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STUDIES ON STEREOSPECIFIC FORMATION OF P-CHIRAL INTERNUCLEOTIDE LINKAGE. SYNTHESIS OF DIASTEREOISOMERIC 2'-DEOXYADENYLYL(3',5')2'-DEOXYADENYLYL S-METHYLPHOSPHOROTHIOATES VIA NUCLEOSIDE HYDROXYL ACTIVATION

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ABSTRACT - Substitution of 4-nitrophenoxy group at phosphorus atom of 5'-O-monomethoxytrityl-2'-deoxyadenosine 3'-O-|O-(4-nitrophenyl)-S-methylphosphorothioate| (4) |(Sp)-4:(Rp)-4=90:10 and (Sp)-4:(Rp)-4=30:70| by base-activated 5'-hydroxyl function of 3'-O-tert-butyldimethylsilyl-2'-deoxyadenosine (5) gives fully protected P-chiral 2'-deoxyadenylyl(3',5') 2'-deoxyadenosine S-methylphosphorothioates (6), |(Sp)-6:(Rp)-6=92:8 and (Sp)-6:(Rp)-6=32:68 respectively|. Demethylation of internucleotide S-methylphosphorothioate group in 6 and deprotection of 3'- and 5'-hydroxyl functions lead to diastereoisomeric mixture of 2'-deoxyadenylyl(3',5') 2'-deoxyadenosine-O,O-phosphorothioates (7).

Oligonucleotides with a chiral centre at phosphorus of internucleotide |^{18}O|-phosphate or phosphorothioate groups are widely used in the studies of stereochemical aspects of action of phosphorolytic enzymes involved in nucleic acids metabolism [1,2]. P-Chiral O-alkyl phosphotriester modified oligonucleotides are valuable tools for investigation of the nucleic acid structure-function relations [3]. An interesting mutation of oligonucleotide analogues are positively charged oligonucleotide triesters used in the interoligonucleotide complex formation studies [4].

There are several established chemical methods of P-chiral oligonucleotides synthesis but all of them require separation of diastereoisomeric oligonucleotides bearing chiral phosphorothioate or O-alkyl phosphate group by means of chromatographic techniques [2]. The separation of P-chiral oligonucleotides is usually laborious, and sometimes hard to achieve [5,6].

The enzymatic synthesis of P-chiral polynucleotides containing 3',5'-phosphorothioate moieties provides one isomer only [2], so does in fact, stereoselective chemical synthesis of dinucleoside monophosphate aryl esters reported recently [7]. In this paper we wish to report a stereospecific approach to the chemical synthesis of both isomers of fully protected P-chiral 2'-deoxyadenylyl(3',5') 2'-deoxyadenosine S-methylphosphorothioate (6)*, and to discuss their further transformations.

RESULTS AND DISCUSSION

A key process in the oligonucleotide synthesis is the condensation of a nucleoside hydroxyl group with the appropriate nucleoside phosphate (phosphite) derivative. The interest in this field has been directed mainly towards the development of highly reactive phosphorylating and phosphitylating agents [8-10].

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*The synthesis of 6 was presented in outline at Sofia, Bulgaria, in September 1985, see: FECS Third International Conference on Chemistry and Biotechnology of Biologically Active Natural Products, Communications, Vol. 4, p. 70.

Another approach involving internucleotide bond formation via nucleoside hydroxyl activation, although well known for several years, has not found wide application mainly due to the forcing reaction conditions and lack of selectivity of the hydroxyl function activation. Recent progress in this field, however [11-13], together with persistent interest in the availability of oligonucleotides with a chiral centre at phosphorus, prompted us to apply this process in our approach to the synthesis of P-chiral oligonucleotides.

The method described herein is based on the substitution of aryloxy group of P-chiral nucleotide component 4 (Sp- and Rp-) by base-activated 5'-hydroxyl function of nucleoside 5. The observation that O,S-dialkyl-O-(4-nitrophenyl)phosphorothioates undergo hydrolysis under alkaline conditions with removal of 4-nitrophenyl but not S-methyl group [14], prompted us to utilize the 5'-protected nucleoside 3'-O-|O-(4-nitrophenyl)-S-methylphosphorothioates| (4) as nucleotide components.

