

Accounts

Toward an Ideal Synthesis of Oligonucleotides: Development of a Novel Phosphoramidite Method with High Capability#

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Several novel methods for reliably carrying out the oligonucleotide synthesis via the phosphoramidite strategy have been developed. The first is the method using allyloxycarbonyl and allyl groups for the protection of nucleoside bases and internucleotide linkages, respectively. These protectors can be cleanly removed under almost neutral conditions with a mixture of an organic Pd(0) complex such as $Pd[P(C_6H_5)_3]_4$ or $Pd_2[(C_6H_5CH{=}CH)_2CO]_3{\cdot}CHCl_3$, and $P(C_6H_5)_3$ in the presence of a nucleophile such as butylammonium formate, diethylammonium hydrogencarbonate, diethylammonium carbonate, or dimedone. A method for the facile oxidation of nucleoside phosphites to phosphates has been developed; this is performed under non-aqueous and non-basic conditions using 2-butanone peroxide in dichloromethane, *t*-butyl hydroperoxide in toluene, or bis(trimethylsilyl) peroxide in dichloromethane. The combined use of the *N*-allyloxycarbonyl/*P*–*O*-allyl-protected method and anhydrous/non-basic oxidation is extremely useful for the synthesis of nucleotide derivatives sensitive to moisture and/or bases, producing them in higher yield and at a greater level of purity than the existing methods using acyl or aroyl and cyanoethyl groups for the protection of nucleoside bases and internucleotide linkages, respectively, and iodine in aqueous pyridine for the oxidation. Further, acid/azole complexes serving as highly useful activators of phosphoramidites have been invented. The reagents include imidazolium triflate, *N*-(phenyl)imidazolium triflate, benzimidazolium triflate, and *N*-(methyl)benzimidazolium triflate, which allow higher-yield synthesis of oligonucleotides than conventionally employed 1*H*-tetrazole and related reagents. For example, synthesis of oligoribonucleotides via the allyloxycarbonyl/allyl-protected strategy has been carried out more efficiently using *N*-(phenyl)imidazolium triflate as a promoter than 5-(ethylthio)-1*H*-tetrazole, which has been found to be the most effective promoter among those currently available for oligoribonucleotide synthesis. Further, imidazolium triflate has opened the door to the extremely convenient and efficient synthesis of oligodeoxyribonucleotides without nucleoside-base protection. This method is obviously advantageous over *N*-protected methods because it eliminates tedious operations and the use of superfluous reagents required for the introduction and removal of the *N*-protecting group; risks of decomposition and loss of product by protection and deprotection are also avoided. Facile liquid-phase preparation of short oligomers has also been developed; this is successfully accomplished using stoichiometric amounts of building blocks by the aid of an equimolar amount of azolium reagent or a catalytic amount of 5-(*p*-nitrophenyl)-1*H*-tetrazole. These two methods are beneficial from an economical point of view, particularly in large-scale synthesis.

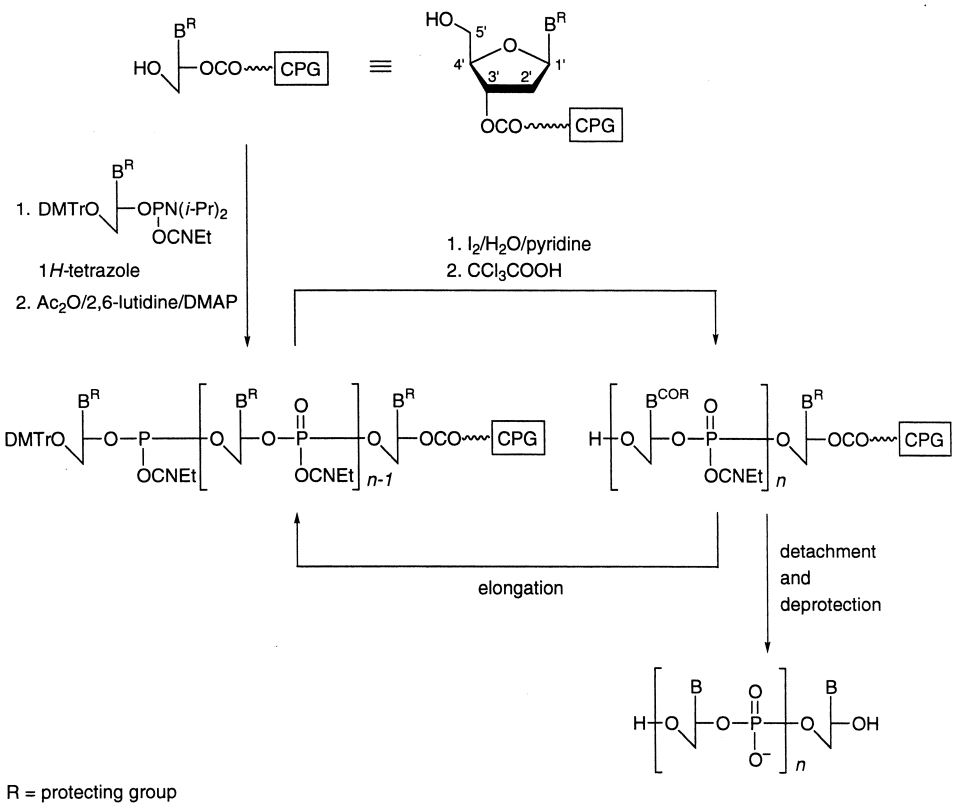
Development of an efficient and reliable method for the chemical synthesis of oligonucleotides[1] is an important subject in nucleic acid research and its applications. In particular, the spread of diversity of the research through its recent advance requires methods capable of preparing oligonucleotides both with and without artificial modifications in high yields and at a high level of purity. Further, we need a method capable of preparing rather long oligomers such as the 20mer, which is the

General notice. In this article, abbreviations are used for the following compounds and substituents: All = allyl; AOC = allyloxycarbonyl; BIT = benzimidazolium triflate; Bz = benzoyl; CNEt = 2-cyanoethyl; CPG = controlled pore glass; CSO = (1*S*)-(+)-(10-camphorylsulfonyl)oxaziridine; dba = dibenzalacetone; DMAP = 4-dimethylaminopyridine; DMF = *N*,*N*-dimethylformamide; DMTr = *p*,*p*′-dimethoxytrityl; ETT = 5-(ethylthio)-1*H*-tetrazole; HMPA = hexamethylphosphoric triamide; Ibu = isobutyryl; IMP = imidazolium perchlorate; IMT = imidazolium triflate; MCPBA = *m*-chloroperbenzoic acid; MMTr = *p*-methoxytrityl; MS = molecular sieves; *N*-AcPhIMT = *N*-(*p*-acetylphenyl)imidazolium triflate; *N*-MeBIT = *N*-(methyl)benzimidazlium triflate; *N*-PhIMP = *N*-(phenyl)imidazolium perchlorate; *N*-PhIMT = *N*-(phenyl)imidazolium triflate; NPT = 5-(*p*-nitrophenyl)-1*H*-tetrazole; OBT = 1-hydroxybenzotriazolyl; OTf = triflate; SATE = 2-(acetylthio)ethyl; TBAF = tetrabutylammonium fluoride; TBDMS = *t*-butyldimethylsilyl; TBHP = *t*-butyl hydroperoxide; Tet = 1-tetrazolyl; TMS = trimethylsilyl.

average size required in a variety of biological applications such as antisense therapy[2] and PCR reactions. There have been a variety of methods[3] reported, including the phosphodiester method,[4] the phosphotriester method,[5] the phosphite method,[6] the phosphoramidite method,[7] and the *H*-phosphonate method.[8] Among these, the phosphoramidite strategy may best meet the above requirements. In particular, this approach is useful for the synthesis of long oligonucleotides with artificially modified backbones, which are not easily prepared by other methods. The artificial analogs include alkyl/arylphosphonates,[9] phosphotriesters,[9,10] phosphoramidates,[11] phosphoramidimidates,[12] phosphorothioates,[2d,13] phosphorodithioates,[14] phosphoroselenates,[15] and boranophosphates,[16] which are attractive as antisense molecules.[17] Thus, the phosphoramidite strategy has been and is being most widely employed in both liquid-phase and solid-phase syntheses of various kinds of oligonucleotides.

The phosphoramidite strategy was originally invented by Caruthers[18] and was later improved by Köster[19] and others.[6] Scheme 1 illustrates the synthetic pathway of the solid-phase synthesis of oligodeoxyribonucleotides on the basis of the representative Köster method. Thus, initially, a fully protected oligonucleotide attached on solid supports is prepared via chain elongation by the repetition of the reaction sequences of (1) condensation of a 5′-*O*-dimethoxytrityl-*N*-acyl/aroyl-protected deoxyribonucleoside 3′-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidite) and a solid-anchored 5′-*O*-free-*N*-acyl/aroyl-protected nucleotide (or oligonucleotide) by the assistance of 1*H*-tetrazole or a related reagent such as NPT, (2) capping of the unreacted 5′-hydroxy using a mixture of acetic anhydride, 2,6-lutidine, and 4-dimethylaminopyridine, (3) oxidation of the resulting nucleoside phosphite intermediate using iodine in aqueous pyridine to the phosphate, and (4) removal of the dimethoxytrityl protector on the hydroxy of the 5′-end by the use of an acid such as dichloroacetic acid or trichloroacetic acid. After finishing the chain elongation, both the deprotection of cyanoethyl protecting groups and detachment of the resulting product is carried out by treatment with concentrated aqueous ammonia at ambient temperature. Finally, the *N*-acyl/aroyl protecting groups are deblocked by heating, usually at 55 °C, for several hours with concentrated aqueous ammonia to obtain the target DNA oligomer.

This method, however, has several drawbacks, the most serious of which involves the use of acyl and aroyl protecting groups for the nucleoside bases. Normally, the synthesis employs benzoyl for adenine, benzoyl or *p*-anisoyl for cytosine, and isobutyryl for guanine, the deprotection of which demands the harsh basic conditions described above, causing various side reactions including a breakage of internucleotide linkages. Further, this strategy cannot be used for the synthesis of compounds with base-labile functions. The 2-cyanoethyl protector for the phosphate moiety also becomes troublesome in some cases. Since this protecting group is quite sensitive to bases, it suffers from undesirable deblocking when basic treatment is necessary during the synthesis. Another drawback is the use of an I_2/H_2O/pyridine mixture for oxidation of the nucleoside phosphite intermediate to the phosphate.[18,19] This oxidation is not favorable to the synthesis of moisture- and/or base-sensi-



Scheme 1. Outline of solid-phase synthesis of oligodeoxyribonucleotides via the Köster method.

