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MASTERARBEIT

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„On the Biodegradation of Fosfomycin and Phosphaisoserine“

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There's three ways to do things, the right way, the wrong way and the way that I do it. (Robert De Niro, Casino)

Be an optimist, at least until they start moving animals in pairs to Cape Kennedy. (Anonymous)

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1. P-C compounds - a short introduction

Phosphorus is an essential nutrient due to its varied functions in metabolic pathways and as a component of macromolecules such as phospholipids, RNA or DNA. It accounts for about 3% of the dry weight of all living organisms and plays a vital role in marine ecosystems where growth and productivity of organisms is limited by the availability of nitrogen, iron and phosphorus.[1,2,3]

In the biosphere, phosphorus is present mostly in its highest oxidation state (+5) as inorganic orthophosphate (P_i), phosphate-containing minerals and organic phosphate esters.[2] Though, over the past decades it has become obvious that other types of organo-phosphorus compounds in lower oxidation states also play important roles in biochemistry. Figure 1.1 (the first digit gives the number of the chapter; the same is true for Schemes) gives an overview of the oxidation states of phosphorus in some families of compounds.

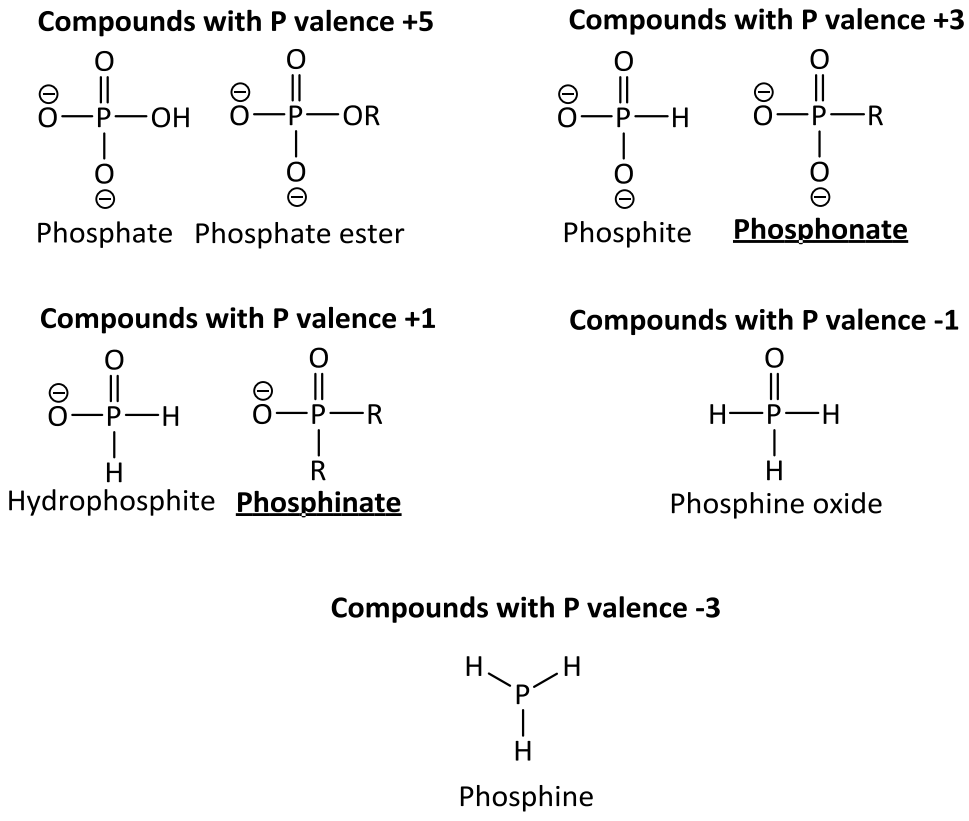


Figure 1.1. Oxidation states of phosphorus.[4]

Phosphonates and phosphinates are characterized by direct, covalent phosphorus to carbon bonds (P-C in phosphonates and C-P-C in phosphinates) instead of the carbon-oxygen-phosphorus (C-O-P) bond which is present in organic phosphate esters.[4]

One of their major characteristics is the high chemical stability of the P-C bond in comparison to the rather labile and reactive O-P bond. P-C compounds are resistant towards hydrolysis under strong acidic and basic conditions at high temperatures, as well as towards photolysis, thermal decomposition and the action of phosphatases.[5]

Although the dissociation energy of a P-C bond is of the same order of magnitude as those for N-P, S-P and O-P bonds (about 60 kcal/mol), it is much more stable. This is due to the much higher activation energy for P-C bond cleavage.[6] Phosphonates with substituents on their α-carbon atom are an exception from the rule and can be hydrolysed more easily.[7]

Aminomethylphosphonic acid was the first synthetic organophosphonate to be synthesised in 1944. However, it was not until 1959 that the first organophosphonate of biogenic origin, 2-aminoethylphosphonic acid (2-AEP, **1.1**) was isolated from sheep rumen protozoa (Figure 1.2).[8] Since then the number of known natural phosphonates has been steadily increasing but is still limited.

Phosphonates are widely distributed in nature and they account for about 20% of the bio-available phosphorus in marine ecosystems.[9] Many lower organisms produce a large variety of phosphonates and phosphinates. They are produced by aquatic and some terrestrial invertebrates, as well as by some insects, mollusks and microorganisms where they are found as conjugates with macromolecules (associated with glycolipids, glycoproteins or polysaccharides). Furthermore, they were identified as a component of phosphonoglycan[10], a major integral part of the cell wall of *Trypanosoma cruzi*, which is the pathogen of Chagas disease. Phosphonates also play a role as cell protection mechanisms if present in outer-membrane structures as they are very stable towards enzymatic degradation. In addition to that they are believed to be involved in certain invertebrate parasite-host recognition mechanisms and to play a role in primitive neuronal systems. In some organisms , such as the sea anemone *Tealia feline* or in the eggs of the snail *Helisoma* phosphonates account for 50 and 95% of the total phosphorus content, respectively.[3] As macro molecule associates they have mainly structural functions.

In addition to those macromolecules, there is also a group of small bioactive molecules of secondary metabolic origin (in most cases microbial) which exhibit antibiotic, antimalarial and herbicidal activity and are therefore of special interest. Phosphonates were also found

in higher organisms, including human-beings, but they are believed to have dietary origins in the latter.[11]

It is speculated that phosphonates and phosphinates, *aka* P-C compounds, were the dominant phosphorus containing species in the reducing atmosphere of primitive times and that they replaced phosphates in early life forms.[12]
Nowadays, both biogenic and synthetic organophosphonates can be found. Even though industry did not see the potential of phosphonates and phosphinates until recently, there is now a wide range of applications for P-C compounds.

As phosphonates are very stable, nearly isosteric to the more labile phosphates and also due to their similarity to carboxylic acids, they have a high potential as antimetabolites by blocking enzymatic processes.[4] Among the most important biogenic phosphonates are 2-AEP (**1.1**), the first phosphonate to be isolated from a natural source; fosfomycin (**1.2**), showing antibiotic activities; phosphinotricin (**1.3**), a non-selective herbicide, and fosmidomycin (**1.4**) which is an antimalarial agent (Figure 1.2).

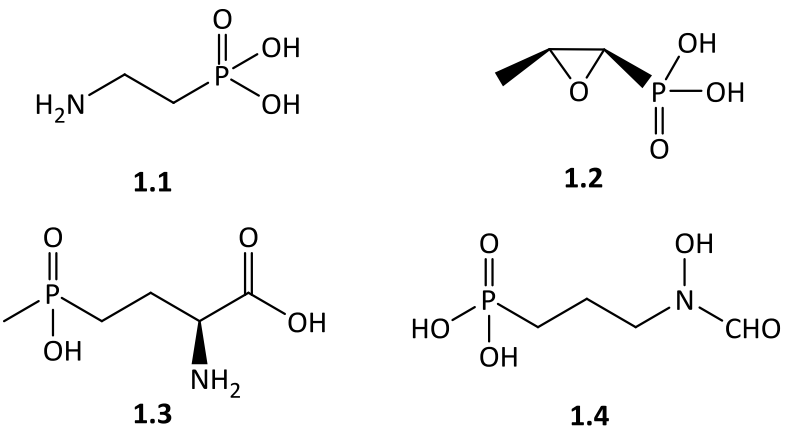
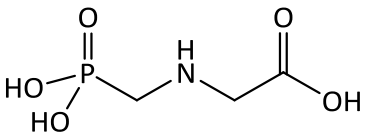
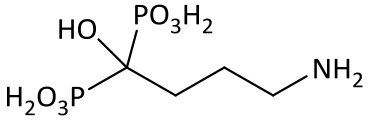


Figure 1.2

The favourable properties of phosphonates of natural origin also led to the development of a huge number of synthetic organophosphonates. They are used as herbicides, insecticides, antiviral agents (phosphonoformate) and flame retardants. Prominent synthetic phosphonates are glyphosate (**1.5**), one of the most widely applied herbicides, and alendronate, an anti-osteoporisis agent (**1.6**) (Figure 1.3).



1.5



1.6

Figure 1.3

A high amount of these synthetic phosphonates is released into the environment and therefore the understanding of their mineralisation is of crucial interest.[3] A closer insight into antibiotic resistance mechanisms is another reason to investigate phosphonic acid biodegradative pathways.

2. Some natural P-C compounds of interest

2.1 2-Aminoethylphosphonic acid (2-AEP)

2-AEP is the most abundant organophosphonate and was hence the first one to be isolated from a natural source, namely protozoa of sheep rumen.[8] Subsequently, 2-AEP was found in many other organisms and it was shown to be a head group of membrane lipids like phosphatidylethanolamine.[4]

These findings led to the discovery of phosphonolipids in many other species, such as coelenterates, gastropods, bivalves, bacteria and plants.

2-AEP is also the most abundant phosphonate species comprised in phosphonoglycans, even though *N*-methylated AEP and different organophosphonates were isolated.

As 2-AEP biosynthesis does not take place in humans but in some pathogens, it is a promising candidate for enzyme inhibition based drugs.[4]

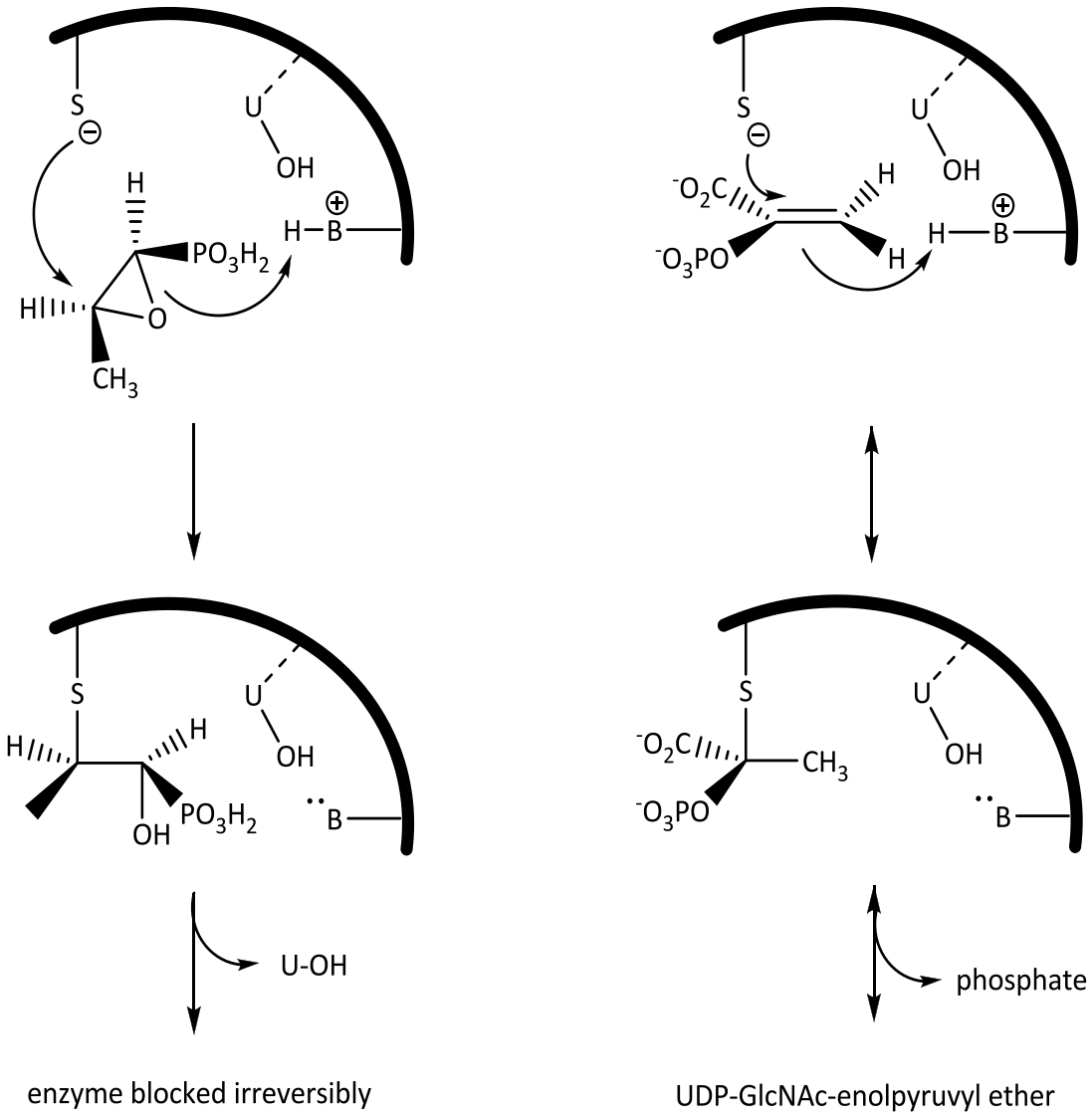
2.2 Fosfomycin

2.2.1 General remarks

Fosfomycin (**1.2**) is the first phosphonate antibiotic isolated from *Streptomyces fradiae*. Afterwards, it was isolated from several other strains of *Streptomyces*. It is effective against Gram-positive as well as against Gram-negative bacteria.[13] The laboratory-synthesis of enantiomerically pure fosfomycin was accomplished in 1989[14] and since then fosfomycin has been used as an intravenously administered antibiotic. It is used in case of β-lactam antibiotic intolerance, but its application is limited to the clinical use (due to the intravenous administration).

2.2.2 Mode of action

Fosfomycin inhibits phosphoenolpyruvate: UDP-*N*-acetyl-glucosamine-3-*O*-enolpyruvyltransferase (pyruvyl transferase)[15], the first enzyme catalysing peptidoglycan synthesis and therefore inhibits cell wall biosynthesis of bacteria.[16] This enzyme links the general nucleotide-sugar metabolism with the synthesis of *N*-acetylmuramyl peptide. It catalyses the reaction of UDP-*N*-acetylglucosamine (UDP-GlcNAc) and phosphoenolpyruvate (PEP) to form an UDP-GlcNAc-3-enolpyruvyl ether. This intermediate is then converted to



Scheme 2.1. Schematic drawing for inactivation of enzyme by fosfomycin (left) compared to the reaction of the enzyme with PEP (right); U stands for UDP*N*GlcNAc.[15]

UDP-*N*-acetylmuramic acid via reduction. Afterwards, a peptide chain is linked to the carboxyl group of the latter and the cell walls of bacteria are formed from these fragments. Fosfomycin blocks this reaction by covalently binding to the enzyme pyruvyl transferase. However, covalent binding of fosfomycin is only possible in the presence of UDP-GlcNAc. The latter is needed as a stabilizer for enzyme/substrate binding between fosfomycin and pyruvyl transferase without covalently reacting with either compound at first.

The antibacterial activity of fosfomycin arises from its structural similarity to PEP. In the active site of pyruvyl tranferase, fosfomycin is oriented so that a proton donor activates the epoxide. This group normally protonates C-3 in the actual substrate PEP. However, with

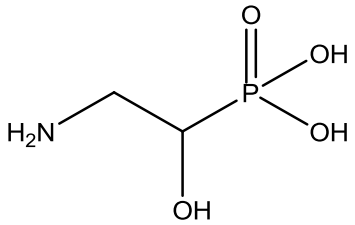
fosfomycin instead of the natural substrate in the active site, this protonation allows the opening of the epoxide by nucleophilic attack of an SH-group of a cysteine residue at C-2 of the antibiotic. This leads to the formation of a covalent bond between the enzyme and fosfomycin. This irreversible binding inactivates the enzyme (Scheme 2.1).

It is important to note that fosfomycin only acts as a structural analogue of PEP concerning this particular enzyme. This arises from the fact that fosfomycin and PEP are not similar in the configuration, but in the sensitivity of the C-2-O bond towards addition of a nucleophile in the active site of the enzyme. Fosfomycin does not have a carboxyl group responsible for strong electrostatic interactions of PEP with other enzymes. Thus, fosfomycin does not resemble PEP sufficiently to act as an inhibitor of other enzymes that use PEP as a substrate (such as pyruvate kinase, enolase or PEP carboxykinase). Furthermore, these enzymes all cleave O-P bonds by their catalytic activity which is not possible in fosfomycin. Therefore, it is not able to act either as a competitive inhibitor or by covalent reaction with these enzymes. This is the reason why fosfomycin does not have any effect on higher organisms such as mammals even though PEP is a ubiquitous primary metabolite.[15]

2.3 Phosphaisoserine

2.3.1 General remarks

The optically active phosphaisoserine (**2.1**), 2-amino-1-hydroxyethylphosphonic acid, is the phosphonic acid analogue of the non-proteinogenic amino acid isoserine (Figure 2.1). It is a



2.1

Figure 2.1

component of the lipophosphonoglycan in the plasma membrane of the protozoon *Acanthamoeba castellanii*.[17]

2.4 Phosphinothricin

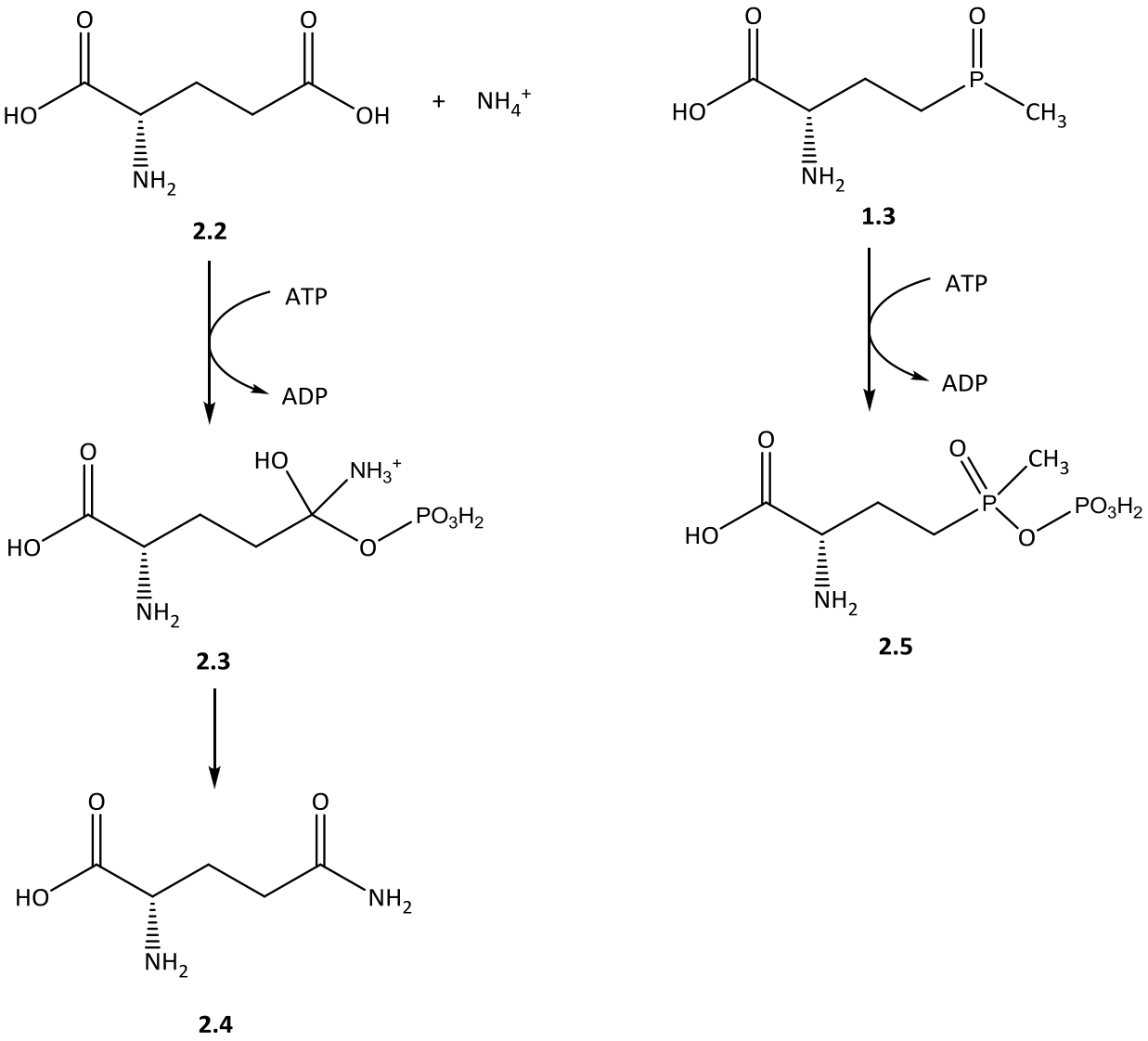
2.4.1 General remarks

2-Amino-4-[hydroxy(methyl)phosphoryl]butanoic acid, phosphinothricin (**1.3**), is produced by *Streptomyces viridochromogenes* and *S. hygroscopicus* as a tripeptide bound to the amino group of alanylalanine. This tripeptide is called bialaphos and used as herbicide. Phosphinothricin has a very low antibiotic potential as it is a structural analogue of glutamate (**2.2**) and therefore its effect can easily be suppressed by the latter. However, it is a very potent[28] but unselective herbicide, often used in combination with transgenic economic plants like corn.

Possessing a C-P-C structural element, the non-proteinogenic amino acid phosphinothricin is special among the natural P-C compounds as it has two phosphorus-to-carbon bonds. Expectedly, the biosynthesis of this substance differs from those for other P-C compounds and is quite complicated. It involves about 20 steps.[4]

2.4.2 Mode of action

Plants need glutamine synthase to keep the concentration of toxic ammonia low. Phosphinothricin efficiently inhibits the formation of glutamine catalysed by glutamine synthase. During glutamine synthesis, the natural substrate of glutamine synthase, glutamate (**2.2**), is phosphorylated by ATP in the first step to give the tetrahedral intermediate **2.3** which is converted to glutamine (**2.4**) in the second step (Scheme 2.2).



Scheme 2.2. Mechanism of glutamate synthase deactivation (right hand side) compared to mechanism of glutamine synthesis by the enzyme (left hand side).

Phosphinothricin (**1.3**) is likewise phosphorylated to give compound **2.5** able to mimick the tetrahedral reaction intermediate and thus blockating the enzyme (Scheme 2.2). As the phosphorylated product of phophinothricin, produced by the plant itself, and not phosphinothricin itself is the active species, its mode of action as herbicide is referred to as "Trojan horse" mechanism.[4]

2.5 Fosmidomycin

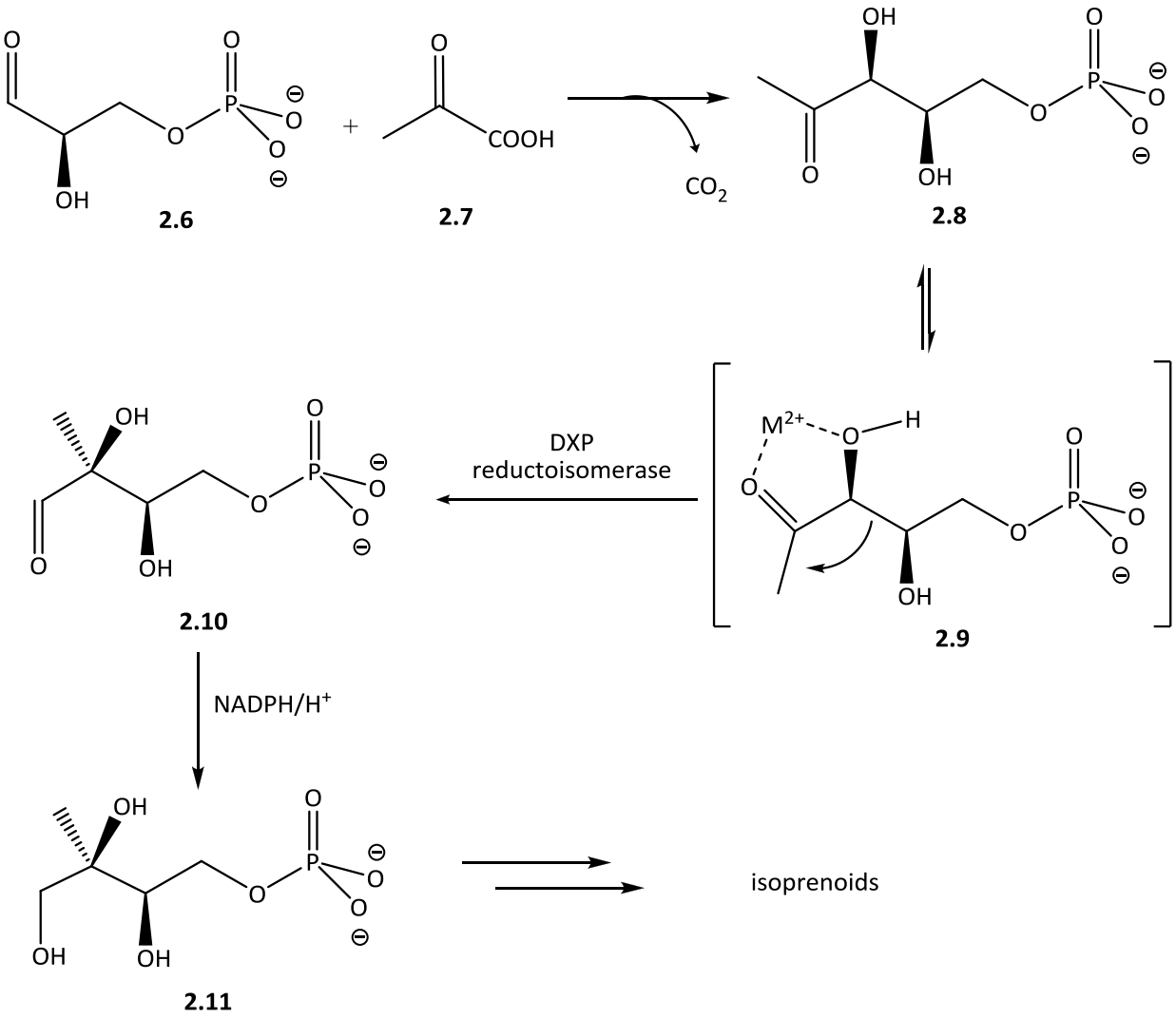
2.5.1 General remarks

Fosmidomycin (**1.4**) is an antimalarial drug which was originally isolated as a natural antibiotic from *Streptomyces lavendulae* in 1980.[18] Its antimalarial activity was not detected until 1998.[19] One of its major advantages is the very low toxicity and high activity against multiresistent strains of malaria pathogens.[20] Disadvantages are its low bioavailability (about 30%) and the low plasma half-life (about 2.5 h) because of the extremely hydrophilic properties. It is tested in combination with other antimalarial agents because of its high relapse rate when used as the only therapeutic agent.[21] Furthermore, derivates and analogues of the natural product are tested.[19]

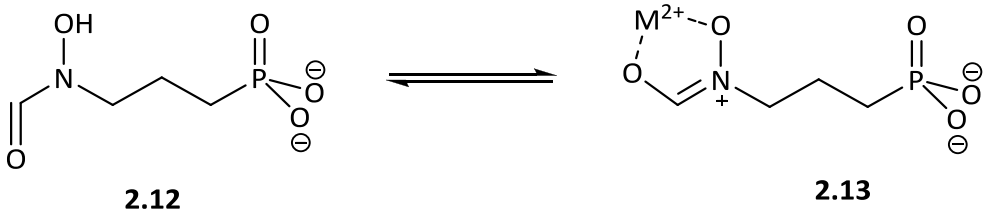
2.5.2 Mode of action

Malaria is caused by protozoal parasites called *Plasmodia.* Four genera are known to infect humans, *P. falciparum* being one of those.[19] Isoprenoids in these organisms are not biosynthesised by the mevalonate, but Rhomer pathway (nonmevalonate pathway) starting from glycerine-aldehyde-3-phosphate (**2.6**, GA3P) and pyruvic acid (**2.7**). This pathway is found in several genera of bacteria, green algae and in chloroplasts of higher plants (Scheme 2.3).

The mode of action of fosmidomycin uses this difference between humans and *Plasmodia* in producing isoprenoides. It inhibits 1-deoxy-D-xylulose 5-phosphate (DXP) reductoisomerase[22], a key enzyme of the Rhomer pathway and therefore blocks the parasite's ability to produce vital isoprenoids. DXP reductoisomerase catalyses the isomerisation and reduction of DXP to 2-*C*-methyl-D-erythritol 4-phosphate (**2.8**, MEP).
Scheme 2.4 shows the inhibition of this enzyme by fosmidomycin.



Scheme 2.3. First steps of Rhomer pathway.



Scheme 2.4. Inhibition of DXP reductoisomerase by fosmidomycin; M^{2+} can either be Mg^{2+} or Mn^{2+}.

It was suggested that although fosmidomycin binds to the active site of this enzyme with a comparatively low affinity, it induces structural changes of the enzyme which increase the enzyme's affinity for the agent. Therefore this phosphonate is a very effective competitive inhibitor of DXP reductoisomerase.

