Preparation of Thymine Dinucleotide Methylphosphonate Analogs via Thymine Methylphosphonofluoridate

Michael C. Pirrung*,[1] and N. Chidambaram

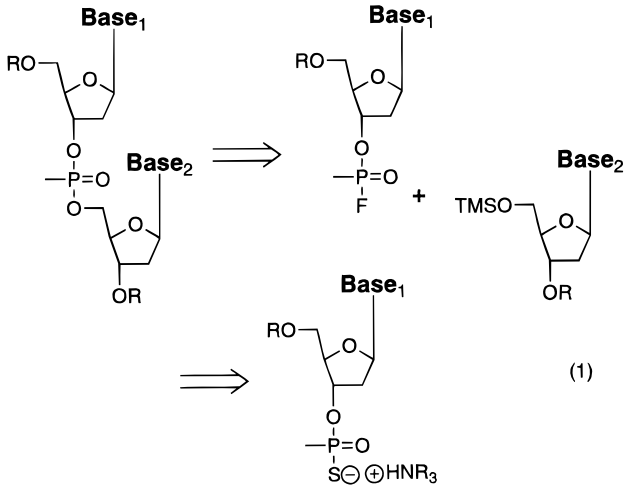
Department of Chemistry, P. M. Gross Chemical Laboratory, Duke University, Durham, North Carolina 27708-0346

Received July 28, 1995

Analogs of the charged and enzymatically-labile phosphodiester bonds in oligonucleotides have been used in the antisense approach to drug development which targets nucleic acids.[2] Methylphosphonates,[3] originally prepared by Miller and Ts'o using phosphonodiester chemistry,[4] can be prepared using either tricoordinate or tetracoordinate phosphorus chemistry.[5] Methods that have been developed recently for the synthesis of methylphosphonate nucleotides have focused on phosphorus(V) derivatives in which active esters of 3′-methylphosphonates are coupled to 5′-alcohols. Stec and co-workers have reported the synthesis of oligomers up to pentamers using DBU/LiCl to couple diastereomerically-pure nucleoside selenomethyl methylphosphonates with the 5′-alcohols of protected nucleosides.[6] The use of hexafluoroisopropyl[7] and *p*-nitrophenyl[8] leaving groups with *t*-BuMgCl as base is also a known coupling protocol.

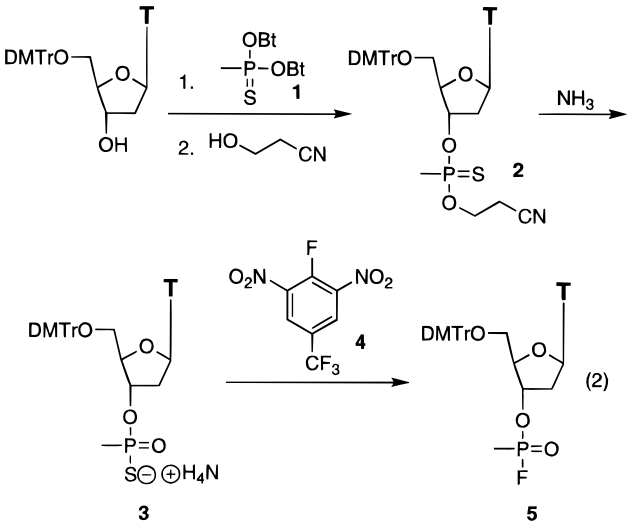
We were interested in developing a method that would allow near "reagent-less" couplings to be conducted in solution to facilitate the preparation of methylphosphonate dinucleotides on a significant scale. We focused on the condensation of 5′-*O*-(trimethylsilyl)nucleosides with a 3′-*O*-methylphosphonofluoridate to produce the dinucleotide and the volatile trimethylsilyl fluoride (eq 1). Methods for the preparation of phosphonofluoridates from phosphonothioates were known,[9] but an efficient method for the preparation of phosphonothioates was needed.

