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Synthesis and structure-activity relationships of new chiral spiro-β-lactams highly active against HIV-1 and *Plasmodium*

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ABSTRACT

The synthesis and antimicrobial activity of new spiro-β-lactams is reported. The design of the new molecules was based on the structural modulation of two previously identified lead spiro-penicillanates with dual activity against HIV and *Plasmodium*. The spiro-β-lactams synthesized were assayed for their *in vitro* activity against HIV-1, providing relevant structure-activity relationship information. Among the tested compounds, two spirocyclopentenyl-β-lactams were identified as having remarkable nanomolar activity against HIV-1. Additionally, the same molecules showed promising antiplasmodial activity, inhibiting both the hepatic and blood stages of *Plasmodium* infection.

1. Introduction

Spiro-β-lactams are a subclass of β-lactams characterized by having an additional ring with the fusion of the two rings in one shared sp^3 carbon. Such feature causes these two rings to be disposed on different planes of the molecule. Given their molecular spatial arrangement, spiro-β-lactams have been widely explored in peptidomimetic chemistry as β-turn mimetics [1,2]. Yet, the interest in spirocyclic β-lactams goes far beyond its specific three-dimensional features. These molecules also show high potential in medicinal chemistry, having a wide spectrum of activity ranging from antimicrobial and antitumoral activity to the inhibition of cholesterol absorption [3–13].

The synthesis of chiral spirocyclic β-lactams derived from 6-aminopenicillanic acid (6-APA) has been explored by our research group, with distinct synthetic approaches leading to different classes of chiral spiro-penicillanates (Scheme 1) [14–17]. The overall synthetic strategy consisted of exploring the reactivity of 6-diazopenicillanates and 6-alkylidenepenicillanates in order to build molecules that kept the penicillanate core and to which a new chiral spiro carbon was added, a structural feature associated with the biological activity of known natural spirocyclic compounds. The 1,3-dipolar cycloaddition between 6-diazopenicillanates and electron-deficient alkenes led to spiropyrazoline-β-lactams, while

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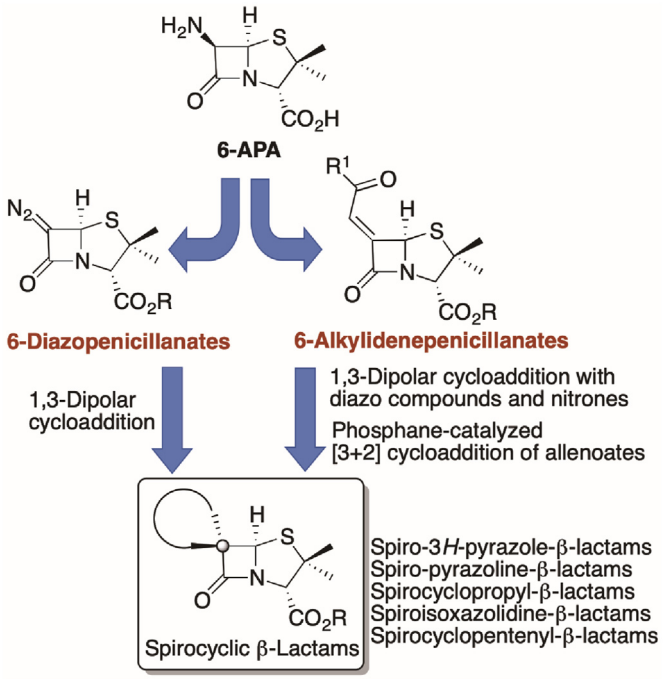


Scheme 1. Reactivity of 6-diazopenicillanates and 6-alkylidenepenicillanates as an approach to new spirocyclic penicillin analogues.

the cycloaddition with electron-deficient alkynes gave spiro-3*H*-pyrazole-β-lactams [14]. Spiropyrazolinepenicillanates were also obtained via stereoselective 1,3-dipolar cycloaddition reaction of 6-alkylidenepenicillanates with diazo compounds [15]. Interestingly, microwave-induced ring contraction of spiro-1-pyrazoline-β-lactams allowed the synthesis of chiral spirocyclopropylpenicillanates [15]. Recently, the synthesis of novel chiral spiroisoxazolidine-penicillanates from the cycloaddition of 6-alkylidenepenicillanates with nitrones has also been reported [16]. Finally, chiral spirocyclopentenyl-β-lactams were synthesized through phosphane-catalyzed [3+2] annulation of allenoates with 6-alkylidenepenicillanates [17].

Among the spiro-β-lactams synthesized by our research group, three were identified as having dual antimicrobial *in vitro* activity against both HIV and *Plasmodium* (Fig. 1) [18]. Spiro-3*H*-pyrazole-β-lactam **3** showed high activity against HIV-1 (IC_{50} = 12 nM). Nevertheless, the most promising molecule was spirocyclopentenyl-β-lactam **1** which presented IC_{50} values of 14 and 8 nM against HIV-1 and HIV-2, respectively, being active also against multidrug resistant HIV strains. Spiro-β-lactam **1** also proved to be highly active against *P. berghei* hepatic infection (IC_{50} = 550 nM) and against the erythrocytic stages of *P. falciparum* (IC_{50} = 430 nM). Beyond its dual antimicrobial activity, molecule **1** appears to affect all stages of the HIV replicative cycle suggesting a complex and new mechanism of action [18]. This result can be regarded as the starting point for the development of new therapeutic alternatives to the current anti-HIV and anti-plasmodial drugs. Despite being a β-lactam, molecule **1** displays no activity against bacteria or yeasts. Altogether, spiro-β-lactam **1** stands out as a very promising lead molecule for further structural modulation on the synthesis of new spiro-β-lactams with an innovative mechanism and activity against HIV and *Plasmodium*.

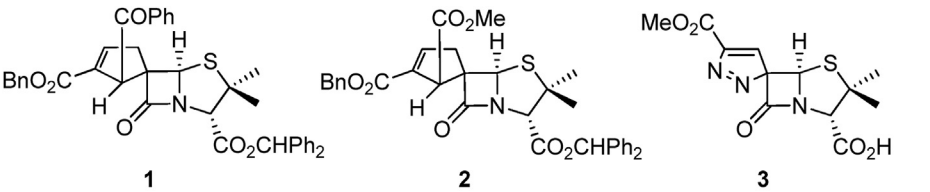
The present work focused on the synthesis of new molecules derived from lead molecules **1** and **3.** The designed structural modulations aimed at the discovery of new active spiro-β-lactams, and the gathering of relevant structure-activity relationships and mechanism of action information (Fig. 2). This rational structure derivatization-based approach led to the synthesis of twenty-three new spiro-β-lactams (see Figure S1), two of them showing submicromolar activity against HIV-1. The same two spiro-β-lactams also displayed a promising activity against *Plasmodium*, inhibiting both the hepatic and erythrocytic stages of the parasite's life cycle.

2. Results and discussion

2.1. Spiro-β-lactams synthesis

The spiro-3*H*-pyrazole-β-lactam **3** was previously reported as highly active against HIV-1 and some structure-activity relationships were identified [6,18]. It was observed that the presence of two methyl ester substituents on the pyrazole ring leads to a decrease of activity. On the other hand, a free carboxylic acid is an important structural feature to ensure bioactivity since the conversion of spiro-3*H*-pyrazole-β-lactam **3** into the corresponding benzhydryl carboxylate led to complete loss of activity. Furthermore, the molecule's bioactivity relies on its β-lactam ring, a structure-activity relationship determined by the lack of activity observed for spiro-lactam analogues where the four-membered β-lactam ring was replaced by a five-membered γ-lactam ring [6]. Finally, by comparing spiro-3*H*-pyrazole-β-lactam **3** and spirocyclopentenyl-β-lactam **1** structures, the two active molecules clearly diverge on their aromatic content, with molecule **1** presenting four phenyl groups while molecule **3** is characterized by the complete absence of phenyl groups in its structure. Such previous considerations were the starting point for further structural modulations of molecule **3**.

Concerning the structure-activity relationship information of the highly promising spirocyclopentenyl-β-lactam **1**, it is only known that replacing the benzoyl group at the cyclopentene ring by a methyl ester causes a decrease of the molecule's bioactivity. Thus,



1
IC_{50} (HIV-1) = 0.014 μM
IC_{50} (HIV-2) = 0.008 μM
IC_{50} (*P. berghei*) = 0.550 μM
IC_{50} (*P. falciparum*) = 0.430 μM

2
IC_{50} (HIV-1) = 0.650 μM
IC_{50} (HIV-2) = 0.510 μM
IC_{50} (*P. berghei*) < 5 μM

3
IC_{50} (HIV-1) = 0.012 μM
IC_{50} (*P. berghei*) < 10 μM

Fig. 1. Molecular structure of lead molecules with i*n vitro* antimicrobial activity against HIV and *Plasmodium*.

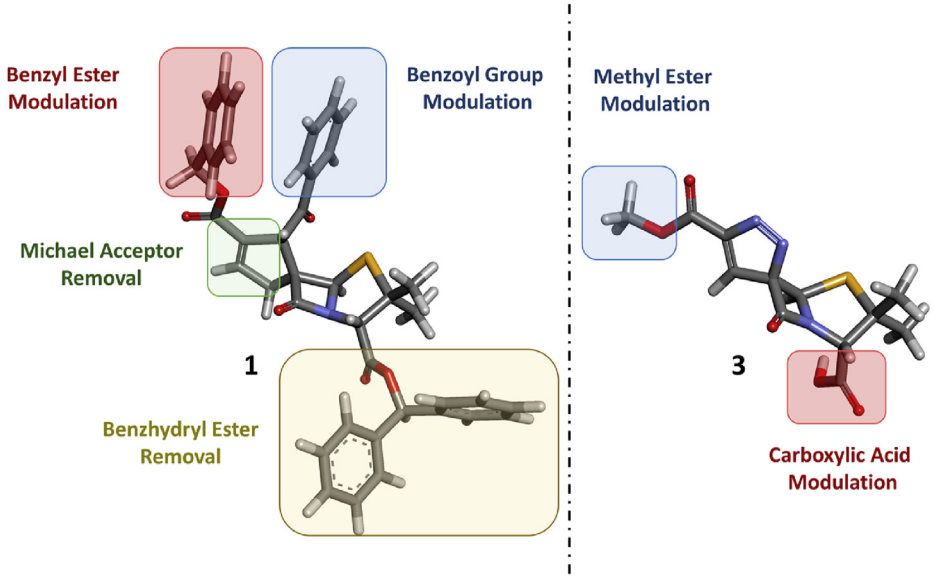


Fig. 2. Designed structural modulation of lead molecules **1** and **3**.

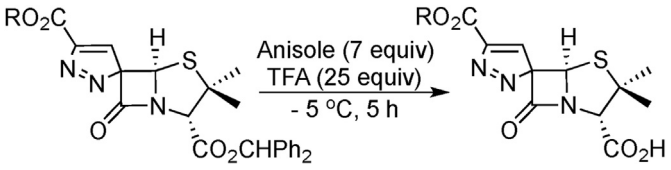
the structural alterations focused mainly on the cyclopentene ring pharmacophoric features (i.e. benzoyl, benzyl ester and Michael acceptor system) although the removal of the benzhydryl ester was also performed to study this specific functional group importance for the molecule's activity.

*2.1.1. Synthesis of spiro-3H-pyrazole-β-lactam analogues of molecule **3***

The first objective of the design of new spiro-3*H*-pyrazole-β-lactam analogues of molecule **3** was the modulation of the methyl ester substituent and the increase of the aromatic features content. To achieve this goal, 1,3-dipolar cycloaddition of 6-diazopenicillanate **4** with selected electron-deficient alkynes was explored (Scheme 2). Following our previously reported synthetic procedure [14], chiral 6-diazopenicillanate **4** reacted with propiolates **5a-d** in dichloromethane (DCM) at reflux to afford four new spiro-3*H*-pyrazole-β-lactams **6a-d**, bearing an ethyl, *tert*-butyl, 2-naphthyl and benzhydryl ester group at the C5 pyrazole ring position and no substituent at C4 position. The reaction of 6-diazopenicillanate **4** with dibenzhydryl but-2-ynedioate (**5e**) was also carried out to afford spiro-3*H*-pyrazole-β-lactam **6e** where the pyrazole ring is substituted with two benzhydryloxy carbonyl substituents. The 1,3-dipolar cycloaddition proved to be regioselective giving the target compounds as single diastereoisomers in moderate to good yields (33–74%).

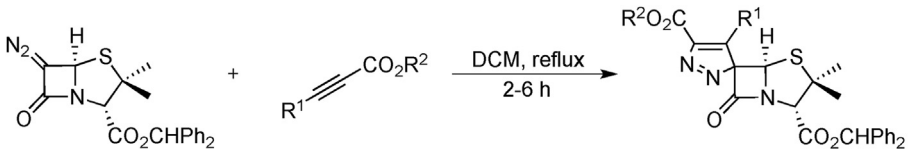
Spiro-β-lactams **6a-d** were then converted to their respective free penicillanic acid derivatives following a previously reported general procedure (Scheme 3) [6,18]. Thus, treatment with anisole and trifluoroacetic acid (TFA) in anhydrous DCM at −5 °C for 5 h, allowed the efficient benzhydryl moiety removal, affording four new spiro-3*H*-pyrazole-penicillanic acids **7a-d** in high yields (81–93%). Noteworthy, is the deprotection of both ester groups in the case of spiro-β-lactam **6d** leading to the dicarboxylic acid derivative **7d**.

The NOESY spectrum of compound **7c** showed cross-peaks between protons H4' (δ = 7.25 ppm) and H5 (δ = 6.65 ppm) corroborating the selectivity observed in the 1,3-dipolar cycloaddition reaction of 6-diazopenicillanate **4** with propiolates. This outcome is in agreement with a previous report on the synthesis of similar



6a R = Et		**7a** R = Et	93%
6b R = *t*-Bu		**7b** R = *t*-Bu	81%
6c R = 2-Naphthyl		**7c** R = 2-Naphthyl	86%
6d R = $CHPh_2$		**7d** R = H	90%

Scheme 3. Spiro-3*H*-pyrazole-penicillanic acids via deprotection of the corresponding esters with anisole and TFA.



