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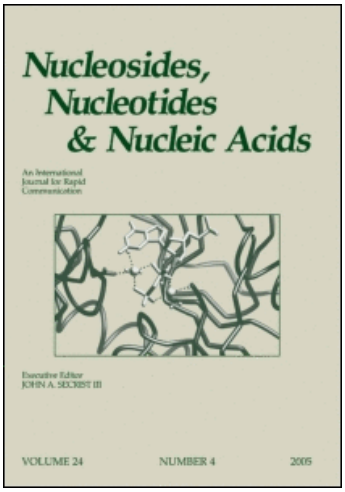

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Structural Investigation of Model Deadenylated Oligonucleotide Phosphorothioate Analog of Isis 2105

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STRUCTURAL INVESTIGATION OF MODEL DEADENYLATED OLIGONUCLEOTIDE PHOSPHOROTHIOATE ANALOG OF ISIS 2105

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Abstract : Structural characteristics of deadenylated oligonucleotide of ISIS 2105 is provided by the chemical synthesis of a model deadenylated analog. The results of molecular mechanics calculations incorporating the abasic site in the duplex are presented.

INTRODUCTION

Our understanding of the nature of nucleic acid-nucleic acid and nucleic acid-protein interactions involved in DNA transcription and RNA translation events has led to the emergence of antisense oligonucleotide technology.[1-25] Several chemical modifications have been reported to yield successful antisense molecules. All these structural modifications are primarily aimed at a) increasing the specificity and binding affinity towards mRNA, b) improving the permeability, uptake, and bioavailability of antisense oligomers, c) conferring resistance to nucleases, d) activating RNase H for cleavage of mRNA, and e) designing antisense compounds that are free from significant toxic effects upon administeration into human beings. Among these modifications, uniformly modified phosphorothioate oligodeoxyribonucleotides have been the first class of compounds to reach the clinic. ISIS 2105, targeted to the E2 mRNA of papillomavirus for the treatment of genital warts,[26] is in pivotal Phase II clinical trials, and ISIS 2922, targeted to CMV mRNA for the treatment of retinal keratitis, is in Phase I clinical testing. Both ISIS 2105 (TTG-CTT-CCA-TCT-TCC-TCG-TC) (20 mer) and ISIS 2922 (GCG-TTT-GCT-CTT-CTT-CTT-GCG) (21 mer) are uniformly modified oligodeoxyribonucleotide phosphorothioates. An important

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factor in the development of these drugs is advancements made in large scale synthesis, purification and characterization of phosphorothioates. During the manufacture of these compounds, such as ISIS 2105, process related impurities are formed due to a) failure of intermediates to detritylate, b) failure of monomers to couple, c) failure of unreacted 5'-hydroxyl group to cap with acetic anhydride, and d) failure of phosphite triester intermediates to sulfurize. An additional impurity class potentially formed as a result of repetitive acidic detritylation step is the deadenylated product. If deadenylation is followed by β-elimination of the apurinic glycosyl moiety, chain cleavage results and the shorter products are both detectable and removable. Large quantities of oligonucleotides are purified using reverse-phase HPLC using dimethoxytrityl group as a liphophilic handle. This is followed by a acidic detritylation step which may lead to deadenylation. Also, under typical storage conditions of the bulk drug substance, the oligomer could undergo deadenylation and may be undetected. In an effort to identify the formation of this trace impurity, we became interested in synthesizing an authentic sample and characterize it.

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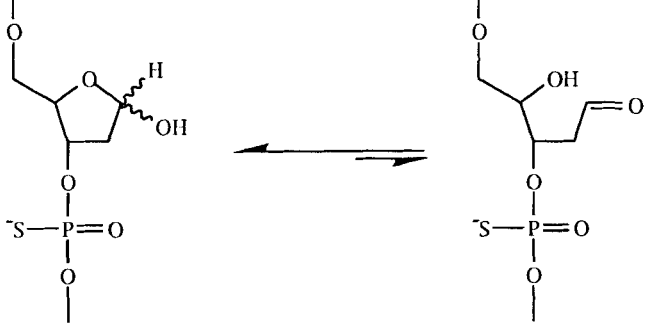
Depurination: Cleavage of the *N*-glycosyl bond between a base and its 2'-deoxyribose moiety in DNA generates an apurinic/apyrimidinic (abasic or AP) site. This phenomenon, which is referred to as depurination or depyrimidination occurs spontaneously under physiological conditions. Lindahl and Nyberg[27] have measured a rate constant for DNA depurination of 4 X 10^{-9}/sec at 70^{0}C and pH 7.4 in a Mg^{2+} containing buffer of physiological ionic strength. Also, it has been shown that purine nucleotides hydrolyze faster by *ca.* 2 orders of magnitude over pyrimidine nucleotides.[28,29]

