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Prodrugs of Biologically Active Phosphate Esters

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Dedicated to Prof. Dr. Bernd Jastorff on the occasion of his 60th birthday

Abstract—Bioactivatable protecting groups represent an enormously powerful tool to increase bioavailability or to generally help deliver drugs to cells. This approach is particularly valuable in the case of biologically active phosphates because of the high intrinsic hydrophilicity and the multitude of biological functions phosphate esters exhibit inside cells. Here, the most prominent masking groups used so far are introduced. The stability and toxicology of the resulting prodrugs is discussed. Finally, this review tries to cover briefly some of the work that describes the usefulness and efficiency of the approach in various application areas.

Introduction

Phosphate esters play a dominant role in the physiology of cells and hence are essential to any organism. Most prominent is the participation of phosphate esters as a structural and functional element in DNA, RNA, and its monomeric building blocks, the occurance as a post-translational signal in proteins and as a head group in phospholipids. Furthermore, carbohydrates are often phosphorylated, sometimes with single phosphates, like in the cyclic nucleotides or in GPI ankers, sometimes heavily, as in inositol polyphosphates and phosphoinositides.

Most of the non-polymeric phosphate esters that occur in cells might be considered lead compounds for the development of drugs, as the majority of them are directly or indirectly involved in intracellular signaling or the assembly of DNA or RNA. In fact, DNA and RNA themselves or fragments thereof, in form of anti-sense oligonucleotides and iRNAs or in gene therapy, are considered useful for the treatment of diseases.

Phosphate esters usually have a low pK_a, often between 1 and 2. Subjected to the physiological pH in the range of 7.0–7.4, the compounds are permanently deprotonated and therefore negatively charged. The negative charge on phosphate esters is responsible for a variety of its properties.[1] First, other negatively charged, or nucleophilic, compounds are repelled due to Coulomb interactions, resulting in a remarkable stability against non-enzymatic hydrolysis with half-lives that are easily in the order of hundreds of years.[2] Second, compounds with phosphates are mostly impermeant to cellular membranes, unless active transport, endo/exocytosis, or viral fusion is involved. Regardless of the nature of the phosphate ester, its spatial distribution is crucial for its function. Therefore, phosphates serve as functional units in biologically active molecules that prevent the spreading of the chemical signal from one cell to the other, thus generating intracellular signals that are regulated or at least controlled.

When investigating these chemical signals or, for instance, the action of a given RNA molecule, it would be desirable to elevate the concentration of that particular phosphate ester inside the living cell. Likewise, there is a great need to introduce modified nucleotides to the cytosol in order to interfere with viral replication. Due to the impermeant nature of the molecules, in some cases the activation relies on intracellular phosphorylation by the cell itself or the compounds have to be administered via comparably violent physical or biochemical methods like electroporation, microinjection, or transfection. These often create large changes in the electrolyte homeostasis and subsequently, artifacts in the physiological results and prohibit the treatment of large populations of cells or whole organs for medicinal purposes. For these reasons, chemists created masking groups to allow the delivery of phosphate or phosphonate esters to the interior of the cell and to increase

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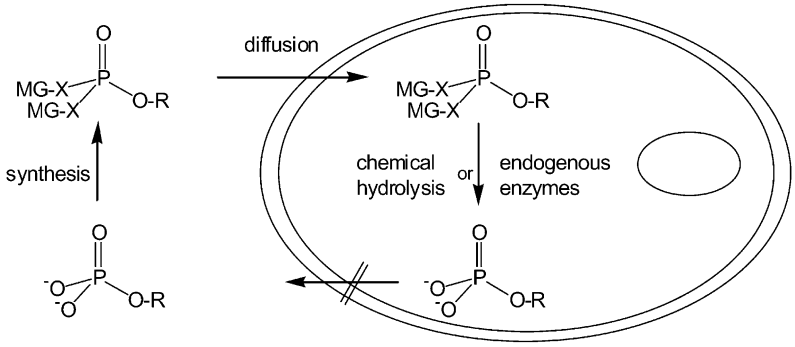


Figure 1. A suitable prodrug concept for phosphate esters: masking groups (MG) that allow diffusion across the plasma membrane are introduced by chemical synthesis. Inside cells the groups are removed and the phosphate ester may accumulate.

bioavailability. The resulting non-charged compounds are usually able to diffuse across the plasma membrane (Fig. 1). Once inside the cells, the masking groups are removed by chemical or enzymatic hydrolysis, regenerating the charged phosphate or phosphonate esters, making the molecule again impermeant to cell membranes and biologically active.

Quite a number of review articles have summarized the work on phosphate prodrugs in the past.[3,4] The bulk of work on prodrugs of phosphates was performed to allow delivery of biologically active nucleoside monophosphates to the cytosol as antiviral or anticancer drugs. Most of this work has already been competently reviewed.[2,5,6] The present paper will therefore predominantly concentrate on the different approaches to the design of phosphate prodrugs and examples of this design for molecules other than nucleoside monophosphates. Nevertheless, a brief update on this highly important area of medicinal chemistry will be included. At this point, I would like to mention that the latter subject is incompletely covered with respect to the literature available.

Chemically Labile Phosphate Protecting Groups

Early attempts to create uncharged phosphate derivatives relied on the synthesis of simple phosphate triesters.[7,8] Because phosphate triesters are uncommon in nature, specific enzymes that may cleave them have not been identified. Hence, the cleavage of the protecting group usually depends exclusively on chemical hydrolysis, although there is some indication that diphenyl phosphonate prodrugs may be converted enzymatically.[9] Phosphate trialkylesters and phosphonate dialkylesters are undoubtly sensitive to nucleophilic attack of hydroxyl ions at the phosphorous atom. The ethyl ester of cyclic adenosine 3′,5′-monophosphate (cAMP) is shown to have a half-life of about 13 days at 56 °C.[7] Faster hydrolysis rates in the min to h range have been found for benzyl esters, especially when electron-donating substituents are attached to the aromatic ring,[8] mainly because the phosphate serves as an excellent leaving group.[10]

Phosphate monoesters, however, have two negative charges to be masked. Because of the stability of the phosphate diester, the hydrolysis of the second protecting group relies on enzymatic catalysis, as by phosphodiesterases. Approaches that employ pure chemical cleavage of phosphate triesters have therefore rarely been successful. Exceptions are diphenyl phosphonates of 9-[2-(phosphonomethoxy)ethoxy]adenine prodrugs[11] and *N*-phosphonomethyl dipeptides as endopeptidase inhibitors.[9] Eventually, Meier introduced a cyclic protecting group that is released by a tandem reaction to circumvent the problem.[12] The *cyclo*Sal(igenyl) group formed a six-membered ring masking the phosphate, hence forming a phenolic and a benzylic phosphate ester (Fig. 2). Since the phenyl ester was more labile, it hydrolysed first, via an attack at the phosphorous. The resulting *o*-hydroxybenzyl group was then spontaneously released by C–O bond cleavage.[13] Substitutions of the aromatic ring allowed fine-tuning of chemical lability. The approach was successfully applied to various anti-HIV-nucleosides[14–16] and the potency of the anti-viral activities was comparable to derivatives with enzymatically cleavable masking groups. The half-life time of electron-donor-substituted *cyclo*Sal groups was 7–35 h in medium containing 10% fetal calf serum.[17] Other antiviral agents were also successfully prepared as uncharged *cyclo*Sal derivatives and tested for potency. For instance, the brivudin prodrug was tested against Epstein–Barr virus.[18,19]

Bioactivatable Phosphate Protecting Groups

Masking groups for the negative charges of phosphates or other functional groups that are cleaved by intracellular enzymes, but are stable outside cells, are of particular interest to medicinal chemists. Intracellular enzymatic hydrolysis ensures rapid conversion of prodrugs to the charged derivative and hence, traps the

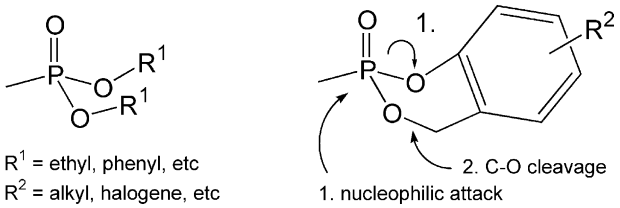


Figure 2. Phosphate triester prodrugs rely on chemical hydrolysis. The cleavage rate is largely dependent on the nature of the residues R.

