Synthesis of 2′,3′-Dideoxyribonucleoside-3′-*N*-(2-oxo-1,3,2-oxathiaphospholanes) and Their Reactions with 5′-OH Nucleosides and Fluoride Ion

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Introduction

During the past few years, Gryaznov et al. reported in numerous papers that analogues of oligodeoxyribonucleotides, where the 3′-oxygen of incorporated deoxyribonucleosides is replaced by an NH function, possess interesting properties of hybridization to complementary strands of DNA and RNA. Moreover, these N3′→P5′ DNA analogues, with relatively low avidity for cellular proteins, appeared to possess high affinity to double-stranded DNA forming stable DNA triplexes.[1] For these reasons, N3′→P5′ oligos became of special interest for their use in antisense[2] and antigene[3] strategies targeting the inhibition of biosynthesis of preselected proteins, coded by corresponding mRNA and DNA. Although several methods of synthesis of N3′→P5′ oligos have been developed,[1b−d,4] their accessibility remains limited. In the search for new preparative methodology, we revisited our interest in the chemistry of 2-alkylamino-2-thio-1,3,2-oxathiaphospholanes.[5b] In the course of our studies on the synthesis, structure, and stereochemistry of base-assisted alcoholysis of diastereomeric (R_P,R_C)- and (S_P,R_C)-1-(α-naphthyl)ethylamino-2-thio-1,3,2-oxathiaphospholanes (**1**), we have found that methanolysis of (R_P,R_C)-**1** in the presence of triethylamine occurred smoothly, providing *N*-1-(α-naphthyl)ethyl-*O*-methylphosphoramidothioate (**2**), which upon treatment with methyl iodide gave (S_P,R_C)-*O*,*S*-dimethyl-*N*-1-(α-naphthyl)ethylphosphoramidothioate.[6] Thus, a new approach to the stereocontrolled synthesis of *O*-alkyl-*N*-alkylphosphoramidothioates has been demonstrated. In light of these results, it was tempting to test the suitability of 2-oxo-1,3,2-oxathiaphospholane analogues of 3′-amino-2′,3′-dideoxyribonucleosides for synthesis of N3′→P5′ oligos.

Results and Discussion

The reaction of 5′-*O*-DMT-base-protected-3′-amino-2′,3′-dideoxyribonucleosides **3a**−**d** with an equimolar amount of 2-chloro-4,4-spiro(pentamethylene-1,3,2-oxathiaphospholane) (**4**)[5d] resulted in the formation of an intermediate phosphoramidite (**5a**−**d**), which was oxidized without isolation by means of *tert*-butyl hydroperoxide (TBHP) into the corresponding 5′-*O*-DMT-base-protected-2′,3′-dideoxyribonucleoside-3′-*N*-(2-oxo-4,4-spiro(pentamethylene-1,3,2-oxathiaphospholane)) (**6a**−**d**, Scheme 1).

Compounds **6a**−**d** have been isolated from the reaction mixture by column chromatography on silica gel as a mixture of diastereomers and have been characterized by ^{31}P NMR and FAB-MS (Table 1).

Condensation of **6a** with an equimolar amount of 3′-*O*-acetylthymidine in the presence of a 5-fold molar excess of triethylamine in acetonitrile solution led, after 24 h, to dithymidine N3′→P5′-phosphoramidate (**7a**, 70% yield). However, when 1,8-diazabicyclo[5.4.0]undec-1-ene (DBU) was used instead of triethylamine, the above condensation process occurred much faster and the desired product **7a** was obtained in 85% yield after 30 min.

This result prompted us to attempt a solid-support synthesis of dinucleoside N3′→P5′ phosphoramidates **8a**−**d** using **6a**−**d** as the substrates (Scheme 2). As described by Brown et al.,[7] DBU partially cleaves the standard succinoyl linker and releases the dinucleotide from the solid support. Therefore, 5′-*O*-DMT-thymidine was immobilized on controlled pore glass via a DBU-resistant sarcosinyl-succinoyl linker.[5c] The optimized conditions for 1 μM solid-phase synthesis were as follows: 20-fold molar excess of oxathiaphospholane monomer **6a**−**d**, 50-fold molar excess of DBU, coupling time 800 s. Compound **8a** was obtained in 93% yield as calculated from the DMT cation assay. In an analogous way, syntheses of compounds **8b**−**d** were performed and the results are presented in Table 2.

