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PREPARATION OF NEW SEMI-SYNTHETIC STEROIDS

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Thesis conducted under supervision of Professor Doctor Jorge António Ribeiro Salvador and co-supervision of Assistant Professor Samuel Martins Silvestre from Faculty of Health Sciences in University of Beira Interior in fulfilment of the requirements for the Master's Degree in Industrial Pharmaceutical Chemistry and presented to the Faculty of Pharmacy of University of Coimbra

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“Don't be afraid of
What your mind conceives
You should make a stand
Stand up for what you believe
And tonight we can truly say
Together we're invincible”

“Invincible” - Muse

Acknowledgements/Agradecimentos

Ao Professor Doutor Jorge Salvador, agradeço por me ter recebido no seio do seu grupo de investigação e por me ter dado a oportunidade de trabalhar com um grupo sensacional, com elevado espírito de equipa e entreajuda. Agradeço-lhe ainda a revisão desta dissertação.

Ao Professor Doutor Samuel Silvestre, o meu mais sincero agradecimento por todo o apoio nesta etapa final e pela minuciosa revisão desta dissertação.

A todos os meus amigos que ajudaram a conseguir concluir este trabalho, principalmente os meus colegas do laboratório: Sofia, Salete, Filipa, Daniela, Judite, Vasco e Carla, o meu mais profundo agradecimento pelo constante apoio, conselhos, boa disposição e por serem das melhores pessoas que já tive o prazer de conhecer. Ao Bruno e à Vanessa, o meu obrigada pela receção e adaptação no laboratório, essenciais para o desenvolvimento do trabalho. Não posso também deixar de agradecer à Dona Graça, à Dona Anabela e à Dona Rosária pelo apoio, carinho e boa disposição com que me receberam. Sem vocês todos, isto não teria sido possível.

À minha família, o meu muito obrigada por ser o pilar fundamental durante esta longa caminhada e por me apoiar incondicionalmente nas minhas escolhas.

A todos os que de alguma forma contribuíram para a finalização desta etapa, o meu sincero agradecimento.

Contents

Abstract

Natural products continue to be an inspirational source for drug discovery. Nevertheless, structural modification in the basic skeleton of these compounds is often necessary to overpass some limitations and optimizing their pharmacological profile. Therefore, the production of semi-synthetic derivatives of NPs has increased in the last decades.

Several studies have shown that chemical modifications in the basic structure of steroids lead to the formation of semi-synthetic derivatives with increased potency and selectivity when compared to the initial molecules. These results have been encouraging the realization of additional studies in this field, with new therapeutic options as the final goal.

Within the steroids, because of their versatility, relatively low price and described medicinal uses, bile acids (BAs) seem promising molecules to perform new semi-synthetic reactions.

In the present work, derivatives of cholic and ursodeoxycholic acids were synthesized. Methylation of cholic acid (CA) was performed with a good yield of 98%. Acetylated BAs were both prepared and isolated with yields until 44%. The acetylated CA was further used to originate urea derivatives as final new compounds, through isocyanate intermediates, with yields of 46 and 51%.

All the compounds were then analysed by Nuclear Magnetic Resonance (NMR) to partially confirm their structure and by melting point analysis to confirm their purity. As future perspectives, it is of utmost interest to evaluate the biological activity of these compounds as well as to conceive different chemical reactions in several BAs, for structural diversification.

Keywords: steroids, bile acids, cholic acid, ursodeoxycholic acid, semi-synthesis, urea

Resumo

Os produtos naturais continuam a ser uma fonte inspiradora para a descoberta de medicamentos. No entanto, a modificação estrutural no esqueleto base desses compostos é frequentemente necessária para superar algumas limitações e otimizar o seu perfil farmacológico. Assim, a produção de derivados semi-sintéticos de produtos naturais tem crescido nas últimas décadas.

Vários estudos demonstraram que modificações químicas na estrutura base de esteroides levam à formação de derivados semi-sintéticos com maior potência e seletividade quando comparados com as moléculas de partida. Estes resultados têm incentivado a realização de estudos adicionais nesta área com vista ao desenvolvimento de novas opções terapêuticas.

Dentro dos esteroides, devido à sua versatilidade, preço relativamente baixo e usos medicinais descritos, os ácidos biliares parecem moléculas promissoras para a realização de novas reações semi-sintéticas.

No presente trabalho, foram sintetizados derivados dos ácidos cólico e ursodesoxicólico. A metilação do ácido cólico foi realizada com um bom rendimento de 98%. Ambos os derivados acetilados destes ácidos biliares foram sintetizados e isolados, com rendimentos até 44%. O ácido cólico acetilado foi utilizado para preparar derivados de ureia como novos compostos finais, através de intermediários de isocianato, com rendimentos de 46 e 51%.

Todos os compostos foram depois analisados através de Ressonância Magnética Nuclear (RMN) para confirmar parcialmente a sua estrutura e da determinação do ponto de fusão para confirmar a sua pureza.

Quanto a perspetivas futuras, é de grande interesse avaliar a atividade biológica destes compostos, bem como preparar reações químicas diferentes em vários ácidos biliares, para diversificação estrutural.

Palavras-chave: esteroides, ácidos biliares, ácido cólico, ácido ursodeoxicólico, semi-síntese, ureia

Abbreviations list

ABCA-1	Transporter ATP-binding cassette A1
ABCG1	Transporter ATP-binding cassette G1
ALS	Amyotrophic lateral sclerosis
ApoA1	Lipid-free apolipoprotein A1
AcOEt	Ethyl acetate
ADME	Absorption, Distribution, Metabolism and Excretion
Ara-C	Cytosine arabinoside
ASBT	Apical sodium-dependent bile acid transporter
BAs	Bile acids
CA	Cholic Acid
$CDCl_3$	Deuterated chloroform
C_6D_6	Deuterated benzene
CYP7A1	Cholesterol 7α-hydroxylase
CYP7B1	25-Hydroxycholesterol 7α-hydroxylase
CYP27A1	Sterol 27-hydroxylase
d	Doublet
DCM	Dichloromethane
DEPT-135	"Distortion less Enhancement by Polarization Transfer" using a 135° decouple pulse
DMAP	4-Dimethylaminopyridine
DMAPP	Dimethylallylpyrophosphate
DMF	Dimethylformamide
DPPA	Diphenyl phosphoryl azide
ER	Estrogen receptors
FCC	Flash Column Chromatography
FDA	Food and Drug Administration
FPP	Farnesyl pyrophosphate
FXR	Farnesoid X Receptor
GCA	Glycocholic acid
GPP	Geranyl Pyrophosphate
HBMEC	Human brain microvascular endothelial cells
HMG-CoA	Hydroxy-3-methylglutaryl-CoA
HSD3B7	3β-hydroxysteroid dehydrogenase type 7
HT screening	High-throughput screening
Hz	Hertz (SI)

IPP	Isopentylpyrophosphate
J	Coupling constant
LCA	Lithocholic acid
LDL	Low density lipoprotein
MeI	Iodomethane
NIH	National Institutes of Health
NMEs	New molecular entities
NMR	Nuclear Magnetic Resonance
NPs	Natural Products
OCs	Oral contraceptives
PCOS	Polycystic ovarian syndrome
PPi	Pyrophosphate
q	Quartet
s	Singlet
SAR	Structure-activity relationship
t	Triplet
TCDCA	Taurochenodeoxycholic acid
TEA	Triethylamine
THF	Tetrahydrofuran
TLC	Thin layer chromatography
UDCA	Ursodeoxycholic acid
ZSDs	Zellweger spectrum disorders
^{13}C NMR	Carbon-13 Nuclear Magnetic Resonance
^{1}H NMR	Proton Nuclear Magnetic Resonance
δ	Chemical shift (NMR)

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I - Introduction

1 - Introduction

1.1 - Natural products in medicinal/pharmaceutical chemistry

Natural products (NPs) have been proving to be a rich source of therapeutic agents throughout history, due to their exceptional structural diversity, contributing to a wide range of action, from antimicrobial to anticancer activity (Dias, Urban e Roessner, 2012; Patridge *et al*., 2015). In fact, the use of substances with antimicrobial activity for topical treatment of wounds and other types of therapy extends back thousands of years before the modern antibiotic era (Moellering Jr., 1995). However, it wasn't until the discovery and isolation of some molecular entities that several NPs turned out to be potential drug leads and started to have an important role in medicinal chemistry and the pharmaceutical market (figure 1.1).

In 1803, the investigation of *Papaver somniferum L.* (opium poppy) resulted in the isolation of several alkaloids including morphine, a commercially important drug. In the 1870s crude morphine was converted to codeine, a painkiller and cough suppressor. In 1853, acetylsalicylic acid (most known as aspirin) was synthesized from salicin, isolated from the tree *Salix alba L.* (Dias, Urban and Roessner, 2012). In 1930, digoxin – a cardiotonic steroid - was first isolated from *Digitalis lanata* and is being used to treat various heart diseases.

Despite plants being known as one of the biggest sources of NPs as drug candidates, fungi and bacteria are significant sources as well. Undoubtedly, one of the most important NPs discoveries derived from a fungus (*Penicillium notatum)* was penicillin G, discovered in 1929 (Dias, Urban and Roessner, 2012). Later, in 1940, an investigation group at Rutgers performed a systematic screening of actinomycetes obtained from soil, leading to the discoveries of several antibiotics such as streptomycin, which prompted pharmaceutical companies to begin screening extracts of actinomycetes and fungi for activities primarily against pathogenic bacteria (Katz and Baltz, 2016).

Animals also have been playing a relevant role as the source of NPs for potential leads. The first notable discovery of biologically active compounds from animals, was the isolation of C-nucleosides with antiviral activity in marine sources. Then, the synthesis of structural analogues led to the development of cytosine arabinoside (Ara-C). as an anticancer agent (Oliver, Ross and Frank, 1994). Another remarkable finding was made in 1929, where cortisone and other steroids were isolated from bovine adrenal glands and allowed the treatment of several health problems including autoimmune diseases and eczema (Saenger, 2010).

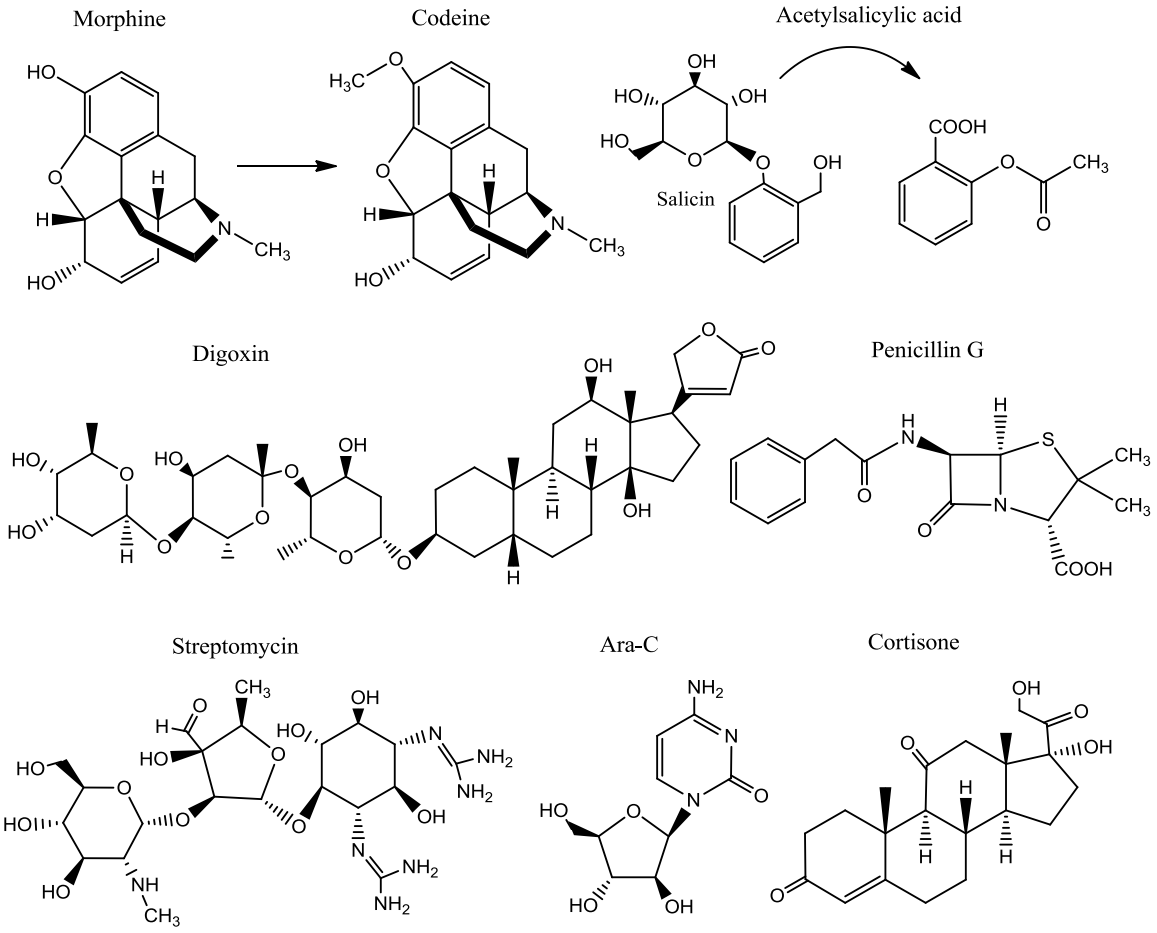


Figure 1.1 - Chemical structures of some of the molecular entities discovered and isolated from natural sources, except Ara-C (semi-synthetic derivative).

Although NPs have unique biological properties that separate them from synthetic products, there are some limitations. In fact, frequently, complex structures, poor stability and poor solubility lead to structural modifications needed to develop novel compounds with improved properties (Yao *et al.*, 2017). Therefore, in the last decades, the total fraction of NPs has diminished whereas the development of semi-synthetic derivatives has gradually increased. In fact, unmodified products dropped to 9,5% of all approved new molecular entities (NMEs) by Food and Drug Administration (FDA), whereas their derivatives doubled to 28%, since 1940 (Patridge *et al.*, 2015). Currently, FDA-approved NMEs reveals that NPs and their derivatives represent over 1/3 of all NMEs which means that they continue to be promising in the medicinal and pharmaceutical field (Patridge *et al.*, 2015). Statistic data of all new approved drugs by FDA from 1981 to 2014 are represented in figure 1.2.

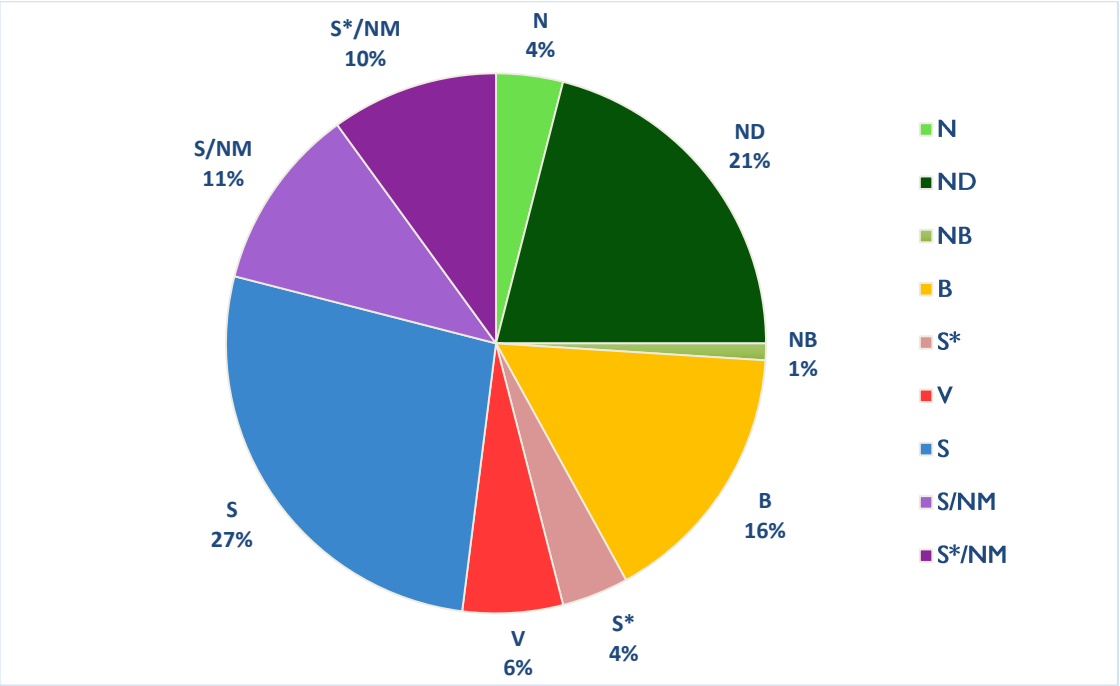


Figure 1.2 - All new approved drugs by FDA from 1981-2014 (n=1562) (adapted from Newman and Cragg, 2016). [B = Biological macromolecule; N = Unaltered natural product; NB = Botanical drug (defined mixture); ND = Natural product derivative; S = Synthetic drug; S* = Synthetic drug (NP pharmacophore), V = Vaccine; /NM = mimic of natural product]

To achieve the best results is essential a multidisciplinary approach using available and new technologies through the different phases of drug discovery, from the extraction to the clinical trials and drug approval (figure 1.3). This will allow the development of next-generation drugs to combat the present and future health challenges (Thomford *et al.*, 2018).

1.2 - Steroids

Despite the importance of bacteria and fungi, NPs from plants and animals, including steroids, have been the go-to source of drugs especially for anticancer and antimicrobial agents (Thomford *et al.*, 2018).

Steroids are a specific class of lipids which share a rigid 4-ring carbon skeleton with three cyclohexanes and one cyclopentane (figure 1.4). The focus of the present work is the family of bile acids (BAs), a class of animal steroids.

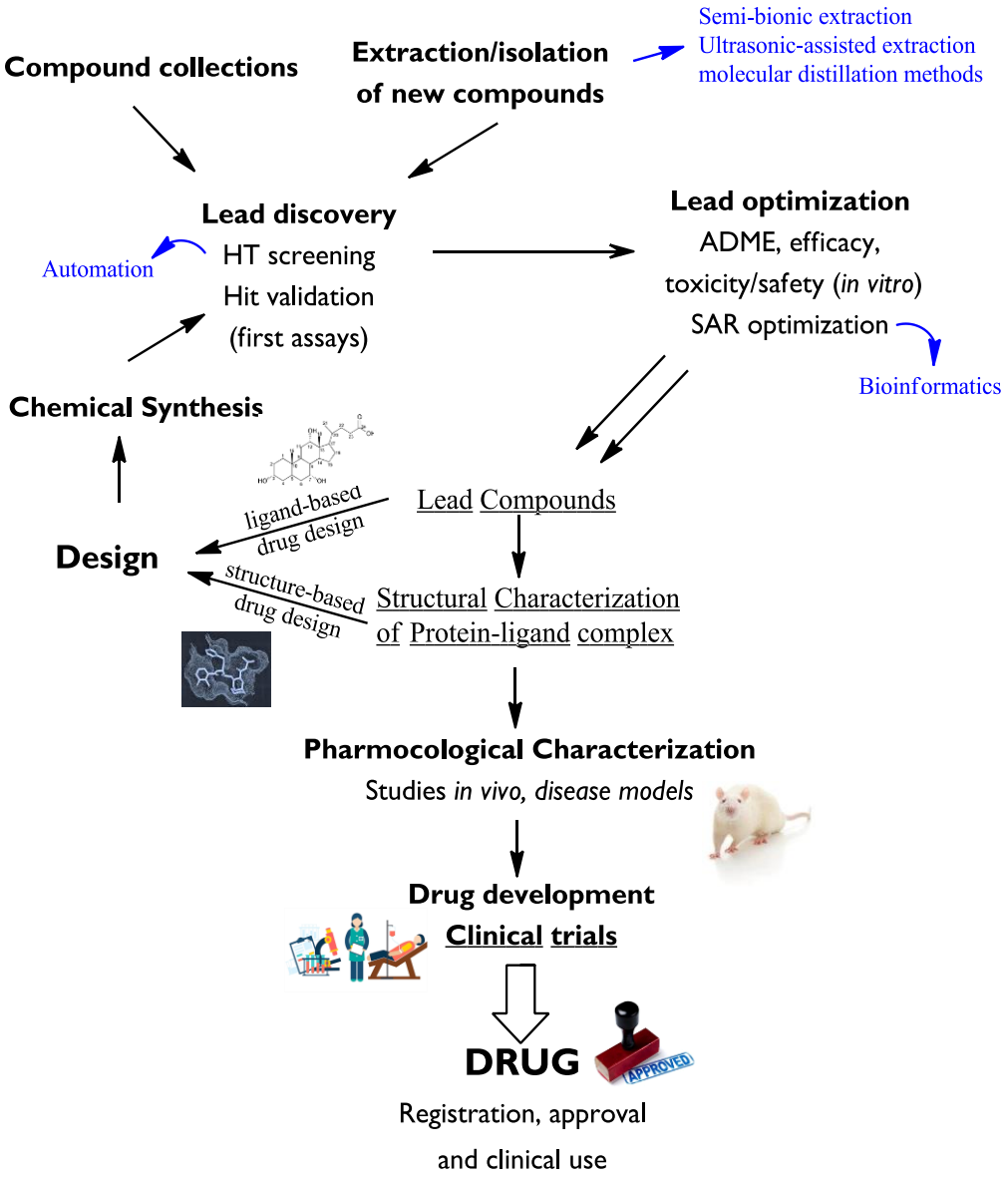


Figure 1.3 - Scheme of the drug discovery and development process. In the scheme are described some of the innovative tools that are already used, such as HT screening through automation, bioinformatics and different techniques for extraction of new compounds (adapted from www.newdrugapprovals.org and Thomford *et al.*, 2018)

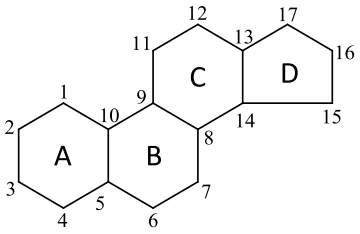


Figure 1.4 - Chemical structure of the steroid nucleus along with the carbon numbering. The four rings are commonly denoted by the capital letters A, B, C and D reading from left to right.

