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Assessment of 4-Nitrogenated Benzyloxymethyl Groups for 2′-Hydroxyl Protection in Solid-Phase RNA Synthesis

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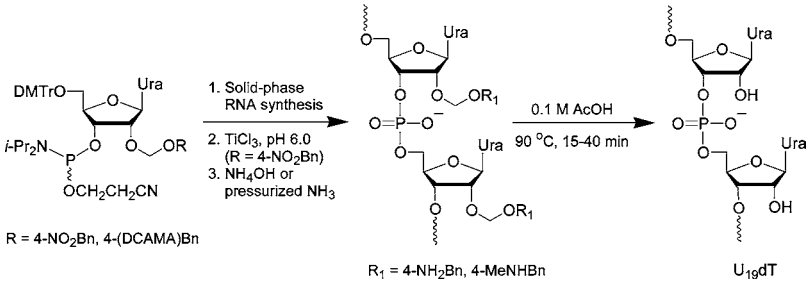
ABSTRACT



The search for a 2′-OH protecting group that would impart ribonucleoside phosphoramidites with coupling kinetics and coupling efficiencies comparable to those of deoxyribonucleoside phosphoramidites led to an assessment of 2′-*O*-(4-nitrogenated benzyloxy)methyl groups through solid-phase RNA synthesis using phosphoramidites 2a–d, 12a, and 14a. These phosphoramidites exhibited rapid and efficient coupling properties. Particularly noteworthy is the cleavage of the 2′-*O*-[4-(*N*-methylamino)benzyloxy]methyl groups in 0.1 M AcOH, which led to U_{19}dT within 15 min at 90 °C.

With the advent of RNA interference as a means to silence gene expression,[1,2] small interfering RNA (siRNA) oligonucleotides have been recognized as powerful tools for targeting mRNAs and eliciting their demises.[3] As a consequence of this discovery, siRNA oligonucleotides consisting of less than 25 nucleotides are now being intensely investigated as potential therapeutic agents for various biomedical indications.[3,4] Such a scrutiny has spurred a renewed interest in the development of rapid and efficient methods for solid-phase RNA synthesis.

A formidable challenge in the preparation of RNA oligonucleotides is designing a 2′-hydroxyl protecting group that would provide ribonucleoside phosphoramidites with coupling kinetics and coupling efficiencies comparable to those of deoxyribonucleoside phosphoramidites. Furthermore,

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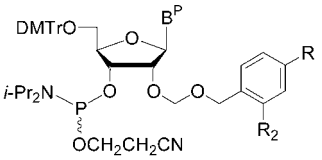
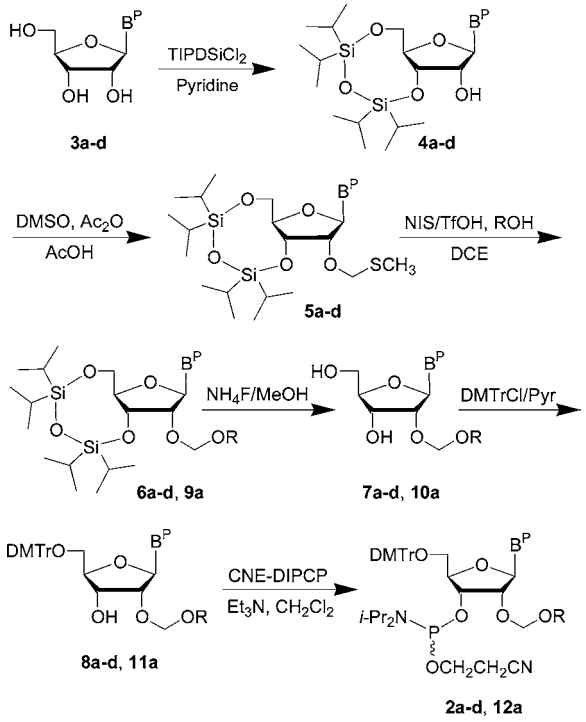
the 2′-OH protecting group must be stable to the reagents and conditions used during solid-phase DNA/RNA synthesis in addition to those required for nucleobase and phosphate deprotection. Last, the 2′-OH protecting group must be cleaved under conditions that will not harm the oligoribonucleotide. Thus, the search for an ideal 2′-OH protecting group in RNA synthesis has been ongoing for decades and has been the subject of several reviews.[5] One notable advance in solid-phase RNA synthesis emerged from the implementation of the 2-nitrobenzyloxymethyl and 4-nitrobenzyloxymethyl (4-NBOM) groups for 2′-hydroxyl protection.[6] Ribonucleoside phosphoramidites functionalized with these 2′-OH protecting groups (**1** and **2**) produced coupling efficiencies exceeding 98% within 2−3 min.[6]



