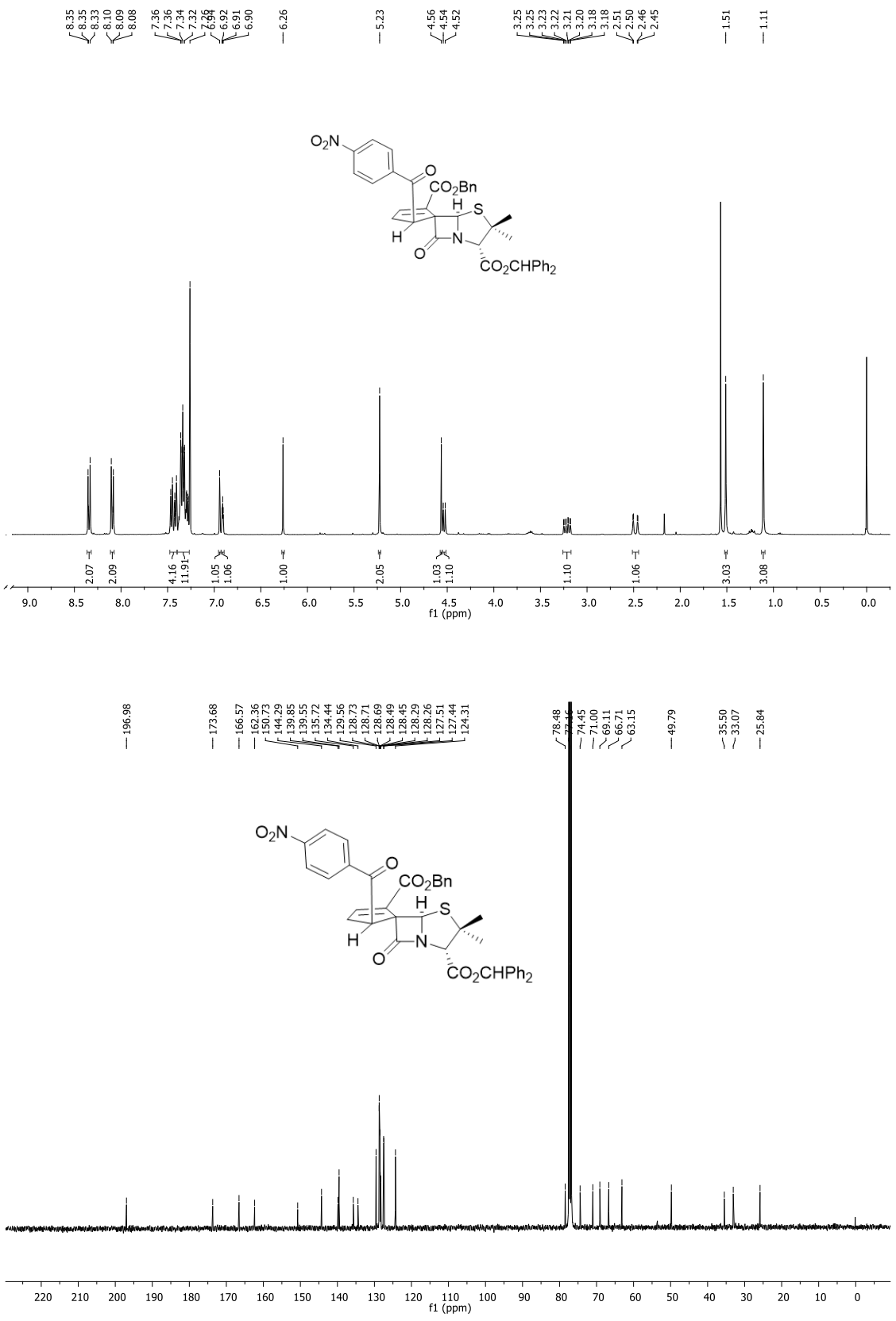


Figure S26: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **2.29d**.

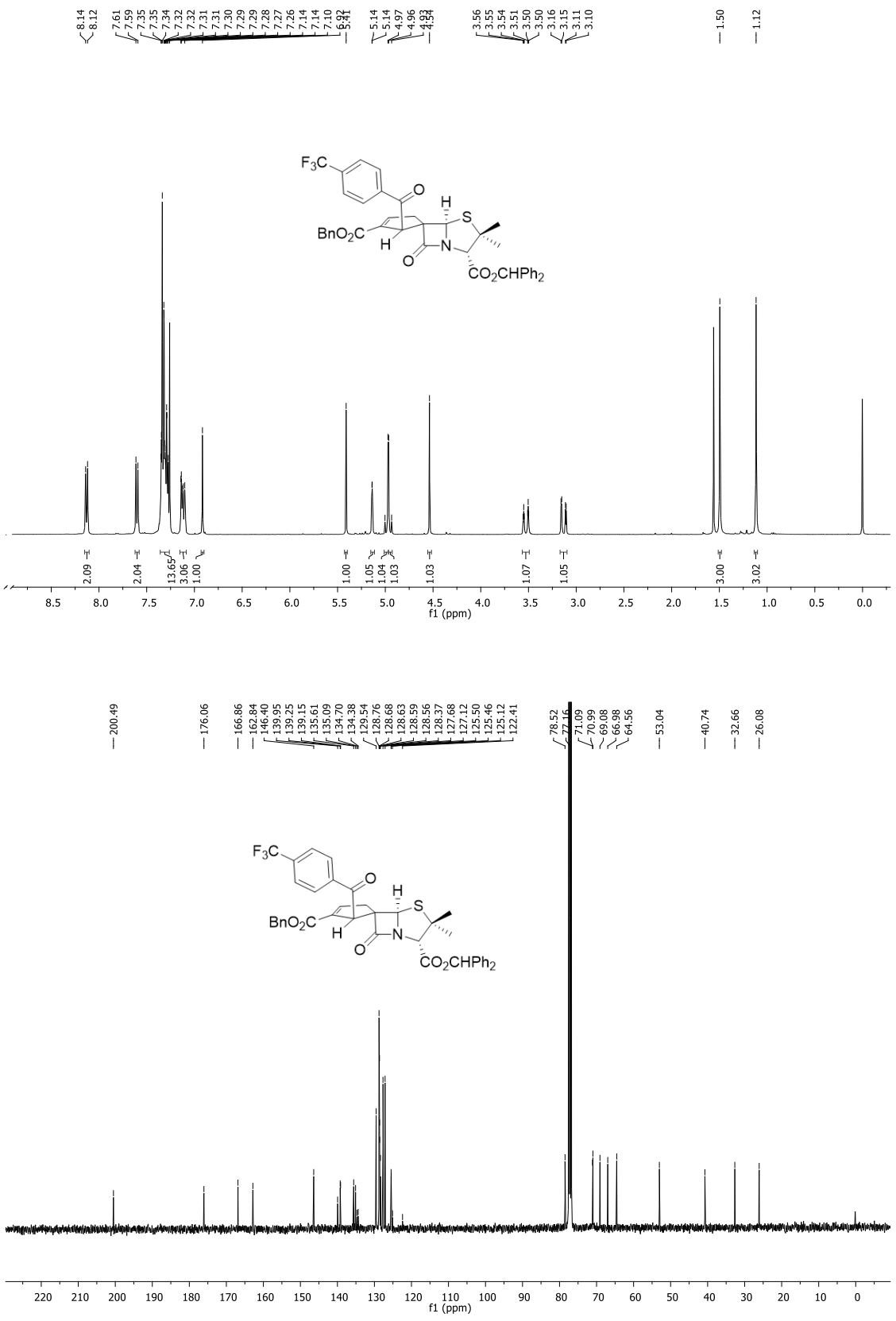


Figure S27: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **2.28e**.

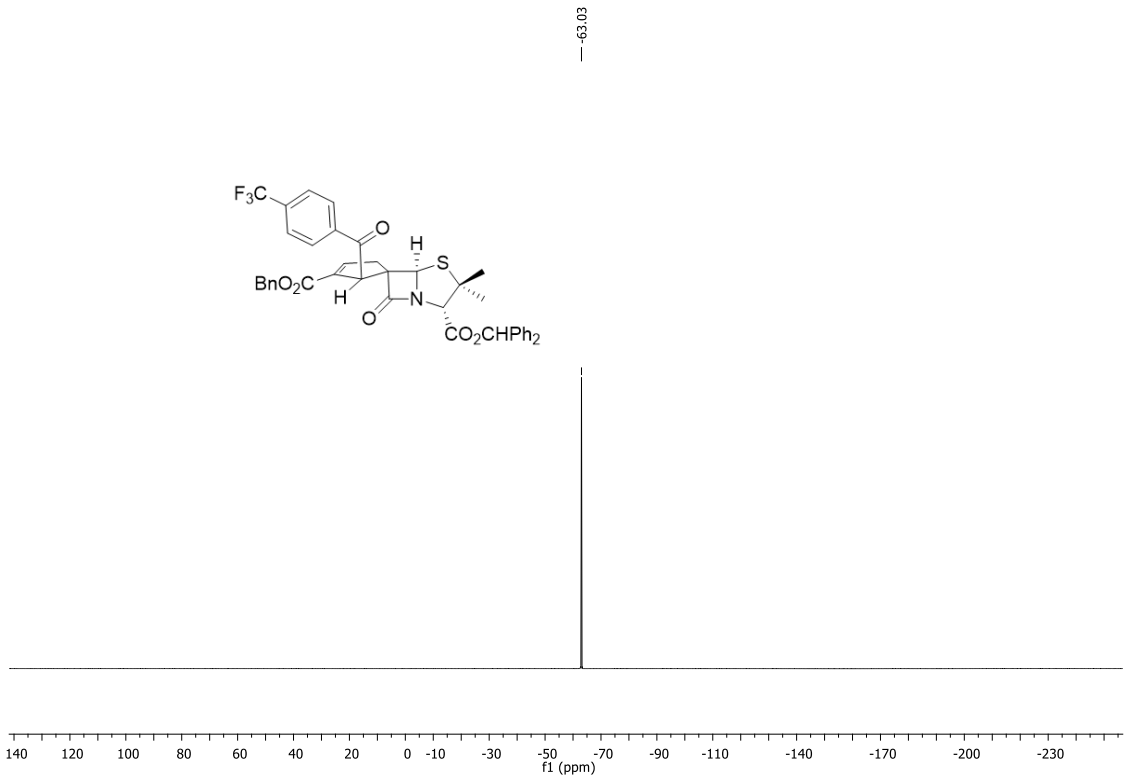


Figure S28: ^{19}F NMR spectrum ($CDCl_3$) of compound **2.28e**.

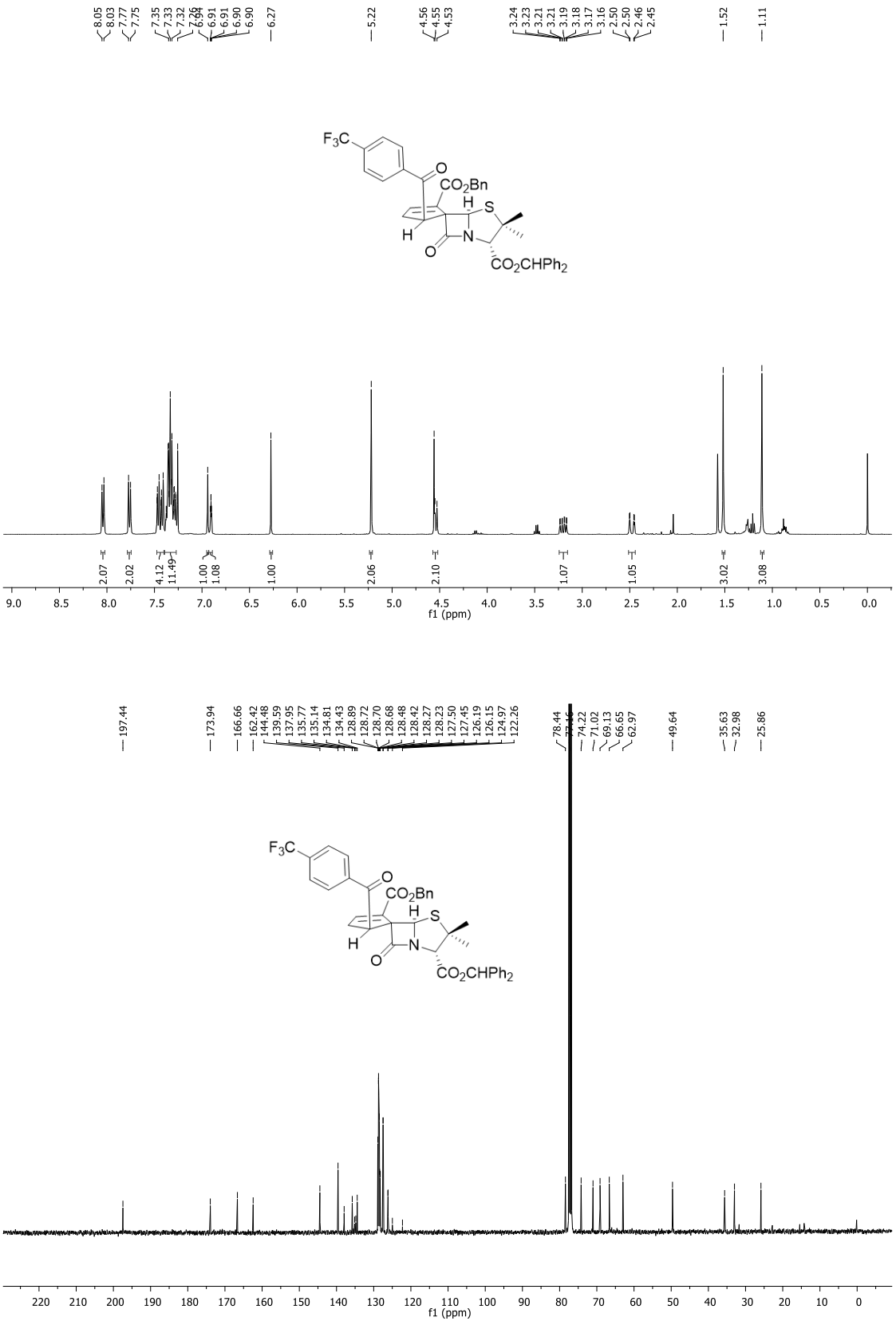


Figure S29: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **2.29e**.

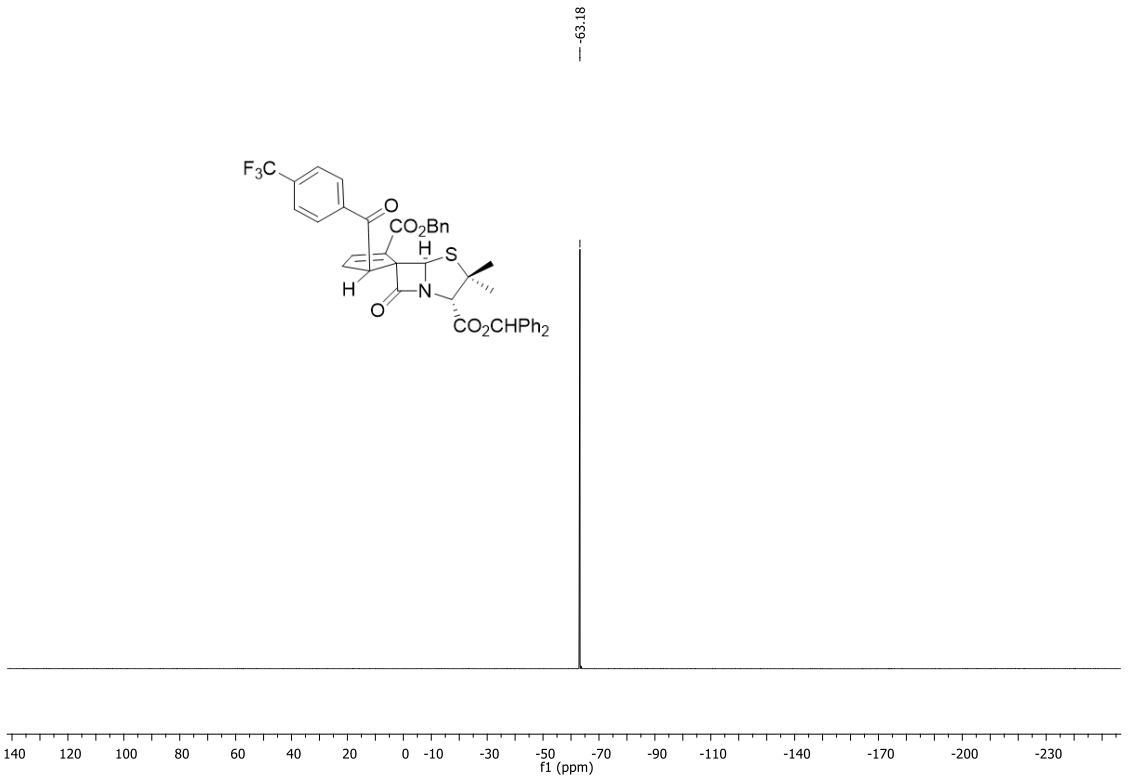


Figure S30: ^{19}F NMR spectrum ($CDCl_3$) of compound **2.29e**.

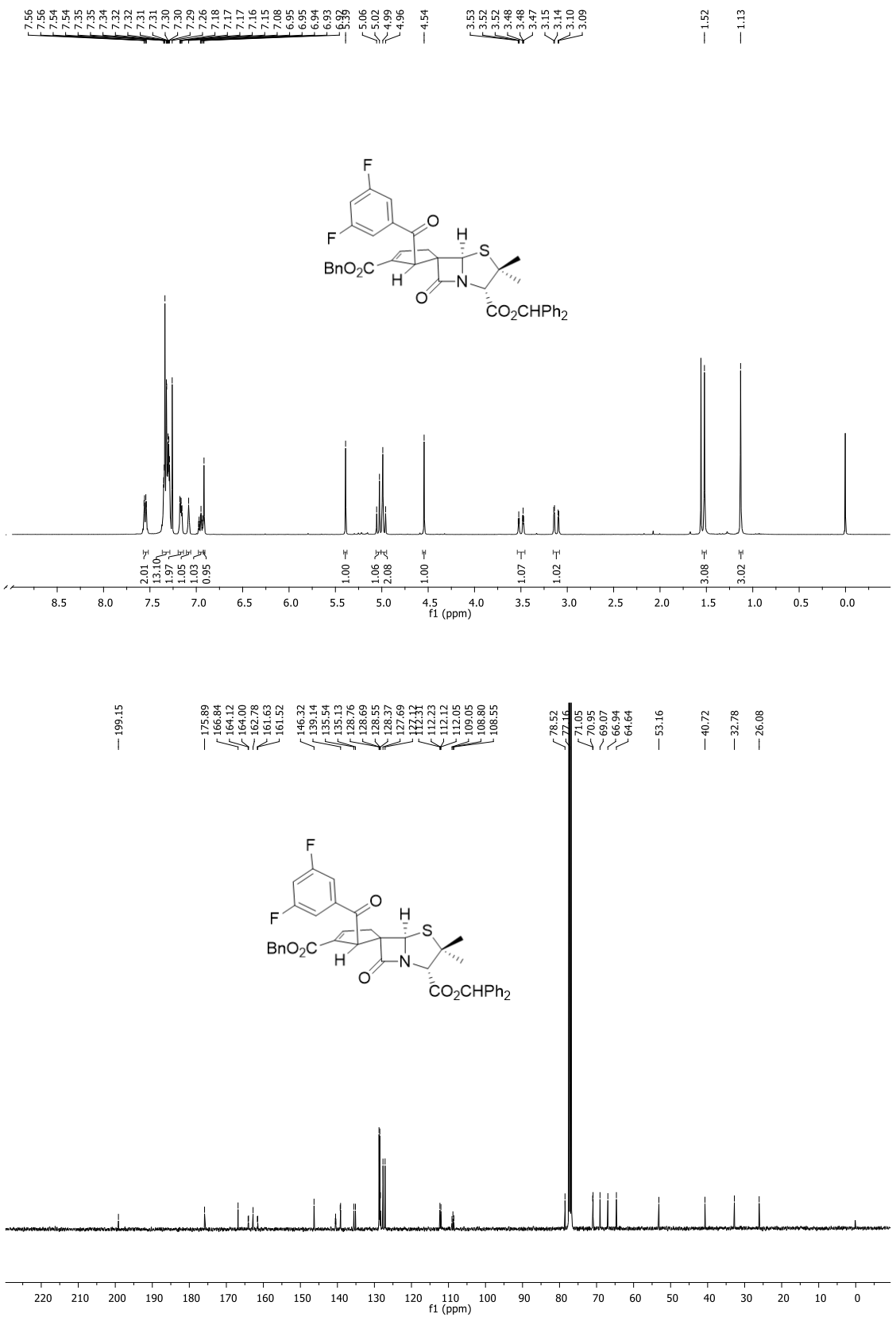


Figure S31: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **2.28f**.

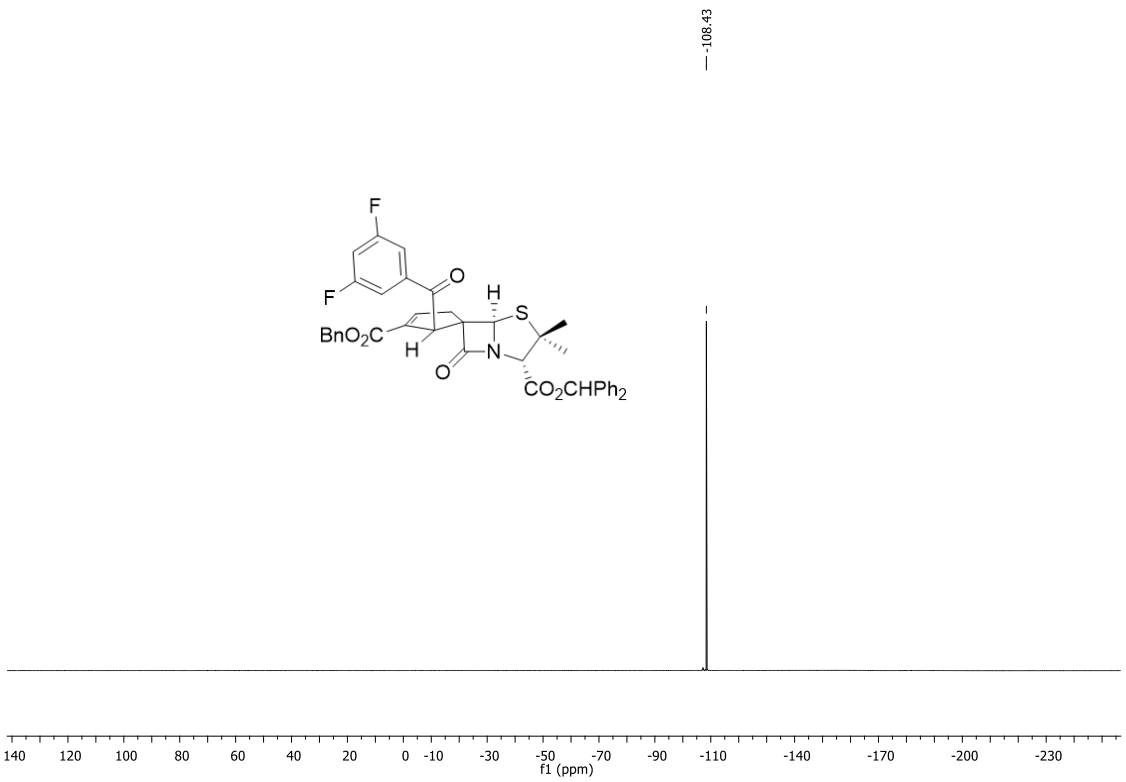


Figure S32: ^{19}F NMR spectrum ($CDCl_3$) of compound **2.28f**.

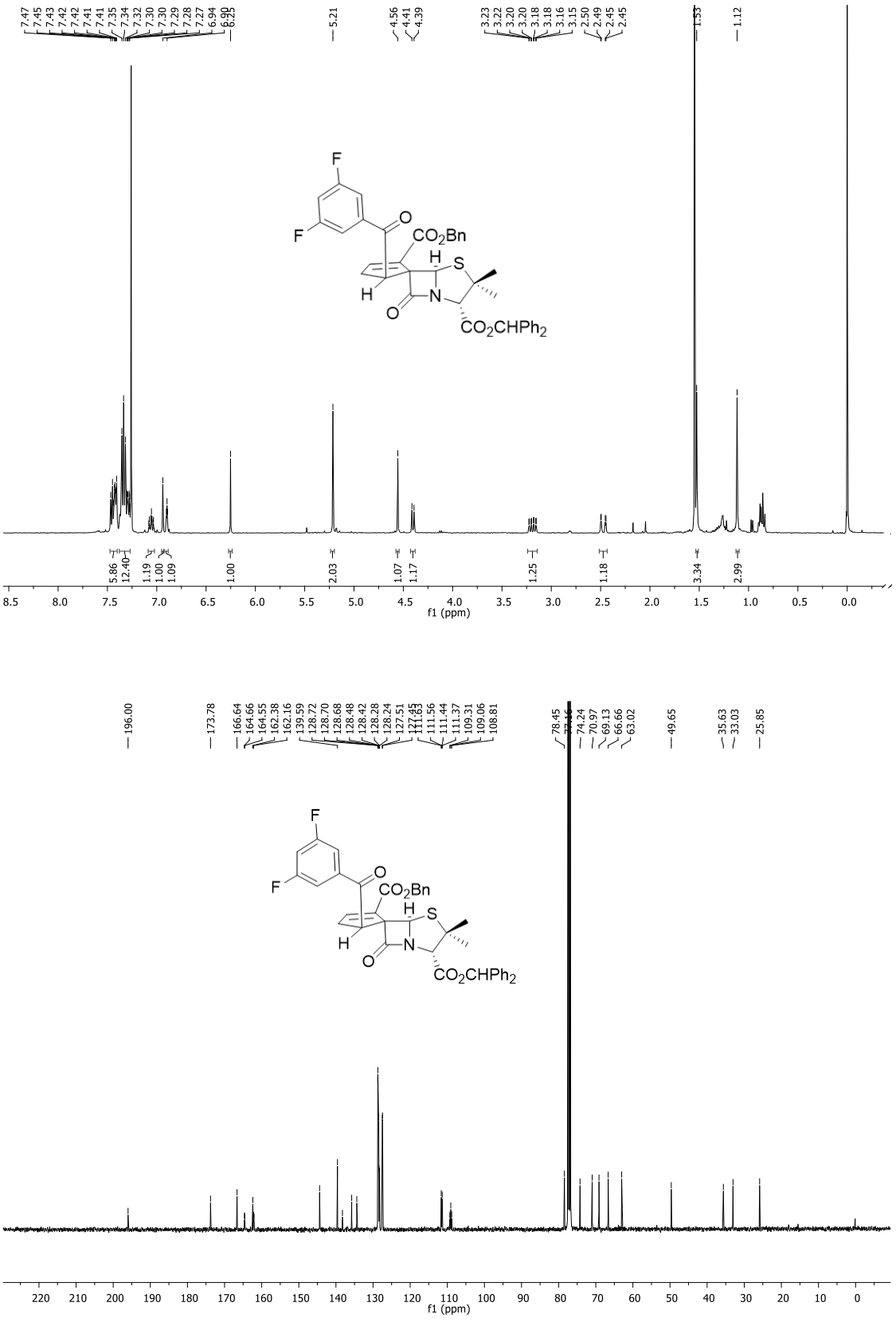


Figure S33: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **2.29f**.

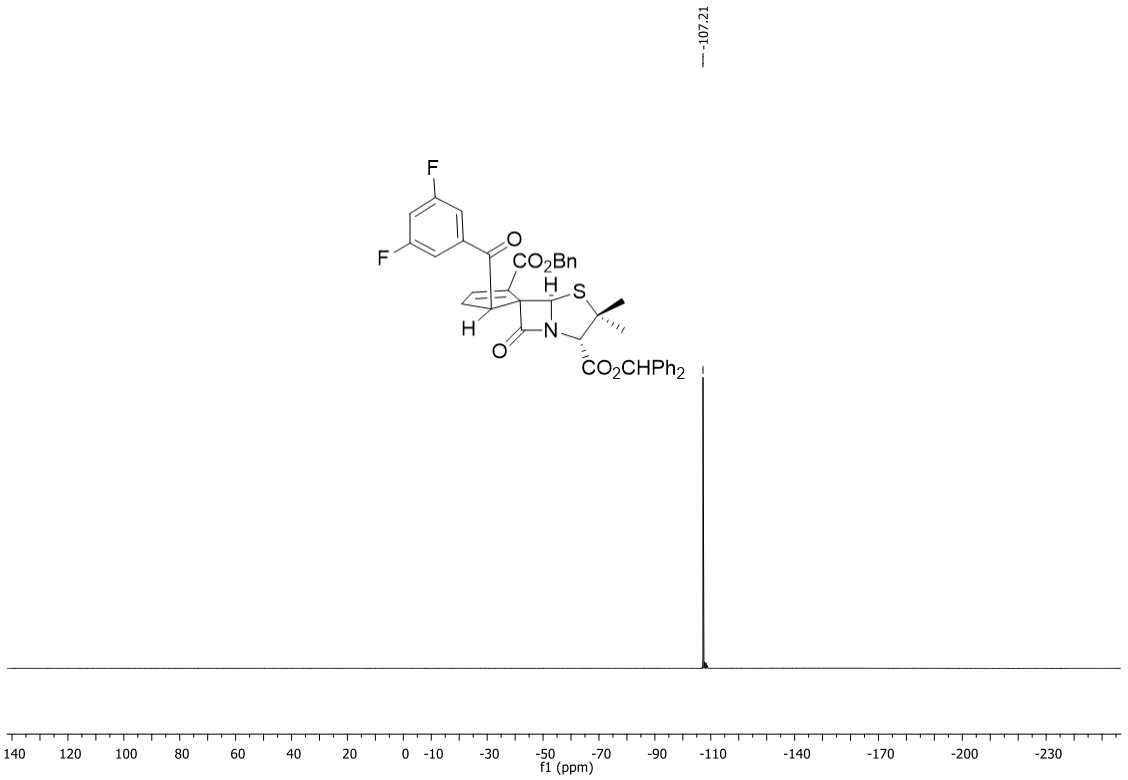


Figure S34: ^{19}F NMR spectrum ($CDCl_3$) of compound **2.29f**.

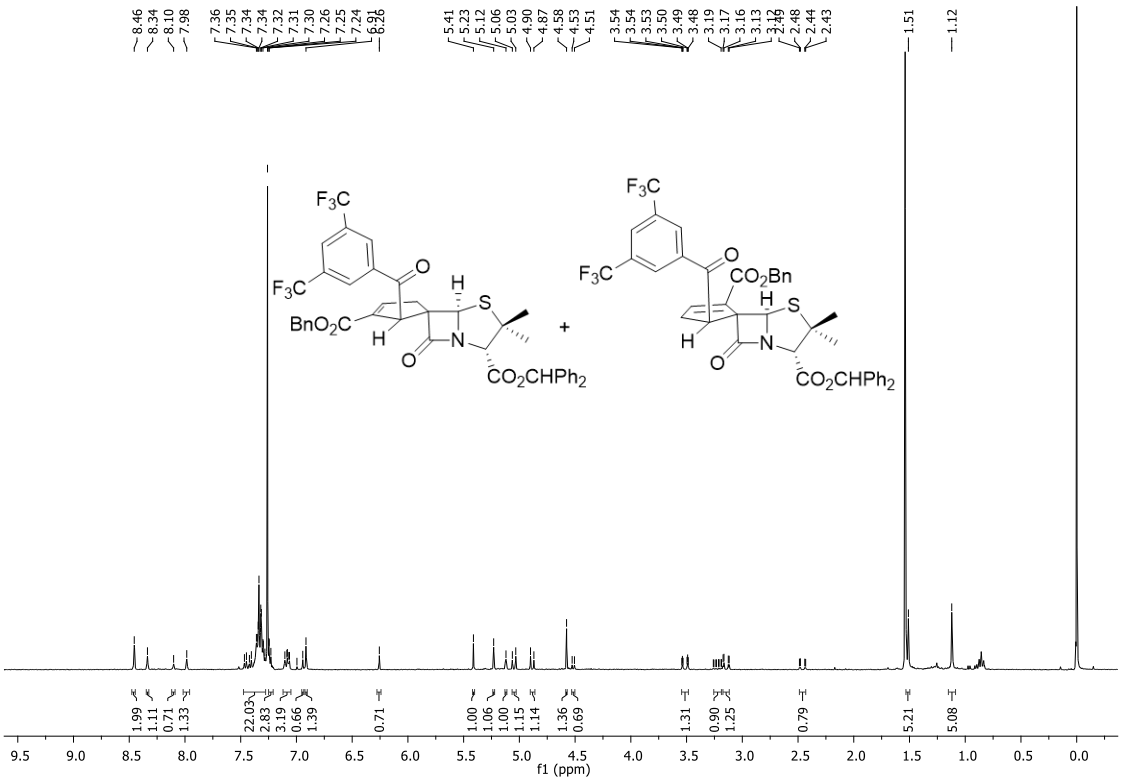


Figure S35: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of mixture **2.28g:2.29g**.

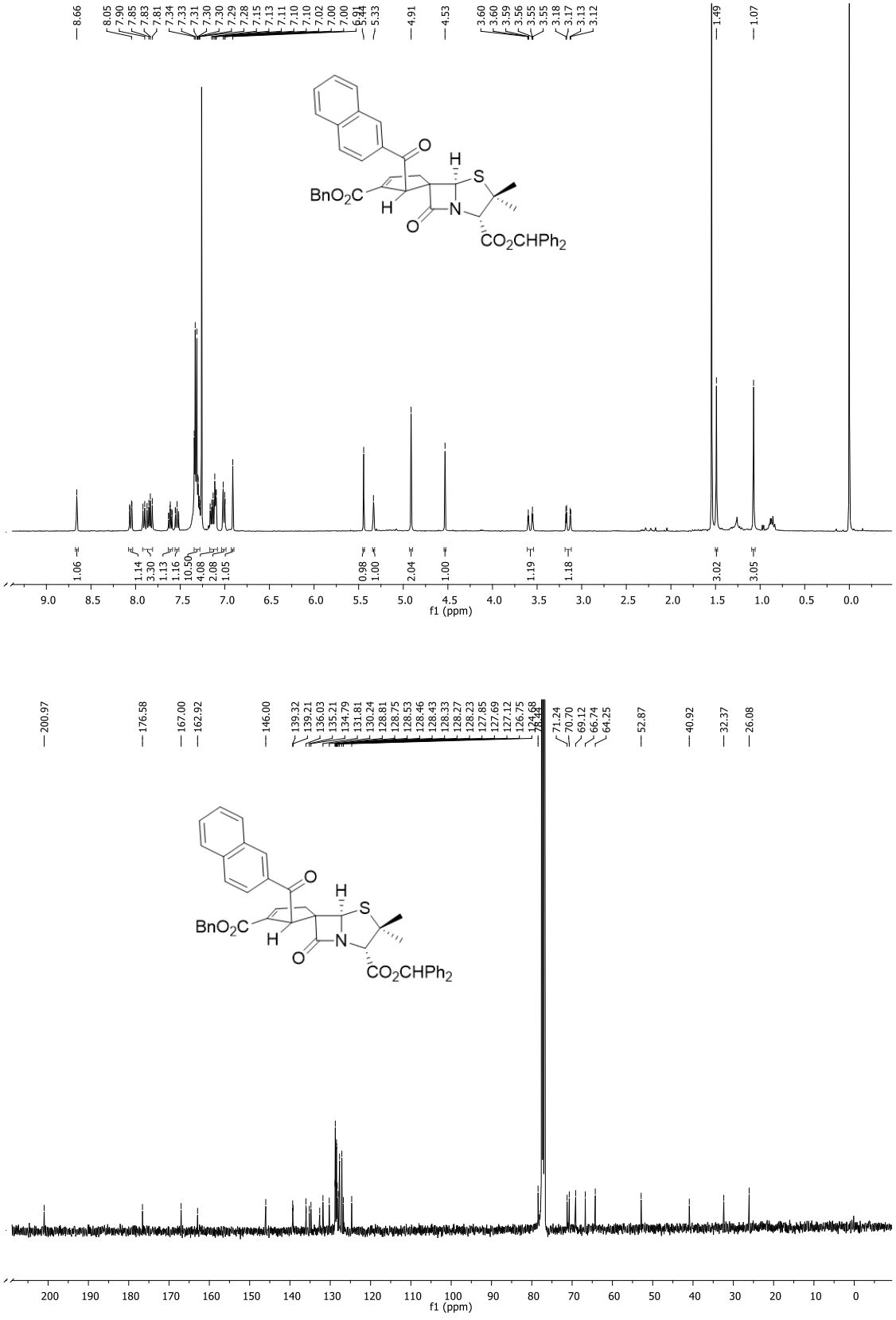


Figure S36: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **2.28h**.

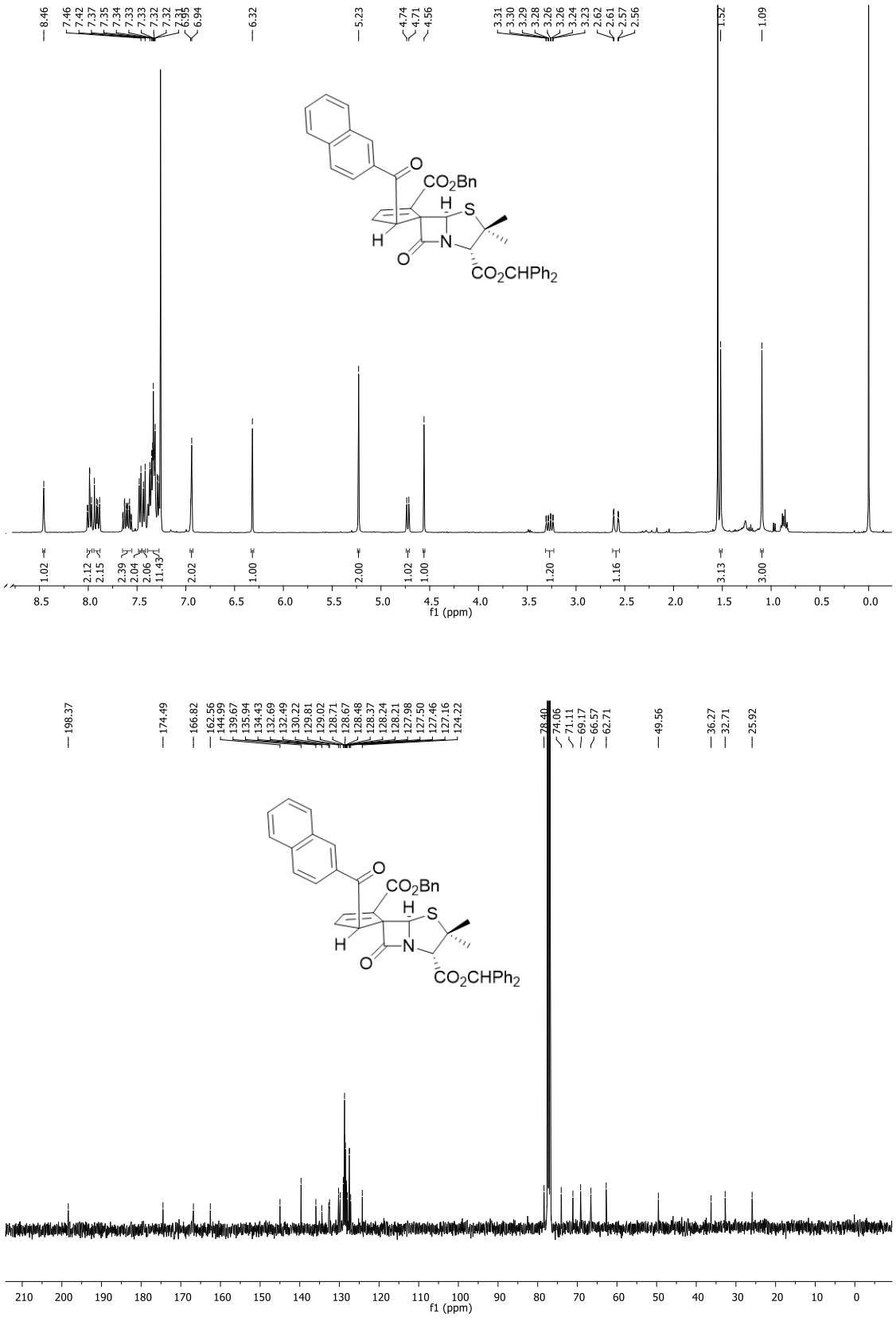


Figure S37: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **2.29h**.

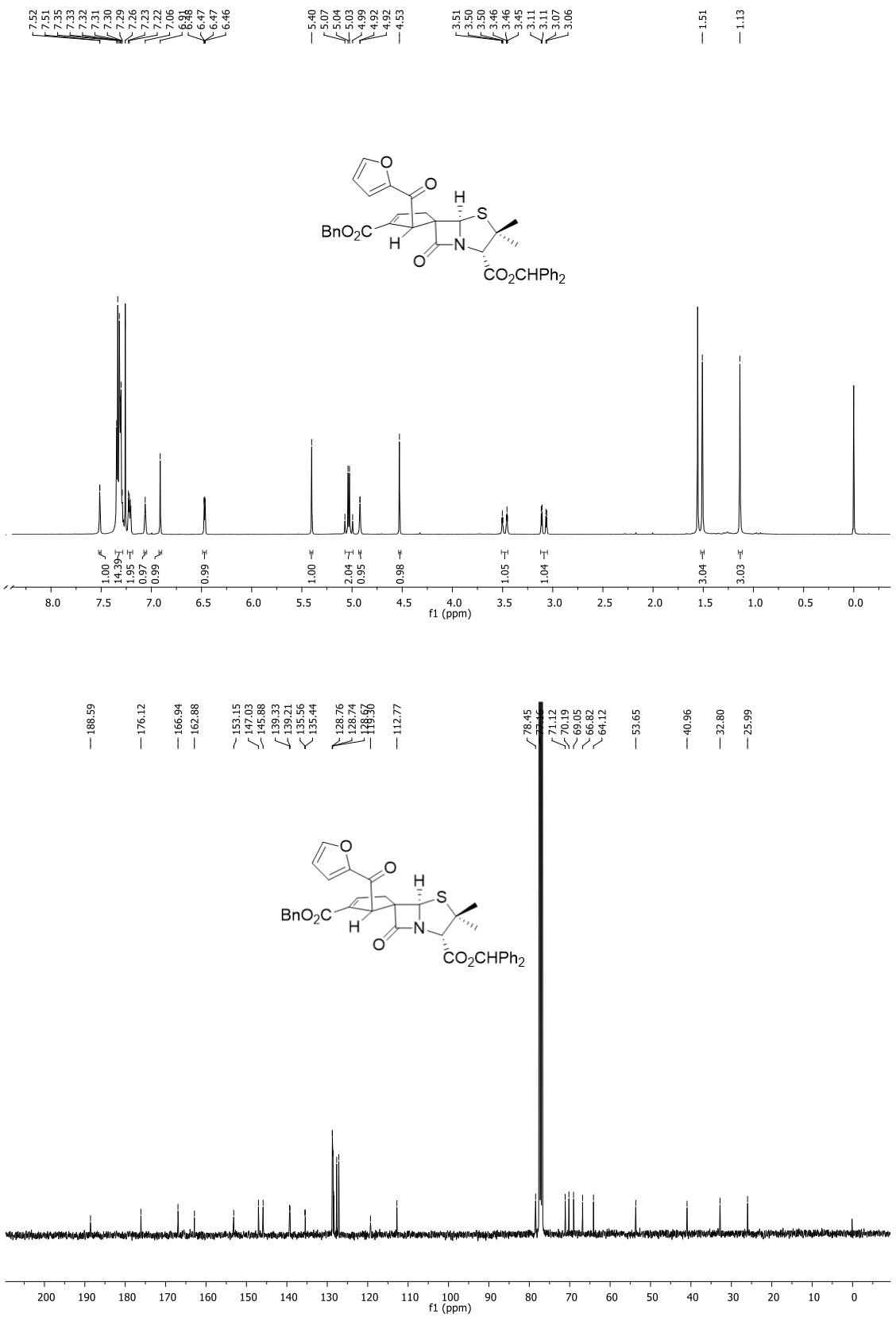


Figure S38: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **2.28i**.

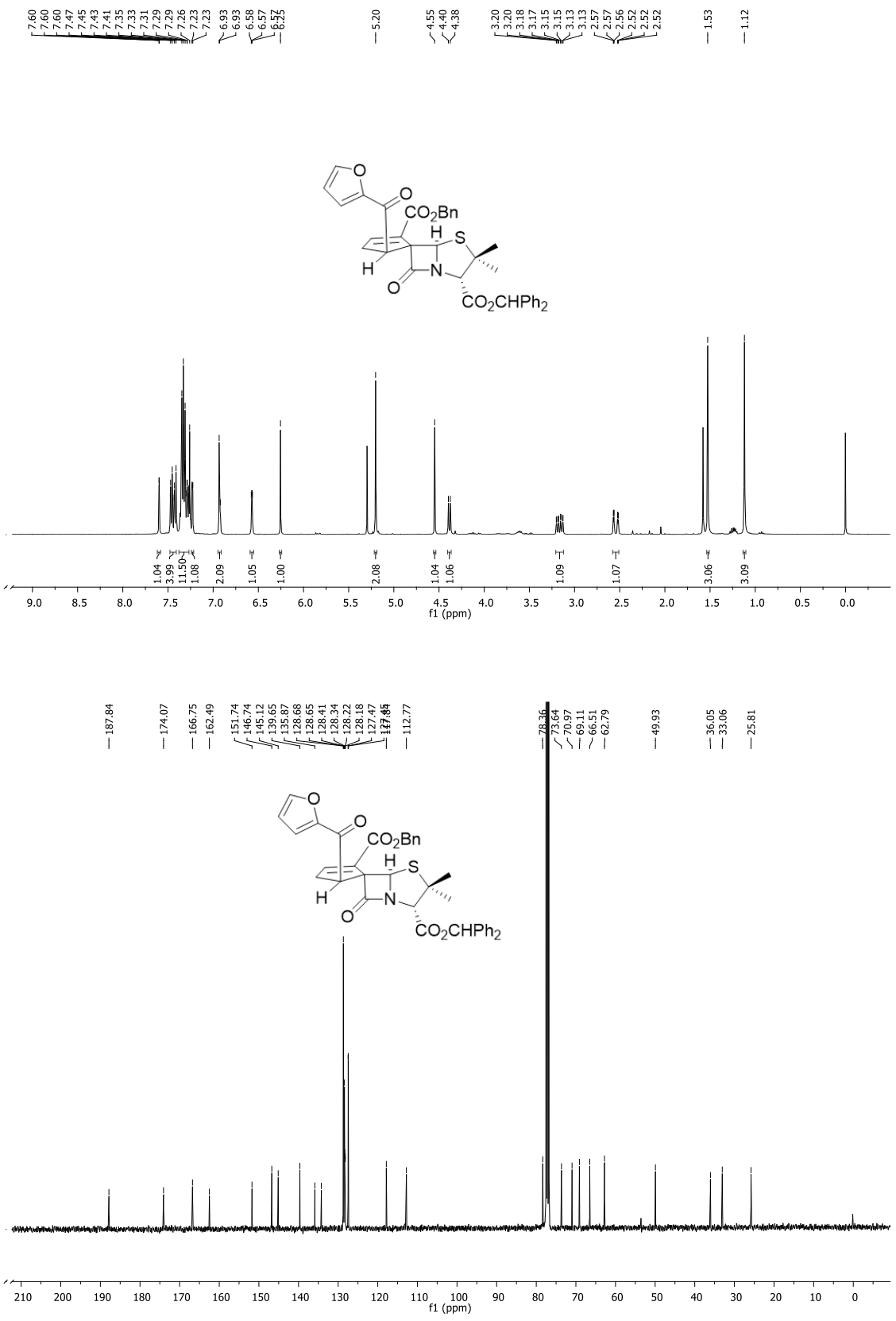


Figure S39: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **2.29i**.

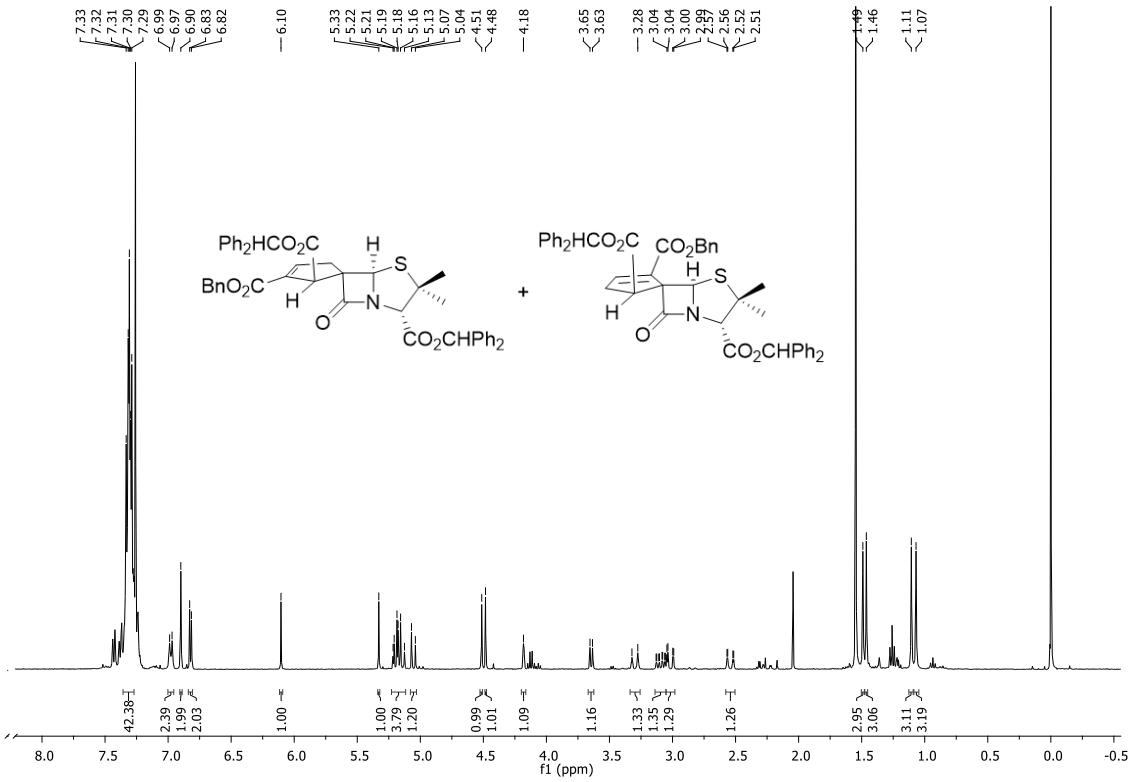


Figure S40: ^{1}H NMR spectrum ($CDCl_3$) of mixture **2.28j: 2.29j**.

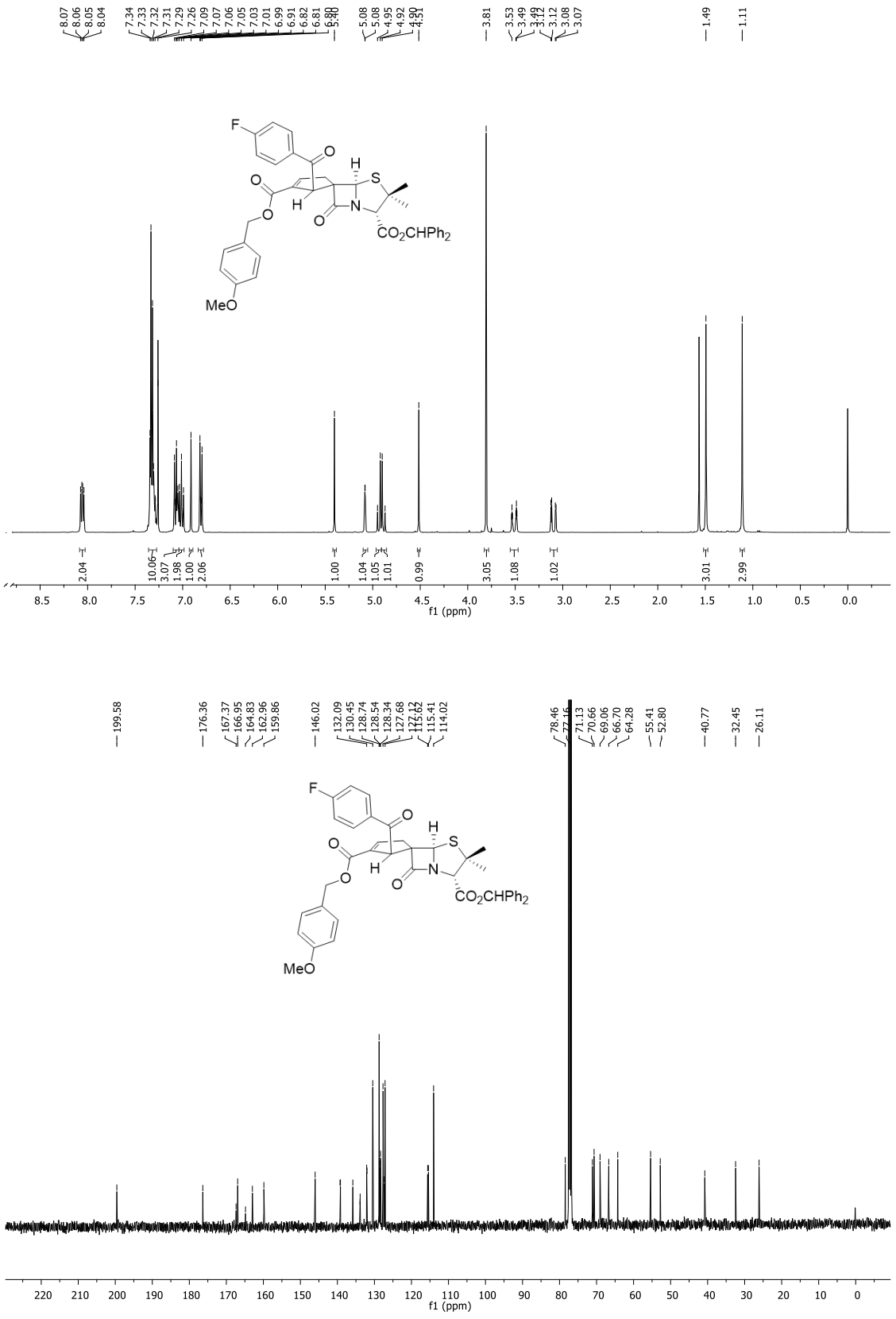


Figure S41: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **2.31a**.

