Recent Trends in Nucleotide Synthesis

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ABSTRACT: Focusing on the recent literature (since 2000), this review outlines the main synthetic approaches for the preparation of 5′-mono-, 5′-di-, and 5′-triphasphorylated nucleosides, also known as nucleotides, as well as several derivatives, namely, cyclic nucleotides and dinucleotides, dinucleoside 5′,5′-polyphosphates, sugar nucleotides, and nucleolipids. Endogenous nucleotides and their analogues can be obtained enzymatically, which is often restricted to natural substrates, or chemically. In chemical synthesis, protected or unprotected nucleosides can be used as the starting material, depending on the nature of the reagents selected from P(III) or P(V) species. Both solution-phase and solid-support syntheses have been developed and are reported here. Although a considerable amount of research has been conducted in this field, further work is required because chemists are still faced with the challenge of developing a universal methodology that is compatible with a large variety of nucleoside analogues.

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Nucleotides, such as S'-mono- and S'-polyphosphorylated nucleosides, and their conjugates are essential biomolecules that play key functional and structural roles in biological systems (Figure 1, Table 1). As an example, they provide the fundamental building blocks in the polymerase-mediated synthesis of nucleic acids. The structural feature common to all of these compounds is the presence of one or two nucleosides that are attached to one or more phosphate groups, with phosphorus in the oxidation state +5. The most versatile nucleotide is adenosine 5'-triphosphate (ATP). It is the primary energy source for cellular functions. Indeed, many reactions that occur in the cell, which range from metabolic transformations to signaling events, are coupled to the hydrolysis of ATP. It is remarkable that the human body turns over its own body weight equivalent in ATP each day. In addition to serving as the major energy currency of cells, ATP also acts as an important allosteric effector in the regulation of cell metabolism.

Cyclic mononucleotides (cNMPs) are single-phosphate-containing compounds characterized by an intramolecular phosphodiester bond between the phosphorus atom and the
Table 1. Biological Functions of Natural Nucleotides and Their Conjugates in Humans

<table>
<thead>
<tr>
<th>compounds</th>
<th>role</th>
<th>purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>nucleotides(^{(d)NMPs, (d)NDPs})</td>
<td>substrates for nucleotide kinases(^{55})</td>
<td>(d)NTP synthesis</td>
</tr>
<tr>
<td></td>
<td>signaling molecules (ADP, UDP)(^{30–38})</td>
<td>activation of G protein-coupled membrane receptors</td>
</tr>
<tr>
<td>(d)NTPs</td>
<td>substrates for DNA or RNA polymerases protein regulators(^{55})</td>
<td>storage and transmission of genetic information (replication/transcription)</td>
</tr>
<tr>
<td></td>
<td>signaling molecules (ATP, UTP)(^{30–38})</td>
<td>activation of G protein-coupled membrane receptors</td>
</tr>
<tr>
<td></td>
<td>cofactors (ATP, UTP)(^{30,39})</td>
<td>activation of G protein-coupled membrane receptors</td>
</tr>
<tr>
<td></td>
<td>energy-rich carriers (ATP, GTP)</td>
<td>transfer of a phosphoryl group to an acceptor molecule</td>
</tr>
<tr>
<td>cyclic nucleotides and cyclic dinucleotides</td>
<td>secondary messengers(^{30,40})</td>
<td>energy supply in biochemical reactions</td>
</tr>
<tr>
<td>dinucleoside S',S'-polyphosphates(^{(d)NMPs, (d)NDPs, (d)NTPs, (2'-deoxy)-ribonucleoside S'-monophosphates, (d)NDPs, (2'-deoxy)-ribonucleoside S'-triphosphates; ADP, adenosine S'-diphosphate; UDP, uridine S'-diphosphate; ATP, adenosine S'-triphosphate; UTP, uridine S'-triphosphate; GTP, guanosine S'-triphosphate; (\text{Ap}_4\text{A}, \text{P}^1\text{S}'-\text{O}-\text{adenosine}-\text{P}^2\text{S}'-\text{O}-\text{adenosine} \text{ tetraphosphate.} )(^{(d)NTP pool homeostasis, control of cell proliferation \text{ and transfer of a phosphoryl group to an acceptor molecule.})</td>
<td>impact on the physiology and pathology of cardiovascular systems</td>
<td></td>
</tr>
<tr>
<td>nicotinamide mononucleotides</td>
<td>enzyme cofactors (NAD, NADH, NADP, NADPH)</td>
<td>activation of G protein-coupled membrane receptors</td>
</tr>
<tr>
<td>flavin mononucleotides</td>
<td>enzyme cofactors (FAD, FADH(_2))</td>
<td>act in redox processes</td>
</tr>
<tr>
<td>sugar nucleotides</td>
<td>glycosyl donors for glycosyl transferases(^{9,10})</td>
<td>biosynthesis of oligo- and polysaccharides</td>
</tr>
<tr>
<td></td>
<td>substrates for UDP galactose 4-epimerase(^{31})</td>
<td>glycosylation of proteins and lipids</td>
</tr>
<tr>
<td></td>
<td>intermediates of carbohydrate metabolism</td>
<td>interconversion of glucosyl and galactosyl groups</td>
</tr>
<tr>
<td></td>
<td>signaling molecules (UDP glucose)(^{36–38})</td>
<td>biosynthesis of deoxysugars, aminodeoxysugars, chain-branched sugar and uronic acids</td>
</tr>
<tr>
<td>nucleolipid(^{19})</td>
<td>intermediates of lipid metabolism ((\text{CDP-DAG}))(^{19})</td>
<td>activation of G protein-coupled membrane receptors</td>
</tr>
</tbody>
</table>

\(^{1}\) \(\text{d)NMPs, (2'-deoxy)ribonucleoside S'-monophosphates; (d)NDPs, (2'-deoxy)ribonucleoside S'-triphosphates; ADP, adenosine S'-diphosphate; UDP, uridine S'-diphosphate; ATP, adenosine S'-triphosphate; UTP, uridine S'-triphosphate; GTP, guanosine S'-triphosphate.} \(\text{Ap}_4\text{A}, \text{P}^1\text{S}'-\text{O}-\text{adenosine}-\text{P}^2\text{S}'-\text{O}-\text{adenosine} \text{ tetraphosphate.} \)\(^{(d)NTP pool homeostasis, control of cell proliferation \text{ and transfer of a phosphoryl group to an acceptor molecule.}\)
including the acyclic and carbocyclic ones. The strategies for the preparation of nucleoside S'-mono-, S'-di-, and S'-triphosphates; 3',S'-cyclic nucleotides; dinucleotides; sugar nucleotides; and nucleolipids are described. Since Burgess and Cook’s comprehensive review on NTPs synthesis, which was published in this journal in 2000, some aspects of these topics have been covered in several reports and chapters. Our objective is to review the synthesis of these nucleotides from a holistic perspective and highlight recent advances. Therefore, we focus on the methodologies reported since 2000, particularly those involving solid-phase and polymer-assisted synthesis. We do not describe the methods for preparing oligonucleotides because they go beyond the scope of this review.

2. STRUCTURAL FEATURES AND PROPERTIES OF NUCLEOTIDES

2.1. Structural and Chemical Properties

2.1.1. Structure and Conformation. Endogenous nucleosides comprise a nitrogen-containing heterocycle, called a nucleobase, connected to a sugar, namely, β-β-d-ribofuranose or β-β-d-ribofuranose. In nucleic acids, the common nucleobases are the purines (adenine and guanine) and the pyrimidines (cytosine; uracil; and thymine, also called S-methyluracil), which are covalently bonded from N1 of pyrimidine or N9 of purine to the anomic carbon (C1') of the β-β-d-ribose in a β-β-d configuration. Natural nucleotides incorporate one to three phosphate groups (Pα, Pβ, and Pγ) attached to the hydroxyl group on the C5' position of the nucleosides (Figure 1).

Extensive studies conducted during the 1960s and 1970s showed that nucleosides and nucleotides can adopt many different conformations in solution that are in a rapid dynamic equilibrium. Background information on natural nucleoside conformational studies is available in the literature. In addition, numerous research groups have been studying the impacts of various modifications/constraints on the puckering preferences of the furanose ring. Furthering our knowledge of nucleotides’ preferred conformations is important because it could help researchers understand and possibly even predict their interactions with various biological targets. Generally speaking, nucleotides are less flexible than their nucleoside counterparts. The conformational behavior of nucleotides can be significantly modified through electrostatic interactions between the nucleobase and the S'-phosphate group and through the alteration of the intramolecular hydrogen bonds involving the nucleobase and the hydroxyl group in the S'-position.

Three main types of structural data are commonly used to describe the conformational properties of these derivatives (Figure 2). First, the glycosyl torsion angle χ (O4'−C1'−N1−C2 for pyrimidine nucleosides and O4'−C1'−N9−C4 for purine nucleosides, Figure 2B and 2C) is related to the rotation about the β-N-glycosidic bond. When the C2 carbonyl of pyrimidines or the N3 of purines lies over the sugar ring, the conformation is called syn; otherwise, it is called anti. In the solid state and in solution, the conformation of natural nucleosides and nucleotides is generally anti. The second parameter is the torsion angle γ (O5'S−C5'S−C4'−C3'), which reflects the position of the S'-hydroxyl group relative to the C3' carbon atom (Figure 2B and 2C). Finally, the phase angle of pseudorotation P and the maximum out-of-plane pucker ηmax are related to the sugar ring conformation.

In mononucleotides, derivatives containing purine tend to adopt the southern (S, C2'-endo, Figure 2D) conformation, whereas the northern (N, C2'-exo, Figure 2A) conformer is predominant in pyrimidine derivatives. Shishkin et al. investigated the conformational equilibrium of natural dNMP using calculations. They concluded that S/anti is the predominant conformation, with the exception of S'-dGMP, which behaved as an S/syn conformer. In biological systems, nucleotides serve as substrates for enzymes in the form of their metal ion complexes. These metals are mostly divalent cations, such as Mg2+, Mn2+, and Zn2+. The dominating conformations in nucleotides have consequences for the metal-ion-binding properties of these compounds, such as the formation of macrochelates.

Stern et al. performed conformational studies on a series of dinucleoside polyphosphates (Np,Np) at physiological pH using various NMR techniques (i.e., 1H, 13C, and 31P NMR spectroscopies, as well as COSY, HMQC, HMBC, and NOESY 2D experiments). Ab initio calculations, supported by NMR data, showed that, in solution, physiologically active dinucleoside polyphosphates are more frequently found in a stacked rather than an extended conformation, and no predominant conformation for the ribose moiety was observed. Recently, the Gao research group investigated the conformational behavior and structural flexibility of purine cyclic dinucleotides in aqueous solution, demonstrating that the phosphodiester backbone (either 2',5'- or 3',5'-) and sugar puckering affect the overall flexibility of CDNs and, consequently, their binding affinities.

2.1.2. Acid and Base Properties. The pKα values of the five main nucleobases in nucleotides are listed in Table 2. At physiological pH, they exist almost completely (>99.99%) in the keto and amino tautomeric forms, as shown in Figure 3. Using 1H and 13C NMR spectroscopies, Velikyan et al. accurately determined the pKα values of the 2'-hydroxyl group in several nucleosides and nucleotides. They showed that the pKα values depend on the ability of the various sugar substituents to
Table 2. pKₐ Values and Spectroscopic Data at pH 7.64 for Endogenous Nucleoside 5′-Monophosphates

<table>
<thead>
<tr>
<th>compound</th>
<th>pKₐ</th>
<th>λ_max (nm)</th>
<th>ε (mM⁻¹ cm⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>N1, 3.74</td>
<td>259</td>
<td>15.04</td>
</tr>
<tr>
<td>GMP</td>
<td>N7, 2.40, N1, 9.40</td>
<td>252</td>
<td>14.09</td>
</tr>
<tr>
<td>CMP</td>
<td>N3, 4.56</td>
<td>271</td>
<td>8.74</td>
</tr>
<tr>
<td>UMP</td>
<td>N3, 9.50</td>
<td>262</td>
<td>9.78</td>
</tr>
<tr>
<td>dTMP</td>
<td>N3, 10.00</td>
<td>267</td>
<td>9.49</td>
</tr>
</tbody>
</table>

Stabilize the 2′-oxygen; for example, the pKₐ value of 2′-OH in AMP (13.38) is greater than that of adenosine (12.17).

The pKₐ values of several phosphate moieties found in biomolecules are indicated in Figure 4. Pyrophosphoric acid corresponds to the anhydride of two orthophosphoric (phosphoric) acid molecules. On the basis of their reported pKₐ values, these inorganic compounds behave as phosphate and pyrophosphate anions at physiological pH. Thus, phosphoric acid can be mono-, di-, or triesterified to give phosphate monoester, diester, or triester derivatives, respectively. Only phosphate monoesters and diesters are naturally occurring, whereas phosphate triesters are exclusively synthetic compounds. In phosphate monoesters such as nucleoside 5′-monophosphates, the two ionizable hydroxyl groups near the phosphorus atom have pKₐ values of 1.6 and 6.6.64

In phosphate diesters, the remaining hydroxyl group is strongly acidic, with pKₐ ≈ 1.5. It is assumed that nature has chosen the canonical bases because they are not ionized under physiological pH, thus allowing Watson–Crick base pairing.68 Conversely, the phosphate moiety in phosphate diesters is ionized under physiological conditions. This enables the dissolution of nucleic acids in aqueous media, stabilizes the backbone against hydrolysis, and also retains the molecules in the cells because they are trapped by the lipidic membrane.69

Using phosphorus nuclear magnetic resonance (31P NMR) spectroscopy, Jancan and Macnaughtan determined the acid dissociation constants of UDP and UDP-sugars.70 The pKₐ values for the diphosphate of UDP and UDP-N-acetyl glucosamine (UDP-GlcNac) were found to be approximately 6.5 for both Pα and Pβ.

