

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

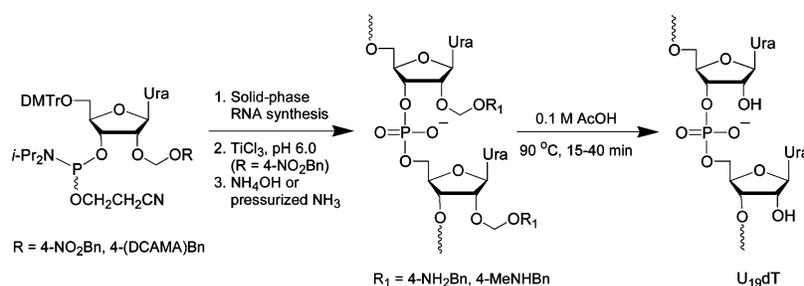
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## ABSTRACT



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<sub>19</sub>dT within 15 min at 90 °C.

With the advent of RNA interference as a means to silence gene expression,<sup>1,2</sup> small interfering RNA (siRNA) oligonucleotides have been recognized as powerful tools for targeting mRNAs and eliciting their demises.<sup>3</sup> 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.<sup>3,4</sup> 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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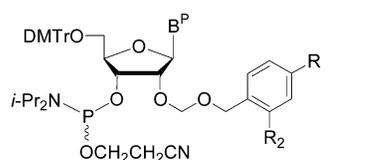
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the 2'-OH protecting group must be stable to the reagents and conditions used during solid-phase DNA/RNA synthesis in addition to those required for nucleobase and phosphate deprotection. Last, the 2'-OH protecting group must be cleaved under conditions 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.<sup>5</sup> 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.<sup>6</sup> Ribonucleoside phosphoramidites functionalized with these 2'-OH protecting groups (**1** and **2**) produced coupling efficiencies exceeding 98% within 2–3 min.<sup>6</sup>

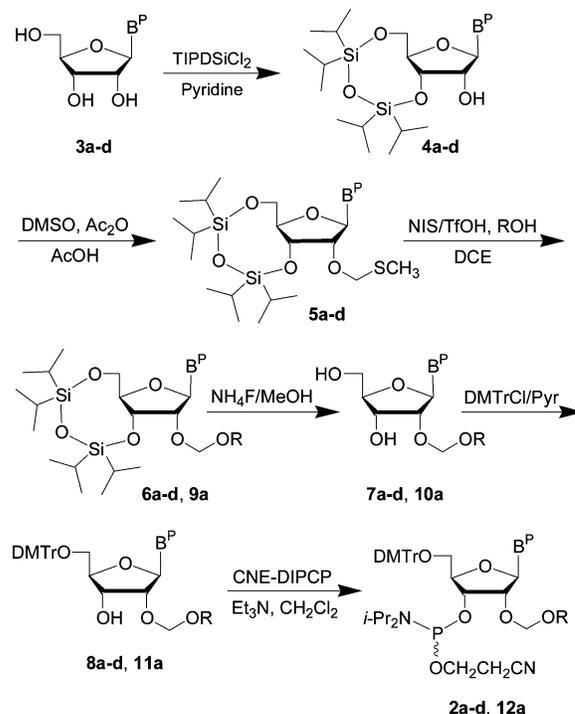


- 1 R = H, R<sub>2</sub> = NO<sub>2</sub>; B<sup>P</sup> = U, C<sup>Bz</sup>, A<sup>Bz</sup>, G<sup>iBu</sup>  
 2 R = NO<sub>2</sub>, R<sub>2</sub> = H; B<sup>P</sup> = U, C<sup>Bz</sup>, A<sup>Bz</sup>, G<sup>iBu</sup>

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,<sup>7</sup> 2'-*O*-[1-(2-cyanoethoxy)]ethyl,<sup>8</sup> 2'-*O*-triisopropylsilyloxymethyl (TIPSOM),<sup>9</sup> and 2'-*O*-(2-cyanoethoxy)methyl (CEM)<sup>10</sup> 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.<sup>9,10</sup> 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,<sup>6c</sup> 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<sup>11</sup> 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.<sup>12</sup>

**Scheme 1.** Synthesis of Phosphoramidites **2a–d** and **12a**<sup>a</sup>



<sup>a</sup> Keys: **2, 6, 7, 8**, R = 4-nitrobenzyl; **9, 10, 11, 12**, R = 4-(*N*-dichloroacetyl-*N*-methyl)aminobenzyl; B<sup>P</sup>, U (a), C<sup>Bz</sup> (b), A<sup>Bz</sup> (c), G<sup>iBu</sup> (d); TIPDSiCl<sub>2</sub>, 1,3-dichloro-1,1,3,3-tetraisopropylidisiloxane; 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<sub>19</sub>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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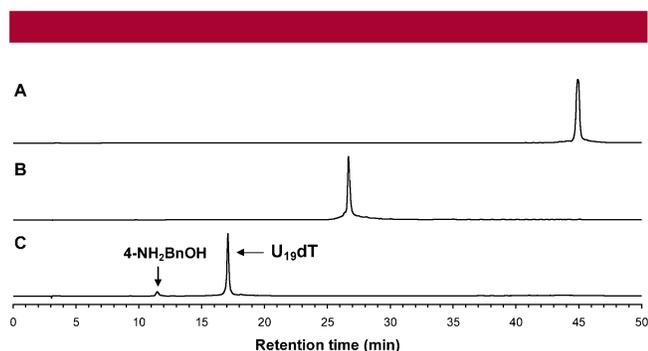
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of which was treated with concentrated  $\text{NH}_4\text{OH}$  for 30 min at 25 °C to remove the phosphate protecting groups and release the 2'-*O*-protected RNA oligonucleotide from the support. The other fraction of the support was suspended in 0.1 M  $\text{TiCl}_3$ <sup>13</sup> (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  $\text{TiCl}_3$ , 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  $\text{NH}_3$  gas.<sup>12</sup> As shown in Figure 1A, the RP-HPLC profile of



