The observation of multivalent complexes of Shiga-like toxin with globotriaoside and the determination of their stoichiometry by nanoelectrospray Fourier-transform ion cyclotron resonance mass spectrometry

Elena N. Kitova, Pavel I. Kitov, David R. Bundle, and John S. Klassen

Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2G2

Received on December 7, 2000; revised on February 6, 2001; accepted on February 12, 2001

We show by nanoelectrospray ionization (nanoES) Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR MS) that it is possible to observe oligosaccharide–protein complexes with dissociation constants in the millimolar range, such as Pk trisaccharide (globotriaoside) complexed with the Shiga-like toxin (SLT) of pathogenic E. coli. It is further demonstrated that nanoES/FT-ICR MS is an exquisite method to study quantitative aspects of the association of mono- and polyvalent oligosaccharide ligands with multimeric proteins, such as the SLTs. At increasing trisaccharide:protein ratios it was shown that the B5 toxin subunit complexes with 5 Pk trisaccharides and only after all 5 copies of site 2 are essentially filled do any of the remaining 10 receptor sites become occupied. From the distribution of bound Pk's at the five binding sites, it was shown that the association constants for each of the five sites and to confirm that binding occurs noncooperatively, the association constants for each site are identical and that compared to site 1, site 2 exhibits a tenfold higher affinity for the globotriaoside synthetic ligand 1. The facile identification of the occupancy of binding sites represents information that is not readily available by other techniques. This sensitive and rapid estimation of association constants for protein–ligand complexes, which are free of unpredictable secondary effects that plague enzyme linked assays, is likely to find wide application.

Key words: protein-oligosaccharide complexes/association constants/stoichiometry/Shiga-like toxin/nanoelectrospray ionization

Introduction

Shiga and cholera toxins belong to the clinically significant subset of bacterial AB5 toxins consisting of an enzymatically active A subunit that gains entry to susceptible mammalian cells following oligosaccharide recognition by the B5 homopentamer (Merritt and Hol, 1995; Lindberg et al., 1987). Shiga toxin produced by Shigella dysenteriae type 1 and the homologous Shiga-like toxins (SLTs) of Escherichia coli, sometimes also called Verotoxins, cause serious clinical complications in humans infected by these organisms (Karmali et al., 1983a,b). Infection by the SLT-producing strain E. coli serotype O157:H7 has been called “hamburger disease” in North America because it often results from eating contaminated hamburger meat, although other foods and contaminated ground water may be the source of infection. In about 10% of cases, infection with SLT-producing E. coli, such as the O157:H7 serotype, leads to serious life-threatening complications as a result of the extraintestinal spread of SLTs (Boyd and Lingwood, 1989; Cleary and Lopez, 1989; Lingwood, 1994). These conditions include damage to erythrocytes (hemolytic anemia), consumption of platelets (thrombocytopenia), and acute renal failure, a triad of symptoms known collectively as hemolytic-uremic syndrome (HUS) (Karmali et al., 1985). A much more serious form of HUS, known as thrombotic thrombocytopenic purpura, can also occur as a result of these SLT-producing E. coli infections (Ashkenazi, 1993; Blecker and Vandenplas, 1994; Keusch and Acheson, 1997). HUS, the direct result of SLT-induced kidney damage, is the major cause of acute pediatric renal failure, and 30% of survivors experience permanent kidney damage (Karmali et al., 1983a,b). The functional toxin receptor on mammalian cells is the glycolipid Gb3 (Lindberg et al., 1987) and the high incidence of HUS in children correlates with the expression of Gb3 in the pediatric renal glomerulus (Boyd and Lingwood, 1989). The cytotoxic A subunit gains entry to the cell only following adherence of the whole toxin to its target receptor, the Gb3 glycolipid. It has been proposed that synthetic Gb3 analogues covalently attached to insoluble silica particles may competitively adsorb toxin from the gut (Armstrong et al., 1991); the adsorbent, Synsorb® P, is currently undergoing phase III clinical trials (Armstrong et al., 1995, 1998).

