Pure & Appl. Chem., Vol. 65, No. 4, pp. 707–714, 1993.
Printed in Great Britain.

Antisense oligonucleotides: Stereocontrolled synthesis of phosphorothioate oligonucleotides

Alina Suska[a], Andrzej Grajkowski[a], Andrzej Wilk[a], Bogdan Uznanski[a], Jerzy Blaszczyk[b], Michal Wieczorek[b] and Wojciech J.Stec[a]

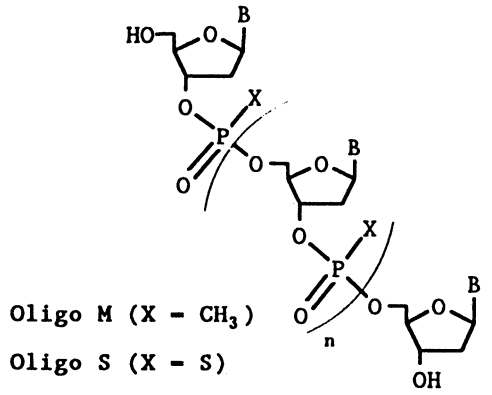
[a]Polish Academy of Sciences, Centre of Molecular & Macromolecular Studies, Department of Bioorganic Chemistry, Sienkiewicza 112, 90-363 Lodz, Poland
[b]Technical University of Lodz, Institute of Technical Biochemistry, Stefanowskiego 4/10, 90-924 Lodz, Poland

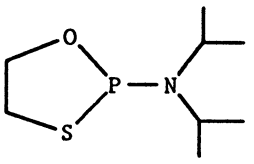
Abstract. A new method of oligo(nucleoside phosphorothioate)s (OligoS) synthesis, based on the oligonucleotide chain extention via DBU-catalyzed reaction of 5'-OH-nucleoside(-tide)with5'-DMT-nucleoside-3'-O-(2-thiono-1.3.2-oxathiaphospholane)s (2) is elaborated. The process of oxathiaphospholane ring opening is shown to be chemo- and stereoselective. The use of separated diastereoisomers of 2 allows the synthesis of OligoS with a predetermined sense of chirality at each internucleotide phosphorothioate function.

INTRODUCTION

Advances in synthetic chemistry and molecular genetics in the past several years have led to new approaches towards the development of new therapeutics (Ref. 1). These new approaches involve blocking genetic messages to turn off the production of disease-causing proteins. The target here is a nucleotide sequence on a single stranded messenger RNA, which encodes for disease-causing proteins, or double stranded DNA from which mRNA is transcribed. Once a target sequence is determined, complementary or antisense DNA sequence that bind via Watson-Crick type hydrogen bonding and deactivate the genetic message can be synthesized. At 16-28 nucleotides long, an antisense oligonucleotide is expected to be highly selective in its ability to recognize and bind to its target sequences, since a single mismatch in the complementary strand can reduce the affinity for hybridization by several orders of magnitude.

Unmodified synthetic oligonucleotides are not expected to function well as potential therapeutics because they are readily degraded by nucleases. Changes in the phosphodiester-sugar backbone, mostly by replacement of non-bridging oxygens at internucleotide phosphates, led to the first-generation compounds such as oligo(nucleoside methanephosphonate)s (OligoM) (Ref. 2) and oligo(nucleoside phosphorothioate)s (OligoS) (Ref. 3). These modifications in chemical structure are being evaluated for stability of oligonucleotide analogues, ability to permeate the cells, toxicity, and affinity to hybridize with a target mRNA. Both OligoM and OligoS may be synthesized by automated techniques. These techniques involve condensation of 5'-OH-nucleoside (bound via its 3'-oxygen with solid support) with 5'-DMT-nucleoside 3'-O-[N,N-dialkyl methanephosphonamidite] (Ref. 2), followed by oxidation, or with 5'-DMT-nucleoside 3'-O-[N,N-diisopropyl O-ß-cyanoethyl phosphoramidite], followed by sulfurization (Ref. 4), respectively. However, the common problem for both classes of oligonucleotide analogues is the lack of effective stereocontrolled methods of their synthesis; existing technology allows the





1

formation of mixtures consisting of 2^n diastereoisomers, where *n* is a number of modified P-chiral internucleotide bonds (Ref. 5).