4

5a R^1 = H, R^2 = Et	**6a** R^1 = H, R^2 = Et	45%
5b R^1 = H, R^2 = *t*-Bu	**6b** R^1 = H, R^2 = *t*-Bu	33%
5c R^1 = H, R^2 = 2-Naphthyl	**6c** R^1 = H, R^2 = 2-Naphthyl	54%
5d R^1 = H, R^2 = $CHPh_2$	**6d** R^1 = H, R^2 = $CHPh_2$	74%
5e R^1 = CO_2CHPh_2, R^2 = $CHPh_2$	**6e** R^1 = CO_2CHPh_2, R^2 = $CHPh_2$	36%

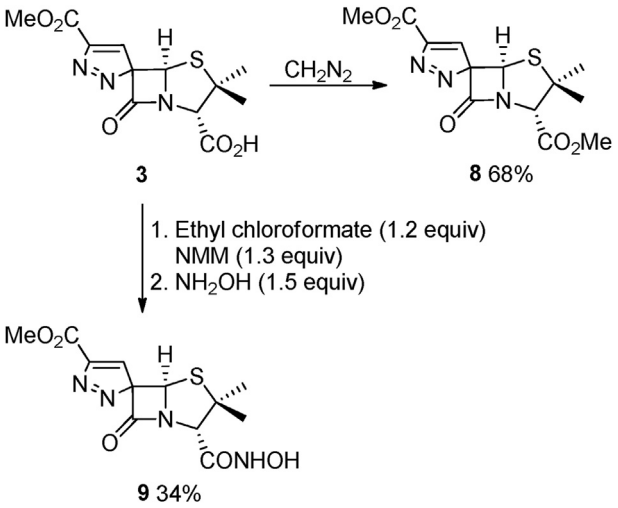
Scheme 2. Chiral spiro-3*H*-pyrazole-β-lactams from 1,3-dipolar cycloaddition reaction of 6-diazopenicillanate **4** with five different electron-deficient alkynes.

spiro-3*H*-pyrazole-β-lactams, with respect not only to regioselectivity, but also to diastereoselectivity which results from the approach of the dipolarophile by the less hindered convex face (α-side) of the 6-diazopenicillanate derivative [14].

Lead spiro-3*H*-pyrazole-β-lactam **3** was also the target to structural modulations on its carboxylic acid feature to study this functional group importance for the observed biological activity (Scheme 4). The first approach involved the conversion of compound **3** into the corresponding methyl spiro-3*H*-pyrazole-penicillanate **8** by treatment with excess of ethereal diazomethane. Spiro-β-lactam **8**, having two methyl ester groups, was isolated in 68% yield. The second structural modification focused on the carboxylic acid functional group conversion into an hydroxamic acid. This structural modulation followed a reported general procedure [19], comprising a first carboxylic acid activation step through anhydride formation, and a subsequent hydroxyamidation step with hydroxylamine, affording hydroxamic acid spiro-β-lactam **9** in moderate yield (34%).

2.1.2. Synthesis of spirocyclopentenyl-β-lactam analogues of molecule *1* and their α-regioisomers

The previously described spirocyclopentenyl-β-lactams were obtained via phosphane-catalyzed [3+2] annulation of allenes, acting as three-carbon synthons, with 6-alkylidenepenicillanates (Scheme 5) [17]. In particular, the reaction of benzyl 2,3-butadienoate (**11**) with 6-alkylidenepenicillanate **10**, in the presence of triphenylphosphine, allowed the synthesis of the lead spirocyclopentenyl-β-lactam **1**.



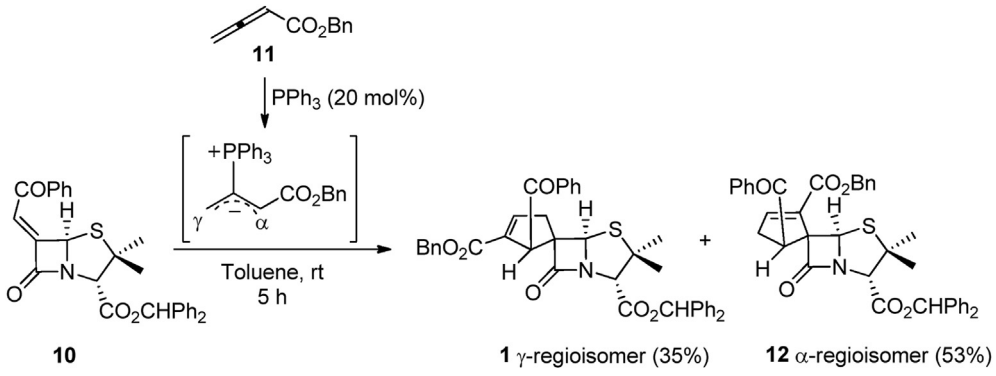
Scheme 4. Structural modulation of molecule **3** carboxylic acid via its conversion into a methyl ester and a hydroxamic acid group.

Active molecules **1** and **2** share the same benzyl ester at position 3 of the cyclopentene ring (Fig. 1). Thus, this position on molecule **1** was the target to structural modulation to understand the importance of the benzyl ester group in the molecule activity.

The first two modulations focused on endowing position 3 of the cyclopentene ring with smaller esters, namely an ethyl and a methyl ester, to obtain molecules **14a** and **14b** (Table 1). Monosubstituted 2,3-butadienoates, such as methyl and ethyl 2,3-butadienoate, are highly volatile and, consequently, difficult to handle. Therefore, we explored 2-butynoates (*e.g.* methyl and ethyl 2-butynoate) as an alternative to allenoates to act as formal 1,3-dipole precursor in the synthesis of the new spirocyclopentenyl-β-lactams. Although the use of 2-butynoates as dipole precursors in phosphane-catalyzed [3+2] annulation reactions is known [20–23], the reactivity towards 6-alkylidenepenicillanates is unexplored.

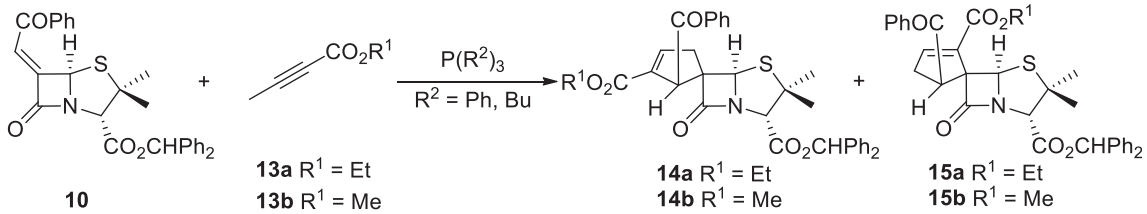
Initially, the reaction of 6-alkylidenepenicillanate **10** with ethyl 2-butynoate (**13a**) was attempted, under the reaction conditions used in the synthesis of molecule **1** with benzyl 2,3-butadienoate (**11**) [17]. However, no products were formed after stirring the reaction mixture for 5 h. In fact, the reaction of 6-alkylidenepenicillanate **10** with ethyl 2-butynoate (**13a**) (1 equiv) and PPh_3 (20 mol%) at room temperature, only showed evidence of the target products in trace amounts after 120 h (Table 1, entry 1). Attempts to improve the reaction outcome by increasing the reaction temperature (entries 2 and 3) or using a more nucleophilic phosphine PBu_3 (entry 4), also proved unfruitful. Interestingly, by increasing the initial amount of ethyl 2-butynoate (**13a**) to 6 equivalents, maintaining the butynoate/phosphine ratio, spirocyclopentenyl-β-lactams **14a** and **15a** were obtained in 30% and 40% yields, respectively, after 120 h (entry 5). We were pleased to observe that changing the butynoate/phosphine molar ratio to 40 mol%, the reaction time could be reduced to 48 h and products **14a** (53%) and **15a** (40%) obtained in 93% overall yield (entry 6). These optimized reaction conditions were then applied to carry out the reaction of 6-alkylidenepenicillanate **10** with methyl 2-butynoate (**13b**) in the presence of PPh_3, affording spirocyclopentenyl-β-lactams **14b** and **15b** in 25% and 49% yield, respectively (entry 7).

2D NOE experiments corroborated the structural assignments of the two regioisomers (*e.g.* **14b** and **15b**) as well as the high stereoselectivity, involving the addition of the formal dipole to the less sterically hindered α-side of the 6-alkylidenepenicillanate. From the analysis of the NOESY spectrum of both compounds it was possible to observe cross-peaks between protons of β-Me (δ = 1.51 ppm) and H1' (δ = 5.15 ppm and δ = 4.55 ppm, for



Scheme 5. Synthesis of lead molecule **1**.

Table 1
Phosphane-catalyzed [3+2] annulation between 2-butynoates and a 6-alkylidenepenicillanate.



Entry	R^1	Butynoate (equiv)	R^2	Phosphine (equiv)	Reaction conditions	Isolated yield (%) 14 + 15
1	Et	1	Ph	0.2	rt, 120 h	Trace Amounts
2	Et	1	Ph	0.2	55 °C, 120 h	Trace Amounts
3	Et	1	Ph	0.2	80 °C, 120 h	Trace Amounts
4	Et	1	Bu	0.2	rt, 120 h	–
5	Et	6	Ph	1.2	rt, 120 h	30 + 40
6	Et	6	Ph	2.4	rt, 48 h	53 + 40
7	Me	6	Ph	2.4	rt, 48 h	25 + 49

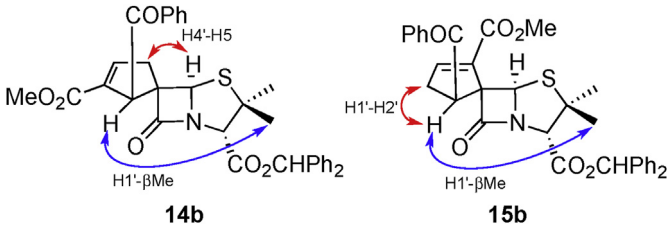


Fig. 3. NOESY correlations of compounds **14b** and **15b**.

compound **14b** and **15b**, respectively). Furthermore, for compound **14b** it was possible to observe NOE between protons H4' ($\delta = 3.12$ and 3.54 ppm) and H5 ($\delta = 5.44$ ppm). In the case of compound **15b** cross-peaks signals between H1' ($\delta = 4.55$ ppm) and the methylene group H2' ($\delta = 2.54$ and 3.17 ppm) were observed and no correlation was observed between the latter and proton H5 (Fig. 3).

In sum, the present phosphane-catalyzed [3+2] annulation between 2-butynoates and 6-alkylidenepenicillanate **10** affords chiral spirocyclopentenyl-β-lactams isolated as single diastereoisomers in high overall yields (74–93%) under mild reaction conditions, demonstrating that small chain 2-butynoates stand out as a plausible alternative to its allenoates counterparts broadening the type of compounds that can be made available by this synthetic methodology.

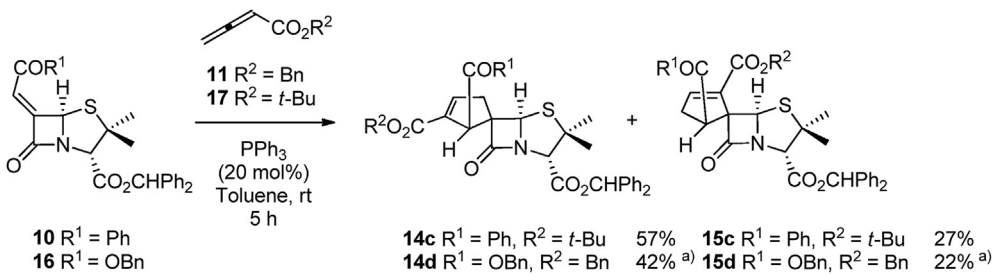
A second structural modulation approach focused on endowing molecule **1** cyclopentene spiro-ring with more bulkier alkyl functional groups consisted on the synthesis of two new chiral spirocyclopentenyl-β-lactams **14c** and **14d**. Molecule **14c** had the benzyl ester at cyclopentene ring replaced by a *tert*-butyl ester, and molecule **14d** had the benzoyl group replaced by a longer chain and less conformationally restrained benzyl ester. Molecules **14c** and **14d**, which were obtained together with the corresponding α-regioisomers **15**, were synthesized following the same general synthetic procedure used to synthesize lead molecule **1** (Scheme 6) [17]. It should be noted that compound **15d** could not be separated from its γ-regioisomer **14d**, although it was possible to isolate spiro-β-lactam **14d** in pure form.

To understand the importance of the benzhydryl ester substituent for the spiro-β-lactam **1**'s anti-HIV activity, this molecule was converted into its carboxylic acid analogue, with TFA and anisole in DCM at −5 °C, affording molecule **18** in moderate yield (59%) (Scheme 7).

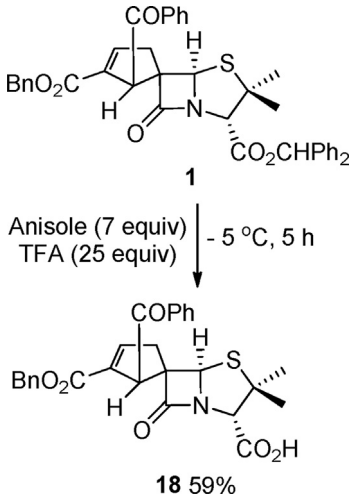
Spirocyclopentenyl-β-lactam **12**, the α-regioisomer of molecule **1**, was also subjected to its benzhydryl ester removal following the same deprotection procedure. Spiro-β-lactam-carboxylic acid **19** was obtained, in its salt form, in 39% yield and was esterified with iodomethane to afford molecule **20** in 31% yield (Scheme 8).

Molecule **1** contains an α,β-unsaturated carbonyl system in its cyclopentene spiro-ring. Such highly electrophilic structural feature is prone to alkylate biomolecules and is usually avoided in drug discovery since it has been associated with lack of molecular target selectivity. However, Michael acceptors are crucial for the bioactivity of several molecules [24,25]. Thus, a 1,3-dipolar cycloaddition based approach was explored to synthesize spirocyclic β-lactams **21** and **23**, two analogues of molecule **1** without the α,β-unsaturated carbonyl system in their structure.

