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Synthesis of High-Quality Antisense Drugs. Addition of Acrylonitrile to Phosphorothioate Oligonucleotides: Adduct Characterization and Avoidance

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Abstract:

It is demonstrated that the acrylonitrile (AN) generated during the ammonolysis step of oligonucleotide manufacture selectively adds to thymine residue present in ISIS 2302 to give a full-length oligonucleotide in which thymine is replaced by an N^3-cyanoethylthymine residue. Treatment of support-bound ISIS 2302 with a solution of triethylamine in CH_3CN before ammonolysis is sufficient to prevent formation of this class of impurity.

Phosphorothioate (PS) oligonucleotides demonstrate the capacity to specifically block protein synthesis in vitro and in vivo via an antisense mechanism.[1] Because of their specificity of binding, their ability to support RNase-H mediated cleavage of hybridized mRNA target sequences, and their enzymatic stability and low toxicity, phosphorothioate oligonucleotides targeted at a variety of human conditions including cancer, psoriasis, and Crohn's disease are currently under clinical investigation.[2] Approval of the antisense oligonucleotide fomivirsen sodium (Vitravene) for the treatment of CMV retinitis in AIDS patients by the FDA promises to herald a new dawn in the treatment of human disease.[3] Further commercial realization of the promise of antisense technology depends largely on the ability to synthesize substantial quantities (hundreds of kilograms) of high-quality oligonucleotide, a need which is met at present by solid-phase synthesis utilizing phosphoramidite chemistry.[4] In brief, oligomerization proceeds by detritylation of a support-bound nucleoside **1** and coupling of a cyanoethyl-protected phosphoramidite to give an intermediate phosphite triester **2** (Scheme 1).[5] Subsequent sulfurization and capping of unreacted hydroxy functionalities completes a synthesis cycle and gives the phosphorothioate triester **3**.

Upon completion of the chain-assembly steps the oligonucleotide is released from the support and deprotected by incubation with ammonium hydroxide. The finished product is obtained after purification (usually by reversed-phase HPLC) and a final detritylation step. Typically, the quality of the oligonucleotide is ascertained by a combination of techniques including capillary gel electrophoresis (CGE),[6] strong anion-exchange (SAX) chromatography,[7] ^{31}P NMR spectroscopy, and electrospray mass spectrometry (ES-MS).[8]

ISIS 2302, d(GCC-CAA-GCT-GGC-ATC-CGT-CA),is a 20-mer phosphorothioate oligodeoxynucleotide currently in a pivotal quality trial for treatment of Crohn's disease. During the course of our studies on optimization of the ISIS 2302 manufacturing process and of solid-phase oligonucleotide synthesis in general, we consistently observed the occurrence of 2−3% of an impurity with characteristic mass 53 amu more than ISIS 2302. Observations made on a number of sequences indicated that impurity levels varied in a sequence-specific manner and were present to a higher degree in

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(2) ISIS 2302 is in a pivotal Phase III trial for Crohn's disease and in Phase II clinical trials for renal transplant rejection, ulcerative colitis, and psoriasis. Affinitak (ISIS 3521) is in pivotal Phase III clinical trials as an anticancer agent. Currently using Amersham Biosciences' OligoProcess, we routinely synthesize phosphorothioate oligodeoxyribonucleotides at scales between 300 and 600 mmol, yielding more than 2 kg of purified drug in a synthesis at the high end of synthesis (viz. 600 mmol scale). On the basis of IP-LC−MS analysis, drug of very good quality is being manufactured here at Isis Pharmaceuticals, Inc.

(3) More than 25 oligonucleotides (both first- and second-generation combined) are being evaluated here at Isis Pharmaceuticals as well as in other places for the treatment of various diseases.

