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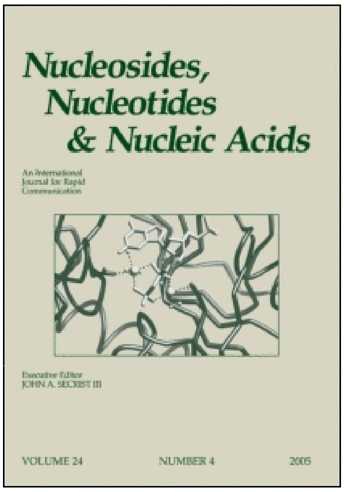

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The First Stereocontrolled Synthesis of Thiooligoribonucleotide: (RpRp)- and (SpSp)-$U_{ps}U_{ps}U$

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THE FIRST STEREOCONTROLLED SYNTHESIS OF THIOOLIGORIBONUCLEOTIDE: (RpRp)- AND (SpSp)-$U_{PS}U_{PS}U$

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ABSTRACT: An approach to the stereocontrolled synthesis of P-homochiral thiooligoribonucleotide: (Rp,Rp)- and (Sp,Sp)-diastereomers of uridinylyl(3',5')uridinylyl(3',5')uridine di(O,O-phosphorothioate) (9) is decribed. The influence of 2'-protection on the efficiency and stereochemistry of the coupling reaction is discussed.

INTRODUCTION

The rapidly increasing interest in the chemistry, molecular biology and biochemistry of RNA is mainly due to the novel properties of RNA molecules discovered in recent studies on the mechanism of maturation of RNA precursors(1,2) and the well known role of RNA in the translation of the genetic code. Additional impact has also been added by recent progress of the antisense approach towards modulation of gene expression (3).

Thus, the need is created for specifically modified oligoribonucleotides as tools for the study of RNA metabolism and structure-function relationships. Phosphorothioates are among the most obvious and thus probably most common used analogues of naturally occurring phosphates(4,5). Despite the fact that there are several well established chemical methods of thiooligodeoxyribonucleotide preparation(4-6), only few examples of thiooligiribonucleotide synthesis have been published so far. The first synthesis of diribonucleotide phosphoromonothioate by

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phosphate triester method was described by Malkiewicz and Smrt(7). A similar approach was utilized by Burgers and Eckstein(8). The phosphite triester methodology was also successfully used for synthesis of a diribonucleotide(9-11) and, more recently, a 2',5'-linked triribonucleotide(12) phosphorothioate analogue. The synthesis of oligo(2'-O-methyl)-ribonucleotides phosphorothioate via H-phosphonate approach has also been performed(13).

One of the most important features of the oligonucleotide analogues obtained by replacement of one of non-bridging oxygens of the internucleotide phosphate group by sulphur or other substituents is the formation of a chirality center at phosphorus. None of the syntheses described above occurs selectively. The oligonucleotide bearing *n* P-chiral centres is obtained as a mixture of 2^n diastereomers, an unsatisfactory result for many investigations. The diastereomeric mixture separation is laborious and difficult to achieve for $n \geq 2$.

In this paper I describe the first, stereospecific synthesis of a phosphorothioate analogue of an oligoribonucleotide. However the bond order and charge localization in nucleoside phosphorothioates are still controversial(14) it seems reasonable to assume that charge distribution, electrostatic potential surface(15,16) and hydration pattern(17,18) of P-stereodefined oligo(nucleoside phosphorothioates) may differ from oligomers with diester phosphates in their internucleotide linkages.

This assumption is further supported by the finding, published only recently, that even the phosphinyl group displays a slight stereochemical preference in its interaction with hydrogen bond donors(19).

Stereospecific methods of the synthesis of P-chiral, P-tactic oligonucleotide analogues may make available compounds useful for the study of nucleic acids interactions with other biomolecules(20), for studies on oligonucleotide hydration(17,18) and may help to design molecules (eg.ribozymes) with improved catalytic activities(21). Stereospecific synthesis may also allow for an improved understanding of antisense oligonucleotide properties in terms of their potential therapeutic applications(22).

EXPERIMENTAL

Column chromatography was performed on silica gel 60, 230-400 mesh from Fluka, TLC was performed on DC-Alufolien silica gel 60 F254 plates

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from E.Merck. Solvents were purchased in the highest available quality and used without further purification or drying. t-BuMgCl was purchased as a 2M solution in THF from Aldrich. UV spectra were recorded with Uvikon 930 spectrometer (Kontron Instruments AG). ^{13}C- and ^{31}P-NMR spectra were recorded on a Bruker AM 250 Spectrometer operating at 62.90 MHz and 101.27 MH_Z respectively. Tetramethylsilane and external trimethyl phosphate were used as the respective references. ^{31}P-NMR chemical shifts were next recalculated against 80% H_3PO_4 standard assuming $\delta(MeO)_3PO$ = -1.0 ppm(23). Shifts downfield from the reference are assigned as positive.

5'-O-Dimethoxytrityl-2'-O-tetrahydropyranyl uridine, high Rf isomer (1).

The title compound was prepared according to the literature procedure(24).

2',3'-O,O-Diacetyl uridine (4).

The title compound was obtained in 80% yield according to described procedure(25) except that instead of triphenylmethyl chloride, 4,4'-dimethoxytrityl chloride was used for transient 5'-OH protection and that all operations were performed at room temperature.

O-(4-Nitrophenyl)-S-(4-nitrobenzyl)phosphorothioate (2).