Nucleoside 5′-triphosphates are in the form of NTP⁺ at physiological pH.71 Indeed, the fully protonated nucleoside 5′-triphosphates release the first two protons from their triphosphate chain at very low pH. The third deprotonation is characterized by pKₐ ≈ 2, whereas the pKₐ of the terminal Pγ phosphate group is approximately 6.7.71

2.1.3. Chemical Stability. It is fascinating how nature has created biomolecules that can be either stable or reactive depending on the prevailing chemical conditions. The behavior of phosphate mono- and diesters is a perfect illustration of this phenomenon. In the absence of a catalyst, the half-life of phosphate monoester dianion hydrolysis is estimated to be 1.1 × 10²⁷ years at 25 °C,72 whereas in the presence of phosphatases, the rate of water attack on the substrate increases by a factor of 10²¹. Similarly, a kinetic study of the uncatalyzed hydrolysis of diphosphate (a model compound used to mimic phosphate diesters) at high temperature resulted in an extrapolated half-life of 30 billion years at 25 °C. The rate of hydrolysis increased by a factor of ~10¹⁷ in the presence of staphylococcal nuclease, a phosphodiesterase.73 Therefore, the strong resistance to spontaneous/chemical hydrolysis of phosphodiester bonds contributes to the stability of genes. The mechanisms underlying the spontaneous or enzymatic hydrolysis of phosphate esters have been reviewed in the literature.74,75–79

The rate profile of the nonenzymatic hydrolysis of pyrophosphoric acid is a complex function closely related to the pH of the medium. The pyrophosphate bond increases in stability as the number of overall negative charges of the derivative increases. The tetra-anion is the form that is most resistant to hydrolysis.75,74

Nucleoside 5′-phosphates, including triphosphates, are stable only in slightly alkaline solution (around pH 8), whereas they are very sensitive under acidic conditions (pH below 5). This results from the stabilization of the negatively charged phosphate anion and contrasts with esters of carboxylic acids. Compared to nucleoside 5′-polynucleotides, dinucleoside 5′,5′-polypophosphates are more stable and have relatively long half-lives.4,80 For example, DNPs are stable during prolonged dry storage at room temperature, whereas mononucleotides must be stored at 0 °C or below. Mononucleotides slowly degrade in solution at neutral pH or lower, but this is not the case for DNPs.80 In 2010, Huhta et al. performed a kinetic study on the chemical cleavage of nucleoside diphosphate sugars.81 Using ADP-glucose and UDP-glucose as model compounds, they showed that these compounds are fairly stable under neutral conditions, even at high temperatures, and that their decomposition is enhanced by acids and bases.

2.2. Analysis

The study of nucleotides and their analogues using NMR techniques can determine the conformation (syn/anti, sugar puckering) and nucleobase properties (tautomerism, H-bonds, and π-stacking interactions), as well as the anomic α/β configuration.82 1H and 13C NMR chemical shifts for natural β-ribonucleoside 5′-triphosphates are reported in Table 3. Nucleotides exhibit relatively simple 1H NMR spectra.83,84 The aromatic protons of the purines, as well as the H6 atoms of the pyrimidines, resonate at low field (δ = 7.62–8.52 ppm) because of the proximity of nitrogen atoms, whereas H5 of the

Figure 3. Tautomeric equilibria of purines and pyrimidines.
pyrimidines occurs at a higher field ($\delta = 5.95-6.34$ ppm). The sugar moieties exhibit a multispin system that ranges from $\delta \sim 6$ ppm ($H_1'$) to $\delta \sim 4$ ppm ($H_5'$, $H_5''$). In addition, the spectra of 2'-deoxynucleotides exhibit the signal of the two hydrogen atoms in the 2'-position and appear as an ABMX system close to $\delta 2.5$ ppm.

Similarly to $^1$H and $^{13}$C, $^{31}$P has a spin quantum number of $1/2$, but in contrast, $^{31}$P is the only natural isotope of the phosphorus element. Although the nucleus has a rather low sensitivity (ca. 7% that of $^1$H), NMR measurements are easy to conduct. $^{31}$P NMR spectroscopy is highly valuable for studying the binding of phosphorus biomolecules to proteins and the structure and dynamics of phosphorylated proteins, as well as for the characterization of phosphorus compounds in metabolic studies and food science. This technique is also very useful for monitoring the course of reactions and characterizing reaction intermediates. The range of chemical shifts ($\delta$) for most organophosphorus compounds covers 700 ppm (from 500 to $-200$ ppm), using 85% $H_3PO_4$ as the external reference at $\delta 0.0$ ppm. For endogenous nucleotides and derivatives, however, the chemical shift values are limited to a narrow range, from 10 to $-30$ ppm, and exhibit high pH and counterion dependences. A set of representative nucleotide spectra are shown in Figure 5. In $^1$H-decoupled $^{31}$P NMR spectroscopy, nucleoside 5'-monophosphates are characterized by a singlet at approximately 4 ppm (Figure 5A), whereas cNMPs (Figure 5B) and NMP-sugars exhibit singlets at approximately $-2$ and $-5$ ppm, respectively. Nucleoside 5'-diphosphates feature a doublet of doublets with a coupling constant of $^{3}J_{\alpha\beta} \approx 20$ Hz (Figure 5C). Nucleoside 5'-triphosphates show three resonance signals at approximately $-10$ ($P_{\alpha}$ doublet, $^{3}J_{\alpha\beta} \approx 20$ Hz), $-11$ ($P_{\mu}$ doublet, $^{3}J_{\alpha\beta} \approx 20$ Hz), and $-22$ ppm ($P_{\rho}$ pseudo triplet) (Figure 5D). It is important to note that the P$_{\mu}$ groups of (d)NTPs can shift between $5$ and $10$ ppm, depending on the pH. Within pyrophosphate diesters, the $^{31}$P nuclei are affected by the protonation states of the phosphate groups and by the types and conformational positions of the substituents. In this regard, pyrophosphate diesters fall into two categories: those characterized by a singlet in the range from $-10$ to $-15$ ppm and those exhibiting two doublets with a $^{31}$P--$^{31}$P scalar coupling value ($^{3}J_{\alpha\beta}$) of $\sim 20$ Hz. The first category includes dinucleoside pyrophosphates, as well as the reduced cofactors NADPH and NADH (Figure 5E). For unsymmetrical pyrophosphate diesters, the singlet indicates that the $^{31}$P nuclei are in similar chemical environments because of the conformational symmetry around the pyrophosphate diesters and the absence of nearby substituents that have a strong influence on the chemical shift, such as aromatic rings or alkyne groups.

The second category encompasses compounds with non-identical phosphorus centers. Thus, if the difference in the $^{31}$P chemical shifts in hertz ($\Delta \nu$) is sufficiently high compared to the $^{3}J_{\alpha\beta}$ coupling constant ($\Delta \nu / J_{\alpha\beta} > 5$), the spectrum will show a characteristic first-order splitting of a doublet of doublets (two spin AX systems). The oxidized cofactors NADP$^+$ and NAD$^+$ (Figures 1 and 5F) and NDP-sugars (UDP-Glc, UDP-Gal, ADP-Glc, GDP-Man) fall into this category. The difference between the reduced and oxidized cofactors is the conjugation...
state of the nicotinamide ring. In the oxidized form, the aromatic conjugation is intact, and the ring current has a major impact on the chemical shifts of nearby nuclei. The orientation of the ring relative to the phosphorus nuclei also affects the direction and amplitude of the chemical shift. Within the reduced cofactor, the nicotinamide ring is not conjugated, and the $^{31}$P nuclei are, therefore, magnetically equivalent.

The electronic properties of nucleosides and nucleotides have been determined for more than 30 years. However, a recent study showed that nearly all of the previously published UV extinction coefficients for nucleotides are inaccurate, with errors of up to 7%. The revised electronic data for natural NMPs are reported in Table 2. Because the sugar and phosphate moieties have no significant UV absorption above 230 nm, nucleotides exhibit UV absorption profiles similar to those of their parent nucleosides with $\lambda_{\text{max}}$ values close to 260 nm and molar extinction coefficients of about 10 mM$^{-1}$ cm$^{-1}$. In addition to NMR and UV spectroscopies, mass spectrometry (MS) is a key tool for the analysis of nucleotides, either as part of nucleic acids or as individual compounds. Recently, exhaustive reviews were published on this subject.

Initially, high-performance liquid chromatography (HPLC) coupled with UV detection and based on ion-exchange (IE) chromatography was used to quantify nucleotides. In the past

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**Figure 5.** $^{31}$P NMR (162 MHz) spectra in D$_2$O of common nucleotides and derivatives as sodium salts.
15 years, liquid chromatography coupled with mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS) has emerged, offering improved selectivity and sensitivity. However, classical IE analytical conditions are often incompatible with mass spectrometric detection, as high concentrations of nonvolatile salts are used in mobile phases. To overcome this drawback, reverse-phase liquid chromatography (RPLC) was developed. In this case, a typical mobile phase consists of a mixture of water and methanol, or acetonitrile, combined with a volatile ion-pairing agent, such as trifluoroacetic acid or N,N-dimethylhexylamine. Alternatively, hydrophilic interaction chromatography (HILIC) has recently generated interest in the analysis of polar compounds, such as nucleotides.

This variant of normal-phase chromatography uses a hydrophilic stationary phase combined with a mixture of water (as the stronger eluting solvent) and methanol or acetonitrile as the eluent. Unlike RPLC, the HILIC gradient elution begins with a low-polarity organic solvent, and analytes are eluted by increasing the water content in the eluent.

### 2.3. Biological Applications

Given the importance of nucleotides and their conjugates in biological processes, considerable research effort has been directed toward developing synthetic routes to gain access to these compounds and structural analogues. Indeed, the structural modification of nucleotides significantly alters their chemical properties and, therefore, their biological activities. The scope of these compounds ranges from mechanistic probes and versatile chemical tools for assay development to therapies and diagnostics.

Nucleoside 5′-triphosphates are key compounds in genome analysis and medicinal diagnostics. Thus, the “dideoxy method” of DNA sequencing, developed by Sanger et al., is based on the DNA polymerase-dependent synthesis of a nucleic acid strand that is complementary to a nucleic acid template. This occurs in the presence of natural dNTPs and 2′,3′-dideoxynucleotides (ddNTPs), which act as chain terminators. The DNA elongation is randomly terminated whenever a ddNTP (ddATP, ddCTP, ddTTP, or ddGTP) is added to the growing oligonucleotide chain. The resulting products have varying lengths and can be separated by size using polyacrylamide gel electrophoresis. Originally, four different reactions were required per template, and each reaction involved a different ddNTP. However, advances in fluorescence detection have made it possible to combine the four terminators into a single reaction using a set of four chain-terminating dideoxynucleotides, each of which is labeled with a different fluorescent dye.

Over the past 10 years, the Sanger method has been partially superseded by several “next-generation” sequencing technologies that offer major increases in cost-effective sequence throughput. Modified (d)NTPs can also be involved in the systemic evolution of ligands by exponential enrichment (SELEX) and other related combinatorial methods of in vitro selection to generate modified catalytic nucleic acids and aptamers for various practical applications.

In addition, (d)NTPs can be applied to pharmacological studies of biologically active nucleoside analogues. Nucleoside analogues (NAs), for example, 3′-azido-2′,3′-dideoxythymidine (AZT, zidovudine), β-L-2′,3′-dideoxy-β-thiacytidine (3TC, lamivudine), and β-D-arabinofuranosylcytosine (araC, cytarabine), are a class of antiviral and anticancer drugs whose mechanism of action involves the interaction of their 5′-phosphorylated derivatives with essential enzymes, such as DNA or RNA polymerases.

Leading to the inhibition of viral replication. In contrast, araC is a cytotoxic drug that incorporates into DNA as its 5′-triphosphate derivative (araCTP), with subsequent DNA strand breaks. Thus, the availability of nucleotide analogues is crucial for biochemical and pharmacological studies.

Another example of the nucleotides’ biological activity is illustrated by di(adenosine) tetraphosphate (Up,U, diquafoxosan), an agonist of the P2Y2 purinergic receptor, which is used to treat dry eye disease (Figure 6). Dinucleotides also appear to be a promising new class of antiplatelet agents. Sugar nucleotides have been synthesized and studied as potential therapeutic candidates, and they may act as inhibitors/promoters of therapeutically significant enzymes. Finally, there is growing interest in nucleolipids as molecular carriers for drug delivery.

### 3. GENERAL CONSIDERATIONS

#### 3.1. Phosphorus

Phosphorus (13P), with the electronic configuration 1s22s22p63s23p3, is in the same group as nitrogen in the periodic table. The oxidation state of phosphorus can range from −3 to +5, but the latter is the only state present in cells. The nomenclature of organophosphorus compounds depends on both the oxidation state of phosphorus and the type of atoms (C, N, O, S) to which it is attached. The main phosphorus functional groups covered in this review are summarized in Table 4.

It is important to note that, in solution, tetracoordinated H-phosphonates are in equilibrium with tricoordinated phosphites (Figure 7). These tautomeric forms have different reactivities; therefore, the corresponding P(III) entities are versatile synthetic precursors in organophosphorus chemistry.

#### 3.2. Purification of Nucleotides

Isolating nucleotides and their analogues still presents a major challenge, because purification by chromatography of the polar...
compounds contaminated with various negatively charged species remains a limiting step. The multiple purification procedures associated with the derivatives’ relatively low chemical stability are time-consuming, require extreme care, and often produce low yields.

Indeed, the crude material obtained after solution synthesis is made up of a complex mixture of nucleotides with different numbers of phosphate groups, as well as byproducts (mostly inorganic phosphates) with similar characteristics. Generally, purification procedures require at least two types of chromatography. The first involves an ion-exchange support that is positively charged, such as diethylaminoethyl (DEAE) Sephadex, where the species are eluted according to the number of charges they have. The order for this elution is as follows: monophosphates, diphosphates, and triphosphates, followed by oligo- and polyphosphates. As the nucleotides often coelute with inorganic phosphates (e.g., phosphate [PO$_4^{3-}$], pyrophosphates [P$_2$O$_7^{2-}$], linear triphosphate [P$_3$O$_{10}^{2-}$], and cyclic trimetaphosphate [P$_3$O$_9^{3-}$]), a second purification step is performed involving reverse-phase chromatography, possibly using a semipreparative HPLC system. In addition to inorganic phosphates, an excess of triethylammonium cations can be observed, which can be attributed to the incomplete removal of triethylamine. During the reverse-phase chromatographic step and/or after the concentration of the combined fractions, these counterions can also be lost.