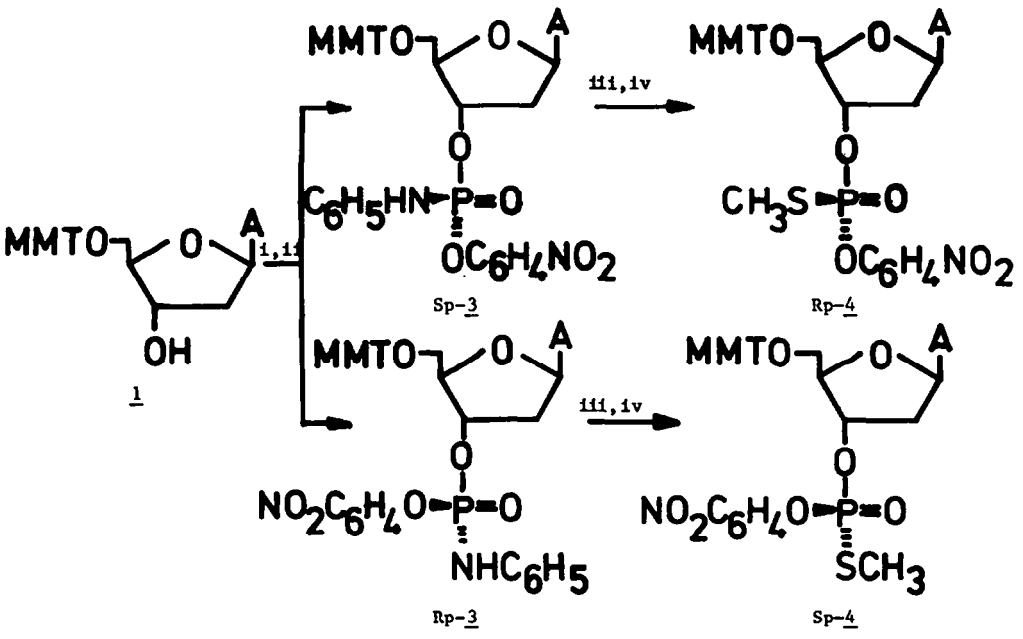
Our original strategy relies upon the use of chiral (but racemic) phosphorylating agent O-(4-nitrophenyl)-N-phenylphosphoramidochloridate (2) for the synthesis of diastereoisomers of 5'-O-monomethoxytrityl-2'-deoxyadenosine 3'-O-|O-(4-nitrophenyl)-N-phenylphosphoramidate| (3) which can be separated by silica gel chromatography. Diastereoisomeric P-anilidates 3 are stereospecifically (retention) converted into 5'-O-monomethoxytrityl-2'-deoxyadenosine 3'-O-|O-(4-nitrophenyl)-S-methylphosphorothioates| |(Rp)-4| and |(Sp)-4|, respectively, by treatment of 3 with sodium hydride and carbon disulphide, followed by alkylation with methyl iodide, according to the method described earlier for thymidine derivatives [15,16]. Substitution of 4-nitrophenoxy group in (Rp)-4 and (Sp)-4 by base-activated 5'-hydroxyl function of nucleoside component 5 should give fully protected P-chiral 2'-deoxyadenylyl(3',5') 2'-deoxyadenosine S-methylphosphorothioates, (Rp)-6 and (Sp)-6. Deprotection of phosphorothioate function and 3'- and 5'-terminal hydroxyl groups in 6 provides 2'-deoxyadenylyl(3',5') 2'-deoxyadenylyl-O,O-phosphorothioate (Rp)-7 and (Sp)-7.

The synthesis of 5'-O-monomethoxytrityl-2'-deoxyadenosine 3'-O-|O-(4-nitrophenyl)-S-methylphosphorothioates|, (Rp)-4 and (Sp)-4.

Treatment of 5'-O-monomethoxytrityl-2'-deoxyadenosine (1) with 50% molar excess of O-(4-nitrophenyl)-N-phenylphosphoramidochloridate (2) in pyridine solution gave two diastereoisomeric phosphoranilidates 3 [17]. They were jointly isolated by means of short-column silica gel chromatography in 50% yield. Diastereoisomerically pure |(Rp)-3| and |(Sp)-3| isomers (TLC criterion) were obtained by subsequent separations by means of column or preparative thin layer silica gel chromatography.

The absolute configuration at phosphorus in both diastereoisomers of 3 was assigned by means of chemical correlation. From earlier studies reported from this, and other Laboratories it is known that 3, after deprotection of 5'-hydroxyl function, undergo cyclization upon treatment with potassium t-butoxide with formation of 2'-deoxyadenosine cyclic 3',5'-phosphoranilidates [17,18]. This process of intramolecular nucleophilic substitution at the phosphorus atom is stereospecific and occurs with inversion of configuration at the phosphorus centre [18,19]. Since the absolute configuration at phosphorus of (Rp)-cdAMP anilidate has been assigned by means of spectroscopic methods and fully confirmed by single crystal X-ray spectroscopy [20], the absolute configuration of its precursor, "low R_f-3" is (Rp), and that of its counterpart "high R_f-3" is (Sp). Finally, this regressive type of stereochemical analysis together with the formerly established stereoretentive nature of the PN→PS conversion [21,22] and due to the fact that S-methylation of obtained phosphorothioates does not affect of the spatial orientation of the substituents at phosphorus let us to assign the absolute configuration to 5'-O-monomethoxytrityl-2'-deoxyadenosine 3'-O-|O-(4-nitrophenyl)-S-methylphosphorothioates| (4) resulting from individual isomers of P-anilidates (Sp)-3 and

(Rp)-3 as shown in Scheme 1.



i/ $(4\text{-}NO_2C_6H_4O)(C_6H_5NH)P(O)Cl$/pyridine, iii/ NaH/CS_2/dioxane-DMF,
ii/ separation of diastereoisomers, iv/ CH_3I/acetone

