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SYNTHESIS OF NUCLEOSIDE METHYLPHOSPHONOTHIOATES

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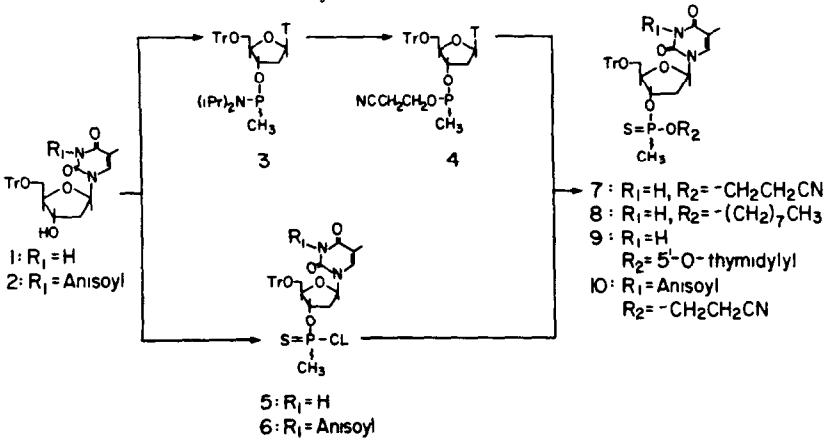
Deoxydinucleotide methylphosphonothioates containing 5'-5' and 3'-5' internucleotide linkages were synthesized regioselectively from nucleosides and methylphosphonothioic dichloride by successive displacement of chlorine atoms. The diastereomers were resolved by hplc.

Recent advances in nucleic acid chemistry have led to the development of several oligonucleotide analogs having chiral phosphorus internucleotide linkages and biochemical properties that are both interesting and different from the corresponding naturally occurring compounds. Because these features may prove useful for a wide range of biochemical and medicinal applications, much current research in the polynucleotide field has focused on the development of facile synthetic routes for the preparation of this group of compounds. Among the more interesting and potentially useful phosphorus derivatives of polynucleotides are the alkylphosphonothioates[1] and alkylphosphonates[2] primarily because these analogs are chiral and do not racemize easily in aqueous solution. Here we report our research directed toward the development of methods for synthesizing nucleoside and dinucleotide alkylphosphonothioates.

Nucleosides used for this study were 5'-O-tritylthymidine (**1**) and 5'-O-trityl-N^3-anisoylthymidine (**2**). The latter compound proved especially valuable primarily because the nucleoside O,O-dialkylmethylphosphonothioate diastereomers of **10** were more easily resolved chromatographically than the corresponding derivatives (compound **7**) of **1**. Compound **2** was synthesized in a one flask procedure from **1** via transient protection of the 3'-hydroxyl group[3] with lithium hexamethyldisilazanide and *t*-butyldimethylsilylchloride in THF (1 hr), subsequent addition of anisoyl chloride and fluoride mediated desilylation.[4]



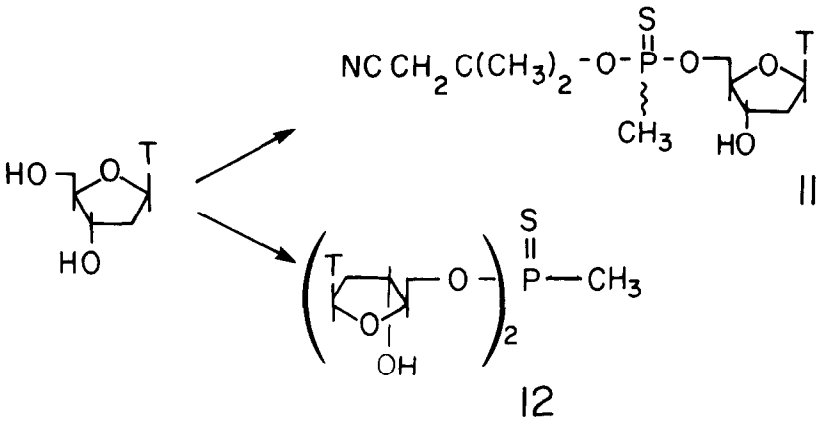
Initial attempts to synthesize dialkylmethylphosphonothioates (**7-10**) were based upon earlier investigations with O-alkylmethylphosphonochloridothioates.[5] When **1** and **2** were reacted with O-cyanoethyl- or O-n-octylmethylphosphonochloridothioates [6-8] in pyridine at room tempera-

ture, the yields of **7**, **8**, and **10** were very low and several uncharacterized side-products were formed. The major limitation of this approach appeared to be the thermal instability of O-alkylmethylphosphonochloridothioates during distillative work-up. The compounds could not be isolated pure for subsequent reactions.

