Review

Nucleoside Di- and Triphosphates as a New Generation of Anti-HIV Pronucleotides. Chemical and Biological Aspects

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Abstract: This review provides a short account of the chemical synthesis of nucleoside di- and triphosphates on a historical background, together with the use of this class of compounds as potential pronucleotides in anti-HIV therapy.

Keywords: nucleoside di- and triphosphates; anti-HIV pronucleotides; synthetic strategies; biological activity

1. Introduction

Phosphorylated nucleosides, collectively called nucleotides, play pivotal roles in various biological processes. Naturally occurring deoxy- and ribonucleoside triphosphates are basic building blocks for the enzymatic synthesis of DNA and RNA and participate also in energy transfer processes, intracellular signaling, and the regulation of proteins’ biological activity [1]. Scientists have long been interested in synthetic nucleotide analogs because they allow the study of complex biological systems and themselves have potential therapeutic and diagnostic value [2,3].

Di- and triphosphates of modified nucleosides are often used as compounds of potential antiviral (e.g., anti-HIV) activity. The core of this class of compounds consists of 2′,3′-dideoxynucleoside 5′-triphosphates (ddN, e.g., AZT, d4T, ddI, ddC, 3TC, FTC), which are inhibitors of reverse transcription (RT) during HIV replication [4,5]. However, direct administration of such compounds to patients is pointless, because as negatively charged molecules they are not effectively transported through the cell membrane [6], which practically precludes their bioavailability [7–9]. The simplest solution would be to administer their prodrugs, dideoxynucleosides, which in vivo would be phosphorylated to the corresponding triphosphates. Although this approach works in many cases (e.g., AZT), it turned out that some dideoxynucleosides (e.g., ddU), which are precursors of the very potent anti-HIV compounds (e.g., ddU 5′-triphosphate), are practically not phosphorylated to nucleoside 5′-monophosphate (NMP) in the cell. Such dideoxynucleosides, when administered in the form of free nucleosides, usually showed no anti-HIV activity [4,5,7,8]. Moreover, AZT also suffers several drawbacks associated with the unfavorable kinetics of its successive in vivo phosphorylations (leading in consequence to serious adverse effects of the AZT therapy) [10] and the existence of the thymidine kinase-deficient cells, in which AZT is not phosphorylated and remains as an inactive prodrug prior to clearance (such cells become reservoirs of viruses inaccessible for the therapy) [11]. Therefore, knowing the limitations of the use of 2′,3′-dideoxynucleosides as a potential anti-HIV therapeutics, the pronucleotide idea was born.

Pronucleotides in their basic concept are electrically neutral phosphate derivatives of biologically active nucleosides, in which protective groups on the phosphate residues
neutralize electrical charges. In such form, phosphorylated nucleosides can easily penetrate cell membranes. Inside the cell, the masking groups are removed via enzymatic and/or chemical hydrolysis, which lead to the release of the corresponding nucleoside 5′-monophosphate (ddNMP). Then, cellular kinases in a stepwise manner phosphorylate ddNMP ultimately to its 2′,3′-dideoxynucleoside 5′-triphosphate (ddNTP), an anti-HIV active species (Scheme 1) [9,12,13].

Scheme 1. A general principle of the monophosphate pronucleotides mode of action.

Initially, when developing the concept of pronucleotides, it was assumed as an absolutely necessary condition that the electric charges in the prodrug molecule were completely masked. Following this paradigm, various pronucleotide strategies were proposed, which were based mainly on esters or amides as potential protective groups for 5′-phosphate moiety of ddNMP (e.g., studies of McGuigan [11,14,15], Imbach [16–19], Meier [20,21], Kraszewski [22,23], and others groups). However, over the years, it has been observed that certain types of pronucleotides endowed with negative charges could also effectively penetrate cell membranes, and showed high biological activity (e.g., the research of the Wagner [24–28] and Kraszewski groups [23,29,30]). This challenged the validity of the generally accepted postulate of electrical neutrality as the basic criterion for the effective transport of pronucleotides through cell membranes, but at the same time it opened up new possibilities for extending the spectrum of the designed pronucleotides to include new ionic structural features.

The pronucleotide strategy for delivering monophosphorylated antiviral nucleosides to the cell did not, however, completely solve the problem of the effective in vivo generation of an active metabolite, ddNTP. Sometimes the enzymatic phosphorylation of 2′,3′-dideoxynucleoside monophosphates to di- and ultimately to triphosphates is very difficult or has undesirable kinetics. Typically, such problems arise during the conversion of a monophosphorylated nucleoside into its corresponding diphosphate. For example, AZT is a good substrate for thymidine kinase, similarly to natural thymidine, but thymidylate kinase converts AZTMP into AZTDP much less efficiently than TMP into TDP [10]. Thus, in such situations it would be advantageous to deliver the pronucleotides to the cells in the form of suitably protected diphosphates of antiviral nucleosides in order to bypass enzymatic phosphorylation to ddNDP. While the final phosphorylation of dideoxynucleoside diphosphates to the corresponding triphosphates is usually fast and effective, it was also tempting to develop triphosphate pronucleotides so that in vivo generation of the final active metabolite would be completely independent of cellular phosphorylation processes.

Despite the apparent advantages of such approaches, the first studies on the use of di- and triphosphate pronucleotides against HIV appeared two decades after the concept of anti-HIV monophosphate pronucleotides emerged [31]. This delay, most likely, was due to synthetic difficulties and the extreme lability of the anhydride bond in uncharged di- and triphosphates, containing masking groups in aqueous media. However, the reports from the Wagner group [24–28], showing that monophosphate pronucleotides with electric charges are both stable and can cross the cell membranes, revived the interest of both
chemists and biologists in $2',3'$-dideoxynucleoside di- and triphosphates as potential pronucleotides.

In this review, we present chemical methods for the formation of the anhydride polyphosphate linkages together with their applications to the synthesis of $2',3'$-dideoxynucleoside di- and triphosphates, as a new generation of anti-HIV pronucleotides.

For recent reviews on the related topics, see also Hollenstein (2012) [32], Hou et al. (2014) [33], Sherstyuk and Abramova (2015) [34], H. Tanaka (2015) [35], Ahmadipour and Miller (2017) [36], Kaczynski and Chmielewski (2017) [37], and Camarasa (2018) [38].

2. Chemical Synthesis of Nucleoside Di- and Triphosphates

Due to the plethora of biological functions of nucleoside di- and triphosphates, it is not surprising that there has been a long-lasting interest in synthetic methods for the efficient preparation of this class of compounds. However, due to the presence of multiple reactive centers in nucleotide derivatives, developing efficient synthetic protocols is still a challenging task.

There are two general synthetic approaches to nucleoside di- and triphosphates. Historically, the first one exploits the chemistry of P(V) compounds and is based on increasing the electrophilicity of the phosphorous center through its activation with condensing agents to make it more susceptible to nucleophilic attack. The second approach makes use of more reactive P(III) compounds, which can be either coupled with a nucleophile and subsequently oxidized, or coupled and oxidized simultaneously in a reaction called an “oxidative coupling”. This opens up additional synthetic possibilities in the preparation of nucleoside di- and triphosphates and their analogs.

2.1. Early Methods for the Preparation of Nucleoside Di- and Triphosphates

The first chemical synthesis of di- and triphosphates served mainly to determine and confirm the structure of nucleotides isolated from biological materials. The pioneering research in this field was carried out by Lord Alexander Todd, who, together with his collaborators, in the late 1940s and early 1950s, published a series of fundamental papers on the synthesis of an anhydride bond in nucleoside di- and triphosphates.