The *bis*(benzotriazolyl) phosphonothioate (**1**) is prepared by treating methylphosphonothioic dichloride with hydroxybenzotriazole by analogy to the results of van Boom, who has prepared *O*,*O*-*bis*(benzotriazolyl) active



(1)

ester derivatives of both alkyl phosphates and methylphosphonate.[10] Compound **1** can be stored as a stock solution and coupled with 5′-*O*-(dimethoxytrityl)thymidine. However, unlike van Boom's example with the P═O compounds, this coupling requires addition of the nucleophilic catalyst DMAP. Hydroxypropionitrile is coupled to the resulting monobenzotriazolyl phosphonothioate in the presence of N-methylimidazole to afford **2** as a mixture of diastereoisomers (eq 2). Treatment of **2**



(2)

with freshly-condensed ammonia in ethanol (∼1:3) for 1 h at room temperature yields salt **3**, which can be fluorinated in a convenient procedure using the commercially-available 4-fluoro-3,5-dinitrobenzotrifluoride (**4**) to give **5**. This material exhibits the expected large one-bond P−F coupling (J = 1049 Hz). The stoichiometry of fluorinating agent and the solvent affect this fluorination reaction, which could be monitored by ^{31}P NMR. When it is performed with 1 equiv of **4** in acetone, the thioester **6**, presumably an intermediate in the overall

(1) Fellow of the John Simon Guggenheim Memorial Foundation, 1994−1995.

(2) Zon, G. *Ann. N.Y. Acad. Sci.* **1990**, *616*, 161−72. Miller, P. S. *Bio/Technology* **1991**, *9*, 358−62. *Antisense Nucleic Acids Proteins*; Mol, J. N. M., Van der Krol, A. R., Eds.; Dekker: New York, 1991. Uhlmann, E.; Peyman, A. *Chem. Rev.* **1990**, *90*, 543−84.

(3) For recent reviews, see: Miller, P. S.; Cushman, C. D.; Levis, J. T. In *Oligonucleotides and Analogues*; Eckstein, F., Ed.; Oxford University Press: Oxford, 1991; pp 137−159. Miller, P. S.; Ts'o, P. O. P.; Hogrefe, R. I.; Reynolds, M. A.; Arnold, L. J., Jr. In *Antisense Research and Applications*; Crooke, S. T., Lebleu, B., Eds.; CRC Press, Inc.: Boca Raton, FL, 1993; pp 189−203.

(4) Miller, P. S.; Yano, J.; Yano, E.; Carroll, C.; Jayaraman, K.; Ts'o, P. O. P. *Biochemistry* **1979**, *18*, 5134.

(5) Vyazovkina, E. V.; Komarova, N. I.; Lebedev, A. V. *Bioorg. Khim.* **1993**, *19*, 86 and references cited therein.

(6) (a) Wozniak, L. A.; Pyzowski, J.; Wieczorek, M.; Stec, W. J. *J. Org. Chem.* **1994**, *59*, 5843. (b) Lesnikowski, Z. J.; Zabawska, D.; Jaworska-Maslanka, M. M.; Schinazi, R. F.; Stec, W. J. *New J. Chem.* **1994**, *18*, 1197.

(7) Lesnikowski, Z. J.; Wolkanin, P. J.; Stec, W. J. In *Proceedings of the Second International Symposium on Phosphorus Chemistry Directed Towards Biology*; Bruzik, K. S., Stec, W. J., Eds.; Elsevier Science Publishers B. V.: Amsterdam, 1987; pp 189−194. Lesnikowski, Z. J.; Wolkanin, P. J.; Stec, W. J. *Tetrahedron Lett.* **1987**, *28*, 5535. Lesnikowski, Z. J.; Jaworska-Maslanka, M. M.; Stec, W. J. *Nucleosides Nucleotides* **1991**, *10*, 733.

(8) Cormier, J. F.; Pannunzio, T. *Tetrahedron Lett.* **1991**, *32*, 7161.

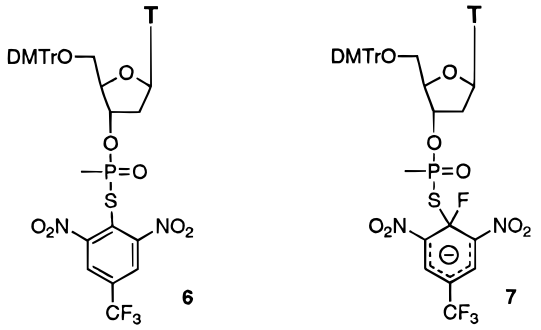
(9) Boter, H. L.; Ooms, A. J. J.; Van Der Berg, G. R.; Van Dijk, C. *Recl. Trav. Chim. Pays-Bas* **1966**, *85*, 147. Benschop, H. P.; De Jong, L. P. A. *Acc. Chem. Res.* **1988**, *21*, 368.

