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Trichloroacetaldehyde modified oligonucleotides

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Abstract—Some commercial batches of dichloroacetic acid (DCA) contain traces of chloral (trichloroacetaldehyde). Using such DCA to effect detritylation during solid-phase oligonucleotide synthesis results in the formation of a family of process impurities in which the atoms of chloral (Cl_3CCHO) are incorporated between the 5′-oxygen and phosphorus atoms of an internucleotide linkage. The structure was elucidated by HPLC with UV and MS detection, digestion of the oligonucleotide, synthesis of model compounds, and ^{1}H and ^{31}P NMR spectroscopy. By understanding the chemistry behind its formation, we are now able to limit levels of this impurity in synthetic oligonucleotides by limiting chloral in DCA.

Synthetic oligonucleotides are widely used in molecular biology and diagnostics. Recently, DNA and RNA analogs have also emerged as potential drugs for treatment of diseases through antisense mechanisms of action.[1–4] The most advanced drug candidates are phosphorothioate oligonucleotides (PS-oligonucleotides) in which one of the non-bridging oxygens of the phosphate diester internucleotide linkage of DNA or RNA is replaced by sulfur. Automated synthesis of PS-oligonucleotides is normally performed on a solid support using commercially available nucleoside phosphoramidites as the starting materials.[5] The typical synthesis cycle (Scheme 1) consists of four chemical reactions that are separated by rinsing steps designed to remove excess reagents. The four reactions are:

- Acid-induced removal of the 4,4′-dimethoxytrityl (DMTr) protecting group to liberate the 5′-hydroxyl group of the support-bound oligonucleotide (reaction *a*).
- Extension of the oligonucleotide chain by coupling of a protected nucleoside phosphoramidite in the presence of a weak acid (reaction *b*).
- Incorporation of a sulfur atom by oxidative sulfurization of the trialkyl phosphite triester intermediate to form a phosphorothioate triester (reaction *c*).
- Capping of unreacted 5′-hydroxyl groups to prevent elongation of failure sequences (reaction *d*).

Repetition of this synthesis cycle allows for assembly of PS-oligonucleotides on scales ranging from a few nanomoles to several hundreds of millimoles. Cleavage and separation of the oligonucleotide from the solid support, deprotection steps, purification and isolation of the final product complete the synthesis (reactions *e*). This synthesis cycle was used to prepare the target PS-oligodeoxyribonucleotide **1**, the nucleotide sequence of which is shown here: PS-d(TCCGTCATCGCTCCTC AGGG).[6] Despite recent advances in oligonucleotide chemistry, the development of improved methods for the synthesis of therapeutic grade oligonucleotides remains an area of intense research.[7–13] During the course of studies aimed at optimizing synthesis of **1**, we observed the occurrence of low levels of an impurity that had a mass of 147 Da more than the desired product. In this report, we describe the structure elucidation of this component and suggest a potential mechanism of formation.

The strong anion exchange (SAX) HPLC chromatogram[14] of a batch of **1** is shown in Figure 1. The chromatogram shows a later-eluting impurity (arrowed) that accounted for 3% of the total UV area.

To facilitate identification of this component, we used preparative SAX chromatography[15] to obtain an enriched sample, which was then analyzed by HPLC with

