Syntheses of Nucleoside Triphosphates

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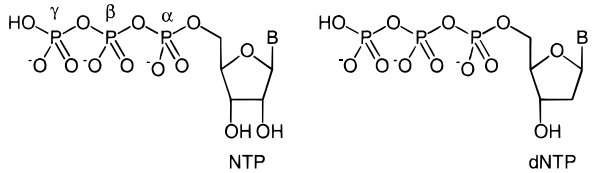
1. Introduction

Nucleoside triphosphates (NTPs and dNTPs, Figure 1) have important therapeutic and diagnostic applications. Experience with syntheses of these

Figure 1



B = heterocyclic base, NTP = ribonucleoside triphosphate
dNTP = 2'-deoxyribonucleoside triphosphate

compounds, and discussions with others working in this area, has led us to the conclusion that the need for a general and high-yielding route to nucleoside

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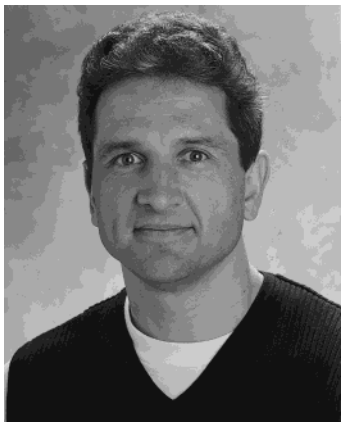
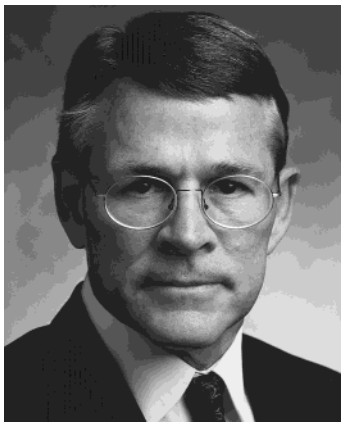


Kevin Burgess is a professor at Texas A&M University, where he has been since September 1992. His research interest focuses on ways in which synthetic organic chemists can help to solve problems in medicinal chemistry and biotechnology. Specific research projects in The Burgess Group include peptidomimetics of protein–protein interactions, combinatorial chemistry, libraries of asymmetric catalysis, and methodology for high-throughput DNA sequencing. It is interest in the latter project that provided motivation to write this review.



Dan Cook, a keep-fit fanatic, was born in 1944 in the farm community of Clovis, NM. On his way to obtaining his doctorate in organic chemistry, under the direction of Raymond N. Castle, he attended the New Mexico Military Institute, Eastern New Mexico University, University of New Mexico, and Brigham Young University. He completed an industrial postdoctoral fellowship with Roland K. Robins at ICN Pharmaceutical in Costa Mesa, CA, in 1970–72, spent 11 years as a medicinal chemist at Warner-Lambert/Parke-Davis Pharmaceutical Research Division in Ann Arbor, MI, and one year at Eastman Kodak Research Laboratories in Rochester, NY. After that he moved to Malvern, PA, with the Eastman Pharmaceutical Division/Sterling Research Group. In 1989, he joined Isis Pharmaceuticals in Carlsbad, CA, where he is now Vice President, Research Chemistry. His research interests include heterocycles, nucleosides, nucleotides, and oligonucleotides as they relate to medicinal applications.

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triphosphates is an unsolved problem. There are methods that work extremely well for some substrates, and there are a few methods that work moderately well for most substrates. However, none of the protocols for making nucleoside triphosphates is universally satisfactory. For instance, there is none that would allow a diverse set of nucleoside triphosphates to be made via a combinatorial or high-throughput parallel synthesis.

This review was written to summarize the most useful approaches that have been used to prepare nucleoside triphosphates. It is not comprehensive since some of the earliest work has clearly been superseded by newer methods, and that earlier work is not included here. Furthermore, we did not attempt to tabulate all the different nucleoside triphosphates that have been made. Instead, the goal of this review is to illustrate the most practical methods for obtaining nucleoside triphosphates and, more importantly, to hint at areas wherein further research might lead to superior approaches.

Syntheses of some nucleoside triphosphate analogues are discussed in this review to illustrate how synthetic methods for obtaining nucleoside triphosphates have been applied in different ways. Distinct methods unique to preparations of analogues may not be cited here. Syntheses of radiolabeled nucleoside triphosphates will only be mentioned if they demonstrate some particular feature of the synthetic strategy.

2. General Practical Considerations

Nucleoside triphosphates are difficult to make, isolate, characterize, and store due to several factors. First, many of the methods for their preparation involve combinations of charged ionic reagents with more lypophilic substrates (e.g., pyrophosphates and protected nucleosides, respectively). Consequently, it is difficult to find appropriate reaction media and the purification procedures must involve isolation of a charged water-soluble product from a mixture of hydrophilic and hydrophobic impurities. Second, nucleoside triphosphates are not particularly robust. It is difficult to make quantitative generalizations about their rates of decomposition, but it is certain that hydrolysis of the triphosphate functionality is accelerated under basic and acidic conditions. Researchers have commented that solid samples of nucleoside triphosphates decompose on standing over a period of days, even at low temperature.[1] In general, nucleoside stability, or lack of it, is probably related to the triphosphate counterions and the local environment of the sample. Triphosphates in the protic form are thought to be relatively unstable, presumably because they lower the pH of unbuffered media in which they are dissolved, accelerating their decomposition. Under neutral conditions, however, they can be stable at reduced temperatures for years, particularly if trialkylammonium counterions are used.

Methods for detecting and isolating triphosphates have improved dramatically since the first chemical syntheses. Indeed, the fact that the pioneers in this area were able to make so much progress without some techniques that are now commonplace provides remarkable illustrations of superb practical expertise combined with dogged perseverance. For example, in 1949 Michelson and Todd reported a synthesis, isolation, and characterization of ATP.[2] Their isolation protocol involved several laborious precipitations of barium salts under controlled pH conditions, nonchromatographic ion exchange procedures, and recrystallization of the corresponding acridinium salt. Characterization of the product was via, "...m.p., mixed m.p., X-ray powder photographs, infra-red absorption spectra, and biological activity of the regenerated free acid...". Later, paper chromatography was used extensively to gauge the purity of triphosphates,[1] but this method has now been largely superseded by HPLC.[3] For the latter, C-18 and ion-exchange columns are preferred. Generally, the eluant will involve a volatile, protic, ammonium salt (e.g., triethylammonium bicarbonate) that can be removed under vacuum to give protonated triphosphates. Chromatographic isolations of triphosphates generally involve gravity chromatography of the crude product on DEAE-A25 cellulose [2-(diethylamino)ethyl-sephadex] using a gradient of increasing triethylammonium bicarbonate in water as the eluant. Product in the fractions is conveniently detected via UV spectroscopy monitoring at 250−280 nm; a flow cell for continuous detection is particularly useful for this purpose. Analytical TLC on silica plates using 1-propanol/H_2O/28% ammonium hydroxide (11:2:7)[4] or 2-propanol/NH_3/H_2O (7:1:2)[5] is also a useful tool. Further purification, if necessary, is usually performed via preparative HPLC. Several freeze-drying cycles may be necessary to remove the ammonium counterions from the final product.

^{31}P NMR spectra of nucleoside triphosphates feature three resonances at around −6, −11, and −22 ppm (in D_2O relative to H_3PO_4 external standard), corresponding to the γ, α, and β phosphorus atoms, respectively. Chemical shift values for nucleoside triphosphates are highly pH and counterion dependent. The ease with which these signals are observed is inversely related to exchange processes that occur on the NMR time scale. These exchange processes, usually involving the triphosphate counterions, can be tempered by buffering the medium. Addition of EDTA to samples is a useful way to suppress exchange processes involving metallic counterions, giving more easily interpretable ^{31}P NMR spectra.[5] Proton NMR spectra of nucleoside triphosphates are also influenced by exchange phenomena. Moreover, inadequate freeze-drying can result in trialkylammonium salt impurities that can obscure and/or overshadow relevant peaks in the spectra. The spectral characteristics of nucleoside triphosphates are also influenced by pH.

