ORGANIC LETTERS

2001
Vol. 3, No. 9
1287−1290

The 2-(*N*-Formyl-*N*-methyl)aminoethyl Group as a Potential Phosphate/Thiophosphate Protecting Group in Solid-Phase Oligodeoxyribonucleotide Synthesis†

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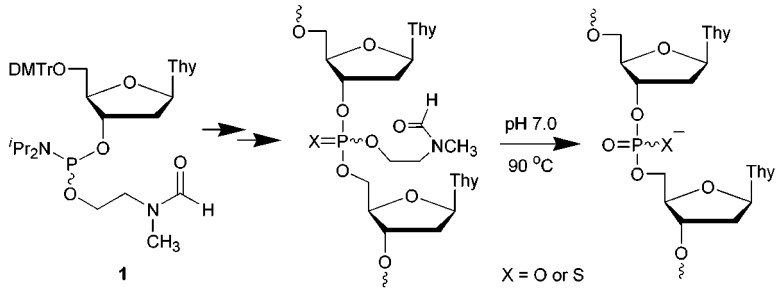
Received January 18, 2001

ABSTRACT



The 2-(*N*-formyl-*N*-methyl)aminoethyl deoxyribonucleoside phosphoramidite 1 has been synthesized and used in the solid-phase synthesis of an octadecathymidylic acid as a cost-efficient monomer for potential application in the preparation of therapeutic oligonucleotides. The 2-(*N*-formyl-*N*-methyl)aminoethyl group can be cleaved from oligonucleotides according to a unique thermolytic cyclodeesterification process at pH 7.0. In addition to being cost-effective, the use of 1 simplifies oligonucleotide postsynthesis processing by eliminating the utilization of concentrated ammonium hydroxide in oligonucleotide deprotection.

Large-scale production of synthetic oligonucleotides for therapeutic indications against cancer and infectious diseases in humans is a challenge in that the methods used for synthesizing oligonucleotides must not generate side products that can chemically modify the biopolymers and must be cost-effective to ensure an affordable cost per dose of oligonucleotide drugs. We have earlier reported a phosphoramidite approach to the solid-phase synthesis of DNA oligonucleotides that employs the 4-[(*N*-(2,2,2-trifluoroacetyl)amino]butyl[1] or the 4-[(*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butyl[1b,2] groups for phosphate/thiophosphate protection. Unlike oligonucleotides carrying the conventional 2-cyanoethyl phosphate/thiophosphate protecting group, deprotection of the 4-[(*N*-(2,2,2-trifluoroacetyl)amino]butyl or 4-[(*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butyl groups from oligonucleotides does not generate potent mutagens, such

† This paper is dedicated to Profs. Marvin H. Caruthers and Wojciech J. Stec on the occasion of their 60th birthdays.

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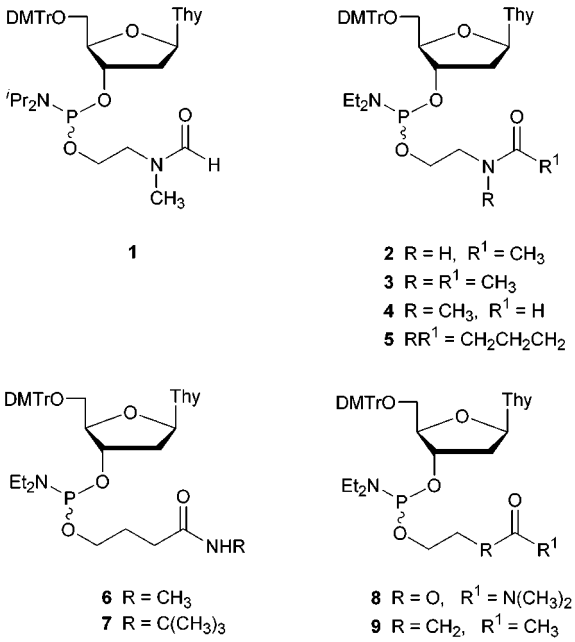
(1) (a) Wilk, A.; Srinivasachar, K.; Beaucage, S. L. *J. Org. Chem.* **1997**, *62*, 6712−6713. (b) Wilk, A.; Grajkowski, A.; Srinivasachar, K.; Beaucage, S. L. *Antisense Nucl. Acid. Drug Devel.* **1999**, *9*, 361−366.

