Satoshi Obika · Mitsuo Sekine *Editors*

Synthesis of Therapeutic Oligonucleotides

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Preface

Nucleic acids have been unambiguously recognized as potential resources for the development of new drugs to treat incurable genetic diseases, as well as functional materials for various applications in bioscience and biotechnology fields. In 2016, a new academic society, the Nucleic Acids Therapeutics Society of Japan, was inaugurated. This society was established based on a previous domestic organization, the Antisense Symposium. In addition, the Society of Nucleic Acids Chemistry was established in 2017 in Japan. During this time, organic chemistry of nucleic acids has gained importance in providing new drugs and materials. In Japan, we have a long history of nucleic acid chemistry studies. The first symposium on this subject was held at Osaka University in 1973 by the late Professor Morio Ikehara. Japan is unique in that large amounts of nucleic acids are produced as by-products of soy sauce and pulp. Therefore, many researchers working in biotechnology companies and universities have actively studied the utilization of such easily accessible natural products for a long period of time. As a result, Japan is one of the world's leading countries in nucleic acid chemistry research.

This book contains the latest research from active researchers in the nucleic acid chemistry field in Japan. Part I reviews recent developments in chemical synthesis of DNA and RNA oligomers and includes practical applications such as large-scale synthesis of DNA and RNA fragments. Part II summarizes new strategies for the synthesis of oligonucleotides modified at the nucleobases, sugar moieties, and phosphodiester linkages; these changes have been developed to improve their original properties such as hybridization affinity for DNA and RNA, as well as resistance to nucleases. The topics discussed in this book would be beneficial to those who want to join nucleic acid chemistry research or to discover more effective nucleic acid drugs in the future. We hope that this book may provide an opportunity for researchers to gain new understanding and inspire new ideas in nucleic acid chemistry research that may eventually lead to novel concepts and techniques.

Suita, Japan — Satoshi Obika
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January 20, 2018

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Part I
Synthesis of Natural Oligonucleotides

Non-protected Synthesis of Oligonucleotides

Akihiro Ohkubo, Kohji Seio, and Mitsuo Sekine

Abstract Much attention has been paid to the development of effective methods for synthesizing modified oligonucleotides that have various functional groups. Recently, we have developed a selective phosphorylation toward the hydroxyl group (*O*-selective phosphorylation), which is named as the proton-block method. An activated phosphite method was also developed to synthesize modified DNA oligonucleotides having alkaline-labile functional groups. The DNA synthesis using the activated phosphite method, which involves a phosphite intermediate generated from the phosphoramidite building block, presents excellent chemoselectivity toward the hydroxyl groups on resins under solid-phase conditions. In addition, the *O*-selectivity of the phosphorylation with P–N bond cleavage using 6-nitro-HOBt is more than 99% in the RNA synthesis without base protection. In this review, we summarize the *O*-selective phosphorylation in DNA and RNA synthesis without base protection and the synthesis of modified oligonucleotides having alkaline-labile functional groups using these new methods.

Keywords *N*-unprotected DNA and RNA synthesis · *O*-selective phosphorylation · Modified oligonucleotides · P–N bond cleavage · Solid-phase synthesis · Silyl type linker

1 Introduction

During the recent two decades, a wide variety of modified oligonucleotides have been synthesized using nucleic acid chemistry [1]. These molecules have proved to be useful for gene therapy [2] and genetic diagnosis [3]. In order to further develop these techniques, there is a need to synthesize epoch-making artificial oligonucleotides that can result in the next generation DNA or RNA technology.

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Recently, modified oligonucleotides having base-labile functional groups have received much attention in the field of nucleic acid research. For example, amino-acyl tRNAs having an unnatural amino acid [4] and 2′-*O*-acyloxymethyl RNAs [5] are expected as the key molecules for protein engineering and down-regulation of gene expression. However, these compounds cannot be efficiently synthesized by using the standard methods of DNA/RNA synthesis because the base-labile ester functions are decomposed by treatment with ammonia in the final step in the solid-phase synthesis.

To overcome this problem, we started our original studies for developing new routes to synthesize the oligonucleotides having base-labile functional groups without base protection. When such strategies were employed, it was expected that *N*-phosphorylated branched oligonucleotides would be generated [6] because the exocyclic amino groups of cytosine and adenine bases are known to react with trivalent phosphorylating reagents. Therefore, we attempted to achieve the highly *O*-selective phosphorylation of the hydroxyl group in DNA and RNA synthesis without base protection [7].

In 1991, R. L. Letsinger first synthesized oligodeoxynucleotides using *N*-unprotected deoxynucleoside 3′-phosphoramidites via the phophoramidite method [8]. Two-step reactions were involved, including condensation and selective cleavage of the once-generated N–P bonds [9]. A year later, R. A. Jones reported the *H*-phosphonate method without base protection using *N*-unprotected deoxynucleoside 3′-H-phosphonate building blocks [10]. However, serious *N*-acylations on the base residues were observed when pivaloyl chloride was used as the condensing agent. In 1997, our research group improved the *H*-phosphonate approach using 2-(benzotriazol-1-yloxy)-1,1-dimethyl-2-pyrrolidin-1-yl-1,3,2-diazaphospholidinium hexafluorophosphate (BOMP) as the effective coupling agent which does not react with the base residues. A DNA 12 mer was thus obtained as shown by the main peak in the HPLC analysis [11]. A year later, Hayakawa and Kataoka reported a procedure for DNA fragments based on the phosphoramidite method without base protection, and a promoter of imidazolium triflate (IMT) was used to activate the deoxynucleoside-3′-phosphoramidite building blocks [12]. However, this method was not applied to the actual synthesis of DNA oligomers because ester exchange reactions might occur during the second treatment step in the condensation.

In this review, we describe our extensive studies on this subject after these precedent efforts.

2 Development of Proton-Block Strategy for the Synthesis of Oligonucleotides Without Base Protection

In the phosphoramidite method for synthesizing oligonucleotides, the chain elongation (phosphorylation) is carried out by activating phosphoramidite building blocks using acidic activators, such as 1*H*-tetrazole (p*K*a = 4.8) on polymer supports [8].

The p*K*a value of activators used in DNA/RNA synthesis varies from 4.5 to 7.0. On the other hand, the pK_{B+H} values of cytosine and adenine bases which have highly nucleophilic amino groups are 4.2 and 3.8, respectively. Therefore, cytosine and adenine can be protonated by using a certain activator having a pK_a value less than 3.8 during the condensation. The nucleophilicity of the resulting protonated amino groups can thus be greatly decreased (proton-block method, Fig. 1). Because the amino group of the guanine base shows the inherently low nucleophilicity, the phosphorylation on the base residue does not occur [9].