Years of basic research have led to the development of new methods for the phosphorylation of alcohols and phenols with dibenzyl chlorophosphate (DBCP). It was found that this reagent could be used for both the phosphorylation of nucleosides and nucleotides (Scheme 2). Thus, when DBCP was allowed to react with a free 5'-OH group of the appropriately masked adenosine, dibenzyl adenosine 5'-monophosphate $1$ was obtained. This compound could be partly deprotected by a careful treatment with diluted sulfuric acid in ethanol to form monobenzylated AMP $2$. The phosphorylation of this intermediate, again with DBCP, followed by hydrogenation, led to the preparation of the first synthetic nucleoside diphosphate, ADP (Scheme 2). It is noteworthy that the intermediate product $3$ appeared to be a mixture of di- and tribenzyl diphosphates $3a$ and $3b$, since one benzyl group was partly lost under the reaction conditions. Fortunately, there was no need for the separation of these products or a conversion of $3a$ into $3b$, since the catalytic hydrogenation of this mixture furnished the desired adenosine 5'-diphosphate (ADP), which was isolated as an acridinium salt. After various modifications of this synthetic protocol, the product could be obtained in a 55% yield [39].
Scheme 2. Synthesis of adenosine 5′-diphosphate (ADP) [39] and adenosine 5′-triphosphate (ATP) [40] according to Todd et al.

Todd et al. [40,41] also developed a method for the preparation of nucleoside 5′-triphosphates. The first synthesized compound was adenosine 5′-triphosphate (ATP), which helped confirm the assumed structure of a compound (tentatively identified as ATP) isolated from muscle extracts 15 years earlier. For this purpose, the aforementioned mixture of di- and tribenzyl diphosphates 3a and 3b was used as the starting material. In the first step, fully protected diphosphate 3a was converted into dibenzyl derivative 3b. For selective monodebenzylation of the terminal phosphate residue (Pβ), a new reagent was developed, N-methylmorpholine [42]. After the short treatment (15 min) of the 3a/3b mixture with this amine, Bn₂ADP 3b could be isolated from the reaction mixture. This was phosphorylated with DBCP to an electrically neutral tetrabenzyl ATP that was hydrogenated in order to remove the masking groups, and to furnish the final ATP as an acridinium salt with 37% yield. The relatively low yield of this method was accounted for by a rapid degradation of the anhydride bond in the completely masked 5′-triphosphate molecule, since adenosine 5′-diphosphate was observed as a by-product [40].

Another nucleotide, which structure was confirmed by chemical synthesis, was uridine 5′-diphosphate (UDP). Initially, an attempt was made to use the same method as for ATP, but it proved to be ineffective as it resulted in large amounts of by-products, prob-
ably due to the more acidic nature of the uracil residue [43]. In this situation, Todd et al. developed a new method for the formation of the P–O–P bond system in this particular compound, starting from benzyl uridine H-phosphonate diester 4 as a substrate (Scheme 3) [44] (preparation of H-phosphonate 4 from 2′,3′-O-isopropylideneuridine and dibenzyl pyrophosphate was described in an accompanying paper, also by Todd et al. [45]). Thus, nucleoside 5′-H-phosphonate 4 was oxidized with N-chlorosuccinimide (NCS) toward the corresponding chlorophosphate 5, which was allowed to react with triethylammonium salt of dibenzyl phosphate 6. This led to the formation of a uridine 5′-diphosphate derivative with a fully masked charge at the phosphate moieties. The benzyl groups were removed by heating with LiCl in ethoxyethanol, while the isopropylidene group, by treatment with an acid. Using the precipitation technique, the product UDP was isolated as a barium salt in a total yield of ca. 25% [44].

![Scheme 3](image)

Scheme 3. Synthesis of uridine 5′-diphosphate (UDP) using uridine 5′-H-phosphonate as a substrate [44].

To sum up this part, the early syntheses of nucleotides developed by Todd et al. focused primarily on the preparation of the appropriate di- or triphosphates of natural nucleosides in order to verify the structural assignment of compounds isolated from biological materials. However, due to many drawbacks, e.g., the significant formation of side-products, low yields, and synthetic inconveniences, these approaches have not found wider application in the synthesis of nucleoside di- and triphosphates.

2.2. Synthesis of Nucleoside Di- and Triphosphates Using Carbodiimides

The limitations of the benzyl chlorophosphate strategy in the synthesis of diphosphates led Khorana et al. to the study of carbodiimides as coupling agents in the condensation of mononucleotides with inorganic phosphate (Scheme 4).
Scheme 4. A mechanism for the formation of nucleoside 5′-diphosphates using dicyclohexylcarbodiimide (DCC) as a condensing agent.

By using the most common carbodiimide, \(N,N′\)-dicyclohexylcarbodiimide (DCC), it was possible to synthesize a number of di- and triphosphates of naturally occurring nucleosides (adenosine, uridine, guanosine, thymidine, and deoxycytidine) in isolated yields of 25–35\% [46,47]. During the initial studies on the reaction mechanism using pyridine (Py) as a solvent, the formation of symmetrical dinucleoside pyrophosphates was observed. It is noteworthy that the desired products, nucleoside diphosphates, were not formed when \(3\)-n-butylamine was present in the reaction mixture. This suggested a crucial role of the protonation of DCC in triggering this reaction. Consistent with this, the presence of strongly basic amines in the reaction mixture prevented the condensation [48]. An interesting observation was that using a 10-fold molar excess of phosphoric acid and a 50-molar excess of DCC over the nucleoside monophosphate favored the formation of nucleoside triphosphates over the corresponding diphosphates. For this, a credible mechanistic explanation was provided [49]. Upon optimization of the method, various NTPs could be obtained in 40–80\% yields, depending on the type of the mononucleotide used as a substrate [49].

Unfortunately, the method was not free from some drawbacks. The main one was the formation of symmetrical diphosphates (inorganic and dinucleoside ones) as a result of the competing dimerization of mononucleotides and inorganic phosphate, and other di- and triphosphates, which complicated the separation process. Currently, this method is not used very often.

2.3. Synthesis of Nucleoside Di- and Triphosphates via Phosphoramidate Intermediates

During studies on the activation of phosphate monoesters, nucleoside phosphoramidates emerged as potential synthetically useful intermediates. Preliminary studies by Khorana and Chambers showed that phosphoramidic acid rapidly hydrolyzed to phosphoric acid in an acidic environment [50]. Under these conditions, the protonation of the amino function apparently transformed it into a good leaving group (ammonia), significantly increasing the electrophilicity of the phosphorus center. This could open up new synthetic possibilities. The expected advantage of the phosphoramidate strategy in the synthesis of di- and triphosphates compared to the chlorophosphate method described above was the possibility of using unprotected phosphate groups, which would reduce the number of possible side reactions, mainly di- and triphosphate hydrolysis, during the deprotection process. An important point was also the relatively low reactivity of phosphoramidates towards alcohols, which should alleviate the need to protect the 2′- and 3′-OH groups in the ribose ring [43,47].

In the initial attempts to synthesize di- and triphosphates by the phosphoramidate method, an unsubstituted amide group P-NH\(_2\) was used. However, the low reactivity of such compounds has limited their use to only a few cases, e.g., ADP, ATP and UDP [51,52]. In order to increase the reactivity of phosphoramidates as well as their solubility in organic
solvents, derivatives of $^2\$ amines were investigated, mainly phosphoromorpholidates and phosphorimidazolides, generated from the corresponding NMPs.