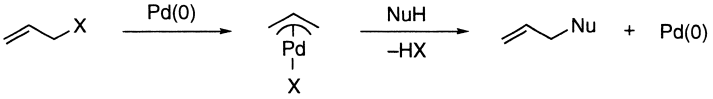
tive compounds. In addition, the aqueous oxidation is not entirely suitable for solid-phase synthesis, as the subsequent reaction must be carried out under anhydrous conditions to prevent the phosphoramidites from hydrolytic decomposition, therefore requiring persistent washing with a suitable solvent to remove water. The use of 1*H*-tetrazole or NPT[20] as a promoter for the condensation of the nucleoside phosphoramidite and the nucleoside is also problematic in some cases. For example, the reactivity of 1*H*-tetrazole is too low to sufficiently activate lowly reactive phosphoramidites, such as derivatives with sterically crowded substituents or nearby electron-withdrawing functionalities. These phosphoramidites are sometimes required as building blocks for the synthesis of oligoribonucleotides with modified backbones, and thus the lack of reactivity limits the scope of the substances prepared. While NPT has good reactivity, its solubility in acetonitrile is very low (ca. 0.1 mol/L).[20a] Therefore, this reagent demands a problematically large volume of the reaction solvent. Therefore, research to develop new methods eliminating these drawbacks has been fostered to render the phosphoramidite strategy more useful and more reliable. This account reports some new strategies invented in our laboratory, improving on the Köster method. In addition, the application of new strategies to the synthesis of some biologically important substances is briefly described.

Allyl-Protected Phosphoramidite Method

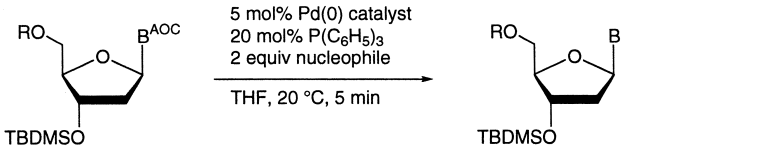
We first attempted to find reliable protectors for internucleotide linkage and nucleoside bases. In order to improve on the acyl/aroyl protection of nucleoside bases in the Köster method, alternative protecting groups have been developed; the representative ones are phenoxyacetyl,[21] isopropoxyacetyl,[22] *t*-butylphenoxyacetyl,[23] 2-(acetoxymethyl)benzoyl,[24] 2-(*t*-butyldiphenylsilyloxymethyl)benzoyl,[25] formamidine,[26] (9-fluorenyl)methoxycarbonyl,[27] and 2-(*p*-nitrophenyl)ethoxycarbonyl.[28] These protectors can be removed under rather mild conditions, but their deprotection still requires basic reagents, thus involving a risk of undesirable decomposition in the synthesis of base-sensitive derivatives. In addition, some of these protectors are too labile to bases, thus risking themselves in response to basic treatment during the synthesis. Further, some cases require a tedious workup and purification to obtain the target nucleotides after deprotection. In contrast, as a protecting group for the phosphate moiety, methyl,[29] (9-fluorenyl)methyl,[30] 2-cyano-1,1-dimethylethyl,[31] 2,2,2-trichloroethyl,[32] 2,2,2-trichloro-1,1-dimethylethyl,[32a,33] 2,2,2-tribromoethyl,[34] 2-(*p*-nitrophenyl)ethyl,[35] 2-(2-pyridyl)ethyl,[36] 2-(methylsulfonyl)ethyl,[37] 2-(phenylsulfonyl)ethyl,[38] *o*-nitrobenzyl,[39] *o*-(*t*-butyl)phenyl,[40] *o*- or *p*-chlorophenyl,[41] and 8-quinolyl,[42] have been found to be functional in place of 2-cyanoethyl, but these protecting groups still have problems. Deprotection of some protecting groups requires basic reagents not easily separable from the target nucleotides. In addition, some of these protectors are not absolutely stable under certain conditions in the synthesis, sometimes suffering decomposition or causing side reactions; *N*-methylation of the thymine base in the case of methyl protection is one example. Thus, development of new, reliable protectors for internucleotide bonds and nucleoside bases removable by a mild, non-basic reaction is strongly demanded.

We were intrigued by Tsuji–Trost organopalladium chemistry[43] in relation to solving this problem. As shown in Scheme 2, an allylic compound having an appropriate leaving group reacts with various nucleophiles in the presence of a Pd(0) catalyst to give a suitable product with release of the leaving group. The nucleophiles include several non-basic substances such as an alcohol, a 1,3-dioxo compound, a mixture of an amine and formic acid or carbonic acid, or a carboxylic acid. However, the interests of most synthetic chemists have been limited to the allylic moieties creating organic frameworks and have not included the fate of leaving groups. In contrast, we saw this reaction from the perspective that the allyl moiety can be removed under mild, non-basic conditions from some functional groups with a good leaving aptitude.

In conjunction with organopalladium chemistry, we were first interested in the AOC group, which was originally developed by Kunz for peptide synthesis,[44] for protection of the nucleoside bases. AOC has been used as a protecting group for some aliphatic amines.[43f] However, to our knowledge, it had not been used for the protection of aromatic amines, probably because protection is difficult, as aromatic amines are not sufficiently nucleophilic to react with the existing AOC reagents. In fact, introduction of the AOC group to nucleoside bases is not easy, and suitable conditions are highly dependent on the structures. For example, the adenine base efficiently undergoes the allyloxycarbonylation through the use of AOC-Cl and *N*-methylimidazole in dichloromethane,[45] AOC-OBT by the assistance of *t*-butyllithium in THF at −78 °C,[46] or AOC-tetrazolide in refluxing THF.[47] The procedure using an AOC-Cl/*N*-methylimidazole mixture is also effective for introducing the AOC protector to the cytosine base.[45] The use of AOC-OBT in pyridine[46] or in the presence of triethylamine in THF[46] is an alternative means of protecting cytidine derivatives. N^2-AOC protection of the guanine base is very complicated. Direct allyloxycarbonylation of the free guanine base requires AOC-Cl and an excess amount of *t*-butylmagnesium chloride in a THF–HMPA mixture.[46] Occasionally, nucleotide synthesis requires protection of not only the N^2-position but also the O^6-position of the guanine base to obtain good results. In this case, allyl serves as a good protector for O^6-position. The introduction of N^2-AOC and O^6-allyl protectors of the guanine moiety was achieved by the following two approaches. One method[48] in-

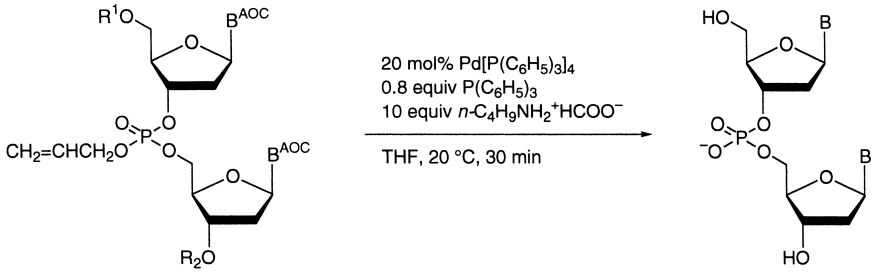


Scheme 2. π-Allyl-palladium reaction.



Pd(0) catalyst = $Pd[P(C_6H_5)_3]_4$, $[Pd_2(dba)_3]CHCl_3$
nucleophile = $(C_2H_5)_2NH$–H_2CO_3, n-$C_4H_9NH_2$–HCOOH, dimedone, HCOOH, CH_3COOH, etc.

Scheme 3. Deprotection of *N*-AOC protection.



Scheme 4. Removal of *P*–*O*-allyl and *N*-AOC protectors.

volves three steps: (1) triethylamine-mediated condensation with 2-mesitylenesulfonyl chloride, forming O^6-sulfonate, (2) the reaction with allyl alcohol, providing the O^6-allyl derivative, and (3) the organomagnesium-assisted N^2-allyloxycarbonylation with AOC-Cl. An alternative pathway[45] consists of (1) O^6-allylation by the Mitsunobu reaction,[49] (2) the Grignard reagent-assisted N^2,N^2-bis-allyloxycarbonylation, and (3) selective removal of one N^2-AOC group using a 0.05 M NaOH/ethanol solution.

The *N*-AOC and the guanosine O^6-allyl protecting groups can be easily removed by the catalytic action of a Pd(0) complex such as $Pd[P(C_6H_5)_3]_4$ or $[Pd_2(dba)_3]\cdot CHCl_3$ in the presence of an almost neutral or slightly acidic nucleophilic agent such as dimedone, formic acid, acetic acid, or a mixture of a primary or secondary amine and formic acid or carbonic acid (Scheme 3).[48] The allylic protectors are also electrochemically deblocked using the Pd(0) catalyst.[50] The allylic protection shows good functional-group compatibility. The allylic protecting groups are quite stable in the presence of an acid such as dichloroacetic acid and trichloroacetic acid, used for removing the MMTr and DMTr protectors, and the fluoride ion, employed for deblocking the TBDMS protector. Further, the AOC/ally-protected adenosine and guanosine derivatives suffer no depurination in response to the acid treatment. In contrast, the palladium reaction does not damage MMTr, DMTr, and TBDMS groups at all.