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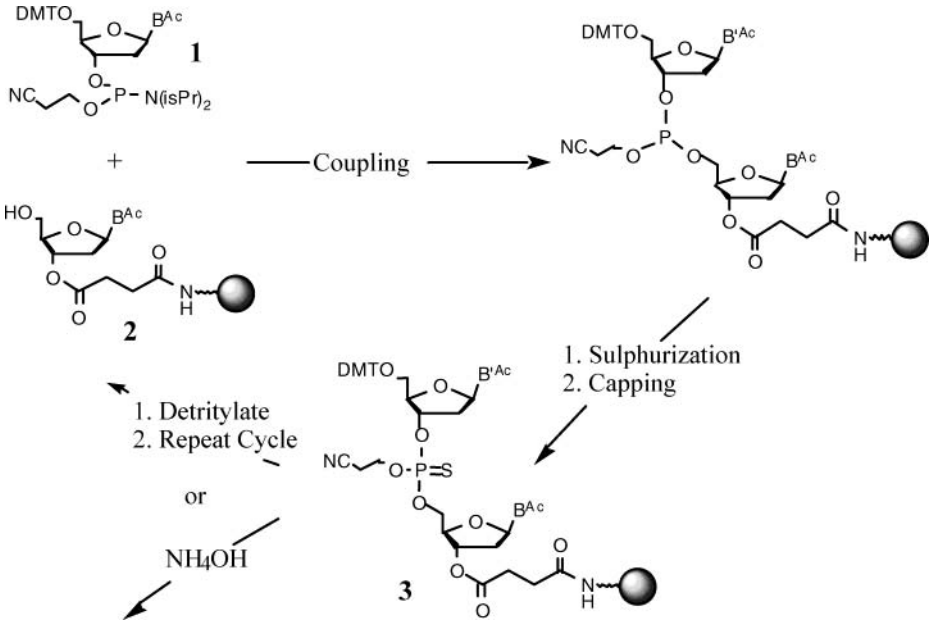
Scheme 1. **Synthesis of phosphorothioate oligonucleotides**[a]



[a] Detritylation = removal of DMT groups, coupling = reaction of phosphoramidite in presence of an activator such as ^{1}H-tetrazole, sulphurization = oxidizing phosphite triester to phosphorothioate triester, capping = blocking of unreacted hydroxyl groups.

thymidine-rich oligonucleotides (ca. 0.5%/thymine residue). The observation that the impurity was associated with thymine residues, coupled with the mass spectroscopy data, led to the tentative postulate that the impurity arose by the adventitious addition of acrylonitrile (AN) to thymine residues present in the oligonucleotide.

During the course of the ammonium hydroxide deprotection it is evident that the cyanoethyl groups protecting the internucleotide linkages are removed by a β-elimination mechanism to generate AN. It seemed possible that the thus formed AN could be captured in a Michael-type[9] fashion by the N^3 atom of thymidine. Alkylation of thymine residues during the course of oligonucleotide synthesis has been described previously. Gait et al noted that thymine residues underwent transmethylation when methyl-protected oligonucleotide triesters were treated with 1,8-diazabicyclo-[5.4.0]-undec-7-ene (DBU).[10] More recently Eritja[11] described similar transalkylation problems associated with DBU treatment of cyanoethyl-protected oligonucleotide triesters although no structural proof was provided. AN is known to alkylate guanine and thymine residues in nucleic acids, giving N^7-2-cyanoethylguanine and N^3-2-cyanoethylthymine residues, respectively, as the main products after acid-induced depurination.[12] Interestingly, reaction with cytosine and adenine residues leads directly to the N^3 and N^1 (and in the case of adenine, N^6 via a Dimroth rearrangement[13,14]) carboxyethyl derivatives (an addition of +73 amu) due to assisted hydrolysis of the nitrile by the proximal exocyclic nitrogen atom.[12] We now report isolation and characterization of the +53 amu impurity from a variety of phosphorothioate oligonucleotides including ISIS 2302. In addition, a simple and effective method to avoid its formation is described.

Results and Discussion

Part 1: Isolation and Characterization. Initial experiments aimed at the isolation and characterization of the impurity were performed on phosphorothioate oligothymidylates. Nonadecathymidyloctadecaphosphorothioic acid $(T_{PS})_{18}T$ was synthesised on a ca. 160 μmol scale under above-described oligonucleotide synthesis conditions. Treatment of the support-bound oligonucleotide with ammonium hydroxide to effect succinate cleavage and phosphate deprotection was followed by detritylation with AcOH−H_2O (1:4 v/v). The crude nonadecamer was analyzed by LC−MS. Selective ion monitoring was used to expand the region encompassing the −4 charge state. Figure 1 shows a main quadruply charged ion with m/z = 1500.6 corresponding to $(T_{PS})_{18}T$. In addition to the monophosphate diester (strand containing one phosphate group by replacement of a sulfur atom by oxygen) of $(T_{PS})_{18}T$ (m/z = 1496.8) and the monosodium adduct (m/z = 1506.2), a molecular ion with m/z = 1514 was also detected. This mass increase (+53 amu) is consistent with the addition of acrylonitrile to a single thymine residue in $(T_{PS})_{18}T$. Integration of the relative peak intensities showed the monocyanoethyl adduct to be present to the extent of ca. 15%.