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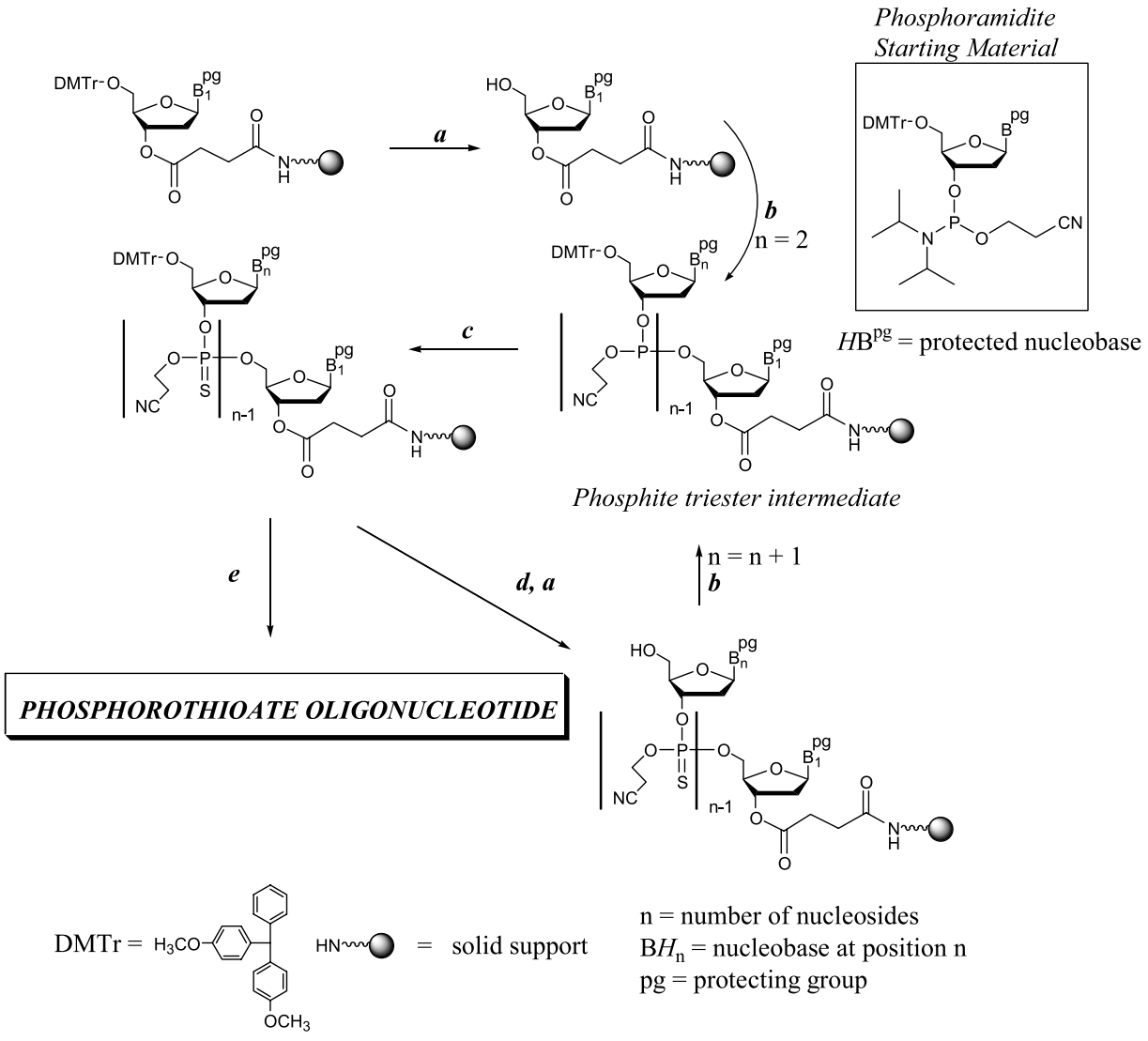

Scheme 1. Typical synthetic scheme for the solid-phase synthesis of phosphorothioate oligonucleotides.

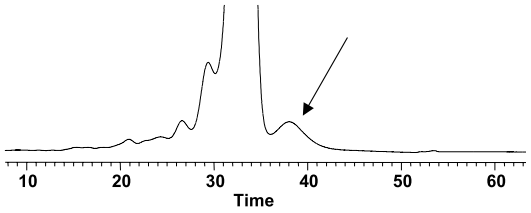

Figure 1. Strong anion exchange chromatogram of a batch of **1**.

UV and MS detection (HPLC–UV–MS).[16] The upper panel of Figure 2 shows the chromatogram obtained by HPLC–UV analysis. Two poorly resolved peaks were visible; the average mass spectrum of the earlier eluting peak (Fig. 2, middle panel) showed signals at m/z = 1269.0 and m/z = 1586.5 attributable to the −5 and −4 charge states, respectively, of **1** (MW Calcd: 6349.6, found 6350.0). The later-eluting peak gave a mass spectrum (Fig. 2, lower panel) that contained signals at m/z = 1298.5 and m/z = 1623.3, attributable to the −5 and −4 charge states of a component with a molecular weight of 6497.4, or 147.4 Da more than that measured for **1**.

