Action of Toluene-*p*-thiol and Triethylamine on Fully Protected Thymidylyl-(3′ → 5′)-thymidine. Possible Occurrence of Thiolate Ion-promoted Internucleotide Cleavage in the Synthesis of Oligonucleotides by the Phosphotriester Approach

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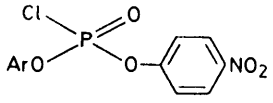
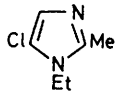
When 5′-*O*-methoxytetrahydropyranylthymidylyl-(3′ → 5′)-3′-*O*-methoxytetrahydropyranylthymidine *o*-chlorophenyl ester (12) is treated with an excess of toluene-*p*-thiol and triethylamine in acetonitrile solution at room temperature, it is cleaved to give 5′-*O*-methoxytetrahydropyranylthymidine 3′-(*o*-chlorophenyl) phosphate (10) and 5′-deoxy-5′-(*p*-tolylthio)-3′-*O*-methoxytetrahydropyranylthymidine (13). The bearing of this result on the synthesis of oligonucleotides by the phosphotriester approach is discussed.

We previously recommended [1] 4-nitrophenyl phenyl and 2-chlorophenyl 4-nitrophenyl phosphorochloridates [1] (1a) and (1b) respectively] as useful monofunctional phosphorylating agents for the first step of the phosphotriester approach to oligonucleotide synthesis. We found [1] that appropriate 5′-protected 2′-deoxyribonucleoside and 2′,5′-protected ribonucleoside derivatives (4a) and (4b), respectively, reacted readily (Scheme 1) with 4-nitrophenyl phenyl phosphorochloridate (1a) in the presence of a 1-alkylimidazole catalyst (2) in pyridine solution to give the corresponding 3′-phosphotriesters (5; Ar = Ph) in high yields. We further found [1] that when the latter (5; Ar = Ph) were treated with an excess (usually tenfold) of toluene-*p*-thiol (3a) and triethylamine in acetonitrile solution for *ca.* 1 h at 20 °C, the triethylammonium salts of the desired protected nucleoside 3′-phenyl phosphates (6; Ar = Ph) were obtained in virtually quantitative yields. The latter could be isolated by precipitation as pure solids, free from the excess of the triethylammonium salt of toluene-*p*-thiol and free from the diaryl sulphide (3b) also formed.



(1) a; Ar = Ph
b; Ar = 2-ClC_6H_4



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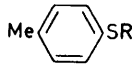
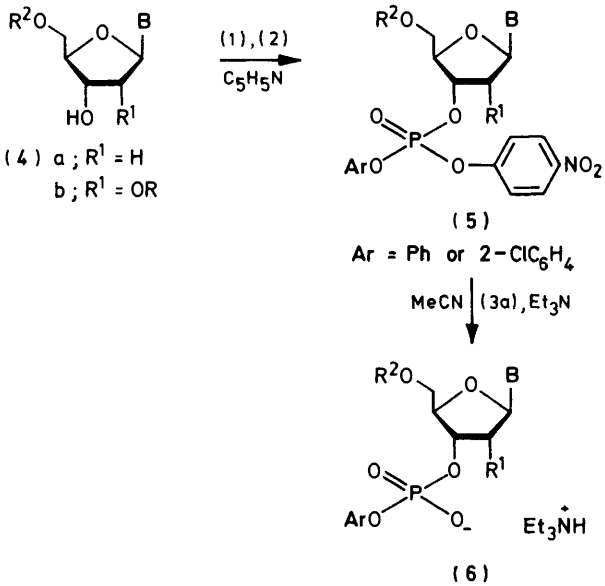


(3) a; R = H
b; R = 4-$O_2NC_6H_4$

The procedure indicated in Scheme 1 is very satisfactory indeed for the conversion of protected nucleoside derivatives (4) into their 3′-phenyl [1] and 3′-(*o*-chlorophenyl) [2] phosphates (6; Ar = Ph and 2-ClC_6H_4, respectively); it is therefore suitable for use in the stepwise synthesis of oligonucleotides and has been used successfully in the preparation of a number of short oligonucleotides both in the deoxyribose [1,2] and ribose [1] series. It has also been used very recently [3] in the synthesis of 5′-*O*-triphosphoryladenylyl-(2′ → 5′)-adenylyl-(2′ → 5′)-adenosine (2—5A), a powerful inhibitor of protein synthesis. However, when an



