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Sequence analysis of phosphorothioate oligonucleotides via matrix-assisted laser desorption ionization time-of-flight mass spectrometry ☆

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Abstract

Modification of the natural phosphodiester backbone of deoxyribooligonucleotides can impart increased biostability via nuclease resistance. Further, uniform incorporation of phosphorothioate linkages renders oligonucleotides highly resistant to reagents traditionally used in sequencing reactions. As a consequence, analytical tests crucial for establishing the identity of such oligonucleotide drugs are less informative. To circumvent this problem, chemical oxidation has been employed for converting the phosphorothioate to the uniform phosphodiester, thereby facilitating enzymatic degradation. Following oxidation, exonucleases which sequentially cleave individual bases from the 3′ or 5′ terminus of the oligonucleotide or base-specific cleavage chemicals were used to facilitate sequence identification of the oligonucleotide. Matrix-assisted laser desorption ionization-time-of-flight/mass spectrometry (MALDI-TOF/MS), previously used to sequence natural phosphodiester DNA, was then used to sequence the chemically oxidized phosphorothioate. Sequential enzymatic cleavage of desulphurized phosphorothioates in combination with MALDI analysis not only provides a viable alternative to radiolabeling as used in conventional sequencing approaches (e.g. Maxam–Gilbert), but also enables rapid sequencing of phosphorothioate oligonucleotides, for routine drug analysis.

Keywords: Matrix-assisted laser desorption ionization (MALDI); Mass spectrometry; Phosphorothioate oligonucleotides; Sequencing; Time-of-flight

1. Introduction

There has been increasing interest in the use of mass spectrometry as a tool for direct mapping and sequence analysis of peptides and modified oligonucleotides or analysis following partial degradation of the analyte through proteolytic or nucleolytic digestion [1–7]. The matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) approach is particularly attractive. Here, ions from a digest mixture are formed upon laser irradiation of the analyte imbedded in a solid matrix. Because, in theory, the majority of ions formed in the source reach the detector, MALDI-TOF provides an extremely sensitive approach for

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detecting sequencing fragments. In addition, the relatively uncomplicated spectrum resulting from soft ionization of the sample facilitates sequence reading.

A particular sequencing challenge is presented, however, by non-native oligonucleotides incorporating backbone modifications. For example, phosphorothioate oligonucleotides (see Fig. 1) useful in antisense drug therapy [8] or oligonucleotides containing methylphosphonate functionalities by design hinder the action of traditional cleavage reagents used in sequencing chemistries. For methylphosphonate oligomers, one sequencing approach has utilized direct tandem MS analysis to extract sequence information by rationalizing product ion spectra of multiply charged precursors [9]. Alternatively, sequence information has been gathered by using MALDI to assign masses to the fragments of methylphosphonate oligodeoxyribonucleotide crude synthesis samples. With the exception of the last four nucleotides in a 15-base sequence, the full parent sequence was deduced from the mass differences between fragments [10]. The successful use of this approach largely depends on the levels of "failure sequences" present in the crude sample. A combination of "bidirectional" enzymatic digestion and MALDI-TOF/MS has been demonstrated as useful for sequencing oligonucleotides incorporating a single 2′-*O*-methyl adenosine moiety [1]. The primary advantage of this approach is that the use of terminus-specific enzymes enables missing sequence information to be pieced together from separate cleavage reactions, thereby reducing the overall analysis time and eliminating labeling steps characteristically involved in most other sequencing approaches.

The therapeutic value of antisense drugs is derived from the sequence-specificity of the oligonucleotide for its target messenger RNA. Consequently, establishing the sequence of the oligonucleotide is crucial for unambiguous identification of the drug [11], confirmation of reaction mechanism, and control of its synthesis. Previously, a modified Maxam–Gilbert method was used to sequence phosphorothioate oligonucleotides [12]; however, a Maxam–Gilbert approach was found to be unsuitable for routine sequencing. Preliminary efforts to *directly* sequence (i.e. without prior desulphurization) phosphorothioate oligonucleotides via MALDI-TOF/MS were partially successful, although extremely harsh conditions and increased digestion time were necessary to force degradation of the oligomer. Thus, desulphurization [13,14] prior to sequencing phosphorothioate oligonucleotides is particularly important for facilitating chemical degradation or enzymolysis.

In this report, a two-part approach to authenticating the sequence of a 20 and 21 base phosphorothioate oligonucleotide drug is described and compared to sequencing via direct enzymolysis. The method employs a 3′- and 5′-exonuclease to digest the *desulphurized* oligonucleotide in a sequential pattern followed temporally by MALDI-TOF/MS analysis of the fragments from both digests. The use of information from both digests provides rapid and complete sequence verification, thereby making this approach suitable for routine analysis of phosphorothioate oligonucleotide drugs during the quality control and stability monitoring stages of drug development.

