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Characterisation of Fungal Lanostane-type Triterpene Acids by Electrospray Ionisation Mass Spectrometry

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Abstract: Lanostane-triterpene acids obtained from the culture of the fungus *Coriolellus malicola* were studied by electrospray mass spectrometry in the negative ion mode using quadrupole time-of-flight and quadrupole ion trap analysers. Despite the differences observed in the mass spectra recorded with these instruments, a set of fragment ions was found to be characteristic of the family, depending on the $\Delta^{7,9(11)}$ or Δ^{8} skeleton and the particular functional group at C-3. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: Mass spectrometry; fungal metabolites; triterpene acids; *Coriolellus malicola*; *Antrodia malicola*; basidiomycete.

INTRODUCTION

Lanostane-type triterpene acids are fungal metabolites, several of which have been reported to possess important and diverse biological activities, such as anti-inflammatory (Cuéllar *et al.*, 1996; Yasukawa *et al.*, 1998; Giner-Larza *et al.*, 2000), antibiotic (Keller *et al.*, 1996), cytotoxic (Gan *et al.*, 1998; Ukiya *et al.*, 2002) and tumour promotion inhibition, particularly suppressing the promoting effect of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Kaminaga *et al.*, 1996; Ukiya *et al.*, 2002). Such cancer chemopreventive properties and promising anti-inflammatory qualities (Kemani Wangun *et al.*, 2004) have renewed interest in these triterpenoids. These compounds are abundant mainly in the Polyporaceae and other fungi and are present as complex mixtures of different compounds. The principal differences between the triperpenic acids are in the location of the double-bond, Δ^{8} or $\Delta^{7,9(11)}$, the functionalities on the skeleton rings and the side chain. Seco-derivatives may also be found (Rösecke and König, 1999; Ukiya *et al.*, 2002). The use of HPLC, isolation of the individual components of the mixture and full NMR analysis are usually required for their identification and structural elucidation.

Mass spectrometry (MS) has not been systematically employed to analyse these compounds or their crude extracts. Usually, electron ionisation is applied to characterise the molecular ion and measure the spectra, but characteristic fragment ions have not been recognised or described, except for secolanostanoids (Rösecke and König, 1999; Ukiya *et al.*, 2002).

As direct infusion electrospray ionisation (ESI) coupled with tandem mass spectrometry has become an effective technique for the structural characterisation of other triterpenoids (Sturm and Stuppner, 2000; Miao *et al.*, 2002), the objective of this study was to investigate the ESI-MS/MS behaviour of lanostane acids for the first time using hybrid quadrupole-time of flight (QTOF) and quadrupole ion traps (QIT) instruments. The goal was therefore to develop a fast yet robust screening method applicable to most common MS/MS instruments.

EXPERIMENTAL

Materials. The triterpenes analysed in this study were isolated from the mycelium of the fungus *C. malicola* (BAFC Culture Collection, FCEN-UBA, CONICET, accession number BAFC 2065). The culture (5 L) was filtered and the mycelium was extracted with ethanol (2 × 500 mL) and ethyl acetate (2 × 500 mL). The resulting extract (782 mg) from the combined extracts was passed through a Sephadex LH-20 column, using methanol as eluent. A subfraction (400 mg) was purified by HPLC (column, YMC C 18, 5 µm, 22.5 × 2.5 cm i.d.; eluent, methanol:water (85:15), 6 mL/min; detection, UV 215 nm, RI), affording **1** (8 mg), **2** (21 mg), **3** (6 mg), **4** (3 mg), **5** (6 mg) and **6** (8 mg). The compounds were identified by full NMR analysis, EIMS and comparison with literature data (Tai *et al.*, 1992, 1995a; Keller *et al.*, 1996). NMR spectra were acquired on a Bruker AM 500 (Bruker BioSpin GmbH, Silberstreifen, Germany).