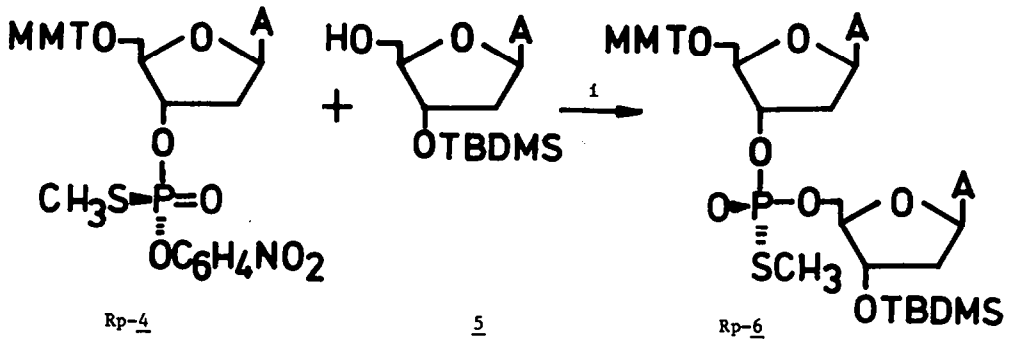
SCHEME 1.

Since the efficiency of separation of diastereoisomeric (Rp)- and (Sp)-3 was moderate only, samples of 3 enriched with one of diastereoisomers have been used for their conversions into diastereoisomeric mixtures (Rp)- and (Sp)-4. Thus the (Sp + Rp)-3 enriched with Sp-isomer has been converted into the mixture containing predominantly (Rp)-4 (Rp:Sp=70:30) while its counterpart enriched with (Rp)-3 has been converted by means of NaH/CS_2 in dioxane and/or DMF solution followed by CH_3I S-alkylation into (Sp + Rp)-4 with predominant (Sp)-4 (Sp : Rp = 90 : 10). It should be pointed out that in all transformations described in this report 6-amino-function of adenylyl residue has been unprotected , what can be partially responsible for lower yields of particular intermediates, 3, 4 and 6.

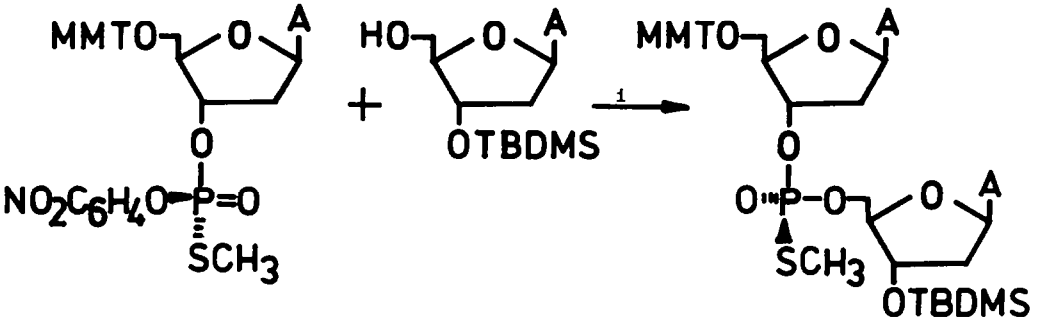
The synthesis of 5'-O-monomethoxytrityl-2'-deoxyadenylyl(3',5')(3'-O-tert-butyldimethylsilyl-2'-deoxyadenosine) S-methylphosphorothioates |(Rp)-6| and |(Sp)-6|

The nucleoside component 3'-O-tert-butyldimethylsilyl-2'-deoxyadenosine (5) was prepared according to the procedure described by Ogilvie et al. [23]. The activation of hydroxyl function of 5 and its further reaction with nucleotide component 4 was performed with minor modifications, according to the procedure of nucleoside phosphates preparation described by Hayakawa et al. [11]. The procedure leading to (Rp)-6 and (Sp)-6 is depicted in Scheme 2. Enriched samples of (Rp)-4 |70% (Rp), 30% (Sp)| and (Sp)-4 |90% (Sp), 10% (Rp); ^{31}P-NMR analysis| were used. To the lithium derivative generated from 5 and n-butyl- or t-butyllithium in THF solution at -60°C, the solution of (Rp)-4 in THF was added at the same temperature. After 4h the reaction was quenched with an excess of pyridinium form of Dowex 50W x 8 or the CH_3COOH in THF solution and the product (Rp)-6 was isolated by means of preparative TLC in 30% yield. (Sp)-4 was converted to (Sp)-6 in 40% yield in a similar way. The desired products

were accompanied by considerable amount of side products, probably due to the phosphorothioylation of adenine moiety. Diastereomeric purities of the resulting 6 were 68% (Rp)-6 and 92% (Sp)-6 as determined by ^{31}P-NMR spectroscopy. This is consistent with the diastereoisomeric purity of starting phosphorothioates (Rp)-4 and (Sp)-4 and implies the stereospecificity of condensation reaction within the limits of the sensitivity of spectroscopic analysis.



Rp-4 5 Rp-6



Sp-4 5 Sp-6

i/ n-BuLi/THF

SCHEME 2.

