Tetrahedron Letters, Vol. 34, No. 40, pp. 6435-6438, 1993
Printed in Great Britain

0040-4039/93 $6.00 + .00
Pergamon Press Ltd

ANALOGUES OF OLIGONUCLEOTIDES CONTAINING 3'-DEOXY-β-D-PSICOTHYMIDINE

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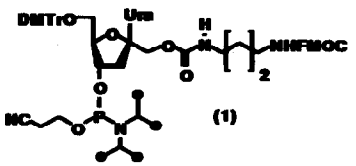
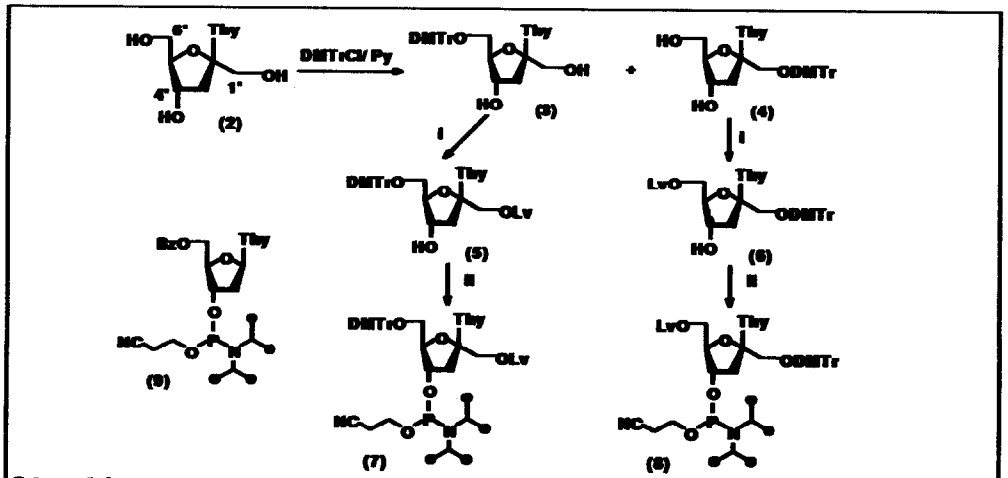
Abstract: Two different building blocks derived from 3'-deoxy-β-D-psicothymidine [1-(3-deoxy-β-D-*erythro*-2-hexulofuranosyl)thymidine] were prepared and used in the synthesis of modified oligonucleotides.

Dan *et al.*[1] recently reported on the preparation of a phosphoramidite, **1**, derived from 3'-deoxy-β-D-psicouridine,[2] the O1´ of which was linked to an *N*-protected aminoalkyl chain *via* a carbamate group. This building block was incorporated into oligonucleotides, and an intercalating group was attached to its amino function. The oligonucleotides obtained were shown to form stable duplexes with complementary strands, the conjugate groups being accommodated in the minor groove. We have approached the same subject in an alternative manner. The two hydroxymethyl functions of the 3´-deoxypsiconucleoside building block were protected with two different groups, both compatible with the usual phosphoroamidite strategy of oligonucleotide synthesis on solid support. This enables derivatisation of the 1´-position during the course of the oligonucleotide synthesis and also selective chain elongation



