5000–5005 Nucleic Acids Research, 1995, Vol. 23, No. 24

Stereodifferentiation—the effect of P chirality of oligo(nucleoside phosphorothioates) on the activity of bacterial RNase H

Maria Koziolkiewicz, Agnieszka Krakowiak, Marek Kwinkowski, Malgorzata Boczkowska and Wojciech J. Stec*

Polish Academy of Sciences, Centre of Molecular and Macromolecular Studies, Department of Bioorganic Chemistry, Sienkiewicza 112, 90-363 Lódz, Poland

Received September 28, 1995; Revised and Accepted November 13, 1995

ABSTRACT

P stereoregular phosphorothioate analogs of pentadecamer 5′-d(AGATGTTTGAGCTCT)-3′ were synthesized by the oxathiaphospholane method. Their diastereomeric purity was assigned by means of enzymatic degradation with nuclease P1 and, independently, with snake venom phosphodiesterase. DNA–RNA hybrids formed by phosphorothioate oligonucleotides (PS-oligos) with the corresponding complementary pentadecaribonucleotide were treated with bacterial RNase H. The DNA–RNA complex containing the PS-oligo of [all-R_P] configuration was found to be more susceptible to RNase H-dependent degradation of the pentadecaribonucleotide compared with hybrids containing either the [all-S_P] counterpart or the so called 'random mixture of diastereomers' of the pentadeca(nucleoside phosphorothioate). This stereodependence of RNase H action was also observed for a polyribonucleotide (475 nt) hybridized with these phosphorothioate oligonucleotides. The results of melting studies of PS-oligo–RNA hybrids allowed a rationalization of the observed stereodifferentiation in terms of the higher stability of heterodimers formed between oligoribonucleotides and [all-R_P]-oligo(nucleoside phosphorothioates), compared with the less stable heterodimers formed with [all-S_P]-oligo(nucleoside phosphorothioates) or the random mixture of diastereomers.

INTRODUCTION

Inhibition of gene expression by the use of synthetic oligonucleotides, broadly known as the 'antisense mRNA approach' (1), the 'antigene approach' (2) or the 'ribozyme approach' (3) is based on the introduction of synthetic oligomers into the cells and their hybridization to targeted fragments of DNA or RNA responsible for biosynthesis of 'unwanted' proteins. Although in the ribozyme approach the mechanism of RNA cleavage is well defined, the mode of action of antisense oligonucleotides is still a matter of dispute (4). The most generally accepted mechanism for their action involves activation of RNase H, which selectively cleaves RNA chains within segments associated with the antisense oligonucleotide (5,6). This explanation can be questioned, because oligo(nucleoside methanephosphonates) complementary to the selected fragments of RNA decrease production of the corresponding proteins without cleavage of the RNA by RNase H (7). Moreover, bacterial strains that overexpress RNase H on treatment with antisense oligo(nucleoside phosphorothioates) (PS-oligos) do not show an increase in antisense response of a targeted gene compared with normal strains (8).

PS-oligos which form duplexes with RNA that are recognized by RNase H (9) are considered the most promising potential therapeutics to date. The selection is based on the close resemblance of PS-oligos to the natural oligonucleotides (isosteric and isoelectronic with natural oligonucleotides), their known resistance to nucleolytic enzymes (10) and their ability to activate RNase H. Our research is focused on the effective chemical synthesis of PS-oligos (11–13) and the precise description of their structure and diastereomeric composition (14). This last issue is more complex than is generally appreciated, because each phosphorothioate unit within oligo(nucleoside phosphorothioates) constitutes a center of asymmetry. Consequently, each PS-oligo prepared by generally used techniques (15) consists of a mixture of 2^n diastereomers, where n is the number of internucleotide phosphorothioate linkages. Moreover, the contribution of each diastereomer cannot be described by the 2^{-n} relationship, because each step of chain elongation is slightly stereoselective (16). Our recently elaborated oxathiaphospholane method for the synthesis of PS-oligos is, thus far, the only one allowing P stereocontrolled chemical synthesis of oligo(nucleoside phosphorothioates) (17,18) longer than tetramers in either the S_P or R_P configuration (19,20). Having this method of synthesis in hand we decided to check the influence of 'chirality' (21) of PS-oligos upon their proclivity towards RNA duplex formation and the response of RNase H towards double-stranded DNA–RNA structures containing PS-oligos of predetermined chiral sense (absolute configuration) at the phosphorus at each internucleotide phosphorothioate function.

