BISPHOSPHONATE PRODRUGS UTILIZING ENDOGENOUS CARRIERS

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ABSTRACT

Targeting of therapeutic agents to a specific site, as well as controlling the rate and time of release, has been intensively investigated and established over the last decades. These studies concerning drug delivery systems led to the formulation of several products that can improve the diffusion across the barriers after drug administration.

For this purpose, the development of strategies of novel drug delivery systems for bisphosphonates had taken hold to improve both the bioavailability and safety. Firstly, they have been used for over a century in the branch of industry and later, in the 1960s, in medicine. Bisphosphonates are synthetic compound analogs to the naturally occurring pyrophosphates. They are characterized by two phosphate groups bound to the same carbon atom, a 'P-C-P' moiety, and two functional groups R^1 and R^2. Mainly, bisphosphonates are effective inhibitors of bone resorption due to calcium disorders, such as osteoporosis, Paget's disease, hypercalcemia of malignancy, multiple myeloma, and bone metastasis. Generally, they can be classified into two groups according to their mechanism of action: non-nitrogen containing bisphosphonates and nitrogen containing bisphosphonates.

However, the therapeutic use of bisphosphonates is restricted by their poor oral bioavailability (less than 1%) due either to their hydrophilic properties and to the bulky and charged phosphonate groups. In order to overcome these issues and enhance the intestinal permeability of bisphosphonates, a prodrug design approach has been evaluated for these compounds. In the literature review of this thesis an overview of some of the available synthetic strategies, which involve the modification of the functional groups with a bioreversible promoiety according to the desired properties in the compound, is presented.

The experimental part is aimed to conjugate bisphosphonates with bile acids in order to improve the oral bioavailability of the bisphosphonate. To optimize the yield, three different synthetic strategies were tested. In all syntheses sodium alendronate was used as the bisphosphonate and deoxycholic acid as the bile acid.

PREFACE

The present thesis was done at the laboratories of Organic Chemistry, Department of Chemistry, University of Jyväskylä from October 2015 to October 2016.

I would like to thank my supervisor Elina Sievänen for the opportunity to work in her research group and guiding me through this interesting topic. The nice working atmosphere, her advices, and encouragements were essential for the development and success of this thesis.

My heartfelt thanks is for my family, to guide me and walk always by my side, to believe in me despite my right or wrong choices, and to continue to encourage me to pursue my dreams.

My deepest thanks is also for my friends, for all the support, the laughs, and the time spent together. I will keep in my heart every single memory.

Truly thanks to everyone who have brought happiness in my life.

Jyväskylä, October 2016.

Carolina Giammei

CONTENTS

ABBREVIATIONS

ADME: Absorption, Distribution, Metabolism and Excretion

ALN: Alendronate

AppCp-type: Non-hydrolyzable ATP Analogs

ASBT: Sodium Dependent Bile Acid Transporter

ATP: Adenosine Triphosphate

BPs: Bisphosphonates

CLOD: Clodronate

CMD: Carboxymethyldextran

DCC: *N*,*N*′-Dicyclohexylcarbodiimide

DDS: Drug Delivery System

DMF: *N*,*N*-Dimethylformamide

EDC: 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide

ETID: Etidronate

FDA: Food and Drug Administration

FPP: Farnesylpyrophosphate

FPPS: Farnesylpyrophosphate Synthase

GGPP: Geranylgeranylpyrophosphate

HAP: Hydroxyapatite

HPCCC: High Performance Countercurrent Chromatography

IBAN: Ibandronate

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

N-BPs: Nitrogen-containing BPs

NER: Neridronate

NHS: *N*-hydroxysuccinimide

ODDS: Osteotropic Dug Delivery System

P_{app}: Apparent Partition Coefficients

PAs: Polyamines

P_{eff}: Effective Permeability Coefficient

PEPT1: Oligopeptide Transporter

PPi: Pyrophosphate

RIS: Risedronate

TbFPPS: *T. brucei* farnesyl pyrophosphate synthase

TcFPPS: *T. cruzi* farnesyl pyrophosphate synthase

TMS: Tetramethylsilane

ZOL: Zolendronate

I LITERATURE PART

1. INTRODUCTION

Development of new therapeutic agents is expensive and time consuming. For this reason, the enhancement of safety and efficacy ratio of existing drugs has taken hold through the formulation of prodrugs.[1] Even though the prodrug concept was known over a century, the definition of a prodrug was introduced for the first time only in the end of the 1950s. Basically, prodrugs are biologically inactive derivatives of the active drugs that, once introduced into the body, must undergo enzymatic and/or chemical transformation in order to become active pharmacological agents. Therefore, prodrugs have become an established tool to improve undesirable properties regarding absorption, distribution, metabolism, and excretion (ADME).[2]

In the literature review of this thesis, after a brief description of drug delivery and prodrug approach, the focus shifts mainly to showing of the promising applicability of the prodrug design regarding a class of organophosphorus compounds known as bisphosphonates.

Even though the knowledge of bisphosphonates dates back to the middle of the 19th century for industrial applications, only later, in the 1960s they started to be used in medicine as treatment for calcium disorders.[3] Bisphosphonates are chemical analogs to the naturally occurring pyrophosphates. Pyrophosphates are characterized by a P-O-P backbone while bisphosphonates present a 'P-C-P' moiety more stable and capable to prevent calcification of tissues. Consequently, bisphosphonates are effective inhibitors of bone resorption due to calcium disorders, such as osteoporosis, Paget's disease, hypercalcemia of malignancy, multiple myeloma, and bone metastasis. Generally, they can be classified into two groups according to their mechanism of action: non-nitrogen containing bisphosphonates and nitrogen containing bisphosphonates.[4,5] The antiresorptive effect of bisphosphonates is strictly correlated to their structure.[6] Studies confirmed that the most potent bisphosphonates were those containing a nitrogen atom at appropriate distance from the 'P-C-P' moiety.[3] Their action is mainly due to the inhibition of farnesylpyrophosphate synthase of mevalonate pathway, an essential pathway for several cellular processes.[7]

However, the therapeutic use of bisphosphonates is restricted by their poor oral bioavailability (less than 1%) due either to their hydrophilic properties and to the bulky and charged phosphonate groups.[8] The absorption of bisphosphonates might be also affected by food, probably due to the conversion into a non-soluble aggregate with a

consequent reduction in absorption. Alternatively, high dose might effect on the intracellular junctions increasing the absorption. Unfortunately, this often leads to side-effects. In order to overcome the bioavailability and safety issues, many strategies of novel drug delivery systems for bisphosphonates have been developed.[9]

The synthetic strategies established to enhance the intestinal permeability of bisphosphonates is based on the reversible modification of one or more ionizable groups.[9] The functional groups that can be modified are OH and/or NH in the side chain or either of the two phosphorous groups according to the desired properties in the compounds.[10]

These functional groups may be acetylated, esterified, or converted to anhydrides. In addition, they may be conjugated with endogenous molecules, such as peptides, amino acids, fatty acids, steroids, carbohydrates, and liposomes.

This thesis presents an overview of some of the synthetic strategies mentioned above in order to overcome the restrictions due to the poor oral bioavailability and safety. The synthetic strategies that will be proposed involve the modification of the functional groups with a bioreversible promoiety. The biological evaluation of bisphosphonate prodrugs as well as future development of the research area are also described.

2. DRUG DISCOVERY AND DEVELOPMENT

2.1 Drug delivery

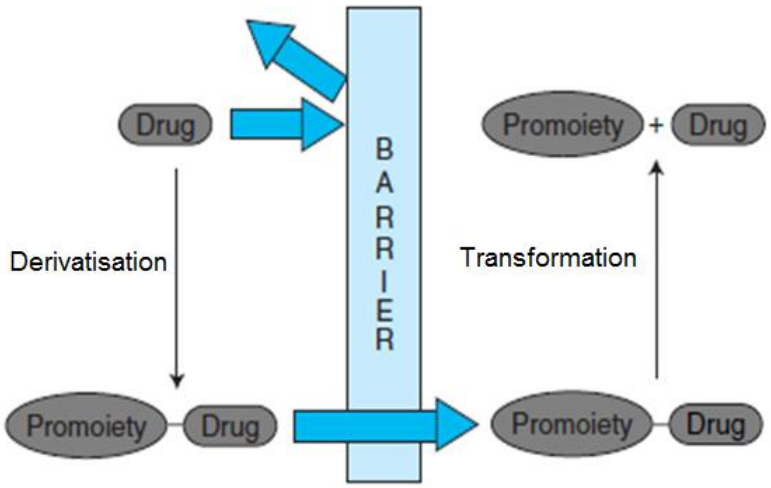
The aim of drug development is to put effort in the improvement of the efficiency of an existing drug instead of a new drug. Indeed, the cost and the effort of the discovery a new drug is really high and time consuming. For this reason drug delivery has taken hold.[11]

Drug delivery is the selective transport of the drug at the site of action by controlling the rate, time, and place of release of drugs in the body. A successful drug delivery system (DDS) is obtained after a thorough evaluation of the physicochemical properties of the drug. Nowadays, the most common routes of administration are oral, parenteral transdermal, and aerosol. Among them the preferred methods are the oral routes for both the formulation (chemically stable, easier to process, and more convenient to administer than liquid formulation) and the tolerance of the patient. Since the delivery method is chosen by the physiochemical properties of the drug, if there is some incompatibilities with the oral dosing then some other routes is taken in consideration.[12] After oral administration, the drug must overcome several barriers to reach its site of action. These barriers are needed in order to the body to inhibit the passage of toxic molecules. The hindrance can be physiological, biochemical, or chemical. In the case of oral administration of a drug, the first barrier is physiological represented by the intestinal mucosa that protects the body from unwanted molecules. The second barrier is the biochemical one composed of metabolizing enzymes able to degrade the drug. Finally, the drug needs to have the optimal physiochemical properties to be able to overcome the biological barriers. A lot of research concentrates on this particular area. One alternative is to chemically modify the drug producing prodrugs.[13]

2.2 Prodrugs

Several therapeutic drugs fail in the clinical application. Undesirable drug properties, such as low oral drug absorption, lack of site specificity, chemical instability, toxicity, bad taste, odor, or pain at injection site hinder the drugs to achieve the site of action. Prodrug strategies have been developed to overcome the pharmacological, pharmaceutical, and pharmacokinetic barriers.[14] Prodrugs are modified drugs covalently linked to a chemical moiety. The task of the chemical moiety, “promoiety”, is to help crossing the barriers. Prodrugs are inactive before the metabolism and act releasing the active drug from its promoiety. Once past the barriers the prodrug goes through a process

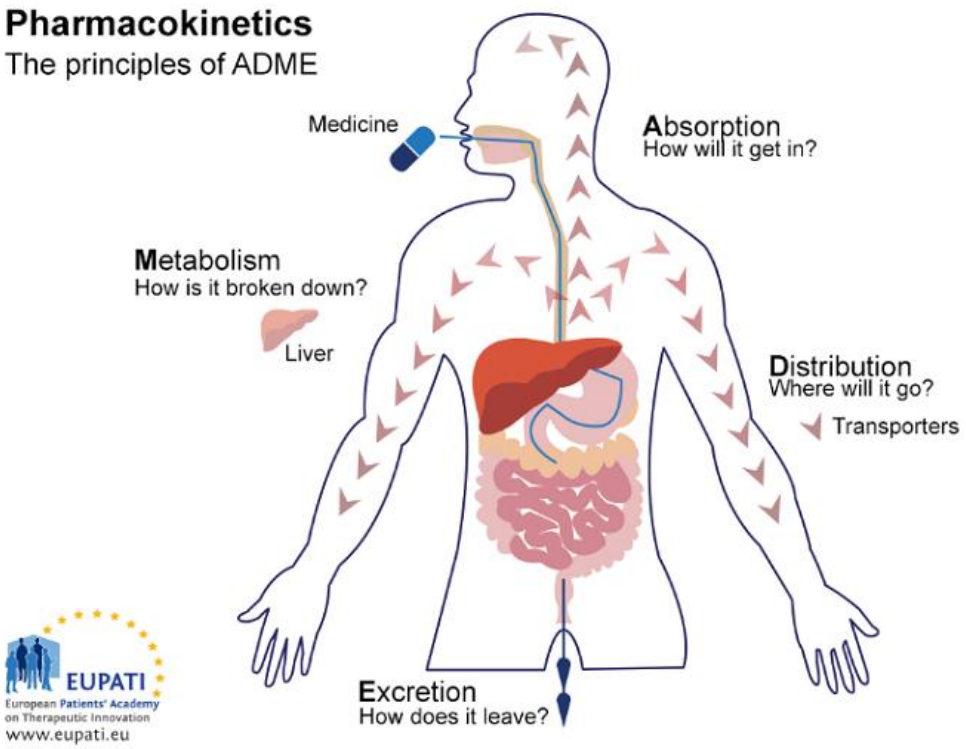
that can be enzymatic or nonenzymatic resulting the drug becoming active to exert its therapeutic effect in the optimal manner.[15] The prodrug concept is well explained by **Scheme 1**.[16]



Scheme 1. Prodrug concept.[16]

2.3 Prodrug approach

As explained earlier, the prodrug concept is an attractive tool to improve undesirable properties regarding absorption, distribution, metabolism, and excretion (ADME), but only in the last decades it has become prominent and outstanding. The prodrug approach is a very versatile strategy that enhances some already discovered pharmacological agents lacking ADME properties (**Scheme 2**[17]).[2]

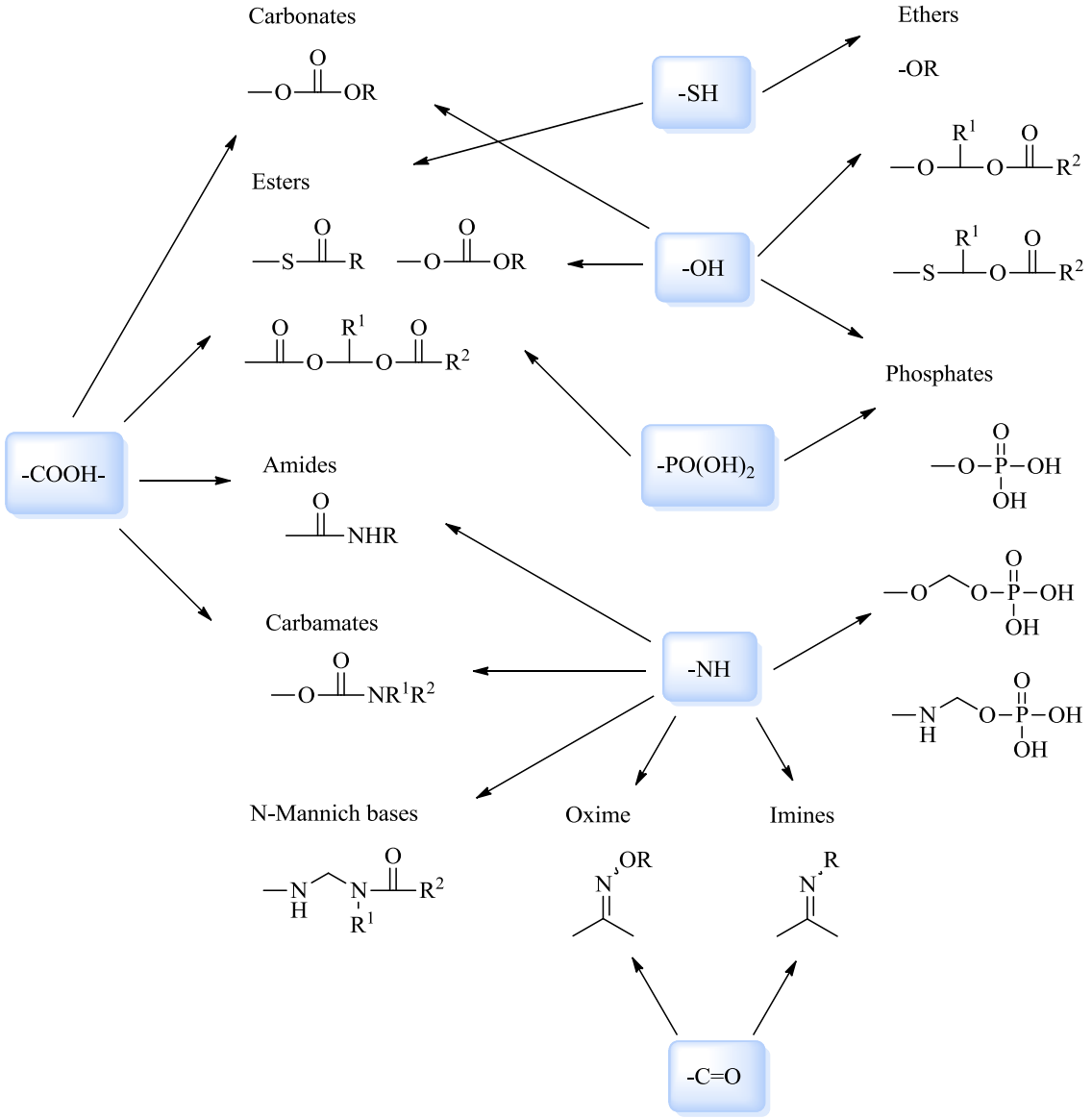


Scheme 2. The principles of ADME concern the interaction of the medicine with the body and vice versa.[17]

The aim of a classical prodrug approach is to overcome physiochemical and biopharmaceutical problems. The drug is covalently modified with hydrophilic (e.g. phosphate, sulfate) or lipophilic (e.g. ester) groups to enhance the solubility and permeability, respectively. This is a non-specific modification lacking site specificity. Instead, the modern prodrug approach, owed to the molecular progress design, consists of pro-moieties covalently linked to the molecule of interest to selectively target transporters or enzymes. Examples of transporters allowing targeting include sodium dependent bile acid transporter (ASBT), oligopeptide transporter (PEPT1), and many others.[18]

2.3.1 Prodrug criteria

When a prodrug is designed, many important factors should be taken into account, such as the functional group subject to derivatization, the safety of the promoiety, ADME properties, and the degradation by-products. Several functional groups, which can be utilized in prodrug formulation, are shown in the **Scheme 3**.[19]



Scheme 3. Common functional groups found in prodrugs.[19]

As was mentioned above this approach has many advantages compared to the conventional drug administration, such as increase the bioavailability, site selectivity and patient tolerance. In addition, it has all the potential to be optimized in order to be an effective method for the treatment of future deseases.[20]

3. BISPHOSPHONATES

3.1 Introduction

The knowledge of bisphosphonates date back to the middle of the 19th century. Their use was mainly industrial since the 1960s when they started to be used in medicine as treatment for calcium disorders.[3]

Herbert Fleisch and Bill Neuman, during their studies about the mechanism of calcification induced by collagen, observed that the body fluids contained inhibitors of calcification.[3] Collagen is a protein present in many tissues that interacts with hydroxyapatite to generate crystals.[21] Hydroxyapatite (HAP) is a calcium phosphate salt [$Ca_{10}(PO_4)_6(OH)_2$] and is the main mineral constituent of the bone. In fact, calcium in the human body is stored in the bone tissues as hydroxyapatite.[22] Since collagen could act as nucleating agent, the inhibitors of calcification are needed to avoid the calcification of all tissue. The inhibitor found in urine was inorganic pyrophosphate (PPi). The studies of the clinical disorders due to the alteration of pyrophosphate helped to understand the metabolism of PPi. PPi is an endogenous regulator, also called 'water softener', utilized by the body to regulate bone mineralization. However, the experiments with pyrophosphates and polyphosphates where successful only when the compounds were injected. Indeed, when they were given by mouth, the compounds become inactive due to the hydrolysis of pyrophosphate in the gastrointestinal tract. Consequently, the research of chemically analogs but more stable compounds than pyrophosphate started. The attempts were successful with compounds called diphosphonates, nowadays known as bisphosphonates. Bisphosphonates, conversely to pyrophosphate, do not breakdown by enzymatic hydrolysis; therefore, they can be used to prevent calcification of tissues. Perhaps the feature that made bisphosphonates so interesting for future applications was their ability to inhibit the dissolution of hydroxyapatite crystal.[5]

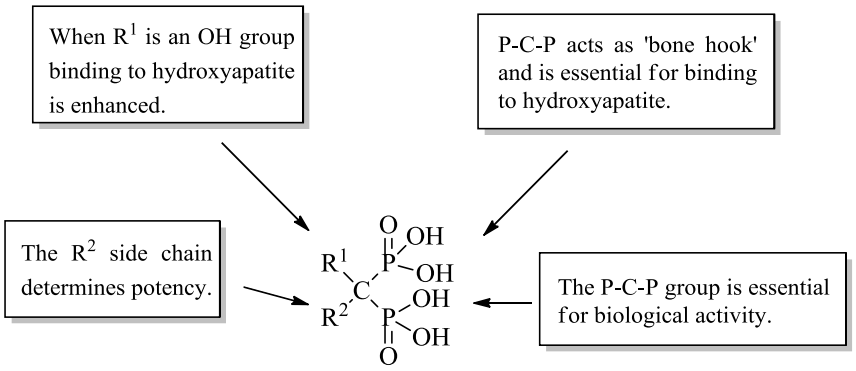
Bisphosphonates are characterized by two phosphates groups bound to the same carbon atom, a ‘P-C-P’ moiety, whereas pyrophosphates replace the geminal carbon atom with an oxygen atom. Bisphosphonates and pyrophosphates are illustrated in **Scheme 4**.[23]

1 **2**

Scheme 4. Bisphosphonic acid (**1**) and pyrophosphoric acid (**2**).[23]

3.2 Chemistry and properties

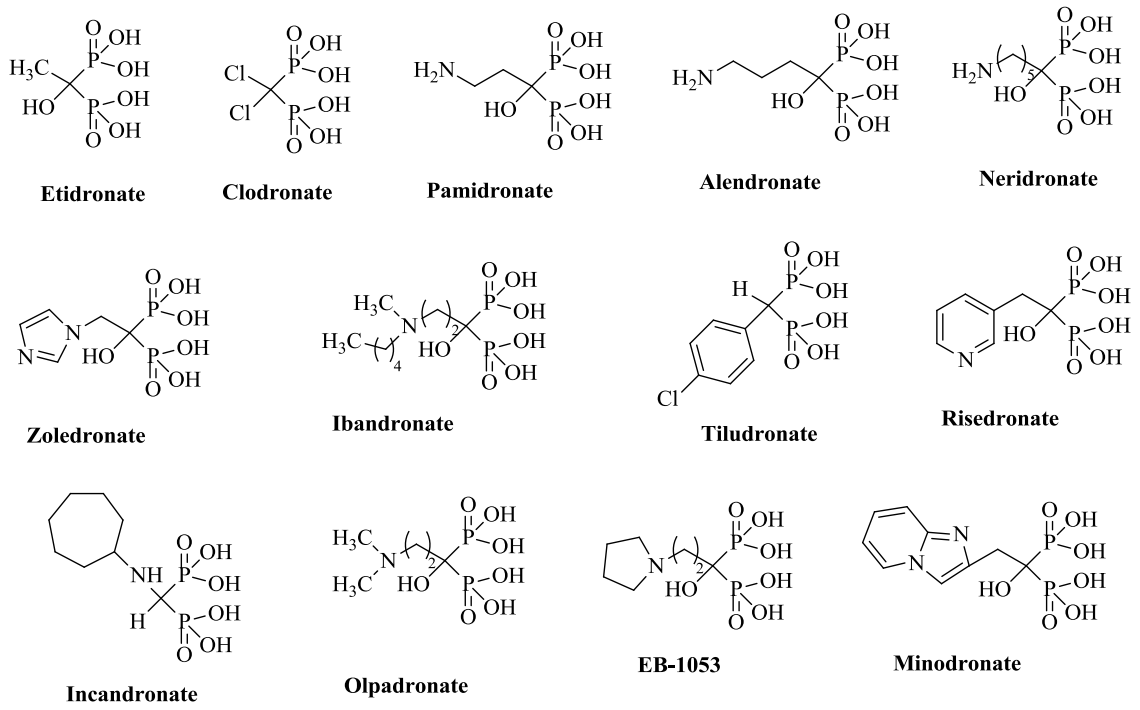
As explained in the introduction, bisphosphonates (BPs) are the chemically corresponding compounds of the naturally occurring pyrophosphate (PPi).[4] The generic structure of bisphosphonates and their functional groups are shown in **Scheme 5**.[6]



