ORGANIC LETTERS

2005
Vol. 7, No. 19
4201−4204

An Efficient Reagent for the Phosphorylation of Deoxyribonucleosides, DNA Oligonucleotides, and Their Thermolytic Analogues

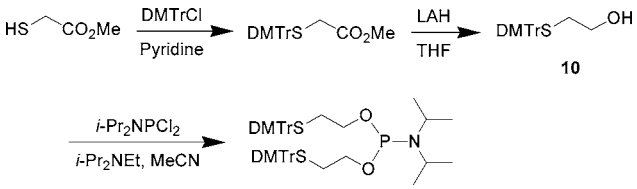
Cristina Ausín, Andrzej Grajkowski, Jacek Cieślak, and Serge L. Beaucage*

Division of Therapeutic Proteins, Center for Drug Evaluation and Research, Food and Drug Administration, 8800 Rockville Pike, Bethesda, Maryland 20892

beaucage@cber.fda.gov

Received July 11, 2005

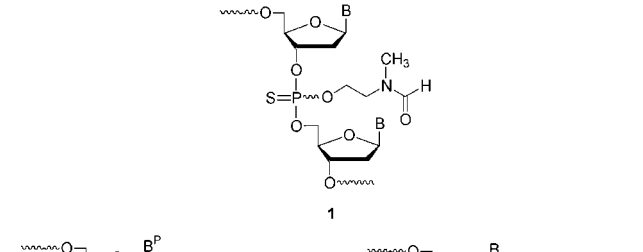
ABSTRACT



The phosphoramidite 11 was prepared in three steps from methyl 2-mercaptoacetate and demonstrated efficiency in the synthesis of conventional 5′-/3′-phosphate/thiophosphate monoester derivatives of 2′-deoxyribonucleosides and DNA oligonucleotides. Moreover, the use of 11 has enabled the preparation of the dinucleoside phosphorothioate analogue 26 in high yields (>95%) with minimal cleavage (<2%) of the thermolytic thiophosphate protecting group.

We recently reported the preparation of CpG-containing DNA oligonucleotides functionalized with 2-(*N*-formyl-*N*-methyl)aminoethyl thiophosphate triesters (**1**) as a new class of thermolytic immunotherapeutic prodrugs.[1] To further expand the potential therapeutic applications of thermolytic oligonucleotide prodrugs, we decided to assess the thermosensitivity of DNA oligonucleotide analogues **2** and **3**.[2] The synthesis of **3** is challenging given that the phosphorylation of each 4-hydroxybutyl thiophosphate triester of oligonucleotide **2** must be performed under conditions that will not induce premature thermolytic cleavage of the internucleotidic phosphorothioated thiophosphate protecting group.

Several phosphorylating reagents (**4**−**9**, Figure 1) have been employed in the preparation of 5′-/3′-phosphate/thiophosphate monoester derivatives of nucleosides, oligonucleotides, and selected polyols.[3−8] Three of these reagents,



B^P = Thymin-1-yl, N^4-benzoylcytosin-1-yl, N^6-benzoyladenin-9-yl or N^2-isobutyrylguanin-9-yl; B = Thymin-1-yl, cytosin-1-yl, adenin-9-yl or guanin-9-yl

(1) Grajkowski, A.; Pedras-Vasconcelos, J.; Wang, V.; Ausín, C.; Hess, S.; Verthelyi, D.; Beaucage, S. L. *Nucleic Acids Res.* **2005**, *33*, 3550−3560.

(2) Grajkowski, A.; Beaucage, S. L. Unpublished results.