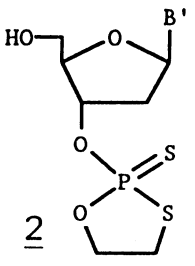
There are several lines of evidence that oligonucleotide analogues of predetermined sense of chirality at phosphorus may possess different physicochemical properties such as hybridization affinity to target, solubility, or cellular uptake.

Moreover, considering P-chiral analogues of oligonucleotides as potential therapeutics, one has to address the possible requirement imposed by regulatory authorities in Europe, Japan and United States in particular, that P-chiral analogues of potential drugs must eventually be obtained as diastereoisomerically pure compounds, at least for comparison of biological properties of pure diastereoisomers with their stereorandom mixtures.

In spite of efforts of many Laboratories (Ref. 6), stereocontrolled methods of synthesis of OligoS of predetermined sense of chirality are still limited to the synthesis of short, mostly di- and trinucleotides (Ref. 7). The aim of this lecture is to present a novel approach for the synthesis of oligo(nucleoside phosphorothioate)s which kindle some hope that they can be prepared in a stereocontrolled manner.

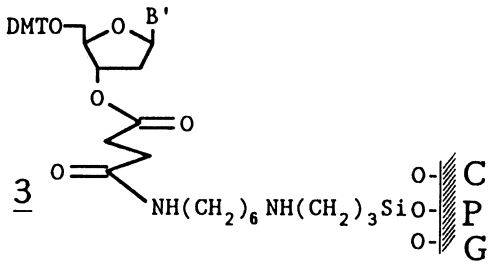
THE OXATHIAPHOSPHOLANE APPROACH TO THE SYNTHESIS OF OLIGO(NUCLEOSIDE PHOSPHOROTHIOATE)S

Earlier results from this laboratory indicated that conversion of P-chiral O,O-dialkyl phosphorothioates into O,O-dialkyl[^{16}O,^{18}O]phosphates by means of alkylene 1,2-[^{18}O]oxides occurs with full retention of configuration at phosphorus (Ref. 8). Furthermore, it has been demonstrated that diastereoisomeric P-chiral O-alkyl-O-aryl phosphorothioates upon treatment with butylene 1,2-[^{18}O]oxide in [^{17}O]water are stereospecifically converted into O-alkyl[^{16}O,^{17}O,^{18}O]phosphates. Mechanistic considerations of this reaction imply that 2-alkoxy-2-oxo-1,3,2-oxathiaphospholane participates as the discrete intermediate undergoing chemoselective and stereospecific hydrolysis by means of [^{17}O]water, followed by elimination of ethylene sulphide. In fact, these studies constituted the first demonstration of the stereochemistry of the process of 1,3,2-oxathiaphospholane ring opening (Ref. 9), and prompted further studies, including the application of 1,3,2-oxathiaphospholanes as phosphorylating reagents (Ref. 10). 2-(N,N-Diisopropylamino)-1,3,2-oxathiaphospholane (1) effectively phosphitylates apropriately protected 3'-OH-nucleosides using 1-H-tetrazole as the catalyst. Nucleoside 3'-O[1,3,2-oxathiaphospholane]s, without isolation, are oxidized by means of elemental sulphur, and the resulting nucleoside 3'-O[2-thiono-1,3,2-oxathiaphospholane]s (2) are isolated in satisfactory yield by silica gel flash-chromatography. Without separation of diastereoisomers, these