However, attempts at elongation of the oligonucleotide chain were not successful. The yield of the second

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Scheme 1

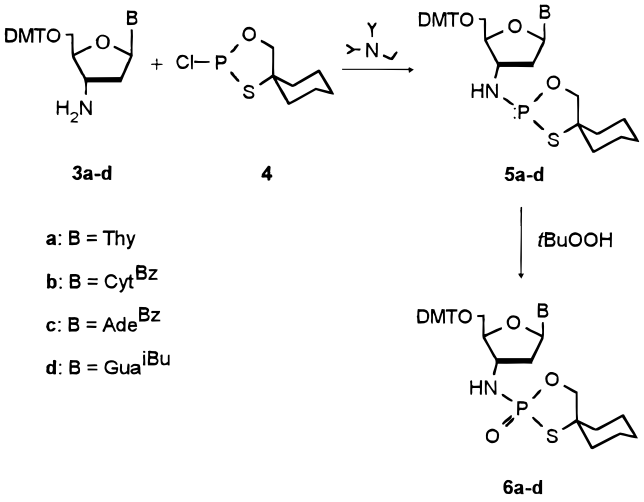

Table 1. Characteristics of Oxathiaphospholane Monomers 6

compd	δ ^{31}P NMR (ppm, C_6D_6)	FAB-MS (M-1)	yield (%)
6a: B = Thy	44.9, 44.7	732.5	79
6b: B = Cyt^{Bz}	44.7, 45.4	823.5	51
6c: B = Ade^{Bz}	46.6, 47.0	847.3	53
6d: B = Gua^{i}Bu	45.3, 46.0	813.0	50

Scheme 2

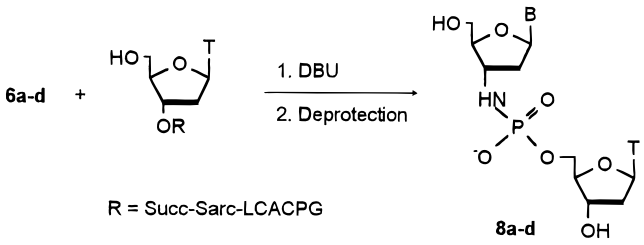

Table 2. Characteristics of Dinucleoside N3′→P5′ Phosphoramidates 8

dinucleotide **8**	δ ^{31}P NMR (ppm, D_2O)	FAB-MS (M-1)	yield (%)
a: $T_{NP}T$	6.7	544.5	93
b: $A_{NP}T$	6.7	553.1	90
c: $C_{NP}T$	6.3	529.0	91
d: $G_{NP}T$	6.9	569.4	89
e: $T_{NPS}T$	59.2, 57.4	560.5	90

coupling step dropped to 50%. According to literature sources, acid-promoted cleavage of the P−N bond in monoester phosphoramidates[8] could have occurred during the process of detritylation [dichloroacetic acid (DCA)] preceding the second coupling step. However, comparison of HPLC chromatograms of dinucleotide $T_{NP}T$ released from the solid support in DMT-on and DMT-off modes[9] indicated that cleavage of the P−N bond did not occur during the treatment of DMT-protected dinucleotide (30 s with 3% DCA in methylene chloride solution).

The same has been proven true by ^{31}P NMR assay. After 4 h incubation of **8a** with 3% DCA in CH_2Cl_2, the ^{31}P NMR spectrum did not indicate the presence of a signal at ca. 0 ppm that could be assigned to thymidine 5′-phosphate, which would be expected as a result of cleavage of the P−N bond.

