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High-energy Collision-induced Dissociation by MALDI TOF/TOF Causes Charge-Remote Fragmentation of Steroid Sulfates

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Abstract

A method for structural elucidation of biomolecules dating to the 1980s utilized high-energy collisions (~10 keV, laboratory frame) that induced charge-remote fragmentations (CRF), a class of fragmentations particularly informative for lipids, steroids, surfactants, and peptides. Unfortunately, the capability for high-energy activation has largely disappeared with the demise of magnetic sector instruments. With the latest designs of tandem time-of-flight mass spectrometers (TOF/TOF), however, this capability is now being restored to coincide with the renewed interest in metabolites and lipids including steroid-sulfates and other steroid metabolites. For these metabolites, structure determinations are required at concentration levels below that appropriate for NMR. To meet this need, we explored CRF with TOF/TOF mass spectrometry for two groups of steroid sulfates, 3-sulfates and 21-sulfates. We demonstrated that the current generation of MALDI TOF/TOF instruments can generate charge-remote-fragmentations for these materials. The resulting collision-induced dissociation (CID) spectra are useful for positional isomer differentiation and very often allow the complete structure determination of the steroid. We also propose a new nomenclature that directly indicates the cleavage sites on the steroid ring with carbon numbers.

Introduction

Steroids are among the main regulators in mammalian physiology [1], and as a result, their metabolites are attractive candidates for conveying many aspects of an animal's status. Steroid sulfates were first recognized as naturally occurring metabolites in 1938 when Schachter and Marrian [2] isolated estrone sulfate from the urine of pregnant mares. Since then, many more steroid sulfates have been isolated from different biological sources and identified. Until the 1960s, they were considered as biologically inactive end-products of metabolism appropriate for elimination [3]. Many efforts have been made since then to search for the physiological role for steroid sulfates, and these efforts revealed the significance of sulfated steroids for structural, signaling and regulatory functions [4–8]. That they are biologically active is made clear by the commonly used drug Premarin, which is a complex mixture of steroid sulfates. Recently, with the emergence of lipidomics and metabolomics, the interest in steroid conjugate analysis is again growing. An example is the work of one of the authors (T. Holy), who found steroid sulfates of the glucocorticoid family

as “social odors” or “pheromones” in mouse urine [9, 10]. Given the low levels of these materials in mammalian fluids (at sub-micro molar concentration in mouse urine [9]), we need improved methods for structure determinations of steroid sulfates to deal with materials at concentrations below the levels at which NMR can be used.

In early studies of analysis of steroids and their conjugates, GC/MS with electron ionization (EI) was the dominant approach [11–13]. Although EI affords rich fragmentation, its utility is limited because most steroids require derivatization to form volatile, thermally stable analytes [14–17], sometimes compromising sensitivity. In the 1980s, the introduction of fast atom bombardment (FAB) [18] and tandem mass spectrometry (MS/MS) [19, 20], afforded a new approach for structural characterizations of steroid conjugates with mass spectrometry, but the approach required magnetic-sector instruments and high collision energies to generate charge-remote fragmentation. The concept of charge-remote (originally called remote-site fragmentation) was first described in 1983 as observed on a unique three-sector mass spectrometer [21, 22]. This instrumentation was later used to demonstrate the charge-remote fragmentations of a series of bile acids and bile salt conjugates [23] and other steroids [24, 25]. Tomer and Gross [26] then reported in 1988 that both charge-remote and charge-proximate fragmentations occur for bile salts, steroid sulfates, and glucuronides, showing that nearly complete structure determination of steroid rings could be achieved. Unfortunately, there has been a marked decline in the application of high-energy CID with the demise of sector instruments and the current dominance of ion traps, QTOFs, and hybrid ion-trap FT spectrometers that employ low-energy CID. Although the latter has been applied over years in structural characterization of organic compounds, including steroid sulfates [10, 27], the structural elucidation is complicated and incomplete because the major fragment ions usually result from losses of small molecules, and MS^3 is often required to get a more complete set of fragment ions. Furthermore, low-energy CID spectra tend to show more rearrangement reactions and are not as reproducible as high-energy spectra. Because low-energy CID strongly depends on instrument characteristics and settings, the prospects of building an MS^2 database for structure searches has become more complex [28].