We examined extensively various compounds as the activators in terms of their pK_a values, solubility, hygroscopicity, and easiness in the activation of *N*-unprotected phosphoramidite monomers. As a result, 4-nitro-6-trifluoromethylbenzotriazol-1-ol ($HO^{nt}Bt$) [13], 5-nitrobenzimidazolium triflate (NBT), and triazolium triflate (TRT) were selected as the activators having pK_a values of 2.70, 2.76, and 2.85, respectively, which might meet the demand of this proton-block method (Fig. 2) [7a].

In order to evaluate the *O*-selectivity of the phosphorylation using these compounds as the activator, the synthesis of dimers (d[AT], d[CT], and d[GT]) were carried out. As shown in Fig. 3, 20 equivalents of *N*-unprotected phosphoramidite unit [7a] and 40 equivalents of an activator were added to the thymidine-loaded highly cross-linked polystyrene (HCP) resin in a mixture of acetonitrile and *N*-methylpyrrolidone (NMP). Because of the low solubility of these activators in acetonitrile and the risk of acid-promoted elimination of the DMTr group, NMP was used as the co-solvent. After the phosphorylation, oxidation, and deprotection of the DMTr group were successively carried out, the oligomers were released from the resin by treatment with 28% NH_4OH. The *O*-selectivity of the phosphorylation was evaluated by using HPLC.

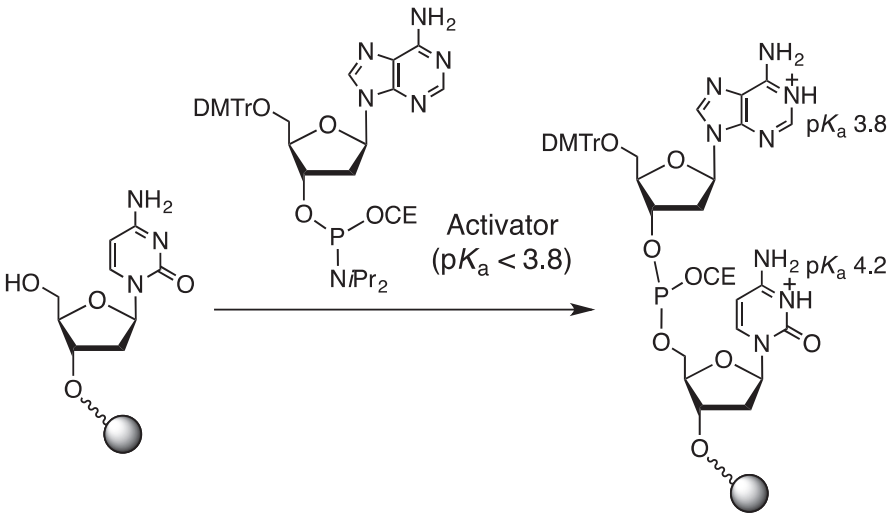


Fig. 1 Proton-block strategy for the synthesis of oligonucleotides without base protection

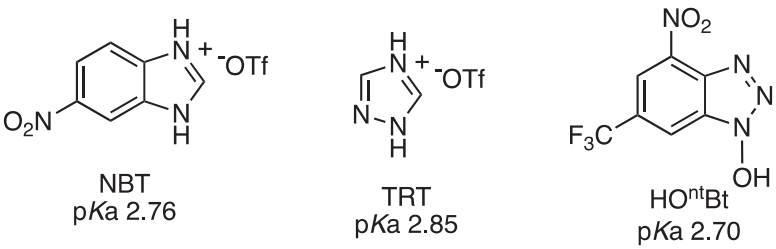


Fig. 2 Chemical structures of activators for the proton-block strategy

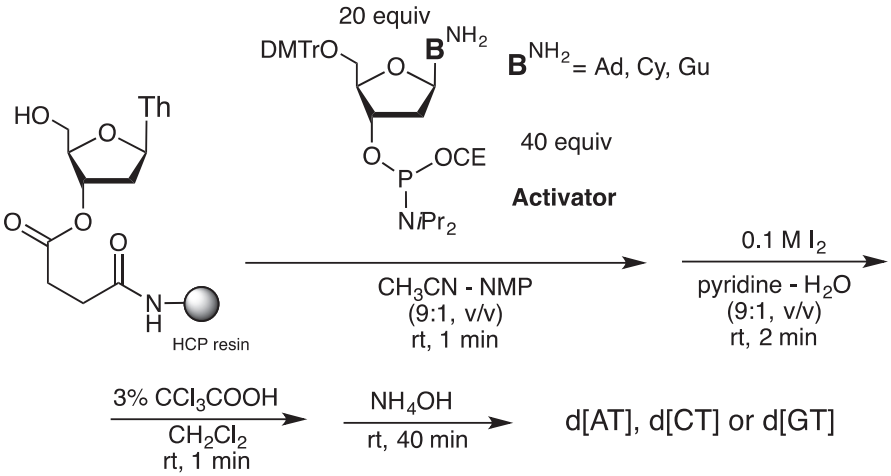


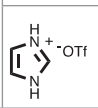
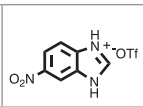
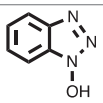
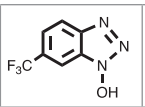
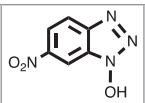
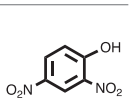
Fig. 3 Synthesis of d[AT], d[CT], and d[GT] using the *N*-unprotected phosphoramidite method

In the synthesis of d[AT] and d[CT] using imidazolium triflate (IMT, pK_a = 7.0) [12, 14], which was reported as the activator having the highest *O*-selectivity, the *O*-selectivities were 77.0% and 82.9%, respectively. In contrast, when HO^{nt}Bt, NBT, and TRT were used instead, the *O*-selectivities of the reactions were considerably improved.

In particular, NBT has the highest selectivity of 99% or more in dimer synthesis (Table 1).

For synthesizing trimers d[TXT] (X = A, C, and G) from the dimers obtained according to the above solid-phase synthesis, the *O*-selectivity of more than 99.8% can be achieved when NBT was used as the activator (Fig. 4). This strategy was applied to the synthesis of d[C_6T] and d[A_6T] in 21% and 25% yields, respectively (Fig. 5). In a similar manner, a DNA 12 mer of d[CAGTCAGTCAGT] was synthesized as the main product in 18% yield [7a]. However, the synthesis of these oligomers is accompanied by a series of minor peaks at later retention time in the anion-exchange HPLC. These minor peaks are assigned to a cluster of undesired *N*-branched oligomers. When IMT was used under similar conditions, these cluster peaks indicated the predominant products.

