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Oligonucleotide Synthesis Involving Deprotection of Amidine-Type Protecting Groups for Nucleobases under Acidic Conditions

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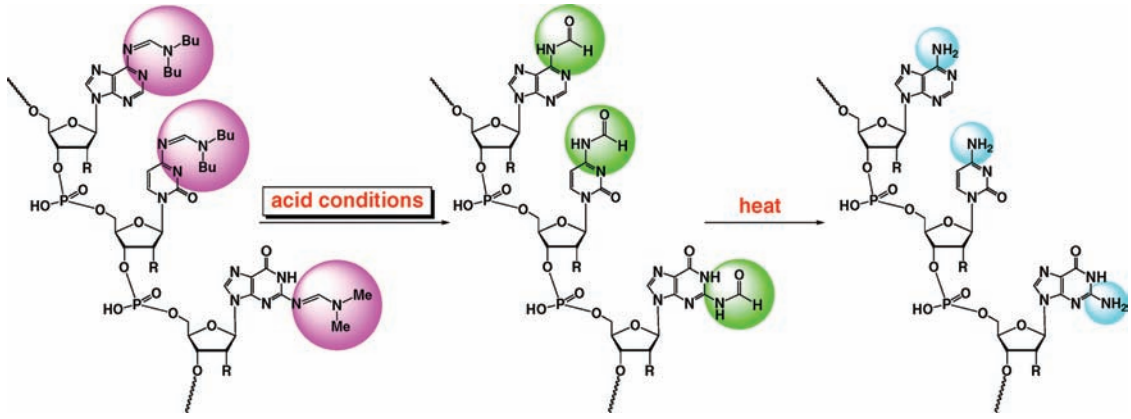
ABSTRACT



Amidine-type protecting groups, i.e., *N,N*-dimethylformamidine (dmf) and *N,N*-dibutylformamidine (dbf) groups, introduced into nucleobases were rapidly removed under mild acidic conditions using imidazolium triflate (IMT) or 1-hydroxybenztriazole (HOBt). This new deprotection strategy allowed a 2′-*O*-methyl-RNA derivative bearing a base-labile group to be efficiently synthesized using a silyl-type linker. It was also found that our new method could be applied to the synthesis of an unmodified RNA oligomer.

Chemical modification plays an important role in improving the properties of natural DNA and RNA molecules, including hybridization, base recognition, and nuclease resistance.[1] In particular, several 2′-modified RNAs, such as 2′-O-alkylated and 2′-fluoro-RNAs, have been applied to gene regulation in antisense, antigene, and RNAi strategies.[2] Further studies for the introduction of additional functional groups into these 2′-modified RNAs are necessary to increase their therapeutic effect in gene therapy. For example, the introduction of acyl-type functional groups into nucleobases has proved to increase hybridization and base-recognition abilities.[3] In contrast, oligonucleotides with acyloxymethyl and acylthioethyl groups as hydroxyl and phosphate group modifiers,

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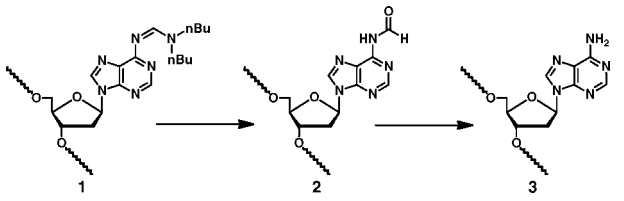


Figure 1. Deprotection of *N*,*N*-dibutylformamidine (dbf) from deoxyadenine (dA) via *N*-formyl-dA formation.

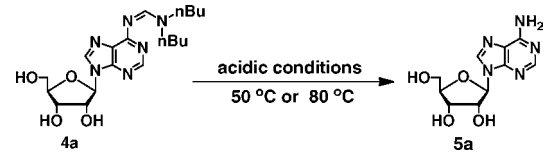
respectively, are expected to be useful as prodrugs.[4] However, the synthesis of 2′-substituted RNAs containing these functional groups through standard RNA synthesis procedures is problematic because of their lack of stability under basic conditions.