Both types of purification steps were reported by Murphy. The technical details for buffer preparation and a description of the ion-exchange supports are available. In a few cases, an additional step has been reported, prior to the separation of the various nucleotides by ion-exchange chromatography, to avoid the presence of multiple counterions in the crude mixture (i.e., (Bu)$_4$N$^+$, (Bu)$_3$NH$^+$, and Et$_3$NH$^+$ cations), which can disrupt the order of elution. These quaternary ammonium cations were replaced by NH$_4^+$ ions using simple percolation through a cation-exchange resin with pure water as the eluting solvent. Recently, Korhonen and co-workers reported the use of a new phosphorylating agent, called PPN pyrophosphate {tris[bis(triphenylphosphoranylidene)ammonium]pyrophosphate} for the synthesis of NTPs and NDPs. In this case, selective precipitation made it possible to eliminate the excess of pyrophosphate and PPN cations and allowed the nucleotides to be isolated directly as sodium salts.

In the particular case of the enzymatic synthesis of nucleoside 5′-triphosphates, Wu et al. developed a purification procedure based on boronate affinity gel. The ribonucleotides were isolated through the formation of a complex between the borate group and the cis-diol of the ribonucleotides. In contrast, for deoxyribonucleotide derivatives, the hydrolysis of excess ATP

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**Table 4. Nomenclature of Phosphorus Functional Groups Covered in This Review**

<table>
<thead>
<tr>
<th>P(III)</th>
<th>P(V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{P} - \text{O}$</td>
<td>$\text{P} - \text{P} - \text{O}$</td>
</tr>
<tr>
<td>phosphate</td>
<td>phosphorimidate</td>
</tr>
<tr>
<td>$\text{P} - \text{H}$</td>
<td>$\text{P} - \text{O} - \text{NR}_2$</td>
</tr>
<tr>
<td>H-phosphonate</td>
<td>phosphoramidate</td>
</tr>
</tbody>
</table>

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**Figure 7.** Tautomeric equilibrium between the H-phosphonate and phosphate forms.

<table>
<thead>
<tr>
<th>Functionalized solid-supports</th>
<th>Soluble-supports</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino Controlled Pore Glass (CPG) 80-120 μmol/g$^{119}$</td>
<td>Carboxypolystyrene resin 3.9 mmol/g$^{129}$</td>
</tr>
<tr>
<td>Bromoacetimidomethyl</td>
<td>Polystyrene 4-methoxystyryl chloride (PS-MMTr-Cl) resin 1.3 mmol/g$^{128}$</td>
</tr>
<tr>
<td>NovaGel resin 0.64 mmol/g$^{117}$</td>
<td>Polystyrene aminomethyl (PS-AM) resin 1.2 mmol/g$^{110-112}$</td>
</tr>
<tr>
<td>PEG$^{135-135}$</td>
<td>Polystyrene chloromethyl resin 1.34 mmol/g$^{117}$</td>
</tr>
<tr>
<td>NovaSyn Tentagel bromoresin 0.35 mmol/g$^{117}$</td>
<td>Polystyrene triphenylphosphine (PS-PP$_3$) resin 3 mmol/g$^{138}$</td>
</tr>
</tbody>
</table>

**Figure 8.** Main supports used for polyphosphorylated nucleoside synthesis and their loadings.

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Chem. Rev. 2016, 116, 7854−7897
(required during the enzymatic phosphorylation) was required. Finally, a last step of purification, using anion-exchange chromatography on Q sepharose FF, was required for all nucleotides.

3.3. Solid-Phase and Polymer-Assisted Nucleotide Synthesis

The different approaches for accessing nucleotides and related compounds involve classical solution synthesis, as well as supported solution synthesis. Supported organic chemistry emerged in the 1960s with the Merrifield method for peptide solid-phase synthesis.121 Anchoring reactants to a support enables reactions to be pushed to completion using an excess of a reagent. In addition, purification of the crude reaction mixture is simplified because the products are immobilized on the support, which means that they can easily be separated from the soluble byproducts and excess reagents by filtration. Alternatively, the anchored entities can also be reagents or catalysts. Supports are characterized by their loading capacity, which refers to the number of anchoring sites per gram of support (mmol/g). To improve the accessibility of the reagents and also to facilitate cleavage for product recovery, a linker is often anchored to the support before immobilization of the reactant. Solid-phase and polymer-assisted syntheses have been the subjects of numerous reviews in the literature.122−125 In this section, we provide a brief overview of the main supports currently used in nucleotide synthesis that involve either solid or soluble supports (Figure 8).

Insoluble resins (typically beads 50−200 μm in diameter) are commonly used as supports for the synthesis of oligonucleotides. Controlled pore glass (CPG) and polystyrene (PS) have proved to be the most suitable. CPG is a rigid nonswelling material that has deep pores where oligonucleotide synthesis takes place. Highly cross-linked PS beads have good moisture-exclusion properties and allow for very efficient oligonucleotide synthesis.

Despite the advantages offered by the solid-phase approach, the heterogeneous reaction conditions can cause other problems, such as nonlinear reaction kinetics, unequal distribution and/or access to the reaction sites, solvation problems, inefficient coupling rates, and difficulties with intermediate analysis.

Soluble polymer-supported synthesis, also termed liquid-phase synthesis, is an alternative method that has the advantages of solid-phase synthesis while working under homogeneous reaction conditions. The crude reaction mixture is usually worked-up by dilution with a solvent that causes the precipitation of the polymer-bound products, which are then isolated by filtration. The most widely used soluble polymers in liquid-phase synthesis are the two homopolymers poly(vinyl alcohol) (PVA) and poly(ethylene glycol) (PEG).122,123,125,139

Room-temperature ionic liquids (RTILs) are salts that are liquid at room temperature and have relatively low viscosities. These compounds have generated increased interest not only as solvents for organic synthesis but also as homogeneous supports thanks to their properties, such as nonflammability, nonvolatility, and greater thermal stability.140−143 They have recently been used for nucleotide supported synthesis.

4. NUCLEOSIDE 5′-MONOPHOSPHATES

Synthetic approaches for the synthesis of nucleoside 5′-monophosphates using both P(III) and P(V) chemistries have been extensively described, and the corresponding intermediates are shown in Figure 9.

4.1. Solution-Phase Synthesis

4.1.1. Synthesis via a Phosphorodichloridate Intermediate. This is the most widely used method for the synthesis of NMPs. In 1967, Yoshikawa and co-workers reported a one-step 5′-monophosphorylation of natural ribonucleosides using phosphoryl chloride in trimethylphosphate at 0−4 °C, followed
by hydrolysis (Scheme 1).144 Yields ranged from 88% to 98%. As shown in Scheme 1, the reaction’s regioselectivity can be explained by the formation of a nucleoside–trialkyl phosphate complex (1).143 Hydrolysis of the phosphorodichloridate intermediate 2 was initially performed with water, leading to the hydrolysis of excess phosphoryl chloride into phosphoric acid with the concomitant release of hydrogen chloride. To prevent the acid-catalyzed cleavage of the glycosidic bond, water was replaced by a TEAB buffer at pH ~7.5. Furthermore, the addition of molecular sieves or 1,8-bis(dimethylamino)naphthalene, alias Proton Sponge, proved to be effective for the synthesis of highly acid-sensitive nucleotides.116,134

In addition to numerous nucleoside analogues, this approach was also applied to the preparation of acyclic NMPs 3–5 and carbocyclic NMPs 6–8, as shown in Figure 10.146–149 The uracil derivatives were synthesized in 50–60% (3a,b) and 10–15% (5a–c) yields.148 Interestingly, the carbocyclic nucleosides (in which a fused cyclopropane moiety constrains the pseudo sugar conformation)146 Indeed, the adenine analogue in the southern (S, 2’-endo) conformation was easily monophosphorylated to produce 6 in 60% yield, whereas, for the derivative in the northern (N, 2’-exo) conformation, the cyclic 3’,5’-nucleotide was isolated as a major product. This was attributed to the close proximity between the 3’-OH and 5’-OH groups.

4.1.2. Synthesis via a Phosphoramidite Intermediate. Vanboom et al. reported that 2,2,2-tribromoethylophosphoromorpholinocloridate (9) was a convenient reagent for synthesizing ribonucleotides.130 The reaction of the base-modified analogues of adenosine 10a–c provided the corresponding phosphoramidilates 11a–c, followed by the in situ removal of the 2,2,2-tribromoethyl protecting group with the Cu–Zn couple. The subsequent acid hydrolysis gave AMP in 65–70% yields (Scheme 2).

4.1.3. Synthesis via a Phosphoester Intermediate. Scheit described the synthesis of TMP and its N-methyl derivative 12 by coupling the 2’,3’-protected nucleosides 13 and 14 with 2-cyanoethyl phosphate in the presence of N,N’-dicyclohexylcarbodiimide (DCC), followed by in situ removal of the cyanoethyl group (Scheme 3).152

More recently, another phosphorylating reagent, 2-O-(4,4’-dimethoxytrityl)ethylsulfonylthexan-2’-yl-phosphate (15), was developed by Taktakhivl and Nair for the phosphorylation of primary and secondary alcohols of deoxyribonucleosides 16–20 in the presence of trisopropylbenzene sulfonyl tetrazolide (TPS-TAZ) (Scheme 4).153 The phosphodiester intermediates 21–25 were deprotected in situ under basic conditions to afford dNMPs in 80–95% yields.

These approaches, involving a phosphodiester as an intermediate, have one drawback: protection is required for the nucleobase, as well as on the 2’- and 3’-hydroxyl groups of the sugar, prior to phosphorylation.

4.1.4. Synthesis via a Phosphotriester Intermediate. This type of synthesis, developed by Kimura et al., involves a Mitsunobu-type coupling between an unprotected nucleoside (thymidine or uridine) and dibenzyl phosphate in the presence of triphenylphosphine and diethylazodicarboxylate.154 The best results were obtained using hexamethylphosphoramide (HMPA) as a solvent (Scheme 5). Subsequent debenzylation of the resulting phosphotriester intermediates 26 and 27 by catalytic hydrogenation provided the corresponding NMPs in 73–78% yields.

Another approach for accessing a phosphotriester intermediate relies on P(III) chemistry and involves the coupling of a protected nucleoside with a phosphoramidite reagent, followed by the in situ oxidation of the resulting phosphate triester. As illustrated in Scheme 6, the uridine and adenosine analogues 28 and 29 reacted first with di-tert-butylxyl N,N-diethylphosphoramidite in the presence of 1H-tetrazole and were then oxidized...
with m-CPBA to produce the corresponding phosphotriesters 30 and 31.146 Finally, both tert-butyl and acetonide groups were removed simultaneously under acidic conditions (using a Dowex resin), and the desired monophosphates 32 and 33 were isolated in 68% and 54% yields, respectively.

The carbocyclic NMPs 34−38 shown in Figure 11 were obtained in three steps according to this synthetic methodology. In several cases, the deprotection step was achieved by treatment with a solution of trifluoroacetic acid (TFA; 5−10%) in dichloromethane.154−156 The 3′-OH function was protected prior to the synthesis of compounds 36 and 37 with a tetrahydropyranyl group, which was then removed together with the tert-butyl groups in the last step.155,156 The yields for this type of phosphotriester approach ranged from 45−69% for compounds 36 and 37 to 97% for compound 38.154

In the late 1990s, Chris Meier introduced a new concept for the intracellular delivery of antiviral nucleotides157−161 based on the use of salicylic alcohols to mask the phosphate group of NMPs. This led to neutral and lipophilic phosphotriesters called cycloSal pronucleotides. This type of phosphate protecting group
is cleaved according to a pH-driven chemical hydrolysis mechanism. Thus, the nucleophilic attack of water or hydroxide anion on the phosphotriester gives rise to a benzyl phosphodiester intermediate that is spontaneously converted into the NMP (Figure 12). By varying the nature of the substituents on the aromatic ring (X, Figure 12), the phosphorus atom’s electrophilicity and the reaction’s kinetics can be finely tuned. Accordingly, acceptor substituents, such as chloro and nitro groups, increase the reactivity of the cycloSal phosphate triesters compared to donor substituents, such as methyl groups.

In addition to their interest as original prodrugs, such phosphotriester derivatives have also been used for the synthesis of polyphosphorylated nucleosides and their conjugates. The coupling of the cycloSal phosphate moiety to the S’-hydroxyl group of a nucleoside is achieved using either P(III) or P(V) chemistry, as shown in Figure 13. The first approach is based on the coupling of a nucleoside with saligenylchlorophosphite, followed by in situ oxidation (Figure 13, path A). Oxone was found to be a better oxidizing agent than t-BuOOH or the mixture I2/pyridine/water for two reasons: (i) it allows the quantitative oxidation of the phosphite triesters into the phosphotriesters and (ii) inorganic salts of Oxone are easily washed out by liquid–liquid extraction.16 The second P(III) pathway involves the reaction of a nucleoside with a phosphoramidite and then the oxidation of the phosphite triester (Figure 13, path B). The third strategy requires the reaction of a nucleoside with a cyclosaligenylphosphorochloridate (Figure 13, path C), whereas the fourth approach involves the prior synthesis of nucleoside phosphorodichloridate, which is then treated with salicylic alcohol (Figure 13, path D). The synthetic approaches using P(III) intermediates are the most
common because P(III) species have greater reactivity than P(V) species.

For the synthesis of carbocyclic NMPs, only P(III) chemistry has been used. Accordingly, carbocyclic nucleosides 39 and 40 were first reacted with saligenylochlorophosphite 41 in the presence of N,N-diisopropylethylamine (DIPEA) to produce the corresponding cycloSal phosphite triesters, which were oxidized in situ. Derivatives 42 and 43 were obtained in 26−46% yields (Scheme 7). After hydrolysis under aqueous basic conditions, compounds 44 and 45 were isolated in 39% and 84% yields, respectively.163

4.1.5. Synthesis via a 5′-H-Phosphonate Intermediate.

H-phosphonate monoesters are relevant P(III) precursors that can be used as substrates for oxidative transformations to provide a variety of phosphate monoesters, such as NMPs.115 This approach provides an alternative for the monophosphorylation of acid-sensitive nucleosides, as shown by the synthesis of 3TC 5′-monophosphate (Scheme 8).164 Nucleoside 5′-H-phosphonate monoesters are easily prepared through the transesterification of diphenyl H-phosphonate with suitably protected nucleosides in pyridine and produce nucleoside phenyl H-phosphonate diesters intermediates.165 The addition of aqueous triethylamine to the reaction mixture results in the rapid hydrolysis of both the excess reagent and the nucleoside phenyl H-phosphonate diester. Accordingly, H-phosphonate monoester 46 was obtained in 95% yield (Scheme 8).