We next converted the phosphorothioate diester linkages of the enriched sample to phosphate diester linkages using an iodine/*N*-methyl imidazole procedure[17] and analyzed the resulting mixture by HPLC–UV–MS.[16] As expected, the average mass spectrum of the main UV component (Fig. 3) showed signals that could be ascribed to the −5 and −4 charge states (m/z = 1207.9 and m/z = 1510.2, respectively) of the phosphate diester analogue of **1** (MW Calcd: 6045.6 Da, found: 6044.7). Also present were signals due to the −5 and −4 charge states (m/z = 1237.4 and m/z = 1547.0, respectively) of a component with a molecular weight of 6192, or 147 Da more than that found for desulfurized **1**. The fact that the mass difference between the two components was the

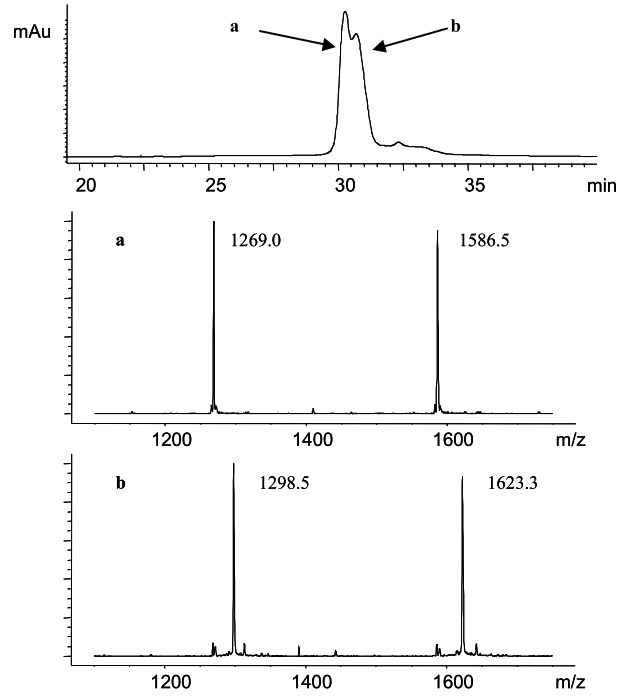

Figure 2. Upper panel: HPLC–UV trace of a sample enriched in the 147 Da impurity. Middle panel: The average mass spectrum under peak a. Lower panel: The average mass spectrum under peak b.

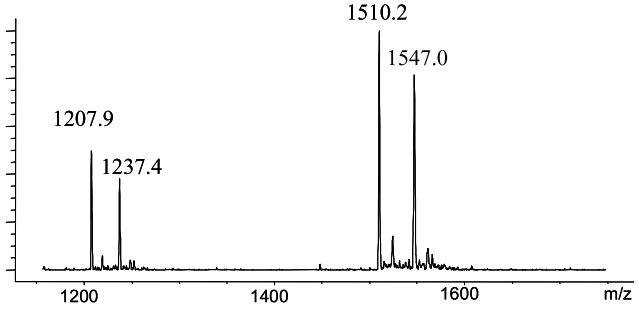

Figure 3. Mass spectrum of the sample enriched in the 147 Da impurity following desulfurization.

same following conversion to their phosphate diester analogues indicated that the impurity, like **1**, possessed 19 phosphorothioate internucleotide linkages.

We obtained further structural information by enzymatically digesting the desulfurized sample using snake venom phosphodiesterase and alkaline phosphatase. HPLC–UV analysis[18] of the digest showed, in addition to the four natural nucleosides [dC (4.6 min), dG (9.2 min), T (11.2 min), and dA (13.3 min)], a number of small, later-eluting peaks (Fig. 4). Particularly interesting were several groups of peaks that eluted between 19 and 25.5 min (labeled **a** to **g**).