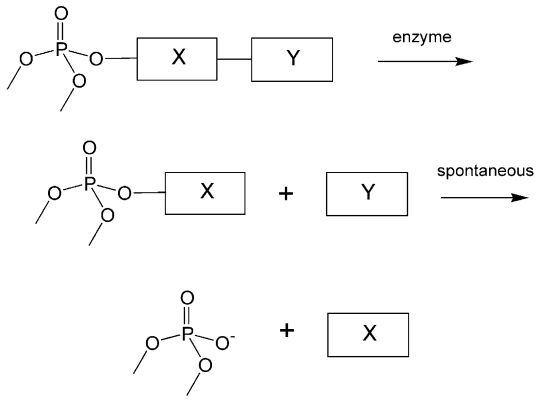


Figure 3. The commonly used 'tripartate' concept features an enzymatic cleavage site in some distance from the phosphorous. Usually a carboxylic ester is employed as enzyme-labile moiety. The resulting intermediate is intrinsically unstable, thus liberating the charged phosphate group.

subsequently charged compound inside the cell (Fig. 1). This technique could be used to cause the accumulation of biologically active compounds inside cells, decreasing the required dose to be administered. However, the selection of bioactivatable protecting groups to choose from is surprisingly small. One reason is that phosphate triesters are not common in cells and therefore nature does not provide 'phosphate triesterases'. The uncharged prodrug should be designed in a way that allows enzymatic cleavage at some distance from the phosphate, followed by the spontaneous release of the linker that connects the phosphate and the cleavage site (Fig. 3). This concept is frequently referred to as the 'tripartate prodrug concept'.[20] However, the rather small number of spontaneously decaying intermediates seems to limit the possibilities of the chemists.

This principle was used very early for penicillins,[21] where an acetoxymethyl (AM) ester group was employed to mask the carboxy group. In this approach, the acetate is enzymatically hydrolyzed and the remaining hydroxymethylene ester of the penicillin would spontaneously fall apart to release penicillin and form formaldehyde. Later, similar groups like pivaloyloxymethyl (POM) or ethoxycarbonyloxyethyl groups were shown to increase the bioavailability of the ß-lactam antibiotics significantly.[22,23] These derivatives are on the market under the names pivampillicin and bacampillicin, respectively. This methodology was transferred to phosphates by Farquhar and co-workers in the early eighties, when acyloxymethyl esters were demonstrated to show suitable half-lives on model compounds.[24,25] Since then a fairly large number of acyloxymethyl and, to a lesser extent, the corresponding methylene-substituted acyloxyalkyl esters substituted at the methylene bridge, have been synthesized (see Fig. 4a and below). The synthesis typically relies on the alkylation of the phosphate by an acyloxyalkyl halogenide, in the presence of a sterically hindered base, in dry organic solvent. In some cases a reaction employing silver salts of the phosphate derivatives is preferred,[25,26] as for the preparation of the tri(acetoxymethyl) ester of inorganic phosphate.[27] In order to avoid the generation of formaldehyde or carboxylic acid, alkoxycarbonyloxyalkyl groups (Fig. 4b) were introduced to the ACE inhibitor fosinopril and the antiviral compound (*R*)-9-[phosphonomethoxy]propyl)adenine (PMPA), respectively.[28]

To increase the distance between the phosphate or phosphonate and the enzymatic hydrolysis site, mainly in light of the more restricted and slow cleavage of phosphate diester intermediates, 4-acetoxybenzyl esters were introduced as bioreversable groups.[10] After cleavage of the acetate, the electron-donating hydroxy group destabilized the benzyl group that would decompose via a benzyl carbonium ion (Fig. 5). The benzyl esters were formed from benzyl alkohols and chloro phosphates and phosphonates, respectively.

Friis and Bundgaard expanded the concept of bringing a larger distance between the phosphate and the site of enzymatic attack. The α-acyloxyalkyl ester prodrug groups were designed in such a way, that after enzymatic cleavage, a hydroxy group that led to a re-esterification, was generated, thereby forming a lactone as a leaving group. After preparing and testing phenyl phosphate model compounds, the [3-(2-acetoxyphenyl)propionyl]oxymethyl group (Fig. 6) appeared to be most promising.[4]

Using similar principles, an even more elaborate masking group was introduced by Glazier et al. A carboxymethyl group attached to the benzylic methylene group (Fig. 7) allowed, presumably, a fast elimination reaction due to the higher acidity of the single benzylic proton and the formation of the stable trans-4-hydroxycinnamic acid or its ester (refs 40 and 45 in ref 2).

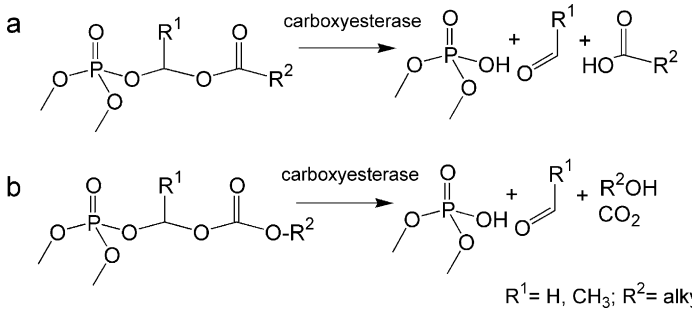


Figure 4. Enzymatic hydrolysis of acyloxyalkyl esters (a) liberates the phosphate and generates an aldehyde, a carboxylate, and two protons. Carbonic diesters (b) produce an alcohol and carbon dioxide next to the aldehyde.

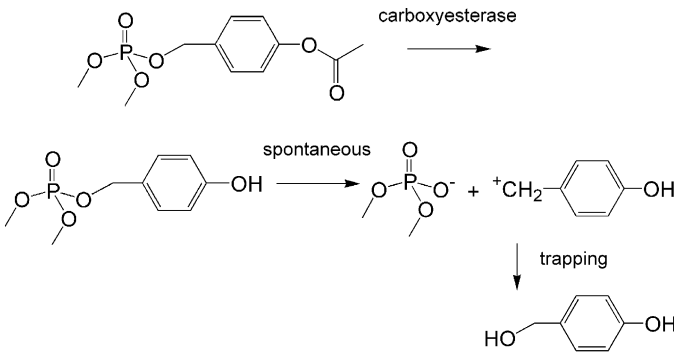


Figure 5. Acetoxybenzyl esters utilize the lability of the 4-hydroxybenzyl ester to allow spontaneous cleavage after enzymatic removal of the acetate.

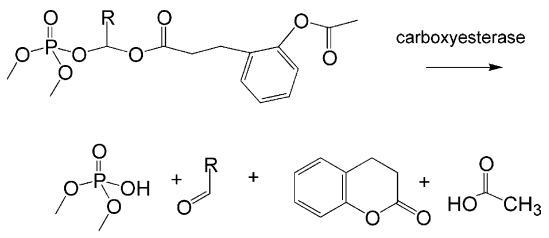


Figure 6. A 'tetra-partate' approach, where the enzymatic hydrolysis is distant to the phosphate, relies on the formation of a phenolic nucleophile and the subsequent formation of a lactone (R = H or alkyl).

