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THE CHEMICAL SYNTHESIS OF THE R_P AND S_P DIASTEREOMERS OF THYMIDYL-(3'-5')THYMIDYL O,O-PHOSPHOROTHIOATE

Bogdan Uznański, Wojciech Niewiarowski, Wojciech J. Stec*

Polish Academy of Sciences, Centre of Molecular and Macromolecular Studies, Department of Bioorganic Chemistry, 90-362 Łódź, Boczna 5, Poland

Abstract: *The synthesis and separation of diastereomers of protected thymidyl-(3'-5')thymidyl O,O-phosphoranilidate (4) allowed to obtain in the stereospecific manner title compounds 5, whose absolute configuration at P atom was assigned enzymatically. $T_{P(S)}T$ diastereomers (5) were obtained independently via "phosphite" procedure.*

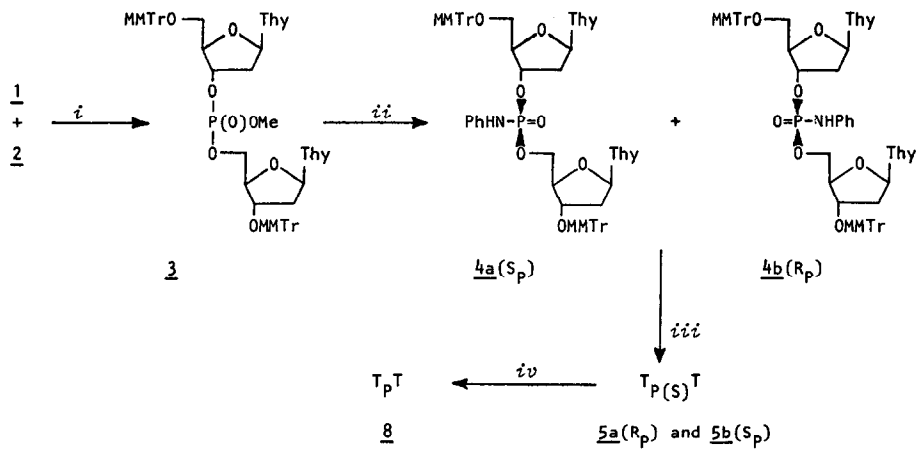
The stereochemical approach to the elucidation of the mode of action of the enzymes responsible for the nucleotidyl- and phosphoryl- transfer reaction led to a growing demand of stereospecific method of the synthesis of nucleoside P-chiral phosphates and phosphorothioates [1].

Although such compounds have been prepared before, there are only two reported examples of the chemical synthesis of the separated diastereomers of uridyl(3'-5')adenyl O,O-phosphorothioate[2] and adenyl(3'-5')adenyl O,O-phosphorothioate [3]. The absolute configurations of diastereomers of the above mentioned compounds were established by their reaction with RNase A [2] and bovine phosphodiesterase [3], respectively. The modified nucleotides which contain chiral P atom have been applied successfuly for the stereochemical study of the action of the enzymes [4] and in genetic engineering [5,6].

To pursue this aim we undertook some efforts to synthesize diastereomers of thymidyl-(3'-5')thymidyl O,O-phosphorothioate ($T_{P(S)}T$) of known configuration at P atom by means of phosphodiesteramidate approach elaborated in this Laboratory [7,8]. The mixtures of unseparated diastereomers of $T_{P(S)}T$ were originally obtained by Eckstein [9], Smrt [10] and on independent way by Leśnikowski et al. [11].

