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STUDIES ON STEREOSPECIFIC FORMATION OF P-CHIRAL INTERNUCLEOTIDE LINKAGE. SYNTHESIS OF (R_P,R_P)- AND (S_P,S_P)-THYMIDYLYL(3',5')THYMIDYLYL(3',5')THYMIDINE DI(O,O-PHOSPHOROTHIOATE) USING 2-NITROBENZYL GROUP AS A NEW S-PROTECTION

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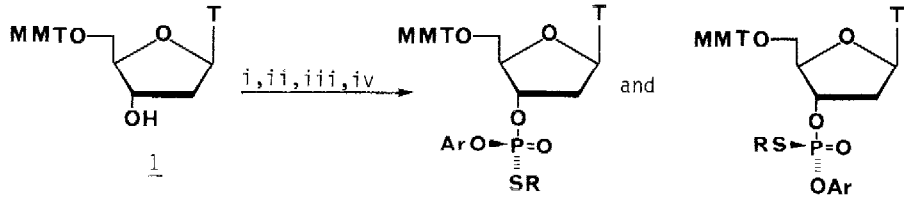
SUMMARY: (R_P,R_P)- and (S_P,S_P)-diastereomers of thymidylyl(3',5')thymidylyl(3',5')thymidine di(O,O-phosphorothioate (6) were prepared in the stereospecific reaction of (R_P)- or (S_P)-isomer of 5'-O-monomethoxytritylthymidine 3'-O-[O-(4-nitrophenyl)-S-(2-nitrobenzyl)phosphorothioate] (2) with 5'-OH activated 3'-O-acetylthymidine and subsequently with 5'-OH deprotected, 5'-OH activated derivative of resulted dinucleotide 3.

Among the chiral at phosphorus oligonucleotide analogues, proved as useful tools in molecular biology, the potential of phosphorothioates[1] and methanephosphonates [2] is most widely explored. Due to the chirality at phosphorus centre they are obtained as a mixture of diastereomers, sometimes enriched in one or another isomer[3]. However, in many studies P-chiral oligonucleotide analogues with defined sense of chirality are desired. Therefore, the mixture of diastereomers have to be separated by a tedious chromatographic techniques[4-6]. Recently we have reported a stereospecific approach to the synthesis of diastereomeric 2'-deoxyadenylyl(3',5')2'-deoxyadenylyl phosphorothioates[7]. Similar methodology was also used for the stereospecific synthesis of thymidylyl(3',5')thymidylyl methanephosphonates[8] and homochiral oligo(thymidine methanephosphonates)[9].

In our earlier attempt to the stereospecific formation of P-chiral phosphorothioate internucleotide linkage we have used methyl group for sulfur protection [7]. Unfortunately, in contrast to demethylation of internucleotide O-methylphosphate by treatment with a mixture of thiophenol and triethylamine in dioxane, which is fast and virtually quantitative reaction[10], the deprotection of thiolo- function of S-methylphosphorothioate under the same reaction conditions, requires several hours and the process is accompanied by side product formation[7]. In a search for a better S-protection of phosphorothioate we decided to utilize 2-nitrobenzyl group. It should be more readily removable than methyl group due to electronegative properties of its 2-nitrophenyl moiety, thus facilitating an attack of thiophenoxide ion on the carbon atom of methylene group. Indeed, we have found that deprotection time is ca 150 times shorter (less than 5 min. instead of 12 h)[11] if 2-nitrobenzyl instead of methyl group is used as protection of thiolo-function of phosphorothioate.