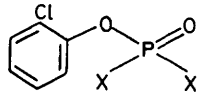
Scheme 1

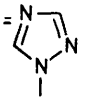
attempt was made [4] to phosphorylate a fully protected dinucleoside phosphate (4; R^2 = protected nucleoside-3′-arylphosphoryl) by this procedure, an undesirable side-reaction, leading to internucleotide cleavage, occurred. We now report the nature of this side-reaction.†

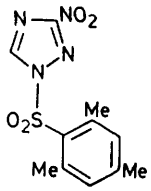
As reported previously,[5] when *o*-chlorophenyl phosphorobis-(1,2,4-triazolide) (7b), which may be prepared by treating *o*-chlorophenyl phosphorodichloridate (7a) with 1,2,4-triazole and triethylamine in acetonitrile, is used in excess, it behaves essentially as a monofunctional phosphorylating agent and, unlike 2-chlorophenyl 4-nitrophenyl phosphorochloridate (1b), it is equally suitable for the phosphorylation of the 3′-hydroxy-functions both of protected nucleoside and oligonucleo-

† This work was presented in outline at Burzenin, Poland, in September 1979; see ' Phosphorus Chemistry Directed Towards Biology ', ed. W. J. Stec, Pergamon, Oxford, 1980, pp. 145—155.

tide derivatives.[5] The fully protected thymidylyl-(3′ → 5′)-thymidine derivative (12), which was required as the substrate in our studies, was prepared by the procedure outlined in Scheme 2. 5′-*O*-Methoxy-

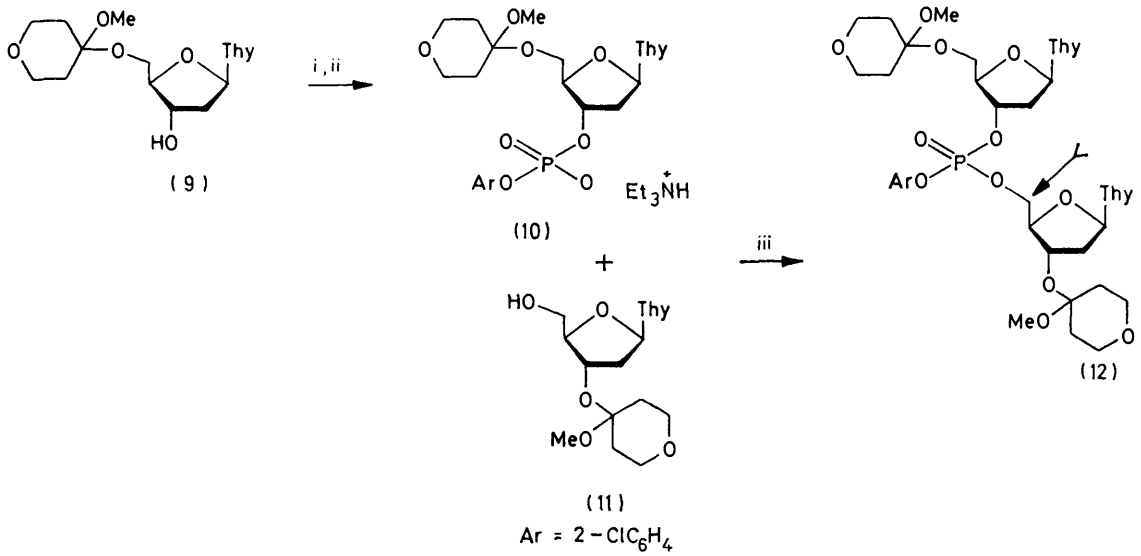


(7) a; X = Cl
b; X = 



(8)

tetrahydropyranylthymidine [6] (9) was treated first with an excess (almost 2.5 mol equiv.) of *o*-chlorophenyl phosphorobis-(1,2,4-triazolide) [5] (7b) in acetonitrile–pyridine solution and, after treatment of the products with aqueous triethylamine, the triethylammonium salt of the intermediate 3′-(*o*-chlorophenyl) phosphate (10) corresponded approximately to those [1] under which the *p*-nitrophenyl group was cleaved from the phosphotriesters (5) to give the corresponding phosphodiesters (6) (Scheme 1) in 1 h. The less polar product obtained from the reaction between (12) and toluene-*p*-thiolate ion was isolated, following chromatography of the products, as a crystalline solid in 72% yield and identified as 5′-deoxy-5′-(*p*-tolylthio)-3′-*O*-methoxytetrahydropyranylthymidine (13) on the basis of analytical and spectroscopic evidence. In order to confirm this structural assignment, (13) was synthesized unambiguously and in 76% yield by treating the 5′-*O*-(toluene-*p*-sulphonyl) derivative of 3′-*O*-methoxytetrahydropyranylthymidine (11) with toluene-*p*-thiol (3a) and triethylamine in acetonitrile. The more polar product obtained from the toluene-*p*-thiolate ion-promoted cleavage of (12) was identified as 5′-*O*-methoxytetrahydropyranylthymidine 3′-(*o*-chlorophenyl) phosphate (10) on the basis of its ^{1}H n.m.r. spectrum. The latter compound (10) could readily be distinguished (^{1}H n.m.r., t.l.c.) from the isomeric 5′-phosphodiester (14)

