Investigation of the ‘n − 1’ impurity in phosphorothioate oligodeoxynucleotides synthesized by the solid-phase β-cyanoethyl phosphoramidite method using stepwise sulfurization

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ABSTRACT

Electrospray ionization mass spectrometry (ESI-MS) of reversed-phase HPLC-purified phosphorothioate oligodeoxynucleotides (S-ODNs), and the single- (‘n − 1’) and double-nucleotide deletion (‘n − 2’) impurities subsequently isolated from them by preparative polyacrylamide gel electrophoresis (PAGE), has provided direct analytical data for the identification of both S-ODN products and their major oligomeric impurities. The ‘n − 1’ impurity seen by PAGE consists of a mixture of all possible single deletion sequences relative to the parent S-ODN (n-mer) and results from repetitive, though minor, imperfections in the synthesis cycle, such as incomplete detritylation, or incomplete coupling followed by incomplete capping or incomplete sulfurization. Therefore each possible ‘n − 1’, ‘n − 2’, and other short-mer sequence is present only in very low abundance. The conversion of the gel-isolated ‘n − 1’ impurity from phosphorothioate to phosphodiester followed by base composition-dependent anion-exchange chromatography allowed for independent confirmation of its heterogeneity and quantitation of its various components. ESI-MS of both S-ODN products and their gel-isolated impurities allowed for this first molecular identification of ‘n − 1’, ‘n − 2’ and other oligomeric impurities in S-ODNs obtained from state-of-the-art solid-phase synthesis and reversed-phase HPLC purification methods.

INTRODUCTION

There has been a continuing interest in the synthesis, properties and applications of analogues of DNA which have internucleoside linkages wherein a non-bridging phosphate oxygen is replaced by sulfur (1,2). Because such phosphorothioate oligonucleotides (S-ODNs) are resistant to degradation by nucleases, form stable duplexes, and are now readily synthesized (3,4), attention has focused more recently on use of S-ODNs as ‘antisense’ agents, which are intended to hybridize to preselected sequences of RNA and thus allow control of genetic expression. This has been studied in a diverse array of systems both in vitro (2) and in vivo (5–7), and a number of S-ODNs are in ongoing clinical trials (8–11). For these pharmaceutical applications, as well as in studies aimed at further refining synthesis of oligonucleotides and their analogues, in general, it is necessary to address issues of product identification and purity analysis, characterization of impurities and reproducibility of synthesis and purification methods (12).

The purity of ODNs with regard to size homogeneity is conventionally assessed by polyacrylamide gel electrophoresis (PAGE), which provides separations of ODNs based on molecular weight assuming a constant ratio of charge to mass (13). This method of separation usually indicates that the desired synthetic oligomer product ‘n’ nucleotides in length is contaminated with shorter oligomers, with the impurity of length ‘n − 1’ generally being the most predominant and progressively truncated oligomers being correspondingly less abundant. Because PAGE is more sensitive to size than it is to sequence (13), it has been heretofore unclear and a matter of speculation as to whether each of these impurity bands is a single compound or a mixture of possibly many compounds of the same oligomer length. Less than full-length, ‘short’ ODNs can be formed by imperfections in the repetitive solid-phase synthesis chemistry, such as incomplete detritylation, or incomplete coupling followed by incomplete capping, or incomplete sulfurization (14). Assuming these failures are low-frequency random events, a complex mixture of ‘n − 1’ S-ODNs is created. Other mechanisms by which shorter than full length ODNs can be formed are oligomer extension after adventitious coupling to exposed functional groups on the solid support (15,16), or acid-catalyzed depurination (17,18), or oligomer chain cleavage during the final treatment with ammonia (19) to achieve deprotection and removal from the solid support. These mechanisms lead to impurities of a particular sequence, rather than a mixture. ‘Short-mers’ generated by any of these hypothetical routes must also contain a DMT protecting group on the 5’ end of the oligomer

