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Stereochemistry of Cleavage of Internucleotide Bonds by *Serratia marcescens* Endonuclease

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Abstract—Endonuclease from *Serratia marcescens* hydrolyzes internucleotide phosphorothioate linkages of R_P configuration with *inversion* of configuration at P-atom. This observation supports a reported architecture of the active site, with 3′-bridging and pro-S_P non-bridging oxygen atoms of the scissile phosphate group involved in direct contact with hydrated magnesium cation, while His-89 activates a water molecule which attacks the phosphorus atom according to a one-step *in-line* mechanism. The presence of a phosphorothioate bond of S_P configuration downstream to that one being cleaved reduces the rate of hydrolysis. This suggests participation of the pro-S_P oxygen atom of that phosphate bond in the mechanism of action of the enzyme, which was not detected in published crystallographic analyses.

Introduction

The extracellular endonuclease of the *Serratia marcescens* belongs to a family of related nucleases whose amino acid sequences are highly conserved among a variety of prokaryotic and eukaryotic organisms including humans.[1,2] The *Serratia* enzyme is the only member of this family for which structural data exists, and it has been extensively studied biochemically.[3,4] On the basis of the structural studies the molecular mechanism of action of this enzyme was recently proposed by Stoddart et al.[5] and, independently, by Miller et al.[6] Both groups proposed the presence of hydrated magnesium ion in the active site and proved the pivotal function of amino acid residues such as Arg-57, His-89, Glu-127, Asn-119, and Asp-86 (Fig. 1A and B), but the arrangement of ligands surrounding the metal ion has not been determined precisely yet. Direct contact between magnesium cation and 3′-oxygen atom of the cleaved phosphate group has been proved by its replacement with sulfur (Fig. 1A, structure a: Z = S) to abolish the cleavage,[5] although involvement of one of two 'non-bridging' oxygens of phosphate in direct contact with the metal ion is still ambiguous.[6] Surprisingly, recent studies revealed that the DNA/RNA nonspecific *Serratia* nuclease and the highly specific intron-encoded I-*Ppo*I homing endonuclease share a structurally conserved catalytic motif consisting of His, Arg and Asn residues and have similar mechanism of nucleolytic action.[7–9] Moreover, X-ray studies on the structure of the co-crystal I-*Ppo*I endonuclease–substrate complex revealed that the conserved His 98 is within hydrogen-bond distance from the scissile phosphate group. According to Flick et al.[9] this His-98 became an obvious candidate to act as a Lewis acid and to stabilize the negative charge on the pentacoordinate phosphorane transition state. Since there was no indication that a water molecule bound to the adjacent phosphate group might act as a nucleophile, authors preferred a model in which a magnesium bound water is deprotonated by a general base and attacks the phosphate. If both *Serratia* and *I-PpoI* endonucleases share the same architecture at the active site for the mechanism of cleavage of the internucleotide bond, only one of two proposed mechanisms, depicted in Figure 1A and B, can be true. The first of them implies that the metal ion interacts with two phosphate oxygen atoms [the bridging 3′-oxygen (Z) and *pro*-S non-bridging oxygen (Y)], with oxygen atom of Asn-119 and with three water molecules. The second mechanism is based on the model where a

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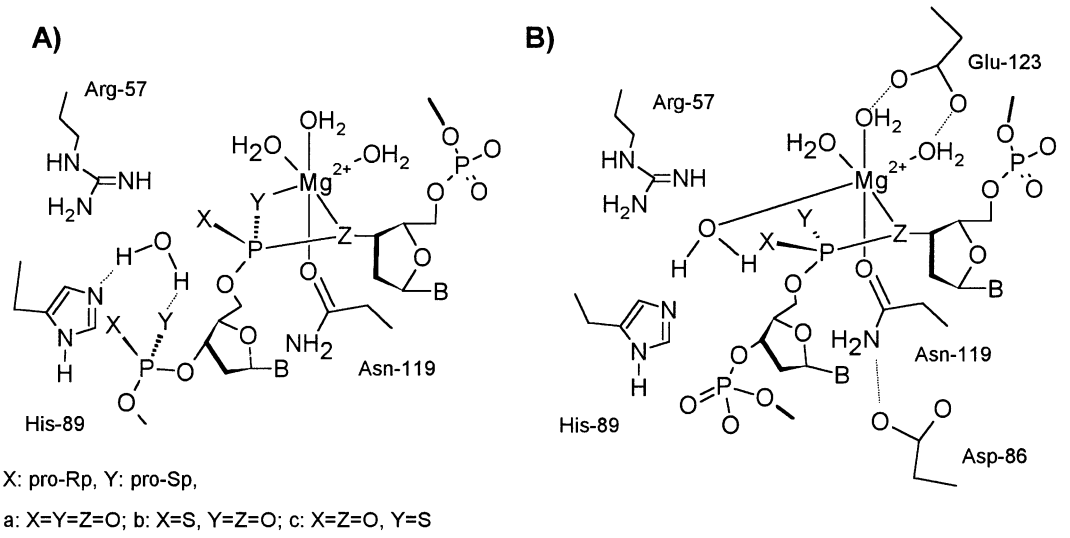