Because H-phosphonate monoesters are more resistant to oxidation than the corresponding H-phosphonate diesters, they need to be converted into trivalent silyl phosphites prior to oxidation. As shown in Scheme 8, the reaction of nucleoside H-
phosphonate monoester \(46\) with excess \(N,\text{O}-\text{bis}(\text{trimethylsilyl})\) acetamide (BSA) leads to the quantitative formation of the corresponding \(\text{bis}(\text{trimethylsilyl})\) phosphate \(47\). The subsequent addition of \((-)(8,8\text{-dichloroacamphorsulfonyl})\text{oxaziridine} (\text{DCSO})\) to the reaction mixture produced the \(\text{bis}(\text{trimethylsilyl})\) phosphotriester \(48\), which was finally hydrolyzed to eliminate the TMS groups (overall yield of 85% from \(46\)).

4.2. Polymer-Supported Synthesis

4.2.1. Synthesis Involving a Polymer-Bound Reagent.

Over the past decade, the Parang research group has developed various synthetic approaches for the regioselective phosphorylation of unprotected nucleosides and carbohydrates, involving \(\text{P(III)}\) chemistry and a polystyrene support. In their first report,\(^{129}\) the immobilized phosphitylating reagent \(49\), prepared by coupling polystyrene-bound \(p\)-hydroxybenzyl alcohol with the commercially available \(2\text{-cyanoethyl}\) \(N,N\text{-bis} propylchlorophosphoramidite, was reacted with uridine in the presence of 1\(H\)-tetrakizole to produce the corresponding polymer-bound phosphate triester \(50\) (Scheme 9). In situ oxidation with \(t\)-BuOOH gave the polymer-bound phosphotriester, which was treated with \(1,8\text{-diazabicyclo}[5.4.0]undec-7\)-ene (DBU) to remove the \(2\text{-cyanoethyl}\) (CNE) protecting group. Finally, basic cleavage of the \(p\)-hydroxybenzyl linker of \(51\) afforded UMP in 67% overall yield.

Because of the cleavage mechanism, the linker was released along with the phosphorylated products, leading to contamination. Extra purification steps were required as a result. To overcome this limitation, two new linkers, involving an amide (\(52\)) or an \(N\)-Boc aminomethyl (\(53\)) bond, were proposed and coupled with \(2\text{-cyanoethyl} N,N\text{-bis} propylchlorophosphoramidite to produce the corresponding polymer-bound phosphitylating reagents \(54\) and \(55\) (Figure 14).\(^{131}\)

This modification involves an additional step (acidic treatment leading to the cleavage of the \(p\)-acetoxybenzyl alcohol linker, Scheme 10) that was not included in the previous reaction sequence (coupling, oxidation, and removal of the CNE group, Scheme 9). It is important to note that, after the final cleavage, the solid-phase resins can be reused for generating the solid-phase phosphitylating reagents. Using this methodology, UMP, dTMP, and AMP were synthesized in overall yields of 53–67%.

The Parang research group also reported an alternative method using two types of polymer-bound oxathiophospholanes, \(56\) and \(57\) (Scheme 11), which were prepared from NovaSyn Tentagel bromo resin and bromoacetamidomethyl NovaGel resin.\(^{156}\) Coupling of \(A\), \(U\), and \(d\)T with \(56\) and \(57\) gave \(\text{P(III)}\) derivatives \(58\) and \(59\), which were further oxidized to produce \(60\) and \(61\), respectively. Cleavage of these cyclic P(V) intermediates with 3-hydroxypropionitrile, followed by treatment with DBU, resulted in the corresponding nucleoside \(5\)'-monophosphates through the elimination of the polymer-bound ethylene episulfide. Accordingly, UMP, dTMP, and AMP were obtained in 67–78% yields from the polymer-bound linkers. This approach was expanded to the synthesis of nucleoside \(5\)'-monophosphorothioates by using 3\(H\)-1,2-benzodithiol-3-one-1,1-dioxide (Beaucage reagent) during the oxidation step.

4.2.2. Synthesis Involving Attachment of the Sugar Moiety to the Support. In 2010, Olivierio et al. developed this approach to prepare \(5\)-amino-1-\(\beta\)-D-ribofuranosylimidazole-4-carboxamide (AICAR) \(5\)'-monophosphate, a biosynthetic precursor of purine nucleotides, and its 4-\(N\)-alkyl derivatives.\(^ {128}\) The solution-phase synthesis of these compounds is difficult to achieve; therefore, the authors set up a high-yielding supported synthesis. This involved the connection of inosine to the solid support through the formation of an acetal bond with the hydroxyl groups in the \(2',3\text{'-positions} (Scheme 12). Because of the nature of anchoring, this approach is restricted to the phosphorylation of ribonucleosides.

The supported nucleoside \(62\) was obtained in three steps: treatment of the \(5\)'-protected inosine \(63\) with 4-(hydroxymethyl)benzaldehyde dimethyl acetal in the presence of catalytic amounts of \(p\)-TsOH, followed by coupling of the resulting intermediate with the polystyrene MMT\(\text{r}Cl\) resin and, finally, removal of the tert-butyldiphenylsilyl (TBDPs) protecting group with ammonium fluoride. Therefore, inosine was bound to the trityl resin through a linker bearing two acid-labile groups, namely, a benzyl tritylether and a \(2',3\text{'-acetal that could be selectively cleaved in anhydrous and aqueous acidic conditions, respectively.

Synthesis of the phosphotriester precursors \(64a\) and \(64b\) was achieved upon treatment of \(62\) with \(\text{bis} (\text{cyanoethyl})\)- or \(\text{bis} (\text{trimethylsilyl}ethyl)\)-\(N,N\text{-bis} propylchlorophosphoramidites in the presence of 1\(H\)-tetrakizole, followed by an oxidation step with \(t\)-BuOOH. Finally, IMP was released from \(64a\) using acidic treatment followed by washing with water. Recovery from \(64b\) required an extra step to eliminate the cyanoethyl protection under basic conditions. In addition to the synthesis of IMP, triesters \(64a\) and \(64b\) also served as starting materials for the selective modifications of the nucleobase in order to form 4-\(N\)-alkyl derivatives of AICAR \(5\)'-monophosphate.

4.2.3. Synthesis Involving Attachment of the Base Moiety to the Support. In 2009, our group first reported a soluble-supported synthesis of cytosine-containing nucleotides.\(^ {133}\) This synthetic strategy was based on the use of a PEG anchored to the nucleobase moiety through a base labile linker (Scheme 13).

Thus, the succinyl linker was introduced onto the soluble support by reacting commercially available \(\text{PEG}_{4000}(\text{OH})_2\) with succinic anhydride in the presence of a catalytic amount of 4-dimethylaminopyridine (DMAP) in pyridine (86% yield).
resulting PEG-(O-succinate), \(65\) was subsequently coupled with the exocyclic amino group of the free nucleosides \(\text{C}, \text{dC}, \text{araC},\) and \(\text{ddC}\) in the presence of the coupling agents \(1\)-hydroxybenzotriazole (HOBt) and DCC to produce \(66-69\). Interestingly, the reactions were easy to monitor with \(^1H\) and \(^{13}C\) NMR spectroscopies. Conversions were quantitative, and yields were \(\sim 85\%\). With one nucleoside at each end of the polymeric chain, the capacity of the synthesized PEG nucleoside was evaluated at 0.5 mmol·g\(^{-1}\).

Finally, regioselective 5′-O-phosphorylation of the nucleosides was achieved using P(V) chemistry according to Yoshikawa’s method, albeit at 35–40 °C,\(^{133,135}\) to produce the compounds \(70-72\). P(III) chemistry was used for the acid-sensitive nucleoside \(\text{ddC}\).\(^{134}\) The supported \(H\)-phosphonate monoester \(73\) was converted to \(74\) by silylation with BSA and final oxidation with \(t\)-BuOOH. The remaining salts and excess reagents were removed by a simple and easy liquid–liquid extraction process. Finally, NMPs were isolated in 60–92% yields after precipitation of the supported nucleotides in cold diethyl ether and then cleavage with concentrated ammonia. Compared to classical solution procedures, this PEG-supported methodology proved to be more efficient and scalable, as well as easier to handle.

### 4.3. Enzymatic Synthesis

In cells, NMPs are synthesized from their nucleoside counterparts by nucleoside kinases, which usually requires ATP as a phosphate donor.\(^{110}\) A few enzymatic procedures have been...
reported for the preparation of natural and modified NMPs starting from the corresponding nucleoside precursors. Although the main advantage of using enzymes (especially kinases) is the regioslectivity of the reaction toward the 5'-OH group, they are somehow selective for their natural substrates. Consequently, this approach might not be applicable to all structural substrate analogues. Therefore, microbial and plant nucleoside phosphotransferases (EC 2.7.1.77; NPase) are preferentially used as biocatalysts for the 5'-monophosphorylation of nucleoside analogues, because of their broader specificity with respect to both the phosphate donors and acceptors.

Barai et al. described the synthesis of (β-D-arabinofuranosyl) nucleoside 5'-monophosphates using whole cells of Erwinia herbicola. This method uses the shift in the equilibrium state of the reaction that occurs during the formation of the desired product when it is precipitated by Zn\(^{2+}\). Under optimal conditions, the conversion of nucleosides into NMP reached 41–91%.

More recently, Cotticelli and Salvetti patented a procedure for the preparation of natural and modified NMPs using either free or immobilized purified enzymes or enzymes contained in cell pastes or cells.

5. NUCLEOSIDE 5'-DIPHOSPHATES

Synthetic approaches for the synthesis of nucleoside 5'-diphosphates involve either P(III) and/or P(V) chemistry. The related intermediates are highlighted in Figure 15. The availability of dry and organic-solvent soluble reagents (i.e., phosphate or pyrophosphate salts) is often a critical factor for successful reactions. To increase both their nucleophilicity and solubility in organic solvents, the corresponding tri- or tetraalkylammonium salts are often preferred over the commercially available sodium salts.

5.1. Solution-Phase Synthesis

5.1.1. Synthesis via a Phosphorodichloridate Intermediate. This one-pot, three-step approach starts with the synthesis of an unprotected nucleoside phosphorodichloridate according to Yoshikawa’s procedure for NMPs (Scheme 1, section 4.1.1), followed by the addition of tetra-n-butyl ammonium phosphate for a short period of time and then hydrolysis with TEAB (Scheme 14). A large panel of diphosphate derivatives of natural and nucleoside analogues have been obtained using this synthetic pathway.

Whereas the formation of a small amount of nucleoside triphosphates, as byproducts, has been reported in some cases, Mishra and Broom were able to obtain NTPs as major products when using excess POCl\(_3\) and tri-n-butyl ammonium phosphate.

5.1.2. Synthesis via a Phosphoramidate Intermediate. This approach is based on the nucleophilic substitution of an NMP, activated as the phosphoramidate monoester by inorganic phosphate salts. It is important to note that nucleoside phosphorothioimidazolates are more reactive than the corresponding phosphoromorpholidates and more permissive when it comes to the choice of solvent.

Since the pioneering work of several groups more than 40 years ago, this approach has become the main strategy for the synthesis of NDPs from NMPs. Investigation of the first step, which involves activation of NMPs with CDI, by \(^{31}\)P NMR spectroscopy revealed carbonation of ribonucleotides during CDI treatment (Scheme 15). Removal of the 2',3'-carbonate protecting group of 7S was easily performed under basic conditions. Finally, the last step involves the is in situ treatment of phosphorimidazolate intermediates with tetra(-n-butyl)-ammonium phosphate or tri(-n-butyl)ammonium phosphate in dimethylformamide (DMF) to produce the diphosphate derivatives.

This method has been applied to the synthesis of a large number of NDP analogues, such as base-modified NDPs, as well as the carbocyclic 76–78 and acyclic 79a–e derivatives shown in Figure 16.

A similar strategy was reported by Kore and Parmar, involving the coupling of the triethylammonium salts of ribonucleoside 5'-diphosphates and imidazole in the presence of triphenylphosphine and 2,2'-dithiodipyridine as activating agents (Scheme 16). The resulting 5'-phosphorimidazolides 80–83 were isolated by precipitation with sodium perchlorate in acetone and filtration. Their treatment with tri(-n-butyl)-ammonium phosphate in DMF in the presence of zinc chloride produced the diphosphates in high yields (95–98%).

