UNIVERSITY OF
EASTERN FINLAND
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SYNTHESIS OF PARTIAL BISPHOSPHONATE ESTERS WITH SINGLE SIDE CHAIN

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Master's thesis

Department of Chemistry

Medicinal Chemistry

401/2012

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Approval date: 28.03.2012

Abstract

Bisphosphonates are well known class of organophosphorus compounds having biological and industrial applications and their development is ongoing. Among other prospectives of bisphosphonates the partial bisphosphonate esters present an interest for use in biological systems with their increased lipophilicity. Current research is aiming to make one more step in chemistry of partial bisphosphonate esters with transport-optimised side chain in order to approach the calcium imaging utility. The literature overview is aimed to show the dialectics of studies in this field and to highlight the major approaches to the synthesis of bisphosphonates. The experimental part is dedicated to the problems of synthesis and purification of individual compounds. The synthesis and isolation of new compounds are described and possible reaction mechanisms are discussed.

Abbreviations

Ala – beta-alanine

ATP- adenosine triphosphate

DIPEA – diisopropylethylamine

DMEBP – P, P'-dimethyl ethenylidene-1,1-bisphosphonate

DMF – dimethyl formamide

FPPS – farnesyl pyrophosphate synthase

GGPPS – geranylgeranyl pyrophosphate synthase

LDA – lithium diisopropylamide

Me-ala – beta-alanine methyl ester

NBP – nitrogen containing bisphosphonates

NNBP – non-nitrogen containing bisphosphonates

OPG – osteoprotogerine

OPGL – osteoprotogerine ligand

PFA – paraformaldehyde

RANK – receptor activator of nuclear factor κB

THF – tetrahydrofurane

TMEBP – tetramethyl ethenylidene-1,1-bisphosphonate

TRANCE – tumor necrosis factor related activation induced cytokine

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1 STRUCTURE OF BISPHOSPHONATES AS A KEY BASIS FOR THEIR APPLICATIONS

1.1. INTRODUCTION

Presented thesis is a part of larger research dedicated to bisphosphonates. This class of organophosphorus compounds is known over a century[1], but was extensively developed during last 50 years owing to their use in technological and medicinal aspects. Today this field of science is still drawing interest of researchers and potential of this class of compounds in other fields is being discovered. The structure of bisphosphonates determines their physicohemical and biochemical properties. The variation of these properties leads to variation in their behavior in biological systems because such systems have developed mechanisms of recognition and utilisation of xenobiotics.

In addition to conventional applications some new potentials for use of bisphosphonates have been proposed that require specific optimized properties and accordingly specific structure features. In the same time direct methods for altering separate moieties in bisphosphonates are not always available. Altogether this present challenges for bisphosphonate development for biological and industrial applications. The literature overview is intended to highlight current knowledge on this chemical class.

1.2. STRUCTURAL ASPECT: TERMS AMBIGUITY AND DIVERSITY

As it unambiguously comes from the name “bisphosphonates”, these compounds are a class of organophosphorous compounds bearing two phosphonate groups. According to IUPAC recommendations term “phosphonic acid” refers to structure ($HP(O)(OH)_2$). Alkylphosphonic acids are characterized by presence of alkyl group binded directly to the central pentavalent atom of phosphorus. The name for such compounds is derived from parent compound[2]. For instance the substitution of hydrogen to ethyl in phosphonic acid gives ethylphosphonic acid. When acidic hydrogen is substituted by carbon or by cations, it is determined by word “phosphonate”, for example dimethyl methylphosphonate or disodium methylphosphonate (Fig. 1).

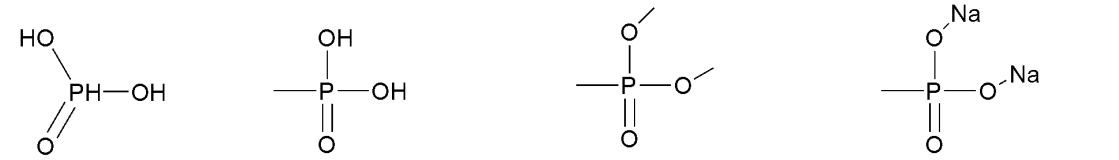


Figure 1. The nomenclature of phosphonic acid and its derivatives.

However in common practice whole class of these compounds is historically called “bisphosphonates” regardless of the substitution of acidic hydrogen. The term “diphosphonates” is erroneous for BPs since it describes compounds bearing two sequentially bonded phosphonic acid residues as it is in inorganic pyrophosphate (Fig. 2)[3]

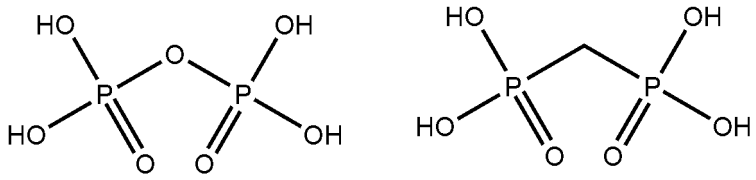


Figure 2. Pyrophosphoric (left) and methylenebisphosphonic (right) acids.

Whereas the name “bisphosphonate” clearly shows the presence of two phosphonate groups in the structure, it does not specify relative positions of these two functions. Historically geminal bisphosphonates were developed most of all, and omission of prefix “gem-” is common in later publications, though non-geminal bisphosphonates are also studied[4,5,6]. Although it is not completely correct to use the term “bisphosphonates” concerning only geminal bisphosphonates and/or bisphosphonic acids, the prefix “*gem*-” and ester content are omitted in this work for the sake of simplicity when a whole drug class is meant.

Even with restrictions mentioned above bisphosphonates present significant chemical space. Firstly two lateral side chains on the central carbon atom may provide opportunity to introduce substituents of all kind involving carbon as well as heteroatoms. In addition to altering of the side chains, the bisphosphonic hydroxy groups are the points for derivation of different chemical nature (Fig. 3).

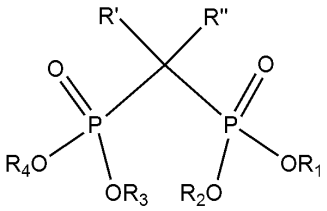


Figure 3. General structure of the geminal bisphosphonates.

Ester groups or cations then can be arranged in symmetrical or asymmetrical fashion. Sources of asymmetry are one to three free acid hydroxyl- and/or non-equivalent substituents (Table 1, general structure on Figure 3). That is why for bisphosphonate with given side chain a net charge, charge distribution and sterical bulk that define physicochemical properties, can vary significantly. For instance bisphosphonic acids are very hydrophilic, while tetraalkyl bisphosphonates can dissolve in non-polar solvents.

Table 1. Examples of reported bisphosphonic acid esters.

Substituents		Reference
R_1=R_4	**R_2=R_3**	Stepinski et al.[7]
H	Octyl, cyclohexyl, Ph	
Me	Octyl, cyclohexyl, Ph	
R_1=R_2=R_3=R_4		
Cyclohexyl, Ph, 3-(Me_3Si)Pr		
R_1=R_2	**R_3=R_4**	Abdou et al.[8]
Et	Me	
H	Me	Barbey et al.[9]
R_1,R_2	**R_3=R_4**	
Dioxalane	Me	
R_1	**R_2=R_3=R_4**	Turhanen et al.[10]
K^+	Me	
Na^+	Ph	
n-hexyl	H	

1.3. KNOWN APPLICATIONS OF BISPHOSPHONATES

Bisphosphonates have four anion centers and they can act as chelator molecules. Chelating properties in case of bisphosphonates are expressed well with divalent metal cations in aqueous media[11]. In a set of competitive gas phase experiments divalent metal ions were substituting the host-structure of bisphosphonate. This model has allowed to find relative gas phase affinity values for these cations towards bisphosphonates used and the calcium ions have shown the highest affinity[12]. Reported instability constants of bisphosphonate metal complexes vary, and that suggests their possible use for selective chelation[13]. This property defines the earliest use of bisphosphonates as agents with a broad range of industrial applications[14] first of all in the metal complexing[15]. In spite of good achievements and industrial scale of use, the research in this field is continued revealing novel or improved target properties of this chemical class[16,17,18,19].

A number of bisphosphonates are nowadays used for therapeutic purposes[20]. Diversity of these drugs (Table 2) is due to lateral chains on the *meso*- atom of carbon while acidic functions are in free form. Within this row a division to nitrogen and non-nitrogen bisphosphonates (NBPs and NNBPs respectively) can be seen. The domination of NBPs can be explained with early recognition of special role of the nitrogen in the side chain in case-by-case search of more potent structures. This structural feature becomes more evident and reasoned as the mechanism of bisphosphonate action in biological systems is taken into account.

Table 2. Bisphosphonates used in clinical therapy of osteoporosis[20]. R_1 and R_2 are substituents at the *meso*-atom of carbon (Figure 3).

Drug	Substituents		Potency[21]
	R_1	R_2	
Etidronate	OH	Me	1
Clodronate	Cl	Cl	10
Tiludronate	H	$-CH_2-S-C_6H_4-Cl$	10
Pamidronate	OH	$-(CH_2)_2NH_2$	10^2
Alendronate	OH	$-(CH_2)_3NH_2$	10^2-10^3
Risedronate	OH	$-CH_2$-3-Py	10^3-10^4
Ibandronate	OH	$-(CH_2)_2N(Me)(n-C_5H_{11})$	10^3-10^4
Zolendronate	OH	$-CH_2$-imidazol-1-yl	$>10^4$
Neridronate	OH	$-(CH_2)_5-NH_2$	--
Minodronate	OH	$-CH_2$-imidazo[1,2-a]pyridin-3-yl	--

Currently all bisphosphonate drugs in clinical use or trial are divided to three generations based on combination of their structure and activity. The first generation includes NNBPs which have relatively low potency and they resemble pyrophosphate most of all bisphosphonates. The second generation are those of NBPs that contain amine nitrogen substituent and have significant increase in potency compared to NNBPs. Tiludronate is included in the second generation. The rest of bisphosphonates belong to the third generation, which is characterized by high potency, heterocycle content and increased lipophilicity at the side chain terminus.

Another utility of bisphosphonates that is widely used nowadays is imaging[22]. The ability of coordinated bisphosphonates to preserve calcium affinity is known[23] and that allows such an imaging complex to localize in the sites of active bone turnover. The SPECT (Single-Photon Emission Computed Tomography) is a widely used technique for gamma-emitting bisphosphonate complexes. Such complexes bisphosphonates form with technetium-99m. This isotope of technetium have an relatively long time of half-life (6 hours), emit gamma-radiation and transform into stable technetium-99 via nuclear transformation[24]. These complexes have shown to be beneficial compared to complexes of different ligands or other labels for skeletal imaging[25,26] though different biodistribution for them is taking place[27]. Altogether it makes remarkably safe, simple and reliable method for observation of dynamics of the bone changes and localization of pathological processes.

1.4. Pharmacokinetics of bisphosphonates

The pharmacokinetics of bisphosphonates is a characteristic complex of properties based on which this class of drugs can be distinguished from many other classes. These properties are summarized in several reviews[28,29,30].

One of characteristic properties of bisphosphonates is their low oral bioavailability. This parameter can be measured by comparison of drug concentration in plasma after its peroral and IV administration during the time the values are above detectable level[31]. Also this parameter for bisphosphonates can be estimated by relative content of labeled drug in bone samples. In this way the bioavailability of alendronate have been estimated for test animals in range of 0.9–1,7%[32]. Another method based on the assumption that bisphosphonate in plasma is either adsorbed to bone or organs or excreted with urine gave bioavailability value of pamidronate less than 1%[27], while etidronate test in animals result in values of 2,5% the least[33]. The extent of bisphosphanates bioavailability in plasma was also found in humans.

The values found according to data obtained via urine monitoring gives 0.3% for pamidronate, 0.7% for alendronate[34], 3–7% for etidronate[35] and via plasma monitoring 1–2% for clodronate[36]. This allows rough comparison of bisphosphonate drugs and elucidation of the drug structure role in biodistribution.

Besides other available mechanisms the paracellular transport way is responsible for bisphosphonates uptake as it is demonstrated experimentally[37,38,39]. In theory the gastrointestinal uptake can be realised in two routes. The transcellular route is described as a transport of drug through biological membranes into epithelial cells from where it is distributed to other cells until it reaches blood medium, but for bulk and negatively charged bisphosphonates it unlikely to take place. The intercellular way is formed by tight junctions of epithelial cells and is available for low-molecular compounds and electrolytes. The selective intake is defined by the brush-border membrane negative charge, electric potential throughout the way and permeating particle size[40,41].

The physicochemical properties of bisphosphonates are crucial for the paracellular transport. These compounds have molecular weight ranging from 176 g/mol for medronate to 322 g/mol for minodronate and since they are acidic they are in anionic form (H_2L^{2-}) at physiological pH[42]. The partition coefficient in octanol/buffer is 0.0017 for pH range 2-11[39]. All in all it results in slow rates of bisphosphonate uptake. The additional effect comes from the divalent ions that are transported via paracellular transport and that hinder passing the tight junction by large anionic molecules. This is supported by dose-dependent studies with additional complexing agents *in vitro*[43,44] and *in vivo*[45]. In accordance with this the cations hinder the tight junctions passages and their removal by chelation improves the drug transport.

Once the bisphosphonate is in plasma it is rapidly absorbed by the skeleton, by kidney and to a small degree by other tissues (Figure 4). This distribution is affected by bone affinity of the drug, rate of bone turnover and renal function[29]. The bisphosphonate which is absorbed by non-calcified tissues is rapidly decaying and removed by urinary excretion. Scaling up of the dose results in larger bisphosphonate deposition and longer release, while distribution of the drug is the same[46,47].

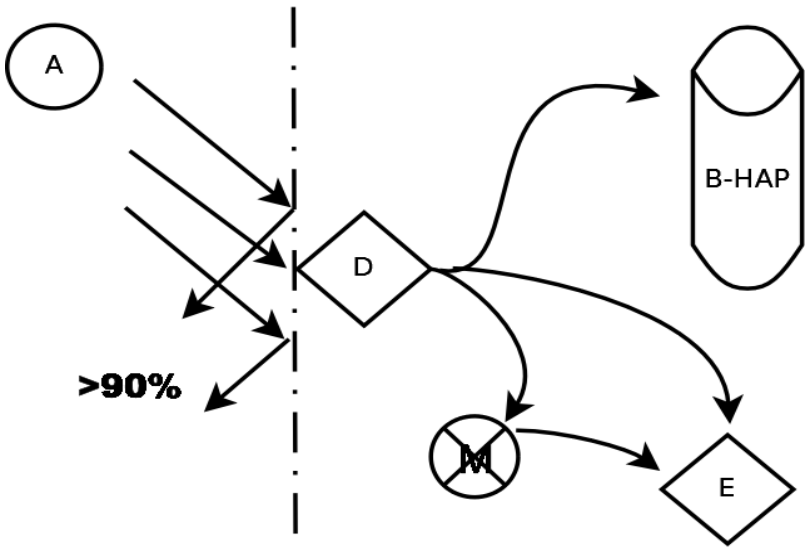


Figure 4. A simplified model of bisphosphonates ADME. A – absorption, D – digestion, M – metabolism, B-HAP – biogenic hydroxyapatite, E – excretion.