To the solution of O-(4-nitrophenyl)dichlorophosphorothioate(26) (10.8g, 40 mM) in dry dioxane (560 mL) a solution of sodium hydroxide (6.4g, 160 mM) in a mixture of water-dioxane (320 mL, 1:1 v/v) was added at room temperature with vigorous stirring in not less than 2.5 h. To the resulting solution of the sodium salt of O-(4-nitrophenyl)phosphorothioate, a solution of 4-nitrobenzylbromide (8.6 g, 40 mM) in dioxane (50 mL) was added. After ca. 0.5 h the dioxane was evaporated from the reaction mixture and resulting aqueous solution of crude 2 concentrated to ca. 250 mL. This was extracted with chloroform (3x100 mL) and was evaporated to dryness. The residue was redissolved in minimal amount of water. The clear solution was added dropwise, with stirring, to acetone (0.8 L), yielding a suspension. The precipitate was filtered and the filtrate, containing 2, was collected and the acetone evaporated. The oily residue was dried by several coevaporations with a mixture of acetone/benzene, then dissolved in ethyl acetate. The insoluble impurities were filtered off, and 2 was precipitated by dropwise addition of the filtrate to benzene, filtered and dried under vacuum. Yield 50-80% (7.8-12.6 g).

TLC: Rf (CH_3CN-H_2O, 9:1), 0.58; UV:(H_2O) λ_{max} 283.0, λ_{min} 237.0; $\delta^{31}P(D_2O)$ 15.60; $\delta^{13}C$ ($CHCl_3$): 4-nitrophenyl: 123.43 (C2,6), 127.97 (C3,5), 145.76 (C4), 159.55 (C1); 4-nitrobenzyl: 36.99 (C methylene), 126.18 (C3,5), 132.41 (C2,6), 148.95 and 149.40 (C1,4); δ^1H (D_2O): 4-nitrobenzyl: 4.0 (2H methylene, J_{PH}=16.4 Hz), 7.33 (H2,6; J_{2H3H}=8.8 Hz), 7.84 (H3,5; J_{3H2H}=8.8 Hz); 4-nitrophenyl: 6.98 (H2,6; δ_{2H3H}=9.2 Hz), 7.93 (H3,5, J_{3H2H}=9.2 Hz).

(Sp)- and (Rp)-Isomers of 5'-O-dimethoxytrityl-2'-O-tetrahydropyranyl uridine 3'-O-[O-(4-nitrophenyl)-S-(4-nitrobenzyl)phosphorothioate] (3).

5'-O-Dimethoxytrityl-2'-O-tetrahydropyranyl uridine (1, high Rf isomer) (5.04g,8 mM) and O-(4-nitrophenyl)-S-(4-nitrobenzyl)phosphorothioate (2) (6.30g,16 mM) and 4-toluenesulphonyl chloride (4.56g,24 mM) were stirred in dry pyridine (400 mL) for ca. 1h at room temperature (TLC control: dichloromethane-acetone, 8:2). The reaction mixture was evaporated to dryness. The residue was coevaporated three fold with toluene, and then dissolved in ethyl acetate (250 mL). The resultant solution was washed first with saturated sodium chloride (2x100 mL), then with water (1x100 mL). The organic layer was dried over sodium sulphate, filtered and evaporated to dryness. The product was then purified and separated into diastereomers on a silica gel column, and eluted with a gradient of 0-5% iso-butanol in water- saturated dichloromethane. The yield of 3 as a 3a-(Sp), 3b-(Rp) and 3-(Sp+Rp) was 40-45% (3.1-3.0 g). The ratio 3a-(Sp)/3b-(Rp)/3-(Sp+Rp) was ca. 2.3:1.7:1. 3-(Sp+Rp) was recycled for repeated isolations of diastereomeric forms of 3.

3a-(Sp): TLC: Rf (CH_2Cl_2-i-BuOH, 97:3) 0.25; UV: (96% C_2H_5OH) λ_{max} 266.0, λ_{min} 235; δ ^{31}P (toluene) 20.02, δ ^{13}C ($CDCl_3$): uracil: 102.82 (C5), 139.65 (C6), 150.49(C2), 163.05(C4); ribosyl: 62.29(C5'), 75.70(C2'), 82.23(C4'), 87.72(C1'), C3'-under $CDCl_3$ signal ca. 77.0; tetrahydropyranyl: 19.67(C4), 25.06(C5), 30.44(C3), 63.77(C6), 98.98(C2); 4-nitrobenzyl: 34.38(C-methylene), 123.89(C3,5), 129.83(C2,6), 143.86 and 147.48(C1,4); 4-nitrophenyl: 120.93(C2,6), 125.54(C3,5), 154.05 and 154.11(C1,4); dimethoxytrityl: 55,19(C-methoxyl), 86.00(C-methylidene), 113.32, 127.32, 128.12, 128.93, 130.31, 134.60, 145.04, 158.80(C-phenyl).

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3b-(Rp):TLC: Rf(CH_2Cl_2-i-BuOH,97:3)0.19; UV:(96% C_2H_5OH)λ_{max}265.0, λ_{min}-247.0; $\delta^{31}P$ (toluene) 19.35; $\delta^{13}C$ ($CDCl_3$): uracil: 102.65(C5), 139.45(C6), 150.43(C2), 163.18(C4); ribosyl: 61.89(C5'), 75.51(C2'), 81.55(C4'), 87.49(C1'), C3'- under $CDCl_3$ signal ca. 77.0; tetrahydropyranyl: 19.15(C4), 24.98(C5), 30.19(C3), 63.09(C6), 98.24(C2); 4-nitrobenzyl: 34.35 (C-methylene), 123.71 (C 3,5), 129.64(C2,6), 143.72 and 147.25(C1,4); 4-nitrophenyl: 121.05(C2,6), 125.39(C3,5), 154.12 and 154.24(C1,4); dimethoxytrityl: 55.05(C-methoxyl), 86.19(C-methylidene), 113.17, 127.18, 127.88, 127.99, 130.06, 134.49, 144.93, 158.66 (C-phenyl).