1.2.1 - Main structural characteristics

Steroids can be divided in several subclasses, considering the steroid nucleus as the template (Kasal, 2010; Lednicer, 2010; Moss *et al.*, 1989). Probably the most relevant subclasses are the following (figure 1.5):

- Estranes (a): frequently the ring A is aromatic; include, for example, 17β-estradiol derivatives found in oral contraceptives.
- Androstanes (b): have 2 angular methyl groups at C10 and C13 and are often called C-19 steroids; include, for example, the androgens which support male reproductive function.
- Pregnanes (c): besides the 2 methyl groups they have a 2-carbon side chain at C17 and include, for example, the sex hormone progesterone and glucocorticoids.
- Cholestanes (d): have a larger side chain at C17 and include sterols such as cholesterol.
- Cholanes (e): the side chain at C17 is shorter than in cholestanes; constitute the structural nucleus of BAs.

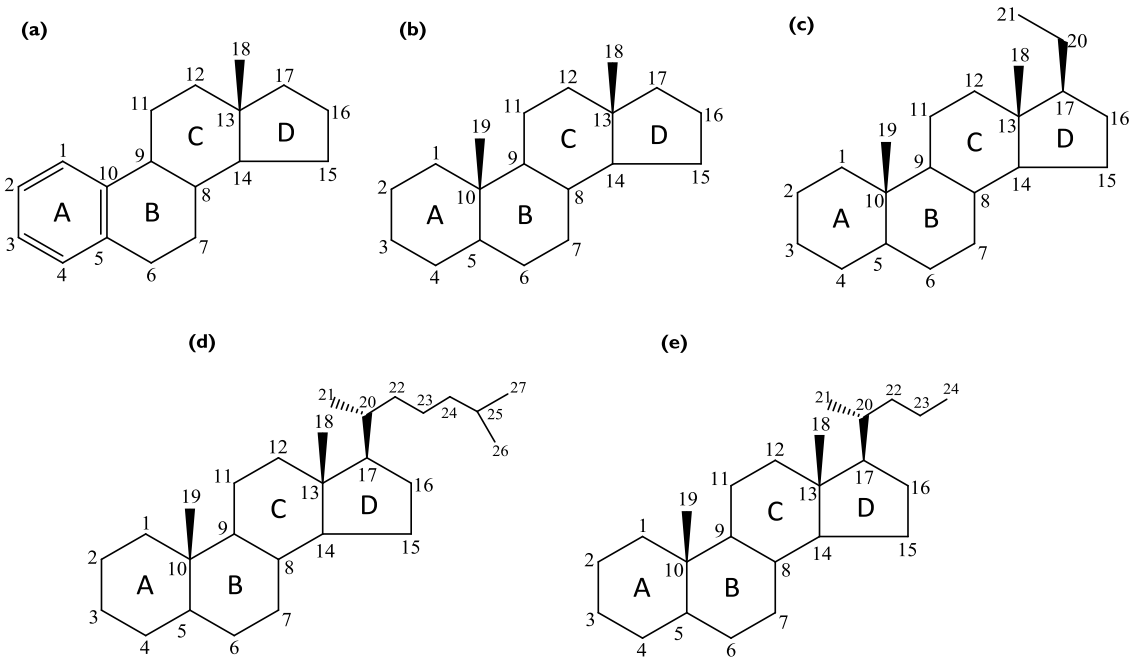


Figure 1.5 - Chemical structures of estranes (a), androstanes (b), pregnanes (c) and cholestanes (d) and cholanes (e) (adapted from Lednicer, 2010).

Another important structural aspect of the steroids is the three-dimensional configuration and the several chiral carbons. In this point, it was defined that substituents groups below the plane are named α and those that are above are designated β (figure 1.6).

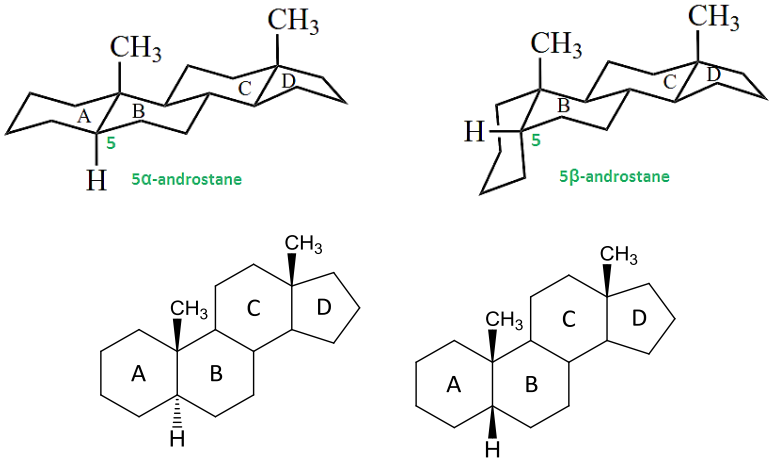


Figure 1.6 - 3D and 2D representation of the androstane nucleus, with the 2 possibilites in C5. Above there's the representation of the chair conformation (adapted from Lednicer, 2010) and below the usual planar drawing. On the left side, the hydrogen atom is under the plan being named 5α-androstane while on the right side it is above the plan which corresponds to the 5β-androstane.

1.2.2 - Biosynthesis and biological importance

Steroid biosynthesis occurs through an anabolic pathway - **mevalonate pathway** - starting from acetyl-CoA and acetoacetyl-CoA as the first two building blocks, which together originate hydroxy-3-methylgutaryl-CoA (HMG-CoA). Then, HMG-CoA is reduced to **mevalonic acid,** which is then sequently phosphorylated and subsequently decarboxylated to form isopentylpyrophosphate (IPP), which can be isomered to dimethylallylpyrophosphate (DMAPP) (Lednicer, 2010). HMG-CoA reductase is the main target of statins, which are lipid-lowering drugs used to prevent cardiovascular disease (figure 1.7).

Afterwards, condensation of IPP with DMAPP produces farnesyl and geranyl intermediates, which originate squalene in the endoplasmic reticulum. Squalene is then activated with the introduction of an epoxide and further cyclized to form lanosterol. Modifications of lanosterol originating other steroids are classified as **steroidogenesis transformations**. After 19 reactions, cholesterol, the central steroid in human biology, is formed (figure 1.8). Besides biosynthesis, cholesterol can be obtained from animal fats consumed in the diet.

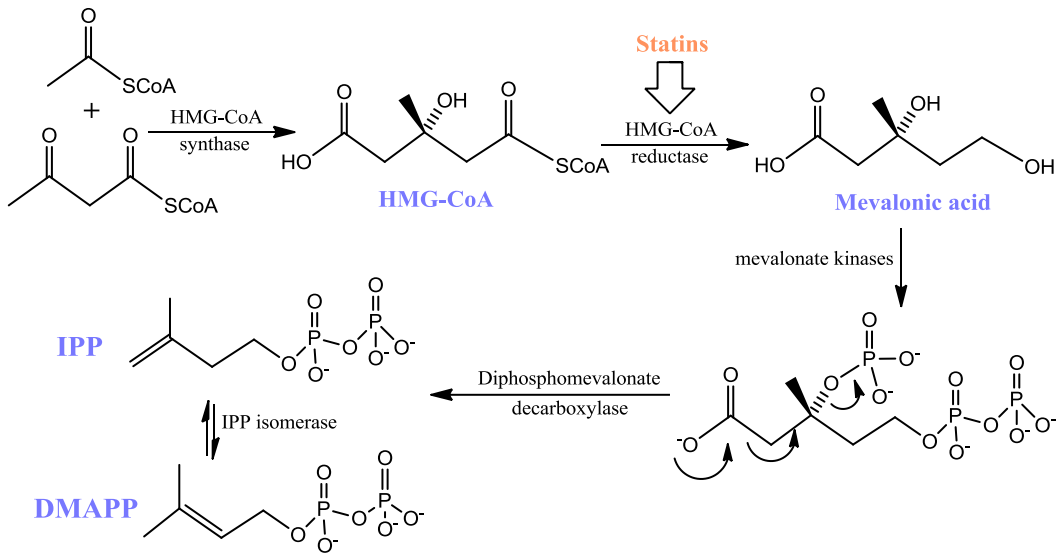


Figure 1.7 - Scheme of isopentenyl pyrophosphate (IPP) and dimethylallylpyrophosphate (DMAPP) biosynthesis (adapted from Lednicer, 2010).

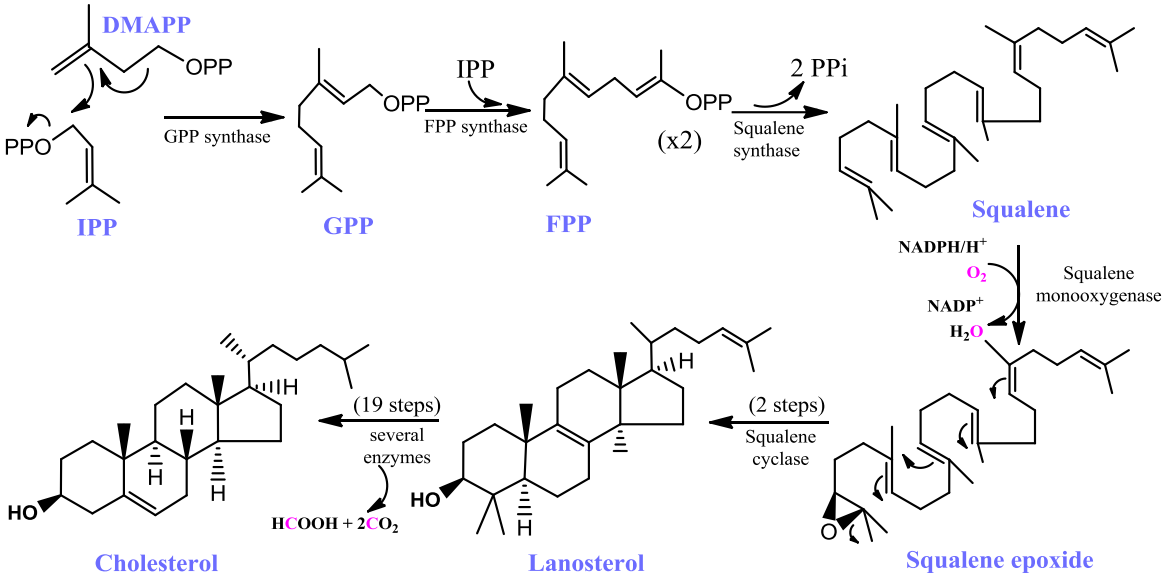


Figure 1.8 - Scheme resume of biochemical steps from IPP and DMAPP to yield cholesterol (adapted from Lednicer, 2010).

Cholesterol, a steroid alcohol, is probably the most abundant steroid in our organism and modulates cell membrane fluidity, being also the starting point for the biosynthesis of **steroid hormones, vitamin D_3 and BAs** (figure 1.9).

Steroid hormones are effector molecules crucial for life processes, since they control various bodily functions at very low concentrations, (Lednicer, 2010) and include:

- Sex hormones: 17β-estradiol and progesterone and testosterone which control female and male reproductive function, respectively.

- Glucocorticoids: include hydrocortisone, produced by the adrenal cortex, which acts on glucose metabolism and stress response.
- Mineralocorticoids: such as aldosterone, which acts directly on the kidney to maintain electrolytes and blood volume.

Vitamin D_3 is an important regulator of bone metabolism, calcium and phosphate homeostasis and its biosynthesis starts in the skin tissue upon exposure of 7-dehydrocholesterol to UVB radiation (Salvador *et al.*, 2013).

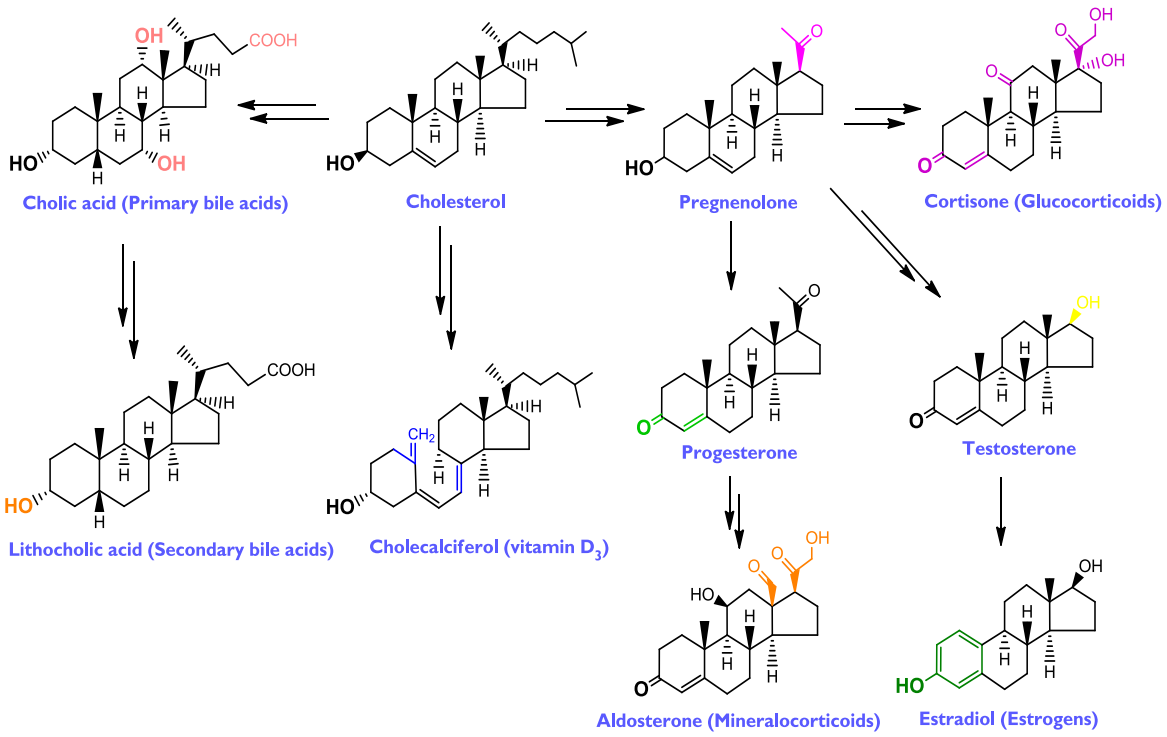


Figure 1.9 - Simplified scheme of stereoidogenesis from cholesterol.

BAs, the focus of the present work, have an important role in fat digestion and absortion and signalling, and will be developed below.

Because of their frequently high lipophilicity, usually steroids can easily diffuse across the plasmatic membrane and bind to nuclear receptors, as described in figure 1.10. This binding and subsequent biochemical events can directly stimulate the transcription of their target genes (GM, 2000). Some steroids can also bind to receptors located in the cell membrane leading to several cellular responses.

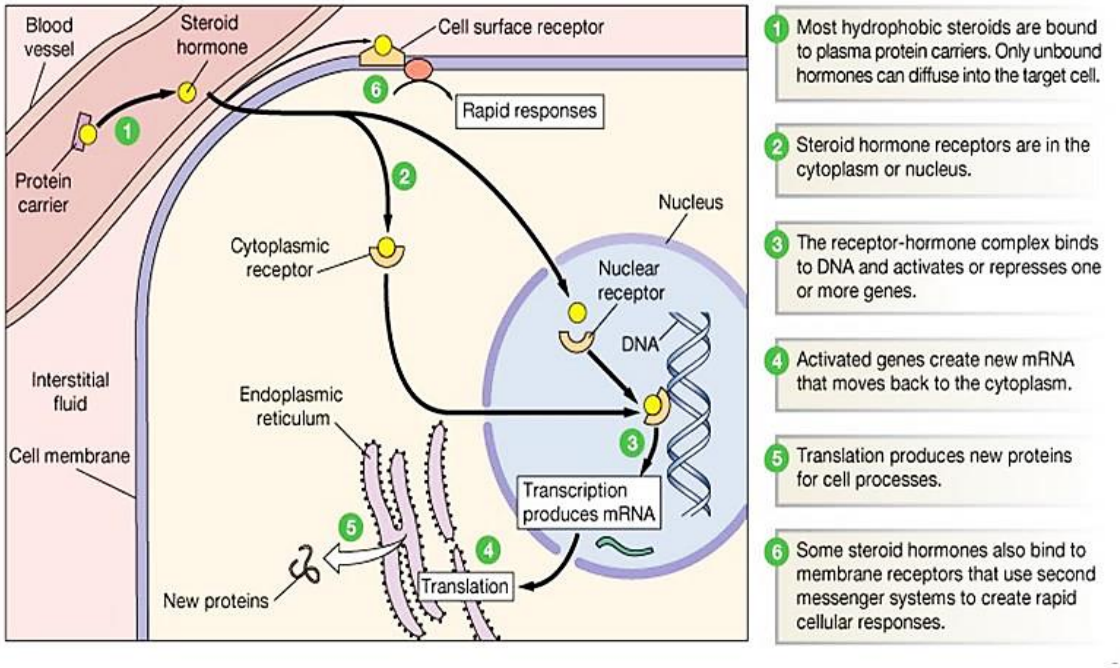


Figure 1.10 - Steroids' general mechanism of action, since their arrival at the cell to the production of new proteins. The new proteins are then normally activated to enzymes with specific functions (Via slideplayer, consulted at 10th august of 2018: https://slideplayer.com/slide/8620305/).

1.2.3 - Pharmacological uses – some examples

Due to their biological and pharmacological properties, steroids are usually considered excellent drug candidates and several of them have been already used in the treatment of many diseases, especially those involving inflammation and compromised immune system. For example, oral glucocorticoids such as prednisolone (figure 1.11) are commonly used in patients with **autoimmune diseases** including rheumatoid arthritis and erythematosus systemic lupus and other **inflammatory diseases** such as inflammatory bowel's and asthma (C. Case-Lo, 2016; Townsend and Saag, 2004).

Once they freely cross the cell membrane into the cytoplasm, they bind to the glucocorticoid receptor, which subsequently binds reversibly to specific DNA promoter or suppressor sites in the nucleus. This can result in either production or transcription inhibition of anti-inflammatory or pro-inflammatory proteins, respectively. Specifically, the anti-inflammatory effect of glucocorticoids is essentially due to the upregulation of annexin, which inhibits the action of phospholipase A2, an enzyme essential to the production of potent mediators of inflammation such as leukotrienes and prostaglandins (Townsend e Saag, 2004; Wallner *et al.*, 1986). In addition, the immunosuppressive effect of these molecules is mainly associated to an influence in the initiation and progression of the T-cell (lymphocyte) cycle (Boumpas *et al.*, 1993).

The sex hormones, estrogens and progestagens, and several similar derivatives, such as medroxyprogesterone acetate (figure 1.11), have pharmacological properties as well, being more frequently used in contraception but also in the treatment of some health problems like **acne** and **polycystic ovarian syndrome (PCOS)** (Melo, de *et al.*, 2017; Palacio-Cardona and Caicedo Borrero, 2017; Patel, 2018).

Several studies also have shown the **anti-tumour activity** of steroids, including 7α-derivatives of estradiol (estrogen antagonists), calcitriol (the vitamin D active form) and BAs, among others (Salvador *et al.*, 2013). For example, antiestrogens, such as fulvestrant (figure 1.11), compete with endogenous estrogens by binding to estrogen receptors (ER) and can be useful in the treatment of estrogen-dependent breast tumours (Wakeling, Dukes e Bowler, 1991). In this context, recent studies reported that fulvestrant induces rapid degradation of the ER, leading to the reduction of their intracellular levels (Moscetti *et al.*, 2017; Osborne, Wakeling and Nicholson, 2004).

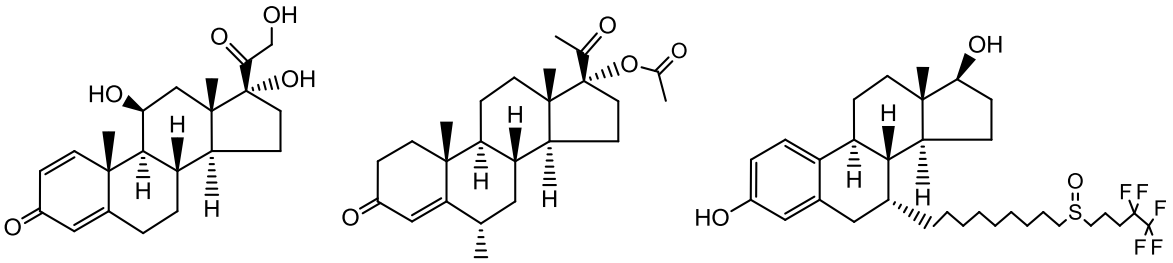


Figure 1.11 - Examples of steroids drugs used in the treatment of rheumatoid arthritis (prednisolone), in oral contraceptives (medroxyprogesterone acetate) and in breast cancer (fulvestrant).

1.3 - Bile acids

1.3.1 - Generalities

Bile acids (BAs) are amphipathic molecules formed from cholesterol. Through the formation of micelles, these molecules are crucial for absorption of lipids, lipid-soluble vitamins and even hydrophobic drugs in the organism (Maldonado-Valderrama *et al.*, 2011; Monte *et al.*, 2009). Their biosynthesis occurs mainly in the liver – primary BAs – and also in the intestine – secondary BAs (figure 1.12) (Ma e Patti, 2014).

