Prodrugs of Nucleoside 5'-Monophosphate Analogues: Overview of the Recent Literature Concerning their Synthesis and Applications

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Abstract: Nucleoside analogues are widely used as anti-infectious and antitumoral agents. However, their clinical use may face limitations associated with their physico-chemical properties, pharmacokinetic parameters, and/or their peculiar mechanisms of action. Indeed, once inside the cells, nucleoside analogues required to be metabolize into their corresponding (poly-)phosphorylated derivatives, mediated by cellular and/or viral kinases, in order to interfere with nucleic acids biosynthesis. Within this activation process, the first-phosphorylation step is often the limiting one and to overcome these limitations numerous prodrug approaches have been proposed. Herein, we will focus on recent literature data (from 2015 and onwards) related to new prodrug strategies, the development of original synthetic approaches and novel applications of nucleotide prodrugs (namely pronucleotides) leading to the intracellular delivery of 5'-monophosphate nucleoside analogues.

Keywords: Pronucleotide, Phosphotriester, Phosphodiester, Phosphoramidate, Phosphorodiamidate, Asymmetric Synthesis, Chemotherapy.

1. INTRODUCTION

Nucleoside analogues represent the frontline treatment for viral infectious diseases [1] and they are also widely used in cancer chemotherapy [2], mainly as antimetabolites. This particularity may be attributed to the mechanism of action of this family of compounds (*i.e.*, interference of the poly-phosphorylated forms of nucleoside derivatives with the nucleic acid biosynthesis) as both viruses and cancer cells survival required the replication of their genetic materials. Thus, to exert their biological activities, once inside cells, nucleoside analogues need to be successively converted to their mono-, di- and triphosphate forms (Fig. **1A**) and one or more of these steps may be of limited efficiency due to poor recognition or affinity of the modified nucleosidic scaffolds for nucleoside and nucleotide kinases in comparison to endogenous nucleoside building blocks.

The prodrug concept applied to nucleoside 5'-monophosphate (5'-mononucleotide, NMP) derivatives has attracted a constant interest over the last decades and has found many applications both in antiviral and antitumoral chemotherapy [2b, 3]. Initially developed to overcome the first-phosphorylation step of nucleoside analogues and/or to improve the bioavailability of these derivatives by masking the two negatively charged oxygen atoms of the 5'-monophosphate group, the resulting neutral compounds can efficiently cross cell membranes (Fig. **1B&1C**). This area of research has now experienced new directions concerning stereoselective synthetic approaches and novel type of activation process. Pronucleotides (PROdrugs + monoNUCLEOTIDE) have also been exploited to explore enzymatic mechanisms and for their application as biological tools.

The aim of this review is to briefly describe the key concepts of the various prodrug approaches applied to 5'-mononucleotides and depending of the chemical structures of the corresponding derivatives (*i.e.*, phosphoramidate di- and monoester, phosphorodiamidate monoester, phosphotriester, and phosphodiester …, Fig. **2**).

We did not intend to provide a historical and chronological account of the research in this area as the readers may refer to abundant literature and reviews previously published during the last two decades [2b, 3a-e, 4]. Thus, we collected recent literature data, from 2015 and onwards, and we focused on pronucleotides designed to deliver the 5'-monophosphorylated nucleoside analogues.

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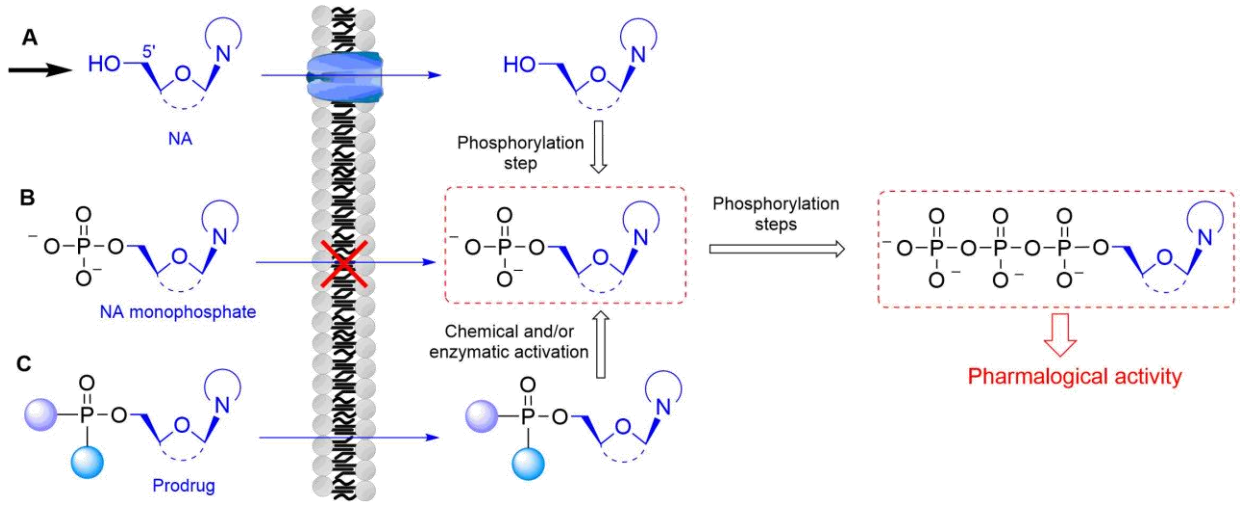


Fig. (1). (A) Overall mechanism of action of nucleoside analogues including their metabolization/activation into their bioactive forms within cells. **(B)** 5'-mononucleotide are not able to cross cell membranes and are catabolized by extracellular phosphatases. **(C)** The use of 5'-mononucleotide prodrugs (pronucleotides) may overcome both limitations associated with cell membrane penetration and first-phosphorylation step.

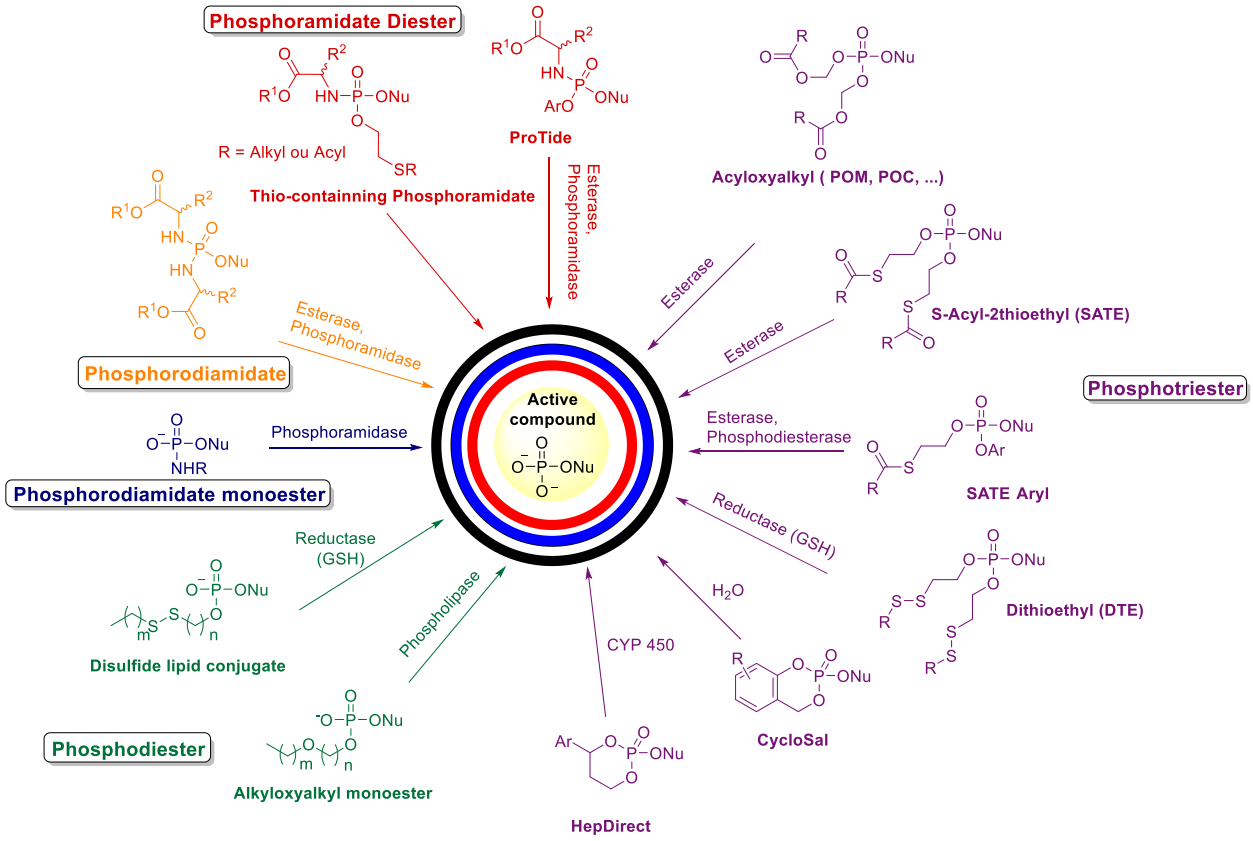


Fig. (2). Main prodrug approaches used to deliver 5'-mononucleotides within cells and belonging to various chemical classes of derivatives (phosphotriester, phosphodiester, phosphoramidate mono- and diester, phosphorodiamidate …).

Figure **2** illustrates generic structures of the most common pronucleotide approaches leading to the intracellular delivery of nucleoside 5'-monophosphate and involving chemical and/or enzymatic-mediated hydrolysis steps. Among them, the most successful one is the so-called ProTide technology (PROdrug + nucleoTIDE) developed by McGuigan and co-workers since the 90s [5], that led to several marketed drugs. As example, Sofosbuvir [6] was the first FDA (US Food and Drug Administration) approved liver-targeting drug for the cure of hepatitis C in 2013 and Remdesivir [4, 7] is a C-nucleoside based ProTide exhibiting broad spectrum antiviral activity against RNA viruses, also licensed by Gilead Sciences. Another strategy that may prove to be promising is the TriPPPro approach proposed by

Meier and co-workers [8], allowing intracellular delivery of nucleoside 5'-triphosphates (the pharmacologically active metabolite of a nucleoside analogue). It is based on the substitution of the two oxygen atoms of the γ-phosphate of the nucleoside 5'-triphosphate by lipophilic groups [9]. Reports and reviews related to the prodrug approaches applied to poly-phosphorylated nucleoside analogues [8, 10] or to acyclophosphonate derivatives [11] may also be gleaned from the literature.

2. Phosphoramidate diester: Synthesis - Therapeutic agents - Biological tools

Nucleoside phosphoramidates [12] are a class of organophosphorus compounds of general formula $(RO)_2P(O)NR'_2$ (R, R' = H, alkyl, aryl, heteroaryl …). In the following sections, we will highlight recent advances in the field of ProTides, the main family of nucleoside phosphoramidate diesters, then derivatives incorporating a promoiety including a sulfur atom, and finally various pronucleotides such as cyclic phosphoramidate and biarylphosphoramidate derivatives.

2.1. ProTides

ProTides incorporate a N-aminoacyl ester and an O-aryloxy-substituted phosphoryl group attached to the 5'-hydroxyl functionality of nucleosides. The aryloxyphosphoramidate platform was first described by the group of C. McGuigan at Cardiff University in the early 1990s [13]. The ProTide approach has been successfully applied to a vast number of nucleoside analogues with antiviral and anticancer activity, leading to three FDA-approved medicines, namely Sofosbuvir (Sovaldi®), tenofovir alafenamide (as fumarate salt, Vemlidy®) and Remdesivir (Veklury®) for antiviral chemotherapy (Fig. **3**). Several ProTides are also under clinical investigation for antitumoral applications. A number of recent reviews have focused on ProTides. [4, 5c-f, 14].

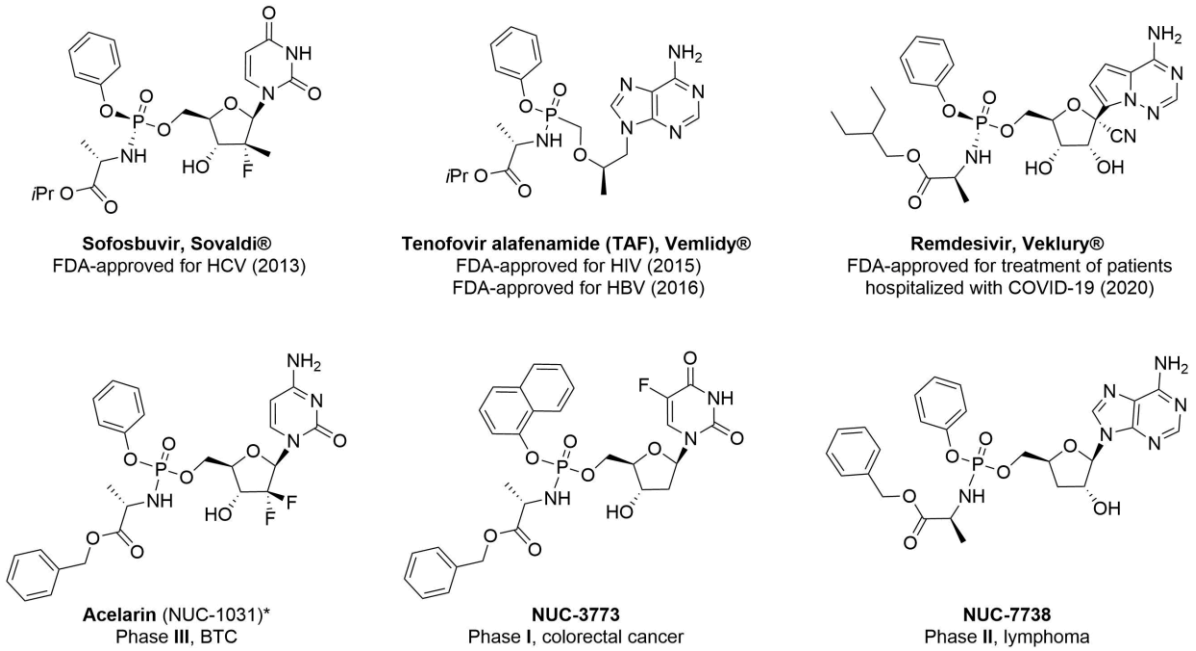


Fig. (3). ProTide derivatives of antiviral and anticancer nucleoside analogues used in the clinics or ongoing clinical studies (*Acelarin, the SP isomer of NUC-1031 has received a fast track designation from the US FDA for the treatment of biliary tract cancer (BTC) in September 2021, however the clinical phase III for the combination of NAC-1031 and cisplatin [15] was discontinued for advanced BTC in march 2022).

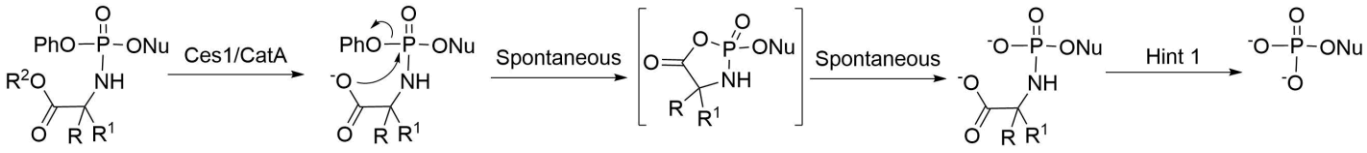
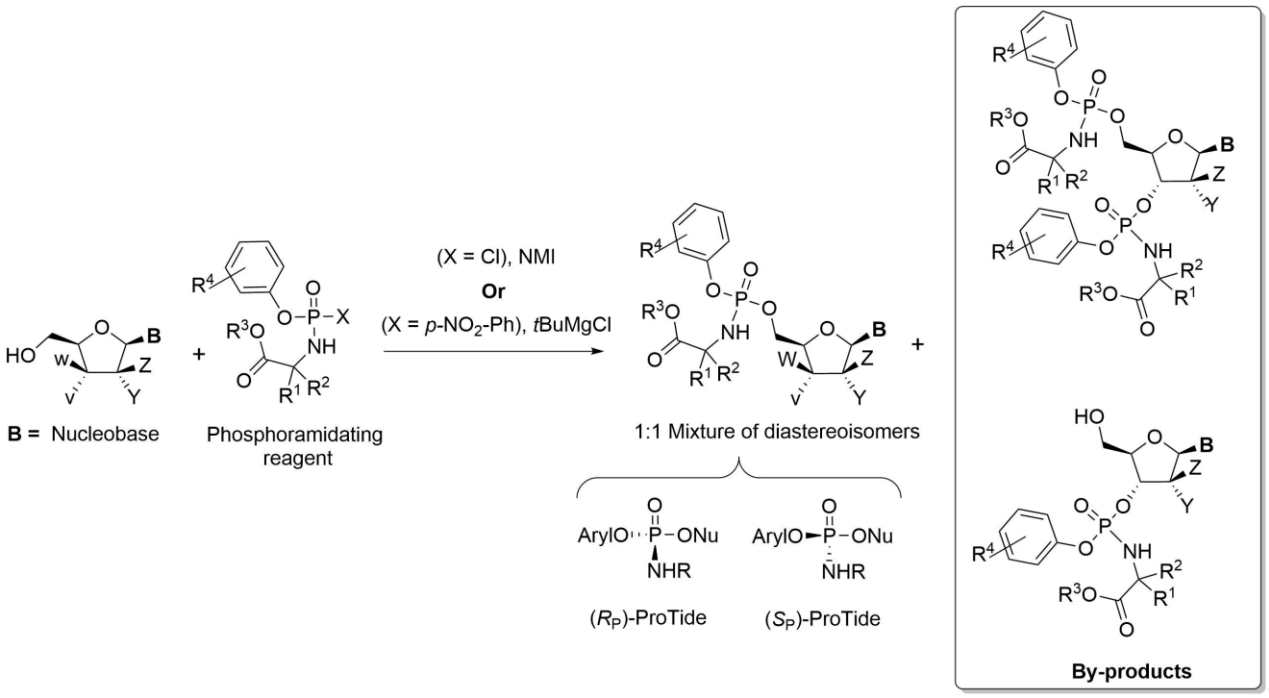


Fig. (4). General mechanism of the bioactivation of ProTides.

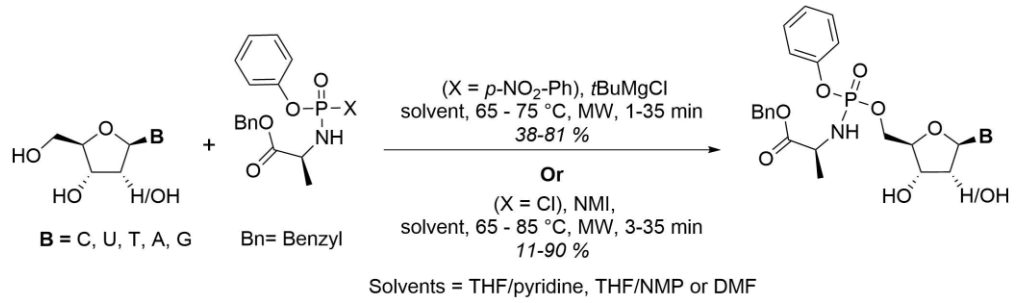
ProTides cross the cell membrane by passive diffusion and therefore do not require a membrane transporter [16]. Their hydrolysis is mediated by carboxylic-ester hydrolases or carboxypeptidase-type enzymes (CPY), leading to the release of the carboxylic acid from the amino acid, then an intramolecular nucleophilic substitution of the aryloxy group by the carboxylate occurred, giving rise to a five-membered cyclic intermediate [17] (Fig. **4**). This intermediate is rapidly hydrolyzed to the corresponding N-aminoacyl phosphoramidate which undergoes P–N bond cleavage, mediated through phosphoramidase activity such as histidine triad protein (Hint1), to ultimately release the 5'-monophosphate of the parent drug. Finally, this last undergoes two successive phosphorylations to generate the biologically active 5'-triphosphate form. Among the various methodologies developed to gain access to ProTides, [3d,

5d] the main one is based on nucleoside phosphoramidation (*i.e.*, the coupling of the 5'-hydroxyl group of the nucleoside analogues to a phosphoramidating reagent) in the presence of the Lewis base *N*-methylimidazole (NMI) or the *tert*-butyl magnesium chloride (*t*-BuMgCl) as Brønsted base (Scheme **1**).

In the first approach, originally pioneered by McGuigan *et al.* [13c], the coupling step is mediated by NMI which activates a phosphoramidochloridate reagent as an imidazolium intermediate. In the second one, the Grignard reagent *t*-BuMgCl acts as a strong base, activating the 5'-hydroxyl group of the nucleoside as its corresponding Mg-alkoxide, followed by the nucleophilic substitution of the phosphorus reagent bearing a *p*-nitrophenyl group as leaving group to yield the phosphoramidate diester. Both of these strategies have been successfully used for the synthesis of numerous ProTides, however their suitability is substrate dependent and can be affected by the nature of the nucleobase and the presence of competing hydroxyl groups. Indeed, while the use of NMI favors the selective phosphorylation of the primary 5'-hydroxyl group of the nucleoside, the "Grignard methodology" is not as selective, thus often leading to the formation of undesired regioisomers and/or bis-phosphoramidates (see the by-products in Scheme **1**). Additional limitations may also be associated with the competitive phosphorylation of some nucleobases and/or poor solubility of the substrate (free nucleoside) in the reaction solvents. To avoid by-products formation, appropriate protection of the sugar hydroxyl groups of the nucleoside analogues prior to the coupling reaction is often required. Thus, reaction conditions for the introduction and the removal of protecting groups must be optimized to be compatible with the nucleoside analogue and the ProTide moiety.



Scheme (1). ProTide "historical" synthetic approaches.



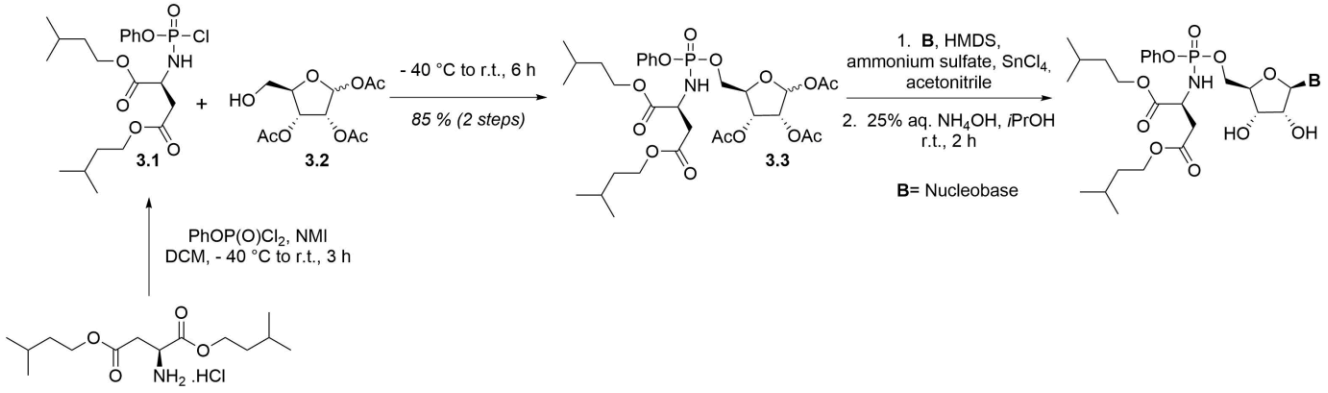
Scheme (2). Preparation of nucleoside phosphoramidates under MAOS.

In addition to the regioselectivity issue, these two procedures (NMI, *t*-BuMgCl, vide supra) generally give rise to the mixture of diastereoisomers (R_P and S_P, 1:1) of the final products due to the chirality at the phosphorus atom (Scheme **1**). Separation of the *P*-diastereoisomers is often a difficult and tedious task to achieve, nevertheless chromatographic separations using reverse-phase or chiral preparative HPLC were found successful in some cases. Therefore, the control of diastereoselectivity has become a major challenge in ProTides synthesis because the *in vitro*

biological activity and the pharmacokinetics have often been found dependent on the configuration of the phosphorus atom, this observation being also generally admitted for other pronucleotide series. For example, the R_P and S_P stereoisomers of Sofosbuvir exhibited EC_{90} values of 7.5 mM and 0.42 mM respectively, in HCV replicon assay, thus showing a ~ 18-fold more potent activity for the S_P diastereoisomer [18]. Therefore, Sofosbuvir has been developed and marketed as a pure single diastereoisomer at the cost of many efforts and high expenses. These advances in ProTide clinical development highlighted the need to develop synthetic methodologies for delivering such chiral derivatives as single diastereoisomers. In the following sections, we will focus on the recent synthetic approaches underlining their strengths and weaknesses.