Formation of abasic sites may occur as a result of chemical agents[30,31] and as intermediates during the normal course of repair mechanisms involving glycosylases.[32-34] Abasic sites have been chemically generated at random sites in DNA by acid[35] and/or heat treatment[27,35] and irradiation.[36,37] AP sites can also be introduced in DNA by some antitumor antibiotics, including bleomycin which binds to DNA and causes breakage of thymine-deoxyribose bonds.[38] The 2'-deoxyribose residue which remains after glycosidic bond hydrolysis exists in



99% 1%

a ring-opened (aldehyde) and ring-closed (furanose) tautomeric equilibrium with the former species highly base labile. Cleavage of the sugar-phosphate backbone at such sites readily occurs via β-elimination reactions. From the chemical point of view, the abasic site corresponds to a ca. 60:40 mixture of β- and α-hemiacetals[39] in tautomeric equilibrium with the ring-opened aldehydic form representing less than 1% of the total.[40] Recently, Celebuski and co-workers have reported the base composition analysis of oligonucleotides containing apurinic sites wherein they have compared tetramers containing abasic site following enzymatic digestion with that of 'normal' tetramers.[41]

Synthesis of oligonucleotides on solid supports on larger scale requires extended reaction and washing times, increasing depurination during the acidic detritylation steps. Final deprotection under basic conditions cleaves the synthesized oligonucleotides at the apurinic sites. The desired synthetic oligonucleotides, bearing a DMT group, can be usually separated from the truncated sequences by reverse-phase HPLC. However, the final step of purification involves removal of the DMT group under acidic conditions and may lead to the formation of AP sites.

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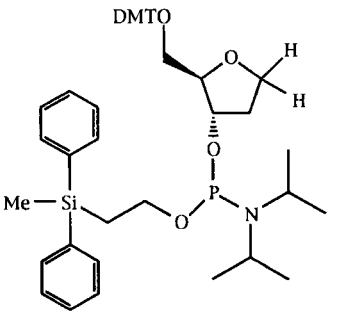
Only a few methods of selectively generating defined AP sites have been reported. One process involves enzymatic hydrolysis of uracil with uracil-DNA glycosylase in synthetic oligodeoxynucleotides containing deoxyuridine.[42,43] A chemical synthesis of short apurinic DNA sequences was reported by Vasseur *et al.*[44,45] However, this method is limited to synthesis of oligodeoxypyrimidines containing apurinic sites. Groebke and Leumann have reported[46] a non-enzymatic method of synthesizing oligodeoxynucleotides with predefined AP sites by protecting the anomeric hydroxyl group of furanose ring as a *tert*-butyldimethylsilyl ether.

During the manufacture of ISIS 2105, we were interested in the identification and characterization of one of the minor undesired products which we expect to be the deadenylated oligonucleotide phosphorothioate of ISIS 2105. The 1',2'-dideoxyribose abasic phosphoramidite shown below was prepared according to the method of Eritja[47] utilizing 2-diphenylmethyl silylethyl (DPSE) as phosphate protecting group.[48,49]



EXPERIMENTAL

NMR

^{31}P NMR spectra were recorded, at ambient temperature, on a Varian Unity 400 MHz spectrometer with an external capillary containing 85% H_3PO_4 used as a reference. Downfield chemical shifts were recorded as positive values for ^{31}P NMR.

Electrospray Mass Spectroscopy

Electrospray mass spectrum was run on a HP 5989A Mass Spectrometer. 2 OD in 100 ml (MeOH/H_2O 1:1) Mode ES-API; Inlet : LC; VCap=3900, VEnd=2700, VCyl=2000, CapEx=-160, EntLens=140V, Xray(HED)=10000, Polarity=negative.

