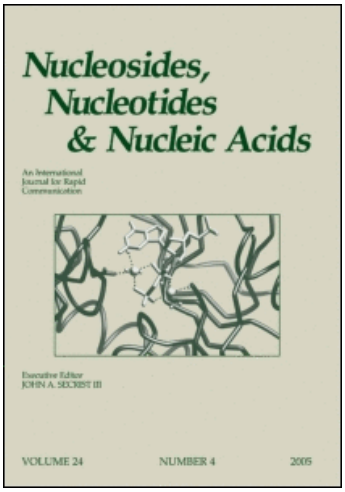

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SYNTHESIS OF ISIS 2105 CONTAINING THE ABASIC SITE MODEL COMPOUND 1,4-ANHYDRO-2-DEOXY-D-RIBITOL

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Abstract

An indirect method for investigating evidence of the formation of full length deadenylated phosphorothioate oligonucleotide (ISIS 2105) as a degradation product is reported through synthesis of its deadenylated analog. Alternatively, use of Leumann's method for the synthesis of authentic deadenylated ISIS 2105 using the *tert*-butyldimethylsilyl protecting group at the anomeric hydroxyl function of the furanose ring failed in our hands.

Introduction

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[1] 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.[2,3]

Formation of abasic sites may occur as a result of chemical agents[2,4] and as intermediates during the normal course of repair mechanisms involving glycosylases.[5-7] Abasic sites have been chemically generated at random sites in DNA by acid[8] and/or heat treatment[1,8] and irradiation.[9,10] 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.[11] The 2'-deoxyribose residue which remains after glycosidic bond hydrolysis exists in a ring-opened (aldehyde) and ring-closed (furanose) tautomeric equilibrium with the former species highly base labile. Clevage of the sugar-phosphate backbone at such sites readily occurs via β-elimination reactions. The exact mechanism of the chain cleavage is not fully understood although the classical work of Livingston[12,13] proposed a straight forward path involving Schiff base formation and subsequent β-elimination, using common carbonyl reagents such as phenylhydrazine, semicarbazide, etc. The situation is not so clear, however, as other mechanisms can be considered owing to the fact that the intermediate α,β-unsaturated compound can react to give a Michael adduct[14] or give rise to unexpected transpositions.[15]

Chemical stability of synthetic oligonucleotides is significantly influenced by the nature of protecting groups on exocyclic amino groups of the heterocyclic bases. The most popular strategy, used in the present oligonucleotide synthesis, employs combination of base-labile protecting groups for phosphorothioate (2-cyanoethyl) and for aglycone residues (N-acyl), and an acid-labile transient protecting group (4,4'-dimetoxytrityl, DMT) for 5'-hydroxyl protection of 2'-deoxyribose. It has been shown that 9-(2'-deoxypentafuranosyl) purines with benzoyl and isobutyryl as exocyclic amino protecting groups, the most commonly used in solid-supported oligonucleotide synthesis, undergo acidic hydrolysis by almost one order of magnitude faster. Synthesis of oligonucleotides on solid support in 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 DMT group, can be usually separated from the truncated sequences by reverse-phase HPLC. However, the final step of purification involves removal of DMT group under acidic condition and may lead to the formation of AP sites. Loss of the heterocyclic base reduces T_m of the DNA duplexes formed by depurinated oligomers[16] and may affect their interactions with proteins.[17]

Owing to this instability, chemical synthesis of DNA fragments containing abasic sites has not been extensively studied. However their mutagenic nature has stimulated this area of research and several chemically stable models of AP sites mimicking both the cyclic as well as the open-chain form of deoxyribose residue have been synthesized and incorporated into oligodeoxynucleotides.[18-22] Although most of the structural aspects[18-26] of such mimics seem to be relevant to the natural system, they certainly do not reflect the chemical reactivity of apurinic DNA.

Only few methods of selectively generating defined AP sites have been reported so far. One process involves enzymatic hydrolysis of uracil with uracil-DNA glycosylase in synthetic oligodeoxynucleotides containing deoxyuridine.[27,28] A chemical synthesis of short apurinic DNA sequences was reported by Vasseur *et al.*[29,30] However, this method is limited to synthesis of oligodeoxypyrimidines containing apurinic sites. Groebke and Leumann have reported[31] 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.