We have also found that the allyl group is useful for protecting the internucleotide linkage.[9,47,51] For example, the nucleoside phosphotriester with an allyl protecting group on the phosphate function and the AOC protector on the nucleoside base is readily deblocked to give the phosphodieter in excellent yield by exposure to a $Pd[P(C_6H_5)_3]_4$ or $[Pd_2(dba)_3]\cdot CHCl_3$ catalyst in the presence of a suitable nucleophile such as an amine, an amine/formic acid or carbonic acid mixture, and dimedone (Scheme 4). Electrochemical removal using a Pd(0) catalyst is also effective.[50]

Based on these results, we next prepared four kinds of phosphoramidites with the AOC and allyl protecting groups, **1**, **2**, **3**, **4**, and **5** (Chart 1), required as monomer units for the synthesis of oligonucleotides, by condensation of the deoxyribonucleoside and $(CH_2{=}CHCH_2O)P[N(i\text{-}C_3H_7)_2]_2$ with the assistance of 1*H*-tetrazole and diisopropylamine.[47,52] These AOC/allyl-protected nucleoside phosphoramidites are stable under ambient conditions.

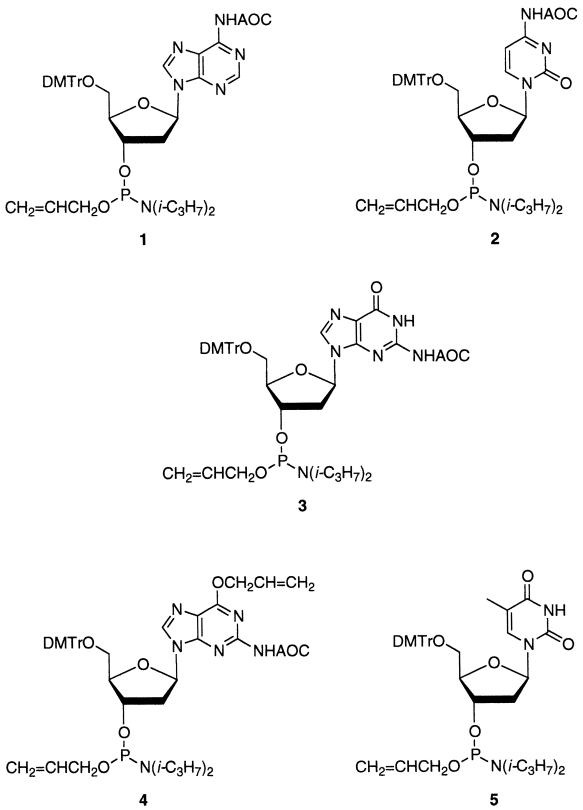


Chart 1.

Meanwhile, we have invented a new means of oxidizing the nucleoside phosphite to the phosphate under anhydrous and neutral conditions through the use of a TBHP/toluene solution in acetonitrile,[53] bis(trimethylsilyl) peroxide in the presence of trimethylsilyl triflate as a catalyst in dichloromethane,[53,54] or 2-butanone peroxide in dichloromethane.[55] These methods can be used more reliably than the I_2/H_2O/pyridine method in both the liquid-phase and solid-phase syntheses and allow for phosphite-specific oxidation without any damage of nucleoside bases and allyl, AOC, and DMTr protectors. In general, the oxidation is accomplished quantitatively. The present methods are also advantageous in some respects over other existing non-aqueous/non-basic tactics using nitrogen dioxide in dichloromethane,[51a,56] MCPBA in dichloromethane,[57] dimethyldioxirane in dichloromethane,[58] and CSO in acetonitrile.[59] For example, nitrogen dioxide is highly toxic, and MCPBA is also toxic. Further, MCPBA generates *m*-chlorobenzoic acid as oxidation progresses and this acid frequently brings about an undesired cleavage of the 5′-*O*-dimethoxytrityl protecting group. Dimethyldioxirane causes undesired oxidative modification of thymine,[60] uracil,[60] and adenine bases.[61] CSO is very expensive. In contrast, the reagents employed in our methods are commercially available at a low price or are easily prepared from inexpensive, non-toxic materials, and bring about no undesirable reactions.

These new tactics stimulated us to examine the solid-phase synthesis of oligodeoxyribonucleotides via the allyl-protected phosphoramidite method, including non-aqueous/non-basic oxidation.[47,62] Synthesis of three kinds of DNA oligomers of different lengths and base sequences: $^{5'}$TATGGGCCTTTTGATAGGATGCTCACCGAGCAAACCAAGAACAACCAGGAGATTTTATT$^{3'}$ (60mer), $^{5'}$TATCGGACACGTAACCCTCCCATGTCGATGCAAATCTTTAACA$^{3'}$ (43mer), and $^{5'}$CAAGTTGATGAACAATACTTAATACCTAAACT$^{3'}$ (32mer), were carried out on a 0.1-μmol scale on CPG (pore size 500 Å) with a long aminoalkyl spacer. The chain elongation was conducted via a reaction cycle similar to that shown in Scheme 1, employing the *N*-AOC/*P*–*O*-allyl-protected nucleoside phosphoramidites, **1**, **2**, **3**, and **5**, NPT in place of 1*H*-tetrazole as the promoter for the condensation, and an anhydrous TBHP/toluene solution in place of an I_2/H_2O/pyridine mixture to oxidize the phosphite intermediate. As a control, synthesis of these nucleotides was also achieved by the Köster method with slight modification, using *N*-acyl,aroyl/*P*–*O*-cyanoethyl-protected nucleoside phosphoramidites (B^R = Ade^{Bz}, Cyt^{Bz}, Gua^{Ibu}, and Thy in Scheme 1), NPT as the promoter, and TBHP for the oxidation.

In the allyl/AOC-protected method, after chain elongation, all allyl and AOC groups were first removed from the resulting protected CPG-linked product on the CPG matrix by treatment with $[Pd_2(dba)_3]\cdot CHCl_3$, triphenylphosphine, and butylammonium formate, and then the fully deprotected target compound was detached from CPG by exposure to concentrated ammonia at 25 °C for 2 h. In contrast, in the synthesis via the Köster method, the fully protected chain-elongation product anchored on the CPG supports was treated with concentrated ammonia at room temperature for 2 h to detach the CPG support and to deblock the cyanoethyl protector from the phosphate moieties, and subsequently *N*-benzoyl and *N*-isobutyryl groups were removed from nucleoside bases by heating with concentrated ammonia at 55 °C for 12 h to obtain the target product.

The bio-image chromatogram of the crude products (Fig. 1) indicates their purity. For example, the 60mer product synthesized by the AOC/allyl-protected method shows a clean profile, whereas the sample prepared by the Köster method shows many spots due to the shorter-sequence oligomers. The analysis indicates that the purity of the former product is approximately 70%; while there are some impurities (total 30%). This purity is much higher than that (at most 20%) of the product obtained by the Köster procedure. A similar superiority of the allyl/AOC-protected strategy to the Köster approach was observed in the synthesis of other samples having 43 and 32 bases. In the AOC/allyl-protected method, the isolation of target oligomers does not require tedious, time-consuming chromatography on ion-exchange columns. All unbound contaminants can be removed by simple washing the CPG-bound material, and the final oligomers are obtained by evaporation of an aqueous solution after detachment from the solid supports. Thus, the risk of decomposition and the loss of product is kept to a minimum, resulting in the target compound being obtained in high yield and at a high level of purity. This operational simplicity is another notable benefit of the method described herein.

Next, the utility of the allyl/AOC-protected phosphoramidite approach was investigated in the synthesis of RNA oligomers using 2′-*O*-(*t*-butyldimethylsilyl)ribonucleoside 3′-(allyl *N*,*N*-diisopropylphosphoramidite)s, **6**, **7**, **8**, and **9**, as monomer units (Chart 2). First, we attempted the synthesis of a 10mer, $^{5'}$ACGACUACUU$^{3'}$, by the use of the ETT[63] promoter, which is to our knowledge the most efficient promoter in constructing the interribonucleotide linkage according to the phosphora-

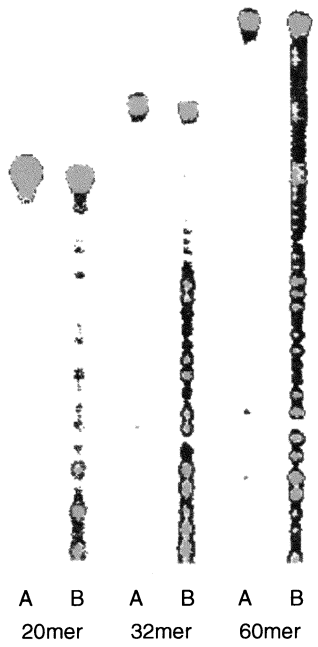


Fig. 1. Bio-image chromatogram of crude products of synthetic DNA oligomers. Lane A: the AOC/allyl-protected method. Lane B: the acyl, aroyl/cyanoethyl-protected method (Köster method).

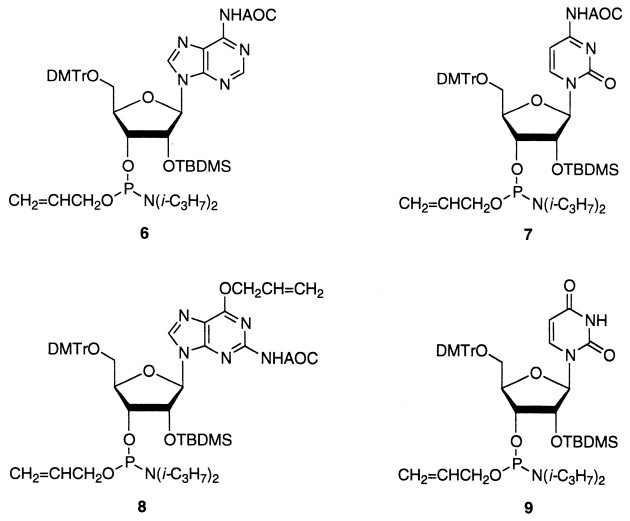


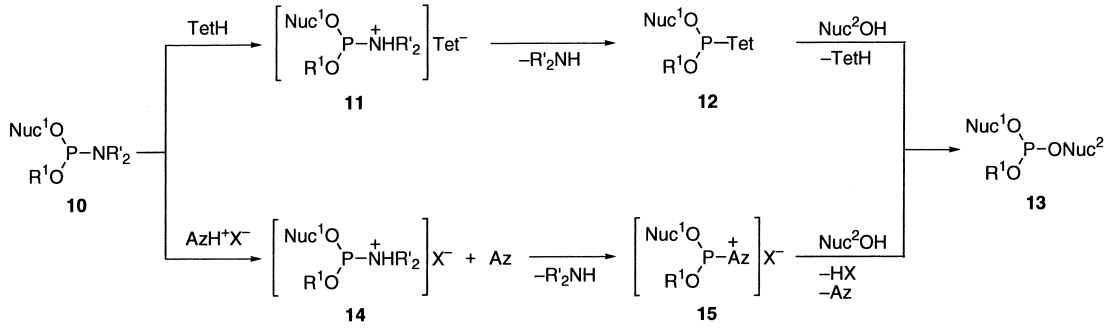
Chart 2.

midite strategy. The synthesis was carried out on a 0.2-μmol scale with a 0.1 M acetonitrile solution of the phosphoramidite monomers and a 0.1 M acetonitrile solution of ETT. The chain elongation was conducted on CPG (pore size, 500 Å) on an Applied Biosystems Model 392 DNA/RNA synthesizer, where TBHP was used in oxidizing the nucleoside phosphite intermediate. However, this attempt did not give the target oligomer in satisfactory yield; the average yield per one-base elongation was 89.6%, attaining an overall yield of only 37.3% (cf. Fig. 4). Such an unacceptable result is obviously caused by the low efficiency of ETT toward the phosphoramidites, **6**, **7**, **8**, and **9**, whose reactivity is decreased by the steric hindrance of the bulky silyl protecting group at the 2′-hydroxy. Therefore, it was clearly necessary to invent new promoters with much higher reactivity to allow for high-yield interribonucleotide-bond construction.