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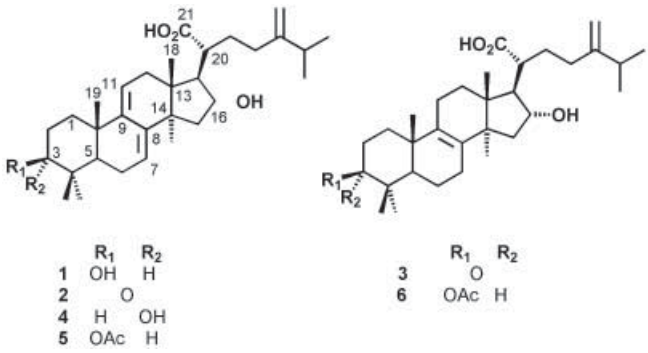

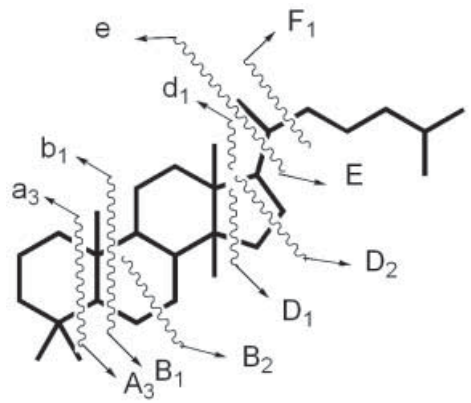

Figure 1 Fragmentation pattern for triterpenes.

Mass spectrometry. ESI-MS were acquired in the negative ion mode from a solution of triterpene (acetonitrile: water 1:1 or dichloromethane:methanol 1:1) with optimal conditions: capillary voltage of 1 kV, a cone voltage of 30 V, extractor voltage of 5 V and a desolvation gas temperature of 100°C. For ESI-MS/MS, the ion of interest was subjected to collision-induced dissociation with argon with a collision energy of 40 eV (QTOF) or 38% (QIT). The instruments employed were a QTOF high resolution–high accuracy mass spectrometer (QTOF 1; Micromass, Manchester, UK) in the Q-orthogonal time-of-flight configuration operating at 7000 mass resolution, and a LCQ Classic mass spectrometer (Thermofisher, San José, CA, USA) equipped with an ESI ionisation interface.

RESULTS AND DISCUSSION

The analysed triterpene acids included Δ^8 and $\Delta^{7,9(11)}$ skeletons and different functionalities on C-3. The negative ion mode ESI mass spectra of compounds **1**–**6** showed a predominant $[M - H]^-$ ion, which was used as the precursor ion for the MS/MS analysis. The nomenclature employed in this paper is that proposed by Griffiths (Griffiths *et al.*, 1996; Griffiths, 2003) for steroids and related compounds (Fig. 1). Briefly, a prime to the left of the fragment-describing letter indicates that the ion is deficient in one hydrogen compared with a fragment formed by a homolytic cleavage at the same point in the molecular ion. In the same way, a prime to the right indicates one more H-atom and two primes indicate two H-atoms.

The ESI-MS/MS show significant differences depending on the instrument employed. As a general description, LCQ spectra were cleaner, with fewer ions, but of higher relative intensity than the QTOF spectra. Some product ion (MS^2) spectra are shown in Figs 2 and 3.

The only fragment common to all of the compounds in both instruments, was the $[M - H - CO_2 - H_2O]^-$ ion. These ions were of very high relative intensity in the 3-hydroxy compounds and of moderate relative intensity in the others (Tables 1–3).

The structurally unspecific fragments $[M - H - H_2O]^-$ and $[M - H - CO_2]^-$ were almost exclusively seen in the LCQ spectra, probably due to the higher collision energy employed using the QTOF, with low and moderate relative abundances, respectively. The $[M - H - CO - H_2O]^-$ fragment was also seen for most compounds with variable intensities depending on the instrument employed, and so are of poor diagnostic value. This latter fragment can also be explained as a loss of formic acid, that is as $[M - H - HCO_2H]^-$, but although this loss has been reported many times (Burinsky and Sides, 2004; Bobeldijk *et al.*, 2005; Furey *et al.*, 2005), it has never been confirmed. Furthermore, the loss of water and carbon monoxide was shown to be thermodynamically and kinetically favoured over the alternative formic acid fragmentation process for the amino acid glycine (O'Hair *et al.*, 2000). However, the loss of formic acid cannot be confidently eliminated, at least as a minor process (Dookeran *et al.*, 1996).

