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RESEARCH ARTICLE

Novel Fragmentation Pathways of Anionic Adducts of Steroids Formed by Electrospray Anion Attachment Involving Regioselective Attachment, Regiospecific Decompositions, Charge-Induced Pathways, and Ion–Dipole Complex Intermediates

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Abstract

The analysis of several bifunctional neutral steroids, 5-α-pregnane diol (5-α-pregnane-3α-20βdiol), estradiol (3,17α-dihydroxy-1,3,5(10)-estratriene), progesterone (4-pregnene-3,20-dione), lupeol (3β-hydroxy-20(29)-lupene), pregnenolone (5-pregnen-3β-ol-20-one), and pregnenolone acetate (5-pregnen-3β-ol-20-one acetate) was accomplished by negative ion electrospray mass spectrometry (ESI-MS) employing adduct formation with various anions: fluoride, bicarbonate, acetate, and chloride. Fluoride yielded higher abundances of anionic adducts and more substantial abundances of deprotonated molecules compared with other investigated anions. Collision-induced dissociation (CID) of precursor $[M+anion]^-$ adducts of these steroids revealed that fluoride adduct $[M+F]^-$ precursors first lose HF to produce $[M-H]^-$ and then undergo consecutive decompositions to yield higher abundances of structurally-informative product ions than the other tested anions. In addition to charge-remote fragmentations, the majority of CID pathways of estradiol are deduced to occur via charge-induced fragmentation. Most interestingly, certain anions exhibit preferential attachment to a specific site on these bifunctional steroid molecules, which we are calling "regioselective anion attachment." Regioselective anion attachment is evidenced by subsequent regiospecific decomposition. Regioselective attachment of fluoride (and acetate) anions to low (and moderate) acidity functional groups of pregnenolone, respectively, is demonstrated using deuterated compounds. Moreover, the formation of unique intermediate ion–dipole complexes leading to novel fragmentation pathways of fluoride adducts of pregnenolone acetate, and bicarbonate adducts of d_4-pregnenolone, are also discussed.

Key words: Steroids, Anion attachment, Electrospray, Mass spectrometry, Regioselective, Regiospecific, Ion–dipole complex, Ion–neutral complex, Charge-induced decomposition

Introduction

Steroids are important compounds that perform essential tasks in nature [1, 2]. These include vital hormone functions that exert a variety of physiological effects in the

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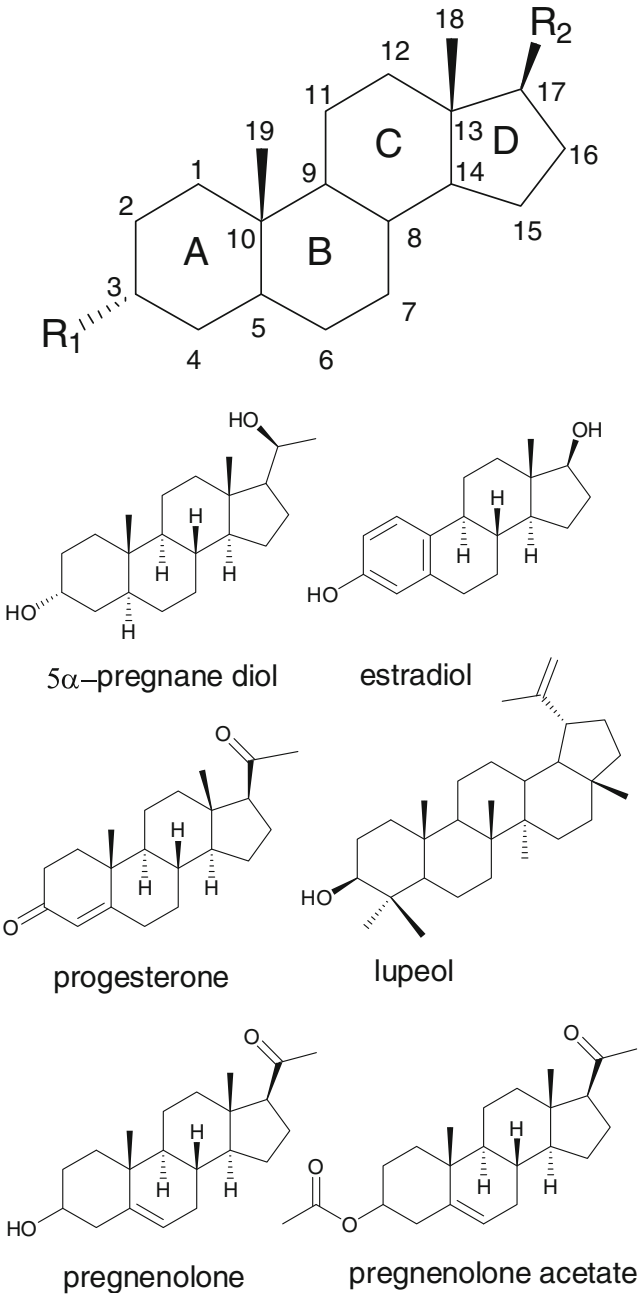
body [3–5]. Steroids facilitate protein synthesis and growth of muscles and bones [6–8]. They are characterized by their backbone, which is composed of three six-membered rings and one five-membered ring, labeled as A, B, C, and D (Scheme 1). The development of suitable analytical techniques for the characterization and analysis of steroids has been on-going for several decades. Mass spectrometry combined with chromatographic techniques has become very popular owing to its sensitivity and selectivity [9]. The analysis of steroids has been carried out by gas chromatography–mass spectrometry (GC-MS) [10–16], liquid chromatography-mass spectrometry (LC-MS) [17–19], electrospray mass spectrometry (ESI-MS) [20–24], atmospheric pressure chemical ionization mass spectrometry (APCI-MS) [25], matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) [26–28], and immunoassay [29]. Although GC-MS approaches are highly sensitive, they have some limitations due to the need for multistep derivatization procedures for these thermally-labile compounds, especially those that contain large numbers of hydroxyl groups [9, 30, 31]. In recent years, ESI-MS has been widely used for steroid analysis [32–34]. Currently, LC coupled to ESI-MS [35–37], ESI-MS/MS, or APCI-MS [38] are the favored techniques to analyze steroid mixtures. These techniques have the advantage of high sensitivity and modest sample preparation requirements. In ESI-MS, however, some of the neutral steroids may not be readily ionized because of the lack of basic or acidic functional groups. The attachment of anions to these neutral steroids offers a viable alternative method to produce anionic adducts and deprotonated molecules.