$T_{NP}T$ (**8a**) was found to be virtually insoluble in acetonitrile. This property probably explains the failure of effective elongation of the oligonucleotide chain by 1,3,2-oxathiaphospholane ring-opening condensation. Several efforts were made to improve the solubility of $T_{NP}T$ by the use of different solvents (DMF, CH_2Cl_2, pyridine, pyridine−CH_3CN) for coupling step. Nevertheless, in no case was the coupling efficiency higher than that obtained in acetonitrile as reaction medium. We, therefore, decided to test the possibility of the use of 2-thio analogues of **6a**. This would involve the formation of dinucleoside N3′→P5′ phosphoramidothioates (**8e**), which were expected to be either more soluble in organic solvents or offer an opportunity of selective S-alkylation of intermediate **8e** of enhanced solubility in organic nonhydroxylic solvents. Thus, compound **6e** has been prepared by analogy to that presented for syntheses of **6a**−**d** (Scheme 1), replacing TBHP with elemental sulfur; **6e** was further used for the synthesis of $T_{NPS}T$ (**8e**, Table 2). While in this case the yield of the first coupling step was relatively high (90%), that of the second coupling dropped to 50%. Attempted alkylation of **8e** with methyl iodide, 4-nitrobenzyl bromide, or 4-nitrobenzyl chloride−NaI−2,6-lutidine did not substantially increase the effectiveness of the process of chain extension. Using a pyridine solution of 4-nitrobenzyl bromide, the coupling efficiency increased up to 75%, but unfortunately, we were unable to find conditions under which a coupling yield would reach at least 90%.

In light of the above findings, we have to conclude that the oxathiaphospholane approach cannot be applied to satisfactory synthesis of N3′→P5′ oligos beyond dinucleotides. Such failure does not result from the lack of efficiency of the 1,3,2-oxathiaphospholane ring-opening condensation process, but most probably is caused by poor solubility of solid-support bound dinucleoside N3′→P5′ phosphoramidates.

As expected,[1m] compound **8e**, either as a mixture of diastereomers or as individual separated isomers, was not hydrolyzed by Nuclease P1 (from *Penicillium citrinum*).

In light of our interest in nucleoside phosphorofluoridates and nucleoside phosphorofluoridothioates,[10] compounds **6a** and **6e** were treated with triethylammonium fluoride in the presence of DBU. Thus, **6a** gave 5′-*O*-DMT-2′,3′-dideoxythymidine-3′-*N*-phosphoramidofluoridate (**9a**) (Scheme 3). The dimethoxytrityl protecting group was removed by the treatment of **9a** with Dowex Wx8 [H^+ form]. The final product, **10a**, was purified by column chromatography on SiO_2 and obtained in 78% yield in a one-pot synthesis. In an analogous way pure **10e** has been obtained from **6e** in 80% yield.

Compound **10e** (as a mixture of two diastereomers) was subjected to enzymatic digestion by means of nuclease P1. In a control experiment without the enzyme it was shown that compound **10e** underwent hydrolysis in the appropriate buffer (see the Experimental Section) at pH 7.2, during 24 h, to 36% extent. Similar experiments with

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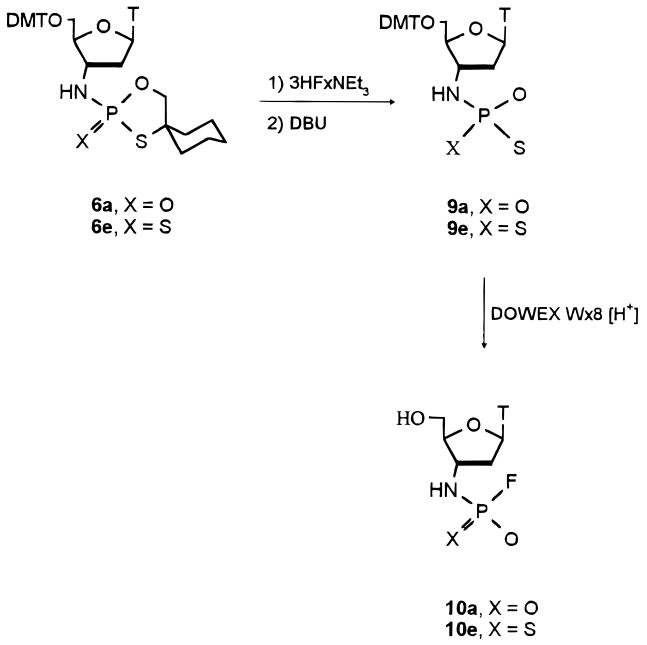
Scheme 3



added nuclease P1 resulted in 40% hydrolysis of the P−F bond in **10e**, as recorded by RP-HPLC. In the same buffer, but at pH 5.0, the P−F bond in **10e** was hydrolytically stable independent of the presence of enzyme. Therefore, it may be concluded that the activity of nuclease P1 toward 2′,3′-dideoxythymidine-3′-*N*-phosphoramidofluoridothioates (**10e**) is either zero or negligible.

