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# Separation of synthetic phosphorothioate oligodeoxynucleotides from their oxygenated (phosphodiester) defect species by strong-anion-exchange high-performance liquid chromatography

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ABSTRACT

A method is described which separates synthetic deoxynucleotide phosphorothioates from their oxygenated (phosphodiester, "P=O") defect species by strong-anion-exchange chromatography, using novel "soft-base" anionic eluents. The method enables the qualitative and quantitative assessment of P=O content in phosphorothioate DNA, and represents a rapid and sample-conserving alternative to $^{31}$P-NMR.

## INTRODUCTION

Synthetic DNA analogues, particularly those which have been modified at the phosphorus internucleotidic linkage, now have become indispensable tools for research in the field of "anti-sense DNA," where blockage of translational gene product (protein) formation can be effected by Watson–Crick binding of a short (15–30 base) strand of DNA to an appropriate complementary sequence within the target mRNA. An analogue showing promise as a potential therapeutic agent is the phosphorothioate [1] congener of DNA, where sulfur replaces the non-bridging oxygen at the pentavalent phosphorus nexus of the polymer (Fig. 1).

The Therapeutics Group at Applied Biosystems has been investigating large-scale synthesis of synthetic DNA, particularly the phosphorothioate analogs. As a potential drug, such material must be rigorously characterized as to its chemical nature, which, in turn, demands stringent analytical criteria for purity (or homogeneity).

High-performance ion-exchange chromatography (IEC) is being increasingly used for relatively rapid qualitative and quantitative profiling of synthetic DNA [2–5]. Agrawal *et al.* [6] have recently reported on a high-performance liquid chromatographic (HPLC) method for characterization of phosphorothioate DNA, though only for octamers. We have extended the use of IEC to the analysis of 20- to 27-mer phosphorothioates, and wish to report a most unusual and unexpected finding. Our synthetic methodology leading to the desired phosphorothioates requires oxidative sulfurization [7,8] at the phosphite linkage during each cycle of synthesis; however, a fractional amount (0.5–1%) of the oxygenated (phosphodiester) species is produced concomitantly. Cumulatively, this incompleteness of sulfurization leads to the formation of significant populations of oligonucleotide phospho-

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S-d($C_{12}G_5T_{10}$) S-d($A_3C_7G_7T_4$)

1 2

S-d($A_1C_8G_2T_9$) S-d($C_{13}G_3T_4$)

3 4

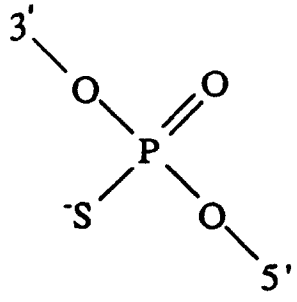



Fig. 1. Empirical formulae for synthetic phosphorothioate DNA used in this study, and detail of phosphorothioate moiety.

rothioates containing phosphoric acid diester ("P=O") linkages. We have developed an ion-exchange method of high-performance chromatographic separation of these various defect species using novel ("soft-base") anionic eluents, and we shall demonstrate the utility of the assay in obtaining accurate profiles of "P=O" contributions to several phosphorothioate synthetic analogues. Relative to $^{31}$P-NMR spectroscopy, the conventional method of ascertaining the molecular environment about the phosphate linkage in DNA [9], our IEC method is (a) readily automated, and (b) requires a thousand-fold less sample.

## EXPERIMENTAL

### *Chemicals and reagents*

All HPLC buffer salts were reagent grade or better supplied by Aldrich (Milwaukee, WI, USA). Acetonitrile (HPLC grade) was from Burdick and Jackson (Muskegon, MI, USA). All gel electrophoresis reagents came from IBI (New Haven, CT, USA). Stains-all dye was from Eastman Kodak (Rochester, NY, USA).

### *DNA synthesis and purification*

Phosphorothioate DNA was prepared on either an Applied Biosystems Model 380B or 390Z Automated DNA Synthesis instrument using standard protocols [10] as suggested by the manufacturer, except with the substitution of 3H-1,2-benzothiol-3-one 1,1-dioxide [7] (or an equivalent sulfur-donor reagent) for iodine, and reversing the normal oxidation-then-cap sequence within each cycle. The crude DNA is subsequently purified by reversed-phase preparative chromatography as its 5'-O-dimethoxytrityl derivative according to published methods [11]. Following detritylation and sodium chloride–ethanol precipitation, the recovered phosphorothioate DNA is freeze-dried to a powered solid.