In 2013, Sun and co-workers described another multistep approach for the synthesis of NDPs and NTPs that involved protected nucleoside 5'-phosphoropiperidates and was promoted by 4,5-dicyanoimidazole (DCI). First, phosphitylation of the carboxybenzyl-protected nucleosides 84 and 85 with benzyl N,N-disopropylchlorophosphoramidite 86 was per-
formed in the presence of 1H-tetrazole, and then oxidative coupling with CCl₄/Et₃N/piperidine produced the phosphoropiperidates 87 and 88 in 75–85% yields over three steps (Scheme 17). Then, both carboxybenzyl (Cbz) and benzyl (Bn) esters of the nucleosidic and phosphoramidate moieties were simultaneously and quantitatively cleaved by mild catalytic hydrogenation. After filtration of the reaction mixture, treatment with excess DICI and bis(tetra-n-butylammonium) hydrogen phosphate made it possible to isolate the corresponding NDPs in 68–79% yields. This strategy was applied to the acyclic antiviral drug acyclovir [9-(2-hydroxyethoxymethyl)guanine, 79a in Figure 16] and enabled the formation of the corresponding diphosphate derivative in 56% overall yield.¹⁷⁸

5.1.3. Synthesis via a Tosylate Intermediate. The Poulter research group reported a diphosphorylation procedure based on the nucleophilic substitution (SN₂) of 5′-tosyl nucleosides 89–93 by tris(tetra-n-butylammonium) pyrophosphate in acetonitrile (Scheme 18).¹⁷⁹ This strategy requires the prior multistep synthesis of 5′-tosyl nucleosides (introduction of N₅-O-protecting groups, tosylation, and removal of the protecting groups in 52–83% overall yields). Yields for the phosphorylation step ranged from 43% to 83%. This approach has been expanded to the synthesis of isosteric methylene-bridged diphosphonate triester precursors.⁸²,¹⁶¹ Because of the high reactivity of phosphotriester reagents, such as chlorophosphites, prior protection of the functional groups of the starting nucleoside (except 5′-OH) is required to avoid side reactions. For example, the synthesis of dTDP is presented in Scheme 20. The 5′-nitrosaligenyl chlorophosphite 106 was first prepared in 70–80% yields by reacting 5-nitrosalicyl alcohol with PCl₃ in pyridine/Et₂O at −20 °C. Coupling to 3′-O-acetyl-2′-deoxythymidine 17 was then performed, and in situ oxidation of the resulting phosphate triester 107 with Ozone produced the phosphotriester 108 in high levels of purity and at reasonable levels of mass recovery without chromatographic purifications through the addition of further volumes of sodium iodide in acetone at 0 °C. Yields for the phosphorylation step ranged from 43% to 83%.

5.1.4. Synthesis via a Mixed P(III)–P(V) Intermediate. Recently, the Jessen research group reported an iterative strategy to access NMPs, NDPs, and NTPs starting from the unprotected nucleosides.²⁷,¹⁸⁰ Its application to the synthesis of natural NDPs is presented in Scheme 19. First, NMPs in the form of their tetra-N-butyl or tris-N-hexyl ammonium salts were regioselectively phosphorylated with the bulky phosphoramidite reagent (iPr)₂NP(OFm)₂ ¹⁸¹ (95) in the presence of various activating agents to selectively produce the mixed P(III)–P(V) anhydrides 96–100. Oxidation to 101–105 and then cleavage of the fluorenlylmethyl protecting groups led to NDPs as piperidium salts in 75–93% yields starting from the NMPs. Ambient temperature and nondry conditions (reagents and solvents) are essential if coupling is to occur selectively because traces of water hydrolyze the excess phosphoramidite reagent and, therefore, prevent side phosphitylation reactions with the sugar’s free hydroxyl groups. In addition to high yields, short reaction times, and simple procedures, product purification is achieved through precipitation upon addition of Et₂O.

5.1.5. Synthesis via a Phosphotriester Intermediate. As with the synthesis of NMPs, NDPs can be obtained from cycloSal phosphotriester precursors.²⁸,¹⁶¹ Because of the high reactivity of P(III) reagents, such as chlorophosphites, prior protection of the functional groups of the starting nucleoside (except 5′-OH) is required to avoid side reactions. For example, the synthesis of dTDP is presented in Scheme 20. The 5′-nitrosaligenyl chlorophosphite 106 was first prepared in 70–80% yields by reacting 5-nitrosalicyl alcohol with PCl₃ in pyridine/Et₂O at −20 °C. Coupling to 3′-O-acetyl-2′-deoxythymidine 17 was then performed, and in situ oxidation of the resulting phosphate triester 107 with Ozone produced the phosphotriester 108 in high levels of purity and at reasonable levels of mass recovery without chromatographic purifications through the addition of further volumes of sodium iodide in acetone at 0 °C.
91% yield over the two steps. Treatment of the latter with bis(tetra-n-butylammonium)phosphate in DMF, followed by removal of the acetyl protection, produced dTDP in 83% yield starting from the protected nucleoside 17.

5.2. Polymer-Supported Synthesis

5.2.1. Synthesis Involving a Polymer-Bound Reagent.
In 2005, Ahmadibeni and Parang generated nucleotides in good yields and purity by using polymer-bound phosphitylating reagents. For (d)NDP synthesis (Scheme 21, \( n = 1 \)), the polystyrene support 52 or 53 (Figure 14, section 4.2.1.) was treated with the diphosphitylating reagent 109a in the presence of 1H-tetrazole to give the polymer-bound diphosphorylating reagents 110a. It is important to note that 109a was obtained in four steps from phosphorus trichloride (97% overall yield) and should be stored at \(-20^\circ C\) (stable for at least 2 weeks under these conditions). Then, unprotected nucleosides were reacted with the polymer-bound reagent 110a in the presence of 1H-tetrazole to yield 111a, 112a, and 113a. Oxidation with t-BuOOH, followed by removal of the cyanoethyl group with DBU, produced the corresponding polymer-bound NDPs, which were released from the support under acidic conditions and then isolated in 64–80% overall yields.

5.2.2. Synthesis Involving Attachment of the Sugar Moiety to the Support.
In 2011, Meier adapted the cycloSal strategy to solid-support syntheses. The general procedure for the synthesis of natural 2′-deoxyribonucleotides consists of three steps, as shown in Scheme 22. The 5′-phosphotriester derivatives, including the succinyl linker attached to the 3′-hydroxyl of the nucleosides, have to be prepared using classical solution synthesis before anchoring. Thus, synthesis of 3′-O-succinyl-nucleotides was performed by reacting succinic acid with the phosphate ester of the nucleoside and the succinyl linker. The resulting 3′-O-succinyl-nucleotides were then anchored to the polystyrene support using a phosphotriester linker. Oxidation of the 5′-phosphate group with t-BuOOH, followed by removal of the cyanoethyl group with DBU, produced the corresponding polymer-bound NDPs, which were released from the support under acidic conditions and then isolated in 64–80% overall yields.
anhydride with the 5′-dimethoxytrityl- (5′-O-DMTr-) protected nucleosides 114–116 and then removing the 5′-O-DMTr group with TFA. The 3′-O-succinyl-nucleosides 117–119 were then converted to cycloSal phosphotriesters 120–122 by phosphitylation with saligenylchlorophosphites 123 or 124, followed by oxidation as previously stated (Figure 13).

Phosphotriester derivatives were finally anchored to the support using classical coupling conditions [with 1-hydroxybenzotriazole (HOBt) or O-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium tetrafluoroborate (TBTU) serving as the coupling agent]. Treatment of 125–127 with excess bis(tetra-n-butylammonium)phosphate followed by cleavage under basic conditions produced the desired (d)NDPs in purities ranging from 44% to 90%. The purity of dCDP was unexpectedly low compared to those of the other (d)NDP because of the formation of a dCDP conjugate. Finally, the desired compounds required further purification using reverse-phase column chromatography (with C18 silica gel).

This strategy was also applied to the synthesis of a single ribonucleotide, UDP, starting from 2′- or 3′-acetylated uridines. After several steps of synthesis in solution (DMTr protection, coupling of succinyl linker, deprotection of 5′-OH, and coupling with the chlorophosphanes), the 2′/3′-O-succinyl-5′-cycloSal-nucleotide was grafted to the support and treated with bis(tetra-n-butylammonium)phosphate and then cleaved under basic conditions to produce UDP.

In 2014, the Jessen research group applied the iterative strategy shown previously in Scheme 19 (section 5.1.4) to the supported synthesis of NDPs using CPG as the solid support. For this purpose, commercially available dG 3′-succinyl-anchored to CPG was first phosphitylated with bis-(fluorenymethyl)disopropylphosphoramidite 95, and after two steps (oxidation with m-CPBA and treatment with 5% piperidine), the CPG-bound dGMP was obtained. One iteration followed by cleavage of the products from CPG under basic conditions produced reasonably pure dGDP.
5.2.3. Synthesis Involving Attachment of the Base Moiety to the Support. This synthesis pathway is an extension of the soluble-supported synthesis of cytosine-NMPs as shown in Scheme 13 (section 4.2.3). Accordingly, the supported nucleoside 5′-monophosphates 71, 72, and 74 were first activated by CDI, and subsequent condensation of the 5′-phosphorimidazolyl intermediates 128–130 with inorganic phosphate was carried out (Scheme 23). PEG-NDPs 131–133 were isolated after purification on reverse-phase RP-C18 column to eliminate residual phosphorus salts. Treatment of the supported diphosphates with concentrated aqueous ammonia, purification by reverse-phase column chromatography, and dialysis yielded the desired NDPs in moderate to good yields (51−96%).

6. NUCLEOSIDE 5′-TRIPHOSPHATES

Because of their crucial role in biological systems, synthetic access to nucleoside 5′-triphosphates has received considerable attention. In 2000, Burgess and Cook published a comprehensive review on (d)NTP synthesis. Since then, several reviews and chapters in books have focused on the subject. However, these reports only partially cover solid support synthesis, and in recent years, a number of relevant synthetic
approaches have also emerged. Similarly to NDPs, synthesis of NTPs relies on P(III) and/or P(V) intermediates (Figure 18) and involves the nucleoside or nucleoside 5′-monophosphates as the starting material. In all cases, anhydrous conditions are essential to avoid the generation of byproducts formed by side reactions with water.

A substantial number of acyclic and carbocyclic nucleoside 5′-triphosphates have been synthesized (134–151 in Figure 19) using either phosphorodichloridate, phosphoramidate, or mixed P(III)–P(V) intermediates. Some examples are reported in the following sections.
6.1. Solution-Phase Synthesis

6.1.1. Synthesis via a Phosphorodichloridate Intermediate. This one-pot, three-step synthesis was initially developed by Ludwig to synthesize ATP and dATP (Scheme 24).184 Unprotected nucleosides were first reacted with phosphoryl chloride in trimethylphosphate according to Yoshikawa’s procedure144 to produce phosphorodichloridate intermediates. Then, treatment with tributylamine and a 5-fold excess of bis(tri-n-butylammonium) pyrophosphate in dry DMF for 1 min led to the cyclic entity 152, which was hydrolyzed with TEAB (pH 7.5) to produce the nucleoside triphosphates in 78–86% yields. To date, this approach has been applied to numerous protected and unprotected nucleosides and their analogues. The corresponding yields of NTPs ranged from poor to high because this reaction is highly dependent on the nature of the substrate. Interestingly, this approach can also be adapted for the synthesis of nucleotide analogues, including modifications on the phosphate moieties. For example, nucleoside \( S′-O-(\alpha\text{-thio}) \)-triphosphate derivatives can be obtained by simply replacing POCI\(_3\) with thio phosphoryl chloride (PSCl\(_3\)).188,189 Nucleoside \((\beta\gamma\text{-imido})\) or \((\beta\gamma\text{-dichloromethylene})\) triphosphates were generated by using imidodiphosphate or dichloromethylenediphosphonate, respectively, instead of pyrophosphate.116,190

As previously stated in section 5.1.1, the use of a large excess of POCI\(_3\) and tri-n-butylammonium phosphate results in the formation of ATP as a major product, instead of ADP.169 In this case, the proposed mechanism involves the formation of a pseudo-ATP intermediate (153, Figure 20), which rearranges itself to form the cyclic triphosphate 152a.

![Image](20.png)

Figure 20. Pseudo-ATP intermediate.169

In 2006, El-Tayeb and co-workers focused on the byproducts formed during the synthesis of base-modified UTP derivatives. They were able to isolate and identify NMP and NDP, as well as 2′,3′-cyclic phosphate and 2′-phosphate NTPs.116 More recently, a detailed analysis was performed by Gillerman and Fischer within the framework of ATP synthesis.191 The various byproducts, identified by \(^{1}H\)-decoupled \(^{31}P\) NMR spectroscopy,

are shown in Figure 21. The spectra of NMP and nucleotides 154–156 show a single signal between 3 and \(-5\) ppm, whereas the spectra of cyclic trimetaphosphate 157 feature a singlet at \(-20\) ppm. The polyphosphorylated compound 158 shows three signals that are characteristic of the 5′-phosphate moiety (\(-10\) ppm for P\(_{n}\), \(-11\) ppm for P\(_{m}\), and \(-23\) ppm for P\(_{b}\)) and a fourth signal at \(+20\) ppm corresponding to the 2′,3′-cyclic phosphate. The major byproduct (ca. 60% yield) was identified as AMP and resulted from the hydrolysis of the nucleoside 5′-phosphorodichloridate intermediate with residual water. In addition, the reaction of DMF with POCI\(_3\) is known to produce the Vilsmeier reagent \([(CH\(_3\))\(_2\)NCHCIP\(_2\)Cl\(_2\)]\), which can further react with pyrophosphate or with the 2′- and 3′-OH groups of 152a to produce byproducts 157 and 158, respectively.

Despite its poor to moderate yields, the Ludwig procedure is one of the most widely used methods for NTP synthesis because of its simplicity (use of unprotected nucleosides, one-pot reaction, short reaction times) and its applicability to a wide range of nucleoside analogues modified either on the base moiety,204,206,207 and/or on the sugar moiety.202,204 Carbocyclic 134 and acyclic 135 and 136 derivatives (Figure 19) have been synthesized in 25–35% yields according to this procedure.

Significant improvements have been made to the Ludwig method since it was first reported in 1987. These include addition of Proton Sponge during the first step of the reaction sequence, even for non-acid-sensitive nucleosides, to increase the rate of phosphorylation,206 and working at a lower temperature while extending the reaction time.191 Accordingly, the ATP/AMP ratio was subsequently improved when the reaction temperature was reduced from 0 to \(-15\) °C and the reaction time was extended to 2 h for the second step. Under these last conditions, neither nucleotide 158 nor cyclic trimetaphosphate 157 were observed. Similar results were obtained for UTP and GTP syntheses, whereas CTP synthesis required prior protection of the exocyclic amine. Thus, natural NTPs were isolated in 54–74% yields.