(10) Marugg, J. E.; de Vroom, E.; Dreef, C. E.; Tromp, M.; van der Marel, G. A.; van Boom, J. H. *Nucl. Acids Res.* **1986**, *14*, 2171.

(11) Attempts to form the diastereomerically-pure phosphonofluoridate from chromatographically-separated diastereomers of **2** by analogy to earlier results in the preparation of optically-active Sarin (isopropyl methylfluorophosphonate)[9] (using picryl fluoride) failed. It is unclear if this is related to the relatively subtle change to fluorinating agent **4** or to the difference between isopropoxy and the nucleoside 3′ oxygen. We were unable to reproduce literature preparations of picryl fluoride.

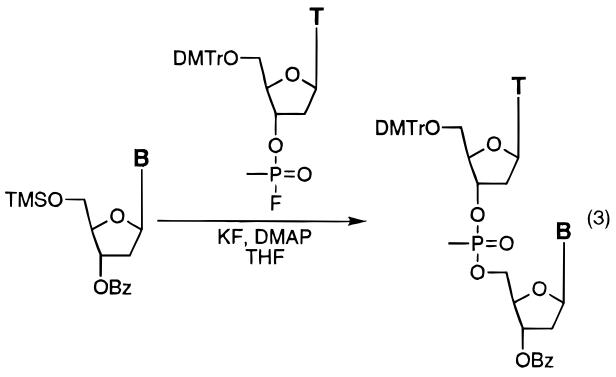
0022-3263/96/1961-1540$12.00/0

fluorination process, also results. In pyridine, it gives **5** and a compound postulated to be Meisenheimer complex **7** [$J_{P-S-C-F}$ = 47 Hz).[11]



To serve as coupling partners for **5**, 3′-*O*-benzoyl-dT and the 3′-*O*-benzoyl-protected nucleosides dC^{Bz} and dA^{Bz} were silylated using hexamethyldisilazane and chlorotrimethylsilane in pyridine at room temperature for 0.25 h[12] to afford 5′-*O*-(trimethylsilyl)deoxynucleosides **8**−**10** in near-quantitative yields. 3′-*O*-Benzoyl(N^2-*i*-Bu)dG was silylated with neat TMSCN to give a mixture of **11** and starting material in a 2:1 ratio.

Thymine dinucleoside methylphosphonate analogs can be prepared by coupling **5** with trimethylsilyl ethers **8**−**11** in the presence of KF[13] and DMAP (useful in preventing detritylation) in THF at 60 °C for 24−48 h (eq 3). Control experiments show that both additives are required, though an explicit role in the reaction has been ascribed to neither. Other fluoride sources (CsF and Bu_4-NF) and milder conditions (with 18-crown-6) do not lead to cleaner products. The isolated yields of the dinucleotides are good (68−79%), and the products are readily obtained in pure form by filtration and chromatography, removing salts and any unreacted starting material. No aqueous workup is required.



8	B=T	**12**	B=T
9	B=C^{Bz}	**13**	B=C^{Bz} **5**
10	B=A^{Bz}	**14**	B=A^{Bz}
11	B=$G^{i\text{-}Bu}$	**15**	B=$G^{i\text{-}Bu}$

Experimental Section

***Bis*-*O*-(1-Benzotriazolyl) Methylphosphonothioate (1).** A solution of methylphosphonothioic dichloride (0.79 mL, 1.12 g, 7.5 mmol, Johnson Matthey) in anhydrous dioxane (15 mL) was added dropwise to a stirring solution of 1-hydroxybenzotriazole (2.05 g, 5.2 mmol, dried in a drying pistol over refluxing toluene) and pyridine (1.5 mL) in anhydrous dioxane (50 mL) at room temperature. The reaction mixture was stirred for 2 h, and the pyridinium hydrochloride salts were removed by filtration under anhydrous conditions to give a stock solution of **1** (0.11M). ^{31}P NMR (dioxane): δ 121.31.

