Overview of the Synthesis of Nucleoside Phosphates and Polyphosphates

Phosphorylated nucleosides play a dominant role in biochemistry. Primary metabolism, DNA replication and repair, RNA synthesis, protein synthesis, signal transduction, polysaccharide biosynthesis, and enzyme regulation are just a handful of processes involving these molecules. Literally thousands of enzymes use these compounds as substrates and/or regulators. The need to obtain such compounds in both labeled and unlabeled forms, as well as a burgeoning need for analogs, has driven the development of a myriad of chemical and enzymatic synthetic approaches. As chemical entities, few molecules possess the wide array of densely packed functionality present in phosphorylated nucleosides. This poses a formidable challenge to the synthetic chemist, one that has not yet been fully overcome. This overview will address some common methods (synthetic and enzymatic) used to construct phosphorylated nucleosides. Particular emphasis is placed upon the advantages, limitations, and generality of the methods. Additionally, the synthesis of phosphorylated analogs such as phosphorylsulfates, phosphoramidates, phosphonates, thiophosphates, and imidophosphates will be addressed. This overview is not intended as a comprehensive review, but rather as a guide to general principles associated with the synthesis of phosphorylated nucleosides.

ISSUES ASSOCIATED WITH NUCLEOSIDE PHOSPHORYLATON

In nature, the conversion of nucleosides to mono-, di-, or triphosphates is accomplished by specific enzyme-catalyzed reactions. Invariably, a nucleoside hydroxyl group functions as a nucleophile in coupling with an electrophilic phosphoryl donor (ATP, phosphoenol pyruvate, acetyl phosphate, and others). Binding constraints in the active site of the enzyme guarantee high regio- and chemoselectivity in the ensuing reaction. During chemical syntheses of nucleoside phosphates, the nucleoside can serve as either an electrophile or a nucleophile. Despite this flexibility, chemical syntheses are fundamentally more problematic than enzymatic ones. Phosphorylation procedures using electrophilic phosphorus reagents generally do not show high regioselectivity. Thus, it is often necessary to use protecting groups for the sugar, adding yet another level of complexity to the synthesis. Side reactions can occur, such as depurination of the nucleoside, phosphorylation of the nucleobase, as well as chemical alteration of nucleobase analogs. Due to their intrinsic reactivity, the synthesis of phosphoanhydride bonds is also synthetically challenging. Phosphate anhydrides are phosphorylating reagents that are readily degraded under acidic conditions. Finally, purification of synthetic nucleotides can be problematic. Ionic reagents, starting materials, and mixtures of regioisomers (2′-, 3′-, 5′-phosphates) can be particularly difficult to separate from the desired product.

In spite of the many potential difficulties associated with nucleoside phosphorylation and polyphosphorylation, a certain amount of success has been achieved in these areas. Given the wealth of phosphorylating reagents available, simple phosphorylation of nucleosides at any of the hydroxyl groups is possible, although selective phosphorylation at any particular hydroxyl group may require specific protection strategies. With sufficient attention to the nature of the nucleobase and protecting groups, the correct choice of phosphorylating agent should lead to a successful and high-yield synthesis. The synthesis of nucleoside diphosphates (NDPs) can also be succesfully performed in high yield. The excellent strategy introduced some time ago by Poulter for nucleophilic displacement of tosylates with tetra-*n*-butylammonium salts of pyrophosphate continues to be a very useful and successful strategy for simple preparation of diphosphates. While this is a multi-step procedure (particularly if protected nucleosides are used), the overall high yields, relative simplicity, and high reliability of this method make it one of the most useful preparative strategies available for the preparation of NDPs.

In contrast, the preparation of nucleoside triphosphates (NTPs) and polyphosphate diesters remains somewhat less satisfactorily addressed. There is considerable hope in this area, however. Properly activated nucleoside monophosphates and their derivatives appear to be good acceptors for pyrophosphate and phosphate esters. The major problems here appear to be the highly variable yields reported for specific methods. This may simply be a consequence of the idiosyncratic nature of nucleoside

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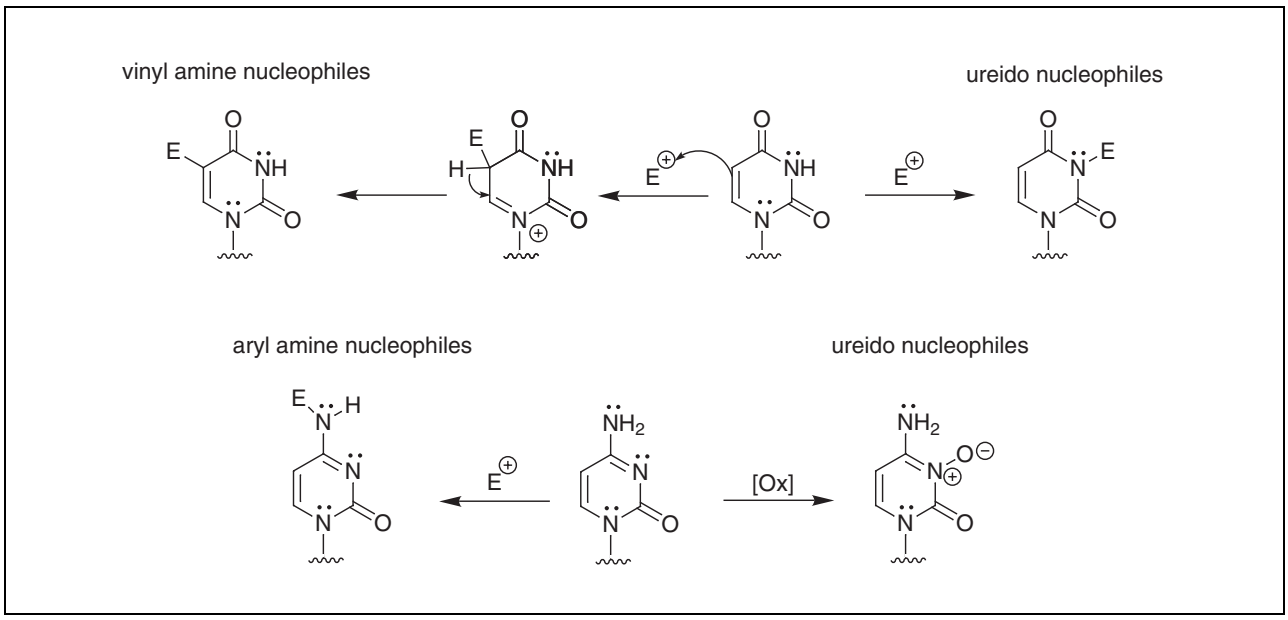

Figure 13.1.1 Some side reactions of nucleobases.

chemistry (in that different nucleosides are chemically very different), or may be related to the inherent difficulty in the reactions themselves. Many laboratories that use these methods are not ideally set up for organic synthesis or have little experience in doing this kind of chemistry. Developing robust chemistry for polyphosphate synthesis that can be routinely performed in a minimum of time, with a minimum number of steps, remains a daunting task for nucleoside chemists.

CHEMICAL REACTIVITY OF NUCLEOSIDES

Nucleosides as Nucleophiles

Both components of nucleosides, the sugar and nucleobase, possess nucleophilic functionality. The hydroxyl groups present in the sugar moiety of nucleosides are more acidic and less nucleophilic than corresponding primary or secondary alcohols in non-nucleosides. The sugar component of a nucleoside ionizes in the pH range of 12 to 13 for ribo- and deoxyribonucleosides (Albert, 1973). The reduced nucleophilicity of sugar hydroxyl groups may permit nucleophilic moieties in the nucleobase to compete for electrophiles. Accordingly, understanding the chemical properties of nucleobases is essential for implementing successful syntheses of nucleoside phosphates.

The traditional nucleobases, as well as common derivatives, share chemical functionality of similar reactivity (Fig. 13.1.1). Aryl and vinyl amines are common nucleophiles present in the nucleobases. Many bases also contain nucleophiles such as lactam, ureido, and guanidinium moieties. These moieties have different ionization characteristics. For this reason, Hayakawa and co-workers (Uchiyama et al., 1993) have grouped the common nucleobases into two different categories based upon their acidity. In group I nucleosides, the sugar hydroxyls are the most acidic functionality present. In group II nucleosides, the nucleobase has functionality more acidic than the sugar hydroxyls. Adenosine and cytidine belong to group I, whereas guanosine, thymidine, and uridine belong to group II. Unfortunately, these groupings do not directly correlate with the nucleophilicity of the nucleobase functionality. Nucleobases have different nucleophilicity towards electrophiles, even under identical reaction conditions. Thus, many phosphorylation procedures rely upon protecting groups (*UNIT 2.1*) to facilitate regioselective phosphorylation.

Nucleosides as Electrophiles

Nucleosides can be phosphorylated by a displacement reaction between phosphate and an electrophilic carbon of a nucleoside. To render a carbon electrophilic, the hydroxyl group must be converted into a leaving group of some kind (e.g., a halogen or sulfonate ester). There are two common approaches to convert the 5′-hydroxyl into an electrophile: (1) conversion to a relatively stable leaving group, or (2) in-situ activation of the 5′-hydroxyl.

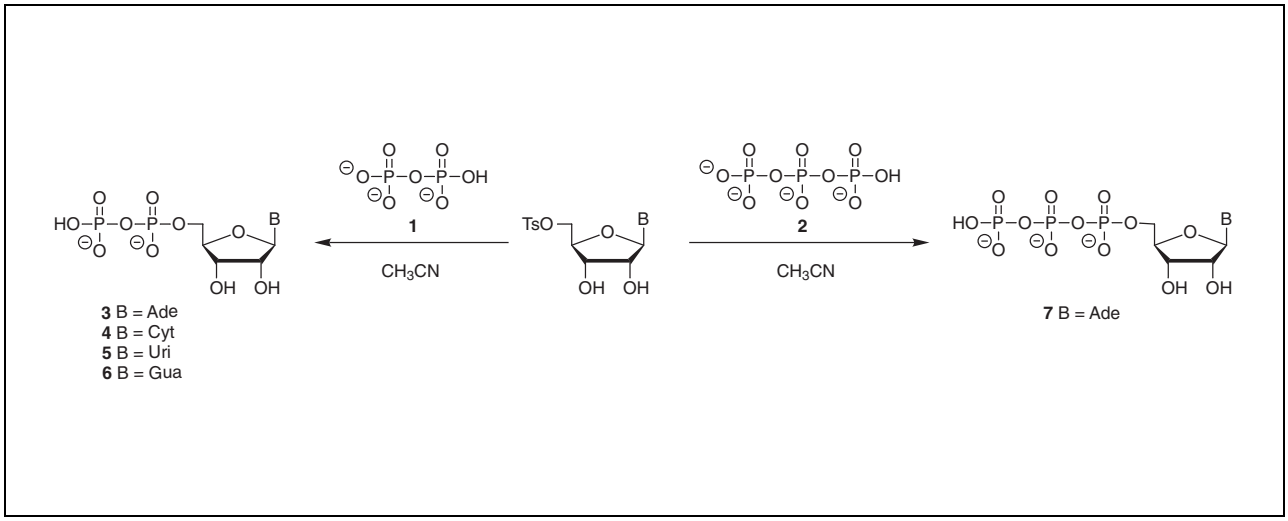

Figure 13.1.2 Nucleosides as electrophiles. Displacement of tosylate (Ts) by various phosphate species.

Nucleotides via stable intermediates

Halogenation is one common method of activating a 5′-hydroxyl group. For example, synthesis of 5′-iodides is readily achieved with methyltriphenoxyphosphonium iodide (MTPI; Verheyden and Moffatt, 1970) as well as a wide variety of other reagents. However, tosylation of the 5′-hydroxyl with toluene sulfonylchloride has proven to be one of the best activation methods. Synthesis of nucleoside 5′-tosylates of adenosine, guanosine, uridine, cytidine, and thymidine has been described (Davisson et al., 1987).

Poulter introduced the synthesis of 5′-nucleoside mono-, di-, and triphosphates by displacement of the tosylate leaving group (Davisson et al., 1987; Wu et al., 2003; also see *UNIT 13.2*). Displacement of tosylate by bis(tetra-*n*-butylammonium) phosphate or tris(tetra-*n*-butylammonium) pyrophosphate (**S.1**) gives the corresponding nucleoside 5′-phosphate or 5′-diphosphate (**S.3-S.6**; Fig. 13.1.2), respectively. Similarly, displacement of tosylate by tetrakis(tetra-*n*-butylammonium) triphosphate (**S.2**) gives the nucleoside 5′-triphosphate. However, this latter reaction has only been useful for the synthesis of ATP (**S.7**; Burgess and Cook, 2000).

To successfully execute a displacement reaction, care must be exercised in preparation and handling of the hygroscopic tetra-*n*-butylammonium phosphate salts. One nice feature of displacement reactions is that either protected or unprotected nucleosides can be phosphorylated. Ketal-protected nucleosides combine with tris(tetra-*n*-butylammonium) pyrophosphate much more rapidly than their unprotected counterparts. Reactions with protected nucleosides are complete in just 2 hours, whereas 2 to 4 days are often necessary with unprotected nucleosides. It is possible that the vicinal diols of ribonucleosides participate in H-bonding with the pyrophosphate reagent, thus slowing the reaction. Additionally, the yields for the displacement reaction are higher for protected nucleosides (Dixit and Poulter, 1984), suggesting that in appropriate cases it may be worthwhile to use protecting groups in spite of the added synthetic steps.

Nucleotides via in-situ activation: The Mitsunobu reaction

The Mitsunobu reaction (Fig. 13.1.3) exemplifies in-situ electrophilic activation of an alcohol functional group (Kimura et al., 1979). Activation is achieved by conversion of alcohol **S.8** to the corresponding alkoxyphosphonium salt (**S.9**). In-situ displacement of the alkoxyphosphonium salt by dibenzylphosphate gives nucleotide triester **S.10** and triphenylphosphine oxide (**S.11**). This reaction is driven by the formation of triphenylphosphine oxide and a strong phosphate ester bond. Deprotection via hydrogenolysis of the benzyl protecting groups affords the nucleoside monophosphate. Protected pyrimidine nucleosides can be phosphorylated in dioxane or tetrahydrofuran (THF), usually at an elevated temperature (60°C). However, phosphorylation of unprotected pyrimidine nucleosides is usually conducted at room temperature in polar aprotic solvents such as *N*,*N*-dimethylformide (DMF) or hexamethylphosphorous triamide (HMPT), in yields exceeding 75% (Kimura et al., 1979).

Syntheses involving purine nucleosides in polar aprotic solvents are complicated by intra-

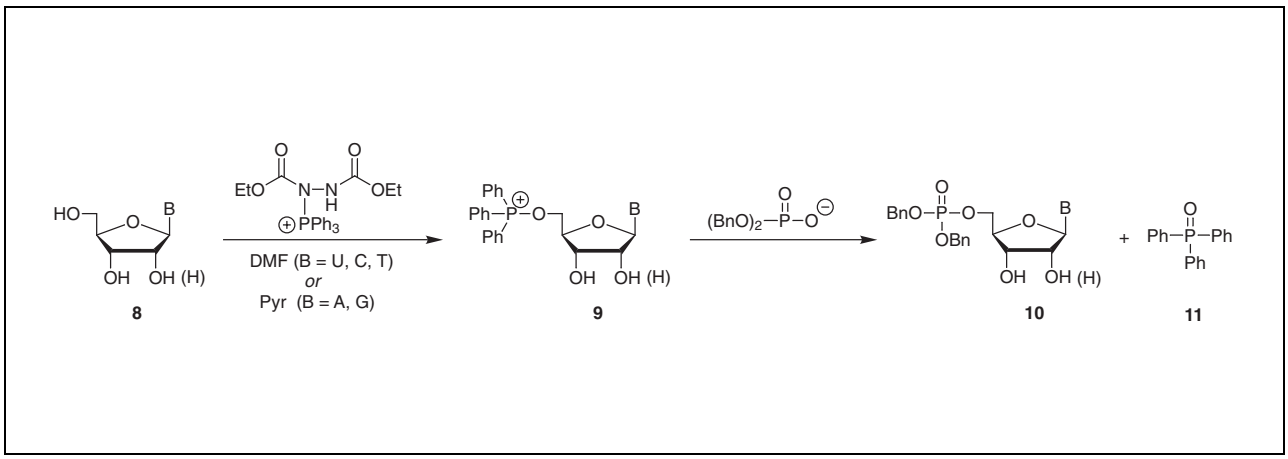

Figure 13.1.3 Electrophilic activation of nucleoside alcohols. The Mitsunobu phosphorylation. Bn, benzyl; DMF, *N,N*-dimethylformamide.

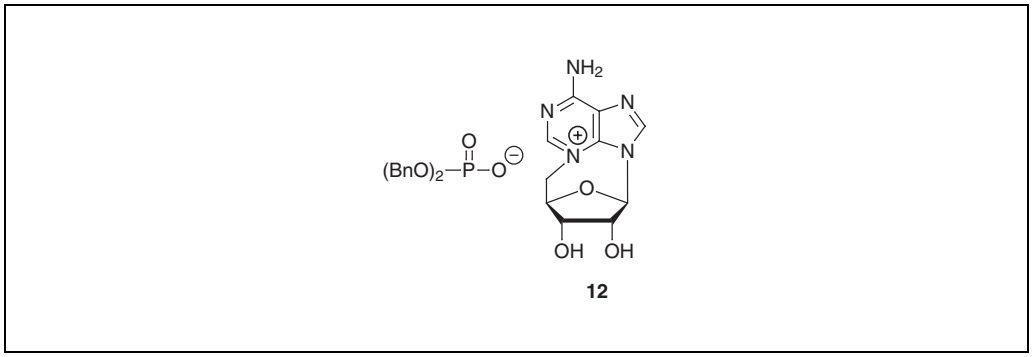

Figure 13.1.4 The N^3,5′-cyclonucleoside salt of adenosine, the product of the Mitsunobu reaction in polar aprotic solvent. Bn, benzyl.

molecular cyclization, forming N^3,5′-cyclonucleoside salts (Fig. 13.1.4). Formation of the purine cyclonucleosides can be avoided by conducting the phosphorylation reaction in anhydrous pyridine (Saady et al., 1995a). Phosphorylation of nucleosides with modified purine nucleobases (e.g., N^6-chloro- and N^6-azidoadenine) are successful in pyridine. However, anhydrous pyridine is not a suitable solvent for phosphorylation of unprotected pyrimidine nucleosides, as low yields are observed (Kimura et al., 1979). Finally, the Mitsunobu reaction is unsuitable for nucleoside 3′-phosphorylation due to competing intramolecular cyclization, resulting in the formation of anhydronucleosides.

CHEMICAL REACTIVITY OF PHOSPHORUS

Phosphorus as a Nucleophile

Phosphorous acids and ethers are ambident nucleophiles (Fig. 13.1.5), reactive on phosphorus or oxygen. Accordingly, the phosphorus coordination number, degree of esterification, and reaction conditions dictate the site of alkylation. These criteria are especially important for three-coordinate compounds of phosphorus, which will be considered first.

Phosphites are three-coordinate (σ^3) phosphorus species containing three total bonds (λ^3) and are exceptionally useful for nucleoside chemistry. Phosphites exist as either a configurationally stable structure or a pair or tautomeric structures, depending upon the extent of esterification (Fig. 13.1.5). Trialkyl phosphites are configurationally stable structures characterized by a nucleophilic phosphorus and are incapable of tautomerizing to an *H*-phosphonate form. These species are easily oxidized by electrophilic oxidants and are subject to air oxidation as well. In contrast, both dialkyl phosphites (**S.15**) and monoalkyl phosphites (**S.16**) exist primarily in the four-coordinate *H*-phosphonate (σ^4,λ^5) tautomer. The *H*-phosphonate tautomer is resistant to oxidation. Therefore, oxidation of four-coordinate *H*-phosphonates proceeds by way of the three-coordinate phosphite tautomer. In con-

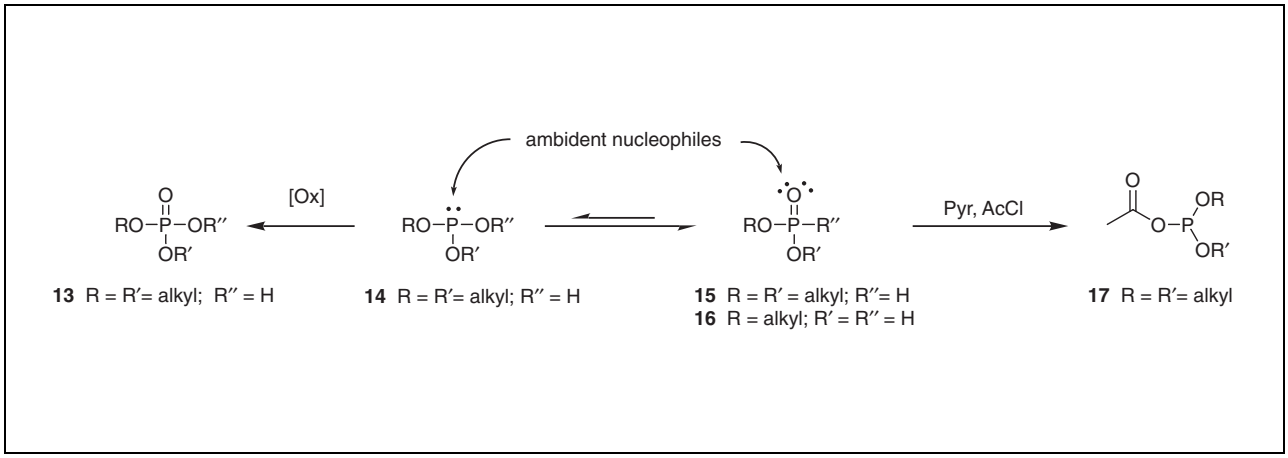

Figure 13.1.5 The principle reactive species of phosphites.

