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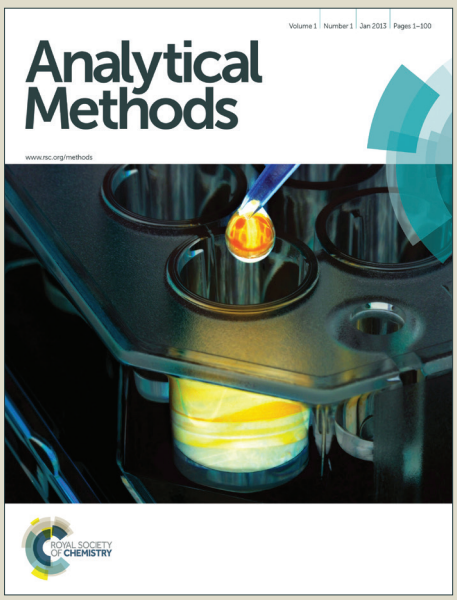

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Rapid Identification of Lichen Compounds based on Structure-Fragmentation Relationship using ESI-MS/MS Analysis

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Abstract

Lichens are symbiotic composites of algae and fungi which produce characteristic secondary metabolites of interest. This study, based on negative mode electrospray quadrupole time-of-flight mass spectrometry (ESI-Qq-TOF-MS/MS) reveals structure-fragmentation relationship of ten metabolites belonging to various chemical classes such as monocyclic phenols, depsides, depsidones and dibenzofurans. Low energy collision induced dissociation tandem mass spectrometric analysis of these deprotonated molecules yielded key fragments due to the loss of neutral components like CO, CO_2, methanol, ethanol, and ethene. Interestingly, odd electron fragments were also observed in sekikaic acid, lobaric acid, and usnic acid as a characteristic fragments. Fragmentation pattern of standard compounds, high resolution analysis and database were used for the rapid identification of compounds in lichen species, *Parmotrema grayana* and *Heterdermia obscurata.* Furthermore MS/MS spectra revealed different fragmentation pathways for different classes of secondary metabolites. Total fifteen compounds were identified from the methanolic and dichloromethane extracts of *Parmotrema grayana*, and *Heterdermia obscurata.*

Key words: Lichen, Secondary metabolites, Electrospray ionization quadrupole time-of-flight mass spectrometry, *Parmotrema grayana*, *Heterdermia obscurata*

1. Introduction

Lichens have great diversity in terms of their physiology and taxonomy and represent a unique division in the plant kingdom. Their use for medicinal purposes dates back to the 18th century and they currently find their place in traditional Chinese, Indian, homeopathic and western herbal medicines. Secondary metabolites in lichens, which are different from their higher plant counterparts, such as polyketides of the type monocyclic aromatic phenol, depsides, depsidones and dibenzofurans contribute to their great medicinal value [1-3]. Studies for the exploration of their potential as medicines has skyrocketed in the past decade and several bioactivities such as antioxidant, antiviral, antibiotic, antitumor, allergenic, antibacterial, antifungal, anti-inflammatory, anticancer and enzyme inhibitory activities have been reported so far [4-8].

There have been many reports for the analysis of these compounds in lichens using various techniques such as thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) [9-11], fast atom bombardment mass spectrometry [12] and liquid chromatography-mass spectrometry (LC-MS) [13-15]. Electrospray tandem mass spectrometry (ESI-MS/MS) is a robust technique for identification and characterization of secondary metabolites in lichens crude extracts. However, characterization and structure elucidation employing ESI-MS/MS requires knowledge and understanding of CID-fragmentation patterns of specific compounds and this account for the need of structure-fragmentation studies.

In continuation of our previous studies on fragmentation patterns of natural products [16-19], this current study presents ESI-QqTOF-MS (negative ion mode) and CID-MS/MS analyses of specific secondary metabolites common to various lichen species. Moreover, this study reveals

characteristic fragmentation patterns for identification of metabolites in the extracts of two lichen species, *Parmotrema grayana*, and *Heterodermia obscurata*.

2.0 Experimental

2.1. Chemicals and reagents

Methanol and chloroform were of HPLC grade, and were purchased from Aldrich-Sigma (USA). Deionized water (Milli-Q) was used throughout the study. Secondary metabolites **1-10** were previously isolated from various species of lichens, including *Parmotrema grayana, Roccella montagnei, Parmotrema cooperi, Heterdermia obscurata* and *Cladonia* species. These metabolites were fully characterized by using spectroscopic techniques, including ^{1}H NMR, ^{13}C NMR and mass spectrometry [5]. Detailed isolation procedure and spectroscopic data of compounds **1-10** are provided in supplementary documents.

2.2. Extraction procedure and sample preparation of *Parmotrema grayana* and *Heterodermia obscurata*

The lichen specimen *Parmotrema grayana* (35 g) were scraped off from the stem bark and *Heterdormia obscurata* (69 g) from rock surface, which were cleaned manually to remove other lichens and air-dried at room temperature for a few days. They were crushed into a powder before extraction. Powdered lichens were sequentially extracted into distilled CH_2Cl_2 and then into MeOH in a bottle shaker (1 L x 3 times). The solvent of the extracts obtained after filteration

was removed under reduced pressure (< 40 °C) using a rotavapor. A CH_2Cl_2 extract (1.0 g from *P. grayana* and 1.8 g from *H. obscurata*) and a brown powdery MeOH extract (10 g from *P. grayana* and 12 g from *H. obscurata*) were obtained.

