Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 7, No. 20, pp. 2651-2656, 1997

0960-894X/97 $17.00 + 0.00

PII: S0960-894X(97)10040-3

DEOXY*XYLO*THYMIDINE 3'-O-PHOSPHOROTHIOATES: SYNTHESIS, STEREOCHEMISTRY AND STEREOCONTROLLED INCORPORATION INTO OLIGOTHYMIDYLATES

Xian-bin Yang, Konrad Misiura, Marek Sochacki and Wojciech J. Stec*
Polish Academy of Science, Centre of Molecular and Macromolecular Studies, Department of Bioorganic Chemistry, Sienkiewicza 112, 90-363 Łódź, Poland

Abstract: New reagent, 5'-O-DMT-*xylo*thymidine 3'-O-(2-thio-4,4-*spiro*pentamethylene-1,3,2-oxathiaphospholane) and its separated diastereomers can be used for stereocontrolled incorporation of *xylo*thymidine 3'-O-phosphorothioate of predetermined sense of P-chirality into desired oligonucleotide constructs.

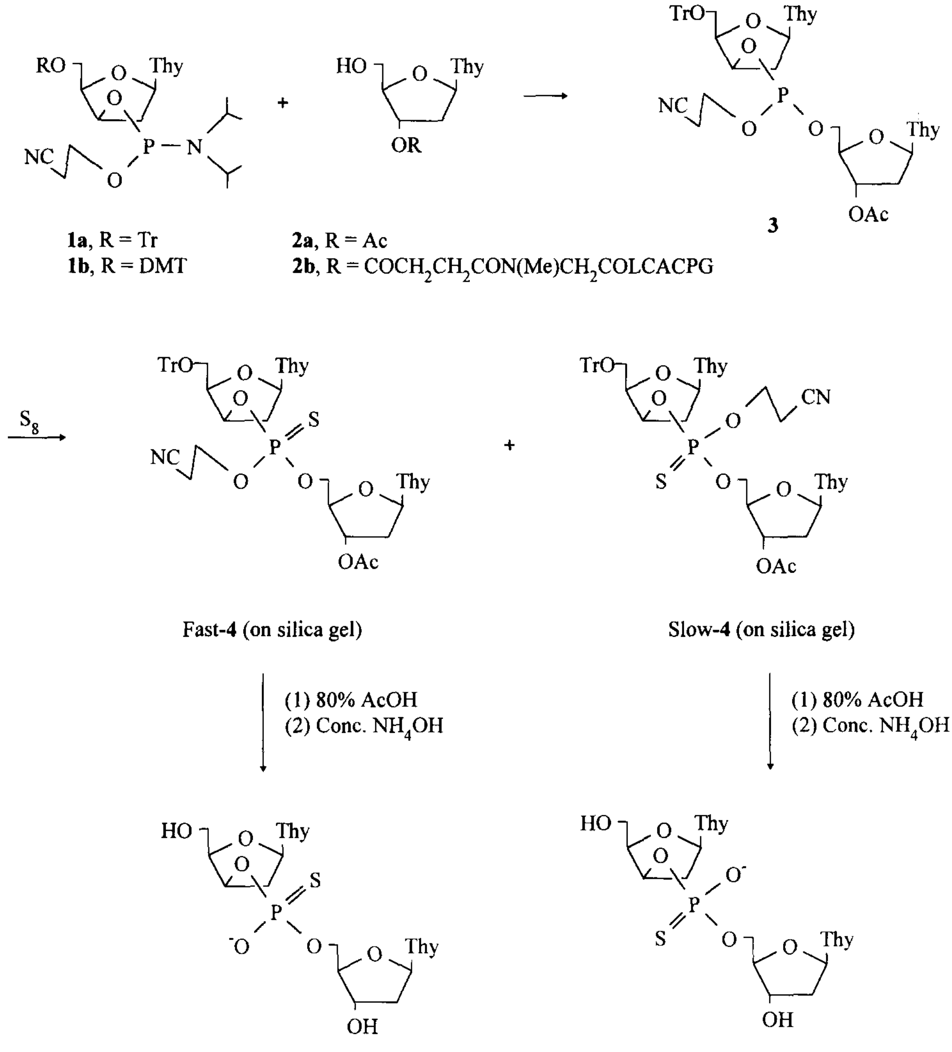
Although an early interest in chemistry of *xylo*nucleosides was kindled by hopes of their use as intermediate in the synthesis of oligonucleotides,[1] most efforts were paid to their use in the synthesis of new therapeutics, such as AZT[2] and others.[3] 3'-O-Activated *xylo*nucleosides, if used as the substrates for oligo(nucleoside phosphorothioate)s synthesis, have shown limited application due to the low nucleophilicity of phosphorothioate ions towards secondary 3'-carbon atom and elimination process accompanying substitution.[4] Oligonucleotides containing incorporated 2'-deoxy*xylo*nucleosides were first prepared by Shabarova[5], and independently by Seela *et al.*[6] Both groups of researchers emphasized lower avidity of oligonucleotides containing incorporated 2'-deoxy*xylo*nucleosides towards complementary DNA or RNA and their enhanced stability against nucleases.

