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SOLID PHASE SYNTHESIS OF OLIGONUCLEOTIDES CONTAINING 3'-THIOTHYMIDINE

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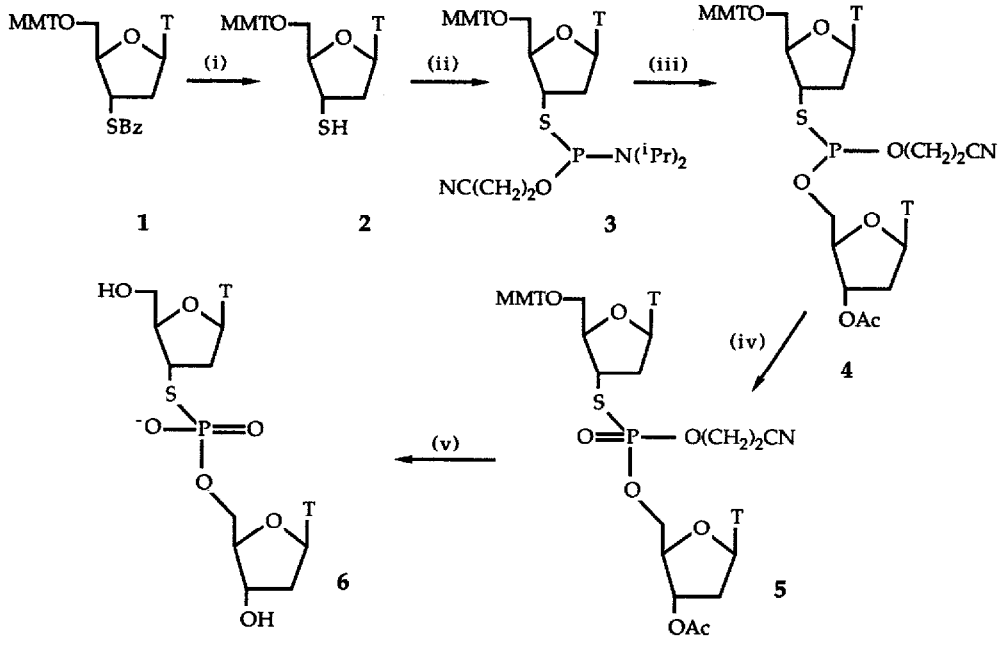
Summary- A 5'-*O*-monomethoxytritylthymidine-3'-*S*-thiophosphoramidite (3) has been used to prepare oligodeoxynucleotides containing 3'-thiothymidine on a solid phase support. The intermediate thiophosphites are most efficiently oxidised using tetrabutylammonium periodate.

Polynucleotide analogues containing modified internucleotide linkages are receiving considerable attention as potential therapeutic agents[1] and tools for the manipulation of DNA[2]. The phosphorothioate[2,3] and phosphorodithioate[4] modifications are particularly attractive since they are isopolar and isosteric with the natural congener. Recently we reported the synthesis and characterisation of 3'-thiothymidylyl(3'-5')thymidine[5] (6); a dithymidine phosphate analogue in which a sulphur atom replaces the 3'-oxygen atom in the phosphodiester linkage. It was noted that 6 was resistant to hydrolysis by nuclease P1, but the phosphorus-sulphur bond could be cleaved under mild oxidative conditions. We now report the preparation of a 5'-*O*-monomethoxytritylthymidine-3'-*S*-thiophosphoramidite (3) and its application to the solid phase synthesis of oligodeoxynucleotides containing 3'-thiothymidine.

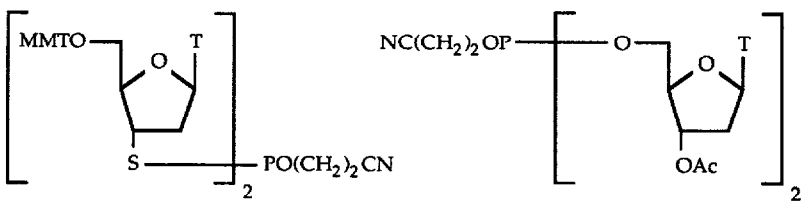
5'-*O*-monomethoxytrityl-3'-*S*-benzoyl-3'-thiothymidine (1) was prepared as previously described[5]. Treatment of 1 (2.4 mmol) in argon saturated ethanol (240 ml) at 5°C with 10N sodium hydroxide (7 ml) gave complete debenzoylation after 50 min (scheme). Silica gel column chromatography afforded 5'-*O*-monomethoxytrityl-3'-thiothymidine (2) in excellent yield (95%) and only minor quantities (3%) of the corresponding disulphide. Reaction of 2 with 2-cyanoethyl-*N,N*-diisopropylaminophosphomonochloridite[6] was perfomed under standard conditions[7] to give the thiophosphoramidite[8] (3; 87%).