HPLC–MS analysis[18] of these regions showed the presence of a number of different compounds that had molecular weights and isotopic distribution patterns consistent with those calculated for a series of dinucleo-

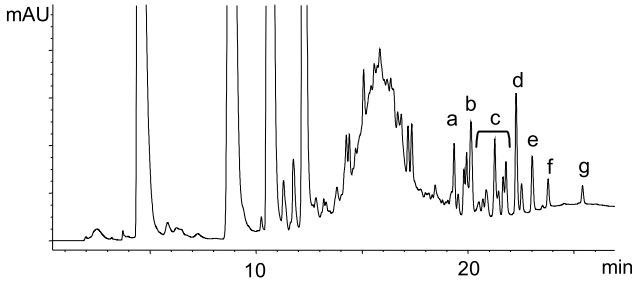

Figure 4. HPLC–UV analysis of the sample enriched in the 147 Da impurity following desulfurization and enzymatic digestion.

side phosphates modified by the addition of two carbon, one hydrogen, one oxygen, and three chlorine atoms.

For example, in region **a**, the observed masses and their distribution were consistent with those calculated for the −1 charge state of d(CpG), or d(GpC), plus C_2HOCl_3 (Fig. 5). Identical signals were noted in region **b**, along with others that could be attributed to modified d(CpC) and d(GpG). Region **c** contained masses attributable to modified d(CpG), d(CpA), d(ApG), and d(TpC)/d(CpT), while masses in regions **d** and **e** indicated the presence of modified d(TpC)/d(CpT). Finally, signals in regions **f** and **g** could be attributed to modified d(GpT) and d(ApT).[19] The simplest way in which the atoms 2C, H, O, and 3Cl can be added to an oligonucleotide is in the form of chloral (trichloroacetaldehyde), the most likely source of which is the DCA that is used to promote detritylation at the beginning of each synthetic cycle (Scheme 1, reaction *a*).[20] Seeking to establish a link between chloral and formation of the 147 Da impurity, we synthesized **1** by standard solid-phase phosphoramidite chemistry, using for the detritylation reaction a solution of DCA in toluene[9] to which we added 300 ppm chloral hydrate.[21] Under these conditions, the support bound oligonucleotide was exposed to approximately 1.2 molar equivalents of chloral over a period of 2 min during each detritylation reaction. Following synthesis and ammonolysis, the crude material

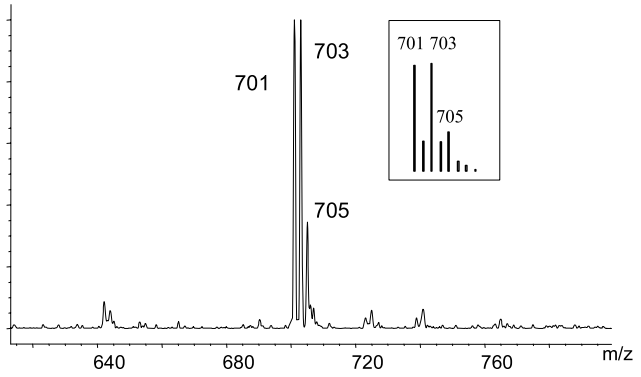

Figure 5. Mass spectrum of region **a** of the UV chromatogram shown in Figure 4. The mass spectrum was consistent with the addition of C_2HOCl_3 to dinucleoside phosphate d(CpG) or d(GpC). Inset: The calculated isotopic distribution pattern for d(CpG) + C_2HOCl_3, $[M-H]^-$.

was analyzed by HPLC–UV–MS.[16] The results of these analyses are shown in Figure 6.

The average mass spectrum under the main UV peak (lower panel) contained a set of signals (m/z = 1661.9, 1329.3, and 1107.7) that corresponded to the −4, −5, and −6 charge states, respectively, of DMTr-protected **1** (MW Calcd: 6651.8, found 6652.4) and another set (m/z = 1698.8, 1358.9, and 1132.2) attributable to the same charge states of the corresponding 147 Da impurity (MW 6799.3). These results confirmed the proposed link between chloral and formation of the 147 Da impurity of **1**. In fact, in this experiment, the extent of modification was sufficient to allow detection of a signal at m/z = 1388.2, consistent with the −5 charge state of a component that had added two molecules of chloral. By extracting and integrating the ion currents due to the −5 charge states of the three components, we estimated they were present in a 1.00:0.37:0.08 ratio, suggesting that about 2% of molecules were modified during each detritylation step under these conditions.[22]

