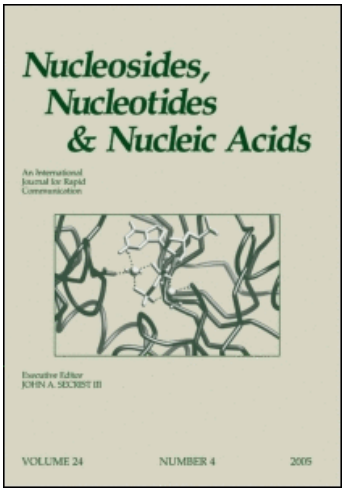


Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Application of Oligonucleoside Methylphosphonates in the Studies on Phosphodiester Hydrolysis by *Serratia* Endonuclease

Tushar Kumar Srivastava[a]; Peter Friedhoff[b]; Alfred Pingoud[b]; S. B. Katti[a]
[a] Division of Biopolymers, Central Drug Research Institute, Lucknow, India [b] Institut für Biochemie, Justus-Liebig-Universität, Giessen, Germany

To cite this Article Srivastava, Tushar Kumar , Friedhoff, Peter , Pingoud, Alfred and Katti, S. B.(1999) 'Application of Oligonucleoside Methylphosphonates in the Studies on Phosphodiester Hydrolysis by *Serratia* Endonuclease', Nucleosides, Nucleotides and Nucleic Acids, 18: 9, 1945 — 1960
To link to this Article: DOI: 10.1080/07328319908044856
URL: http://dx.doi.org/10.1080/07328319908044856

NUCLEOSIDES & NUCLEOTIDES, 18(9), 1945–1960 (1999)

APPLICATION OF OLIGONUCLEOSIDE METHYLPHOSPHONATES IN THE STUDIES ON PHOSPHODIESTER HYDROLYSIS BY *SERRATIA* ENDONUCLEASE#

Tushar Kumar Srivastava[1], Peter Friedhoff[2], Alfred Pingoud[2] and S.B. Katti[1*]

[1]Division of Biopolymers, Central Drug Research Institute, Lucknow, India.
[2]Institut für Biochemie, Justus-Liebig-Universität, D-35392 Giessen, Germany.

ABSTRACT : The endonuclease from *Serratia marcescens* is a non-specific enzyme that cleaves single and double stranded RNA and DNA. It accepts a phosphorylated pentanucleotide as a minimal substrate which is cleaved in the presence of Mg^{2+} at the second phosphodiester linkage. The present study is aimed at understanding the role of electrostatic and hydrogen bond interactions in phosphodiester hydrolysis. Towards this objective, six pentadeoxyadenylates with single stereoregular methylphosphonate substitution within this minimal substrate (2a-4b) were synthesized following a protocol described here. These modified oligonucleotides were used as substrates for the *Serratia* nuclease. The enzyme interaction studies revealed that the enzyme failed to hydrolyze any of the methylphosphonate analogues suggesting the importance of negative charge and/or hydrogen bond acceptors in binding and cleavage of its substrate. Based on these results and available site-directed mutagenesis as well as structural data, a model for nucleic acid binding by *Serratia* nuclease is proposed.

INTRODUCTION

There has been a renewed interest in the chemical synthesis of oligonucleoside methylphosphonate (P-Me) analogues because of their application as antisense therapeutic agents[1,2] and as probes in DNA-protein interaction studies[3,4]. There are two major problems associated with the synthesis of P-Me oligos and their chimeric analogues. The published procedures lead to diastereomeric mixture of Rp and Sp isomers[5]. So far, the

CDRI Communication No. : 5777

only stereoregular P-Me prepared, longer than dimers, are the all Rp and all Sp tetrathymidylates[6]. Secondly, the presently employed deblocking protocols are either tedious or may lead to hydrolysis of the P-Me linkage[7]. This report describes improved synthesis of pentamers (2a/b-4a/b) containing methylphosphonate linkages at preselected positions, their characterization by negative ion fast atom bombardment mass spectrometry and enzymatic studies with the extracellular *Serratia marcescens* nuclease. This enzyme is a non-specific endonuclease which cleaves single and double stranded RNA and DNA with very high catalytic activity and produces 5'-phosphorylated di-, tri- and tetranucleotides[8]. Its minimal deoxynucleotide substrate is d(pNpNpNpNpN) which is cleaved preferentially between the second and third nucleotide residue[9]. In order to find out whether a negative charge and/or a hydrogen bond acceptor at the phosphate residue attacked by the enzyme or at the adjacent phosphate residues are required by *Serratia* nuclease, we have synthesized in addition to the unmodified pentamer, six pentadeoxyadenylates carrying a methylphosphonate substitution in Rp- and Sp configuration, respectively, at the second, third or fourth phosphate residue (TABLE I). These pentamers were phosphorylated at their 5'-end and then analyzed for their suitability as substrates for *Serratia* nuclease. Our data show that -as expected- a pentamer carrying a methylphosphonate substitution at the position of preferential attack is not cleaved by the enzyme. However, also the other modified pentamers are not accepted as substrates, demonstrating the importance of electrostatic and/or hydrogen bond interactions for the cleavage of nucleic acids by this non-specific endonuclease. This result supports a model for the interaction between *Serratia* nuclease derived from a comparison of the crystal structure of this enzyme[10] with the co-crystal structure of the homing endonuclease I-*Ppo*I and its DNA substrate[11]

