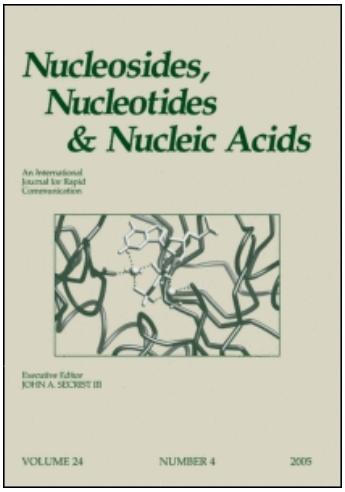

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Jan Kehler[a]; Ask Püschl[a]; Otto Dahl[a]
[a] Department of Chemistry, The H. C. Ørsted Institute, University of Copenhagen, Copenhagen, Denmark

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SOLUTION PHASE SYNTHESIS OF DITHYMIDINE PHOSPHORODITHIOATE USING NEW S-PROTECTING GROUPS IN COMBINATION WITH A CHEMOSELECTIVE COUPLING REAGENT (PyNOP)

Jan Kehler, Ask Püschl and Otto Dahl*

Department of Chemistry, The H. C. Ørsted Institute, University of Copenhagen, Universitetsparken 5, DK-2100 Copenhagen, Denmark.

ABSTRACT. A method for the synthesis of *O*-thymidin-3'-yl *S*-alkyl dithiophosphate monomers **1** with different *S*-protecting groups has been developed. These have been used for solution phase synthesis of dithymidine phosphorodithioate by a new phosphotriester method. Coupling reactions are fast (15 min.) and the products are free from phosphorothioate contaminations.

Oligodeoxynucleoside phosphorodithioates, which are of interest as inhibitors of viral gene expression[1] have been prepared by various methods[2-5]. We have recently published a triester method (a modified HOBt-method) for the preparation of phosphorodithioates using either solution phase chemistry[4a] or solid phase synthesis[4b] which is suitable for large scale synthesis of phosphorodithioates free of phosphorothioate impurities and for semiautomatic solid phase synthesis. But due to the poor stability of the activated monomers and their great sensitivity towards water, a classical phosphotriester strategy (see Fig. 1) using stable monomers and excess coupling reagent would be more ideal. Caruthers *et al.* have published such a phosphotriester method for the solution phase synthesis of deoxynucleoside phosphorodithioates[5], but the method gave variable amounts of phosphorothioate impurities (**4** in Fig. 1). Very recently Stec *et al.* have extended their previously published dithiaphospholane approach[2a] into a fully automated method[2b]. Froehler and Matteucci[6] introduced the idea of using a catalytic phosphate protection group in oligonucleotide synthesis and used 2-(1-methylimidazol-2-yl)phenyl as an efficient catalytic phosphate protecting group thereby taking advantage of the great rate acceleration achieved by

anchimeric (neighbouring group) assistance. Efimov *et al.*[7] used (2-pyridyl)methyl *N*-oxides as catalytic phosphate protecting groups. Very recently the use of 4-methoxy-1-oxido-2-picolyl as a catalytic protecting group has been applied to the synthesis of alkylphosphonates by Stawinski *et al.*[8]. To the best of our knowledge catalytic protecting groups have not yet been used in the synthesis of deoxynucleoside phosphorothioates or phosphorodithioates.

We now wish to report the use of a phosphotriester method (Fig. 1) for the solution phase synthesis of dithymidine phosphorodithioates **3** using new *S*-protecting groups in combination with 6-nitrobenzotriazol-1-yl-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate[9] (PyNOP) **5** or 4-nitro-6-trifluoromethylbenzotriazol-1-yl-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate[10] (PyFNOP) **6** (see Fig. 2) as chemoselective coupling reagents.

Important properties of an *S*-protecting group are whether 1) it gives any rate acceleration in the coupling step (i.e. function as a catalytic protecting group) and 2) it can be selectively removed without side reactions. In order to find selectively removable *S*-protecting groups we treated fully protected dithymidine phosphorodithioates **3a-3f** with thiophenolate ions and determined the amount of side products (Fig. 3).