The first method made use of phosphoromorpholidates of type 7 (Scheme 5) as reactive intermediates generated in situ, which were further reacted with tetra-$n$-butylammonium salts of mono- or diphosphates in pyridine to provide nucleoside di- or triphosphates, respectively [51], although the latter were obtained only in moderate yields (<60%). Further studies have showed that pyridine used as a solvent stimulates equilibrium between nucleoside triphosphate (NTP) and diophosphate (NDP), and its replacement by DMSO raised the yields of NTPs to >80% (Scheme 5) [53]. This method of synthesizing di- and triphosphates, developed by Moffatt and Khorana in the early 1960s, was rather universal and is still in use today; it works well with a variety of nucleoside substrates, and is also used to make phosphonate mimics of the triphosphate group [54–56].

![Scheme 5](image)

**Scheme 5.** Synthesis of NTP via phosphoromorpholidates, according to Moffatt and Khorana [53].

It is worth noting that in 2013, Sun et al. described a novel variant of this approach, in which the reactive phosphoramidate intermediate 8 was obtained from a suitably protected nucleoside and benzyl $N,N$-diisopropylchlorophosphoramidite 9 (Scheme 6). The phosphitylation was promoted by 1H-tetrazole and the obtained phosphoramidate intermediate (structure not shown) was hydrolyzed to benzyl nucleoside H-phosphonate diester, which was subjected to oxidative coupling using the $CCl_3/Et_3N/piperidine$ system to afford phosphoropiperidate 8. Then, all benzyl groups (Bn and Cbz) were removed by hydrogenation, and the unprotected phosphoramidate was treated with tris(tetrabutylammonium) salts of phosphoric or pyrophosphoric acids in the presence of 4,5-dicyanoimidazole (DCI), which acted as a mild proton donor that catalyzed the substitution reaction at the phosphorus center. This chemistry was used for the preparation of ribo- and deoxynucleoside di- and triphosphates in good yields (68–81%) [57].

![Scheme 6](image)

**Scheme 6.** Synthesis of nucleoside di- and triphosphates via phosphoropiperidate as an intermediate [57].

Another route to nucleoside di- and triphosphates was proposed by Cramer et al., who showed that phosphorimidazolides 10 (Scheme 7) could also be easily generated by the reaction of nucleoside phosphates with carbonyldimidazole (CDI) (Scheme 7), and may be useful intermediates in the synthesis of nucleoside pyrophosphates [58–60]. The in situ-generated phosphorimidazolides of type 10 easily reacted with various phosphate nucleophiles, leading to NDP or NTP. The reaction occurred under mild conditions and
did not require the use of protective groups in the nucleoside moiety. This synthetic protocol was further developed by Hoard and Ott, who applied it to the preparation of 5′-O-triphosphates of deoxynucleosides and oligodeoxynucleotides. The yields of isolated products reached 70%, although in some cases were as low as 20% [61]. Phosphorimidazolides 10 intermediates were used also for the synthesis of nucleotide derivatives, with various natural and unnatural sugar residues at the Pβ phosphate moiety (Scheme 7) [62].

![Scheme 7. Synthesis of various nucleoside di- and triphosphates via phosphorimidazolide intermediates 10.](image)

An effective modification of this approach was proposed by Kore and colleagues [63]. For the formation of phosphorimidazolide 10 they used an imidazole/triphenylphosphine/2,2′-dithiopyridine reagent system (B, Scheme 7), which was previously developed by Mukaiyama et al. [64]. After the generation of nucleotide derivative 10, inorganic phosphate and a catalyst (zinc chloride, to increase the leaving group ability) were added. The condensation to NDP was usually complete within a few hours, and the final products were formed in very good yields (95-97%) [63].

Currently, there are several methods available for the synthesis of di- and triphosphates from the corresponding phosphate monoesters, differing in the choice of the reagent used to generate phosphorimidazolide 10 [3,65]. One of the recent proposals is 2-imidazolyl-1,3-dimethylimidazinium chloride [66] or hexafluorophosphate [2] (ImIm, C, Scheme 7), which were both prepared in situ and were used in the synthesis of NDPs, NTPs, symmetrical bis-5′,5′-O-dinucleoside diphosphates, and nucleoside di- and triphosphates with various sugar residues attached to the terminal phosphate. The advantage of this strategy is that unprotected nucleotides (commercially available sodium or potassium salts) can be used as starting materials, and the reactions can take place in an aqueous medium. The yields of this method are 20–60%.

To sum up, in most of the methods based on the activation of a phosphorus center in the form of phosphoramidates, the transformation of the substrate into the desired product (NDP or NTP) takes quite a long time (in some cases even several days), and the yield of isolated compounds is not always satisfactory.

### 2.4. Synthesis of Diphosphates Using 5′-O-Tosylated Nucleosides

A completely different synthetic approach was proposed in the mid-1980s by the Poulter group. They developed a method for the synthesis of nucleoside 5′-diphosphates based on a one-step nucleophilic substitution (SN2) of the 5′-O-tosyl group in nucleosides 11 in acetonitrile by tetrabutylammonium salt of pyrophosphate (Scheme 8). This method allowed the synthesis of different NDPs in a yield range of 43% to 83%, depending on the type of a nucleoside used. It was also successful in the preparation of 5′-diphosphate analogs containing a methylene group in the bridging position of the anhydride bond [67].
A significant improvement of the discussed strategy was made by Hodgson and collaborators [68,69]. In their approach, instead of typical tri- or tetraammonium salt of pyrophosphate, they used tris{(bis(triphenylphosphoranylidene)ammonium} pyrophosphate (PPN, Figure 1) [68]. This modification alleviated a persistent problem of alkylammonium pyrophosphates: extreme hygroscopicity and the formation of heavily water-clathrated structures. In contrast, PPN pyrophosphate is not hygroscopic and can be easily obtained by precipitation as a dry powder. After the formation of the nucleoside 5′-diphosphates, excess PPN pyrophosphate can be precipitated, which greatly facilitates the isolation of products [69].

Figure 1. Tris{(bis(triphenylphosphoranylidene)ammonium} pyrophosphate (PPN).

2.5. Synthesis of Nucleoside Di- and Triphosphates via Dichlorophosphate Intermediate

Ludwig and co-workers described another method for the synthesis of the P–O–P bond. They used the Yoshikawa’s protocol [70], the reaction of unprotected nucleosides with POCl₃ in trimethyl phosphate as a solvent, to generate reactive dichlorophosphate intermediate. After adding tetraalkylammonium phosphate salts and hydrolysis in triethylammonium bicarbonate (TEAB) buffer (pH 7.5), various nucleoside di- or triphosphates could be obtained (Scheme 9) [71]. Ludwig’s approach was recently optimized using modern techniques to control the progress of the reaction and the application of liquid chromatography to isolate the products (NTPs, 51–74% yields) [72].

Interestingly, nucleoside 5′-O-dichlorophosphates of type 12 were prepared previously using tetrachloropyrophosphate (P₂O₅Cl₄) as a phosphorylating agent; however, they were not converted into pyrophosphates, but hydrolyzed to nucleoside 5′-monophosphates [73,74].