RESULTS AND DISCUSSION

Synthesis of methylphosphonate substituted oligomers

The synthesis of oligonucleotides (2a/b-4a/b) reported in the present study has been executed in two steps. The first step involves synthesis, resolution and phosphitylation at the 3'-OH of DMT-dA^{Bz}p(Me)dA^{Bz}-OH (7a/b-9a/b). Oligonucleotide synthesis was accomplished in the second step followed by 5'-phosphorylation. The

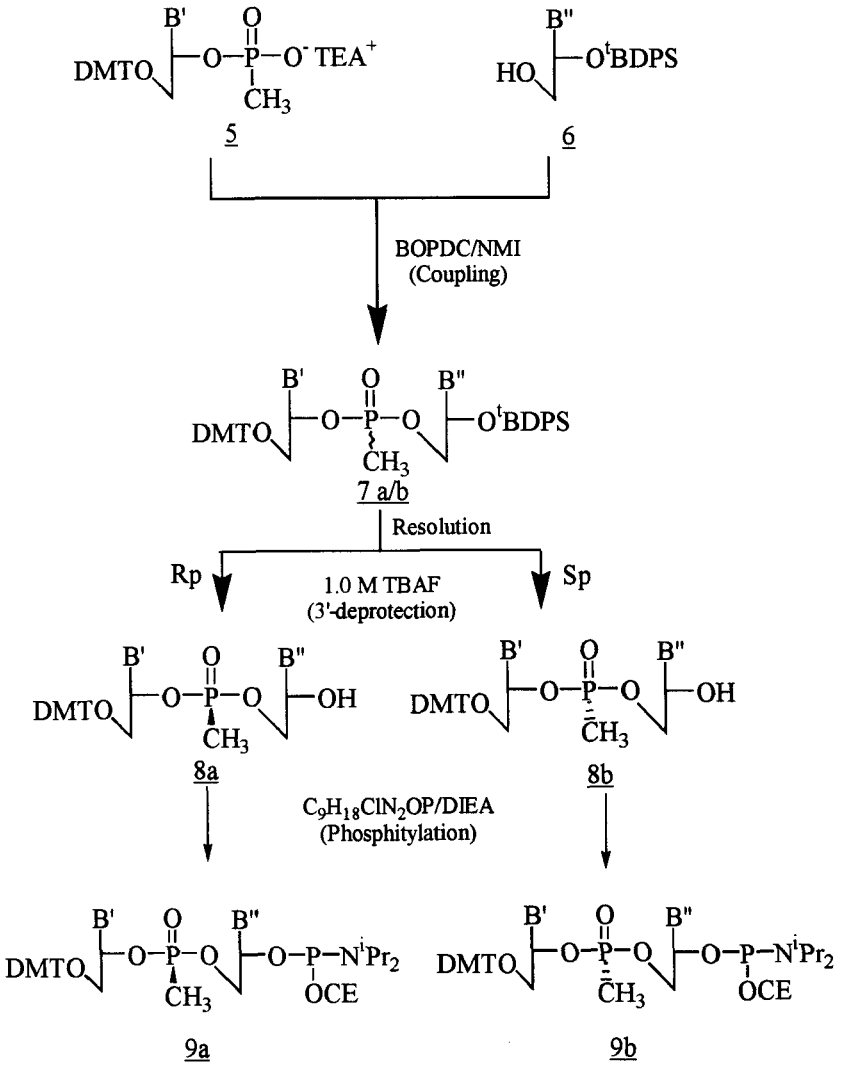
TABLE I : Pentadeoxyadenylates synthesized as substrates for *Serratia* nuclease

Oligo	Isomer	Compound No.
5′-d[ApApApApA]-3′	-------	1 [1′]
5′-d[Ap(Me)ApApApA]-3′	Rp isomer	2a [2′a]
5′-d[Ap(Me)ApApApA]-3′	Sp isomer	2b [2′b]
5′-d[ApAp(Me)ApApA]-3′	Rp isomer	3a [3′a]
5′-d[ApAp(Me)ApApA]-3′	Sp isomer	3b [3′b]
5′-d[ApApAp(Me)ApA]-3′	Rp isomer	4a [4′a]
5′-d[ApApAp(Me)ApA]-3′	Sp isomer	4b [4′b]

The numbers in parentheses indicate the corresponding 5′-phosphorylated oligomer.

synthetic route for the preparation of dimeric synthons (9a/b) is outlined in SCHEME I. The suitably protected monomer was phosphonylated following a published procedure[5]. The methylphosphonate salt was coupled with the hydroxyl component in the presence of bis(2-oxo-3-oxazolidinyl)phosphinic chloride (BOPDC), as a coupling reagent[12]. At this stage, the diastereomers (Rp and Sp) were resolved by flash silica gel column chromatography. Subsequently the 3′-silyl protecting group was removed by tetrabutylammoniumfluoride (TBAF). The resulting dimer (8a/b) was phosphitylated with 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite to give the 3′-phosphoramidite dinucleoside methylphosphonate (9a/b). Prior to phosphitylation, the isomeric purity of the dinucleoside methylphosphonate was confirmed by ^{31}P NMR spectroscopy. Earlier, it has been shown that the phosphorous chemical shift in the case of Rp methylphosphonate appears at a slightly higher field as compared to the corresponding Sp isomer[13]. Accordingly, the isomer (8a) having higher mobility on the silica gel TLC exhibiting a singlet at δ 32.65 ppm was assigned to the Rp isomer while the slower moving isomer (8b) showing singlet at δ 33.01 ppm was assigned to the Sp isomer.

