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Solid Phase Synthesis of Oligodeoxynucleoside Phosphorodithioates by a Phosphotriester Method using a Chemoselective Coupling Reagent

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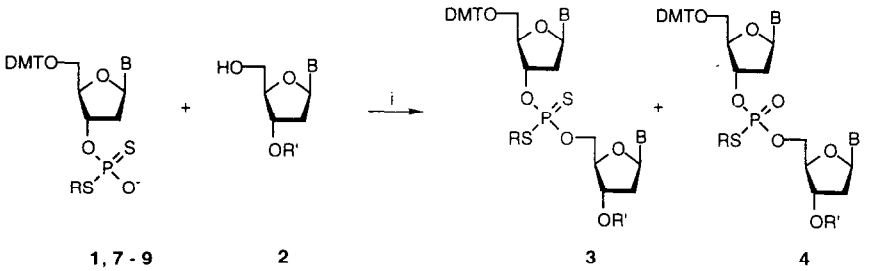
Abstract. A phosphotriester method has been developed for solid phase synthesis of oligodeoxynucleoside phosphorodithioates. Couplings are performed by chemoselective oxygen activation of protected nucleoside dithiophosphate anions **1**, **7**, **8**, **9** with 4-nitro-6-trifluoromethylbenzotriazol-1-yl-oxy-tris(pyrrolidine)-phosphonium hexafluorophosphate (PyFNOP). Under optimised conditions coupling yields are above 95 % for 10 - 20 min couplings, and the products after deblocking are free from phosphorothioate contaminations (detection limit 0.5%). Copyright © 1996 Elsevier Science Ltd

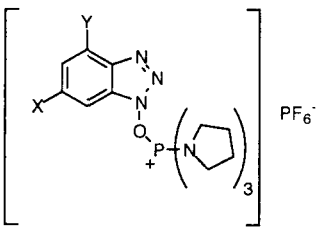
Several methods to prepare oligodeoxynucleoside phosphorodithioates, which are of interest as inhibitors of viral gene expression, have been published [1-5]. However, most of these methods give products that are inseparable mixtures of full length phosphorodithioates containing variable amounts of phosphorothioate linkages. A recently published phosphotriester method (a modified Hobt-method) [4] gives phosphorodithioates free of phosphorothioate contaminations, but the poor stability of the activated Hobt monomers and their great sensitivity towards water makes the method less suitable for automated solid phase synthesis. A classical phosphotriester strategy which use stable monomers and excess coupling reagent (see **Figure 1**) would be more ideal. Caruthers *et al.* [5] have published such a method but only for solution phase synthesis, and the products were contaminated with at least 1 % of phosphorothioates. The dithiaphospholane approach of Stec *et al.* [2] is a promising solid phase method, but gives products containing 2-3% phosphorothioate linkages.

We now wish to report the use of a phosphotriester method (**Figure 1**) for the solid phase synthesis of oligodeoxynucleoside phosphorodithioates using protected nucleoside dithiophosphate monomers **1a-1c** [6] and **7a - 9a** and 6-nitrobenzotriazol-1-yl-oxy-tris(pyrrolidine)phosphonium hexafluorophosphate (PyNOP) [7] **5** or 4-nitro-6-trifluoromethylbenzotriazol-1-yl-oxy-tris(pyrrolidine)phosphonium hexafluorophosphate (PyFNOP) [8,9] **6** as chemoselective and very active coupling reagents.

The coupling conditions were optimised using solution phase chemistry. In our experience the coupling reaction has to be completed in a few min in solution in order to be suitable for solid phase synthesis. Coupling reactions with nucleoside *di*thiophosphates proceed more slowly than coupling reactions with nucleoside *mono*thiophosphates which again react more slowly than nucleoside phosphates. The previously used coupling reagents for synthesis of deoxynucleoside phosphorodithioates [5] were to slow to be suitable for solid phase synthesis and furthermore they were not chemoselective. Three different strategies can be followed in order to obtain higher coupling rates: 1) Development of more reactive coupling reagents, 2) development of more efficient catalysts or 3) development of catalytic protection groups. An earlier attempt to prepare catalytic sulphur protection groups failed [6] so we decided to try to increase the coupling rates by using more active coupling reagents. This strategy also has the advantage that it can be combined with improvement in chemoselectivity. Thus we have found, that the phosphonium based coupling reagent PyNOP [7] **5** and PyFNOP [8,9] **6** (see **Figure 1**) are

highly chemoselective when used in solution phase chemistry, primarily activating the hard oxygen atom of **1** and thereby avoiding the formation of phosphorothioates **4** [6].