### *NMR*

$^{31}$P-NMR was performed on the following two instruments with their respective operating conditions: (1) JEOL GSX-500; resonance frequency, 202.45 MHz; acquisition time, 0.655 s; pulse delay, 6 s; pulse width, 5 $\mu$s (45°) and (2) Varian Unity 3000; resonance frequency, 121.42 MHz; acquisition time, 1.6 s; pulse delay, 0 s; pulse width, 11 $\mu$s (90°).

### *Ion-exchange chromatography*

The HPLC instrumentation was as follows: Series 410 BIO LC System, ISS-200 Auto-sampler, Model 1020 Data System (Perkin-Elmer, Norwalk, CT, USA); 759A UV Detector (Applied Biosystems, Foster City, CA, USA). The columns were PL-SAX (Polymer Labs., Church Stretton, UK) strong-anion-exchanger, 10 $\mu$m, 100 Å porosity, 15 cm × 7.5 mm I.D., and Nucleogen-DEAE 60-7 (Macherey-Nagel, Düren, Germany), 7 $\mu$m, 60 Å porosity, 12.5 cm × 4 mm I.D. Mobile phases: A, 50 m*M* ammonium phosphate pH 8.2–$CH_3CN$ (95:5, v/v); B, 1.5 *M* potassium bromide in 50 m*M* ammonium phosphate pH 6.7–$CH_3CN$ (80:20, v/v); C, $CH_3CN$; D, 1.0 *M* sodium thiocyanate in 50 m*M* ammonium phosphate pH 8.2. Separation of

the DNA species was achieved by gradient elution, and the various gradient profiles used are summarized in Table I. The UV monitor was set at 260 nm for gradients I and III and 280 nm for gradient II.

*Gel electrophoresis*

Purified deoxyoligonucleotides (*ca.* 0.05–0.1 optical density units (O.D.)/lane) were analyzed by polyacrylamide gel electrophoresis (PAGE) on 15 × 15 cm gels (20%T, 5%C[a]) using 40 m*M* Tris–borate buffer at pH 8.3. Bands were visualized by staining with Stains-all according to the manufacturer's suggested protocol[b] and quantified by scanning densitometry using a Model 300S Computing Densitometer (Molecular Dynamics, Sunnyvale, CA, USA).

TABLE I

GRADIENT PROGRAMS USED IN THIS STUDY

Mobile phases are identified in the Experimental section.

| Gradient | Time (min) | Flow-rate (ml/min) | Mobile phase (%) | | | |
|---|---|---|---|---|---|---|
| | | | A | B | C | D |
| I | Start | 1.5 | 50 | 30 | 20 | – |
| | 48 | 1.5 | 0 | 80 | 20 | – |
| | 4 | 1.5 | 0 | 80 | 20 | – |
| | 10 | 2.0 | 50 | 30 | 20 | – |
| II | Start | 1.5 | 50 | – | 20 | 30 |
| | 48 | 1.5 | 10 | – | 20 | 70 |
| | 4 | 1.5 | 10 | – | 20 | 70 |
| | 10 | 2.0 | 50 | – | 20 | 30 |
| III | Start | 1.5 | 80 | 0 | 20 | – |
| | 70 | 1.5 | 0 | 80 | 20 | – |
| | 10 | 2.0 | 80 | 0 | 20 | – |

## RESULTS AND DISCUSSION

Our orifinal intent was to evaluate various silica- and polymer-based anionic exchange media for their suitability in assessing synthetic phosphorothioate chain-length homogeneity. We assumed only marginal and not unexpected chromatographic differences between the phosphorothioates and their respective phosphodiester congeners; essentially the phosphorothioates as a class are more lipophilic than phosphodiesters [6,12], and somewhat stronger retention of the former was anticipated. However, phosphorothioates **1**–**4** *could not* be eluted from either weak- (WAX) or strong- (SAX) anion-exchange supports using conventional salt gradients, *e.g.* 0–2 *M* sodium chloride, 0–1.5 *M* ammonium sulfate, etc. Neither increasing the mole-fraction of the organic modifier nor varying the type of modifier (acetonitrile, methanol, formamide) had any effect on elution profiles. Conventional denaturants such as 7 *M* urea also were without effect. The corresponding phosphodiester compounds eluted smoothly as expected, however (data not shown).