Figure 1. Mechanistic schemes for cleavage of internucleotide bonds by *Serratia marcescens* endonuclease based on mechanisms discussed in refs 5 and 7 (panel A) and in ref 6 (panel B).

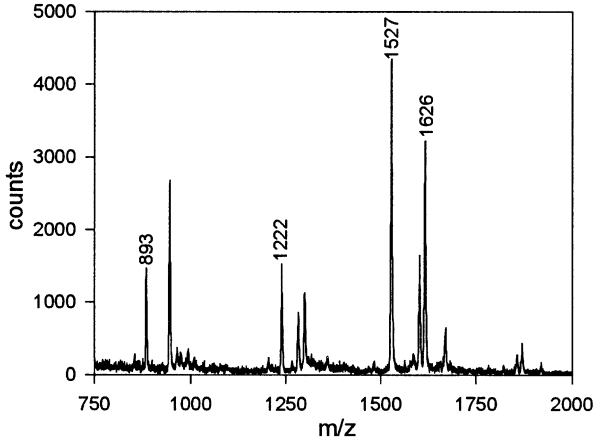


Figure 2. MALDI-TOF mass spectrometry analysis for degradation of **1a** with *Serratia* endonuclease in unlabeled water. The spectrum was recorded in a linear mode. Other spectral parameters as in Experimental.

fourth water molecule is involved in direct contact with magnesium, rather than non-bridging oxygen.[6] The latter model implies that one of four water molecules coordinated by magnesium acts as a nucleophile attacking the phosphorus atom. Although in recent studies the *in-line* direct S_N2 type mechanism of P–O3′ bond cleavage (leading to *inversion* of configuration at phosphorus) is postulated,[5,7,8] the stereochemistry of that process has not been verified. In the course of our studies on the assignment of diastereomeric composition of phosphorothioate analogues of oligonucleotides (PS-Oligo) prepared by stereocontrolled oxathiaphospholane methodology,[10] we have found that *Serratia* nuclease is stereoselective towards internucleotide phosphorothioate linkages of R_P configuration.[10,11] Surprisingly, this observation and its possible mechanistic implications have not been taken into account in previously reported considerations noted above. In our opinion, the observed stereoselectivity of *Serratia* endonuclease indicates the validity of the mechanistic alternative depicted in Figure 1A. The non-bridging *pro*-S_P oxygen atom (Y) must be involved in the contact with magnesium ion, since its replacement by sulfur abolishes the activity of the enzyme (structure c; X = Z = O, Y = S), while elemental substitution of *pro*-R_P oxygen by sulfur (structure b; X = S, Y = Z = O) retains nuclease activity. In other words, the involvement of non-bridging oxygen in contact with metal ion is a critical discriminatory factor for selectivity of the *Serratia* enzyme towards phosphorothioate analogues, while the mechanism depicted in Figure 1B does not justify this phenomenon. To gain an additional proof for mechanistic description of the activity of this enzyme, the stereochemistry of phosphorothioate bond cleavage has been elucidated, as presented below.