Oligonucleotide phosphorothioate S-d(TTGCTTCCATCTTCCTCGTC) (ISIS 2105) is being developed as a drug for treatment of genital warts. During this complex synthetic and purification process, we were interested in the identification of one of the possible side-products which we expect to be the deadenylated oligonucleotide phosphorothioate. We wanted to chemically synthesize this oligomer and establish its structure. The method of Groebke and Leumann gave only scission product and hence, we sought an indirect way of establishing the structure of deadenylated ISIS 2105 and herein we report our results.

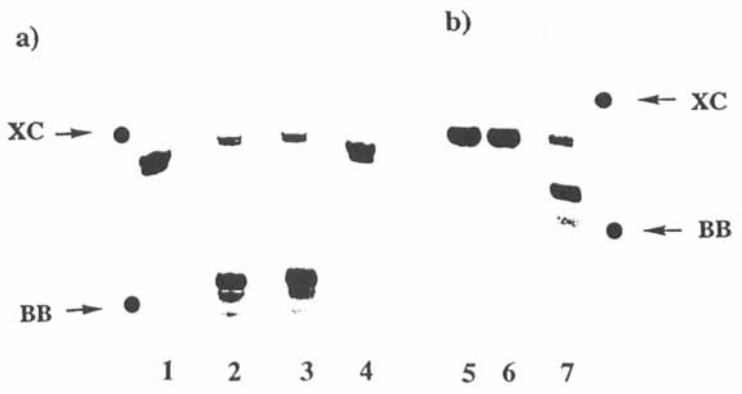

Fig. 1. PAGE analysis of : a) ISIS 2105 (lane 1), b) the product of attempted synthesis of the deadenylated analog of ISIS 2105 (lanes 2,3), c) analysis of ISIS 2105 (lane 4), d) the model analog of deadenylated ISIS 2105 (lanes 5,6) and e) lenght marker (20, 16 and 12-mer) (lane 7). Bromophenol Blue (BB) and Xylene Cyanol (XC) were used as length markers. Oligonucleotides were visualized by staining in Stains-All.

Results and Discussion

The synthesis of 1-O-[*tert*-butyldimethylsilyl]-3'-O-[(2-cyanoethoxy)(N,N-diisopropylamino)phosphino]-2'-deoxy-5'-O-(4,4'-dimethoxytriphenylmethyl)-α-D-ribofuranose was carried as reported earlier.[31] The oligonucleotide phosphorothioate was synthesized in 2-μmol scale. Standard synthons: 5'-Dimethoxytrityl-N^2-isobutyryldeoxyguanosine-3'-(2-cyanoethyl-*N,N*-diisopropyl) phosphoramidite, 5'-dimethoxytrityl-N^4-benzoyldeoxycytidine-3'-(2-cyanoethyl-*N,N*-diisopropyl)phosphoramidite (0.1M) and abasic phosphoramidite (0.2M) were used as solutions in acetonitrile. At the end of synthesis, the oligonucleotide was detached from the solid support with 30% aqueous ammonium hydroxide and all the protecting groups removed by incubation at 55^{0}C for 12h. Analysis of the synthesized oligonucleotide using 20% polyacrylamide gel along with the reference sample of ISIS 2105 is shown in Fig. 1.

In attempts to achieve the more stable analog of deadenylated phosphorothioate oligonucleotide, synthesis of abasic site monomer was carried out as follows: 1,4-anhydro-2-deoxy-D-ribitol was synthesized by acid-catalyzed dehydration of 2-deoxy-D-ribitol which was prepared by borohydride reduction of the commercially

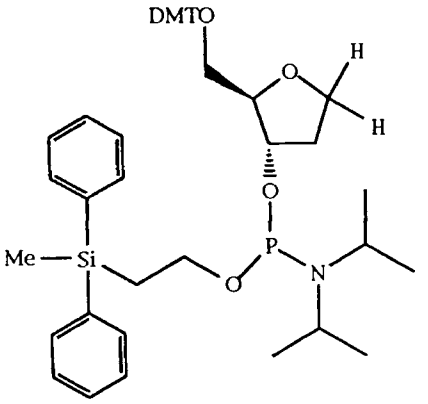

Fig. 2. 2-Diphenylmethylsilylethyl protected phosphoramidite used in the synthesis.

available 2-deoxy-D-ribose. Dimethoxytritylation followed by phosphitylation[32] using our newly developed 2-diphenylmethylsilyl ethyl *N,N*-diisopropylphosphoramidite under standard conditions gave the abasic phosphoramidite in 76% yield. This phosphate protecting group has been found to be equally effective as the cyanoethoxy group in the synthesis of oligodeoxyribonucleotides.