2. Experimental

2.1. Materials

Phosphodiesterase I (SVPD) (100 U) was purchased from United States Biochemicals (Cleveland, OH). Calf spleen phosphodiesterase (2 U ml^{-1}) was obtained from Boehringer Mannheim (Germany). ACS reagent grade iodine (100%) was purchased from Sigma. Omnisolv® tetrahydrofuran was obtained from EM Science (Gibbstown, NJ), and 99+% redistilled 1-methylimidazole was purchased from Aldrich (Milwaukee, WI).

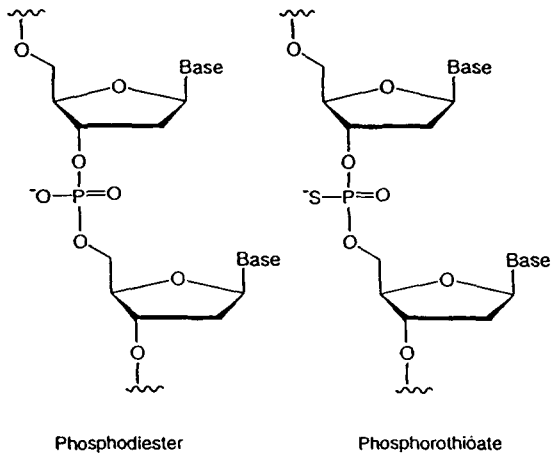

Fig. 1. Pictorial diagram of a representative phosphodiester and phosphorothioate oligonucleotide.

2.2. Mass spectrometry of phosphorothioate oligonucleotide

Mass spectra were recorded on either a Voyager™ (PerSeptive Biosystems, Cambridge, MA) linear time-of-flight mass spectrometer or on an LDI 1700 (Linear Scientific Inc., Reno, NV) time-of-flight mass spectrometer. Measurements by the Voyager instrument used an external calibration sample of either a 21-base oligonucleotide or $d(T)_{14}$. An external calibration sample containing a mixture of $d(T)_5$, $d(T)_{10}$, $d(T)_{15}$ and $d(T)_{20}$ was employed for experiments on the LDI 1700 instrument. A nitrogen laser (337 nm) was used in both instruments. The laser power was estimated at 10^6 W cm^{-2} (4–10 μJ per pulse) on the LDI system, and at 1.8–2.2 μJ per pulse on the Voyager™ system. Measurements were performed in the negative ion mode.

2.3. Preparation of matrix

The sample matrix was prepared by mixing 10 μl of a 0.5 M solution of 2,4,6-trihydroxyacetophenone (Aldrich, Milwaukee, WI) in ethanol (Quantum Chemical Corp., Tuscola, IL) with 5 μl of a 0.10 M aqueous solution of diammonium hydrogen citrate (Aldrich, Milwaukee, WI).

2.4. Oxidation of the phosphorothioate oligonucleotide via reaction with THF/water/methylimidazole/iodine

A solution containing 1.0 ml of THF–water–methylimidazole (16:4:1, v/v/v) and 10 mg of iodine was added to 10.0 OD_{260} of the dry phosphorothioate oligonucleotide and allowed to react for 2.0 h at 37 °C. The extent of oxidation has previously been followed by RP-HPLC and determined to be about 94% effective under these reaction conditions. The sample was diluted with 200 μl water, and iodine was then removed by passing the sample through a size exclusion column (slurry packed with Biogel). Samples were collected at 1.5 ml intervals using water treated with four drops of ammonia per liter of water (pH ≈ 10.3). The phosphodiester oligonucleotide was eluted between 18 and 21 ml. Approximately 60% recovery of the sample was possible. Samples containing the phosphodiester were collected and dried using a Savant Automatic Speed Vac system (Farmingdale, NY). Water was then added to bring the total oligonucleotide concentration to within 5–10 OD_{260} ml^{-1}. This procedure also effectively desalted the samples through replacement of sodium ions by ammonium ions.

2.5. Preparation of enzymatic reaction mixtures

A 0.05 unit μl^{-1} stock solution of snake venom phosphodiesterase and 0.001 unit μl^{-1} stock solution of calf spleen phosphodiesterase were prepared and stored at 4 °C. Sequencing of the oligonucleotide was achieved through time-controlled enzymatic digestion. Digestions were performed in a 1.5 ml centrifuge tube inserted in a 37 °C water bath. Although digestion time may be improved by the use of salts or buffers, only water was employed in order to minimize the formation of possible salts adducts. A single enzyme was used to digest the sample either from the 3′ or the 5′ terminus. Sequence information beyond the 16th base was increasingly difficult to acquire over a reasonable period of time using a single enzyme. Therefore, the opposite enzyme was introduced to the oligonucleotide in a separate reaction to retrieve information about the sequence of the missing terminal bases.