* To whom correspondence should be addressed

Table 1. Oligonucleotide constructs used for RNase H studies

	Sequence	Internucleotide linkage
PO-**1**	5′-d(AGA TGT TTG AGC TCT)-3′	Phosphate
PO-**2**	5′-d(AGA GCT CAA ACA TCT)-3′	Phosphate
[mix]-PS-**3**	5′-d(AGA TGT TTG AGC TCT)-3′	Phosphorothioate
[all-*R*p]-PS-**3**	5′-d(AGA TGT TTG AGC TCT)-3′	Phosphorothioate
[all-*S*p]-PS-**3**	5′-d(AGA TGT TTG AGC TCT)-3′	Phosphorothioate
PO-**5**	5′-r(AGA GCU CAA ACA UCU)-3′	Phosphate
PO-**6**	Polyribonucleotide (475 nt) (26, J. Jarosz, unpublished results)	Phosphate
PO-**7**	Polyribonucleotide (231 nt) (26, J. Jarosz, unpublished results)	Phosphate

MATERIALS AND METHODS

All reagents were of analytical grade. Snake venom phosphodiesterase (EC 3.1.15.1) and ribonuclease H (EC 3.1.26.4) were purchased from Boehringer Mannheim (Germany). Nuclease P1 (EC 3.1.30.1) and glycogen (from oyster) were obtained from Sigma (St Louis, MO). T7 RNA polymerase (EC 2.7.7.6) and ribonuclease inhibitor (RNasin) were purchased from Fermentas (Vilnius, Lithuania). T4 polynucleotide kinase (EC 2.7.1.78) was obtained from Amersham (Little Chalfont, UK).

Syntheses

The pentadecadeoxyribonucleotide PO-**1** and complementary sequence PO-**2** (Table 1) were synthesized by the phosphoramidite method (22) on an ABI 391 DNA Synthesizer using the standard ABI protocol and reagents. The same instrument was used for the synthesis of pentadeca(deoxyribonucleoside phosphorothioates) [mix]-PS-**3**, [all-R_P]-PS-**3** and [all-S_P]-PS-**3** (all of the same sequence as PO-**1**), which were prepared by means of the oxathiaphospholane method (18,23).

5′-*O*-Dimethoxytrityl(DMT)-nucleoside-3′-*O*-(2-thi no-1,3,2-oxathiaphospholanes) (**4**) [nucleoside = thymidine (Thy), N^6-benzoyl adenosine (Ade^{Bz}), N^4-benzoyl cytidine (Cyt^{Bz}) or N^2-isobutyryl-O^6-phenylcarbamoyl guanosine ($Gua^{iBu,DPC}$)] were prepared as described elsewhere (24) and separated into their P epimers by column chromatography on silica gel 60H (Merck, Germany) using ethyl acetate or butyl acetate as an eluent. The purity of separated diastereomers of **4** was determined by ^{31}P NMR and was shown to be >98%. The 5′-*O*-DMT base-protected (except Thy) nucleosides were bound to controlled pore glass through a long chain alkylamine–sarcosinyl–succinyl linker (25). The protocol for 1 μmol scale synthesis involved a 20-fold molar excess of **4** and a 200-fold molar excess of 1.8-diazabicyclo[5,4,0]undec-7-ene (DBU) per single coupling, with a coupling time of 220 s. The average repetitive yield was 94%, as measured by DMT cation assay. The oligomers were isolated using a two step reversed-phase high performance liquid chromatography purification [ODS-Hypersil column, 300 × 4.6 mm, flow rate 1.5 ml/min, 0–40% CH_3CN/0.1 M triethylammonium bicarbonate (TEAB), 0.75%/min]. The preparative yield was as follows: PO-**1**, 25 A_{260} U (95%); [mix]-PS-**3**, 9.8 A_{260} U (95%); [all-R_P]-PS-**3**, 7.0 A_{260} U (92%); [all-S_P]-PS-**3**, 8.4 A_{260} U (95%). Numbers in parentheses indicate the chain integrity as measured by densitometry of polyacrylamide gel electrophoresis (PAGE) analyzed 5′-^{32}P-labeled samples (Fig. 1).

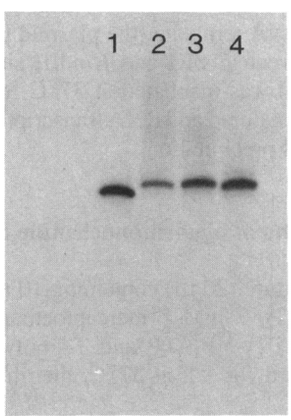

Figure 1. Purity of the PO-**1** and PS-**3** constructs. Lane 1, PO-**1**; lane 2, [mix]-PS-**3**; lane 3, [all-S_P]-PS-**3**; lane 4, [all-R_P]-PS-**3**. Oligomers were analyzed on 20% polyacrylamide–7 M urea gels as described in Materials and Methods.