We next sought to produce simple model compounds that we anticipated would facilitate characterization by NMR spectroscopy. Treatment of the four, support-bound 2′-deoxynucleosides with a solution of chloral hydrate (0.15% w/v) in DCA and toluene, followed by ammonolysis at 60 °C for 5 h, however, gave only unmodified nucleosides (data not shown). Reasoning that steps subsequent to the detritylation reaction might somehow stabilize the chloral adduct, we treated thymidine-loaded support under the same conditions, this time following the detritylation reaction with coupling, using 5′-*O*-DMTr-N^6-benzoyl-2′-deoxyadenosine-3′-(2-cyanoethyl)-*N*,*N*-bis(diisopropyl) phosphoramidite, oxidation and detritylation steps. The support-bound material was treated with ammonium hydroxide and the crude products analyzed by HPLC–UV–MS[18] (Fig. 7).

The HPLC–UV chromatogram of the reaction mixture showed, in addition to d(ApT) (t_R = 15.0 min), the presence of two later-eluting impurities (t_R = 23.2 and 25.2 min) that accounted for 23% of the total UV area (Fig. 7, upper panel). Mass spectral analysis of the t_R = 23.2 min component (Fig. 7, lower panel) showed signals at m/z = 700, 702, and 704 that were consistent with those expected for d(ApT) following addition of chloral. The signals at m/z = 565 and 567, and at m/z = 574 and 576, respectively, were attributed to loss of adenine and thymine in the gas phase.[23] The average mass spectra of the t_R = 23.2 min and 25.2 min components were identical. The chromatographic, UV, and mass spectral properties of these components and the modified d(ApT) dimer detected following desulfurization and enzymatic digestion of the sample enriched in the 147 Da impurity of **1** were also identical. With the identity of the synthetic and authentic dimers confirmed as the same, it was necessary to isolate sufficient quantify of the former to allow an unambiguous structural determination by ^{1}H and ^{31}P NMR analysis.[24] The results of these analyses led to the structure shown in Chart 1.

The spectra were consistent with those expected of a diastereoisomeric mixture of compounds in which the atoms of chloral are inserted between the 5′-oxygen and phosphorus atoms of the internucleotide linkage of 2′-deoxyadenylyl-(3′ → 5′)thymidine as pictured in Chart 1.[25] The acetal proton of each diastereoisomer appeared as a doublet at 5.58 and 5.51 ppm ($^3J_{PH}$ = 7.6 and 7.1 Hz, respectively).

Chloral reacts with the sulfur atom of phosphorothioate diesters and α-thiotriphosphates to give the corresponding phosphates,[26] with amides and amines to give the corresponding hemiaminals,[27,28] and with alcohols and diols to give the corresponding hemiacetals[29] and acetals,[30] respectively. In the present case based in solid-