Table 1 The selectivity of condensation in the *N*-unprotected phosphoramidite method

	Ratio of the desired product					
						
Product	IMT	NBT	HOBt	HO^{d}Bt	HO^{n}Bt	DNP
d[AT]	77.0	99.2	99.7	99.3	98.8	97.1
d[CT]	82.9	99.0	99.9	98.9	99.8	99.5
d[GT]	>99.9	>99.9	>99.9	>99.9	>99.9	>99.9
d[TAT]	90.5	>99.9	>99.9	99.1	97.5	99.6
d[TCT]	9.7	99.8	>99.9	98.7	97.2	99.4
d[TGT]	>99.9	>99.9	>99.9	>99.9	>99.9	>99.9

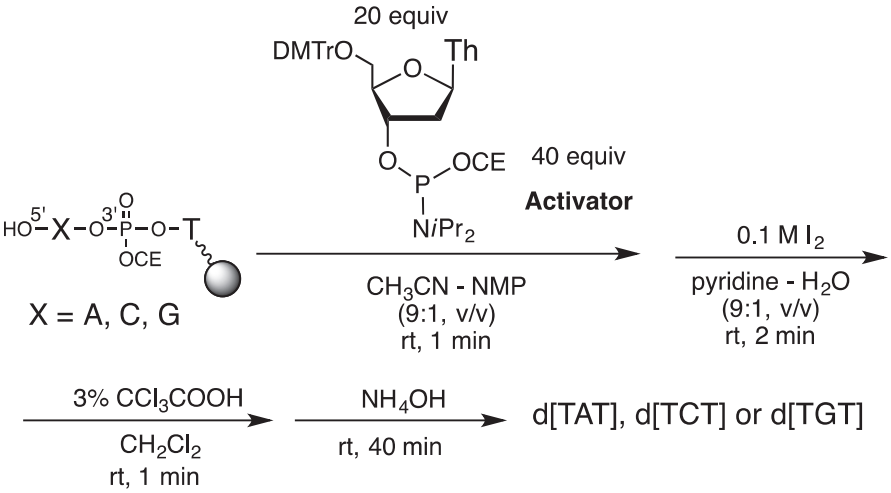


Fig. 4 Synthesis of d[TAT], d[TCT], and d[TGT] using the *N*-unprotected phosphoramidite method

3 Development of the Activated Phosphite Method Using *N*-Unprotected Phosphoramidites

Wada reported the *H*-phosphonate method for the synthesis of oligodeoxynucleotides using BOMP, a new type of phosphonium salt, as the condensing agent [11a]. The predominant *O*-selectivity observed in this method was explained by the strong MO interaction between the resulting trivalent phosphite triester intermediates and the 5′-terminal OH group in the growing DNA chain (Fig. 6).

Therefore, it can be speculated that the *O*-selectivity could be considerably improved if such a phosphite triester intermediate can be formed from the phosphoramidite building blocks.

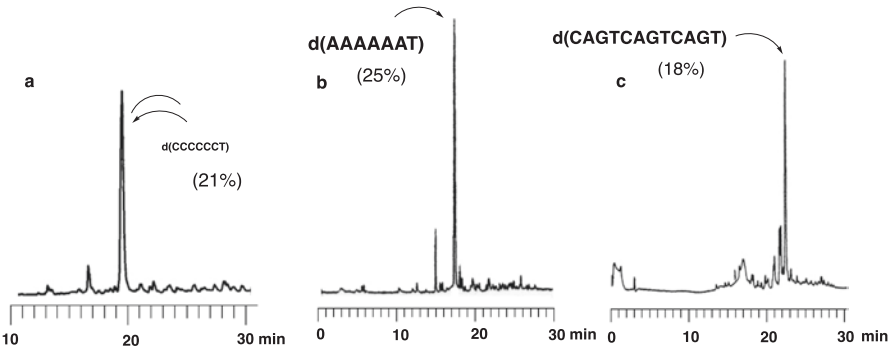


Fig. 5 The anion-exchange HPLC profiles of the crude mixtures in the synthesis of oligodeoxynucleotides

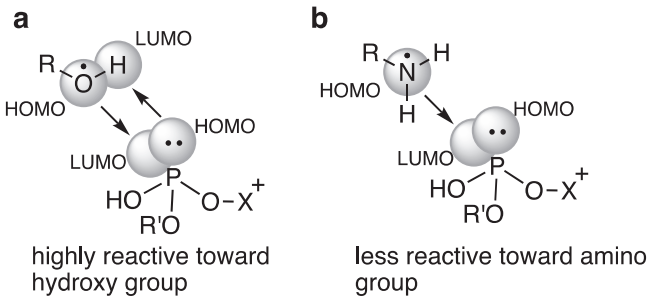


Fig. 6 The reaction selectivity in unprotected *H*-phosphonate method using BOMP

We examined a number of alcohol derivatives having acidic protons as the promoters (Fig. 7). The use of 1-hydroxybenzotriazole (HOBt, pK_a = 5.4) showed an excellent *O*-selectivity of more than 99.7% in the synthesis of d[XT] and d[TXT], as listed in Table 1 [7b]. d[CAGTCAGTCAGT] was synthesized in 36% yield. The HPLC analysis suggested that *N*-branched oligomers were negligibly formed in the synthesis.

More acidic reagents, such as 1-hydroxy-6-torifluoromethylbenzotriazole (HO^{tf}Bt, p*K*a = 4.3) and 1-hydroxy-6-nitrobenzotriazole (HO^{n}Bt, p*K*a = 3.5), decrease the *O*-selectivity. Interestingly, 2, 4-dinitrophenol (p*K*a = 4.1) still shows high *O*-selectivity [7c]. However, when this approach was employed using a DNA synthesizer, HO^{tf}Bt showed better results than HOBt. Using HO^{tf}Bt, α-d[TC*TTC*C*TTC*TTT] (61%, C*: 5-methyl-C), and d[CAGTCAGTCAGT] (32%) can be synthesized in satisfied yields [7c]. These oligomers were synthesized using the succinate linker on the highly cross-linked polystyrene (HCP) which was proved to be the best resin among the tested ones. However, this linker could be cleaved by ammonia. For synthesizing oligomers having base-labile functional groups, cleavable linkers under conditions rather than basic ones were required. Therefore, we used a silyl type of linker (Fig. 8a) [15].