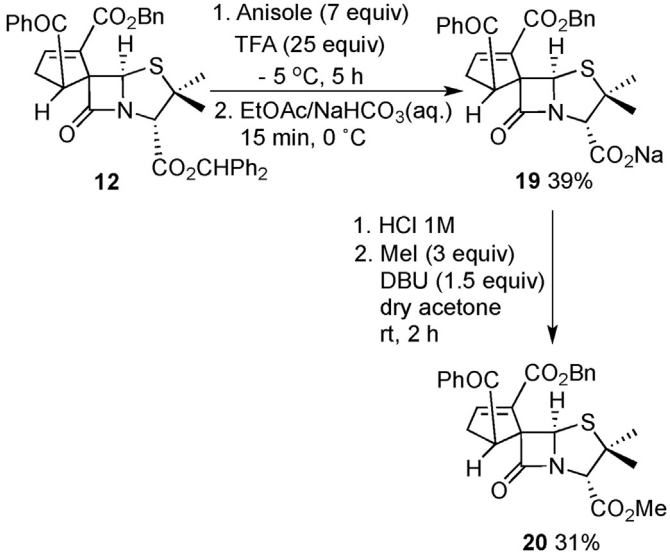
Chiral molecule **21** was synthesized carrying out the 1,3-dipolar



Scheme 6. Synthesis of new chiral spirocyclopentenyl-β-lactams through phosphane-catalyzed [3+2] annulation between allenoates and 6-alkylidenepenicillanates. [a)] Yield determined by ^{1}H NMR.



Scheme 7. Molecule **1** ester deprotection to its spirocyclopentenyl-β-lactam-carboxylic acid analogue, using anisole and TFA.

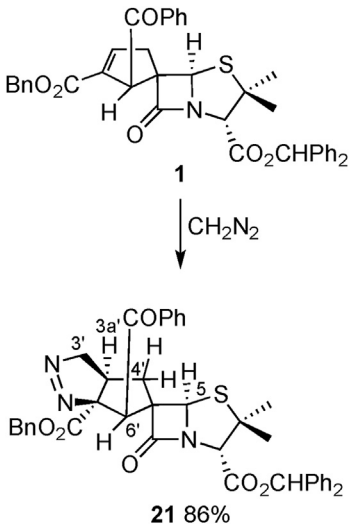


Scheme 8. Structural modulation of molecule **12** targeting the benzhydryl ester.

cycloaddition between diazomethane and molecule **1**, with its α,β-unsaturated carbonyl system serving as dipolarophile (Scheme 9). The reaction proved to be highly regio- and stereoselective affording spirocyclic β-lactam **21** as single stereoisomer and in high yield (86%).

The full structural assignment of compound **21** was based on NMR data, in particular by observing the signal's multiplicity of protons H3a′, H4′ and H3′ in the ^{1}H NMR spectrum and the corresponding correlations in the COSY spectrum. It is noteworthy that the observed regioselectivity is in agreement with the one expected from the cycloaddition of diazoalkanes with acrylates [26–28]. From the analysis of HSQC spectrum, it was possible to assign the diastereotopic protons H4' (δ = 2.17 and 3.22 ppm). On the other hand, the NOESY spectrum showed cross-peaks between proton H5 (δ = 5.20 ppm) and one of the diastereotopic protons (H4′, δ = 3.22 ppm) and cross-peaks were also observed between the other diastereotopic proton (H4′, δ = 2.17 ppm) and proton H3a' (δ = 3.15 ppm).

Spiro-β-lactam **23** was obtained in 30% yield by reacting 6-alkylidenepenicillanate **10** with benzyl diazoacetate (**22**) acting as

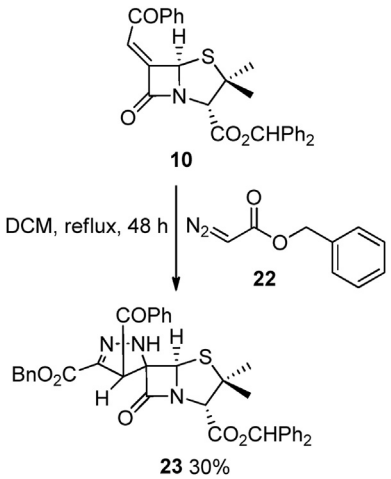


Scheme 9. 1,3-Dipolar cycloaddition between lead molecule **1** and diazomethane.

1,3-dipole (Scheme 10). It has been previously reported that the cycloaddition of 6-alkylidenepenicillanates with diazo compounds (diphenyldiazomethane, phenyldiazomethane and diazomethane) can lead to the synthesis of spiro-1-pyrazolinepenicillanates and/or spiro-2-pyrazolinepenicillanates [15,29,30]. On the other hand, the unambiguous structural assignment of compound **23** was also required to determine the regioselectivity.

The molecule structure was determined based on several 2D NMR studies. In the analysis of the HSQC spectrum, no connectivity was observed between the proton with δ = 6.24 ppm, confirming that this proton is attached to a nitrogen atom. Therefore, the possibility of having a spiro-1-pyrazolinepenicillanate derivative was ruled out. Nevertheless, depending on the regioselectivity two isomeric spiro-2-pyrazolinepenicillanates, molecules **23** and **24**, could still be formed (Fig. 4).

From the analysis of the NOESY spectrum, cross-peaks were observed between protons of the β-methyl group of the penicillanate core (βMe, δ = 1.52 ppm) and the proton of the 2-pyrazoline ring (H4′, δ = 5.65 ppm). Due to the butterfly-like structure of penicillanates, this correlation could be observed for both structures **23** and **24**, as previously described for other



Scheme 10. 1,3-Dipolar cycloaddition between alkylidene **10** and benzyl diazoacetate (**22**).

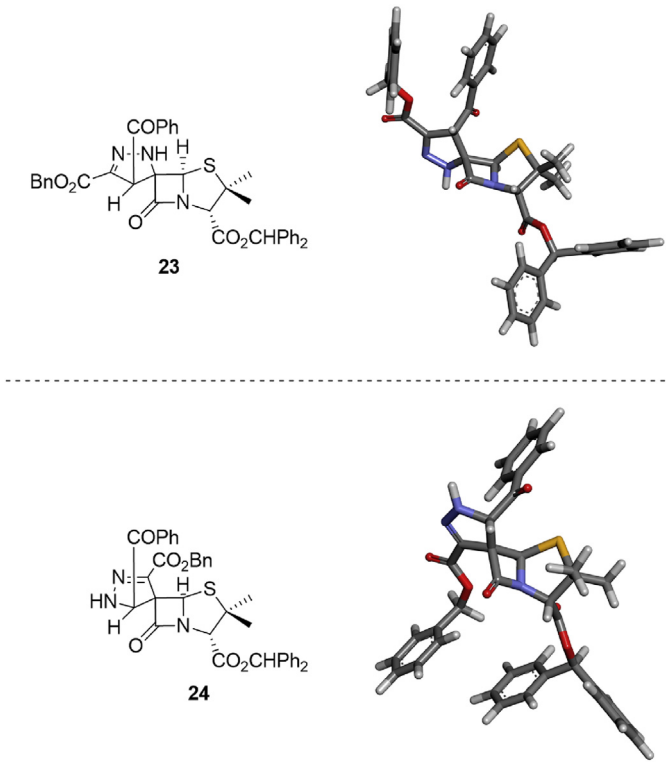


Fig. 4. Optimized geometries (B3LYP/6-31G(*d*) level) of structures rationalized as possible products of the 1,3-dipolar cycloaddition between alkylidene **10** and benzyl diazoacetate.

spiropenicillanates [14–17]. However, internuclear distances and the corresponding NOE intensities could allow to distinguish the two structures. Therefore, quantum chemical calculations, at the density functional theory (DFT) level (B3LYP/6-31G(d)) were carried out and internuclear distance values were estimated from the optimized structures of compounds **23** and **24**. In the case of spiropenicillanate **23**, the estimated distances between βMe-H4′ and H4′-H(Ar) were 5.005 Å and 3.831 Å, respectively, which is in agreement with the observed NOEs, where cross-peaks of βMe-H4′ showed lower intensity than the ones of H4′-H(Ar). Moreover, internuclear distances βMe-H4′ and H4′-H(Ar) for compound **24** (3.034 Å and 5.542 Å, respectively) should lead to NOEs with opposite intensities. On the other hand, for structure **24** NOE would be expected between the methylene group of the benzyl ester with proton H5 which was not observed. Finally, the calculated energy values for the optimized geometries revealed that structure **23** was estimated to be 12.04 kJ/mol more stable than structure **24**. Considering all the above, the structure of compound **23** was unequivocally assigned.

2.2. Biological evaluation

2.2.1. Spiro-β-lactams anti-HIV activity

The *in vitro* cytotoxicity (CC_{50}) of the synthesized spirocyclic β-lactams was assessed in TZM-bl cells, prior to determining the molecules anti-HIV activity. Among the set of twenty-three molecules, only molecule **23** showed significant cytotoxicity (CC_{50} = 17.16 μM). The remaining spiro-β-lactams have acceptable CC_{50} values, ranging from 33.55 μM (molecule **6e**) to 101.38 μM (molecule **7d**) (Table 2). These results are consistent with previous studies of our research group which indicated that the penicillin-derived spiro-3*H*-pyrazole-β-lactams and spirocyclopentenyl-β-lactams have low *in vitro* toxicity [18].

Table 2
CC_{50} values and maximum percentage of inhibition of an HIV-1 isolate (strain SG3.1) of the synthesized spiro-β-lactams.

Compound	CC_{50} (μM)	MPI (10 μg/ml)
6a	46.85	0%
6b	55.14	0%
6c	49.10	0%
6d	54.39	0%
6e	33.55	0%
7a	83.81	0%
7b	79.00	0%
7c	68.60	0%
7d	101.38	0%
8	89.75	0%
9	76.79	0%
14a	58.95	99%
14b	53.21	99%
14c	44.39	0%
14d	43.22	0%
15a	57.09	0%
15b	54.88	0%
15c	43.25	0%
18	60.33	0%
19	56.65	0%
20	55.38	0%
21	41.83	0%
23	17.16	–

The *in vitro* antiviral activity of the twenty-two non-cytotoxic spiro-β-lactams was evaluated at a single drug concentration (10 μg/mL) in a single-round viral infectivity assay against a HIV-1 isolate, resulting in the identification of two spirocyclopentenyl-β-lactams (**14a** and **14b**) with high antiviral activity. Molecules **14a** and **14b** are derived from spirocyclopentenyl-β-lactam **1** and both exhibited a maximum percentage of inhibition (MPI) of 99%. None of the remaining spiro-β-lactams showed antiviral activity. Given the promising results, the IC_{50} and IC_{90} values of **14a** and **14b** against HIV-1 strain SG3.1 were determined. Both molecules presented sub-micromolar IC_{50} and IC_{90} values (Fig. 5). Spiro-β-lactam **14b** showed a remarkable IC_{50} value of 0.058 μM and an IC_{90} value of 0.233 μM. Molecule **14a** showed lower, yet still considerable, activity with IC_{50} and IC_{90} values of 0.305 and 0.479 μM, respectively. The discovery of these two new highly potent molecules reinforce the potential of spiro-β-lactams in general, and spirocyclopentenyl-β-lactams in particular, as highly promising agents against HIV-1.

2.2.2. Spiro-β-lactams antiplasmodial activity

Aside from its notable anti-HIV activity, lead spirociclopentenyl-β-lactam **1** also presents a promising submicromolar activity against both the hepatic (IC_{50} *P. berghei* = 0.550 μM) and blood (IC_{50} *P. falciparum* = 0.430 μM) stages of *Plasmodium* infection [18]. Thus, the antiplasmodial activity of its analogues **14a** and **14b** was also evaluated and their IC_{50} values against *P. berghei* hepatic stage and *P. falciparum* erythrocytic stage were determined (Fig. 6).

Both molecules showed promising activity against *P. berghei* hepatic stages, with spiro-β-lactams **14a** and **14b** displaying IC_{50} values of 1.924 ± 0.240 μM and 2.289 ± 0.151 μM, respectively. Yet, the two compounds proved to be more active against the erythrocytic stage of *Plasmodium* infection, with IC_{50} values of 0.844 ± 0.036 μM and 1.255 ± 0.003 μM, respectively. None of the tested compounds displayed cytotoxicity against the Huh-7 host cells employed in the hepatic stage assays.

It is noteworthy that the dual bioactivities of the two spiro-β-lactams display a trend inversion, since **14b** displays higher anti-HIV activity than **14a**, while the opposite is observed for the molecules' anti-plasmodial activities. Nevertheless, these results

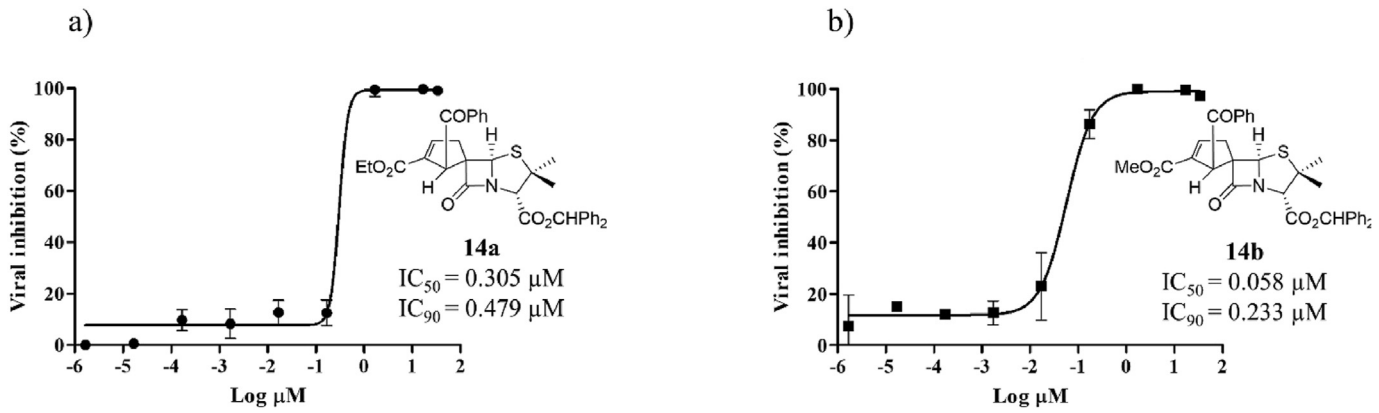


Fig. 5. Activity of spirocyclopentenyl-β-lactams **14a** and **14b** against HIV-1.

emphasize the high potential of the present class of spiro-cyclopentenyl-β-lactams as therapeutic agents with dual antimicrobial activity.