The presence of N^3-cyanoethylthymine residues was confirmed by desulphurization and digestion of $(T_{PS})_{18}T$ with snake venom phosphodiesterase (SVPD) and alkaline phosphatase (AP). RP-HPLC analysis of the products showed that, in addition to thymidine (R_T = 15 min), a later-eluting product (R_T = 25 min) was also present (Figure 2). Mass spectroscopic analysis of the latter material and co-injection

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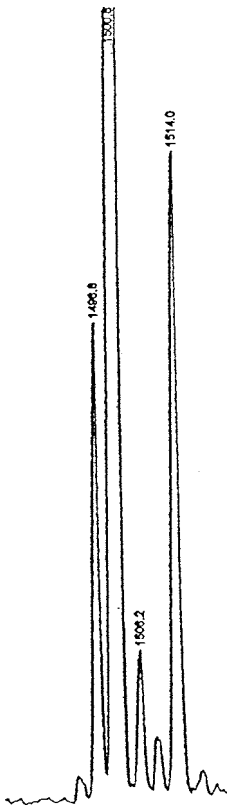

Figure 1. **MS of phosphorothioate oligodeoxyribonucleotide nonadecamer $(T_{PS})_{18}T$.**

with an authentic sample confirmed its identity as N^3-cyanoethylthymidine.

The stability of N^3-cyanoethylthymidine towards NH_4OH was investigated by incubating an ammoniacal solution at 60 °C for 16 h. RP-HPLC analysis of the products (Figure 3) indicated that ca. 12 area % of the starting material was converted back to thymidine, while a further 8 area % was converted into an another compound whose identity was not established (possibly the corresponding amide or acid). These results indicate that N^3-cyanoethylthymidine[17] and, by analogy, N^3-cyanoethylthymine residues present in oligonucleotides are relatively stable toward the unblocking conditions.

Attempts were also made to look for products arising from the addition of AN to adenine, cytidine, and guanine residues. The extent of reaction was markedly less for these species. Results obtained from LC−MS analysis of the products arising from incubation of ammonium hydroxide solutions of $d[(A_{PS})_{18}A]$, $d[(C_{PS})_{18}C]$, $d[(G_{PS})_{18}G]$, and $d[(T_{PS}C)_9T]$ under a variety of conditions including elevated temperature are contrasted with results obtained from $(T_{PS})_{18}T$ in Table 1.

Table 1 shows that low levels of an impurity having a mass of 53 amu greater than full length oligonucleotide were also present in samples of $d[(A_{PS})_{18}A]$ and $d[(C_{PS})_{18}C]$ that were unblocked under standard conditions (row 1, Table 1). Addition of AN to the deprotection mixtures increased these levels by a factor of 2. Interestingly, if $d[(C_{PS})_{18}C]$ was treated with NH_4OH for 5 h at 60 °C to remove the N^4-benzoyl protecting groups (a standard group used to protect exocyclic amino group of deoxycytidine, deoxyadenosine) prior to addition of AN, the amount of adduct was seen to increase to ca. 12%. No + 53 amu impurity was seen in samples of $d[(G_{PS})_{18}G]$ unblocked under any of the above conditions, and no adducts having a mass of 73 amu greater than full-length oligonucleotide were ever observed. Under normal unblocking conditions low levels of adduct were observed in $d[(A_{PS})_{18}A]$ and $d[(C_{PS})_{18}C]$. In both cases levels were much lower than those observed in $(T_{PS})_{18}T$ (row 1, Table 1). In addition, LC−MS analysis of desulfurized and enzymatically digested $d[(C_{PS})_{18}C]$ and $d[(T_{PS}C)_9T]$ indicated the presence of low levels of a nucleoside having a mass 53 amu greater than that of 2′-deoxycytidine.[5] Replacement of 10 out of the 19 cytosine residues with thymine in $d[(T_{PS}C)_9T]$ increased adduct level by a factor of ca. 7.

It should be remembered that AN has very limited stability in NH_4OH (half-life is ca. 10 min at 25 °C in ND_4OD[15]) and that for base modification to occur, the base must react effectively with AN before its destruction by NH_4OH. Apparently, under these conditions, only thymine residues react quickly enough to undergo cyanoethylation to any great extent. These rate differences add credence to the idea that in a mixed-base sequence (e.g. ISIS 2302) AN will add selectively to thymine residues. It appears that this selectivity is, at least in part, due to the fact that cytosine residues are protected by a benzoyl group on N^4 during the early stages of ammonolysis. Table 1 shows that while addition of AN to ammoniacal solutions of $d[(C_{PS})_{18}C]$ increased the amount of adduct moderately (from 1.3 to 2.5%), addition of AN after removal of the benzoyl protecting groups resulted in ca. 10-fold increase in the amount of adduct formed. Further selectivity no doubt arises because of the acidity of thymine residues ($pK_a = 9.79$, dC, $pK_a = 4.25$; dA, $pK_a = 1.75$; dG, $pK_a = 1.6$)[16] with the consequence that under the ammonolysis they would be expected to be at least partially deprotonated.