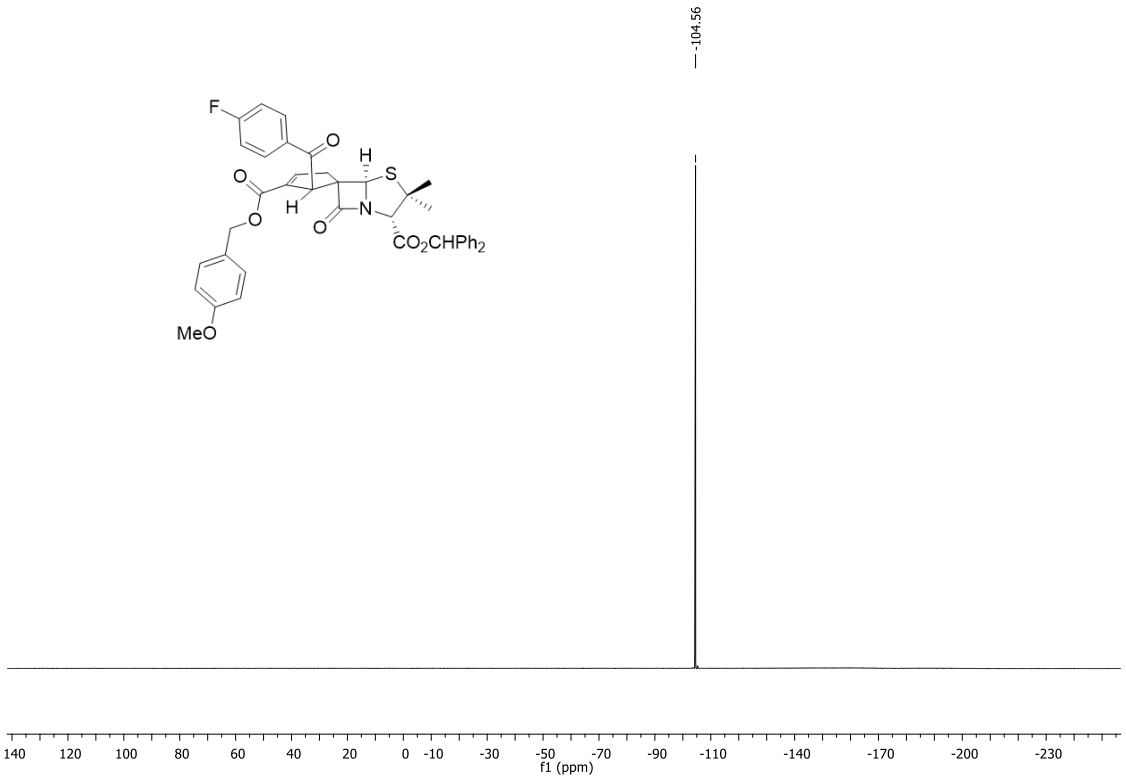


Figure S42: ^{19}F NMR spectrum ($CDCl_3$) of compound **2.31a**.

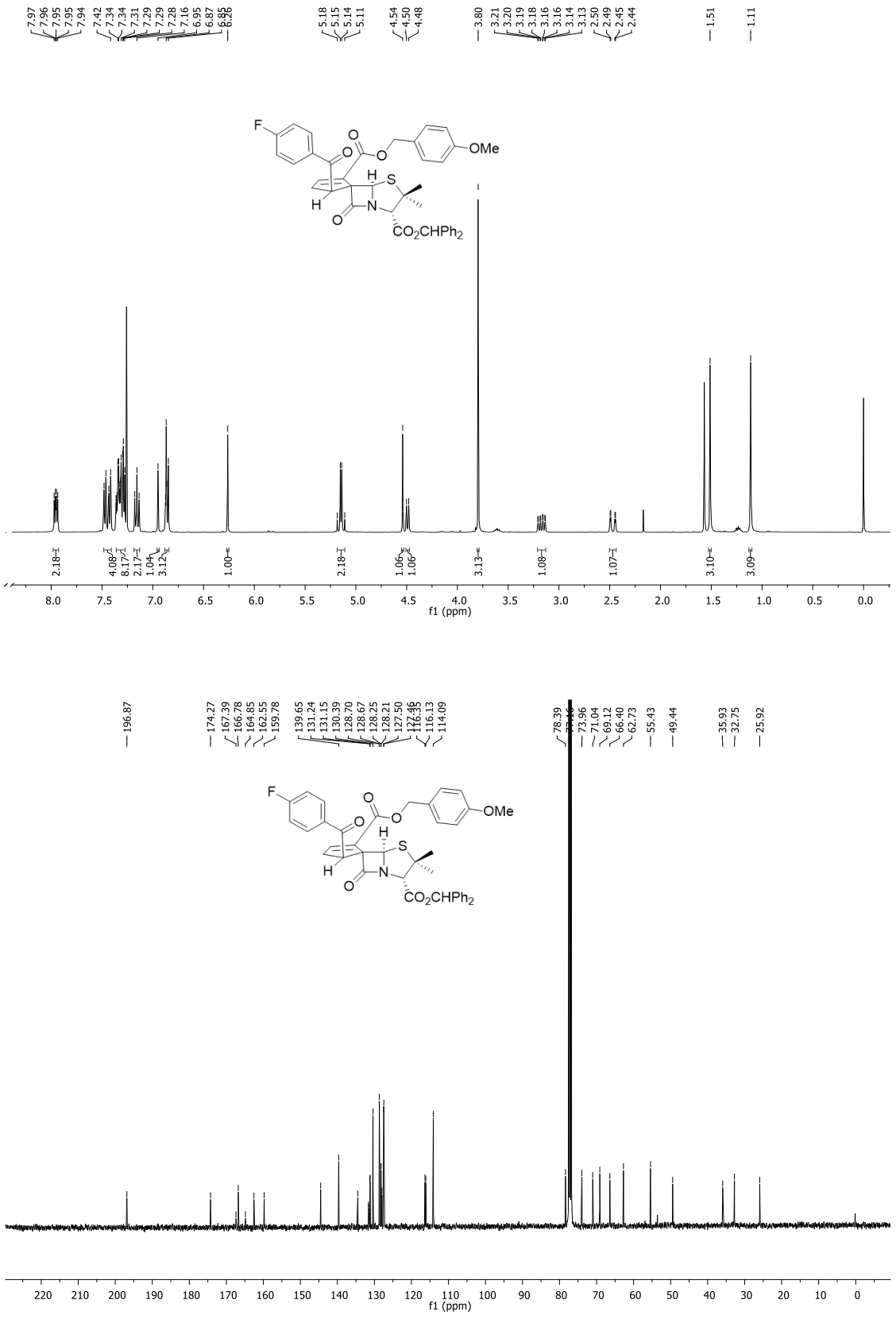


Figure S43: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **2.32a**.

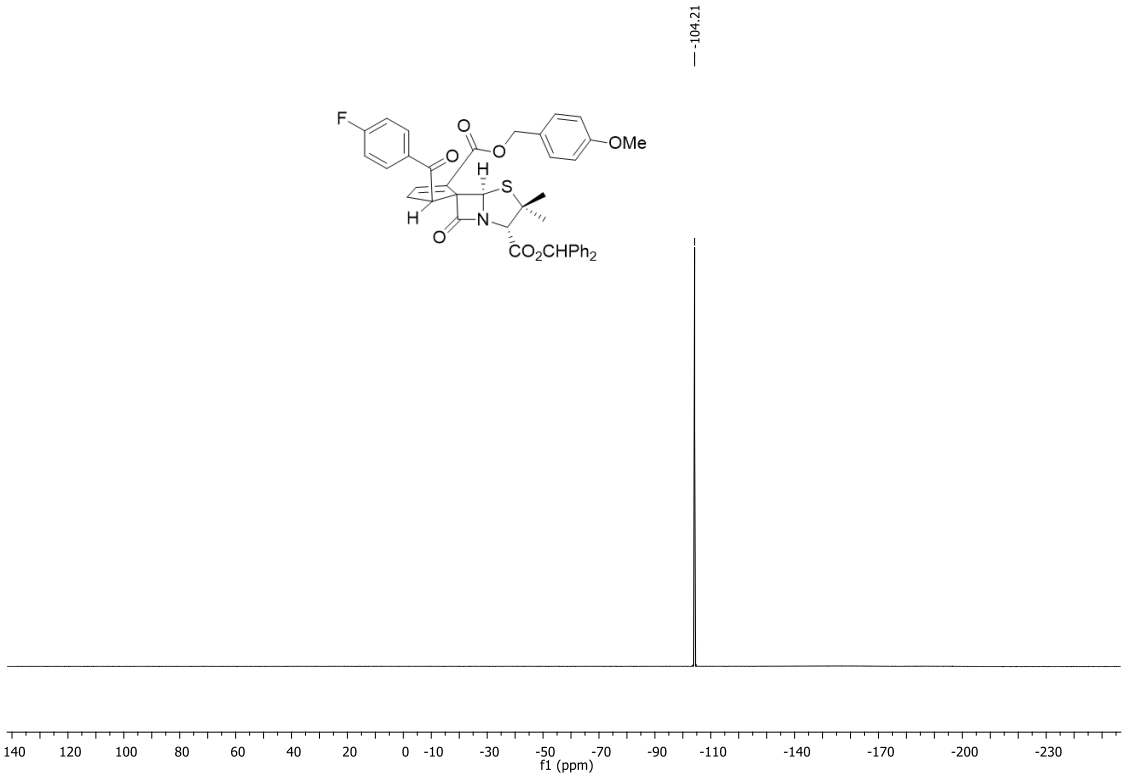


Figure S44: ^{19}F NMR spectrum ($CDCl_3$) of compound **2.32a**.

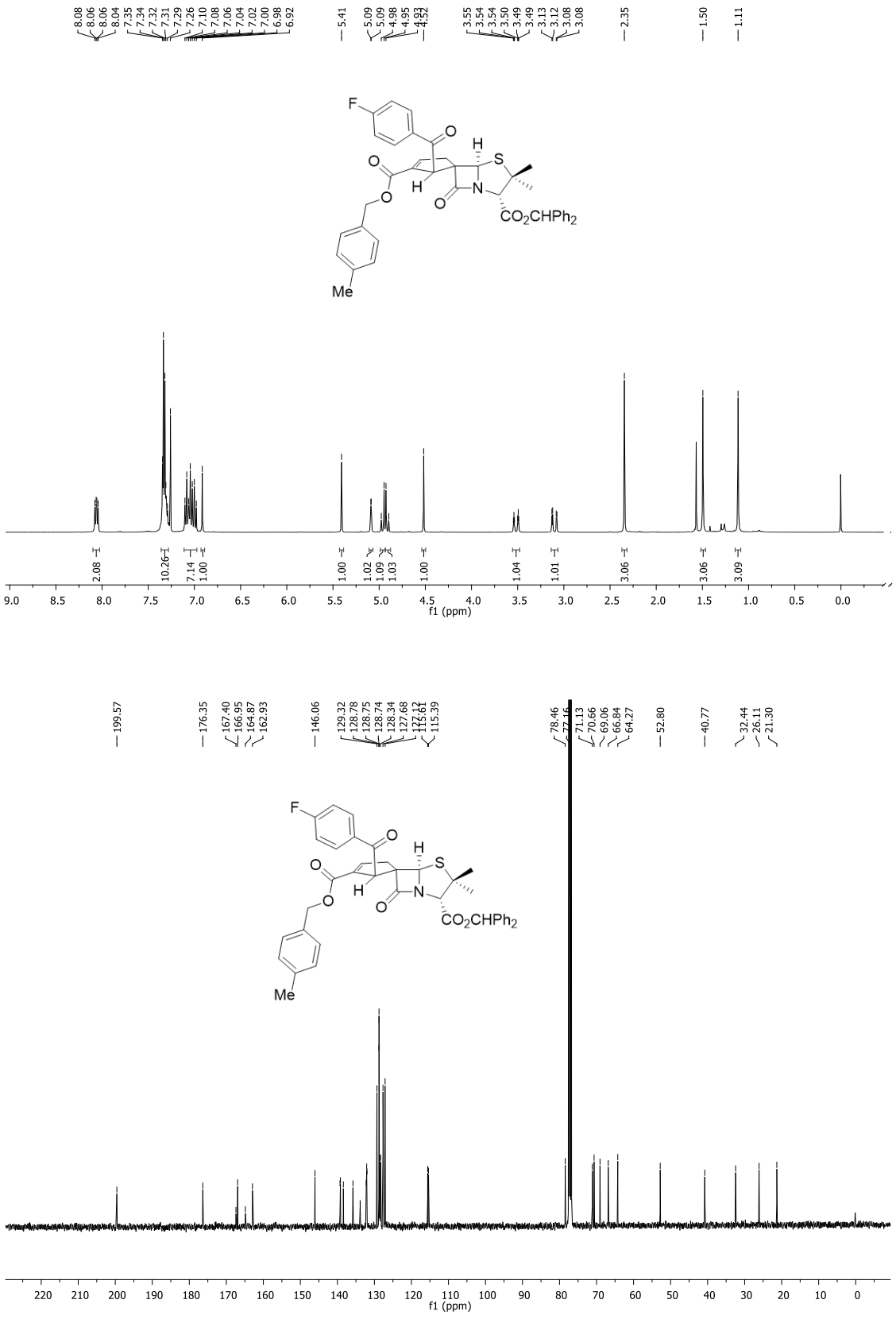


Figure S45: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **2.31b**.

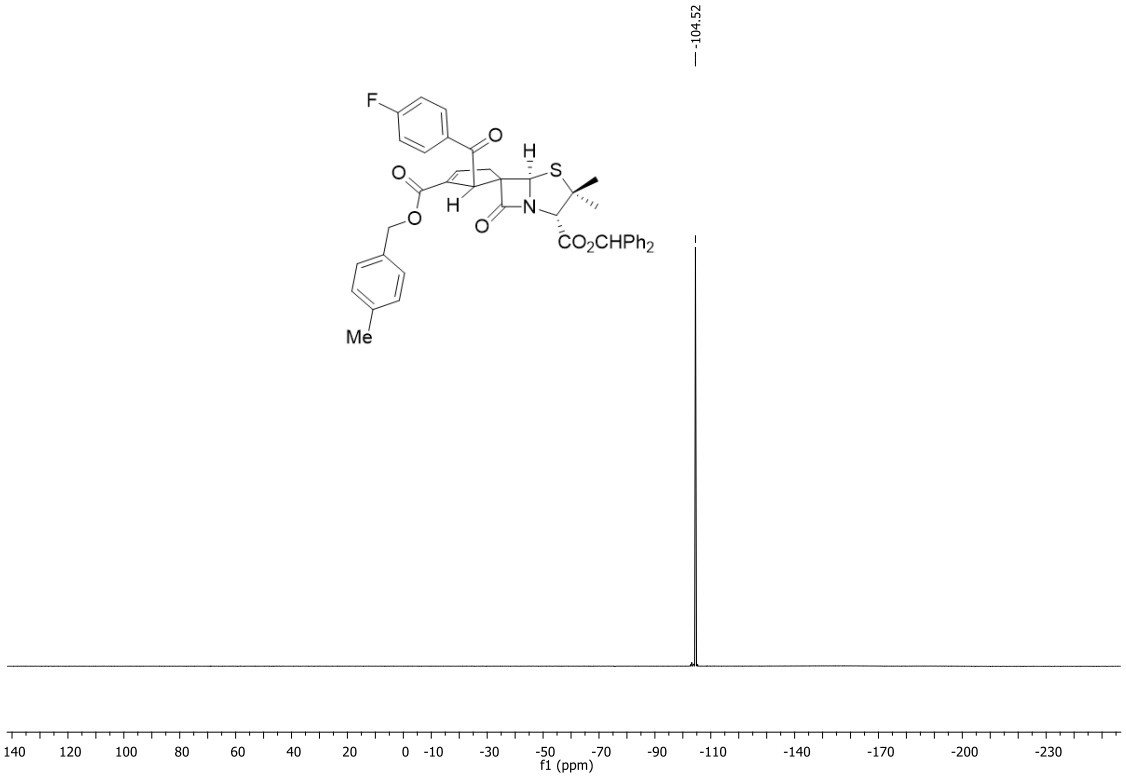


Figure S46: ^{19}F NMR spectrum ($CDCl_3$) of compound **2.31b**.

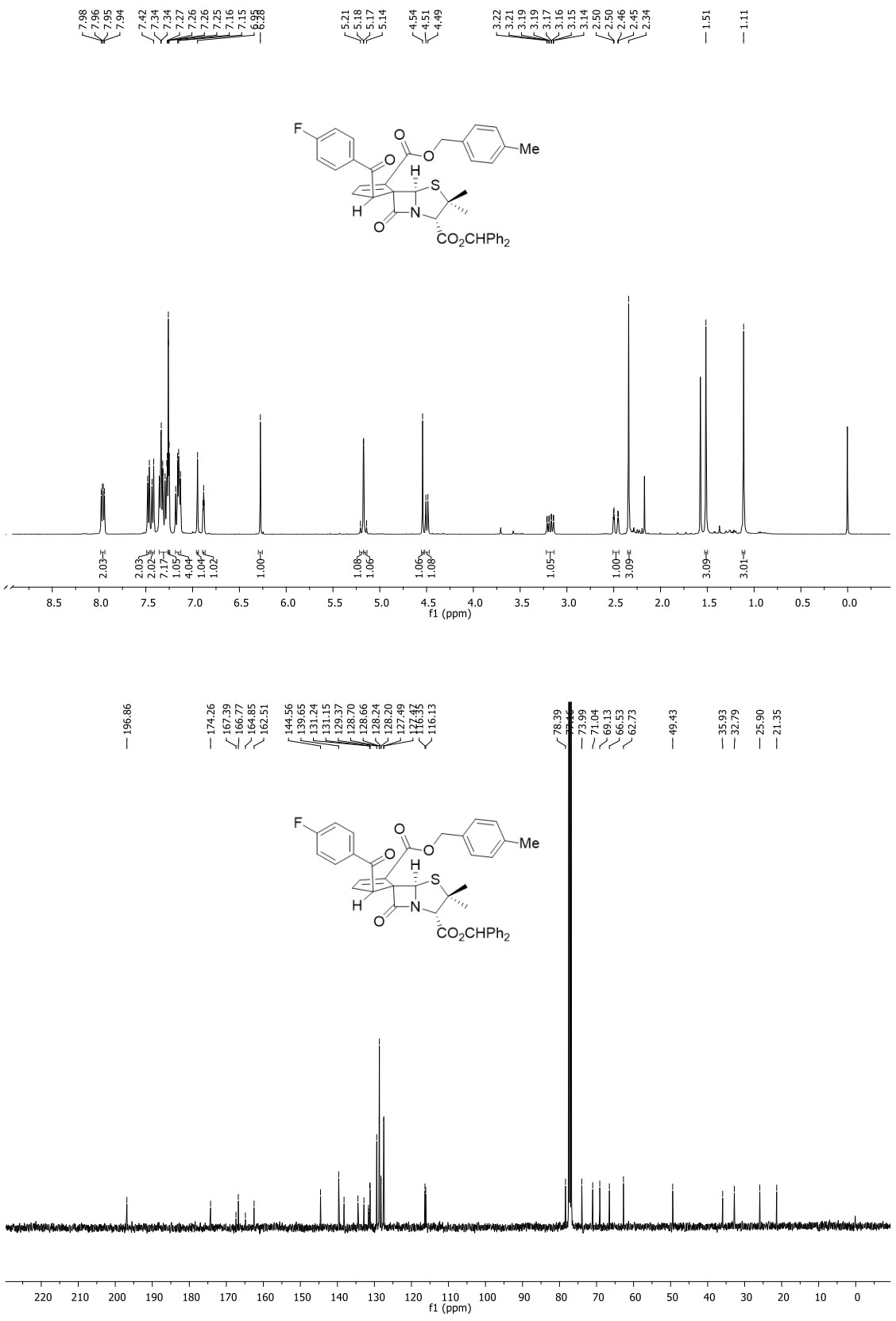


Figure S47: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **2.32b**.

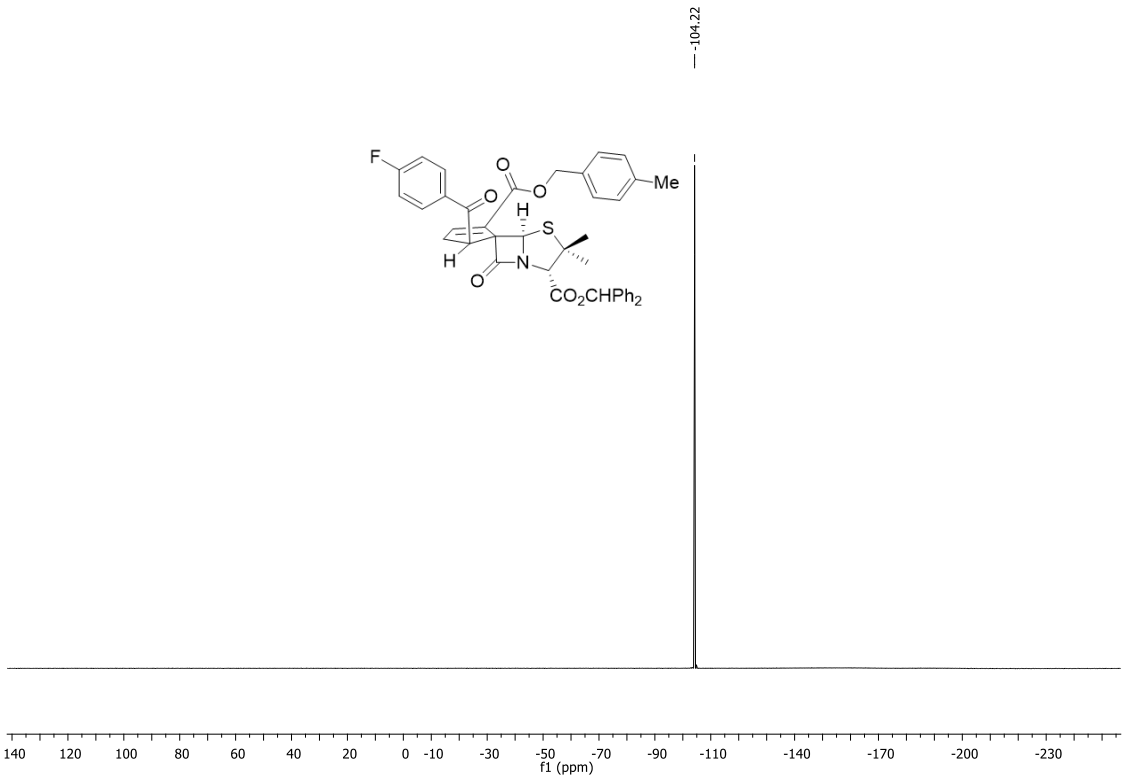


Figure S48: ^{19}F NMR spectrum ($CDCl_3$) of compound **2.32b**.

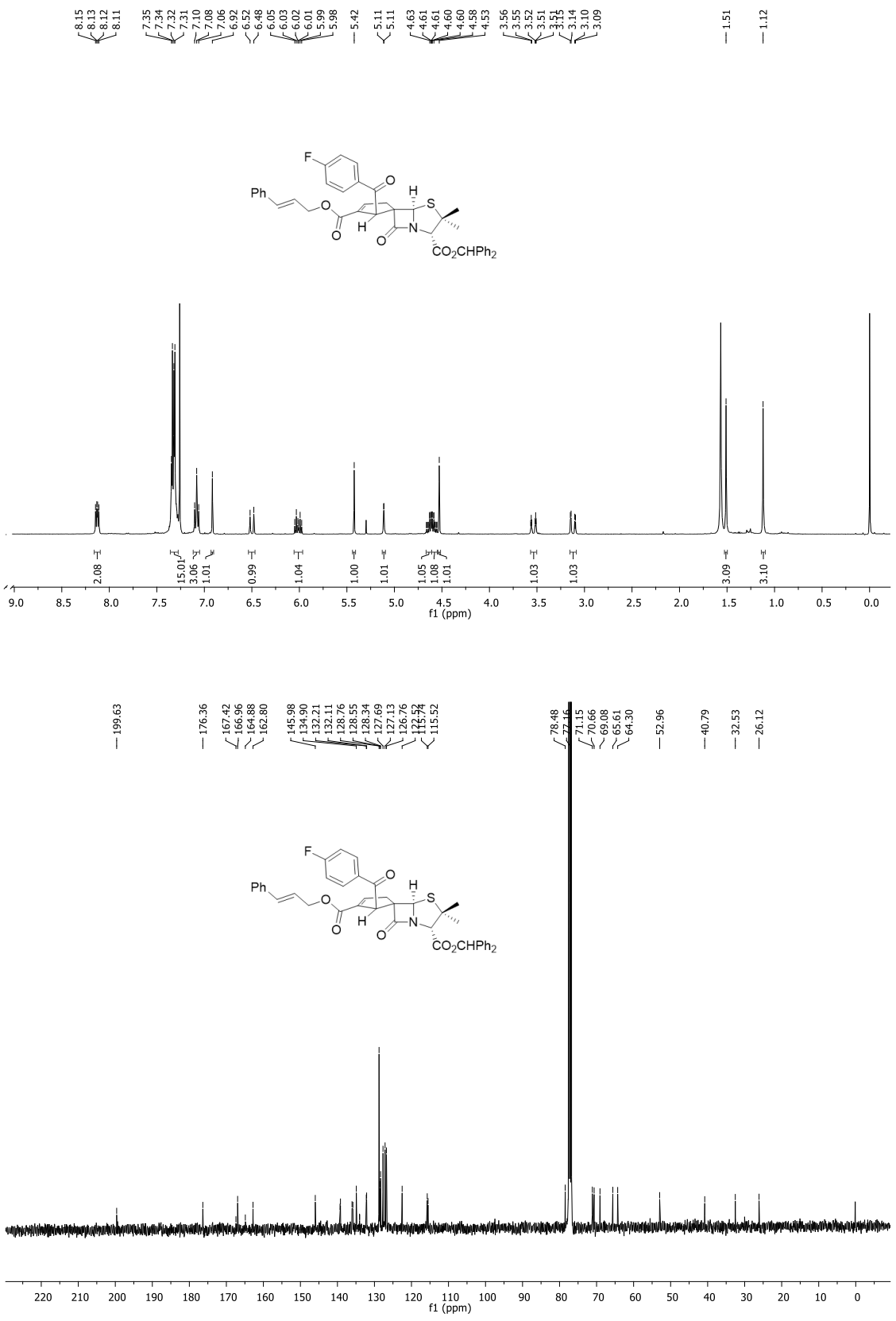


Figure S49: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **2.31c**.

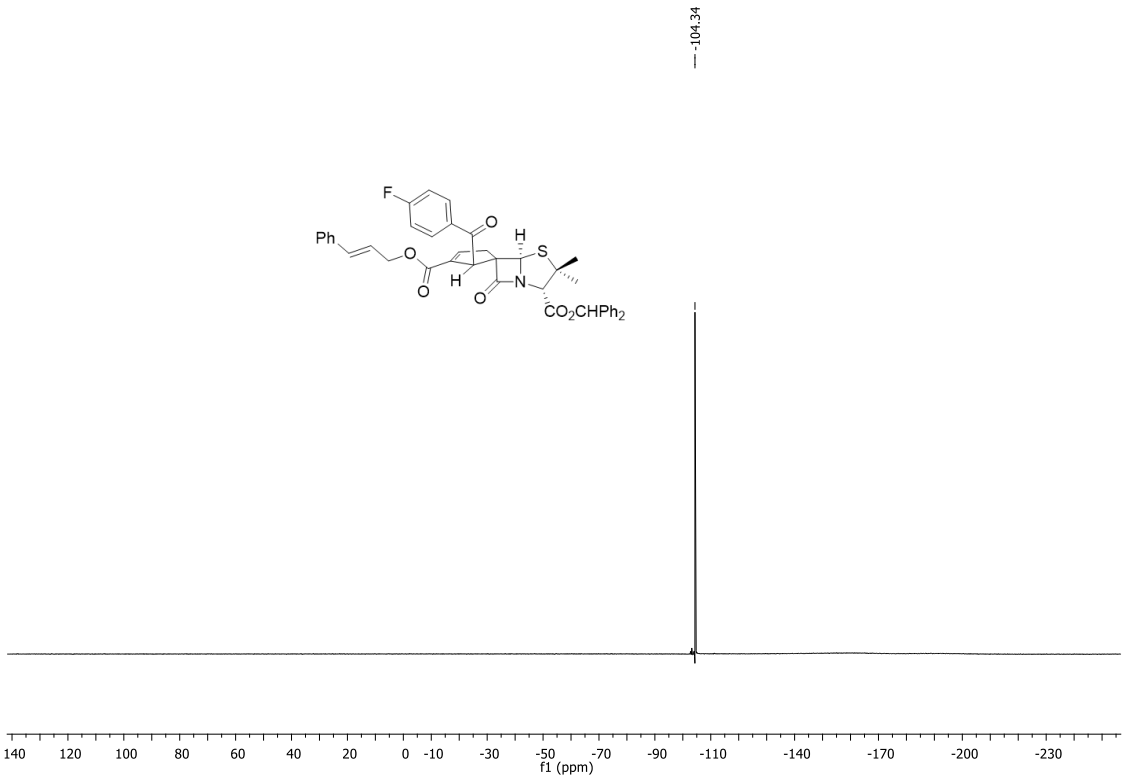


Figure S50: ^{19}F NMR spectrum ($CDCl_3$) of compound **2.31c**.

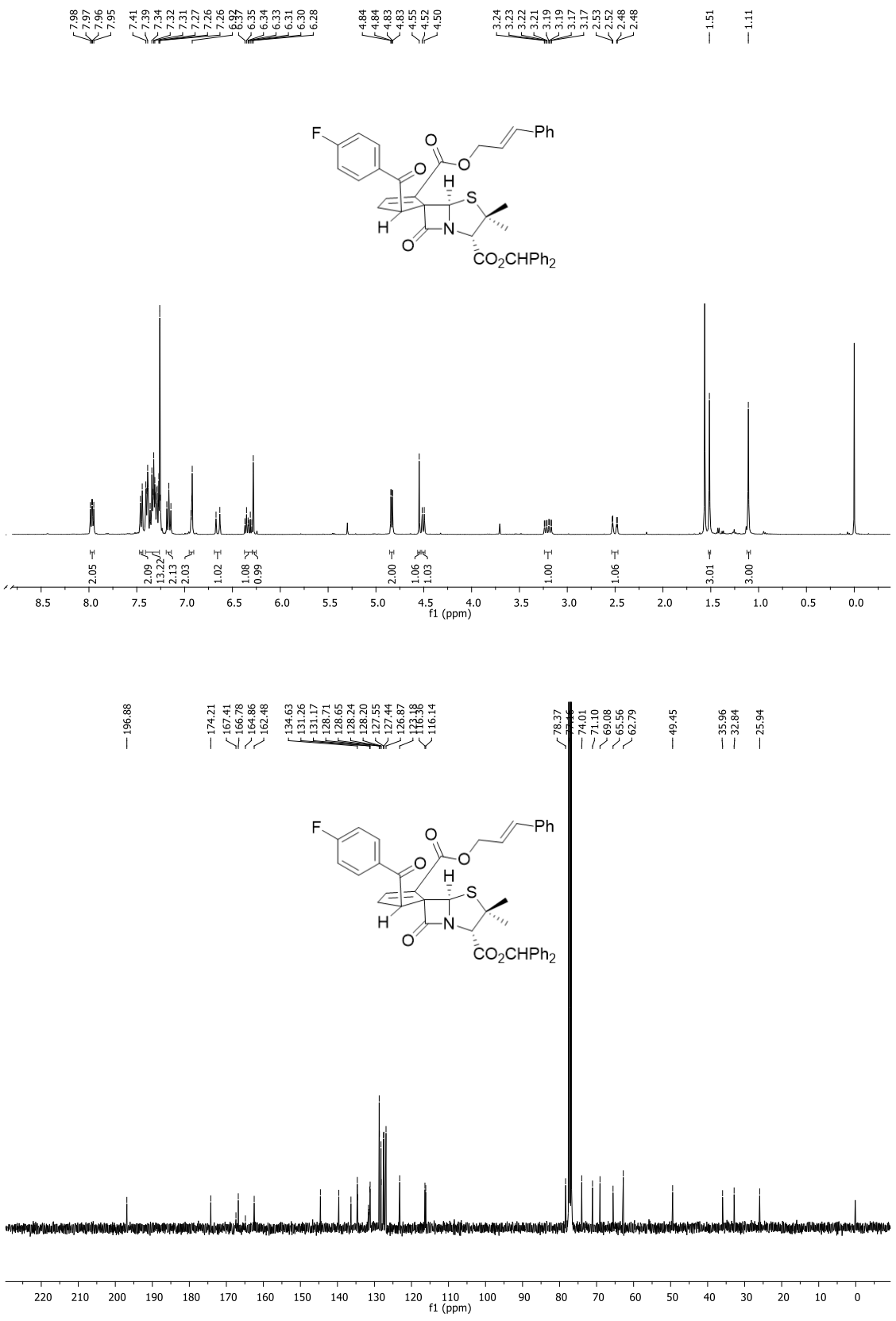


Figure S51: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **2.32c**.

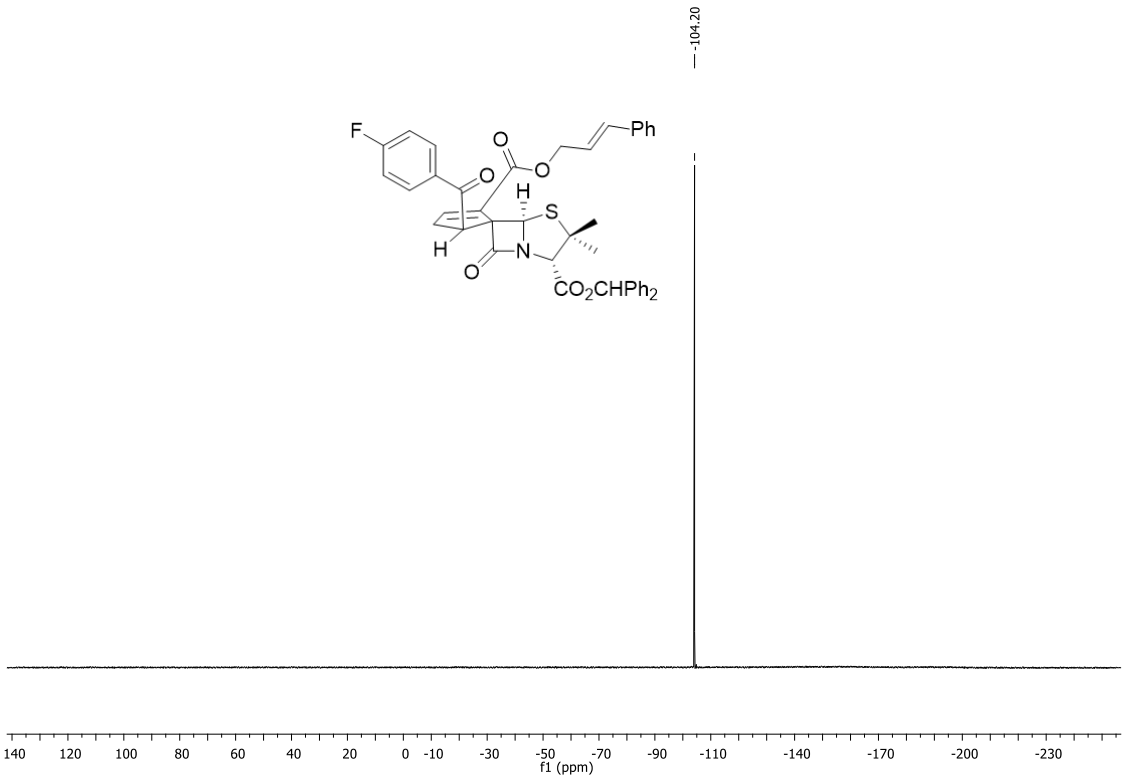


Figure S52: ^{19}F NMR spectrum ($CDCl_3$) of compound **2.32c**.

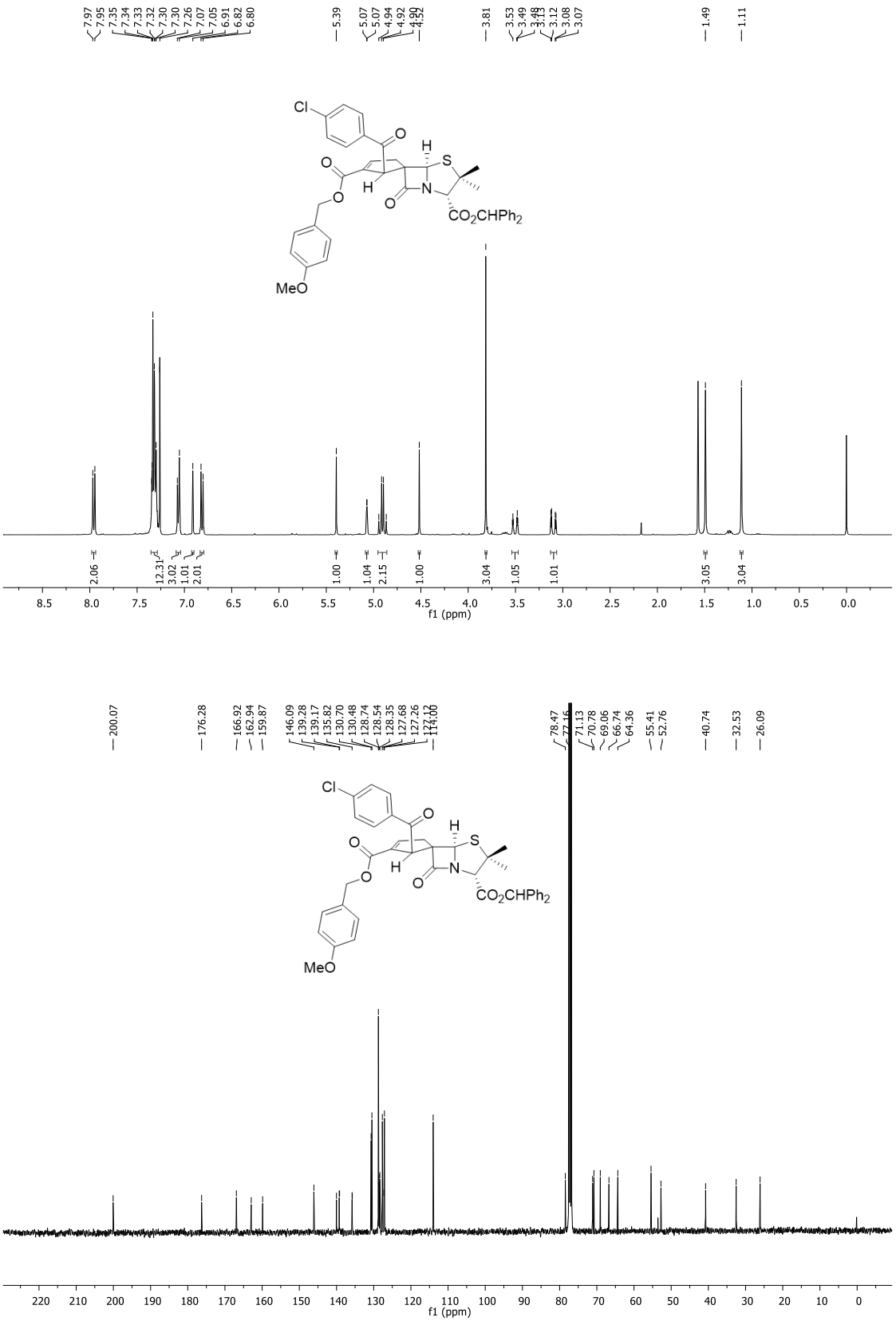


Figure S53: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **2.31d**.

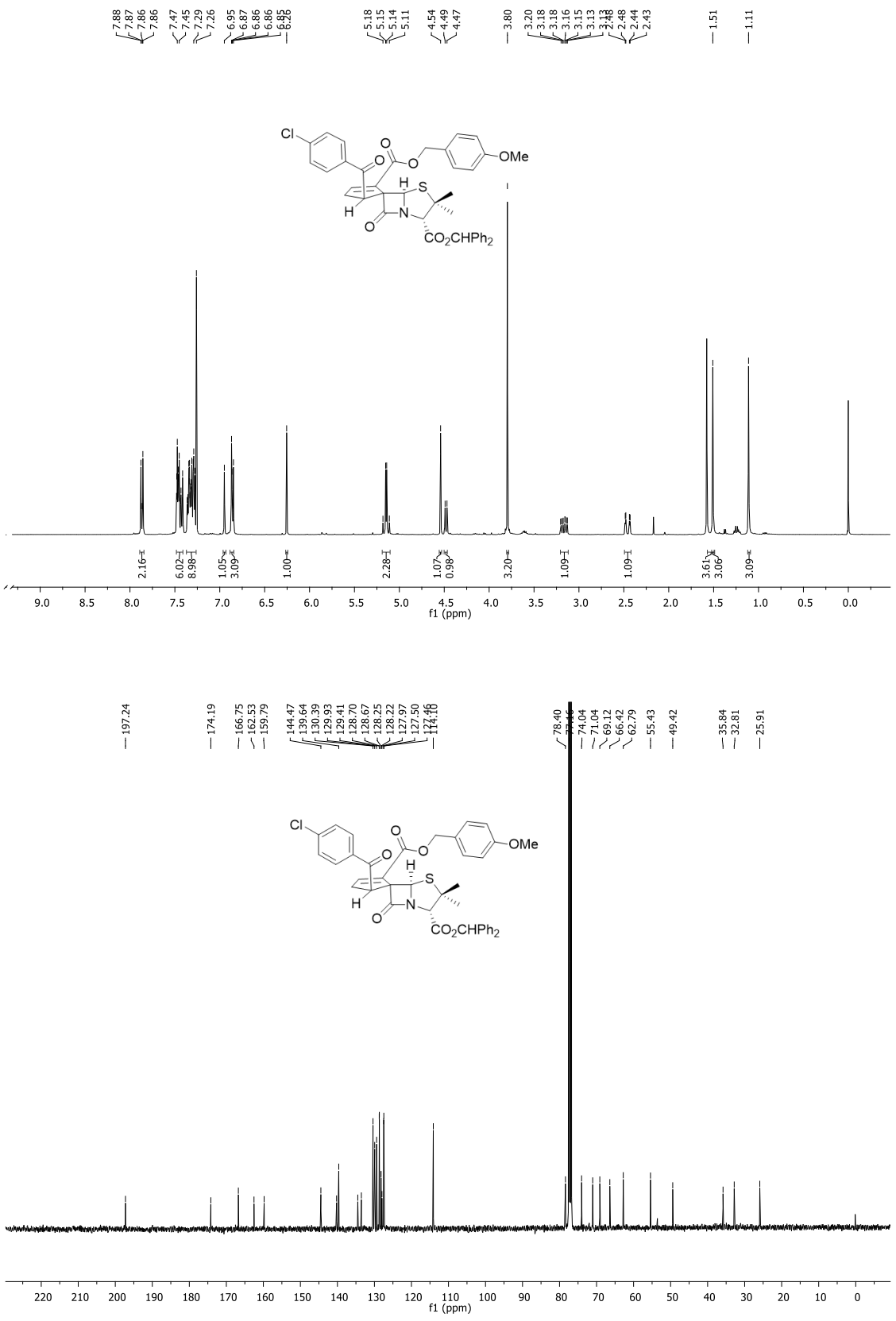


Figure S54: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **2.32d**.

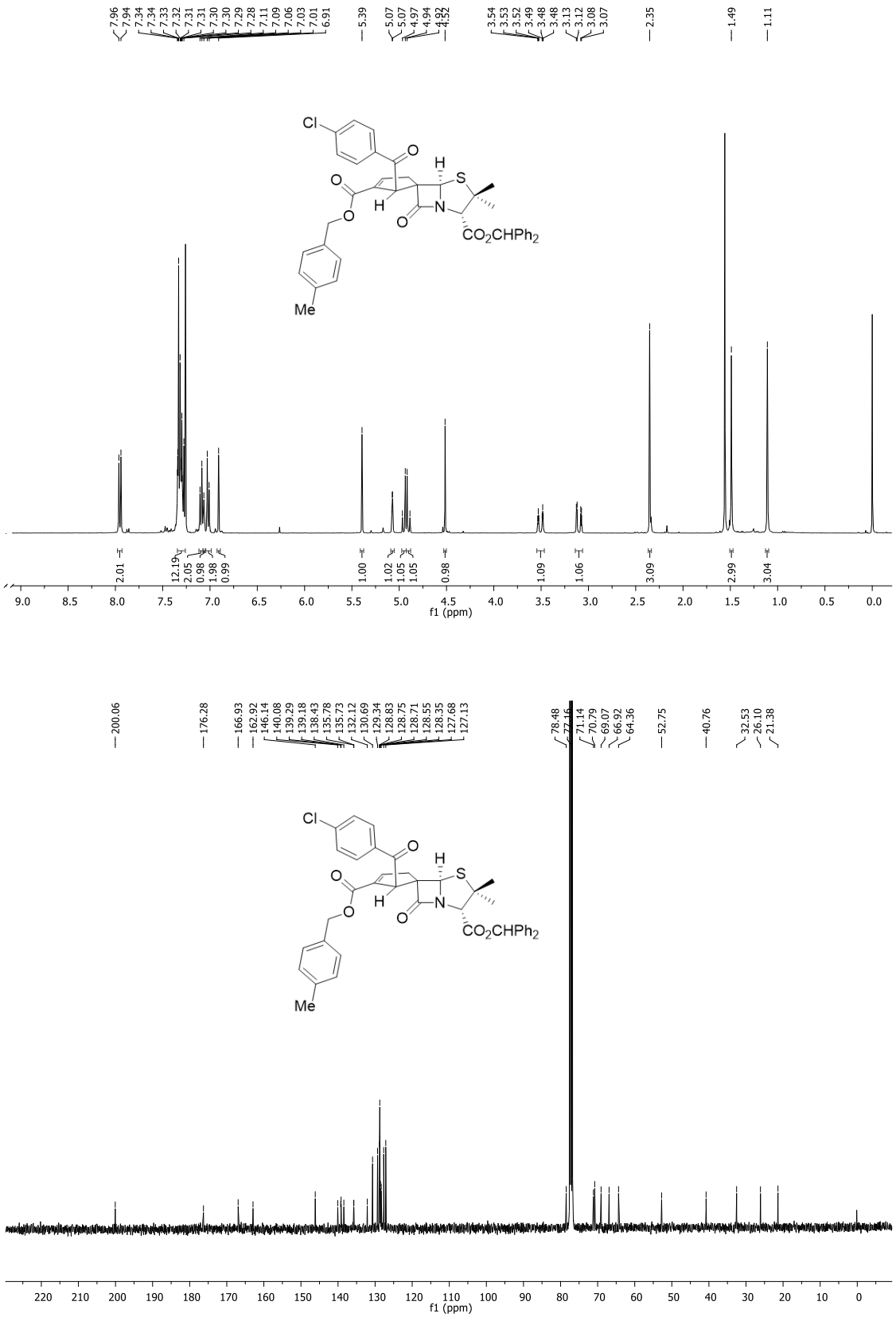


Figure S55: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **2.31e**.

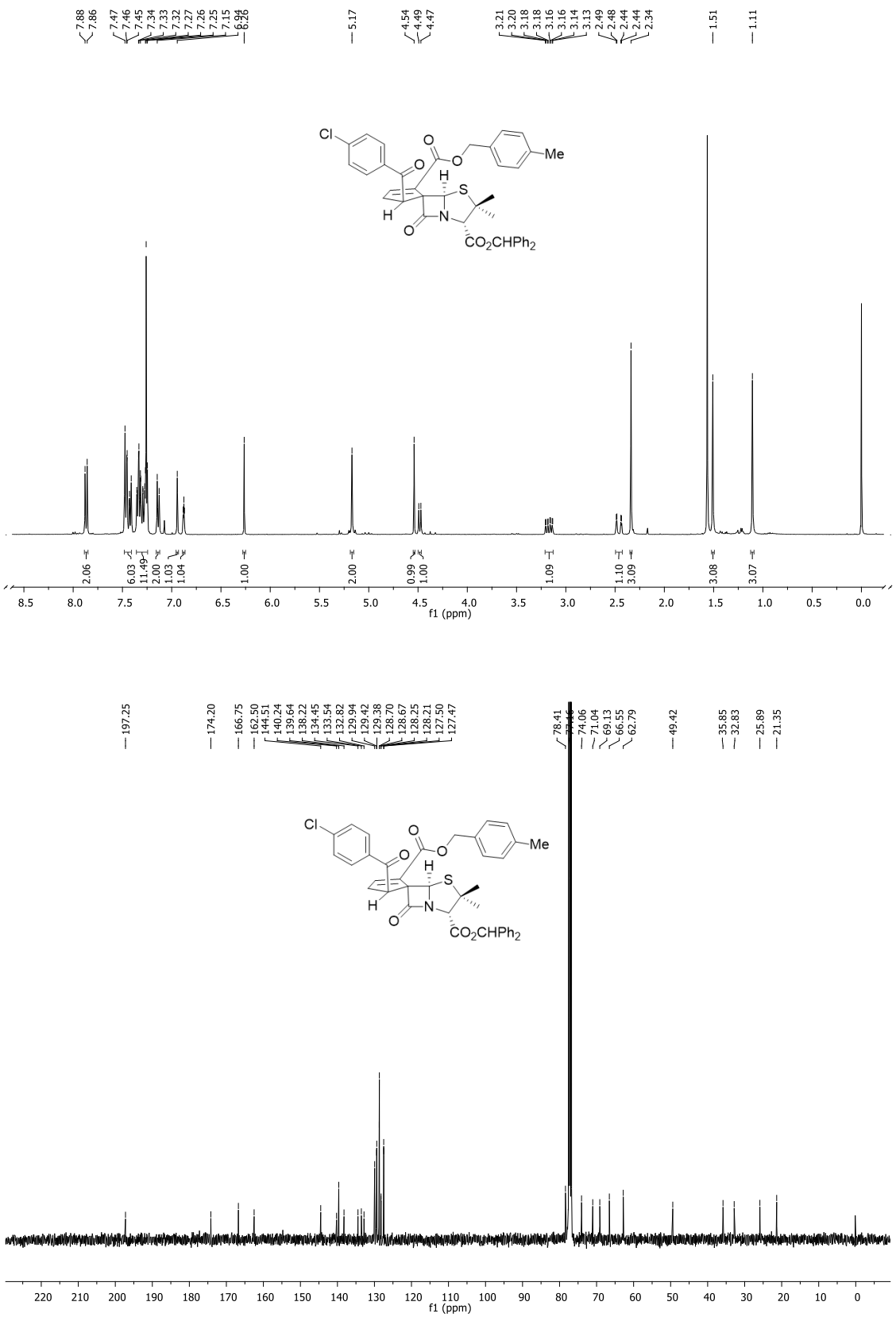


Figure S56: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **2.32e**.

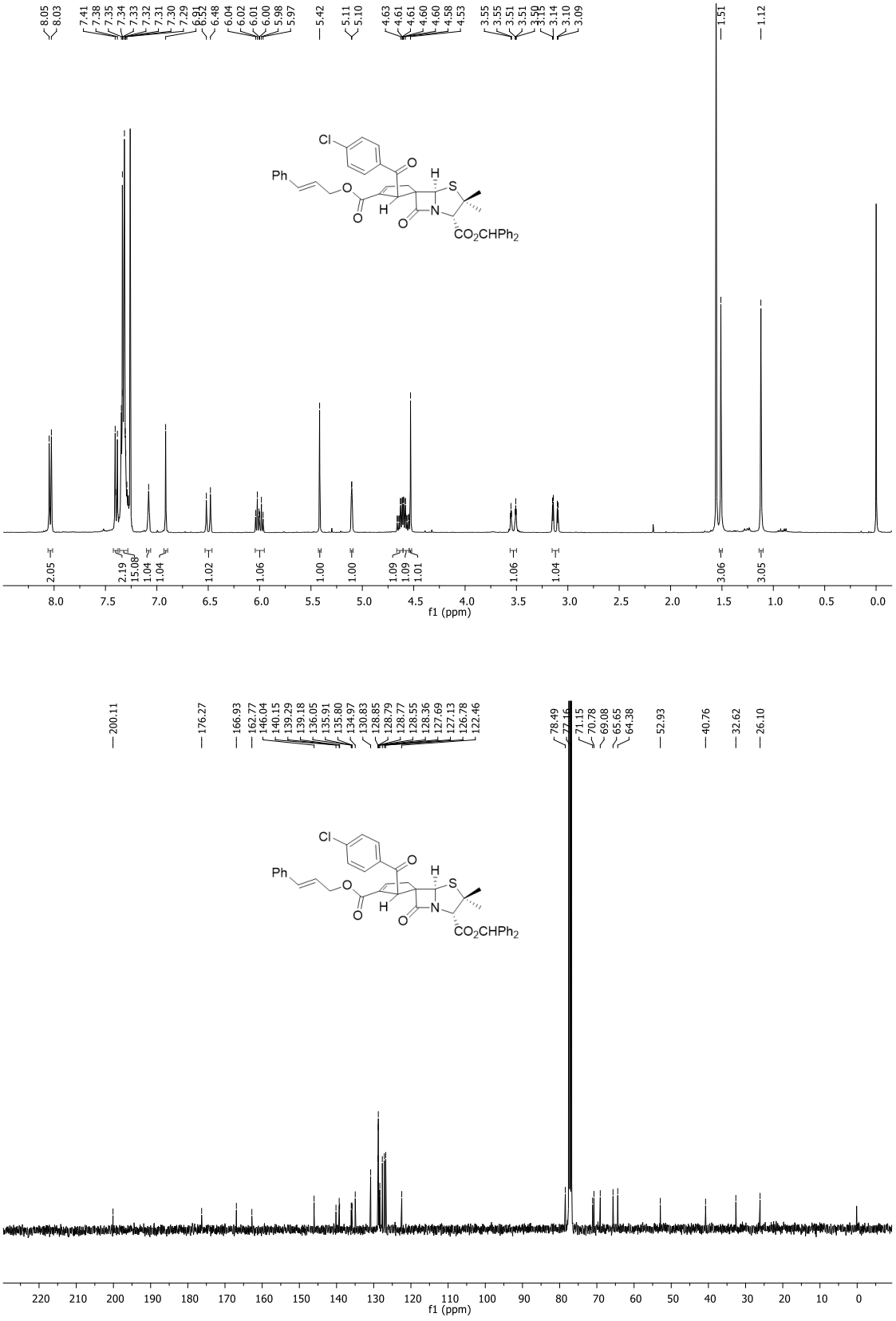


Figure S57: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **2.31f**.

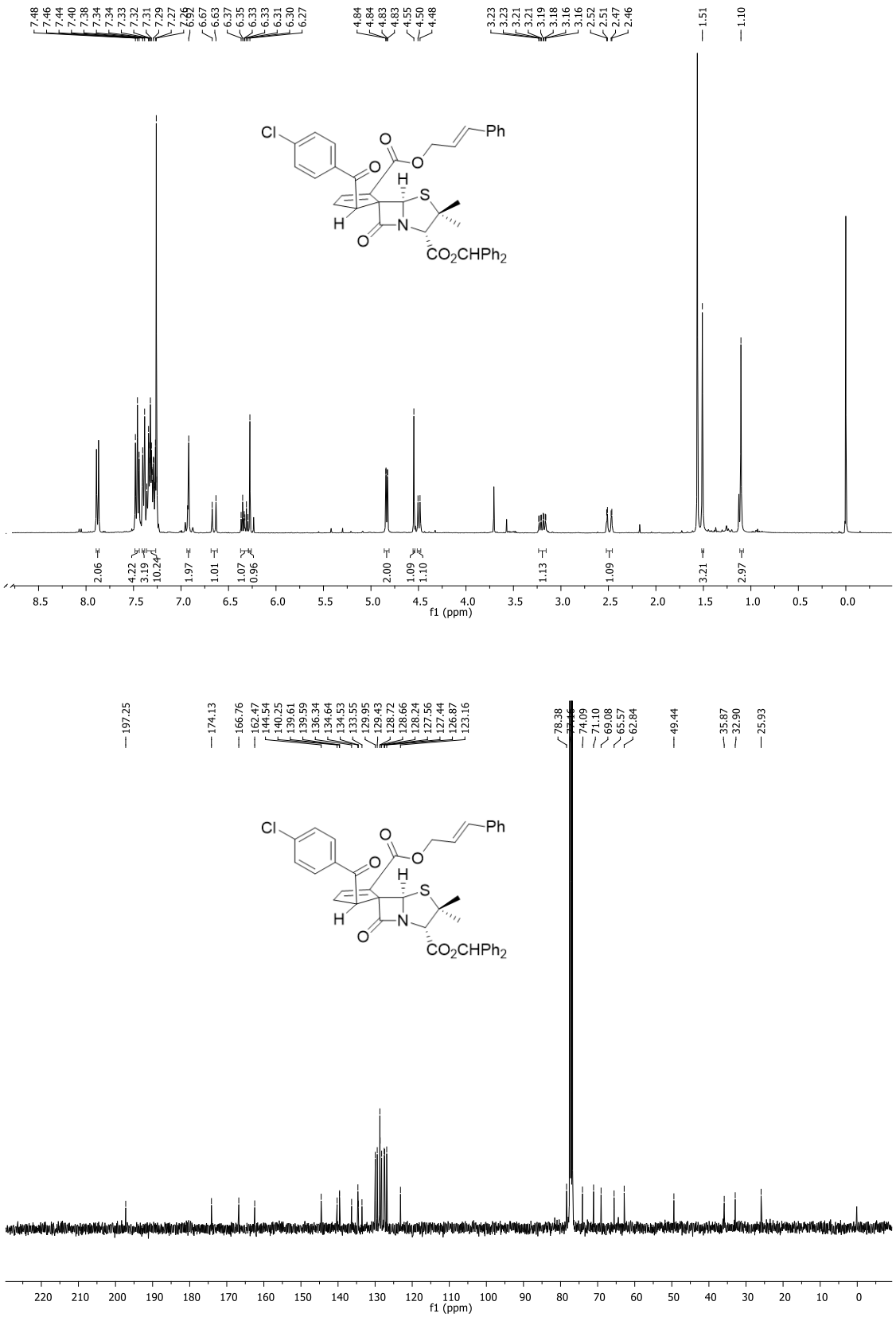


Figure S58: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **2.32f**.

