Research Article

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Rapid identification of triterpenoid sulfates and hydroxy fatty acids including two new constituents from *Tydemania expeditionis* by liquid chromatography–mass spectrometry

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Tydemania expeditionis **Weber–van Bosse (Udoteaceae) is a weakly calcified green alga. In the present paper, liquid chromatography coupled with photodiode array detection and electrospray mass spectrometry was developed to identify the fingerprint components. A total of four triterpenoid sulfates and three hydroxy fatty acids in the ethyl acetate fraction of the crude extract were structurally characterized on the basis of retention time, online UV spectrum, and mass fragmentation pattern. Furthermore, a detailed liquid chromatography–mass spectrometry analysis revealed two new hydroxy fatty acids, which were then prepared and characterized by extensive nuclear magnetic resonance (NMR) analyses. The proposed method provides a scientific and technical platform for the rapid identification of triterpenoid sulfates and hydroxy fatty acids in similar marine algae and terrestrial plants. Copyright © 2011 John Wiley & Sons, Ltd.**

Keywords: *Tydemania expeditionis*; liquid chromatography–mass spectrometry; triterpenoid sulfate; hydroxy fatty acid; fingerprinting analysis

INTRODUCTION

Triterpenoid sulfates are featured by the covalent linkage from a triterpenoid moiety to one or more inorganic sulfate groups via C–O–S bonds. Compounds of this class have been found in both terrestrial plants (e.g. *Fagonia arabica*[1] and *Schefflera heptaphylla*[2]) and marine organisms (e.g. *Tricleocarpa fragilis*[3] and *Penicillus capitatus*[4]). Some of them have been found to show antiviral[2] and anticancer activities.[5]

Hydroxy fatty acids are characterized by the presence of one or more hydroxyl groups in the fatty acid chain. Although this kind of compounds were found to exist in terrestrial plants, marine alga, animals, and microorganisms, they are less common as compared with common fatty acids. Because of the hydroxylation, hydroxy fatty acids are significantly more polar than the corresponding fatty acid and possessed unique biological activities, for example, antineoplastic[6] and [7] activities, and some of them can be used as probes for diagnosing diseases.[8]

Tydemania expeditionis Weber–van Bosse (Udoteaceae) is a weakly calcified green alga. It is distributed in the tropical Pacific and Indian Oceans. Previous phytochemical studies on this species led to the isolation of norcyloartene triterpenoids,[9] linear diterpenoid,[10] and cycloartanol sulfates with inhibitory activity against the $pp60^{v\text{-}rsc}$ protein tyrosine kinase.[11] Our recent study on this alga resulted in the isolation and identification of four triterpenoid sulfates with antifungal activity[12] and three hydroxy fatty acids with inhibitory activities against a range of cancer cells.[13] Furthermore, we found that both the triterpenoid sulfates and the hydroxy fatty acids showed characteristic fragmentation patterns and can be used to search for similar components from marine or terrestrial sources.

Liquid chromatography–mass spectrometry (LC–MS) is a hyphenated technique that combines both liquid chromatographic separation and structure identification on the basis of mass spectrometry. Although this technique has been widely used for the fingerprinting analyses of carotenoids,[14] isoflavonoids,[15] and toxins[16,17] in marine algae, the analyses of triterpenoid sulfates and hydroxy fatty acids were not reported. Herein, we report the fragmentation patterns of both kinds of compounds and the rapid identification of these compounds from *T. expeditionis* by LC–MS analysis.

EXPERIMENTAL

Chemicals and reagents

Liquid chromatography–grade acetonitrile (Tedia, Fairfield, CA) was used for the high-performance liquid chromatography (HPLC) analysis. Water prepared with a Millipore Milli-Q SP

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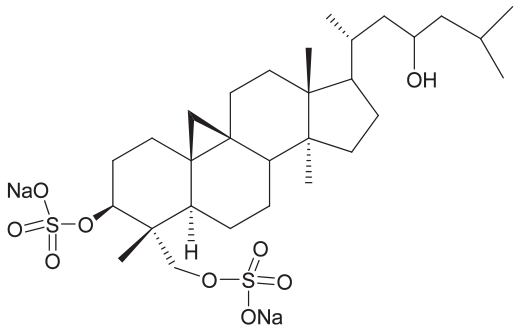
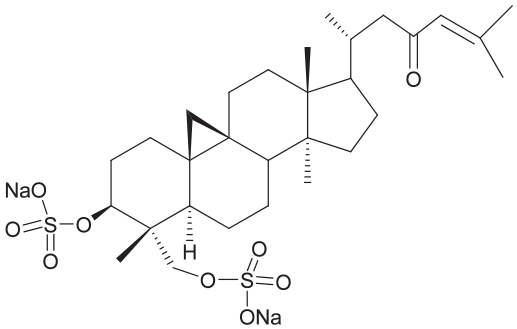
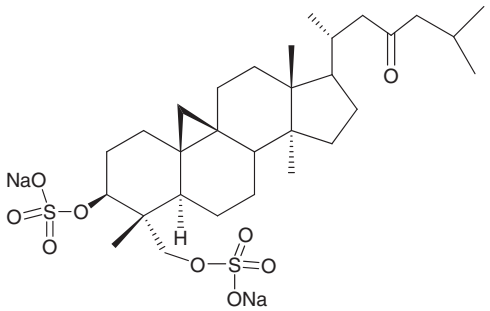