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in order for them to co-elute with the desired full-length n-mer product during 'trityl-on' reversed-phase HPLC purification (17). The analysis of ODNs by electrospray ionization mass spectrometry (ESI-MS) (20–25) shows considerable promise due to its ability to provide precise mass values, high resolution and little fragmentation, which is especially important for the identification of impurities. We describe here the characterization of two S-ODNs currently involved in clinical trials: LR-3523, a 20mer for either ex vivo or systemic treatments of acute myelogenous leukemia (7) and LR-3001 (c-myb), a 24mer for analogous treatments of chronic myelogenous leukemia (5,6). ESI-MS of the 'trityl-on' HPLC-purified final product and the 'n – 1' and 'n – 2' impurities subsequently isolated from it by PAGE has provided direct analytical data for the structure confirmation (26) of S-ODN products and identification of their major oligomeric impurities. The novel conversion from phosphorothioate to phosphodiester followed by base composition-dependent anion-exchange HPLC analysis (27) was used to quantitate and further characterize the 'n – 1' impurities.

MATERIALS AND METHODS

Gel electrophoresis reagents were from IBI. Stains-all dye and 4-(N,N-dimethylamino)pyridine (DMAP) were from Eastman Kodak. Reagent grade sodium chloride, sodium hydroxide and sodium thiosulfate were from Mallinckrodt. Ammonium acetate and iodine were from J. T. Baker. Ethanol was from Quantum and THF and deuterium oxide were from Aldrich. DNA synthesis reagents, oligonucleotide purification columns (OPC) and 2M triethylammonium acetate (TEAA) were purchased from Perkin Elmer/Applied Biosystems Division. Anionitrile, methylene chloride and pyridine were from Burdick and Jackson. Sephadex G-25 was from Pharmacia. 31P NMR was performed on a Varian Unity 300 with a resonance frequency of 121.42 MHz. Ion exchange chromatography (IEC) of S-ODNs was performed as reported elsewhere (28). Reversed-phase HPLC was performed using a Perkin Elmer Series 410 LC system, 757 Absorbance Detector and LCI-100 Laboratory Computing Integrator. A Polymer Labs PLRP-S column (0.46 × 25 cm) with a gradient of 5–25% A over 30 min (A = CH3CN; B = 0.1 M TEAA/2% CH3CN/pH 7.0) was used. The flow rate was 1.0 ml/min and detection was at 260 nm. IEC of phosphodiester ODNs was performed on a Dionex DX-300 HPLC system equipped with a Dionex NucleoPac PA-100 column (0.4 × 25 cm). A gradient of 0–30% B over 30 min (A = 0.01 M NaOH/0.01 M NaCl; B = A + 1.5 M NaCl) was used. The flow rate was 1.0 ml/min and detection was at 260 nm.

DNA synthesis and purification

Phosphorothioate DNA was prepared on either an Applied Biosystems Model 380B (1 μmol) or 390Z (275–450 μmol) automated DNA Synthesizer using standard phosphoramidite protocols (3,4), except for the substitution of bis(O,O-diisopropoxy phosphinothioyl) disulfide (S-tetra) for the iodine and capping after, instead of before, sulfurization (29,30). Either CPG or Tentagel™ was used as the solid support. The crude DNA was purified as its 5′-O-DMT derivative according to published procedures (3,4). The DNA was detritylated with aqueous acetic acid, precipitated using sodium chloride and ethanol (3,4), and finally lyophilized to a powdered solid. Phosphodiester ODNs and the 3′-thiophosphate S-ODN (LR-3434) were synthesized according to published procedures (31).

Polyacrylamide gel electrophoresis (PAGE)

Purified S-ODNs [0.02–0.08 optical density units (OD)/lane] were analyzed by PAGE on 15 × 15 cm × 0.75 mm analytical gels (20% total polyacrylamide, 5% cross-linked; 7 M urea) using 40 mM Tris-borate buffer at pH 8.3. Bands were visualized by staining with a 0.006% solution of Stains-all™ in 1:1 formamide:deionized water for 8–16 h. The bands were quantified by scanning densitometry using a Model 300S Computing Densitomer (Molecular Dynamics).