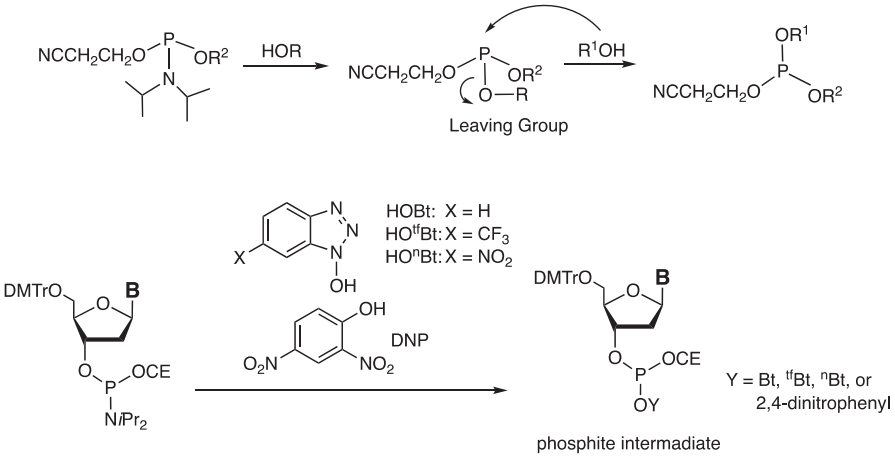


Fig. 7 Activated phosphite triester method

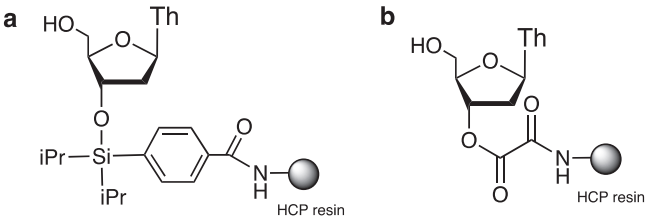


Fig. 8 Chemical structures of T-loaded HCP resins having silyl (**a**) and oxalyl (**b**) linkers

Thus, d[GC^{ac}ATCAGC^{ac}C^{ac}TCAT] having three C^{ac} bases (Fig. 10a) was synthesized in 33% yield using this linker which can be removed by treatment with Bu_4NF under neutral conditions (Fig. 9a) [7c]. Moreover, further improvement for the coupling conditions was conducted using the post-treatment of the P–N bonds on the base residues by BIT after every condensation. This improved procedure gave d[$C_5T_5(CT)_5$] in a high yield of 81%, indicated by the considerably simplified peak in HPLC (Fig. 9b). A long oligomer of d[$T_2A_5T_2AT_2A_3T_2AT_2$] was also obtained in a satisfactory yield (24%) using this improved procedure (Fig. 9c) [7c].

As one application of this method, we demonstrated the synthesis of oligodeoxynucleotides containing a dithymidine hydroxymethylphosphonate residue [$Tp_{(CH2OH)}T$] [16]. Such a residue was labile toward ammonia which was used for deprotecting the usual acyl protecting groups on the base residues. Ammonia, however, was sufficiently resistant under the conditions of 5% $PrNH_2$ in MeOH for 30 min, which was used for the cleavage of the base-labile oxalyl linker (Fig. 8b) [16]. Thus, d[pTxTpCpTxTpCpCpTxTpCpT], which contained many lipophilic species of the diastereomeric TxT units, was obtained using the oxalyl linker. This modified oligomer showed a strong triple strand formation with a hairpin DNA 34 mer [16] (Fig. 10).

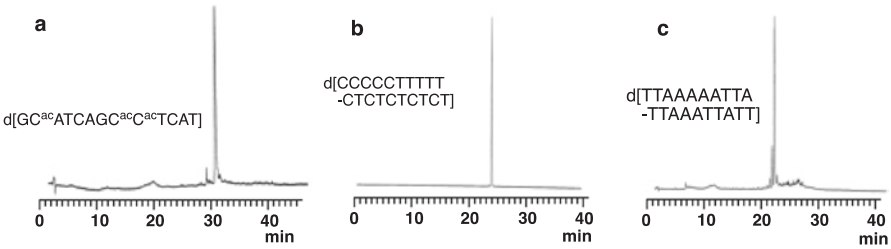


Fig. 9 The anion-exchange HPLC profiles of the crude mixtures in the synthesis of (**a**) d[GC^{ac}ATCAGC^{ac}C^{ac}TCAT], (**b**) d[C_5T_5CTCTCTCT], and (**c**) d[TTAAAAATTATTAAATTATT]

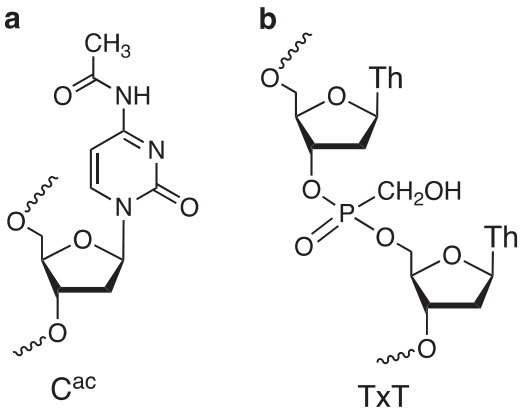


Fig. 10 Chemical structures of 2′-deoxy-*N*-acetylcytidine (**a**) and the hydroxymethylphosphonate (**b**) linkage

In place of the silyl linker (Fig. 8a) which is cleavable by Bu_4NF, we designed a new type of silyl linker containing a triazole ring, which can be formed by the reaction between the azido group and the acetylene group attached to the HCP resin, as shown in Fig. 11 [17].

The activated phosphite method is proved to be useful for synthesizing the oligodeoxynucleotides containing a cytosine N-oxide or an adenine N-oxide base. As the synthetic intermediates, the partially protected oligodeoxynucleotide derivatives, which incorporate the *N*-unprotected deoxyadenosine or deoxycytidine, can be produced on the HCP resin with the combination of *N*-protected and *N*-unprotected deoxynucleoside phosphoramidite units. Oxidation of these intermediates with mCPBA, followed by deprotection and cleavage of the linker, generates oligodeoxynucleotides having adenine-N-oxide or cytosine-N-oxide site specifically. The biological properties of DNA having such base-oxidized species were unknown at that time, although they can be formed via oxidation using hydrogen peroxide in cells. The synthesis of this type of oligomers was achieved using the *N*-unprotected deoxycytidine or deoxyadenosine phosphoramidite building block along with the

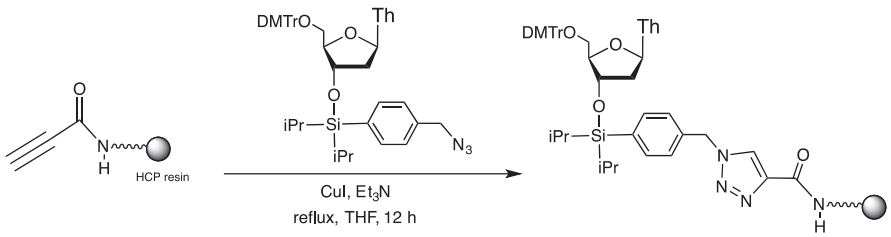


Fig. 11 A silyl type of linker constructed by Huisgen reaction

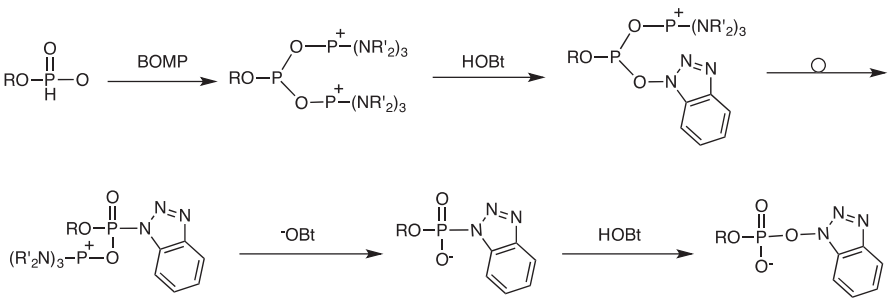


Fig. 12 Rearrangement of the phosphite intermediates in the *H*-phosphonate method

usual thymidine and (4-isopropylphenoxy)acetyldeoxyguanosine units. It is now clear that such modified oligomers can bind the complementary DNA strand correctly without the formation of mismatched base pairs [18].