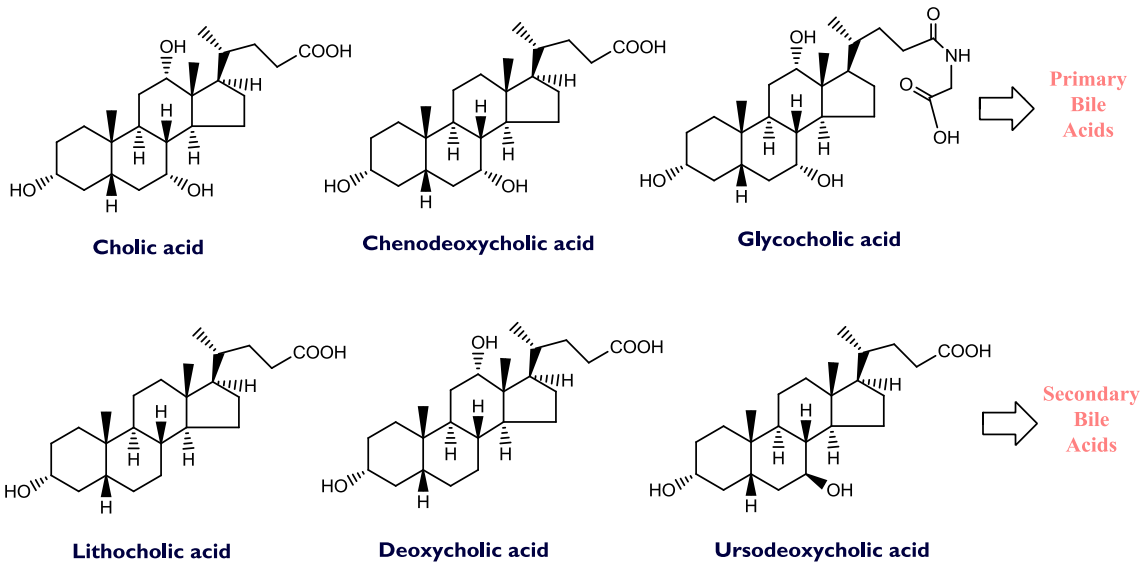


Figure 1.12 - Some examples of primary and secondary BAs.

1.3.2 - Biosynthesis

In the liver, the biosynthesis occurs through two major pathways, as described in figure 1.13: the classic (or neutral pathway) and the alternative (or acidic pathway), corresponding to about 90% and 10% of total bile acid production, respectively (Li e Chiang, 2014).

Primary BAs can react with amino acids in an enzymatic reaction and the products exist as salts and are usually named bile salts. Glycocholic acid (GCA) is one example of a conjugation product between cholic acid (CA) and glycine. BAs are usually charged molecules, having increased solubility in comparison with their non-ionized counterparts under most physiological pH ranges (Li and Chiang, 2014).

Once produced in the liver, BAs are stored in the gallbladder and are excreted into the duodenum in response to feeding, contributing to digestion and absorption of lipids and lipid-soluble vitamins (Li and Chiang, 2014; Ma and Patti, 2014). In the intestine, primary BAs can be deconjugated, dehydroxylated or epimerised by gut microbiota to produce secondary BAs like lithocholic acid (LCA) and ursodeoxycholic acid (UDCA) (Faustino *et al.*, 2016; Ridlon and Bajaj, 2015). The reason why this happens remains unclear but it is believed that gut bacteria benefit from it by acquiring glycine and taurine for subsequent metabolism, and by using BAs as sinks for the disposal of electrons from fermentation (Dawson and Karpen, 2015; Philipp, 2011). Most BAs can diffuse through the membrane (passive diffusion), while others are transported by specific proteins (ASBT), being reabsorbed and taken by the liver (enterohepatic circulation) (Faustino *et al.*, 2016). In fact, it is estimated that about 90–95% of BAs are reabsorbed in the intestine with minimal daily loss in the faeces (Li and Chiang, 2014).

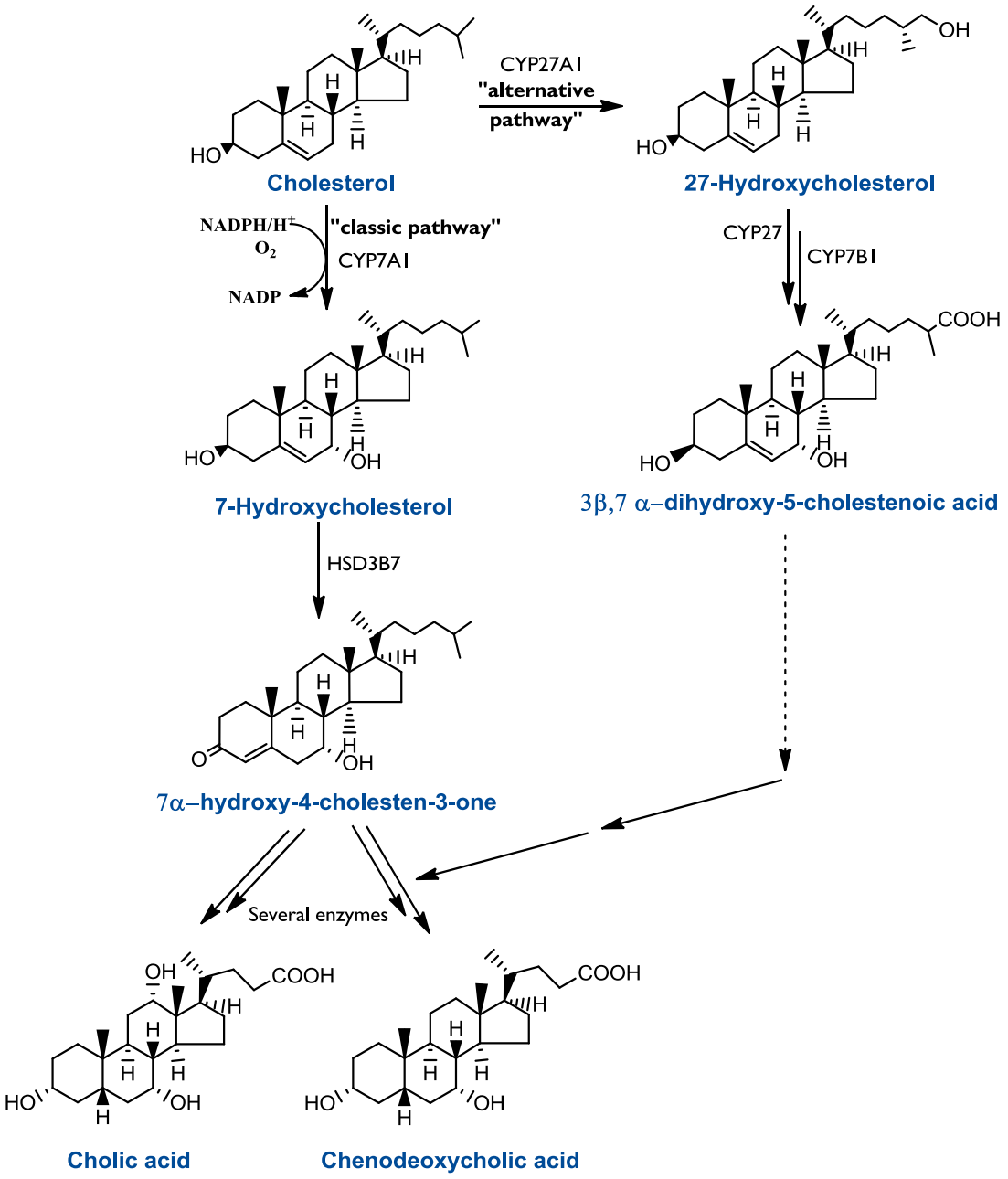


Figure 1.13 - Schematic representation of primary BAs biosynthesis from cholesterol, through the classic and alternative pathways (adapted from Chiang, 1998; Li and Chiang, 2014).

Therefore, BAs undergo multistep biotransformations usually catalysed by enzymes in order to produce several bioactive compounds that have different biochemical purposes, being promising compounds in the pharmaceutical/medicinal field.

1.3.3 - Main pharmacological applications

Because of their biochemical properties, as well as their availability and relatively low cost, BAs have already been used in the treatment of several diseases and have become attractive building blocks for the design of novel drugs (Faustino *et al.*, 2016).

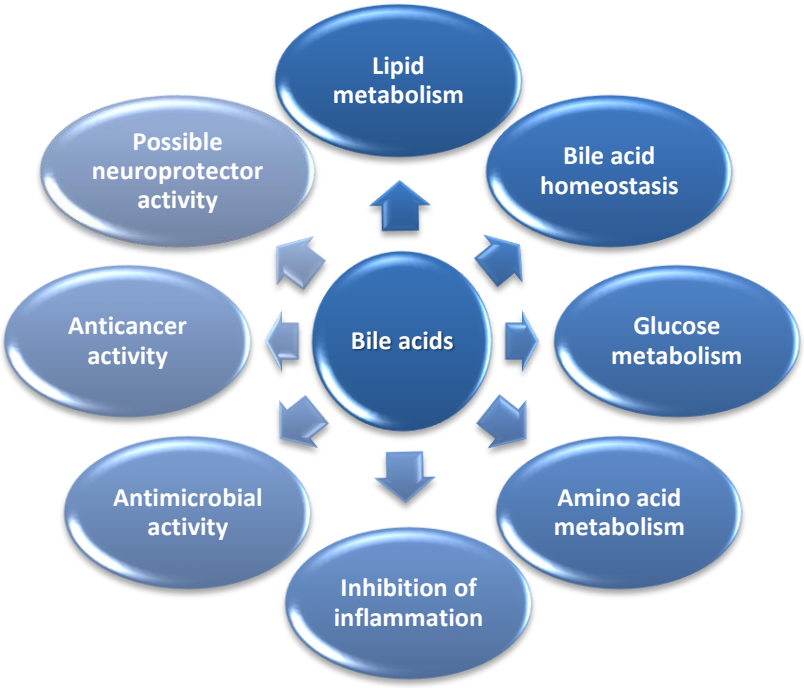
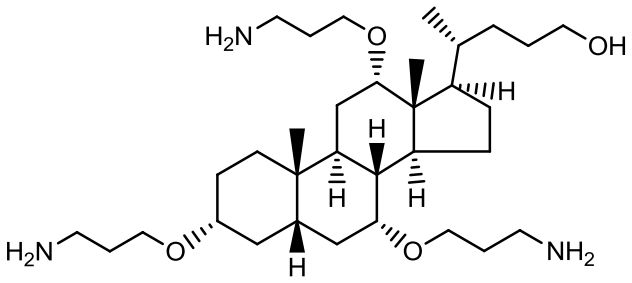


Figure 1.14 - Schematic resume of the main pharmacological properties of BAs (from Faustino *et al.*, 2016; Li and Chiang, 2014).

1.3.3.1 - Antimicrobial activity

Besides helping in fat digestion and absorption, it is thought that BAs have antimicrobial activity, controlling intestinal microflora. In fact, by damaging of the bacterial cell membrane, bacteria outgrowth is inhibited by these compounds. (Dobson, Maxwell e Ramsubhag, 2018; Inagaki *et al.*, 2005; Kurdi *et al.*, 2006)

On the other hand, antibacterial activity can be induced through electrostatic interactions with lipopolysaccharides on bacterial outer membranes, leading to its permeabilization to hydrophobic antibiotics. For example, a series of CA derivatives with appended ceragenins (figure 1.15) showed potent activity against a broad range of bacteria (Lai *et al.*, 2008).



CSA-8

Figure 1.15 - Chemical structure of one of the ceragenins of the colic acid (CSA-8) (from Lai *et al.*, 2008).

1.3.3.2 – FXR activation

Similarly to hormones, these molecules are also signalling activators of many receptors, especially the Farnesoid X Receptor (FXR) in liver metabolism. FXR regulate the expression of genes involved in bile acids, lipids, glucose and amino acids metabolism. In addition, FXR activation has been shown to inhibit the inflammatory response in the liver, being an important target for the treatment of several liver diseases (Massafra *et al.*, 2018).

Recent data showed that oral treatment with CA hinders the formation of hepatotoxic C27-BAs by inhibition of cholesterol 7α-hydroxylase (CYP7A1) through FXR activation. Accumulation of these compounds has been associated to **liver disease in Zellweger spectrum disorders (ZSDs)**, a group of rare, genetic and multisystemic disorders (Braverman *et al.*, 2016; Heubi, Setchell and Bove, 2018; Klouwer *et al.*, 2015).

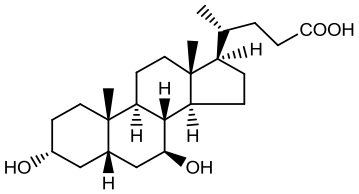
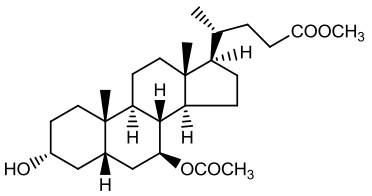
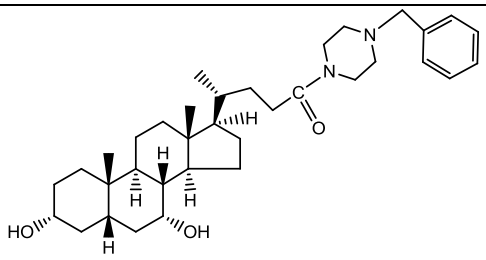
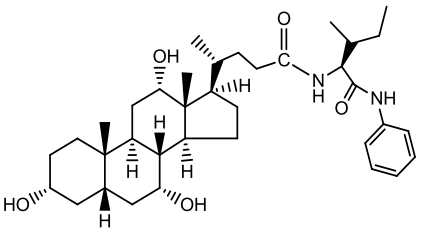
1.3.3.3 - Anticancer activity

According to NIH (www.nih.gov), cancer is one of the leading causes of death worldwide and is far from being completely understood. Recent scientific data has shown that BAs and their derivatives also have an important role in several types of cancer by inducing apoptosis or inhibiting cell growth, as resumed in table 1.1 (Agarwal *et al.*, 2015; Faustino *et al.*, 2016). For example:

- UDCA was found to suppress proliferation of **colon cancer cells**, to decrease stem cell formation in **pancreatic cancer cells** and it was associated with reduced risk of **colorectal adenoma** (Huang *et al.*, 2016; Kim *et al.*, 2017, 2017). Additionally, an ester derivative of UDCA showed **antihepatoma** effects (Xu *et al.*, 2014).
- Amide conjugates of CDCA also proved to be effective in multiple myeloma cancer cell line and in several **colon adenocarcinoma cell lines** (Brossard *et al.*, 2014).

- Some CA amide derivatives showed higher activity against **glioblastoma and breast cancer cell lines** in comparison to already existing chemotherapy drugs like cisplatin and doxorubicin (Agarwal *et al.*, 2015).
- GCA and Taurochenodeoxycholic (TCDCA) acid were recently identified as phenotypic biomarkers in **cholangiocarcinoma (bile duct cancer)** (Song *et al.*, 2018).

Table 1.1 - Resume of some bile acid and derivative compounds with their respective chemical structure and type of cancer in which they act.

Bile acid and derivative compounds	Type of cancer
 UDCA	Colon cancer (Kim *et al.*, 2017) Pancreatic cancer (Kim *et al.*, 2017) Colorectal adenoma (Huang *et al.*, 2016)
 UDCA ester derivative	Hepatoma (Xu *et al.*, 2014)
 CDCA amide derivative	Colon adenocarcinoma (Brossard *et al.*, 2014)
 CA amide derivative	Breast cancer and Glioblastoma (Agarwal *et al.*, 2015)

1.3.3.4 - Neurodegenerative diseases – a future target

Although UDCA and TCDCA are mainly being used clinically for the treatment of certain cholestatic liver diseases, studies suggested that the application of these compounds in neurodegenerative disorders is also an attractive area of research. Besides their anti-apoptotic and cytoprotective properties, both UDCA and TCDCA showed to permeate to the blood-brain barrier endothelium, crossing the human brain microvascular endothelial cells (HBMEC) *in vitro* (Palmela *et al.*, 2015). Besides that, they were detected in the brain parenchyma after injection in rodents and in the cerebrospinal fluid after oral administration in patients with amyotrophic lateral sclerosis (ALS) (Amaral *et al.*, 2009; Ramalho *et al.*, 2008; Vang *et al.*, 2014).

1.3.4 – Semi-synthetic approaches in bile acids

Inside the organism, BAs suffer different biochemical enzymatic reactions. This results in the production of several bioactive compounds with different pharmacological effects, namely, in metabolic diseases and antibacterial and anticancer, as referred. Nevertheless, many of these compounds face some limitations concerning their pharmacological applications, including low potency, selectivity and side effects. Therefore, it is of the utmost interest to perform structural modifications in these molecules to obtain relevant structure-activity relationships (SAR) data in order to improve these points and develop new and improved future drugs.

Despite the possibility to obtain these molecules by total synthesis, BA derivatives are usually prepared by semi-synthesis, starting with easily accessible starting materials obtained from natural sources.

1.3.4.1 - Protecting groups reactions

In a semi-synthetic approach, at least two points must be considered: the chemical groups to be introduced and/or modified, and the groups to be protected in order to successfully achieve it. Besides, highly selective and efficient reactions are crucial to increase the yield of target compounds.

Most of BAs only have hydroxyl and carboxyl reacting groups attached to their basic hydrocarbonated skeleton. The two most common reactions to protect these groups are methylation of the carboxyl's and acetylation of hydroxyl(s)' (Sartori *et al.*, 2004).

Methylation can be performed using dimethylformamide (DMF) as the polar solvent, potassium carbonate for the carboxylic acid activation and iodomethane (MeI) as the reagent

(Chakraborti, Basak and Grover, 1999). Hydroxyl groups are frequently protected by the introduction of an acetate group (-$OCOCH_3$). The acetylation of alcohols represents an important reaction for the synthetic organic chemistry and it is usually performed by using acyl chlorides or the corresponding anhydrides in the presence of a base such as triethylamine or pyridine (Sartori *et al.*, 2004). However, faster reaction rates can be achieved by adding 4-(dimethylamino)pyridine (DMAP) or 4-pyrrolidinopyridine as co-catalyst (Höfle e Steglich, 1972). The mechanism of the reactions will be summarized in chapter 3.

In addition, it is known that, sometimes, a simple functional group change is enough to significantly increase the potency of a compound. In fact, and as an example, a preliminary SAR analysis of UDCA derivatives with antihepatoma effects (table 1.1) suggested that acetylation of hydroxyl group at C-7 is critical to the anticancer activity and low toxicity to normal liver cells (Xu *et al.*, 2014). Besides that, glucocorticoids were reported to show better anti-inflammatory activity after the introduction of a single C-16 α-methyl (He *et al.*, 2014). Therefore, even with the main purpose of protecting groups, reactions such as acetylation and methylation, in steroids, can also improve the potency of these compounds.

1.3.4.2 - Synthesis of bile acid urea derivatives

Several recent studies reported the introduction of urea in the skeleton of terpenoids and steroids, to increase their biological potency and/or selectivity. For instance, some urea derivatives of several terpenoids showed to be selectively cytotoxic and induced apoptosis in cancer cells (Sommerwerk *et al.*, 2016). In addition, urea and carbamate derivatives of celastrol (also a terpenoid) showed enhanced selectivity and potency as cytotoxic compounds relatively to celastrol itself (Figueiredo *et al.*, 2017, 2017). Moreover, diosgenin, a steroidal sapogenin, showed better antioxidant and antiproliferative profile when urea groups were introduced in its skeleton (Romero-Hernandez et al., 2015). Other urea derivatives of steroids showed better antibacterial activity than some drugs already used (Khan, Singh e Saleem, 2008).

Within bile acids, it was only reported the formation of two urea derivatives in cholic acid series, through an isocyanate, as a highly efficient strategy (Luo, Chen e Zhu, 2007). The reaction was performed via Curtius rearrangement of acyl azide intermediates, using diphenyl phosphoryl azide (DPPA). To our knowledge, no other urea derivatives of BAs were described before. Therefore, the main purpose of this work is to synthesize some urea BA derivatives in some BAs, for posterior biological evaluation.

In our semi-synthetic approach, in the presence of a base, the BA carboxylic acid is treated with an azide and the Curtius rearrangement occurs through thermal decomposition, as a concerted process, affording an isocyanate. This group can react with amines, alcohols or water, leading to ureas, carbamates and amines, respectively, as shown in figure 1.17 (Czako, 2005; Ninomiya, Shioiri and Yamada, 1974).

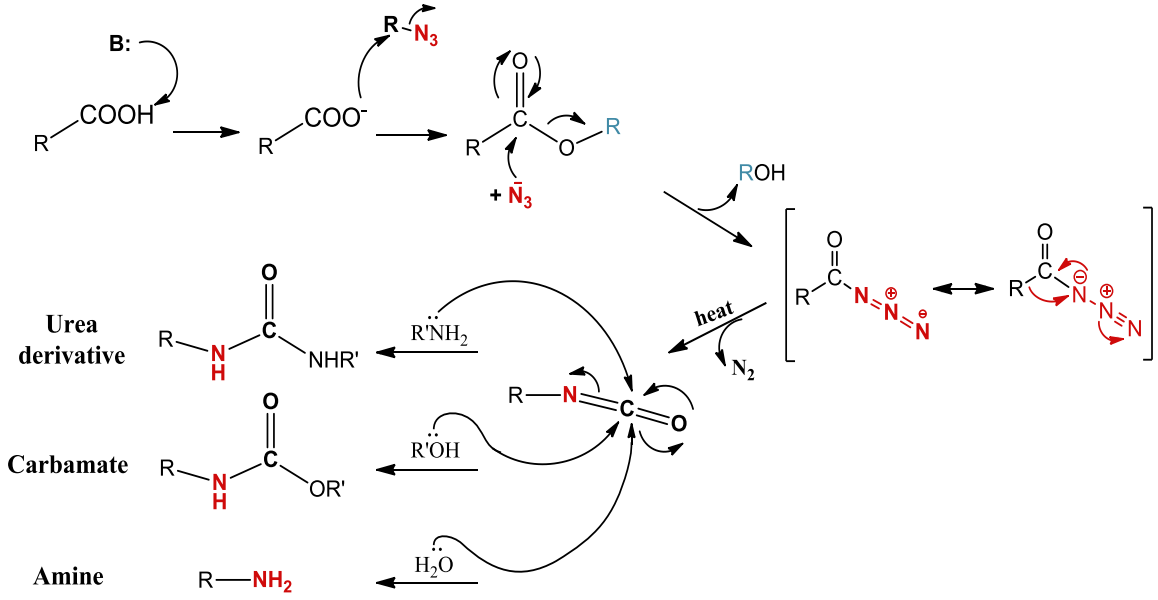


Figure 1.16 - General reaction mechanism through isocyanate reactions from carboxylic acids (adapted from Czako, 2005).

2 - Objectives

2 - Objectives

As mentioned in chapter 1, NPs continue to be an inspirational source for drug discovery. Nevertheless, structural modifications are frequently necessary to overpass some limitations, namely to increase their potency and selectivity and, consequently, decreasing the side effects. Therefore, the production of semi-synthetic NP derivatives has increased in the last decades.