1.5. BISPHOSPHONATES AS DRUGS FOR BONE DISEASES

Bisphosphonates have been known for over a century[1], but their relevancy to the calcium homeostasis was shown only about 50 years ago[48]. Today this class of drug is a treatment of choice for patients with bone resorption diseases and Paget's disease. This chapter is intended to show the evolution of views on bisphosphonate action on bone homeostasis. The main target organ for this action is the bone and brief highlight of this topic appears to be essential for understanding of bone-related processes.

The living bone is a porous structure of fluid, living cells and mineral that is closely related to hydroxyapatite. In normal healthy organism the bone undergoes continuous renewal process[49]. The bone remodeling function is executed on the cellular level by iterative removal and the formation of the mineral on available bone surfaces. Osteoclasts and osteoblasts are two different lineages of cells that are responsible for mineral removal and regeneration respectively[50]. Osteoblasts are recruited from mesenchymal (or stromal) stem cells and they mainly form a mineralisation matrix[51] and regulate osteoclast activity[52]. Osteoclasts are of leucocyte-related origin and they mobilize the bone mineral and the matrix. The mineral resorption occurs via acidification of individual osteoclast site[53]. The bone cells form aggregates – so-called bone remodeling units – in which osteoclasts are on the moving frontier and the osteoblasts are following osteoclasts in formed depressions. Remodeling process is normally in an equilibrium state unless it is shifted by some system or local factors[54]. The lack of knowledge about physiology of bone on the cellular level is explained by difficulties of observing living bone *in vivo*.

The history of bisphosphonates studies in biological field began in late 1960s from the studies of Herbert Fleisch pioneer team. In 1966 they demonstrated that urine and plasma components have inhibitory effect *in vitro* on calcium phosphate precipitation. An inorganic pyrophosphate was recognized as one of the natural factors responsible for calcium homeostasis. *In vivo* this compound has shown such an effect on ectopic sites of calcification but no effect was observed on the other sites of calcium phosphate turnover[55]. This fact was explained by lability of pyrophosphate to phosphatase degradation and led to experiments with already known structural analogs – bisphosphonates. Bisphosphonates with their P–C–P backbone have proved to be stable in aqueous media by then in industry[15]. Following the expectations, *in vivo* these compounds have been stable against phosphatase as well[48,56]. According with the major application of bisphosphonates in more common osteoporosis treatment literature overview will highlight bisphosphonates in cases of bone resorption. Nevertheless the pathologic mineralization phenomena such as Paget's disease, ossification or urinary stones cases give additional opportunity to demonstrate an effect of bisphosphonates on the mineral balance of the body.

The mode of bisphosphonate action on calcium deposition/resorption was reconsidered as studies on this class of drugs were becoming more sophisticated. The data from different stages of study clearly reflect different, sometimes controversial views on the subject. As of 2011 after long and extensive research it appears to be clear what are the ways the bisphosphonates affect bone resorption and integer image can be made[57]. As a consequence of this general assumptions may be done to systematically overview major interactions of bisphosphonates at resorption sites. In simplified model a complex role of bisphosphonates can be represented as independent interactions on the cellular or molecular level (Figure 4).

1.5.1. HYDROXYAPATITE AS A MODEL FOR BISPHOSPHONATE-BONE INTERACTIONS

The molecular level of bisphosphonates action (A, Figure 4) was the first to be proposed. In straightforward approximation the mineral resorption inhibition can be explained by chelating properties of bisphosphonate molecules. There are studies that confirm that hydroxyapatite surface stability is strongly dependent on the surrounding medium[58]. However a contribution of bisphosphonates to the mineral resorption via solution effects (i.e. without binding to the mineral surface) is not reported if extreme dosages are not taken into an account[59] This is consistent with major effect attributed to bisphosphonates in industrial applications as described in previous chapter.

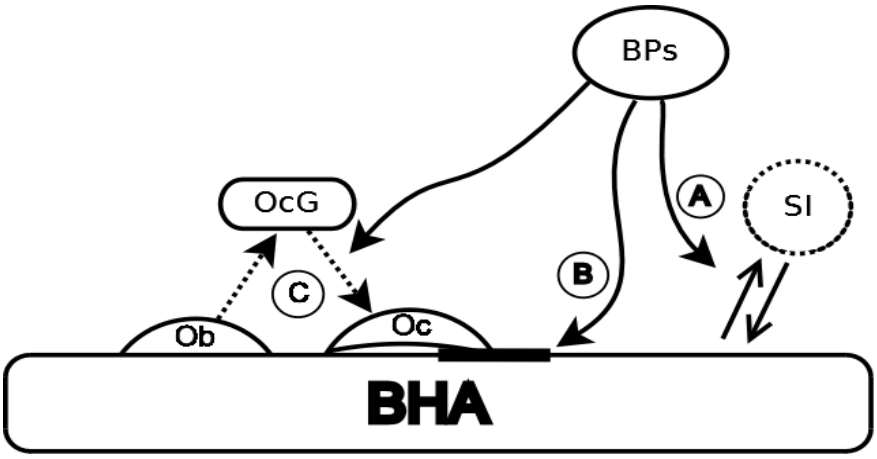


Figure 4. Bisphosphonates and a bone resorption. Abbreviations: BPs - bisphosphonates, BHA - biogenic hydroxyapatite, SI - solvated ions, Oc - steoclasts, Ob - osteoblasts, OcG - osteoclastogenesis.

On the other hand bisphosphonates immediately bind to a mineral surface. Possible effects are more evident. Here two hypotheses of binding can be proposed. Firstly it can be a formation of layer of bisphosphonate that isolate outer media from the mineral. Secondly an incorporation of bisphosphonate into the mineral will provide an increased stability against hydrolysis of the formed non-homogenic structure. The surface binding and structural incorporation are not necessarily exclusive processes. A layer of chelating molecules can be theoretically included in the mineral structure provided such layer will have a low density allowing mineral components to precipitate. These hypotheses, although not confirmed directly, are consistent with experimental results. For instance it is shown that bisphosphonates form a continuous layer on the surface of hydroxyapatite as a crystal-growth poison[60] As for incorporation in solid structure it was observed for alendronate *in vivo*[61]. It is possible to state at the current state of study that regardless on the exact mode of binding of bisphosphonates they are accumulated in the bone.

The biodistribution of bisphosphonates is such that bone tissues are exposed to these compounds in high degree[28]. Theoretical studies show a basis of binding of these compounds to hydroxy apatite depending on the binding surface[62] and the chelating structure[63]. Bisphosphonates are believed to be bidentate or tridentate chelating molecules depending on the α-hydroxyl functionality[64,65,66].

According to mentioned effects bisphosphonates, regardless to the mechanism the bone resorption is inhibited, primarily bind to the surface of the bone. The hydroxyapatite bone affinity therefore determines the accumulation of the given structure in target tissue and can be used to pre-evaluate the drug candidate[67,68,69].

Consequently related parameter of the bisphosphonate release makes possible prediction of pharmacokinetics of these drugs.

First discoveries have revealed that bisphosphonates can inhibit ectopic mineralisation and suppress the bone resorption *in vivo* and it was consistent with early views on factors of these compounds action. However attempt to find a relationship of bisphosphonates resorption inhibition *in vivo* and *in vitro* on the one hand and purely physicochemical prevention of hydroxyapatite resorption on another did not give clear result. It was suggested that bisphosphonates have a cellular mechanism of action[70].

To conclude hydroxyapatite gives an insight to the properties of the bone and the bisphosphonates. Hydroxyapatite gives possibility to pre-evaluate new drug candidates that are targeting bone. But as any model hydroxyapatite has limitations. The differences of the bone and inorganic hydroxyapatite structure are so far not brought up and the observation of cellular effects requires more complex models.

1.5.2. Do bisphosphonate cytotoxic and anti-resorption effects correlate?

Cellular nature of bone remodeling suggests interference of bisphosphonates with specialized cells (B, C Figure 4.). Early basis for this idea was a study demonstrating the ability of bisphosphonates to affect rodent calvaria cells cytotoxically *in vitro* and *in vivo*[70]. Good correlation was found also between cytotoxic and anti-resorbing action of bisphosphonates as an indication of the role of cytotoxic effect in target property[71]. The latter of mentioned experiments have been set with amoebae *Dictyostelium discoideum* thus cytotoxic effect of bisphosphonates is not exceptional for bone cells though *in vivo* in mammals their selective action might be determined by their biodistribution.

The concept of cytotoxic effect of bisphosphonates can explain inhibition of bone resorption whether the target was only osteoclastic or both types of bone cells (B, C, Figure 4.). In theory the induction of bone cells apoptosis as such should affect bone cells equally. However a real selectivity for cytotoxic effect is influenced by drug localisation. Osteoclasts have been shown to release bisphosphonate from bone during acidification[61]. This suggests that bisphosphonates are affecting osteoclast cells in the resorption lacuna and an exceptionally high exposure to the drug takes place. In full consistence with this the observation by Coxon *et al*[72]. show that osteoclasts are affected by major part of bisphosphonate during bone dissolution while non-resorbing cells receive minor part of the compound.

The direct introduction of bisphosphonates into the bone macrophages from solution most likely takes place like in case of Caco-2 cells[73,74]. In the same time it is known that the concentration of these drugs in biological media outside of resorption site is low. Thus action via biological media on the cellular targets especially those of the osteoblasts should be highly specific to have an effect.

Biochemical targets of bisphosphonates in conditions when they induce apoptosis are known to a representative degree. Here the division to NBPs and NNBPs becomes justified when diverse effects of these subclasses of bisphosphonates are observed. Possibly the first evidence in this question was obtained in 1988 when Klein *et al*. found that medronate (methylene-1,1-bisphosphonic acid) is metabolised into analogues of adenosine triphosphate (ATP)[75]. Studies of Rogers *et al.* have shown that other NNBPs (etidronate, clodronate) are also metabolized into analogues of ATP[76,77]. These biogenic analogues mimicking regular ATP are known to be enzymatically stable[78]. This fact together with essential role of ATP in cell metabolism can explain the cytotoxic effect of these NNBPs[79]. NNBP tiludronate is reported to have specific effect on osteoclast ATP-ase in acidification volume though the fact of incorporation of tiludronate into ATP analogues remains unclear[26].

Molecular targets of NBPs are also known. The involvement of NBPs in a protein isoprenylation part of mevalonate pathway was demonstrated in several studies[81,82]. This pathway has been shown earlier to utilize pyrophosphate substrates[83]. The farnesyl pyrophosphate synthase (FPPS) has been identified as the main target of bisphosphonic ligands. This enzyme is a key part of the mevalonate pathway that is essential for protein isoprenylation. The GTPases which are involved in many cellular functions are isoprenylated along this pathway. Therefore the blocking of GTPases isoprenylation leads in extreme case to apoptosis of osteoclast. The downstream enzyme geranylgeranyl pyrophosphate synthase is also reported to be inhibited by bisphosphonates though to a lesser degree[84]. The recognition of FPPS and more generally mevalonate pathway as anti-resorption target led to a simple models. Available stamms of *Dictyostelium discoideum* and more specific human FPPS proved to be efficient. The order of potency of NBP against FPPS corresponds to that of anti-resorbtion (Table 3).

Table 3. The comparative list of bisphosphonate drugs activity.

Bisphosphonate drug	IC_{50} (nM), human recombinant FPPS[85]	Potency relative to etidronate[21]
Pamidronate	200	10^2
Alendronate	50	10^2-10^3
Ibandrobate	20	10^3-10^4
Risendronate	10	10^3-10^4
Zolendronate	3	$>10^4$

The concept of apoptosis of the osteoclasts as a mode of bisphosphonates action is reasoned and well described. In practice the inhibition of bone resorption can happen without osteoclast apoptosis. Such results were derived from the experiment involving NBPs as well as NNBPs in dose-response studies. It has been shown that whereas NNBPs affect osteoclast functions mainly through apoptosis induction, NBPs are able to exhibit the target property without inducing apoptosis[86]. Moreover in certain observations bisphosphonates have been shown to prevent artificially induced apoptosis[87]. Answering the heading question osteoclast apoptosis leads to reduction of bone resorption as it happens in case of NNBPs and NBPs in higher doses, but this is not a necessary condition for a target effect on the bone resorption.

1.5.3. Osteoblast related action of bisphosphonates

There are evidences that osteoblasts regulate the resorption activity of osteoclasts by a signalling system-osteoprotogerin[88] which is identified also as a cell survival factor[89]. This signalling factor was identified in different studies as an osteoprotogerin (OPG), RANK (receptor activator of nuclear factor-κB)[90], TRANCE (tumor necrosis factor (TNF) related activation induced cytokine)[91]. Along with this factor there is a corresponding ligand factor (OPGL) that is involved in different regulation patterns. Besides all this factor mediates osteoclasts' differentiation and activity[92]. The ligand and OPG factor bind to each other and the balance of OPG and OPGL define how much the osteoclast promotion signal is expressed (C, Figure 4). In such a way the regulation of osteoclasts activity is supposed to happen normally[93]. NBPs have been found to affect this mechanism by inducing the expression of OPG[88,94]. The increase in OPG expression consequently might lead to reduction of bone resorption and an increase in cell survival.

The pharmacophore of bisphosphonates can be virtually divided to P–C–P moiety (where P stands for phosphonate group) which is strongly limited in modification and side chains where the variations are more possible. The bone affinity and FPPS (and to a less extent GGPPS) inhibition both should be expressed in a potent structure for increase of bone resorption inhibition. The P–C–P part is essential for binding to the bone mineral since its geometry is optimal for binding to calcium and this property is described above. Addition of the hydroxyl group in gem position to phosphonic groups increases bone affinity as well[95]. As for the side chain of more potent NBPs it also has a positive effect in bone binding due to nucleophilic nitrogen[63]. The interaction of bisphosphonates with FPPS is defined by successful resembling of dimethylallyl pyrophosphate substrate (Figure 5.). From the crystal structures of protein-ligand complex it can be seen that side chain besides nitrogen region also has a lipophilic region that is responsible for dimethylallyl chain mimicking[96]. This also can be concluded from equal activities of zolendronate and more lipophilic minodronate[85].