It became apparent from initial experiments that the thiophosphoramidite was less reactive under standard coupling conditions than the corresponding 3'-*O*-phosphoramidite. For example, under conditions previously established as giving quantitative conversion of the 3'-*O*-phosphoramidite to the 3',5'-dinucleoside phosphite (tetrazole (4 equivalents), *N,N*-dimethylaminopyridine[9] (DMAP) (0.8 equivalent) and 3'-*O*-acetylthymidine (1.1 equivalents) in acetonitrile at room temperature for 8 min) greater than 95% (estimated by ^{31}P nmr) of 3 remained. Variations on this procedure gave differing yields of the two symmetrical phosphites (7 and 8), but no resonances attributable to the two diastereomers of the desired thiophosphite (4) were observed.



Scheme; (i) sodium hydroxide; (ii) 2-cyanoethyl-*N*,*N*-diisopropylaminophosphomonochloridite; (iii) 3'-*O*-acetylthymidine, 5-(*p*-nitrophenyl)tetrazole; (iv) oxidant; (v) *t*-butylamine, 80% aqueous acetic acid, conc. aqueous ammonia.

The use of a more acidic activating agent such as 5-(*p*-nitrophenyl)tetrazole[10] gave, in addition to the symmetrical phosphites, ^{31}P signals corresponding to the thiophosphite **4** and conditions were developed to maximise this product. Thus, a solution of 3'-*O*-acetylthymidine (0.12 mmol) in acetonitrile (1.6 ml) was added dropwise over 20 min to a stirred solution of **3** (0.34 mmol) in the same solvent (0.9 ml) saturated with 5-(*p*-nitrophenyl)tetrazole. The symmetrical phosphites appear to result from side reactions that occur on activation of the thiophosphoramidite. For example, treatment of **3** with 5-(*p*-nitrophenyl)tetrazole in the absence of 3'-*O*-acetylthymidine gave a significant yield of **2**. This result is consistent with displacement of the 3'-thionucleoside from the phosphorus centre by the activating agent and would account for formation of both **7** and **8**. Formation of **8** would also result from the analogous reaction of the thiophosphite **4**.



7 **8**

In situ oxidation of **4** was performed by initially quenching the reaction with 2,6-lutidine (0.1 ml) followed by the addition of the oxidant either tetrabutylammonium (TBA) oxone[11] (2 equivalents) or TBA periodate[12] (2 equivalents) in dichloromethane. The fully protected dimer **5**[13] was isolated in yields of 56%[14] and 75%

respectively based on 3'-*O*-acetylthymidine. Deprotection of **5** was performed under standard conditions (legend to the **Scheme**).

Solid phase synthesis of d(TpTpTspTpT) (the central thymidine residue is replaced by 3'-thiothymidine) was performed on controlled pore glass[15] using a continual flow bench synthsizer [16] according to the protocol outlined in the **table** .

Table . Reaction cycle.

Reaction	Reagents	Time (min)
Capping	6.5% DMAP in THF/acetic anhydride/2,6-lutidine (75:15:10)	3
Wash	Acetonitrile (MeCN)	2
Wash	1,2-Dichloroethane (DCE)	1
Deprotection	5% Trichloroacetic acid/DCE	3
Wash	DCE	1
Wash	MeCN	4
Couple	Stop flow	
Wash	MeCN	1
Oxidation	1.0 mM TBA periodate in DCM/MeCN/2,6-lutidine (5:5:2)	3
Wash	MeCN	4