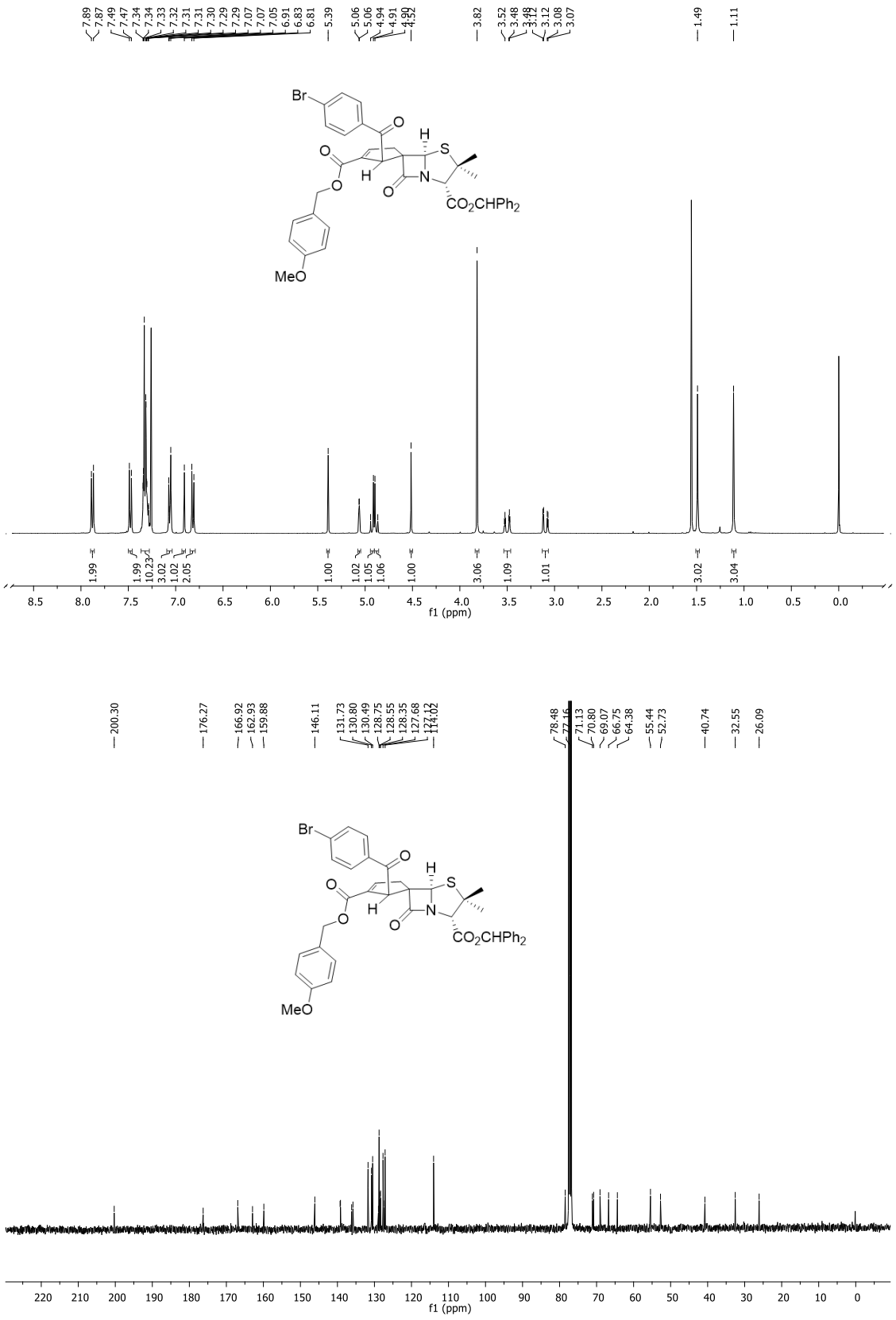


Figure S59: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **2.31g**.

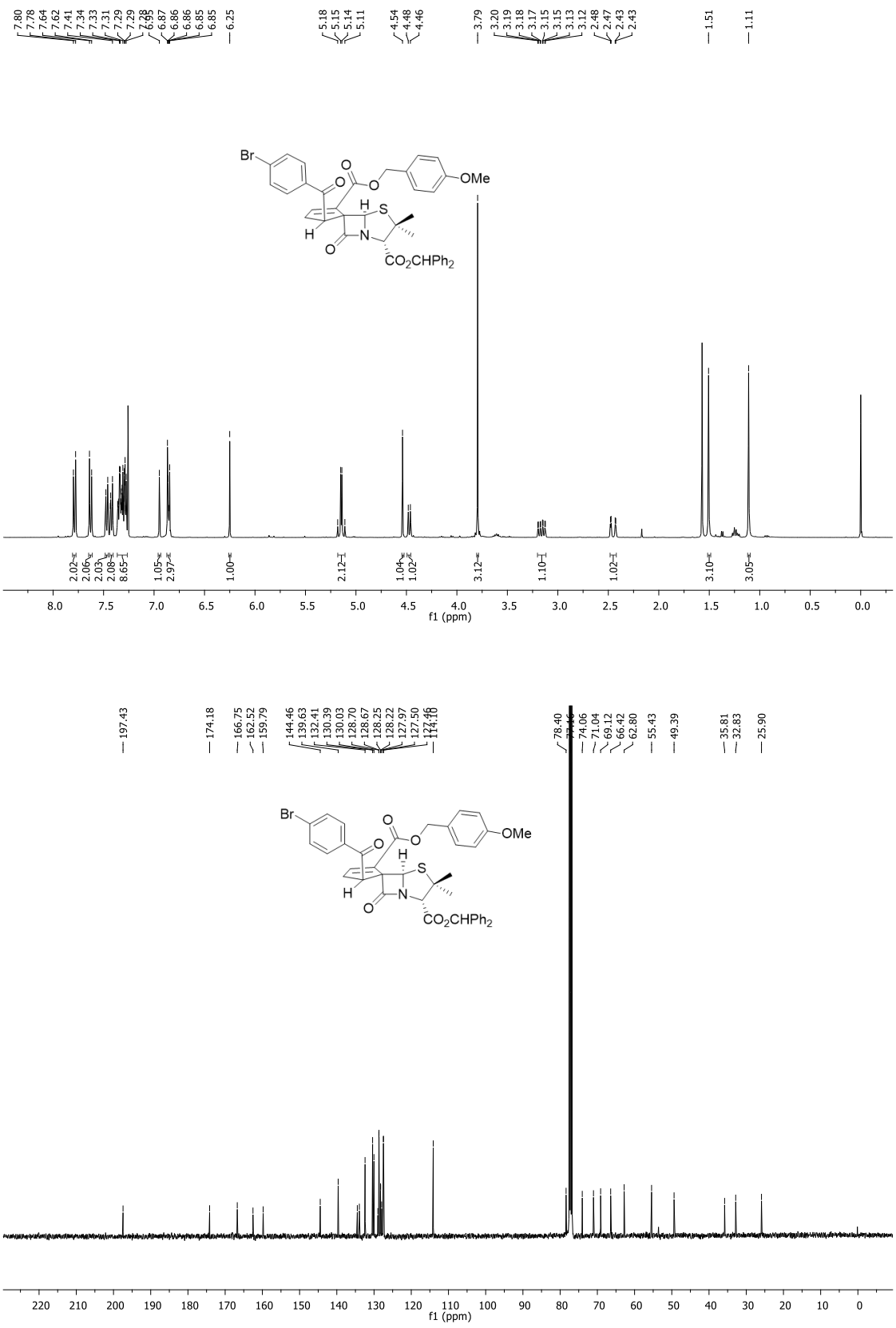


Figure S60: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **2.32g**.

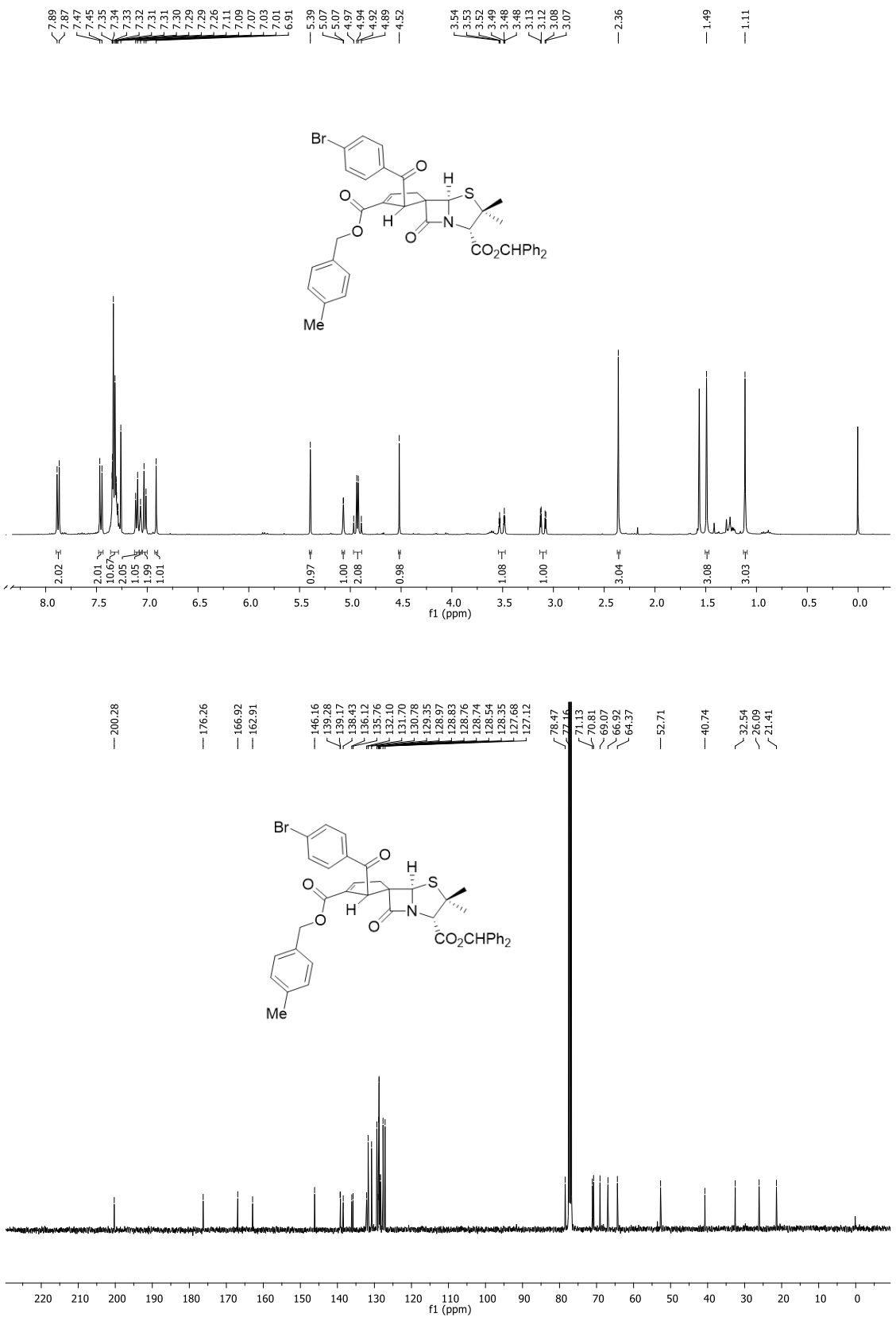


Figure S61: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **2.31h**.

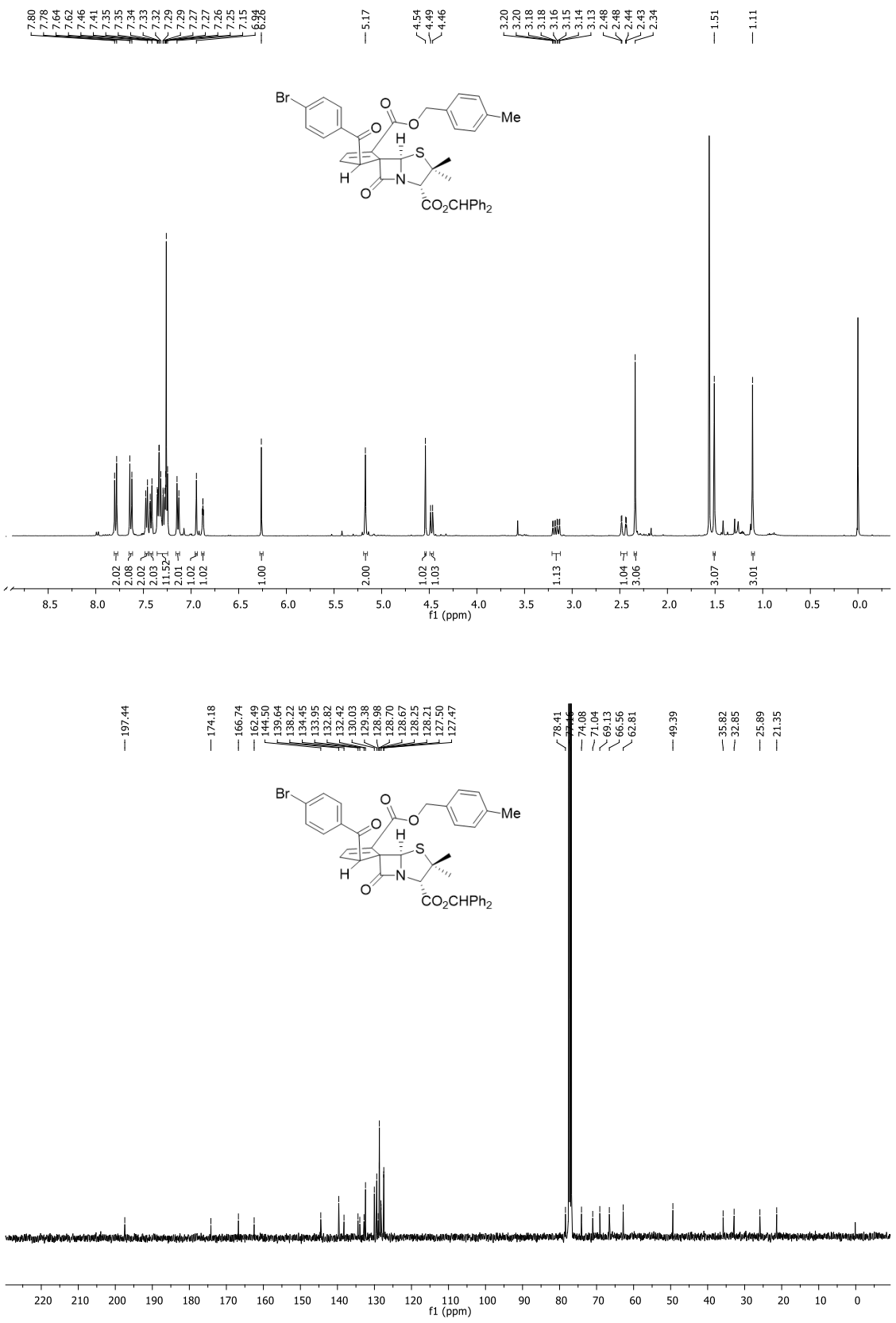


Figure S62: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **2.32h**.

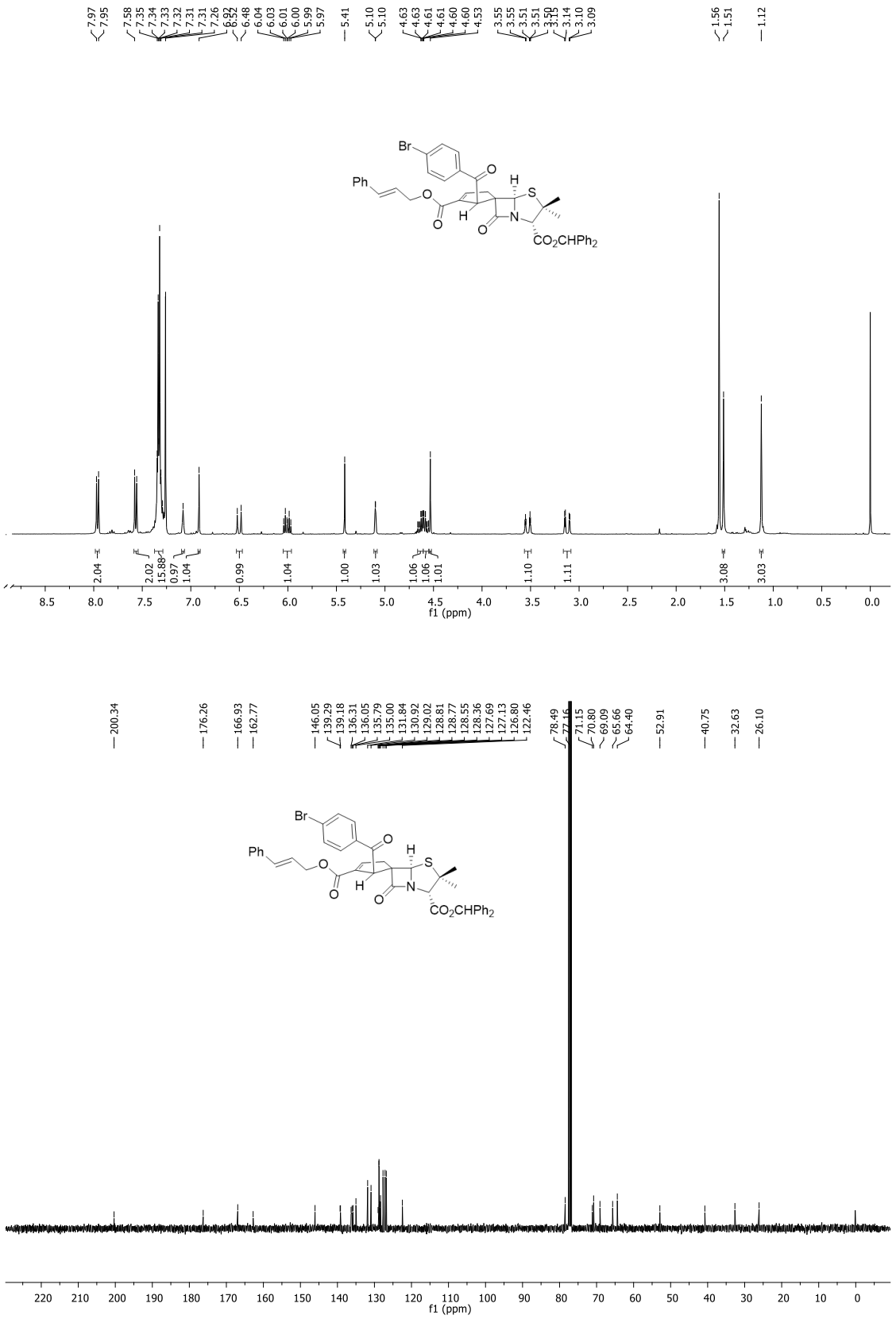


Figure S63: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **2.31i**.

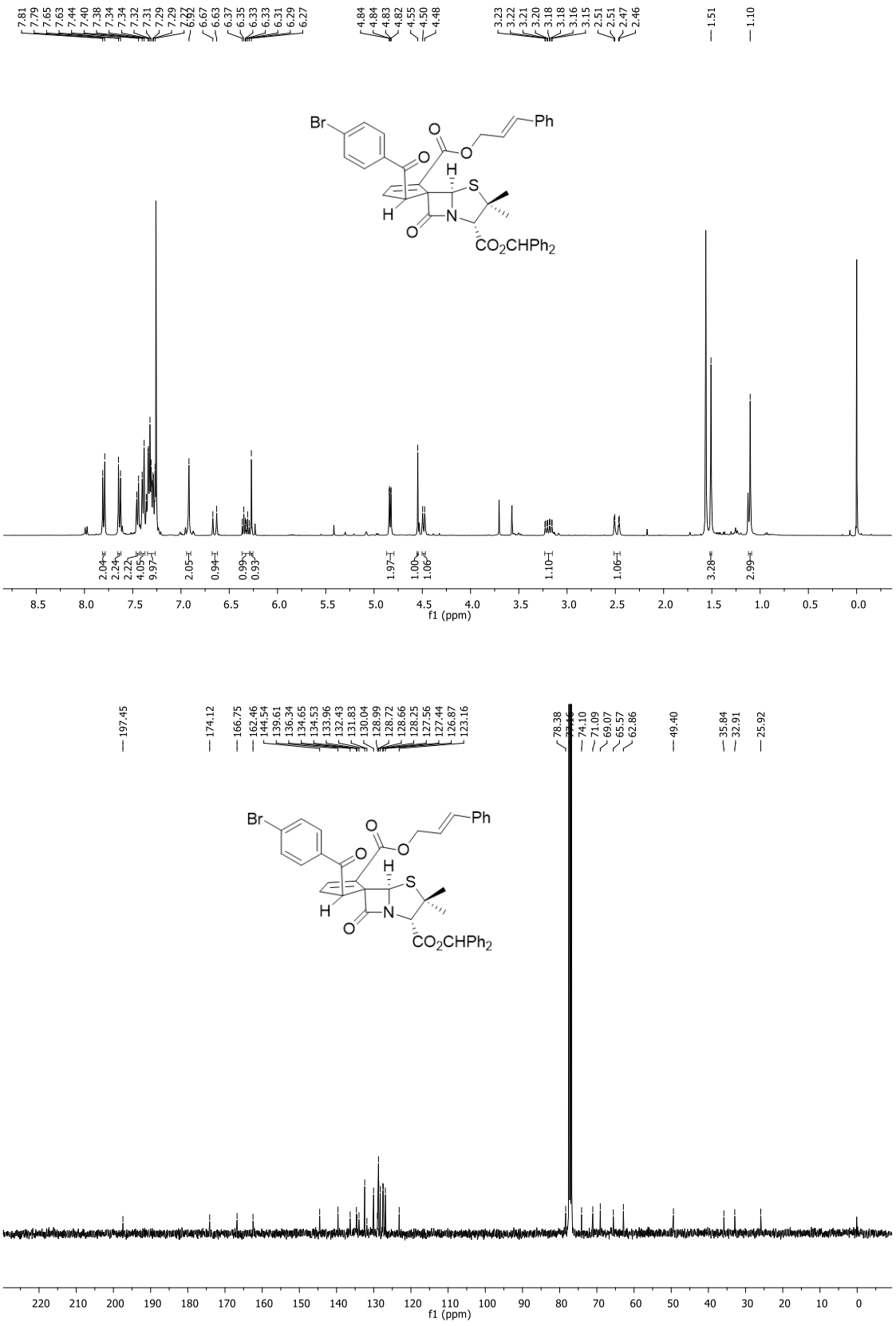


Figure S64: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **2.32i**.

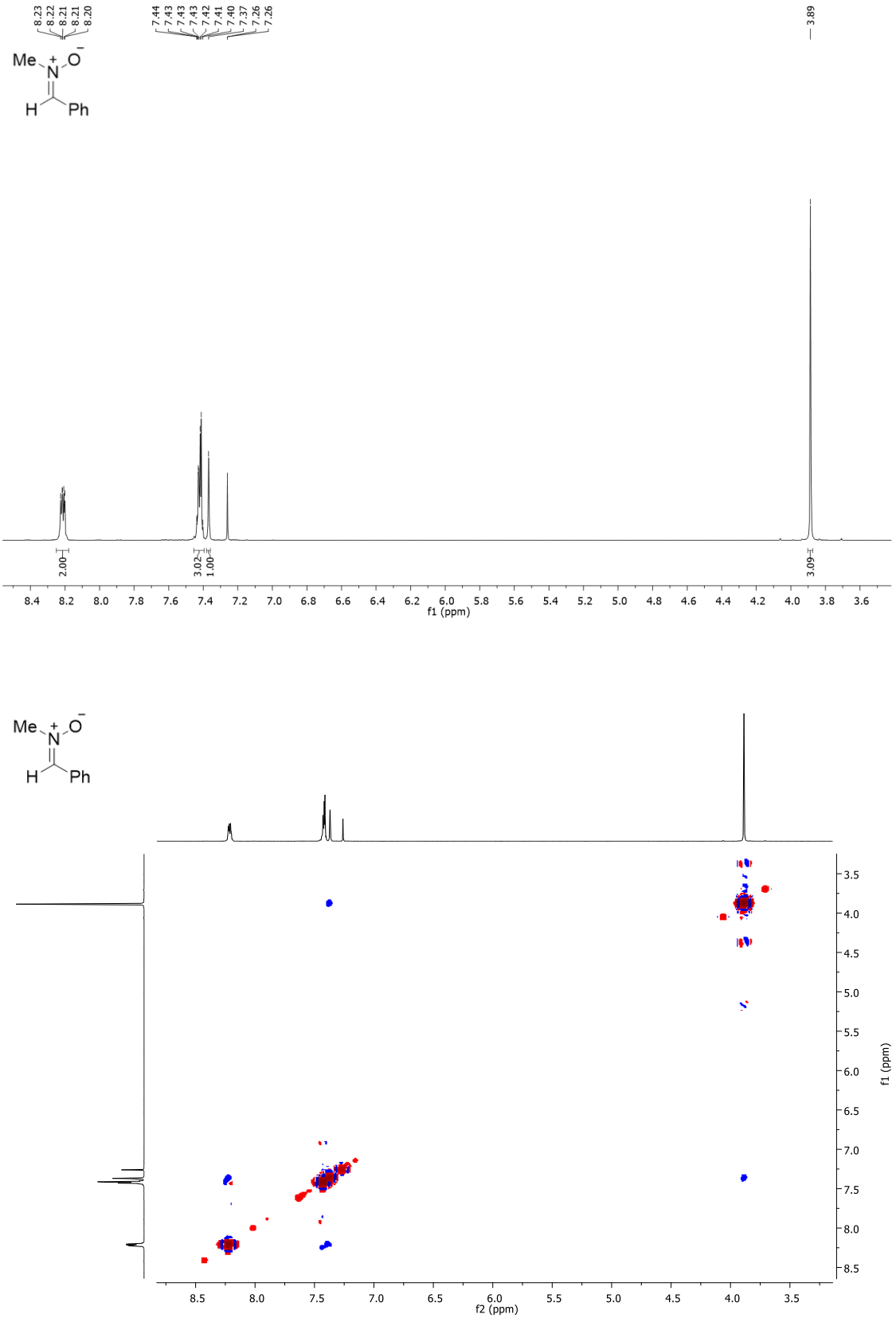


Figure S65: ^{1}H NMR and NOESY spectra of compound **3.41a (**$CDCl_3$).

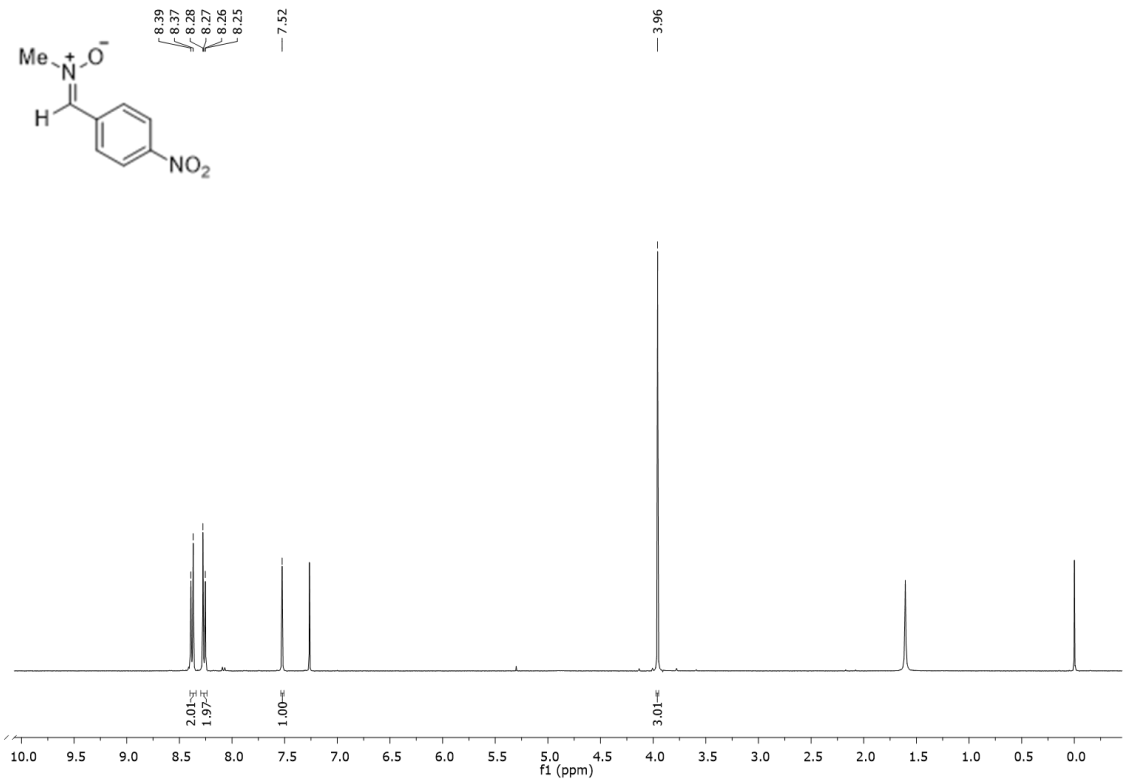


Figure S66: ^{1}H NMR spectrum of compound **3.41b (**$CDCl_3$).

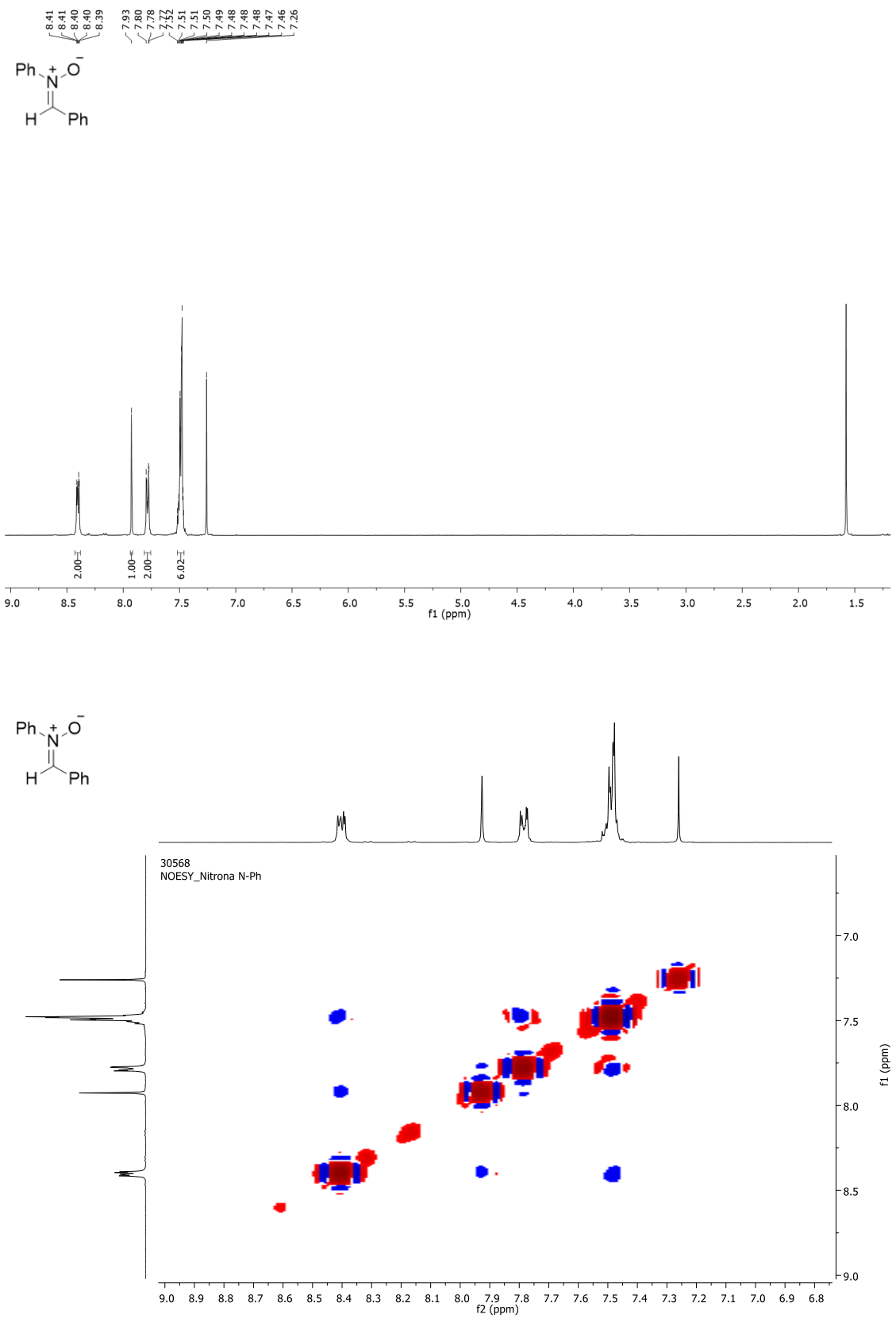


Figure S67: ^{1}H NMR and NOESY spectra of compound **3.41d (**$CDCl_3$).

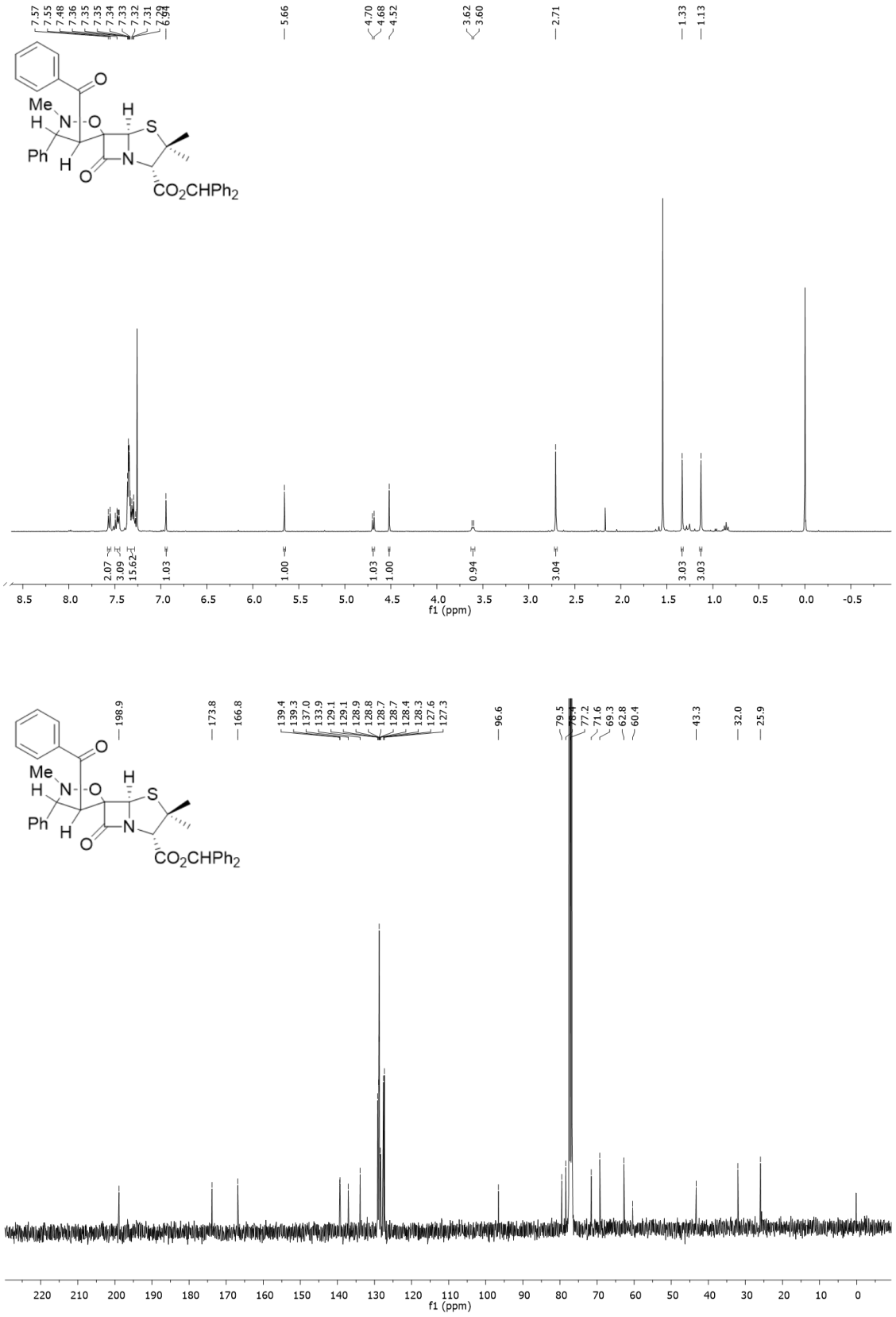


Figure S68: ^{1}H and ^{13}C NMR spectra of compound **3.43a (**$CDCl_3$).

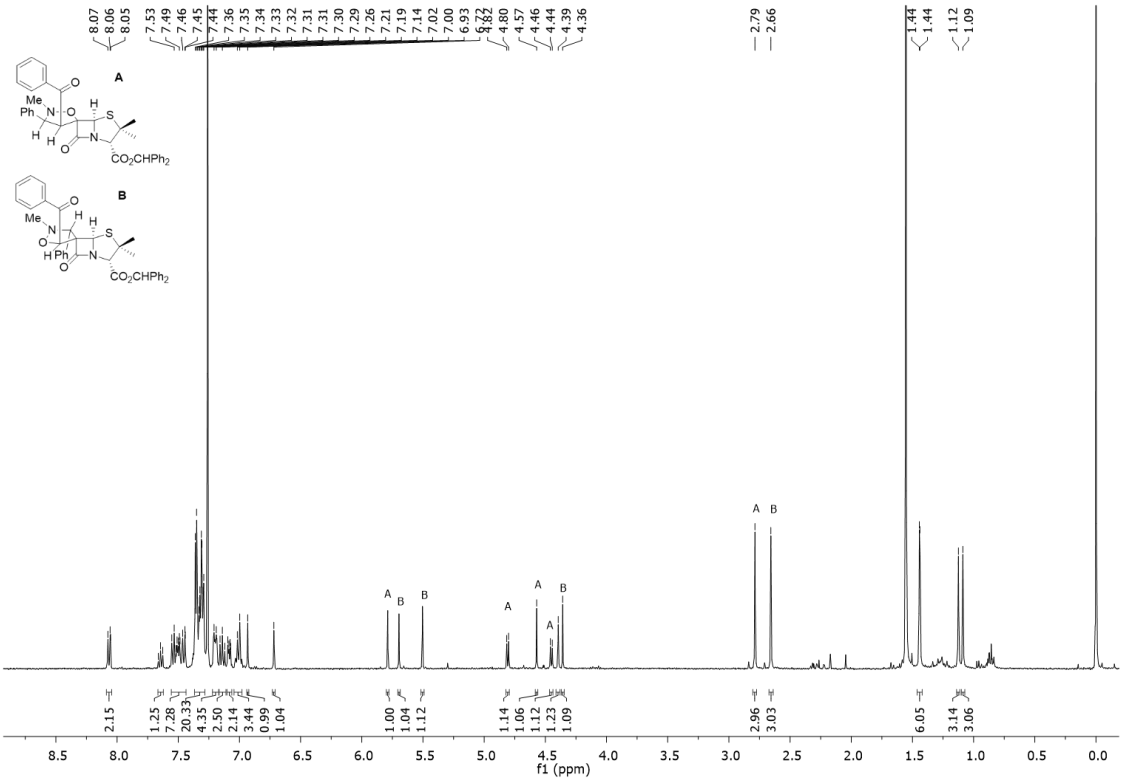


Figure S69: ^{1}H NMR spectrum of compound **3.44a** (A) and **3.45a** (B) **(**$CDCl_3$).

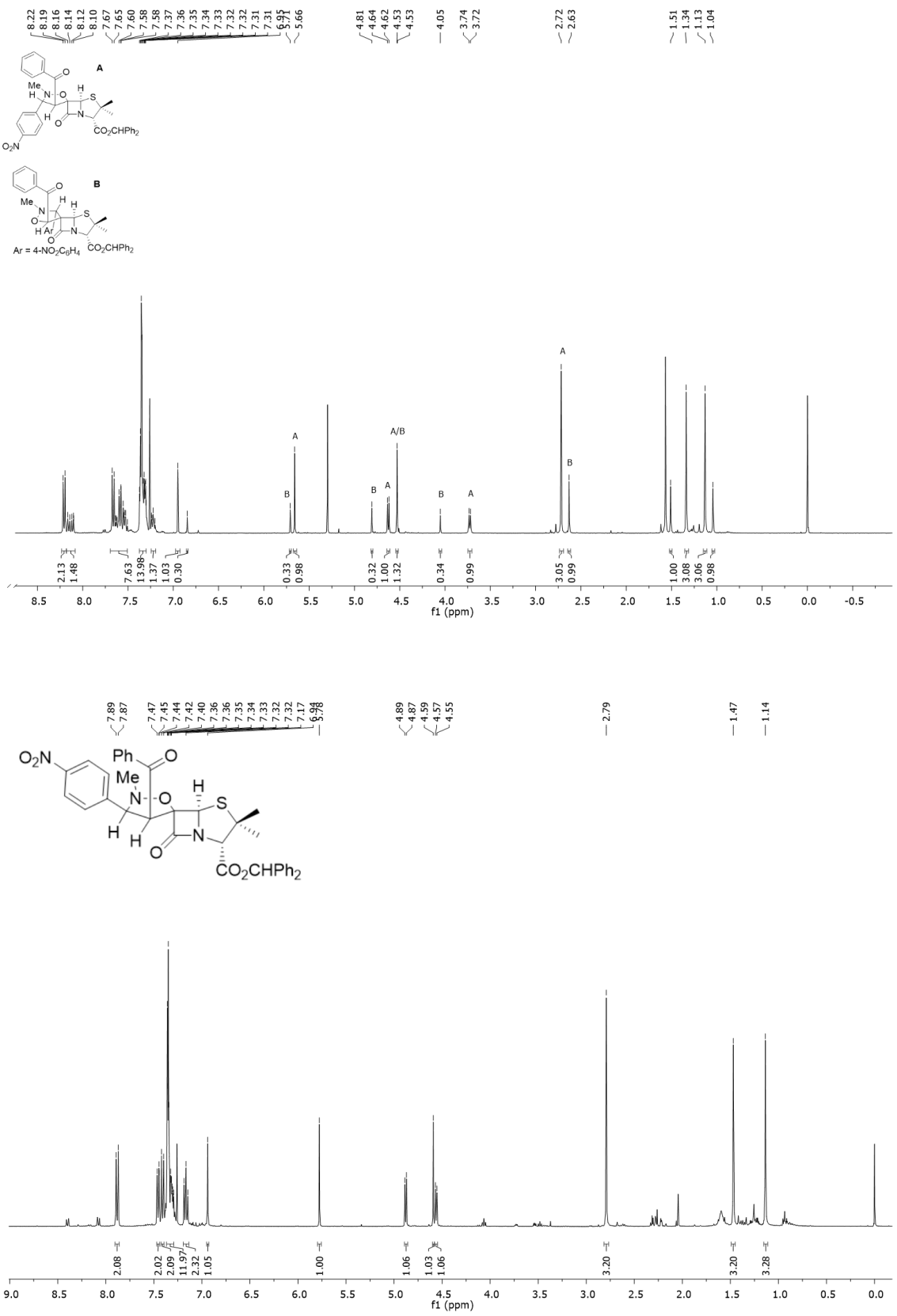


Figure S70: ^{1}H NMR spectrum of compound **3.43b** (A) and **3.45b** (B) (up) and **3.44b** (down) **(**$CDCl_3$).

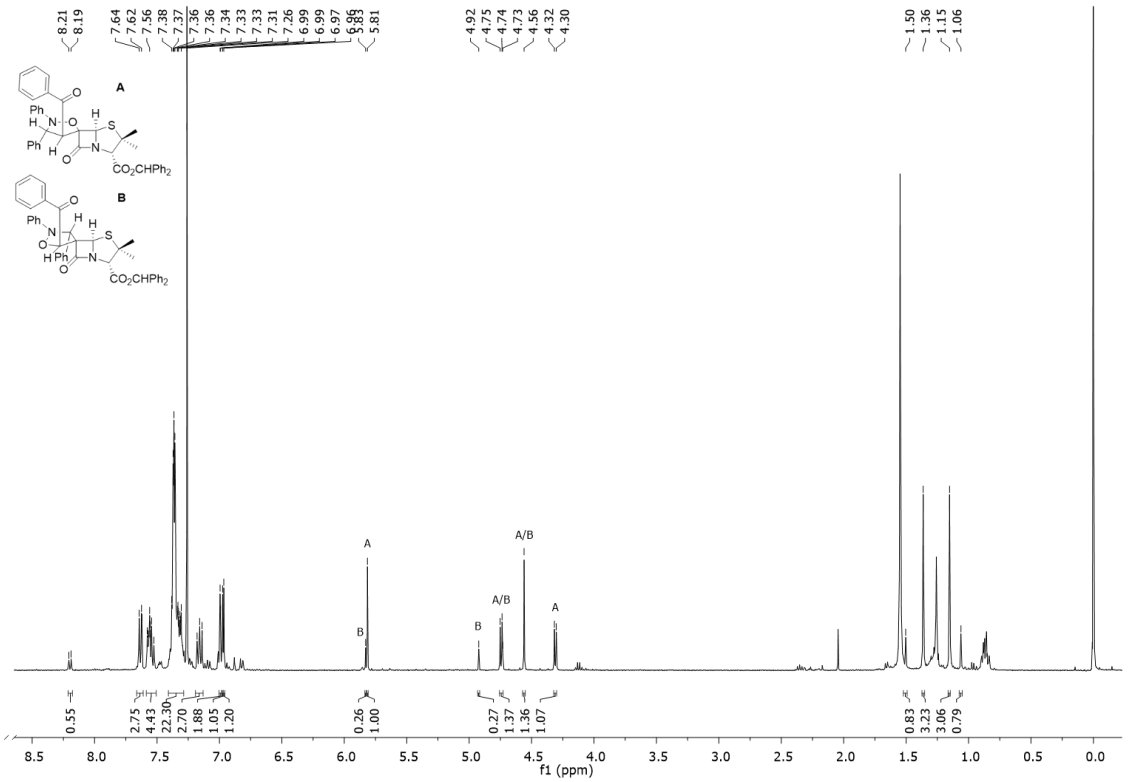


Figure S71: ^{1}H NMR spectrum of compound **3.43c** (A) and **3.45c** (B) **(**$CDCl_3$).

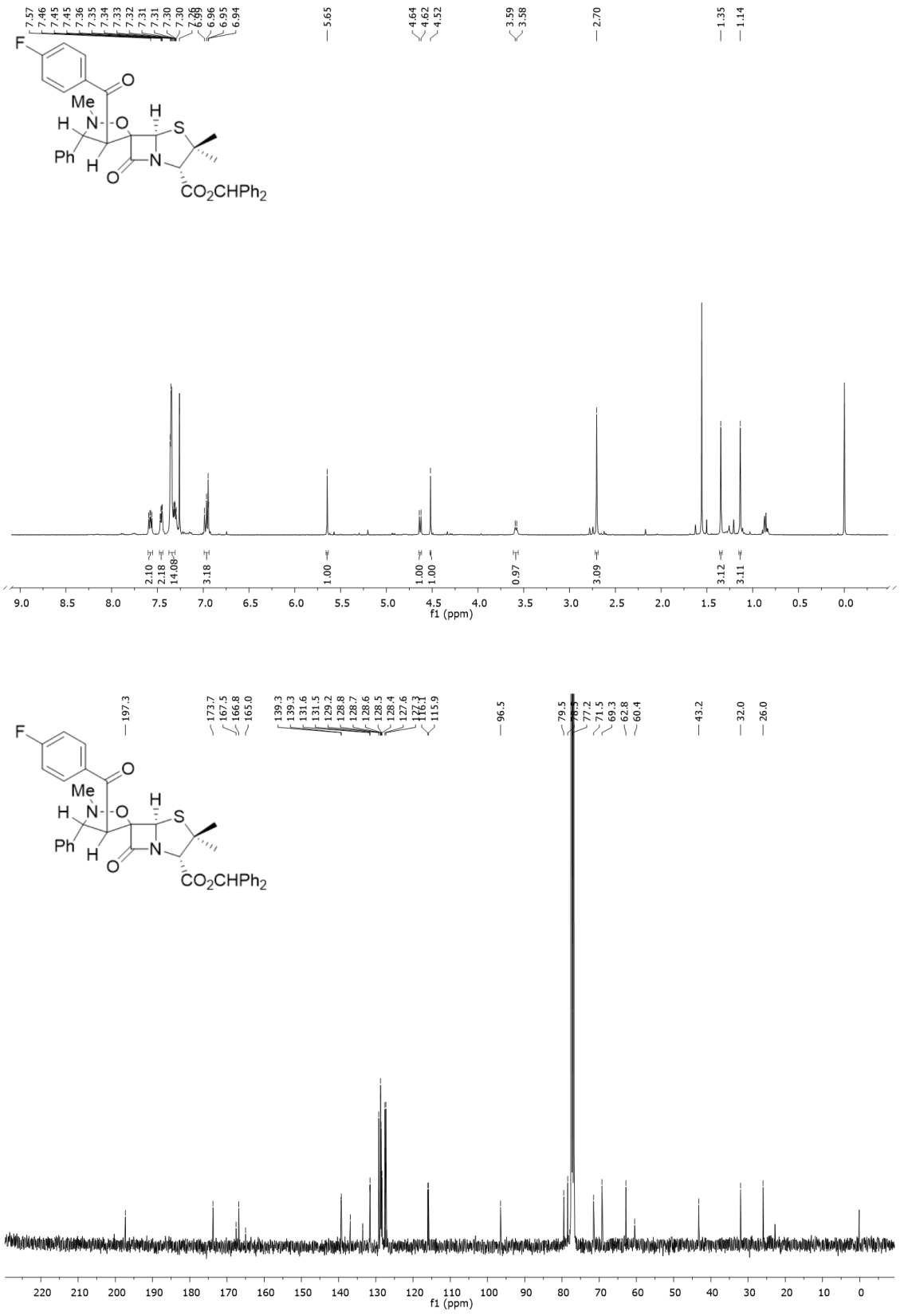


Figure S72: ^{1}H and ^{13}C NMR spectra of compound **3.47a (**$CDCl_3$).

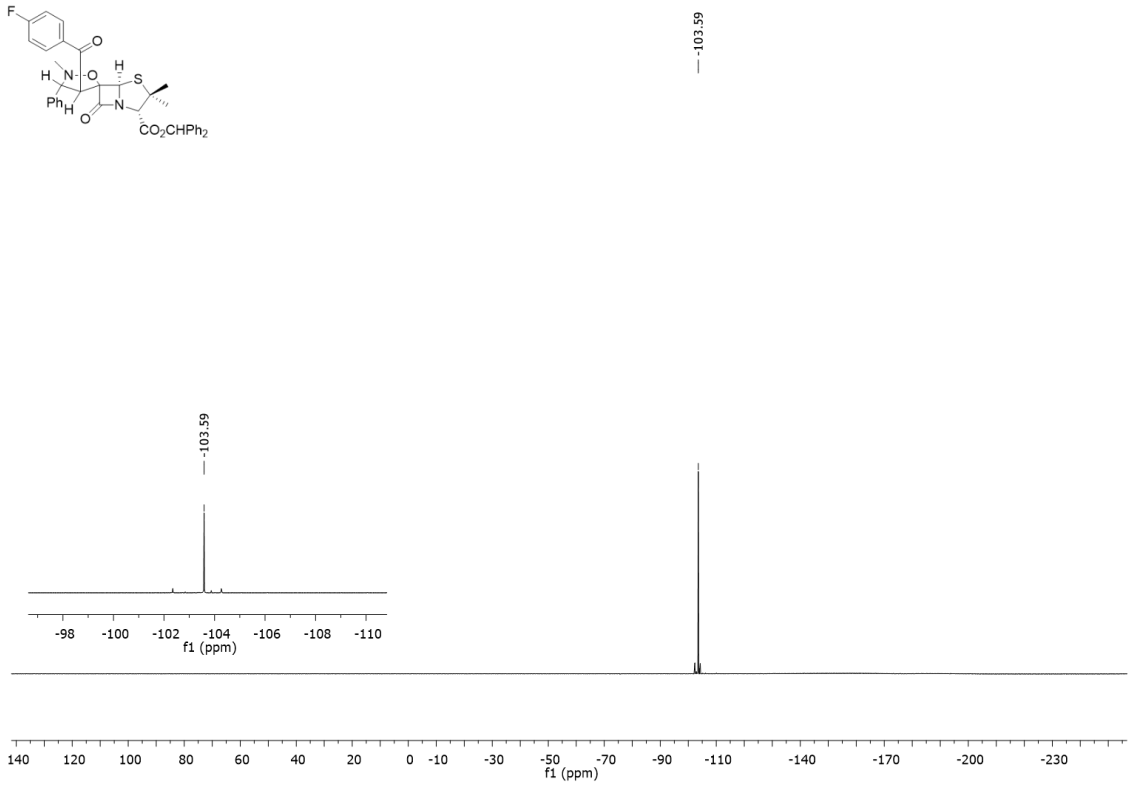


Figure S73: ^{19}F spectrum of compound **3.47a (**$CDCl_3$).