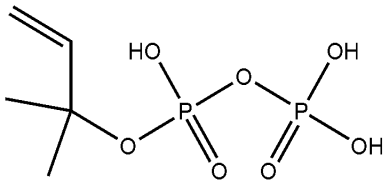


Figure 5. Dimethylallyl pyrophosphate - a natural FPPS ligand

To conclude the chapter bisphosphonates have a diverse mode of action depending on their structure, dose and cells involved. From the recent reviews it appears that there is a consensus on what mode of action for NBPs and NNBPs contribute more or less to resorption inhibition[97,57]. Bisphosphonates are binding to the bone surface and are released during osteolysis. NNBPs are metabolised to relatively inert ATP-analogues and thus exhibit cytotoxic action reducing the number of resorption sites. NBPs inhibit farnesyl pyrophosphate synthase reducing the activity of osteoclasts in extreme case inducing their apoptosis.

1.6. BISPHOSPHONATES: NEW CONCEPTS

The pharmacokinetical/pharmacodynamical profile of bisphosphonates is rather specific. Generally bisphosphonic acids are poorly absorbed in gastro-intestinal tract, then rapidly distibuted in plasma so that with minor exceptions one part binds to the skeleton and another is excreted by kidneys. The bisphosphonate that is binded to the bone is then released during normal or pathological bone resorp-

tion. Depending on the structure of the released compound it is either metabolised to ATP analogue or it interact with enzymes of protein isoprenylation, mainly with FPPS. The more detailed description of the mentioned subjects is available in two previous sections.

Along the way described above there are limitations on the way of bisphosphonic compounds. Firstly, the molecular weight and explicit negative charge limit the uptake and paracellular transport of these compounds, reducing their amount in plasma. Secondly, the bone affinity determines the extent of bisphosphonate bone sorption. Bisphosphonates are typically not reaching high concentrations in soft tissues excluding kidney[29,46,47]. In the same time bisphosphonates posses several properties that can be of use in parts of the body that are distant from the skeleton or renal function. For instance, bisphosphonates have a known potency against both bone related and unrelated tumor processes[98,99,100]. The activity against protozoan parasites *Trypanosoma cruzi* and *Toxoplasma gondii* that act in internal organs is also evaluated[101,102,103]. These directions of bisphosphonate activity on the first place and bone-related utilities (e.g. Paget's disease and osteoporosis treatment, skeletal imaging) suffer severe drawback from poor availability of bisphosphonates in plasma and more specifically in bone-distant tissues.

Since every significantly active xenobiotic compound expresses its potency against its target in micro- and nanomolar range the bioavailability is a crucial factor of improving overall potency of the drug. A prodrug is a derivative of known biologically active compound with improved absorption and digestion that is metabolised into an active drug in proximity to its biological target. There is a constant interest to the improvement absorption, digestion, metabolism and excretion (ADME) properties of drugs so that a prodrug methodology is developed as well. A prodrug concept has been applied to various drugs with aspirin being the classical example and since then the modification techniques became more sophisticated[104].

In case of bisphosphonate drugs a successful prodrug strategy for the side chain of NBPs resulting in better oral absorption is known[105,106]. In the same time for potent compounds, when the side chain is a determinant of a target properties varying this region might mean a high price in inhibition as such. Another point against varying of the bisphosphonate lateral chains is possibility the conjugation of this bone targeting scaffold with other biologically active compounds in order to reach selective effect of latter. The concept of bone-targeting bisphosphonate conjugation is continuously studied[107,108,105] and general image of the subject is made in reviews[109,110].

On the other hand the phosphonate groups play a major role in physicochemical properties of compounds in question and their modification appears to be most efficient in respect to the balance of target specific and transport optimised properties. An acid charge masking by esterification is a well known method within phosphate type prodrug approaches[111]. This simple method allows biologically active phosphorous compounds to reach higher drug efficacy though with lesser effect that with more complicated kinds of masking[112]. In case of bisphosphonates the charge masking is expected to improve membrane permeation as well[113]. There is an optimal number of the ester groups since tetraester bisphosphonate will have a poor water solubility. Technetium-99m imaging in organs require relatively lipophilic complexes as shown with various ligands[114], thus for imaging purposes an optimum between complexing and drug-like properties should be found. Technetium-99m might also contribute to decrease of anionic character of bisphosphonate[115] and the results of recent study suggest that symmetrical bisphosphonate diesters have reduced bone affinity compared to free bisphosphonic acids[116]. However, the persistence of such compounds in plasma is yet to be evaluated.

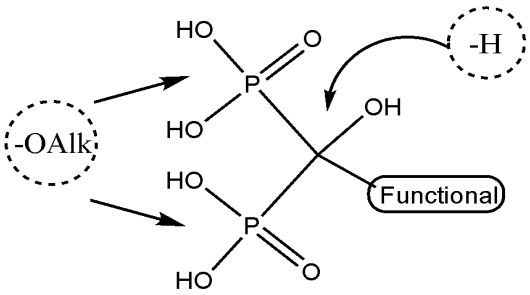


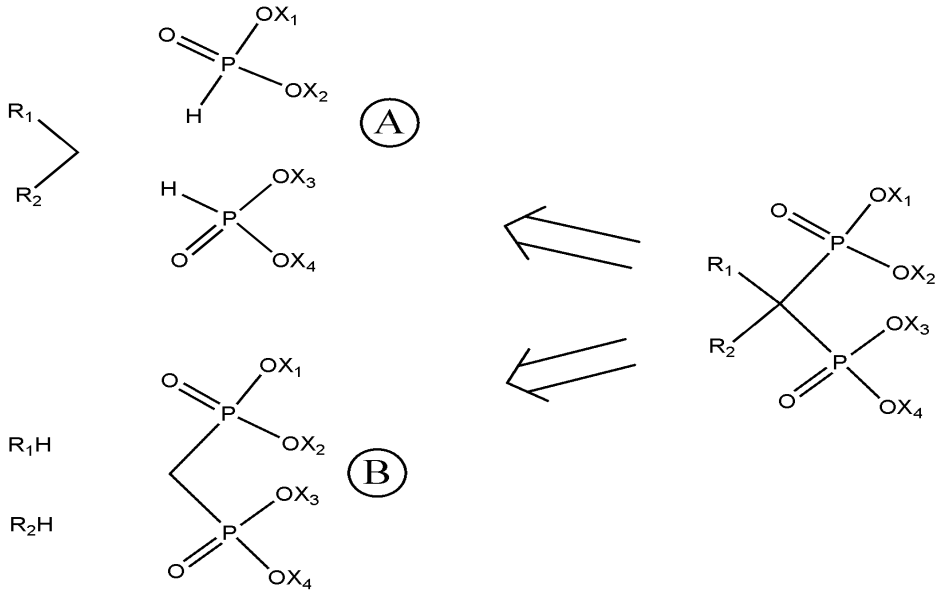
Figure 6. Bisphosphonate structure optimisation

To conclude a symmetrical masking of two hydroxyl groups in a bisphosphonate appears to be a reasoned compromise between all the range of properties of the bisphosphonate class. This method, once developed, might bring a benefit in bioavailability with remaining variety of potential uses and could be a universal tool for development of this class of compounds.

1.7. Synthetic approaches to bisphosphonates

The first synthesis of bisphosphonates dates back to 1865[117]. A simple deduction of the P–C–P backbone suggests two main approaches to bisphosphonates as a class. The first one is an assembly of P–C–P backbone from separate reactive spe-

cies allowing the carbon part to bear some useful moiety (A, Scheme 1). Another one divides the P–C–P backbone and the side chain formation (B, Scheme 1)

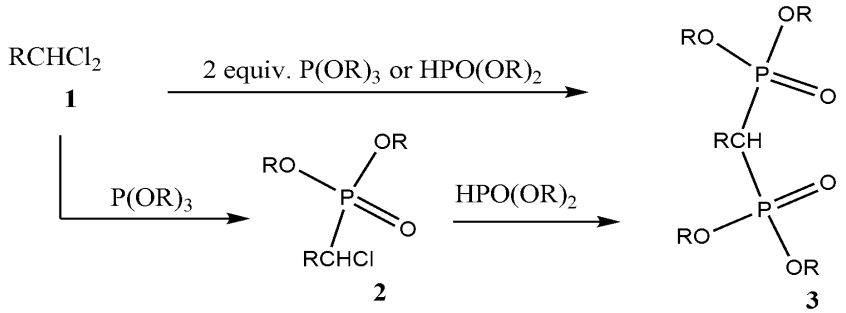


Scheme 1. Two types of approach to bisphosphonates

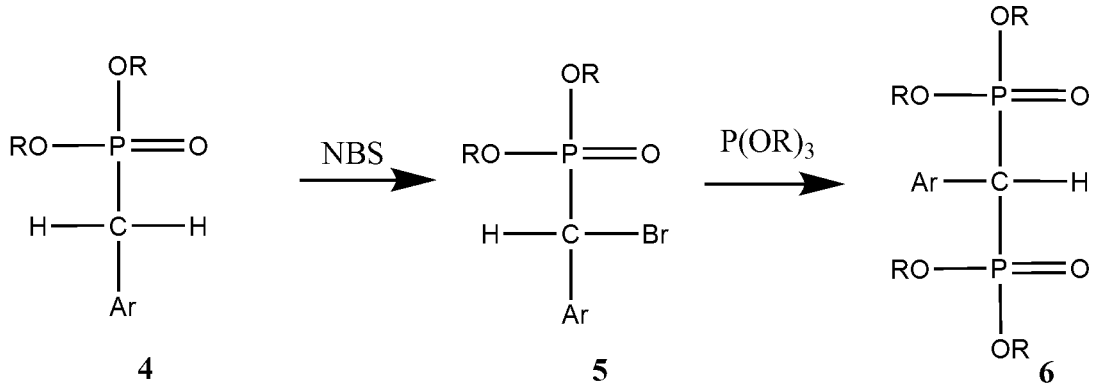
1.7.1. SYNTHESES INVOLVING P–C–P BACKBONE ASSEMBLY

The classical approach via Michaelis-Arbusov reaction is based on the reaction of halogenated substrate (**1**, Scheme 2) with trialkylphosphite and subsequent reaction of formed phosphonate (**2**) with reactive dialkyl phosphonate. For instance Binderup[118] has described such a method of synthesis from a dihalogenated starting material. Interestingly, a target bisphosphonate (**3**) can be obtained by treatment with each of individual phosphonating agents alone, though dialkyl phosphonates lead to a lesser yield of **3**.

A reactive starting material for introduction of the second phosphonate group can be prepared by α-halogenation[119]. After this the phosphonation proceeds smoothly (Scheme 3).



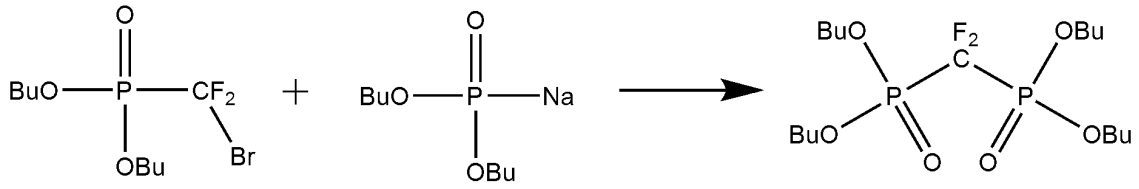
Scheme 2



4 **5** **6**

Scheme 3

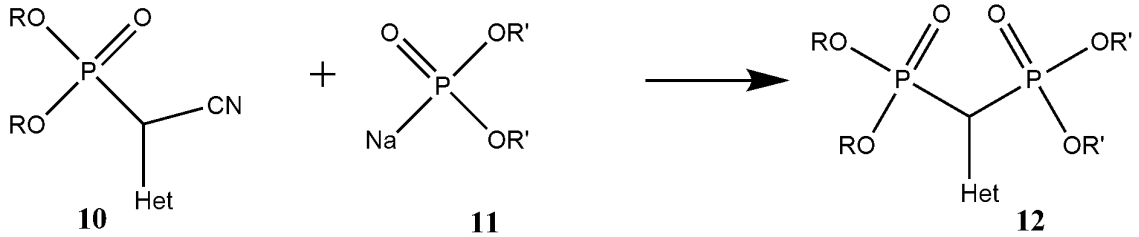
The sharing of α-position by fluorine and easy leaving halogen (**7**, Scheme 4.) results in cleavage of latter during the reaction allowing a synthesis of more lipophilic P–C–P part (**9**)[120]. In the same time using perhalogenmethane CF_2Hal_2 results in lesser yield and significant side reactions[120].



7 **8** **9**

Scheme 4

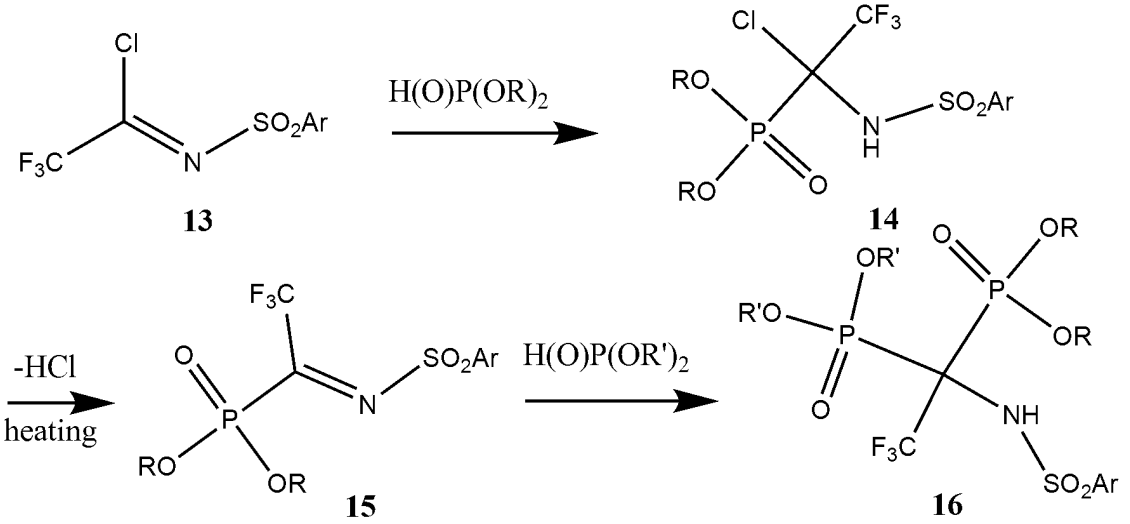
As shown by Abdou *et al*.[8] a pseudohalogen group exhibits analogous reactivity and can be utilized for second stage phosphonation (Scheme 5). That particular study have shown an approach to asymmetrical bisphosphonates.