(2) Wilk, A.; Grajkowski, A.; Phillips, L. R.; Beaucage, S. L. *J. Org. Chem.* **1999**, *64*, 7515−7522.

10.1021/ol0156852
Published on Web 04/05/2001

as acrylonitrile, which can alkylate DNA. However, minor drawbacks such as the cost of 4-aminobutan-1-ol[1] and the lack of commercial sources for 4-[(*N*-methyl)amino]butan-1-ol[2] prompted us to investigate phosphate/thiophosphate protecting groups that are more cost-effective and would permit the production of oligonucleotides of a quality comparable to those that carry 4-[(*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butyl phosphate/thiophosphate protecting groups. We have recently reported that the 2-[*N*-(2-fluoroacetyl)amino]-1-phenylethyl thiophosphate protecting group can be efficiently removed from oligonucleotides in the presence or *absence* of concentrated ammonium hydroxide.[3,4] On the basis of these findings, we wish to report preliminary results of our search for cost-effective phosphate/thiophosphate protecting groups that can be removed from oligonucleotides under neutral conditions to simplify postsynthesis processing. In this regard, the preparation of deoxyribonucleoside phosphoramidite **1** (Scheme 1) carrying a P(III) 2-(*N*-formyl-*N*-methyl)aminoethoxy group is described along with its use in the solid-phase synthesis of an octadecathymidylic acid.

Scheme 1[a]



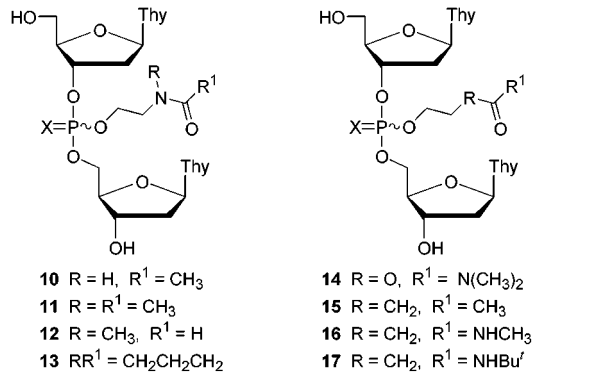
[a] DMTr, 4,4′-dimethoxytrityl; Thy, thymin-1-yl; ^{i}Pr, isopropyl; Et, ethyl.

Alcohol derivatives that have been investigated in this work as potential phosphate/thiophosphate protecting groups include the following: *N*-acetylethanolamine,[5] 2-(*N*-acetyl-*N*-methyl)aminoethanol,[6] 2-(*N*-formyl-*N*-methyl)aminoethanol,[7] 1-(2-hydroxyethyl)-2-pyrrolidinone,[5] *N*-methyl-4-hydroxybutyramide,[2] *N*-*tert*-butyl-4-hydroxybutyramide,[8] *N*,*N*-dimethyl-1-hydroxyethylcarbamate,[9,10] and 3-acetyl-1-propanol.[5]

Typically, equimolar amounts of 5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxythymidine, hexaethylphosphorus triamide, and diethylammonium tetrazolide are mixed in dry acetonitrile for 30 min at 25 °C. The deoxyribonucleoside 3′-*O*-phosphordiamidite that is generated in situ is then reacted with an equimolar amount of any of the above alcohol derivatives, and the reaction mixture is left stirring for at least 6 h at ambient temperature.[1a] The resulting deoxyribonucleoside phosphoramidites **2**−**9** (Scheme 1) are purified by silica gel chromatography and isolated as white foams in yields generally exceeding 90%.[11]