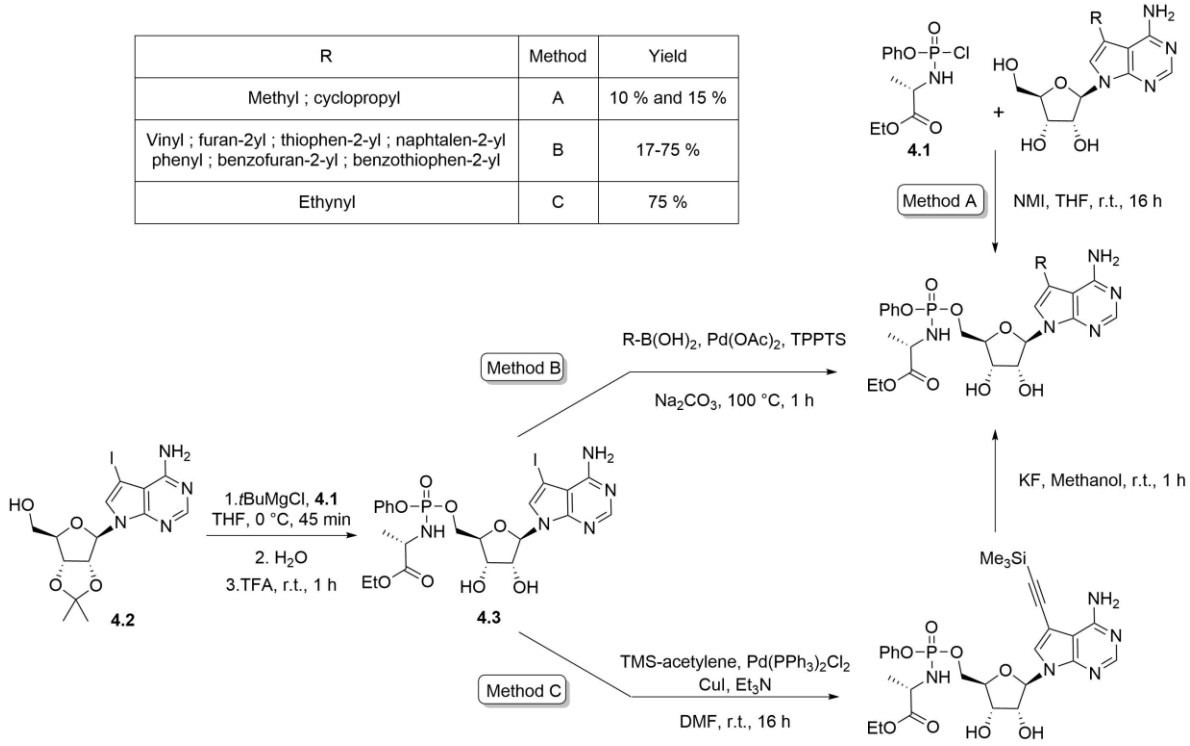
In 2019, Bordoni and co-workers [19] reported a microwave-assisted organic synthesis (MAOS) to obtain ProTides starting from unprotected nucleosides and minimizing the formation of the bis-phosphorylation byproducts. The phosphoramidation was performed using *t*-BuMgCl or NMI exploiting the cooling-while-heating microwave technology (Scheme **2**). Under these conditions, the temperature was kept constant during the microwave irradiation experiment. The outcome of the reactions were compared to those with conventional thermal heating. This methodology has been successfully applied to natural ribo- and 2'-deoxyribonucleosides. Yields of the 5'-ProTides ranged from 11% to 90%, with a huge shortening of the reaction time compared to classical solution synthesis (2–150 times decrease).

As mentioned before, the lack of 5′-selectivity often requires the introduction of protecting groups on the hydroxyls of the sugar scaffold prior to the phosphoramidation. In 2016, Herdewijn and co-workers [20] reported the use of a sugar derivative including the ProTide moiety as key intermediate for the preparation of a nucleobase-modified ProTide library. Briefly, treatment of phenyl phosphorodichloridate with the diisoamyl ester of L-aspartic acid hydrochloride in the presence of NMI gave rise to phosphorochloridate 3.1 (Scheme **3**). Then, its coupling with the 1,2,3-triacetylated D-ribose derivative 3.2 afforded the ProTide sugar 3.3, isolated in 85% yield as mixture of four isomers (i.e. R_P or S_P, α or β at the anomeric center). The glycosylation step was performed according to the Vorbrüggen coupling conditions. Optimization highlighted the use of hexamethyldisilazane (HMDS) as the silylating agent in the presence of a catalytic amount of ammonium sulfate, together with tin(IV) chloride as Lewis acid. This strategy was applied to the preparation of several sugar-protected pyrimidine and purine nucleotides, including non-natural nucleobases and affording exclusively the β-anomers in good to excellent yields. Finally, selective O-deacetylation was performed in basic conditions, where the diisoamyl ester and phenoxy promoieties were not affected. Compared to classical procedures, this method offers an access to a wide variety of nucleobase-modified ProTides in a two-step process.



Scheme (3). Synthesis of a "ProTide sugar" as key intermediate for the preparation of a nucleobase-modified library of ProTide derivatives.

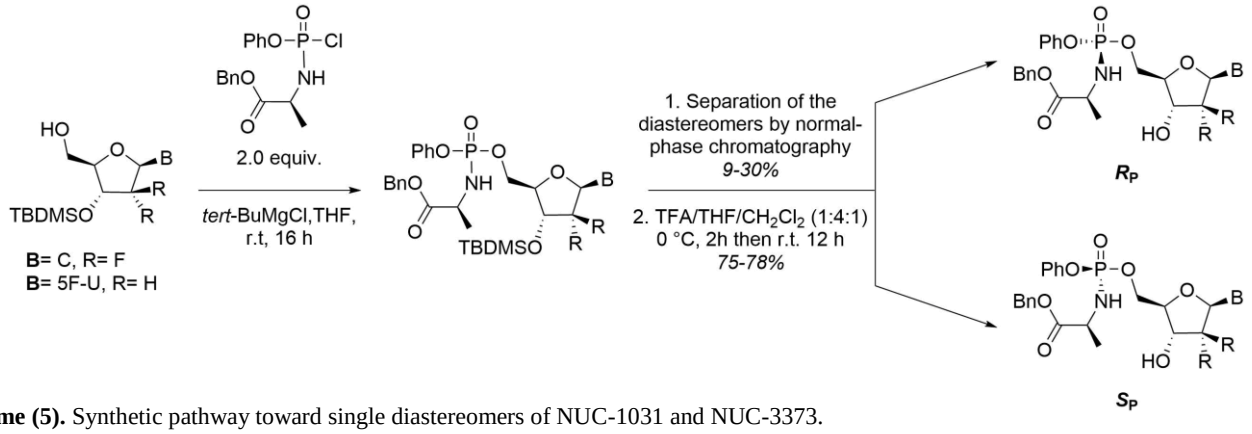
R	Method	Yield
Methyl ; cyclopropyl	A	10 % and 15 %
Vinyl ; furan-2yl ; thiophen-2-yl ; naphtalen-2-yl phenyl ; benzofuran-2-yl ; benzothiophen-2-yl	B	17-75 %
Ethynyl	C	75 %



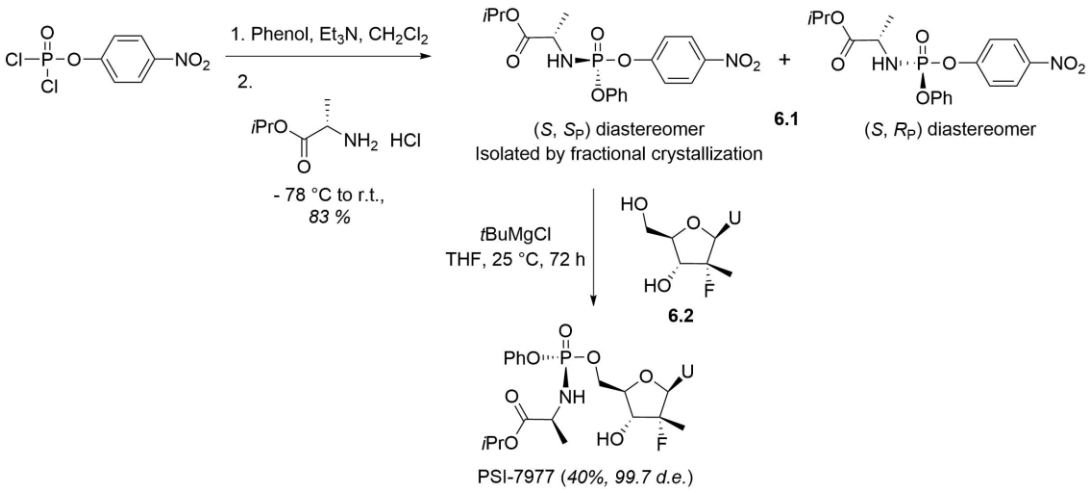
Scheme (4). Synthetic pathways to ProTides of 7-substituted 7-deazapurine ribonucleosides.

In 2021, the Hocek's group [21] reported the preparation of 7-substituted 7-deazapurine ribonucleosides, as well as their prodrugs (see also section **5**). ProTides bearing methyl and cyclopropyl groups into the position 7 of 6-amino-7-deazapurine derivatives were obtained by 5'-phosphoramidation of the free nucleosides with phosphochloridate **4.1** in the presence NMI (Scheme **4**, method A). The other derivatives were obtained from a common iodinated intermediate **4.3**, which was prepared by selective 5'-phosphoramidation of 2',3'-isopropylidene nucleoside **4.2** with **4.1** in the presence of *t*-BuMgCl followed by acidic deprotection. The Suzuki-Miyaura cross-coupling reaction of 4.3 with vinyl- or arylboronic acids gave rise to the desired 7-vinyl or 7-aryl derivatives in moderate yields due to partial hydrolysis of the promoiety (Scheme **4**, method B). Synthesis of the ethynyl derivative was achieved via Sonogashira coupling of 4.3 with TMS-acetylene, followed by subsequent desilylation with KF in MeOH (Scheme **4**, method C).

In 2021, Slusarczyck and co-workers [22] reported an alternative route toward the synthesis of single diastereoisomers of NUC-1031 (namely Acelarin) and NUC-3373, the ProTides of gemcitabine and floxuridine, respectively. Instead of performing the coupling reaction between the 3′-Boc-protected nucleoside analogues and the appropriate phosphorochloridate in the presence of either Grignard reagent or NMI, they developed a 3'-TBDMS protected route and separated the mixture of the two diastereomers at the level of the 3′-protected ProTides by using silica gel column chromatography (Scheme **5**), albeit in low yields. Further removal of the TBDMS group of each separated isomer afforded NUC-1031 and NUC-3373 as single isomers. In addition, the NUC-1031 diastereomers were obtained in crystalline form, allowing the assignment of the stereochemistry at the phosphorus center via X-ray crystallography.

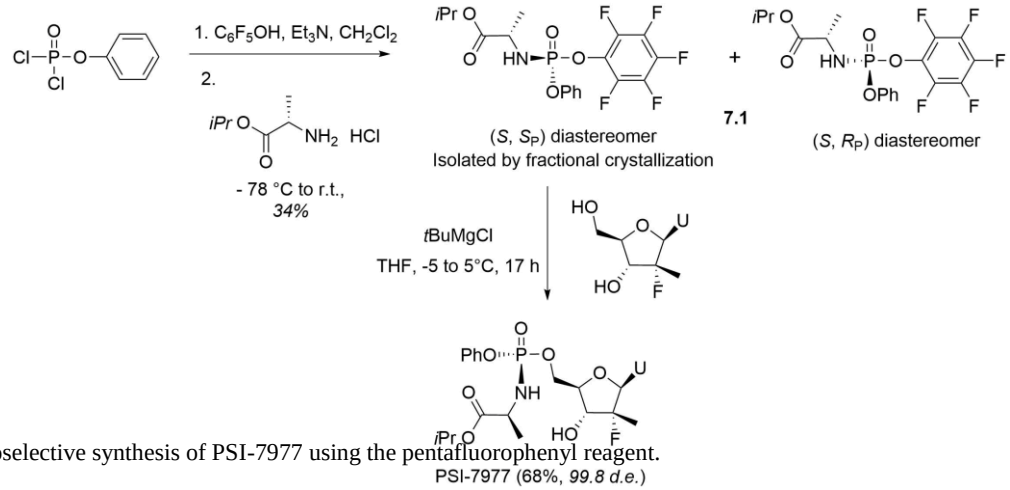


Scheme (5). Synthetic pathway toward single diastereomers of NUC-1031 and NUC-3373.

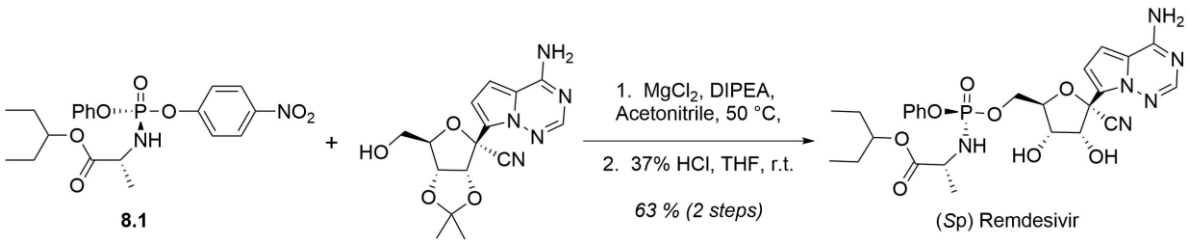


Scheme (6). Stereoselective synthesis of PSI-7977 using the p-nitrophenol reagent.

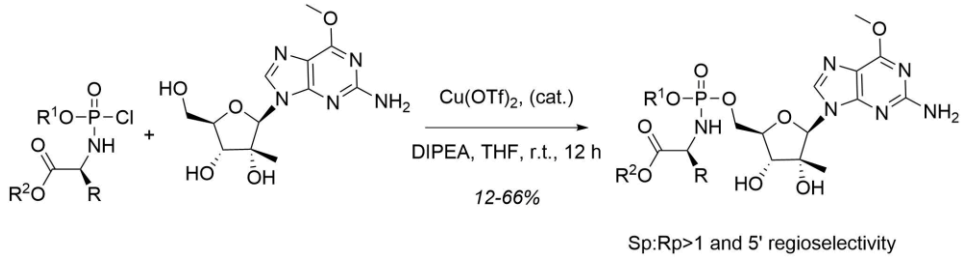
As already mentioned, the stereochemistry at phosphorus can substantially influence the biological properties of the resulting prodrugs, underlining the importance to obtain stereochemically defined derivatives. Common synthetic procedures for the synthesis of ProTides do not consider the chirality at the phosphorus atom and most often a mixture of diastereoisomers (R_P and S_P, 1:1) is isolated. Thus, separation of diastereomeric mixtures by fractional crystallization or chromatography have been proposed but are still tedious. To address this issue, diastereoselective approaches to access *P*-chirogenic ProTides have emerged in recent years. The first reports involved the use of chiral auxiliaries [23] or chiral nonracemic phosphorylating agents. [24]. In 2011, Ross and co-workers [18, 24] reported the efficient preparation of diastereomerically pure ProTides using single diastereomer phosphoramidating reagents bearing *p*-nitrophenyl or pentafluorophenyl as leaving groups (Schemes **6** and **7**). The nitrophenyl reagent **6.1** was obtained as a mixture of diastereomers R_P and S_P (1:1) by reacting commercial *p*-nitrophenyl dichlorophosphate with phenol in the presence of triethylamine, followed by coupling with L-alanine isopropyl ester hydrochloride (Scheme 6). The S_P-diastereoisomer was isolated by crystallization with 96% diastereomeric excess and 44% yield. Considering the change in stereochemical nomenclature priorities, the nucleophilic substitution at the phosphorus center of the S_P phosphoramidating reagent by the nucleoside analogue **6.2** afforded the S_P ProTide (PSI-7977) with high diastereoisomeric excess (*d.e.*). On the other hand, the pentafluorophenyl phosphoramidate reagent **7.1** was prepared by reacting phenyl dichlorophosphate with the amino acid ester, followed by the addition of pentafluorophenol (Scheme **7**). Its S_P diastereoisomer was isolated by fractional crystallization and allowed a multigram scale synthesis of PSI-7977 in 68% yield and high diastereomeric purity. Similarly, the S_P diastereomer of the phosphoramidate reagent **8.1** was readily resolved from the corresponding *P*-diastereoisomeric mixture through crystallization [7a, 25] (Scheme **8**), allowing the preparation of optically pure GS-5734 (later named Remdesivir). The main drawback of these synthetic pathways is that only one diastereoisomer of the bi(*O*-aryl)-*N*-aminoacyl-phosphoramidate intermediate is used for the synthesis of the desired optically pure ProTide, consequently the yield cannot be superior higher than 50%. Moreover, while effective in the described examples, it should be noted that some reports [26] indicate that these phosphoramidating reagents are sensitive and decompose easily, with racemization of the phosphorus stereocenter occurring.



Scheme (7). Stereoselective synthesis of PSI-7977 using the pentafluorophenyl reagent.



Scheme (8). Preparation of optically pure Remdesivir.



Scheme (9). Synthesis of ProTides under transition metal catalyzed conditions (best results were obtained for R= methyl, R1= naphthyl and R2= neopentyl).

In 2015, Pertusati and McGuigan [26a] reported the synthesis of diastereomeric-enriched mixtures of ProTides via copper-catalyzed reaction. Coupling of the 2'-C-methyl-6-*O*-methyl guanosine with various phosphochloridates in the presence of $Cu(OTf)_2$ as catalyst resulted in the formation of the corresponding ProTides in 12−66% yield (Scheme **9**). The results showed that bulky substituents on the phosphochloridate intermediate impact the stereoselectivity of the reaction, favoring the formation of the S_P diastereoisomer over the R_P one. The highest diastereoselectivity ratio (8.3:1, S_P/R_P) was observed for the bulky neopentyloxy-L-alanyl phosphorochloridate bearing a naphthyl group as aryloxy moiety. This methodology was applied to the synthesis of ProTides of Clofarabine (**5.1**), Nelarabine (**5.2**) and 2'-deoxy-2'-fluorouridine (**5.3**), (Fig. **5**). It is noteworthy that phosphoramidate **5.2** was obtained with opposite diastereoselectivity (S_P/R_P, 1:3.5). The reaction conditions were further optimized for the pyrimidine containing nucleoside and in this case copper(I) acetate proved to be the best catalyst in terms of diatereoselectivity, affording **5.3** in modest yield. Even so, a catalytic system to prepare diastereomeric-enriched mixtures of ProTides was identified, this synthetic methodology still suffers from long reaction time (8–12 h), substrate variability and in most cases modest yields. In addition to stereoselective coupling, another valorization of P(V) species has been proposed and involves a dynamic kinetic asymmetric transformation (DyKAT) of achiral species (Fig. **6**) [27].

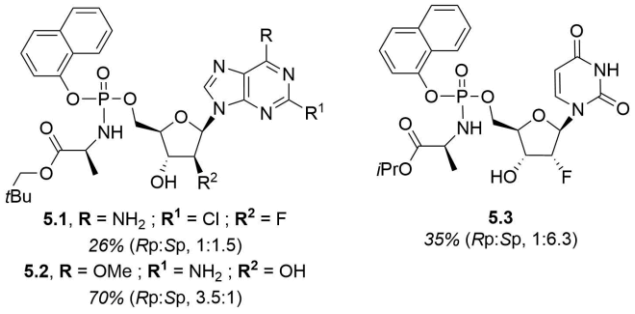


Figure (5). Purine (**5.1** and **5.2**) and pyrimidine (**5.3**) containing ProTides prepared using copper catalysts.

In 2017, Simmons and co-workers [28] reported a highly regio- and diastereoselective method to access ProTides of ribonucleosides in excellent yields without using a 3′-protecting group. Based on the Ross's approach [24], this strategy was improved by the use of Me_2AlCl as Lewis acid to catalyze the substitution reaction and to control regioselectivity (Fig. **7**). This efficient coupling approach was successfully applied for the diastereoselective synthesis of twenty one purine and pyrimidine ProTides including pharmaceutically relevant ones. The average isolated yield for six relevant ProTides was twice more than the one obtained with previously existing methodologies.

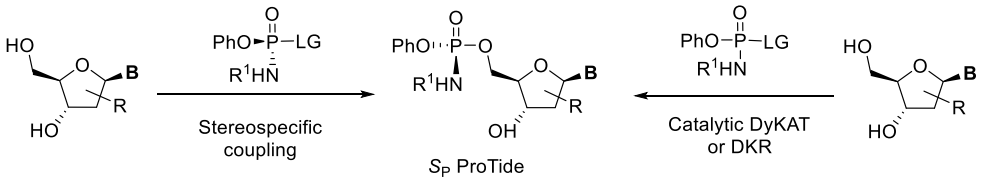


Figure (6). Approaches to the stereoselective synthesis of ProTides (LG= leaving group; R= various substituents; DyKAT, dynamic kinetic asymmetric transformation; DKR, dynamic kinetic resolution).

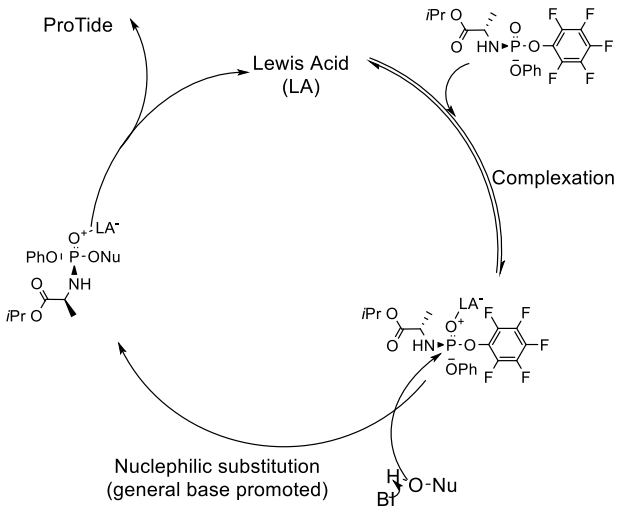
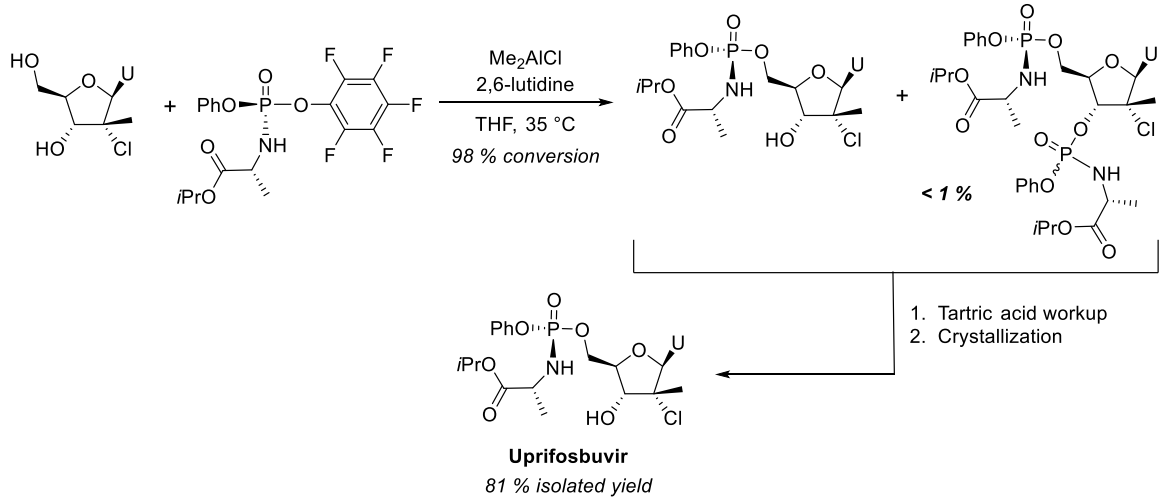


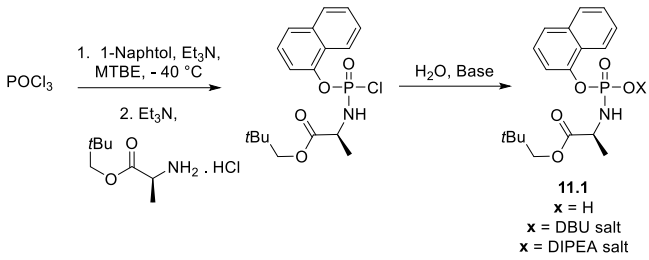
Figure (7). Proposed mechanism for the synthesis of ProTides catalyzed by aluminum-based-Lewis acids.

A few years later, the same research group [29] applied this synthetic pathway for the preparation of Uprifosbuvir (MK-3682), a clinical candidate for the treatment of hepatitis C (Scheme **10**). This compound was previously obtained according to conventional procedures (*t*-BuMgCl) [24] in the course of early clinical studies, but with low selectivity (mono- versus bisphosphorylation) and in 45% overall yield. Thus, the phosphoramidation reaction catalyzed by Me_2AlCl was further optimized in order to avoid pyridine as solvent and the chromatography steps. These lasts were both incompatible with production of an active pharmaceutical ingredient (API). Several aluminum-based Lewis acids, bases and solvents were screened. [30] The optimal conditions (0.5 eq Me_2AlCl, 2,6-lutidine in THF) provided to be highly regioselective (ratio mono versus bis-substitution, 320:1) and diastereoselective (*d.r.*, 101:1), thus avoiding the tedious workup associated with the *t*-BuMgCl protocol and leading to approximately a two-fold increase in isolated yield. This approach was successfully adapted to a multikilogram-scale process, including the safe handling of Me_2AlCl, and considering all specification for the use of API in late-stage clinical trials (Scheme **10**).

In 2015, Tran and co-workers [26b] reported the diastereoselective coupling of 2'-C-methyl-6-O-methyl guanosine to a phosphoramidic acid derivative via dynamic kinetic resolution (DKR). The phosphoramidic acid **11.1** was obtained by hydrolysis of its chlorophosphoramidate counterpart (Scheme **11**) and bases, such as DBU or diisopropylethylamine, were required to stabilize this reagent in solution. The coupling of unprotected nucleoside and the DBU salt of **11.1** was optimized using different peptides coupling reagents as well as several chiral and achiral bases, which resulted in the selection of HATU and quinine as the best combination (Scheme **12**). In particular, quinine was found crucial for maximizing the diastereomeric ratio. A mechanistic study, supported by ^{31}P NMR, was undertaken to explain this result and it was shown to proceed through a dynamic kinetic resolution of a 1:1 mixture of activated phosphate ester diastereoisomers, which means that i) the interconversion of diastereoisomers of the activated esters (*S*,*S*) and (*S*,*R*) is rapid and ii) the reaction of the nucleoside with one diastereoisomer occurs with a significantly higher rate than with the other diastereoisomer (Scheme **12**).