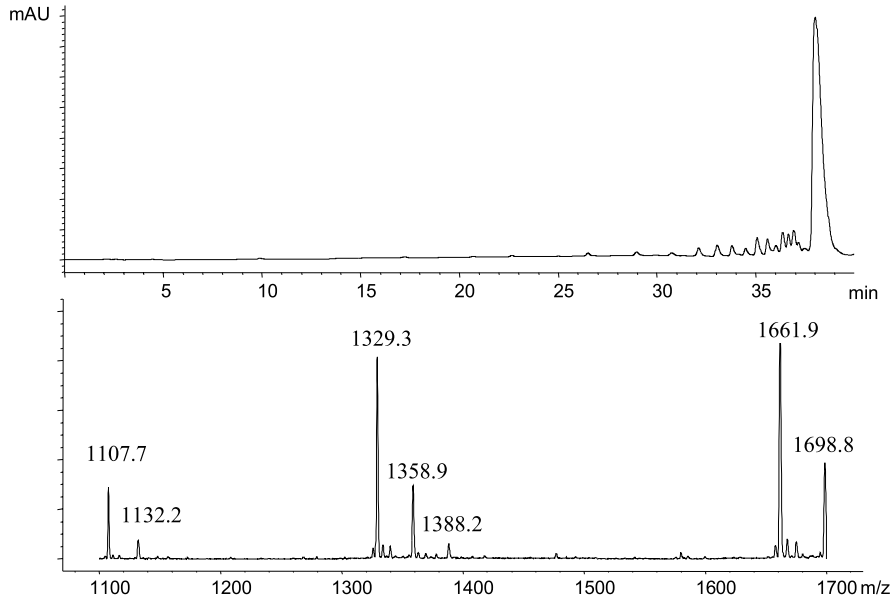

Figure 6. Upper panel: HPLC–UV chromatogram of crude DMTr-protected **1** synthesized in the presence of chloral. Lower panel: The average mass spectrum of the main UV peak.

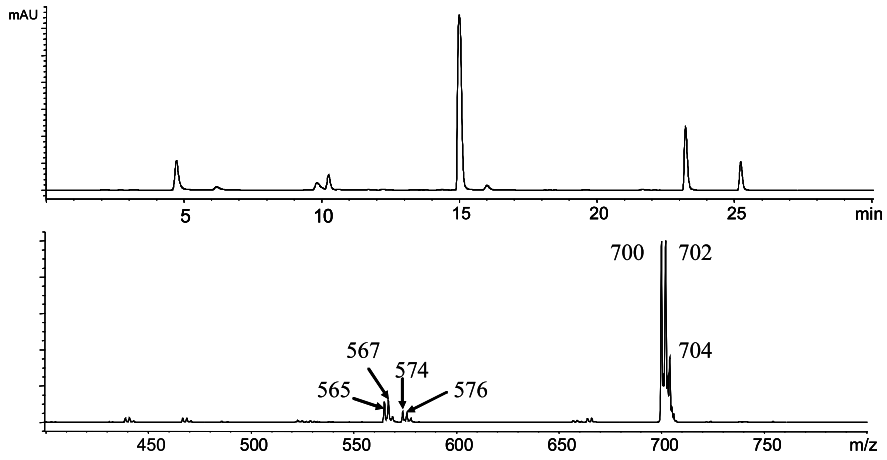

Figure 7. Upper panel: HPLC–UV trace of d(ApT) made in the presence of chloral hydrate. Lower panel: Average mass spectrum of the t_R = 23.2 min component.

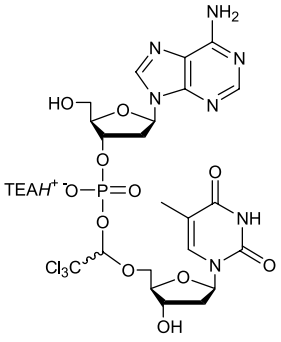

Chart 1. Structure of chloral-modified d(ApT).

phase oligonucleotide chemistry, we provide evidence suggesting that low levels of chloral in the DCA used to promote detritylation lead to the formation of a family of oligonucleotides in which chloral is inserted between the 5′-oxygen and phosphorus atoms of an internucleotide linkage. Most probably this reaction proceeds by way of an acid-catalyzed acetalation reaction that gives a 5′-terminal hemiacetal, the hydroxyl function of which can react further with an activated phosphoramidite in the subsequent coupling reaction. The coupling step stabilizes the product toward ammonia treatment, presumably by preventing degradation via the haloform reaction, while the electron-withdrawing nature of the trichloromethyl group prevents hydrolysis during subsequent acidic steps.[31]