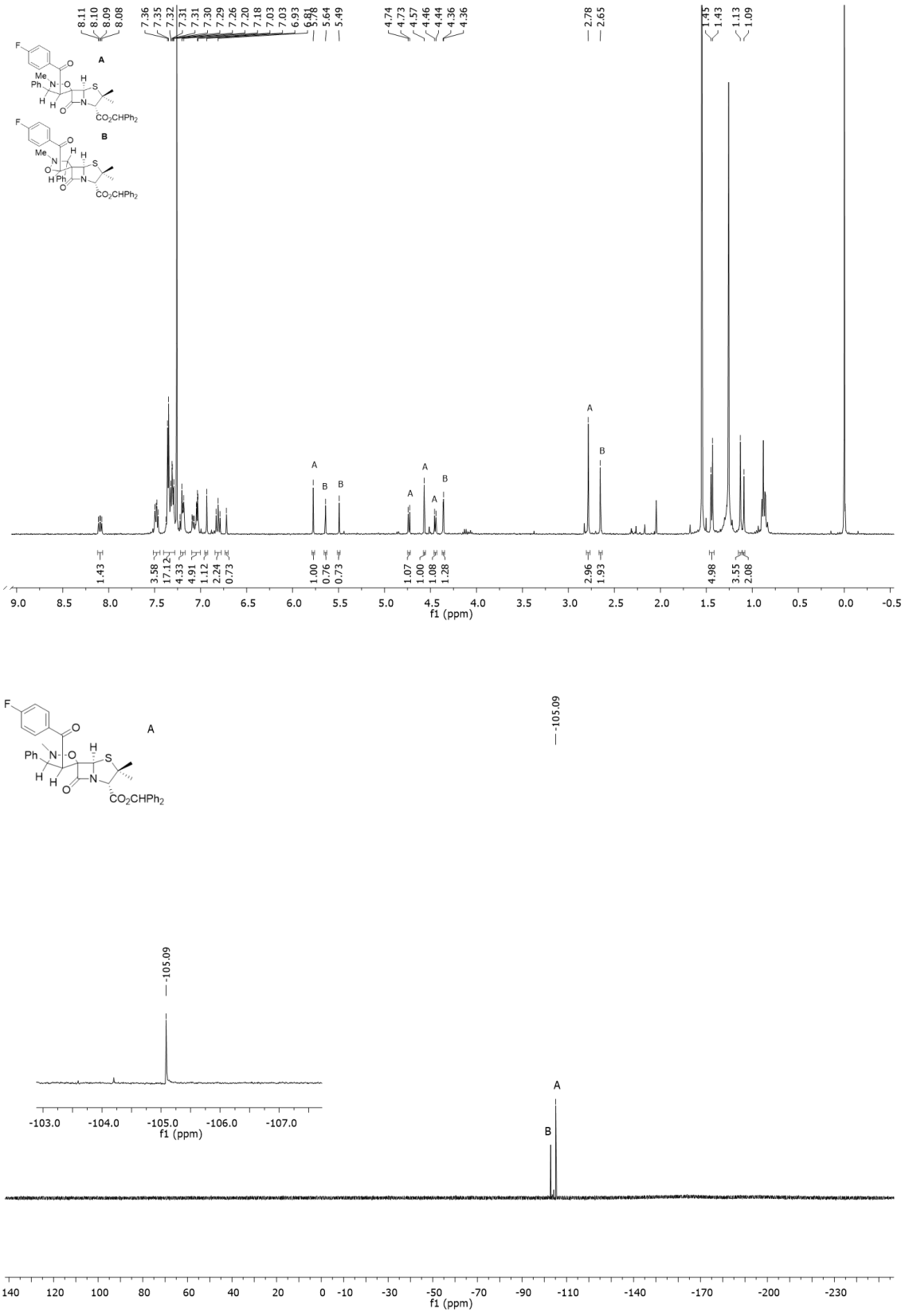


Figure S74: ^{1}H NMR and ^{19}F spectra of compound **3.48a** (A) and **3.49a** (B) **(**$CDCl_3$).

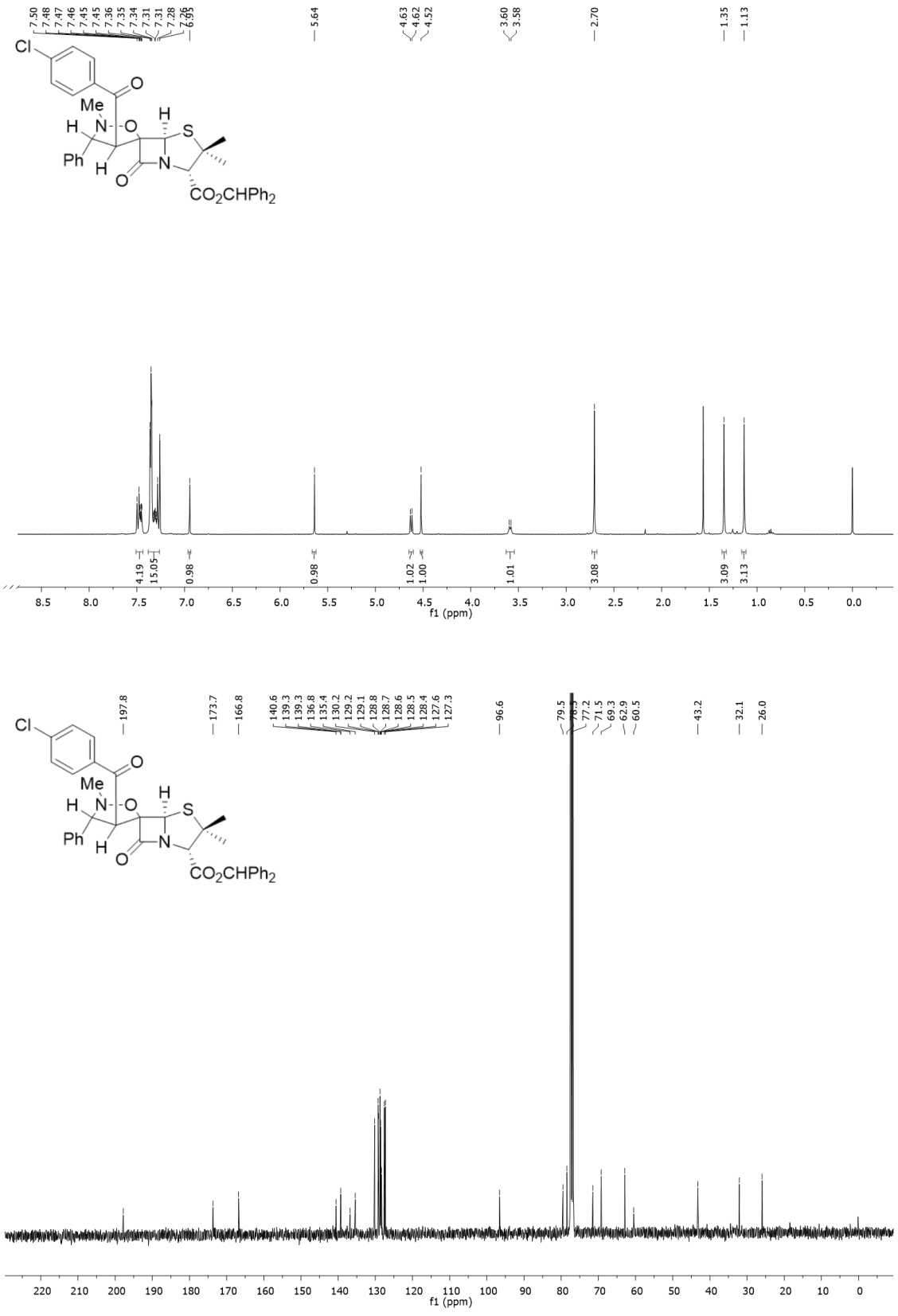


Figure S75: ^{1}H and ^{13}C NMR spectra of compound **3.47b (**$CDCl_3$).

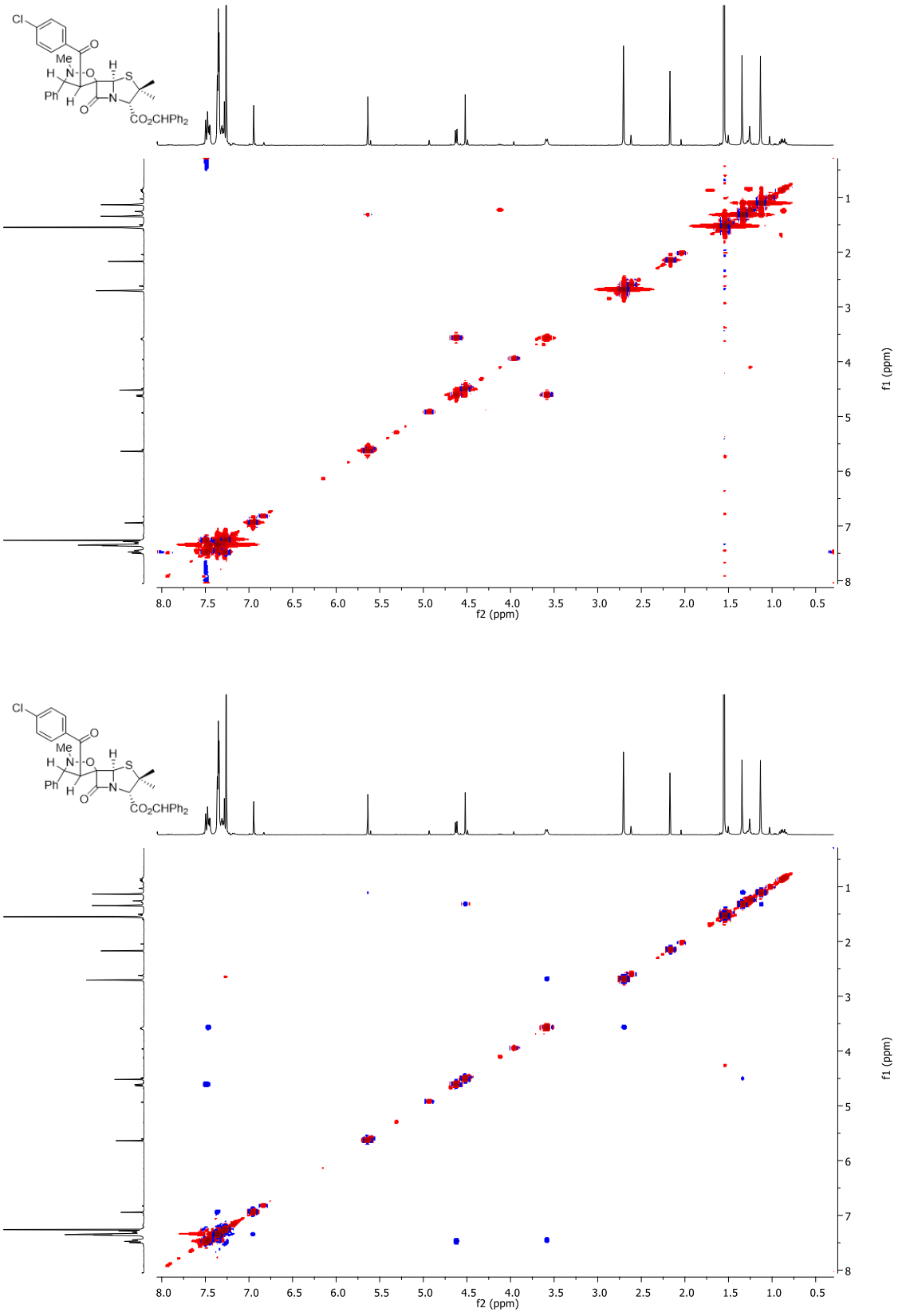


Figure S76: COSY and NOESY spectra of compound **3.47b (**$CDCl_3$).

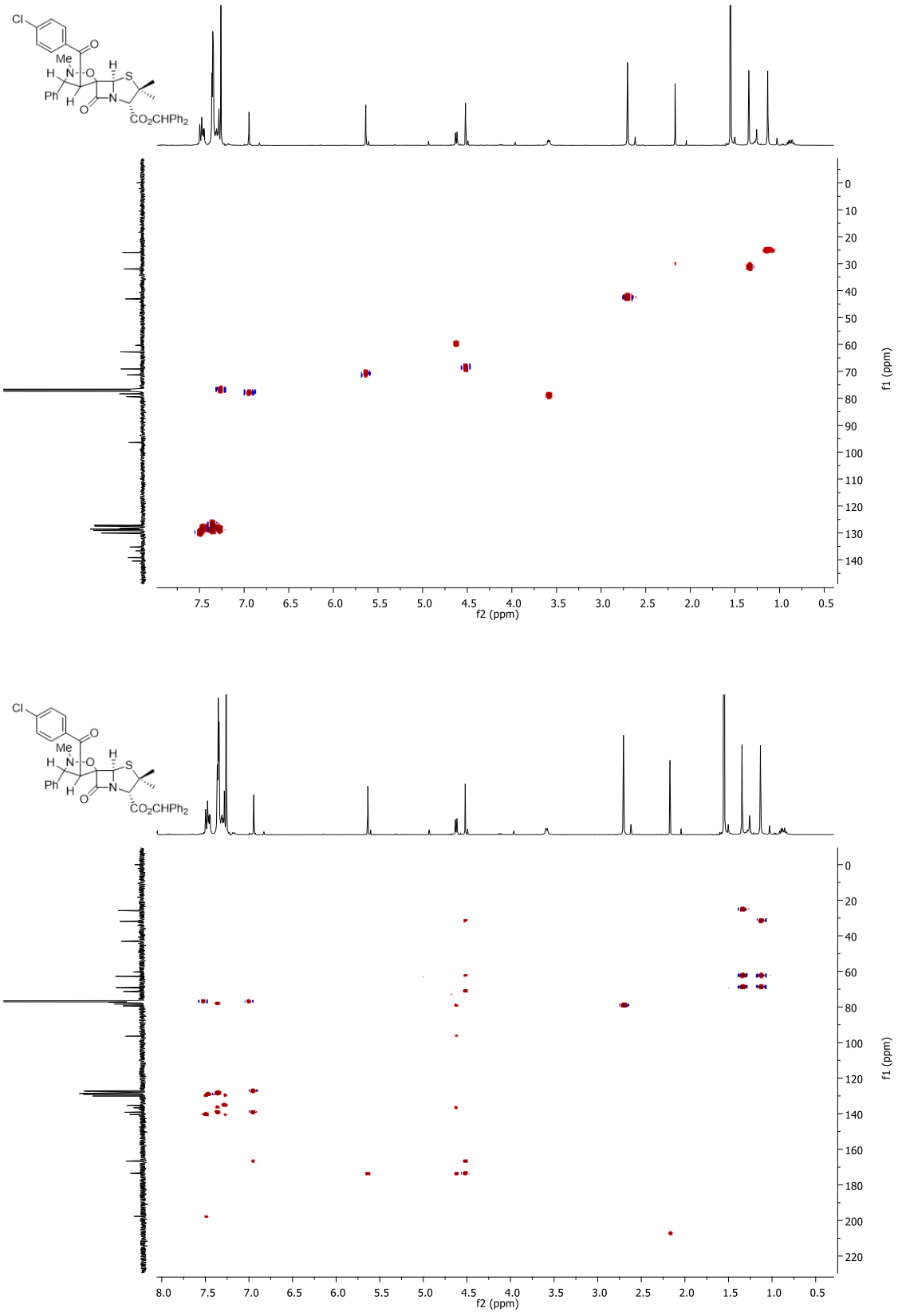


Figure S77: HSQC and HMBC spectra of compound **3.47b (**$CDCl_3$).

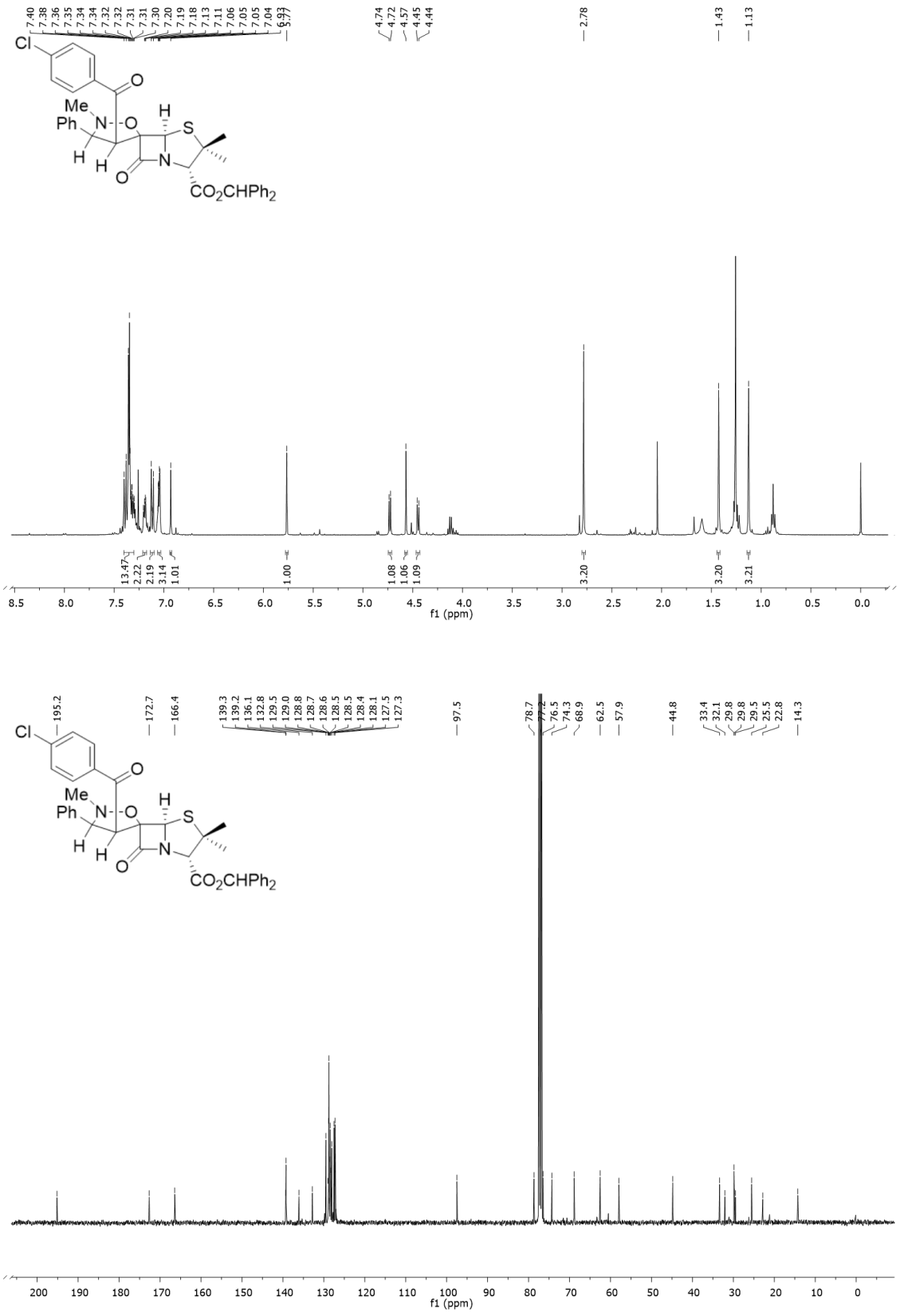


Figure S78: ^{1}H and ^{13}C NMR spectra of compound **3.48b (**$CDCl_3$**).**

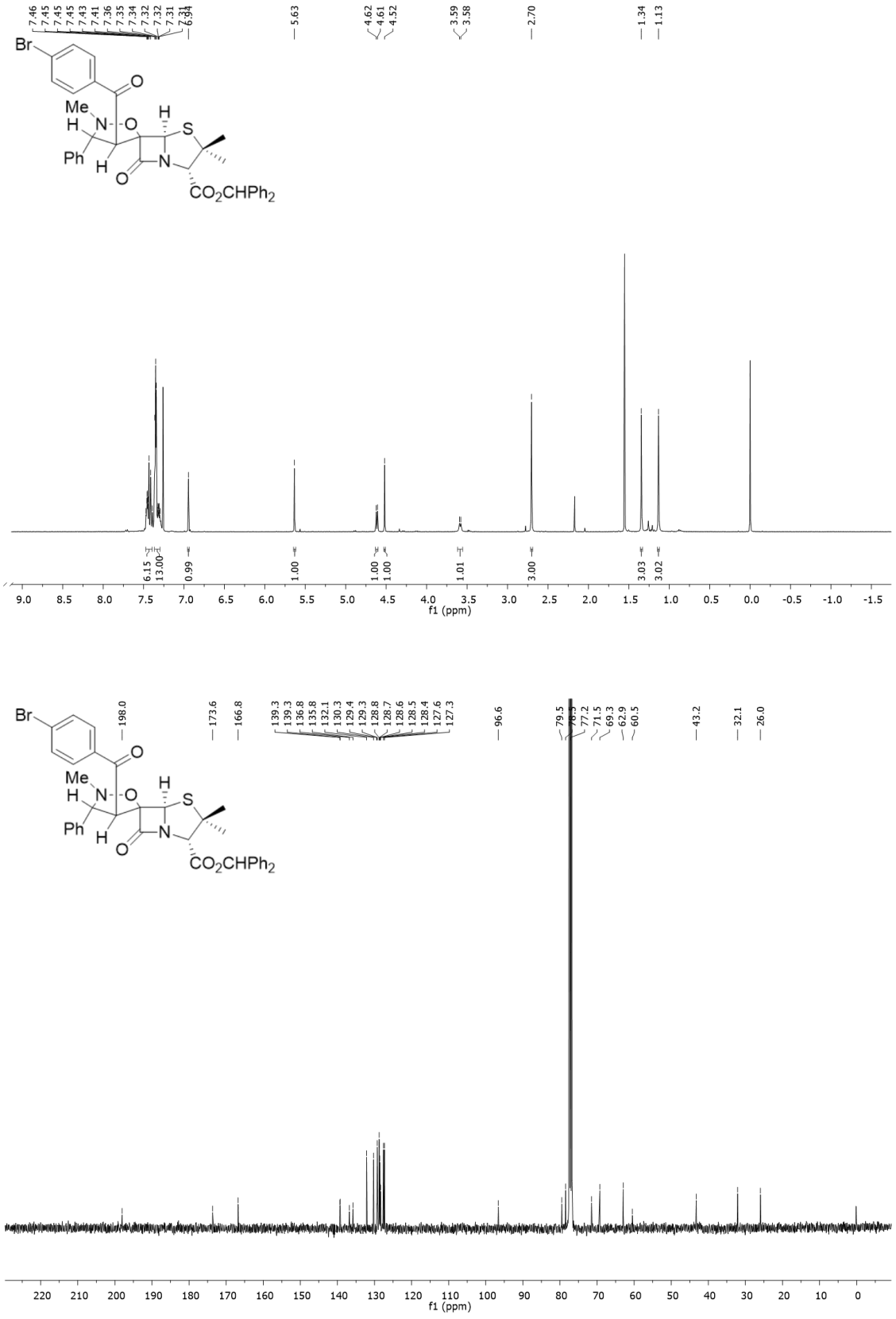


Figure S79: ^{1}H and ^{13}C NMR spectra of compound **3.47c (**$CDCl_3$**).**

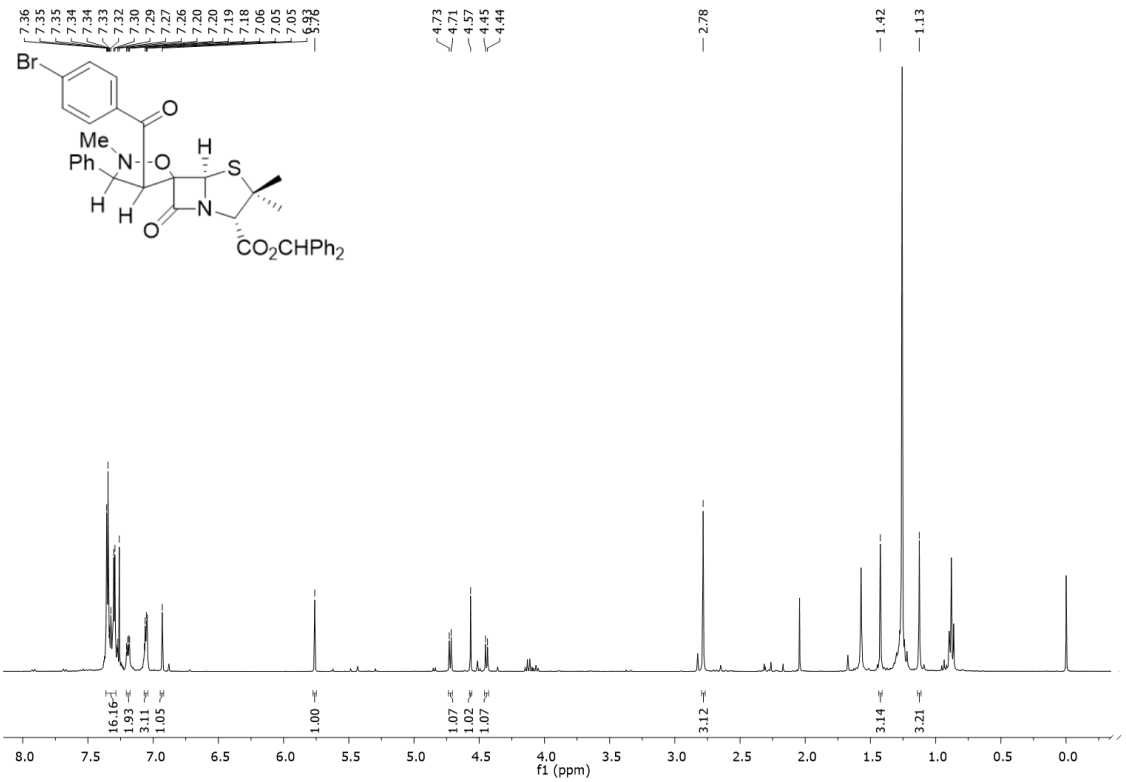


Figure S80: ^{1}H NMR spectrum of compound **3.48b (**$CDCl_3$).

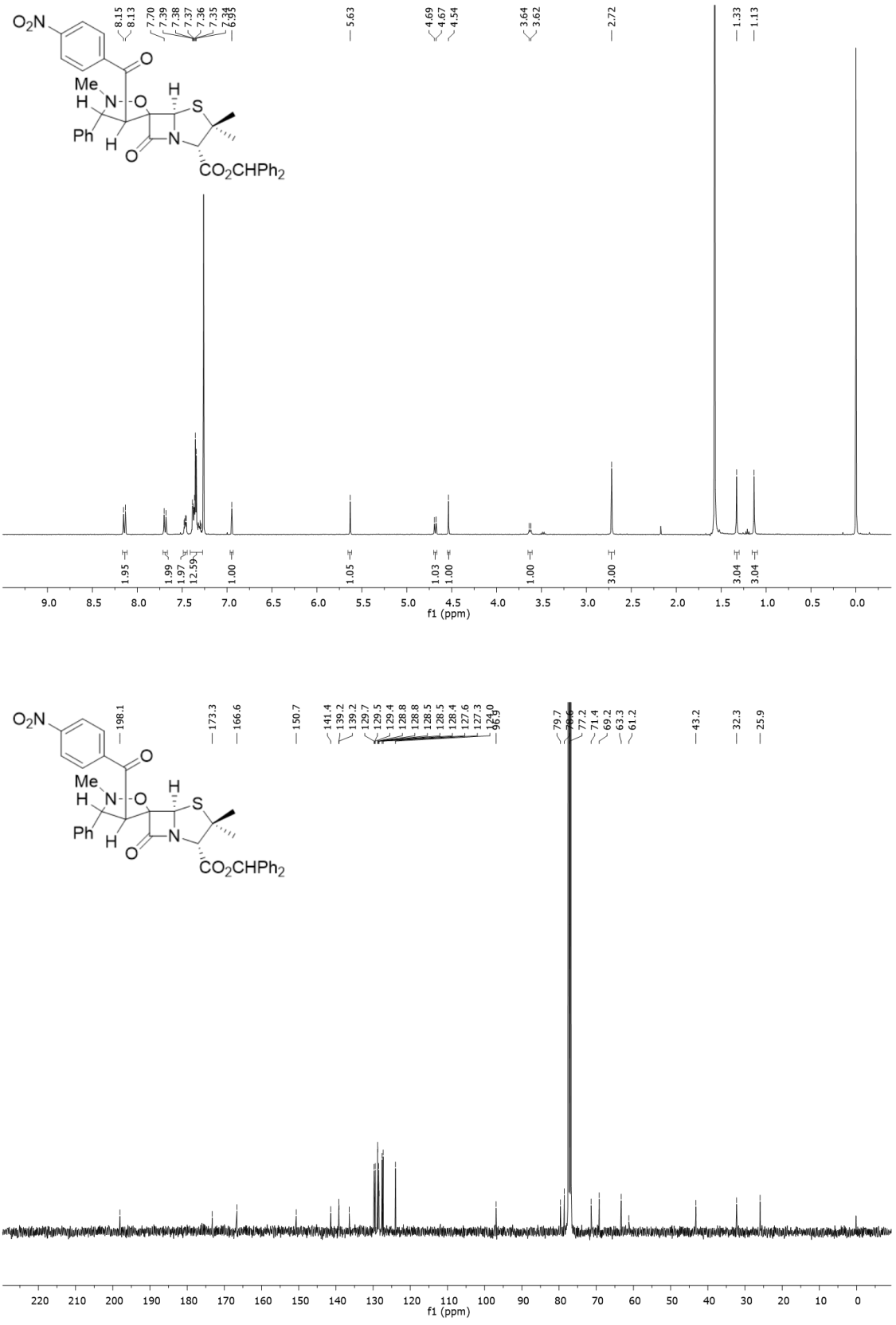


Figure S81: ^{1}H and ^{13}C NMR spectra of compound **3.47d (**$CDCl_3$**)**.

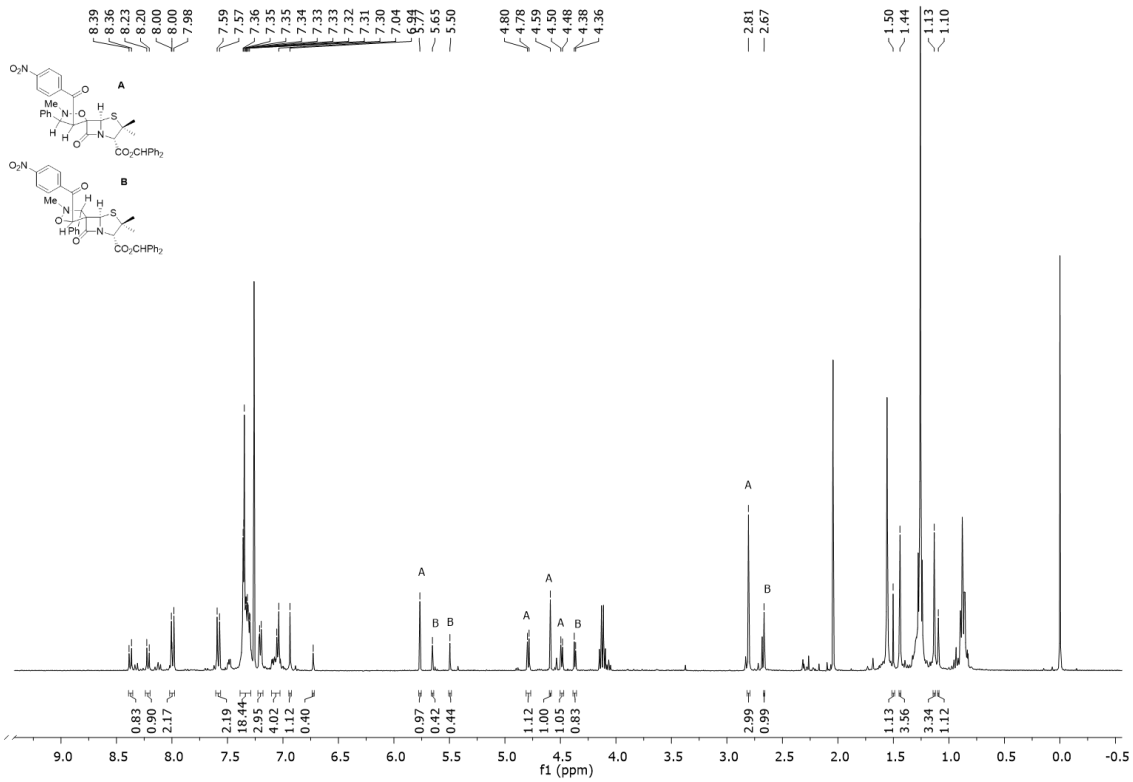


Figure S82: ^{1}H NMR spectrum of compound **3.48d** (A) and **3.49d** (B) **(**$CDCl_3$).

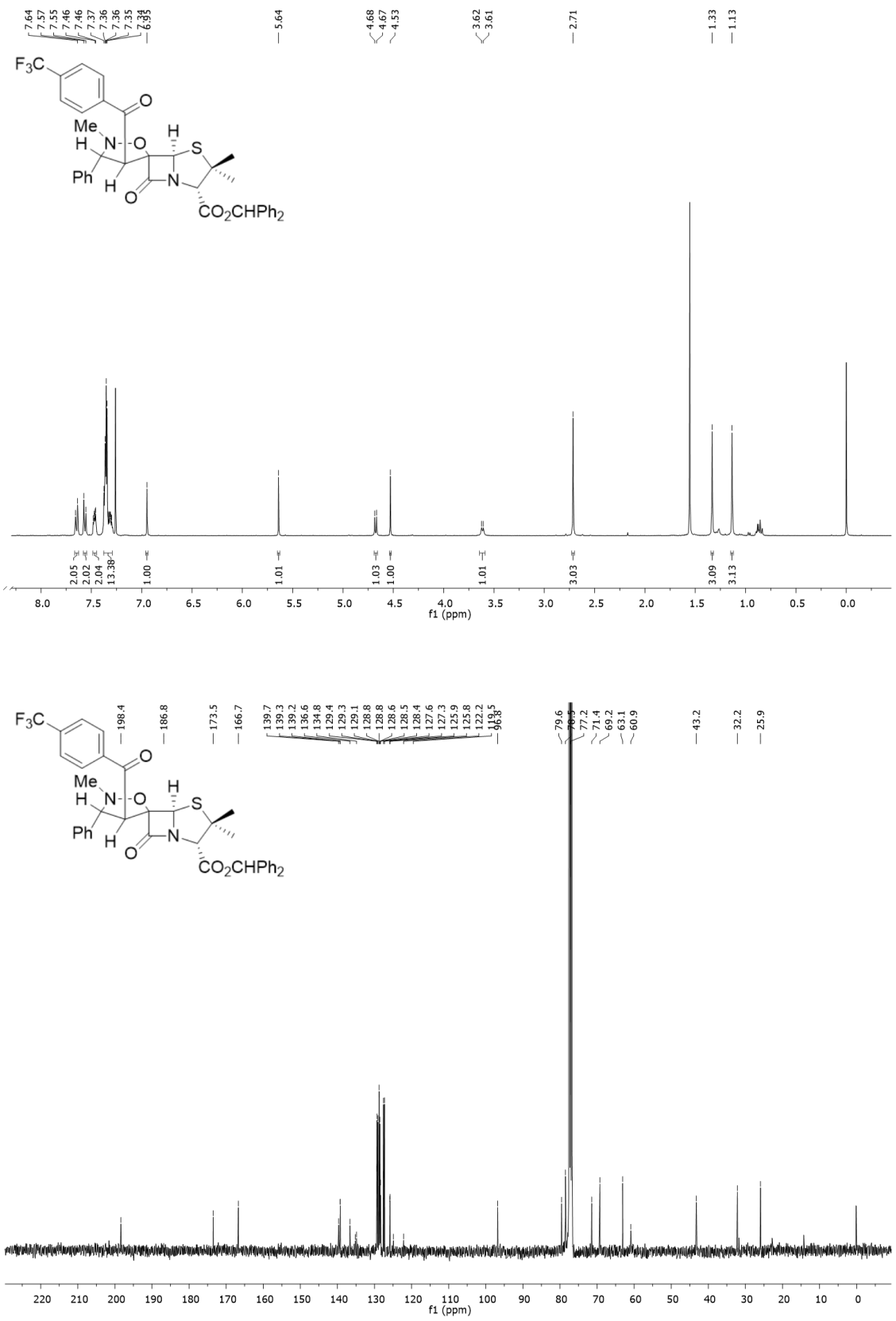


Figure S83: ^{1}H and ^{13}C NMR spectra of compound **3.47e (**$CDCl_3$).

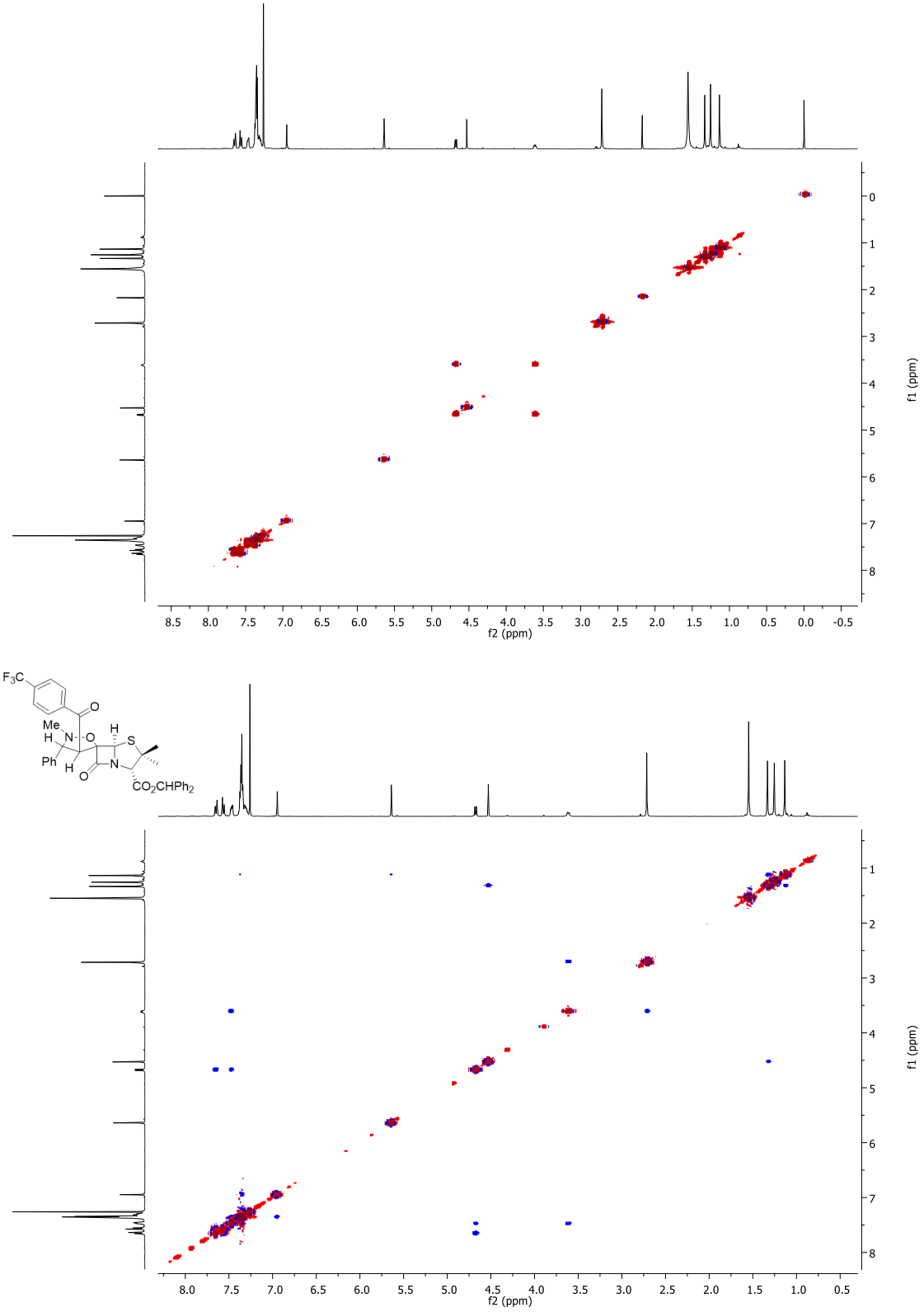


Figure S84: COSY and NOESY spectra of compound **3.47e (**$CDCl_3$).

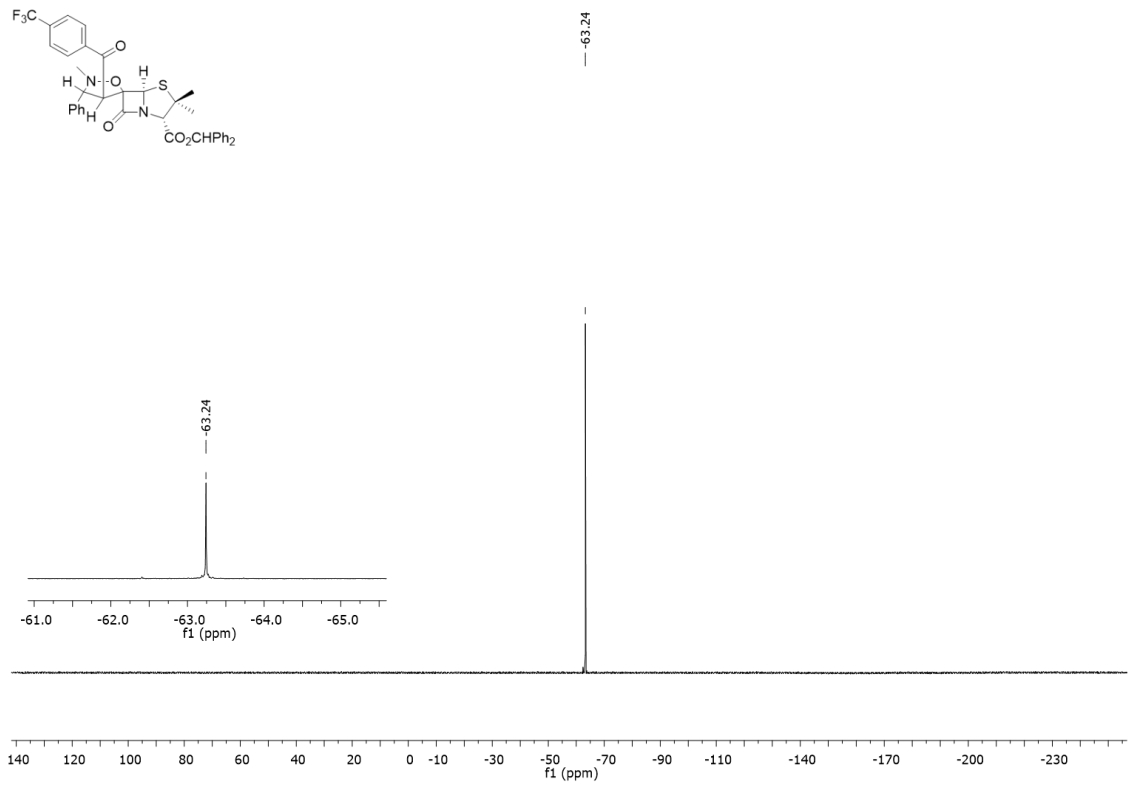


Figure S85: ^{19}F spectrum of compound **3.47e (**$CDCl_3$).

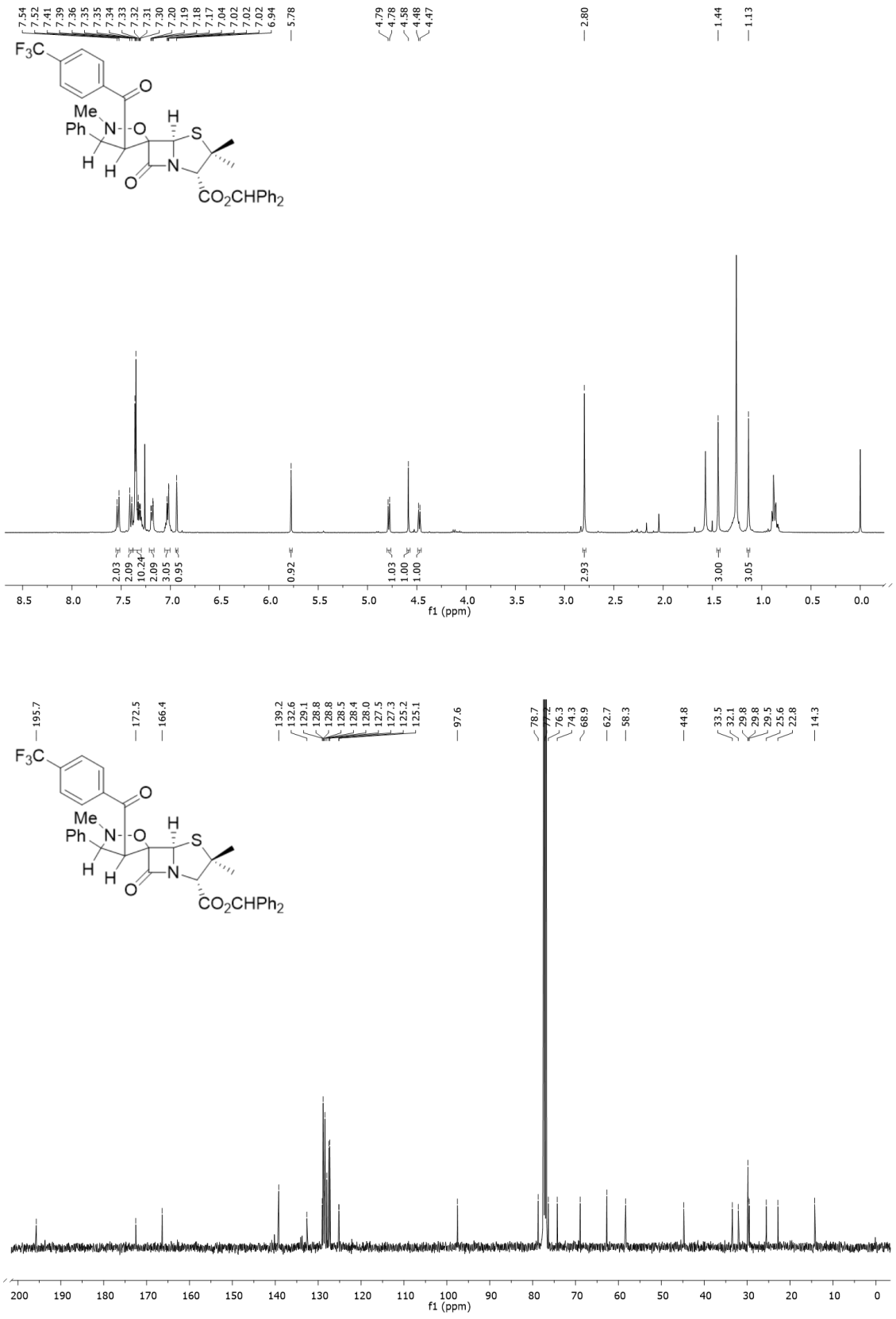


Figure S86: ^{1}H and ^{13}C NMR spectra of compound **3.48e (**$CDCl_3$**).**

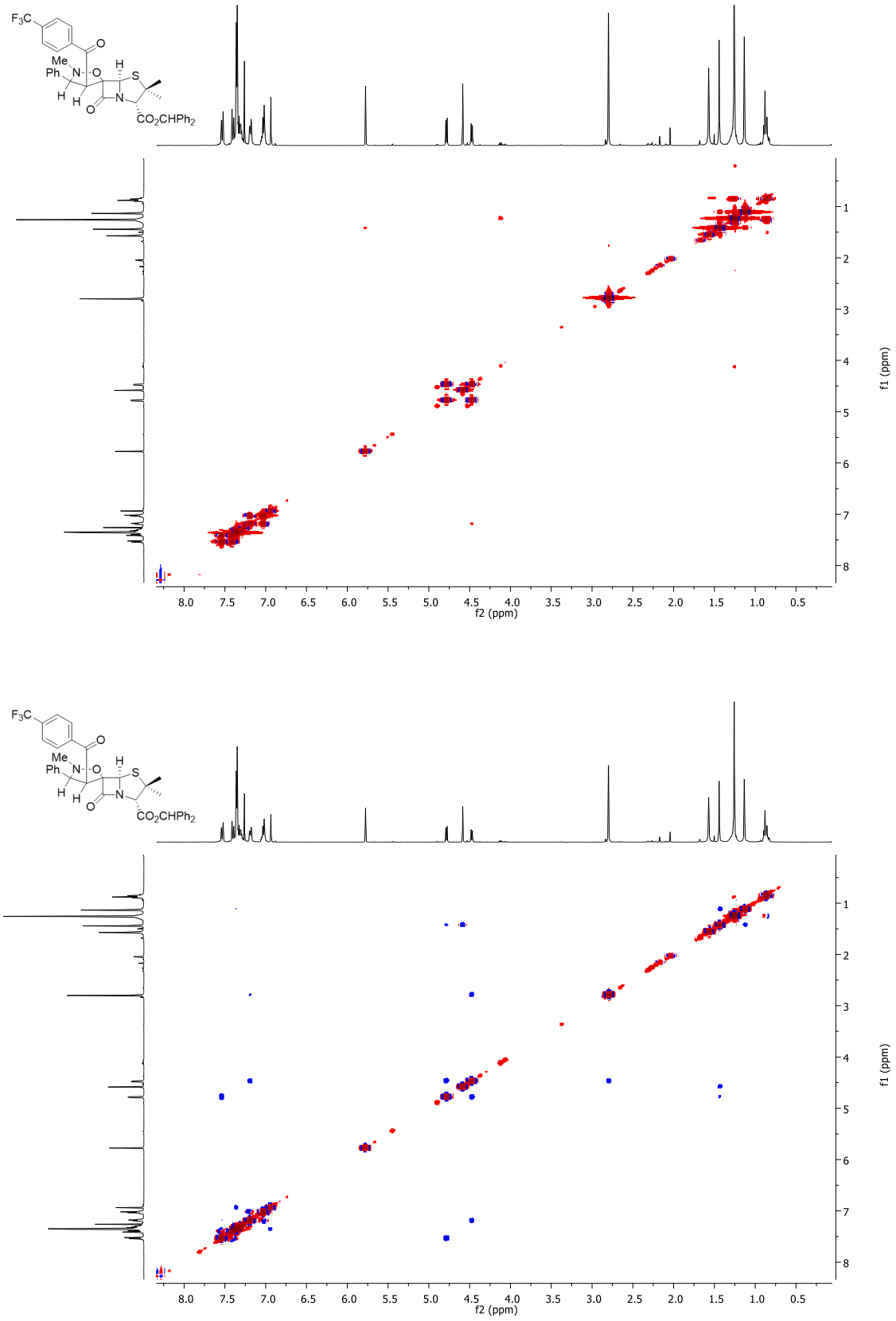


Figure S87: COSY and NOESY spectra of compound **3.48e (**$CDCl_3$).

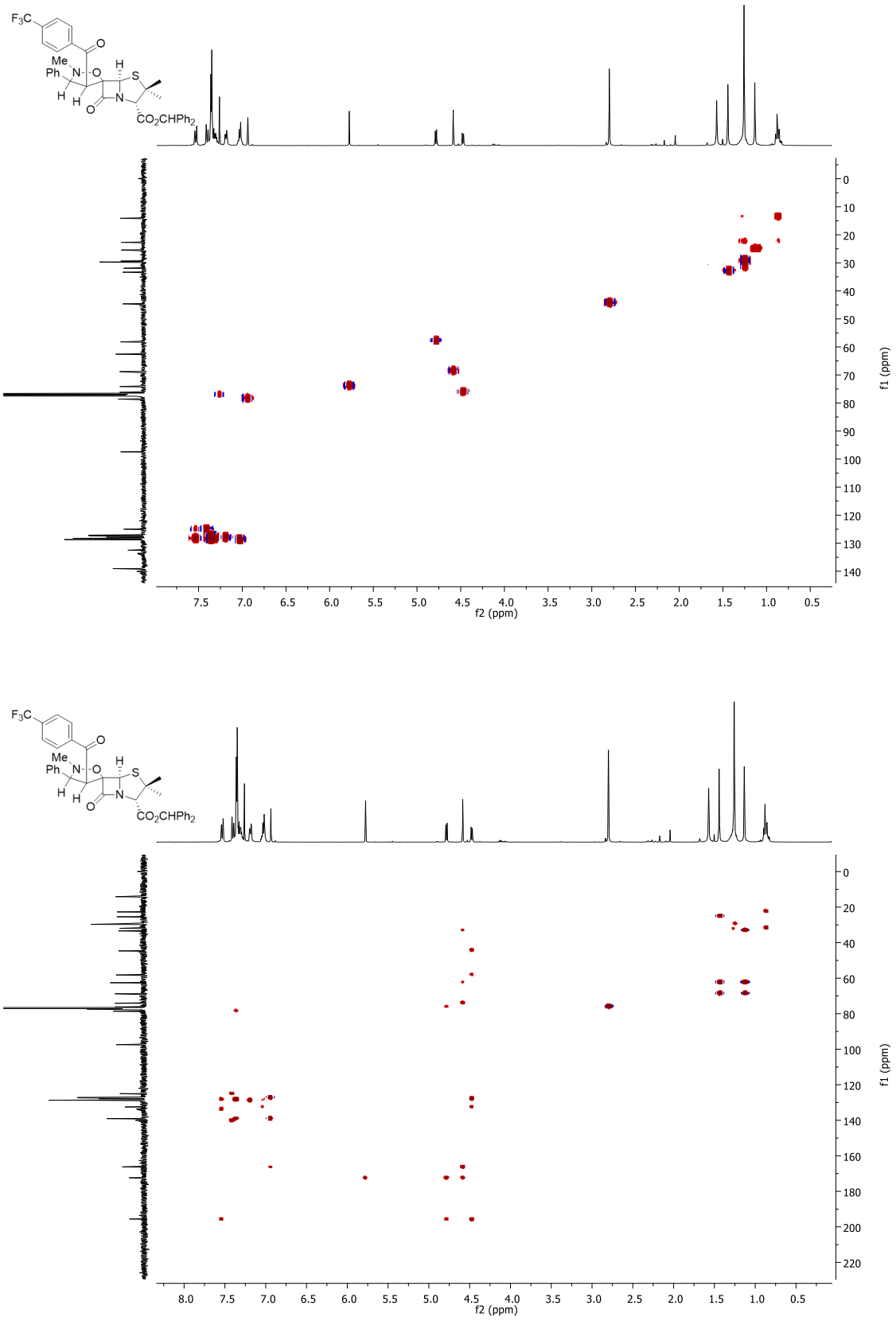


Figure S88: HSQC and HMBC spectra of compound **3.48e (**$CDCl_3$).

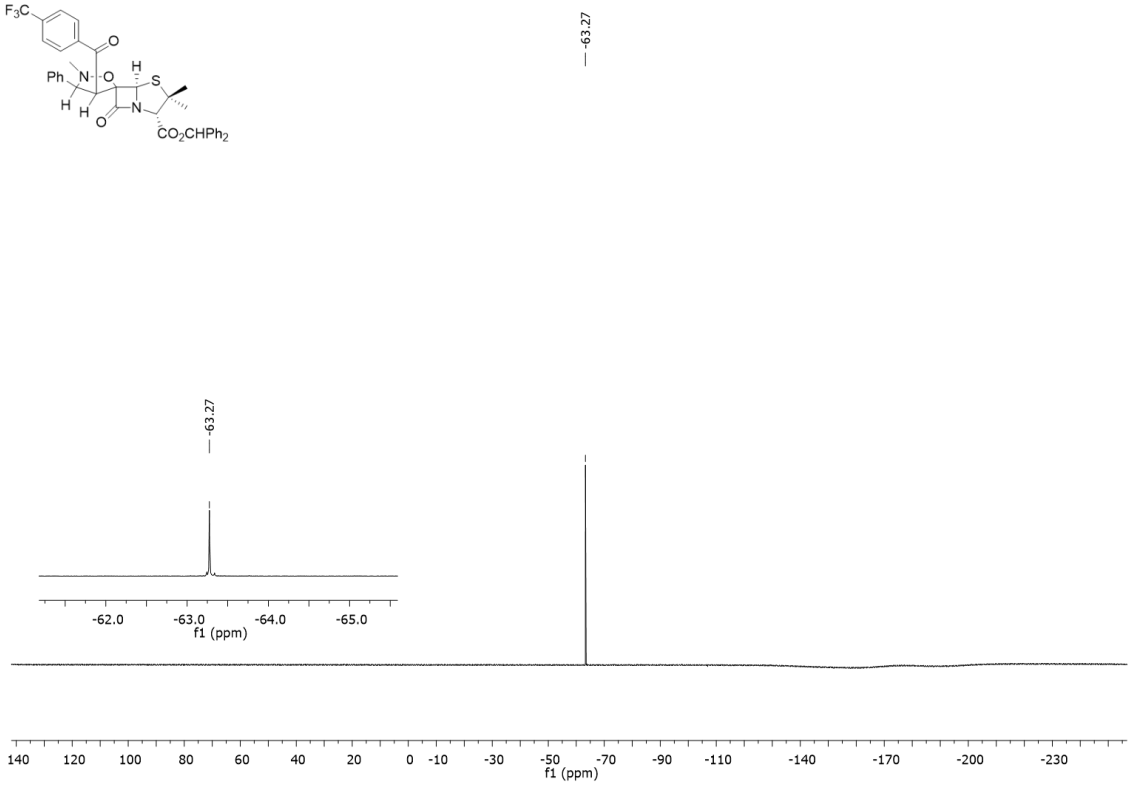


Figure S89: ^{19}F spectrum of compound **3.48e (**$CDCl_3$).