The pentadecaribonucleotide 5′-r(AGAGCUCAAACAUCU)-3′ (PO-**5**, sequence complementary to that of PO-**1** and PS-**3**) was a gift from Dr S. Tam (Hofmann-La Roche, Nutley, NJ); its purity was >98%, as analyzed by PAGE. Polyribonucleotide PO-**6** (475 nt) was obtained by *in vitro* transcription using plasmid pT7-7* containing the interleukin-2 (Il-2) gene inserted between *Eco*RI and *Hin*dIII recognition sites. The plasmid pT7-7* was constructed from plasmid pT7-7 (26) by replacement of the 23 nt fragment (nt 22964–22987) with the sequence 5′-d(TAGAATTCCATATG)-3′ (J. Jarosz, unpublished results).

Proof of diastereomeric purity of PS-oligos (3)

Diastereomeric purity of the [all-S_P]-PS-**3** was assessed using R_P-specific snake venom phosphodiesterase (svPDE) (27,28). The samples of corresponding oligonucleotides [mix]-PS-**3**, [all-R_P]-PS-**3** and [all-S_P]-PS-**3** were incubated with svPDE at 37°C for 24 h. In a typical experiment the reaction mixture (60 μl) contained 3 μg svPDE, 25 mM Tris–HCl, pH 8.5, 4 mM $MgCl_2$ and 1–2 μg (0.2–0.3 nmol) of the corresponding PS-oligo

labeled at the 5′-end with ^{32}P. Aliquots of 10 µl taken after 0, 4, 8, 16 and 24 h were heat denatured and analyzed by means of 20% polyacrylamide–7 M urea gels.

Diastereomeric purity of [all-R_P]-PS-**3** was assessed by means of S_P-specific nuclease P1 (29). The samples corresponding to constructs [mix]-PS-**3**, [all-R_P]-PS-**3** and [all-S_P]-PS-**3** were incubated with this enzyme at 20°C for 24 h. In a typical experiment the reaction mixture (60 µl) contained 0.05 µg nuclease P1, 100 mM Tris–HCl, pH 7.2, 1 mM $ZnCl_2$ and 1–2 µg (0.2–0.3 nmol) 5′-^{32}P labeled oligonucleotide. Aliquots of 10 µl taken after 0, 4, 8, 16 and 24 h were heat denatured and analyzed by means of 20% polyacrylamide–7 M urea gels.

In vitro transcription

The incubation mixture (50 µl) containing 40 mM Tris–HCl, pH 8.0, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol (DTT), 0.4 mM each nucleoside 5′-triphosphate (NTP) (N = A, U, C or G), [α-^{32}P]CTP, bovine serum albumin (5 µg), RNasin (75 U), 1.72 µg DNA template (the plasmid pT7-7* containing the Il-2 gene between *Eco*RI and *Hin*dIII sites) and T7 RNA polymerase (20 U) was incubated at 37°C for 1.5 h. Then the sample was heat denatured and RNA transcript PO-**6** (475 nt) was isolated by ethanol precipitation.

5′-^{32}P-End-labeling of oligoribonucleotide PO-5

An incubation mixture (20 µl) containing 10 mM Tris–HCl, pH 8.5, 10 mM $MgCl_2$, 7 mM β-mercaptoethanol, 35 µM PO-**5** (0.7 nmol), 15 µCi [γ-^{32}P]ATP and T4 polynucleotide kinase (5 U) was incubated for 1 h at 37°C, then heat denatured and stored at –70°C.

RNase H-catalyzed hydrolysis of oligoribonucleotide PO-5

The incubation mixture (10 µl) containing 20 mM Hepes–KOH, 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 35 pmol 5′-^{32}P-end-labeled PO-**5** and 35 or 105 pmol PO-**1** or the corresponding PS-**3** was heated to 75°C (2 min) and then slowly cooled to 20°C (1.5 h). A 10 µl aliquot of RNase H (1 U) in the same buffer was then added to the incubation mixture. After 15, 30 or 45 min incubation at 37°C (or 28°C) products were isolated in the presence of 4 µg glycogen by ethanol precipitation, dissolved in the loading buffer (formamide, xylene cyanol and bromophenol blue) and loaded onto a 20% polyacrylamide–7 M urea gel.

RNase H-catalyzed cleavage of polyribonucleotide PO-6 hybridized with PO-1, [mix]-PS-3, [all-R_P]-PS-3 or [all-S_P]-PS-3

An incubation mixture (10 µl) containing 130 mM NH_4Cl, ~100 pmol transcript PO-**6** and the corresponding construct PO-**1** or PS-**3** (100 pmol) was heated for 5 min at 95°C and then quickly cooled on ice (5 min). Then a 10 µl solution containing 130 mM NH_4Cl, 10 mM Tris, pH 7.5, 10 mM $MgCl_2$, 5% sucrose and RNase H (1 U) was added to the mixture containing complexes PO-**6**–PO-**1** or PO-**6**–PS-**3**. The total volume of the reaction mixture was 20 µl. After 45 min incubation at 37°C RNA was isolated by ethanol precipitation and analyzed on 7% polyacrylamide–7 M urea gels.