Scheme 5. Bisphosphonate (**1**): generic structure and functional groups.[6]

Bisphosphonates, compared to pyrophosphate, have two additional groups in their structure called R^1 and R^2. These groups can be modified according to the properties desired in the compound. When R^1 is substituted with a hydroxyl group, the bonding affinity for the bone mineral is elevated. This is probably due to the stability of the links that the hydroxyl groups can establish producing a chelate effect by a tridentate binding. The amino group behaves similarly to the hydroxyl group. On the other hand if the R^1 group has other substitutions, the bonding affinity is lower owing to bidentate binding rather than a tridentate. In addition, R^2 group is needed for the potency and efficacy of BPs to act as proper antiresorptive agents. Antiresorptive agents are used as therapy to reduce bone loss affecting the osteoclasts activity.[4]

In S**cheme 6** are presented some of the most effective BPs studied and under clinical development after the 1980s.[3]



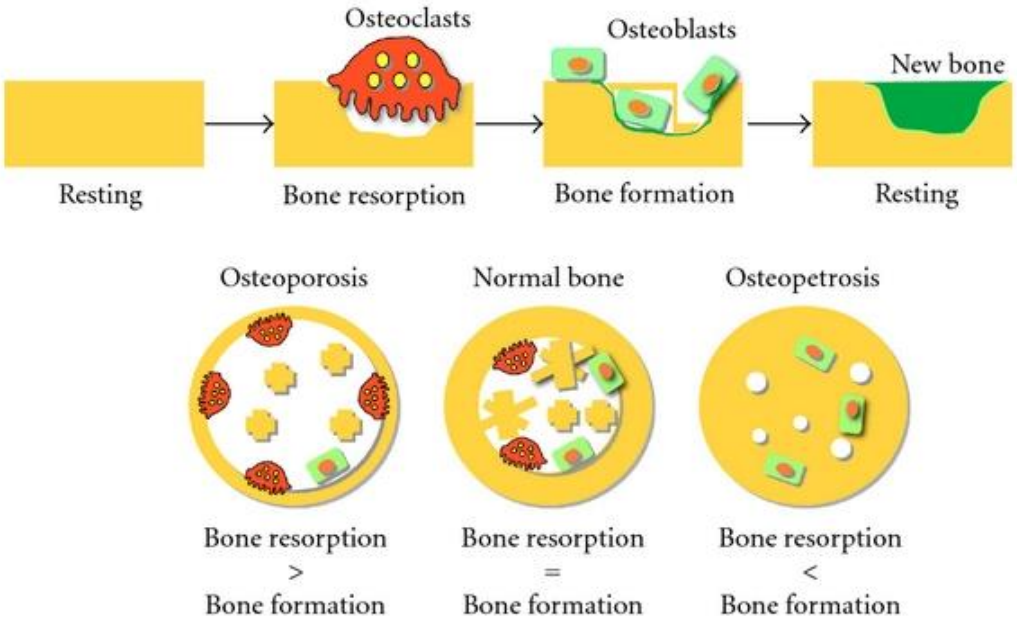
Scheme 6. Bisphosphonates studied and under clinical development after the 1980s.[3]

These compounds were synthesized in order to understand and optimize the antiresorptive effect. The most potent BPs were those containing a nitrogen atom in the R^2 side chain with the nitrogen atom at appropriate distance from the P-C-P group.[3]

In summary, there is a strong correlation between the bisphosphonate structure and its antiresorptive effect based on two properties of the BP's structure; the P-C-P moiety acting as 'bone hook' combined with the hydroxyl group at the R^1 position are fundamental for binding HAP. In other words, they are necessary for a rapid and efficient uptake and distribution of BPs on the mineral surface of the bones. This property of BPs can not be achieved with monophosphates or with P-C-C-P or P-N-P compounds. The R^2 side chain then determines the biochemical activity towards the targets.[6]

The knowledge of the structural properties enhanced a better understanding of how the BPs work. They do not work by simple retention on the bone surface preventing the hydroxyapatite (HAP) dissolution by physicochemical mechanism. Instead, they must interfere on osteoclast activity by cellular effect inhibiting bone resorption.[24]

Osteoclasts, as well as osteoblasts, are involved in the bone remodeling cycle illustrated in **Scheme 7**.[25]



Scheme 7. Bone remodeling cycle: the osteoblasts are involved in new bone formation while osteoclasts are required in bone resorption. If the balance between bone formation and resorption is lost, the subject could be affected by osteoporosis and osteopetrosis.[25]

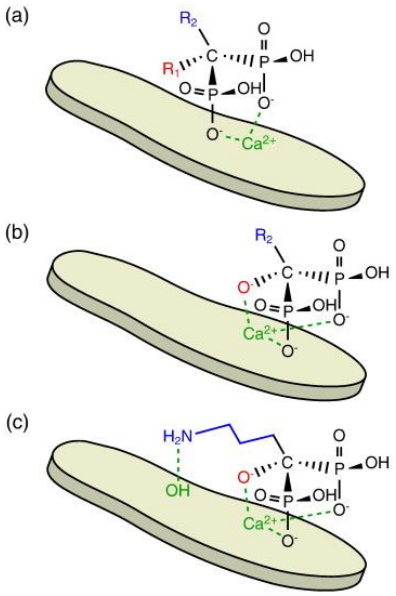
3.3 Classification and mechanism of action

Several generation of bisphosphonates have been studied and they can be classified into two groups according to their mechanism of action: non-nitrogen containing BPs and nitrogen containing BPs (*N*-BPs).

They inhibit bone resorption based on two features: mineral binding affinity and altering cellular metabolism.

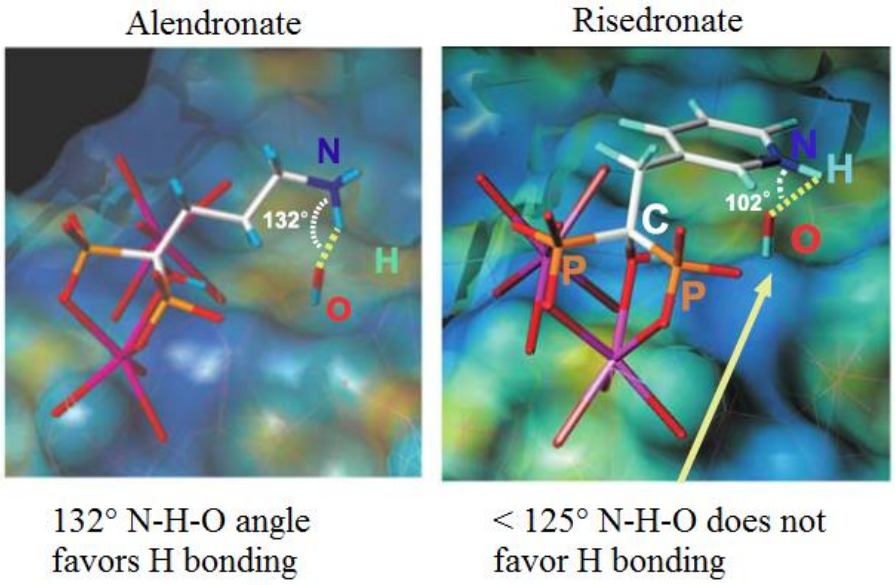
Regarding the mineral binding property, it has been established that it depends on the interaction between the structure of BPs and HAP. As explained early and returning briefly to structure of BPs, HAP binding is enhances by the P-C-P 'bone hook' and OH or NH_2 at the R^1 site. In addition, a nitrogen atom in the alkyl side chain or heterocyclic ring at the R^2 site increase the binding affinity. This is due to the possibility to establish

hydrogen bonds on the HAP surface.[26] The molecular structure of BPs with the binding affinity to HAP is shown in **Scheme 8**.[27]



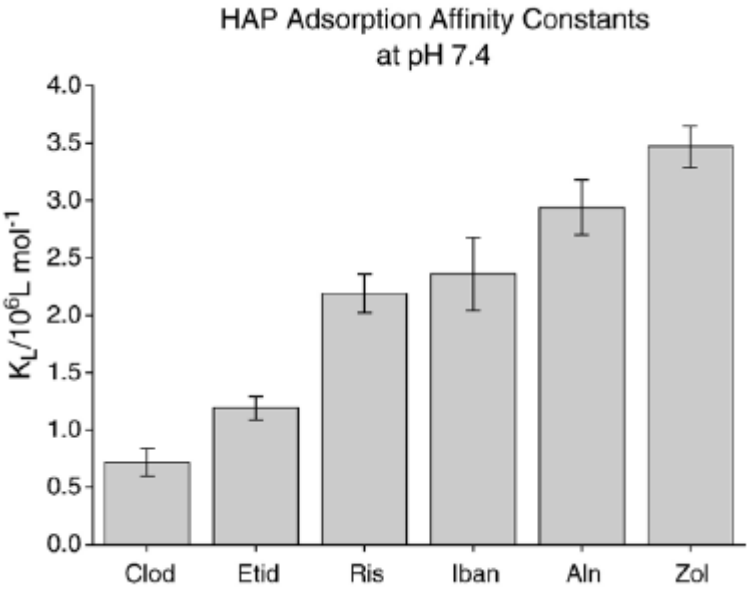
Scheme 8. Schematic representation of BPs binding HAP surface, showing (a) bidentate binding involving the two phosphonates, (b) tridentate binding due to the presence of OH at R^1 side group, and (c) additional interaction from a nitrogen atom at R^2 side group (e.g. alendronate).[27]

An angle ≥ 125° and a N-O distance of ~ 3 Å are required to optimize the N-H-O hydrogen bond. As illustrated in **Scheme 9**, alendronate is capable to form N-H-O bond unlike risedronate in which the nitrogen has not the correct orientation to interact properly for the hydrogen bonding.[26]



Scheme 9. Alendronate and risedronate binding residues of HAP.[26]

Therefore, the R^2 site affect the kinetic binding of BPs to HAP and the influence is significant if at R^1 there are different functional groups than –OH group. This is shown in **Scheme 10** for six bisphosphonates currently in use.[28]

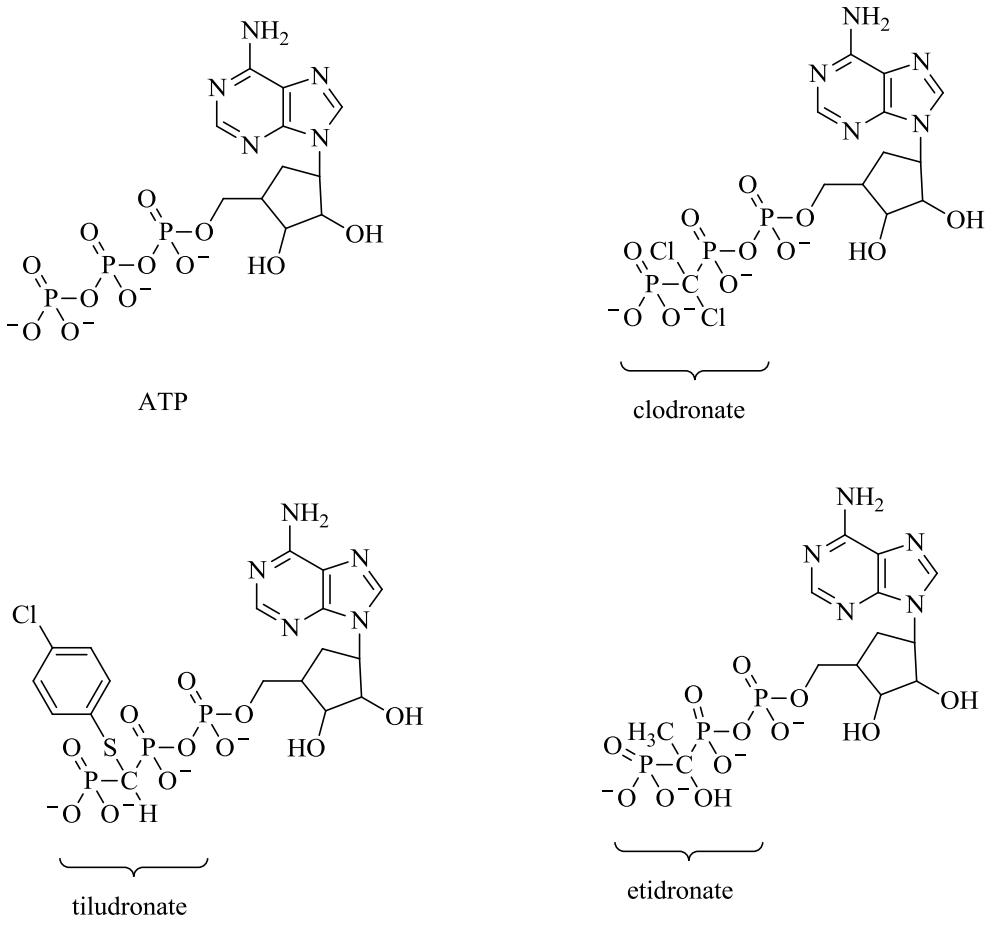


Scheme 10. HAP adsorption affinity constant for clodronate (CLOD), etidronate (ETID), risedronate (RIS), ibandronate (IBAN), alendronate (ALN) and zolendronate (ZOL).[28]

Accordingly with **Scheme 10** the rank order of BPs affinity to HAP is clodronate< etidronate< risedronate< ibandronate< alendronate< zolendronate.[28]

In terms of altering cellular metabolism, unlike the simple BPs that target osteoclasts causing apoptosis, N-BPs interfere on osteoclasts activity but they do not necessary induce osteoclasts' apoptosis.[29]

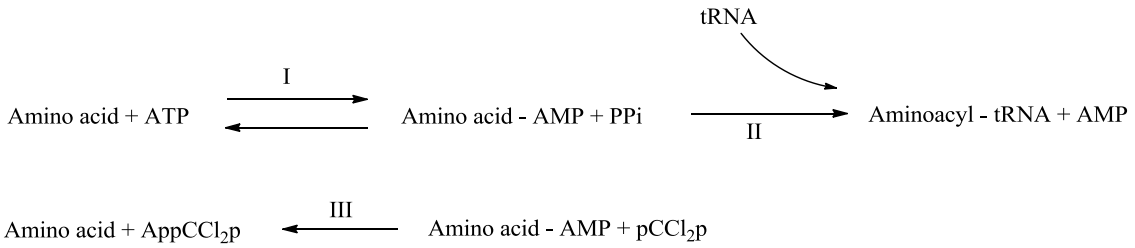
Simple BPs, such as clodronate, etidronate, and tiludronate, closely resemble PPi in structure. They are incorporated from the phosphate chain into compounds containing analogs of ATP becoming non-hydrolyzable (AppCp-type) as illustrated in **Scheme 11**.[30]



Scheme 11. The structure of ATP and the AppCp-type metabolites of clodronate, etidronate, and tiludronate.[30]

In **Scheme 12** the reaction for the formation of an AppCp-type metabolite of clodronate (AppCCl2p), one type of the non-hydrolyzable ATP analogs, is explained. The first

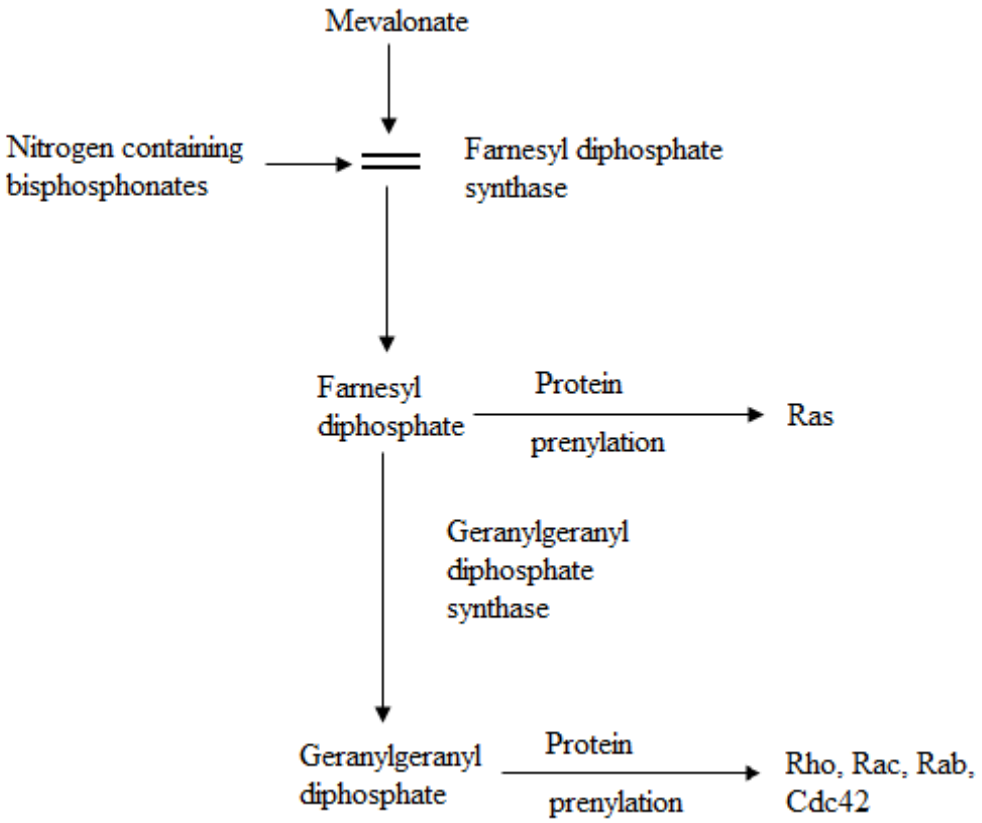
reaction is reversible and involves a condensation of an amino acid with ATP (Appp) to form an amino acid-AMP releasing PPi. Then the amino acid-AMP reacts with a molecule of tRNA to generate an aminoacyl-tRNA and AMP. As was mentioned above, since simple BPs resemble PPi, clodronate (pCCl2p) can replace PPi to form a non-hydrolyzable ATP analogs containing clodronate (AppCCl2p).[30]



Scheme 12. AppCp-type metabolite of clodronate (AppCCl2p).[30]

The accumulation of these non-hydrolyzable ATP analogues (AppCp-type) have a toxic effect on cell functions inducing apoptosis.

Unlike non-nitrogen containing BPs, N-BPs such as risedronate, ibandronate, alendronate, and zoledronate, are most potent inhibitors. They act by altering the cytoskeleton of osteoclasts. Apoptosis may eventually occur even if it is not strictly necessary for inhibition of bone resorption.[31] This action is mainly due to the inhibition of farnesylpyrophosphate synthase (FPPS) of the mevalonate pathway (**Scheme 13**[32]), an essential pathway for several cellular processes.[7]



Scheme 13. Mevalonate pathway.[32]

Inhibition of FPPS does not allow the formation of farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP). FPP and GGPP are isoprenoid lipids necessary for a proteins' modification (prenylation). Among these proteins there are also GTP-binding proteins Ras, Rho, Rac**,** and Rab. This process is required for several cell functions and the small GTPases cause changes in their activity and may induce cell death and therefore inhibit bone resorption.[7]

Despite BPs are attractive antiresorptive agents for the treatment of diseases characterized by increased bone resorption such as osteoporosis, there are still some mechanistic issues to be clarified.[33] Moreover, their poor bioavailability sets restrictions for their use.[34]

3.5 Improving the bioavailability of bisphosphonates

As was mentioned in the previous chapter, BPs are effective in inhibiting bone resorption due to skeletal disorders, such as osteoporosis, Paget's disease, hypercalcemia of

malignancy, multiple myeloma, and bone metastasis. A demanding inconvenience associated with the clinical use of BPs is their poor bioavailability.[35]

The poor bioavailability of BPs is due both to their hydrophilic properties, and the bulky and charged phosphonate groups. Indeed, they hinder the absorption in the gastrointestinal tract after oral administration. After poor oral absorption (generally around 1%), only about 50% of the absorbed BPs are then taken up by the bone while the remainders are eliminated in the urine leading to a total uptake of about 0.5% of the administered BPs to the bone.[8]

The absorption of BPs might be also affected by food, probably due to the conversion into a non-soluble aggregate with a consequent reduction in absorption. The drug may also form complexes that at high dose affect the intracellular junctions increasing the absorption. Unfortunately, this often leads to side-effects. These reasons have enhanced the interest in the development of strategies of novel drug delivery systems for BPs to improve both the bioavailability and safety.[9]

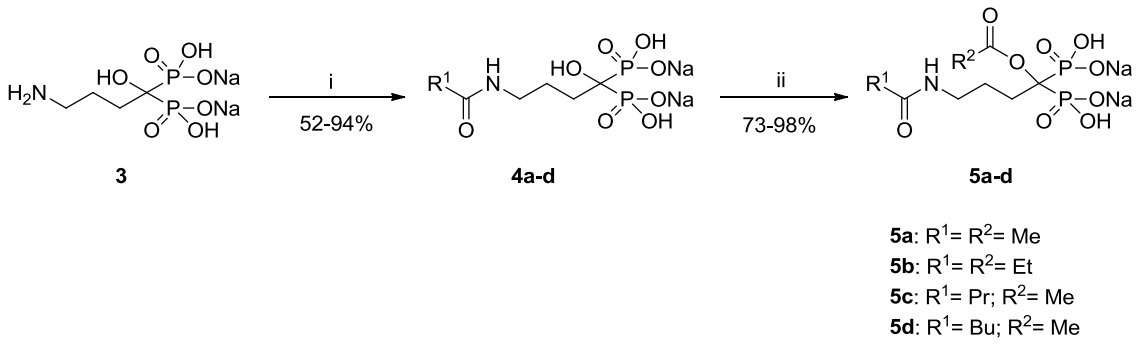
Oral drugs depend on the apparent partition coefficients (P_{app}) and when log P_{app} = 2-3 the transportation through the cells is achieved. The problem with BPs is a low log P_{app}, which is, however, possible to overcome through the prodrug technique. Indeed, one or more ionizable groups available in BPs can be replaced with a bioreversible promoiety. The BPs' functional groups that can be modified are OH and/or NH in the side chain or either of the two phosphorous groups.[10]

3.5.1 Synthetic strategies for improving the bioavailability of bisphosphonates

Several approaches have been investigated to improve the bioavailability of BPs.[9]

The synthetic strategies established to enhance the intestinal permeability of BPs is based on the reversible modification of one or more ionizable groups.[9]