Scheme 1. Steroid nomenclature and structures of investigated bi-functional neutral steroids

Upon addition of ammonium acetate and ammonium formate into the mobile phase, previous chromatography/ESI-MS studies of steroids have reported the formation of deprotonated molecules and the corresponding anionic adducts in negative ion ESI-MS. When using an LC mobile phase of methanol/water/ammonium acetate (1 mM) and 0.001 % acetic acid, Pozo et al. [39] reported the formation of $[M+CH_3COO]^-$ for several anabolic steroids. Antignac et al. [40] observed $[M+CH_3COO]^-$ formation from corticosteroids in biological matrices of cattle when employing a methanol/water/1 % acetic acid mobile phase. From a mobile phase of acetonitrile/water/formic acid (0.01 %), Pozo et al. [41] reported $[M+HCOO]^-$ when performing analysis of corticosteroid metabolites in urine. Tolgysea et al. [42] documented that when acetate buffer is employed to adjust the pH in preparation for APCI-MS/MS of corticosteroids, more intense precursor ions were formed in the negative ion mode than in the positive ion mode. The formation of acetate adduct has been reported as common for anabolic steroids and corticosteroids, which have hydroxyl groups in the steroid skeleton [40]. As described in the above references, it is clear that the yields of these adduct ions depend on the type and amount of additives in the mobile phase.

Our previous experience with electrospray anion attachment targeting neutral compounds [43–52] led us to examine the possibility of creating anionic adducts of neutral steroids with different anions and, subsequently, studying the fragmentation patterns. Formation of the deprotonated molecule is a two-step procedure in which the neutral precursor first reacts with anion to form the anionic adduct, $[M+anion]^-$ followed by decomposition of this adduct under collision to form $[M - H]^-$. Success in producing deprotonated forms of neutral steroids depends upon the kinetics of the abstraction of protons and the stability of the formed $[M - H]^-$ species. Adduct formation occurs between the introduced anion and electron-deficient atoms on the molecule of interest. For

example, the H atom on a hydroxyl group is a good candidate site for anion attachment thereby forming a proton-bound mixed dimer of anions of the form $[M - H]^-....H^+....A^-$ [44]. The stability of this type of anionic adduct depends on factors such as the gas-phase basicity (GB) of the deprotonated analyte vs. that of the attaching anion; when the two are well-matched, adduct stability has been shown to improve [46]. If an anion has the ability to form multiple sites of hydrogen bonding, this has also been shown to improve adduct stability [47].

In this study, we reinforce and build upon the use of anion attachment as a complement to $[M - H]^-$ formation in negative ion ESI-MS for the analysis of neutral steroids that often show poor yields of $[M - H]^-$ in ESI-MS. We investigate in detail the decomposition of anionic adducts of bifunctional steroids (Scheme 1), especially fluoride adducts of these steroids [52], in view of broadening the ability to obtain structural information from anionic adducts of these weakly acidic steroids.

Experimental

Materials

Methanol, ammonium fluoride, ammonium bicarbonate, ammonium acetate, ammonium chloride, and all neutral steroids were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Solvents were all HPLC grade and were used without further purification. The quadruply deuterated analogue of pregnenolone (17, 21, 21, 21-d_4-pregnenolone) was obtained from Cambridge Isotopes Inc., (Andover, MA, USA). The concentration of the stock solutions of steroids was 1 mM in methanol:water 9:1. Stock solutions of salts of the respective anions (1 mM) were also prepared in methanol:water 9:1. Individual working solutions of each steroid were obtained by diluting the steroid stock solution with methanol:water (9:1) containing 1 % (wt/wt) ammonium salt of the anion.

Mass Spectrometry

All mass spectrometry experiments were performed on a triple quadrupole ion trapping tandem mass spectrometer (Applied Biosystems 3200 Q Trap LC MS/MS, Foster City, CA, USA) equipped with a standard Turbo V electrospray source. Collision-induced dissociation (CID) experiments were carried out at ~3–4 mTorr pressure of collision gas. Sample solutions were introduced into the mass spectrometer at a flow rate of 5 μL/min using nitrogen as both nebulizing gas and drying gas; the capillary voltage was −4500 V. Mass spectrometric conditions were optimized for $[M+anion]^-$ signals before acquiring data using standard solutions of estradiol, lupeol, progesterone, pregnenolone, 5-α-pregnane diol, and pregnenolone acetate.

Results and Discussions

It is important to note at the outset that most neutral steroids are not charged in aqueous solutions near neutral pH because of the high pK_a and low pK_b of these steroids. Lacking strong acidic or basic functionalities, these steroids tend to exhibit poor ESI-MS responses. Building upon our previous experience with anion attachment in negative ion ESI-MS, various anions were tested for their abilities to form anionic adducts with neutral steroids containing two functional groups with the goal of improving the mass spectral response.

Selection of Anion for Attachment

A judicious choice of anion can offer success in promoting negative ion ESI-MS signals and in obtaining structurally-informative fragmentation in tandem mass spectrometry experiments. Various anions (i.e., fluoride, bicarbonate, acetate, and chloride (listed in order of decreasing GB, corresponding to increasing acidity of the neutral acid form, see Table 1) were tested for their abilities to form adducts with neutral steroids: 5α-pregnane diol, estradiol, lupeol, progesterone, pregnenolone, and pregnenolone acetate.

Table 1. Signals from Formation and Decomposition of Steroid Adducts with Inorganic Anions[a]

Steroid	F^-		HCO_3^-		CH_3COO^-		Cl^-	
	GB*=1530[54,56]		GB=1458[57]		GB=1429[54,55]		GB=1373[55]	
	$[M–H]^{-b}$	$[M+A]^{-c}$	$[M–H]^{-b}$	$[M+A]^{-c}$	$[M–H]^{-b}$	$[M+A]^{-c}$	$[M–H]^{-b}$	$[M+A]^{-c}$
5α-pregnane	+++	+++	+++	+++	+	+++	nd	++++
Estradiol	++++	++++	+++	+++	+++	+++	+++	+++
Progesterone	+++	++++	++	+++	++	+++	nd	+++
Lupeol	+++	++++	++	+++	++	+++	nd	+++
Pregnenolone	++++	++++	+++	+++	+++	+++	nd	++++
Pregnenolone acetate	+++	++++	+++	+++	+++	++++	nd	+++

* Gas phase basicity (GB) values of conjugate bases are given in kJ/mol.
[a] Ammonium salts are used as the anion source.
[b] Indicates intensity of $[steroid - H]^-$ peak.
[c] Indicates intensity of $[steroid+anion]^-$ peak.
+ Indicates absolute intensity $>10^5$, ++ indicates absolute intensity $>10^6$, +++ indicates absolute intensity $>10^7$, ++++ indicates absolute intensity $>10^8$.
nd=not detected.