The main advantage of Ludwig’s approach lies in the use of unprotected nucleosides as substrates. Unfortunately, this method, due to a strong activation of the phosphorus center in intermediate 12, required difficult-to-control conditions to give satisfactory results [72]. In order to overcome these drawbacks, Hodgson and co-workers proposed using PPN pyrophosphate (Figure 1), which indeed remedied some of these problems [68]. Finally,
replacing POCl₃ with PSCl₃ in the first stage of the synthesis allowed them to obtain various α-thiotriphosphate analogs of NTPs (dAZTPαS, dGTPαS, UTPαS, dTTPαS) in 26–60% yields [75,76].

In order to obtain analogs of P–CH₂–P diphosphates, Darzynkiewicz et al. [77] used the phosphorylation of nucleosides under Yoshikawa’s conditions with methylenebis(phosphonic dichloride) 13 bearing a methylene bridge between two phosphorus centers (Scheme 10). Compound 13 was found to be more reactive than POCl₃, apparently due to the presence of the -CH₂- group in the bridging position, which could not donate electrons by back donation and thus increased the electrophilicity of the phosphorus center. After the generation of trichlorodiphophonate 14 and its hydrolysis in TEAB buffer (pH 7.5), analogs of nucleoside diphosphates (P–CH₂–P), which were difficult to access in any other way, were obtained in good yields (approx. 70%) [77].

![Scheme 10. Synthesis of nucleoside pyrophosphonate analogs with the methylene group in the bridging position, developed by Darzynkiewicz et al. [77].](image)

2.6. Synthesis of Nucleoside Di- and Triphosphates via Cyclic Phosphite Triester Intermediates

The previously discussed methods for di- and triphosphate synthesis were based on the use of reagents containing the phosphorus atom in the V oxidation state (P(V) compounds). At the end of the 1980s, Ludwig and Eckstein developed a different approach for the preparation of nucleoside triphosphates and theirs α-thio-analogs, which was based on the reactivity of P(III)–P(V) mixed anhydrides (Scheme 11). In this method, nucleosides must be properly protected due to their non-regioselective phosphitylation with salicylchlorophosphite 15. In the first stage of the synthesis, nucleoside cyclic phosphite 16 is formed, which undergoes a reaction with tributylammonium salt of pyrophosphate and is transformed into mixed P(III)–P(V) “trimeta” anhydride 17, a key intermediate for the whole process. Its oxidation, followed by hydrolysis and deprotection, gives the final product, the corresponding nucleoside 5′-triphosphate (Scheme 11) [78].

![Scheme 11. Synthesis of NTP and NTPαS using the Ludwig and Eckstein method [78].](image)
It is postulated that the first intermediate 16 undergoes a nucleophilic attack at the phosphorus center by pyrophosphate with the departure of a carboxylate, followed by the ring’s closure as a result of an intramolecular substitution of the aryloxy group [78]. The formed mixed anhydride 17 is then oxidized with iodine/water or sulfur, leading to nucleoside cyclic triphosphates 18 or their thio derivatives. Hydrolysis of these trimetaphosphates intermediates, followed by their deprotection (under basic or acidic conditions, depending on the protecting groups used), leads to the formation of nucleoside triphosphates in the ribo- and deoxy- series (oxidation with I2/H2O system) or their α-thio-analogs (oxidation with elemental sulfur). The ring opening proceeds with full regioselectivity towards NTPs/NTPαsSs without the formation of branched isomers νNTPs/νNTPαsSs (Scheme 11). After DEAE-Sephadex chromatography, the products were obtained in 60–75% yields. Due to the high reactivity of salicylchlorophosphite 15 it is important to maintain strictly anhydrous conditions to minimize the formation of undesired side-products [65].

Exploring the further synthetic possibilities of this method, Ludwig and Eckstein proposed the use of thiopyrophosphate salts (Figure 2) as a nucleophile for the reaction with cyclic intermediate 16. This, combined with the oxidation of the P(III) center by sulfur, provided access to the P,P-dithio analogs of nucleoside 5′-triposphates. The ring opening was not fully chemoselective, and the formation of ca. 15% of Pα,Pγ-dithio isomer was also observed. This by-product could be removed by selective acidic hydrolysis toward nucleoside thiodiphosphate, followed by DEAE-Sephadex chromatography [79].

Figure 2. Thiopyrophosphate used as a nucleophile in the Ludwig and Eckstein method [79].

The above-described one-pot, three-steps protocol is currently one of the most popular methods for the preparation of nucleoside 5′-triposphates NTPs and their various analogs [32].

2.7. Synthesis of Nucleoside Di- and Triphosphates Using Cyclic Phosphate Triesters

In 2009, Meier et al. described a new, effective method for the synthesis of nucleoside di- and triphosphates based on his earlier concept of the cycloSal group [80]. CycloSal nucleotides, developed for the purpose of the pronucleotide approach, are derivatives of NMPs in which the phosphate group is esterified with salicyl alcohol (or “saligenin”), forming cyclic phosphate triesters of type 19 (Scheme 12) [20,21]. In the reaction with monophosphate or diphosphate tetrabutylammonium salts, these species form the corresponding NDPs or NTPs in 40–80% yields. The starting material, cycloSal triester 19, can be obtained by the reaction of phosphorus trichloride (PCl3) with 2-(hydroxymethyl)-4-nitro(chloro)phenol, followed by oxidative condensation with a protected nucleoside. The reactivity of these compounds can be modulated by the introduction of –NO2 or –Cl substituents in the aromatic ring, which, due to the electron-withdrawing effect, makes the phosphorus center more electrophilic and therefore more susceptible to nucleophilic attack. The Meier group also used compound 19 for the preparation of nucleoside 5′-diphosphate analogs with various sugar residues at the terminal phosphorus atom [81]. The method is rather straightforward, although the yields in some cases are moderate [80].

Scheme 12. Synthesis of nucleoside di- and triphosphates via the cycloSal approach [80].
2.8. Synthesis of Nucleoside Di- and Triphosphates via Mixed P(III)–P(V) Anhydrides

One of the more recently proposed method for the synthesis of nucleoside di- and triphosphates was described by Jessen et al., which uses the high reactivity of P(III)–P(V) mixed anhydrides generated in situ (Scheme 13). In the first stage, an unprotected nucleoside phosphate (NMP) is allowed to react with a slight excess (1.1–1.4 equiv.) of bis(fluorenylmethyl) phosphoramidite \(20\) in the presence of a tetrazole-type of activator, leading to the formation of mixed anhydride \(21\), which was then oxidized in situ. After the removal of the phosphate-protecting groups (\(\beta\)-elimination under very mild conditions), nucleoside 5′-diphosphates could be obtained in 75–93% yields as piperidinium salts. If NDP is used as a substrate, the method permits the synthesis of the corresponding nucleoside 5′-triphosphates. Interestingly, despite the known high reactivity of phosphoramidites, no phosphitylation of the 3′-OH group of nucleosides with reagent \(20\) was observed, probably due to the steric hindrance imparted by two fluorenylmethyl groups [82].

![Scheme 13. Synthesis of nucleoside diphosphates using the mixed P(III)–P(V) anhydrides [82].](image)

This approach has many advantages, including, among others, the use of unprotected NMPs, short reaction times, the ease of purification of the products, and good yields. However, as in other methods based on P(III)’s chemistry, strictly anhydrous conditions are crucial for the successful reactions, as traces of water lead to the formation of various by-products.