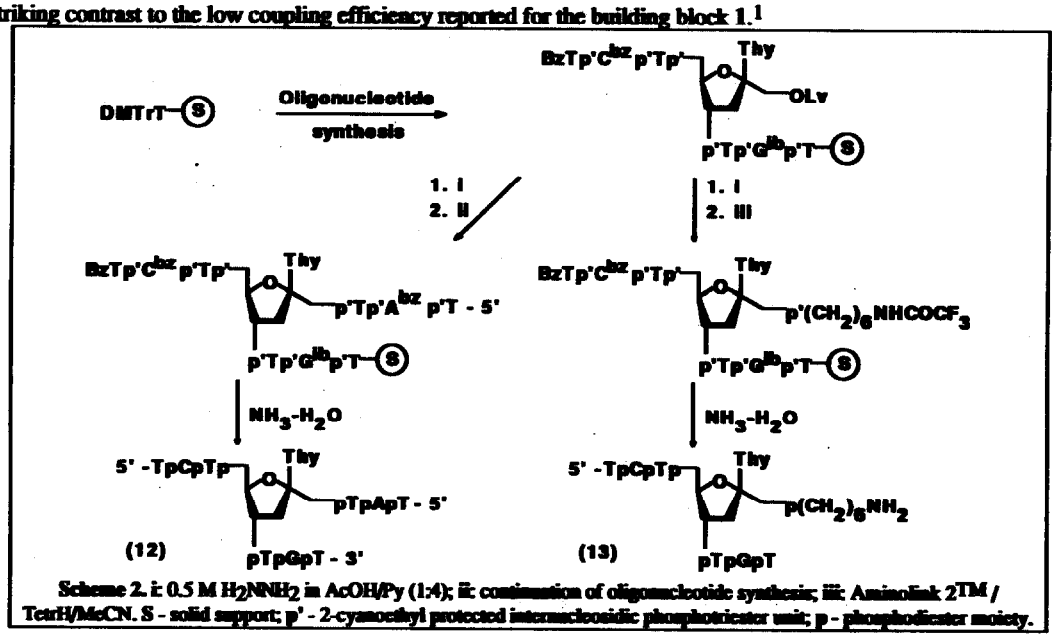


Scheme 1. i: Lv_2O / Py / dioxane; ii: 2-cyanoethyl *N,N,N´,N´*-tetraisopropylphosphorodiamidite/ TetrH/ MeCN. DMTr - 4,4'-dimethoxytrityl; Lv - levulinyl (4-oxopentanoyl).

via either O1′or O6′, *i.e.* preparation of branched oligonucleotides. 3′-Deoxy-β-D-psicothymidine,[3] (2), having a normal DNA base instead of uracil, was used to construct the appropriate building blocks, 7 and 8.

The synthesis of appropriately protected psicothymidine phosphoroamidites is outlined in Scheme 1. Dimethoxytritylation of 3'-deoxy-β-D-psicothymidine (Ψ, 2)[3] gave a mixture of 1'- and 6'-*O*-protected nucleosides (3,4), which were separated by silica gel chromatography.[4] Acylation of 3 and 4 with levulinic anhydride[5] in dioxane - pyridine led in both cases to a mixture of two monoacylated and one diacylated product. Isolation by silica gel chromatography afforded 6'-*O*-(4,4'-dimethoxytrityl)-1'-*O*-levulinyl-3'-deoxy-β-D-psicothymidine (5) and its regioisomer 6 in moderate yields.[6] Finally, treatment of 5 and 6 with 2-cyanoethyl *N,N,N′,N′*-tetraisopropylphosphorodiamidite[7] (1.25 equiv.) in acetonitrile in the presence of 1H-tetrazole (1.0 equiv.) followed by aqueous work-up and precipitation from toluene with cold hexane, gave the phosphoramidites 7 and 8 as white powders.[8] 5'-*O*-Benzoylthymidine 3'-*O*-(2-cyanoethyl *N,N*-diisopropylphosphoamidite) (9) was prepared analogously starting from 5'-*O*-benzoylthymidine.[9]

In order to demonstrate the applicability of 7 in preparation of modified oligonucleotides, the model sequences 5'-TpCpT(3'-p-6')Ψ(4'-p-5')TpGpT-3' (10) and 5'-TpApTpTpCpT(3'-p-6')Ψ(4'-p-5')TpGpT-3' (11) were synthesised. The oligonucleotides were assembled on an Applied Biosystems 392 DNA Synthesizer (0.2 μmol scale) according to the recommended protocol. No difference in coupling yields (>98% as determined by trityl assay) was detected between compound 7 and commercial phosphoramidites of unmodified nucleosides, striking contrast to the low coupling efficiency reported for the building block 1.[1]



Scheme 2. i: 0.5 M H_2NNH_2 in AcOH/Py (1:4); ii: continuation of oligonucleotide synthesis; iii: Aminolink 2™ / TetrH/MeCN. S - solid support; p' - 2-cyanoethyl protected internucleosidic phosphotriester unit; p - phosphodiester moiety.