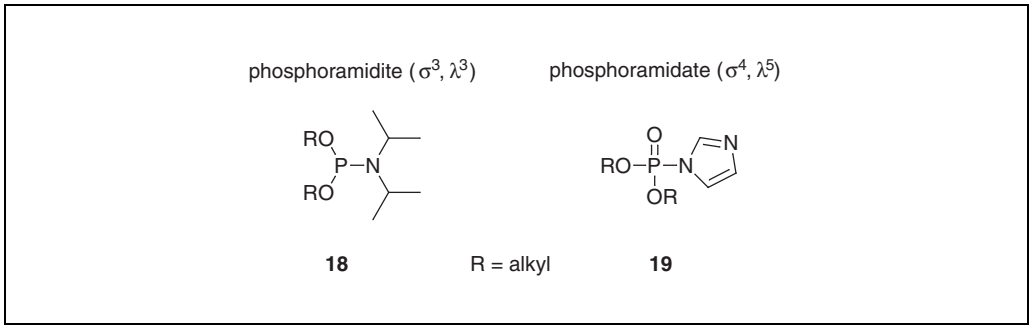

Figure 13.1.6 Examples of electrophilic amino-phosphorus compounds important in nucleoside chemistry.

trast to oxidation, *O*-alkylation occurs primarily through the *H*-phosphonate tautomer.

Acylation of *H*-phosphonates is affected by the strength of the base used. In the presence of weak bases such as pyridine, mono- and dialkyl phosphites are typically *O*-acylated forming *O*-acylphosphite ester intermediates (**S.17**). Transesterification of *O*-acylphosphite esters readily occurs in alcoholic solvents. However, in the presence of a strong base, competitive *P*-acylation of the phosphonates can occur, forming acylphosphonates (*UNIT 3.4*). Acylphosphonates readily degrade without undergoing transesterification.

Another phosphorus nucleophile is the four-coordinate, five-bond (σ^4,λ^5) phosphoric acid. Unlike *H*-phosphonates, phosphoric acid is exclusively *O*-alkylated. However, *O*-alkylation of phosphoric acid has found only modest application in the synthesis of nucleoside phosphates, due to the limited solubility of phosphoric acid in organic solvents. Biphasic solvent systems and phase-transfer catalysts have been used to facilitate phosphorylation. Alkylammonium salts of phosphoric acid are quite soluble in organic solvents and are often used in nucleotide synthesis (see Nucleosides via stable intermediates).

Phosphorus as an Electrophile

There are two coordination states common to electrophilic phosphorus, the phosphite (three-coordinate) and phosphate (four-coordinate) states. Two important amine-containing phosphorus compounds that can function as electrophiles are phosphoramidites (σ^3,λ^3) and phosphoramidates (σ^4,λ^5) (Fig. 13.1.6). Phosphoramidites are three-coordinate species with sigma bonds to two alkoxy groups and an amino functionality. Phosphoramidates are four-coordinate species containing two alkoxy groups and an amino group bound to a phosphoryl group. The amino moiety of these electrophilic phosphorus species has a P-N bond that is considerably weaker than the P-O bonds (70 versus 86 kcal/mol; Corbridge, 1995). Further, the amino moieties retain their basicity due to weak p_π-d_π bonding interactions. The amino moieties can become activated under acidic conditions to become good leaving groups. However, the basicity of the amino group can be affected by delocalization of the nitrogen

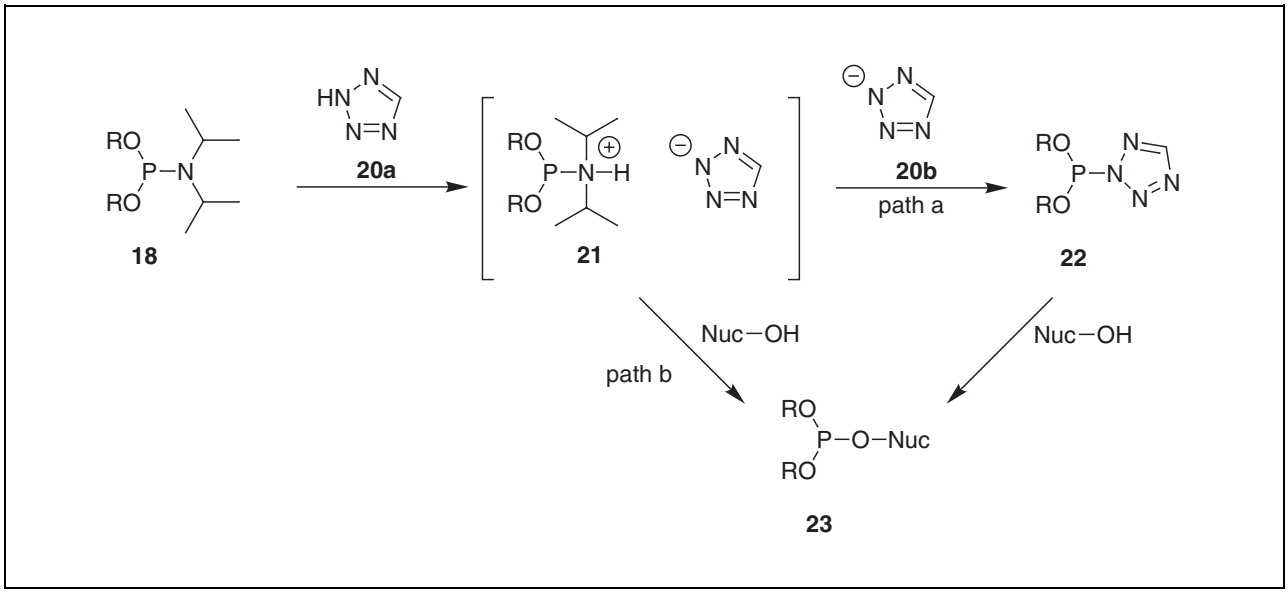

Figure 13.1.7 Mechanistic rationale of *N,N*-diisopropyl dialkyl phosphoramidite coupling with a nucleoside.

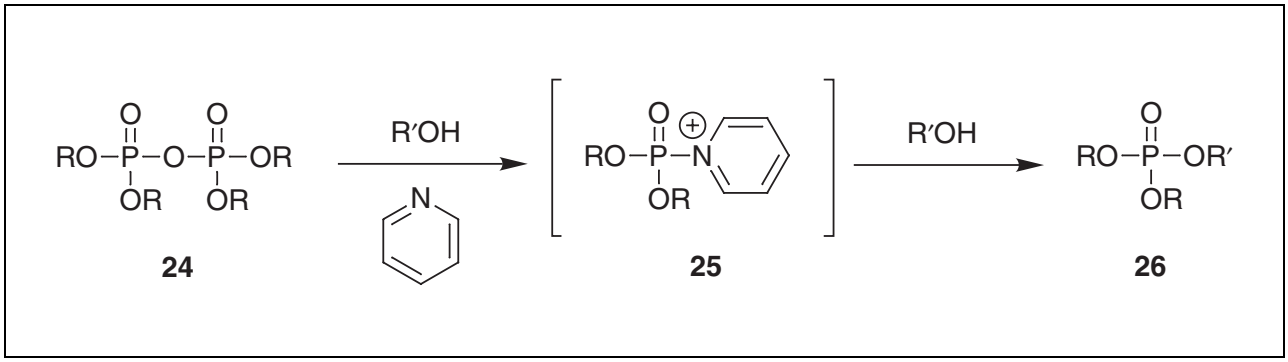

Figure 13.1.8 Tetrakis(alkyl) pyrophosphates break down in pyridine to active phosphorylating agents.

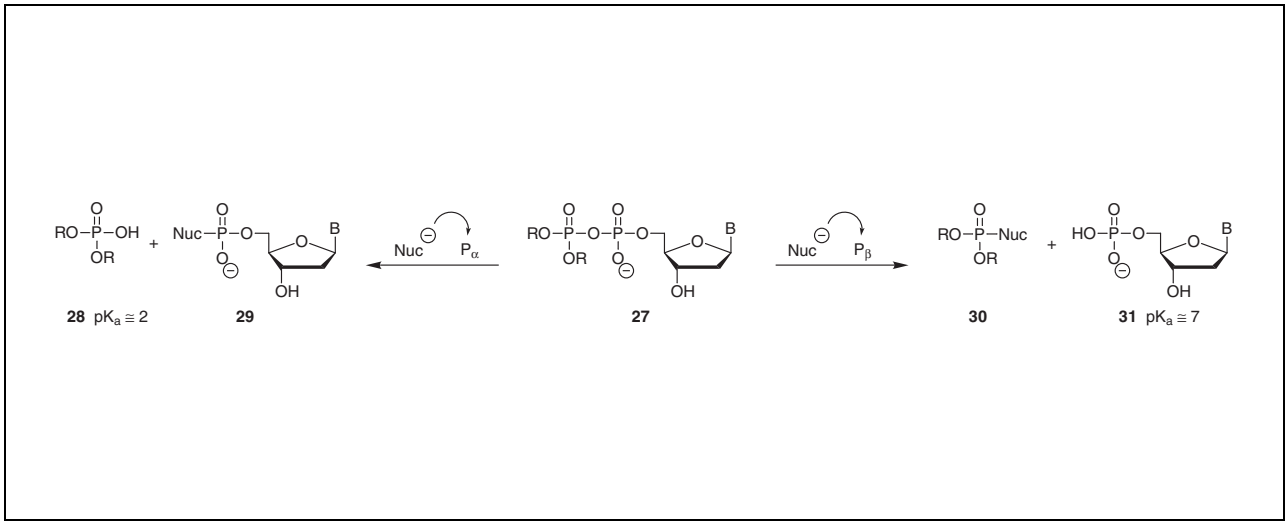

Figure 13.1.9 Selectivity in nucleophilic attack upon tris(substituted) nucleoside pyrophosphates.

lone pair into aryl substitution on the amine, engendering stability under acidic conditions. Accordingly, aliphatic amines are essential. Diisopropylamine is widely used owing to its ease and purity in preparation, as well as the balance between its chemical stability and reactivity (McBride and Caruthers, 1983). In phosphoramidate chemistry, the amino moiety is often morpholine or imidazole.

Phosphoramidates can directly combine with nucleophiles, but do so very sluggishly. In the presence of catalysts such as acidic azoles or divalent metals, phosphoramidates react much more readily with nucleophiles. Azoles such as imidazole, tetrazole, 1-methylimidazole, 4,5-dicyanoimidazole, and other nitro-substituted variations are useful as acidic catalysts. Unlike phosphoramidates (σ^4,λ^5), phosphoramidites (σ^3,λ^3) require activation by an acidic azole (e.g., 1*H*-tetrazole **S.20a**, pK_a = 4.76) to form two electrophilic intermediates: protonated phosphoramidite **S.21** and tetrazolide **S.22** (Fig. 13.1.7). Protonated phosphoramidite **S.21** can react with tetrazole anion **S.20b** to form a tetrazolide intermediate (path a) or can directly capture a nucleoside (path b). Interestingly, breakdown of phosphoramidite **S.18** is zero order in alcohol and second order in tetrazole (Nurminen et al., 1998).

Other common electrophilic phosphorus motifs are phosphorus halides and phosphate anhydrides. Unlike phosphorus amines, phosphorous halides and phosphate anhydrides do not require activation for rapid reaction with nucleophiles. The general structure of a tetrakis(alkyl) pyrophosphate **S.24** is shown in Figure 13.1.8. Because the reaction of an alcohol with tetrakis(alkyl) pyrophosphates is sluggish, a base is generally used to reduce the time of reaction. Pyridine cleaves tetrakis(alkyl) pyrophosphates at either phosphoryl center, forming dialkyl phosphate and the phosphoryl-pyridinium species **S.25**, an active phosphorylating agent.

Unlike tetrakis(alkyl) pyrophosphate, in tris(substituted) nucleoside pyrophosphates (**S.27**; Fig. 13.1.9) either phosphoryl center (P_α or P_β) can be attacked by a nucleophile. The selectivity is based upon which phosphoryl center is the better leaving group. A phosphoryl center becomes a better leaving group when it is part of the more acidic phosphate (e.g., **S.28** versus **S.31**). Increased electron withdrawing characteristics of the alkyl substituents will make a phosphate more acidic and, hence, a better leaving group. Therefore, the phosphoryl center of the weaker acid (i.e., **S.29**) is attacked by nucleophiles in tris(substituted) nucleoside pyrophosphates (Fig. 13.1.9).

In principle, phosphates such as phosphoric acid and its cyclic-ester trimer (trimetaphosphate) contain electrophilic phosphoryl centers and should undergo transesterification reactions with alcohols. In practice, this reaction finds limited application. However, the reaction of trimetaphosphate with unprotected ribonucleosides is noteworthy. Monophosphorylation of either the 2′- or 3′-hydroxyl group of unprotected ribonucleosides (A, C, G, U) can be achieved in aqueous base. The reaction requires elevated temperatures if sodium trimetaphosphate is used (Tsuhako et al., 1984), but can be conducted at room temperature with tris(tetramethylammonium) trimetaphosphate (Saffhill, 1970). Regardless of which trimetaphosphate is used, prolonged reaction times are required (up to 4 days). Aside from the prolonged reaction times, the reaction suffers from a lack of regioselectivity. Mixtures of 2′-monophosphate and 3′-monophosphate nucleosides are obtained. Interestingly, deoxyribonucleosides are not phosphorylated by trimetaphosphate.

REAGENTS USED IN SYNTHETIC NUCLEOTIDE SYNTHESIS

Phosphorus Oxychloride

Phosphorylation of nucleosides with phosphorus oxychloride ($POCl_3$) initially yields a nucleoside phosphorodichloridate. Phosphorodichloridates such as **S.32** (Fig. 13.1.10) can be hydrolyzed, providing the nucleoside monophosphate (not shown). The reaction is conducted in a trialkylphosphate solvent with an excess of $POCl_3$ at a low temperature (–5°C). However, phosphorylation under these conditions leads to mixtures of 5′- and 2′(3′)-nucleoside phosphates. Protection of the vicinal diols in ribonucleosides alleviates regioselectivity problems. Thus, phosphorylation of ketal-protected nucleosides proceeds in high yield (Yoshikawa et al., 1967). Unprotected nucleosides can be regioselectively 5′-phosphorylated by modifying the reaction conditions described above. The addition of water to the phosphorylating reagent results in selective 5′-phosphorylation of unprotected nucleosides in moderate to high yield (Yoshikawa et al., 1967). Despite the acidity of the reaction medium, depurination has not been reported to be a significant side-reaction.

Phosphorus oxychloride is also used to synthesize nucleoside triphosphates. However, construction of nucleoside diphosphates has

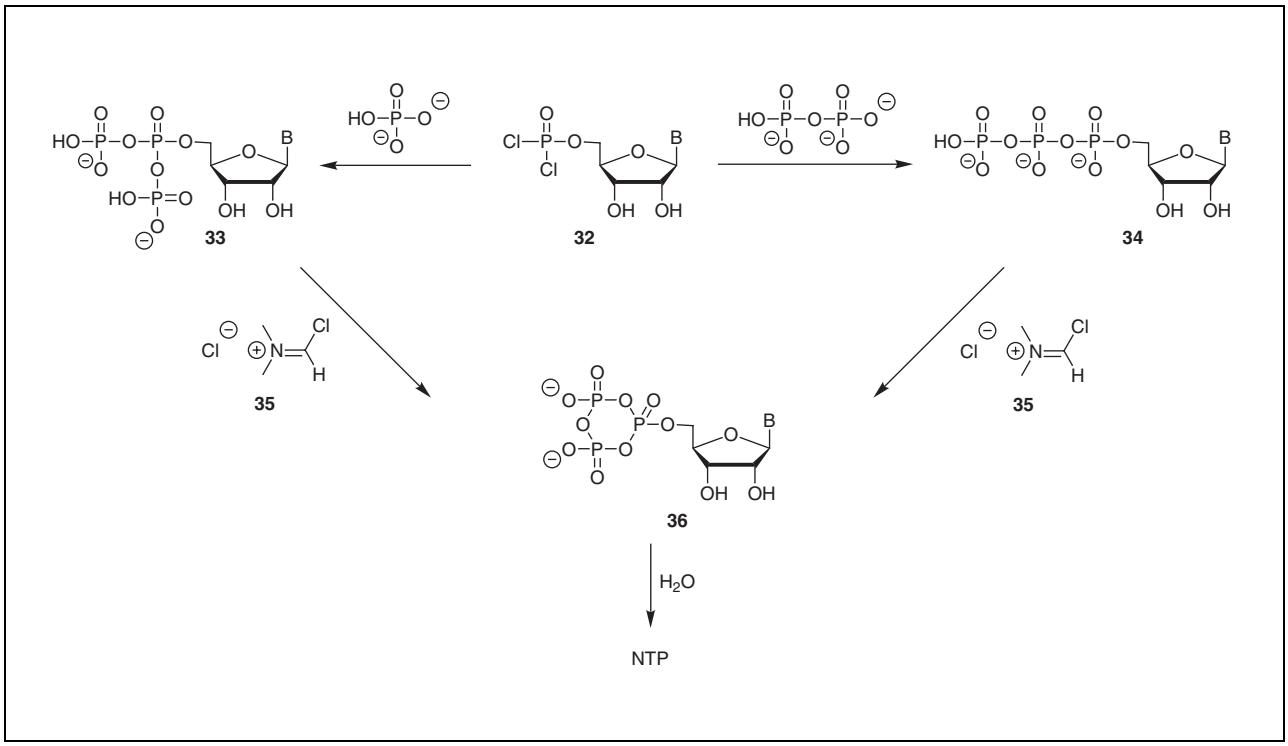

Figure 13.1.10 Synthesis of nucleoside triphosphates from phosphorodichloridates via trimetaphosphate intermediates.

not been reported with this reagent. To understand why, it is beneficial to understand the mechanistic rationale behind the synthesis of nucleoside triphosphates using $POCl_3$.

As shown in Figure 13.1.10, nucleoside triphosphates are synthesized by adding a tributylamine/DMF solution of either inorganic *ortho*-phosphate (Mishra and Broom, 1991) or pyrophosphate (Ludwig, 1987) to the nucleoside phosphorodichloridate **S.32**. *ortho*-Phosphate or pyrophosphate displaces chloride from nucleoside phosphorodichloridate **S.32**, forming intermediate **S.33** or **S.34**, respectively. Excess $POCl_3$ present in the reaction medium combines with DMF to form the Vilsmeier reagent (**S.35**), which promotes the dehydration of intermediates **S.33** and **S.34**, resulting in the formation of nucleoside 5′-trimetaphosphate (**S.36**; Glonek et al., 1974). Hydrolysis of the trimetaphosphate intermediate under buffered conditions gives the NTP as a stable product.

Phosphorylation of nucleosides containing non-natural nucleobases is possible with the $POCl_3$/trialkylphosphate reagent combination (Nairne et al., 2002). However, modifications of the nucleobase that are sensitive to acid (e.g., alkynyl substituents) are not always compatible with the $POCl_3$ reagent. Addition of the proton sponge 1,8-bis(dimethylamino)naphthalene has been reported to mitigate side-reactions promoted by the acidity of the reaction medium. Unexpectedly, the addition of the proton sponge reduced the reaction time for phosphorylation from 12 hours to 2 hours (Kovacs and Otvos, 1988). Finally, the $POCl_3$ methodology is not particularly suitable for selectively introducing radiolabels at the P_β or P_γ phosphoryl of nucleoside triphosphates. This is because hydrolysis of trimetaphosphate **S.36** is nonselective. Hydrolysis can occur at either the P_β or $P_{\beta'}$ position, leading to a mixture of products.

