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Mass Spectrometric Studies on 17β-Estradiol-17-fatty Acid Esters: Evidence for the Formation of Anion–Dipole Intermediates

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The behaviour towards low collision energy processes (eV range) of $[M - H]^-$ prepared under negative ion chemical ionization (NICI) ammonia conditions from 17β-estradiol-17-fatty acid esters has been investigated. From such bifunctional compounds containing two acidic sites (i.e. phenol and ester groups), two isomeric forms (i.e. phenoxide and enolate forms) characterize the $[M - H]^-$ ion structures, whose distribution depends on the ion preparation mode. Here NICI (ammonia) provides both phenoxide and enolate forms as the $[M - H]^-$ species. This behaviour contrasts with the regioselectivity observed for proton abstraction from phenol under NICI (N_2O) and fast atom bombardment conditions. Production of both phenoxide and enolate forms in NICI (ammonia) is demonstrated under NICI (ND_3) conditions in which DO-labelled $[M_d - H]^-$ enolate ions are produced in a similar yield to unlabelled $[M_d - D]^-$ phenoxide ions. Collisionally activated dissociation (CAD) spectra of both isomeric deprotonated molecules differ strongly by the presence of two different pairs of complementary daughter ions, suggesting that these ionic species are unconvertible. This is due to a steric hindrance effect on the long-distance proton transfer. A mechanistic investigation on the formation of fragment ion pairs produced under CAD was performed with various deuterium-labelled molecules. From these experiments, evidence is provided for molecular isomerizations into ion–dipole complexes (prior to dissociation) which are structurally dependent on the initial charge location. Direct dissociation of these intermediates competes with the occurrence of exothermic proton transfer(s) yielding the formation of other isomeric intermediate forms. The orientation of these proton transfers is dictated by the relative acidities of both moieties of the complex.

INTRODUCTION

Steroids and lipids form a group of complex molecules of immense biological importance, which pose interesting challenges for structural investigations and, for this reason, they have been extensively studied by mass spectrometry.[1–3] Several studies on such compounds have been described, in particular for polar and thermally labile derivatives such as steroid conjugates (e.g. sulphates, glucuronides and glycosides). This class of underivatized conjugates have been analysed under various desorption conditions (e.g. desorption chemical ionization (DCI),[4] fast atom bombardment (FAB),[5] plasma desorption (PD)[6] and laser desorption (LD)[7]). Under negative ion conditions, it has been shown that the detection of molecular species (i.e. $[M - H]^-$) was strongly improved over the comparative positive ion mode.[8]

Tandem mass spectrometry (MS/MS) combined with desorption modes has been shown to be a useful method for the structural analysis of these non-volatile complex compounds and to determine glycosidic steroid positions[5,9,10] and glycosidic sequences. Concerning conjugates containing very acidic sites (e.g. sulphates)[10] studied by FABMS/MS, additional structural information can be obtained under high-energy collision conditions (keV range). Indeed, steroid skeleton decompositions take place via remote charge mechanisms rather than from pathways resulting from charge-promoted decompositions. This behaviour also characterizes 17-hydroxy-3-sulphate steroids bearing a negative charge at the sulphate site.[10] The presence of the hydroxyl group did not seem to influence the orientation of the decompositions. Note that such processes, which take place on glucuronic acid conjugates or glycoside derivatives, were partially hindered because charge-promoted cleavages occur competitively, leading to glycoside linkage cleavages.

Among the different classes of known steroid conjugates, steroid esters such as fatty acid esters have been less extensively investigated.[8,11] Under negative ion chemical ionization (NIC) (OH^-) conditions, they yield, in addition to $[M - H]^-$, complementary fragment ions (i.e. $RCOO^-$ and $[M - H - RCOOH]^-$) considered to be the most interesting structural information. A preliminary study on the decompositions induced by low collision energy (eV range)[12] from deprotonated 17β-estradiol-17-fatty acid esters has shown that the ester function was mainly concerned with the cleavage processes. Further, from these bifunctional compounds, the negative charge location (at phenol or/and ester sites) was strongly dependent on the mode of obtaining $[M - H]^-$ since the two functions are

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characterized by different acidities. Comparison of low-energy collisionally activated dissociation (CAD) spectra of $[M - H]^-$ according to the ion preparation mode reveals that decomposition pathways are influenced by this factor. Indeed, two decomposition modes (charge-promoted and remote-charge fragmentations) take place according to the charge location (i.e. at the phenol or ester position). This was interpreted as resulting from non-occurrence of the proton migration from the acidic site to the deprotonated position, since the two groups are not neighbours. In this preliminary study, several ambiguities appeared in the mechanistic understanding of the formation of these fragment ions according to their origins, since the distribution of the inconvertible $[M - H]^-$ isomeric species has been shown to depend on the mode of ion preparation.[13]

In this paper, we report an investigation of the fragmentations of $[M - H]^-$ species prepared from 17β-estradiol-17-fatty acid esters (palmitate and stearate esters, relative molecular mass (RMM) = 510 and 538, respectively) in a triple quadrupole instrument under different $[M - H]^-$ preparation modes (FAB and NICI experiments). Such compounds, containing functional groups at long distances, constitute an interesting model to investigate the role played by the different deprotonated sites which must not interact (sterically remote) and the fragile ester bond linking the side-chain to the steroid moiety in the D ring in the orientation of different competitive $[M - H]^-$ decompositions under eV range CAD (20 and 200 eV as E_{lab}) conditions, via either charge-induced decompositions or remote-charge processes. Based on the examination of CAD spectra of deprotonated 17β-estradiol-17-fatty acid esters dideuterated at C(16), C(2′) and C(3′) positions, $[M_{d2} - H]^-$, (and from OD-labelled estradiol sterarate esters prepared under NICI (ND_3) conditions), additional evidence is provided for molecular isomerization into the ion–dipole complexes,[12] whose structure depends on the location of the deprotonated site.[13]

EXPERIMENTAL

Synthesis

Estradiol esters. 17β-Estradiol-17-palmitate and -17-stearate were prepared according to the method described by Mellon-Nussbaum *et al.*[14] A 1 g amount of 17β-estradiol (Sigma) was dissolved in 10 ml of anhydrous pyridine (0 °C) under nitrogen and 2 ml of palmitoyl (or stearoyl) chloride (Sigma) were added. The mixture was stirred at room temperature for 12 h. It was then acidified (10% HCl) and the solution was extracted three times with diethyl ether (30 ml). The organic phase was washed with 10% aqueous sodium hydrogencarbonate ($NaHCO_3$) and then water until neutral pH. 17β-Estradiol-3,17-dipalmitate (or distearate), the major reaction product, was hydrolysed with $NaHCO_3$ to cleave the labile phenolic ester as follows: the ethereal solution was evaporated to dryness and the resulting oil was dissolved in benzene (3 ml), to which 100 ml of methanol and 500 mg of $NaHCO_3$ were added. The mixture was heated overnight (50–55 °C), diluted with diethyl ether and washed with water. The organic phase was dried over anhydrous sodium sulphate, filtered and the solvent removed *in vacuo*. The product was purified by chromatography on a silica gel column. The column was eluted with isooctane–benzene, first 1:1 (v/v) and then 1:3 (v/v). The estradiol palmitate or stearate was then eluted with benzene (yield 50–60%).