With the exception of the (2-pyridyl)methyl *N*-oxide dimer **3f**, the only side products detected by ^{31}P-NMR were the *S*-protected thymidine dithiophosphate monomers **1a-1f** formed by cleavage of the internucleotide bridge by attack of thiophenolate ions at the 5'-position of the dimer **3**[11]. The (2-pyridyl)methyl protecting groups in **3d** and **3e** gave rise to unacceptable high 5'-cleavage when deprotected. Thiophenolate treatment of (2-pyridyl)methyl *N*-oxide dimer **3f** unfortunately gave rise to phosphorothioate **4** and phosphate impurities which is clearly unacceptable. In the case of **3c** 0.8 % 5'-cleavage was detected. The *S*-2,4-dichlorobenzyl protecting group of **3a** has previously been reported to be removed without any side reactions [4a,12], however careful analysis showed 1.2 % 5'-cleavage in agreement with recent results by Barber *et al.* for phosphorothioates[13b]. This 5'-cleavage was largely avoided in case of **3b** when 4-chloro-2-nitrobenzyl was used as the *S*-protecting group.

The *S*-protected thymidine dithiophosphate monomers **1a-c** are stable, easy to handle and easy to synthesise pure and in high yields as shown in Fig. 4. Although the *S*-2,4-dichlorobenzyl thymidine dithiophosphate monomer **1a** has been synthesised from commercially available phosphoramidite **9** by reaction with 2,4-dichlorobenzyl mercaptan in the presence of tetrazole and subsequent oxidation with sulphur[5], it is very difficult to obtain **1a** pure by this method necessitating careful column chromatography[13a,b]. Furthermore we could not introduce the (*N*-methylimidazol-2-yl)methyl *S*-protecting group in **1c** by this method due to unclarified side reactions. We therefore developed a more general strategy for

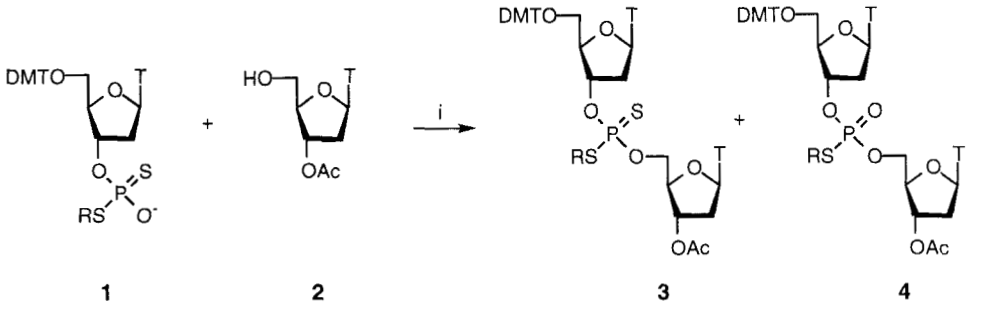


Fig. 1. Synthesis of the dithymidine phosphorodithioate **3** by a phosphotriester method and the phosphorothioate impurity **4**. DMT is 4,4'-dimethoxytrityl, Ac is acetyl, T is thymine, R is a protecting group as in Fig. 3. i) coupling reagent.

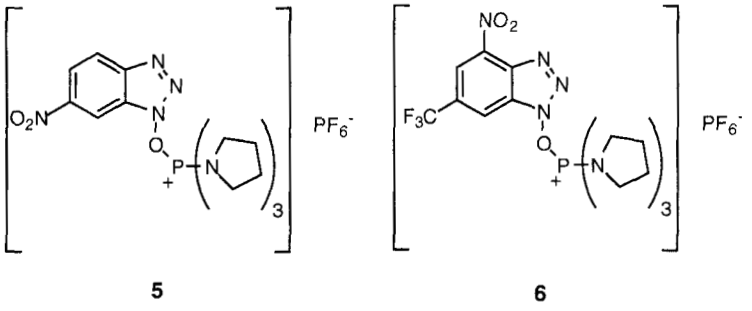
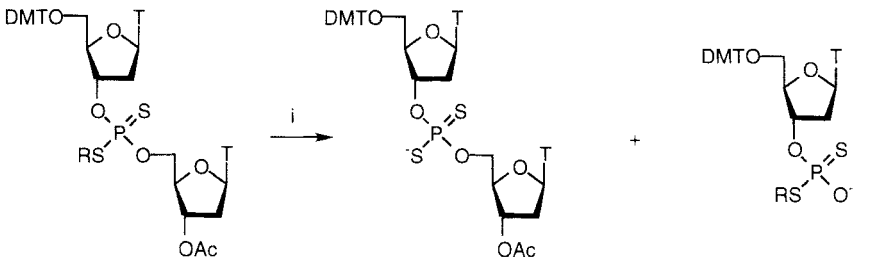