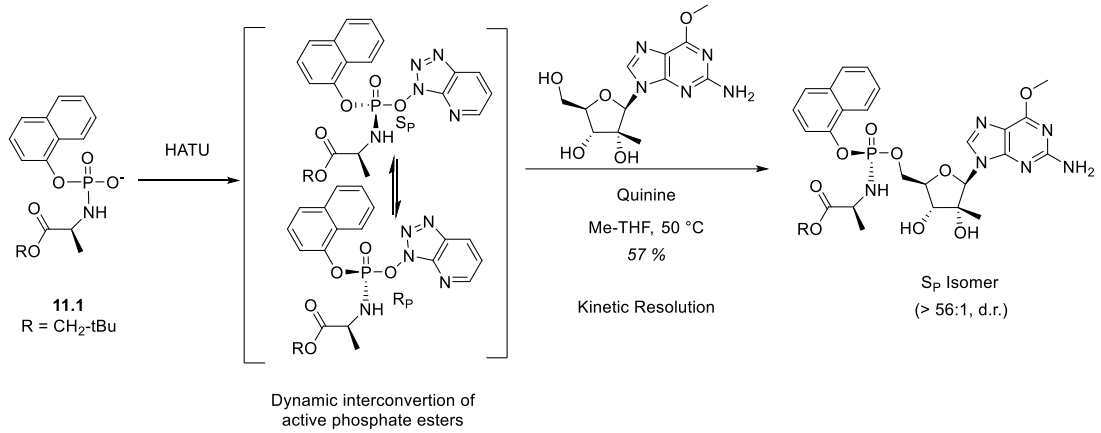


Scheme (10). Optimized synthesis of Uprifosbuvir at multi-kilogram scale.

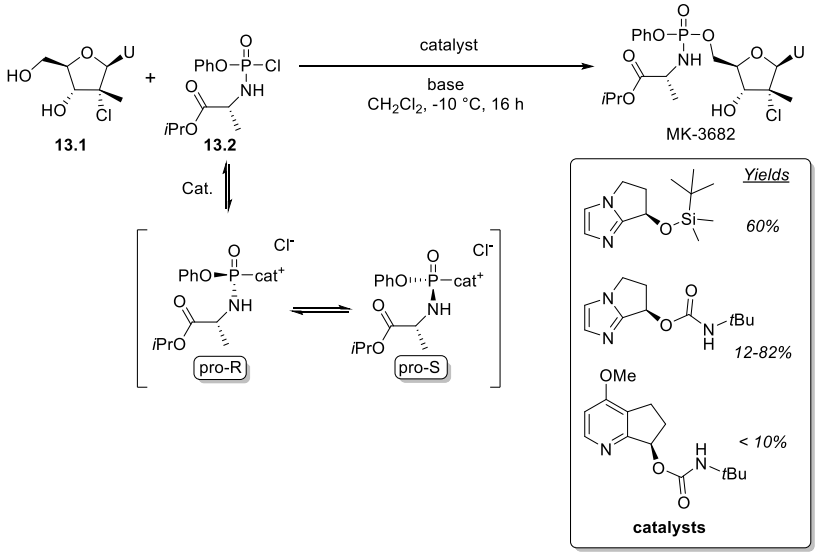


Scheme (11). Preparation of the various salts of phosphoramidic acid.

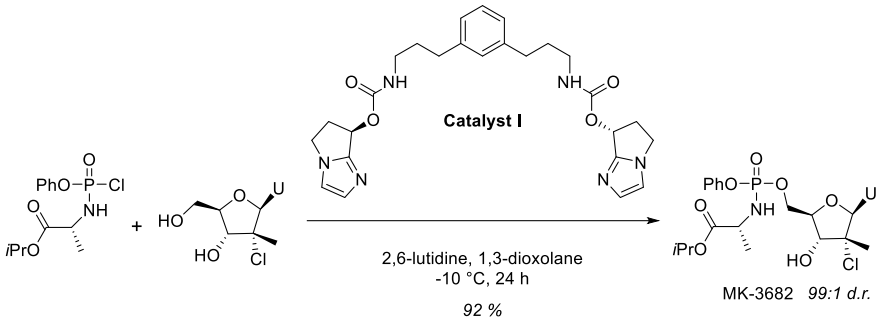
In 2017, DiRocco and co-workers [31] reported an improved stereoselective approach using small molecule catalysts to enhance the stereoselectivity of the phosphoramidation through a dynamic stereoselective process. The authors studied the reaction of nucleoside **13.1** with chlorophosphoramidate **13.2** to obtain MK-3682 (Scheme **13**), currently in late-stage clinical trials for the treatment of HCV infection. Under conditions originally reported by McGuigan (NMI and 2,6-lutidine), the desired product was obtained in 49% yield, with a diastereoisomeric ratio of 52:48 (R_P/S_P) [13b]. Thus, a variety of chiral catalysts were examined and the outcome of the reactions, as well as 1H and ^{31}P NMR studies, indicated that the chlorophosphoramidate **13.2** is in rapid equilibrium with the activated species (pro-*R* and pro-*S*) and that P-O bond formation is the turnover-limiting step (Scheme **13**). Then, several optimization steps associated with computational studies led to the design of catalyst **I**, a double-head small-molecule containing a central 1,3-phenyl group (Scheme **14**). It was shown that this catalyst lowers barriers to P–O bond formation and P(V) inversion but it is turnover-limited by the rate of catalyst addition to the chlorophosphoramidate. The general applicability of this catalyst was demonstrated on a broad range of therapeutically relevant nucleosides (Fig. **8**) and their corresponding ProTides were obtained with high diastereoselectivity.



Scheme (12). Diastereoselective coupling via dynamic kinetic resolution.



Scheme (13). Diastereoselective coupling via dynamic kinetic resolution.



Scheme (14). Optimized conditions and best catalyst for the preparation of MK-3682.

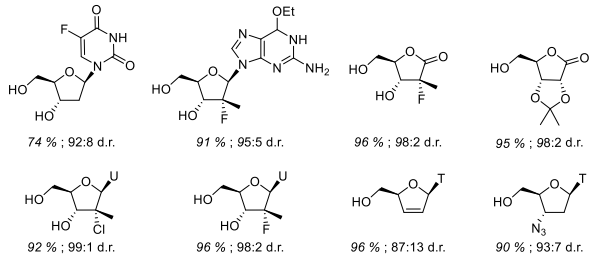
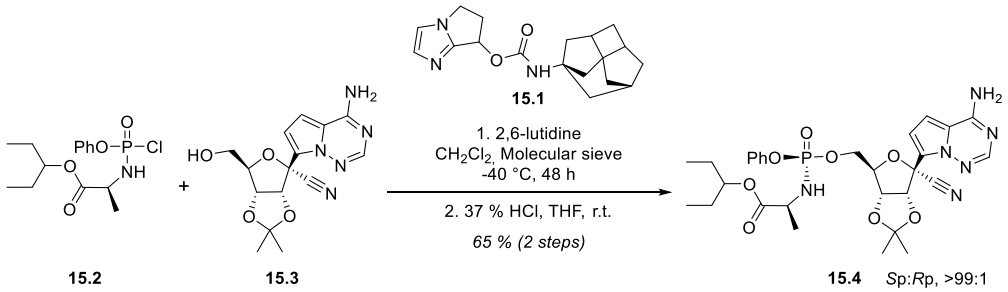
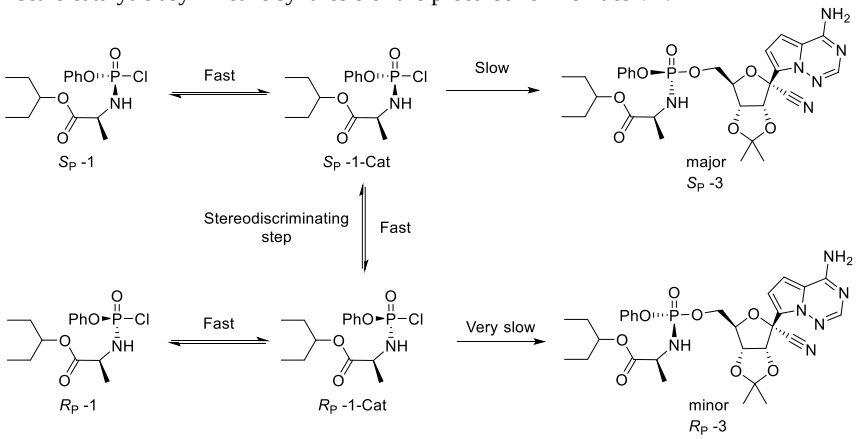


Figure (8). Scope of the phosphoramidation catalyzed by catalyst I.

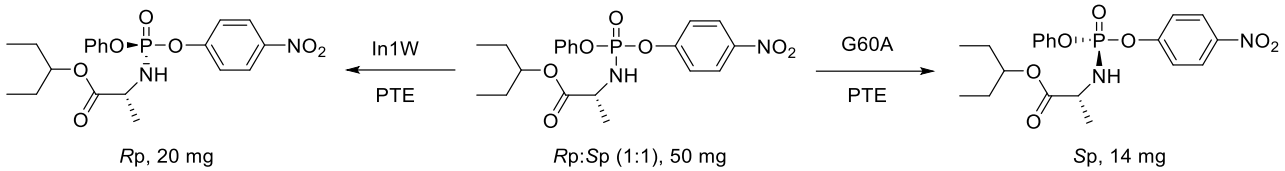
In the same line, the Zhang's group reported over the past decade the catalytic asymmetric conversion [32] of racemic phosphoryl chloride to phosphoramidate using chiral bicyclic imidazole organocatalysts. Some of them were screened by Di Rocco *et al.* [31] as discussed previously (Scheme **13**). In 2020, the same group [32] described the first highly efficient catalytic asymmetric synthesis of Remdesivir using the bicyclic imidazole catalyst **15.1** (Scheme **15**) via a dynamic kinetic asymmetric transformation (DyKAT). Accordingly, the coupling of the chlorophosphoramidate **15.2** with protected nucleoside **15.3** in the presence of 2,6-lutidine and **15.1** leads to the formation of protected ProTide 15.4 (Scheme **15**), which was then converted to Remdesivir. When performed on a 10-gram scale, a 89% isolated yield with diastereoisomeric ratio (>99:1, S_P/R_P) was obtained for the coupling step, demonstrating its potential for industrial application. Moreover, the catalyst could be easily recovered after column chromatography and recycled. Mechanistic studies revealed that the chiral bicyclic imidazole skeleton combined with the adamantinyl-substituted carbamate group of the catalyst are both required for the DyKAT (Scheme **16**) to proceed smoothly with high reactivity and excellent stereoselectivity.



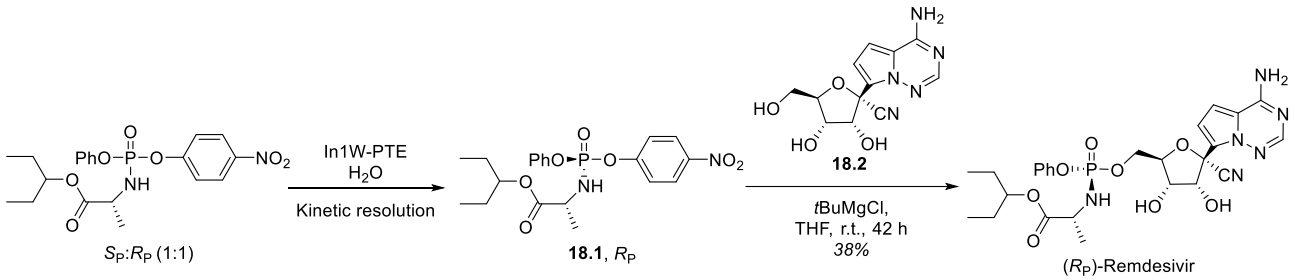
Scheme (15). The gram-scale catalytic asymmetric synthesis of the precursor of Remdesivir.



Scheme (16). Proposed mechanism for the bicyclic imidazole-catalyzed DyKAT using **15.1** as catalyst for the preparation of Remdesivir.



Scheme (17). Enzyme-catalyzed kinetic resolution of chiral precursors for ProTides synthesis.



Scheme (18). Synthesis of Remdesivir via enzymatic catalytic kinetic resolution of the biaryl phosphoramidate reagent.

Alternatively, the Raushel's group [33] investigated the preparation of diastereomerically pure ProTides using an enzyme-catalyzed kinetic resolution of the chiral precursors. Briefly, a phosphotriesterase from *Pseudomonas diminuta* (PTE) has showed a broad substrate specificity and excellent stereoselectivity, allowing its use in chiral resolution procedure with various phosphorus-containing compounds. To challenge the use of PTE for the preparation of pure diastereoisomers of building blocks, several variants were screened. Starting from a 1:1 mixture of p-nitrophenyl phosphoramidate diester, the G60A-PTE and In1W-PTE variants were selected as they display a ~165- to 1400-fold preference for each of the two diastereoisomers (Scheme **17**). In this approach, the undesired diastereoisomer is preferentially hydrolyzed by the required enzyme, allowing to isolate the remaining one. As illustrated in Scheme **18**, this approach was successfully applied to the preparation of the (R_P)-diastereomer of Remdesivir in two steps [34]. First, the chemoenzymatic strategy involves the In1W-PTE variant to isolate the R_P-precursor **18.1**, which is directly coupled with the nucleoside analogue **18.2** leading to Remdesivir.

In recent years, a large variety of ProTides were synthesized and evaluated as antiviral agents (Fig. **9**). Their preparation was performed according to standard procedures, mostly by phosphoramidation in the presence of the Grignard reagent *t*-BuMgCl. Most often, diastereoisomers were purified by preparative HPLC or column chromatography on silica gel. It is not our purpose to present an exhaustive list but rather to exemplify the wide application of the ProTide approach.

In 2017, C.B. Dousson and co-workers [35] reported the synthesis and biological evaluation of several ProTide derivatives of 2'−α−chloro-2'-β-C-methyluridine. Among them, compound IDX21437 (Fig. **9**) showed high levels of active nucleoside triphosphate (a potent inhibitor of the HCV NS5B RNA-dependent RNA polymerase, RdRp) in mouse liver and in human hepatocytes, despite showing very weak activity in the in vitro replicon cell assay.

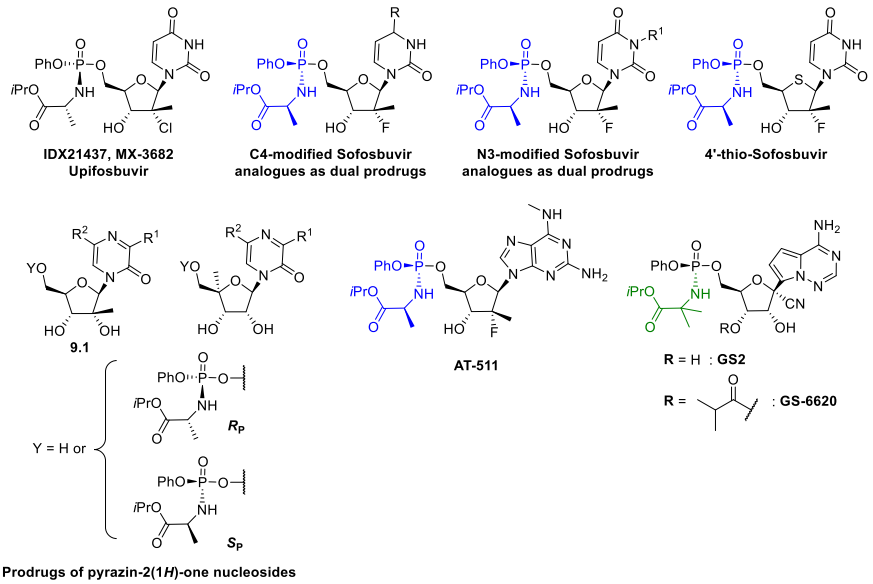


Figure (9). Selection of ProTides studied as anti-HCV agents.

In 2019, Guo and co-workers [36] described the preparation of analogues of Sofosbuvir including C4 or N3 modifications, as well as pyrazin-2(1*H*)-one pronucleotides (Fig. **9**), and their potential application as anti-HCV agents. Within the last series, the 2'-C-methyl nucleosidic derivative **9.1** with R^1=$CONH_2$, and R^2 = Cl was the best anti-HCV inhibitor with an EC_{50} value of 7.3 μM.

In 2022, the G.J. Miller's group [37] reported the synthesis and the biological evaluation of 4'-thioanalogues of Sofosbuvir (Fig. **9**). While substitution of the intracyclic oxygen may sometine be of interest, in this case a 100-fold decrease of the in vitro activity was observed in the HCV replicon assay.

In 2020, Good and co-workers [38] reported the preclinical study of AT-527, the hemi-sulfate salt of AT-511 (Fig. **9**), with potent pan-genotypic activity against HCV. Compared to AT-511, AT-527 exhibited increased aqueous solubility, thus enhancing oral absorption. In vivo data, shown that AT-527 delivered high levels of the corresponding nucleoside 5'-triphosphate in the liver after oral administration to both rats and monkeys. The same group also reported in 2021 the potent in vitro activity of AT-511 against various human coronaviruses, including SARS-CoV-2 [39]. On the basis of these encouraging results, AT-527 has entered clinical development for the treatment of hepatitis C (Phase III) and CoViD-19 infection (Phase II).

In recent years, the Gilead company, which is actively involved in the development of antiviral ProTides (*e.g.*, Sofosbuvir and Remdesivir), reported in-depth studies [40] related to the metabolism and pharmacology of Sofosbuvir and GS2 (Fig. **9**), an anti-HCV nucleotide prodrug containing a nonproteinogenic amino acid. Compared to GS-6620, GS2 prodrug showed improved oral delivery in the clinic. Regarding Sofosbuvir, its pharmacokinetic profiles in rodent and non-rodent animal models were assessed and compared using the clinical liver exposure as a benchmark. At human equivalent dose, the hepatic concentrations of the corresponding NTPs in dog and PXB mouse were comparable to those observed in the human liver. In these species, high and sustained levels of NTP were observed in both primary hepatocytes in vitro and the liver *in vivo*.

In 2017, Lagrutta and co-workers [41] examined the impact of diastereochemistry (D/L-alanine, *R*/*S*-phosphoryl) of HCV-NS5B ProTides, in vitro and in vivo, on the adverse cardiac drug-drug interaction (DDI) with amiodarone. For this purpose, they compared uridine analogues (Fig. **10**) displaying D-ala, R_P stereochemistry (MNI-2, MNI-4 and MK-3682) and their L-ala, S_P counterparts (Sofosbuvir, MNI-1, and MNI-3, respectively). They demonstrated that cardiac DDI between these ProTides and amiodarone is directly related to diastereochemistry. Thus, metabolization process and pharmacological effect in cardiomyocytes appeared dependent on the combination of L-ala and S_P stereochemistry.

In 2019, McGuigan's group [42] described the synthesis of a series of polyfluoroaromatic ProTides of stavudine (d4T), an anti-HIV agent. ProTides, prepared using previously published procedures, were isolated as diastereoisomeric mixture and their antiviral activity was evaluated in vitro against HIV-1 and HIV-2. The introduction of different polyfluorinated substituents (4-CF_3/3-CF_3/3-SF_5, Fig. **11**, derivatives **11.1**) onto the aryl counterpart would affect the lipophilic and electronegative properties, as well as their anti-HIV activity and toxicity profiles. All ProTides showed markedly enhanced antiviral activity compared to the parent nucleoside, the non-fluorinated derivative being as potent as d4T. The ProTide featuring a meta-pentafluorosulfanyl (3-SF_5) substituent was identified as promising lead, with low nanomolar antiviral activity (IC_{50} = 30 nM, HIV-1 and IC_{50} = 36 nM, HIV-2), and tenfold more potency than d4T. Moreover, it exhibited the best selectivity index.

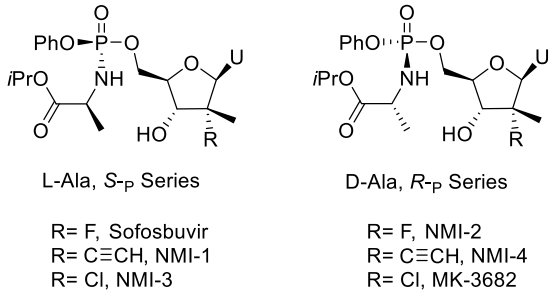


Figure (10). Compounds studied to determine the impact of stereochemistry, of both the amino-acid residue and at the phosphorus, on adverse cardiac drug-drug interaction with amiodarone.

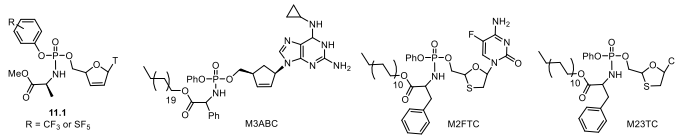


Figure (11). Various ProTides studied as anti-HIV or anti-HBV agents.

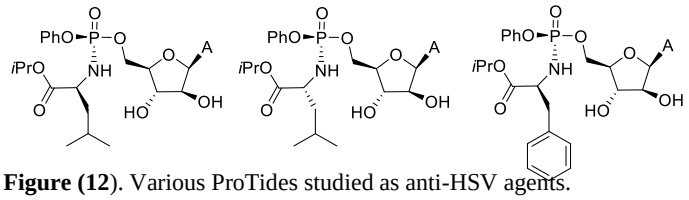


Figure (12). Various ProTides studied as anti-HSV agents.

On the other hand, the Edagwa's group reported the preparation and the study of nanoformulation of ProTide derivatives of Abacavir (ABC) [43]; Lamivudine (3TC) [44], and Emtricitabine (FTC) [45] as long acting slow effective release antiretroviral therapy. The objective is to mask the nucleoside 5'-monophosphates with hydrophobic amino aryl esters to improve plasma drug stability, membrane penetration and to allow encapsulation. Their synthesis was performed according to the "Grignard methodology". These studies show that M3ABC, M2FTC and M23TC (Fig. **11**), encapsulated into poloxamer coated drug nanoparticles, improve human monocyte-derived macrophages MDM drug uptake, sustained retention and anti-HIV activities for up to one month. The evaluation of the anti-HBV activity of M23TC nanoparticles in humanized liver mice provides evidence that a nanoformulated long-acting 3TC prodrug could provide sustained anti-HBV responses after a single treatment.

In 2021, Z. Wang and coworkers [46] examined ProTide derivatives of Vidarabine (AraA) in order to propose an alternative for Acyclovir-resistant/TK-deficient patients subjected to HSV-1 infections. Indeed, AraA follows a different bioactivation pathway compared to acyclovir, being phosphorylated by cellular kinases. In addition, conversion to a prodrug may limit the catabolism of AraA by adenosine deaminase. Synthesis of sixteen ProTides of AraA, including isopropyl esters of a large variety of D- or L-amino-acids was performed and antiviral activities of all compounds were evaluated in HepG2, Huh7 and Vero cell cultures. Three derivatives (Fig.**12**) exhibited dose-dependent inhibition of HSV replication and EC_{50} values in the low micromolar range. Eventually, they shown that bioactivation of the ProTides does not occur in Vero cells presumably because this cell line does not express the required enzymes.

As mentioned before, the aryloxyphosphoramidate prodrug strategy has also been applied successfully in oncology, as evidenced by NUC-1031 (Acelarin) [47] and NUC-3373, the prodrugs of gemcitabine and floxuridine respectively (Fig. **3**). In 2021, Slusarczyck and co-workers [22] reported in vitro biological data for single RP and SP isomers of NUC-1031 and NUC-3373 as well as their ability to target cancer stem cells. The in vitro cytotoxic activities of the individual diastereomers were found to be similar to their diastereomeric mixtures. Acelarin, the SP isomer of NUC-1031 has received a fast track designation from the US-FDA for the treatment of biliary tract cancer (BTC) in September 2021. However, the clinical phase III for the combination of NAC-1031 and cisplatin [15] was discontinued for advanced BTC in march 2022. In the meantime, NUC-7738 (Fig. **3**, the ProTide derivative of cordycepin) has also been proposed in order to overcome the susceptibility of the natural nucleoside cordycepin to adenosine deaminase [48]. It is currently under clinical evaluation as anticancer agent for patients with advanced solid tumors.

Besides their importance in the field of cancer and virology, ProTides are of potential interest as treatments for neurodegenerative diseases. Indeed, the activation of PINK1 (PTEN-induced kinase 1) has emerged as a useful strategy to induce and to maintain neuroprotective effects, an approach that would be suitable for treating Parkinson's disease. For example, N^6-furfuryladenine (kinetin) being shown to activate PINK1 in cells, the Mehellou's group [49] reported the synthesis (using a previously described procedure) and application of kinetin riboside ProTides to by-pass two important activation steps of kinetin (*i.e.*, glycosylation and the first phosphorylation step) (Fig. **13**). Such derivatives were able to activate PINK1 in cells independently of the mitochondrial depolarization. The ProTide technology has also been applied in the field of rare diseases, such as mitochondrial DNA depletion syndrome (MDS). Thus CERC-913 (a ProTide of an analogue of dG, undisclosed structure) was able to by-pass DGUOK deficiency in vitro and led to a significant increase of the mtDNA content [50].

In addition to their interest as therapeutic agents, ProTides have also been used as tools for delivering intracellularly nucleosidic probes, this last being especially useful to get an inventory of AMPylated proteins. In 2020, The Sieber's group [51] reported two studies related to AMPylation, in which adenosine triphosphate (ATP) donates an adenosine 5′-monophosphate (AMP) moiety to a protein's Ser, Tyr, or Thr side-chain. This prevalent posttranslational modification of proteins is regulating protein activity and is crucial for signaling pathways. Using a *N*-6-propargyl adenosine (N6pA) ProTide as probe, a chemical-proteomic approach was performed for identification of protein AMPylation in living cells (Fig. **14**).