The crystal structure of the SLT-1 B5 pentamer complexed with a Gb3 trisaccharide analogue has been solved at 2.8 Å resolution (Ling et al., 1998). Three saccharide binding sites are found in each 7.7 kDa subunit. The subunits associate noncovalently to form a doughnut-shaped B5-pentamer with 15 saccharide binding sites aligned on one face of the toxin. The A subunit is attached to the opposite surface of the B5 pentamer (Stein et al., 1992; Fraser et al., 1994), thereby allowing all 15 sites to engage cell surface receptors. Each of the three binding sites interacts with the saccharide moiety of Gb3 in a distinct manner such that sites 1, 2, and 3 bury varying proportions of the trisaccharide epitope (Ling et al., 1998). The relative importance of the three binding sites observed by

1To whom correspondence should be addressed

© 2001 Oxford University Press
crystallography and their contribution toward biological activity is the subject of some speculation. Solution binding studies by titration microcalorimetry (St. Hilaire et al., 1994) or by nuclear magnetic resonance (NMR) studies (Shimizu et al., 1998) suggest that site 2 provides the crucial recognition, with minor contributions from sites 1 and 3. Based on computer modeling studies and receptor analogue binding data, site 1 was concluded to be the major Gb₃ binding site (Nyholm et al., 1996). The involvement of multiple sites was invoked on the basis of mutagenesis and crystal structure data (Ling et al., 1998; Bast et al., 1999).

We recently reported the design, synthesis, and crystal structure of a multivalent subnanomolar inhibitor complexed with SLT-1 B₅ pentamer (Kitov et al., 2000). The inhibitor design based on the published crystal structure placed two trisaccharide receptors at the tips of each of five spacer arms to permit the simultaneous engagement of sites 1 and 2 in all five B subunits. Although the inhibitor successfully embraced the toxin surface and placed the P⁵ trisaccharides in each B subunit, the bivalent ligand at the end of each arm occupied only site 2 of a single B subunit and does not bridge sites 1 and 2 as planned. Instead the remaining five copies of the P⁵ trisaccharide complexed a second molecule of toxin again via saccharide receptor binding to site 2.

During studies of oligosaccharide binding to single chain antibody using nanoelectrospray ionization (nanoES) Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR MS), we realized that this technique could provide unique data on the occupancy of the different binding sites of SLT-1. We demonstrate here the application nanoES/FT-ICR MS to delineate aspects of the self-association of the multimeric protein, SLT, as well as the stoichiometry and affinity of its specific association with various analogs of its natural receptor ligand, globotriaose [α-D-Galp(1→4)β-D-Galp(1→4)β-D-Glcp], also known as P⁵ trisaccharide.

Results

Detection of the intact AB₅ holotoxin and B₅ homopentameter in the gas phase

The individual A and B subunits but not the intact holotoxin have been observed by electrospray–mass spectrometry (ES-MS) (Kondo et al., 1997). Therefore, we first demonstrated that the SLT-1 holotoxin is preserved during the nanoES process and observed in the mass spectrum. At neutral pH only a weak ion signal, corresponding to polyprotonated B₅ pentamer, is present in the nanoES spectrum of SLT-1. No A subunit nor holotoxin ions are observed. The gaseous holotoxin ions are, in fact, believed to be produced by the nanoES process but at mass-to-charge ratios (m/z) that exceed the current capabilities of our mass spectrometer (>5000 m/z). This assumption is supported by the fact that the protonated holotoxin ions, (AB₅ + nH)ⁿ⁺ = AB₅ⁿ⁺ (where n = 18 to 20), are observed by acidifying the solution to pH 3.5 (Figure 1). It has been widely recognized that acidification leads to an increase in the charge states observed for gaseous proteins and protein complexes, resulting from acid-induced conformational changes (Konermann and Douglas, 1997). Denaturation of the subunits would result in an increase in the solvent-exposed surface area of the protein and the exposure of basic sites (Arg and Lys) that are unavailable in the native state. In addition, the less compact subunits are able to accommodate a greater number of charges in the gas phase (Schnier et al., 1995; De la Mora, 2000). NMR studies have shown that the B₅ pentamer undergoes a conformational change at pH 4 (Richardson et al., 1997), resulting in a loss of binding affinity for P⁵ trisaccharide (Scheme 1). The appearance of A and B subunits, along with B₃ ions, indicates that the conformational changes also weaken the intersubunit interactions and results in partial decomposition of the hexamer. Further reduction of pH, to a value of ~1, results in the loss of the quaternary structure of the holotoxin and the observation of only species corresponding to the individual A and B subunits. These results demonstrate that the intact holotoxin is easily transferred to the gas phase by nanoES. However, due to the low charge states generated at neutral pH, the holotoxin ions are beyond the m/z range of our instrument and could not be used to study oligosaccharides binding. Instead, our binding studies were conducted using the homopentameric B₅ molecule, a recombinant protein expressed in E. coli (Ramotar et al., 1990).