Changing the eluting anion to either bromide or thiocyanate did in fact promote elution of the phosphorothioates under gradient conditions (Fig. 2). The profiles of compounds **1** and **2** suggested substantial presence of so-called "deletion" or "failure" sequences (a consequence of poor coupling yields and/or incomplete detritylation at the 5′-terminus during each cycle of automated solid-phase synthesis [10]; however, when compound **2** ("high P=O", four components by IEC) was analyzed by PAGE–scanning densitometry, essentially a single band was detected (Fig. 3), inconsistent with a sus-

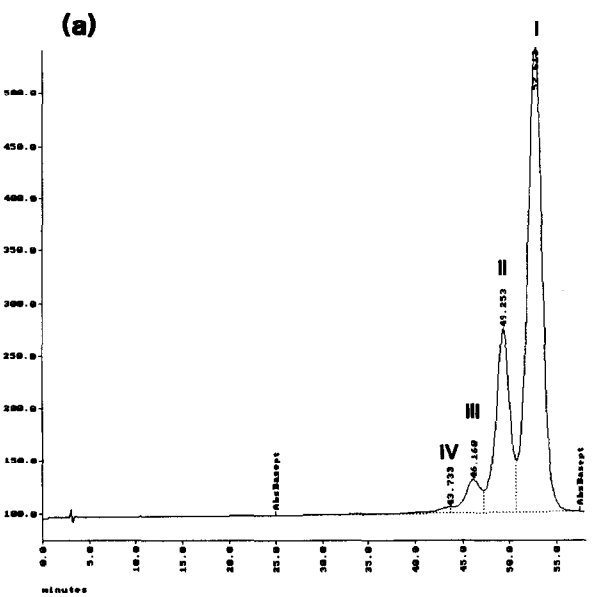



Fig. 2.

*(Continued on p. 38)*

[a] T = [g acrylamide + g N,N′-methylenebisacrylamide (Bis)]/100 ml solution; C = g Bis/% T.

[b] Kodak Product Publication Number JJ-11.