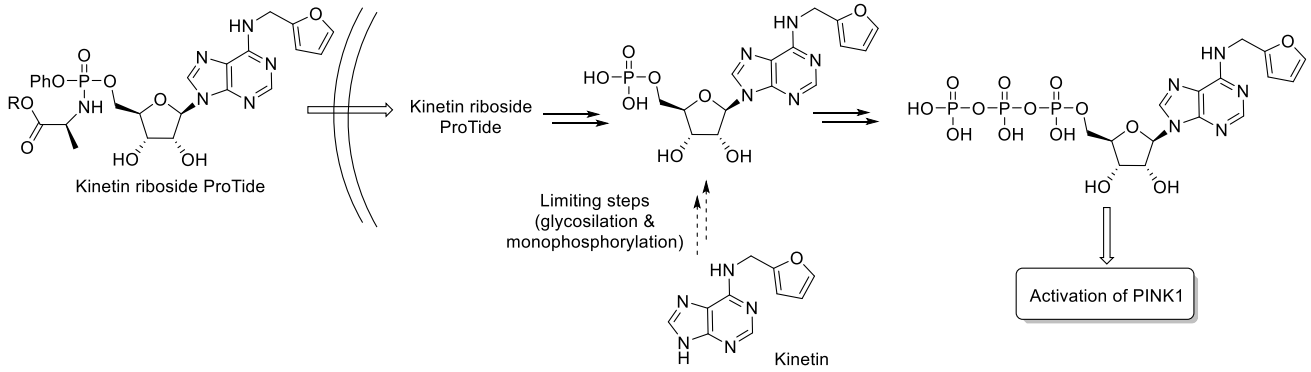


Figure (13). Application of the ProTide approach to kinetin for potential treatments of neurodegenerative diseases.

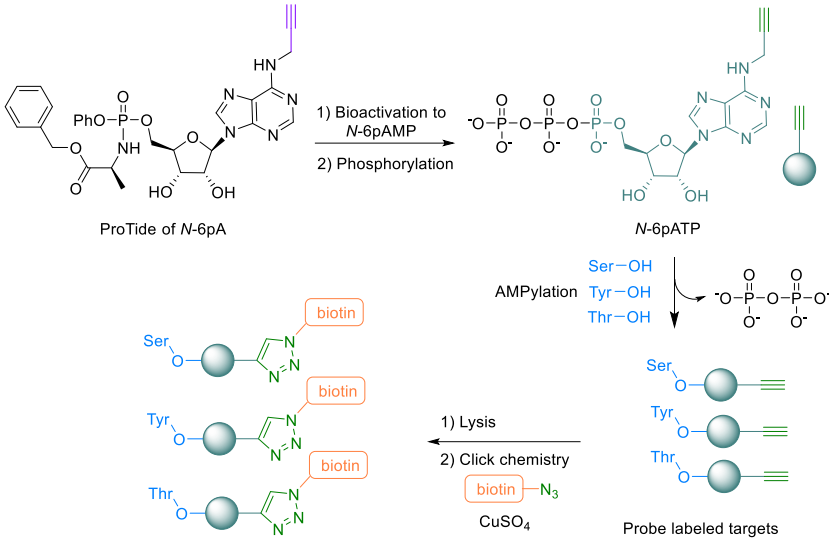


Figure (14). Application of the ProTide approach to N-6-propargyl adenosine to identify protein AMPylation in living cells.

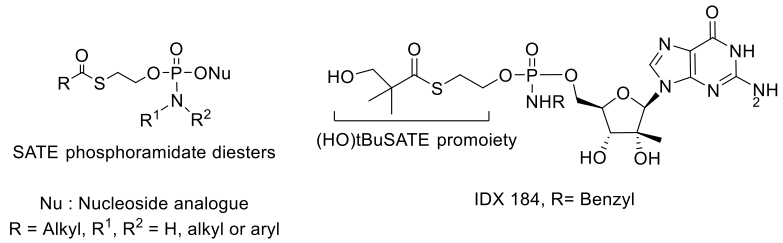


Figure (15). Generic structures of the SATE phosphoramidate diesters and IDX 184 (adapted from [54]).

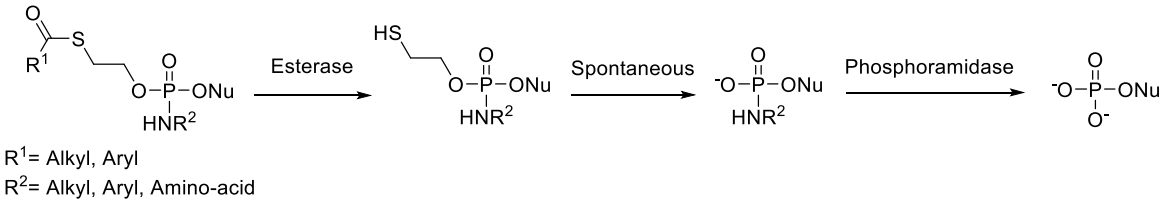


Figure (16). General mechanism of the bioactivation of SATE phosphoramidate diesters

Synthesis of the pronucleotide of N6pA was performed according to phosphoramidation with tBuMgCl. The presence of the alkyne group allowed to performed click chemistry with a rhodamine-biotin-azide tag, which resulted in intracellular probe visualization using immunohistochemistry. Thanks to this approach, AMPylated proteins were identified in various cell types such as cancer lines, human derived stem cells, as well as during bacterial infection. Moreover, the authors implemented the chemical-proteomic method for deciphering AMPylated host proteins in situ during bacterial infection of HeLa cells with Vibrio parahaemolyticus. Overall, the methodology provides a reliable detection of host AMPylation in situ and thus a versatile tool in monitoring infection processes.

2.2. Phosphoramidates incorporating a sulfur atom:

SATE (*S*-acyl-2-thioethyl) phosphoramidate diesters were first described by our laboratory in the 2000s (Fig. **15**) [52]. Once in the cell, these derivatives are enzymatically metabolized to deliver the nucleoside 5'-monophosphate, which is further converted to the active 5'-triphosphate form of the nucleoside analogue. Among these, IDX 184 was the first anti-HCV pronucleotide that entered clinical evaluations [3f, 53] but its development was unfortunately stopped in 2012. More recently, the Wagner's group has proposed a novel series of pronucleotides [55], based on the anchimerically activation process and incorporating a 2-(methylthio)ethyl group and tryptamine as phosphate protecting moieties (Fig. **18**).

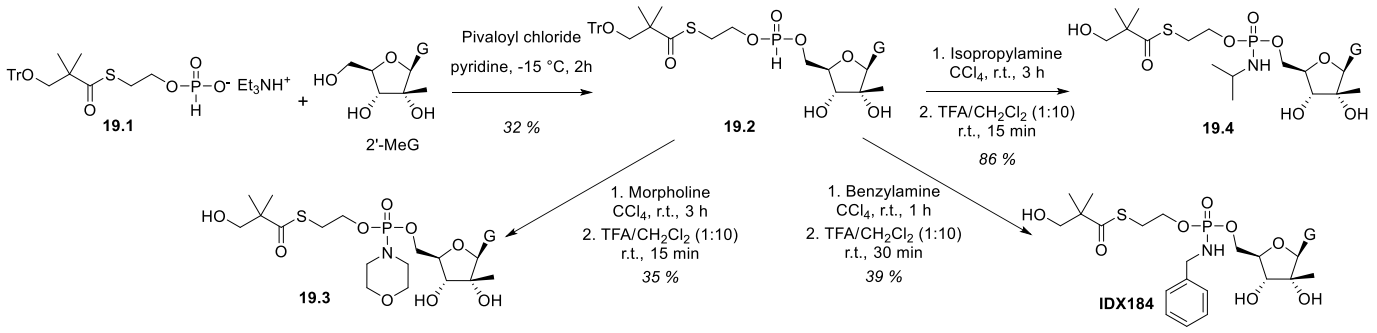
2.2.1. SATE phosphoramidate diesters

Previously, our group has showed that *S*-pivaloyl-2-thioethyl (*t*BuSATE) phosphoramidate diester derivatives of 3′-azido-2′,3′-dideoxy-thymidine (AZT), used as an anti-HIV nucleoside model, acted as true 5'-mononucleotide prodrugs in cell culture experiments [52]. Based on stability studies, a decomposition pathway for such SATE phosphoramidate diester derivatives was proposed and involved two enzymatic hydrolytic steps, the first being catalyzed by an esterase and the second one by a phosphoramidase (Fig. **16**).

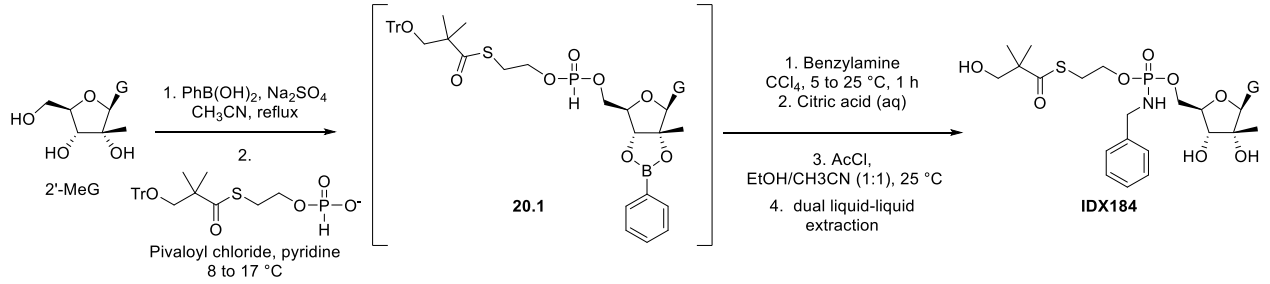
In 2015, G. Gosselin and coworkers [53] reported the synthesis and the study of SATE phosphoramidate diester derivatives of 2′-C-methyl guanosine (2′-MeG) as potential anti-HCV agents. The pronucleotides including benzylamine, morpholine or isopropylamine as amino residues were obtained in two steps (Scheme **19**) by coupling the nucleoside analogue (2′-MeG) with the required SATE

H-phosphonate monoester derivative **19.1** to afford a common nucleoside *H*-phosphonate diester intermediate **19.2**. Then, implementation of an oxidative Atherton–Todd amination reaction with benzylamine, morpholine or isopropylamine in CCl_4 gave rise to the corresponding phosphoramidate diesters, which were treated in acidic conditions to afford the desired IDX184 and its analogues **19.3** and **19.4**. All these pronucleotides were isolated as a mixture of diastereoisomers (1:1) that could not be separated during purification by reverse phase column chromatography. The three derivatives (IDX184, EC_{50} = 0.2 μM; compound 19.3, EC50 = 1.4 μM and compound 19.4, EC_{50} = 0.9 μM) showed better anti-HCV activities than the parent nucleoside (2′-MeG, EC_{50} = 3.2 μM). Thus, the more lipophilic derivative (IDX184) was found to be the most potent. Although the *in vitro* HCV replicon assay activities of the optically pure R_P and S_P diastereomers were close, they displayed marked physical differences.

Later on, a scalable synthesis of IDX-184 was developed and involved the use of phenylboronic acid (PBA) as to transient protecting group of the 2′,3′-hydroxyls of the nucleoside analogue. [56] Briefly, the free nucleoside 2′-MeG was protected with PBA, then coupled with H-phosphonate salt **19.1** in presence of pivaloyl chloride and pyridine to afford the H-phosphonate diester **20.1** (Scheme **20**), which was in situ treated with benzylamine and carbon tetrachloride according to the Atherton–Todd reaction. The PBA protecting group was removed using aqueous citric acid and detritylation was achieved with dry HCl. The 40–45% overall yield of IDX184 from 2′-MeG resulted in a 3-fold increase relative to the original unprotected approach (Scheme **19**). Therefore, this approach was implemented for the preparation of the first kilogram batches of IDX184. A number of improvements were required to scale up the procedure (cleavage of the cyclic boronate with aqueous citric acid, and detritylation with AcCl/EtOH). This process allowed to produce 20 kg of drug substance in 59% overall yield, with >99% purity in the requisite 1:1 diastereomeric ratio.



Scheme (19). Synthetic pathway to IDX184 and analogues.



Scheme (20). Process developed for the production of IDX184 on a multi-kilogram scale.

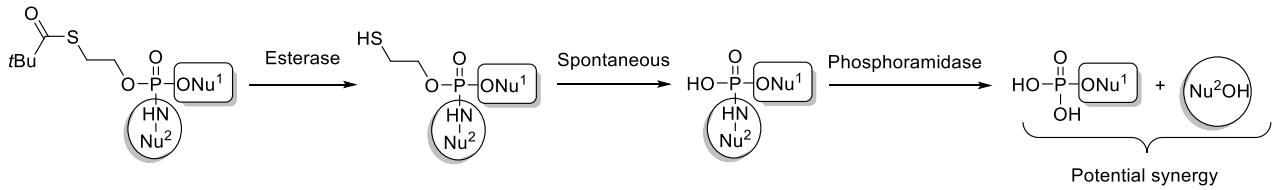
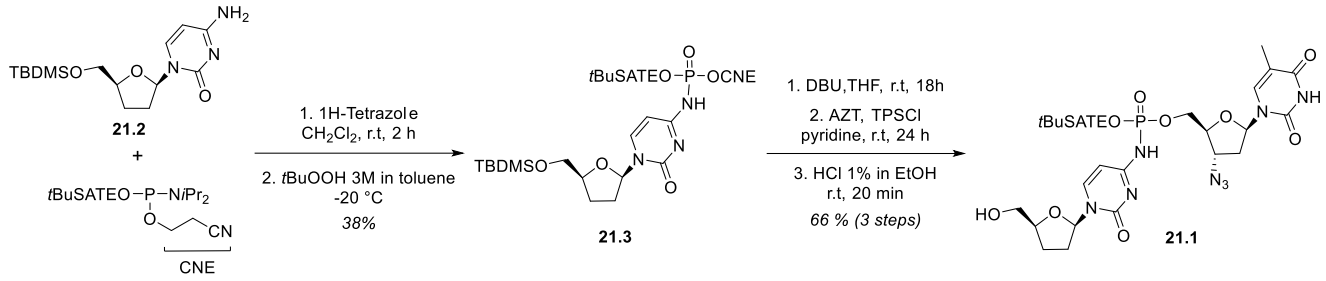
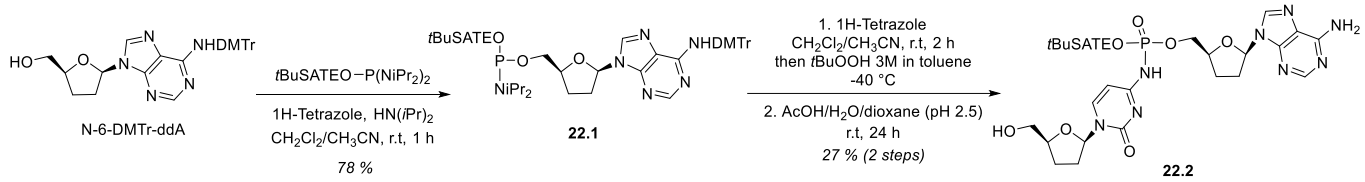


Figure (17). Design of dual prodrugs for the intracellular delivery of two bioactive nucleoside analogues.



Scheme (21). Synthetic pathway to AZT/ddC dual prodrug.



Scheme (22). Synthetic pathway to ddA/ddC dual prodrug.

In 2021, our group reported an original strategy to gain access to a novel series of phosphoramidate diesters as dual prodrugs [54]. In addition to the SATE promoiety, these derivatives incorporate two different anti-HIV nucleoside analogues (namely AZT and 2′,3′-dideoxycytidine (ddC) or ddC and 2′,3′-dideoxyadenosine (ddA)) linked to the phosphorus atom respectively by the sugar residue and the exocyclic amino function of the nucleobase (Fig. **17**). The rational for the design of such constructs was to deliver into cells two bioactive entities, one of which as a 5'-mononucleotide and the second as a free nucleoside, with the aim to observe synergistic effect. The desired compound **21.1** was prepared through a synthetic procedure involving the phosphitylation of 5'-silylated nucleoside 21.2, followed by in situ oxidation with *t*BuOOH to give rise to the N-4-phosphoramidate 21.3 in 38% yield (Scheme **21**). Then, the cyanoethyl (CNE) group was removed by using DBU to afford a phosphoroamidate monoester intermediate, which was condensed with AZT in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride (TPSCl), leading to the phosphorylated intermediate in 83% yield over two steps. Subsequent acidic treatment gave rise to the desired pronucleotide **21.1** in 80% yield. This strategy was also applied to the synthesis of a phosphoramidate derivative bearing ddA instead of AZT (Scheme **22**). Nucleoside 5'-*O*-phosphoramidite **22.1** was obtained in 78% yield by reacting N^6-DMTr-ddA with the *S*-pivaloyl-2-thioethyl *N*,*N*-bis(diisopropylamino)phosphine. Then, coupling with ddC in presence of 1*H*-tetrazole, followed by in situ oxidation with *t*BuOOH gave rise to the fully protected phosphoramidate in 41% yield. Finally, a smooth acidic treatment led to the pronucleotide **22.2** in 67% yield. The anti-HIV activities of the dual prodrugs **21.1** and **22.2** were determined and compared with their related nucleoside analogues. In human T4-lymphoblastoid cells, the derivative **21.1** incorporating both AZT and ddC exhibited remarkable antiviral activity with an EC_{50} in the nanomolar range and without additional cytotoxicity. In addition, similar EC50 values were observed in both CEM/TK$^-$ and CEM-SS cell lines, thus demonstrating the successful release of the 5′-mononucleotide of AZT within cells. In addition, these two pronucleotides showed higher selectivity index compared to the equimolar mixture of their constitutive nucleoside analogues.

2.2.1. SATE phosphoramidate diesters

Although widely successful, several drawbacks of the ProTide strategy [4, 57] limit its utility for the delivery of significant levels of nucleotide analogs in tissues other than the liver. In order to broaden the use of these pronucleotides, Wagner's research group [55, 58] has proposed a novel strategy that address the limitation of the ProTide promoiety. This approach, referred to as "anchimerically Hint1-activated ProTides (AHA-ProTides)", is based on anchimerically activated phosphoramidate derivatives, incorporating a cleavable 2-(methylthio)ethyl substituent and a Hint1-cleavable tryptamine as phosphate protecting groups (Fig. **18**). The prodrug is activated by a sulfur mediated intramolecular nucleophilic substitution to yield a monoester phosphoramidate, which gets hydrolyzed by Hint1 to release the 5'-mononucleotide.

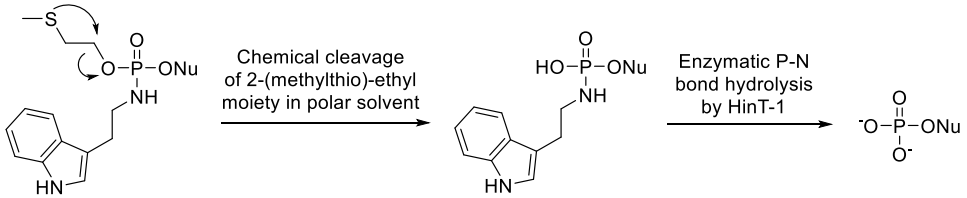
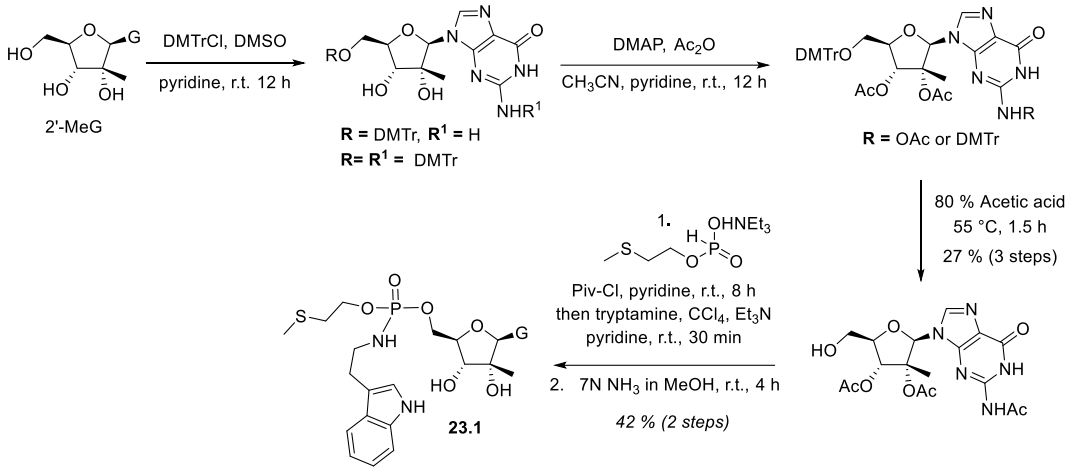


Figure (18). Proposed mechanism for the activation of 2-(methylthio)ethyl phosphoramidate diester



Scheme (23). Synthesis of the AHA-ProTide derivative of 2′-MeG using an *H*-phosphonate approach.

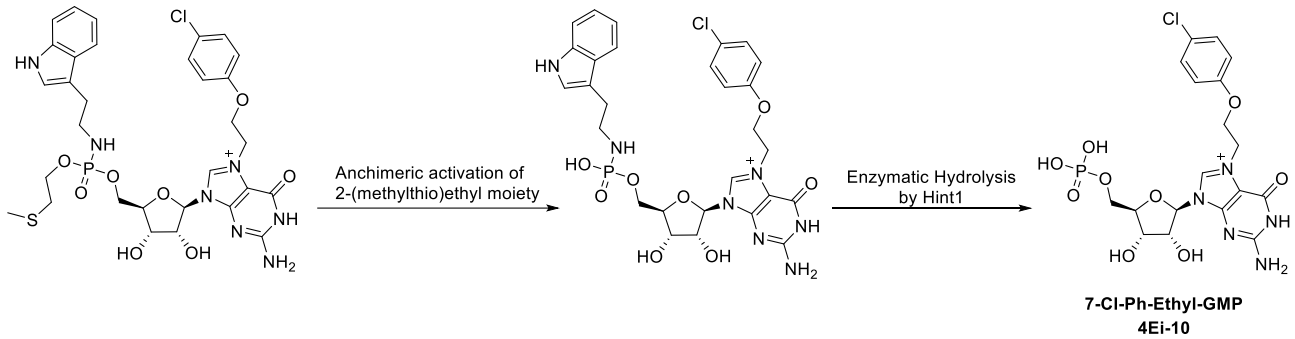
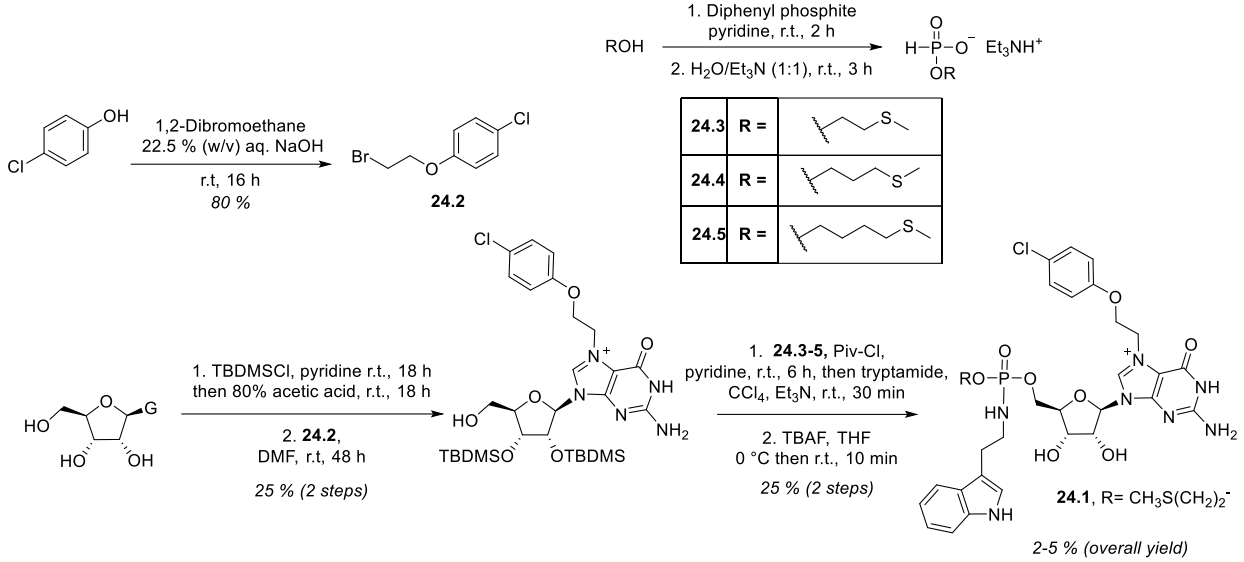


Figure (19). Proposed mechanism for the intracellular release of 4Ei-10 from the corresponding pronucleotides.

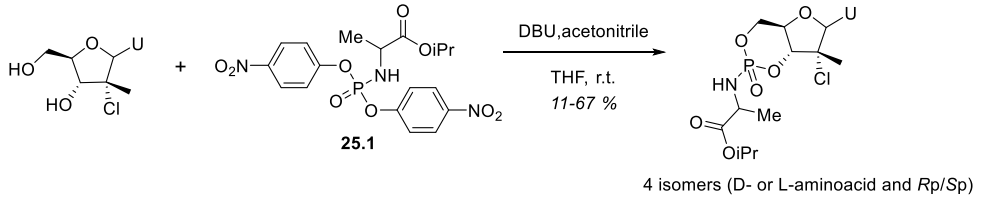
Anchimerically activated phosphoramidate derivatives proved to be useful for 5'-mononucleotide delivery in both antiviral [55a, 58a] and antitumoral [55b, 59] fields. Accordingly, the 2′-C-β-methyl guanosine phosphoramidate diester **23.1** was obtained using standard *H*-phosphonate chemistry (Scheme **23**) and was found to be more potent than the parental nucleoside against Dengue virus 2 (DENV-2) [55a] and Zika virus (ZKV) [58a], while displaying no cytotoxicity. Another anchimerically activatable pronucleotide, shown in Fig. **19**, was developed by Wagner's group in order to release into cells the N-7-(p-chlorophenoxyethyl)guanosine monophosphate (4Ei-10), a nucleotide antagonist of the eukaryotic translation initiator factor (eIF4E) [55b]. The binding of 4Ei-10 to eIF4E blocks eIF4E from interfering with the mRNA cap, and therefore inhibits the initiation of translation [59]. Treatment of malignant mesothelioma with 4Ei-10 led to decreased cell proliferation, increased sensitivity to pemetrexed and altered expression of malignancy-related proteins. Synthesis of pronucleotide **24.1** and analogues was performed using standard H-phosphonate chemistry (Scheme **24**) and low overall yields.