NanoES of an aqueous solution of SLT-1(B₅)(10⁻⁵ M) at pH 3.5. The individual A and B subunits but not the intact holotoxin have been observed by electrospray–mass spectrometry (ES-MS) (Kondo et al., 1997). Therefore, we first demonstrated that the SLT-1 holotoxin is preserved during the nanoES process and observed in the mass spectrum. At neutral pH only a weak ion signal, corresponding to polyprotonated B₅ pentamer, is present in the nanoES spectrum of SLT-1. No A subunit nor holotoxin ions are observed. The gaseous holotoxin ions are, in fact, believed to be produced by the nanoES process but at mass-to-charge ratios (m/z) that exceed the current capabilities of our mass spectrometer (>5000 m/z). This assumption is supported by the fact that the protonated holotoxin ions, (AB₅ + nH)ⁿ⁺ = AB₅ⁿ⁺ (where n = 18 to 20), are observed by acidifying the solution to pH 3.5 (Figure 1). It has been widely recognized that acidification leads to an increase in the charge states observed for gaseous proteins and protein complexes, resulting from acid-induced conformational changes (Konermann and Douglas, 1997). Denaturation of the subunits would result in an increase in the solvent-exposed surface area of the protein and the exposure of basic sites (Arg and Lys) that are unavailable in the native state. In addition, the less compact subunits are able to accommodate a greater number of charges in the gas phase (Schnier et al., 1995; De la Mora, 2000). NMR studies have shown that the B₅ pentamer undergoes a conformational change at pH 4 (Richardson et al., 1997), resulting in a loss of binding affinity for P⁵ trisaccharide (Scheme 1). The appearance of A and B subunits, along with B₃ ions, indicates that the conformational changes also weaken the intersubunit interactions and results in partial decomposition of the hexamer. Further reduction of pH, to a value of ~1, results in the loss of the quaternary structure of the holotoxin and the observation of only species corresponding to the individual A and B subunits. These results demonstrate that the intact holotoxin is easily transferred to the gas phase by nanoES. However, due to the low charge states generated at neutral pH, the holotoxin ions are beyond the m/z range of our instrument and could not be used to study oligosaccharides binding. Instead, our binding studies were conducted using the homopentameric B₅ molecule, a recombinant protein expressed in E. coli (Ramotar et al., 1990).
Multivalent complexes of Shiga-like toxin with globotriaoside

Complexes of $P^a$ and $B_5$ homopentamer

We investigated the complexation of 1 by SLT-1($B_5$) at various ligand concentrations and detected protonated ions for free $B_5$ and a limited range of bound species, $B_5$•1$_n$. NanoES was performed on aqueous solutions (pH 7.2) containing $5 \times 10^{-6}$ M of SLT-1($B_5$) and different concentrations of 1 (45, 154, and 310 µM). Using "gentle" sampling conditions, which minimize collisional heating of the ions during sampling into the mass spectrometer, the complexes of SLT-1($B_5$) and 1 are readily observed (Figure 3A–C). As expected, the degree of complexation increases with the concentration of the ligand. At the highest concentration studied, up to five and perhaps six trisaccharides are attached to the $B_5$ pentamer (Figure 3C). From the solution NMR studies, we expect five major binding sites and the possible contribution of the five minor sites at high ligand concentrations (following saturation of "site 2"). Microcalorimetry studies have revealed that binding of 1 to SLT-1($B_5$) is noncooperative and the binding constant ($K_{assoc}$ = $1.5 \pm 0.5 \times 10^3$ M$^{-1}$) is equivalent for the attachment at all five primary binding sites. Using this binding constant for "site 2" and an estimated value for "site 1" ($K_{assoc} = 0.15 \times K_{assoc}$) the distribution of $B_5$•1$_n$ complexes was calculated for each ligand concentration. The predicted distributions are shown together with the mass spectra in Figure 3. Despite the rather poor signal-to-noise ratio, particularly at the highest ligand concentration, the experimental distributions agree exceptionally well with the calculated distribution. In fact, the binding constant determined directly from the relative ion intensities for the addition of the first five ligands, averaged over the two charge states, is $2 \times 10^3$ M$^{-1}$, a value in excellent agreement with the isothermal titration calorimetry-derived figure (St. Hilaire et al., 1994).