2.3. Structure-activity relationships

2.3.1. Spiro-3H-pyrazole-β-lactams derived from lead molecule **3**

The antimicrobial activity of β-lactams is commonly related to their capability to acylate macromolecules of the pathogenic agents [31]. This β-lactam reactivity is associated with the 2-azetidione ring strain, which makes the ring opening an energy favourable event. Moreover, the presence of a nearby ionizable functional group, such as a carboxylic acid, is also crucial for penicillin antibacterial activity since it acts as a catalyst of the ring opening within its target bacterial transpeptidase enzyme (PBP) [32] active site [33,34]. This catalytic behaviour relies on a preliminary carboxylic acid protonation step which selectively occurs in the enzyme pocket. Once protonated, the carboxylic acid establishes an internal H-bond with the nitrogen atom of the lactam ring, withdrawing electronic density from the amide bond and weakening it, causing the β-lactam ring to became highly reactive toward nucleophiles. Despite its penicillin-core with a free carboxylic acid, it is noteworthy that molecule **3** showed no antibacterial activity. Yet, this fact can be related with the presence of a spiro-ring in spirocyclic β-lactam. Such structural feature considerably increases the steric hindrance around the 2-azetidione ring, being plausible that it can prevent an effective fitting of spiro-β-lactam **3** inside penicillin's target PBP enzyme active site. Regardless of molecule **3**'s lack of antibacterial activity, our previously reported results suggest that the β-lactam ring is somehow a crucial structural feature for the bioactivity of spirocyclic pyrazole-β-lactam **3** against HIV-1. In fact, the molecule five-membered ring γ-lactam analogue showed no relevant activity against HIV-1 [6]. On the other hand, spiro-β-lactam **3** analogue containing a benzhydryl ester instead of the free carboxylic acid proved to be inactive against the same virus [18].

In the present work, several structural modulations were performed using the active spiro-3*H*-pyrazole-β-lactam **3** as lead molecule to synthesize new spiro-3*H*-pyrazole-β-lactam derivatives with potential activity against HIV-1. Such structural modulations also intent to serve the purpose of gathering more structure-activity information to understand molecule **3** mechanism of action. These structural derivatizations focused on both the thiazolidine's carboxylic acid and the ester functional group present on the pyrazole ring.

It was previously shown that esterification of molecule **3** with a benzhydryl group leads to the molecule's loss of activity [18]. Yet, since the benzhydryl group is highly bulky, the molecule's loss of activity could be exclusively associated with stereo hindrance in the molecule-target interaction, instead of the absence of an ionizable group on the β-lactam ring nearby. Thus, to effectively understand the importance of the carboxylic acid group, this structural feature was converted to a methyl ester, the least bulky ester, affording molecule **8**. To study if a replacement of the carboxylic group by another ionizable group would preserve the molecule's bioactivity, the carboxylic acid was also converted to an hydroxamic acid, resulting in molecule **9**. Both these carboxylic acid modulation strategies led to inactive molecules, indicating that the carboxylic acid is crucial for bioactivity. Furthermore, all the new spiro-3*H*-pyrazole-β-lactam derivatives **6a-d** containing a benzhydryl ester on the thiazolidine moiety showed no activity against HIV-1. These results corroborate our previous assumptions about the importance of the β-lactam ring and the carboxylic acid group, leading us to speculate whether the molecule exerts its activity through an acylation-based mechanism.

The methyl ester functional group present at position 5 of the 3*H*-pyrazole ring was also the target of structural modulations giving four new acid spiropyrazole-β-lactams **7a-d**, analogues of our previously reported active molecule **3**. The structural changes were performed either by increasing the size of the ester (to an ethyl, naphthyl or *tert*-butyl ester) or by replacing it by a carboxylic acid. None of the new spiropyrazole-β-lactams showed activity against HIV-1. The lack of activity of molecules containing larger esters is thought to be related with the increase of bulkiness which can prevent the molecule's effective interaction with its putative molecular target. However, one must be more cautious when rationalizing the effect of replacing the ester group of the 3*H*-pyrazole ring by a carboxylic acid group. The fact that this molecule shows no activity against HIV-1 notwithstanding it is a close structural analogue of the active molecule **3**, may be solely related to the absence of a hydrophobic feature, such as a methyl ester which may be relevant for molecule-target interactions. It should also be noted that the presence of two carboxylic groups can render the molecule unable to cross membrane barriers due to its formal charge at physiologic pH, preventing the molecule from accessing its putative molecular target. Nevertheless, these results demonstrate the importance of the methyl ester for the activity of the template molecule.

Finally, the synthesis of spiropyrazole-β-lactams containing benzhydryl carboxylate groups on their 3*H*-pyrazole ring involved a synthetic strategy to endow the molecules with more aromatic rings, pharmacophoric features which are present in the highly active spirocyclopentenyl-β-lactam **1** spiro-ring. However, this structural modulation was unsuccessful as the two molecules **6d** and **6e** proved to be inactive against HIV-1.

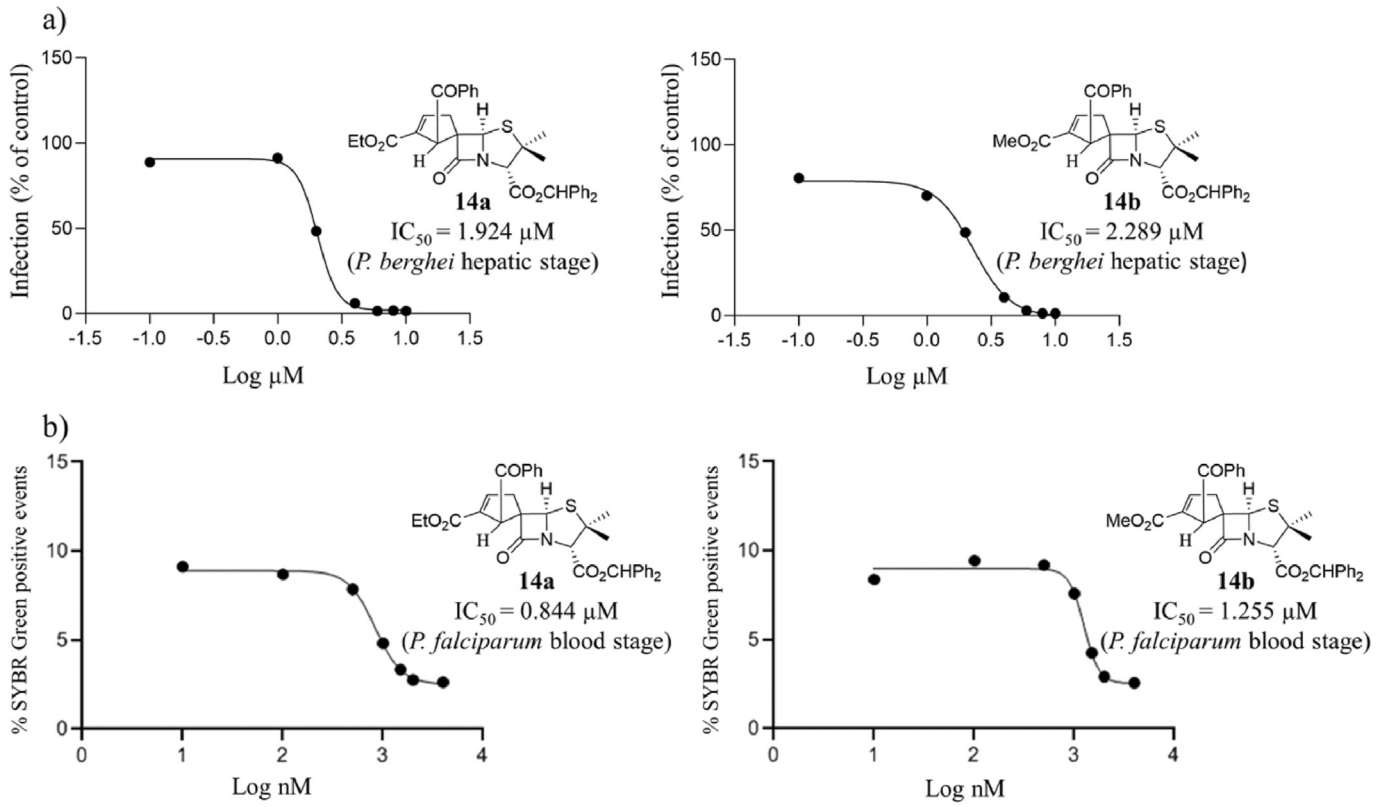


Fig. 6. *In vitro* activity of spirocyclopentenyl-β-lactams **14a** and **14b** against a) hepatic *P. berghei* infection; and b) *P. falciparum* erythrocytic infection. The IC_{50} values were calculated as an average value of two independent assays and DMSO was used as control.

Altogether, the new structure-activity relationship results regarding analogues of the spiro-3*H*-pyrazole-β-lactam lead molecule **3** reveal the importance of the methyl ester group presence at the 3*H*-pyrazole ring and also supports the initial proposal about this molecule activity relying on its β-lactam ring and thiazolidine's carboxylic acid.

2.3.2. Spirocyclopentyl-β-lactams derived from lead molecule **1**

By exploring the synthesis of chiral spirocyclopentenyl-β-lactams through phosphane-catalyzed [3+2] annulation of allenoates with 6-alkylidenepenicillanates, our research group identified the two spirocyclopentenyl-β-lactams **1** and **2** with remarkable activity against both HIV-1 and HIV-2. These two molecules are both γ-regioisomers containing a benzyl ester at position 3 of the spirocyclopentene-ring, which were formed along with the corresponding α-regioisomers (see Fig. 1 and Scheme 5) [17]. The most active spirocyclopentenyl-β-lactam **1**, with an IC_{50} against HIV-1 of 0.014 μM, was used as template for structural modulation focused mainly on the spirocyclopentene-ring pharmacophoric features although the modulation of the benzhydryl ester was also performed.

In order to understand the importance of benzhydryl ester at position 3 of the lead spirocyclopentenyl-β-lactam **1**, the molecule's benzhydryl group was removed, affording an analogue containing an unprotected carboxylic acid. The resultant molecule **18** showed no activity against HIV-1. The fact that the benzhydryl ester presence is vital for the anti-HIV activity of spirocyclopentenyl-β-lactam **1**, while the presence of the same group leads to loss of activity of the spiro-3*H*-pyrazole-β-lactam derivatives, suggests that the two types of spiro-β-lactams have different molecular targets and/or mechanisms of action.

The benzhydryl ester group of molecule **12**, the α-regioisomer of molecule **1**, was also the target of structural modulations. In agreement with the previously observed lack of anti-HIV-1 activity of spirocyclopentenyl-β-lactam **12**, the present work confirms that α-regioisomers **15a**-**d**, as well as their derivatives **19** and **20,** were not active against HIV-1.

The active spirocyclopentenyl-β-lactams **1** and **2** diverged on the substituent on position 2 of the spirocyclopentene ring, molecule **1** has a benzoyl group while molecule **2** has a methyl ester group. A new analogue **14d** containing a benzyl ester at the same position was synthesized and assayed for its anti-HIV-1 activity. The rational for the design of this molecule was to introduce a substituent combining the ester functionality with an aromatic feature. However, the molecule showed no activity against the virus. This can be explained considering that the benzyl ester is less conformationally restrained than the benzoyl group which may prevent a favourable interaction between the molecule and its potential target.

A 1,3-dipolar cycloaddition-based approach was applied in the synthesis of spirocyclic β-lactam **23**, an analogue of molecule **1** corresponding to the removal of the Michael acceptor system (see Scheme 10). However, this spirocyclic β-lactam proved to be inactive against HIV-1.

The benzyl ester substituent of spirocyclopentenyl-β-lactam **1** was also subjected to structural modulation. This group was replaced by a methyl, an ethyl and a *tert*-butyl ester, affording spirocyclopentenyl-β-lactams **14a**-**14c**. Spirocyclopentenyl-β-lactam **14c** containing a *tert*-butyl ester showed no activity. Interestingly, spirocyclopentenyl-β-lactams **14a** (with an ethyl ester) and **14b** (with a methyl ester) proved to be two highly active compounds (IC_{50} < 0.4 μM) against HIV-1.

Although the new active molecules **14a** and **14b** are slightly less active than molecule **1**, their IC_{50} values are still remarkable. These results suggest that the presence of the benzyl ester improves the molecule activity but this position also tolerates less hindered substituents, such as methyl or ethyl ester groups, without causing an abrupt or even complete loss of antiviral activity. On the contrary, the presence of a bulky *tert*-butyl ester substituent at the same position, cause a total loss of activity. Nevertheless, the fact that spiro-β-lactam **14b** displays an activity against HIV-1 of the same order of magnitude as lead molecule **1**, is a relevant achievement since the replacement of the benzyl group for a methyl group reduces the molecule hydrophobicity and aromatic content, turning spirocyclic β-lactam **14b** a more "medchem friendly" molecule than lead molecule **1** [35–37]. The fact that spiro-β-lactams **14a** and **14b** show a high structural resemblance with molecule **1** led us to speculate that they share the same anti-HIV mechanism of action.

Finally, the results concerning the activity against *Plasmodium* in both hepatic and blood stage of molecules **14a** and **14b** are particularly relevant. Two new spiro-β-lactams with potent activity against both malaria and HIV infectious agents have been identified which can be regarded a breakthrough in the fight against both infections. Interestingly, a direct correlation is observed between molecules' bioactivity against both hepatic and erythrocytic stage of *Plasmodium* (**1** > **14a** > **14b**) and the increasing of the chain length of the ester substituent at their spirocyclopentene ring (Bn > Et > Me).