1 mg of dichloromethane and methanolic extracts were dissolved in 1 mL of chloroform and methanol, respectively. Working dilutions of dichloromethane extract was prepared in 50:50 methanol-chloroform, while methanolic extract was further diluted with methanol. Analysis was performed by electrospray ionization (ESI) and collision-induced dissociation (CID), negative ion mode, on Qq-TOF-MS/MS instrument (QSTAR XL mass spectrometers Applied Biosystem/MDS Sciex, Darmstadt, Germany).

2.3. Electrospray ionization-mass spectrometry

All secondary metabolites, except compounds **8-10** were dissolved in chloroform and methanol mixture (1:1), while compounds **8-10** were dissolved in methanol (1 μg/mL each) and analyzed by electrospray ionization (ESI) and collision-induced dissociation (CID) negative ion mode on QqTOF-MS/MS instrument (QSTAR XL mass spectrometer Applied Biosystem/ MDS Sciex, Darmstadt, Germany) at room temperature. High purity nitrogen gas was used as the curtain and collision gas which was delivered from Peak Scientific nitrogen generator. The ESI interface conditions were as follows: ion spray capillary voltage of −4200 V, curtain gas flow rate 25 L min^{-1}, nebulizer gas flow rate 15 L min-^{1}, DP1 -60 V, DP2 -15 V, and focusing potential of −200 V. The collision energy was swept from -5 to -40 eV for MS/MS analysis. Calibration were performed using internal calibration process, and samples were introduced into the mass

spectrometer using a Harvard syringe pump (Holliston, MA, USA) at a flow rate of 5 µL/ min. 0.2 ng/µL taurochloric acid was used as an internal calibrant.

Computational studies were performed using DFT at the B3LYP level with 6-31G⁄ basis set in Spartan 08 v 1.2.0 (Wavefunction, CA, USA) to investigate the most probable deprotonation site in methyl-*β*-orcinolcarboxylate (**3**), atranorin (**5**), lobaric acid (**9**), and usnic acid (**10**) by utilizing the previously established strategy [16]. Theoretical fragmentations of deprotonated secondary metabolites were studied by using ACD/MS Fragmenter software (ACD Labs).

3. Results and Discussion

Ten common secondary metabolites **1-10** (Fig. 1.) of lichens were investigated by negative ESI-QTOF-MS/MS for the development of a structure-fragmentation relationship. Structure and HR-ESI-MS data of these compounds are presented in Fig. 1 and Table1 respectively, while the HR-ESI-MS data of all fragment ions are presented in supplementary Table S1 (see supporting documents).

3.1. Collision energy optimization and computational studies

Relative intensities of selected product ions of $[M-H]^-$ *versus* collision energy ranging from -5 to -40 eV (with stepping up of 5 eV each time) were plotted for methyl-*β*-orcinolcarboxylate (**3**) monocyclic aromatic phenol, depside atranorin (**5**), depsidone lobaric acid (**9**), and dibenzofuran usnic acid (**10**). These compounds were taken as a representative for the four different groups of secondary metabolites (Fig. 2.). The optimum collision energy (CE) for recording product ion

spectra of methyl-*β*-orcinolcarboxylate (**3**), atranorin (**5**), usnic acid (**9**), and lobaric acid (**10**) were found to be -30, -15, -30, and -35 eV, respectively. Peak ions abundance and fragmentation pathways of secondary metabolites of lichens were considerably influenced by the variation in collision energy. ACD/MS Fragmenter software and computational studies were used to assist the elucidation of the fragmentation patterns. *In silico* studies were predicted deprotonation site. Minimum energy conformation of the neutral molecules was firstly optimized and then every possible deprotonation site was separately analyzed after optimization. The two hydroxyl sites of methyl-*β*-orcinolcarboxylate (**3**) were evaluated for deprotonation, however no major differences in energy was found. In atranorin (**5**), three possible hydroxyl oxygen at C-2, C-4 and C-2' were evaluated for the deprotonation. Hydroxyl oxygen at C-2 showed minimum energy so it was identified as the most favourable site for deprotonation, while hydroxyl oxygen at C-4 was the second most favourable sites. In lobaric acid (**9**), two possible depronated sites were evaluated, which includes the carboxylate site at C-7', and the hydroxyl site at C-2'. It was found that the carboxylic site is most favourable, as compared to hydroxyl site for deprotonation due to minimum energy (-1570.80727 hartree). In case of usnic acid (**10**), two possible deprotonated sites were calculated which includes the hydroxyl sites at C-6 (A) and C-8 (B). It was found that C-6 (-1221.57046 hartree) possess minimum energy as compared to C-8 (-1221.56900 Hartree). Calculated energies for selected secondary metabolites are shown in Table 2.