In previous studies of oligonucleotide synthesis, various protecting groups have been reported for nucleobase amino groups.[5] Among them, acyl groups, such as acetyl, benzoyl, and isobutyryl, have been commonly used in the phosphoramidite approach to avoid side reactions associated with the nucleobase amino groups. These acyl groups can be removed by treatment with aqueous ammonia but are incompatible with the synthesis of oligonucleotides bearing base-labile functional groups.

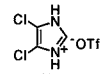
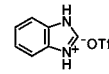
Recently, we developed a new method called "activated phosphite method without base protection" for the synthesis of base-labile modified oligomers.[6] In this method, the *O*-selectivity of the internucleotidic bond formation in DNA synthesis reached more than 99% using 6-nitroHOBt as an activator of *N*-unprotected deoxynucleoside 3′-phosphoramidite units. This high selectivity results from the low reactivity of phosphite triester intermediates toward the exocyclic amino groups of the adenine and cytosine base moieties compared to the 5′-hydroxyl group. However, the RNA synthesis required an additional P(III)−N bond cleavage step after each condensation reaction because of the low *O*-selectivity (93−95%) of coupling steps involving RNA monomer building blocks.[7] In addition, the purification of desired oligomers from shorter oligomers missing one and two nucleotide residues was difficult because the capping step using acetic anhydride was omitted to avoid undesired *N*-acetylations.

In this study, we synthesized an 2′-OMe-RNA oligomer containing an *N*-benzoyladenosine derivative as a representative base-labile model compound. To achieve this synthesis, our interest was focused on the unique property of the formyl group attached to the base amino groups. In a previous study

Table 1. Deprotection of dbf Groups Incorporated in rA under Acidic Conditions



4a 5a

entry	conditions			time (h)[a]	
	reagent (5 equiv)	solvent	pH	50 °C	80 °C
1	——	80% AcOH aq	——	10	3
2	——	10 mM sodium citrate buffer	2.0	9	2
3		MeOH-H_2O (9:1, v/v)	0.8	6.5	3
4		MeOH-H_2O (9:1, v/v)	1.4	6	2.5
5		MeOH-H_2O (9:1, v/v)	2.1	4.5	1.5
6		MeOH-H_2O (9:1, v/v)	2.5	3	0.5
7		MeOH-H_2O (9:1, v/v)	2.7	1.8	0.7
8		MeOH-H_2O (9:1, v/v)	2.9	1	0.3
9		MeOH-H_2O (9:1, v/v)	5.3	4	1

[a] Estimated by TLC analysis.

(unpublished data), a modified DNA oligomer **2** having a 6-*N*-formyl-dA was rapidly converted to the unmodified oligomer **3** by simple heating under neutral conditions, as shown in Figure 1. In contrast, the formyl group was reported to form from the *N*,*N*-dimethylformamidine group under acidic conditions.[8] *N*,*N*-Dialkylformamidine groups have been usually used as base protecting groups that are removed by aqueous ammonia treatment.

In this paper, we propose a new method for RNA synthesis involving the deprotection of amidine-type groups under acidic conditions.

First, the deprotection of the *N*,*N*-dibutylformamidine (dbf) group was examined using 6-*N*-(*N*,*N*-dibutylformamidine)adenosine **4a** (Table 1) and its deoxy counterpart **4b** (Table S1, Supporting Information) under various acidic conditions. This dbf group is well-known as an adenine and cytosine protecting group in DNA and RNA synthesis.[9] When **4a** was treated with 80% AcOH and heated in a 10 mM sodium citrate buffer at 50 °C for 9−10 h, it was completely converted to **5a** without decomposition (entries 1 and 2 in Table 1). Subsequently, deprotection of the dbf group by