The analog of ISIS 2105, containing the abasic site d($T_{PS}T_{PS}G_{PS}C_{PS}T_{PS}T_{PS}C_{PS}C_{PS}A^{*}{}_{PS}T_{PS}C_{PS}T_{PS}T_{PS}C_{PS}C_{PS}T_{PS}C_{PS}G_{PS}T_{PS}C$) (A*= apurinic site) was prepared by conventional solid-phase synthesis using controlled-pore glass as a solid support. The efficiency of synthesis was monitored by measuring the absorbance of dimethoxytrityl cation released during the deprotection steps. The overall yield of synthesis was 98.5% and the incorporation of ribitol derivative was achieved in 97.5% yield. Crude oligonucleotide product contained 1.1% of the phosphodiester linkages (based on ^{31}P NMR). After removal of protecting groups, the oligonucleotide was purified using PAGE (20% acrylamide, 7M urea)(Fig.1.) and desalted by gel filtration on a Bio-Gel P-4 column.

The deconvoluted electrospray mass spectrum of analyzed products are shown in Fig. 3. 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 main peaks

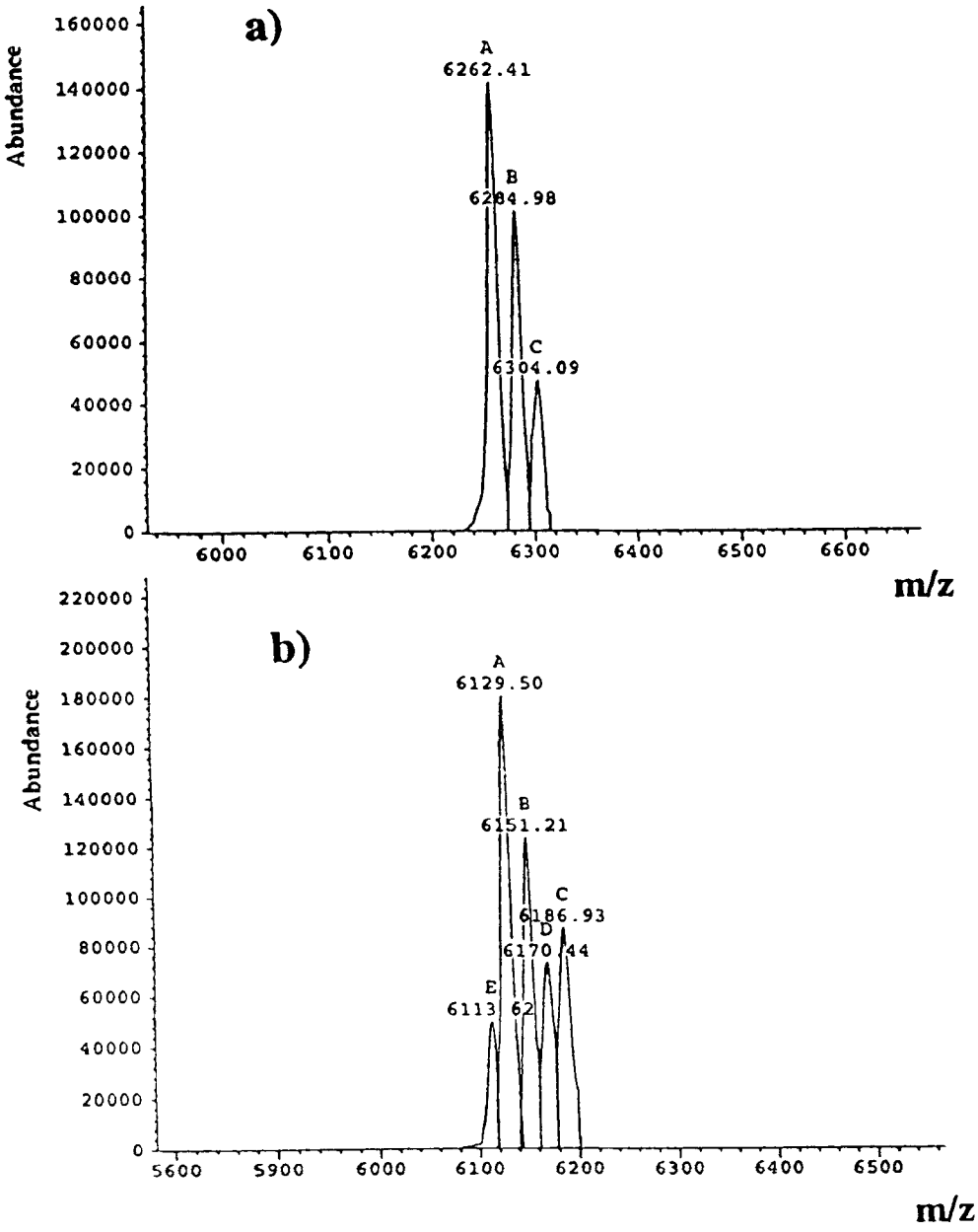

Fig 3. Deconvoluted electrospray mass spectrum of ISIS 2105 (a) and the model analog of deadenylated ISIS 2105 (b).

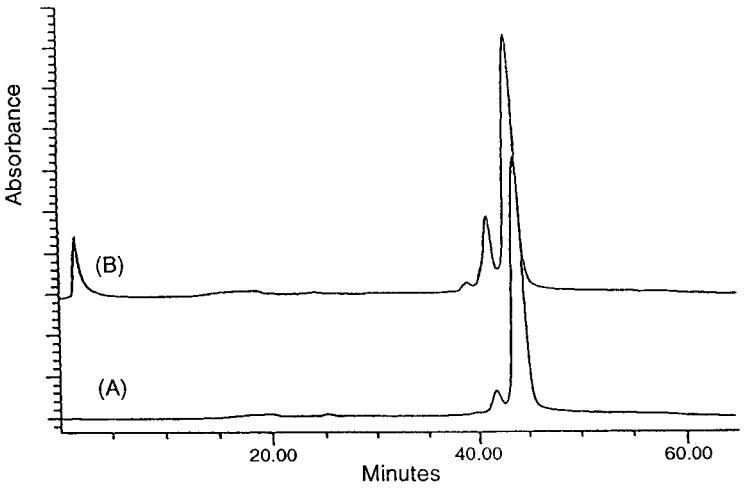

Fig 4. HPLC analysis of ISIS 2105 (A) and the model deadenylated analog of ISIS 2105 (B) were performed on GEN-PAK FAX column, using a linear gradient of buffer A and buffer B (0% B for 5'; 0-100% B for 45'). Buffer A = 2M NaCl in 50 mM Tris/methanol, 90/10, pH10, and buffer B = 50 mM Tris/methanol, 90/10, pH10.