2.6. Digestions

1 μl of calf spleen phosphodiesterase (CSP, for a 5′–3′ digestion) stock or snake venom phosphodiesterase (SVP, for a 3′–5′ digestion) stock was added to 20 μl of the oligonucleotide stock solution. 1 μl samples were removed from the reaction mixture periodically and added to 15 μl of the matrix described above. The sample and matrix components were thoroughly mixed by vortexing. 1.0 μl of this mixture (corresponding to 3.5 pmol from a 10 OD_{260} ml^{-1} stock) was then applied to a stainless steel sample plate allowing the sample to dry at room temperature.

3. Results and discussion

Sequence analysis of phosphorothioate oligonucleotide drug substances is essential for establishing quality and confirming absolute identity of the drug [11]. For natural phosphodiester oligonucleotides, sequencing has traditionally been achieved through the Sanger or Maxam–Gilbert methods. In contrast, the sta-

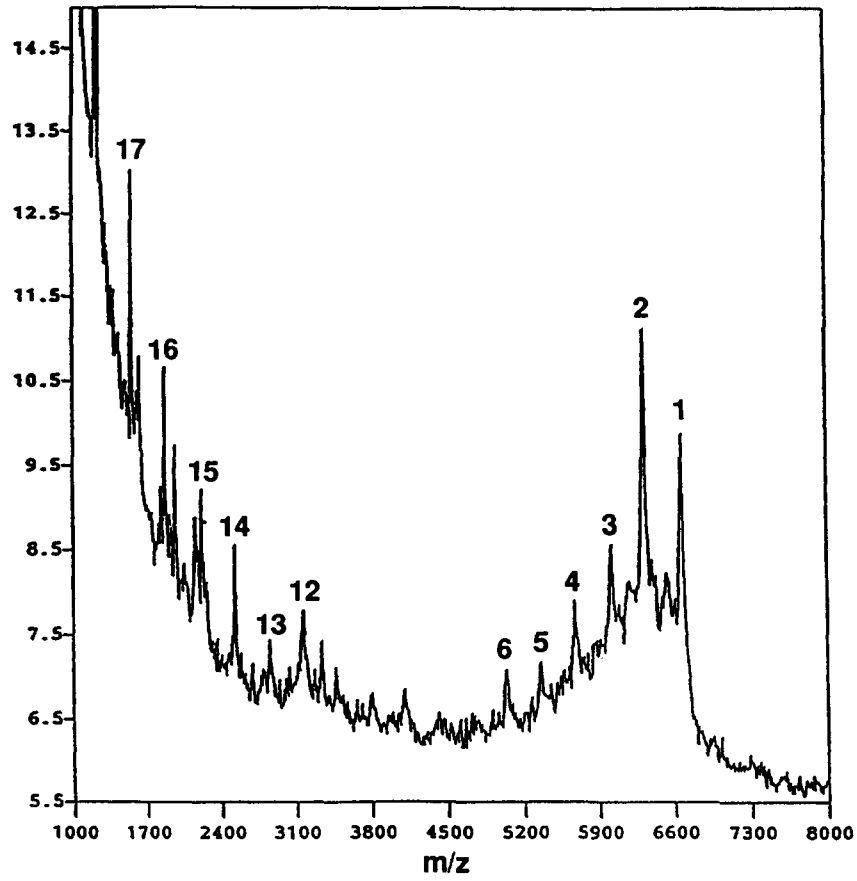

Fig. 2. MALDI-TOF mass spectra (LDI-1700 mass spectrometer) of 3′-exonuclease (SVP) direct digestion fragments of ISIS 2922 phosphorothioate oligonucleotide sampled following overnight digestion at 55 °C.