In this communication we wish to present the synthesis of oligonucleotides with incorporated *xylo*thymidine linked with 3'-O-phosphorothioate function. It was of interest to check to what extent the replacement of internucleotide phosphate by stereogenic phosphorothioate linkage(s) will influence upon the physicochemical properties and enzymatic stability of oligonucleotide constructs possessing incorporated *xylo*thymidine phosphorothioate functions. 5'-O-Trityl-*xylo*thymidine 3'-O-acetylthymidyl (3',5')-O-2-cyanoethylphosphorothioate (**4**) was obtained as a mixture of two diastereomers according to phosphoramidite methodology represented in **Scheme 1**. Condensation of 5'-O-trityl-*xylo*thymidine 3'-O-(2-cyanoethyl)-N,N-diisopropylphosphoramidite (**1a**)[7] with 3'-O-acetylthymidine (**2a**) in the presence of tetrazole was performed in acetonitrile solution. The ratio of reagents **1a**:**2a** was 1.3:1. Intermediate 3'-O-(2-cyanoethyl) phosphite (**3**) without isolation was sulfurized with elemental sulfur and resulting fully protected dimer **4** formed as a mixture of two diastereomers (ratio 56:44, ^{31}P-NMR assay), was applied onto silica gel column (silica gel: 230-400 mesh; solvent system: CH_2Cl_2, then a gradient of 0-2% methanol in chloroform) for purification and diastereomers separation. Purity of diastereomers of **4** (Fast-eluted, Fast-**4**, yield 30% and Slow-eluted, Slow-**4**, yield 15%) were confirmed by means of ^{31}P-NMR: Fast-**4**, R_f 0.32 (on silicagel plates 60 F_{254}, solvent system $CHCl_3$:EtOH, 19:1), δ_p 67.59 ppm ($CDCl_3$); Slow-**4**, R_f 0.18, δ_p 66.74 ppm ($CDCl_3$).[8] Each diastereomer Fast-**4** and Slow-**4** was deprotected by sequential treatment with 80% acetic acid (three days at r.t.) followed by concentrated ammonia at 55°C for 16 h, respectively.

* E-mail: wjstec@bio.cbmm.lodz.pl; Fax no: (48-42) 81 54 83

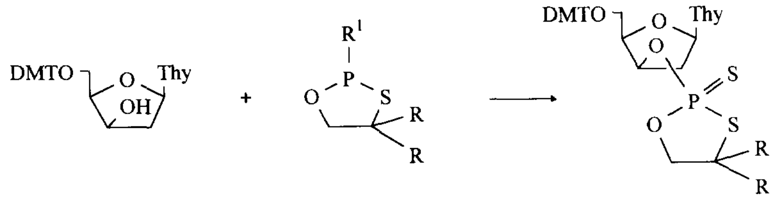
Thus, deprotection of Fast-**4** (silicagel) gave Fast-**5** (RP-HPLC); R_t 11.92 min, δ_p 59.30 ppm (CD_3OD+D_2O), FAB MS, -VE, m/z 561 $(M)^-$; Slow-**4** (silicagel) gave Slow-**5** (RP-HPLC); R_t, 12.36 min, δ_p 57.47 ppm ($CD_3OD+ D_2O$), FAB MS,-VE, m/z 561 $(M)^-$. (Scheme 1).

