ORGANIC LETTERS

2007
Vol. 9, No. 4
671−674

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

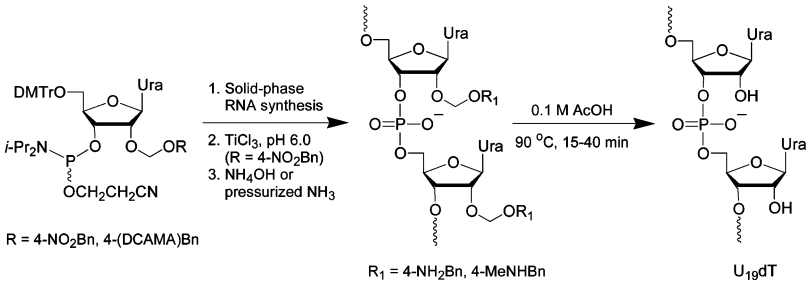
Jacek Cieślak,[†] Jon S. Kauffman,[‡] Michelle J. Kolodziejski,[‡] John R. Lloyd,[§] and Serge L. Beaucage*,[†]

Division of Therapeutic Proteins, Center for Drug Evaluation and Research, Food and Drug Administration, 8800 Rockville Pike, Bethesda, Maryland 20892, Lancaster Laboratories, 2425 New Holland Pike, Lancaster, Pennsylvania 17605, and Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, The National Institutes of Health, 9000 Rockville Pike, Bethesda, Maryland 20892

serge.beaucage@fda.hhs.gov

Received December 9, 2006

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.

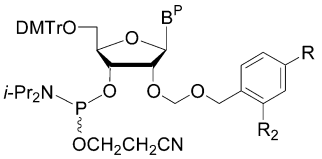
A formidable challenge in the preparation of RNA oligonucleotides is designing a 2′-hydroxyl protecting group that would provide ribonucleoside phosphoramidites with coupling kinetics and coupling efficiencies comparable to those of deoxyribonucleoside phosphoramidites. Furthermore,

[†] Food and Drug Administration.
[‡] Lancaster Laboratories.
[§] The National Institutes of Health.

(1) Fire, A.; Xu, S.; Montgomery, M. K.; Kostas, S. A.; Driver, S. E.; Mello, C. C. *Nature* **1998**, *391*, 806−811.
(2) Elbashir, S. M.; Harborth, J.; Lendeckel, W.; Yalcin, A.; Weber, K.; Tuschl, T. *Nature* **2001**, *411*, 494−498.
(3) Dorsett, Y.; Tuschl, T. *Nat. Rev. Drug Discovery* **2004**, *3*, 318−329.
(4) Manoharan, M. *Curr. Opin. Chem. Biol.* **2004**, *8*, 570−579.

10.1021/ol0629824 CCC: $37.00
Published on Web 01/26/2007

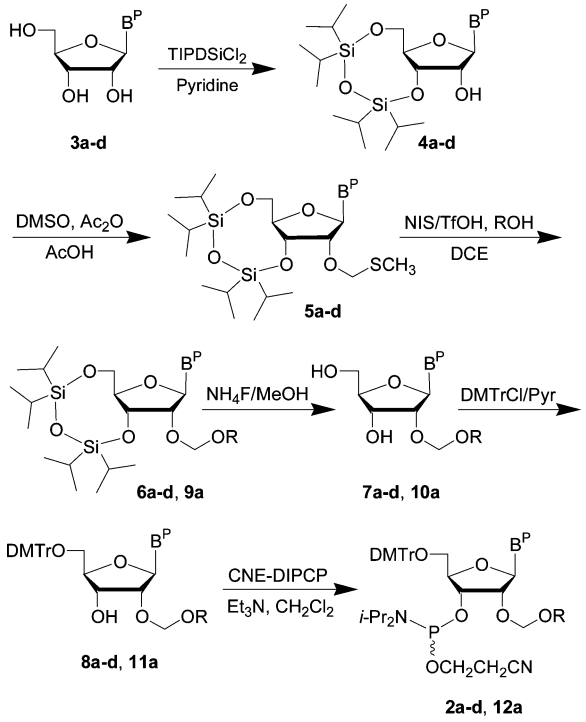
the 2′-OH protecting group must be stable to the reagents and conditions used during solid-phase DNA/RNA synthesis in addition to those required for nucleobase and phosphate deprotection. Last, the 2′-OH protecting group must be cleaved under conditions that will not harm the oligoribonucleotide. Thus, 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

(5) (a) Reese, C. B. *Org. Biomol. Chem.* **2005**, *3*, 3851−3868. (b) Muller, S.; Wolf, G.; Ivanov, S. A. *Curr. Org. Synth.* **2004**, *1*, 293−307. (c) Marshall, W. S.; Kaiser, R. J. *Curr. Opin. Chem. Biol.* **2004**, *8*, 222−229. (d) Reese, C. B. *Tetrahedon* **2002**, *58*, 8893−8920. (e) Reese, C. B. In *Current Protocols in Nucleic Acid Chemistry*; Beaucage, S. L., Bergstrom, D. E., Glick, G. D., Jones, R. A., Eds.; John Wiley & Sons: New York, 2000; Vol. I, pp 2.2.1−2.2.24. (f) Pitsch, S. *Chimia* **2001**, *55*, 320−324. (g) Beaucage, S. L.; Iyer, R. P. *Tetrahedron* **1992**, *48*, 2223−2311.