It is difficult to make generalities regarding nucleoside triphosphate acidities because they are complicated by the composition of the heterocyclic base and by the presence of metal ions in solution. Measurements on the linear inorganic triphosphate unit have been made, but these are not particularly helpful.[6] If the fully protonated form of a nucleoside triphosphate is represented by H_4NTP, then the

transitions to the mono- and dianions, H_3NTP^- and H_2NTP^{2-}, take place at very low pH values, such that functionality on the heterocyclic base is also likely to be protonated. The next deprotonation to the $HNTP^{3-}$ form is likely to be competitive with deprotonation of the heterocyclic base. Transition of the trianions to the tetranionic state NTP^{4-} takes place at pH values very close to 7.0.[7]

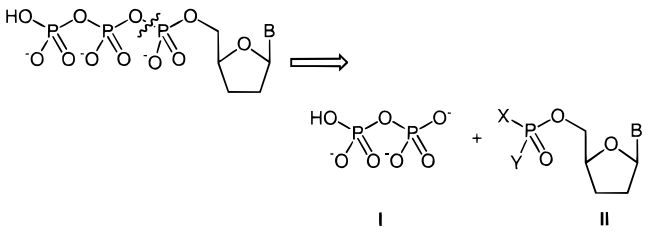
Fast-atom bombardment (FAB) has gained prevalence in mass spectrometry of nucleoside triphosphates. However, a direct comparison of FAB and MALDI (matrix-assisted laser desorption ionization) analyses of ATP, GTP, TTP, and CTP revealed the latter technique gave less fragmentation and required less sample.[8] The matrices used in these particular FAB and MALDI experiments were diethanolamine/triethanolamine and 2,4,6-triacetoxyacetophenone, respectively. Both experiments were run in the negative-ion mode.

Some recent examples of preparations of nucleoside triphosphates give excellent experimental procedures from which many practical tips can be learned.[4]

3. Syntheses via Nucleophilic Attack of Pyrophosphate on an Activated Nucleoside Monophosphate

The most widely used syntheses of nucleoside triphosphates involve the disconnection shown in Figure 2.

Figure 2



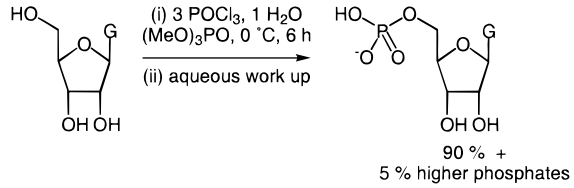
Pyrophosphate salts **I** are commercially available, like tri-*n*-butylammonium pyrophosphate from Sigma Co., but the activated nucleoside monophosphate **II** must be prepared.

3.1. Reactions Involving Dichlorophosphates

Early work on the phosphorylation of nucleosides using phosphorus oxytrichloride was hampered by lack of regioselectivity. An innovation was made by Yoshikawa and co-workers who discovered that the rate of phosphorylation of nucleosides was accelerated by using trimethyl- or triethylphosphate as the solvent.[9] Two attributes of trialkylphosphate solvents are notable. First, unlike some of the media used in earlier work, they tend to dissolve the nucleoside and reagents giving homogeneous solutions. Second, interaction of the trialkylphosphate with $POCl_3$ is thought to form an active intermediate that has been compared to the complex formed between DMF and $POCl_3$.[10]

Yoshikawa's initial studies were performed on 2′,3′-*O*-isopropylidene-protected NTPs, but later they found selective reaction at the 5′-hydroxyl was possible for unprotected NTPs and dNTPs. An acidic medium was reported to be critical for suppressing formation of 2′- and/or 3′-phosphates; Yoshikawa's procedure was to add controlled amounts of water to form HCl in situ. However, subsequent work (vide infra) reveals that good regioselectivities can be obtained when the medium is slightly basic, so the relationship between regioselectivity of phosphorylation and pH remains unclear. The example shown in Scheme 1 is typical of the results reported by Yoshikawa's group.

Scheme 1

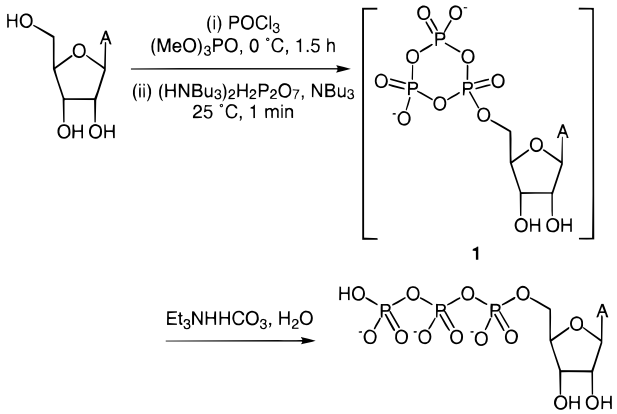


Earlier references describe the selectivity of monophosphorylation via Yoshikawa's method to be high, e.g., for 5′-selective monophosphorylation of a diribonucleotide.[11] However, it appears that the selectivity is seldom perfect, and contemporary analytical methods would show this more clearly than was previously possible.[12]

Yoshikawa and co-workers also used pyrophosphoryl chloride [$Cl_2P(O)OP(O)Cl_2$] in place of $P(O)Cl_3$ but reported no significant advantages. Almost concurrently, another group used dichlorophosphoric acid anhydride to phosphorylate nucleosides in media other than trialkyl phosphates (e.g., 2-chlorophenol). Some highly selective reactions were found, but nonselective examples were also observed.[13,14] This methodology has not been as widely used as the corresponding reactions in trialkylphosphates.

An attractive feature of Yoshikawa's monophosphorylation method is that the chlorophosphate intermediate, before hydrolysis, can be used directly in reactions with the pyrophosphate ion. Thus, Ludwig,[15] and others working independently,[16] generated nucleoside dichlorophoshates via Yoshikawa's procedure and then added bis(tri-*n*-butylammonium) pyrophosphate in dry DMF. Ludwig noted that decreased yields were obtained if an amine base was not added. After 1 min, the reaction was quenched with triethylammonium bicarbonate buffer and worked up in the usual way (Scheme 2).

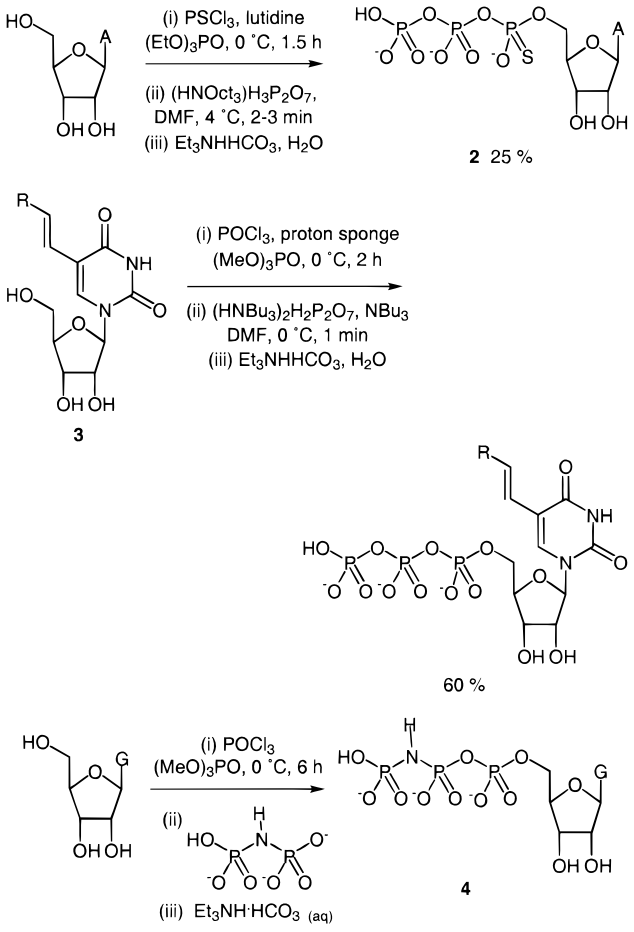
Scheme 2



Several features of the procedure shown in Scheme 2 deserve further comment. First, water was not added in the $POCl_3$ phosphorylation, even though Yoshikawa et al. had indicated that the HCl generated by doing this increases the selectivity for 5′-phosphorylation. Second, Ludwig indicates that incomplete selectivity in the first phosphorylation step may give rise to impurities. Finally, it was proposed that the alkyl trimetaphosphate intermediates like **1** were formed immediately prior to the hydrolysis step. Trimetaphosphates are produced from activated triphosphate derivatives under anhydrous conditions and are readily hydrolyzed in the presence of water.[2,17−19] Detailed procedures for particular applications of Ludwig's procedure have been reported.[20]