10 **11** **12**

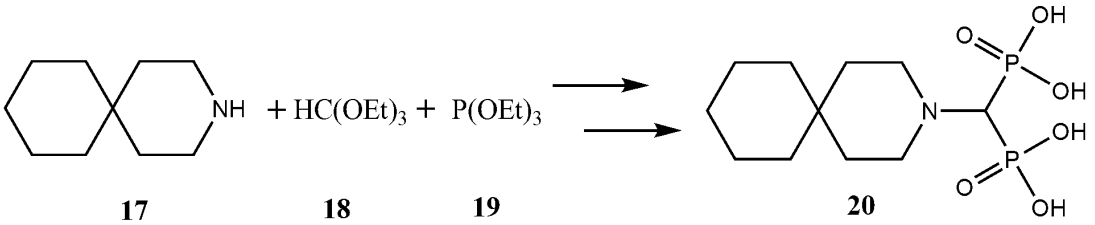
Scheme 5

When a target product requires a presence of nitrogen and trifluoromethyl on the *meso*-atom of bisphosphonate (**16**, Scheme 6) the trifluoro-acetimidoyl chloride (13) can be used[121,122,123]. The phosphonation itself occurs without halogen cleavage.



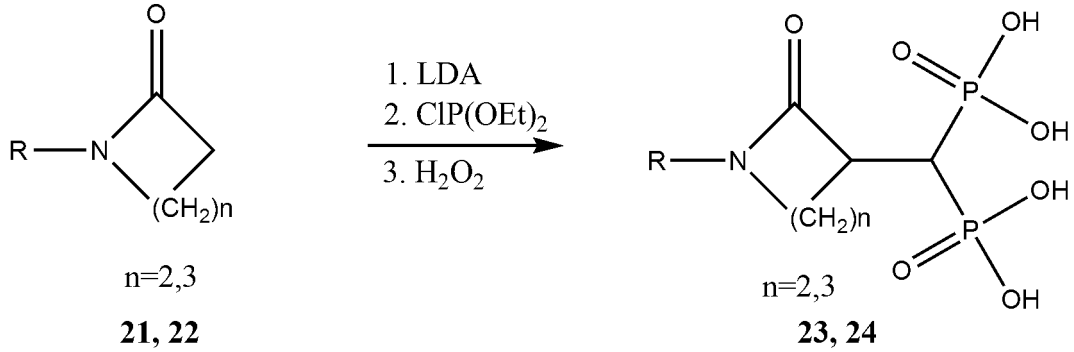
Scheme 6

An α-nitrogen functionality is also provided by reaction of amine with triethyl-orthoformate and phosphonating agent in one pot synthesis[122] (Scheme 7). A further highlight of aminobisphosphonates synthesis is provided by the review by Romanenko *et al*[124].



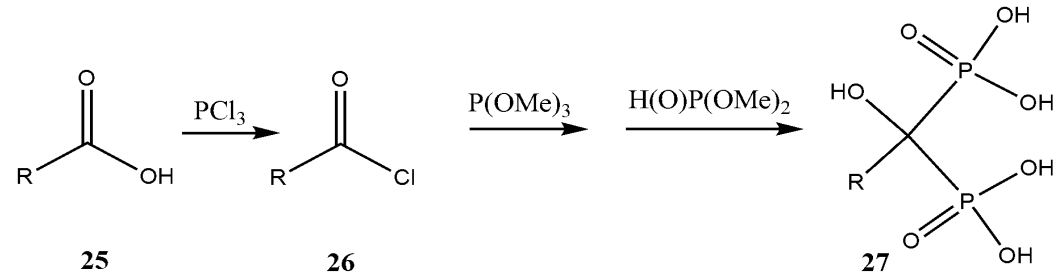
Scheme 7

A diethyl phosphorochloridite have proved to be an efficient phosphonating agent in reactions with linear and alicyclic carbonyl substrates[125] (Scheme 8).



Scheme 8

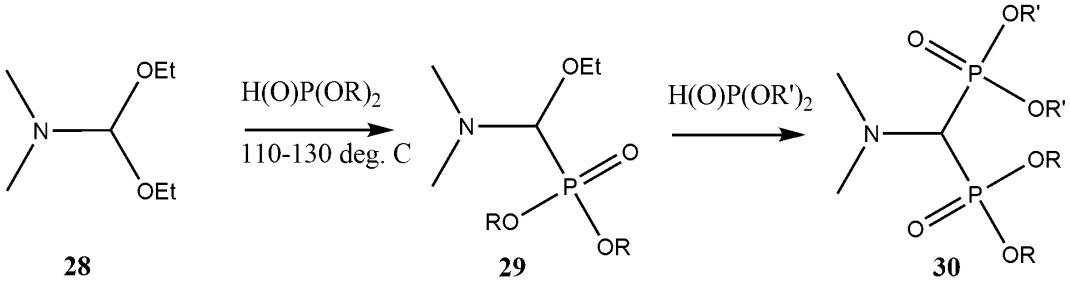
Other carbonyl substrates can be used for bisphosphonate design as well. For instance carboxylic acids were treated with phosphorus trichloride and then introduced in reaction with trialkyl phosphinite and dialkyl phosphonate yielding 1-hydroxy-methylene-1,1-bisphosphonate (**27**) with pre-defined side chain[11] (Scheme 9).



25 **26** **27**

Scheme 9

An interesting approach is provided by use of dimethyl formamide acetal material (**28**). The reaction with phosphonating agent is going stepwise allowing a synthesis of asymmetrical bisphosphonates (**30**)[126] (Scheme 10).

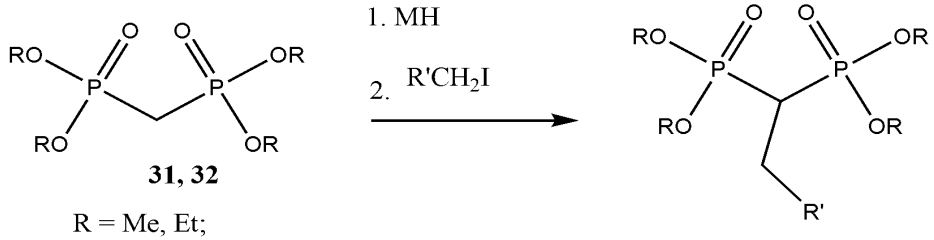


28 **29** **30**

Scheme 10

1.7.2. SYNTHESES UTILISING READY P–C–P BACKBONE

The synthesis starting from methylene-1,1-bisphosphonate starting material (**31**, **32**) is widely used. The hydrogen atom in *meso*-position of bisphosphonate structure shows acidic properties and under metal hydride treatment leads to formation of reactive anion. This property is utilised in reactions with halogenated substrates[127,128] (Scheme 11) and with activated π-systems[129] (Scheme 12).

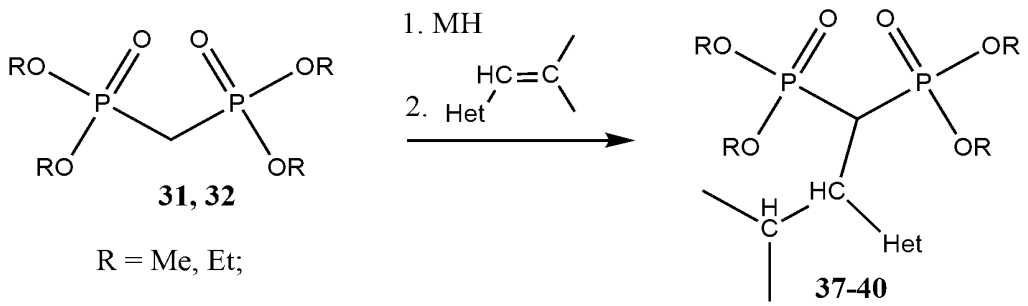


31, 32

R = Me, Et;
R' = farnesylmethyl, 2-(1-iso-indolinone)-ethyl

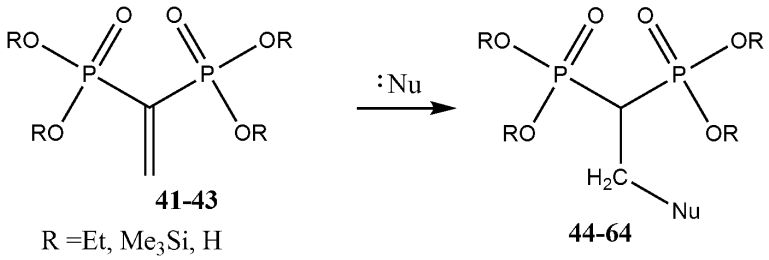
33-36

Scheme 11



Scheme 12

Method based on Michael addition of ethenylene-1,1-bisphosphonates (**41-43**) allows synthesis of ethylidene-1,1-bisphosphonates (**44-64**). The reports regarding the Michael addition of methylene-1,1-bisphosphonates differ in bisphosphonate protection and attacking nucleophile (Scheme 13). Methyl-, ethyl-, or a trimethylsilyl- protective groups are removed after the target structure is obtained. Nucleophile may be oxygen, sulfur, and more commonly nitrogen. The diversity of bisphosphonate Michael additions is expressed in Tables 4 and 5. Based on reported results it can be proposed that important factors are nucleophilicity of the attacking molecule, protection of phosphonate groups and protection-dependent reaction media.



Scheme 13

Table 4.

N	Nucleophile	Protection group, R	Yield, %	Reference
44	H_2O	Et	87	Bailly *et al.*[130]
45	HS-Ph		90	Hutchinson *et al.*[131]
46	HS-Et			
47	HS-Pr			
48	H_2N-$(CH_2)_4$-NH_2		93	Szajnman *et al.*[101]
49	H_2N-Bu		92	
50	H_2N-iPr		79	
51	H_2N-tBu		71	
52	Cyclohexylamine		66	

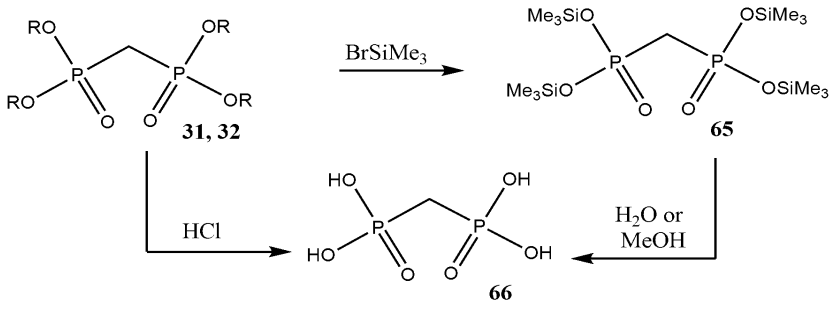
Table 5.

N	Nucleophile	Protection group, R	Yield (acid form), %	Reference
53	Cyclopropylamine	Me_3Si	93	Schevchuk *et al.*[116]
54	H_2N-tBu		99	
55	Cyclohexylamine		99	
56	3-amino-pyridine		75	
57	2-aminopyridine		75	
58	H_2N-Me	OH	94	Alfer'ev *et al.*[117]
59	H_2N-Et		71	
60	H_2N-C($CH_2OH)_3$		49	
61	$HNEt_2$		85	
62	Piperidine		95	
63	Glycine		100	
64	β-alanine		95	

1.7.3. METHODS OF ALTERING THE BISPHOSPHONATE PROTECTION

Various forms of bisphosphonic acid esters are reported (Table 1). Possibly the main reason for well described bisphosphonate acid esters chemistry is that the starting materials for the synthesis of bisphosphonate structure as such are phosphites and phosphonate acid esters. Once the P–C–P backbone and laterial chains are formed the protection groups are removed by one of methods.

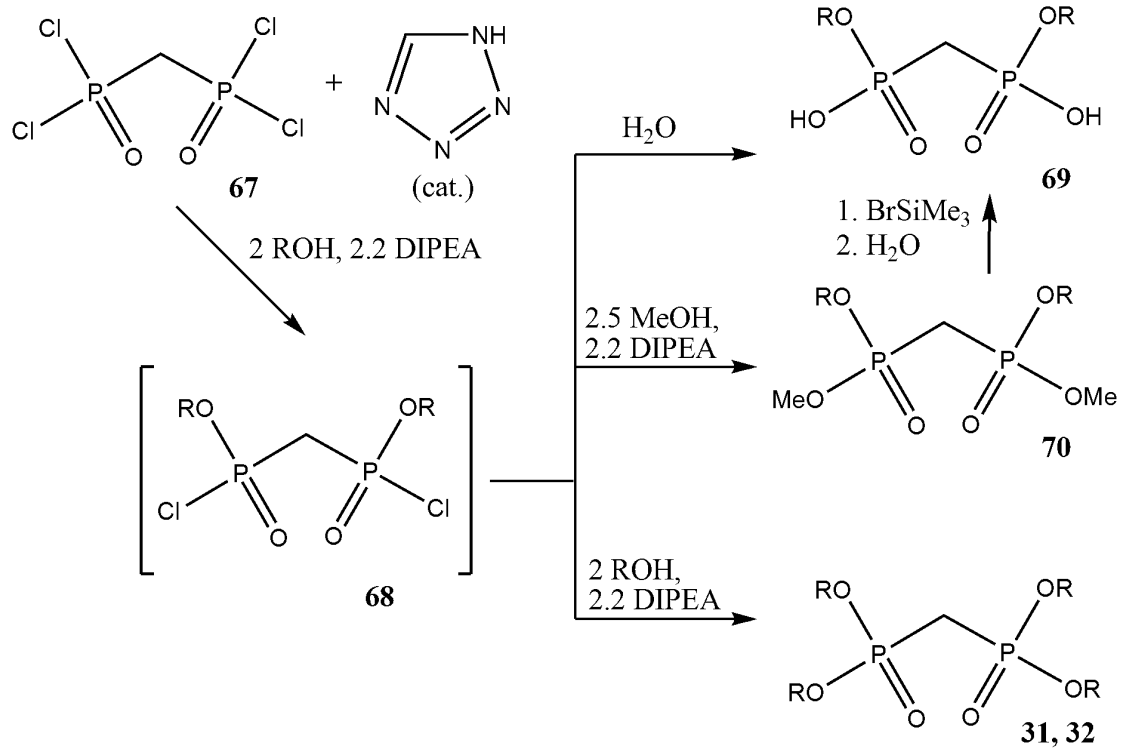
Generally the simplest from synthesis point of view are full homoleptic bisphosphonate esters and bisphosphonic acids since former do not require additional transformations after the P–C–P backbone is ready and latter are obtained with simple procedures. The typical method in literature is an acid hydrolysis,[132,8,101] and more mild method utilising bromotrimethylsilane exists as well[131,133](Scheme 14).