2

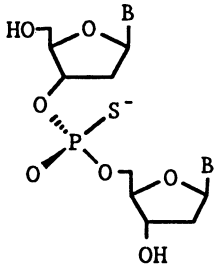
DMT = 4,4'-dimethoxytrityl
B' = Thy, Ade^{Bz}, Gua^{iBu}, Cyt^{Bz}



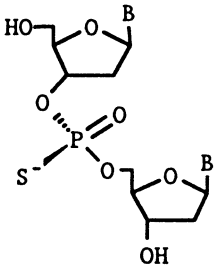
3

DMT = 4,4'-dimethoxytrityl
B' = Thy, Ade^{Bz}, Gua^{iBu}, Cyt^{Bz}

compounds are treated with 5'-OH-nucleosides bound via 3'-oxygen to solid support (succinyl-LCA-CPG-linker) (3) (Ref. 11).
In the presence of triethylamine, independent of the reaction time, dinucleoside 3',5'-phosphorothioates (5) are formed, but in a yields of only ca. 5%. However, introduction of a strong organic base such as 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU) increases the efficiency of internucleotide phosphorothioate bond formation to the level of ca. 95%. Because unseparated diastereoisomeric mixtures of 2 are used, the diastereosiomeric ratio of the resulting dinucleoside 3',5'-phosphorothioates



[Rp] - 5



[Sp] - 5

(5), after release from the solid support, is ca. 1:1. Further experiments demonstrated that chain extention of OligoS can be performed if :

a) rigorous anhydrous conditions are applied;
b) DBU - resistant linker to the solid support (4), such as that described by T.Brown (Ref. 12), is introduced for immobilization of the first (from the 3'-end) nucleoside;
c) the molar ratio of 2:DBU:5'-OH immobilized nucleoside is 10:300:1;
d) each coupling step is followed by careful washing of the column with anhydrous CH_3CN; and
e) capping of unreacted, immobilized 5'-OH-nucleosides is performed by means of acetic anhydride - N-methylimidazole. Figure 1 summarizes the steps for assembling of [all-Sp]-OligoS.

The synthesis of $d(N_{PS})_nN$ requires n cycles according to above protocol, followed by cleavage from the support/base deblocking (25% NH_4OH, 18 h, 55°C).
First manually, and then using an ABI 380B DNA synthesizer, several OligoS were obtained; representative examples are as follows: $d(C_{PS})_4C$ (6), $d(T_{PS})_7T$ (7), $d(G_{PS}G_{PS}A_{PS}A_{PS}T_{PS}T_{PS}C_{PS}C)$ (8),
$(T_{PS}C_{PS}G_{PS}T_{PS}C_{PS}G_{PS}C_{PS}T_{PS}G_{PS}T_{PS}C_{PS}T_{PS}C_{PS}C_{PS}G_{PS}C_{PS}T_{PS}T_{PS}C_{PS}T_{PS}C_{PS}C_{PS}T_{PS}G_{PS}C_{PS}C_{PS}A)$ (9) (Ref. 13).