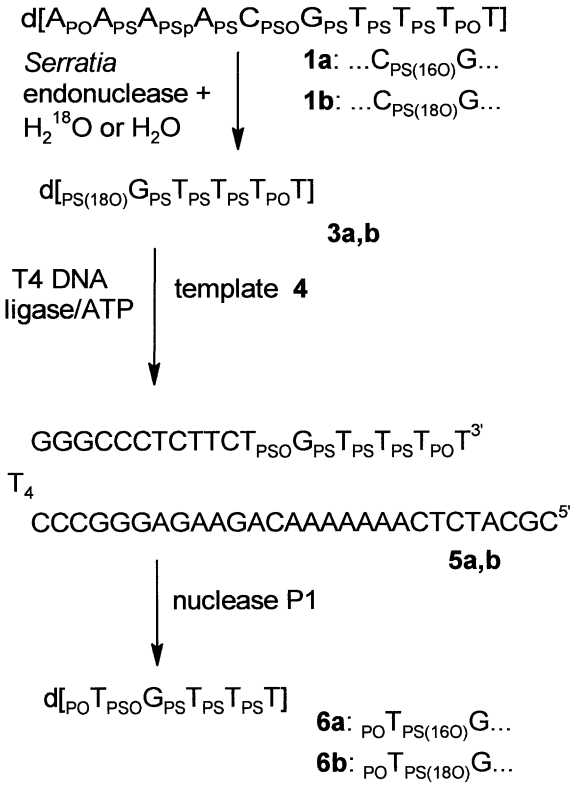
Results and Discussion

In numerous studies on the mechanism of nucleases and phosphotransferases, stereochemical evidence allowed one to distinguish between *one-step* (inversion of configuration, enzyme-assistance in nucleophilic substitution at P atom) and *two-step* (retention, involvement of enzyme-substrate intermediate) modes of enzyme action.[12] On the basis of existing data, the involvement of an enzyme-substrate intermediate in the case of *Serratia* endonuclease has not been claimed, although the position of His 89 within hydrogen-bond distance from the scissile phosphate group has been noticed. On the other hand, direct in-line attack of water on phosphorus involved in a strained four-membered ring (P–O–Mg–O3′) via a pentacoordinate transition state does not necessarily result in inversion of configuration. Large angle distortions noticed by Stoddart in his studies on mutant I-*Ppo*I protein (His98Ala)[5] allow for involvement of a tetrahedral pyramid transition state that collapses with unpredictable stereochemistry.[13] That uncertainity can be solved by hydrolysis of the oligonucleotide substrate containing a phosphorothioate internucleotide bond of R_P-configuration with *Serratia*

endonuclease in [^{18}O]water, followed by determination of the absolute configuration of the P-chiral 5′-*O*-[^{18}O]phosphorothioate in the resulting oligonucleotide product.[14] Earlier designed methodology involved enzymatic digestion of this product, isolation of the resulting nucleoside 5′-*O*-[^{18}O]phosphorothioate, its enzymatic conversion to the corresponding nucleoside α-[^{18}O]thiotriphosphate, and final stereochemical analysis based on precise measurement of isotope chemical shift effect in ^{31}P NMR experiment.[15] One of the serious limitations of this procedure is the demand for the use of a few mg amounts of final material for NMR analysis. That obstacle can be avoided using the methodology presented recently by Mizuuchi et al.[16] It has been demonstrated that 5′-*O*-[^{18}O]phosphorothioylated oligonucleotide generated during the cleavage of parent oligomer by the endonuclease of interest in [^{18}O]water can be ligated to the 3′-end of an appropriate oligonucleotide. In the ligation process, assisted by T4 DNA ligase with the participation of ATP, one of the oxygen atoms of the 5′-terminal phosphate group is adenylated and then removed from the resulting oligonucleotide product upon attack of the 3′-OH group of the other oligonucleotide. According to Mizuuchi, the ligation of a 5′-*O*-phosphorothioylated DNA is stereoselective and results in formation of an R_P-internucleotide phosphorothioate bond with the sulfur atom always present at the non-bridging position.[16] Depending upon the chirality of the ligated 5′-phosphorothioyl group, either an ^{16}O or ^{18}O atom becomes the bridging atom in the adenylated intermediate, and is finally lost from the ligated oligonucleotide product.[17] MALDI-TOF mass spectrometry allows one to determine whether or not the ^{18}O atom incorporated during the cleavage of the initial substrate is retained in the ligation product. The ligation product contains usually 40–50 nucleotides. Therefore, before mass spectrometry analysis can be done, it should be digested by S_P-stereoselective nuclease P1 to generate a dinucleotide containing the phosphorothioate linkage of the R_P configuration. Thus, for an initial substrate carrying an R_P phosphorothioate at the scissile bond and cleaved by the nuclease of interest in [^{18}O]water, the loss of ^{18}O atom from the ligated (and cleaved by nP1) product indicates the inversion of configuration, most likely resulting from a one-step mechanism of enzymatic hydrolysis.