1 R = H, R_2 = NO_2; B^P = U, C^{Bz}, A^{Bz}, G^{iBu}
2 R = NO_2, R_2 = H; B^P = U, C^{Bz}, A^{Bz}, G^{iBu}

Such impressive coupling rates, relative to those of the 2′-*O*-*tert*-butyldimethylsilyl ribonucleoside phosphoramidites (∼10 min), were presumably due to the flexibility of the benzyloxymethyl group, which lessened the steric demand around the activated phosphoramidite entity. These findings were influential given that the 2′-*O*-substituted 1-(benzyloxy)ethyl,[7] 2′-*O*-[1-(2-cyanoethoxy)]ethyl,[8] 2′-*O*-triisopropylsilyloxymethyl (TIPSOM),[9] and 2′-*O*-(2-cyanoethoxy)-methyl (CEM)[10] ribonucleoside phosphoramidites were since reported to share structural homologies with phosphoramidite **2**. More specifically, the 2′-*O*-TIPSOM and 2′-*O*-CEM ribonucleoside phosphoramidites were claimed to exhibit coupling reaction kinetics and coupling efficiencies comparable to those of DNA phosphoramidites.[9,10] These findings prompted us to investigate further the use of 2′-*O*-(4-NBOM) ribonucleoside phosphoramidites for solid-phase RNA synthesis and to develop a different method for the deprotection of 2′-*O*-(4-NBOM) RNA oligonucleotides. We rationalized that, instead of using fluoride ions for cleavage of the 2′-*O*-(4-NBOM) group,[6c] converting its 4-nitro group to the electron-donating 4-amino function would facilitate the cleavage of the 2′-*O*-acetal through formation of an iminoquinone methide[11] intermediate and elimination of formaldehyde. Our investigations began with the synthesis of 2′-*O*-(4-NBOM) uridine (**7a**) and of its phosphoramidite **2a** as depicted in Scheme 1.[12]

Scheme 1. Synthesis of Phosphoramidites **2a**−**d** and **12a**[a]



[a] Keys: **2**, **6**, **7**, **8**, R = 4-nitrobenzyl; **9**, **10**, **11**, **12**, R = 4-(*N*-dichloroacetyl-*N*-methyl)aminobenzyl; B^P, U (a), C^{Bz} (b), A^{Bz} (c), G^{iBu} (d); $TIPDSiCl_2$, 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane; NIS, *N*-iodosuccinimide; TfOH, trifluoromethanesulfonic acid; DCE, 1,2-dichloroethane; DMTrCl, 4,4′-dimethoxytrityl chloride; Pyr, pyridine; CNE-DIPCP, 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite.

Automated solid-phase synthesis of a chimeric polyuridylic acid (U_{19}dT), as a model RNA oligonucleotide, was conducted using commercial long-chain alkylamine controlled-pore glass covalently linked to 5′-*O*-DMTr-dT through a 3′-*O*-succinyl linker. Phosphoramidite **2a** was dissolved in dry MeCN to a concentration of 0.15 M and activated with 0.25 M 5-ethylthio-1*H*-tetrazole in MeCN. The coupling time was set to 3 min. Upon completion of the oligonucleotide chain assembly, the solid support was split into two fractions, one

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(12) Experimental details and literature references are reported in the Supporting Information.