corresponding to the molecular ions, in both cases, are accompanied by additional peaks due to incomplete exchange of Na^+ ions for NH_4^+ 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. 4A) and its deadenylated analog (Fig. 4B) had similar retention times of 43.79 and 43.19 min. respectively, which confirmed the length of the synthesized analog.

In conclusion, the model analog of deadenylated ISIS 2105 has been synthesized and characterized using electrospray mass spectrometry, ion-exchange HPLC and ^{31}P NMR. It is our believe that the physico-chemical characteristics of the synthesized compound can be utilized for evaluation of the purification protocol used by us for large scale synthesis.

Materials and Methods

Automated synthesis of abasic ISIS 2105 was performed on an Applied Biosystems DNA synthesizer (model 394 B) by the

phosphoramidite method using the standard commercially available reagents. The standard phosphoramidites were purchased from Glen Research. Sulfurization of the oligonucleotide was performed using Beaucage reagent.[33] Analytical and preparative gel electrophoresis were accomplished using 20% acrylamide gel containing 7.5M urea. BIO-GEL P-4 (medium, 130 μm, exclusion limit 4x 10^4 Daltons) was from Bio-Rad. Analysis of the products and preparation of samples for MS was performed using HPLC system from Waters. 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. ^{31}P NMR spectra were recorded, at ambient temperature, on a Varian Unity 400 MHz spectrometer using 85% H_3PO_4 as external reference. Downfield chemical shifts were recorded as positive values for ^{31}P NMR. Electrospray mass spectrum was run on a HP 5989A Mass Spectrometer.