Chemicals and reagents

All HPLC buffer salts were reagent grade and supplied by Aldrich (Milwaukee, WI). Acetonitrile (HPLC grade) was purchased from Mallinkrocdt. All gel electrophoresis reagents were purchased from Boheringer Mannheim Biochemicals. Stains-all dye was from Eastman Kodak. The standard phosphoramidites were purchased from Glen Research. Sulfurization of the oligonucleotide was performed using 3H-1,2-benzodithiole-3-one 1,1-dioxide[11,50]

DNA synthesis and purification

ISIS 2105 was synthesized on a Millgen/Biosearch 8800 synthesizer. The model analog of deadenylated ISIS 2105 and its DNA complement were synthesized on an Applied Biosystems Model 394B automated DNA synthesizer.

Ion-exchange chromatography

A Waters Millennium 2010 Chromatography Manager as solvent programmer, a Waters 996M Photodiode Array Detector, a NEC 486/33i microprocessor-controlled data system were employed. GEN-PAK FAX column (100 x 4,6 mm) and ODS Hypersil column (5 μm, 100 x 4,6 mm) were from Waters. Samples were analyzed at 25^{0}C on a GEN-PAK FAX column, using a linear gradient of buffer A and buffer B (0% B for 5'; 0-100% B for 45'). Buffer A = 2M NaCl in 50 mM Tris/methanol, 90/10, pH10, and buffer B = 50 mM Tris/methanol, 90/10, pH, 10. Flow rate 0.7ml/min.

Gel Electrophoresis

Analytical and preparative gel electrophoresis was accomplished using 1mm thick 20% acrylamide gel (containing 7.5 M urea) at 50-55^{0}C. Samples were run at 40 watts (ca. 1000-1100 volts). Position of the oligonucleotide was identified in the UV light by placing gel on the preparative silica gel plate with

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a fluorescent marker. Gel containing full-length product was cut out using scalpel, transferred to the eppendorf tube, grinded using a micropippete tip and covered with sterilized water (150μl). The tubes were vortexed, incubated for 60 min. at 55^{0}C and finally centrifuged. The product obtained was desalted by gel filtration, using column (V=26 ml) containing BIO-GEL P-4 (medium, 130 mm, exclusion limit 4x 10^4 Daltons) and evaporated to dryness at 25^{0}C.

Capillary Gel Electrophoresis

Electrophoretic data were acquired using a Beckman 2000 or 5000 P/ACE instrument operated at an applied voltage of 14.1 kV. The column temperature was maintained at 30^{0}C and detection was at 254 or 265 nm. Polyacrylamide Gel columns U100P (Catalog #338480) with an effective column length of 40 cm and the running buffer tris-Borate/Urea (Catalog #338481) were purchased from Beckman Instruments, Fullerton, CA. Electrokinetic injections were made at an injection voltage of 7 kV for 30 and 20 seconds for 3 and 10 μ g/mL samples, respectively.

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Melting Temperature

Absorbance *versus* temperature curves were measured at 260 nm using Hewlett-Packard spectrophotometer 8452A and temperature controling unit 89090A. Oligonucleotide phosphorothioate ISIS 2105 and its deadenylated analog were hybridized with complementary DNA sequence. The buffer contained 100 mM Na^+, 10 mM phosphate, and 0.1 mM EDTA, pH 7. Oligonucleotide concentration was 3 μM of each strand and the reported melting temperatures are averages of three experiments.

RESULTS AND DISCUSSION

The sequence of ISIS 2105 is S-d(TTGCTTCCATCTTCCTCGTC) and that of the model deadenylated ISIS 2105 is S-d(TTGCTTCCA*TCTTCCTCGTC) where A* denotes the site of abasic site. The synthesized oligonucleotides were purified using PAGE and desalted by gel filtration on a Bio-Gel P-4 column. Fig. 1 shows the PAGE analysis of ISIS 2105 (lane 1), the abasic ISIS 2105 (lane 2,3) and length marker (20, 16, and 12-mer) (lane 4).

Fig 2 shows the deconvoluted electrospray mass spectrum of the two samples. For electrospray mass spectral analysis, both the oligonucleotides were immobilized on a reverse-phase column (ODS Hypersil, 5 μm, 100 x 4.6 mm), washed with 0.05M solution of ammonium formate (5 column vol.), 5M solution of ammonium formate (14 column vol.), 0.05M solution of ammonium formate (22 column vol.) and eluted with acetonitrile. Fractions containing samples were combined, evaporated, resuspended in water (200 μl), and used for analysis.