Several studies have shown that steroids share a wide range of biochemical and pharmacological properties, being promising compounds in drug discovery. Because of their versatility, relatively low price and described medicinal uses, BAs were the type of steroids selected for the present work.

Inside the organism, BAs are structurally modified, originating several bioactive compounds. These, along with laboratory-made semi-synthetic derivatives, have been shown to have relevant pharmacological effects, namely as antibacterial and anticancer agents. However, drug discovery is an ascending field, always looking for better Lead compounds with higher potency and selectivity. Therefore, the design and chemical synthesis of new compounds for posterior biological activity assays is a very important challenge. Specifically, the present work aims to develop several urea BA derivatives for further biological evaluation. For this, the following tasks were performed:

1- **Chemical synthesis** and subsequent **purification** by Flash Column Chromatography (FCC) or Preparative Thin Layer Chromatography (Preparative TLC) of relevant BAs derivatives through different protecting groups reactions and isocyanate intermediates.

2- **Structural elucidation** of the semi-synthetic compounds by ^{1}H, ^{13}C and DEPT-135 Nuclear Magnetic Resonance (NMR).

3- **Purity determination** through melting point analysis.

3- Results and Discussion

3 - Results and Discussion

3.1 – Introduction of protecting groups

3.1.1 - Methylation of cholic acid

As mentioned in chapter 1, methylation is the most common and simple reaction to protect carboxylic groups and can also have a positive impact in the bioactivity of the molecule (He et al., 2014; Sartori et al., 2004). In fact, this chemical modification increases the lipophilicity of the parent compound and can enhance its cell membrane permeability.

The carboxylic acid methylation of cholic acid (figure 3.1) was performed using dimethylformamide (DMF) as polar solvent, potassium carbonate for carboxylic acid activation and iodomethane (MeI) as methylation reagent (Chakraborti, Basak and Grover, 1999).

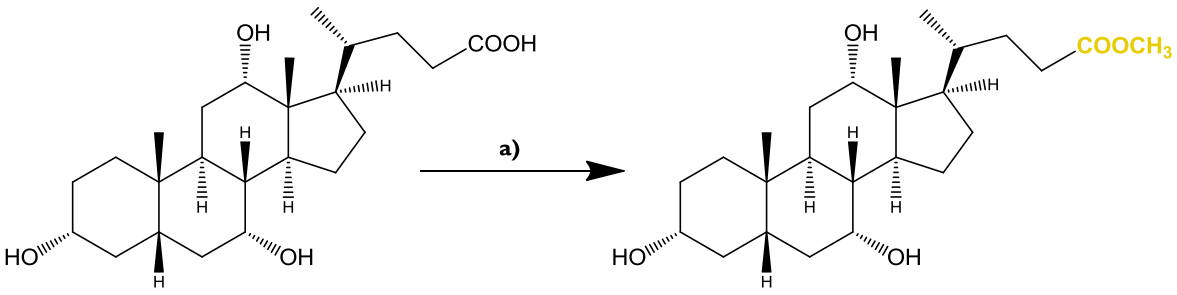


Figure 3.1 - Methylation of cholic acid. Reagents and conditions: a) MeI, DMF, K_2CO_3, r.t., anhydrous conditions, 1h30.

The general mechanism of the reaction is represented in figure 3.2. Potassium carbonate behaves as a basic reagent, activating the carboxylic acid group. Then, a nucleophile substitution takes place by the carboxylate, attacking the methyl group and iodide, a good leaving group, is released.

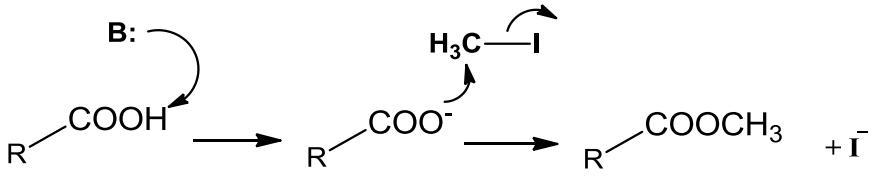


Figure 3.2 - Schematic representation of the methylation reaction's mechanism.

The ^{1}H NMR spectrum of compound 3.1 (*SpectraBase*, 2018) showed a singlet signal at **δ** = 3.66 ppm assigned to the methyl ester protons (figure 3.3). The signals at **δ** = 0.68, 0.88,

0.97 and 0.98 ppm are consistent with the protons from the methyl groups (two singlets and one doublet).

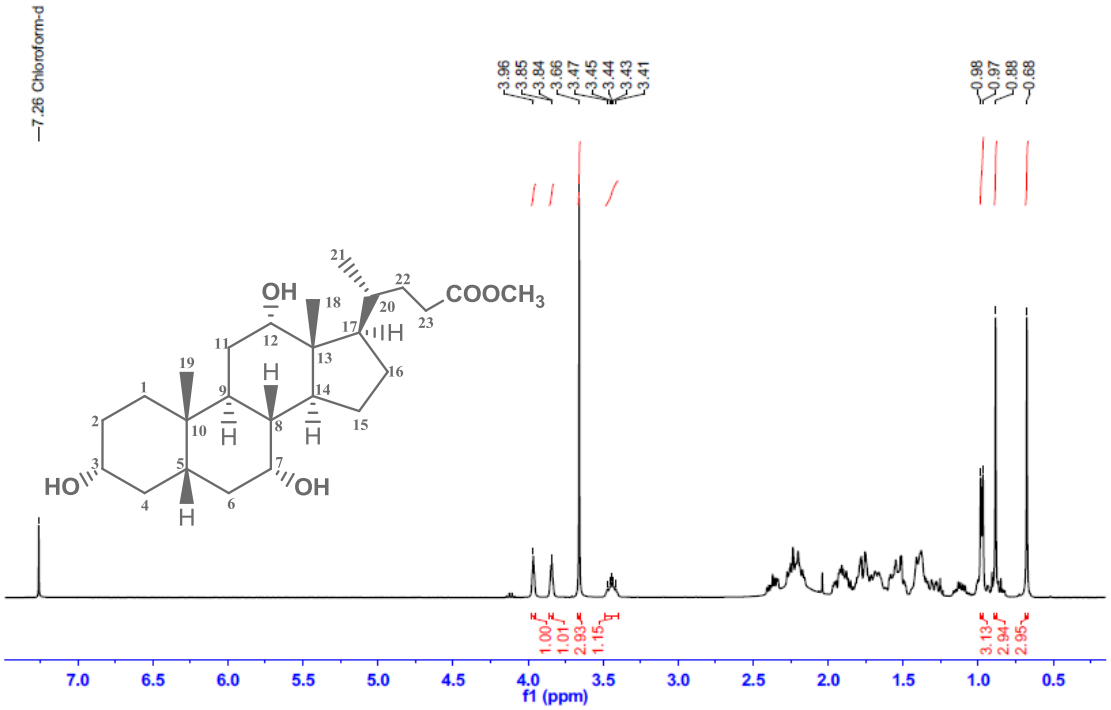


Figure 3.3 – ^{1}H NMR spectrum of compound 3.1. The signal at δ = 3.66 is concordant with methyl ester protons.

The ^{13}C NMR spectrum (figure 3.4), already reported (Iida *et al.*, 1983), confirms the presence of 25 carbons which is in accordance with the expected chemical formula of the compound. The signal at **δ** = 174.79 is concordant with the C=O of the methyl carboxylate.

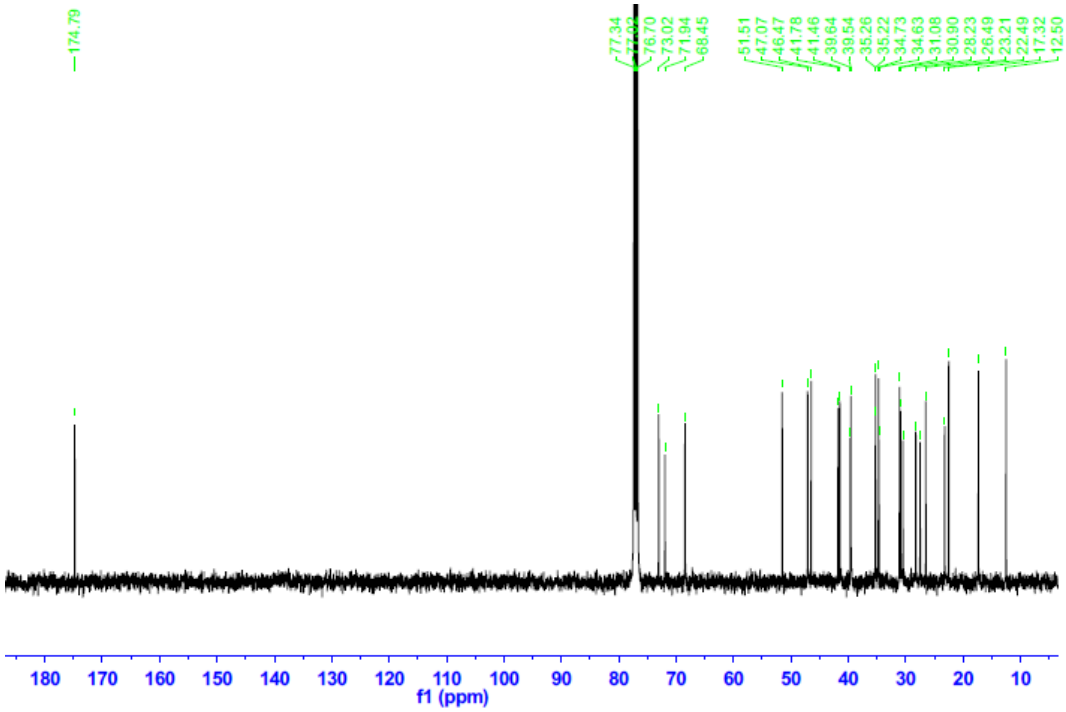


Figure 3.4 - ^{13}C NMR spectrum of compound 3.1 revealing the presence of 25 carbons.

Furthermore, the DEPT-135 spectrum (figure 3.5) show 13 primary (CH_3) and tertiary (CH) carbons and 9 secondary carbons (CH_2).

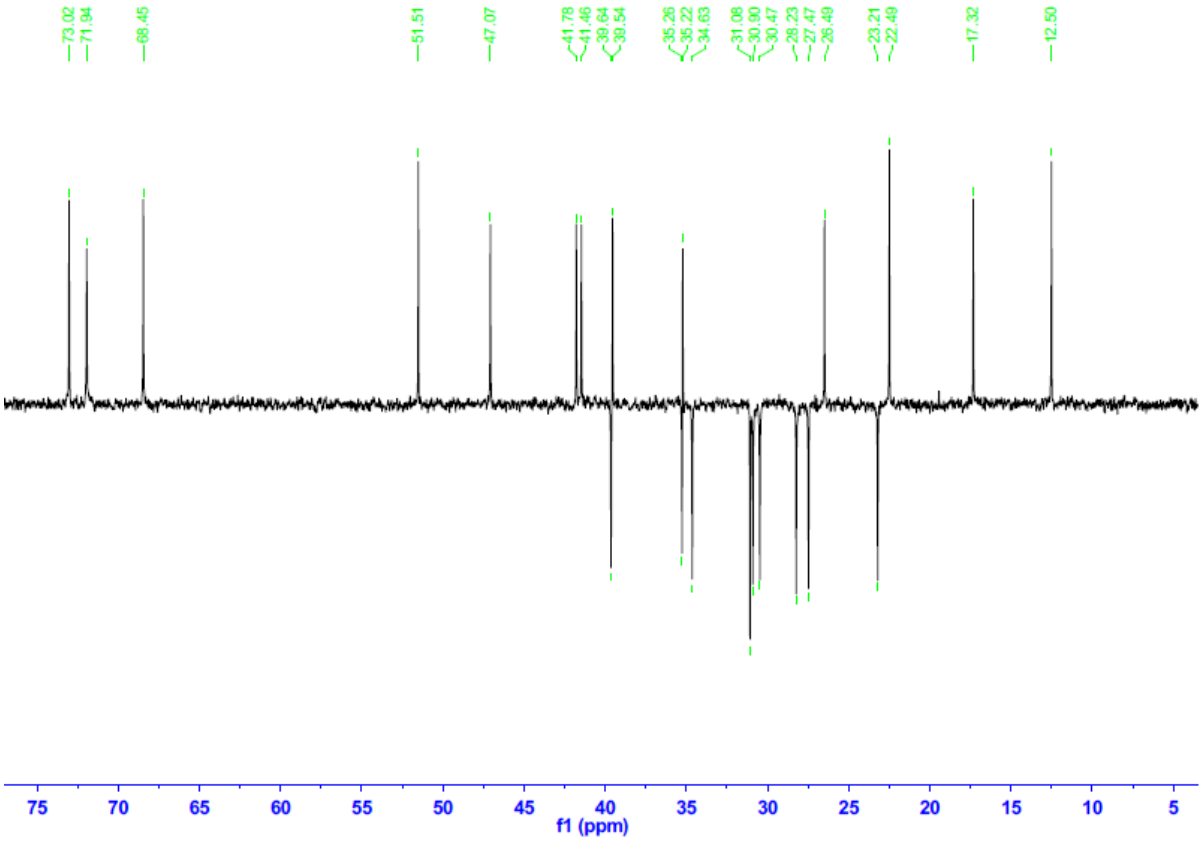


Figure 3.5 - DEPT-135 spectrum of compound 3.1 revealing 13 up signals and 9 down signals.

3.1.2 - Acetylation of cholic acid

The acetylation of cholic acid (CA) (Kannan *et al.*, 2001) was performed using tetrahydrofuran (THF) as the solvent, DMAP as the catalyst and acetic anhydride (Ac_2O) as the reagent, to introduce the acetyl groups in this BA skeleton. The reaction was performed at 66°C (reflux) in order to facilitate the acetylation in C-12, which sometimes is difficult because of the usual stereochemical impairment on C-ring of steroids. However, even in reflux conditions, the reaction wasn't complete and two different acetylated compounds 3.2 and 3.3 (figure 3.6) were isolated by Flash Column Chromatography (FCC).

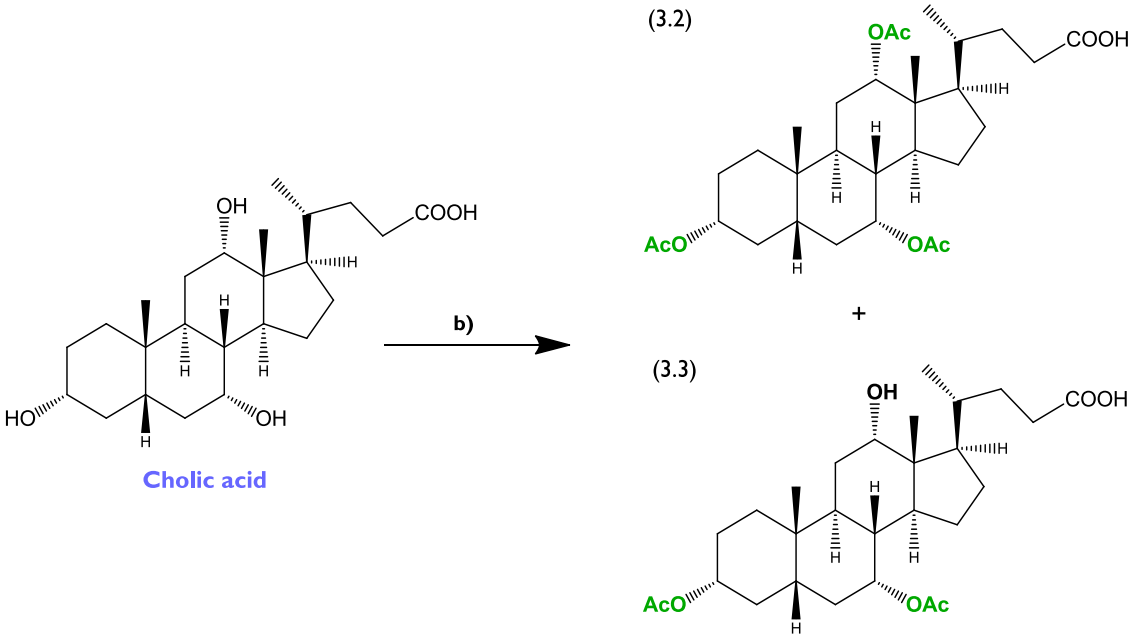


Figure 3.6 - Acetylation of cholic acid. Reagents and conditions: b) Ac_2O, DMAP, THF, 66°C (reflux), anhydrous conditions, 2h30.

The currently accepted mechanism for this transformation (Xu et al., 2005) involves the pre-equilibrium formation of an acyl pyridinium cation through reaction of DMAP with the acyl donor (figure 3.7). The hydroxyl group then reacts with the acylated catalyst to form the ester product together with the deactivated (protonated) catalyst.

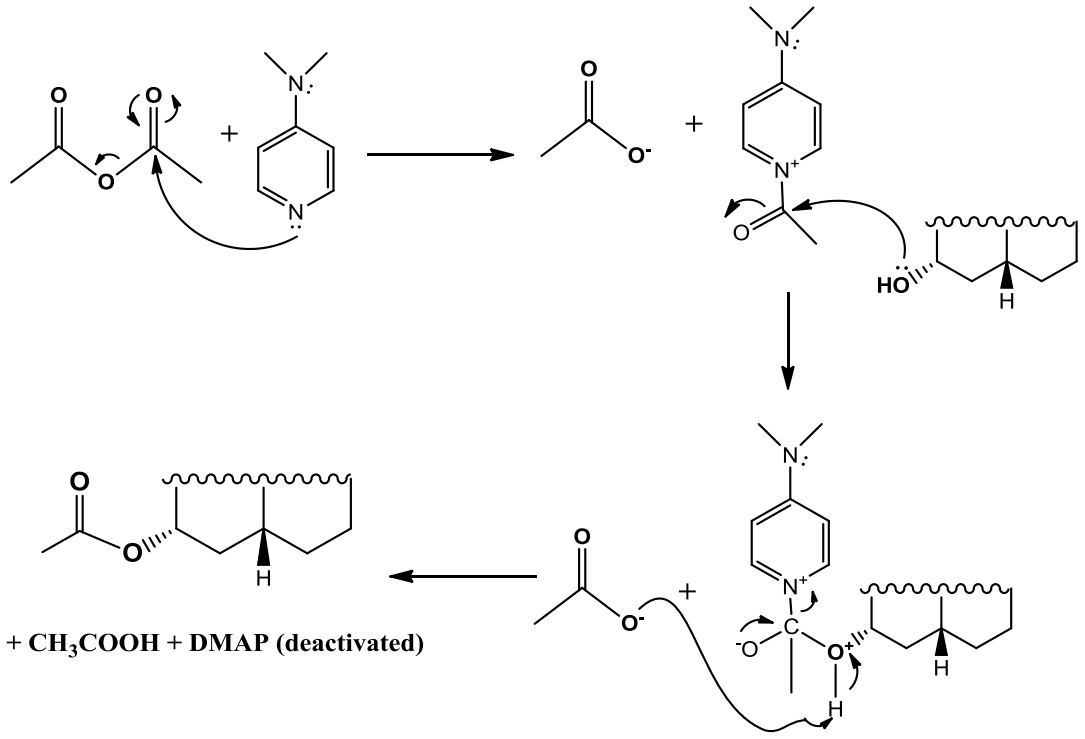


Figure 3.7 - Simplified mechanism of acetylation with DMAP and acetate anhydride (adapted from Xu *et al.*, 2005).

To confirm the structure of the compounds, a sample of both were analysed by NMR. Firstly, the ^{1}H NMR spectrum of compound 3.2 is represented in figure 3.8.

The 3 signals with highest chemical shift (δ) at δ=5.06, near 4.89 and between 4.51 to 4.59 ppm (figure 3.9) were originated by each proton of the carbon attached directly to an oxygen (C-3, C-7 and C-12). These relatively high δ values are due to the electronegativity of the oxygens of the acetate groups. Table 3.1 resumes the correspondence of the 3 signals with higher δ values to each carbon's protons

Table 3.1 - Correspondence of the 3 signals with higher δ values to each carbon's protons

• δ value (ppm)	Proton-Carbon	Number of equivalent neighboring protons
5.06	H-12	2
4.89	H-7	3
4.51-4.59	H-3	4

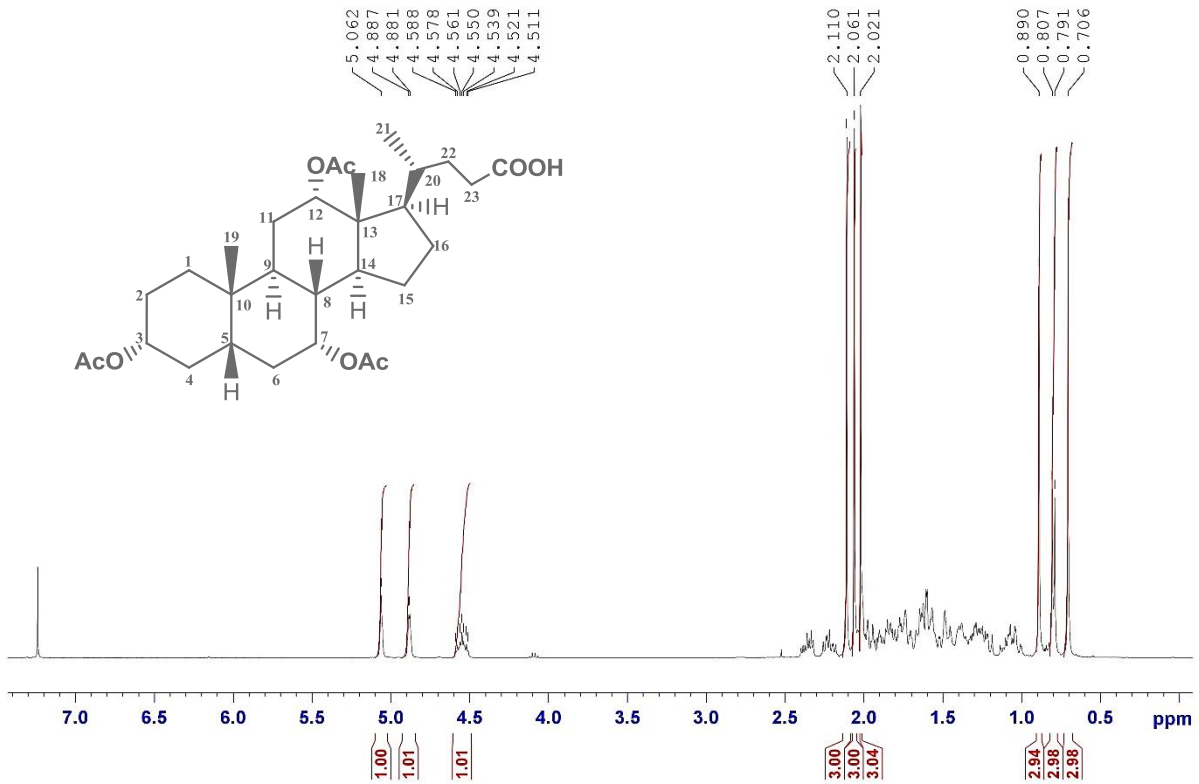


Figure 3.8 - ^{1}H NMR spectrum of compound 3.2 as one the products of cholic acid acetylation.