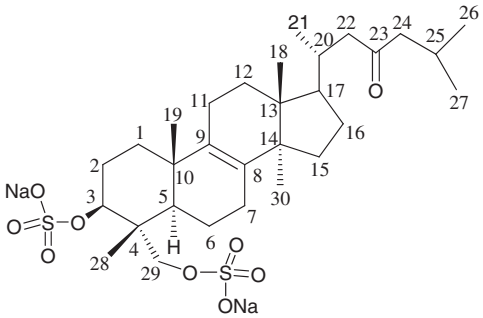
cycloartan-3,23,29-triol 3,29-disodium sulfate (**1**)



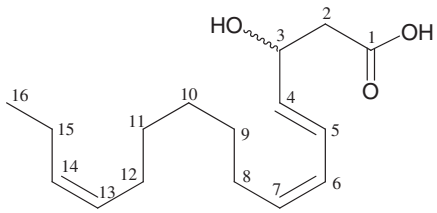
cycloart-24-en-3,29-diol-23-one 3,29-disodium sulfate (**2**)



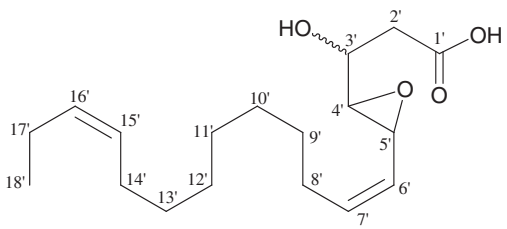
cycloartan-3,29-diol-23-one 3,29-disodium sulfate (**3**)



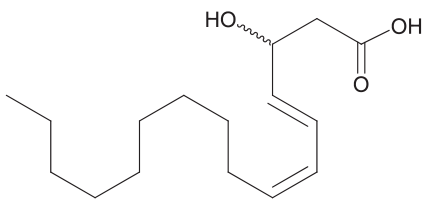
lanost-8-en-3,29-diol-23-one 3,29-disodium sulfate (**4**)



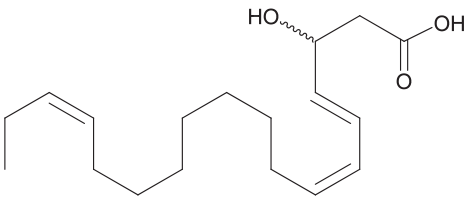
3(ζ)-hydroxy-hexadeca-4(*E*),6(*Z*),13(*Z*)-trienoic acid (**5**)



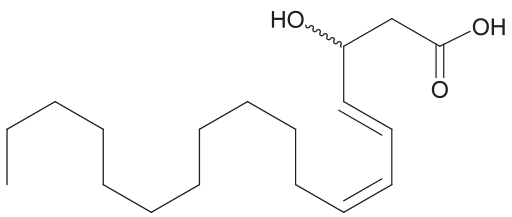
3(ζ)-hydroxy-octadeca-4,5-cis-epoxy-6(*Z*),15(*Z*)-dienoic acid (**6**)



3(ζ)-hydroxy-hexadeca-4(*E*),6(*Z*)-dienoic acid (**7**)



3(ζ)-hydroxy-octadeca-4(*E*),6(*Z*),15(*Z*)-trienoic acid (**8**)



3(ζ)-hydroxy-octadeca-4(*E*),6(*Z*)-dienoic acid (**9**)

Figure 1. Structural formulae of compounds **1–9**.

purification system (Millipore, France) was used during sample preparation procedures and HPLC analyses. Cycloartan-3,23,29-triol 3,29-disodium sulfate (compound **1**) and 3(ζ)-hydroxy-octadeca-4(*E*),6(*Z*),15(*Z*)-trienoic acid (compound **8**) were chosen as standards (Fig. 1), which were isolated from *T. expeditionis* in our laboratory and were identified on the basis of spectral (NMR and MS) analysis in combination with X-ray crystallographic analysis.[12,13]

Collection of samples

T. expeditionis was collected in the Kadavu Province of Fiji (GPS location: 18° 46.370′ S and 178°27.746′ E). Specimens (G-2004-06-45) were identified by Dr Posa Skelton at the University of South Pacific and deposited at the University of the South Pacific.

Sample preparation

The pulverized alga (100 g) was extracted under supersonic conditions with methanol (500 mL) for an hour. The extracting process was repeated three times. The extraction solutions were combined, filtered, and evaporated under vacuum to afford the crude extract, which is suspended in distilled water and extracted by hexane, ethyl acetate, and butanol successively. The ethyl acetate fraction was dissolved in methanol to make a solution of 1 mg/mL. The solution was filtered through a 0.22-μm PTFE syringe filter, and an aliquot of filtrate (10 μL) was injected into the LC–MS instrument for analysis.

Equipment and chromatographic conditions

High-performance liquid chromatography analyses and online UV spectra were acquired on a Waters 2690 system consisting of a vacuum degasser, a quaternary pump, an autosampler, and a Diode-array detector (DAD). Separation was achieved with an Alltech Alltima C18 (Alltech Associate Inc., Deerfield, Illinois, USA) reversed-phase column (3 μm, 2.1 × 150 mm). The mobile phase used was water (A) and acetonitrile (B). Binary gradient was programmed as follows: 0–53 min, linear 10 → 70% B; 53–60 min, linear 70 → 100% B; and 60–63 min, linear 100 → 10% B. UV detections were at 215 and 235 nm. The effluent from HPLC directly entered the electrospray ionization (ESI) source.