The introduction of TOF/TOF instruments, specifically designed for high-energy CID [29, 30], has triggered a “renaissance” of interest in high-energy fragmentation processes. MALDI coupled to an appropriate TOF/TOF-instrument can accommodate the need for sufficient mass resolving power, precursor-ion selection, and high collision energies for the structural analysis of biomolecules [31–34]. The first evidence comes from Trimpin et al. [31] who showed that metal-cationized fatty acids desorbed from a solvent-free preparation undergo CRF in a TOF/TOF. Additional evidence is from Allmeier and Pittenauer [33, 34] who induced CRF of trialkylglycerols (TAGs). Other applications of MALDI TOF/TOF were also reported for carbohydrates and peptides [35, 36]. Whereas fatty acids have been investigated by MALDI TOF/TOF and their CID spectra are well understood, steroids and their conjugates have been far less investigated [37]. Here, we demonstrate, for the first time to our knowledge, the application of MALDI TOF/TOF instrumentation to induce CRF and enable structural characterization of sulfated steroids. The results are comparable to those obtained on the older tandem magnetic sector instruments and strongly suggest that TOF/TOF instruments offer a new opportunity for lipid and steroid structure-determination studies and for lipidomics in general.

Experiment

Materials

All steroids and sulfated steroids, cholesteryl sulfate, 3 β -hydroxy-5-pregnen-20-one-3-sulfate (termed SS395, for sulfated steroid 395, the number of which is the molecular mass of $[M - H]^-$), etiocholan-3 α -ol-11,17-dione sulfate (SS383), 4-pregnen-11 β ,21-diol-3,20-dione-21-sulfate (SS425), 4-pregnen-11 β , 17,21-triol-3,20-dione-21-sulfate (SS441), 4-pregnen-17,21-diol-3,11,20-trione-21-sulfate (SS439), 5 β -pregnan-11 β ,21-diol-3,20-dione, and 4-pregnen-11 β , 20 β ,21-triol-3-one were purchased from Steraloids Inc (Newport, RI), and dissolved in methanol to give a 5 mM stock solution. SS427 and iSS427 were prepared by sulfating 5 β -pregnan-11 β , 21-diol-3,20-dione and 4-pregnen-11 β ,20 β ,21-triol-3-one as described previously [38]. The structures of all steroid sulfates used are listed in Figure 1. All other chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MO).

Mass Spectrometry

A MALDI-TOF/TOF mass spectrometer (SpiralTOFTM, JEOL Ltd., Tokyo, Japan) was used for all measurements. This MALDI-TOF/TOF mass spectrometer has unique multi-turn spiral ion optics with a 17 m long flight path to provide ultrahigh mass resolving power for MALDI-TOF measurements [39–41]. An ion gate positioned at the 15 m point in the spiral ion flight path provided mono-isotopic precursor-ion isolation for tandem mass spectrometry upon high-energy CID [42, 43]. Selected monoisotopic precursor ions undergo 20 kV collisions with the helium collision gas at a pressure of 1.7×10^{-4} Pa. Ions exiting the collision chamber underwent 9 kV post-acceleration and focusing to ensure efficient detection of low m/z fragments.

A 349 nm Nd-YLF laser operated at a laser repetition rate of 1 kHz was used to desorb and ionize the samples. The extraction delay was optimized to 50 nsec. α -Cyano-4-hydroxycinnamic acid (CHCA) matrix was dissolved in 1:1 water/acetonitrile containing 0.1% trifluoroacetic acid at a concentration of 10 mg/mL (Trifluoroacetic acid, normally removed for the negative-ion mode detection, caused the signal intensity to decrease by two fold compared to its absence. It was utilized here to show its compatibility with negative-ion mode detection and to keep open the possibility for adoption when analyzing a biological sample mixture in both the positive- and negative-ion modes.). The steroid sulfate and matrix solutions were mixed together 1:1 by volume. A 1.0 μ L aliquot of this mixture was deposited onto the MALDI target plate for analysis with the MALDI-TOF/TOF mass spectrometer.