Fig. 2. PyNOP **5** and PyFNOP **6**

the synthesis of *S*-protected thymidine dithiophosphate monomers (such as **1a-1c**, Fig. 4). The phosphoramidite **9** was treated with hydrogen sulphide in the presence of tetrazole, oxidised with sulphur to the phosphorodithioate **11**[14] which in the presence of sodium iodide could be quantitatively *S*-alkylated to the dithiophosphotriesters **12a-12c**, with 2,4-dichlorobenzyl chloride, 4-chloro-2-nitrobenzyl chloride or 2-chloromethyl-*N*-methylimidazole hydrochloride[15] respectively. When **12c** was synthesised it was crucial not to use excess of the alkylating reagent, as this lead to further alkylation and hence destruction of **12c**. The dithiophosphotriesters **12a-12c** were deprotected with triethylamine to give quantitatively the thymidine dithiophosphate monomers **1a-1c**.

Couplings of **1a-1c** with **2** were performed in pyridine with the addition of a coupling reagent and, apart from **1c**, N-methylimidazole. The coupling reagents 2,4,6-



3a-3f **7** **1a-1f**

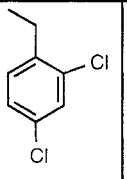
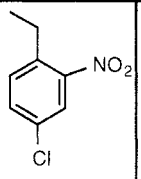
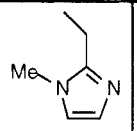
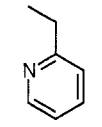
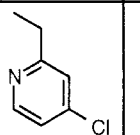
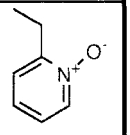
	a	b	c	d	e	f
R						
5'-cleavage	1.2 %	0.3 %	0.8 %	3.0 %	1.0%	0.4 %[a)]

Fig. 3. Deprotection and concomitant cleavage of the internucleoside linkage (5'-cleavage) in the phosphorodithioate dimers **3a-3f**. i) Reactions were performed by dissolving the fully protected dimers **3a-3f** (0.1 mmol) in pyridine/triethylamine/thiophenol (0.1 ml : 0.1 ml : 0.1 ml). Dimers **3a-3c** were synthesised by the method shown in Fig. 1. Dimers **3d-3f** were synthesised by alkylation of dimer **7**[4a] with the corresponding alkyl chloride. The amount of 5'-cleavage was determined by ^{31}P-NMR, detection limit 0.2 %.
a) Phosphorothioate **4** (1 %) and phosphate (1 %) were also detected.

triisopropylbenzensulfonyl chloride (TIPSCl) and **5** (Fig. 2) gave the results shown in Table 1.

TIPSCl is not a very chemoselective coupling reagent and therefore gave rise to variable and unacceptable amounts of phosphorothioate impurities **4**. Prompted by a recent publication by Holm *et al.*[9], describing the chemoselective synthesis of thioamides, we used the phosphorus based coupling reagent **5** as we expected it to be chemoselective, primarily activating the hard oxygen and thereby avoiding the formation of the phosphorothioate impurity **4**. As shown in Table 1 PyNOP indeed was completely chemoselective, and gave rise to pure dithymidine phosphorodithioates **3a-3c**, independent of the type of *S*-protecting group. Furthermore PyNOP and TIPSCl are equal in reactivity.

1b reacted very slowly with TIPSCl when *N*-methylimidazole was not added (37% conversion to **3b** + **4b** in 24 h.). This should be compared with **1c** which under similar