The ESI–MS spectra were acquired in negative ion modes on a Micromass ZQ 2000 instrument. The mass spectrometry detector parameters were set as follows: ion source temperature, 100 °C; desolvation temperature, 400 °C; spray voltage, 3.5 kV; cone voltage, 40.0 V; full scan (*m/z* 50–700) with 500 ms collection time; desolvation gas, 300 L/h; cone gas, 50 L/h; and optimized relative collision energies, 50%.

Peaks **1** and **8** were identified by direct comparison with the standard compounds. For peaks **2–7** and **9** with no available standards for reference, the identities were assigned by comparing the retention time and the molecular weight of chemicals in the literature and by interpretation of the mass spectra. For the new compounds **5** and **6**, extensive NMR spectral analyses were further used to support the structural elucidation.

Semipreparative HPLC

Semipreparative HPLC was performed on a Waters 2695 system equipped with 2996 diode-array UV detection. The mixture of compounds 5 and 6 (retention time 42.2–43.0 min) was purified with an Agilent Zorbax SB-C_{18} (Agilent Technologies Inc., Wilmington, Delaware, USA) reversed-phase column (5 μm, 9.4 × 250 mm) using the same gradient of aqueous acetonitrile as that used in the LC–MS analysis with detection at 215 nm.

NMR spectroscopy

NMR spectra were acquired on a Bruker DRX-500 spectrometer equipped with a 5-mm broadband probe. All NMR spectra were recorded at ambient temperature in CD_3OD and were referenced to the residual light solvent (δ_H = 3.31 and δ_C = 49.0 for methanol). Chemical shifts were given on the δ scale. For ^1H NMR, 64 transients were acquired with 1.0 s of relaxation delay and 10,330.6 Hz of spectral width. For ^{13}C NMR, 17,554 transients were acquired with 2.0 s of relaxation delay and 30,030.0 Hz of spectral width. Both the ^1H–^1H magnitude-mode ge-2D COSY spectrum and the phase-sensitive ge-2D HSQC spectrum were acquired over the 1K data points in F2 and the 256 data points in F1, using standard Bruker library pulse sequence (cosygpqf and hsqcetgpsi2, respectively). The magnitude-mode ge-2D Heteronuclear Multiple Bond Correlation (HMBC) spectrum was recorded over the 2K data points in F2 and the 256 data points in F1, also using the standard Bruker pulse program (hmbcgplpndqf).

RESULTS AND DISCUSSION

Fragmentation behavior of pure triterpenoid sulfate and hydroxy fatty acid

The authentic samples of cycloartan-3,23,29-triol 3,29-disodium sulfate (compound **1**) [13] and 3(ζ)-hydroxy-octadeca-4(*E*),6(*Z*),15

Table 1. Main fragment ions observed by the negative ESI–MS analysis of compounds 1–9 from *Tydemania expeditionis*

Peak	t_R min	Molecular weight	UV λ_{max} (nm)	Fragment ions (*m/z*)	Identification
1	19.1	664	—	309, 641, 619, 539, 521	Cycloartan-3,23,29-triol 3,29-disodium sulfate
2	19.8	660	235	307, 637, 615, 535, 517	Cycloart-24-en-3,29-diol-23-one 3,29-disodium sulfate
3	21.3	662	—	308, 639, 617, 537, 519	Cycloartan-3,29-diol-23-one 3,29-disodium sulfate
4	22.1	662	215	308, 639, 617, 537, 519	Lanosta-8-en-3,29-diol-23-one 3,29-disodium sulfate
5	42.7	266	234	265, 247	3(ζ)-Hydroxy-hexadeca-4(*E*),6(*Z*),13(*Z*)-trienoic acid
6	42.9	310	—	309. 291	3(ζ)-Hydroxy-octadeca-4,5-cis-epoxy-6(*Z*),15(*Z*)-dienoic acid
7	45.9	268	233	267, 249	3(ζ)-Hydroxy-hexadeca-4(*E*),6(*Z*)-dienoic acid
8	49.9	294	235	293, 275	3(ζ)-Hydroxy-octadeca-4(*E*),6(*Z*),15(*Z*)-trienoic acid
9	53.4	296	234	295, 277	3(ζ)-Hydroxy-octadeca-4(*E*),6(*Z*)-dienoic acid

"—" indicates no significant absorption between 200 and 400 nm.

(*Z*)-trienoic acid (compound **8**) [12] were studied by ESI–MS in the negative mode.

Compound **1**, as a typical example of the cycloartane-type triterpenoid sulfate, gave the base peak $[M–46]^{2-}$ at *m/z* 309, a characteristic double charge ion for the disulfates, by the loss of two sodium ions. Compound **1** formed a precursor ion at *m/z* 641 $[M–Na]^-$ by the loss of a sodium ion, which further fragmented into a secondary ion, at *m/z* 619, by the loss of the second sodium ion followed by the capture of a proton. Then fragment ions at *m/z* 539, 521 (Table 1) were successively generated by the loss of a neutral sulfur trioxide and a water molecules from the secondary ion. It was interesting to note that the protonation of sulfate compound **1**, after the loss of sodium ion, might result from the surface enrichment of electrolytically produced protons in the ionization droplets, which was similar to the protonation of caffeine in neutral solution.[18] The mass spectrum of compound **1** is shown in Fig. 2a, and the fragmentation mechanism is proposed in Fig. 2b.