Oligonucleotides with all phosphate linkages (1) and P-Me oligonucleotides (2a/b-4a/b) were synthesized on an automated DNA synthesizer that employed conventional β-



SCHEME I : Synthesis of dimer

cyanoethyl chemistry using the DMT-off protocol[14]. The manufacturer's recommended protocol and standard chemistry cycle for the β-cyanoethyl reagents were used except for the inclusion of methylphosphonate linkage. This was incorporated as a dimer thus leading to the synthesis of chimeric oligonucleotides with stereochemically defined methylphosphonate linkages at preselected positions. Following the completion of the synthesis, the oligo was deblocked from the solid support by treating it with 30% aq.

ammonia at 18°C for 36 h. Each of the pentamer thus obtained was purified by reversed phase HPLC.

Stability of the methylphosphonate linkage

As mentioned earlier, lability of methylphosphonate linkage to ammonia poses a serious problem in the deblocking and purification steps. Although some modified deblocking protocols have been reported in the literature[7,15] but due to some practical problems associated with these procedures we have investigated this problem using dithymidyl methylphosphonate as a model. The dimer was kept in 30% aq. ammonia at 18° and 24°C and the aliquots were taken at different time intervals between 2-48 h. Further, the same reaction mixture was heated at 65°C for 6 h, as this is the condition routinely used for the deblocking of phosphate and base protecting groups in the oligonucleotide synthesis. Analytes were subjected to RP-18 HPLC and the results indicated that at 18°C, even after 48 h, methylphosphonate is hydrolyzed only to the extent of nearly 5% while at 24°C extent of hydrolysis is more. Upon heating the same reaction mixture at 65°C, there is a substantial cleavage of the P-Me linkage. The hydrolytic products correspond to the thymidine-3′/5′-methylphosphonate and thymidine, as seen in co-HPLC (data not shown). Further, this reaction condition was extended to 5′-OH-C^{Bz}p(Me)G^{Ib}-3′-OH dimer in order to see whether base protecting groups can be completely deblocked. When the reaction product was subjected to negative ion FAB MS, a molecular ion peak at m/z 553 corresponding to the dimer 5′-OH-Cp(Me)G-3′-OH appeared while there was no peak corresponding to the starting dimer or the intermediates having either of the base protecting groups in the aliquot taken out after 36 h (data not shown). Thus we have demonstrated that the deblocking protocol (30% aq. ammonia at 18°C for 36 h) reported here can be safely used for hybrid oligos containing phosphate and methylphosphonate linkages.

Characterization of the methylphosphonate substituted oligomers

The presence of the methylphosphonate linkage was confirmed by heating the pentamers (2a/b-4a/b, 0.1 A_{260} unit each) with 30% aq. ammonia at 65°C for 6 h This treatment is known to selectively hydrolyze the methylphosphonate linkage without effecting the phosphodiester linkage[16] Aliquots from the reaction mixture of each

TABLE II : HPLC data of pentamers (before and after ammonia hydrolysis)

Oligo	Rt (min)	Rt (min) of hydrolytic fragments		
1	15.72	****	****	****
2a	17.50	13.10	14.40	15.90
2b	18.10	14.10	15.34	16.44
3a	16.90	14.36	14.90	15.40
3b	17.76	13.92	14.52	15.20
4a	16.82	13.79	14.70	15.30
4b	17.50	13.60	15.00	15.60

pentamer were subjected to reversed phase HPLC wherein, peaks corresponding to the hydrolytic products due to cleavage at the methylphosphonate linkage along with a residual peak for the pentamer (TABLE II) appeared. Under similar conditions, pentamer-1 with all phosphodiester linkages, was not hydrolyzed.

Further verification of the correctness of the chemical structure was corroborated by the negative ion fast atom bombardment mass spectroscopy. Earlier we have shown that this technique could be used for sequencing of oligonucleotides containing nonionic phosphate groups[17]. As expected, pentamer-1 exhibited a molecular ion peak at 2 mass units higher than the pentamers 2a/b-4a/b. Ions having appropriate m/z values were observed for the molecular ions and for fragments characteristic of the phosphodiester cleavage[18] (TABLE III). A schematic representation of the probable fragmentation for only a representative pentamer (3a/b) is shown in FIG I. Since all the pentamers have homopolymeric sequence, it is difficult to assign fragment ions coming from either 5′- or 3′-side cleavage. Nevertheless, major fragments could be assigned from the spectra thus confirming the sequence of the oligomers reported here.