The internucleotide bond is formed in the stereospecific substitution of aryloxy group of P-chiral nucleotide component, 5'-O-monomethoxytritylthymidine 3'-O-[O-(4-nitrophenyl)-S-(2-nitrobenzyl)phosphorothioate] (2) by base activated 5'-hydroxyl function of 3'-O-acethylthymidine or 5'-deprotected oligonucleotide 3 (SCHEME II). The synthesis of 2 is outlined in Scheme I. The phosphorylation of 1 with O-(4-nitrophenyl)-N-phenylamidochloridate and PN->PS conversion of resulted phosphoranilidates into phosphorothioates were performed according to the method described earlier[12,13]. The crude mixture of diastereomeric phosphorothioates, obtained in 75% yield,

was S-alkylated without further purification by treatment with two equivalents of 2-nitrobenzyl bromide in acetone solution. Crude product of alkylation was obtained in 80% yield. The diastereomers of 2 were separated into individual species [2a, $\delta^{31}P$ 24.99 and 2b, $\delta^{31}P$ 24.64 ppm (C_6H_6)] by means of column chromatography on silica gel using acetone-chloroform as eluting solvent [19]. Some losses of 2 during chromatography were observed, probably due to its partial decomposition.



1

i/ (ArO)(PhNH)P(O)Cl/pyridine
ii/ NaH/CS_2/dioxane
iii/ RBr/acetone
iv/ separation of diastereomers

(Sp)-2a (Rp)-2b

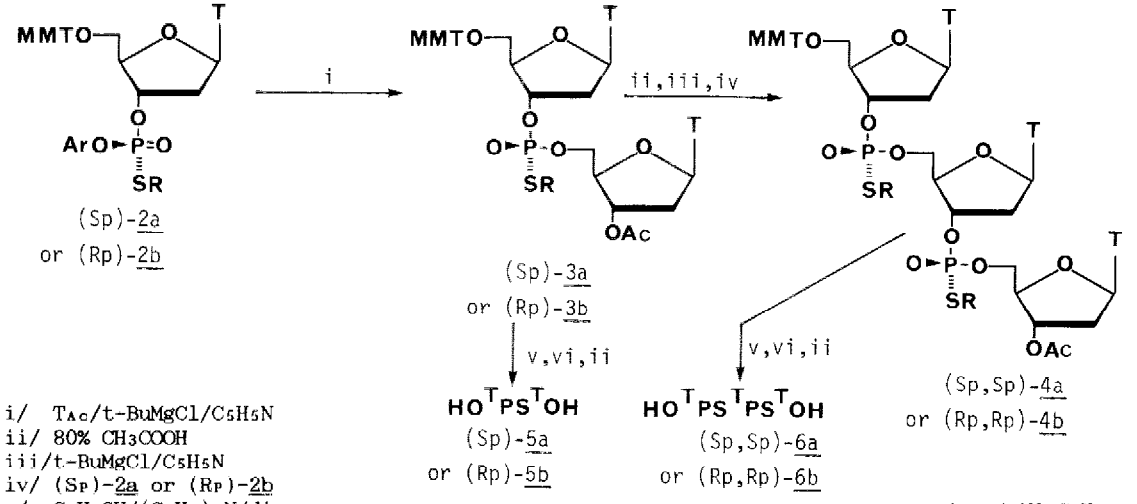
Ar= 4-$NO_2C_6H_4$-
R = 2-$NO_2C_6H_4CH_2$-

SCHEME I

Assuming, that the relationship between absolute configuration at phosphorus and the chemical shift in ^{31}P-NMR observed for diastereomeric 5'-O-monomethoxytrityl-2'-deoxyadenosine 3'-O-[O-(4-nitrophenyl)-S-methylphosphorothioates] described previously[15] will also hold for diastereomeric pair of 2 we have assigned the S_P-configuration for the isomer of 2a absorbing at lower field in ^{31}P-NMR. The activation of the hydroxyl function of nucleoside component, 3'-acetylthymidine, and its further reaction with (S_P)-2a or (R_P)-2b, were carried out similarly as described earlier[8] but anhydrous pyridine has been used as the reaction medium. Thus, the 2,15M solution of t-butylmagnesium chloride (10% molar excess) in dry THF was added under argon to the solution of 3'-O-acetylthymidine in dry pyridine. To the resulted suspension of 5'-hydroxyl activated nucleoside (30% molar excess) a solution of (S_P)-2a, or (R_P)-2b containing 15% (S_P)-2a, in pyridine was added and the reaction mixture was stirred at room temperature for 8h. The reaction is extremely water-sensitive and must be carried out under an inert atmosphere. The efficiency of coupling reaction leading to dinucleotides 3 was 50-95% as determined by ^{31}P-NMR spectra of the crude post-reaction mixture. The only side-product observed ($\delta^{31}P$ 18.87 ppm) resulted probably from the monomer 2 hydrolysis. The diastereomeric purity of 3a originating from (S_P)-2a was ca 100% (no signal of another isomer was observed) and that of 3b, originating from (R_P)-2b/(S_P)-2a mixture was ca 75%. Taking into account accuracy of measurements (±5%) it is consistent with the diastereomeric purities of substrates 2, and speaks for stereospecificity of the coupling reaction.