Phosphorochloridites and Phosphorochloridates

In general, both phosphorochloridite [$(RO)_2PCl$] and phosphorochloridate [$(RO)_2POCl$] reagents are *O*-protected. There are many protecting groups that have been developed. In the case of phosphorochloridite reagents, the protecting groups are removed after oxidation of the phosphite triester. Therefore, protecting groups need to be stable to oxidants such as peroxides, organic peracids, and molecular halogens. Protecting groups have been developed that are labile to acid, base, or alternate conditions (e.g., reductive fission or photolysis). The functionality inherent in the nucleoside will determine which protecting group is most suitable for any given application. Nucleosides with modifications in the nucleobase require the most judicious

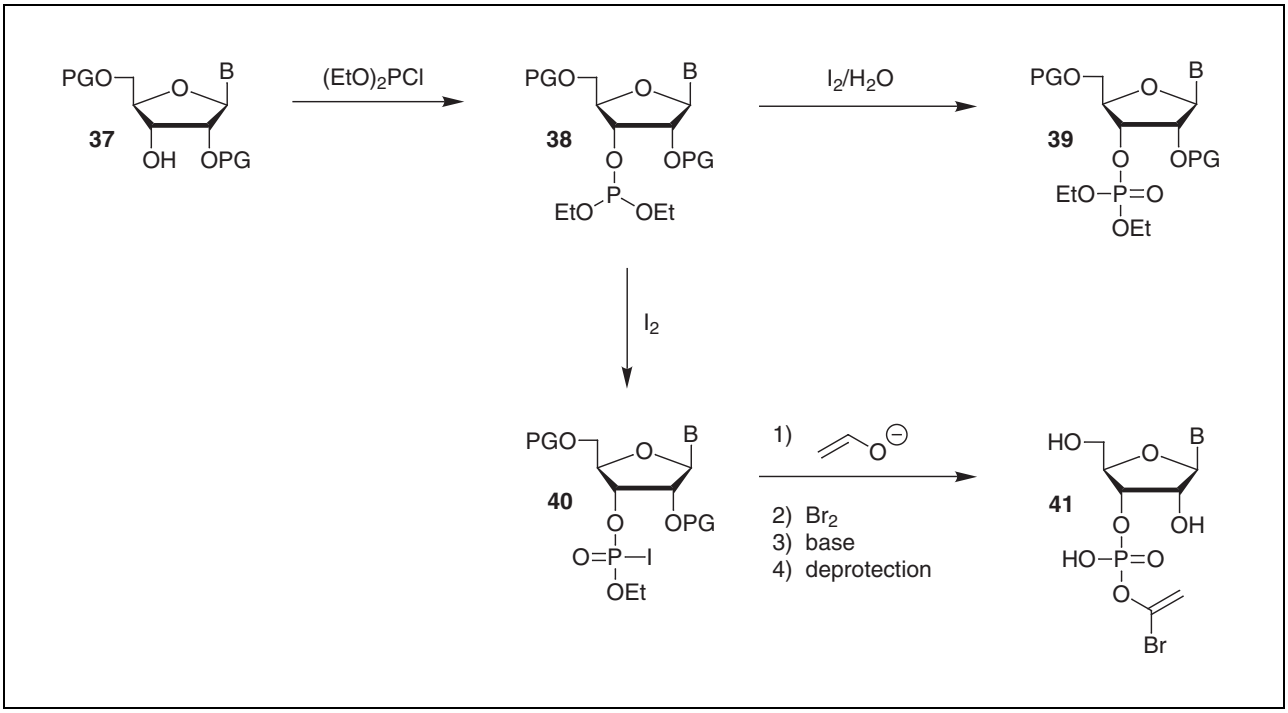

Figure 13.1.11 Phosphitylation as a method for NMP synthesis. PG, protecting group.

choice of protecting group, as the common nucleobases (A, C, T, G, U) are fairly compatible with many deprotection conditions.

Phosphorochloridite reagents can be used to synthesize nucleoside monophosphates. The general strategy is outlined in Figure 13.1.11. Phosphitylation of protected nucleoside **S.37** with diethyl phosphorochloridite produces the phosphite triester **S.38**. Oxidation of the phosphite triester is usually effected by peroxide, organic peracid, or aqueous iodine. Following oxidation, the trialkyl phosphate **S.39** is then deprotected to provide the nucleoside monophosphate (not shown). However, phosphite intermediate **S.38** can be oxidized with iodine in situ to give halophosphate **S.40**. Various nucleophiles can be trapped by **S.40**, and appropriate deprotection then affords the nucleoside diester **S.41**. This dual phosphitylation/phosphorylation strategy is useful for preparation of nucleoside diesters that are inaccessible by standard phosphoramidite methodology, such as nucleoside-enol diester **S.41** (Stowell and Widlanski, 1995).

The synthesis of nucleoside monophosphates via phosphorochloridites is conceptually straightforward, but can be experimentally challenging. First, phosphorochloridites are air-sensitive reagents that are usually prepared in low yield and purified by vacuum distillation. Second, phosphite triesters are labile towards acid. This can be problematic if the phosphite triester is purified by silica-gel chromatography. Including triethylamine (1% to 2%) in the eluent, however, usually prevents significant decomposition of the phosphite triester during purification. Last, phosphite triesters can air-oxidize, which is essentially an irreversible chemical modification.

Phosphorochloridites can be used to synthesize nucleoside triphosphates as well. In Figure 13.1.12, phosphitylation of a protected nucleoside (**S.42**) with salicyl phosphorochloridite (**S.43**) is carried out in pyridine/dioxane or pyridine/DMF. The phosphite triesters produced are diastereomers and are not isolated; rather, transesterification of the salicyl moiety is effected by adding a solution of bis(tri-*n*-butylammonium) pyrophosphate/tributylamine in DMF. Complete transesterification leads to the formation of the cyclic phosphite species **S.45**. Oxidation of phosphite **S.45** with aqueous iodine produces nucleoside 5′-trimetaphosphate. Concomitant hydrolysis of the nucleoside 5′-trimetaphosphate yields protected nucleoside triphosphates as the product. The ester protecting groups used to protect the nucleoside are removed by aminolysis. By this process, TTP was synthesized from the corresponding protected nucleoside in 72% yield (Ludwig and Eckstein, 1989).

Phosphorochloridite reagents are known to phosphitylate nucleobases at ambient temperatures but not at low temperatures (–78°C; Imai

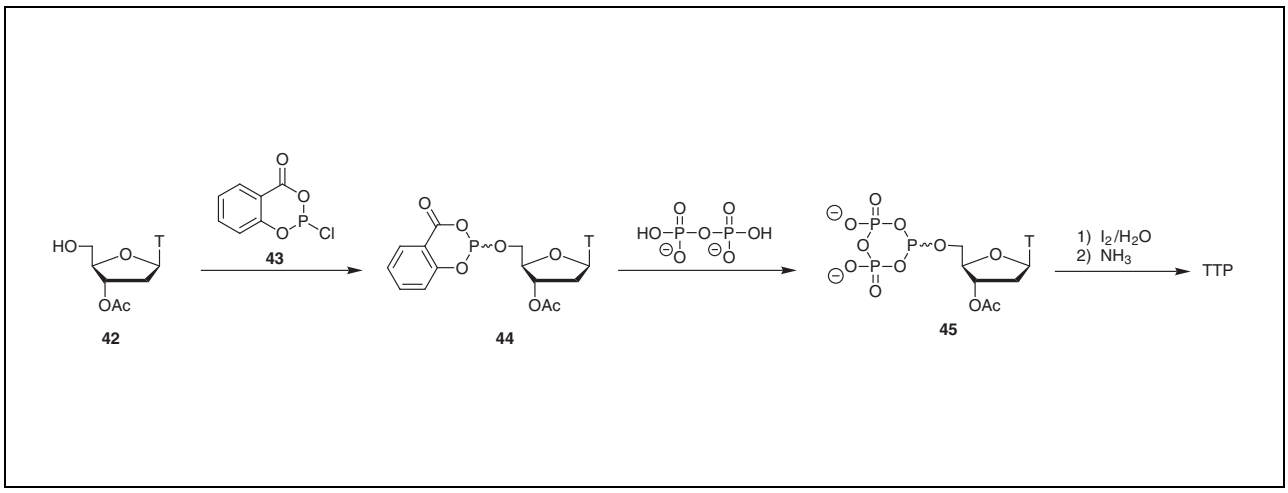

Figure 13.1.12 Salicyl phosphorochloridite. A useful reagent for NTP synthesis.

and Torrence, 1981). Given that temperature is a factor in nucleobase phosphitylation, the experimental conditions under which salicyl phosphorochloridite is used may promote nucleobase phosphitylation. The extent to which salicyl phosphorochloridite may phosphitylate nucleobases has not been thoroughly investigated. Experimental data in the original paper do not conclusively rule out nucleobase phosphitylation as a source of reduced yield. As an aside, *N*-protected nucleobases are recommended during construction of nucleoside *H*-phosphonates using salicyl phosphorochloridite (*UNIT 2.6*).

Nucleobase phosphitylation can be of particular concern when constructing nucleotides that have nucleobase modifications. Typical yields for phosphorylation of nucleosides with modified nucleobases are <45%, with yields in the 15% to 30% range being common (Jurczyk et al., 1999; Trevisiol et al., 2000). Nucleobase phosphitylation may also be a concern when using salicyl phosphorochloridite with oligodeoxyribonucleotides. Recent application of the salicyl phosphorochloridite methodology on solid support provided a low yield (15% to 30%) of the desired 5′-triphosphate-capped oligonucleotide (Lebedev et al., 2001). Finally, due to the intermediacy of the nucleoside 5′-trimetaphosphate, this methodology is unlikely to be used for selective radiolabeling of nucleoside triphosphates at the P_β or P_γ positions.

Phosphorochloridates share many similarities with phosphorochloridites. For example, many of the same protecting groups are used and similar side reactions are observed. However, phosphorochloridates provide direct access to nucleoside dialkyl-phosphates without the need for oxidation. Reactions with phosphorochloridates can be sluggish, requiring prolonged reaction times. The reaction time can be reduced through use of a nucleophilic catalyst such as 1-methylimidazole or iodide ion (Stromberg and Stawinski, 1987). These catalysts function by forming a more electrophilic phosphorylating agent. However, increasing the electrophilicity of the phosphorochloridate also results in increased nucleobase phosphorylation. Protecting groups that are strongly electron withdrawing also increase the electrophilicity of the phosphorochloridate.

Unlike their three-coordinate counterparts, four-coordinate phosphorochloridates can be used to synthesize nucleoside 3′- or 5′-monophosphates from *N*-unprotected nucleosides. Chemo- and regioselective phosphorylation can be affected through "functional group activation" (Uchiyama et al., 1993). Treatment of an appropriate hydroxyl-protected, *N*-unprotected nucleoside with *tert*-butylmagnesium chloride results in the formation of a nucleoside alkoxide (Fig. 13.1.13). Group I nucleosides (adenosine and cytidine) require only one equivalent of strong base, whereas group II nucleosides (guanosine, thymidine, uridine) require two equivalents of base. Upon treatment of the alkoxide with phosphorochloridate, the nucleoside-dialkyl-phosphate is obtained in high yield. Competition experiments demonstrate the preferential deprotonation of the hydroxyl functionality over nucleobase functionality for group I nucleosides. This deprotonation is expected because the sugar hydroxyl is more acidic than the nucleobase functionality.

Chemoselectivity for group II nucleosides is not explicable based upon acidity. The 3′-magnesium alkoxide bis-protected thymidine **S.54** (Fig. 13.1.14) is preferentially phospho-

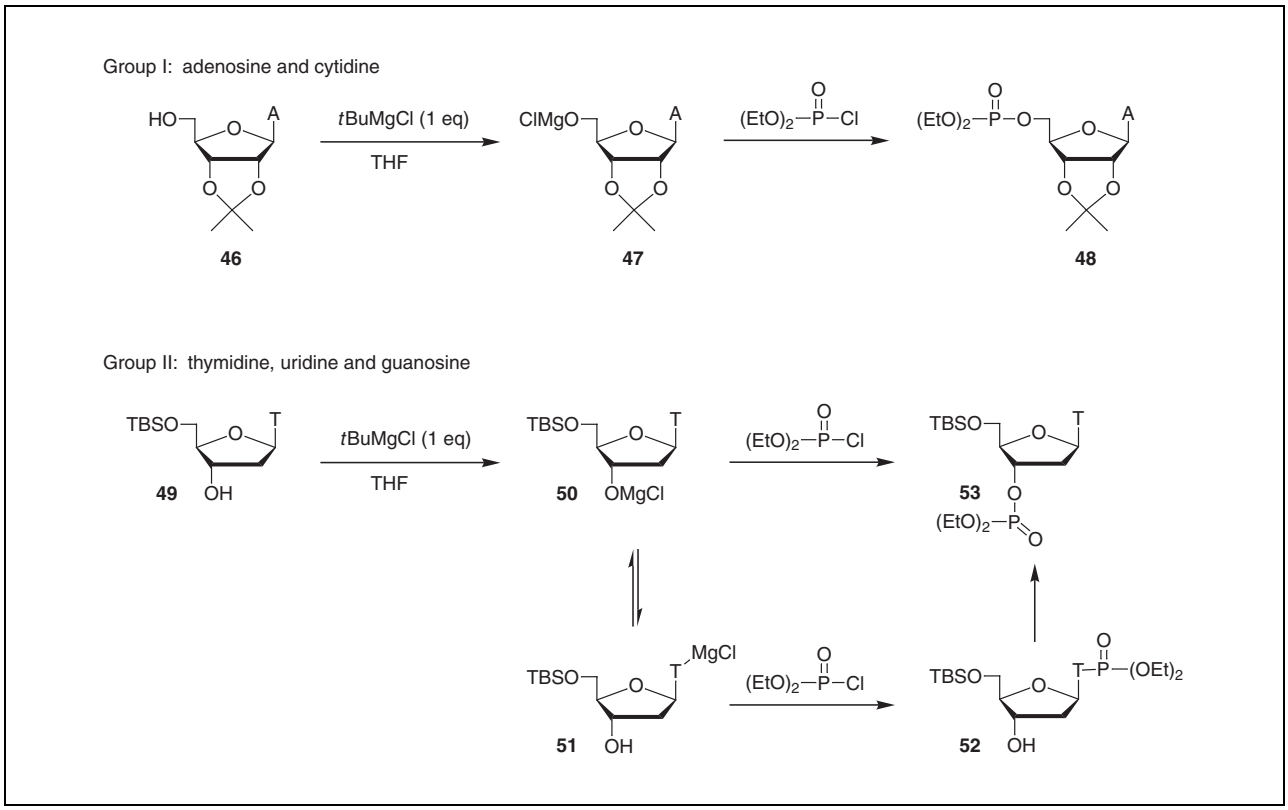

Figure 13.1.13 Grignard-promoted chemoselective phosphorylations based upon acidity (group I) or reversibility (group II). TBS, *tert*-butyldimethylsilyl.

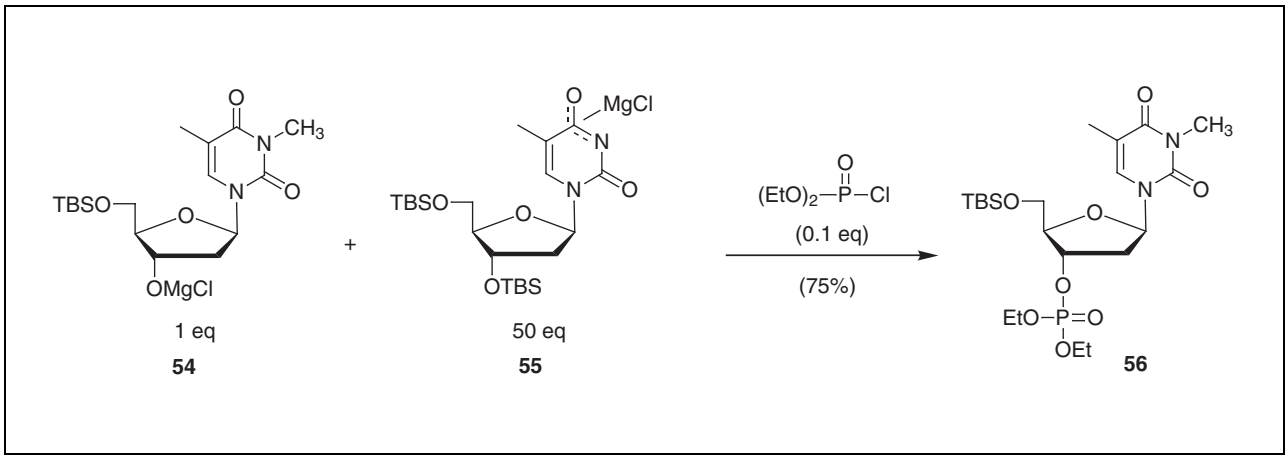

Figure 13.1.14 Competition experiment establishing alkoxide as the more reactive phosphorochloridate acceptor. TBS, *tert*-butyldimethylsilyl.

rylated by a limiting amount of diethyl phosphorochloridate in the presence of a 50-fold excess of N^5-magnesium iminoxide bis-protected thymidine **S.55**. This result corresponds to the more basic magnesium alkoxide being phosphorylated in preference to the weaker magnesium iminoxide base. Interestingly, treatment of 5′-protected thymidine **S.49** with one equivalent of base results in phosphorylation of the iminoxide functionality (Fig. 13.1.13). However, formation of phosphoramidate **S.52** is reversible. Intermolecular interconversion provides exclusively the *O*-phosphorylated product **S.53** after 96 hr.

Phosphoramidites and Phosphoramidates

Phosphoramidites (σ^3,λ^3) can be used for the synthesis of NMPs, although their most popular application is found in the synthesis of oligodeoxyribonucleotides (*UNIT 3.3*). Unlike phosphoramidates (σ^4,λ^5), the construction of a nucleotide from phosphoramidites requires a minimum of two synthetic steps. The first step

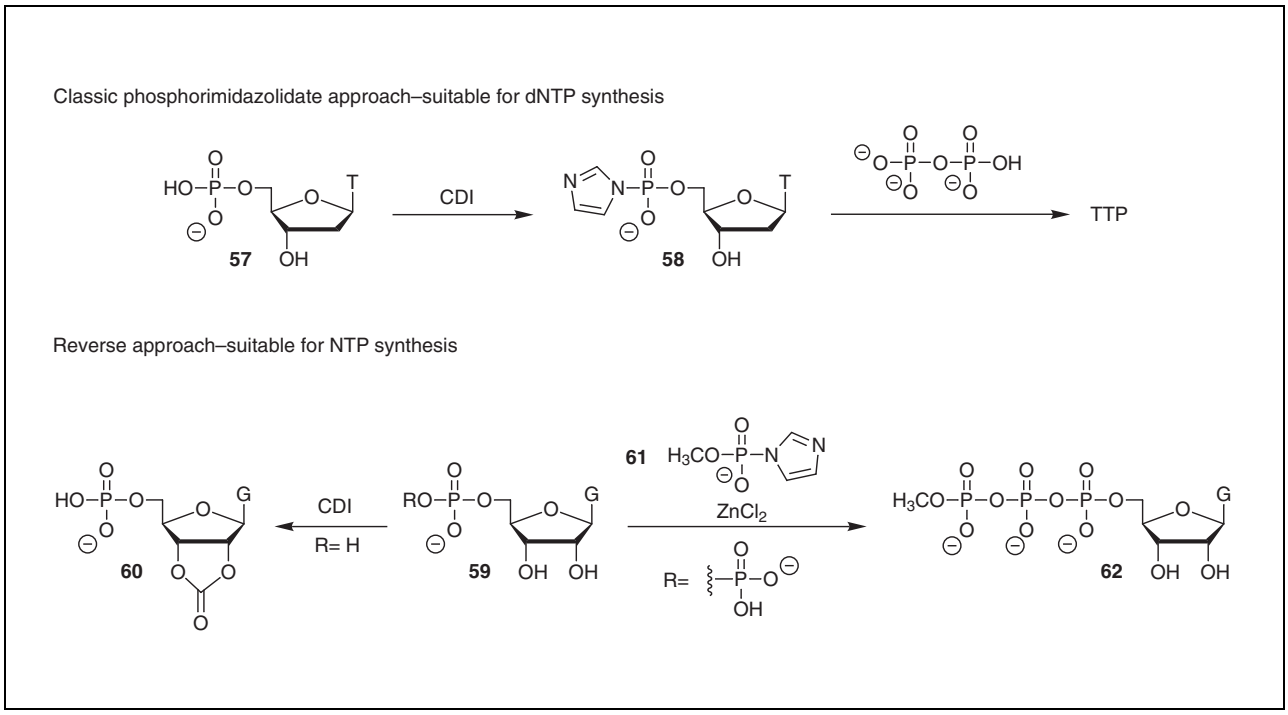

Figure 13.1.15 Phosphorimidazolidates as a phosphorylation strategy. CDI, carbonyldiimidazole.