Enzyme studies

Recently we have shown that the substitution of phosphate by methylphosphonate residues within the recognition sequence -GATATC- of the restriction endonuclease

TABLE III : FAB MS data of pentamers

Oligo	$[M-H]^-$	Fragment ions			
1	1502	1269	956	643	***
2a/b	1500	1267	***	643	641
3a/b	1500	1267	954	643	641
4a/b	1500	1267	954	643	641

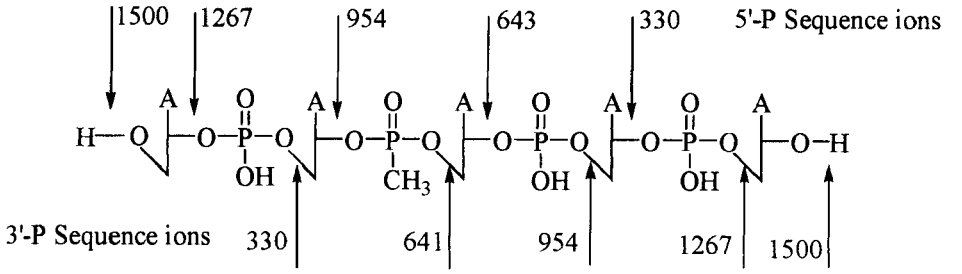


FIG I : Shorthand structure of 3a/b showing its fragmentation pattern

*Eco*RV leads to substrate analogues that are not cleaved by this highly specific enzyme with one exception : the substitution at the last position in the Sp-configuration is cleaved by the enzyme but not the Rp-diastereomer[4]. We were interested in extending our studies to *Serratia marcescens* nuclease. This enzyme in contrast to *Eco*RV is a very non-specific nuclease that accept single and double stranded RNA and DNA with little sequence preferences[19] as substrates and cleaves them in the presence of Mg ions. The end products of the cleavage of poly- and oligodeoxyadenylic acids by the *Serratia* nuclease are mainly di- and trinucleotides, to a smaller extent also tetranucleotides[9]. The phosphorylated pentadeoxyadenylic acid, d(pApApApApA), is cleaved almost exclusively at the second phosphodiester bond, the corresponding tetradeoxyadenylic acid, d(pApApApA), also at this position but only at very high enzyme concentrations and with a very slow rate. Thus an oligonucleotide with five phosphate residues seems to be the minimal substrate for the

TABLE IV : Cleavage of pentadeoxyadenylic acids by the *Serratia* nuclease

Oligonucleotide	Rate of cleavage[#] [$\mu Mol^{-1}s^{-1}$]
1′	1.5
2′a	0.0
2′b	0.0
3′a	0.0
3′b	0.0
4′a	0.0
4′b	0.0

[#]determined at an oligonucleotide concentration of 0.5 μM

non-specific endonuclease from *Serratia marcescens*, as proposed by L'vova *et al*[20]. In line with these results is the finding reported here that the substitution at any position of the phosphate by a methylphosphonate prevents cleavage by the *Serratia* nuclease (TABLE IV).

This result differs considerably from the results of cleavage experiments with pentadeoxyadenylic acids in which individual phosphates were substituted by phosphorothioates; all of these modified oligonucleotides were cleaved, albeit at different positions and with different rates[9]. This allows to conclude that a negative charge and/or hydrogen bond acceptor are required at the scissile phosphate as well as the flanking phosphates. Presumably, in each position the non-bridging oxygen atoms, one of which carries a negative charge, are required for the binding and the correct positioning of the substrate. From the crystal structure[10] it is clear that the nucleic acid binding site of the *Serratia* nuclease is defined by basic amino acid residues, two of which are located very close to the active site, Arg 87 and Arg 131, and one presumably being part of the active site, Arg 57[21,22]. If these basic amino acid residues of the *Serratia* nuclease are replaced by alanine, as done in site directed mutagenesis[21,23], or the negatively charged phosphate residues in the substrate substituted by the uncharged methylphosphonate residues, as done here, a productive interaction between enzyme and substrate is impaired or prevented.

A model for the interaction between *Serratia* nuclease and nucleic acids

So far a co-crystal structure of *Serratia* nuclease and its nucleic acid substrate has not been determined. This has impeded the elucidation of the precise mechanism of action