Scheme 14

The bisphosphonate tetraester have been shown to form from bisphosphonic acid upon treatment with orthoformate[134] and from bishosphonic acid chloroanhydride upon treatment with alcohol[135]. However an isolation of partial esters as a semi-products of total esterification or hydrolysis appears to be sub-optimal way[135].

An interesting approach to partial (**69**), homoleptic (**31**, **32**) and mixed (**70**) symmetrical bisphosphonate esters was demonstrated by Stepinski *et al.*[7]. With tetrazole catalysis, a stochiometrically controlled[136] reaction takes place and two or four ester groups can be introduced to bisphosphonate scaffold (Scheme 15).



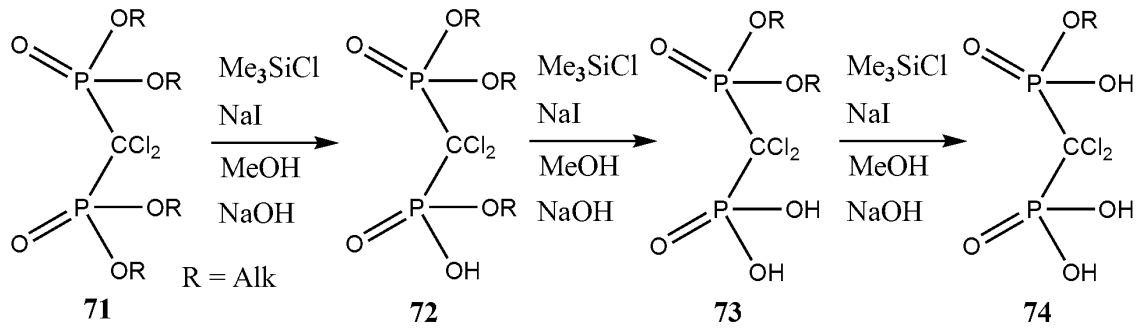
Scheme 15

Partial bisphosphonate esters can be formed from fully protected bisphosphonates by treatment with selective reagents. This way provides a choice of protection positions to be cleaved than hydrolysis method does. For instance in series of studies[135,137,10] it has been shown that depending on the target esterification and a starting bisphosphonate ester a specific method can be applied.

For stepwise removal of ester groups in asymmetrical fashion a stochiometrically controlled treatment by trimethylsilylcloride and sodium iodide in basic methanol can be chosen[135] (Scheme 16).

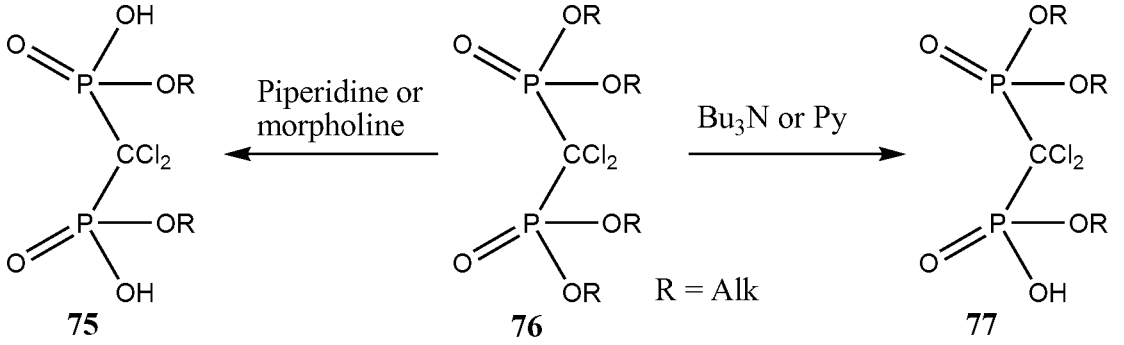
The application of amines results in selective cleavage of ester groups[137]: one group upon treatment with tertiary or aromatic amine (**77**, Scheme 17) or two

groups upon treatment with secondary amine (**75**). If more harsh conditions are provided a treatment by pyridine gives a P,P'-dialkylbisphosphonate (**78**, Scheme 18) while treatment with morpholine gives bisphosphonate monoesters (**80**).



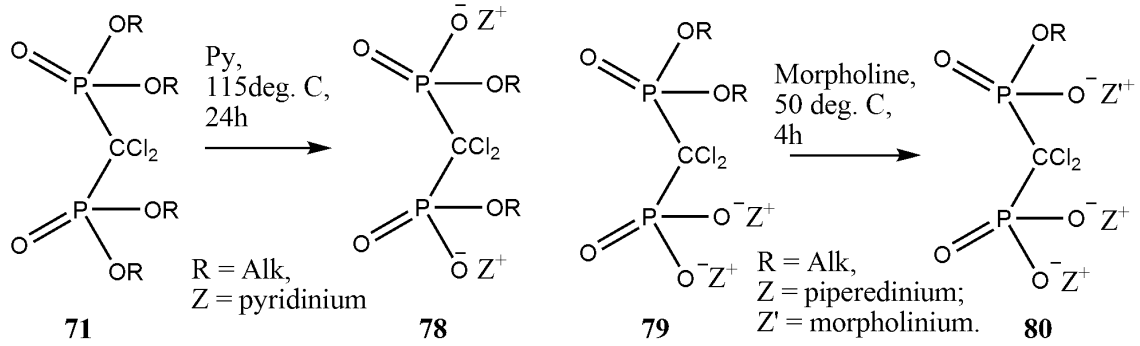
71 **72** **73** **74**

Scheme 16



75 **76** **77**

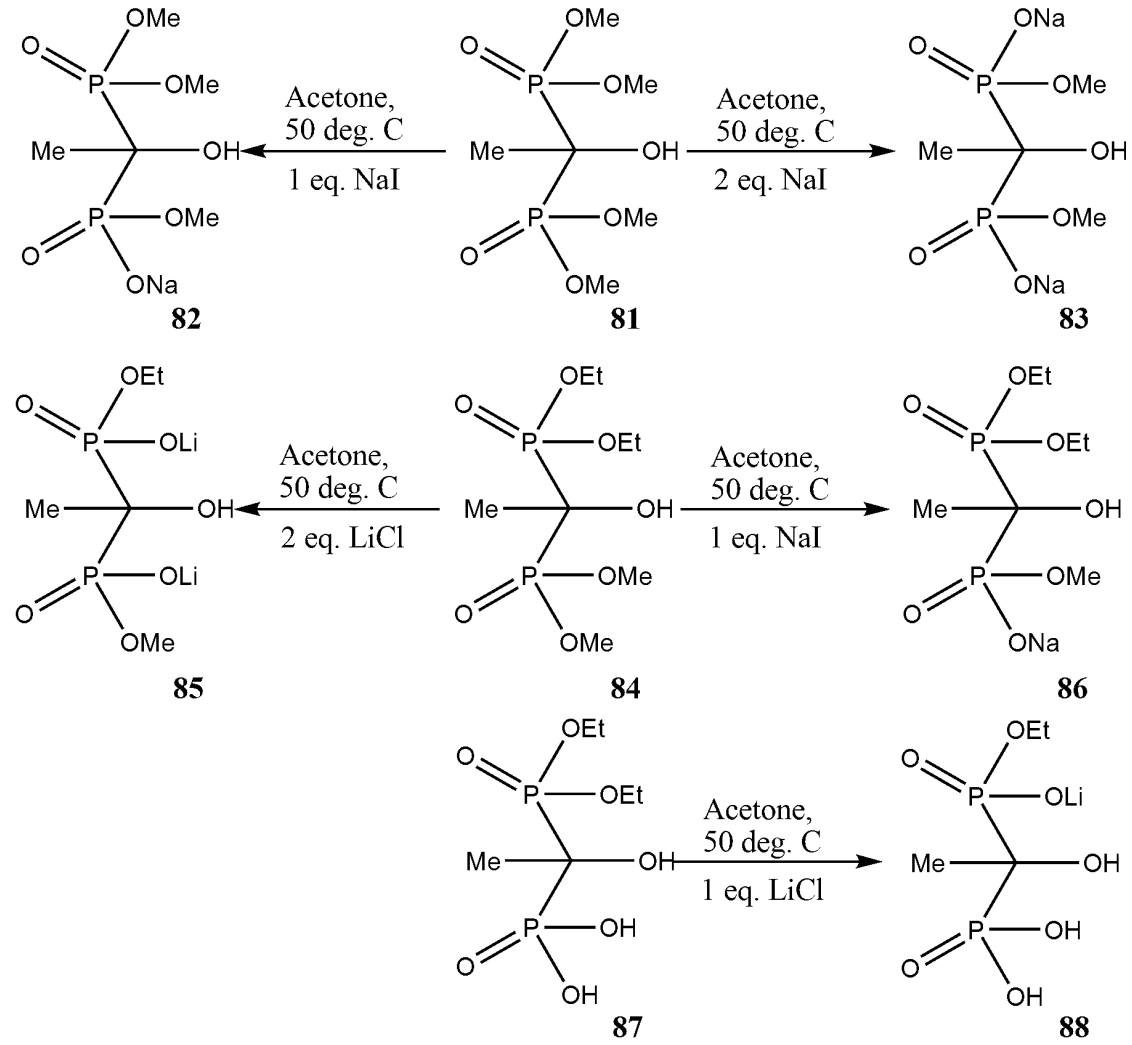
Scheme 17



71 **78** **79** **80**

Scheme 18

Besides trimethylsilylhalogens and amines the metal halogen salts act as a selective deprotection agents. In mild conditions sodium iodide has been found to be selective methyl cleavage reagent and use of lithium chloride results in more general deprotection[10](Scheme 19).



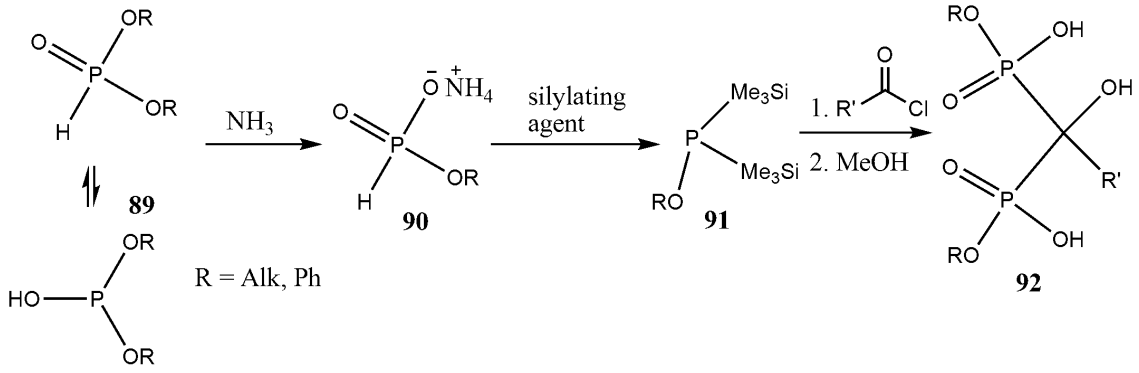
82 **81** **83**

85 **84** **86**

87 **88**

Scheme 19

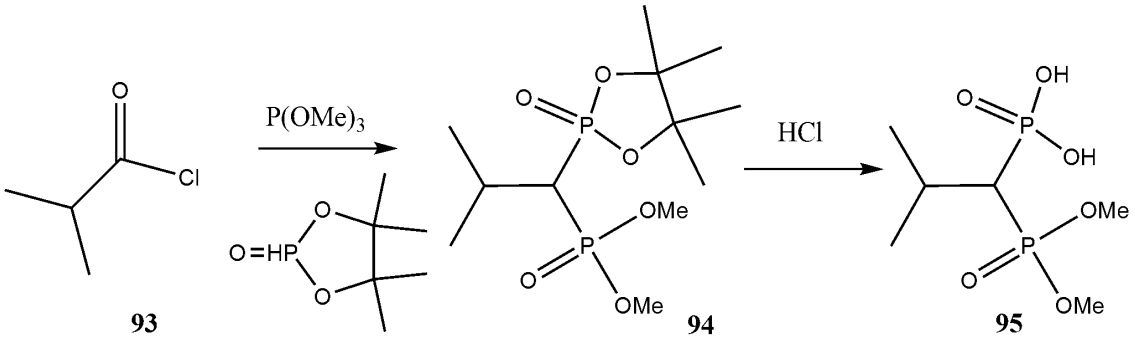
The methods shown above reflect an approach utilizing a ready P–C–P backbone. However a synthesis of bisphosphonates with pre-defined protection is also possible. A set of protection groups in P,P'-dialkylbisphosphonates (**92**, Scheme 20) have been achieved with use of bis(trimethylsilyl)alkylphosphite (**91**) in four step method[138].



89 **90** **91** **92**

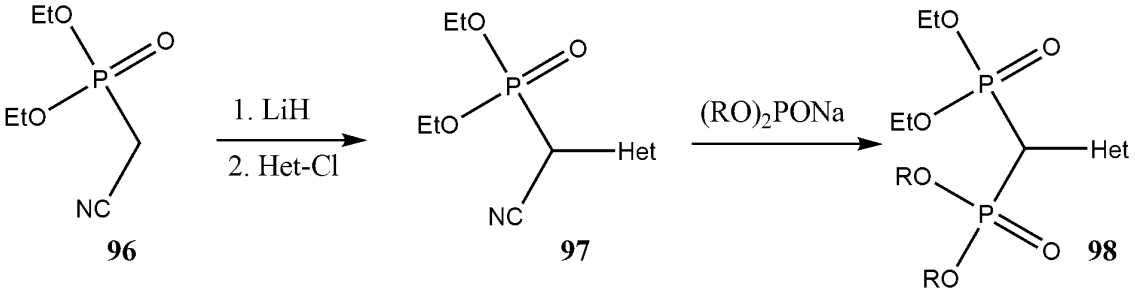
Scheme 20

The synthesis of asymmetrical bisphosphonate diesters becomes possible with application of different monophosphorous species for the Michaelis-Arbuzov reaction. In this methodology trimethylphosphate and 4,4,5,5-tetramethyl-[1,2,3]-dioxaphospholane 2-oxide form bisphosphonate tetraester with two differently protected hydrolytically labile groups (**94**) [9] (Scheme 21).