Preparative gels were run as above except using gels which were 20 × 20 cm × 1.5 mm and loading 2 OD/lane. The S-ODN bands were visualized by placing the gel on a fluorescent TLC plate and illuminating it with 254 nm ultraviolet light. The desired bands were cut out and eluted by the crush and soak method (13) using 0.5 M ammonium acetate/pH 7.5. The gel purified S-ODNs were desalted on oligonucleotide purification columns (OPC) using the protocol provided by the manufacturer.

Conversion of S-ODNs to the ammonium salt

10 M ammonium acetate (0.2 ml) was added to the S-ODN dissolved in water (0.2 ml, 25 OD/ml). The mixture was vortexed well and allowed to sit for 1 h before precipitating it with 1 ml ethanol. The samples were cooled to −20°C, centrifuged and the supernatant aspirated. This procedure was repeated two to three times. The pellet was dissolved in 0.5 ml deionized H2O and the amount of DNA was quantitated by absorbance at A260. Smaller amounts of S-ODNs were precipitated using proportionally less of all reagents and generally required 10 vol ethanol for good recoveries.

Mass spectrometry

Purified S-ODNs in the ammonium form (20–50 μM) were mixed with acetonitrile to bring the final organic concentration to 10%. The solutions were infused at 3–4 μl/min into the Ionspray interface of a PE-Sciex API III triple quadrupole mass spectrometer. The Ionspray voltage was −3400 V and the orifice voltage was set to −45 V. Data were acquired in the multichannel averaging mode with 20–70 scans summed per spectrum. Raw data displaying multiple charges in the mass-to-charge dimension were transformed to a simple mass scale with the Reconstruct program. The masses were calculated from the m/z data using the Hypermass program and the standard deviations ranged from 0.3 to 1.6 Da.

Oxidative desulfurization of S-ODNs

To 200 μl S-ODN dissolved in water (10 OD, 50 OD/ml) was added 150 μl 1 M N-methylimidazole (NMI)/THF; and 75 μl 0.5 M iodine dissolved in 85:15 1 M NMI/THF:H2O. The solution was allowed to stand 2 min and then 30 μl 1 M DMAP/THF was added. After 2.5 h the reaction was quenched with 50 μl 1 M aqueous sodium thiosulfate and desalted on Sephadex G-25. The conversion to phosphodiester was verified by reverse phase HPLC, which gave a retention time of 25.4 min for the S-ODNs and 15.5 min for the ODNs. The purity of the ODNs with regard to chain length was determined by PAGE.
4-6% The purity band is repetitive cycle (32). Contiguous repeated, deletion and ('Stains-all'), of being to a synthesized LR-3523, authentic reference of the LR-3434 -LR-3434 together S-ODNs (Fig. PAGE mobility same scenarios.

weight determination (lane 2756 Nucleic 1); (b) the statistically weighed mixture (LR-3187-LR-3197) of synthetic 'n -1' S-ODNs (lane 2); (c) LR-3344 (lane 3); (d) the PAGE-purified 'n -1' impurity from the CPG synthesis of LR-3523; (e) the PAGE-purified 'n -1' or (f) 'n -2' impurity from the Tentagel™ synthesis of LR-3523; (g) LR-3523 (0.08 OD) synthesized on Tentagel™. The S-ODNs were visualized with Stains-all™.

RESULTS

Reference compounds and PAGE

The purity of S-ODNs is routinely determined by sequential PAGE separation, visualization of the resultant bands with a dye ('Stains-all'), and then integration using laser scanning densitometry (32). Typical gel profiles at different loading densities are shown in Figure 1, lanes 1 and 7, for the case of LR-3523 synthesized on CPG and Tentagel™, respectively. The 'n -1' band is the predominant impurity and is typically in the range of 4-6% for CPG and 1.5-3% for Tentagel™.