3. Synthetic phosphonates

The field of application for synthetic phosphonates is vast. They are used in agriculture because of their herbicidal (glyphosate) and fungicidal activity. They are used in medicine as antiviral agents (phosphonoacetic acid) and as diphosphonates having an effect on osteoporosis. Furthermore, industry takes advantage of organophosphonates as flame retardants, detergent additives and catalysts.[11]

Phosphonoacetic acid or Fosfonet, for example, is a synthetic antiviral agent. Its natural occurrence could not be confirmed yet, but it is quite likely that it is produced by microorganisms possessing transaminases but no P-C cleaving enzymes. In such microorganisms, phosphonoacetic acid is likely formed as a degradation product of 2-AEP. Formation of phosphonoacetic acid could accompany phosphonoacetaldehyde (PAA) detoxification in those microbes.[3]

3.1 Glyphosate

3.1.1 General remarks

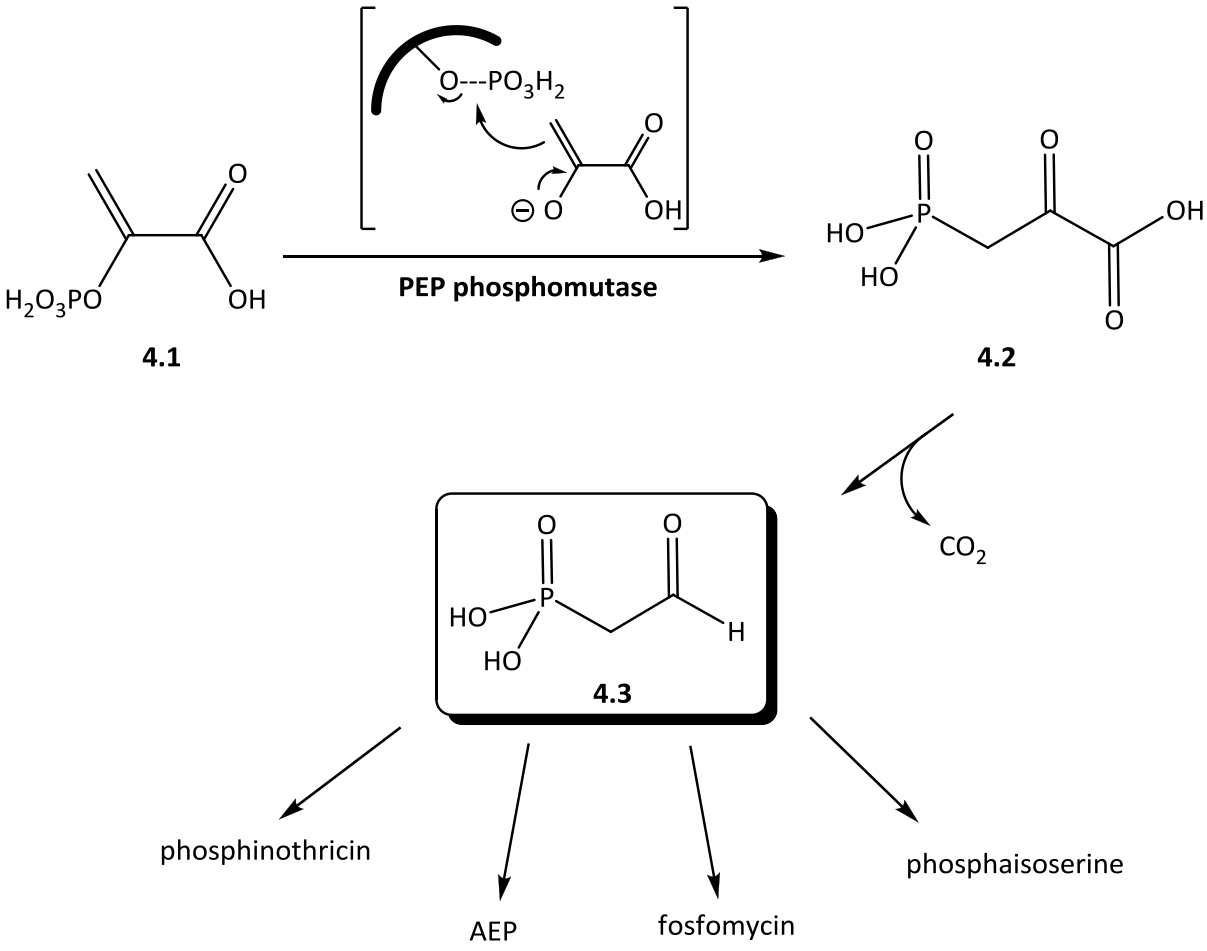
Glyphosate (**1.5**) is probably the most important synthetic organophosphonate. It was first produced as an herbicide by Monsanto under the commercial name “Roundup” in 1974. It is used all over the world today. It has a very low toxicity (LD_{50} (rats) > 5g/kg) and is not harmful to mammals.[23]

3.1.2 Mode of action

Glyphosate is a non-selective total herbicide, which blocks the shikimic acid pathway by inhibiting the 5-enol-pyruvyl-shikimate-3-phosphate synthase (EPSPS). It is a transition state analogue of PEP, the natural substrate of this enzyme. EPSPS catalyses the reaction of shikimate-3-phosphate to chorismate, which is a key intermediate for the biosynthesis of aromatic amino acids. Therefore, glyphosate blocks the biosynthesis of phenylalanine, tyrosine and tryptophan and phenylpropanoids in plants. Resistance mechanisms are known.[23] It is used with transgenic plants such as soybeans, canola and corn.

4. Biosynthesis of P-C compounds

Up to now the biosynthesis of only three biogenic phosphonates has been unravelled in detail. These are 2-AEP, fosfomycin and in part bialaphos (a tripeptide containing phosphinothricin and two molecules of alanine) numbers, then you don't need explanation. It is assumed that the first step of the biosynthesis of all natural products containing a P-C bond is the rearrangement of phosphoenolpyruvate (**4.1**, PEP) to phosphonopyruvic acid (**4.2**) (Scheme 4.1). This rearrangement was first suggested in 1969.[24]



Scheme 4.1. First steps of phosphonate biosynthesis, which are postulated to be similar for all members of this substance family.

PEP, a ubiquitous primary metabolite, is rearranged to phosphonopyruvic acid (**4.2**, PnPy) via an intramolecular phosphoryl transfer from the oxygen to the carbon atom. The catalysing enzyme is called phosphoenolpyruvate phosphomutase (PEPPM).[25,26] This reaction probably

involves a covalent phosphoenzyme species, which has not yet been identified, and 2-oxopropanoic acid enolate as intermediates.[27]

A common point in phosphonate biosynthesis is the highly unfavourable first step. PnPy has to be metabolised immediately after its formation as the reaction equilibrium for its formation lies 500-fold at the side of PEP.

The formed PnPy species is in most cases immediately decarboxylated by PnPy decarboxylase to give phosphonoacetaldehyde (**4.3,** PAA).[28] This trimeric enzyme is thiamine pyrophosphate and Mg^{2+} dependent.[25,29]

All phosphonate biosynthetic pathways comprise reactions similar to the ones used in primary metabolism. For example some steps of bialaphos biosynthesis are quite similar to glycolysis and some steps of FR900098 and bialaphos biosynthesis resemble the tricarboxylic acid cycle (TCA-cycle). Similarities between P-C compounds and their carboxylic analogues partly allow their conversion via the "normal" enzymes, however, enzymes specialized on phosphonates have evolved.[4] All phosphonates are derived from phosphonoacetaldehyde.

Of course, there are exceptions to the rule. In the biosynthesis of the fosmidomycin analogue FR900098, a condensation reaction between PnPy and acetyl-SCoA accounts for the necessary exergonic second reaction step via acetyl-SCoA hydrolysis instead of a decarboxylation reaction.[4]

4.1 Biosynthesis of 2-AEP

The biosynthesis of 2-AEP only requires three enzymes which have already been characterized from different species. There is no known organism, from which all three proteins have been isolated.[29]

Early *in vivo* P-labeling experiments led to the conclusion, that 2-AEP is derived from phosphoenolpyruvate (PEP). However, this could only be confirmed by the isolation of the respective enzyme in 1988 and following *in vitro* studies.[30,31]

The first enzyme in the biosynthetic pathway leading to 2-AEP was shown to be phosphoenolpyruvate mutase (PEP mutase or PEP phosphomutase). It catalyses the transformation of PEP to phosphonopyruvic acid via an intramolecular phosphoryl transfer. The equilibrium of this reaction is clearly on the side of PEP. As retention of stereochemistry at the phosphorus atom was observed, a mechanism involving a phosphoenzyme intermediate was proposed. This intermediate would be formed with inversion of

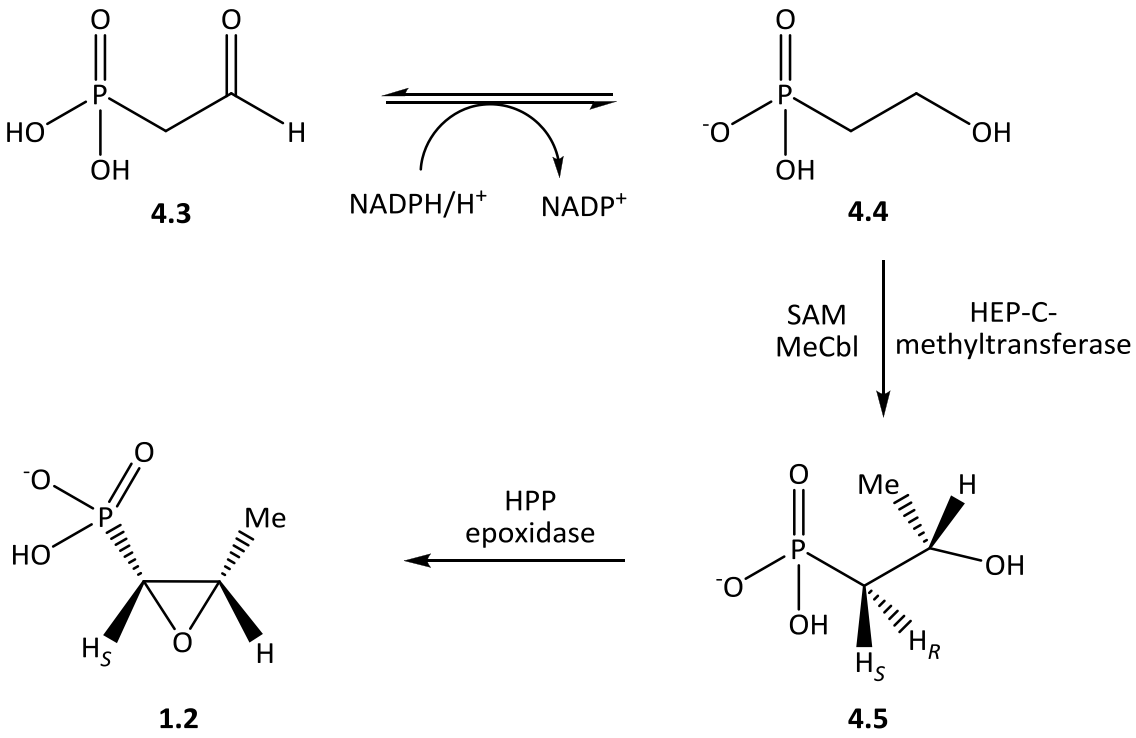
configuration and the following attack of enolpyruvate would again give an inversion leading to a net retention of configuration for the overall reaction. This mechanism could not yet be confirmed and others were proposed. For example, a dissociative process generating metaphosphate, which is kept in place by active site residues of the enzyme, as an intermediate, which could account for the same stereochemical outcome.[32]

Phosphonopyruvic acid has to be used immediately after its formation as already mentioned in the general chapter on phosphonate biosynthesis. Therefore PAA is formed via decarboxylation.

The last required enzyme is an aminotransferase, which up to now has not been characterized from 2-AEP producing organisms, but only from 2-AEP degrading ones. It is called AEP-transaminase and is a member of the family of PLP-dependant enzymes.[4]

4.2 Biosynthesis of fosfomycin

The first two steps of the biosynthesis of fosfomycin and 2-AEP are the same. The assumption that all phosphonates are derived from PEP is equally true for fosfomycin. The methyl group of fosfomycin was originally thought to be directly transferred to PAA from methylcobalamine (MeCbl).[33] This mechanism would have involved a formal methyl anion, which is in contrast to the generally presumed transfer of the methyl group of MeCbl as a methyl cation. More recent studies, which are consistent with all former experiments, suggest that PAA (**4.3**) is reduced to hydroxyethylphosphonic acid (**4.4**, HEP) prior to methylation (Scheme 4.2).[34]



Scheme 4.2. Fosfomycin biosynthesis.

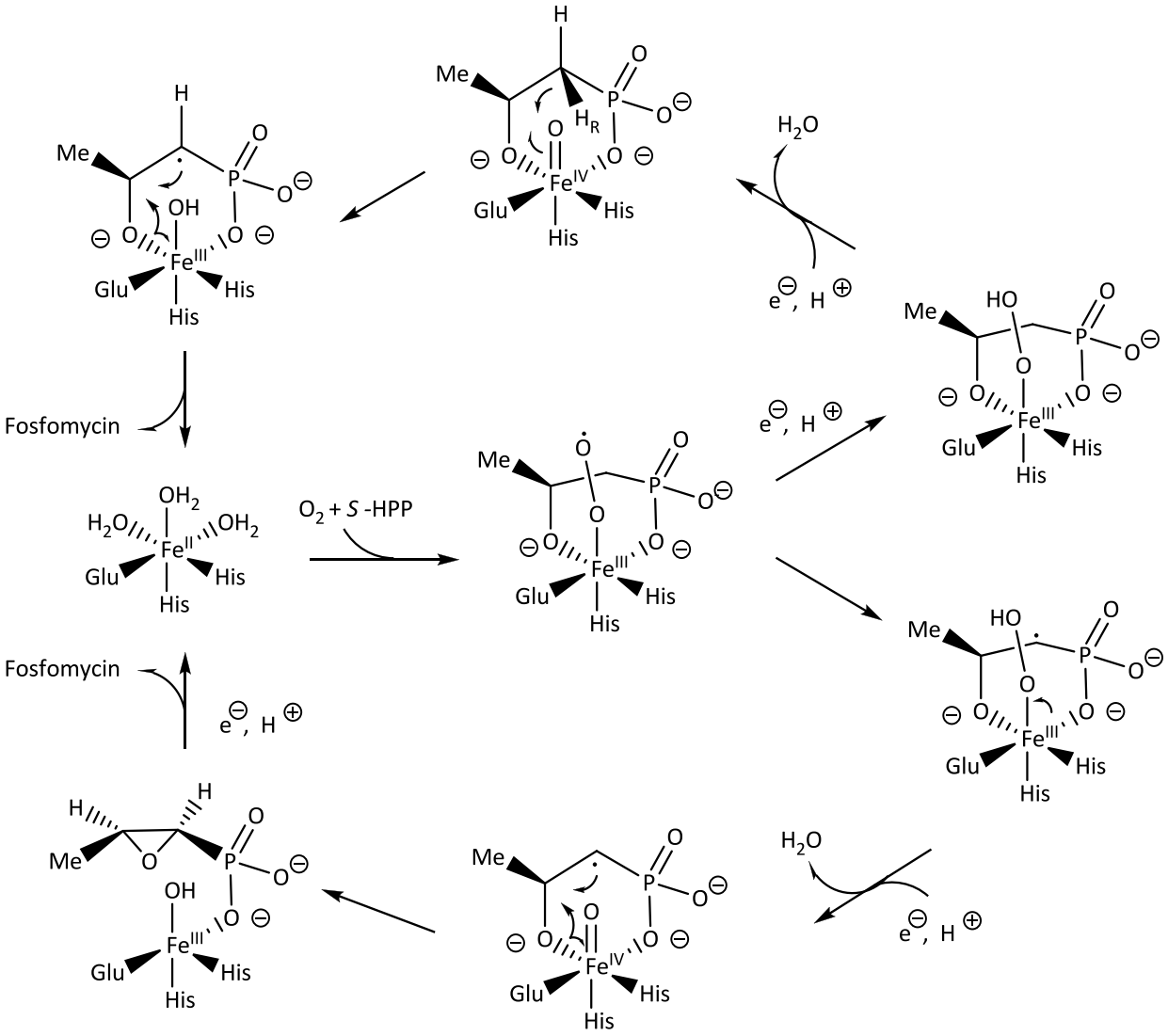
The enzyme performing the reduction (PAA reductase) of PAA was identified as an Fe^{III}-dependent alcohol dehydrogenase needing NADPH as a cosubstrate.

The methyl transfer to HEP is proposed to involve *S*-adenosylmethionine (SAM) and a reduced [4Fe-4S]-cluster. These two components may be involved in the formation of an adenosyl radical (by one electron transfer from the iron-sulfur cluster to SAM) able to abstract the *pro*-(*R)*-hydrogen atom from C-2 of HEP. This is in agreement with the mechanism of other MeCbl dependent enzymes. The generated HEP radical can easily be methylated by MeCbl with transfer of a methyl radical to give 2-hydroxypropylphosphonic acid (**4.5**).[4,35]

The last step of fosfomycin biosynthesis is the formation of the epoxide ring. This reaction is catalysed by a very unusual mononuclear nonheme iron (II) monoxygenase[36] which requires $NADH/H^+$, [37] molecular O_2 and a reductase (the biological one has not yet been identified). Unlike most enzymes of this type, there is no α-ketoglutarate dependence. It is called hydroxypropylphosphonic acid (HPP) epoxidase and is a homotetramer with one iron atom per unit.[38] ^{18}O-Labeling studies revealed that the oxirane oxygen atom is not derived from atmospheric dioxygen.[39] However, ^{18}O-labeling of HPP (**4.5**) yielded labeled fosfomycin as a

product. This led to the conclusion that the epoxide ring in fosfomycin is formed in a unique way via dehydrogenation of the secondary alcohol HPP.[4]

The two mechanisms proposed for this reaction differ in the exact moment for the electron transfer reaction, but both include radical substrate intermediates (Scheme 4.3). The configuration at C-1 is inverted during formation of the C-O bond.



Scheme 4.3. Suggested radical mechanisms for HPP epoxidase, the last enzyme in fosfomycin biosynthesis.[40]

The substrate binding in the active site of the enzyme occurs via both the hydroxyl and the PO_3^{2-} groups of HPP. This bidentate coordination induces a conformational change of the

enzyme leading to a kind of "closing" of the active site. This was suggested to protect reactive species which are formed as intermediates during the reaction.[4]

4.3 Biosynthesis of phosphaisoserine

Phosphaisoserine is produced by *Acanthamoeba castellanii* via hydroxylation of 2-AEP. Insertion of oxygen into a C-H bond at C-1 by a monoxygenase could be demonstrated with labeling experiments. Whether this insertion takes place before or after integration of AEP into the lipophosphonoglycan remains elusive. [41] This was the first example of a hydroxylase with a P-C compound as its natural substrate. The hydrogen atom abstracted during the course of this reaction could be identified as the *pro*-(*S*)-hydrogen atom by D-labeling. As 1-hydroxy-AEP has (*R*)-configuration[17], the insertion takes place with retention of configuration.[42] The unknown hydroxylase was suggested to be a cytochrome P_{450} monooxygense. Enzymes of this type are known to perform insertions of oxygen atoms into C-H bonds with retention of configuration and a weak primary kinetic isotopic effect (as can be found for phosphaisoserine).[17,41]

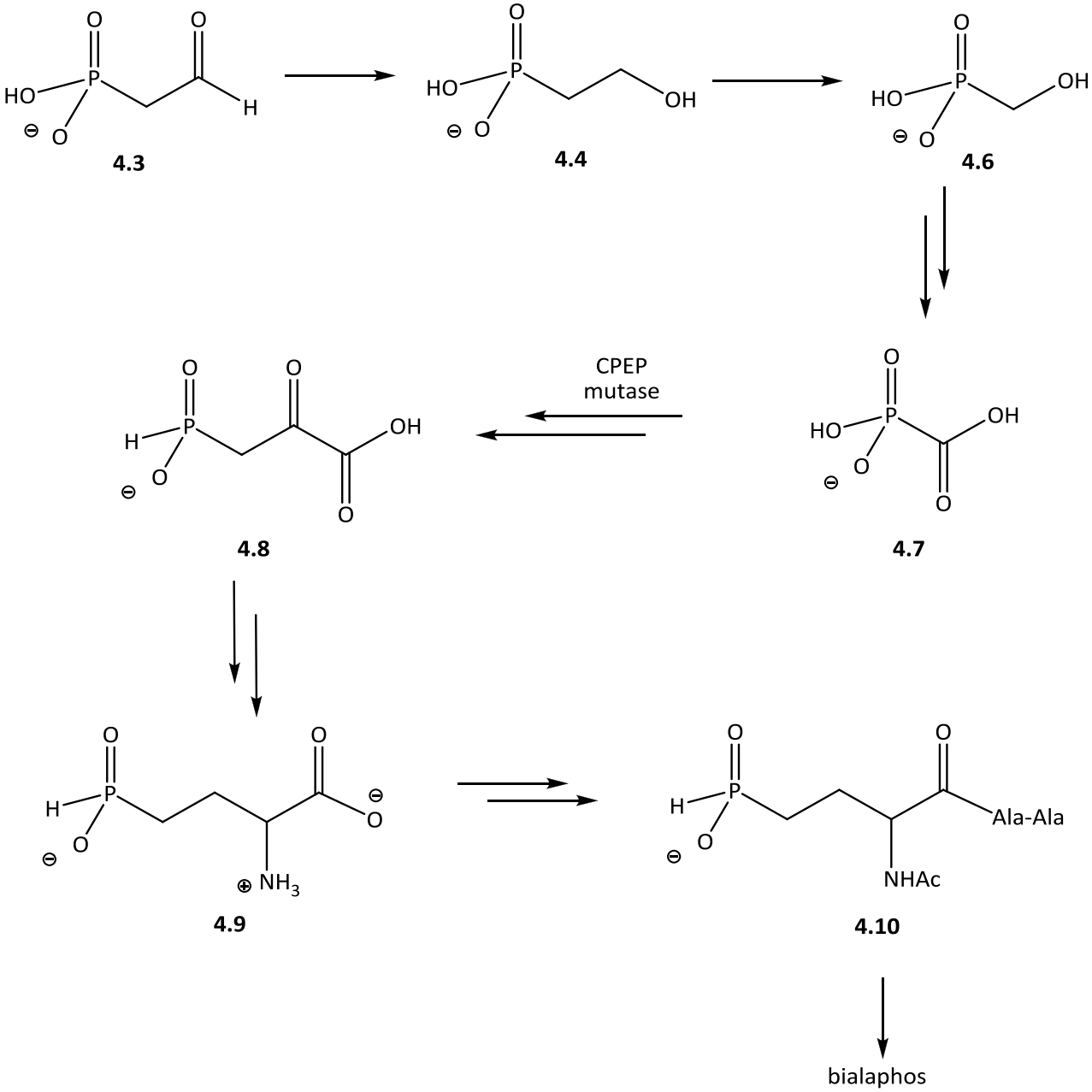
4.4 Biosynthesis of phosphinothricin

The research concerning the biosynthesis of this compound mainly focused on the formation of the P-C bonds. Some interesting steps will be discussed in this section. The whole biosynthetic pathway summarised in Scheme 4.4 can be found in the literature.[4,16]

As already discussed in the general section about the biosynthesis of phosphonates, the first two steps of phosphinothricin formation involve the rearrangement of PEP to PnPy (**4.2**) followed by decarboxylation to give phosphonoacetaldehyde (**4.3**) (Scheme 4.4). These common reactions are followed by the reduction of PAA to 2-hydroxyethylphosphonic acid (**4.4**). HEP is afterwards converted to hydroxymethylphosphonate (**4.6**, HMP) by an enzyme called HEP dioxygenase (HEPD), a mononuclear non-heme iron (II) enzyme.[43] Since no reductact is needed for this reaction, it is safe to assume that all four electrons needed for the reduction of one O_2-molecule stem from HEP. Experiments showed that carbon atom C-2 is eliminated as formate.

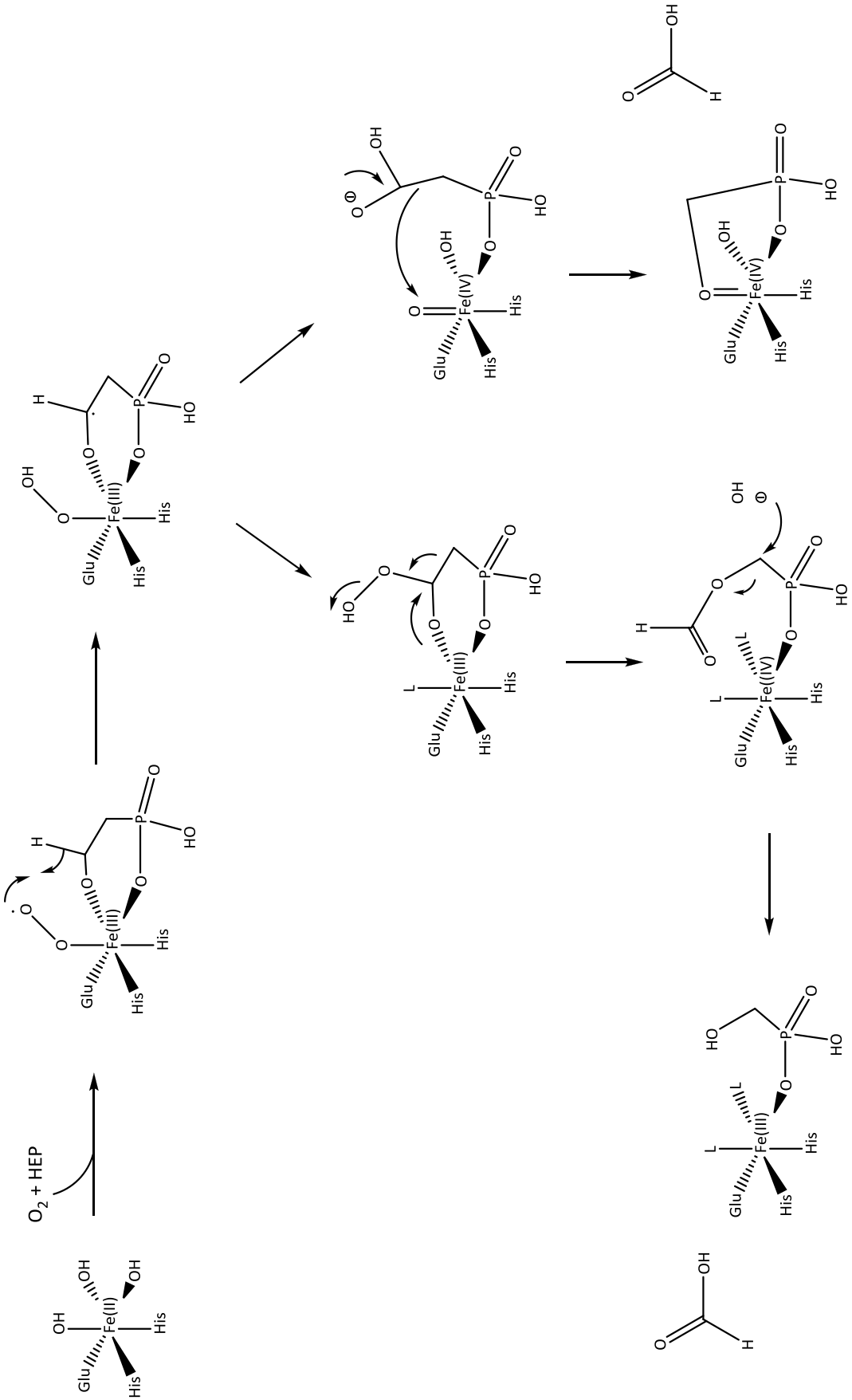
Two mechanisms have been proposed for this conversion (Scheme 4.5). A P-C bond is cleaved here instead of the C-C bond in PEP rearrangement.[4]

HMP is afterwards oxidized in two steps to the corresponding carboxylic acid phosphonoformate (**4.7**) by a $NAD(P)^+$ dependent alcohol dehydrogenase. However, the respective enzyme has not been identified yet.



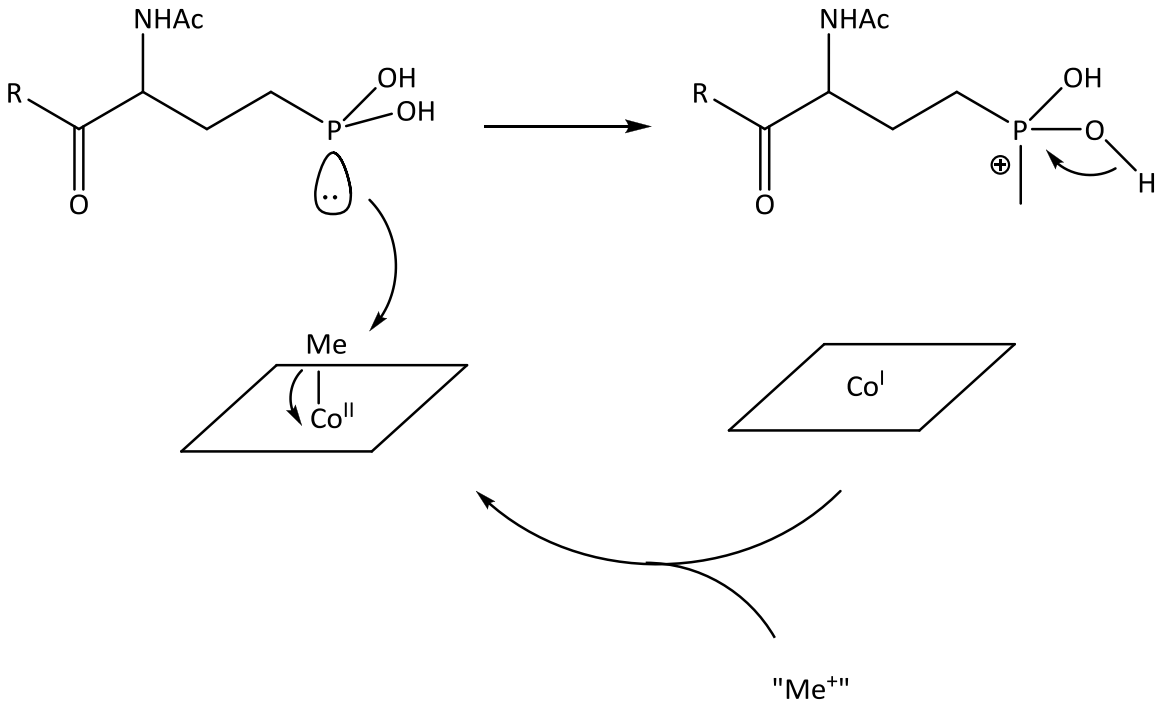
Scheme 4.4. Overview of phosphinothricin biosynthesis.

This is followed by a transformation to carboxyphosphoenolpyruvate which is subsequently converted to phosphinopyruvate (**4.8**) in a reaction catalysed by CPEP mutase. This rearrangment follows a similar mechanism as the conversion of PEP to PnPy catalysed by PEP mutase. CPEP mutase catalyses the deacarboxylation and yields phosphinopyruvate.



Scheme 4.5. Suggested mechanisms for the elimination of a CH_2 group to form HMP as a substrate.[4]

In the next steps of bialaphos biosynthesis, 4-(hydroxyhydrophosphoryl)-2-oxobutanoic acid, which is subsequently transaminated to demethylphosphinothricin, is generated (**4.9**, DMPT). Acetylation in this case is a self-protection mechanism of phosphinothricin producing organisms against its toxic metabolic product. The methyl group is transferred from MeCbl to the *N*-acetylated form of the tripeptide. This suggestion was underpinned by the fact that microorganisms grown without cobalt as trace element accumulated DMPT (**4.9**) as well as the corresponding tripeptide. Labeling studies with $^{14}CH_3$-cobalamine led to the same result.[16]

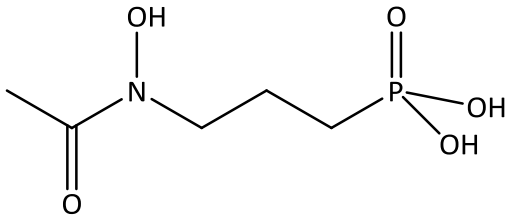


Scheme 4.6. Proposed mechanism for methyl transfer from MeCbl to DMPT.[4]

Two mechanisms have been proposed for the methyl transfer. The first one involves a formal CH_3^+ transfer Co^I in cobalamine (Scheme 4.6). This species could afterwards be re-methylated by a methyl donor. It is the simplest possible mechanism for this methyl tranfer.[4] The second mechanism involves radicals and needs an Fe-S cluster as well as S-adenosylmethionine (SAM) as cofactors.[16]

4.5 Biosynthesis of FR900098

FR900098 (**4.11**) belongs to the same group of 3-(*N*-hydroxyamino)propylphosphonic acids as fosmidomycin (**1.4**) (Figure 4.1).