5′-*O*-(Dimethoxytrityl)thymidine 3′-*O*-(2″-cyanoethyl methylphosphonothioate) (2).[14] 5′-*O*-(Dimethoxytrityl)thymidine (1.09 g, 2 mmol) was dissolved in anhydrous pyridine (5 mL) and evaporated to dryness. This material was dissolved in CH_3CN (15 mL) and transferred through a cannula to a flask containing a solution of **1** (22 mL, 1.21 mmol) and DMAP (0.27 g, 2.2 mmol, dried in a drying pistol over refluxing toluene). After 0.5 h, 3-hydroxypropionitrile (0.35 g, 5 mmol, 0.34 mL) was added followed by *N*-methylimidazole (0.8 mL, 10 mmol). Stirring was continued for 2 h. Solvents were removed *in vacuo*, and the residue was dissolved in CH_2Cl_2 (40 mL), washed with 0.1 M triethylammonium bicarbonate (15 mL), and water (15 mL), dried over $MgSO_4$, and concentrated. The crude compound was purified by flash chromatography (CH_2Cl_2:EtOH 98:2) to afford **2** (1.12g, 81%) as a mixture of diastereoisomers. R_f: 0.49, 0.53 (EtOH:$CHCl_3$ 6:94). ^{1}H NMR ($CDCl_3$): δ 8.48 (s, 1H), 8.44 (s, 1H), 7.60 (s, 1H), 6.46 (m, 1H), 6.43 (m, 1H), 5.45 (m, 1H), 4.21 (d, J = 2.2 Hz, 1H), 3.81 (s, 6H), 3.46 (m, 2H), 2.77 (t, J = 6.2 Hz, 2H), 2.56 (m, 1H), 2.46 (m, 1H), 1.91 (d, J = 14.1 Hz, 3H), 1.86 (d, J =14.4 Hz, 3H), 1.48 (s, 3H), 1.46 (s, 3H). ^{31}P NMR ($CDCl_3$): δ 98.30, 98.77 (identical to literature).

5′-*O*-(Dimethoxytrityl)thymidine 3′-*O*-Methylphosphonothioic Acid Ammonium Salt (3). Freshly-condensed NH_3 in EtOH (∼1:3, 3 mL) was added to **2** (0.054 g, 0.078 mmol), and the solution was stirred for 1 h at room temperature to afford **3**. ^{31}P NMR (EtOH): δ 77.38, 78.19. This material was used without further purification.

5′-*O*-(Dimethoxytrityl)thymidine 3′-*O*-Methylphosphonofluoridate (5). The salt obtained from the above reaction (0.078 mmol) was dried over anhydrous pyridine and dissolved in acetone (2 mL). Compound **4** (0.04 g, 0.16 mmol, Marshallton Research Labs) was added, and the reaction mixture was stirred at room temperature for 1−1.5 h. Progress of the reaction was monitored by phosphorus NMR. ^{31}P NMR (acetone): δ 31.84 (d, J = 1048.42 Hz), 31.90 (d, J = 1049.46 Hz). Evidence for the elemental composition of this compound could not be obtained because of its instability.[15]

5′-*O*-(Dimethoxytrityl)thymidine 3′-O-((*S*-(2″,6″-Dinitro-4″-(trifluoromethyl)phenyl)methylphosphonothioate) (6). ^{31}P NMR (acetone): δ 90.85, 91.36. Evidence for the elemental composition of this compound could not be obtained because of its instability.

General Procedure for the Preparation of 5′-*O*-(Trimethylsilyl)-3′-*O*-benzoyl deoxynucleosides 8−10. Hexamethyldisilazane (1.2 mL, 0.92 g, 5.69 mmol) and chlorotrimethylsilane (0.6 mL, 0.51 g, 4.72 mmol) were added to 3′-*O*-benzoylthymidine[14] (0.4 g, 1.16 mmol) in pyridine (4 mL) and stirred at room temperature for 0.25 h. The pyridine was removed *in vacuo*, and its final traces were removed by coevaporating with toluene (10 mL). Diethyl ether (75 mL) or CH_2Cl_2 (40 mL) was added to precipitate the products.