For a 20mer S-ODN such as LR-3523, there are 20 possible 'n -1' S-ODNs (19mers) based on defect scenarios for the repetitive cycle of synthesis chemistry discussed above. However, when a product n-mer has a sequence in which a base is contingously repeated, deletion of this base at any position in the contiguous repeat generates the same 'n-1' species. Therefore, because of this degeneracy, there are only 11 unique 'n -1' S-ODNs for LR-3523. These 11 'n -1' deletion sequences relative to LR-3523 (LR-3187 - LR-3197, Table 1) were independently synthesized together with the 3'-thiophosphate derivative (LR-3434) of the 'n -1' missing the 3'-terminal base. These authentic reference compounds were analyzed by 20% PAGE both individually and as a statistically-weighed mixture prepared under the assumption that every base in the sequence has the same probability of being deleted according to the aforementioned defect scenarios. It can be seen in Figure 1 that the mixture of all 11 synthetic 'n -1' S-ODNs (lane 2) ran as a single band and had the same PAGE mobility as the PAGE-purified 'n -1' band isolated from LR-3523, synthesized either on CPG (lane 4) or Tentagel™ (lane 5). The 'n -1' with the 3'-terminal thiophosphate (LR-3434; Fig. 1, lane 3) had the same PAGE mobility as these 'n -1' S-ODNs (Fig. 1, lane 2) despite its additional negative charge.

ESI-MS

ESI-MS has been recently found (20-25) to be very useful for the molecular weight determination of ODNs. ESI-MS in the negative ion mode give a series of peaks that correspond to partially deprotonated (M - nH)-n ions, from which the fully-protonated, neutral molecular mass (M) can be calculated by means of a mathematical algorithm (PE Sciex Hypermass program). Both S-ODNs and unmodified, phosphodiester-linked ODNs are generally isolated as the sodium salt for biological studies; however, sodium ions cause severe problems in ESI-MS because of the formation of adduct peaks, i.e. replacement of protons with sodium ions which produces another series of peaks that make peak assignments much more difficult and significantly reduces signal to noise. Therefore, an important requirement for obtaining the parent ion molecular weight by this method is the exchange of sodium atoms with a volatile proton donor counterion, such as ammonium. This exchange in the case of unmodified, phosphodiester ODNs has been recently accomplished by precipitation of the ODNs with ethanol in the presence of ammonium acetate (22).

In the presently described study, S-ODNs were found to be generally more difficult to precipitate as the ammonium salt than were phosphodiester ODNs; consequently, the S-ODNs generally required the use of 2-4-fold more ethanol in order to obtain good recoveries, which was especially important in sample-limited cases.

C-Rich example (20mer)

The negative ion ESI-MS of LR-3523 synthesized on CPG is shown in Figure 2A (found: 6207.2 Da; calculated: 6207.0 Da). The families of much lower intensity ions seen in this spectrum are due, in part, to impurities in LR-3523; these impurities are generally easier to visualize in the reconstructed mass spectrum (Fig. 2B). For instance, the peaks corresponding to LR-3523 containing either one or two phosphodiester (PO) linkages instead of phosphorothioate (PS) linkages are present, with found molecular weights of 6190.5 Da (calculated: 6190.9 Da) and 6174.0 Da (calculated: 6174.9 Da), respectively. The amounts of these PO-defect species were determined by IEC, as described elsewhere (28), and based on this information, we believe that, in general, impurities in the S-ODN products are detectable in the ESI-MS if they are at least ~1 mol-% of the material analyzed.

Perusal of Table 1 indicates that there are five constitutively isomeric LR-3523 'n -1' sequences in which one of the 13 2'-deoxyctydine 3'-thiophosphate nucleotide moieties has been deleted; however, being isomeric they have the same molecular weight. In the reconstructed mass spectrum of LR-3523 (Fig. 2B), a peak corresponding to this mass is visible (found: 5902.0 Da; calculated: 5901.7 Da). A second peak identified as LR-3523 missing a thymidine 3'-thiophosphate is also seen in the spectrum (found: 5887.5 Da; calculated: 5887.5 Da). The other 'n -1' S-ODN corresponding to deletion of a 2'-deoxyguanosine 3'-thiophosphate is apparently too low in relative abundance (<1%) to be detected.