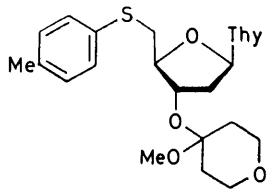


SCHEME 2 *Reagents:* i, (7b)/MeCN–C_5H_5N; ii, Et_3N–H_2O; iii, (8)/C_5H_6N

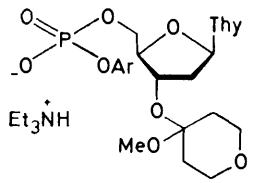
was obtained in virtually quantitative yield. The latter compound (10) was then allowed to react with a stoicheiometric quantity of 3′-*O*-methoxytetrahydropyranylthymidine [7] (11) in the presence of an excess (2.5 mol equiv.) of 1-(mesitylene-2-sulphonyl)-3-nitro-1,2,4-triazole [3a,8] (8) in anhydrous pyridine solution to give the fully protected dinucleoside phosphate (12) which, following chromatography of the products, was isolated as a pure colourless solid in 68% yield.

When a solution (0.075M) of the fully protected dinucleoside phosphate (12) in acetonitrile was treated with toluene-*p*-thiol (3a) (13 mol equiv.) and triethylamine (12 mol equiv.) at room temperature, t.l.c. revealed that, after 5 h, *ca.* 50% of the starting material (12) had been converted into one slightly less polar and one much more polar product and that, after 70 h, no starting material whatsoever remained. The reaction conditions which was prepared by phosphorylating 3′-*O*-methoxytetrahydropyranylthymidine (11) with *o*-chlorophenyl phosphorobis-(1,2,4-triazolide) (7b).

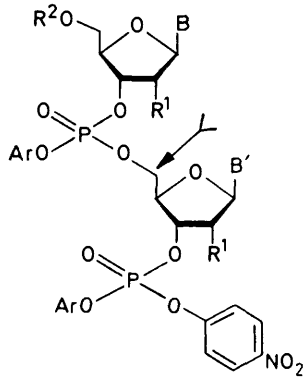
The reaction between (12) and toluene-*p*-thiolate ion would appear to involve attack, by an S_N2 mechanism, of the latter nucleophile on C-5′ adjacent to the phosphotriester group of (12) (*i.e.* at the carbon atom indicated by the arrow) to give the products (10) and (13). It should be noted that the cleavage reaction appears to be *ca.* 10% complete under the conditions required (1 h at 20 °C) [1] to remove the *p*-nitrophenyl group from the intermediate phosphotriester (5) in the phosphorylation procedure (Scheme 1) involving aryl 4-nitrophenyl phosphorochloridates (1). Thus, phosphorylation of the 3′-hydroxy-functions even of dinucleoside phosphates [which would lead to fully-protected intermediates of the types (15a) and (15b) in the deoxyribose and



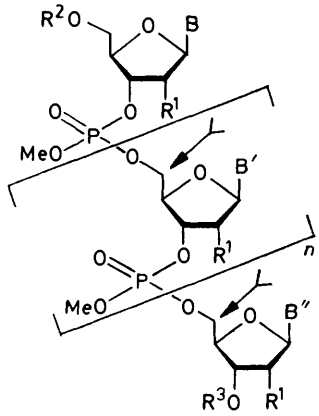
(13)



(14)



(15) a ; R^1 = H
b ; R^1 = OR



(16) a ; R^1 = H
b ; R^1 = OR

ribose series respectively] by this procedure would not be feasible [4] as significant cleavage at C-5′ [indicated by the arrow in (15)] would occur during the toluene-*p*-thiolate ion-promoted removal of the *p*-nitrophenyl group. It follows that this phosphorylation procedure is unsuitable for use in the block synthesis of oligonucleotides.