(Sp)- and (Rp)-Isomers of 5'-O-dimethoxytrityl-2'-O-tetrahydropyranyl-uridinylyl (3',5') 2',3'-O,O-diacetyluridine[S-(4-nitrobenzyl)phosphorothioate (5).

2',3'-O.O-Diacetyluridine (4) (0.15 g, 0.45 mM) was dissolved in dry CH_3CN (4.5 mL) and t-BuMgCl (0.65 mL of 2M solution in THF, 1.30 mM) was added with vigorous stirring. To the resultant white suspension of activated nucleoside 4, was added, after 5 min., the solution of monomer 3a-(Sp) or 3b-(Rp) (0.3 g,. 0.3 mM) in dry THF (1.5 mL). The reaction mixture was left overnight at room temperature (TLC control: CH_2Cl_2-i-BuOH, 9:1), then quenched with acetic acid (0.8 mL of 2M solution in THF, 1.60 mM) and diluted with dichloromethane (12 mL). The dichloromethane solution was washed with H_2O till the excess of 4 was removed, then dried over sodium sulphate and evaporated to dryness. Dinucleotides 5a and 5b were purified by means of silica gel column chromatography using 0-8% i-BuOH in CH_2Cl_2, as a eluting solvent system. The preparative yield of dinucleotides 5 was 15-25% (0.052-0.088 g).

5a-(Sp): TLC: Rf (CH_2Cl_2-i-BuOH, 9:1) 0.39; UV: (96% C_2H_5OH) λ_{max} 262.0, 237.0, λ_{min} 246.0; δ ^{31}P (CH_2Cl_2) 23.20; δ ^{13}C ($CDCl_3$): uracil (Up and pU): 102.63, 103.40(2C5), 139.60, 139.94(2C6), 150.33, 150.50(2C2), 162.89, 163.13(2C4); ribosyl (Up): 61.93(C5'), 75.88(C2'), 81.49(C4'), 87.25(C1'), C3'- under $CDCl_3$ signal, ca. 77.0; ribosyl (pU): 66.68(C5'), 69.96(C3'), 72.45(C2'), 80.48(C4'), 86.72(C1'); acetyl: 20.18, 20.38(2C-methyl), 169.54(2C-carbonyl); tetrahydropyranyl: 19.62(C4), 25.07(C5), 30.45(C3), 63.50(C6), 98.59(C2); 4-nitrobenzyl: 34.16(C-methylene), 123.93(C3,5), 129.72(C2,6), 143.84 and 147.39(C1,4); dimethoxytrityl: 55.20(C-methoxyl), 87.54(C-methylidene); 113.28, 127.28, 127.98, 130.21, 134.75, 143.95, 158.76(C-phenyl).

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5b-(Rp): TLC: Rf (CH_2Cl_2-i-BuOH, 9:1) 0.53; UV: (96% C_2H_5OH) λ_{max} 262.0, 237.0, λ_{min} 246.0; δ ^{31}P (CH_2Cl_2) 24.24; δ ^{13}C ($CDCl_3$): uracil (Up and pU): 102.80, 103.58(2C5), 139.67, 139.78(2C6), 150.32, 150.64(2C2), 163.01, 163.14(2C4); ribosyl (Up): 62.36(C5'), 75.68(C2'), 80.42(C4'), 87.93(C1'), C3'- under $CDCl_3$ signal, ca. 77.00; ribosyl (pU): 66.35(C5'), 70.03(C3'), 72.54(C2'), 80.20(C4'), 87.67(C1'); acetyl: 20.35(2C-methyl), 169.57(2C-carbonyl); tetrahydropyranyl: 20.00(C4), 25.00(C5), 30.46(C3), 64.01(C6), 99.25(C2); 4-nitrobenzyl: 33.87(C-methylene); 123.95(C3,5); 129.84(C2,6), 143.92 and 147.43(C1,4); dimethoxytrityl: 55.20(C-methoxyl), 85.76(C-methylidene; 113.31, 127.28, 127.99, 130.24, 134.73, 144.15, 158.74(C-phenyl).

(SpSp)- and (RpRp)-Isomers of 5'-O-dimethoxytrityl-2'-O-tetrahydropyranyluridinylyl (3',5') 2'-O-tetrahydropyranyluridinylyl (3',5') 2',3'-O.O- diacetyluridine di{[O,O-(S-4-nitrobenzyl)phosphorothioate]} (7).

a. 5'-OH Detritylation step

Dinucleotide 5a or 5b (53.5 mg, 45 μM) was treated with dichloroacetic acid in dichloromethane (4 mL of 0.15 M solution) at room temperature(27). The reaction progress was monitored by means of TLC (CH_2Cl_2-CH_3OH, 3:1). After ca. 15-25 min the reaction mixture was washed with water (2x3 mL). The organic layer was separated and dried over sodium sulphate. The product was next precipitated by dropwise addition of the dichloromethane solution to a mixture of petroleum ether-diethyl ether (8:2). The precipitation was repeated twice, and then the 5'-OH unprotected product was centrifuged and dried under vacuum. The yield was 70-90% (27.4-35.2 mg).