Using 5α-pregnane diol (5 μM) as an example, with no anion added to the solution, deprotonated 5α-pregnane diol was barely detectable by negative ion ESI-MS (Figure 1a) because of the very low acidity of the two hydroxyl groups on the molecule. However, when 0.1 % (wt/wt) NH_4F was added to the solution, formation of $[M - H]^-$ and $[M+F]^-$ adduct ions was observed in high yield (Figure 1b). The abundance of $[M+F]^-$ is highest at low collision offset voltages (20–40 V), whereas elevated cone voltages promote nozzle-skimmer fragmentation. Low collision offsets allowed $[M+F]^-$ detection of 5α-pregnane diol at 100 nM concentration with 1.5 nmol consumed during acquisition.

Fluoride, bicarbonate, acetate, and chloride anions, each readily formed anionic adducts, $[M+A]^-$ with all the steroids tested, and the corresponding signal intensities are shown in Table 1. Excellent ESI-MS adduct signals were obtained from each of the four anions, indicating that stable proton-bound mixed dimers of anions could be formed in each case. The lifetime of such adducts increases when one or more deprotonated form(s) of the steroid has a GB that is close to that of the attaching anion [46]. If there is a "mismatch" in terms of the gas-phase basicities of the two anions comprising the proton-bound mixed dimer, then the adduct will decompose rapidly.

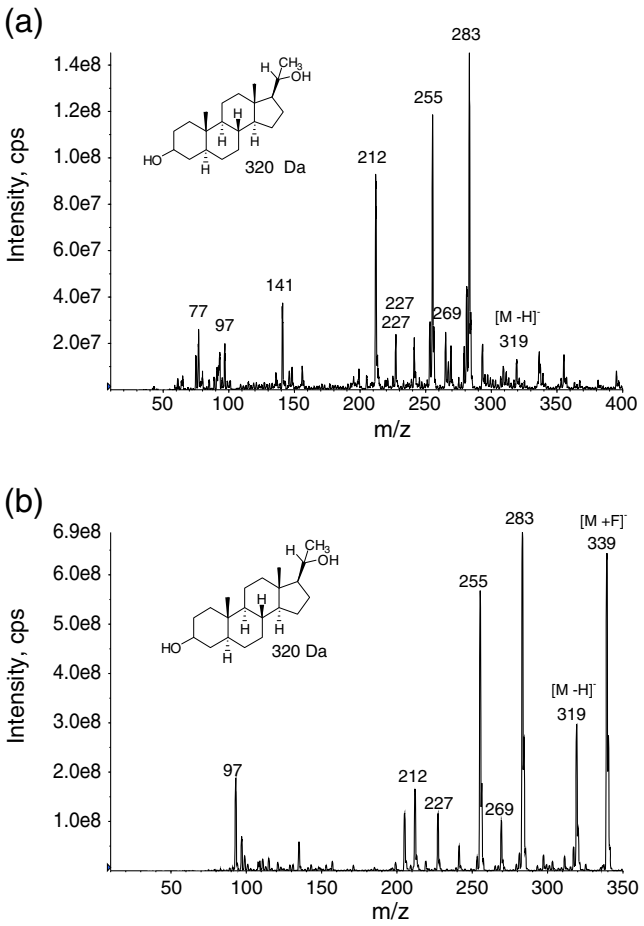


Figure 1. Negative ion ES mass spectrum of 5 μM 5-α-pregnane diol: **(a)** in the absence of additives, note low signal level of $[M - H]^-$; **(b)** in the presence of NH_4F, allowing facile $[M+F]^-$ detection

ESI-MS/MS of Anionic Adduct Precursors of Steroids

These same anions were tested for their abilities to provide structurally-informative fragment ions in MS/MS experiments examining decompositions of anionic adduct precursors. Experiments with chloride and acetate showed that while these two anions can form adduct ions in high intensities in negative mode electrospray, upon CID, chloride adducts produce only chloride ion (*m/z* 35), whereas acetate adducts primarily produce acetate ions (*m/z* 59) along with a few very minor steroid-containing product ions. Figure 2a shows the example of predominate chloride departure from the chloride adduct of estradiol. The weak GB of chloride renders it incapable of pulling off the weakly acidic phenolic hydrogen to form $[M - H]^-$ and, therefore, structural information cannot be obtained.

In MS/MS investigations of gas-phase decomposition of precursor adduct ions, only $[M+F]^-$ gave strictly steroid-related product ions (i.e., no F^- observed). The very high GB of F^- imposes a strong attraction for protons leading to rapid loss of HF, thus leaving $[M - H]^-$ to possibly undergo consecutive decompositions. At very low-energy CID, formation of $[M - H]^-$ was observed to dominate the product ion mass spectrum. As the collision energy was raised, more fragment ions were observed due to consecutive fragmentation of the $[M - H]^-$ ion (Figure 2b). Figure 2c shows CID products obtained from the acetate adduct of estradiol. Owing to its rather low GB, acetate is only capable of abstracting the phenolic hydrogen (the most acidic proton of estradiol) in low yield; acetate ion (*m/z* 59) is observed to dominate the CID mass spectrum. We show this example because the CID products are clearly formed from consecutive decompositions of $[M - H]^-$ deprotonated at the phenoxide group. In addition to fluoride and acetate, bicarbonate (GB between that of fluoride and acetate) was also capable of providing informative fragment ions in high yield upon CID of the $[M+HCO_3]^-$ adduct.

In the high *m/z* range (*m/z* >250) typical charge-remote fragmentation patterns [32] were observed for the examined steroids (see Table 1) and were consistent with side chain bond cleavages. In ESI-MS/MS experiments, all tested steroids gave rise to dominant fragment ions derived from the losses of H_2, CH_4, or H_2O to produce $[M - H_2]^-$, $[M - CH_4]^-$, $[M - H_2O]^-$, and $[M - H_2 - CH_4]^-$. When leaving as CH_4, the CH_3 group presumably originated from either C-13 or C-10 (except for lupeol, which has four additional methyl groups) with a vicinal H removed in the process to form a double bond. If more than one CH_3 group is present on the steroid, the subsequent release of a second CH_4 group was observed.