Around **δ** = 2 ppm, three evident singlets, each corresponding to three protons, are observed, which are consistent with the three acetylated groups (figure 3.9).

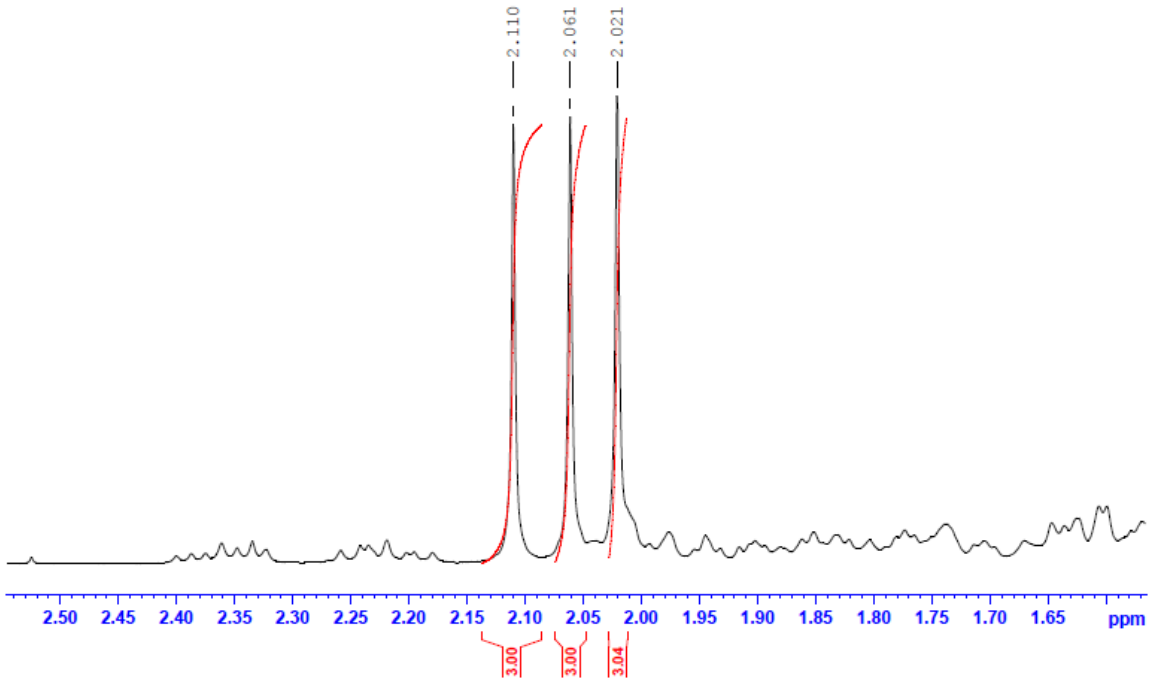


Figure 3.9 - Zoom in of the 1H NMR spectrum of compound 3.2. The 3 signals correspond to the protons of acetate groups [**δ** 2.11 (*s*, 3H); 2.06 (*s*, 3H); 2.02 (*s*, 3H)].

The signals at δ = 0.89, 0.79-0.81 and 0.71 ppm can be attributed to the three non-acetyl methyl protons (figure 3.10). The doublet at 0.79-0,81 was originated by three equivalent methyl protons neighbours of a CH group and, therefore, corresponds to the C-21 methyl protons. Although additional structure analysis is required, the signal at δ=0.89 is probable to be assigned to C-18 methyl protons, because proton shifts move downfield when electronegative substituents are attached to the same or an adjacent carbon (Reich, 2018).

Furthermore, the number of signals in the ^{13}C NMR spectrum (figure 3.11) is consistent with the number of carbons of this compound, which correspond to 30 in total. Besides, the four signals around δ = 170.0 and δ = 180.0 ppm are assigned to the four carbonyl groups present in the compound (three from the acetatyloxy groups and one from the carboxylic acid group) and the three signals at around δ = 70.0, with higher chemical shift in comparison to the remaining carbons, are consistent with the three carbons (C-3, C-7, C-12) directly linked to the acetyloxy groups.

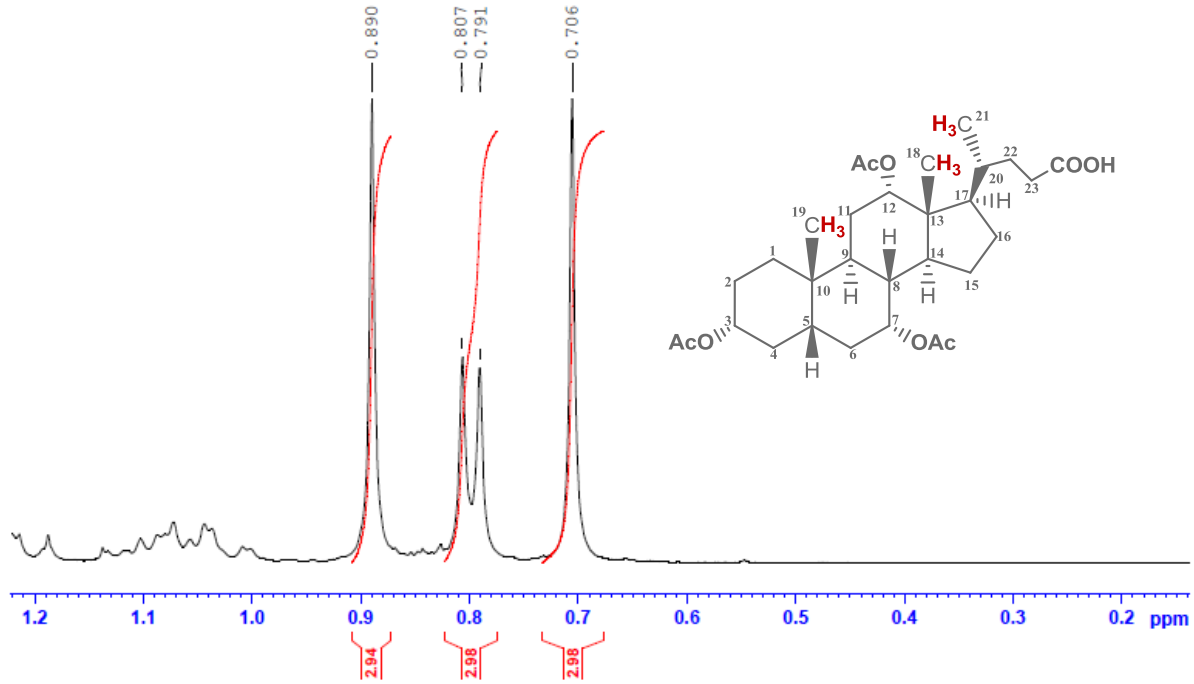


Figure 3.10 - Zoom in of the 3 signals correspondent to the protons of the three methyl groups in compound 3.2 [δ0.89 (*s*, 3H); 0.80 (*d*, 3H); 0.71 (*s*, 3H)].

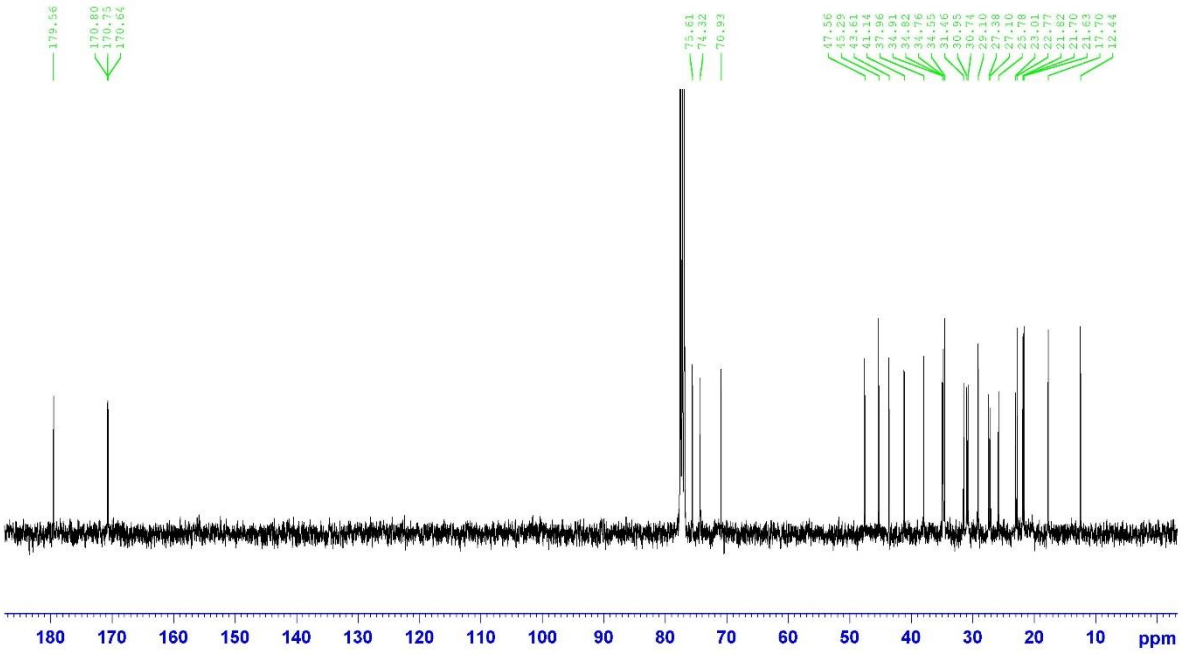


Figure 3.11 - ^{13}C NMR spectrum of the compound 3.2 with 30 signals correspondent to the 30 total carbons of the molecule.

In the DEPT-135 spectrum (figure 3.12), 15 signals, corresponding to both CH_3 and CH of the compound, and 9 signals, corresponding to CH_2, are detected.

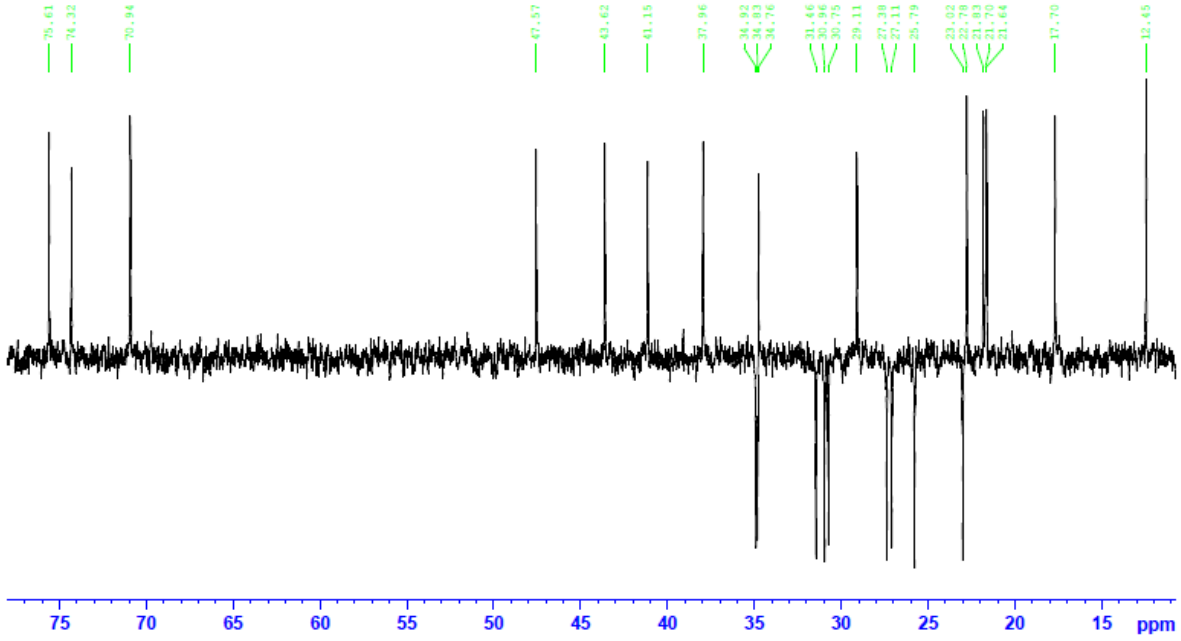


Figure 3.12 - DEPT-135 spectrum of compound 3.2.

The spectra of the second compound 3.3 are relatively similar, since the only difference between structures 3.2 and 3.3 is the lack of the acetyl group in C-12 of compound 3.3. Therefore, in the ^{1}H NMR spectrum (figure 3.13) only 2 signals at δ=2.01 and δ=2.04 ppm consistent with the two acetate groups can be observed.

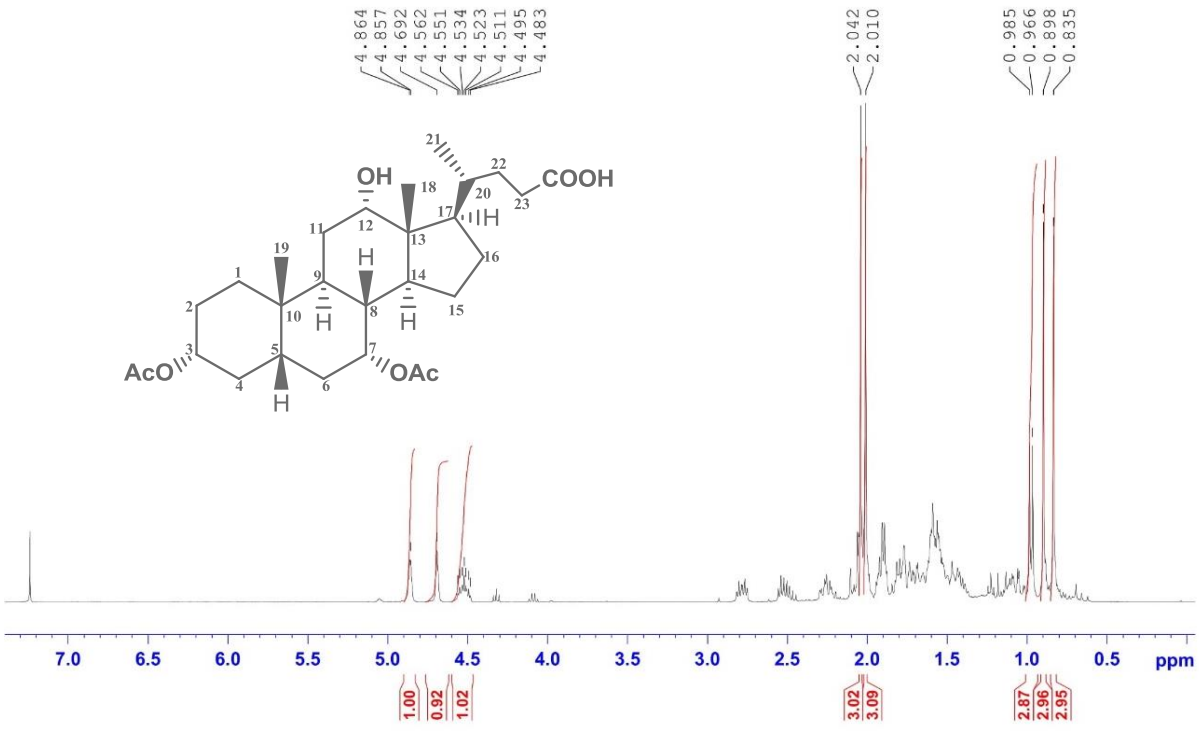


Figure 3.13 - Complete ^{1}H NMR spectrum of compound 3.3.

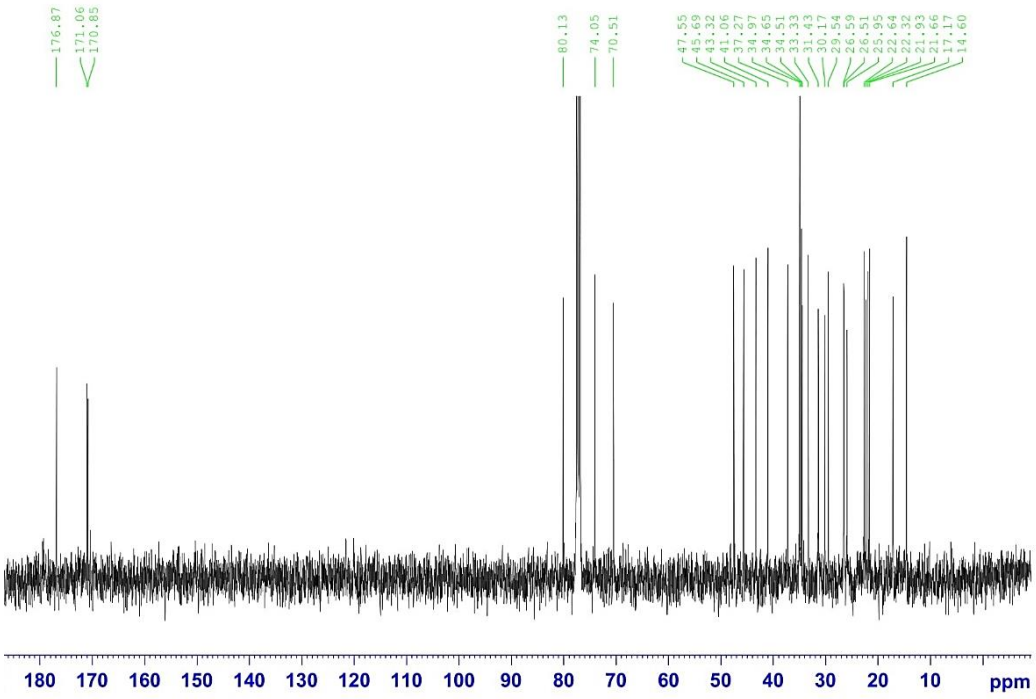


Figure 3.14 - ^{13}C NMR spectrum of compound 3.3 with 27 signals (Note: δ=34.98 ppm corresponds to 2C).

The total number of carbons of this compound is 28 (two less carbons because of the acetate group missing) which is concordant to the ^{13}C NMR spectrum, in which appears 27 signals, but the signal at δ=34.98 ppm corresponds to two carbons (figure 3.14). In addition, only three signals attributable to carbonyls were detected. In the DEPT-135 spectrum (figure 3.15) was detected one less signal than in the corresponding spectrum for compound 3.2.

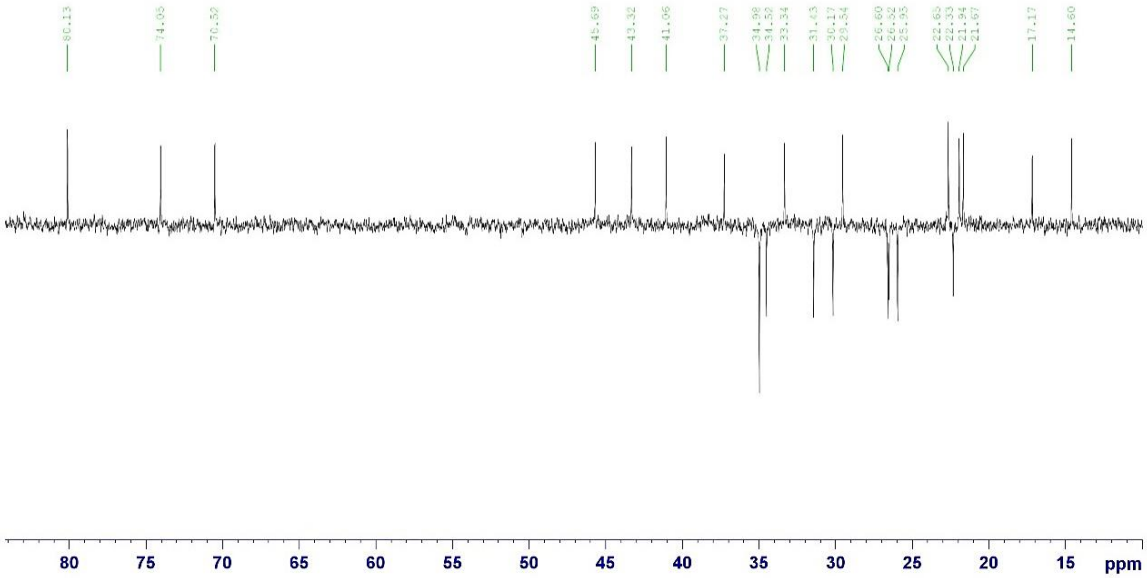
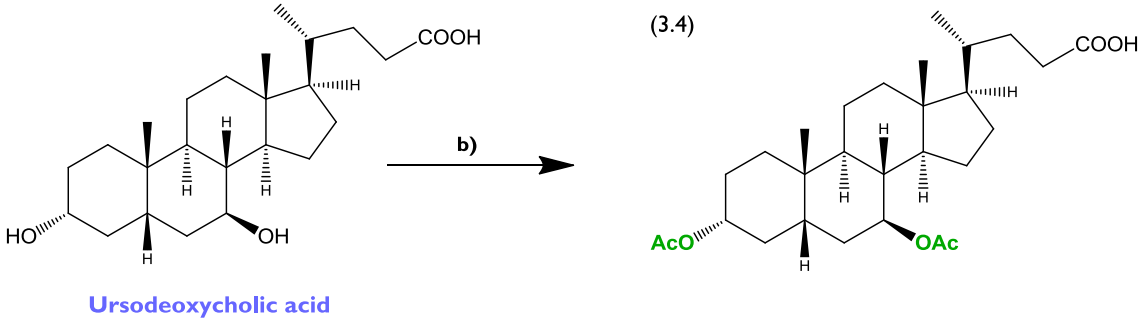


Figure 3.15 - DEPT-135 spectrum of compound 3.3 shows 14 signals up and 8 down (Note: δ=34.98 ppm corresponds to 2 CH_2 groups).