Fig. 2. NanoES spectra of 10 µM aqueous solution of SLT-1($B_5$) at (A) pH 7.2 and (B) pH 3.5.

Fig. 3. NanoES spectra of the +12 and +13 charge states of SLT-1($B_5$) (5.0 µM) obtained at three different concentrations of $P^a$ trisaccharide glycoside 1: (A) 44.6 µM; (B) 154 µM; (C) 310 µM. Indicated on the mass spectrum is the number (n) of $P^a$ molecules bound to the $B_5$ pentamer ($B_5$($P^a$)$_n$). Shown beside each spectrum is the distribution of complexes calculated based on the binding constant determined in solution for site 2 ($K_{assoc} = 1.5 \pm 0.5 \times 10^3$ M$^{-1}$) and an estimated binding constant for site 1 ($K_{site1} = 0.15 \times K_{assoc}$).

Complexes of $B_5$ and multivalent oligosaccharides

We have extended this approach to other oligosaccharides for which the binding data have been obtained by competitive solid phase assays (Kitov et al., 2000). Oligosaccharides 2 and 3 (see Schemes 2 and 3; Kitov and Bundle, unpublished data) are divalent and decavalent ligands, capable of engaging in multiple interactions with the toxin molecule(s).

Based on the measured ion intensities obtained at neutral pH, the binding affinities were evaluated. For 2, a sixfold increase in binding affinity ($K_{assoc} = 9 \times 10^3$ M$^{-1}$), compared with that of 1 is calculated from the MS data. This increase in affinity can
be explained by interaction of the second Pk trisaccharide moiety with the weaker binding site 1. However, the magnitude of the binding constant is much smaller than the 40-fold increase in activity determined by enzyme-linked immunosorbent assay (Kitov et al., 2000). The origin of the discrepancy in the binding affinity is not known, and additional studies are needed. The decameric molecule 3 was also examined. At equimolar concentrations (3 × 10^{-6} M) of SLT-1(B5) and 3 only the 1:1 complex was observed and no unbound B5 ions were detected (Figure 4). Although this prevents us from measuring the binding affinity, it can be estimated to be >10^6 M^{-1} (calculated based on a lower limit of 2 for signal-to-noise ratio necessary to positively identify the unbound toxin). At pH 4, significant 2:1 B5•3 complex can be detected in the spectrum (Figure 5), consistent with the crystal structure for this complex (Kitov et al., 2000).
Fig. 5. NanoES mass spectrum of an aqueous solution containing 3 µM of SLT-1(B₅) and 3 µM STARFISH 3 acidified with acetic acid to pH 4.

Discussion

The excellent quantitative agreement with the solution composition, predicted from microcalorimetry data, observed in the nanoES spectra for solutions of B₅ and trisaccharide 1 is a significant result. ES-MS has previously been used to measure solution binding stoichiometry and affinity for a variety of biomolecular complexes, such as protein–protein and protein–ligand complexes (Ayed et al., 1998), protein–oligonucleotide complexes (Greig et al., 1995) and peptide and RNA-binding antibiotics (Jorgensen et al., 1998; Sannes-Lowery et al., 2000). With the exception of recent binding measurements reported for small molecule–RNA complexes with dissociation constants in the millimolar range (Griffey et al., 2000), quantitative ES-MS studies have been restricted to moderately or strongly bound complexes, with dissociation constants in the micro- to nanomolar range. To our knowledge, this is the first report of the direct determination by ES-MS of the binding stoichiometry and affinity for a protein–ligand complex with dissociation constant in the millimolar range.