The molecular ion of ISIS 2105 appears at 6262.41(-H + Na^+) and that of the model deadenylated analog of ISIS 2105 appears at 6129.5(-H + Na^+). The

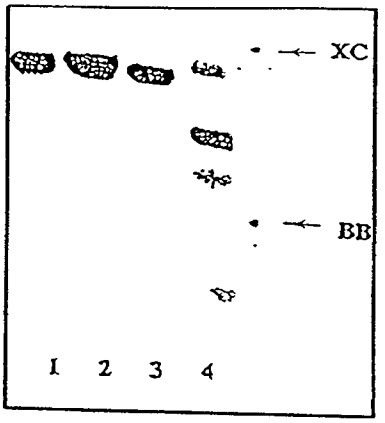

Fig. 1 PAGE analysis of a) ISIS 2105 (lane 1), the model analog of deadenylated ISIS 2105 (lane 2,3) and length marker (20, 16, and 12-mer) (lane 4). XC = Xylene cyanol; BB = Bromophenol blue.

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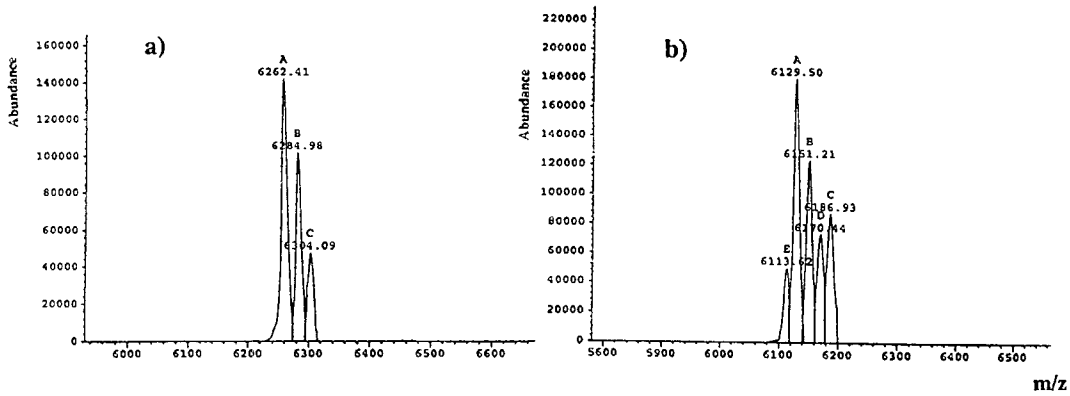

Fig. 2 Deconvoluted electrospray mass spectrum of ISIS 2105 (A) and the analog of model deadenylated ISIS 2105 (B).

main peaks corresponding to the molecular ions, in both cases, are accompanied by additional peaks due to incomplete exchange of Na^+ ions for $NH4^+$ ions.

Because of their diastereomeric and liphophilic nature, oligonucleotide phosphorothioates are difficult to analyze on a reversed-phase HPLC. Therefore ISIS 2105 and its deadenylated analog were analyzed using a strong anion-exchange column. Under these conditions ISIS 2105 (Fig. 3A) and its deadenylated analog (Fig. 3B) had similar retention times of 43.79 and 43.19 min. respectively, which confirmed the length of the synthesized oligomer. The faster eluting peak corresponds to the randomly distributed phosphodiester of the synthesized oligomer.