Other initial attempts to synthesize compounds **7-10** were based upon modifications of earlier reports on the synthesis of nucleoside methylphosphonates via phosphonamidite intermediates.[9,10] The methylphosphonamidite (**3**) was prepared by condensation of 1 mmol of **1** with bis (N,N-diisopropylamino)methylphosphine[11] (1.5 mmol) in the presence of 1 g anhydrous Dowex-50WX-4 (pyridinium form)[12] and acetonitrile (12 hr). The yield of **3** was 68% after flash chromatography (toluene:ethylacetate:triethylamine; 65:30:5, v/v/v) and precipitation into pentane.[13] Formation of a symmetrical 3'-3' dinucleoside methylphosphonite was not observed on an analytical tlc plate. Activation of **3** with tetrazole,[10] condensation with cyanoethanol, and subsequent oxidation with elemental sulfur in CS_2 leads mainly to decomposition products due presumably to the lability of **4** to moisture, heat and transesterification.[10,14]

Successful syntheses of **7-10** were completed using methylphosphonothioic dichloride. Investigations with this reagent were prompted by earlier research indicating that the chlorine substituents could be selectively displaced by alkoxy groups.[5] Protected deoxynucleoside (**1** or **2**, 0.5 mmol) was first treated with methylphosphonothioic dichloride (224 mg, 1.5 mmol) and anhydrouspyridine (161 µl, 2 mmol) in dry CH_2CL_2 (5 ml)[15] for 17 hr in order to obtain quantitatively either **5** (^{31}P NMR δ 97.0) or **6**, (^{31}P NMR δ 97.3). Dimerization to form the 3'-O-bis-(thymidilyl) methylphosphonothioate derivative was not observed. Addition of 6 molar equivalents of the appropriate primary alcohol in anhydrous pyridine (2 ml) gave (4hr) **7**, **8** or **10** in 70-76% yield after flash column chromatography and precipitation into pentane. Of considerable interest was the relative separability of the diastereomers of **7**, **8**, and **10** via flash column chromatography or preparative tlc. It was observed that the diastereomers of **8** and **10** could be separated whereas the diastereomers of **7** were not resolved using either procedure.[16-18]

Of particular interest was the observation that the alkoxymethylchloridothioates react regioselectively with deoxynucleosides. Initially this was shown using O-α,α-dimethy-β-cyanoethylmethyl phosphonochloridothioate, a sterically bulky model compound. This reagent was synthesized by reacting 3,3-dimethyl-3-hydroxypropionitrile (247 µl, 2 mmol) with methylphosphonothioic dichloride (223 mg, 1.5 mmol) in the presence of N-methylimidazole (159 µl, 2 mmol) for two hours at 35°C. ^{31}P NMR of the reaction mixture showed one peak at 87.1 ppm having 85% of the total integral value of all ^{31}P containing components. When this phosphonothioating reagent was reacted with thymidine (986 mg, 4 mmol) in pyridine (12 ml) for 48 hr, only compound **11** was formed (56%).[19] Extension of this observation led to the unexpected result that the symmetrical **12** was formed in quantitative yield[20] when thymidine (987 mg, 4 mmol) was reacted with methylphosphonothioic dichloride (149 mg, 1 mmol) in anhydrous pyridine (2 ml) and 20 ml CH_2Cl_2. A regioselective synthesis strategy was therefore used to synthesize **9**. The first step was preparation of **5**. Methylphosphonothioic dichloride (223 mg, 1.5 mmol), **1** (242 mg, 0.5 mmol), and anhydrous pyridine (162 µl, 2 mmol) in dry CH_2CL_2 (5 ml) were allowed to react for 17 hr. Thymidine (986 mg, 4 mmol) in anhydrous pyridine (2 ml) was added and the reaction allowed to continue (3 hr). The reaction mixture was extracted twice with water to remove **12**. The organic