b. Coupling step

(Sp)- or (Rp)-Isomer of 5'-OH-deprotected dinucleotide (22.0 mg 25 μM) was dissolved in dry CH_3CN (450 μL), and t-BuMgCl (80 μL of 2 M solution in THF, 160 μM) was added with vigorous stirring. To the resulting suspension of 5'-OH activated dinucleotide the solution of monomer 3a-(Sp) or 3b-(Rp) (50 mg, 50 μM) in dry THF (225 μL) was added. The reaction mixture was left for 24 h at room temperature (TLC control: CH_2Cl_2-CH_3OH, 9:1), quenched with acetic acid (135 μL of 2M solution in THF,270 μM) then diluted with dichloromethane (3 mL). The dichloromethane solution was washed with H_2O (3x1.5 mL), then dried over sodium sulphate and evaporated to dryness. Crude 7 was redissolved in dichloro-

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methane and precipitated from petroleum ether. Finally, protected trinucleotide was purified by means of silica gel column chromatography using 0-6% i-BuOH in CH_2Cl_2 followed by 4-6% CH_3OH in CH_2Cl_2 as eluting solvent systems. The preparative yield was 10% (4.5 mg).

7a-(SpSp): TLC: Rf ($CHCl_3$-CH_3OH, 9:1) 0.46; UV: (96% C_2H_5OH) λ_{max} 261.0, 239.0; λ_{min} 243.0; δ ^{31}P (CH_2Cl_2) 23.28, 22.37.

7b-(RpRp): TLC: Rf ($CHCl_3$-CH_3OH, 9:1) 0.46; UV: (96% C_2H_5OH) λ_{max} 262.0, 239.0, λ_{min} 243.0; δ ^{31}P(CH_2Cl_2) 23.99, 23.66.

Deprotection of oligonucleotides 5 and 7.

a. Removal of 4-nitrobenzyl-group

Individual isomers of 5 or 7 (ca. 5 μM) were dissolved in dioxane (200 μL) then thiophenol (200 μL) and triethylamine (300 μL) were added. The reaction was performed at room temperature. After 15 min (TLC control: dichloromethane-methanole, 9:1), the reaction mixture was evaporated under reduced pressure. The oily residue was then redissolved in dichloromethane (0.4 mL) and added dropwise into hexane. The procedure was repeated twice and allows the efficient removal of thiophenol. The precipitate was next washed with hexane and dried under reduced pressure.

b. Removal of 2',3'-O.O-acetyl groups

S-Deprotected oligonucleotides (ca. 5μM) were dissolved in ethyl alcohol (400 μL), and concentrated aqueous ammonia solution (400 μL) was added. The reaction progress was monitored by means of TLC (acetonitrile-water, 9:1 as developing solvent system) and when the reaction completed (ca. 1.5h) it was evaporated to dryness.

c. Removal of 5'-O-dimethoxytrityl group

S- and 2',3'-O.O-deprotected oligonucleotides (ca. 5μM) obtained as described above in procedure a and b, were dissolved in dioxane (50 μL), and 80% acetic acid (450 μL) was added. The reaction was monitored by TLC (acetonitrile-water, 9:1). After 15 min acetic acid was removed by coevaporation with n-BuOH, yielding a dry residue.

d. Removal of 2'-O-tetrahydropyranyl group

2'-O-Tetrahydropyranyl protected, -SH and 2',3',5'-OH free oligonucleotides were dissolved in ethyl alcohol (50 μL) then water (445 μL) and 1M HCl (5μL) were added. The reaction mixture was left at room temperature overnight. The post-reaction mixture was loaded directly on two

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10x20 cm cellulose plates (0.1 mm) and developed with i-PrOH/NH_3aq/H_2O (7:1:2). The deprotected oligonucleotides were next eluted from cellulose by means of water alkalized with a drop of concentrated ammonia water solution. The yield was 10-20 A_{260} units.

For enzymatic digestion samples of 1 A_{260} unit were additionally passed through RP-HPLC column using following conditions: ODS Hypersil 5μ, 4.6x300 mm column, gradient: from 5% to 20% CH_3CN in 0.1 M TBAF, flow rate 1.5 *mL/min.*

6a-(Sp): TLC: Rf (silica gel, i-PrOH-NH_3aq-H_2O, 7:1:2), 0.63; Rf (cellulose, i-PrOH-NH_3aq-H_2O, 7:1:2) 0.29; UV: (H_2O) λ_{max} 260.0 nm, λ_{min} 232.0 nm, HPLC: Rt (conditions as above) 8.15 min.

6b-(Rp): TLC: Rf (silica gel, i-PrOH-NH_3aq-H_2O, 7:1:2) 0.63; Rf (cellulose, i-PrOH-NH_3aq-H_2O, 7:1:2) 0.29; UV: (H_2O) λ_{max} 260.0 nm, λ_{min} 230 nm. HPLC: Rt (conditions as above) 6.98 min.

9a-(SpSp): TLC: Rf (silica gel, i-PrOH-NH_3aq-H_2O, 7:1:2) 0.61; Rf (cellulose, i-PrOH-NH_3aq-H_2O, 7:1:2) 0.10; UV: (H_2O) λ_{max} 260.0 nm, λ_{min} 238 nm, HPLC: Rt (conditions as above) 11.17 min.