Several examples of "one-pot, three-step" formation of triphosphates are shown in Scheme 3. The first

Scheme 3



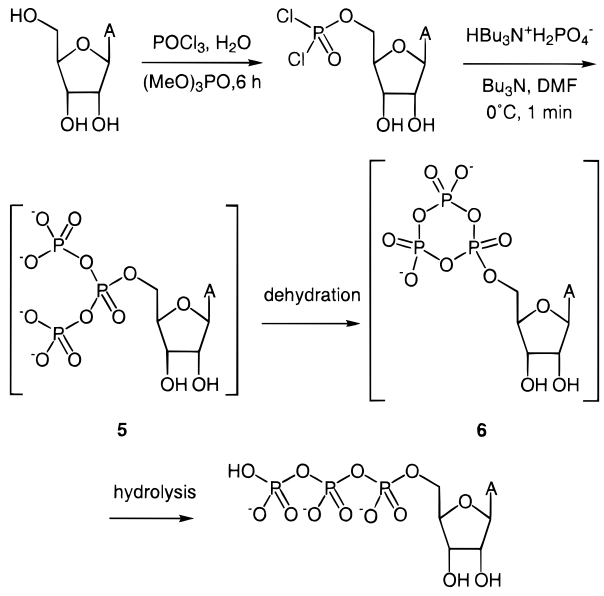
illustrates that the methodology can be applied to syntheses of nucleoside 5′-*O*-(1-thiotriphosphates) (prepared as a mixture of diastereoisomers), e.g., **2**;[21,22] however, the yield obtained in this transformation was moderate. More recently, 5′-*O*-(1-thiotriphosphate)-2′-deoxyadenosine was prepared in a similar manner in 42% yield, and a detailed experimental procedure was given.[23] The second example shown in Scheme 3 features the alkene-functionalized nucleoside **3**. That alkene functionality was shown to react with HCl in the medium when Yoshikawa's phosphorylation conditions were used.[24] Consequently, 1,8-bis(dimethylamino)naphthalene (proton sponge) was added to facilitate phosphorylation under basic conditions. No problems with regioselectivity were reported, and triphosphates were isolated in ca. 60% yield using this modification. The third transformation in Scheme 3 is one in which Ludwig used an imidodiphosphate instead of pyrophosphate in the second step to generate nucleoside β,γ-imidotriphosphates **4**.[25]

A modification of Yoshikawa's procedure that uses $PSCl_3$ to generate 1-thiotriphosphates has also been reported.[26]

Despite the popularity of nucleoside triphosphate syntheses that involve reaction of nucleosides with $POCl_3$ then with pyrophosphate, the method is not perfect and is not successful for all nucleoside derivatives. Careful analyses of reactions to monitor formation of intermediates has shown that the reaction times are highly variable depending on the type of nucleoside used.[16] It has also been noted that the first step is not perfectly selective for the 5′-hydroxyl group and that both the possible monophosphorylation products and the diphosphorylation products can be generated. This is a particularly serious concern when the corrresponding byproducts can skew data obtained from bioassays featuring the products.[12]

Treatment of activated nucleoside monophosphate with phosphate ions (rather than pyrophosphate) has been reported to give nucleoside triphosphates. For instance, guanosine has been treated with DCC in pyridine, then with excess 85% phosphoric acid, to give GTP.[27] Similarly, treatment of the adenosine 5′-dichlorophosphoramidate with phosphate was reported to give ATP.[28] It was proposed that the latter reaction proceeded via the *pseudo*-ATP **5**,[29] dehydration to the monoalkyl trimetaphosphate **6** mediated by excess $POCl_3$, and then hydrolysis (Scheme 4).[19]

Scheme 4



However, it is possible that diphosphate formed in situ under the reaction conditions, then added to the activated phosphate derivative. Certainly, speculation regarding intermediates such as **6** preceded

advanced spectroscopic techniques that might have been used to establish their presence.

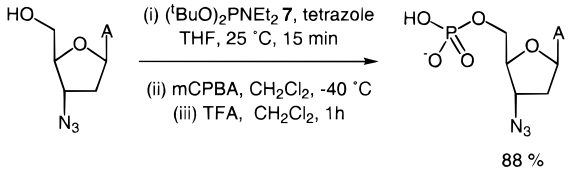
3.2. Reactions Involving Nucleoside Phosphoramidates

It is difficult to ascertain the efficiency of the monophosphorylation step in the "one-pot, three-step" triphosphorylations discussed in the last section. This is not a problem, however, if the nucleoside monophosphates are isolated en route to nucleoside triphosphates.

Natural dNTP nucleoside monophosphates are obtained on a large scale by hydrolysis of salmon sperm, which is very rich in DNA. Chemical methods must be used, however, for preparation of analogues or derivatives.[30,31]

There are several methods for 5′-phosphorylation of nucleosides besides direct reaction of nucleosides with $POCl_3$ then hydrolysis.[32] Some of the alternatives have not been fully exploited in multistep syntheses of nucleoside triphosphates. For instance, 5′-phosphates can be introduced via the Mitsunobu reaction,[33−35] though application of this type of transformation in syntheses of nucleoside triphosphates is uncommon (vide infra). Similarly, phosphoramidites are obvious reagents for the formation of 5′-nucleoside monophosphates, as illustrated in Scheme 5[36] in which the hindered phosphoramidite **7**[37] is used.

Scheme 5

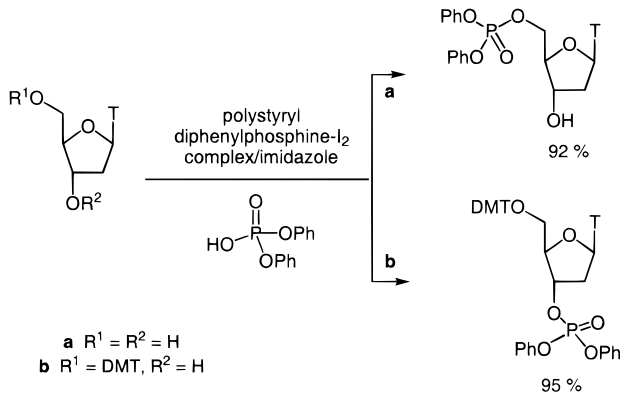


Approaches to phosphorylation of organic compounds were reviewed in 1977.[38] More recently, several interesting nucleoside phosphorylation procedures have been described. A method presented as being a highly *O*-selective phosphorylation of nucleosides features *tert*-butylmagnesium chloride (or similar reagents) for hydroxyl activation. Phosphorylated nucleosides were reported to be formed in yields exceeding 90% via this procedure.[39] A mild and regioselective phosphorylation of nucleosides was achieved by direct coupling of nucleosides with a suitable phosphorylating agent in the presence of the supported activation agent: polystyryl diphenylphosphine−I_2 complex (Scheme 6).[40] This appears to be one of the first reports of a direct, solid-phase phosphorylation, and it may have potential applications in combinatorial chemistry/parallel syntheses.