The $\Delta^{7,9(11)}$ compounds (**1**, **2**, **4**) showed a relative intense fragment ion corresponding to $[M - H - 60]^-$. Two possible mechanisms can be proposed to explain its origin. The first implies the loss of carbon dioxide and methane from one of the angular methyl groups. In particular, the elimination of the 18-CH_3 group from the metastable molecular ion, accompanied by the release of large amounts of translational energy, was reported for steroids (Zaretskii *et al.*, 1983, 1986) upon electron ionisation. The authors also stated (Zaretskii *et al.*, 1983) that the strained trans-C/D (14 α) ring junction would tend to enhance the elimination of the 18-CH_3 group. In this case, both skeletons are 14 α, but the product ion should be highly conjugated for $\Delta^{7,9(11)}$ compounds. The loss of methane is a common observation in MS/MS of cardenolides (Crockett, 1990) and other compounds (Lin *et al.*, 1996; Limb *et al.*, 1999; Langouët *et al.*, 2002) employing FAB or ESI, and high-energy CID. The second alternative mechanism would involve the concerted loss of the 18-CH_3 and the carboxyl group as acetic acid, but the proximities of these groups are not different enough in both skeletons to confidently explain a concerted elimination. Again, the high stability of the conjugated product

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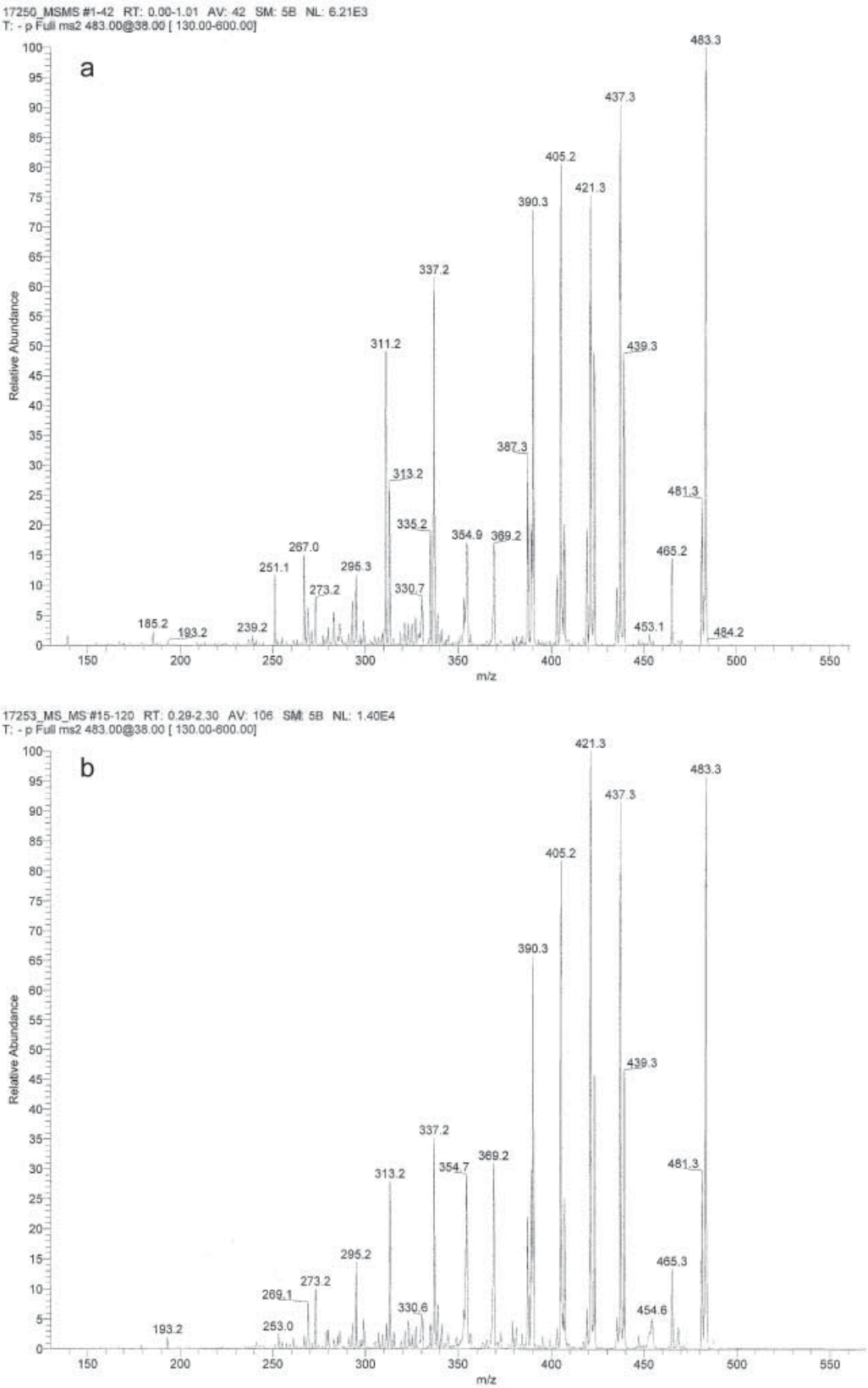