2.3.2.1. In silico minimum energy conformations calculation. Quantum chemical calculations, at the DFT level of theory, were carried out to explore the structure and the preferred conformations of active spiro-β-lactams **14a** and **14b** by comparison with lead molecule **1**. As expected, due to the high structural resemblance between the three molecules, the conformational study confirmed a very similar conformation at their minimum energies (Fig. 7). This result also supports our expectation that molecules **14a** and **14b** share multi-stage and new anti-HIV mechanisms of action similar to those reported for spirocyclopentenyl-β-lactam **1** [18].

3. Conclusion

Structural modulation of lead compounds with antimicrobial activity against both HIV and *P. berghei* hepatic stage was performed, leading to the synthesis of twenty-three new spirocyclic β-lactams. These new molecules were assayed for its cytotoxicity and activity against HIV-1, providing relevant information concerning this class of anti-HIV agents' structure-activity relationship. Among the assayed molecules, two new spirocyclopentenyl-β-lactams were identified as having an outstanding activity against HIV-1 (IC_{50} < 0.4 μM). Remarkably, the same two molecules also showed promising antiplasmodial activity, inhibiting both the hepatic (IC_{50} < 2.3 μM) and blood (IC_{50} < 1.3 μM) stages of *Plasmodium* infection.

Altogether, our present results open avenues for new rational structural modifications aiming at new bioactive spirocyclic lactams and, above all, strengthen the potentiality of the spiro-β-lactams class as innovative and highly promising agents with dual activity against both HIV and *Plasmodium*.

4. Materials and methods

4.1. Chemistry

Thin-layer chromatography (TLC) analyses were performed using precoated silica gel plates. Flash column chromatography was performed with silica gel 60 as the stationary phase. ^{1}H Nuclear magnetic resonance (NMR) spectra (400 MHz) and ^{13}C NMR spectra (100 MHz) were recorded in $CDCl_3$, CD_3OD or hexadeuterated dimethylsulfoxide (DMSO-d_6). Chemical shifts are expressed in parts per million (ppm) relatively to internal tetramethylsilane (TMS) and coupling constants (*J*) are expressed in hertz (Hz). Infrared spectra (IR) were recorded in a Fourier Transform spectrometer coupled with a diamond Attenuated Total Reflectance (ATR) sampling accessory. Elemental analyses were carried out with an Elemental Vario Micro Cube analyser. High-resolution mass spectra (HRMS) were obtained on a TOF VG Autospect M spectrometer with electrospray ionization (ESI). Melting points (mp) were determined in open glass capillaries. Optical rotations were measured on an Optical Activity AA-5 electrical polarimeter. 6-Diazopenicillanate **4** [14], 6-alkylidenepenicillanates **10** [17] and **16** [16], diazomethane [38], allenoates **11** [39], and **17** [40], and diphenylmethyl propiolate **5d** [41] were prepared as described in the literature. Acetylene dicarboxylic acid, propiolates **5a-c,** ethyl-2-butynoate (**13a**), methyl-2-butynoate (**13b**) and benzyl diazoacetate (**22**) were purchased and used as such.

Dibenzhydryl but-2-ynedioate (**5e**). To a stirred solution of acetylene dicarboxylic acid (171 mg, 1.50 mmol) in methanol (2.5 mL) a solution of diphenyldiazomethane (583 mg, 3.00 mmol) in dichloromethane (10 mL) was added dropwise. After stirring for 24 h at room temperature, a second portion of diphenyldiazomethane (292 mg, 1.50 mmol) was added and the reaction mixture was stirred for another 20 h. The solvent was removed under reduced pressure. Purification by flash chromatography [ethyl

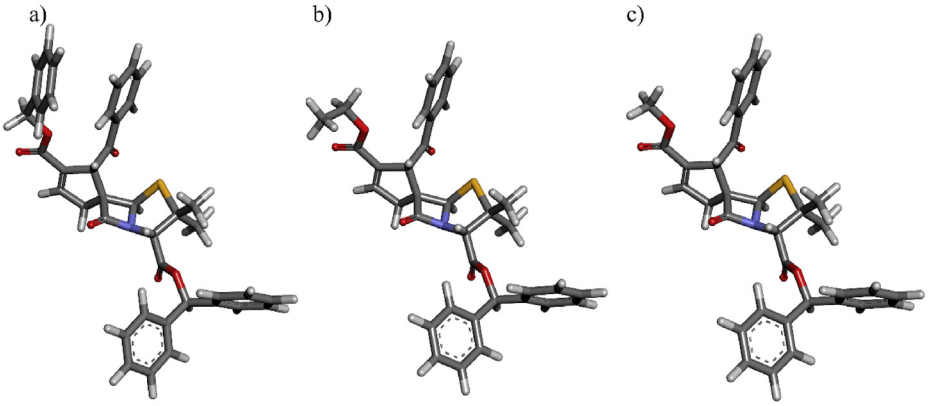


Fig. 7. Optimized geometries of bioactive spiro-β-lactam a) **1**, b) **14a** and c) **14b**.

acetate/hexane (1:9)] gave dibenzhydryl but-2-ynedioate **5e** as a colorless solid (317 mg, 1.13 mmol, 75%). ^{1}H NMR δ ($CDCl_3$): 6.97 (s, 2H), 7.30–7.36 (m, 20H); ^{13}C NMR δ ($CDCl_3$): 75.3, 79.9, 127.3, 128.6, 128.8, 138.6, 151.1.

General procedure for the 1,3-dipolar cycloaddition of 6-diazopenicillanate 4 with propiolates 5. A solution of the appropriate propiolate (1.5 equiv) in dry dichloromethane (2 mL) was added to a solution of 6-diazopenicillanate **4** (1 equiv) in dry dichloromethane (5 mL). The reaction mixture was stirred at 45 °C under nitrogen atmosphere, monitored by TLC. Upon completion, the solvent was removed under reduced pressure and the product was purified by flash chromatography [ethyl acetate/hexane] or by crystallization with a mixture of ethyl acetate/hexane.

Benzhydryl spiro[penicillanate-6,3'-(5-ethoxycarbonyl-3H-pyrazole)] (**6a**). Obtained from 6-diazopenicillanate **4** (939 mg, 2.39 mmol) and ethyl propiolate (**5a**) (351 mg, 3.58 mmol) as described in the general procedure (reaction time: 4 h). Purification by flash chromatography [ethyl acetate/hexane (1:3)] gave spiro-β-lactam **6a** as a fluffy yellow solid (524 mg, 1.07 mmol, 45%). mp 58.7–60.4 °C; $[\alpha]_D^{25} = +270$ (*c* 0.5 in CH_2Cl_2); IR (ATR) ν: 976, 1018, 1133, 1203, 1312, 1449, 1559, 1719 and 1780 cm^{-1}; ^{1}H NMR δ ($CDCl_3$): 1.33 (s, 3H), 1.41 (t, $J = 7.2$ Hz, 3H), 1.56 (s, 3H), 4.43 (q, $J = 7.2$ Hz, 2H), 4.81 (s, 1H, 3-H), 6.34 (s, 1H, 5-H), 6.85 (d, $J = 0.8$ Hz, 1H), 7.01 (s, 1H), 7.32–7.38 (m, 10H); ^{13}C NMR δ ($CDCl_3$): 14.4, 26.0, 32.1, 60.6, 62.0, 62.4, 69.2, 79.1, 104.5, 127.1, 127.9, 128.5, 128.7, 128.8, 128.9, 138.9, 139.0, 145.7, 149.7, 152.2, 161.5, 166.9; Anal. Calcd for $C_{26}H_{25}N_3O_5S$: C, 63.53; H, 5.13; N, 8.85; S, 6.52. Found: C, 63.91; H, 5.31; N, 8.49; S, 6.57.

Benzhydryl spiro[penicillanate-6,3'-(5-tert-butoxycarbonyl-3H-pyrazole)] (**6b**). Obtained from 6-diazopenicillanate **4** (469 mg, 1.19 mmol) and *tert*-butyl propiolate (**5b**) (226 mg, 1.79 mmol) as described in the general procedure (reaction time: 6 h). Purification by flash chromatography [ethyl acetate/hexane (1:6)] gave spiro-β-lactam **6b** as a colorless solid (203 mg, 0.39 mmol, 33%). mp 156.5–159.0 °C; $[\alpha]_D^{25} = +260$ (*c* 0.5 in CH_2Cl_2); IR (ATR) ν: 977, 1026, 1153, 1208, 1243, 1317, 1449, 1559, 1711, 1735 and 1772 cm^{-1}; ^{1}H NMR δ ($CDCl_3$): 1.32 (s, 3H), 1.55 (s, 3), 1.61 (s, 9H), 4.82 (s, 1H), 6.33 (s, 1H), 6.79 (d, $J = 0.4$ Hz, 1H), 7.00 (s, 1H), 7.31–7.38 (m, 10H); ^{13}C NMR δ ($CDCl_3$): 26.0, 28.3, 32.2, 60.6, 62.3, 69.2, 79.1, 83.1, 104.4, 127.1, 127.8, 128.5, 128.7, 128.8, 128.8, 138.9, 139.0, 145.6, 150.0, 153.7, 160.7, 167.0; HRMS (ESI-TOF) *m/z*: $[M+H]^+$ Calcd for $C_{28}H_{30}N_3O_5S$ 520.1901; Found 520.1902.

Benzhydryl spiro[penicillanate-6,3'-(5-(2-naphthoxy)carbonyl-3H-pyrazole)] (**6c**). Obtained from 6-diazopenicillanate **4** (295 mg, 0.75 mmol) and 2-naphthyl propiolate (**8c**) (220 mg, 1.12 mmol) as described in the general procedure (reaction time: 3 h). The solvent was removed under reduced pressure and a mixture of ethyl acetate/hexane (1:3) was added and left at − 5 °C overnight. The product precipitates and was filtered, affording the spiro-β-lactam **6c** as a colorless solid (237 mg, 0.40 mmol, 54%). mp 210.5–212.3 °C; $[\alpha]_D^{25} = +270$ (*c* 0.5 in CH_2Cl_2); IR (ATR) ν: 978, 1074, 1183, 1210, 1331, 1457, 1576, 1731, 1758 and 1776 cm^{-1}; ^{1}H NMR δ ($CDCl_3$): 1.36 (s, 3H), 1.61 (s, 3), 4.86 (s, 1H), 6.41 (s, 1H), 7.03 (d, $J = 0.8$ Hz, 1H), 7.04 (s, 1H), 7.31–7.40 (m, 11H), 7.47–7.54 (m, 2H, Ar), 7.72 (d, $J = 2.4$ Hz, 1H), 7.83–7.89 (m, 2H), 7.90 (d, $J = 9.2$ Hz, 1H); ^{13}C NMR δ ($CDCl_3$): 26.0, 32.1, 60.6, 62.5, 69.2, 79.2, 105.1, 118.8, 121.0, 126.1, 126.8, 127.1, 127.9, 127.9, 128.5, 128.7, 128.8, 128.9, 129.7, 131.8, 133.9, 138.9, 139.0, 146.0, 148.1, 149.6, 151.4, 160.11, 166.9; Anal. Calcd for $C_{34}H_{27}N_3O_5S$: C, 69.25; H, 4.62; N, 7.13; S, 5.44. Found: C, 69.50; H, 4.36; N, 7.22; S, 5.30.

Benzhydryl spiro[penicillanate-6,3'-(5-benzhydryloxycarbonyl-3H-pyrazole)] (**6d**). Obtained from 6-diazopenicillanate **4** (751 mg, 1.91 mmol) and benzhydryl propiolate (**5d**) (350 mg, 1.48 mmol) as described in the general procedure (reaction time: 3 h). Purification by flash chromatography [ethyl acetate/hexane (1:4)] gave spiro-β-lactam **6d** as a fluffy colorless solid (691 mg, 1.10 mmol, 74%). mp 82.5–84.3 °C; $[\alpha]_D^{25} = +230$ (*c* 0.5 in CH_2Cl_2); IR (ATR) ν: 982, 1096, 1176, 1309, 1449, 1494, 1724 and 1781 cm^{-1}; ^{1}H NMR δ ($CDCl_3$): 1.33 (s, 3H), 1.56 (s, 3H), 4.82 (s, 1H), 6.34 (s, 1H), 6.89 (d, $J = 0.8$ Hz, 1H), 7.01 (s, 1H), 7.15 (s, 1H), 7.29–7.38 (m, 16H), 7.44–7.46 (m, 4H); ^{13}C NMR δ ($CDCl_3$): 26.0, 32.2, 60.5, 62.4, 69.2, 78.3, 79.1, 104.8, 127.1, 127.4, 127.8, 128.3, 128.5, 128.7, 128.8, 128.9, 138.9, 139.0, 139.7, 145.8, 149.9, 152.0, 160.6, 166.9; Anal. Calcd for $C_{37}H_{31}N_3O_5S$: C, 70.57; H, 4.96; N, 6.67; S, 5.09. Found: C, 70.14; H, 4.96; N, 6.48; S, 4.82.

Benzhydryl spiro[penicillanate-6,3'-(4,5-dibenzhydryloxycarbonyl-3H-pyrazole)] (**6e**). Obtained from 6-diazopenicillanate **4** (751 mg, 1.91 mmol) and dibenzhydryl but-2-ynedioate (**5e**) (592 mg, 2.46 mmol) as described in the general procedure (reaction time: 2 h). Purification by flash chromatography [ethyl acetate/hexane (1:4)] gave spiro-β-lactam **6e** as a fluffy yellow solid (574 mg, 0.68 mmol, 36%). mp 87.4–88.3 °C; $[\alpha]_D^{25} = +160$ (*c* 0.5 in CH_2Cl_2); IR (ATR) ν: 946, 1063, 1131, 1175, 1290, 1449, 1585, 1735 and 1789 cm^{-1}; 1H NMR δ ($CDCl_3$): 1.30 (s, 3), 1.55 (s, 3), 4.80 (s, 1H), 6.42 (s, 1H), 7.00 (s, 1H), 7.02 (s, 1H), 7.12 (s, 1H), 7.27–7.37 (m, 26H), 7.40–7.43 (m, 4H); ^{13}C NMR δ ($CDCl_3$): 25.9, 32.3, 61.3, 62.1, 69.5, 78.7, 79.2, 79.3, 110.7, 127.1, 127.3, 127.4, 127.8, 128.2, 128.3, 128.5, 128.7, 128.7, 128.8, 128.9, 138.9, 139.5, 139.5, 139.6, 149.3, 150.1, 151.0, 158.9, 159.9, 166.5; Anal. Calcd for $C_{51}H_{41}N_3O_7S$: C, 72.93; H, 4.92; N, 5.00; S, 3.82. Found: C, 72.84; H, 4.79; N, 4.89; S, 3.71.