Scheme 2 describes the preparation of a branched oligonucleotide, 12, and an aminoalkyl derivatised oligonucleotide, 13. The syntheses were started by assembling the chain 5'-BzTp'C^{bz}p'T(3'-p'-6')Ψ(4'-p'-5')Tp'G^{ib}p'T-3'. 5′-*O*-Benzoylated building block, 9, was used to terminate the elongation of this chain. The column was removed from the synthesiser, and the 1'-*O*-levulinyl protection of psicothymidine was cleaved by treating the solid support with 0.5 M hydrazine hydrate in Py/AcOH (4:1 v/v; 6 ml) for 20 min., and washing the column with EtOH and acetonitrile. After the column was reinstalled to the synthesiser, the second branch,

5'-Tp'A^{bz}p'T, was assembled starting from the liberated O1´ of the psicothymidine residue. Analogously, condensation with aminolink 2™ completed the assembly of the aminoalkyl derivatised oligonucleotide **13**.[10]

Phosphoramidite **8** was used also to prepare the same branched oligonucleotide **12** and the sequence 5'-TpApT(3'-p-1')Ψ(4'-p-5')TpGpT-3' (**14**) possessing only the O1´- branch. In contrast to **7**, both **8** and the next phosphoramidite T were coupled for 6 min. The strategy of assembling **12** was similar to that described above for **7**. First the chain 5'-Tp'A^{bz}p'T(3'-p'-1')Ψ(4'-p'-5')Tp'Tp'G^{ib}p'T was assembled, and, after cleavage of the 6'-*O*-levulinyl group, 5'-Tp'C^{bz}p'T-3' branch was attached to the 6'-*O* of psicothymidine residue. After the syntheses were completed, the standard procedure of ammoniolytic deprotection was used. The oligonucleotides (**10-14**) were isolated by an anion exchange HPLC.[11] Chromatographical profile of crude **12** is shown in figure 1 as an illustrative example. After purification by RP HPLC[12] and desalting by gel filtration[13] the oligonucleotides were characterised by PAGE (Fig. 2.) and HPLC (Table 1.).

Table 1. Retention times of **11-14** relative to **10**.

	Relative retention time (min)					
	10*	**11**	**12a**	**12b**	**13**	**14**
Ion exchange[11]	18.6	+5.2	+5.2	+5.2	-0.7	0.0
Rev. phase[12]	29.1	+3.0	+0.9	+0.9	-0.5	-0.4

* Absolute retention time (min); **a**: prepared by using **7**; **b**: prepared by using **8**. **12a** and **12b** coeluted both on ion exchange and RP columns.

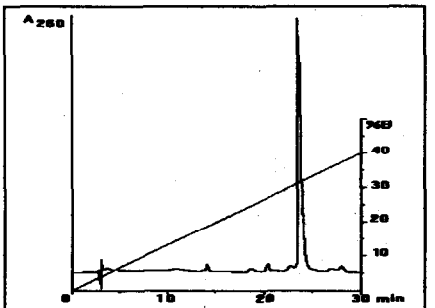
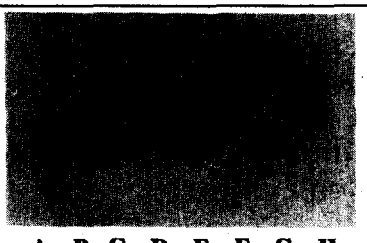


Fig. 1. Ion Exchange HPLC Profile of **12** (crude reaction mixture).