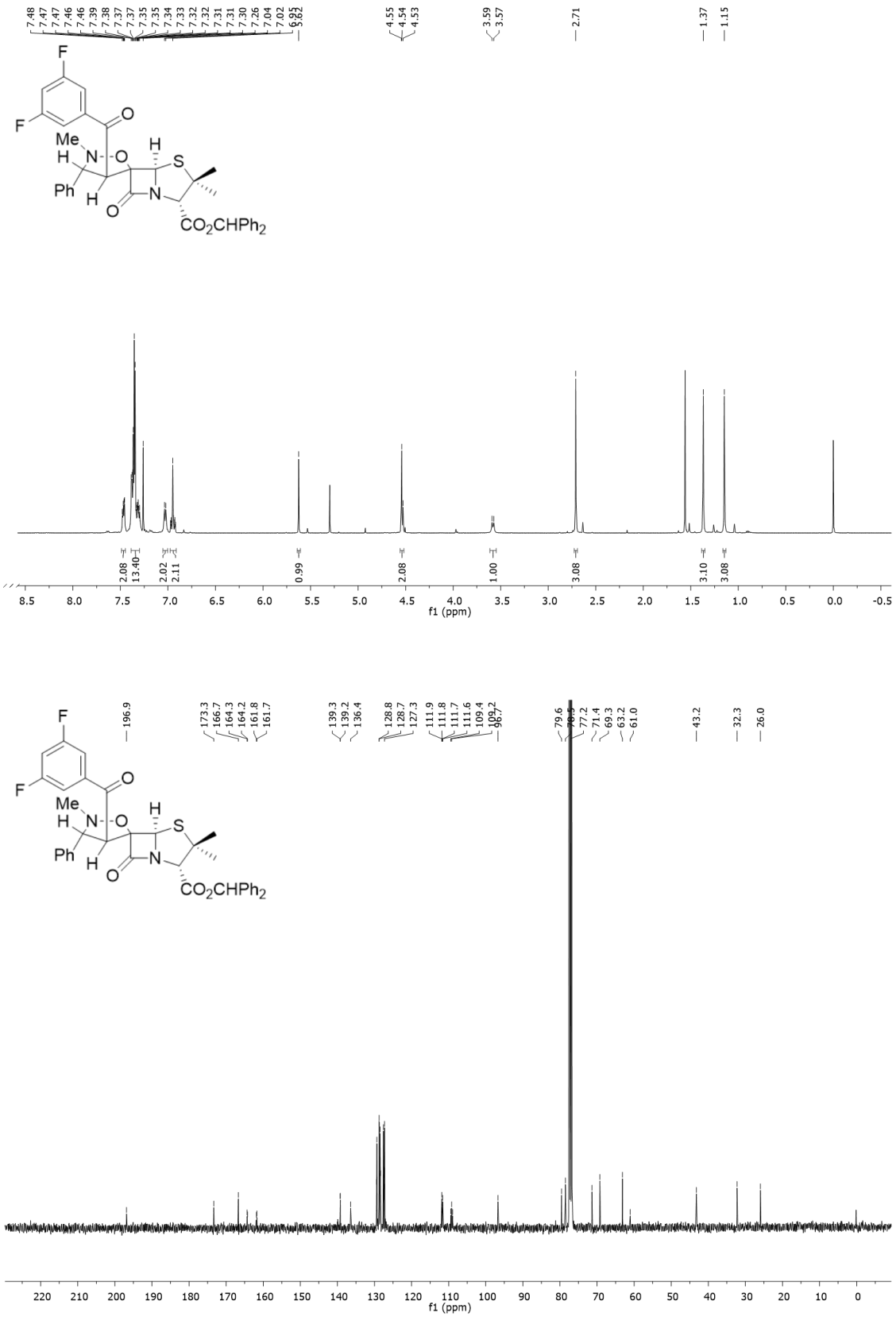


Figure S90: ^{1}H and ^{13}C NMR spectra of compound **3.47f (**$CDCl_3$).

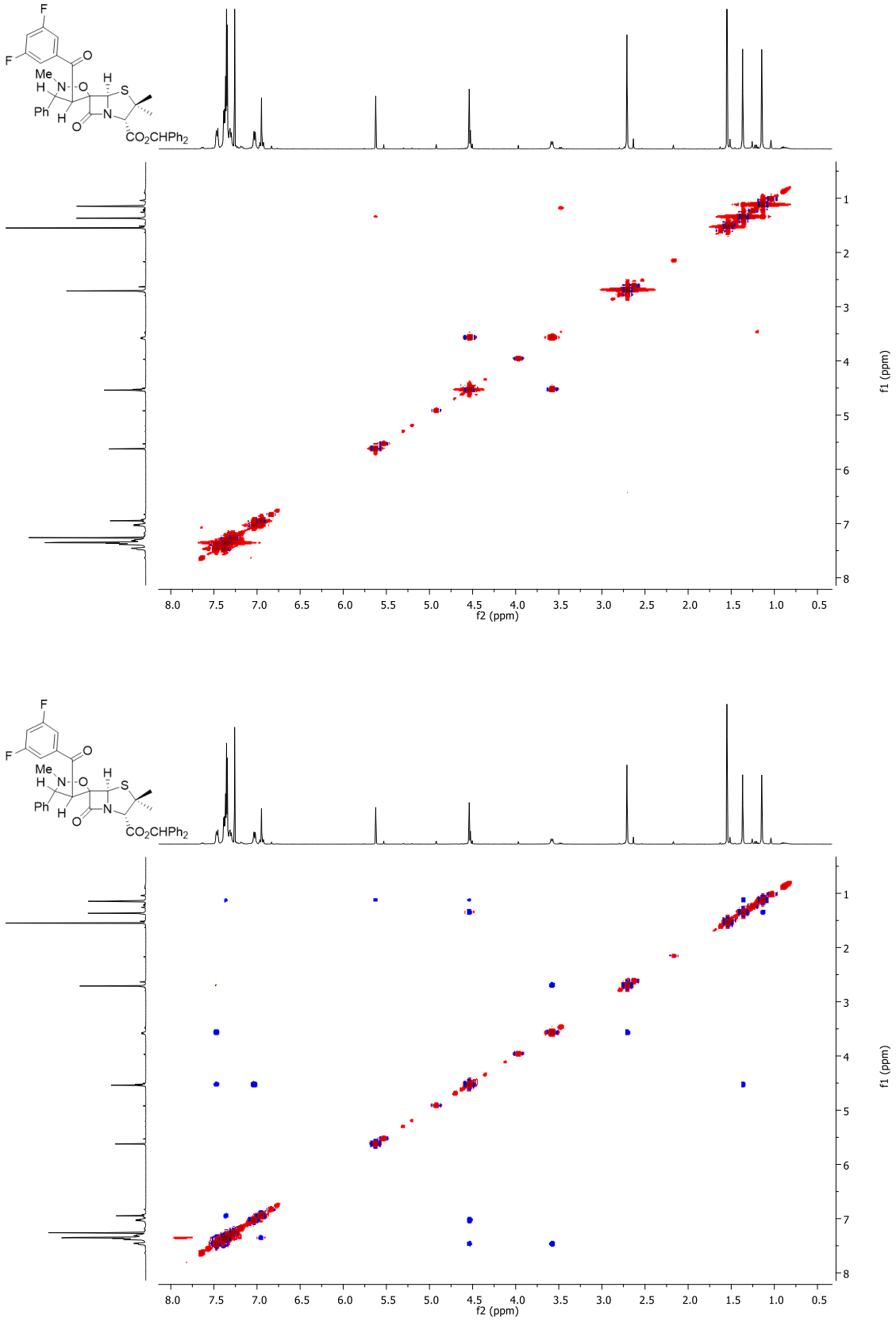
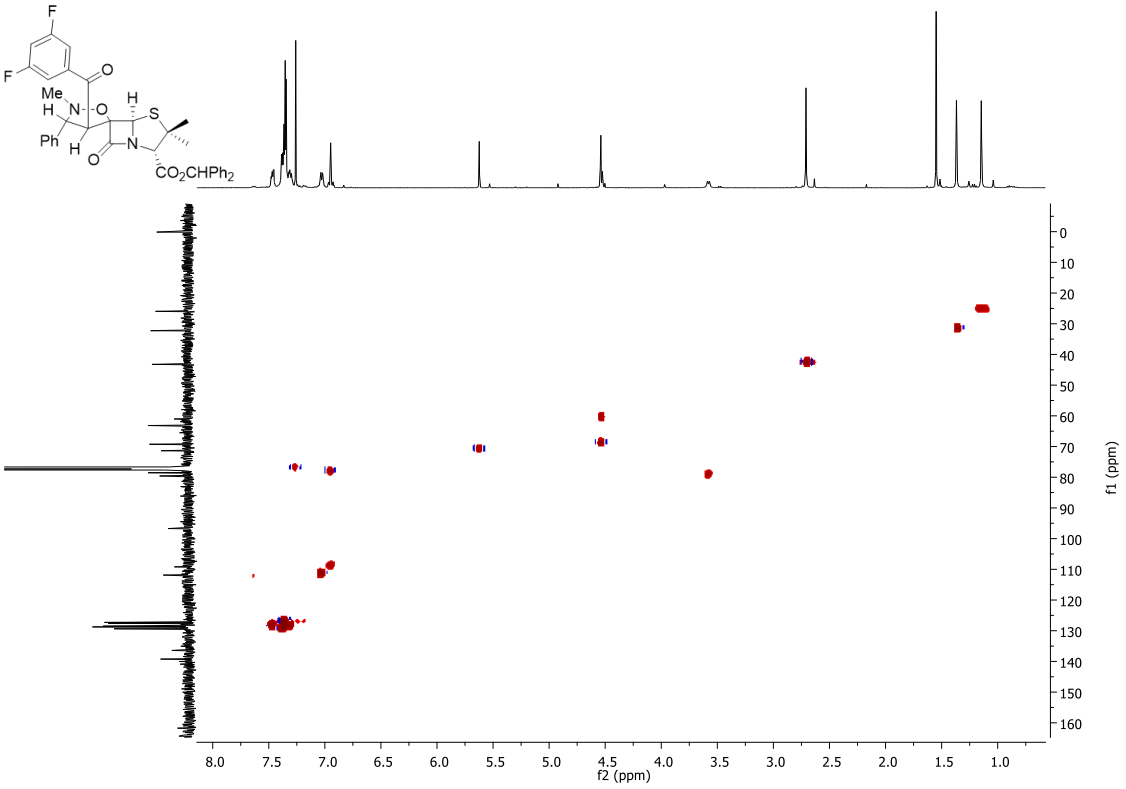


Figure S91: COSY and NOESY spectra of compound **3.47f (**$CDCl_3$).



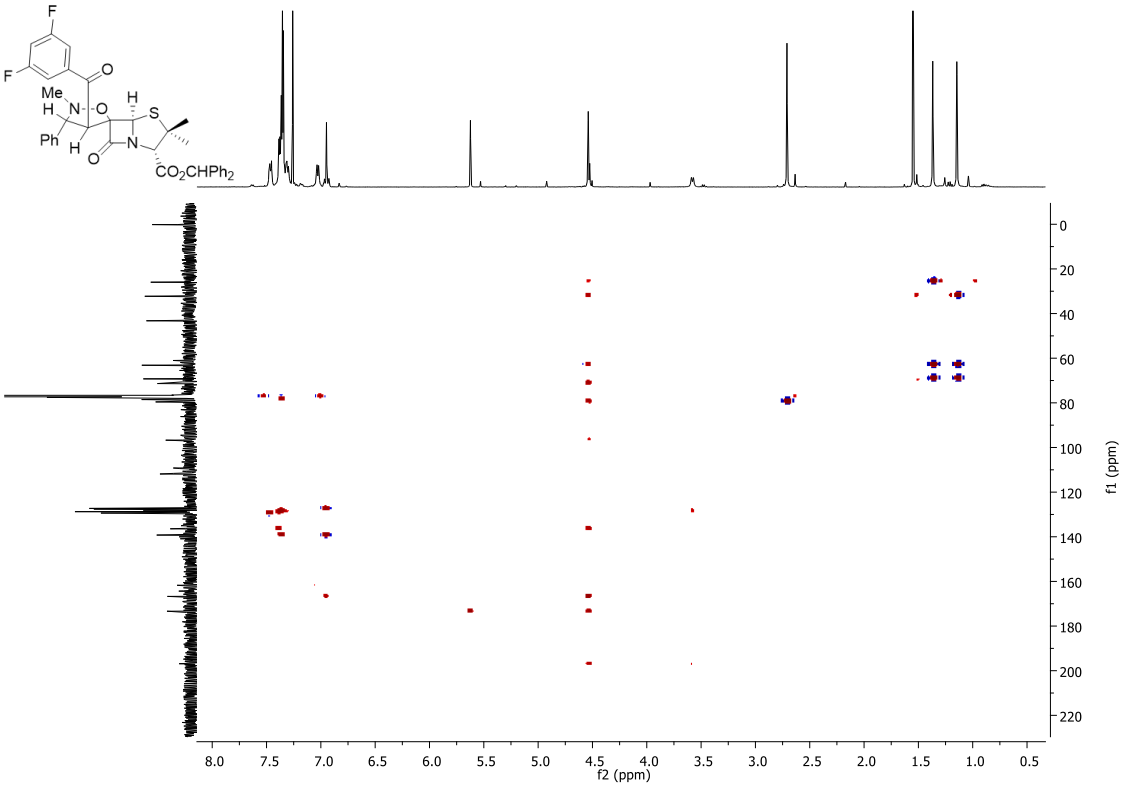


Figure S92: HSQC and HMBC spectra of compound **3.47f (**$CDCl_3$).

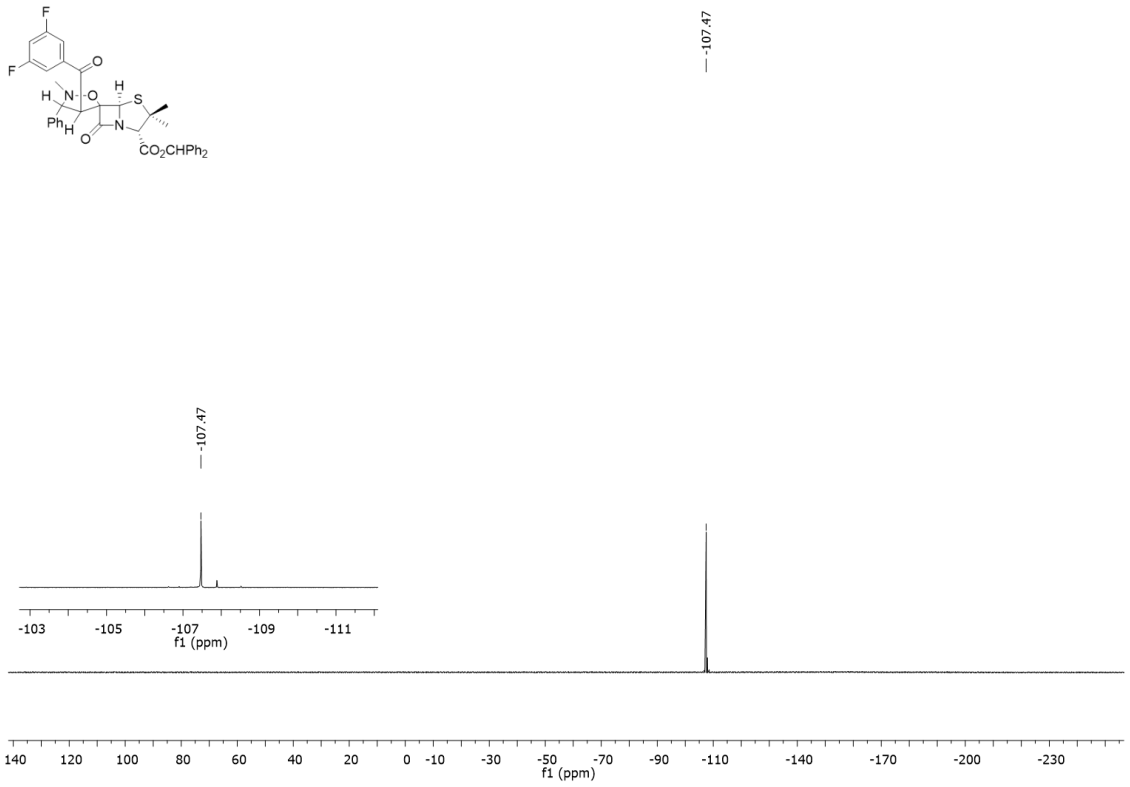


Figure S93: ^{19}F spectrum of compound **3.47f (**$CDCl_3$).

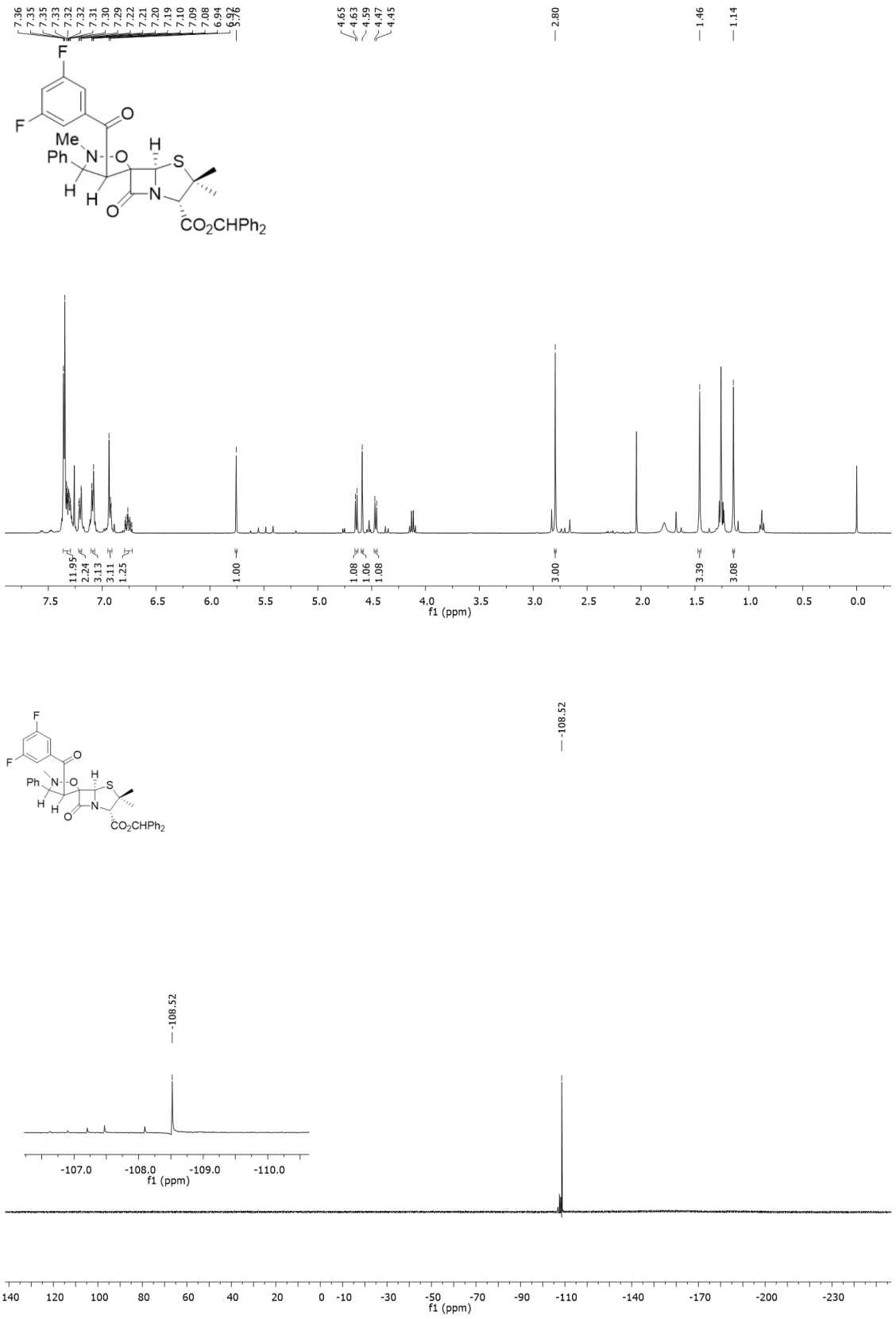


Figure S94: ^{1}H NMR and ^{19}F spectra of compound **3.48f (**$CDCl_3$).

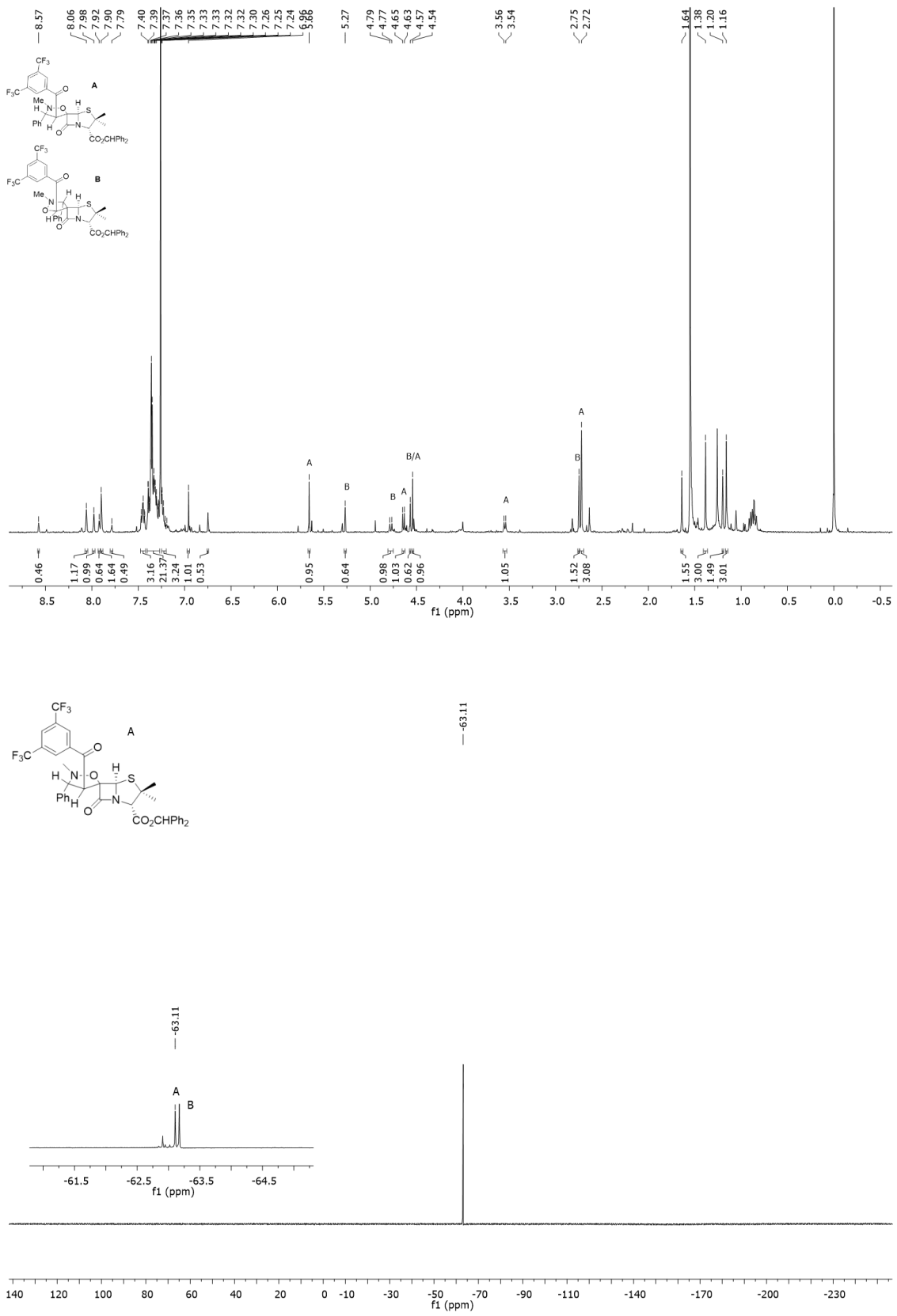


Figure S95: ^{1}H and ^{19}F NMR spectra of compound **3.47g** (A) and **3.49g** (B) **(**$CDCl_3$).

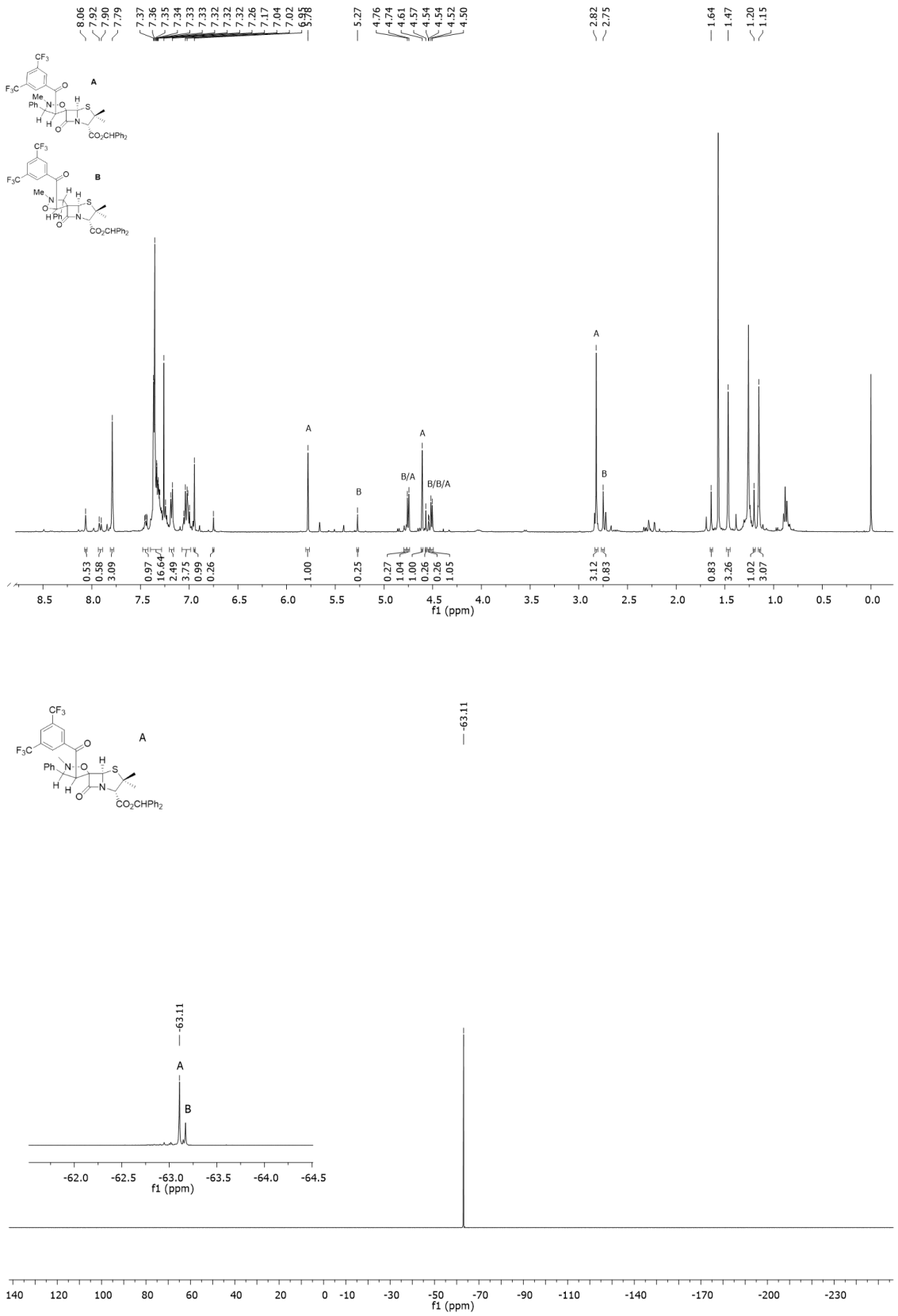


Figure S96: ^{1}H and ^{19}F NMR spectra of compound **3.48g** (A) and **3.49g** (B) **(**$CDCl_3$).

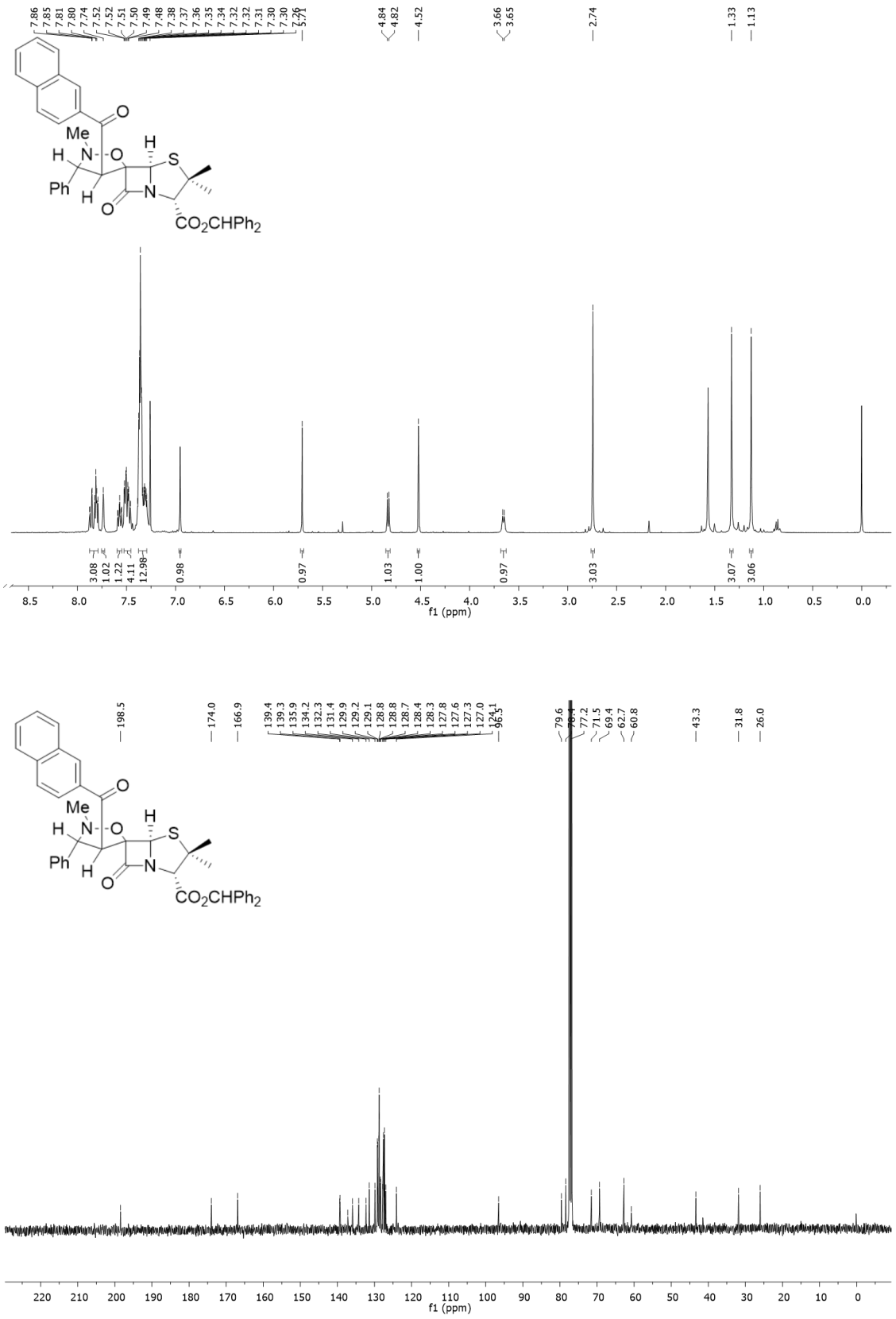


Figure S97: ^{1}H and ^{13}C NMR spectra of compound **3.47h (**$CDCl_3$**)**.

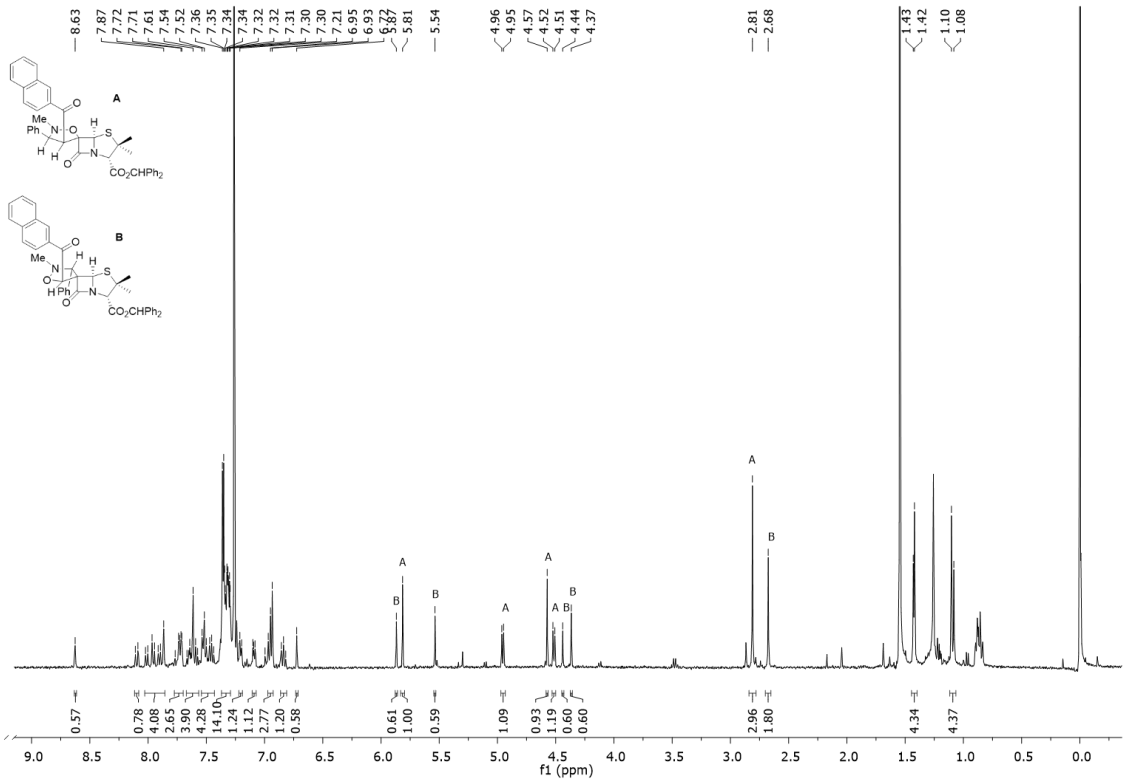


Figure S98: ^{1}H NMR spectrum of compound **3.48h** (A) and **3.49h** (B) **(**$CDCl_3$).

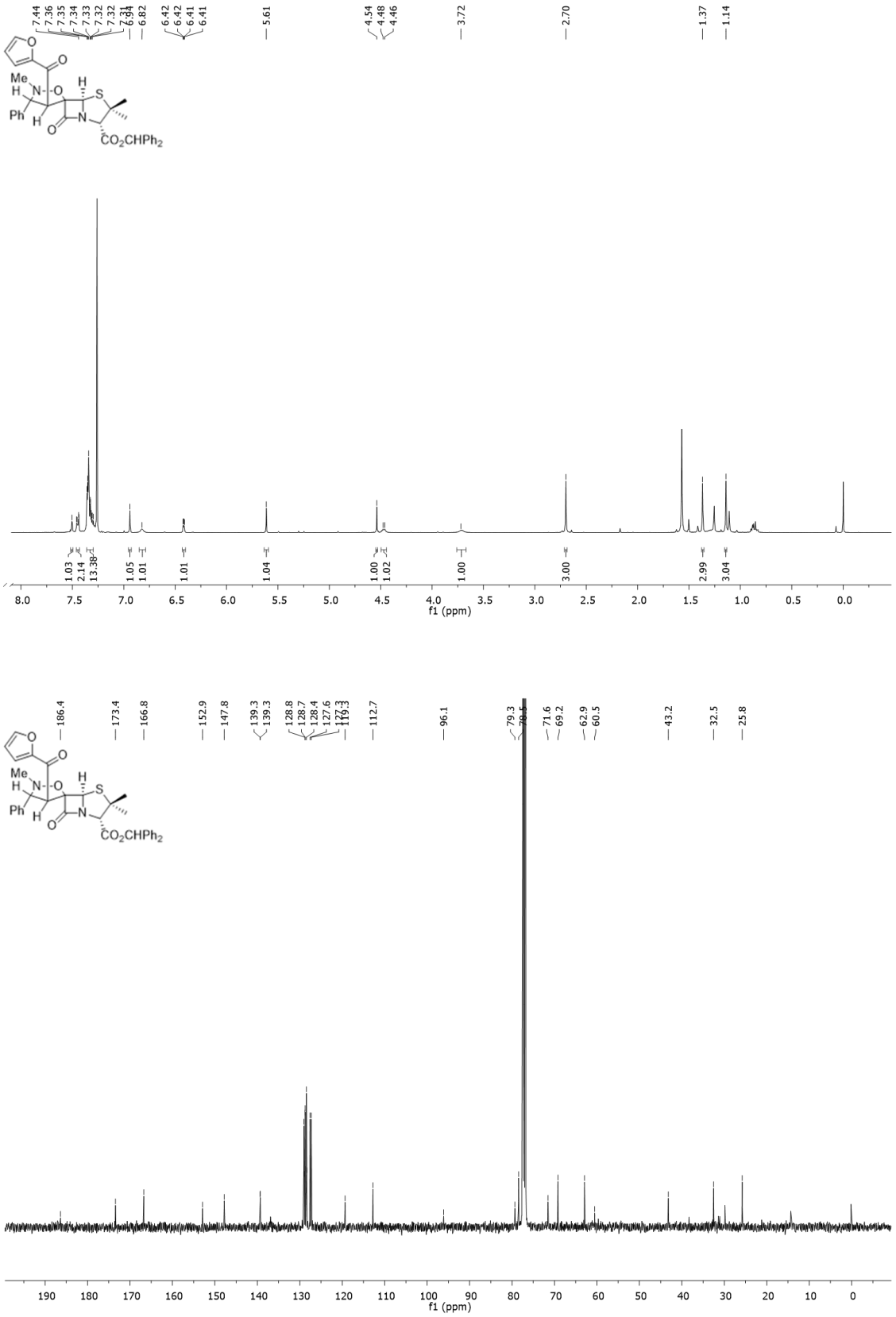


Figure S99: ^{1}H and ^{13}C NMR spectra of compound **3.47i (**$CDCl_3$).

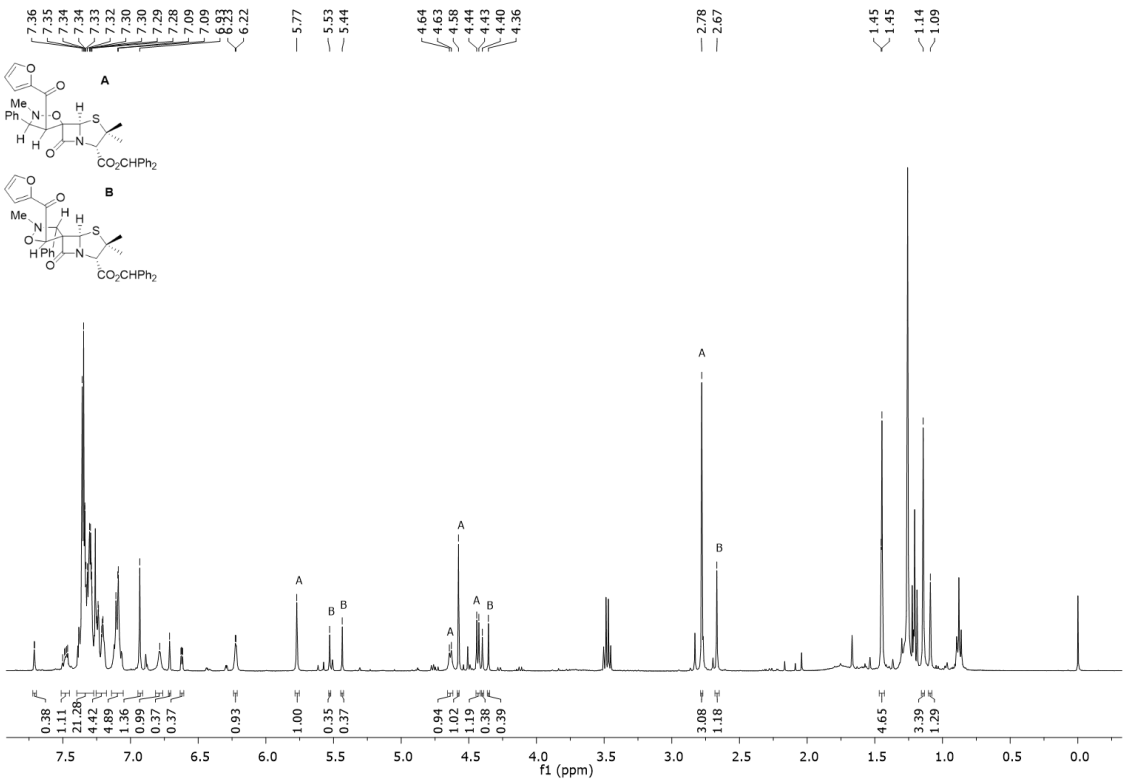


Figure S100: ^{1}H NMR spectrum of compound **3.48i** (A) and **3.49i** (B) **(**$CDCl_3$).

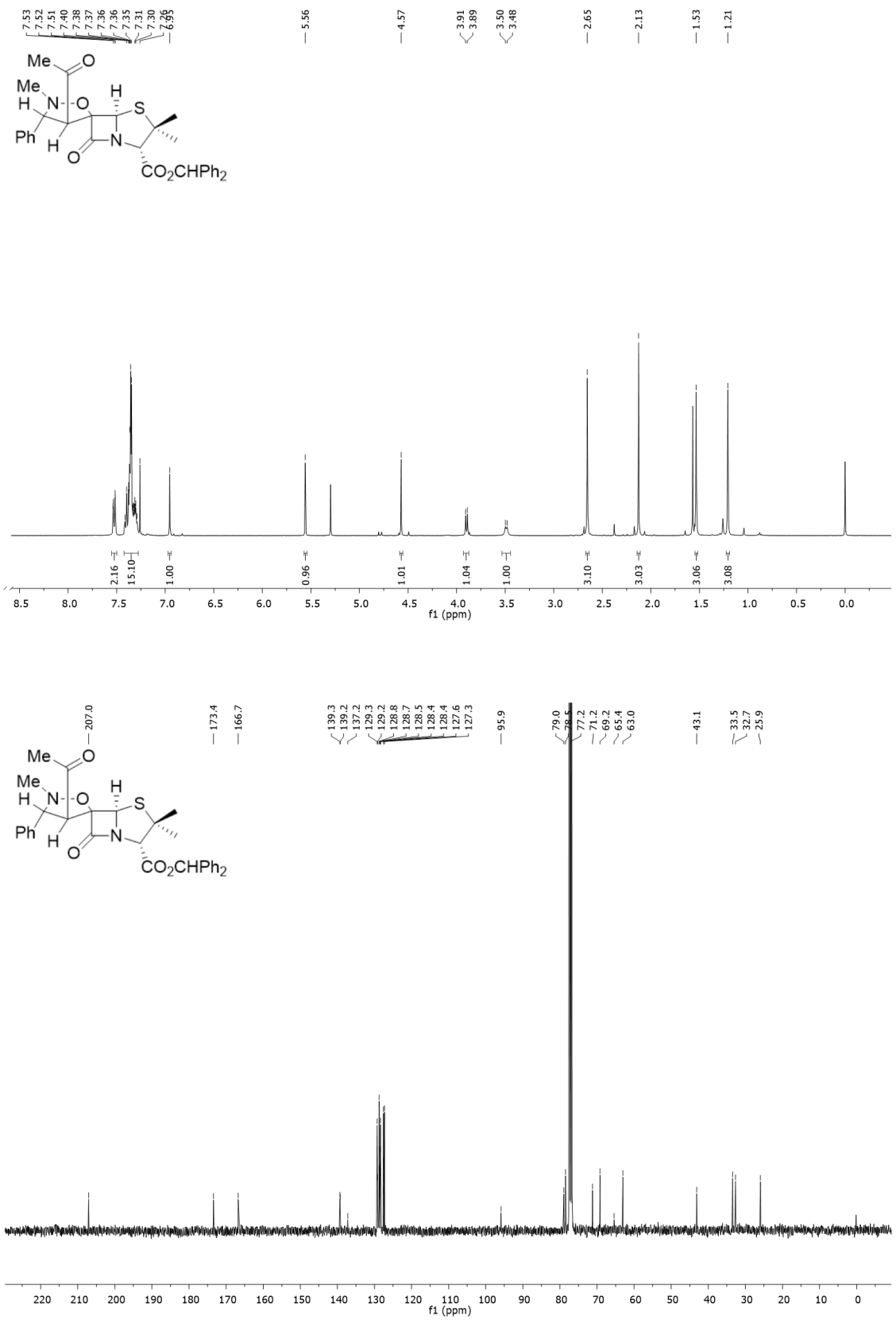


Figure S101: ^{1}H and ^{13}C NMR spectra of compound **3.47j (**$CDCl_3$).

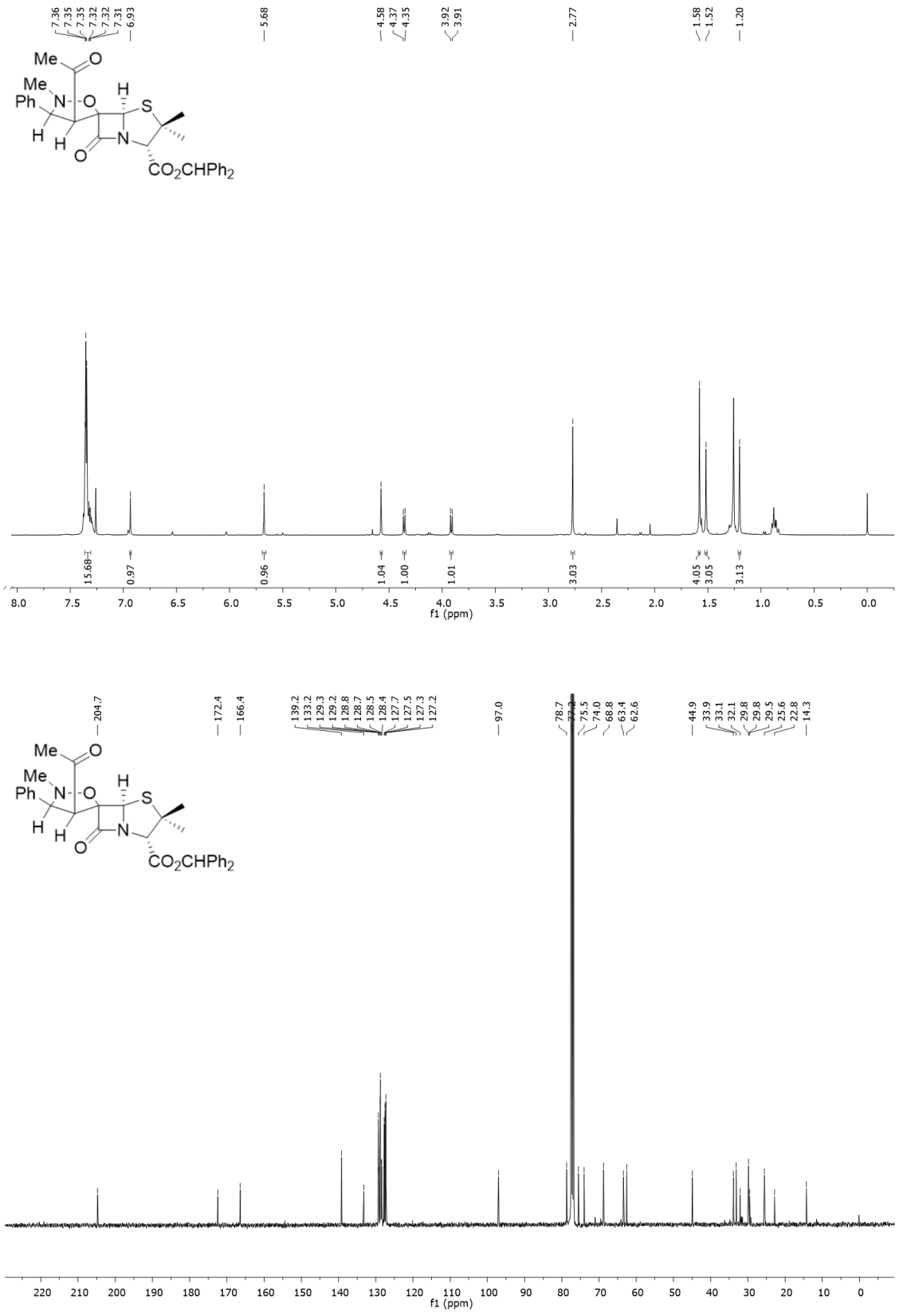


Figure S102: ^{1}H and ^{13}C NMR spectra of compound **3.48j (**$CDCl_3$).

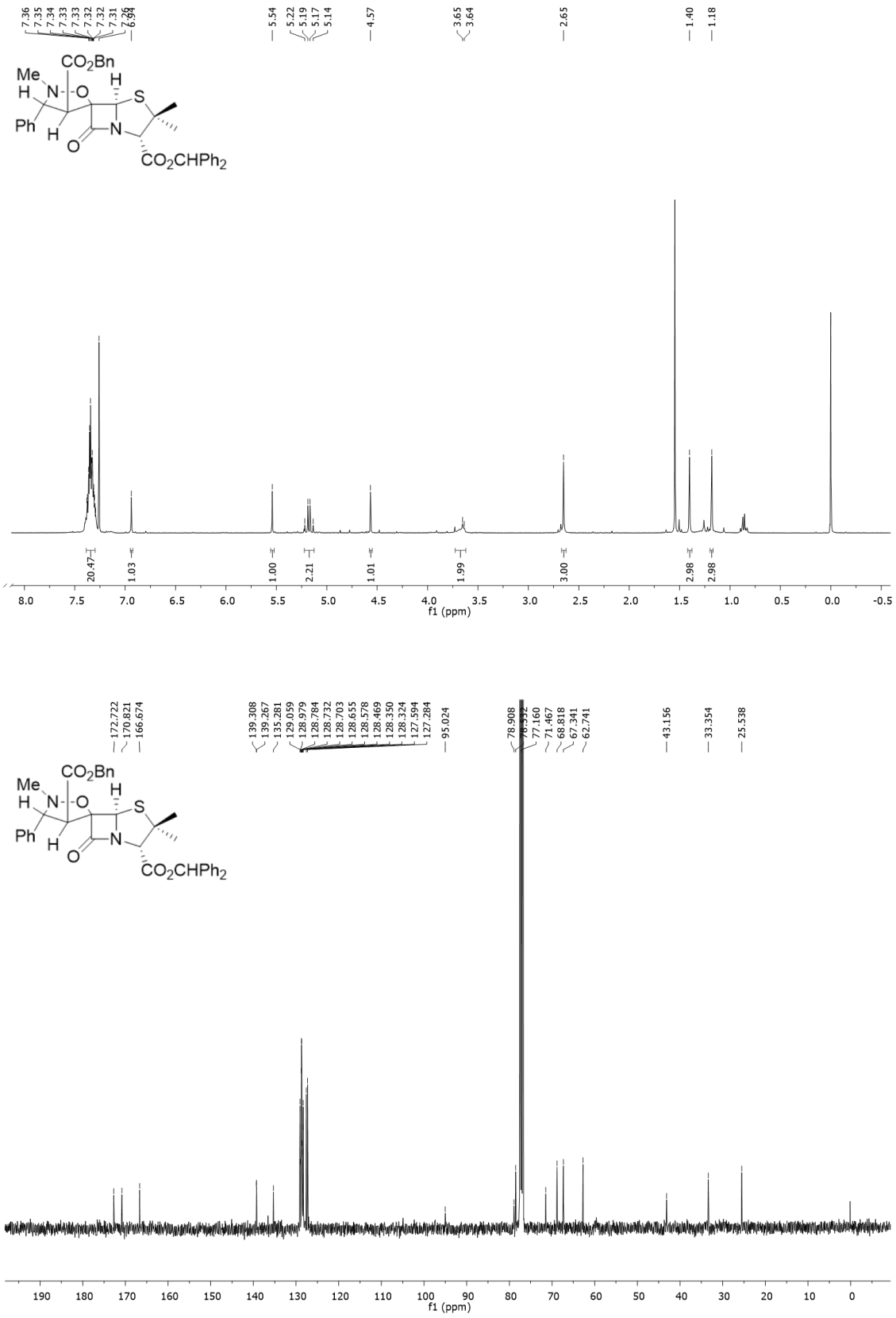


Figure S103: ^{1}H and ^{13}C NMR spectra of compound **3.47k (**$CDCl_3$).

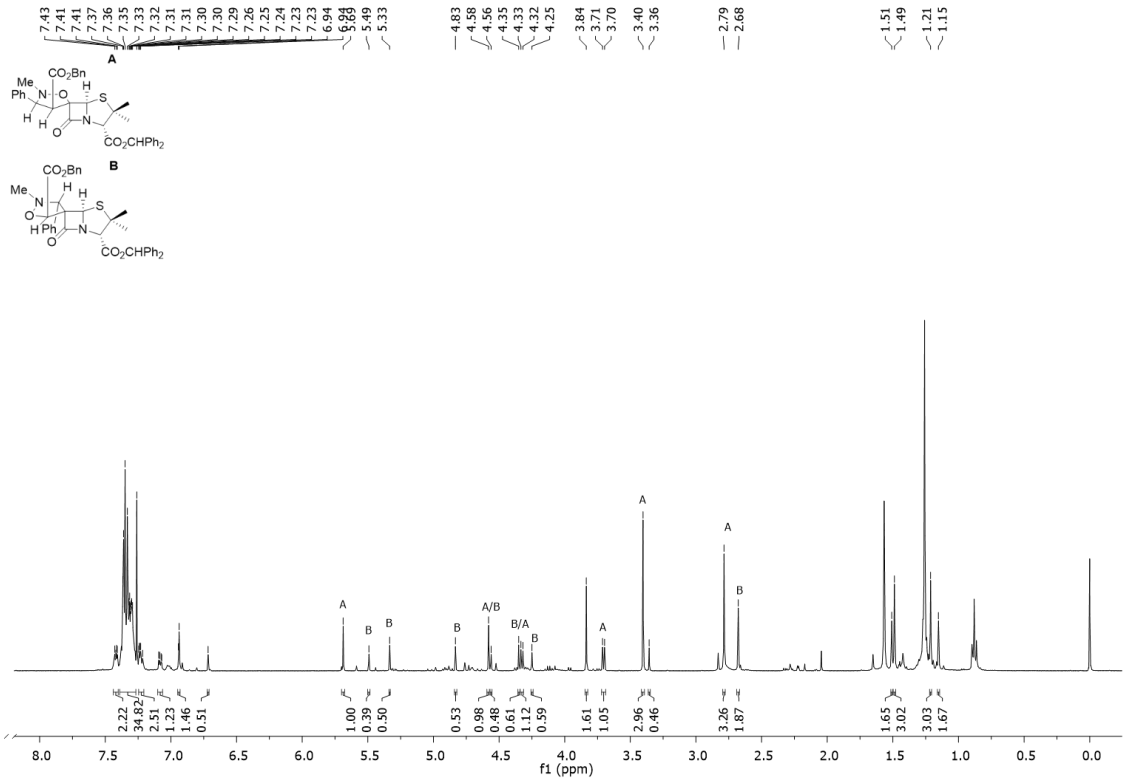


Figure S104: ^{1}H NMR spectrum of compound **3.48k** (A) and **3.49k** (B) **(**$CDCl_3$).