4 Mechanism of the Activated Phosphite Method

In the *N*-unprotected phosphoramidite method, the reaction is considered to proceed via a phosphite triester intermediate. On the other hand, the intermediate, which is supposed as a plausible intermediate in the above-mentioned *H*-phosphonate method, is a kind of phosphite triester having a phosphonium cation (Fig. 12) [11a].

This intermediate is rapidly converted via an N–O rearrangement to give a phosphoramidate derivative, which might also react with the 5′-terminal HO group of the growing chain on the polymer support and give directly a five-valent phosphotriester. Therefore, there is a possibility that the phosphite intermediate in the activated phosphite method might be transferred to a phosphoramidate derivative (Fig. 13).

To confirm the presence of the rearrangement, we examined the synthesis of Tp(S)T using sulfurization in place of oxidation, as shown in Fig. 13. Consequently, the ratio of TpT/Tp(S)T was 4.9:95.1. When IMT was used, the ratio was decreased to 1.4:98.6 because of air oxidation. Therefore, the actual rearrangement in the phosphite triester method using HOBt was estimated to be a ratio of 3.5% [7c] (Fig. 14).

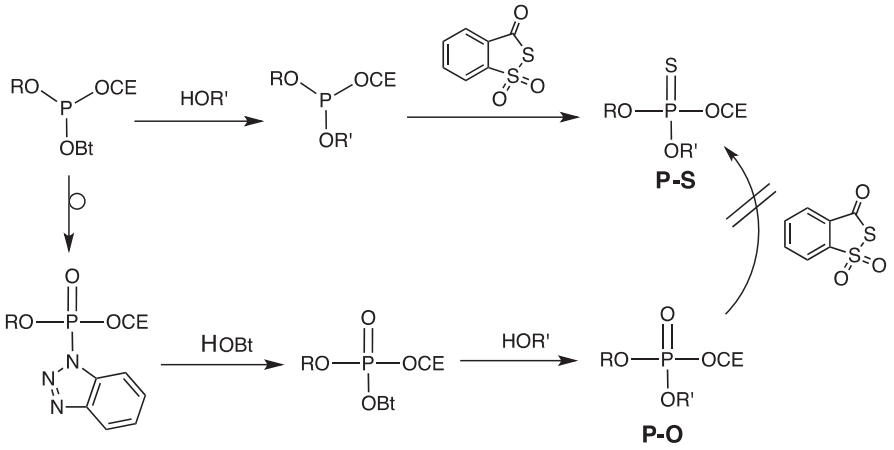


Fig. 13 Reaction mechanism of the phosphorylation in the activated phosphite method

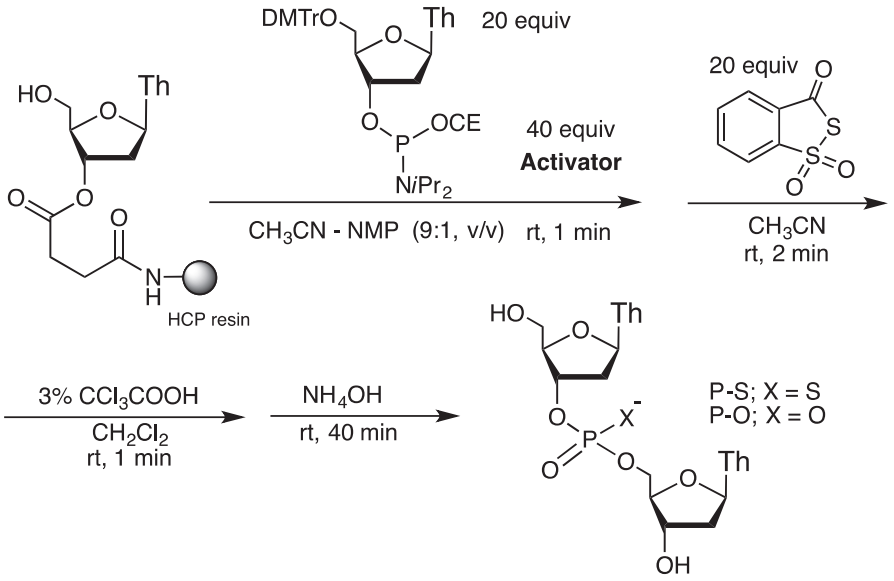


Fig. 14 Synthesis of the phosphorothioate TT dimer

5 Synthesis of RNA Oligomers Using the Activated Phosphite Method

For the synthesis of RNA, we applied our original procedure used for DNA synthesis to achieve this goal, using *N*-unprotected ribonucleotide building blocks. For this purpose, we examined the synthesis of AmpT and CmpT using

2′-*O*-methyladenosine and 2′-*O*-methylcytidine phosphoramidite units. It turned out that the efficiency of the condensation decreased greatly. Therefore, our efforts were focused on a condensation mode involving a two-step treatment initially introduced by R. L. Letsinger [9]. In this procedure, the post-treatment of the resulting *N*-branched species with a more acidic promoter was carried out after the condensation was finished, as shown in Fig. 15 [19]. In the first treatment, we employed a powerful activator, i.e., benzimidazolinium triflate (BIT) developed by Hayakawa. The resulting *N*-branched DNA oligomer species were treated using several HOBt derivatives as well as pyridine-HCl in the presence of aniline (Letsinger's choice). It was found that the use of HO^{n}Bt gave the best result, and the efficiency of the P–N bond cleavage remained at the level of more than 99%. Once the resulting phosphite ester derivative represented by ROP(OCe)(O^{n}Bt) could be converted to an inert five-valent species ROP(O)(OCe)(O^{n}Bt) via an inherent rearrangement as reported by us, we can obtain only the desired linear DNA chain at the final process.

According to this improved procedure, we also examined dimer synthesis using several kinds of 2′-O-masked adenosine and cytidine phosphoramidite building blocks. In all cases, the P–N bond cleavage was achieved in more than 99%, when the time for the second treatment was extended to 2 min. Using these conditions, we synthesized r[UUUUCUUUUU] as the almost single peak in HPLC. Starting from the C-loaded HCP resin having the silyl linker mentioned above, we also succeeded in synthesizing an RNA 25 mer of r[UAGAAGUG^{ac}CAUACUAG^{ac}UGAGUUUGC], where two base-labile *N*-acetylguanine bases were introduced into the oligomer. This synthesis was conducted in 14% yield [20].

