International Journal of Mass Spectrometry 278 (2008) 38–49

Contents lists available at ScienceDirect

International Journal of Mass Spectrometry

journal homepage: www.elsevier.com/locate/ijms

Analysis of triptophenolide and its related compounds from *Tripterygium wilfordii Hook.f* by electrospray ionization tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 10 June 2008
Received in revised form 28 July 2008
Accepted 7 August 2008
Available online 20 August 2008

Keywords:
Triptophenolide
(−)ESI–MS/MS
Bond dissociation energy
Fragmentation mechanism
Metabolite

ABSTRACT

Triptophenolide and its related compounds from *Tripterygium wilfordii Hook.f* is a kind of diterpenoids which shows anti-inflammatory activity. To study the metabolites of triptophenolide related compounds, the fragmentation mechanisms of them were investigated by using negative electrospray tandem mass spectrometry. With the aid of high resolution of ESI–QTOF–MS/MS, the fragmentation mechanisms of six diterpenoid compounds were systematically investigated. The fragmentation behavior mainly depends on what substituent groups the benzyl C ring bears. If there is a hydroxyl group on the position of C14, loss of CH_4 is dominating. However, the successive loss of two CH_3 radicals is predominant when the hydroxyl group of O14 is methylated. The lactone ring is prone to be dissociated to loss of CO, CO_2 and $C_2H_2O_2$ molecules. The pericyclic reaction can occur on A ring if there is an active hydrogen resides on C ring. Furthermore, one metabolite of compound A1 was confirmed by cytochrome P450 in vitro and the structure was proposed by tandem mass experiment together with the fragmentation mechanisms of this type of compounds.

1. Introduction

Tripterygium Wilfordii Hook F (TWHF) is a traditional Chinese herb grown in the south of China, and it has long been used for the treatment of various immune and inflammatory diseases including rheumatoid arthritis, nephritis, systemic lupus erythematous, skin disorders and so on [1–3]. It has been reported that the main active constituents of the herb are diterpenes, triterpenes and alkaloid compounds [3,4]. For example, triptophenolide is a kind of diterpenoids which shows anti-inflammatory activity and obvious inhibiting effects on lymphocyte and IgG [5]. It is also used as starting material to synthesize triptolide and triptonide [6,7] which have potent antitumor, anti-inflammatory, immunosuppressive, and anti-fertile activities [8–11].

However, to our best knowledge, there are no articles revolving on the study of the metabolites of triptophenolide related compounds. High performance liquid chromatography (HPLC) coupled with tandem mass spectrometry is a powerful tool in characterization and identification of active components in Chinese medicines and their metabolites by dissociation of the parent ions into small daughter ions without further isolation and purification [12–15]. Previously, we analyzed the steroid saponins [16], hederagenin saponins, sesquiterpene oligoglycoside [17], aconite alkaloids [18], paeoniflorin and their derivatives [19] by using tandem mass spectrometry. Since there are few articles concerning the study of the fragmentation mechanism of triptophenolide and its related compounds using electrospray ionization (ESI) source, the fragmentation mechanism of six diterpenoid compounds (Fig. 1) were investigated. Last, a metabolite of compound A1 was found, and its structure was presumed by tandem mass spectrometry.

2. Experimental

2.1. Chemicals and solutions

Unless specified otherwise, all chemicals and solvents are of analytical reagent grade and were obtained from Chengdu Chemical Factory (Chengdu, China). HPLC-grade methanol is from Aldrich. Water was purified using a Milli-Q system. HEPES was obtained from Bochringer Mannheim GmbH. Rats F344 were bought from Shanghai SLAC laboratory animal CO. Ltd. (2007-0006). Collagenase was purchased from Invitrogen corporation.

Compound A1, A2, B1 and B2 were separated and purified from crude extract of *Tripterygium wilfordii Hook.f* by high-speed

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doi:10.1016/j.ijms.2008.08.007

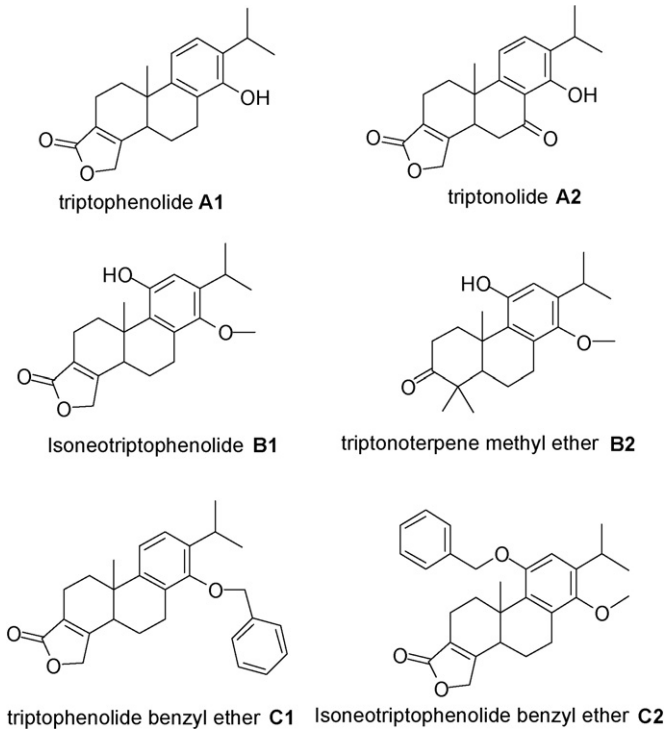


Fig. 1. The structures of the six triptophenolide related compounds.

countercurrent chromatography (HSCCC) [20]. The solvent system consisted of *n*-hexane–ethyl acetate–methanol–water (3:2:3:2, v/v). Compound C1 was synthesized by the following procedures: A1 was added to anhydrous acetone, and then refluxed with benzyl bromide, potassium iodide and potassium carbonate for 4 h. The solvent was evaporated and the precipitant was dissolved in ethyl acetate, and then washed by water. Compound C2 was synthesized from B1 by the same procedures as C1. Their structures were identified by electrospray ionization–quadrupole time of flight–mass spectrometry (ESI–QTOF–MS), ^{1}H NMR and ^{13}C NMR. Data of compound C1: ESI–TOF–MS (*m*/*z*): $[M-H]^-$ 401.2125, molecular formula: $C_{27}H_{29}O_3$. ^{1}H NMR (400 MHz, $CDCl_3$): 7.53–7.19 (7 H, m), 5.12 (2 H, s), 4.82 (2 H, m), 3.40 (1 H, sept, *J* = 7 Hz), 3.03 (1 H, m), 1.28 (6H, d, *J* = 7 Hz), 1.06 (3H, s). ^{13}C NMR (400 MHz, $CDCl_3$): 174.1, 162.9, 154.1, 144.2, 139.5, 137.6, 128.5, 128.2, 127.9, 127.3, 125.0, 124.2, 120.4, 74.5, 70.4, 41.1, 36.4, 32.7, 26.3, 24.0, 23.9, 23.0, 22.2, 19.8, 18.2. Data of compound C2: ESI–TOF–MS (*m*/*z*): $[M-H]^-$ 431.2224, molecular formula: $C_{28}H_{31}O_4$. ^{1}H NMR (400 MHz, $CDCl_3$): 7.25–7.45 (5 H, m), 6.69 (1 H, s), 5.10 (2 H, s), 4.75(2 H, m), 3.69(3 H, s), 3.29(1 H, sept, *J* = 7 Hz), 1.20(6 H, d, *J* = 7 Hz), 1.07 (3H, s). ^{13}C NMR (400 MHz, $CDCl_3$): 174.3, 163.2, 154.2, 149.3, 139.4, 137.3, 131.5, 130.6, 128.6, 127.8, 127.21, 125.2, 108.5, 70.6, 70.5, 60.7, 44.3, 37.5, 31.3, 26.4, 25.6, 23.8, 23.7, 19.4, 18.6, 17.5. The nuclear magnetic resonance (NMR) spectrometer used here is a BRUKER Avance 400 NMR system.

2.2. ESI–QTOF–MS/MS

Tandem mass experiments of the six triptophenolide related compounds were performed on an ESI–QTOF–mass spectrometer (Micromass, UK) equipped with an interchangeable ESI Z-spray source. MassLynx 4.1 software (Waters) was used for system control and data processing. The samples were dissolved in methanol and introduced via a syringe pump at a flow rate of 10 μl/min. Nitrogen was used as nebulizing gas, desolvation gas, and cone curtain gas. The QTOF–MS source parameters were set as follows: The spray voltage, 2800 V in the negative mode; sample cone, 40 V; extract cone, 4 V; source temperature, 90 °C; desolvation temperature, 250 °C; cone gas flow, 100 l/h; and desolvation gas flow, 500 l/h. The microchannel plate detector was operated at 2100 V. An *m*/*z* range of 50–600 was recorded at every 1.0 s with an interscan time of 0.02 s for QTOF–MS analysis. In the tandem mass mode, argon was chosen as collision gas with the collision cell gas pressure at 10 psi, and the collision energy was raised from 6 to 30 eV for QTOF–MS/MS analysis.

2.3. Calculations

All the calculations were carried out using the Gaussian 03 [21] program. Geometries of the related ions and radical species were optimized at the B3LYP/6-31G(d) level, and there structures were verified by frequency calculations at the same level. Single-point energies on the optimized structures were computed with B3P86 and 6–311 + G(d,p) basis set, which had been reported to be promising in energetic calculations. Zero-point energy was considered and scaled by 0.9806. The bond orders and natural charges were calculated by NBO3.1 in the B3LYP/6-31G(d) level.