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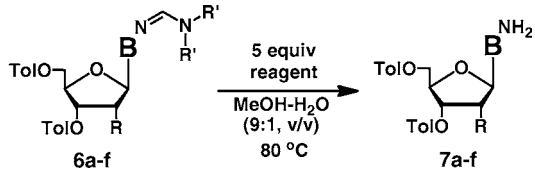
Table 2. Deprotection of dbf and dmf Groups in *O*-Toluoylnucleosides Using HOBt or IMT



a; B = Ad, R = OTol, R' = Bu
b; B = Ad, R = H, R' = Bu
c; B = Cy, R = OTol, R' = Bu
d; B = Cy, R = H, R' = Bu
e; B = Gu, R = OTol, R' = Me
f; B = Gu, R = H, R' = Me
Tol = toluoyl

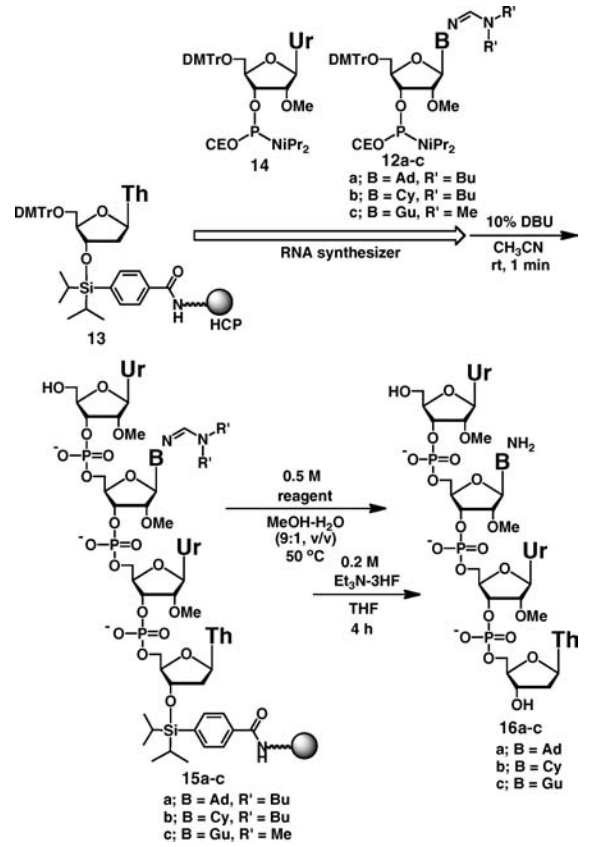
a; B = Ad, R = OTol
b; B = Ad, R = H
c; B = Cy, R = OTol
d; B = Cy, R = H
e; B = Gu, R = OTol
f; B = Gu, R = H

entry	starting compd	B	reagent	time (h)	yield (%)
1	**6a**	Ad	HOBt	0.5	98
2	**6a**	Ad	IMT	1	87
3	**6b**	Ad	HOBt	0.2	92
4	**6b**	Ad	IMT	1.5	quant
5	**6c**	Cy	HOBt	0.5	quant
6	**6c**	Cy	IMT	1.5	81
7	**6d**	Cy	HOBt	0.2	90
8	**6d**	Cy	IMT	1.5	quant
9	**6e**	Gu	HOBt	1	87
10	**6e**	Gu	IMT	22	68
11	**6f**	Gu	HOBt	1.3	68
12	**6f**	Gu	IMT	24	70