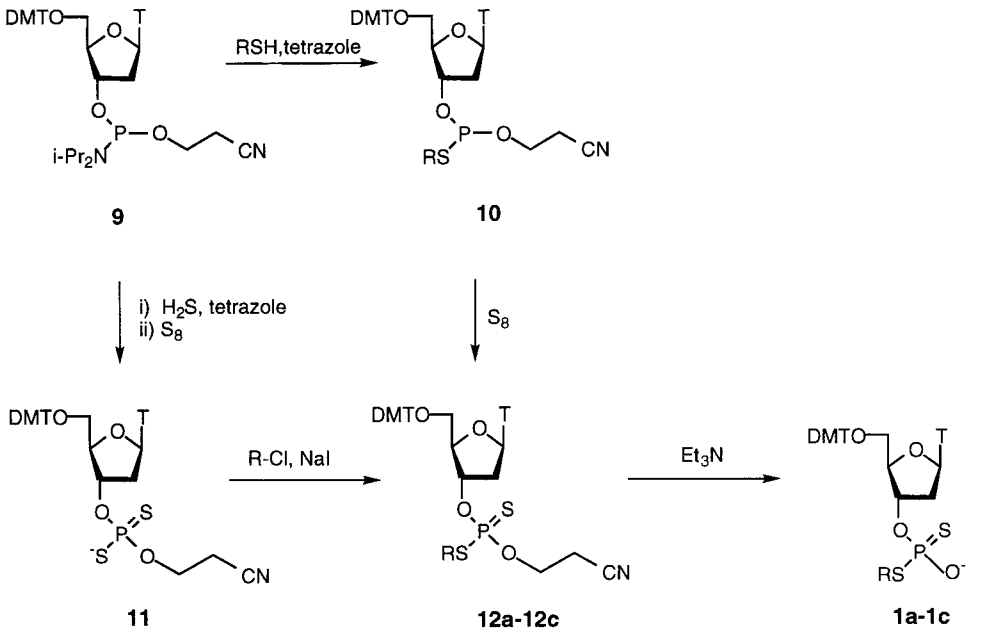


Fig. 4. Synthesis of the thymidine phosphorodithioate monomers **1a-1c**. DMT is 4,4'-dimethoxytrityl, Ac is acetyl, T is thymine, R is a protection group as in Fig. 3.

conditions was converted to **3c** in 30 min. (Table 1). We believe that the observed rate enhancement for **1c** is due to an anchimeric (neighbouring group) effect from the participation of the nucleophilic protecting group in a five-membered cyclic transition state. However, this modest rate acceleration lead us to abandon the use of **1c** for further coupling reactions.

In order to further enhance the coupling rate we also investigated the even more reactive coupling reagent PyFNOP **6**. Like PyNOP, PyFNOP was completely chemoselective, and with **1b** the coupling reaction was complete in less than 4 min. However in some experiments using PyFNOP the yields were lowered 10-15 % due to yet unclarified side reactions resulting in a by-product (^{31}P-NMR δ 88 ppm). Nevertheless such short coupling times seems appropriate for solid phase synthesis, and work is in progress to develop the method for all four bases and on a solid support.

In conclusion we have studied a phosphotriester method for the synthesis of dinucleoside phosphorodithioates using new selectively removable *S*-protecting groups and chemoselective coupling reagents. For solution phase synthesis of dithymidine phosphorodithioate the best conditions seems to be **1b** in combination with **5**.

Table 1. Coupling of the thymidine dithiophosphate monomers **1a-1c** with 3'-acetylthymidine to give TT dimers **4**.

monomer		TIPSCl	PyNOP
1a [a]	% POS (**4**)	2.3 %	0 % [c]
	coupling time	15 min.	15 min.
1b [a]	% POS (**4**)	3.5 %	0 % [c]
	coupling time	15 min.	15 min.
1c [b]	% POS (**4**)	26 %	n.d. [d]
	coupling time	30 min.	n.d. [d]

a) Reaction conditions: **1** (0.030 mmol), **2** (0.045 mmol) and coupling reagent (0.060 mmol) were dissolved in dry pyridine (0.15 ml) in a dry NMR tube. *N*-methylimidazole (NMI, 0.015 ml) was added and the reaction was followed by ^{31}P-NMR. Coupling times (approximate) are for > 95% conversion. b) Reaction conditions as in a) but without NMI. c) Detection limit 0.2 %. d) n.d. = Not determined.