T_m measurement

The samples were dissolved in 10 mM sodium cacodylate, pH 7.4, containing 10 mM $MgCl_2$ and 70 mM NaCl. Oligomer concentrations were 2.4 µM for d[$(A_{PS})_{11}$A] and $(T_{PS})_{11}$T (18) and 2 µM for oligomers [all-R_P]-, [all-S_P]- and [mix]-PS-**3**. The equimolar mixture of the template (dA_{12}, T_{12}, U_{12}, oligodeoxyribonucleotide PO-**2** or oligoribonucleotide PO-**5**) and corresponding phosphorothioate oligonucleotide {$(T_{PS})_{11}$T, d[$(A_{PS})_{11}$A], (18) [mix]-PS-**3**, [all-S_P]-PS-**3** or [all-R_P]-PS-**3** respectively} was heated at 75°C for 2 min and then slowly (2 h) cooled to 2°C. The melting profiles were recorded at 260 nm using a GBC UV/VIS 916 spectrophotometer equipped with a Thermocell unit. The insulated cell compartment was warmed from 2 to 80°C at a rate of 0.2°C/min.

RESULTS

For our studies we have selected the sequence 5′-d[AGATGTTT-GAGCTCT]-3′, complementary to the central fragment of mRNA transcribed from the Il-2 gene inserted into plasmid pT-7-7*. The polyribonucleotide PO-**6** (475 nt) transcribed *in vitro* from the full-length Il-2 gene contains the targeted sequence in positions 161–175. The second polyribonucleotide PO-**7** (231 nt) was transcribed *in vitro* from the same DNA template cleaved by *Sac*I restrictase to obtain the size marker for the expected product of RNase H-dependent cleavage of polyribonucleotide PO-**6**. The pentadecamers PS-**3**, possessing phosphorothioate functions at each internucleotide linkage, were prepared by the oxathiaphospholane methodology. The oligomer [mix]-PS-**3** (a mixture of all possible diastereomers) was prepared from the unseparated diastereomeric mixture of the corresponding monomer **4**. The stereochemically defined isomers [all-R_P]-PS-**3** and [all-S_P]-PS-**3**, possessing all phosphorus atoms of R_P or S_P absolute configuration respectively (23), were synthesized starting from SLOW- or FAST-eluted diastereomers of the corresponding **4**. The diastereomeric purity of stereoregular oligomers has been assigned after 5′-^{32}P-end-labeling and enzymatic digestion with nuclease P1 and, independently, with snake venom phosphodiesterase (svPDE). Diastereomeric purity of [all-R_P]-PS-**3** was determined to be 72% as a result of its exhaustive treatment with nuclease P1 (24 h). Treatment of this construct with svPDE caused complete degradation after 1 h. The same enzyme caused 28% degradation of [all-S_P]-PS-**3** and, therefore, the diastereomeric purity of [all-S_P]-PS-**3** was determined to be 72%. Nuclease P1 caused complete hydrolysis of this last construct after 1 h. Oligomers PO-**1** or [mix]-PS-**3**, [all-R_P]-PS-**3** and [all-S_P]-PS-**3** respectively were separately added to ^{32}P-labeled pentadecaribonucleotide PO-**5** and the corresponding mixtures heated to 75°C. After slow cooling to 25°C the hybrids PO-**1**–PO-**5**, [mix]-PS-**3**–PO-**5**, [all-R_P]-PS-**3**–PO-**5** and [all-S_P]-PS-**3**–PO-**5** were treated with RNase H (45 min at 37 or 28°C). The resulting digests were analyzed by PAGE followed by densitometric analysis of autoradiograms (Fig. 2A and B). The quantitative data are collected in Table 2.