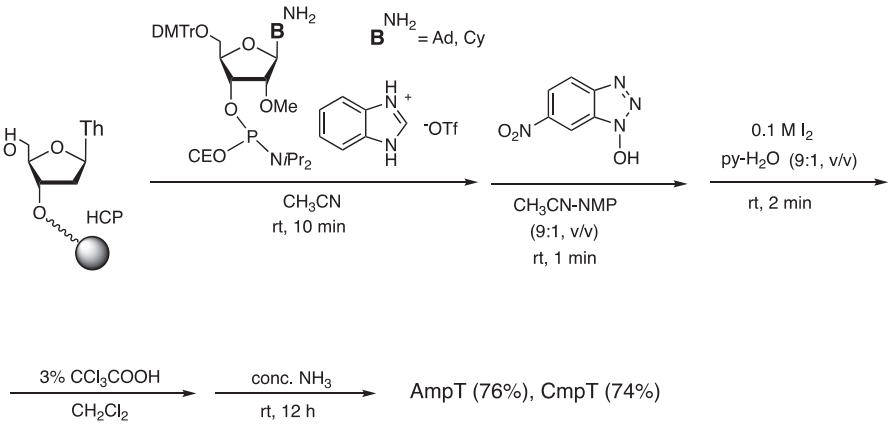


Fig. 15 Synthesis of the phosphorothioate TT dimer

6 Synthesis of Phosphoramidite Monomer Building Blocks

In the synthesis of oligonucleotides without base protection, N-unprotected monomer building blocks were required and therefore synthesized according to several methods. Since the selective 5′-*O*-dimethoxytritylation of deoxynucleosides has been reported by several research groups, direct 3′-*O*-phosphitylation of 5′-*O*-DMTr-deoxynucleosides has also been developed to obtain the *N*-unprotected monomer units. This process seems to be ideal, but some drawbacks occur in the two-step procedure. The selective 5′-*O*-dimethoxytritylation was not so good because simultaneous 3′-*O*-dimethoxytritylation to some extent occurred. The purification of the desired product thus became a little challenging. Moreover, the yield of the 5′-*O*-DMTr-deoxyguanosine was not satisfied. From the practical viewpoint, we have developed an alternative to the usual method. On the basis of our own experience that tervalent phosphoramidite derivatives were rather stable under basic conditions compared with the corresponding five-valent phosphoramidate derivatives, we found a simple treatment of the conventional deoxyribonucleoside phosphoramidite monomers using methylamine in THF [19, 21].

7 Conclusion

A number of methods for synthesizing DNA and RNA have been reported using the well-established phosphoramidite method up to date [8]. Under the restricted conditions of this standard method, it is difficult to synthesize oligonucleotides containing base-labile functional groups. Usually, such base-labile functional groups have been introduced into DNA or RNA by post-modification using amino groups that can react with acylating agents containing the base-sensitive group. Our activated phosphite method now enables us to synthesize the base-labile modified oligonucleotide derivatives in a more straightforward manner. Actually, the synthesis of oligonucleotides incorporating lipid structures using our original strategy has been reported [22].

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Various Coupling Agents in the Phosphoramidite Method for Oligonucleotide Synthesis

Masaki Tsukamoto and Yoshihiro Hayakawa

Abstract This review selects some representative coupling agents used for internucleotide bond formation reactions in the phosphoramidite method, which is now the most widely employed method for the chemical synthesis of oligodeoxyribonucleotides and oligoribonucleotides, and it describes their utility, efficiency, and drawbacks. Moreover, the mechanism of the coupling of the nucleoside phosphoramidite and nucleoside promoted by the coupling agent is discussed in some cases. The selected coupling agents are 1*H*-tetrazole, 5-ethylthio-1*H*-tetrazole (ETT), 5-benzylthio-1*H*-tetrazole (BTT), 5-[3,5-bis(trifluoromethyl)phenyl]-1*H*-tetrazole (Activator 42), 4,5-dicyanoimidazole (DCI), certain carboxylic acids, and various acid/azole complexes such as benzimidazolium triflate (BIT) and saccharin 1-methylimidazole (SMI).

Keywords Synthesis of oligonucleotides · Phosphoramidite method · 1*H*-Tetrazole · Acid/azole complexes · DNA oligomer · RNA oligomer

1 Introduction

In response to the recent progress in the life sciences, the importance of the chemical synthesis of DNA and RNA oligomers, i.e., oligonucleotides, has increased noticeably, because it is no exaggeration to say that research in the life sciences always uses chemically synthesized oligomers. In the chemical synthesis of oligonucleotides, a key stage is the construction of an internucleotide linkage [1–3]. Thus far, many methods have been reported for the formation of internucleotide bonds, such as the phosphodiester method developed by Khorana [4, 5], the

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phosphotriester method devised by Letsinger [6, 7] and Reese [2, 8], the phosphite method developed by Letsinger [9, 10], the phosphoramidite method devised by Caruthers [11–15], the *H*-phosphonate method as originally developed by Todd [16, 17], and the improved version devised by Froehler [18] and Stawinski [19]. Figure 1 outlines the modes of internucleotide bond formation in these methods.

Among the existing methods, the phosphoramidite method [1, 11–15] is the most widely employed at present, because this method has many advantages over the other methods. For example, nucleoside phosphoramidites, which are used as building blocks in the phosphoramidite method, are easily prepared and stable in storage. In addition, the phosphoramidite method generally enables higher speed and higher yield in internucleotide bond formation compared with the other methods. This factor is a great advantage in the synthesis of long oligonucleotides. Therefore, the phosphoramidite method has been applied not only to the synthesis of oligodeoxyribonucleotides but also to that of oligoribonucleotides and has enabled the production of a variety of biologically important substances, such as primers for the polymerase chain reaction, oligonucleotide probes, oligonucleotide medicines, and cyclic dinucleotides [20–30].