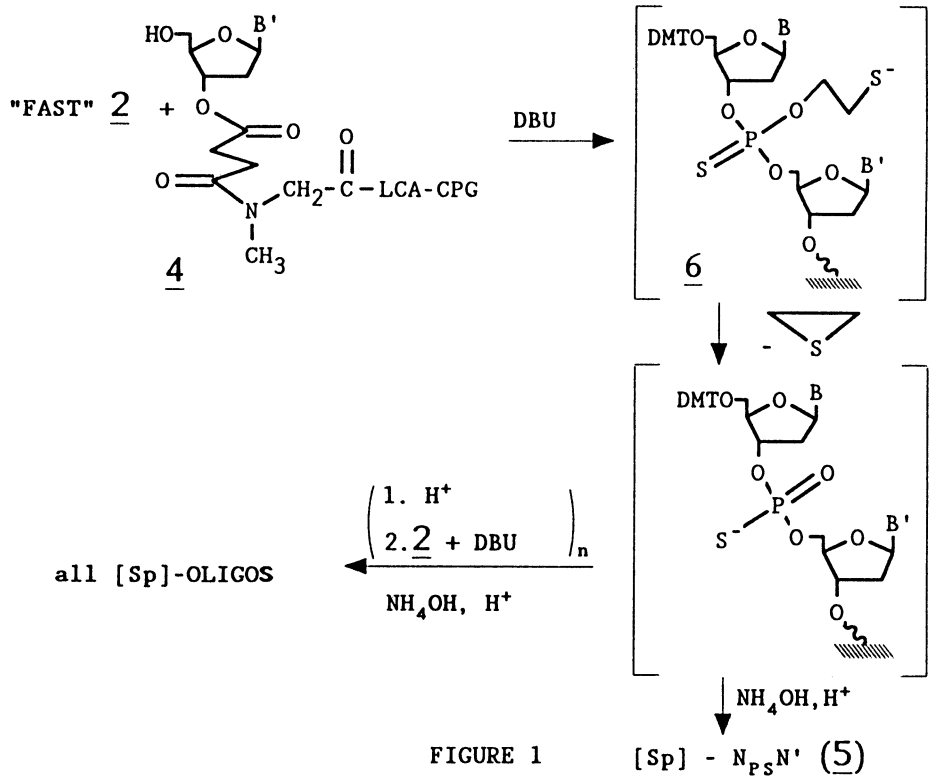


FIGURE 1

The protocol for synthesis of OligoS on a solid support involves:

Purpose	Reagent or Solvent		Time (min)
DETRITYLATION	Dichloroacetic acid in CH_2Cl_2 (2:98, v/v)	2 ml	1.5
WASH	Acetonitrile	5 ml	2
COUPLING	Activated nucleotide in acetonitrile*		10
WASH	Acetonitrile	5 ml	2
CAPPING	Acetic anhydride/2,6-lutidine/THF (10:10:80, v/v) N-methylimidazol/THF (16:84, v/v)	1 ml	2
WASH	Acetonitrile	5 ml	1

* For 1 μmol synthesis scale, 2 M DBU in pyridine (100 μl) and 0.1 M 5'-O-DMT-deoxynucleoside 3'-O-(2-thio-1,3,2-oxathiaphospholane) (50 μl) in acetonitrile is used.

Isolation of OligoS involves 2-step HPLC purification, as used for standard oligonucleotide synthesis, according to the protocols described earlier (Ref. 14). Because the yield of a single coupling is on the level of 95%, as assigned by trityl cation assay, the preparative yield depends markedly on the sequence and purification efficiency. ^{31}P NMR spectra indicate that OligoS are not contaminated with species bearing internucleotide phosphate bonds, and PAGE-analysis indicates satisfactory purity, albeit in the case of $d[C_{PS}]_9C$ small amounts of $d[C_{PS}]_8C$ and $d[C_{PS}]_7C$ have been detected by means of capillary electrophoresis.

STEREOCONTROLLED SYNTHESIS OF OLIGOS

Owing to chirality of the deoxyribose moiety and stereogenicity of the phosphorus atom, protected nucleoside 3'-O-2-thiono-1,3,2-oxathiaphospholanes (2) constitute diastereoisomeric species, and thus their potential application to stereocontrolled synthesis of OligoS requires their separation. This has been achieved by means of the silica gel column chromatography using ethyl acetate or n-butyl acetate as an eluent. Diastereoisomerically pure 2 were obtained and characterized as "Fast"- and "Slow"-eluted isomers by means of ^{31}P NMR, mass spectrometry and TLC-chromatographic mobility. Reactions of "Fast"-eluted isomers of 2 with immobilized 5'-OH nucleosides, under conditions described above, all led to exclusive formation of dinucleoside 3',5'-phosphorothioates of Sp-configuration, while "Slow"-eluted 2 gave exclusively their Rp-epimers. Stereospecificity of the coupling step is between 98-100%. Diastereoisomeric purity was assigned by means of RP-HPLC and the products were identified by comparison with authentic samples prepared as described earlier (Ref. 4). With the assumption that each coupling step occurs with the same sense of stereospecificity, the following assignments of oligonucleotide stereochemistry have been made:

[all-Rp]-d$(C_{PS})_3$C (10), *15.30*[a]	[all-Rp]-d$(T_{PS})_7$T (7), *16.75*[a]
[all-Sp]-d$(C_{PS})_3$C (10), *18.00*	[all-Sp]-d$(T_{PS})_7$T (7), *18.00*
[all-Rp]-d$(C_{PS})_4$C (6), *16.50*	[all-Rp]-d$(A_{PS})_{11}$A (12), *18.00*
[all-Sp]-d$(C_{PS})_4$C (6), *19.80*	[all-Sp]-d$(A_{PS})_{11}$A (12), *19.30*
[all-Rp]-d$(C_{PS})_9$C (11), *12.50*	[all-Rp]-d[$G_{PS}G_{PS}A_{PS}A_{PS}T_{PS}T_{PS}C_{PS}C$] (8), *16.00*
all-Sp]-d$(C_{PS})_9$C (11), *15.50*	[all-Sp]-d[$G_{PS}G_{PS}A_{PS}A_{PS}T_{PS}T_{PS}C_{PS}C$] (8), *16.30*

[a]*Retention time (in minutes) on ODS-Hypersil (5 μ) column (4.7 mmx30cm), flow rate 1.5 ml/min; linear gradient of acetonitrile 0-20% (ca.1%/min) vs 0.1M TEAB.*

Because to date there are no methods available which allow assignment of the diastereoisomeric purities of OligoS, we have applied, in the case of homopolymers, enzyme-assisted hydrolysis as a test of diastereoisomeric purity. It was well established that nuclease P1 selectively digests a phosphorothioate internucleotide bond of Sp-configuration (Ref. 15), whereas snake venom phosphodiesterase (svPDE) assists the selective hydrolysis of an internucleotide phosphorothioate bond of Rp-configuration (Ref. 16). Using parallel hydrolysis of [all-Rp]-d$(C_{PS})_4$C with these enzymes, and treatment of the resulting digests with alkaline phosphatase (AP), we have found that svPDE/AP treatment led to dC and minute amounts of [Rp]-d$(C_{PS}C)$, while nuclease P1/AP digests contained only unchanged substrate. Action of svPDE/AP on [all-Sp]-d$(C_{PS})_4$C (6) left undigested substrate, but nuclease P1/AP digestion led exclusively to dC. Product analysis was performed by means of RP-HPLC. Cross experiments with mixtures containing [all-Rp]-6:[all-Sp]-6 = 95:5 and 5:95, respectively, resulted in the expected ratio of undigested substrates and products.

Similar digestion experiments were performed for both [all-Rp] and [all-Sp]-isoforms of d$(A_{PS})_{11}$A, and the results confirmed the assumption concerning stereochemical integrity of the oligonucleotides under investigation. Further studies, including X-ray and NMR techniques, are in progress.

MELTING TEMPERATURE EXPERIMENTS

One of the crucial questions to be answered concerns the influence of "tacticity" of OligoS on their hybridization with a complementary strand (template) of natural oligonucleotide. Earlier studies with self-complementary sequences d(GGAATTCC) containing a phosphorothioate function in different positions have shown that the