SCHEME 1



Independently, diastereomers Fast-**5** and Slow-**5** were prepared on an alternate route, according to oxathiaphospholane method recently developed in this Laboratory.[9] (Schemes 2 and 3).

SCHEME 2

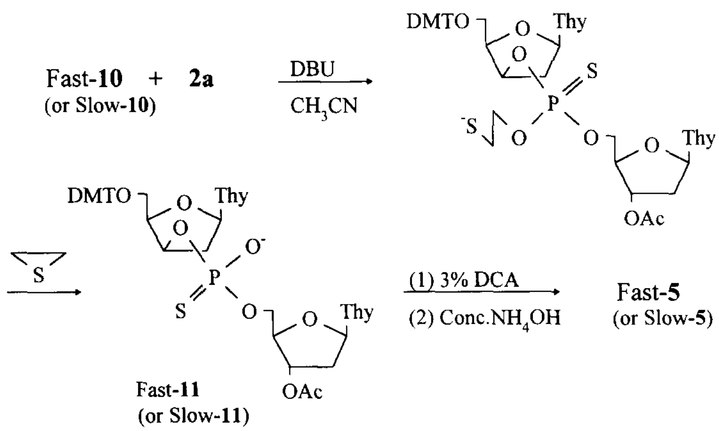


6   7, R = H, R^1 = N(i-Pr)$_2$; 8, R = -(CH$_2$)-$_5$, R^1 = Cl   9, R = H; 10, R = -(CH$_2$)-$_5$

Reaction of 5'-O-DMT-*xylo*thymidine (**6**) with 2-N,N-diisopropylamino-1,3,2-oxathiaphospholane[8] (**7**) in the presence of 1.5 molar equivalent of tetrazole in dry acetonitrile, or with 2-chloro-4,4-*spiro*-pentamethylene-1,3,2-oxathiaphospholane (**8**)[10] in the presence of 5 molar equivalents of diisopropylethylamine in dichloromethane (phosphitylation), followed by addition of elemental sulfur, gave a mixture of diastereomers **9** or **10**, respectively. Chromatographic purification on silicagel column (230-400 mesh) gave **9** or **10** in 54% and 72% yield, respectively, as the mixture of two diastereomers in the ratio 55:45 (^{31}P-NMR assay). Any attempts for separation of diastereomers of 5'-O-DMT-*xylo*thymidine 3'-O-(2-thio-1,3,2-oxathiaphospholane) (**9**) by means of silica gel column chromatography have failed. However, separation of the diastereomers of **10** was easily achieved by applying the sample onto a column (30x6 cm) containing Kieselgel 60H. The column was eluted with a mixture of ethyl acetate and petroleum ether (1:1, v/v).

Fast-eluted, Fast-**10** [R_f 0.30 (silicagel plates 60 F_{254}, developing system: ethyl acetate-petroleum ether, 1:1), δ_p 104.07 ppm (C_6D_6), FAB MS, -VE, m/z 749 $(M-1)^-$] was isolated with the yield 32%; Slow-eluted,

SCHEME 3



Slow-**10** ,R_f 0.24, δ_p 105.85 ppm (C_6D_6), FAB MS-VE, m/z 749 ($M-1^-$) was isolated with 30% yield. The condensation of each isomer of **10** (Fast- or Slow-eluted) with 3'-O-acetyl-thymidine (**2a**) in the presence of 1 molar equivalent of 1,4-diazabicyclo[5,4,0]-undec-7-ene (DBU) performed at room temperature (reaction time 30 min) led to the Fast-**11** and Slow-**11**, respectively. After removal of protective groups with 3% DCA in dichloromethane followed by a concentrated ammonia products **5** were analyzed by RP-HPLC: from Fast-**10** (diast. excess 87.0%, ^{31}P-NMR assay) Fast-**5** (d.e. 87.6%, RP-HPLC assay) was obtained. Pure Slow-**10** was converted to pure Slow-**5** with the full stereospecificity. The corresponding pairs of diastereomers of **5** obtained on both alternative routes have shown to be identical by RP-HPLC analysis and FAB MS data.