3.2. Fragmentation pattern of secondary metabolites of lichens

ESI-QqTOF-MS (negative mode) scan of monocyclic phenol derivatives, including orsellinic acid (**1**), methylorsellinate (**2**), methyl-*β*-orcinolcarboxylate (**3**), and ethylhaematomate (**4**)

showed $[M-H]^-$ peak at *m/z* 167.0349, 181.0498, 195.0657, and 223.0610, respectively. $[M-H]^-$ ions of all compounds were subjected to CID-MS analyses. Orsellinic acid (**1**) easily decarboxylate yielding $[M-H-44]^-$ as a characteristic product ion at *m/z* 123, which further fragmented to *m/z* 79 and 81 through the loss of CO_2 and C_2H_2O, respectively. Compounds **2** and **3** showed removal of methanol from $[M-H]^-$ at *m/z* 149 and 163, respectively. Sequential loss of methanol and CO_2 from $[M-H]^-$ were also observed in compounds **2** and **3** yielding ions at *m/z* 105 and 119, respectively. The ESI-MS/MS showed $[M-H]^-$ ions of more substituted phenols such as compound **4** where it easily fragment with collision energy of -30 eV and yields information-rich mass spectra. CID-MS/MS analysis of compound **4** produce product ion at *m/z* 177, corresponding to the loss of ethanol while another fragment ion was observed at *m/z* 195 due to loss of ethene. The product ion at *m/z* 151 was produced from *m/z* 195 by the loss of CO_2. Sequential loss of CO and CO_2 from product ion at *m/z* 195 yielded ions at *m/z* 167 and 123, respectively (Fig. 3D). Subsequent fragmentation and mass spectrum of compounds **1**, **2**, **3**, and **4** at -30 eV are shown in Fig. 3. APCI-MS/MS spectra of ethyl hematommate (**4**) has been reported, however no fragmentation patterns were given [13].

ESI-QqTOF-MS (negative mode) scan of depsides, including atranorin (**5**), lecanoric acid (**6**), erythrin (**7**) and sekikaic acid (**8**), showed $[M-H]^-$ peak at *m/z* 373.0947, 317.0670, 421.1134 and 417.1565, respectively. Depsides usually give characteristic fragment ion peak due to the cleavage of ester bond. Major product ions of atranorin (**5**) appeared at CE -15 eV, corresponding to $[M-H-C_9H_5O_4]^-$ and $[M-H-C_{10}H_{11}O_4]^-$ at *m/z* 177 and 195, respectively. Loss of methanol from *m/z* 195 yields the product ion at *m/z* 163 [13]. CID-MS/MS analyses of lecanoric acid (**6**) and erythrin (**7**) also showed the product ions at *m/z* 167 and 271, again due to the cleavage of ester bond with the removal of ring A as a neutral moiety. The product ion at *m/z* 149

in both compounds **6** and **7** was also produced by ester bond cleavage, but this time ring B removed as neutral molecule. Loss of CO_2 from *m/z* 167 yields an ion at *m/z* 123. Compound **7** showed an additional loss of $C_4H_8O_2$ due to the substitution on C-7', in comparison to compound **6**. The CID-MS/MS spectra and proposed mechanistic fragmentation pathway of atranorin (**5**), lecanoric acid (**6**), and erythrin (**7**) are shown in Fig. 4.

Detailed examination of compound **8** can be differentiated in comparison of compounds **5**, **6** and **7** on the basis of more alkyl and methoxy groups attached to the rings. $[M-H]^-$ ion of compound **8** at *m/z* 417 yielded fragment ion at *m/z* 225 which resulted again from the cleavage of the ester bond while ring A cleaved as neutral molecule. Loss of CO_2 from *m/z* 225 also showed the fragment ion at *m/z* 181. Another product ions at *m/z* 209, corresponding to $[M-H-C_{11}H_{11}O_3]^-$, was observed which further decarboxylated and yielded a fragment ion at *m/z* 165. Interestingly, sekikaic acid (**8**) also showed the loss of the C_3H_7 group, providing an $[M-43]^{\cdot-}$ radical anion at *m/z* 122. Methyl from the methoxy group was also removed as a radical and produce anion radical at *m/z* 150. Interestingly, this way has already been reported in other type of compounds [20, 21]. The CID-MS/MS spectra and proposed mechanistic fragmentation pathway of sekikaic acid (**8**) are shown in Fig. 5. Fast atom bombardment MS/MS (-ve) analysis of sekikaic acid (**8**) has been reported, however, neither radical anion nor detailed fragmentation pattern was described [12].

The spectra generated for lobaric acid (**9**) (a representative of depsidone) by electrospray mass spectrometry in the negative ion mode gave the deprotonated molecule $[M-H]^-$ at *m/z* 455.1691. Major product ions of lobaric acid (**9**) appeared at CE -30 eV, corresponding to $[M+H-CO_2]^-$ and

$[M+H-2CO_2]^-$ at *m/z* 411 and 367, respectively. Along with the *m/z* 367 peak $[M+H-2CO_2]^-$, the fragmentation of lobaric acid also produced a radical anion with *m/z* 352 ($[M-2CO_2-15]^-$) by losing a CH_3 group from the *m/z* 367 product ion. The removal of methyl radical has been reported from methoxy groups of phenyl ring under ESI-MS/MS (-ve) conditions [20, 21]. Along with the loss of CH_3 radical, a product ion at *m/z* 296, was also observed due to the loss of C_5H_{11} from *m/z* 367 peak. MS/MS analyses of other depsidones have been reported, indicating the loss of CO_2 as a characteristic fragment of depsidones class, however, no radical formation has been reported [14]. The MS/MS spectra and mechanistic fragmentation pathway of lobaric acid (**9**) are shown in Fig. 6.