Scheme 21

A stepwise method developed by Abdou *et al*. involves Horner-Wittig reagents[8] (Scheme 22).



Scheme 22

The methods shown above form a basis for synthesis of various bisphosphonate esters on diverse stages and from diverse starting points. The choice of particular method therefore should be consistent with a synthetic strategy for a given target bisphosphonate.

1.8. THE AIM OF THE RESEARCH

The literature review shows that bisphosphonates in biological applications were developed mainly in bone selectivity and/or specific enzymes inhibition, whereas lesser attention was made for improvement of their ADME properties.

The current study is aimed to the synthesis of methylene-1,1-bisphosphonates diesters bearing a conjugation function in lateral chain as universal mean for bisphosphonate development and for transport purposes.

2 EXPERIMENTAL PART

2.1. METHODS AND MATERIALS

2.1.1. GENERAL

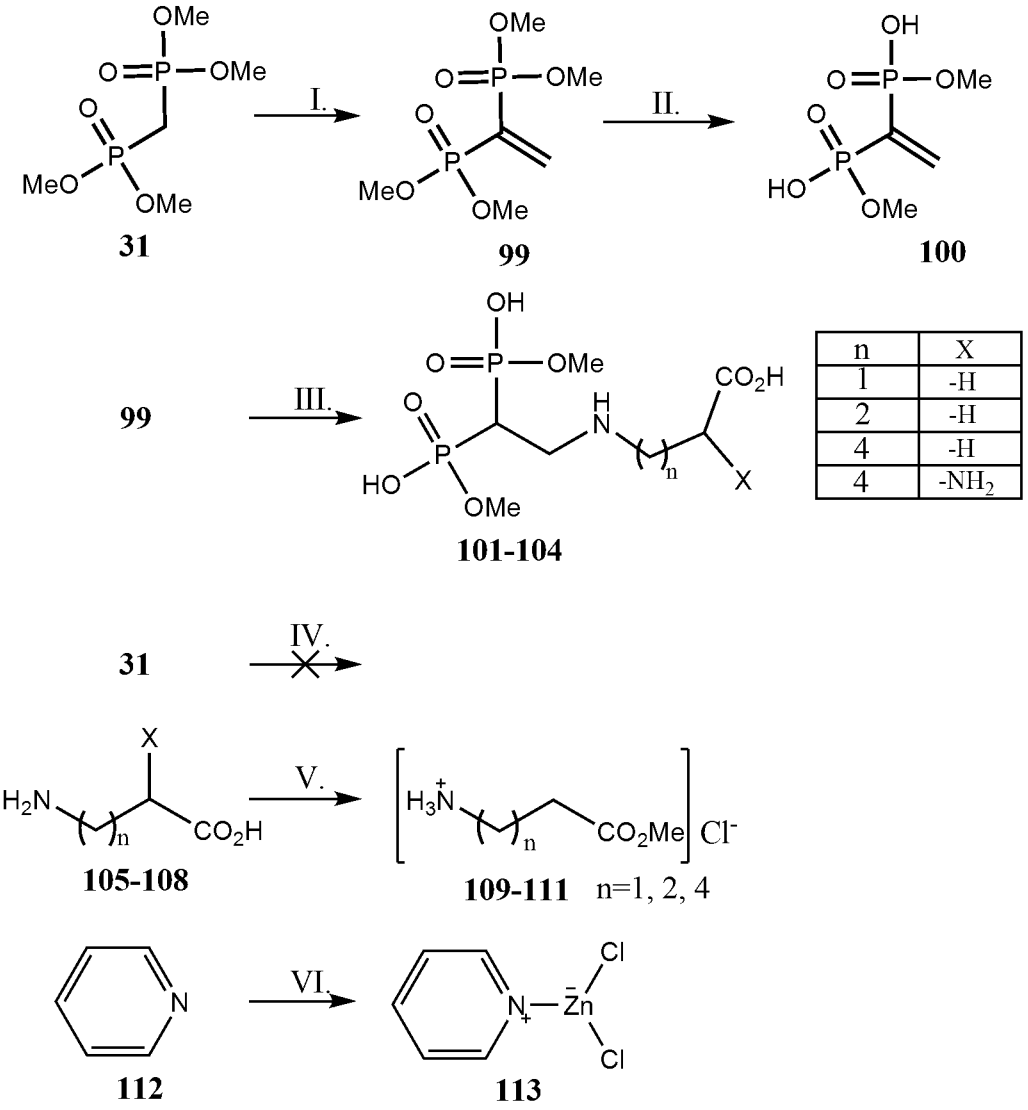
The basic method for reaction observation and compound analysis was ^{1}H, ^{31}P and ^{13}C NMR spectroscopy. TLC was used for purity evaluation when it was possible. The NMR was acquired using Bruker Avance 500 DRX instrument with working frequency of 500.13 Hz, 202.46 Hz and 125.77 Hz for ^{1}H, ^{31}P and ^{13}C nuclei respectively. The ^{31}P and ^{13}C NMR spectra were acquired in proton decoupled mode. Spectra were routinely obtained in deuterium oxide at neutral pH if other is not specified with referencing of the signals to sodium 3-(trimethylsilyl)-1-propanesulfonate (TSP, 0 ppm). The coupling constants are reported in Hz. The related coupling constants are reported if the difference in values does not exceed 0.1 Hz.

2.1.2. MATERIALS

Solvents used in reactions were used as supplied. When required dry solvents were prepared by distillation over drying agents and used under dry argon medium. Tetramethyl-methylenebisphosphonate (98%) was supplied by ABCR, Karlsruhe, Germany and used as is. Tetramethyl-ethenylidene-1,1-bisphosphonate and P,P'-Dimethyl-ethenylidene-1,1-bisphosphonate were prepared according to the literature methods of Degenhardt et al.[123] and Turhanen et al.[10] respectively; the purity not less than 90 % estimated by NMR. 6-Aminocaproic acid (99+%) was supplied by Acros Organics, beta-alanine (98%), gamma-aminobutyric acid (97%) were supplied by Aldrich and L-lysine(98% monohydrate) was supplied by Sigma. Free aminoacids were mostly used, but for experiments with protected aminoacids methyl esters of beta-alanine, gamma-aminobutyric and 6-aminocaproic acids were synthesized by facile method[139] using trimethylsilyl bromide (97 %, Aldrich) or trimethylsilyl chloride (99 %, Aldrich). For reactions in DMF pyridinium dichlorozincate complex (1M solution in DMF, prepared and stored separately in dry conditions) was used in order to dissolve free aminoacids[140]. Silica gel 60 (Merck) was used for routine column chromatography. Strong acid Amberlyst 15 and strong base Dowex 1x8 resins were implied for ion exchange experiments. TLC plates were Merck silica gel 60 F254 and neutral aluminium oxide 60 F_{254} developed with iodine or observed with 366nm UV irradiation.

2.1.3. PREPARED COMPOUNDS

A summary of transformations performed in the study is shown in the Scheme 23.



Scheme 23. I – Degenhardt et al.[142]; II – Turhanen et al.[10], III – present study IV – Houghton et al.[147], V – Li, Sha[139], VI – Ryadnov et al[140].

Tetramethyl ethenylidene-1,1-bisphosphonate (99, TMEBP)

Paraformaldehyde (3.90 g, 129.9 mmol) and diethylamine (1.88 g, 25.7 mmol) were dissolved in 50 ml of dry methanol with heating under dry argon. Clear solution was cooled down and tetramethyl methylenebisphosphonate (6.00 g, 25.8 mmol) in 10 ml of dry methanol was added. The mixture was refluxed for 2 hours and the solvent was evaporated. Then 10 ml of dry toluene was added and the mixture was concentrated until complete removal of solvent. The latter procedure was repeated to ensure removal of all methanol. Then 50 ml of toluene and catalytic amount of *p*-toluenesulfonic acid were

added and the mixture was refluxed with Dean-Stark trap overnight. The resulting mixture was distilled under reduced pressure (121-137 °C/2.9-1.2 mbar) yielding 4.63g (72.4 %, purity est. 90%). ^{1}H NMR ($CDCl_3$): 7.03 (2H, m, =C<u>H</u>$_2$); 3.80 (12H, m, OC<u>H</u>$_3$); ^{31}P NMR ($CDCl_3$): 16.7 (s); ^{13}C NMR ($CDCl_3$): 150.6 (s, $=CH_2$); 130.4 (t, $^{1}J_{CP}$ = 167.6, PCP); 53.5 (t, $^{3}J_{CP}$ = 2.89, OCH_3);

P,P'-dimethyl ethenylidene-1,1-bisphosphonic acid (100, DMEBP)

Tetramethyl ethenylidene-1,1-bisphosphonate (**2**, 0.500 g, 2.07 mmol) was dissolved in 3 ml of acetone, then sodium iodide (0.609 g, 4.15 mmol) was added. The mixture was refluxed for 3 hours and then formed precipitate was filtered, washed with acetone and dried in vacuum until constant weight. Yield 0.295 g (55.3 %) ^{1}H NMR: 3.53 (6H, m, OC<u>H</u>$_3$); 6.55 (2H, m, =C<u>H</u>$_2$). ^{31}P NMR: 14.5 (s). ^{13}C NMR: 142.42 (s, CH_2); 52.46 (s, OCH_3).

General procedures for synthesis of protected aminoacids[139]

Trimethylsilyl chloride or bromide(2 equiv.) was slowly added to dry aminoacid (1 equiv.) in flask with stirring. Contact of reaction mixture with metal and air moisture was avoided. When the mixture became clear an excess of methanol was added. A precipitate of protected aminoacid was filtered, washed with acetone until visibly clean and dried in vacuum until constant weight.

Beta-alanine methyl ester hydrochloride (109)

Trimethylsilyl chloride (2.173 g, 20 mmol) was slowly added to β-alanine (0.891 g, 10 mmol) and stirred until clear solution was formed. Then 10 ml of methanol was added, precipitate was filtered, washed with 20 ml of acetone and dried in vacuum until constant weight. Yield 0.859 g (61.6 %) ^{1}H NMR: 3.69 (3H, s, -CO_2-C<u>H</u>$_3$); 3.24 (2H, m, -C<u>H</u>$_2$-CO_2-); 2.77 (2H, m, -C<u>H</u>$_2$-NH_2).

Gamma-amino butyric acid methyl ester hydrobromide (110)

Trimethylsilyl bromide (97 %, 3.1567 g, 20 mmol) was slowly added to γ-aminobutyric acid (1.012 g, 10 mmol) and stirred until clear solution was formed. Then 10 ml of methanol was added, precipitate was filtered, washed with 20 ml of acetone and dried in vacuum until constant weight. Yield 0.525 g (26.5 %) ^{1}H NMR: 3.72 (3H, s, -CO_2-C<u>H</u>$_3$); 3.05 (2H, m, -C<u>H</u>$_2$-CO_2-); 2.54 (2H, m, -C<u>H</u>$_2$-NH_2); 1.97 (2H, m, C<u>H</u>$_2$).

6-Aminocaproic acid methyl ester hydrobromide (111)

Trimethylsilyl bromide (97 %, 3.1567 g, 20 mmol) was slowly added to 6-aminocaproic acid (1.3117 g, 10 mmol) and stirred until clear solution was formed. Then 10 ml of methanol was added, precipitate was filtered, washed with 20 ml of acetone and dried in vacuum until constant weight. Yield 0.629 g (27.8 %) ^{1}H NMR: 3.70 (3H, s, -CO_2-C<u>H</u>$_3$);

3.00 (2H, m, -C<u>H</u>$_2$-CO$_2$-); 2.43 (2H, m, -C<u>H</u>$_2$-NH$_2$); 1.67 (4H, m, -C<u>H</u>$_2$-CH$_2$-C<u>H</u>$_2$-); 1.41 (2H, m, -CH$_2$-C<u>H</u>$_2$-CH$_2$-).

Pyridinium dichlorozincate (113)[140]

Dry zinc chloride (13.632 g, 100 mmol) was dissolved in 100 ml of diethyl ether and kept at 0 °C while a solution of pyridine (8.226 g, 104 mmol) in 75 ml of ether was added dropwise with stirring. The precipitate was filtered, washed with 100 ml of ether and dried in vacuum until constant weight. Resulting white solid was dissolved in DMF to obtain 1M solution and stored in dry place. Yield 14.814 g (68.8 %) ^{1}H NMR: 8.56 (2H, m, -N-C<u>H</u>-CH-CH-); 7.97 (1H, m, -N-CH-CH-C<u>H</u>-); 7.54 (2H, m, -N-CH-C<u>H</u>-CH-).

N-(2,2-bis(hydroxy(methoxy)phosphoryl)ethyl) β-alanine (101)

Beta-alanine acid (0.055 g, 0.617 mmol) was dissolved in 4 ml of pyridinium dichlorozincate DMF solution (1M) at 70°C. Then 1 ml of tetramethyl ethenylidene-1,1-bisphosphonate (**2**, 0.150 g, 0.614 mmol) DMF solution was added. Reaction mixture was stirred at 70°C for 72 hours. DMF was removed in vacuum and the residue was dissolved in 5 ml of warm water and 5 ml of methanol was added. Precipitate was collected and dried in vacuum until constant weight. Crystallization was repeated two more times. Resulting pale yellow solid was dried in vacuum. Yield 0.034 g (18.1 %) ^{1}H NMR: 3.7 (d, 6H, d, $^3J_{HP}$=10.83, OC<u>H</u>$_3$); 3.5 (2H, td, $^3J_{HP}$=14.70, $^3J_{HH}$=6.89, -P-CH(C<u>H</u>$_2$)-P-); 3.4 (2H, t, $^3J_{HH}$=6.0, -C<u>H</u>$_2$-CO$_2$H); 2.9 (2H, t, $^3J_{HH}$=6.1, -C<u>H</u>$_2$-NH-); 2.7 (1H, tt, $^2J_{HP}$=21.6, $^3J_{HH}$=13.6, -P-C<u>H</u>(CH$_2$)-P-). ^{31}P NMR: 19.0 (s). ^{13}C NMR: 174.6 (s, <u>C</u>O$_2$H); 52.7 (d, $^2J_{CP}$=5.78, O<u>C</u>H$_3$); 45.4 (t, $^2J_{CP}$ =3, CH<u>C</u>H$_2$); 43.8 (s, NH<u>C</u>H$_2$); 34.0 (t, $^1J_{CP}$=122, -P-CH-P-); 30.3 (s, <u>C</u>H$_2$CO$_2$H).