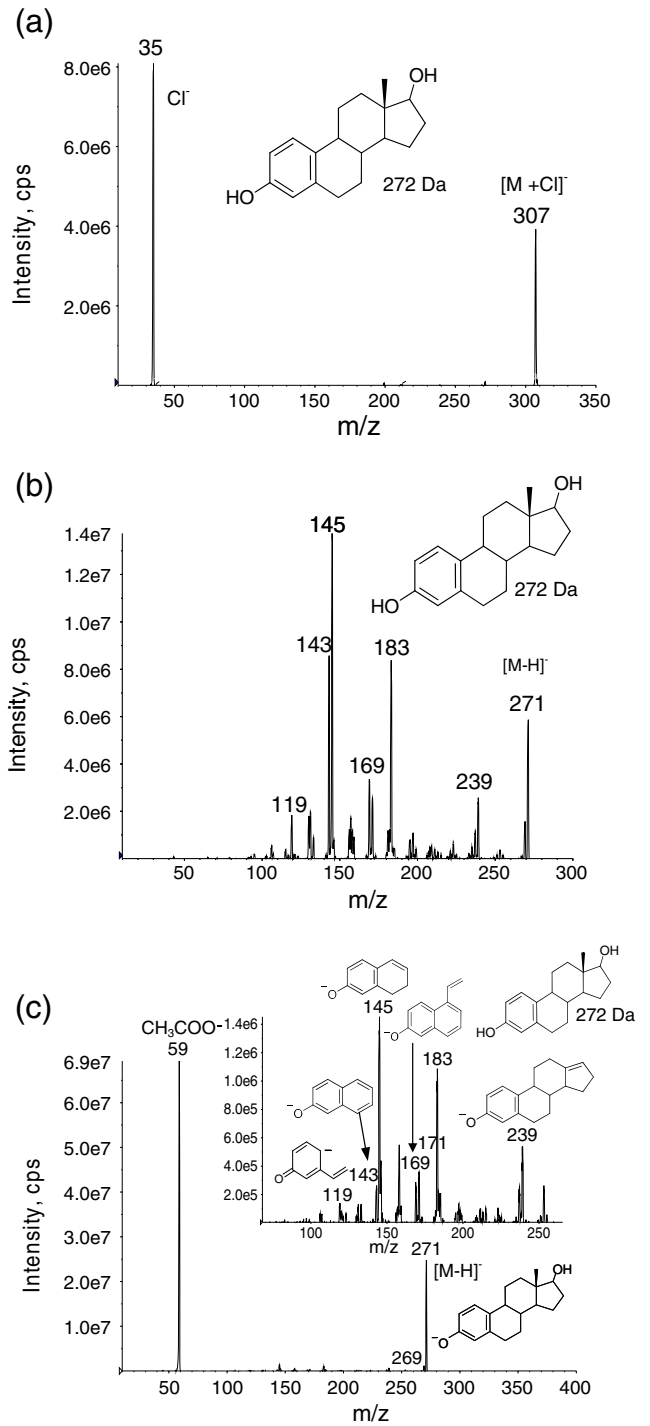


Figure 2. Negative mode ES-MS/MS product ion mass spectra of anionic adduct precursors of estradiol: **(a)** chloride adduct at *m/z* 307; **(b)** fluoride adduct at *m/z* 291; **(c)** acetate adduct at *m/z* 331

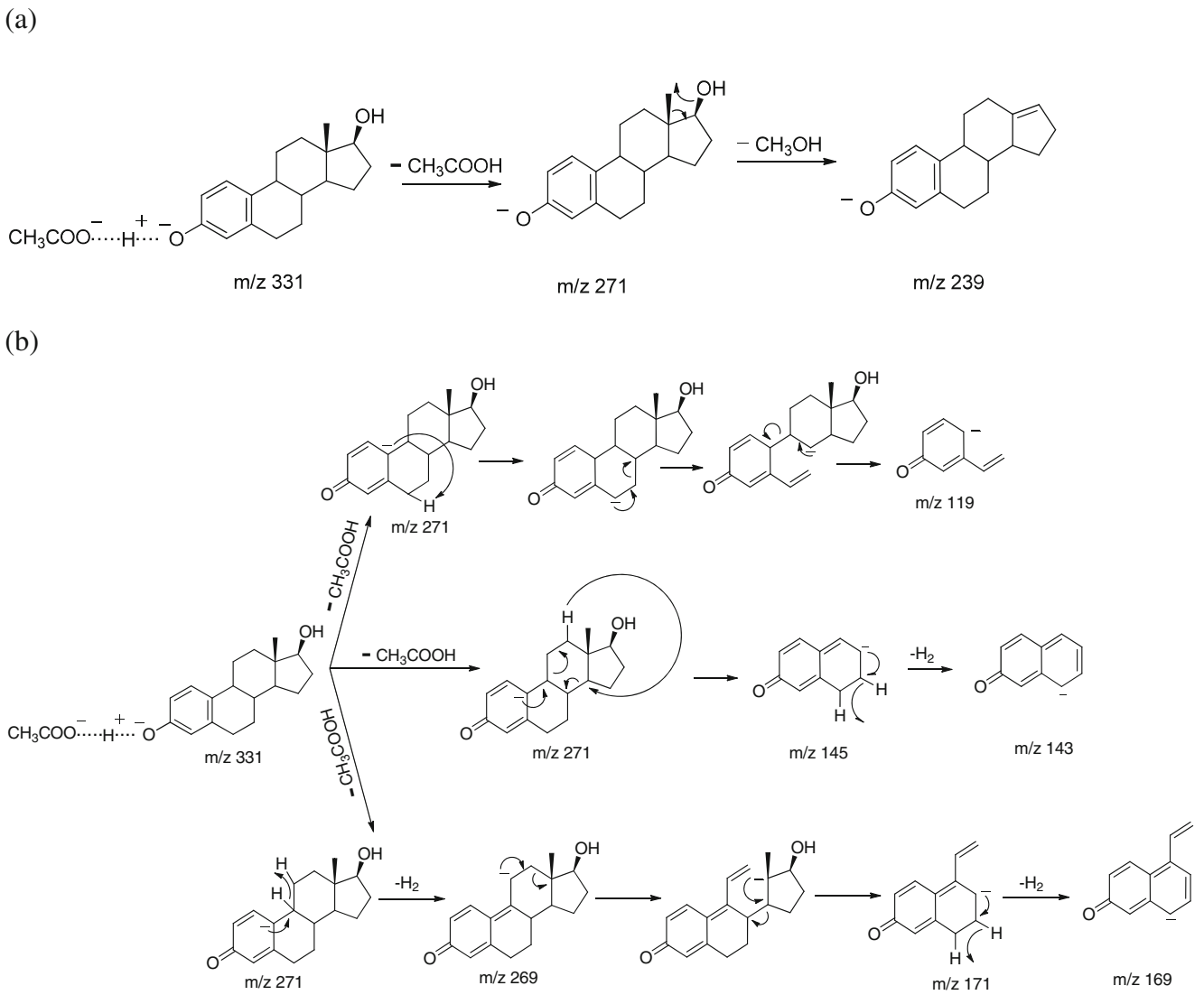
Novel Charge-Induced Fragmentation Pathways of Estradiol