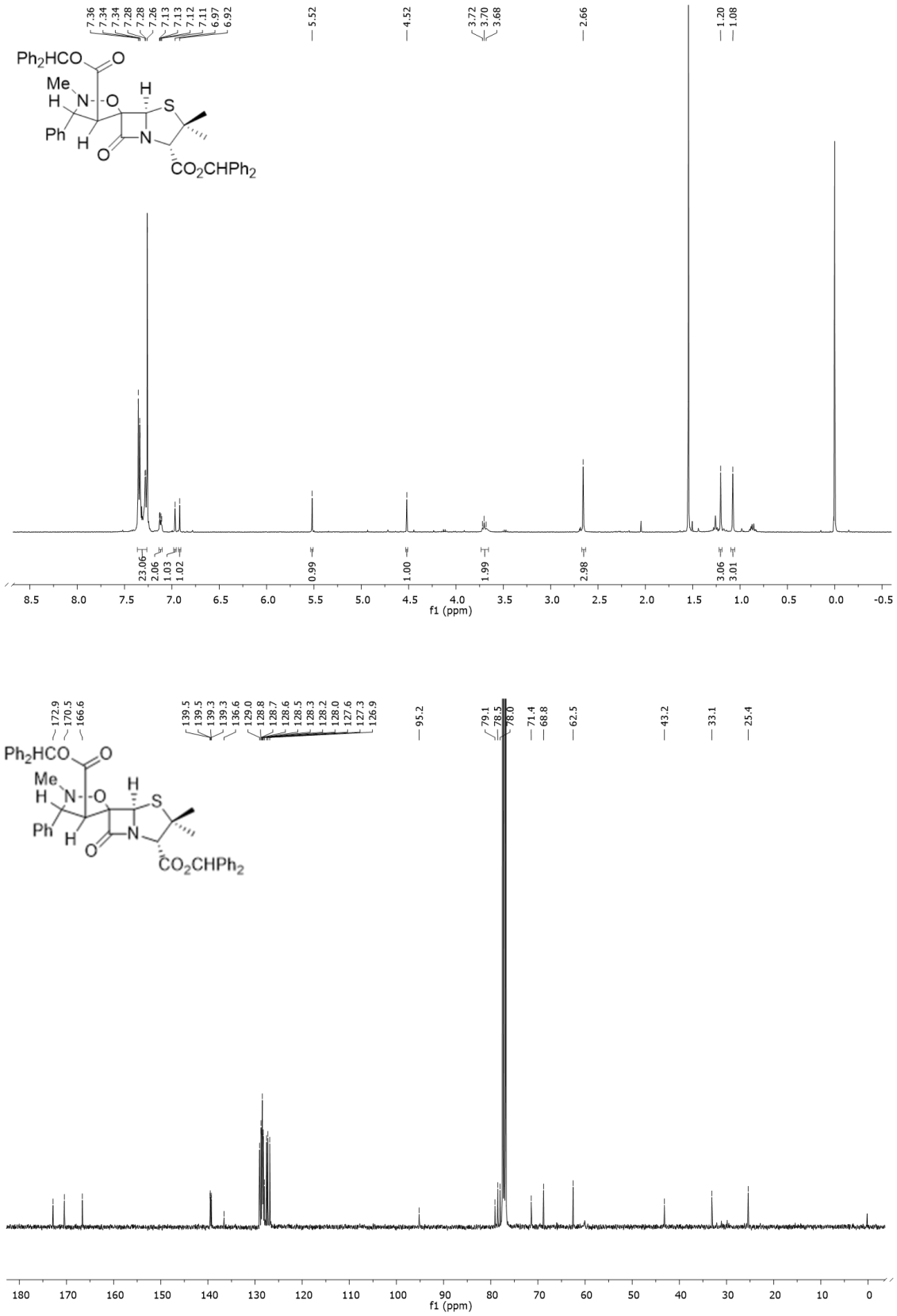


Figure S105: ^{1}H and ^{13}C NMR spectra of compound **3.47l (**$CDCl_3$).

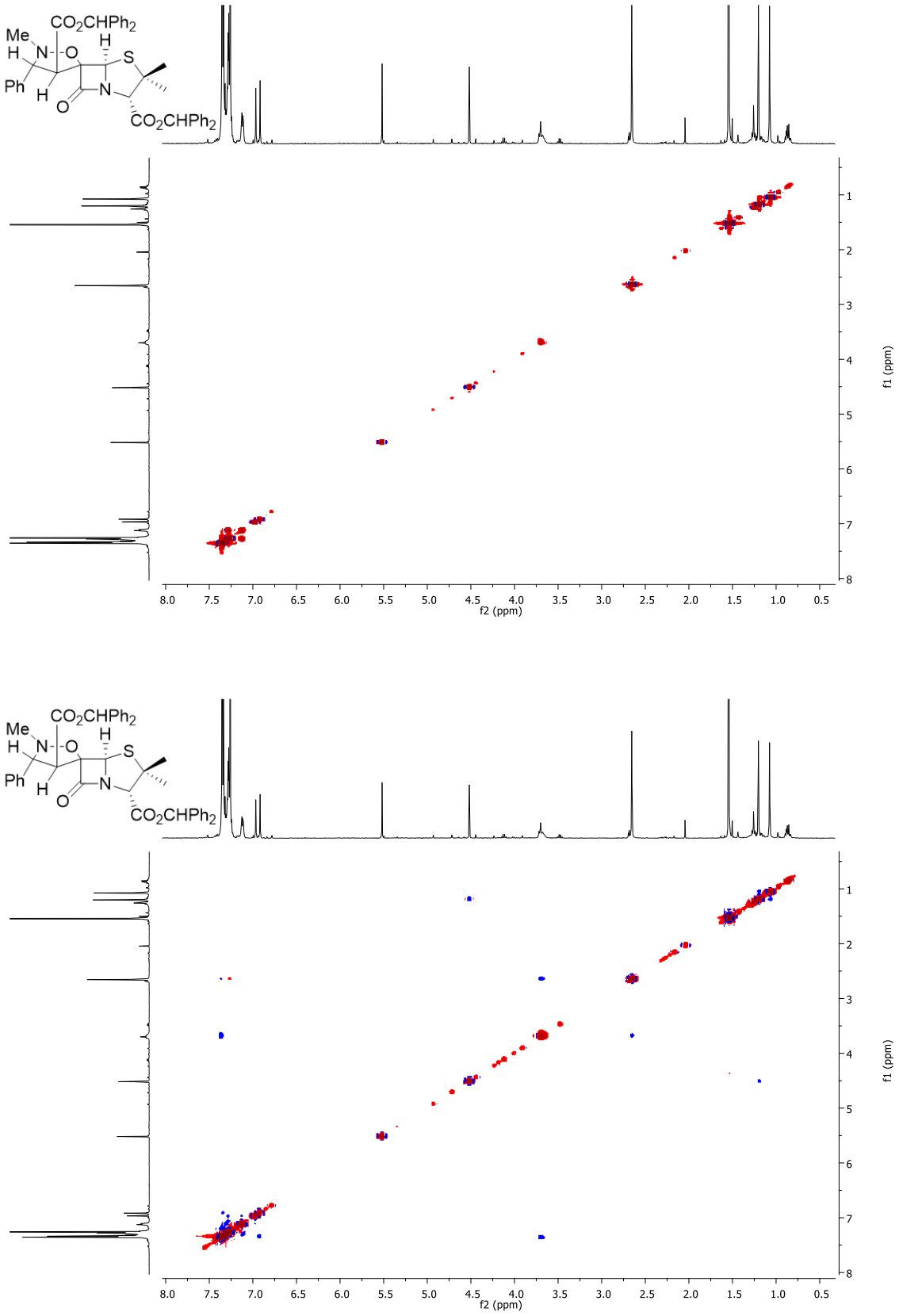


Figure S106: COSY and NOESY spectra of compound **3.47l (**$CDCl_3$).

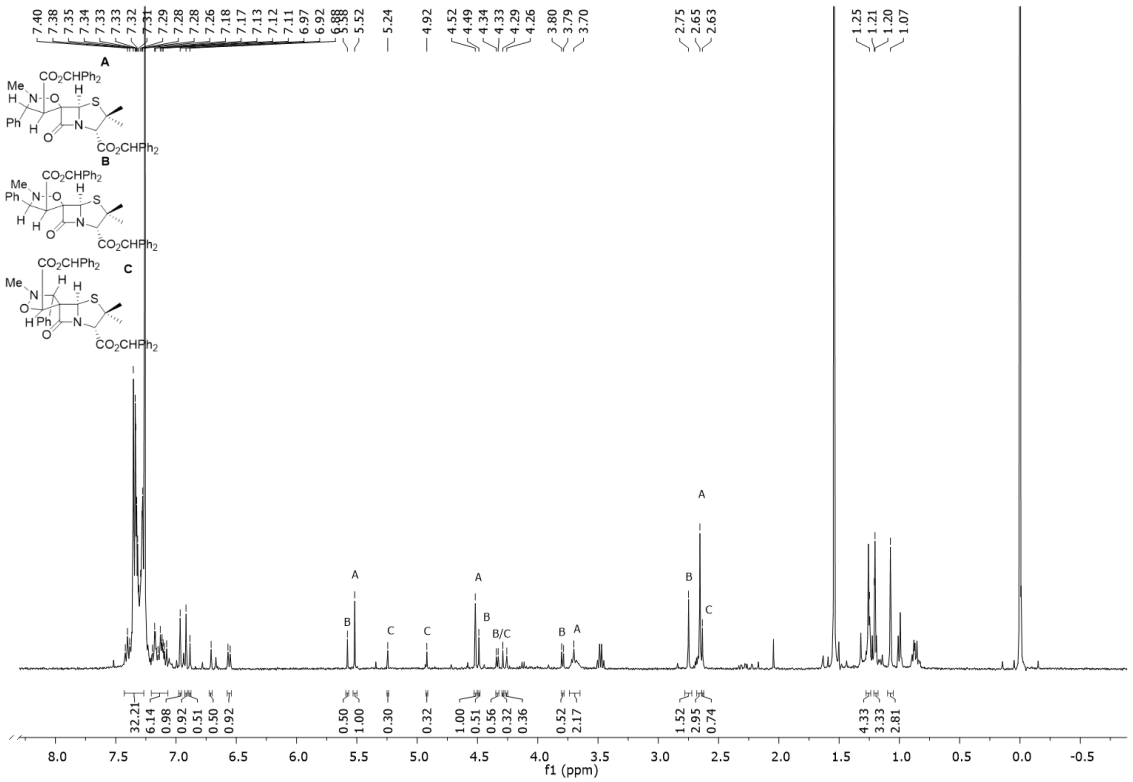


Figure S107: ^{1}H NMR spectrum of compound **3.47l** (A), **3.48l** (B) and **3.49l** (C) **(**$CDCl_3$**).**

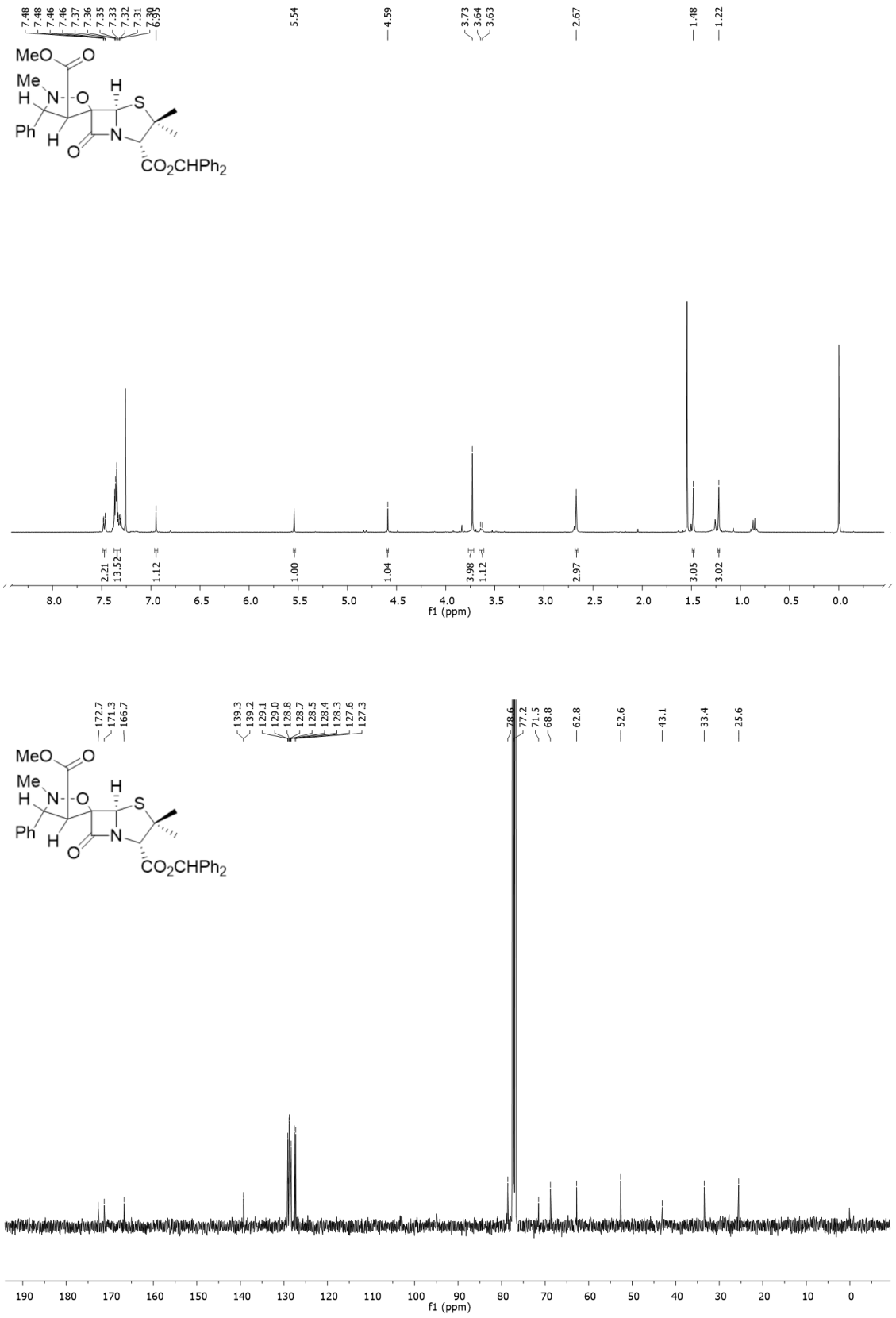


Figure S108: ^{1}H and ^{13}C NMR spectra of compound **3.47m (**$CDCl_3$).

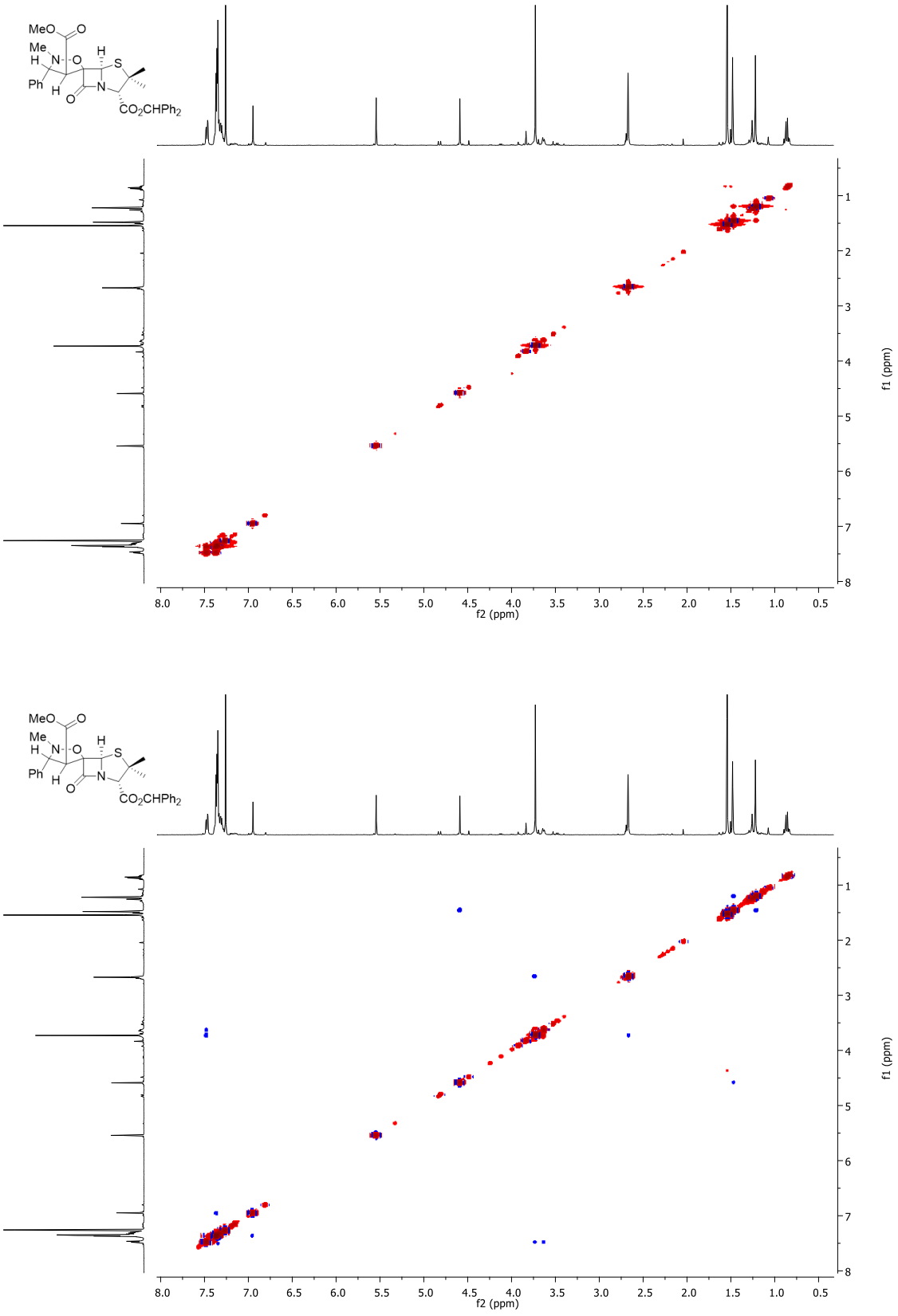


Figure S109: COSY and NOESY spectra of compound **3.47m (**$CDCl_3$).

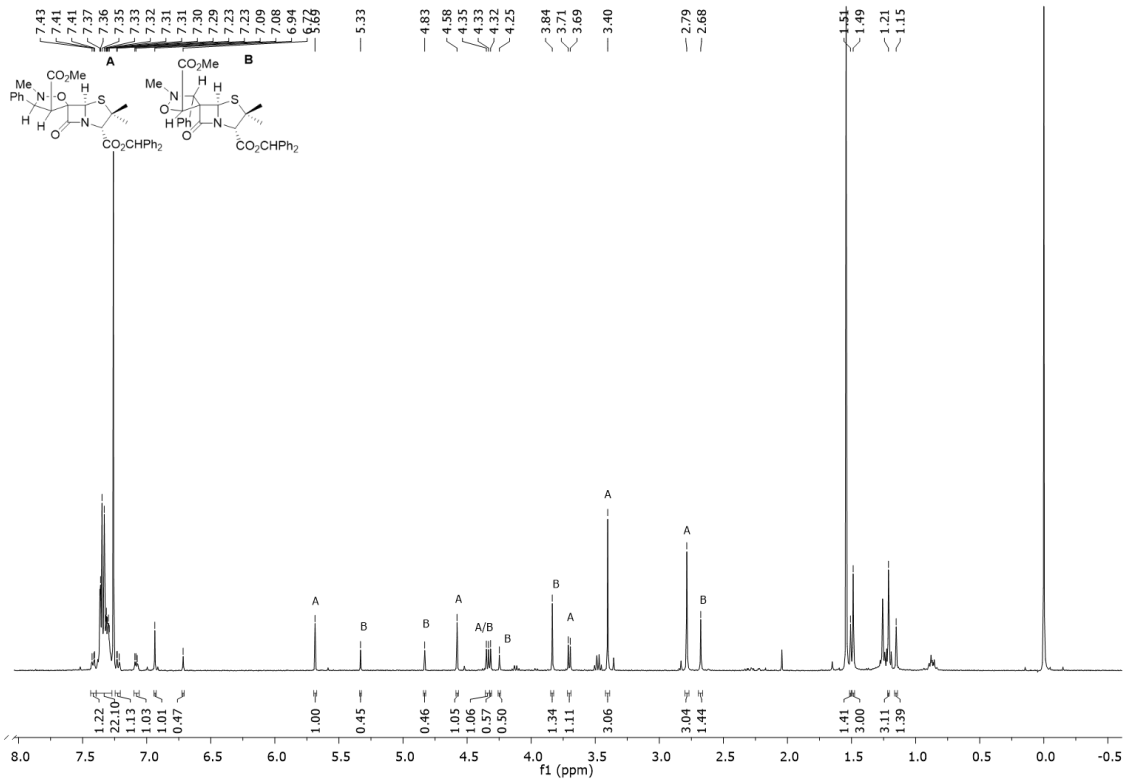


Figure S110: ^{1}H NMR spectrum of compound **3.48m** (A) and **3.49m** (B) **(**$CDCl_3$).

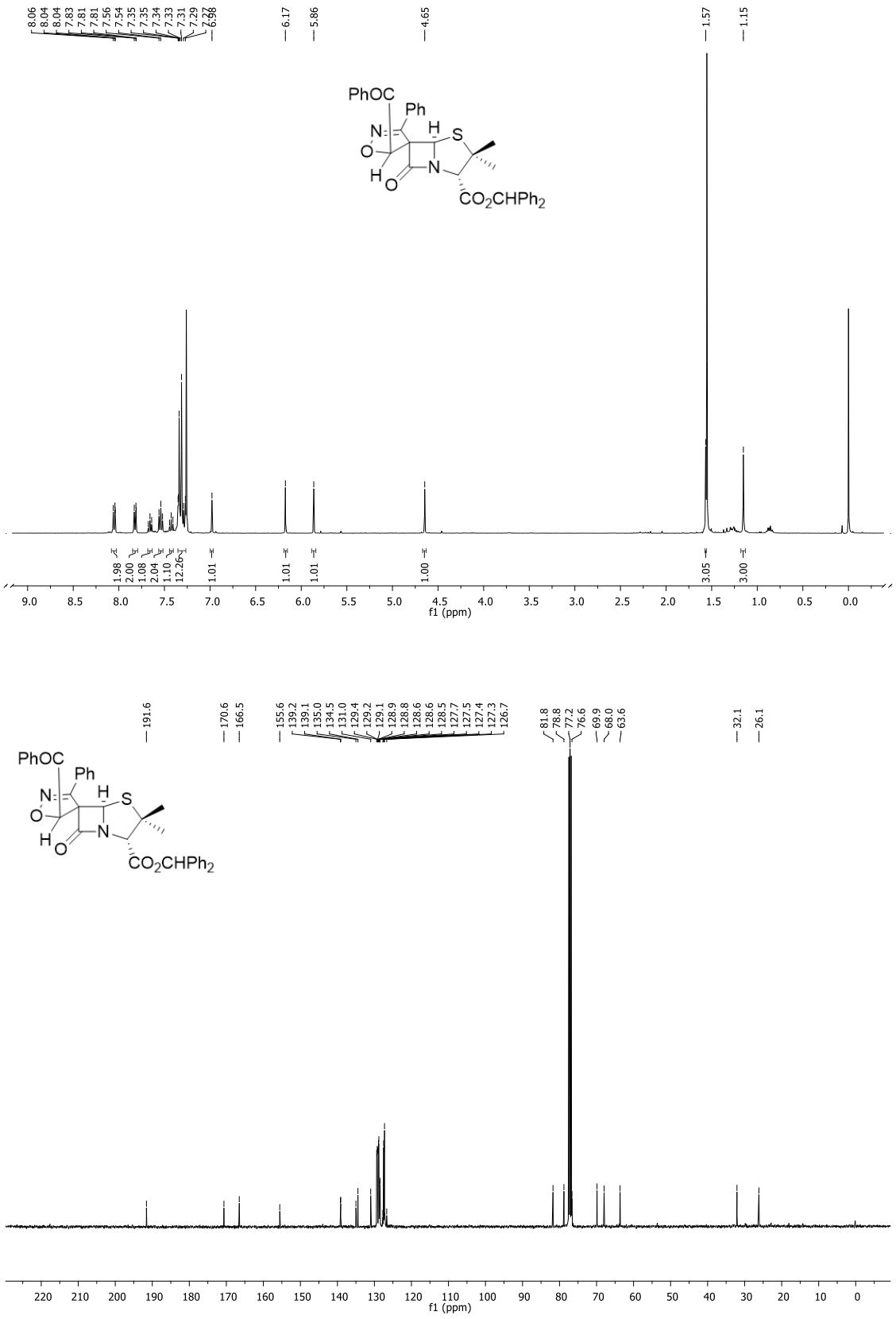


Figure S111: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **4.51a**.

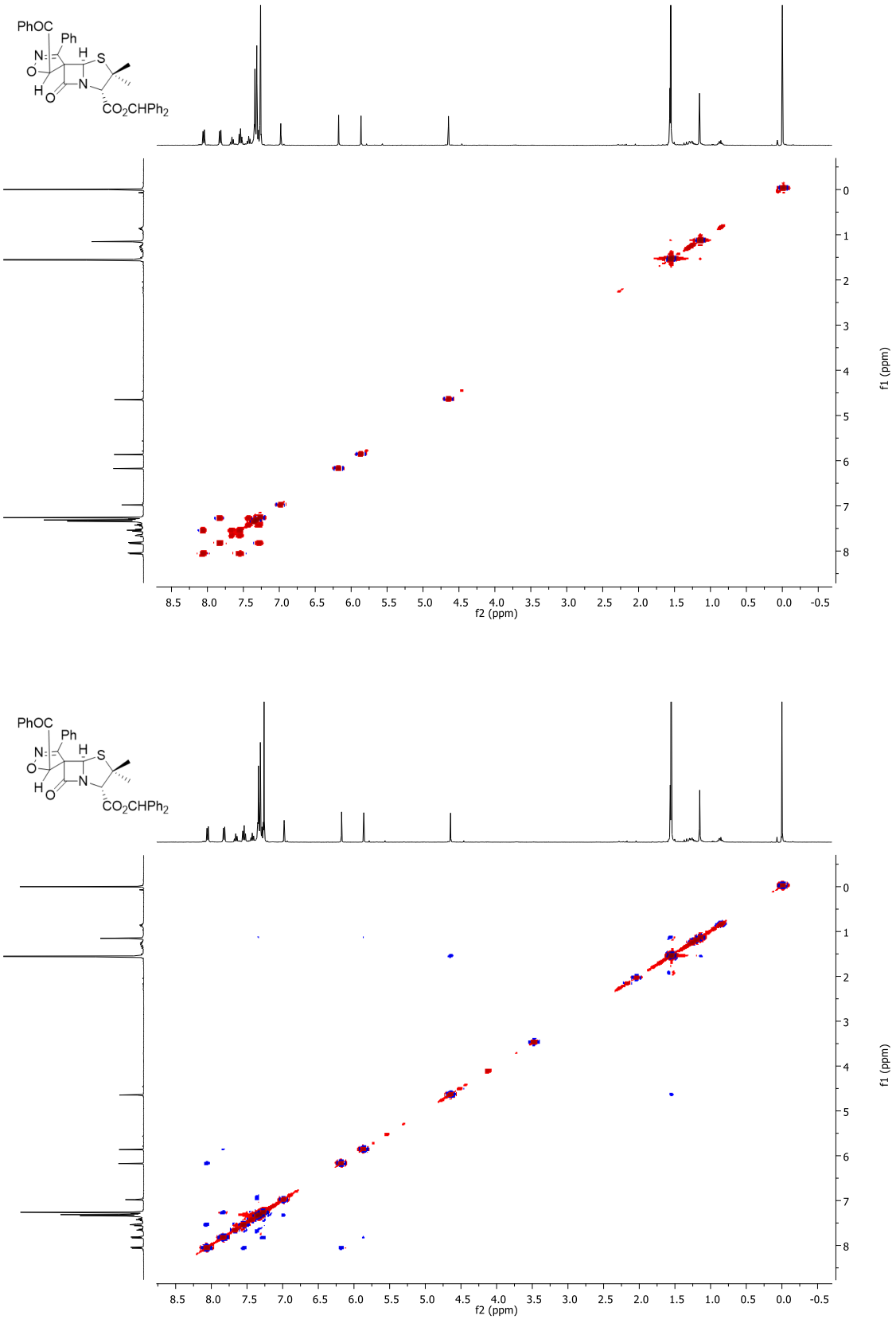


Figure S112: COSY and NOESY spectra ($CDCl_3$) of compound **4.51a**.

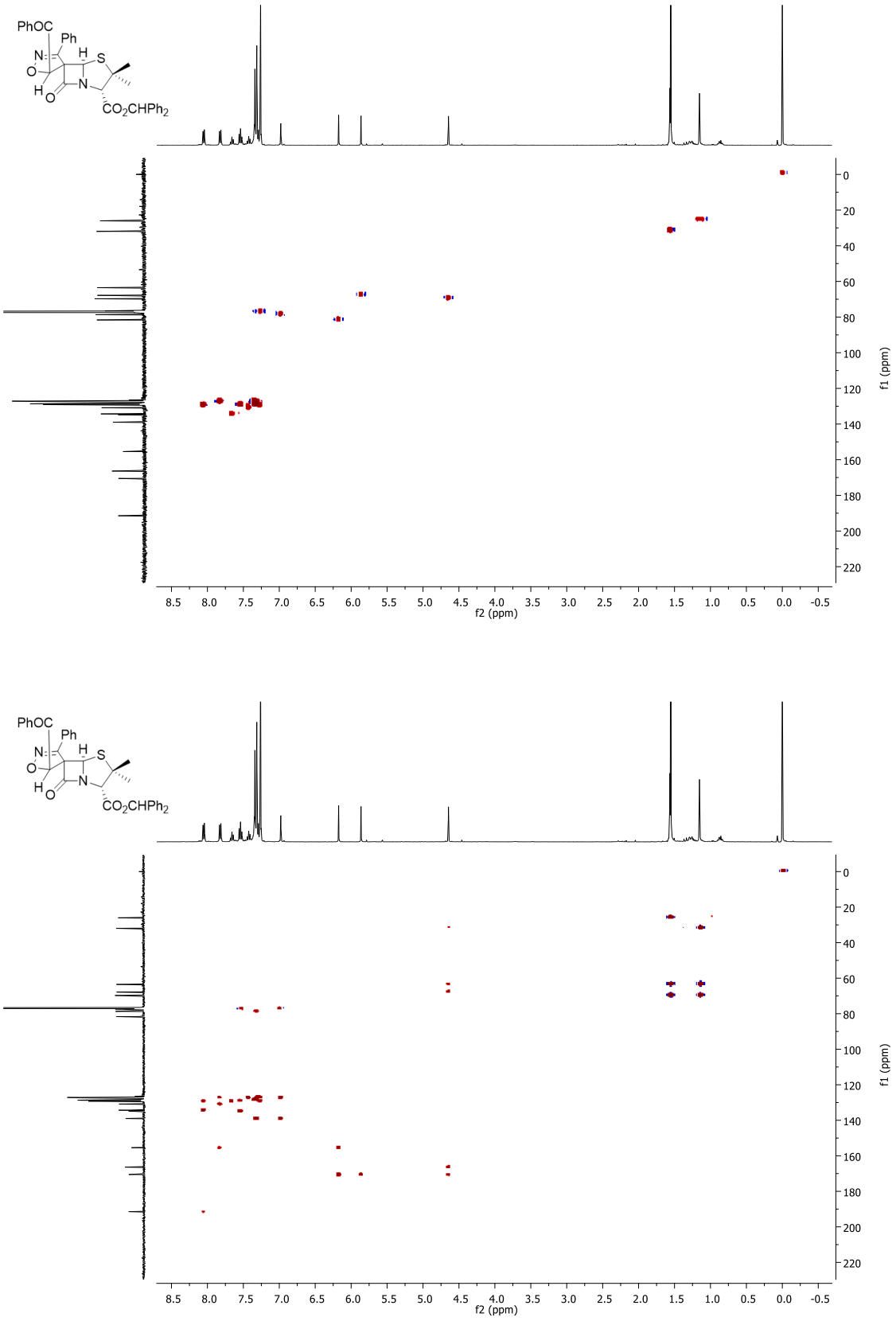


Figure S113: HSQC and HMBC spectra ($CDCl_3$) of compound **4.51a**.

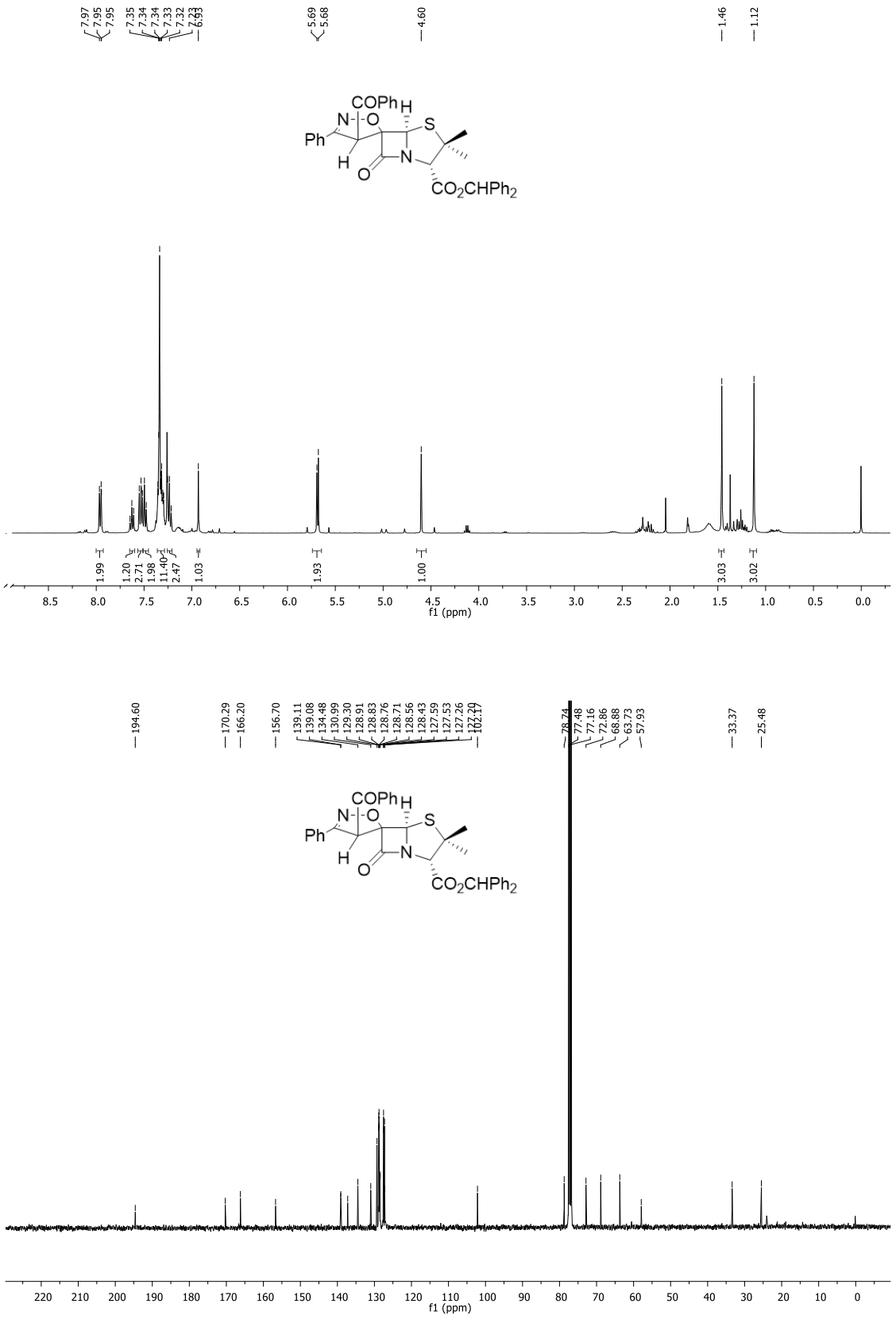


Figure S114: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **4.52a**.

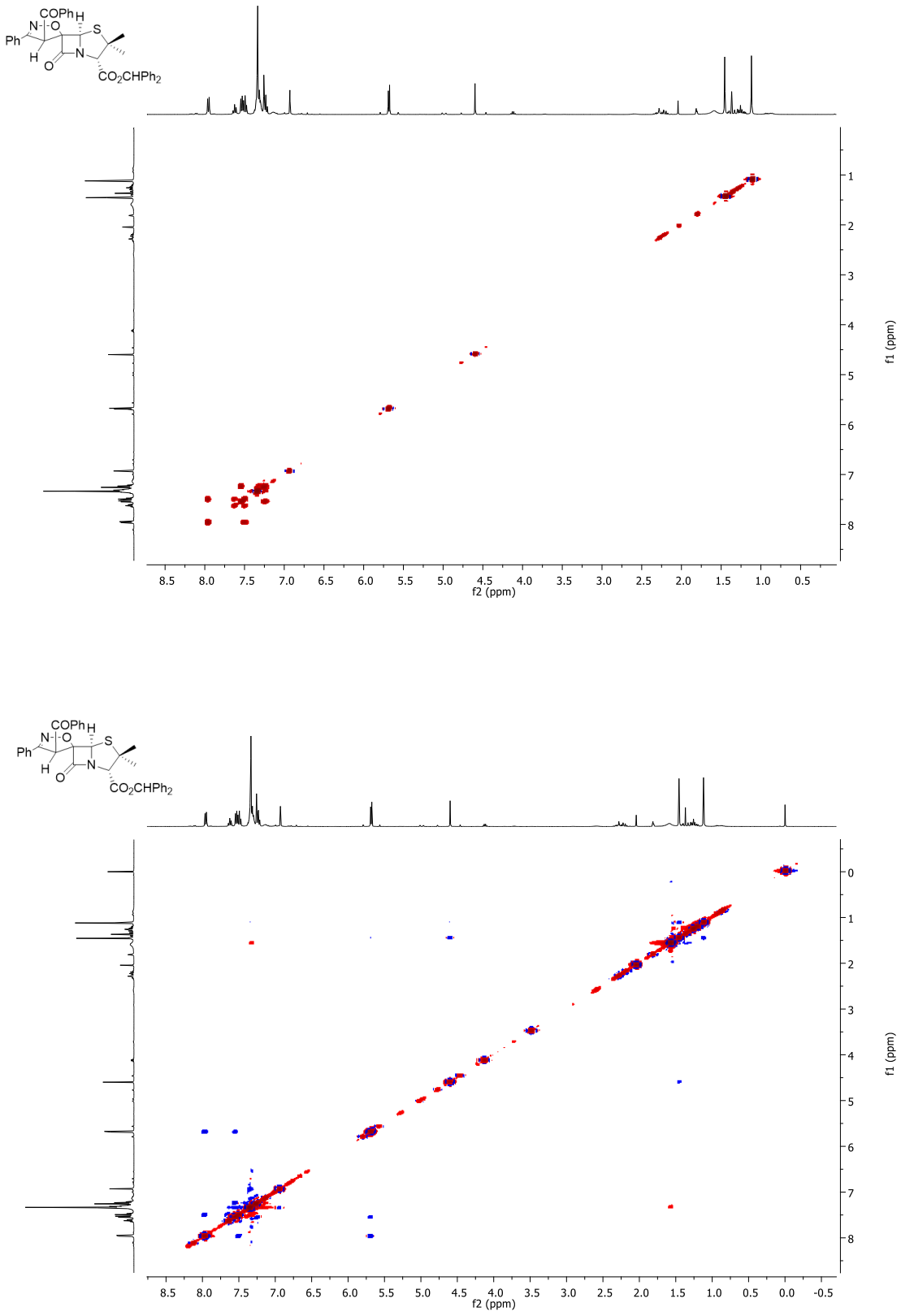


Figure S115: COSY and NOESY spectra ($CDCl_3$) of compound **4.52a**.

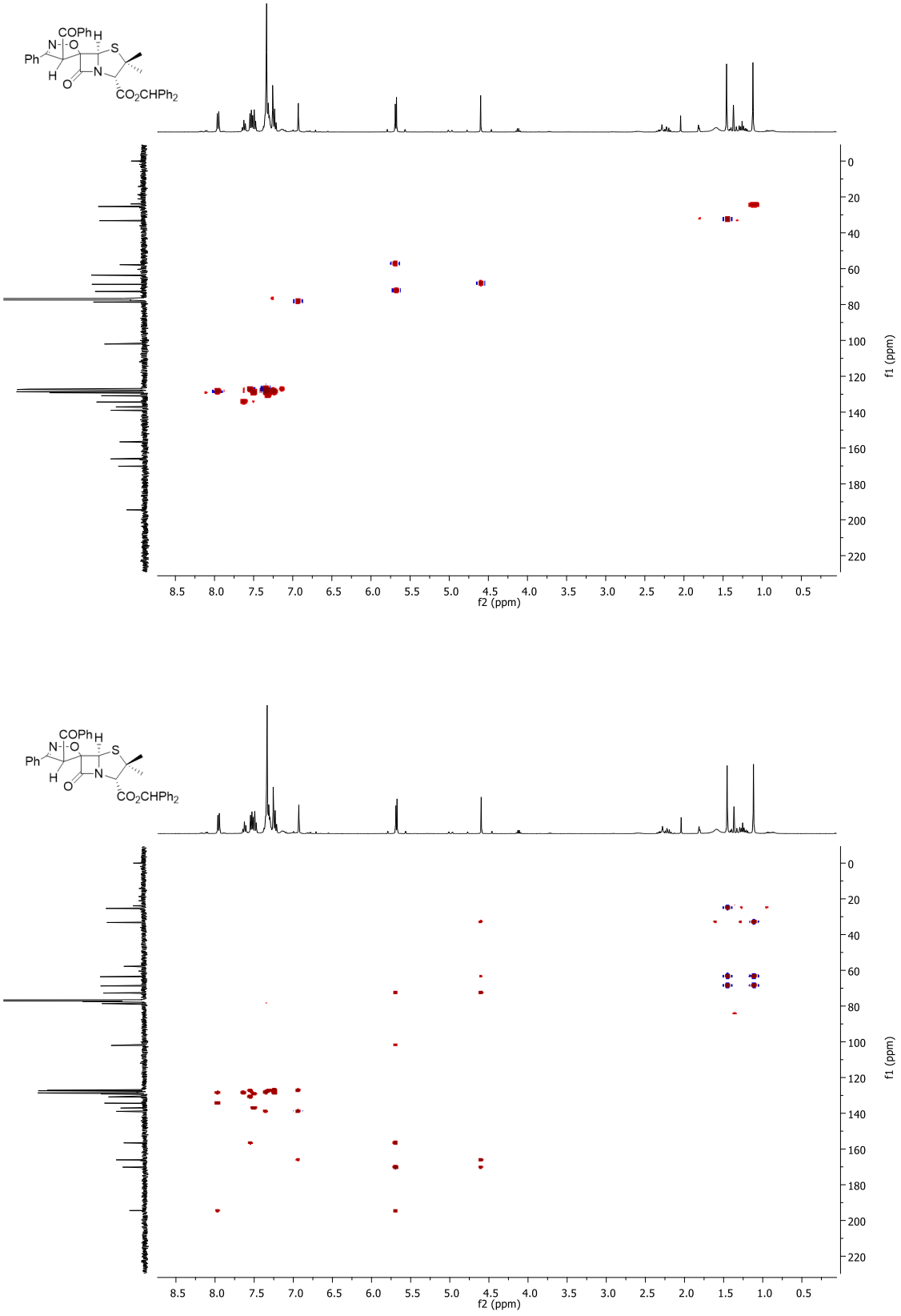


Figure S116: HSQC and HMBC spectra ($CDCl_3$) of compound **4.52a**.

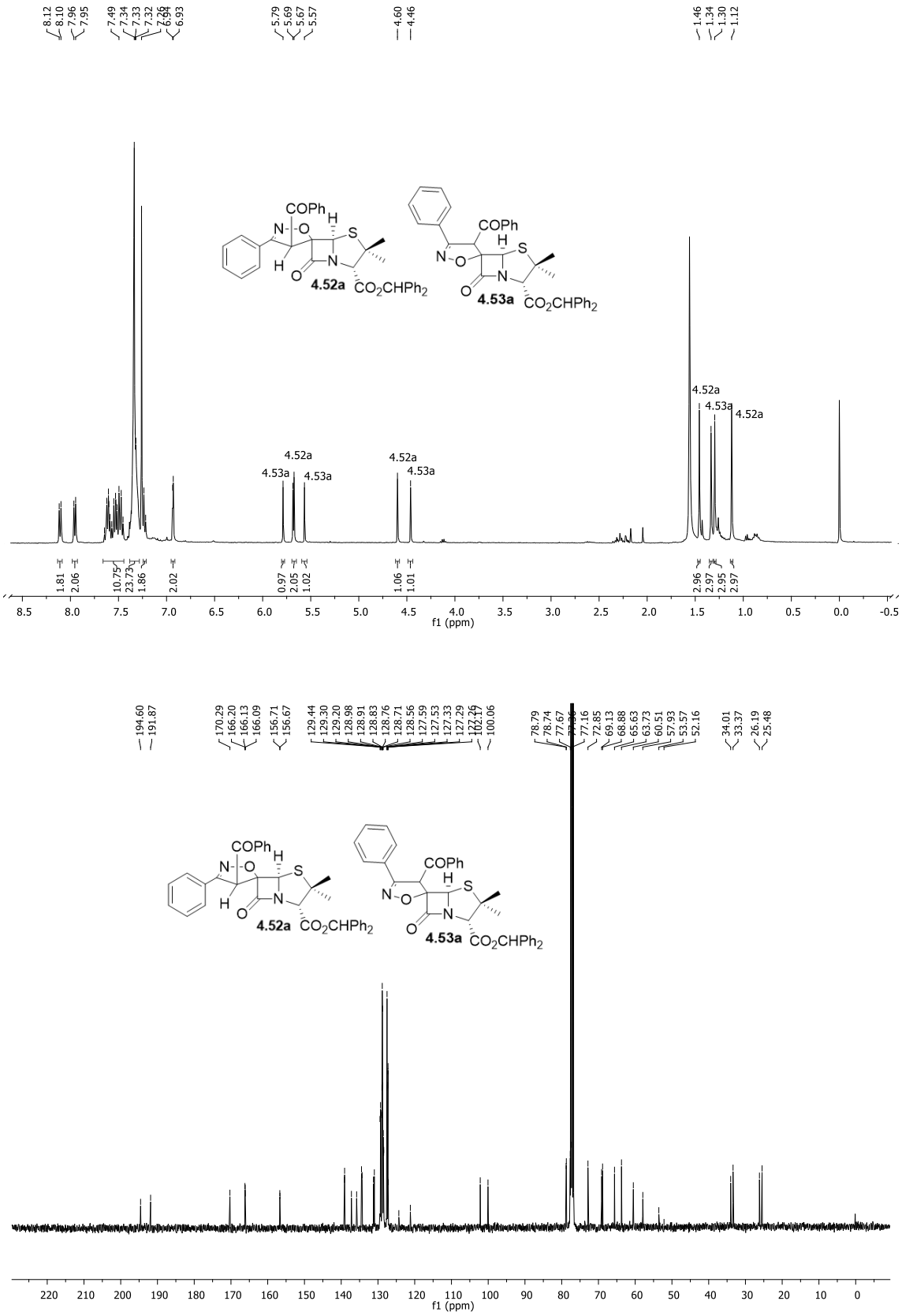


Figure S117: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **4.52a/4.53a**.

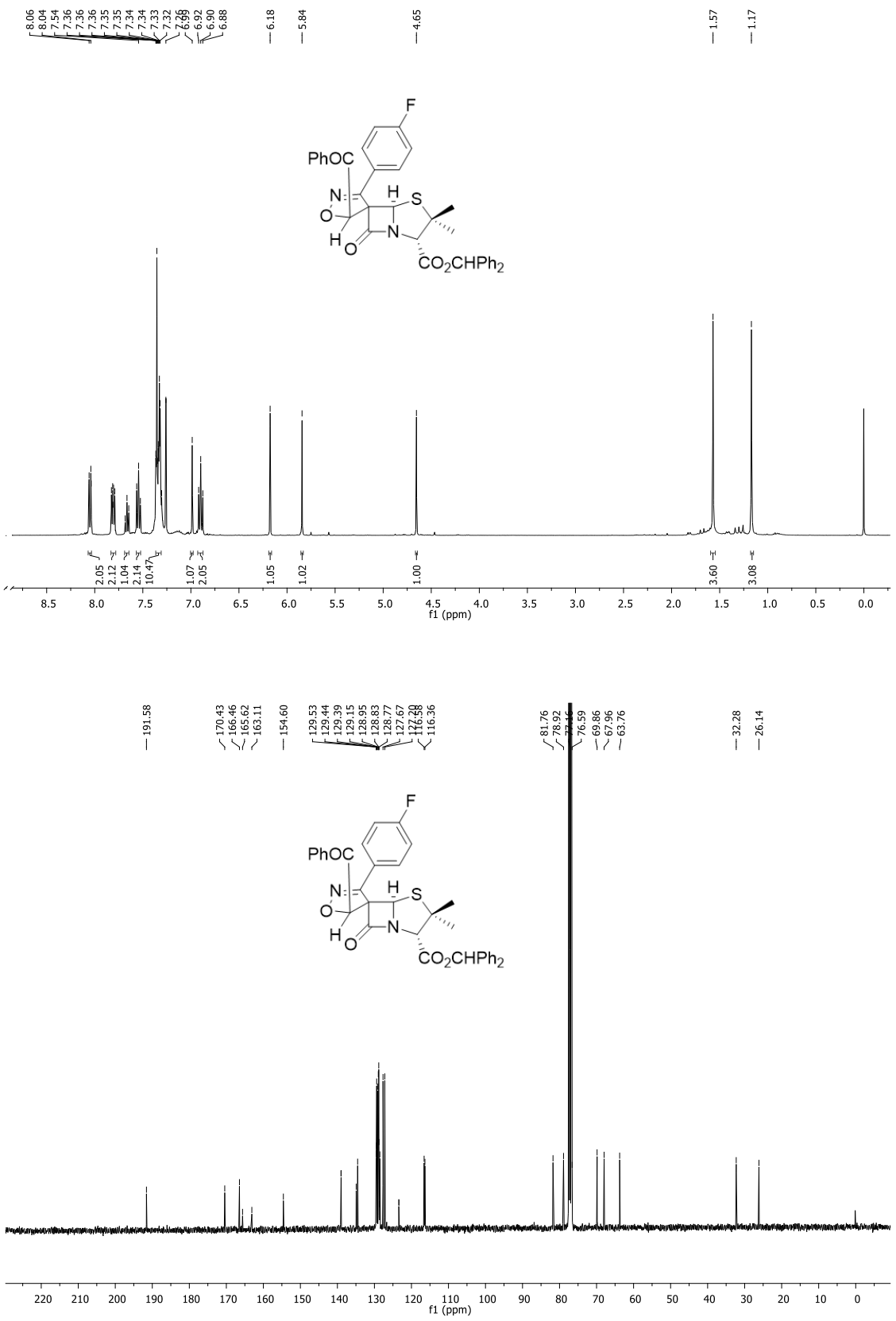


Figure S118: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **4.51b**.

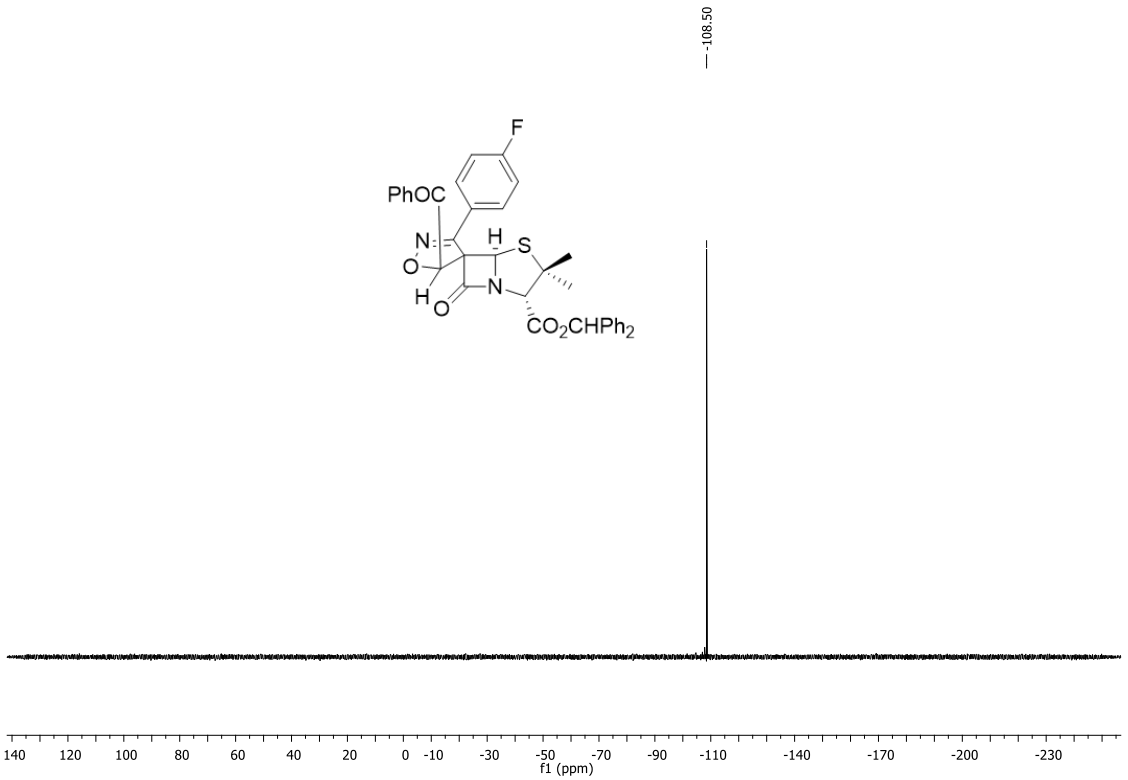


Figure S119: ^{19}F NMR spectrum ($CDCl_3$) of compound **4.51b**.