N-(2,2-bis(hydroxy(methoxy)phosphoryl)ethyl) γ-aminobutyric acid (102) Gamma-aminobutyric acid (0.063 g, 0.615 mmol) was dissolved in 4 ml of pyridinium dichlorozincate DMF solution (1M) at 70°C. Then 1 ml of tetramethyl ethenylidene-1,1-bisphosphonate (**2**, 0.150 g, 0.614 mmol) DMF solution was added. Reaction mixture was stirred at 70°C for 72 hours. DMF was removed in vacuum and the residue was dissolved in 5 ml of warm water and 5 ml of methanol was added. Precipitate was collected and dried in vacuum. Crystallization was repeated two more times. Resulting pale yellow hygroscopic solid was dried in vacuum until constant weight. Yield 0.027 g (12.5 %) ^{1}H NMR 3.7 (6H, d, $^3J_{HP}$=10.8, OC<u>H</u>$_3$); 3.5 (2H, td, $^3J_{HH}$=7.0, $^3J_{HP}$=15.1, -P-CH(C<u>H</u>$_2$)-P-); 3.0 (2H, m, -C<u>H</u>$_2$-CO$_2$H); 2.8 (1H, tt, $^2J_{HP}$=22.5, $^3J_{HH}$=7.0, -P-C<u>H</u>(CH$_2$)-P-); 2.5 (2H, t, $^3J_{HH}$=7.0, -C<u>H</u>$_2$-NH); 2.0 (2H, quint, $^3J_{HH}$=7.0, -CH$_2$-). ^{31}P NMR 19.5. (br. s).

N-(2,2-bis(hydroxy(methoxy)phosphoryl)ethyl) 6-aminocaproic acid (103)

6-Aminocaproic acid (0.081 g, 0.615 mmol) was dissolved in 4 ml of pyridinium dichlorozincate DMF solution (1M) at 70°C. Then 1 ml of tetramethyl ethenylidene-1,1-bisphosphonate (**2**, 0.150 g, 0.614 mmol) DMF solution was added. Reaction mixture was stirred at 70°C for 72 hours. DMF was removed in vacuum and the residue was dissolved in 5 ml of warm water and 5 ml of methanol was added. Precipitate was collected and dried in vacuum. Crystallization was repeated two more times. Resulting white hygroscopic solid was dried in vacuum until constant weight. Yield 0.053 g (18.4 %, purity est. 90 %) ^{1}H NMR 3,7 (6H, d, $^{3}J_{HP}$=10.7, OC$\underline{H}_3$); 3.5 (2H, td, $^{3}J_{HH}$=7.0, $^{3}J_{HP}$=14.5, -P-CH(C$\underline{H}_2$)-P-); 3.1 (2H, m, -C$\underline{H}_2$-CO_2H); 2.8 (1H, tt, $^{2}J_{HP}$=22.2, $^{3}J_{HH}$=7.0, -P-C$\underline{H}$(CH_2)-P-); 2.4 (2H, m, -C$\underline{H}_2$-NH); 1.8 (2H, m, -CH_2-CH_2-C$\underline{H}_2$-CH_2-CO_2H); 1.7 (2H, m, -C$\underline{H}_2$-CH_2-CH_2-CH_2-CO_2H); 1.4 (2H, m, -CH_2-C$\underline{H}_2$-CH_2-CH_2-CO_2H). ^{31}P NMR 19.5 (br. s).

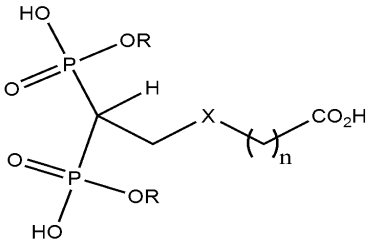
N-(2,2-bis(hydroxy(methoxy)phosphoryl)ethyl) L-lysine (104)

L-lysine (0.090 g, 0.615 mmol) was dissolved in 4 ml of pyridinium dichlorozincate DMF solution (1M) at 70°C. Then 1 ml of tetramethyl ethenylidene-1,1-bisphosphonate (**2**, 0.150 g, 0.614 mmol) DMF solution was added. Reaction mixture was stirred at 70°C for 72 hours. DMF was removed in vacuum and the residue was dissolved in 5 ml of warm water and 5 ml of methanol was added. Precipitate was collected and dried in vacuum. Crystallization was repeated two more times. Resulting white hygroscopic solid was dried in vacuum until constant weight. Yield 0.032 g (11.6 %, purity est. ≤80 %) ^{1}H NMR 1.6 (2H, m, -CH_2-C$\underline{H}_2$-CH_2-CH_2-CO_2H); 1.8 (2H, m, -C$\underline{H}_2$-CH_2-CH_2-CH_2-CO_2H); 2.0 (2H, m, -CH_2-C$\underline{H}_2$-C$\underline{H}_2$-CH_2-CO_2H); 2.8 (1H, dist. tt, $^{2}J_{HP}$=21.5, -P-C$\underline{H}$(CH_2)-P-); 3.0 (2H, m, -C$\underline{H}_2$-NH); 3.2 (1H, m, -C$\underline{H}$(NH_2)-CO_2H); 3.5 (2H, m, -P-CH(C$\underline{H}_2$)-P-); 3,7 (6H, d, $^{3}J_{HP}$=10.2, OC$\underline{H}_3$). ^{31}P NMR 19.5 (br. s).

2.2. Results and discussion

2.2.1. Synthesis planning

The target structural features suggest that the reasonable strategy of synthesis utilize a ready P–C–P scaffold. It narrows the choice of synthetic methods to carbanion method or Michael addition (Scheme 24) that are based on tetraalkyl methylene-1,1-bisphosphonic acid (**98**) as a starting material.

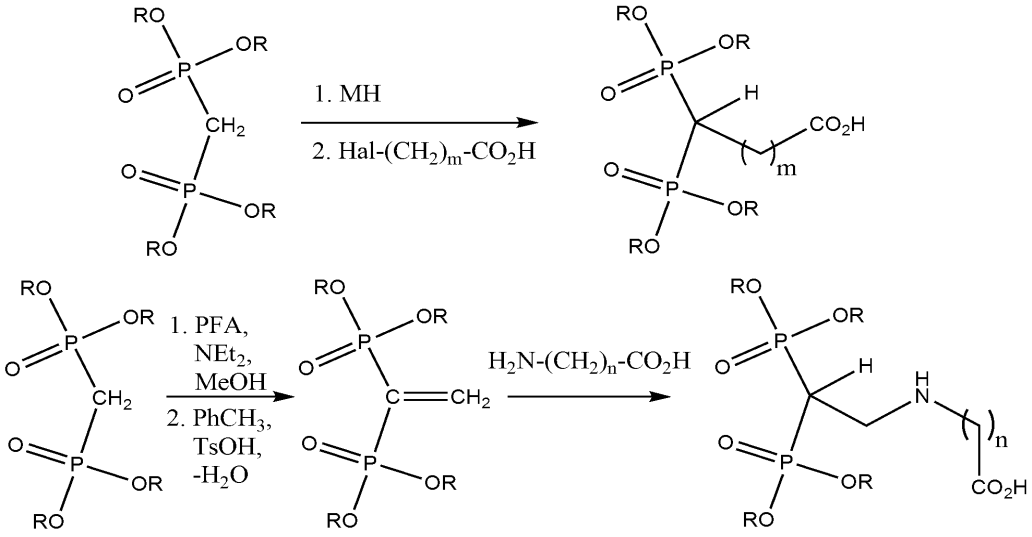


	X	n
I	CH_2	0
II	CH_2	2
III	NH	2
IV	NH	4
V	NH	5

Figure 7

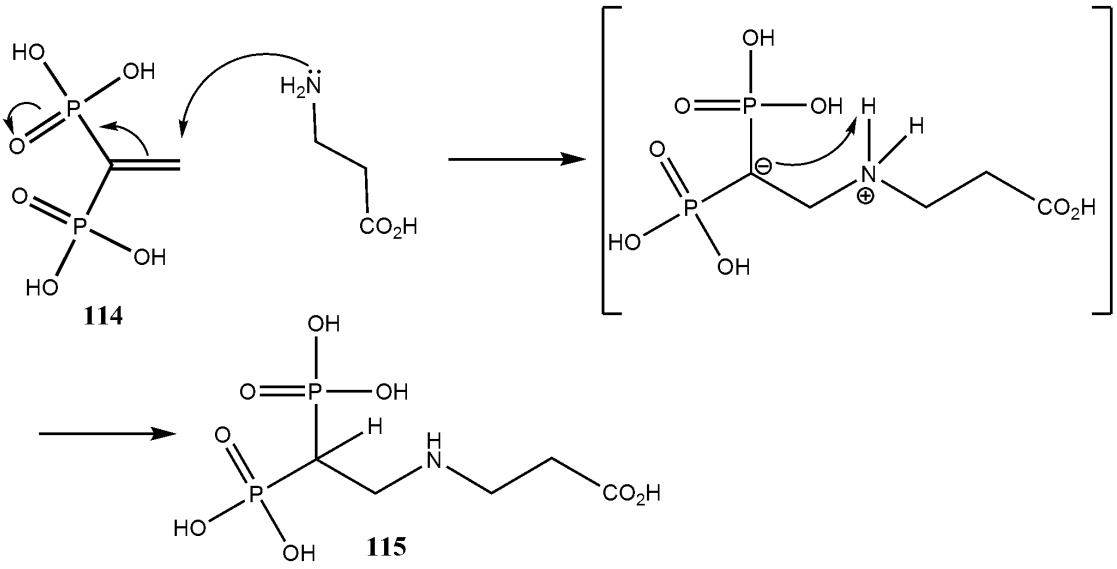
To assess these synthetic paths a preliminary set of experiments has been performed. Following the first method a 3-bromo-propionic acid ester was expected to react with tetramethyl methylene-1,1-bisphosphonic acid (**98**) in presence of sodium hydride in dry THF. This reaction have proved to be reliable if strong metal hydrides and very dry media are used[128,127]. Unfortunately the repeated experiments with the sodium hydride have not led to expected products of conjugation. The development of this synthesis might give desired compounds, but the optimal conditions then might present a practical challenge.

The evaluation of Michael addition method was done in experiments with tetramethyl ethenylidene-1,1-bisphosphonic acid (TMEBP, **99**) and tert-butyl glycine hydrochloride in methanol. As a result a conjugate was observed by ^{1}H NMR (though it was not isolated) and this have shown a potential of method for optimization.



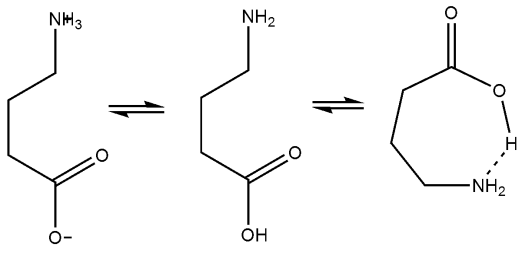
Scheme 24.

A priori the nucleophilic addition of ethenylidene-1,1-bisphosphonate and amino group is driven by electron deficiency on terminal carbon of double bond and by nucleophilicity of amino-reagent (Scheme 25). Typically the polar protic solvents are used to dissolve amino acids due to their limited scope of solubility especially in their free state. This, in turn leads to a high degree of protonation of amino acid N-terminus and its shift to non-reactive state. Moreover omega-amino acids can theoretically exist in acyclic zwitterionic or in intramolecular hydrogen bonded state which also can contribute to reduction of their reactivity as N-nucleophiles (Scheme 26).

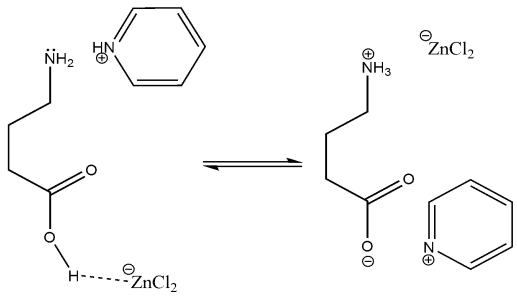


Scheme 25

The general base catalysis appears to be suitable to maintain a neutral state of N-terminus of amino acid, but the P–C–P bonds are labile at pH values higher than 12[137] and the use of tertiary or secondary amines with tetramethyl methylene-1,1-bisphosphonic acid (**1**) necessarily implies bisphosphonate partial deprotection[137]. These considerations led to the search of aprotic reaction medium for free aminoacids. In literature data such media are suggested by Ryadnov *et al.*[140] and consists of DMF solution of pyridinium complexes of Lewis acids. Although the precise mode of amino acid solvation in such media is not known i.e. whether dissolved amino acid is in zwitterion form (Scheme 27) or not, so far that is only way[141] to perform reactions of free amino acids in aprotic polar solvents.



Scheme 26

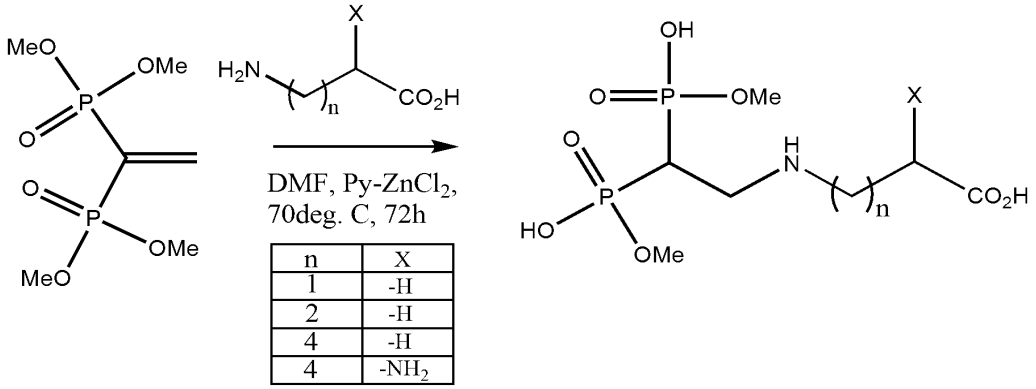


Scheme 27

An additional complexity to the synthesis is gained by necessity of symmetrical deprotection of bisphosphonate on one of synthesis stages. This can be done by one of the methods described in section 1.7.3. An inorganic salt was applied to TMEBP (**2**) with formation of dimethyl ethenylidene-1,1-bisphosphonate sodium

salt (DMEBP, **3**) as a pale-brown solid. DMEBP can be used as an intermediate product of synthesis and in the present study it was used for a test reactions.