9b-(RpRp): TLC: Rf (silica gel, i-PrOH-NH_3aq-H_2O, 7:1:2) 0.61; Rf (cellulose, i-PrOH-NH_3aq-H_2O, 7:1:2) 6.10; UV: (H_2O), λ_{max} 260.0 nm, λ_{min} 235 nm; HPLC: Rt (condition as above) 9.70 min.

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Enzymatic digestion of oligonucleotides 6 and 9 with nuclease P1.

To water solution of suitable isomer of oligonucleotide 6 or 9 (0.4 H_{260}, 30 μl) mixed with 0.1M TRIS-HCl buffer, pH 7.2 (10 μl), containing 1 mM $ZnCl_2$, nuclease P1 (Pharmacia, lot ND85219; 10 u, 20 μl) was added. After 15h at room temperature the reaction mixture was analysed by means of TLC (i-PrOH-NH_{3aq}-H_2O, 7:1:2).

DISCUSSION

Recently, we have reported on a stereospecific approach to the synthesis of phosphorothioate(28,29) and methanephosphonate(30-33) oligodeoxyribonucleotide "P-tactic" analogues, with a defined chirality at phosphorus.

Our original concept relies upon the transesterification reaction besed on the nucleophilic substitution of aryloxy group in diastereomerically pure, P-chiral monomers, by a base activated 5'-hydroxyl function of nucleoside or growing oligonucleotide chain.

In this paper I describe a broadening of our method to include the synthesis of short, P-chiral phosphorothioate oligoribonucleotides. Uridinylyl(3',5')uridinylyl(3',5')uridine phosphorothioate - a phosphorothioate analogue of the phenylalanine codon - was chosen as a model system (FIGURE 1).

As a nucleoside substrate, 5'-O-dimethoxytrityl-2'-O-tetrahydropyranyl uridine (1) was used. Because of the chirality of the tetrahydropyranyl group, the tetrahydropyranyl nucleoside derivatives are obtained as a mixture of two diastereomers which can be separated chromatographically into individual species(24). Only the high R_f isomer was utilized so as to avoid the generation of a complex diastereomeric mixture, which may be produced subsequent to the thiophosphorylation step which generates an additional centre of chirality at phosphorus.

The monomer, 5'-O-dimethoxytrityl-2'-O-tetrahydropyranyluridine 3'-O-[O-(4-nitrophenyl)-S-(4-nitrobenzyl)phosphorothioate] 3 was obtained as a mixture of diastereomers which were next separated into individual (Sp)-3 and (Rp)-3 species by means of silica-gel column chromatography. In our earlier attempts to synthesize monomers of type 3 we used the multi-step procedure, which involves phosphorylation of a suitable protected nucleoside with O-(4-nitrophenyl)-N-phenylamidochloridate followed by PN->PS conversion of the resultant phosphoranilidate into phosphorothioate, followed in turn by S-alkylation(28,29).

Here, I propose an improved, one step procedure of the synthesis of 3, based upon the direct thiophosphorylation of a 2',5'-protected nucleoside with O-(4-nitrophenyl)-S-(4-nitrobenzyl)phosphorothioate (2) in the presence of 4-toluenesulfonyl chloride, as a new thiophosphorylating agent.

Phosphorothioate 2 was prepared essentially according to a modified method described earlier for O-(4-chlorophenyl)-S-(N-monomethoxytritylaminoethyl)phosphorothioate(34). The method rests upon the hydrolysis of O-(4-nitrophenyl)dichlorophosphorothioate(26) with an aqueous sodium hydroxide solution followed by alkylation of the resultant O-(4-nitrophenyl)phosphorothioate with 4-nitrobenzyl bromide. Careful pH-control in the course of both steps is essential.

It is worth mentioning that 2 can be sufficiently purified by a simple precipitation procedure only, yielding a chromatographically homogenous product.

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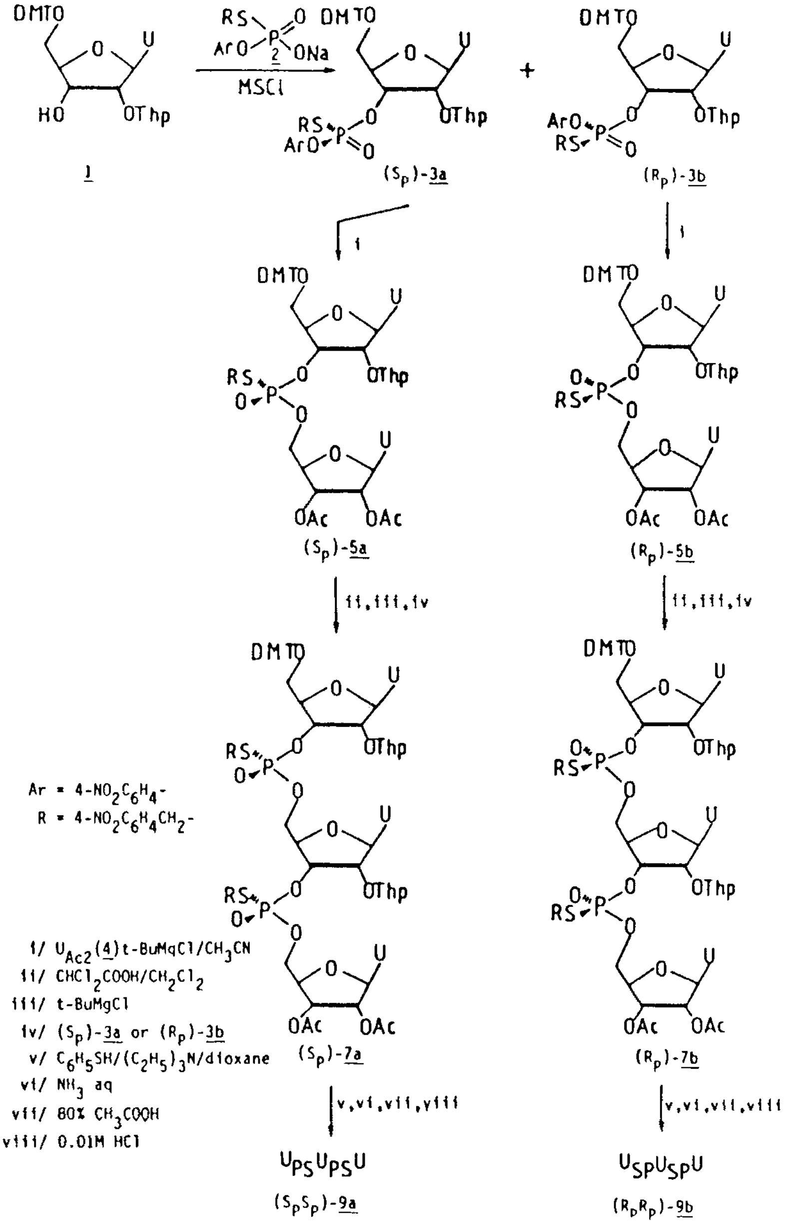