[16,16-D_2]-17β-Estradiol-17-stearate. [16,16-D_2]-17β-Estradiol-17-stearate was prepared according to a method proposed by Dehennin *et al.*[15] Estrone (100 mg) was dissolved in MeOD (10 ml) and NaOD (9 M in D_2O, final concentration 0.5 M) was added. The solution was then heated for 24 h at 60 °C. The mixture was neutralized with DCl (6 M in D_2O) and then filtered to eliminate the excess of precipitated sodium chloride. Reduction of the 17-keto group was carried out by adding of $NaBH_4$ (50 mg) and leaving the reaction mixture at room temperature for 30 min. The mixture was evaporated to dryness. The residue was dissolved in ethyl acetate, washed with water and purified by chromatography on silica gel 60 (Merck). [16,16-D_2]-17β-Estradiol was eluted with benzene–ethanol (85:15, v/v) and then acylated according to the previously described procedure (yield 50%).

17β-Estradiol-17-[2′,2′-D_2]stearate. For the preparation of [2,2-D_2]stearic acid, methyl stearate (300 mg) was refluxed for 18 h in dry MeOD (10 ml) previously made alkaline with sodium metal (30 mg), in a slow current of nitrogen, with a calcium chloride guard tube, as described by Vetter *et al.*[16] The reaction mixture was cooled to room temperature and deuterium oxide (3 ml) was added. Further refluxing under the same conditions yielded [2,2-D_2]stearic acid (265 mg).

For the preparation of [2,2-D_2]stearic acid chloride, [2,2-D_2]stearic acid was dissolved in benzene (2.5 ml, sodium dried) and phosphorus pentachloride (210 mg) was added. The solution was heated for 2 h at 60–80 °C under a nitrogen atmosphere. [α-D_2]stearic acid chloride was then obtained after removing the solvent under reduced pressure. The product was used immediately without further purification.

The condensation of [2,2-D_2]stearic acid chloride with estradiol was carried out according to the previously described method to give 17β-estradiol-17-[2′,2′-D_2]stearate (yield 50%)

17β-Estradiol-17-[3′,3′-D_2]stearate. Preparation of [1,1-D_2]cetyl alcohol. Palmitic acid (1 g) was dissolved in anhydrous diethyl ether (50 ml) in a reaction flask with a reflux condenser. With continuous stirring, $LiAlD_4$ was carefully added. After reaction for 1.5 h at room temperature, the reaction mixture was cooled to 0 °C and NH_4Cl (10% solution, 30–40 ml) was slowly added. The precipitate formed was filtered and washed (water). The ethereal phase, was dried over anhydrous sodium sulphate and the ether removed *in vacuo* to leave white, sticky [1,1-D_2]cetyl alcohol (yield 89%).

Preparation of [1,1-D_2]-1-chlorohexadecane from [1,1-D_2]cetyl alcohol. [1,1-D_2]cetyl alcohol (0.9 g) was

dissolved in 2 ml of dry pyridine. To this stirred solution was added 0.8 ml of thionyl chloride very slowly and the solution was refluxed for 1 h. The mixture was then cooled and extracted with diethyl ether. The ether layer was washed with water and 0.1 M NaOH, dried over anhydrous calcium chloride and evaporated to dryness. A 0.5 g amount of yellow–brownish oily product was obtained (55% yield).

Preparation of [3,3-D_2]stearic acid. Sodium (100 mg) was dissolved in 20 ml of dry ethanol and the mixture was warmed until a clear solution was obtained. Diethyl malonate (0.6 g) was added and the solution was refluxed to give diethyl malonate sodium salt. To this solution, [1,1-D_2]-1-chlorohexadecane was added. Part of the ethanol was removed by distillation, and slight precipitation of the salt was seen, which increased on cooling the mixture. After filtration, KOH solution (0.45 g in 10 ml of water) was added and the reaction mixture was refluxed for 4 h. Then, after neutralization of this alkaline solution, 5 ml of concentrated hydrochloric acid were added and the mixture was heated, whereby decarboxylation of one of the COOH groups occurred, thus yielding [3,3-D_2]stearic acid (yield 46%).

Preparation of 17β-estradiol-17-[3′,3′-D_2]stearate. The methods described for the preparation of [2,2-D_2] stearic acid chloride and subsequently 17β-estradiol-17-[2′,2′-D_2]stearate were used successfully (yield 50%).

Mass spectrometry

Mass spectra (MS and MS/MS) were measured on a triple quadrupole mass spectrometer (R 30-10, Nermag, France).,

FAB experiments. The mass spectrometer was fitted with a FAB gun (M-Scan, UK) and krypton gas was used for bombardment, operating at ~8 kV accelerating voltage and 1–2 mA as discharge current. The samples were prepared by mixing 3 μl of a sample solution (1 μg μl^{-1} in methanol) with *N*-benzoylimidazole–triethanolamine (1 : 1 v/v) as the matrix.

NICI experiments. The NICI mass spectra were obtained using a direct introduction probe (DCI) where 1 μl of sample solution (1 μg μl^{-1} in methanol) was placed on the tungsten filament. The following source operating conditions were used: emission current, 100 mA; repeller voltage, 10 V; source temperature, 180 °C, and reagent gas presssure, 2×10^{-4} Torr (source housing presssure) for NICI (NH_3) (or ND_3, obtained from CEA, France) experiments and 5×10^{-5} Torr for NICI (N_2O) experiments (1 Torr = 133.3 Pa).

MS/MS experiments. From a selected mean beam, CAD spectra were obtained using argon as collision gas either at 5×10^{-1} Torr (measured just inside the collision cell housing) for yielding multiple collision conditions or at 2×10^{-2} Torr (single collision conditions) under variable E_{lab} values (Q_2 potential relative to source potential varying between 10 and 200 V), e.g. E_{lab} = 200 eV was used for the major experiments.

The scan rate was 1 s for each spectrum recorded using a PDP 11/73 (Sidar data system). Each reported CAD spectrum is an average of at least 50 consecutive scans which were selected for optimum signal-to-noise ratios.

RESULTS AND DISCUSSION

Influence of the mode of preparation of $[M - H]^-$ ions on the CAD spectra

Under negative ion FAB conditions, different matrices such as glycerol, thioglycerol, *m*-nitrobenzyl alcohol, di- and triethanolamine and magic bullet were employed without obtaining advantageously large $[M - H]^-$ abundances. However, by using a mixture of triethanolamine and *N*-benzoylimidazole (NBI) as the matrix, the 17β-estradiol palmitate ester **1** gave intense $[M - H]^-$ ions at *m/z* 509 in high yield, accompanied by a sole fragment ion at *m/z* 255 (base peak).