of which was treated with concentrated NH_4OH for 30 min at 25 °C to remove the phosphate protecting groups and release the 2′-*O*-protected RNA oligonucleotide from the support. The other fraction of the support was suspended in 0.1 M $TiCl_3$[13] (pH 6.0) for 1 h at 25 °C to reduce the 2′-*O*-(4-NBOM) groups to the corresponding 2′-*O*-(4-aminobenzyloxy)methyl (4-ABOM) groups. After washing away residual $TiCl_3$, removal of the phosphate protecting groups and release of the 2′-*O*-(4-ABOM) RNA oligonucleotide from the support were effected upon exposure to pressurized NH_3 gas.[12] As shown in Figure 1A, the RP-HPLC profile of

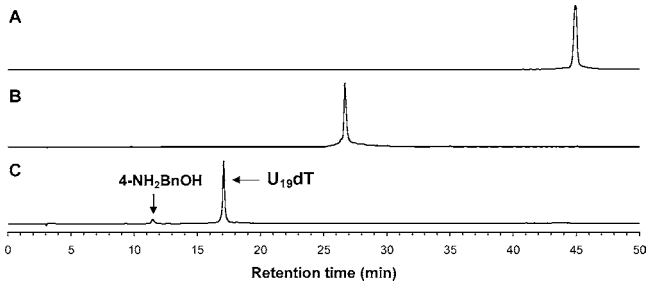

Figure 1. RP-HPLC profiles of unpurified 2′-*O*-protected/-deprotected U_{19}dT. **A**: [2′-*O*-(4-NBOM) U]$_{19}$dT. **B**: [2′-*O*-(4-ABOM) U]$_{19}$dT obtained from the $TiCl_3$-mediated reduction of [2′-*O*-(4-NBOM) U]$_{19}$dT at pH 6.0. **C**: U_{19}dT obtained from the thermolytic deprotection of [2′-*O*-(4-ABOM) U]$_{19}$dT in 0.1 M AcOH at 90 °C. Conditions: see Supporting Information.

the unpurified RNA oligonucleotide protected with 2′-*O*-(4-NBOM) groups is reflective of the coupling efficiency of phosphoramidite **2a**, which averaged 99%. Reduction of the 2′-*O*-(4-NBOM) group to its 2′-*O*-(4-ABOM) derivative was efficient and clean, as illustrated in Figure 1B. The 2′-*O*-(4-ABOM) group is stable under both neutral (0.1 M triethylammonium acetate buffer, pH 7.0, 90 °C, 3 h) and basic (concentrated NH_4OH, 55 °C, 16 h) conditions. Thus, the 2′-*O*-(4-ABOM) group and its homologues (vide infra) are ideal for protecting RNA oligonucleotides against ubiquitous ribonucleases under normal handling and storage conditions. Complete removal of the 2′-*O*-(4-ABOM) group is however achieved within 40 min upon heating the oligonucleotide at 90 °C in 0.1 M AcOH. The RP-HPLC profile of U_{19}dT (Figure 1C) did not reveal substantial chain cleavage.[14]

As anticipated, a peak (t_R = 11.5 min) corresponding to 4-aminobenzyl alcohol was detected. This aminoalcohol was produced from the cleavage of the 2′-*O*-(4-ABOM) group, presumably through formation of an iminoquinone methide intermediate followed by immediate hydration.

Unpurified U_{19}dT was completely digested to uridine and thymidine upon incubation with snake venom phosphodiesterase and bacterial alkaline phosphatase. RP-HPLC analysis of the digest did not indicate any nucleobase modifications. Encouraged by these results, the 2′-*O*-(4-NBOM) phosphoramidite derivatives **2b**−**d** were prepared as described in Scheme 1.[12] These phosphoramidites were employed in the solid-phase synthesis of an oligoribonucleotide (20-mer) and were comparable to phosphoramidite **2a** in terms of coupling kinetics and coupling efficiencies. However, the $TiCl_3$-mediated reduction of the 2′-*O*-(4-NBOM)-protected 20-mer was not as efficient as that achieved with [2′-*O*-(4-NBOM)U]$_{19}$dT and resulted in a product of inferior quality.[15] Such an apparent deficiency in the reductive capacity of $TiCl_3$ appears related to the presence of nucleobases other than uracil and thus precluded its routine use in solid-phase RNA synthesis.