Other procedures for monophosphorylation that may be useful for nucleosides include phosphorylation of nucleosides with trialkyl phosphites in the presence of iodine.[41] This gives dialkyl esters that can be selectively dealkylated using bromotrimethylsilane.[41,42]

The literature shows that useful conditions for activation of nucleoside monophosphates for reaction

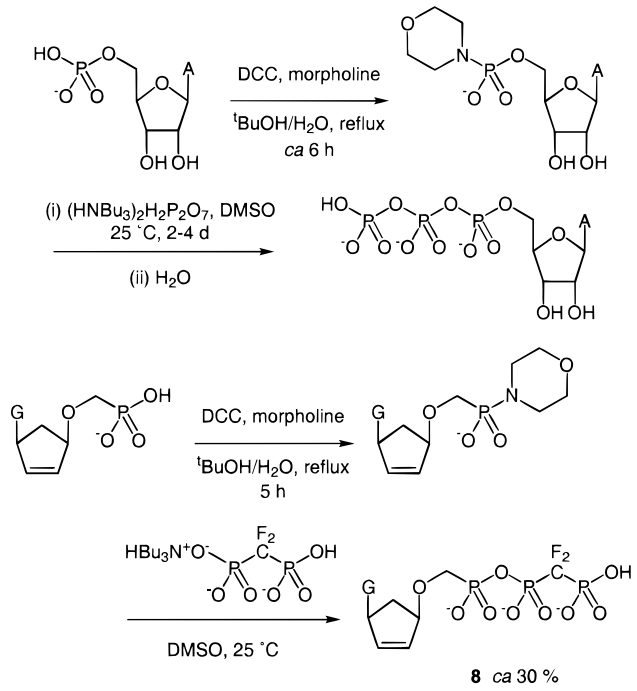
Scheme 6



with pyrophosphates took considerable effort to develop. Early studies focused on use of DCC in pyridine for activation, but mono-, di-, and inorganic polyphosphates were formed in addition to the desired triphosphates.[43,44]

Two improvements to the protocol for DCC activation of monophosphates transformed this approach into a truly practical procedure. The first was to add an amine, e.g., morpholine, to the system resulting in formation of an intermediate phosphoramidate. A complication in this reaction is competing addition of the amine to the DCC giving a guanidine that inhibits the formation of the phosphoramidate. Nevertheless, conditions were devised to suppress the detrimental effects of this side-reaction.[45] The second development came after it was realized that the triphosphate undergoes disproportionation reactions in pyridine. Thus, the triphosphate concentration would peak and then decline giving diphosphates after extended reaction times. Moffat realized this and found that the disproportionation reaction did not occur in other solvents, notably DMSO.[46] Scheme 7 shows the details of the

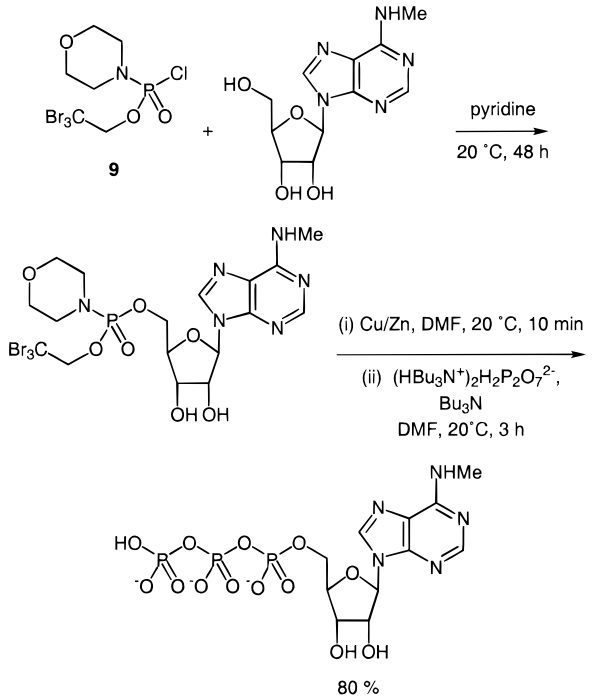
Scheme 7



original synthesis of ATP via this method, and yields of 73−80% were obtained for various other nucleosides. Preparation of the difluoromethylenebisphosphonate of a carbocyclic nucleoside triphosphate, i.e., compound **8**,[47] is also shown. This transformation illustrates one of the many different analogue types that can be prepared by modification of Moffat's procedure.[48−52]

2,2,2-Tribromoethyl morpholinochlorophosphate (**9**) has been used as an alternative reagent for generating phosphoramidate in nucleoside triphosphate syntheses.[53] Its reaction with N^6-methyl adenosine was shown to proceed with high selectivity for the 5′-hydroxyl group. Reductive removal of the *O*-protecting group, then reaction pyrophosphate with the resulting phosphoramidate, gave a methylated ATP in high yield (Scheme 8).

Scheme 8

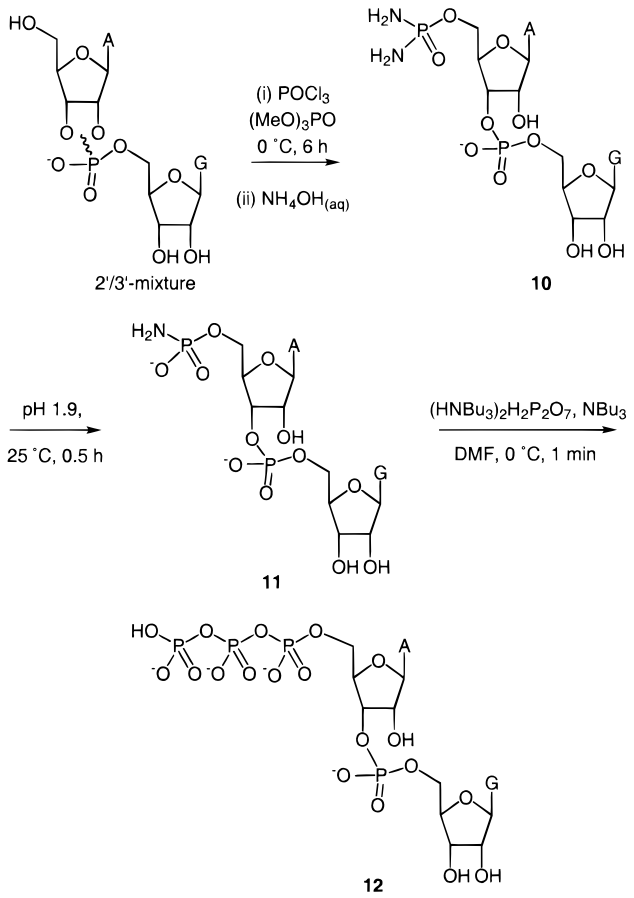


A simple, two-step method to prepare 5′-triphosphate derivatives of 3′−5′-diribonucleoside phosphates (e.g., 5′-pppApG) was described by Tomasz et al.[54] A mixture of 2′- and 3′-diribonucleoside phosphates were 5′-phosphorylated by Yoshikawa's $POCl_3$ procedure, and the resulting chlorophosphate esters were converted into the corresponding 5′-phosphordiamidates using aqueous ammonium hydroxide. The 2′−5′ linked material **10** was then separated from this mixture. Acid hydrolysis selectively removed one of the two amide groups to provide the 5′-phosphoramidate **11**.[55] This was converted into the desired 5′-triphosphate **12** using bis-(tri-*n*-butyl)ammonium pyrophosphate (Scheme 9).[2]

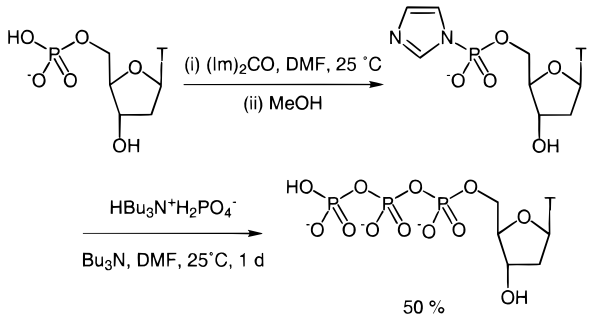
3.3. Reactions Involving Other Activated Nucleoside Monophosphates

Phosphoroimidazolidates result from activation of nucleoside monophosphates with 1,1′-carbonyldiimidazole.[56−58] Hoard and Ott's original research on this transformation featured syntheses of triphosphates from dNTPs and oligomers capped with dNTP residues. Yields ranged between 70% and 20%.[59] An example is shown in Scheme 10.

Scheme 9



Scheme 10



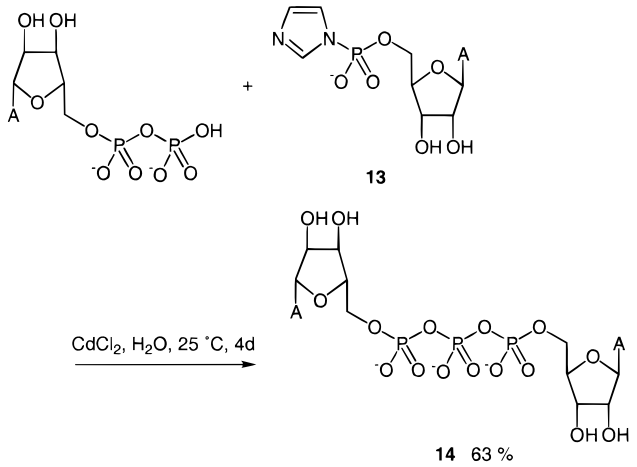
Complications in the Hoard−Ott procedure have been reported when NTPs (rather than dNTPs as in Scheme 10) were activated with 1,1′-carbonyldiimidazole.[60] This phosgene equivalent reacted to give cyclic carbonates that were carried through as impurities in the triphosphorylation procedure (Scheme 11). However, removal of the carbonate functionality with ammonia is possible.