10.1021/ol0516263
Published on Web 08/23/2005

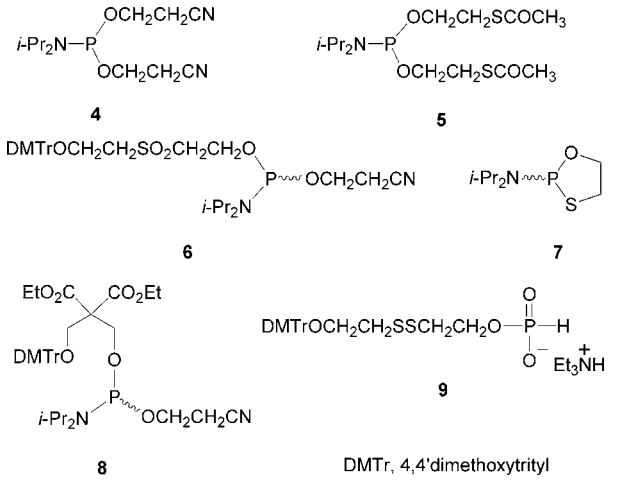


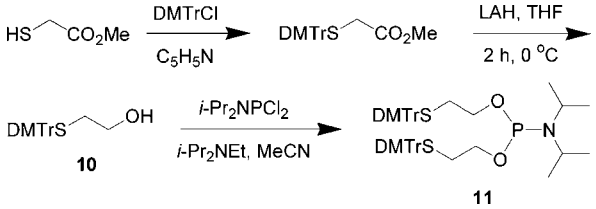
Figure 1. Reagents for the phosphorylation of nucleosides and oligonucleotides.

namely, **4**,[3] **6**,[4] and **7**,[5] require elevated temperature conditions (concd NH_4OH, 55−60 °C) that are incompatible with the preparation of oligonucleotides functionalized with thermosensitive phosphotriester groups. Furthermore, the coupling efficiency of **4**, **5**,[6] and **7** cannot be easily monitored because each reagent is devoid of any reporter group.

Whereas reagent **8**[7] also requires an elevated temperature to produce phosphorothioate monoester **26** within a reasonable period of time, the *H*-phosphonate reagent **9**[8] is incompatible with automated phosphoramidite chemistry for solid-phase oligonucleotide synthesis. Given the limitations of reagents **4**−**9** in the context of our studies, we decided to develop a phosphorylating reagent that would be: (*i*) compatible with automated phosphoramidite chemistry; (*ii*) functionalized with a reporter group to permit accurate evaluation of its coupling efficiency; and (*iii*) capable of generating thiophosphate monoester derivatives of oligonucleotides, such as in **3**, under mild temperature conditions (∼23 °C) to prevent premature thermolytic cleavage of these thiophosphate protecting groups.

The phosphorylating agent **11** was designed to fulfill all of the above requirements and was prepared in three steps from methyl 2-mercaptoacetate (Scheme 1). Specifically, methyl 2-mercaptoacetate was first functionalized with the DMTr reporter group upon reaction with DMTrCl in

Scheme 1. Synthesis of Phosphoramidite **11**



pyridine,[9] affording methyl *S*-(4,4-dimethoxytrityl)-2-mercaptoacetate. The crude ester was then treated with $LiAlH_4$ in THF to give **10** in 90% yield.[10] Condensation of **10** with i-Pr_2NPCl_2 and i-Pr_2NEt in anhydrous MeCN proceeded smoothly, as indicated by ^{31}P NMR analysis of the reaction mixture. Complete conversion of i-Pr_2NPCl_2 (δ_P 170 ppm) to the phosphoramidite **11** (δ_P 148 ppm) occurred within 2 h at 25 °C. Purification of the reaction product was accomplished by silica gel chromatography, affording **11** in an isolated yield of 82%. The parameters for optimal coupling efficiency of **11** were first investigated by performing manual syntheses of 5′-phosphate/thiophosphate monoester derivatives of commercial deoxyribonucleosides covalently attached to controlled-pore glass (CPG) through a 3′-*O*-succinyl linker (**12a**−**d**, Scheme 2). Typically, a 0.1 M solution of activated **11** in MeCN was mixed with 5′-*O*-detritylated **12a**−**d** for 3 min. A treatment with 0.1 M ethyl(methyl)dioxirane[11,12] in CH_2Cl_2 for 1 min or 0.05 M 3*H*-1,2-benzodithiol-3-one-1,1-dioxide[13] in MeCN for 2 min was performed and resulted in the formation of **13a**−**d** or **14a**−**d** in yields exceeding 95%. Exposure of **13a**−**d** or **14a**−**d** to 3% TCA in CH_2Cl_2 for 9 min and then to a solution of 1.2% (w/v) DTT and 5% (v/v) i-Pr_2NEt in H_2O for 1 h at ambient temperature produced **15a**−**d** or **16a**−**d**. Subsequent reaction with $MeNH_2$ gas (∼2.5 bar) for 30 min or concd NH_4OH for 10 h at 55 °C cleaved the nucleobase protecting groups and released **17a**−**d** or **18a**−**d** from the solid support. When **12a**−**d** is replaced with **19a**−**d** under identical conditions, the corresponding deoxyribonucleoside 3′-phosphate/thiophosphate monoesters **20a**−**d** or **21a**−**d** are produced in yields comparable (±3%) to those obtained when employing **6** or **8** as the phosphorylating reagent. The phosphate monoesters **17a**−**d** and **20a**−**d** were analyzed by RP-HPLC (data shown in the Supporting Information) and exhibited chromatographic profiles identical to those of authentic deoxyribonucleoside 5′-monophosphates or 3′-monophosphates obtained from commercial sources. To further assess the scope and limitations of **11** as a phosphorylating reagent, the preparation of oligonucleotide 5′-phosphate/thiophosphate monoesters was undertaken. Specifically, the automated