According to a proposed mechanism of the 1*H*-tetrazole-promoted condensation of a nucleoside phosphoramidite and a nucleoside (Scheme 5),[52a,64] 1*H*-tetrazole (TetH in Scheme 5) first acts as an acid activating the phosphoramidite **10** by protonation to form **11**. Subsequently, **11** undergoes a nucleophilic attack by the tetrazolide anion (Tet^- in Scheme 2), generated in the first step, giving the phosphorotetrazolidite **12**. Finally, this species reacts with a nucleoside (NucOH) to provide the target dinucleoside phosphite **13**. In this process,[64a] the rate-determining step is the formation of **12**, i.e., the reaction between the protonated phosphoramidite and the tetrazolide anion. Therefore, in order to achieve high reaction rate, the promoter must be sufficiently acidic, thus providing high concentrations of the protonated phosphoramidite species; in addition the conjugate base of the promoter must be sufficiently nucleophilic to react smoothly with **11**.

1*H*-Tetrazole and its conjugate base, the tetrazolide anion, meet these requirements though inadequately, and thus 1*H*-tetrazole serves as a promoter. However, for the following reasons, it is fundamentally inconsistent to require an HX-type promoter such as 1*H*-tetrazole to have a high grade of both these two characters together. The use of a strong acid as HX is necessary to fulfill the first requirement, but, in this case, the conjugate base X^- is likely only weakly nucleophilic, not fulfilling the second requirement. In contrast, if X^- is strongly nucleophilic, HX would be a weak acid, not meeting the first requirement. Therefore, we concluded that it is difficult to find a more reactive promoter than 1*H*-tetrazole among HX-type compounds, and accordingly attempted to investigate different types of reagents. The reagent we designed is the salt of a strong acid (HX) and an azole (Az) with good nucleophilicity. This salt would be sufficiently acidic to activate the phosphoramidite, producing a high concentration of the protonated phosphoramidite **14**, and this activation would include the formation of a sufficient amount of the free azole (see Scheme 5). Of more importance in the reaction with the salt is that the resulting azole (but not the conjugate base X^- of the acid) may act as a reacting species toward the protonated phosphoramidite to allow rapid generation of the intermediate **15** (rate-determining step). Consequently, we expected that the acid/azole complex would have higher reactivity than 1*H*-tetrazole, serving as an efficient promoter for the phosphoramidite approach.

In actuality, we discovered several azolium salts,[65] including *N*-PhIMT,[65b] *N*-MeBIT, BIT,[66] *N*-PhIMP, IMP, and *N*-Ac-PhIMT, with higher reactivity than 1*H*-tetrazole, ETT, and some known azolium salt-related reagents, including *N*-(methyl)imidazolium triflate,[67] pyridinium tetrafluoroborate (PyTFB),[14b] a pyridinium chloride-imidazole mixture,[68] and *N*-methylanilinium trifluoroacetate.[69] Figure 2 shows the relative



NucOH = nucleoside

Scheme 5. Mechanism of condensation of a nucleoside phosphoramidite and a nucleoside by the aid of a promoter.

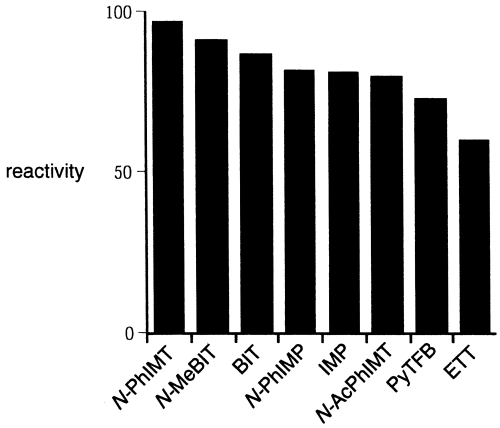


Fig. 2. Reactivity of promoters toward a ribonucleoside 3′-phosphoramidite.

reactivities of the new azolium reagents and some others; the reactivity was evaluated based on the yield of the product in the reaction of the ribonucleoside phosphoramidite **7** and 5′-*O*-free-N^6,2′,3′-*O*-tris(allyloxycarbonyl)adenosine at 25 °C for 1 min, with 0.02 mmol each of the two reactants and the promoter being in a 0.1 M solution in acetonitrile.[70]

We therefore examined the solid-phase synthesis of the 10mer, $^{5'}$ACGACUACUU$^{3'}$, via a method using the acid/azole complex in place of ETT as the promoter; the chain elongation was conducted in a manner similar to that described above for the case using ETT. Indeed, some azolium promoters allowed for a higher yield than ETT. *N*-PhIMT was among the best, giving a 99.4% average coupling yield per elongation cycle, thus a 94.4% overall coupling yield.[70] The target oligonucleotide was isolated by successive treatment with (1) a mixture of $[Pd_2(dba)_3]\cdot CHCl_3$ and $(C_6H_5)_3P$ in the presence of diethylammonium carbonate in THF at 50 °C for 60 min, with (2) concentrated ammonia at 25 °C for 60 min, and with (3) a neat triethylamine/3HF complex[71] at 25 °C for 6 h. Figure 3 shows the HPLC profile of the crude products obtained using *N*-PhIMT and ETT as the promoter; this analysis showed that the former method permits a nicer synthesis. The method using *N*-PhIMT is also effective for the synthesis of longer oligomers such as $^{5'}$AGCUACGUGACUACUACUUU$^{3'}$ (20mer), which was prepared with a 98.8% average coupling yield (78.8% overall coupling yield); the purity is fairly good in the crude form as shown in Fig. 3. Incidentally, some azolium promoters are also effective in the synthesis of oligodeoxyribonucleotides using suitably *N*-protected deoxyribonucleoside 3′-(allyl *N*,*N*-diisopropylphosphoramidite)s or -(cyanoethyl *N*,*N*-diisopropylphosphoramidite)s, and the 2′–5′-linked oligoribonucleotide using 3′-*O*-(*t*-butyldimethylsilyl)ribonucleoside 2′-(allyl *N*,*N*-diisopropylphosphoramidite)s.[70] An example of oligodeoxyribonucleotide synthesis achieved by the allyl-protected phosphoramidite method using BIT as the promoter is given below (see Fig. 4).

The allyl/AOC-protected phosphoramidite method, sometimes involving anhydrous/non-basic oxidation, has been applied to the synthesis of a variety of biologically important and attractive nucleic acid-related compounds. For example, we prepared the following compounds (Chart 3) in the liquid phase (some preparations were achieved in collaboration with other groups): *O*-(cytidine-5′-monophosphono)-*N*-acetylneuraminic acid (CMP-Neu5Ac) (**16**)[72] acting as a source of sialic acid in the sialyltransferase-catalyzed biosynthesis of sialyl oiligosaccharides;[73] adenylyl(2′–5′)adenylyl(2′–5′)adenosine (2–5A core) (**17**)[70] with strong antiviral activity;[74] the branched RNA trimer **18**[51d,75] recognized as the substance playing an integral role in eukaryotic splicing;[76] the 2′-*C*-cyano-2′-deoxy-1-β-D-*arabino*-pentafuranosylcytosine (CNDAC) 3′-phosphate derivative **19**,[77] which is a key compound for elucidating the mechanism of the self-strand cleavage of CNDAC-incorporated DNA.[78] Further, the solid-phase ap-

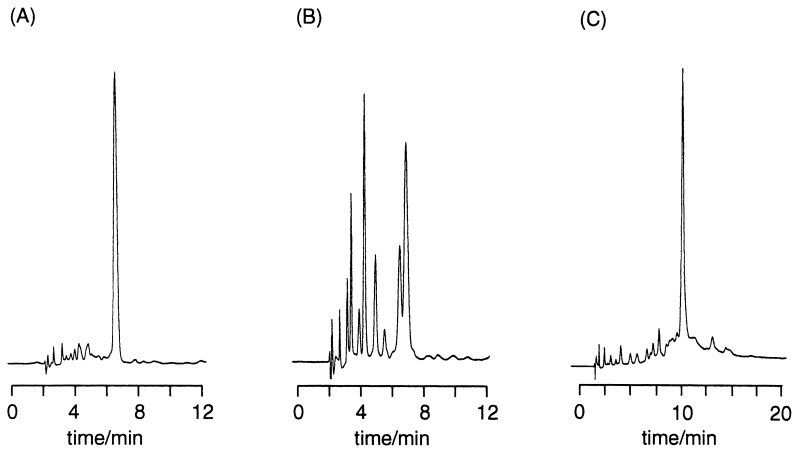


Fig. 3. HPLC profiles of crude products of (A) ACGACUACUU prepared using *N*-PhIMT as the promoter, (B) ACGACUACUU prepared using ETT as the promoter, and (C) AGCUACGUGACUACUACUUU prepared using *N*-PhIMT as the promoter. Conditions: COSMOSIL 5C18-MS column (4.6 × 250 mm); buffer A: 5% acetonitrile–0.1 M ammonium acetate; buffer B: 25% acetonitrile–0.1 M ammonium acetate; gradient: (A), (B) linear 0 to 12.5% B in 30 min; (C) linear 0 to 50% B in 30 min; detection: 260 nm; flow rate: 1.0 mL/min; temperature: 40 °C.