A bidental protecting group was developed based on substituted 1,3,2-dioxaphosphorinane-2-yl derivatives (Fig. 8).[29] The enzyme-labile ester was located at the α-position to the cyclophosphate oxygen. Upon cleavage, the group spontaneously formed an aldehyde, which was in turn subject to β-elimination. This reaction generated acrolein, thus liberating the desired phosphate monoester in a single enzymatic activation step. In this tandem reaction an enzymatic attack on a negatively charged species, namely a phosphate diester, was avoided. The drawback of this method is certainly the generation of several new stereogenic centers that makes analysis, as well as kinetic studies with the mixture of diastereomers, more difficult. The synthesis required the formation of 1-(pivaloyloxy)-1,3-dichloropropane and its reaction with silver salts of the phosphate ester. After deprotection, the cyclic phosphate might be used as a phosphorylating agent under Mitsunobu conditions.[29]

Another approach that relies on an intramolecular elimination reaction, employs thioethanol derivatives. An enzymatically liberated thiolate attacks the α-carbon of the phosphate ester and releases the phosphate spontaneously, thereby eliminating thiirane.[30,31] As enzymatically removable moieties either *S*-(2-hydroxyethyl)sulfidyl groups or *S*-acyl groups were used. The *S*-[(2-hydroxyethyl)sulfidyl]-2-thioethyl (DTE, Fig. 9a) esters were subjected to a reductase-mediated activation process.[32] A variety of esterase labile *S*-acyl-2-thioethyl (SATE, Fig. 9b) esters were prepared and tested, exhibiting different lipophilicity and hence biological activity (see below).[31]

The advantage in synthesizing various SATE groups is that the bioactivatable group is stable enough to allow the preparation of a P(III) phosphitylating reagent and

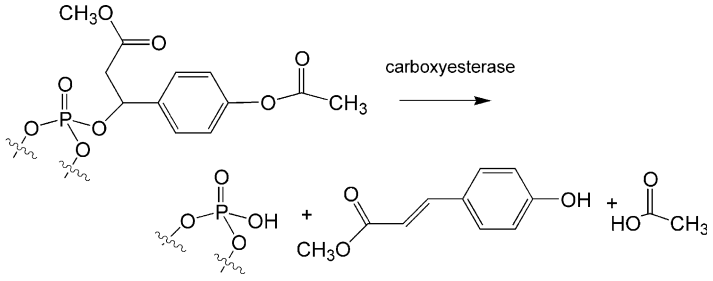


Figure 7. Increased acidity at the α-position was intended to increase spontaneous cleavage rates after enzymatic activation.

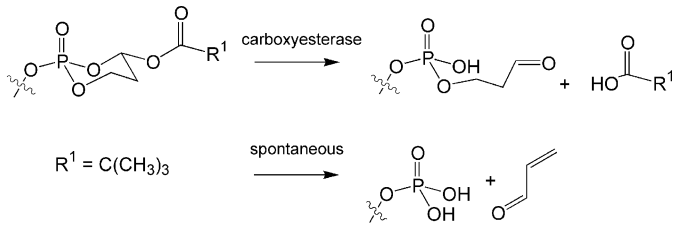
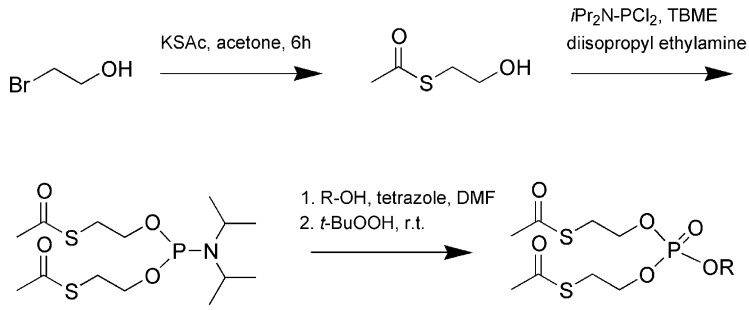


Figure 8. This cyclic protecting group liberates the phosphate monoester via an enzymatically induced tandem reaction.



Scheme 1. Stepwise building of the bis(SATE)phosphoramidite reagent. This allows for the introduction of a masking group-bearing phosphate in a single step.

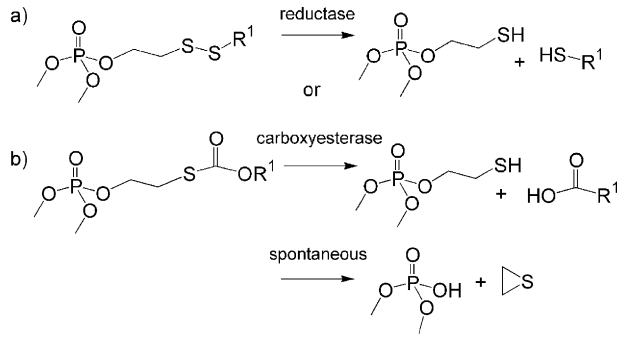


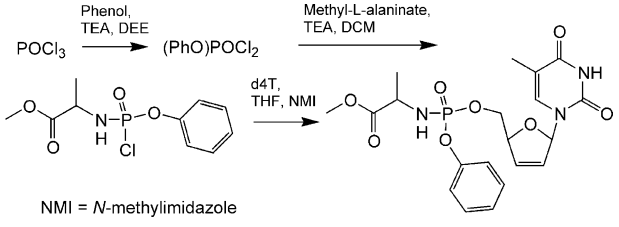
Figure 9. Both, the *S*-[(2-hydroxyethyl)sulfidyl]-2-thioethyl (DTE) group (a) and the *S*-acyl-2-thioethyl (SATE) group (b) give a ethylene thiol that spontaneously decays by forming a thiirane.

subsequent introduction of the phosphate triester in one final procedure (Scheme 1).

A similar synthetic approach was successful in introducing a 2-hydroxyethyl 1-β-D-thioglucoside phosphotriester group and its acetyl esters to AZT. These groups were designed to be bioactivatable by glycosidases. However, hydrolysis of the glycosidic bond was not found.[33]

A particular class of enzyme-labile protecting groups was developed for the delivery of antiviral nucleoside prodrugs: a combination of aryl ester and amino acid phosphoramidate (Scheme 2). The amino acid usually carried a methyl ester.

Upon intracellular enzymatic hydrolysis, the carboxylate would nucleophilically attack the phosphorous leading to the release of the aromatic moiety. Surprisingly, electron-donating or -accepting substituents on the aryl group did not influence the release rate in a predictable way.[30,34] The increase in lipophilicity, however, was suspected to be responsible for enhanced antiviral potency.[34]

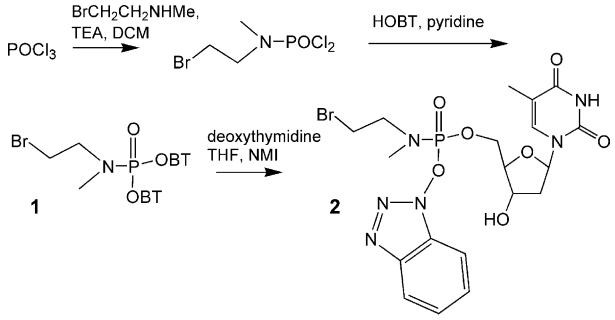


Scheme 2. Synthetic pathway to the aryl alaninyl phosphoramidate prodrug of 2′,3′-dideoxy-2′,3′-didehydro thymidine (d4T).[34,35]