The mass spectrum of 3(ζ)-hydroxy-octadeca-4(*E*),6(*Z*),15(*Z*)-trienoic acid (compound **8**) showed $[M–H]^-$ at *m/z* 293 as the base peak, a common deprotonation ion for the carboxylic acid, and $[M–H–H_2O]^-$ by further loss of a water molecule at *m/z* 275, indicative of the presence of a hydroxy group. The mass spectrum of compound **8** is shown in Fig. 3a, and the fragmentation mechanism is proposed in Fig. 3b.

Identification of triterpenoid sulfates and hydroxy fatty acids in fingerprint chromatograms

Online LC–MS was used to determine the constituents in the ethyl acetate fraction of the crude extracts of *T. expeditionis*. The fragmentation rules of the triterpenoid sulfate **1** and hydroxy fatty acid **8** could be used to characterize similar structures. The LC profiles of *T. expeditionis* included nine major peaks **1–9** and are shown in Fig. 4. Peaks **1** and **8** were identified as cycloartan-3,23,29-triol 3,29-disodium sulfate and 3(ζ)-hydroxy-octadeca-4(*E*),6(*Z*),15(*Z*)-trienoic acid, respectively, by direct comparison with the standard compounds (Table 1).

Peaks **2–4** showed similar fragmentation patterns as peak **1**. All of them gave the characteristic double charge ion by the loss of two sodium ions as the base peak and formed the precursor ion by the loss of a sodium ion, which further fragmented into a secondary ion by the loss of the second sodium ion followed by the capture of a proton. Fragment ions due to the sequential loss of a neutral sulfur trioxide and water molecule from the secondary ions were also observed (Table 1). With regard to compound **2**, these characteristic fragments were at *m/z* 307, 637, 615, 535, and 517, respectively, indicating that the molecular weight of compound **2** was four units smaller than that of compound **1**. Likewise, the mass spectra of compounds **3** and **4** were characterized by the ions at *m/z* 308, 639, 617, 537, and 519, indicating that they possessed the same molecular weight, which was two units smaller than compound **1** (Table 1). In addition, peak **2** showed strong UV absorbance at both 215 and 235 nm, peak **4** showed strong UV absorbance at only 215 nm, and peak **3** showed only very weak UV absorbance at both wavelengths. Accordingly, peaks **2–4** could be elucidated as cycloart-24-en-3,29-diol-23-one 3,29-disodium sulfate (peak **2**), cycloartan-3,29-diol-23-one 3,29-disodium sulfate (peak **3**), and lanosta-8-en-3,29-diol-23-oxo-3,29-disodium sulfate (peak **4**) by referring the retention time, the molecular

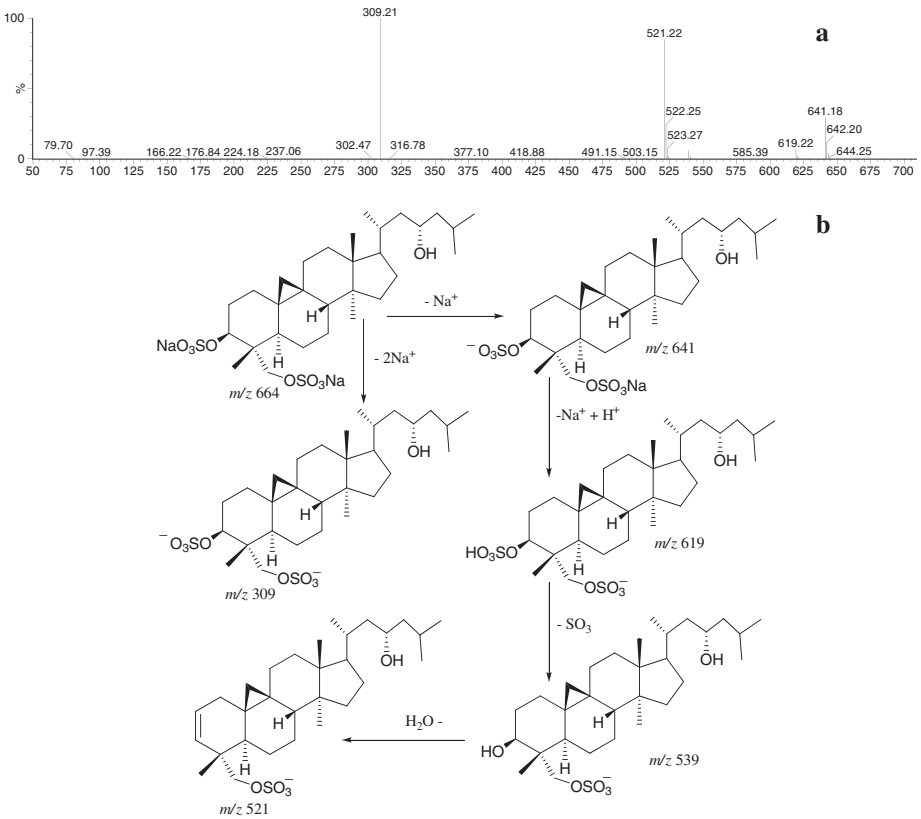


Figure 2. (a) The ESI-negative ion mass spectrum of compound **1** and (b) the proposed fragmentation pathway.