bility of phosphorothioate oligonucleotides to chemical and enzymatic cleavage limits the quality of information gained from these same approaches [12]. Further, such analyses are typically very time-consuming and require use of radioactive substances, rendering them unsuitable for more routine sequence analysis. Recently, Pieles, et al. [1] described the utility of MALDI/MS for sequence analysis of modified oligonucleotides incorporating a 2′-*O*-methyl functionality. The less destructive laser desorption ionization of MALDI-TOF/MS makes it well-suited to analysis of oligonucleotide cleavage products from enzymolysis, since it leaves these fragments essentially intact. Although enzymolysis is reported to be inefficient for phosphorothioate-backboned oligonucleotides, it was hypothesized that the higher sensitivity of mass spectrometry (relative to that for slab gel separation of radiolabeled phosphorothioate oligonucleotide material) might allow adequate detection of cleavage products from even a few successful digestion events. Fig. 2 depicts a representative mass spectrum of the $(M-H)^{-1}$ fragments produced from direct digestion of ISIS 2922 *phosphorothioate* oligonucleotide at the 3′ terminus. Extended exposure of the phosphorothioate to snake venom phosphodiesterase and increased incubation temperature were necessary to promote even partial cleavage. Interpretation of the mass spectrum enabled identification of oligonucleotide bases cleaved from fragments 1–5 and 12–16 (see Table 1). Although the mass difference of 330.2 u between fragment 13 and 14 corresponds to the mass of a dA_S, assignment of base # 13 as a T_S was supported by information from base composition analysis of ISIS 2922 which confirmed the absence of dA_S in the sequence. It is important to emphasize that generally only confirmatory information about an anticipated sequence is necessary; however, for these as well as for unknown sequences, other information from supporting identity tests typically already exists prior to sequencing.

Table 1
Cleavage products for ISIS 2922 phosphorothioate following direct SVP digestion

Peak	Sequence	Calc. [a] mass (u)	Exp. [a] mass (u)	Δ Mass [a,b] (u)
1	5′-d(GCGTTTGCTCTTCTTCTTGCG)	6682.5	6673.3	
2	5′-d(GCGTTTGCTCTTCTTCTTGC)	6337.5	6328.5	344.8
3	5′-d(GCGTTTGCTCTTCTTCTTG)	6032.4	6023.9	304.6
4	5′-d(GCGTTTGCTCTTCTTCTT)	5687.4	5676.1	347.8
5	5′-d(GCGTTTGCTCTTCTTCT)	5367.4	5357.7	318.4
6	5′-d(GCGTTTGCTCTTCTTC)	5047.4	5037.5	320.1
12	5′-d(GCGTTTGCTC)	3157.2	3165.4	1872.1
13	5′-d(GCGTTTGCT)	2852.2	2856.3	309.2
14	5′-d(GCGTTTGC)	2532.2	2526.1	330.2
15	5′-d(GCGTTTG)	2227.1	2220.3	305.8
16	5′-d(GCGTTT)	1882.1	1876.3	344.0
17	5′-d(GCGTT)	1562.1	1556.9	319.5

[a] Exp. = experimentally determined mass. Calc. = theoretical mass for each fragment ΔMass = difference between consecutive experimentally determined fragments.
[b] Average experimental mass observed for phosphorothioate nucleotides: dC_S 306.5 ± 2.4 ($n = 3$), dG_S 345.5 ± 2.0 ($n = 3$), T_S 322.1 ± 5.5 ($n = 4$).

Information is absent for the six internal bases of ISIS 2922 lost from fragments 6–11 which are essential for completing the sequence. Further, assignment of bases cleaved from fragments 17–20 was not accomplished, since digestion with a 5′-exonuclease (CSP) proved unsuccessful. Comparison of theoretical masses with those obtained experimentally demonstrates that the phosphorothioate linkage does, in fact, remain intact. The difference in signal intensities across separate regions of the mass spectrum confirms some dependence of ionization efficiency on base composition and/or sequence and homogeneity of the sample, as recently reported by Wang and Biemann [15] for MALDI-TOF/MS analysis of modified oligonucleotides. Fitzgerald et al. [6] observed that the presence of smaller fragments in the sample tended to decrease the efficiency of desorption/ionization for larger fragments. Base composition or sequence may also have some influence on the efficiency of enzymatic action. The addition of magnesium has been reported to improve digestion efficiency of phosphorothioate oligonucleotides [16], although this has not yet been evaluated in the current ISIS 2922 sequencing protocol.

In contrast, following oxidation of ISIS 2922 to its corresponding phosphodiester analog, straightforward full sequence analysis was accomplished in 28 min. The spectra shown in Fig. 3 depict the sequencing approach used. Assignment of bases 2–12 was facilitated by 3′-exonuclease digestion. Identification of bases 21–9 was then confirmed from the 5′ terminus using CSP. The identity of the 3′ terminal base was established by calculating the difference between the mass assigned to fragment peak 2 (6037.2 u) in Fig. 3(a) and to the parent fragment peak 21 (6370.7 u) in Fig. 3(c).