The phosphoramidites **2**−**9** are then activated upon reaction with 1*H*-tetrazole and manually coupled to 5′-unprotected thymidine covalently attached to long chain alkylamine controlled pore glass (LCAA-CPG). After standard aqueous iodine oxidation or sulfurization,[12] the dinucleoside phosphotriesters **10**−**17** (Scheme 2) are released from LCAA-CPG by treatment with pressurized methylamine gas[13] for 3 min at 25 °C. The crude phosphotriesters are eluted from the LCAA-CPG column with a solution of 40% acetonitrile in 0.1 M triethylammonium acetate pH 7.0 and then purified by reversed-phase (RP) HPLC.[14] Removal of the phosphate protecting groups from purified dinucleoside phosphotriesters **10**−**12** and **15**−**17** in aqueous solvents, without the aid of

Scheme 2[a]



[a] X = O or S.

(3) Wilk, A.; Grajkowski, A.; Phillips, L. R.; Beaucage, S. L. *J. Am. Chem. Soc.* **2000**, *122*, 2149−2156.

(4) During the course of our investigations, the use of 2-amidoethyl groups for thiophosphate protection has appeared in the patent literature, see: Cheruvallath, Z. S.; Capaldi, D. C.; Ravikumar, V. T.; Cole, D. L. U.S. Patent 5,959,099. These protecting groups are reportedly removed from oligonucleotides by treatment with ammonium hydroxide.

(5) Commercially available from Aldrich.

(6) Saegusa, T.; Kobayashi, S.; Yamada, A. *Makromol. Chem.* **1976**, *177*, 2271−2283.

(7) Shibanuma, T.; Iwanami, M.; Fujimoto, M.; Takenaka, T.; Murakami, M. *Chem Pharm. Bull.* **1980**, *28*, 2609−2613.

(8) Prepared according to the folowing: Bigg, D. C. H.; Lesimple, P. *Synthesis* **1992**, 277−278.

(9) Prepared according to the following: Probst, J.; Kolb, G. *Makromol. Chem.* **1976**, *177*, 2681−2695.

(10) The 2-[(1-naphthyl)carbamoyloxy]ethyl group has recently been reported for phosphate protection, see: Guzaev, A. P.; Manoharan, M. *Tetrahedron Lett.* **2000**, *41*, 5623−5626.

(11) The deoxyribonucleoside phosphoramidites **2**−**9** were characterized by ^{31}P NMR spectroscopy. Selected spectra are reported in the Supporting Information.

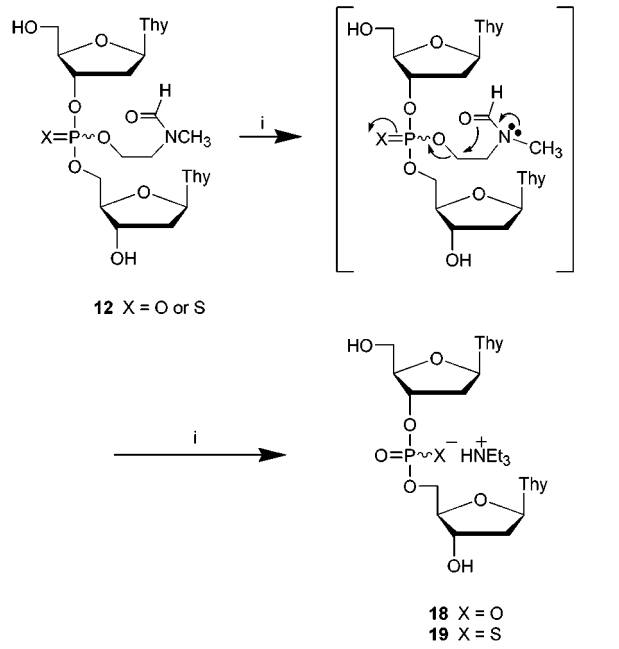
concentrated ammonium hydroxide, occurs in less than 3 h at ~90 °C, affording the corresponding dithymidylyl monophosphate **18** in essentially quantitative yields.[15]