Coordination of metal ions to phosphoroimidazolidates may increase their electrophilicity, and this concept has been used in nucleoside triphosphate syntheses. Thus reaction of nucleoside phosphoroimidazolidates **13** in neutral aqueous solution was

Scheme 11

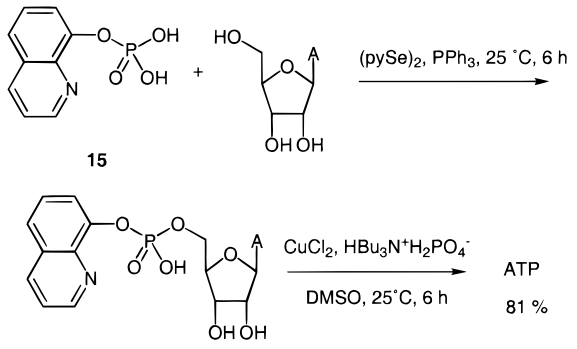
accelerated by $MnCl_2$, and $CdCl_2$. Relatively low yields of NTPs were formed when inorganic pyrophosphate was used as a nucleophile, but a good yield of the dinucleoside triphosphate **14** was formed (Scheme 12).

Scheme 12



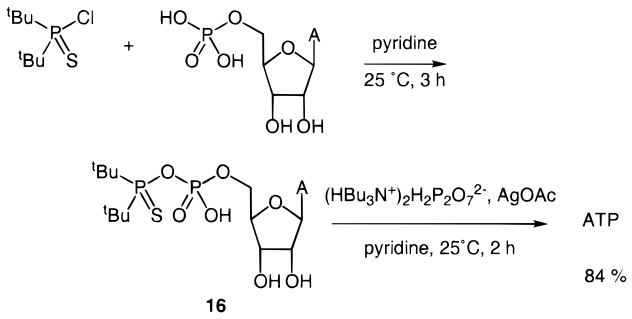
Activation by coordination was also used in a synthesis of nucleoside triphosphates from 8-quinolyl phosphates (Scheme 13).[61] The requisite 8-quinolyl diesters were prepared by reaction of the 8-quinolyl monophosphate **15** with triphenylphosphine/2,2′-dipyridyl diselenide. The purified intermediate was activated for reaction with pyrophosphate by addition of copper (2+) in DMSO; the products were isolated in 73−83% yields based on the intermediate.

Scheme 13



Formation of di-*tert*-butylphosphinethionic anhydrides such as **16** provides another method for activating nucleoside phosphates for reaction with pyrophosphate. They were formed in nearly quantitative yield in the reaction shown in Scheme 14.[62] Silver ions are used to enhance the reactivity of these phosphorus-based mixed anhydrides as indicated.

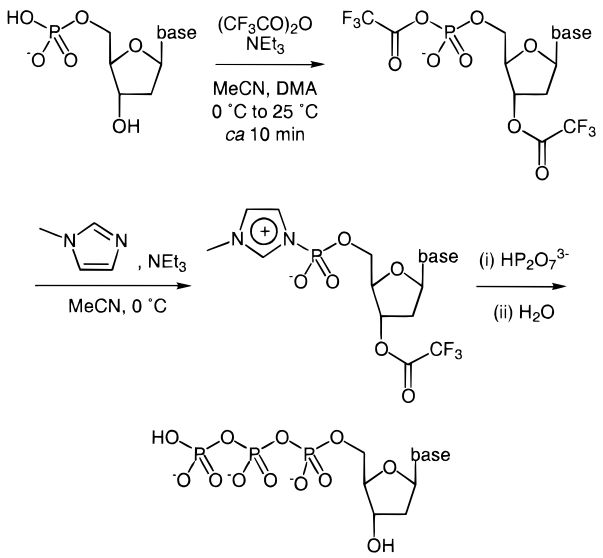
Scheme 14



The method shown above is similar to that described in older work by Michelson in which A 5′-phosphate is activated with $(PhO)_2P(O)Cl$ to form the corresponding mixed anhydride.[63] Diphenyl phosphate is a better leaving group than the nucleoside 5′-phosphate, hence reaction with pyrophosphate nucleophile gives the corresponding triphosphate. Only a slight excess of pyrophosphate is required, so the product is not contaminated by large excess of this material. This method has not been widely used in recent syntheses of nucleoside triphosphates but has no obvious and serious disadvantages. Indeed, a similar methodology has been used to form trihphosphates functionalized at the γ-phosphorus with fluoride, azides, and with alkoxides.[64,65]

Trifluoroacetic anhydride has also been used for activation of nucleoside monophosphates.[66] This procedure, summarized in Scheme 15, features *N*-methylimidazole or 4-(dimethylamino)pyridine (4-DMAP) as a carrier for the phosphorylation process. Yields of 89−92% were reported for A, T, G, anc C. This paper appeared in the Russian literature in 1996 and has not been used extensively, but it may be useful in practice.

Scheme 15

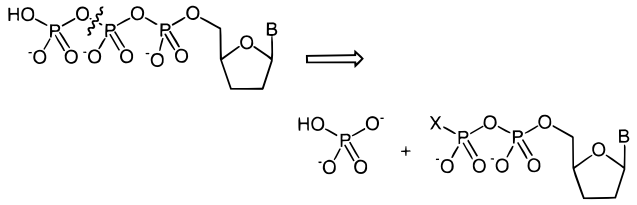


4. Syntheses via Nucleophilic Attack of Phosphate on an Activated Nucleoside Pyrophosphate

There are surprisingly few nucleoside triphosphate syntheses that proceed via the disconnection indi-

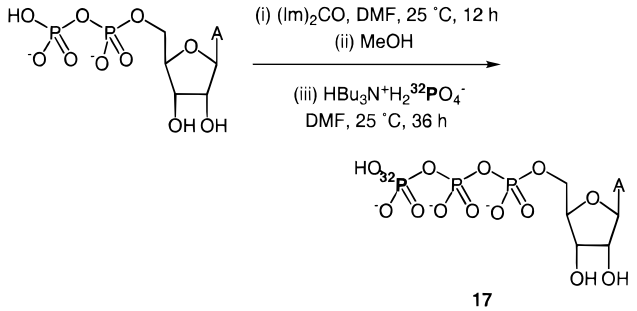
cated in Figure 3. Obstacles that must be overcome in this type of approach include activation of the correct phosphorus atom (β not α) without disrupting other functionality in the nucleoside unit. However, neither of these potential difficulties is discussed in the sparse literature that exists on this subject.

Figure 3



The phosphate/nucleoside diphosphate disconnection indicated above is an obvious route to ^{32}P-labeled nucleoside triphosphates and has been used for exactly that application (Scheme 16).[67] Several aspects of this synthesis are interesting in the light of work already discussed in this review. First, 1,1′-carbonyldiimidazole is known to form cyclic carbonate byproducts with ribonucleotides,[68] but none were detected in this particular synthesis. Second, activation at both the α- and β-phosphorus atoms of the diphosphate is possible. If partial activation had occurred at the α-position, then this should lead to *pseudo*-ATP **5** (vide supra) which might possibly dehydrate and rearrange to ATP via a metatriphosphate intermediate **1**. However, the ^{32}P label introduced to prepare radioactive product also acts as a mechanistic probe in this experiment. All of the label was shown to reside in the γ-phosphate of product **17** via a specific enzyme-mediated transfer of the γ-phosphate to glucose, then the radioactivity in the glucose-6-phosphate and in the residual ADP was measured. Only the glucose-6-phosphate was radioactive, providing evidence against activation at the α-phosphate of the starting material.