Residual oxidation reagents and limitations due to the presence of matrix components prevent assignment of bases below 1160 u. Additionally, the signal/noise level was influenced by the presence of salt adducts formed and the number of samplings per location on the plate. Occasionally, salt adducts, partially oxidized material, or $(M - 2H)^{-2}$ ions may dominate certain regions of interest; however, it is straightforward to distinguish interfering peaks on the basis of mass differences expected between related phosphodiester oligonucleotide fragments (i.e. 289–329 u). Further, since the masses of individual bases differ by at least 9 u, there is sufficient resolution to distinguish one base from another. However, in cases where base assignment was difficult owing to a base mass that fell between expected values, spectra were either reacquired at that time point or overlapping mass information from the following time point was used to confirm base identity. The low-mass $(M - 2H)^{-2}$ region observed in Figs. 3(b) and 3(c) can also be used to assign base identity. Mass differences between these doubly charged ions, if multiplied by a factor of two, may be used to verify the sequence information from the singly charged ions using the Voyager instrument, although this was not ob-

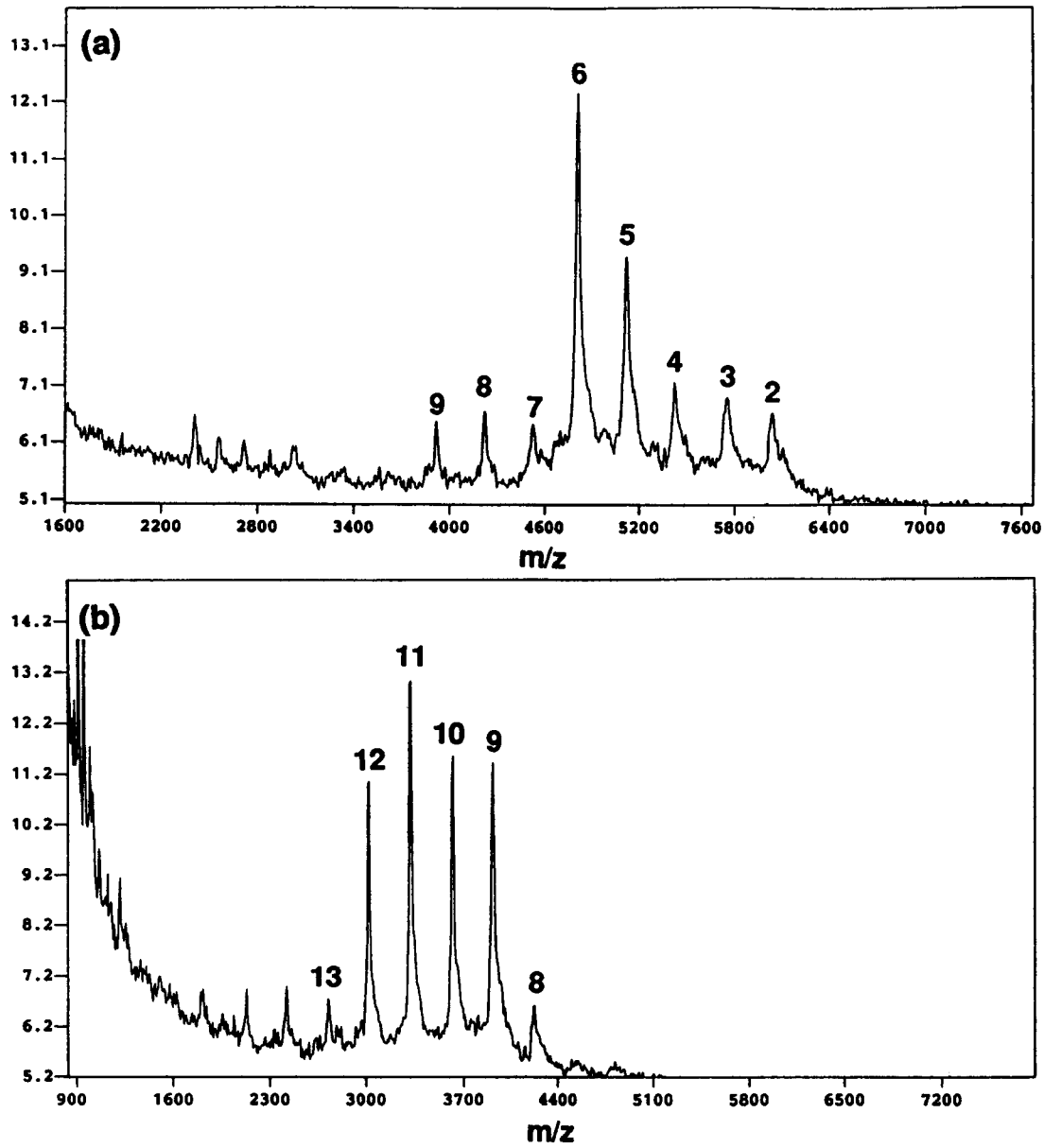

Fig. 3(a) and (b).

served for the LDI 1700 data. Table 2 lists the $(M-H)^{-1}$ fragment compositions for ISIS 2922. The relative standard deviation of experimental fragment masses from calculated masses ranged between 0.09 and 0.18% with an average error of 0.14% ($n = 23$). Average experimental values for individual bases (dC = 289.8 u, dG = 329.5 u and T = 305.1 u) agreed very well with theoretical values (dC = 289.2 u, dG = 329.2 u and T = 304.2 u).