2.3. Miscellaneous

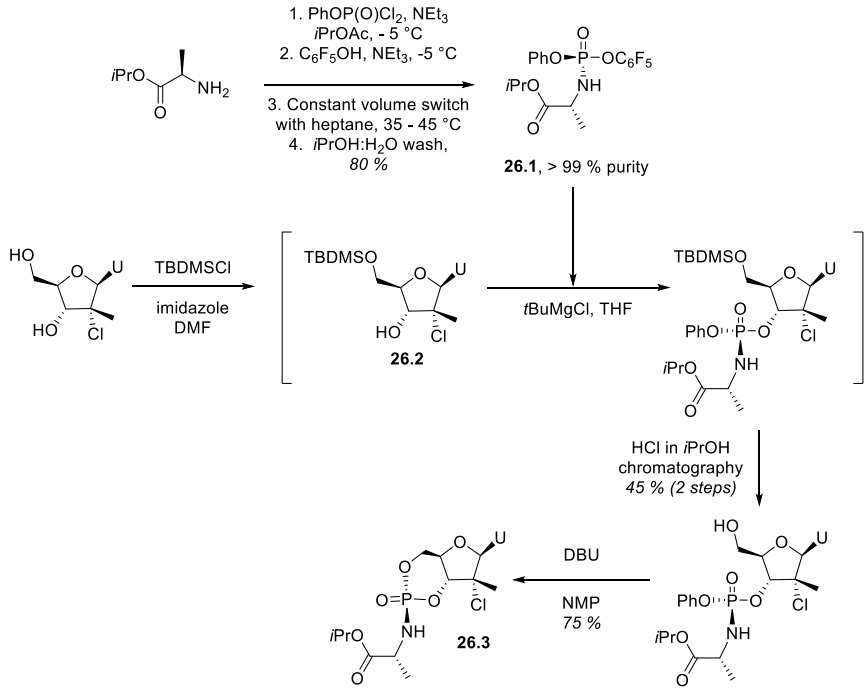
Initially reported by Meppen and co-workers [60] in 2009, 3',5'-cyclic mononucleoside phosphoramidate diesters have also been viewed with interest [61]. In 2017, Dousson and coworkers [35] applied this approach to 2'-α-chloro-2'-β-C-methyluridine aiming at discovering potent liver-targeted HCV polymerase inhibitors. Synthesis was performed using a previously described one-step method involving phosphoramidating reagent **25.1** (scheme **25**). All 4 isomers including either D- or L-alanyl isopropyl ester and R_P and S_P diastereoisomers were separated by preparative HPLC or by column chromatography on silica gel. Single crystals were isolated for derivatives incorporating D-amino acid and X-ray structure determination allowed to assign *P*-stereochemistry. As observed for the corresponding ProTides, the D -alanine derivatives led to high levels of NTP in the liver using a mice model.



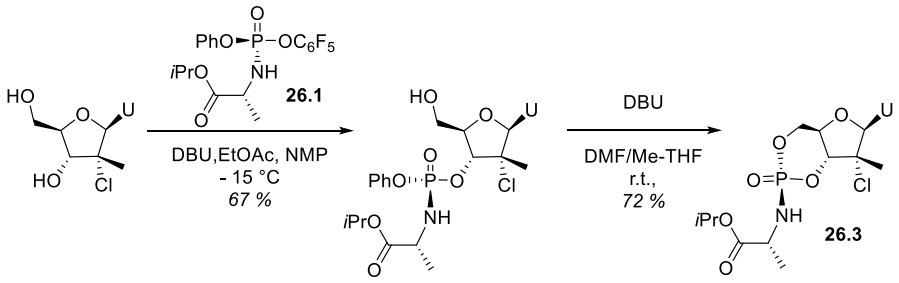
Scheme (24). Synthesis of anchimerically activatable pronucleotides of N7-(*p*-chlorophenoxyethyl)guanosine.



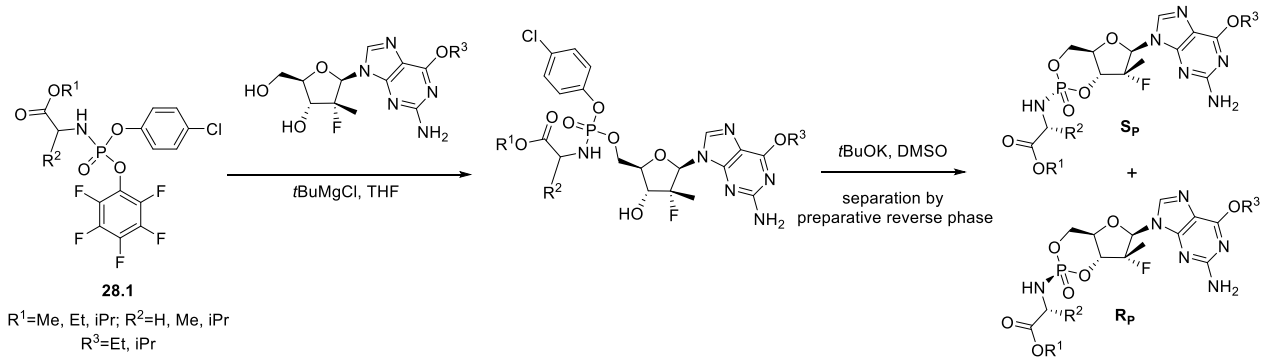
Scheme (25). Synthesis of 3',5'-cyclic mononucleoside pronucleotides of 2'-α-chloro-2'-β-C-methyluridine.



Scheme (26). Isolation of the (*R*,*R*) phosphoramidate diester 26.1 via DKR crystallization and its subsequent use for the preparation of cyclic phosphoramidate diester 26.3.



Scheme (27). Protecting-group-free route to cyclic phosphoramidate diester **26.3**.

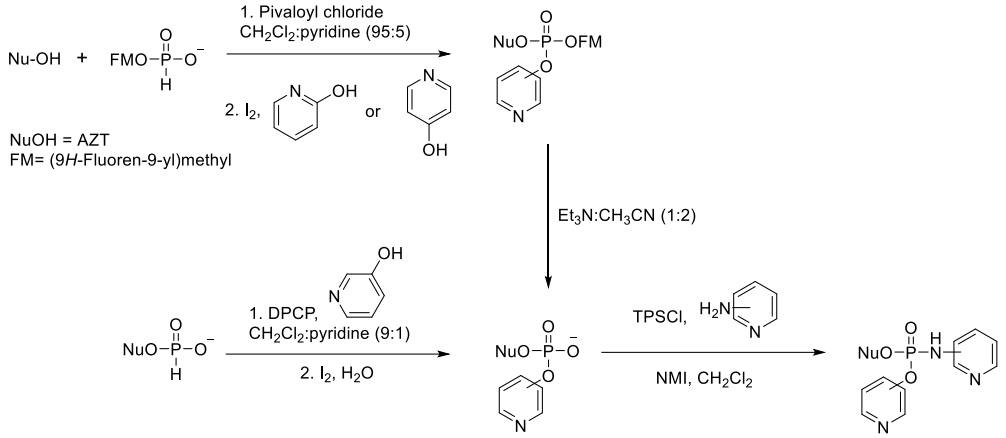


Scheme (28). Synthetic pathway to cyclic phosphoramidate prodrugs of 6-O-alkyl-2'-□-fluoro-2'-□-C-methyl guanosine.

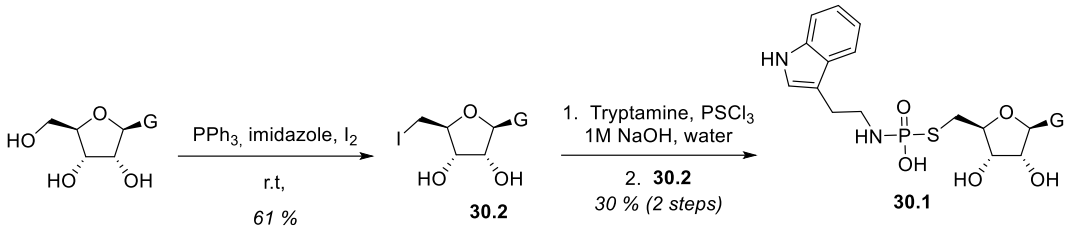
In 2018, R. K. Orr *et al.* [62] reported the synthesis of the same cyclic prodrug through a green and sustainable approach (Scheme **26**). In the course of this study, an efficient dynamic crystallization process was developed to obtain the (*R*,*R*) phosphoramidate diastereoisomer **26.1** in 80% yield and 99% purity. An original and highly stereoselective double inversion strategy was set up, involving 5'-protected nucleoside analogue **26.2**, then 3'-phosphoramidation with optically pure reagent **26.1**, followed by deprotection and intramolecular cyclisation in the presence of DBU (Scheme **26**). This strategy was further improved to gain access to kilogram scale of pronucleotide **26.3**. Accordingly, a protecting-group-free route was developed through a DBU-promoted selective 3'-phosphorylation reaction affording 3'-substituted phosphoramidate **27.1** (Scheme **27**). Based on NMR and computational studies, this unusual regioselectivity was explained by the formation of a critical hydrogen bond between the 5'-OH and uridine carbonyl in position 2, therefore hampering the reactivity at the 5'-hydroxyl. Finally, cyclisation was performed using DBU in DMF/Me-THF. Finally, this two steps procedure was reduced to a one-pot process by slowly adding DBU to a solution of the nucleoside and reagent **26.1** in acetonitrile, at low temperature over 2h and then warming to 0°C for 18h, giving rise to **26.3** in 66% yield.

In 2020, R. Karuna *et al.* [63] reported the preparation and anti-dengue virus activities of cyclic phosphoramidate derivatives of 2'-α-fluoro-2'-β-C-methyl guanosine (Scheme **28**). These prodrugs were prepared by coupling the nucleoside analogue with the phosphoramidating reagents **28.1** in presence of *t*-BuMgCl and then intramolecular cyclisation was performed in presence of *t*BuOK. The corresponding cyclic phosphoramidate diesters were obtained as diastereomeric mixture. They identify a potent prodrug 28.2 (R_P isomer incorporating L-alanyl ethylester) producing high level of NTP in peripheral blood mononuclear cells (PBMCs) and exhibiting in vivo efficiency in mice and dog models. However, the preclinical safety results ("no observed adverse effect level" (NOAEL) could not be achieved in dogs) suspended its development.

In 2019, the Stawinski's group [64] has designed bi(heteroaryl) phosphoramidate diester derivatives of AZT and ddU as potential anti-HIV pronucleotides. As no enzymatic activity is known to hydrolyze phosphoramidate diesters, the first step in the decomposition process of these derivatives is expected to be the nucleophilic attack of water onto the phosphorus atom and may led to either (or both) the cleavage of the P-N or the P-O bonds. After numerous attempts, three different synthetic pathways, involving *H*-phosphonate chemistry, were tested for the preparation of such derivatives, as illustrated in Scheme **29** for AZT derivatives. Analysis of the biological data were puzzling and a few derivatives were able to inhibit HIV-replication with EC_{50} in the nanomolar range. Further investigations are still required to probe the mechanism of action of such compounds.



Scheme (29). Example of the synthetic routes to obtain bi(heteroaryl) phosphoramidate diesters derivatives of AZT.



Scheme (30). Synthesis of tryptamine containing 5'thio-guanosine phosphoramidate **30.1**.

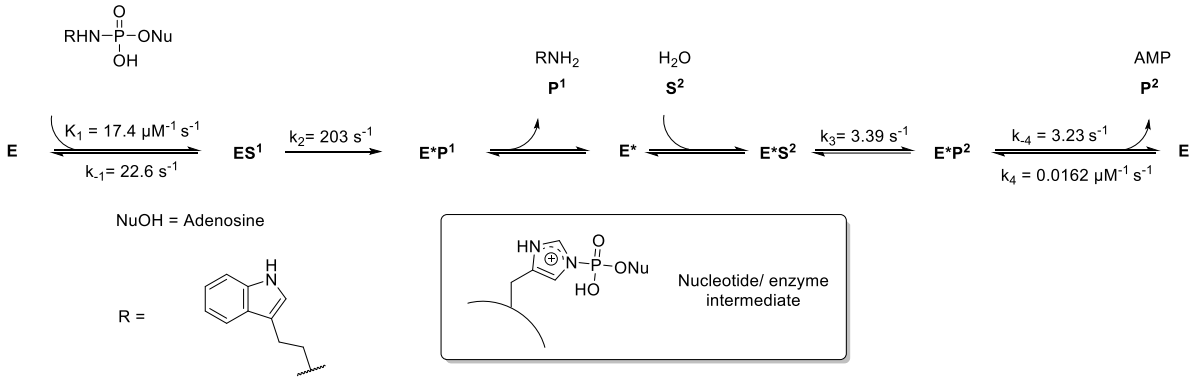


Figure (20). Kinetic parameters and enzyme/substrate complexes proposed by C.R. Wagner and co-workers, adapted from [68].

3. Phosphoramidate monoester: Synthesis - Therapeutic agents - Biological tools

C.R. Wagner and co-workers have been involved in the design, synthesis and study of nucleoside phosphoramidate monoester as prodrugs of 5'-mononucleotides since the 90s [65]. They were also interested in deciphering the mechanism of hydrolysis of such derivatives by human histidine triad nucleotide binding protein 1 (hHint1) and probing its substrate specificity [66]. More recently, they reported anchimerically activated phosphoramidate diester derivatives (see previous section) [55]. By using fluorogenic nucleoside tryptamine thiophosphoramidate (compound **30.1**, Scheme **30**) as substrate and cryo-crystallography, they were able to trapped nucleotide / enzyme intermediates previously suggested [67] (Fig. **20**) and investigated the hHint1-catalyzed hydrolysis of phosphoramidate nucleoside monoester. Synthesis of compound **30.1** was performed in two steps, starting from guanosine, with 30% overall yield (Scheme **30**). Briefly, treatment of the free nucleoside by triphenylphosphine, imidazole and iodine afforded 5'-iodo guanosine **30.2** which was substituted by tryptamine thiophosphoramidate, generated beforehand from tryptamine and thiophosphoryl trichloride in basic aqueous conditions. In the meantime, they also reported an extensive study of the substrate selectivity of hHint enzymes by using enzyme kinetics, X-ray crystallography, and isothermal titration calorimetry with both wild-type and inactive mutant enzymes. [69] The studied nucleoside phosphoramidate monoesters (Fig. **21**) were obtained by coupling the 5'-mononucleotide to various amino-derivatives in presence of carbodiimide (EDC.HCl) as previously described [66c].

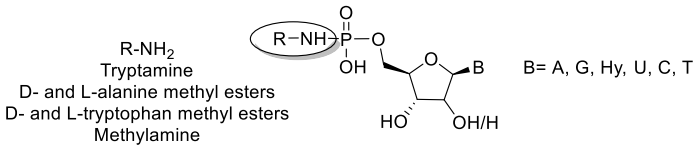
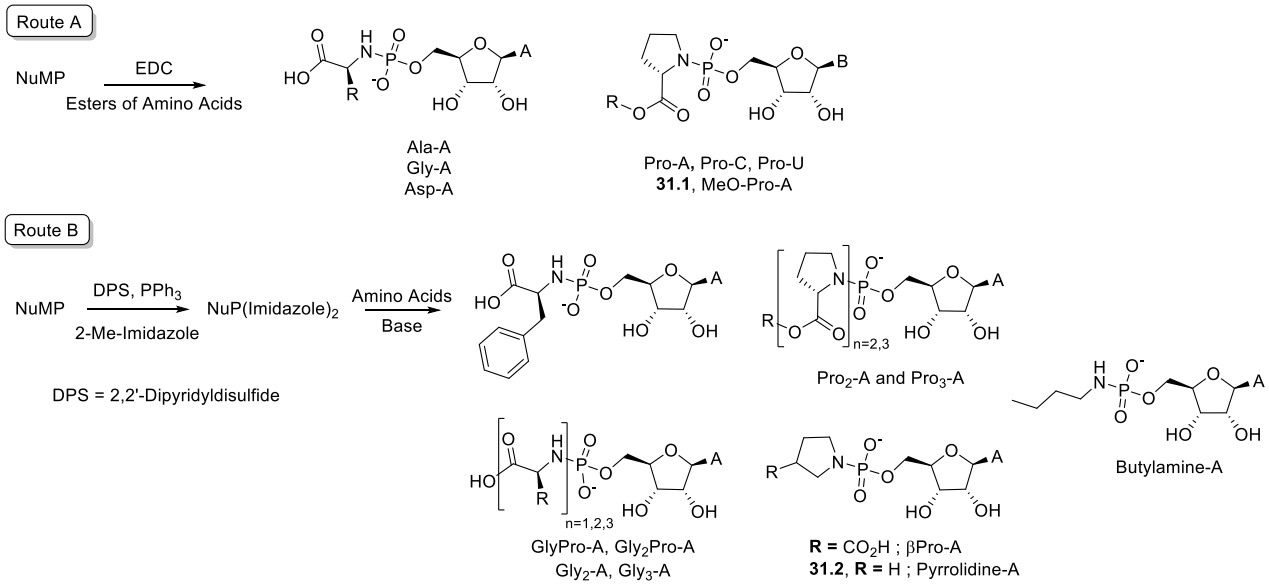


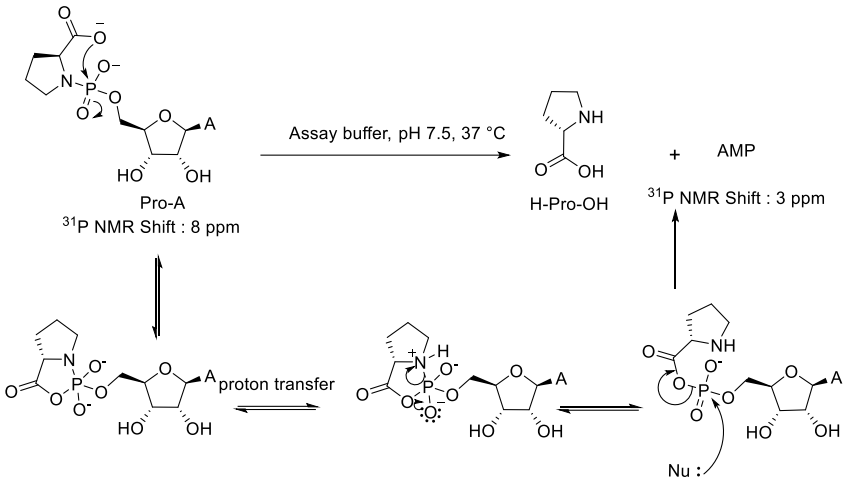
Figure (21). Generic structure of the derivatives used to challenge hHint1 substrate selectivity. Adapted from [69].



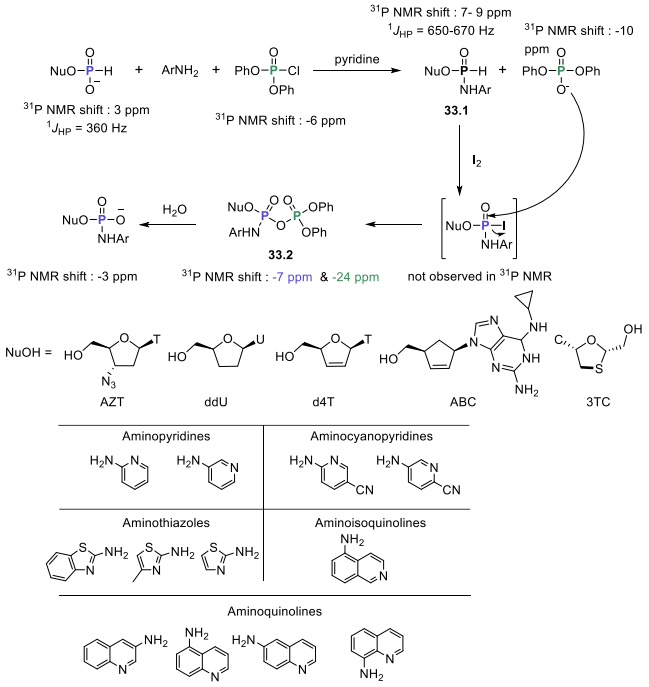
Scheme (31). Synthetic pathways to nucleoside 5'-phospohramidate monoesters containing various amino-acids, di- or tripeptides, pyrrolidine or n-butylamine.

Triciribine and cidofovir were also used as representative of nucleoside and acyclic nucleoside analogues, respectively. Minimal structural basis for binding into the active site included the presence of a phosphate or phosphate mimic (making extensive hydrogen bonding contacts with the protein) and the five member-ring of the sugar. Diversity of the nucleobase (with preference for purine ones) is somehow accepted as binding modes are similar but changes were observed in the nature and the number of contacts. Finally, among the amino counterpart, which is directly related to the design of ProTides and phosphoramidate diester prodrugs, tryptamine exhibited the most efficient catalysis as it does not generate steric hindrance in the vicinity of the phosphoramidate group.

Recently, D. Jovanovic and co-workers [70] became interested in studying the enzyme-free release of 5'-mononucleotide from nucleoside 5'-phosphoramidate monoesters. The latter include α-amino-acids, β-proline, pyrrolidine, n-butylamine or tripeptides as amino-residues and adenosine, cytosine and uridine as nucleosidic counter-part (Scheme **31**). Depending of the nature of the amino residue the free 5'-mononucleotides were either directly coupled with the amino-acid esters in presence of EDC (route **A**) or first converted into their corresponding phosphorimidazolates in presence of triphenylphosphine, 2,2'-dipyridyl-1,1'-disulfide and 2-methylimidazole and then substituted by the required amines in basic conditions (route **B**). Hydrolysis reactions were monitored by ^{31}P NMR (in 1-ethylimidazole buffer at pH 7.5 and 0°C or 37°C), as both starting material and 5'-mononucleotide showed distinct displacements, respectively at ~8 and 3 ppm. The results demonstrated a strong effect of the nature of the amino-acid residue, with proline containing derivatives being hydrolyzed much faster ($t_{1/2}$ < 5 h) than all other derivatives ($t_{1/2}$ > 3 days), whatever the nature of the nucleobases, except for proline methylester phosphoramidate (compound **31.1**). The short half-life observed for derivative 31.2 ($t_{1/2}$ ~ 8 h), containing pyrrolidine and in the same range as for proline phosphoramidates, highlighted the importance of the cyclic secondary amine structure. A possible mechanism for the hydrolysis of prolinyl phosphoramidates (Scheme **32**) was also proposed on the basis of crystallographic data obtained from various RNase A-phosphoramidate complexes.

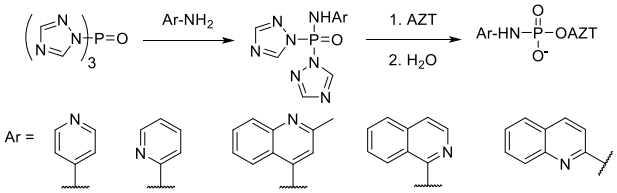


Scheme (32). Hydrolysis step of prolinyl phosphoramidate monoester of adenosine and proposed mechanism.



Scheme (33). Synthetic pathways for N-heteroaryl phosphoramidate monoesters of various antiviral nucleoside analogues.

Following their work on AZT (*N*-pyridyl)-phosphoramidate monoesters [71], the group of A. Kraszewski and M. Sobkowski proposed lipophilic (*N*-heteroaryl)phosphoramidate derivatives as potential anti-HIV agents [72]. Heterocyclic amines were selected on the basis of lipophilic input and pK_a as both parameters may influence the cellular permeability of the resulting phosphoramidate monoester and the equilibrium of their respective neutral or protonated forms under physiological pH. A first series of derivatives (Scheme **33**), incorporating commercially available isomers of aminopyridines, amino-cyanopyridines, aminothiazoles, aminoquinolines and amino-isoquinolines…, were obtained using *H*-phosphonate chemistry and an optimized one-pot two-stage process. Briefly, the nucleoside *H*-phosphonate monoesters were condensed with the selected heteroaryl amines in presence of diphenyl chlorophosphite, affording *H*-phosphonamidate intermediates **33.1**. The latter were in situ oxidized in presence of iodine to the desired phosphoramidate monoesters.



Scheme (34). One-pot synthesis N-heteroaryl phosphoramidate monoesters of AZT.