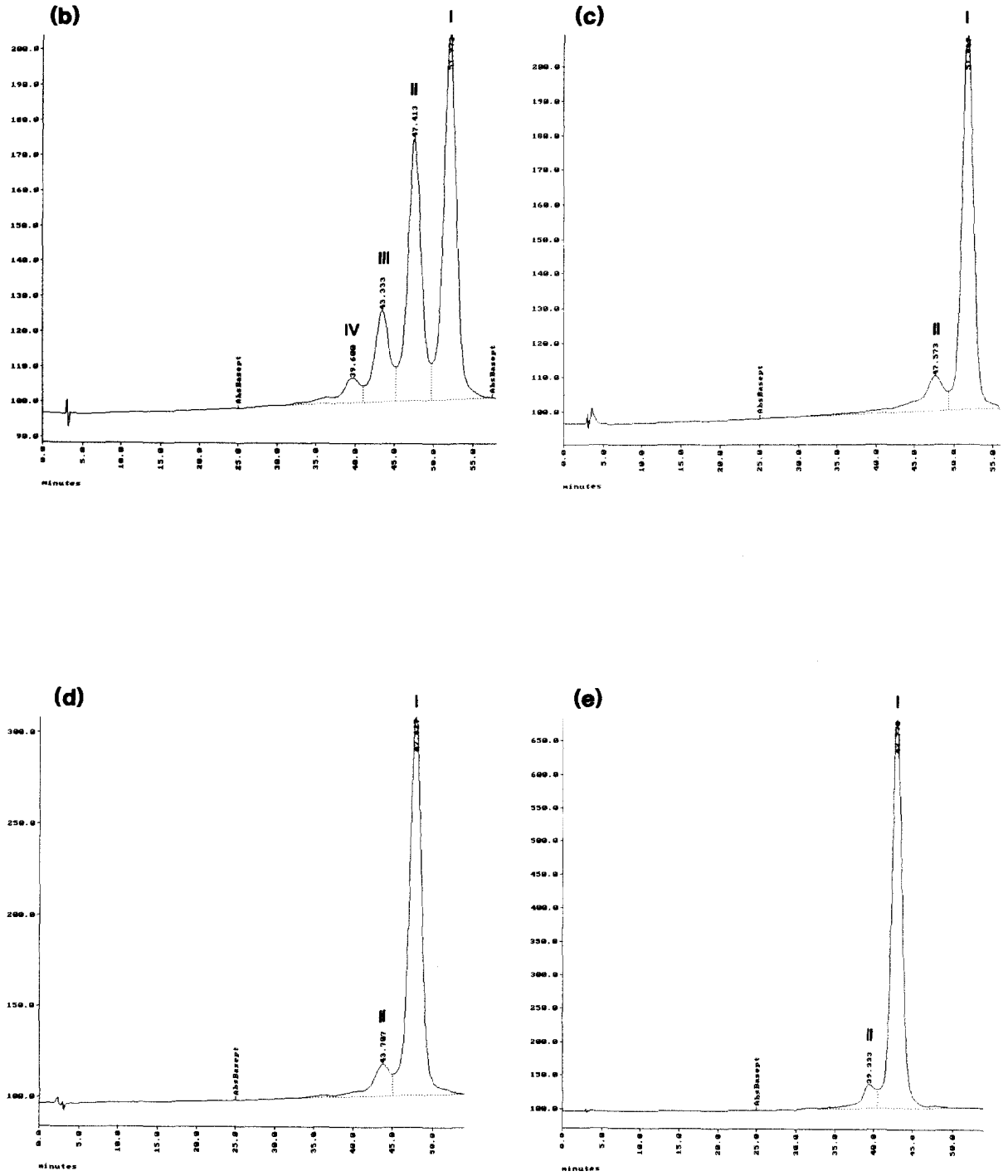
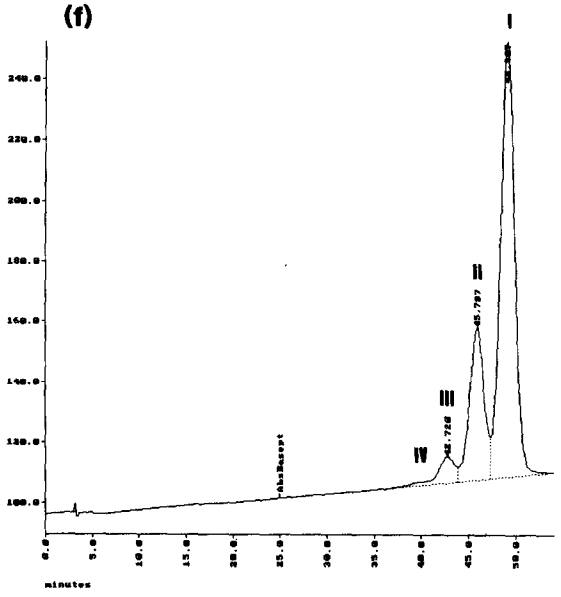



Fig. 2.



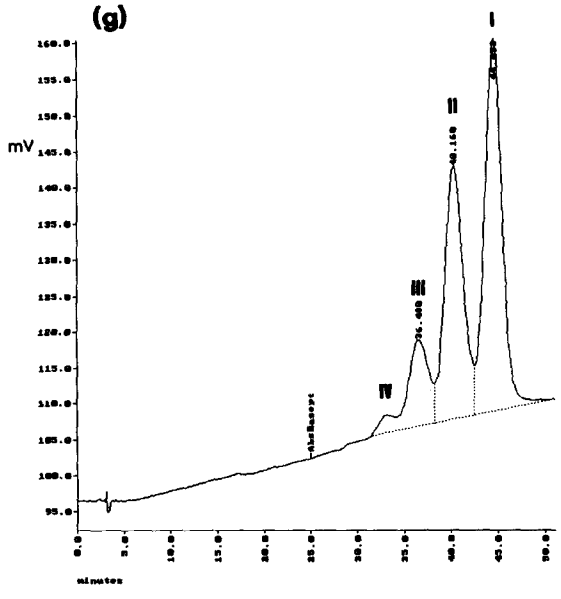




Fig. 2. IEC chromatograms of phosphorothioate DNA: (a) **1**, (b) **2**, "high P=O", (c) **2**, "low P=O," (d) **3**, (e) **4**, conditions per gradient I; (f) **1**, (g) **2**, "high P=O", conditions per gradient III. Roman numerals indicate constitutive peaks of each analogue (see Tables I and II). Column: PL-SAX at 1.5 ml/min flow-rate. Approximately 10–20 $\mu$g of DNA was injected on-column per analysis.