Because ISIS 2922 does not contain deoxyadenosine, a number of other phosphorothioate and natural phosphodiester oligonucleotides were evaluated to demonstrate that this procedure is capable of adequately identifying deoxyadenosine. Fig. 4 and Table 3 combine information from the cleavage products of a sequential 3′-exonuclease digestion with that from a 5′-exonuclease digestion of oxidized ISIS 2105. Deoxyadenosine in ISIS 2105 is detected as base 12, having a mass difference of 310.9 u which corresponds to the theoretical mass of 313.1 u. Again, the average error of experimentally observed fragment masses relative to calculated values was 0.05% ($n = 22$), with the standard deviation ranging from 0.02 to 0.11%.

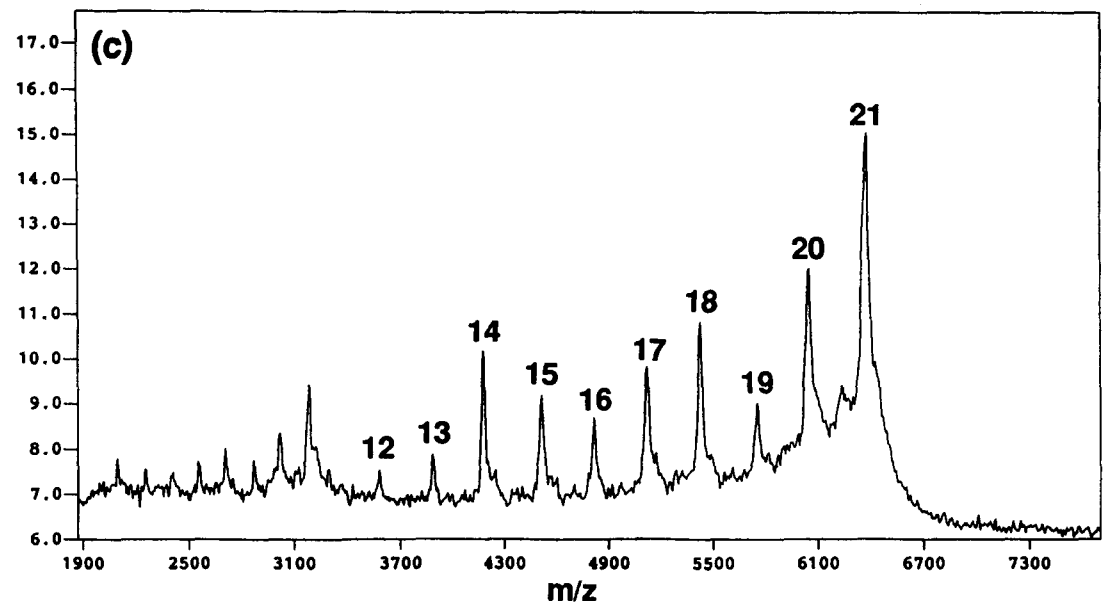

Fig. 3. MALDI-TOF mass spectra (LDI-1700 mass spectrometer) of 3'-exonuclease (SVP) digestion fragments of oxidized ISIS 2922 sampled at (a) $t = 7$ min and (b) $t = 20$ min, and 5'-exonuclease (CSP) digestion fragments of oxidized ISIS 2922 sampled at (c) $t = 8$ min. The base cleaved from fragment $1 \rightarrow 2$ is identified by calculating the mass difference between fragment 2 (Exp. mass = 6037.5 u) in (a) and the parent (Exp. mass = 6370.7 u) as assigned to fragment 21 in (c).

Table 2
Cleavage products of ISIS 2922 following oxidation to the phosphodiester analog