2.9. Synthesis of Nucleoside Di- and Triphosphates via Phosphobetaines

Further developing the phosphoramidate strategy, Borch and colleagues proposed a new way to activate the phosphorus center in the form of a highly reactive pyrrolidinium intermediate \(22\) (Scheme 14). The pyrrolidinium group in this betaine can be effectively substituted by phosphate nucleophiles, leading to the formation of nucleoside 5′-di- and triphosphates, as well as disubstituted 5′-diphosphates bearing sugar residues on the \(P_\beta\) phosphates (Scheme 14) [83,84]. The reaction times were rather short; for example, 1 h for the reaction with carbohydrate phosphates or 10 min for the formation of nucleoside triphosphates. For carbohydrate–nucleoside diphosphates, the formation of small amounts of nucleoside monophosphates was observed as a side product, presumably due to the presence of traces of water in the reaction system [83]. Despite the promising good yields, this method is not frequently used, probably due to the time-consuming and rather laborious synthesis of the starting phosphoramidate diester \(23\) [32].
Another type of a phosphobetaine, which potentially can be used in the synthesis of di- and triphosphates, is nucleoside N-pyridiniumphosphonate 25 (Scheme 15). The formation of such compounds (which may be considered as a pyridine adduct to nucleoside metaphosphate) was postulated by Michelson in 1960s during studies on the activation of phosphate monoesters [85], but for many years they did not find a practical application in the synthesis of phosphate anhydrides, mainly due to the lack of an effective method for the generation of this type of zwitterions. At the end of the 1990s, Stawinski and co-workers developed an efficient and simple method for the formation of phosphobetaine of type 25 from H-phosphonate monoesters 26, and showed its high reactivity towards different nucleophiles, e.g., alcohols, amines, and fluorides [86–88]. Drawing on these findings, in 2008, Sun et al. used phosphobetaine 25 for the preparation of NTPs by reacting it with a tetrabutylammonium salt of pyrophosphate (Scheme 15). While the yields of isolated products were moderate (26–41%), the conversion of H-phosphonates 26 into NTPs was simple and rapid, as was the purification of the products [89].

Scheme 15. Synthesis of NTPs via N-pyridiniumphosphonate intermediates of type 25 [89].

The same research group took advantage of the ease of the in situ generation of N-pyridiniumphosphonate 25 and its high susceptibility to nucleophilic substitution in the synthesis of symmetric dinucleoside diphosphates. To this end, the generated zwitterion 25 was reacted with 1 equiv. of water to furnish in good yields (70–75%) ribo-homodinucleotides (27a, Figure 3) as well as homodinucleotides containing nucleosides of known antiviral activity (AZT and d4T derivatives 27b; Figure 3). [90,91]
Another type of a phosphobetaine, which potentially can be used in the synthesis of phosphate anhydrides, mainly due to the lack of an effective method for the generation of this type of zwitterions. At the end of the 1990s, Stawinski described the use of orthophosphoric acid instead of lipophilic phosphate salts (e.g., tetrabutylammonium salts) greatly simplified the purification process of the NDPs’ synthesis [92].

3. Anti-HIV Pronucleotides—Nucleotide Reverse-Transcriptase Inhibitors (NtRTIs)

The antiviral activity of anti-HIV pronucleotides is based on the presence in its structure of 2′,3′-dideoxynucleosides (ddNs), which, due to the lack of the 3′-OH function in the dideoxynucleoside moiety, terminates the process of reverse transcription after the incorporation of a nucleotide analog into the growing viral DNA chain. This inhibits the synthesis of viral DNA and prevents virus replication (Scheme 16) [93]. Modified nucleosides are usually not biologically active as such, and must be phosphorylated in the cell to the appropriate 5′-triphosphate in order to exert a therapeutic effect [4].

![Figure 3. Homodinucleotides obtained by Sun et al. via pyridinium phosphobetaine intermediates.](image)

Recently, Romanowska et al. described the use of orthophosphoric acid as a nucleophile in the reaction with N-pyridiniumphosphonate 25 as a simple and convenient method for the preparation of various ribo- and deoxyribonucleoside diphosphates and their analogs. The use of orthophosphoric acid instead of lipophilic phosphate salts (e.g., tetrabutylammonium salts) greatly simplified the purification process of the NDPs’ synthesis [92].

Scheme 16. A general mechanism for the inhibition of HIV replication via termination of the viral DNA chain synthesis catalyzed by the viral reverse transcriptase.

The efficacy of 2′,3′-dideoxynucleosides in antiretroviral therapy is significantly hampered by their limited bioavailability and complex metabolic pathways [4]. In order to increase the therapeutic effect of ddNs, their administration in a phosphorylated form (i.e., as nucleotides) seemed to be a solution to bypass the first, often chimeric, phase of the phosphorylation cascade. Unfortunately, under physiological conditions nucleoside phosphates are ionized, which prevents their permeation through charged cell membranes. Moreover, biological membranes are rich in phosphatases, which rapidly hydrolyze the P–O–C ester linkages, leading to the reconstitution of the parent nucleosides. This would jeopardize the whole concept of improving the pharmacokinetics of a potential drug in this way [6,94–96].

Due to these limitations, the idea of pronucleotides was born, according to which antiviral nucleotides should be administered in a form able to cross the cell membranes. Once
inside the cell, the therapeutic nucleotide would be released through the use of chemical or enzymatic processes. Pronucleotides are thus prodrugs, which are not biologically active per se, but after chemical and/or enzymatic transformations, can generate a precursor of biologically active compound, the corresponding ddNMP (Scheme 1). Initially, the pronucleotide approach assumed the delivery of suitably masked 2',3'-dideoxynucleoside monophosphates, and over two decades, many monophosphate pronucleotide strategies were proposed and experimentally verified. In contrast, the concept of using protected nucleoside di- or triphosphates as possible prodrugs has long been of much less interest. This was mainly due to the fact that fully protected di- and triphosphates are unstable in the aqueous environment due to their high susceptibility to the hydrolysis of the uncharged P–O–P anhydride bond that basically excluded their use as therapeutic agents [97]. Only recently, it has emerged that partially protected pronucleotides are also apparently capable of entering the cell, as evidenced by their high antiviral activity. This opened a possibility of also exploring compounds with a di- or triphosphate skeleton as potential pronucleotides.

The second part of this review will be focused on dedicated synthetic strategies that have been developed to prepare nucleoside di- and triphosphates useful in anti-HIV therapy as prodrugs (pronucleotide analogs of di- and triphosphates), along with the biological evaluation of the synthesized compounds.

3.1. Diphosphate Esters of 2',3'-Dideoxynucleosides

At the onset, the strategy of di- and triphosphate prodrugs was developed mainly for AZT derivatives, since for this particular 2',3'-dideoxynucleoside, the second stage of phosphorylation (conversion of AZTMP to AZTDP) is the most problematic and is a source of the acute toxicity of the AZT therapy [7,8].

Studies on diphosphate pronucleotides date back to early 1990s and the works of Hostetler and collaborators, who introduced lipidic moieties at the terminal phosphate residue of AZTDP and ddCDP, and evaluated the anti-HIV potential of such phospholipid conjugates (compounds of type 28, Scheme 17) [98–100]. It should be noted, however, that these compounds were designed as precursors of ddN monophosphates, not their diphosphates. In order to obtain these derivatives, suitable phosphoromorpholidate derivatives (originally introduced by Khorana et al.) [101] were coupled with glycerophospholipids to give diphosphates of type 28.