diastereoisomer of Rp-configuration (the sulphur atom oriented inward the double helix) destabilizes the duplex much stronger than the phosphorothioate of Sp-configuration (outward orientation of sulphur with respect to the double helix), but that the decrease of Tm was sequence-dependent (Ref. 18). Stein and Cohen performed systematic studies and proved that, in general, the value Tm for dA_{28}-dT_{28} duplexes are ca. 15-20°C lower for the OligoS in relation to the normal (unmodified) oligonucleotide duplexes of equal length (Ref. 5). All OligoS used in these studies were obtained via the H-phosphonate/sulphurization methodology (Ref. 19). Lee obtained enzymatically (Ref. 20) several [all-Rp]-OligoS, according to the method described by Eckstein (Ref. 21), and found that Tm values for the hybride OligoS/complementary Oligo are several degrees (5-20°C) lower than those measured for unmodified parent duplexes. Because OligoS of [all-Sp]-configuration were unavailable, we have selected dA_{12}-dT_{12} as an initial model system and Tm measurements for $d(A_{PS})_{11}A$-dT_{12} were performed under "low salt" and "high salt" conditions.

Heteroduplex	Tm [°C]	
dT_{12}/dA_{12}	36.3[a]	41.4[b]
$dT_{12}/d(A_{PS})_{11}A$ "random"	33.2	35.7
$dT_{12}/d(A_{PS})_{11}A$ [all-Rp]	16.6	25.2
$dT_{12}/d(A_{PS})_{11}A$ [all-Sp]	33.5	38.5

[a] *Solutions contained 0.25 A_{260} OD of each component per 1mL in 50 mM HEPES/KOH, pH7.0, containing 10mM $MgCl_2$ and 70mM NaCl.*

[b] *as above but under higher NaCl concentration (800 mM).*

The results have clearly shown the influence of "tacticity" of $d(A_{PS})_{11}A$ on its hybridization with dodecathymidylic acid indicating that stereoregular oligo(nucleoside phosphorothioate) of [all-Sp]-configuration binds to the template much stronger than its [all-Rp]-counterpart. This effect has been rationalized by a steric factor. It has been demonstrated earlier that introduction of sulphur to adenosine cyclic-3',5'-phosphate increases its "molecular volume" ca. 8% with respect to that calculated for the molecule of parent cAMP (Ref. 22). Moreover, destabilization may result also from the stronger repulsive effect between two negatively charged strands since, according to Frey (Ref. 23), the charge within phosphorothioate anion is localized on sulphur, thus bringing negatively charged elements closer to negatively charged phosphate on the complementary strand. In the hybrids formed between [all-Sp]-$d(A_{PS})_{11}A$ and dT_{12} sulphur atoms are all directed outward from the double helix, so both repulsive effects, steric and electrostatic, are minimized. An intriguing problem was posed by the Tm value measured for the aforementioned phosphorothioate obtained under non-stereocontrolled routine conditions (Ref. 5). It has been accepted that this procedure should lead to a mixture of 2^n (2048) diastereoisomers. The fact that the Tm value for random $d(A_{PS})_{11}A$-dT_{12} is closer to that of [all-Sp]-$d(A_{PS})A$-dT_{12} requires further elucidation, but one may speculate that:

a) the Tm parameter, whose physical meaning is that a 50% population of oligonucleotides is associated with template, while another 50% is dissociated (single strands), is in reality assigned for the "best fit" population, or

b) the so called "stereorandom mixture" of diastereoisomers of $d(A_{PS})_{11}A$, due to some stereoselectivity of the process of phosphoramidite coupling (followed by stereospecific sulphurization) is, in fact, enriched to a meaningful extent with phosphorothioate population of Sp-configuration. Elucidation of this problem requires further studies.

STEREOCHEMISTRY OF THE 1,3,2-OXATHIAPHOSPHOLANE RING OPENING PROCESS

As presented above, DBU-assisted alcoholysis of nucleoside-3'-O(2-thiono-1,3,2-oxathiaphospholanes) (2) occurs in high yield and with complete stereospecificity. Although the absolute configuration of the resulting internucleotide phosphorothioates could be assigned by enzymatic digestion, the absolute configuration of the substrate was unknown (Ref. 10). Because our attempts of growing suitable crystals of 2 for X-ray analysis have failed, we decided to construct the model compounds, namely 2-N-α-naphthyl-ethylamino-2-thiono-1,3,2-oxathiaphospholanes (13) and O,S-dimethyl-N-α-naphthylethyl phosphoramidothioates (14) (Ref. 24) in order to determine model absolute configuration.