FIG. 1. Synthesis of monomers, 5'-O-dimethoxytrityl-2'-O-tetrahydropyranyl uridine 3'-O-[O-(4-nitrophenyl)-S-(4-nitrobenzyl)phosphorothioate] (3) and individual homochiral diastereomers (SpSp) and (RpRp) of tri(uridine phosphorothioate) (9).

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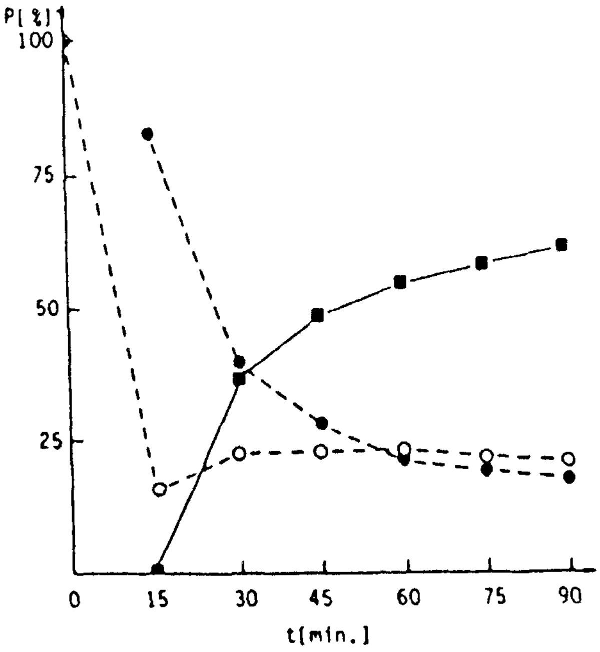

FIG. 2. The kinetic curves of the reaction of 5'-O -dimethoxytrityl-2'-O-tetrahydropyranyl uridine (1) and 4-toluenesulfonyl chloride acivated O-(4-nitrophenyl)-S-(4-nitrobenzyl)phosphorothioate (2) in pyridine. 2:○- -○,active form of 2 (see discussion):●- -●,5'-O-dimexytrityl-2'-O-tetrahydropyranyl uridine 3'-O-[-4-nitrophenyl-S-(4-nitrobenzyl)phosphorothioate](3):■ —■. t= 0 min. - addition of 4-toluenesulfonyl chloride to 2, t=15 min. - addition of 1 into activated 2. P[%] - percentage of the P-consisting reaction components as mesured by ^{31}P-NMR.

Thiophosphorylation of 1 with 2 was performed in the presence of 4-toluenesulfonyl chloride as an activating agent(35). Kinetics of the thiophosphorylation process was followed by means of ^{31}P-NMR and is depicted in FIGURE 2. The representative ^{31}P-NMR spectra are shown in FIGURE 3. First, reaction of 2 with the activating agent in anhydrous pyridine results in the rapid decrease of the 2 ^{31}P signal intensity at

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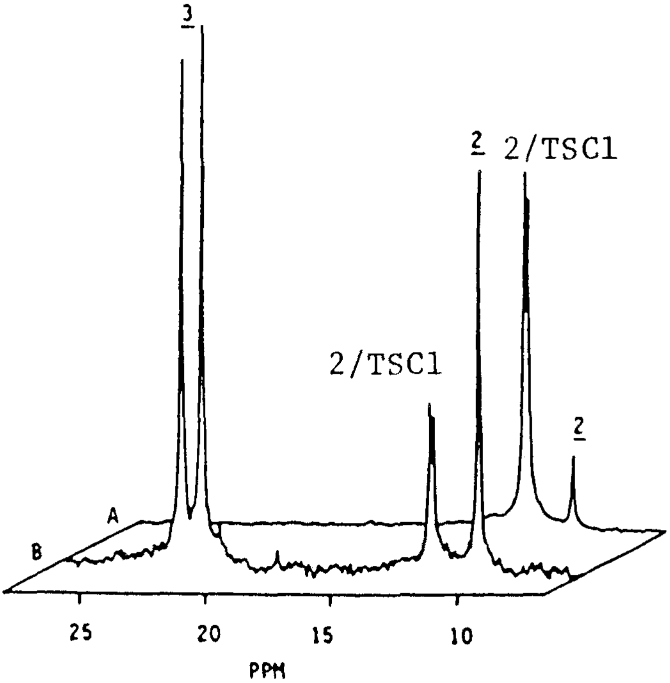

FIG. 3. A: ^{31}P-NMR spectra (101.27 MHz) of mixture of O-(4-nitrophenyl)-S-(4-nitrobenzyl)phosphorothioate and 4-toluenesulfonyl chloride after 15 min. (2/TSCl) and B: 2/TSCl/5'-O-dimethoxytrityl-2'-O-tetrahydropyranyl uridine 3'-O-[O-(4-nitrophenyl)-S-(4-nitrobenzyl) phosphorothioate]. Chemical shifts are given in ppm (δ) relative to 85% H_3PO_4.