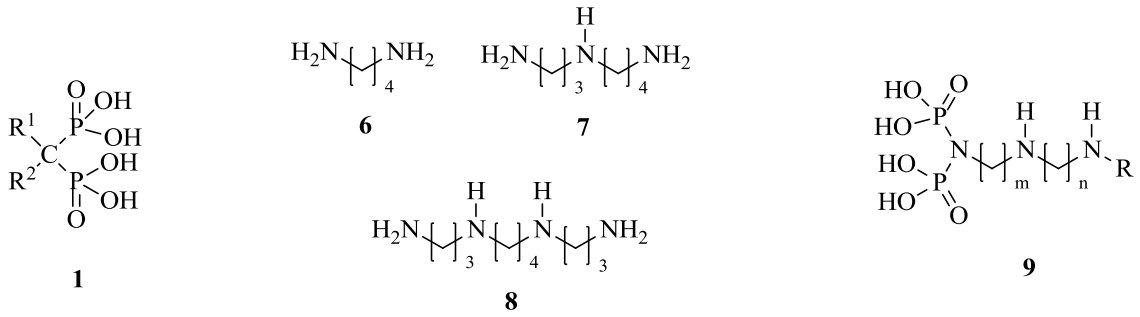
Turhanen *et al.*[36] developed a synthesis to improve the lipophilicity of *N*-BPs masking the amino and the hydroxyl groups with acetyl moieties. (1-Alkanoyloxy-4-alkanoylaminobutylidene)-1,1-bisphosphonic acid derivates were synthesized starting from (1-hydroxy-4-aminobutylidene)-1,1-bisphosphonic acid disodium salt (disodium alendronate) as shown in **Scheme 14**.[36]



Scheme 14. Synthesis of (1-alkanoyloxy-4-alkanoylaminobutylidene)-1,1-bisphosphonic acid derivatives (**5a-d**). Conditions: i) $(R^1CO)_2O$ in H_2O; ii) $(R^2CO)_2O$ then H_2O (if necessary).[36]

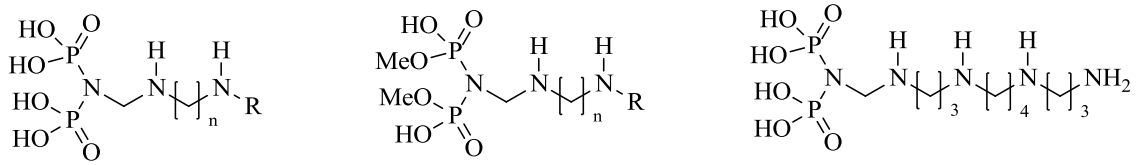
The features of these synthesized compounds, such as lipophilicity and solubility, appear to be enhanced if compared with alendronate. In order to consider these derivates as potential prodrug of alendronate more biological evaluations need to be carried out.[36]

Sankala *et al.*,[37] for one, have investigated the properties of bisphosphonate-polyamine conjugates (BP-PA) (**Scheme 15**).[37]



Scheme 15. General structure of bisphosphonic acid (**1**), natural polyamines namely putrescine (**6**), spermidine (**7**), and spermine (**8**), and bisphosphonate polyamine conjugates (**9**).[37]

Natural polyamines (PAs) are potential drug carriers, which is why BP-PAs were synthesized (**Scheme 16**) and their affinities for HAP evaluated.[37]



9a: n=3
9b: n=4

9c: n=4

9d

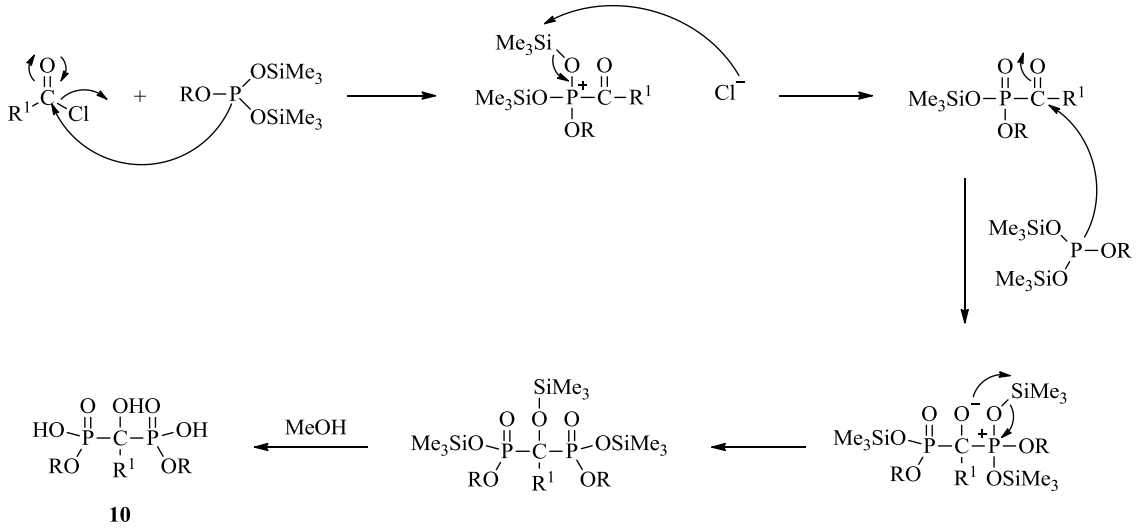
Scheme 16. Novel synthesized BP-PA conjugates (**9a-d**).[37]

As shown in **Table 1**, *N*-BP-PAs have higher affinity for HAP than etidronate chosen as control compound. In addition, the binding activity is influenced by the length between the geminal carbon and the first nitrogen atom in the PA chain.[37]

Table 1. The relative affinity of etidronate and BP-PA conjugates (**9a-d**) to hydroxyapatite after one hour incubation at room temperature.[37]

Compound	**Affinity to hydroxyapatite (%)**
Etidronate	30±9
9a	83±6
9b	72±3
9c	53±8
9d	72±19

The synthesis proposed by Montil *et al.*[38] involves the side chains modification of 1-hydroxyl-1,1-bisphosphonate into diesters utilizing various alkyl or aryl substituents. The key of this synthesis (**Scheme 17**), which gives good yields, was to use silylated phosphites and acid chloride. The silylated product needs to be hydrolyze, as last step, to give the corresponding aromatic or aliphatic 1-hydroxyl-1,1-bisphosphonate diester **10**.[38]

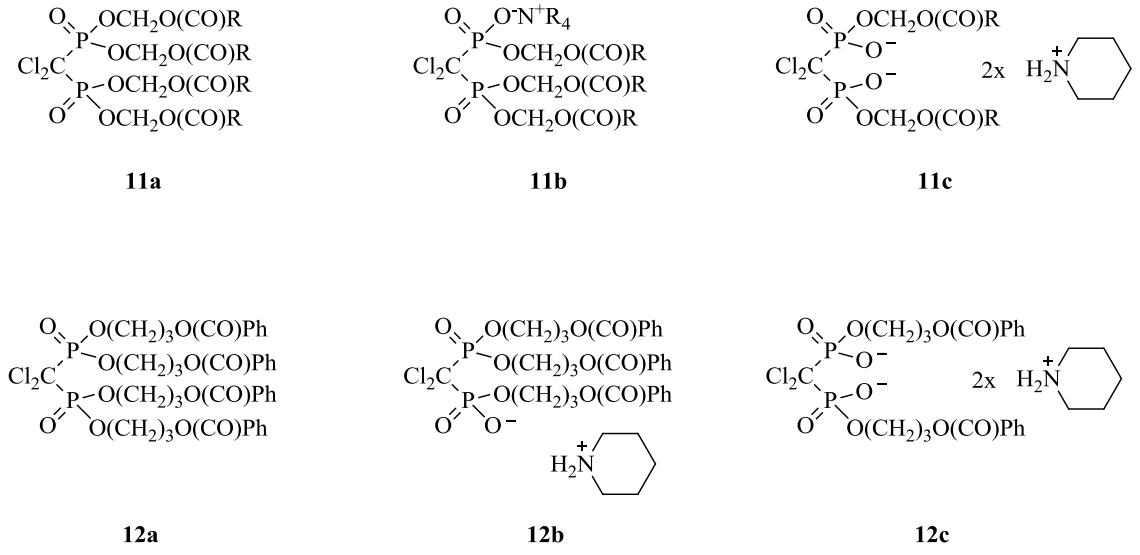


Scheme 17. Synthesis of aromatic and aliphatic 1-hydroxyl-1,1-bisphosphonate diester (**10**).[38]

3.5.2 Bisphosphonate-ester derivates

In order to improve the poor lipophilicity of BPs several paracellular pathways of transport have been investigated.[39]

Niemi *et al.*[39] synthesized and evaluated the properties of pivaloyloxymethyl esters and benzoyloxypropyl esters of clodronic acid (**Scheme 18**).[39]



Scheme 18. Pivaloyloxymethyl esters (**11a-c**) and benzoyloxypropyl esters (**12a-c**).[39]

The latter could not be considered as a bioreversible prodrugs since they did not release the parent drug through enzymatic or chemical process. On the other hand, pivaloyloxymethyl esters of clodronic acid gave better results when evaluated *in vitro*. Particularly, tetra-, tri-, and P,P'-dipivaloyloxymethyl esters of clodronic acid (**Scheme 18**) were synthesized and analyzed.[39]

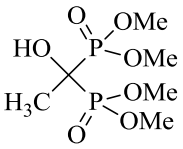
According to **Table 2** all the pivaloyloxymethyl esters proved to be more lipophilic than clodronate (log $P_{app} \leq -5.4$).[39]

Table 2. Apparent Partition Coefficients (log P_{app}) for pivaloyloxymethyl esters of clodronic acid (**11a-c**).[39]

Compound	**log P_{app} pH 7.4**
11a (tetra)	7.4
11b (tri)	1.56 ± 0.02
11c (di)	-2.13 ± 0.01

Even though it was possible to increase the lipophilicity, also the analysis of the other data needs to be considered. The stability in phosphate buffer solution and the enzymatic hydrolysis have shown that the pivaloyloxymethyl ester with the most potential among the three synthesized ones is tripivaloyloxymethyl ester **11b**. Indeed, it has an optimal log P_{app}, acceptable water solubility, and it hydrolyzes to clodronic acid.[39]

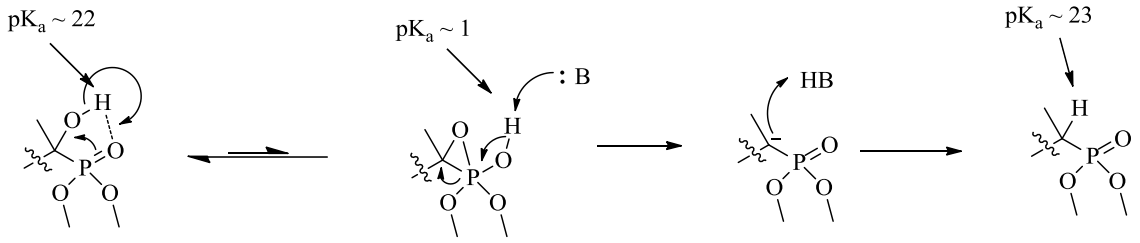
These successful findings were also applied for the design of a prodrug that could release etidronic acid. A tetramethyl ester of etidronic acid was synthesized (**Scheme 19**).[40]



13

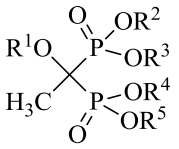
Scheme 19. Tetramethyl ester of etidronic acid (**13**).[40]

It was observed that this compound did not react to give neither tri- or dimethyl ester of etidronic acid nor the parent drug. Instead, it was found that tetramethyl ester of etidronic was isomerized in a phosphate buffer at pH 7.4. The isomerization involves the rearrangement of the P-C-P moiety to a P-C-O-P as illustrated in **Scheme 20**.[40]



Scheme 20. The mechanism of isomerization of tetramethyl ester of etidronic acid.[40]

For this reason unlike with clodronate, when etidronate prodrugs were designed, the alcohol group in the bridging carbon needed to be taken into account. The isomerization was prevented through acylation of the hydroxyl group attached to the geminal carbon atom. Regarding the phosphonate hydroxyl groups, pivaloyloxymethyl group was chosen as a pro-group candidate since it was proven promising from the previous studies with clodronate. Consequently, acetylated pivaloyloxymethyl esters of etidronic acid (**Scheme 21**) were synthesized and evaluated *in vitro*.[40]



14

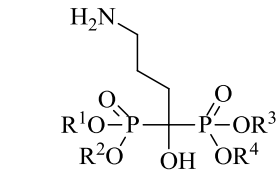
Compound	R^1	R^2	R^3	R^4	R^5
Etidronate	H	H	Na	Na	H
14a	H	Me	Me	Me	Me
14b	H	Me	Na	Na	Me
14c	H	Me	Me	K	Me
14d	-$COCH_3$	Me	Me	Me	Me
14e	-$CO(CH_2)_2CH_3$	H	H	H	H
14f	-$CO(CH_2)_6CH_3$	H	H	H	H
14g	-$COCH_3$	Na	Na	Na	Na
14h	-$COCH_3$	Me	Na	Na	Me
14i	-$COCH_3$	POM[a]	POM	POM	POM
14j	-$COCH_3$	POM	POM	POM	X[b]

a: –$CH_2O(CO)C(CH_3)_3$; b: *N*-pivaloyloxymethyl-*N*,*N*,*N*-triethyl ammonium salt.

Scheme 21. Acetylated pivaloyloxymethyl esters of etidronic acid (**14a-j**).[40]

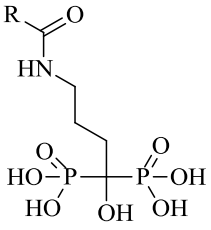
The obtained results showed that the most potential prodrug candidates for etidronate was acetylated tripivaloyloxymethyl ester of etidronic acid because it had satisfactory aqueous solubility and lipophilicity, and it released etidronate acceptably.[40]

Investigations *in vitro* and *in vivo* of alendronate derivates as potential prodrugs have been made by Vachal *et al*. The potential alendronate prodrugs are shown in **Scheme 22**.[41]

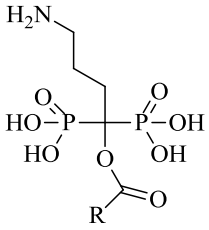


R^1, R^2 and R^3,R^4 cyclic or acyclic

15



16



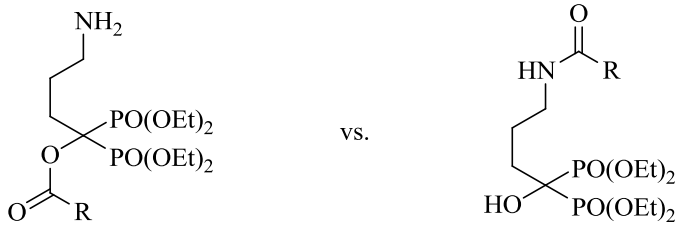
17

Scheme 22. Potential alendronate prodrugs: tetraalkyl alendronate (**15**), *N*-acylalendronic acid (**16**), and *O*-acylalendronic acid (**17**).[41]

Tetraalkyl alendronates and *N*-acylalendronc acid lack the zwitterionic nature of *N*-BPs. They thus increase the lipophilicity of the molecule. *O*-Acylalendronates have a limited stability.[41]

Regarding tetraester alendronate derivatives the rearrangement of the 1-hydroxy-1,1-bisphosphonate to 1-phosphonate-1-phosphate was observed. The observation was consistent with the studies conducted by Niemi *et al.*[40] about esterification of etidronate. Furthermore, it was not possible to carry out the tetraesterification of alendronate with the previously known methods, since in addition to an alcohol group in the bridging carbon a reactive amino group is also present in the molecule. For this reason, a new synthetic strategy to design tetraalkyl alendronates was successfully completed. It was then observed *in vitro* that this class of derivatives can not be considered as alendronate prodrugs since they rearrange instead of hydrolysing to the parent drug.[41]

Also *O*-acylatealendronate derivatives were synthesized but it was found that an intramolecular O→N acyl transfer converted tetraalkyl *O*-acylalendronate into a tetraalkyl *N*-acylalendronate (**Scheme 23**).[41]

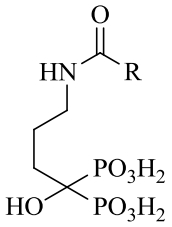


18
expected, not observed

19
only product

Scheme 23. The expected tetraalkyl *O*-acylalendronate (**18**) was not observed due to an intramolecular O→N acyl transfer to give tetraalkyl *N*-acylalendronate (**19**).[41]

Despite this synthesis has a number of drawbacks, the O→N acyl transfer may be useful for the synthesis of *N*-acylalendronates. The optimized conditions, which provide the protection of hydroxyl group by silylation, allowed prepraration of *N*-acylalendronic acid with a good yield (**Scheme 24**).[41]



19a-b

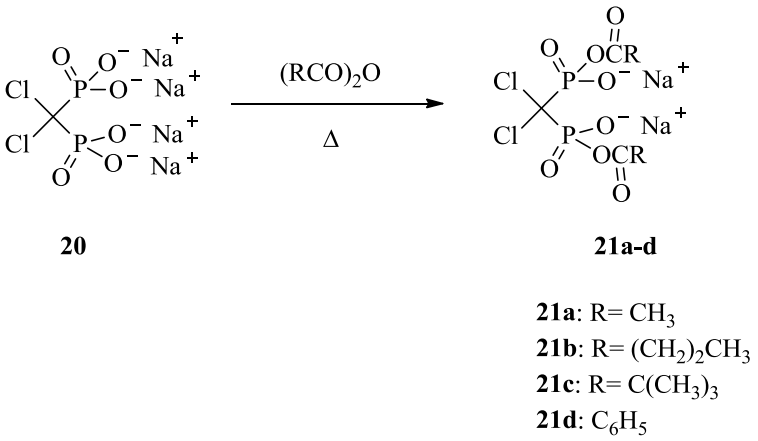
19a: RCO= n-C13H37CO, 78%
19b: RCO= Ac, 87%

Scheme 24. *N*-acylalendronic acids (**19a-b**).[41]

Since *in vitro* experiments did not increase understanding of the behavior of prodrug conversion, *in vivo* experiments on group of rats were carried out. Even though these investigated potential alendronate prodrugs did not provide a marked increase of bioavailability, *N*-myristoylalendronic acid seems to be a promising prodrug with 25% of the parent drug released.[41]

3.5.3 Bisphosphonate-dianhydride derivates

Another way of preparing bioreversible prodrugs from bisphosphonates is to convert ionizable groups into dianhydrides. An example is reported by Ahlmark *et al.*[42], who converted two ionizable groups of clodronate into dianhydrides. As illustrated in **Scheme 25**, four bioreversible prodrugs of clodronate with different steric hindrance were synthesized.[42]



Scheme 25. Bioreversible prodrugs of clodronate (**21a-d**).[42]

These novel dianhydride derivates are more lipophilic than clodronate although there is not significant differences between their log P_{app} values, as shown in **Table 3**.[42]

Table 3. Log P_{app} values of clodronic acid dianhydrides (**21a-d**).[42]

Compound	**log P_{app}**	
	pH 2.0	**pH 7.4**
21a	-1.4	-2.2
21b	-1.9	-2.3
21c	-2.0	-2.3
21d	-1.5	-2.3

This is probably due to the dianhydride promoieties because the substitution of more than two phosphonate groups is believed to increase the log P_{app}. In conclusion, these novel

clodronic acid dianhydrides are potential prodrugs because they are stable toward chemical hydrolysis but they release the parent drug enzymatically.[42]

3.6 Conjugation with endogenous molecules

As explained earlier, the oral drug administration is the most preferred and acceptable way even though the gastrointestinal tract presents several barriers, such as biochemical and physical ones. These barriers can cause low drug bioavailability. It follows that overcoming these barriers is one of the challenges in efficient drug delivery. Many strategies have been developed to improve the absorption of drugs after oral administration. These strategies include the conjugation of the drug with a carrier system. This allows to target the drug exclusively to the site which requires the treatment.[43]

The main tools of drug targeting use receptors or ligands to direct the drugs to sites of action. The receptor acts as molecular target to which the drug will be directed, while the ligand represents a seeking section. The ligands can be exogenous or endogenous. Although exogenous ligands can be site-selective, they may produce immunological issues, such as non-biocompatibility or non-immunogenicity. On the other hand, endogenous ligands are sensitive, specific, and effective in site-targeting. Furthermore, they can exert their function either in their native or engineered form.[44]

Consequently, the employment of endogenous ligands in drug delivery systems offers enormous benefits both for the design of carriers and for the patient compliance.[44]

Following the idea of Paul Ehrlich, according to which an ideal drug acts with a specific distribution towards the target, the development of ‘magic bullets’ for bone diseases started to take hold. In particular, in the early 1960s efforts for the use of endogenous molecules with pyrophosphates, which were afterwards replaced with bisphosphonates, were set.[45]

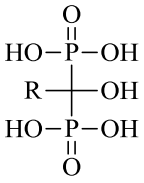
The prodrug studies regarding BPs can be divided in two types of approaches. One approach involves targeted carrier systems where the promoieties can be linked either to phosphorous atoms or to the side chain of BPs. Instead, on the other approach the BPs themselves act as promoieties for other drugs.[10]

Indeed, BPs, when conjugated to drugs, can act as carriers targeting the attached drugs to bone tissue due to the high affinity of BPs for HAP.[27] This method is called Osteotropic Drug Delivery System (ODDS) and it covers the design of ODDS via BPs after

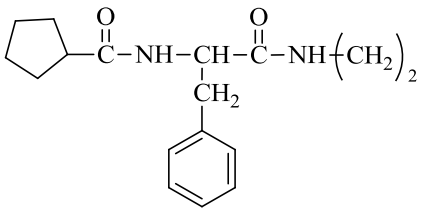
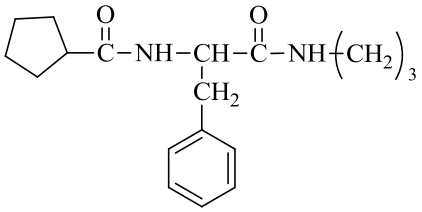
considering the drawbacks of the cases such as the lipophilicity and the precipitability of bisphosphonate-calcium complexes.[46]

3.6.1 Bisphosphonate-peptidyl derivates

Conjugating bisphosphonates with peptides aims at increasing peptide permeability through the intestinal mucosa. This is equipped with brush-border that mediates the di/tri-peptide absorption. Golomb *et al.*[47] formulated peptidyl-bisphosphonate prodrugs targeting the brushborder. In particular, pamidronate and alendronate were converted into prolyl-phenylalanyl-pamidronate (Pro-Phe-pamidronate) and prolyl-phenylalanyl-alendronate (Pro-Phe-alendronate) as shown in **Scheme 26**.[47]



22

Compound	R
Pamidronate	$NH_2CH_2CH_2$
Alendronate	$NH_2CH_2CH_2CH_2$
Pro-Phe-pamidronate	
Pro-Phe-alendronate	

Scheme 26. Structure of pamidronate, alendronate, Pro-Phe-pamidronate, and Pro-Phe-alendronate.[47]

Different types of measurements about concentration in bone tissue of these peptidyl prodrugs were made to estimate their absorption. The bioavailability of Pro-Phe-pamidronate and Pro-Phe-alendronate was estimated by the concentration accumulated in bone and urine. It was found that the values of the parent drugs were lower than those of the prodrugs as shown in **Table 4**.[47]