Scheme 16



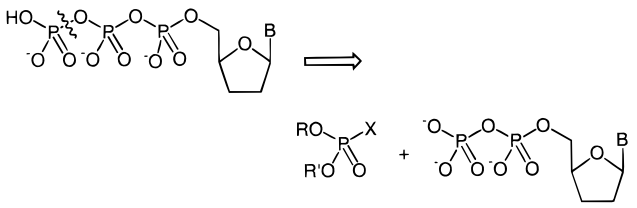
A reported procedure that is similar to the one shown in Scheme 16 involved activation of nucleoside 5′-diphosphates using ethyl chloroformate. The resulting mixed anhydrides were reacted with [^{31}P]-triethylammonium orthophosphate to provide γ-labeled ATP.[69]

Some biochemical methods (summarized in the final section of this review) also convert diphosphates into triphosphates via the disconnection mentioned in Figure 4.

5. Syntheses via Nucleophilic Attack of Nucleoside Diphosphate on an Activated Phosphate Synthon

The reactions discussed in the previous section involve a diphosphate electrophile and a phosphate nucleophile, but these roles could be reversed as indicated in Figure 4.

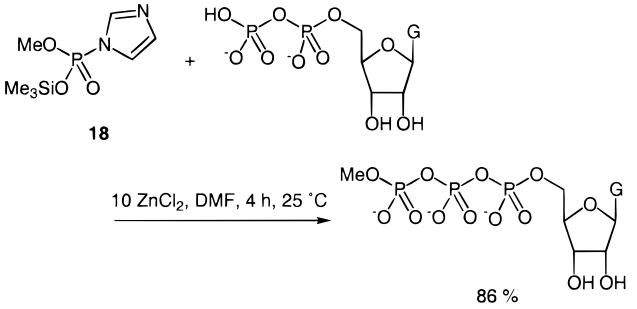
Figure 4



The disconnection shown in Figure 4 was the basis of early work by Todd and co-workers in which a benzyl-protected nucleoside diphosphate was reacted with dibenzyl chlorophosphate[70] or benzyl phosphoramidate.[71] At the end of this procedure, the benzyl protecting groups were removed via hydrogenolysis. It is interesting that contemporary syntheses of nucleoside triphosphates tend to involve coupling of unprotected phosphates. However, protected derivatives are more lipophilic, hence they are somewhat more easily purified. It might be opportune for a commercial supplier to invest the effort required to market a few key protected reagents designed for syntheses of masked nucleoside triphosphates.

A recent synthesis of γ-methyl GTP provides an illustration of the approach outlined in Figure 4.[72] The methyl phosphorimidazolide **18** was prepared especially for this synthesis. Reaction of this with guanosine diphosphate was unsatisfactory unless electrophilic metal ions were added; the most suitable metal salt found to mediate this reaction was zinc dichloride (Scheme 17).

Scheme 17



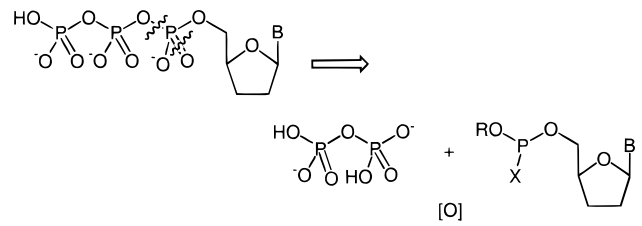
Reagents similar to **18** have also been used to prepare triphosphate analogues from the corresponding diphosphates.[73]

Some biochemical methods (summarized in the final section of this review) also convert diphosphates into triphosphates via the disconnection mentioned in Figure 4.[74]

6. Syntheses Involving Activated Phosphites or Phosphoramidites Derived from Nucleosides

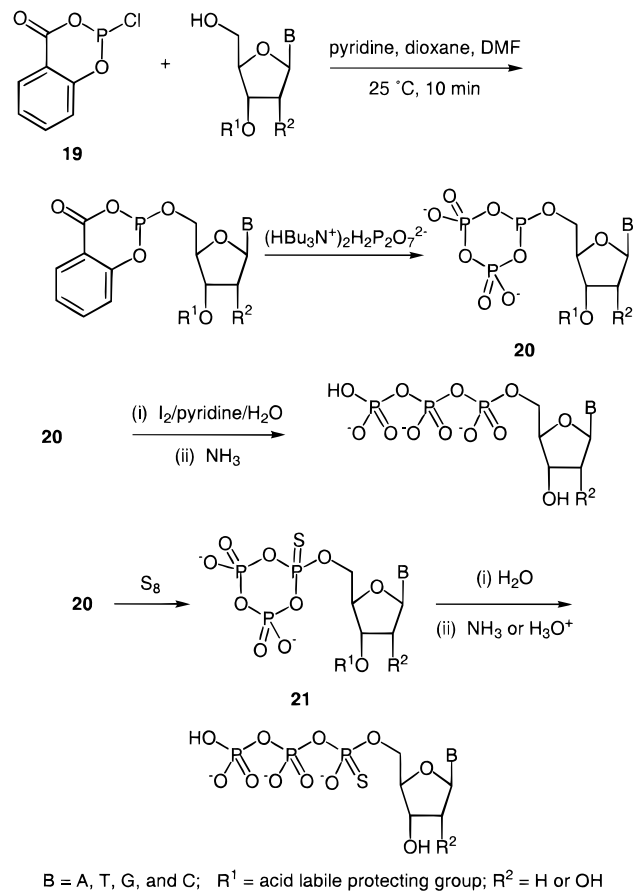
A set of extremely useful nucleoside triphosphate syntheses conforms to the disconnections shown in Figure 5.

Figure 5



The most important paper concerning the approach illustrated above is one by Ludwig and Eckstein.[5] They reacted nucleoside derivatives with 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (**19**). Reagent **19** had previously been used to form nucleoside *H*-phosphonates.[75] This reaction gave an activated phosphite that was reacted with pyrophosphate to form the cyclic intermediate **20** that was then hydrolyzed/oxidized to give the corresponding triphosphate (Scheme 18). Modification of the oxidation conditions also facilitated syntheses of 5′-*O*-(1-thiotriphosphate)s as mixtures of diastereomers. It was also shown that protection of nucleobase functionality for A, T, G, and C was not required, but selectivity for the 5′-hydroxyl in the initial phosphitylation step was marginal if the 3′- (and presumably the 2′-) hydroxyl was not protected.

Scheme 18



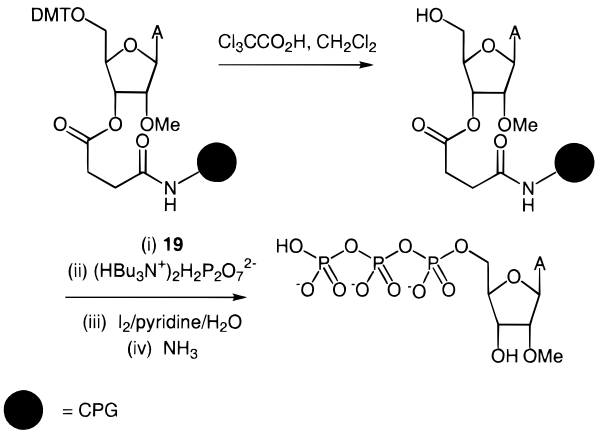
B = A, T, G, and C; R^1 = acid labile protecting group; R^2 = H or OH

Ludwig and Eckstein used ^{31}P NMR to follow the course of the reactions shown in Scheme 18. Consequently, there is good evidence for the intermediates shown above, even though this is a one-flask protocol, hence the intermediates were not isolated. Interestingly, hydrolysis/ring opening of the 1-*O*-alkyl-1-thiometatriphosphate intermediate **21** could occur at a phosphate or at the thiophosphate functionality. It was shown by $H_2{}^{18}O$-labeling experiments that the hydrolysis occurs exclusively at phosphate (label observed in the γ-phosphate of the product). This is rational since α-phosphothioates are better leaving groups than phosphates.