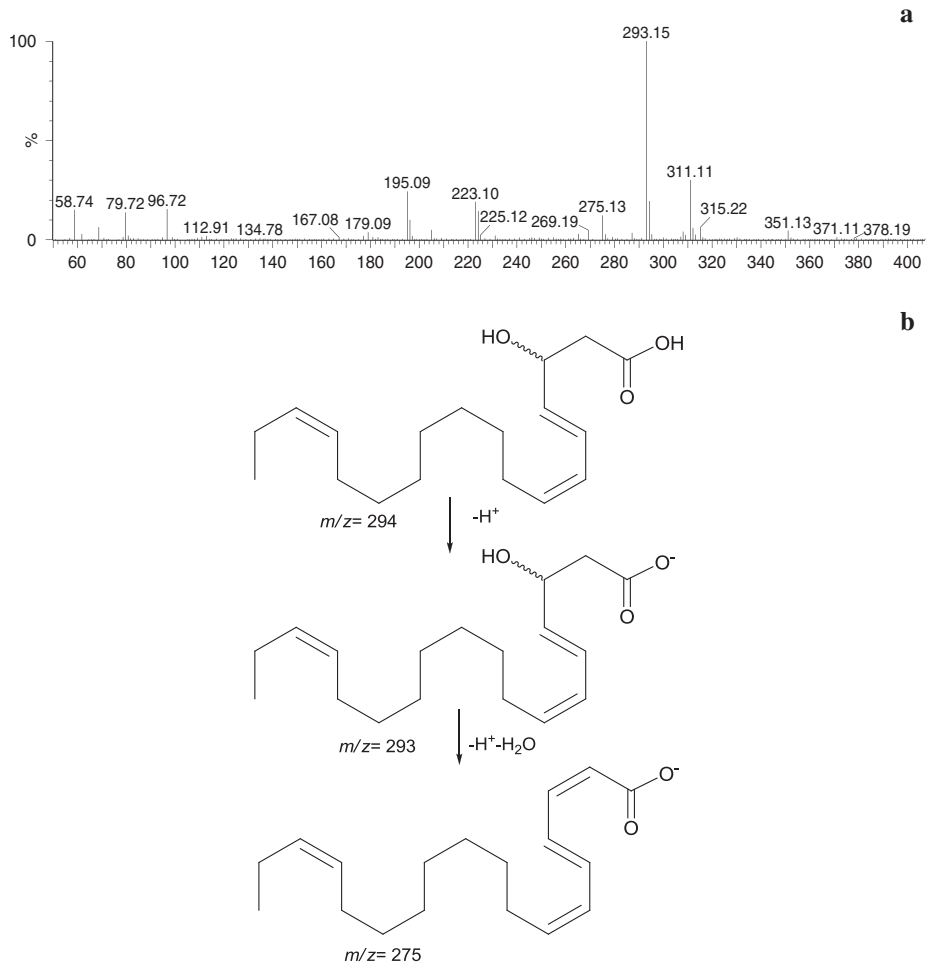


Figure 3. (a) The ESI-negative ion mass spectrum of compound **8** and (b) the proposed fragmentation pathway

weight, the fragmentation pattern, and the UV spectra to the literature.[13]

Peaks **7** and **9** showed similar fragmentation patterns as that of peak **8**. Both of them formed precursor ion by the loss of a proton and further fragment ions by the loss of a water molecule. The characteristic ions for peak **7** were $[M–H]^-$ at *m/z* 267 and $[M–H–H_2O]^-$ at *m/z* 249, indicating a molecular weight of 268. The corresponding ions for peak **9** were $[M–H]^-$ at *m/z* 295 and $[M–H–H_2O]^-$ at *m/z* 277, indicating a molecular weight of 296. Both peaks **7** and **9** showed strong UV absorbance at both 215 and 235 nm, indicating the presence of a chromophore in the molecules. Referring to the literature,[12] peaks **7** and **9** could be identified as 3(ζ)-hydroxy-hexadeca-4(*E*),6(*Z*)-dienoic acid and 3(ζ)-hydroxy-octadeca-4(*E*),6(*Z*)-dienoic acid, respectively.

The retention times of peaks **5** and **6** were very close to each other. Peak **5** showed strong UV absorbance at both 215 and 235 nm, indicating the presence of a chromophore in the molecule. In contrast, peak **6** only had very weak absorption at both wavelengths. The negative ESI spectra of peaks **5** and **6** showed similar $[M–H]^-$ and $[M–H–H_2O]^-$ peaks (265 and 247 for peak **5**; 309 and 291 for peak 6) as those of peaks 7–9, suggesting that they were hydroxy fatty acids. The molecular weight of peak **5** is 266, which is two units less than that of peak **7**, suggesting the presence of an additional double bond as compared with peak **7**. The molecular weight of peak **6** is 310, which is 16 units larger than that of peak **8**, suggesting that one of the double bond in peak 8 might be oxidized into an epoxide ring. Thus, a detailed LC–MS analysis revealed two new hydroxyl fatty acids.

To further confirm the new structures of peaks **5** and **6**, we used semipreparative HPLC to collect the peaks. Because of the close retention time, they were analyzed as a mixture. The quantity ratio was inferred to be 1:1, as shown by the similar intensity of the hydroxylated proton signals (δ = 4.10 and 4.16) in the ^{1}H NMR spectrum (Fig. 5).