Results and Discussion

We used MALDI TOF/TOF to investigate the fragmentation of two groups of steroid sulfates, namely 3-sulfates and 21-sulfates. These two groups were chosen because hydroxyl sites on neutral steroids suitable for sulfonation by steroid sulfotransferase are commonly present at carbons 3 and 21 (or 17) for C-21 steroids (or C-19 steroids). Furthermore, they have the sulfate group fixed at either end of the steroid [44], allowing us to demonstrate the charge-remote nature of the fragmentation. The fixed charge at one end of the precursor ion after ionization should allow CRF to be induced remotely as the predominant reaction

channel under high-energy CID for both 3-sulfates and 21-sulfates. With the fixed sulfate group, these steroid sulfates can be readily desorbed and ionized by MALDI as $[M-H]^-$ species (Figure 2a shows one example of the negative-ion mass spectrum of SS441). With the SpiralTOF, accurate mass measurements are achieved in MS1 scans, and monoisotopic precursor-ion selection is made possible for fragmentation (Fig 2b), allowing straightforward interpretation of the product-ion spectra (TOF/TOF). The major fragmentations for members of each subclass are similar and often involve through-ring cleavages, as is described below.

Fragmentation nomenclature

Given that this fragmentation by through-ring cleavages may be general, we provide a new nomenclature designating the steroid ring cleavages. Shown for cholesteryl sulfate (Table 1) is the nomenclature, where each cleavage is named for the ring in which it occurs and the positions of two carbon atoms constituting the two bonds that must be broken to cause a through-ring cleavage (the designated carbon atoms are to the left of the cleavage, and ring A is on the left, ring D on the right, as per convention). For example, a cleavage that breaks ring B at C9-C10 and C7-C8 is designated as $^{7,10}B$. In addition to major ring cleavages, high-mass ions are formed by cleavages of the side chain attached to ring D, remote from the charge site (Figure 3). Cleavages occurring at the side chain are named by using the two carbon positions at the C-C bond being cleaved. For example, the cleavage at bond C20-C22 is named as $^{20,22}S$ ("S" refers to the side-chain).

3-Sulfates

We investigated three steroids substituted with sulfate at the 3-position (A ring) by MALDI TOF/TOF and obtained highly informative MS/MS data, with rich fragmentation occurring through the steroid ring (Table 1). As indicated in the product-ion spectra (Table 1 and Figure 3), 3-sulfates undergo predominantly charge-remote fragmentation upon high-energy collisions, providing information about the nature of the substituent and its location, especially if the substituent is an alkyl chain. Taking cholesteryl sulfate as an example, we find rich fragmentation of the ring structure and the side chain at C17 (Figure 3a) to give a detailed "footprint" of the ring junctions and the side-chain branching of this compound. For example, the mass difference for the B-ring cleavage, $^{7,10}B$, and the C ring cleavage, $^{8,9}C$, is 26 Da, which corresponds to C_2H_2 and shows that the ring junction is unsubstituted. Comparing the three 3-sulfates, we find that processes $^{2,3}A$, $^{8,9}C$, and $^{13,14}D$ occur for the three compounds, and the mass differences between $^{2,3}A$ and $^{8,9}C$ are 120 Da (C_9H_{12}) for cholesteryl sulfate and SS395 whereas for SS383, the difference is 122 Da (C_9H_{14}), consistent with the absence of a double bond in the latter. Similarly, the differences between $^{8,9}C$ and $^{13,14}D$ are consistent with the substitution patterns (Table 1 and Figure 3). For cholesteryl sulfate and SS383, the differences between $^{13,14}D$ and $^{13,15}D$ are 14 Da, as expected whereas for SS395, the $^{13,15}D$ fragment is missing, and replaced by an ion m/z 323 formed by loss of C_4H_8O by an unknown mechanism. On the contrary, the appearance of product ions from cleavages across the B ring are significantly different for SS383 and the other two, mainly because the C5-C6 bond is a single bond for SS383 and a double bond for the other two (Figure 3a and 3b). A fragment ion is formed by the nearby cleavage, $^{7,10}B$, of both cholesteryl sulfate and SS395, although this fragment is missing in the product-ion