While the cleavage of phosphate protecting groups from **10**−**17** proceeds cleanly, deprotection of the parent thiophosphate protecting groups from **11**, **13**, and **15** under identical conditions produces dithymidylyl phosphorothioate **19** along with 10% to 20% of the native phosphodiester **18**. Such an extent of desulfurization precludes the use of these thiophosphate protecting groups in routine oligonucleoside phosphorothioate syntheses. However, heating the dinucleoside thiophosphate triesters **10**, **12**, **14**, **16**, and **17** under conditions identical to those used for the related phosphotriesters results in complete removal of the thiophosphate protecting groups without significant desulfurization (<0.5%) of **19**.[16] These findings raise interesting questions about the deprotection mechanism of these thiophosphate protecting groups.

Preliminary mechanistic studies using **12** as a model strongly suggest that the presence of water is required for rapid phosphate/thiophosphate deprotection;[17] a tentative mechanism involving cyclodeesterification[3] of the dinucleoside phosphotriester is proposed in Scheme 3.[18] Whether the proposed phosphate/thiophosphate deprotection mechanism applies to the dinucleoside phosphotriesters **10**, **11**, and **13**−**17** remains to be determined and will be addressed at a later date.

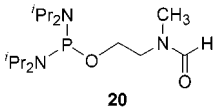
Since the groups used for phosphate/thiophosphate protection of **10**, **12**, **14**, **16**, and **17** are easily removed under neutral conditions, the deoxyribonucleoside phosphoramidite

Scheme 3[a]



[a] Conditions: (i) 0.1 M triethylammonium acetate pH 7.0, 90 °C, 3 h.

1 was selected as a model and used in the solid-phase synthesis of an octadecanucleotide. The synthesis of **1** begins with the preparation of the phosphordiamidite **20**. Thus, addition of anhydrous *N*,*N*-diisopropylamine to a solution of freshly distilled phosphorus trichloride in dry benzene gives bis(*N*,*N*-diisopropylamino)chlorophosphine, which is then immediately reacted with 2-(*N*-formyl-*N*-methyl)-aminoethan-1-ol. ^{31}P NMR analysis of the reaction mixture indicates that the formation of the phosphordiamidite **20** (δ_P 118.0 and 118.7 ppm) is nearly complete (~96%) after 2 h at 25 °C. After workup, the phosphinylating reagent is purified from hydrolysis side products by silica gel chromatography and is isolated as an oil in 73% yield.



20

Phosphinylation of 5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxythymidine with **20** is performed essentially as reported by Barone et al.[19] and affords the deoxyribonucleoside phosphoramidite **1**. The crude phosphoramidite is purified by silica gel chromatography and isolated as a white foam in 92% yield.[20] The pure deoxyribonucleoside phosphoramidite **1** is then used in the solid-phase synthesis of an oligonucleotide (18-mer) according to standard protocols.[21] The 5′-deprotected oligonucleoside phosphotriester is released from

(12) The sulfurization reaction is effected by 3*H*-1,2-benzodithiol-3-one 1,1-dioxide, see: (a) Beaucage, S. L.; Iyer, R. P.; Egan, W.; Regan, J. B. *Ann. New York Acad. Sci.* **1990**, *616*, 483−485. (b) Iyer, R. P.; Phillips, L. R.; Egan, W.; Regan, J. B.; Beaucage, S. L. *J. Org. Chem.* **1990**, *55*, 4693−4699. (c) Regan, J. B.; Phillips, L. R.; Beaucage, S. L. *Org. Prep. Proc. Int.* **1992**, *24*, 488−492.

(13) Boal, J. H.; Wilk, A.; Harindranath, N.; Max, E. E.; Kempe, T.; Beaucage, S. L. *Nucl. Acids Res.* **1996**, *24*, 3115−3117.