The finding of +53 amu adducts in homoadenylates and homocytidylates is contradictory to reports in the literature which state that only carboxyethyl adducts (+73 amu) of adenine and cytosine residues are ever encountered.[12] Although the adenine and cytosine adducts above have not been characterized, the results indicate (at least for cytosine residues) that AN adds to the base and not to, for example, the free 3′-hydroxyl. The dramatic increases in adduct levels upon preincubation of $d[(C_{PS})_{18}C]$ with NH_4OH before AN addition, coupled with the fact that $d[(A_{PS})_{18}A]$ does not show a similar increase, also support addition of AN to the base moiety rather than a sugar hydroxyl. The fact that the adduct has a molecular weight of 53 amu and not, as reported,[12] 73 amu more than that of full-length can be interpreted in one

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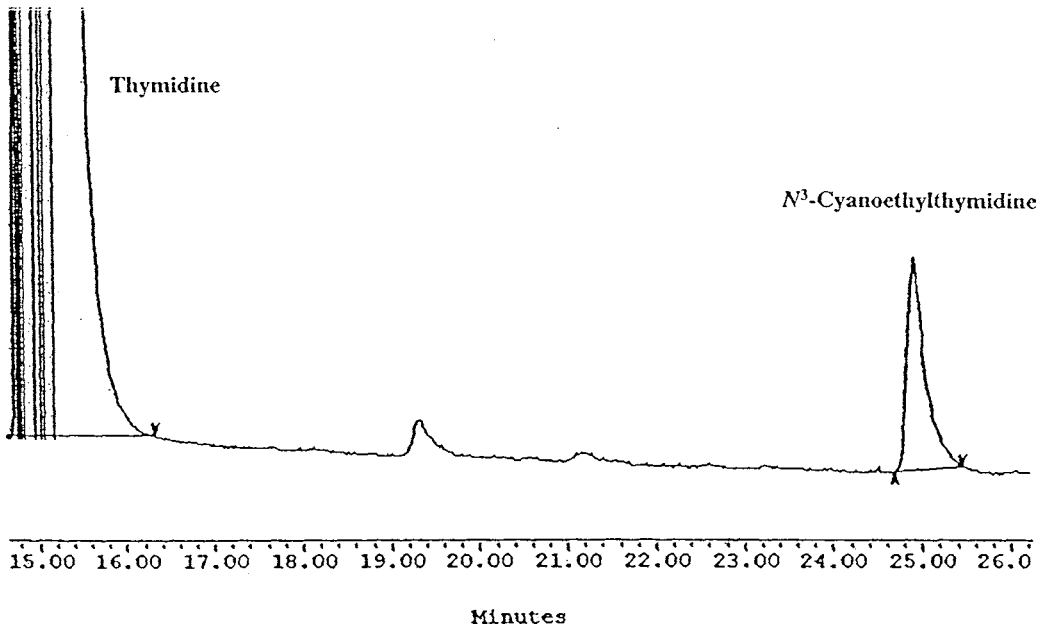


Figure 2. **RP-HPLC of enzymatic digest of phosphorothioate oligodeoxyribonucleotide nonadecamer $(T_{PS})_{18}T$.**

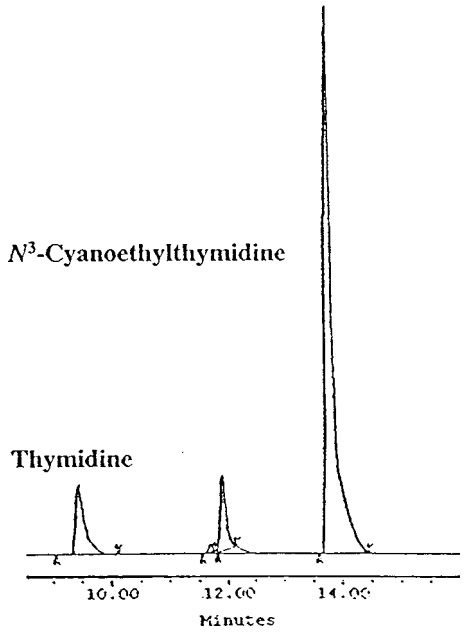


Figure 3. **RP-HPLC explainaing the stability of N^3-cyanoethylthymidine toward ammonium hydroxide.**

of two ways. First it may be that, under the specific conditions studied, cyanoethyl adducts of adenine and cytosine are stable. Alternatively, one could postulate that the initially formed cyanoethyl adducts do in fact hydrolyze to the corresponding carboxyethyl adducts as reported,[12] but that these adducts are themselves unstable and cyclize to form the lactam structures **4** and **5** (Figure 4).