The ionization of neutral estradiol is not very efficient in conventional negative ion ESI-MS, resulting in a low abundance of $[M - H]^-$. Because estradiol possesses a phenolic hydrogen, one might expect an elevated acidity compared with other steroids that have a saturated A ring. Nonetheless, CID of abundant chloride adducts yields only Cl^- (Figure 2a), thus indicating that chloride has a weaker GB than the phenoxide site of deprotonated estradiol. Addition of fluoride, however, can readily generate $[M+F]^-$ which, under CID, can produce a wealth of fragment ions (Figure 2b). Acetate adducts produced fewer steroid fragment ions under CID (Figure 2c); the peak corresponding to $[M - H - 32]^-$ has been assigned as loss of CH_3OH involving the C-18 methyl group and the hydroxyl group at C-17. This loss of methanol is proposed to occur by charge-remote fragmentation, after initial loss of acetic acid (phenolic hydrogen abstraction) from $[M+CH_3COO]^-$ (Scheme 2a). Analogous methanol loss involving the C-18 methyl group and a C-17 hydroxyl function was shown to occur from stanozolol in the positive ion mode [53].

Under low-energy CID, the $[M+CH_3COO]^-$ of estradiol produced other product ion peaks at *m/z* 119, 143/145, 169/171, 183, 239, and 269 (Figure 2c). Some of these peaks have been assigned in the literature as products of charge-remote fragmentation of the B, C, and D rings [32]. In contrast to previous reports, we attribute most decompositions of estradiol to charge-induced fragmentation pathways (Scheme 2b). Charge migration via HOMO electron delocalization (see Supplemental Material) can substantially activate hydrogen transfers and ring opening leading to highly conjugated product ions. Among the observed peaks, *m/z* 119, 143/145, 169/171, and 269 have been rationalized to form via charge-induced fragmentation (Scheme 2b). Charge-induced fragmentation starts from deprotonation of the phenolic hydrogen. As shown in Scheme 2b, the negative charge initially localized to the phenolic oxygen can shift to C-10 of the aromatic A ring. The negative charge localized on C-10 is key to initiating charge-induced fragmentation, involving proton transfer followed by B ring opening resulting in the highly conjugated *m/z* 119. Formation of a double bond between C-10 and C-9 can lead to C ring opening followed by H transfer to yield *m/z* 145, which can subsequently lose H_2 to form *m/z* 143. Formation of highly conjugated *m/z* 171 and 169, can also occur by charge-induced pathways.

Regioselective Anion Attachment

It was somewhat unexpected that all four of the anions listed in Table 1, with widely differing gas-phase basicities, would give such strong adduct signals. This is because we are accustomed to observing only a narrow range of gas-phase basicities matching the deprotonated form of the most acidic site on the molecule [46]. Comparing the product ion spectra of chloride, acetate, bicarbonate, and fluoride adduct precursors of pregnenolone (5-pregnen-3β-ol-20-one), CID of the chloride adduct of pregnenolone yields chloride anion as the only product ion, hence, it is not useful for structural



Scheme 2. Mechanism of [estradiol+CH_3COO]$^-$ decomposition: **(a)** methanol loss by charge remote fragmentation after initial loss of CH_3COOH; **(b)** charge-induced decomposition leading to product ions at *m/z* 271, 119, 145, 143, 269, 171, and 169

characterization. The acetate adduct of pregnenolone produces a very small peak corresponding to [pregnenolone-H]$^-$ (after losing CH_3COOH) and a few other small peaks (e.g., *m/z* 97 and 283) (Figure 3a) along with the predominant acetate anion peak. By contrast, upon CID, the fluoride adduct of pregnenolone precursor readily produces a variety of product ions at *m/z* 299, 255, 161, 135, 123, 69, 57, and 41 (Figure 3c). Lastly, under low-energy CID, bicarbonate adducts of pregnenolone decompose to give product ions with intermediate intensities (i.e., between fluoride and acetate (Figure 3b)); because bicarbonate ion (*m/z* 61) dominates in the spectrum, the efficiency of production of structurally-informative product ions suffers compared to the fluoride case.

A remarkable observation was the fact that dissociation of the fluoride adduct gave different fragment ions compared to those observed in the decomposition of acetate adduct. This implies that the two series of product ions are not coming from the decomposition of same [M – H]$^-$ ion. Pregnenolone has one moderately acidic hydrogen at the cyclohexanol group and also four weakly acidic hydrogens located alpha to the carbonyl group of the cyclopentane ring. To elucidate the implications of this difference in proton acidity at opposite ends of the bifunctional molecule, anion attachment experiments were repeated using this time a quadruply deuterated analogue of pregnenolone (17,21,21,21-d_4-pregnenolone).

Surprisingly, fluoride adducts of d_4-pregnenolone selectively lost DF to produce *m/z* 318, [d_4-pregnenolone-D]$^-$ along with product ions containing two or three deuterium atoms (*m/z* 302, 301, 183, 164, 126, 60, 59, and 42) (Figure 3d). It should be noted that all product ions can be assigned as consecutive decomposition products of the [d_4-pregnenolone-D]$^-$ precursor. This result indicates that the key to explaining CID product ions lies in understanding the initial site of deprotonation. Figure 4 displays a direct