The structure of 6 was confirmed by conversion of products 6 into 2'-deoxyadenylyl(3',5') 2'-deoxyadenosine in following reaction sequence: i/ 80% CH_3COOH for MMTr [24], ii/ 0.1 N NaOH in dioxane-H_2O (1:3) for CH_3S- [25], iii/ 1M TBAF in THF for TBDMS [26]. The resulting dinucleotide d(ApA) was treated with snake venom and spleen phosphodiesterase to afford dA and pdA or dAp respectively.

The assignment of absolute configuration at phosphorus in 6 was achieved by the spectroscopic comparison (^{31}P-NMR) with authentic samples of (Rp)- and (Sp)-isomers of 6 prepared on an independent way (*vide infra*).

The absolute configuration at P in diastereoisomeric 6 was established independently by their direct conversion into diastereoisomeric 2'-deoxyadenylyl(3',5') 2'-deoxyadenylyl phosphorothioates (7) and enzymatic digestion with nuclease P1 (E.C. 3.1.30.1).
The resistance of dinucleoside phosphorothioate towards hydrolysis in the presence of nuclease P1 is indicative of the (Rp)-configuration, while facile hydrolysis indicates the (Sp)-configuration [27,28]. On the basis of the above criterion we were able to assign the (Rp)-configuration to the compounds 7 and 6 originating from (Rp)-4, and (Sp)-configuration to the other isomers of 7 and 6, obtained from (Sp)-4. The stereochemical analysis discussed above

let us to assign the absolute configuration at phosphorus in substrates 4 and products 6 of the condensation reactions; this in turn allowed us to conclude that since the (Rp)-isomer of 4 gave (Rp)-isomer of 6, and (Sp)-4 gave (Sp)-6, the condensation leading from 4 to 6 proceeds with inversion of configuration at the phosphorus atom of 4. The independent synthesis of 6 was performed as follows: the fully protected dinucleotide 6-N-benzoyl-5'-O-monomethoxytrityl-2'-deoxyadenylyl(3',5')-(6-N-benzoyl-3'-O-tert-butyldimethylsilyl-2'-deoxyadenylyl) O-methylphosphorothioate (11) was prepared according to the literature method [29] as a mixture of two diastereoisomers which were further separated by means of preparative TLC.
Their absolute configuration at phosphorus was assigned enzymatically [27] after the removal of the protecting groups under standard conditions. The reference samples of 6 with known configuration at P were prepared from pure (Rp)- and (Sp)-diastereoisomers of 11 after removing the methyl and benzoyl protecting groups by treatment with dioxane-triethylamine-thiophenol (2:2:1) mixture [30], followed by ammonia saturated methanol and further S-alkylation with methyl iodide as described above for the preparation of 4.

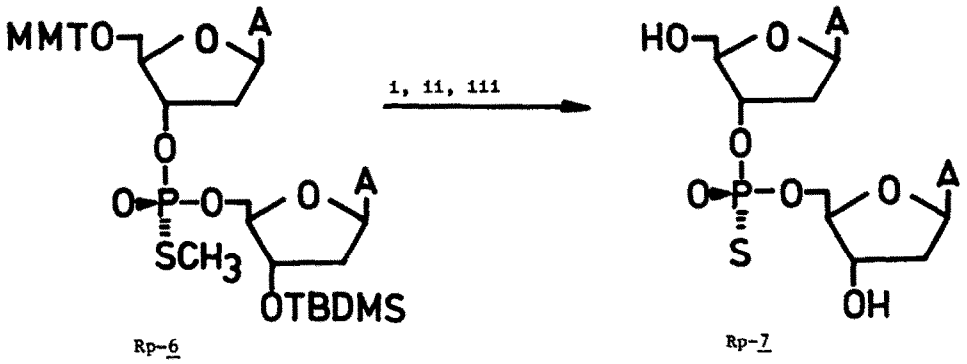
The conversion of (Rp)-6 and (Sp)-6 into 2'-deoxyadenylyl(3',5') 2'-deoxyadenylyl phosphorothioates |(Rp)-7| and |(Sp)-7|

The particular problem concerning 6 → 7 conversion is related to the methyl protection of internucleotide phosphorothioate group in 6. There are several S-alkyl [31] and S-aryl [32] nucleoside phosphorothioates known, but there are only a few examples of the S-protected dinucleoside phosphorothioates. In the cases described, where sulphur was bearing β-cyanoethyl group, the deprotection was achieved under very mild basic conditions [33-35].
In our case the removal of methyl group from phosphorothioate sulphur atom was much more difficult, since 6 represents an example of ambident electrophile, and any nucleophile can attack the phosphorus and three adjacent carbon centres. There are also reports in the literature indicating that sulphur of phosphorothioate moiety can be attacked as well [36].