Design of the target oligonucleotide

The strategy developed by Mizuuchi is readily applicable for assignment of the stereochemical course of degradation of phosphorothioate modified oligonucleotides by sequence-specific endonucleases.[16,18,19] The *Serratia* endonuclease is not sequence-specific, and typically the product of degradation of PS-Oligos consists of a mixture of several 5′-*O*-phosphorothioylated fragments. This disallows for straightforward adaptation of analytical protocol described by Mizuuchi. A prerequisite for the use of this method is the endonucleolytic degradation of the substrate at a single internucleotide phosphorothioate bond, thus enabling a precise annealing experiment. Moreover, the resulting PS-Oligo has to be long enough to form a substrate accepted by T4 DNA ligase (i.e., a thermodynamically stable duplex with a template). Fortunately, earlier observations indicated some sequence or structural preferences of the *Serratia* enzyme. For example, Pingoud and co-workers showed that the *Serratia* enzyme cleaves preferentially the internucleotide linkage within d[CG] dinucleotide flanked with oligo-dA and oligo-dT stretches.[20] However, our attempt to use as the substrate a single modified analogue of a sequence d[AAAAC$_{PS}$GTTTT] (**1**) (containing a single internucleotide phosphorothioate bond between dC and dG) was unsuccessful, as the enzyme hydrolyzed two unmodified bonds dA2-dA3 and dA4-dC5, rather than the phosphorothioate linkage. To prevent this unwanted cleavage, another decanucleotide of a sequence [Mix-PS]-d[$A_{PO}A_{PS}A_{PS}A_{PS}C_{PS}G_{PS}T_{PS}T_{PS}T_{PO}T$] (**1a**) was synthesized. The descriptor Mix-PS means that compound **1a** has all phosphorothioate linkages of randomal configuration. This substrate was cleaved by *Serratia* endonuclease predominantly at the internucleotide bond between dC and dG. MALDI-TOF analysis confirmed the presence of two expected main products d[$A_{PO}A_{PS}A_{PS}A_{PS}C$] (*m*/*z* 1527) (**2**) and d[$_{PS}G_{PS}T_{PS}T_{PS}T_{PO}T$] (*m*/*z* 1626) (**3**), albeit the reaction mixture contained also other products, for example d[$A_{PO}A_{PS}A$] (*m*/*z* 893) and d[$A_{PO}A_{PS}A_{PS}A$] (*m*/*z* 1222) (Fig. 2). The oligonucleotide **1a** was then used for *Serratia*-assisted hydrolysis with [^{18}O]water (60 atom% ^{18}O) (Scheme 1). Crucial for the intended stereochemical analysis, the pentamer d[$^{18}_{OPS}G_{PS}T_{PS}T_{PS}T_{PO}T$] (**3a**, *m*/*z* 1626/1628, Fig. 3A) was formed with ca. 10% yield and was isolated by RP-HPLC (an ODS-Hypersil



Scheme 1.

column). Enzymatic hydrolysis of the pentamer **3a** with nuclease P1 resulted in cleavage of the 3′-terminal phosphodiester bond providing as a product the tetramer d[^{18}$_{OPS}$$G_{PS}T_{PS}T_{PS}T$] (*m*/*z* 1322/1324), which did not undergo further degradation. This means that in isolated **3a** all internucleotide phosphorothioate linkages are of R_P configuration. Similar analysis of isolated oligomer **2** revealed that this product has the R_P phosphorothioate linkage between dA4 and dC5. Observed stereochemical preferences of the enzyme provide additional information with respect to enzyme-substrate interactions taking place outside of the scissile bond region. Such interactions can be probed using stereodefined phosphorothioate oligomers with consecutive bonds of predetermined configurations. The substrate stereochemical preferences of the *Serratia* enzyme provide an explanation for the relatively low efficiency of hydrolysis of the substrate **1a**, consisting of the stereorandom mixture of diastereomers. Efficiency of hydrolysis increases if the scissile R_P-bond is surrounded by phosphorothioate internucleotide bonds of the R_P-configuration. Since the sequence d[...$A_{PS}C_{PS}G_{PS}T$...], apparently involved in interaction of **1a** with the enzyme during cleavage of the d[$C_{PS}G$] bond, exists as a mixture of $2^3 = 8$ diastereomers, only 1/8 of **1a** has this motif of All-R_P configuration. Nonetheless, a fraction of **1a** with one or two S_P-phosphorothioates outside of the d[...$A_{PS}C_{PS}G_{PS}T$...] segment was also degraded by *Serratia* enzyme, but because of their low content, the corresponding products were not collected during HPLC analysis.