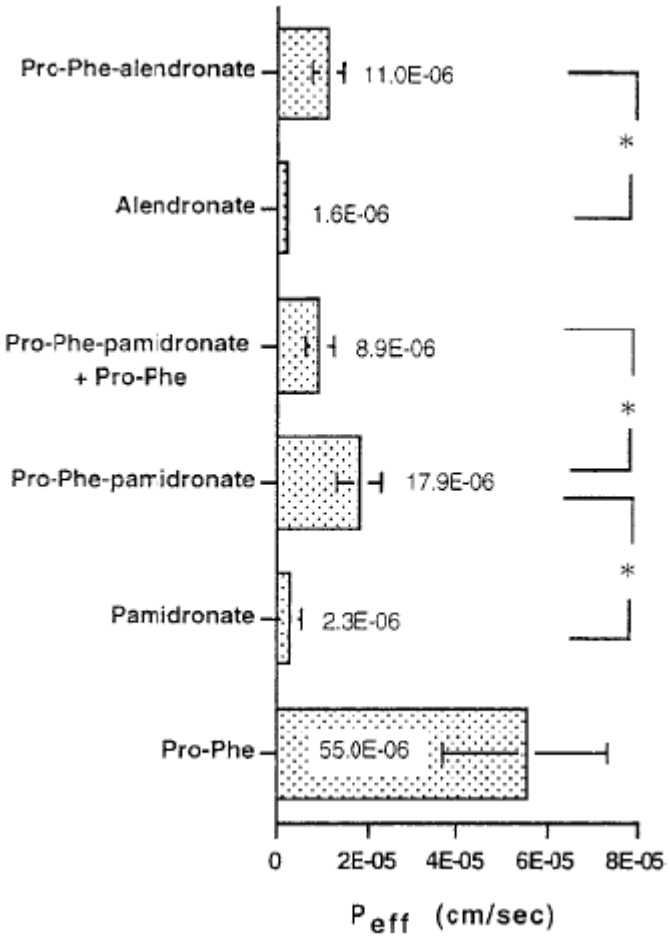
Table 4. Oral bioavailability of the Pro-Phe-bisphosphonate prodrug and the relative parent drug in bone and urine 24h following administration.[47]

Tissue	**Pro-Phe-[^{14}C]-alendronate**	**Alendronate**	**Pro-Phe-[^{14}C]-pamidronate**	**Pamidronate**
Urine	3.10	1.65	3.17	1.74
Tibia	1.74	0.52	1.65	0.44

To understand the mechanism of prodrug absorption different tests were carried out in rats such as the determination of the site of prodrug absorption, the effective permeability coefficients and the time-dependent uptake.[47]

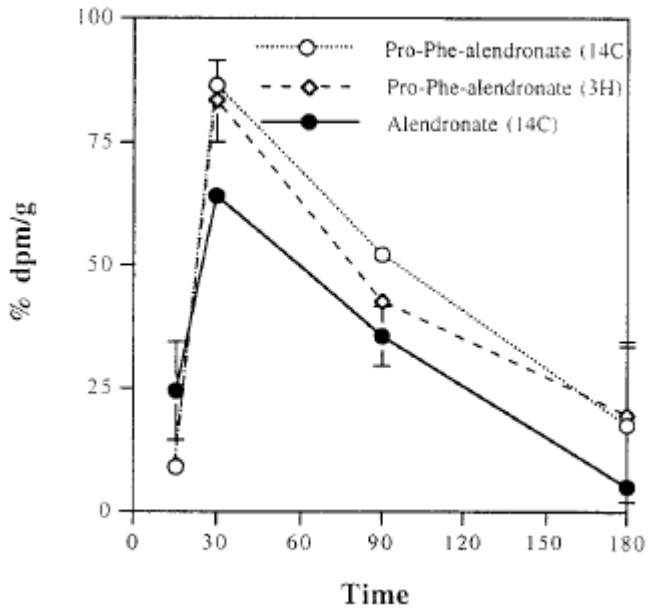
To determine the site of prodrug absorption the amount of ^{14}C in bone was studied in pylorus-ligated rats after oral administration of Pro-Phe-pamidronate and pamidronate.[47]

The effective permeability coefficient (P_{eff}) was studied and compared among the parent drug (pamidronate and alendronate), the dipeptide (Pro-Phe), and the prodrugs (Pro-Phe-pamidronate and Pro-Phe-alendronate). As shown in **Scheme 27** the P_{eff} values of Pro-Phe-pamidronate and Pro-Phe-alendronate were higher than those of the parent drugs.[47]



Scheme 27. The effective permeability coefficients (P_{eff}) for the parent drug (pamidronate and alendronate), the dipeptide (Pro-Phe), and the prodrugs (Pro-Phe-pamidronate/alendronate).[47]

The time-dependent uptake was determined for Pro-Phe-alendronate and alendronate. As illustrated in **Scheme 28** the amounts of ^{14}C and ^{3}H for the prodrug in the intestinal wall of rats was higher than that of the parent drug at all times.[47]



Scheme 28. Time-dependent uptake of Pho-Phe-alendronate and alendronate in the intestine after oral administration.[47]

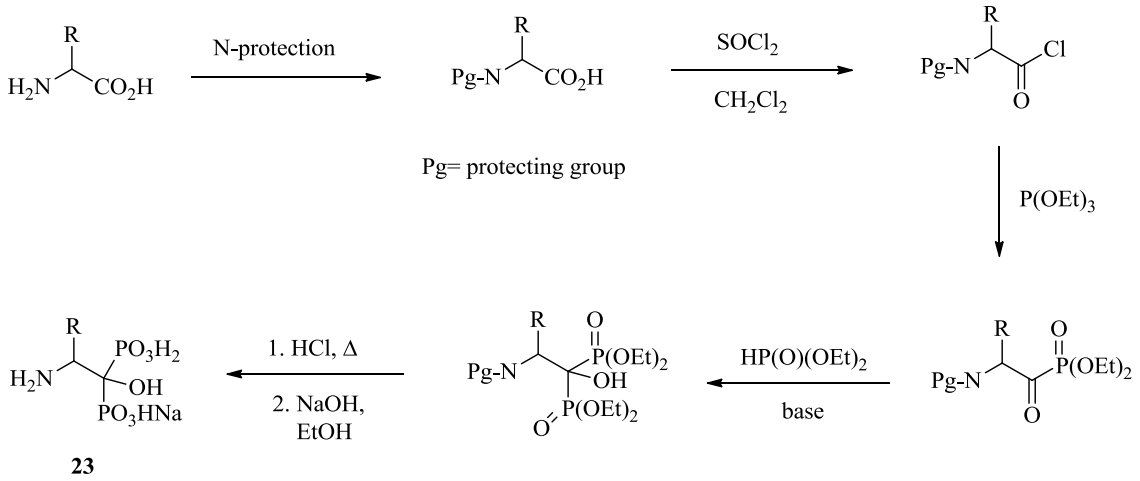
This means that the affinity of the prodrug for the intestinal tissue is higher than that for the parent drug. Nevertheless, experiments about biological activities are indispensable. For this purpose the inhibition of HAP formation and dissolution were evaluated. It was found that the prodrugs inhibited the calcification of tissues showing a so-called 'crystal poison' effect. Regarding the inhibition of HAP dissolution the prodrugs exhibited an antiresorptive activity.[47]

In conclusion, according to the results oral bioavailability of BPs had clearly increased owing to the peptidyl prodrug approach.[47]

3.6.2 Bisphosphonate-amino acid conjugates

The synthesis and the anti-resorptive activity of several α-amino acid derived bisphosphonates have been described by Mizrahi *et al.*[48]

First, the protection of the amino group (-NH_2) was required and phthalimide was used as a protecting group. Second, after the activation of the carboxylic acid (-COOH) group, the ester-protected BP conjugates were achieved through two steps (**Scheme 29**).[48]



Scheme 29. Synthetic route for amino acid derived bisphosphonates (**23**).[48]

It was found out that the synthesis of BPs (**23**) resulted in a mixture with the analogs phosphate-phosphonate rearranged products. The reaction rate between the formation of the BP and its corresponding rearrangement product highly depended on the hindrance of the β-substituent in the amino acid. For example, in the reaction with glycine, the rapid formation of BP conjugate quickly rearranged to the phosphate-phosphonate by-product.

Several novel BP salts (**23**) were prepared using this method as shown in **Table 5**.[48]

Table 5. Novel bisphosphonate salts (**23a-m**).[48]

Compound	**Amino acid**	**R**	**Yield (%)**
23a	Ala	CH_2Me	20
23b	Pro	Cyclic	40
23c	Phe	CH_2Ph	12.5
23d	Leu	CH_2(i-Pr)	45
23e	Ile	$CH(Me)CH_2Me$	Impure
23f	Trp	CH_2(indole)	31
23g	Val	$CH(Me)_2$	Mixture
23h	Met	$(CH_2)_2SMe$	42, mixture
23i	Asp	CH_2CO_2H	7
23j	Glu	$(CH_2)_2CO_2H$	24
23k	Lys	$(CH_2)_4NH_2$	15
23l	Om		14
23m	Alendronate[a]		43

a: leading to Alendronate

The synthesized BP conjugates, after purification through column chromatography, were hydrolyzed to remove the ester and phthalimide groups. The obtained pure BP-amino acid conjugates were finally isolated for biological tests as mono sodium salts. None of the conjugates, apart from phenylalanine **23c**, leucine **23d**, and glutamine **23j** bisphosphonates, showed any activity regarding the inhibition of bone resorption compared to the action of alendronate sodium salt (**Table 6**).[48]

Table 6. Activity in inhibition of bone resorption of BP-amino acid conjugates (**23a-l**).[48]

Compound	**Original amino acid**	**Inhibition at 0.1 mg P/Kg (%)**
23a	Ala	1.7 ± 21.3
23b	Pro	0
23c	Phe	26.5 ± 8.2
23d	Leu	19.7 ± 23
23f	Trp	0
23g	Val	0
23h	Met	0
23i	Asp	0
23j	Glu	33.3 ± 18.2
23k	Lys	0
23l	Om	0

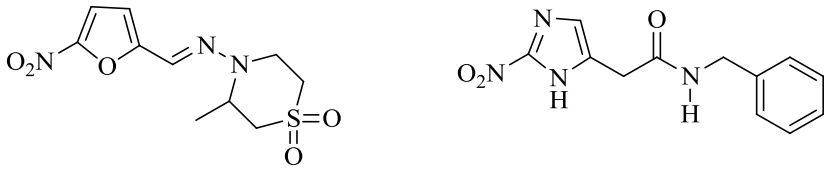
The explanation of this inactivity might be due to the increase and uniform distribution of polarity owing to the presence of amino group at β- and γ-positions decreasing the antiresorptive activity. In addition, the larger steric hindrance of the 'bone hook', fundamental for binding HAP, probably affected the inactivity.[48]

3.6.3 Bisphosphonate-fatty acid conjugates

The activity of a series of BPs derived from fatty acids have been investigated by Szajnman *et al.*[49-53]

Besides being effective for the inhibition of bone resorption and the treatment of other bone disorders, it was found out that BP-fatty acid conjugates resulted to be potent inhibitors of pathogenic trypanosomatids such as *Trypanosoma cruzi* and *Trypanosoma brucei*. *Trypanosoma cruzi* is a parasitic protozoan that causes diseases, such as American trypanosomiasis also known as Chagas's disease. Once the parasites are hosted by the mammal they invade the tissues, specifically cardiac muscle and gastrointestinal tract. Chemotherapy available for the treatment of Chagas's disease such as nifurtimox and

benznidazole (**Scheme 30**[50]), are non-specific and unsafe drugs since they are carcinogenic in animals. For these reasons interest in developing more efficient and safer drugs than those currently available has evolved. [49]

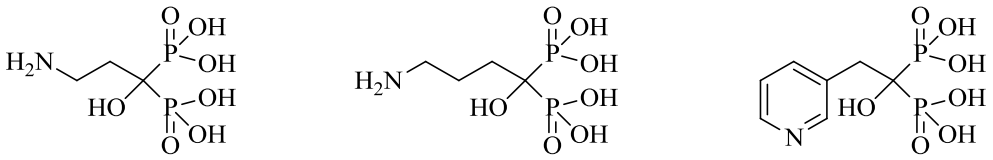


24 **25**

Scheme 30. Drugs for the treatment of Chagas's disease: nifurtimox (**24**) and benznidazole (**25**).[50]

Understanding the biochemistry and physiology of *T. cruzi* enhanced the identification of targets for drug delivery. For endurance of the trypanosomatids protein prenylation is essential. Bearing in mind that the molecular target of BPs is FPPS, an enzyme in charge of the formation of the substrate for protein prenylation, the use of BPs as inhibitors of *T. cruzi* was successfully achieved.[52] In addition, since BP derivatives are approved for clinical use by the U.S. Food and Drug Administration (FDA), they verifiably possess low toxicity.[49]

N-BPs, such as pamidronate, alendronate, and risedronate (**Scheme 31**), were shown to be able to inhibit the proliferation of *T. cruzi in vitro* and *in vivo*.[49]

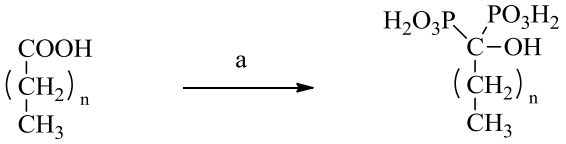


26 **27** **28**

Scheme 31. Pamidronate (**26**), alendronate (**27**), and risedronate (**28**) inhibit the proliferation of *T. cruzi*.[49]

These results emphasized the efficiency of BPs as therapeutic agents against Chagas's disease. For understanding the effect of the nitrogen atom on the biological activity of BPs, fatty acid-derived bisphosphonates lacking nitrogen were synthesized and biologically evaluated.[52]

The 1-hydroxyalkyl-1,1-bisphosphonates were prepared form commercially available fatty acids as shown in **Scheme 32**.[49]



29 **30**

Fatty acid	N	Bisphosphonate	n	Yield (%)
29a	2	**30a**	2	99
29b	4	**30b**	4	92
29c	5	**30c**	5	84
29d	6	**30d**	6	82
29e	7	**30e**	7	77
29f	8	**30f**	8	74
29g	12	**30g**	12	67

Scheme 32. 1-Hydroxyalkyl-1,1-bisphosphonates (**30a-g**) synthesized form commercially available fatty acids (**29a-g**). Reagents: (a) (i) H_3PO_3, PCl_3, $PhSO_3H$, 65 °C, 16h, (ii) H_2O, 100 °C, 5h.[49]

The IC_{50} values of 1-hydroxy-1,1-bisphosphonates were estimated.[49] IC_{50} is the inhibitory concentration of a drug required to reduce a process by 50%.[54]

Gem-bisphosphonates are potent and competitive inhibitors of farnesyl pyrophosphate synthase of *T. cruzi* (TcFPPS).[51] As it was explained before the inhibition of FPPS prevents formation of farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP), which in turn prevent prenylation of the proteins. Prenylation, for one, is

required for many cell functions and activity.[7] Thus, inhibition of this process gives rise to inhibition of *T. cruzi* growth.[52]

Therefore, some of the synthesized conjugates were confirmed to be potent inhibitors of the relevant form of the parasite, performing IC_{50} values at the low micromolar level. For example, compound **30b** has an IC_{50} value of 1.94 μM meaning that it has a high degree of efficiency as inhibitor of TcFPPS. In addition, the other *gem*-BPs exhibit a satisfying IC_{50} values (**Table 7**) and can be considered as potent inhibitors against *T. cruzi* proliferation.[51]

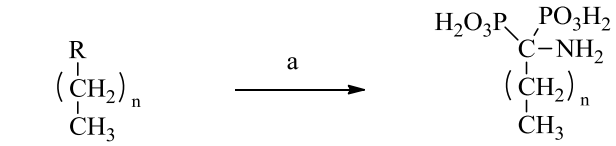
Table 7. IC_{50} values for *gem*-bisphosphonates (**30a-e**) against TcFPPS activity.[51]

Bisphosphonate	**n**	**IC_{50} (μM)**
30a	2	42.83
30b	4	1.94
30c	5	2.37
30d	6	9.36
30e	7	8.45

Despite of recognizing the molecular target of these new BP-fatty acid conjugates, the effect of the hydroxyl group at the C-1 position of BPs on biological activity was investigated. For this reason 1,1-bisphosphonates (lacking OH in position C-1) were prepared and evaluated. It was found that also this set of BP-fatty acid conjugates was effective inhibiting TcFPPS, although slightly less efficiently than 1-hydroxy-1,1-bisphosphonates. This difference in activity may be due to the absence of a site to coordinate Mg^{2+} ion. In other words, 1-hydroxy-1,1-bisphosphonate derivatives can coordinate Mg^{2+} with a tridentate binding while 1,1-bisphosphonates (lacking the OH-group) have only two coordination sites. Furthermore, it was proved that 1-hydroxy-1,1-bisphosphonates were potent inhibitors also against farnesyl pyrophosphate synthase from *T. brucei* (TbFPPS), the parasite liable for the sleeping sickness, a disease that targets the central nervous system.[51]

Keeping in mind that BPs with three functional groups at C-1 able to bind substrates in a tridentate manner are more potent inhibitors than those containing only two (or one),

replacement of the hydroxyl group at C-1 by an amino group was investigated. The synthesis (**Scheme 33**) can be started either from the corresponding cyano or amide derivatives.[52]



R= CN or $C(O)NH_2$

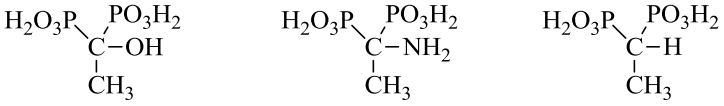
31 **32**

1-Amino-1,1-bisphosphonate	n	a	Yield (%)
32a	0	MeCN, H_3PO_3, 130°C, 12 h	51
32b	1	CH_3CH_2CN, H_3PO_3, 135°C, 12 h	70
32c	2	(i) $CH_3CH_2CH_2CN$, H_3PO_3, $PhSO_3H$, 65°C, 10 min; (ii) PCl_3, 70°C, 6 h	53
32d	3	(i) $CH_3(CH_2)_3CN$, H_3PO_3, $PhSO_3H$, 65°C, 10 min; (ii) PCl_3, 85°C, 16 h	25
32e	4	(i) $CH_3(CH_2)_4CN$, H_3PO_3, $PhSO_3H$, 70°C, 10 min; (ii) PCl_3, 90°C, 16 h	15
32f	5	(i) $CH_3(CH_2)_4CH_2C(O)NH_2$, H_3PO_3, $PhSO_3H$, 65°C, 10 min; (ii) PCl_3, 90°C, 16 h	10

Scheme 33. 1-Amino-1,1-bisphosphonates (**32a-f**) derived from fatty acids. The synthesis was started from the appropriate nitrile with exception for compound **32f**, for which a corresponding amide was used.[52]

1-Amino-1,1-bisphosphonates derived from fatty acids resulted to be even more effective inhibitors against TcFPPS than 1-hydroxyl-1,1-bisphosphonates. For example, compound **32e** exhibited an IC_{50} value of 0.38 μM making it a potent antiproliferative agent against the parasite.[52]

Moreover, the ability of three BP-fatty acid derivatives differing only by one substituent at C-1 (**Scheme 34**) to coordinate Mg^{2+} ion was investigated.[52]

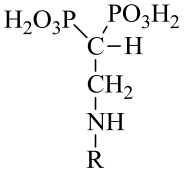


30 **32** **33**

Scheme 34. Chemical structure of 1-hydroxy (**30**), 1-amino (**32**), and alkyl-1,1-bisphosphonates (**33**) studied to compare their ability to coordinate Mg^{2+}.[52]

Compounds **30** and **32** exhibited stronger efficacy to coordinate Mg^{2+} ion than compound **33**. As it was discussed above, this efficacy of compounds **30** and **32** is due to the opportunity to coordinate ions in a tridentate manner instead of bidentate as in compound **33**. Consequently, these results confirmed the expectations.[52]

In yet another study the activity of a series of 2-alkylaminoethyl-1,1-bisphosphonates (**Scheme 35**) against *T. cruzi* was examined.



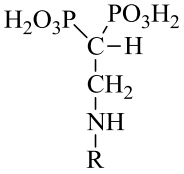
34

Compound	R	Yield (%)
34a	*n*-propyl	94
34b	*n*-butyl	92
34c	*n*-pentyl	98
34d	*n*-hexyl	85
34e	*n*-heptyl	91
34f	*n*-octyl	78
34g	3-methyl-but-1-yl	88
34h	*tert*-butyl	71
34i	cyclohexyl	66
34j	prop-2-yl	79
34k	but-2-yl	80
34l	2-methylprop-1-yl	68
34m	pyrrolidin-1-yl	77
34n	piperidin-1-yl	84

Scheme 35. 2-Alkylaminoethyl-1,1-bisphosphonates (**34a-n**) with R= alkyl group.[50]

Most of the synthesized 2-alkylaminoethyl *gem*-bisphosphonates were active against *T. cruzi*, showing a high selectivity for the inhibition of the enzymatic activity of TcFPPS. Compounds **34c** and **34d** were the most potent compounds among this new family of BP-fatty acid derivatives.[50]

In addition, long chain analogs of 2-alkylaminoethyl-1,1-bisphosphonates (**Scheme 36**) were proved to be potent inhibitors against *T. cruzi*.[53]



34

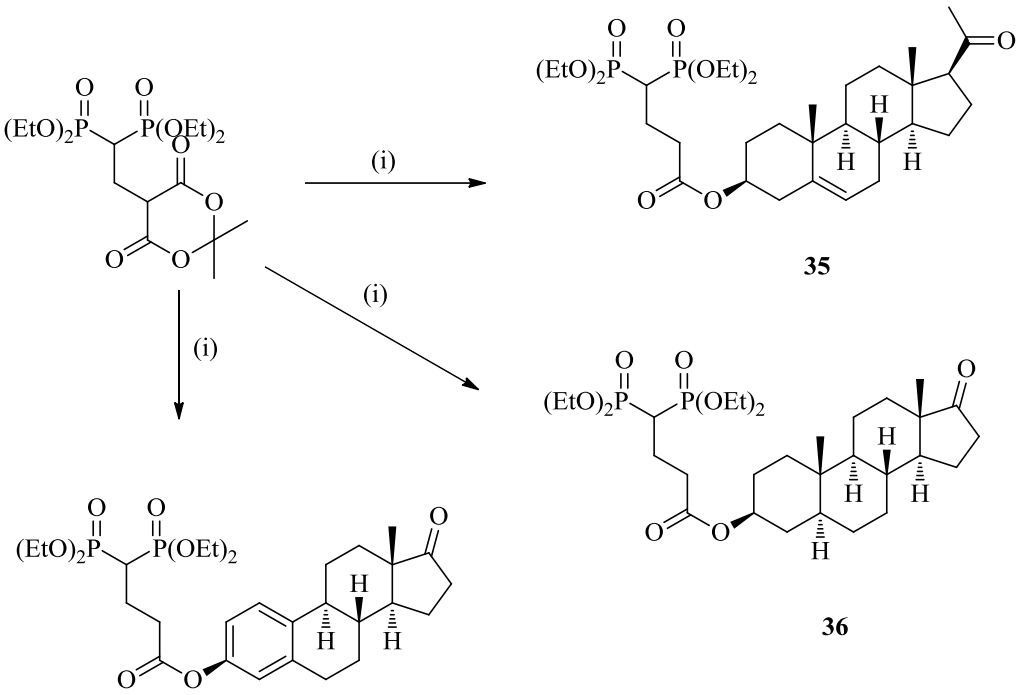
Compound	R
34o	benzyl
34p	*n*-nonyl
34q	*n*-decyl
34r	*n*-undecyl
34s	*n*-dodecyl
34t	*n*-tetradecyl
34u	*n*-hexadecyl
34v	*n*-octadecyl

Scheme 36. 2-Alkylaminoethyl-1,1-bisphosphonates (**34o-v**).[53]