The substrate, 5'-monomethoxytritylthymidyl(3'-5')-3'-monomethoxytritylthymidyl O-methyl phosphate (3) was obtained according to the method described in the literature [12]. Thus, 5'-monomethoxytritylthymidine (1) was condensed with 3'-monomethoxytritylthymidine (2) by means of $MeOPCl_2$ in THF solution in the presence of collidine and intermediary phosphite was oxidized by means of I_2/H_2O/THF mixture. The yield of 3 was 82%. Any attempts of separation of 3 into individual diastereomers have failed. The O-methyl group was removed after overnight storage of 3 in t-$BuNH_2$ solution [13]. 5'-monomethoxytritylthymidyl(3'-5')-3'-monomethoxytritylthymidyl phosphate, obtained in 91% yield, was converted into its pyridi-

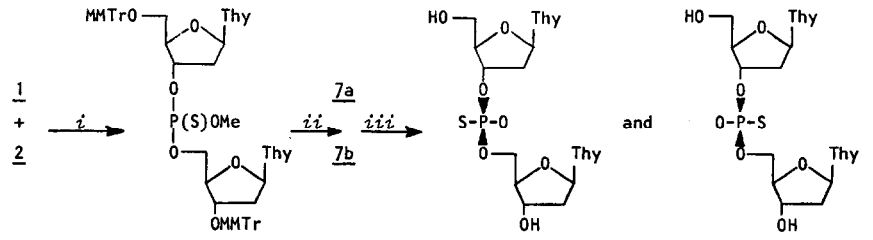
nium salt using Dowex (Py^+) column and, under conditions of Appel's reaction (Ph_3P-CCl_4--$PhNH_2$) [14], this last compound was transformed into the mixture of 5'-monomethoxytritylthymidyl(3'-5')-3'-monomethoxytritylthymidyl phosphoranilidates (4a and 4b). These diastereomers were easily separated on preparative TLC (silicagel plates 60 F_{254}, solvent system $CHCl_3$--96%EtOH, 100:6, triple development). The purities of the diastereomers of 4a (yield 28%) and 4b (yield 25%) were confirmed by means of ^{31}P-NMR: 4a, R_f 0.32, *"fast"* (silicagel plates 60 F_{254}, solvent system $CHCl_3$-96%EtOH, 100:6), *δ2.3 ppm*($CHCl_3$); 4b, R_f 0.28, *"slow"*, *δ1.8 ppm* ($CHCl_3$). Each of the diastereomers 4a and 4b was separately converted with NaH/CS_2 in DMF solution into 5'-monomethoxytritylthymidyl(3'-5')-3'-monomethoxytritylthymidyl O,O-phosphorothioates; thus, from *"fast"* 4a compound 5a', *δ52.3 ppm*($CHCl_3$), was obtained in 85% yield. *"Slow"* 4b was converted to compound 5b', *δ54.3 ppm*($CHCl_3$) in the yield of 76%. Both 5a' and 5b' after treatment with 80% acetic acid, gave desired diastereomers of title compound 5a and 5b, in yield 70% and 72%, respectively (Scheme 1).



i - $MeOPCl_2$/THF/collidine; I_2/H_2O. *ii* - t-$BuNH_2$; $CCl_4/Ph_3P/PhNH_2$; separation of diastereomers. *iii* - NaH/CS_2/DMF; 80% AcOH. *iv* - ClCN/H_2O/THF;

The same compounds were obtained on alternative route, analogous to that reported earlier for the preparation of $U_{P(S)}A$ [2] and $A_{P(S)}A$ [3]. This approach is depicted in Scheme 2. Thus 1 and 2 were condensed by means of methyl phosphorodichloridite in THF solution in the presence of elemental sulfur, and resulting 5'-monomethoxytritylthymidyl(3'-5')-3'-monomethoxytritylthymidyl-O-methyl O,O-phosphorothioate (6) was isolated as crude product in 96% yield, and separated by short column filtration (silicagel 230-400 mesh, eluent system EtOAc-benzene-iPrOH, 1:2:0.2) into diastereomers: 6a *"fast"*, *δ69.5 ppm*($CHCl_3$), yield 24%; and 6b *"slow"*, *δ69.2 ppm*($CHCl_3$), yield 25%. The reaction of each isomer of 6 with t-butylamine [13] led to the products of demethylation (7, retention of configuration at P atom), which were identified by means of ^{31}P-NMR: 7a, *"fast"*, *δ52.6 ppm*($CHCl_3$); 7b, *"slow"*, *δ54.9 ppm*($CHCl_3$). These methyl-t-butylammonium salts of 5'-monomethoxytritylthymidyl(3'-5')-3'-monomethoxytritylthymidyl O,O-

SCHEME 2.