spectrum of SS383 (Figure 3c). Besides B-ring cleavages, cleavages of the C ring show the expected differences among the three compounds. For example, $^{8,11}\text{C}$ is absent in SS383 fragmentation, consistent with the presence of the C11 keto group that prevents this cleavage (Table 1), and the differences between $^{8,9}\text{C}$ and $^{8,11}\text{C}$ for cholesteryl sulfate and SS395 is 14 Da, consistent with a CH_2 group at position 11. This detailed analysis of ring cleavages was not presented in the early papers on CRF of steroids, and the prospects for a nearly complete structural analysis were not understood.

Importantly, branching sites on the side chain are easily determined by examining the pattern for $\text{C}_n\text{H}_{2n+2}$ eliminations, because cleavages happening at either side of a branch point are more facile than those occurring via double cleavages at a branch point.

In addition to the common fragment ions due to ring or side-chain cleavage, some unexpected fragments are also formed; these are consistent with our early findings on CRF of steroid sulfates [26]. For example, besides the losses of neutral molecules in which the C17 substituent is cleaved with H-transfer, cleavages also occur to eliminate directly a CH_3 radical, presumably forming a distonic ion, rather than H transfer and elimination of neutral molecules (this cleavage is termed $^{17,18}\text{S}$, as shown in Table 1). The mechanism for this cleavage was previously proposed to occur through a six-member transition state (Scheme 1 in ref 26). Although obtaining detailed mechanistic data is not the purpose of this paper and must await future work, we show the fragmentation for clarity. Another example is the fragmentation of the three 3-sulfates to undergo a CH_4 loss upon CID. This process most likely arises from the loss of an angular methyl group (C18 or C19) with H rearrangement and indicates the presence of methyl substituents.

21-Sulfates

We investigated five different compounds with different substitutions on the ring or the side chain, as listed in Table 2. Similar to 3-sulfates, 21-sulfates give rich charge-remote fragmentation as cross-ring cleavages and of the side chain upon high-energy CID (Figure 4). Ions of m/z 80 and 97 are also abundant in product-ion spectra of 21-sulfates, representing SO_3^- and HSO_4^- from releases of small, charged sulfur-containing groups. Although we adopted the same nomenclature for all the cleavages of 21-sulfates as for the 3-sulfates, the designations for 21-sulfates are of portions of the steroid that are lost as neutrals whereas for the 3-sulfates, they are part of the fragment ion. The scheme in Table 2 shows an example of the cleavages of SS427.

The CID spectra of $[\text{M} - \text{H}]^-$ ions can be used to delineate clearly the locations and type of different substitutions. For example, the positional isomers, iSS427 and SS427 differ in the position of a double bond; iSS427 has the double bond between C4 and C5, whereas SS427 has the unsaturation located at C20 as a keto group. As a result, their product-ion spectra (Figure 4a and 4b) show that ions formed by $^{1,4}\text{A}$ cleavage are among the most abundant fragments formed from SS427 but undetectable for iSS427. The same types of fragmentations involving rings B, C, and D occur for both compounds, but they show a consistent mass shift of 2 Da. In addition, the C20 keto groups in SS427 and in SS425 (Figure 4b and 4c) prevent the $^{17,20}\text{S}$ cleavage, but replacement of the keto group with C20-OH in iSS427 promotes this fragmentation and gives the ion of m/z 139 (Figure 4a). An m/z

139 ion is observed, however, although less abundant, in the product-ion spectra of SS441 and SS439 (Figure 4d and 4e), indicating that the effect of a C20 keto group on $^{17,20}\text{S}$ is eased when substituted by a hydroxyl group at C17 (the only difference between SS441 and SS425). Although SS441 differs from SS425 only by the C17 hydroxyl group, it undergoes fewer cleavages of the C and D rings, suggesting that a C17 hydroxyl group suppresses cleavages of the C and D ring. Likewise, SS439 also undergoes nearly no cleavages across the C and D ring under CID.