The $[M - H]^-$ ionic current produced was sufficiently high to investigate the decompositions of this ion under collision processes. At E_{lab} = 200 eV (under multiple collision conditions), the deprotonated estradiol palmitate ion (*m/z* 509) mainly decomposes into two pairs of complementary ions (i.e. a major one at *m/z* 253–255 and a very minor one at *m/z* 271–237, Fig.1(a)). In addition, the *m/z* 145 fragment ion appears at E_{lab} = 200 eV and is not detected at 20 eV collision energy (CAD spectrum not reported here). Note that the collision energy changes do not significantly influence the trend of the *m/z* 253/*m/z* 255 and *m/z* 271/*m/z* 237 ion abundance ratios.

From the stearate ester **2**, containing a longer side-chain ester (i.e. two additional methylene groups on the fatty acid ester part), collisional decompositions (E_{lab} = 200 eV, Table1) yield the same daughter ions at *m/z* 145 and *m/z* 253 and an additional peak at *m/z* 283 (corresponding to *m/z* 255 shifted by 28 u) as intense peaks. A pair of complementary fragment ions at *m/z* 265 and *m/z* 271 also appears with low abundance.

The *m/z* 255 (for palmitate **1**) and *m/z* 283 (for stearate **2**) daughter ions correspond to a carboxylate

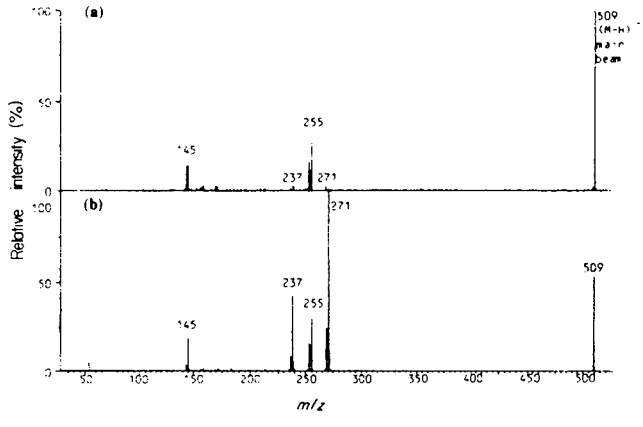


Figure 1. CAD spectra (E_{lab} = 200 eV, multiple collision conditions) of $[M - H]^-$ (*m/z* 509) prepared under (a) NI-FAB and (b) NICI(NH_3) conditions from 17β-estradiol palmitate ester.

Table 1. Abundances of main fragment ions produced by collision (E_{lab} = 200 eV, multiple collision conditions) from [M − H]⁻ (*m/z* 537) prepared under either FAB or NICI(NH_3) conditions from the stearate ester

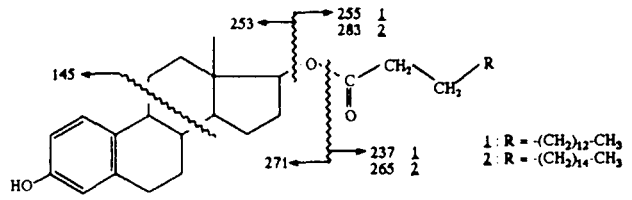
Mode of ion preparation	Daughter ion abundances (%)[a]					
	m/z 283	*m/z* 271	*m/z* 270	*m/z* 265	*m/z* 253	*m/z* 145
NI-FAB	47	5	1	2	31	14
NICI(NH_3)	10	54	5	22	6	3

[a] Abundances relative to the total fragment ion current (100%).

$CH_3(CH_2)_nCOO^-$ ion structure (i.e. with n = 14 and 16) due to charge retention on the side-chain, whereas the *m/z* 145 and 253 ions (unchanged on modifying the side-chain length) correspond to the steroid skeleton moiety fragmentation and to the estra-1,3,5(10),16-tetraen-3-ol, respectively (Scheme 1).

The [M − H]⁻ ions prepared under NICI(NH_3) conditions are characterized by a different CAD spectrum (Fig. 1(b) for palmitate and Table 1 for stearate) since the *m/z* 253 and 255 (or 283) ions are disfavoured compared with the complementary fragment ions at *m/z* 271 and 237 from the estradiol palmitate ester (and *m/z* 271 and 265 from stearate), which are enhanced. Note that the relative trend of the fragment ion species abundances (i.e. *m/z* 237 < *m/z* 271 and *m/z* 253 < *m/z* 255, Figures 1(a) and 1(b)) measured in the CAD spectrum of deprotonated palmitate is not strongly affected by the [M − H]⁻ preparation mode. A similar feature characterizes the daughter ion abundances measured from deprotonated stearate ester (i.e. *m/z* 265 < *m/z* 271 and *m/z* 253 < *m/z* 283, Table 1).[12] The *m/z* 237 and 265 fragment ions correspond to the ynolate $CH_3(CH_2)_{n-1}C{\equiv}C{-}O^-$ structure (with n = 14 and 16) and *m/z* 271 is the deprotonated estradiol species (Scheme 1).

The dependence of the CAD spectrum on the preparation mode (FAB or NICI) of selected ions should be explained by considering that the initial internal energy carried by the deprotonated molecules is influenced by the preparation mode[17] and/or the proton abstraction selectivity differs in FAB and NICI, according to the relative acidities of the mobile protons. The influence of the initial internal energy of [M − H]⁻ was ruled out because the CAD spectra characteristics are preserved by changing the collision energy value. Hence only the deprotonation selectivity must explain the differences observed in the CAD spectra of [M − H]⁻ prepared under various conditions.



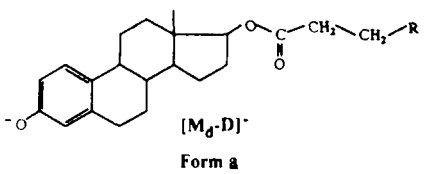
Scheme 1. Conventional cleavages of [M − H]⁻ ions formed in NI-FAB.

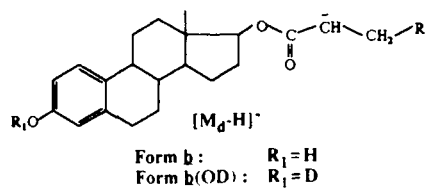
Deprotonation sites determined under labelled FAB and NICI conditions

In order to determine the deprotonation site(s) in FAB from estradiol stearate and palmitate esters, labelling conditions were used (i.e. D_2O + NBI mixture as matrix in FAB and ND_3 as reagent gas in NICI) for the preparation of OD-labelled molecules yielding deprotonated species denoted $[M_d - D]^-$ and $[M_d - H]^-$ ions, according to the deprotonated site involved (phenol site, form *a*, or enolizable site, form *b*-(OD), respectively, Scheme 2).

Under FAB conditions, exclusively $[M_d - D]^-$ are produced (i.e. *m/z* 509 and 537 ions for estradiol palmitate and stearate esters, respectively). The absence of labelled deprotonated molecules (i.e. $[M_d - H]^-$) indicates that, in the matrix, deprotonation occurs regiospecifically at the A-ring phenol site (without possible H/D exchange at the enolizable position), yielding the unlabelled *a* form (Scheme 2). This was expected, since the desorbed ions are preformed in the matrix[18] and the deprotonation is oriented by the relative pK_a of the different acidic groups. Thus, the phenoxy *a* species must be more favoured than the *b* enolate (or *b*-(OD) form under labelling conditions), because the phenol pK_a value is lower than that of the ester. In this case, negative charge being selectively located at the phenol site, the formation of the carboxylate $CH_3(CH_2)_nCOO^-$ ions (i.e. *m/z* 255 and 283 for palmitate and stearate, respectively) is not promoted by the negative charge. Indeed, cleavage of the C(17)—O bond with charge retention on the side-chain involves a proton transfer from the enolizable site to the phenoxy site of the *a* form, which is sterically ruled out.