The finding that AN reacts substantially faster with thymine residues than with the three remaining bases indicates that the 2−3 area % of adduct peak detected in ISIS 2302 is composed mainly of N^3-cyanoethylthymine containing full-length oligonucleotide. It seems reasonable to treat each of the three thymine residues in ISIS 2302 as equivalent and postulate that the monoalkylated impurity peak is an equimolar mixture of three compounds.

Part II: Avoiding Formation of Cyanoethyl Adducts. With the identity of the impurity confirmed it became of interest to devise a method to avoid its formation. Initial efforts were centered on two main strategies. First, deprotection of the oligonucleotide in the presence of a reagent reactive towards acrylonitrile (i.e., a scavenger) would be expected to reduce the percentage of thymidine residues that undergo alkylation. In a second scenario, deprotection of the internucleotide linkages could be performed under conditions under which the liberated acrylonitrile did not react with thymidine residues.

Addition of acrylonitrile scavengers to the deprotection mixture raised several concerns. First, one must select a reagent that adds exclusively to acrylonitrile and not to any other functionality. Second, it must be demonstrated that adduct can be completely removed from the final product. Despite some initial success with some scavengers such as thymine, we felt that these concerns would be difficult to resolve and so concomitantly turned to the second strategy.

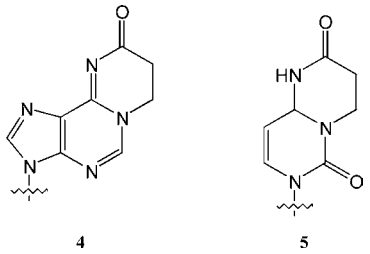
In essence, the second strategy involved treatment of the support-bound oligonucleotide with a solution of a base in a suitable solvent. The appropriate base would cleave cyanoethyl groups at a reasonable rate while not attacking the succinate ester tethering the oligonucleotide to the solid support. Initial screening experiments performed (with amines such as triethylamine, tributylamine, diethylamine, and diisopropylamine and in solvents such as acetonitrile, toluene, and dioxane) in solution at the dimer level led us to believe that a 1:1 (v/v) mixture of triethylamine (TEA) in acetonitrile would constitute a suitable reagent system.

The scavenging condition was extended to deprotection of nonadecathymidyloctadecaphosphorothioic acid $(T_{PS})_{18}T$. Following complete oligomerization, the support-bound oligonucleotide was washed with a solution of TEA−CH_3-CN (1:1 v/v) at a linear flow rate of 0.8 cm min^{-1} for 4 h. The eluate was collected as four consecutive 1-h fractions and analyzed for the presence of acrylonitrile by gas

Table 1

deprotection conditions	% of +53 amu impurity[a]				
	$d[(A_{PS})_{18}A]$	$d[(C_{PS})_{18}C]$	$d[(G_{PS})_{18}G]$	$d[(T_{PS}C)_9T]$	$(T_{PS})_{18}T$
NH_4OH[b]	1.5	1.3	N.D.[e]	10	15
NH_4OH, AN[c]	2.5	2.5	N.D.	20	−
NH_4OH then NH_4OH, AN[d]	2.6	12	N.D.	−	−

[a] Per cent of +53 amu impurity relative to full-length oligonucleotide as judged by LC−MS. [b] 50 mg support-bound oligo treated with NH_4OH (1 mL) for 16 h at 60 °C. [c] 50 mg support-bound oligo treated with NH_4OH (10 μL) for 16 h at 60 °C. [d] 50 mg support-bound oligo treated with NH_4OH (1 mL) for 5 h at 60 °C then with NH_4OH (1 mL) and AN (10 μL) for 16 h at 60 °C°. [e] N.D. = not detected.



4 5

Figure 4. **Cyclic adducts of bases.**

chromatography (GC). Approximately 90% of the theoretical amount of acrylonitrile was contained within the first fraction, and the reaction was essentially complete after 4 h. After washing the support-bound material briefly with CH_3CN the oligonucleotide was released from the solid support by treatment with ammonium hydroxide in the normal fashion. Detritylation followed by LC−MS analysis revealed the crude product to be free of cyanoethylated thymine residues (Figure 5).