Another improved procedure for solution-phase synthesis of (d)NTPs through a phosphorodichloridate intermediate was reported by Kore et al.207,208 Initially developed for the synthesis of dNTPs,207 this protection-free and gram-scale procedure involves the use of (1) trimethylphosphate as the solvent for the phosphorodichloridate formation; (2) acetonitrile as the solvent in the second step of the reaction, instead of DMF, to prevent the formation of the Vilsmeier–Haack complex; and (3) sub-stoichiometric amounts of bis(tri-n-butylammonium) pyrophosphate. Both steps were performed at \(-5\) °C. Under these
optimized conditions, the five natural dNTPs were prepared in 65–70% yields. This protocol was successfully exemplified on base-modified dNTPs. \(^\text{209–212}\) It is important to note that the monophosphorylation reaction of ribonucleosides does not require a base in the reaction medium, unlike the successful monophosphorylation reaction of a deoxyribonucleoside, which requires a carefully chosen base. \(^\text{213}\)

6.1.2. Synthesis via a Phosphoramidate Intermediate. Similarly to NDPs (section 5.1.2), NTPs can be synthesized through the nucleophilic substitution of nucleoside 5′-phosphoramidates using inorganic pyrophosphate. Two options should be considered for the phosphoramidate approach: (1) the synthesis of a nucleoside phosphoromorpholidate intermediate by coupling NMP with morpholine in the presence of DCC, followed by nucleophilic substitution with tributylammonium pyrophosphate \(^\text{171,214}\) (for example, synthesis of the carbocyclic \(^\text{143}\)); \(^\text{215}\) and (2) the use of reagent 9, mentioned previously (Scheme 2, section 4.1.2.), followed by the removal of the 2,2,2-tribromoethyl protecting group and then treatment with tributylammonium pyrophosphate in DMF (81–85% yields). \(^\text{150}\)

Another method introduced by Hoard and Ott relies on a phosphorimidazolate intermediate that is formed by reacting NMP with CDI in DMF. \(^\text{216}\) Under these conditions and when using ribonucleoside analogues, the cyclic carbonate function introduced onto the 2′,3′-hydroxyl groups can be removed by a final treatment with triethylamine in aqueous methanol (Scheme 15, section 5.1.2.). This approach has also been applied to the synthesis of various carbocyclic derivatives \(^\text{137–141 and 144–147,146,149,154,175}\). As illustrated in Scheme 25, the triphosphate 142 was isolated in 61% yield starting from 159. \(^\text{154}\)

As for the protocol detailed in Scheme 16 (see section 5.1.2.), nucleoside phosphorimidazolates could be prepared by coupling NMPs with N-methylimidazole to produce the corresponding phosphorimidazolate intermediates compared to the phosphorodichloridate approach. \(^\text{203}\)

Bogachev developed a one-pot, three-step procedure using trifluoroacetic anhydride (TFAA) as the activating agent to convert dNMPs to dNTPs. \(^\text{217}\) TFAA is highly reactive toward the acylation of phosphate, hydroxyl, and amino groups but is easily eliminated from the reaction mixtures. Thus, the in situ activation and protection of dNMPs with a large excess of TFAA in the presence of a tertiary amine in acetonitrile were performed, and the remaining excess TFAA was removed in a vacuum. This step is highly sensitive to moisture. The resulting mixed anhydrides were treated using N-methylimidazole to produce the corresponding phosphorimidazolaminyl carbonate intermediates. Substitution with pyrophosphate and removal of the trifluoroacetyl protecting groups afforded dATP, dTTP, dGTP, and dCTP in 89–92% yields. It is important to note that the reaction time did not exceed 2–5 min for any given step.

In 2004, the Borch research group reported an original strategy involving the in situ generation of pyrrolidinium phosphoramidate species as shown in Scheme 26. \(^\text{218}\) The starting materials phosphoramidate diesters \(^\text{160–163}\) were obtained beforehand using a one-pot reaction. Natural and modified pyrimidine-containing nucleotides were synthesized using this one-pot, two-step procedure. Initially, this involved the catalytic hydrolysis of the phosphoramidate diesters \(^\text{160–163}\) and the spontaneous rearrangement [intramolecular cyclization of the N-(3-chlorobutyl), N-methyl moiety] of the phosphoramidate monoesters \(^\text{164–167}\), leading to the formation of the pyrrolidinium phosphoramidate intermediates \(^\text{168–171}\). Then, treatment with tris(tetra-n-butylammonium) hydrogen pyrophosphate produced dTTP, dUTP, dGTP, and some analogues
in yields ranging from 55% to 77%. The advantages of this method include short reaction times and easy purification steps. However, additional steps of synthesis were required to prepare the starting materials.

In 2008, Sun and co-workers reported a one-pot procedure to prepare NTPs from nucleoside 5′-H-phosphonate monoesters through pyridinium phosphoramidate intermediates (Scheme 27).219 Nucleoside H-phosphonates 172−175 were obtained in three steps and in 71−85% yields by treating the 2′,3′-isopropylidene nucleosides 176−179 with either salicylchlorophosphite 180 or phosphorus trichloride, followed by hydrolysis with aqueous triethylamine and removal of the protecting group.219,220 Silylation of the H-phosphonate monoesters with TMSCl in pyridine, followed by oxidation with iodine and substitution of the resulting pyridinium phosphoramidates with pyrophosphate, produced NTPs in 26−53% yields starting from 172−175.

An alternative approach, mentioned previously, involves nucleoside S′-phosphoropiperidate intermediates (Scheme 17, section 5.1.2). It was adapted for the synthesis of NTP by replacing bis(tetra-n-butylammonium) hydrogen phosphate with tris(tetra-n-butylammonium) hydrogen pyrophosphate in the last step of the reaction sequence.177,221 Silylation of the H-phosphonate monoesters with TMSCl in pyridine, followed by oxidation with iodine and substitution of the resulting pyridinium phosphoramidates with pyrophosphate, produced NTPs in 26−53% yields starting from 172−175.

6.1.3. Synthesis via a Mixed P(III)−P(V) Intermediate. In 1989, Ludwig and Eckstein described a one-pot synthetic approach to synthesize dNDPs, based on the phosphitylation of protected nucleosides with salicylchlorophosphite.222 Anhydrous conditions are essential to prevent the formation of nucleoside H-phosphonates. A detailed description of this method, including crucial experimental details, was reported in a recent video article.107 Scheme 28 illustrates this methodology for dTTP synthesis.

The reaction of 3′-O-acetylthymidine 17 with 2-chloro-1,3,2-benzodioxaphosphorin-4-one 180 in pyridine/dioxane produced a first intermediate 181 as two diastereoisomers, characterized by 31P NMR spectroscopy with two signals at 127.6 and 126.3 ppm. Treatment with bis(tri-n-butylammonium) pyrophosphate produced the corresponding cyclic intermediate 182, which shows two quartets at about −19 ppm for P(V), along with a triplet centered at 105.5 ppm for P(III). Oxidation of the trivalent phosphorus with an aqueous pyridine solution of iodine led to Pα oxidation and concomitant ring opening. Removal of the acetate protecting group using ammonia generated dTTP in 72% yield.222 This methodology can be applied to ribonucleosides, but both 2′- and 3′-hydroxyl functions require protection. In addition, for adenine, guanine, and cytosine nucleobases, the exocyclic amino groups must be protected to prevent any side reactions.222 This method has also been applied to the synthesis of base-modified NTPs108,223−225 and sugar-modified NTPs.224

In 2011−2013, the Huang research group reported a method based on the hypothesis that a bulky phosphitylation reagent would react selectively with the primary 5′-OH group versus the secondary 2′- and 3′-OH groups,185,186,226,227 thus avoiding extra...
steps of synthesis (introduction and removal of protecting groups). New reagent 183 was generated in situ through the reaction of salicylic chlorophosphite 180 with excess tetra(n-butylammonium) pyrophosphate (Scheme 29). Regioselective phosphitylation of the 5′-position of unprotected nucleosides generated the cyclic intermediates 184−187, which were oxidized by iodine in pyridine and water and subsequently hydrolyzed to give NTPs. Conversion was estimated to be about 10−50% of the crude material, and HPLC purification was subsequently carried out. This method was recently applied to the synthesis of ATP analogues.228

As an alternative phosphitylating reagent to salicylchlorophosphite, which is difficult to handle because of its high reactivity and hygroscopicity, Li and co-workers used bis(diisopropylamino)chlorophosphine to prepare the phosphorobis(amidite) derivative 188 (Scheme 30).229 Phosphitylation of 189 was carried out in the presence of DMAP and DIPEA. The corresponding phosphorobis(amidite) 188 was treated with pyrophosphate organic salts in the presence of 1H-tetrazole to form the cyclic intermediate 190. The reaction was monitored by 31P NMR spectroscopy. During the course of the reaction, the singlet at 127.72 ppm [corresponding to the phosphorobis(amidite) entity] was replaced by a triplet at 105.73 ppm for P(III), along with a doublet at −20.73 for P(V). This step was completed in 15 min after the addition of 4 equiv of the coupling agent. Then, oxidation of 190 with an aqueous solution of iodine led to the isolation of the acyclothymidine 5′-triphosphate 149 in 48% yield.

Finally, using the iterative strategy (coupling, oxidation, deprotection) developed by the Jessen research group and previously discussed in section 5.1.4, it is possible to access not only (d)NDPs (Scheme 19), but also (d)NTPs, as shown in Scheme 31.27,180

Interestingly, all previously cited methods involving a mixed P(III)−P(V) intermediate for nucleoside 5′-triphosphate synthesis can also be used to access analogues containing...
modifications at the α-phosphate, such as 5′-(α-P-thiotriphosphates), 5′-(α-P-boranotriphosphates), and 5′-(α-P-selenotriphosphates). 185,186,222,229

6.1.4. Synthesis via a Phosphotriester Intermediate. In 2009, Meier and co-workers expanded the use of the cycloSal phosphate triester 108 as an intermediate for the synthesis of (d)NTPs.82,161 Scheme 32 illustrates the synthetic pathway for dTTP. Details of the preparation of the starting material 108 were previously described in section 5.1.5 (Scheme 20). The reaction of 108 with pyrophosphate in DMF, followed by the removal of the acetyl group, generated dTTP in 80% yield. As stated before for the synthesis of (d)NDPs, this approach requires the introduction of N,O-protecting groups on the nucleoside moiety because of the high reactivity of the reagents involved in the phosphitylation reaction. Under these restrictions, ATP, GTP, CTP, and UTP were obtained in 40–65% yields, and carbocyclic nucleotides 150 and 151 (Figure 19) were synthesized in 18–19% yields.163

6.1.5. Synthesis via a Phosphosulfonyl Intermediate. In 2012, the Taylor research group described a procedure for the synthesis of nucleoside polyphosphates and their conjugates using sulfonylimidazolium salts as key reagents.230 Interestingly, this procedure appears to be useful for activating nucleoside mono-, di-, and triphosphates, and the nucleoside functionalities do not require prior protection. Sulfonylimidazolium salt 191 was prepared in high yield by reacting phenylsulfonylimidazole with methyl triflate in ether (Scheme 33). Treatment of the tetra- n-butylammonium salts of NMPs with 191 in the presence of N-methylimidazole (NMI) or N,N′-diisopropylethylamine (DIPEA) as a base, followed by displacement with bis(tetra-n-butyrammonium) pyrophosphate, produced high yields (84–90%) of NTPs.
6.2. Solid-Supported Synthesis

6.2.1. Synthesis Involving a Polymer-Bound Reagent.

By analogy with the synthesis of (d)NDPs (see section 5.2.1), nucleoside 5′-triphosphates can be obtained using the polystyrene-bound phosphitylating reagent 110b (Scheme 21, n = 2). Coupling of the unprotected nucleosides (A, T, U, AZT) with the supported reagent, oxidation with t-BuOOH, then subsequent removal of the cyanoethyl groups with DBU, and final cleavage of the polymer-bound compounds under acidic conditions generated the corresponding (d)NTPs in 60-79% yields. In 2007, in a patent, Bradley and Bruckler reported the preparation of an immobilized phosphitylating reagent and its application to the synthesis of (d)NTPs.
2′-O-methyl ribonucleoside triphosphates 198–201 were obtained in yields of 60–65% after phosphitylation, oxidation, and removal of the base protecting group, along with cleavage of the products from the support by heating with aqueous ammonia.

The Schoetzau research group described the synthesis of 2′- and 3′-amino nucleoside-5′-triphosphates using a polymer-bound triphenylphosphine resin as the support.138 No linker was required because immobilization of the azidonucleosides to the support was performed according to the Staudinger reaction. This reaction occurs between an azide and triphenylphosphine (TPP), which, under the loss of nitrogen, results in the formation of an iminophosphorane intermediate 202 (Scheme 36). In the presence of water, this intermediate is hydrolyzed to produce a primary amine and triphenylphosphine oxide.

The synthesis of 3′-amino dNTPs 203 and 204 is shown in Scheme 37. The 3′-azido-2′-deoxynucleosides 205 and 206 were first coupled to the commercially available polystyrene–TPP resin 207 to form supported iminophosphoranes 208 and 209, which were treated following the Ludwig–Eckstein procedure to produce 210 and 211 and then cleaved under basic conditions to yield the 3′-amino dNTPs 203 and 204. Yields, determined by HPLC, ranged from 70% to 75%. In contrast to the situation for the solution-phase synthesis, the 31P NMR spectra of the final products did not show the presence of side products, such as pyrophosphate or inorganic cyclic triphosphate, which were eliminated during the washing step before the oxidation step. However, the main drawback of this approach is that it is limited to the use of azidonucleosides as substrates and to the preparation of 2′- or 3′-amino NTPs.

As previously detailed for (d)NDP synthesis (see section S.2.2), supported cycloSal phosphotriester intermediates can also be used to prepare (d)NTPs. This involves replacing phosphate salts with pyrophosphate salts as the nucleophile in the last
step. In dTTP synthesis, the replacement of tetra-n-butylammonium phosphate with tetra-n-butylammonium pyrophosphate during the last step of the synthetic pathway, shown in Scheme 22, led to the formation of dTTP of 90% purity.

6.2.3. Synthesis Involving Attachment of the Base Moiety to the Support. Both strategies reported in this section were conducted in a homogeneous solution. The PEG-supported synthesis of cytosine-(d)NDPs was adapted to the synthesis of the corresponding cytosine-(d)NTPs by using tri-n-butylammonium pyrophosphate instead of the phosphate salt (Scheme 38). After cleavage of the products from the support, purification on a reverse-phase column, and then dialysis, CTP, dCTP, ddCTP, araCTP, and 3TCTP were obtained in 35−92% yields and 76−87% purities. Remarkably, the use of this supported methodology led to a 5-fold increase in yield for araCTP compared to the Hoard−Ott solution-phase synthesis.