General procedure for the benzhydryl ester deprotection. Anisole (7 equiv) and TFA (25 equiv) were added to a solution of the appropriate spiro-3*H*-pyrazole-β-lactam **6a-d** or spiro-β-lactam **1** (1 equiv) in dry dichloromethane (2 mL). The reaction mixture was stirred at −5 °C under nitrogen atmosphere for 5 h. After this time, the mixture was diluted with cold diethyl ether (10 mL) and the solvent was evaporated. The co-evaporation with diethyl ether was repeated five times. The residue was dissolved in ethyl acetate (5 mL), a saturated solution of $NaHCO_3$ (15 mL) was added and the mixture was stirred for 15 min at 0 °C. The mixture was partitioned between water (5 mL) and ethyl acetate (20 mL). The two layers were separated and the aqueous layer was extracted with ethyl acetate (2 x 20 mL). The aqueous layer was acidified to pH 3 in an ice bath with HCl 1 N and extracted with ethyl acetate (3 x 20 mL). The combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure to give the target acid.

Spiro[penicillanic-6,3'-(5-ethoxycarbonyl-3H-pyrazole)] acid (**7a**). Obtained from spiro-3*H*-pyrazole-β-lactam **6a** (196 mg, 0.40 mmol) as a low melting point yellow solid (120 mg, 0.37 mmol, 93%). $[\alpha]_D^{25} = +320$ (*c* 0.25 in acetone); IR (ATR) ν: 976, 1013, 1134, 1203, 1312, 1370, 1457, 1559, 1719, 1777, 2974 and 3148 cm^{-1}; ^{1}H NMR δ ($CDCl_3$): 1.41 (t, $J = 7.2$ Hz, 3H), 1.64 (s, 3H), 1.68 (s, 3H), 4.44 (q, $J = 7.2$ Hz, 2H), 4.72 (s, 1H), 6.33 (s, 1H), 6.87 (s, 1H); ^{13}C NMR δ (DMSO-d_6): 14.1, 25.7, 30.3, 60.0, 61.1, 61.2, 68.8, 104.7, 146.1, 149.6, 150.8, 160.9, 168.7; HRMS (ESI-TOF) *m/z*: $[M+H]^+$ Calcd for $C_{21}H_{18}N_3O_5S$ 326.0818; Found 326.0805.

Spiro[penicillanic-6,3'-(5-tert-butoxycarbonyl-3H-pyrazole)] acid (**7b**). Obtained from spiro-3*H*-pyrazole-β-lactam **6b** (252 mg, 0.48 mmol) as a light brown solid (139 mg, 0.37 mmol, 81%). mp 139.2–142.0 °C; $[\alpha]_D^{25} = +330$ (*c* 0.35 in acetone); IR (ATR) ν: 979, 1134, 1153, 1210, 1313, 1368, 1457, 1559, 1713, 1773, 2933 and 2970 cm^{-1}; ^{1}H NMR δ ($CDCl_3$): 1.61 (s, 9H), 1.62 (s, 3H), 1.66 (s, 3H), 4.73 (s, 1H), 6.32 (s, 1H), 6.80 (d, $J = 0.8$ Hz, 1H); ^{13}C NMR δ ($CDCl_3$): 26.3, 28.3, 31.7, 60.5, 61.9, 69.0, 83.3, 104.5, 145.4,150.0, 153.6, 160.7, 171.4; HRMS (ESI-TOF) *m/z*: $[M+H]^+$ Calcd for $C_{15}H_{20}N_3O_5S$ 354.1118; Found 354.1131.

Spiro[penicillanic-6,3'-(5-(2-naphthoxy)carbonyl-3H-pyrazole)]

acid (**7c**). Obtained from spiro-3*H*-pyrazole-β-lactam **6c** (237 mg, 0.40 mmol). Compound **7c** was crystalized by the addition of cold diethyl ether and obtained as a colorless solid (144 mg, 0.34 mmol, 86%). mp > 209.5 °C (with decomposition); $[\alpha]_D^{25} = +340$ (*c* 0.25 in CH_2Cl_2); IR (ATR) *ν*: 977, 1069, 1146, 1180, 1322, 1369, 1449, 1507, 1601, 1722, 1773, 2480 and 3197 cm^{-1}; ^1H NMR *δ* (DMSO-d_6): 1.57 (s, 3H), 1.60 (s, 3H), 4.63 (s, 1H), 6.64 (s, 1H), 7.25 (d, *J* = 0.4 Hz, 1H), 7.50 (dd, *J* = 9.2 and 2.4 Hz, 1H) 7.54–7.61 (m, 2H), 7.87 (d, *J* = 2.4 Hz, 1H), 7.95–8.01 (m, 2H), 8.04 (d, *J* = 8.8 Hz, 1H); ^{13}C NMR *δ* (DMSO-d_6): 25.7, 30.5, 60.1, 61.1, 68.9, 105.5, 118.7, 121.3, 126.1, 126.9, 127.6, 127.8, 129.6, 131.2, 133.3, 146.5, 147.7, 149.5, 149.9, 159.7, 168.7; HRMS (ESI-TOF) *m/z*: $[M+H]^+$ Calcd for $C_{13}H_{16}N_3O_5S$ 424.0962; Found 424.0980.

Spiro[penicillanic-6,3'-(5-hydroxycarbonyl-3H-pyrazole)] acid (**7d**). Obtained from spiro-3*H*-pyrazole-β-lactam **6d** (315 mg, 0.50 mmol) as a low melting point light brown solid (134 mg, 0.45 mmol, 90%). $[\alpha]_D^{25} = +300$ (*c* 0.25 in acetone); IR (ATR) *ν*: 977, 1133, 1188, 1312, 1373, 1465, 1559, 1719, 1773, 2935 and 2972 cm^{-1}; ^1H NMR *δ* (DMSO-d_6): 1.55 (s, 3H), 1.57 (s, 3H), 4.56 (s, 1H), 6.55 (s, 1H), 6.95 (d, *J* = 0.4 Hz, 1H); ^{13}C NMR *δ* (DMSO-d_6): 25.8, 30.4, 60.0, 61.1, 68.9, 104.7, 146.0, 149.8, 152.0, 162.4, 168.8; HRMS (ESI-TOF) *m/z*: $[M+H]^+$ Calcd for $C_{11}H_{12}N_3O_5S$ 298.0492; Found 298.0499.

(1'R,6R)-Spiro[penicillanic-6,1'-(2-benzoyl-3-benzyloxycarbonyl(cyclopent-3-enyl))] acid (**18**). Obtained from spiro-β-lactam **1** (0.25 g, 0.38 mmol) as a low melting point brown solid (111 mg, 0.23 mmol, 59%). $[\alpha]_D^{25} = +350$ (*c* 0.5 in CH_2Cl_2); IR (ATR) *ν*: 1000, 1063, 1104, 1200, 1330, 1371, 1448, 1667, 1711 and 2973 cm^{-1}; ^1H NMR *δ* ($CDCl_3$): 1.42 (s, 3H), 1.54 (s, 3H), 3.13 (dd, *J* = 18.4 and 3.2 Hz, 1H), 3.55 (dt, *J* = 18.8 and 2.0 Hz, 1H), 4.42 (s, 1H), 4.94 (d, *J* = 12.2 Hz, 2H), 4.98 (d, *J* = 12.2 Hz, 2H), 5.17 (d, *J* = 0.8 Hz, 1H), 5.39 (s, 1H), 7.06 (br s, 1H), 7.12–7.14 (m, 2H) 7.27–7.29 (m, 3H), 7.38–7.42 (m, 2H), 7.53–7.57 (m, 1H), 8.05–8.07 (m, 2H); ^{13}C NMR *δ* ($CDCl_3$): 26.6, 31.3, 40.8, 52.9, 63.9, 66.8, 69.0, 70.1, 70.7, 128.4, 128.5, 128.5, 128.6, 129.5, 133.7, 135.4, 135.8, 137.2, 145.9, 162.9, 171.9, 176.9, 201.1; HRMS (ESI-TOF) *m/z*: $[M+H]^+$ Calcd for $C_{27}H_{26}NO_6S$ 492.1475; Found 492.1460.

Methyl spiro[penicillanate-6,3'-(5-methoxycarbonyl-3H-pyrazole)] (**8**). Ethereal diazomethane was added dropwise, in excess, to a solution of spiro-β-lactam **3** (100 mg, 0.32 mmol) in dichloromethane (5 mL) at 0 °C. The reaction mixture was manually stirred and monitored by TLC. Excess of diazomethane was purged with nitrogen and the product was purified by flash chromatography [ethyl acetate/hexane (2:3)]. Spiro-β-lactam **8** was obtained as a low melting point colorless solid (71 mg, 0.22 mmol, 68%). $[\alpha]_D^{25} = +390$ (*c* 0.5 in CH_2Cl_2); IR (ATR) *ν*: 988, 1029, 1134, 1203, 1311, 1363, 1458, 1559, 1722, 1777 and 2957 cm^{-1}; ^1H NMR *δ* ($CDCl_3$): 1.54 (s, 3H), 1.58 (s, 3H), 3.83 (s, 3H), 3.94 (s, 3H), 4.69 (s, 1H), 6.35 (s, 1H), 6.85 (s, 1H); ^{13}C NMR *δ* ($CDCl_3$): 26.3, 31.9, 52.7, 52.8, 60.4, 62.1, 69.1, 104.4, 145.6, 149.5, 151.7, 161.9, 168.2; HRMS (ESI-TOF) *m/z*: $[M+H]^+$ Calcd for $C_{13}H_{16}N_3O_5S$ 326.0805; Found 326.0808.

(2S,5R)-Methyl 2-(hydroxycarbamoyl)-3,3-dimethyl-7-oxo-4-thia-1-azaspiro[bicyclo[3.2.0]heptane-6,3'-pyrazole]-5'-carboxylate (**9**). Ethyl chloroformate (49 mg, 0.46 mmol) and *N*-methylmorpholine (NMM) (50 mg, 0.49 mmol) were added to a solution of spiro-β-lactam **3** (118 mg, 0.38 mmol) in diethyl ether (10 mL) at 0 °C. The reaction was stirred for 20 min. The precipitate solid was filtered and the filtrate was added to freshly prepared hydroxylamine [42] (19 mg, 0.57 mmol) in methanol (4.5 mL). The reaction mixture was stirred at room temperature for 15 min. The solvent was evaporated off, and the residue was purified by flash chromatography with ethyl acetate/hexane (2:1) and ethyl acetate/methanol (9:1). Spiro-β-lactam **9** was obtained as a colorless solid (42 mg, 0.13 mmol, 34%). mp > 190.2 °C (with decomposition); $[\alpha]_D^{25} = +200$ (*c* 0.25 in acetone); IR (ATR) *ν*: 996, 1238, 1327, 1361, 1576, 1654, 1681, 1726, 1768, 2954, 3185 and 3284 cm^{-1}; ^1H NMR *δ* (CD_3OD): 1.62 (s, 3H), 1.63 (s, 3H), 3.94 (s, 3H), 4.44 (s, 1H), 6.47 (s, 1H), 6.95 (s, 1H); ^{13}C NMR *δ* (CD_3OD): 26.1, 31.0, 53.0, 61.7, 62.9, 69.5, 105.3, 147.6, 151.5, 152.6, 163.1, 166.3; HRMS (ESI-TOF) *m/z*: $[M+H]^+$ Calcd for $C_{12}H_{15}N_4O_5S$ 327.0758; Found 327.0758.

General procedure for the phosphane-catalyzed [3+2] annulation of 2-butynoates 13 with 6-alkylidenepenicillanate 10. A solution of the appropriate butynoate **13** (0.94 mmol) in toluene (2 mL) was added to a solution of 6-alkylidenepenicillanate **10** (75 mg, 0.16 mmol) and PPh_3 (98 mg, 0.37 mmol) in toluene (3 mL). The reaction mixture was stirred at room temperature under nitrogen for 48 h. Upon completion, the solvent was removed under reduced pressure and the crude product was purified by flash chromatography [ethyl acetate/hexane].

(1'R,6R)-Benzhydryl spiro[penicillanate-6,1'-(2-benzoyl-3-ethoxycarbonyl(cyclopent-3-enyl))] (**14a**) and *(1'R,6R)-Benzhydryl spiro[penicillanate-6,1'-(2-benzoyl-5-ethoxycarbonyl(cyclopent-4-enyl))]* (**15a**). Obtained from ethyl 2-butynoate (**13a**) (104 mg, 0.94 mmol) and 6-alkylidenepenicillanate **10** (75 mg, 0.16 mmol) as described in the general procedure. Purification of the crude product by flash chromatography [(ethyl acetate/hexane (1:4) and (1:3)] gave, in order of elution, **14a** as a low melting point yellow solid (49 mg, 0.08 mmol, 53%) and **15a** as a low melting point colorless solid (37 mg, 0.06 mmol, 40%).