A typical reaction sequence in the solid-phase synthesis of a DNA oligomer via the phosphoramidite method is illustrated in Fig. 2 [3, 12–15]. First, the 5′-*O*-(4,4′-dimethoxytrityl) (DMTr) protecting group of a nucleoside is removed by an organic acid, such as dichloroacetic acid or trichloroacetic acid, to afford the nucleoside with a free 5′-hydroxy group (detritylation). Then, the product is reacted with a suitably protected deoxyribonucleoside phosphoramidite with the aid of a coupling agent (coupling). The 5′-hydroxy group that undergoes no reaction is capped with an acetyl group using acetic anhydride and pyridine (capping). The resulting phosphite triester is converted into the corresponding phosphate using a suitable oxidizing agent, such as iodine/H_2O/pyridine or *tert*-butyl hydroperoxide (TBHP)

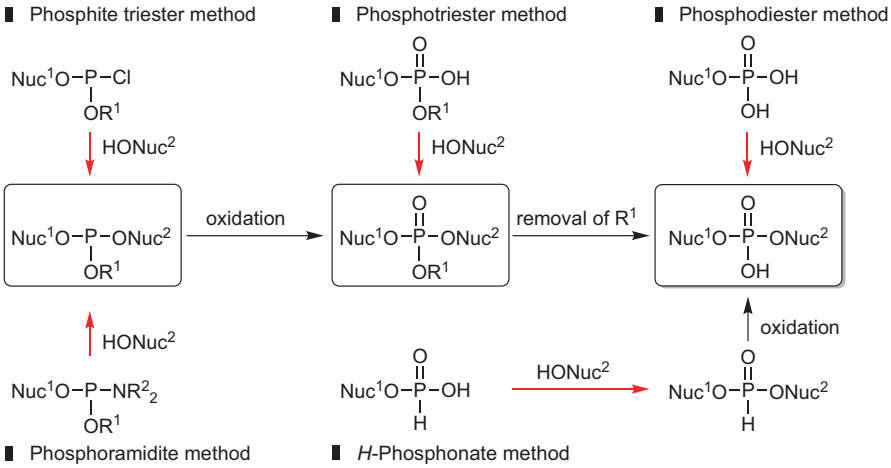


Fig. 1 Outline of representative methods of internucleotide bond formation, where the phosphate esters are shown in the acid form for simplicity

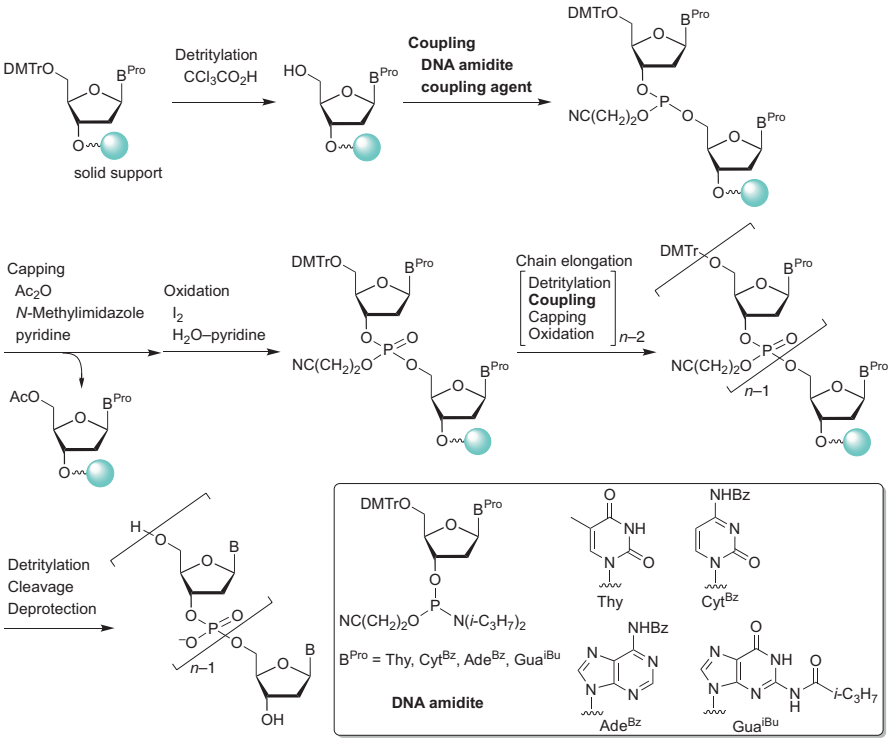


Fig. 2 Reaction sequence of solid-phase synthesis of oligodeoxyribonucleotides by the phosphoramidite method

[31] (oxidation). This four-step procedure, namely, detritylation, coupling, capping, and oxidation, is repeated until the nucleotide reaches the desired length (chain elongation). Finally, the fully protected oligonucleotide on the solid support is subsequently treated with an acid, which removes the 5′-*O*-DMTr protecting group, and with aqueous ammonia, which simultaneously detaches the product from the solid support and removes all the protecting groups to afford the target deprotected oligonucleotide.

The synthesis of oligoribonucleotides (RNA oligomers) is achieved in a similar manner. In the synthesis of ribonucleotides, protection of the 2′-hydroxy group is necessary. A 2′-*O*-*tert*-butyldimethylsilyl (TBDMS) group is most frequently employed for protection, as shown in Fig. 3 [3, 32, 33].

As mentioned above, the most important step in this pathway is the construction of the internucleotide linkage. In the phosphoramidite method, this is achieved on the basis of condensation of a nucleoside with a free 5′-hydroxy group and a nucleoside phosphoramidite assisted by an activator of the phosphoramidite, in other words, a coupling agent or promoter. Therefore, the invention of coupling agents with high efficiency is very important and a lot of effort has been devoted to research in this area so far. As a result, a number of coupling agents have been developed. Among these, this review selects several reagents with high utility and potential and

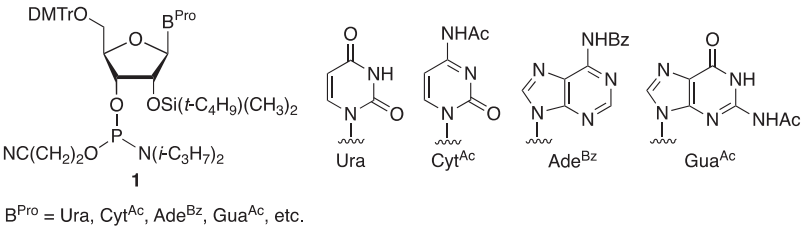


Fig. 3 Representative phosphoramidite building blocks for the synthesis of RNA derivatives

discusses the merits and demerits of these reagents, not merely in the solution-phase synthesis but also in the solid-phase synthesis of DNA and RNA oligomers [14, 32–38]. The selected coupling agents are 1*H*-tetrazole and its derivatives [5-ethylthio-1*H*-tetrazole (ETT), 5-benzylthio-1*H*-tetrazole (BTT), and 5-[3,5-bis (trifluoromethyl)phenyl]-1*H*-tetrazole (Activator 42)], 4,5-dicyanoimidazole (DCI), some carboxylic acids, and various acid/azole complexes [benzimidazolium triflate (BIT) and related compounds, and saccharin 1-methylimidazole (SMI)]. For some coupling agents, it is also discussed how the reagent activates the phosphoramidite and promotes the reaction.

Although there are two different types of phosphoramidite method, i.e., the method using nucleoside phosphoramidites with protected nucleobases (the *N*-protected method) and the method using nucleoside phosphoramidites with unprotected nucleobases (the *N*-unprotected method), this section only deals with examples of the *N*-protected method, because the *N*-unprotected method [39, 40] is reviewed in detail in the previous section.