EXPERIMENTAL

5'-*O*-DMT-thymidine was from Cruachem, 2,4-dichlorobenzyl chloride, 4-chloro-2-nitrobenzyl chloride, and 2-chloromethylpyridine hydrochloride were from Aldrich. Dimer **7**[4a], 4-chloro-2-chloromethylpyridine[16], 2-chloromethylpyridine-*N*-oxide[17,18], 2-chloromethyl-*N*-methylimidazole hydrochloride[15], *N*-methyl-2(-mercaptomethyl) imidazole[20], 2,4-dichlorobenzyl mercaptan[4], PyNOP[9], PyFNOP[10,19], 1-hydroxy-4-nitro-6-trifluoromethylbenzotriazole[19], 1-hydroxy-6-nitrobenzotriazole[4b], 3'-*O*-acetylthymidine **2**[21] and *O*-[5'-*O*-DMT-thymidin-3'-yl] *O*-(β-cyanoethyl) phosphorodithioate triethylammonium salt (**11**)[14] were prepared as published. Acetonitrile (LAB-SCAN), dichloromethane (LAB-SCAN), pyridine (LAB-SCAN) and *N*-methylimidazole (Aldrich) were dried over 4Å molecular sieves (GRACE type 512). TLC (eluent dichloromethane/methanol 95:5: v/v) was performed on silica 60 (Merck 5554 aluminium sheet), column chromatography on silica 60 (Merck 9385). ^{31}P-NMR spectra were obtained on a JEOL FX 90 Q spectrometer at 36.24 MHz for ^{31}P in 5 mm tubes; chemical shifts are positive in the low-field direction, with external 85 % phosphoric acid as reference. ^{1}H spectra were recorded at 400 MHz, on a Varian XL-400 spectrometer, with internal reference TMS. FAB MS spectra were obtained on a JEOL JMS-HX 110/HX 110A using nitrobenzylalcohol as matrix.

General procedure for the preparation of *O*-[5'-*O*-(4,4'-Dimethoxytrityl)-thymidin-3'-yl] *O*-(β-cyanoethyl) *S*-(alkyl) phosphorodithioates (12a-12c). To a stirred solution of the phosphorothioate diester (**11**)[14] in dry acetonitrile (10 ml/mmol) was added 2,6-lutidine (5 mol eq.), NaI (2-4 mol eq.) and alkyl chloride (1-4 mol eq.). The reaction mixture was stirred at room temperature and the progress of the reaction was monitored by ^{31}P-NMR and TLC. When the reaction was complete (less than 1 h.), the reaction mixture was evaporated *in vacuo* (only **12a** and **12b**), the residue dissolved in CH_2Cl_2 (100 ml/mmol), the solution washed with saturated $NaHCO_3$ (30 ml/mmol) and brine (50 ml/mmol), dried over Na_2SO_4, filtered, and the solvent removed *in vacuo*. The oily residue or foam was purified by silica gel column chromatography using an eluent of 3-5% methanol (v/v) in CH_2Cl_2. Columns were washed with the eluent containing 1% pyridine before use. Fractions containing the product were combined and evaporated *in vacuo* to a white foam.

O-[5'-O-(4,4'-Dimethoxytrityl)-thymidin-3'-yl] O-(β-cyanoethyl) S-(2,4-dichlorobenzyl) phosphorodithioate (12a). The reaction was carried out in the presence of 4 eq. 2,4-dichlorobenzyl chloride and 2 eq. NaI. Yield: 94%. TLC: R_f = 0.53. ^{31}P-NMR ($CDCl_3$): δ 94.7 and 94.5 (Lit.[5]: δ 95.8 and 95.5). MS (FAB^+): 869 $[M+H]^+$. According to TLC the product still contained traces of 2,6-lutidine which was most conveniently removed in the next step.

O-[5'-O-(4,4'-Dimethoxytrityl)-thymidin-3'-yl] O-(β-cyanoethyl) S-(4-chloro-2-nitrobenzyl) phosphorodithioate (12b). The reaction was carried out in the presence of 2 eq. 4-chloro-2-nitrobenzyl chloride and 2 eq. NaI. Yield: 90%. TLC: R_f = 0.47. ^{31}P-NMR ($CDCl_3$): δ 94.2 (diastereoisomers not resolved). MS (FAB^+): 880 $[M+H]^+$. According to TLC the product still contained traces of 2,6-lutidine which was most conveniently removed in the next step.