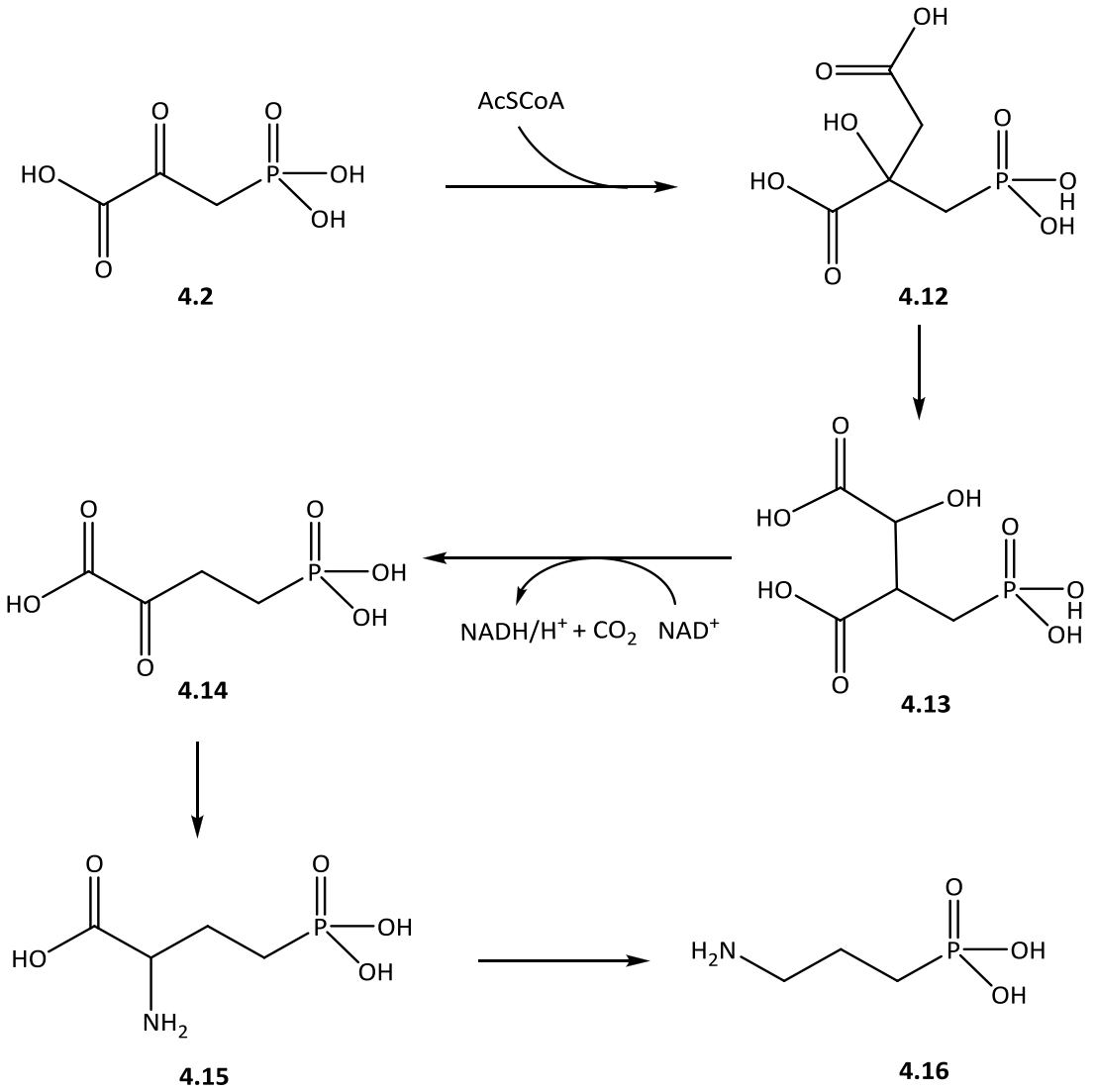


4.11

Figure 4.1

It is produced by *Streptomyces lavendulae* and exhibits antimalarial activity. As the biosynthesis of fosmidomycin still has to be elucidated, the biosynthetic pathway suggested for FR900098, which is currently investigated, is presented here instead.[26] It is assumed that the biosynthetic pathways leading to these two compounds will show similarities.

The first step of FR900098 biosynthesis, according to the above stated general mechanism, is the rearrangement of PEP to phosphonopyruvate (**4.2**). However, the following step is not catalyzed by a decarboxylase as in the case of other natural phosphonates. Instead, genes encoding enzymes known from the tricarboxylic acid (TCA) cycle were found in the FR900098 gene cluster. A homolog of homocitrate synthase accounts for the needed exergonic step by catalysing the condensation of PnPy with acetyl-SCoA to form 2-phosphonomethylmalate (**4.12**) (Scheme 4.7).



Scheme 4.7. Proposed biosynthesis of FR900098.

This transformation could be proved *in vivo*.[26] Dicarboxylate **4.12** is then converted to the isomer **4.13**. Subsequently, this intermediate is assumed to be oxidized and decarboxylated to give 2-oxo-4-phosphonobutyrate (**4.14**) in reactions analogous to the ones of the TCA cycle. Transamination by a yet unknown enzyme gives α-amino acid **4.15** which is decarboxylated to γ-aminophosphonic acid **4.16**. This amine is acetylated and hydroxylated *in vitro* to give FR900098 (**4.11**), though the exact mechanisms of these steps remain to be unravelled.[4]

5. Biodegradation of phosphonates - general remarks

5.1 P-C bond cleavage - phosphonates as phosphorus source

Up to now, the biosynthetic pathways leading to the formation of P-C compounds have not been studied in detail except for some substances of industrial or medicinal importance. However, some general conclusions on the basis of comparative studies can already be drawn and similarities between the biodegradation of certain compounds have already been determined.

In contrast to that, very little is known about the mineralisation of phosphonic acid derivates. This knowledge is nevertheless important for our comprehension of the natural P-cycle and the fate of millions of tons of phosphonates released each year into the environment by agriculture and industry. A detailed knowledge about the biological pathways by which phosphonates are consumed and degraded in nature will provide us with new insights into resistence phenomena against herbicides, antibiotics and other agents.

The first proof for the use of phosphonates as phosphorus source of living organisms was the growth of *E. coli* on alkylphosphonic acids such as methyl- or ethylphosphonic acid which was reported in 1963.[44]

Although P_i is the preferred phosphorus source of living organisms, bacteria have developed pathways to metabolise phosphonates and hence use them as an additional or alternative phosphorus source.

So far, three mechanisms for phosphonate metabolism are known. There are two pathways for bacteria using phosphonates as a phosphorus source only. These two metabolic routes are called “phosphonatase” and “P-C lyase” pathway.[45] They are both regulated via the so-called *pho*-regulon and are only relevant in case of P_i starvation. The third biodegradative way allows the use of phosphonates as P, C and N sources and is independent of the phosphate concentration. Interestingly, this pathway is not regulated via the *pho*-regulon. Numerous earlier studies have suggested that phosphonate breakdown is under the strict control of the *pho*-regulon and P-C compounds can therefore only be used as a phosphorus source.[46] Later studies revealed that there are metabolic pathways for phosphonate breakdown found in some bacterial strains such as *Pseudomonas fluorescens* 23F [47], *Rhizobium huakuii* PMY1[48] or *Burkholderia cepacia* [49] that are independent of the phosphate level.

5.2 The pho *regulon*

There is a certain number of genes that are expressed in bacteria in the case of phosphate starvation. These genes are also expressed in an environment where phosphate is present, but their expression is upregulated drastically (up to 1500-fold), if the environmental phosphate level is low. The total of these genes, also called *psi* genes (phosphate starvation inducible genes), comprise the so called *pho*-regulon.

The expression of this gene set is feedback regulated by the level of available P_i. The regulation is controlled by a signalling cascade. First, the environmental phosphorus is taken up by a phosphorus transport system. If P_i is the phosphorus source, it is taken up either by the high or the low affinity phosphate transporter. Then phosphate is incorporated into ATP inside the cell. If the extracellular phosphate concentration falls below 4 µmol/L, a protein, called *PhoR* (located on the cell surface membrane), autophosphorylates using ATP as a phosphate source. *PhoR* is called the P_i sensor of this signal transduction pathway. It also accounts for P_i repression if there is excess phosphate.

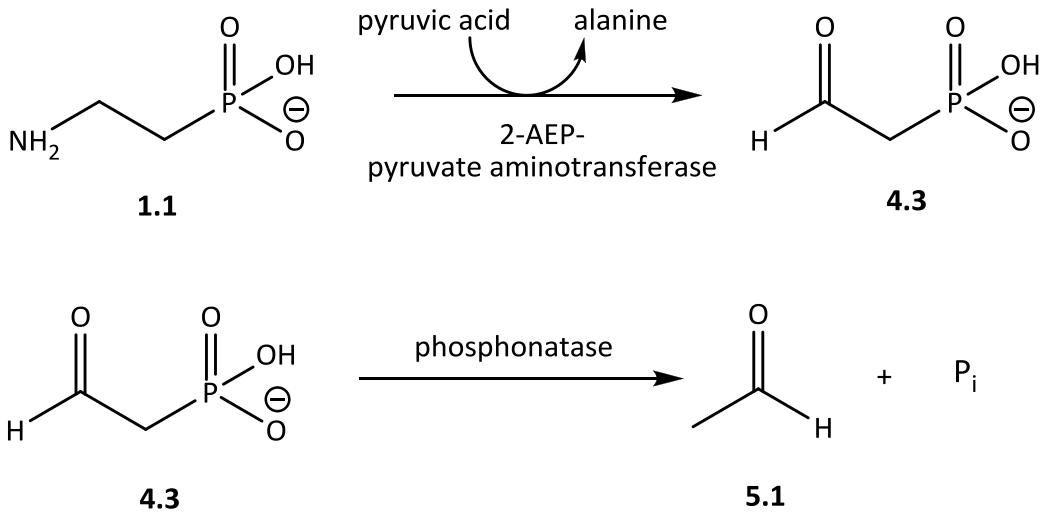
Activated *PhoR* has kinase activity and subsequently phosphorylates the regulatory protein PhoB which, when phosphorylated, activates the transcription of the *psi* genes. Phosphorylated *PhoB* binds to the promoter region, the so called *pho* box, of the *pho* regulon and therewith starts the transcription. Such a signalling cascade has been observed in several microorganisms such as *E.coli*, *P. aeruginosa*, *B. subtilis* and *Klebsiella pneumoniae.* Similar pathways could be observed in *Rhizobium meliloti*, *Mycobacterium leprae* and *Agrobacterium tumefaciens.*[2,7]

If there is excess phosphate available, whether originating from an external source or produced by P-C bond cleavage, the *psi* genes are downregulated and hence phosphonates can only be used as a phosphorus source in situations of phosphate starvation. Therefore, microorganisms metabolising phosphonates under the control of the *pho* regulon cannot use it as a carbon source because the production of phosphate due to P-C bond cleavage inhibits further breakdown of phosphonate.[25,7]

5.3 Three pathways

5.3.1 Phosphonatase pathway

The phosphonatase-pathway has very narrow substrate specificity - it acts only on AEP. The first step of this pathway involves a transamination reaction of 2-AEP using pyruvic acid as an acceptor of the amino group to give phosphonoacetaldehyde (**4.3**) (Scheme 5.1).[25] Therefore, 2-AEP can be used as a nitrogen source in this pathway. This first step is followed by the formation of an imine between a lysine residue in the active site of the enzyme and the generated aldehyde. This destabilizes the P-C bond by an electron withdrawing effect and thus allows for its formal hydrolysis. It was shown that this fission takes place with net retention of configuration at the P atom and yields P_i and acetaldehyde (**5.1**).[50]
The enzyme catalysing this step is called phosphonoacetaldehyde phosphonohydrolase or phosphonatase in short.



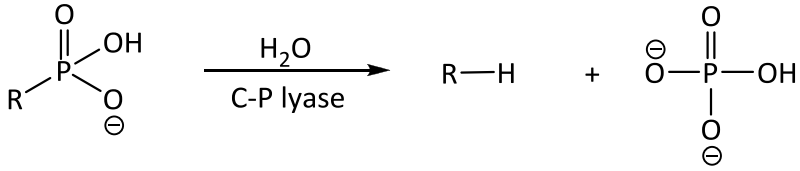
1.1 **4.3** **4.3** **5.1**

Scheme 5.1. Phosphonate breakdown via the phosphonatase pathway.[2]

Microorganisms use phosphonates as a sole phosphorus source via this *pho*-regulon controlled pathway and not as a carbon or energy source. The bacterial strain *Pseudomonas putida* NG2 is the only known exception where 2-AEP breakdown via the phosphonatase pathway is not regulated via the *pho* regulon, but is directly dependent on the presence of the substrate. The corresponding enzymes have been isolated for example from *Bacillus cerus, Enterobacter aerogenes, Salmonella typhimurium* and *Pseudomonas aeruginosa.*[7]

5.3.2 P-C lyase pathway

The characteristics of this pathway are a direct dephosphonation (via a redox process) and very broad substrate specificity. It acts on substituted as well as on unsubstituted phosphonates and is the most widespread pathway for phosphonate degradation in bacteria.[2] P-C bond cleavage via this mechanism leads to racemisation at the carbon atom. As P-C lyase activity could not yet be detected *in vitro*, the mechanism on the molecular level is still unknown. It was suggested that direct P-C bond fission by a so-called P-C lyase will give the corresponding hydrocarbon and phosphite, which is subsequently oxidised to give orthophosphate (Scheme 5.2). This assumption is underpinned by the fact that mutants lacking the ability to metabolise phosphonates equally lose the ability to assimilate phosphite.[51] Furthermore, this lack of *in vitro* P-C lyase activity was assigned to the fact that at least one component of the responsible enzyme complex is membrane bound, which is common for redox enzymes.



Scheme 5.2. Phosphonate breakdown via the P-C lyase pathway.[2]

In vivo studies revealed that the hydrocarbon fragment generated by this cleavage mechanism to be the hydrocarbon directly derived from the phosphonate. The formed phosphorus species has not yet been identified as the release of P_i in cell-free systems could not yet be detected. This can have two reasons: either the produced phosphate is consumed directly and converted into another phosphorus species, or the primary phosphorus species is not P_i.[7]

Genes for this pathway have been found in *E.coli*[52], *Enterobacter aerogenes*[45] and *Rhizobium meliloti*[53] to name a few. So far, no substrates of biogenic origin are known for this pathway with the exception of 2-AEP (**1.1**), which is also a substrate of the phosphonatase-pathway.[7] It was suggested that the function of this pathway may originally be the generation of ribose-phosphonate esters, which are products of the degradation cycle of phosphonates.[3] This route is under the control of the *pho* regulon too.

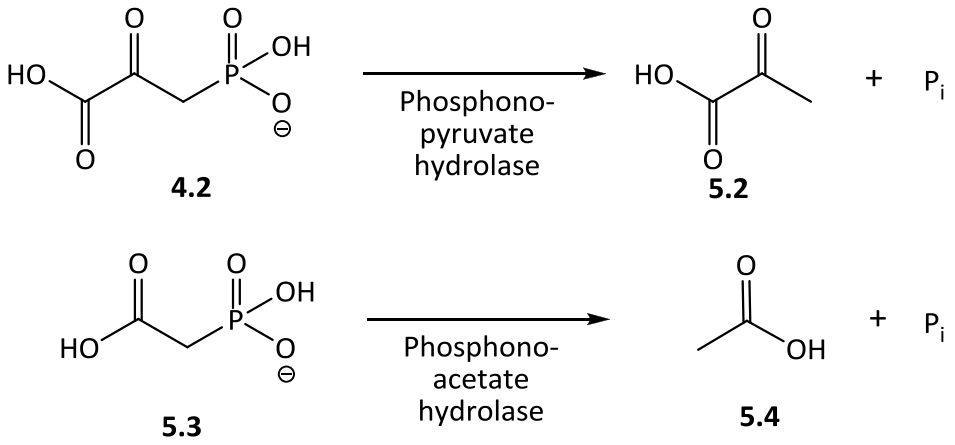
Originally only one type of P-C lyases was postulated, but the suggestion that a variety of enzymes of this type exists could be confirmed by *in silico* analysis. It was revealed by the fact that there are significant differences between the gene clusters of P-C lyases in different prokaryotes.[54]

5.3.3 Phosphonopyruvate - and phosphonoacetate hydrolase pathways

If P-C cleavage of xenobiotic phosphonates (phosphonates of synthetic origin) only occured by P-C lyases, manmade phosphonates would pose an enormous problem. Their biodegradation would be blocked by the suppression of their mineralisation by ubiquitous P_i.[3]

The third pathway for phosphonate degradation is different from the two earlier discussed routes regarding two facts: it is independent of the phosphate status of the cell and it is substrate-inducible. Therefore, the discovery of this pathway broke with the general rule of phosphonate breakdown being dependent on phosphate starvation. Hence, microorganisms can use phosphonates as carbon and phosphorus source via this pathway. In the case of *Pseudomonas fluorescens* 23F and *Burkholderia cepacia* Pa16 it could be shown that substrate uptake is not suppressed by the release of inorganic phosphate due to P-C bond hydrolysis. Also, the use of fosfomycin as a phosphorus and carbon source by *Rhizobium huakuii* PMY1 was observed.[46] These strains do not show feedback inhibition[25] and can use organophosphonates as carbon and energy, rather than phosphorus source. However, both fission products, the carbon and the phosphorus containing one, *can* be used by the organism as a nutrient.[55]

There are two enzymes known catalysing *pho*-regulon idependent pathways. The first, phosphonopyruvate hydrolase, cleaves phosphonopyruvic acid (**4.2**) to give P_i and pyruvic acid (**5.2**) in the breakdown of phosphonoalanine, a component of cell membranes in many, especially marine, organisms. *Burkholderia* sp. can use the L-enantiomer of phosphoalanine as a C and P source (Scheme 5.3).



Scheme 5.3. Phosphonate breakdown via *pho*-regulon independent pathways.[2]

The second, phosphonoacetate hydrolase from *Pseudomonas fluorescens* 23F degrades phosphonoacetate (**5.3**) to give P_i and acetate (**5.4**). [2]

6. Details on the biodegradation of some P-C compounds

6.1 Biodegradation of 2-AEP

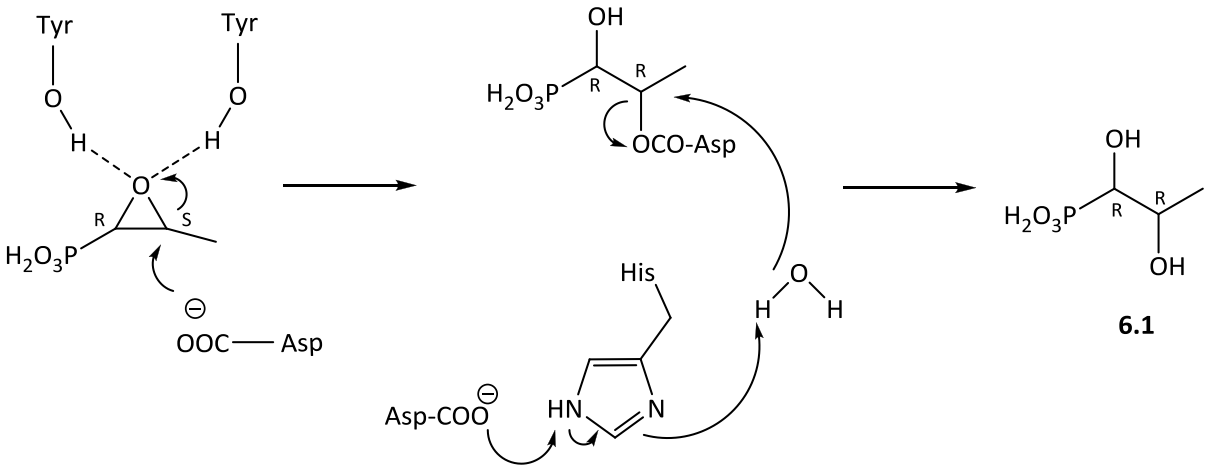
The first stept in 2-AEP biodegradation is transamination of the latter to give PAA. The latter is then cleaved to give inorganic phosphate and acetaldehyde. These two steps are catalysed by two enzymes, called 2-AEP:pyruvate aminotransferase and phosphonoacetaldehyde hydrolase (phosphonatase), respectively. The second enzyme has been studied from several bacterial strains including *Bacillus*, *Pseudomonas*, *Enterobacter* and S*almonella* strains. This degradative pathway is under control of the *pho*-regulon. However, a *pho* independent degradation has been discovered in *Pseudomonas putida.*[46] Mechanistically, an active site lysine forms a Schiff's base intermediate with PAA that leads to a destabilisation of the P-C bond and subsequent bond cleavage.

6.2 Biodegradation of fosfomycin

The bacterium *Rhizobium huakuii* PMY1 is able to use fosfomycin as a phosphorus source independent of the phosphate status of the cell and not under control of the *pho*-regulon. Other members of the *Rhizobiaceae* family have been shown to be capable of metabolising synthetic phosphonates like glyphosate. However, the mineralisation was under the strict control of the *pho*-regulon and therefore restricted to the use of organophosphonates as a phosphorus source. Use of fosfomycin as carbon, energy and phosphorus source by these microorganisms has first been reported in 1998.[56] Cleavage of its P-C bond is possibly responsible for the resistance to fosfomycin as an antibiotic. Consequently, elucidation of the biodegradative pathway of fosfomycin is interesting. As *Rhizobiaceae* can be found everywhere in the biosphere, they may contribute to the rapid detoxification of glyphosate and other herbicides of the organophosphonate type. It has not been possible yet to demonstrate *in vitro* P-C bond cleavage activity by cell-extracts from these microorganisms.[56] This failure is attributed to the fact that the responsible enzyme system is membrane bound.

To elucidate the stepwise biodegradation of fosfomycin, putative intermediates of the enzymatic metabolism were synthesized. It was planned to investigate whether they support growth of *R. huakuii* PMY1 or released P_i in cell-extracts in the case of phosphonates.

Previous experiments revealed the first step of fosfomycin breakdown not to be the P-C bond cleavage, but a modification of the carbon skeleton. Direct P-C bond cleavage would result in inorganic phosphate and *(S)*-methyloxirane which was not considered a suitable compound for testing as it is very volatile (bp 35 °C). However, the oxirane could be hydrolysed by an enzyme in the cells and hence 1,2-propanediol was tested as a metabolite. Neither the racemate nor the (*R*)-enantiomer supported growth of *R. huakuii* PMY1. However, 1,2-dihydroxypropylphosphonic acid (**6.1**) which would result from the epoxide ring opening before P-C bond cleavage supported growth (Scheme 6.1).



Scheme 6.1. Proposed mechanism for epoxide hydrolase acting on fosfomycin as substrate.

As only the *(*1*R,*2*R*)-stereoisomer and none of the three others was metabolised by *R. huakuii* PMY1, the first step of fosfomycin biodegradation was suggested to be epoxide hydrolysis.

Only formal attack of a water molecule at C-2 would invert the configuration and would give the (1*R,*2*R*)-1,2-dihydroxypropylphosphonic acid since fosfomycin has (*1R,2S*)-configuration. So far, the crystal structures of three epoxide hydrolases from three different organisms have been obtained. As their active sites are quite similar, they are believed to act in the same way on their substrates. Two tyrosine residues activate the epoxide by forming hydrogen bonds to the oxygen atom reminiscent of acidic hydrolysis. The attacking nucleophile is the CO_2^- group of an active site aspartate, forming an enzyme bound ester with the substrate. This intermediate is hydrolysed by attack of a water molecule to give the (1*R,*2*R*)-diol. The water molecule itself is activated by a catalytic diade, involving aspartate and histidine.[57] Attack of OH^- at the carbonyl group of the ester releases the diol.

However, nucleophilic attack at C-1 would result in the *(1S,2S)*-enantiomer. Interestingly, FosX proteins from *M. loti* and *Listeria* catalyze the metal ion assisted ring opening of fosfomycin by water at C-1, giving the (1*S*,2*S*)-diol, as a genomic encoded mechanism of resistance.[58]

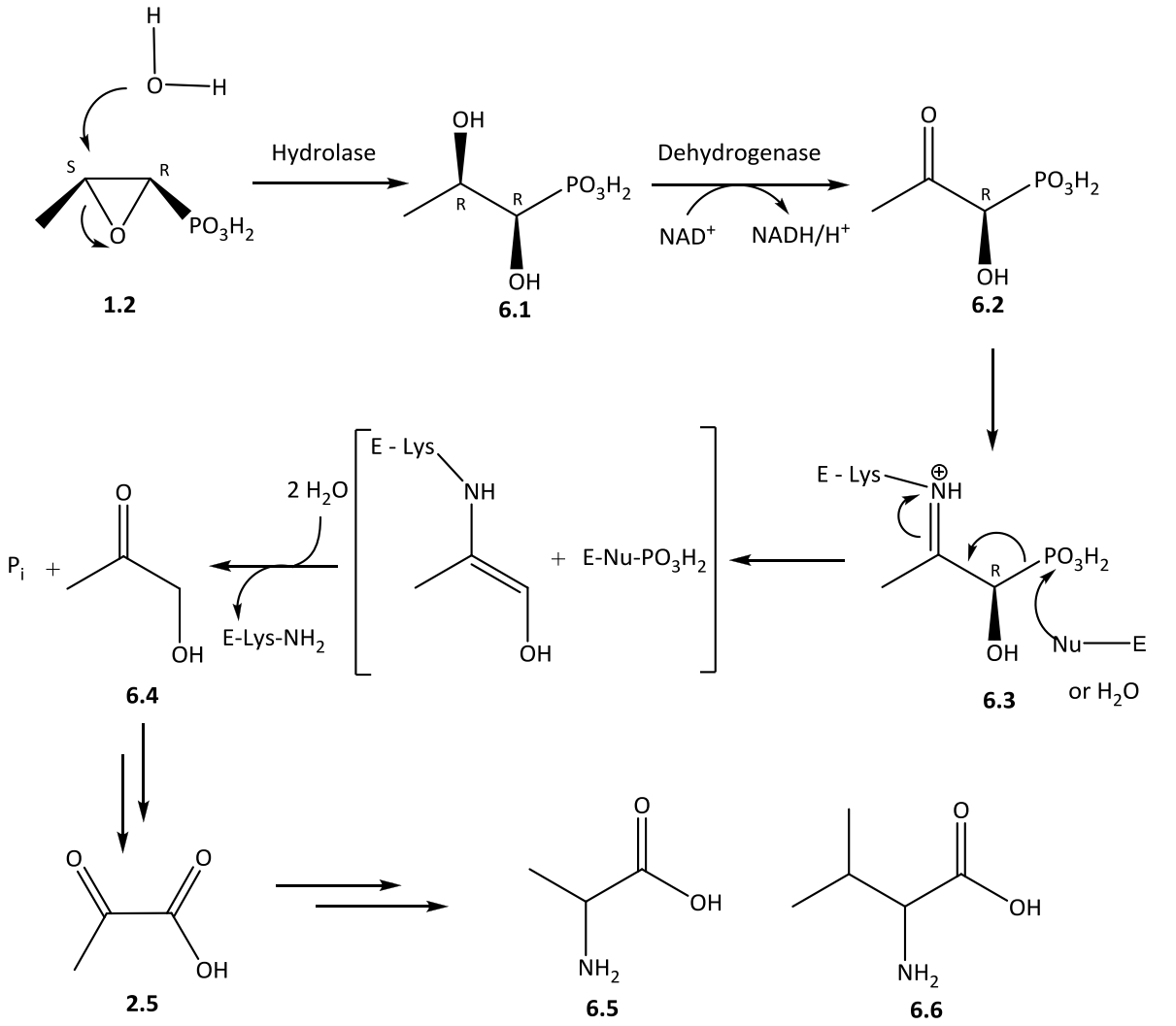
These results also lead to the conclusion that the second step of the pathway cannot be P-C bond cleavage either, because this would again produce 1,2-propanediol which is not metabolised. So, another side chain modification has to follow. Suggestions involved dehydration at either C-1 or C-2 to give the corresponding oxo-propylphosphonic acids or dehydrogenation on either position to give hydroxy-oxopropylphosphonic acids. However, α-hydroxyalkyl- and α-oxoalkylphosphonates show decreased stability of the P-C bonds towards basic hydrolysis. The former give the corresponding aldehydes and phosphite whereas the latter give carboxylic acids and again phosphite. *R. huakuii* PMY1 cannot use phosphite as a phosphorus source. Neither direct use nor phosphite oxidase activity could be detected. Therefore, these two possibilities had to be discarded too.[46]

R. huakuii grows well on hydroxyacetone (**6.4**) and lactic acid, but not on acetone. Therefore, oxidation of the hydroxyl group at C-2 to give 1-hydroxy-2-oxopropylphosphonic acid (**6.2**) was believed to be the next step in fosfomycin metabolism (Scheme 6.2). This putative intermediate was synthesized as a racemate. Unfortunately, it was not metabolised by *R. huakuii* PMY1 but released P_i in a cell-extract. To account for the fact that hydroxyacetone is readily degraded by *R. huakuii* PMY1, two possibilities were considered. Either the 2-oxopropylphosphonic acid did not enter the cells when supplied in the growth medium, or the (*S*)-enantiomer inhibited the degradation of the (*R*)-enantiomer, which is more likely to be the natural product.[46]

Furthermore, it was assumed that hydroxyacetone generated from 1-hydroxy-2-oxopropylphosphonic acid via P-C bond cleavage is oxidized to pyruvic acid. This acid may then be incorporated into amino acids. To test this assumption [C-1-^{13}C]-labeled (1*R**,2*R**)-1,2-dihydroxypropylphosphonic acid was synthesized. Feeding experiments showed a high incorporation rate for this compound into the amino acids alanine (**6.5**) and valine (**6.6**), isolated from the cell hydrolysate unsing HPLC and investigated by NMR spectroscopy. This result was taken as evidence that the P-C bond of 1-hydroxy-2-oxo-propylphosphonic acid (**6.2**) is split.

It could also be shown that the C_3-fragment stays intact as long as it is attached to phosphorus. Hence the carbon fragment released has to contain three carbon atoms. However, their substitution pattern is still unknown.

The cleavage of the P-C bond itself could show similarity to the biodegradation of 2-AEP. There, PAA is formed via transamination and subsequently the P-C bond is cleaved. The



Scheme 6.2. Proposed pathway of fosfomycin biodegradation.

catalysing enzyme for the second step is a phosphonatase with a side chain lysine residue in its active site which is essential for the catalytic cycle. The amino group of lysine forms iminium cation **6.3** with the carbonyl group of ketone **6.2**, facilitating P-C bond cleavage by attack of an enzyme nucleophile (or less likely a water molecule) at the P atom. The protonated Schiff base has an electron sink at C-2 that accepts the electrons of the P-C bond. After bond fission the enzyme bound intermediates, the enamine and the phosphorylated

enzyme, are hydrolysed, finally producing acetaldehyde and inorganic phosphate, respectively.