Large-Scale Synthesis of Phosphorothioate Oligonucleotides. With a suitable reagent system in hand, attention was turned to the ISIS 2302 sequence. Synthesis was performed at the 150 mmol scale on an OligoProcess DNA/RNA synthesizer. Following complete chain assembly, the support-bound oligonucleotide was again washed with a v/v solution of TEA in CH_3CN at a linear flow rate of 0.8 cm min^{-1} for 1 h. After being allowed to remain in contact with above solution for an additional period of 12 h, the oligonucleotide was cleaved from support and deprotected with ammonium hydroxide in the usual manner. The crude synthetic oligonucleotide was purified and detritylated in the normal fashion and then analyzed for the presence of cyanoethyl adducts by LC−MS. Parts a and b of Figure 6 show the mass spectra obtained from untreated and TEA-treated ISIS 2302, respectively. In the untreated control the +53 amu peak is clearly seen at m/z = 1604, while inspection of the TEA treated material shows this peak to be absent.

Multiple phosphorothioate oligonucleotides of 20-mer in length are routinely being synthesized at Isis Pharmaceuticals at 300−600 mmol scale using the above reagent system to generate high-quality antisense drugs for clinical evaluations as well as potentially for the market.

Experimental Section

General. Reversed-phase high-performance liquid chromatography (RP-HPLC) was performed using a Waters 600E controller set at 254 nm equipped with a Waters 717 autosampler. UV spectra were recorded on a HP 84524A diode array spectrophotometer. Gas chromatography was performed on an HP 5590 gas chromatograph using a J&W DB1 Megabore column (30 mm × 0.53 mm, 5 μ) heated from 40 to 220 °C at a rate of 10 °C/min. The injector and detector temperatures were held at 220 and 280 °C, respectively. HPLC−MS analyses were performed using a YMC ODS AQ column (2 mm × 250 mm), eluted under a gradient of CH_3CN in triethylammonium acetate (TEAA) (20 mM, pH 7), attached to an HP MSD 1100 spectrometer fitted with an electrospray source. Spectra were recorded in the negative ionization mode using a mass window of ca. 200 amu centered about the M-4 charge state.

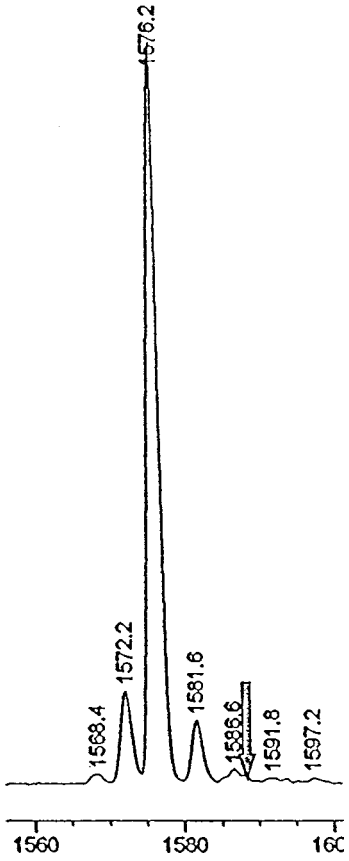


Figure 5. **MS of TEA treated DMT $(T_{PS})_{18}T$.**

5′-*O*-(4,4′-Dimethoxytrityl)-N^3-(2-cyanoethyl)thymidine (6). 5′-*O*-(4,4′-Dimethoxytrityl)thymidine[17] (10.5 g, 19.4 mmol) was dissolved in a mixture of THF and CH_3CN (3:5 v/v, 80 mL), and acrylonitrile (3.1 g, 58 mmol) and tetra-*n*-butylammonium hydroxide (0.3 mL of a 40% w/w solution in water) were added; the mixture was heated at 60 °C for

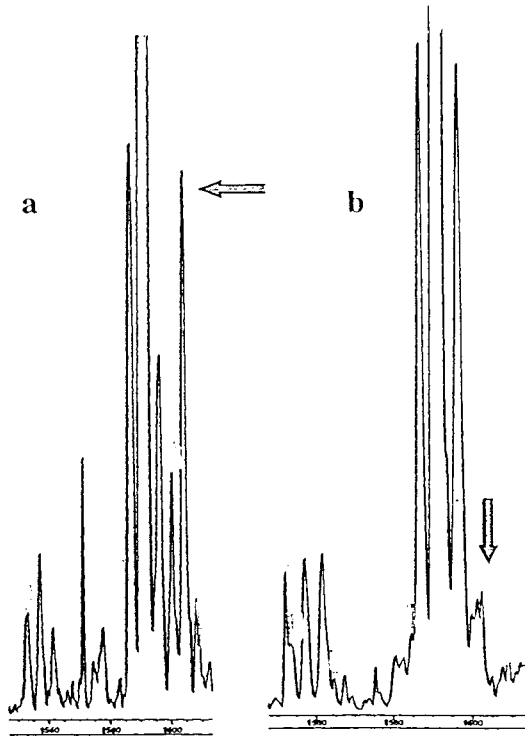


Figure 6. **MS of TEA untreated (a) and treated (b) ISIS 2302.**

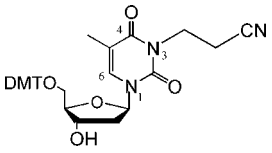


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Figure 7. ***N*^{3}-Cyanoethylthymidine.**