2.4. Liver perfusion and Isolation of hepatocytes

Male F344 rats weighing 180–220 g were fasted for 18–24 h before operation. Isolation of hepatocytes from rats was performed by the collagenase according to the method of Williamson [22]. Rats were anesthetized with pentobarbital sodium (10 mg/100 g body weight), and liver was perfused (5 min, rate, 50 ml/min) via the inferior portal vein with perfusion medium I (containing sodium chloride 142 mM, chloratum kalium 6.7 mM, hydroxyethyl piperazine ethanesulfonic acid (HEPES) 10 mM and natrium hydroxydatum 5.5 mM pH 7.4) which had been presaturated with carbogen (oxygen/CO_2 5%). The liver was digested using perfusion medium II (containing collagenase 0.05%, sodium chloride 67 mM, chloratum kalium 6.7 mM, calcium chloride 5 mM, HEPES 100 mM and natrium hydroxydatum 66 mM pH 7.6). Liver tissue was gently disrupted and washed through a sieve (200 μm mesh size) with liver cell washing medium (containing sodium chloride 142 mM, chloratum kalium 6.7 mM, HEPES 10 mM, calcium chloride 1.2 mM and natrium hydroxydatum 5.5 mM pH 7.4).

Hepatocytes were washed three times and centrifuged (3 min, 60 × *g*, 4 °C). The entire procedure of perfusion and isolation did not exceed 30 min. Cells were suspended in Dulbecco minimum essential medium (DMEM) with 2% fetal bovine serum and determined by counting in a hemocytometer. Hepatocyte viability was above 90% indicated by trypan blue exclusion assay.

2.5. Incubations with hepatocytes

Fresh isolated cells (1×10^6 cells per ml) were suspended in DMEM with 2% fetal bovine serum 2 ml, and incubated in a slowly shaking incubator (80–100 oscillations/min, 37 °C). Compound A1 dissolved in dimethyl sulfoxide (DMSO) was added to the suspension to a final concentration of 50 μg/ml. The max concentration of DMSO less than 0.1% would not damage the cell viability [23]. Control incubations including A1 with heat-inactivated cells or with vehicle only were also conducted.

Termination of hepatocyte incubation was operated by putting the vials into liquid nitrogen after 10, 30, 60, 120 min. Cell activity decreased between 60% and 40% of initial values indicated by trypan blue exclusion test.

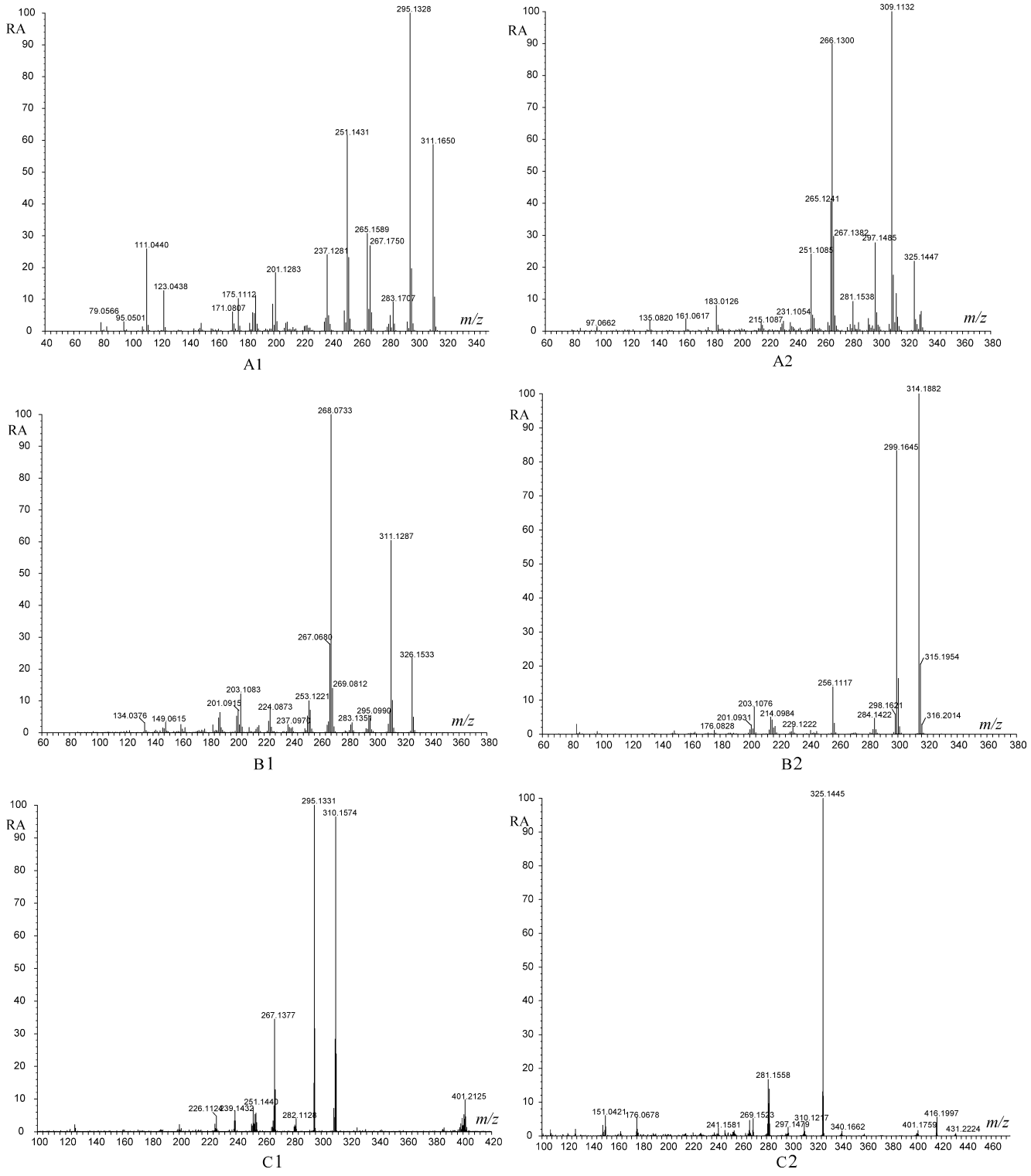


Fig. 2. The tandem mass spectrometry of A1–C2 acquired on ESI–QTOF–MS/MS.

2.6. Extraction of the metabolites

Samples were rapidly defrosted, and then extracted four times with ethyl acetate (same volume of samples). The organic layer was combined and evaporated to dryness under nitrogen. The deposits were dissolved by methanol and water (150 μl, 6:4 v/v), and were analyzed by ultra performance liquid chromatography (UPLC) ESI–MS immediately.

2.7. UPLC/ESI–MS/MS

Sample extracts from metabolite of compound A1 were analyzed with an Acquity UPLC™ system connected in-line to a Quatrro Premier XE triple quadrupole mass spectrometer (Micromass, UK). Samples were introduced via an autosampler and reversed-phase chromatography was carried out at 25 °C with an Waters BEH C18 1.7 μm 2.1 mm × 50 mm column (Waters, Acquity UPLC™). Mobile

phase A: water, phase B: methanol. Chromatography was performed at 0.2 ml/min and starting at 50% B. Over 8 min phase B was ramped linearly to 90%.

The eluent from the HPLC system was connected directly to the ESI interface of the Quatrro Premier XE triple quadrupole mass spectrometer. The mass spectrometer was operated in negative ionization mode for all analyses and the mass range of 50–1000 amu was scanned. MS/MS experiments were conducted by Data Dependent Analysis method. The desolvation gas was nitrogen and its temperature was kept at 150 °C. The collision energy was set at 30 V and the spray voltage at 2.8 kV in the negative mode. The data was processed by Masslynx 4.1 software.