Figure 2 ESI (–) QIT product ion spectra of $[M - H]^-$ of compounds **1** (a) and **4** (b).

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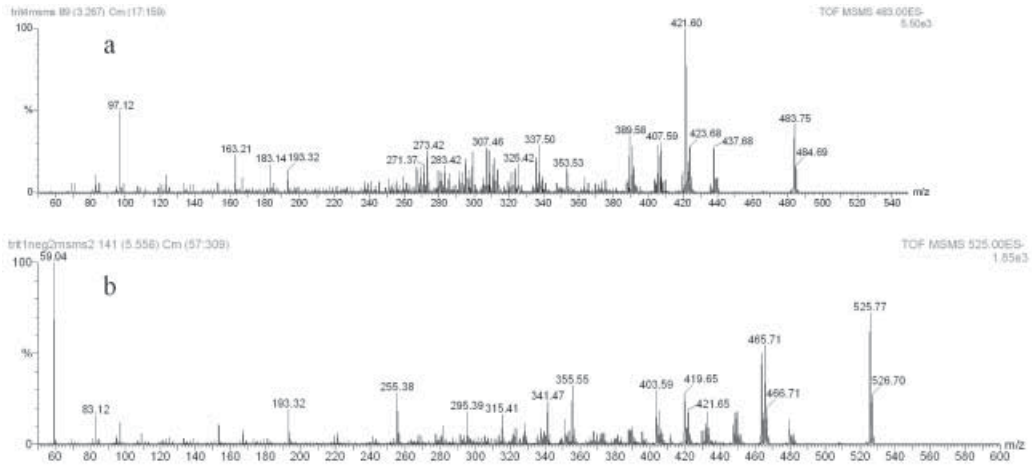


Figure 3 ESI (–) QTOF product ion spectra of $[M - H]^-$ of compounds **1** (a) and **5** (b).

Table 1 Characteristic ions observed in the negative ESI MS/MS spectrum of compounds **1** and **4**

	1[a]	**1**[b]	**4**[a]	**4**[b]
$[M - H]^-$	483 (42)	483 (100)	483 (100)	483 (95)
$[M - H - H_2O]^-$	—	465 (14)	—	465 (12)
$[M - H - CO_2]^-$	439 (<10)	439 (48)	439 (<10)	439 (48)
$[M - H - CO - H_2O]^-$	437 (25)	437 (90)	437 (38)	437 (90)
$[M - H - 60]^-$	423 (25)	423 (48)	423 (38)	423 (45)
$[M - H - CO_2 - H_2O]^-$	421 (100)	421 (75)	421 (92)	421 (100)
$[M - H - 76]^-$	407 (28)	407 (20)	407 (50)	407 (24)
$[M - H - 60 - H_2O]^-$	405 (26)	405 (80)	405 (35)	405 (81)
	390 (25)	390 (72)	390 (32)	390 (65)
$[^{'}g\text{-}CO_2 - H_2O]^-$[c]	337 (25)	337 (62)	337 (30)	337 (35)
$[M - H - 60 - sch]^-$[d]	313 (13)	313 (25)	313 (20)	313 (27)
$[^{'}e_1\text{-}H_2O]^-$	311 (22)	311 (49)	311 (15)	—
$[M - H - 60 - sch - H_2O]^-$[d]	295 (20)	295 (12)	295 (22)	295 (15)
$[^{'}D_1\text{-}H_2O]^-$	193 (12)	193 (1)	193 (15)	—
$[^{'}D_1\text{-}CO]^-$	183 (18)	—	—	—
F_1^-	97 (50)	[e]	97 (31)	[e]

[a] Recorded in QTOF; [b] recorded in QIT; [c] formation of this distonic radical can be explained by the fragmentation of the side chain. This ion is accompanied by a satellite one mass unit below, corresponding to terminally unsaturated anion.