Condensation of monomer Fast-**10** and Slow-**10** with thymidine immobilized *via* DBU-resistant succinoyl-sarcosinyl linker (**2b**) on controlled pore glass[11] was also investigated and proved to give the desired dinucleotide Fast-**5** and Slow-**5**, respectively. To achieve satisfactory yield for coupling step (94%) the high molar excess of monomers Fast-**10** or Slow-**10** (*ca* 40-50 fold) and DBU (200-fold) was necessary to complete the reaction in 40 min.

The absolute configuration at phosphorus in both diastereomers Fast-**5** and Slow-**5** was tentatively assigned on the basis of results of enzymic degradation. Snake-venom phosphodiesterase (svPDE) preferentially cleaves phosphorothioate diesters of [R_P]-configuration. [R_P]-Dithymidine 3',5'-phosphorothioate is hydrolysed to the thymidine 5'-phosphorothioate, while [S_P]- diester is completely resistant toward svPDE-assisted hydrolysis.[12] Thus, each diastereomer Fast-**5** and Slow-**5** was separately treated with this enzyme in 100 mM Tris-HCl buffer containing 20 mM $MgCl_2$, pH 8.0, and after overnight incubation at 37°C only the Slow-**5** (RP-HPLC) was partly hydrolysed to the thymidine 5'-phosphorothioate and *xylo*thymidine, what allowed us to assign the absolute configuration of this substrate as [R_P]; under identical conditions the Fast-**5** was completely resistant towards hydrolysis; therefore, the absolute configuration of this diastereomer should be [S_P]. For the purpose described below it is worth mentioning that Fast-**5** of [S_P]-configuration was prepared from Fast-**10**, while Slow-**5** of [R_P]-configuration was obtained from Slow-**10**.

In the following studies we obtained the dodecanucleotides containing *xylo*thymidine incorporated in the central position. Oligonucleotides T_{12} (**12**) and A_{12} (**13**) were synthesized with an automatic synthesizer by means of phosphoramidite method.[13] The $^{5'}$TTTTT*xyloT*$_{PO}$TTTTTT$^{3'}$ (T_5*xyloT*$_{PO}T_6$, **14**) and R_P/S_P-$^{5'}$TTTTT*xyloT*$_{PS}$TTTTTT$^{3'}$ (T_5*xyloT*$_{PS}T_6$, mix-**15**) were synthesized with an automatic synthesizer *via* phosphoramidite method using phosphoramidite **1b** in 7th cycle of synthesis of **14** and mix-**15**. Any attempts to separate diastereomers T_5*xyloT*$_{PS}T_6$ (mix-**15**) into [S_P]-T_5*xyloT*$_{PS}T_6$ ([S_P]-**15**) and [R_P]-T_5*xyloT*$_{PS}T_6$ ([R_P]-**15**) by means of RP-HPLC have failed. Similar difficulty was observed during attempts of separation of diastereomers of 5'-$T_5T_{PS}T_6$.[14] Therefore, [S_P]-**15** and [R_P]-**15** were synthesized by combining phosphoramidite and oxathiaphospholane methods.[10] The sequence T_6 (starting from 3'-T_1) was prepared by standard phosphoramidite method using ABI 392A DNA Synthesizer (1 μmole scale). Bound to the solid support *via* succinoyl-sarcosinyl linker T_6 was treated with 150 μl of solution of DBU (*ca* 200 μmol) in acetonitrile and Fast-**10** or Slow-**10**, respectively, for 40 min by manual operation. The following sequences T_5 in [S_P]-**15** and [R_P]-**15** were completed *via* oxathiaphospholane method using 5'-O-DMT-*xylo*thymidine-3'-O-(2-oxo-4,4-*spiro*pentamethylene-1.3.2-oxathiaphospholane [**16**, ^{31}P-NMR, δ_P: 44.61 and 44.13 ppm (CD_3CN), ratio 60:40].[10] The coupling yield between T_6 and Fast-**10** and Slow-**10** (switch from phosphoramidite method to oxathiaphospholane method) was 43% or 34%, respectively (DMT^+ assay).