O-[5'-O-(4,4'-Dimethoxytrityl)-thymidin-3'-yl] O-(β-cyanoethyl) S-(N-methyl-2-imidazolyl)methyl phosphorodithioate (12c). The reaction was carried out in the presence of 1.05 eq. 2-chloromethyl-N-methylimidazole hydrochloride[15], and 4 eq. NaI. Yield: 93%. TLC: R_f = 0.10. ^{31}P-NMR ($CDCl_3$): δ 93.0 and 92.8. MS (FAB^+): 805 $[M+H]^+$.

General procedure for the removal of the cyanoethyl group in the preparation of triethylammonium O-[5'-O-(4,4'-Dimethoxytrityl)-thymidin-3'-yl] S-(alkyl phosphorodithioates (1a-1c). **12a-c** were dissolved in anhydrous triethylamine/dichloromethane (1:4 v/v, 5 ml/mmol) and the reaction mixture was stirred under nitrogen at room temperature overnight and then evaporated *in vacuo*. The residue was

dissolved in a small amount of dichloromethane and precipitated at 0° C from petroleum ether to give a white powder. The precipitate was dried *in vacuo* and used without further purification except **1c**, which was purified by chromatography using an eluent of methanol/triethylamine/dichloromethane/ethyl acetate (12:1:49:38).

Triethylammonium *O*-[5'-*O*-(4,4'-Dimethoxytrityl)-thymidin-3'-yl] *S*-(2,4-dichlorobenzyl) phosphorodithioate (1a). Yield: 85% (based on **11**). MS (FAB$^-$): 813 [M-triethylammonium]$^-$. ^{31}P-NMR ($CDCl_3$): δ 73.9 and 72.5.

Triethylammonium *O*-[5'-*O*-(4,4'-Dimethoxytrityl)-thymidin-3'-yl] *S*-(4-chloro-2-nitrobenzyl) phosphorodithioate (1b). Yield: 78 % (based on **11**). MS (FAB$^-$): 824 [M-triethylammonium]$^-$. ^{31}P-NMR ($CDCl_3$): δ 72.9 and 71.9.

Triethylammonium *O*-[5'-*O*-(4,4'-Dimethoxytrityl)-thymidin-3'-yl] *S*-(*N*-methyl-2-imidazolyl)methyl phosphorodithioate (1c). Yield: 72% (based on **11**). MS (FAB$^-$): 749 [M-triethylammonium]$^-$. ^{31}P-NMR ($CDCl_3$): δ 71.8 and 71.4 ppm.

Dimer 3b. Triethylammonium *O*-[5'-*O*-(4,4'-Dimethoxytrityl)-thymidin-3'-yl] *S*-(4-chloro-2-nitrobenzyl) phosphorodithioate (**1b**, 0.10 mmol) and 3'-*O*-acetylthymidine (**2**, 0.12 mmol) were evaporated from dry pyridine (1.0 ml) and redissolved in dry pyridine (0.5 ml). PyNOP (**5**, 0.20 mmol) was added and then *N*-methylimidazole (1.0 mmol). The reaction was quenched after 30 min. by addition of sat. $NaHCO_3$ (5 ml) and the reaction mixture was partitioned between AcOEt (50 ml) and sat. $NaHCO_3$ (25 ml). The organic phase was washed with brine (2 x 25 ml), dried over Na_2SO_4, and evaporated *in vacuo* to an oil. The protected dithymidine phosphorodithioate dimer **3b** was purified by column chromatography (silica gel; eluent AcOEt: MeOH; (99:1)). Yield: 72%. MS (FAB$^+$): 1093.5 $[M+H]^+$. TLC: R_f = 0.28. ^{31}P-NMR ($CDCl_3$): δ 96.1 and 95.3. ^{1}H-NMR ($CDCl_3$): δ 9.5 (2H, s br, 2 x NH), 8.0-7.1 (12H, m, arom + 2 x H6), 6.76 (4H, d, arom H), 6.35-6.2 (2H, m, H-1'), 5.33 (1H, m, H-3'), 5.02 (1H, m, H3'), 4.5-4.0 (6H, m, CH_2S + 2 x H-4' + 2 x H-5'), 3.71 (6H, s, CH_3O) 3.4-3.25 (2H, m, H5'), 2.6-1.95 (4H, m, 4 x H-2'), 2.02 (3H, s, $COCH_3$), 1.82 (3H, d, CH_3 (thymine)), 1.40 (3H, d, CH_3 (thymine)).

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