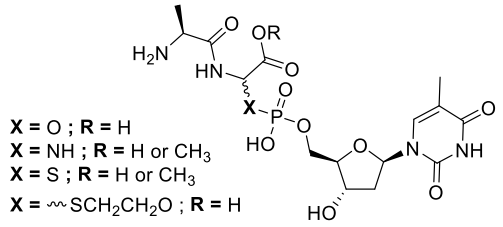
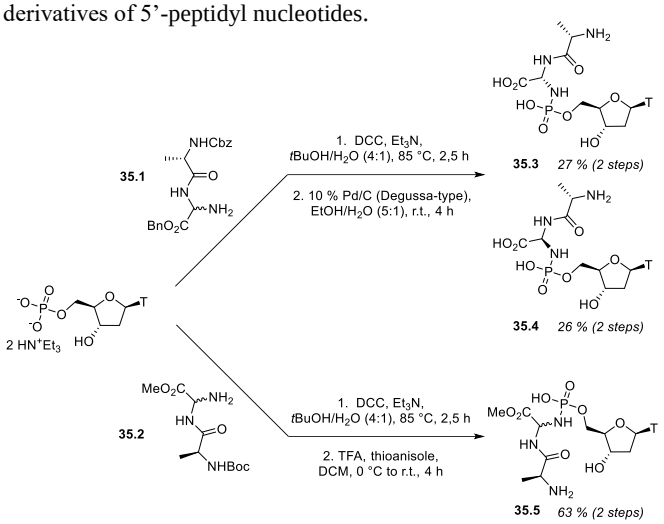


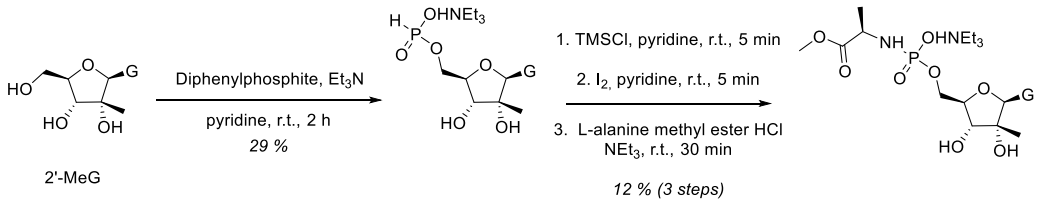
Figure (22). Structures of L-Ala-Gly-dTMP conjugates as model derivatives of 5'-peptidyl nucleotides.



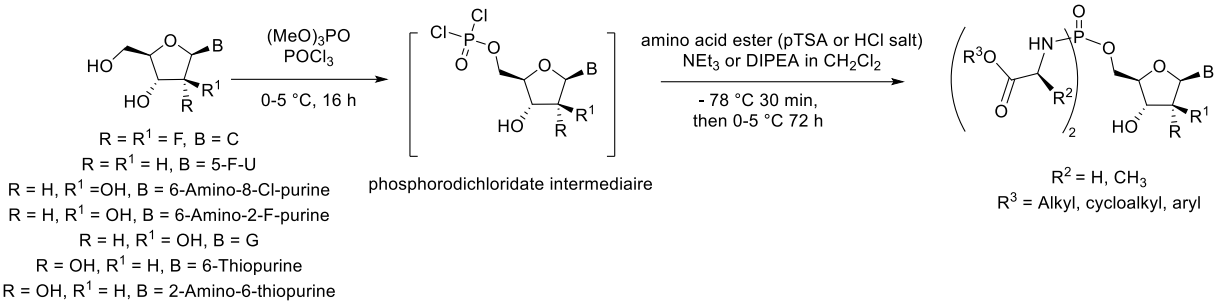
Scheme (35). Synthetic pathways to phosphoaminal derivatives of 2'-deoxythymidine.

In addition, ^{31}P NMR monitoring of the reactions allowed the authors to investigate the mechanism of the reaction and to propose the formation of a mixed pyrophosphoramidate **33.2** as possible intermediate (Scheme **33**). The second series of derivatives including AZT and amines with pK_a > 7 (Scheme **34**) were obtained using a P(V) approach and the tris(triazolyl)phosphoramidate as starting materiel. This last was successively condensed with the required amines and AZT, then hydrolysis was carried out. Stability of all synthesized compounds was evaluated in RPMI containing 10% fetal bovine serum and all derivatives appeared stable ($t_{1/2}$ > 3 days), excepted for the AZT derivatives containing thiazoles and ddU derivatives containing 3-aminoquinoline or methylthiazole. In addition, most of the AZT (*N*-heteroaryl)phosphoramidates exhibited potent anti-HIV-1 activities (EC_{50} values in the nanomolar range) whereas ddU, d4T, ABC, and 3TC derivatives were less actives (EC_{50} values in the micromolar range). As correlation between decomposition of the studied phosphoramidate diesters into 5'-mononucleotide and antiviral activity was not clearly evidenced, the authors proposed two scenarios, either the release of the free nucleoside analogue and/or the 2',3'-dideoxynucleoside (*N*-heteroaryl)phosphoramidate monoesters that may serve as direct substrates for HIV-reverse transcriptase, as suggested by the results of P. Herdewijn group [73]. Indeed, during the last decade, P. Herdewijn and co-workers have reported the ability of 5'-aminoacyl-mononucleotides to readily interfere with the nucleic acid polymerization process [73-74]. When incorporating L-aspartic acid, L-histidine or iminodipropionic acid the corresponding 2'-deoxyadenosine phosphoramidate monoesters have been shown to act as efficient alternatives to NTPs during DNA synthesis. Recently, they reported the synthesis and the study of 5'-peptidyl nucleotides (Fig. **22**), including phosphodiester, phosphoramidate or phosphorothioate bonds [75]. Two synthetic pathways were explored, both involving a nucleophilic substitution reaction of a leaving group that could be carried either by the dipeptide or by the nucleotide counterparts. Synthesis of the nucleoside phosphodiesters (Fig. **22**, X=O, R=H and X= $S(CH_2)_2O$, R=H) was a challenging task. Phosphohemithioaminal ester derivatives (Fig**. 22**, X=S, R=H or CH_3) were readily prepared from selective *S*-alkylation of *N*-Boc-L-alanyl-D,L-2-acetoxyglycine methylester with thymidine 5'-*O*-phosphorothioate (obtained beforehand as triethylammonium salt using an adapted oxathiaphospholane approach previously described in the literature [76]) and then acidic treatment. Finally, extensive chemistry was developped to prepare the required peptidyl precursors (Scheme **35**, compounds **35.1** and **35.2**), which were coupled to thymidine 5'-monophosphate (as triethylammonium salt) in presence of DCC in order to obtain the phosphoaminals of (L-alanyl-D,L-2-aminoglycine)-TMP (compounds **35.3** and **35.4**) and of (L-alanyl- D,L-2-aminoglycine methyl ester)- TMP (compound **35.5**). Among all compounds, only the phosphoaminal ester analogues 35.3 and 35.4 were significantly incorporated by the tested microbial DNA polymerases and may be considered as potential triphosphate mimics. One should note that the diastereoisomer **35.3** exhibited better substrate properties than **35.4**. These results point out that dipeptides behave as good leaving groups for DNA polymerization and highlight the potential of such constructs as valuable biological tools and prodrug.

As part of the structure–activity relationship studies concerning pronucleotides of 2′-C-methylguanosine (see sections 2.2.1 and 5), G. Gosselin and coworkers also obtained the corresponding phosphoramidate monoester derivative including the L -alanine methyl ester (Scheme **36**) [53]. This last was obtained in low yields from the nucleoside 5'-*H*-phosphonate derivative as starting material, prepared by reacting the free nucleoside analogue with diphenylphosphite, successively treated with trimethylsilyl chloride and iodine, and then the amino-acid ester. In comparison to the lead compound IDX184 (see section 2.2.1), the phosphoramidate monoester derivative did not showed significant anti-HCV activity in the replicon assay.



Scheme (36). Synthesis of the 2′-C-methylguanosine phosphoramidate monoester derivative.



Scheme (37). Synthetic pathway to various phosphorodiamidate monoester derivatives.

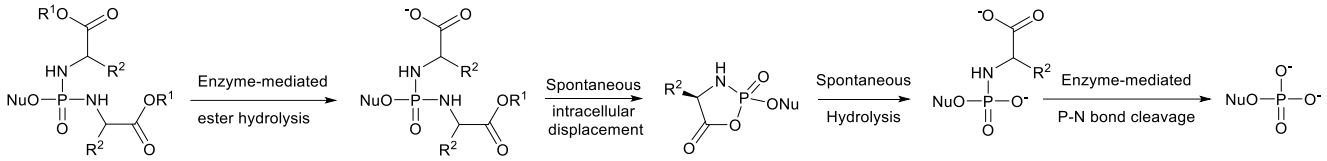
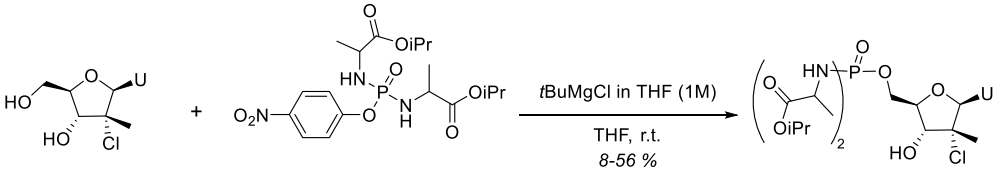


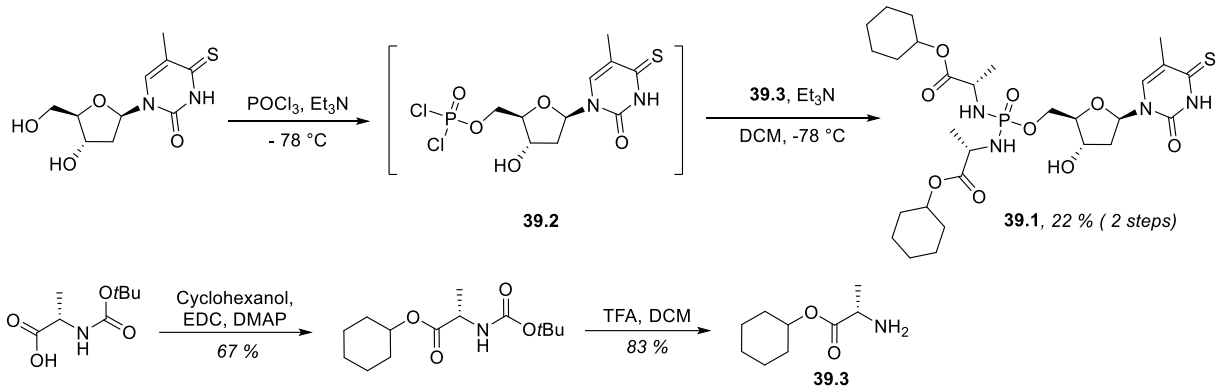
Figure (23). Proposed mechanism for the bioactivation of phosphorodiamidate monoester, involving both enzyme-mediated and spontaneous hydrolytic steps.

4. Phosphorodiamidate: Synthesis - Therapeutic agents - Biological tools

In 2011, C. McGuigan and co-workers reported the first extensive SAR concerning nucleoside phosphorodiamidate monoesters, including D- and L-amino acids (mainly L-alanyl esters) or simple amines, as novel series of anti-HCV pronucleotides [77]. When the two amino-residues are identical, the so called symmetrical phosphorodiamidates present an achiral phosphorus and thus offer a significant advantage over other prodrug approaches leading to diastereoisomeric mixtures. In addition, when natural amino acids are used, their release during the pronucleotide decomposition process should not lead to additional toxicity. The same group also reported the application of the symmetrical phosphorodiamidate strategy to antiviral and anticancer nucleoside analogues [78]. Briefly, these compounds may be obtained in a one-pot two step reaction, where the nucleoside phosphorodichloridate is firstly generated and then the substitution of the chlorine atoms by amino residues afforded the expected phosphorodiamidate derivatives (Scheme **37**). Depending of the nature of the nucleoside analogues (especially its susceptibility to acidic media) the first step corresponds to the Yoshikawa [79] phosphorylation using phosphorus oxychloride, in the presence of triethylamine or not, and then addition of an excess of the appropriate amino acid ester as *p*-toluene sulfonate salt, still in the presence of triethylamine or diisopropylethylamine. The cytotoxic activity of the sixteen derivatives was evaluated in a panel of tumour cell lines, in comparison to their parent nucleoside analogues. All studied compounds appeared as potent or less potent than the parent nucleosides, and the authors hypothesized that this results may be associated either to unfavorable cellular uptake and/or suceptibility to drug efflux (especially for lipophilic compounds and metabolites), and/or default during the bioactivation process leading to the release of the 5'-mononucleotide. Thus, stability of the gemcitabine containing pronucleotides was studied in presence of carboxypeptidase Y, using ^{31}P NMR monitoring, in order to support the previously proposed mechanism of bioactivation for phosphorodiamidate derivatives [77-78] and involving four putative steps (Fig. **23**). Briefly, the first step is mediated by esterase and/or carboxypeptidase activity leading to the hydrolysis of one or the two ester groups from the amino-acid residues, then a spontaneous intramolecular attack of the carboxylate anion on the phosphorus occurs and results in the formation of a five-membered ring phosphoranhydride (intermediate not observed by ^{31}P NMR). Finally, spontaneous hydrolysis of this last affords a nucleoside phosphoramidate monoester which is expected to be converted into the 5'-mononucleotide through a phosphoramidase activity as already observed in the ProTide approach.



Scheme (38). Synthesis of phosphorodiamidate derivatives of 2'-α-chloro-2'-β-C-methyluridine including D or L-alanine isopropyl esters.



Scheme (39). Synthesis of phosphorodiamidate derivative of 2'-deoxy- 4-thiothymidine.

Recently, the phosphorodiamidate approach has been applied to various anti-HCV nucleoside analogues by other groups [35, 80]. When comparing in vitro activities and level of the corresponding NTP produced *in vivo* of phosphorodiamidate derivatives of 2'-α-chloro-2'-β-C-methyluridine (Scheme **38**) [35] marked differences were observed between the two pronucleotides including L- or D-alanine esters. While the D-derivative was inactive in the replicon assay, it provided reasonable level of NTP in the liver. This last result supports the hypothesis that a different metabolism, specific to the D-amino acid promoiety, is likely occurring in the liver in comparison to the HCV replicon cells system.

In 2018, A.K. Hedger *et al.* [81] proposed to use this type of pronucleotides, including 4-thiothymidine as nucleoside counterpart (Scheme **39**), to design a biological tool for cellular DNA labelling. Once incorporated into DNA the 4-thiothymidine would present the advantage to be easily converted into 5-methyl-2'-deoxycytidine in basic conditions. Whereas the conversion of 4-thiothymidine into 5-methyl-2'-deoxycytidine was evidenced for 12-mer oligonucleotides, the result on PCR products was less convincing. Thus, the phosphorodiamidate derivative of 4-thiothymidine 39.1 was obtained in modest yield following a one-pot two-step reaction involving a dichlorophosphoridate intermediate 39.2 and its condensation with cyclohexyl-L-alaninate **39.3**. In a cellular context, neither incubation of the free nucleoside or the pronucleotide led to significant incorporation of 4-thiothymidine into DNA.

5. Phosphotriester: Synthesis - Therapeutic agents - Biological tools

The phosphotriester scaffold is one of the historical structure reported for the design and the synthesis of pronucleotides, in addition symmetrical phosphoesters do not induce the formation of diastereoisomeric mixture associated with a chiral center at the phosphorus atom. Several approaches have been proposed and involved the pivaloyloxymethyl (POM), the isopropyloxycarbonyl (POC), the S-acyl thioethyl (SATE) and the *S*-[(hydroxyethyl)sulfinyl)]-2-thioethyl (DTE) promoieties (Fig. **2**). The success of this symmetrical pronucleotide approaches is associated with its application to antiviral acyclonucleoside phosphonates and including two marketed drugs, *i.e.*, the adefovir dipivoxil (bis(POM)PMEA, Hepsera®) [82] and tenofovir disoproxil fumarate (bis(POC)PMPA or TDF, Viread®) [83]. Their mechanisms of decomposition involved either an esterase or a reductase mediated hydrolysis step (Fig. **24**) and then a spontaneous chemical process leading to the formation of formaldehyde or ethylene sulfide. The two protecting groups bore by the 5'-monophosphate function may also be different, leading to "asymmetrical" derivatives, such as SATE-aryl phosphotriesters (Fig. **2**) [84].

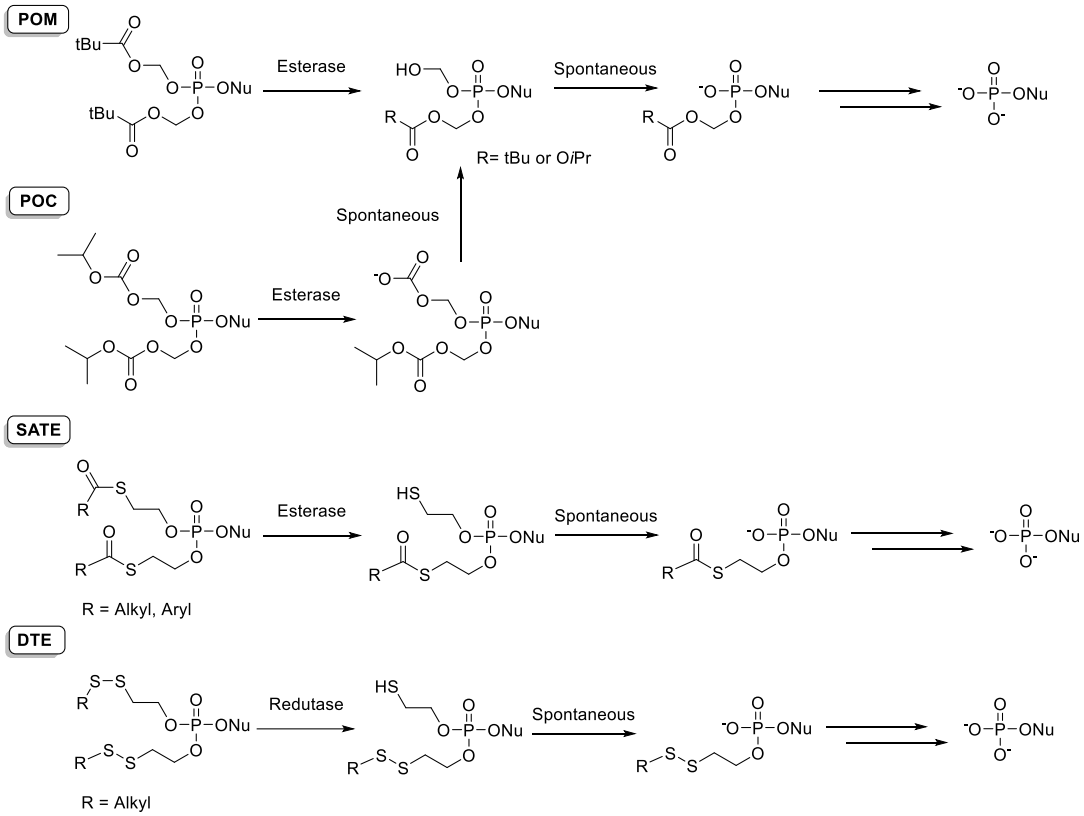


Figure (24). General bioactivation processes of pronucleotides including POM, POC, SATE and DTE promoieties.

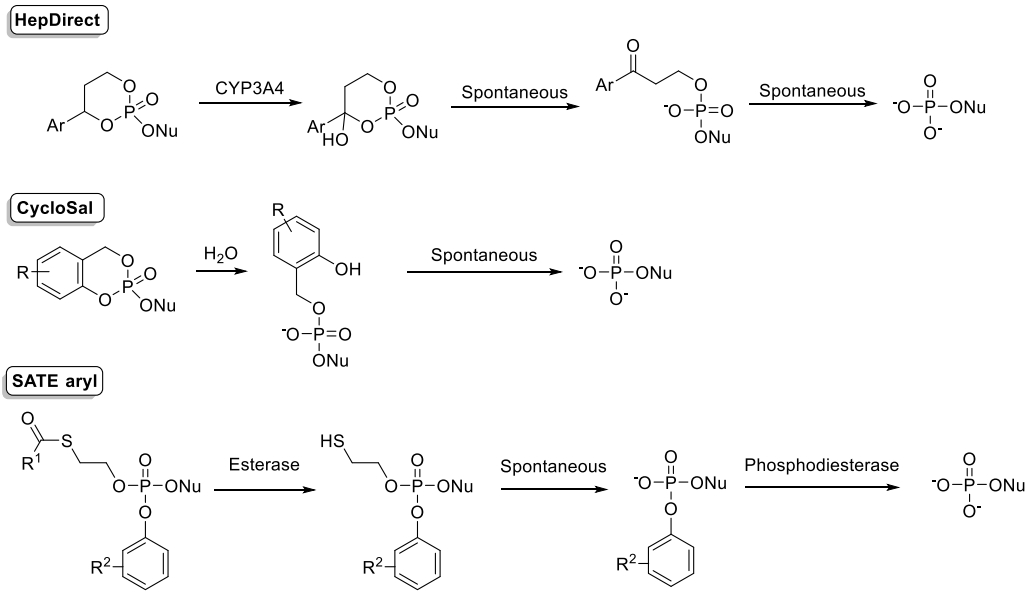
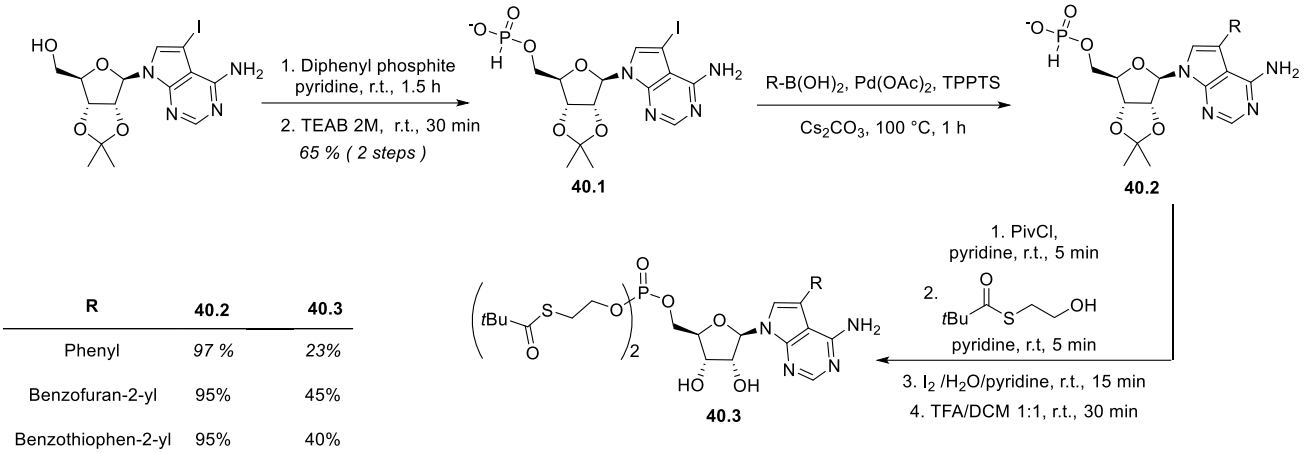


Figure (25). General bioactivation process of pronucleotides including HepDirect, CycloSaL and SATE aryl-promoieties

Asymmetrical pronucleotides also include two series of cyclic-phosphotriesters that have been extensively developed such as the HepDirect [85] and the CycloSaL [86] prodrugs (Fig. **2**), respectively designed by Erion and coworkers and the Meier's group. In the HepDirect approach (Fig. **25**), the phenyl dioxaphosphinane oxide ring is cleaved through a cytochrome P450-catalyzed oxidation resulting in the liver-targeted drug delivery of the 5'-mononucleotide. The CycloSal strategy is a conceptually different pronucleotide system, where a salicylic alcohol is used as promoiety, relying on a chemical process for the release of the 5'-mononucleotide. Finally, the proposed decomposition mechanism of the SATE-aryl phosphotriester derivative is involving two different enzymatic systems, *i.e.*, esterase and phosphodiesterase, respectively.



R	40.2	40.3
Phenyl	*97 %*	*23%*
Benzofuran-2-yl	95%	45%
Benzothiophen-2-yl	95%	40%
Naphtalen-2-yl	98%	30%

Scheme (40). Synthesis of bis(SATE)phosphotriester derivatives using *H*-phosphonate chemistry and nucleobase diversification.

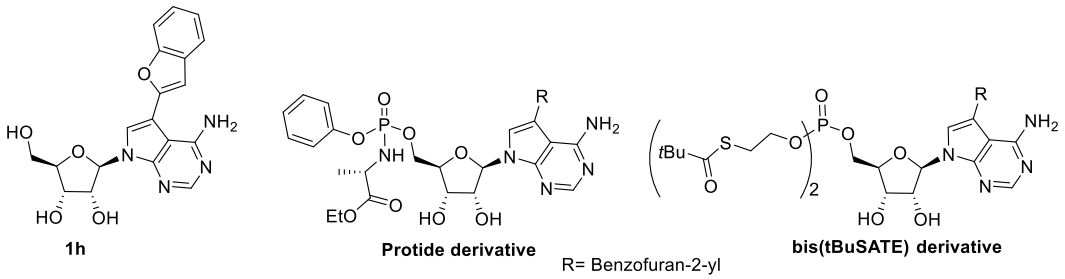
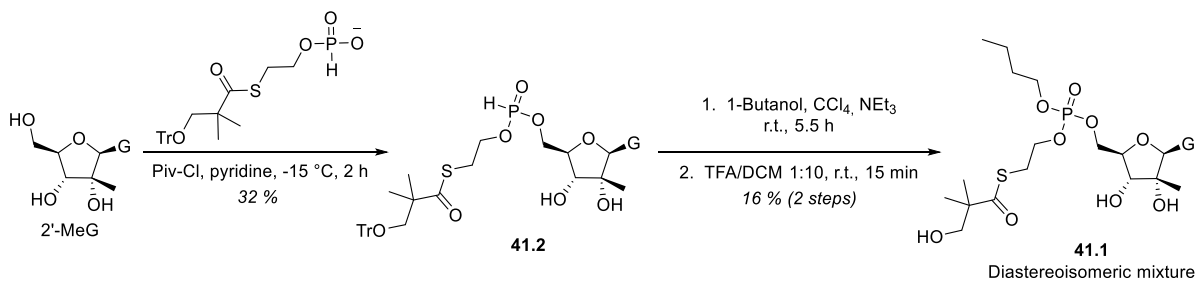


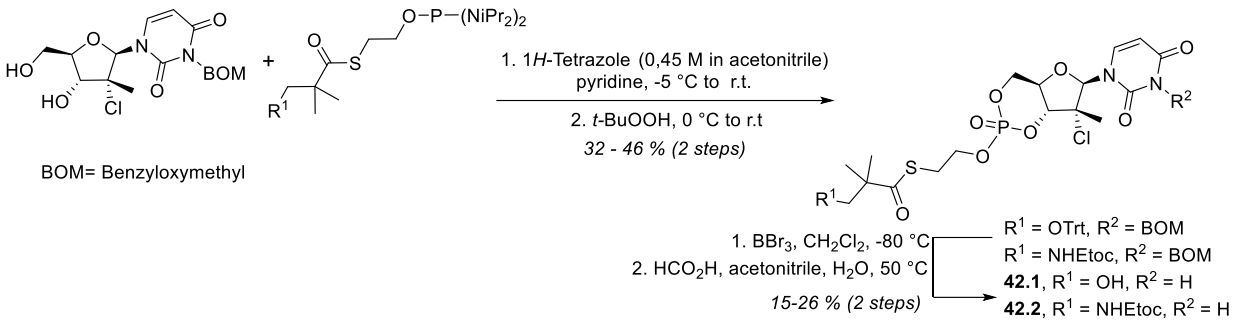
Figure (26). Derivatives selected for further biological evaluation and ability to increase NMP cell delivery.