proach has allowed for the synthesis of several biologically important oligonucleotides. These include nucleotide-peptide conjugates with the base-labile phosphodiester linkers **20** and **21**,[79] which are considered to play decisive roles in important biological processes such as viral replication, and DNA oligomers with modified backbones at selected position(s), such as phosphonodiester,[9] phosphotriester,[9] and phosphorothiodiester,[13b] which then act as attractive antisense molecules. Another notable example is the oligodeoxyribonucleotide **22**[80] with the enzymolabile SATE group on the internucleosidic phosphodiester linkage, which was designed by Imbach to increase the cellular uptake and nuclease stability of oligonucleotides and is expected to deliver the parent oligonucleotides inside the cell by enzymatic hydrolysis.[81] Most of these syntheses were found to be more efficiently achieved by the present *N*-AOC/*P*–*O*-allyl-protected phosphoramidite method than by other existing methods. For example, **19**, **20**, and **21** could not be prepared in satisfactory yields by the *N*-acyl,aroyl/*P*–*O*-cyanoethyl-protected phosphoramidite method, as these compounds undergo β-elimination of phosphoric acid under the basic conditions used for the removal of protecting groups; in these syntheses, use of the anhydrous, non-basic oxidation with TBHP or 2-butanone peroxide was found to be another key to obtain the target products in high yield. A more remarkable example showing the utility of the AOC/allyl-protected strategy is the synthesis of **22**. This substance is extremely labile to bases, thus forbidding the use of not only acyl and 2-cyanoethyl protections, but also a succinyl or oxalyl linker on a solid support, which generally requires basic treatment for deprotection and product detachment. I_2/H_2O/pyridine oxidation also cannot be employed in this case. To our surprise, **22** was completely decomposed even when diethylammonium hydrogencarbonate or butylammonium formate was used as a nucleophile in the Pd(0)-mediated deprotection of *N*-AOC and *P*-*O*-allyl protecting groups. Thus, the synthesis was performed using dimedone as the nucleophile for the deprotection, TBHP for oxidation of the phosphite intermediate, and a photo-labile group as the linker[82] between the product and the solid support.

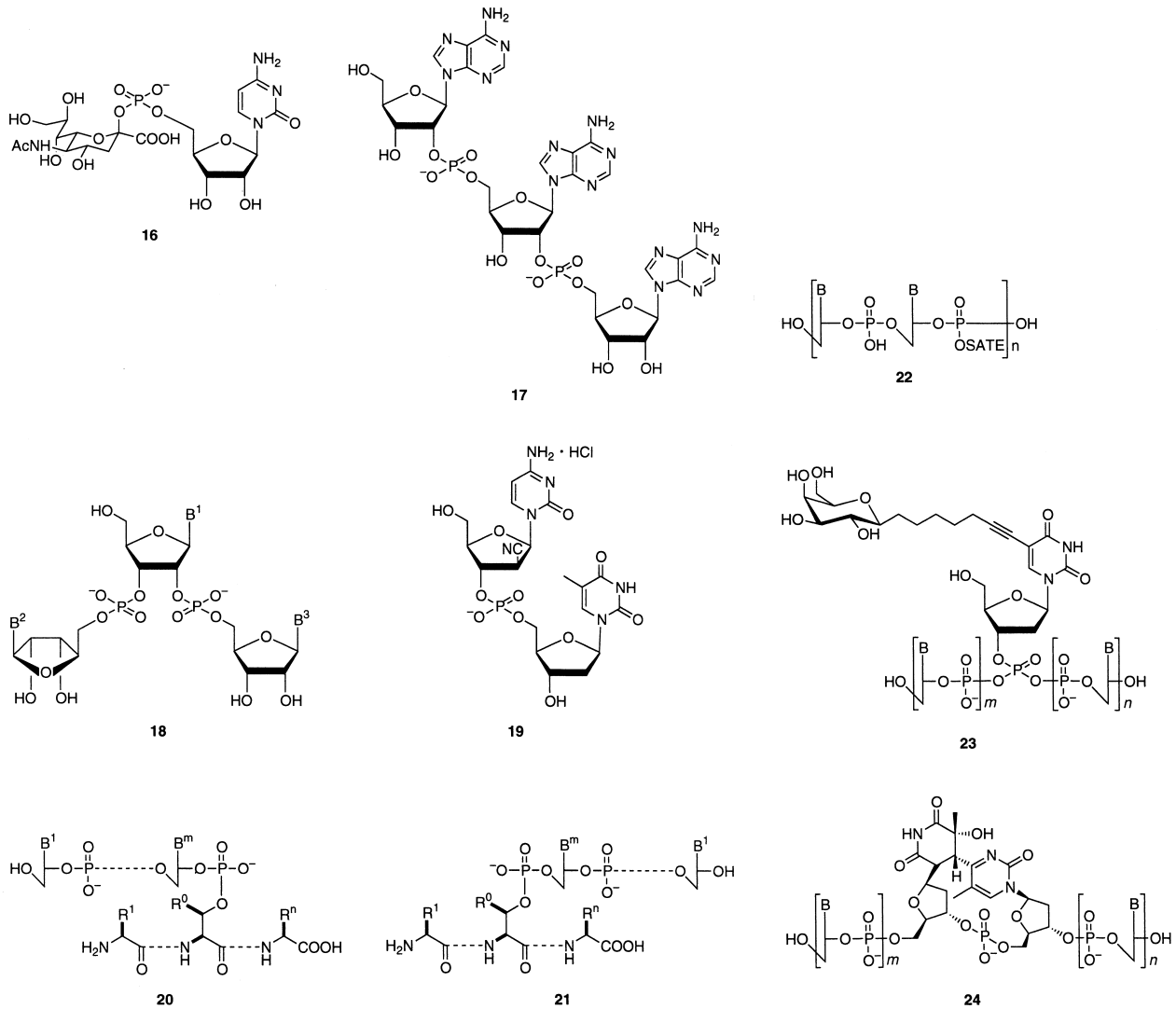


Chart 3.

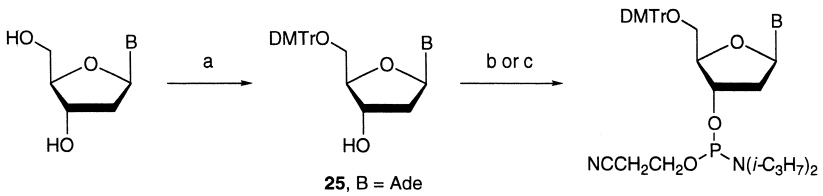
In addition, the newly invented acid/azole-complex promoters such as BIT and *N*-PhIMT were found to show superiority to 1*H*-tetrazole in the synthesis of some biologically important nucleotide-related compounds, including the following: CMP-Neu5Ac;[70,83] the 2-5A core;[70,84] site-specific glycosylated oligodeoxyribonucleotides such as **23**,[85a] which are expected to be useful not only as mimics of naturally occurring glycosylated DNA involved in the protection of phages from nucleases in infected host cells and in the regulation of gene expression, but also as biomedical and clinical tools to apply to the characteristic functions of both DNA and carbohydrates;[86] and the (6–4) photoproduct-containing oligonucleotide **24**,[87a] which is the most important form of DNA damaged by irradiation of UV light and would serve as a key compound in biological and biochemical studies in the fields of mutagenesis and DNA repair.[88]

N-Unprotected Phosphoramidite Method

In the synthesis of nucleotides, it seems reasonable and possibly indispensable to protect the highly reactive amino functions of adenine, cytosine, and guanine bases (*N*-protection), to prevent undesired reactions. However, *N*-protection is undesirable because it decreases the efficiency and applicability of the synthesis, as described below. The introduction of protecting groups commonly requires tedious and multi-step reactions using expensive reagents. In addition, their deprotection very often causes a loss of product, as described above. Further, the reagents required in these two steps limit the range of functional groups that can be tolerated in the synthesis. *N*-Protected deoxyadenosine and deoxyguanosine derivatives easily suffer depurination under acidic conditions, which are generally required for removal of the 5′-*O*-DMTr protecting group.[89] This problem is particularly serious in compounds with acyl/aroyl protectors. For example, in a 2% dichloroacetic acid/dichloromethane solution at 25 °C, *N*-benzoyl- and *N*-phenoxyacetyldeoxyadenosines decompose with half-lives of ca. 5 and 25 min, respectively.[89a] In contrast, the *N*-free deoxyadenosine derivative is rather stable under similar conditions and the half-life is ca. 5 h. Therefore, it would be ideal if *N*-protection could be avoided.

Some pioneering works on the *N*-unprotected phosphoramidite method have been reported, but these variations on the method are not generally and practically usable. The method presented by Gryaznov and Letsinger may be among the best, as it allows for the solid-phase preparation of oligomers by means of nucleoside phosphoramidite methyl esters as monomers and a mixture of pyridinium hydrochloride and imidazole as a condensing agent.[68] However, this method has some drawbacks and limitations. For example, pyridinium hydrochloride is very hygroscopic and thus not suitable for syntheses requiring anhydrous conditions. The mixed activator is useful for methyl phosphoramidites, but not effective for widely employed allyl or 2-cyanoethyl *N*,*N*-diisopropylphosphoramidites. In addition, the methyl phosphate prepared as an synthetic intermediate has a risk of causing undesired N^3-methylation of the thymine base. Further, undesired *N*-phosphitylation of deoxyadenosine and deoxycytidine takes place to some extent in this procedure. Thus, treatment with a pyridine hydrochloride–aniline mixture to remove the *N*-phosphityl group is required to obtain a high level of purity in the products. In addition, the yield per one-base elongation is not sufficiently high for the synthesis of long oligonucleotides. Indeed, according to the reported results, this approach is useful for the preparation of some simple and short oligomers such as d(C_{13}T), yielding a high-quality product. However, the method is not sufficiently effective for the synthesis of rather long heterooligomers such as $^{5'}$ACACCCAATTATGAAAATGG$^{3'}$ (20mer); this compound was produced in a rather low yield. There is another method employing *o*-chlorophenyl phosphoromorpholidites as amidites and *N*-methylanilinium trifluoroacetate as an activator,[69] but this method is not applicable to the preparation of long oligomers. Actually, only a few examples of the solution-phase preparation of short nucleotides such as dimers have been carried out by this approach. Thus, our next challenge was to develop a more general and useful synthesis of oligonucleotides via the *N*-unprotected phosphoramidite method, eliminating the drawbacks of the existing approaches, and we have indeed met this challenge by the discovery of some new tactics.