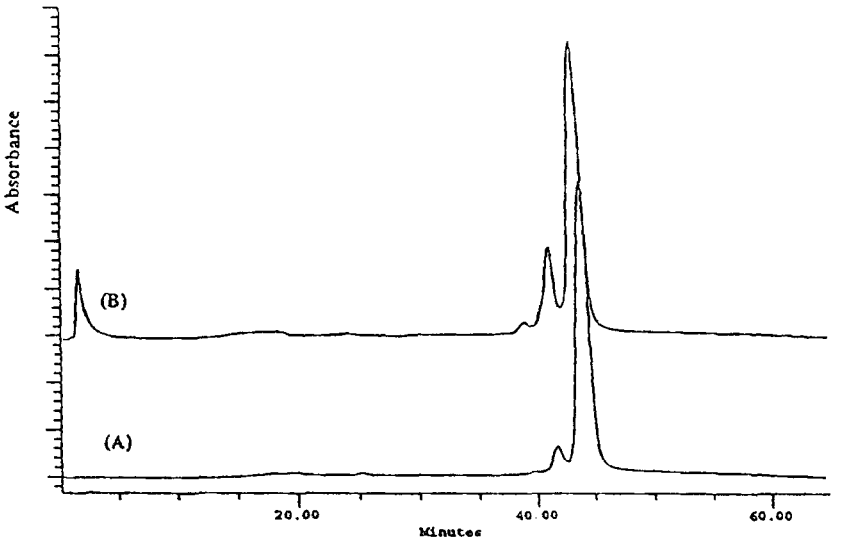

Fig. 3 HPLC analysis of ISIS 2105 (A) and the model deadenylated analog of ISIS 2105 (B) were performed on GEN-PAK FAX column, using a linear gradient of buffer A and buffer B (0% B for 5'; 0-100% B for 45'). Buffer A = 2M NaCl in 50 mM Tris/methanol, 90/10, pH10, and buffer B = 50 mM Tris/methanol, 90/10, pH10.

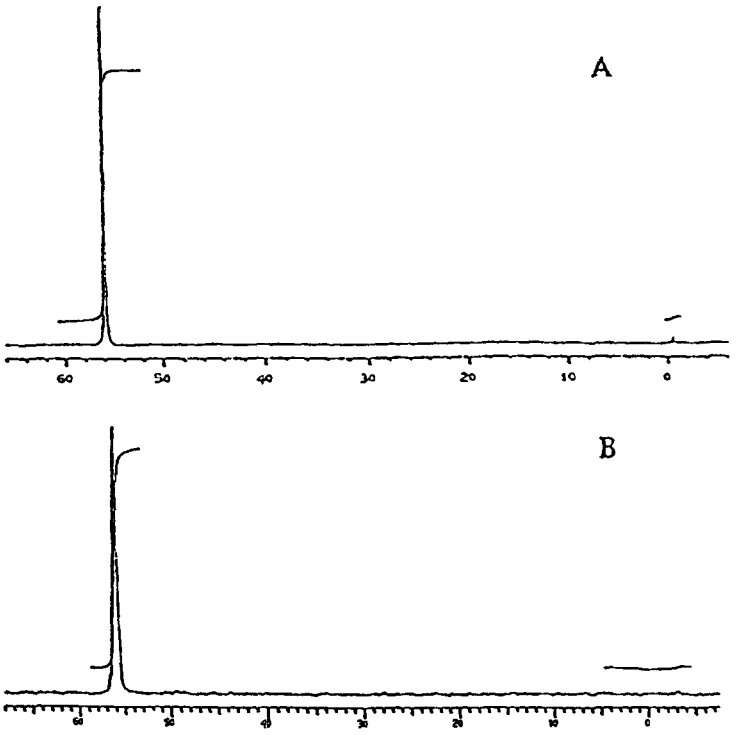

Fig. 4 ^{31}P NMR spectrum of ISIS 2105 (A) and the deadenylated analog of ISIS 2105 (B). Purified products contained 0.4% and 1.1% of phosphodiester linkages respectively.