5 PyNOP; X = NO_2, Y = H
6 PyFNOP; X = CF_3, Y = NO_2

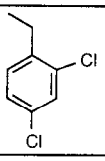
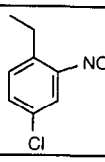
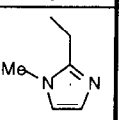
	a	b	c
R			
DMT-yield	> 98 %	97.5%	95%
% POS	0	1	10

Figure 1: Synthesis of a dithymidine phosphorodithioate **3** by the phosphotriester method and the phosphorothioate impurity **4**. DMT is 4,4'-dimethoxytrityl, R' is acetyl or a linker to TentaGel solid support, for **1** - **4** B is thymine, for 7 B is *N*-4-benzoyl cytosine, for **8** B is *N*-6-benzoyl adenine, for **9** B is *N*-2-isobutyryl guanine, R is given in the table. i) Coupling reagent. The table shows the average coupling yields for solid phase synthesis of a pentamer deoxythymidine phosphorodithioate using PyFNOP **6** as coupling reagent and the amount of phosphorothioate contamination (**4**) in the product. Conditions are as specified in the general procedure.

Since the couplings proceeded very fast and completely chemoselective in solution when using PyFNOP and the nucleoside monomers **1a-c** [6] (coupling to **3a-c** were completed within 4 min), this method should be suitable for solid phase synthesis of oligodeoxynucleoside phosphorodithioates. To test this we synthesised three differently *S*-protected pentamer deoxythymidine phosphorodithioates on a TentaGel solid support using PyFNOP as the coupling reagent. Couplings proceeded with more than 95% coupling yield in 10 min, but after deprotection the products from **1b** and **1c** were surprisingly contaminated with respectively 1 % and 10 % phosphoromonothioate (see **Figure 1**). However, the product from **1a** was without discernible amounts (^{31}P NMR, detection limit 0.5 %) of phosphoromonothioates.

In order to investigate the loss in chemoselectivity further we then performed a solution phase experiment simulating the solid phase synthesis conditions. Nucleoside monomer **1c** was preactivated with PyFNOP in pyridine for 4 min and then 3'-O-acetylthymidine **2** was added. Under these reaction conditions the product **3c** was contaminated with 8 % phosphoromonothioate **4c**. In other words scrambling occurred and chemoselectivity was partly lost when **1c** was preactivated. The loss in chemoselectivity was found to be dependent of the reactivity of the coupling reagent. Thus when the same experiment was performed using the less reactive PyNOP only 1 % contamination with phosphoromonothioate **4c** was seen. Furthermore the chemoselectivity was found to be

dependent on the type of *S*-protection group. When **1b** was preactivated with PyFNOP as above **3b** was contaminated with 2% phosphoromonothioate **4b**, and **1a** when preactivated with PyFNOP in the same manner gave **3a** without discernible amount of the phosphoromonothioate **4a** (^{31}P NMR, detection limit 0.2 %). Attempts to minimise the effects of premixing **1b**, or **1c** with PyNOP or PyFNOP by mixing the components on the column were unsuccessful. From the above results we conclude, that although the S-protecting groups of **1b** and **1c** are removed more selectively than the 2.4-dichlorobenzyl group of **1a** from the products with thiophenolate ions, [6] the monomer **1a** is preferred for solid phase synthesis when the amount of phosphoromonothioate contamination is to be kept small. Thus when **1a** was used as the nucleoside monomer with PyFNOP as the coupling reagent the pentamer deoxythymidine phosphorodithioate after deprotection showed no phosphoromonothioate contamination (^{31}P NMR, detection limit 0.5%) and stepwise coupling yields were very high (> 98 %).

An octamer deoxythymidine phosphorodithioate was prepared from **1a** and **6** using the general coupling procedure below (average coupling yield 97.2%), and the product with DMT-on was deprotected and cleaved from the solid support as described. ^{31}P NMR of the crude oligomer showed no phosphoromonothioate contamination (see **Figure 2a**).

The method could be extended to the solid phase synthesis of mixed base sequences. An octamer deoxynucleoside phosphorodithioate with the sequence d(GCTAGCTA) was prepared from **1a**, **7a-9a** [10] and **6**, and the product with DMT-on was deprotected and cleaved from the solid support as described. ^{31}P NMR of the crude oligomer showed no phosphoromonothioate contamination (see **Figure 2b**). Coupling yields were lower for the mixed base sequence as is well known for oligonucleotide synthesis on TentaGel supports [11], but average coupling yields were raised to > 95 % for the octamer d(GCTAGCTA) when double couplings were performed. However lower coupling yields were observed for other sequences. The crude products were purified using a Hamilton PRP-1 HPLC column and fully deprotected using standard conditions [4a].