(14) Selected RP-HPLC chromatograms recorded before and after phosphotriester deprotection are provided in the Supporting Information.

(15) Under these conditions, phosphate deprotection of the dinucleoside phosphotriesters **13** and **14** is complete within 16 and 4 h, respectively.

(16) It should also be noted that heating **17** for an extended period of time (>12 h) in 0.1 M triethylammonium acetate pH 7.0 at 90 °C will result in significant desulfurization of **19** (~10%). This problem can essentially be eliminated by selecting 1X phosphate buffered saline (PBS) pH 7.4 as a buffer formulation for thermolytic thiophosphate deprotection.

(17) Heating the dinucleoside phosphotriester **12** for 3 h at ~90 °C in MeCN containing 50 ppm water affords only ~15% phosphate deprotection. Complete phosphate deprotection is accomplished within 3 h ($t_{1/2}$ ~ 20 min) in 0.1 M triethylammonium acetate pH 7.0 and is consistent with a pseudo-first-order kinetic pathway. The enhanced rate of deprotection does not however result from water attacking the phosphate function and releasing 2-(*N*-formyl-*N*-methyl)aminoethanol. This argument is supported by the fact that hydrolysis of triethyl phosphate in 0.1 M triethylammonium acetate pH 7.0 for 8 h at ~90 °C gives only ~1% diethyl phosphate, thereby exhibiting a phosphate deprotection rate considerably slower ($t_{1/2}$ ~ 600 h) than that of **12**. Data are shown in the Supporting Information.

(18) Removal of thiophosphate protecting groups that are structurally related to **11** or **13** has also been reported by others, see: (a) Iyer, R. P.; Yu, D.; Devlin, T.; Ho, N.-H.; Agrawal, S. *J. Org. Chem.* **1995**, *60*, 5388−5389. (b) Iyer, R. P.; Guo, M. J.; Yu, D.; Agrawal, S. *Tetrahedron Lett.* **1998**, *39*, 2491−2494. (c) Wang, J.-C.; Just, G. *Tetrahedron Lett.* **1997**, *38*, 3797−3800. (d) Wang, J.-C.; Just, G. *J. Org. Chem.* **1999**, *64*, 8090−8097. (e) Guzaev, A. P.; Manoharan, M. *J. Am. Chem. Soc.* **2001**, *123*, 783−793. Unfortunately, deprotection of **11** or **13** in the presence or absence of concentrated ammonium hydroxide leads to desulfurization of the dinucleoside phosphorothioate **19** in unacceptable levels.

(19) Barone, A. D.; Tang, J.-T.; Caruthers, M. H. *Nucl. Acids Res.* **1984**, *12*, 4051−4061.

(20) The detailed preparation of **20** and **1** along with characterization data are provided in the Supporting Information.

LCAA-CPG by the use of pressurized methylamine gas and eluted from the solid support with a solution of 40% acetonitrile in 0.1 M triethylammonium acetate pH 7.0. The solution is heated for 3 h at 90 °C to ensure complete phosphate deprotection. The crude oligonucleotide dT_{18} was analyzed by polyacrylamide gel electrophoresis under denaturing conditions along with a control dT_{18} that was prepared from a commercial 2-cyanoethyl phosphoramidite monomer and deprotected by treatment with concentrated ammonium hydroxide. Upon staining with Stains-all,[5] the gel revealed that dT_{18} prepared from phosphoramidite **1** is of quality equal, if not better, than that of dT_{18} prepared from the parent 2-cyanoethyl phosphoramidite monomer. The crude dT_{18} prepared from **1** was further characterized upon incubation with snake venom phosphodiesterase and bacterial alkaline phosphatase. Analysis of the hydrolysate by RP-HPLC does not show nucleobase modification and/or incomplete oligonucleotide deprotection.