3. Results and discussion

3.1. ESI–QTOF–MS/MS analysis of compound A1 and A2

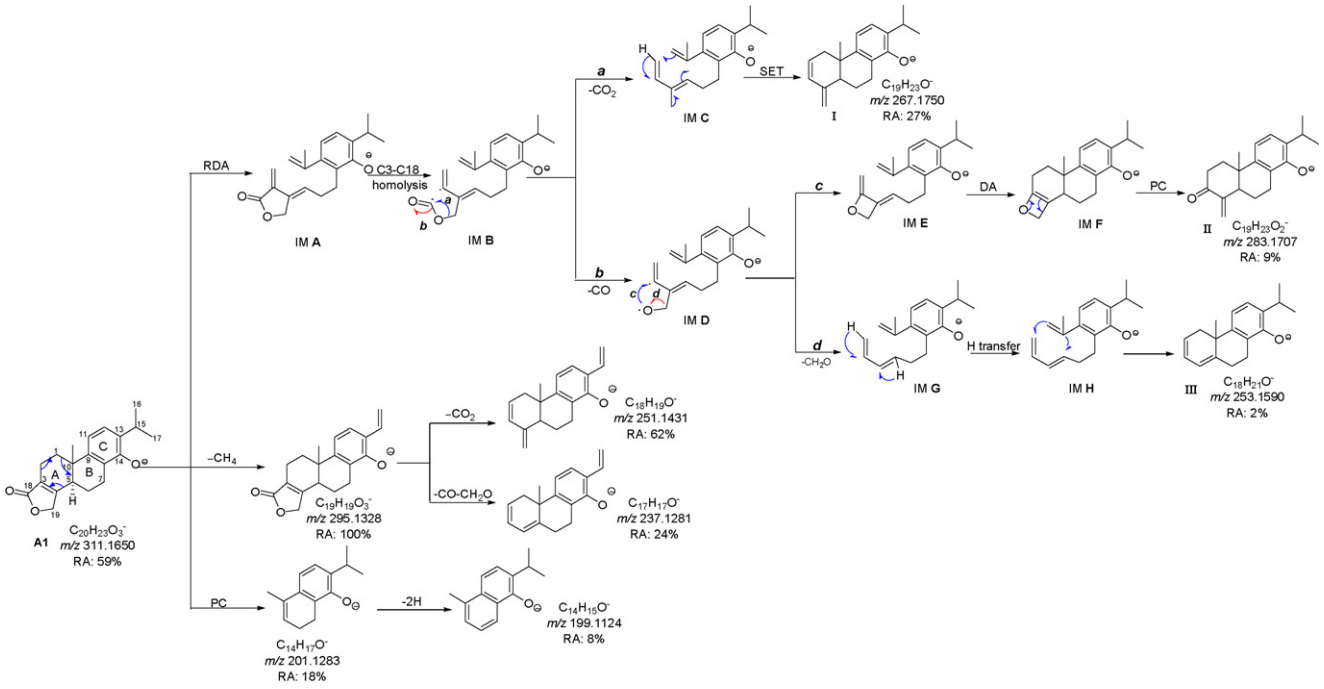
Due to the acidity of phenolic hydroxyl group on C ring, ESI negative mode was used to study their fragmentation behavior. In the full scan mode of compound A1, deprotoned parent ion at *m*/*z* 311.1650 was detected. In the further tandem mass experiment of A1 (Fig. 2), there were eight major fragment ions (Table 1). Through analysis, three fragmentation pathways of A1 were concluded (Scheme 1). The first pathway corresponded to fission of lactone ring. To begin with, retro-Diels-Alder (RDA) reaction might occur on the A ring to produce the intermediate ion IM **A**. Then, in collision with argon, the C3—C18 bond was homolyzed to form the intermediate ion IM **B** with two radicals left on C3 and C18, respectively. Subsequently, induced by the C18 radical, two major fragmentation pathways would occur. Firstly, in pathway ***a***, C19—O bond was broken to produce the intermediate ion IM **C** accompanied by loss of a molecule of CO_2. Next, hydrogen radical on C2 was transferred to C3 followed by a series of single electron transfers (SET) on bonds of C1—C10, C4—C5 and C19—C4 to give rise to the stable daughter ion I at *m*/*z* 267.1750.

Pathway ***b*** corresponded to fission of C18—O bond, which leading to the formation of ion IM **D** together with the loss of a molecule of CO. Then, the radical on O19 would couple to C3 radical to produce the ion IM **E** (path ***c***) or induce the fission of C4—C19 bond to produce the ion IM **G** accompanied by the loss of CH_2O group (path ***d***). In path ***c***, A ring of IM **E** was rebuild to form the ion IM **F** via a Diels-Alder (DA) reaction. Due to the big tension of IM **F**, pericyclic (PC) reaction would occur on the four-membered ring to produce the stable daughter ion II at *m*/*z* 283.1707. In path ***d***, IM **G** was converted to IM **H** through H transfers from C2 and C5 to C3 and C4, respectively. Finally, radicals on C2 and C5 would be combined with C1 and C10 to produce the daughter ion III at *m*/*z* 253.1590.

The second pathway corresponded to loss of a molecule of CH_4 from the parent ion to form the ion at *m*/*z* 295.1328. Because there are two methyl groups on A1, to further confirm which one is apt to lose, the (−)ESI tandem mass experiment of 2-isopropylphenol was conducted. It showed an intensive base peak corresponding to loss of CH_4 group, indicating that the methyl of isopropyl is prone to be eliminated with its vicinal hydrogen. Next, due to the homolysis of C18—C3 bond, CO_2 or $C_2H_2O_2$ ($CO + CH_2O$) groups were eliminated to produce the ions at *m*/*z* 251.1431 and 237.1281, respectively.

The third dissociation pathway corresponded to the PC reaction on A ring to produce the ion at *m*/*z* 201.1283, followed by loss of two hydrogen atoms from C6 and C7 to form a more stable ion at *m*/*z* 199.1124 with conjugated naphthalene system.

Because the molecular structure of A2 is similar to A1, the fragmentation mechanisms of A2 are similar to A1 (Scheme 2). In the MS mode, parent ion was observed at *m*/*z* 325.1447. Due to an extra carbonyl group on C7, three different fragmentation modes were observed when compared with A1. Firstly, the relative abundance of fragment ion at *m*/*z* 297.1485 was increased which can be ascribed to additional loss of a CO from B ring besides the elimination of CO from the lactone ring. Secondly, there was no ion corresponding to successive loss of two hydrogen atoms from the ion at *m*/*z* 215.1087 derived from the PC reaction of A ring, which could be rationalized by the isomerization of carbonyl group to enol form to produce the naphthalene structure. Thirdly, one hydrogen radical would be eliminated from the ion at *m*/*z* 267.1382 to give rise to



Scheme 1. The proposed fragmentation pathways of compound A1 in (−)ESI–MS/MS experiment. RA, relative abundance.

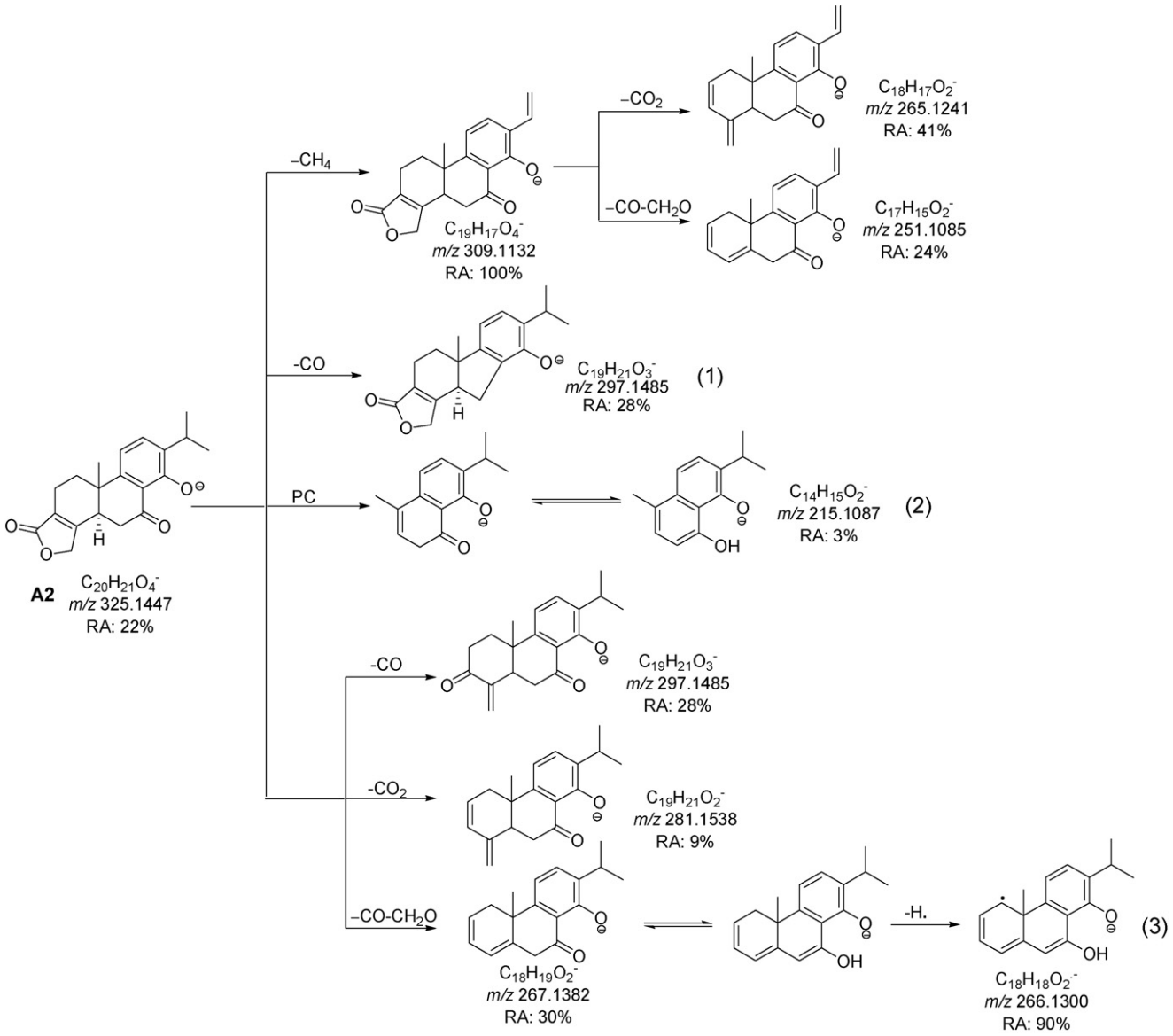
Table 1
Accurate mass data of fragment ions derived from the compound A1–C2 obtained by ESI–QTOF–MS/MS