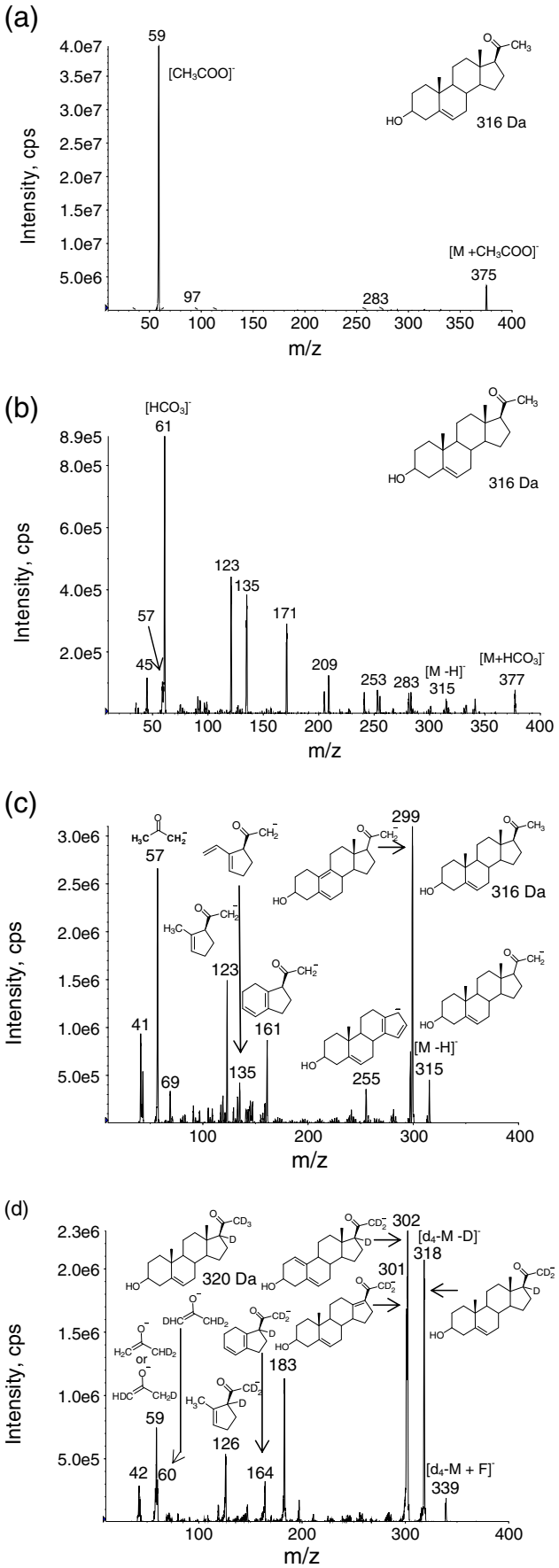


Figure 3. Negative mode ES-MS/MS product ion mass spectra of: **(a)** acetate adduct; **(b)** bicarbonate adduct, **(c)** fluoride adduct of pregnenolone precursors; **(d)** fluoride adduct of d_4-pregnenolone precursor

comparison of product ions appearing in the region of $[M - H]^-$ for decompositions of three anionic adducts of d_4-pregnenolone. The adduct of acetate anion (the anion of lowest GB) selectively lost neutral acetic acid to form $[d_4$-pregnenolone-H$]^-$ at *m/z* 319 (Figure 4a). This clearly indicates that the A-ring hydroxyl proton on pregnenolone

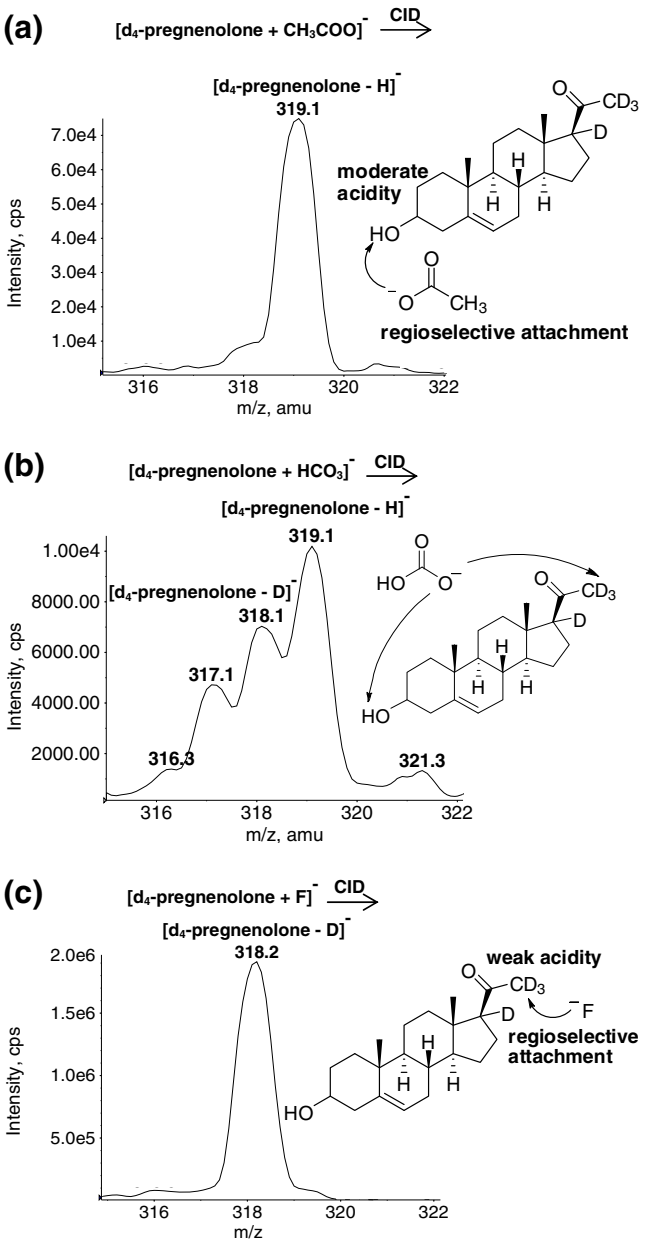


Figure 4. Blow-up of negative mode electrospray CID product ion mass spectra showing *m/z* 315–322 (region of deprotonated molecules) from precursor adducts of d_4-pregnenolone: **(a)** acetate adduct at *m/z* 379; **(b)** bicarbonate adduct at *m/z* 381; **(c)** fluoride adduct at *m/z* 339

has the highest gas-phase acidity, and it is the only proton that acetate can hope to (inefficiently) abstract. A close-up look at the first step in fluoride adduct decomposition confirms that [d_4-pregnenolone-D]$^-$ at *m/z* 318 is selectively formed (Figure 4c). Bicarbonate ion, however, produced both *m/z* 319 [d_4-pregnenolone-H]$^-$ and *m/z* 318 [d_4-pregnenolone-D]$^-$ as a result of the competing losses of HF and DF, respectively (Figure 4b). These results serve as proof that acetate and fluoride ions have different preferred sites of attachment at opposite ends of the bifunctional steroid. We are calling this preferential attachment to a specific site "regioselective anion attachment." The subsequent "regiospecific" decompositions illustrated in Figure 4 provide unequivocal evidence for regioselective stability of the acetate and fluoride adducts.