Hence this particular behaviour towards collision processes suggests the following questions: (i) by which





Scheme 2. Isomeric *a* and *b* structures for deprotonated molecule.

mechanisms are the fragment ions generated from the phenoxy *a* species?; (ii) is deprotonation in the gas phase (i.e. under NICI (NH_3) conditions) also regiospecific at the phenol site such as would be expected from the higher acidity of phenol by about 46 kJ mol^{-1} compared with the esters[19-21]*?; and (iii) which CAD process orientation takes place if the deprotonation occurs from the enolizable site?

In order to examine these questions, deprotonated labelled molecules were prepared under NICI (ND_3) conditions. Under the labelling conditions,[22] neutral–neutral H/D exchange ($M + ND_3 \rightarrow M_d + ND_2H$) occurs at the phenol site. These labelled M_d molecules can be competitively deprotonated at the phenol and/or enolizable positions to produce two different anion species (Eqn (1)).

Actually, the NICI (ND_3) mass spectrum of estradiol palmitate ester displays signals at *m/z* 509 $[M_d - D]^-$ (55%), and *m/z* 510, $[M_d - H]^-$ (45% after subtraction of the natural ^{13}C contribution), indicating that both isomeric deprotonated *a* and *b*-(OD) forms coexist in similar abundances in NICI (ND_3) (and thus in NICI (NH_3)).

$$M_d + ND_2^- \begin{cases} \rightarrow [M_d - D]^- + ND_3 \quad \text{(a)} \\ \quad \text{(form } a\text{)} \\ \rightarrow [M_d - H]^- + ND_2H \quad \text{(b)} \\ \quad \text{(form } b\text{-(OD))} \end{cases} \qquad (1)$$

Note that the absence of *m/z* 511 ions shows that no H/D exchange occur at the enolizable site under these NICI conditions. Analogous results are obtained from the estradiol stearate ester, since both *m/z* 537 and 538 ions are observed in similar abundances. On the other hand, the different fingerprints displayed in the CAD spectra of $[M - H]^-$ ions formed in FAB and NICI can be interpreted by assuming that under the latter conditions both isomeric molecular species i.e. forms *a* and *b*, Scheme 2) coexist without $a \rightleftarrows b$ interconversion.

This assumption was evidenced first by investigation of the CAD spectra of $[M_d - H]^-$ (*m/z* 510) and $[M_d - D]^-$ (*m/z* 509) ions (Table 2) prepared under NICI (ND_3) conditions in the case of estradiol palmitate ester. Indeed, they display different trends in the fragment ion abundances characterizing the charge location in the deprotonated molecule. For instance, superimposable fingerprints characterize the $[M_d - D]^-$ decompositions (phenoxy *a* form, *m/z* 509, Scheme 2) and those of $[M - H]^-$ (*m/z* 509) prepared under FAB conditions (Fig. 1(a)), since both CAD spectra present *m/z* 253 and 255 as major fragment ions. Alternatively, the CAD spectrum of $[M_d - H]^-$ (*m/z* 510, enolate *b*-(OD) form) displays intense signals at *m/z* 237 and 271 (Table 2). From estradiol stearate ester, the $[M_d - D]^-$ (*m/z* 537) decompositions mainly yield *m/z* 253 and 283 (Table 2), as observed in CAD of $[M - H]^-$ ions prepared under FAB conditions (Table 1). This situation contrasts with the $[M_d - H]^-$ ion (*m/z* 539) which fragments into *m/z* 265 and 271 ions.

The respective behaviours of $[M_d - H]^-$ and $[M_d - D]^-$ towards collision processes are preserved by lowering the collision energies of $E_{lab} = 10$ eV. Note that from $[M_d - H]^-$ (*m/z* 510) of estradiol palmitate, a partial shift of the *m/z* 255 and 253 fragment ions by 1 u occurs, owing to the natural ^{13}C isotopic contribution of $[M_d - D]^-$ ions (~30% in *m/z* 510). The absence of labelled fragment ions at *m/z* 238 and 272 obtained from the enolate $[M_d - H]^-$ ion (*m/z* 510) must be emphasized. From the CAD spectra of $[M_d - D]^-$ (*m/z* 537) and $[M_d - H]^-$ (*m/z* 538) of estradiol stearate ester (Table 2), a similar behaviour is observed.

All these experimental results provide evidence that in NICI (NH_3) (or ND_3), the gas-phase deprotonation was not regioselective and occurred on both acidic sites of these hydroxy ester compounds, via competitive exothermic proton transfers to the NH_2^- reactive anion because the acidity of ammonia,[19] $\Delta G_{acid}(NH_3) = 1657$ kJ mol^{-1}, is much lower than that of the phenol and ester acidities ($\Delta G_{acid}(C_6H_5OH) = 1432$ kJ mol^{-1} and $\Delta G_{acid}(CH_3(CH_2)_nCOOR) = 1478$ kJ mol^{-1})[19] (see earlier footnote regarding estimated acidities). The similar yields observed for the formation of both isomeric $[M - H]^-$ anions (i.e. phenoxy *a* and enolate *b*

* Acidities of long-chain compounds were estimated to be higher by 50 kJ mol^{-1} than those of the smaller molecule containing the same function. This increment was appreciated from the acidity change measured from long chain alcohols.[20,21] This acidity increase is due to hydrocarbon chain coiling, which gives rise to a better negative charge solvation. Thus, from $\Delta G_{acid}(CH_3COOCH_3) = 1528$ kJ mol^{-1}, $\Delta G_{acid}(CH_3COOH) = 1429$ kJ mol^{-1} and $\Delta G_{acid}(H_2C{=}C{=}O) = 1497$ kJ mol^{-1}, the acidities of long-chain compounds can be estimated to be as follows: $\Delta G_{acid}(CH_3(CH_2)_nCOOR) = 1478$ kJ mol^{-1}, $\Delta G_{acid}(CH_3(CH_2)_nCOOH) = 1379$ kJ mol^{-1} and $\Delta G_{acid}(CH_3(CH_2)_{(n-1)}HC{=}C{=}O) = 1447$ kJ mol^{-1}.