for this enzyme, in spite of the fact that the amino acid residues that are involved in the catalysis are known from a detailed mutational analysis[21,23]. Recently, however, the co-crystal structure of the homing endonuclease I-*Ppo*I and its double stranded DNA substrate has been determined[11]. A comparison of the structures of the *Serratia* nuclease and I-*Ppo*I showed that these enzyme share a common catalytic center (Friedhoff *et al* communicated), a finding supported by experiments which demonstrated that I-*Ppo*I like *Serratia* nuclease cleaves the artificial substrate deoxythymidine-3′,5′-bis-(*p*-nitrophenyl phosphate) at identical position and with similar rates (Friedhoff *et al* communicated). Intriguingly, in the superimposed structures of I-*Ppo*I and *Serratia* nuclease, the DNA bound to I-*Ppo*I is not clashing into *Serratia* nuclease, but rather fits smoothly into the active site of this enzyme. FIG II shows the active site of this enzyme with the essential His 89, Asn 119 and Glu 127 residues, as well as a trinucleotide piece of the DNA from the I-*Ppo*I-DNA complex, as it is obtained by superposition of the active site residues of the two nucleases. FIG II now demonstrates that three Arg residues, Arg 57, Arg 87 and Arg 131 of *Serratia* nuclease are in close proximity to the phosphate attacked (Arg 57) as well as to the 5′-(Arg 131) and 3′-adjacent phosphate (Arg 87), respectively (TABLE V). These three arginine residues were shown to be very important for the nucleolytic activity of the *Serratia* nuclease : Arg 57, 87 and 131, when replaced by alanine yield variants with only 0.35, 0.18 and 3% residual activity compared to the wild type enzyme[21,23]. In very good agreement with the model presented in FIG II is the experimental finding reported in this paper that in the minimal substrate neither the phosphate attacked nor the 5′- and 3′-adjacent phosphate can be substituted by a methylphosphonate without loss of susceptibility for cleavage by *Serratia* nuclease. Obviously, the negative charge and/or hydrogen bonding capacity of the two non-bridging oxygen atoms at these three phosphate residues are required for the binding and correct positioning of the substrate to make it susceptible to hydrolytic attack by *Serratia* nuclease. Thus, the result that a phosphorylated pentadeoxyadenylates substituted at three different positions by methylphosphonate are not accepted as substrates by *Serratia* nuclease, which cannot be interpreted when considered alone, is easily rationalized in conjunction with site-directed mutagenesis data and structural comparisons, and supports a model for the binding of nucleic acid substrates to *Serratia* nuclease.

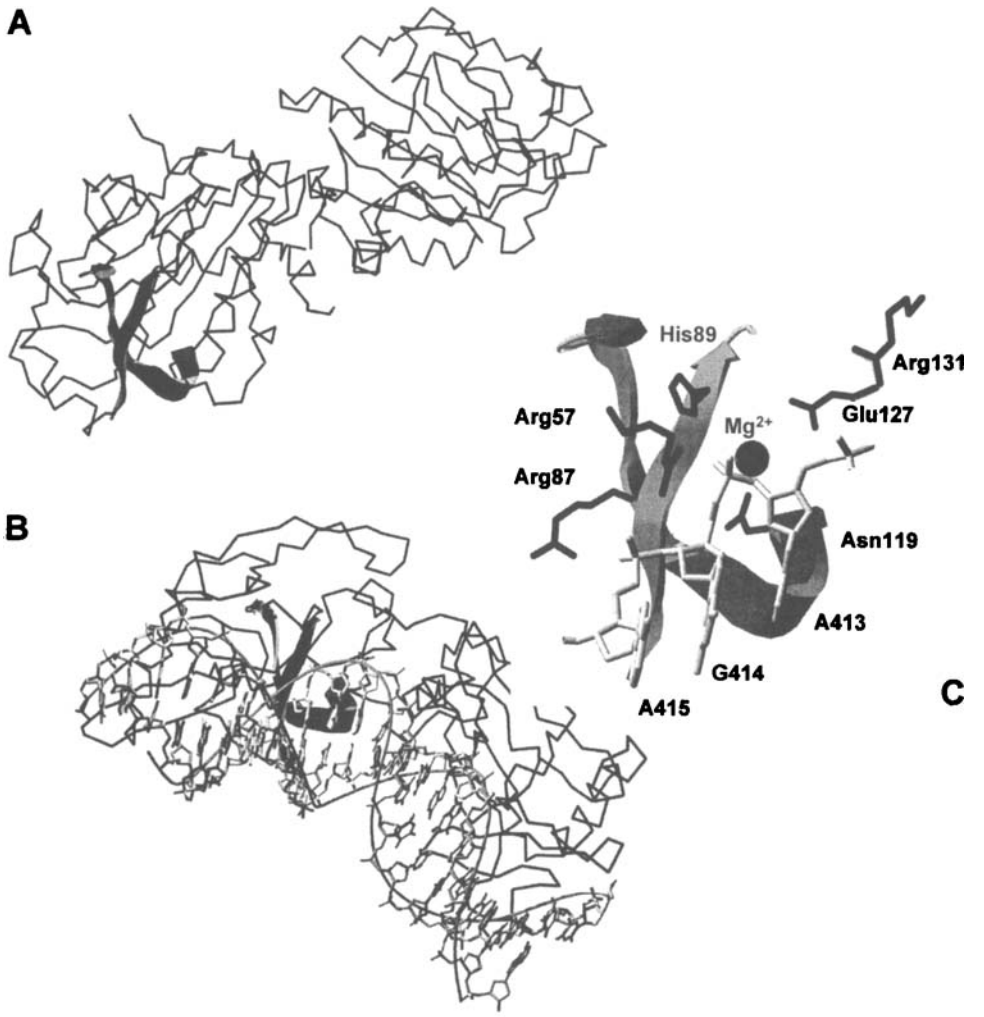


Fig II: A model for the *Serratia* nuclease DNA binding site based on the superposition of residues K84-P92 and N106-N119 of *Serratia* nuclease (PDB code: 1smn) with T93-H101 and H106-N119 of I-*Ppo*I (PDB code : 1a74)