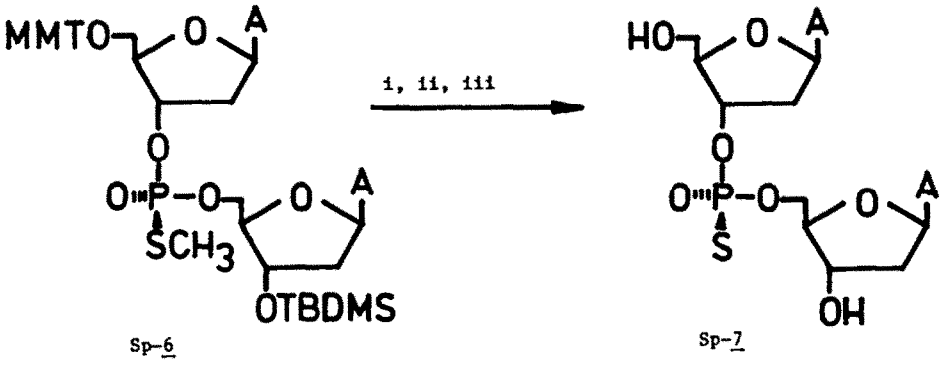
$$-\overset{|}{\underset{|}{P}}=S \xrightarrow[ii/EtS^-]{i/CF_3SO_3Me} -\overset{|}{\underset{|}{P}}\div$$

Due to the known affinity of thiolate anion to sp^3 - hybridized carbon atom, we decided to use thiophenolate ion for conversion of 6 (used as diastereoisomeric mixture of Rp and Sp isomers) into 7 [30]. It was achieved, indeed, in the following reaction sequence: i/ dioxane--triethylamine-thiophenol (2:2:1) [30], ii/ 1M tetrabutylammonium fluoride in THF [26], iii/ 2% toluene-p-sulphonic acid in $CHCl_3$-CH_3OH (7:3) [37].
Resulting 2'-deoxyadenylyl(3',5') 2'-deoxyadenylyl phosphorothioates (7) were isolated by means of preparative TLC.
We have found that in contrast to demethylation of internucleotide O-methylphosphate [30,38], which is fast and virtually quantitative reaction, the deprotection of thiolo-function of 6 requires several hours and overall process is accompanied by side product formation. After 12 h at room temperature we detected by ^{31}P-NMR the presence of ca 80% of desired product 8, accompanied by ca 20% of 5'-O-monomethoxytrityl-2'-deoxyadenosine 3'-O-(S-methylphosphorothioate (9). 5'-Deoxy-5'-phenylthio-3'-O-tertbutyldimethylsilyl-2'-deoxyadenosine (10) was isolated as the second side product by means of preparative TLC.
The structural assignment of 9 was confirmed comparing its ^{31}P-NMR shift value with that of authentic sample of 9 prepared by alkaline hydrolysis of 4. The structure of 10 was established by MS-analysis.
The formation of 9 and 10 in the reaction of 6 with thiophenoxide anion suggests that demethylation of 6 is accompanied by a competetive attack of the nucleophile on C-5' adjacent to the phosphotriester group of 6. This is consistent with earlier observation by Reese et al.

that reaction between fully protected dithymidine arylphosphate and thiophenoxide ion in acetonitrile is accompanied by the internucleotide bond cleavage [39].



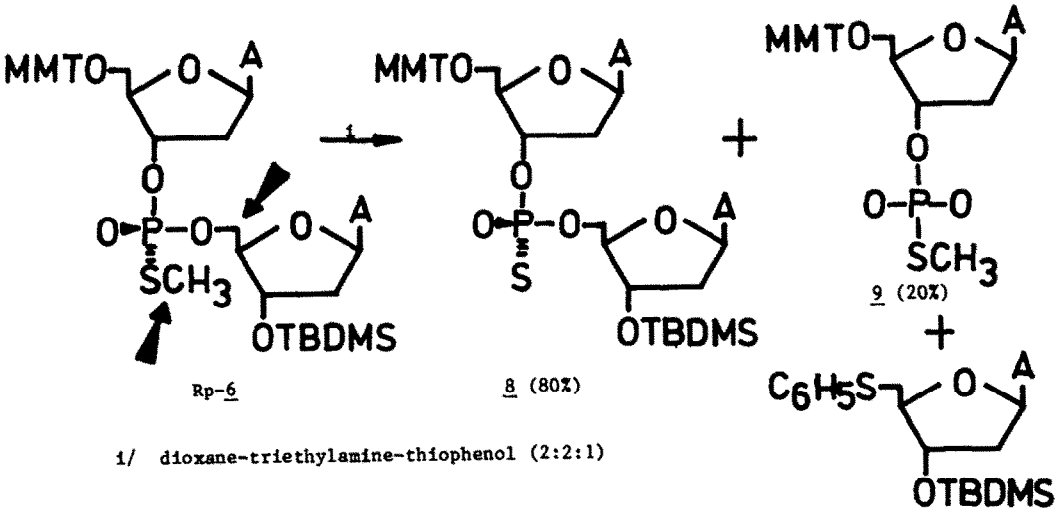
Rp-6 Rp-7



Sp-6 Sp-7

i/ dioxane-triethylamine-thiophenol (2:2:1)
ii/ 1M TBAF/THF
iii/ 2% TsOH/$CHCl_3$-CH_3OH (7:3)

SCHEME 3.