Table 2. CAD spectra (200 eV as E_{lab}, single collision conditions) of $[M_d - D]^-$ (*m/z* 509) and $[M_d - H]^-$ (*m/z* 510) ions of estradiol palmitate ester and $[M_d - D]^-$ (*m/z* 537) and $[M_d - H]^-$ (*m/z* 538) ions of estradiol stearate ester produced under NICI (ND_3) conditions

		Daughter ion abundances (%)[a]								
Ester	Precursor ions	*m/z* 270	*m/z* 271	*m/z* 256	*m/z* 255	*m/z* 254	*m/z* 253	*m/z* 237	*m/z* 146	*m/z* 145
Palmitate	$[M_d - D]^-$, *m/z* 509	1	9[b]	—	45	—	27	8[b]	—	10
	$[M_d - H]^-$, *m/z* 510	4	42	3[c]	6[c]	2[c]	3[c]	32	1[c]	7[c]
		Daughter ion abundances (%)[a]								
		m/z 284	*m/z* 283	*m/z* 270	*m/z* 271	*m/z* 265	*m/z* 254	*m/z* 253	*m/z* 146	*m/z* 145
Stearate	$[M_d - D]^-$, *m/z* 537	—	30	2	8[b]	5[b]	—	36	—	20
	$[M_d - H]^-$, *m/z* 538	2[c]	4[c]	6	52	26	2[c]	4[c]	1[c]	3[c]

[a] Abundances relative to the total fragment ion current (100%).
[b] Fragment ions due to an uncompleted labelling at phenol site.
[c] Fragment ions due to the $[M_d - D]^-$ natural ^{13}C isotopic contribution.

forms) can be expected since the difference in acidity between phenol and ester is ~46 kJ mol^{-1}, a small difference relative to the proton abstraction exothermicity (179 kJ mol^{-1}).

Actually, the observed similarities between phenoxy and enolate ion abundances suggests that the long-chain ester acidity was underestimated, unless this means that a similar cross-section characterizes the proton abstraction from phenol and ester (owing to the very exothermic character of the deprotonation, i.e. 180 kJ mol^{-1}, estimated value).[19] This gas-phase behaviour differs strongly from that observed in solution where intermolecular solvation (matrix) plays a large role in the deprotonation.

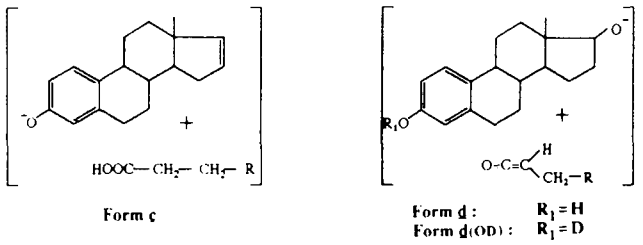
In NICI, the use of N_2O as reagent gas yielding the $O^{-\bullet}$ ion, a weaker base than NH_2^- by 83 kJ mol^{-1} ($\Delta G_{acid}(OH^\bullet)$ = 1574 kJ mol^{-1}),[19] also provides $[M - H]^-$ ions with high abundances. These ions are characterized by CAD spectra (Table 3) having similar fingerprints to those obtained for deprotonated molecules generated in FAB (Fig. 1(a) and Table 1). This experimental result means that proton abstraction is a regioselective reaction at the phenol site leading to the phenoxy *a*, $[M - H]^-$, form rather than the enolate *b* form in NICI (N_2O).

This behaviour towards the $O^{-\bullet}$ reagent is unexpected because it is generally assumed that deprotonation reactions are thermodynamically controlled.[23] Therefore, enolate ion should be produced because its formation is exothermic by ~96 kJ mol^{-1} (cf. 179 kJ mol^{-1} obtained with ammonia). However, this orientation must be ruled out from the experimental results. Hence, the enhanced regiospecificity obtained by decreasing the deprotonation exothermicity should mean that proton abstraction from such large substrates occurs with kinetic control.[24] However, this particular orientation of proton abstraction from such large-sized compounds is actually unclear.

Decomposition mechanisms of $[M - H]^-$: evidence for ion–dipole intermediate formation

The preliminary study[12,13] demonstrated that OD-labelled daughter ions were not produced from decomposition of the enolate $[M_d - H]^-$ ion (i.e. *b*-(OD) form, at *m/z* 510 and 538 for estradiol palmitate and stearate, respectively). This result suggested that most likely, prior to dissociation, $[M_d - H]^-$ isomerized into ion–induced dipole intermediates in which the anion part contained an exchangeable OD site (i.e. the *d*-(OD) form, Scheme 3).

The concept that some ion decompositions occur through ion–dipole complex formation is now well established.[25] In particular, from amino steroid molecular ions, Longevialle and Botter[26] gave direct evidence for $M^{+\bullet}$ isomerization into an ion–dipole complex prior to decomposition. From such intermediates, proton transfer from the neutral to the ionic part appears to be possible without long-distance hindrance between the functional groups (the complex lifetime permits the ion rotation around the steroid skeleton).[27] Isomerization into ion–dipole complexes has also been proposed to explain decompositions of various negative ions such as aliphatic (or alicyclic) alcohols,[28] ketones,[29] esters,[30] glycosides[31,32] and peptides.[33] However, for larger molecules such as steroid derivatives, no direct evidence was given for the formation of these intermediates, and such molecular anion isomerization was only suggested.



Scheme 3. Isomeric ion–dipole *c* and *d* structures produced by isomerizations of the isomeric phenoxy $[M_d - D]^-$ and enolate $[M_d - H]^-$ ions.

In the case of the compounds studied here (i.e. estradiol palmitate and stearate esters), isomerization of deprotonated molecules into ion–dipole complexes is expected to rationalize the loss of labelling from the decompositions of labelled $[M_d - H]^-$ ion (enolate *b*-(OD) form). Additional evidence for such an interpretation is provided by studying the behaviours of various labelled estradiol stearate esters towards collisions.

Mechanism of formation of *m/z* 271–237 fragment ions for palmitate ester (or *m/z* 271–265 for stearate) from labelled *b* form enolate isomerized into an ion–dipole

From the previous discussion of CAD spectra, it has been emphasized that (i) decompositions of the phenoxy $[M_d - D]^-$ ions (*m/z* 509 and 537, prepared under NICI (ND_3) conditions) specifically yield the pairs of complementary fragment ions at *m/z* 253–255 and *m/z* 253–283 for palmitate and stearate esters, respectively; (ii) the enolate $[M_d - H]^-$ ions (*m/z* 510 and 538) fragment, under collision, into different pairs of complementary ions at *m/z* 271–237 (for palmitate) and at *m/z* 271–265 (for stearate) where the OD labelling is not preserved; this finding suggested a molecular isomerization into an ion–dipole intermediate (Scheme 3); and (iii) from the latter selected species, the presence of the ion

Table 3. Abundances of main fragment ions produced by collision (E_{lab} = 200 eV, multiple collision conditions) on $[M - H]^-$ prepared under NICI (N_2O) conditions from palmitate and stearate esters

Ester	Precursor ions	Fragment ions[a]					
Palmitate	$[M - H]^-$, *m/z* 509	270 (<1)	271 (3)	255 (61)	253 (29)	237 (2)	145 (5)
Stearate	$[M - H]^-$, *m/z* 537	283 (50)	270 (<1)	271 (6)	265 (3)	253 (31)	145 (9)

[a] *m/z*, relative to the total fragment ion current; relative ion abundances are given in parentheses.

pairs at *m/z* 253–255 (and *m/z* 254–256) and *m/z* 253–283 (and *m/z* 254–284) was attributed to the natural ^{13}C contribution of $[M_d - D]^-$ ions.