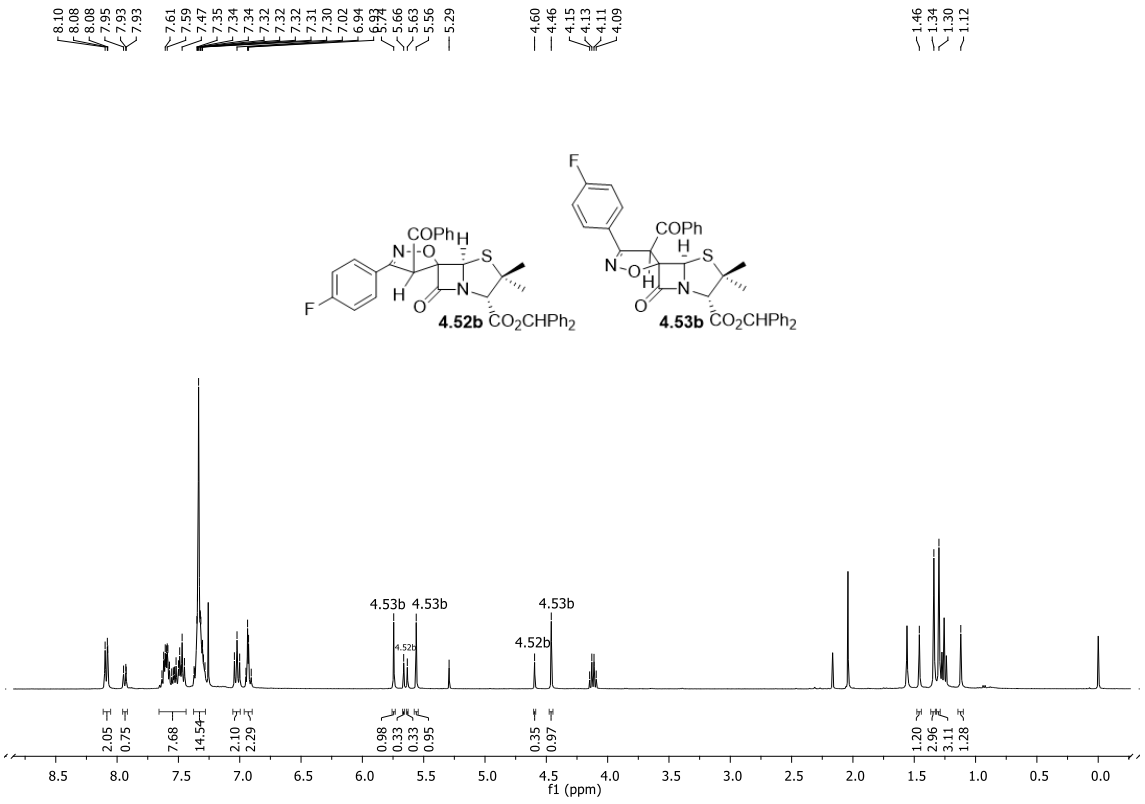


Figure S120: ^{1}H NMR spectrum ($CDCl_3$) of compound **4.52b**/**4.53b**.

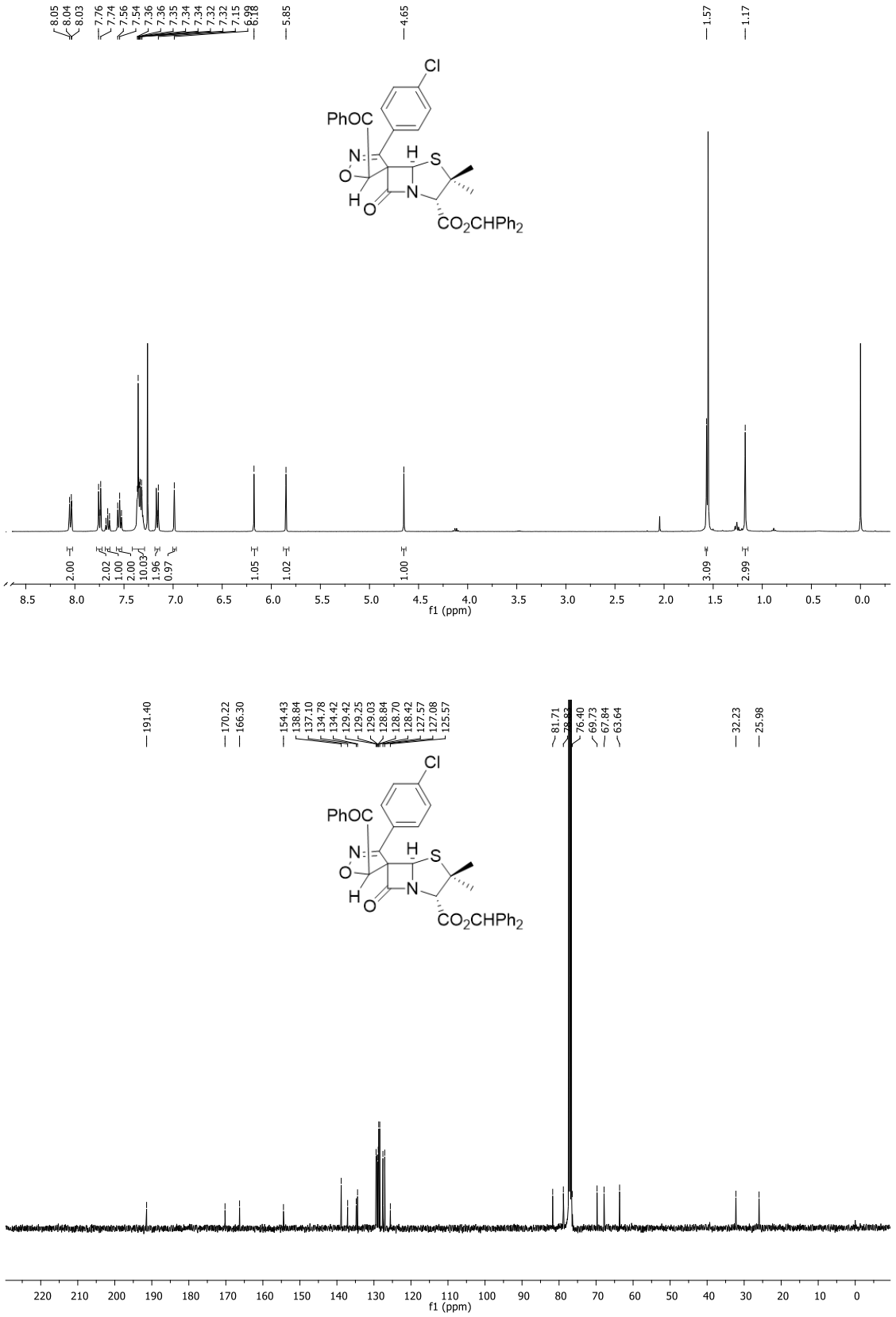


Figure S121: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **4.51c**.

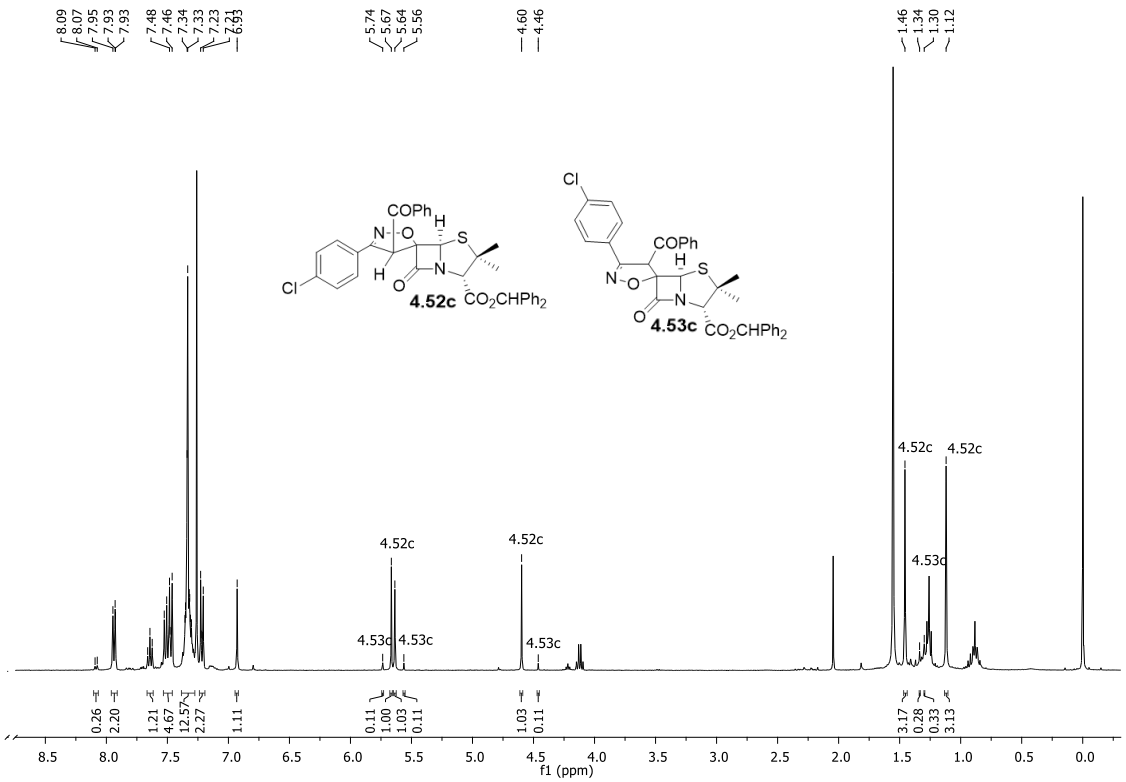


Figure S122: ^{1}H NMR spectrum ($CDCl_3$) of compound **4.52c**/**4.53c**.

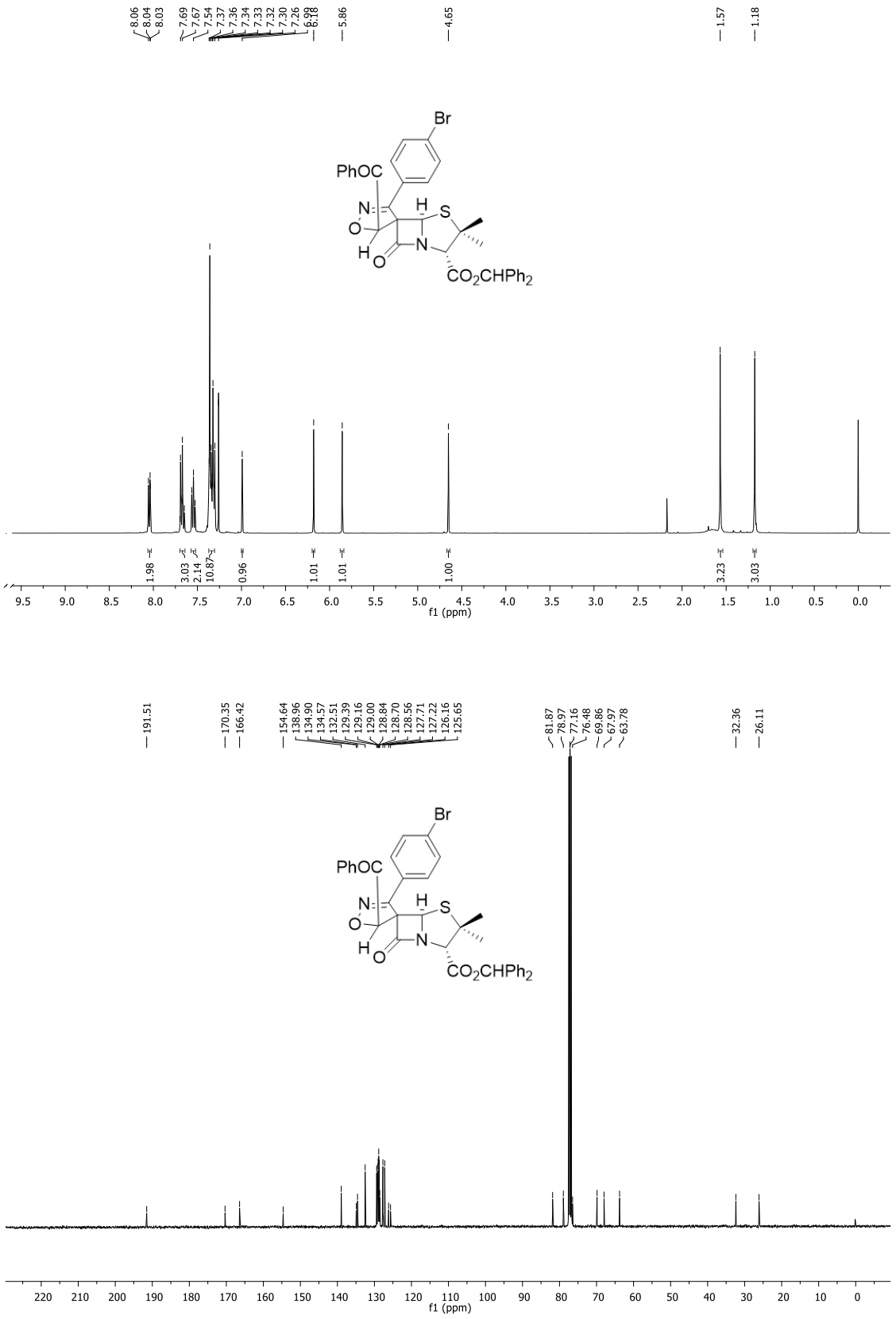


Figure S123: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **4.51d**.

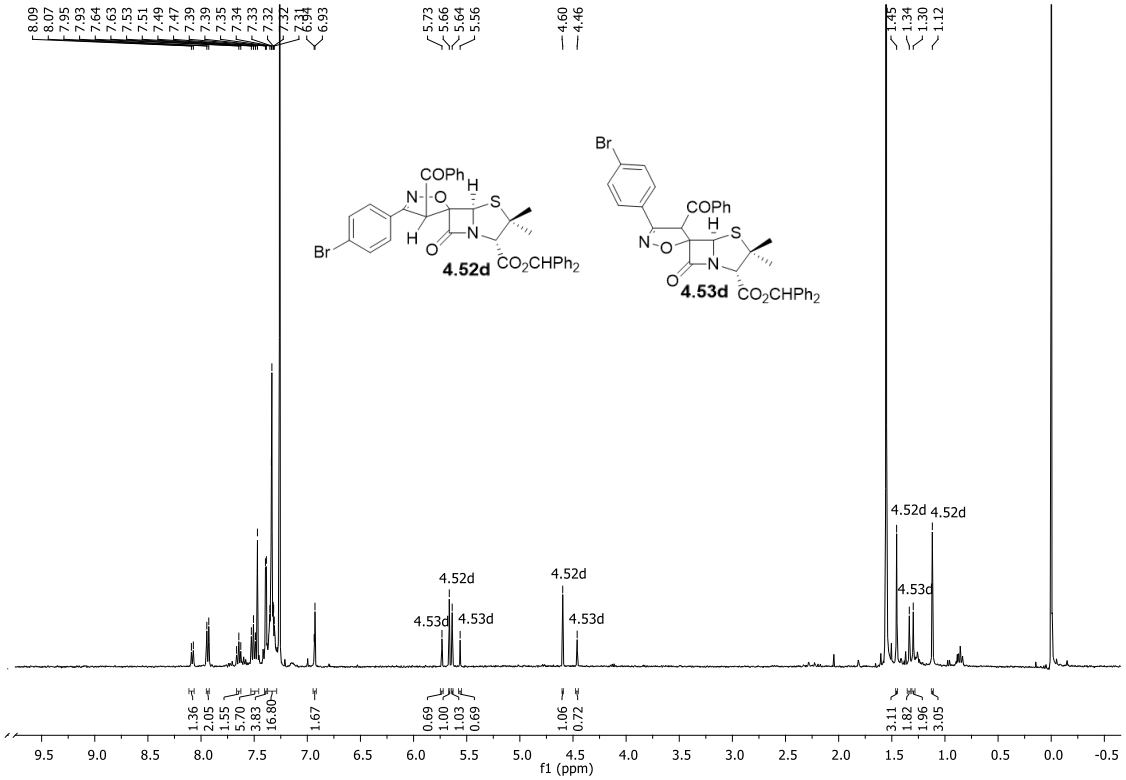


Figure S124: ^{1}H NMR spectrum ($CDCl_3$) of compound **4.52d**/**4.53d**.

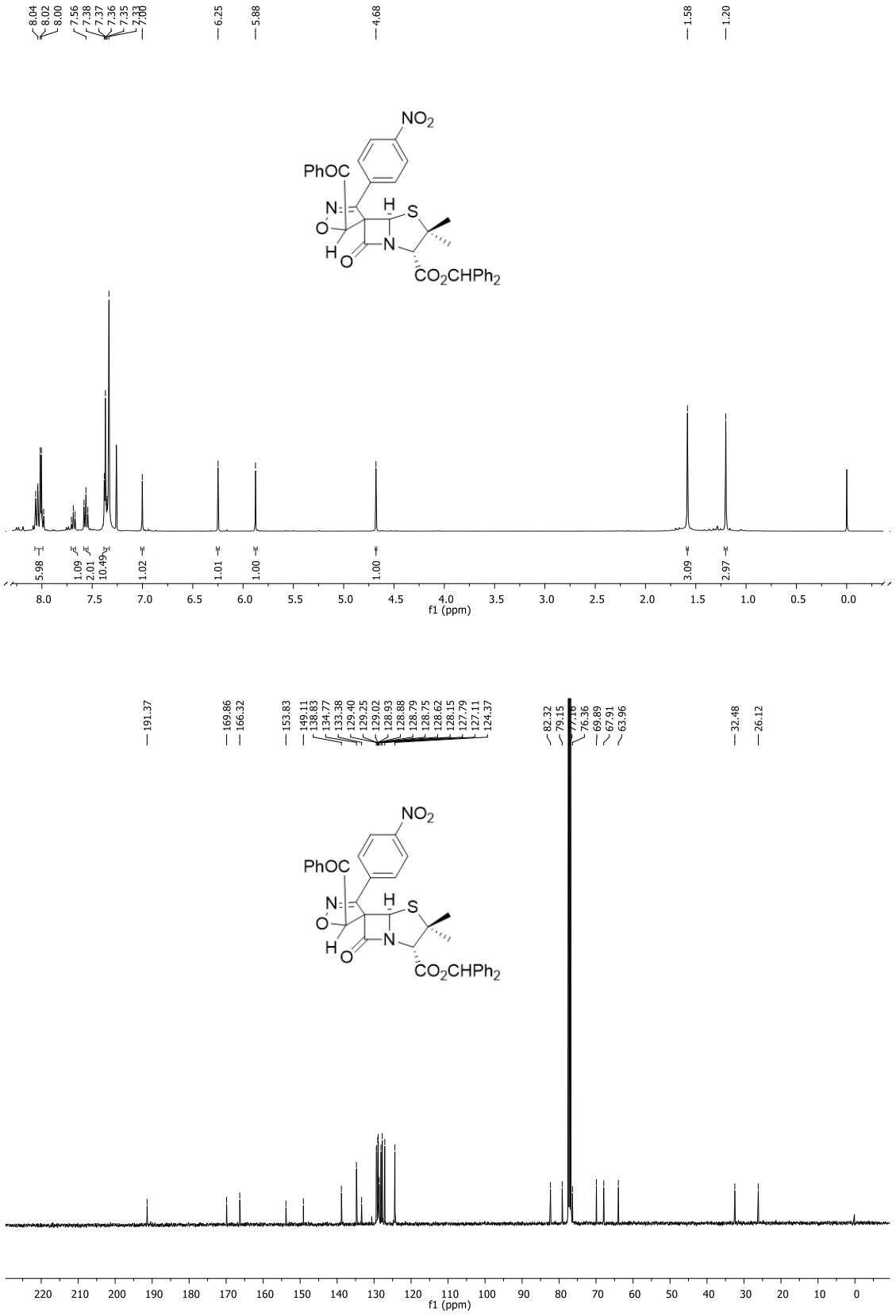


Figure S125: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **4.51e**.

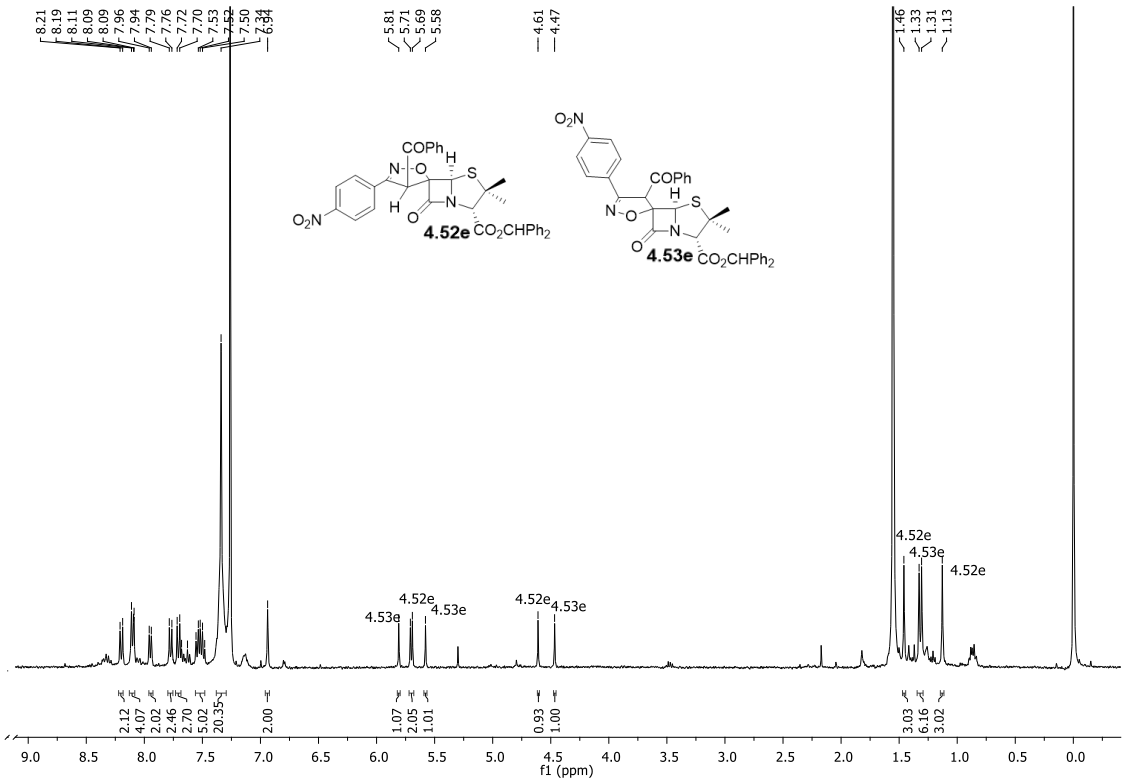


Figure S126: ^{1}H NMR spectrum ($CDCl_3$) of compound **4.52e**/**4.53e**.

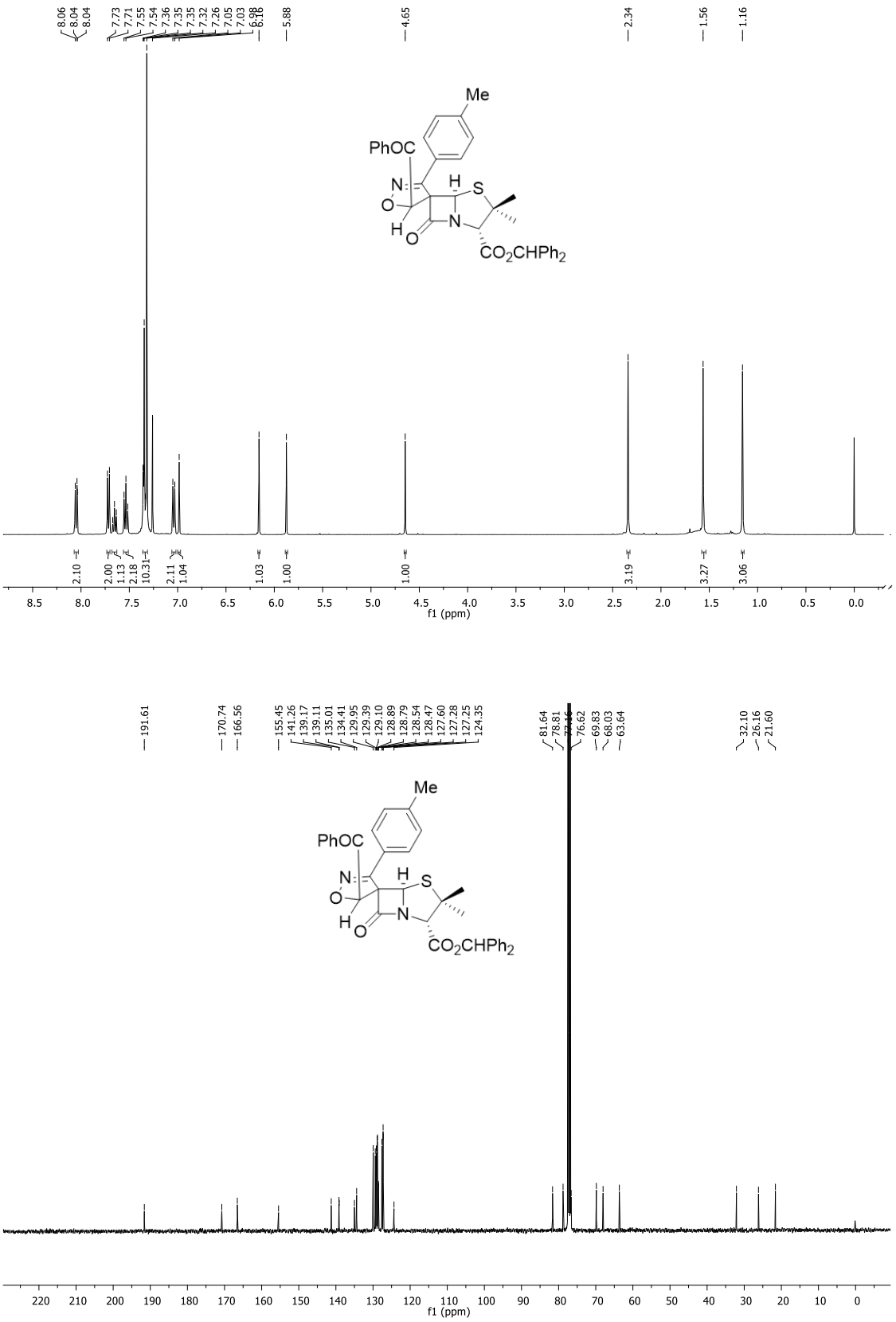


Figure S127: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **4.51f**.

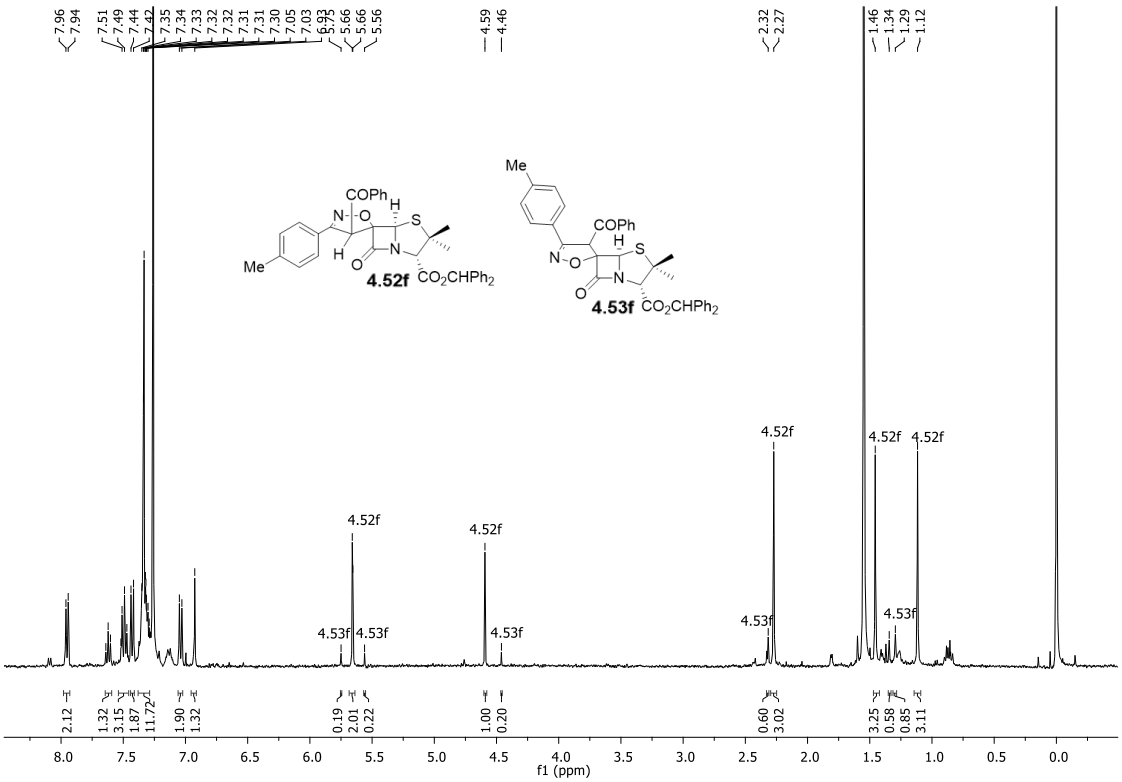


Figure S128: ^{1}H NMR spectrum ($CDCl_3$) of compound **4.52f**/**4.53f**.

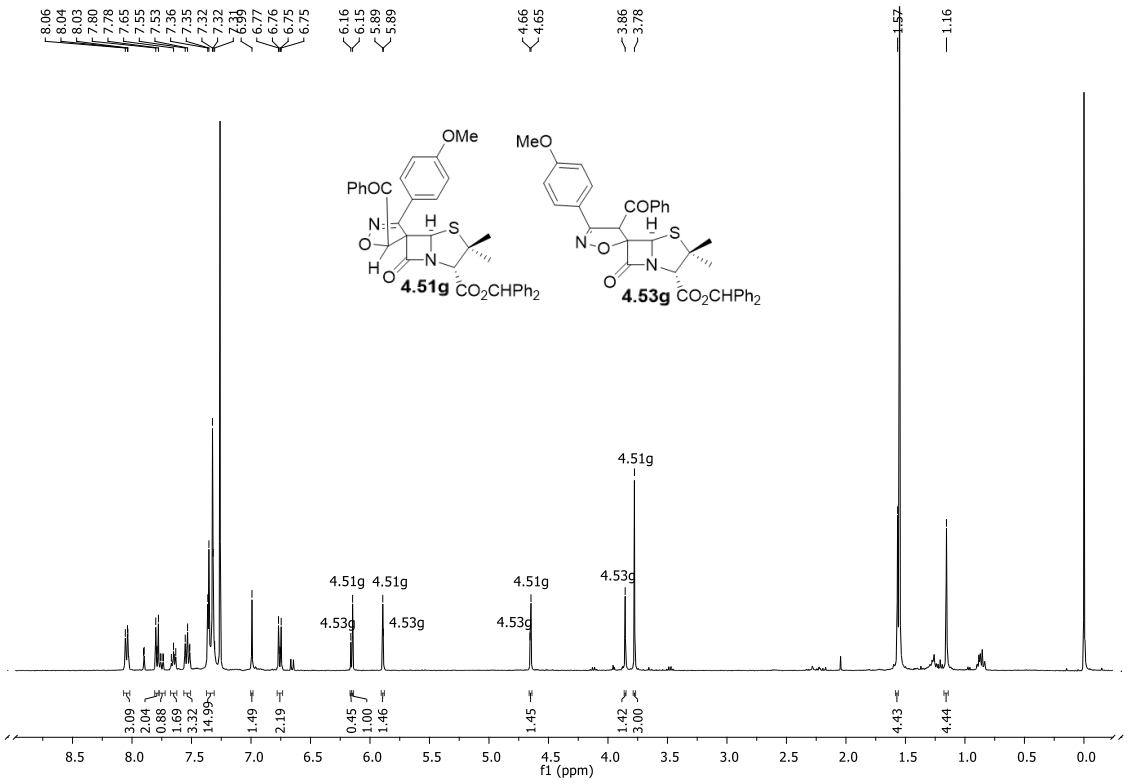


Figure S129: ^{1}H NMR spectrum ($CDCl_3$) of compound **4.51g/4.53g**.

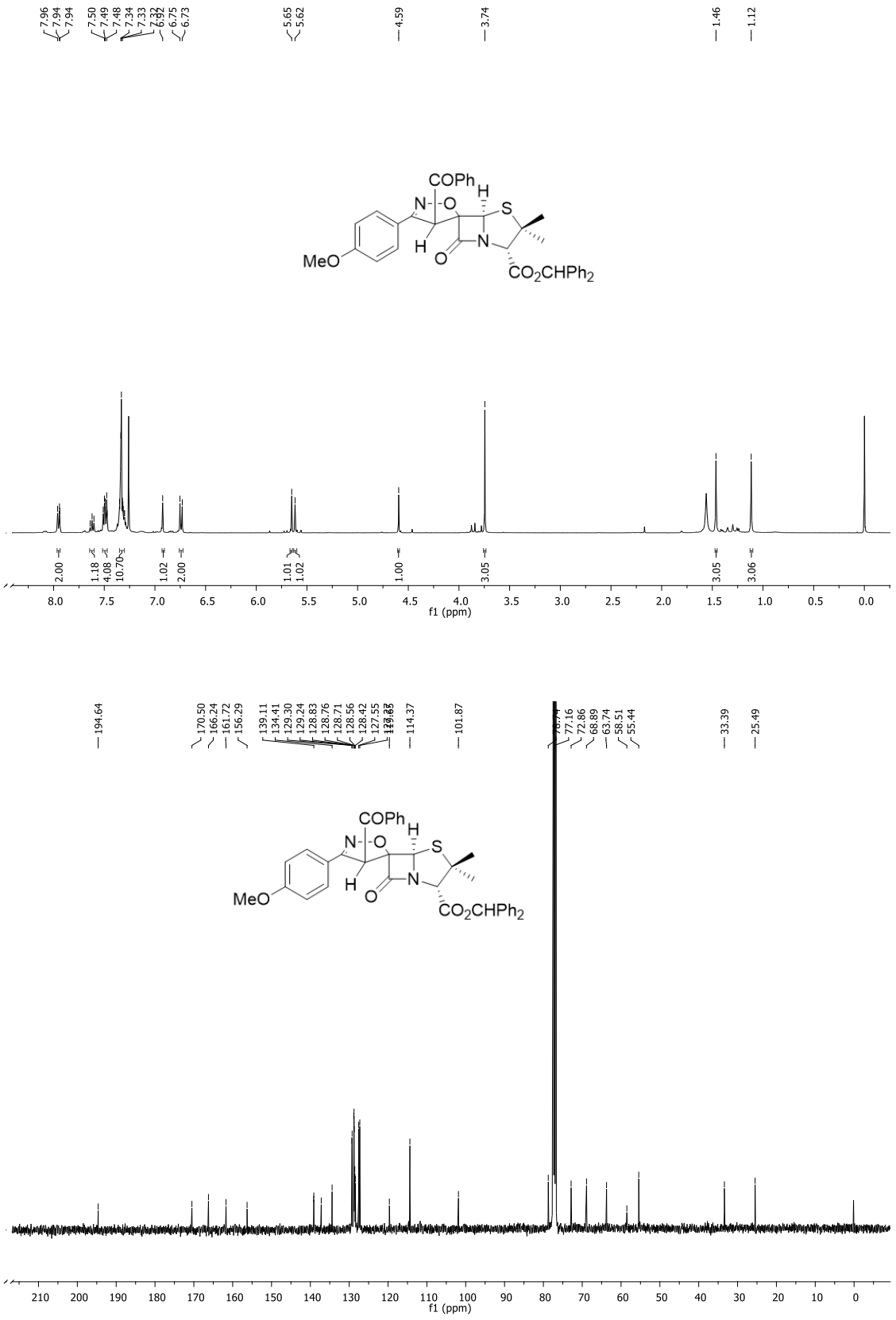


Figure S130: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **4.52g**.

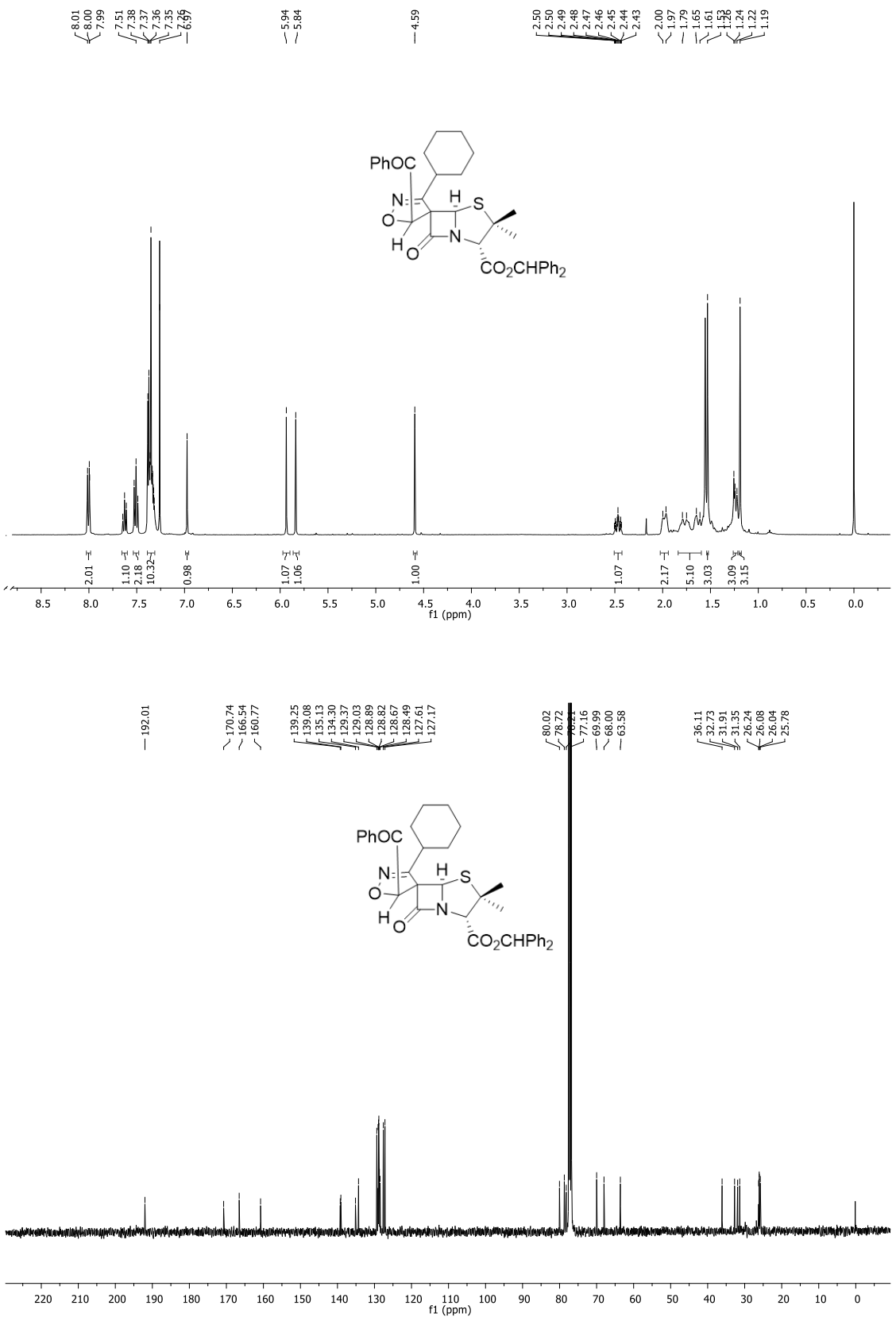


Figure S131: ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of compound **4.51h**.

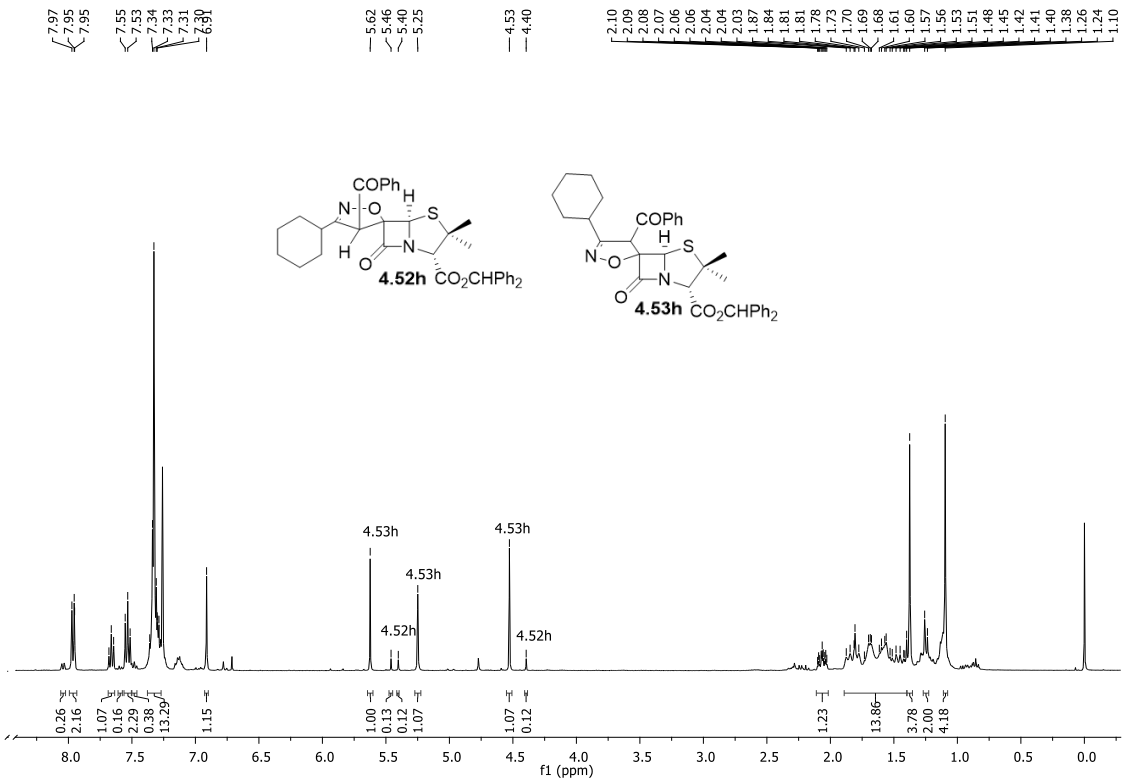


Figure S132: ^{1}H NMR spectrum ($CDCl_3$) of compound **4.52h/4.53h**.

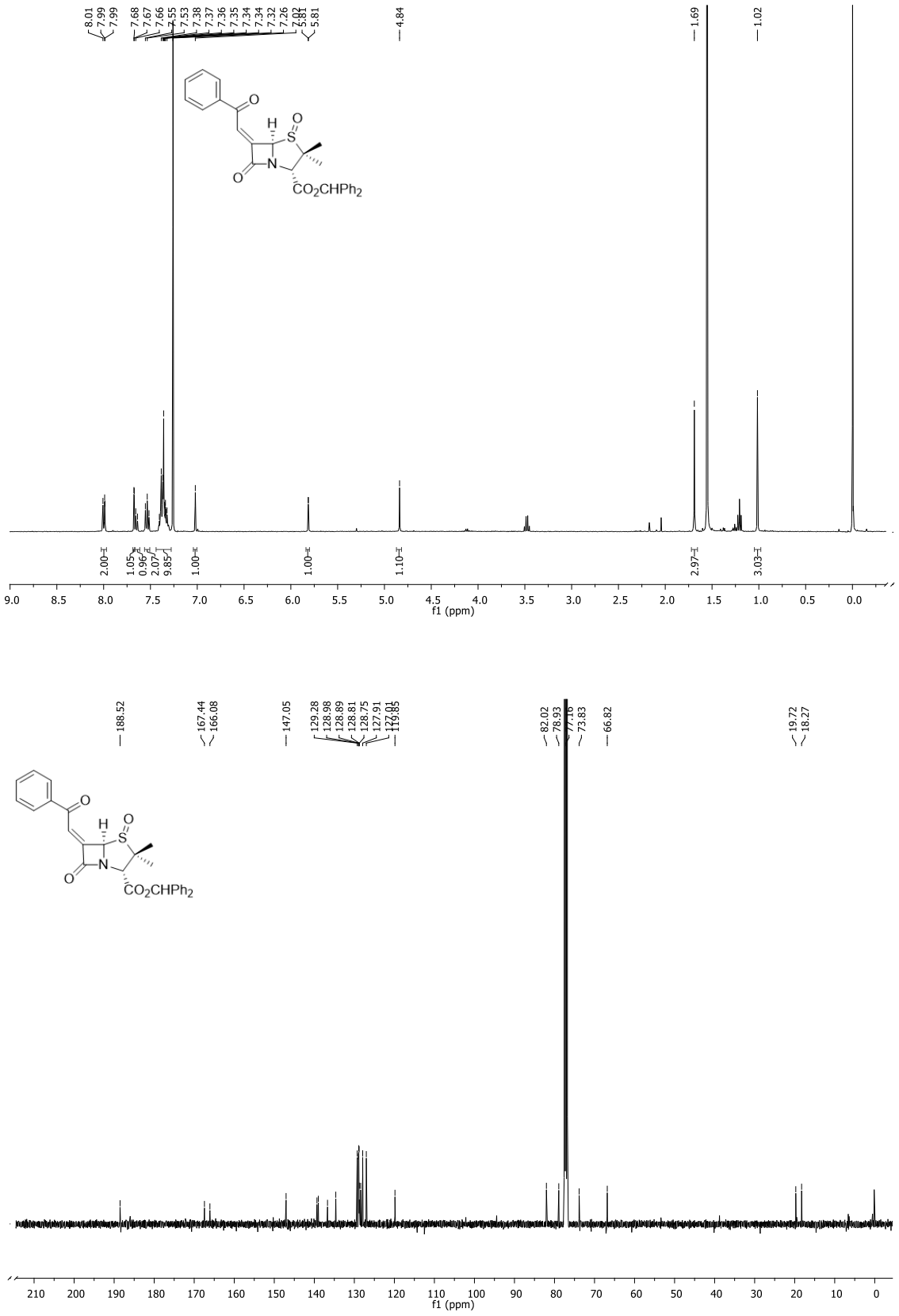


Figure S133: ^{1}H and ^{13}C NMR spectra of compound **5.2a (**$CDCl_3$).

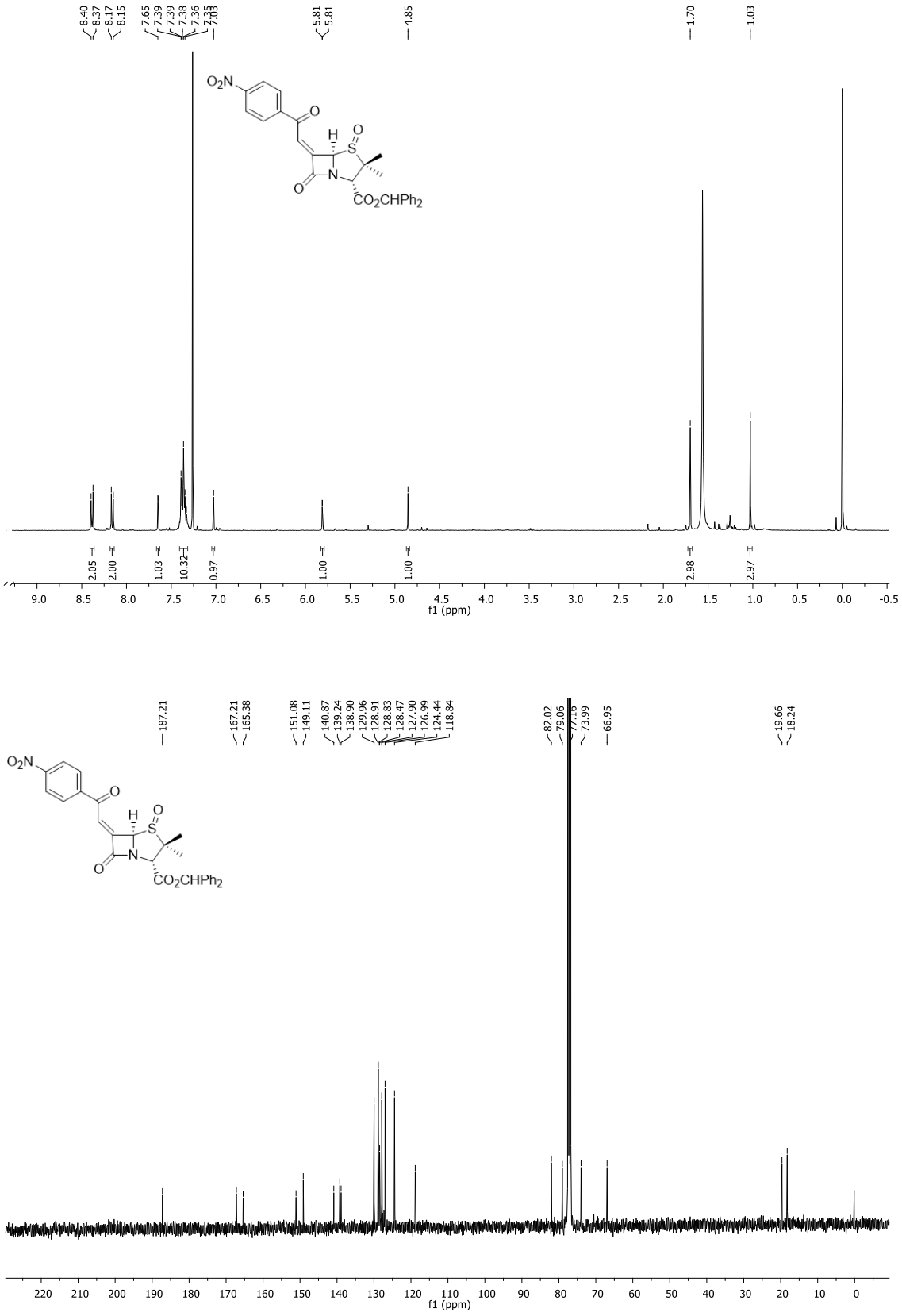


Figure S134: ^{1}H and ^{13}C NMR spectra of compound **5.2b (**$CDCl_3$).

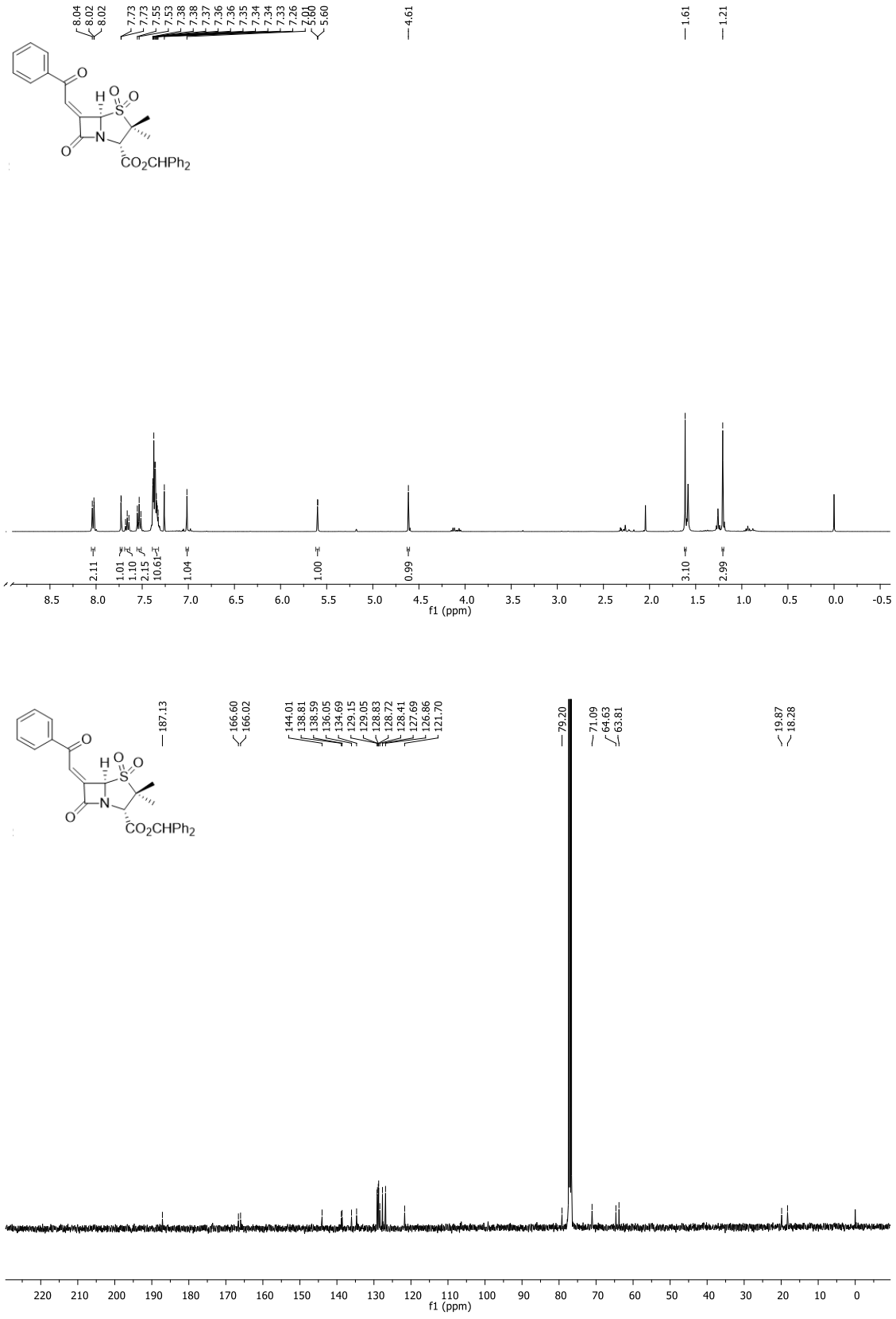


Figure S135: ^{1}H and ^{13}C NMR spectra of compound **5.3a (**$CDCl_3$**)**.