The average coupling yield for continued syntheses of dodecamers using **16** (conditions and reagents excess as above) were 95% and 75%, respectively (DMT^+ assay). Isolation of products **12-15** was achieved by conventional work-up. Oligonucleotides were removed from the support by washing the column with 1 mL of concentrated ammonia at room temperature for 0.5-1 h. The 5'-protected oligonucleotides were then purified by RP-HPLC, and the detritylated compounds (80% AcOH in H_2O) were again submitted to RP-HPLC and lyophilized. Purity of each dodecanucleotide was ca. 98% (PAGE analysis, 20% polyacrylamide gel). Electrophoretic mobility of modified oligonucleotides was the same as that of T_{12}.

Table 1. Formulas and melting temperatures (Tm) of heteroduplexes.

Heteroduplex	Duplex structure	Abbreviation	Tm[a] (°C)
13/12	5'-TTTTTTTTTTTT-3' 3'-AAAAAAAAAAAA-5'	A_{12}/T_{12}	39.8
13/14	5'-TTTTT*xyloT*$_{PO}$TTTTTT-3' 3'-AAAAA-A-AAAAAA-5'	A_{12}/T_5*xyloT*$_{PO}T_6$	29.3
13/mix-**15**	5'-TTTTT*xyloT*$_{PS}$TTTTTT-3' 3'-AAAAA-A-AAAAAA-5'	A_{12}/[Mix]-T_5*xyloT*$_{PS}T_6$	27.6
13/[S_P]-**15**	[S_P]-5'-TTTTT*xyloT*$_{PS}$TTTTTT-3' 3'-AAAAA-A-AAAAAA-5'	A_{12}/[S_P]-T_5*xyloT*$_{PS}T_6$	27.8
13/[R_P]-**15**	[R_P]-5'-TTTTT*xyloT*$_{PS}$TTTTTT-3' 3'-AAAAA-A-AAAAAA-5'	A_{12}/[R_P]-T_5*xyloP*$_{PS}T_6$	28.3

[a] The Tm values of the duplexes at 0.053 mM for each strand were measured in 10 mM Tris-HCl buffer, pH 7.5, containing 10 mM $MgCl_2$ and 100 mM NaCl by heating from 12°C-90°C at 0.3°C/min and measuring the UV absorbance at 260 nm.

Oligonucleotides **14** and mix-**15**, R_P-**15** and S_P-**15** were characterized by treatment with nuclease P1 at 37°C for 3 h, followed by alkaline phosphatase at 37°C for 30 min.[15]

Products of hydrolysis of oligonucleotides were analyzed by RP-HPLC providing thymidine and *xyloT*$_{PO}$T, mix-**5**, [R_P]-**5** and [S_P]-**5**, respectively. Dodecathymidylates **14** and **15** form a stable 1:1 complexes with A_{12} and their melting characteristics, compared with that of A_{12}/T_{12}, are included in Table 1. It is interesting to notice that secondary structures of heteroduplexes formed by parent A_{12}/T_{12}, A_{12}/**14** and A_{12}/**15**, independently upon absolute configuration of incorporated **5** according to CD criterion[16] are the same.

Although an incorporation of single *xylo*thymidine has a dramatic effect on the Tm value of the duplexes, as pointed out earlier by Seela *et al.*,[17] phosphorothioate internucleotide bond following *xylo*thymidine has negligible influence upon Tm, independently upon absolute configuration at phosphorus. Such observation supports the conclusion that introduction of *xylo*thymidine induce structural distortions prevailing these caused by replacement of one of two non-bridging oxygen atoms of internucletide phosphate by sulfur and stereochemical consequences thereof.