Such assumptions (i.e. (ii) and (iii)) can be evidenced from CAD studies of deprotonated molecules labelled at various positions (i.e. at the C(2'), C(3') and C(16) sites. Under NICI (NH_3) conditions, these dideuterated molecules can be competitively deprotonated at phenol and enolizable positions (with or without loss of one D atom), giving rise to the formation of both phenoxide and enolate parent ions, located in the mass spectra either at the same *m/z* value (i.e. *m/z* 539, denoted $[M_{d2} - D]^-$ from labelled **2**-(16,16-d_2) and **2**-(3',3'-d_2) stearates, or at *m/z* 538 (i.e. enolate $[M_{d2}\text{-}D]^-$ ion corrsponding to the *b*-(2'-*d*) form) and *m/z* 539 (i.e. phenoxy $[M_{d2} - H]^-$ ion corresponding to the *a*-(2',2'-d_2) form) from the labelled **2**-(2',2'-d_2) stearate.

Note that from the latter labelled compound, there is no interference between the enolate $[M_{d2} - D]^-$ ion (*b*-(2'-*d*) form) and the natural ^{13}C contribution of phenoxide $[M_{d2} - H]^-$ ions (*a*-(2',2'-d_2) form), since they are located at different *m/z* ratios (i.e. *m/z* 538 and 539, respectively), contrary to the situation previously mentioned in NICI (ND_3) experiments with phenol site-labelled compounds. Then, from CAD spectra of both labelled $[M_{d2} - D]^-$ and $[M_{d2} - H]^-$ ions (for **2**-(2',2'-d_2) stearate, Table 4) complementary information should be provided since the parent *m/z* 538 ion ($[M_{d2} - D]^-$ is exclusively composed of the enolate *b*-(2'-*d*) form.

Decompositions of both deprotonated **2**-(2',2'-d_2) molecules (Table 4) reveal that the selected $[M_{d2} - D]^-$ ion (i.e. enolate *b*-(2'-*d*) form) exclusively yields one pair of complementary ions at *m/z* 272 (85%) and 265 (100%), whereas the second pair (i.e. *m/z* 253 and 283) was not detected (Table 4). This confirms the assumptions made in order to rationalize the signals at *m/z* 253 (and *m/z* 254) and *m/z* 283 (and *m/z* 284), displayed by the CAD spectrum of $[M_d - H]^-$ (*m/z* 538, prepared from **2** under NICI (ND_3) conditions), which has been assumed to be due to the natural ^{13}C contribution of $[M_d - D]^-$ of stearate ester. Further, the non-occurrence of the *m/z* 266 fragment ion formation during decompositions of the enolate *b*-(2'*d*) ion (*m/z* 538) demonstrates that the deuterium at C(2') is lost, and that the specific formation of the ynolate $CH_3(CH_2)_{15}C{\equiv}C{-}O^-$ (*m/z* 265) fragment ion takes place. Alternatively, the deuterium lost mainly appears on the fragment ion at *m/z* 272 (i.e. deprotonated estradiol alkoxide containing one D).

Analysis of the $[M_{d2} - H]^-$ CAD spectrum, *m/z* 539 (mixture of both the enolate and the phenoxide forms), prepared by deprotonation of the labelled **2**-(3',3'-d_2) stearate, shows that the deuterium atom at C(3') was not concerned with the charge-induced ester group cleavage (i.e. the C(1')O—OC(17) bond, Scheme 4), in contrast with that occurring from labelled ester at the C(2') position. A similar finding is provided by studying the decompositions of deprotonated labelled **2**-(16,16-d_2) molecule, since deuterium at C(16) was not lost during the formation of *m/z* 273 and 265 ions.

The different previous findings concerning the specific shifting (or not) of the previous fragment ions, formed from the enolate form, allows us to give details of the mechanistic formation of these daughter ions by considering the following stepwise pathway rather than a direct fragmentation: (i) cleavage of ester bond induced by negative charge yields isomerization of the enolate ion into the ion–dipole complex (isomeric *d* form), composed by a neutral ketene and deprotonated estradiol with an alkoxide site at C(17); (ii) proton (or deuteron) is transferred from the C(2) position of the ketene to the alkoxide site, yielding an isomeric *d'* form (constituted by neutral estradiol and ynolate species), which by dissociation can yield ynolate fragment ions *m/z* 265 (from **2**-(OD), **2**-(2',2'-d_2) and **2**-(16,16-d_2)) and *m/z* 267 (from **2**-(3',3'-d_2)); and/or (iii) this *d'* intermediate can isomerize by proton (or deuteron) migration from the phenol site to the ynolate species, giving rise to the formation of an isomeric *d''* complex. This intermediate can fragment into a deprotonated estradiol bearing the negative charge at the phenolic oxygen, yielding fragments ions at *m/z* 271 (for **2**-(OD) and **2**-(3',3'-d_2)), *m/z* 272 from **2**-(2',2'-d_2) and *m/z* 273 from **2**-(16,16-d_2).

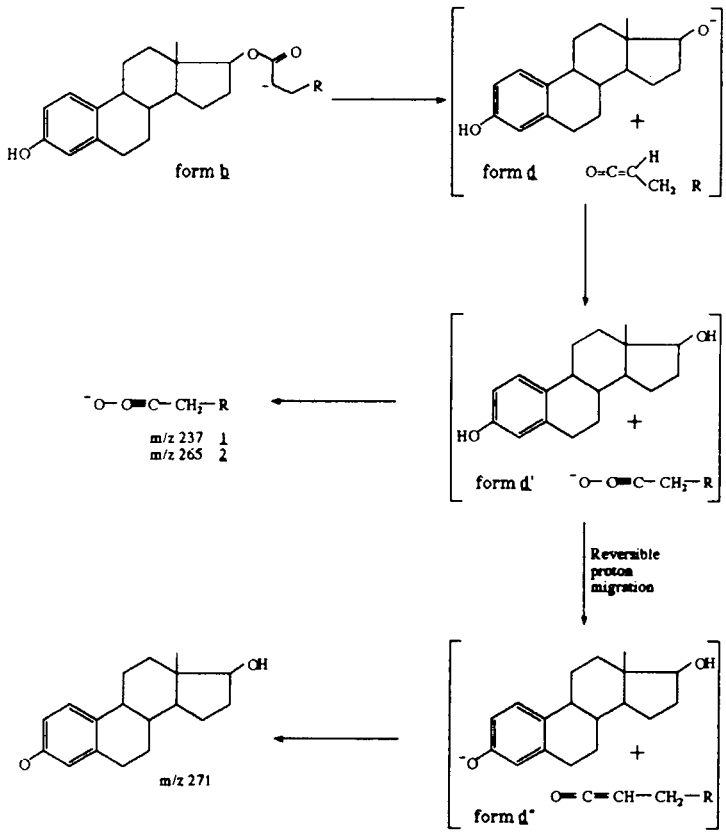
The different proton migrations are oriented by their exothermic character through the competitive pathways. The first proton transfer produced within the *d* intermediate can be explained by considering that $\Delta G_{acid}(CH_3(CH_2)_nHC{=}C{=}O)$ may be close to the estimated 1447 kJ mol^{-1} value.[19–21] This ΔG_{acid} value being lower than that characterizing the secondary alcohol bound to the five-membered D ring (assumed to be similar to ΔG_{acid}(methylcyclohexan-2-ol = 1521 kJ mol^{-1}),[34] proton migration from neutral ketene to alkoxide is then favoured, yielding a *d'* intermediate.