Finally, phosphotriester pronucleotides have also been obtained from 3',5'-cyclic-monophosphate using POM, POC, SATE or alkyl groups as sole masking unit [87]. Preparation of such derivatives has been comprehensively reviewed by Pradere *et al.* in 2014 [3d].

The Hocek's group [21] has recently applied the bis(SATE) and the SATE aryl approaches to 7-substituted 7-deazapurine ribonucleosides as potential inhibitors of emerging RNA viruses. Interestingly, they reported two different synthetic pathways to prepare the phosphotriester and the ProTide derivatives. For this last, the Suzuki-Miyaura cross-coupling step was performed on the iodinated nucleoside phosphoramidate diester (see section 2, Scheme **4**), whereas for the SATE derivatives the introduction of the bulky aryl groups at position 7 of the deazapurines was carried out on the iodinated nucleoside *H*-phosphonate monoester **40.1** (Scheme **40**), which was then converted into the bis(SATE)derivatives **40.3** in two steps (conversion of the *H*-phosphonate monoester into the phosphite triester and then *in situ* oxidation). All derivatives were evaluated for their potential antiviral activity against RNA viruses in vitro. Surprisingly, none of the prodrugs exhibited better activity than the parent compounds and did not reduce the cytotoxicity observed for some of them. To get insight into the mechanism of action of these series of derivatives, the authors compared the intracellular release of the 5'-nucleoside monophosphate (resulting from cellular uptake and then metabolization process) after incubation of selected derivatives (Fig. **26**). The data suggested that the bis(*t*BuSATE) pronucleotide of 7-benzofuran-2-yl 7-deazaadenosine was the best construct to deliver the NMP intracellularly but did not correlate with the *in vitro* antiviral evaluation, thus suggesting that formation of the NMP may not be the only limiting step in the mechanism of action of this series of 7-substituted 7-deazapurine ribonucleosides.



Scheme (41). Synthesis of the SATE phosphotriester derivative of 2'-C-methyl-β-D-guanosine.

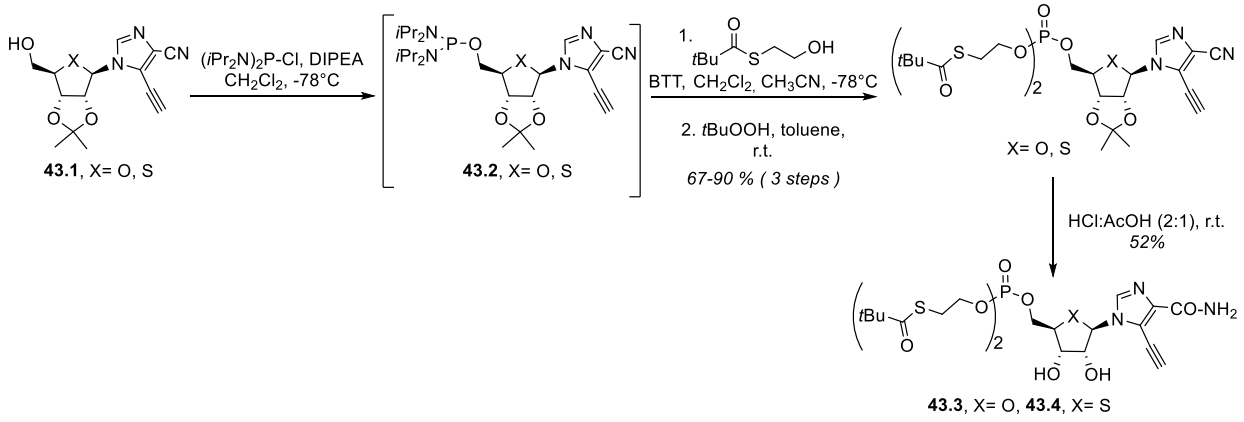


Scheme (42). Synthesis of the SATE 3',5'-cyclic-phosphotriester derivatives of 2'-α-chloro-2'-β-C-methyl-β-D-uridine.

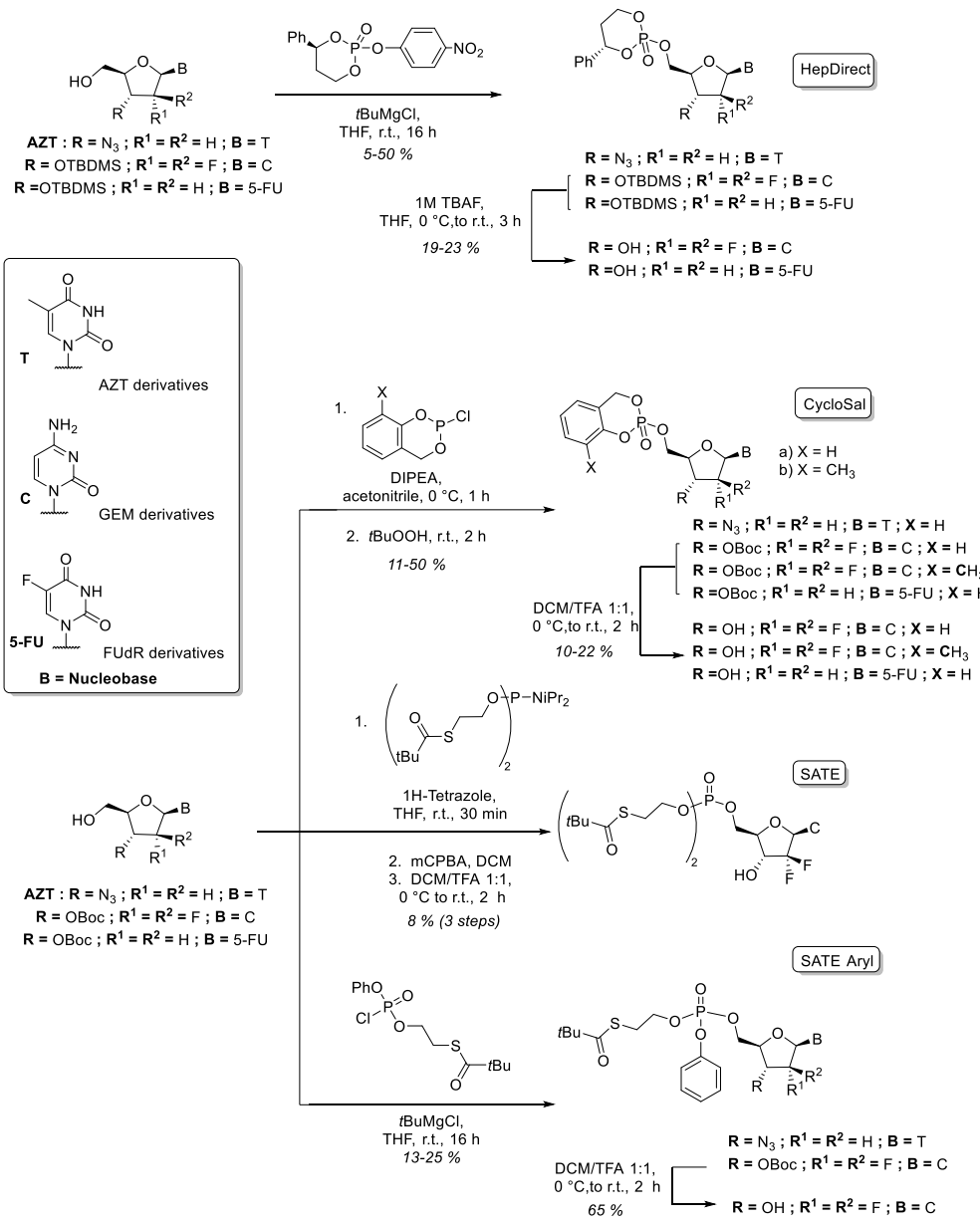
As part of a large SAR study aiming to discover potent liver-targeted HCV polymerase inhibitors, the synthesis of phosphotriester prodrugs of 2'-C-methyl-β-D-ribonucleoside analogues [35, 53] was undertaken (Scheme **41**). Thus, the SATE phosphotriester derivative **41.1** was obtained as a diastereoisomeric mixture in three steps and low overall yield, using the free 2'-MeG as starting material and *H*-phosphonate chemistry. Briefly, the synthetic pathway involved pivaloyl chloride as coupling agent to afford H-phosphonate diester intermediate **41.2** and then the Atherton-Todd oxidation in presence of n-butanol. The decomposition pathway of phosphotriester derivative **41.1** was hypothesized to involve first an esterase-mediated hydrolysis of the SATE promoiety and then a phosphodiesterase activity which may cleave either the P-O-butyl (release of the desired NMP) or the P-5′-O-Nucleoside (release of the nucleoside) bonds. When comparing the *in vitro* anti-HCV activities in replicon assay of compound **41.1** and of the clinical candidate IDX184 (section **1.2.**, Fig. **14**), a 15-fold decrease of the EC_{50} value was observed, suggesting that the release of the 5′-monophosphate derivative of 2′-MeG could be less efficient for **41.1** than for IDX184. Further works concerned the application of various pronucleotide approaches to 2'-α-chloro-2'-β-C-methyl branched uridine [35] and among them SATE phosphotriester derivatives of the 5',3'-cyclic mononucleotide were obtained (compounds **42.1** and **42.2**, Scheme **42**). These lasts were obtained by coupling the required phosphorobisamidites with the *N*-3-benzyloxymethyl (BOM) protected nucleoside and affording the cyclic phosphite triester, *in situ* oxidized, and then removal of the protecting groups. Comparative evaluation showed potent anti-HCV activity *in vitro*, with sub micromolar EC_{50} value, and higher NTP levels produced in the liver than for the corresponding SATE-phosphoramidate derivative.

In 2022, the group of N. Minakawa reported the synthesis and anti-Dengue virus activity of EICAR (5-ethynyl-(1-β-D-ribofuranosyl)imidazole-4-carboxamide) and 4'thio-EICAR pronucleotides. As illustrated in Scheme **43**, a one-pot three steps synthetic pathway has been developed starting from the 4-cyanoimidazole derivatives **43.1** in order to avoid side-reaction on the 4-carboxamide group of the nucleoside analogues. Thus, phosphytilation of the 5'-hydroxyl group was carried out at low temperature and intermediates **43.2** were directly coupled with S-pivaloylthioethanol in presence of 5-(benzylthio)-1*H*-tetrazole (BTT) affording the bis(SATE) phosphite triester which was oxidized in situ. The protected phosphotriester were treated in acidic conditions to remove the isopropylidene group and generate the 4-carboxamide group. Comparative evaluation of compounds **43.3** and **43.4** shown that EICAR and its pronucleotide **43.3** exhibited similar activity in vitro, whereas pronucleotide **43.4** appeared more potent than 4'-thioEICAR. This last observation suggested that applied to a 4'-thioanalogue the SATE approach allows to overcome the low efficiency of the monophosphorylation step.

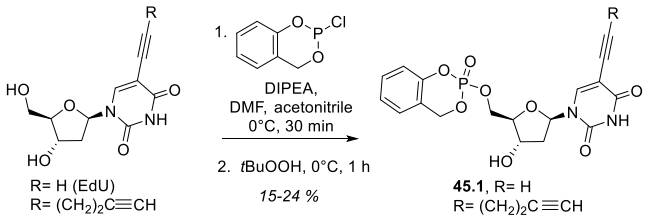
In 2020, Pertusati and co-workers [88] synthesized a set of ten mononucleoside phosphotriester-based prodrugs (belonging to HepDirect, CycloSal, SATE and SATE aryl series, Scheme **44**) of well-known antiviral and antitumoral nucleoside analogues and investigated their antibacterial and antibiofilm activities.



Scheme (43). Synthesis of the bis(SATE) phosphotriester derivatives of EICAR and its 4'thio-analogue.



Scheme (44). Synthesis of various phosphotriester type prodrugs of AZT, GEM and FUdR.

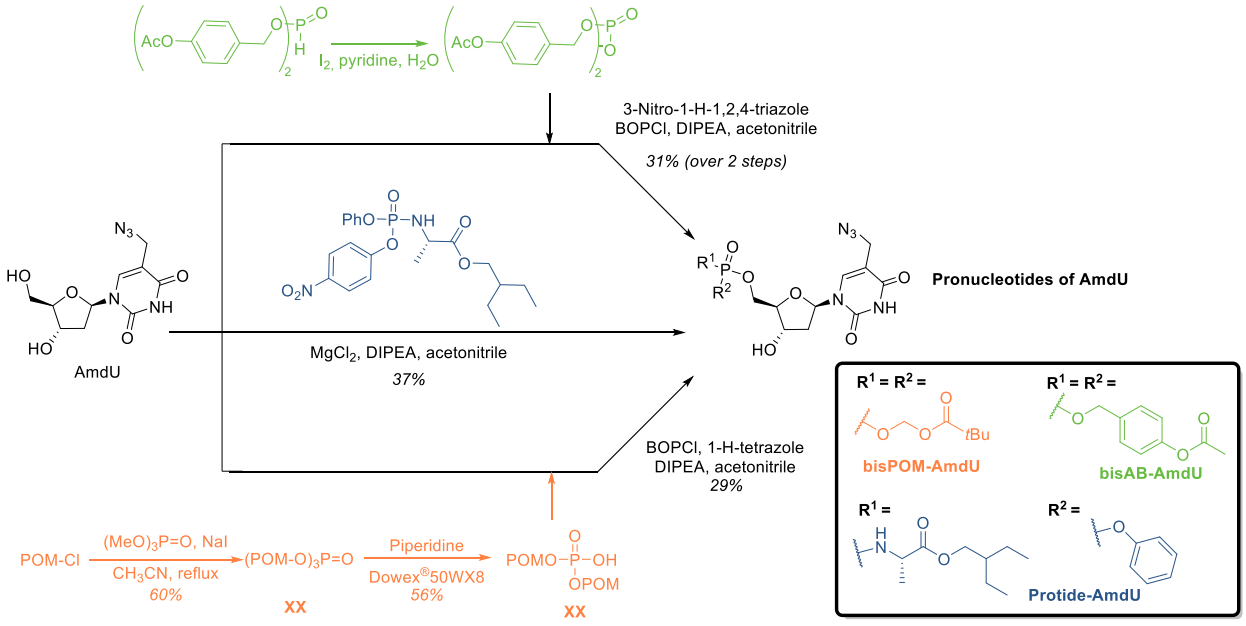


Scheme (45). Synthesis of CycloSal derivatives of EdU and 5-(1,7-octadiynyl)-2'-deoxyuridine.

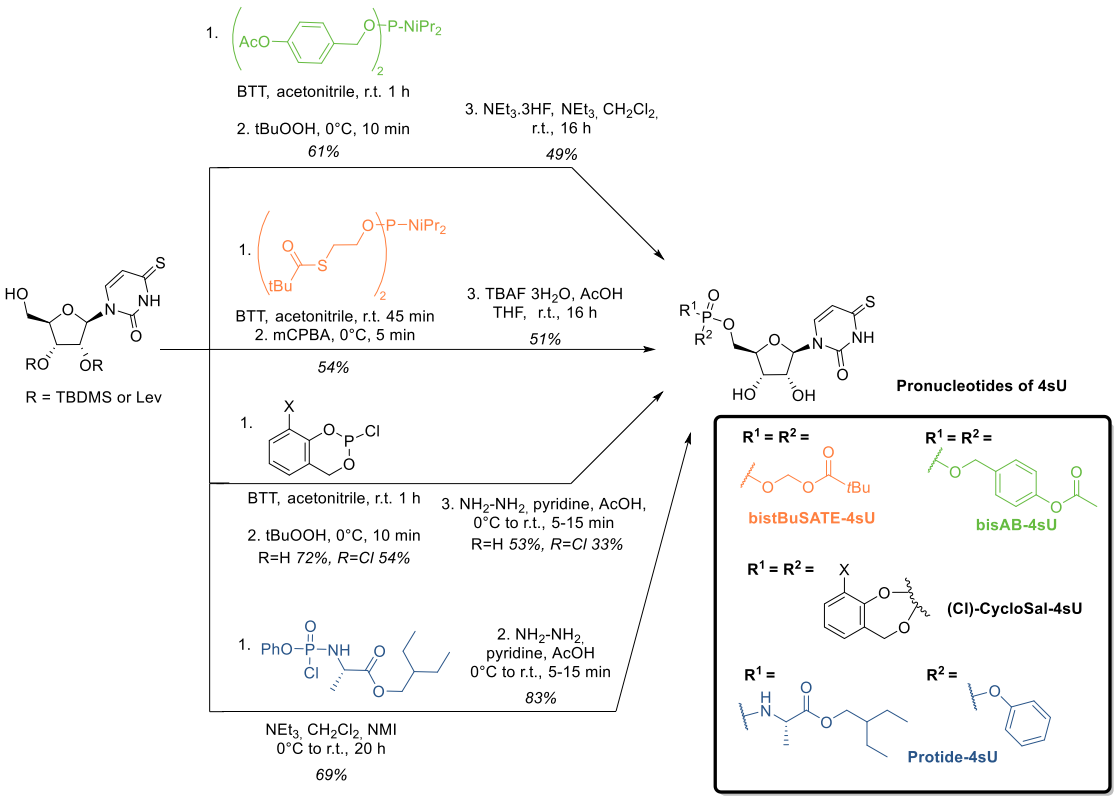
In the context of antibacterial drug discovery, for which crossing of the bacterial cell membrane is still a challenge, the use of lipophilic pronucleotides to overcome both bacterial cell wall permeation and/or first- kinase phosphorylation step appears as a relevant drug-repurposing approach. The various pronucleotides (Scheme **44**) were obtained using previously published procedures [3d] from 3′-azido-3′-deoxythymidine (AZT) or the 3'-*O*-protected derivatives of 2′,2′-difluoro-2′-deoxycytidine (GEM) and 5-fluoro-2′-deoxyuridine (FUdR). The antimicrobial potential of the newly synthesized phosphotriesters was evaluated against a large panel of Gram-positive and -negative pathogens in comparison to the parent nucleoside analogues. In addition, the *in vitro* cytotoxicity was also determined on seven different tumor cell lines (pancreatic adenocarcinoma, chronic and acute myeloid leukemia and acute lymphoblastic leukemia, colorectal and lung carcinoma). Overall, all the studied pronucleotides exhibited lower antibacterial activity compared to the parent nucleosides against selected Gram-positive bacteria, whereas no significant antibacterial effect was found against Gram-negative bacteria. These results support the hypothesis that these derivatives may not be able

to cross the bacterial cell wall of the tested pathogens, and/or cannot be decomposed efficiently, presumably by lack of the required enzymatic activities (esterases for SATE and oxidases for HepDirect prodrugs). Indeed, CycloSaL-prodrugs, for which the cleavage pathway is based on chemical hydrolysis, showed modest (still lower than the nucleoside analogues) antibacterial activity against a few gram-positive bacterial strains. Concerning the inhibition of the biofilm formation, many pronucleotides showed preventive activity. Among them, the CycloSal derivative of GEM and of FUdR, and HepDirect derivative of FUdR exhibited the best preventive antibiofilm activity but were biofilm specific.

In addition to their interest as therapeutic agents, pronucleotide approaches have also been used as tools for delivering intracellularly nucleotidic probes, this last being especially useful for labelling DNA or RNA. Quantitative monitoring of DNA biosynthesis is widely used to study and to decipher cell cycle events, and other significant biological outcomes associated with the effect of drugs on proliferating cells. Thus, the groups of Poulsen [89] and Luedtke [90] have extended the use of phosphotriester pronucleotides as powerful tools for biorthogonal ligation reactions, using either the copper-catalyzed (CuAAC) or the strain-promote (SPAAC) azide-alkyne cycloaddition reactions for cross-linking DNA. Three nucleoside analogues, the 5-ethynyl-2'-deoxyuridine (EdU), the 5-(1,7-octadiynyl)-2'-deoxyuridine [89] and the 5-(azidomethyl)-2'-deoxyuridine (AmdU) [91] were considered as substrate for pronucleotide design, as they offer a non-radioactive alternative to the historical use of [3H]-thymidine. The CycloSal derivatives (Scheme **45**) were prepared by coupling the unprotected nucleoside analogues with salicyl chlorophosphite in presence of DIEA and then the cyclic phosphite intermediate was in situ oxidized with tert-butyl hydroperoxide. Because of the presence of the azide group in AmdU, the synthesis of 5'-bispivaloyloxymethyl (POM), 5'-bis-(4-acetoxybenzyl) (AB) and ProTide derivatives (Scheme **46**) was envisaged by using P(V) instead of P(III) chemistry. The benefit of the pronucleotide approaches applied to 5-modified nucleoside analogues is to bypass both the need for thymidine kinase processing and the reliance on nucleoside active transport mechanisms, for cellular uptake of the polar parent compound. The efficiency of cellular DNA labelling for EdU, 5-(1,7-octadiynyl)-2'-deoxyuridine, their 3'-acetylated and 3',5'-diacetylated derivatives as well as their CycloSal pronucleotides (Scheme **45**) was compared and evaluated in proliferating cells, using Alexa Fluor 488 azide in CuAAC reaction conditions. [89] Quantitative labeling was observed for EdU, its 3'-acetylated derivative and prodrug 45.1 when incubated at 20 µM for 24 h, whereas none of the 5-(1,7-octadiynyl)-2'-deoxyuridine derivatives have been effective. Further comparative study of EdU and compound **45.1** (varying the incubation time at 10 µM concentration) demonstrated that the CycloSal pronucleotide was the most efficient tool to deliver the probe. Concerning AmdU and its prodrugs (Scheme **46**, their DNA metabolic incorporation was evaluated in Hela cells after 24h incubation at 10 µM concentration. The Alexa Fluor 594 alkyne was used to stain the cells in CuAAC reaction conditions [90a]. The POM-AmdU derivative lead to the best results, whereas weak or very low staining was observed for AB-AmdU, for AmdU and its ProTide derivative, respectively. The authors attributed this observation to the lower stability of AB-AmdU in extracellular media in comparison to POM-AmdU. Then, efficiency of POM-AmdU and the parent nucleoside were challenged in vivo using zebrafish models, either using microinjection into the cytosol of one-cell stage zebrafish or by simply soaking the zebrafish larvae into an aqueous solution of the studied compound. Again, POM-AmdU appeared as the best compound, allowing biodistribution of the probe into proliferating cells throughout the body and overcoming the first-phosphorylation step during metabolization of AmdU into its corresponding triphosphate form.

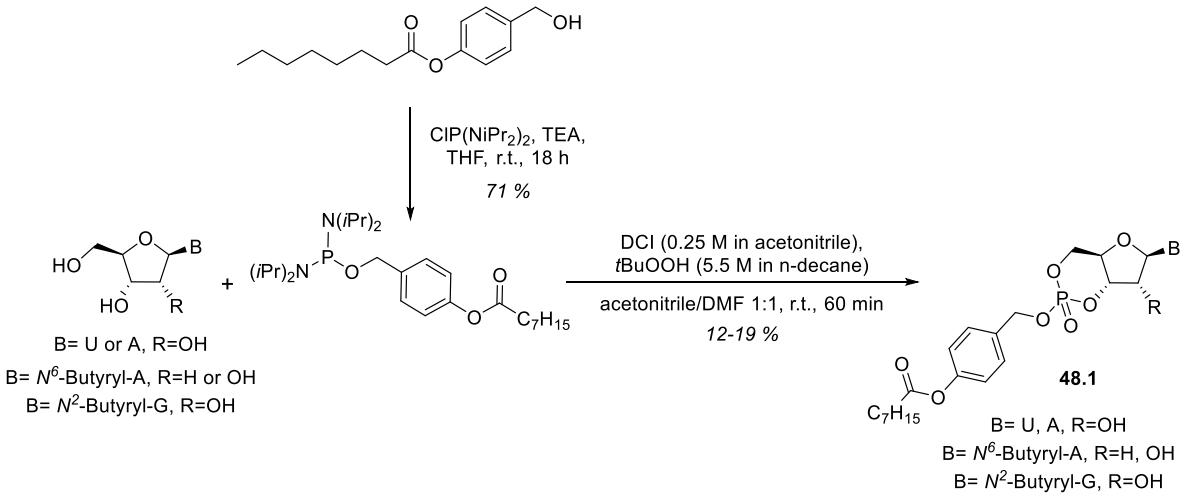


Scheme (46). Synthesis of various pronucleotides of AmdU.



Scheme (47). Synthesis of various pronucleotides of 4sU.

Recently, S. Moreno and coworkers reported the application of pronucleotide approaches to 4-thiouridine (4sU) for RNA labelling and to study cellular RNA dynamics [92]. A first set of derivatives, including acetyloxybenzyl (AB), S-pivaloyl-2-thioethyl (*t*BuSATE) and CycloSaL promoieties was obtained using P(III) chemistry (Scheme **47**) from 2',3'-protected 4sU. 5-(Benzylthio)-1H-tetrazole (BTT) was used as coupling agent and the oxidation step was performed in presence of tbutyl hydroperoxide or 3-chloroperbenzoic acid (mCPBA) at low temperature to avoid the oxidation of 4-thiouracil. Among all derivatives, only the bis(AB)phosphotriester exhibited a similar level of incorporation as 4sU, despite that some cytotoxicity was observed in the HEK293T cell line used for the metabolic labelling experiments. Concerning the other series of pronucleotides low labeling capacity was observed, presumably associated with the slower release of the 5'-monophosphate of 4sU in comparison to the bis(AB) prodrug.