Compound	Measured mass	Attribution	Molecular formula	Theoretical mass	Delta (ppm)
A1	311.1650	$[M-H]^-$	$C_{20}H_{23}O_3^-$	311.1653	−0.96
	295.1328	$[M-H-CH_4]^-$	$C_{19}H_{19}O_3^-$	295.1340	−4.06
	283.1707	$[M-H-CO]^-$	$C_{19}H_{23}O_2^-$	283.1704	1.06
	267.1750	$[M-H-CO_2]^-$	$C_{19}H_{23}O^-$	267.1749	0.37
	265.1589	$[M-H-CO-H_2O]^-$	$C_{19}H_{21}O^-$	265.1588	0.38
	253.1590	$[M-H-CO-CH_2O]^-$	$C_{18}H_{21}O^-$	253.1598	−3.16
	251.1431	$[M-H-CH_4-CO_2]^-$	$C_{18}H_{19}O^-$	251.1441	−3.98
	237.1281	$[M-H-CH_4-C_2H_2O_2]^-$	$C_{17}H_{17}O^-$	237.1285	−1.69
	201.1283	$[M-H-C_6H_6O_2]^-$	$C_{14}H_{17}O^-$	201.1285	−0.99
	199.1124	$[M-H-C_6H_6O_2-H_2]^-$	$C_{14}H_{15}O^-$	199.1128	−2.01
A2	325.1447	$[M-H]^-$	$C_{20}H_{21}O_4^-$	325.1443	1.23
	309.1132	$[M-H-CH_4]^-$	$C_{19}H_{17}O_4^-$	309.1134	−0.65
	297.1485	$[M-H-CO]^-$	$C_{19}H_{21}O_3^-$	297.1496	−3.70
	281.1538	$[M-H-CO_2]^-$	$C_{19}H_{21}O_2^-$	281.1547	−3.20
	267.1382	$[M-H-C_2H_2O_2]^-$	$C_{18}H_{19}O_2^-$	267.1391	−3.37
	266.1300	$[M-H-C_2H_2O_2-H]^-$	$C_{18}H_{18}O_2^-$	266.1312	−4.51
	265.1241	$[M-H-CH_4-CO_2]^-$	$C_{18}H_{17}O_2^-$	265.1240	0.38
	251.1085	$[M-H-CH_4-C_2H_2O_2]^-$	$C_{17}H_{15}O_2^-$	251.1078	2.79
	215.1087	$[M-H-C_6H_6O_2]^-$	$C_{14}H_{15}O_2^-$	215.1078	4.18
B1	341.1758	$[M-H]^-$	$C_{21}H_{25}O_4^-$	341.1759	−0.29
	326.1533	$[M-H-CH_3]^-$	$C_{20}H_{22}O_4^-$	326.1524	2.76
	311.1287	$[M-H-2CH_3]^-$	$C_{19}H_{19}O_4^-$	311.1289	−0.64
	296.1065	$[M-H-3CH_3]^-$	$C_{18}H_{16}O_4^-$	296.1054	3.71
	295.0990	$[M-H-3CH_3-H]^-$	$C_{18}H_{15}O_4^-$	295.0976	4.74
	283.1351	$[M-H-2CH_3-CO]^-$	$C_{18}H_{19}O_3^-$	283.1340	3.88
	268.0733	$[M-H-3CH_3-C_2H_4]^-$	$C_{16}H_{12}O_4^-$	268.0741	−2.98
	267.0680	$[M-H-3CH_3-C_2H_4-H]^-$	$C_{16}H_{11}O_4^-$	267.0663	6.37
	267.1384	$[M-H-2CH_3-CO_2]^-$	$C_{18}H_{19}O_2^-$	267.1391	−2.62
	253.1221	$[M-H-2CH_3-C_2H_2O_2]^-$	$C_{17}H_{17}O_2^-$	253.1234	−5.14
	203.1083	$[M-H-2CH_3-C_6H_6O_2]^-$	$C_{13}H_{15}O_2^-$	203.1078	2.46
	201.0915	$[M-H-C_6H_6O_2-C_2H_4]^-$	$C_{13}H_{13}O_2^-$	201.0921	−2.98
B2	329.2122	$[M-H]^-$	$C_{21}H_{29}O_3^-$	329.2124	−0.61
	314.1882	$[M-H-CH_3]^-$	$C_{20}H_{26}O_3^-$	314.1889	2.23
	299.1645	$[M-H-2CH_3]^-$	$C_{19}H_{23}O_3^-$	299.1653	−2.67
	284.1422	$[M-H-3CH_3]^-$	$C_{18}H_{20}O_3^-$	284.1418	1.40
	256.1117	$[M-H-3CH_3-C_2H_4]^-$	$C_{16}H_{16}O_3^-$	256.1105	4.68
	203.1076	$[M-H-C_6H_{10}O-C_2H_4]^-$	$C_{13}H_{15}O_2^-$	203.1078	−0.98
	201.0931	$[M-H-2CH_3-C_6H_{10}O]^-$	$C_{13}H_{13}O_2^-$	201.0921	4.97
C1	401.2125	$[M-H]^-$	$C_{27}H_{29}O_3^-$	401.2127	−0.50
	310.1574	$[M-H-PhCH_2]^-$	$C_{20}H_{22}O_3^-$	310.1575	−0.32
	295.1331	$[M-H-PhCH_2-CH_3]^-$	$C_{19}H_{19}O_3^-$	295.1340	−3.05
	267.1377	$[M-H-PhCH_2-CH_3-CO]^-$	$C_{18}H_{19}O_2^-$	267.1391	−5.24
	251.1440	$[M-H-PhCH_2-CH_3-CO_2]^-$	$C_{18}H_{19}O^-$	251.1441	−0.40
	239.1432	$[M-H-PhCH_2-CH_3-2CO]^-$	$C_{17}H_{19}O^-$	239.1441	−3.76
	237.1273	$[M-H-PhCH_2-CH_3-C_2H_2O_2]^-$	$C_{17}H_{17}O^-$	237.1285	−5.06
C2	431.2224	$[M-H]^-$	$C_{28}H_{31}O_4^-$	431.2229	−1.16
	416.1997	$[M-H-CH_3]^-$	$C_{27}H_{28}O_4^-$	416.1993	0.96
	401.1759	$[M-H-2CH_3]^-$	$C_{26}H_{25}O_4^-$	401.1758	0.25
	340.1662	$[M-H-PhCH_2]^-$	$C_{21}H_{24}O_4^-$	340.1680	−5.29
	325.1445	$[M-H-PhCH_2-CH_3]^-$	$C_{20}H_{21}O_4^-$	325.1448	−0.92
	310.1211	$[M-H-PhCH_2-2CH_3]^-$	$C_{19}H_{18}O_4^-$	310.1215	−1.29
	297.1479	$[M-H-PhCH_2-CH_3-CO]^-$	$C_{19}H_{21}O_3^-$	297.1496	−5.72
	281.1558	$[M-H-PhCH_2-CH_3-CO_2]^-$	$C_{19}H_{21}O_2^-$	281.1574	−5.69
	269.1523	$[M-H-PhCH_2-CH_3-2CO]^-$	$C_{18}H_{21}O_2^-$	269.1547	−8.55
	241.1581	$[M-H-PhCH_2-CH_3-3CO]^-$	$C_{17}H_{21}O^-$	241.1598	−7.05

the more stable ion at *m*/*z* 266.1300 with extended conjugated ring system.

3.2. ESI–QTOF–MS/MS analysis of compound B1 and B2

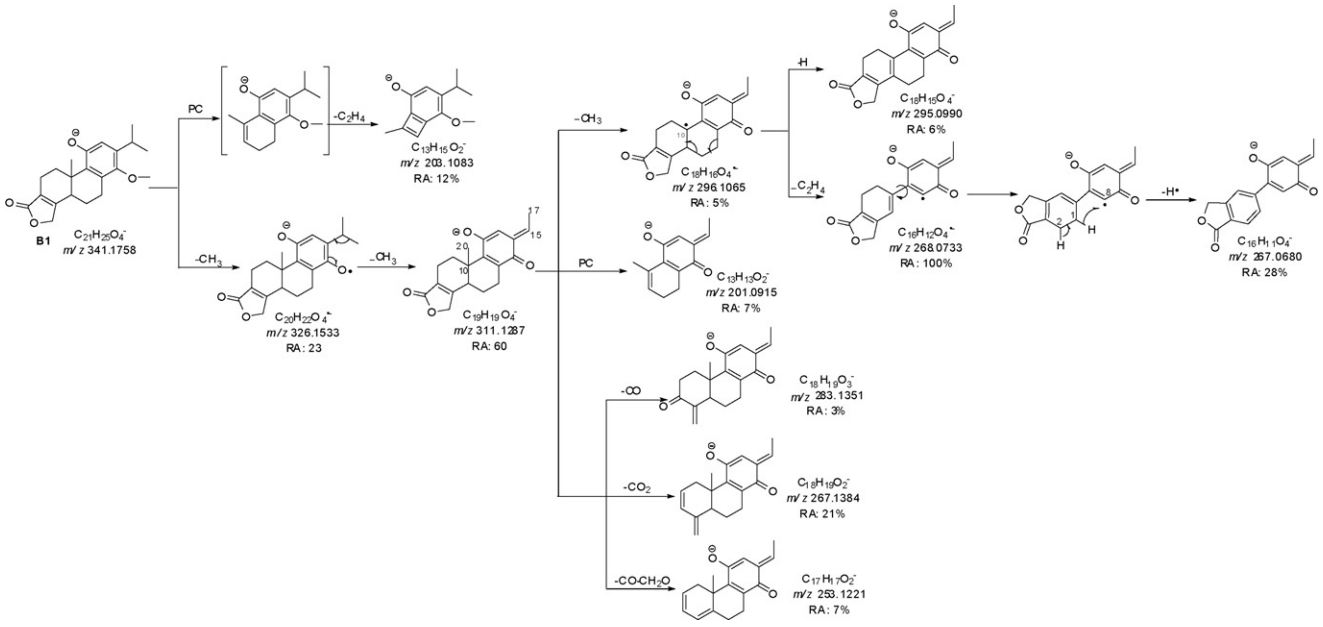
In the (−)ESI full scan mode of compound B1, parent ion at *m*/*z* 341.1758 was found. When it was subjected to tandem mass experiments, two major fragmentation pathways were observed (Scheme 3). Firstly, the PC reaction of A ring followed by loss of a molecule of C_2H_4 on B ring would produce the ion at *m*/*z* 203.1083. Secondly, one CH_3 radical not CH_4 molecule was eliminated to form the daughter ion at *m*/*z* 326.1533. Why? Compared to A1, there is an extra hydroxyl group on C11 and, more important, the hydroxyl of C14 is methylated. Because there was no loss of CH_4 in (+)ESI–MS/MS of 2-isopropylphenol and compound A1 (data not shown), the ortho oxygen anion should play the key role. Previously, Tumas et al. have conducted a systematic investigation of the infrared multiple photon (IRMP) photochemically induced decompositions of a series of alkoxide anions ***a*** and provided a stepwise mechanism for the formal 1,2-elimination of neutral fragments based on kinetic isotope effects and reactivity patterns, which involving initial heterolytic cleavage to an intermediate anion–ketone complex followed by proton transfer to give the ultimate product (Scheme 4a) [24]. Furthermore, Stringer et al have