The Ludwig and Eckstein procedure has proved to be useful for preparations of a wide range of nucleoside derivatives. Synthesis of nucleoside 5′-*O*-(1,3-dithiotriphosphate)s and 5′-*O*-(1,1-dithiotriphosphate)s as a separable mixture was accomplished by treatment of the cyclic thiometatriphosphate intermediate **21** with Li_2S in pyridine/dioxane.[76] The 5′-*O*-(1,1-dithiotriphosphate) was obtained as the sole product if DMF was used as the solvent. In another modification, intermediates related to compound **20** have been reacted with BH_3 to give boronated nucleoside products.[77]

Two solid-phase syntheses of nucleoside triphosphates and a solid-phase synthesis of 5′-triphosphates of oligonucleotides, all based on the Ludwig−Eckstein method, have been reported. The first featured 5′-*O*-dimethoxytrityl (DMT) protected, 2′-*O*-methylribonucleoside derivatives anchored to controlled pore glass (CPG) via a succinimidyl linkage (Scheme 19).[78] Removal of the DMT group with trichloroacetic acid gave an anchored nucleoside ready for triphosphorylation under the Ludwig−Eckstein conditions. Finally, the product was cleaved from the resin via hydrolysis with aqueous ammonia at 50 °C for 2 h. These conditions may seem harsh for nucleoside triphosphates, nevertheless the products were isolated in 60−65% yields. Similar transformations using sulfur as an oxidant gave the corresponding 5′-*O*-(1-thiotriphosphate)s in 40−45% yield.

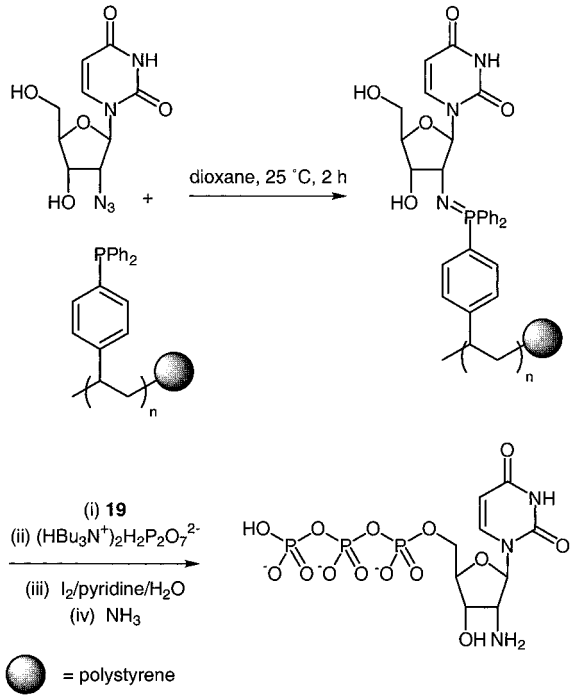
Scheme 19



In another solid-phase variant of the Ludwig−Eckstein procedure, phosphine-functionalized polystyrene resin was used for the second solid-phase synthesis of nucleoside triphosphates.[79] Polystyrene-based resins tend to have higher loading than CPG, but it was found that side products were formed if

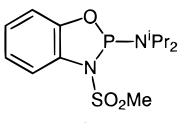
the resin was not capped down to a loading of 0.7 mmol/g. That loading, however, is still much higher than typical loadings for controlled pore glass. The starting material nucleosides were 2′- or 3′-azido derivatives wherein the azide reacted with the supported phosphine to anchor the nucleoside to the support (Scheme 20). Ludwig−Eckstein elaboration to the 5′-*O*-triphosphates and hydrolysis in concentrated ammonia gave the products in overall yields of 70−75%. The net effect of this protocol is a Staudinger reduction[80]/triphosphorylation. An attribute of this methodology is that nucleosides having naked 3′-hydroxyl functionalities reacted without complication. The observed regioselectivity could be due to the polymer sterically shielding the 3′-OH from phosphitylation; alternatively, it is conceivable that a cyclic intermediate involving the 3′- and 5′-oxygen atoms could be involved.

Scheme 20



Supported oligoribonucleotides, prepared on controlled pore glass via standard phosphoramidite chemistry, have also been transformed to triphosphates via the Ludwig−Eckstein procedure. The products were removed from the support under the usual basic conditions and then purified on a polyacrylamide gel.[81] This procedure was developed for biological applications in which relatively small amounts of material are required.

Phosphoramidites are used routinely for automated DNA syntheses,[82] so it is surprising that they have found very little application in syntheses of nucleoside triphosphates. One report describes investigation of reagent **22** in phosphorylating protocols for nucleosides, but triphosphates were incidental products in this study.[83]

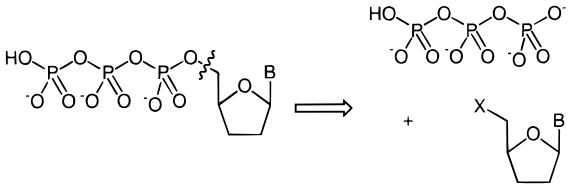


22

7. Syntheses Involving Direct Displacement of 5′-O-Leaving Groups by Triphosphate Nucleophiles

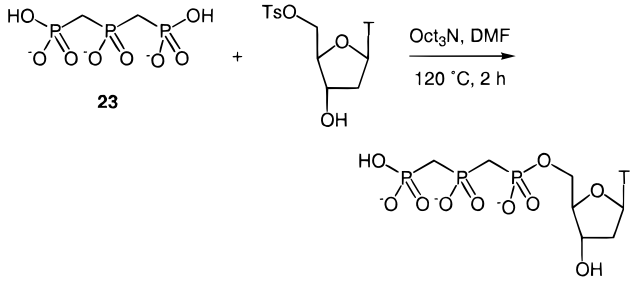
Direct nucleophilic substitution of leaving groups with triphosphate ions and triphosphate analogues is possible (Figure 6), but this transformation has been rarely used in practice.

Figure 6



This type of reaction was first performed[84] using the phosphinic−bisphosphonic acid **23**,[85,86] which is more stable than triphosphate ions. Thus, the nucleophilic displacement was performed by heating **23** with 5′-*O*-tosyl thymidine in DMF (Scheme 21).

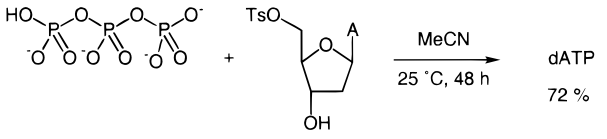
Scheme 21



23

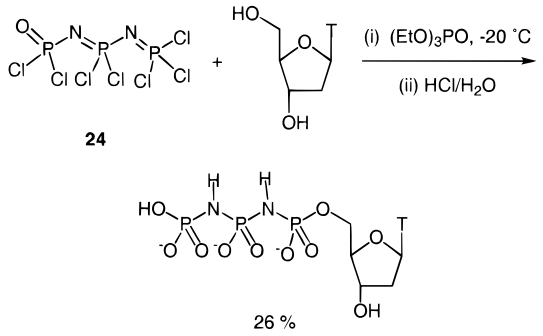
Reaction of tetrabasic triphosphate with 5′-*O*-tosyl adenosine in acetonitrile at room temperature has been used in a synthesis of ATP.[87,88] Unfortunately, this rather simple reaction to prepare triphosphates has only been demonstrated to be practical for adenosine (Scheme 22).

Scheme 22



The above reactions involve a triphosphate or triphosphate analogue nucleophile and a 5′-nucleoside electrophile, but as noted in section 4, these roles could be reversed. This approach has been used to prepare several triphosphate-analogues that incorporate nitrogen into the triphosphate moiety. For instance, the 5′-hydroxyl of thymidine was selectively reacted with the iminotriphosphate precursor [*P*,*P*-dichloro(dichlorophosphinyl)phosphinimyl]phosphorimidic trichloride (**24**) under the Yoshikawa $POCl_3$ phosphorylation conditions as indicated in Scheme 23.[89]

Scheme 23

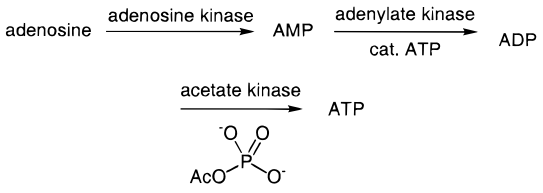


8. Biocatalytic Methods

Enzyme-mediated syntheses of nucleoside triphosphates are ideal for certain applications and of limited value for others. These methods are extremely useful for natural triphosphates, especially if the product can be used as a crude mixture or coupled with another enzyme system for immediate application. Biocatalytic methods also can be applicable to nucleoside triphosphates with unnatural base and/or sugar residues, but development of protocols must involve exploratory work with different enzymes to find ones that will tolerate the unnatural substrates involved. It may be that no such biocatalytic system can be found. Consequently, enzyme-mediated syntheses of unnatural nucleoside triphosphates are only cost-effective if the expected advantages of this approach are likely to offset the costs of the additional development time required. Chemical methods are probably more suitable than enzymatic methods for obtaining small amounts of totally new nucleoside triphosphates that may not be substrates for enzyme-mediated reactions.