In the first place, the *N*-unprotected strategy requires efficient preparation of the *N*-free-5′-*O*-(dimethoxytrityl)nucleosides, **25**, **26**, **27**, and **28**. The most favorable way of obtaining these derivatives is obviously direct dimethoxytritylation of the 5′-hydroxy of parent nucleosides. Before the start of our study, some methods had been developed for preparing the *N*-free-5′-*O*-dimethoxytritylated derivatives of deoxyadenosine,[90] deoxycytidine,[91] and thymidine,[92] in a single step from the parent nucleosides, though the yields were not particularly high. In contrast, there had been no method developed for the one-step preparation of the deoxyguanosine derivative **27** through 5′-*O*-selective tritylation of deoxyguanosine, since tritylations of the 5′-hydroxy and the N^2-amino function of the guanine base occur at a comparable rate. Thus, **27** had been prepared via three steps: (1) protection of the amino function of the guanine base by a dimethylamidine group using (dimethoxy)(dimethylamino)methane in methanol,[89b,89d] (2) dimethoxytritylation of the 5′-hydroxy group using dimethoxytrityl chloride in pyridine, and (3) removal of the amidine protector using aqueous pyridine. Each step requires a troublesome purification of product, and, accordingly, the target product **27** was obtained in a low yield (63% at highest). In addition, (dimethoxy)(dimethylamino)methane employed in the first step is expensive. Therefore, it was necessary to develop a convenient, economical method for the preparation of **25**, **26**, **27**, and **28**, particularly **27**. This problem was resolved by the discovery of a novel method for 5′-*O*-selective dimethoxytritylation of parent nucleosides through the use of *p*,*p*′-dimethoxytrityl chloride in the presence of a 1:1:1 mixture of methanesulfonic acid, diisopropylethylamine, and imidazole (2.0 mol amt. each) in DMF (Scheme 6).[93] This procedure can generally be applied to deoxyadenosine, deoxycytidine, deoxyguanosine, and thymidine to give the corresponding *N*-free-5′-*O*-dimethoxytrityl derivatives, **25**–**28**, in favorable yields. For example, **27** was prepared in 78% yield (versus 63% in the previous three-step method described above). Other derivatives were also obtained in higher yields than those obtained by existing methods: **25** (86% versus 77%),[90] **26** (85% versus 70%),[91] and **28** (89% versus 85%).[92] The present approach is superior to exist-



25, B = Ade
26, B = Cyt
27, B = Gua
28, B = Thy

29, B = Ade
30, B = Cyt
31, B = Gua
32, B = Thy

a: DMTr-Cl (1 equiv), imidazole/CH_3SO_3H/$C_2H_5N(i\text{-}C_3H_7)_2$ (2 equiv each)/DMF, 25 °C
b: $(NCCH_2CH_2O)P[N(i\text{-}C_3H_7)_2]Cl$, $C_2H_5N(i\text{-}C_3H_7)_2/CH_2Cl_2$, –78 to 25 °C
c: $(NCCH_2CH_2O)P[N(i\text{-}C_3H_7)_2]_2$, IMT/$CH_2Cl_2$, 25 °C

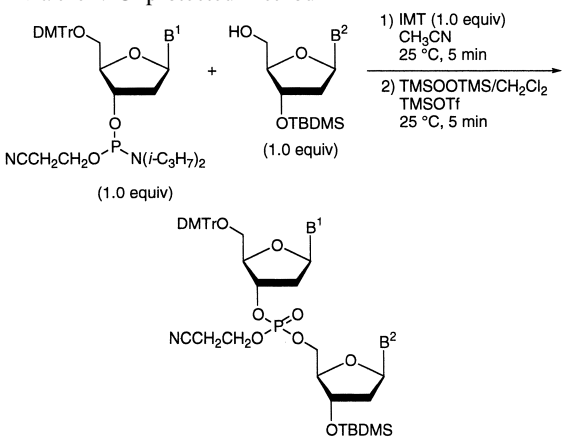
Scheme 6. Preparation of *N*-free-5′-*O*-dimethoxytrityl-2′- deoxyribonucleoside 3′-phosphoramidites.

ing methods with respects to the high product yields, wide generality, operational simplicity, and the low cost of reagents.

Subsequently, preparation of *N*-free-5′-*O*-dimethoxytrityl-2′-deoxyribonucleoside 3′-phosphoramidites as the monomer units requisite for the synthesis was carried out as follows. Initially, we planned to use allyl protection for the phosphate moiety, and thus representatively tried to prepare a deoxyadenosine 3′-(allyl *N*,*N*-diisopropylphosphoramidite) by two approaches, i.e., condensation of **25** and $(CH_2{=}CHCH_2O)P[N(i\text{-}C_3H_7)_2]Cl$ by the aid of diisopropylethylamine or $(CH_2{=}CHCH_2O)P[N(i\text{-}C_3H_7)_2]_2$ by a promoter such as IMT. However, these two approaches were unsuccessful, bringing about side reactions at the unprotected amino function. Accordingly, the plan was changed so that 2-cyanoethyl was used to protect the phosphate function. The preparation of *N*-free nucleoside 3′-(cyanoethyl phosphoramidite)s was actually accomplished by two approaches, i.e., condensation of **25**, **26**, **27**, and **28** with $(NCCH_2CH_2O)P[N(i\text{-}C_3H_7)_2]Cl$ in THF with the assistance of *N*,*N*-diisopropylethylamine (−78 to 25 °C) or with $(NCCH_2CH_2O)P[N(i\text{-}C_3H_7)_2]_2$ promoted by IMT in dichloromethane (25 °C) (Scheme 6).[94] Thus, four kinds of *N*-free deoxyribonucleoside 3′-(cyanoethyl phosphoramidite)s, **29**, **30**, **31**, and **32**, were obtained in $>$ 95% isolated yields. The *N*-free compounds have stability similar to that of the *N*-protected derivatives, and no decrease in their quality was observed in response to storage for several months under standard conditions.

The next problem, which is the most difficult but also the most important in the *N*-unprotected strategy, is acquisition of an appropriate promoter allowing for condensation of the phosphoramidite and the *N*-free nucleoside in a perfectly or almost *O*-selective manner. Fortunately, we found that IMT serves as such a reagent during the above-mentioned development of acid/azole-complex reagents. In the liquid phase, IMT allowed perfectly *O*-selective condensation of an *N*- and 5′-*O*-free nucleoside and one of the *N*-free nucleoside 3′-(cyanoethyl phosphoramidite)s, **29**, **30**, **31**, and **32** (Chart 4), when IMT and the reactants were employed in stoichiometric amounts; the reaction followed by oxidation with bis(trimethylsilyl) peroxide or TBHP gave the desired dinucleoside phosphate as the sole product in 93–98% isolated yield.[95] Several examples of dideoxyribonucleoside phosphates prepared via the *N*-unprotected method in the liquid phase are listed in Table 1. In contrast, in the synthesis on solid supports, which requires excess equivalents of the phosphoramidite and the promoter in relation to the nucleoside, deoxyadenosine and deoxycytidine derivatives suffered from undesirable phosphitylation at their amino functions. According to the preliminarily achieved synthesis of a dimer using 25 mol amt. each of the phosphoramidite and IMT in relation to the nucleoside on TenataGel® solid supports, ca. 4 and 8% of the *N*-phosphitylation was observed on adenine and cytosine bases, respectively (similar results have been observed in the above-described Letsinger's method using pyridinium hydrochloride and imidazole as a condensing agent[68]). However, this difficulty was fortunately cleared up by finding that the *N*-phosphitylated products could be completely converted to the corresponding *N*-free derivatives without any undesired reactions such as depurination and cleavage

Table 1. Preparation of Dideoxyribonucleoside Phosphate via the *N*-Unprotected Method



B^1	B^2	product yield/%[a)]
Ade	Ade	95
Ade	Cyt	93
Ade	Thy	95
Cyt	Ade	96
Cyt	Cyt	95
Cyt	Thy	94
Gua	Ade	96
Gua	Thy	98
Thy	Ade	98

a) Isolated yield.

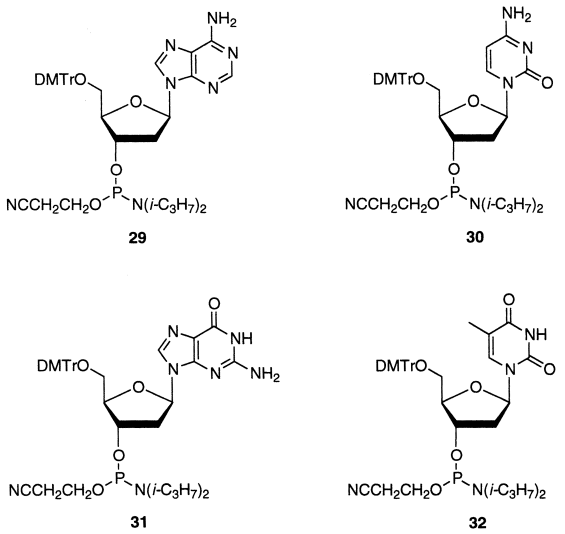


Chart 4.