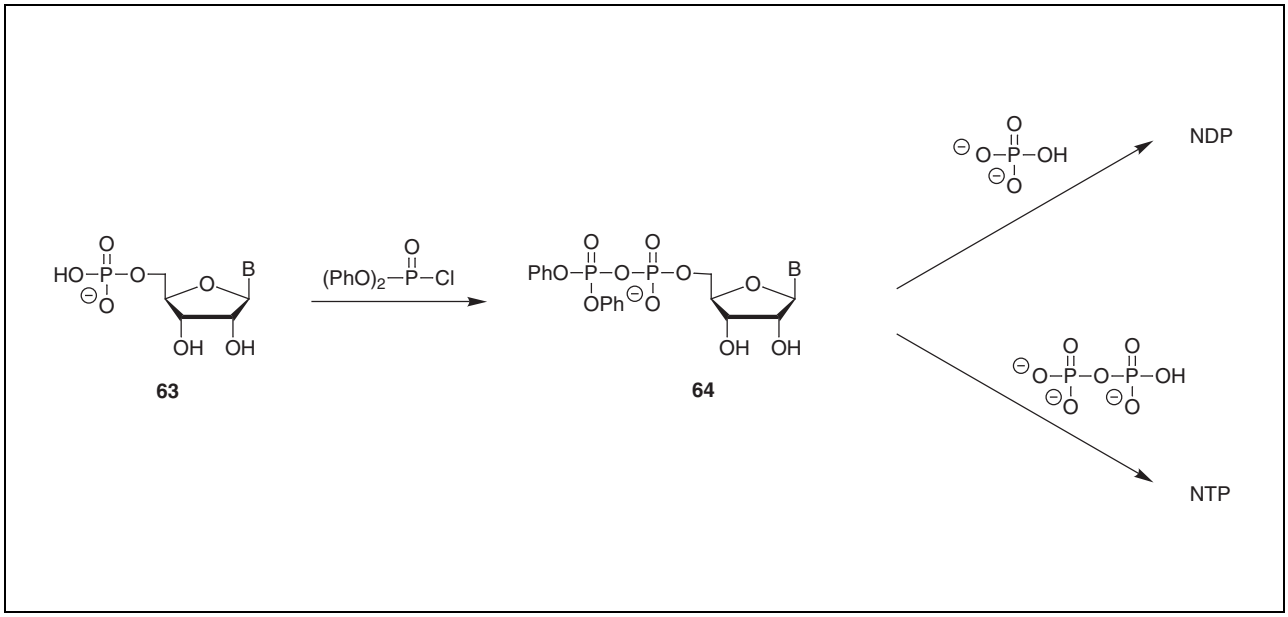

Figure 13.1.16 The anion exchange strategy for synthesis of NDPs and NTPs.

is the coupling of a phosphoramidite to a nucleoside, forming a nucleoside-dialkyl-phosphite, followed by oxidation to form the nucleoside-dialkyl-phosphate. A nucleophilic functionality in the nucleobase may be oxidized under such conditions. The N1 and N3 positions of adenosine and cytidine, respectively, are examples of nucleobase moieties prone to oxidation. Fortunately, appreciable oxidation of the nucleobase is not observed when mild oxidants are used (e.g., I_2/H_2O or *t*BuOOH).

Phosphoramidate NMPs are finding recent application as NMP prodrugs (Zemlicka, 2002), as well as precursors for constructing NDPs and NTPs. A classic method for the synthesis of dNTPs via nucleoside phosphoramidates involves the activation of an NMP with carbonyldiimidazole (CDI) to give the nucleoside phosphorimidazolidate **S.58**

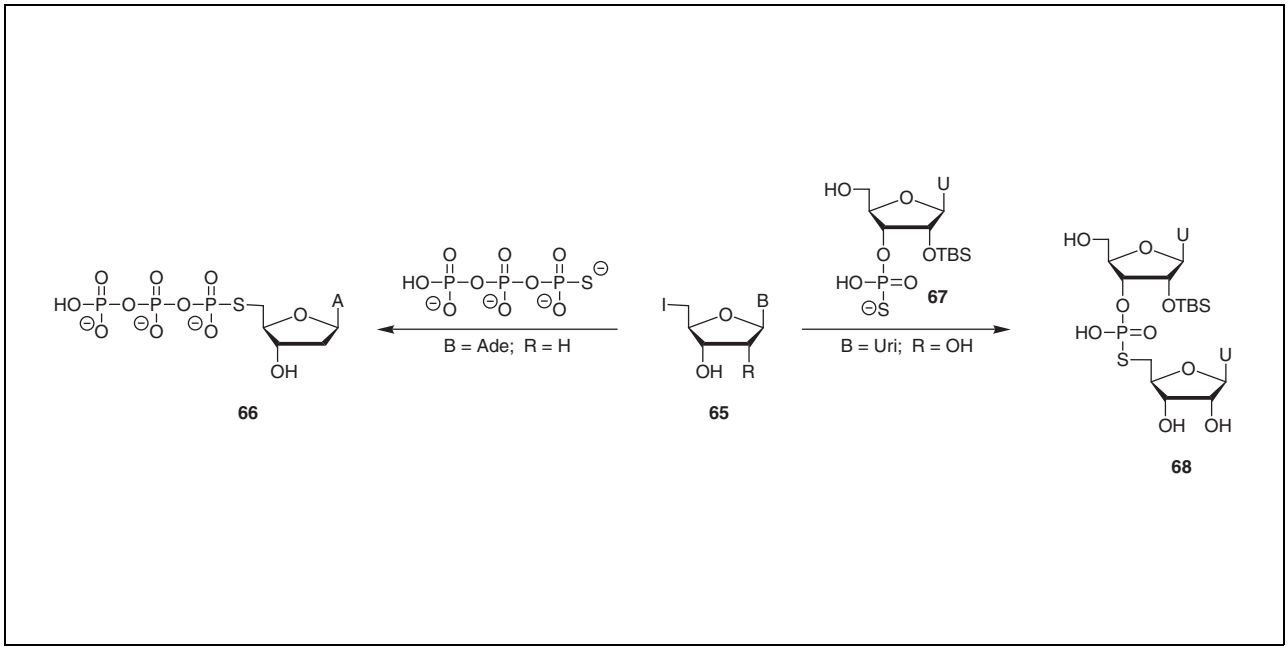

Figure 13.1.17 Synthesis of nucleoside phosphorothiol esters by the displacement strategy. TBS, *tert*-butyldimethylsilyl.

(Fig. 13.1.15). Addition of acidic pyrophosphate activates the phosphorimidazolidate toward nucleophilic displacement by pyrophosphate, giving the dNTP (Hoard and Ott, 1965). This method is rarely used for the synthesis of ribonucleoside triphosphates. Ribonucleosides with unprotected vicinal diols (**S.59**) readily form cyclic carbonates (**S.60**) when treated with CDI. Additionally, nucleoside phosphorimidazolidates can react sluggishly with phosphate or pyrophosphate, requiring prolonged reaction times or the use of catalysts such as 1-methylimidazole or divalent cations (Mn^{II}, Cd^{II}). Reversal of the general strategy, such that the NDP is the nucleophile and the phosphorimidazolidate (e.g., **S.61**) is the electrophile, results in a high yield of the desired NTP without the need for long reaction times. This strategy has been used with success for the synthesis of P_γ-methyl-capped guanosine 5′-triphosphates (e.g., **S.62**) in the presence of $ZnCl_2$ (Kadokura et al., 1997).

Di- and Triphosphates from Monophosphates: Use of Phosphorus Anhydrides

Michelson (1964) described a general method termed "anion exchange" for the synthesis of NDP, NTP, and P^1-nucleoside-5′-P^2-sugars. The method is compatible with unprotected nucleotides and gives good yields. In short, an alkylammonium NMP is phosphorylated with diphenyl phosphorochloridate (Fig. 13.1.16). The resultant nucleoside mixed phosphoanhydride (**S.64**) is exposed to the alkylammonium salt of phosphate or pyrophosphate in pyridine solution to give the NDP or NTP, respectively. The reaction is quite selective for the displacement of diphenylphosphate from the mixed phosphoanhydride **S.64**. The presence of the anionic oxygen on the P_α of **S.64** renders the nucleoside phosphate a weaker acid than diphenylphosphate. The product pyrophosphate is resistant to cleavage because the product possesses relatively poor leaving groups.

NUCLEOTIDE ANALOGS

Analogs may be derived by modification of the sugar, base, or phosphorus component of a nucleotide. Phosphate analogs have great utility for studying the mechanism and stereospecificity of enzyme-catalyzed reactions and for investigating biochemical pathways. Sugar and nucleobase analogs are also used widely as therapeutic agents and mechanistic probes. A method for synthesis of the triphosphate of modified nucleosides by a variation of the Yoshikawa procedure is presented in *UNIT 1.5*. The following section will focus on methodologies and issues associated with the synthesis of analogs of the phosphorus component of nucleotides.

Thio-Phosphorus Derivatives

Synthesis of thio-phosphorus derivatives of nucleotides are particularly problematic. The sulfur atom can occupy either a bridging (phos-

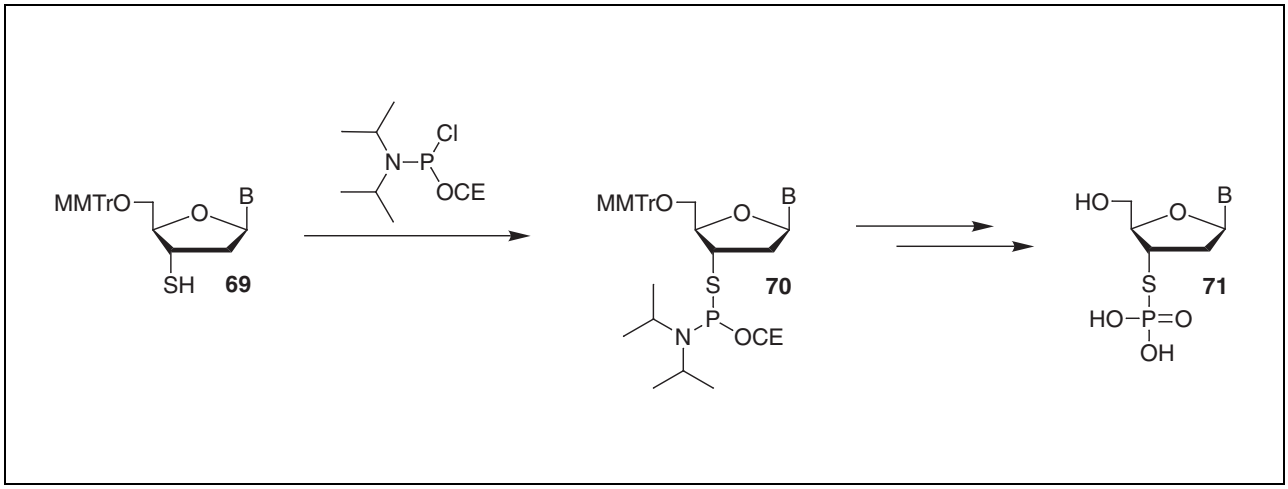

Figure 13.1.18 A phosphoramidite approach toward the synthesis of phosphorothiol esters. CE, cyanoethyl; MMTr, 4-monomethoxytrityl.

phorothiol ester) or a non-bridging (phosphorothioate) position in the phosphate component of the nucleotide. To appreciate the difficulties associated with thio-phosphorus nucleotide synthesis, a brief discussion of the properties of thio-phosphorus acids is warranted.

Thio-phosphorus acids have similar acidity to the corresponding phosphate. For example, diethyl phosphate and *O,O*-diethyl phosphorothioate have pK_a's of 1.37 and 1.49, respectively (Quin, 2000). There are several competing factors that affect the acidity of a phosphorothioate. Although the sulfur atom is larger and should stabilize the conjugate base better than oxygen, competing effects such as solvation, substituent effects, and bond strengths also affect the acidity of thio-phosphorus acids.

Synthesis of nucleoside phosphorothiol esters

Bridging phosphorothiol esters are attractive as phosphate surrogates because they are achiral, thus reducing molecular complexity and easing purification. Several methods have been used to access these compounds, including phosphoramidite methodology and displacement reactions at electrophilic 5′-carbons. However, the majority of bridging phosphorothiol esters are synthesized in oligonucleotides. Nevertheless, the methods should be applicable to the synthesis of nucleosides containing a phosphorothiol ester linkage.

Both nucleoside 5′-tosylates and 5′-iodides have been displaced with phosphorothioates. The reactions are usually conducted in DMF and require prolonged reaction times (24 to 48 hr). Both purine and pyrimidine bases have been used with or without base protection. Syntheses of 5′-thiotriphosphates of uridine and adenosine have been described. Unlike the synthesis of ATP, synthesis of 2′,5′-dideoxyadenosine-5′-thiotriphosphate (**S.66**; Fig. 13.1.17) by displacement of the 5′-iodide of nucleoside **S.65** was accomplished in poor yield (21%). A similar yield was reported for 5′-deoxyuridine-5′-thiotriphosphate (19%; Patel and Eckstein, 1997). Construction of dinucleotides containing a 5′-thioester linkage (e.g., **S.68**) have also been carried out by displacement reactions. Yields are similar for displacement of 5′-tosylates or 5′-iodides by de-

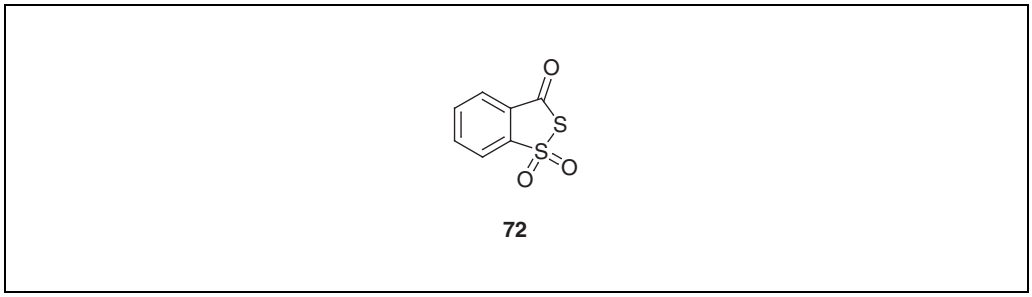

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Figure 13.1.19 3*H*-1,2-Benzodithiol-3-one 1,1-dioxide. A soluble alternative to elemental sulfur.

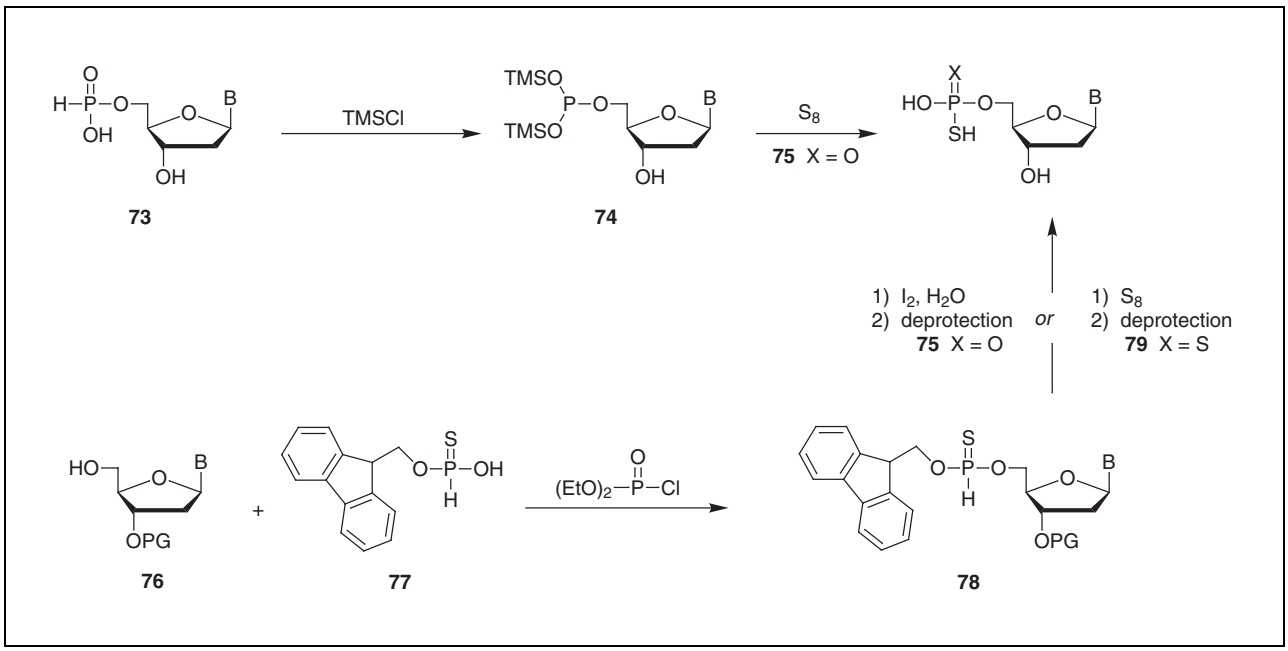

Figure 13.1.20 Synthesis of phosphorothioate nucleosides from *H*-phosphonate precursors. PG, protecting group; TMS, trimethylsilyl.

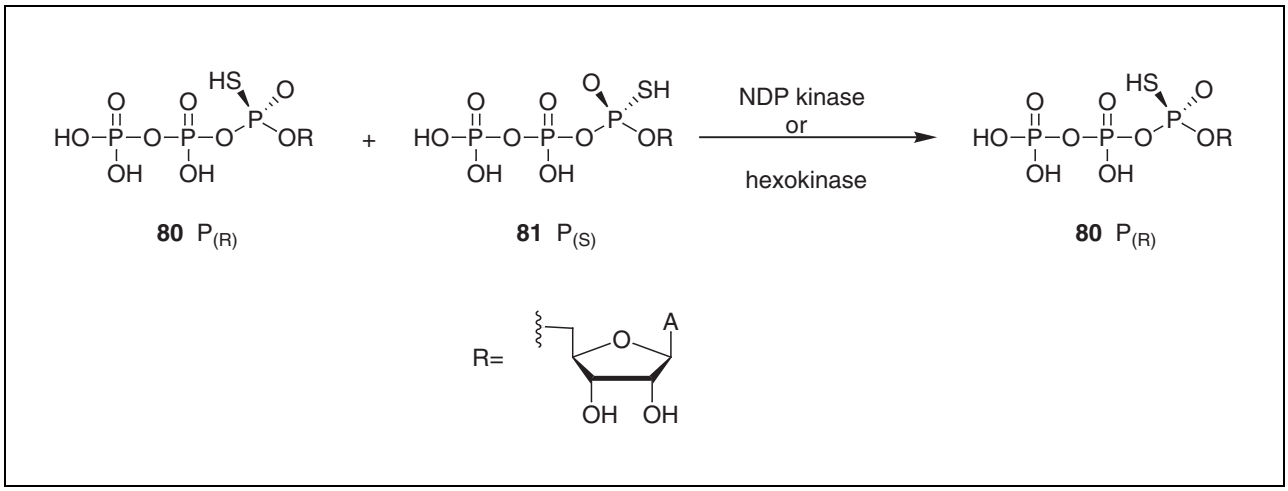

Figure 13.1.21 Selective enzymatic degradation of contaminating diastereomers is a common method to separate mixtures of $P_{(R)}$ and $P_{(S)}$ triphosphorothioates.

oxyribo- and ribonucleoside 3′-phosphorothioates, respectively (Chladek and Nagyvary, 1972; Thomson et al., 1996).

The phosphoramidite approach appears to be a more viable entry into 3′- or 5′-thionucleotides. However, most applications using phosphoramidites are relevant to the construction of oligonucleotides containing bridging phosphorothiol esters and not 5′-thionucleotides. One approach to obtain bridging phosphorothiol esters is outlined in Figure 13.1.18 (Cosstick and Vyle, 1990). Modification of this approach should be suitable for high-yield syntheses of 3′- or 5′-thionucleotides, although this avenue has not been fully explored.

Synthesis of nucleoside phosphorothioates

Nucleoside phosphorothioates are commonly accessed either by oxidation of trialkyl phosphites with sulfur, or by condensation with protected thiophosphoryl agents. Hydrolysis of the alkyl protecting groups produces the nucleotide phosphorothioate.