Both the ^{1}H and the ^{13}C NMR spectra of the mixture indicated 10 olefinic methines (δ_H = 5.3–6.5, δ_C = 123.7–136.7), two hydroxy methines ($\delta_{H\text{-}3}$ = 4.10, *dd*, *J*=12.5, 6.0 Hz, $\delta_{C\text{-}3}$ = 73.3 and $\delta_{H\text{-}3'}$ = 4.16, *ddd*, *J*=2.5, 6.0, 8.5 Hz, $\delta_{C\text{-}3'}$ = 69.3), two epoxide methines ($\delta_{H\text{-}4'}$ = 2.76, *dd*, *J*=2.0, 6.0 Hz, $\delta_{C\text{-}4'}$ = 62.4 and $\delta_{H\text{-}5'}$ = 2.87, *dt*, *J*=2.0, 6.0 Hz, $\delta_{C\text{-}5'}$ = 57.0), one methylene envelope (δ_H = 1.33, δ_C = 28.6–30.7), and two terminal methyls group (δ_H = 0.95, *t*, *J*=8.0 Hz, δ_C = 14.5 and 14.6). Thus, the ^{1}H and the ^{13}C NMR spectra of the mixture were very similar to those of peak **8**, which was identified previously from this alga.[12]

Bearing the mass spectrometry of peaks **5** and **6** in hand, six olefinic methines and one hydroxy methine could be assigned to peak **5**. Because of the similar NMR and UV spectra and mass fragmentation behavior to peak **8**, peak **5** was inferred to have

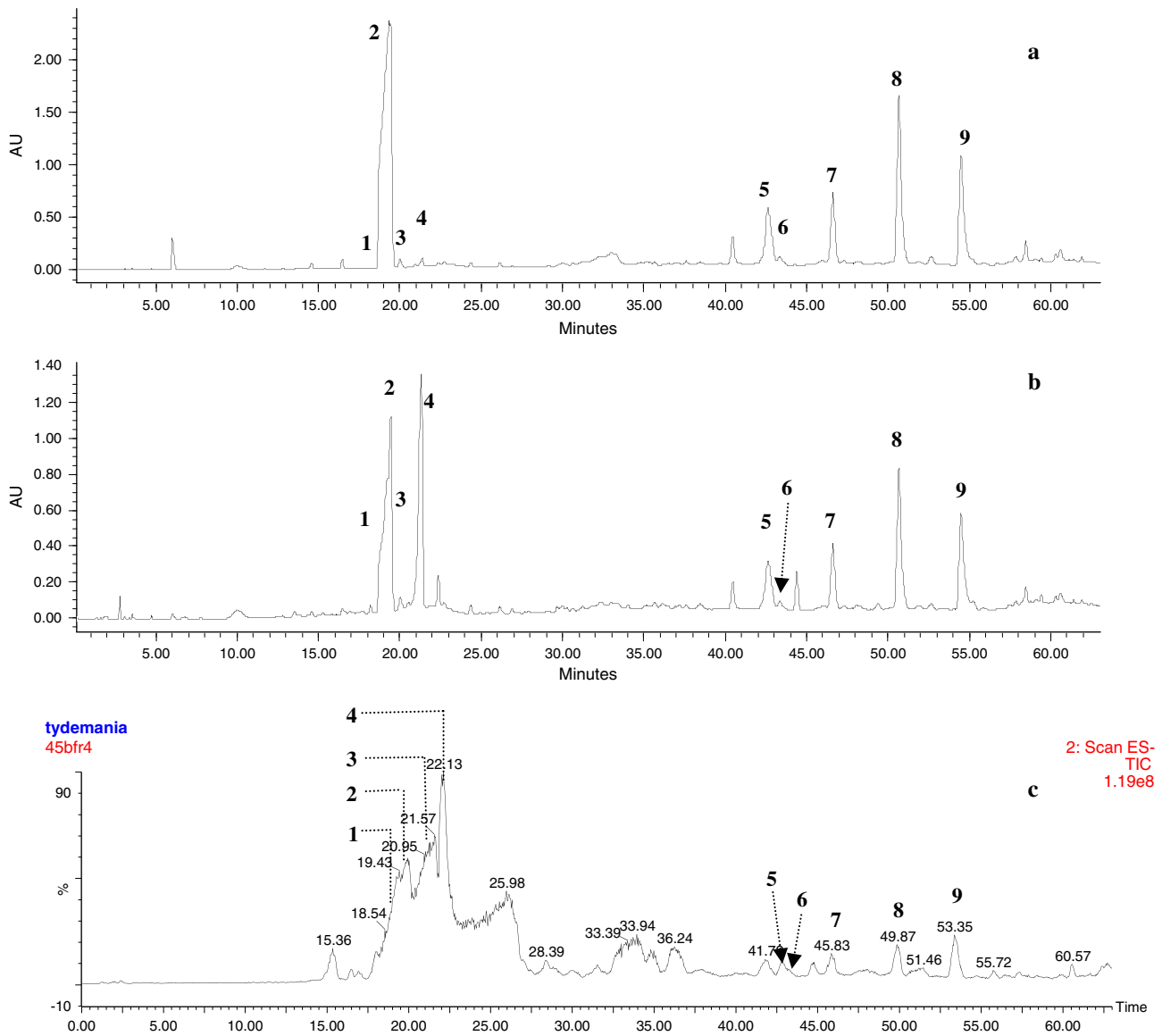


Figure 4. HPLC chromatograms at (a) 235 nm and (b) 215 nm and (c) the LC–MS trace of the ethyl acetate fraction of *T. expeditionis*.

the same double bond pattern in the molecule. Accordingly, peak **5** was tentatively assigned as 3(ζ)-hydroxy-hexadeca-4(*E*),6(*Z*),13(*Z*)-trienoic acid.