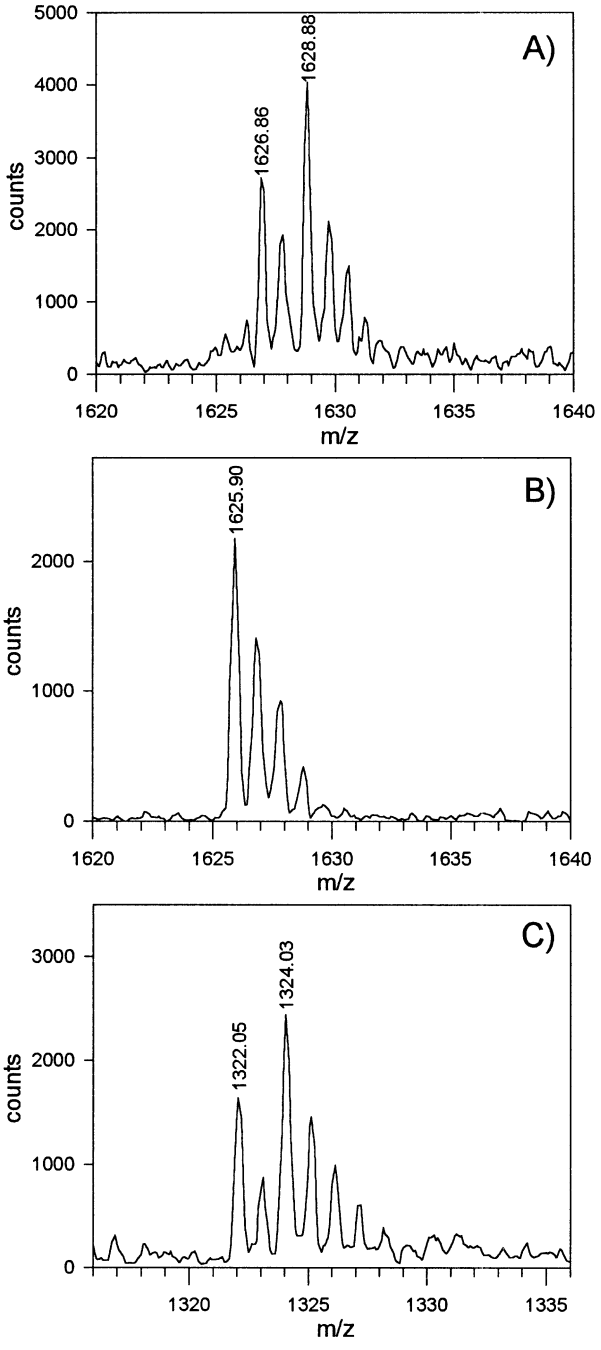


Figure 3. MALDI-TOF mass spectrometry analysis for oligomers **3a** (panel A), **6a** (panel B), and **7a** (panel C). Spectral parameters as described in Experimental.

Ligation of the 5′-*O*-phosphorothioylated pentamer

For further stereochemical analysis, the pentamer **3a** was ligated by T4 DNA ligase to the 3′-end of a 44-base long 'hairpin' oligonucleotide template 5′-d[CGCATCTCAAAAAAACAGAAGAGGGCCCTT-TTGGGCCCTCTTCT]-3′ (**4**), possessing a single-stranded 5′-end part complementary to **3a**.[16] The ligation process required special conditions (see Experimental), because this short oligomer contained a 5′-terminal phosphorothioyl moiety, instead of a phosphoryl one, and three internucleotide phosphorothioate linkages destabilizing a duplex to be formed with a template for efficient ligation.[21] Independent ligation experiments performed with 5′-*O*-phosphorylated stereoregular [All-Rp-PS]- and [All-Sp-PS]-d[A_4] proved the same activity of T4 DNA ligase, independently of absolute configuration of internucleotide phosphorothioates (data not shown). In the ligation reaction 80% of the template was elongated with the formation of the expected 49-base product **5a** (Scheme 1), which was proved by polyacrylamide gel electrophoresis and HPLC. The oligonucleotide **5a** was isolated from the ligation mixture by RP-HPLC and digested with nuclease P1 furnishing the pentamer d[$_{PO}T_{PS}G_{PS}T_{PS}T_{PS}T$] (**6a**) which without isolation was analyzed by MALDI-TOF mass spectrometry. The peak at *m*/*z* 1626, corresponding to unlabeled **6**, was not accompanied by a peak at *m*/*z* 1628 (Fig. 3B). This loss of ^{18}O-isotope indicates inversion of configuration of P-atom during the *Serratia*-catalyzed hydrolysis of **1a**.