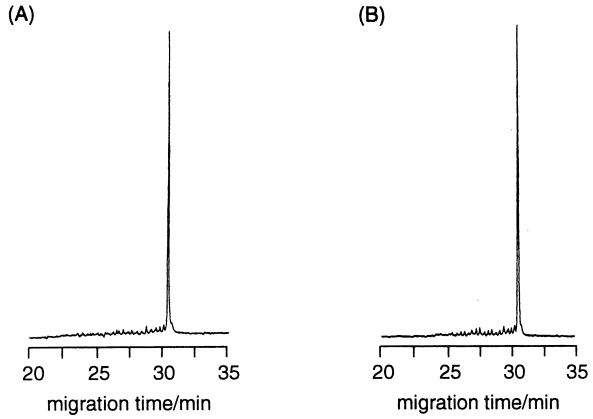


Fig. 4. Capillary gel elelctrophoresis profiles of the crude products of **35**. (A) Prepared via the *N*-unprotected method; (B) prepared via the *N*-AOC/*P*–*O*-allyl-protected method.

of the internucleotidic phosphite linkage by brief treatment with BIT (50 mol amt.) in methanol. In contrast, guanine and thymine bases suffered no undesired reaction. Accordingly, the synthesis via the *N*-unprotected strategy was accomplished in a formal sense through the overall procedure.

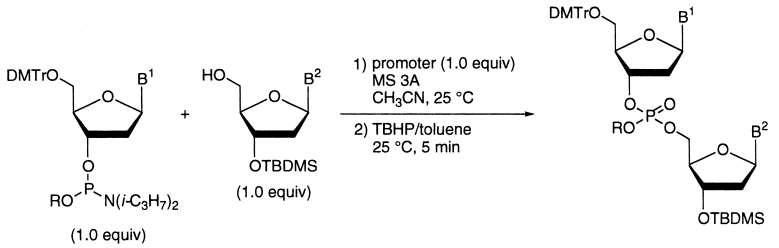
Base on these results, we investigated the solid-phase synthesis of four oligonucleotides: $^{5'}$GTCACGACGTTGTAAAACGAC$^{3'}$ (**33**) (21mer), $^{5'}$CAGGAAACAGCTATGACCATG$^{3'}$ (**34**) (21mer), $^{5'}$CAAGTTGATGAACAATACTTCATACCTAAACT$^{3'}$ (**35**) (32mer), and $^{5'}$TATGGGCCTTTTGATAGGATGCTCACCGAGCAAAACCAAGAACAACCAGGAGATTTTATT$^{3'}$ (**36**) (60mer).[94] The nucleotide-chain construction was conducted according to the reaction sequence similar to Scheme 1 using **29**, **30**, **31**, and **32** as monomer units, IMT as the activator, and TBHP for oxidation of the phosphite intermediate; in this process, the step for the above-mentioned removal of *N*-phosphityl group by treatment with a BIT/methanol mixture was inserted between steps of the condensation with the phosphoramidite and the oxidation of the phosphite intermediate. The average yield per one-base elongation in all the syntheses was $>$ 99.8%. After finishing the chain elongation, removal of the cyanoethyl protectors and detachment of the product from the solid support was achieved by treatment with concentrated ammonia (180 min). Isolated yields of the target oligomers were 72% for **33**, 74% for **34**, 78% for **35**, and 54% for **36**. These oligomers had satisfactorily high purity in their crude forms, comparable to that of the products prepared via the *N*-AOC/*P*–*O*-allyl-protected method. Figure 4 illustrates capillary gel electrophoresis profiles of the crude products of **35** prepared by the present *N*-unprotected method and the *N*-AOC/*P*–*O*-allyl-protected method using BIT as the promoter on high-cross linked polystyrene resins.

Consequently, we developed an efficient synthesis of oligodeoxyribonucleotides via the *N*-unprotected phosphoramidite method, that includes two key reactions of the highly chemoselective condensation of an *N*-unprotected nucleoside phosphoramidite and an *N*-unprotected nucleoside promoted by IMT as well as the quantitative removal of a phosphityl group on the amino groups of the adenine and cytosine bases by treatment with a mixture of BIT and methanol. Since the development of a nucleotide synthesis via the *N*-unprotected method is an extremely attractive and important research subject, a number of nucleotide synthetic chemists have reported several possible strategies,[95] including the *H*-phosphonate method,[96] the phosphite method,[97] and the phosphotriester method via hydroxy activation.[32h,62,98] However, according to reported results, these methods are only effective in the synthesis of rather short oligomers. Thus, the method disclosed by us here may be the most useful and reliable among *N*-unprotected methods reported until now.

Some Other Methods

We have also created some useful tools for large-scale liquid-phase synthesis. Liquid-phase synthesis is suitable for large-scale preparation of relatively short-sequence nucleotides and is usually employed for this purpose. In large-scale synthesis, the use of phosphoramidites in excess is a serious economical disadvantage, as nucleoside phosphoramidites are generally very expensive. In addition, even when the reaction is carried out quantitatively, the phosphoramidites used in excess result in the formation of by-products that must then be removed by troublesome operations. It is therefore important that an unnecessarily excess amount of the nucleoside phosphoramidite is avoided in large-scale, liquid-phase synthesis. However, the standard synthesis normally uses 2–4 molar amounts of the phosphoramidite and a promoter such as 1*H*-tetrazole.[99] One reason why such an excess of reactants is used may be that the reactivity of 1*H*-tetrazole is too low to achieve a smooth and high-yield reaction with stoichiometrical use. In contrast, we have developed a method utilizing the above-described acid/azole-complex reagent, that allows for efficient synthesis using equimolar amounts of a nucleoside phosphoramidite, a nucleoside, and a promoter. The azolium salts[65] include *N*-PhIMT,[65b] *N*-MeBIT, BIT,[66] *N*-PhIMP, etc., and the phosphoramidites include deoxyribonucleoside 3′-(allyl *N*,*N*-diisopropylphosphoramidite)s or 3′-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidite), and 3′-*O*-(*t*-butyldimethylsilyl)ribonucleoside 2′-(allyl phosphoramidite)s, or 2′-*O*-(*t*-butyldimethylsilyl)ribonucleoside 3′-(allyl phosphoramidite)s. For example, *N*-PhIMT generally completes the reaction of the deox-

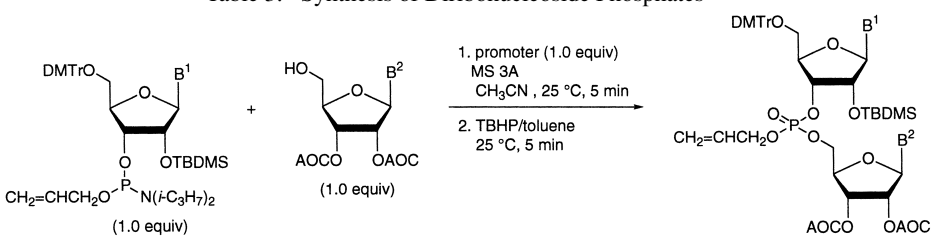
Table 2. Synthesis of Dideoxyribonucleoside Phosphates[a)]



promoter	B^1	B^2	R	reaction time/min	product yield/%[b)]
N-PhIMT	Thy	Thy	All	1	100, 99[c)]
N-PhIMT	Ade^{AOC}	Thy	All	5	100, 99[c)]
N-PhIMT	Cyt^{AOC}	Ade^{AOC}	All	1	91
N-PhIMT	$Gua^{All,AOC}$	Ade^{AOC}	All	5	98, $>$ 95[c)]
N-PhIMT	Ade^{Bz}	Gua^{ibu}	CH_2CH_2CN	5	86
N-PhIMT	Cyt^{Ac}	Thy	CH_2CH_2CN	3	88
N-PhIMT	Gua^{ibu}	Thy	CH_2CH_2CN	10	96, 89[c)]
N-PhIMT	Thy	Thy	CH_2CH_2CN	3	97
N-AcPhIMT	Thy	Thy	All	1	93
N-AcPhIMT	Cyt^{AOC}	Ade^{AOC}	All	1	91, 87[c)]
N-AcPhIMT	$Gua^{All,AOC}$	Ade^{AOC}	All	5	94
N-AcPhIMT	Gua^{ibu}	Thy	CH_2CH_2CN	10	93
N-AcPhIMT	Thy	Thy	CH_2CH_2CN	3	91
BIT	Thy	Thy	All	1	95, 91[c)]
BIT	$Gua^{All,AOC}$	Ade^{AOC}	All	5	91
BIT	Gua^{ibu}	Thy	CH_2CH_2CN	10	92
BIT	Thy	Thy	CH_2CH_2CN	3	95
N-MeBIT	Thy	Thy	All	1	92
N-MeBIT	Cyt^{AOC}	Ade^{AOC}	All	1	90
N-MeBIT	$Gua^{All,AOC}$	Ade^{AOC}	All	5	93

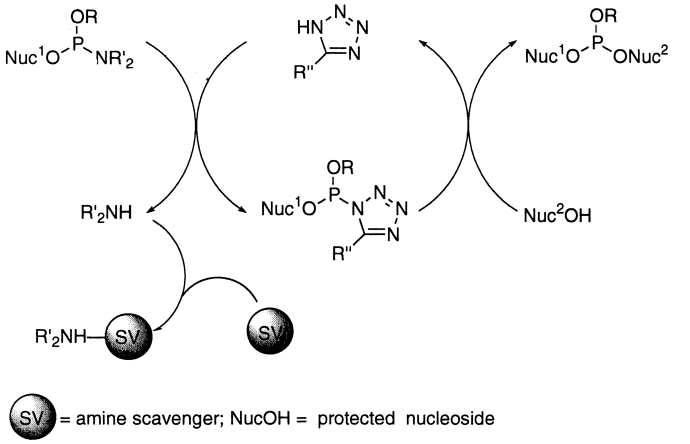
a) The preparation was carried out on a 0.1-mmol scale. b) Determined by ^{31}P NMR analysis unless otherwise noted. c) Isolated yield.