Finally, these two series of 2-alkylaminoethyl *gem*-bisphosphonates showed a broad range of activity since they were effective also against *Toxoplasma gondii*.[50, 53]

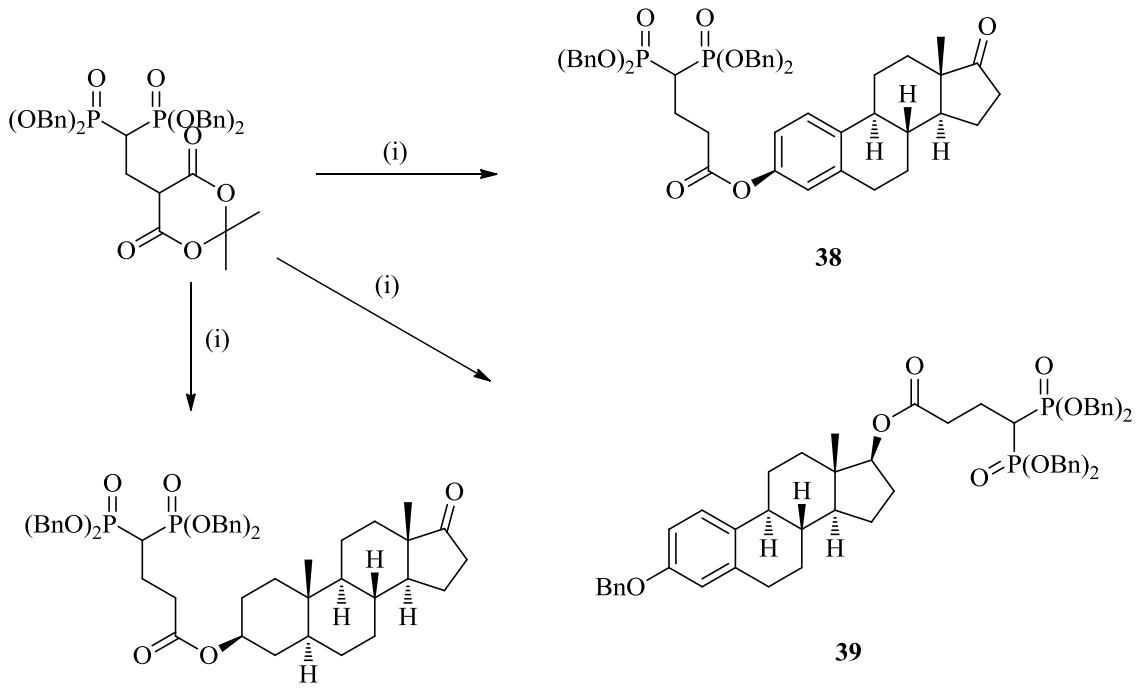
3.6.4 Bisphosphonate-steroid conjugates

Estradiols are biologically active compounds belonging to the class of steroid derivatives. They can be conjugated to BPs to regulate bone remodeling while BPs act as bone-targeting moieties thereby preventing side effects in other tissues. In the design of these compounds, it is important to evaluate the features of the link between the BPs and estradiols. This bond should be neither too stable nor too labile. For this reason, Page *et al.*[55] synthesized bisphosphonic acid-steroid conjugates linking them through a carboxylic ester bond. The linkage can occur at the 3 or 17 position of the steroid. The coupling between preactivated BPs with pregnenolone, oestrone, and *trans*-androsterone were successful (**Scheme 37**).[55]



Scheme 37. Pregnenolone (**35**) (87 %), oestrone (**36**) (77 %), and *trans*-androsterone (**37**) (99 %) linked with preactivated BPs.[55]

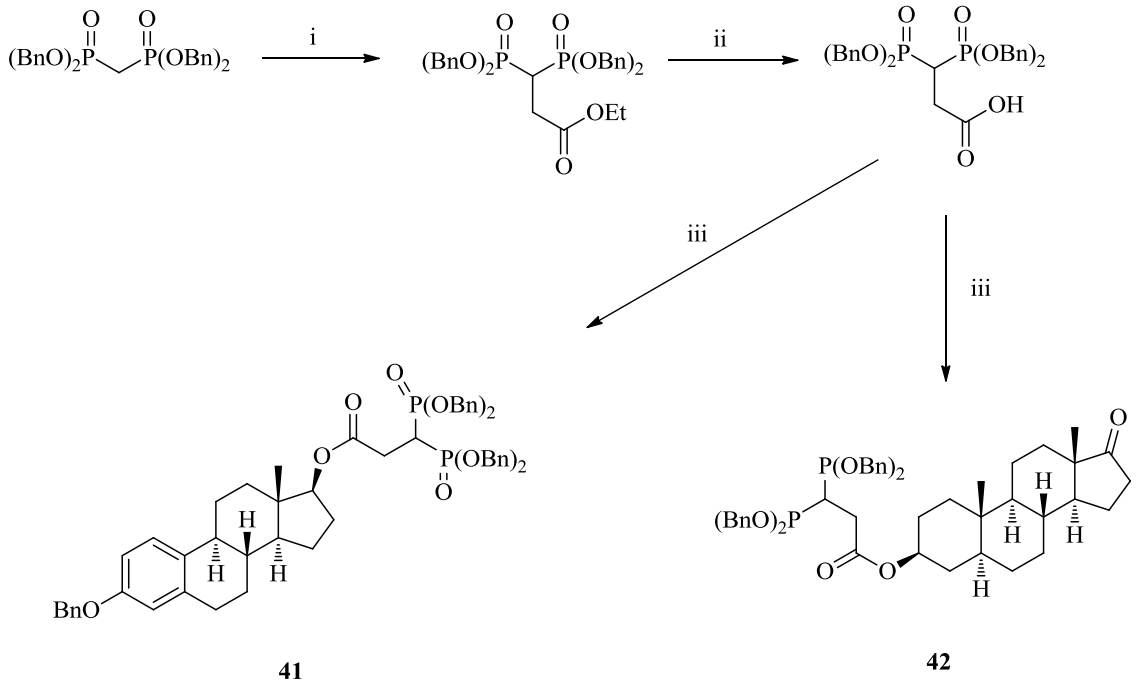
The synthesis of the modified BPs involve Meldrum's acid to activate the carboxylic acid. The same procedure was applied to tetrabenzyl methylenebisphosphonates which, after the activation, achieved the reaction with oestrone, *trans*-androsterone, and 3-benzyl-17*β*-oestradiol to give the conjugated compounds (**Scheme 38**).[55]



(i) Steroid, toluene, Δ

Scheme 38. Oestrone (**38**) (60 %), *trans*-androsterone (**39**) (86 %), and 3-benzyl-17*β*-oestradiol (**40**) (76 %) linked with preactivated BPs.[55]

Other two syntheses were carried out between 3,3-bis(dibenzyloxyphosphoryl)-propanoic acid and *trans*-androsterone and oestrone giving the corresponding BP conjugates **41** and **42** (**Scheme 39**).[55]



(i) NaH, THF, ethyl bromoacetate;
(ii) KOH, MeOH/H_2O;
(iii) steroid, DCC, DMAP, CH_2Cl_2

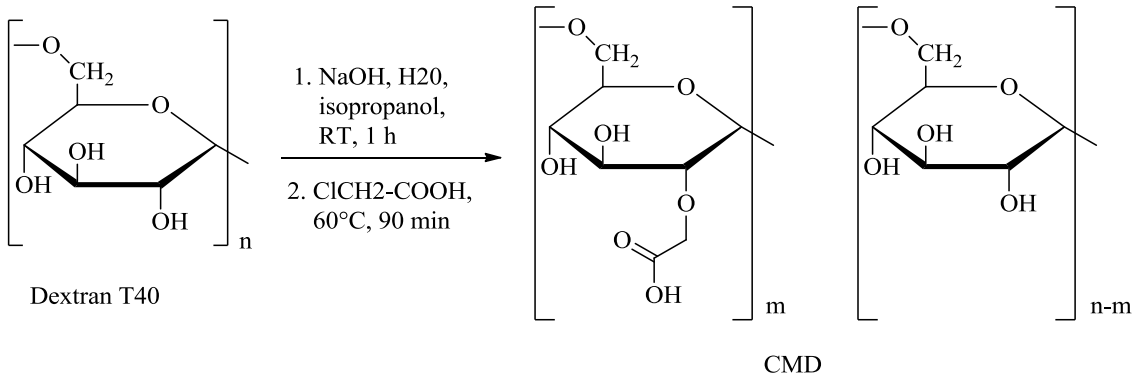
Scheme 39. Synthesis between 3,3-bis(dibenzyloxyphosphoryl)propanoic acid and *trans*-androsterone and oestrone giving the corresponding BP conjugates (**41**) and (**42**).[55]

The hydrogenolysis of these steroidal bisphosphonates gave the corresponding bisphosphonic acid-steroid conjugates with different ranges of lability.[55]

3.6.5 Bisphosphonate-saccharide conjugates

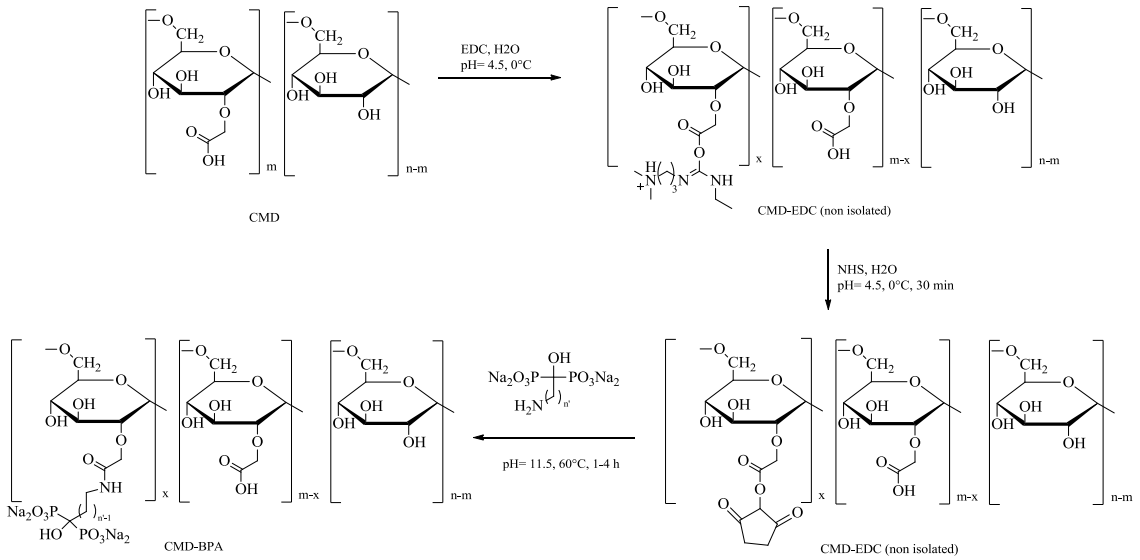
Despite their poor bioavailability in the organism after oral administration, BPs are considered the most important and potent therapeutic agent for diseases related to bones due to their affinity for HAP, the main component of bones. Moreover, it was recently observed that BPs show also interesting anticancer activity. The efficiency of *N*-BPs against tumor cell proliferation of various origins has been established. It is not clear yet if they affect directly by inhibiting tumor cells or indirectly via antiresorptive activity.[56]

However, Migianu-Griffoni *et al.*[57] designed and synthesized new *N*-BP conjugates with a polysaccharide. The polysaccharide used was dextran modified via a carboxymethylation to obtain a carboxymethyldextran (CMD) (**Scheme 40**).[57]



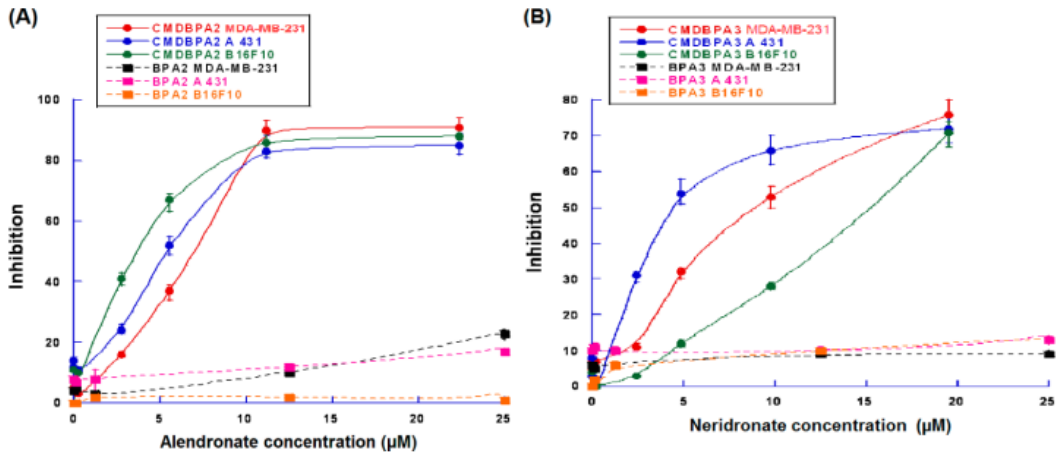
Scheme 40. Synthesis of CMD.[57]

N-BPs, such as alendronate (ALN) and neridronate (NER), were coupled through the amino group with the carboxylic group of CMD. The synthesis was carried out without the need of protecting groups because the amino group is more nucleophilic than the alcohol one. Thus, the coupling between the -COOH group of CMD and the $–NH_2$ group of BP was selective. As shown in **Scheme 41** the synthesis is based on three steps. First, the activation of the carboxylic acid group with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC), a water-soluble activator. Second, the transesterification reaction with *N*-hydroxysuccinimide (NHS), and finally, the reaction between CMD-NHS and *N*-BPs.[57]



Scheme 41. Reaction of carboxymethyldextran (CMD) with nitrogen-containing BPs to obtain more efficient bioconjugates, CMD-BPs.[57]

This new class of bisphosphonate-dextran conjugates were evaluated both *in vivo* and *in vitro*. The inhibition of tumor cell proliferation was investigated *in vitro* on three cell lines: MDA-MB-231 (highly metastatic human breast tumor cells), A431 (epidermoid carcinoma), and B16F10 (melanoma). Between the newly synthesized bioconjugates, CMD-ALN showed a total inhibition of cell proliferation on all the cell lines (**Scheme 42**).[57]



Scheme 42. *In vitro* investigation on antiproliferative activity against tumor cells of (A) carboxymethyldextran-alendronate (CMD-ALN) and alendronate (ALN); and (B) carboxymethyldextran-neridronate (CMD-NER) and neridronate (NER) on three cell lines: MDA-MB-231 (highly metastatic human breast tumor cells), A431 (epidermoid carcinoma), and B16F10 (melanoma).[57]

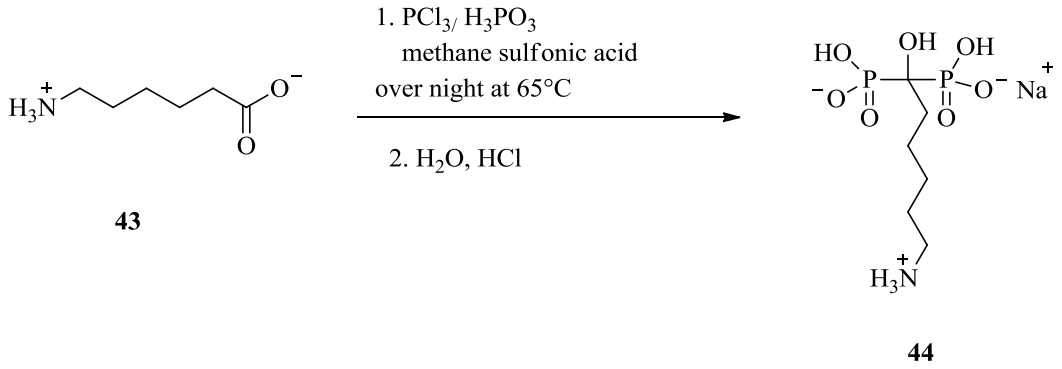
Whereas CMD alone did not show any antiproliferative activity, CMD-ALN exhibited an irreversible loss of cell growth inhibiting the proliferation. For this reason, CMD-ALN was also biologically evaluated *in vivo* in mice with MDA-MB-231. The results showed a greater inhibition for CMD-ALN than free ALN alone.[57]

In conclusion, the use of carboxymethyldextran, having a strong affinity for tumor cells, as a carrier, improves the bioavailability of anticancerous BPs.[57]

3.6.6 Bisphosphonate-liposome conjugates

BPs, as was mentioned above, in addition to being potent therapeutic agents of bone-related diseases, have proven to be efficient inhibitors of growth, attachment, and invasion of tumor cells of various origins.[58]

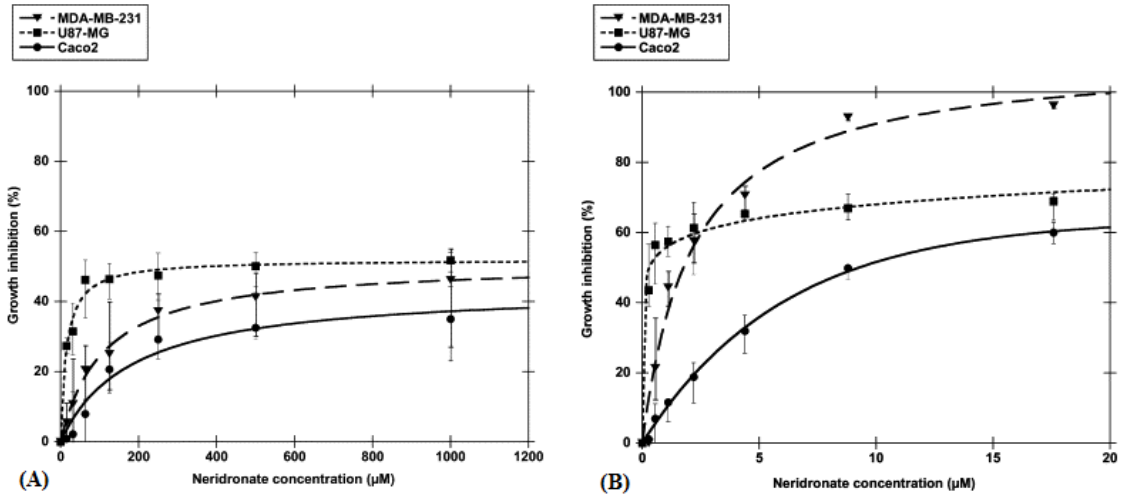
However, due to BPs' own poor bioavailability and probable toxicity with increased doses, potential candidates with better delivery properties and lesser toxicity were studied. Among the candidates, Chebbi *et al.*[59] synthesized and evaluated the delivery of BPs through liposomal carriage. They focused on *N*-BPs, particularly on neridronate (NER). NER was synthesized as shown in **Scheme 43** using a one-pot reaction and then encapsulated in liposomes.[59]



Scheme 43. Synthesis of neridronate (**44**) from the corresponding carboxylic acid precursor (**43**).[59]

The activity of the synthesized conjugate was tested *in vitro* on three carcinoma cell lines: a breast carcinoma (MDA-MB-231), a brain carcinoma (U87-MG) and a colon carcinoma (Caco2). MTT assay was used to evaluate the cell viability.[59] MTT is a colorimetric assay based on the cleavage of tetrazolium salts into purple formazan crystals by mitochondrial enzymes.[59, 60]

The three cell lines were treated either with free NER or with NER entrapped in liposomes. Free NER resulted poor inhibitor of cancer cells, whereas, liposome-NER conjugate showed total inhibition, especially for MDA-MB-231 as illustrated in **Scheme 44**.[59]



Scheme 44. MTT growth inhibition measurements performed after a 72 h incubation of MDA-MB-231, U87-MG and Caco2 cell lines with (**A**) free neridronate and (**B**) liposomal neridronate.[59]

More assays, such as migration and invasion were performed to confirm the results regarding the strong activity of neridronate-liposome conjugates as inhibitors of tumor cells. These assays give information about the ability of cancer cells to migrate and invade other tissues. It was found that neridronate-liposome conjugate showed suppressive activity on carcinoma cell mobility *in vitro* while free neridronate did not.

Despite the liposomal delivery system is promising, it needs to be tested *in vivo* as well as with other BPs.[59]

4 CONCLUSIONS

Discovery new drugs entails high cost and effort. Consequently, drug delivery has taken hold to improve the performance of existing drugs. A successful drug delivery system transports the drug at the site of action by controlling the rate, time, and place of release of drugs in the body. Among the common routes of administration, such as oral, parental, transdermal, and aerosol, the oral one is preferred for both the formulation and tolerance of the patient. A drawback about oral administration is to overcome the barriers, needed to prevent the transition of toxic molecules, in order to reach the site of action of the drug itself. A lot of research focuses on the design of prodrugs in order to allow the passage through the barriers. For this purpose a "promoiety" is covalently linked to the drug. The prodrug, inactive before the metabolism, releases the active drug from its promoiety which exerts its therapeutic effect in the optimal manner. This approach has several advantages compared to the conventional drug administration, such as increase the bioavailability, site selectivity, and patient tolerance.

The knowledge of bisphosphonates date back to the middle of 19^{th} century and their use was mainly for manufacturing. Later, in the 1960s they started to be used in medicine as treatment for calcium disorders. In fact, bisphosphonates are effective inhibitors of bone resorption due to skeletal disorders, such as osteoporosis, Paget's disease, hypercalcemia of malignancy, multiple myeloma, and bone metastasis.

The structure of a bisphosphonate is similar to the naturally occurring pyrophosphate. The antiresorptive effect of bisphosphonates is strongly correlated to their structure. They are characterized by two phosphate groups bound to the same carbon atom, a 'P-C-P' moiety, which confers more stability to the compounds than pyrophosphate. Moreover, they have two functional groups in their structure called R^1 and R^2, which can be modified according to the desired properties in the compound. The P-C-P moiety is indispensable for biological activity being a 'bone hook' for binding to hydroxyapatite. In addition, when R^1 is a hydroxyl group, the binding to hydroxyapatite is enhanced, and R^2 can be used to determine the potency and the efficacy. Probably, this is due to enabling the chelate effect by a tridentate binding. The tridentate binding is achievable for the presence of two phosphate groups, OH at R^1 side group, and an appropriate functional group at R^2 position, such as amino group.

Generally, bisphosphonates can be classified into two groups according to their mechanism of action: non-nitrogen containing bisphosphonates and nitrogen containing bisphosphonates. Their inhibition against bone resorption is based on two features:

mineral binding affinity and altering cellular metabolism. The mineral binding property depends on the interaction between the structure of bisphosphonates and hydroxyapatite. The mutation of cellular metabolism occurs through apoptosis regarding the simple bisphosphonates and through inhibition of osteoclasts activity regarding the nitrogen containing bisphosphonates. When nitrogen containing bisphosphonates interfere with osteoclast activity, apoptosis may eventually occur even though it is not strictly necessary for inhibition of bone resorption. This action is mainly due to the inhibition of farnesylpyrophosphate synthase of mevalonate pathway, an essential pathway for several cellular processes.

Despite bisphosphonates are attractive antiresorptive agents for the treatment of skeletal diseases, their poor bioavailability sets restrictions for their use. This issue is due either for their hydrophilic properties and for the bulky and charged phosphonate groups. Effectively, they hinder the absorption in the gastrointestinal tract and the oral bioavailability after administration is less than 1%. In addition, it is also necessary to consider the unpleasant side effects as inconvenience associated with the clinical use of bisphosphonates. These reasons have enhanced the interest in the development of strategies of novel drug delivery systems for bisphosphonates to improve both the bioavailability and safety. In the prodrug design, one or more ionizable groups available in bisphosphonate can be replaced with a bioreversible promoiety. The functional groups that can be modified are OH and/or NH in the side chain or either of the two phosphorous groups.