Scheme 2. The proposed fragmentation pathways of compound A2 in (−)ESI–MS/MS experiment. 1–3 are different fragmentation patterns compared with compound A1. RA, relative abundance.

conducted the collision activated mass experiment of the heptan-4-one enolate ion ***b***, which shows major elimination of methane through stepwise process via a six-center state by application of double isotope labeling technique (Scheme 4b) [25]. Inspired by their works, we proposed that compound A1 (***c***) might be subjected to the similar process when loss of a molecule of methane. Firstly, Due to the negative charge on the O anion and double electron transfer (DET), the C—CH_3 bond of the ortho isopropyl group was elongated, weakened and then heterolyzed in collision with the argon gas, producing the anion–ketone complex. The proton of the other CH_3 group on isopropyl group was transferred to the CH_3 anion, thus a molecule of methane was expelled. At this time, the negative charge on terminal C atom was transferred to the oxygen by DET, producing the ultimate stable daughter ion (Scheme 4c). As for compound B1, negative charge could not be transferred from meta oxygen anion to C—CH_3 by DET, thus the C—CH_3 bond would not be activated. To further validate our assumption, a series of calculation was conducted. Through geometry optimization (Fig. 3), the bond length (BL) and bond order (BO) of C—CH_3 bond of isopropyl group were obtained. The bond of C15—C16 of A1 is 0.1565 nm, which is longer than that of compound B1 (0.1548 nm), and the bond strength of C15—C16 of A1 (BO: 0.9458) is weaker than that of B1 (BO: 0.9894). In addition, as for compound B1, due to the small BO of the bond O14—CH_3 (0.8308), it was believed that the CH_3 radical on O14 is prone to be lost from the parent ion to produce the ion at *m*/*z* 326.1533.

Next, ion at *m*/*z* 311.1287 corresponded to loss of another CH_3 radical, which could be easily interpreted as the SET from O14 to isopropyl group (Scheme 3). Furthermore, there were three major fragmentation pathways. Firstly, CH_3 radical on C10 was eliminated to produce the ion at *m*/*z* 296.1065, which facilitated the loss of C_2H_4 group via the homolysis of C5—C6 and C7—C8 bonds to give rise to the daughter ion at *m*/*z* 268.0733. Because the radical on C8 is not stable, the hydrogen atom on C1 was transferred to C8 followed by loss of another hydrogen atom on C2 to form the stable benzyl ring ion at *m*/*z* 267.0680. The C10 radical might also induce the elimination of a hydrogen atom from C5 to produce the ion at *m*/*z* 295.0990. It should be pointed out that the ion at *m*/*z* 267 and *m*/*z*



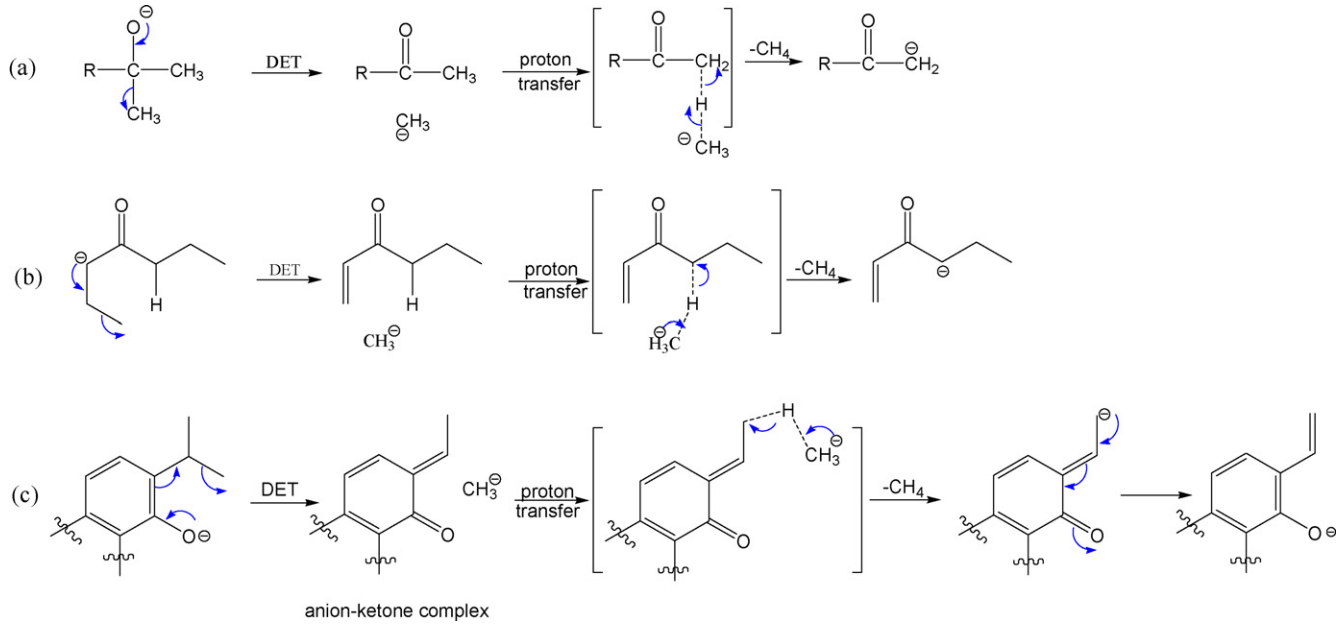
Scheme 3. The proposed fragmentation pathways of compound B1 in (−)ESI–MS/MS experiment. RA, relative abundance.

268 could also be formed by the other two pathways (Scheme 5). In pathway II, CH_3 radical on C10 was eliminated from the precursor ion at *m*/*z* 326 to produce the ion IIA, followed by loss of the isopropyl group to produce the ion IIB. However, the energy of IIA and IIB is higher than that of IA and IC by 39 kJ/mol and 56 kJ/mol, respectively. Next, in pathway III, *m*/*z* 267 originated from loss of CH_4 on C10 followed by loss of the isopropyl group from the precursor ion **PI**. However, the energy of IIIB is higher than that of ID by 231 kJ/mol, so these two pathways are not favorable.

Secondly, the PC reaction of A ring led to the formation of ion at *m*/*z* 201.0915. Because loss of two hydrogen atoms would not give rise to the more stable naphthalene system, ion at *m*/*z* 199 like compound **A1** was not detected. Thirdly, there also existed the fragment ions at *m*/*z* 283.1351, *m*/*z* 267.1384 and *m*/*z* 253.1221 corresponding to homolysis of C3—C18 bond. Compound B2 possesses identical substituent groups on C ring. When it was subjected to tandem mass experiment, it produced the similar fragmentation pathways. However, without the lactone ring, no ions corresponding to loss of CO and CO_2 were detected. Apart from that, due to the quaternary carbon of C4, it is impossible for ion at *m*/*z* 256.1117 to form the benzyl group through hydrogen transfer from C1 to C8 followed by loss of hydrogen atom on C2 as compound B1. So, no ion at *m*/*z* 255 was detected (Scheme 6).

3.3. ESI–QTOF–MS/MS analysis of compound C1 and C2

Triptophenolide ether compounds also exist in the plant. Because we did not acquire them, its related compound C1 and



Scheme 4. Mechanisms proposed for the methane elimination from parent ions a–c.