(3) (a) Uhlmann, E.; Engels, J. *Tetrahedron Lett.* **1986**, *27*, 1023−1026. (b) Thuong, N. T.; Chassignol, M. *Tetrahedron Lett.* **1987**, *28*, 4157−4160. (c) Szczepanik, M. B.; Désaubry, L.; Johnson, R. A. *Tetrahedron Lett.* **1998**, *39*, 7455−7458.

(4) (a) Horn, T.; Urdea, M. S. *Tetrahedron Lett.* **1986**, *27*, 4705−4708. (b) Horn, T.; Allen, J. S.; Urdea, M. S. *Nucleosides Nucleotides* **1987**, *6*, 335−340.

(5) Olesiak, M.; Krajewska, D.; Wasilewska, E.; Korczyński, D.; Baraniak, J.; Okruszek, A.; Stec, W. J. *Synlett* **2002**, 967−971.

(6) (a) Lefebvre, I.; Périgaud, C.; Pompon, A.; Aubertin, A.-M.; Girardet, J.-L.; Kirn, A.; Gosselin, G.; Imbach, J.-L. *J. Med. Chem.* **1995**, *38*, 3941−3950. (b) Tosquellas, G.; Alvarez, K.; Dell'Aquila, C.; Morvan, F.; Vasseur, J.-J.; Imbach, J.-L.; Rayner, B. *Nucleic Acids Res.* **1998**, *26*, 2069−2074.

(7) Guzaev, A.; Salo, H.; Azhayev, A.; Lönnberg, H. *Tetrahedron* **1995**, *51*, 9375−9384.

(8) Lartia, R.; Asseline, U. *Tetrahedron Lett.* **2004**, *45*, 5949−5952.

(9) Rademann, J.; Schmidt, R. R. *J. Org. Chem.* **1997**, *62*, 3650−3653.

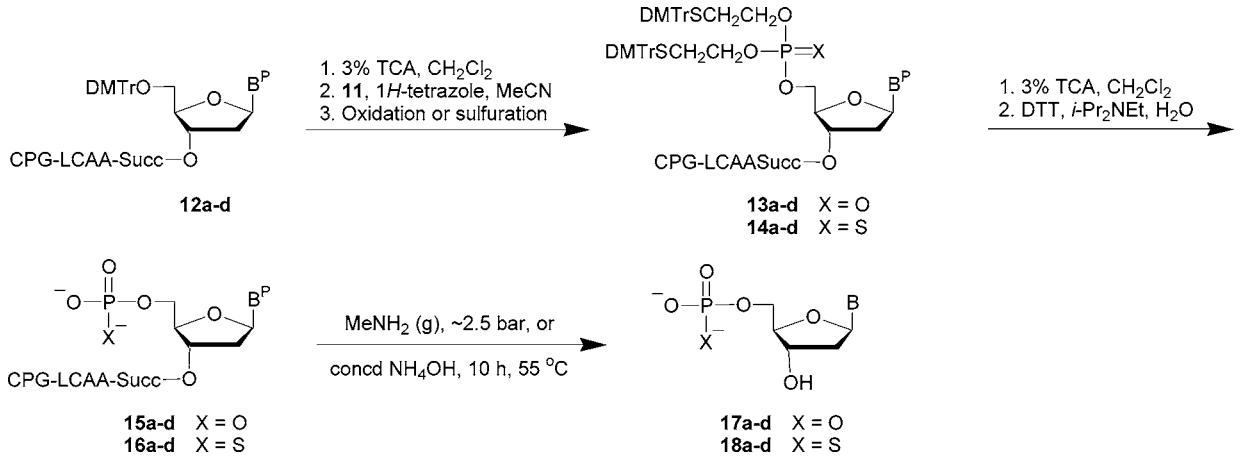
(10) The detailed preparation and characterization of **10** and **11** is provided in the Supporting Information.