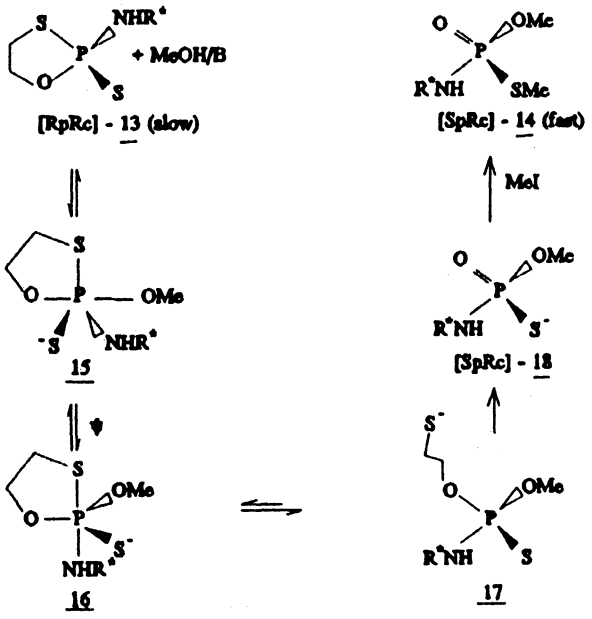
[RpRc] - 13 — [SpRc] - 14

[RpRc] - 13 , δ_{31_P} 94.3 ppm — [SpRc] - 14 , δ_{31_P} 34.65 ppm

mp. 148°- 149°C , $[\alpha]_D^{20}$ - - 40° — mp. 146°- 147°C , $[\alpha]_D^{20}$ - - 29.4°

Both 13 and 14 were obtained from R(+)-1-α-naphthyl-ethylamine and were separated and characterized as diastereoisomerically pure compounds. "Slow"-migrating diastereoisomers of both compounds 13 and 14 gave crystals suitable for X-ray analysis and were shown to possess the [Rp,Rc]-configuration. Therefore, their "Fast"-migrating counterparts must possess the [Sp,Rc]-configuration, respectively. DBU-catalyzed methanolysis of [Rp,Rc]-13 (monitored by ^{31}P NMR) gave a compound, which after alkylation with MeI appeared to be [Sp,Rc]-14. A similar experiment with [Rp,Rc]-:[Sp,Rc]-13 = 79:21 resulted in the formation of 14 in the ratio [Rp,Rc]-:[Sp,Rc]-14 = 23:77. On the basis of these result we could elucidate the stereochemical course of events: an attack of methanol on the molecule of 13 occurs from the side opposite to endocyclic P-O bond.

The resulting discrete intermediate 15 with a TBP structure must undergo a pseudorotation process which places the cleavable P-S bond in an apical position (16). Collapse of intermediate 16 followed by ethylene sulphide elimination gives



rise to the formation of 18 which, after alkylation, gives product 14. The overall process occurs with retention of configuration at phosphorus.
Retrospective stereochemical analysis of the process of DBU-catalysed alcoholysis of nucleoside 3'-O-(2-thiono-1,3,2-oxathiaphospholanes) allowed us to propose the absolute configuration for "Fast"-eluted isomer of 2 as being of [Rp], while that for "Slow"-eluted 2 was therefore assigned as [Sp]. Moreover, X-ray analysis of 13 also allows the conclusion that the driving force for alcoholysis of the 1,3,2-oxathiaphospholanes is due to strain effects of the oxathiaphospholane ring (the angle S-P-O = 97°) and that the kinetically fast elimination of ethylene sulphide forbids the reversibility of 16=17 step (Ref. 24).

Acknowledgements Authors are indebted to Prof. Victor Snieckus (The Guelph-Waterloo Centre, Canada) and Dr. G.Zon (ABI, Foster City, Cal.) for careful reading of the manuscript, comments and corrections.