Scheme (48). Synthesis of octanoyloxybenzyl phosphotriester derivatives as prodrugs of cNMPs.

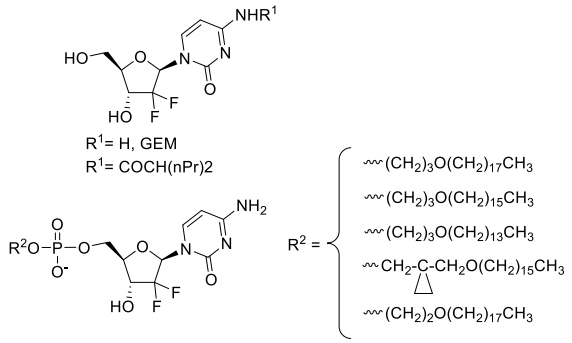
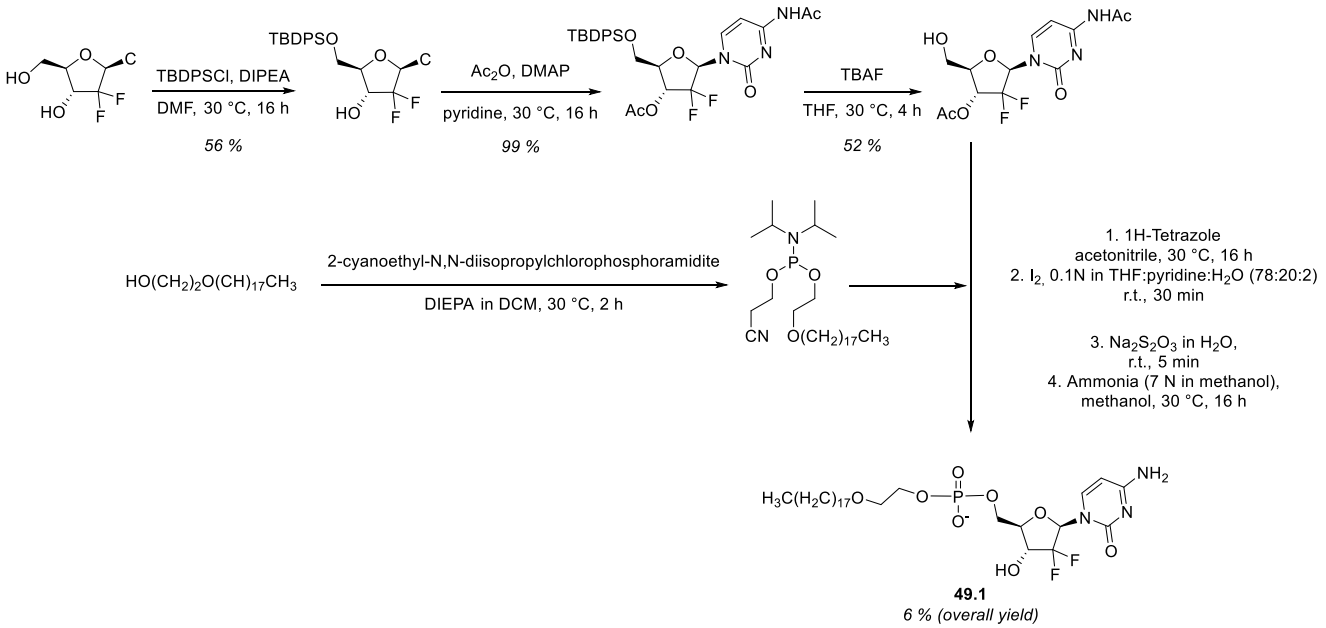


Figure (27). Structures of gemcitabine (GEM), its pronucleoside and phosphodiester derivatives.

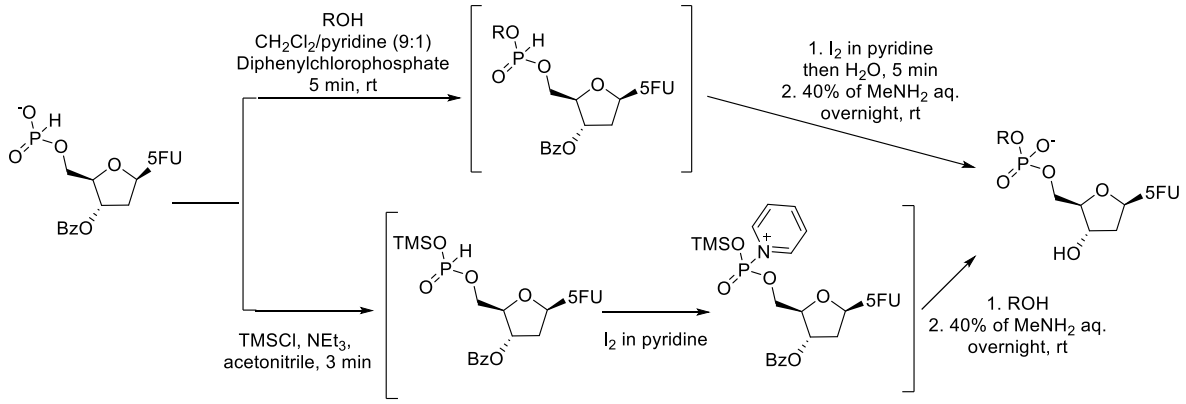
Another application of the pronucleotide approach for designing biological tools has been reported by C. Meier's group with the aim to deliver nucleoside 3',5'-cyclic monophosphate (cNMP) within cells and to perform non-invasive cell assays [93]. The synthetic pathways envisaged involved P(III) chemistry, using bis(*N*,*N*-diisopropylamino)-4-octanoyloxybenzyl phosphorodiamidite [94] and 4,5-dicyanoimidazole as coupling agent (Scheme **48**). Despite the optimization of the reaction conditions, the required cyclic phosphotriesters **48.1** were isolated in low yields. The chemical and enzymatic stabilities of these derivatives were evaluated in PBS buffer and in presence of pig liver esterase to mimic physiological conditions and the esterase-mediated hydrolysis of the promoiety. On the basis of this study, they were considered to be stable enough for performing cell-based assays. Thus, the use of the octanoyloxybenzyl promoiety to mask the negative charge of cNMPs has allowed to demonstrate that such derivatives (Scheme **48**) are valuable tools to delivery cNMPs within cells and to perform non-invasive cell assays.

6. Phosphodiester: Synthesis - Therapeutic agents - Biological tools

In order to overcome the extensive first-pass metabolization of Gemcitabine and as alternative to its *N*-4-valproic acid pronucleoside, the H. Zhang's group [95] has designed and studied a series of phosphodiester derivatives of gemcitabine, incorporating lipophilic chains, as potential pronucleotide for oral administration (Fig. **27**). The desired derivatives were obtained in six steps and low yield, using phosphoramidite chemistry, as illustrated for compound **49.1** (Scheme **49**). Comparative evaluation of their antiproliferative activities on a panel of tumor cell lines allowed the identification of compound **49.1** as potent antitumoral agent. Then, the intracellular concentration of this compound was determined in different cancer cells and its cellular uptake appeared temperature-dependent but likely not relying onto the human equilibrative nucleoside transporter (hENT), that is often involved in resistance mechanisms associated with nucleoside-based chemotherapy.



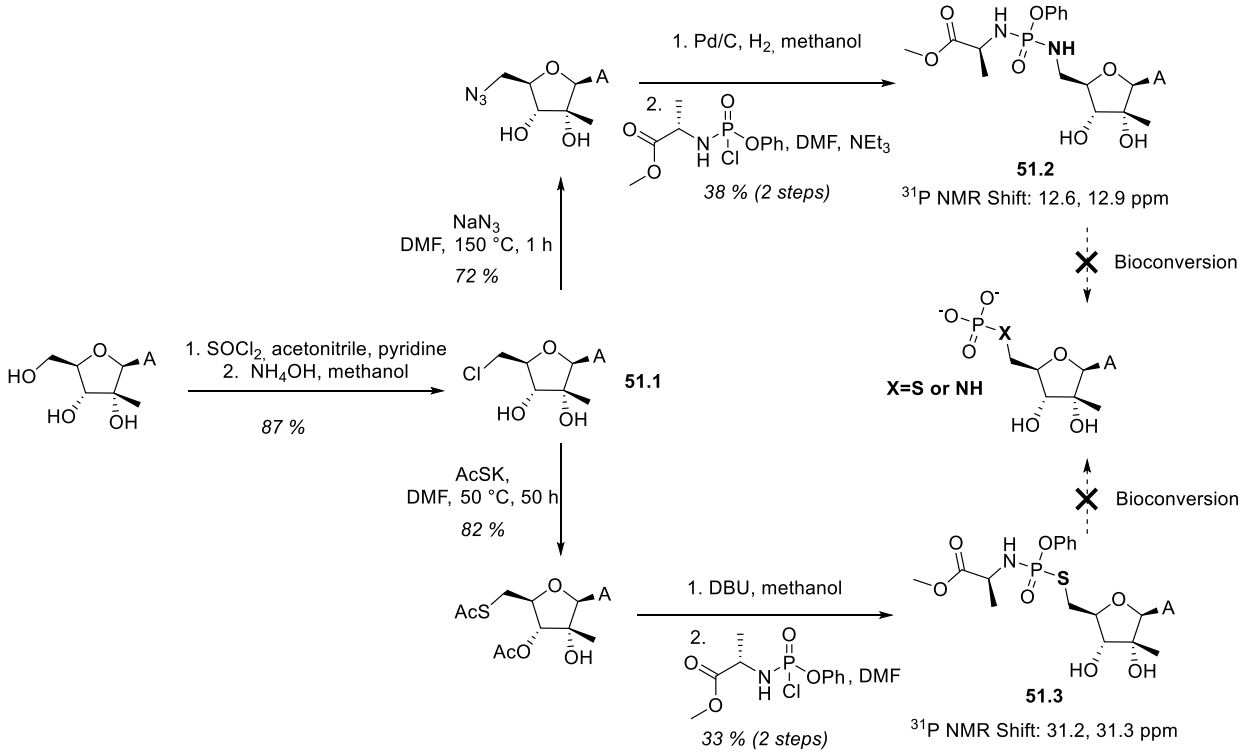
Scheme (49). Example of the synthetic pathway to lipophilic phosphodiester derivative of GEM.



Scheme (50). Synthetic pathways for zwitterionic phosphodiester derivatives of 5-fluoro-2′-deoxyuridine (R= phenyl, 4-nonylphenyl, pyridin-3-yl, quinolin-6-yl, *N*-methylpyridinium-3-yl, *N* -decylpyridinium-3-yl, *N*-benzylpyridinium-3-yl, *N* -(2-ethoxy-2-oxoethyl)pyridinium-3-yl, *N* -(2-ethoxy-2-oxoethyl)quinolinium-6-yl, 2-(*N*, *N*, *N* -trimethylaminium)ethyl, *N*, *N* -dimethylpiperidinium-4-yl, 2-(*N* -benzyl- *N*, *N* -dimethylaminium)ethyl, *N* -benzyltropanium-3-yl.

The authors hypothesized a combination of passive diffusion and active uptake. Additional *in vitro* metabolic studies [95b] shown that compound **49.1** was stable in human and rat plasma and subjected to intensive metabolism in liver and intestinal microsomes. Further *in vivo* studies of compound **49.1** have shown promising results in mice models, with similar efficacy after oral administration as compared to gemcitabine, but administrated intraperitoneally.

As original constructs, the group of A. Kraszewski and J. Stawinski has proposed various electrically neutral pronucleotides as potential antiviral or antitumoral agents [96]. Among them, zwitterionic phosphodiester derivatives of 5-fluoro-2′-deoxyuridine appeared as promising antiglioma agents [97]. The rationale behinds this approach is based on the cellular internalization of phospholipids and that this process may represent a differentiating factor between healthy and malignant cells. The common structural motif of the targeted derivatives is the presence of an aromatic amine, that is quaternarized by the addition of a lipophilic chain to generate permanent zwitterions. All compounds were obtained by using *H*-phosphonate chemistry (two different one-pot routes depending of the promoiety to be introduced) and the *H*-phosphonate monoester of 3'-*O*-benzoyl-5-fluoro-2′-deoxyuridine as starting material (Scheme **50**). Comparative evaluation of the cytotoxicity in different cancer cell lines and healthy cells highlighted the potential of derivatives including a pyridinium or a quinolinium substituent. Stability of model derivatives in cell culture media and human serum demonstrated the release of the 5'-mononucleotide, presumably through phosphodiesterase activity.



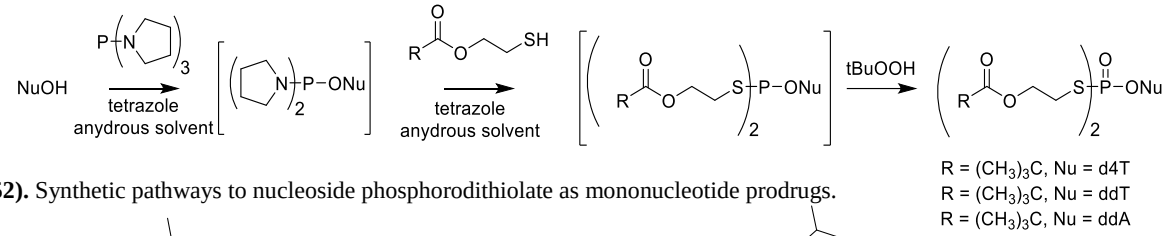
Scheme (51). Synthesis of Protide variants including 5'-N or 5'-S-2'-C-methyl-β-D-adenosine.

7. Miscellaneous

Herein, we have gathered the works concerning structures which were not included in the pronucleotides' series previously mentioned (see introduction section, Fig. **2**).

In 2020, the group of R. Nencka reported the synthesis and the study of ProTide variants including 5'-amino- or 5'-thio-2'-C-methyl-β-D-adenosine as potential antiviral agents. [57]. They hypothesized that the substitution of the oxygen atom in the 5' position of the nucleoside counterpart by a nitrogen or a sulfur atom would increase the metabolic stability of the resulting compounds. Synthetic pathways to the desired compounds involved the same key intermediate, the 5'-chloro-2'-C-methyl-β-D-adenosine **51.1** (Scheme **50**), which was readily converted to the 5'-amino-nucleoside analogue. The latter was then condensed with the (*O*-phenyl)-*N*-alanyl-methyl ester chlorophosphate to afford compound **51.2** in modest yield. Synthesis of the thio-analogue **51.3** required optimization for the removal of the acetyl group and the DBU salt was directly engaged into the coupling step. As no significant antiviral effect was observed for these compounds against a large panel of RNA and DNA viruses, the authors investigated both their enzymatic stability towards carboxypeptidase Y (that is responible for the first step of the activation of protides) and the formation of the poly-phosphorylated nucleoside analogue in cell extracts. Unfortunatly, none of the expected (poly)-phosphorylated forms were observed, thus supporting the lack of biological activity and the fact that these original scaffolds cannot be considered as 5'-mononucleotide prodrugs.

Very recently, our group has proposed a novel *O*-pivaloyl-2-oxyethyl biolabile phosphate protecting group, namely isoSATE [98], where the oxygen and sulfur atoms of the known SATE promoiety have been switched. The corresponding nucleoside phosphorodithiolate derivatives of d4T, ddT and 2',3'-dideoxyadenosine (ddA) were obtained using P(III) chemistry and following an original one-pot three-step procedure (Scheme **52**). Briefly, the nucleoside analogue is coupled with the tris(pyrrolidino)phosphine in presence of 1*H*-tetrazole, this last is converted into a nucleoside phosphorodithioite intermediate which is in situ oxidized. All tested compounds were able to inhibit the HIV replication in vitro, demonstrating their ability to act as mononucleotide prodrugs and remarkably the ddA derivative exhibited a very potent antiretroviral effect with EC_{50} values in the nanomolar range. In primary monocytes/macrophages, this last was 500 times more potent in inhibiting HIV replication (EC_{50} 0.23 pM) than the parent nucleoside (ddA) and the selectivity index of the prodrug was fifty times higher than for parent nucleoside.



Scheme (52). Synthetic pathways to nucleoside phosphorodithiolate as mononucleotide prodrugs.

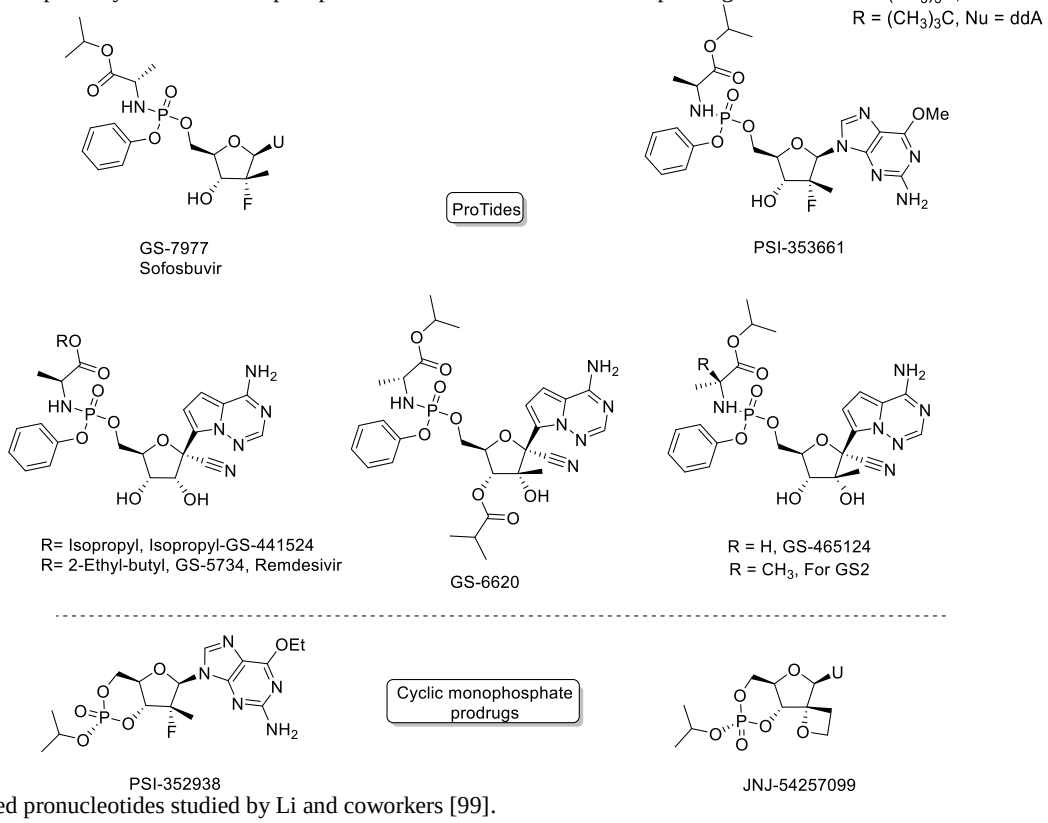


Figure (28). Selected pronucleotides studied by Li and coworkers [99].

In the context of the Sars-Cov-2 pandemic, Li and coworkers [99] have studied the expression of nucleotide-prodrug-activating enzymes [i.e. carboxylesterase 1 (CES1), cathepsin A (CTSA), cytochrome P450 (CYP3A4), …] in different human organs and tissues (including intestine, liver, kidney and lung) by using absolute quantitative proteomics. Among the seventy one serine hydrolases detected in these human tissues, CES1 was the most abundant one in the liver and to a less extend (7-fold lower) in the lung. Low levels of expression were observed in the intestine and kidney. The CTSA protein (the main enzymatic activity responsible for ProTide hydrolysis) level was highest in the lung, followed by the kidney, liver, and intestine. As expected, the CYP3A4 (the unique enzyme responsible for the first step of activation of 3',5'-cyclic phosphotriesters) expression level was high in the liver, 2-fold lower in the intestine, and scarcely detected in the lung and kidney. In this respect, various nucleoside or nucleotide prodrugs were studied including ProTides and 3',5'-cyclic phosphotriesters (Fig. **28**) and their stabilities were predicted. Obviously, the liver was found to be the main and primary site for metabolizing orally-administrated pronucleotides. These data are valuable to understand and eventually predict the activation of the pronucleotides and may serve as a basis for determining the administration route in addition to cell permeability, and/or for future design of tissue-specific prodrugs, according to the pathology of interest.

CONCLUSION

The concept of prodrug has been widely used to overcome limitations associated with drugs since its first definition by A. Albert back in 1958. It appears particularly useful in the field of antiviral and antitumoral nucleoside analogues, as this class of compounds faces low diffusion through biological membranes, cellular kinase-dependent phosphorylation steps before to reach their target (i.e. mainly cellular or viral polymerases). Many approaches have been proposed to mask the monophosphate charges by enzymatic or chemo-labile protecting groups, named promoieties, and most of them allowed the intracellular delivery of the corresponding 5'-mononucleotide in cell culture experiments. A few were also successful in vivo, such as the ProTide, the SATE phosphoramidate, HepDirect and POM/POC derivatives, thus leading to an increase of the nucleoside 5'-triphosphate level in the targeted organ. The advances in ProTide clinical development highlighted the need to develop synthetic methodologies for delivering such chiral derivatives as single diastereoisomers and many works have been performed in organic chemistry to solve this issue. New developments concern the application to other diseases than cancers or viral infections as well as the use of pronucleotides as biological tools. Concerning this last, phosphotriester derivatives seem to fill up the requirements in terms of lipophilicity and stability. Up to now, the different promoities proposed may not allow the effective delivery of the 5'-mononucleotides in tissues other than the liver, so this might be the principal limitation to the application of pronucleotide approaches to a wide panel of diseases. Therefore, more research is still need to reach this goal.

LIST OF ABBREVIATIONS

3TC = Lamivudine
AB = Acetyloxybenzyl
ABC = Abacavir
AHA = Anchimerically Hint1-Activated
AmdU = 5-(azidomethyl)-2'-deoxyuridine
API = Active Pharmaceutical Ingredient
AZT = Azidothymidine
BTT = 5-(benzylthio)-1H-tetrazole
CNE = Cyanoethyl
CoViD = Coronavirus disease
CPY = Carboxypeptidase Y
CuAAC = Cu-catalyzed Azide-Alkyne Cycloaddition
d4T = stavudine
DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene
DCC = 1,3-dicyclohexylcarbodiimide
ddA = 2′,3′-dideoxyadenosine
ddC = 2′,3′-dideoxycytidine
DDI = Drug-Drug Interaction
ddT = 2′,3′-dideoxythymidine
ddU = 2′,3′-dideoxyuridine
DENV = Dengue virus
DGUOK = Deoxyguanosine Kinase
DKR = Dynamic Kinetic Resolution
DMF = *N*,*N*-dimethylformamide
DMTr = 4,4'-dimethoxytrityl
DNA = Deoxyribonucleic Acid
DTE = *S*-[(hydroxyethyl)sulfinyl)]-2-thioethyl
DyKAT = Dynamic Kinetic Asymmetric Transformation
EDC = 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
EdU = 5-ethynyl-2'-deoxyuridine
EICAR = 5-ethynyl-(1-β-D-ribofuranosyl)imidazole-4-carboxamide
FDA = US Food and Drug Administration
FUdR = 5-fluoro-2′-deoxyruridine
FTC = Emtricitabine
GEM = gemcitabine
HCV = Hepatitis C virus
hENT = Human Equilibrative Nucleoside Transporter
HIV = Human Immunodeficiency Virus
HMDS = Hexamethyldisilazane
HPLC = High Performance Liquid Chromatography
HSV = Herpes Simplex Virus
IC_{50} = half maximal inhibitory concentration
LG = Leaving Group
MAOS = Microwave-Assisted Organic Synthesis
mCPBA = meta-Chloroperoxybenzoic acid
MDM = Monocyte-Derived Macrophages
MDS = Mitochondrial DNA Depletion Syndrome
mRNA = Messenger Ribonucleic Acid
mt DNA = Mitochondrial Deoxyribonucleic Acid
MW = Microwave
NMI = *N*-methylimidazole
NMR = Nuclear Magnetic Resonance
NOAEL = No Observed Adverse Effect Level
NMP = Nucleoside 5'-monophosphate
NTP = Nucleoside 5'-triphosphate
PBA = Phenylboronic Acid
PBMCs = Peripheral Blood Mononuclear Cells
PCR = Polymerase Chain Reaction

POC = Isopropyloxycarbonyl
POM = Pivaloyloxymethyl
PTE = *Pseudomonas diminuta*
RNA = Ribonucleic Acid
RNase = Ribonuclease
SAR = Structure–Activity Relationship
SARS-CoV-2 = Severe Acute Respiratory Syndrome Coronavirus 2
SATE = *S*-acyl-2-thioethyl
SPAAC = Strain-Promoted Azide-Alkyne Cycloaddition
TBDMS = *tert*-butyldiméthylsilyle
TDF = Tenofovir Disoproxil Fumarate
TFA = Trifluoroacetic Acid
THF = Tetrahydrofuran
TMS = Trimethylsilyl
TPPTS = 3,3′,3″-Phosphanetriyltris(benzenesulfonic acid) trisodium salt
TPSCl = 2,4,6-triisopropylbenzenesulfonyl chloride
ZKV = Zika Virus

CONFLICT OF INTEREST

All authors read and approved the final manuscript and declare that they have no conflict of interest.

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N.V. is particularly grateful to the University of Montpellier for a PhD fellowship. Herein, we aim to present an updated look (from to 2015 and onwards) to the main advances in the field of pronucleotides designed to deliver nucleoside 5'-monophosphate within cells. We apologize for any unintentionally omitted works.

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