(11) Kataoka, M.; Hattori, A.; Okino, S.; Hyodo, M.; Asano, M.; Kawai, R.; Hayakawa, Y. *Org. Lett.* **2001**, *3*, 815−818.

(12) The use of ethy(methyl)dioxirane as an oxidant over iodine or *tert*-butyl hydroperoxide produced **17a**−**d** in higher yields.

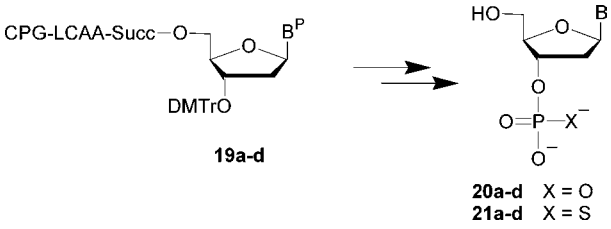
(13) (a) Iyer, R. P.; Phillips, L. R.; Egan, W.; Regan, J. B.; Beaucage, S. L. *J. Org. Chem.* **1990**, *55*, 4693−4699. (b) Regan, J. B.; Phillips, L. R.; Beaucage, S. L. *Org. Prep. Proc. Int.* **1992**, *24*, 488−492.

Scheme 2. Synthesis of 5′-Phosphate/Thiophosphate Monoester Derivatives of Deoxyribonucleosides[a]



[a] Keys: CPG-LCAA-Succ, succinyl long-chain alkylamine controlled-pore glass; TCA, trichloroacetic acid; DTT, dithiothreitol. B^P, **a** = thymin-1-yl, **b** = N^4-benzoylcytosin-1-yl, **c** = N^6-benzoyladenin-9-yl, **d** = N^2-isobutyrylguanin-9-yl; B, **a** = thymin-1-yl, **b** = cytosin-1-yl, **c** = adenin-9-yl, **d** = guanin-9-yl.

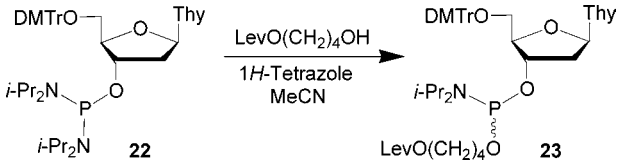
solid-phase syntheses of $_{P}$d($T_PA_PC_PG$) and $_{PS}$d($T_{PS}A_{PS}C_{PS}G$) and that of their 5′-unphosphorylated congeners d($T_PA_PC_PG$) and d($T_{PS}A_{PS}C_{PS}G$) were performed according to a modified synthesis protocol.[14] Upon completion of the syntheses, a manual detritylation of the 5′-phosphate/thiophosphate triester derivative of the solid-phase-linked tetranucleotides was performed along with the required DTT treatment and final nucleobase deprotection steps. The tetranucleotides were released from the support during nucleobase deprotection. RP-HPLC analysis of the deprotected oligonucleotides demonstrated that the terminal 5′-phosphate/thiophosphate monoesters were formed in yields exceeding 95%.[15]