Expulsions of neutral molecules, CH_4 or CO , are dominant reactions for most 21-sulfates being studied. The loss of CH_4 is similar to that in 3-sulfates, which likely involves an angular methyl group. Loss of CO is also a charge-remote cleavage on the A ring of 21-sulfates, which occurs similarly at the D ring of SS383, a 3-sulfate steroid. The other two 3-sulfates undergo no such fragmentation owing to the lack of keto groups on the ring system. Besides losses of CH_4 and CO , SS441 also gives abundant ions by the loss of H_2O , which are followed by other consecutive fragmentations such as $^{2,3}\text{A}$ and $^{1,3}\text{A}$, as indicated in Table 2.

We wish to note that the cleavages are not always as simple as indicated by the dashed lines. Sometimes the cleavage occurs as designated (e.g., $^{1,3}\text{A}$, $^{1,4}\text{A}$, $^{7,10}\text{B}$, $^{8,11}\text{C}$, $^{8,12}\text{C}$, and $^{13,15}\text{D}$) whereas other cleavages occur as designated but are accompanied by additional H transfer(s). Consider, for example, $^{2,3}\text{A}$. A simple through-ring cleavage would give loss of $\text{C}_2\text{H}_2\text{O}$, but the actual fragmentation is to lose $\text{C}_2\text{H}_4\text{O}$. The mechanistic reasons for these differences are interesting and remain to be elucidated. Nevertheless, structural assignments can still be made in the absence of a complete understanding of the ion chemistry.

Approximate Detection Limits

In addition to studying the effectiveness of MS/MS fragmentation for structure elucidation, we evaluated the detection limit of the 3- and 21-sulfates on this MALDI TOF/TOF instrument, requiring sufficient fragmentation information for structure characterization. We took one example from each group of steroids, cholesteryl sulfate and SS425, and evaluated the signal-to-noise ratio (S/N) of the base peaks in their product-ion spectra when different amounts of sample were applied to the MALDI plate. As shown in Table 3, S/N of the base peak at m/z 311 in the product-ion mass spectrum of cholesteryl sulfate decreased from 200 to 80 when the applied sample amount is reduced from 25 pmol to 4 pmol. Further reduction of the sample applied to 1 pmol caused the S/N for detecting the ion of m/z 331 to decrease to 20. Although the intensity for the base peak detection is still acceptable with 1 pmol of sample application, some of lower-abundance product ions disappeared below the S/N at this condition (e.g., the ion of m/z 217), making a structure assignment incomplete. Similarly, for SS425, S/N of the base peak at m/z 219 decreased from 500 to 60 in the product-ion spectrum when the sample applied was decreased from 25 to 1 pmol; further, the lower abundance fragment ions began to disappear with 1 pmol of sample application. Although the S/N tracks only approximately the applied sample quantity, we can conclude that the approximate detection limit sufficient for structure assignment of steroid sulfates is low pmol levels, with some variations for different samples. This low sample-size requirement demonstrates the applicability of MALDI TOF/TOF for analyzing biological

samples where only trace amounts can be isolated (e.g., SS425 was shown to exist in mouse urine at micro-molar concentrations in a previous study [9]).

Conclusion

The effectiveness of charge-remote-fragmentation for primary structure identification of lipids and steroids has been known for some time. The approach can determine locations of double bonds, identify various functional groups, locate branching and ring junctions directly from product-ion spectra. Instrumentation and ionization methods traditionally used for CRF, however, have become rare, diminishing the importance of CRF in structural studies. It continues to be recognized and invoked, however, as one class of fragmentation, a “thermal-like” process that occurs with little or no involvement of a charge or radical site. Low-energy MS/MS fragmentation of steroid sulfates [38] produce fewer cross-ring cleavages and is less informative than the spectra from high-energy activation, which induces extensive fragmentation across the steroid ring at the ring junctures.