2 Coupling Agents in the Phosphoramidite Method

2.1 1H-Tetrazole and Its Derivatives

2.1.1 1*H*-Tetrazole

1*H*-Tetrazole (TetH) [41] was the first phosphoramidite coupling agent to be invented. The historical background of this promoter has been described in several articles [12–15]. TetH displays high reactivity toward the phosphoramidites of all kinds of 2′-deoxyribonucleosides and generally accomplishes the condensation in a short period and in high yield. For example, a 1 μmol scale reaction of a deoxyribonucleoside *N*,*N*-diisopropylphosphoramidite and a deoxyribonucleoside with a free 5′-hydroxy group on a solid support is usually completed in 30 s (Fig. 2) to give the coupling product in >99% yield [42]. The reaction time varies depending on the scale of the reaction. On a larger scale, a longer reaction time is required for completion. For example, a reaction time of 5 min is necessary for the reaction on a 160 μmol scale [43]. The method using TetH as the promoter has been applied to the production of DNA microarrays [15, 44, 45].

TetH

Because TetH is a rather strong acid, with a pK_a value comparable to that of acetic acid, TetH causes decomposition of the 5′-*O*-DMTr protecting group to a substantial extent. If this decomposition takes place during chain elongation of an oligonucleotide, it causes formation of oligomer by-products [(n+1)-mer, (n+2)-mer, etc.] that are longer than the desired length of the oligonucleotide (n-mer). This unwanted detritylation is suppressed by the addition of a basic compound, such as *N*-methylimidazole (NMI). The use of a mixed solution of 0.1 M NMI and 0.45 M TetH in acetonitrile is effective in decreasing the extent of unwanted detritylation and enables synthesis of 51 mer DNA on a 0.35 mmol scale in an average coupling yield of 98.3%, resulting in an overall yield of 41% [46].

The addition of NMI to TetH not only decreases the extent of unwanted detritylation but also accelerates the coupling reaction. The latter role of NMI is described in Sect. 2.4.

In comparison with the phosphoramidites of deoxyribonucleosides, those of ribonucleosides are generally less reactive, in particular in cases in which the protecting group on the 2′-hydroxy group is bulky, such as TBDMS (Fig. 3). The reactivity of TetH is not quite high enough to activate the less reactive ribonucleoside phosphoramidites. Thus, when TetH is used as the promoter in the coupling reaction using 2′-*O*-TBDMS-protected ribonucleoside phosphoramidites, the reaction sometimes requires rather a long time for completion. For example, the reaction of **1** (Fig. 3, B^{Pro} = Ura, Cyt^{Bz}, Ade^{Bz}, Gua^{iBu}) with the aid of TetH requires 12 min for completion, being achieved in 97–99% yield [47]; in this coupling reaction, prolongation of the reaction time does not improve the yield of the desired product but increases the formation of by-products [48]. Therefore, the more reactive TetH derivatives shown below have been invented and employed for the synthesis of RNA oligomers using ribonucleoside phosphoramidites as building blocks.

In order to invent more reactive promoters, it is helpful to know the mechanism of TetH-assisted condensation of nucleoside phosphoramidite and nucleoside. Therefore, here is a brief description of the mechanism that was proposed on the basis of kinetic and NMR studies of the condensation [49, 50]. As shown in Fig. 4, TetH initially acts as an acid that protonates the phosphoramidite **A** to form the ammonium species **B**. At the same time, the tetrazolide anion, Tet^-, is formed. Subsequently, the resulting species **B** undergoes nucleophilic attack by Tet^- to give the phosphorotetrazolide **C**. Finally, nucleophilic substitution occurs between **C** and the nucleoside (Nuc^2OH) to afford the coupling product, i.e., the phosphite triester **D**. In this process, the rate-determining step (RDS) is the reaction between **B** and Tet^- to form **C**. The formation of the intermediate **C** was actually confirmed by a ^{31}P NMR study that monitored the reaction using 5′-*O*-DMTr-thymidine, 3′-[(methyl)-(*N*,*N*-dialkyl)]-phosphoramidites [49–52]. Thus, the elucidated mechanism suggests that a compound that has appropriate acidity and has a conjugate base with high nucleophilicity will act as an effective promoter. On the basis of this

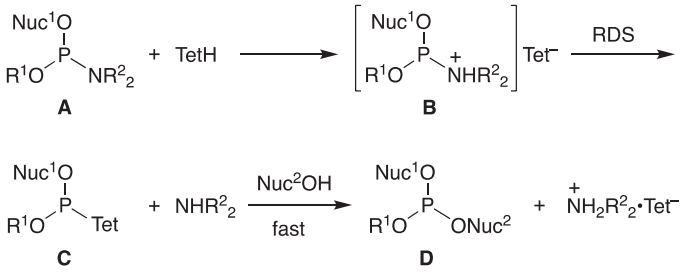
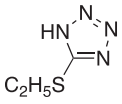


Fig. 4 Proposed mechanism of coupling reaction of a nucleoside phosphoramidite and a nucleoside promoted by TetH

consideration, several promoters have been developed that have higher reactivities than TetH. The compounds described in the following subsections are representative promoters with higher reactivities than TetH.

2.1.2 5-Ethylthio-1*H*-tetrazole

5-Ethylthio-1*H*-tetrazole (ETT), which is prepared from ethyl thiocyanate and sodium azide [53, 54], is a coupling agent with higher reactivity than TetH. This compound is now commercially available.



ETT

ETT accomplishes the coupling reaction with an average yield of 96.2–98.1% in the synthesis of DNA oligomers, such as 18 mers to 34 mers, on a TentaGel support on scales of 25 μmol–1 mmol [55]. The coupling yield is 1–2% higher than that in the case where TetH is used as the promoter.

The efficiency of ETT is also observed to be higher than that of TetH in the synthesis of RNA oligomers employing 2′-*O*-TBDMS-ribonucleoside phosphoramidites [Fig. 3, B^{Pro} = Ura, Cyt^{Ac}, Ade^{Pac}, Gua^{iPrPac} (Pac = phenoxyacetyl; iPrPac = 4-isopropylphenoxyacetyl)]. For example, in the synthesis of an oligoribonucleotide 36 mer on a 0.2 μmol scale, when ETT is used as a 0.25 M solution in acetonitrile, each coupling reaction in the chain elongation process is accomplished in 465 s (ca. 7.8 min) in an average coupling yield of 97.5% to provide the target oligonucleotide in an overall isolated yield of 45% [33]. On the other hand, when a 0.45 M solution of TetH in acetonitrile is used as the promoter for the coupling reaction, which is carried out for the same period as above, the synthesis gives the target product in an isolated yield of only 34%. The synthesis of RNA oligomers shorter than 36 mers on scales of 2.5–25 μmol with ETT as the promoter is also reported and, in this case, the coupling reaction is performed with an average yield of 97.5–99% [48].