By using the ^{1}H–^{1}H COSY spectrum (Fig. 6), the hydroxylated methine signal δ 4.16 (H-3′) and the epoxide signals δ 2.76 (H-4′) and δ 2.87 (H-5′) could be assigned to peak **6**. H-4′ showed cross peaks with H-3′, which suggested that the epoxide ring was adjacent to the hydroxy group at C-3′. In the HMBC spectrum (Fig. 7), the epoxide proton H-4′ showed correlations to C-3′ (δ 69.3), which further confirmed that the epoxide ring was adjacent to the hydroxy group at C-3′. Accordingly, the structure of peak **6** was established as 3(ζ)-hydroxy-octadeca-4,5-*cis*-epoxy-6(*Z*),15(*Z*)-dienoic acid.

CONCLUSIONS

For the first time, the fragmentation behavior of triterpenoid sulfate and hydroxy fatty acid was investigated using negative ion ESI–MS analyses on the standard compounds, and the corresponding fragmentation rules were used to identify other triterpenoid sulfates and hydroxy fatty acids in the fingerprint of *T. expeditionis*. The fragmentation patterns of triterpenoid sulfates and hydroxy fatty acids were characteristic and reproducible and could be used for the rapid characterization of these kinds of compounds in other similar algae or terrestrial plants. Furthermore, a detailed LC–MS analysis revealed two new hydroxy fatty acids. These two compounds were characterized by extensive NMR analyses. The results of the current investigation showed that LC–MS could also be used for the rapid discovery of new constituents in the marine algae for phytochemical study.

Acknowledgements

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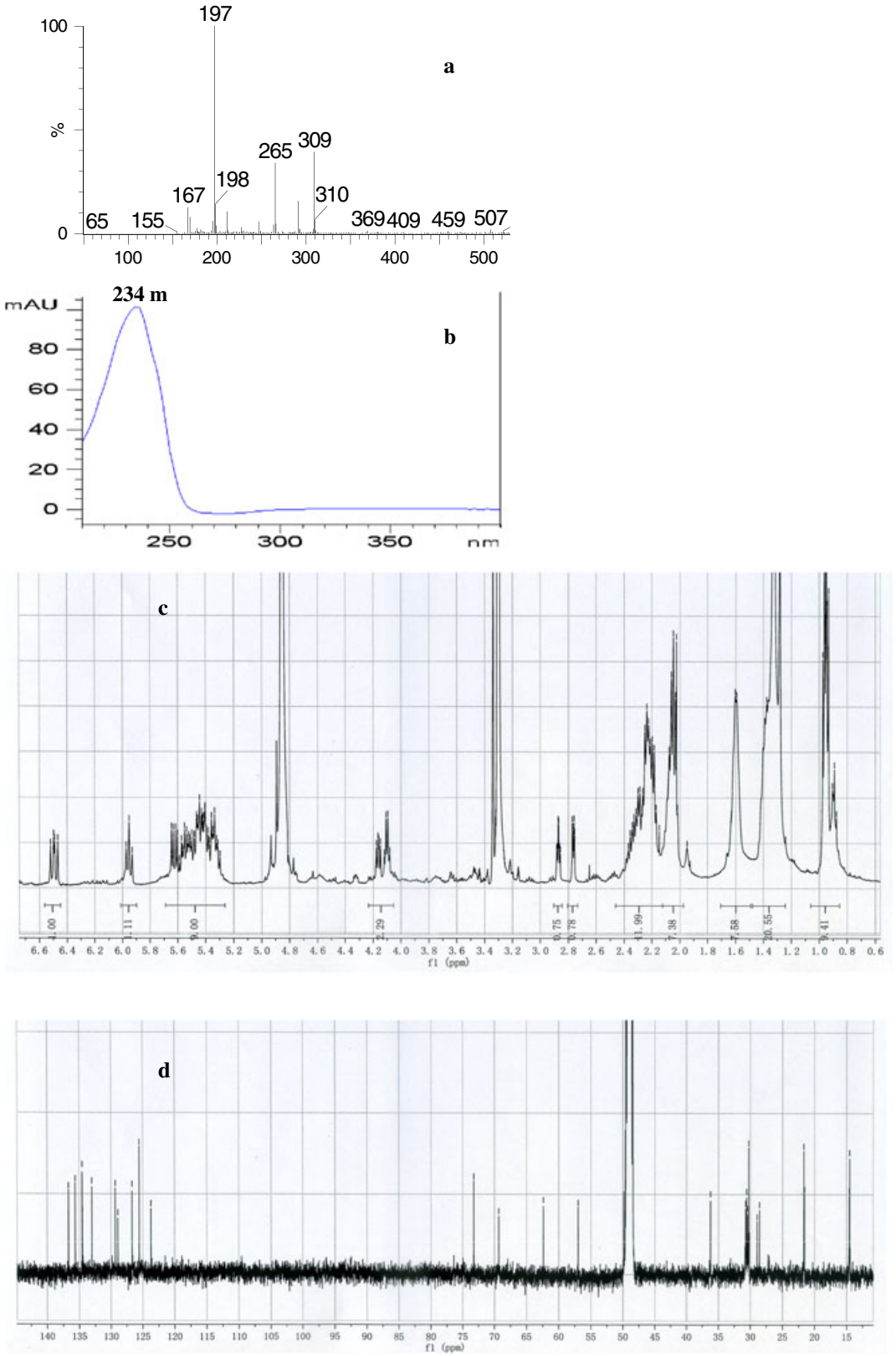

Figure 5. (a) The negative ESI–MS, (b) the UV spectrum, (c) the ^{1}H NMR, and (d) the ^{13}C NMR of mixtures **5** and **6**.