16 h. The cooled products were concentrated under vacuum; a solution of the residue in $CHCl_3$ (200 mL) was washed with water (2 × 100 mL), dried (Na_2SO_4), and the $CHCl_3$ was evaporated. The residual oil was purified by chromatography on silica gel, and concentration of the fractions eluted with $CHCl_3$−MeOH (49:1 v/v) gave the *title compound* (**6**) (3.5 g, 6 mmol, 30%) as a colorless glass[17] (Figure 7).

***N*^{3}-(2-Cyanoethyl)thymidine.** 5′-*O*-(4,4′-Dimethoxytrityl)-*N*^{3}-(2-cyanoethyl)thymidine (0.5 g, 0.8 mmol) was dissolved in AcOH−MeOH (1:1 v/v, 20 mL). After 2 h the products were concentrated, and the residue was purified by chromatography on silica gel. Evaporation of the fractions eluted with $CHCl_3$−MeOH (19:1 to 9:1 v/v) gave the *title compound* (0.21 g, 0.7 mmol, 90%) as a colorless gum which slowly crystallized upon standing.[17] FAB-HRMS: calcd for $C_{13}H_{17}N_3O_5$ $(M + H)^+$ 296.1247, found 296.1230.

Oligonucleotide Synthesis. Oligonucleotide synthesis[4a] was carried out on an Amersham Biosciences OligoPilot II DNA/RNA synthesizer. All sequences were synthesized on a ca. 165 μmol scale using Primer support (ca. 90 μmol/g, Amersham Biosciences). Detritylation was achieved using a 3% v/v solution of dichloroacetic acid in toluene for a contact time of 5 min (flow rate = 12.5 mL/min). Commercial phosphoramidites, (2 equivalents per coupling) purchased from Pierce (www.piercebc.com), were dissolved to a nominal concentration of 0.2 M in anhydrous CH_3CN and activated with a 0.45 M solution of tetrazole in CH_3CN (amidite:tetrazole = 1:3 v/v). The coupling/recycling time was set to 5 min. Sulfurization was effected using a 0.2 M solution of phenylacetyldisulphide (PADS, Acharya Chemicals, Mumbai, India) in picoline−CH_3CN (1:1 v/v, 1.25 column volumes for a contact time of 2 min).[18] Capping reagents were made to recommended Pharmacia composition: Cap A: *N*-methylimidazole−CH_3CN (1:4 v/v); Cap B: acetic anhydride−pyridine−CH_3CN (2:3:5, v/v/v).

Base Treatment and Ammonolysis of Crude Support-bound Oligonucleotides. Cyanoethyl protecting groups were removed prior to ammonolysis by treating support-bound oligonucleotide (ca. 50 mg) with triethylamine−acetonitrile (TEA- CH_3CN) (1:1 v/v, 1 mL) for 12−16 h at 25 °C. Alternatively, a solution of the same mixture was passed through the synthesis column at a linear flow rate of 0.8 cm/min using a small peristaltic pump. After 1 h the pump was turned off and the column allowed to remain in contact with the solution for an additional 12−16 h. In both cases the support-bound oligonucleotide was then washed with CH_3CN and dried under vacuum. Ammonolysis was carried out by suspending the partially protected, support-bound oligonucleotide (50 mg) in concentrated aqueous ammonium hydroxide (28%, NH_4OH; d 0.88, 1 mL) and heating the mixture at 60 °C for 12−16 h. The cooled products were then filtered, and the support was washed with EtOH−water (1:1 v/v, 1 mL). The combined filtrate and washings were concentrated in a SpeedVac.

Rate of Removal of Cyanoethyl Groups. The rate of removal of cyanoethyl groups from phosphorothioate oligonucleotides was estimated by passing a solution of TEA−CH_3CN (1/1 v/v) through support-bound phosphorothioate homothymidine octadecamer $(T_{PS})_{18}T$ contained within the synthesis column at a flow rate of 0.8 cm/min. Four fractions of equal volume were collected over a period of 4 h and the fractions analyzed by GC for the presence of acrylonitrile.