In 2014, Kore and Parang reported an ionic-tag-assisted synthesis of dNTPs (Scheme 38). The ionic liquid 1-carboxymethyl-3-methylimidazolium tetrafluoroborate (CMMIMBF$_4$), shown in Figure 8, was synthesized in a one-pot, two-step procedure starting from bromoacetic acid and methylimidazole. Coupling conditions of cytosine with PEG-(O-succinate)$_n$ as reported by Crauste et al. were applied to selectively anchor the exocyclic amino groups of dC and dA with the ionic liquids (Scheme 38). Upon reaction completion, ionic-liquid-tagged 2'-deoxynucleosides 212 and 213 were oiled out by the addition of ethyl acetate, leaving excess organics and unreacted materials in the top organic layer. Triphosphorylation was performed according to the Ludwig method. Finally, removal of the ionic tag with aqueous ammonium hydroxide produced dCTP and dATP in 72% and 69% yields, respectively.

6.3. Enzymatic Synthesis

As previously mentioned, biocatalytic approaches are useful for the synthesis of natural nucleotides. However, they are less efficient when applied to the synthesis of their analogues, which contain unnatural base and/or sugar residues, because of the selectivity of enzymes toward their natural substrates. Whole cell systems, crude enzyme preparations, and purified enzymes have been used to synthesize natural (d)NTPs and some of their labeled analogues, such as 32P-labeled compounds. The following examples focus specifically on the recent literature.

In 2003, the Davisson research group combined chemical and enzymatic methods to synthesize azole carboxamide (d)NTPs, by taking advantage of the poor selectivity of nucleoside diphosphate kinase toward these substrates. Indeed, P(III) or P(V) chemical strategies proved to be inefficient in accessing these types of NTP analogues. The chemical synthesis of the diphosphate derivatives 214−216 was first carried out in solution in 70−91% yields. It involved selective 5'-tosylation of the nucleoside analogues 217−219, followed by the displacement of the 5'-tosylate group with tris(tetra-n-butylammonium) pyrophosphate (Scheme 39A). Regarding the ribo derivatives, 2',3'-methoxymethylidene group was introduced before the tosylation step and was removed before the enzymatic reaction (Scheme 39B). Two enzymatic conditions were developed: (i) For dNTP analogues 220−222, ATP served as the phosphate donor and nucleoside diphosphate kinase as the catalyst; this system was coupled with the use of pyruvate kinase and phosphoenolpyruvate (PEP) as an ATP regeneration system (Scheme 39A). (ii) In contrast, for NTP analogues 223 and 224, phosphorylation was performed by pyruvate kinase, with PEP as the phosphate donor (Scheme 39B).

In 2007, Hennig et al. described the enzymatic synthesis of 5-fluorouridine and 5-fluorocytidine 5'-triphosphates SF-UTP (225) and SF-CTP (226), respectively, using either the corresponding heterocycle and ribose or the nucleoside analogue as starting materials. As shown in Scheme 40, the enzymatic process involved a cascade of reactions, such as the formation of monophosphates 227 and 228 from 5-fluorouracil by uracil phosphoribosyltransferase (urp$^*$) or from 5-fluorocytidine phosphorylation by uridine kinase (udk), respectively. Two subsequent phosphorylation steps under the control of pyrimidine-specific monophosphate kinase (pyrH) and pyruvate kinase (pyrK) produced the triphosphates 225 and 226.
process provided access to all nucleotidic entities, namely, mono-, di-, and triphosphorylated derivatives of 5-fluorouridine and 5-fluorocytidine.

7. MISCELLANEOUS

The following sections cover the synthesis of cyclic nucleotides, dinucleoside 5′,5′-polyphosphates, and nucleotide conjugates. We highlight the emerging strategies that have been reviewed in recent years (e.g., cyclic dinucleotides). We do not include the synthesis of nucleoside 5′-tetra- and 5′-pentaphosphates or nucleoside cyclopyrophosphates because readers can refer to summary reviews published in 2015.

7.1. Cyclic Nucleotides

Cyclic nucleotides differ from regular nucleoside 5′-monophosphates in that they have an intramolecular phosphodiester bridge connecting two hydroxyl groups of the same sugar moiety (Figure 1). The Khorana research group reported the first synthesis of nucleoside 3′,5′-cyclic phosphates, based on the reaction of NMPs with DCC. Later, such compounds were obtained by the phosphorylation of unprotected nucleosides with phosphorus oxychloride in trialkyl phosphate, followed by the dilution of the reaction mixture in a large volume of potassium hydroxide solution (Scheme 41), leading to quantitative formation of a diasteromeric mixture of cyclic phosphonate diesters. In the presence of pivaloyl chloride resulted in the rapid and concomitant cyclization and hydrolysis of the chlorophospho-

Scheme 41

concomitant cyclization and hydrolysis of the chlorophosphodiester intermediate. This one-pot procedure led to the formation of the title compounds 229 and 230 in 35–49% yields. In 2013, Rozniewska et al. reported an alternative pathway involving nucleoside 3′,5′-cyclic H-phosphonates as key intermediates (Scheme 42). This approach requires prior protection of the exocyclic amines of Ade, Gua, and Cyt and then synthesis of the corresponding nucleoside 3′-H-phosphate monooesters 231–234. Thus, treatment of the starting materials in the presence of pivaloyl chloride resulted in the rapid and quantitative formation of a diastereomeric mixture of cyclic H-phosphonate diesters 235–238. In situ oxidation with iodine in pyridine, followed by hydrolysis and removal of the protecting group (except for Thy) under basic conditions, produced the desired 3′,5′-cyclic NMPs 239–242 in 65–82% yields (calculated on the basis of the nucleoside 3′-H-phosphate as the starting material).

It is important to note that solid-phase synthesis of cyclic nucleotides has not been described so far.

7.2. Cyclic Dinucleotides

In cyclic dinucleotides (cDNs), two nucleosides are intercon-

Scheme 42

connected through two 3′–S′ phosphodiester bounds. Naturally occurring cyclic dinucleotides are symmetrical, as shown by cyclic di晷anosine monophosphate (c-di-GMP) and its adenosine counterpart c-di-AMP (Figure 1). Enzymatic synthesis is restricted to endogenous compounds (such as c-di-GMP and c-di-AMP), whereas chemical synthesis generates structural diversity. The synthetic strategies for accessing cyclic dinucleotides and their analogues, as well as their biological properties, have been reviewed in recent years. briefly, synthetic cDNs are prepared either by nucleotide cyclization or by stereospecific base insertion on a cyclic bis(3′–5′)-sugar phosphate. The first and most widespread approach comprises several strategies shown in Figure 22: (A) intramolecular cyclization of a 5′-monophosphate-3′,5′-dinucleotide; (B) dimerization of a nucleoside 3′- or 5′-monophosphate to produce symmetric derivatives only; (C) intramolecular cyclization of a 3′,5′-dinucleotide bearing a phosphate, phosphodiester, or H-phosphate group at the 3′-end; and (D) intramolecular cyclization of a 3′,5′-phosphotriester dinucleoside.

Solid-phase synthesis has only been reported within the framework of strategy C, using Tentagel or CPG polymers as solid supports. Briefly, the required cyclic dinucleotide was obtained either from a nucleoside 3′-phosphate, whose nucleobase is anchored through its exocyclic amino group to the support, or from a nucleotide whose 3′-phosphate group is linked to the support. This last approach is more common, as it is independent of the nature of the nucleobase moiety.

7.3. Dinucleoside 5′,5′-Polyphosphates

Physiological dinucleoside 5′,5′-polyphosphates (DNPs), commonly abbreviated as Np,Np, contain two ribonucleosides that are linked at the 5′-position of their sugar moiety through n phosphate groups (Figure 1). The first compound belonging to this family to be identified in mammals was the P10′-O-adenosine-P5′-O-adenosine tetraphosphate (ApA). Since then, 16 additional DNPs have been isolated from human tissues and characterized.

Both enzymatic and chemical approaches can be used for preparing DNPs. The former are restricted to naturally occurring compounds and small-scale syntheses. For example, synthesis of dinucleoside tri- and tetraphosphates has been described using an engineered diadenosine triphosphate hydrolase as a catalyst. Because of the limitations inherent to enzymatic catalysis, DNPs are most often obtained chemically by extending the methods for the synthesis of nucleotides described previously. It is important to note that yields drop.
significantly as the length of the polyphosphate chain increases.242

One widely used method involves the activation of a 5′-nucleotide (NMP, NDP, or NTP) as its trialkylammonium salt with CDI in DMF, followed by in situ condensation with the other 5′-nucleotide (tributylammonium salt of NMP, NDP, or NTP) or pyrophosphate to form the required DNPs containing two to five bridging phosphate groups in 10–60% yields.62,80,243−245 Alternatively, the activation of an NTP with DCC produces a cyclic nucleoside 5′-trimetaphosphate, which can react with an NMP or an NDP to produce the corresponding DNP.62,80,243,245 Whereas CDI is preferentially used for the synthesis of dinucleoside diphosphates (50−60% yields) and dinucleoside triphosphates (20−30% yields), DCC has the greatest conversion rates and highest isolated yields for dinucleoside tetraphosphates.80,245

A recent variant of the CDI method consists of preparing P1,P2-di(1-imidazolyl)pyrophosphate 243 (Scheme 43), which then reacts with AMP or UMP to generate the symmetrical 5′,5′-dinucleoside tetraphosphates 244 and 245 in 74% and 61% yields, respectively.246

Alternatively, DNPs can be obtained in 50−70% yields through a phosphoramidite approach described by the Jessen research group (Scheme 31, section 6.1.3) and generated Ap4U and Up2U in 36% and 83% yields, respectively.249

Figure 22. Retrosynthetic pathways for the synthesis of cyclic nucleotides via nucleotide cyclization.

Scheme 43

Figure 23. Retrosynthetic pathways for the synthesis of cyclic nucleotides via nucleotide cyclization.

A few methods based on P(III) chemistry and initially developed for the synthesis of nucleotides were adapted to the synthesis of DNPs by introducing minor modifications in the last steps of the reaction sequence (Figure 23). The first pathway (Figure 23, path A) is based on Ludwig and Eckstein’s procedure for NTPs (Scheme 28, section 6.1.3), involving a salicylphosphite intermediate. Oxidation of the cyclic P(III)—P(V) intermediate under anhydrous conditions, followed by reaction with an NMP or NDP in dry DMF catalyzed by ZnCl2 and then removal of the acetyl groups under basic conditions, generated the corresponding DNPs derivatives. Accordingly, Np4N and Np5N were synthesized in 47−85% yields.138 The second pathway (Figure 23, path B), performed either in solution or on a support,137 relies on a cycloSal phosphotriester intermediate, as reported previously for NDPs (Scheme 20, section 5.1.5; Scheme 22, section 5.2.2) and NTPs (Scheme 32, section 6.1.4). Phosphate or pyrophosphate salts were replaced by nucleotides to form the corresponding DNPs, after deprotection of the 3′-hydroxyl group. Yields for Up2T and Ap4T were 60% and 52%, respectively.82

The third approach (Figure 23, path C) is derived from the approach developed for NTPs (Scheme 27, section 6.1.2) and makes it possible to access symmetrical dinucleoside diphosphates in 68−91% yields starting from the nucleoside 5′-H-phosphonates.177 The fourth pathway (Figure 23, path D) is based on the reaction of unprotected nucleosides with polymer-bound phosphitylating reagents, which are structurally similar to those developed for the synthesis of NDPs and NTPs (Scheme 21, section 5.2.1). Symmetrical DNPs were obtained in 59−68% yields, starting from the supported reagent.249 The last pathway (Figure 23, path E) is based on the iterative phosphoramidite approach described by the Jessen research group (Scheme 31, section 6.1.3) and generated Ap4U and Up2U in 36% and 83% yields, respectively.

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Recently, the Taylor research group reported the synthesis of symmetrical dinucleoside di- and tetraphosphates using sulfonyl imidazolium salt \( \text{191} \) as a reagent (Scheme 44).\(^2\)\(^{30}\) Compared to the synthesis of NTPs illustrated in Scheme 33 (section 6.1.5) and using either NMP or NDP as the starting material, the reactions were performed in the presence of \( \text{191} \), NMI, and \( \text{MgCl}_2 \) in DMF and generated the corresponding dimers \( \text{246} \)–\( \text{248} \). The mixed DNPs \( \text{249} \)–\( \text{253} \) could also be obtained in high yields, starting from NMPs or NTPs and using various NMPs, NDPs, and NTPs as acceptors (Scheme 44).

The Taylor research group also reported an original method for preparing dinucleoside pentaphosphates (Np₅N) using cyclic trimetaphosphate \( \text{157} \) (Scheme 45) as the phosphorylating agent.\(^2\)\(^{50}\) The reaction of the tri(tetra-\( n \)-butylammonium) salt of \( \text{157} \) with \( \text{191} \) or mesitylenesulfonyl chloride (MstCl, \( \text{254} \)) in the presence of NMI in DMF, followed by the addition of an NMP, produced \( \text{Np₅N} \) in \( \text{77–85\%} \) yields. In 2016, this strategy was adapted for the synthesis of NTPs by reacting \( \text{157} \) with MstCl in the presence of DABCO for 1 min at room temperature and then adding 2′,3′-acetylated nucleosides followed by phtalimide.\(^2\)\(^{51}\) After removal of the protecting groups under basic conditions, satisfactory yields of NTPs were obtained. The precise mechanism involved in the triphosphorylation step has not yet been elucidated.

Finally, the approach developed by the Sun research group for the solution-phase synthesis of NDPs, which involves the activation of a nucleoside 5′-phosphoropiperidate with DCI (Scheme 17, section 5.1.2), was adapted for the synthesis of DNPs.\(^2\)\(^{42},252,253\) As shown in Scheme 46, the reaction of nucleoside 5′-phosphoropiperidates with nucleotides in the presence of excess DCI led to the formation of more than 20 symmetrical and mixed DNPs with moderate to high yields.\(^2\)\(^{42},253\) Symmetrical DNPs could also be obtained by reacting nucleoside 5′-phosphoropiperidates with substoichiometric amounts of inorganic phosphate, pyrophosphate, or triphosphate in the presence of excess DCI.\(^2\)\(^{32}\) Compared to other methods, this approach has a shorter reaction time and higher isolated yields.