After 20 h incubation in the presence of T4 DNA ligase, the unreacted pentamer d[$_{PS(18O)}G_{PS}T_{PS}T_{PS}T_{PO}T$], used in 3-fold molar excess for the ligation process, was recovered by RP-HPLC and treated with nuclease P1 to yield the tetramer d[$_{PS(18O)}G_{PS}T_{PS}T_{PS}T$] (**7a**). MALDI-TOF mass spectrometry analysis of **7a** confirmed the unchanged content of ^{18}O-oxygen atom (Fig. 3C), demonstrating that the observed loss of ^{18}O in **6a** resulted from the actual ligation process performed by T4 DNA ligase.

Isotopomeric [^{18}O]PS-oligonucleotide as the substrate for the *Serratia* nuclease

The results presented above indicate that *Serratia* endonuclease cleaves the R_P-internucleotide phosphorothioate bond by a one-step *in-line* mechanism with inversion of configuration at phosphorus. We decided to strenghten that conclusion in cross-check experiments by using the methodology developed recently in our laboratory.[22] Using the oxathiaphospholane monomers[22,23] the target oligonucleotide d[$A_{PO}A_{PS\text{-}Rp,Sp}A_{PS\text{-}Rp,Sp}A_{PS\text{-}Rp}C_{PS(18O)\text{-}Rp}G_{PS\text{-}Rp}T_{PS\text{-}Rp}T_{PS\text{-}Rp,Sp}T_{PO}T$] (**1b**) was synthesized containing four stereodefined internucleotide phosphorothioates of R_P-configuration. Their location along the oligonucleotide chain was based on the result of preliminary studies on the stereochemical preferences of the *Serratia* nuclease (vide supra). The scissile internucleotide bond of the R_P-configuration (between dC and dG units) was additionally labeled in the non-bridging position with the ^{18}O- oxygen (47 atom% ^{18}O) due to the use of isotopically labeled *N*-benzoylated cytidyl oxathiaphospholane monomer.[22] The oligomer **1b** consisted of the mixture of eight diastereomers due to the presence of three internucleotide linkages of undefined stereochemistry, marked as PS-R_P,S_P. These diastereomers were separated by means of RP-HPLC into three fractions. Their digestions with nP1 revealed that the fraction which eluted first had the highest content of internucleotide R_P-phosphorothioates, while the last peak eluted contained the oligomers with the maximal number of the Sp-phosphorothioates. These fractions were separately degraded in unlabeled water by the *Serratia* endonuclease, and because all diastereomers of oligomer **1b** had the d[...$A_{PS}C_{PS}G_{PS}T$...] motif of All-R_P configuration, the efficiency of hydrolysis was almost 100%. The ^{18}O-labeled oligomer d[$_{PS(18O)}$ $G_{PS\text{-}Rp}T_{PS\text{-}Rp}T_{PS\text{-}Rp}T_{PO}T$] (**3b**), possessing the 5′-end [^{18}O]phosphorothioate moiety of the absolute configuration opposite to that in **3a**, was isolated by RP-HPLC and converted into the corresponding d[$_{PO}T_{PS(18O)\text{-}Rp}G$ $_{PS\text{-}Rp}T$ $_{PS\text{-}Rp}T$ $_{PS\text{-}Rp}T$] (**6b**) in a way analogous to **3a**. MALDI-TOF mass spectra of 3**b** (Fig. 4A) and **6b** (Fig. 4B) showed identical pattern of peaks at *m*/*z* 1626 and 1628 corresponding to unlabeled and labeled oligomers. The retention of ^{18}O-oxygen in **6b** confirmed that the *Serratia* endonuclease cleaves the internucleotide phosphorothioate via *in-line* mechanism.