Table 3. Synthesis of Diribonucleoside Phosphates[a)]



promoter	B^1	B^2	reaction time/min	product yield/%[b)]
N-PhIMT	Ade^{AOC}	Ade^{AOC}	10	95
N-PhIMT	Cyt^{AOC}	Ade^{AOC}	15	96
N-PhIMT	Cyt^{AOC}	Ura	15	97
N-PhIMT	$Gua^{All,AOC}$	Cyt^{AOC}	15	96
N-PhIMT	Ura	Ura	15	96
BIT	Ade^{AOC}	Ade^{AOC}	10	91
BIT	Cyt^{AOC}	Ura	15	87
BIT	$Gua^{All,AOC}$	Cyt^{AOC}	15	91
BIT	Ura	Ura	15	93
N-MeBIT	Ade^{AOC}	Ade^{AOC}	10	91
N-MeBIT	Cyt^{AOC}	Ura	15	88
N-MeBIT	$Gua^{All,AOC}$	Cyt^{AOC}	15	91
N-MeBIT	Ura	Ura	15	93

a) The preparation was carried out on a 0.1-mmol scale. b) Isolated yield.



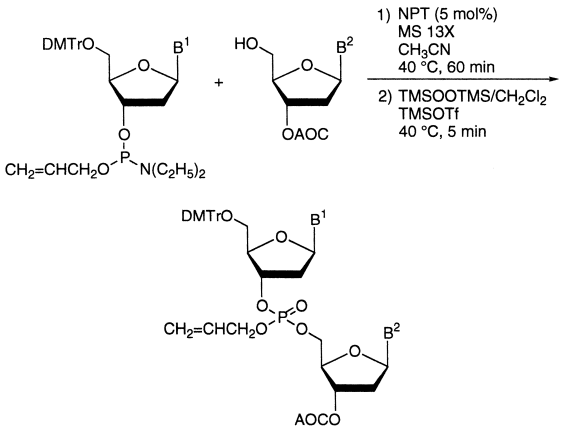
Scheme 7. The phosphoramidite method using a promoter in a catalytic manner.

yribonucleoside 3′-(allyl phosphoramidite) and a 5′-*O*-free deoxyribonucleoside, where equimolar amounts (0.1 mmol) of the reactants and the promoter are employed as a 0.1 M solution in acetonitrile in the presence of powdery MS 3A, at 25 °C for 5 min; the subsequent oxidation with 2-butanone peroxide furnishes the desired dinucleoside phosphate in $>$ 90% yield. Further, *N*-PhIMT allows for the stoichiometric reaction of the ribonucleoside 2′- or 3′-phosphoramidite and the 5′-*O*-free nucleoside in a 0.1 M acetonitrile solution in the presence of MS 3A. The reaction was achieved under similar conditions within 20 min, and the subsequent TBHP oxidation gave the diribonucleoside phosphate in $>$ 95% yield. Tables 2 and 3 summarize several examples of preparation of dinucleoside phosphates according to the azolium salt-promoted strategy.

In the synthesis of deoxyribonucleotides, the *N*-unprotected method using IMT as a promoter, which allows the synthesis to go forward with stoichiometric use of the phosphoramidite, the nucleoside, and the promoter to give the desired products in excellent yields, is thought to be the most efficient. However, the *N*-unprotected approach is currently not applicable to the synthesis of ribonucleotides (see Table 1).

Another tactic useful for large-scale, liquid-phase synthesis is a method allowing for the condensation of a nucleoside phosphoramidite and a nucleoside by the catalytic use of NPT as a promoter. The existing method usually uses a stoichiometric or sometimes an excess amount of NPT. However, the stoichiometric or excess use of NPT is a serious problem in the large-scale synthesis because NPT is poorly soluble in acetonitrile, thus making necessary an unacceptably large volume of the solvent. Further, this promoter is rather expensive and potentially explosive. Therefore, it is necessary to find a way to use NPT in a catalytic manner in the large-scale synthesis. The reason why NPT cannot act catalytically in the condensation of a nucleoside phosphoramidite and a nucleoside can be explained as follows. In this reaction, the acidic tetrazole acts not only as an activator of the phosphoramidite, but also a scavenger of the generated amine, where the scavenger takes precedence over the activator because the amine is a stronger base than the phosphoramidite. Hence, a stoichiometric amount of the tetrazole is the minimum required for completion of the reaction. Here, if the generated amine can be removed by the use of a scavenger, NPT would catalytically promote the condensation as shown in Scheme 7. This ideal was actually achieved using MS 13X (pore size 10 Å; particle size ca. 2 μm) as the amine scavenger.[100] The NPT-catalyzed approach can be applied to syntheses of both deoxyribonucleotides and ribonucleotides. In the synthesis, selection of the phosphoramidite is important for accomplishing the reaction at a reasonable rate and for obtaining the desired product in high yield. In the synthesis of deoxyribonucleotides, the use of *N*,*N*-diethylphosphoramidites is the most effective; the condensation is generally completed within 60 min at 40 °C. In the synthesis of ribonucleotides, the use of *N*,*N*-diisopropylphosphoramidites is recommended. The reaction is generally finished for 8 h at the acetonitrile reflux temperature. In these

Table 4. Preparation of Dinucleoside Phosphates via the Phosphoramidite Approach Using NPT as the Catalytic Promoter



B^1	B^2	product yield/%[a)]
Ade^{AOC}	Cyt^{AOC}	97
Cyt^{AOC}	Cyt^{AOC}	96
$Gua^{All,AOC}$	Cyt^{AOC}	92
Thy	Cyt^{AOC}	98
Ade^{AOC}	Thy	97
Cyt^{AOC}	Thy	97
$Gua^{All,AOC}$	Thy	97
Thy	Thy	99

a) Isolated yield.

syntheses, it is sufficient to use the phosphoramidite in an almost stoichiometric amount (1.05 mol amt.) relative to the nucleoside. Some deoxyribonucleotides synthesized by the method using NPT in a catalytic manner are shown in Table 4. This NPT-catalyzed method allows for the production of 8mers, at the longest, in the synthesis of deoxyribonucleotides.

Conclusion and Perspective

We have made some improvements in the chemical synthesis of DNA and RNA oligomers and related compounds via the phosphoramidite method. The improved methods are obviously advantageous compared to the existing ones with respects to capability, practicability, and efficiency. First, the *N*-AOC/*P*–*O*-allyl-protected phosphoramidite method, which was developed on the basis of organopalladium chemistry, has allowed for the efficient synthesis of not only normal DNA and RNA oligomers, but also a variety of artificial derivatives, particularly those with base-sensitive structure and functions. In addition to the above-described examples prepared in our laboratory, this strategy with or without combined use of anhydrous/non-basic oxidation using TBHP, bis(trimethylsilyl) peroxide, or 2-butanone peroxide has been successfully employed by other groups in syntheses of various nucleic acid-related compounds. These compounds include 9,10-epoxybezo[α]pyrene 7,7-diol adducts at the exocyclic N^2-amino group of deoxyguanosine, which are important authentic samples for the investigation of the carcinogenecity of polycyclic aromatic hydrocarbons, including benzo[α]pyrene;[101] cyclic oligonucleotides for testing the ability to mimic c-di-GMP as activators of cellulose synthase and substrates for a Ca^{2+}-sensitive phosphodiesterase, PDE-A;[102] directly and indirectly linked peptide–oligodeoxyribonucleotide conjugates playing decisive roles in important biological processes like viral replication and being attractive as antisense molecules;[103] pyranosyl RNAs used for a series of studies on the chemistry of homo-DNA, the constitutionally simplified model system of hexopyranosyl-(6′→3′)-oligonucleotide systems as potentially natural nucleic-acid alternatives in the context of a chemical aetiology of nucleic acid structure;[104] α-bicyclo-DNA oligomers, including a rigid nucleoside analog in which the centers C(3′) and C(5′) of the natural deoxyribonucleosides are connected by an ethano bridge, which have been designed to investigate the hypothesis that oligonucleotides derived therefrom have single-stranded structures that are more preorganized for duplex formation and, thus, have the potential to form more stable duplexes with natural RNA and DNA complements;[105] a DNA oligomer that site-specifically contains alkaline labile and mutagenic and/or cytotoxic (5*R*)-5,6-dihydro-5-hydroxythymidine, the substrate required to determine the effects of mutated nucleosides on the function of polymerase and to repair enzymes in vitro and in vivo;[106] a nucleoside hapten with a [P(O)–O–N] linkage eliciting catalytic antibodies;[107] 2′-deoxy-3-isoadenosine-incorporated DNA oligomers used as a key substrate for a model study to determine how the hydrolytically susceptible 3-isoadenosine unit can be incorporated into an oligodeoxyribonucleotide sequence in either a 3′ or 5′ sense;[108] *N*-acetylcytidine-incorporated RNA oligomers present at conserved positions in tRNAs from numerous phylogenetic sources and in the small ribosormal subunit of eukaryotes;[109] a covalently linked dimensional analog of dA·dT, which is an attractive compound for determining the interactions between deoxyadenosine and deoxythymidine units in place with template dA–dT, which is a dimensional mimic of a fixed dA·dT base pair.[110] Further, the AOC/allyl-protected phosphoramidite method has been applied to solid-phase synthesis of DNA oligomers with some functional groups such as CH_2COOH and $PO_3(CH_2)_nCOOH$ on the 3′-hydroxy groups due to the use of a linker containing a photo-labile *o*-nitrobenzyl,[111] mild base-labile 3-amino-1,2-propanediol,[112] or Pd(0)-labile allyloxy[113] moiety in place of a conventionally used long(alkylamino)succinyl[114] or -oxalyl[10c] linker. This method permits the synthesis of short oligonucleotide libraries attached to the solid support through their 5′-ends.[71c] Another important feature of the AOC/allyl-protected method is that it allows for the direct preparation of solid-anchored DNA oligomers, whose applicability is enormous; the possibilities include affinity chromatography for analysis and isolation of specific DNA and RNA sequences enrichment of desired genes in a cDNA library, solid-phase amplification of DNA, purification of DNA binding proteins and diagnosis of infections. Since the *N*-unprotected method does not have a long history, the utility of this method has not been widely demonstrated. However, this method is obviously advantageous over *N*-protected methods in many respects, including versatility, applicability, practicability, and operational simplicity. Thus, the potential of this strategy seems to be enormous. The azolium salt-promoted method and the NPT-catalyzed method, which allow for the synthesis to take place with a stoichiometric use of building blocks, is also a notable improvement from an economical point of view in the large-scale synthesis. A wide use of these approaches is expected in future.

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