### 7.4. Sugar Nucleotides

Sugar nucleotides are important intermediates in carbohydrate metabolism and function, given that they act as activated glycosyl donors in most glycosyl transferase-catalyzed glycosylations.\(^10\) Nucleoside diphosphate sugars (NDP-sugars), such as UDP-\( \alpha \)-D-galactose, and nucleoside monophosphate sugars (NMP-sugars), such as CMP-sialic acid, form two subgroups of natural conjugates.\(^9,10\) To provide an overview, eukaryotic glycoproteins and glycolipids are synthesized from nine sugar-nucleotide donors, most of which are NDP-sugars. The chemical and enzymatic syntheses of these compounds have been described comprehensively in recent reviews and articles.\(^32,34,254–259\) In this section, we focus specifically on the more relevant or recent progress achieved in the chemical synthesis of NDP-sugars.

NDP-sugars are mainly synthesized through the glycosylation of an NDP with a sugar activated entity (Figure 24, path A) or by coupling a sugar-1-phosphate with an activated NMP (Figure 24,
path B). In most cases, the reaction sequence does not require the presence of a protecting group on the nucleobase. When the NDP glycosylation approach is used, the yields are usually low, and in many cases, the stereochemistry at the anomeric center of the sugar in the final products cannot be controlled.

Regarding path B, a number of methods described for NDP or NTP synthesis have been adapted for the preparation of a variety of NDP-sugars, by substituting the phosphate or the pyrophosphate reagent with a sugar phosphate. These methods rely on intermediates, as already mentioned, such as phosphoromorpholidates, phosphorimidazolates, phosphoramidates, phosphosulfonyl derivatives, mixed P(III)−P(V) anhydrides, and cycloSal phosphotriesters.

In 2012, Tanaka et al. made a breakthrough in the chemical synthesis of NDP-sugars, by performing the coupling of a sugar-1-phosphate with a nucleotide in water (Scheme 47). Interestingly, phosphorylated reagents do not need to be converted into their trialkyl- or tetraalkylammonium cations because their commercially available sodium (NMP) and potassium salts (sugar-1-phosphate) are water-soluble. The key
intermediate is 2-imidazolyl-1,3-dimethylimidazolinium chloride (ImIm), formed in situ by mixing 2-chloro-1,3-dimethylimidazolinium chloride (DMC) and imidazole. It can activate an NMP into its corresponding phosphoroimidazolidate (Scheme 47). Optimization procedures showed that the sugar-1-phosphate salt must be added to the reaction mixture 1 h after activation of the NMP (Scheme 48) to minimize both the activation of sugar-1-phosphate by ImIm and the formation of symmetrical dinucleoside diphosphate as a byproduct.

This strategy made it possible to synthesize NDP-sugars 255–257 in 23–35% isolated yields (determined by UV and based on the sugar-1-phosphates). Interestingly, the compounds require no further purification for use as a source of glycosyl donors for preparative oligosaccharide synthesis via glycosyltransferase.32,96

### 7.5. Nucleolipids

Nucleolipids (Figure 1) combine a nucleobase, nucleoside, nucleotide, or oligonucleotide with a lipophilic moiety.12,16 Compared to the nucleotide analogues described so far, only a few methods have been reported to access nucleotide–lipid conjugates. These methods have been covered in several reviews over the past decade.12,15,16,34,114 The phosphoramidite and phosphoramidate strategies have been predominantly used for their chemical synthesis. For example, nucleoside diacylglycerol monophosphates were synthesized through the condensation of 3′-O-protected nucleosides with diacylglycerol phosphorami-
dyes, followed by oxidation with iodine and then deprotection. In the case of phosphoramidates (usually phosphoromorpholidates), two options can be considered: the reaction of a lipid monophosphate with a nucleoside 5′-phosphoramidate or the reaction of an NMP with a lipid phosphoramidate. Alternatively, nucleolipids can be obtained by condensing an acyl phosphate/pyrophosphate with a free nucleoside/nucleotide in the presence of DCC or by coupling an activated carboxylic acid with a nucleotide.

8. CONCLUSIONS

After decades of research to develop efficient, easy, and universal synthetic protocols to obtain mononucleoside polyphosphates and their analogues, several reasonably effective strategies and methods have emerged for the preparation of target compounds from both nucleosides and nucleotides. Researchers continue their investigations in this field because of the considerable therapeutic potential of nucleoside analogues and their active forms, nucleoside 5′-triphosphates.

Synthetic approaches can rely on P(V) and/or P(III) chemistry. The use of protected, partially protected, and even unprotected starting materials can provide the expected nucleotide derivatives in satisfactory to high yields. Whereas the condensation of activated P(V) species (such as P-morpholidates, P-imidazolides, P-imidazolium ions, and others) with phosphates has been successfully developed and can be performed without the use of protecting groups, only a few examples exist for P(III)-based couplings. These phosphorylation reactions often require long reaction times and high amounts of excess reagents and provide low to moderate yields. It is important to point out that the available methods work well with many but not all substrates. Consequently, the search for an ideal synthesis goes on. In comparison, P(III) chemistry can usually be conducted using almost equimolar amounts of the reactants. The high speed and efficiency of coupling have been well established in the context of oligonucleotide chemistry. Thus, couplings based on P(III) chemistry could, theoretically, deliver the desired compounds with satisfactory yields, easy purifications, and efficient coupling rates. However, competitive yields are only obtained when working under strictly dry conditions. Indeed, for many synthetic approaches, involving either P(V) or P(III) intermediates, it is crucial that the reactions be carried out under rigorously dry conditions. Otherwise, huge amounts of nucleotide side products are generated, together with several byproducts, such as organic polyphosphorylated salts.

An ideal synthesis would involve little or no excess reagents and activators, short reaction times, the absence of protecting groups on the starting material, simple conditions (i.e., nondry solvents and reagents, open flasks, room temperature, etc.), high yields, and simple purification procedures. This last point is especially important because the separation of polar nucleotide derivatives from polar contaminants is far more difficult and sluggish than the purification of nonpolar compounds using normal-phase column chromatography. Moreover, as water and buffers are used in the elution during reverse-phase or ion-exchange chromatography, repeated freeze-drying is required. This prolongs the overall process and is problematic in the case of labile P-anhydrides.

In the course of work on this problem, some prospective approaches have recently been proposed, most of which are based on the supported synthesis of oligonucleotides. Unfortunately, no universally applicable and high-yielding procedure exists for the anchoring and phosphorylation of unprotected nucleosides.

Finding a general method that is compatible with a huge range of nucleoside analogues remains a challenge. Indeed, some methods are well suited for certain nucleosides and produce high yields, but when applied to other nucleosides, yields decrease significantly.

Until recently, the preparation of unsymmetrical disubstituted pyro- and polyphosphate derivatives in satisfactory yields presented significant difficulties. Nevertheless, the increasing interest in such compounds as substrates, cofactors, and regulators for a wide range of important biochemical processes calls for research on new synthetic strategies and activating reagents. One approach sets out to obtain nucleoside 5′,5′-polyphosphate derivatives and terminal-phosphate-labeled nucleoside 5′-polyphosphates. The protocols, which are based on unprotected nucleotides as parent compounds, look promising, despite moderate yields in some cases. It is because of the absence of nucleotide protection that laborious and time-consuming process are often required. The thorough selection of activating reagents, solvents, and conjugation strategies might help increase yields and shorten reaction times in approaches based on the activation of phosphate-containing derivatives. In addition, these approaches often have poor reproducibility because of the difficulty in maintaining anhydrous reaction conditions when using hygroscopic substrates and reagents.

Research in the field of nucleic acids has been and will continue to be a major driving force in the development of phosphorylation chemistry. Although it could be argued that many of the problems associated with P-anhydride synthesis have been solved, some issues have yet to be addressed, and new challenges are emerging.

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Notes
The authors declare no competing financial interest.

Biographies

Béatrice Roy graduated in 1989 from Ecole Européenne des Hautes Industries Chimiques de Strasbourg. In 1993, she was awarded a Ph.D. in Organic Chemistry from the University Joseph Fourier (UJF, Grenoble, France), where she worked under the supervision of Professor Marc Fontecave on the design of new inhibitors of ribonucleotide reductase. She pursued postdoctoral studies first at the UJF and then at the University Paris Sud (Orsay, France). In 1997, she held a position of Assistant Professor in Professor G. Lemaire’s group at the University Paris Sud. In 2002, she joined Professor C. Perigaud’s research group (Montpellier, France) and was appointed by the University of Montpellier in 2007. She was a visiting faculty member in 2011 and 2012 at the University of Canterbury in Christchurch, New Zealand. Her research interests focus on the synthesis and analysis of nucleoside analogues and their polyphosphorylated entities.

Anaïs Depaix was awarded her Master’s degree in 2014 in Organic Chemistry by the University Joseph Fourier (UJF, Grenoble, France). In October 2014, she began her Ph.D. studies at the University of Montpellier (France) as part of the Nucleosides & Phosphorylated Effectors group. She is working on the development of a new synthetic methodology for polyphosphorylated nucleosides.
Christian Périgaud received his Master’s and Ph.D. (1991) degrees in Chemistry from the University of Montpellier (France), where he worked with Professor J. L. Imbach and Dr. G. Gosselin on the synthesis of nucleoside analogues. Then, Dr. Périgaud held a postdoctoral position at the University of Alabama in Birmingham, working with Professor J.-P. Sommadossi. When he returned to France in 1994, he joined the University of Montpellier (France) as an Assistant Professor. Nominated Full Professor in 2001, he was the group leader of the Nucleosides & Phosphorylated Effectors research team until 2014. His main areas of expertise and interest concern the organic and medicinal chemistry of nucleosides, nucleotides, and related prodrugs, as well as their analysis. He is coauthor of over 100 publications and eight patents.

Suzanne Peyrottes studied organic chemistry at the University of Montpellier (France). In 1992, she joined the Laboratory of Prof. J. L. Imbach (Montpellier, France) and obtained her Ph.D. in 1995 under the direction of Dr J. J. Vasseur, working on the synthesis and study of modified oligonucleotides. In 1996, she joined the group Dr. M. J. Gait at MRC (Cambridge, U.K.) as a Postdoctoral Fellow, where she worked on the solid-phase synthesis of oligonucleotide–peptide conjugates. In 1998, she joined the laboratory of Dr G. Gosselin (Montpellier, France) to focus on the synthesis of antitumoral mononucleotide prodrugs. A year later, she was appointed by CNRS as senior scientist. Today, she is research director at CNRS (France) and is working on the design of potential therapeutic agents to treat infections and cancers, in addition to a new synthetic methodology related to nucleic acid components. She is coauthor of about 50 publications and five patents.

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ABBREVIATIONS

3TC \( \beta \)-1′,2′,3′-dideoxy-3′-thiacytidine
ADP adenosine 5′-diphosphate
AICAR 5′-amino-1′,2′-ribofuranosylimidazole-4-carboxamide
AMP adenosine 5′-monophosphate
AraC \( \beta \)-1′,2′-arabinofuranosylcytosine
ATP adenosine 5′-triphosphate
AZT 3′-azido-2′,3′-dideoxythymidine
cAMP adenosine 3′,5′-cyclic monophosphate
CBz carboxybenzyl
CDI 1′,1′-carbonyldimidazole
c-di-AMP cyclic diadenosine monophosphate
cDN cyclic dinucleotide
cGMP guanosine 3′,5′-cyclic monophosphate
cNMPs cyclic nucleoside monophosphates
COSY correlation spectroscopy
CPG controlled pore glass
cycloSal cyclosaligenyl
DABCO 1,4-diazabicyclo[2.2.2]octane
DBU 1,8-diazabicyclo[5.4.0]undec-7-ene
DCC \( N,N′ \)-dicyclohexylcarbodiimide
DCI 4,5-dicyanomimidazole
DCSO \((−)-8,8′-dichloroamphophylsulfonfyl\)-oxaziridine
dDC 2′,3′-dideoxyctydine
DIC \( N,N′ \)-disopropylcarbodiimide
DIPEA \( N,N′ \)-disopropylethylamine
DMAP 4-(dimethylamino)pyridine
DNP5 dinucleoside polyphosphates
IE ion exchange
FADH2/FAD flavin adenine dinucleotide
GPCRs G-protein-coupled receptors
HMBC heteronuclear multiple-bond correlation
HMQCs heteronuclear multiple-quantum coherence
HMPA hexamethylphosphoramide
HOBt 1-hydroxybenzotriazole
ImIm 2-imidazolyl-1,3-dimethylimidazolinium chloride
HPLC high-performance liquid chromatography
LC-MS liquid chromatography coupled with mass spectrometry
LC-MS/MS liquid chromatography tandem mass spectrometry
LC-UV liquid chromatography with UV detection
m-CPBA \( \text{meta}-\text{chloroperoxybenzoic acid} \)
MMTCl resin polystyrene monomethoxyxtrimyl chloride resin
MstCl mesitylenesulfonyl chloride
NA nucleoside analogue
NADH/NAD+ nicotinamide adenine dinucleotide
NADPH, NADP+ nicotinamide adenine dinucleotide phosphate
NBD nucleoside diphosphate
NMP nucleoside 5′-monophosphate
NOESY nuclear Overhauser effect spectroscopy
PEG polyethylene glycol
PS polystyrene
PS-AM polystyrene aminomethyl
PVA poly(vinyl alcohol)
PvCl pivaloyl chloride
RTILs room-temperature ionic liquids
SELEX systemic evolution of ligands by exponential enrichment
TBDDS tert-butylidiphenylsilyl
TEAB triethylammonium hydrogen carbonate
TFAD trifluoroacetic acid
TFAMA trifluoroacetic anhydride
dTMP thymidine 5′-monophosphate
TMS trimethylsilyl
TPP triphenylphosphine
TPPO triphenylphosphine oxide
TPS-TAZ triisopropylbenzenesulfonyl tetrazolide
TriMP cyclic trimetaphosphate
UMP uridine 5′-monophosphate

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