In contrast to the two types of 'n -1' impurities found above by direct analysis of LR-3523, the reconstructed mass spectrum of the 'n -1' impurity gel-isolated from LR-3523 synthesized on CPG was shown to contain masses representing all three possible types of 'n -1' species (Fig. 3). The most intense peak, which represents a molecular weight of 5901.2 Da (calculated: 5901.7 Da), is due to LR-3523 with a 2'-deoxyctydine 3'-thiophosphate group deleted. Two less intense peaks seen in Figure 3 have molecular weights of 5886.0 Da, which was identified as the 'n -1' impurity of LR-3523 missing a thymidine 3'-thiophosphate (calculated: 5885.7 Da), and 5862.2 Da, which was identified as the 'n -1' impurity of LR-3523.
missing a 2'-deoxyguanosine 3'-thiophosphate (calculated: 5861.7 Da). The absence of a peak corresponding to a molecular weight of 5998 Da in the reconstructed mass spectrum of this gel-isolated 'n – 1' band indicated that the 3'-thiophosphate derivative of LR-3523 missing its 3'-terminal deoxycytidine is not a detectable component. The mass spectrum of the independently synthesized and subsequently gel purified 3'-thiophosphate derivative of LR-3523 (LR-3434) verified that neither the gel purification nor the ionization process cleaves the terminal thiophosphate group (found: 5998 Da; calculated: 5997.8 Da). Therefore, if there is oligomer chain extension derived from adventitious phosphitylation of available functional groups on the solid support and this produces an 'n – 1' impurity, it must have a terminal 3'-hydroxyl, rather than a 3'-thiophosphate end group.

The gel-purified 'n – 1' sample was found by PAGE analysis to contain -15% of the 'n – 2' impurity. The major components of this 'n – 2' impurity determined from the mass spectrum are the LR-3523 sequence which is missing two 2'-deoxycytidine 3'-thiophosphate moieties (found: 5596.0 Da; calculated: 5596.5 Da), and the LR-3523 sequence which is missing one 2'-deoxycytidine
3'-thiophosphate moiety (found: 5581.5 Da; calculated: 5581.5 Da). The mass spectrum of a gel-purified sample of the ‘n – 2’ impurity band shows the aforementioned peaks and, because of the increased abundance, an additional peak for LR-3523 missing two thymidine 3'-thiophosphate moieties (found: 5565.3 Da; calculated: 5566.5 Da) (data not shown). The components of the gel-purified ‘n – 1’ impurity from the Tentagel™ synthesis of LR-3523 were identical by mass spectral analysis (data not shown) to those found above for the CPG synthesis. In general, there is no discernible influence of the solid-support on the distribution of individual oligomeric impurities other than the relative percentage of the total ‘n – 1’ impurity.

**G-Rich example (24mer)**

Having identified the aforementioned oligomeric impurities in the pyrimidine-rich (85%) LR-3523 20mer product, it was of interest to determine whether analogous kinds of impurities would be found in the G-rich (42%) LR-3001 24mer product. The mass found for LR-3001 synthesized on Tentagel™ is 7777.5 Da (calculated: 7777.2 Da). In this sample of LR-3001 product containing 1.5% ‘n – 1’ impurity by PAGE, no ions representing any of the four possible types of single 2'-deoxynucleotide 3'-thiophosphate deletions could be detected as ‘n – 1’ impurities, in contrast to what had been found above with LR-3523. Similar to what was found above for LR-3523, the reconstructed mass spectrum (data not shown) of the gel-isolated ‘n – 1’ impurity from the LR-3001 product contains peaks which correspond to the LR-3001 sequence missing either one 2'-deoxycytidine 3'-thiophosphate (found: 7473.2 Da; calculated: 7471.9 Da), or one thymidine 3'-thiophosphate (found: 7457.0 Da; calculated: 7456.9 Da), or one 2'-deoxyguanosine 3'-thiophosphate (found: 7431.5 Da; calculated: 7431.9 Da). Because there is only one deA moiety in the 24mer LR-3001 sequence, it was reasonable to presume that the S-ODN missing one 2'-deoxyadenosine 3'-thiophosphate was present at too low of a relative abundance to be detected.