After standard work-up, the dinucleotides 3[16] were purified by means of preparative TLC on silica gel plates using 5% CH_3OH in $CHCl_3$ as eluting solvent system. They were next deprotected in the following reaction sequence: i/C_6H_5SH/(C_2H_5)$_3$N/dioxane (2:2:1)[10], ii/ 25%NH_3aq/CH_3OH (3:1)[17], iii/ 80% CH_3COOH[18] yielding dithymidine phosphorothioates 5. The crude 5 were purified by means of preparative TLC on cellulose plates developed in i-PrOH/NH_3aq/H_2O (7:1:2) as solvent system. The absolute configuration at phosphorus in diastereomers of 5[19] was determined by means of chemical shift criterion in ^{31}P-NMR .The comparison of ^{31}P-NMR spectral data of individual isomers of 5 with those described in literature [20,21] allowed us to ascribe S_P-configuration to 5a originating from (S_P)-2a absorbing at higher field, and R_P-configuration to

this originating from (R_P)-2b and absorbing at lower field, respectively. Assuming that the coupling reaction between monomer 2 and 5'-activated nucleoside leading to 3 occurs most probably with inversion of configuration at phosphorus ,the assignments of absolute configuration in 2, 3 and 5, based on ^{31}P-NMR chemical shift criterion, are consistent. Additionally, the absolute configuration at phosphorus in diastereomers of 5 was established independently by their enzymatic digestion with nuclease P1(E.C.3.1.30.1)[22].



SCHEME II

On the basis of the enzymatic criterion we were able to assign the S_P-configuration to diastereomer of 5a originating from (S_P)-2a and R_P-configuration to the other diastereomer of 5b obtained from (R_P)-2a. This correlation is consistent with above assignment done by means of chemical shift in ^{31}P-NMR criterion, and confirms inversion of configuration at phosphorus of 2 in coupling step.

For the synthesis of 5'-O-monomethoxytritylthymidylyl(3',5')thymidylyl(3',5')(3'-O-acetylthymidine) di{O,O-[S-(2-nitrobenzyl)phosphorothioate]}(4a) and (4b), diastereomerically pure 3 were used. Deprotection of 5'-hydroxyl function of (S_P)-3 and (R_P)-3 was achieved by treatment of fully protected dinucleotide 3 with 80% acetic acid for 3h[18]. Simple repeated precipitation procedure yielded detritylated, chromatographically homogenous product suitable for the next coupling reaction without further purification. Fully protected trinucleotides (S_P,S_P)-4a and (R_P,R_P)-4b were prepared by the reaction between 5'-deprotected, 5'-activated derivative of dinucleotide (S_P)-3a or (R_P)-3b and diastereomerically pure monomer (S_P)-2a or (R_P)-2b, respectively. The general procedure was the same as for synthesis of 3, except that a 50% molar excess of t-butylmagnesium chloride for activation of 5'-deprotected dinucleotide 3 and equimolar amount of monomer (S_P)-2a or (R_P)-2b for coupling reaction, were used. The efficiency of coupling reaction leading to trinucleotides 4 was somewhat lower than for 3 and was up to 50% for 4a and up to 60% for 4b as estimated by means of ^{31}P-NMR. The work-up and purification of 4[23] were the same as described above for 3. The removal of S-protecting 2-nitrobenzyl group achieved in the reaction with thiophenolate ion[10] was near quantitative. The 3'- and 5'-hydroxyl function deprotection was performed as for 3, according to the literature methods [17,18]. Assuming, consistently, inversion of configuration at phosphorus of 2 in second coupling step, the (S_P,S_P)- and (R_P,R_P)-configuration were ascribed to suitable trinucleotides 4a and 4b and sub-