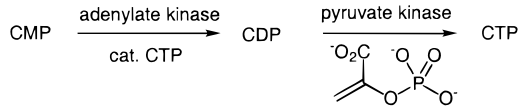
Adenosine has been converted in 98% yield into a 30 mM solution of ATP using a mixture of three enzymes: adenosine kinase, adenylate kinase, and acetate kinase (Scheme 24).[90] A schematic of the process is illustrated below; ATP is required as a catalytic cofactor for the conversion of AMP into ADP by adenylate kinase. Overall, the efficacy of this method depends on the cost/availability of the enzymes involved and immobilization techniques to allow for their recovery.

Scheme 24



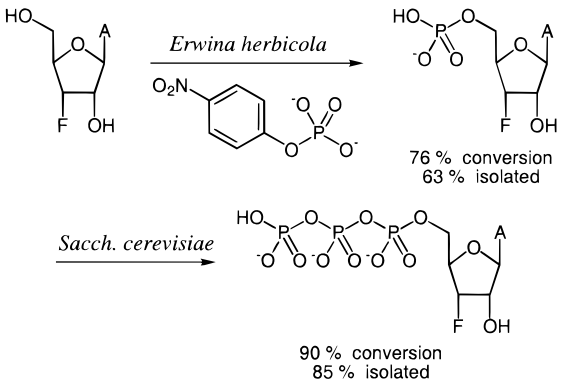
Assumptions regarding the specificity of enzymes should be checked to evaluate different possible routes to the desired product. In the synthesis shown in Scheme 25, lack of availability of a kinase specific for phosphorylation of CMP led to application of the adenylate kinase in the same pathway.[91] Prior to this study it had been reported that adenylate kinase is highly specific for adenosine nucleosides.

Scheme 25



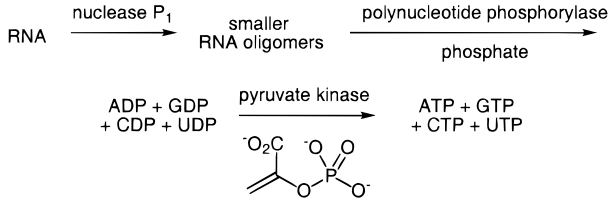
Whole cell systems and crude enzyme preparations have been used instead of isolated enzymes to form triphosphorylating mixtures.[92] For instance, a range of natural and fluorinated nucleosides were phosphorylated using whole cells from *Erwina herbicola* bacteria and extracts from Baker's yeast cells, *Sacch. cerevisiae*.[93] Possible complications that arise when using whole cell systems include competing undesirable enzyme-mediated processes, e.g., from adenosine deaminase activity. This was not observed in the sequence shown in Scheme 26, however.

Scheme 26



Nucleoside triphosphates are often used as mixtures (e.g., ATP, GTP, CTP, UTP). A very useful route to such nucleoside triphosphate "broths" is via degradation of RNA. In the example depicted in Scheme 27, RNA was partially degraded by a nuclease, the resulting oligomers were converted to NDPs using a phosphorylase and then to NTPs via a pyruvate kinase. In fact, this sequence was coupled to another series of enzyme-mediated reactions in which the product NTPs were used to produce nucleoside diphosphate sugars.[94]

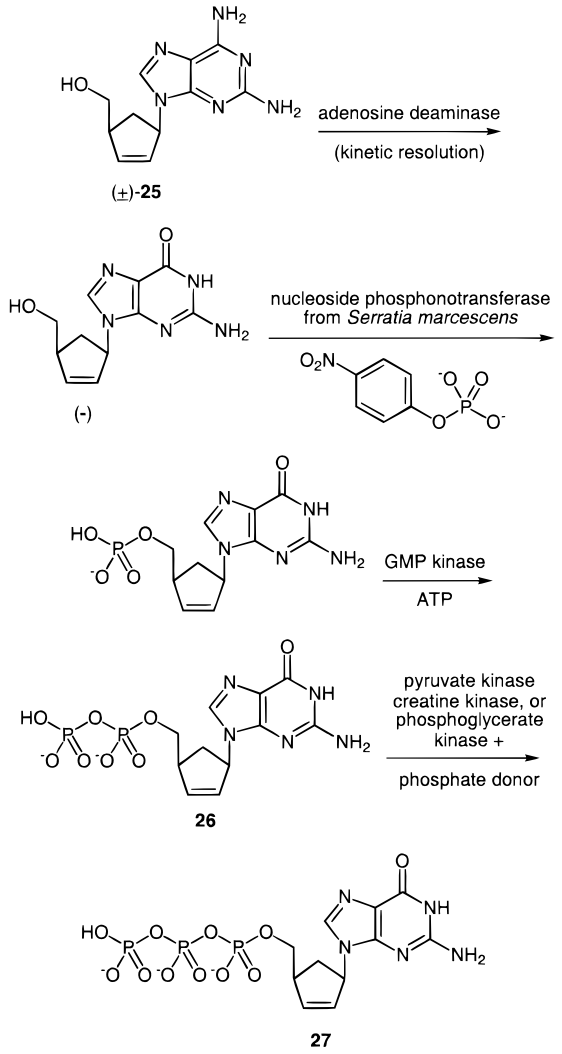
Scheme 27



Enzymatic routes can facilitate resolution of nucleoside analogues into enantiomers. A study with the anti-HIV compound *carbovir* illustrates several different aspects of enzyme selectivity. This work was primarily concerned with the selectivity of various enzymes for *carbovir*, but the preparation of the products was also cited.[95] The racemic starting material **25** was resolved using adenosine deaminase,[96] simultaneously converted to *carbovir*, and then subjected to two sequential phosphorylation reactions to form the diphosphate **26**. That diphosphate was used

in a study with several phosphorylating systems to investigate their selectivity toward this substrate. Several enzymes were found to give the corresponding triphosphate **27** (Scheme 28).

Scheme 28



Various kinases have been used in biocatalytic conversions of nucleoside diphosphates to the corresponding triphosphates, and these methods can be synthetically useful.[74] Similarly, α-^{32}P-labeled triphosphates can be formed by a combination of chemical and biocatalytic reactions,[97]

9. Conclusions

No method for preparing nucleoside triphosphates is suitable for all nucleobase derivatives. This is unfortunate because in this evolving age of genomic drug discovery, the need for nucleoside triphosphates will increase. Current applications for these materials include DNA sequencing, therapeutic nucleoside inhibitors of polymerases, and in vitro transcription procedures to prepare aptamers; this list is likely to expand.

Landmarks in the development of contemporary syntheses of nucleoside triphosphates include: Yoshikawa's $POCl_3$ phosphorylation procedure to give nucleoside monophosphates (section 3.1); Ludwig's "one-pot, three-step" triphosphorylation procedure (Scheme 2); various protocols for activating monophosphates then reacting them with pyrophosphate, e.g., Bogachev's procedure (Scheme 15); and the Ludwig−Eckstein procedure involving an activated phosphite (section 6, Scheme 18)

Chemists wishing to prepare nucleoside triphosphates with a minimum amount of effort should refer to the procedures listed above.

Some research innovations could greatly facilitate syntheses of nucleoside triphosphates. Improved solid-phase syntheses amenable to automation or genuinely combinatorial approaches would be particularly timely. Direct, one-step triphosphorylation procedures, for instance, wherein a triphosphate entity is added as a nucleophile, would also be valuable. Perhaps a rate-limiting feature of development of new methods is availability of protected and/or stabilized triphosphate and diphosphate building blocks. We feel that Todd's early work along these lines has not been paid sufficient attention. Finally, enzymatic procedures have proved to be excellent for biochemical purposes and for some syntheses of purified nucleoside triphosphates. This is another area of potential development.

In summary, methods for syntheses of nucleoside triphosphates evolved rapidly in the 1950s, but efforts in this area have tapered off, and for the past decade they have languished. A revival of interest in preparations of these important molecules would be appropriate.

10. Acknowledgments

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