Alternatively, an active site metal ion, for example Zn^2+, could catalyse the cleavage of the P-C bond. This could lead to the formation of a metal-complexed enolate, which would be an equally good leaving group and hence promote P-C bond cleavage. These two mechanisms are proposed by drawing parallels between P-C bond cleavage and C-3-C-4 bond cleavage in fructose-1,6-bisphosphate by aldolases in the glycolytic cycle. They are grouped into two types on the basis of their catalytic sites, involving either a lysine (and thus an enamine as leaving group) or a Zn^{2+} ion (and therefore an enolate as leaving group).[59,60] Furthermore, P-C bond fission mechanisms involving radicals have also to be taken into account. They could proceed via a phosphoryl radical, which could undergo fragmentation to an alkyl radical and metaphosphate. The radical could subsequently be converted to the corresponding alkane by transfer of an electron and a proton (or an equivalent thereof) and metaphosphate. The latter will give P_i by addition of water.[61]

6.3 Biodegradation of synthetic phosphonates

Glyphosate is not toxic to the environment because of rapid binding to soil and microbial degradation.[25] The most important degradation product is aminomethylphosphonic acid (AMPA) which leads to the conclusion that the first step of mineralisation of glyphosate is C-N bond cleavage via an enamine species.[25] This has been studied with bacteria isolated from waste water containing glyphosate. Hydrolysis of the C-N bond gives glyoxylate, which can be used by the malate synthase and AMPA synthase. The enzyme catalysing this first degradative step is called glyphosate oxidoreductase, a flavoprotein functioning under aerobic and anaerobic conditions.

Another biodegradative pathway for glyphosate used by most bacteria involves initial P-C bond cleavage.[25] The fission products are P_i and sarcosine (*N*-methylglycine).[62]

In general, the breakdown of synthetic phosphonates is effected by P-C lyases (see chapter 5.3.2). A variety of microorganisms can cleave P-C bonds of a broad spectrum of synthetic alkyl- and arylphosphonates. However, only little is known about the exact mechanisms of their detoxification and their fate in the global phosphorus cycle.

7. Applied strategies

7.1 Influences of fluorine on enzymatic processes

7.1.1 Properties of fluorine

The van der Waals radius of fluorine is 1.47 Å. This lies between the values for hydrogen (1.2 Å) and oxygen (1.57 Å).[63] However, replacing a hydroxyl group by a fluorine atom is often less tolerated by enzymes than replacing it by a hydrogen atom.

Oxygen and fluorine are very similar in size, but the electronic effects of both atoms are very different. Electrostatic interactions of fluorine are weaker than those exhibited by oxygen. Therefore the hydrogen bonding ability of a fluoro-substituted enzyme substrate is reduced compared to the natural substrate. As hydrogen bonds and other electrostatic interactions are of crucial importance for enzyme/substrate interactions, fluorine is often not able to mimic hydroxyl groups properly.[64]

However, replacing the smaller hydrogen atom by a fluorine atom is often tolerated well and does not have an influence on substrate/enzyme binding. Fluorine is bioisosteric to hydrogen.[65] X-Ray studies revealed that there is only little distortion in the packing of monofluoro-substituted stearic acids.[66]

Furthermore, fluorine is the most electronegative element. This is also an important criterion having an effect on its interaction with biologically relevant structures and enzymes.

7.1.2 Replacement of CH, CH_2 and CH_3 by CF, CF_2 and CF_3

Replacement of one hydrogen atom has usually only little influence on the binding of a substrate by the metabolising enzyme. Many monofluoro-substituted analogues of naturally occurring compounds have already been tested concerning their interactions with enzymes. Many of them are readily accepted by enzymes in place of their non-fluorinated analogues.

Substitution of a CH_2 by a CF_2 group has a larger influence on the molecule and is more likely to induce conformational changes (and increased conformational flexibility) in the substrate. This leads to lowered melting points and a widening of the angle in aliphatic chains from the tetrahedral angle of 109° to about 115°.

The trifluoromethyl group has only little in common with an ordinary methyl group. It resembles an isopropyl group more than a CH_3 group considering the molar volume.[67] The van der Waals hemisphere of a CF_3 group is about 2.5 times larger than that of a CH_3 group.[68] Thus, replacement of three hydrogen atoms by fluorine atoms already induces large differences in intramolecular interactions.

7.1.3 Mechanistic enzyme studies using fluorinated metabolites

Mono-, di- or trifluorinated analogues of substrates for enzymes are useful tools for the investigation of enzymatic reaction mechanisms. Such surrogates, as for example fluoroacetyl-SCoA or fluoropyruvate are readily accepted by enzymes such as malate or citrate synthase or by L-lactate dehydrogenase, respectively.[69] However, the fact that the fluorinated substrates are bound to the active site of an enzyme does not necessarily mean that they are transformed. There is nevertheless a huge difference in electronegativity between hydrogen and fluorine that often leads to an irreversible inhibition of an enzyme. Another possible outcome is that the fluoro-substituted substrate is transformed first by one enzyme, but the resulting product cannot be metabolised any further. This is the case for fluoroacetyl-SCoA in the Krebs cycle. It is readily converted to 2-fluorocitrate that inhibits the following enzyme of the Krebs cycle (aconitase). Therefore, fluoroacetic acid is highly toxic.[64]

Fluorinated substrate mimetics leading to irreversible enzyme inhibition are called “suicide substrates” for the corresponding enzymes. They are useful tools when it comes to studying enzyme mechanisms.

One possibility to use fluorinated substrate mimetics for these purposes is to observe whether any metabolic product accumulates in the presence of the mimetic in the medium of cell cultures. The cells can also be lysed to analyze the metabolic products which are not released into the medium. In any case, if the mimetic blocks an enzyme, the substrate for this enzyme will accumulate as it cannot be transformed any further. This allows isolation of intermediates of biosynthetic or biodegradative pathways, which are otherwise converted too rapidly to have any chance to analyze them. Isolation of metabolic intermediates is like taking snapshots of the biological pathway of interest. It makes it possible to have a closer look at biological transformations of which we otherwise only know the product and the starting material, but do not have any idea of what happens in between. This knowledge is

important as it provides us with means to interfere with biological pathways. Hence, it enables us to design active components with a high degree of specificity.

7.2 Fluorinated phosphonates

7.2.1 Applications

Phosphonates are often used as phosphate mimetics leading to an inhibition of enzyme activity. This is due to the increased stability of the P-C bond towards hydrolysis compared to the O-P bond in phosphates.[65] They are also often effective as mimetics of the tetrahedral intermediate of carboxylic acids during enzymatic transformations.[2] This principle is used for mechanistic studies concerning enzymes and also for medicinal and industrial applications.[64] A special modification of this technique involves replacement of the O-P bond by a CF_2-P bond instead of CH_2-P bond. Additional fluorine atoms compensate the electronegativity loss due to replacing oxygen by a carbon atom. And indeed, CF_2-phosphonates are very good phosphate mimetics in most cases.[64]

7.2.2 Synthetic strategies to generate fluoro-substituted phosphonates

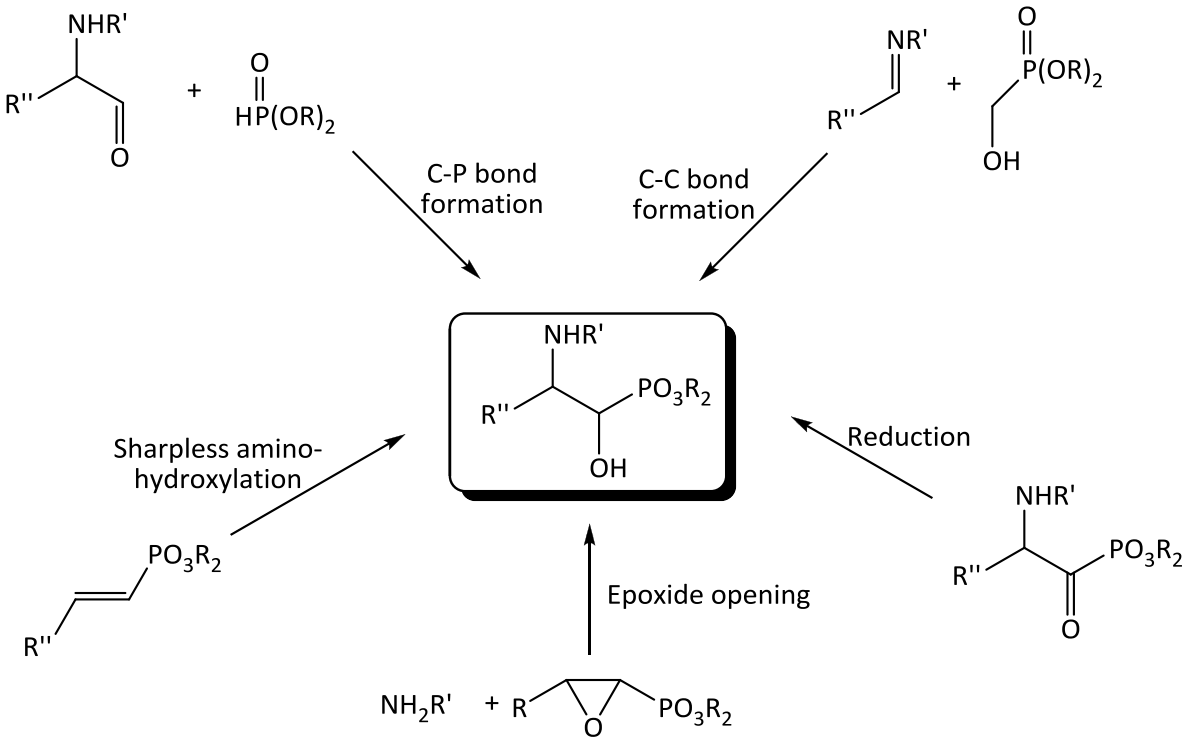
The field of fluorophosphonate chemistry is still very young. The most common synthetic approaches towards fluorinated phosphonates can be divided into six groups:

- direct synthesis from fluorinated-haloalkanes with phosphorous 3^+ species via Arbuzov-type and Michaelis-Becker reactions
- the electrophilic fluorination of phosphonate carbanions
- nucleophilic fluorination of functionalized phosphonates (substituted with leaving groups of high nucleofugicity)
- synthesis from fluorinated phosphonate carbanions
- synthesis via addition reactions with transition metal catalysis
- synthesis via radical reactions.[65]

7.2 Synthetic strategies towards α-hydroxy-β-aminophosphonic acids

7.2.1 General strategies

They interact very selectively with many biological systems and are of growing interest as analogues of the corresponding α-hydroxy-β-amino acids. Several synthetic strategies have been developed for their synthesis (Scheme 7.1). These strategies include Sharpless asymmetric amino-hydroxylation of unsaturated phosphonates, ammonolysis of the corresponding oxiranes, reduction of α-oxo-β-aminophosphonates, reaction of phosphites with the corresponding aldehydes or by reacting α-hydroxyphosphonates with imines. Enzymatic approaches are known too. α-Hydroxyphosphonates are often biologically active and inhibit enzymes.[70] Not surprisingly, the biological effect is strongly dependent on the configuration at C-1. Therefore, the preparation of enantiomerically pure α-hydroxy-β-aminophosphonates is mandatory but difficult.[71]

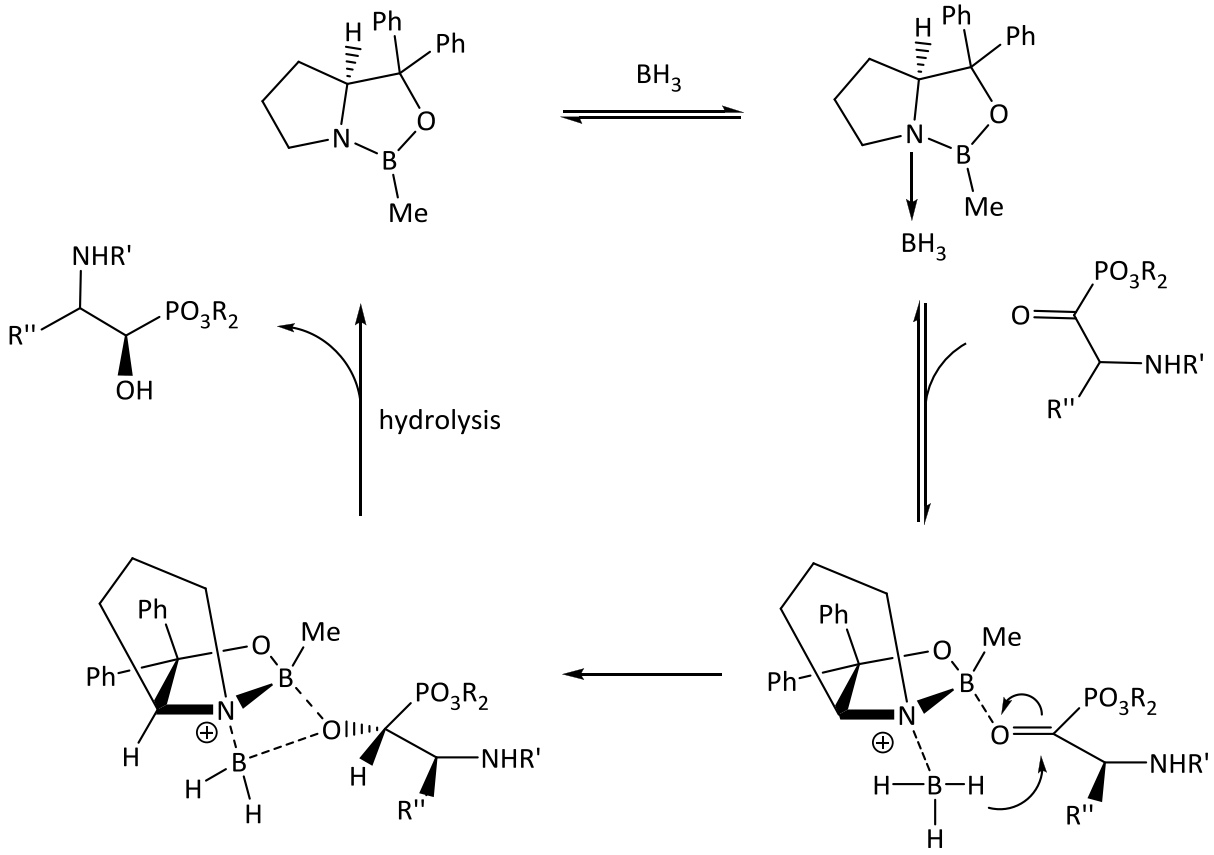


Scheme 7.1. Approaches to α-hydroxy-β-aminophosphonates.

7.2.2 Enantioselective reduction of α-oxophosphonates with chiral oxazaborolidine catalysts

Chiral α-hydroxyphosphonates have been generated by several methods, starting in part from already chiral substrates. Enzymatic resolution was another possibility to access chiral, nonracemic α-hydroxyphosphonates described in literature.[70]

The synthesis of α-hydroxyphosphonates from an achiral substrate, namely the corresponding α-oxophosphonates, is a totally different approach. This can be accomplished by reduction of α-oxophosphonate with borane reagents in the presence of chiral oxazaborolidine catalysts (Scheme 7.2).



Scheme 7.2. Reduction of α-oxophosphonates with borane and (*R*)-2-methyl-oxazaborolidine as catalyst.[72]

This method, referred to as CBS-reduction (Corey-Bakshi-Shibata), was first used for the reduction of 1-aryl-1-oxoalkylphosphonates in 1995.[70]

The approach was first used in 1999 for the synthesis of entiomerically pure α-hydroxy-β-aminophosphonates.[73] The enantiomeric excess of the products obtained by this reduction

method resulted from the pre-coordination of the actual reductant and the ketone to the catalyst. Thus addition of the hydride ion can only happen from one side of the prochiral carbonyl group. The degree to which this reaction is enantioselective is very much dependent on the substrate and reflects steric effects.[70] Formation of the reaction complex forces the delivery of the hydride ion to the carbonyl group to happen from one distinct side.

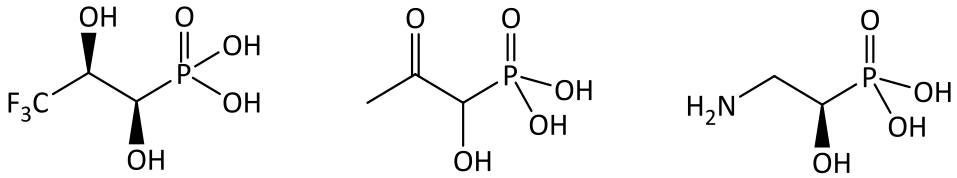
8. Results and discussion

8.1 Aim of this Master Thesis

The topic of my master thesis was the synthesis of various organic compounds, mainly phosphonates, in order to study the biodegradation of natural products containing a phosphorus-carbon bond. Compounds of this type play a crucial role in medicine (the antibiotic fosfomycin), agriculture (the herbicidal phosphinothricin) and other fields already discussed in the previous chapters.

I focused on the synthesis of compounds useful for studying the mineralisation of fosfomycin and (*R*)-2-amino-1-hydroxyethylphosphonic acid produced by amoeba.

3,3,3-Trifluoro-1,2-dihydroxypropylphosphonic acid (**8.1**), a fluorine-containing analogue of a postulated intermediate in the biodegradative pathway of fosfomycin, was prepared (Figure 8.1). Its biodegradation will be evaluated later.



(1*R**,2*R**)-**8.1** **6.2** (*R*)-**2.1**

Figure 8.1. Structures of target compounds.

Fluorinated compounds are useful tools for unravelling metabolic processes as they can efficiently block certain enzymatic key steps.

Moreover, a putative intermediate of fosfomycin biodegradation was prepared. Both enantiomers of 1-hydroxy-2-oxopropylphosphonic acid (**6.2**) were synthesised. They are needed to test which one is converted by a cell-extract from *R. huakuii* PMY1 to hydroxyacetone and orthophosphate.

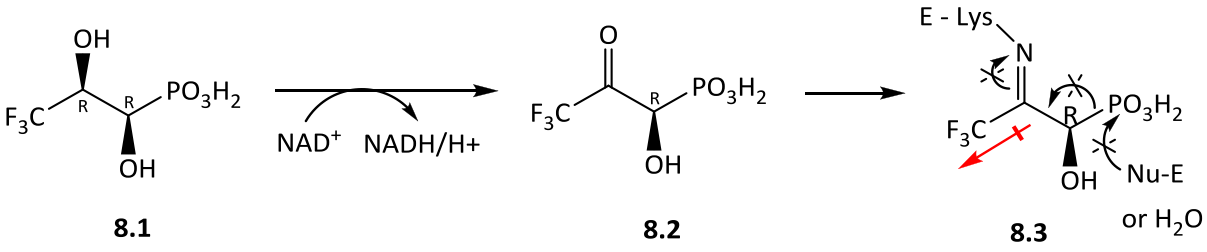
Furthermore, a new synthetic route to enantiomerically pure (*R*)-2-amino-1-hydroxy-ethylphosphonic acid [(*R*)-**2.1**] was developed. Additionally, the synthetic route to the racemic compound was optimised to maximise the yield. Gram quantities of the racemate and the (*R*)-enantiomer were synthesised. These phosphonic acidswill be needed to study their biodegradation.

8.2 3,3,3-Trifluoro-1,2-dihydroxypropylphosphonic acid

8.2.1 Intended use of (1R,2R*)-3,3,3-trifluoro-1,2-dihydroxypropylphosphonic acid*

One target compound to be synthesised during the work for this master thesis was *(1R*,2R*)*-3,3,3-trifluoro-1,2-dihydroxypropylphosphonic acid [*(1R*,2R*)*-**8.1**]. It was synthesised in the racemic form containing both the (1*R*,2*R*) and the (1*S*,2*S*) enantiomer. This will be indicated by asterisk signs throughout the text.

This phosphonate was prepared to study the step in the biodegradation of fosfomycin in which the P-C bond is cleaved. The current hypothesis concerning the biosynthetic pathway of this natural phosphonate is discussed in chapter 6.2. It is assumed that the fission of the P-C bond in the mineralisation of fosfomycin follows a mechanism similar to the one proposed for the biodegradation of 2-AEP. Presumably, P-C bond cleavage involves formation of an iminium ion between the ε-amino group of an active site lysine and the carbonyl group of 1-hydroxy-2-oxopropylphosphonic acid with possibly (*R*)-configuration. This weakens the P-C-bond so that the attack of an enzyme nucleophile or of a water molecule on the phosphorus atom induces P-C fission. This leads to the formation of phosphate and hydroxyacetone after hydrolysis of the enzyme bound intermediates (phosphoenzyme and enamine). However, if (1*R**,2*R**)-3,3,3-trifluoro-1,2-dihydroxypropylphosphonic acid [*(1R*,2R*)*-**8.1**] is present instead of (1*R*,2*R*)-1,2-dihydroxypropylphosphonic acid, it will probably be oxidised to the corresponding 1-hydroxy-2-oxopropylphosphonic acid (**8.2**) (Scheme 8.1).



8.1 **8.2** **8.3**

Scheme 8.1. Possible inhibition of P-C cleavage activity.

This substrate will then form imine **8.3** with the amino group of lysine. Protonation might not be possible because the nitrogen atom of the imine is not basic enough because of the strong electron withdrawing effect of the CF_3 group. Consequently, no iminium cation is

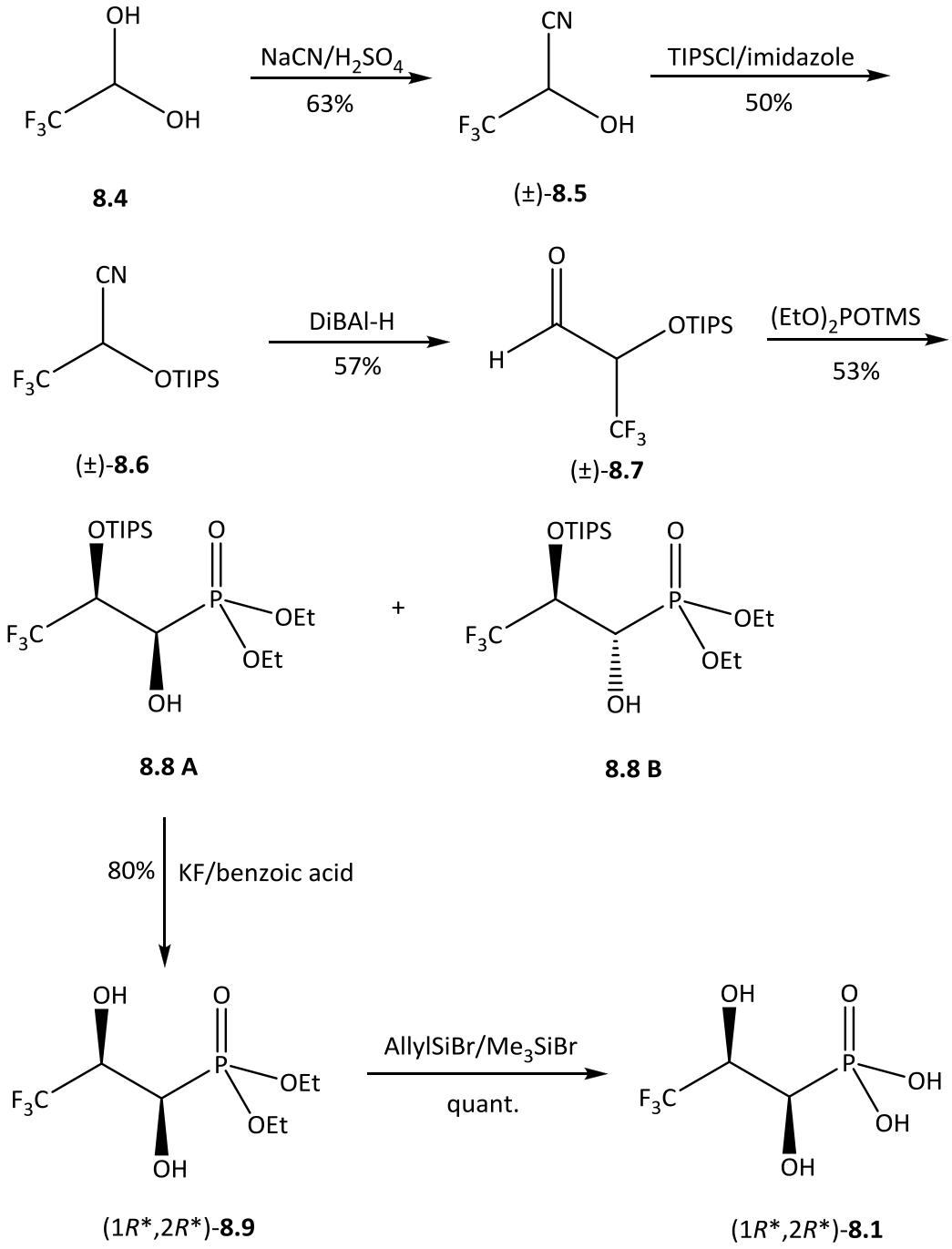
formed and cleavage of the P-C bond is blocked. Therefore, feeding 3,3,3-trifluoro-1,2-dihydroxypropylphosphonic acid (**8.1**) with the right stereochemistry, the (1*R*,2*R*)-enantiomer, to *R. huakuii* PMY 1 degrading fosfomycin should lead to the accumulation of 3,3,3-trifluoro-1-hydroxy-2-oxopropylphosphonic acid (**8.2**). If this could be proved, it would be an important indication in favour of the current hypothesis for the biodegradation of fosfomycin.

8.2.2 Synthesis of (1*R**,2*R**)*-3,3,3-trifluoro-1,2-dihydroxypropylphosphonic acid*

Former attempts by other members of the group to produce trifluoroacetaldehyde as starting material for this synthesis did not yield satisfying results.[74] Now, the synthetic route for the preparation of the phosphonate uses commercially available trifluoroacetaldehyde hydrate (**8.4**) as starting material (Scheme 8.2).

This reagent was converted to 3,3,3-trifluoropropanenitrile [(±)-**8.5**] by reaction with sodium cyanide at 0°C under highly acidic conditions. The product was extracted from the reaction mixture with ethyl acetate. Unfortunately, there was always quite a high amount of ethyl acetate present after concentration under reduced pressure. It was therefore tried to remove this impurity by microdistillation, which did not have the desired effect. The ethyl acetate to product ratio could only be reduced to about 1:1 at worst and to about 1:3 at best. It was feared that ethyl acetate might have an influence on the following reaction, but this was not the case. Therefore, the method for the preparation of the hydroxynitrile was not optimised and the impure product was used without any further purification. The product yield was calculated to be 63 mol% from the composition as determined by ^{1}H NMR spectroscopy.

In the next step the hydroxyl group of the 2-hydroxynitrile was protected with a triisopropylsilyl (TIPS) protective group using TIPS-Cl/imidazole as reagents in DMF as solvent (16 h at room temperature, 2 h at 50°C). The silylation should be performed as soon as possible as the silyl ether is more stable than the hydroxynitrile. This finding was confirmed by the fact that the best yield (50%) of silylated nitrile (±)-**8.6** was obtained when the hydroxynitrile was silylated immediately after preparation. When the hydroxynitrile was kept at 4°C for one week in the fridge, the yield dropped to 38%.



Scheme 8.2. Synthetic route to compound (1*R**,2*R**)-**8.1**.

The protected nitrile was reduced with DiBAl-H to give the corresponding aldehyde (±)-**8.7**. This reaction was carried out at -78°C under argon in dry toluene, the solvent of choice. The product was isolated by extraction with Et_2O after the addition of an aqueous solution of NH_4Cl and sulphuric acid to the reaction mixture. The desired aldehyde was obtained in 57% yield. The ^1H NMR spectrum of the crude product showed that the major side product was a species derived from the TIPS protective group. It was found that the formation of the side

product can be reduced, if the reaction is quenched at -78°C instead of -40°C. A low temperature hence favoured the formation of the desired product. As the aldehyde tended to tail on TLC plates regardless of which eluent was used, column chromatography was not used to purify it.

The crude aldehyde was directly converted to diethyl 3,3,3-trifluoro-1-hydroxy-2-(triisopropylsiloxy)propylphosphonate (**8.8**). Therefore, diethyl trimethylsilyl phosphite was added to a solution of the aldehyde at -78°C. To remove the TMS protective group, a workup with ethanol/HCl followed. The mixture of diastereomeric hydroxyphosphonates was separated by flash column chromatography. All impurities could be removed. The desired (1*R**,2*R**)-diastereomer **8.8 A** and the (1*R**,2*S**)-diastereomer **8.8 B** were obtained in yields of 43% and 10%, respectively. The configurations were assigned analogously to the unfluorinated compounds.

In principle only the (1*R*,2*R*)-enantiomer is needed for the biological test. It is known from previous experiments with (1*R**,2*R**)-1,2-dihydroxypropylphosphonic acid, that only the (1*R*,2*R*)-enantiomer is metabolised. I suppose that in analogy to this result, the (1*S*,2*S*)-enantiomer of the fluorinated analogue will not interfere because it is not oxidised to the ketone. The (1*R**,2*S**)-diastereomer was stored, not deprotected. However, the (1*R**,2*R**)-configured substance had to be deprotected by removing the TIPS group from the oxygen atom and the ethyl groups from the phosphonate group to get the corresponding free phosphonic acid (**8.1**).

The desilylation was the most difficult step of this reaction sequence. Evidently, the CF_3 group retarded the removal of the TIPS group because of its electron withdrawing effect. Therefore, the procedure used for the deprotection of (1*R**,2*R**)-diethyl 1-hydroxy-2-(triisopropylsiloxy)propylphosphonate could not be applied to the fluorinated analogue.

Different reaction conditions were tried to accomplish the desilylation (Table 8.1).

Entry	Conditions	Solvent	Temp./°C	Time/h	Product Yield/mol%
1	HF (40%)	CH_3CN	reflux	6	4
2	HF (40%)	CH_3CN	50	24	17
3	TBAF + CH_3CO_2H	THF	-30	6	71
4	KF + $C_6H_5CO_2H$	CH_3CN	75	24	80

Table 8.1. Reaction conditions for removal of TIPS.

As the yield for the third and the fourth option were comparable (entries 3 and 4; 71 and 80% respectively), the easiest procedure for deblocking of the silyl ether, method 4, was chosen for all following experiments. No aqueous work up was necessary. The cooled reaction mixture was filtered and the eluate was concentrated under reduced pressure. The residue could be purified easily by flash column chromatography to give a white, crystalline solid that was pure as judged by NMR spectroscopy and microanalysis.
The reaction conditions for the deprotection with HF (entries 1 and 2) were too harsh. Part of the product was destroyed and therefore the yields were very low. Higher temperatures and longer reaction times did not improve the results significantly. As a better method was found, aqueous HF was not used as it is quite precarious to work with it.