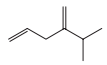
[d] Sch is the loss of 110 u from the side chain as,  ; [e] not detected.

Table 2 Characteristic ions observed in the negative ESI MS/MS spectrum of compounds **2** and **3**

	2[a]	**2**[b]	**3**[a]	**3**[b]
$[M - H]^-$	481 (34)	481 (100)	483 (10)	483 (39)
$[M - H - H_2O]^-$	—	463 (5)	—	465 (7)
$[M - H - CO_2]^-$	437 (<5)	437 (21)	—	439 (17)
$[M - H - CO - H_2O]^-$	435 (12)	435 (18)	—	437 (64)
$[M - H - 60]^-$[c]	421 (21)	421 (18)	423 (<5)	423 (5)
$[M - H - CO_2 - H_2O]^-$	419 (17)	419 (23)	421 (22)	421 (100)
	388 (21)	388 (21)	—	—
$[^{'}g\text{-}CO_2 - H_2O]^-$	335 (10)	335 (11)	—	337 (21)
$[^{'}e_1\text{-}H_2O]^-$	311 (19)	311 (29)	—	—
$[M - H - 60 - sch - H_2O]^-$[d]	293 (3)	293 (6)	—	—
F_1^-	97 (100)	[e]	97 (100)	[e]

[a] Recorded in QTOF; [b] recorded in QIT; [c] formation of this distonic radical can be explained by the fragmentation of the side chain. This ion is accompanied by a satellite one mass unit below, corresponding to terminally unsaturated anion.

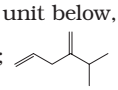
[d] Sch is the loss of 110 u from the side chain as;  [e] not detected.

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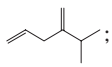
Table 3 Characteristic ions observed in the negative ESI MS/MS spectrum of compounds **5** and **6**

	5[a]	**5**[b]	**6**[a]	**6**[b]
$[M - H]^-$	525 (75)	525 (100)	527 (100)	527 (100)
$[M - H - CO_2]^-$	481 (<5)	—	483 (31)	483 (7)
$[M - H - CO - H_2O]^-$	479 (<15)	—	481 (18)	481 (40)
$[M - H - AcOH]^-$	465 (53)	465 (15)	467 (<10)	467 (40)
$[M - H - CO_2 - H_2O]^-$	463 (50)	463 (10)	465 (24)	465 (76)
$[M - H - AcOH - CO_2]^-$	421 (15)	—	—	—
$[M - H - AcOH - CO - H_2O]^-$	419 (28)	—	—	—
$[M - H - AcOH - CO_2 - H_2O]^-$	403 (29)	—	—	—
$[M - H - AcOH - sch]^-$[c]	355 (30)	355 (7)	—	—
$[^{\prime}B_3 - CO_2 - H_2O]^-$	255 (29)	—	—	—
$[^{\prime}D_1 - H_2O]^-$	193 (20)	—	193 (<5)	—
F_1^-	97 (12)	[d]	97 (13)	[d]
AcO^-	59 (100)	[d]	—	[d]

[a] Recorded in QTOF; [b] recorded in QIT; [c] sch is the loss of 110 u from the side chain as  ; [d] not detected.