![Scheme 17. A postulated hydrolysis pathways for glyceride nucleoside diphosphates in the cell.](image)

It was argued that glyceride nucleoside diphosphates, due to the presence of highly lipophilic acyl groups, should easily penetrate the cell membrane. Hostetler et al. predicted an intracellular metabolism of these compounds to be, by analogy to natural glycerolipids,
initiated by phospholipase A or lysophospholipase. This should lead to the formation of a derivative of type 29, which then can be degraded by another enzyme (phosphodiesterase) to the corresponding ddNMP (path A, Scheme 17). Alternatively, compound 28 with the P–O–P anhydride bond could be directly hydrolyzed by cellular pyrophosphatases to generate nucleoside monophosphate and phosphatidic acid (path B, Scheme 17) [100].

Studies on the stability of the pyrophosphate anhydride bond in pyrophosphate 28 showed that path B of ddNMP formation was preferred [99]. The potential therapeutic value of the obtained diphosphates was rather disappointing, as the antiviral activity of the AZTDP derivative of type 28 (R = C\textsubscript{15}H\textsubscript{31}) in CEM cells was lower than that of AZT alone (EC\textsubscript{50} = 7 \mu M vs. 0.2 \mu M, respectively) [100].

These studies were followed a few years later by Hong and collaborators. They reported on the synthesis of alkyl and thiaalkyl glyceride ether derivatives of type 30 (Figure 4) [102]. These compounds were synthesized via nucleoside 5′-phosphoromorpholidates that were subjected to condensation with the corresponding glycerophospholipids. All the investigated compounds had a low cytotoxicity (CC\textsubscript{50} > 200 \mu M), and the thioether derivative 30a showed greater anti-HIV activity (EC\textsubscript{50} < 0.58 \mu M) than the ether analog 30b (EC\textsubscript{50} = 57 \mu M) [102].

![Figure 4. Lipophilic nucleoside diphosphate analogs designed by Hong et al. [102].](image)

At the same time, Huynh Dinh and collaborators designed mixed carboxylic–phosphoric anhydrides of type 31 as potential pronucleotides (Figure 5). These derivatives contain acyl groups with long alky chains at the terminal phosphate moiety, which should provide appropriate lipophilicity, similarly to the above glycerolipid analogs [103,104]. Nucleotide derivatives of type 31 were synthetized from the corresponding carboxylic acids and nucleoside phosphates using the carbodiimide approach for the formation of an anhydride bond.

![Figure 5. Examples of nucleoside monophosphate triphosphate pronucleotides with a structural motif of the mixed carboxylic–phosphoric anhydrides [103,104].](image)

Derivatives of type 31 were assumed to penetrate cell membranes and release ddNMP, ddNDP or ddNTP for n = 1, 2 or 3, respectively, as a result of the expected preferential hydrolysis of the mixed carboxylic–phosphoric acid anhydride bond (C–O–P) over the pyrophosphate bond (P–O–P). Indeed, this cleavage order was confirmed in both triethylammonium acetate buffer at physiological pH and in RPMI; however, in the last medium the reaction was so rapid (t\textsubscript{1/2} < 2 h) that the lipophilic compounds 31 apparently did not have enough time to enter the cells [104]. The biological evaluation of the activity of the AZT analogs of 31 showed that their EC\textsubscript{50} values were of the same order as for the parent AZT nucleoside, and for the d4T derivatives, even being 100 times lower than that for d4T nucleoside.

The strategy, based on the introduction of long aliphatic chains into the nucleotides, was extended to alkoxyalkyl derivatives of acyclic nucleoside phosphonates (ANPs) by Ruiz et al., who developed derivatives of type 32 as antiviral agents (Figure 6) [105]. In the
synthesis of this class of compounds, alkoxyalkyl phosphoromorpholodiates were used as intermediates that were coupled with the appropriate C-phosphonates of antiviral acyclic nucleoside analogs, adefovir and cidovir.

![Figure 6. Lipophilic acyclic nucleoside phosphonates (ANP) analogs 32 designed by Ruiz et al.](image)

In vitro biological assays showed that analog 32a-HDP inhibited HIV replication much better (EC₅₀ = 0.003 μM) than adefovir alone (EC₅₀ = 1.3 μM); unfortunately, its cytotoxicity in MT-2 cells was clearly higher (CC₅₀ = 0.018 μM vs. CC₅₀ = 157 μM), which resulted in the SI₅₀ selectivity index for this compound being only 6 (SI₅₀ = 121 for adefovir) [105]. Interestingly, adefovir esterified with the HDG group, having similar cytotoxicity, showed extremely high activity against HIV, EC₅₀ = 0.02 nM, which gave SI₅₀ = 3000. Cidovir derivatives were tested against HCMV, HSV-1, vaccinia, and cowpox infections. Of the five compounds tested in this series, the pyrophosphate derivative was found to be the superior in one case. Derivative 32b-ODE showed the strongest inhibition of HSV (EC₅₀ = 0.2 nM), while being moderately cytotoxic (EC₅₀ = 9.5 μM), which resulted in SI₅₀ ≈ 500,000. No information on the possible mechanism of action of this type of pronucleotides was provided by the authors.

The most comprehensive and extensive research on di- and triphosphate pronucleotides was carried out by Meier and co-workers. In 2008, the first attempts were made to adapt the cycloSal approach of pronucleotides to diphosphate derivatives [106]. This choice seemed justified considering the promising biological results of the cycloSal-NMP derivatives [21]. Unfortunately, under hydrolytic conditions, compounds of type 33 (Scheme 18) hydrolyzed with cleavage of the P–O–P bond (path B), resulting mainly in the release of ddNMP rather than the expected ddNDP (path A) [106]. Thus, cycloSal-NDP derivatives appeared to be precursors of the same nucleoside monophosphates as the simpler cycloSal-NMP constructs.

![Scheme 18. The expected (A) and the actual (B) hydrolysis pathways of cycloSal-NDP pronucleotides.](image)

Due to the unfavorable course of the hydrolysis of cycloSal-NDP 33, Meier et al. developed another pronucleotide strategy, called DiPPro. In this approach, the terminal phosphate group of pronucleotide 34 was protected with stable benzyl-type groups, which required the action of cellular enzymes to release unmasked phosphate. This allowed them to avoid the unfavorable nucleophilic attack on the phosphorus center (Scheme 19) [106]. The protecting groups contained a carboxyester moiety that was prone to cleavage by cellular carboxylesterases, releasing the labile 4-hydroxybenzyl ester in intermediate 35, which spontaneously collapsed to diphosphate 36. The second benzyl group could be removed by the same mechanism or could be cleaved directly by phosphoesterases. This mode of action was analogous to the bis(4-acyloxybenzyl)-pronucleotide strategy proposed...
by Freeman et al. in 1993 to achieve the in-cell formation of ddMPs, which did not receive much attention at that time [107]. For DiPPro pronucleotides 34, it was indeed found that the desired ddNDPs were formed during incubation in cell extracts [106]. Although this route was dominant, some ddNMPs formation was also observed [108,109].