Peak	Sequence	Calc. [a] mass (u)	Exp. [a] mass (u)	Δ Mass [a,b] (u)
SVP digestion				
1	5'-d(GCGTTTGCTCTTCTTCTTGCG)	6361.5	6370.7	
2	5'-d(GCGTTTGCTCTTCTTCTTGC)	6032.3	6037.5	333.2
3	5'-d(GCGTTTGCTCTTCTTCTTG)	5743.1	5751.8	285.7
4	5'-d(GCGTTTGCTCTTCTTCTT)	5413.9	5423.3	328.5
5	5'-d(GCGTTTGCTCTTCTTCT)	5109.7	5117.4	305.9
6	5'-d(GCGTTTGCTCTTCTTC)	4805.5	4812.3	305.1
7	5'-d(GCGTTTGCTCTTCTT)	4516.3	4522.4	289.9
8	5'-d(GCGTTTGCTCTTCT)	4212.1	4217.8	304.6
9	5'-d(GCGTTTGCTCTTC)	3907.9	3913.9	303.9
10	5'-d(GCGTTTGCTCTT)	3618.7	3623.3	290.6
11	5'-d(GCGTTTGCTCT)	3314.5	3318.5	304.8
12	5'-d(GCGTTTGCTC)	3010.3	3014.1	304.4
13	5'-d(GCGTTTGCT)	2721.1	2723.2	290.9
CSP digestion				
21	3'-d(GCGTTCTTCTTCTCGTTTGCG)	6361.5	6370.7	
20	3'-d(GCGTTCTTCTTCTCGTTTGC)	6032.3	6043.3	327.7
19	3'-d(GCGTTCTTCTTCTCGTTTG)	5743.1	5750.5	292.8
18	3'-d(GCGTTCTTCTTCTCGTTT)	5413.9	5421.8	328.7
17	3'-d(GCGTTCTTCTTCTCGTT)	5109.7	5117.7	304.1
16	3'-d(GCGTTCTTCTTCTCGT)	4805.5	4812.4	305.3
15	3'-d(GCGTTCTTCTTCTCG)	4501.3	4506.9	305.5
14	3'-d(GCGTTCTTCTTCTC)	4172.1	4177.6	329.3
13	3'-d(GCGTTCTTCTTCT)	3882.9	3889.0	288.6
12	3'-d(GCGTTCTTCTTC)	3578.7	3582.0	307.0

[a] Exp. = experimentally determined mass. Calc. = theoretical mass for each fragment. ΔMass = difference between consecutive experimentally determined fragment masses.
[b] Average experimental values for bases: dC 289.8 ± 2.4 ($n = 6$), dG 329.3 ± 2.2 ($n = 5$), T 305.1 ± 0.9 ($n = 10$).

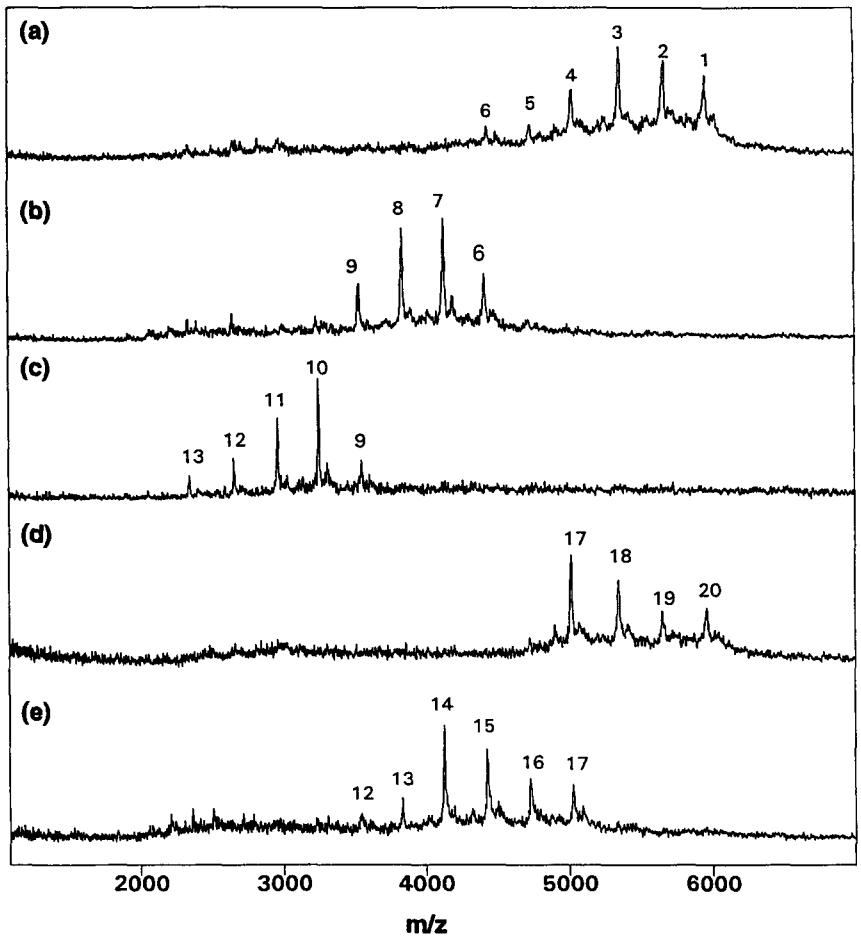

Fig. 4. MALDI-TOF mass spectra (Voyager mass spectrometer) of 3′-exonuclease (SVP) digestion fragments of oxidized ISIS 2105 sampled at (a) $t = 10$ min, (b) $t = 30$ min and (c) $t = 50$ min, and of 5′-exonuclease (CSP) digestion fragments of oxidized ISIS 2105 sampled at (d) $t = 1$ min and (e) 5 min.