The negative ion mode electrospray mass spectrometry of usnic acid (**10**) (a representative for dibenzofuran) gave the deprotonated molecule $[M-H]^-$ at *m/z* 343.0807. Formation of odd electron ion at *m/z* 328, corresponding to $[M-H-CH_3]^{\cdot-}$, produced from an even electron ion at *m/z* 343 in low collision energy [12]. Loss of methyl radical forms stable benzylic radical fragment anion. Fragment at *m/z* 259 is proposed to be generated from *m/z* 343 through retro Diel-Alder cleavage of ring C. Fragment at *m/z* 231 was due to the loss of CO from *m/z* 259. The MS/MS spectrum and mechanistic fragmentation pathway of usnic acid (**10**) are shown in Fig. 7.

3.3. ESI-MS/MS analyses of *Parmotrema grayana* and *Heterdermia obscurata* extracts

The full ESI-QqTOF-MS scan of methanolic and dichloromethane extracts of *Parmotrema grayana* and *Heterodermia obscurata* (negative mode) showed the presence of secondary metabolites as deprotonated $[M-H]^-$ molecular ions (Fig. 8.). Compounds were identified based

on HR-ESI-MS analyses, MS/MS analysis of the $[M-H]^-$ peaks, and database search using Dictionary of Natural Products (DNP). All compounds were searched in the updated DNP (version 23.1) on the basis of their exact molecular masses (without deprotonation) and respective molecular formulae. The MS/MS data yielded the diagnostic ions and losses, which were helpful for the identification of secondary metabolites. Deprotonated ions **III**, **IV**, **VIII**, **XII**, **XVI**, and **XX** were assigned as references for compounds **1**, **6**, **3**, **8**, **5**, and **2**, respectively.

In dichloromethane extract of *Parmotrema grayana*, orsellinic acid (**1**), lecanoric acid (**6**) and methyl-*β*-orcinolcarboxylate (**3**) were identified, while in methanolic extract, sekikaic acid (**8**) was identified. Compounds **3** and **5** were identified in dichloromethane extract of *Heterodermia obscurata.* Methylorsellinate (**2**), methyl-*β*-orcinolcarboxylate (**3**), lecanoric acid (**6**) and sekikaic acid (**8**) were identified in methanolic extract of *Heterodermia obscurata.* List of identified compounds and their MS/MS fragments are presented in Table 3.

Identification of nine peaks, other than reference compounds, are briefly discussed below. Deprotonated ion **I** at *m/z* 123.0444, corresponding to the molecular formula $C_7H_8O_2$ (without deprotonation) was searched in the DNP (MW 124 Da) and identified as orcinol, which has already been reported from the same species [5]. Compound **II** was identified as 4-hydroxy-1, 3-benzenedicarboxaldehyde also belongs to a monocyclic phenol derivative and has been isolated from *Eriostemon myoporoides* [22]. Deprotonated ion **V**, *m/z* 467.0987, corresponding to the molecular formula $C_{24}H_{20}O_{10}$ (without deprotonation) was searched in DNP (MW 468 Da) and identified as gyrophoric acid [12, 23] which was also confirmed using the exact mass and

MS/MS fragments. Compound **IV** at *m/z* 150.0669, corresponding to formula $C_9H_{11}O_2$, gave no hits in DNP search.

Deprotonated ion **VII**, at *m/z* 209.0824 corresponding to the formula $C_{11}H_{14}O_4$ (without deprotonation) was identified as divaricatinic acid using DNP search (MW 210 Da) [24]. The MS/MS data of this deprotonated ion showed product ions at *m/z* 165, 150, and 122. The *m/z* 165 was due to the the neutral loss of CO_2 from parent ion at *m/z* 209. The characteristic loss of CH_3 radical from *m/z* 165 to give *m/z* 150 possesses methoxy group attached to ring. Exact mass measurement and MS/MS analysis of *m/z* 209 also supported the identification of divaricatinic acid. Compound **VIII**, with a molecular formula $C_{10}H_{14}O_2$ (without deprotonation) was also searched in DNP. This search resulted in many compounds, out of which only one compound 3-methoxy-5-propylphenol was identified from lichen as a source however it was also reported from plant source [25]. The MS/MS data was almost identical to compound **VII**, with a difference of carboxylic acid group. Characteristic peaks at *m/z* 150 and 122 also showed the presence of methyl and propyl groups, respectively.

Deprotonated ion **XIII** at *m/z* 375.1067, corresponding to the molecular formula $C_{19}H_{20}O_8$ (without deprotonation), was searched in the DNP (MW 376 Da). This gave three hits from lichen origin, in which one compound, 9-hydroxy barbatic acid belongs to class depsides and others two compounds, placodiolic acid and pseudoplacodiolic acid belong to dibenzofuran class. Placodiolic acid and pseudoplacodiolic acid are isomers. MS/MS data of compound **XIII** gave ions *m/z* 231, 259, 299, and 343. On the basis of fragmentation pattern of compound **XIII**, it was proposed a similar structure as that of usnic acid, only difference being a methoxy group attached to C-13. The parent ion at *m/z* 375 yielded fragment ion at *m/z* 343 due to loss of methanol. The MS/MS data and losses matched with both the isomers which showed the neutral

loss of methanol, CO indicating the presence of methoxy group and fragments ion at *m/z* 259 produced through retro Diel-Alder cleavage. Therefore, its identified as one of the two, placodiolic acid and pseudoplacodiolic acids, two isomers which are difficult to distinguish using mass spectrometry.