The intermediate mixed anhydride spontaneously hydrolyzed to give the phosphoramidate. The existence of an intracellular phosphoramidase that mediates the observed formation of the nucleoside monoesters seem probable, but is still speculative.[34,37–40] Metabolic pathways starting with a chemical hydrolysis of the aryl ester seemed to participate in the prodrug delivery only to a minor extent. There is now a fairly large amount of work going into variations of the aryl,[34,41] the amino acid,[36,42] and the amino acid ester moieties.[42,43] The synthesis of phosphoramidates started with the monosubstitution of $POCl_3$ with the aryl alcohol followed by treatment with the amino acid ester hydrochloride in the presence of triethylamine. The phosphorochloridate was finally used to phosphorylate the 5′-hydroxy group or its equivalent of the nucleoside derivative (Scheme 2). Overall yields were reported to be in the 30–50% range under conditions where *N*-methylimidazole alone was used[41] and up to 79% when pyridine/*N*-methylimidazole was employed. In some cases, *O*-phosphorylation of the base was observed.[44]

Very recently, novel nucleoside phosphoramidate prodrugs were introduced which bear haloethyl or piperidyl substituents and which were combined with an activated phosphate ester, for instance a benzotriazolyl ester.[45,46]

The haloethyl methyl phosphoramidate **1** was prepared from $POCl_3$ by stepwise reaction with the secondary amine and 1-hydroxybenzotriazole (HOBT) to give the uncharged thymidyl phosphoramidate **2** (Scheme 3). As a result of experiments under artificial conditions, the activation mechanism of **2** was explained to be a mixture of aziridinium ion formation and non-specific



Scheme 3. Synthesis of anti-proliferative dTMP prodrugs, that provide an aziridine precursor and that use benzotriazolyl (OBT) or nitrofurylmethyl (not shown) moieties as delivery groups.

hydrolysis of the latter, to the desired thymidine monophosphate or the ethanolamino derivative, derived from substitution of the bromine. In the case of the uncharged compound **2**, very short half-lives were observed and the nucleophile HOBT seemed to form various intermediates.

It should be mentioned that the aziridine-forming, and hence cytotoxic activity, of nitroheterocyclic phosphoramidates is the desired biological activity for prodrugs that rely on the bio-reduction of the nitro group to release and, in that way deliver, the cytotoxic phosphoramide mustards to hypoxic cells.[47]

It appeared to be a surprise that the benzotriazolyl piperidyl phosphoramidate analogue of thymidine significantly inhibited the growth of L1210 leukemia cells, despite the lack of the haloethyl group.[46] The fast hydrolysis of the benzotriazolyl ester was, likely, followed by the HOBT-catalyzed conversion of the piperidyl phosphoramidate to TMP. The authors alternatively suspected that an endogenous phosphoramidase was responsible and necessary for the biological activity in living cells.[45]

Stability and Toxicity

The principle of prodrug action requires sufficient stability of the masked compound to ensure that the cells or the tissue being investigated are reached. The design of enzyme-activatable protecting groups should therefore ensure that these groups are unaffected by enzymes in plasma, blood, or other body fluids. Once the phosphate-bearing compounds enter cells, the masking groups should be rapidly broken down by endogenous intracellular enzymes, predominantly by various esterases or, more rarely, by phosphodiesterases, reductases, amidases, or other enzymes. Furthermore, stability data are essential for the prediction of shelf-life and form of administration, when a new prodrug is on its way from the lab to the patient.

From the vast amount of stability data for the many different compounds, only a representative selection of results from the various classes of masking groups are presented here.

Acyloxyalkyl groups

The general trend for these groups shows, that the bulkier esters lead to masking groups that become more stable against chemical hydrolysis. The groups are generally more stable in an acidic environment than at neutral pH. Bulkier groups seem to reduce the sensitivity to nucleophilic attack and hence, shift the pH stability maximum to neutral pH.[25] Furthermore, electron-donating groups added to the methylene bridge between the phosphate and ester moiety decrease the stability. Finally and not unexpectedly, acyloxyalkyl esters of phosphonates are more stable than comparable phosphate esters. These trends were already established when early model compounds were presented. The bis(acetoxymethyl) ester (AM-ester) of phenylphosphate showed a half-life of 3.2 h in phosphate buffer at pH 7.4, compared to 14 h for the bis(pivaloyloxymethyl) ester (POM ester).[25] A detailed study on the stabilities of various phenylphosphate and one phenylphosphonate prodrug models has previously been published by Friis and Bundgaard.[4] The half-life of bis(acetoxymethyl) phenylphosphonate in HEPES buffer, at pH 7.4, was significantly higher than for the phenylphosphate (23 days),[27] due to the generally higher stability of phosphonate diesters compared to phosphate triesters.[48]

When POM esters were used to mask antitumor nucleotides like 2′-deoxy-5-fluorouridine 5′-monophosphate (FdUMP), the resulting uncharged compound **3** was remarkably stable at low pH ($t_{1/2} > 100$ h) and even at pH 7.4 stability was fairly high ($t_{1/2} = 40$ h).[49] A similar value ($t_{1/2} = 36$ h) was determined for the AM-ester of di-$O^{2'}$,N^6-butyryl cyclic adenosine 3′,5′-monophosphate.[27] When incubated with mouse plasma (25% in phosphate buffer), the bisPOM ester of FdUMP (**3**) was hydrolyzed within less than 5 min (Fig. 10). The resulting POM ester **4** had a considerably longer half-life (72 min).[49] Similar differences were found for the hydrolysis of acyloxybenzyl esters of methyl phosphonates.[50] Chemical stability was very pronounced, with half-lives over 24 h for the phosphonate diesters, and beyond 150 h at pH 8 for the phosphonate monoesters. With human plasma under physiological conditions (pH 7.4, 37 °C) the phosphonate diester was readily degraded (6–9 min, depending on the acyloxy group) to the corresponding monoesters, which in turn were fairly stable with half-lives of roughly 9 h. Remarkably, the pivaloyloxybenzyl ester was significantly more stable (154 h). It should be pointed out that hydrolysis data, generated with isolated enzymes showed very different results in this case. Due to the many different enzymes and preparations described in literature, none of the enzyme-derived data is referred to here. Hydrolysis studies with *S*-acyloxymethyl esters of phosphorthioates showed enormous stability of these compounds in buffer. More importantly, the enzymatic conversion in human serum yielded the corresponding thioates fairly rapidly without significant loss of sulfur, except when using sterically more hindered and hence more slowly hydrolyzed acyloxymethyl esters. Generally, the R_P derivatives of dinucleoside phosphothioates were hydrolyzed 3–6 times more slowly than the S_P isomers.[51]

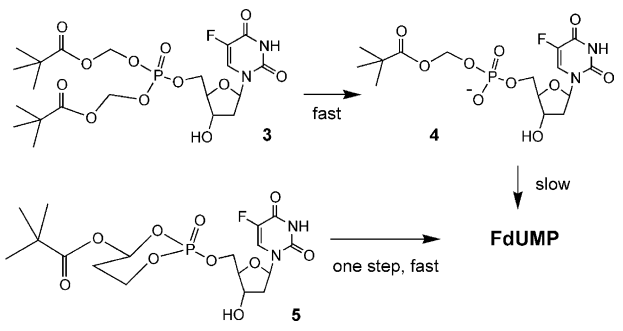


Figure 10. Prodrugs of 5-fluoro-2′-deoxy-uridine monophosphate (FdUMP). **5** produced FdUMP with a half-life of 30 min while the two-step degradation of **3** showed required more than 70 min to produce 50% FdUMP.

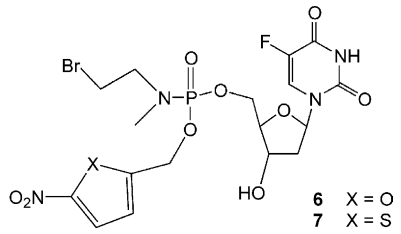


Figure 11.