The resulting dihydroxyphosphonate (1*R**,2*R**)-**8.9** was deprotected at phosphorus using bromotrimethylsilane in 1,2-dichloroethane at room temperature for two days. Allyltrimethylsilane was added to the reaction mixture to remove HBr which was formed by the reaction of the hydroxyl group with TMSBr. After concentration of the reaction mixture under reduced pressure, water and ethanol were added to the residue to hydrolyse the generated trimethylsilylated compound. This gave the free phosphonic acid (1*R**,2*R**)-**8.1**. No purification was necessary. The acid was converted to the corresponding ammonium salt by dissolving the free acid in water, adding a few drops of concentrated ammonia and lyophilising the resulting solution. A crystalline product which can be stored and used later directly was formed. The yield was assumed to be 95%, starting from TIPS-unprotected (1*R**,2*R**)-**8.9**.

In summary, the synthesis of the ammonium salt of (1*R**,2*R**)-3,3,3-trifluoro-1,2-dihydroxypropylphosphonic acid [(1*R**,2*R**)-**8.1**] could be accomplished by a sequence of six steps in an overall yield of 6% (see Scheme 8.2). The most critical of these steps were the

silylation and the formation of the P-C bond. (1*R**,2*S**)-diethyl 3,3,3-trifluoro-1-hydroxy-2-(triisopropylsiloxy)propylphosphonate (**8.8 B**) was formed as a side product in 4 steps and with a poor overall yield of 2%. It was stored at this stage and may be deprotected later if needed.

8.3 1-Hydroxy-2-oxopropylphosphonic acid

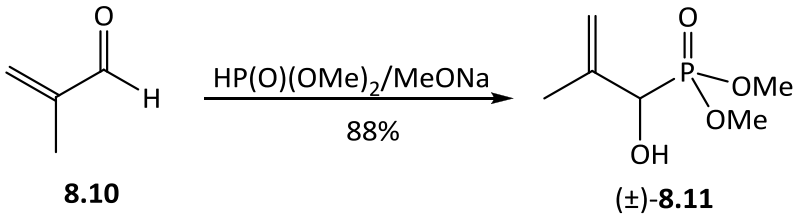
8.3.1 Intended use for 1-hydroxy-2-oxopropylphosphonic acid

(*R*)-1-Hydroxy-2-oxopropylphosphonic acid (**6.2**) is very likely a naturally occurring phosphonic acid and a putative intermediate of fosfomycin biodegradation. It has already been prepared as a racemate and was tested for release of orthophosphate by a culture and a cell-extract from *R. huakuii* PMY1. The racemic mixture of this compound did not support growth of the cell culture, but released P_i in the cell-extract. It was concluded that either its uptake by the microorganism did not take place or that the (*S*)-enantiomer inhibited growth.[75]

Therefore, the synthesis of the two enantiomers of 1-hydroxy-2-oxopropylphosphonic acid was undertaken to test the metabolism of the two enantiomers separately. This could help to exclude the latter possibility. The (*R*)-enantiomer is assumed to be the natural substrate for the enzyme cleaving the P-C bond involved in fosfomycin biodegradation.[75]

8.3.2 Synthesis of 1-hydroxy-2-oxo-propylphosphonic acid

The starting material for the synthesis of **6.2** was methacrylaldehyde (**8.10**) which was reacted with dimethyl phosphite to give (±)-**8.11** (Scheme 8.3). A catalytic amount of a



8.10 (±)-**8.11**

Scheme 8.3. Synthesis of (±)-**8.11**.

saturated solution of sodium methoxide in methanol was needed to induce the addition of dimethyl phosphite to the carbonyl group. The reaction was carried out at -35°C and the crude product was sufficiently pure for the next reaction step. Thus, no purification was needed. The yield was 88%, which is very satisfying. However, if the starting aldehyde was not pure, the yield decreased drastically.

Dimethyl 1-hydroxy-2-methyl-2-propenylphosphonate [(±)-**8.11**] was formed as a racemic mixture. To separate the two enantiomers, chemical resolution had to be performed. The α-hydroxyphosphonate was derivatised with Noe's reagents (lactol and dimer of lactol). In principle, this reaction can give four diastereomeric acetals, but only three of these could be detected by ^{1}H NMR spectroscopy (Figure 8.2). Anticipating analytical results, the two axially substituted acetals **8.12 A** and **8.12 B** derived from (*R*)- and (*S*)-hydroxyphosphonate, respectively, were formed preferentially because of the anomeric effect. Only one equatorially substituted acetal **8.13,** derived from the (*S*)-hydroxyphosphonate was generated as a side product.

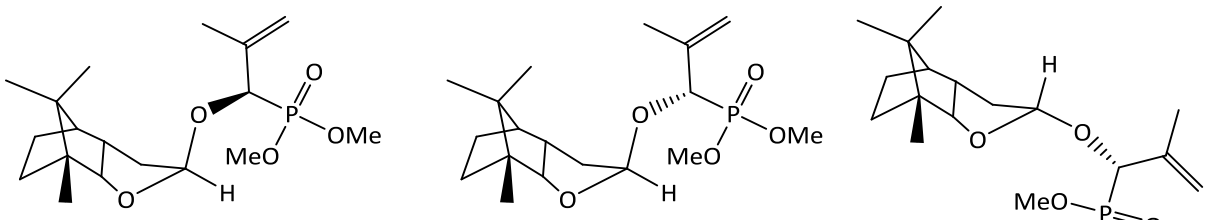
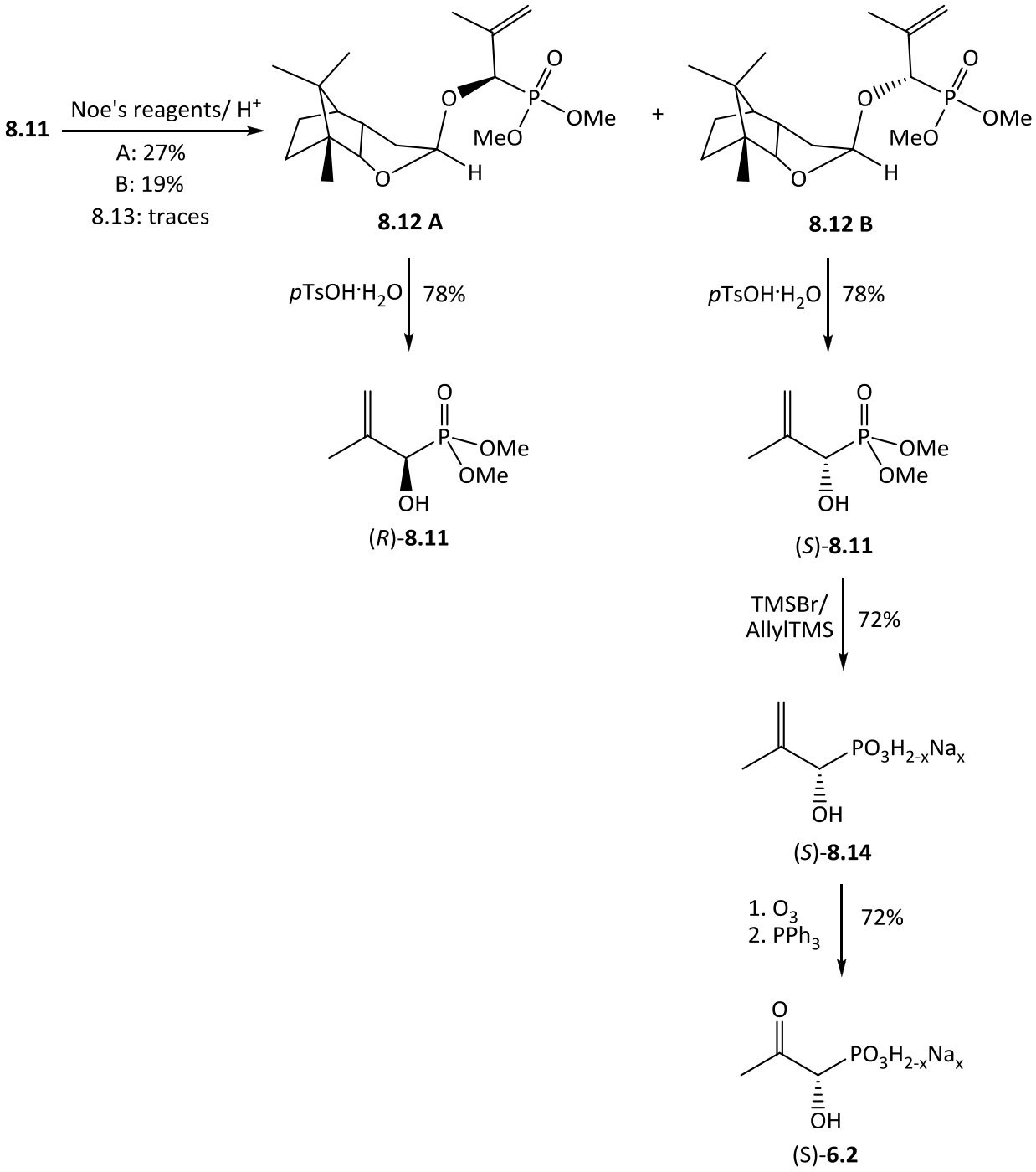


Figure 8.2. Observed acetals derived from (±)-**8.11** with Noe's reagents.

Of these three diastereomers, only the two axially substituted ones were isolated. These could be separated by flash column chromatography. Acetal **8.13** was the least polar of the three diastereomers. Therefore, flash chromatography was performed very carefully (slow elution of compounds) to prevent co-elution with acetal **8.12 A** moving just behind **8.13**. Afterwards the chiral auxiliary was removed again and the two enantiomers of **8.11** were obtained.

Two chiral auxiliaries were tried for the derivatisation. First, the dimer [(+)-MBF-O-MBF] of Noe's lactol was used (Scheme 8.4). This yielded 24% of the diastereomer containing the



Scheme 8.4. Preparation of (*R*)- and (*S*)-1-hydroxy-2-oxopropylphosphonic acid from **8.11**.

(*R*)-α-hydroxyphosphonate and 11% of the diastereomer containing the (*S*)-α-hydroxyphosphonate. They were separated by flash chromatography as already mentioned. Furthermore, the formation of the acetals was also carried out with Noe's lactol [(+)-MBF-OH], which gave slightly higher yields of 27 and 19% for **8.12 A** and **8.12 B**, respectively, compared to the dimer of Noe´s lactol.

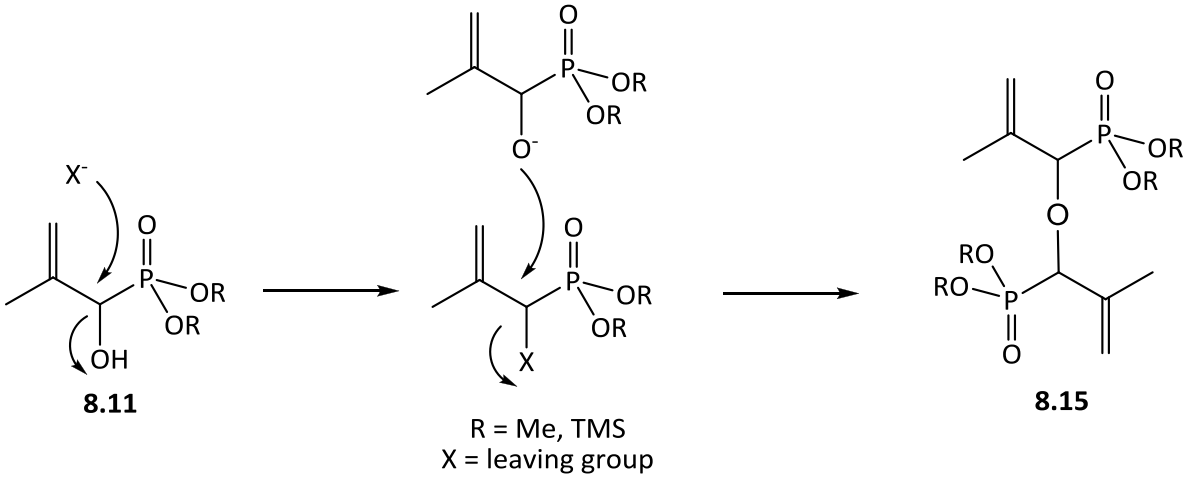
As both reactions are acid-catalysed, the addition of small amounts of *para*-toluenesulfonic acid monohydrate (*p*-TsOH·H_2O) was necessary. This method of chemical resolution had already been used for α-hydroxyphosphonates.[76]

Deprotection of the two acetals **8.12 A** and **8.12 B** gave the (*R*)- and (S)-enantiomer of α-hydroxyphosphonate **8.11**, respectively, anticipating subsequent analytical results. *p*-TsOH·H_2O was added to a solution of a diastereomerin a mixture of CH_2Cl_2 and methanol. After stirring at room temperature, the α-hydroxyphosphonate was isolated and purified by flash chromatography. The yield for both deprotections was 78%.
The absolute configuration of the optically active α-hydroxyphosphonates was determined by ^{1}H and ^{31}P NMR spectroscopy of their (*R*)-Mosher esters. The phosphorus of the (*R*)-Mosher ester derived from (+)-**8.11**, which was obtained from acetal **8.12 A**, resonated at higher field than the phosphorus of the (*R*)-Mosher ester derived from (-)-**8.11** (obtained from **8.12 B**). Therefore, the dextrorotary α-hydroxyphoshonate has (*R*)-configuration, the levorotary one (*S*), in line with previous findings. This assignment is supported by the ^{1}H NMR spectra of the Mosher esters (for details see experimental part). [77,78]

The enantiomerically pure α-hydroxyphosphonates obtained by resolution had to be deprotected at phosphorus to get the corresponding free phosphonic acids. As the enantiomerically pure starting materials for this deprotection were very precious, the removal of the methyl groups was optimised with the racemic compound first. This step was best performed by a classic method in phosphonate chemistry using bromotrimethylsilane (TMSBr) and allyltrimethylsilane (allylTMS). I expected no problems for this step and was therefore surprised by the result.
A side reaction, probably due to the allylic character of the position next to the phosphorus atom, occured. A phosphonate of this type has not been deprotected by this method yet. NMR spectra (^{1}H, ^{13}C and ^{31}P) revealed that the desired product **8.14** was accompanied by a high amount of an unknown impurity (product/impurity = 1:0.25). First I thought that this impurity could be due to the formation of HBr during demethylation, which may cause a S_N2-type exchange of the hydroxyl group by Br^-. Therefore, I argued that its formation should be easily suppressible by first adding allyltrimethylsilane to the reaction mixture, used to trap HBr, and secondly bromotrimethylsilane. (As in the first experiment bromotrimethylsilane

was added before allyltrimethylsilane) Unfortunately, this did not affect the amount of the unknown impurity formed.

A closer look at the ^{13}C and ^{1}H NMR shift values revealed that the signals of the impurity differed only slightly from those of the desired product. However, the signal for C-1 in the ^{13}C NMR spectrum was shifted to the lower field more than expected for a carbon atom with bromine instead of an oxygen atom as substituent. The finding that there were no additional carbon or hydrogen signals present in the unknown compound compared to the deprotected species led to the conclusion that the impurity was presumably dimer **8.15** (Scheme 8.5). A possible mechanism for its formation is given in Scheme 8.5. According to NMR measurements, this dimeric species was already formed before aqueous workup. Unfortunately, this assumption could not be verified as it was not possible to isolate **8.15** as tetramethyl ester after esterification of the mixture of crude acids with diazomethane.



Scheme 8.5. Mechanism for formation of dimer **8.15**.

However, I tried to suppress its formation by lowering the reaction temperature, replacing 1,2 dichloroethane (DCE) by CCl_4 as solvent and using freshly distilled bromotrimethylsilane. Purification of TMSBr seemed necessary to remove traces of iron bromide formed by corrosion of the sure seal cap of the reagent bottle. Thus, the amount of the dimer could be reduced to 3 mol%. Table 8.2 gives an overview of the reaction conditions that were tried.

Entry	Addition of reagents	Solvent	Temp./°C	Reaction time/h	TMSBr	Product : Dimer[a]
1	1ST TMSBr	DCE	24	6	not distilled	1 : 0.24
2	1ST allylTMS	DCE	24	3	not distilled	1 : 0.50
3	together	DCE	0	3	not distilled	1 : 0.10
4	together	DCE	0	1	not distilled	1 : 0.03
5	together	CCl_4	0	3	not distilled	1 : 0.07
6	together	DCE	-20	1	distilled	1 : 0.13
7	together	DCE	0	1	distilled	1 : 0.08

Table 8.2. Conditions for deprotection of phosphonate group; [a] Determined by ^{31}P NMR spectroscopy.

Although none of the conditions given in Table 8.2 furnished only the desired product, a small sample of the enantiomerically pure (*S*)-α-hydroxyphosphonate **8.11** was deprotected at -10°C using the best conditions according to Table 8.2 (entry 4). Surprisingly, no side product could be detected by NMR spectroscopy. I attribute this finding to the fact that this substrate was homogenous as it had been purified by flash chromatography. However, the crude (±)-α-hydroxyphosphonate was regarded as "pure enough" for the next reaction step, but contained some minor impurities which were not identified. Possibly, these impurities could have an influence on the formation of dimer **8.15**. The yield for the racemic sodium salt **8.14** was virtually quantitative, whereas deprotection of the small sample of (*S*)-**8.11** gave only 72% of the deprotected product.

The last step in the reaction sequence leading to the sodium salt of 1-hydroxy-2-oxopropylphosphonate was ozonolysis of the allylphosphonate **8.14** to give the corresponding 2-oxophosphonate **6.2**. Initially, the racemic free acid was ozonolysed in a mixture of water and methanol at -78°C. The reaction mixture was just concentrated in vacuo and the residue was dissolved in water. The pH of the resulting strongly acidic solution was adjusted to 7 with 1 M NaOH and the solution was lyophilised afterwards. The product contained a large amount of formate (oxophosphonate/formate = 1 : 0.46) and phosphate (oxophosphonate/P_i = 1 : 0.53), as well as some minor impurities. Thus a large amount of the

starting material was destroyed by the ozonolysis. A shorter reaction time and a lower reaction temperature (-95°C) did not affect product distribution significantly. However, I found that ozonolysis of the sodium salt of allylphophonic acid under slightly basic conditions (pH = 8.0 of solution before ozonolysis), instead of neutral ones, and the addition of an equimolar amount of PPh_3 at -78°C after ozonolysis suppressed the formation of both side products efficiently. Only 2 mol% of formate were formed and no phosphate could be detected at all. However, the other and minor side products were still present. The aqueous solution of the sodium salt of (±)-**6.2** was lyophilised after extractive removal of the Ph_3P and Ph_3PO. Analogously, enantiomer (*S*)-**6.2** could be prepared with an overall yield of 9% in five steps.

8.3.3 Stability of the sodium salts of (±) and (S)-1-hydroxy-2-oxopropylphosphonic acid towards racemisation in aqueous media

The sodium salts of (*R*)- and (*S*)-1-hydroxy-2-oxopropylphosphonic acid will be used to study cleavage of their P-C bonds by cell-extracts from *R. huakuii* PMY1. Therefore, it is essential to know whether the enantiomers are labile towards racemisation in aqueous media at neutral pH. The mechanism for racemisation is abstraction of the proton at C-1, due to its acidity caused be the carbonyl and phosphonate group and reprotonation from the medium.

To study racemisation, a sample of racemic oxophosphonate **6.2** was dissolved in D_2O and the signal ratio of the hydrogen at C-1 and the hydrogens of the methyl group were observed as a function of time at pH 7, 8.5 and higher. If racemisation via enolate ion formation occurs by the proposed mechanism, H/D exchange would result. These experiments, which are described in more detail in chapter 9.3 in the experimental section, showed that the 2-oxophosphonate is sufficiently stable at pH 7 and pH 8.5 towards racemisation. Racemisation as determined by ^{1}H NMR spectroscopy was fairly low (10-12%) within 24 hours at pH 7. However, after 5 days the α-proton had undergone a complete H/D exchange.
At higher pH (> 8.5) the racemisation proceeded with a higher rate and after 6 h already 50% of the protons at C-1 had been exchanged for deuterium.

Furthermore, the oxophosphonate to phosphate ratio was determined by ^{31}P NMR spectroscopy under acidic conditions (pH ~ 4-5) as well to see whether P-C bond cleavage

occurs at low pH. As the phosphate/phosphonate ratio increased only insignificantly within 24 h, the P-C bond in 2-oxophosphonate is virtually chemically stable under acidic conditions. Therefore, stereochemical and chemical instability will not interfere when the enantiomers of (*R*)- and (*S*)-1-hydroxy-2-oxopropylphosphonte will be tested as substrates for the enzyme splitting the P-C bond in the biodegradative pathway of fosfomycin.

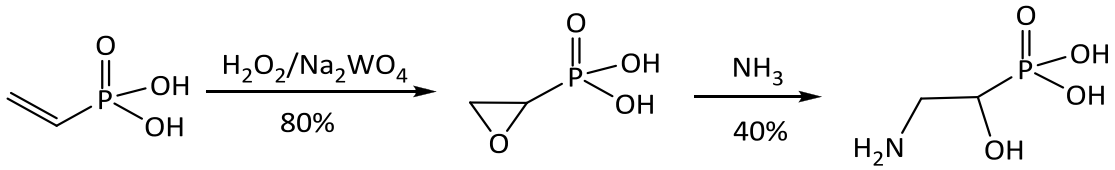
8.4 2-Amino-1-hydroxyethylphosphonic acid (phosphaisoserine)

8.4.1 Purpose of the synthesis

2-Amino-1-hydroxyethylphosphonic acid (**2.1**) was to be synthesised both as racemate and as (*R*)-enantiomer, which is the naturally occurring phosphonic acid analogue of isoserine.[79] These compounds are needed as substrates for the isolation of microorganisms degrading this natural phosphonic acid. Fairly large quantities, 20 g of the racemate and 5 g of the (*R*)-enantiomer, are needed for the beginning.

Different synthetic strategies towards 2-amino-1-hydroxyethylphosphonic acids have been described in chapter 7.2. Of those methods the asymmetric Sharpless amino-hydroxylation of vinylphosphonic acid did not seem to be attractive. Literature points out that the yields for *α,ß*-unsaturated phosphonates are significantly lower than for *α,ß*-unsaturated esters. Furthermore, only phosphonates containing an aromatic substituent in *β*-position to the phosphonate group were amino-hydroxylated.[80]

I envisaged the synthesis of racemic phosphaisoserine starting from commercially available vinylphosphonic acid (**8.16**) comprising two steps - epoxidation and ammonolysis of the epoxide formed (Scheme 8.6). It is known that *cis*-1-propenylphosphonic acid



8.16 (±)-**8.17** (±)-**2.1**

Scheme 8.6. Synthesis of racemic phosphaisoserine

can be epoxidised with hydrogen peroxide in the presence of sodium tungstate as catalyst. The ammonolyis of cis-1,2-epoxypropylphosphonate has been reported as well.[81,82] These two procedures were adapted to vinylphosphonic acid.

This approach could be used to prepare the chiral, nonracemic 2-amino-1-hydroxyethylphosphonic acids too if chiral, nonracemic epoxides were used in place of the racemic epoxide. However, the enantiomeric excess of the product obtained by this method was limited by the enantiomeric excess of the epoxyphosphonate used as starting material for the ammonolysis. Unfortunately, the enantioselective epoxidation of 1-propenylphosphonate proceeds with moderate to low ee.[83] Thus, a different approach had to be used for the synthesis of (*R*)-2-amino-1-hydroxyethylphosphonic acid of high ee. Enantioselective reduction of an appropriate α-oxophosphonate with boranes in the presence of an oxazaborolidine catalyst turned out to be the method of choice.

8.4.2 Optimisation of synthesis of racemic phosphaisoserine

The first step of the synthesis of racemic phosphaisoserine was the epoxidation of the triethylammonium salt of vinylphosphonic acid (**8.16**). The oxidising agent was hydrogen peroxide. A catalytic amount of sodium tungstate, a common catalyst for the epoxidation of electron poor alkenes,[82] and a spatula tip of EDTA were added as well. Addition of $NaHCO_3$ instead of tungstate which has been used for the epoxidation of unsaturated geminal di-phosphonates did not give the desired product.[84] Different reaction conditions were tested to maximise the conversion of the unsaturated phosphonic acid to the epoxide and to minimise the formation of side products.

The major side product was found to be 1,2-dihydroxyethylphosphonic acid, the diol, by the addition of a small amount of authentic cyclohexylammonium salt of racemic 1,2-dihydroxyethylphosphonic acid to the NMR sample of the reaction mixture. A set of signals in the ^{1}H NMR spectrum which was already present before, increased in intensity. The same was true for one peak in the ^{31}P NMR spectrum. As no new additional signals showed up, the side product could be identified unambiguously.

This side product was formed by hydrolytic opening of the oxirane ring of epoxyethylphosphonic acid. Its formation is a function of the reaction temperature, pH and reaction time. If the epoxidation was carried out at higher temperatures, but for shorter reaction times, the formation of the side product could be suppressed considerably. After a

reaction time of three hours, only a small amount of the side product was visible in NMR spectra of the reaction mixture. Unfortunately, the conversion of vinylphosphonic acid to the epoxide did not exceed 50% after such a short reaction time. Therefore, the reaction time was increased which favoured diol formation. However, I found that the formation of 1,2-dihydroxyethylphosphonic acid was insignificant if the reaction was performed at room temperature. Table 8.3 summarises the experiments which were performed to optimise this step.

Entry	Temp./°C	Time/h	equiv. of NEt_3	Product[a]	Starting material[a]	Diol[a]
1	40	24	1.27	1	0.31	0.05
2	60	24	1.30	1	0.38	0.14
3	60	6	1.29	1	0.33	0.10
4	25	72	1.30	1	0.22	0.04
5	30	23	1.00	1	0.14	0.07
6	30	23	0.75	1	0.29	0.53
7	25	72	1.16	1	0.20	0.05

Table 8.3. Reaction conditions for the epoxidation of vinylphosphonic acid; [a] Determined by ^{1}H and ^{31}P NMR spectroscopy.

The best result was achieved if the epoxidation was performed under slightly basic conditions with about 1.15 equivalents of NEt_3 (as base) at room temperature for three days, although such a long reaction time is disadvantageous. Obviously, the pH was another factor influencing the outcome of the transformation. The fact that the crude epoxide could be used without prior purification for the next step was definitely an advantage.

The second step was the opening of the epoxide under basic conditions with concentrated ammonia to give 2-amino-1-hydroxyethylphosphonic acid (±)-**2.1**. The temperature was determining the yield. If it was opened at 70°C for 24 h, the yield was about 32%. However, if the reaction was carried out at room temperature for three days, the yield was increased to 40%. The range of improvement for the second step was definitely not as large as for the first reaction step. The longer reaction time was acceptable because of the improved yield. The aminophosphonic acid was isolated by ion-exchange chromatography on Dowex 50W/H^+ using water as eluent. Fractions were collected and analysed by TLC (thin layer chromatography). Acids (1,2-dihydroxyethylphosphonic acid, vinylphoshonic acid and other acids) were not retained and were eluted first. They were followed by the

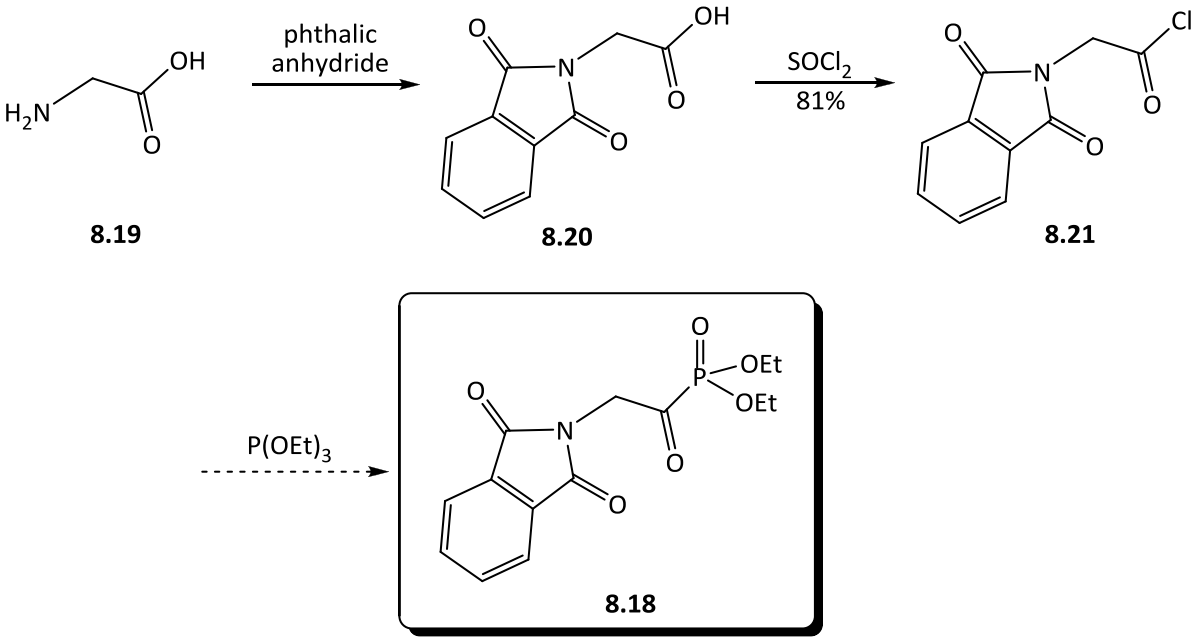
aminohydroxyphosphonic acid (±)-**2.1**. Ninhydrin-positive fractions were pooled, concentrated under reduced pressure and crystallised from water/ethanol.

The epoxide opening by attack of ammonia was expected to occur for steric reasons (highly) regioselectively at C-2. To check whether ammonia also attacked at C-1 to give phosphaserine, all fractions of the ion exchange chromatography including the forerun and the fractions collected after elution of the desired product were analysed by TLC and by NMR spectroscopy. Spiking of the NMR samples with authentic phosphaserine and recording NMR spectra demonstrated that none contained phosphaserine. Therefore, the epoxide ring was opened stereospecificly.

In summary, the synthesis of racemic phosphaisoerine was achieved by a two step protocol using vinylphosphonic acid (**8.16**) as starting material (Scheme 8.6). This unsaturated phosphonic acid was converted to the corresponding triethylammonium salt and then epoxidised with hydrogen peroxide in the presence of sodium tungstate. The yield for epoxidation was highly dependent on the temperature as well as on the pH of the reaction mixture. Slightly basic conditions and room temperature proved to give the best results concerning conversion (up to 80%). The stereospecific opening of epoxide (±)-**8.17** with ammonia proved also to be temperature dependent and could be optimised, so that the isolated product yield was 50%. The overall yield for this two step sequence was 40%.

*8.4.3 The synthesis of (*R*)-2-amino-1-hydroxyethylphosphonic acid [(R)-phosphaisoserine]*

The synthesis of (*R*)-phosphaisoserine [(*R*)-**2.1**] was accomplished using a different synthetic route. It involved the preparation of diethyl 1-oxo-2-phthalimidoethylphosphonate (**8.18**) as key intermediate (Scheme 8.7).



8.19 **8.20** **8.21** **8.18**

Scheme 8.7. Envisaged route to key intermediate **8.18**.