Compound **XIV**, corresponding to the molecular formula $C_{21}H_{24}O_7$ was identified as divaricatic acid which belong to class depsides [26], and had been reported from the *Parmotrema grayana* [5]. MS/MS data showed a very intense peak at *m/z* 195 due to the cleavage of ester bond, and loss of CO_2 from *m/z* 195 to give fragment ion at *m/z* 105. FAB-MS/MS spectra of divaricatic acid has been reported earlier [12]. Molecular formula $C_{19}H_{16}O_8Cl$ (deprotonated form) of compound **XV** at *m/z* 407.0515 was assigned on the basis of accurate mass measurement and identified as chloroatranorin which was also supported by isotopic pattern. MS/MS fragments pattern of compound **XV** is similar to that of other depsides. Compound **XVIII** corresponding to the molecular formula $C_8H_8O_3$ (without deprotonation) was searched in DNP. This resulted many hits but only one hit from lichen which was atranol. Compounds **XVII** and **XIX** are tentatively proposed as on the basis of loss of CO_2 and accurate mass measurements, belonging to monocyclic phenol class.

4. Conclusion:

We report here the fragmentation pattern of ten common secondary metabolites, isolated from different lichen species, by using ESI-QqTOF-MS/MS. Diagnostic structure-fragmentation relationships were developed. Low energy collision induced dissociation tandem mass spectrometry provided key fragments of various classes of lichens compounds. In addition to this useful fragmentation patterns, and unusual radical anion formation are observed in compounds

belonging to depsides, depsidone and dibenzofuran classes of lichen. This generalization of structure-fragmentation relationship (SFR) was applied for the identification of compounds in crude extracts of lichens. Total fifteen compounds were putatively identified from two lichen species, *i.e. Parmotrema grayana* and *Heterdermia obscurata*. This study further highlights the importance of direct infusion mass spectrometry for rapid identification of secondary metabolites in complex mixtures.

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Figure and Table Captions

Figure1. Structures of secondary metabolites isolated from lichens.

Figure 2. Relative abundance of characteristic product ions *vs* collision energy of methyl-β-orcinolcarboxylate (A), atranorin (B), lobaric acid (C), usnic acid (D).

Figure 3. CID-MS/MS Spectra of [M-H]- for of orsellinic acid (**1**, A), methylorsellinate (**2**, B), methyl-β-orcinolcarboxylate (**3**, C), and ethylhaematomate (**4**, D), and proposed fragmentation pathway of compounds **1-4**.

Figure 4. CID-MS/MS Spectra of [M-H]- of atranorin (**5**, E), lecanoric acid (**6**, F), and erythrin (**7**, G), and proposed fragmentation pathway of compounds **5-7**.

Figure 5. CID-MS/MS spectra of $[M-H]^-$ and proposed fragmentation pathway of sekikaic acid (**8**, H).

Figure 6. CID-MS/MS spectra of $[M-H]^-$ and proposed fragmentation pathway of lobaric acid (**9**, I).

Figure 7. CID-MS/MS Spectra of $[M-H]^-$ and proposed fragmentation pathway of usnic acid (**10**, J).

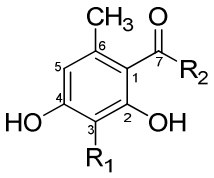
Figure 8. Full scan (-) ESI-QqTOF mass spectra of secondary metabolites of (A) dichloromethane extract and (B) methanolic extract of *Parmotrema grayana*, (C) dichloromethane extract and (D) methanolic extract of *Heterdermia obscurata*.

Table 1. HR-ESI-MS data of secondary metabolites isolated from lichens.

Table 2. Calculated energy for deprotonated representive secondary metabolites at basis set 6-31G*.

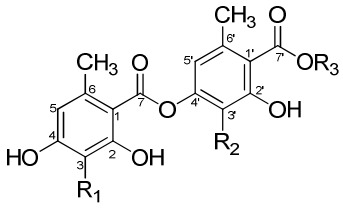
Table 3. Compounds identified by ESI-QqTOF-MS/MS analysis of *Parmotrema grayana* and *Heterdermia obscurata*.

1. Monocyclic phenol derivative

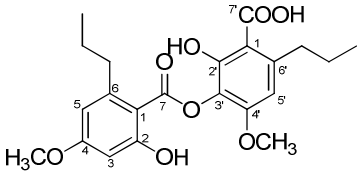


Orsellinic acid **(1)** $R_1 = H, R_2 = OH$
Methylorsellinate **(2)** $R_1 = H, R_2 = OCH_3$
Methyl-β-orcinolcarboxylate **(3)** $R_1 = CH_3, R_2 = OCH_3$
Ethylhaematomate **(4)** $R_1 = CHO, R_2 = OC_2H_5$

2. Depsides

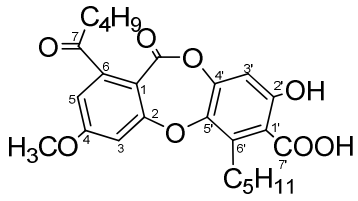


Atranorin **(5)** $R_1 = CHO, R_2 = CH_3, R_3 = CH_3$
Lecanoric acid **(6)** $R_1 = H, R_2 = H, R_3 = H$
Erythrin **(7)** $R_1 = H, R_2 = H, R_3 = CH_2CH(OH)CH(OH)CH_2OH$



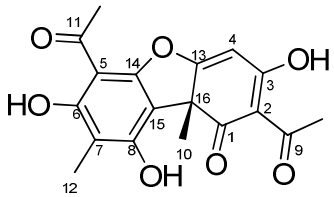
Sekikaic acid **(8)**

3. Depsidones



Lobaric acid **(9)**

4. Dibenzofurans



Usnic Acid **(10)**

Figure 1. Structures of secondary metabolites isolated from lichens.