A B C D E F G H
Fig. 2. Autoradiogram of 20% PAGE **A,G**: 8-nt. marker; **B**: **10**; **C**: **12**; **D**: **13**; **E**: **14**; **F**: **11**; **H**: 10-nt. marker.

The attempts to digest the oligonucleotides **10-12** and **14** with phosphodiesterase I or II in the presence of alkaline phosphatase failed. By contrast, when **10** and **11** were treated with the mixture of these enzymes, nucleosides in the expected ratios were obtained. Under the same conditions **14** gave the expected nucleosides and an additional product which was tentatively assigned as a dimer Ψ(1´-p-3´)T. Furthermore, the enzymatic digestion of **12** gave two additional products along with nucleosides expected. In all likelihood these are a "fork-like" trimer T(3´-p-6´)Ψ(1´-p-3´)T and a dimer Ψ(1'-p-3´)T. These findings demonstrate the stability of the phosphodiester bonds of psicothymidine against nucleases.

Acknowledgments. Financial support from the Research Council for Natural Sciences, the Academy of Finland, is gratefully acknowledged.

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submitted.

4. Compound **3**: 56% as colourless foam. ^{1}H NMR ($CDCl_3$): 11.42 (1H, s, N3-H); 7.64 (1H, s, H-6); 7.43-6.70 (13H, m, arom.); 5.08 (1H, t, OH-1'); 5.03 (1H, d, $J_{4',4'\text{-OH}}$ = 3.91 Hz, OH-4'); 4.06 (1H, m, H-4'); 4.02 (1H, m, H-5'); 3.85 (1H, dd, $J_{1',1'\text{-OH}}$ = 6.34 Hz, $J_{1',1''}$ = 11.23 Hz, H-1'); 3.74 (6H, s, OCH_3); 3.55 (1H, dd, $J_{1'',1'\text{-OH}}$ = 6.34 Hz, $J_{1',1''}$ = 11.23 Hz, H-1"); 3.16 (1H, dd, $J_{5',6'}$ = 3.91 Hz, $J_{6',6''}$ = 10.25 Hz, H-6'); 3.05 (1H, dd, $J_{5',6''}$ = 5.37 Hz, $J_{6',6''}$ = 10.25 Hz, H-6"); 2.63 (1H, dd, $J_{3'',4'}$ = 2.92 Hz, $J_{3',3''}$ = 14.16 Hz, H-3"); 2.30 (1H, dd, $J_{3',4'}$ = 6.48 Hz, $J_{3',3''}$ = 14.16 Hz, H-3'); 1.81 (3H, s, CH_3-5). Compound **4**: 16% of colourless foam. ^{1}H NMR ($CDCl_3$): 11.07 (1H, s, N3-H); 7.84 (1H, s, H-6); 7.32-6.82 (13H, m, arom.); 5.01 (1H, d, $J_{4',4'\text{-OH}}$ = 3.42 Hz, OH-4'); 4.88 (1H, t, $J_{6',6'\text{-OH}}$ = $J_{6'',6'\text{-OH}}$ = 5.13 Hz, OH-6'); 4.05 (1H, m, H-4'); 3.96 (1H, m, H-5'); 3.73 (6H, s, OCH_3); 3.52 (2H, m, H-6' and H-6"); 3.38 (1H, d, $J_{1',1''}$ = 9.28 Hz, H-1'); 3.19 (1H, d, $J_{1',1''}$ = 9.28 Hz, H-1"); 2.66 (1H, dd, $J_{3'',4'}$ < 1 Hz, $J_{3',3''}$ = 14.65 Hz, H-3"); 2.22 (1H, dd, $J_{3',4'}$ = 6.83 Hz, $J_{3',3''}$ = 14.65 Hz, H-3'); 1.87 (3H, s, CH_3-5).

5. Levulinic anhydride was prepared *in situ* by overnigth treatment of levulinic acid (10 mmol) with DCC (5 mmol) in dry dioxane (10 ml).