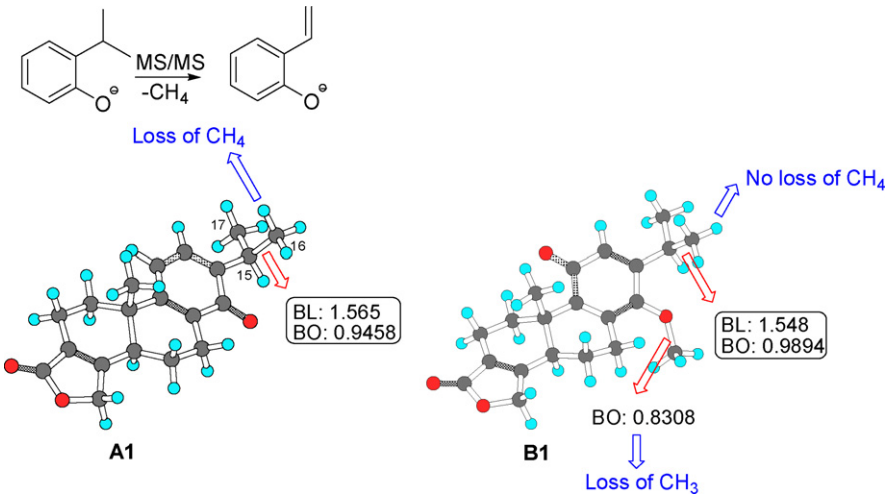
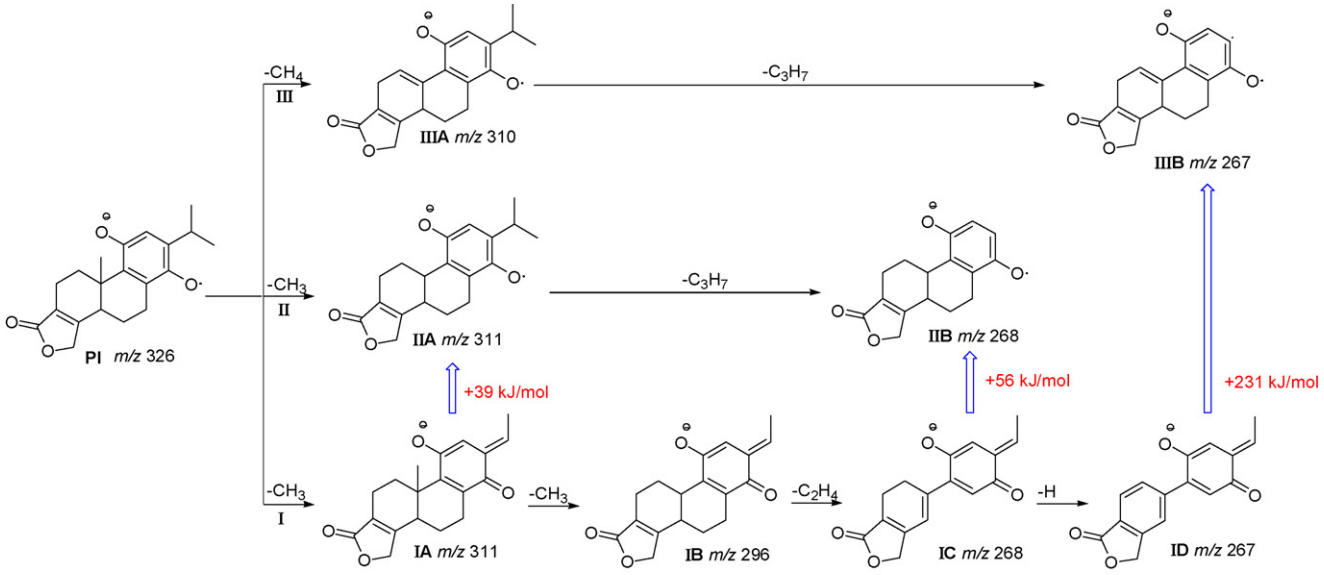
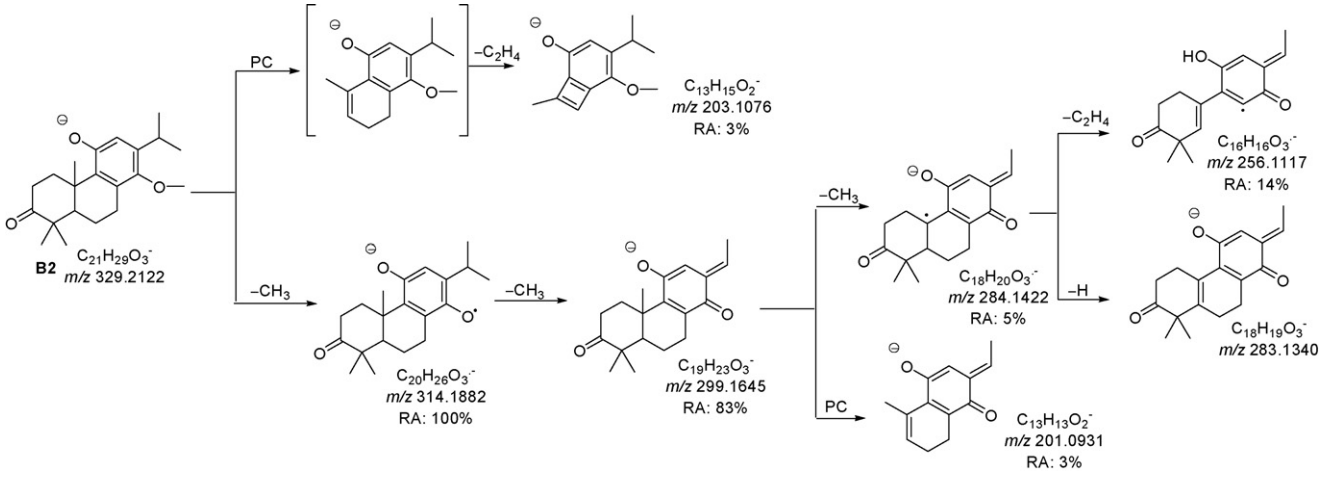


Fig. 3. The bond length (BL) and bond order (BO) of C15—C16 bond of compound A1 and B1 together with BO of C14—O bond of B1.



Scheme 5. The other two possible fragmentation pathways corresponding to formation of ions at *m*/*z* 267 and *m*/*z* 268.



Scheme 6. The proposed fragmentation pathways of compound B2 in (−)ESI–MS/MS experiment. RA, relative abundance.

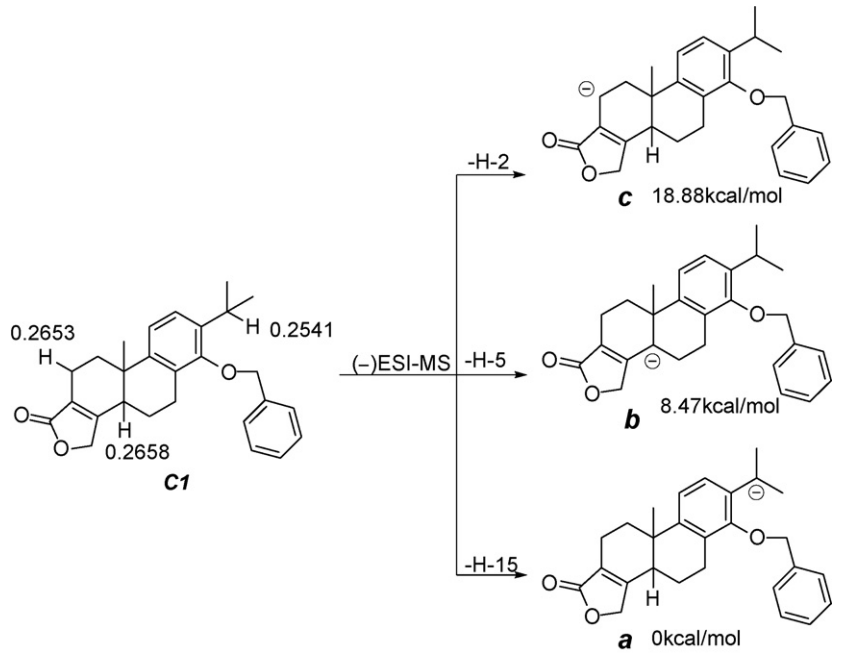
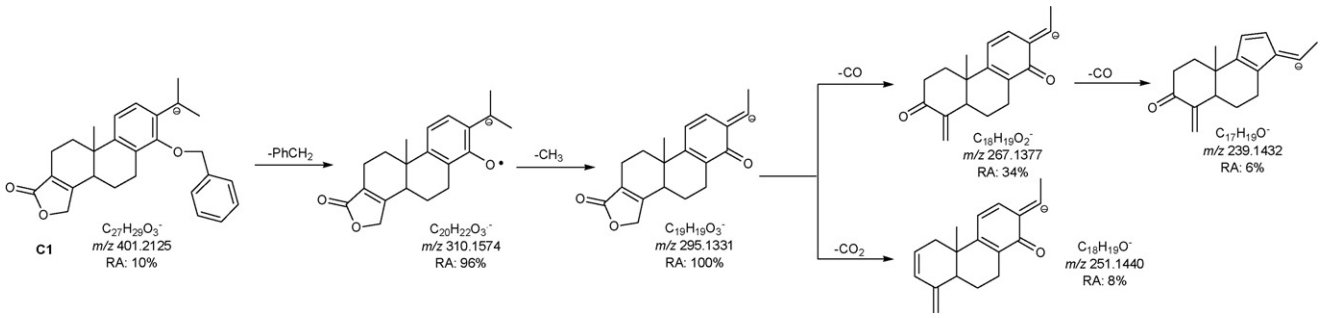
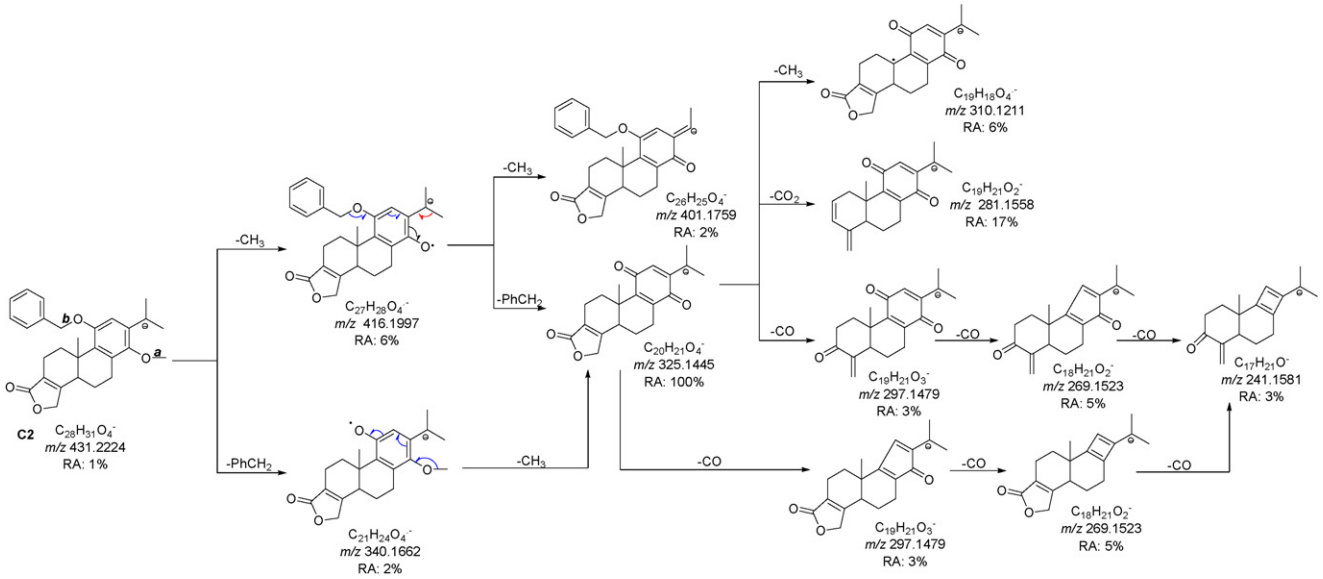