7.96 ppm and the subsequent appearance of two intense signals at 9.95 and 9.80 ppm. When nucleoside 2 is next added, the fast disappearance of the signal at 9.95 and 9.80 ppm, and appearance of two resonances at 20.05 and 19.24 assigned to monomer 3 are observed.

By analogy to the monomer activation process in the phosphotriester method of oligonucleotide synthesis(36,37), one can suppose that the active thiophosphorylating intermediate characterized by δ ^{31}P 9.95 and 9.80 ppm is a symmetrical bis[O-(4-nitrophenyl)-S-(4-nitrobenzyl)]-

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pyrothiophosphate originating from the initially formed 4-toluenesulfonic acid - thiophosphate mixed anhydride(38).

The ^{31}P NMR spectra are consistent with this assumption since two signals at 9.95 and 9.80 ppm can be attributed to RR and SS P-enantiomeric- and RS and SR P-mezo- forms of pyrothiophosphate. Alternatively, for a mixed anhydride formed as an P-enantiomeric mixture, only one signal should be observed.

No other signals, which could be assigned to, eg., mixed anhydride-pyridine adducts, were detected.

The efficiency of the thiophosphorylation reaction, as judged by means of ^{31}P NMR, is nearly quantitative. The lower preparative yield of 3 may be caused by its partial decomposition during chromatographic purification and diastereomers separation.

For the stereospecific synthesis of di- and triribonucleotides the methodology successfully applied in the deoxyribo- series was used(29,32) (FIGURE 1).

Two main questions were addressed in the course of the present study:

1) Is the internucleotide bond formation stereospecific in the presence of a protected 2'-OH function as was previously observed in the deoxyribo-series?

2) Is the coupling reaction efficient under the conditions proposed, in spite of steric hindrance generated by the protected 2'-OH function?

Dinucleotides 5'-O-dimethoxytrityl-2'-O-tetrahydropyranyluridinylyl-(3',5')2',3'-O,O-bisacetyluridine [S-(4-nitrobenzyl)phosphorothioate] (Sp)-5 and (Rp)-5 were obtained by the reaction of 2',3'-O,O-diacetyl uridine (50% molar excess relative to 3), 5'-OH activated with t-BuMgCl, and a suitable Rp- or Sp-isomer of monomer 3. As a solvent a mixture of CH_3CN and THF was used.

Removal of the 5'-DMT group in diastereomerically pure dinucleotides 5 and subsequent 5'-OH activation followed by the reaction with Rp- or Sp-isomer of monomer 3 (100% molar excess relative to 5'-deprotected 5) leads to trinucleotides 5'-O-dimethoxytrityl-2'-O-tetrahydropyranuluridinylyl(3',5')2'-O-tetrahydropyranyluridinylyl(3',5')2',3'-O,O-diacetyluridine di{O,O-[S-(4-nitrobenzyl)phosphorothioate]} (SpSp)-7 and (RpR)-7 (FIGURE 1).

In both cases, dinucleotide 5 synthesis, and chain elongation leading to 7, the coupling reaction appears stereospecific as estimated by ^{31}P-NMR.

Thus, one may conclude that steric hindrance generated by the 2'-O-tetrahydropyranyl group does not change the stereoinvertive nature of the condensation reaction under the conditions studied. It seems also reasonable to assume that for the other 2'-O-protecting groups the stereospecificity of the coupling step will be also preserved.

The efficiency of the coupling reaction is, however, affected by the steric hindrance caused by the 2'-protection. The rate of the reaction is reduced by about three-fold relative to the deoxyribo-series(29). It may reflect a general problem in the chemical synthesis of oligoribonucleotides caused by the presence of the 2'-hydroxyl group in the ribose ring requiring selective protection during the formation of the internucleotide linkage. This is illustrated by the decreasing reactivity of the monomer type of fully protected nucleoside 3'-O-(N,N-diisopropylamino)(2-cyanoethoxy)phosphite in the coupling reaction, in the respective ratio 1, 0.7, 0.4 and 0.1 for 2'-deoxy, 2'-OCH_3, 2'-O-tetrahydropyranyl and 2'-O-t-butyldimethylsilylyl(39). Although more studies are necessary to optimize the coupling reaction it should be pointed out that the reaction as designed proceeds with only small amount of by product formation which allows easy recovery and recycling of unreacted nucleotide components.

The oligonucleotides 5 and 7 were deprotected according to the literature procedures in following reaction sequence: 1/ dioxane-triethylamine-thiophenol (2:2:1) for 4-$NO_2C_6H_4CH_2S$-(29), ii/ concentrated NH_3aq-C_2H_5OH for $CH_3C(O)$-(40), iii/ 80% CH_3COOH for DMT(41), iv/ 0.01 M HCl in H_2O-C_2H_5OH (9:1) for Thp(42). The resulting oligonucleotides were next purified by TLC and/or HPLC.