3.1.3 - Acetylation of ursodeoxycholic acid

Ursodeoxycholic acid was also acetylated. The reaction conditions were similar to those used in the acetylation of cholic acid (figure 3.16).



Ursodeoxycholic acid

Figure 3.16 - Acetylation of ursodeoxycholic acid. Reagents and conditions: b) Ac_2O, DMAP, THF, r.t., anhydrous conditions, 3h.

NMR analyses of compound 3.4 showed similar results to the observed with compound 3.3, once they share an analogous structure. The ^1H NMR spectrum is represented in figure 3.17. The 2 multiplets at around δ = 4.60 - 4.69 and around δ = 4.70 - 4.78 ppm can be attributed to each proton of the carbon directly attached to an acetyloxy group (C-3 and C-7). The signals corresponding to the protons of the acetyl methyl's are consistently observed as two singlets at δ = 2.00 ppm and δ = 1,96 ppm. The other signals are similar to the previously described compounds.

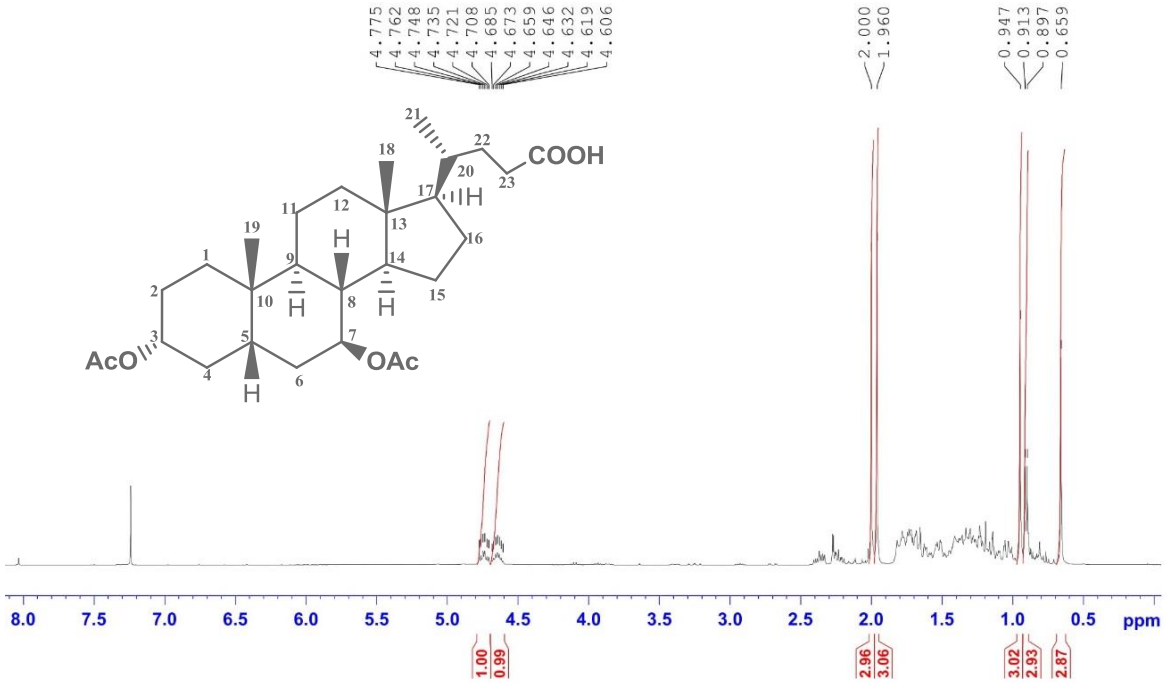


Figure 3.17 – Complete ^1H NMR spectrum of compound 3.4.

The ^{13}C NMR spectrum consistently shows 28 carbons of compound 3.4, as shown in figure 3.18, appearing 26 signals but the signals δ=170.85 and δ=33.08 are assigned to two carbons.

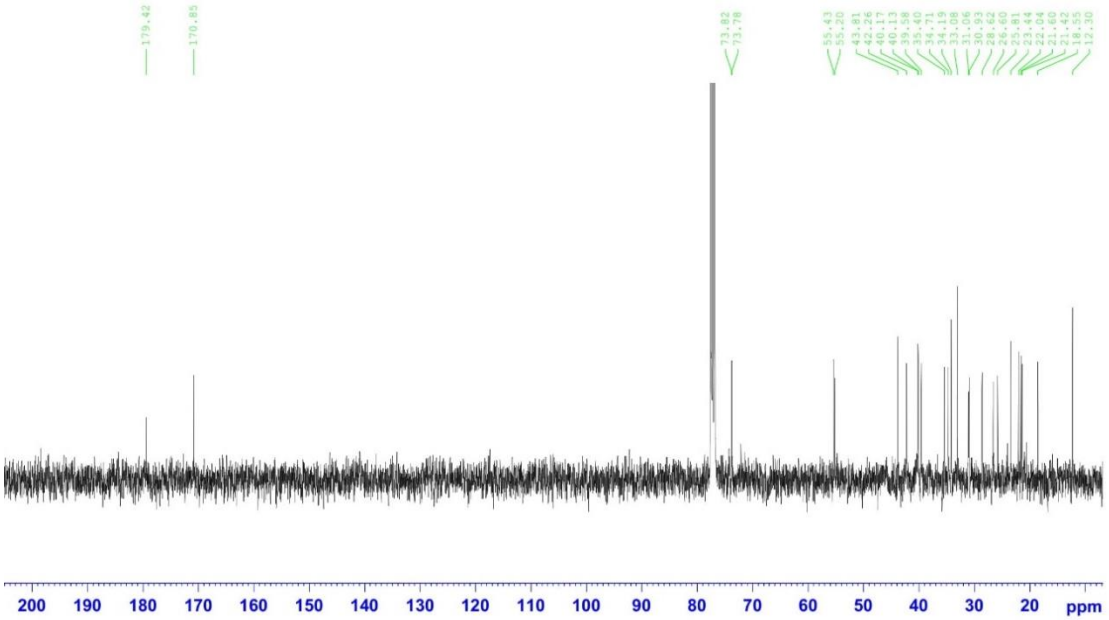


Figure 3.18 - ^{13}C NMR spectrum of compound 3.4 with 26 signals (Note: δ=170.85 and δ=33.08 corresponds to 2C each).

The DEPT-135 spectrum (figure 3.19) shows 15 signals, corresponding to both CH_3 and CH of the compound and 9 signals corresponding to CH_2.

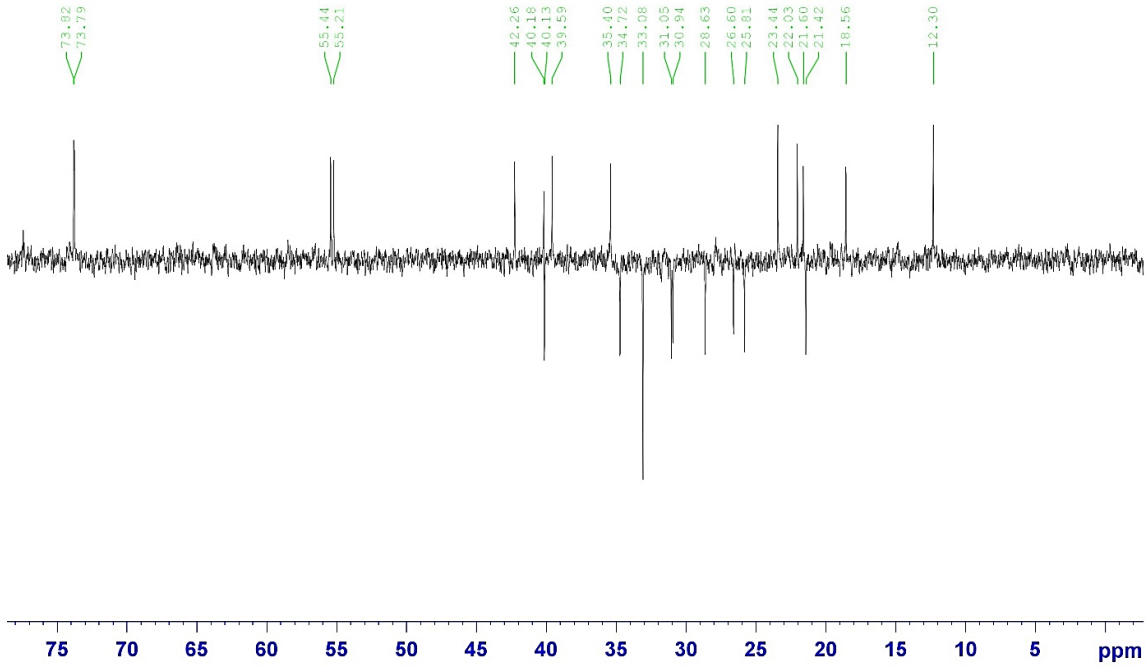


Figure 3.19 - DEPT-135 spectrum of compound (3.4) shows 14 signals up and 8 down (Note: δ=33.08 corresponds to 2 CH_2).

3.2 - Urea derivatives

3.2.1 – Urea derivatives in cholic acid

After the protection of the hydroxyl groups, urea bile acid derivatives were synthesized. The general reaction with the respective conditions is represented in figure 3.20.

The carboxylic acid in C-24 is activated in the presence of triethylamine (TEA), a basic reagent. Then, the carboxylate is treated with diphenylphosphoryl azide (DPPA), occurring two nucleophilic substitution reactions. Curtius rearrangement occurs through thermal decomposition, producing the isocyanate compound. The isocyanate then can react with amines, originating urea derivatives as shown in figure 3.20 (Czako, 2005; Ninomiya, Shioiri and Yamada, 1974).

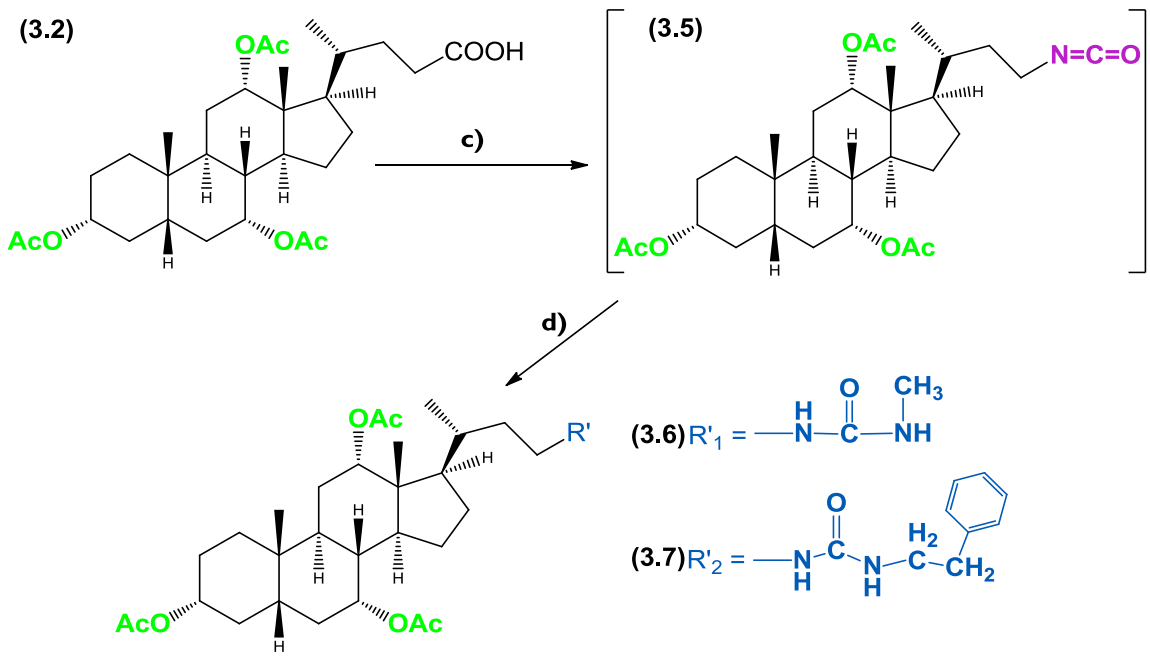


Figure 3.20 - Formation of urea derivatives in cholic acid through isocyanate reactions. Reagents and conditions: c) DPPA, TEA, toluene, 111°C (reflux), N_2; d) respective amine reagent, DCM, 30°C, N_2.

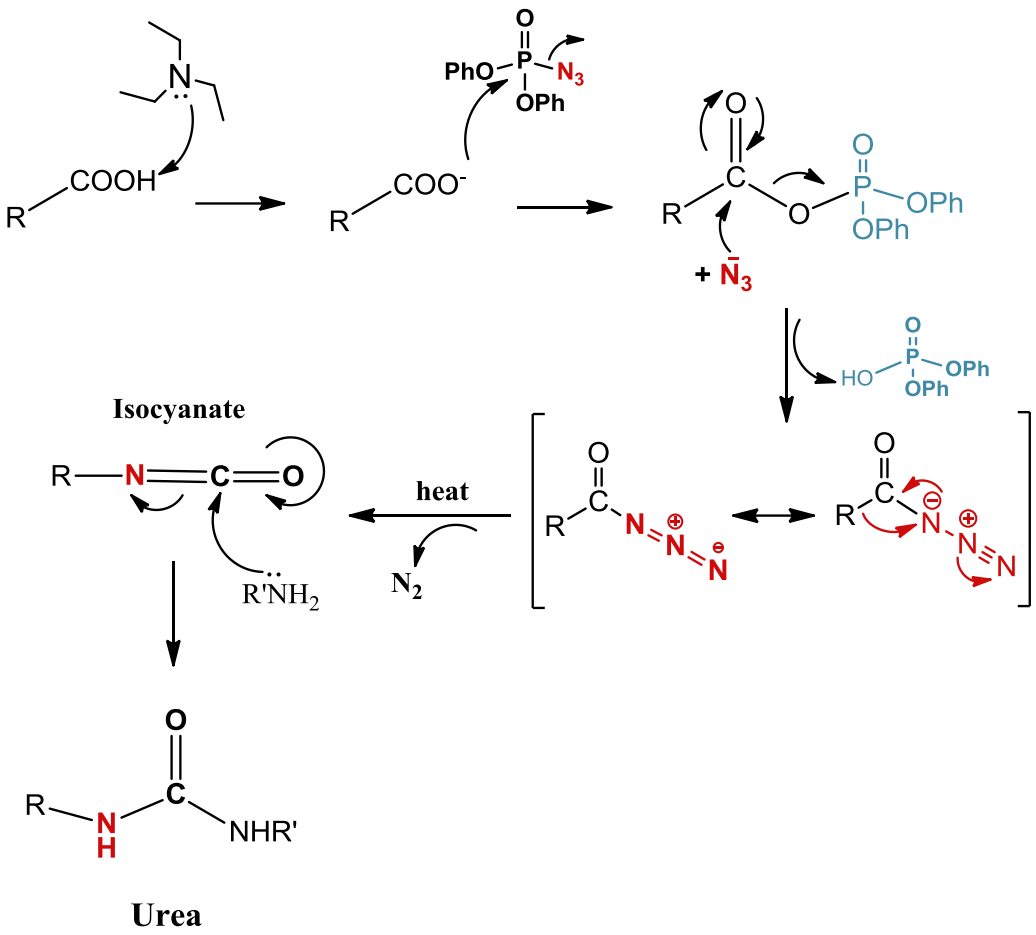


Figure 3.21 - Scheme of the reaction mechanism from the carboxylic acid to the isocyanate, using TEA and DPPA.

3.2.1.1 – Isocyanate introduction

Although the isocyanate 3.5 is a reaction intermediate, it was isolated by FCC for posterior characterization. Its ^{1}H NMR spectrum is represented in figure 3.22. The three signals, corresponding to three protons each, between δ = 1.50 ppm and δ = 2.00 ppm are consistent with three acetate groups. In addition, the three signals, again integrating three protons each, until δ = 1.00 ppm are originated by the three protons of each non-acetyl methyl. The three signals between δ = 4.50 ppm and δ = 5.50 ppm can be attributed to each proton bound to C-3, C-7 and C-12, as with happens for compound 3.2. The signals near δ = 3.00 ppm were assigned to the protons of the carbon directly attached to the isocyanate group (figure 3.23). This interesting signal splitting is the **spin-spin coupling**, when there are magnetic interactions between neighbouring non-equivalent NMR-active nuclei (Soderberg, 2017).

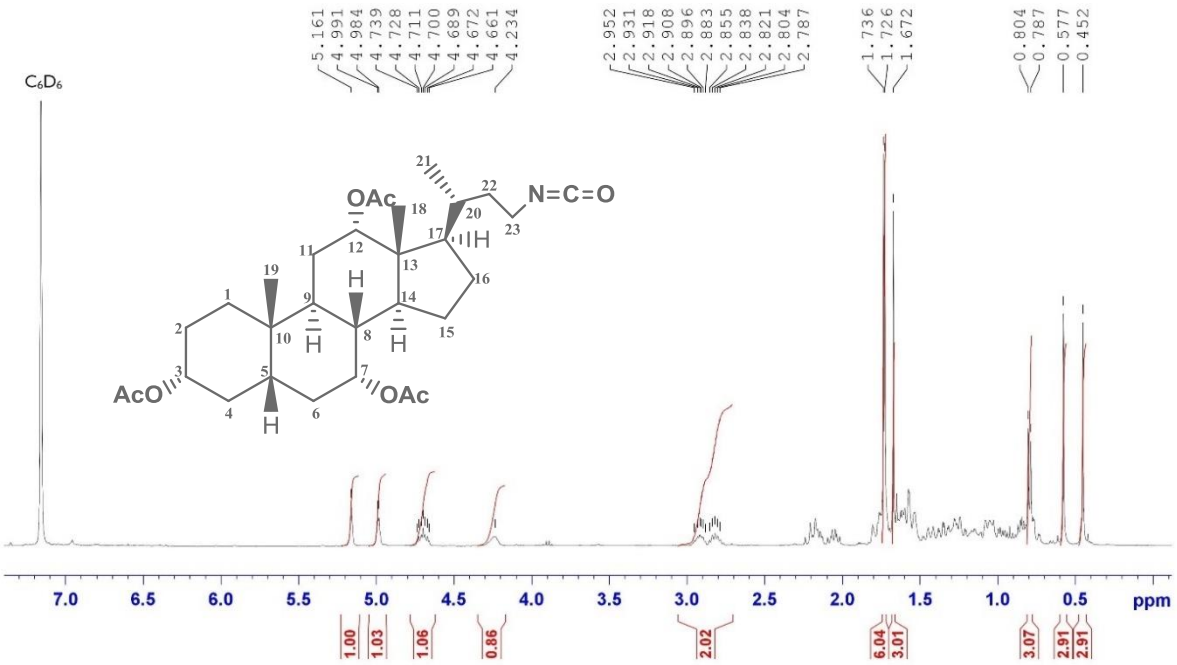


Figure 3.22 - ^{1}H NMR spectrum of compound 3.5, the intermediate for urea formation.

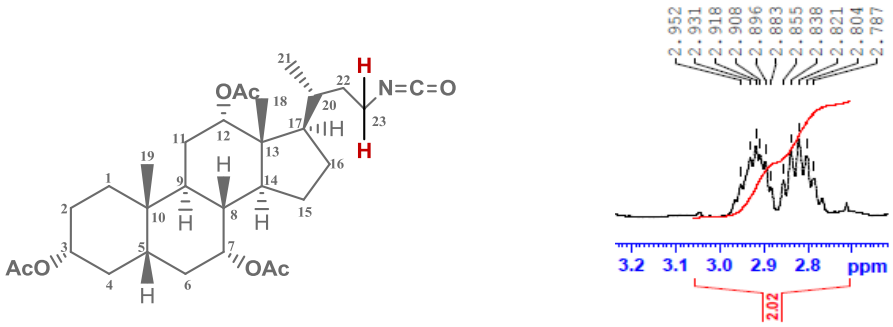


Figure 3.23 - ^{1}H NMR spectrum's zoom in of the signal correspondent to the protons of the carbon directly attached to the isocyanate group.

The ^{13}C NMR spectrum (figure 3.24) confirms the 30 carbons of the compound, with 29 signals, being the signal at δ=169.92 ppm correspondent to two carbons. The DEPT-135 spectrum (figure 3.25) shows 15 signals, corresponding to both CH_3 and CH of the compound and 9 signals corresponding to CH_2.

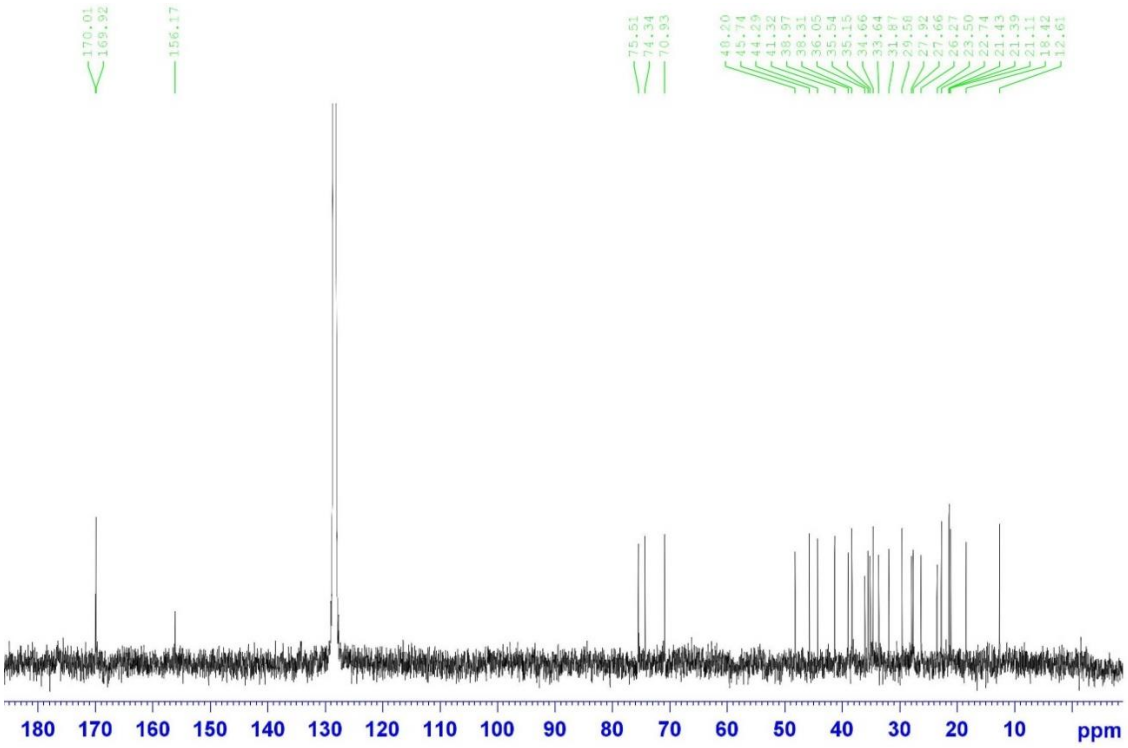


Figure 3.24 - ^{13}C NMR spectrum of compound 3.5 with 29 signals (Note: δ=169.92 ppm corresponds to 2C).