The potential application of **11** to the 5′-phosphorylation of a much larger oligonucleotide was also investigated. The solid-phase syntheses of $_{P}$d($A_PT_PC_PC_PG_PT_PA_PG_PC_PT_PA_P$-$A_PG_PG_PT_PC_PA_PT_PG_PC$), $_{PS}$d($A_{PS}T_{PS}C_{PS}C_{PS}G_{PS}T_{PS}A_{PS}G_{PS}C_{PS}T_{PS}$-$A_{PS}A_{PS}G_{PS}G_{PS}T_{PS}C_{PS}A_{PS}T_{PS}G_{PS}C$), and that of their 5′-unphosphorylated counterparts were accomplished in a manner identical to that of the tetranucleotides.[14] RP-HPLC analysis of the fully deprotected 20-mers indicates that the use of **11** led to the formation of oligonucleotides 5′-phosphate/thiophosphate in yields exceeding 95%.[15] In addition to MALDI-TOF mass spectrometry characterization, the identity of the oligonucleotide 5′-phosphate monoester derivative was further confirmed by conversion to its 5′-unphosphorylated homologue upon reaction with bacterial alkaline phosphatase (data shown in the Supporting Information). These results underscore the use of **11** as an efficient reagent in the preparation of 5′-phosphate/thiophosphate monoester derivatives of deoxyribonucleosides and DNA oligonucleotides.

The application of **11** to the preparation of thermolytic oligonucleotides through the use of a dinucleotide model was then evaluated. The phosphoramidite **23** was first prepared by reacting the diamidite **22**[16] with an equimolar amount of 4-hydroxybutyl levulinate[17] in the presence of 1*H*-tetrazole in MeCN (Scheme 3). Crude **23** was purified by silica gel chromatography and was characterized by ^{31}P NMR spectroscopy and high-resolution mass spectrometry. Condensation of purified **23** (20 molar equiv) with detritylated **12a** and 1*H*-tetrazole (40 molar equiv) in MeCN, followed by exposure to 0.05 M 3*H*-1,2-benzodithiol-3-one-1,1-dioxide in MeCN, afforded the solid-phase-linked dinucleoside

Scheme 3. Preparation of Phosphoramidite **23**[a]



[a] Keys: Lev, levulinyl; Thy, thymin-1-yl.

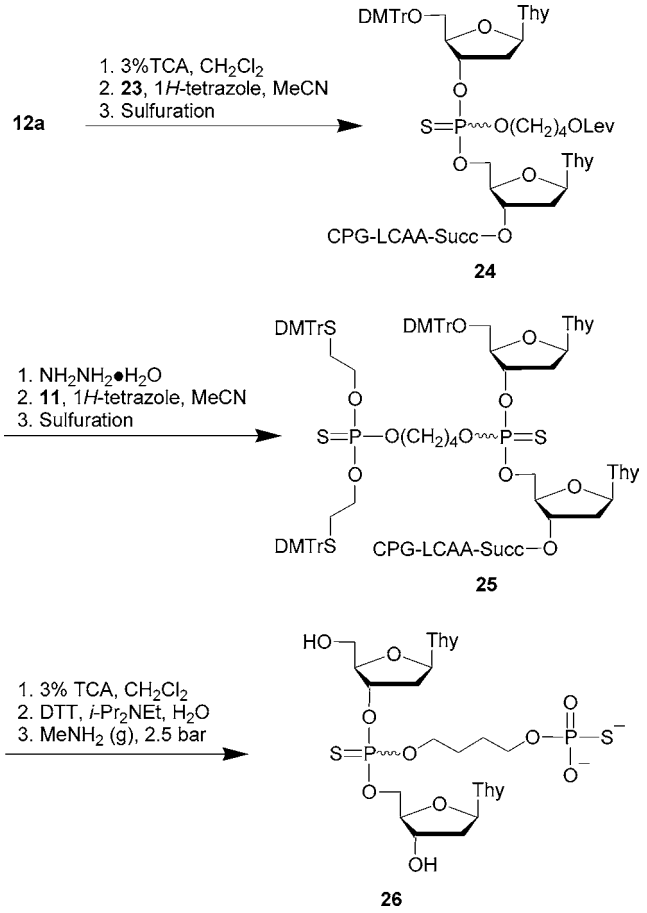
(14) Experimental parameters and conditions for the automated synthesis of 5′-phosphorylated oligonucleotides are provided in the Supporting Information.

(15) RP-HPLC profiles of crude and deprotected oligonucleotides are shown in the Supporting Information along with the MALDI-TOF mass spectral analysis of each oligonucleotide.