5′-*O*-(Trimethylsilyl)-3′-*O*-benzoylthymidine (8) (0.46 g, 95%). Mp: 169.9−171.9 °C. R_f: 0.56 (EtOH:$CHCl_3$ (6:94)). ^{1}H NMR ($CDCl_3$): δ 9.55 (s, 1H), 8.05 (d, J = 7.3 Hz, 2H), 7.73 (s, 1H), 7.60 (br t, J = 7.5, 7.3 Hz, 1H), 7.47 (br t, J = 7.7, 7.5 Hz, 2H), 6.51 (dd, J = 9.0, 5.5 Hz, 1H), 5.48 (d, J = 5.7 Hz, 1H), 4.27 (s, 1H), 3.93 (d, J = 1.9 Hz, 2H), 2.26, 2.55 (m, 2H), 1.94 (s, 3H), 0.20 (s, 9H). HRMS calcd for $C_{20}H_{27}N_2O_6Si$ [M + 1]: 419.526, found 419.1638.

5′-*O*-(Trimethylsilyl)-N^4,3′-*O*-dibenzoyl-2′-deoxycytidine (9) (0.21 g, 95%), colorless solid, mp dec ∼180 °C. R_f: 0.52 (EtOH:$CHCl_3$ 6:94). ^{1}H NMR ($CDCl_3$): δ 8.47 (d, J = 7.5 Hz, 1H), 8.06 (d, J = 7.2 Hz, 1H), 7.92 (d, J = 7.5 Hz, 1H), 7.64−7.46 (m, 7H), 6.53 (dd, J = 8, 6 Hz, 1H), 5.52 (d, J = 6.1 Hz, 1H), 4.40 (d, J = 1.8 Hz, 1H), 3.98 (d, J = 1.7 Hz, 2H), 2.29,

(12) Sweeley, C. C.; Bentley, R.; Makita, M.; Wells, W. W. *J. Am. Chem. Soc.* **1963**, *85*, 2497.

(13) Phosphorofluoridates have been coupled with silanes using KF promotion: Semchenko, F. M.; Eremin, O. G.; Martynov, B. I. *Zh. Obshch. Khim.* **1992**, *62*, 473.

(14) Niewiarowski, W.; Lesnikowski, Z. J.; Wilk, A.; Guga, P.; Okruszek, A.; Uznanski, B.; Stec, W. J. *Acta Biochim. Pol.* **1987**, *34*, 217. Lebedev, A. V.; Rife, J. P.; Wickstron, E. *Tetrahedron Lett.* **1990**, *31*, 855.

(15) Misiura, K.; Pietrasiak, D.; Stec, W. J. *J. Chem. Soc., Chem. Commun.* **1995**, 613−614.

2.91 (m, 2H), 0.19 (s, 9H). HRMS calcd for $C_{26}H_{30}N_3O_6Si$ [M + 1]: 508.636, found 508.1898.

5′-*O*-(Trimethylsilyl)-*N*^{6},3′-*O*-dibenzoyl-2′-deoxyadenosine (10) (0.25 g, 97%), pale yellow solid, mp 143.5−144.5 °C. R_f: 0.45 (EtOH:$CHCl_3$ 6:94). ^{1}H NMR ($CDCl_3$): δ 9.07 (br s, 1H), 8.84 (s, 1H), 8.52 (s, 1H), 6.76 (dd, J = 7.9, 6.4 Hz, 1H), 5.69 (dd, J = 3.0, 1.6 Hz, 1H), 4.45 (d, J = 1.6 Hz, 1H), 3.97 (d, J = 2.4 Hz, 2H), 2.86 (m, 2H), 0.21 (s, 9H). HRMS calcd for $C_{27}H_{30}N_5O_5Si$ [M + 1]: 532.686, found 532.2021.

5′-*O*-(Trimethylsilyl)-*N*^{2}-isobutyroyl-3′-*O*-benzoyldeoxyguanosine (11). Cyanotrimethylsilane (0.13 mL, 0.094 g, 0.95 mmol) was added to 3′-*O*-benzoyldeoxyguanosine[16] (0.42 g, 0.95 mmol), and the solution was stirred at room temperature under nitrogen for 0.5 h. The gas outlet was passed through sodium hydroxide to trap the released hydrocyanic acid. TLC showed starting material, and ^{1}H NMR showed a starting material to silyl ether ratio of 1:2. R_f: 0.45 (EtOH:$CHCl_3$ 6:94). ^{1}H NMR ($CDCl_3$): δ 8.36 (s, 1H), 6.35 (m, 1H), 5.67 (d, J = 5.73 Hz, 1H), 4.38 (d, J = 1.96 Hz, 1H), 3.88 (m, 2H), 2.83 (m, 1H), 2.67 (m, 2H), 1.28 (m, 6H), 0.15 (s, 9H). HRMS calcd for $C_{24}H_{32}N_5O_6Si$ [M + 1]: 514.626, found 514.2114. Attempts to purify this compound by column chromatography or preparative TLC were not successful, and it was used as obtained.