6a(R_P) and 6b(S_P) 5a(R_P) 5b(S_P)

i - $MeOPCl_2/S_8$/THF/collidine. *ii* - separation of diastereomers; t-$BuNH_2$. *iii* - 80% AcOH.

phosphorothioates (7) were converted, after removal of the protecting groups with 80% acetic acid followed by column chromatography on DEAE Sephadex A-25 and Dowex (H^+) and neutralisation with 0.1N NaOH solution, into corresponding sodium salts 5a, $\delta 55.6$ *ppm* and 5b, $\delta 55.1$ *ppm*.

The corresponding diastereomers of dithymidyl phosphorothioate obtained on both alternative routes have shown to be identical by HPLC (5a has shorter retention time, eluent system MeOH-H_2O, 15:85). The oxidation of 5a and 5b by means of cyanogen chloride with an excess of water in the presence of collidine in THF gave thymidyl(3'-5')thymidyl phosphate (8) which was digested by snake venom phosphodiesterase, as expected, to thymidine 5'-phosphate and thymidine.

The absolute configuration at phosphorus in both diastereomers 5a and 5b has been assigned enzymatically. It has been established that phosphodiesterase from snake venom (E.C. 3.1.4.1.) possesses stereoselective hydrolytic activity towards phosphorothioate diesters of R_P configuration [15]. Each diastereomer 5a and 5b was separately treated with this enzyme in 100 mM Tris-AcOH, 20 mM $MgCl_2$ buffer, pH 8.0, and after overnight incubation at 37°C, the products of digestion were identified by means of ^{31}P-NMR, HPLC and TLC criteria (TLC, silicagel plates 60 F_{254}, developing system CH_3CN-100 mM Tris-AcOH, pH 8.0, 10:1). Only diastereomer 5a was hydrolysed to the thymidine 5'-phosphorothioate ($\delta 43.1$ *ppm*), what allowed us to establish the absolute configuration of this substrate as R_P. The products of the enzymatic oxidation were also observed in this process, in agreement with previous reports [2,3]. Under these conditions diastereomer 5b was completely resistant towards hydrolysis.

In ^{31}P-NMR spectrum the signal corresponding to R_P-$T_{P(S)}T$ (5a) occurs at lower field than that of S_P-$T_{P(S)}T$ (5b), what in comparison with the data presented in other works[2,3,4] may suggest that chemical shift parameter is indicative of absolute configuration at phosphorus, if both diastereomers of $N_{P(S)}N$ are available.

It should be noticed that the known stereospecificity of PN→PS conversion, which

takes place with retention of configuration at P atom [7], allowed us to assign the absolute configuration of intermediate dithymidyl phosphoranilidates (4a-S_P and 4b-R_P). Also, the retention of configuration at P atom in the process of conversion of 6 to 5 allowed us to assign the absolute configuration to 6a as R_P and to 6b as S_P.

In the light of demonstrated conversion of phosphoranilidates into $|^{18}O|$phosphates by means of NaH/$|^{18}O|CO_2$ [16] or NaH/$|^{18}O|C_6H_5CHO$ [17], phosphoranilidates 4a and 4b can be applied for stereospecific preparation of diastereomers of thymidyl(3'-5')thymidyl $|^{18}O|$phosphate. These last compounds can be also obtained from 5a and 5b using one of the stereospecific procedures for PS→P$|^{18}O|$ conversion reported only recently by Frey [18], Eckstein [19] and Lowe [20].

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