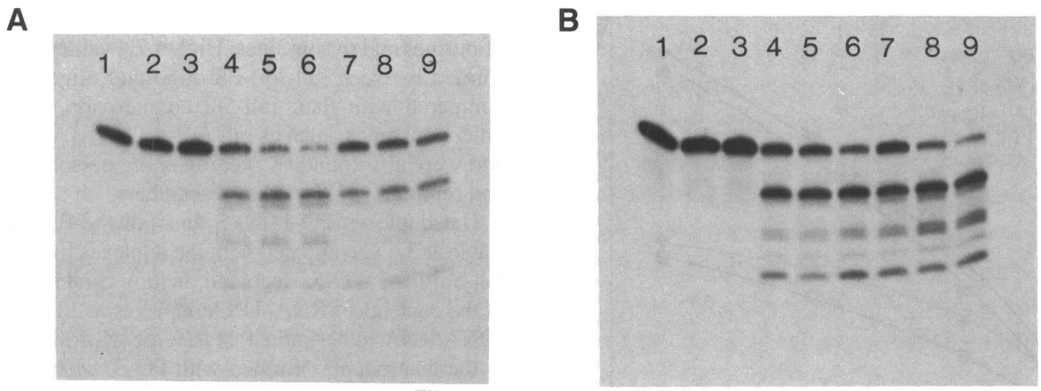

Figure 2. (**A**) RNase H-catalyzed degradation of pentadecaribonucleotide **5** associated with complementary PO-**1** or [mix]-PS-**3** oligomers (temperature 37°C, molar ratio of **5**:**1** or **5**:**3** 1:1). Lane 1, PO-**5**; lane 2, PO-**5** + RNase H; lane 3, PO-**5** + PO-**1**, without RNase H; lanes 4–6, PO-**5** + PO-**1** + RNase H, time 15, 30 and 45 min; lanes 7–9, PO-**5** + [mix]-PS-**3** + RNase H, time 15, 30 and 45 min. (**B**) RNase H-catalyzed degradation of pentadecaribonucleotide **5** associated with complementary [all-S_P]-PS-**3** or [all-R_P]-PS-**3** oligomers (temperature 37°C, molar ratio of **5**:**1** or **5**:**3** 1:1). Lane 1, PO-**5**; lane 2, PO-**5** + RNase H; lane 3, PO-**5** + PO-**1**, without RNase H; lanes 4–6, PO-**5** + [all-S_P]-PS-**3** + RNase H, time 15, 30 and 45 min; lanes 7–9, PO-**5** + [all-R_P]-PS-**3** + RNase H, time 15, 30 and 45 min.

Table 2. Percentage of RNase H-catalyzed degradation of pentadecaribonucleotide PO-**5** and polyribonucleotide PO-**6** associated with oligonucleotides PO-**1** or PS-**3**

RNA component (RNA:DNA molar ratio)	Incubation temperature (°C)	DNA component PO-**1**	[mix]-PS-**3**	[all-R_P]-PS-**3**	[all-S_P]-PS-**3**
PO-**5** (1:1)	37	87	53	89	52
PO-**5** (1:3)	37	96	83	96	75
	28	80	65	86	35
PO-**6** (1:1)	37	100	80	100	80

Digestion of PS-oligo–RNA duplexes with RNase H has shown that the enzyme is more effective against RNA involved in heteroduplexes formed with [all-R_P]-PS-oligo (see Table 2). After 45 min incubation at 37°C oligoribonucleotide PO-**5** associated with oligodeoxyribonucleotide PO-**1** or [all-R_P]-PS-**3** (molar ratio 1:1) was almost completely digested, while PO-**5** complexed under the same conditions with [mix-]-PS-**3** or [all-S_P]-PS-**3** was hydrolyzed 53 and 52% respectively (Fig. 3). A 3-fold molar excess of phosphorothioate constructs PS-**3**, complementary to PO-**5**, caused less pronounced stereodependence of activity of RNase H. Within 45 min at 37°C nearly complete degradation of PO-**5** complexed with PO-**1** or [all-R_P]-PS-**3** was observed, while 20% of PO-**5** involved in heteroduplexes with [mix]-PS-**3** or [all-S_P]-PS-**3** remained. It is interesting that recognition of heteroduplexes [all-R_P]-PS-**3**–PO-**5** and PO-**1**–PO-**5** by RNase H, as measured by the extent of RNA cleavage, is the same.

It should be emphasized that the use of a 15mer PS-oligo non-complementary to PO-**5** did not cause any degradation of PO-**5** by RNase H (data not shown). The effectiveness of RNase H towards polyribonucleotide associated with PO-**1**, [mix]-PS-**3**, [all-R_P]-PS-**3** or [all-S_P]-PS-**3** respectively has been measured independently using mRNA (475 nt) transcribed by T7 RNA polymerase from the Il-2 gene (Table 2). After 45 min incubation with RNase H we found that polyribonucleotide PO-**6** associated with [mix]-PS-**3** or [all-S_P]-PS-**3** was 80% degraded, while the same substrate hybridized with PO-**1** or [all-R_P]-PS-**3** was 100% hydrolyzed.

DISCUSSION

In order to rationalize the observed stereodifferentiation of RNase H-catalyzed cleavage of oligoribonucleotide PO-**5**, as well as polyribonucleotide PO-**6**, we have performed melting studies of [mix]-PS-**3**, [all-R_P]-PS-**3** and [all-S_P]-PS-**3** hybridized to complementary pentadecadeoxyribonucleotide PO-**2** and, independently, to pentadecaribonucleotide PO-**5**. The results are shown in Table 3. Inspection clearly indicates that affinity of the phosphorothioate oligonucleotide [all-R_P]-PS-**3** for oligoribonucleotide PO-**5** is higher (T_m 52°C) than that of [mix]-PS-**3** and [all-S_P]-PS-**3** (T_m 47 and 44°C respectively), albeit still lower than that of PO-**1** (T_m 59°C). This result is the opposite of that predicted from earlier observations (30), as well as of that based upon the results of molecular mechanics calculations performed for simulated complementary heteroduplexes [all-R_P]- and [all-S_P]-phosphorothioate–oligodeoxyribonucleotide (31). Assuming the B conformation for PS-oligo–oligodeoxyribonucleotide complexes and negative charge localization at the phosphorothioate sulfur atom (32) the [all-S_P] isomer was expected to bind more tightly, because the sulfur atom is directed 'outward' from the double helix (30), allowing for better separation of the negative charges. Also, lower steric hindrance was expected, since the sulfur atom is sterically more demanding than an oxygen atom (33). These theoretical considerations have been supported by some experimental data. For example, the [all-S_P]-d[$(A_{PS})_{11}A$]–T_{12} duplex is more stable than the duplex containing the [all-R_P] counterpart