11

12

layer was concentrated to a dry foam and the product, a mixture of diastereomers, was fractionated from side-products and starting material by reversed phase tlc (acetone: H_2O; 3:7, v/v). The diastereomers of **9** were then separated by reverse phase hplc (CH_3CN:H_2O; 3:2, v/v) with an overall isolated yield of 56% from **1**, and characterized.[21]

These results outline a pathway for synthesizing thymidine dinucleotide methylphosphonothioates having a 3'-5' internucleotide linkage. Extension of this synthetic route to the other deoxynucleosides followed by their incorporation into DNA should now be possible.

Acknowledgements

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4. F. W. Adamiak, M. Z. Barciszewska, E. Biala, K. Grzeskowiak, R. Kierzek, A. Kraszewski, W. T. Markiewicz and W. Wiewiorowski, *Nucleic Acids Res.* **3**, 3397-4015 (1976).
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6. ^{31}P NMR and ^{1}H NMR for all compounds were recorded in $CDCl_3$ unless otherwise specified. For ^{31}P NMR, 85% H_3PO_4 was the external reference. UV spectra were recorded in dichloromethane. R_f values refer to separation on silica gel tlc plates unless otherwise specified. All isomer fractionations on hplc were performed using a 250 mm X 10 mm Econosil C-18 column from Altech Applied Science.
7. O-β-Cyanoethylmethylphosphonochloridothioate. Mass Spectrum, 183 (M^+), 148 $(M-Cl)^+$; ^{31}P NMR δ 97.7; ^{1}H NMR δ 4.4 (M, α-CH_2), 2.9 (q, β-CH_2), 1.9-25 (ABX, 3, P-CH_3).
8. O-*n*-Octylmethylphosphonochloridothioate. ^{1}H NMR:δ 3.95 (d, α-CH_2 of n-octyl), 1.8-1.1 (m, CH_2 of n-octyl, P-CH_3), 0.88 (CH_3 of n-octyl).
9. M. A. Dorman, S. A. Noble, L. J. McBride and M. H. Caruthers, *Tetrahedron* **40**, 95-102 (1984).
10. A. Jäger and J. Engels, *Tetrahedron Lett* **25**, 1437-1440 (1984).
11. Bis (N,N-diisopropylamino)methylphosphine. ^{31}P NMR δ 131.6; ^{1}H NMR δ 4.5 (Hβ, $CH(CH_3)_2$), 3.3 (d J_{HP} = 18Hz, p-CH_3), 1.4 (d, $CH_3)_2$).