Dimer structures of (A) *Serratia* nuclease and (B) the I-*Ppo*I×DNA complex; the residues that can be readily superimposed in both enzymes are rendered as ribbons in one subunit only. (C) Blow up of the active site of *Serratia* nuclease showing the conserved arginine residues 57, 87 and 131, as well as the catalytically important residues His89, Asn119 and Glu127. The essential Mg^{2+} ion cofactor (from the unpublished structure of *Serratia* nuclease with Mg^{2+} but without substrate) is shown as a black sphere. The residues (A413 G414 A415) of the substrate DNA of I-*Ppo*I are also shown. Note the good fit of the conserved arginine residue with respect to the phosphate backbone.

TABLE V : Distances of amino acid residues of *Serratia* nuclease to DNA substrate of I-*Ppo*I in the superimposed structures (Å)[#]

Atom 1			Atom 2			Distance
Arg	57	NE	GUA	414	O1P	3.3
Arg	57	NE	GUA	414	O2P	5.3
Arg	57	NH1	GUA	414	O1P	4.9
Arg	57	NH1	GUA	414	O2P	7.3
Arg	57	NH2	GUA	414	O1P	3.0
Arg	57	NH2	GUA	414	O2P	5.6
Arg	87	NE	ADE	415	O1P	4.7
Arg	87	NE	ADE	415	O2P	4.3
Arg	87	NH1	ADE	415	O1P	3.6
Arg	87	NH1	ADE	415	O2P	2.8
Arg	87	NH2	ADE	415	O1P	5.7
Arg	87	NH2	ADE	415	O2P	5.0
Arg	131	NE	ADE	413	O1P	5.8
Arg	131	NE	ADE	413	O2P	5.7
Arg	131	NH1	ADE	413	O1P	5.2
Arg	131	NH1	ADE	413	O2P	5.3
Arg	131	NH2	ADE	413	O1P	3.8
Arg	131	NH2	ADE	413	O2P	3.4
His	89	ND1	GUA	414	P	4.1
Mg	21		GUA	414	O2P	4.3
Mg	21		GUA	414	O1P	2.7
Mg	21		GUA	414	O3	2.7

[#]Notations of atoms are taken from the *Serratia* nuclease or I-*Ppo*I-DNA complex structure, respectively. According to the model in FIG II, residue GUA 414 contains the scissile phosphate, residues ADE 415 and ADE 413 contain the 3′- and 5′-adjacent phosphate, respectively. NE, NH1 and NH2 denote the nitrogen atoms of the guanidinium moiety, ND1 one of the imidazole nitrogens, D1P, D2P and D3 the pro-S-, pro-R-oxygen and one of the non-bridging oxygens.

CONCLUSION

It is evident from the foregoing discussion that we have developed a simple and efficient method for the synthesis of chimeric oligonucleotides containing stereochemically defined methylphosphonate linkages. These oligomers are amicable to negative ion FAB MS sequencing like the all phosphate oligomers. Cleavage experiments with *Serratia* nuclease and methylphosphonate substituted oligonucleotides demonstrated that the negative charges and/or hydrogen bond acceptors on the phosphate to be attacked as well as on the neighboring phosphates are required for binding and correct orientation of the substrate in the active site of this enzyme. These results together with the results of structural and mutational studies on *Serratia* nuclease allow now to put forward a model for the interaction of *Serratia* nuclease with its nucleic acid substrate.

EXPERIMENTAL

All solvents were freshly distilled and dried before use. Nucleosides were purchased from Cruachem, Scotland. *Serratia* nuclease was cloned and expressed in *Escherichia coli*[24]. TLC was carried out on Merck DC-Alufolien Kieselgel 60 F_{254}, 0.2 mm precoated plates obtained from E. Merck, Germany, reversed phase TLC was performed on RP-18 plates (solvent-30% acetone in water) obtained from Macherey-Nagel, Germany and DEAE cellulose TLC plates were obtained from Sigma Chemical Company. HPLC was carried out on an Applied Biosystems HPLC instrument using Merck 50983 Lichrospher 100 RP-18, 0.5 μM, 250 X 4 mm column using a linear gradient of acetonitrile in 0.1M triethylammonium acetate buffer (pH 7.2). ^{1}H NMR spectra and ^{31}P NMR spectra were recorded on a Bruker WM-400 (operating frequency 400 MHz) or Bruker AVANCE DRX 300 (operating frequency 300 MHz for ^{1}H and 121.5 MHz for ^{31}P) spectrometer using 1% H_3PO_4 in D_2O as an external standard for ^{31}P NMR spectra. FAB mass spectra were obtained from a Jeol SX-102/DA 6000 double focusing mass spectrometer. All UV measurements were carried out on a Perkin Elmer (Lambda 15) UV/VIS spectrophotometer. Solid phase synthesis was performed on a Pharmacia Gene Assembler.