Degradation of the 4-pivaloyloxy-1,3,2-dioxaphosphorinan-2-yl group of the 5-fluoro-uridine monophosphate derivative **5** was expected to be devoid of the slower second ester hydrolysis mentioned above. Consequently, the desired FdUMP built up rapidly when **5** was treated with mouse plasma. After 30 min at 37 °C (pH 7.4), 50% of the prodrug **5** and 50% of FdUMP was present, whereas the chemical stability of **5** in aqueous media was about 30 h.[29]

SATE groups and phosphoramidates

The more recently introduced masking groups SATE (*S*-acyl-2-thioethyl) and the heterogeneous phenylmethoxyalaninyl phosphoramidates were compared in regard to their stability by Valette et al.[30] The bis(*S*-acetyl-2-thioethyl) derivatives of AZT monophosphate and dideoxy isoadenosine monophosphate showed half-lives of 9 and 8 h in culture medium and only a few min for the former and 20 min for the latter in cell extracts.[31] The corresponding bis(*S*-pivaloyl-2-thioethyl) derivatives were more stable by about one order of magnitude. An increase in size of the ethylene bridge also led to more stable derivatives, such as the bis(*S*-pivaloyl-2-thiobutyl)[52] and bis(*S*-pivaloyl-2-thioisopropyl)[53] derivatives of AZT that were prepared. Surprisingly, the generation of the parent nucleoside monophosphate from the bis(*S*-acetyl-2-thioethyl) dideoxy isoadenosine derivative was also slow, probably due to different metabolic pathways.[30] This showed that the parent drug might exhibit significant influences on the metabolism of SATE prodrugs. Under identical conditions, the phenylmethoxyalaninyl phosphoramidates were generally more stable than the SATE groups with half-lives of 56 h in culture medium, and 1 h in cell extracts. Nevertheless, anti-HIV activity and cytotoxicity of both types of dideoxy isoadenosine prodrugs were comparable.[30]

The reductase-sensitive nitrofuryl phosphoramidate **6** of 5-fluoro-uridine exhibited a half-life of 18 h under cell assay conditions in the presence of horse serum.[46] The nitrothienyl phosphordiamidate **7** was stable for more than 12 h (Fig. 11), but the nitrothienyl group was rapidly removed in cacodylate buffer in the presence of dithionite.[47] Under the same conditions the deoxythymidyl phosphoramidate **2** lost its 1-hydroxybenzotriazole ester within an few min.[45]

t-Butyl-SATE groups and the corresponding *O*-ester derivatives were attached to various dithymidine phosphorothioates and dithioates. The stability in culture medium was greatly enhanced due to the lesser susceptibility of the phosphorous to nucleophilic attack. In cell extract containing esterases, the picture was less clear, although in general SATE groups attached to a phosphate were slightly more stable than the phosphorthioate and dithioate triesters.[54] A pro-oligonucleotide with 12 Me-SATE-protected phosphorthioate triesters was stable against most sera for several days and showed only slow deprotection in cell extracts ($t_{1/2} = 10$ h).[55]

Toxicity

Most bioactivatable masking groups release more or less toxic compounds upon degradation. Acyloxymethyl esters produce the corresponding carboxylic acid, formaldehyde, and protons when hydrolyzed. Carboxylic acids are likely to enter metabolic pathways or to leave cells rapidly and most cells are well equipped to maintain a stable pH. It is the liberation of formaldehyde that usually raises concern, due to its potentially carcinogenic properties.

However, recent studies show that natural formaldhyde levels in human plasma were fairly high with levels around 100 μM and that free formaldehyde inside cells spontaneously and non-enzymatically reacts with glutathione, which is abundant at a concentration around 5 mM.[56] These results suggest that peak levels of formaldehyde might be harmful due to its ability to form Schiff's bases, but that lower levels of formaldehyde may be better tolerated by man than previously thought. It should be mentioned that some drugs that carry acyloxymethyl esters, like Pivampenicellin, are on the market. Presumably, acyloxyalkyl esters that generate acetaldehyde would be more easily accepted, due to familiarly high levels after alcohol consumption. However, the drawback of acyloxyethyl groups is the lower stability and the introduction of new stereogenic centers that might lead to an increased number of diastereomers. Acyloxybenzyl esters would avoid this problem but the di- and especially the mono-acyloxybenzyl esters of AZT were shown to be more toxic than AZT itself in some cell lines.[57]

Very little is known about the thiirane generated after hydrolysis and spontaneous decomposition of the SATE-type protecting group. The toxicity values generated with masked nucleotides might often reflect increased cytotoxic effects of the nucleotide itself, due to the more rapid and massive administration of the compound to the cytosol.

Phosphonate and Phosphinate Prodrugs

Like phosphates, phosphonates are permanently negatively charged under physiological conditions and the preparation of bioavailable derivatives is an important task. Accordingly, a variety of pharmacologically interesting phosphonates and bisphosphonates has been modified and tested.

Considerable ground has been covered on the synthesis and evaluation of model compounds and small antiviral

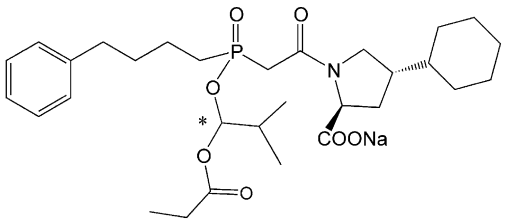


Figure 12. The orally active antihypertensive fosinopril required a acyloxyalkyl ester for sufficient bioavailability. Fosinopril is absorbed in the proximal intestine. Ester hydrolysis generates the active fosinoprilat.

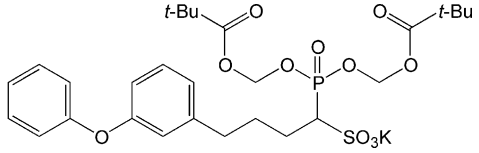


Figure 13. The bis(POM) derivative of this α-phosphonosulfonic acid inhibited cholesterol biosynthesis in rats with an ED_{50} of 3.5 μmol/kg.[25]

phosphonates, like phosphonoacetate or phosphonoformate (forscanet).[4,10,50,58–60] Various acyloxymethyl prodrugs of the related (hydroxy-2-naphthalenylmethyl)phosphonic acid have been probed as inhibitors of the insulin receptor tyrosine kinase and hence, the biological effects of insulin.[61] Also, phosphonate and phosphinate amino acid and peptide mimetica with bioactivatable protecting groups, have received enormous attention as endopeptidase inhibitors. One example is the phosphinate-containing drug, fosinopril, which was equipped with a propionoxy 1-isobutyryl group (Fig. 12) that masks the phosphinate group.[62] This prodrug is on the market as an angiotensin converting enzyme (ACE) inhibitor to treat hypertension. A *N*-phosphonomethyl phosphonate inhibitor of neutral endopeptidase (CGS 24592) was also intended to treat hypertension.[9] The largest amount of work has been spent on the preparation of prodrugs from 9-[2-(phosphonomethoxy)ethyl]adenine (PMEA). This drug possesses significant antiviral activity against HIV,[63,64] herpes simplex virus, and many others but lacks bioavailability in its charged form.[65] The bis(POM) ester[66] and its less hindered diacyloxymethyl relatives[67] are about 200 times more active than PMEA itself. The bis(SATE) ester derivative shows comparable activity, but higher stability against serum and gastric juices and is favored over the bis(dithiodiethyl) derivative.[68] Another promising approach is the use of isopropyloxycarbonyloxymethyl (POC) groups to mask the phosphonates of PMEA and its relative (*R*)-9-[2-(phosphonomethoxy)propyl]-adenine (PMPA). The hydrolysis of the POC group liberated no carboxylic acids and increased the anti-HIV activity up to 35-fold.[28,69]

Simple diphenyl esters of phosphonates have been used successfully twice: To create *N*-phosphonomethyl dipeptide prodrugs as endopeptidase inhibitors[9] and to generate uncharged PMEA derivatives.[11]