General Procedure for the Synthesis of Dinucleoside Methylphosphonates. Acetone was evaporated from the 5′-silyl ether (0.075 mmol) under vacuum, and the resulting material was handled in an inert atmosphere box. THF (2 mL) was added, and the dissolved material was added to **5** (0.063 g, 0.15 mmol) in a V-Vial, with 2 × 1 mL washings of THF to transfer all of the material. KF (0.022 g, 0.38 mmol) and DMAP (0.27 g, 0.22 mmol) were added. The vial was sealed tightly and stirred on a heating block at 60 °C, with reaction monitoring by ^{31}P NMR. After the reaction was complete, the mixture was filtered through a small pad of Celite which was then washed with CH_2Cl_2:EtOH (90:10). The filtrate was concentrated to a crude material that was purified by flash chromatography using EtOH:$CHCl_3$ (2−6%) to obtain the pure dinucleotide.

T-T (12)[6a] (0.057 g, 79%). R_f: 0.36 (EtOH:$CHCl_3$ 6:94). Faster: ^{1}H NMR ($CDCl_3$) δ 1.54 (d, J = 17.7 Hz, 3H); ^{31}P NMR ($CDCl_3$) δ 32.78. Slower: ^{1}H NMR ($CDCl_3$) δ 1.59 (d, J = 17.4 Hz, 3H); ^{31}P NMR ($CDCl_3$) δ 32.20.

T-C (13)[17] (0.057 g, 70%). R_f: 0.52, 0.56 (EtOH:$CHCl_3$ 10:90). Faster: ^{1}H NMR ($CDCl_3$) δ 1.52 (d, J = 17.4 Hz, 3H); ^{31}P NMR ($CDCl_3$) δ 32.58. Slower: ^{1}H NMR ($CDCl_3$) δ 1.60 (d, J = 17.4 Hz, 3H); ^{31}P NMR ($CDCl_3$) δ 33.10.

T-A (14)[18] (0.054 g, 68%). R_f: 0.54, 0.58 (EtOH:$CHCl_3$ 10:90). Faster: ^{1}H NMR ($CDCl_3$) δ 1.48 (d, J = 17.4 Hz, 3H); ^{31}P NMR ($CDCl_3$) δ 32.45. Slower: ^{1}H NMR ($CDCl_3$) δ 1.54 (d, J = 17.4 Hz, 3H); ^{31}P NMR ($CDCl_3$) δ 32.91.

T-G (15)[6a] (0.075 g, 72%). R_f: 0.55, 0.59 (EtOH:$CHCl_3$ 12:88). Faster: ^{1}H NMR ($CDCl_3$) δ 1.46 (d, J = 17.4 Hz, 3H); ^{31}P NMR ($CDCl_3$) δ 33.71. Slower: ^{1}H NMR ($CDCl_3$) δ 1.55 (d, J = 17.4 Hz, 3H); ^{31}P NMR ($CDCl_3$) δ 32.13.

Acknowledgment. Financial support was provided by Genta, Inc.

Supporting Information Available: NMR spectra of compounds **8**−**11** (4 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

JO951395L

(16) de Rooji, J. F. M.; Wille-Hazelger, G.; van Deursen, P. H.; Serdin, J.; van Boom, J. H. *Recl. Trav. Chim. Pays.-Bas* **1979**, *98*, 537.

(17) Vyazovkina, E. V.; Engels, J. W.; Lebedev, A. V. *Bioorg. Khim.* **1993**, *19*, 197.

(18) Kan, L. S.; Cheng, D. M.; Miller, P. S.; Yano, J.; Ts'o, P. O. P. *Biochemistry* **1980**, *19*, 2122.