The difficulty in applying ES-MS to such weakly interacting complexes is the high analyte concentration required to produce a significant amount of complex in solution. High concentrations of analyte in the ES solution often leads to observation of nonspecific complexes, resulting from the formation of random intermolecular interactions as the ES droplet shrinks due to evaporation of solvent. The presence of such complexes in the mass spectrum obscures the true solution composition. In addition to the possibility of the formation of nonspecific complexes, decomposition of protein–oligosaccharide complexes by collisional heating, which occurs during sampling into the mass spectrometer, may also influence the spectrum. The effects of in-source dissociation will be most significant for complexes that are weakly bound in the gas phase and prone to facile decomposition. The fact that quantitative agreement was observed between the solution composition and the relative ion abundance in the mass spectra suggests that neither nonspecific binding nor decomposition of the complexes in the gas phase was significant. The presence of nonspecific binding is normally identified by the disappearance of complexes on reduction of the analyte concentration. In the present case, the dissociation constant determined from the mass spectrometric data was found to be independent of the concentration of 1, which was varied from 45 to 310 µM. Therefore, if nonspecific complexes are produced by the nanoES process, they do not survive long enough to be detected.

We have also investigated the thermal decomposition kinetics for B₅•(Pk)₅ complexes using the blackbody infrared radiative dissociation (BIRD) technique (Price et al., 1996). This study (Felitsyn et al., unpublished data) revealed that the complexes are quite robust in the gas phase, presumably due to the retention of the protein–sugar hydrogen bonds originally present in solution and, perhaps, the formation of additional interactions. For example, at relatively high temperatures (~170°C) loss of a subunit is more facile than loss of Pk from protonated B₅•(Pk)₅ ions. Therefore, Pk trisaccharides that are bound specifically are unlikely to be lost during introduction into the mass spectrometer. However, B₅•(Pk) complexes that originate from nonspecific binding, via the formation of random interactions during the desolvation process, may be much more labile in the gas phase and easily decomposed during sampling. If this is the case, these weakly bound complexes may be filtered out in the source, leaving only the more stable, specific complexes. In an effort to address this question, we are extending our BIRD studies to evaluate the stability of other protein–oligosaccharide complexes.

The mass spectrometric data obtained for SLT-1(B₅) and 1 allowed us to establish that the binding sites of each subunit operate in a noncooperative manner and that preferential occupation of all five copies of the toxin site 2 occurs prior to occupancy of site 1. The relative importance of the three different binding sites has been a matter of debate in the literature. Our results, which indicate the presence of only one major binding site per subunit, are in agreement with the solution NMR studies (Shimizu et al., 1998). Furthermore, based on the relative abundance of bound and unbound B₅, we were able to determine the binding constant for attachment of the first five Pk’s at site 2. The mass spectrometric data also suggest that the Pk affinity for site 2 is 10-fold higher than for site 1.

NanoES/FT-ICR MS is an exquisite method to study the association of mono- and polyvalent oligosaccharide ligands with multimeric proteins, such as SLTs. It permits the facile identification of the occupancy of binding sites, information that is not readily available by other techniques. Its high-resolution capability is ideally suited to the observation of interactions between a large protein receptor and a relatively small oligosaccharide ligand. The sensitive and rapid estimation of association constants for protein–ligand complexes, which are free of unpredictable secondary effects that plague enzyme linked assays, is likely to find wide application.

Materials and methods

Holotoxin, homopentamer, and oligosaccharides

The SLT Type 1 (holotoxin) and homopentamer SLT-1(B₅) (Ramotar et al., 1990) were generously provided by G. Armstrong. Both proteins were purified according to the procedure described by Mulvey et al. (1998). Sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis (SDS–PAGE) in 12% gel with reducing agent (dithiothreitol) was performed for SLT-1(B₅) and produced a single band corresponding to an approximate molecular weight of 8000 Da, consistent with the
molecular weight of the B subunit calculated from the amino acid sequence (7688.3 Da). SDS–PAGE of the holotoxin showed two bands, corresponding to the A (32,210 Da) and B subunits. All protein samples were dialyzed against deionized water and lyophilized prior to MS analysis. Analysis of an acidified (pH 3.5) aqueous solution of SLT-1(B5) by ES-MS confirmed the purity of the protein sample. The measured molecular weight of B subunit (7688 ± 2 Da) is in excellent agreement with the sequence-derived value. Mass spectra obtained for the holotoxin in acidified solution (pH 3.5) exhibited two series of peaks, corresponding to the charge state. Mass spectra showed two bands, corresponding to the A (32,210 Da) and B subunits. All protein samples were dialyzed against deionized water and lyophilized prior to MS analysis. Analysis of an acidified (pH 3.5) aqueous solution of SLT-1(B5) by ES-MS confirmed the purity of the protein sample. The measured molecular weight of B subunit (7688 ± 2 Da) was in excellent agreement with the sequence-derived value. Mass spectra obtained for the holotoxin in acidified solution (pH 3.5) exhibited two series of peaks, corresponding to the charge state. Mass spectra confirmed the purity of the protein sample. The measured molecular weight of B subunit (7688 ± 2 Da) was in excellent agreement with the sequence-derived value. Mass spectra obtained for the holotoxin in acidified solution (pH 3.5) exhibited two series of peaks, corresponding to the charge state.