This compound was chosen as it could easily be transformed into phosphaisoserine by reducing the carbonyl group and deblocking both the amino and phosphonic acid group. With this key intermediate in hands, I reasoned to have four options to obtain the chiral, nonracemic hydroxyphosphonate:

- Enantioselective reduction of the 1-oxo group with borane dimethyl sulfide, $BH_3{*}(CH_3)_2S$, and an oxazaborolidine catalyst to give the desired α-hydroxyphosphonate.
- Enantioselective reduction with catecholborane and an oxazaborolidine catalyst to give the desired enantiomer.
- Reduction to the racemate, esterification of the α-hydroxyphosphonate and enzyme-catalysed resolution with a lipase to obtain the (*R*)-enantiomer.
- Reduction to the racemate followed by derivatisation with a chiral auxiliary to give a pair of diastereomers which can be separated by flash chromatography and finally deprotected.

As there was more than one way leading to the desired (*R*)-α-hydroxyphosphonate **2.1**, α-oxophosphonate **8.18** met my expectations as key intermediate. Many transformations can

be tried with it without having to synthesise a new starting material for each one first. This was saving time as well as resources.

The synthesis of the key intermediate was envisaged to be accomplished with glycine (**8.19**) as starting material. The amino group of this proteinogenic amino acid was protected with phthalic anhydride. The conversion was done solvent free by heating a well stirred mixture of phthalic anhydride and glycine at 160°C. Phthalimidoacetic acid (**8.20**) was formed and purified by crystallisation. Then, the acid was refluxed in thionyl chloride for 1.5 h to convert it to the corresponding acid chloride **8.21**. The crude product could again be purified just by crystallisation. The overall yield for the two steps starting from cheap glycine was 82%.

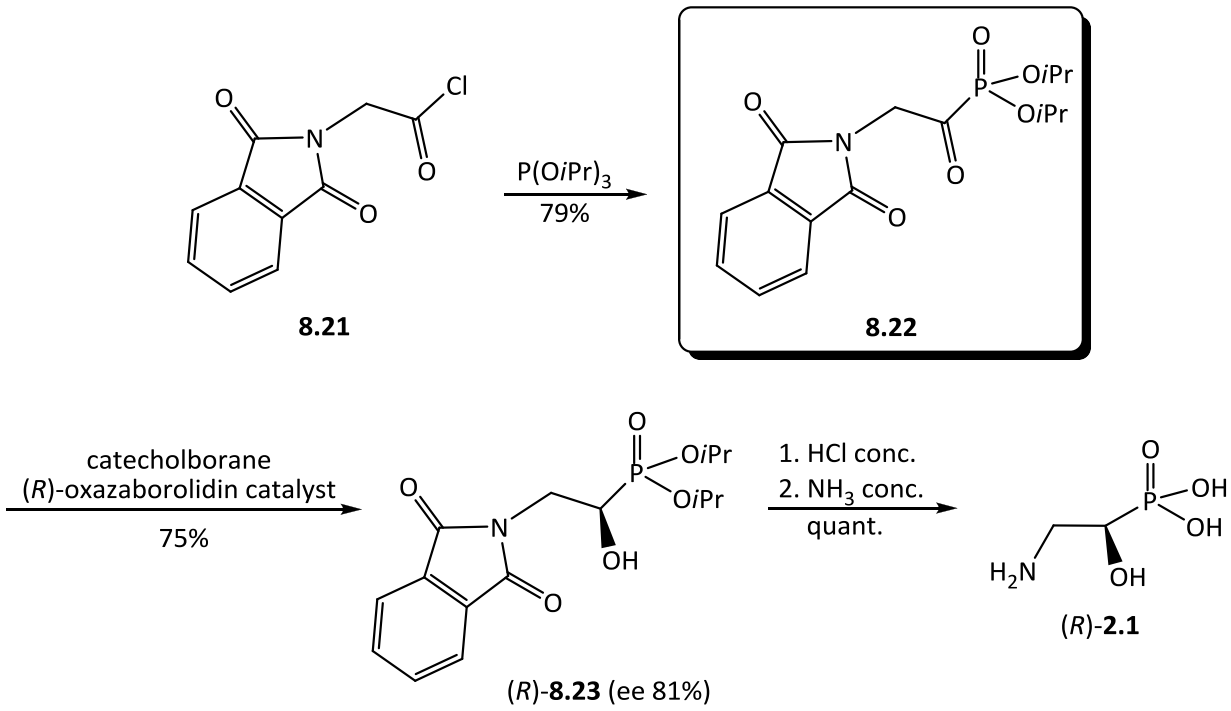
Next, the acid chloride was reacted with triethyl phosphite [$P(OEt)_3$] to give oxophosphonate **8.18**. Unfortunately, this Arbuzov reaction always gave mixtures of several (seven and more) products. The molar ratios of the products were estimated by analysis of the ^{31}P NMR spectra recorded from the reaction mixture. The reaction conditions which were tried are compiled in Table 8.4.

Entry	temp./°C	time/h	solvent	Outcome
1	25	2	THF	3 major and many side products
2	25	0.5	toluene	mixture of at least 8 products
3	0	1.5	toluene	7 products
4	0	1	CH_2Cl_2	at least 8 products
5	25	1	CH_2Cl_2	59 mol% of the desired product

Table 8.4. Reaction conditions for Arbuzov reaction.

The best reaction conditions gave a mixture consisting of 59 mol% of the desired α-oxophosphonate **8.18** (entry 5), but it could not be forced to crystallise. A one pot synthesis for these three steps was not successful either.[85]

As ethyl phosphonate **8.18** could not be isolated from these reaction mixtures, I replaced triethyl phosphite [$P(OEt)_3$] by triisopropyl phosphite [($P(O\textit{i}Pr)_3$] and generated diisopropyl 1-oxo-*2*-phthalimidoethylphosphonate (**8.22**) as an alternative key intermediate (Scheme 8.8).



Scheme 8.8. Synthesis of (*R*)-2-amino-1-hydroxyethylphosphonic acid from glycine.

The results for the Arbuzov reactions are summarised in Table 8.5.

Entry	temp./°C	time/h	solvent	% product in reaction mixture[a]
1	25	1.25	toluene	70
2	0	1	toluene	73
3	0	1	CH_3CN	45
4	0	1	CH_2Cl_2	71
5	-20	2.25	CH_2Cl_2	45
6	25	0.5	CH_2Cl_2	79

Table 8.5. Reaction conditions for Arbuzov reaction between $P(OiPr)_3$ and phthalimidoacetyl chloride; [a] determined by ^{31}P NMR.

This Arbuzov reaction appeared to be highly dependent on the temperature and the solvent used. In contrast to the transformation with triethyl phosphite, it produced satisfying amounts of the desired α-oxophosphonate **8.22**. However, the biggest problem after optimisation of the reaction conditions was the purification of the product. As the crude product decomposed on silica gel, flash chromatography was not appropriate for that purpose. Nevertheless, if the procedure was carried out quickly, a satisfactory yield of 60% of the desired phosphonate could be obtained.

By chance, I found that all impurities were readily soluble in ethyl acetate at room temperature, but the α-oxophosphonate was only in the boiling solvent. Therefore, the α-oxophosphonate could be crystallised easily from ethyl acetate. The pale yellow crystals obtained were homogenous by IR, NMR spectroscopy and microanalysis. The Arbuzov reaction carried out in dry CH_2Cl_2 at room temperature for 30 minutes and followed by recrystallisation of the product from ethyl acetate yielded 80% of the diisopropyl α-oxophosphonate **8.22**.

The new key intermediate was reduced using two different agents. At first, borane dimethyl-sulfide and (*R*)-2-methyl-oxazaborolidine (as catalyst) were used in different solvents and at different temperatures. These experiments showed that lower temperatures definitely produce α-hydroxyphosphonates of high ee. However, it was not possible to generate α-hydroxyphosphonates with an ee of more than 53% by reduction under these conditions. Interestingly, temperatures below -50°C did not bring about a higher selectivity. One experiment was performed using (*R*)-2-butyl-oxazaborolidine as catalyst to find out whether the longer butyl group at boron induced an additional selectivity compared to the methyl group. This was not the case. The results of the various reductions with borane dimethyl-sulfide are shown in Table 8.6.

Entry	catalyst[a]	temp/°C	time/h	solvent	Yield/%	ee/%
1	2-methyl	25	4.5	THF	57	30
2	2-methyl	0	5	THF	75	34
3	2-methyl	-20	2.5	THF	86	36
4	2-methyl	-50	3	THF	68	52
5	2-methyl	-78	3	THF	62	53
6	2-butyl	-20	2.5	THF	82	22

Table 8.6. Reduction of **8.22** with borane dimethyl-sulfide and chiral catalyst; [a] (*R*)-oxazaborolidine.

The second reducing agent tested was catecholborane showing higher selectivity than borane dimethyl-sulfide. And indeed, this reagent produced the desired α-hydroxy-phosphonate (*R*)-**8.23** with enantiomeric excesses of up to 81%. Again, these ees were dependent on the reaction temperature as well as on the solvent. In this case (*R*)-2-butyl-oxazaborolidin did not increase the ee of the desired enantiomer as was the case for the first

reducing agent. Catecholborane was added to a mixture of the starting material and the catalyst at -78°C. Afterwards, the flask with the reaction mixture was stored at a temperature given in Table 8.8. The results for the reduction experiments with catecholborane are summarised in Table 8.8.

Entry	catalyst[a]	temp/°C[b]	time/h[c]	solvent	Yield/%	ee/%
1	2-methyl	-25	5.5	toluene	75	81
2	2-methyl	-25	5.5	THF	38	23
3	2-methyl	-45	6	THF	49	25
4	2-methyl	-50	7	toluene	62	74
5	2-butyl	-25	6	toluene	65	81

Table 8.7. Reduction of oxophosphonate **8.22** with catecholborane; [a] (*R*)-oxazaborolidine, [b] Temperature at which flask was stored after mixing reagents at -78°C, [c] Duration of storage.

Thus, the best result for the reduction could be obtained with catecholborane as reducing agent and (*R*)-2-methyl-oxazaborolidine as catalyst at -25°C (entry 1). The yield of the (*R*)-α-hydroxyphosphonate **8.23** was 75% and the ee 81%. The use of the 2-butyl-oxazaborolidine catalyst did not improve the ee and lowered the yield.

The reduced compound could be purified easily by flash chromatography on silica gel. Catecholborane in general tends to tail on silica gel, but this was not the case here. The pure product readily crystallised. Unfortunately, it was not possible to increase its ee by crystallisation as the racemate seemed to crystallise first. Therefore, we wanted to try to increase the ee using crystallisation after the deprotection of the amino and phosphonate moieties.

The α-hydroxyphosphonate was first deprotected by refluxing with 6 M HCl for 16 h to remove the isopropyl groups. The reaction mixture was concentrated under reduced pressure and the crude residue was stirred with concentrated ammonia at 50°C for another 16 h. The crude (ammonium salts of the) phosphonic and phthalic acid could be purified in the same way as the racemate by ion exchange chromatography. Deprotection and purification proceeded virtually quantitatively. The enantiomeric excess of starting material and product was the same as determined by HPLC on chiral stationary phases (Chiracel OD-H, hexane/isopropyl alcohol 9:1 for hydroxyphoshonate and QN-AX PF210108_1 (2009), methanol/TEA phosphate buffer 2 M (pH 4) 9:1 for aminophosphonic acid after

derivatisation with 2,4-dinitrobenzylchloroformate).[86] However, it was proved to be useful to extract the phthalic acid with ethyl acetate prior to the purification of the phosphonic acid with Dowex 50W/H^+. Deblocking of the amino group with hydrazine did not give satisfying amounts of the desired product.

As the isolated 2-amino-1-hydroxyethylphosphonic acid is levorotary, it has (*R*)-configuration which is also the configuration of the natural product. Unfortunately, it was not possible to increase the ee of the purified 2-amino-1-hydroxyethylphosphonic acid by crystallisation from water/ethanol. The racemate seemed to crystallise first.

In summary, the synthesis of (*R*)-2-amino-1-hydroxyethylphosphonic acid (**2.1**) could be accomplished in 6 steps starting from glycine with an overall yield of 49%. The best ee was 81% (in favour of the desired configuration). The most critical steps were the bond formation between the acid chloride and the phosphite, which could be overcome by using triisopropyl phosphite, and the enantioselective reduction of the diisopropyl α-oxophosphonate **8.22**.

9. Experimental Section

9.1 General Remarks

NMR spectra were recorded either on a Bruker DRX 600 (^{1}H: 600.13 MHz, ^{13}C: 150.92 MHz, ^{31}P: 242.94 MHz), Bruker Avance DRX 400 (^{1}H: 400.13 MHz, ^{13}C: 100.61 MHz, ^{31}P: 161.98 MHz) or a Bruker AV 400 (^{1}H: 400.27 MHz, ^{13}C: 100.65 MHz, ^{31}P: 162.03 MHz) spectrometer. Residual solvent proton peaks ($CHCl_3$: δ_H = 7.24 ppm; HOD: δ_H = 4.80 ppm; *d_6*-acetone CD_2H: δ_H = 2.05 ppm; *d_8*-toluene CHD_2: δ_H = 2.09 ppm) or $CDCl_3$ (δ_C = 77.23 ppm)/ d_8-toluene (δ_C = 21.40 ppm) were used to reference the spectra.

IR spectra were measured on a Bruker VERTEX 70 IR spectrometer either as films on a silicon disc or as ATR spectra.

Flash (column) chromatography was carried out with Merck silica gel 60 (230-400 mesh). Reaction progress and flash chromatography were monitored by TLC on Merck silica gel 60 F_{254} plates (0.25 mm thick). The spots were visualized by UV and/or dipping the plate into a molybdate solution [25 g of $(NH_4)_6Mo_7O_{24}$×4 H_2O and 1 g of $Ce(SO_4)_2$×4 H_2O in 500 mL of 10% aqueous H_2SO_4] followed by heating with a heat gun. Compounds containing an amino group were made visible by dipping into a ninhydrin solution (0.2% ninhydrin in ethanol 98%) again followed by heating the TLC plate with a heat gun.

Melting points are generally uncorrected and were determined on a Leica Galen III Reichert Thermovar.

Optical rotations were measured on a Perkin-Elmer 341 polarimeter in a 1 dm cell at 20°C.

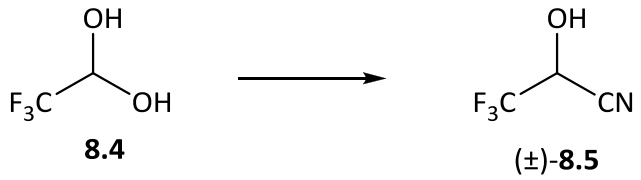
Enantiomeric excesses of chiral, nonracemic α-hydroxyphosphonate **8.23** and 2-amino-1-hydroxyethylphosphonic acid (**2.1**) were determined via HPLC on chiral stationary phases (for **8.23**: Chiracel-OD column, solvent: hexanes/isopropanol 9:1; for **2.1**: QN-AX PF210108_1 (2009), solvent: methanol/TEA phosphate buffer 2M (pH 4) 9:1).[87]

THF and Et_2O were dried by refluxing over potassium and $LiAlH_4$, respectively, and distillation before use. CCl_4 and 1,2 dichloroethane were distilled from P_2O_5 and stored over molecular sieves (4 Å). All other chemicals were used as purchased from Sigma-Aldrich, Acros, Fluka or Merck.

All yields are average values for optimised reaction conditions.

9.2 3,3,3-Trifluoro-1,2-dihydroxypropylphosphonic acid

*9.2.1 (±)-3,3,3-Trifluoro-2-hydroxypropanenitrile [(±)-**8.5**]*



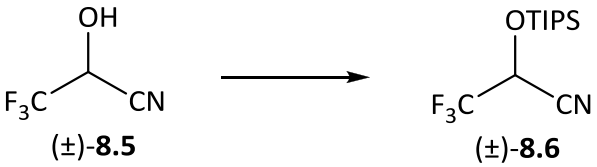
8.4 (±)-**8.5**

Sodium cyanide (1.862 g, 38 mmol) was dissolved in water (10 mL) and the stirred solution was cooled to 0°C. After dropwise addition of trifluoroacetaldehyde hydrate (**8.4**, 3.064 g, 20 mmol, Alfa Aesar, 75% in water) and H_2SO_4 (13 mL, 3 M), the solution was stirred for 24 h at ambient temperature. The reaction mixture was extracted with ethyl acetate (4 x 20 mL). The combined organic layers were dried (Na_2SO_4), concentrated under reduced pressure and dried in vacuo. The residue was purified by distillation (45 mm Hg/90-98°C) to yield hydroxynitrile **8.5** (2.961 g) as a colourless liquid which contained the desired product (53 mol% as judged by ^{1}H NMR) as well as ethyl acetate.[88] The crude product was used for the following reaction without further purification.

^{1}H NMR (400.27 MHz, $CDCl_3$, 4Mar2911/290 [KS1-007]): δ = 4.85 (br. s, 3H, 2 x OH and CH).

^{1}H NMR (400.27 MHz, D_2O, 4Mar2911/350 [KS1-007 + D_2O]): δ = 4.77 (q, J = 5.8 Hz, 1H, CH).

*9.2.2 (±)-3,3,3-Trifluoro-2-(triisopropylsiloxy)propanenitrile [(±)-**8.6**]*



(±)-**8.5** (±)-**8.6**

3,3,3-Trifluoro-2-hydroxypropanenitrile [(±)-**8.5**, 1.489 g, 11.9 mmol, 53mol% hydroxynitrile)] was dissolved in dry DMF (12 mL) under argon at ambient temperature. After addition of imidazole (2.395 g, 35.5 mmol) and TIPS-Cl (3.429 g, 17.8 mmol) stirring of the solution was continued for 15 h at room temperature. Water (50 mL) was added after stirring the mixture for another two h at 50°C and the reaction mixture was extracted with diisopropyl ether (3 x 20 mL). The combined organic layers were washed with water (30 mL), dried (Na_2SO_4) and concentrated under reduced pressure. The residue was purified by flash

chromatography (hexanes/CH_2Cl_2 = 4:1, R_f = 0.67) to give silyl ether **(±)-8.6** (1.779 g, 6.3 mmol, 50%) as a colourless oil.[89]

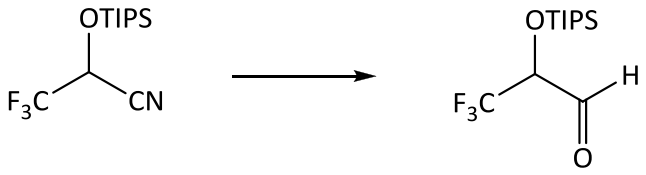
^{1}H NMR (400.27 MHz, $CDCl_3$, 4Mar3011/370 [KS1-008]): δ = 4.88 (q, J = 5.2 Hz, 1H, CF_3CH), 1.25-1.14 (m, 3H, 3xCHSi), 1.10, 1.08 (overlapping doublets, J = 5.7 Hz, J = 5.6 Hz, 18H, total of 6x CH_3 of TIPS).

^{13}C NMR (100.65 MHz, $CDCl_3$): δ = 121.05 (q, J = 283.3 Hz, CF_3), 123.54 (s, CN), 63.08 (q, J = 38.2 Hz, CHO), 17.47 (s, 6C, 6 x CH_3 of TIPS), 11.85 (s, 3C, 3 x CHSi).

IR (ATR): ν = 3407, 2949, 1703, 1347, 1266, 1204, 1153, 1109 cm^{-1}.

Anal. calcd. for $C_{12}H_{22}F_3NOSi$ (281.39): C 51.22%, H 7.88%, N 4.98%; found: C 51.70%, H 7.92%,N 4.88%.

*9.2.3 (±)-3,3,3-Trifluoro-2-(triisopropylsiloxy)propane [(±)-**8.7**]*

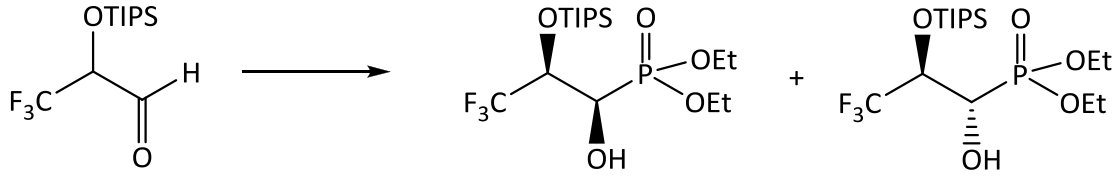


(±)-**8.6** (±)-**8.7**

DiBAl-H (4.92 mL, 1 M solution in hexane) was added to a stirred solution of (±)-3,3,3-trifluoro-2-(triisopropylsiloxy)propanenitrile [(±)-**8.6**, 1.141 g, 4.1 mmol] in dry toluene (12 mL) at -78°C under argon and stirring was continued for 1 h. Then Et_2O (30 mL), a saturated aqueous solution of NH_4Cl (30 mL) and H_2SO_4 (20 mL, 1.5 M) were added to the cooled solution and the resulting two-phase mixture was stirred at ambient temperature for 20 min. The phases were separated and the aqueous layer was extracted with Et_2O (3 x 20 mL). The combined organic layers were washed with water (30 mL), dried ($MgSO_4$) and concentrated under reduced pressure. The residue was dissolved in dry toluene (20 mL) and concentrated under reduced pressure to remove residual water. The oily residue was dried in vacuo to yield aldehyde **(±)-8.7** (0.934 g, 80%) as an oil. The crude product was used without further purification in the following step. As it seemed not to be sufficiently stable, no column chromatography was performed.

^{1}H NMR (400.27 MHz, $CDCl_3$, 4Apr0611 [KS1-012]): δ = 9.55 (quin, J = 2.1 Hz, 1H, CHO), 4.36 (qd, J = 7.0 Hz, 1.9 Hz, 1H, CF_3CH), 1.15-1.05 (m, 3H, 3x CHSi), 1.04 (d, J = 4.3 Hz, 9H, 3xCH_3 of TIPS), 1.01 (d, J = 4.3 Hz, 9H, 3xCH_3 of TIPS).

9.2. 4 (±)-(1R,2R*)- and (1R*,2S*)-Diethyl 3,3,3-trifluoro-1-hydroxy-2-(triisopropylsilyloxy)-propylphosphonate [(1R*,2R*)- and (1R*,2S*)-**8.8**]*



(±)-**8.7** *(1R*,2R*)*-**8.8** *(1S*,2R*)*-**8.8**

Diethyl trimethylsilyl phosphite (1.451 g, 6.9 mmol, 1.58 mL) was added dropwise to a stirred solution of (±)-3,3,3-trifluoro-2-(triisopropylsiloxy)propanal [(±)-**8.7**, 1.612 g, 5.7 mmol] under an argon atmosphere at -78°C in dry toluene (20 mL). The solution was allowed to warm up to room temperature over night, concentrated under reduced pressure and dried in vacuo. The residual oil was dissolved in ethanol (30 mL) and concentrated HCl (11 drops) was added. The solution was stirred for 2 h at ambient temperature followed by concentration under reduced pressure. Water (10 mL) was added andthe mixture was extracted with ethyl acetate (3 x 20 mL). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure to yield a crude mixture of (1*R**,2*R**)-**8.8** and (1*S**,2*R**)-**8.8**.

The diastereomers were separated by flash chromatography (hexanes/acetone = 3:1, R_f (1*R**,2*R**- **8.8**) = 0.21, R_f (*1S*,2R**-**8.8**) = 0.13) to yield (1*R**,2*R**)-**8.8** (0.858 g, 2.0 mmol, 35% from starting silylated nitrile) and (1*S**,2*R**)-**8.8** (0.154 g, 0.4 mmol, 7% from starting nitrile). The products were crystallized from hexanes.

(1*R**,2*R**)-**8.8:** mp 63-64°C.

^{1}H NMR (400.13 MHz, $CDCl_3$, Apr1511/51 [KS1-013A]): δ = 4.58 (quint, J = 6.8 Hz, 1.1 Hz, 1H, CF_3CH), 4.25 (m, 4H, 2 x OCH_2), 4.07 (m, J = 10.6 Hz, 1H, CHP), 2.84 (d, J = 9.8 Hz, 1H, OH), 1.33 (t, J = 7.1 Hz, 6H, 2 x CH_3), 1.28 - 1.17 (m, 3H, 3 x CHSi), 1.11 (d, J = 3.5 Hz) and 1.09 (d, J = 3.5 Hz, total of 18H, 6 x CH_3 of TIPS).

^{13}C NMR (100.61 MHz, $CDCl_3$, Apr1511/50 [KS1-013A]): δ = 124.3 (qd, J = 284.9 Hz, 21.9 Hz, CF_3), 69.6 (qd, J = 31.1 Hz, 3.2 Hz, CF_3-C), 66.1 (qd, J = 169.3 Hz, 1.9 Hz, CHP), 63.6 (d, J = 7.6

Hz, OCH_2), 62.8 (d, *J* = 7.6 Hz, OCH_2), 18.03 (s, 3C, 3 x CH_3 of TIPS), 18.01 (s, 3C, 3 x CH_3 of TIPS), 16.7 (d, *J* = 5.7 Hz, CH_3), 16.7 (d, *J* = 6.0 Hz, CH_3), 13.0 (s, 3C, CHSi).

^{31}P NMR (162.03 MHz, $CDCl_3$, 4Apr0811/511): δ = 19.6 (q, *J* = 3.9 Hz).

IR (Si, [KS1-013A]): ν = 3268, 2945, 2869, 1462, 1264, 1164, 1139, 1025, 971 cm $^{-1}$.

Anal. calcd. for $C_{16}H_{34}F_3O_5Psi$ (422.51): C 45.49%, H 8.11%; found: C 45.53%, H 8.05%.

(1*S**,2*R**)-**8.8:** mp 51-52°C.

^{1}H NMR (400.13 MHz, $CDCl_3$, Apr1511/41 [KS1-013B]): δ = 4.56 - 4.46 (m, 1H, CF_3CH), 4.25-4.11 (m, 5H, 2 x OCH_2 and CHP), 2.90 (br s, 1H, OH), 1.33 (td, *J* = 7.0 Hz, 0.3 Hz, 6H, 2 x CH_3), 1.20 - 1.03 (m, 21H, 3 x TIPS).

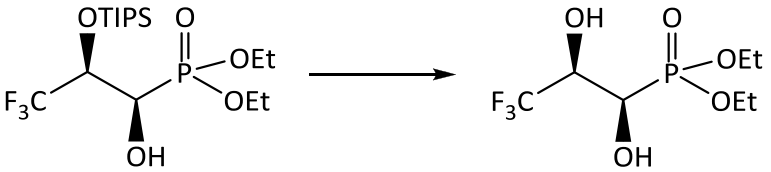
^{13}C NMR (100.61 MHz, $CDCl_3$, Apr1511/40 [KS1-013B]): δ = 124.1 (qd, *J* = 284.1 Hz, 3.4 Hz, CF_3), 72.2 (qd, *J* = 30.9 Hz, 6.3 Hz, CF_3C), 70.5 (d, *J* = 161.2 Hz, CHP), 63.4 (d, *J* = 5.8 Hz, OCH_2), 63.3 (d, *J* = 6.9 Hz, OCH_2), 18.1 (s, 3C, 3 x CH_3 of TIPS), 18.0 (s, 3C, 3 x CH_3 of TIPS), 16.6 (d, *J* = 9.0 Hz, 2C, 2 x CH_3), 12.7 (s, 3C, 3 x CHSi).

^{31}P NMR (162.03 MHz, $CDCl_3$, Apr1511/42 [KS1-013B]): δ = 18.5 (s).

IR (Si): ν = 3266, 2946, 2870, 1467, 1262, 1177, 1140, 1054, 1026 cm^{-1}.

Anal. calcd. for $C_{16}H_{34}F_3O_5PSi$ (422.51): C 45.49%, H 8.11%, O 18.93%; found: C 45.70%, H 7.97%, O 18.78%

9.2.5 (1R,2R*)-Diethyl 3,3,3-trifluoro-1,2-dihydroxypropylphosphonate [(1R*,2R*)-**8.9**] (Method A)*



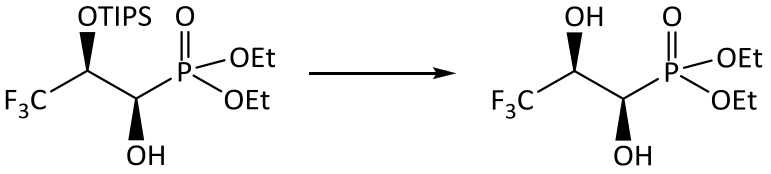
(*1R*,2R**)-**8.8** (*1R*,2R**)-**8.9**

The silylated phosphonate [(*1R*,2R**)-**8.8**, 0.775 g, 1.8 mmol] was dissolved in dry acetonitrile (40 mL) together with sodium fluoride (0.521 g, 9.0 mmol) and benzoic acid (1.098 g, 9.0 mmol). The resulting mixture was stirred for 16 h at 70°C under argon atmosphere. The cooled solution was concentrated under reduced pressure and the crude product was purified by flash chromatography (ethyl acetate, R_f = 0.45) to yield diol

(*1R**,*2R**)-**8.9** (0.477 g, 1.7 mmol, 94%). The product was crystallised from hexanes/CH_2Cl_2 to give colourless needles;[90] mp 38-40°C.

^{1}H NMR (400.13 MHz, $CDCl_3$, Mar2111/20 [KS1-005]): δ = 5.40 (br. s, 1H, OH), 4.75 (br. s, 1H, OH), 4.32-4.14 (m, 6H, 2 x OCH_2, CF_3CH and CHP), 1.34 (t, *J* = 7.1 Hz, 6H, 2 x CH_3).
^{13}C NMR (100.61 MHz, $CDCl_3$, Mar2111/22 [KS1-005]): δ = 124.4 (qd, *J* = 283.7 Hz, 22.8Hz, CF_3), 69.3 (qd, *J* = 31.2 Hz, 3.1 Hz, CF_3-C), 66.8 (qd, *J* = 166.7 Hz, 1.1 Hz, CH-P), 64.3 (d, *J* = 7.0 Hz, OCH_2), 63.9 (d, *J* = 7.4 Hz, OCH_2), 16.6 (d, *J* = 5.3 Hz, CH_3), 16.5 (d, *J* = 5.3 Hz, CH_3).
^{31}P NMR (161.98 MHz, $CDCl_3$, Mar2111/21 [KS1-005]): δ = 21.8 (q, *J* = 4.4 Hz).
IR (Si, [KS1-005]): ν = 3255, 2985, 1276, 1260, 1166, 1137, 1029 cm^{-1}.
Anal. calcd. for $C_7H_{14}O_5F_3P$ (265.19): C 31.59%, H 5.30%, O 30.06%; found: C 31.43%, H 5.18%, O 29.84%

9.2.6 (1R,2R*)-Diethyl 3,3,3-trifluoro-1,2-dihydroxypropylphosphonate [(1R*,2R*)-**8.9**] (Method B)*

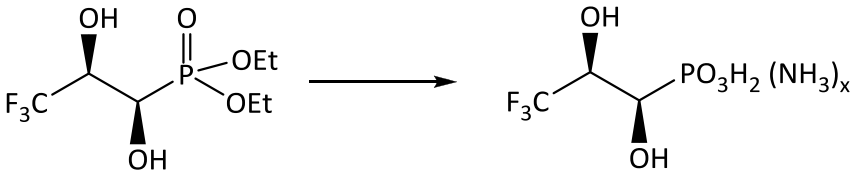


(*1R**,*2R**)-**8.8** (*1R**,*2R**)-**8.9**

Tetra-*n*-butylammonium fluoride (0.5 mL, 1M solution in THF) and acetic acid (0.03 mL, 98%) were added to a stirred solution of (*1R**,*2R**)-**8.8** (0.100 g, 0.24 mmol) in dry THF (3 mL) at -30°C. Stirring was continued for 6 h and then the solution was allowed to warm up to ambient temperature in the cooling bath. The reaction mixture was diluted with ethyl acetate (10 mL) and a saturated aqueous solution of $NaHCO_3$ (6 mL). The layers were separated, the organic phase was extracted with a saturated aqueous solution of $NaHCO_3$ (10 mL) and brine (10 mL), dried ($MgSO_4$) and concentrated under reduced pressure. The crude product was purified by flash chromatography (ethyl acetate/hexanes = 3:1, R_f = 0.38) to yield dihydroxyphosphonate (*1R**,*2R**)-**8.9** (0.045 g, 0.17 mmol, 71%)[91].