The GB of an anion clearly plays an important role in achieving regioselective attachment. For an adduct ion composed of a deprotonated steroid and a small inorganic anion each bound to a central proton, the adduct ion is more stable if the GBs of the two components of the mixed dimer are closely matched. Results shown in Figure 4 lead to the conclusion that the GB of acetate anion (1429 kJ/mol) [54, 55] must be reasonably matched with pregnenolone deprotonated at the hydroxyl site in order to bind and survive as an adduct ion. The fluoride adduct has a long lifetime only when attached to the acetyl site of pregnenolone, again as the result of the reasonably matched GBs of fluoride (1530 kJ/mol) [54, 56] and pregnenolone deprotonated alpha to the carbonyl group of the cyclopentane ring. If fluoride attaches instead to the hydroxyl site of pregnenolone in the ion source, HF loss is apparently so rapid that this type of adduct cannot survive to the collision cell. The GB of bicarbonate anion (1458 kJ/mol) [57] is in between that of acetate and fluoride, and its intermediate GB matches reasonably well with the GBs of pregnenolone deprotonated at either the hydroxyl site or the acetyl site. The fact that the [d_4-pregnenolone-H]$^-$ (*m/z* 319) peak is substantially larger than the [d_4-pregnenolone-D]$^-$ peak at *m/z* 318 in Figure 4b suggests that bicarbonate's GB is more closely matched with pregnenolone deprotonated at the hydroxyl site.

Further evidence of regioselective fluoride attachment at an acetyl hydrogen of pregnenolone comes from the decomposition of the acetyl group to produce *m/z* 57 (Figure 3c) [52]. This ion becomes shifted to *m/z* 60 in the analogous decomposition of the [d_4-pregnenolone+F]$^-$ adduct (Figure 3d). Neither chloride nor acetate ion is basic enough to remove the proton from the acetyl group, and CID of the chloride or acetate adduct of pregnenolone does not produce *m/z* 57. Moreover, neither chloride nor acetate adducts of d_4-pregnenolone produced a product ion peak at *m/z* 60. However, bicarbonate ion, having a GB in between those of fluoride and acetate, may remove the proton from the acetyl group of pregnenolone in low yield, and thus does produce a very small peak at *m/z* 57 (Figure 3b).

Progesterone differs from pregnenolone in that the C-3 hydroxyl group of the latter is oxidized to the carbonyl and a double bond is shifted (Scheme 1). Upon low-energy CID, the fluoride adduct of progesterone also produced an intense [M – H]$^-$ ion (*m/z* 313) and a variety of consecutive decomposition products at *m/z* 297/295, 241, 171, 145, 135, 123, and 57 (Figure 5a). The appearance of *m/z* 57 from CID of [progesterone+F]$^-$ adds further support to our prior assignment of *m/z* 57 as originating from decomposition of the acetyl group in pregnenolone.

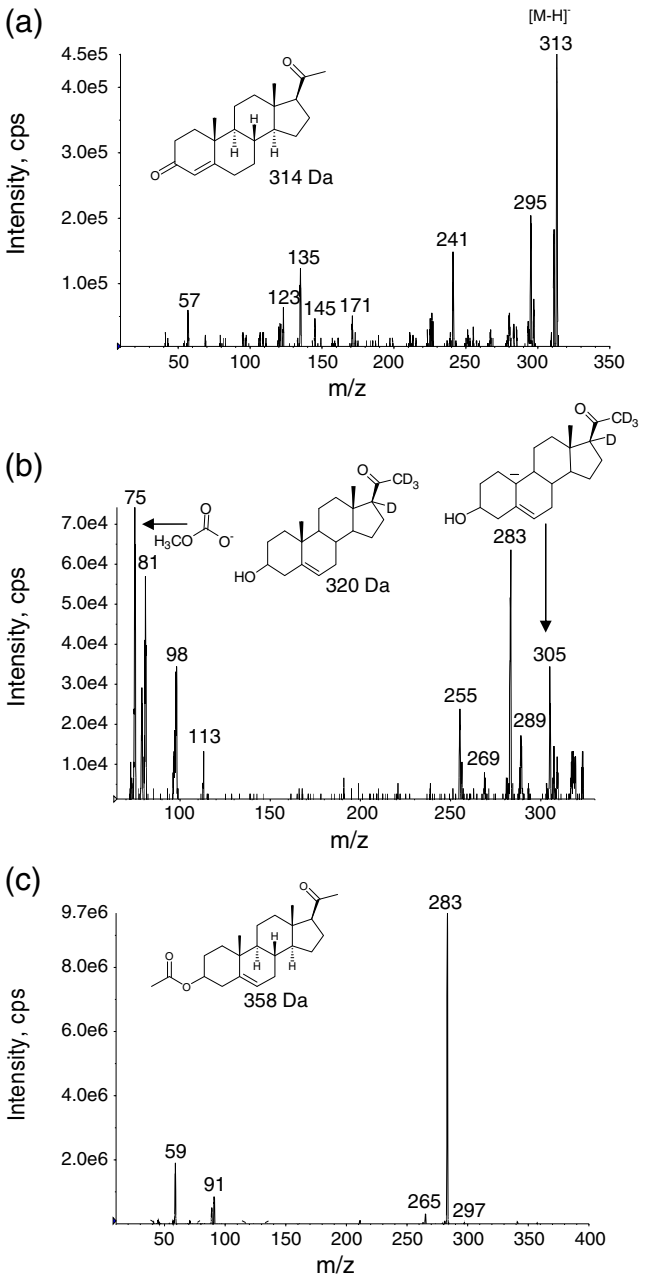
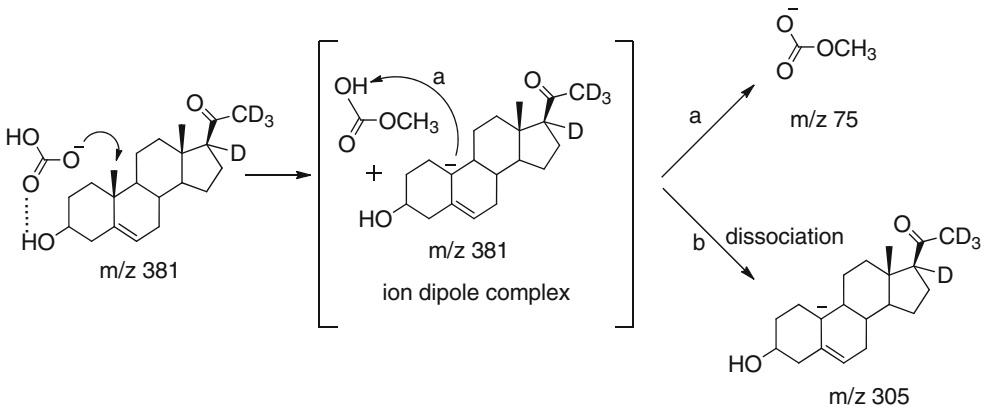


Figure 5. Negative mode ES-MS/MS product ion mass spectra of the following precursors: **(a)** fluoride adduct of progesterone; **(b)** bicarbonate adduct of d_4-pregnenolone; **(c)** fluoride adduct of pregnenolone acetate