The ability to induce CRF coupled with the advantage of monoisotopic precursor-ion selection afforded by new TOF/TOF instruments, permits relatively straight-forward interpretation of product-ion spectra because all product ions are monoisotopic. These characteristic ions formed under high-energy CID are nearly identical to those observed many years ago on tandem sector instruments when the sample was introduced by FAB ionization. Cleavages through the steroid rings occur whether the steroid is a 3- or a 21-sulfate, consistent with the classification that the processes are “charge-remote” as would be expected because the intervening rigid steroid ring makes it impossible for the charge site to interact with the reaction site. Although the earlier measurements on steroids proved that the fragmentation is indeed charge-remote and showed the nature of the remote side chain, this work also shows that there is more information in these spectra (e.g., on ring junctions and substituent locations).

In spite of the commonality of ring cleavages, 3-sulfates can be easily differentiated from 21-sulfates by the low m/z product ions. For example, 3-sulfates give a product ion of m/z 123 by $^{2,3}A$ cleavage, whereas 21-sulfates give product ions of m/z 110 arising from the side-chain cleavage, $^{20,21}S$. 3-Sulfates containing alkyl side chains on the D ring can also be differentiated from 21-sulfates, because the former is rich in fragments in the high mass range, owing to cleavages of the side chain, to give product ions that reflect its structure and locate the branch points. Locating functional groups is also straightforward by considering the mass differences between nearby cleavages. An important caveat is that certain substitutions on the ring (e.g., a C11 keto group) can prevent nearby fragmentations. In this case, the absence of cleavages is also informative and should be taken into account when elucidating unknown steroid structures.

We project that an important application of this approach will be in imaging steroids, lipids, and related materials.

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Figure 1.
Structures of steroid sulfates investigated here.

Figure 2.
Monoisotopic precursor ion selection of SS441 for TOF/TOF measurements in Spiral TOF.

Figure 3. High-energy CID fragmentation in the negative-ion mode of 3-sulfates: a. cholesteryl sulfate, b. SS395, c. SS383. The highly broadened $[M-H]^-$ signals indicate this precursor is off scale presumably because the product-ion yield is low and dispersed over many fragments.

Figure 4.

High-energy CID fragmentation in the negative-ion mode of 21-sulfates: a. iSS427, b. SS427 c, SS425 d, SS441, and e. SS439. The highly broadened $[M-H]^-$ signals indicate this precursor is off scale presumably because the product-ion yield is low and dispersed over many fragments.

Table 1

Major fragment ions from high-energy CID of [M - H]⁻ ions of 3-sulfates.

Steroid sulfate	SO ₃ ⁻ HSO ₃ ⁻	2,3A	5,10B	6,10B	7,10B	8,9C	8,11C	13,14D	13,15D	17,18S	17,18S
Cholesteryl sulfate	80,97	123	N/A	N/A	217	243	257	311	325	337	337
SS395	80,97	123	N/A	N/A	217	243	257	311	N/A	337	337
SS383	80,97	123	191	204	N/A	245	N/A	327	341	N/A	N/A

Major fragment ions from high-energy CID of $[M - H]^-$ ions of 21-sulfates.

Steroid sulfate	2,3-A	1,3-A	1,4-A	5,10-B	6,10-B	7,10-B	8,9-C	8,11-C	8,12-C	13,14-D	13,15-D	17,20-S	20,21-S
iS8427	383	371	N/A	317	303	291	263	235	221	180	167	139	110
SS427	383	371	357	315	301	288	261	233	219	178	165	N/A	110
SS425	381	369	N/A	315	301	289	261	233	219	178	165	N/A	110
SS441	381	367	N/A	331	317	305	N/A	N/A	235	N/A	N/A	139	110
SS439	395	383	N/A	329	N/A	303	N/A	N/A	235	N/A	181	139	110

***^{2,3}A and ^{1,3}A product ions from SS441 is combined with a consecutive lost of H₂O, which is revealed in m/z.

Signal-to-noise ratios of base peaks in the product-ion spectra of cholesteryl sulfate and SS425 as a function of the amount of sample applied

Table 3

Steroid sulfate	Cholesteryl sulfate					SS425				
Base peak (m/z)	311					219				
Applied amount (pmol)	25	10	4	1	25	10	4	1		
S/N	200	100	80	20	500	400	200	60		