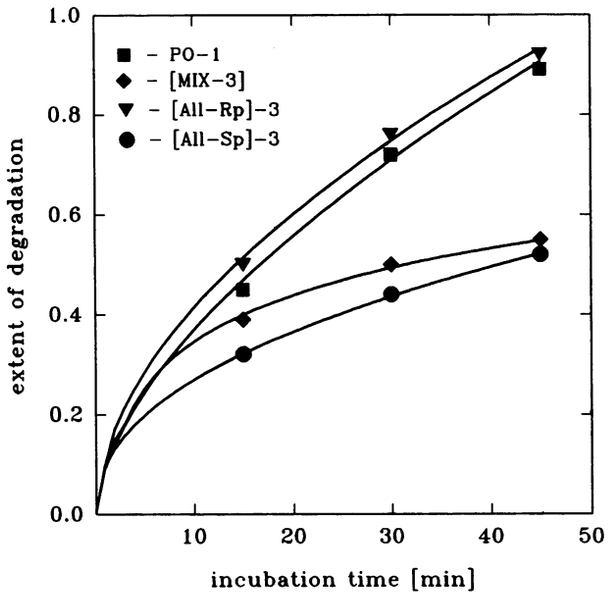

Figure 3. Time course of RNase H-catalyzed hydrolysis of pentadecaribonucleotide PO-**5**. Temperature 37°C, molar ratio of **5**:1 or **5**:3 1:1.

(Table 4). However, the order of stability of duplexes containing [all-*R*P] and [all-*S*P] isomers of $(T_{PS})_{11}T$ is the opposite. Thus the stability of PS-oligo–DNA heteroduplexes seems to be dependent upon sulfur atom orientation, on the sequence of the modified strand and the purine/pyrimidine composition. There is no difference in stability between DNA–DNA duplexes involving [mix]-PS-**3**, [all-*R*P]-PS-**3** or [all-*S*P]-PS-**3** and PO-**2**. The higher stability of PS-oligo–RNA duplexes containing [all-*R*P] isomers seems to be more dependent on the stereochemistry around the P atom than upon the sequence of the modified strand or the content of purines and pyrimidines. Higher T_m values for heteroduplexes formed between [all-*R*P]-PS-oligo and oligoribonucleotides, as compared with their [all-*S*P] counterparts, were observed for other constructs, such as [all-*R*P]-d[$(A_{PS})_{11}$A]–U_{12} (see Table 4) and were also found (K. Fearon *et al.*, personal communication) and reported (34) by other researchers.

The duplexes PO-**1**–PO-**5** and [all-*R*P]-PS-**3**–PO-**5** are more stable at 37°C compared with the duplexes [mix]-PS-**3**–PO-**5** and [all-*S*P]-PS-**3**–PO-**5**. Recorded melting curves allowed estimation of the contents of RNA–DNA duplexes at 28 and 37°C. At 28°C (DNA:RNA molar ratio 1:1) 92% of [all-*R*P]-PS-**3** was involved in the heteroduplex formed with PO-**5**, while the corresponding numbers for [mix-]-PS-**3** and [all-*S*P]-PS-**3** were 87 and 86% respectively (Table 3). If duplex stability is the only reason for the different extents of RNA cleavage at 37°C these differences should be less profound at 28°C. However, the results presented in Table 2 indicate that under these conditions the trend of stereodependence is the same, although the extent of RNA cleavage was lower. We found that the oligomer PO-**5** complexed with [all-*R*P]-PS-**3** was degraded by 86%, while the same substrate hybridized with [mix]-PS-**3** and [all-*S*P]-PS-**3** was 65 and 35% hydrolyzed respectively. These results may suggest that the higher stability of the duplex formed with [all-*R*P]-PS-**3** is not the only reason for the stereodifferentiated activity of RNase H. Other factors, such as different conformational requirements of stereoregular PS-oligos which are involved in DNA–RNA duplexes, may be responsible for the altered recognition of hybrids by RNase H. However, preliminary CD studies (data not shown) do not show any differences in CD curves recorded for DNA–RNA duplexes formed with PS-oligos of opposite chiral sense. Studies of the influence of P chirality of phosphorothioate oligonucleotides on the kinetics of RNase H-catalyzed cleavage of RNA complexed with PS-oligos of predetermined chirality are in progress.