![Scheme 19](image)

**Scheme 19.** A postulated mechanism for the releasing of nucleoside diphosphates from DiPPro pronucleotides [106].

Initially, the RC(O) acyl moieties of both benzyl-protecting groups at the Pβ center were identical, and the chain length of R, which correlated with the lipophilicity of R, was found to significantly affect the hydrolytic properties of DiPPro pronucleotides 34. The anti-HIV activity of the d4T derivatives of 34 was similar or slightly worse than that of d4T in CEM/0 cells; however, in TK- cells, in which d4T is completely inactive, diphosphates 34 (R = heptyl or phenyl) retained their activity. In contrast, DiPPro with R = Me was probably too polar to penetrate the cell membrane, and the one with R = tBu was speculated to be resistant to the second deprotection step (i.e., 36 → d4TDP) [106]. These results, along with the finding that the amount of undesirable monophosphate formed from pronucleotides 34 closely correlated with the increasing chain length of the acyl residue, prompted the Meier group to design unsymmetrical DiPPro derivatives 34a (Figure 7), which contained two different masking groups at the Pβ phosphate, differing in the length of the carbon chains of the acyl residues [108,109]. The R1 group with a short aliphatic chain should undergo rapid hydrolysis in the presence of appropriate cellular enzymes, thus preventing cleavage of the P–O–P bond. In contrast, the acyl substituent in the second benzyl group, with a long carbon chain, should ensure the adequate lipophilicity of the molecule. When tested under various experimental conditions, the unsymmetrical compounds 34a produced significantly less NMPs and more NDPs in all instances compared to the previous symmetrical DiPPro compounds 34.

![Figure 7](image)

**Figure 7.** Structures of the developed pronucleotides of DiPPro type [108].
The Meier group also designed bis(benzylxybenzyl) derivatives of type 34b (Figure 7), containing in their structure electron-withdrawing and electron-donating substituents in the para position of the benzyl residues. Stability studies in cell extract revealed that derivatives of type 34b with strong electron-withdrawing groups (e.g., CF₃, CN, NO₂) released only or mostly the expected ddNDP [110].

Studies on the therapeutical parameters of d4T derivatives of type 34 have shown that these compounds had similar antiviral activities to the parent nucleosides [106]. Most of these analogs were effective in inhibiting HIV replication, and in some cases improved biological activity, as compared to free nucleosides, was even apparent. Moreover, a high antiviral activity was also observed in CEM/TK- cells, which supported the pronucleotide mode of action for these compounds [108]. Among the investigated compounds, the most active were analogs of type 34b [110].

For the synthesis of various types of DiPPro pronucleotides, the Meier group used a combination of P(III) and P(V) chemistries. The phosphitylating reagent 37 bearing two identical benzyl groups could be prepared relatively simply by reacting dichloro-N,N-diisopropylaminophosphoramidite with 2 equiv. of an appropriate benzyl alcohol [106,110,111]. In the case of an unsymmetrical version of reagent 37, this approach failed, and a more sophisticated protocol had to be developed. To this end, the first benzyl alcohol was reacted with PCl₃ to give benzyl phosphorodichloridite intermediate, which was converted into the bis(N,N-diisopropylamidite) derivative, and subjected to the reaction with the second benzyl alcohol. The reaction of phosphoramidite 37 with tetraalkylammonium salt of a monophosphate or a diphosphate of antiviral nucleoside (e.g., d4T) resulted in the mixed P(III)–P(V) anhydride 38, the oxidation of which with tert-butyl hydroperoxide (tBuOOH) led to the desired NDP, with the masking groups at the terminal phosphate, in 31–41% yields (Scheme 20) [112,113].

![Scheme 20. Synthesis of pronucleotides of the DiPPro type using the mixed anhydrides P(III)–P(V) method [106,108,110,112].](image)

### 3.2. Triphosphate Esters of 2',3'-Dideoxynucleosides

It has been long believed that the delivery of a triphosphate prodrug to the cell is impossible due to the high polarity of such compounds and the inherent lability of the anhydride bond in fully masked NTP. Additionally, due to susceptibility to phosphatases, it was assumed that these compounds are short-lived in vivo [109]. Despite these concerns, the Meier group, encouraged by the positive results of the DiPPro approach, synthesized triphosphate analogs, called TriPPPPro pronucleotides, with a similar arrangement of masking groups on the terminal P₆-phosphate residue as for the DiPPro derivatives, expecting their metabolism to lead directly to biologically active ddNTPs, without the involvement of cellular kinases (Scheme 21) [38,113–115].
In the initial studies, symmetrical acyloxybenzyl-masking groups with C1–C17 alkyl, alkoxy, and aminooxy chains were used (compound of type 39a, R1 = R2, Scheme 21) [113–115]. These compounds were synthesized analogously to the nucleoside diphosphonate analogs discussed above (Scheme 20), using ddNDPs (prepared via the cycloSal approach, cf. Scheme 12) instead of ddNMPs, as the nucleotidic components [113]. In addition, triphosphates of type 40 (Nucl = d4T) with only one benzyl-masking group, the expected metabolites of compounds 39, were prepared to determine the stability under various experimental conditions. Since the route via a partial hydrolysis of the corresponding acyl derivatives, analogously to monobenzyl compounds in the DiPPPPro series [111], was not very effective, monobenzyl TriPPPPro’s 40 were obtained (albeit with moderate yields) by reacting benzyl cycloSal esters with ddNDPs [113].

It was found that dibenzyl TriPPPPro’s 39 (Nucl = d4T) bearing alkyl chains up to C11 were efficiently cleaved enzymatically to monobenzyl derivatives 40, and then to the expected d4TNTPs. Although their anti-HIV activity and cytotoxicity in CEM cells were similar to that of the parent nucleoside, these compounds retained their full anti-HIV potency in TK+ cells, where d4T was totally inactive [113]. These results demonstrated that ionic nucleoside triphosphates with the appropriate lipophilic masking groups can enter cells and deliver an active nucleotide, probably in the form of the corresponding nucleoside triphosphate.

In follow-up studies, Meier et al. described an alternative method for the preparation of pronucleotides of type 39 (Scheme 22) [114,115]. A key reagent in this method, the pyrophosphorylating agent of type 41, was obtained by the transesterification of diphenyl H-phosphonate [116] with the appropriate benzyl alcohol, followed by chlorination with N-chlorosuccinimide and the reaction with inorganic phosphate. Its activation with trifluoroacetic anhydride and the subsequent reactions with N-methylimidazole and ddNMPs led to TriPPPPro-nucleotides in 35–85% yields.

![Scheme 21](image1)

**Scheme 21.** The TriPPPPro approach to a kinase-bypassing delivery of ddNTPs to the cell.

![Scheme 22](image2)

**Scheme 22.** H-Phosphonate approach to the preparation of TriPPPPro-nucleotides 39 [115].
The previous results on the highly selective conversion of unsymmetrical DiPPPros into the corresponding ddNDPs encouraged Meier's group to apply the same strategy also to TriPPPPro-nucleotides. They synthesized a series of triphosphate derivatives of type 39b (Figure 8), in which one benzyl group contained an acyl with a short (C2–C6) alkyl chain, while the other benzyl group, an acyl with a long (C14–C17) alkyl chain, or triethyleneglycol linked via succinic or glutaric diester [117]. The shorter-chain acyl group was expected to be more susceptible to enzymatic hydrolysis than the longer-chain acyl group, thus providing the better pharmacokinetics of the pronucleotide. However, these expectations did not come true. Hydrolysis studies of such compounds revealed that apparently, there was no selective cleavage between the short-chain acyl ester and the long-chain acyl ester groups (in contrast to the DiPPPPro derivatives, vide supra), and thus unsymmetrical pronucleotides of type 39b did not have any advantage over the simpler, symmetrical TriPPPPros 39a (R1 = R2) in terms of their anti-HIV activity. The same was observed also for TriPPPPros 39 containing other known or potential antiviral nucleosides, e.g., AZT, AZU, ddC, ddG, ddI, carba-T, FddClU, FTC, 3TC, Abacavir, Carbovir, BVdU, which showed appreciable anti-HIV activity both in TK+ and TK− cells [114,115,118].