sequently to 6a and 6b[24] originating from (S_P)-2a and (S_P)-3a, and(R_P)-2b and (R_P)-3b, respectively.

The absolute configuration assignment at phosphorus of homochiral trinucleotides 6a and 6b was confirmed by their comparison by means of HPLC[26] with original samples obtained independently according to literature method based on nonstereospecific synthesis and separation of diastereomers[4,25].

The stereospecific synthesis of longer oligo(nucleoside phosphorothioates) with defined sense of chirality at phosphorus is now under study.

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14. (S_P)-2a; TLC: 0.28[$CHCl_3$-$(CH_3)_2CO$(90:10)], UV:λ_{min}247, λ_{max}268 nm(96% C_2H_5OH), δ^{31}P24,99 ppm(C_6H_6). (R_P)-2b; TLC: 0.24[$CHCl_3$-$(CH_3)_2CO$(90:10)], UV: λ_{min}245,λ_{max}266 nm(96% C_2H_5OH), δ^{31}P24.64 ppm(C_6H_6).
15. Isomer of 5'-O-monomethoxytrityl-2'-deoxyadenosine 3'-O-[O-(4-nitrophenyl)-S-methyl-phosphorothioate] absorbing at lower field has S_P- while its counterpart absorbing at higher field has R_P- configuration [7].
16. (S_P)-3a; TLC: 0.29[$CHCl_3$-CH_3OH(95:5)], UV: λ_{min}245, λ_{max}264 nm(96% C_2H_5OH), δ^{31}P28.46 ppm($CHCl_3$). (R_P)-3b; TLC: 0.34[$CHCl_3$-CH_3OH(95:5)], UV: λ_{min}245, λ_{max}264nm (96%C_2H_5OH), δ^{31}P28.52 ppm ($CHCl_3$).
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19. (S_P)-5a; TLC: 0.54[i-PrOH-NH_3aq-H_2O(7:1:2)], UV: λ_{min}237, λ_{max}266 nm(96% C_2H_5OH), δ^{31}P55.97 ppm(D_2O). (R_P)-5b; TLC: 0.54[i-PrOH-NH_3aq-H_2O(7:1:2)], UV: λ_{min}236, λ_{max}266 nm(96% C_2H_5OH), δ^{31}P56.30(D_2O)
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23. (S_PS_P)-4a; TLC: 0.31[$CHCl_3$-CH_3OH(94:6)], UV: λ_{min}254, λ_{max}258 nm(96% C_2H_5OH),δ^{31}P29.49 ppm($CDCl_3$).(R_PR_P)-4b; TLC: 0.37[$CHCl_3$-CH_3OH(94-6)], UV: λ_{max}258 nm(96% C_2H_5OH), δ^{31}P30.01, 29.54 ppm($CDCl_3$)
24. (S_PS_P)-6a; TLC: 0.39[CH_3CN-H_2O(90:10)], UV: λ_{min}237, λ_{max}262 nm(96% C_2H_5OH), δ^{31}P55.32, 55.27 ppm(D_2O).(R_PR_P)-6b; TLC: 0.40[CH_3CN-H_2O(90:10), UV: λ_{min}243, λ_{max}259 nm(96% C_2H_5OH), δ^{31}P55.48, 55.43 ppm(D_2O)[25].
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26. ODS Hypersil 5µ, 4.6x300mm column, linear gradient from 5% to 40% CH_3CN in 0.1M TEAB, 0.4%CH_3CN/min.

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