Table 3..Stability of DNA–RNA and DNA–DNA duplexes formed with oligomer PO-**1** and its phosphorothioate analog **3**

DNA component	RNA template **5** T_m (°C)[a]	Content of DNA–RNA duplex; α (%)[b] at 37°C	at 28°C	DNA template **2** T_m (°C)[a]
PO-**1**	59	93	96	61
[mix]-PS-**3**	47	78	87	52
[all-*R*P]-PS-**3**	52	86	92	52
[all-*S*P]-PS-**3**	44	75	86	52

[a]Error range ±0.5°C.
[b]Calculated using the Van't Hoff method according to Breslauer (36).

Table 4. Results of T_m studies of PS-oligo–DNA and PS-oligo–RNA heteroduplexes[a]

DNA template Duplex	T_m (°C)[b]	Duplex	T_m (°C)[b]	RNA template Duplex	T_m (°C)[b]
T_{12}–dA_{12}	36.0	dA_{12}–T_{12}	36.0	dA_{12}–U_{12}	23.0
[all-*S*P]-$(T_{PS})_{11}T$–dA_{12}	14.0	[all-*S*P]-d[$(A_{PS})_{11}$A]–T_{12}	34.0	[all-*S*P]-d[$(A_{PS})_{11}$A–U_{12}	18.0
[mix]-$(T_{PS})_{11}T$–dA_{12}	17.0	[mix]-d[$(A_{PS})_{11}$A]–T_{12}	31.0	[mix]-d[$(A_{PS}]_{11}$A]–U_{12}	29.0
[all-*R*P]-$(T_{PS})_{11}T$–dA_{12}	18.0	[all-*R*P]-d[$(A_{PS})_{11}$A]–T_{12}	29.0	[all-*R*P]-d[$(A_{PS})_{11}$A]–U_{12}	37.0

[a]The synthesis of stereoregular PS-oligos presented in this Table is described in Stec *et al.* (18).
[b]Error range ±0.5°C.

The results of RNase H-catalyzed cleavage of polyribonucleotide PO-**6** (Table 2) indicate that the extent of degradation is higher than that observed for pentadecaribonucleotide PO-**5**. However, in both cases (compounds PO-**5** and PO-**6**) DNA–RNA duplexes containing [all-R_P]-PS-**3** are better substrates for RNase H than those containing [mix]-PS-**3** or [all-S_P]-PS-**3**, although the extent of degradation seems to be dependent upon the length and the primary and secondary structure of the RNA substrate. The same trend of the stereodependence of RNase H activity towards [all-R_P]-PS-oligo and [mix]-PS-oligo was also observed by Tang *et al.* (34)

If an idealistic assumption of the antisense mRNA strategy implies complete inactivation of all copies of RNA responsible for biosynthesis of unwanted proteins, [all-R_P]-PS-oligos seem to be promising as potential therapeutics. On the other hand, our data (M. Koziolkiewicz, manuscript in preparation) indicate that [all-R_P]-PS-oligos are much better substrates for plasma 3′-exonuclease than [mix]-PS-oligos, while [all-S_P]-PS-oligos are completely resistant to this enzyme. Perhaps, for the practical application of [all-R_P]-PS-oligos, their 3′-ends should be protected by phosphorothioate linkages of [S_P] configuration or by achiral phosphorodithioates (35). Synthesis of appropriate constructs is under way.

ACKNOWLEDGEMENTS

This paper is dedicated to Professor David Shugar on the occasion of his 80th birthday. The authors are indebted to Drs Steve Tam and Willy Bannwarth (Hoffmann La Roche) for their generous gift of PO-**5** and U_{12} and to Mrs Wieslawa Goss for her excellent technical assistance. Words of appreciation are directed to Professor Perry A. Frey (Institute of Enzymes, Madison, WI) and Professor Michael Blackburn (Sheffield University, UK) for their assistance during preparation of this manuscript and valuable comments. Studies presented in this communication were financially supported by the State Committee for Scientific Research, grants 6 6286 92 03 and 6 P203 007 05.