The specific shift of *m/z* 271 to 272 from **2**-(2',2'-d_2) indicates that this migration occurring, the $d \rightarrow d'$ isomerization seems to be not reversible (exothermicity estimated to be 74 kJ mol^{-1}). Alternatively, this means that the rate constant for the direct dissociation of the *d* intermediate is lower than that of the exothermic

Table 4. Labelling incorporation[a] in the *m/z* 253, 265, 271 and 283 fragment ions observed from CAD spectra of $[M_{d2} - H]^-$ and $[M_{d2} - D]^-$ ions prepared under NICI (NH_3) conditions from various labelled stearate 2-(2',2'-d_2), 2(3',3'-d_2) and 2(16,16-d_2) esters

	2-(2',2'-d_2)		2-(3',3'-d_2)	2-(16,16-d_2)
Ion (*m/z*)	$[M_{d2} - D]^-$ (*m/z* 538)	$[M_{d2} - H]^-$ (*m/z* 539)	$[M_{d2} - H]^-$ (*m/z* 539)	$[M_{d2} - H]^-$ (*m/z* 539)
253	—	100	100	—
254	—	—	—	79
255	—	—	—	21
265	100	57[c]	—	100
266	—	43[c]	—	—
267	—	—	100	—
270[b]	—	5[b]	10[b]	—
271	13	62[c]	90	2
272	87	43[c]	—	18
273	—	—	—	78
283	—	—	—	100
284	—	—	—	—
285	—	100	100	—

[a] Normalized ion intensities (%) relative to ionic current of each zone.
[b] Ion *m/z* 270 observed from unlabelled parent ions.
[c] Due to the natural ^{13}C contribution of $[M_{d2} - D]^-$.



Scheme 4. Enolate parent ion isomerization into ion–dipole *d* complex and formation of fragment ions at *m/z* 265 and 271.

proton abstraction yielding the $d \rightarrow d'$ isomerization. Concerning the second proton migration from the phenol to the ynolate (i.e. $d' \rightarrow d''$ isomerization), the situation is different. Indeed, from the enolate $[M_d - H]^-$ ion (formed under NICI (ND_3) conditions from stearate ester), phenoxide *m/z* 271 fragment ion is specifically produced. This suggests that the rate constant of the direct *d'* dissociation is comparable to that of the $d' \rightarrow d''$ isomerization, so this reaction is reversible whereas exothermicity of this proton migration is close to 15 kJ mol^{-1} (estimated from $\Delta G_{acid}(CH_3(CH_2)_nHC{=}C{=}O) = 1447$ kJ mol^{-1} and $\Delta G_{acid}(C_6H_5OH) = 1432$ kJ mol^{-1}).[19–21] The occurrence of this proton migration indicates that most likely the energy barrier must be low for this reversible isomerization.[35]

A similar stepwise decomposition can be proposed to explain the formation of the ynolate *m/z* 237 and phenoxide *m/z* 271 fragment ions from estradiol palmitate esters **1** (*m/z* 509) and the labelled **1**-(OD) derivative (enolate *b*(OD) form, *m/z* 510).

Mechanism of formation of *m/z* 253–255 fragment ions for palmitate ester (or *m/z* 253–283 for stearate) from labelled *a* form phenoxide isomerized into an ion–dipole

Decompositions of phenoxide *a* parent species (i.e. $[M_d - D]^-$ ions, *m/z* 509 and 537, produced under NICI (ND_3) from palmitate and stearate esters, respectively) lead to the formation of complementary pairs of ions (*m/z* 253–255 and *m/z* 253–283, respectively) due to the ester group cleavage (i.e. the C(2')COO—C(17) bond). The structure of the common fragment *m/z* 253 ions corresponds to the estra-1,3,5(10),16-tetraen-3-phenoxide, whereas carboxylate ions characterize the structure of the *m/z* 255 and 283 fragment ions.

In contrast to the charge-promoted ester bond cleavage from the enolate *b* form as shown previously, phenoxide *a* form decompositions are not induced by the negative charge located at C(3) (phenol site), since this charged site is not sterically in close proximity to the ester group at C(17). This particular fragmentation, competitively leading to a pair of complementary daughter ions (i.e. estratetraenephenoxide and carboxylate ions) can be considered as produced *via* a quasi-thermal mechanism (or remote-charge decomposition). Therefore, since the charge is located at the phenol, the ester bond cleavage needs an H (or D) transfer from the steroid skeleton to the carboxylic site, *via* for instance, a six-membered transition state, leading to the formation of a carboxylic acid (i.e. palmitic or stearic acid) as the neutral and estratetraenephenoxide (*m/z* 253) as the anion. Nevertheless, the production of the carboxylate ions (*m/z* 255 and 283) implies a second proton transfer from the carboxylic acid to the phenoxide site, which can take place if an ion–dipole intermediate (isomeric *c* form) is formed prior to dissociation.

The study of the fragment ion shifting displayed in the CAD spectra of the labelled stearate esters (i.e. **2**-(2',2-d_2), **2**-(3',3'-d_2) and **2**-(16,16-d_2)) may provide information on the ester fragmentation mechanism by demonstrating the origin of the proton transferred during the isomerization of the phenoxide *a*-(d_2) parent ion into the ion–dipole *c* intermediate. First, the selected phenoxide $[M_{d2} - H]^-$ ion (*m/z* 539, generated

from 2-(2',2'-d_2) under NICI (NH_3)) fragments into *m/z* 253 and 285 (Table 4) without any loss of labelling on the side-chain. However, the selected *m/z* 539 ions being a mixture of phenoxide *a*-(2',2'-d_2) and enolate *b*-(^{13}C,2'-*d*) (natural ^{13}C contribution of *b*-(2'-*d*), *m/z* 538) ions, additional fragment ions are yielded due to the decompositions of the enolate ^{13}C species. The CAD spectrum of $[M_{d2} - H]^-$ (unseparated mixture of phenoxide and enolate ions) of 2-(3',3'-d_2) reveals a similar feature since the labelling at C(3') is preserved on the carboxylate ion (*m/z* 285) and the estratetraenephenoxide *m/z* 253 ion is not shifted.