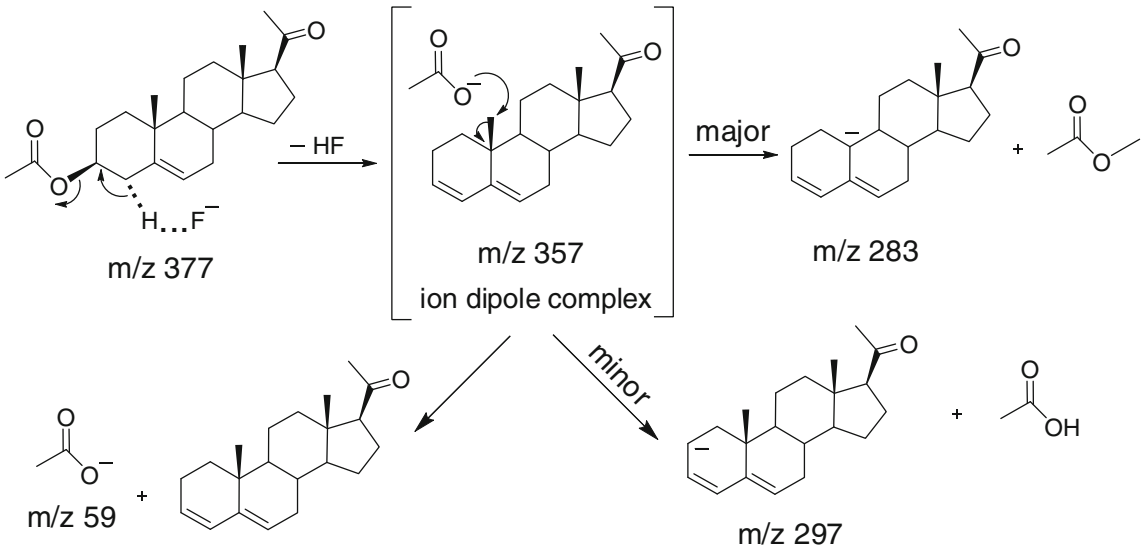


Scheme 3. Bicarbonate ion initially adducted to hydroxyl group of d_4-pregnenolone undergoes migration and captures a methyl cation forming ion–dipole complex of *m/z* 381. Proton transfer may then occur to form *m/z* 75 (base peak) or the ion–dipole complex may directly dissociate to *m/z* 305

Novel Decomposition Pathways Passing Through an Ion–Dipole Complex

During low-energy decomposition reactions, ion–dipole complexes [58, 59] (also known as ion–neutral complexes) [60, 61] may be formed when, following bond cleavage, an attraction remains between the charge of the fragment ion and the dipole of the newly formed neutral molecule. This electrostatic interaction is often sufficiently long-lived on the mass spectrometric time scale to permit further low-energy reaction (often a rearrangement). Negative ion ESI-MS/MS experiments investigating CID of bicarbonate adducts of d_4-pregnenolone (Figure 5b) or fluoride adducts of pregnenolone acetate (Figure 5c) present two clear examples where the interpretation of CID mass spectra can be readily rationalized by considering the formation of intermediate ion–dipole complexes. In the case of the bicarbonate adduct of d_4-pregnenolone, bicarbonate ion initially interacting with the A-ring (C-3) hydroxyl group can migrate toward the methyl group at C-10 and capture the methyl cation (Scheme 3). The neutral $CH_3OC(O)OH$ thus formed stays in close proximity to the negatively charged steroid that benefits from charge delocalization via conjugation. This ion–dipole complex may then either dissociate directly to form *m/z* 305 or undergo proton transfer from the carboxylic acid site to the steroid, thereby forming *m/z* 75 (Scheme 3).

In the second example, the same methyl group at C-10 is again implicated. Initially, fluoride adduct precursors of pregnenolone acetate lose HF to form [pregnenolone acetate – H]$^-$. This deprotonation is likely to occur at C-4 (alpha to



Scheme 4. HF loss from fluoride adduct of pregnenolone acetate forming ion–dipole complex of *m/z* 357, which may undergo methyl cation transfer (forming *m/z* 283, base peak), direct dissociation (forming *m/z* 59), or low yield proton transfer (forming *m/z* 297)

the double bond, Scheme 4), leading to the formation of a new double bond between C-3 and C-4 of the A ring with the CH_3COO^- group obtaining the charge. This CH_3COO^- then forms an ion–dipole complex with the remaining neutral steroid. From this ion–dipole complex, several events may occur. Analogous to the previous example, CH_3COO^- may migrate toward the CH_3 group located on C-10 and capture the methyl cation thus forming neutral CH_3COOCH_3 (methyl acetate) while concomitantly leaving behind the resonance-stabilized *m/z* 283 as the major product ion (Scheme 4). Alternatively, from the ion–dipole complex, direct proton transfer from C-2 (or perhaps C-7) to CH_3COO^- forms acetic acid (neutral) and *m/z* 297 in low yield. Lastly, the direct dissociation of the ion–dipole complex to form CH_3COO^- at *m/z* 59 can also occur.

Conclusion

Attachment of anions to neutral steroid molecules is developed here as a viable alternative method to improve the sensitivity for analysis of neutral steroids by negative ion electrospray mass spectrometry. Anion attachment, followed by MS/MS employing low-energy CID, has proven to be an excellent method for the structural characterization of neutral steroids. Success in obtaining $[M - H]^-$ and structurally-informative consecutive decomposition products from anionic adduct precursors of steroids depends upon judicious choice of the anion. We have found that the investigated bifunctional steroids reliably form adduct ions with fluoride, bicarbonate, acetate, and chloride anions. However, only fluoride and bicarbonate provide abundant structurally-informative fragment ions upon CID, with fluoride adducts offering the highest yields. Low-energy CID of fluoride adducts of estradiol produced a wealth of product ions, which have been interpreted as charge-induced fragmentations. CID product ions derived from precursor bicarbonate adducts of d_4-pregnenolone or fluoride adducts of pregnenolone acetate are rationalized to have formed via passage through unique ion–dipole complexes. Results obtained from deuterated starting materials unequivocally demonstrate that different anions exhibit preferential attachment to different specific sites on pregnenolone (i.e., "regioselective anion attachment") based on the matching of the GB of the anion and the deprotonated form of the particular site of the molecule involved in adduct formation. Clear evidence for regioselective attachment is provided by subsequent "regiospecific" decompositions. The applicability of this approach for the detection of other neutral compounds and the presence of neutral steroids in biological fluids are on-going.

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