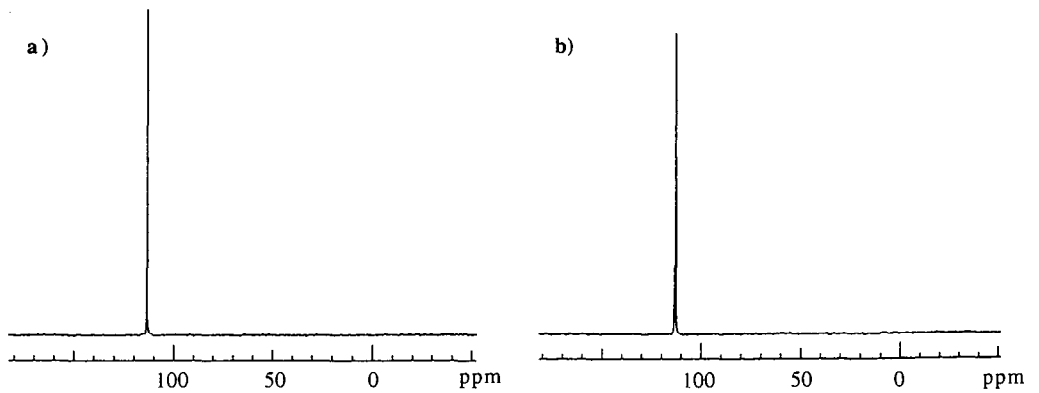


Figure 2: a) ^{31}P NMR (D_2O) of the crude octamer deoxythymidine phosphorodithioate with DMT-on.
b) ^{31}P NMR (D_2O) of the crude octamer d(GCTAGCTA) phosphorodithioate with DMT-on.

There has previously been reported base modifications, mainly of thymine, when Hobt-derived condensing reagents were used for oligonucleotide synthesis [12,13]. We therefore checked this using the method of Reese *et al.* [12] and van Boom *et al.* [13] and found that the use of PyNOP or PyFNOP did not lead to modifications of deoxythymidine in agreement with an earlier report on this type of coupling reagents [14].

In conclusion we have developed a phosphotriester method for the solid phase synthesis of oligodeoxynucleoside phosphorodithioates using the *S*-2,4-dichlorobenzyl protected nucleoside dithiophosphate monomers **1a**, **7a** - **9a**, and 4-nitro-6-trifluoromethylbenzotriazol-1-yl-oxy-tris(pyrrolidine)phosphonium hexafluorophosphate (PyFNOP) **6** as a completely chemoselective and very active coupling reagent. Coupling

yields are high (> 95 %), coupling times are short and the products after deprotection are free from phosphoromonothioate contaminations (detection limit 0.5%).

General procedure for couplings on solid phase.
In a Biosearch 1 µmol column, ca. 5 mg TentaGel-support (Rapp Polymere, corresponding to 1 µmol nucleoside) was detritylated using an ABI 360B DNA synthesiser (3% CCl_3COOH in CH_2Cl_2, 60 sec) and washed (acetonitrile, 60 sec). Triethylammonium O-[5'-O-(4,4'-dimethoxytritylnucleosid-3'-yl] S-protected phosphorodithioate (**1**, **7**, **8** or **9**, 30 µmol) and PyNOP or PyFNOP (90 µmol) were dissolved in dry acetonitrile (0.15 ml) in a dry flask under nitrogen, dry N-methylimidazole (25 µl, 0.3 mmol) was added and the solution injected onto the column using a polypropylene syringe. The reagents were left in contact with the support for 10 min. The column was washed with acetonitrile (5 ml), flushed with argon, capped (acetic anhydride, N-methylimidazole, THF, 30 sec), detritylated, and the coupling efficiency monitored by measuring the absorption of the released DMT-cation at 499 nm.

General procedure for deprotection and cleavage of the oligomer from the solid support.
The solid support was treated in the column with a mixture of thiophenol, anhydrous pyridine and triethylamine (0.2 ml/0.2 ml/0.2 ml) for 120 min. The solid support was washed with dry pyridine (2 times 5 ml) and dried. The oligomer was cleaved from the support by treatment with concentrated ammonia containing 0.01 M EDTA [15] for 2h at rt and deprotected by heating the ammonia solution at 55 °C for 6 h.

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10. **7a-9a** were prepared in the same way as **1a** [6], **7a**: ^{31}P NMR: $\delta_P(CDCl_3)$ 74.3 & 72.9 ppm, FAB$^-$ MS: 903.8 ($M\text{-}Et_3N^+H$), **8a**: ^{31}P NMR: $\delta_P(CDCl_3)$ 73.8 & 73.2 ppm, FAB$^-$ MS: 928 ($M\text{-}Et_3N^+H$), **9a**: ^{31}P NMR: $\delta_P(DMSO\text{-}d_6)$ 68.1 & 67.4 ppm, FAB$^-$ MS: 910 ($M\text{-}Et_3N^+H$).
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