Nucleoside trialkylphosphites are commonly accessed via phosphoramidite methodology (see Phosphoramidites and Phosphoramidates). Oxidation of the trialkyl

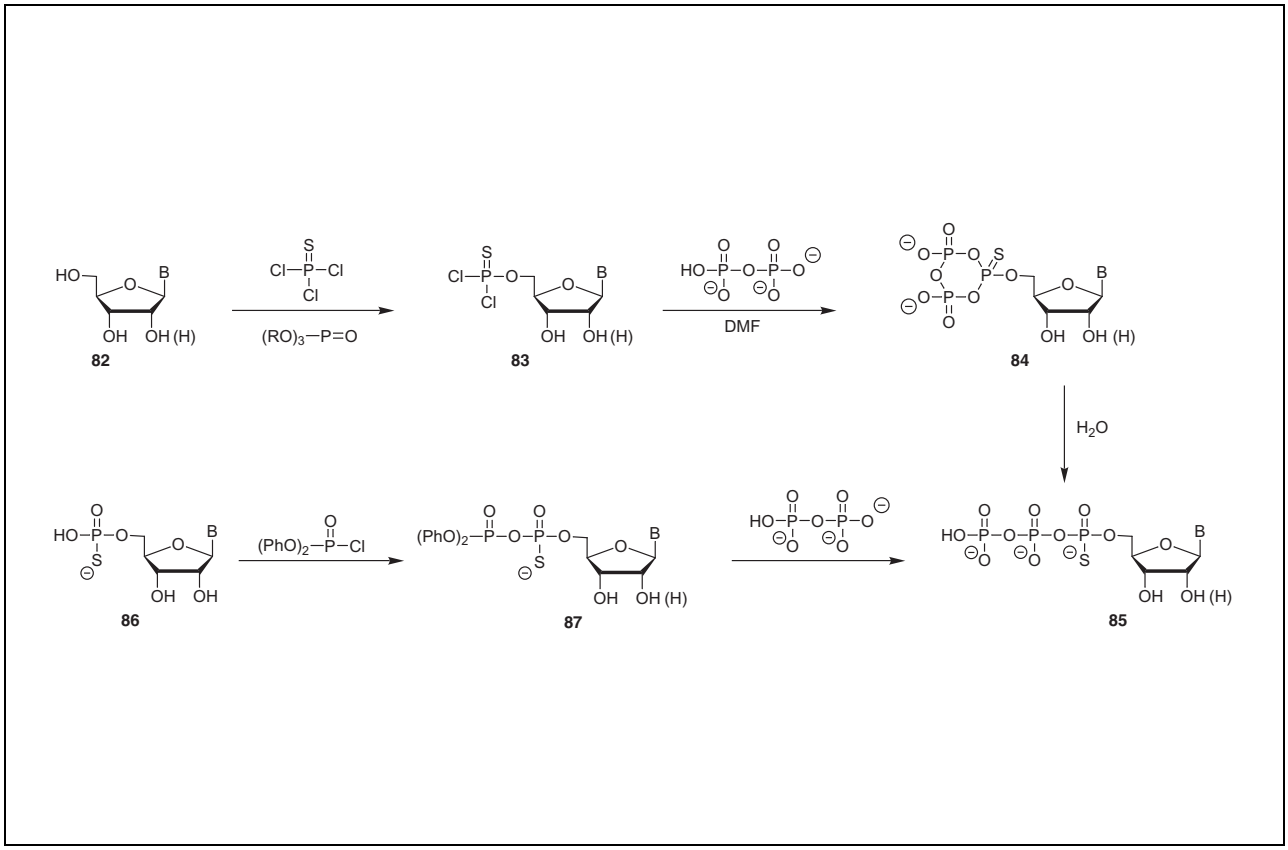

Figure 13.1.22 Synthesis of 5′-(1-thio)triphosphates via anion exchange or through thiophosphorochloridates.

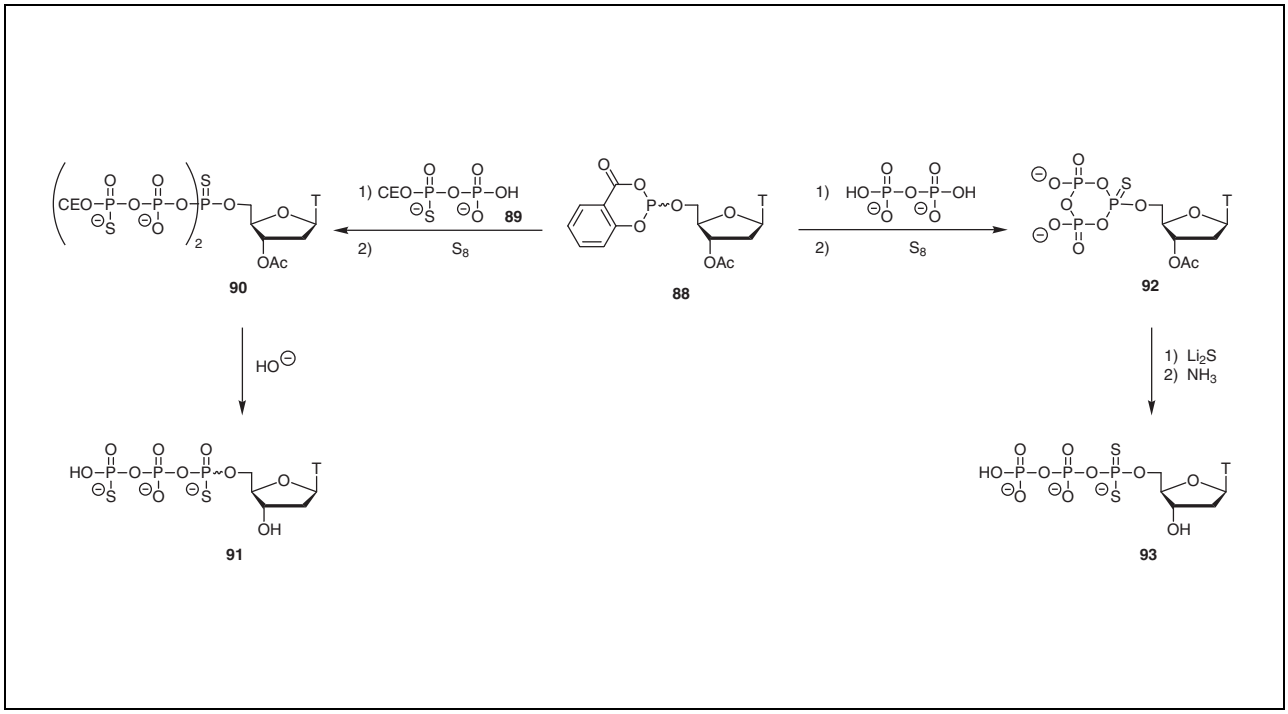

Figure 13.1.23 Salicyl phosphorochloridite provides versatility. Chiral and achiral thio-phosphorus analogs can be synthesized from a common precursor. CE, cyanoethyl.

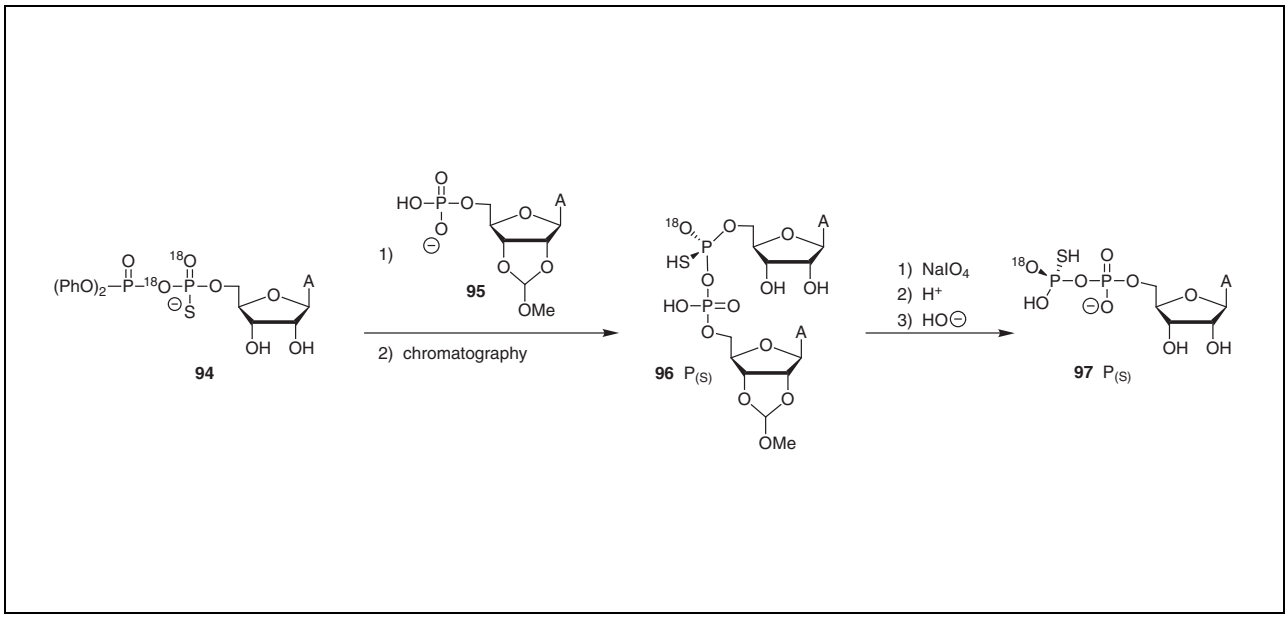

Figure 13.1.24 Synthesis of terminal chiral phosphates of known stereochemical configuration.

phosphite can be carried out with elemental sulfur. However, oxidation with elemental sulfur is sluggish due to poor solubility of the sulfur oxidant. Alternative sulfur oxidants such as 3*H*-1,2-benzodithiol-3-one 1,1,-dioxide (**S.72**; Fig. 13.1.19) have been introduced to eliminate the solubility problems associated with elemental sulfur (Iyer et al., 1990). Unlike elemental sulfur, **S.72** is soluble in acetonitrile, oxidizes phosphites readily, and does not modify nucleobases on prolonged exposure (Regan et al., 1992). However, the full scope and utility of oxidant **S.72** has yet to be assessed. It is not known, for example, whether unprotected nucleosides are compatible with this reagent.

Another method to access nucleoside phosphorothioates is by oxidation of *H*-phosphonates (Fig. 13.1.20). Thus, phosphorous acid can be condensed with an unprotected nucleoside in the presence of *N,N′*-ditolylcarbodiimide to give predominately the nucleoside 5′-*H*-phosphonate **S.73**. Silylation of the nucleoside 5′-*H*-phosphonate with trimethylsilyl chloride (TMSCl) forms the intermediate silylphosphite **S.74**. Oxidation with elemental sulfur in pyridine then gives the nucleoside 5′-phosphorothioate **S.75** (Chen and Benkovic, 1983). Although no protecting groups are necessary, this method is limited by the long reaction times needed for the condensation (~3 days) and the low yields of the nucleoside 5′-*H*-phosphonates produced (30% to 64%). Additionally, some nucleoside di-*H*-phosphonates are produced as byproducts.

An alternate procedure to access 5′-phosphorothioates makes use of 9-fluorenemethyl *H*-phosphonothioate **S.77**. This reagent condenses readily (<3 min) with protected nucleosides in the presence of diethyl phosphorochloridate to give nucleoside *H*-phosphonothioate diester **S.78** (Jankowska et al., 1997). The salient feature of this method is that phosphorothioates can be accessed via aqueous iodine oxidation of *H*-phosphonothioate **S.78**, or phosphorodithioates (**S.79**) can be obtained by sulfur oxidation of **S.78**. The method is suitable for many deoxyribonucleosides and gives good to high yields of the phosphorothioates and phosphorodithioates (Jankowska et al., 1998).

Although nucleoside 5′-phosphorothioates are more complex to synthesize than nucleoside 5′-phosphates, the synthesis of nucleoside 5′-di- and 5′-triphosphorothioates is even more complicated. Esterification of a nucleoside 5′-phosphorothioate results in the formation of a new chiral center. The majority of chemical methods available to access nucleoside 5′-di- and 5′-triphosphorothioates are nonstereoselective. Therefore, a mixture of $P_{(S)}$ and $P_{(R)}$ diastereomers are produced during the synthesis. Separation of the diastereomers by conventional chromatography is non-trivial. One popular strategy for obtaining a pure diastereomer is to enzymatically degrade the contaminating isomer (Fig. 13.1.21). For example, both nucleoside diphosphate kinase and hexokinase selectively hydrolyze the $P_{(S)}$ isomer of

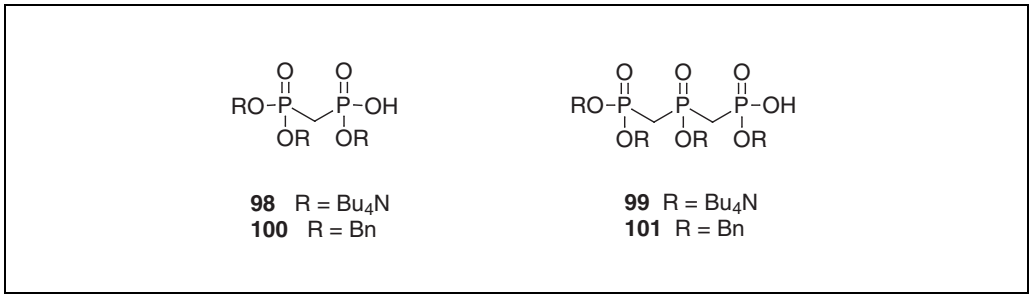

Figure 13.1.25 Structural variations of phosphonates make them suitable for use with either displacement strategies or Mitsunobu reaction. Bn, benzyl; Bu, butyl.

ATPαS leaving the pure $P_{(R)}$ isomer of ATPαS untouched (Eckstein, 1979).

Despite being nonstereoselective, many chemical methods used to synthesize nucleoside triphosphates are applicable to the construction of nucleoside 5′-(1-thio)triphosphates. Unprotected nucleosides can be mixed with thiophosphoryl trichloride in a trialkylphosphate solvent to form the corresponding thiophosphorodichloridate (**S.83**; Fig. 13.1.22). Introduction of pyrophosphate to the reaction mixture results in formation of the nucleoside thio-trimetaphosphate **S.84**. Hydrolysis of thio-trimetaphosphate **S.84** produces the nucleoside 5′-(1-thio)triphosphate **S.85**. This method has been applied to the synthesis of several nucleosides, including dA, dG, T, and U. Although it is an expedient route to nucleoside 5′-(1-thio)triphosphates, yields are typically low (Arabshahi and Frey, 1994).

Michelson's technique of anion exchange has also been applied to the synthesis of nucleoside 5′-(1-thio)triphosphates (Fig. 13.1.22). Thus, activation of AMPS and GMPS (**S.86**) with diphenyl phosphorochloridate followed by displacement with pyrophosphate gives the ATPαS and GTPαS in reasonable yield (Chen et al., 1983).

The salicyl phosphorochloridite methodology has been applied to the synthesis of nucleoside 5′-(1,3-bisthio)triphosphates and nucleoside 5′-(1,1-dithio)triphosphates (Fig. 13.1.23; Ludwig and Eckstein, 1991). Transesterification of nucleoside phosphite triester **S.88** with excess of P^1-*O*-(cyanoethyl)-P^1-thiopyrophosphate (**S.89**) followed by oxidation with elemental sulfur gives the branched pentaphosphate **S.90**. Selective hydrolysis along with concomitant deprotection of the cyanoethyl protecting group occurs under basic conditions to give thymidine 5′-(1,3-bisthio)triphosphate **S.91**. Selectivity for the hydrolysis follows the principles outlined earlier (see Phosphorus as an Electrophile).

For construction of nucleoside 5′-(1,1-dithio)triphosphates, transesterification of nucleoside phosphite triester **S.88** with pyrophosphate followed by oxidation with elemental sulfur leads to the formation of thiotrimetaphosphate **S.92**. Selective cleavage of thiotrimetaphosphate **S.92** at P_α occurs in DMF with lithium sulfide. Selectivity is not observed when pyridine/dioxane is used as the solvent. Aminolysis of the protecting groups then provides thymidine 5′-(1,1-dithio)triphosphate **S.93**. Although the method produces **S.93** in very low yield (15%), **S.93** may serve as a good achiral phosphorothioate derivative.

Frey has introduced a very elegant method for the synthesis of ATP with chiral α, β, or γ terminal phosphates of known configuration. The phosphates are rendered chiral by containing sulfur and heavy oxygen (^{18}O). Thus, adenosine 5′-(1-thio,1-^{18}O)diphosphorothioate, adenosine 5′-(2-thio,2-^{18}O)diphosphorothioate, and adenosine 5′-(3-thio,3-^{18}O)triphosphorothioate have been synthesized (Richard et al., 1978). The basic strategy is to selectively thiophosphorylate adenosine with thiophosphoryl chloride followed by hydrolysis with $H_2{}^{18}O$, yielding **S.94** (Fig. 13.1.24). Michelson's anion-exchange reaction is used to displace diphenylphosphate with 2′,3′-methoxy-methylidene AMP (**S.95**), providing diadenosine **S.96**. Separation of the diastereomers at this point ensures configurational purity of the phosphorus stereocenter in the product. Sodium periodate oxidation of the unprotected vicinal diols of **S.96** cascades into complete degradation of the adenosine moiety, providing (2-thio)diphosphorothioate **S.97**. Similar methodology is used to obtain adenosine 5′-(1-thio,1-^{18}O)diphosphorothioate and adenosine 5′-(3-thio,3-^{18}O)triphosphorothioate.

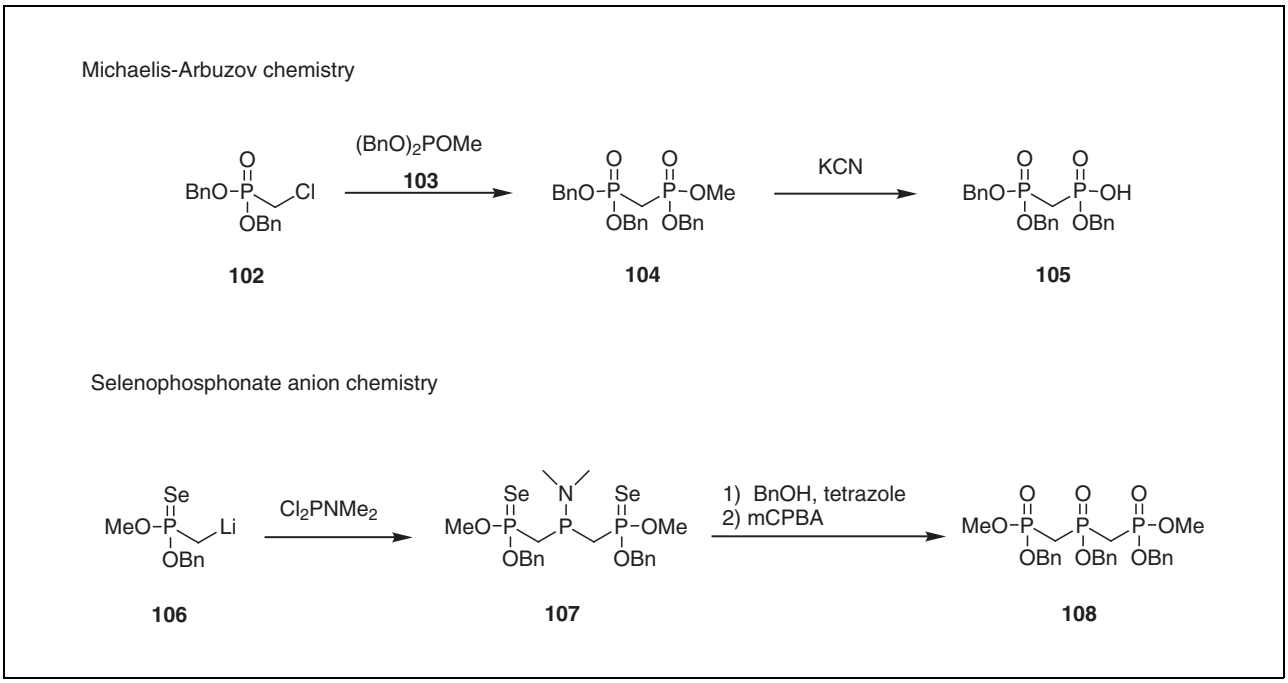

Figure 13.1.26 Strategies to access phosphonates. mCPBA, *meta*-chloroperoxybenzoic acid.

Phosphonate Derivatives

Replacing a bridging oxygen with a methylene functionality in pyrophosphate produces a pyrophosphonate (Fig. 13.1.25). The bond dissociation energy for a C-P bond is 65 kcal/mol, which is considerably less than the P-O bond dissociation energy (86 kcal/mol; Corbridge, 1995). Despite this, pyrophosphonates have considerably more thermal stability than phosphoanhydrides. In addition, pyrophosphonates are more resistant to hydrolysis, a consequence arising from the reduced electrophilicity of the phosphoryl group when bonded to the methylene unit. However, pyrophosphonate is neither a geometric nor an electronic isostere of pyrophosphate. The oxygen-oxygen nonbonded distance in a pyrophosphonate is ~16% greater than in a pyrophosphate. Pyrophosphonate is not capable of accepting a hydrogen bond to the bridging atom like pyrophosphate can. Despite these differences, nucleoside pyrophosphonates are used as enzymatically stable, nonhydrolyzable surrogates for nucleoside pyrophosphates (Engel, 1977).

Nucleoside di- and triphosphonates have been prepared either with a single bridging methylene unit replacing the bridging α-β or β-γ oxygens, or with total oxygen replacement in these bridging positions. Direct displacement reactions conducted on nucleoside 5′-tosylates with tris(tetrabutylammonium) pyrophosphonate (**S.98**) give the nucleoside 5′-pyrophosphonates in good yield (Dixit et al., 1984). Similarly, displacement of 5′-tosylates with bis-methylene triphosphonates (**S.99**) give the nucleoside analogs with α-β and β-γ bridging methylenes (Stock, 1979). Significantly better yields are obtained for the displacement reaction when performed in acetonitrile at room temperature, as opposed to heating in DMF, although acetonitrile requires longer reaction times. The nice feature about accessing nucleoside phosphonates in this fashion is that no protecting groups are needed on either the nucleoside or the phosphonate.