Finally, attention should be drawn to the fact that the methyl group has been used for the protection of internucleotide linkages in oligoribo- [9,10] and oligodeoxyribo- [11,12] nucleotide synthesis and that demethylation of the protected oligonucleotides has been accomplished [9,11] by treatment with thiophenol and triethylamine in dioxan solution. This strategy has also been used in the synthesis of a di-ribonucleoside phosphorothioate.[13] No mention was made in any of these reports [9,11,13] of the occurrence of internucleotide cleavage. However, the reaction conditions required for the thiophenate ion-promoted removal of methyl groups appear [9] to be similar to those required [1] for the removal of a *p*-nitrophenyl group by toluene-*p*-thiolate ion. The possibility therefore exists that demethylation of a fully protected oligodeoxyribo- or oligoribo-nucleotide (16a) or (16b) will be accompanied by thiophenate ion attack occurring to a certain extent at each C-5′ adjacent to a phosphotriester group [*i.e.* at all the carbon atoms indicated by arrows in (16)]. Indeed, it is reasonable to conclude that it would always be wise to exercise caution in the unblocking of methyl-protected oligonucleotides as the use [10,12] of nucleophiles other than thiolate ions could also lead to the competitive occurrence of internucleotide cleavage.

EXPERIMENTAL

^{1}H N.m.r. spectra were measured at 90 and 250 MHz, respectively, with Bruker HFX 90 and WH 250 spectrometers; tetramethylsilane was used as an internal standard. ^{31}P N.m.r. spectra were measured at 36.4 MHz with a Bruker HFX 90 spectrometer; 85% orthophosphoric acid was used as an external standard. U.v. absorption spectra were measured with a Cary 17 recording spectrophotometer. I.r. spectra were measured with a Perkin-Elmer 257 spectrometer.

Merck silica gel 60 F_{254} plates and DC-Alufolien cellulose F_{254} sheets were used for t.l.c. Reeve Angel silica gel and Merck silica gel H were used for short-column chromatography. Acetonitrile, triethylamine, and pyridine were dried by heating, under reflux, with calcium hydride for 3—5 h; these solvents were then distilled at atmospheric pressure. Acetonitrile and pyridine were stored over 4A molecular sieves.

Triethylammonium Salt of 5′-O-*Methoxytetrahydropyranylthymidine* 3′-(o-*Chlorophenyl*) *Phosphate* (10).—1,2,4-Triazole (0.35 g, 5.0 mmol), acetonitrile (2.5 ml), and triethylamine (0.71 ml, 5.1 mmol) were added to a stirred solution of *o*-chlorophenyl phosphorodichloridate (0.60 g, 2.44 mmol) in acetonitrile (2.5 ml) under anhydrous conditions at room temperature. After 15 min, 5′-*O*-methoxytetrahydropyranylthymidine [6] (0.356 g, 1.0 mmol) and pyridine (5 ml) were added and, after a further 20 min, the mixture was treated with a solution of triethylamine (0.88 ml, 6.3 mmol) and water (0.3 ml, 16.7 mmol) in pyridine (2 ml). After 10 min, the products were poured into saturated aqueous sodium hydrogen carbonate (30 ml) and the resulting mixture was extracted with chloroform (5 × 40 ml). The combined chloroform extracts were washed with water (*ca.* 40 ml) and evaporated under reduced pressure to give a glassy residue (0.648 g); δ [$(CD_3)_2SO$; 250 MHz] 1.17 (9 H, t, *J* 7.3 Hz), 1.67 (4 H, m), 1.77 (3 H, s), 2.21 (2 H, m), 3.07 (9 H, m), 3.48 (6 H, m), 4.08 (1 H, m), 4.74 (1 H, m), 6.16 (1 H, dd, *J* 5.9 and 8.5 Hz), 6.98 (1 H, m), 7.23 (1 H, m), 7.38 (1 H, d, *J* 7.6 Hz), 7.51 (1 H, s), and 7.67 (1 H, d, *J* 8.2 Hz); R_F[$CHCl_3$–MeOH (4 : 1 v/v)] 0.41.