Rp-6 8 (80%) 10

i/ dioxane-triethylamine-thiophenol (2:2:1)

SCHEME 4.

CONCLUSIONS

Phosphorothioate analogues of oligonucleotides have proved to be valuable tools for mechanistic studies of action of enzymes responsible for phosphoryl and nucleotidyl transfer. Dinucleoside phosphorothioates have been used for better comprehension of the mode of action of such enzymes as pancreatic ribonuclease [40,41], bacteriophage T7 induced DNA polymerase [42], nuclease P1 [27], and they were prepared by non-stereospecific synthesis followed by the chromatographic separation of constitutional diastereoisomers. However, it was ascertained only recently that existing methodologies are sometimes inefficient, especially for the phosphorothioate analogues of longer oligonucleotides than dimers, since separation of diastereoisomeric species even by means of the reversed-phase HPLC have failed [6]. For that reason, the search for new, stereospecific methods of synthesis of P-diastereoisomeric analogues of oligonucleotides is a continuous goal of several Laboratories [3,43,44]. The results presented in this paper point to one of the possibilities of stereospecific formation of internucleotide phosphorothioate moieties using readily available mononucleotide precursors 4 of defined structure. Treatment of nucleotide component 4 used as an enriched mixture of |(Rp)-4| and |(Sp)-4| isomers with activated nucleoside component 5 gives an enriched mixture of P-chiral dinucleotides |(Rp)-6| and |(Sp)-6|. The results obtained imply that nucleophilic substitution at phosphorus of 4 proceeds stereospecifically and with inversion of configuration at the phosphorus atom. Although at the moment this methodology seems to be impractical with respect to the synthesis of long-sequence oligonucleotide analogues, it demonstrates a strategy which will be further improved by better selection of S-protective groups, better selection of leaving group, and milder activation conditions for the process of the nucleophilic substitution.

EXPERIMENTAL

^{31}P-NMR spectra were recorded with a Jeol FX60 spectrometer operating at 24.3 MHz, using solns as indicated, with 85% H_3PO_4 as external standard. Positive chemical shift values are assigned for compounds absorbing at lower field than H_3PO_4. UV spectra were recorded with Specord UV-VIS spectrometer (Carl-Zeiss, Jena). Mass spectra (EI) were obtained by means of LKB-2091. TLC was performed on silica gel F254 plates (E.Merck). Column chromatography was performed on silica gel 230-400 mesh (E.Merck). The following developing solvent systems were applied: S_1, $CHCl_3$-MeOH (9:1); S_2, $CHCl_3$-Me_2CO (10:3); S_3, n-BuOH-AcOH-H_2O (5:2:3); S_4, iPrOH -NH_{3aq}-H_2O (7:1:2).

Solvents were of commercial grade and were dried and distilled before use. Pyridine was dried over KOH, refluxed with $KMnO_4$, distilled, dried over CaH_2, and redistilled. Fraction boiling at 114-116°C was collected and stored over granulated CaH_2. THF was dried over $NaBH_4/CaH_2$, distilled, dried over potassium, and redistilled under argon before use. Dioxane was dried over KOH, then $NaBH_4/CaH_2$, distilled, dried over NaH, and redistilled before use. Diisopropyl ether was dried over KOH, and distilled before use. NaH was used as 50% suspension in mineral oil. All evaporations under reduced pressure were performed at bath temp. not exceeding 40°C. Snake venom phosphodiesterase from Crotalus terr. (1 mg/ml suspension in glycerine) and phosphodiesterase from calf spleen (2 mg/ml suspension in glycerine) were obtained from Boehringer Mannheim GmbH (W.Germany), nuclease P1 (2 mg/ml solution in water) was purchased as lyophylized powder from Sigma.

O-(4-Nitrophenyl)-N-phenylphosphoramidochloridate (2) [47] was prepared according to the method described by Zielinski and Lesnikowski [45], 5'-O-monomethoxytrityl-2'-deoxyadenosine (1) was obtained by modified procedure described by Zemlicka et al. [46], 3'-O-tert-butyldimethylsilyl-2'-deoxyadenosine was prepared as described by Ogilvie et al. [23].

5'-O-Monomethoxytrityl-2'-deoxyadenosine 3'-O-|O-(4-nitrophenyl)-N-phenylphosphoramidate| (3)

The soln of 1 (5.2 g, 10 mmol) in pyridine was evaporated to dryness. The operation was repeated twice and the residue was dissolved in pyridine (100 ml). Into this soln 2 (6.2 g, 20 mmol) was added and the mixture was left at room temp. for 20 h protected from moisture. Then, water (150 ml) was added and after 0.5 h product was extracted with $CHCl_3$ (4 x 50 ml). The organic layer was separated and dried over $MgSO_4$. Solvents were evaporated and the oily residue was coevaporated with pyridine (2 x 10 ml) and toluene (2 x 10 ml). Diastereoisomeric mixture of 3 was isolated by means of short-column chromatography on silica gel (250 g). Product was eluted with $CHCl_3$-Me_2CO (2:1) followed by $CHCl_3$-MeOH (95:5) and $CHCl_3$-MeOH (9:1). The efficiency of separation was monitored on TLC plates. Fractions containing the desired product were pooled together, solvents were evaporated and residue was dissolved in benzene, and this soln was added dropwise into n-hexane. The ppt was filtered