**Figure 1.** RP-HPLC profiles of unpurified 2'-*O*-protected/deprotected  $\text{U}_{19}\text{dT}$ . **A:** [2'-*O*-(4-NBOM)  $\text{U}_{19}\text{dT}$ ]. **B:** [2'-*O*-(4-ABOM)  $\text{U}_{19}\text{dT}$ ] obtained from the  $\text{TiCl}_3$ -mediated reduction of [2'-*O*-(4-NBOM)  $\text{U}_{19}\text{dT}$ ] at pH 6.0. **C:**  $\text{U}_{19}\text{dT}$  obtained from the thermolytic deprotection of [2'-*O*-(4-ABOM)  $\text{U}_{19}\text{dT}$ ] in 0.1 M  $\text{AcOH}$  at 90 °C. Conditions: see Supporting Information.

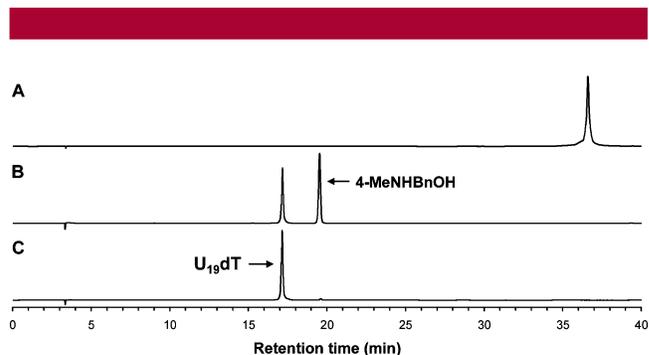
the unpurified RNA oligonucleotide protected with 2'-*O*-(4-NBOM) groups is reflective of the coupling efficiency of phosphoramidite **2a**, which averaged 99%. Reduction of the 2'-*O*-(4-NBOM) group to its 2'-*O*-(4-ABOM) derivative was efficient and clean, as illustrated in Figure 1B. The 2'-*O*-(4-ABOM) group is stable under both neutral (0.1 M triethylammonium acetate buffer, pH 7.0, 90 °C, 3 h) and basic (concentrated  $\text{NH}_4\text{OH}$ , 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  $\text{AcOH}$ . The RP-HPLC profile of  $\text{U}_{19}\text{dT}$  (Figure 1C) did not reveal substantial chain cleavage.<sup>14</sup>

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  $\text{U}_{19}\text{dT}$  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 modifica-

tions. Encouraged by these results, the 2'-*O*-(4-NBOM) phosphoramidite derivatives **2b–d** were prepared as described in Scheme 1.<sup>12</sup> 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  $\text{TiCl}_3$ -mediated reduction of the 2'-*O*-(4-NBOM)-protected 20-mer was not as efficient as that achieved with [2'-*O*-(4-NBOM)  $\text{U}_{19}\text{dT}$ ] and resulted in a product of inferior quality.<sup>15</sup> Such an apparent deficiency in the reductive capacity of  $\text{TiCl}_3$  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)  $\text{U}_{19}\text{dT}$ ], 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<sup>16</sup> was found optimal for *N*-protection of the 2'-*O*-(4-MABOM) acetal. Thus, 4-(*N*-methylaminobenzyl alcohol was *O*-silylated upon reaction with  $\text{Me}_3\text{SiCl}$  and then *N*-acylated with dichloroacetic anhydride to give 4-(*N*-dichloroacetyl-*N*-methylamino)benzyl alcohol after hydrolytic workup.<sup>12</sup> 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.<sup>12</sup> Phosphoramidite **12a** was employed in the solid-phase synthesis of  $\text{U}_{19}\text{dT}$  under conditions identical to those described when using **2a**. The 5'-*O*-dedimethoxytritylated solid-phase linked oligoribonucleotide was exposed to concentrated  $\text{NH}_4\text{OH}$  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-



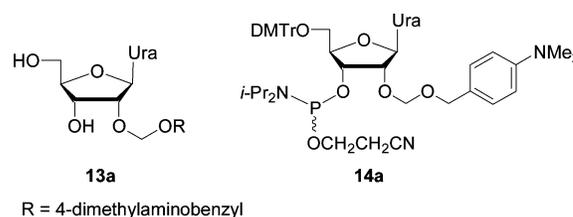
**Figure 2.** RP-HPLC profiles of unpurified 2'-*O*-protected/deprotected  $\text{U}_{19}\text{dT}$ . **A:** [2'-*O*-(4-MABOM)  $\text{U}_{19}\text{dT}$ ]. **B:** [2'-*O*-(4-MABOM)  $\text{U}_{19}\text{dT}$ ] in 0.1 M  $\text{AcOH}$ , 90 °C, 15 min. **C:** Material of profile B after multiple  $\text{Et}_2\text{O}$  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<sub>19</sub>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<sub>19</sub>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.<sup>12</sup> 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<sup>17</sup> 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<sub>3</sub> (pH 6.0) and was then reacted with formaldehyde in the presence of NaBH<sub>3</sub>CN and ZnCl<sub>2</sub> in MeOH<sup>18</sup> 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.<sup>12</sup> The solid-phase synthesis of dinucleotide UdT was carried out employ-

ing **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.<sup>17</sup> An optimized



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; <sup>1</sup>H and <sup>13</sup>C NMR spectra of **7a–d**, **10a**, and **13a**; <sup>31</sup>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<sub>19</sub>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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