The spectra were identical to those reported for the compound prepared by Method A.

9.2.7 Ammonium salt of (1R, 2R*)-3,3,3-trifluoro-1,2-dihydroxypropylphosphonic acid [(1R*,2R*)-**8.1**]*



(*1R**,*2R**)-**8.9** (*1R**,*2R**)-**8.1**

A mixture of dihydroxyphosphonate (*1R**,*2R**)-**8.9** (0.330 g, 1.2 mmol), bromotrimethylsilane (1.470 g, 9.6 mmol, 1.27 mL) and allyltrimethylsilane (0.069 g, 0.6 mmol, 0.10 mL) in 1,2-dichloroethane (7.5 mL) was stirred for 3 d at ambient temperature. Afterwards the volatile components were removed under reduced pressure (0.5 mm Hg/room temperature, then 35°C). The residual liquid was dissolved in water/ethanol (10 mL, 1:1) and stirred for 3 h at room temperature. The solution was concentrated again to give a white powdery solid. Water (3 mL) and a concentrated ammonia solution (5 drops, 25%) were added. The solution was lyophilised to give the ammonium salt of (*1R**,*2R**)-**8.1** (0.219g, 99%); mp 172-175°C.

^{1}H NMR (400.13 MHz, D_2O, Mar3011/60 [KS1-006]): δ = 4.42 (qdd, *J* = 7.6 Hz, *J* = 4.8 Hz, *J* = 0.9 Hz, 1H, CF_3CH), 3.99 (dd, *J* = 13.0 Hz, 0.8 Hz, 1H, CHP).

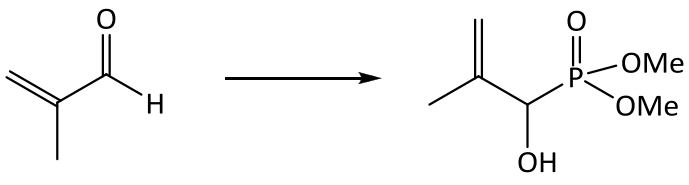
^{13}C NMR (100.61 MHz, D_2O, Mar3011/61 [KS1-006]): δ = 125.3 (qd, *J* = 282.2 Hz, 19.1 Hz, CF_3C), 69.7 (qd, *J* = 30.3 Hz, 1.8 Hz, CF_3), 66.6 (d, *J* = 147.6 Hz, CHP).

^{31}P NMR (161.98 MHz, D_2O, Mar3011/62 [KS1-006]): δ = 15.7 (s).

IR (ATR, [KS1-016]): ν = 3019, 2816, 1437, 1264, 1114, 1038, 970 cm^{-1}.

9.3 (±)-1-Hydroxy-2-oxopropylphosphonic acid

*9.3.1 (±)-Dimethyl 1-hydroxy-2-propenylphosphonate [(±)-**8.11**]*



(±)-**8.10** (±)-**8.11**

A saturated solution (0.1 mL) of NaOMe in methanol was added to a stirred mixture of methacroleine (**8.10**, 0.701 g, 10.0 mmol, 0.83 mL) and dimethyl phosphite (0.781 g, 10.0 mmol, 0.65 mL) in Et_2O (20 mL) under an argon atmosphere at -35°C. After stirring for 30 min at -35°C concentrated H_2SO_4 (9 drops) was added. The reaction mixture was allowed to warm to room temperature and the solvent was removed under reduced pressure. The crude product was dissolved in toluene/CH_2Cl_2 (10 mL, 1:1) filtered and dried in vacuo to yield hydroxyallylphosphonate (±)-**8.11** (1.579 g, 8.8 mmol, 88%) as colourless oil.[92] The product was used for the following reaction without further purification. For future experiments it might be useful to add H_2SO_4 in a quantity sufficient to neutralise the base and purify the crude α-hydroxyphosphonate by flash chromatography.

The recorded spectra are identical to those reported in the literature.[92]

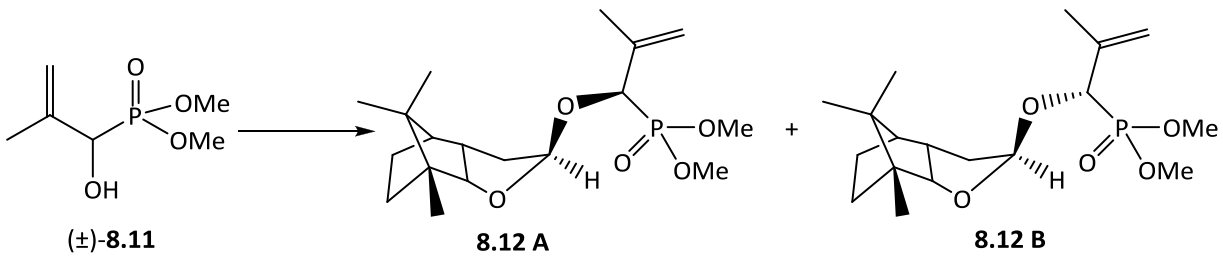
^{1}H NMR (400.13 MHz, $CDCl_3$): δ = 5.17 (dq, *J* = 4.9 Hz, 1.0 Hz, 1H, CH=), 5.05 (dq, *J* = 2.5 Hz, 1.0 Hz, 1H, CH=), 4.43 (dd, *J* = 12.8 Hz, 5.9 Hz, 1H, CHP), 4.01 (dd, *J* = 9.4 Hz, 5.9 Hz, 1H, OH), 3.788 and 3.785 (2 d, *J* = 10.8 Hz, 2 x 3H, 2 x OCH_3), 1.87 (dt, *J* = 3.8 Hz, 3H, CH_3).

^{13}C NMR (100.61 MHz, $CDCl_3$): δ = 140.8 (d, *J* = 3.8 Hz, C_q), 113.9 (d, *J* = 11.1 Hz, CH_2), 71.8 (d, *J* = 157.1 Hz, CH-P), 53.7 (d, *J* = 6.9 Hz, OCH_3), 53.6 (d, *J* = 7.1 Hz, OCH_3), 19.5 (d, *J* = 2.2 Hz, CH_3).

^{31}P NMR (161.98, $CDCl_3$, Jul2611/10 [KS2-008]): δ = 24.0 (s, integration: 1.00), 27.5 (s, integration: 0.07).

IR (ATR, [KS2-008]): ν = 3260, 2960, 1460, 1223, 1184, 1058, 1023, 905 cm^{-1}.

*9.3.2 Acetals of (±)-dimethyl 1-hydroxy-2-methylallylphosphonate (**8.12 A** and **8.12 B**) derived from Noe´s lactol and its dimer*



(±)-**8.11** **8.12 A** **8.12 B**

After stirring the crude α-hydroxyphosphonate [(±)-**8.11**, 2.748 g, 15 mmol] over molecular sieves (4Å) in dry CH_2Cl_2 (60 mL) for 15 min, the solution was cooled to 0°C. (+)-Noe´s lactol [(+)-MBF-OH, 2.944g, 15 mmol], *p*-toluenesulfonic acid monohydrate (0.094 g, 0.6 mmol)

were added and stirring was continued for 1 h. The molecular sieves were removed by filtration, the reaction mixture was extracted with a saturated aqueous solution of $NaHCO_3$ (3 x 20 mL), the organic layer was separated and dried ($MgSO_4$). The solution was concentrated under reduced pressure. The crude mixture was separated by flash chromatography [gradient: ethyl acetate/PE (1:1) → ethyl acetate, R_f (A) = 0.36 and R_f (B) = 0.29 in ethyl acetate] to yield diastereomers **8.12 A** (1.452 g, 4.1 mmol, 27 %) and **8.12 B** (1.005 g, 2.8 mmol, 19%) as oils.[76]

The acetalisation with (+)-MBF-O-MBF was performed similarly, except that only 0.5 equivalents relative to hydroxyphosphonate were used.

Diastereomer (1*R**,2*R**)-**8.12**: ^{1}H NMR (400.27 MHz, d_8-toluene, 4Jun0711/460 [KS2-006A]): δ = 5.17 (br d, *J* = 4.7 Hz, 1H, OCHO), 5.15 - 5.10 (m, 1H, CH=), 5.02 (sept, *J* = 1.6 Hz, 1C, CH=), 4.68 (d, *J* = 18.7 Hz, 1H CHO), 4.43 (dd, *J* = 9.5 Hz, 1.6 Hz, 1H, CHP), 3.53 (d, *J* = 10.4 Hz, 3H, OCH_3), 3.48 (d, *J* = 10.5 Hz, 3H, OCH_3), 2.88 - 2.77 (m, 1H, CH), 1.96 - 1.93 (m, 3H, CH_3C=), 1.90 - 1.82 (m, 1H, CH), 1.76 - 1.61 (m, 2H, 2 x CH), 1.50 - 1.35 (m, 2H, 2 x CH), 1.27 (ddd, *J* = 13.5 Hz, 9.1 Hz, 4.2 Hz, 1H, CH), 1.11 (tdd, *J* = 12.2 Hz, 3.8 Hz, 1.5 Hz, 1H, CH), 0.90 (s, 3H, CH_3), 0.89 (s, 3H, CH_3), 0.81 (s, 3H, CH_3).

^{13}C NMR (100.61 MHz, d_8-toluene): δ = 140.9 (s, 1C, C_q), 117.0 (d, *J* = 12.2 Hz, CH_2=), 106.8 (d, *J* = 12.2 Hz, OCHO), 90.9 (s, CHO), 75.5 (d, *J* = 169.0 Hz, CHP), 54.1 (d, *J* = 6.1 Hz, $POCH_3$), 53.8 (s, C_q), 53.4 (d, *J* = 6.1 Hz, $POCH_3$), 49.8 (s, C_q), 48.7 (s, CH), 41.5 (s, CH), 33.4 (s, CH_2), 27.8 (s, CH_2), 21.9 (s, CH_3), 21.6 (s, CH_2), 20.6 (s, CH_3), 19.7 (s, CH_3), 16.0 (s, CH_3).

^{31}P NMR (162.03 MHz, d_8-toluene, 4Jun0711/461 [KS2-006A]): δ = 22.1 (s).

IR (Si): ν = 2954, 1451, 1262, 1181, 1040 cm^{-1}.

Diastereomer (1*R**,2*S**)-**8.12**: ^{1}H NMR (400.27 MHz, d_8-toluene, 4May3111/30 [KS2-002B]): δ = 5.64 (d, *J* = 4.7 Hz, 1H, OCHO), 5.23 - 5.18 (m, 1H, CH=), 4.97 - 4.87 (m, 1H CH=), 4.49 (d, *J* = 13.3 Hz, 1H, CHO), 4.25 (dd, *J* = 9.5 Hz, 1.4 Hz, 1H, CH-P), 3.50 (d, *J* = 10.6 Hz, 3H, OCH_3), 3.47 (d, *J* = 10.6 Hz, 3H, OCH_3), 2.86 - 2.76 (m, 1H, CH), 1.97 - 1.92 (m, 3H, CH_3C=), 1.90 - 1.67 (m, 3H, CH_3), 1.47 - 1.33 (m, 2H, 2 x CH), 1.32 - 1.23 (m, 1H, CH), 1.15 - 1.05 (m, 1H, CH), 0.85 (s, 3H, CH_3), 0.83 (s, 3H, CH_3), 0.78 (s, 3H, CH_3).

^{13}C NMR (100.61 MHz, d_8-toluene): δ = 142.6 (s, =C_q), 114.6 (d, *J* = 10.7 Hz, CH_2), 110.3 (d, *J* = 5.4 Hz, OCHO), 91.3 (s, CHO), 76.7 (d, *J* = 161.4 Hz, CHP), 53.721 (d, *J* = 6.9 Hz, 2C, 2 x OCH_3),

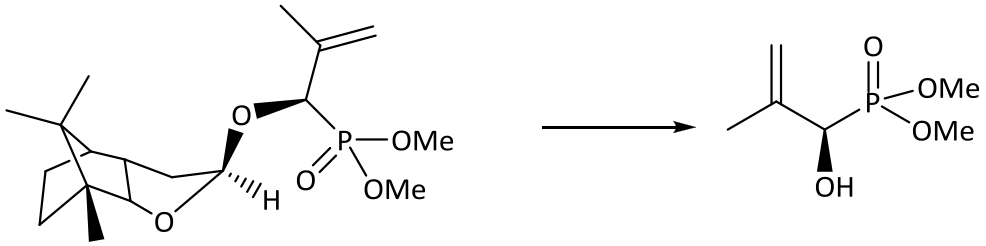
53.720 (s, C_q), 53.71 (d, J = 6.9 Hz, OCH_3), 49.77 (s, C_q), 48.75 (s, CH), 41.5 (s, CH), 33.81 (s, CH_2), 27.8 (s, CH_2), 21.9 (s, CH_3), 21.8 (s, CH_2), 21.0 (s, CH_3), 19.7 (s, CH_3), 15.8 (CH_3).

^{31}P NMR (162.32 MHz, d_8-toluene, 4May3111/31 [KS2-002B]): δ = 22.6 (s, integration: 1.00, desired product), 25.9 (s, integration: 0.06), 25.9 (s, integration: 0.04).

IR (Si): ν = 2954, 1458, 1259, 1182, 1099, 1033, 985 cm^{-1}.

*9.3.3 (R)- and (S)-Dimethyl 1-hydroxy-2-methylpropylphosphonate [(R)- and (S)-**8.11**]*

*Deprotection of diastereomer **8.12 A**:*



8.12 A (*R*)-**8.11**

p-Toluenesulfonic acid monohydrate (0.062 g, 0.4 mmol) was added to a stirred solution of diastereomer **8.12 A** (1.452 g, 4.1 mmol) in dry CH_2Cl_2 (30 mL) and dry methanol (9 mL) at room temperature. The resulting solution was stirred for 2.5 h before addition of a small amount of solid $NaHCO_3$. Stirring was continued for 10 min, the reaction mixture was filtered and concentrated under reduced pressure. The residue was purified by flash chromatography (ethyl acetate, R_f = 0.23) to give (*R*)-**8.11** (0.598 g, 3.3 mmol, 80%) as a colourless oil. The product was crystallized from diisopropyl ether to give colourless crystals; $[\alpha]_D^{20}$ = +2.81 (*c* 0.93, acetone).

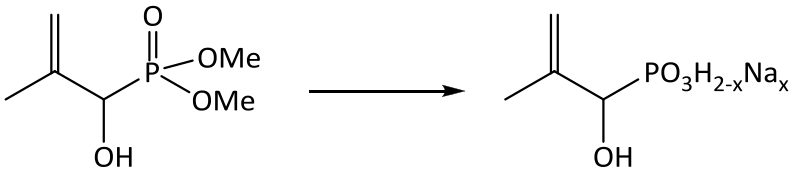
The NMR data are identical to those of the racemic compound.

*Deprotection of diastereomer **8.12 B**:*

Similarly, diastereomer **8.12 B** (1.005 g, 2.8 mmol) was deprotected to give α-hydroxyphosphonate (*S*)-**8.11** (0.424 g, 2.4 mmol, 86%). The product was crystallized from diisopropyl ether; $[\alpha]_D^{20}$ = -2.80 (*c* 0.97, acetone), mp 47-49°C.

The NMR data are identical to those of the racemic compound.

*9.3.4 Sodium salt of (±)- and (S)-1-hydroxy-2-methylpropylphosphonic acid [(±)- and (S)-**8.14**]*



(±)-**8.11** (±)-**8.14**

*Removal of methyl groups from racemic **8.11**:*

Dimethyl phosphonate (±)-**8.11** (0.410 g, 2.3 mmol) was dissolved in 1,2-dichloroethane (2.3 mL) under an argon atmosphere at 0°C. Allyltrimethylsilane (0.503 g, 4.4 mmol, 0.7 mL) and bromotrimethylsilane (1.183 g, 7.7 mmol, 1.02 mL) were added simultaneously to the stirred solution and stirring was maintained for 1 h at 0°C. All volatile components were removed under reduced pressure (1 mbar) at room temperature and the residue was dissolved in H_2O (10 mL). After adjusting the pH to 7 with 1 M NaOH, the aqueous solution was lyophilised to yield a sodium salt of (±)-**8.14** as a white solid (0.451 g, 2.3 mmol, 99%).[93]

^{1}H NMR (400.27 MHz, D_2O, 4Jul1311/70 [KS2-013 Lyo]): δ = 5.08 (d, J = 2.0 Hz, 1H, CH=), 5.01 (s, 1H, CH=), 4.24 (d, J = 13.6 Hz, 1H, CHP), 1.90 (s, 3H, CH_3).
^{13}C NMR (100.61 MHz, D_2O, 4Jun2411/70 [KS2-013]): δ = 144.4 (d, J = 3.2 Hz, C_q), 112.6 (d, J = 9.8 Hz, CH_2=), 74.4 (d, J = 150.0 Hz, CHP), 19.7 (d, J = 1.5 Hz, CH_3).
^{31}P NMR (162.03 MHz, D_2O, 4Jul1311/71 [KS2-013]): δ = 15.9 (s, integration: 1.00, desired product), 11.5 (s, integration: 0.03, unknown impurity), 19.6 (s, integration: 0.05, unknown impurity).
IR (ATR, [KS2-009]): ν = 3216, 1645, 1449, 1376, 1154, 1061,892 cm^{-1}.

It was assumed that the impurity with signal integration 0.03 in the ^{31}P NMR spectrum was caused by the dimeric species (see chapter 8.3.2).

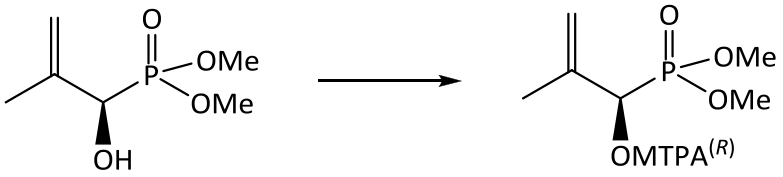
*Removal of methyl groups from α-hydroxyphosphonate (S)-**8.11**:*

Similarly, the corresponding sodium salt of (*S*)-phosphonic acid (*S*)-**8.14** (0.184 g, 0.9 mmol, 72%) was obtained from phosphonate (*S*)-**8.11** (0.235 g, 1.3 mmol), $[\alpha]_D^{20}$ = +3.8 (*c* 0.99, water).

^{1}H NMR (400.27 MHz, D_2O, 4Jul2011/30 [KS2-019]): δ = 5.10 (br. d, *J* = 2.9 Hz, 1H, CH=), 5.00 (br. s, 1H, CH=), 4.28 (d, *J* = 13.9 Hz, 1H, CH-P), 1.90 (s, 3H, CH_3).
^{13}C NMR (100.65 MHz, D_2O, 4Jul2611/42 [KS2-019]): δ = 143.8 (d, *J* = 3.5 Hz, C_q), 112.3 (d, *J* = 9.5 Hz, CH_2), 73.9 (d, *J* = 149.5 Hz, CHP), 19.2 (d, *J* = 1.5 Hz, CH_3).
^{31}P NMR (162.02 MHz, D_2O, 4Jul2011/31 [KS2-019]): δ = 16.4 (s).
IR (ATR, [KS2-019]): ν = 3226, 2832, 2729, 2324, 1648, 1128, 1075, 1021, 986, 892 cm^{-1}.

The impurities which were present in the sodium salt of racemic phosphonate **8.14** could not be detected for the sodium salt of the (*S*)-enantiomer. Therefore, I concluded that those impurities were derived from the impurity in the starting α-hydroxyphosphonate, which was not present in the enantiomerically pure starting material as it was purified by column chromatography prior to use contrary to the racemate.

*Preparation of (R)-Mosher esters exemplified for Mosher esters of (+)-hydroxyphosphonate **8.11**:*



(*R*)-**8.11** **9.1**

A solution of (+)-hydroxyphosphonate **8.11** (0.0216 g, 0.12 mmol, derived from diastereomer **8.12 A,** dried by coevaporation with toluene), dry pyridine (1 mL), dry CH_2Cl_2 (0.5 mL) and (*S*)-MTPACl (0.4 mL, 0.5 M solution in dry CH_2Cl_2, 0.2 mmol) was left at room temperature for 18 h. No spot of remaining hydroxyphosphonate was visible on TLC (hexanes/ethyl acetate = 1:1). Water (5 mL) and HCl (5 mL, 2 M) were added and the mixture was stirred for 10 min. It was extracted with CH_2Cl_2 (3 x 10 mL). The combined organic layers were washed

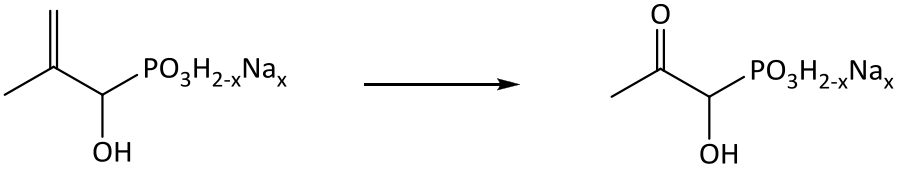
with a saturated aqueous solution of $NaHCO_3$ and water, dried (Na_2SO_4) and concentrated under reduced pressure. The residue was purified by flash chromatography (hexanes/ethyl acetate = 1:1, R_f = 0.60) to give the (*R*)-Mosher ester (0.037 g, 78%) as an oil.[93]

^{1}H NMR (400.13 MHz, $CDCl_3$), significant signals: δ = 3.57 (q, *J* = 1.0 Hz, 3H, OCH_3), 1.81 (br. s, 3H, CH_3C=).
^{31}P NMR (161.98 MHz, $CDCl_3$): δ = 19.73 (s).

Similarly, α-hydroxyphosphonate (-)-**8.11** derived from diastereomer 8.12 B was derivatised to give (*R*)-Mosher ester (0.031 g, 65%) as an oil.

^{1}H NMR (400.13 MHz, $CDCl_3$), significant signals: δ = 3.54 (q, *J* = 1.0 Hz, 3H, OCH_3), 1.91 (br. s, 3H, CH_3C=).
^{31}P NMR (161.98 MHz, $CDCl_3$): δ = 19.27 (s).

*9.3.5 Sodium salt of (±)- and (S)-1-hydroxy-2-oxopropylphosphonic acid [(S)-**6.2**]*



(*S*)-**8.14** (*S*)-**6.2**

*Ozonolysis of sodium salt of (±)-**8.14**:*

The sodium salt of α-hydroxyallylphosphonic acid (±)-**8.14** (0.349 g, 1.7 mmol) was dissolved in H_2O (1.7 mL) and the solution was diluted with methanol (17 mL). Ozone was bubbled through the stirred solution at -78°C for 4 min until the reaction mixture turned cobalt blue. Afterwards air was bubbled through the solution to remove excess ozone. PPh_3 (0.445 g, 1.7 mmol) dissolved in CH_2Cl_2 (2 mL), was added and stirring was continued at -78°C for 10 min. The mixture was allowed to warm up to room temperature. This was followed by removing the solvent under reduced pressure. The residue was dissolved in water (15 mL), CH_2Cl_2 (15 mL) was added, the phases were separated and the aqueous layer was extracted with CH_2Cl_2

(3 x 15 mL). Residual organic solvents were removed from the aqueous phase under reduced pressure and its pH was adjusted to pH 7 with NaOH (1M). Finally, the resulting solution was lyophilised to yield a sodium salt of 1-hydroxy-2-oxopropylphosphonate [(±)-**6.2**, 0.355 g, 1.7 mmol, 99%] as white, powdery solid.[93]

^{1}H NMR (400.27 MHz, D_2O, 4Jul2911/250 [KS2-023 0h]): δ = 4.64 (d, J = 18.7 Hz, 1H, CHP), 2.38 (s, 3H, CH_3).
^{13}C NMR (100.65, D_2O, 4Jul2911/252 [KS2-023 0h]): δ = 212.3 (d, J = 7.0 Hz, C=O), 80.0 (d, J = 120.4 Hz, CHP), 27.1 (s, CH_3).
^{31}P NMR (162.03 MHz, D_2O, 4Jul2911/251 [KS2-023 0h]): δ = 19.17 (s, integration: 0.06), 13.02 (s, integration: 0.01), 9.14 (s, integration: 1.00, desired product), 5.43 (s, integration: 0.11), 4.63 (s, integration: 0.04), 2.20 (s, integration: 0.05, phosphate).
IR (ATR, [KS2-023]): ν = 3179, 1689, 1417, 1358, 1076, 964 cm^{-1}.

Stability of racemic 1-hydroxy-2-oxo-propylphosphonic acid towards racemisation at pH 7:

A sample of compound (±)-**6.2** was analysed by NMR spectroscopy. Racemisation occurring due to the acidity of the proton in α-position to both the oxo- and the phosphonic acid group was investigated. Therefore, it was dissolved in D_2O and the ratio of the methyl group (δ = 3.07 ppm, which was not supposed to undergo H/D exchange) and the signal assigned to the CHP group (δ = 4.63 ppm, d, J = 18.7 Hz, 1H) was measured as a function of time. (The methyl group protons are supposed not to undergo H/D exchange, but the CHP proton is likely to exchange.) If H/D-exchange occurred, the ratio of the ^{1}H NMR resonances of CH_3 to CHP should increase with time (Table 9.1).

Entry	Reaction time	integration of CH_3	integration of CHP
1	0 h	3	0.97
2	6 h	3	0.96
3	24 h	3	0.88
4	5 days	3	0.00

Table 9.1. Stability of (±)-**6.2** towards racemisation at pH 7.

This clearly shows that racemisation due to the acidity of the hydrogen atom of the CHP group is a very slow process (about 10% in 24 h). However, after 5 days H/D exchange was nearly complete.

Stability of racemic 1-hydroxy-2-oxo-propylphosphonic acid towards racemisation under basic conditions:

The pH of a small sample of sodium salt of **6.2** was adjusted to pH 8.5 with 1M NaOH in H_2O. Water was removed and the sample was dissolved in D_2O. The integration ratio between the formate proton (formate forms during the reaction and is therefore present in the resulting reaction mixture) and the CHP proton was observed as a function of time. The principle is the same as already explained for pH 7, but this time the formate proton integral was used as a reference value instead of the methyl group integral. It is not likely to undergo H/D-exchange either (Table 9.2).

Entry	Time after adjustment of pH	Integration of formate proton	Integration of CHP proton
1	0 h	1.00	2.00
2	2 h	1.00	1.88
3	5 h	1.00	1.76

Table 9.2. Stability of (±)-**6.2** towards racemisation at pH 8.5.

These measurements show that the compound is sufficiently stable over the intended testing period in a solution of slightly basic pH.

Afterwards one drop of 1M NaOD in D_2O was added to the sample and the same ratio was analysed.

With this measurement it was possible to show that racemisation does occur at pH higher than 8.5 within a shorter period (Table 9.3).

Entry	Time after addition of NaOD	Integration of formate proton	Integration of CHP proton
1	0 h	1.00	2.00
2	16 h	1.00	0.82

Table 9.3. Stability of (±)-**6.2** towards racemisation at pH > 8.5.

However, the rate of H/D exchange proved to be much slower than originally expected.

Stability of racemic 1-hydroxy-2-oxopropylphosphonic acid towards cleavage of the P-C bond under acidic conditions:

One drop of DCl in D_2O was added to an NMR sample of **6.2** in D_2O and the ratio of phosphate, which was present from the beginning (δ = 1.58 ppm), to product was observed by ^{31}P NMR spectroscopy. As the data shows, P-C bond cleavage is negligible (5% at most in 25h):

Entry	Time after addition of DCl	Integration of phosphate signal	Integration of product signal
1	before addition	0.61	1
2	0 h	0.59	1
3	2 h	0.61	1
4	5 h	0.62	1
5	25 h	0.67	1

Table 8.4. Chemical stability of (±)-**6.2**

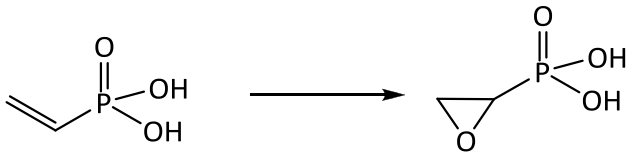
*Ozonolysis of (S)-**8.14**:*

Similarly, the sodium salt of (*S*)-1-hydroxy-2-methyl-2-propenylphosphonic acid was ozonolysed and gave the sodium salt of (*S*)-**6.2** (140 mg, 0.7 mmol, 99%) as white powdery solid.

^1H NMR (400.27 MHz, D_2O, 4Aug3111/130 [KS2-025]): δ = 4.65 (d, *J* = 19.0 Hz, 1H, CHP), 2.38 (s, 3H, CH_3).
^{31}P NMR (162.03 MHz, D_2O), 4Aug3111/131 [KS2-025]: δ = 9.4 (s, 1.00, desired product), 3.7 (s, 0.02, unknown byproduct), 1.17 (s, 0.05, phosphate).
IR (ATR, [KS2-023]): ν = 3171, 1690, 1357, 1070, 964 cm^{-1}.
$[\alpha]_D^{20}$ = +94.8 (*c* 1.01), when left for 5 h at room temperature: $[\alpha]_D^{20}$ = +93.2 (*c* 1.01), for 24 h: $[\alpha]_D^{20}$ = +85.2 (c 1.01).

9.4 2-Amino-1-hydroxyethylphosphonic acid

*9.4.1 (±)-Oxiran-2-yl-phosphonic acid [(±)-**8.17**]*



8.16 (±)-**8.17**

Vinylphosphonic acid (**8.16**, 3.995 g, 37 mmol, 90% in water) was dissolved in isopropyl alcohol (18 mL), NEt_3 (4.380 g, 43 mmol, 6 mL) was added to the solution at room temperature. $K_3EDTA \times 2\ H_2O$ (0.075 g, 0.2 mmol) , $Na_2WO_4 \times 2\ H_2O$ (0.627 g, 1.9 mmol) and H_2O_2 (9.5 mL, 30%) were added to the stirred solution. After 4 h a second portion of H_2O_2 (9.5 mL, 30%) was added and the mixture was kept at ambient temperature for 3 d.