Acknowledgments: This project was financially assisted by the State Committee for Scientific Research, grant no. 4.PO5F.023.10 (to W.J.S).

References and Notes

1. Zemlicka, J.; Smrt, J. *Tetrahedron Lett.* **1964**, 5, 2081; Mizuno, Y.; Sasaki, T. *J.Am.Chem.Soc.*, **1966**, 88, 863.
2. Mitsuya, H.; Weinhold, K.J.; Furman, P.A.; St. Clair, M.H.; Lehrman, S.N.; Gallo, R.C.; Bolognesi, D.; Barry, D.W.; Broder, S. *Proc.Natl.Acad.Sci. USA* **1985,** 82, 7096.
3. Balzarini, J.; Kang, G.J.; Dalal, M.; Herdewijn, P.; De Clercq, E.; Broder, S.; Johns, D.G. *Mol.Pharmacol.* **1987**, 32, 162.
4. Yang, Xian-bin; Misiura, K.; Stec, W.J. - submitted for publication.
5. Sokolova, N.I.; Dolinnaya, N.G.; Krynetskaya, N.F.; Shabarova, Z.A. *Nucleosides & Nucleotides* **1990**, 9, 515.
6. Rosemeyer, H.; Seela, F. *Helv.Chim.Acta,* **1991**, 74, 748.
7. ^{31}P-NMR(81MHz, $CDCl_3$): δ_P=151.92 (49.1%), 148.51 (50.9%).
8. The isolated total yield of **4** was 62%.
9. Stec, W.J.; Grajkowski, A.; Kobylańska, A.; Karwowski, B.; Koziołkiewicz, M.; Misiura, K.; Okruszek, A; Wilk, A.; Guga, P.; Boczkowska, M. *J.Am.Chem.Soc.* **1995**, 117, 12019.
10. Karwowski, B.; Guga, P.; Stec. W.J. *The IXth European Symposium on Organic Chemistry*, Warszawa, Poland, June 18-23, **1995**, PC6; Stec, W.J.; Karwowski, B.; Guga, P.; Koziołkiewicz, M.; Boczkowska, M.; Wieczorek, M.W.; Błaszczyk, J. - submitted for publication.
11. Brown, T.; Pritchard, C.E.; Turner, G.; Salisbury, S.A. *J.Chem.Soc.Chem. Commun.* **1989**, 891.
12. Burgers, P.M.J.; Eckstein, F. *Proc.Natl.Acad.Sci. USA* **1978**, 75, 4798. Uznański, B.; Niewiarowski, W.; Stec, W.J. *Tetrahedron Lett.* **1982**, 23, 4289.
13. *Oligonucleotides and Analogues: Apractical Approach.* Ed. Eckstein, F.; IRL Press, Oxford **1991**.
14. Koziolkiewicz, *M. Ph.D. Thesis.* Polish Academy of Sciences, Centre of Molecular and Macromolecular Studies, Sienkiewicza 112, 90-363, Lodz, Poland, **1989**, p.190.
15. A solution of **14**, mix-**15**, [S_P]-**15** and [R_P]-**15** (*ca.* 0.2 OD unit) in 100 mM Tris-HCl buffer containing nuclease P_1 (3 ug) (pH 7.2, 1 mM Zn^+) was incubated at 37°C for 3h, then was treated with alkaline phosphatase at 37°C for 30 min, respectively. The protein was denatured on heat-block (95-100°C) for 2 min. The products were analyzed by RP-HPLC *via* coinjection with thymidine and corresponding dimer.
16. CD spectra of heteroduplexes (see Table 1) were recorded at 12°C in 10 mM Tris-HCl buffer, pH 7.5 containing 10 mM $MgCl_2$ and 100 mM NaCl.
17. Rosemeyer, H.; Krecmerova, M.; Seela, F. *Helv.Chim.Acta,* **1991**, 74, 2054.

(*Received in Belgium* 27 *May* 1997; *accepted* 17 *September* 1997)