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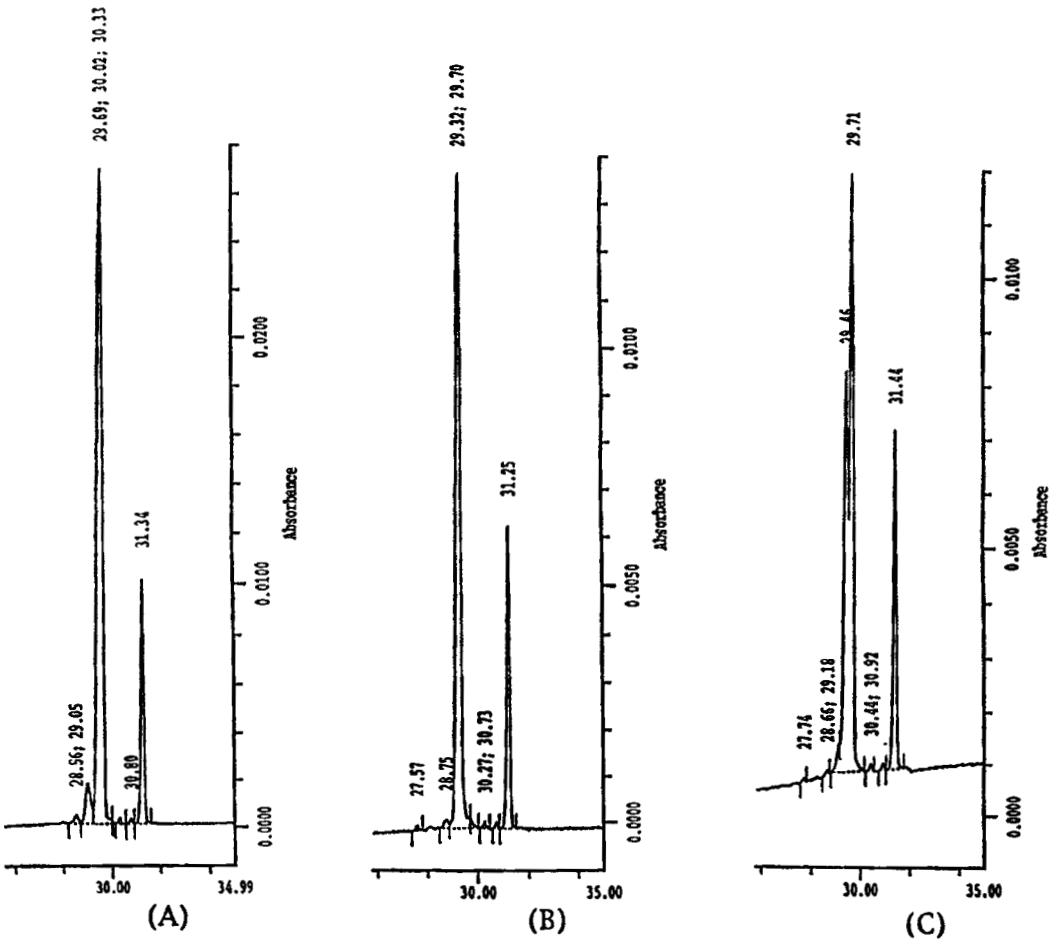

Fig. 5 Capillary Gel Electrophoresis (CGE) of (A) ISIS 2105, (B) the model analog of deadenylated ISIS 2105 and (C) coinjection of ISIS 2105 and the depurinated analog.

Fig. 4 shows the ^{31}P NMR spectrum of ISIS 2105 and the model compound of deadenylated ISIS 2105.

Fig. 5 shows the capillary gel electrophoresis (CGE) of ISIS 2105, the model analog of deadenylated ISIS 2105 and a coinjection of both the oligos. Homothymidine 23-mer was used as internal reference in all cases. Even though the electropherogram shows that the two oligomers have slightly different mobility, due to the presence of relatively larger quantities of (n-1)-mers which also have equal mobility as the deadenylated product, it may be difficult to identify the formation of depurinated product in the bulk drug substance.

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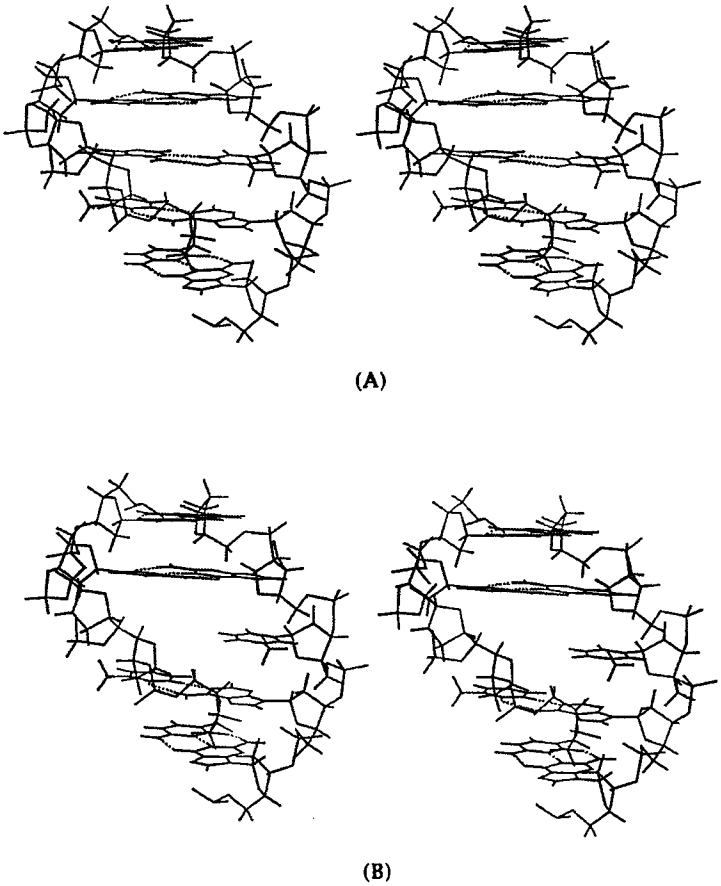