Fig. 4. The charges of compound C1 and the energies of anion compounds ***a***, ***b*** and ***c*** corresponding to loss of H-15 hydrogen, loss of H-5 hydrogen and loss of H-2 hydrogen, respectively.



Scheme 7. The proposed fragmentation pathways of compound C1 in (−)ESI–MS/MS experiment. RA, relative abundance.



Scheme 8. The proposed fragmentation pathways of compound C2 in (−)ESI–MS/MS experiment. RA, relative abundance.

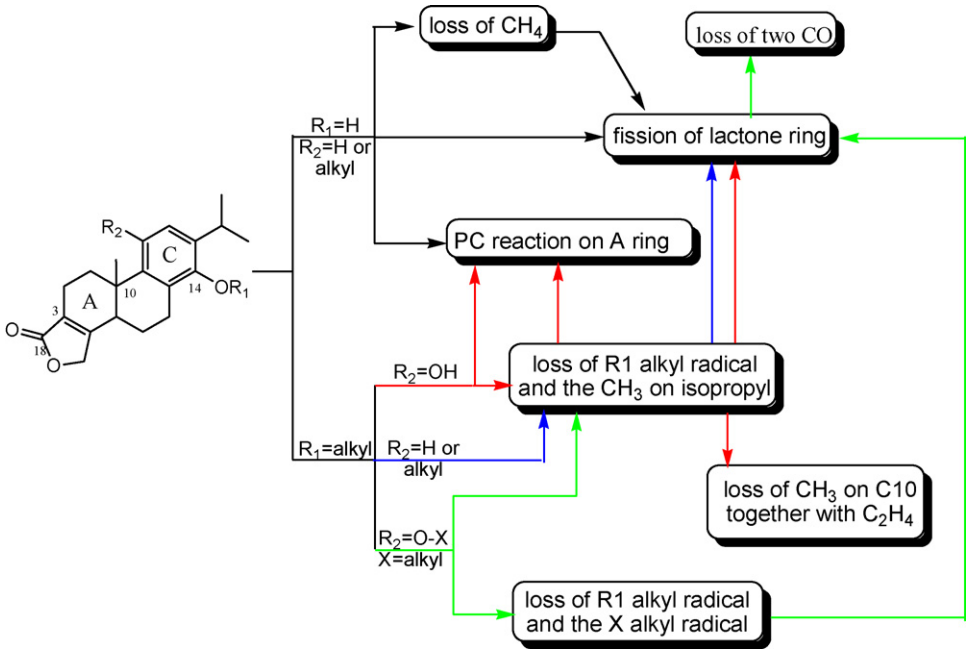


Fig. 5. The fragmentation pathways of triptophenolide related compounds decided by the substituents on benzyl C ring.

C2 were synthesized from **A1** and **B1** with $PhCH_2Br$. Without the acid hydrogen on C ring, the signal of them in the ESI negative mode is weak. Through calculation, the atom partial charges on the H15 (0.2541), H5 (0.2658) and H2 (0.2653) are more positive (except for the hydrogen atoms on benzyl group) (Fig. 4). So in the powerful negative electro field, the H15, H5 and H2 protons may be apt to be eliminated, with the negative charge left on C15, C5 and C2 to form the ions ***a***, ***b*** and ***c***, respectively. Through structural optimization and energy calculation, the ***a*** ion is more stable than ***b*** ion by 8.47 kcal/mol, and more stable than ***c*** ion by 18.88 kcal/mol. So the major negative charge might be located on C15. In the full scan mode, compound C1 was observed at *m*/*z* 401.2125. The most abundant fragment ions at *m*/*z* 310.1574 and 295.1331 corresponded to homolysis of O14—$PhCH_2$ bond and successive loss of a CH_3 on C15 via SET, respectively. Next, due to the lactone ring, CO and CO_2 were eliminated from the precursor ion at *m*/*z* 295.1331 to produce the ions at *m*/*z* 267.1377 and *m*/*z* 251.1440, respectively. The ion at *m*/*z* 239.1432 corresponded to loss of another CO from the precursor ion at *m*/*z* 267.1345 (Scheme 7). As for compound C2, parent ion at *m*/*z* 431.2224 was detected in the full scan mode. In tandem mass experiment, due to the homolysis of ether bonds ***a*** and ***b***, fragment ions at *m*/*z* 416.1997 and *m*/*z* 340.1662 were observed. Next, the O14 radical of the ion at *m*/*z* 416 would trigger a series of SET to produce

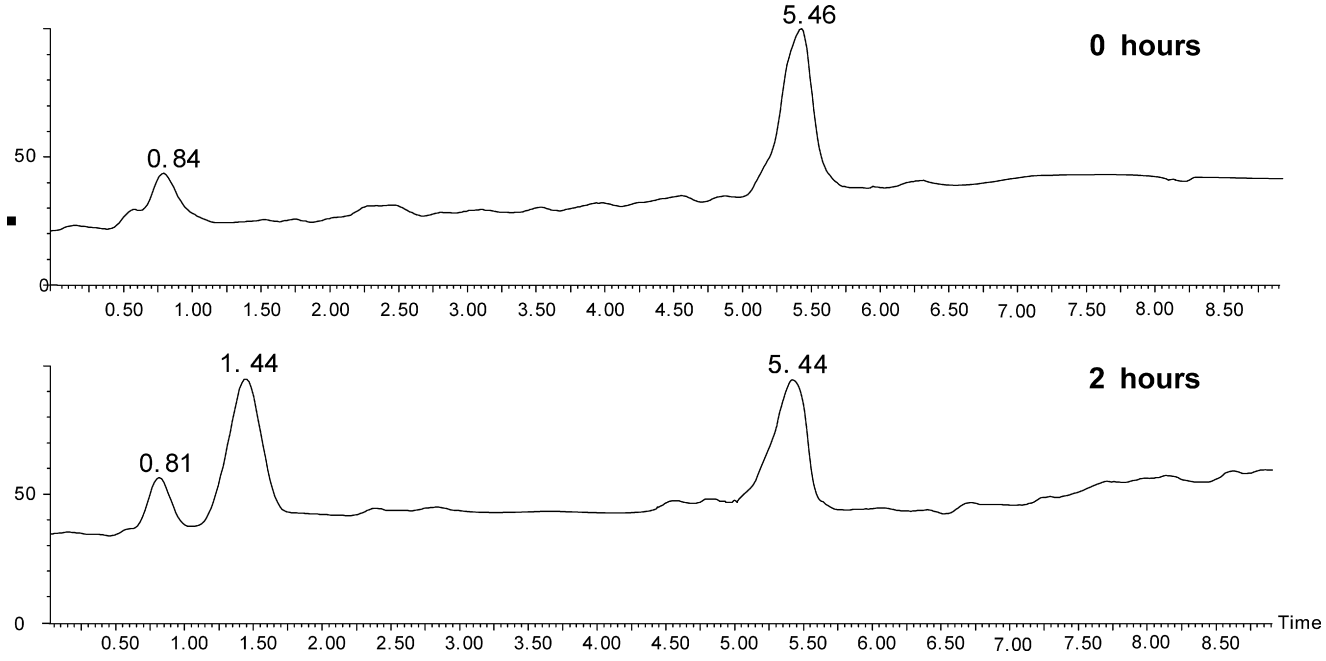


Fig. 6. UPLC/ESI–MS spectrum of metabolite control sample and metabolite for 2 h of compound A1.