Data for compound **14a**: $[\alpha]_D^{25} = +340$ (*c* 0.5 in CH_2Cl_2); IR (ATR) *ν*: 986, 1103, 1175, 1236, 1368, 1448, 1669, 1710, 1740 and 1773 cm^{-1}; ^1H NMR *δ* ($CDCl_3$): 1.01 (t, *J* = 7.2 Hz, 3H) 1.12 (s, 3H), 1.53 (s, 3H), 3.11 (dd, *J* = 18.4 and 3.2 Hz, 1H), 3.53 (dt, *J* = 18.4 and 2.4 Hz, 1H), 3.92–4.03 (m, 2H), 4.54 (s, 1H), 5.15 (br s, 1H), 5.44 (s, 1H), 6.92 (s, 1H), 7.02 (br s, 1H), 7.27–7.36 (m, 10H), 7.45–7.49 (m, 2H), 7.56–7.60 (m, 1H), 8.08–8.10 (m, 2H); ^{13}C NMR *δ* ($CDCl_3$): 13.9, 26.1, 32.6, 40.7, 52.9, 60.8, 64.2, 69.1, 70.7, 71.2, 78.4, 127.1, 127.7, 128.3, 128.4, 128.5, 128.7, 128.7, 129.4, 133.6, 136.6, 137.7, 139.2, 139.3, 145.3, 163.0, 167.0, 176.5, 201.4; Anal. Calcd for $C_{35}H_{33}NO_6S$: C, 70.57; H, 5.58; N, 2.35; S, 5.38. Found: C, 70.58; H, 5.99; N, 2.61; S, 5.01.

Data for compound **15a**: $[\alpha]_D^{25} = +400$ (*c* 0.25 in CH_2Cl_2); IR (ATR) *ν*: 974, 1041, 1121, 1152, 1177, 1200, 1263, 1306, 1333, 1449, 1667, 1724, 1741 and 1772 cm^{-1}; ^1H NMR *δ* ($CDCl_3$): 1.13 (s, 3H), 1.26 (t, *J* = 7.2Hz, 3H) 1.52 (s, 3H), 2.51 (dd, *J* = 18.8 and 2.4 Hz, 1H), 3.18 (ddd, *J* = 18.8, 9.2 and 2.0 Hz, 1H), 4.22 (q, *J* = 7.2 Hz, 2H), 4.54–4.56 (m, 1H), 4.55 (s, 1H), 6.27 (s, 1H), 6.88 (dd, *J* = 2.8 and 2.0 Hz, 1H), 6.95 (s, 1H), 7.28–7.36 (m, 6H), 7.40–7.51 (m, 6H), 7.58–7.62 (m, 1H), 7.93–7.95 (m, 2H); ^{13}C NMR *δ* ($CDCl_3$): 14.2, 25.9, 32.9, 35.9, 49.5, 60.9, 62.6, 69.1, 71.2, 74.0, 78.3, 127.4, 127.5, 128.2, 128.2, 128.6, 128.6, 128.7, 129.0, 133.6, 134.8, 135.2, 139.6, 139.6, 144.2, 162.8, 166.8, 174.5, 198.4; HRMS (ESI-TOF) *m/z*: $[M+H]^+$ Calcd for $C_{35}H_{34}NO_6S$ 596.2101; Found 596.2123.

(1'R,6R)-Benzhydryl spiro[penicillanate-6,1'-(2-benzoyl-3-methoxycarbonyl(cyclopent-3-enyl))] (**14b**) and *(1'R,6R)-Benzhydryl spiro[penicillanate-6,1'-(2-benzoyl-5-methoxycarbonyl(cyclopent-4-enyl))]* (**15b**). Obtained from methyl 2-butynoate (**13b**) (91 mg, 0.94 mmol) and 6-alkylidenepenicillanate **10** (75 mg, 0.16 mmol) as described in the general procedure. Purification of the crude product by flash chromatography [(ethyl acetate/hexane (1:4)] gave, in order of elution, **14b** as a fluffy colorless solid (23 mg, 0.04 mmol, 25%) and **15b** as a colorless solid (44 mg, 0.08 mmol, 49%).

Data for compound **14b**: mp 92.2–94.0 °C; $[\alpha]_D^{25} = +390$ (*c* 0.5 in CH_2Cl_2); IR (ATR) *ν*: 954, 1000, 1107, 1203, 1284, 1434, 1448, 1665, 1715, 1740 and 1771 cm^{-1}; ^1H NMR *δ* ($CDCl_3$): 1.12 (s, 3H), 1.52 (s, 3H), 3.11 (dd, *J* = 18.4 and 2.8 Hz, 1H), 3.51–3.57 (m, 1H), 3.53 (s, 3H), 4.53 (s, 1H), 5.16 (dd, *J* = 2.4 and 1.2 Hz, 1H), 5.44 (s, 1H), 6.92 (s,

1H), 7.01 (br s, 1H), 7.27–7.35 (m, 10H), 7.46–7.50 (m, 2H), 7.57–7.60 (m, 1H), 8.09–8.10 (m, 2H); ^{13}C NMR δ ($CDCl_3$): 26.1, 32.6, 40.7, 51.7, 53.1, 64.2, 69.1, 70.7, 71.2, 78.4, 127.1, 127.7, 128.3, 128.4, 128.5, 128.7, 129.4, 133.6, 135.9, 137.5, 139.2, 139.3, 145.5, 163.4, 167.0, 176.5, 201.2; Anal. Calcd for $C_{34}H_{31}NO_6S$: C, 70.20; H, 5.37; N, 2.41; S, 5.51. Found: C, 70.54; H, 5.69; N, 2.40; S, 5.62.

Data for compound **15b**: mp 163.4–165.4 °C; $[\alpha]_D^{25} = +470$ (*c* 0.5 in CH_2Cl_2); IR (ATR) ν: 976, 1021, 1040, 1126, 1178, 1200, 1265, 1306, 1336, 1434, 1449, 1665, 1729 and 1772; ^{1}H NMR δ ($CDCl_3$): 1.13 (s, 3H), 1.52 (s, 3H), 2.52 (dd, *J* = 18.4 and 2.0 Hz, 1H), 3.19 (ddd, *J* = 18.4, 9.2 and 2.0 Hz, 1H), 3.70 (s, 3H), 4.55–4.57 (m, 1H), 4.55 (s, 1H), 6.26 (s, 1H), 6.88 (dd, *J* = 2.8 and 2.4 Hz, 1H), 6.96 (s, 1H), 7.27–7.37 (m, 6H), 7.41–7.51 (m, 6H), 7.59–7.62 (m, 1H), 7.93–7.95 (m, 2H); ^{13}C NMR δ ($CDCl_3$): 25.9, 33.1, 35.9, 49.5, 51.8, 62.7, 69.1, 71.2, 74.0, 78.4, 127.4, 127.5, 128.2, 128.6, 128.6, 128.7, 129.1, 133.7, 134.5, 135.2, 139.6, 144.6, 163.2, 166.9, 174.3, 198.4; Anal. Calcd for $C_{34}H_{31}NO_6S$: C, 70.20; H, 5.37; N, 2.41; S, 5.51. Found: C, 70.35; H, 5.67; N, 2.39; S, 5.60.

General procedure for the phosphane-catalyzed [3+2] annulation of allenoates with 6-alkylidenepenicillanates. A solution of the appropriate allenoate (0.50 mmol) in toluene (2 mL) was added to a solution of the appropriate 6-alkylidenepenicillanate (0.50 mmol) and PPh_3 (20 mol%) in toluene (3 mL). The reaction mixture was stirred at room temperature under nitrogen for 5 h. Upon completion, the solvent was removed under reduced pressure and the crude product was purified by flash chromatography [ethyl acetate/hexane].

(1′R,6R)-Benzhydryl spiro[penicillanate-6,1′-(2-benzoyl-3-tert-butoxycarbonyl(cyclopent-3-enyl))] (**14c**) and *(1′R,6R)-Benzhydryl spiro[penicillanate-6,1′-(2-benzoyl-5-tert-butoxycarbonyl(cyclopent-4-enyl))]* (**15c**). Obtained from *tert*-butyl 2,3-butadienoate (**17**) (70 mg, 0.50 mmol) and 6-alkylidenepenicillanate **10** (241 mg, 0.50 mmol) as described in the general procedure. Purification of the crude product by flash chromatography [(ethyl acetate/hexane (1:7) and (1:6)] gave, in order of elution, **14c** as a colorless solid (186 mg, 0.30 mmol, 57%) and **15c** as a colorless solid (83 mg, 0.13 mmol, 27%).

Data for compound **14c**: mp > 192.0 °C (with decomposition); $[\alpha]_D^{25} = +320$ (*c* 0.5 in CH_2Cl_2); IR (ATR) ν: 997, 1109, 1174, 1244, 1287, 1344, 1667, 1748 and 1769 cm^{-1}; ^{1}H NMR δ ($CDCl_3$): 1.12 (s, 3H), 1.21 (s, 9H), 1.54 (s, 3H), 3.09 (dd, *J* = 18.4 and 3.2 Hz, 1H), 3.48 (dt, *J* = 18.4 and 2.4 Hz, 1H), 4.53 (s, 1H), 5.10 (d, *J* = 1.2 Hz, 1H), 5.42 (s, 1H), 6.91–6.92 (m, 2H), 7.27–7.35 (m, 10H), 7.44–7.48 (m, 2H), 7.55–7.59 (m, 1H), 8.04–8.06 (m, 2H); ^{13}C NMR δ ($CDCl_3$): 26.1, 27.9, 31.1, 32.6, 40.7, 53.0, 64.2, 69.1, 70.7, 71.3, 78.4, 81.5, 127.1, 127.7, 128.3, 128.5, 128.5, 128.7, 128.8, 129.4, 133.4, 137.7, 138.0, 139.2, 139.3, 144.2, 162.3, 167.0, 176.6, 201.7; Anal. Calcd for $C_{37}H_{37}NO_6S$: C, 71.24; H, 5.98; N, 2.21; S, 5.14. Found: C, 71.51; H, 6.09; N, 2.25; S, 5.12.

Data for compound **15c**: mp 84.0–86.1 °C; $[\alpha]_D^{25} = +450$ (*c* 0.5 in CH_2Cl_2); IR (ATR) ν: 980, 1018, 1126, 1173, 1210, 1254, 1311, 1448, 1676, 1707 and 1763 cm^{-1}; ^{1}H NMR δ ($CDCl_3$): 1.15 (s, 3H), 1.48 (s, 9H), 1.51 (s, 3H), 2.47 (dd, *J* = 18.4 and 2.4 Hz, 1H), 3.15 (ddd, *J* = 18.4, 9.2 and 2.0 Hz, 1H), 4.53–4.55 (m, 1H), 4.54 (s, 1H), 6.23 (s, 1H), 6.78 (dd, *J* = 3.2 and 2.4 Hz, 1H), 6.94 (s, 1H), 7.27–7.35 (m, 6H), 7.41–7.51 (m, 6H), 7.58–7.62 (m, 1H), 7.93–7.95 (m, 2H); ^{13}C NMR δ ($CDCl_3$): 25.9, 28.2, 32.8, 35.6, 49.8, 62.4, 69.1, 71.3, 74.0, 78.4, 81.7, 127.4, 127.4, 128.2, 128.2, 128.6, 128.7, 128.7, 129.0, 133.6, 135.3, 136.2, 139.7, 143.4, 162.1, 166.9, 174.9, 198.4; Anal. Calcd for $C_{37}H_{37}NO_6S$: C, 71.24; H, 5.98; N, 2.21; S, 5.14. Found: C, 71.51; H, 6.07; N, 2.24; S, 4.94.

(1′R,6R)-Benzhydryl spiro[penicillanate-6,1′-(2,3-dibenzyloxycarbonyl(cyclopent-3-enyl))] (**14d**) and *(1′R,6R)-Benzhydryl spiro[penicillanate-6,1′-(2,5-dibenzyloxycarbonyl(cyclopent-4-enyl))]* (**15d**). Obtained from benzyl 2,3-butadienoate (**11**) (87 mg, 0.50 mmol) and 6-alkylidenepenicillanate **16** (256 mg, 0.50 mmol) as described in the general procedure. Purification of the crude product by flash chromatography [(ethyl acetate/hexane (1:6)] gave a mixture of regioisomers **14d/15d** in 64% overall yield. A small portion of **14d** was isolated pure as a low melting point colorless solid (36 mg) and fully characterized, however compound **15d** could not be isolated in its pure form.

Data for compound **14d**: $[\alpha]_D^{25} = +200$ (*c* 1 in CH_2Cl_2); IR (ATR) ν: 984, 1080, 1103, 1165, 1200, 1236, 1329, 1374, 1455, 1496, 1636, 1719, 1774, 2965 and 3032 cm^{-1}; ^{1}H NMR δ ($CDCl_3$): 1.20 (s, 3H), 1.55 (s, 3H), 3.03 (dd, *J* = 18.4 and 2.8 Hz, 1H), 3.29 (dt, *J* = 18.4 and 2.4 Hz, 1H), 4.10–4.12 (m, 1H), 4.52 (s, 1H), 5.06 (d, *J* = 12.4 Hz, 1H), 5.12–5.15 (m, 3H), 5.39 (s, 1H), 6.92 (s, 1H), 7.00 (br s, 1H), 7.29–7.36 (m, 20H); ^{13}C NMR δ ($CDCl_3$): 26.1, 33.0, 40.1, 52.5, 63.6, 66.7, 67.4, 68.1, 68.8, 71.0, 78.5, 127.1, 127.7, 128.3, 128.4, 128.5, 128.5, 128.6, 128.7, 128.8, 134.4, 135.5, 135.7, 139.2, 139.3, 145.4, 162.6, 167.0, 171.1, 175.4; HRMS (ESI-TOF) *m/z*: $[M+H]^+$ Calcd for $C_{41}H_{38}NO_7S$ 688.2363; Found 688.2345.

Data for compound **15d:** ^{1}H NMR δ ($CDCl_3$): 1.16 (s, 3H), 1.55 (s, 3H), 2.59 (dd, *J* = 18.8 and 2.4 Hz, 1H), 3.01–3.12 (m, 1H), 3.59 (d, *J* = 7.6 Hz, 1H), 4.55 (s, 1H), 5.04–5.21 (m, 4H), 6.15 (s, 1H), 6.93 (s, 1H), 6.98–7.00 (s, 1H), 7.27–7.36 (m, 16H), 7.40–7.46 (m, 4H).