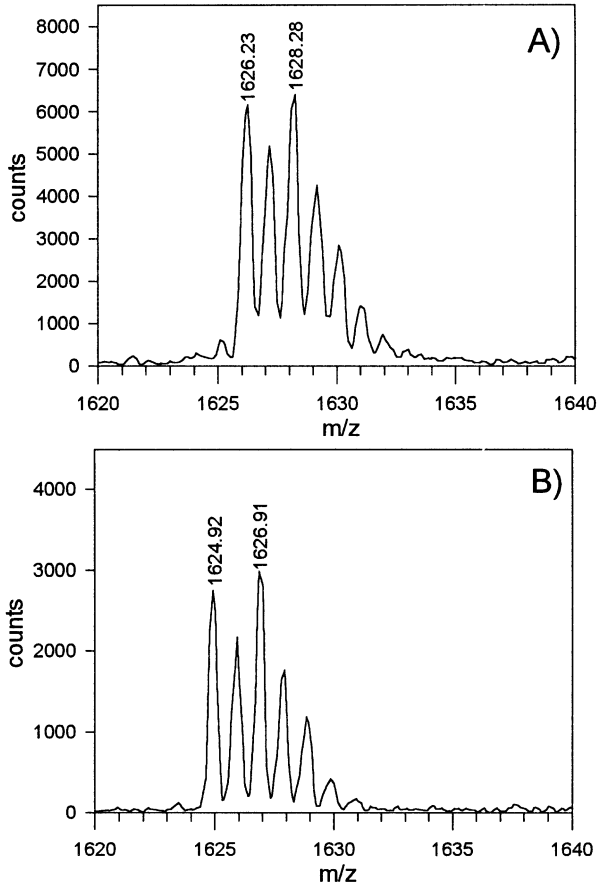


Figure 4. MALDI-TOF mass spectrometry analysis for oligomers **3b** (panel A) and **6b** (panel B). Spectral parameters as described in Experimental.

The results presented demonstrate that the T4 DNA ligase-based method for determination of the stereochemistry of enzymatic DNA cleavage can be applied not only to sequence-specific restriction endonucleases, but also to sequence nonspecific enzymes. However, in the latter case it is necessary to find an oligonucleotide sequence and/or reaction conditions in which the substrate is cleaved preferably at a single internucleotide phosphorothioate bond with sufficient efficacy. The use of oligonucleotides containing several internucleotide phosphorothioate linkages can be useful for construction of such substrates, although in this case the search for conditions suitable for efficient action of T4 DNA ligase, intended to join the resulting short phosphorothioate oligonucleotides with appropriate oligonucleotide template, may be quite a challenging task.

Conclusions

Presented in this work is evidence for the stereoinvertive mode of action of the *Serratia* enzyme to support the architecture of the active site as depicted in Figure 1A. Beyond that, our results emphasize the contribution of the phosphate group downstream to that one being cleaved, in the functioning of either the active site or binding domain of the enzyme, not detected during crystallographic analysis.[4,5] Friedhoff et al. observed that the rate of cleavage of pentaadenylates with a single phosphorothioate modification was influenced by their stereochemistry. The S_P-phosphorothioate modification located downstream to the scissile bond reduces the rate of cleavage, while the presence of R_P-phosphorothioate does not affect the rate of hydrolysis compared to unmodified oligomer.[3] Based on Friedhoff's data and our results concerning the stereochemistry of internucleotide phosphorothioate linkages in **3**, it can be hypothesized that the *pro-S*-oxygen of the 3′-neighbor phosphate acts as the acceptor of hydrogen atom of the water molecule activated by His-89. If replaced by sulfur, the location of water in the proximity of the attacked phosphorus may be distorted due to changed

steric requirements. Also the ability of the sulfur atom of a phosphorothioate moiety to serve as a hydrogen bond acceptor[24] is altered as compared to non-bridging oxygen atoms in a phosphate bond, which may affect the rate of hydrolysis. Such a hypothesis is in agreement with an involvement of the 3′-adjacent internucleotide bond and His-98 in activation of water molecule attacking phosphorus in the nucleolytic cleavage by I-*Ppo*I endonuclease, emphasized by Mannino et al.[25] Further studies verifying this hypothesis are in progress.

Experimental

Enzymes and chemicals

Endonuclease from *Serratia marcescens* was purchased from Sigma (St Louis, MO). T4 DNA ligase was obtained from Amersham (Bucks, UK). Nuclease P1 was obtained from Pharmacia LKB (Uppsala, Sweden). [^{18}O]water was purchased from ICON Isotopes (Summit, NJ).

Chemical synthesis of oligonucleotides

The synthesis of unmodified oligonucleotides and [Mix]-oligo(nucleoside phosphorothioate)s was performed on an ABI 394 DNA synthesizer (Applied Biosystems, Inc.; Foster City, CA) at a 1 μmol scale using a standard phosphoramidite or phosphoramidite/sulfurization protocol, respectively.