treatment with acid−azole or acid−pyridine complexes was examined at 50 °C (entries 3−6 and 9). Interestingly, the deprotection rate using benzimidazolium triflate (BIT, entry 6) was higher than with 4,5-dichloroimidazolium triflate (dcIMT, entry 3), 1,2,4-trizazolium triflate (TRT, entry 4), and pyridinium chloride (PyHCl, entry 5), despite a higher pH value for BIT compared to dcIMT, TRT, and PyHCl. These results suggest that the important factors were not only the acidity of reagents and solvents used to hydrolyze the amidine group by conversion of the dbf group into a formyl group but also the nucleophilicity of the reagents used to deprotect the formyl group. Surprisingly, the deprotection rate under mild acidic conditions using imidazolium triflate (IMT, pH 5.3, entry 9) was also higher than that using PyHCl. This two-step deprotection strategy might be useful for nucleoside and nucleotide chemistry because the depurination and decomposition of various functional groups might be suppressed under these mild acidic conditions. On the other hand, deprotections using 1*H*-tetrazole and 1-hydroxybenztriazole (HOBt) were found to be faster than with the acidic−azole complexes (entries 6 and 9). In particular, the dbf group was removed in only 1 h by treatment with HOBt.

Deprotection results obtained for **4b** (entries 1 and 2 in Table S1, Supporting Information) using BIT, HOBt, and IMT were similar to those of **4a**. However, depurination was observed under acidic conditions using PyHCl (entry 1 in Table S1, Supporting Information). Moreover, an increase in reaction temperature from 50 to 80 °C was found to significantly accelerate the deprotection in all cases.

Table 3. Deprotection of dbf and dmf Groups Incorporated into Nucleotides Using HOBt or IMT on HCP Resins



entry	B	R′	reagent	time[a] (h)
1	Ad	Bu	HOBt	12
2	Ad	Bu	IMT	4.5
3	Cy	Bu	HOBt	3.5
4	Cy	Bu	IMT	1
5	Gu	Me	HOBt	3.5
6	Gu	Me	IMT	24

[a] Estimated by RP-HPLC analysis.

Next, the deprotection of dbf and *N*,*N*-dimethylformamidine (dmf) groups from *O*-toluoyl (Tol) nucleoside derivatives **6a**−**f** using HOBt and IMT under acidic conditions was investigated. These results are summarized in Table 2. The formamidine group was rapidly deprotected without decomposition of the ester groups and depurinaton by treatment with HOBt for all nucleobases (entries 1, 3, 5, 7, 9, and 11). The dbf group introduced into adenine and cytosine bases was removed within 1.5 h by treatment with IMT, although reactions using IMT were slower than those using HOBt (entries 2, 4, 6, and 8). However, the removal of the dmf group from guanine was very slow. Reaction times were 22 and 24 h for compounds **6e** and **6f**, respectively (entries 10 and 12). The deprotection time might be extended because 2-*N*-formylguanine is stable under conditions using IMT.

Subsequently, the stability of silyl-type protecting groups under acidic conditions using IMT was examined (Scheme S1, Supporting Information). The dbf group was removed

from 2′,3′,5′-*O*-tris(*tert*-butyldimethylsilyl)adenosine (tri-TBDMS-A) derivative **8** by treatment with IMT without decomposition of the silyl-type linker. The isolated yield of the desired product **9** was 81%. These results indicate that the acyl- and silyl-type protecting groups are stable under conditions for removal of formamidine-type protecting groups from nucleobases.

In addition, we investigated the deprotection of dbf and dmf groups incorporated into polymer-supported oligonucleotides. Phosphoramidite units **12a**−**c** were easily synthesized by reacting *N*,*N*-dialkylformamide dimethyl acetal (3 equiv) with *N*-unprotected phosphoramidite units **11a**−**c**,[10] which were derived from *N*-acylated phosphoramidite units **10a**−**c** (Scheme S2, Supporting Information). After chain elongation using phosphoramidite derivatives **12a**−**c** and **14**, the removal of 2-cyanoethyl groups on T-loaded highly cross-linked polystyrene (HCP) resins having a silyl-type linker and the deprotection of dbf and dmf groups using HOBt and IMT were successively carried out (Table 3). The dbf or dmf group was removed from the adenine base over prolonged periods of time at 50 °C (12 h, entry 1) but was deprotected in only 3.5 h from cytosine and guanine bases using HOBt (entries 3 and 5). The slow deprotection of the dbf group from the adenine base results from the slow hydrolysis of the amidine group into a formyl group. The dbf group on the adenine and cytosine bases was deprotected within 4.5 h using IMT (entries 2 and 4). On the other hand, the deprotection of the dmf group on the guanine base required a prolonged time (24 h, entry 6) similar to the deprotection of guanosine monomers **6e** and **6f**.