The Mitsunobu reaction has also been used in the construction of nucleoside phosphonates. Unlike the displacement methodology, protected nucleosides as well as protected phosphonates are usually used under Mitsunobu conditions. The reaction has been perfected such that both purine and pyrimidine nucleosides can be used without significant competing cyclization to the anhydro-nucleoside. Phosphorylation of protected nucleosides via the Mitsunobu reaction gives higher yields with tris(benzyl) pyrophosphonate (**S.100**) than with tetrakis(benzyl) bis-methylene triphosphonate (**S.101**). The yields for the phosphorylation step with tris(benzyl) pyrophosphonate are comparable to the displacement method on comparable nucleosides. Slightly better yields for the phosphorylation step with tetrakis(benzyl) bis-methylene triphosphonate are observed for the Mitsunobu reaction than with the

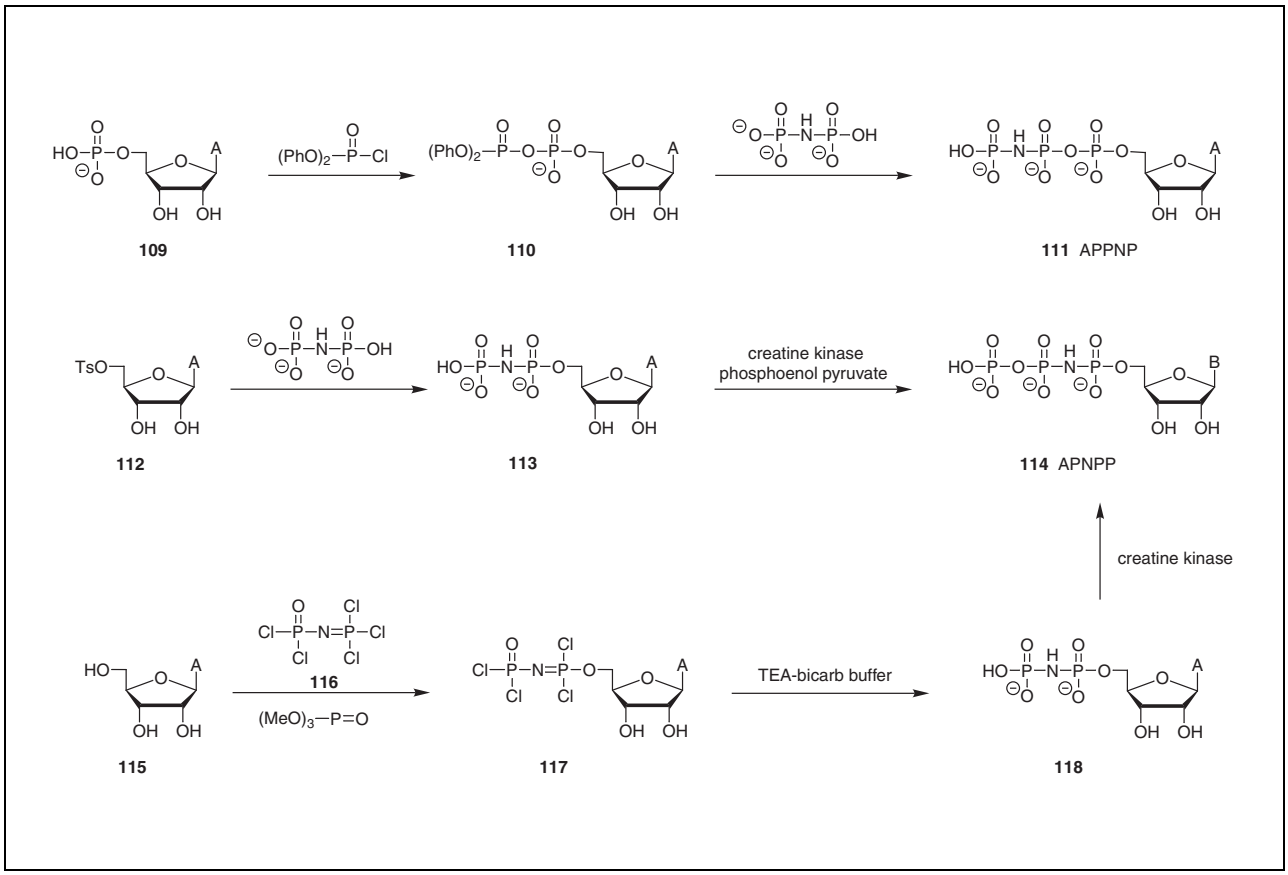

Figure 13.1.27 The common strategies used to access imidophosphate derivatives of adenosine. TEA, triethylamine.

displacement method. Deprotection of the nucleoside benzyl-protected phosphonates is routinely performed with trimethylsilyl bromide or catalytic hydrogenolysis. Either method seems to work equally well.

Protected pyrophosphonates can be constructed by the Michaelis-Arbuzov reaction (Saady et al., 1995b) or by phosphorylation of methane selenophosphonate anions (Eymery et al., 1999). The Michaelis-Arbuzov reaction allows ready access to tris(benzyl) pyrophosphonate (**S.105**; Fig. 13.1.26). However, experimental reproducibility of the Michaelis-Arbuzov reaction can be tricky. Recently, selenophosphonate anion chemistry has been developed that allows efficient access to bis-methylene triphosphonates (Klein et al., 2002). Methaneselenophosphonate anion **S.106** couples with dichlorophosphoroamidite producing selenophosphoramidite **S.107**. Owing to the reduced basicity of the selenophosphoryl moiety, competing deprotonation of the selenophosphoramidite intermediates is not observed. Tetrazole-promoted esterification of phosphoramidite **S.107** is followed by oxidation to give bis-methylene triphosphonate **S.108**. The diacid form of triphosphonate **S.108** is revealed upon selective demethylation with potassium cyanide.

Imidophosphate Derivatives

The chemistry of the imidodiphosphate and diimidotriphosphate functional groups closely mirrors that of the phosphoramidate functional group (see Phosphoramidites and Phosphoramidates). Thus, imidodiphosphates and diimidotriphosphates are stable in alkaline media, but hydrolyze in acidic media (Tomasz et al., 1988). Despite its acid lability, the imidophosphate functional group has been used in nucleotides as a phosphate analog. When either the α/β or β/γ oxygen of ATP is replaced with nitrogen, the result is adenosine imidotriphosphate, abbreviated APNPP and APPNP, respectively. If both the α/β and β/γ oxygens are replaced by nitrogen, the result is adenosine diimidotriphosphate, APNPNP.

Adenosine imidotriphosphate mimics ATP for many applications. This mimicry may arise because the physical properties of the imidodiphosphate and pyrophosphate functional groups are quite similar (Larsen et al., 1969).

Although imidodiphosphate is not a geometric isostere in the truest sense, the bond angle and bond lengths are very close to those of pyrophosphate. For example, the P-N-P and P-O-P bond angles are 127.2° and 128.6°, respectively. The bond lengths are also very similar: 1.68 Å (P-N) and 1.63 Å (P-O). Unlike a phosphate ester linkage, an imidophosphate can, in principle, accept or donate a H-bond. However, the shortened P-N bond length suggests partial P=N characteristic of the imidophosphate, which mitigates the H-bond donating and accepting abilities. Various nucleosides with the imidophosphate structural motif are inhibitors of HIV-RT1 (Li et al., 1996) and ATP/GTP phosphatases (Batra et al., 1987), but are substrates for *E. coli* alkaline phosphatase (Yount et al., 1971).

Nucleoside imidophosphates are widely used in their triphosphate form. As a result, synthetic methods have focused principally on the triphosphate form as the target. There are several approaches to synthesize nucleoside imidophosphates, and no single approach is superior. All methods give similar, yet variable, yields for attachment of the imidophosphate functionality to the nucleoside.

Synthesis of nucleoside-β,γ-imidotriphosphates can be accomplished by Michelson's anion-displacement methodology (Yount et al., 1971). Activation of AMP with diphenyl phosphorochloridate followed by displacement of diphenyl phosphate with imidodiphosphate gives APPNP (**S.111**; Fig. 13.1.27). This method has been used successfully to incorporate heavy nuclides of oxygen and nitrogen into the phosphate component by the labeling of imidodiphosphate (Reynolds et al., 1983).

The preparation of nucleoside-α,β-imidotriphosphates is generally carried out in a two-step procedure. The nucleoside-α,β-imidodiphosphate is chemically synthesized and then enzymatically phosphorylated. Displacement of the tosylate leaving group in adenosine 5′-tosylate **S.112** by tris(tetrabutylammonium) imidodiphosphate gives access to adenosine-α,β-imidodiphosphate **S.113** (Ma et al., 1988). Enzymatic phosphorylation of **S.113** by creatine kinase using phosphoenol pyruvate as the phosphoryl donor provides access to APNPP (**S.114**). An alternate strategy uses imidophosphorylation of unprotected nucleosides with trichloro-[(dichlorophosphoryl)imido]phosphorane (**S.116**; Tomasz et al., 1988). Both ribo- and deoxyribonucleosides have been imidophosphorylated using phosphorane **S.116**. Phosphorane **S.116** is also a good chlorinating reagent, however, and yields nucleoside 5′-chlorides as byproducts. Many purine and pyrimidine nucleoside-α,β-imidodiphosphates obtained through imidophosphorylation have been converted to their triphosphate counterparts using creatine kinase (Li et al., 1996).

Access to nucleoside-α,β-β,γ-diimidotriphosphates is very challenging; more so than imidotriphosphates. In fact, no satisfactory method is yet available. Adenosine-α,β-β,γ-diimidotriphosphate has been accessed by reaction between adenosine 5′-tosylate and the tetrabutylammonium salt of diimidotriphosphate in acetonitrile (Ma et al., 1990). The desired product was obtained in poor yield (7%) along with another diimidotriphosphate, $AP(NP)_2$ (6%).

Several imidophosphate derivatives of adenosine and guanosine are commercially available. However, use of commercial preparations requires HPLC purification prior to use to remove the contaminating phosphoramidate APPN (Penningroth et al., 1980; Batra et al., 1987). APPN does not reappear after prolonged storage (8 months) of repurified APPNP, suggesting the phosphoramidate contaminant is a byproduct of preparation.

^{32}P-Radiolabeled Derivatives

This section will focus on radiolabeled compounds containing ^{32}P modifications to the phosphate component of nucleotides. There are several important provisos to consider when choosing a method to synthesize radiolabeled derivatives of nucleotides. First is the location of the radiolabel. Second is the availability of reagents in labeled form. Third is the time required for synthesis to be complete. Because the half-life of ^{32}P is 14 days, prolonged syntheses reduce the specific activity of the labeled nucleotide. Taken together, these provisos place severe restrictions on the methodologies that are available to synthesize radiolabeled nucleotides.

Nucleotides with radiolabeled phosphates are commercially available for common nucleoside mono- and triphosphates (C, G, T, dA, and others). The nucleotide triphosphates are widely available with $^{32}P_\alpha$ and $^{32}P_\gamma$ labels. However, there is a conspicuous lack of $^{32}P_\beta$-labeled triphosphates. Therefore, synthetic methods are necessary to obtain $^{32}P_\beta$-labeled nucleotides. Synthetic methods are also necessary to obtain radiolabeled mono-, di-, and triphosphates of nucleosides with sugar and/or nucleobase modifications. There are many methods available for the synthesis of nucleo-

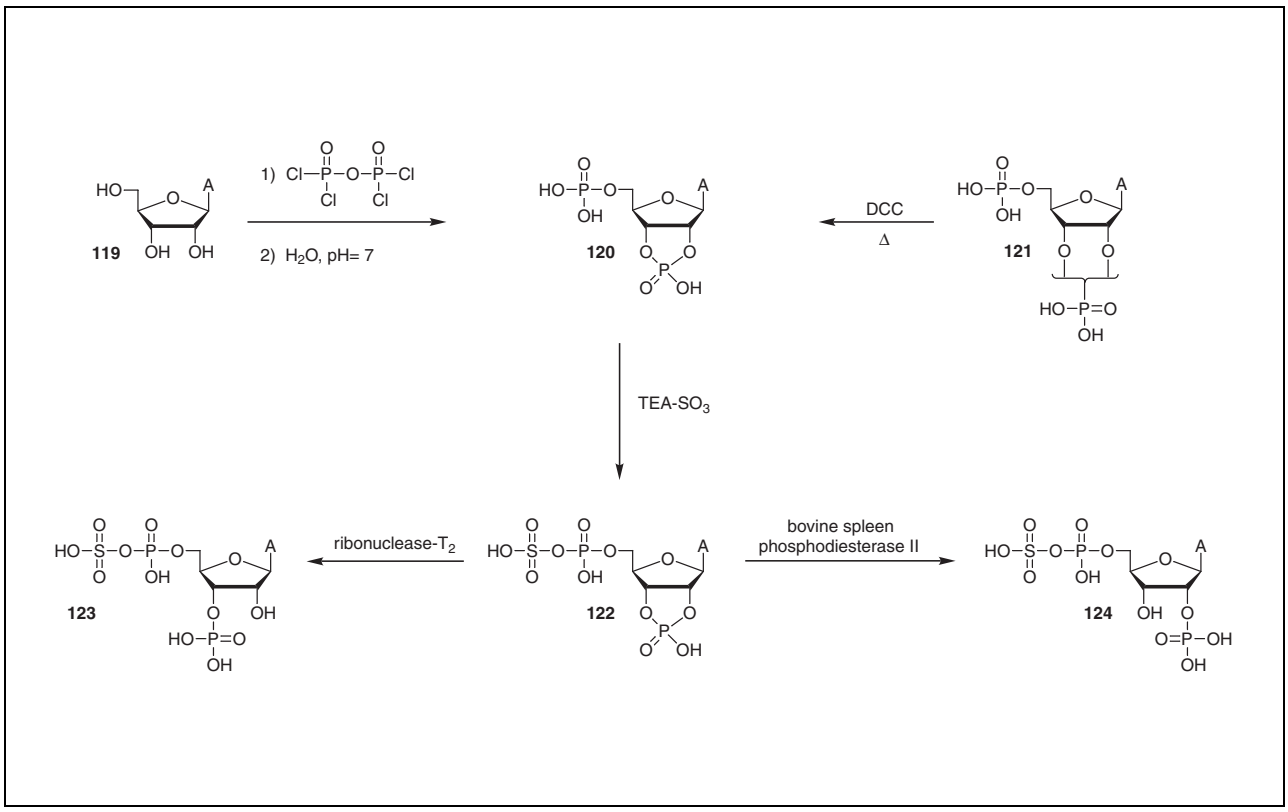

Figure 13.1.28 Combination methodology for the synthesis of PAPS. DCC, dicyclohexylcarbodiimide; TEA-SO_3, triethylamine-*N*-sulfonic acid.

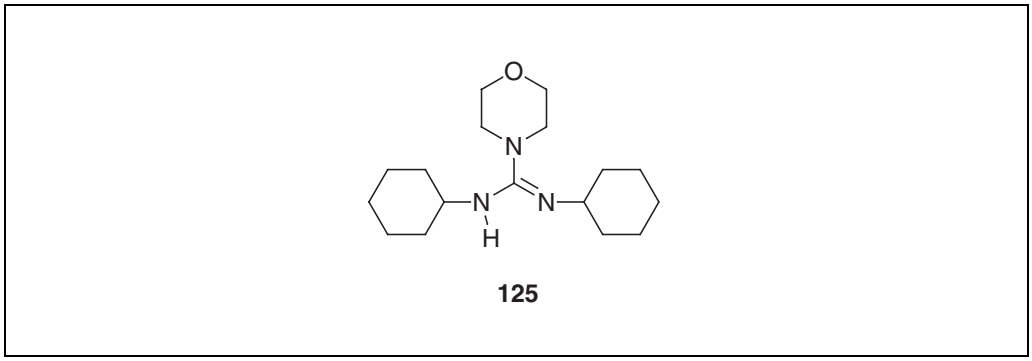

Figure 13.1.29 4-Morpholine-*N,N'*-dicyclohexyl carboxamidine. A useful base for increasing solubility of NMPs.

tides with ^{31}P that cannot be used with ^{32}P, because ^{32}P is not available in as many coordination states as ^{31}P. The most readily available form of ^{32}P is an aqueous solution of *ortho*-phosphoric acid. Consequently, many of the phosphorylation reactions with ^{32}P are conducted in dimethyl sulfoxide (DMSO), dimethylformamide (DMF), or water.

Nucleoside phosphate mixed anhydrides and nucleoside phosphoramidates are electrophilic phosphorus species that are suitable for use with ^{32}P-*ortho*-phosphoric acid. Treatment of NDPs with ethylchloroformate produces a nucleoside phosphate mixed anhydride. Reaction of the mixed anhydride with ^{32}P-*ortho*-phosphoric acid produces the ^{32}P-labeled nucleoside triphosphate. This method is compatible with various NDPs (A, C, G, U) for the construction of $^{32}P_{\gamma}$-labeled NTPs. The reaction is complete in a short period of time, but requires extensive handling of the ^{32}P reagent (Janecka et al., 1980). This method does not allow for the construction of $^{32}P_{\beta}$-labeled NDPs, as the NMP mixed anhydride does not react to an appreciable extent with phosphoric acid (Michelson, 1964).

The phosphoramidate approach relies upon ready access to the nucleoside phosphormor-

pholidate. Condensation of AMP or ADP with morpholine in the presence of dicyclohexylcarbodiimide (DCC) gives the corresponding nucleoside phosphormorpholidates. $^{32}P_{\beta}$- or $^{32}P_{\gamma}$-labeled ATP can be obtained from AMP- and ADP-morpholidate, respectively. Reaction of AMP-morpholidate with $^{32}P_{\beta}$-ρ-nitrobenzyl diphosphate gives $^{32}P_{\beta}$-labeled ATP in low yield. Reaction between ^{32}P-*ortho*-phosphoric acid and ADP-morpholidate gives $^{32}P_{\gamma}$-labeled ATP in good yield. The nice feature of this method is that commercially available unprotected nucleosides can be used. A drawback of the method is the vast excess of reagents used throughout the process. Both DCC and ^{32}P-*ortho*-phosphoric acid are used in excess. This is disadvantageous because the urea byproduct of DCC can be difficult to separate from nucleotides, and excess ^{32}P reagent contributes to additional radioactive waste. Additionally, the reaction requires prolonged reaction times, which results in reduced activity of the ^{32}P-labeled nucleotides (Werhli et al., 1965).

Introduction of the ^{32}P label at the P_{α} of NDP or NTP is possible by condensation of a 2′,3′-isopropylidene ribonucleoside with ^{32}P-*ortho*-phosphoric acid in the presence of trichloroacetonitrile and triethylamine. Following the removal of the protecting group, the ^{32}P-labeled monophosphate can be phosphorylated with (2-cyanoethyl)phosphoryl- or (2-cyanoethyl)pyrophosphoryl imidazolidate, providing $^{32}P_{\alpha}$-NDPs and $^{32}P_{\alpha}$-NTPs, respectively (Symons, 1970). Extension of this methodology with unprotected nucleosides is possible, although the first condensation step produces a mixture of 2′(3′)- and 5′-nucleoside monophosphates. This mixture can be selectively phosphorylated with enzymes to yield the desired $^{32}P_{\alpha}$-NTPs or $^{32}P_{\alpha}$-dNTPs (Symons, 1974).

In addition to the purely chemical and mixed chemical/enzymatic syntheses of $^{32}P_{\alpha}$-NTPs, a complete enzyme-mediated process has been developed. The enzymatic process has the advantages that the reaction is performed in the container that the ^{32}P is shipped in and the intermediates do not need to be purified. A consequence, though, is that the final purification can be difficult (Walseth and Johnson, 1979; Walseth et al., 1991).

As with any enzymatic synthesis of nucleotides, if the phosphoryl donor is ATP, contamination of the product NTPs with ATP is possible. This is particularly problematic with ^{32}P-labeled ATP, as the specific activity can be reduced significantly (Furuichi and Shatkin, 1977). This can be addressed by use of alternate phosphoryl donors such as phosphoenol pyruvate or acetyl phosphate. Enzyme systems have been developed for the synthesis of $^{32}P_{\beta}$-NTPs from phosphorylation of NMPs (Furuichi et al., 1977) and by polynucleotide digestion with polynucleotide phosphorylase (Kaufmann et al., 1980).

3′-PHOSPHOADENOSINE-5′-PHOSPHOSULFATE (PAPS)

3′-Phosphoadenosine-5′-phosphosulfate (PAPS) is the sulfate source used by sulfotransferases for the sulfation of steroids, drugs, proteins, and saccharides (Klaassen et al., 1997). PAPS has been synthesized for direct use as a cofactor and to study biological processes. Methodologies developed for the synthesis of PAPS reflect the application. Methods combining both chemical synthesis and enzymes have been detailed. Synthetic methods typically have fallen short of providing PAPS in pure form, as both the 2′- and 3′-phosphate regioisomers are produced. Thus, synthesis of PAPS by pure synthetic methods will not be addressed.