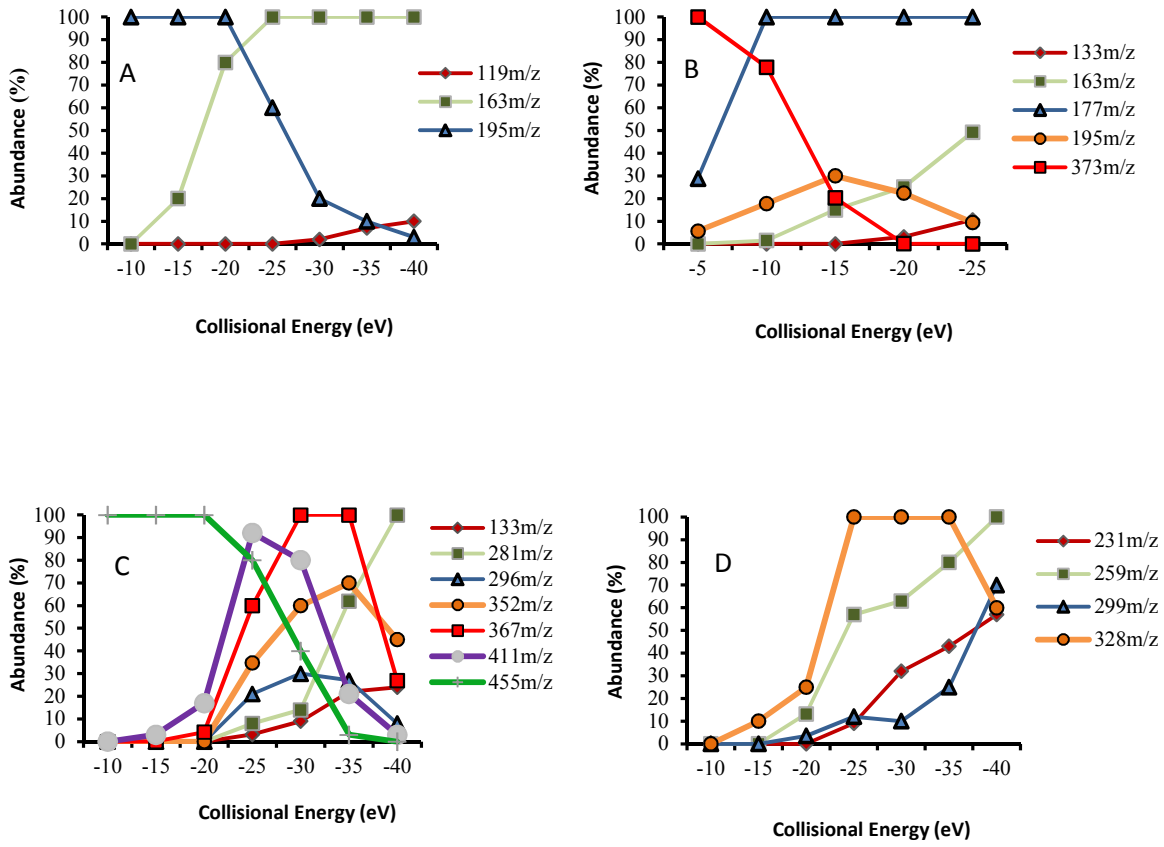


Figure 2. Relative abundance of characteristic product ions *vs* collision energy of methyl-*β*-orcinolcarboxylate (A), atranorin (B), lobaric acid (C), and usnic acid (D).