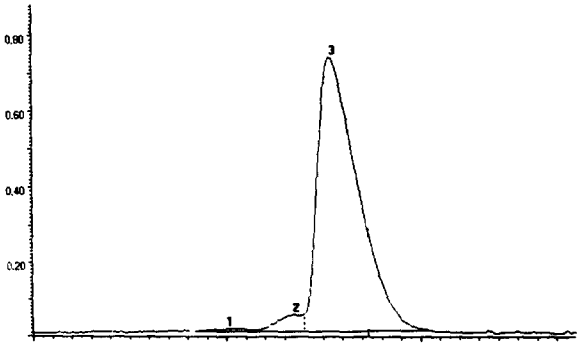



Fig. 3. Computing densitometric scan of compound **2** ("high P=O," see Fig. 2b) following slab-gel electrophoresis and staining. Migration is from right (top) to left (bottom). Band 3 = full-length 21-mer (97%); band 2 = 20-mer ($n-1$) impurity (2.8%); band 1 = 19-mer ($n-2$) impurity (0.2%).

pected high degree of chain-length heterogeneity. IEC was clearly revealing heterogeneity within the molecule, though *not* as a consequence of length variability.

*Isolation and $^{31}$P-NMR spectroscopy of IEC-resolved components*

To examine further the nature of the constitutive elements forming **1** and **2**, approximately 50 mg of each were taken for preparatieve IEC, where 10 mg aliquots were chromatographed on the 15 cm × 7.6 mm I.D. PL-SAX column using Gradient II as described under Experimental. The largest three components (peaks I, II and III) from each compound

TABLE II

DETERMINATION BY $^{31}$P-NMR OF % P=O OCCURRING IN CONSTITUTIVE SPECIES OF SELECTED PHOSPHOROTHIOATE DNA ANALOGUES (**1** AND **2**)

| Peak | %P=O | | | |
|---|---|---|---|---|
| | Found | | Theoretical | |
| | **1** | **2** | **1** | **2** |
| I | 0.0[a] | 0.1[a] | 0.0 | 0.0 |
| II | 5.1[a] | 3.6[b] | 3.8 (1/26) | 5.0 (1/20) |
| III | 8.8[a] | 7.7[b] | 7.7 (2/26) | 10.0 (2/20) |
| IV | N.D.[c] | N.D. | 11.5 (3/26) | 15.0 (3/20) |

[a] 500 MHz, quantified by peak height.
[b] 300 MHz, quantified by resonance integral (area).
[c] N.D. = Not determined.