Many synthetic strategies have been investigated to enhance the intestinal permeability of bisphosphonates. For instance, masking the amino and the hydroxyl groups of nitrogen containing bisphosphonates with acetyl moieties improved the lipophilicity and the solubility of the compounds. Nitrogen containing bisphosphonates conjugated with polyamine resulted to have higher affinity for hydroxyapatite. The side chain modification of 1-hydroxyl-1,1-bisphopshonate into diesters utilizing various alkyl or aryl substituents was also advantageous. Pivaloyloxymethyl ester and benzoyloxypropyl ester of clodronic acid were also synthesized and evaluated. In contrast to benzoyloxypropyl ester, which did not release the parent drug, pivaloyloxymethyl ester gave better results establishing more lipophilicity than clodronate. Similarly, since tetramethyl ester of etidronic acid did not release the parent drug, acetylated pivaloyloxymethyl esters of etidronic acid were synthesized and evaluated. Satisfactory aqueous solubility and lipophilicity, and acceptable release of etidronate, were found.

Furthermore, alendronate derivatives were designed and optimized with promising potential as prodrugs. In addition, bisphosphonate bioreversible prodrugs can be formulated converting two ionizable groups into dianhydrides. For example, four prodrugs of clodronate were synthesized with different steric hindrance. They showed more lipophilicity than clodronate releasing the parent drug enzymatically.

Among all the strategies applied for a better drug targeting, the employment of endogenous ligands in drug delivery systems offers enormous benefits both for the design of carriers and for the patient compliance. There are two types of approaches regarding bisphosphonate prodrugs. On the one hand, targeted carrier systems link the bisphosphonates either to phosphorous atoms or to the side chain to improve their efficacy as drug. On the other hand, bisphosphonates themselves act as promoieties of carrier systems for a better delivery of other drugs.

Oral bioavailability clearly increased when bisphosphonates were conjugated to peptidyl moieties. In fact, the affinity of the prodrugs for the intestinal tissue were higher than those of the parent drugs with also better effective permeability coefficients. However, the prodrugs inhibited the calcification of tissues showing a so called ‘crystal poison’ effect. Although, α-amino acid derived bisphosphonates were not all so successful. Apart from leucine, phenylalanine, and glutamine bisphosphonates, none of the other conjugates showed any antiresorptive activity. This inactivity might be influenced by two factors: the larger steric hindrance of the ‘bone hook’ and the increasing of polarity due to the presence of amino groups which decrease the antiresorptive activity. Moreover, bisphosphonate-fatty acid conjugates, such as 1-hydroxyalkyl-1,1-bisphosphonates, 1,1-bisphosphonates, 1-amino-1,1-bisphosphonates, and 2-alkyllaminoethyl-1,1-bisphosphonates, were synthesized and biologically evaluated. They resulted to be potent inhibitors of Trypanosomatidae family, parasitic protozoans that cause diseases, such as Chagas’s disease and sleeping sickness. Bisphosphonates can also achieve bone-seeking target if conjugated to steroids, such as estradiol, thereby preventing side effect in other tissues. Despite being potent therapeutic agents for diseases related to bones, it was recently observed that bisphosphonates show interesting anticancer activity. Therefore, a new class of nitrogen containing bisphosphonates conjugated with a polysaccharide were synthesized and evaluated both *in vivo* and *in vitro*. The investigation about antiproliferative activity of tumor cells gave promising results. For instance, carboxymethyldextran-alendronate exhibited an irreversible loss of cell growth inhibiting the proliferation. Other derivatives that displayed efficient anticancer activity but need

still to be tested *in vivo*, are nitrogen containing bisphosphonates conjugated with liposomes. Particularly, neridronate-liposome derivate showed encouraging suppressive activity on carcinoma cell mobility.

In conclusion, it has been clearly shown that bisphosphonate prodrugs increase either the absorption of the bisphosphonate acting as drug or the delivery of other drugs.

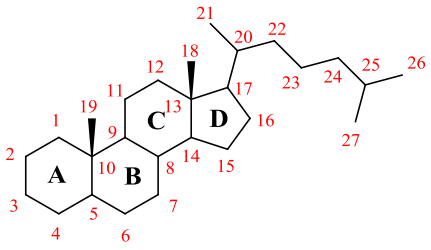
II EXPERIMENTAL PART

5. INTRODUCTION

This chapter follows on from the previous one, which examined how a prodrug approach increases the bioavailability and safety of bisphosphonates using endogenous molecules as carriers.

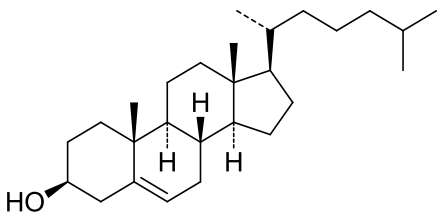
This introductory section provides a brief overview of steroids and particularly of bile acids and their potentiality to act as carriers. It then goes on to the experimental section and results.

Steroids, derived terpenoids, represent a wide family of natural products which play an important role in drug development due to their biological activity. The ring lettering and atom numbering of steroids are illustrated in **Scheme 45**.[61]



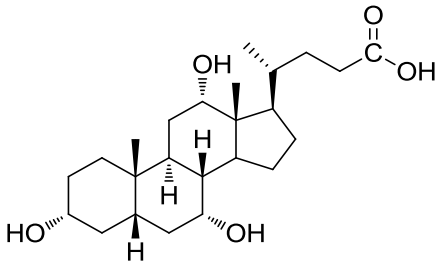
Scheme 45. General structure and skeleton of steroid compounds.[61]

Cholesterol is one of the best known steroid and, as it is shown in **Scheme 46**, typifies the fundamental structure of this class of compounds.[61]



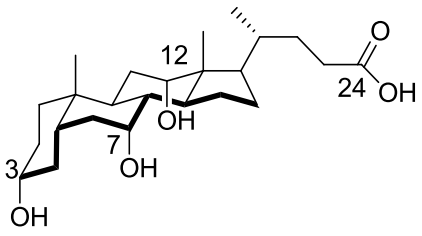
Scheme 46. Structure of cholesterol.[61]

Steroids are characterized by a tetracyclic ring system consisting of three fused six-membered and one five-membered ring.[61] The functional groups are mostly located at the C-3, C-7, C-10, C-11, C-12, and C-17 positions.[62] Based on the modification from the common structural skeleton, it is possible to achieve a broad range of natural products of great biological interest. Among them stand out bile acids, steroidal surfactant derivatives.[61] Bile acids are C_{24} skeletal compounds, e.g. cholic acid (**Scheme 47**), consisting of a rigid steroid nucleus and a short aliphatic side chain.[63]



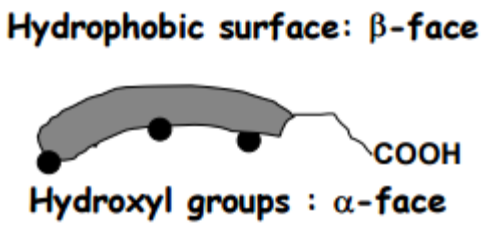
Scheme 47. Structure of cholic acid.[63]

The features that make bile acids different to one another are basically due to the side chain structure, the stereochemistry of A/B rings, and the hydroxyl groups in the steroid nucleus. In the higher vertebrates, the steroidal nucleus is curved due to the *cis*-fused configuration of A and B rings, as shown in **Scheme 48**.[63]



Scheme 48. Cholic acid in *cis* A/B ring configuration.[63]

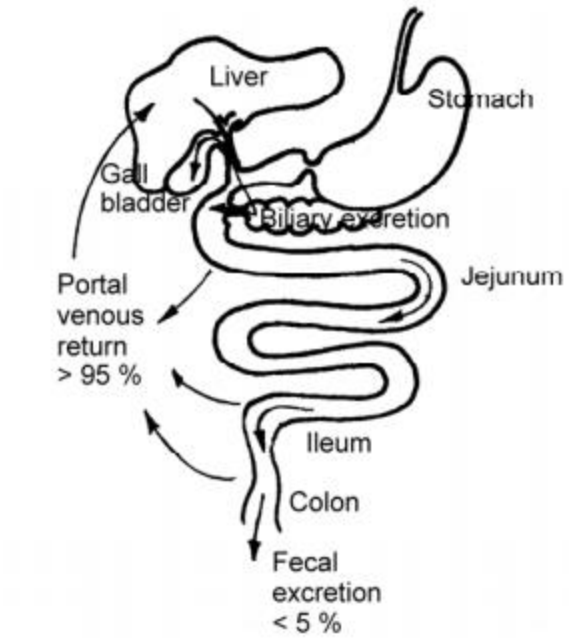
Furthermore, in lower vertebrates, the A/B ring system can show *trans*-fused configuration. Accordingly to the *cis* A/B ring configuration, bile acids present a concave and a convex sides called α- and β-faces, respectively, as illustrated in **Scheme 49**.[63]



Scheme 49. Concave α-face and convex β-face of bile acids.[63]

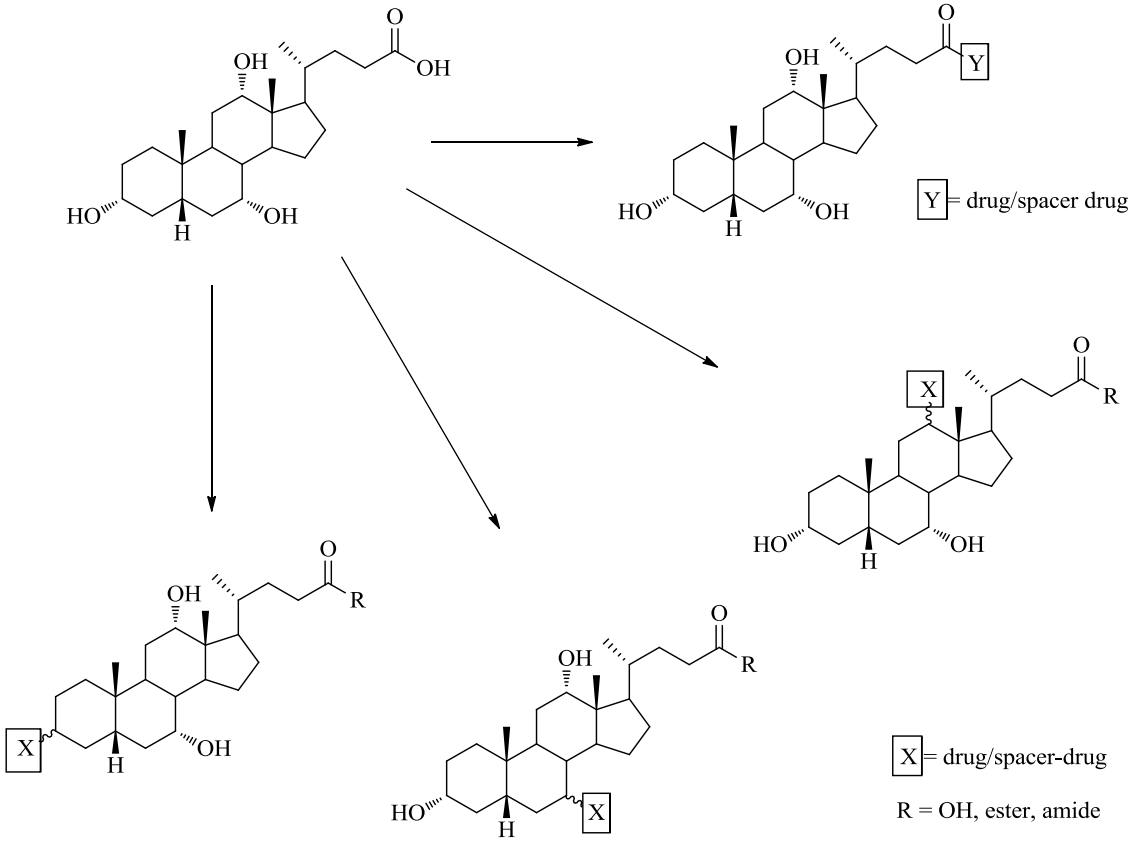
The concave α-side results to be hydrophilic due to the presence of hydroxyl groups while the convex β-side results to be hydrophobic for the presence of alkyl groups.[63] This polar lower α-face in contrast with the non-polar β-face cause amphiphilic properties in bile acids. For this reason, they are able to form micelles and to act as detergents.[61] The features explained above, in addition to being endogenous, easily modifiable, available, and cheap compounds, make bile acids attractive tools for pharmacological applications as potential carriers.[62]

Bile acids are metabolic products of cholesterol and they are responsible for the solubilization and absorption of lipids and lipid-soluble vitamins. Bile is stored in the gall bladder and, after food intake, it is secreted into the small intestine. Here, bile acids exert the function of digestion and resorption of the nutrients. These nutrients, such as fat, fatty acids, and lipid-soluble vitamins, are insoluble in water. It follows that, in order to allow their uptake, they are dispersed in micelles of bile acids and lipids. At this point, bile acids are almost completely reabsorbed by both passive transport, in the small intestine, and active transport, in the ileum. These processes are responsible of the bile acids' way back to the liver for re-circulation *via* the portal vein. The entire mechanism of transport of bile acids is known as entherohepatic circulation (**Scheme 50**) and it happens 6-15 times per day.[63, 64]



Scheme 50. Enterohepatic circulation of bile acids.[65]

Because of the high specificity and capacity of bile acid transport systems described above, the development of the research for drug-bile acid conjugates has taken hold. Research efforts have been focused on specific drugs in order to target the liver and to enhance the intestinal absorption of poorly or non-absorbed drugs.[63, 64] For the purpose of a successful drug design, the following properties of bile acids need to be preserved: the negatively charged side chain, the *cis* A/B ring configuration, and at least one axial hydroxyl group on the steroid skeleton at position C-3, C-7, or C-12.[64] Several drugs can be attached either to the steroidal backbone or to the side chain directly or *via* specific linkers at positions C-3, C-7, C-12, or C-24, as shown in **Scheme 51**.[65]



Scheme 51. Structures of potential bile acid derivatives.[65]

A variety of bile acid derivatives can be prepared modifying these functional groups: the carboxylic acid group may be esterified, amidated, reduced, or acylated; the hydroxyl groups in the steroidal nucleus may be subjected to etherification, oxidation, in addition to esterification and reduction.[65]

However, the aptitude of these compounds to form bile-salt micelles is one of the significant properties of bile acids.[63] By taking advantage of their unique physiology and their specific transport processes, bile acids have become important and enhanced systems for drug delivery.[64]

6 AIM OF THE WORK

The aim of this work was to conjugate bisphosphonates with bile acids in order to improve the oral bioavailability of the bisphosphonate. To optimize the yield, three different synthetic strategies were tested. In all syntheses sodium alendronate was used as the bisphosphonate and deoxycholic acid as the bile acid.

In the first synthesis the reactivity of the carboxylic acid group of the bile acid was increased by converting it to an anhydride,[66] which then reacted with the amino group of the Na-alendronate to obtain the desired conjugate.

In the second synthesis the bile acid was activated with *N*-hydroxysuccinimide,[67] after which the active intermediate was allowed to react with Na-alendronate.

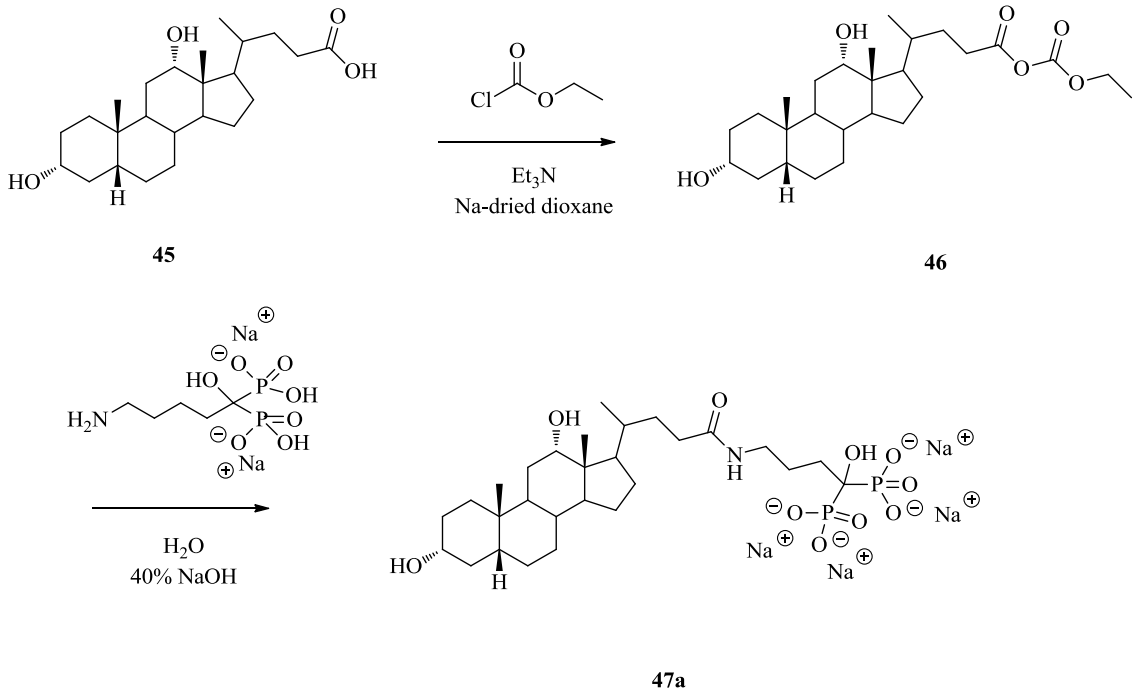
Finally, the bile acid was converted to the most active derivative of carboxylic group, namely acid chloride. First the hydroxyl groups were protected with trifluoroacetic acid anhydride and then the carboxyl group converted to acid chloride with $SOCl_2$.[68] Once the bile acid is activated, the reaction with Na-alendronate shall proceed smoothly.

The bile acid-alendronate conjugates were characterized using NMR spectroscopy and mass spectrometry.

7 SYNTHESES

7.1 Synthesis of deoxycholic acid-alendronate through an anhydride as the intermediate

Deoxycholic acid-alendronate conjugate was prepared through the reaction shown in **Scheme 52**, which demanded an anhydride as the intermediate.[66]



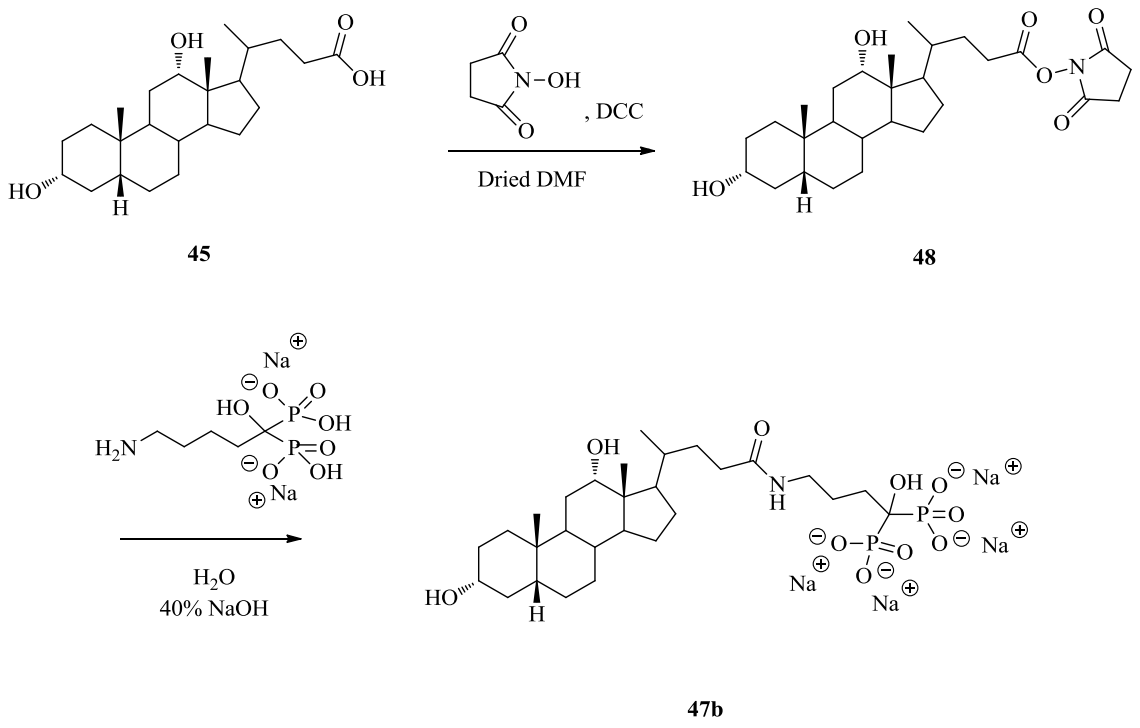
Scheme 52. General procedure for the synthesis of deoxycholic acid-alendronate conjugate through an anhydride as the intermediate.[66]

The reaction between deoxycholic acid and Na-alendronate was performed after the activation of the bile acid through the corresponding anhydride **46**. The formation of the obtained product was confirmed after 48h with ^{31}P NMR. The work-up of the crude product was performed as follows: the residue was washed with diethyl ether to eliminate any unreacted compound. Then the precipitate was dissolved in ethanol and filtered since the product should be soluble in ethanol. The ethanol solution was treated with ion exchange resin to remove any unreacted Na-alendronate and to obtain the deoxycholic acid-alendronate conjugate.

The deoxycholic acid-alendronate conjugate was characterized by ^{31}P and ^{1}H NMR.

7.2 Synthesis of deoxycholic acid-alendronate through succinimido deoxycholate as the intermediate

The second method used to prepare deoxycholic acid-alendronate conjugate is shown in **Scheme 53**. It was foreseen the formation of succinimido deoxycholate **48** as an intermediate.[67]



Scheme 53. General procedure for the synthesis of deoxycholic acid-alendronate conjugate through succinimido deoxycholate as the intermediate.

After the activation of the bile acid by preparation of the corresponding succinimido ester, the more active but impure intermediate was tried to be purified by column chromatography. Several attempts were made with different amounts of methanol in chloroform as eluent with no results. The nature of the crude product was checked with ^{1}H and ^{13}C NMR as well with MS. From these analyses it was possible to gather that the succinimido deoxycholate had reacted with the eluent in the column during the purification. Other eluents, such as ethyl acetate in dichloromethane, were tested through TLC with no success either.

Therefore, the reaction between impure succinimido deoxycholate and Na-alendronate was performed. The progress of the reaction was checked after 24, 48, and 72 h by ^{31}P and ^{1}H NMR but no achievement was confirmed.