In conclusion, to check the scope and limitation of the 1,3,2-oxathiaphospholane ring-opening condensation process, hitherto successfully used for the synthesis of oligodeoxyribonucleotides and the stereocontrolled synthesis of oligo(deoxyribonucleoside phosphorothioate)s as chimeric PO/PS-oligos,[5d] 5′-*O*-DMT-2′,3′-dideoxyribonucleoside-3′-*N*(2-oxo-4,4-spiro(pentamethylene-1,3,2-oxathiaphospholanes)) **6a**−**d** and their 2-thio analogue **6e** were prepared and used in DBU-assisted condensation with 5′-OH-nucleosides. The resulting dinucleoside N3′→P5′ phosphoramidates and dithymidine N3′→P5′ phosphoramidothioates formed in ca. 90% yield appear to be insoluble in nonhydroxylic solvents. Thus, further chain elongation was not possible. Compounds **6a** and **6e** were found to react with fluoride ion to give 2′,3′-dideoxythymidine-3′-*N*-phosphoramidofluoridate (**10a**) or the corresponding 3′-*N*-phosphoramidofluoridothioates (**10e**).

Experimental Section

General Methods. Deoxyribonucleosides were purchased from Pharma Waldhof (Germany). Acetonitrile and 1,4-diazabicyclo[5.4.0]undec-7-ene (DBU) were supplied by Merck (Germany). Acetonitrile to be used as solvent for DBU and oxathiaphospholane monomers was dried over P_2O_5 (5 g/L) and distilled through a Vigreux column in an atmosphere of dry argon. Nuclease P1 (nP1, EC 3.1.30.1) was purchased from Sigma.

5′-*O*-DMT-base-protected-3′-amino-2′,3′-dideoxyribonucleoside (**3a**−**d**)[1c] and 2-chloro-4,4-spiro(pentamethylene-1,3,2-oxathiaphospholane) (**4**)[5d] were obtained according to the procedures described. HPLC analyses were performed on a Waters Millennium liquid chromatography system, with a Supelco LC-18 column (2.1 × 250 mm), 0.3 mL/min flow rate; buffer A, 0.05 M TEAB pH 7.5; buffer B, 40% CH_3CN in 0.05 M TEAB; gradient from 0 to 100% B over 35 min.

Since *O,N*-trialkyl phosphoramidofluoridates are known to be extremely toxic,[11] special caution was taken when working with compounds **10** because of their unknown physiological activity!

Reaction of Protected 3′-Amino-2′,3′-dideoxyribonucleoside with 2-Chloro-4,4-spiro(pentamethylene-1,3,2-oxathiaphospholane): General Procedure. To the solution of appropriately protected 3′-amino-2′,3′-dideoxyribonucleoside (1 mmol; dA^{Bz}, $dG^{i}Bu$, T, or dC^{Bz}) and diisopropylethylamine (0.19 mL, 1.1 mmol) in dry acetonitrile (2 mL) was added 2-chloro-4,4-spiro(pentamethylene-1,3,2-oxathiaphospholane) (0.231 g, 1.1. mmol) dropwise at room temperature. After 2 h, an equimolar amount of *tert*-butyl hydroperoxide (0.02 mL of 5 M solution in C_6H_6) was added and the reaction mixture kept at room temperature for 1 h. Solvent was evaporated to dryness, and the residue was chromatographed on a column (0→3% methanol in chloroform) to give compounds **6a**−**d**. Yields, ^{31}P NMR chemical shifts, and FAB-MS data of compounds **6a**−**d** are given in Table 1.

5′-*O*-DMT-2′,3′-dideoxythymidine-3′-*N*-(2-thio-4,4-spiro-(pentamethylene-1,3,2-oxathiaphospholane)) (6e). To a solution of 5′-*O*-DMT-3′-amino-2′,3′-dideoxythymidine (0.55 g, 1 mmol) and diisopropylethylamine (0.19 mL, 1.1 mmol) in dry acetonitrile (2 mL) were added elemental sulfur (0.05 g, 1.5 mmol) and 2-chloro-4,4-spiro(pentamethylene-1,3,2-oxathiaphospholane) (0.231 g, 1.1 mmol). Stirring was continued 12 h and the excess sulfur filtered off. After evaporation of solvent, the residue was purified by column chromatography (0→3% methanol in chloroform). Compound **6e** was isolated as a mixture of diastereomers in 82% yield [^{31}P NMR (C_6D_6) δ 96.2 and 95.6 ppm; FAB (M − 1) MS *m/z* 748.5].