Triethylammonium Salt of 3′-O-*Methoxytetrahydropyranylthymidine* 5′-(o-*Chlorophenyl*)*Phosphate* (14).—1,2,4-Triazole (0.175 g, 2.5 mmol), acetonitrile (1.5 ml), and triethylamine (0.36 ml, 2.6 mmol) were added to a stirred solution of *o*-chlorophenyl phosphorodichloridate (0.30 g, 1.2 mmol) in acetonitrile (1.25 ml) under anhydrous conditions at room temperature. After 15 min, 3′-*O*-methoxytetrahydropyranylthymidine (0.178 g, 0.5 mmol) and pyridine (1.25 ml) were added and, after a further 20 min, the mixture was treated with a solution of triethylamine (0.44 ml, 3.15 mmol) and water (0.15 ml, 8.3 mmol) in pyridine (1 ml). After 10 min, the products were poured into aqueous sodium hydro-

gen carbonate (20 ml) and worked up according to the procedure described for the isomeric 3′-phosphate. A solution of the glassy residue obtained in chloroform (5 ml) was added to light petroleum (b.p. 30—40 °C) (200 ml) and the precipitated product was collected by centrifugation (0.20 g, 62%), δ [$(CD_3)_2SO$; 250 MHz] 1.06 (9 H, t, *J* 7.3 Hz), 1.67 (4 H, m), 1.77 (3 H, s), 2.13 (2 H, m), 2.76 (6 H, m), 3.09 (3 H, s), 3.54 (4 H, m), 3.92 (2 H, m), 4.02 (1 H, m), 4.39 (1 H, s), 6.18 (1 H, t, *J* 7.1 Hz), and 7.79 (1 H, s); R_F [$CHCl_3$–MeOH (4 : 1 v/v)] 0.17.

5′-O-*Methoxytetrahydropyranylthymidylyl*-(3′ → 5′)-3′-O-*methoxytetrahydropyranylthymidine* o-*Chlorophenyl Ester* (12).—1-(Mesitylene-2-sulphonyl)-3-nitro-1,2,4-triazole [8] (0.741 g, 2.5 mmol) was added to a magnetically stirred anhydrous solution of the triethylammonium salt of 5′-*O*-methoxytetrahydropyranylthymidine 3′-(*o*-chlorophenyl) phosphate (0.648 g, 1.0 mmol) and 3′-*O*-methoxytetrahydropyranylthymidine [7] (0.356 g, 1.0 mmol) in pyridine (10 ml) at room temperature. After 20 min, water (0.3 ml) was added and, after a further 5 min, the products were poured into saturated aqueous sodium hydrogen carbonate (40 ml) and the resultant mixture was extracted with chloroform (3 × 50 ml). The dried combined chloroform extracts were concentrated under reduced pressure and the residue was chromatographed on a short column of Merck silica gel H (15 g). Elution of the column with chloroform–ethanol (94 : 6 v/v) and evaporation of the appropriate fractions gave the desired product as a glass. When a solution of the latter material in chloroform (5 ml) was added to stirred light petroleum (b.p. 30—40 °C) (250 ml), the product was precipitated as a colourless solid (0.60 g, 68%); R_F [$CHCl_3$–MeOH (9 : 1 v/v)] 0.48 and 0.51; δ_P [$(CD_3)_2SO$] — 7.71.

Reaction between 5′-O-*Methoxytetrahydropyranylthymidylyl*-(3′ → 5′)-3′-O-*methoxytetrahydropyranylthymidine* o-*Chlorophenyl Ester* (12), *Toluene*-p-*thiol, and Triethylamine in Acetonitrile Solution.*—Triethylamine (1.0 ml, 7.2 mmol) was added to a magnetically stirred solution of the fully protected dinucleoside phosphate (12) (0.531 g, 0.6 mmol) and toluene-*p*-thiol (0.97 g, 7.8 mmol) in anhydrous acetonitrile (8 ml) at room temperature. The reaction was allowed to proceed under N_2. After 5 h, t.l.c. [$CHCl_3$–MeOH (9 : 1 v/v)] revealed that the starting material (12) (R_F 0.48, 0.51) was *ca.* 50% consumed and that two products (R_F 0.59 and *ca.* 0) had been formed. The reaction was allowed to proceed for a total of 70 h, after which time no starting material (12) remained. The products were then evaporated under reduced pressure, the residue redissolved in acetonitrile and the solution re-evaporated. The residue was then partitioned between chloroform and water and the dried ($MgSO_4$) chloroform layer was concentrated under reduced pressure. The residue was chromatographed on a column of Merck silica gel H (5 g) which was eluted first with chloroform–ethanol (49 : 1 v/v) and then with chloroform–ethanol (4 : 1 v/v). Evaporation of the appropriate fractions, eluted with chloroform–ethanol (49 : 1 v/v) gave a glass. When the latter material was dissolved in chloroform (5 ml) and the solution added to stirred light petroleum (b.p. 30—40 °C) (200 ml), a precipitate was obtained. Crystallization of the precipitate from aqueous ethanol gave 5′-*deoxy*-5′-(p-*tolylthio*)-3′-O-*methoxytetrahydropyranylthymidine* (Found: C, 57.5; H, 6.5; N, 6.0. $C_{23}H_{30}N_2O_6S{\cdot}H_2O$ requires C, 57.5; H, 6.7; N, 5.8%) as crystals, m.p. 93—94 °C (0.20 g, 72%); δ ($CDCl_3$; 90 MHz) 1.80 (7 H, m), 2.0—2.5 (5 H, m), 3.19 (3 H, s), 3.22 (2 H, m), 3.66 (4 H, m), 4.22 (1 H, m), 4.46 (1 H, m), 6.25 (1 H, t, *J* 6.6 Hz), 7.0—7.35 (5 H, m), and 9.20br (1 H, s); R_F [$CHCl_3$–MeOH (9 : 1 v/v)] 0.59.