12. Dowex 50W-X4 (pyridinium form) was washed successively with anhydrous pyridine, dry acetonitrile and anhydrous ethyl ether. The polymer was then dried *in vacuo* over P_2O_5/KOH. Before formation of the amidite, the resin was co-evaporated 5 times with dry pyridine.
13. 5'-O-Tritylthymidine-3'-O-methylphosphono N,N-diisopropylamidite (**3**). FAB^+ mass spectrum, 630 (M^+), 467 (M - $OP(CH_3)(NCH(CH_3)_2)^+$, 386 (M - trityl)$^+$, 243 (trityl)$^+$; ^{31}P NMR δ 119.9; ^1H NMR δ 8.62 (s, H_6), 7.52-7.25 (m, trityl), 6.43 (q, H_1'), 4.50 (m, H_3'), 1.42 (s, 5-CH_3), 1.2-0.76 (m, P-CH_3, CH_3 of $CH(CH_3)_2$).
14. J. Nielson, J. E. Marugg, J. H. van Boom, J. Honnens, M. Taagaard, O. Dahl, *J. Chem. Res.*, 26-27 (1986).
15. Pyridine was distilled successively over toluene sulfonylchloride and barium oxide (3X) to remove secondary amines. CH_2Cl_2 was distilled from CaH_2.
16. 5'-O-Tritylthymidine 3'-O(O-β-cyanoethyl)methylphosphonothioate (**7**). FAB^+ Mass spectrum, 632 (M + 1)$^+$, 649 (M + H_2O)$^+$, 654 (M + Na)$^+$; FAB^- mass spectrum 577 (M-2-(CH_2 = C(H)CN))$^{--}$; ^{31}P NMR δ 98.8, 98.4 (I = 1:1.2); ^1H NMR δ 8.08 (s, H_3), 7.57 (s, H_6), 7.43-7.27 m, trityl), 6.45 (q, H_1'), 5.51 (m, H_3'), 4.21 (m, αCH_2 of $-CH_2CH_2CN$, H_4'), 2.78-2.52 (m, βCH_2 of $-CH_2CH_2CN$, H_2'), 1.89, 1.85 (d, J_{HP} = 15.5 Hz, P-CH_3), 1.57 (s, 5-CH_3); UV λ_{max} = 234, 266 nm; R_f = 0.33, 0.29 (CH_3CCl_3/CH_3OH, 9/1, v/v).
17. 5'-O-Tritylthymidine 3'-O-(O-*n*-octyl)methylphosphonothioate (**8**). Isomer A: FAB^+ mass spectrum, 689 (M + 1 + H_2O)$^+$, 713 (M + Na)$^+$; ^{31}P NMR δ 96.8; ^1H NMR δ 8.12 (s, H_3), 7.59 (s, H_3), 7.59 (s, H_6), 7.44-7.24 (m, trityl), 6.44 (q, H_1'), 5.53 (m, H_3'), 4.20 (m, H_4'), 4.1-3.96 (m, αCH_2 of *n*-octyl), 3.52-3.45 (m, H_5'), 2.54-2.4 (m, H_2'), 1.78 (d, J_{HP} = 15.5 Hz, P-CH_3), 1.42 (s, 5-CH_3), 1.87-1.23 (m, CH_2 of *n*-octyl), 0.87 (m, CH_3 of *n*-octyl); UV λ_{max} = 234,266 nm; R_f = 0.41 (CH_3CCl_3/CH_3OH, 95/5, v/v). Isomer B: ^{31}P NMR δ 97.1; ^1H NMR δ 8.3 (s, H_3), 7.58 (s, H_6), 7.43-7.21 (m, trityl), 6.45 (q, H_1'), 5.52 (m, H_3'), 4.21 (m, H_4'), 3.97-3.72 (m, αCH_2 of *n*-octyl), 3.47-3.37 (m, H_5'), 2.52-2.41 (m, H_2'), 1.84 (d, J_{HP} = 15.3 Hz, P-CH_3), 1.43 (s, 5-CH_3) 1.75-1.23 (m, CH_2 of *n*-octyl), 0.87 (m, CH_3 of *n*-octyl); R_f = (CH_3CCl_3/CH_3OH, 95/5, v/v).
18. 5'-O-Trityl-N^3-anisoylthymidine 3'-O(O-β-cyanoethyl)methylphosphonothioate (**10**). Isomer A: FAB^+ mass spectrum, 804 (M + K)$^+$, 788 (M + Na)$^+$; ^{31}P NMR δ 98.4; ^1H NMR δ 7.9 (AB, J_{HH} = 8.8 Hz, OH of anisoyl), 6.42 (t, H_1'), 5.47 (m, H_3'), 4.2 (m, H_4'), 4.16-4.09 (m, αCH_2 of $-CH_2CH_2CN$), 3.85 (s, CH_3 of anisoyl), 3.46 (m, H_5'), 2.42-2.11 (m, βCH_2 of $-CH_2CH_2CN$, H_2'), 1.85 (d, J_{HP} = 15.5 