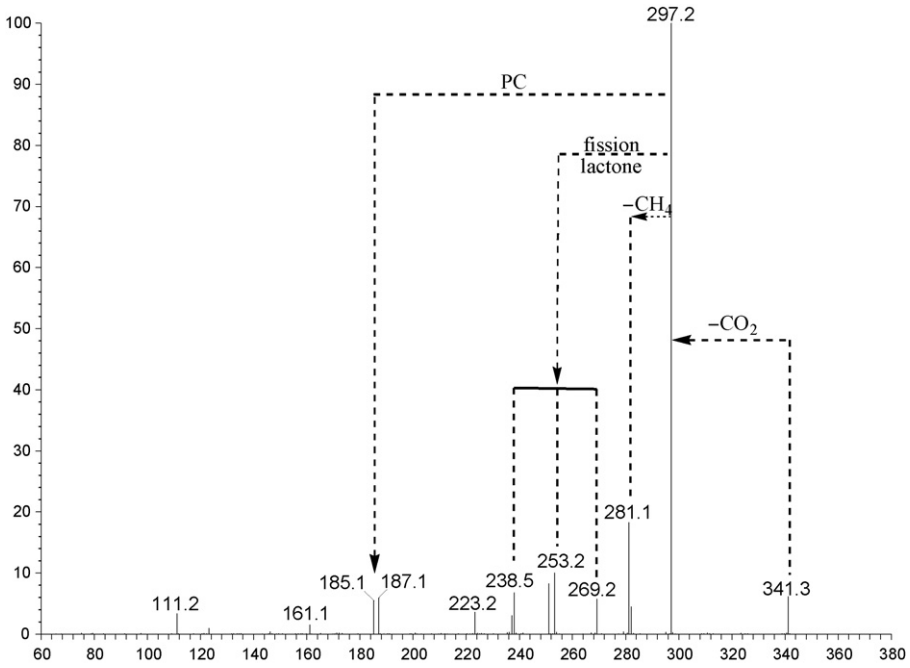
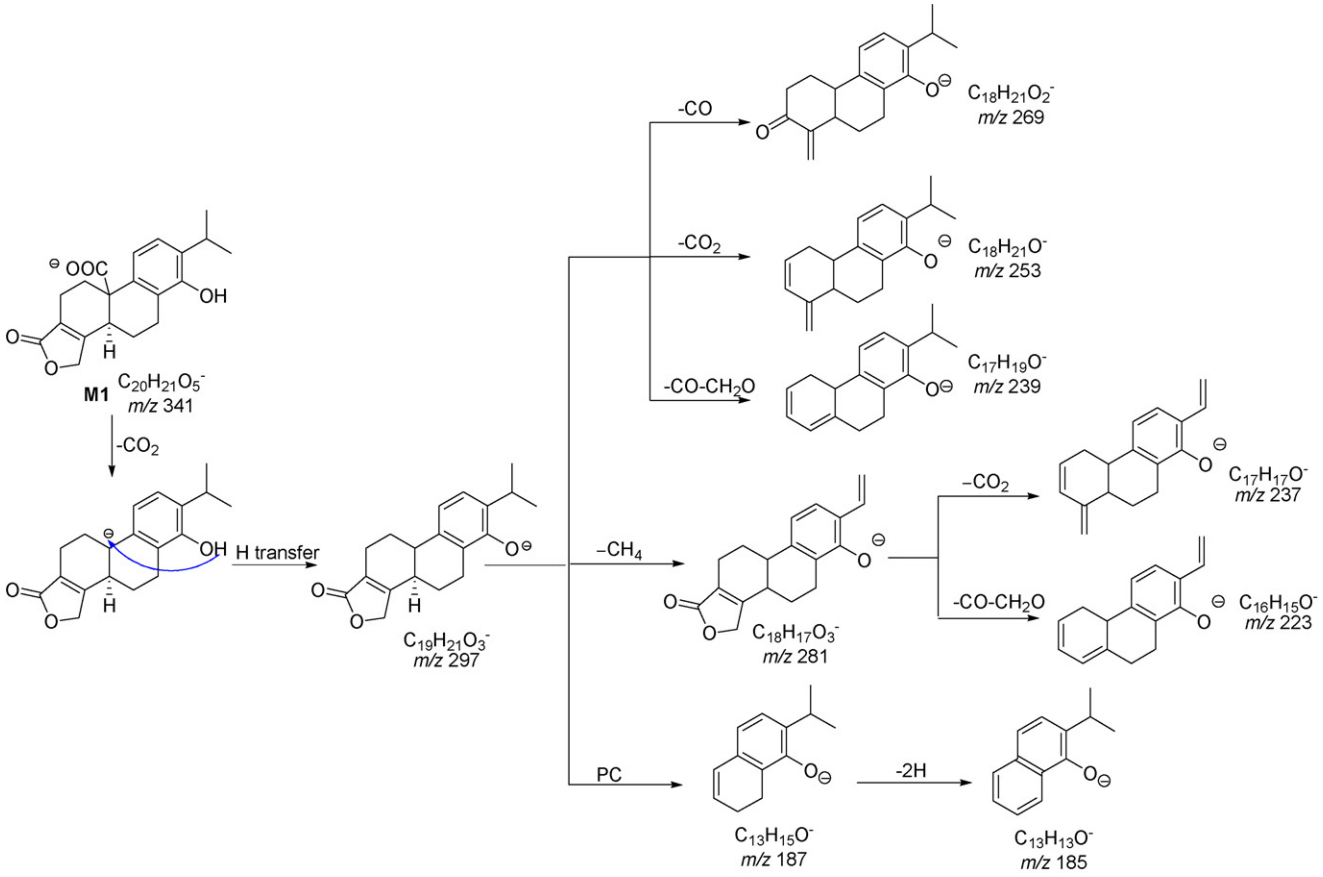


Fig. 7. Tandem mass spectrum of M1 (RT: 1.44 min) ion at *m*/*z* 341.

the ion at *m*/*z* 401.1759 and *m*/*z* 325.1445, while the O11 radical of ion at *m*/*z* 340.1662 would only induce the homolysis of the ether bond ***a*** to produce the ion at *m*/*z* 325.1445. Due to the lactone ring, CO or CO_2 would be eliminated from the precursor ion at *m*/*z* 325.1445 to form the ion at *m*/*z* 297.1479 and *m*/*z* 281.1558. Apart from that, CH_3 radical might also be eliminated from C10 of ion at *m*/*z* 325.1445 to produce the ion at *m*/*z* 310.1211. Because of the newly formed benzoquinone moiety, successive loss of two CO from precursor ion at *m*/*z* 325.1445 and *m*/*z* 297.1479 would occur (Scheme 8).



Scheme 9. The proposed fragmentation pathways of compound M1 in (−)ESI–MS/MS experiment.

3.4. Fragmentation mechanism conclusion

As for the triptophenolide related compounds, the fragmentation pathways mainly depend on what substituent groups the benzyl C ring bears (Fig. 5). Firstly, if there is an active hydrogen on O14, loss of CH_4 from isopropyl group, the PC reaction on A ring and fission of lactone ring will occur. Secondly, if R_1 is alkyl group, there are three variations: first, when R_2 is hydroxyl group, loss of R_1 alkyl radical and the CH_3 from isopropyl group will happen, followed by three fragmentation pathways including loss of CH_3 on C10 together with a molecule of C_2H_4, PC reaction on A ring and fission of lactone ring. Second, if R_2 is hydrogen or alkyl, loss of R_1 radical and the CH_3 will occur followed by dissociation of lactone ring. Finally, if R_2 is O–X (X = alkyl), loss of R_1 alkyl radical and X radical followed by fission of lactone ring were dominant; moreover, loss of two CO can be observed due to the newly formed benzoquinone moiety.

3.5. Identification of metabolites of compound A1 by ESI–MS/MS

Firstly, a series of tandem mass experiments of A1–C2 were done by using ESI–trpiple quadrupole mass spectrometer, which produced the similar fragment ions as those of ESI–TOF mass spectrometer. So we can use the above fragmentation laws to predict the metabolites in UPLC/ESI–trpiple quadrupole mass spectrometer. Through comparison of UPLC/ESI–MS spectra of the control sample (0 h) and the metabolite sample (2 h) from incubation with hepatocytes (Fig. 6), a new peak at 1.44 min was found. In the full scan mode, the parent ion M1 at *m*/*z* 341 was found. To further investigate its structure, tandem mass experiment was done to afford the daughter ions shown in Fig. 7. Firstly, the most abundant ion at *m*/*z* 297 corresponded to loss of CO_2 from the parent ion, indicating that a carboxyl group was introduced to **A1**. Because ion at *m*/*z* 281 ascribed to loss of CH_4 from the precursor ion at *m*/*z* 297 was observed, the benzyl C ring and isopropyl group might not be modified by P450. Furthermore, there are ions corresponding to loss of CO, CO_2 and $C_2H_2O_2$ indicating that the lactone ring was not affected. Finally, ions at *m*/*z* 187 and 185, which were decreased by 14 Da compared to *m*/*z* 201 and 199 formed by PC reaction of **A1**, should be ascribed to loss of CH_2 group on C10 position. So it was assumed that the CH_3 on C10 should be oxidized to carboxyl group by P450 followed by loss of CO_2 under tandem mass condition to produce the most abundant ion at *m*/*z* 297. The corresponding fragmentation pathways of M1 were depicted in Scheme 9.

4. Conclusion

Through negative ESI–QTOF–MS/MS analysis and DFT calculation, fragmentation mechanisms of triptophenolide related compounds were concluded. The fragmentation behavior mainly depends on what substituent groups the benzyl C ring bears. If there is a hydroxyl group on the position of C14, loss of CH_4 is dominating. However, the successive loss of two CH_3 radicals is predominant when the hydroxyl group of O14 is methylated. The lactone ring is prone to be dissociated to loss of CO, CO_2 and $C_2H_2O_2$ molecules. The PC reaction will occur on A ring if there is an active hydrogen resides on C ring. In addition, the metabolite of A1 was detected by UPLC/ESI–MS and its structure was primarily identified as C10 carboxylated of **A1** by tandem mass spectrometry. In the future work, metabolite experiments of other triptophenolide related compounds will be done.

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