Oligonucleotide Purification and Final Detritylation. Crude DMT protected (DMT-on) oligonucleotide (ca. 2000 A_{260} units) was dissolved in water and purified by RP-HPLC using a Waters NovaPak C18 column (3.9 mm × 300 mm) eluted under a gradient of CH_3CN in 0.1 M TEAA (pH 7). The solution of DMT-on material (eluted with CH_3CN−0.1 M TEAA, 1:1 v/v) was collected and concentrated. The residue was dissolved in water to a concentration of ca. 1000 A_{260} units/mL, and 3.5 volumes of NaOAc (pH 3.0, 0.1 M) was added. The products were incubated at room temperature for 1 h, and then 2.5 M NaOAc (pH 7.5) was added to a final concentration of ca. 0.35 M. The oligonucleotide was precipitated by addition of 5 volumes of cold (−20 °C) EtOH and collected by centrifugation.[19]

Desulfurization and Enzymatic Digestion. RP-HPLC-purified ISIS 2302 (10 A_{260} units) in water (100 μL) was added to 1 mL of a solution of iodine (0.2 g) and

(18) (a) Cheruvallath, Z. S.; Wheeler, P. D.; Cole, D. L.; Ravikumar, V. T. *Nucleosides Nucleotides* **1999**, 18, 484. (b) Cheruvallath, Z. S.; Carty, R. L.; Moore, M. N.; Capaldi, D. C.; Krotz, A. H.; Wheeler, P. D.; Turney, B. J.; Craig, S. R.; Gaus, H. J.; Scozzari, A. N.; Cole, D. L.; Ravikumar, V. T. *Org. Process Res. Dev.* **2000**, *4*, 199.

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N-methylimidazole (1 ml) in water (3 mL) and THF (16 mL). The products were incubated at 37 °C for 3 h and then concentrated to a volume of ca. 100 μL. Water (1 mL) was added and the mixture centrifuged. The supernatant was removed and concentrated to a volume of ca. 100 μL. An aqueous NaOAc solution (2.5 M, pH 7.5, 20 μL) was added and the DNA precipitated by addition of ethanol (1 mL). The DNA was washed with ethanol (1 mL) and dried. Snake venom phosphodiesterase (SVPDE) [5 μL of a standard solution in 100 mM trisHCl, 10 mM with respect to $MgCl_2$, pH 8.3 (2 units mL^{-1})] and alkaline phosphatase (AP) [5 μL of a standard solution in the same buffer (100 units mL^{-1})] were added to a solution of the oligonucleotide in 100 mM tris-HCl (tris(hydroxymethyl)aminomethane hydrochloride), 10 mM with respect to $MgCl_2$, pH 8.3 (200 μL), and the mixture was incubated at 37 °C for 16 h. The products were analyzed by RP-HPLC using a Phenomenex Luna C18 (5 μ) column eluted under a linear gradient of methanol in water (7 to 100% over 30 min).

Conclusions

Acrylonitrile (AN) adds to nucleobases of DNA under forcing conditions. The present study revealed that AN generated under the ammonolysis conditions employed for deprotection of synthetic phosphorothioate oligonucleotides is also capable of adduct formation with DNA bases. Studies on a variety of sequences indicated that, while AN is capable of adding to adenine, cytidine, and thymine residues, only the latter react quickly enough to form high levels of cyanoethyl adduct. This selectivity can be explained in part by the instability of AN in NH_4OH, by the relatively high acidity of thymine residues, and at least in the case of cytidine residues, by the fact that at the onset of ammonolysis these residues are protected by a benzoyl group. In the case of thymine the adduct has been identified as N^3-cyanoethylthymine and shown to be quite stable towards the ammonolysis conditions. The observation of +53 amu adducts in synthetic homopolymers of 2′-deoxyadenosine and 2′-deoxycytosine under standard conditions was unexpected; the structures of these adducts have not yet been determined. No evidence of addition to guanine residues in terms either of adduct formation or increased levels of depurination was observed. The inherent rate differences between the bases mean that in ISIS 2302 the vast majority of the ca. 2−3% of +53 adduct observed is composed of three compounds, and in each compound one of the three thymine residues is replaced by a N^3-cyanoethylthymine. A simple method to avoid cyanoethyl adduct formation has been developed. It was shown that removal of the cyanoethyl protecting groups with TEA in CH_3CN at room temperature prior to heating in ammonium hydroxide results in the production of synthetic oligonucleotides free from cyanoethylated thymine residues. This simple and cost-effective methodology has become part of our routine, large-scale oligonucleotide manufacturing process.

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