![Figure 8. Examples of general structures of TriPPPPro-nucleotides (39a–e) synthesized by the Meier group [113,117,119].](image-url)

This approach was applied also for the preparation of the other unsymmetrical TriPPPPros described later in the text. Products 39b–d were obtained in 23–78% yields. Although originally developed for the triphosphorylation of d4T, it was used also for the synthesis of TriPPPPro prodrugs of a number of known or potential antiviral nucleosides—AZT, AZU, ddC, ddG, ddI, carba-T, FddClU, FTC, 3TC, Abacavir, Carbovir, BVdU, and several
their regioisomers and derivatives. Appreciably, most of these triphosphate pronucleotides were found to be active against HIV in both TK+ and TK- cells [114,115,118].

Although TriPPPPro of type 39b apparently acted as true pronucleotides, the authors considered that their rate of conversion into the corresponding nucleoside triphosphates was too high and this compromised their anti-HIV potential. To modulate this process, specifically, the rate of the generation of monobenzyl intermediates of type 40 (Scheme 21) from 39b precursors, one or both of the acyloxybenzyl groups of symmetrical TriPPPpronucleotides 39 were replaced with the alkoxyacryloxybenzyl ones (compounds 39c and 39d, Figure 8) [119]. It was argued that triphosphate derivatives of type 39c and 39d will still act as pronucleotides for nucleoside triphosphates (Scheme 23), but since the carbonate diesters should be less susceptible to degradation by esterases than the acyl esters, this would secure slower generation of, and provide higher cellular stability in, the monobenzyl intermediates (e.g., 40b, Scheme 23). Additionally, alkyl substituents R1–R3 of various chain lengths were used for the optimal cell membrane permeability and the kinetics of formation of d4TTP in the cell.

Scheme 23. Unsymmetrical dibenzyl prodrugs 39c/d and their assumed cellular metabolism to d4TTP [119].

For the preparation of such unsymmetrical TriPPPPros 39c/d, an analogous H-phosphonate strategy to that depicted in Scheme 22 was used [119]. By these means, variants 39e of TriPPPPros were also prepared, in which one of the benzyl groups was replaced with 2-cyanoethyl to enable the formation of monobenzyl intermediate 40b via a β-elimination process.

TriPPPPro pronucleotides of type 39d had similar anti-HIV activity as d4T in normal cells (EC50 ≈ 0.2–0.3 μM) and also in TK- cells (EC50 ≈ 1–3 μM), confirming their pronucleotide mode of action. Interestingly, pronucleotides 39c were more active than 39d by roughly an order of magnitude, both in TK+ and TK- cells. The highest activity (EC50 = 0.005 μM for TK+ and 0.1 μM for TK-) was found for 39c with R1 = Et and R2 = C16H33. Bioassay studies were also carried out for the monobenzyl triphosphates of type 40b (bearing C12 and C16 alkyl chains), and these showed practically the same activity as that of compounds 39c. These clearly demonstrated that ionic compounds with the proper lipophilic handles can efficiently migrate through the cell membrane [119].

Pronucleotides of type 39c (R1 = Et or Bu; R2 = C16H33) but bearing other than d4T antiviral nucleoside residues were also prepared, and showed similar or even slightly better activities against HIV-1 and HIV-2 than the parent nucleosides in both TK+ and TK- cells [118].

Despite the promising results obtained with pronucleotides TriPPPPro of type 39 (bearing the benzyl groups derivatized with both carboxy and/or carbonate ester groups),
detailed analyses revealed some drawbacks of these constructs. The stability studies in enzymatic media showed that although the compounds 39 were admittedly liberated of the desired NTPs, their rapid intracellular dephosphorylation compromised their pronucleotide mode of action. Moreover, a careful scrutiny of the possible sources of the observed cytotoxicity strongly suggested that the released ddNTPs were not only substrates for the viral reverse transcriptase (RT), but also to some extent for cellular polymerases, particularly for the mitochondrial DNA polymerase \( \gamma \) [120].

To remedy these problems, Meier et al. developed another type of unsymmetrical triphosphate pronucleotides 43 (Figure 9) that contained only one biodegradable acyloxybenzyl group, as in the previous constructs (R = C1–C15), in combination with the simple alkyl phosphate esters of different chain length (C4, C15, C18). The aim of this was, on the one hand, to ensure the adequate lipophilicity of the pronucleotides, which would enable the efficient crossing of cell membranes, and, on the other hand, to protect the triphosphate residue against dephosphorylation by the presence of a more difficult-to-cleave \( \gamma \)-alkyl group. It was also expected that the presence of the \( \gamma \)-alkyl group would prevent the binding of such triphosphate analogs by cellular polymerases, but not by the more promiscuous viral RT enzyme.

![Figure 9. Structure of triphosphate pronucleotides 43 containing alkyl chains at the P\textsubscript{\( \gamma \)} moiety [120].](image)

For the synthesis of prodrugs 43, the same H-phosphonate method (Scheme 22) as for prodrugs 39b–d (yields 33–63%) was used, and the expected products of their cellular metabolism, \( \gamma \)-alkyl-NTPs 44, were obtained via \( \beta \)-elimination of the cyanoethyl group. The synthesized pronucleotides 43 were found to successfully enter the cells, followed by a selective cleavage of the benzyl group. The formed \( \gamma \)-alkyl-NTPs 44 appeared to be stable in cell extracts, as expected, and acted as substrates for the viral RT only. This selectivity can form the basis for fighting viral infection in the body without interfering with the vital functions of the cell. Almost all pronucleotides 43 obtained were slightly more active against HIV-1 and HIV-2 than the parent nucleoside d4T, and some of them retained their full activity in the TK\textsuperscript{−} cells. The \( \gamma \)-alkyl-NTPs 44 were similarly active in normal CEM/0 cells, while their anti-HIV activity in TK\textsuperscript{−} cells varied dramatically depending on the alkyl chain length. Thus, for derivatives 44 with \( R = C4 \) or \( C11 \), no anti-HIV activity was observed, while for \( R = C18 \), the antiviral activity became very high (EC\textsubscript{50} = 0.05 \( \mu \)M) [120].

4. Conclusions

The biological importance of phosphorylated nucleosides has been the driving force behind the chemical studies on phosphorus-containing natural products. Lord Todd’s seminal work in the middle of the last century, honored with the Nobel Prize in Chemistry in 1957, not only helped elucidate the chemical structure of first nucleoside di- and triphosphates isolated from natural sources (e.g., ATP, UTP, etc.), but also was instrumental in the development of the future chemical synthesis of oligonucleotide and the emergence of chemical biology. While most of the preparative chemical methods developed by this group are now primarily of historical value, they laid the foundation for nucleotide chemistry as a separate field of phosphorus chemistry. After several decades of research, the chemical synthesis of nucleoside di- and triphosphates is still not a trivial task, and the
search for new synthesis strategies, more efficient and simpler methods, and simplified purification procedures, is still valid. In recent years, we have witnessed a growing interest in nucleoside di- and triphosphate derivatives and their analogs, for which new methods based, inter alia, on the activation of P(V) compounds, or the use of the reactivity of P(III) derivatives, have been developed and reviewed in this paper. This time, the stimuli came from extensive research on pronucleotides as potential antiviral and anti-tumor agents. This called for novel structural variants of the nucleoside di- and triphosphates, and it seems that chemistry will meet these new challenges again.

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