A different trend characterizes the decompositions, under collision conditions, of $[M_{d2} - H]^-$ (*m/z* 539) prepared from 2-(16,16-d_2) since the C(16) labelling is partially lost (i.e. production of the estratetraenephenoxide fragment ion at *m/z* 254 for 79%), whereas the signal corresponding to the carboxylate ion remains unshifted at *m/z* 283. The latter result suggests that (i) the first proton transfer mainly occurs from the C(16) position to induce the ester cleavage into ion–dipole *c* intermediate (composed of estratetraenephenoxide and OD-labelled carboxylic acid, Scheme 5) and (ii) the OD labelling is not preserved during the generation of the carboxylate ions. Alternatively, this means that within the *c* form, this intermediate can dissociate into estratetraenephenoxide *m/z* 254 ion or isomerize into the *c'* form by deuteron transfer from neutral OD-labelled carboxylic acid to the phenoxide site. This ion–dipole *c'* complex can fragment giving rise to the formation of the unlabelled carboxylate *m/z* 283 ion (Scheme 5).

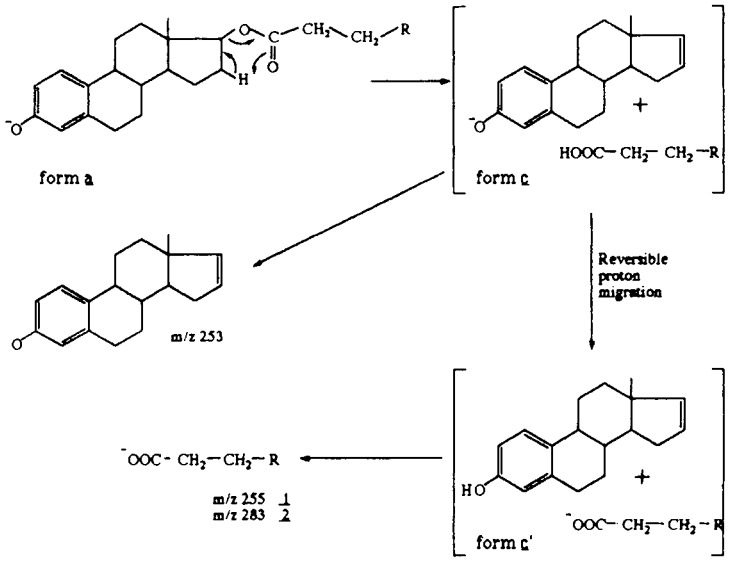
Such a behaviour, which is characteristic of a stepwise pathway for the decomposition of phenoxide *a*-(16,16-d_2) parent ions, can be considered as a possible reversible $c \rightleftharpoons c'$ process, where the dissociation is oriented by the relative acidities of phenol and carboxylic acid. The phenol acidity being close to ~1432 kJ mol^{-1} (or slightly lower), this value must be compared with that of long-chain carboxylic acids, which is estimated[19] (see earlier footnote regarding estimated activities) to be close to 1379 kJ mol^{-1} (value calculated from $\Delta G_{acid}(CH_3CO_2H)$ = 1429 kJ mol^{-1}).[19] This value suggests that the abundance of the carboxylate ion (*m/z* 283 from stearate ester) must be higher than that of the estratetraenephenoxide ion (*m/z* 253). This situation is observed in the CAD spectrum of the deprotonated stearate ester (Table 1) since the peak at *m/z* 253 is less intense than that at *m/z* 283.

Similar mechanisms may explain the decomposition of phenoxide parent ions generated from palmitate ester. In particular, as previously discussed, it was expected that the CAD spectrum of $[M - H]^-$ must display a peak at *m/z* 255 (carboxylate ion) of higher intensity than the signal at *m/z* 253 (estratetraenephenoxide ion), and this situation was experimentally observed (Fig. 1(a)).

CONCLUSION

17β-Estradiol-17-fatty acid ester derivatives (as palmitate and stearate esters) possess two different acidic sites: phenol group at the A-ring C(3) position and fatty acid ester group at C_{17} of the D ring. These functions are not sufficiently close to allow direct interaction. According to the negative ion preparation mode (FAB, NICI (NH_3) and NICI (N_2O), the $[M - H]^-$ ions produced submitted to collision processes (in the eV energy range) yield pairs of complementary fragment ions characteristic of the parent $[M - H]^-$ ion structure (i.e. phenoxide and enolate forms corresponding to the different negative charge locations).

A similar fingerprint characterizes the CAD spectra of $[M - H]^-$ solely composed of phenoxide species (FAB and NICI (N_2O) experiments). These spectra contrast with that of the $[M - H]^-$ ions prepared under NICI (NH_3), which are constituted by a mixture of isomeric phenoxide and enolate forms. The dependence of the negative charge location on the gas-phase reagent (and matrix) has been attributed to the basic strength of the reagent ion: the weaker ion (i.e. $O^{-\bullet}$) yielding a regioselective formation of phenoxide form, in contrast to the stronger ion (i.e. NH_2^-), which gives rise to competitive formation of both phenoxide and enolate forms in comparable abundances. Regioselectivity of deprotonation also characterizes the reaction produced in a



Scheme 5. Phenoxide parent ion isomerization into ion–dipole *c* complex and formation of fragment ions at *m/z* 253 and 283.

liquid matrix under FAB conditions, where mainly the pK_a value orients the proton transfer process from the phenol position.

The non-occurrence of isomerization of the enolate form into the phenoxide form (due to proton migration equilibrium between both distant functional groups) is supported by NICI (ND_3) experiments. These NICI conditions provide both isomeric phenoxide $[M_d - D]^-$ and enolate $[M_d - H]^-$ forms in similar abundances. Each isomeric parent ion produced decomposes, under CAD conditions, into one specific pair of diagnostic fragment ions. In particular, specific fragment ions concerning the side-chain ester are either an ynolate fragment ion from the enolate $[M_d - H]^-$ decomposition or a carboxylate daughter ion derived from the phenoxide $[M_d - D]^-$ species. Production of these fragment ions takes place via stepwise processes depending on the charge location.

Unlabelled estradiol phenoxide fragment ions obtained from decompositions of the enolate $[M_d - H]^-$ ions (OD-labelled stearate and palmitate, under NICI (ND_3)) give the first evidence of this molecular isomerization into an ion–dipole complex (composed of estradiol alkoxide at C(17) and a long-chain neutral ketene) prior to the $[M_d - H]^-$ fragmentation. Alternatively, complementary evidence is provided from the CAD spectrum of enolate $[M_{d2} - D]^-$ (stearate labelled at an enolizable position) which decomposes into an unlabelled ynolate fragment ion. The analysis of the shift for each pair of complementary fragment ions displayed in the CAD spectra of various labelled deprotonated molecules (containing labelling at phenol, C(16), C(2') and C(3') positions) confirms the assumption concerning the specific formation of ion–dipole intermediates according to the charge location. This demonstrates that the dissociation of the different intermediates occurs via transfer of either one (from phenoxide $[M - H]^-$ form) or two protons (from enolate $[M - H]^-$ species). Prior to dissociation of each complex species, proton transfer orientation (and reversibility) from the neutral to the anionic part is influenced by the relative acidity of each component of the intermediate. If one moiety (in the case of neutral estradiol) of the ion–dipole is constituted by two acidic sites, the rate constant for proton transfer to the alkoxide site is higher, i.e. regioselective, from the most acidic position (phenol site). On the other hand, in this case, the proton transfer from ketene to alkoxide at C(17) is not reversible.

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