Furthermore, we synthesized a 2′-OMe-RNA-DNA chimeric oligomer (**17**), which is a 21-base siRNA analogue of Maitogen−protein kinase 14[11] and a base-labile 2′-OMe-RNA oligomer (**18**) having a 6-*N*-benzoyladenine base (A^{bz}) by deprotection of amidine-type groups under acidic conditions. In these syntheses, IMT was chosen because of its milder acidity compared to HOBt. Oligomer chain elongation was performed on HCP resins having a silyl-type linker[12] according to the general procedure involving the capping step using Ac_2O. When the resin-bound 21-mer oligomer protected with dbf and dmf groups was treated with IMT for 24 h after removal of the 2-cyanoethyl groups, ca. 70% of product **17** was unexpectedly found to be released from the resin because of Si−O bond cleavage. A solution containing the released oligomer was therefore mixed with a solution obtained by treating the residual resin with 0.2 M Et_3N−3HF in THF for 4 h. HPLC analysis of the resulting mixture showed that compound **17** was obtained as the main product and was isolated in 18% yield (Figure 2a). Figure 2b shows the anion exchange HPLC profile of the crude mixture obtained for the synthesis of 2′-OMe-RNA oligomer **18**. The desired oligonucleotide was obtained as the main product, although several peaks of byproducts containing the oligomer containing an acyl group were observed in HPLC and MALDI-TOF mass spectroscopy (Supporting Information). Oligomer **18** was isolated in 19% yield and was characterized by MALDI-TOF mass spectroscopy.

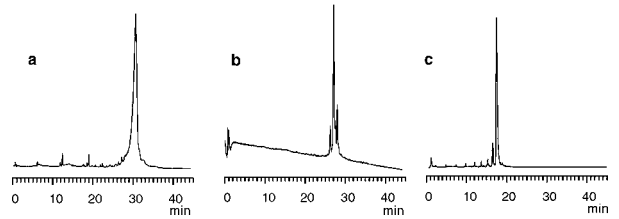


Figure 2. Anion-exchange HPLC profiles of crude mixtures obtained by RNA−DNA chimeric and RNA oligomer syntheses: (a) 2′-OMe-[CCUACAGAGAACUGCGGUU]-TT (**17**), (b) 2′-OMe-[GAUACA^{bz}UUGACCU] (**18**), and (c) r[CUCUCUCUCU]-T (**19**).

Finally, this method was applied to the synthesis of an unmodified RNA oligomer (**19**). The triisopropylsilyloxymethyl (TOM) group was chosen to protect the 2′-hydroxyl group, since the TBDMS group was unstable even under weak acidic conditions because of the neighboring 3′-phosphate group.[13] Figure 2c shows the anion-exchange HPLC profile of the crude mixture obtained from the synthesis of RNA oligomer **19** using 2′-TOM-C^{dbf} phosporamidite **20**. The desired oligonucleotide was obtained as the main product and was isolated in 19% yield. No 3′→2′ isomerization of the internucleotidic phosphodiester linkages of oligomer **19** was confirmed by its complete digestion with nuclease P1.[14] These results demonstrate that our new method can be applied to the synthesis of unmodified RNA oligomers.

In summary, we developed a new method for the deprotection of dbf and dmf groups on nucleosides and nucleotides under acidic conditions using HOBt and IMT. Moreover, our new strategy allowed the first synthesis of an RNA oligomer carrying a base-labile acyl group on adenine. These results have prompted us to study the introduction of various base-labile functional groups into 2′-substituted and unmodified RNA. These additional studies are now underway.

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Supporting Information Available: Experimental procedures and full spectroscopic data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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