off, washed with n-pentane and dried under reduced pressure. Yield of 3 as a mixture of diastereoisomers was 55%. Rechromatography using a higher gel/sample ratio yielded pure |(Rp)-3| and |(Sp)-3| isomers as well as |(Rp)-3 + (Sp)-3| mixture.
(Sp)-3, TLC: Rf(S_1) 0.57; UV: λ_{max} 265.3 nm, λ_{min} 247.5 nm (96% C_2H_5OH); δ_{31P} -3.87 ppm($CHCl_3$)
(Rp)-3, TLC: Rf(S_1) 0.48; UV:λ_{max} 265.3 nm, λ_{min} 247.5nm (96% C_2H_5OH?; δ_{31P} -3.77 ppm($CHCl_3$).

5'-O-Monomethoxytrityl-2'-deoxyadenosine 3'-O-|O-(4-nitrophenyl)-S-methylphosphorothioate| (4)

Phosphoranilidate 3 (1.2 g, 1.5 mmol) was dissolved in dioxane or dioxane-DMF (15 ml) and NaH (0.14 g, 3.0 mmol) was added. This mixture was stirred for 15 min at room temp. and then treated with CS_2 (6 ml). The reaction was controlled by means of TLC (S_1). After the substrate disappeared, the mixture was cooled on CO_2-i-PrOH bath and an excess of pyridinium form of Dowex 50 Wx8 was added (pH 8). The ion exchange resin was filtered off and washed with pyridine. Combined solns were evaporated. Oily residue was coevaporated with pyridine (2 x 5 ml) and toluene (2 x 5 ml), dissolved in $CHCl_3$ and added dropwise into diisopropyl ether. The ppt was washed with n-pentane and dried under reduced pressure. Product of PN→PS conversion (0.9 g, 1.1 mmol) was dissolved without further purification in acetone and CH_3I (125 µl, 2 mmol) was added. The mixture was left for 20 h at room temp. (TLC control, S_1) then solvent was evaporated . The residue was redissolved in $CHCl_3$ (20 ml) and washed with H_2O (3 x 10 ml). The organic layer was dried over $MgSO_4$ and $CHCl_3$ evaporated. The product was dissolved in acetone-benzene then those soln was added dropwise into n-hexane. The ppt was washed with n-pentane and dried under reduced pressure. Resulting 4 was purified by means of short-column silica gel chromatography. $CHCl_3$-MeOH (95:5) as a solvent system was used. Yield 50-55%.
(Rp)-4, TLC: Rf(S_1) 0.60, Rf(S_2) 0.42; UV:λ_{max} 265.3 nm,λ_{min} 247.5 nm,δ_{31P} 25.02 ppm($CHCl_3$)
(Sp)-4, TLC: Rf(S_1) 0.60, Rf(S_2) 0.42; UV:λ_{max} 265.3 nm,λ_{min} 247.5 nm,δ_{31P} 25.26 ppm($CHCl_3$).

5'-O-Monomethoxytrityl-2'-deoxyadenylyl(3',5')(3'-O-tert-butyldimethylsilyl-2'-deoxyadenylyl)-S-methylphosphorothioate (6)

The solution of n-BuLi in hexane (0.09 ml, 0.14 mmol) was added under argon to the THF (1.5 ml) solution of 5 (0.045 g, 0.12 mmol) at -60°C (CO_2-i-PrOH bath). After 15 min., to the resulting heterogenous mixture, at the same temp., the solution of 4 (0.06 g, 0.08 mmol) in THF (1.5 ml) was added. After 4 h the reaction was quenched with CH_3COOH-THF solution and/or an excess of pyridinium form of Dowex 50Wx8 (pH 8). The ion exchange resin was filtered off and washed with pyridine and THF. Combined solns were evaporated. Residue was coevaporated with pyridine (3 x 2 ml) and toluene (3 x 2 ml), then dissolved in $CHCl_3$ and dropped into n-pentane. The ppt was dried under reduced pressure and desired product was isolated by means of preparative TLC (S_1). For the eluation of product from the silica gel, the solvent system $CHCl_3$-MeOH (1:1) was used. After solvent evaporation 6 was redissolved in $CHCl_3$ and precipitated as described above. Yield 30-40%.
(Rp)-6, TLC: Rf(S_1) 0.30; UV:λ_{max} 260.5 nm, λ_{min} 244.0 nm (96% C_2H_5OH),δ_{31P} 29.20ppm($CHCl_3$)
(Sp)-6, TLC: Rf(S_1) 0.30; UV:λ_{max} 260.5 nm, λ_{min} 244.0 nm (96% C_2H_5OH),δ_{31P} 29.05ppm($CHCl_3$).