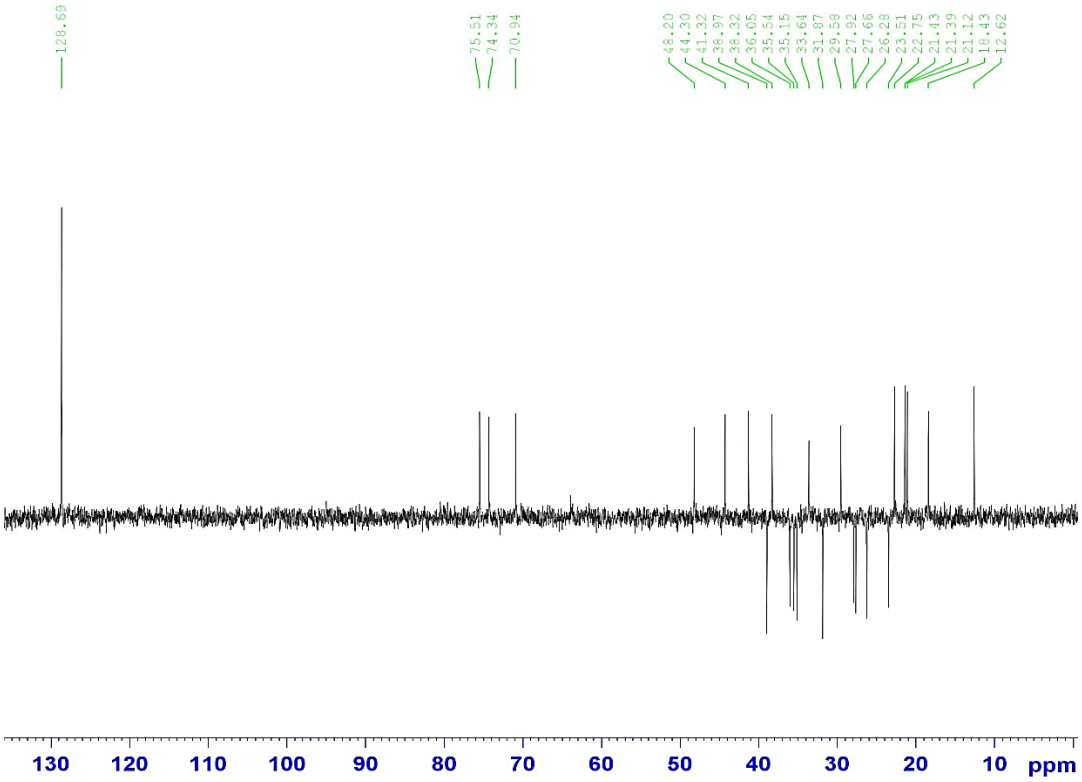


Figure 3.25 - DEPT-135 spectrum of compound 3.5 with 15 signals up and 9 signals down.

3.2.1.2 – Methyl urea

The methyl urea (compound 3.6) was obtained according to the reaction conditions indicated in figure 3.21 and purified by FCC, with a yield of 46%.

In the ^{1}H NMR spectrum (figure 3.26) the signal at 2.17 ppm can be attributed to the N-CH_3 protons. Additional analytical methods, such as mass spectrometry or elemental analysis, are required to confirm the presence of the methyl urea group.

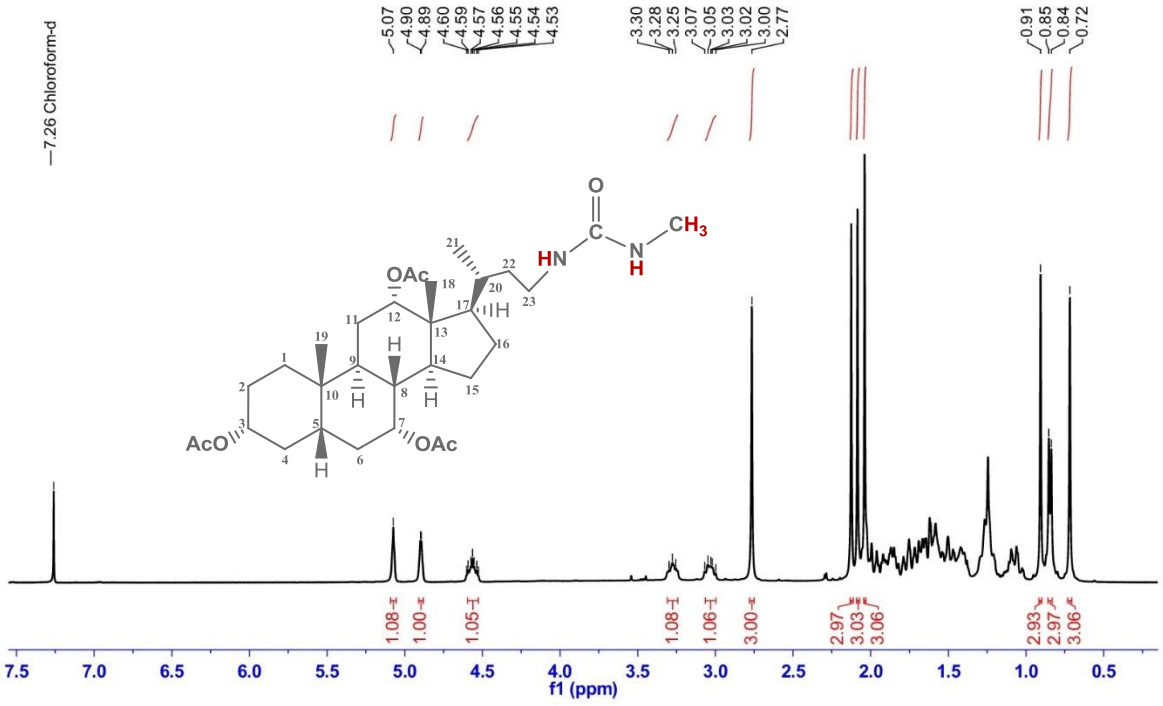


Figure 3.26 - ^{1}H NMR spectrum of compound 3.6.

In the ^{13}C NMR spectrum (figure 3.27) only 28 signals can be detected where the values at δ=170.57 and δ=21.49 ppm correspond to two C=O and two C, respectively. The carbonyl carbon of the methyl urea is missing, possibly because of the insufficient time of accumulation of the spectrum. The DEPT-135 spectrum (figure 3.29) shows an additional signal in comparison to the DEPT of compound 3.5, correspondent to an additional CH_3, confirming the introduction of the urea methyl group.

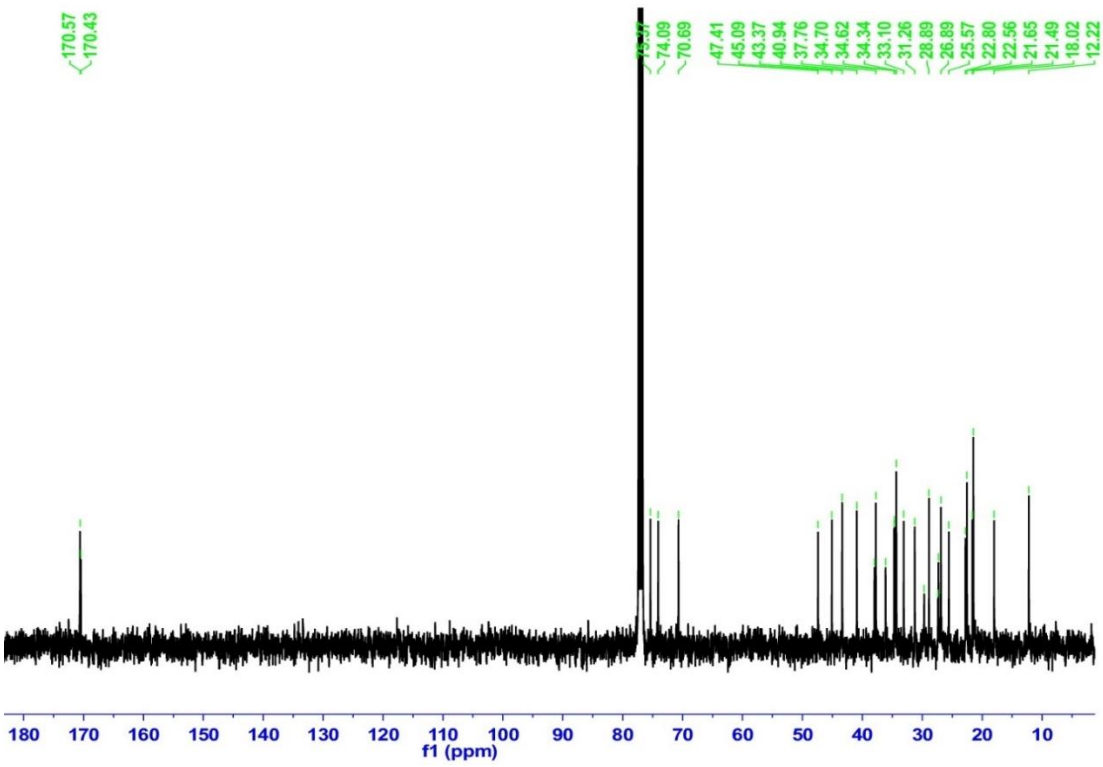


Figure 3.27 - ^{13}C NMR spectrum of compound 3.6, which shows 28 signals (Note: δ=170.57 ppm and δ= 21.49 corresponds to 2 C=O and 2 C, respectively; the fourth C=O is missing).

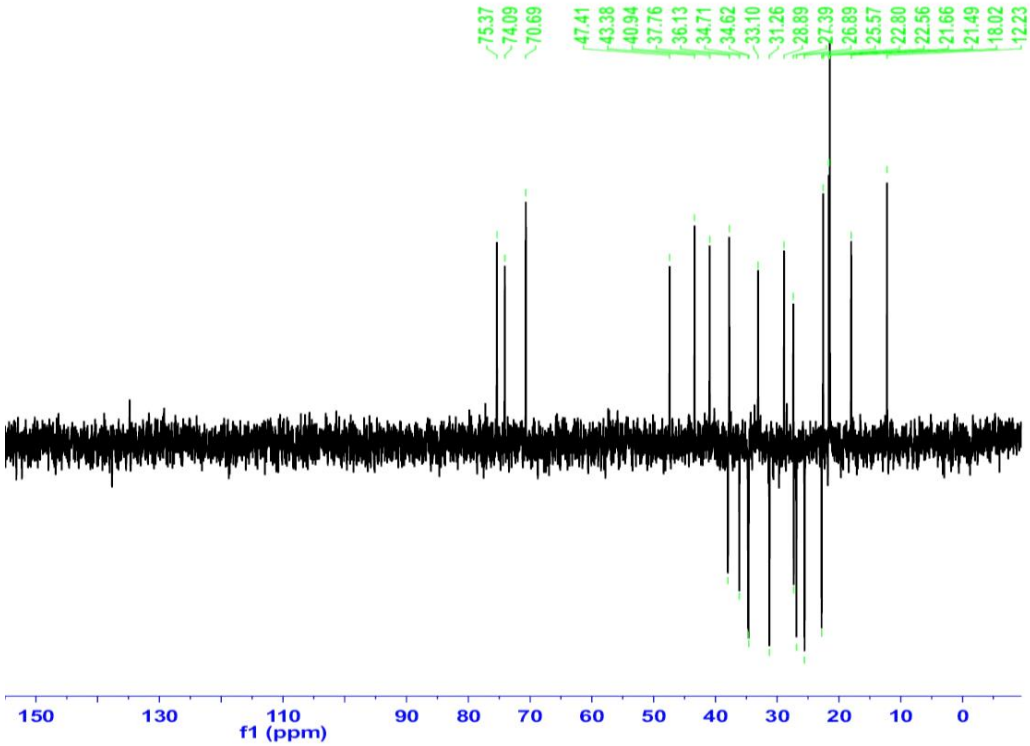


Figure 3.28 - DEPT-135 spectrum of compound 3.6 shows one additional signal up in comparison to isocyanate, which corresponds to CH_3, confirming the introduction of the urea methyl group (Note: δ=21.49 ppm corresponds to 2 C).

3.2.1.3 – Phenylethyl urea

The phenylethyl urea (compound 3.7) was prepared according to the reaction conditions indicated in figure 3.21 and was purified by Preparative TLC, since it has an aromatic group, with a yield of 51%.

The signals appearing between δ=7.00 and δ=7.50 ppm in the ^{1}H NMR spectrum (figure 3.29) are consistent with the presence of an aromatic group. The signal between δ=3.42 and δ=3.45 and the signal between δ=2.79 and δ=2.97 are attributed to the protons of each CH_2 of the ethyl group which is between the urea and the phenyl. Additional analytical methods are required as well, to confirm the presence of the phenylethyl urea group.

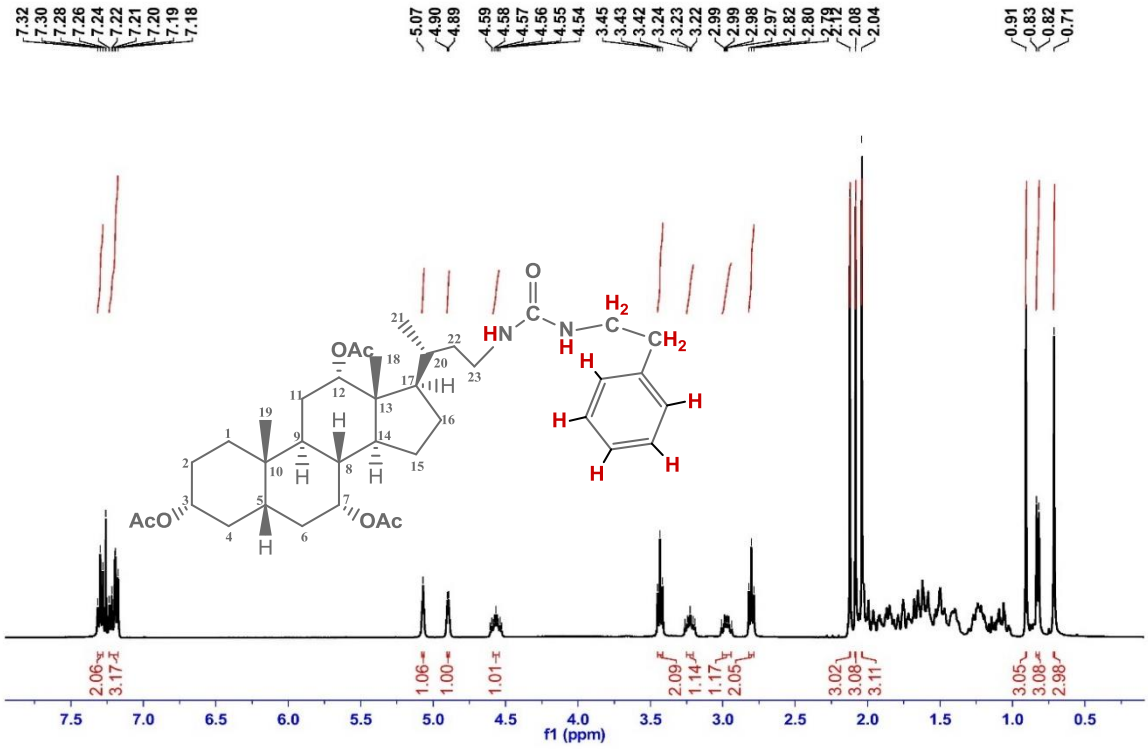


Figure 3.29 - ^{1}H NMR spectrum of compound 3.7.

In the ^{13}C NMR spectrum (figure 3.30) only 36 signals are detected but, of these, two signals (at δ = 128.82 and δ = 128.65 ppm) correspond to two carbons each (equivalent aromatic carbons). The other two aromatic carbons appear at δ = 139.05 and δ = 126.50 ppm and the signal of the urea carbonyl is at δ = 158.15, which sums a total of 38 carbons (30 + 6 from the benzyl + 2 from the ethyl group).

The DEPT-135 spectrum (figure 3.31) shows 18 signals associated to CH_3 and CH (2 of them are assigned to two carbons) and 11 corresponding to CH_2.

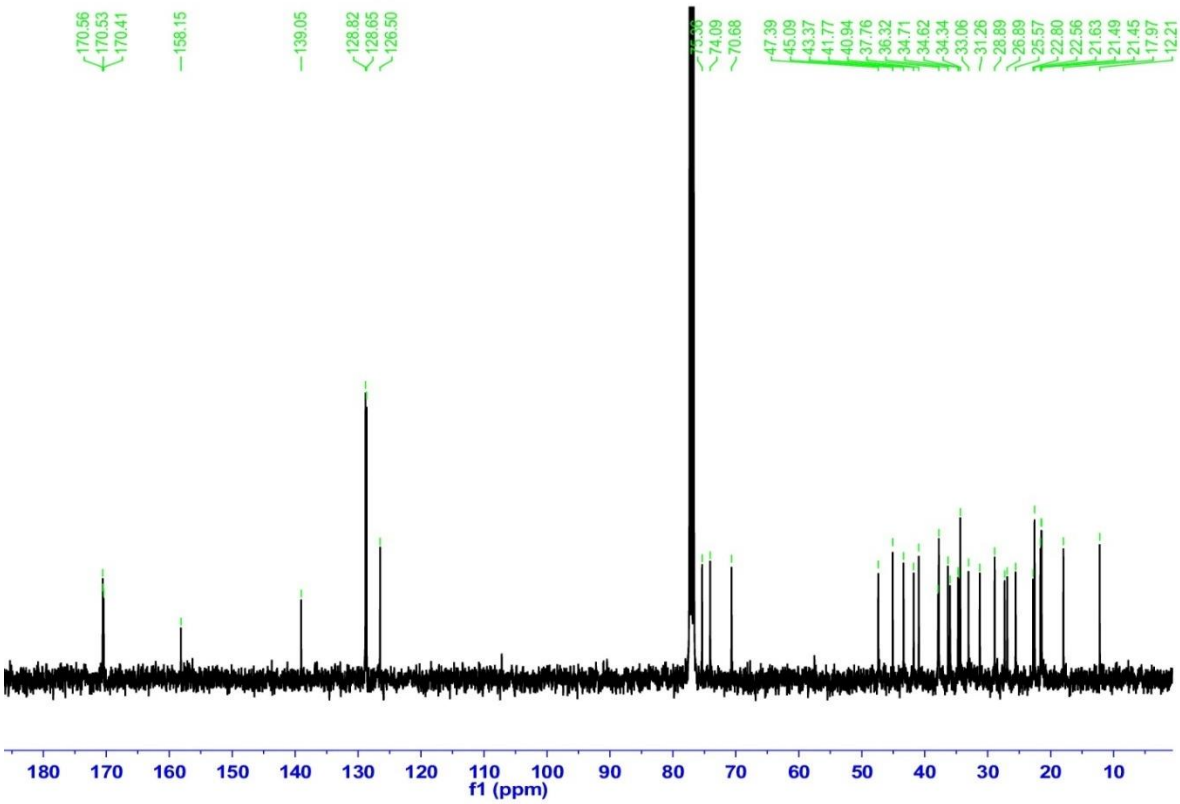


Figure 3.30 - ^{13}C NMR spectrum of compound 3.7 with a total of 38 carbons. (Note: δ = 128.82 and δ = 128.65 ppm correspond to 2C).

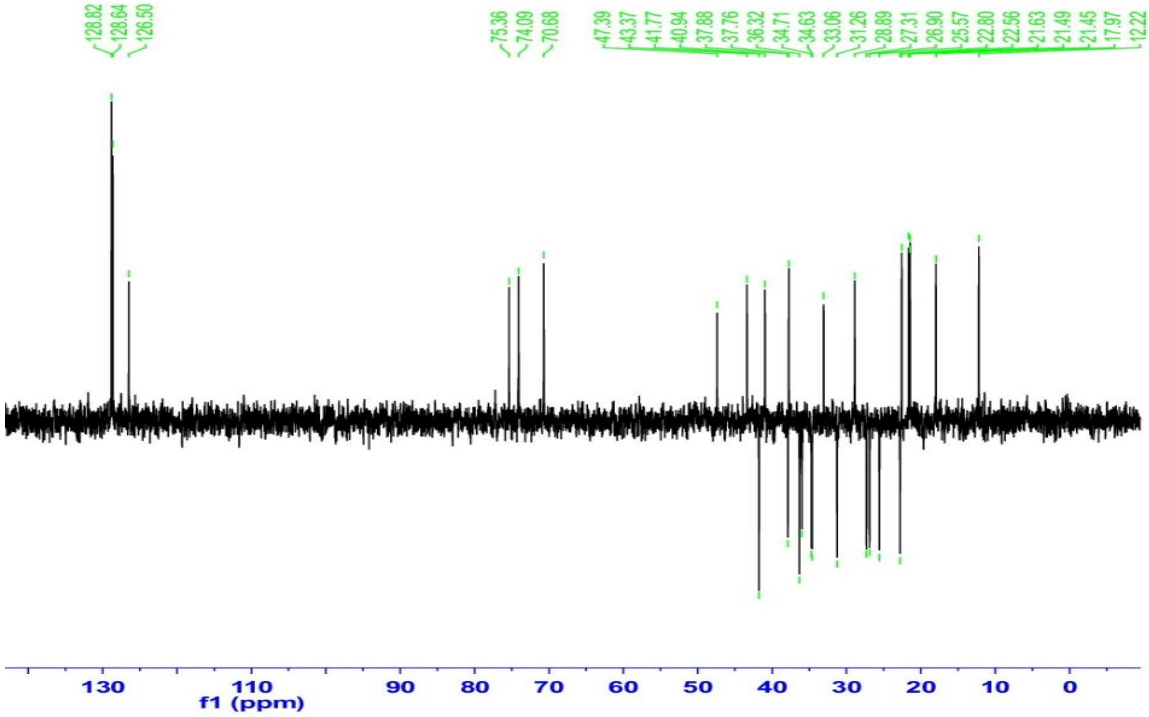


Figure 3.31 - DEPT-135 spectrum of compound 3.7 (Note: δ = 128.82 and δ = 128.65 ppm correspond to 2C).