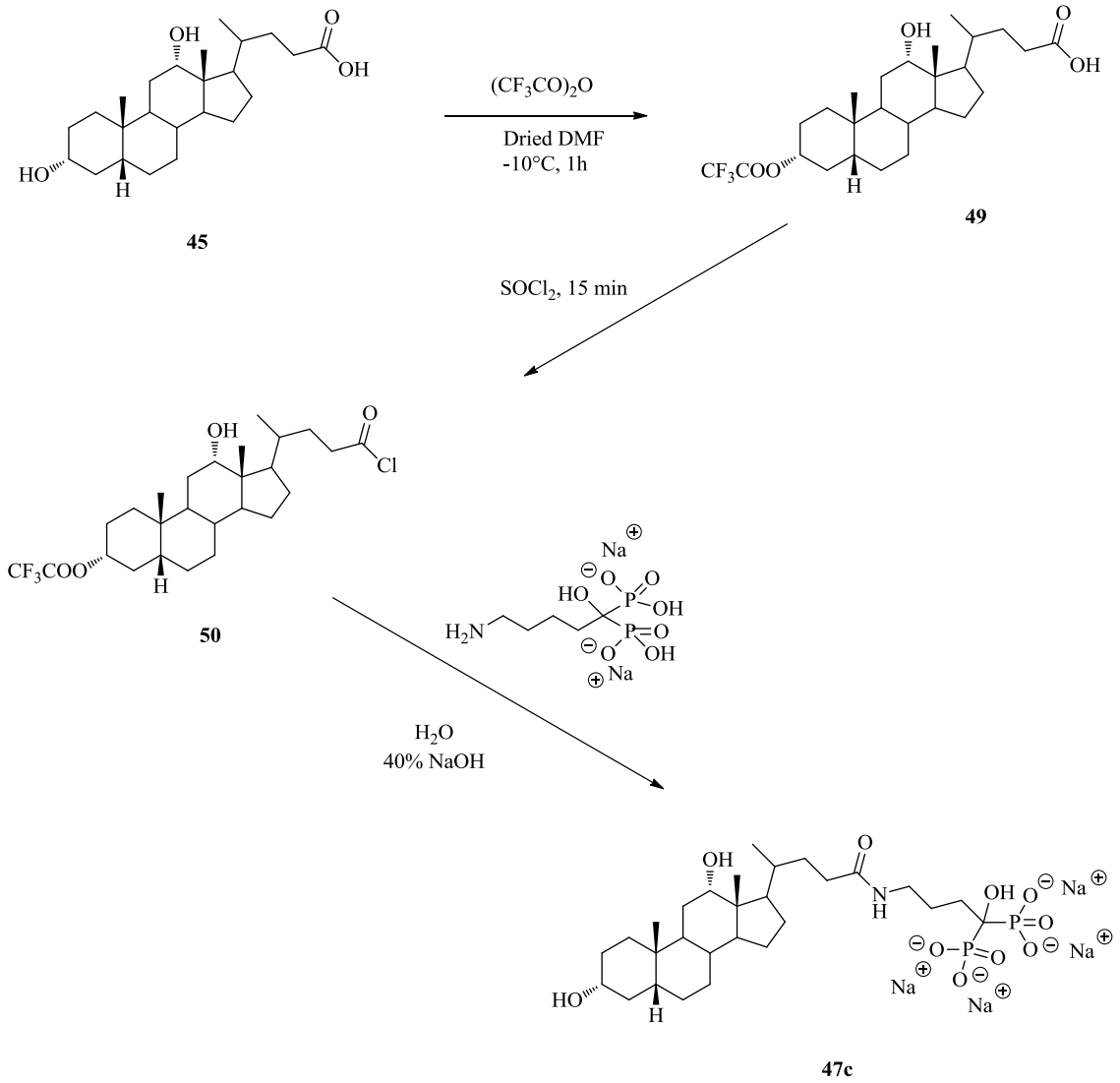
In order to enable the reaction between the succinimido ester-activated bile acid and Na-alendronate a purification technique was essential. For this reason, the impure succinimido deoxycholate was decided to purify through a liquid-liquid

($CHCl_3$/$NaHCO_3$) extraction. After the extraction, the intermediate was obtained pure as confirmed with NMR.

The reaction between the pure succinimido deoxycholate and Na-alendronate was then performed and the progress of the reaction checked with ^{31}P NMR. The crude product was then purified. First a liquid-liquid ($CHCl_3$/$NaHCO_3$) extraction was performed but the proton NMR spectrum showed still some impurities. Second a treatment with ion exchange resin followed by extraction was tried and the product was checked by NMR as well by MS with no satisfying results. However, it was possible to gather that the reaction had taken place although it was not obtained as pure. For this purpose, the impure deoxycholic acid-alendronate conjugate was sent to the University of Eastern Finland to be purified with high performance countercurrent chromatography (HPCCC), and to study its potential to act as a prodrug by hydrolysis experiments.

7.3 Synthesis of deoxycholic acid-alendronate through an acyl chloride as the intermediate

In the third method, the bile acid was converted to the most active of the carboxylic acid derivatives **50**, namely acid chloride, which was then allowed to react with Na-alendronate as shown in **Scheme 54**.[68]



Scheme 54. General procedure for the synthesis of deoxycholic acid-alendronate conjugate through trifluoroacetate derivative of deoxycholic acid chloride as the intermediate.

First the hydroxyl groups were protected with trifluoroacetic acid anhydride and then the carboxyl group converted to acid chloride with $SOCl_2$. According to the carboxylic acid derivative reactivity, once the carboxyl group of the bile acid was modified with the more reactive acyl chloride, the reaction with Na-alendronate is supposed proceed smoothly. Na-alendronate was thus added to the solution with the activated deoxycholic acid to perform the reaction. After 48 h, the reaction was checked with ^{31}P NMR. Surprisingly, it was noted that the reaction showed no progress at all.

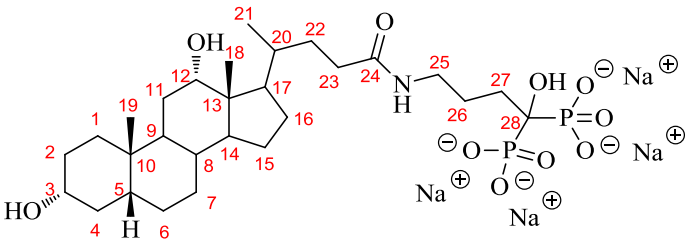
8 ANALYSES

8.1 NMR spectroscopy

NMR spectra were measured with Bruker Avance III 300 MHz (^{1}H NMR 300 MHz, ^{31}P NMR 121.5 MHz) and Bruker Avance 400 MHz (^{13}C NMR 100 MHz). The resonant frequencies were referenced to the signals of residual δ(**H**DO) = 4.80 ppm or δ(C**H**D_2OD) = 4.78 ppm from internal TMS.[69] The analysis were carried out in the laboratory of Organic Chemistry, University of Jyväskylä.

NMR spectroscopy is a well-suited method in order to provide structural information about bile-acid alendronate conjugates. Moreover, NMR is a reliable, sensitive, and non-invasive technique.

The deoxycholic acid-alendronate conjugates (**Scheme 55**) were characterized by ^{1}H, ^{13}C, and ^{31}P analysis.



47

Scheme 55. Deoxycholic acid-alendronate conjugate (**47**).

Regarding the reaction shown in **Scheme 52**, it was possible to confirm the success of the reaction by ^{31}P NMR (**Appendix 1**). The signal of the unreacted Na-alendronate appears at approximately δ = 19.36 ppm. Because only one signal at δ = 19.78 ppm was observed, it was concluded that deoxycholic acid-alendronate without any unreacted Na-alendronate was obtained. The ^{1}H NMR spectrum (**Appendix 2**) supported the formation of the desired product, even though some impurities were observed at approx. 3.5 and 4.0 ppm. These are most probably due to an ethyl group of a reactant. The signal of CH_3-18 at δ = 0.60 ppm exists as two peaks probably due to the presence of some unreacted deoxycholic acid, which despite of efforts could not be removed from the product. The methyl groups CH_3-19 and CH_3-21 were observed at 0.83 and 0.90 ppm, respectively.

The 3β and 12β hydrogens were observed at 3.4 and 3.85 ppm, respectively. Two groups of multiplets belonging to CH_2-23 and CH_2-25 were detected between 2.1 and 2.4 ppm. These multiplets are more deshielded than the ones belonging to CH_2-26 and CH_2-27 due to the proximity of the amide. CH_2-26 and CH_2-27 were identified among those peaks to appear between 1.0 and 1.9 ppm. The solvent was not completely removed either evidenced by the three peaks belonging to DMF at 2.8, 2.9, and 7.9 ppm.

Regarding the synthetic strategy shown in **Scheme 53**, again it was possible to confirm the success of the reaction by ^{31}P NMR (**Appendix 3**). Actually, the phosphorus spectrum showed two peaks at δ=19.36 and 19.60 ppm meaning that there was some unreacted Na-alendronate mixed with deoxycholic acid-alendronate. In fact, the signal at 19.36 ppm corresponds to the unreacted Na-alendronate while the one at 19.60 ppm belongs to the deoxycholic acid-alendronate conjugate. Despite the fact the product was not obtained as pure, by ^{1}H NMR (**Appendix 4**) the presence of deoxycholic acid-alendronate was established. The signal of CH_3-18 at δ = 0.70 ppm appeared as two peaks because of impurities, most probably unreacted deoxycholic acid. The methyl group CH_3-19 was detected as expected as a singlet at δ = 0.91 ppm. On the other hand, the signal observed at δ = 1.0 ppm corresponded CH_3-21 from more than one deoxycholic acid derivative (compare with CH_3-18 above). The 3β and 12β hydrogens were identified at approx. 3.5 and 4.0 ppm, respectively. The multiplets belonging to CH_2-23 and CH_2-25, and CH_2-26 and CH_2-27 were overlapped by impurities. However, it was possible to assign them among the signals between 2.1 and 2.4 ppm and 1.1 and 2.0 ppm, respectively. The solvent was not completely removed this time either as it was possible to observe the three characteristic peaks at 2.8, 2.9, and 7.9 ppm belonging to DMF. The ^{13}C NMR spectrum (**Appendix 5**) supported the presence of the O=C-NH- bond by showing the chemical shift of the carbonyl carbon at approx. 171 ppm. Moreover, O=C-OH carbonyl was detected at 180 ppm, confirming the presence of the unreacted deoxycholic acid.

Regarding the synthesis shown in **Scheme 54**, the ^{31}P NMR (**Appendix 6**) established no progress for this method. The signal was detected at δ= 19.24, which belongs to the unreacted Na-alendronate.

8.2 Mass Spectrometry

The mass spectrometric analyses were carried out with Micromass LCT ESI-TOF instrument in the laboratory of Organic Chemistry, University of Jyväskylä.

In the mass spectrum of the deoxycholic acid-alendronate, measured in the previous study[66], it was not possible to establish whether the final compound was an amide or an ester derivative, since the molecular weights of the two products are exactly the same.

However, mass spectrometry can be used in analyzing the different purification methods used for synthesizing deoxycholic acid-alendronate by the reaction illustrated in **Scheme 53**.

Particularly, after the attempts of purification of succinimido deoxycholate through column chromatography, the crude product was checked with both NMR and MS.

In order to analyze the product with mass spectrometer, 1.05 mg of product was weighed in a glass vial and 1000 μL of $CHCl_3$ was added to obtain 1 mM stock solution. After that 100 μL from the stock solution was pipetted in an Eppendorf tube containing 900 μL of MeOH to obtain 100 μM of sample solution.

The spectrum of the selected fraction (**Appendix 7**) showed one peak at *m/z* 442.7099. Probably, this peak corresponds to a methyl ester deoxycholate $[M+2H_2O]^+$, since the exact mass of succinimido deoxycholate is 489.31 g mol^{-1}. Similarly, the peak at *m/z* 442.7099 was observed in the spectrum of another fraction (**Appendix 8**). Again, it was supposed to be a methyl ester deoxycholate $[M+2H_2O]^+$, while the other peaks can be identified as reagents. The results support the assumption according to which the ester bond conjugating the succinimido part to deoxycholic acid had hydrolyzed and the free deoxycholic acid further reacted with methanol in the eluent in the column during the purification producing methyl deoxycholate (see Chapter 7.2).

9 REAGENTS

The reagents used for the experiments, and their manufacturer as well as purity are listed in **Table 8**.

Table 8. List of reagents with corresponding manufacturer and purity.

Reagent	**Manufacturer**	**Purity (%)**
Amberlite	BDH	---
Chloroform	Fisher Chemical	99.98
Deoxycholic acid	Sigma	≥ 99.0
Diethyl ether	VWR Chemical	100
Dimethylformamide	VWR Chemical	100
Ethanol	Altia Oyj	Min. 99.5
Ethyl chloroformate	Merk	97
Magnesium sulfate	Sigma-Aldrich	99.0-101.0
Na-Alendronate	University of Eastern Finland	---
N-Hydroxysuccinimide	Aldrich	98
N,N'-Dicyclohexylcarbodiimide	Fluka	≥ 99.0
Sodium hydroxide	Sigma-Aldrich	≥ 98
Thionyl chloride	Riedel-De Han	≥ 98
Triethylamine	Merk	99
Trifluoroacetic acid anhydride	Merk	99

10 PROCEDURES

10.1 Deoxycholic acid-alendronate through an anhydride as intermediate

In a round-bottomed three-necked flask with an adjustable thermometer, reflux condenser (with $CaCl_2$-tube at the top), and dropping funnel, a mixture of deoxycholic acid (0.40 g, 1.00 mmol) and dried DMF (30 mL) is cooled on an ice-water bath to +10 ˚C. Freshly distilled triethylamine (0.467 mL) is slowly added to the solution through a dropping funnel with vigorous stirring, followed by a dropwise addition of freshly distilled ethyl chloroformate (0.319 mL) in DMF (3 mL). The ice-water bath is removed and the mixture is stirred at room temperature for 30 minutes. At the same time, in a two necked flask equipped with reflux condenser (with $CaCl_2$-tube at the top), Na-alendronate (0.99 g, 3.23 mmol) is dissolved in deionized water (10 mL), and the mixture is stirred in an ice-bath until the solution becomes clear. It is followed by a dropwise addition of 40% NaOH (0.390 mL). The mixture is stirred at room temperature for 30 minutes. Then, the tetrasodium alendronate in water is added dropwise to the freshly prepared activated bile acid. The mixture is stirred in the oil bath at 80° C for 48 h.

The product is filtered with the pump system and the volatiles are evaporated off. The crude product obtained is dissolved in Et_2O (20 mL) and the precipitate filtered. The precipitate is allowed to dry for a while, after which it is dissolved in absolute EtOH. The crude product is treated with ion exchange resin in a beaker, and the resin filtered off. The volatiles are evaporated off. The purity of the product is checked by ^{1}H, ^{13}C, and ^{31}P NMR.

Yield: 0.10 g (0.14 mmol, 14.36 %).

10.2 Deoxycholic acid-alendronate through a succinimido deoxycholate as intermediate

In a round-bottomed two-necked flask equipped with reflux condenser (with $CaCl_2$-tube at the top), and dropping funnel, a mixture of deoxycholic acid (6.04 g, 15.39 mmol) and *N*-hydroxysuccinimide (2.01 g, 17.50 mmol) were dissolved in dried DMF (40 mL). To this solution was added DCC (3.74 g, 18.12 mmol) and the mixture was stirred at 60° C for 15 h.

After that, the mixture was filtered to remove the solid precipitate, dicyclohexylurea. The filtrate was mixed with ice-water (500 mL) and the white solid obtained was filtered off, washed with ice-water, and dried in vacuum.

The impure succinimido deoxycholate (4.01 g, 8.19 mmol) was dissolved in $CHCl_3$ (80 mL) and the $CHCl_3$ layer was first washed with sat. aq. $NaHCO_3$ (4 x 40 mL), then with H_2O (2 x 20 mL), and finally dried with $MgSO_4$. The purity of the product is checked by ^1H NMR. Pure succinimido deoxycholate (1.71 g, 3.49 mmol).

In a two necked flask equipped with reflux condenser (with $CaCl_2$-tube at the top), Na-alendronate (1.09 g, 3.54 mmol) is dissolved in deionized water (10 mL), and the mixture is stirred in an ice-bath until the solution becomes clear. It is followed by a dropwise addition of 40% NaOH (0.390 mL). The mixture is stirred at room temperature for 30 minutes. Then, the succinimido deoxycholate (0.52 g, 1.06 mmol) in DMF (20 mL) is added dropwise to the solution. The mixture is stirred in the oil bath at 100° C for 24 h.

The product is characterized using ^{31}P, ^1H, and ^{13}C NMR spectroscopy.

Yield: 0.29 g (0.41 mmol, 38.79 %)

10.3 Deoxycholic acid-alendronate through a acyl chloride as intermediate

In a round-bottomed three-necked flask with an adjustable thermometer, reflux condenser (with $CaCl_2$-tube at the top), and dropping funnel, a mixture of deoxycholic acid (1.07 g, 2.73 mmol) and dried DMF (26 mL) is cooled to -10 ˚C in an ice-salt bath. Freshly distilled trifloroacetic acid anhydride (7 mL) is added in 15 min to the solution through a dropping funnel with stirring. The mixture was kept at -10 °C for 1 h and stirred for 2 h at room temperature. Then the mixture was purred into Et_2O/ice (40 mL: 10 g). The organic layer was washed with water (20 mL), with sat. aq. $NaHCO_3$ solution until the water solution gave an alkaline reaction, with brine (20 mL), dried $MgSO_4$ and evaporated to dryness.

Yield: 0.39 g (0.80 mmol, 29.32 %)

The product (the protected bile acid) (0.39 g, 0.80 mmol) and freshly distilled $SOCl_2$ (5 mL) was refluxed for 15 min and the excess of $SOCl_2$ was distilled off. The crude product was dissolved in $CHCl_3$ (10 mL) and evaporated to dryness under vacuum.

Yield: 0.41 g (0.81 mmol, ~100 %)

In a two-necked flask equipped with reflux condenser (with $CaCl_2$-tube at the top), Na-alendronate (1.06 g, 3.45 mmol) is dissolved in deionized water (10 mL), and the mixture is stirred in an ice-bath until the solution becomes clear. It is followed by a dropwise addition of 40% NaOH (0.390 mL). The mixture is stirred at room temperature for 30 minutes. Then, the tetrasodium alendronate in water is added dropwise to the freshly prepared activated bile acid in DMF (20 mL). The mixture is stirred in the oil bath at 100° C for 48 h.

The purity of the product is checked by ^{31}P NMR.

11 SUMMARY

This project was undertaken to design deoxycholic acid-alendronate conjugates and evaluate three synthetic strategies involving different intermediates.

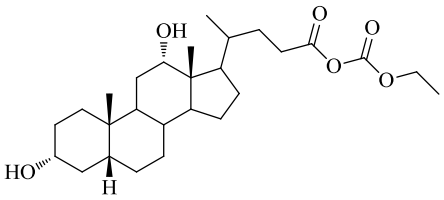
Generally, the synthesis between Na-alendronate and the anhydride of the corresponding deoxycholic acid as the intermediate was successful. Nonetheless, a second synthetic strategy was tried due to the low yield of the final product (14 %).

In the second method, the experiment required a reaction between a more active intermediate, namely succinimido deoxycholate, and Na-alendronate. The synthesis of the compound resulted in a mixture of products. In other words, the reaction had taken place although the product was not obtained as pure. The yield of the impure product was 39 %, which is far more than the yield obtained by the first reaction. This amount is, however, affected by reagents and residual solvents impossible to remove from the final product. After several ineffective attempts to purify the product, the impure deoxycholic acid-alendronate conjugate was sent to the University of Eastern Finland to be purified with high performance countercurrent chromatography (HPCCC). Further studies, such as hydrolysis experiments, need to be carried out in order to establish whether deoxycholic acid-alendronate conjugate can act as a prodrug.

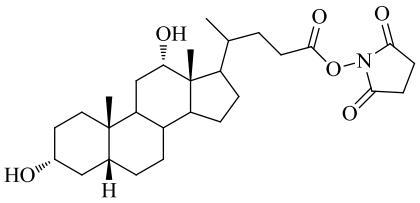
In the third method, the reaction between the acyl chloride of the analogs deoxycholic acid and Na-alendronate was against expectations not achieved.

The current work provides further insights for future research regarding the synthesis of bisphosphonates conjugated with the series of bile acids through a succinimido intermediate.

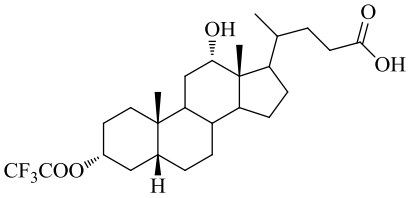
12 SYNTHESIZED COMPOUNDS



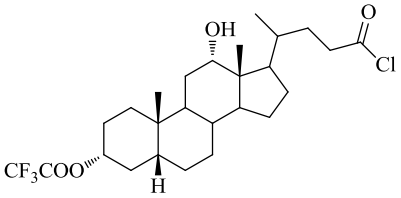
3α-Hydroxy-5β-deoxycholan-24-oyl Ethyloxy Formic Anhydride



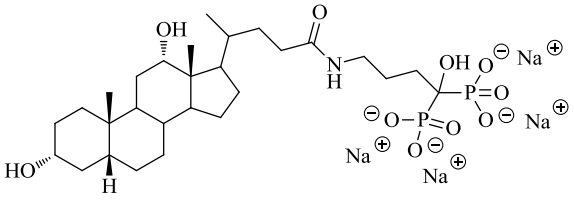
3α-Hydroxy-5β-deoxycholan-24-oyl Succinimido Ester



3α-Trifluoroacetoxy-5β-deoxycholan-24-oic Acid



3α-Trifluoroacetoxy-5β-deoxycholan-24-oyl Chloride



3α-Hydroxy-5β-deoxycholan-24-oyl Tetrasodiumalendronate Amide

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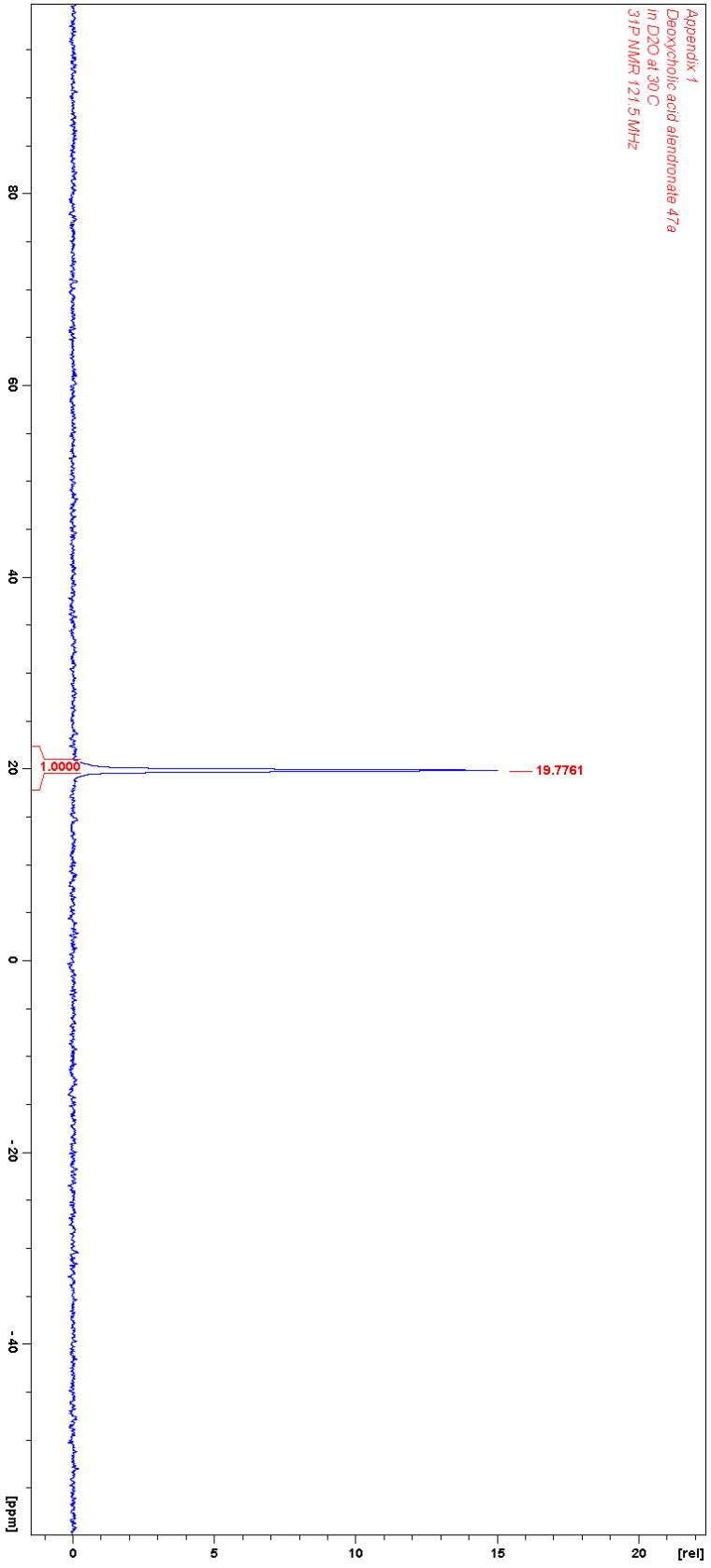
APPENDICES