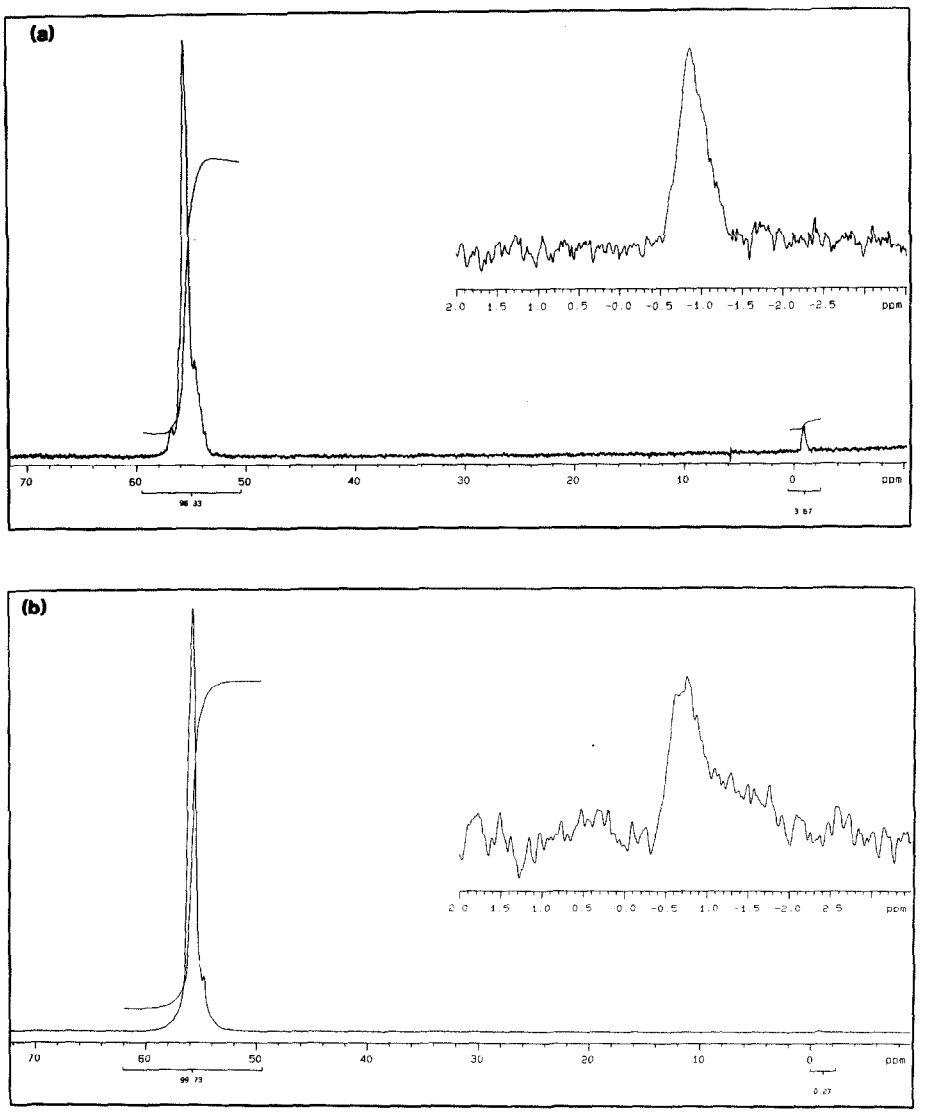



Fig. 4. $^{31}$P-NMR spectra of (a) **2**, "high P=O," and (b) **3**. Instrument: Varian Unity 3000, conditions per Experimental. Signal at 54–56 ppm is phosphorothiate P=S, and signal at −1 to −3 ppm is phosphodiester P=O.

were accumulated until 2–12 mg each were recovered. Following desalting and quantification by UV spectrometry the isolated materials (80–95% pure) were lyophilized, then reconstituted in HPLC-grade water or deuterium oxide for $^{31}$P-NMR studies. Table II summarizes the results for Peaks I, II and III of **1** and **2**, respectively.

Resonances in the $^{31}$P-NMR are well-resolved and conclusive for, (1) $(RO)_2$ P=O($S^-$), 50–60 ppm, and (2) $(RO)_2$ P=O($O^-$), (−)5–0 ppm [13]. Reference $^{31}$P-NMR Spectra for **1** and **2** are shown in Fig. 4. These data clearly show that the IEC profiles are in fact indicating the presence of oxygenated defect species of each parent phosphorothioate, and moreover, the distribution of these defect species can be described mathematically as terms of the binomial expansion series, $(x+y)^n$.

TABLE III

COMPARISONS BETWEEN CALCULATED AND ACTUAL VALUES OF OXYGENATION DISTRIBUTION AT INTERNUCLEOTIDIC PHOSPHATE LINKAGES OF SEVERAL DNA PHOSPHOROTHIOATE ANALOGUES

Calculated values were found by solving the first four terms of the general equation $(x + y)^n$ as developed in the text, where $x$ (P=S) and $y$ (P=O) were obtained from $^{31}$P-NMR[a], and actual values were determined by IEC.