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Fig. 6 Stereo-view of a portion of energy minimized structures of (A) ISIS 2105 and (B) model deadenylated analog of ISIS 2105.

Molecular Mechanics Calculations

We have performed molecular mechanics calculations to investigate the effect of destabilization due to the abasic site on the duplex formation. All computations were performed on a Silicon Graphics Indigo2 Extreme workstation. QUANTA and CHARMM molecular modeling software package was used in our investigation. Duplexes containing a) adenine b) -OH and c) -H at the anomeric position which represent the antisense strand were model built and energy-minimized using the CHARMM force field[51] using a distant dependent dielectric method. Portions of minimized structures of ISIS 2105 and deadenylated analog of ISIS 2105 around the abasic site are shown in Fig. 6. Stacking energies of the bases in the antisense strand were computed and plotted as shown in Fig. 7. Unfavorable stacking interactions were observed at

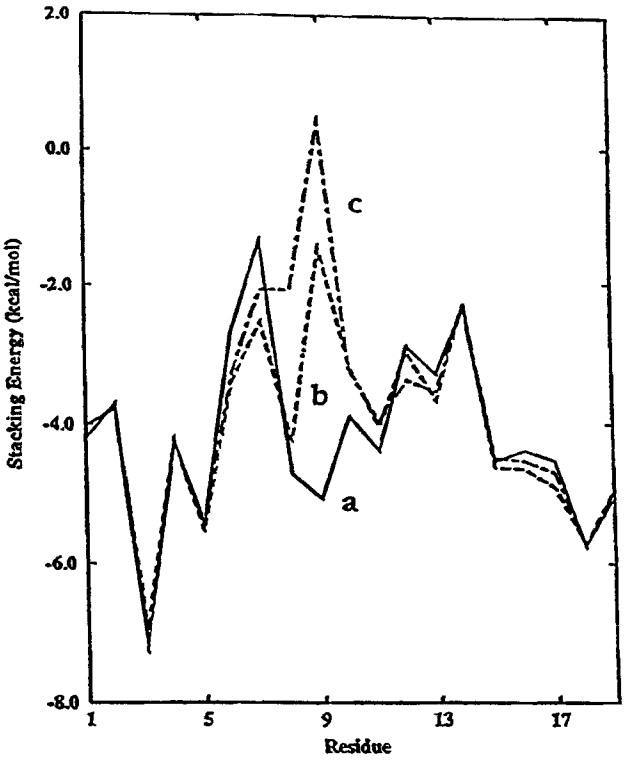

Fig. 7 Stacking energy is plotted as a function of residue numbers of the antisense strand of the duplex containing (a) adenine, (b) -OH and (c) -H at the anomeric position.

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the abasic sites. However, no significant difference was observed between -H and -OH substitution.

Melting Temperature

T_m measurements for ISIS 2105 and the model analog of deadenylated ISIS 2105 were performed and found to have 52.4 ± 0.6 and 40.8 ± 0.6 degrees respectively, giving rise to a ΔT_m of -11.6 ± 0.6 degrees. The unfavorable stacking interactions observed at the abasic site of the deadenylated analog in terms of molecular mechanics calculations is amply supported by the decrease in melting temperature.

CONCLUSION

The model analog of deadenylated ISIS 2105 has been characterized using electrospray mass spectrometry, ion-exchange HPLC, capillary gel

electrophoresis, polyacrylamide gel electrophoresis, and ^{31}P NMR. In the context of above results it is clear that low-concentration of the deadenylated product may not be identifiable in the bulk drug substance. Also, toxic studies using this bulk drug substance have shown no serious side effects implying that the deadenylated product is not a major concern.

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