(1′R,6R)-Sodium spiro[penicillanate-6,1′-(2-benzoyl-5-benzyloxycarbonyl(cyclopent-4-enyl))] (**19**). Anisole (0.58 g, 5.32 mmol) and TFA (2.17 g, 19.02 mmol) were added to a solution of spirocyclopentenyl-β-lactam **12** (0.50 g, 0.76 mmol) in dry dichloromethane (8 mL). The reaction mixture was stirred at −5 °C under nitrogen atmosphere for 5 h. The mixture was diluted with cold diethyl ether (10 mL) and the solvent was removed under reduced pressure. The co-evaporation with diethyl ether was repeated five times. The residue was dissolved in ethyl acetate (5 mL), a saturated solution of $NaHCO_3$ (15 mL) was added and the mixture was stirred for 15 min at 0 °C. The mixture was partitioned between water (5 mL) and ethyl acetate (20 mL). The product precipitated in the interphase of the two layers, was filtered and washed sequentially with deionized water and diethyl ether. Compound **19** was obtained as a colorless solid (124 mg, 0.24 mmol, 32%). mp > 175.0 °C (with decomposition); $[\alpha]_D^{25} = +360$ (*c* 0.25 in THF); IR (ATR) ν: 949, 1023, 1043, 1136, 1214, 1267, 1337, 1402, 1454, 1591, 1669, 1721, 1749 and 2970 cm^{-1}; ^{1}H NMR δ (DMSO-d_6): 1.34 (s, 3H), 1.38 (s, 3H), 2.32 (dd, *J* = 18.8 and 2.8 Hz, 1H), 3.06 (dd, *J* = 18.0 and 9.6 Hz, 1H), 3.90 (s, 1H), 4.54 (d, *J* = 8.8 Hz, 1H) 5.20 (d, *J* = 12.6 Hz, 1H), 5.25 (d, *J* = 12.6 Hz, 1H), 5.91 (s, 1H), 6.85 (br s, 1H), 7.32–7.41 (m, 3H), 7.48–7.55 (m, 4H), 7.63–7.66 (m, 1H), 7.99 (d, *J* = 7.6 Hz, 2H); ^{13}C NMR δ (DMSO-d_6): 26.5, 30.7, 33.1, 35.5, 49.0, 61.9, 65.7, 69.7, 72.5, 72.7, 128.1, 128.3, 128.4, 128.5, 128.9, 133.4, 134.0, 134.9, 136.0, 145.0, 162.4, 169.6, 172.7, 198.4; HRMS (ESI-TOF) *m/z*: $[M+H]^+$ Calcd for $C_{27}H_{25}NNaO_6S$ 514.1295; Found 514.1298.

(1′R,6R)-Methyl spiro[penicillanate-6,1′-(2-benzoyl-5-benzyloxycarbonyl(cyclopent-4-enyl))] (**20**). Compound **19** (157 mg, 0.31 mmol) was suspended in a mixture of ethyl acetate (5 mL) and saturated solution of $NaHCO_3$ (15 mL) at 0 °C. The mixture was stirred until complete dissolution, and then partitioned between deionized water (5 mL) and ethyl acetate (20 mL). The two layers were then separated and the aqueous layer was extracted with ethyl acetate (2 x 20 mL). The aqueous layer was acidified to pH 3 in an ice bath with HCl (1 N) and extracted with ethyl acetate (3 x 20 mL). The combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The residue was then

dissolved in dry acetone (5 mL) and MeI (128 mg, 0.91 mmol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (69 mg, 0.45 mmol) were added. The reaction mixture was stirred at room temperature under nitrogen atmosphere for 2 h, monitored by TLC. The solvent was removed under reduced pressure and the crude product was purified by flash chromatography [(ethyl acetate/hexane (1:3)]. Spiro-β-lactam **20** was obtained as a fluffy colorless solid (48 mg 0.10 mmol, 31%). mp 58.5–60.3 °C; $[\alpha]_D^{25} = +470$ (*c* 1 in CH_2Cl_2); IR (ATR) *ν*: 999, 1022, 1121, 1154, 1208, 1233, 1264, 1308, 1448, 1624, 1676, 1711, 1763 and 2953 cm^{-1}; ^1H NMR *δ* ($CDCl_3$): 1.37 (s, 3H), 1.54 (s, 3H), 2.50 (dd, *J* = 18.4 and 2.0 Hz, 1H), 3.19 (ddd, *J* = 18.4, 9.2 and 2.0 Hz, 1H), 3.73 (s, 3H) 4.47 (s, 1H), 4.56 (d, *J* = 8.4 Hz, 1H) 5.24 (d, *J* = 12.4 Hz, 1H); 5.28 (d, *J* = 12.4 Hz, 1H), 6.18 (s, 1H), 6.92 (dd, *J* = 2.8 and 2.0 Hz, 1H), 7.32–7.42 (m, 5H), 7.47–7.51 (m, 2H), 7.58–7.62 (m, 1H), 7.92–7.94 (m, 2H); ^{13}C NMR *δ* ($CDCl_3$): 26.5, 32.2, 36.0, 49.6, 52.4, 62.4, 66.7, 69.0, 70.8, 73.6, 128.4, 128.4, 128.6, 128.7, 129.0, 133.7, 134.4, 135.1, 135.8, 145.2, 162.7, 168.2, 174.3, 198.2; HRMS (ESI-TOF) *m/z*: $[M+H]^+$ Calcd for $C_{28}H_{28}NO_6S$ 506.1632; Found 506.1644.

(3a′R,6R,6a′S)-Benzhydryl spiro[penicillanate-6,5′-(6-benzoyl-6a-benzyloxycarbonyl-(3a,3,6,6a-tetrahydrocyclopenta[c]pyrazole))] (**21**). Ethereal diazomethane was added in excess to a solution of spiro-β-lactam **1** (0.200 g, 0.304 mmol) in dry dichloromethane (5 mL) at 0 °C. The reaction mixture was manually stirred and monitored by TLC. Upon completion, the excess of diazomethane was purged with nitrogen and the crude product was purified by flash chromatography [(ethyl acetate/hexane (1:3) and (1:2)]. Spiro-β-lactam **21** was obtained as a colorless solid (182 mg, 0.26 mmol, 86% yield). mp 139.6–142.0 °C; $[\alpha]_D^{25} = +270$ (*c* 0.5 in CH_2Cl_2); IR (ATR) *ν*: 972, 1020, 1093, 1151, 1177, 1200, 1235, 1276, 11307, 1332, 1448, 1491, 1684, 1735 and 1763 cm^{-1}; ^1H NMR *δ* ($CDCl_3$): 1.07 (s, 3H, 2α-Me), 1.45 (s, 3H, 2β-Me), 2.17 (d, *J* = 13.4 Hz, 1H, H-4′), 3.13 (dt, *J* = 8.8 and 3.6 Hz, 1H, H-3a′) 3.23 (dd, *J* = 13.4 and 9.6 Hz, 1H, H-4′), 4.44 (s, 1H, H-3), 4.44 (d, *J* = 12.4 Hz, 1H, C*H*$_2$Ph), 4.75 (d, *J* = 12.4 Hz, 1H, C*H*$_2$Ph), 4.82 (dd, *J* = 14.8 and 8.8 Hz, 2H, H-3′), 5.08 (s, 1H, H-6′), 5.20 (s, 1H, H-5) 6.88 (s, 1H, C*H*Ph$_2$), 7.00–7.02 (m, 2H, Ar*H*), 7.21–7.34 (m, 13H, Ar*H*), 7.41–7.45 (m, 2H, Ar*H*), 7.55–7.58 (m, 1H, Ar*H*), 8.06–8.09 (m, 2H, Ar*H*); ^{13}C NMR *δ* ($CDCl_3$): 26.1, 32.2, 38.4, 41.8, 53.4, 64.3, 67.7, 69.4, 69.5, 70.6, 78.4, 87.3, 110.9, 127.1, 127.7, 128.2, 128.3, 128.5, 128.5, 128.6, 128.7, 129.1, 133.7, 134.4, 137.8, 139.2, 139.3, 167.0, 167.2, 174.75, 198.4; Anal. Calcd for $C_{41}H_{37}N_3O_6S$: C, 70.37; H, 5.33; N, 6.00; S, 4.58. Found: C, 70.74; H, 5.64; N, 5.93; S, 4.73.

Benzhydryl spiro[penicillanate-6,5′-(4-benzoyl-3-benzyloxycarbonyl-2-pyrazoline)] (**23**). A solution of benzyl diazoacetate (**22**) (164 mg, 0.93 mmol) in dry dichloromethane (3 mL) was added dropwise under nitrogen atmosphere to a solution of 6-alkylidenepenicillanate **10** (0.250 g, 0.517 mmol) in dry dichloromethane (2 mL). After complete addition of benzyl diazoacetate, the mixture was stirred at reflux for 48 h. The solvent was removed under reduced pressure and the crude product was purified by flash chromatography [(ethyl acetate/hexane (1:4)] to give spiro-β-lactam **23** as a yellow solid (102 mg, 0.16 mmol, 30%). mp 142.6–144.3 °C; $[\alpha]_D^{25} = +630$ (*c* 0.5 in CH_2Cl_2); IR (ATR) *ν*: 980, 1045, 1144, 1216, 1261, 1333, 1448, 1668, 1701, 1729, 1774 and 3303 cm^{-1}; ^1H NMR *δ* ($CDCl_3$): 1.12 (s, 3H, 2αMe), 1.52 (s, 3H, 2βMe), 4.59 (s, 1H, H-3), 5.30 (d, *J* = 12.2 Hz, 1H, C*H*$_2$Ph), 5.33 (d, *J* = 12.2 Hz, 1H, C*H*$_2$Ph), 5.65 (s, 1H, H-4′), 6.22 (br s, 1H, N*H*), 6.29 (s, 1H, H-5) 6.95 (s, 1H, C*H*Ph$_2$), 7.29–7.36 (m, 9H, Ar*H*), 7.40–7.43 (m, 6H, Ar*H*), 7.53–7.57 (m, 2H, Ar*H*), 7.65–7.69 (m, 1H, Ar*H*), 7.95–7.97 (m, 2H, Ar*H*); ^{13}C NMR *δ* ($CDCl_3$): 25.9, 32.9, 63.4, 67.0, 67.3, 69.4, 69.6, 73.8, 78.6, 127.4, 127.5, 128.3, 128.3, 128.6, 128.7, 128.7, 128.8, 129.6, 134.4, 134.8, 135.3, 139.5, 139.6, 160.5, 166.4, 170.8, 191.8; Anal. Calcd for $C_{38}H_{33}NO_6S$: C, 69.18; H, 5.04; N, 6.37; S, 4.86. Found: C, 68.87; H, 5.23; N, 6.25; S, 4.90.

4.2. Biological evaluation

4.2.1. Cell lines

TZM-bl cells (AIDS Research and Reference Reagent Program, National Institutes of Health, USA) were cultured in complete growth medium consisting 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 37 °C in 5% CO_2.

4.2.2. Viruses and titration

The HIV-1 SG3.1 is a reference subtype B strain that uses the CXCR4 coreceptor. It was obtained by transfection of HEK293T cells with pSG3.1 plasmid using jetPrime transfection reagent (Polyplus-transfection SA, Illkirch, France) according to the instructions of the manufacturer. The 50% Tissue Culture Infectious Dose (TCID50) of each virus was determined in a single-round viral infectivity assay using a luciferase reporter gene assay in TZM-bl cells [43,44] and calculated using the statistical method of Reed and Muench.

4.2.3. 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. Medium controls (only growth medium), cell controls (cells without test compound) and cytotoxicity controls (a compound that kills cells) were employed in each assay. The cytotoxicity of each test compound was expressed by the 50% cytotoxic concentration (CC_{50}), corresponding to the concentration of compound causing a 50% decrease of cellular viability.

4.2.4. Anti-HIV assays

The antiviral activity of test compounds was determined in a single-round viral infectivity assay using TZM-bl reporter cells, as previously described [43–45]. Briefly, TZM-bl cells were infected with 200 TCID50 of HIV-1 in the presence of serial fold dilutions of the compounds in the growth medium, supplemented with diethylaminoethyl-dextran (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 instructions of the manufacturer. For each compound dilution, the assay was set up in triplicate wells. Virus controls, cell controls and inhibitors controls (drugs with a known action against each virus) were employed. 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%. IC_{50} and IC_{90} were estimated by the sigmoidal dose-response (variable slope) equation in Prism version 5.01 for windows (GraphPad Software, USA).

4.2.5. Evaluation of hepatic stage anti-plasmodial activity

The inhibitory activity of test compounds on *in vitro* hepatic infection by *P. berghei* was determined by comparing the parasite load in compound- and solvent-treated *P. berghei*-infected Huh7 cells. Infection load was assessed by measurement of luminescence intensity in Huh-7 cells infected with a firefly luciferase-expressing *P. berghei* line, as previously described [29,30]. Briefly, Huh-7 cells were seeded in 96-well plates (1.0×10^4 cells per well) the day

before drug treatment and infection. The medium was replaced by medium containing the appropriate concentration of each compound approximately 1 h prior to infection with sporozoites freshly obtained through disruption of salivary glands of infected female *Anopheles stephensi* mosquitoes. Sporozoite addition was followed by centrifugation at 1700×*g* for 5 min. Parasite infection load was measured 48 h after infection by a bioluminescence assay (Biotium). Cell confluence, a surrogate measure of compound toxicity to Huh7 cells, was measured by fluorescence measurements using the AlamarBlue assay, according to the manufacturer's instructions. IC_{50} of compounds **14a** and **14b** was determined by evaluating their activity at 7 different concentrations ranging from 0.1 to 10 μM.

4.2.6. Evaluation of blood stage anti-plasmodial activity

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 compounds **14a** and **14b** 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 stained sample was analysed by flow cytometry. Approximately 100,000 events were analysed in each flow cytometry measurement. Two independent experiments were performed and all samples were analysed in triplicate.

4.3. Minimum energy calculations

Quantum chemical calculations were carried out to explore the structure and the preferred conformations of molecules **1**, **8a** and **8b**. One initial low-energy conformer of each molecule was generated using the Open Eye Omega software [46,47]. These low-energy structures were then optimized at the DFT level of theory, using the B3LYP hybrid functional [48–50] and the standard 6-31G(d) basis set. All calculations were performed using the GAMESS program package [51]. Graphical representations were produced with Discovery Studio Visualizer v20.1.0.19295 software [52]. The optimized structures are depicted in Fig. (6). Energy values and Cartesian coordinates are given in Supporting Information.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2021.113439.

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