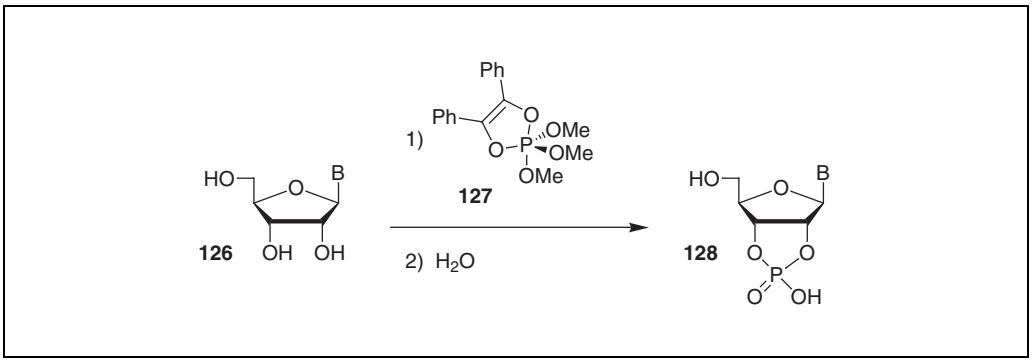

Figure 13.1.30 Selective transesterification of unprotected nucleosides by oxyphosphorane leads to regioselective formation of 2′,3′-cyclic phosphates.

Enzymatic Methods

Pure enzymatic synthesis of PAPS is particularly suitable when PAPS is a cofactor used in sulfation reactions. In these instances a large quantity of PAPS is not necessary. Rather, what is needed is a means to reconvert PAP back to the coenzyme PAPS. Systems have been developed that directly convert PAP to PAPS (Burkart et al., 1999) or that accomplish this conversion through several enzymatic steps (i.e., PAP → AP → APS → PAPS; Lin et al., 1995). The one real disadvantage of this methodology is its level of sophistication. Although very elegant, multienzymatic regeneration of PAPS requires construction of a plasmid, as well as overexpression and purification of several enzymes (Burkart et al., 1999). These procedures are routine in molecular biology laboratories, but may be intimidating to the synthetic chemist.

Combination Methods

Figure 13.1.28 summarizes methods to obtain PAPS by combination methodology. The key to several combination methods is the availability of 2′,3′-cyclic phosphoadenosine 5′-phosphosulfate (**S.122**). Several approaches are available to access this intermediate. Adenosine (**S.119**) can be phosphorylated with pyrophosphoryl chloride followed by hydrolysis to give 2′,3′-cyclic phosphoadenosine 5′-phosphate (**S.120**; Horwitz et al., 1977). Alternately, cyclic phosphate **S.120** can be obtained by cyclizing a commercial mixture of 2′,5′- and 3′,5′-bis(phospho)adenosine (**S.121**) with DCC at elevated temperature (Sekura, 1981). Sulfation of cyclic phosphate **S.120** is most commonly effected with triethylamine-*N*-sulfonic acid (Horwitz et al., 1980; Sekura, 1981). 5′-Phosphosulfate nucleosides are quite prone to hydrolysis and decomposition. Therefore, yields for the sulfation step tend to vary (46% to 75%). Cyclic phosphate **S.122** can be cleaved by either bovine spleen phosphodiesterase II or ribonuclease-T_2 to provide 2′- or 3′-phosphoadenoside-5′-phosphosulfate, respectively (Horwitz et al., 1980; Sekura, 1981). The enzymatic cleavage of cyclic phosphates is tolerant to minor structural modifications of the nucleobase (Horwitz et al., 1981).

CYCLIC NUCLEOSIDE PHOSPHATES AND PHOSPHOROTHIOATES

Two important types of nucleoside cyclic phosphates are 2′,3′- and 3′,5′-cyclic phosphates. There are two common approaches to obtain these cyclic phosphates: cyclization by dehydration or cyclization by nucleophilic attack on electrophilic phosphorus. The latter is often promoted by adding base to the reaction medium.

Cyclization by dehydration is commonly affected with DCC, although chloroformate reagents and trifluoroacetic anhydride have also been used. Preparation of cAMP, cCMP, cGMP, and cUMP have been carried out by heating the corresponding NMP-4-morpholine-*N,N′*-dicyclohexylcarboxamidinium salts in pyridine/DCC (Smith et al., 1961). Unlike tri-*n*-butylamine or tri-*n*-octylamine, the base 4-morpholine-*N,N′*-dicyclohexylcarboxamidine **S.125** (Fig. 13.1.29) imparts better solubility upon nucleoside monophosphates at high temperatures. Despite the better solubility of the carboxamidinium salts, guanosine and cytidine need *N*-benzoyl protecting groups for full dissolution. The yields for this reaction are generally good.

DCC-promoted dehydration can also be used for the synthesis of cyclic-2′,3′-phosphates. Cyclization of a commercial mixture of 2′,5′- and 3′,5′-bis(phospho)adenosine with DCC at elevated temperature provides cyclic-2′,3′-phosphoadenosine-5′-phosphate (**S.120**; Fig. 13.1.28; Sekura, 1981). Similarly, reaction of unprotected nucleosides (A, C, G, U) with pyrophosphoryl chloride followed by buffered hydrolysis (pH = 7) results in the formation of cyclic-2′,3′-phosphonucleoside-5′-phosphates (Fig. 13.1.28; Simoncsits and Tomasz, 1975). This reaction is successful for adenosine, cytidine, and guanosine. However, cyclic-2′,3′-phosphouridine-5′-phosphate is obtained in low yield via this procedure. These reactions have the inherent drawback that the bis-phosphate is produced instead of pure cyclic-2′,3′-phosphate. This limitation has been overcome by the use of the five-coordinate oxyphosphorane **S.127** (σ^5,λ^5) instead of pyrophosphoryl chloride. Treatment of an unprotected nucleoside with oxyphosphorane **S.127** in pyridine followed by hydrolysis provides the nucleoside cyclic-2′,3′-phosphate (**S.128**; Fig. 13.1.30). In this manner, both adenosine and cytidine cyclic-2′,3′-phosphates have been regioselectively synthesized in high yield (Chen et al., 1997).

Entrée into 3′,5′-cyclic phosphates is possible through base-promoted cyclization of phosphorodichloridates. Treatment of an unprotected nucleoside with $POCl_3$ produces a phosphorodichloridate that is hydrolyzed with an aqueous solution of KOH in acetonitrile at 0°C

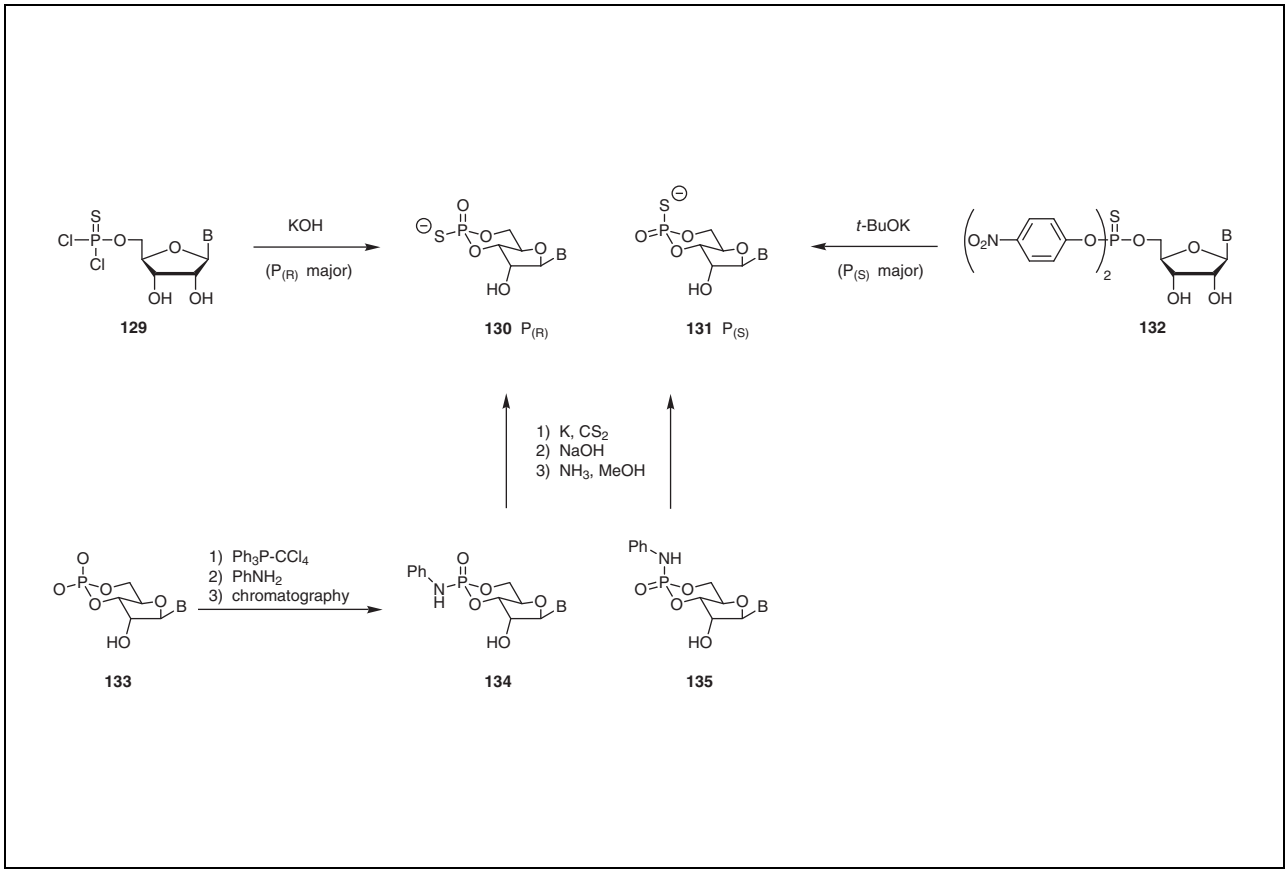

Figure 13.1.31 Synthesis of cyclic phosphorothioates.

to produce the cyclic phosphate in low yield (22% to 49%). This procedure varies slightly from the Yoshikawa procedure (Fig. 13.1.10) in that water is omitted from the reaction milieu during formation of the phosphorodichloridate. Omission of water during the initial phosphorylation step furnishes mixtures of nucleoside-2′(3′)-5′-diphosphates (Yoshikawa et al., 1967). Therefore, the reduced yields obtained for this cyclization reaction may be due to lack of regio-control in the initial phosphorylation step.

As secondary messengers, nucleoside cyclic-3′,5′-phosphates are important in many biological pathways. Investigation of the pathways could benefit from the ready availability of nucleoside cyclic phosphate analogs that have increased enzymatic and hydrolytic stability. Phosphorothioate derivatives of cyclic-3′,5′-phosphates such as cAMPS are, in fact, hydrolyzed more slowly than the parent phosphate (Eckstein, 1979). Additionally, the stereochemical course of enzyme-catalyzed reactions has been determined by using nucleoside cyclic-2′,3′-phosphorothioates (Usher et al., 1970).

The synthesis of cAMPS and other cyclic phosphorothioates is difficult because the phosphorothioate functional group is part of a six-membered ring. The phosphorus center is therefore stereogenic. Both the $P_{(R)}$ and $P_{(S)}$ stereoisomers are configurationally stable. As shown in Figure 13.1.31, cyclic-3′,5′-phosphorothioates can be accessed by base-promoted cyclization of phosphorothiodichloridates (**S.129**; Genieser et al., 1988) or nucleoside 5′-bis(ρ-nitrophenyl)phosphorothioates (**S.132**; Eckstein and Kutzke, 1986). Cyclic phosphorothioates synthesized by these methods are mixtures of phosphorus stereoisomers. Interestingly, ester displacement reactions give predominately the $P_{(S)}$ stereoisomer, whereas chloride displacement results in primarily the $P_{(R)}$ stereoisomer (Fig. 13.1.31). Resolution of the individual stereoisomers from these reactions is possible, but the process is tedious and the yields are generally poor.

An alternate method to access the individual stereoisomers is through the stereospecific Horner-Wadsworth-Emmons reaction of nucleoside phosphoranilidates (**S.134** or **S.135**) with potassium reduced carbon disulfide (Fig. 13.1.31; Stec, 1983). This approach has the

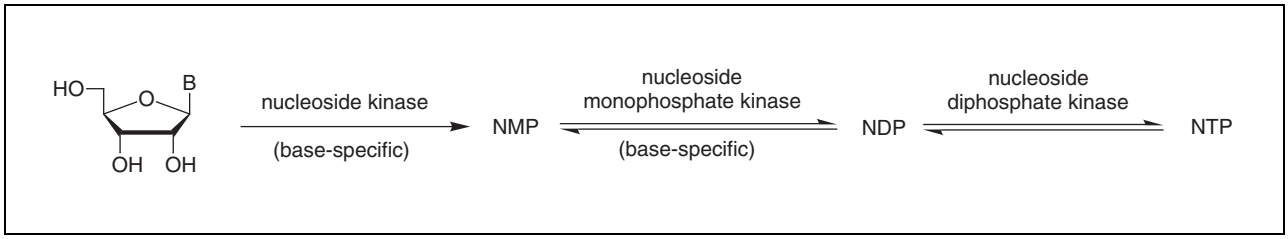

Figure 13.1.32 Enzyme-catalyzed phosphorylation of nucleosides proceeds in a stepwise fashion.

advantage that commercially available nucleoside cyclic phosphates can be used. The reaction sequence is outlined in Figure 13.1.31 and has been applied to adenosine, cytidine, guanosine, and uridine. Additionally, the separation of the nucleoside phosphoranilidate stereoisomers is easier than separation of the nucleoside cyclic phosphorothioates. However, the chemical synthesis of the nucleoside phosphoranilidates proceeds in low yield.

ENZYMATIC NUCLEOTIDE SYNTHESIS

In principle, enzymes can be used to synthesize NMPs, NDPs, or NTPs. However, cell-free enzyme-catalyzed reactions are most routinely used for the synthesis of NTPs. Enzymes catalyze the phosphorylation of nucleosides to NTP in a stepwise fashion by the successive action of nucleoside kinase, nucleoside monophosphate kinase, and nucleoside diphosphate kinase (Fig. 13.1.32). The first two enzymes in this pathway are nucleoside specific, whereas, nucleoside diphosphate kinase is not nucleotide specific.

Nucleoside kinase, nucleoside monophosphate kinase, and nucleoside diphosphate kinase primarily use ATP as the phosphate donor. Therefore, ATP has to be supplied or regenerated, depending upon scale, during the nucleotide syntheses (Chenault et al., 1988). Due to the use of ATP as phosphate donor, pure nucleotide product can be difficult to obtain. Pure NTP product (where N = C, T, U, G) can be especially difficult to separate from ATP.

Purity of product NTPs can be increased by several modifications of the general enzymatic scheme outlined in Figure 13.1.32. Use of commercially available NMPs and regeneration of catalytic amounts of ATP are two approaches to reduce contamination by ATP/ADP. Another way is to use alternate enzymes/phosphate donors to effect the phosphorylation. For example, by choosing conditions that favor the backwards reactions of pyruvate or acetate kinase, phosphoenol pyruvate and acetyl phosphate can be used as phosphate donors. Phosphoenol pyruvate is more stable in solution than acetyl phosphate and is used extensively for the phosphorylation of NDPs. Hydrolytically stable phosphate donors are especially important when the nucleoside is phosphorylated at slow rates (such as the case with modified nucleosides).

Nucleosides containing modified nucleobases cannot always be phosphorylated using synthetic methods due to incompatibility with the reaction conditions. Enzymatic phosphorylation offers an alternative to synthetic methods because the reaction conditions are generally mild and phosphorylations are regio- and chemoselective. However, there has been limited success for enzyme-mediated phosphorylation of modified nucleosides. Unnatural purine- and pyrimidine-like nucleobases are phosphorylated with reduced efficiency (k_{cat}/K_m) by *D. melanogaster* deoxynucleoside kinase (by a factor of 10^2 to 10^4; Wu et al., 2002).

Phosphorylation of sugar-modified nucleosides can also be problematic. The kinases that phosphorylate AZT to AZT-TP do so with reduced efficiency (Van Rompay et al., 2000). For example, NDP kinase phosphorylates AZT-DP with reduced efficiency (a factor of 10^3 to 10^4; Schneider et al., 2001). This is interesting because NDP kinase has little specificity for either the nucleobase or the ribose or deoxyribose of natural NDPs. However, critical H-bonds are provided by the 3′-hydroxyl group to stabilize a conformation of the nucleotide that facilitates phosphorylation. Thus, a minor modification in the architecture of the nucleoside (3′-OH to 3′-N_3) results in the destabilization of the active conformation of the nucleotide within the kinase.

In contrast to the problems associated with enzymatic phosphorylation of AZT-DP, NDP kinase has been used successfully to phosphorylate nucleoside diphosphates containing nonnatural nucleobases (Wu et al., 2003). Diphosphates of azole carboxamide deoxyribonucleosides (or azole carboxamide

ribonucleosides) are phosphorylated by NDP kinase using ATP as the phosphate donor (see *UNIT 13.2*). Good to high yields of the desired azole carboxamide nucleoside triphosphates are obtained. Precursor diphosphates are obtained by way of Poulter's tosylate displacement methodology. This combination synthetic/enzymatic method is notable for several reasons. First, the method is of interest for its potential as a general means to phosphorylate nucleoside diphosphates containing non-natural nucleobases. Second, the method addresses contamination of the desired nucleoside triphosphate product with ATP/ADP. Use of an ATP regeneration system involving phosphoenol pyruvate and pyruvate kinase minimizes the amount of ATP in the reaction milieu. Nevertheless, contaminating ATP/ADP is conveniently removed by retention on a boronate affinity gel column. The boronate affinity gel forms a complex with the vicinal diols of ribonucleotides; thus, azole carboxamide deoxyribonucleotide products conveniently pass through the gel.

There are two issues unique to enzyme-catalyzed phosphorylations that are not encountered during chemical syntheses: enzyme stability and product inhibition. The source of the enzyme can greatly affect the required reaction conditions. For example, acetate kinase from *E. coli* contains a thiol group that can be oxidized. To prevent inactivation due to oxidation, reactions are often conducted in the presence of a reducing agent (DTT) or under an inert atmosphere. Interestingly, acetate kinase from *B. stearothermophilus* cannot be autooxidized because it does not contain a thiol group (Kim and Whitesides, 1987).

Aside from enzyme inactivation, inhibition of the enzymes can also cause a decrease in the reaction rate. The phosphorylation reactions catalyzed by NMP and NDP kinases are reversible. The consequence of this reversibility is that a large buildup of NDP can inhibit NMP kinase. Thus, the rate at which NMP kinase produces NDP product slows dramatically. Product inhibition is another complicating factor when designing enzyme-catalyzed phosphorylations.

STATE OF THE ART

In a 1958 Nobel Prize speech, Sir Alexander Todd made the following observation, "we are thus still seeking an ideal method for unsymmetrical pyrophosphate synthesis" (Todd, 1958). Despite the passage of almost 50 years, the challenge of an "ideal method" for nucleotide synthesis has yet to be satisfactorily met. Nominally, an ideal method permits the regio- and chemoselective phosphorylation of all nucleosides and nucleoside analogs (base or sugar modifications alike). Additionally, an ideal method should facilitate the synthesis of any phosphate-chain analog (thio-, borano-, imido-, and others) from common intermediates or an appropriate reagent system.

This overview has presented some common methods and reagents used to construct nucleotides. Despite the elegant design of the many methods available, it is clear that no single method is "ideal" under the criteria outlined above. This is due in part to the variation in chemical properties of the nucleobases. Chemoselectivity may be improved by transiently masking the nucleophilic moieties of the nucleobases. Alternately, finding conditions that selectively degrade any phosphorylated (phosphitylated) nucleobase moieties shows potential for a general solution. Progress has been made on chemoselective phosphitylation of *N*-unprotected nucleosides using phosphoramidite reagents. Both transient protection via protonation of basic nucleobases (Sekine et al., 2003) and selective cleavage of phosphitylated nucleobases (Gryaznov and Letsinger, 1991) have been successfully employed in deoxyoligonucleotide syntheses. Modification of these methods may ultimately be useful for the synthesis of nucleoside mono-, di-, and triphosphates. However, a general solution for the regio- and chemoselective phosphorylation of nucleosides and analogs without invoking the use of protecting groups is a very difficult problem that awaits a general solution.

LITERATURE CITED

Albert, A. 1973. Ionization constants of pyrimidines and purines. *In* Synthetic Procedures in Nucleic Acid Chemistry (W.W. Zorbach and R.S. Tipson, eds.) pp. 1-46. Wiley-Interscience, New York.