The search for an analogue of the 2′-*O*-(4-ABOM) group that would permit solid-phase RNA synthesis and produce an oligonucleotide homologous to [2′-*O*-(4-ABOM) U]$_{19}$dT, when using standard reagents and conditions, was initiated. The 4-(*N*-methylaminobenzyloxy)methyl (4-MABOM) group was identified as a close homologue of the 2′-*O*-(4-ABOM) group. Much like nucleobases, the 4-MABOM group must be *N*-protected during solid-phase oligonucleotide synthesis but should revert to its native state under the conditions employed for oligonucleotide deprotection. The dichloroacetyl group[16] was found optimal for *N*-protection of the 2′-*O*-(4-MABOM) acetal. Thus, 4-*N*-methylaminobenzyl alcohol was *O*-silylated upon reaction with Me_3SiCl and then *N*-acylated with dichloroacetic anhydride to give 4-(*N*-dichloroacetyl-*N*-methylamino)benzyl alcohol after hydrolytic workup.[12] The use of this alcohol in the preparation of **9a** from **5a** and conversion of **9a** to phosphoramidite **12a** were accomplished as outlined in Scheme 1.[12] Phosphoramidite **12a** was employed in the solid-phase synthesis of U_{19}dT under conditions identical to those described when using **2a**. The 5′-*O*-dedimethoxytritylated solid-phase linked oligoribonucleotide was exposed to concentrated NH_4OH for 5 h at 55 °C to: (i) cleave the *N*-dichloroacetyl groups from the 2′-*O*-(4-MABOM) acetals; (ii) remove the phosphate protecting groups; and (iii) release the 2′-*O*-(4-MABOM)-protected RNA oligonucleotide from the support. RP-HPLC analysis of the RNA oligonucleotide (Figure 2A) indicates that the phosphoramidite **12a** is as efficient as **2a** in solid-

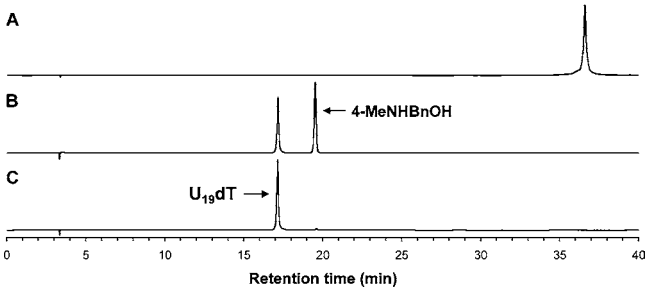

Figure 2. RP-HPLC profiles of unpurified 2′-*O*-protected/-deprotected U_{19}dT. **A**: [2′-*O*-(4-MABOM) U]$_{19}$dT. **B**: [2′-*O*-(4-MABOM) U]$_{19}$dT in 0.1 M AcOH, 90 °C, 15 min. **C**: Material of profile B after multiple Et_2O extractions. Conditions: see Supporting Information.

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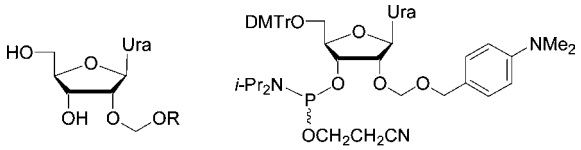
phase RNA synthesis in terms of purity. Although the 2′-*O*-(4-MABOM) group is as stable as the 2′-*O*-(4-ABOM) group under neutral and basic conditions, its complete cleavage under acidic conditions is faster than that of the 2′-*O*-(4-ABOM) group by a factor of ∼3. The RP-HPLC profile of U_{19}dT shown in Figure 2B,C is comparable to that of Figure 1C. An RP-HPLC peak corresponding to 4-(*N*-methylamino)benzyl alcohol was detected, as expected, from the cleavage of the 2′-*O*-(4-MABOM) group. Unpurified U_{19}dT was completely digested by snake venom phosphodiesterase and bacterial alkaline phosphatase to uridine and thymidine without apparent nucleobase modifications as judged by RP-HPLC analysis of the digest.[12] This approach to solid-phase RNA synthesis is attractive given its similarity to solid-phase DNA synthesis in regard to the nucleobase and phosphate protecting groups being used and also in regard to the coupling rate and coupling efficiency of **12a**, which are comparable to those of deoxyribonucleoside phosphoramidites. Moreover, the 2′-*O*-(4-MABOM) group is deprotected under mild acidic conditions similar to those reported by others[17] in the production of commercial RNA oligonucleotides.