(6) (a) Schwartz, M. E.; Breaker, R. R.; Asteriadis, G. T.; deBear, J. S.; Gough, G. R. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 1019−1024. (b) Miller, T. J.; Schwartz, M. E.; Gough, G. R. In *Current Protocols in Nucleic Acid Chemistry*; Beaucage, S. L., Bergstrom, D. E., Glick, G. D., Jones, R. A., Eds.; John Wiley & Sons: New York, 2000; Vol. I, pp 2.5.1−2.5.36 and pp 3.7.1−3.7.8. (c) Gough, G. R.; Miller, T. J.; Mantick, N. A. *Tetrahedron Lett.* **1996**, *37*, 981−982.

(7) (a) Matysiak, S.; Fitznar, H.-P.; Schnell, R.; Pfleiderer, W. *Helv. Chim. Acta* **1998**, *81*, 1545−1566. (b) Matysiak, S.; Pfleiderer, W. *Helv. Chim. Acta* **2001**, *84*, 1066−1085.

(8) Umemoto, T.; Wada, T. *Tetrahedron Lett.* **2004**, *45*, 9529−9531.

(9) (a) Pitsch, S.; Weiss, P. A.; Jenny, L.; Stutz, A.; Wu, X. *Helv. Chim. Acta* **2001**, *84*, 3773−3795. (b) Pitsch, S.; Weiss, P. A. In *Current Protocols in Nucleic Acid Chemistry*; Beaucage, S. L., Bergstrom, D. E., Glick, G. D., Jones, R. A., Eds.; John Wiley & Sons: New York, 2000; Vol. I, pp 3.8.1−3.8.15.

(10) Ohgi, T.; Masutomi, Y.; Ishiyama, K.; Kitagawa, H.; Shiba, Y.; Yano, J. *Org. Lett.* **2005**, *7*, 3477−3480.

(11) Carl, P. L.; Chakravarty, P. K.; Katzenellenbogen, J. A. *J. Med. Chem.* **1981**, *24*, 479−480.

(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

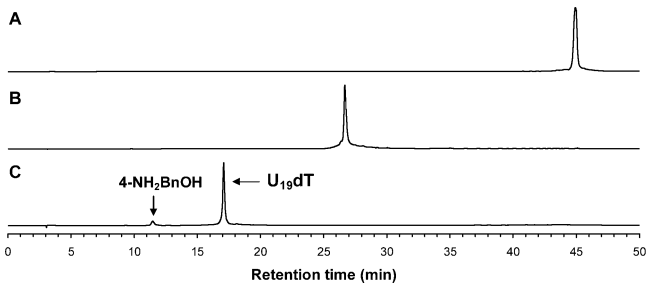


Figure 1. RP-HPLC profiles of unpurified 2′-*O*-protected/-deprotected U_{19}dT. **A**: [2′-*O*-(4-NBOM) U]$_{19}$dT. **B**: [2′-*O*-(4-ABOM) U]$_{19}$dT obtained from the $TiCl_3$-mediated reduction of [2′-*O*-(4-NBOM) U]$_{19}$dT at pH 6.0. **C**: U_{19}dT obtained from the thermolytic deprotection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

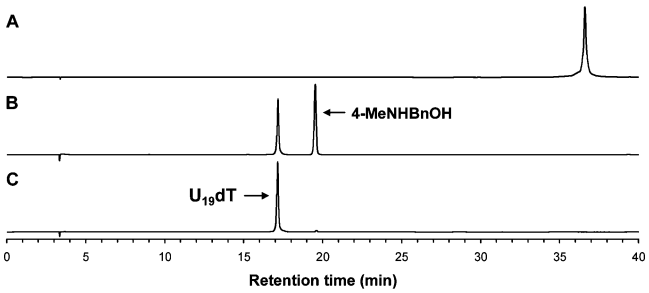
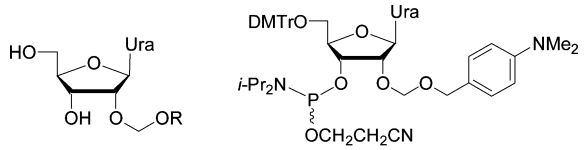


Figure 2. RP-HPLC profiles of unpurified 2′-*O*-protected/-deprotected U_{19}dT. **A**: [2′-*O*-(4-MABOM) U]$_{19}$dT. **B**: [2′-*O*-(4-MABOM) U]$_{19}$dT in 0.1 M AcOH, 90 °C, 15 min. **C**: Material of profile B after multiple Et_2O extractions. Conditions: see Supporting Information.

(13) Somei, M.; Kato, K.; Inoue, S. *Chem. Pharm. Bull.* **1980**, *28*, 2515−2518.

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.

OL0629824

(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.

(16) Goody, R. S.; Walker, R. T. *Tetrahedron Lett.* **1967**, *8*, 289−291.

(17) Scaringe, S. A. *Methods* **2001**, *23*, 206−217.

(18) Kim, S.; Oh, C. H.; Ko, J. S.; Ahn, K. H.; Kim, Y. J. *J. Org. Chem.* **1985**, *50*, 1927−1932.