Arabshahi, A. and Frey, P.A. 1994. A simplified procedure for synthesizing nucleoside 1-thiotriphosphate: dATPαS, dGTPαS, UTPαS, and dTTPαS. *Biochem. Biophys. Res. Commun.* 204:150-155.

Batra, J.K., Lin, C.M., and Hamel, E. 1987. Nucleotide interconversions in microtubule protein preparations, a significant complication for accurate measurement of GTP hydrolysis in the presence of adenosine 5′-(β,γ-imidotriphosphate). *Biochemistry* 26:5925-5931.

Burgess, K. and Cook, D. 2000. Syntheses of nucleoside triphosphates. *Chem. Rev.* 100:2047-2059.

Burkart, M.D., Izumi, M., and Wong, C.-H. 1999. Enzymatic regeneration of 3′-phosphoadenosine-5′-phosphosulfate using aryl sulfotransferase for the preparative enzymatic synthesis of sulfated carbohydrates. *Angew. Chem. Int. Ed. Engl.* 38:2747-2750.

Chen, J.-T. and Benkovic, S.J. 1983. Synthesis and separation of diastereomers of deoxynucleoside 5′-*O*-(1-thio)triphosphates. *Nucl. Acids Res.* 11:3737-3751.

Chen, X., Zhang, N.-J., Li, Y.-M., Jiang, Y., Zhang, X., and Zhao, Y.-F. 1997. Direct phosphorylation of nucleosides by oxyphosphorane. *Tetrahedron Lett.* 38:1615-1618.

Chenault, H.K., Simon, E.S., and Whitesides, G.M. 1988. Cofactor regeneration for enzyme-catalysed synthesis. *In* Biotechnology and Genetic Engineering Reviews (G.E. Russell, ed.) pp. 221-270. Intercept, Wimborne, Dorset, England.

Chladek, S. and Nagyvary, J. 1972. Nucleophilic reactions of some nucleoside phosphorothioates. *J. Am. Chem. Soc.* 94:2079-2085.

Corbridge, D.E.C. 1995. Phosphorous. An Outline of its Chemistry, Biochemistry and Uses. Elsevier, Amsterdam.

Cosstick, R. and Vyle, J.S. 1990. Synthesis and properties of dithymidine phosphate analogues containing 3′-thiothymidine. *Nucl. Acids Res.* 18:829-835.

Davisson, V.J., Davis, D.R, Dixit, V.M., and Poulter, C.D. 1987. Synthesis of nucleotide 5′-diphosphates from 5′-*O*-tosyl nucleosides. *J. Org. Chem.* 52:1794-1801.

Dixit, V.M. and Poulter, C.D. 1984. Convenient syntheses of adenosine 5′-diphosphate, adenosine 5′-methylenediphosphonate, and adenosine 5′-triphosphate. *Tetrahedron Lett.* 25:4055-4058.

Eckstein, F. 1979. Phosphorothioate analogues of nucleotides. *Acc. Chem. Res.* 12:204-210.

Eckstein, F. and Kutzke, U. 1986. Synthesis of nucleoside 3′,5′-cyclic phosphorothioates. *Tetrahedron Lett.* 27:1657-1660.

Engel, R. 1977. Phosphonates as analogues of natural phosphates. *Chem. Rev.* 77:349-367.

Eymery, F., Iorga, B., and Savignac, P. 1999. Synthesis of phosphonates by nucleophilic substitution at phosphorus: The S_N P(V) reaction. *Tetrahedron* 55:13109-13150.

Furuichi, Y. and Shatkin, A.J. 1977. A simple method for the preparation of [β-^{32}P]purine nucleoside triphosphates. *Nucl. Acids Res.* 4:3341-3355.

Genieser, H.-G., Dostmann, W., Bottin, U., Butt, E., and Jastorff, B. 1988. Synthesis of nucleoside-3′,5′-cyclic phosphorothioates by cyclothiophosphorylation of unprotected nucleosides. *Tetrahedron Lett.* 29:2803-2804.

Glonek, T., Kleps, R.A., and Myers, T.C. 1974. Cyclization of the phosphate side chain of adenosine triphosphate: Formation of monoadenosine 5′-trimetaphosphate. *Science* 185:352-354.

Gryaznov, S.M. and Letsinger, R.L. 1991. Synthesis of oligonucleotides via monomers with unprotected bases. *J. Am. Chem. Soc.* 113:5876-5877.

Hoard, D.E. and Ott, D.G. 1965. Conversion of mono- and oligodeoxyribonucleotides to 5′-triphosphates. *J. Am. Chem. Soc.* 87:1785-1788.

Horwitz, J.P., Neenan, J.P., Misra, R.S., Rozhin, J., Huo, A., and Philips, K.D. 1977. Studies on bovine adrenal estrogen sulfotransferase III. Facile synthesis of 3′-phospho- and 2′-phosphoadenosine 5′-phosphosulfate. *Biochim. Biophys. Acta* 480:376-381.

Horwitz, J.P., Misra, R.S., Rozhin, J., Helmer, S., Bhuta, A., and Brooks, S.C. 1980. Studies on bovine adrenal estrogen sulfotransferase. V. Synthesis and assay of analogs of 3′-phosphoadenosine 5′-phosphosulfate as cosubstrates for estrogen sulfurylation. *Biochim. Biophys. Acta* 613:85-94.

Horwitz, J.P., Neenan, J.P., Misra, R.S., Rozhin, J., Huo, A., and Philips, K.D. May 1981.U.S. patent 4,266,048. Synthesis of Analogs of 3′-Phosphoadenosine 5′-Phosphosulfate (PAPS). USPTO Patent Full-Text and Image Database. USA, The United States of America as represented by the Department of Health.

Imai, J. and Torrence, P.F. 1981. Bis(2,2,2-trichloroethyl) phosphorochloridite as a reagent for the phosphorylation of oligonucleotides: Preparation of 5′-phosphorylated 2′,5′-oligoadenylates. *J. Org. Chem.* 46:4015-4021.

Iyer, R., Phillips, L.R., Egan, W., Regan, J.B., and Beaucage, S.L. 1990. The automated synthesis of sulfur-containing oligodeoxyribonucleotides using 3*H*-1,2-benzodithiol-3-one 1,1-dioxide as a sulfur-transfer reagent. *J. Org. Chem.* 55:4693-4699.

Janecka, A., Panusz, H., Pankowski, J., and Koziolkiewicz, W. 1980. Chemical synthesis of nucleoside-γ-[^{32}P]triphosphates of high specific activity. *Prep. Biochem.* 10:27-35.

Jankowska, J., Cieslak, J., and Kraszewski, A. 1997. 9-Fluorenemethyl *H*-phosphonothioate, a versatile reagent for the preparation of *H*-phosphonothioate, phosphorothioate, and phosphorodithioate monoesters. *Tetrahedron Lett.* 38:2007-2010.

Jankowska, J., Sobkowska, A., Cieslak, J., Sobkowski, M., Krazewski, A., Stawinski, J., and Shugar, D. 1998. Nucleoside *H*-phosphonates. 18. Synthesis of unprotected nucleoside 5′-*H*-phosphonates and nucleoside 5′-*H*-phosphonothioates and their conversion into the 5′-phosphorothioate and 5′-phosphorodithioate monoesters. *J. Org. Chem.* 63:8150-8156.

Jurczyk, S.C., Kodra, J.T., Park, J.-H., Benner, S.A., and Battersby, T.R. 1999. Synthesis of 2′-deoxyisoguanosine 5′-triphosphate and 2′-deoxy-5-methylisocytidine 5′-triphosphate. *Helv. Chim. Acta* 82:1005-1015.

Kadokura, M., Wada, T., Urashima, C., and Sekine, M. 1997. Efficient synthesis of γ-methyl-capped guanosine 5′-triphosphate as a 5′-terminal unique structure of U6 RNA via a new triphosphate bond formation involving activation of methyl phosphorimidazolidate using $ZnCl_2$ as a catalyst in DMF under anhydrous conditions. *Tetrahedron Lett.* 38:8359-8362.

Kaufmann, G., Choder, M., and Groner, Y. 1980. Synthesis of carrier-free β-^{32}P-nucleoside-triphosphate in almost quantitative yields. *Anal. Biochem.* 109:198-202.

Kim, M.-J. and Whitesides, G.M. 1987. Enzyme-catalyzed synthesis of nucleoside triphosphates from nucleoside monophosphates. *Appl. Biochem. Biotechnol.* 16:95-108.

Kimura, J., Fujisawa, Y., Yoshizawa, T., Fukuda, K., and Mitsunobu, O. 1979. Studies on nucleosides and nucleotides. VII. Preparation of pyrimidine nucleoside 5′-phosphates and N^3,5′-purine cyclonucleosides by selective activation of the 5′-hydroxyl group. *Bull. Chem. Soc. Jpn.* 52:1191-1196.

Klaassen, C.D. and Boles, J.W. 1997. The importance of 3′-phosphoadenosine 5′-phosphosulfate (PAPS) in the regulation of sulfation. *FASEB J.* 11:404-418.

Klein, E., Mons, S., Valleix, A., Mioskowski, C., and Lebeau, L. 2002. Synthesis of enzymatically and chemically non-hydrolyzable analogues of dinucleoside triphosphate Ap_3A and Gp_3G. *J. Org. Chem.* 67:146-153.

Kovacs, T. and Otvos, L. 1988. Simple synthesis of 5-vinyl- and 5-ethynyl-2′-deoxyuridine-5′-triphosphates. *Tetrahedron Lett.* 29:4525-4528.

Larsen, M., Willett, R., and Yount, R.G. 1969. Imidodiphosphate and pyrophosphate: Possible biological significance of similar structures. *Science* 166:1510-1511.

Lebedev, A.V., Koukhareva, I.I., Beck, T., and Vaghefi, M.M. 2001. Preparation of oligodeoxynucleotide 5′-triphosphates using solid support approach. *Nucleosides Nucleotides Nucleic Acids* 20:1403-1409.

Li, R., Muscate, A., and Kenyon, G.L. 1996. Synthesis, characterization, and inhibitory activities of nucleoside α,β-imido triphosphate analogues on human immunodeficiency virus-1 reverse transcriptase. *Bioorg. Chem.* 24:251-261.

Lin, C.-H., Shen, G.-J., Garcia-Junceda, E., and Wong, C.-H. 1995. Enzymatic synthesis and regeneration of 3′-phosphoadenosine 5′-phosphosulfate (PAPS) for regioselective sulfation of oligosaccharides. *J. Am. Chem. Soc.* 117:8031-8032.

Ludwig, J. 1987. A simple one flask synthesis of nucleoside 5′-triphosphates from unprotected nucleosides via nucleoside 5′-cyclotriphosphates. *In* Biophosphates and Their Analogues—Synthesis, Structure, Metabolism and Activity (K.S. Bruzik and W.J. Stec, eds.) pp. 201-204. Elsevier Science Publishers B V, Amsterdam.

Ludwig, J. and Eckstein, F. 1989. Rapid and efficient synthesis of nucleoside 5′-*O*-(1-thiotriphosphates), 5′-triphosphates and 2′,3′-cyclophosphorothioates using 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one. *J. Org. Chem.* 54:631-635.

Ludwig, J. and Eckstein, F. 1991. Synthesis of nucleoside 5′-*O*-(1,3-dithiotriphosphates) and 5′-*O*-(1,1-dithiotriphosphates). *J. Org. Chem.* 56:1777-1783.

Ma, Q.-F., Babbitt, P.C., and Kenyon, G.L. 1988. Adenosine 5′-[α,β-imido]triphosphate, a substrate for T7 RNA polymerase and rabbit muscle creatine kinase. *J. Am. Chem. Soc.* 110:4060-4061.

Ma, Q.-F., Kenyon, G.L., and Markham, G.D. 1990. Specificity of S-adenosylmethionine synthetase for ATP analogues mono- and disubstituted in bridging positions of the polyphosphate chain. *Biochemistry* 29:1412-1416.

McBride, L.J. and Caruthers, M.H. 1983. An investigation of several deoxynucleoside phosphoramidites. *Tetrahedron Lett.* 24:245-248.

Michelson, A.M. 1964. Synthesis of nucleotide anhydrides by anion exchange. *Biochim. Biophys. Acta* 91:1-13.

Mishra, N.C. and Broom, A.D. 1991. A novel synthesis of nucleoside 5′-triphosphates. *J. Chem. Soc., Chem. Comm.* 18:1276-1277.

Nairne, R.J.D., Pickering, L., and Smith, C.L. 2002. Synthesis of pyrrole carboxamide nucleotide triphosphates—putative labeled nucleotide analogues. *Tetrahedron Lett.* 43:2289-2291.

Nurminen, E.J., Mattinen, J.K., and Lonnberg, H. 1998. Kinetics and mechanism of tetrazole-catalyzed phosphoramidite alcoholysis. *J. Chem. Soc., Perkin Trans.* 2:1621-1628.

Patel, B.K. and Eckstein, F. 1997. 5′-Deoxy-5′-thioribonucleoside-5′-triphosphates. *Tetrahedron Lett.* 38:1021-1024.

Penningroth, S.M., Olehnik, K., and Cheung, A. 1980. ATP formation from adenyl-5′-yl imidodiphosphate, a nonhydrolyzable ATP analog. *J. Biol. Chem.* 255:9545-9548.

Quin, L.D. 2000. Organophosphorus Chemistry. Wiley-Interscience, New York.

Regan, J.B., Phillips, L.R., and Beaucage, S.L. 1992. Large-scale preparation of the sulfur-transfer reagent 3*H*-1,2-benzodithiol-3-one 1,1-dioxide. *Org. Prep. Proc. Int.* 24:488-492.

Reynolds, M.A., Gerlt, J.A., Demou, P.C., Oppenheimer, N.J., and Kenyon, G.L. 1983. ^{15}N and ^{17}O NMR studies of the proton binding sites in imidodiphosphate, tetraethyl imidodiphosphate, and adenylyl imidodiphosphate. *J. Am. Chem. Soc.* 105:6475-6481.

Richard, J.P., Ho, H.-T., and Frey, P.A. 1978. Synthesis of nucleoside [^{18}O]pyrophosphorothioates with chiral [^{18}O]phosphorothioate groups of known configuration. Stereochemical orientations of enzymatic phosphorylations of chiral [^{18}O]phosphorothioates. *J. Am. Chem. Soc.* 100:7756-7757.

Saady, M., Lebeau, L., and Mioskowski, C. 1995a. Synthesis of adenosine-5′-phosphates and 5′-alkylphosphonates via the Mitsunobu reaction. *Tetrahedron Lett.* 36:2239-2242.

Saady, M., Lebeau, L., and Mioskowski, C. 1995b. Synthesis of di- and triphosphate ester analogs via a modified Michaelis-Arbuzov reaction. *Tetrahedron Lett.* 36:5183-5186.

Saffhill, R. 1970. Selective phosphorylation of the *cis*-2′,3′-diol of unprotected ribonucleosides with trimetaphosphate in aqueous solution. *J. Org. Chem.* 35:2881-2883.

Schneider, B., Babolat, M., Xu, Y.W., Janin, J., Vernon, M., and Deville-Bonne, D. 2001. Mechanism of phosphoryl transfer by nucleoside diphosphate kinase. *Eur. J. Biochem.* 268:1964-1971.

Sekine, M., Ohkubo, A., and Seio, K. 2003. Proton-block strategy for the synthesis of oligodeoxynucleotides without base protection, capping reaction and P-N bond cleavage reaction. *J. Org. Chem.* 68:5478-5492.

Sekura, R.D. 1981. [53] Adenosine 3′-phosphate 5′-phosphosulfate. *In* Detoxification and Drug Metabolism: Conjugation and Related Systems (W.B. Jakoby, ed.) pp. 413-415. Academic Press, New York.

Simoncsits, A. and Tomasz, J. 1975. Simple one-step synthesis of ribonucleoside 2′,3′-cyclic phosphate 5′-phosphates. *Biochim. Biophys. Acta* 395:74-79.

Smith, M., Drummond, G.I., and Khorana, H.G. 1961. Cyclic phosphates. IV. Ribonucleoside-3′,5′ cyclic phosphates. A general method of synthesis and some properties. *J. Am. Chem. Soc.* 83:698-706.

Stec, W.J. 1983. Wadsworth-Emmons reaction revisited. *Acc. Chem. Res.* 16:411-417.

Stock, J.A. 1979. Synthesis of phosphonate analogues of thymidine di- and triphosphate from 5′-*O*-toluenesulfonylthymidine. *J. Org. Chem.* 44:3997-4000.

Stowell, J.K. and Widlanski, T.S. 1995. Mechanism-based inactiviation of ribonuclease A. *J. Org. Chem.* 60:6930-6936.

Stromberg, R. and Stawinski, J. 1987. Iodide and iodine catalysed phosphorylation of nucleosides by phosphorodiester derivatives. *Nucleosides & Nucleotides* 6:815-820.

Symons, R.H. 1970. Practical methods for the routine chemical synthesis of ^{32}P-labelled nucleoside di- and triphosphates. *Biochim. Biophys. Acta* 209:296-305.

Symons, R.H. 1974. Synthesis of [α-^{32}P]ribo- and deoxyribonucleoside 5′-triphosphates. *Methods Enzymol.* 29:102-115.

Thomson, J.B., Patel, B.K., Jimenez, V., Eckart, K., and Eckstein, F. 1996. Synthesis and properties of diuridine phosphate analogues containing thio and amino modifications. *J. Org. Chem.* 61:6273-6281.

Todd, A. 1958. Synthesis in the study of nucleotides. *Science* 127:787-792.

Tomasz, J., Vaghefi, M.M., Ratsep, P.C., Willis, R.C., and Robins, R.K. 1988. Nucleoside imidodiphosphate synthesis and biological activities. *Nucl. Acids Res.* 16:8645-8664.

Trevisiol, E., Defrancq, E., Lhomme, J., Laayoun, A., and Cros, P. 2000. Synthesis of nucleoside triphosphates that contain an aminooxy function for "Post-Amplification Labeling". *Eur. J. Org. Chem.* 1:211-217.

Tsuhako, M., Fujimoto, M., Ohashi, S., Nariai, H., and Motooka, I. 1984. Phosphorylation of nucleosides with sodium *cyclo*-triphosphate. *Bull. Chem. Soc. Jpn.* 57:3274-3280.

Uchiyama, M., Aso, Y., Noyori, R., and Hayakawa, Y. 1993. *O*-selective phosphorylation of nucleosides without *N*-protection. *J. Org. Chem.* 58:373-379.

Usher, D.A., Richardson, D.I. Jr., and Eckstein, F. 1970. Absolute stereochemistry of the second step of ribonuclease action. *Nature* 228:663-665.

Van Rompay, A.R., Johansson, M., and Karlsson, A. 2000. Phosphorylation of nucleosides and nucleoside analogs by mammalian nucleoside monophosphate kinases. *Pharmacol. Ther.* 87:189-198.

Verheyden, J.P.H. and Moffatt, J.G. 1970. Halo sugar nucleosides. I. Iodination of the primary hydroxyl groups of nucleosides with methyltriphenoxyphosphonium iodide. *J. Org. Chem.* 35:2319-2326.

Walseth, T.F. and Johnson, R.A. 1979. The enzymatic preparation of [α-^{32}P]nucleoside triphosphates, cyclic [^{32}P]AMP, and cyclic [^{32}P]GMP. *Biochim. Biophys. Acta* 526:11-31.

Walseth, T.F., Yuen, P.S.T., and Moos, M.C. Jr. 1991. Preparation of α-^{32}P-labeled nucleoside triphosphates, nicotinamide adenine dinucleotide, and cyclic nucleotides for use in determining adenylyl and guanylyl cyclases and cyclic nucleotide phosphodiesterase. *Methods Enzymol.* 195:29-44.

Werhli, W.E., Verheydden, D.L.M., and Moffatt, J.G. 1965. Dismutation reactions of nucleoside polyphosphates. II. Specific chemical syntheses of α-, β-, and γ-^{32}P-nucleoside 5′-triphosphates. *J. Am. Chem. Soc.* 87:2265-2277.

Wu, Y., Fa, M., Tae, E.L., Schultz, P.G., and Romesberg, F.E. 2002. Enzymatic phosphorylation of unnatural nucleosides. *J. Am. Chem. Soc.* 124:14626-14630.

Wu, W., Bergstrom, D.E., and Davisson, V.J. 2003. A combination chemical and enzymatic approach for the preparation of azole carboxamide nucleoside triphosphate. *J. Org. Chem.* 68:3860-3865.

Yoshikawa, M., Kato, T., and Takenishi, T. 1967. A novel method for phosphorylation of nucleosides to 5′-nucleotides. *Tetrahedron Lett.* 50:5065-5068.

Yount, R.G., Babcock, D., Ballantyne, W., and Ojala, D. 1971. Adenylyl imidodiphosphate, an adenosine triphosphate analog containing a P-N-P linkage. *Biochemistry* 10:2484-2489.

Zemlicka, J. 2002. Lipophilic phosphoramidates as antiviral pronucleotides. *Biochim. Biophys. Acta* 1587:276-286.

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