"In Solution" Synthesis of 5′-*O*-DMT-3′-*O*-acetyldithymidyl-(N3′→P5′)-phosphoramidates (7a). 5′-*O*-DMT-2′,3′-dideoxythymidine-3′-*N*-(2-oxo-4,4-spiro(pentamethylene-1,3,2-oxathiaphospholane)) (0.367 g, 0.5 mmol) was dissolved in dry acetonitrile (1 mL), and to this solution were added a solution of 3′-*O*-acetylthymidine (0.142 g, 0.5 mmol) and DBU (0.15 g, 1 mmol) in dry acetonitrile (1 mL). After 30 min, the reaction mixture was concentrated under reduced pressure and the residue dissolved in chloroform and purified on PTLC plates using chloroform−methanol (9:1) as the developing system. Compound **7a** was obtained in 85% yield [^{31}P NMR ($CDCl_3$) δ 7.1 ppm; FAB (M − 1) MS *m/z* 888.5].

Solid-Phase Synthesis of Compounds 8a−e. Reactions were carried out manually by the syringe technique using 5′-*O*-DMT-3′-Succ-Sarc-LCACPG-thymidine (1 mmol). A single cycle of chain elongation was as follows: detritylation, 3% dichloroacetic acid in methylene chloride (5 mL); time 120 s; wash, acetonitrile (5 mL); coupling, 0.2 M acetonitrile solution of **6** (20 mmol, 0.1 mL) and DBU (0.05 mL of 1 M solution in CH_3CN), freshly premixed; time 800 s; wash, acetonitrile (2 mL), methylene chloride (2 mL), and acetonitrile (3 mL); capping, acetic anhydride/DMAP/2,6-lutidine/tetrahydrofuran (0.15 mL, 2 min); wash, acetonitrile (5 mL).

The cleavage from the support and nucleobase deprotection were performed by means of ammonia at 55 °C for 8 h. The yields (calculated from the DMT cation assay), ^{31}P NMR chemical shifts, and FAB-MS data of compounds **8a**−**e** are given in Table 2.

Compound **8e** has been separated into individual isomers by means of RP-HPLC (retention times: 16.98 and 17.77 min).

2′,3′-Dideoxythymidine-3′-*N*-phosphoramidofluoridate (10a). Compound **6a** (0.074 g, 0.1 mmol) was dissolved in dry acetonitrile (0.5 mL), and 3HF·NEt_3 (0.017 mL, 0.1 mmol) followed by DBU (0.015 mL, 0.1 mmol) was added. After 1 h, the reaction mixture was concentrated and the residue dissolved in methanol−H_2O (v/v 1:1, 1 mL) and applied to a Dowex Wx8 [H^+ form] column (4 mL). The column was eluted with methanol−H_2O (v/v 1:1). Solvents were evaporated in vacuo to afford crude **10a**. The solid was dissolved in chloroform (1 mL) and purified on PTLC plates (chloroform−methanol−NEt_3 90:10:1). Compound **10a** was obtained in 78% yield [^{31}P NMR ($CDCl_3$) δ 4.0 ppm, $^1J_{P-F}$ 918 Hz, FAB (M − 1) MS *m/z* 322.5].

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2′,3′-Dideoxythymidine-3′-*N*-phosphoramidofluoridothioates (10e). In an analogous way, compound **10e** has been obtained [80% yield starting from **6e**, ^{31}P NMR ($CDCl_3$) δ 60.85 and 60.51 ppm; $^1J_{P-F}$ 1010 and 1004 Hz; FAB (M − 1) MS *m*/*z* 338.5].

Hydrolysis with Nuclease P1. General Procedure. In a typical experiment, the reaction mixture (100 mL) containing 0.015−0.15 mg of nuclease P1, 100 mM Tris-Cl (pH 7.2), 1 mM $ZnCl_2$, and nucleotide (0.2 OD) was incubated at 37 °C; 50 mL aliquots taken after 1, 12, 24, and 44 h were heat-denaturated and analyzed by HPLC.

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