(16) Marugg, J. E.; Burik, A.; Tromp, M.; van der Marel, G. A.; van Boom, J. H. *Tetrahedron Lett.* **1986**, *27*, 2271−2274.

(17) Tsukamoto, H.; Kondo, Y. *Synlett* **2003**, 1061−1063.

Scheme 4. Synthesis of the Thermolytic Dinucleotide **26**



thiophosphate triester **24** (Scheme 4). Hydrazinolysis of **24** followed by phosphitylation with activated **11** gave **25** in ~98% yield after standard sulfuration effected by 0.05 M 3*H*-1,2-benzodithiol-3-one-1,1-dioxide in MeCN. Complete detritylation of **25** followed by treatment with pressurized methylamine gas afforded the thermolytic dinucleotide **26** with minimal thermolytic cleavage of the thiophosphate protecting group; less than 2% of the dinucleoside phosphorothioate diester $T_{PS}T$ was detected by RP-HPLC analysis of crude **26**.[18] The use of **8** instead of **11** led to the relatively sluggish formation of **26** at ambient temperature.[18] Attempts to accelerate the reaction upon heating resulted in extensive conversion of **26** to $T_{PS}T$. This experiment underscored the usefulness of **11** in the preparation of dinucleotides and, potentially, oligonucleotides functionalized with thermolytic alkylphosphorothioate monoester for thiophosphate protection. To this end, the synthesis of thermolytic DNA oligonucleotides **3** from the precursor oligonucleotides **2** is underway using **11** as the phosphorylating reagent. The results of this work will be reported in due course.

In summary, the phosphorylating reagent **11** was designed specifically for the functionalization of oligonucleotides analogous to **2** with phosphorothioate monoesters. The challenge is to efficiently produce thermolytic oligonucleotides **3** under conditions that would not induce premature cleavage of the thiophosphate protecting group. A dinucleotide model demonstrated the efficiency and uniqueness of reagent **11** in generating **26** with minimum thermolytic cleavage of the thiophosphate protecting group. Phosphorylating reagent **11** is also efficient in the preparation of deoxyribonucleoside 5′-/3′-phosphate/thiophosphate monoesters and DNA oligonucleotide 5′-phosphate/thiophosphate monoesters. Thus, by virtue of its attributes,[19] **11** is a valuable addition to the collection of phosphorylating reagents for functionalization of nucleosides and oligonucleotides.

Acknowledgment. This research is supported in part by an appointment to the Postgraduate Research Participation Program at the Center for Drug 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: Materials and methods; preparation of **10** and **11**; ^{31}P NMR spectrum of **11** in MeCN; general procedures for the preparation of **17a**−**d**, **18a**−**d**, **20a**−**d**, and **21a**−**d** using solid-phase techniques; RP-HPLC profiles of **17a**−**d**, **18a**−**d**, **20a**−**d**, and **21a**−**d**; comparative RP-HPLC profiles of **17d**, **18b**, **20c**, and **21b** that were prepared using **6**, **8**, or **11** as phosphorylating reagents; tables of RP-HPLC retention times for **17a**−**d**, **18a**−**d**, **20a**−**d**, and **21a**−**d**; solid-phase synthesis of oligonucleotide 5′-phosphate/thiophosphate monoesters along with RP-HPLC profiles, and MALDI-TOF analysis of each oligonucleotide; 5′-dephosphorylation of an oligonucleotide 5′-phosphate monoester catalyzed by bacterial alkaline phosphatase; synthesis of **23** and **26**; ^{31}P NMR spectrum of **26** in H_2O; RP-HPLC analysis of **26** and that of its thermal conversion to $T_{PS}T$. This material is available free of charge via the Internet at http://pubs.acs.org.

OL0516263

(18) Experimental details for the preparation of **26** from **24** using either **11** or **8** are provided in the Supporting Information along with relevant RP-HPLC profiles, MALDI-TOF mass spectral analysis, and ^{31}P NMR data.

(19) In addition to its coupling efficiency and enhanced reporting ability (two DMTr groups per mole), **11** is stable as a 0.05 M solution in MeCN over a period of 25 days at 25 °C. Under these storage conditions, only 15% decomposition was detected by ^{31}P NMR spectroscopy.