Not unexpectedly, phosphonates have quite a variety of pharmacological applications. For instance, α-phosphonosulfonic acids (α-PSAs) and the corresponding 1,1-bisphosphonates are potent inhibitors of squalene synthase, an essential enzyme in the biosynthesis of cholesterol.[70,71] Since the bisphosphonates showed some hepatoxicity in mice, only the α-PSAs were prepared as bis(acyloxymethyl) or other bis(acyloxyalkyl) esters. These prodrugs (Fig. 13) exhibit largely improved oral potency as cholesterol lowering agents in animal models.[26] The negative charge of the sulfonic acid group was retained to maintain solubility. Bisphosphonates are commonly used to mimic diphosphates as shown above. Another major application is the treatment of osteoporosis, as a range of simple bisphosphonates like clodronate or etidronate (Fig. 14) are able to inhibit bone resorption.[72,73] A drawback is the limited bioavailability. Therefore, bioreversible prodrugs of these bisphosphonates in the form of mixed anhydrides[74] or acyloxymethyl esters[75] were recently synthesized and tested. Most promising was a compound with three POM esters and a lipophilic counterion that exhibited a log P_{app} of 0.6, while the tetrakisPOM ester showed a value of 7.4.[75]

Finally, the simple phenoxyethyl bisphosphonate L-690,330 (Fig. 14) was shown to be a potent inhibitor of the inositol monophosphatase, in vitro.[76] The effects on cortical inositol monophosphate accumulation were in the micromolar range in vivo, when four POM groups were employed. Interestingly, the tris(POM)ester was similarly active, while di- and monoesters were lower in activity by several orders of magnitude.[77]

Nucleotide Prodrugs

The foremost targets in the development of prodrugs, thus far, are nucleoside monophosphates and the large variety of modified derivatives. The resulting uncharged compounds are sometimes referred to as 'pro-nucleotides'.[2] The general mode of action of these nucleotides is to inhibit viral reverse transcriptases, or to function as DNA chain terminators. Many nucleotide prodrugs are used as antitumor drugs in chemotherapy against HIV or Herpes. The design is straight forward. Usually, regular base moieties like adenine, uracil, hypoxanthine, or thymidine are attached to strongly altered ribose backbones that do not permit DNA chain elongation, due to the lack of the 3′-hydroxy group. The most prominent examples are 3′-azido-2′,3′-dideoxythymidine (AZT,

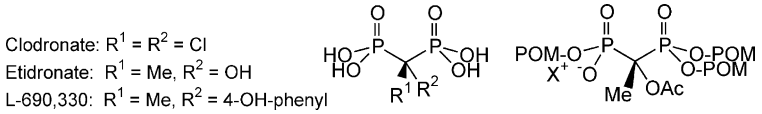


Figure 14. Bisphosphonate prodrugs of etidronic acid. Note, that one charge was maintained to ensure water solubility and oral bioavailability.

Zidovudine, Retrovir®), 2′,3′-dideoxyadenosine (ddA), 2′,3′-dideoxy-2′,3′-didehydro thymidine (d4T, Stavudine, Zerit®), and 9-(2-hydroxyethyl-methyl)guanine (Acyclovir). However, the alterations prevent an efficient phosphorylation of the nucleosides to the monophosphates and subsequently to the desired 5′-triphosphates that are to be incorporated into DNA, thus terminating the growth of the macromolecule.[78,79] Therefore, prodrugs of the 5′-monophosphates have been prime targets in the past decade and their preparation and use was extensively reviewed before.[2,5] The current review is, hence, restricted to some of the more recent developments in the field of pro-nucleotides, namely to phosphoramidates, the cyclosaligenyl (cycloSal) and S-acyl-2-thioethyl (SATE) derivatives.

L-Alaninyl-d4TMP phosphotriesters (Scheme 2) represent the prototypes of the phosphoramidate-based nucleotide prodrug.[37] One of the particularities of the phosphoramidate approach is the difference in anti-HIV efficacy between the AZT and d4T prodrugs. A closer investigation of the respective phosphoramidate prodrugs in HIV-infected thymidine kinase-deficient cell cultures, showed that the main metabolite formed from the L-alaninyl-AZTMP intermediate was the free nucleoside, while L-alaninyl-d4TMP was efficiently converted to the active d4T-monophosphate.[42] A wide variety of antiviral applications was shown for phenylmethylphosphoralaninate derivatives of the *Z*-methylenecyclopropane analogues of nucleosides. Most promising against HIV and hepatitis B virus (HBV) appears to be the 2,6-diaminopurine analogue (Fig. 15). The compound was somewhat less active against other viruses, such as the Epstein–Barr virus (EBV), but showed little cytotoxicity.[44] 2-Amino-6-methoxypurine and 2-amino-6-cyclopropylaminopurine prodrugs were favorable in oral applications against murine cytomegalovirus.[80] The *E*-isomers were mostly inactive.

Other phosphoramidate prodrugs, in particular of the thymidylate synthase inhibitor FdUMP, are directed towards inhibiting cell growth of L1210 mouse leukemia cells, and might serve as anticancer agents.[81] The most potent prodrugs of this series described so far contain a delivery group and a masking group attached to the phosphate and inhibit cell proliferation in the nanomolar range.[46] The compounds consist of a haloalkylamine group that is able to cyclize in order to create a suitable leaving group. The delivery group needs to be enzymatically reduced in order to generate the phosphoramidate anion.[47,81] Since solid tumors are known to provide a reducing environment, the described type of prodrug is believed to allow for tumor-selective action. Nitroheterocyclic phosphoramidate prodrugs (Fig. 11), that rely predominantly on the alkylating activity, have been adapted to this aim in recent efforts.[82]

A currently established procedure to administer antiviral nucleotide derivatives to cells is through the use of the cyclosaligenyl group (see above).[13] All of the commonly used antiviral nucleosides such as AZT,[14] d4T,[17] ddA, and d4A[16] were prepared as masked prodrug nucleotides in order to by-pass the low affinity of the nucleosides towards the respective nucleoside kinase. The approach is generally very successful and generates for example, 10 times higher concentrations of the active thymidine triphosphate, than the nucleoside d4T in living cells.[83] In some lymphocytes, however, especially in those devoid of the thymidine kinase, the AZT monophosphate prodrug failed to generate antivirally active metabolites.[84] The masking tool predominantly employed was the 3-methyl-2-hydroxybenzyl alcohol group. Recently, the method was expanded to 2′-fluoro-2′,3′-dideoxyadenosine derivatives[85] and acylic nucleoside analogues.[86] An interesting new approach uses 5-[(*E*)-2-bromovinyl]-2′-deoxyuridine (BVDU or Brivudin), a well-known inhibitor of herpes virus replication. The 3-methyl *cyclo*Sal derivative of BVDU (4 μM) (Fig. 14) was more active than acyclovir (7 μM) in inhibiting Epstein-Barr virus DNA synthesis in P3HR-1 cells, while BVDU itself was inactive. 3′-*O*-Acyl derivatives were not effective whereas α-amino acid esters retained activity.[19,87]

S-Acyl-2-thioethyl (SATE) groups are widely used by Imbach and co-workers, predominantly for antiviral nucleotides. In addition, other biologically active phosphate esters, like phosphopeptides, were also successfully masked.[88] It was shown in 1995, that the bis(SATE)phosphotriester derivative of AZT was more effective in inhibiting HIV-1 replication in CEM lymphocytes than the corresponding bis(DTE) or bis(POM) compounds, yet was less active than AZT itself.[31] In order to develop compounds that are active in the submicromolar range, more lipophilic SATE groups like the *S*-pivaloyl-2-thioethyl group, in combination with β-L-ddAMP (normally a rapidly metabolized nucleotide), yielded prodrugs that inhibited HIV replication at EC_{50} values around 2 nM and hepatitis B replication at 80 nM extracellular concentration.[89] The same modification of the phosphate proved beneficial for acyclovir[90] as well as the monophosphate of racemic 9-(4′-hydroxy-1′,2′-butadienyl)adenine (adenallene) and increased potency in lymphocytes by about a factor of 50 compared to the nucleoside analogues.[91] Nucleotides based on AZT that were prepared with mixed leaving groups