Synthesis of DMT-dA^{Bz}p(Me)dA^{Bz}-OH (8a/b)

Triethylammonium salt of 5′-*O*-(4,4′-dimethoxytrityl)-N^6-benzoyl-2′-deoxyadenosine methylphosphonate[5] (5:1.1 mmol, 919 mg), N^6-benzoyl-3′-*O*-

tert.butyldiphenylsilyl-2′-deoxyadenosine[25] (6:1.0 mmol, 593 mg) and *N*-methylimidazole (3.3 mmol, 260 µl) were azeotroped with anhydrous pyridine. The residue was dissolved in anhydrous pyridine (15 ml). To this was added BOPDC (3.3 mmol, 840 mg) and the reaction mixture stirred at room temperature for 15 minutes. After the completion of the reaction, the reaction mixture was concentrated under reduced pressure. The residue was dissolved in dichloromethane and washed with aq. sodium bicarbonate, water, brine, dried over anhydrous sodium sulphate and concentrated under reduced pressure. The crude diastereomers were resolved by flash silica gel column chromatography using a linear gradient of methanol (0-8%) in benzene. Appropriate fractions containing only one diastereomer were pooled and concentrated. The product was precipitated from chloroform and petroleum ether. Subsequently, the individual diastereomers (0.2 mmol, 262 mg) were stirred with 1 M solution of TBAF in THF (0.3 mmol, 300 µl) for 30 min at 15°C. After completion of the reaction, the reaction mixture was concentrated under reduced pressure and diluted with dichloromethane. The dichloromethane solution was washed with water, dried over anhydrous sodium sulphate and concentrated under reduced pressure to a gummy residue. The gummy residue was purified by flash silica gel column chromatography using a linear gradient of 0-10% methanol in chloroform. Fully protected dimers (7a/b) showed a prominent molecular ion $[M+H]^+$ peak at 1311 whereas the dimers with free 3′-OH (8a/b), showed a molecular ion $[M-H]^-$ peak at 1071, in the negative ion FAB MS. A singlet at δ 31.98 ppm for Rp isomer (7a) and a singlet at δ 32.74 ppm for Sp isomer (7b) was observed in ^{31}P NMR of the fully protected dimers whereas a singlet at δ 32.65 ppm for Rp isomer (8a) and a singlet at δ 33.01 ppm for Sp isomer (8b) was observed in case of dimer with free 3′-OH terminal.

^1H NMR (9a) ($CDCl_3$) δ ppm : 1.55 (d, 3H, P-CH_3, J= 17 Hz); 3.76 (s, 6H, OCH_3, DMT); 6.45 (t, 1H, H-1′, adenosine); 6.55 (t, 1H, H-1′, adenosine); 8.13 (s, 1H, H-2, adenosine); 8.21 (s, 1H, H-2, adenosine); 8.68 (s, 1H, H-8, adenosine); 8.73 (s, 1H, H-8, adenosine).

Phosphitylation of the dimers

Predried 8a or 8b (0.1 mmol, 107 mg) was dissolved in dry dichloromethane (1 ml). To this was added dry *N,N*-diisopropylethylamine (0.4 mmol, 69.7 µl) followed by 2-cyanoethyl-*N,N*,-diisopropylchlorophosphoramidite (0.2 mmol, 44.6 µl). The reaction, on completion, was quenched with absolute methanol (0.5 ml) and then diluted with 10%

(v/v) triethylamine in ethyl acetate (10 ml). The organic layer was washed with chilled 5% aq. bicarbonate, water and brine, dried over sodium sulphate and concentrated to foam. The foam was dissolved in dry toluene and precipitated in cold, dry hexane with stirring. ^{31}P NMR ($CDCl_3$) δ ppm : Rp (9a) : 32.0 (s, methyl phosphonate); 149.5 and 149.6 (phosphoramidite)

Sp (9b) : 32.3 (s, methyl phosphonate); 149.6 and 149.7 (phosphoramidite)

Synthesis, deblocking and purification of the pentamers

Synthesis of the pentamers was carried out on a Pharmacia Gene Assembler using the standard protocol for phosphoramidite chemistry of oligonucleotide synthesis. All syntheses were performed on 0.2 μmol scale and on lcaa-CPG support. Coupling of the dimer unit was done twice to achieve maximum coupling efficiency. Deblocking of fully protected CPG bound pentamers was performed by treating them separately with 30% aq. ammonia at 18°C for 36 h. Completely deblocked pentamers were purified to homogeneity by reversed phase HPLC using a linear gradient of 0-30% acetonitrile in 0.1M triethylammonium acetate buffer (pH 7.2). 5-6 A_{260} units of each pentamer were obtained after purification.

5'-OH Phosphorylation

Oligodeoxynucleotides were labeled using [γ-^{32}P]ATP and T4-polynucleotide kinase. ADP and unreacted ATP were removed after the reaction by gel filtration using NAP-10 columns (Pharmacia) to obtain 1'-4'a/b.