The assignment of the absolute configuration at phosphorus of monomers 3 and oligonucleotides 5 (6) and 7 (9) is crucial for the discussion of the possible relationship between the stereochemistry of the internucleotide linkage and the physicochemical properties of the monomers 3 and dinucleotides 5, and monomers 3 and unmodified oligonucleotide. This was done by chemical correlation between uridine cyclic 3',5'-O,O-phosphorothioate 10 as shown in FIGURE 4. In the course of the present study it was found that conversion of 3 into 10 and 3 into 5 is stereospecific. The latter was performed according to the procedure described above for coupling reaction. The conversion of monomer 3 into uridine cyclic 3',5'-phosphorothioates 10 was performed as follows. The

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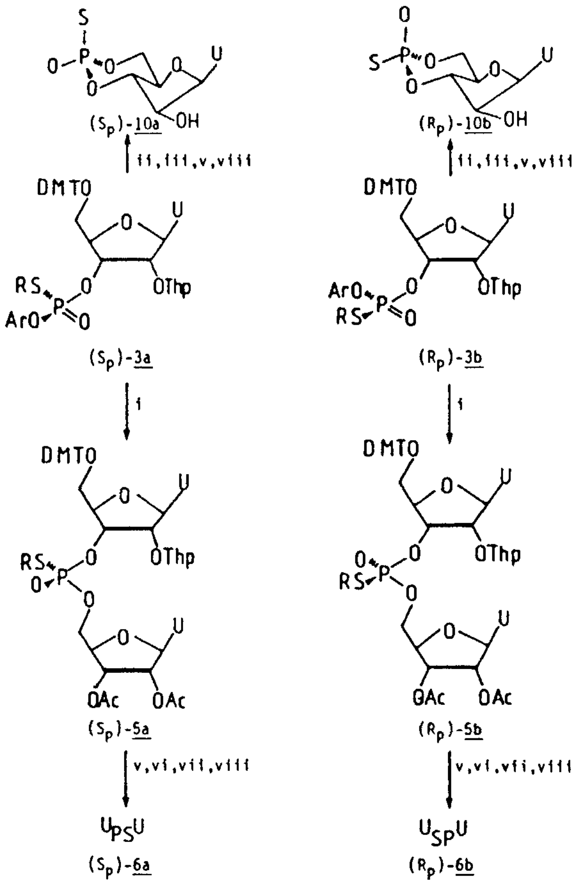

i/ U_{AcZ} (4)/t-BuMgCl/CH_3CN
ii/ $CHCl_2COOH/CH_2Cl_2$
iii/ t-BuMgCl
v/ $C_6H_5SH/(C_2H_5)_3N$/dioxane
vi/ NH_3 aq
vii/ 80% CH_3COOH
viii/ 0.01M HCl

Ar = 4-$NO_2C_6H_4$-
R = 4-$NO_2C_6H_4CH_2$-

FIG. 4. Assignment of the absolute configuration at phosphorus of monomers 3 and dinucleotides 5 (6).

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5'-DMT group was removed in reaction of diastereomerically enriched mixture of 3 (FAST-3/SLOW-3=70:30) with dichloroacetic acid in dichloromethane(27). Cyclization of 5'-deprotected 3 was next performed via 5'-OH base activation. As the reaction catalyst, the t-BuMgCl was used similarly as for the coupling reaction. The cyclization was nearly quantitative and yielded an enriched mixture of diastereomeric 2'-O-tetrahydropyranyluridine 3',5'-(S-4-nitrobenzyl)phosphorothioate which was next S- and 2'-OH deprotected as described above for oligonucleotides 5 and 7, yielding 10. The ratio of 10 originating from FAST-3/10 originating from SLOW-3, was close to the original ratio of monomer 3, as judged by ^{31}P-NMR measurements. The experimental details of the t-BuMgCl catalyzed cyclization reaction will be described elsewhere. Taking advantage of the fact that the absolute configuration of 10 was established earlier(43) and assuming that the stereospecific reaction leading to 5 and 10 respectively occurs most probably with inversion of configuration at the phosphorus atom of monomer 3(44), the absolute configuration was assigned first for 3 and next for 5. Thus the Sp-configuration may be ascribed to the phosphorus in FAST-migrating 3 leading to (Sp)-10 and Rp-configuration for its SLOW-migrating counterpart. Consequently, the Sp-configuration was assigned to 5 originating from (Sp)-3 and Rp-configuration to 5 originating from (Rp)-3. Additionally the absolute configuration at phosphorus of the diastereomers of 5 was established independently, after their deprotection, by enzymatic digestion with nuclease P1 (E.C. 3.1.30.1)(45). This correlation is consistent with the above assignment done by means of ^{31}P-NMR chemical shift criteria and confirms inversion of configuration at phosphorus of 3, in the coupling as well as in the cyclization step.

Since there is no reason to assume that the stereochemistry of the coupling reaction leading to dinucleotide 5 is different from that of the elongation reaction leading to trinucleotide 7, the absolute configuration of both diastereomers of 7 may also be ascribed.

In conclusion, an approach to stereospecific synthesis of short thiooligoribonucleotides was proposed. The absolute configuration at phosphorus of monomers and oligonucleotide products was established. This work may allow incorporation of thiooligoribonucleotide, P-stereodefined domains into natural and synthetic ribonucleic acids via T4 RNA catalyzed reaction(46).

This work, too, is in progress in our laboratory.

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