Concentration of the fractions eluted with chloroform–ethanol (4 : 1 v/v) gave 5′-*O*-methoxytetrahydropyranylthymidine 3′-(*o*-chlorophenyl) phosphate (0.075 g, 19%), identical (t.l.c., ^1H n.m.r.) to the material prepared directly (see above) from 5′-*O*-methoxytetrahydropyranylthymidine.

Preparation of 5′-*Deoxy*-5′-(p-*tolylthio*)-3′-O-*Methoxytetrahydropyranylthymidine* (13) *from* 3′-O-*Methoxytetrahydropyranylthymidine.*—A solution of 3′-*O*-methoxytetrahydropyranylthymidine [7] (0.60 g, 1.68 mmol), toluene-*p*-sulphonyl chloride (0.481 g, 2.5 mmol) in anhydrous pyridine (3 ml) was stirred at room temperature. After 16 h, saturated aqueous sodium hydrogen carbonate was added and, after a further 10 min, the products were partitioned between chloroform and saturated aqueous sodium hydrogen carbonate. The dried ($MgSO_4$) chloroform layer was concentrated under reduced pressure and the residue was chromatographed on a short column of Reeve Angel silica gel (40 g). Evaporation of the appropriate fractions, eluted with chloroform–ethanol (97 : 3 v/v), gave 3′-*O*-methoxytetrahydropyranyl-5′-*O*-(*p*-tolylsulphonyl)thymidine as a glass homogeneous on t.l.c. [R_F 0.55, $CHCl_3$–MeOH (9 : 1 v/v)] (0.82 g, 96%); δ ($CDCl_3$; 90 MHz) 1.74 (4 H, m), 1.95 (1 H, d, *J* 1.2 Hz), 2.28 (2 H, m), 2.46 (3 H, s), 3.17 (3 H, s), 3.68 (4 H, m), 4.0—4.25 (3 H, m), 4.49 (1 H, m), 6.30 (1 H, t, *J* 6.8 Hz), 7.2—7.45 (3 H, m), 7.79 (2 H, d, *J* 8.5 Hz), and 9.58br (1 H, s).

Triethylamine (0.60 g, 6.0 mmol) was added to an anhydrous solution of toluene-*p*-thiol (0.745 g, 6.0 mmol) and the above 5′-*O*-(toluene-*p*-sulphonyl) derivative in acetonitrile (2 ml), and the reaction solution was stirred in an atmosphere of argon at room temperature for 50 h. The products were then concentrated under reduced pressure and the residue was purified by short-column chromatography on Reeve Angel silica gel (40 g). Evaporation of the appropriate fractions, eluted with chloroform–ethanol (49 : 1 v/v) gave a glass which crystallized from aqueous ethanol to give 5′-deoxy-5′-(*p*-tolylthio)-3′-*O*-methoxytetrahydropyranylthymidine, m.p. 91—92 °C (0.42 g, 76%). The latter compound was identical (t.l.c., n.m.r.) to the product obtained (above) from the reaction between the fully protected thymidylyl-(3′ → 5′)-thymidine (12), toluene-*p*-thiol and triethylamine.

We thank the S.R.C. and I.C.I. Corporate Laboratory (C.A.S.E. Studentship to R. C. T.) for generous financial support.

[1/073 *Received*, 19*th January*, 1981]

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