5-O-(4,4'-dimethoxytriphenylmethyl)-1,4-anhydro-2-deoxy-D-ribitol-3'-O-[(2-diphenylmethylsilylethoxy)(*N*,*N*-diisopropylamino)phosphoramidite

A solution of 5-O-(4,4'-dimethoxytriphenylmethyl)-1,4-anhydro-2-deoxy-D-ribitol (0.841 g, 2 mmol) in dichloromethane (5 ml) was evaporated and the residue subsequently dried at 0.5 Torr for 2h. Under argon, tetrazole (0.112 g, 1.6 mmol) was added. The residue was suspended in dichloromethane (20 ml), and a solution of (2-diphenylmethylsilylethoxy)bis(*N*,*N*-diisopropylamino)phosphine[30] (2.08 g, 4.4 mmol) in dichloromethane (10 ml) was added dropwise within 2 min. with a syringe at room temperature. After stirring for 2h, the reaction mixture was filtered and the filtrate diluted with ethyl acetate (50 ml), washed with ice-cold saturated sodium bicarbonate (15 ml), brine and dried ($MgSO_4$). The concentrated crude material was purified by flash-chromatography with hexane/ethylacetate (1:4) containing 1% triethylamine. The product containing fractions were combined and evaporated to afford a pale-yellow viscous liquid. (1.33 g, 84%). TLC (hexane:ethyl acetate 2:1): R_f 0.15. ^{31}P-NMR ($CDCl_3$): 145.003, 145.194 ppm (mixture of two diastereo isomers).

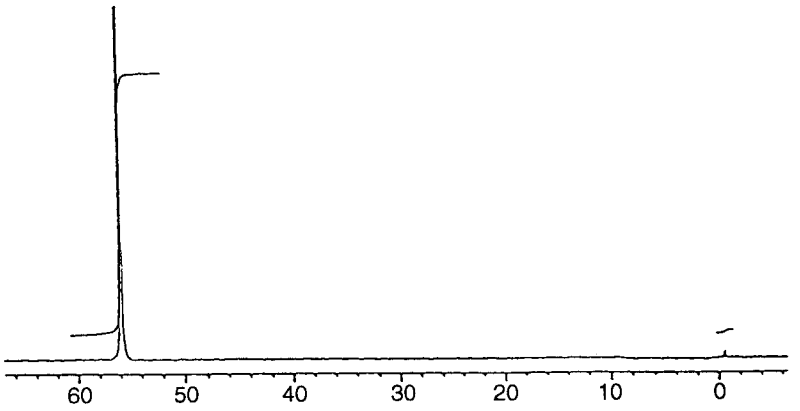

Fig. 5. ^{31}P NMR spectrum of ISIS 2105 containing the abasic site. Purified product contained 98.2% of the phosphorothioatediester linkages (δ =56 ppm).

Oligonucleotide Synthesis:

Deadenylated analog of ISIS 2105 was synthesized in 2 μmole scale (2x1 μmole) on controlled pore glass (CPG), using Applied Biosystems DNA synthesizer (model 394B) and phosphoroamidite chemistry. Standard synthons were prepared as a 0.1M solutions in CH_3CN. Beaucage reagent (0.2M solution in CH_3CN) was used[33] for sulfurization. Synthesis of oligonucleotide was performed using mixed-cycle option (switching automatically between standard sulfurization cycle and modified sulfurization cycle, used for incorporation of the abasic phosphoroamidite). In both cycles sulfurization was followed by capping[33]. Due to application of the more active 3H-1,2-benzodithiol-3-one 1,1-dioxide as a sulfurizing agent, sulfurization time for the standard cycle, was changed to 120 seconds. Abasic synthon was delivered to the column using port number 5. In order to ensure efficient incorporation, 5'-DMT-(1,4-anhydro-2-deoxy-D-ribitol-3'-O-phosphoramidite was prepared as 0.2M solution. Also, the duration of coupling and sulfurization reactions was extended to 5 and 8 minutes, respectively.

At the end of synthesis, the oligomer was deprotected under standard conditions involving decyanoethylation of phosphorothio-triesters, deprotection of heterocyclic bases and cleavage from the support with concentrated ammonia (55^0C, 12h) giving 220 O.D. of crude product. Deprotected oligonucleotide was separated from the

failure sequences using polyacrylamide gel electrophoresis (20% gel, 7.5M urea). Full-length product was cut out of the gel and eluted with water. Fractions obtained from elution was applied on a column containing Bio-Gel P-2 (V_O=6 ml), eluted with water and evaporated to dryness at room temperature. ^{31}P NMR (D_2O) of the purified product is shown in Fig. 5.

Preparation of the sample for MS analysis.

Deadenylated ISIS 2105 reconstituted in water was 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 sample were combined, evaporated, resuspended in water (200 μl) and used for analysis.

Acknowledgments

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