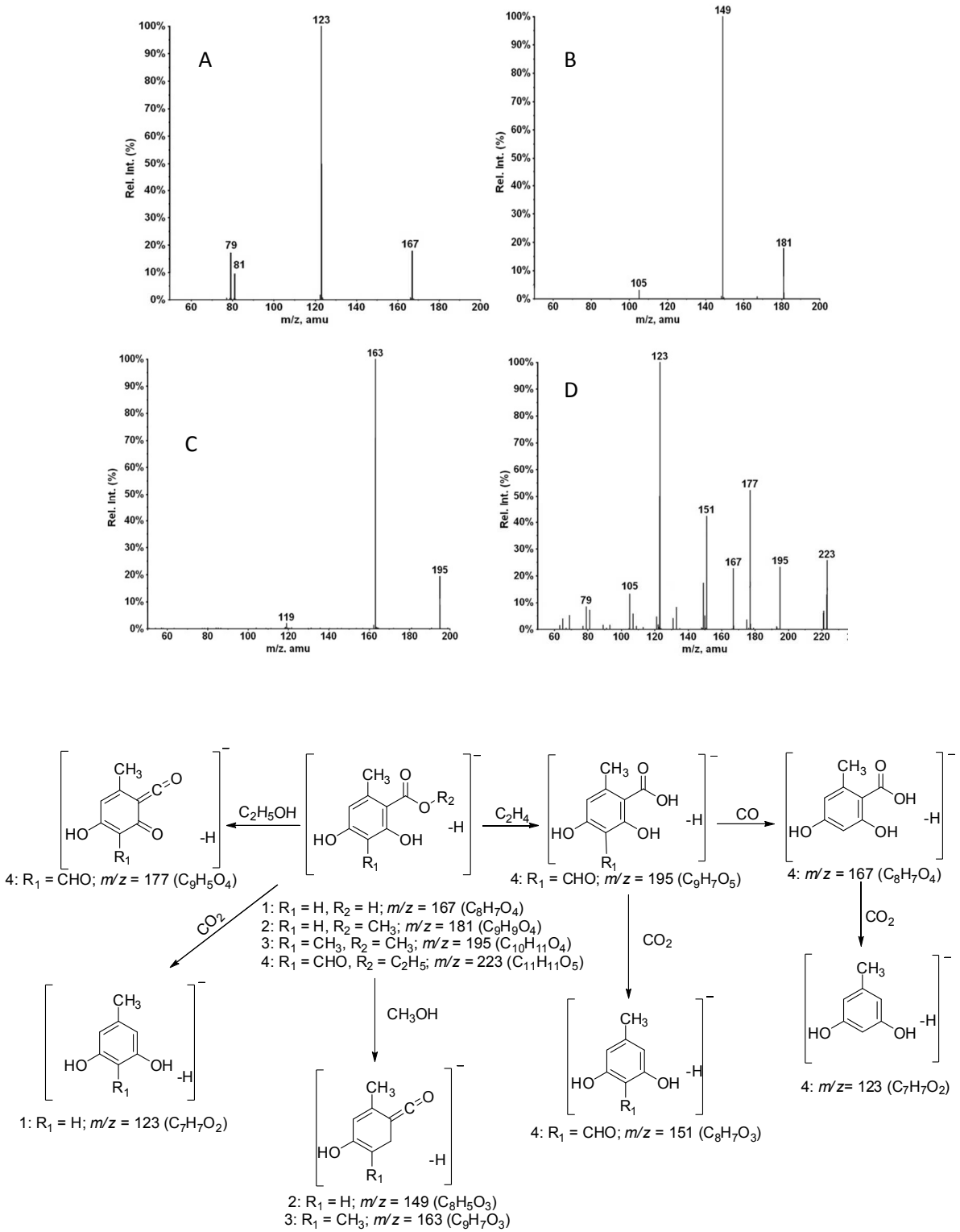


Figure 3. CID-MS/MS Spectra of $[M-H]^-$ for of orsellinic acid (**1**, A), methylorsellinate (**2**, B), methyl-*β*-orcinolcarboxylate (**3**, C), and ethylhaematomate (**4**, D), and proposed fragmentation pathway of compounds **1-4**.