The above observations, of course, suggest that some commercially available lots of DCA are contaminated with traces of chloral. By our initial SAX analysis of **1** (Fig. 1), we estimated that the 147 Da impurity was present at about the 3% level, much less than the approximately 40% that was formed by adding 300 ppm of chloral hydrate to the detritylation solution. This suggested that the DCA used in our initial synthesis of **1** contained a very small amount of chloral. Unfortunately, we did not have access to this particular lot of acid and so were unable to determine whether it contained chloral. A survey of our drug substance inventory, however, revealed several batches of phosphorothioate oligonucleotides other than **1** that also contained the corresponding 147 Da impurity, for which we were able to locate retained samples of DCA. Analysis of these lots by gas chromatography confirmed they contained traces of chloral.[32] The amount of 147 Da adduct formed will undoubtedly depend on the level of chloral, the concentration of DCA in the detritylation solution, the volume of solution used, and the detritylation time. Our results employing 10% w/v DCA in toluene[9] suggest that using acid containing 100 ppm chloral (or 10 ppm of the detritylation solution) leads to slightly less than 1% of the 147 Da impurity under our standard detritylation parameters. We have also demonstrated that atmospheric pressure distillation of DCA is sufficient to reduce chloral to undetectable levels.[33]

The above results highlight the power of HPLC–UV–MS techniques for the routine analysis of phosphorothioate oligonucleotides intended for use as therapeutics. In addition, they indicate that the synthesis of high quality oligonucleotides is dependent not only on a thorough understanding of the manufacturing process, but also on detailed knowledge and control of the raw material supply chain. While it is certain that the quality of most drug substances, including **1**, benefit from post-synthetic purification steps, it is often the case that avoiding formation of an impurity is a more efficient strategy. Because of the work described in this paper, we are now able to employ this second approach, and currently control levels of the 147 Da by controlling chloral in DCA.