REFERENCES

1. G.Zon, Pharmaceutical Res. 5, 539 (1988).
2. P.S.Miller, Biotechnology 9, 358 (1991).
3. G.Zon, W.J.Stec, A Practical Approach, F.Eckstein, Ed., IRL Press, Oxford, 1991, 87.
4. W.J.Stec, G.Zon, W.Egan, B.Stec, J.Am.Chem.Soc. 106, 6077 (1984).
5. C.A.Stein, S.Cohen, in Molecular and Structural Biology, Vol. 12, Macmillan Press, London, 1989, 97.
6. W.J.Stec, G.Zon, Tetrahedron Lett. 25, 5279 (1984); M.Fujii, K.Ozaki, A.Kume, M.Sekine, T.Hata, Tetrahedron Lett. 26, 935 (1986); R.Cosstick, D.M.Williams, Nucleic Acids Res. 15, 9921 (1987).
7. F.Eckstein, Ann.Rev.Biochem. 54, 367 (1985); Z.J.Lesnikowski, M.M.Jaworska, Tetrahedron Lett. 30, 3821 (1989).
8. A.Okruszek, P.Guga, W.J.Stec, in Biophosphates and Their Analogues-Synthesis, Structure, Metabolism and Activity, K.S.Bruzik, W.J.Stec, Eds., Elsevier Science Publishers B.V., Amsterdam, 1987, 247.
9. A.Okruszek, P.Guga, W.J.Stec, Heteroatom Chem. 2, 561 (1991).
10. W.J.Stec, A.Grajkowski, M.Koziolkiewicz, B.Uznanski, Nucleic Acids Res. 19, 5883 (1991).
11. M.H.Caruthers, Science 230, 281 (1985).
12. T.Brown, C.E.Pritchard, G.Turner, S.A.Salisbury, J.Chem.Soc., Chem.Commun., 891 (1989).
13. M.Matsukura, K.Shinozuka, G.Zon, H.Mitsuya, M.Reitz, J.S.Cohen, S.Broder, Proc.Natl.Acad.Sci. USA. 84, 7706 (1987).
14. W.J.Stec, G.Zon, B.Uznanski, Journal of Chromatohraphy 326, 263 (1985).
15. B.V.L.Potter, B.A.Connolly, F.Eckstein, Biochemistry 22, 1369 (1983).
16. P.M.J.Burgers, F.Eckstein, D.H.Hunneman, J.Biol.Chem. 254, 7476 (1979).
17. F.R.Bryant, S.J.Benkovic, Biochemisty 18, 2825 (1979).
18. G.Zon, M.F.Summers, K.A.Gallo, K.-L.Shao, M.Koziolkiewicz, B.Uznanski, W.J.Stec, in Biophosphates and Their Analogues - Synthesis, Structure, Metabolism and Activity, K.S.Bruzik, W.J.Stec, Eds., Elsevier Science Publishers B.V., Amsterdam, 1987, 165.
19. B.C.Froehler, Tetrahedron Lett. 27, 5575 (1986).
20. L.J.P.Latimer, K.Hampel, J.S.Lee, Nucleic Acids Res. 17, 1549 (1989).
21. F.Eckstein, H.Gindl, Eur.J.Biochem. 13, 558 (1970);F.Eckstein, W.Armstrong, H.Sternbach, Biochemistry 73, 2987 (1976);
22. L.H.P.Botelho, L.C.Webster, J.D.Rothermel, J.Baraniak, W.J.Stec, J.Biol.Chem 263, 5301 (1988).
23. J.Baraniak, P.A.Frey, J.Am.Chem.Soc. 110, 4059 (1988).
24. B.Uznanski, A.Grajkowski, B.Krzyzanowska, A.Kazmierkowska, W.J.Stec, M.W.Wieczorek, J.Blaszczyk, J.Am.Chem.Soc., submitted for publication