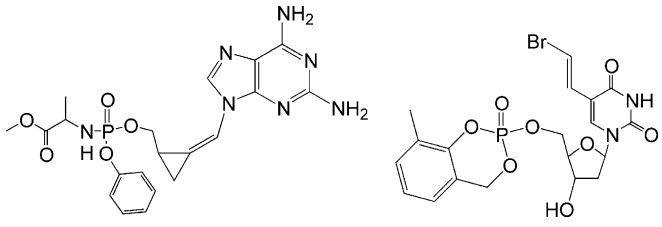


Figure 15. The phosphoamidate prodrug of a 2,6-diaminopurine methylenecyclopropane nucleoside analogue and the cycloSal prodrug of brivudin.

on the phosphate moiety, varying from fluorine[92] to phenyl and tyrosine groups.[93]

The combined results show that the increase in bioavailability, due to the various pronucleotide approaches compared to unmasked antiviral nucleosides, is certainly helpful. However, only when the technique is combined with the careful design or selection of the nucleoside, will sufficiently potent derivatives be derived from the efforts.

Cyclic Nucleotide Prodrugs

cAMP has been a prime target for the conversion into prodrug forms due to its well investigated biological function. Furthermore, only one negative charge needs to be masked in the case of cyclic nucleotides. The above-mentioned ethyl and benzyl esters[8] were the starting point for developing photolyzable ('caged') derivatives of cAMP, in the form of its o-nitrobenzyl esters.[94] Recently, caged cyclic nucleotide derivatives, employing coumarin groups to mask the phosphate, were introduced.[95,96] To create bioactivatable derivatives, alkylation of N^6,$O^{2'}$-dibutyryl-cAMP (Bt_2cAMP) with acetoxymethyl bromide (AM-Br), in the presence of diisopropylethylamine, was employed to give Bt_2cAMP acetoxymethyl ester (Bt_2cAMP/AM).[27] As expected, Bt_2cAMP/AM was several hundred-fold more potent than Bt_2cAMP in different applications, such as the activation of epithelial chloride secretion or the aggregation of dye from fish melanophores.[27] Threshhold concentrations for biological activity in the extracellular medium were as low as 10 nM when synergistic effects of cAMP and calcium signaling pathways were investigated.[97] cAMP/AM, a derivative without butyrates was less potent but had the advantage of being able to generate cAMP itself, thus allowing a transient cAMP signal inside cells, after the supply from the extracellular medium was cut by a washing procedure.[98] More recently, cAMP derivatives[99] and cGMP derivatives[100] modified in the 8-position, as well as a cAMP-phosphothioate derivative,[101] were prepared as AM esters and successfully tested. The synthesis of cGMP acetoxymethyl ester (cGMP/AM) derivatives was significantly more difficult. cGMP/AM inhibited platelet aggregation with an EC_{50} of 1 μM (Schultz, Makings, Tsien, unpublished results). This compound was also shown to induce long-term-potentiation of rat hippocampal neurons.[102] Many of the named compounds are now commercially available.

Oligonucleotide Prodrugs

Synthetic antisense oligonucleotides represent a widely accepted approach to interfere with RNA or DNA, thus inhibiting various intracellular processes for therapeutic or analytic purposes. Regular oligonucleotides have various negative charges that prohibit penetration of cellular membranes. To increase bioavailability and to prevent degradation in the extracellular space a large variety of oligonucleotides modified at the phosphate groups have been prepared.[103] However, the resulting phosphortriesters are chiral and apparently cannot be synthesized stereoselectively in the desired configuration, namely the one that allows hybridization with the target nucleotide. Accordingly, in an early approach, acyloxymethyl esters of model oligonucleotide thioates were prepared,[51,104] but no biological results were reported. More recently, Imbach and co-workers introduced the solid-phase synthesis of SATE-equipped model dodecathymidines using a photolabile linker.[55] This approach has been adapted to other nucleotides by employing photolabile protecting groups that mask the exocyclic amino groups.[105] Other model studies used γ-aminobutyric acid as an enzyme-labile group for the pro-nucleotide approach.[106]

Although quite a bit of ground has been covered towards a prodrug approach for oligonucleotides, the successful use by exhibiting increased biological activity of an actual antisense oligonucleotide still needs to be demonstrated.

Inositol Phosphate Prodrugs

Nucleotides usually have only a single phosphate that needs masking. Some other signaling molecules, especially inositol polyphosphates, require the modification of numerous charged groups. Initial attempts to convert inositol phosphates into prodrug molecules focused on *myo*-inositol 1,4,5-trisphosphate [**8**, Ins(1,4,5)P_3, Fig. 16a], because of its known function in regulating intracellular calcium levels. The attempt to directly introduce bioactivatable groups led to the formation of cyclic phosphates.

Therefore, total synthesis is required to selectively protect the hydroxy groups, perform the phosphorylation, and finally to introduce acyloxymethyl esters. In the early attempts, the hydroxy groups were protected with butyrates and the phosphates with acetoxymethyl (AM) esters to give compound **9**.[107] However, this product was unreliable in its ability to elevate intracellular calcium concentrations. This was probably due to the fact that the generation of Ins(1,4,5)P_3 levels by intracellular esterases was slower than the metabolism of the Ins(1,4,5)P_3 generated by prodrug delivery. More successful were derivatives like **10** that avoided use of the slowly hydrolyzing butyrates (Fig. 16b) and carried propionoxymethyl or butyryloxymethyl esters instead of AM esters (Fig. 16c). These derivatives were able to reliably elevate calcium levels to a steady plateau.[107] Another approach is the introduction of an additional phosphate at the 2-OH position that generates the metabolically more stable 1,2,4,5-tetrakisphosphate. Its membrane-permeant AM-ester (**11**, Fig. 17a) induced a long-lasting calcium plateau in PC12 cells.[108] Propionoxymethyl (PM) esters reduced the required extracellular dose by about a factor of 10 to 10 μM (Gillandt and Schultz, unpublished results). However, many calcium signals are usually transient. To address this problem, Li and co-workers introduced a photochemically removable nitrobenzyl group to an essential hydroxy

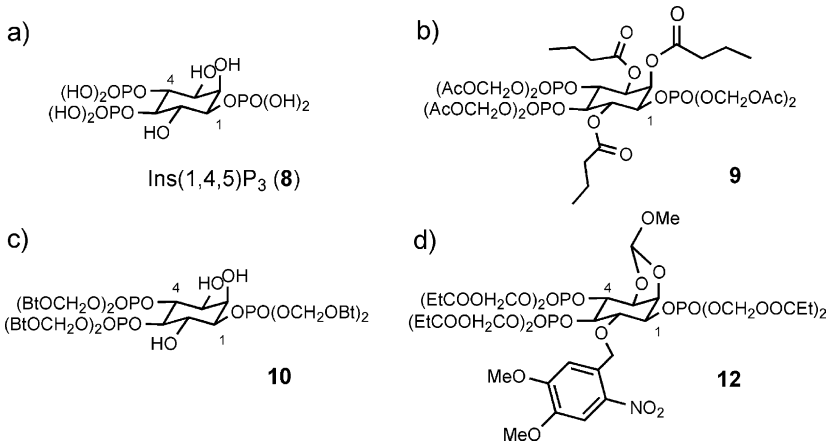


Figure 16. *myo*-Inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] and various membrane-permeant derivatives. Note the different acyloxymethyl esters used to adjust for overall lipophilicity (Bt = COC_3H_7).