REFERENCES

1 Zon,G. (1988) *Pharm. Res.*, **5**, 539–549.
2 Thuong,N.T. and Helene,C. (1993) *Angew. Chem. Int. Edn English*, **32**, 666–690.
3 Sarver,N., Cantin,E.M., Chang,P.S., Zaia,J.A., Ladne,P.A., Stephens,D.A. and Rossi,J.J. (1990) *Science*, **247**, 1222–1225.
4 Stein,C.A. and Cheng,Y.-C. (1993) *Science*, **261**, 1004–1012.
5 Walder,R.Y. and Walder,J.A. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 5011–5015.
6 Yang,W., Hendrickson,W.A., Crouch,R.J. and Satow,Y. (1990) *Science*, **249**, 1398–1405.
7 Miller,P.S. (1989) In Cohen,J.S. (ed.), *Oligonucleotides: Antisense Inhibitors of Gene Expression*. Macmillan Press, London, UK, pp. 79–95.
8 Rosolen,A., Kyle,E., Chavany,C.,Bergan,K., Kalman,E.T.,Crouch,K. and Neckers,L. (1993) *Biochimie*, **75**, 79–87.
9 Furdon,P.J., Dominski,Z. and Kole,R. (1989) *Nucleic Acids Res.*, **17**, 9193–9204.
10 Eckstein,F. (1985) *Annu. Rev. Biochem.*, **54**, 367–402.
11 Stec,W.J., Zon,G., Egan,W. and Stec,B. (1984) *J. Am. Chem. Soc.*, **106**, 6077–6079.
12 Stec,W.J., Uznanski,B., Wilk,A., Hirschbein,B.L., Fearon,K.L. and Bergot,B.L. (1993) *Tetrahedron Lett.*, **34**, 5317–5320.
13 Okruszek,A. Sierzchala,A., Sochacki,M. and Stec,W.J. (1992) *Tetrahedron Lett.*, **33**, 7585–7588.
14 Wilk,A. and Stec,W.J. (1995) *Nucleic Acids Res.*, **23**, 530–534.
15 Zon,G. and Stec,W.J., (1991) In Eckstein,F. (ed.), *Oligonucleotides and Analogues: A Practical Approach*. IRL Press, Oxford, UK, pp. 87–108.
16 Stec,W.J. and WilkA. (1994) *Angew. Chem. Int. Edn English*, **33**, 709–722.
17 Stec,W.J., Grajkowski,A., Koziolkiewicz,M. and Uznanski,B. (1991) *Nucleic Acids Res.*, **19**, 5883–5888.
18 Stec,W.J., Grajkowski,A., Kobylanska,A., Karwowski,B., Koziolkiewicz,M., Misiura,K., Okruszek,A., Wilk,A., Guga,P., Boczkowska,M. (1995) *J. Am. Chem. Soc.*, accepted for publication.
19 Lesnikowski,Z.J and Jaworska-Maslanka,M. (1989) *Tetrahedron Lett.*, **30**, 3821–3824.
20 Murakami,A., Tamura,Y., Wada,H. and Makino,K. (1994) *Anal. Biochem.*, **233**, 285–290.
21 Buda,A.B., Heide,T.A. and Mislow,K. (1992) *Angew. Chem. Int. Edn English*, **31**, 987–1007.
22 Caruthers,M.H. (1990) *Acc. Chem. Res.*, **24**, 278–284.
23 Stec,W.J. (1991) *Nucleic Acids Symp. Ser.*, **25**, 171–172.
24 Stec,W.J. and Lesnikowski,Z.J. (1993) In Agrawal,S. (ed.), *Oligonucleotide Protocols*. Humana Press Inc., Totowa, NJ, pp. 285–314.
25 Brown,T., Pritchard,C.E., Turner,G. and Salisbury,S.A. (1989) *J. Chem. Soc. Chem. Commun.*, 891–893.
26 Tabor,S. and Richardson,C.C. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 1074–1078.
27 Eckstein,F., Burgers,P.M.J. and Hunneman,P.H. (1979) *J. Biol. Chem.*, **254**, 7476–7478.
28 Benkovic,S.J. and Bryant,T.R. (1979) *Biochemistry*, **18**, 2825–2828.
29 Potter,B.V.L., Connolly,B.A. and Eckstein,F. (1983) *Biochemistry*, **22**, 1369–1377.
30 Zon,G., Summers,M.F., Gallo,K.A., Shao,K.-L., Koziolkiewicz,M., Uznanski,B. and Stec,W.J. (1987) In Bruzik,K. and Stec,W.J. (eds), *Biophosphates and Their Analogues—Synthesis, Structure, Metabolism and Activity*. Elsevier, Amsterdam, The Netherlands, pp. 165–178.
31 Jaroszewski,J.W., Syi,J.-L., Maizel,J. and Cohen,J.S. (1992) *Anticancer Drug Design*, **7**, 253–262.
32 Frey,P.A. and Sammons,R.D. (1985) *Science*, **228**, 541–545.
33 Botelho,L.H.P., Webster,L.C., Rothemel,J.D., Baraniak,J. and Stec,W.J. (1988) *J. Biol. Chem.*, **263**, 5301–5305.
34 Tang,J., Roskey,A., Ying,L. and Agrawal,S. (1994) *Nucleosides Nucleotides*, **14**, 985–989.
35 Marshall,W.S. and Caruthers,M.H. (1993) *Science*, **259**, 1564–1570.
36 Breslauer,K. (1994) In Agrawal,S. (ed.), *Protocols for Oligonucleotide Conjugates*. Humana Press Inc., Totowa, NJ, pp. 347–372.