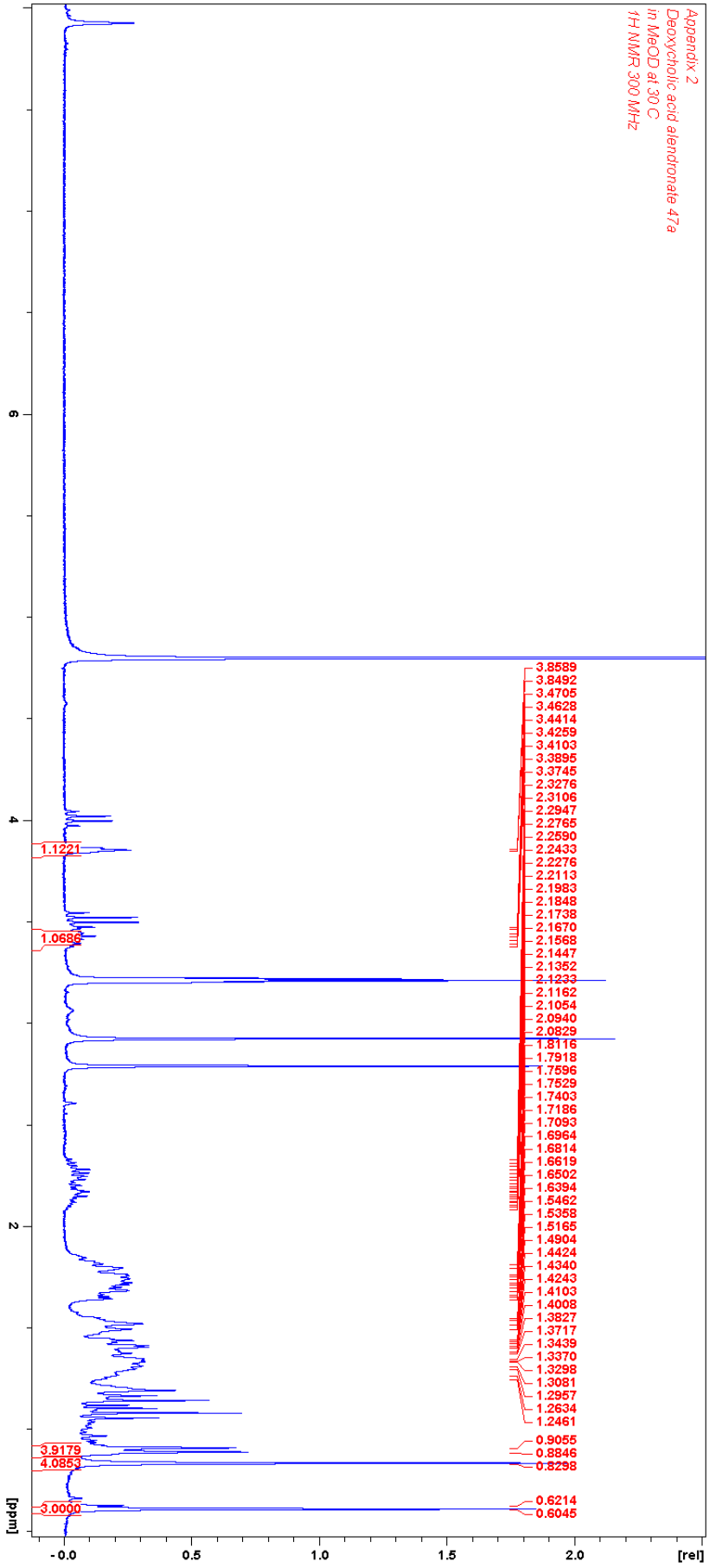
Appendix 1

^{31}P NMR of deoxycholic acid alendronate **47a**.



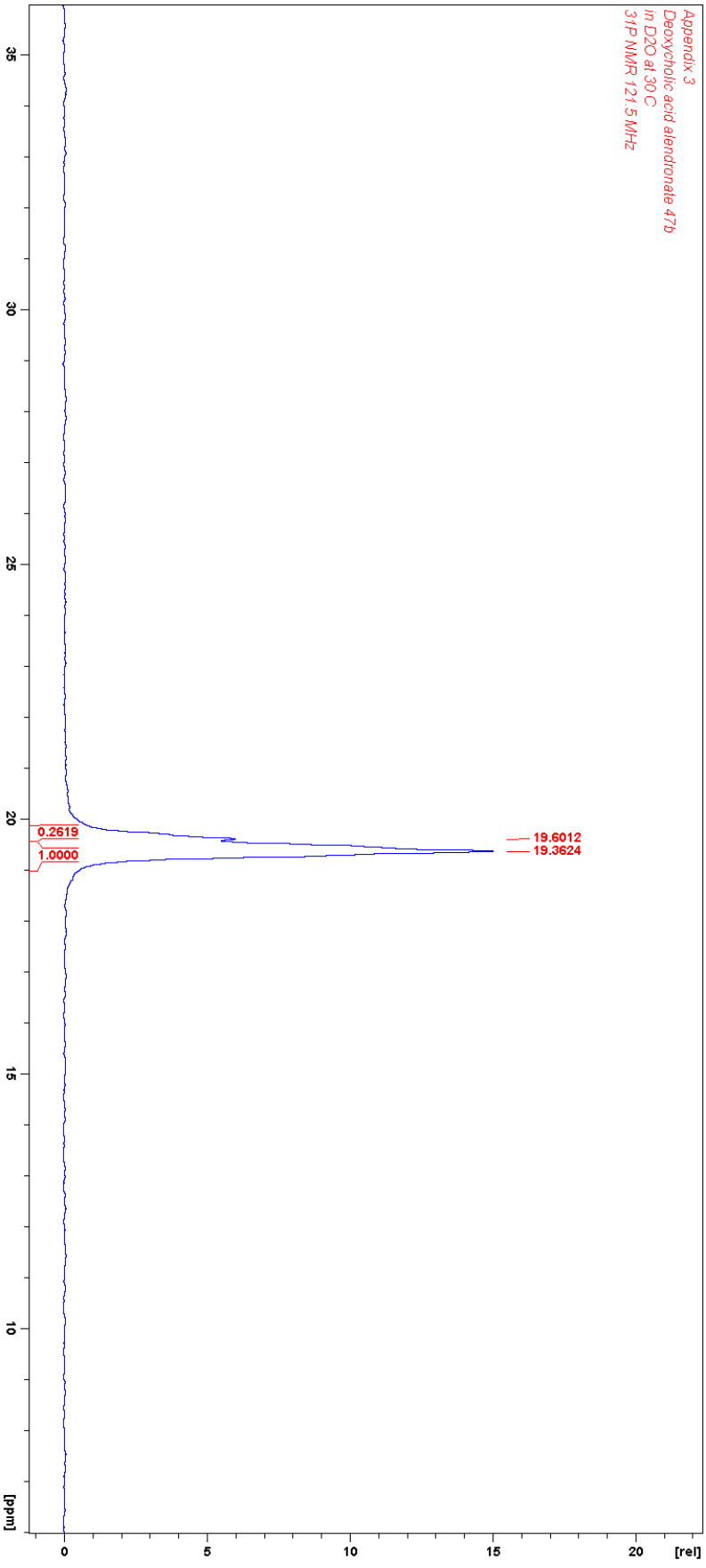
Appendix 2

^{1}H NMR of deoxycholic acid alendronate **47a**.



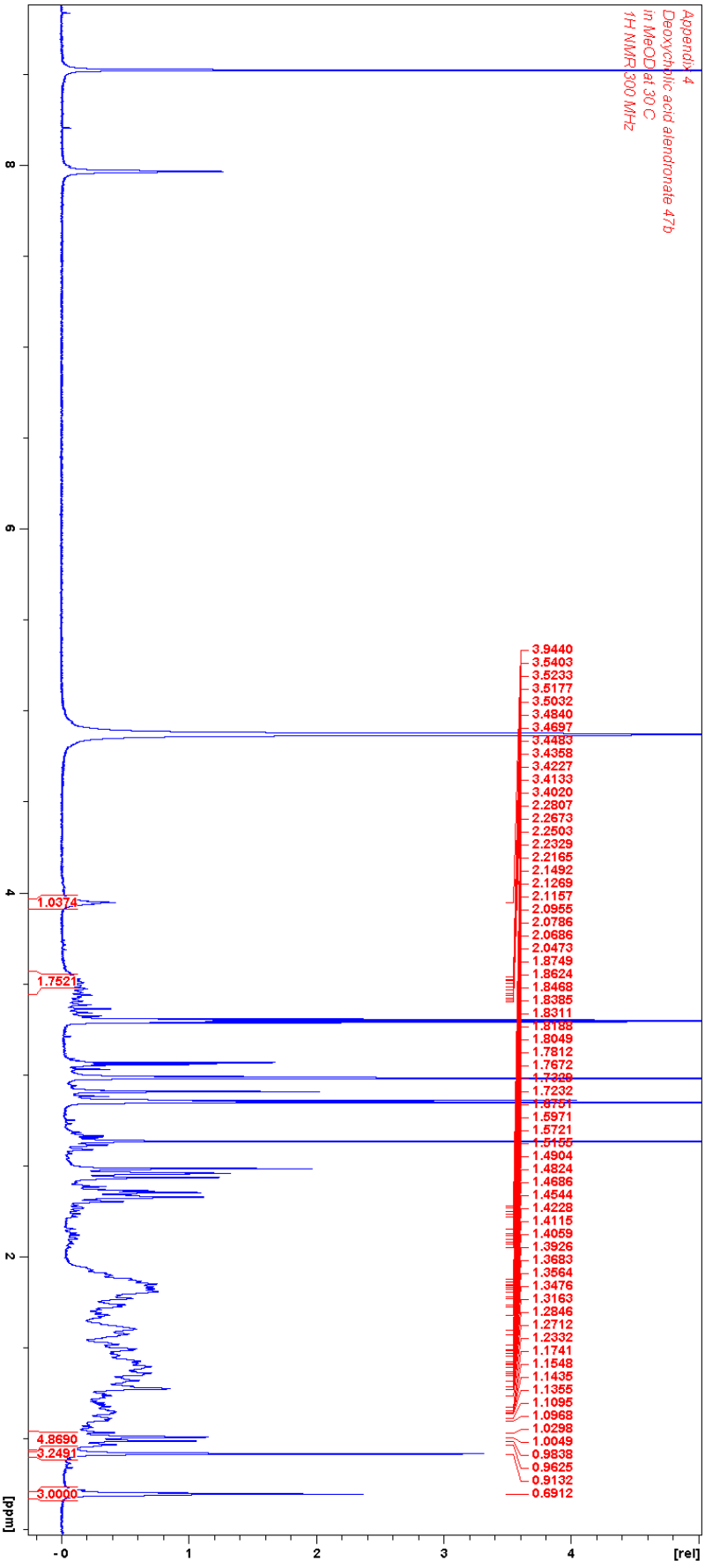
Appendix 3.

^{31}P NMR of deoxycholic acid alendronate **47b**.



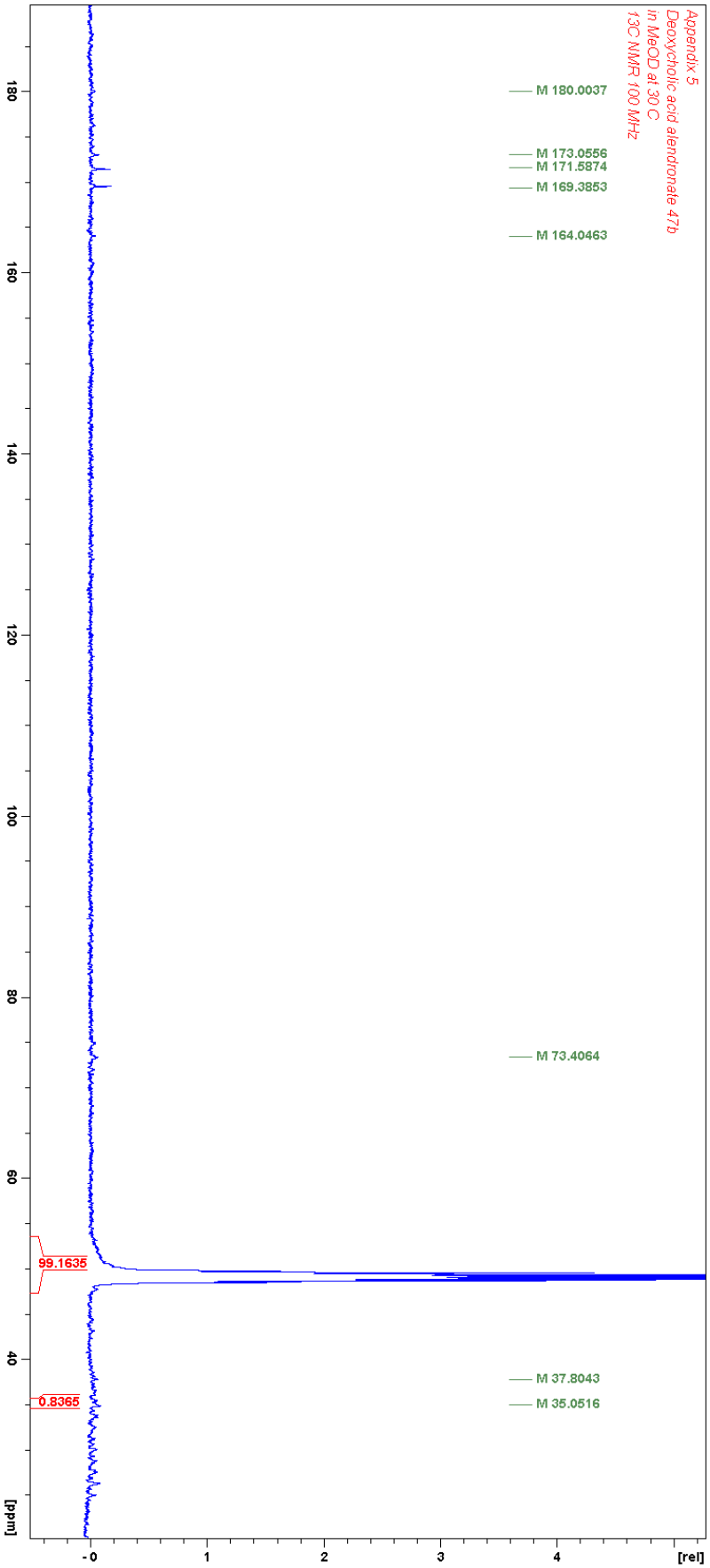
Appendix 4

^{1}H NMR of deoxycholic acid alendronate **47b**.



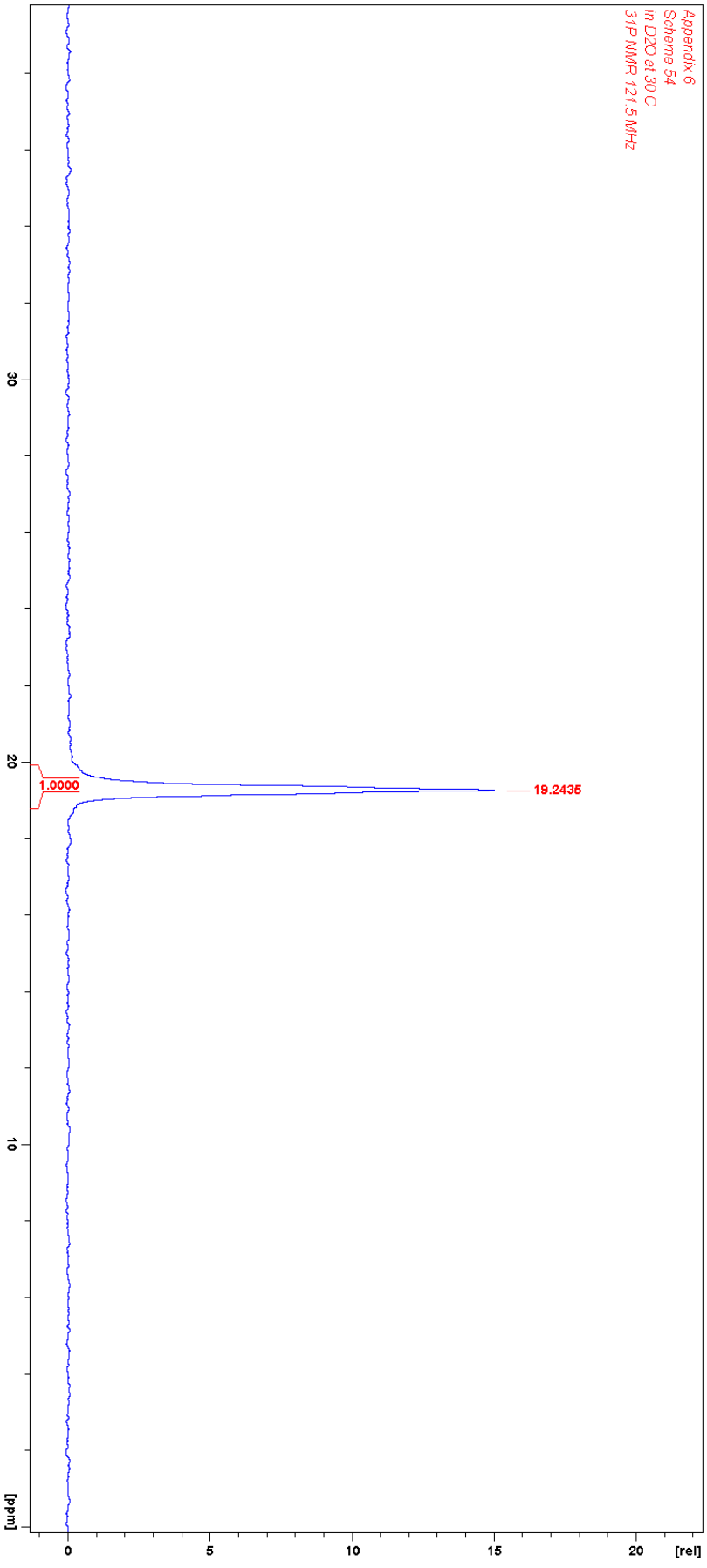
Appendix 5

^{13}C NMR of deoxycholic acid alendronate **47b**.



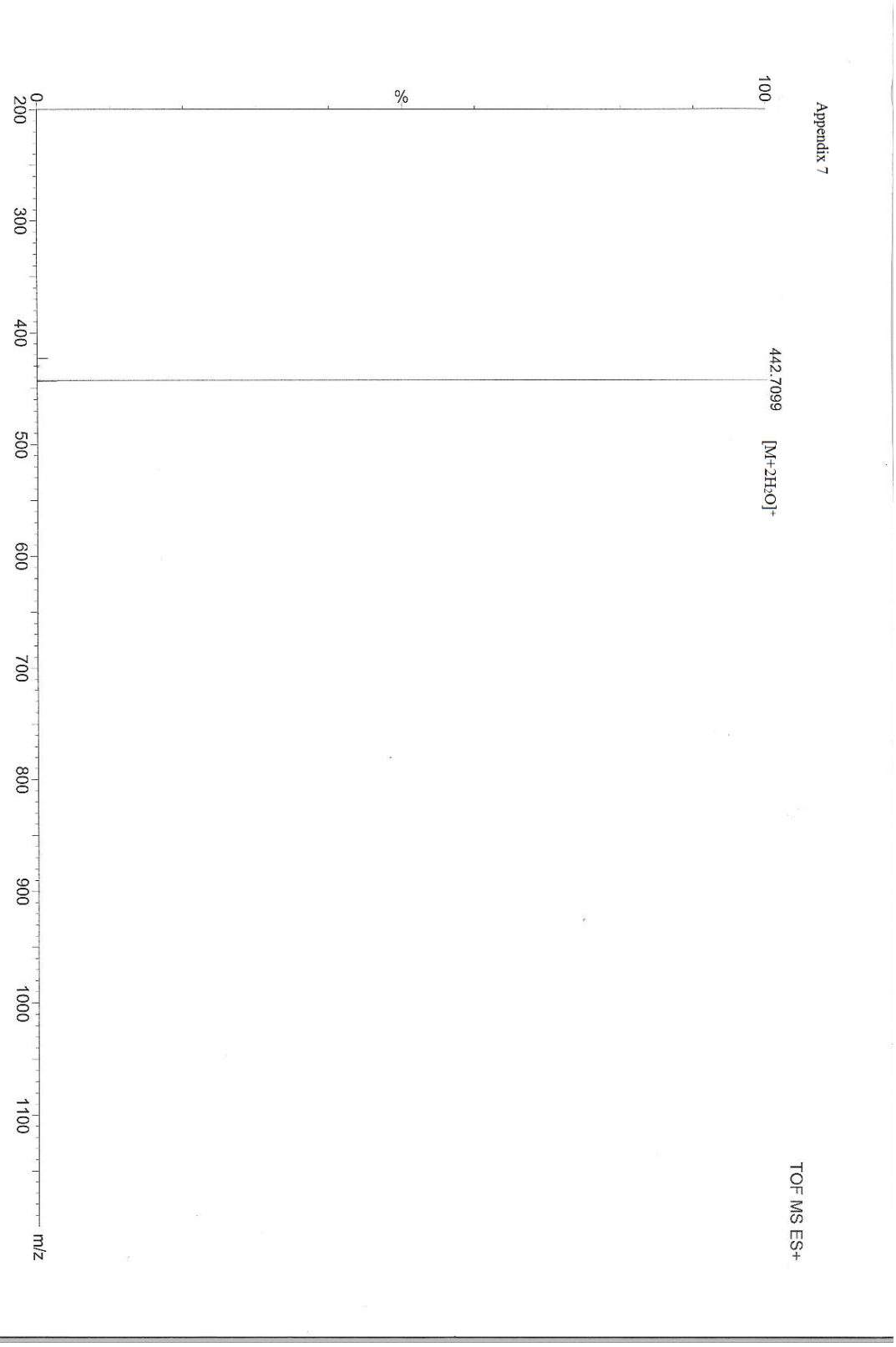
Appendix 6

^{31}P NMR of unreacted alendronate of the reaction shown in **Scheme 54**.



Appendix 7

MS of methyl ester deoxycholate



Appendix 8

MS of methyl ester deoxycholate