The synthesis of stereodefined [PS]-oligonucleotide **1b** was performed manually. The first nucleoside units were anchored to the solid support by a sarcosinyl linker. Appropriately protected deoxynucleosidyl monomers possessing 3′-*O*-(2-thio-'spiro'-4,4-pentamethylene-1,3,2-oxathiaphospholane) moiety were synthesized and separated chromatographically into pure diastereomers. Isotopically labeled cytidyl monomer, necessary for synthesis of stereodefined PS^{18}O internucleotide bond, was obtained using phosphitylating reagent 2-chloro-'spiro'-4,4-pentamethylene-1,3,2-[^{18}O]oxathiaphospholane (47 atom% ^{18}O) carrying an isotope label in endocyclic position.[22] The protocol for the synthesis has been published elsewhere.[23]

All synthesized oligomers were purified either by two-step RP-HPLC (DMT-on and DMT-off, an ODS Hypersil column) or by ion-exchange chromatography (a DNAPack PA 100 column, Dionex; Austin, TX) and their purity and identity was assessed by polyacrylamide gel electrophoresis and MALDI TOF mass spectrometry.

Enzymatic reactions

The *Serratia*-catalyzed cleavage of the decanucleotide **1** was carried out using 10 nmol of the substrate and 150 units of the enzyme in 100 μL of buffer containing 25 mM Tris–Cl (pH 8.5) and 5 mM $MgCl_2$. For the reactions performed with either random mixture of diastereomers (**1a**) or its stereodefined analogue **1b**, the same buffer but 1500 units of enzyme were employed. The reactions were carried in the presence of either [^{16}O] water or [^{18}O] water (60 atom% ^{18}O). The mixtures were incubated at 37 °C for 30 min. The extent of the cleavage reaction was 10% for the oligomer **1a** and 100% for **1b**, as assessed from HPLC analysis. The enzymatic reaction was stopped by heating at 95 °C for 5 min. Then, the reaction mixtures were analyzed by RP-HPLC (ODS Hypersil 5-μ column) and the pentamers **2** and **3** were isolated using the linear gradient 0–20% CH_3CN/0.1 M TEAB (triethylammonium bicarbonate), pH 7.4, 0.66%/min at a flow-rate of 1 mL/min. Under these conditions, the pentamers **2a** and **2b** were eluted at 23.5 min, while the pentamers **3a** and **3b** were eluted at 25.6 min. The collected material, after evaporation of eluting buffer, was used for further treatment with nuclease P1 or for the ligation with T4 DNA ligase.

Cleavage with nuclease P1 was carried out in 40 μL of buffer containing 100 mM Tris–Cl (pH 7.2) and 1 mM $ZnCl_2$ in the presence of 2 μg of the enzyme. The reaction mixtures were incubated at 20 °C for 16 h. After quenching the reactions by heating at 95 °C for 2 min, the samples were evaporated and, without any purification, analyzed by mass spectrometry.

Before ligation, the template **4** (350 pmol) was added to 30 μL of ligation buffer containing 66 mM Tris–Cl (pH 7.6), 6.6 mM $MgCl_2$, 10 mM DTT and 66 μM ATP, and this mixture was heated at 95 °C for 2 min. After slow cooling down to the room temperature, the oligomer **3** (1 nmol) and the T4 DNA ligase (30 units) were added. The mixtures were incubated at 6 °C for 20 h. Then, the ligated products **5** and non-ligated oligomers **3** were isolated by RP-HPLC using the linear gradient 0–30% CH_3CN/0.1 M TEAB, pH 7.4, 1.2%/min at a flow-rate of 1 mL/min. Under these conditions the ligation product **5** was eluted at 16.3 min, while the unligated substrate **3** was eluted at 17.8 min. The eluted oligonucleotides samples were evaporated and then treated with nuclease P1 as described above.

Mass spectrometry

Mass spectrometry analyses have been performed using a Voyager-Elite MALDI-TOF mass spectrometer (PerSeptive Biosystems Inc., Framingham, MA, USA) equipped with delayed extraction. Typical conditions included 20 kV acceleration voltage and nitrogen laser pulse (wavelength 337 nm). High resolution negative-ion spectra were recorded in reflector mode. Two-point external calibration has been made with dT_3 and dT_6 as standards. The matrix used consisted of 2,4,6-trihydroxyacetophenone (10 mg/mL in ethanol) and diammonium hydrogen citrate (50 mg/mL in acetonitrile/water 1:1) mixed in 8:1 ratio. Purified samples (0.005–0.01 OD/μL) were dissolved in acetonitrile/water (1:1), mixed with matrix in 1:1 ratio on the surface of a sample plate and left for crystallization at room temperature. The spectra have been obtained with mass spectrometer resolution about 3000 (defined as relation: mass/width at half height of the mass peak) allowing for observation of isotope peaks distribution.

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