6. Compound **5**: 30.8% as colourless foam. ^{1}H NMR ($CDCl_3$): 9.07 (1H, s, N3-H); 7.62 (1H, s, H-6); 7.43-6.83 (13H, m, arom.); 4.64 (1H, d, $J_{1',1''}$ = 11.48 Hz, H-1'); 4.44 (1H, m, H-4'); 4.35 (1H, d, $J_{1',1''}$ = 11.48 Hz, H-1"); 4.26 (1H, m, H-5'); 3.80 (6H, s, OCH_3); 3.31 (1H, dd, $J_{5',6'}$ = 4.40 Hz, $J_{6',6''}$ = 10.25 Hz, H-6'); 3.26 (1H, dd, $J_{5',6''}$ = 4.40 Hz, $J_{6',6''}$ = 10.25 Hz, H-6"); 3.09 (1H, dd, $J_{3'',4'}$ = 2.44 Hz, $J_{3',3''}$ = 14.65 Hz, H-3"); 2.65 (1H, ddd, $J_{A,M}$ = 6.35 Hz, $J_{A,L}$ = 7.32 Hz, $J_{A,B}$ = 18.55 Hz, $CH_3COC\text{-}H_A$); 2.59-2.53 (2H, m, H-3', $CH_3COC\text{-}H_B$); 2.46 (1H, ddd, $J_{B,L}$ = 5.86 Hz, $J_{A,L}$ = 7.32 Hz, $J_{L,M}$ = 17.57 Hz, $OCOC\text{-}H_L$); 2.33 (1H, ddd, $J_{B,M}$ = 6.35 Hz, $J_{A,M}$ = 6.35 Hz, $J_{L,M}$ = 17.57 Hz, $OCOC\text{-}H_M$); 2.09 (3H, s, CH_3CO); 1.91 (3H, s, CH_3-5). Compound **6**: 32.6% as colourless foam. ^{1}H NMR ($CDCl_3$): 11.40 (1H, s, N3-H); 7.50 (1H, s, H-6); 7.45-6.75 (13H, m, arom.); 4.43 (1H, m, H-4'); 4.10 (1H, m, H-5'); 3.80 (6H, s, OCH_3); 3.51 (1H, d, $J_{1',1''}$ = 9.28 Hz, H-1'); 3.34 (1H, d, $J_{1',1''}$ = 9.28 Hz, H-1"); 3.05-2.30 (8H, m, H-6', H-3', H-3", $(CH_2)_2$); 2.21 (3H, s, CH_3CO); 1.97 (3H, s, CH_3-5).

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8. Compound **7**: ^{31}P NMR ($CDCl_3$): 148.87 (0.5 P); 148.64 (0.5 P); TLC (Kieselgel 60 F_{254}, CH_2Cl_2-MeOH 19:1): R_f 0.34, 0.40. Compound **8**: ^{31}P NMR ($CDCl_3$): 149.15 (0.5 P); 149.03 (0.5 P); TLC (Kieselgel 60 F_{254}, CH_2Cl_2- MeOH 19:1): R_f 0.43, 0.48.

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11. Ion exchange HPLC was performed on a Synchropak AX-300 column (6.5 µm, 4.6x250 mm) at flow rate 1.0 ml/min using a linear gradient from 0 to 40% of B for 40 min. (A = 0.03 M KH_2PO_4 in 50% aqueous formamide, pH 5.60; B = 0.6 M $(NH_4)_2SO_4$ in buffer A, pH 5.60).

12. Reversed phase HPLC was performed on a Nucleosil 120-5C18 column (5 µm, 4.0x250 mm) at flow rate 1.0 ml/min using a linear gradient from 0 to 40% B for 40 min. (A = 0.1 M ammonium acetate buffer; B = 0.1 M ammonium acetate buffer containing 50% acetonitrile).

13. TSKgel G2000SW (7.5 x 300 mm, Toso Haas); flow rate 1.0 ml/min of water.

(Received in UK 5 July 1993; accepted 23 July 1993)