**Conversion from phosphorothioate to phosphodiester and analysis by anion exchange chromatography**

In principle, anion exchange chromatography (IEC) at pH 12 can be used to distinguish and quantitate phosphodiester ODNs of the same oligomer length but with different base composition because of the different charge states of the bases at this pH (27). This procedure can not apparently be used for S-ODNs which are tightly bound to the ion-exchange resin under these conditions. Certain specific IEC conditions have been devised (28) for elution of S-ODNs, but do not lend themselves to the aforementioned type of analysis. S-ODNs can, however, be converted to phosphodiester ODNs with only a small amount of chain scission by using a solution of iodine, 4-(N,N-dimethylamino)pyridine and N-methylimidazole in THF/water. This mild oxidative desulfurization of S-ODNs to ODNs was therefore exploited in order to further characterize the oligomeric impurities in S-ODNs, as described here.

LR-3523 product derived from CPG or Tentagel™ was subjected to oxidative desulfurization in parallel with a set of the independently synthesized ‘n – 1’ versions of LR-3523 (Table 1), as well as the gel-purified ‘n – 1’ band obtained from preparative PAGE of these LR-3523 products. That essentially complete desulfurization was achieved with little chain scission was established by

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**Figure 3.** Reconstructed mass spectrum of the PAGE-purified ‘n – 1’ impurity from LR-3523 synthesized on CPG, which shows all three possible types of ‘n – 1’ and two types of ‘n – 2’ impurities, as discussed in the text. The peak at 5967.0 Da is an adduct of unknown identity.
PAGE and reverse-phase HPLC analyses prior to anion exchange chromatography. The resultant mixture of all possible synthetic ‘n – 1’ ODNs gave rise to four major peaks or envelopes of peaks (Fig. 4A), which were subsequently identified by retention time comparisons following separate injection of each synthetic ‘n – 1’ ODN. The two ‘n – 1’ ODNs missing a 2'-deoxyguanosine 3'-phosphate moiety elute first as a single peak at 16.06 min, followed by the four ODNs missing a 2'-thymidine 3'-phosphate moiety which appear as an overlapping pair of peaks at 16.35 min. The five compounds missing one 2'-deoxycytidine 3'-phosphate elute as two overlapping peaks, with the major peak at 17.34 min and the minor peak at 17.92 min. The latter minor peak was further identified as the ODN sequence missing nucleotide-unit 15 (LR-3190, Table 1), based on its retention time. It was not obvious why this particular ODN elutes later than all of the others; however, the above order of elution is consistent with theory (27). IEC of the ODNs obtained by desulfurization of the gel-isolated ‘n – 1’ band for LR-3523 made on either CPG (Fig. 4B) or Tentagel™ (Fig. 4C) each showed a profile virtually identical to that given by the synthetic mixture of ‘n – 1’ ODNs (Fig. 4A). The only peak present in the HPLC profiles of the gel-isolated ‘n – 1’ samples that is not in the synthetic mixture was found to co-elute with two representative synthetic samples of LR-3523 missing two 2'-deoxycytidine 3'-thiophosphate moieties at 17.02 min (LR-4383 and LR-4384; Table 1). Subsequent PAGE analysis confirmed that these samples contain ~15% of phosphodiester-linked ‘n – 2’ impurity, which had apparently been co-collected with the ‘n – 1’ band during preparative PAGE in the original S-ODN form. These compositional data derived from IEC of desulfurized samples of LR-3523 agreed very well with the mass spectral data discussed above for this S-ODN, which indicated that this sequential desulfurization-anion exchange HPLC method could provide a more conventional approach for identification and quantitation of the ‘n – 1’ impurities in an S-ODN to the extent that resolution was achievable.