In a typical procedure for the introduction of the 3'-thiothymidine residue, **3** (70 μmol) was dissolved in acetonitrile (0.5 ml) saturated with 5-(*p*-nitrophenyl)tetrazole and injected, over a period of 8 min, into the column containing the solid support (82 mg, capacity: 36 μmol/g). The coupling was then repeated on half this scale over a period of 4 min. The coupling efficiency was approximately 80%, as determined by monitoring the release of the trityl cations. Thymidine residues were introduced as the 5'-DMT protected 3'-*O*-(2-cyanoethyl)-*N*,*N*-diisopropylaminophosphites using a standard activation procedure[15]: coupling solutions containing nucleoside phosphoramidite (40 μmol) and tetrazole (125 μmol) in acetonitrile (0.45 ml) were injected into the column over a period of 6 min. Phosphate deprotection and cleavage from the support was effected by treatment with concentrated ammonia solution overnight at 47°c. The crude DMT-protected pentamer was purified by reverse phase hplc (**Figure 1**) and finally, the dimethoxytrityl group was removed by a treatment with 80% aqueous acetic acid for 1 h at room temperature. The ^{31}P nmr spectrum of pure d(TpTpTspTpT) showed resonances at 16.84 and -1.25 ppm attributable to the 3'-*S*-phosphorothioate and phosphate groups respectively (**Figure 2**). Digestion with nuclease P1 in the presence of alkaline phosphatase gave thymidine and 3'-thiothymidine in the expected ratio of 4:1[17]. It is interesting to note that under the same digestion conditions **6** is almost completely resistant to hydrolysis. Treatment of d(TpTpTspTpT) with aqueous silver nitrate (30 mM) at room temperature for 1 h, gave clean and quantitative cleavage of the phosphorus-sulphur bond to yield d(pTpT) and the silver salt of the corresponding thiol.

More recently we have prepared a self-complementary dodecamer d(GACGATsATCGTC) containing the recognition sequence (underlined) for the restriction endonuclease *Eco*R V, in which a 3'-thiothymidine residue is placed at the

cleavage site. Using the restriction enzyme under conditions which gave complete hydrolysis of the natural dodecamer within 5 min, there was no observable cleavage of the modified dodecamer in 24 h.

These results demonstrate that oligodeoxynucleotides containing a 3'-thiothymidine residue can be prepared from 5'-*O*-monomethoxytritylthymidine-3'-*S*-thiophosphoramidites using chemistry that is compatible with automated solid phase synthesis. The ease with which the modified linkage is cleaved in the presence of silver ions suggests that these analogues may be of considerable interest in the nicking and manipulation of DNA.

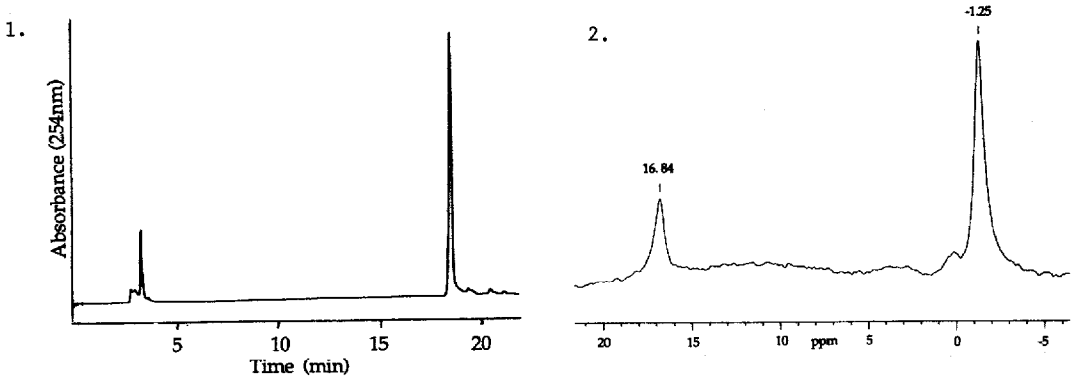


Figure 1. Hplc analysis of crude DMT-d(TpTpTspTpT) (elution using a gradient (30 min) of 12 to 65 % MeCN in 100 mM triethylammonium acetate pH 6.5); **Figure 2.** ^{31}P nmr spectrum of d(TpTpTspTpT) (5 mM oligodeoxynucleotide in D_2O).

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8. **3**; FAB$^+$ mass spectrum 731 (M+H); ^{31}P nmr ($CDCl_3$) δ 164.4 and 159.9.
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14. Reaction conditions not yet optimized.
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16. Omnifit manual bench synthsizer see B. S. Sproat and M. J. Gait, *Ibid.*, pp. 83-115.
17. During the long incubation that is necessary to effect complete hydrolysis to the constituent nucleosides the 3'-thiothymidine is partially oxidised to the corresponding disulphide.

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