Enzyme studies

Cleavage reactions were performed in 50 mM Tris/HCl (pH 8.0), 5 mM $MgCl_2$ at 25°C. The concentrations of oligodeoxynucleotides (1'-4'a/b) and the *Serratia* nuclease were 0.5 and 0.1 μM, respectively. Reactions were stopped by pipetting 1 μl aliquots onto a DEAE-cellulose thin-layer plate which was subsequently subjected to homochromatography. The thin-layer chromatograms were analyzed using an instant imager (Canberra-Packard).

ACKNOWLEDGEMENT

We thank Dr. K.L. Krause and Dr. B.L. Stoddard for supplying coordinates prior to publication. Grateful acknowledgement is made to RSIC and National Parasite Bank,

CDRI for providing the spectral data and DNA synthesizer facility. One of us (TKS) thanks CSIR for the financial assistance as SRF. Work in AP's laboratory has been supported by a grant from the Deutsche Forschungsgemeinschaft (Pi 122/9-2) and the Fonds der Chemischen Industrie. AP would like to acknowledge the receipt of a travel grant within the DAAD-CSIR exchange program.

REFERENCES

1. Miller, P.S., *Biotechnology* **1991** ***9***, 358-362.
2. Agarwal, S. and Iyer, R.P., *Curr. Op. Biotech.* **1995** ***6***, 12-27.
3. Noble, S.A., Fisher, E.F. and Caruthers, M.H., *Nucleic Acids Res.* **1984** ***12***, 3387-3404.
4. Lanio, T., Selent, U., Wenz, C., Wende, W., Schulz, A., Adiraj, M., Katti, S.B., and Pingoud, A., *Protein Engineering* **1996** ***9***, 1005-1010.
5. Agarwal, K.L. and Riftina, F., *Nucleic Acids Res.* **1979** ***6***, 3009-3024.
6. Lesnikowski, Z.J., Wolkami, P.J. and Stec, W.J., *Nucleic Acids Res.* **1988** ***16***, 11675-11689.
7. Hogrefe, R.I., Vaghefi, M.M., Reynolds, M.A., Young, K.M. and Arnold, L.J. Jr., *Nucleic Acids Res.* **1993** ***21***, 2031-2038.
8. Benedik, M.J.; Strych, U. *FEMS Microbiology Letters* **1998** ***165***, 1-13.
9. Friedhoff, P.; Meiss, G.; Kolmes, B.; Piper, U.; Gimadutdinow, O.; Urbanke, C.; Pingoud, A. *Eur. J. Biochem.* **1996a** ***241***, 572.
10. Miller, M.D.; Tanner, J.; Alpaugh, M.; Benedik, M.J.; Krause, K.L. *Nat. Struct. Biol.* **1994** ***1***, 461.
11. Flick, K.E.; Jurica, M.S.; Monnat, R.T., Jr.; Stoddard, B.L. *Nature* **1998** ***394***, 96-101.
12. Katti, S.B. and Agarwal, K.L., *Tetrahedron Lett.* **1985** ***26***, 2547-2550.
13. Loschner, T. and Engels, J.W., *Nucleic Acids Res.* **1990** ***18***, 5083-5088.
14. Gait, M.J., *Oligonucleotide Synthesis - A Practical Approach*, pp 35-81, IRL Press Limited, Oxford 19.
15. Habus, I.; Devlin, T.; Iyer, R.P.; Agrawal, S. *Bioorg. Med. Chem. Lett.* **1996** ***6***, 1393.
16. Miller, P.S., Agris, C.H., Murakami, A., Reddy, P.M., Spitz, S.A. and Ts'o, P.O., *Nucleic Acids Res.* **1983** ***11***, 6225-6242.

17. Madhusudanan, K.P., Katti, S.B. and Hashmi, S.A.N., *Org. Mass Spectrometry* **1993** ***28***, 970-976.

18. Grotjahn, L., Frank, R. and Blöcker, H., *Nucleic Acids Res.* **1982** ***10***, 4671-4678.

19. Meiss, G.; Friedhoff, P.; Hahn, M.; Gimadutdinow, O.; Pingoud, A. *Biochemistry* **1995** ***34***, 11979-11988.

20. L'vova, T.H.; Arrosimova-amel-ianchik, N.M.; Tatarskaia, R.I. *Biokhimiia* **1976** ***41***, 2077.

21. Friedhoff, P.; Kolmes, B.; Gimadutdinow, O.; Wende, W.; Krause, K.L.; Pingoud, A. *Nucleic Acids Res.* **1996b** ***24***, 2632.

22. Kolmes, B.; Franke, I.; Friedhoff, P.; Pingoud, A. *FEBS Lett.* **1996** ***397***, 343.

23. Friedhoff, P.; Gimadutdinow, O.; Pingoud, A. *Nucleic Acids Res.* **1994b** ***22***, 3280-3287.

24. Friedhoff, P.; Gimadutdinow, O.; Rüter, T.; Wende, W.; Urbanke, C.; Thole, H.; Pingoud, A. *Protein Expression Purif.* **1994** ***5***, 37.

25. Hashmi, S.A.N., *Ph.D. Thesis* **1994**, Lucknow University, Lucknow, India.

Received 12/1/98
Accepted 4/7/99