| Compound | P=S[a] | $(P=S)_n$, Peak I (%) | | $(P=S)_{n-1}$, $(P=O)$, Peak II (%) | | $(P=S)_{n-2}$, $(P=O)_2$, Peak III (%) | | $(P=S)_{n-3}$, $(P=O)_3$, Peak IV (%) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Calc. | Found | Calc. | Found | Calc. | Found | Calc. | Found |
| 1 | 99.3 | 83.3 | 83.7 | 15.3 | 14.1 | 1.35 | 1.9 | 0.07 | 0.3 |
| 1 | 98.3 | 64.03 | 64.5 | 28.8 | 29.0 | 6.2 | 5.6 | 0.9 | 0.8 |
| 1 | 98.0 | 59.1 | 58.1 | 31.4 | 29.4 | 8 | 9.8 | 1.3 | 2.7 |
| 2 | 99.6 | 92.3 | 90.0 | 7.4 | 7.9 | 0.3 | $\leqslant 2$ | $\leqslant 0.1$ | $<0.5$ |
| 2 | 97.3 | 57.8 | 52.4 | 32.1 | 33.5 | 8.5 | 11.3 | 1.4 | 2.8 |
| 3 | 99.7 | 94.4 | 93.8 | 5.4 | 5.9 | $<0.2$ | $<0.5$ | $<0.1$ | $<0.5$ |
| 4 | 99.7 | 94.4 | 93.0 | 5.4 | 7.0 | $<0.2$ | $<0.5$ | $<0.1$ | $<0.5$ |

[a] From $^{31}$P-NMR, where % P=S = 100 × integral area (P=S)/integral area [(P=S) + (P=O)], and % P=O = 100 × integral area (P=O)/integral area [(P=S) + (P=O)].

*Mathematical depiction of peak profiles*

Our chemical pathway for phosphorothioate DNA synthesis requires oxidative sulfurization at the tervalent phosphorus (phosphite) intermediate at each cycle of deoxynucleotide monomer addition. Due to adventitious chemical side-reactions, a certain (presumably constant) proportion of phosphite is also converted to the oxo-form [8] per cycle. Letting $x$ and $y$ be the mole-fractions of phosphorothioate and phosphoric acid diester linkages, respectively, then for a 27-mer (26 internucleotidic linkages) $(x+y)^{26} = x^{26} + 26x^{25}y + 325\,x^{24}y^2 + 2600\,x^{23}y^3 + \ldots$ etc. At 98% overall sulfurization ($x = 0.98$) and 2% oxygenation ($y = 0.02$), the numerical values of the first four terms in the binomial expansion are 0.591 + 0.341 + 0.08 + 0.013. The first term represents the relative abundance of the fully thiolated (P(=S) material, the second term represents the relative abundance of the aggregate of all species containing a *single* oxygen, the third term represents all species containing *two* oxygens, the fourth term represents all species containing *three* oxygens, and so forth. Further correlations between $^{31}$P-NMR and IEC data are shown in Table III, where very good agreement between expected and actual IEC profiles was obtained. Therefore, by examination of the IEC profile of a synthetic phosphorothioate DNA, one may be able to compute the *net* or *aggregate* P=O contribution to the molecule using the general formula: $\%P=S = (\text{peak height}/100)^{1/n-1} \times 100$, where $n$ = number of deoxynucleotide residues and peak height is that of the last eluted peak in the chromatogram, and $\%P=O = 100 - (\%P=S)$.

*General comments*

The ability of bromide and thiocyanate to elute smoothly phosphorothioate DNA (16 to 27 bases) from WAX or SAX media, as opposed to the general ineffectiveness of traditional high-salt gradients, may be rationalized by invoking the principle of "hard" *vs.* "soft" bases [14] (or nucleophiles). The sulfur anion is regarded as a "soft" base [15], and, indeed, the net negative charge residing within each phosphorothioate moiety appears to be principally located on sulfur [16]. This suggests that the strong ion-pair formed between the thiolo (−) and quaternary ammonium (+) exchanger can be most efficiently disrupted by a "soft" competitive anionic species, such as thiocyanate or bromide. However, we did note that the latter salts were equally effective (at low molarity) in displacing "natural" phosphodiester congeners of compounds **1–4** (data not shown), implying additional applications for these systems in ion-exchange analysis of synthetic deoxyoligonucleotides.

## CONCLUSIONS

We have devised a rapid quantitative and qualitative means for determining the extent of oxygenation (phosphodiester) present in deoxyoligonucleotide phosphorothioate analogues synthesized via contemporary automated solid-phase modalities. The method requires less than 1 O.D. ($< 3\ \mu g$) DNA per analysis, and *ca.* 1 h analysis cycle. $^{31}P$-NMR assay, on the other hand, requires typically 20 mg material and a minimum of 2–4 h scanning time for reasonable signal-to-noise peak detection and integration (at the $\leqslant 1\%$ P=O level).

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