4 - Conclusions

4 - Conclusions

The synthesis of several BA derivatives such as acetylated and methylated compounds and cholic acid urea derivatives was successfully achieved. Reactions of protection were needed in order to obtain the final compounds with no formation of secondary products and with better yields.

NMR spectra allowed to partially elucidate the chemical structure, although other analytical methods, including mass spectrometry and elementar analysis are required to confirm the structure of the new compounds.

As future perspectives, it is of the utmost interest to evaluate the biological activity of these compounds namely through *in vitro* assays as well as to apply other different chemical reactions in several BAs, for structural diversification and obtain relevant structure activity relationship data.

5 - Experimental procedures

5 - Experimental procedures

5.1 - General aspects

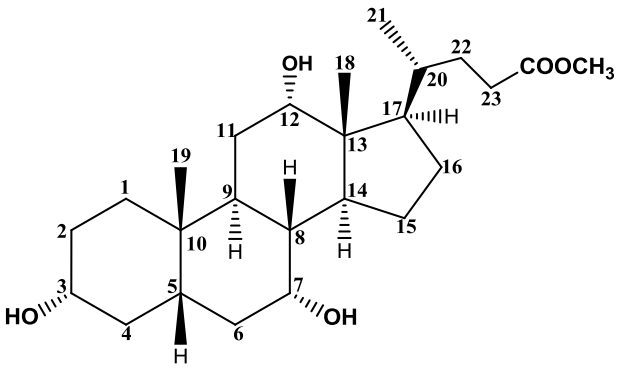
The evolution of chemical reactions was analysed by thin layer chromatography (TLC), using Merck's 60 F_{254} silica plaques with aluminum support. The plaques were then revealed by heating, after applying a mixture of sulfuric acid and ethanol as the revealing solution (5:95). Most of the compounds were purified by flash column cromatography (FCC), using silica-gel Kieselgel 60 (230-400 mesh, Merck). Some of the compounds have aromatic groups and were purified using Preparative TLC with Kieselgel 60H_{F254}/Kieselgel 60G.

Nuclear magnetic resonance spectra were obtained with Bruker Avance III spectrometers, operating at 400 MHz (^{1}H NMR) and 100 MHz (^{13}C NMR). Deuterated chloroform ($CDCl_3$) or deuterated benzene (C_6D_6) were the solvents used, chemical shift (δ) values are represented in parts per million (ppm) and the coupling constants (J) are expressed in Hz. The standard values used were δ 7.24 in ^{1}H NMR and δ 77.23 in ^{13}C NMR, corresponding to $CDCl_3$. Melting points were measured using a BUCHI melting point B-540 apparatus.

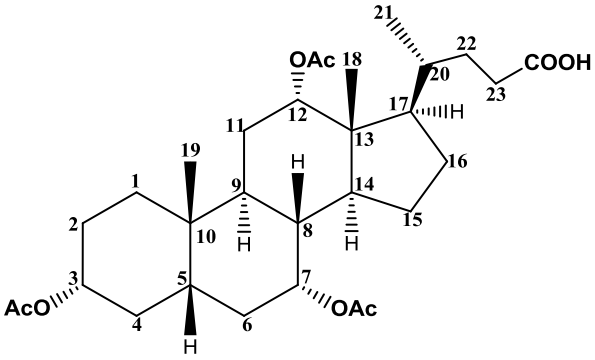
Compounds, solvents and reagents:

Cholic acid (CA), Ursodesoxycholic acid (UDCA), 4-dimetilaminopyridine (DMAP), acetic anhydride [$(CH_3CO)_2O$], Tetrahydrofuran (THF), potassium carbonate, methyl iodide (CH_3I), dimethylformamide (DMF), diphenyl phosphoryl azide (DPPA), toluene, triethylamine (TEA), dichloromethane (DCM), phenylethylamine, methylamine, ethyl acetate and HCl were purchased from Merck & Co and VWR Portugal. Sodium bicarbonate ($NaHCO_3$) and sodium chloride (NaCl) were obtained commercially and prepared in the laboratory for work-up washing solutions. The dry solvents used were previously dried according to usual procedures (Armarego and Chai, 2003).

5.2 – Chemical synthesis

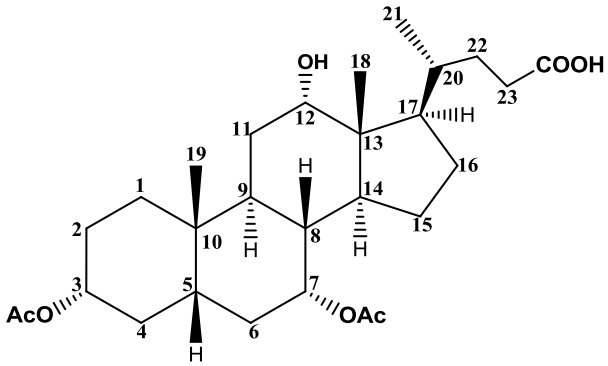


Methyl 3α,7α,12α-Trihydroxy-5β-cholan-24-oate (3.1): To a stirred solution of cholic acid (1) (250.4 mg, 0.613 mmol) and anhydrous potassium carbonate (211.5 mg, 1.532 mmol, 2.5 eq.) in DMF (6 mL), methyl iodide (0.08 mL; 1.226 mmol, 2 eq.) was added. The reaction mixture was stirred at room temperature in anhydrous conditions. After 1h and 30min, it was dispersed by water (60 mL) and extracted with ethyl acetate (3x60mL). The resulting organic phase was washed with 5% aqueous HCl (2x60 mL), 10% aqueous $NaHCO_3$, (2x60 mL), water (2x60 mL) and brine (60 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum to afford (3.1) as a white powder (261.4 mg, 98%). Mp: 77-80,1°C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H 3.96 (*m*, 1H, H-12), 3.85 (*m*, 1H, H-7), 3.66 (*s*, 3H, COOC<u>H</u>$_3$), 3.44 (*m*, 1H, H-3), 0.98 (*d*, *J* = 6,16 Hz, 3H, H-21), 0.88 (s, 3H, H-19), 0.68 (s, 3H, H-18). ^{13}C NMR ($CDCl_3$, 100 MHz): δ_C 174.79 (<u>C</u>OOCH$_3$, C-24), 73.02, 71.94, 68.45, 51.51 (COO<u>C</u>H$_3$), 47.07, 46.47, 41.78, 41.46, 39.64, 39.54, 35.26, 35.22, 34.73, 34.63, 31.03, 30.90, 30.47, 28.23, 27.47, 26.49, 23.21, 22.49, 17.32, 12.50.

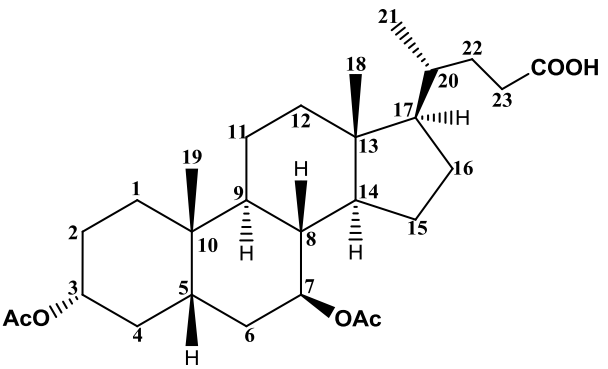


3α, 7α, 12α-triacetoxy-5β-cholane-24-oic acid (3.2): To a stirred solution of cholic acid (350.5 mg, 0.857 mmol) in dry THF (12.5 mL), acetic anhydride (2.4 mL; 25.74 mmol, 30 eq.) and a catalytic amount of DMAP (105.2 mg) were added. The mixture was stirred at 66°C in anhydrous conditions. After 2h and 30min, the reaction mixture was evaporated under reduced pressure to remove the organic phase. The obtained crude was dispersed by water (50 mL) and extracted with ethyl acetate (3×50 mL). The resulting organic phase was washed with 5% aqueous HCl (2×50 mL), 10% aqueous $NaHCO_3$ (2×50 mL), water (50 mL) and brine (50 mL), dried over Na_2SO_4, filtered and concentrated under vacuum. The crude solid was later purified by flash column chromatography to afford compound (3.2) as a white powder (155mg, 44%). Mp: 92-94,5°C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.06 (*s*, 1H, H-12); 4.89 (*m*, 1H, H-7); 4.55 (*m*, 1H, H-3); 2.11 (*s*, 3H, OCOC<u>H</u>$_3$); 2.06 (*s*, 3H, OCOC<u>H</u>$_3$); 2.02 (*s*, 3H,

OCOC$\underline{H}_3$); 0.89 (*s*, 3H, C$\underline{H}_3$); 0.80 (*d*, J=6.37 Hz, 3H, C$\underline{H}_3$); 0.71 (*s*, 3H, C$\underline{H}_3$). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 179.56 (C$\underline{O}$OH), 170.80 (C$\underline{O}$OCH$_3$), 170.75 (C$\underline{O}$OCH$_3$), 170.64 (C$\underline{O}$OCH$_3$), 75.61, 74.32, 70.93, 47.56, 45.29, 43.61, 41.14, 37.96, 34.91, 34.82, 34.76, 34.55, 31.46, 30.95, 30.74, 29.10, 27.38, 27.10, 25.78, 23.01, 22.77, 21.82, 21.70, 21.63, 17.70, 12.44.

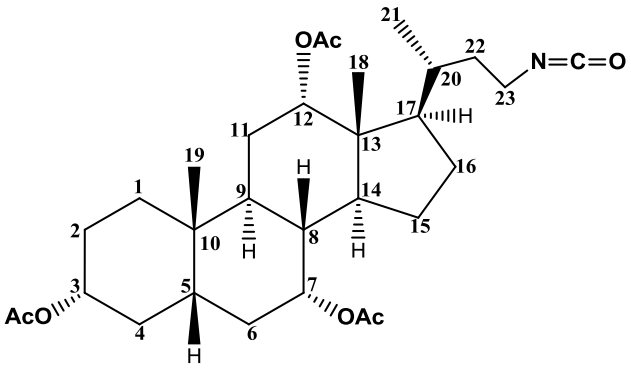


3α,7α-diacetoxy-12α-hidroxy-5β-cholane-24-oic acid (3.3): using the same method described for (3.2), the crude solid was purified by flash column chromatography to afford compound (3.3) as a white powder (17.9mg, 5%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 4.86 (*m*, 1H); 4.69 (*s*, 1H); 4.52 (*m*, 1H); 2.04 (*s*, 3H, OCOC$\underline{H}_3$); 2.01 (*s*, 3H, OCOC$\underline{H}_3$); 0.98 (*d*, J=7.41 Hz, 3H, C$\underline{H}_3$); 0.90 (*s*, 3H, C$\underline{H}_3$); 0.84 (*s*, 3H, C$\underline{H}_3$). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 176.87, 171.06 (C$\underline{O}$OCH$_3$), 170.85 (C$\underline{O}$OCH$_3$)), 80.13, 74.05, 70.51, 47.55, 45.69, 43.32, 41.06, 37.27, 34.97 (2C), 34.65, 34.51, 33.33, 31.43, 30.17, 29.54, 26.59, 26.51, 25.95, 22.64, 22.32, 21.93, 21.66, 17.17, 14.60.

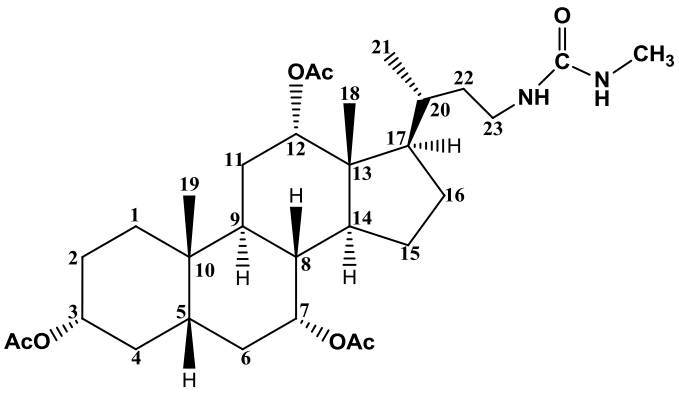


3α,7β-diacetoxy-5β-cholane-24-oic acid (3.4): according to the method described for (3.2), using ursodeoxycholic acid (600 mg, 1,53 mmol), THF (22.4 mL), acetic anhydride (0.9 mL; 9.18 mmol, 6 eq.) and DMAP (60 mg). The mixture was stirred at room temperature in anhydrous conditions. After 3h, the reaction mixture was evaporated under reduced pressure to remove the organic phase. The obtained crude was dispersed by water (60 mL) and extracted with ethyl acetate (3×60 mL). The resulting organic phase was washed with 5% aqueous HCl (2×60 mL), 10% aqueous $NaHCO_3$ (2×60 mL), water (60 mL) and brine (60 mL), dried over Na_2SO_4, filtered and concentrated under vacuum. An amount of the crude solid was isolated (120 mg) and purified by flash column chromatography to afford compound (3.4) as a white powder (30mg, 25%). Mp: 90-91°C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 4.74 (*m*, 1H, C$\underline{H}$OAc); 4.65 (*m*, 1H, C$\underline{H}$OAc); 2.00 (*s*, 3H, OCOC$\underline{H}_3$); 1.96 (*s*, 3H, OCOC$\underline{H}_3$); 0.95 (*s*, 3H, C$\underline{H}_3$); 0.90 (*d*, J=7.40 Hz, 3H, C$\underline{H}_3$); 0.66 (*s*, 3H, C$\underline{H}_3$). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 176.87,

171.06 (<u>C</u>OOCH$_3$), 170.85 (<u>C</u>OOCH$_3$)), 80.13, 74.05, 70.51, 47.55, 45.69, 43.32, 41.06, 37.27, 34.97 (2C), 34.65, 34.51, 33.33, 31.43, 30.17, 29.54, 26.59, 26.51, 25.95, 22.64, 22.32, 21.93, 21.66, 17.17, 14.60.



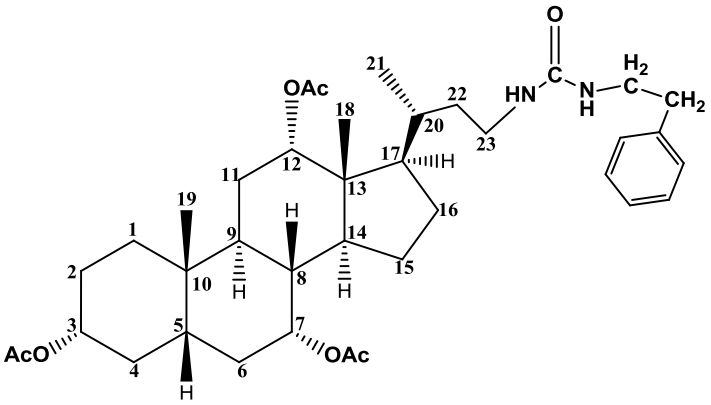
3α, 7α, 12α-triacetoxy-5β-cholane-24-isocyanate (3.5): To a stirred solution of compound (3.2) in dry toluene (100 mg, 0,187 mmol), catalytic amounts of dry triethylamine and DPPA were added. The reaction mixture was stirred at 111°C, under N_2 atmosphere and left overnight. Then, the solvent was evaporated under reduced pressure. The organic phase was dispersed in water (40 mL), extracted with ethyl acetate (3x40mL) and then washed with 5% aqueous HCl (2×40 mL), 10% aqueous $NaHCO_3$ (2×40 mL), water (40 mL) and brine (40 mL). Then, it was dried over Na_2SO_4, filtered and concentrated under vacuum. The crude solid was later purified by flash column chromatography to afford compound (3.5) as a white powder (15%). ^{1}H NMR (C_6D_6, 400 MHz): δ 5.16 (*s*, 1H, H-12); 4.99 (*m*, 1H, H-7); 4.70 (*m*, 1H, H-3); 2.88 (*m*, 2H, C<u>H</u>$_2$NCO); 1.74 (*s*, 3H, OCOC<u>H</u>$_3$); 1.73 (*s*, 3H, OCOC<u>H</u>$_3$); 1.67 (*s*, 3H, OCOC<u>H</u>$_3$); 0.79 (*d*, J=6.35 Hz, 3H, C<u>H</u>$_3$); 0.58 (*s*, 3H, C<u>H</u>$_3$); 0.45 (*s*, 3H, C<u>H</u>$_3$). ^{13}C NMR (100 MHz, C_6D_6): 170.01 (<u>C</u>OOCH$_3$), 169.92 (2C-(<u>C</u>OOCH$_3$)), 156.17, 75.51, 74.34, 70.93, 48.20, 45.74, 44.29, 41.32, 38.97, 38.31, 36.05, 35.54, 35.15, 34.66, 33.64, 31.87, 29.58, 27.92, 27.66, 26.27, 23.50, 22.74, 21.43, 21.39, 21.11, 18.42, 12.61.



3α, 7α, 12α-triacetoxy-5β-cholane-24-methylurea (3.6): according to the method described for (3.5), to a stirred solution of (3.2) (80,7 mg; 0,151 mmol) in dry toluene (2,1 mL), catalytic amounts of dry triethylamine and DPPA were added. The reaction mixture was stirred at 111°C, under N_2 atmosphere, and left overnight. Then, the solvent was evaporated under reduced pressure. To the resulting crude, dry DCM (3 mL) and methylamine (0,05 mL; 1,13 mmol) were added and left reacting, under N_2 atmosphere, at 30°C. After 5h, the solvent was evaporated under reduced pressure. The organic phase was

dispersed in water (30 mL), extracted with ethyl acetate (3x30mL) and then washed with 5% aqueous HCl (2×30 mL), 10% aqueous $NaHCO_3$ (2×30 mL), water (30 mL) and brine (30 mL). Then, it was dried over Na_2SO_4, filtered and concentrated under vacuum. The crude solid was later purified by flash column chromatography to afford compound (3.6) as a white powder (46%). Mp: 104-106,5°C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.07 (*s*, 1H, H-12); 4.90 (*m*, 1H, H-7); 4.56 (*m*, 1H, H-3); 2.77 (*s*, 3H, NHC<u>H</u>$_3$); 2.13 (*s*, 3H, OCOC<u>H</u>$_3$); 2.08 (*s*, 3H, OCOC<u>H</u>$_3$); 2.04 (*s*, 3H, OCOC<u>H</u>$_3$); 0.91 (*s*, 3H, C<u>H</u>$_3$); 0.85 (*d*, J=6.40 Hz, 3H, C<u>H</u>$_3$); 0.72 (*s*, 3H, C<u>H</u>$_3$).

^{13}C NMR (100 MHz, $CDCl_3$): δ 170.57(2C-(<u>C</u>OOCH$_3$)), 170.43 (<u>C</u>OOCH$_3$), 75.37, 74.09, 70.69, 47.41, 45.09, 43.37. 40.94, 37.97, 37.76, 36.12, 34.70, 34.62, 34.34, 33.10, 31.26, 29.69, 28.89, 27.39, 27.32, 26.89, 25.57, 22.80, 22.56, 21.65, 21.49, 18.02, 12.22.



3α, 7α, 12α-triacetoxy-5β-cholane-24-phenethylurea (3.7): according to the method described for (3.6), using phenylethylamine (0,06 mL; 0,48 mmol). After 4h30, the solvent was evaporated under reduced pressure. The organic phase was dispersed in water (30 mL), extracted with ethyl acetate (3x30mL) and then washed with 5% aqueous HCl (2×30 mL), 10% aqueous $NaHCO_3$ (2×30 mL), water (30 mL) and brine (30 mL). Then, it was dried over Na_2SO_4, filtered and concentrated under vacuum. The crude solid was later purified by Preparative TLC to afford compound (3.7) as a white powder (51%). Mp: 103-105°C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.28 (*m*, 2H, Ar-H); 7.20 (*m*, 3H, Ar-H); 5.07 (*s*, 1H, H-12); 4.90 (*m*, 1H, H-7); 4.57 (*m*, 1H, H-3); 3.43 (*t*, J =6.98 Hz, 2H, Ar-C<u>H</u>$_2$); 2.80 (*t*, J =6.78 Hz, 2H, CONHC<u>H</u>$_2$); 2.12 (*s*, 3H, OCOC<u>H</u>$_3$); 2.08 (*s*, 3H, OCOC<u>H</u>$_3$); 2.04 (*s*, 3H, OCOC<u>H</u>$_3$); 0.91 (*s*, 3H, C<u>H</u>$_3$); 0.83 (*d*, J=6.59 Hz, 3H, C<u>H</u>$_3$); 0.71 (*s*, 3H, C<u>H</u>$_3$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.56 (<u>C</u>OOCH3), 170.53 (<u>C</u>OOCH$_3$), 170.41 (<u>C</u>OOCH$_3$), 158.15 (NH<u>C</u>ONH), 139.05, 128.82 (2C), 128.65 (2C), 126.50, 75.36, 74.09, 70.68, 47.39, 45.09, 43.37, 41.77, 40.94, 37.88, 37.76, 36.32, 35.99, 34.71, 34.62, 34.34, 33.06, 31.26, 28.89, 27.31, 26.89, 25.57, 22.80, 22.56, 21.63, 21.49, 21.45, 17.97, 12.21.

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