These data demonstrate that the 2-(*N*-formyl-*N*-methyl)-aminoethyl group can be used as a cost-effective phosphate/thiophosphate protecting group in solid-phase syntheses of oligothymidylic acids.[22] We are currently studying the application of this group to the functionalization of the remaining three deoxyribonucleoside phosphoramidites needed for synthesizing DNA oligonucleotides composed of mixed nucleobases. The results of these studies will be reported in due course.

The removal of 2-(*N*-formyl-*N*-methyl)aminoethyl groups from DNA oligonucleotides, in the absence of concentrated ammonium hydroxide, is advantageous in that the handling of hazardous ammonium hydroxide can be completely eliminated from oligonucleotide processing. This advantage should certainly be appreciated when performing large-scale therapeutic oligonucleotide syntheses, as large quantities of concentrated ammonium hydroxide are needed to release oligonucleotides from the solid support and for nucleobase deprotection. Furthermore, the deprotection of 2-(*N*-formyl-*N*-methyl)aminoethyl phosphate/thiophosphate protecting groups under the thermolytic conditions described herein does not lead to detectable alkylation or any other modification of thymine.

The use of the 2-(*N*-formyl-*N*-methyl)aminoethyl group for phosphate/thiophosphate protection can potentially be advantageous in the synthesis of oligoribonucleotides and those oligonucleotides carrying nucleobases that are sensitive to concentrated ammonium hydroxide but not to anhydrous pressurized ammonia gas.

Finally, the application of the 2-(*N*-acetyl)aminoethyl) and 3-[(*N*-*tert*-butyl)carboxamido]-1-propyl groups (as in **10** and **17**, respectively) to phosphate/thiophosphate protection along with the 2-(*N*-acetyl-*N*-methyl)aminoethyl and 3-acetyl-1-propyl groups (as in **11** and **15**, respectively) for phosphate protection are being further investigated in the development of versatile, cost-effective, and alkylation-free methods for the preparation of therapeutic oligonucleotides. The results of these investigations will be reported in full in the near future.

Acknowledgment. This research was supported in part by an appointment to the Postgraduate Research Participation Program at the Center for Biologics Evaluation and Research administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and the U.S. Food and Drug Administration.

Supporting Information Available: ^{31}P NMR spectra of the deoxyribonucleoside phosphoramidites **3**−**7** and **9**. RP-HPLC chromatograms of purified dinucleoside phosphotriesters **10**−**17** along with those chromatograms recorded after phosphate/thiophosphate deprotection. Kinetic profile of the thermolytic phosphate/thiophosphate deprotection of **12** and **13** in aqueous solvents. ^{31}P NMR spectra of the hydrolysis of triethyl phosphate. Detailed preparation and characterization of the phosphordiamidite **20**, deoxyribonucleoside phosphoramidite **1**, and their congeners. Photograph of the stained polyacrylamide gel displaying the purity of crude dT_{18}, which was synthesized from phosphoramidite **1**. HPLC chromatogram of crude $d(AG)_{10}$ that was prepared from 2-(*N*-formyl-*N*-methyl)aminoethyl deoxyribonucleoside phosphoramidites. HPLC chromatogram of the digestion of crude dT_{18} and $d(AG)_{10}$ effected by snake venom phosphodiesterase and bacterial alkaline phosphatase. This material is available free of charge via the Internet at http://pubs.acs.org.

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(21) The concentration of **1** in dry acetonitrile was increased to 0.2 M. The wait time required for the coupling step and the oxidation reaction was extended to 150 and 60 s, respectively.

(22) The solid-phase synthesis of $d(AG)_{10}$ using the 2-(*N*-formyl-*N*-methyl)aminoethyl group for phosphate protection has also been successful. A RP-HPLC chromatogram of crude $d(AG)_{10}$ is shown in the Supporting Information. Digestion of crude $d(AG)_{10}$ by snake venom phosphodiesterase and bacterial alkaline phosphatase does not show incomplete oligonucleotide deprotection and does not reveal any detectable nucleobase modification on the basis of RP-HPLC analysis of the digest (see Supporting Information).