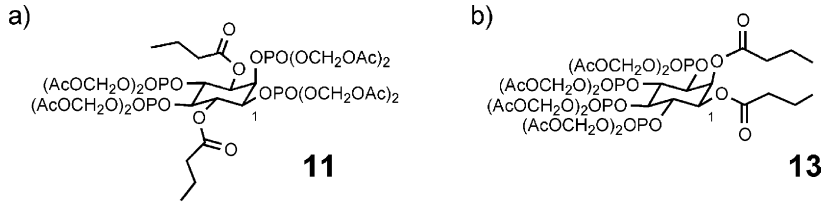


Figure 17. Membrane-permeant derivatives of *myo*-inositol 1,2,4,5- and 3,4,5,6-tetrakisphosphates, that elevate intracellular calcium levels (a) or inhibit epithelial chloride secretion (b), respectively.

group of Ins(1,4,5)P_3 (Fig. 16d).[109] The combination of the photoremovable 'cage'-group and bioactivatable acyloxymethyl esters in compound **12** has been shown to allow the generation of a sequence of short, transient calcium spikes in astrocytoma cells. In this way it has been demonstrated that a particular pattern of calcium spikes is necessary to induce gene expression.[109] The prodrug served as a unique tool that allowed investigation of intracellular events in a time-resolved manner.

The preparation of bioactivatable derivatives of other inositol polyphosphate isomers has been the starting point to determine the function of these orphan messengers in the regulation of epithelial chloride secretion. The octakis(acetoxymethyl) ester of 1,2-di-*O*-butyryl-*myo*-inositol 3,4,5,6-tetrakisphosphate (**13**) (Fig. 17b) uncoupled the chloride secretion from the intracellular calcium signal, thus defining Ins(3,4,5,6)P_4 as a downstream shut-off messenger for calcium-mediated chloride secretion (CaMCS).[110,111] Incubations of T84 epithelial cells with **13** (200 μM) gave intracellular Ins(3,4,5,6)P_4 levels of about 4 μM after 30 min. In the meantime, the use of propionoxymethyl esters and other modifications permitted the use of this kind of compounds in much lower concentrations.[112]

One of the sources of natural Ins(3,4,5,6)P_4 is most likely the pentakisphosphate Ins(1,3,4,5,6)P_5, one of the most abundant inositol polyphosphates in cells. Since there exists an equilibrium between the Ins(3,4,5,6)P_4 and the pentakisphosphate which is regulated by Ins(1,3,4)P_3, a membrane-permeant hexakis(acetoxymethyl)ester derivative of the latter was prepared. It has been shown that, after preincubation with the membrane-permeant Ins(1,3,4)P_3 derivative, Ins(3,4,5,6)P_4 levels in pancreatoma cells are significantly increased.[113] Furthermore, the hexakis(acetoxymethyl)ester of Ins(1,3,4)P_3 was able to reduce CaMCS of epithelial cells, presumably via elevated Ins(3,4,5,6)P_4 levels.[114]

Ins(1,4,5,6)P_4, the enantiomer of Ins(3,4,5,6)P_4, does not appear to be involved in the regulation of epithelial chloride secretion, because its membrane-permeant derivatives were successfully used as negative controls in the experiments mentioned above. Surprisingly, the previously known inhibitory effect of epidermal growth factor (EGF) on chloride secretion was partially blocked by the membrane-permeant derivative of Ins(1,4,5,6)P_4.[115]

The ability to selectively increase the concentration of an inositol phosphate by simply adding the membrane-permeant derivatives to living cells opens the intriguing possibility of studying the currently unknown physiological role of these potential signaling molecules. From a pharmaceutical standpoint any inositol polyphosphate could be considered a lead structure for the development of future drugs, simply, because this is one of the few examples of natural small molecules interfering with events downstream of receptors. Structurally modified inositol polyphosphate derivatives, especially of Ins(3,4,5,6)P_4, were prepared as membrane-permeant acyloxymethyl esters in attempts to generate compounds that positively influence chloride secretion from lung epithelia to treat cystic fibrosis.[116,117] In fact, some compounds allowed for multiple activation of epithelial

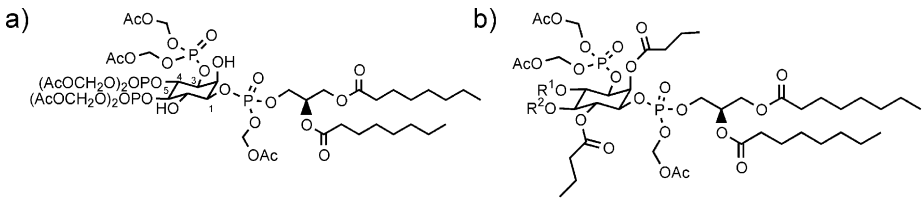


Figure 18. Membrane-permeant derivatives of 3-OH-phosphorylated phosphoinositides. R^1 and R^2 were $OPO(OCH_2OAc)_2$ or butyrate.

chloride channels and also reduced the pathologically high sodium absorption of nasal epithelia from cystic fibrosis patients.[118,119]

Phosphoinositide Prodrugs

The products of phosphoinositide 3-OH kinases[120,121] represent one of the currently most exciting groups of small intracellular signaling molecules. Their formation can be activated by both, tyrosine kinase and G-protein dependent, pathways and appears to be fairly transient. Downstream targets are various protein kinases and other proteins that have phosphoinositide-binding domains such as pleckstrin homology domains.[122] Prominent examples are protein kinase B (Akt)[123] and phospholipase Cδ1. Furthermore, phosphoinositides are recognized by adaptor proteins that are involved in endo- and exocytosis.[122,124–126] Membrane-permeant derivatives of phosphoinositides were synthesized to help elucidate the intracellular function of the putative signaling molecules in a more physiological context, to dissect the signaling pathways of growth factors and other first messengers, and to determine which of the metabolically connected 3-OH-phosphorylated phosphoinositides is the key player within a given signaling cascade. As for inositol phosphates, the preparation of the membrane-permeant derivatives required total synthesis starting from inositol. Jiang et al. synthesized the heptakis(acetoxymethyl) ester of phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3, Fig. 18a] with a dioctanoyl- and a dilaurylglycerol moiety, respectively.[127]

Simultaneously, our lab prepared a similar PtdIns(3,4,5)P_3 derivative with a dipalmitoyl glycerol and an additional butyrate at the 6-OH-group, by a different synthetic pathway.[108] The compounds of both groups were tested jointly in order to examine the effect on epithelial chloride secretion. It was shown that extracellular addition of PtdIns(3,4,5)P_3 levels mimicked the inhibitory effects of EGF on chloride secretion.[115] Furthermore, membrane-permeant PtdIns(3,4,5)P_3 derivatives activated deoxyglucose uptake in adipocytes[127] and neutrophil migration.[128] More recently, we prepared acetoxymethyl esters of a variety of 3-OH-phosphorylated phosphoinositides. In order to prevent phosphate migration during the enzymatic acetoxymethyl ester hydrolysis all inositol hydroxy groups were masked with butyrates (Fig. 18b). The specificity of the messenger function of PtdIns(3,4,5)P_3 over PtdIns(3,4)P_2 and PtdIns(3,5)P_2 in inhibiting epithelial chloride secretion of human nasal epithelia cells was demonstrated.[129]

Conclusions

Despite the huge amount of work published on phosphate-containing prodrug candidates the results are still unsatisfactory for patients. With the exception of some penicillins and the ACE inhibitors mentioned above the bioactivatable compounds remain in the experimental stage. Even for effective anti-HIV or anti-proliferative prodrugs no full size toxicological studies have been published so far. However, the results of these studies would be badly needed to allow the community to focus on the most successful and compliable masking groups available. It will be interesting to see which of the various approaches will finally allow oral or topical availability and will eventually enter the market.

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