Hz, P-CH_3), 1.45 (s, 5-CH_3), UV λ_{max} = 228, 286 nm; R_f = 0.29 (toluene/ethyl acetate, 4/1, v/v). Isomer B: ^{31}P NMR δ 98.8; ^1H NMR δ 7.9 (AB, J_{HH} = 7 Hz OH of anisoyl), 7.68 (s, H_6), 7.46-7.26 (m, trityl), 6.95 (AB, H_{meta} of anisoyl), 6.43 (q, H_1'), 5.5 (m, H_3'), 4.32-42 (m, αCH_2 of $-CH_2CH_2CN$, H_4'), 3.51-3.46 (m, H_5'), 2.74 (m, H_2'), 1.81 (d, J_{HP} = 15.6 Hz, P-CH_3), 1.45 (s, 5-CH_3); UV λ_{max} = 232, 286 nm; R_f = 0.21 (toluene/ethyl acetate, 4/1, v/v).
19. Thymidine 5'-O-(O-α,α-dimethyl-β-cyanoethyl)methylphosphonothioate (**11**). FAB^+ mass spectrum, 418 (M + 1); FAB^- mass spectrum, 452 (M - 4 + K)$^{3-}$, 416 (M - 1)$^-$, 335 (M -1 - $(CH_3)_2C$ = C(H)(CN)); ^{31}P NMR δ 88.43; ^1H NMR δ 8.12 (s, H_3), 7.36 (s, H_6), 6.31 (t, J_{HH} = 6.3 Hz, H_1'), 4.49 (m, H_3'), 4.33-4.25 (m, H_5'), 4.13 (m, H_4'), 1.96 (m, βCH_2 of α,α-dimethylcyanoethyl), 1.91, 1.89 (d, J_{HP} = 15.3 Hz, P-CH_3 for both diastereomers), 1.69 (s, 5-CH_3), 1.58 (s, CH_3 of α,α-dimethylcyanoethyl); UV λ_{max} 266 nm; R_f = (CH_3CCl_3/CH_3OH, 4/1, v/v).
20. Bis(5'-O-thymidylyl)methylphosphonothioate (**12**). FAB^+ mass spectrum, 562 (M + 1)$^+$, 583 (M + Na)$^+$; FAB^- mass spectrum, 559 (M - 1)$^-$, 595 (M + Cl)$^-$; ^{31}P NMR(DMSO) δ 97.8; ^1H NMR δ 7.45 (s, H_6), 623 (t, J_{HH} = 6.8, H_1'), 5.43 (d, 3'-OH, exchangeable with D_2O), 4.25 (m, H_5'), 3.95 (m, H_3'), 2.13 (m, H_2'), 1.9 (d, J_{HP} = 15.3 Hz, P-CH_3), 1.8 (s, 5-CH_3); UV λ_{max} = 266 nm; R_f = 0.25 (acetone/H_2O, 3/7, v/v) on C-18 silica.
21. 5'-O-Tritylthymidine 3'-O(5'-O-thymidylyl)methylphosphonothioate (**9**). Isomer A: FAB^+ mass spectrum, 803 (M)$^+$, 467 (5'-O-trityl-3'-O^2-anhydrothymidine)$^+$, 243 (thymidine +1)$^+$; FAB^- mass spectrum, 801 (M - 2)$^{--}$, 577 (M - thymidine)$^-$, 335 (M -5'-O-tritylthymidine); ^{31}P NMR δ 95.9; ^1H NMR δ 8.52 (s, H_3), 7.69 (m, H_6), 7.51 (m, H_6), 7.19-7.25 (m, trityl), 6.39 (q, H_1'), 6.19 (t, J_{HH} = 6.4 Hz, H_1'), 5.44 (m, H_3'), 4.35 (m, H_3'), 3.94 (m, H_5'), 3.48-3.39 (m, H_5'), 2.53-2.29 (m, H_2'), 1.9 (s, 5-CH_3), 1.89 (d, J_{HP} = 15.3 Hz, P-CH_3), 1.45 (s, 5'-CH_3), UV λ_{max} = 233, 266; R_f = (CH_3CCl_3/CH_3OH, 4/1, v/v); hplc retention time = 20.4 min at 2 ml/min on C-18 (acetonitrile/H_2O, 3/2, v/v). Isomer B: ^{31}P NMR δ 94.6; ^1H NMR δ 9.4 (s, H_3), 8.9 (s, H_3'), 7.68, 7.51 (two multiplets, H_6), 7.50 (s, H_6), 7.4-7.21 (m, trityl), 6.31 (q, H_1'), 6.2 (t, H_1), 5.44 (m, H_3'), 4.52 (m, H_3'), 4.26 (m, H_5'), 4.18 (m, H_4'), 4.12 (m, H_4'), 3.42 (m, H_5'), 2.65-2.3 (m, H_2'), 1.91 (s, 5-CH_3), 1.81 (d, J_{HP} = 15.4 Hz), 1.47 (s, 5'-CH_3), R_f = (CH_3CCl_3/CH_3OH, 4/1, v/v); hplc retention time = 28.1 min at 2 ml/min on C-18 (acetonitrile/H_2O, 3/2, v/v).

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