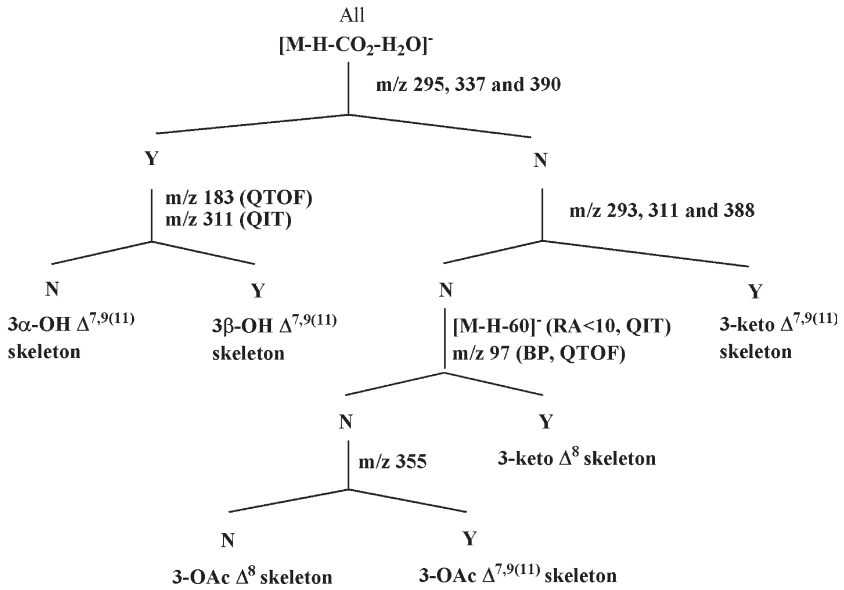


Figure 4 Identification scheme for the studied triterpenes.

ion would be the driving force for fragmentation. Further studies should be performed in order to explain this unexpected, but structurally diagnostic, fragmentation. A further loss of methane, that yields the ions of *m/z* 407 and 405 for **1**, **4** and **2**, respectively, would account for the formation of a more stable conjugated system.

Another diagnostic fragment ion, characteristic of the 3-hydroxy (or acetoxy)-$\Delta^{7,9(11)}$ compounds, is that of the ion at *m/z* 295. It could be produced from the parent ion at *m/z* 423 by the loss of the side chain (*m/z* 313) and then water. Remarkably, this ion has been reported many times in the EI spectra of this type of compound (Tai *et al.*, 1995a, b; Keller *et al.*, 1996), although its structure has never been reported or proposed.

Differentiation could easily be made between compounds with a $\Delta^{7,9(11)}$ skeleton. In general, 3-keto derivatives show ESI-MS/MS with less fragmentation, and the characteristic fragments were shifted 2 *m/z* units lower when compared with the corresponding 3-hydroxy compounds. Examples are the abundant ions at *m/z* 337 and 390 in 3-hydroxy derivatives, which are shifted to *m/z* 335 and 388, respectively, in the 3-keto derivative (Tables 1 and 2). The ion at *m/z* 97 (QTOF) was of higher intensity, and was the base peak in the case of 3-keto compounds **2** and **3** regardless of the skeleton. This low-mass F_1^- ion is obviously related exclusively to the side chain and therefore the presence of other chains should alter its observation.

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Differentiation between the epimers 3β and 3α-hydroxy, compounds **1** and **4**, was clearly achieved. The ion $[^{'}e_1\text{-}H_2O]^-$ at *m/z* 311 was observed exclusively in the ESI-MS/MS of compound **1** (LCQ), just as for the ion $[^{'}D_1\text{-}CO]^-$ at *m/z* 183, observed using the QTOF only. Fragments D, d, E, e and F are very common in the EI spectra of triterpenoids (Audier *et al.*, 1966; Hasan *et al.*, 1987) and steroids (Griffiths *et al.*, 1996).

A remarkable difference was observed between acetates **5** and **6**. The acetate anion of *m/z* 59 was not detected and $[M - H - AcOH]^-$ was observed with very low abundance in the QTOF ESI-MS/MS of **6**. The fragment ions $[M - H - CO_2]^-$ and $[M - H - CO - H_2O]^-$ were absent in the LCQ ESI-MS/MS spectrum of **5**. Low mass fragment ions were obviously not detected in the LCQ.

Differences between mass spectra recorded with different instruments are known to occur (Cabrera *et al.*, 2000; Josephs and Sanders, 2004; Hsu and Turk, 2005). This fact was particularly considered when spectral libraries were developed. Different designs of instruments, particularly ionisation sources and/or the difference in the residence time, collision energies and the efficiency of collisional excitation of ions, particularly for the ion trap instrument, could explain the observed differences.

In conclusion, the ESI-MS(/MS) analysis of lanostane-type triterpene acids was performed employing two tandem instruments with different configurations for MS/MS, QTOF and QIT. In both cases, the spectra showed similar fragmentation patterns, although some characteristic ions were instrument-dependent. A complete identification of the isolated compounds was performed, allowing the clear differentiation between Δ^8 and $\Delta^{7,9(11)}$ skeletons, 3-keto vs 3-hydroxy compounds and 3α vs 3β-hydroxy derivatives (Fig. 4).

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