The first trial of the reaction in DMF:pyridinium dichlorozincate solution have shown: (a) the reaction proceeds upon heating within a period of three days, (b) the product bisphosphonate has ionic nature – hygroscopic, dissolves easily in water and precipitates after addition of acetone, methanol or ethanol, (c) the product bisphosphonate is a conjugate of target structure. The products were isolated with iterative precipitation in water – water:methanol system with moderate yields.



n	X
1	-H
2	-H
4	-H
4	$-NH_2$

Scheme 28

2.2.2. DEVELOPING THE REACTION

The reaction of tetramethyl ethenylidene-1,1-bisphosphonate with four amino acids in DMF:pyridinium dichlorozincate medium led to a target compounds with moderate yields. The observation of the process at moderate heating (60-70°C) by ^{1}H NMR has shown that the reasonable degree of conversion is achieved after 72 hours.

In order to find an optimal temperature mode a reaction mixture (Scheme 28, n=1) was observed at five temperature points by ^{1}H and ^{31}P NMR the latter being characteristic. In a simple approach it has been shown that a starting material undergoes a transformation at 60-70°C within one hour (Figure 7). It is a matter of discussion what kind of transformation takes place during this rapid change. The fact that a significant conversion of TMEBP into conjugate product (**3a-3d**) was observed only after several hours at 70°C (the rest conditions being the same) lead to assumption that the nucleophilic attack of amino acid is a rate-limiting step while previous steps are fast. On the other hand a clear difference of chemical shifts of DMEBP and intermediate and singlet signal of latter mean that a chemical

environment of both phosphorus atoms is changed during this rapid transformation. This suggests a deprotection step and formation of intermediate partial ester pyridinium salt to be the first step of the reaction. However this hypothesis is to be evaluated, for instance via synthesis of proposed intermediate in control test.

In proposed mechanism a pyridinium complex is responsible for methyl cleavage and N-methyl pyridinium cation is formed. During a methyl cleavage a negatively charged zinc chloride is donating an electron to the deprotected oxygen acting as a promoting agent. If such a step takes place then an isolation of N-methyl pyridinium salts (**A**, **B**, Scheme 29) might be possible. Once the intermediate diester (**B**) is formed a slow stage of nucleophile addition is following. The adduct (**C**) is probably losing pyridinium cations during the isolation since corresponding ^{1}H NMR signals are observed in crude product, but strongly decay when the purification is done.

The control reactions of free amino acids with TMEBP are impossible due to insolubility of former in DMF. The control reactions were set with O-methyl amino acids hydrobromides in presence of equimolar diisopropylethylamine in DMF with and without pyridinium dichlorozincate. Opposite to expected in these reactions conjugate structures were not observed. That was probably due to a lower rate of reaction or effects of the media that might have complex acid-base equilibria.

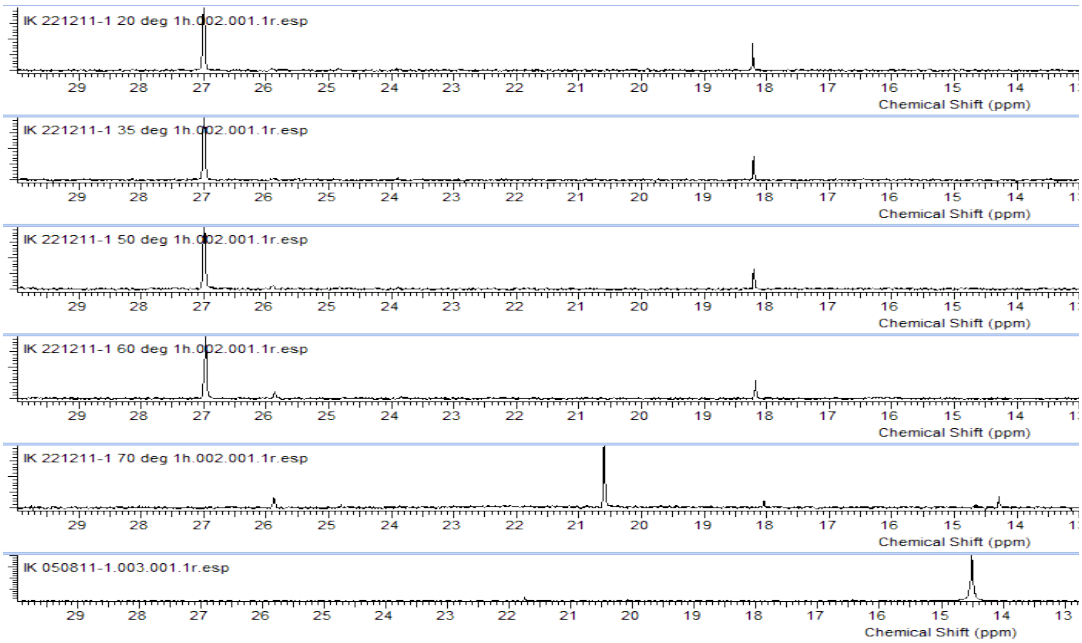
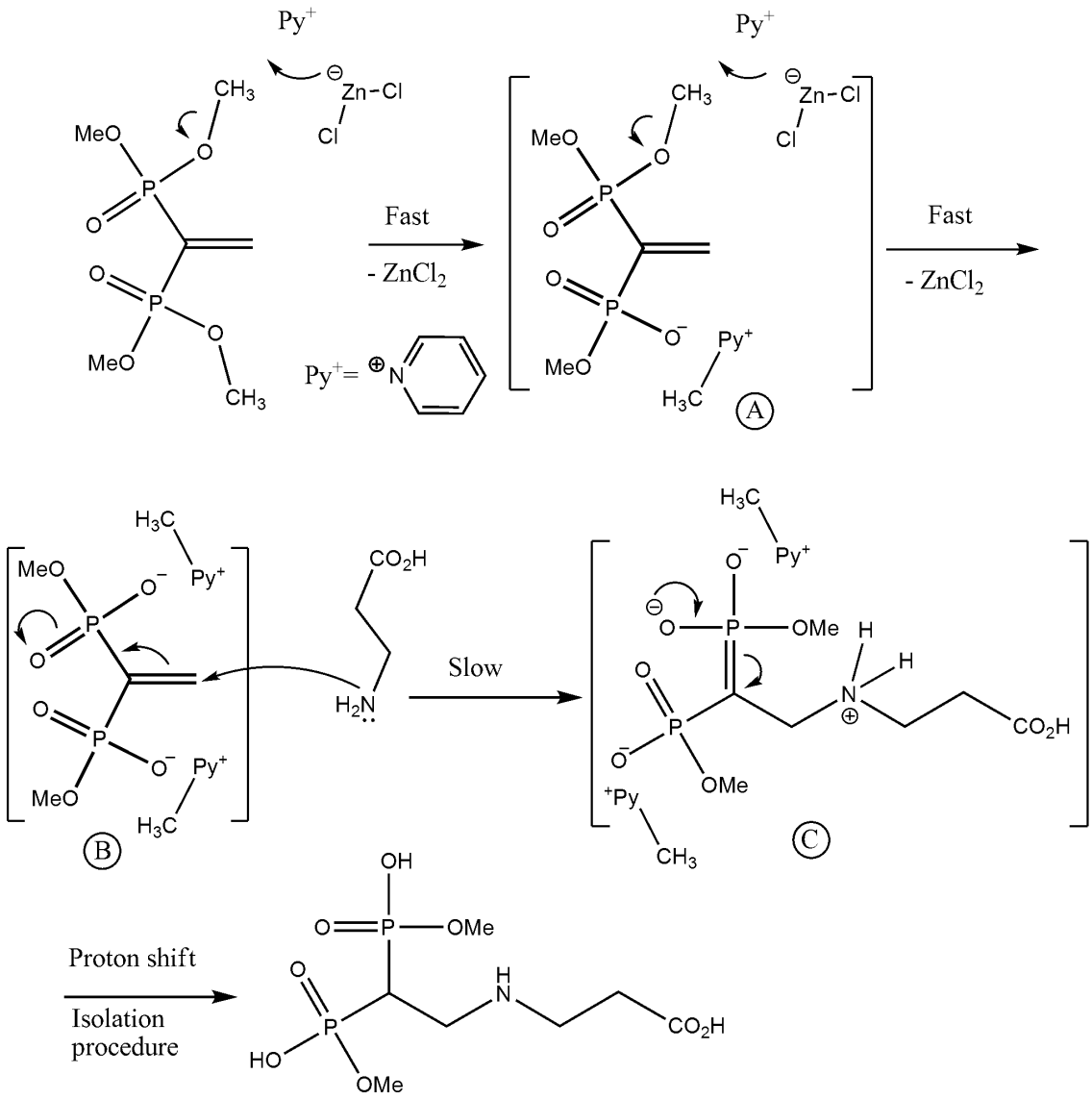


Figure 9. ^{31}P NMR. From the top to the bottom: 20°, 35°, 50°, 60°, 70°C after 1 hour each. The bottom spectrum is a DMEBP reference.



Scheme 29. A suggested mechanism of reaction

An additional series of reactions was set to extend the synthesis to more common methanol and water. Protected amino acids (**109-111**) were tested for their reactivity with TMEBP (**99**) and DMEBP (**100**) in methanol, while free amino acids (**105-108**) were tested with these bisphosphonates in water. The reaction of TMEBP with methyl β-alanine in methanol and free β-alanine in water produced some product of double bond conjugations as observed by ^{1}H NMR, but these compounds were not isolated. In contrast to tetraester (**99**) the DMEBP (**100**) have produced a conjugate (observed in the same way) only with a methyl β-alanine in methanol.

2.2.3. PURIFICATION TECHNIQUES

The method applied for a crude isolation of amino acid conjugated DMEBP was based on manipulation of the solvatation capability of the water solution. As the procedure was iteratively repeated the purity of the compounds was increasing. However the target product might be soluble to some extent in methanol-water systems as well as some impurities might have the same properties in these solution thus the method is suffering from both selectivity and target compound losses. This in turn distort the yield comparison and quantitation and restrict the development of the reaction based on subtle differences.

The chromatography is the basic method routinely used for separation of complex mixtures of compounds in laboratory practice. In need for reliable preparation and analysis tools for bisphosphonate drugs several methods were developed[142]. Thin-layer chromatography was used for monitoring chemical reactions. In all experiments a normal phase stationary phase - silica and neutral alumina - was strongly retaining a bisphosphonate (detected in 366 nm UV irradiation) and the rest of components could be observed in elution line. The apparent strength of polar interactions with stationary phase in case of protected amino acids and their possible products was as strong and the interaction is attributed to the amino group of the components. Possible solution for silica based chromatography can be use of functionally modified silica[143] and/or a “reversed” stationary phase[144].

The ion exchange method of separation is typical for bisphosphonates owing to their strong anionic character[145,146]. This approach is applicable to the obtained bisphosphonate diesters bearing two additional ionic centers – a carboxylic and a secondary amino groups. Consequently a cation and anion exchange can take place for these compounds. Additionally the cation exchange can be used to liberate ammonium cation that might exist in the product bisphosphonate for a selective purification. The anion-exchange should utilize the differences in acidity of phosphonic and carboxylic components when phosphorous species preserve anionic form at lower pH values. This idea was tested in a single experiment with strong base Dowex 1x8 resin in a batch method with a set of HCl:KCl buffers. The buffer pH was discretely varying from 6 to 1,5 units and final sample was extracted with 1M hydrochloric acid. Unfortunately the samples had a strong concentrations of inorganic components of buffer and reliable observations could not be made. The test of cation exchange was done with strong acid Amberlyst 15 resin in a column method with pre-treatment of the column by NaOH, but the bisphosphonate was retained by the resin probably because of amino group interactions. Another reason

might be a degradation of bisphosphonate in strong basic environment of resin. As a result it can be concluded that ion exchange interactions take place, however for the practically relevant performance the type of resin and the method should be optimized.

2.2.4. FURTHER RESEARCH PROBLEMS

During the study a synthetic path to P,P'-dimethyl ethenylidene-1,1-bisphosphonic acid and ω-amino acids conjugates was found. The study has revealed several problems related to the synthesis of bisphosphonate-amino acid conjugate P,P'-diesters, which solution will certainly improve presented synthetic path.

The current yields show that the reaction conditions are sub-optimal and the conversion degree is low. Elucidation of role of the pyridine complex reagent and the solvent in the reaction mechanism will provide a stronger rational basis of search. The conjugation of protected and free amino acids with TMEBP and DMEBP might be observed in water and methanol if general base catalysis is tested for applicability.

The problem of reproducible yielding of zwitterionic bisphosphonate conjugates present a challenge in practical field. The reversed phase chromatography will probably suffer from lack of retention of bisphosphonate products. Ion exchange methods along with studies of acidity constants (e. g. by ^{1}H, ^{13}C and ^{31}P NMR observed titration) can provide a good selectivity based on properties of individual conjugate.

Besides problems mentioned above the compounds synthesised require an assessment in one of proposed applications – as an imaging agent or transport moiety or in co-transport with other transport units.

3 ACKNOWLEDGEMENTS

The present thesis is a product of hours spent in laboratory, extensive literature research, discussion of separate problems faced and, of course, of trials and errors. Within the time I was working on the topic of my thesis I was in environment of hard working, thinking and positive people which contribution for the presented study cannot be counted but can be gladly appreciated.

I feel gratefulness for the great experience offered by Master's Degree Programme for Research Chemists as a merit of Professor Tapani Pakkanen. Thanks to this educational programme I have expanded my scope of skills and knowledge.

I would like to thank my supervisors Professor Jouko Vepsäläinen and MSc Elina Sankala for introducing me a world of organophosphorous compounds, supporting me and providing advices for the challenges I have faced along my way.

There is a number of people I met and worked with during my project that I would like to thank and just listing them would make a pretty full page. Thank you!

I am thankful to my family and my dear people that support me in my studies and inspire me in moments of discouragement. Your support is essential for me. Thank you!

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