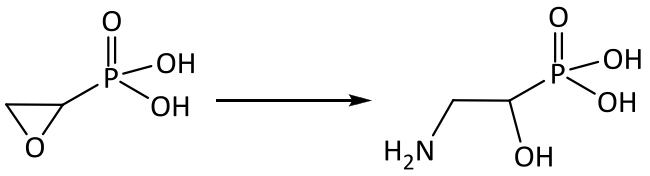
^1H and ^{31}P NMR revealed the degree of conversion of vinylphosphonic acid to the corresponding epoxide **(±)-8.17** to be 80 mol%. The formation of 1,2-dihydroxyethylphosphonic acid, which was the major side product if the reaction was carried out at higher temperatures could be reduced to 4 mol% when the epoxidation was performed at room temperature for 3 d.

A spatula tip of MnO_2 was added to destroy excess H_2O_2. The mixture was filtered (celite moistened with ethanol) and concentrated under reduced pressure. The crude epoxide (±)-**8.17** was used without further purification for the following step.[82]

^1H NMR of the reaction mixture before workup (400.27 MHz, D_2O + NaOD, 4Mar1411/60 [KS3-001 roh 4]): *δ(vinylphosphonic acid)* = 6.28 - 6.12 (m, 1H, CHP), 6.03 - 5.78 (m, 2H, CH_2). *δ(epoxide)* = 3.51 (q, *J* = 7.0 Hz, 1H, CHP), 3.08 - 3.02 (m, 1H, CH_2), 3.00-2.91 (m, 1H, CH_2).

^{31}P NMR of the reaction mixture before workup (162.03 MHz, D_2O + NaOD): 14.5 (s, caused by diol, 4%), 11.0 (s, epoxide, 80%), 10.6 (s, vinylphosphonic acid, 16%).

*9.4.2 (±)-Phosphaisoserine [(±)-**2.1**]*



(±)-**8.17** (±)-**2.1**

The crude triethylammonium salt of the epoxyphosphonic acid (±)-**8.17** (30 mmol) was dissolved in an aqueous ammonia solution (50 mL, 25%) and stirred for 3 d at room temperature. The reaction mixture was then concentrated under reduced pressure and the residue purified by ion exchange chromatography (Dowex 50W/H^+, water) to yield (±)-2-amino-1-hydroxyethylphosphonic acid (±)-**2.1** (2.092 g, 15 mmol, 50%) as crystalline solid; [94] TLC: isopropanol/H_2O/NH_3(25%) = 6:3:2, R_f = 0.18. (±)-Phosphaisoserine was crystallised from water/ethanol to give colourless, cubic crystals; mp 273°C (decomposition).

^{1}H NMR (400.13 MHz, D_2O, Mar2411/44 [KS3-002]): δ = 4.00 (td, J = 9.9 Hz, 3.3 Hz, 1H, CHP), 3.31 (ABXP system, J_{AB} = 13.3 Hz, J_{HH} = 6.5 Hz, J_{HP} = 3.3 Hz; J_{AB} = 13.3 Hz, J_{HH} = 9.9 Hz, J_{HP} = 6.3 Hz, 2H, CH_2N).

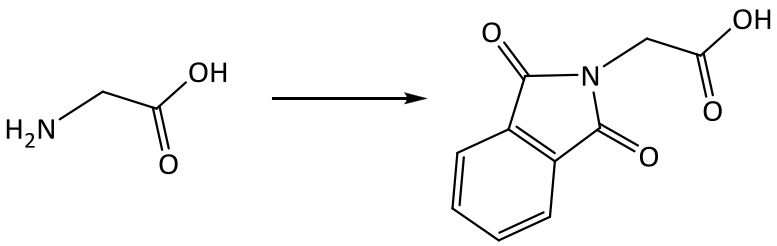
^{13}C NMR (100.61 MHz, D_2O, Mar2411/40 [KS3-002]): δ = 65.7 (d, J = 155.3 Hz, CH-P), 41.9 (d, J = 8.72 Hz, CH_2).

^{31}P (162.03 MHz, D_2O, Mar2411/45 [KS3-002]): δ = 15.0 (s).

IR (ATR, [KS3-021]): ν = 3138, 2837, 2659, 2578, 2270, 1628, 1538, 1076, 914 cm^{-1}.

Anal. calcd. for $C_2H_8NO_4P$ (141.05): C 17.03%, H 5.72%, N 9.93%; found: C 17.25%, H 5.48%, N 9.70%.

*9.4.3 Phthalimidoacetic acid (**8.20**)*

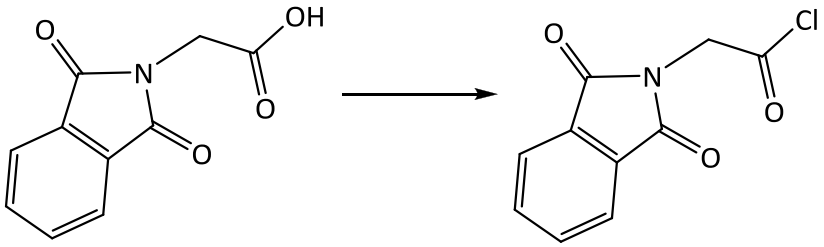


8.19 **8.20**

A stirred mixture of glycine (**8.19**, 6.890 g, 93 mmol) and phthalic anhydride (14.050 g, 95 mmol) was heated to 180°C. It melted. The reaction mixture was kept at that temperature for 10 min (or until the mixture solidifies again). On cooling the brown crystalline solid was crystallized from water to give pure **8.20** (17.173 g, 83 mmol, 89%) as colourless needles;[95] mp 190 - 192°C (literature: mp 192°C [95]).

^{1}H NMR (400.27 MHz, d_6-acetone, 4Apr1311/170 [KS3-013/1 Mutterlauge]): 11.50 (br. s, 1H, COOH), 7.95 - 7.87 (sym m, 4H, CH_{arom}), 4.44 (s, 2H, CH_2).

*9.4.4 Phthalimidoacetyl chloride (**8.21**)*

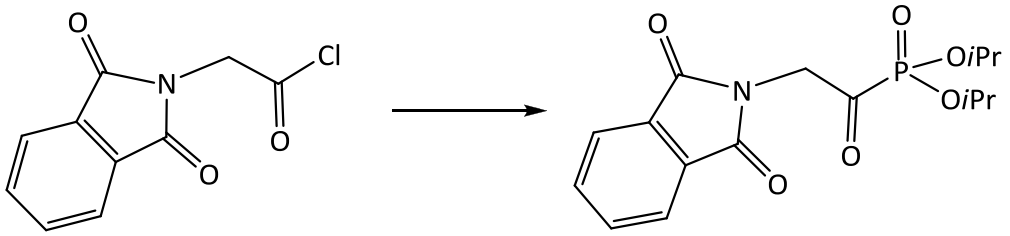


8.20 **8.21**

Phthalimidoacetic acid (**8.20**, 17.173 g, 84 mmol) was refluxed in $SOCl_2$ (20 mL) for 1.5 h. Excess $SOCl_2$ was removed under reduced pressure at room temperature and the residue was crystallised from hexanes/toluene to yield acid chloride **8.21 (**16.910 g, 76 mmol, 90%) as pale yellow crystals;[85] mp 80-82°C.

^{1}H NMR (400.27 MHz, $CDCl_3$, May0511/81 [KS3-023]): 7.92 - 7.86 (m, 2H, CH_{arom}), 7.80 -7.74 (m, 2H, CH_{arom}), 4.80 (s, 2H, CH_2).
^{13}C NMR (100.61 MHz, $CDCl_3$, May0511/80 [KS3-023]): 169.3 (s, C=O), 166.8 (s, 2 x C=O), 134.9 (s, 2C, CH_{arom}), 131.84 (s, 2C, 2 x C_{arom}), 124.2 (s, 2C, 2 x CH_{arom}), 47.8 (s, CH_2).
IR (Si, [KS3-016]): ν = 2936, 1803, 1776, 1722, 1404, 1381, 1110, 999, 939 cm^{-1}.

*9.4.5 Diisopropyl 1-oxo-2-phthalimidoethylphosphonate (**8.22**)*



8.21 **8.22**

Acid chloride **8.21** (16.246 g, 73 mmol) was dissolved in dry CH_2Cl_2 (75 mL). Triisopropyl phosphite (19.0 mL, 16.034 g, 77 mmol) was added and the resulting orange solution was stirred at room temperature for 30 min. The reaction mixture was concentrated under reduced pressure and the crude α-oxo-phosphonate was crystallised from hexanes/diisopropyl ether to give homogenous α-oxo-phosphonate **8.22** (20.677 g, 57 mmol, 80%) as slightly yellow crystals; mp 88-90°C.

^{1}H NMR (400.13 MHz, $CDCl_3$, May0511/91 [KS3-028B]): δ = 7.88 -7.82 (m, 2H, CH_{arom}), 7.75 - 7.69 (m, 2H, CH_{arom}), 4.88 (d, *J* = 3.6 Hz, 2H, CH_2), 4.81 (septd, *J* = 7.1 Hz, 6.2 Hz, 2H, 2 x OCH), 1.38 (d, *J* = 6.2 Hz, 6H, 2 x CH_3), 1.37 (d, *J* = 6.2 Hz, 6H, 2 x CH_3).

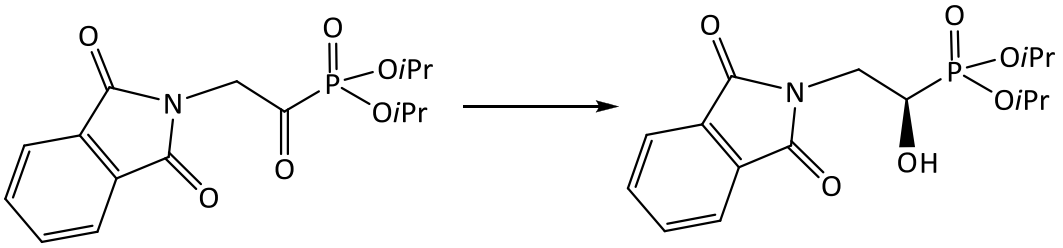
^{13}C NMR (100.61 MHz, $CDCl_3$, May0511/90 [KS3-028B]): δ = 204.4 (s, *J* = 177.5 Hz, CO), 167.5 (s, 2C, 2 x C(O)N), 134.5 (s, 2C, 2 x CH_{arom}), 132.3 (s, 2C, 2 x C_{arom}), 123.8 (s, 2C, 2 x CH_{arom}), 74.2 (d, *J* = 7.3 Hz, 2C, 2 x OCH), 48.1 (d, *J* = 69.3 Hz, CH_2), 24.3 (d, *J* = 3.7 Hz, 2C, 2 x CH_3), 24.0 (s, *J* = 4.8 Hz, 2C, 2 x CH_3).

^{31}P NMR (161.98 MHz, $CDCl_3$, May0511/92 [KS3-028B]): δ = -4.6 (s).

IR (Si): ν = 2983, 2935, 1779, 1723, 1468, 1411, 1387, 1263, 1100, 996 cm^{-1}.

Anal. calcd. for $C_{16}H_{20}NO_6P$ (353.31): C 54.39%, H 5.71%, N 3.96%, O 27.17%; found: C 54.44%, H 5.63%, N 3.92%, O 26.92%.

*9.4.6 (*R*)- and (±)-Diisopropyl 1-hydroxy-phthalimido-ethylphosphonate [(R)-**8.23**] (Method A)*



8.22 (*R*)-**8.23**

*Synthesis of (R)-**8.23**:*

(*R*)-2-Methyloxazaborolidine (0.2 mL, 1M solution in dry toluene, commercial reagent) was added to a stirred solution of the α-oxophosphonate (**8.22**, 0.645 g, 1.8 mmol) in dry THF (3 mL) under an argon atmosphere at -50°C. Borane dimethyl sulphide (1.2 mL, 1M solution in dry THF) was added slowly with a syringe pump (0.1 mm/min) over a period of 3 h. The reaction mixture was allowed to warm up to room temperature and afterwards methanol (2 mL) was added. The solvent was evaporated under reduced pressure and the residue was purified by flash chromatography (ethyl acetate, R_f = 0.48) to give (*R*)-enantiomer of α-hydroxyphosphonate (*R*)-**8.23** (0.436 g, 1.2 mmol, 68%, ee = 52% by HPLC on chiral column) as colourless crystals.[96,70]

^{1}H NMR (400.27 MHz, $CDCl_3$, 4Jul0511/210 [KS3-063]): δ = 7.87 - 7.81 (m, 2H, 2 x CH_{arom}), 7.74 - 7.67 (m, 2H, 2 x CH_{arom}), 4.82 - 4.68 (m, 2H, 2 x OCH), 4.22-4.06 (m, 2H, CH_2), 4.02 - 3.93 (m, 1H, CHP), 3.16 (br. s, 1H, OH), 1.36 (d, *J* = 6.2 Hz, 3H, CH_3), 1.32 (d, *J* = 6.2 Hz, 3H, CH_3), 1.31 (d, *J* = 6.3 Hz, 3H, CH_3), 1.30 (d, *J* = 6.3 Hz, 3H, CH_3). *Oodelally*

^{13}C (100.61 MHz, $CDCl_3$, Jul2711/10 [KS3-063]): δ = 168.8 (s, 2C, C=O), 134.3 (s, 2C, CH_{arom}), 132.3 (s, 2C, C_{arom}), 123.7 (s, 2C, CH_{arom}), 72.08 (d, *J* = 7.3 Hz, OCH), 72.1 (d, *J* = 7.4 Hz, OCH) , 66.9 (d, *J* = 161.7 Hz, CHP), 40.3 (d, *J* = 7.2 Hz, CH_2), 24.33 (d, *J* = 2.0 Hz, CH_3), 24.30 (d, *J* = 2.4 Hz, CH_3), 24.2 (d, *J* = 5.1 Hz, CH_3), 24.1 (d, *J* = 4.8 Hz, CH_3).

^{31}P (162.03 MHz, $CDCl_3$, 4Jul0511/211 [KS3-063]): δ = 19.0 (s, integration: 1.00, desired product), 4.4 (s, integration: 0.01, phosphite).

IR (ATR, [KS3-049]): ν = 3275, 1709, 1397, 1210, 977 cm^{-1}.

Anal. cald. for $C_{16}H_{22}NO_6P$ (355.33): C 54.08%, H 6.24%, N 3.94%, O 27.02%; found: C 54.04%, H 6.13%, N 3.90%, O 26.80%

Analytical HPLC: Chiralcel OD-H, hexanes/isopropanol 90:10, (*R)*-enantiomer: t_R = 7.29 min, (*S)*-enantiomer: t_R = 8.70 min; ee 52%.

Comment: The (*R*)-Mosher of the chiral, nonracemic α-hydroxyphosphonate could not be prepared to determine the ee as the hydroxy group is too hindered to be esterified with (*S*)-Mosher chloride even at higher temperatures (50°C) and by prolonged heating (3 d). However, ^{31}P NMR spectra of the resulting reaction mixtures were used to qualitatively prove excess of the desired enantiomer.

The two enantiomers of the racemate were separated by preparative HPLC on a chiral stationary phase (Chiralcel OD-H) to determine their melting points, spectra and optical rotations. Their spectroscopic data were identical.

(*R*)-**8.23**

$[a]_D^{20}$ = - 19.7 (c 0.965, CH_2Cl_2)

mp 120-122°C (*iso*prpanol)

^{1}H NMR (600.13 MHz, $CDCl_3$, 6Sep0111/11 [KS3-040 R]): δ = 7.84 - 7.81 (m, 2H, 2 x CH_{arom}), 7.71 - 7.68 (m, 2H, 2 x CH_{arom}), 4.79 - 4.69 (m, 2H, 2 x OCH), 4.18 (X part of ABXP-system, ddd, *J* = 9.7 Hz, *J* = 7.6 Hz, *J* = 3.3 Hz, 1H, CHP), 4.02 (ABXP-system, A-part:, J_{AB} = 14.5 Hz, *J* = 9.7 Hz, *J* = 7.9 Hz; B-part: J_{AB} = 14.5 Hz, *J* = 6.8 Hz, *J* = 3.3 Hz, 2H, CH_2N), 1.35 (d, *J* = 6.5 Hz, 3H, CH_3), 1.31 (d, *J* = 6.3 Hz, 6H, 2 x CH_3), 1.28 (d, *J* = 6.1 Hz, 3H, CH_3).

^{13}C (150.90 MHz, $CDCl_3$, 6Sep011/10 [KS3-040 R]: δ = 168.7 (s, 2C, C=O), 134.3 (s, 2C, CH_{arom}), 132.2 (s, 2C, C_{arom}), 123.6 (s, 2c, CH_{arom}), 72.1 (d, *J* = 6.3 Hz, OCH), 72.0 (d, *J* = 6.6 Hz, OCH), 66.7 (d, *J* = 162.1 Hz, CHP), 40.2 (d, *J* = 7.4 Hz, CH_2N), 24.3 (d, *J* = 3.5 Hz, 2C, 2 x CH_3), 24.13 (d, *J* = 4.5 Hz, CH_3), 23.06 (d, *J* = 4.5 Hz, CH_3).

^{31}P (242.93 MHz, $CDCl_3$, 6Sep0111/12 [KS3-040 R]): δ = 20.4 (s).

IR (ATR, [KS3-040 R]): ν = 3266, 2980, 1708, 1397, 1209, 977 cm^{-1}.

(*S*)-**8.23**

$[a]_D^{20}$ = + 18.4 (c 1.040, CH_2Cl_2)

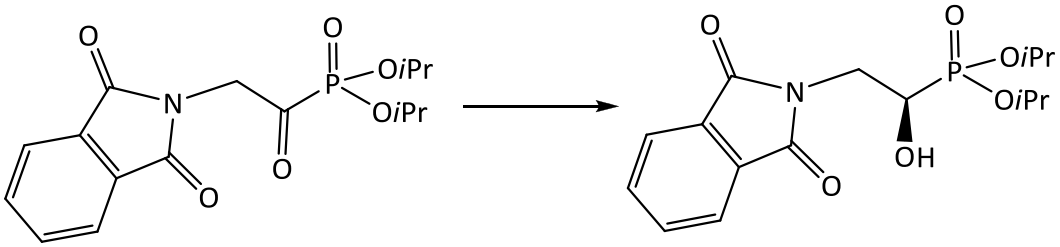
mp 121-123°C (*iso*propanol)

*Synthesis of (±)-**8.23**:*

(±)-Diisopropyl 1-hydroxy-2-phthalimidoethylphosphonate [(±)-**8.23**, 0.106 g, 33%] was prepared from α-oxophosphonate **8.22** (0.321 g, 0.91 mmol) using the same method as for the chiral, nonracemic one except that (*R*)-2-methyl-oxazaborolidine was omitted.

The recorded NMR spectra are identical to those of (*R*)-**8.23**.

*9.4.7 (R)-Diisopropyl 1-hydroxy-2-phthalimidoethylphosphonate [(R)-**8.23**] (Method B)*



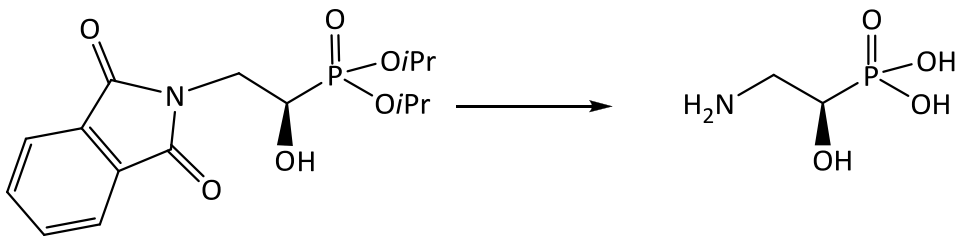
8.22 (*R*)-**8.23**

(*R*)-2-Methyloxazaborolidine (2.2 mL, 1M solution in toluene) was added to a stirred solution of α-oxophosphonate **8.22** (6.540 g, 18.5 mmol) under argon atmosphere at room temperature. The mixture was cooled to -78°C immediately and catecholborane (2.446 g, 20.4 mmol, 2.2 mL) was added in one portion. The reaction mixture was stored at -23°C for 5 h and was then allowed to warm up to room temperature. It was diluted with Et_2O (200 mL) and extracted with a saturated aqueous solution of $NaHCO_3$ (5 x 50 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure.

The crude hydroxyphosphonate was purified by flash column chromatography (ethyl acetate, R_f = 0.53) to yield chiral, nonracemic (*R*)-**8.22** (5.369 g, 15.1 mmol, 82%, ee 84% by HPLC).[70,96]

The ee could not be increased by crystallisation as the racemate crystallised slightly more readily and the desired (*R*)-enantiomer was enriched in the mother liquor.

*9.4.8 (R)-Phosphaisoserine [(R)-**2.1**]*



(*R*)-**8.22** (*R*)-**2.1**

A mixture of protected (*R*)-phosphaisoserine [(*R*)-**8.23**, 5.369 g, 15.1 mmol, ee 78%] and HCl (100 mL, 6M) was refluxed for 18 h. The reaction mixture was cooled and subsequently concentrated under reduced pressure. The residue was dried in vacuo and then dissolved in ammonia solution (100 mL, 25%) The resulting mixture was stirred at 50°C for 24 h to give (*R*)-2-amino-1-hydroxyethylphosphonic acid [(*R*)-**2.1**] in admixture with a phthalic acid derivative, possibly phthalimide.

The solution was concentrated under reduced pressure, the residue dissolved in water (10 mL) and extracted with ethyl acetate (3 x 20 mL). The aqueous phase was separated and concentrated under reduced pressure. The crude product was applied to a column filled with Dowex 50W/H^+ (water as eluent: TLC: R_f = 0.19, *i*PrOH/H_2O/NH_3(25%) = 6:3:2, ninhydrin reagent for detection) to give (*R*)-phosphaisoserine [(*R*)-**2.1**. 1.833 g, 13.0 mmol, 86%]; ee will have to be determined.

Recorded NMR spectra and melting point were identical to that of (±)-**2.1**.

10. Summary

Organophosphonates, compounds containing a direct P-C bond, play an important role in medicine agriculture and industry. They can be mineralised and used as an additional or sole phosphorus source by microorganisms including some pathogens but not vertebrates.
Their biodegradation is of crucial interest as it helps to understand their role in the global phosphorus redox cycle and to get a closer insight into their mode of action. Much work has already been done to clarify the biosynthesis of several phosphonates, showing remarkable and unexpected results. However, only little is known about their biodegradation.

In the course of my master thesis I have synthesised several phosphonates which will be used to study the biodegradation of the antibiotic fosfomycin and 2-amino-1-hydroxyethylphosphonic acid, a cell component of amoeba.
The 3,3,3-trifluoro analogue of (1*R**,2*R**)-1,2-dihydroxypropylphosphonic acid was synthesised using trifluoroacetaldehyde hydrate as starting material which was converted to the corresponding cyanohydrine. Its hydroxyl group was TIPS-protected and the resulting α-silyloxynitrile was reduced with DIBAl-H to give racemic 3,3,3-trifluoro-2-triisopropylsilyloxypropanal. Then, diethyl trimethylsilyl phosphite was added to the carbonyl group to form a P-C bond. The two resulting diastereomers were separated by flash chromatography and only the one with (1*R**,2*R**)-configuration was completely deprotected to give the desired compound as a racemate. Its synthesis could be accomplished by a six step reaction sequence with an overall yield of 6%.
Furthermore, (±)- and (*S*)-1-hydroxy-2-oxopropylphosphonic acid were synthesised in three and five steps, respectively. Starting from methacroleine, racemic dimethyl 1-hydroxy-2-methyl-2-propenylphosphonate was prepared. Its two enantiomers were obtained by chemical resolution with Noe's reagents. The racemate and the (*S*)-enantiomer of this α-hydroxyphosphonate were converted to the sodium salts of the acids and ozonolysed to give β-oxophosphonates. It was found by measuring the H/D exchange at C-1 by ^{1}H NMR spectroscopy and the optical rotation that racemisation at neutral pH in 24 h is negligible.
A new synthetic route to (±)-2-amino-1-hydroxyethylphosphonic acid comprising two steps was optimised. Epoxidation of commercially available vinylphosphonic acid followed by ammonolysis of the epoxide yielded 40% of the target compound.

Finally, (*R*)-2-amino-1-hydroxyethylphosphonic acid was synthesised in six steps with an ee of 81% and an overall yield of 49%. The starting material, phthalimidoacetic acid, was prepared from glycine and was converted to the acid chloride which was subsequently reacted with triisopropyl phosphite. The key step of this synthesis was enantioselective reduction of the thus formed α-oxophosphonate using catecholborane and (*R*)-2-methyloxazaborolidine as catalyst. Removal of the protective groups finished the synthesis of the compound needed in g quantities for biodegradative studies.

11. Zusammenfassung

Organophosphonate, Verbindungen mit einer direkten P-C-Bindung, spielen in der Medizin, Landwirtschaft und Industrie eine wichtige Rolle. Sie können von Mikroorganismen, einige pathogene eingeschlossen, nicht aber von Wirbeltieren, mineralisiert und als zusätzliche oder alleinige Phosphorquelle verwendet werden. Ihr biologischer Abbau ist von entscheidender Bedeutung, da er hilft, ihre Rolle im globalen Phosphor-Redox-Zyklus zu verstehen und ein tieferes Verständnis ihrer Wirkungweise zu gewinnen. Verschiedene Arbeitsgruppen haben sich bereits mit der Aufklärung der Biosynthese einiger spezieller Phosphonate beschäftigt und dabei bemerkenswerte und gleichzeitig erstaunliche Ergebnisse erhalten. Die Biodegradation von Phosphonsäuren ist hingegen noch immer kaum erforscht.

Im Rahmen meiner Masterarbeit habe ich verschiedene Phosphonate synthetisiert, die bei Studien zur Aufklärung der Biodegradation von Fosfomycin und der 2-Amino-1-Hhydroxyethylphosphonsäure eingesetzt werden sollen.

Das 3,3,3-Trifluor-substituierte Analogon der (1*R**,2*R**)-1,2-Dihydroxypropylphosphonsäure wurde ausgehend von Trifluoracetaldehyd-Hydrat synthetisiert, das in das Cyanhydrin überführt wurde. Seine Hydroxygruppe wurde TIPS-geschützt und das resultierende α-silyloxy-substituierte Nitril wurde mit DIBAl-H zum Aldehyd reduziert. Dann wurde Diethyltrimmethylsilylphosphit an die Carbonylgruppe addiert, um die P-C-Bindung zu bilden. Die beiden resultierenden Diastereomere wurden mittels Flash-Chromatography getrennt und vollständig deblockiert. Die gewünschte Verbindung entstand als Racemat, dessen Synthese in sechs Schritten in einer Gesamtausbeute von 6% gelang.

Des Weiteren wurde racemische und enantiomerenreine (*S*)-1-Hydroxy-2-oxopropylphosphonsäure in drei bzw. fünf Schritten synthetisiert. Ausgehend von Methancrolein wurde racemisches Dimethyl 1-hydroxy-2-methyl-2-propenylphoshonat hergestellt. Seine beiden Enantiomere wurden durch chemische Racematspaltung mit Noe Reagentien erhalten. Das Racemat und das (S)-Enantiomer dieses α-Hydroxyphosphonats wurden in die Natriumsalze der Säuren überführt, die mittels Ozonolyse die β-Oxophosphonate lieferten. Durch Messung des H/D-Austausches am C-1 und des Drehwerts

wurde gefunden, dass die Racemisierung bei neutralem pH innerhalb von 24 h unbedeutend ist.

Darüber hinaus wurde eine neue zweistufige Synthese für (±)-2-Amino-1-hydroxyethylphosphonsäure ausgehend von kommerziell erhältlicher Vinylphosphonsäure optimiert. Diese wurde epoxidiert und das entstandene Epoxid anschließend mit Ammoniak regioselektiv geöffnet. Dies liefert die gewünschte Zielverbindung mit einer 40%igen Ausbeute.

Schließlich wurde (*R*)-2-Amino-1-hydroxyethylphosphonsäure in einer sechsstufigen Synthesesequenz mit einer Gesamtausbeute von 49% und einem ee von 81% hergestellt. Das Ausgangmaterial, die Phthalimidoessigsäure, die aus Glycin hergestellt und in das Säurechlorid überführt wurde, wurde mit Triisopropylphosphit umgesetzt. Der Schlüsselschritt dieser Synthese ist die enantioselective Reduktion des α-Oxophosphonates mit Catecholboran und (*R*)-2-Methyloxazaborolidin als Katalysator. Abspaltung der Schutzgruppen schloss die Synthese der Verbindung, die in Gramm-Mengen für Abbaustudien benötigt wird, ab.

12. Curriculum Vitae

Name: Schiessl Katharina

Degree: Bakk. rer. nat.

Date of Birth: 01.03.1988

Place of Birth: Eisenstadt, Austria

Education	
Sept. 1998 - July 2006	**BG/BRG/BORG Eisenstadt** Matura passed with honours
Oct. 2006 - June 2009 	***Bachelor's program*** in ***Chemistry***, Universität Wien *Degree:* Bakk. rer. nat. (Bakkalaurea der Naturwissenschaften) *Title of Bachelor Thesis:* „Ruthenium-Nitrosyl-Komplexe und ihre Verwendung in Medizin, Technik und moderner Synthese“ *Passed:* with honours (average grade: 1.26) *Supervisor:* Ao. Univ.-Prof. Dr. Vladimir B. Arion
Oct. 2009 - Sept 2011 	***Master's program*** in ***Chemistry***, Universität Wien *Planned Degree:* MSc (Master of Science) *Emphasis on:* Organic Chemistry, Inorganic Chemistry and Theoretical Chemistry *Titel of Master Thesis:* “On the Biodegradation of Fosfomycin and Phosphaisoserine” *Supervisor:* Ao. Univ.-Prof. Dr. Friedrich Hammerschmidt *Average grade:* 1.07

Experiences	
July 2004 & 2005	**Bank Burgenland Leasing GmbH** various activities for the accounting department
July 2006 & April 2007	**Investigation, Research & Consulting Center** design of concentration tests
August 2006	**ARC Seibersdorf research GmbH**, department: Life Sciences chemical analysis of food and other consumer goods

August 2007	**AIT Austrian Institute of Technology**, department: SENS (Sustainable Energy Systems)
Aug. 2008 & 2009	**Wiener Zeitung GmbH** Logistic assistant
June 2010	**Universität Wien** research assistant of Ao. Univ.-Prof. Dr. F. Hammerschmidt project work on organophosphonates
Aug.-Sept. 2010	**Katholieke Universiteit Leuven**, Belgium scientific project on the "Electronic structure of osmium(IV) complexes with azole ligands as potential antitumor agents studied by CASSCF/CASPT2-SO-RASSI ab initio methods"
Oct. 2010 - Jan. 2011	**Universität Wien** tutor in the practical course: "Spezielle Synthesechemie"

Publications	
Instanbul, 2009	*poster:* "Electronic Structure of $[RuCl_3(Hind)_2NO]$", ESERA Conference

Achievements	
May 2006	Landeswettbewerb des Landes Burgenland, 32. Österreichische Chemieolympiade (3rd price)
Feb. 2006	16. Burgenländischer Fremdsprachenwettbewerb AHS, Französisch (2nd price)
Feb. 2008, Jan. 2010 & Jan. 2011	merit scholarship from Universität Wien (average grade: 1.15, 1.24 and 1.12, respectively)

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