Oxidation of phosphorothioate oligonucleotides to their intact phosphodiester analogs prior to enzymolysis facilitates cleavage and reduces overall oligomer sequencing time. Instruments using a stainless steel multisample holder similar to the Voyager may allow up to 100 digest samplings to be loaded and effectively inactivated by the matrix components. Through such a design, potentially more samples may be digested in a given day and analyzed as time permits. These elements are essential for sequencing on a routine basis, and therefore enable analysis of bulk drug substances at various time points of interest during and following synthesis.

4. Conclusion

It has been shown that sequential enzymatic cleavage in combination with MALDI-TOF analysis provides accurate confirmation of sequence for phosphorothioate oligonucleotides and a viable alternative to more time-consuming traditional methods of sequencing. It is interesting to note that the relative intensities of signal responses for individual fragments differ with location of the laser on the sample spot, suggesting that heterogeneity of the sample and matrix are important for signal reproducibility. However, with better control of sample/matrix heterogeneity, it is conceivable that a combination of information from unique mass differences and signal intensities for a few bases of a sequence at a particular time point might provide a basis for pattern recognition that could potentially replace current base-by-base identification. This feature of sequencing by enzymatic digestion will be explored in further detail. Additionally, it was noted that digestion with CSP was much more efficient than with SVP on phosphodiester oligonucleotides

Table 3
Cleavage products of ISIS 2105 following oxidation to the phosphodiester analog

Peak	Sequence	Calc. [a] mass (u)	Exp. [a] mass (u)	Δ Mass [a,b] (u)
SVP digestion				
1	5′-d(TTGCTTCCATCTTCCTCGTC)	5959.9	5955.8	
2	5′-d(TTGCTTCCATCTTCCTCGT)	5670.7	5668.7	287.1
3	5′-d(TTGCTTCCATCTTCCTCG)	5366.5	5363.2	305.5
4	5′-d(TTGCTTCCATCTTCCTC)	5037.3	5033.6	329.6
5	5′-d(TTGCTTCCATCTTCCT)	4748.1	4742.9	290.7
6	5′-d(TTGCTTCCATCTTCC)	4443.9	4442.0	300.9
7	5′-d(TTGCTTCCATCTTC)	4154.8	4154.3	287.7
8	5′-d(TTGCTTCCATCTT)	3865.6	3864.1	290.2
9	5′-d(TTGCTTCCATCT)	3561.4	3560.3	303.8
10	5′-d(TTGCTTCCATC)	3257.2	3256.4	303.9
11	5′-d(TTGCTTCCAT)	2968.0	2967.0	289.4
12	5′-d(TTGCTTCCA)	2663.8	2661.1	305.9
13	5′-d(TTGCTTCC)	2350.6	2350.2	310.9
CSP digestion				
20	3′-d(CTGCTCCTTCTACCTTCGTT)	5959.9	5957.8	
19	3′-d(CTGCTCCTTCTACCTTCGT)	5655.7	5649.7	308.1
18	3′-d(CTGCTCCTTCTACCTTCG)	5351.5	5347.2	302.5
17	3′-d(CTGCTCCTTCTACCTTC)	5022.3	5018.7	328.5
16	3′-d(CTGCTCCTTCTACCTT)	4733.1	4735.3	283.4
15	3′-d(CTGCTCCTTCTACCT)	4428.9	4430.7	304.6
14	3′-d(CTGCTCCTTCTACC)	4124.7	4126.1	304.6
13	3′-d(CTGCTCCTTCTAC)	3835.6	3834.7	291.4
12	3′-d(CTGCTCCTTCTA)	3546.4	3548.4	286.3

[a] Exp. = experimentally determined mass. Calc. = theoretical mass for each fragment. ΔMass = difference between consecutive experimentally determined fragment masses.
[b] Average experimental values for bases are: dC 288.3 ± 2.7 ($n = 8$), dG 329.1 ± 0.8 ($n = 2$), and T 304.4 ± 2.1 ($n = 9$).

and that the speed of digestion varied along the chain, suggesting that the enzyme kinetics is also sequence dependent. Snake venom phosphodiesterase, however, does appear to be more aggressive in digesting 2′-*O*-methyladenosine and phosphorothioate moieties. Further understanding of the kinetics will allow optimization of analysis time.

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