**DISCUSSION**

Negative ESI-MS was found to provide molecular mass data on S-ODNs with a very high degree of accuracy (± 0.02%), provided that samples were in the ammonium form and substantially free of interfering counter ions such as sodium. This allowed for what we believe is the first molecular identification of ‘n – 1’, ‘n – 2’ and other oligomeric impurities in S-ODNs obtained from state-of-the-art solid-phase synthesis and reversed-phase HPLC purification methods. Of the two cases reported here, only when the ‘n – 1’ impurities were seen in aggregate at >2% by PAGE could any of the individual ‘n – 1’ impurities be directly detected in the mass spectrum of the product.

The mass spectra of the gel-isolated ‘n – 1’ impurities from LR-3523 and LR-3001 unambiguously demonstrated that these impurities were not single species. The presence of ‘n – 1’ S-ODNs comprising each of the theoretical types of base deletion clearly shows the existence of internal base deletions, which heretofore has not been demonstrated for solid-phase synthesis by the phosphoramidite method. Such internal deletions can only be caused by imperfections in the repetitive cycle of synthesis chemistry. The IEC of the ‘n – 1’ S-ODNs comprised from LR-3523 by preparative PAGE and then converted by oxidative desulfurization to a phosphodiester-linked ODN, provided independent confirmation of heterogeneity of the ‘n – 1’ impurity and, moreover, allowed for quantitation of each type of deletion. In this case, the amount of each type of deletion qualitatively agreed with (i) the theoretical value for each type of deletion, determined by assuming that every base in the LR-3523 sequence had the same probability of being deleted (Table 2), and (ii) the relative

![Figure 4](https://example.com/figure4.png)

Figure 4. Ion exchange chromatograms of the following S-ODNs converted to ODNs: the statistically weighted mixture of synthetic ‘n – 1’ sequences relative to LR-3523 (LR-3187-LR-3197) (A); the PAGE-purified ‘n – 1’ impurity from LR-3523 synthesized on (B) CPG or (C) Tentagel™. The peaks assignments are given in the text.
intensities of each type of ion in the mass spectra (Fig. 3). The relative intensities of the 'n - 1' ions in the mass spectrum of the 'n - 1' band isolated from LR-3001 by preparative PAGE are also qualitatively in agreement with the theoretical values determined for the types of deletions in the LR-3001 sequence.

Table 2.

<table>
<thead>
<tr>
<th>Name</th>
<th>Base Number(s) Deleted</th>
<th>Sequence and 3'-&gt;5' Base Number</th>
<th>Statistical Weighting for &quot;n-1&quot; mixture</th>
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</thead>
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<tr>
<td>LR-3523</td>
<td>none</td>
<td>20 19 18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1</td>
<td>none</td>
</tr>
</tbody>
</table>
types of impurities nor the processes that lead to their formation had
been heretofore well-characterized, which underscores the value of
the presently described mass spectrometric method. Knowing the
identity of these impurities allows for the systematic study of new
or improved synthesis and purification procedures. For example, the
‘n − 1’ impurity may be reduced by the development of (i) a more
efficient (or stable) capping agent, (ii) a more effective sulfurization
agent, or (iii) feedback conductivity monitoring of the detritylation
which becomes longer on a large scale. Regardless of whether such
improvements are achieved, the structural information derived from
ESI-MS on (short-mer) process impurities enables the investigator
to obtain through synthesis working quantities of these materials in
order to better assess their chemical and biological properties in
the course of pre-clinical evaluation of the respective parent S-ODNs.
In fact, the US Food and Drug Administration requires this type of
complete characterization of related structural impurities for all
drugs. Finally, it will be interesting to determine whether ESI-MS
can be extended to identification and quantitation of the metabolites
or conjugates of S-ODNs and other ODN analogues in conjunction with
in vivo studies of these compounds as antisense, antigen, or
antiprotein therapeutic agents.

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