R. W. J.), the Fundamental Research Funds for the Central Universities (grant no. 21609202), Guangdong High Level Talent Scheme and the U.S. National Institutes of Health's International Cooperative Biodiversity Groups program (grant no. U01-TW007401).

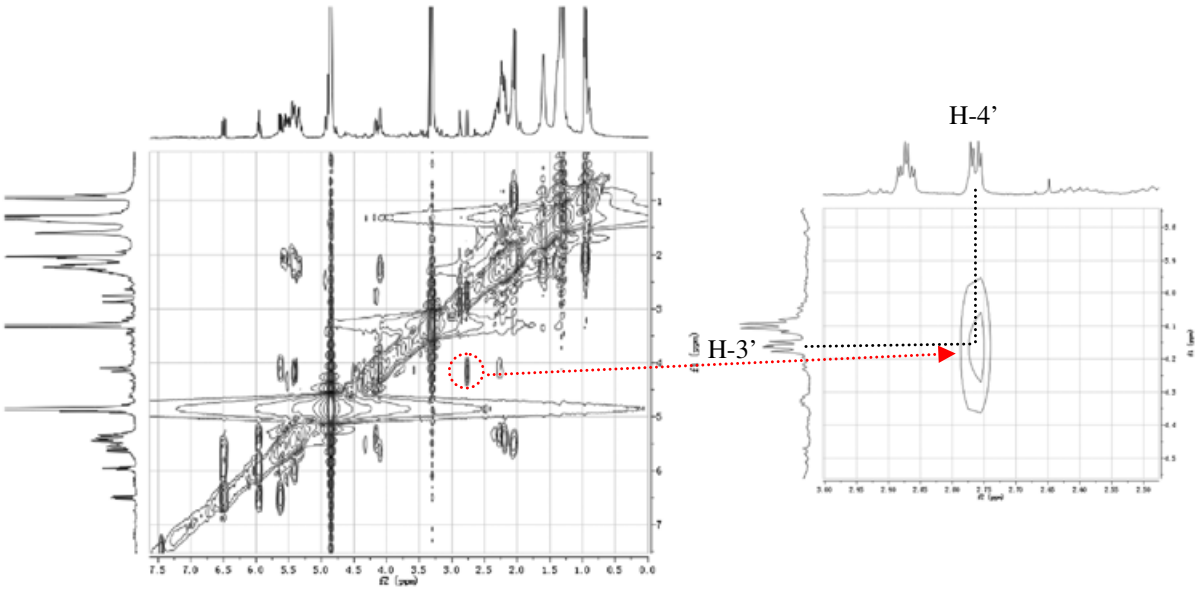

Figure 6. The ^{1}H–^{1}H COSY spectrum of mixtures **5** and **6** showing the correlation between H-4′ and H-3′.

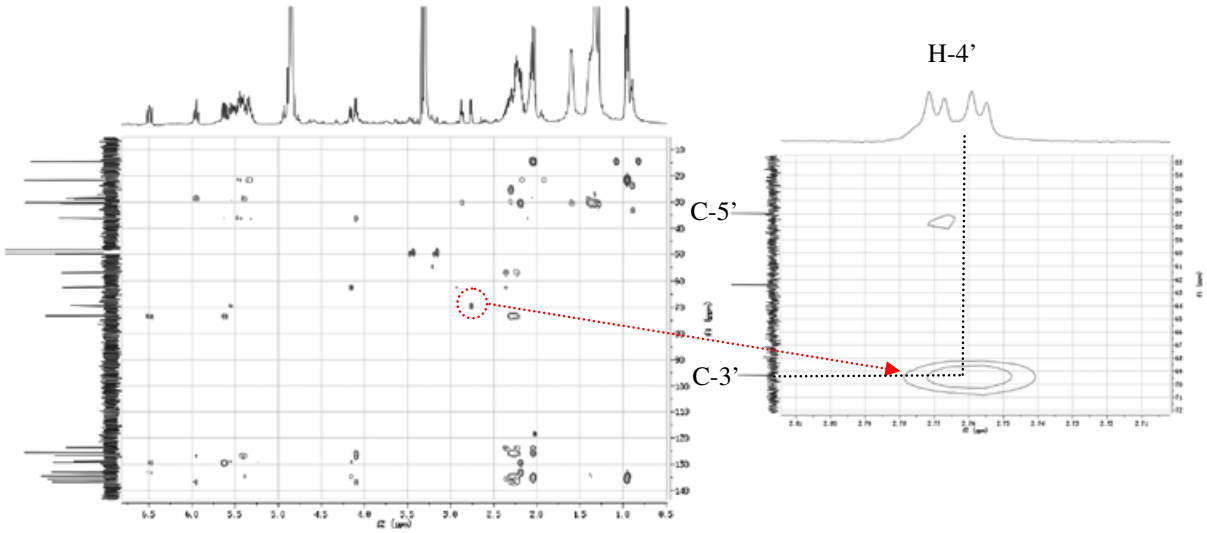

Figure 7. The HMBC spectrum of mixtures **5** and **6** showing the correlation between H-4′ and C-3′.

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