The trimethylsilyl glycoside of Pα trisaccharide 1 was synthesized as described by Kihlberg et al. (1989) and purified by reverse phase high-performance liquid chromatography. Stock solution with 2 mg/ml concentration in deionized water was used. Synthesis of divalent tethered compound 2 and the decavalent STARFISH molecule 3 will be described elsewhere (Kitov and Bundle, unpublished data). A stock solutions of SLT and SLT-1 B5-pentamer, in aqueous 50 mM ammonia bicarbonate (pH 7.2), were prepared at a concentration of 1 mg/ml. Acetic acid was used to reduce pH, as required.

**Mass spectrometry**

Mass spectra were obtained using a Bruker 47e ApexII FT-ICR mass spectrometer equipped with electrospray source (Analytica, Branford). To facilitate the observation of weakly bound noncovalent complexes, the glass sampling capillary in the source was replaced with a stainless steel desolvation capillary (0.43 mm i.d.) which was operated at ~150°C. NanoES was performed using aluminosilicate capillaries (0.68 mm i.d.), pulled to approximately 20 µm o.d. and 1–10 µm i.d. at one end using a micropipette puller (Sutter Instrument Co.). The nanospray tips were positioned approximately 1 mm from the sampling capillary using a microelectrode holder (Warner Instrument Inc.). The electric field required to spray the solution was established by applying a voltage of 800–1000 V to a platinum wire inserted inside glass tip. The solution flow rate ranged from 5 to 60 nl/min depending on diameter of the nanoelectrospray tip, electrospray voltage, and composition of the solution.

The droplets formed at atmospheric pressure in the interface between the nanoeIS tip and the metal capillary were introduced to the vacuum system of mass spectrometer. Solvent evaporation from the droplets or microsolvated complexes was assisted by thermal heating provided by sampling capillary. The ion/gas jet sampled by the capillary (52 V) was transmitted through a skimmer (4 V) and stored, electrosstatically, in the hexapole. Ions were accumulated in hexapole for 2–15 s, depending on the ion intensities, then ejected and accelerated by using high potential (~2700 V) through the fringing field of the 4.7 Tesla magnet, decelerated, and introduced into the ion cell and detected.

The ion intensities observed for the toxin–oligosaccharide complexes were found to be very sensitive to electrospray and source conditions (nanospray tip dimensions, solution flow rate, temperature of metal capillary, applied voltages, and storage time ions in hexapole). The strongest ion signals for complexes were observed at a relatively low electrospray voltage (~800 V) and a low solution flow rate (~5–10 nl/min). Increasing the solution flow rate required longer storage times in hexapole (up to 15 s) to generate comparable ion abundances.

Data acquisition was controlled by SGI R5000 computer running the Bruker Daltonics XMass software version 5.0. Mass spectra were obtained using standard experimental sequences with chirp broadband excitation. The time-domain signal, consisting of the sum of 10–500 transients containing 128 K data points per transient, was subjected to one zero-fill prior to Fourier transformation. The number of transients acquired varied in different experiments depending on signal-to-noise ratio.

**Acknowledgments**

Financial support for this work was generously provided by the Natural Sciences and Engineering Research Council of Canada, the Canadian Bacterial Disease Network, and the University of Alberta. We thank Paola Marcato and Dr. Glen Armstrong for samples of the SLT holotoxin and B5 subunit and Mrs. Joanna Sadowska for technical assistance.

**Abbreviations**

BIRD, blackbody infrared radiative dissociation; ES-MS, electrospray–mass spectrometry; FT-ICR MS, Fourier-transform ion cyclotron resonance mass spectrometry; HUS, hemolytic–uremic syndrome; nanoES, nanoelectrospray ionization; NMR, nuclear magnetic resonance; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SLT, Shiga-like toxin.

**References**