2'-Deoxyadenylyl(3',5') 2'-deoxyadenylyl phosphorothioate (7)

Diastereoisomeric mixture of 6 (10 mg, 0.010 mmol) was dissolved in dioxane (2.5 ml) then triethylamine (2.5 ml) and thiophenol (1.25 ml) were added. After 30h (TLC and ^{31}P-NMR control) solvents were evaporated, residue was redissolved in $CHCl_3$ and this soln was added dropwise into n-pentane. The precipitation procedure was repeated fourfold. To thiophenol--treated 6, the solution of TBAF (0.026 g) in THF (0.14 ml) was added. After 0.5 h at room temp. an excess of pyridinium form of Dowex 50Wx8 was added. The ion exchange resin was filtered off and washed with THF (3 x 0.5 ml) and pyridine (3 x 0.5 ml). Combined solns were evaporated, oily residue was coevaporated with pyridine (2 x 0.5 ml) and toluene (2 x 0.5 ml) then dissolved in $CHCl_3$ and added dropwise into pentane. Thiophenol and TBAF treated 6 was dissolved in 2% of toluene-p-sulphonic acid monohydrate in $CHCl_3$-MeOH (7:3, 0.35 ml). After 15 min at room temp. resulting 7 was isolated by means of TLC on cellulose F254 plates (E.Merck, S_3). For the eluation of product from the cellulose, water was used.
Yield: 35 A_{260} units; TLC: Rf (S_3, cellulose F_{254} plates, E.Merck) 0.44, Rf (S_4, cellulose F_{254} plates, E.Merck) 0.59; UV: max 259.4 nm, min 228.4 nm; ^{31}P 55.75 ppm (D_2O).
No differences in chromatographic mobilities, and chemical shifts in ^{31}P-NMR spectra for (Rp)- and (Sp)- isomers of 7 were observed under conditions applied. The (Rp)- and (Sp)-isomers of 7 can be distinquished by means of HPLC: µBondapak (9 x 300 mm, buffer 10% CH_3CN in 0.1 M triethylammonium acetate, pH 7.0). At flow rate 1.5 ml/min the following retention times were observed: 7.5 min, for (Rp)-7 and 10.5 min for (Sp)-7

Conversion of 5'-O-monomethoxytrityl-2'-deoxyadenosine 3'-O-|O-(4-nitrophenyl)-N-phenylphosphoramidate| (3) into 2'-deoxyadenosine cyclic 3',5'-phosphoranilidates (11)

The corresponding single diastereoisomer of 3 (0.036 g, 0.045 mmol) was added into the soln of 2% of toluene-p-sulphonic acid monohydrate in $CHCl_3$-MeOH (7:3, 1.0 ml) and the mixture was stirred at room temp. for 15 min. The mixture was washed with 0.1 M $KHCO_3$ (2 x 0.5 ml). The combined aqueous layers were extracted with $CHCl_3$ (3 x 0.2 ml) and combined organic fractions were dried over $MgSO_4$. Solvents were evaporated and the residue was dissolved in pyridine. Pyridine was evaporated and the residue was coevaporated with toluene. Product was dissolved in $CHCl_3$ and the soln was added dropwise into n-hexane. The ppt was

washed with n-pentane and dried under reduced pressure.
The solution of corresponding diastereoisomer of 2'-deoxyadenosine 3'-O-[O-(4-nitrophenyl)-N-phenylphosphoramidate] (0.02 g, 0.038 mmol) in dry N,N-dimethylacetamide (0.4 ml) was added to 10 molar excess of freshly prepared potassium tert-butoxide. The reaction mixture was left for 20 h at room temp., protected from the moisture. After cooling to -70°C an excess of Dowex 50Wx8 (pyridinium form) was added until pH 7.0. The resin was filtered off and washed with pyridine (3 x 0.5 ml). Combined filtrates were evaporated to dryness and the residue was coevaporated with pyridine (3 x 0.5 ml). The residue was redissolved in pyridine (0.4 ml) and ^{31}P-NMR spectra were recorded: Rp-11: δ^{31}_{P}-3.83; Sp-11: δ^{31}_{P}0.72 (C_5H_5N)[17].

Enzymatic assay

a/ Structure assignment of d(ApA).
To water solution (75 µl) of d(ApA) obtained from 6, 0.1 M TRIS-HCl buffer, pH 8.0 (20 µl), containing 15 mM $MgCl_2$, and snake venom phosphodiesterase (5 µl) were added; alternatively to water solution (75 µl) of d(ApA), mixed with 0.1 M AcONa buffer, pH 5.0 (20 µl), containing 1 mM $ZnCl_2$, bovine spleen phosphodiesterase (5 µl) was added.
After 1 h at room temp. TLC analysis on cellulose F_{254} and Kiesel gel F_{254} plates (E.Merck, S_3, S_4) was performed. Only dAp or pdA and dA were detected.

b/ Assignment of the absolute configuration at phosphorus atom in 7.
To water soln of enriched sample of 7 (ca 40% Rp, 60% Sp, 0.25 A_{260}, 25 µl) mixed with 0.1 M AcONa buffer, pH 5.0 (25 µl), containing 1 mM $ZnCl_2$, nuclease P1 (5 µl) was added. After 10 h at room temp. the reaction mixture was analysed by means of HPLC under conditions described above.

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