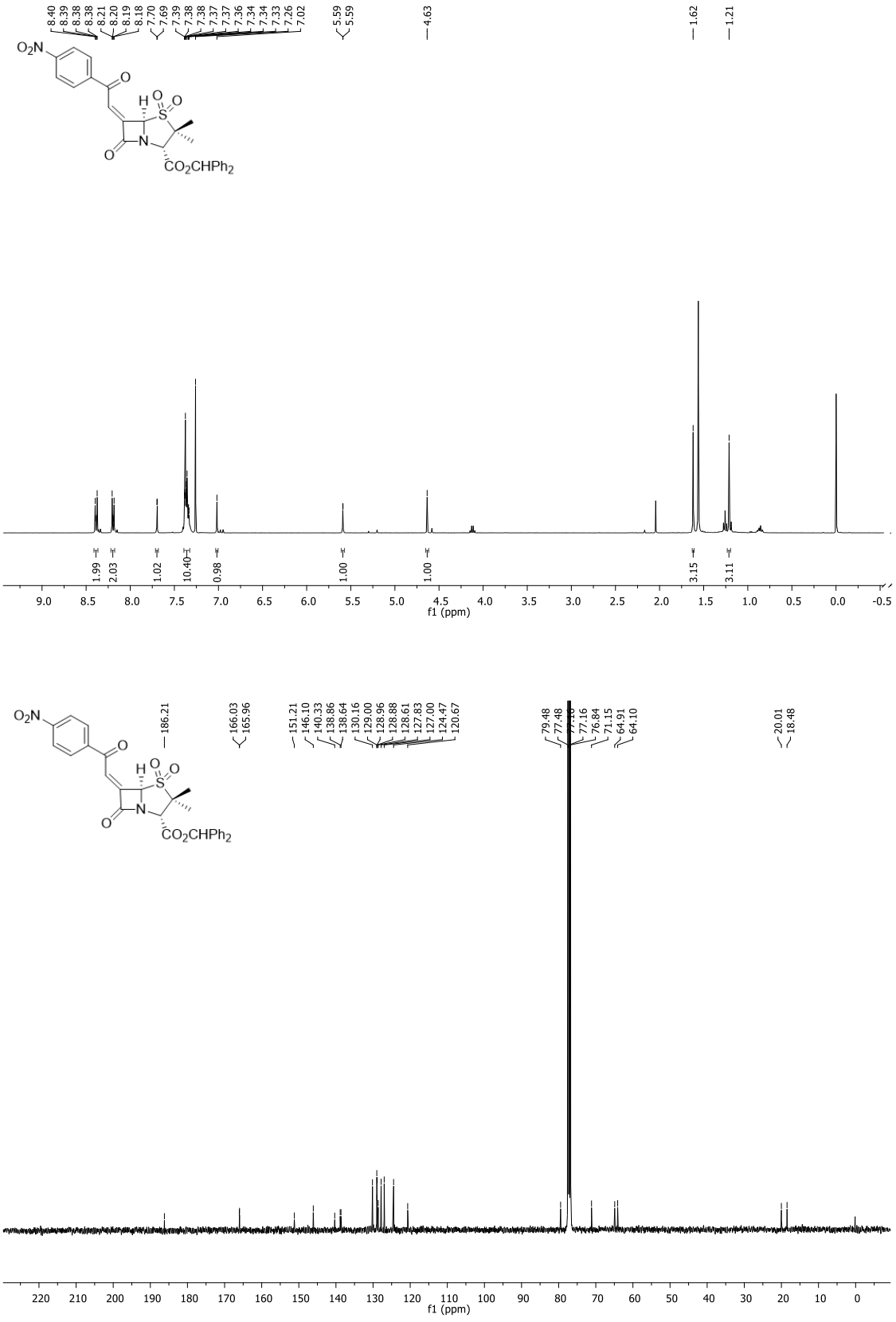


Figure S136: ^{1}H and ^{13}C NMR spectra of compound **5.3b (**$CDCl_3$**).**

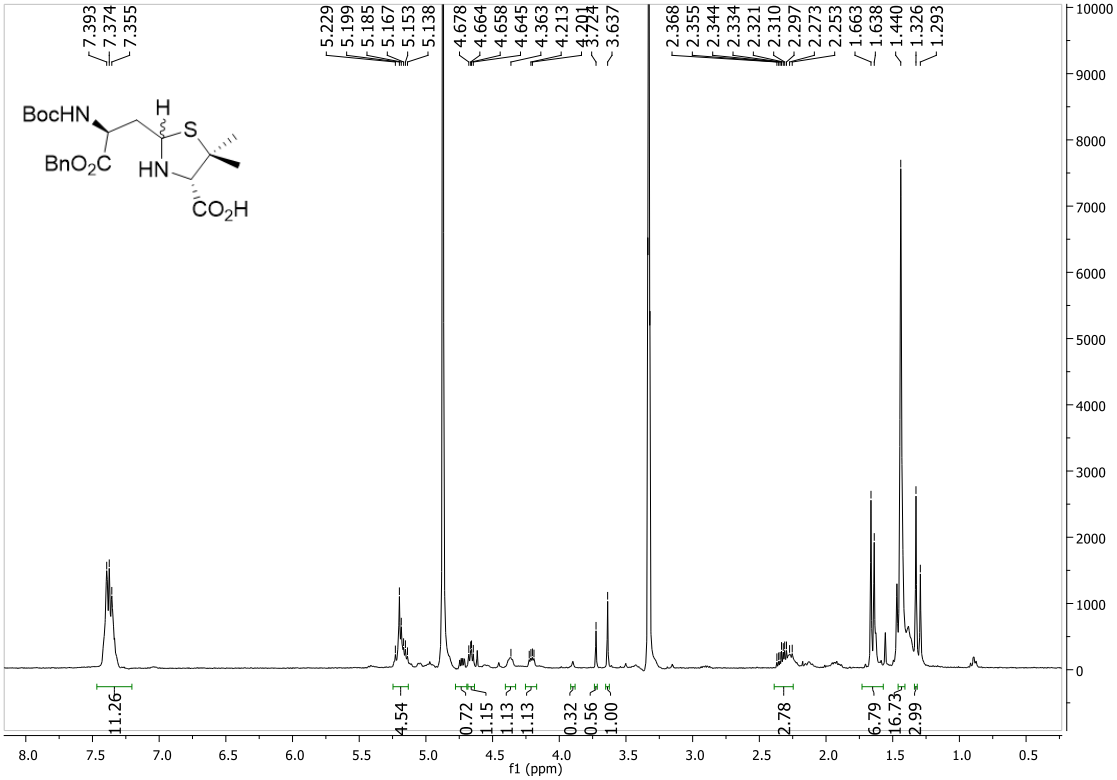


Figure S137: ^{1}H NMR spectra of compound **5.6 (**CD_3OD).

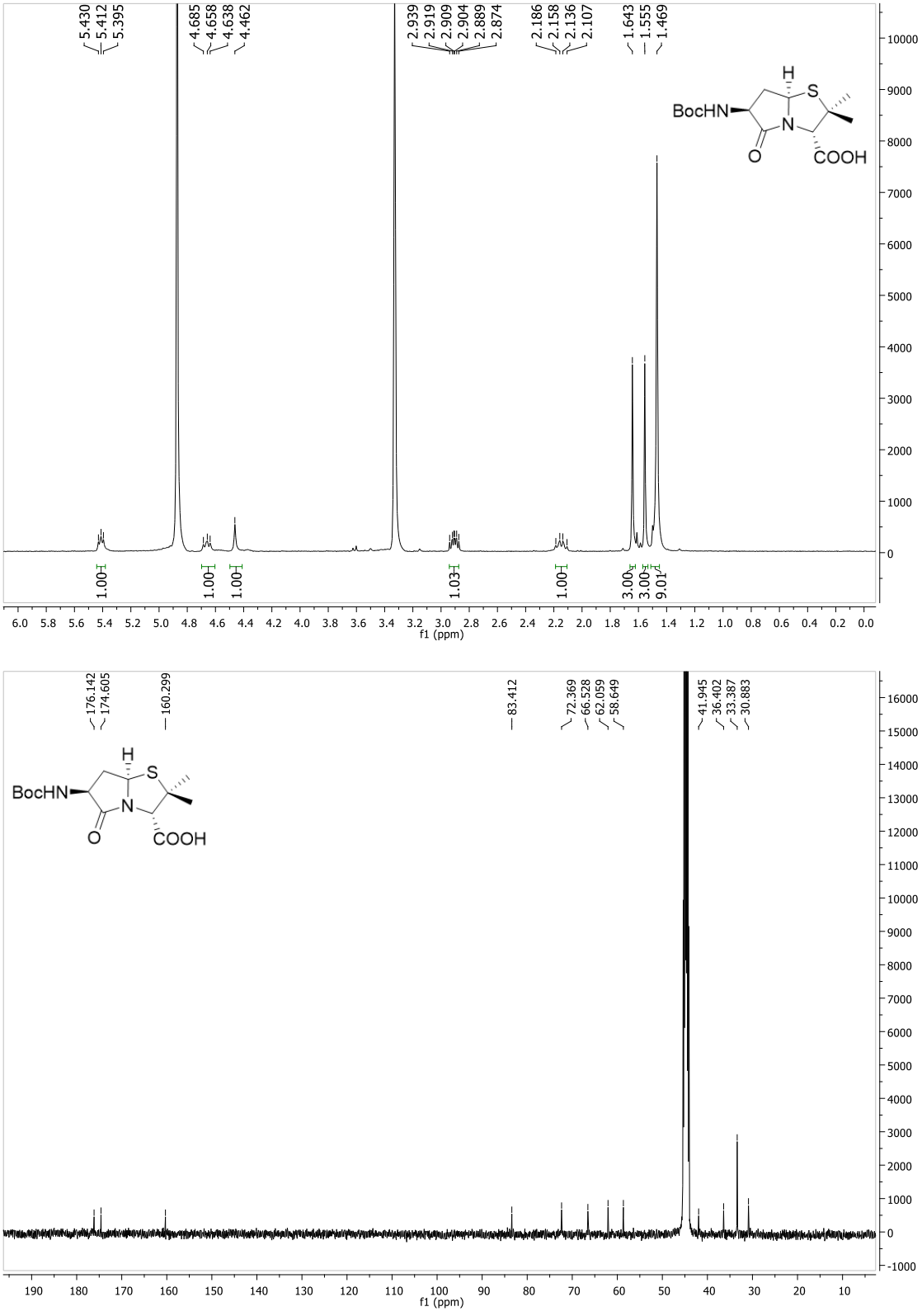


Figure S138: ^{1}H and ^{13}C NMR spectra of compound **5.7 (**CD_3OD).

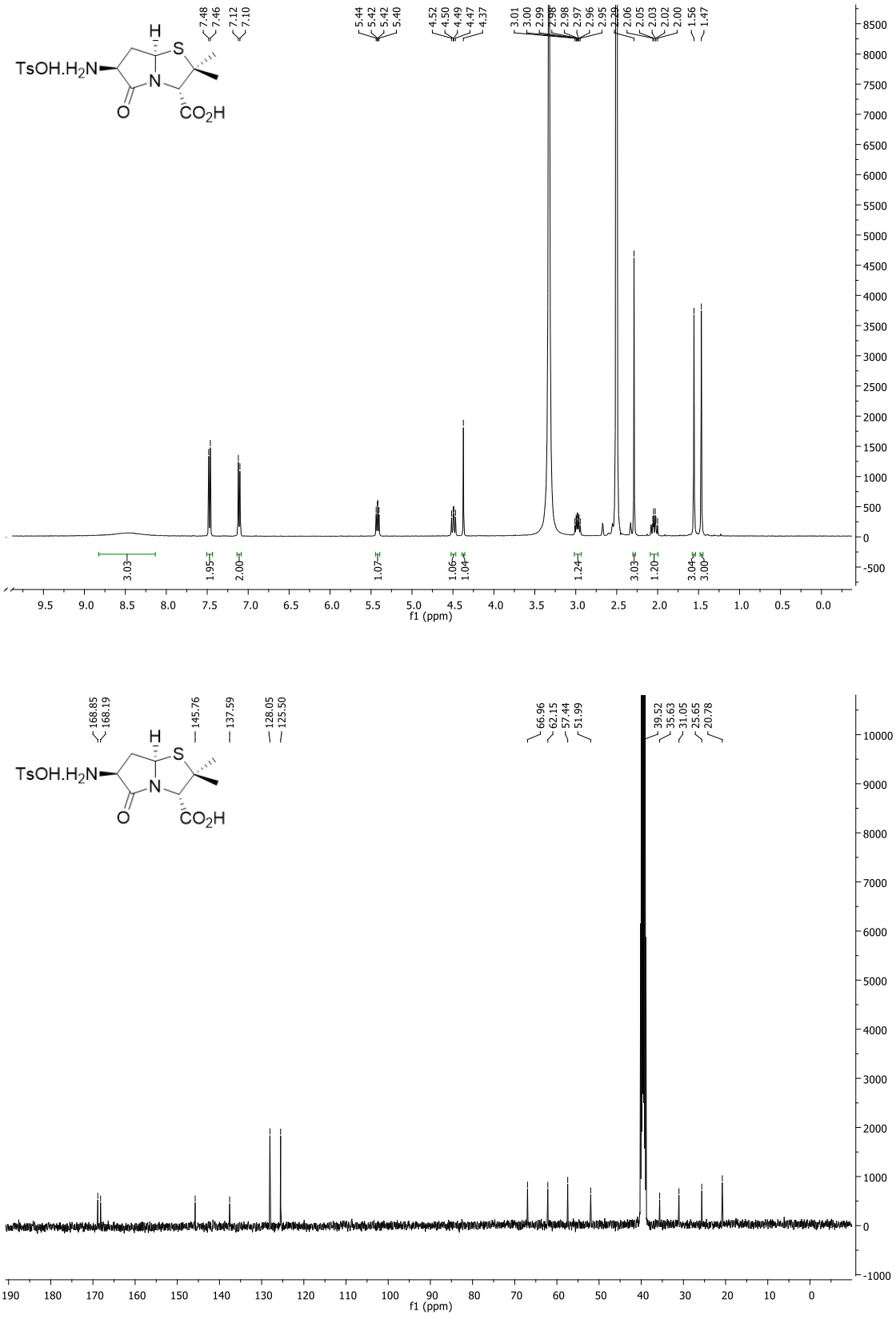


Figure S139: ^{1}H and ^{13}C NMR spectra of compound **5.8 (**DMSO-d_6).

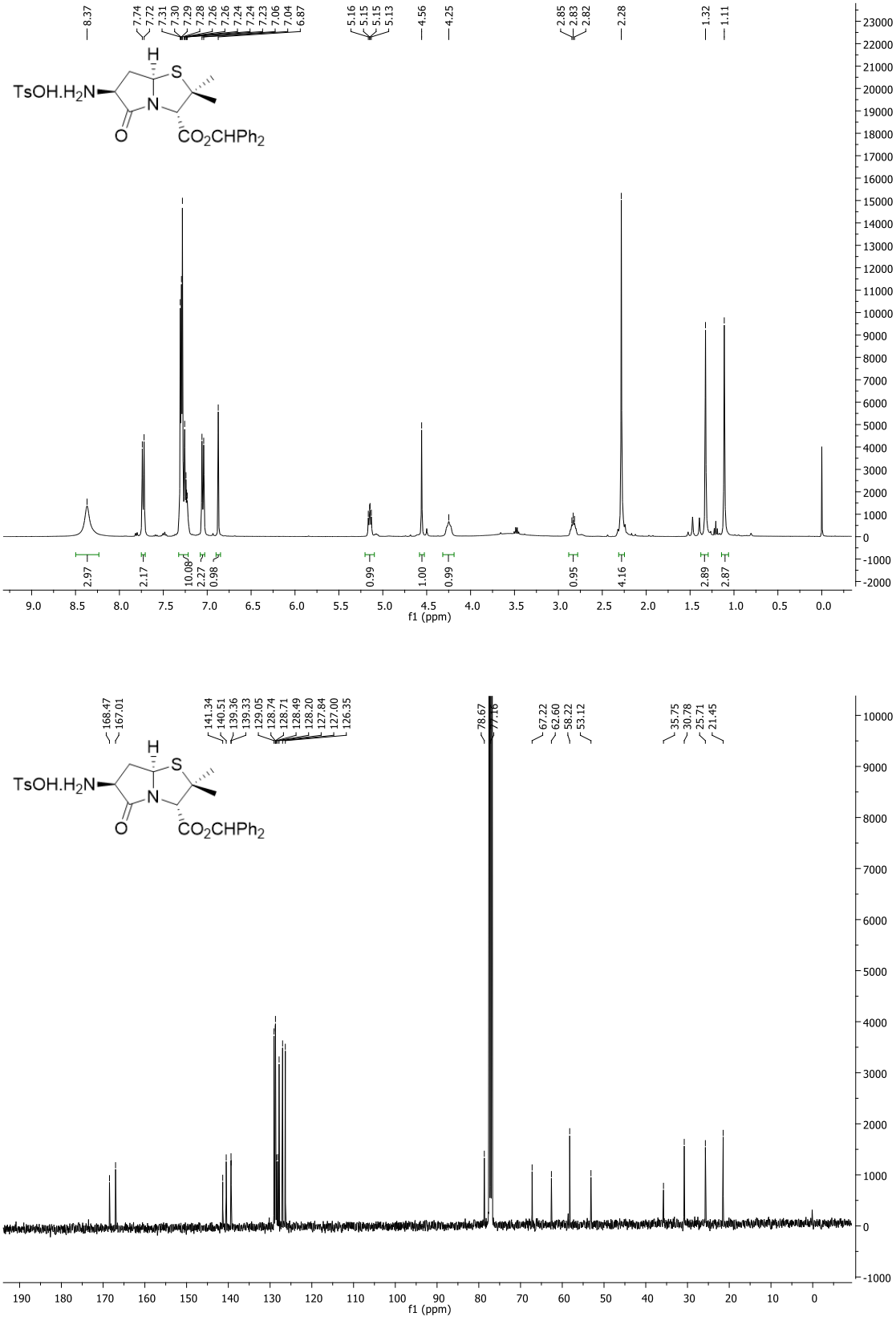


Figure S140: ^{1}H and ^{13}C NMR spectra of compound **5.9 (**$CDCl_3$).

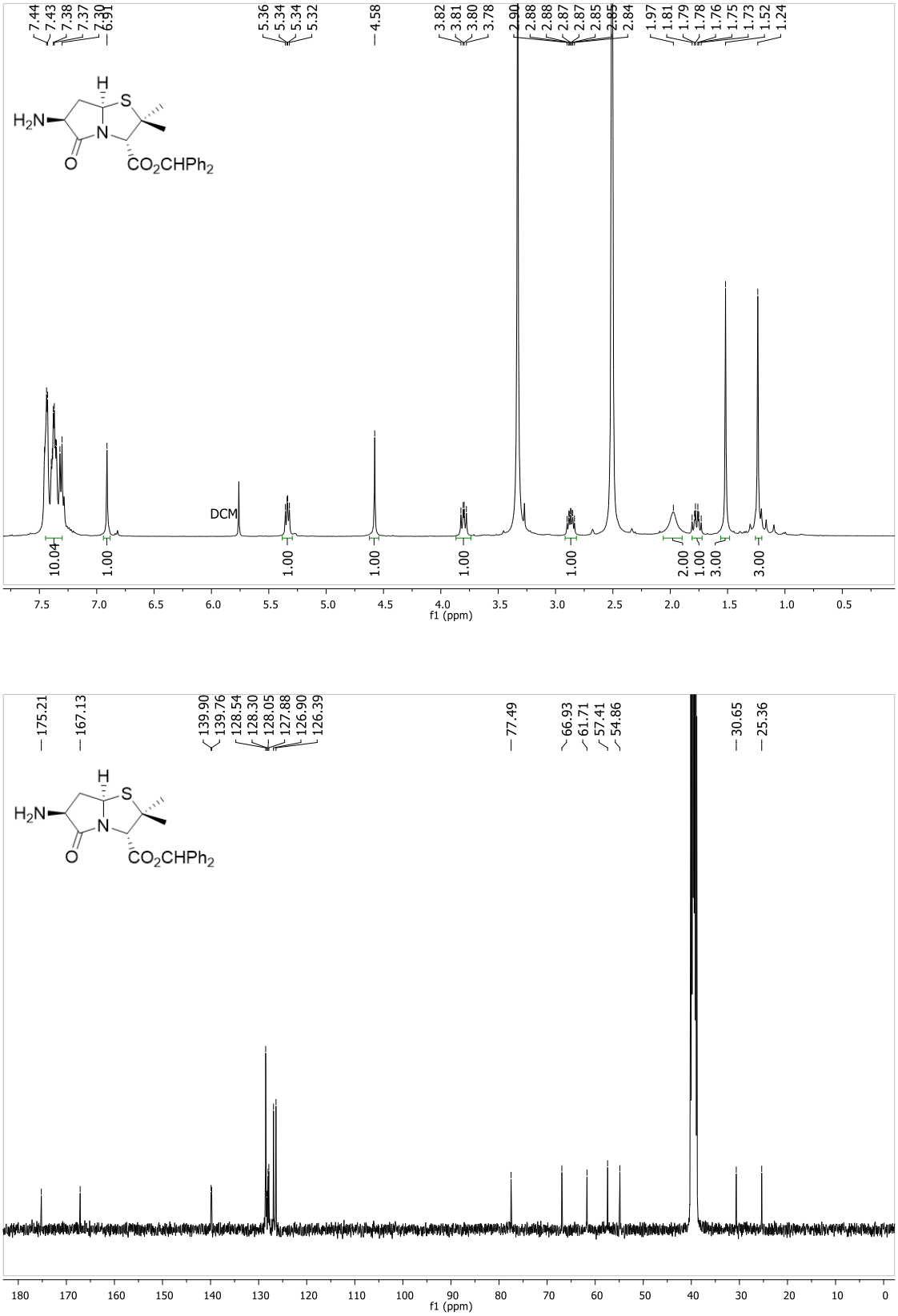


Figure S141: ^{1}H and ^{13}C NMR spectra of compound **5.10 (**DMSO-d_6).

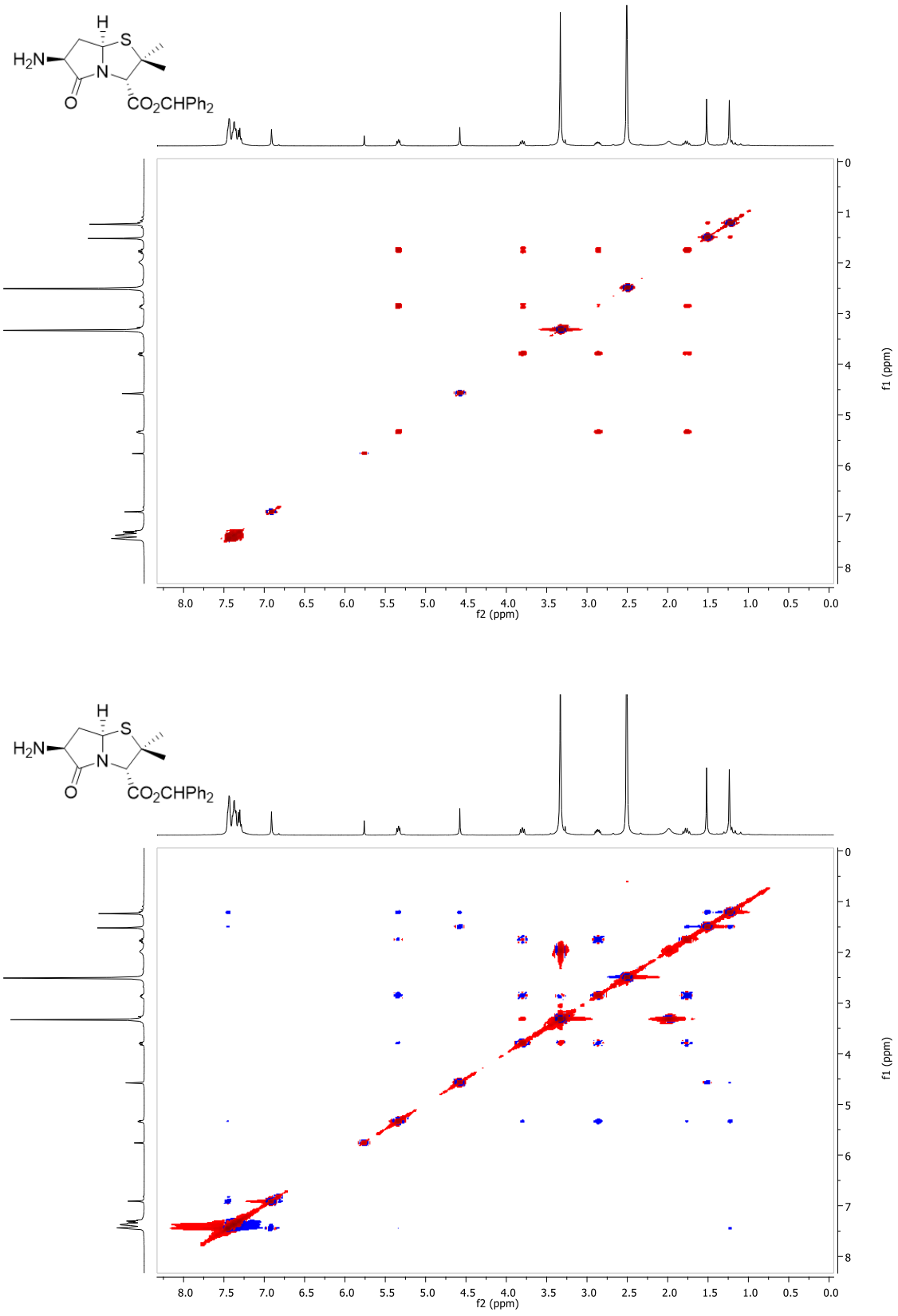


Figure S142: COSY and NOESY spectra of compound **5.10 (**DMSO-d_6).

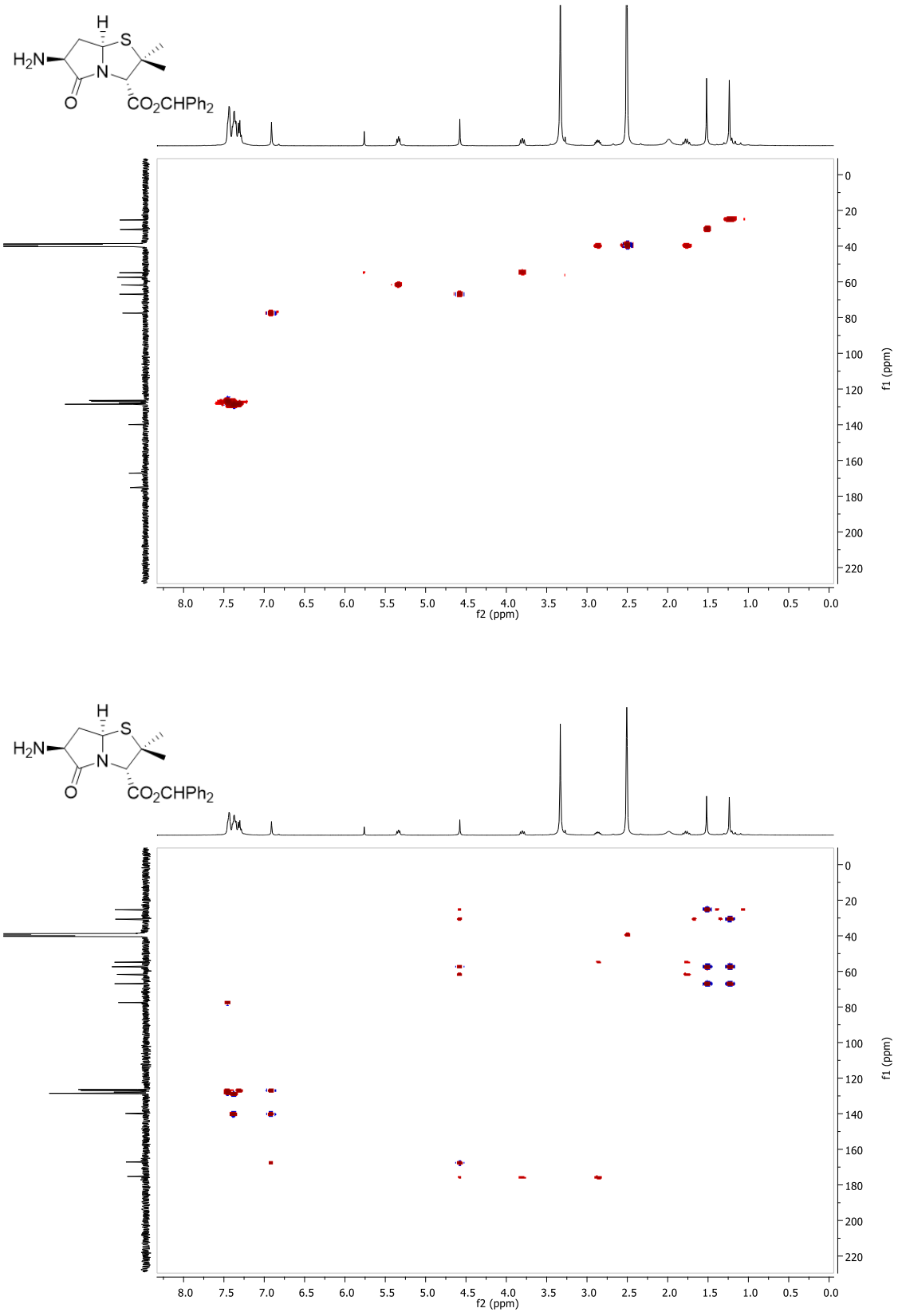


Figure S143: HSQC and HMBC spectra of compound **5.10 (**DMSO-d_6).

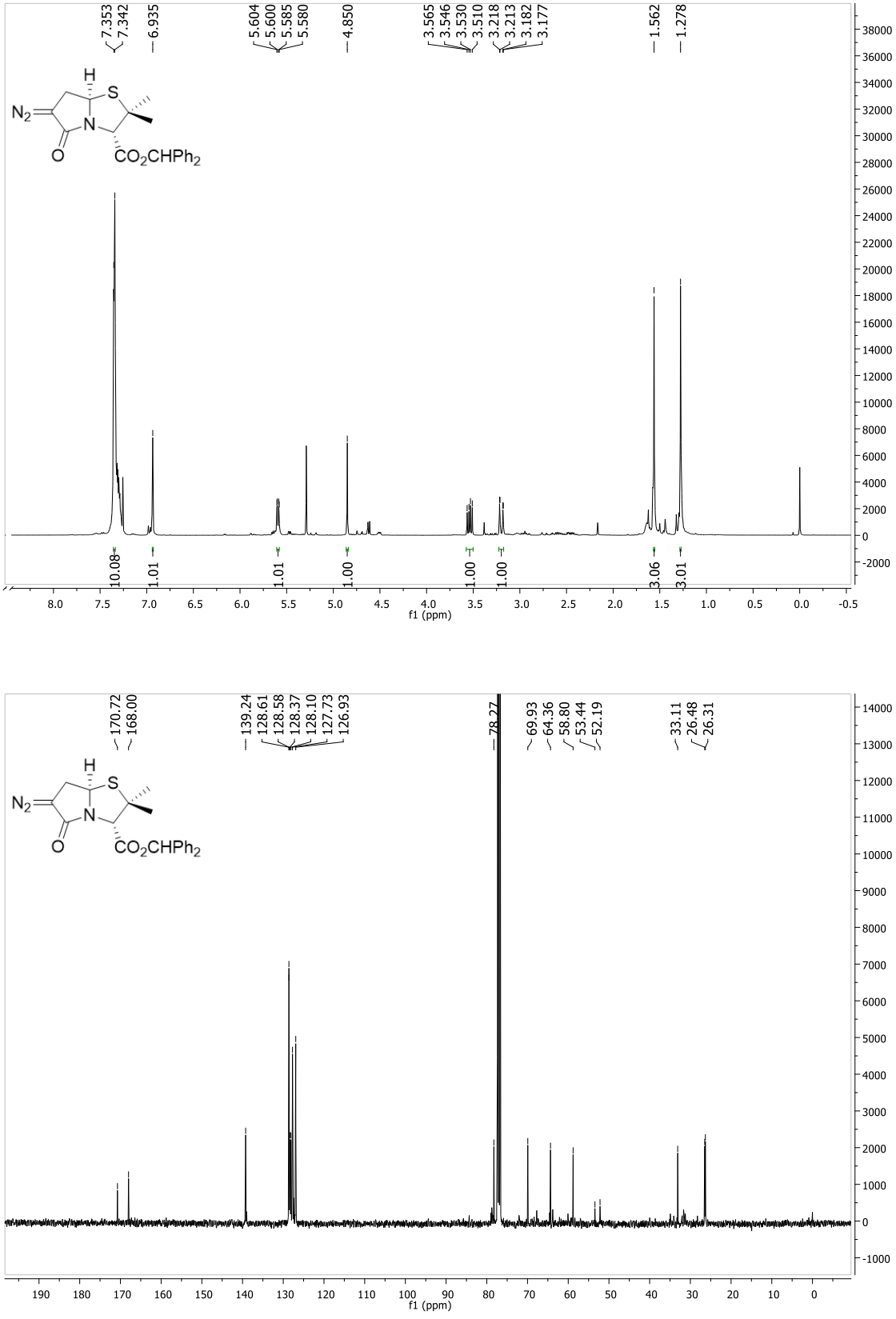


Figure S144: ^{1}H and ^{13}C NMR spectra of compound **5.11** ($CDCl_3$).

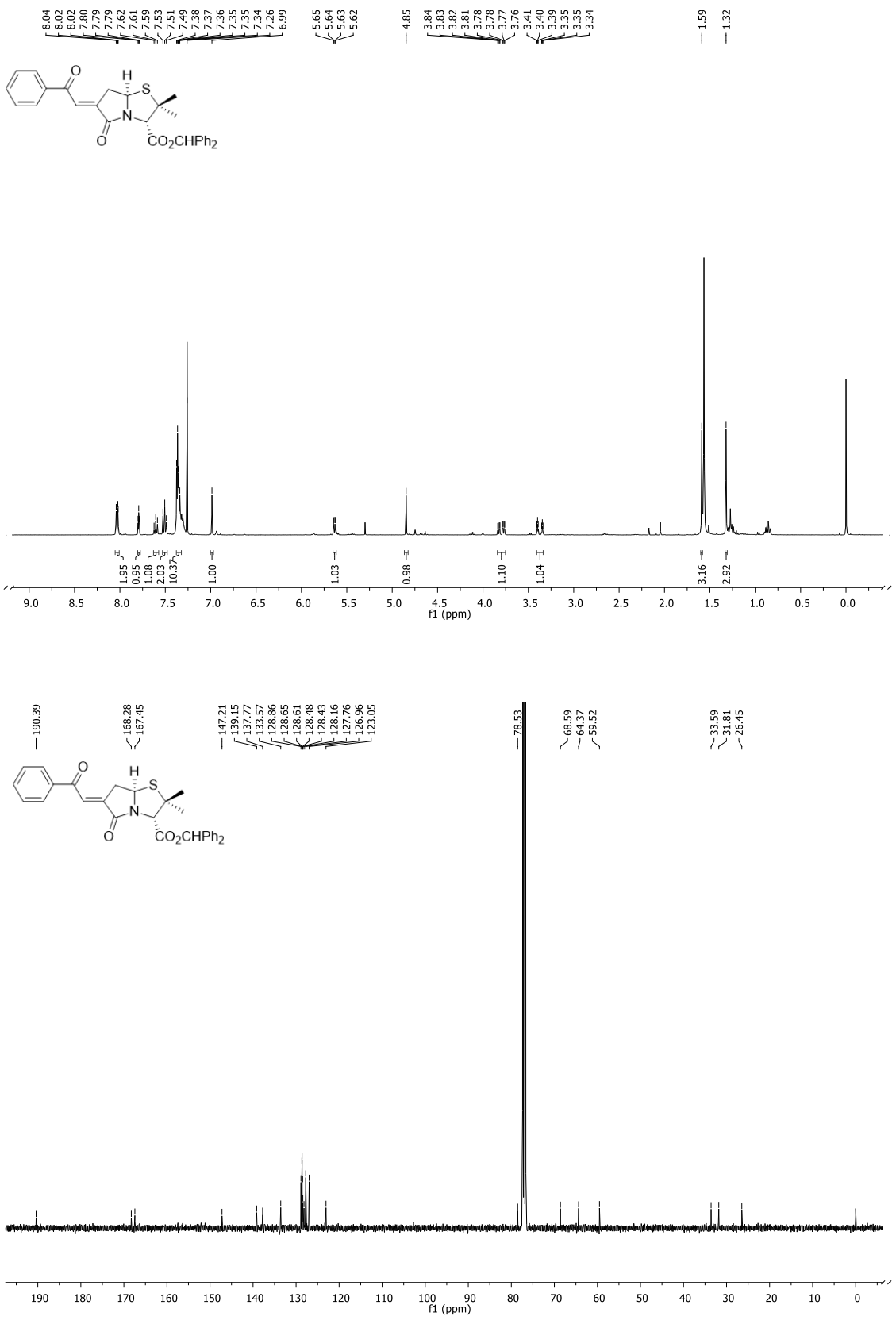


Figure S145: ^{1}H and ^{13}C NMR spectra of compound **5.13a (**$CDCl_3$).

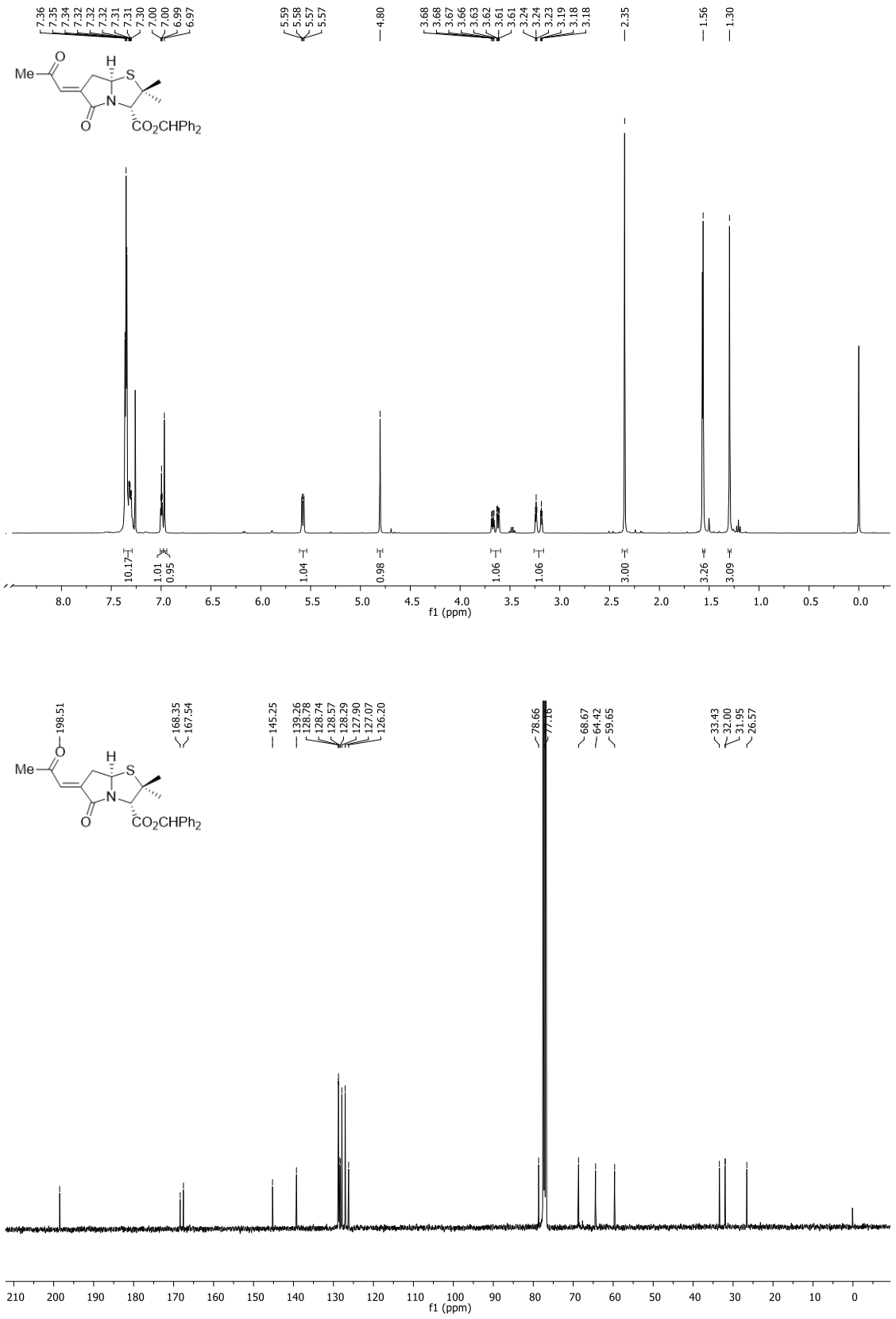


Figure S146: ^{1}H and ^{13}C NMR spectra of compound **5.13b (**$CDCl_3$).

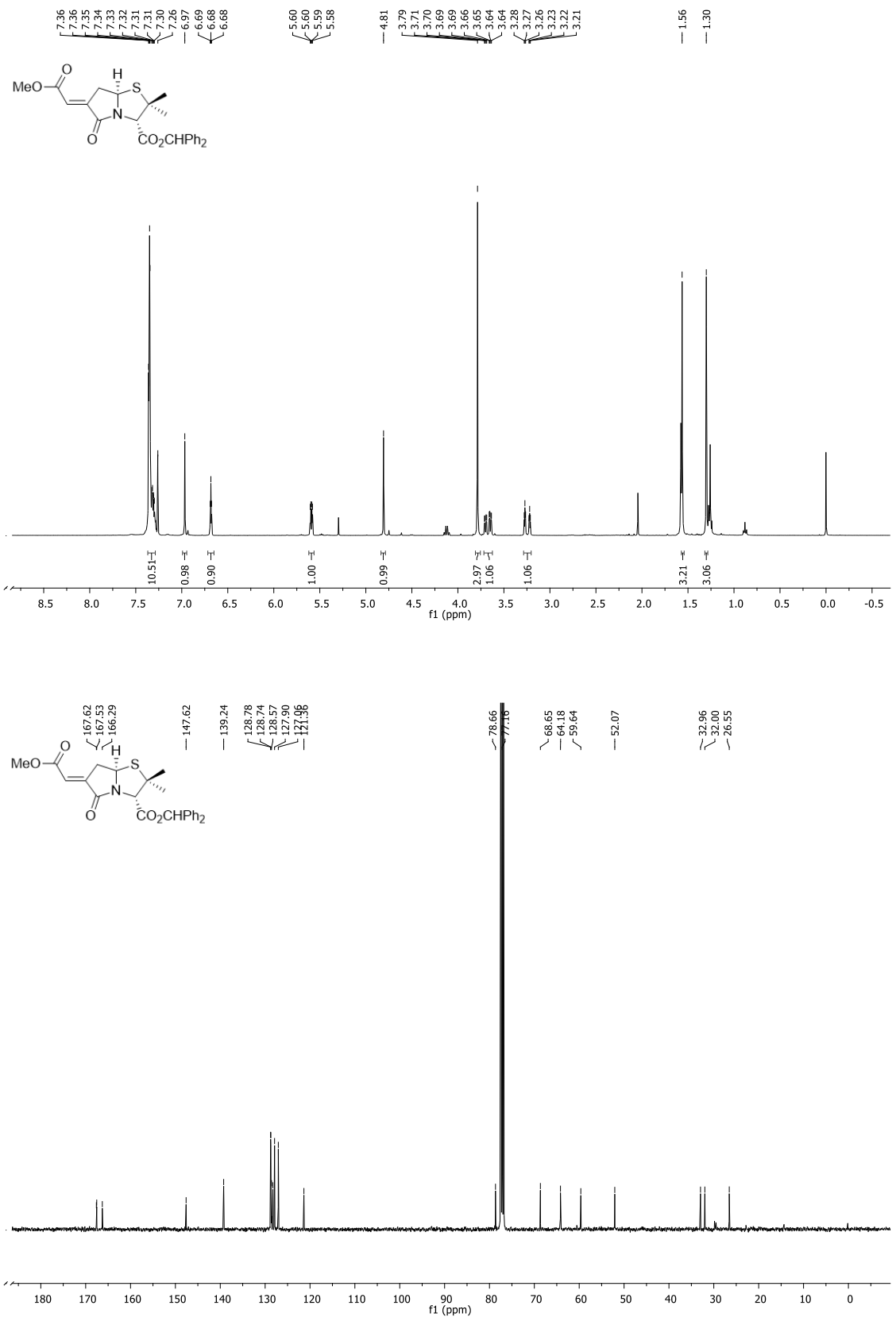


Figure S147: ^{1}H and ^{13}C NMR spectra of compound **5.13c (**$CDCl_3$).

Appendix 2. Theoretical Calculations

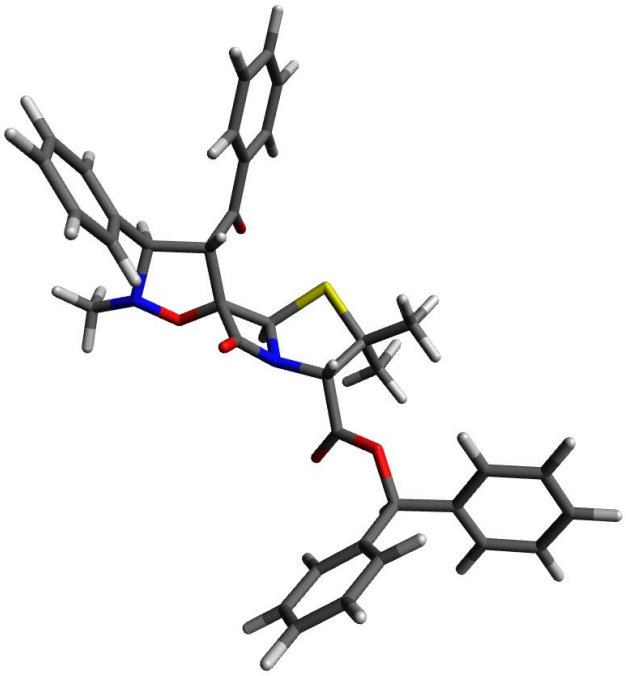


Figure S148. Optimized geometry (B3LYP/6-31G*) of compound **3.43a**. Color code: gray refers to carbon, red to oxygen, blue to nitrogen, white to hydrogen and yellow to sulphur atoms.

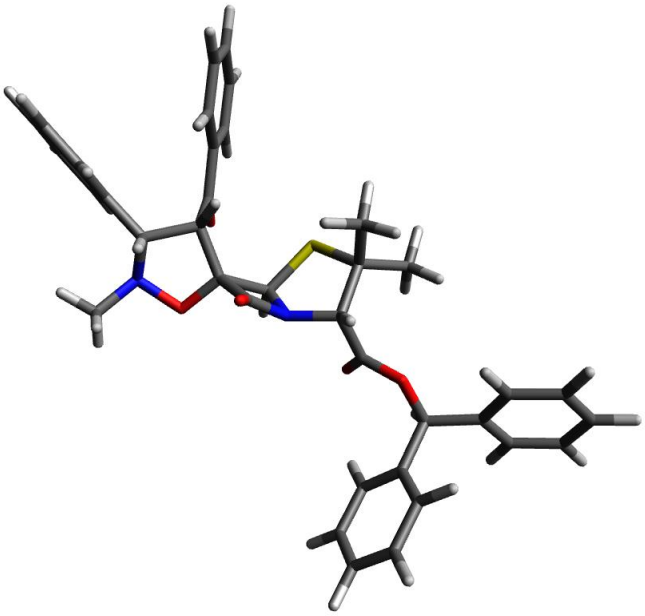


Figure S149. Optimized geometry (B3LYP/6-31G*) of compound **3.44a**. Color code: gray refers to carbon, red to oxygen, blue to nitrogen, white to hydrogen and yellow to sulphur atoms.

Table S1. Electronic energies of compounds **3.43a** and **3.44a**, calculated at the B3LYP/6-31G(d) level of theory.

Compound	E/hartree
3.43a	-2312.9499818932
3.44a	-2312.9473054458

Cartesian coordinates (Å) obtained from the B3LYP/6-31G(d) calculations

Compound **3.43a**

C	-3.7353856247	2.6696733469	5.0999525295
C	-3.6184617690	5.8401810901	-0.9627676662
C	-2.0637049046	-9.0144680863	-2.3574230780
C	-0.7291361843	-3.8069074544	-7.3234995442
C	-3.9779421669	2.8485267843	3.7371009169
C	-2.4964016051	2.1836639026	5.5318131602
C	-3.3337922555	4.6698959294	-1.6735122763
C	-2.8525487589	6.1759075672	0.1540531316
C	-0.7936274940	-8.9746033604	-2.9374615231
C	-2.7247756753	-7.8211107862	-2.0638236467
C	0.4941257694	-3.6345781437	-6.6737480543
C	-1.7772265666	-4.4551931638	-6.6660885580
C	-2.9892843269	2.5383772283	2.8024522157
C	-1.5076407226	1.8796156204	4.6028877314
C	-2.2905419062	3.8370483563	-1.2712174262
C	-1.8053841238	5.3434021713	0.5580637021
C	-0.1906403476	-7.7485594704	-3.2185281265
C	-2.1223443366	-6.5934277695	-2.3482450068
C	0.6676928139	-4.1071834722	-5.3723138106
C	-1.6028612379	-4.9312715343	-5.3667903506
C	-1.7429326646	2.0496543982	3.2267919225
C	-1.5185421199	4.1680787586	-0.1455458774
C	-0.8480173624	-6.5460477266	-2.9279777995
C	-0.3792223176	-4.7591658657	-4.7065790543
C	-0.5798316304	0.1412797373	-1.2182860856
C	-0.6291680880	1.7028222440	2.2896908738
C	0.1929736322	-3.2670203547	-1.9361717151
C	-0.3946403522	3.2576836225	0.2999461421
C	-0.8402754953	1.8250836237	0.7780401517
C	-0.4944290047	-2.3535905671	-0.9180961213
C	0.6002871677	-0.4937240154	0.3907659471
C	0.0958262315	0.9143532248	-0.0439736585

C	-0.4188124923	-2.9395216803	0.5497985621
C	0.8371571419	-3.8000799400	0.7780712118
C	-1.6827839290	-3.7391435455	0.8907596005
C	1.5719630067	3.9414818639	-1.0154184667
C	-0.1677484352	-5.2357869302	-3.2759734582
N	0.1006970327	-1.0284991905	-0.8915282870
N	0.5102985921	2.9607896297	-0.8411374693
O	-1.4415576817	0.3607113030	-2.0368124254
O	0.4652206829	1.3588880818	2.7190298012
O	1.3434437258	-3.1717982009	-2.2954443265
O	1.1804001370	1.7291923446	-0.4288415791
O	-0.6729761120	-4.2210332378	-2.3445491245
S	-0.3415530800	-1.4311667481	1.6634550552
H	-4.5089681141	2.9082220388	5.8256511071
H	-4.4325272014	6.4869228797	-1.2804458619
H	-2.5326958360	-9.9691739073	-2.1337976344
H	-0.8647574206	-3.4392446657	-8.3374250210
H	-4.9373377395	3.2302558620	3.3984938403
H	-2.3059492934	2.0432722473	6.5926776739
H	-3.9254337185	4.4056681800	-2.5462952625
H	-3.0654040915	7.0854384236	0.7099229881
H	-0.2683361450	-9.8986284854	-3.1648803468
H	-3.7122297118	-7.8421890394	-1.6096141301
H	1.3147488178	-3.1308662089	-7.1783435090
H	-2.7314309017	-4.5965152385	-7.1671845885
H	-3.1945453399	2.6954867228	1.7489875389
H	-0.5385202216	1.5036740205	4.9144492895
H	-2.0657172446	2.9278658426	-1.8223269687
H	-1.2078413974	5.6071554188	1.4281328466
H	0.7991199684	-7.7234705833	-3.6694294381
H	-2.6372666461	-5.6676206306	-2.1126854812
H	1.6125257224	-3.9543230933	-4.8587099992
H	-2.4175967489	-5.4486832975	-4.8685335091
H	0.1706688692	3.7341862309	1.1173215326
H	-1.8789740708	1.6362500930	0.5039574965
H	-1.5435159536	-2.2499276909	-1.2163595432
H	1.6782443267	-0.5649830787	0.5394747717
H	0.9121300921	-4.0715140630	1.8349466386
H	1.7530644195	-3.2766737797	0.4895215833
H	0.7785687168	-4.7271731685	0.1938277920
H	-1.6406166862	-4.1075568588	1.9216220944
H	-1.7633196025	-4.6078609931	0.2276795450
H	-2.5841812183	-3.1279522277	0.7795145390
H	2.2145164788	3.6209636713	-1.8393198136
H	2.1801255364	4.0710931032	-0.1056849234
H	1.1019816553	4.8928233418	-1.2814549805
H	0.9065755382	-5.3216506506	-3.0953084063

Compound **3.44a**

C	-2.4372580868	4.4221755563	-4.1787679398
C	2.6206074143	6.1571227281	-2.2998923201
C	-4.2175421674	-8.8410976439	0.7416269836
C	1.5537018372	-6.5712787938	4.5618407114
C	-1.2815061232	3.6569027752	-4.3414475401
C	-3.0019930148	4.5704647494	-2.9077784838
C	3.1822576013	5.0085979896	-2.8597057045
C	1.8258946883	6.0512738497	-1.1559476242
C	-4.3215181713	-8.4376708137	2.0754676378
C	-3.3725734490	-8.1460378630	-0.1235237873
C	0.7550114871	-5.4928867256	4.9456891810
C	1.2223565391	-7.3100690395	3.4231200000
C	-0.6924305192	3.0354388183	-3.2402665624
C	-2.4127678482	3.9544152223	-1.8088551438
C	2.9471346076	3.7619453010	-2.2769068420
C	1.5846845177	4.8053586822	-0.5768265724
C	-3.5845376872	-7.3477871917	2.5354608948
C	-2.6312069231	-7.0547416086	0.3382131380
C	-0.3721904423	-5.1554584162	4.1947314799
C	0.0945385738	-6.9752379918	2.6743048813
C	-1.2520708228	3.1763648430	-1.9608700069
C	2.1398382411	3.6458647677	-1.1383434314
C	-2.7311422927	-6.6462133743	1.6730622419
C	-0.7125740777	-5.8949309916	3.0544218145
C	0.9618315377	-0.9104556540	-0.1855236518
C	-0.6869622335	2.5379713686	-0.7290743239
C	-1.4606789871	-3.3149621316	1.3300815856
C	1.8884117052	2.2770407351	-0.5392545020
C	0.5150963755	1.5912615987	-0.8427597628
C	-0.9534066946	-2.5868592114	0.0846467607
C	-0.7680484233	-0.1922282024	0.7764101291
C	0.5286718532	0.5241529766	0.2595069552
C	-2.1126303797	-1.9886173475	-0.8093237650
C	-3.4548077364	-2.7173527240	-0.6451827169
C	-1.6707283857	-2.0147521878	-2.2830588174
C	3.2151890737	2.3423740840	1.5182985171
C	-1.9309683928	-5.4876526943	2.2384306436
N	-0.1188843814	-1.4753056649	0.4856087158
N	1.8821297678	2.3199991241	0.9286803304
O	1.8366986997	-1.3868184416	-0.8703050658
O	-1.1746047125	2.7639419040	0.3697573761
O	-1.7937826689	-2.7610570141	2.3544313365
O	1.3308516034	1.0109494463	1.3163418258

O	-1.4934652224	-4.6465505056	1.1201183889
S	-2.3196427686	-0.2071951295	-0.2373208011
H	-2.8966552650	4.9028405951	-5.0389085824
H	2.8062951989	7.1299449168	-2.7480041000
H	-4.7947404432	-9.6883971860	0.3805791727
H	2.4307572433	-6.8360423098	5.1468342693
H	-0.8353206175	3.5439780979	-5.3259486303
H	-3.9014925220	5.1664774754	-2.7772521858
H	3.8118907060	5.0817754473	-3.7428502076
H	1.3911616175	6.9426373080	-0.7108278368
H	-4.9805254354	-8.9691002264	2.7572095663
H	-3.2868362799	-8.4512458505	-1.1635826311
H	1.0083744770	-4.9123239732	5.8288173344
H	1.8404144882	-8.1513558694	3.1202937837
H	0.2148343101	2.4597932052	-3.3889328476
H	-2.8344824733	4.0556108756	-0.8141839719
H	3.3989344073	2.8698199238	-2.7064604268
H	0.9744174856	4.7213611731	0.3167041726
H	-3.6687490038	-7.0385593583	3.5752099117
H	-1.9796885650	-6.5143016265	-0.3405314740
H	-0.9852976400	-4.3051805154	4.4800038068
H	-0.1669095030	-7.5599839650	1.7968621186
H	2.6602072437	1.5777328550	-0.9087535114
H	0.5528989512	1.1240158579	-1.8279568495
H	-0.3518870239	-3.2787536929	-0.5102103052
H	-1.0029655959	-0.0116171623	1.8237902195
H	-4.2117163608	-2.2660152385	-1.2953634377
H	-3.8196418550	-2.6610549455	0.3839724518
H	-3.3559344731	-3.7723319328	-0.9283473221
H	-2.3756774236	-1.4555144290	-2.9065289227
H	-1.6377053408	-3.0498110876	-2.6495271147
H	-0.6770627170	-1.5760488679	-2.4159167434
H	3.1169281906	2.2727813804	2.6045049456
H	3.6764636817	3.3023335525	1.2678169711
H	3.8512700544	1.5182554018	1.1576041340
H	-2.5782430283	-4.8695189868	2.8653680796