Acknowledgments

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14. Analytical SAX chromatography was performed using a Resource Q column (1 mL, Pharmacia). The column was held at 60 °C and eluted over 40 min with a linear gradient of NaCl (1–2 M) in 0.1 M sodium phosphate (pH 11.5).
15. Preparative SAX chromatography was performed using a Q15HR column (50 × 100 mm, Waters). The sample was loaded onto the column in 0.1 M sodium phosphate (pH 11.5), then eluted with a gradient of NaCl in methanol and 0.1 M sodium phosphate (pH 11.5).
16. HPLC–UV–MS analysis of oligonucleotides was performed on an Agilent MSD1100 instrument. Separation was achieved using a C18 column held at 50 °C and eluted with a gradient of acetonitrile in aqueous tripentylammonium acetate. The UV detector was set at 260 nm and mass spectra recorded from $m/z = 1000$ to 1700 in the negative ionization mode.
17. Isolated impurity (10 A_{260} units) in water (100 μL) was added to 1 mL of solution of iodine (0.2 g) and *N*-methylimidazole (1 mL) in water (3 mL) and THF (16 mL). The products were incubated at 37 °C for 3 h, then concentrated to a volume of 100 μL. Water (1 mL) was added and the mixture centrifuged. The supernatant was removed and concentrated to a volume of 100 μL. Aqueous NaOAc solution (2.5 M, pH 7.5, 20 μL) was added and the products precipitated by addition of ethanol (1 mL) then washed with ethanol (1 mL) and dried. Snake venom phosphodiesterase [from *crotalus adamanteus*, 5 μL of a solution in 100 mM Tris–HCl, 10 mM with respect to $MgCl_2$, pH 8.3 (2 U/mL)] and alkaline phosphatase [AP, 5 μL of a solution in the same buffer (100 U/mL)] were added to a solution of the precipitate in 100 mM Tris–HCl (10 mM with respect to $MgCl_2$, pH 8.3, 200 μL) and the mixture incubated at 37 °C for 16 h. (Schuette, J. M.; Srivatsa, G. S.; Cole, D. L. *J. Pharm. Biomed. Anal.* **1994**, 12, 1345).
18. HPLC–UV–MS analysis of enzyme digest samples was performed on an Agilent MSD1100 instrument. Separation was achieved using a C18 column held at 40 °C and eluted with a gradient of acetonitrile in aqueous triethylammonium formate. The UV detector was set at 260 nm and mass spectra recorded from $m/z = 400$ to 800 in the negative ionization mode.
19. **1** can give rise to the following dinucleoside phosphates with the stated frequencies: d(CpG), 2; d(CpC), 2; d(GpG), 2; d(TpC), 5; d(CpA), 2; d(GpC), 1; d(GpT), 1; d(ApT), 1; d(CpT), 2; d(ApG), 1. Of these ten, d(CpG) and d(GpC) are isobaric, as are d(TpC) and d(CpT).
20. DCA is commonly produced commercially by hydrolysis of dichloroacetyl chloride, which itself may contain low levels of chloral (V.T. Ravikumar, personal communication).
21. **1** was synthesized on a Pharmacia OligoPilot I DNA/RNA synthesizer using 30 μmol of Primer HL 30 2′-deoxyguanosine loaded support. Detritylation was effected by treatment with a 3% v/v solution of dichloroacetic acid in toluene (Ref. 9) containing 300 ppm chloral hydrate for 2 min at a flow rate of 10 mL/min. Phosphoramidites were dissolved to a nominal concentration of 0.2 M in anhydrous CH_3CN and activated with two volumes of a 0.45 M solution of 1-*H*-tetrazole in CH_3CN; couplings were performed in the recycle mode with a contact time of 5 min. Sulfurization was achieved by treatment with 4 mL of a 0.2 M solution of phenylacetyl disulfide in CH_3CN-3-picoline (1:1 v/v) for a contact time of 1 min (Ref. 7). Capping was performed using 4 mL of a 1:1 v/v mixture of acetic anhydride in CH_3CN (1:4 v/v) and *N*-methylimidazole-pyridine in CH_3CN (2:3:5 v/v/v) for a contact time of 1 min. Following chain assembly, the support-bound oligonucleotide was treated with concentrated ammonium hydroxide (NH_4OH, 5 mL) for 8 h at 60 °C. The products were filtered and the filtrate evaporated under reduced pressure. The residue was dissolved in water (1 mL) and an aliquot (0.2 mL) purified by reversed-phase HPLC. Concentration of the fractions eluted with 60% CH_3CN in 0.1 M TEAA and detritylation of the residue (0.1 M sodium acetate pH 3, 40 min, 25 °C), followed by precipitation using 5 volumes of cold (−20 °C) ethanol gave the product as a white solid.
22. Assuming reaction occurs equally during each detritylation step, the products will be distributed according to the binomial expansion $(x + y)$,[19] where x and y are the decimal fractions of chains that do not and do react, respectively during each step. For example, if 2% of chains are modified during each detritylation step, the ratio unmodified:singly modified:doubly modified is calculated at 1:0.38:0.07.
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24. The crude product (ca. 1.2 mmol) in water (50 mL) was loaded onto a C18 column and the column eluted with a gradient of acetonitrile in 20 mM NH_4OAc. The fraction eluted with 25–35% acetonitrile was concentrated under reduced pressure. A solution of the residue in water (10 mL) was freeze-dried to give chloral modified d(ApT) as a colorless glass (185 mg).
25. ^{1}H NMR (CD_3OD) δ: 8.30 and 8.20 (1H total, s, dA H_8), 8.16 and 8.13 (1H total, s, dA H_2), 7.70 and 7.60 (1H total, d, $J = 1.2$ and 1.1 Hz, respectively, T H_6) 6.46–6.28 (2H, m, dA $H_{1'}$ and T $H_{1'}$), 5.67 and 5.61 (1H total, d, $^3J_{PH} = 7.5$ and 7.2 Hz, respectively, acetal CH), 5.15–5.05 (1H, m, dA $H_{3'}$), 4.68–3.99 (7H, m, dA $H_{4'}$, T $H_{3'}$, T $H_{4'}$ and T H_5), 3.85–3.80 (2H, m, dA H_5), 2.96–2.63 (2H, m, dA $H_{2'}$), 2.24–2.13 (2H, m, T $H_{2'}$), 1.88 and 1.77 (3H total, d, $J = 1.1$ and 1.1 Hz, T-CH_3) ^{31}P NMR (CD_3OD) δ: −3.44 and −3.82 HRMS $[MH]^+$ Calcd for $C_{22}H_{27}N_7O_{11}Cl_3P$: 702.0644, found: 702.0642.

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31. Trichloroacetal based protecting groups are resistant to acid hydrolysis and are normally cleaved by reduction (see Ref. 30).
32. Chloral levels ranged from none detected (limit of detection = 3 ppm) to 120 ppm.
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