To further assess 4-nitrogenated benzyloxymethyl groups for 2′-OH protection in solid-phase RNA synthesis, replacement of the 4-*N*-dichloroacetyl group in phosphoramidite **12a** with a methyl group was considered. Such a modification should functionally simplify the 2′-*O*-acetal protection and accelerate its subsequent cleavage considering the strong electron-donating ability of the 4-dimethylamino group. To evaluate this rationale, **7a** was converted to 2′-*O*-ABOM uridine upon treatment with 0.1 M $TiCl_3$ (pH 6.0) and was then reacted with formaldehyde in the presence of $NaBH_3CN$ and $ZnCl_2$ in MeOH[18] to give **13a**. 5′-*O*-Dimethoxytritylation and 3′-*O*-phosphinylation of **13a** were achieved as described for the preparation of **2a** and **12a** affording phosphoramidite **14a** in similar yields.[12] The solid-phase synthesis of dinucleotide UdT was carried out employing **14a** with the intent of evaluating the deprotection kinetics of the 2′-*O*-(4-dimethylamino)benzyloxymethyl (4-DABOM) group. Upon release of the dinucleotide from the support and subsequent treatment with 0.1 M AcOH at 90 °C, cleavage of the 2′-*O*-(4-DABOM) group occurred, unexpectedly, at a rate slower than that of the 2′-*O*-(4-MABOM) group (15 min) but comparable to that of the 2′-*O*-(4-ABOM) group (40 min). While attempts at improving the deprotection kinetics of the 2′-*O*-(4-DABOM) group are underway, our assessment of the 4-nitrogenated benzyloxymethyl groups investigated so far favors the use of phosphoramidites functionalized with the 4-(*N*-dichloroacetyl-*N*-methylamino)-benzyloxymethyl group in solid-phase RNA synthesis. Given that the coupling rate and coupling efficiency of **12a** are similar to those of **2a** or **14a**, it is anticipated that RNA oligonucleotides prepared via **12a**−**d** will be deprotected under conditions identical to those used for DNA oligonucleotides with the exception of the 2′-*O*-(4-MABOM) groups, which will be removed rapidly under acidic conditions essentially as described in the literature.[17] An optimized



13a **14a**

R = 4-dimethylaminobenzyl

method for the solid-phase synthesis of RNA oligonucleotides through the use of phosphoramidites **12a**−**d** is currently being developed. The details of this optimized method will be reported in due course.

Supporting Information Available: Details on the synthesis and characterization of the compounds prepared according to Scheme 1; ^{1}H and ^{13}C NMR spectra of **7a**−**d**, **10a**, and **13a**; ^{31}P NMR spectra of **2a**−**d**, **12a**, and **14a**; expanded Figures 1 and 2; RP-HPLC profiles of the oligonucleotides that were prepared using **2a**−**d**; RP-HPLC profile of the enzymatic digest of U_{19}dT that was prepared from **12a**; PAGE analysis of a commercial oligoribonucleotide that was heated in 0.1 M AcOH for up to 40 min at 90 °C. This material is available free of charge via the Internet at http://pubs.acs.org.

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(14) Heating commercial AUCCGUAGCUAAGGUCAUCGU for up to 40 min in 0.1 M AcOH at 90 °C did not result in significant chain cleavage as estimated by polyacrylamide gel electrophoresis analysis. Data shown in the Supporting Information.

(15) See Chart 1B,C of the Supporting Information.

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