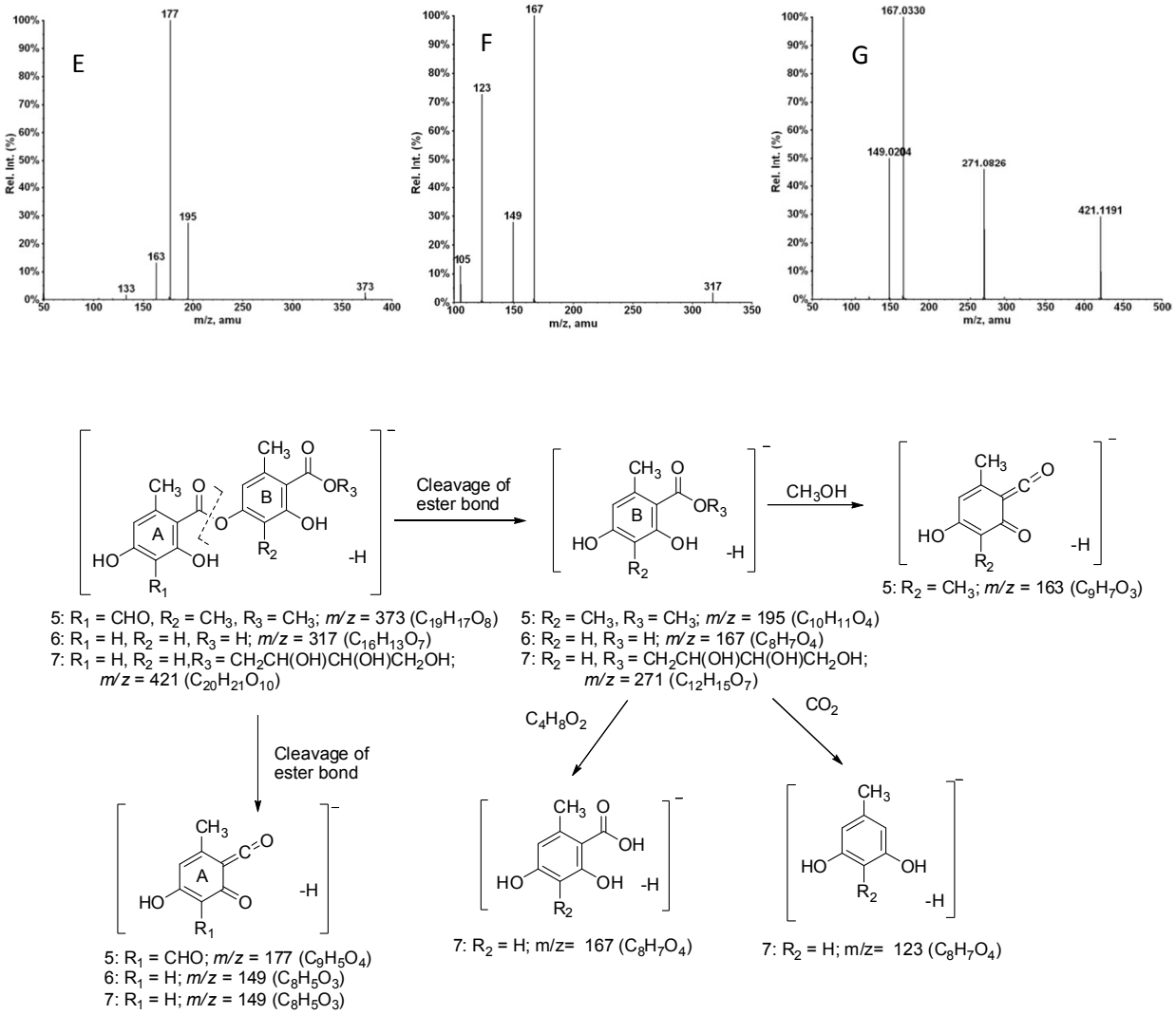


Figure 4. CID-MS/MS Spectra of $[M-H]^-$ of atranorin (**5**, E), lecanoric acid (**6**, F), and erythrin (**7**, G), and proposed fragmentation pathway of compounds **5**-**7**.

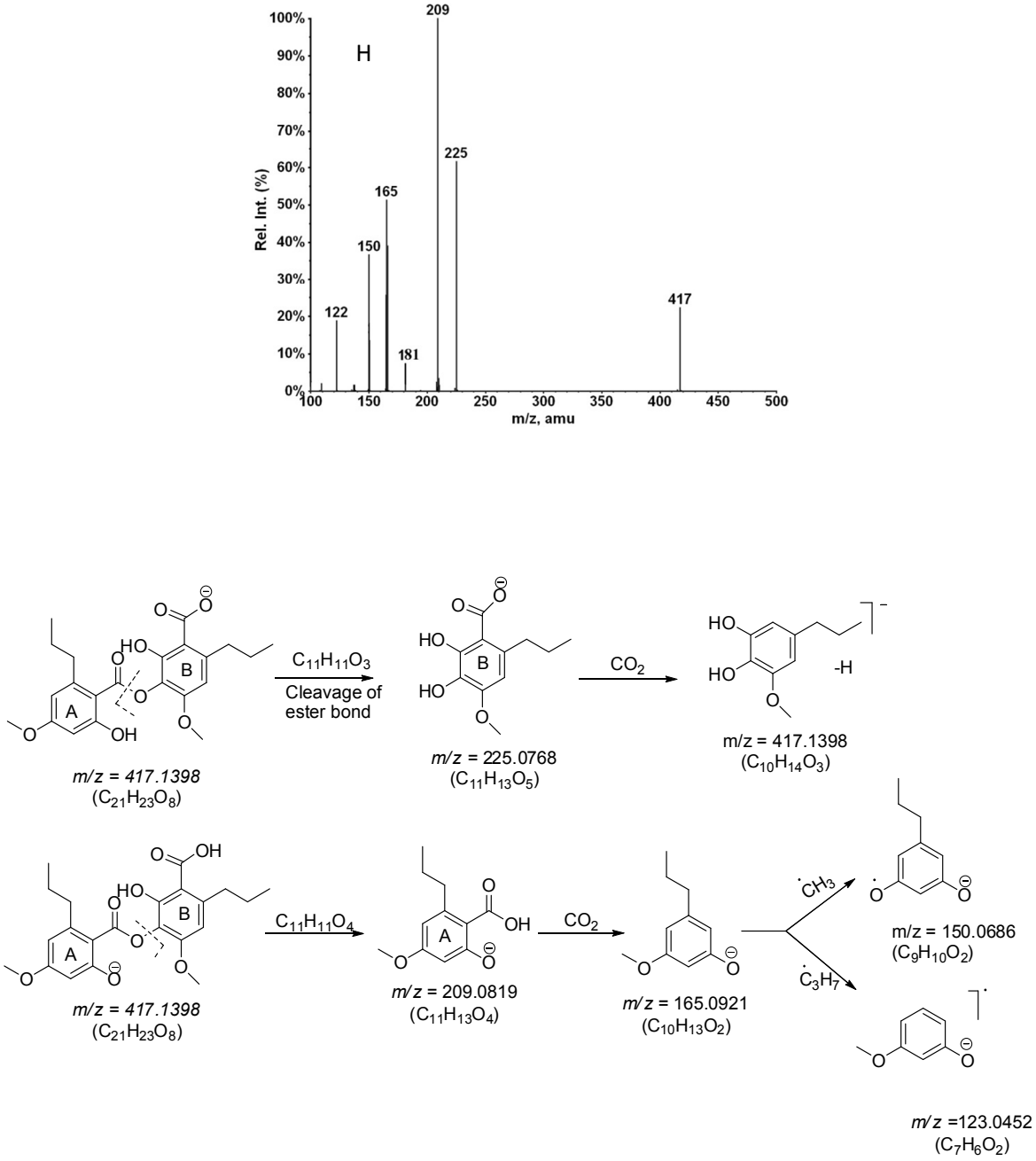


Figure 5. CID-MS/MS Spectra of $[M-H]^-$ and proposed fragmentation pathway of sekikaic acid (**8**, H).

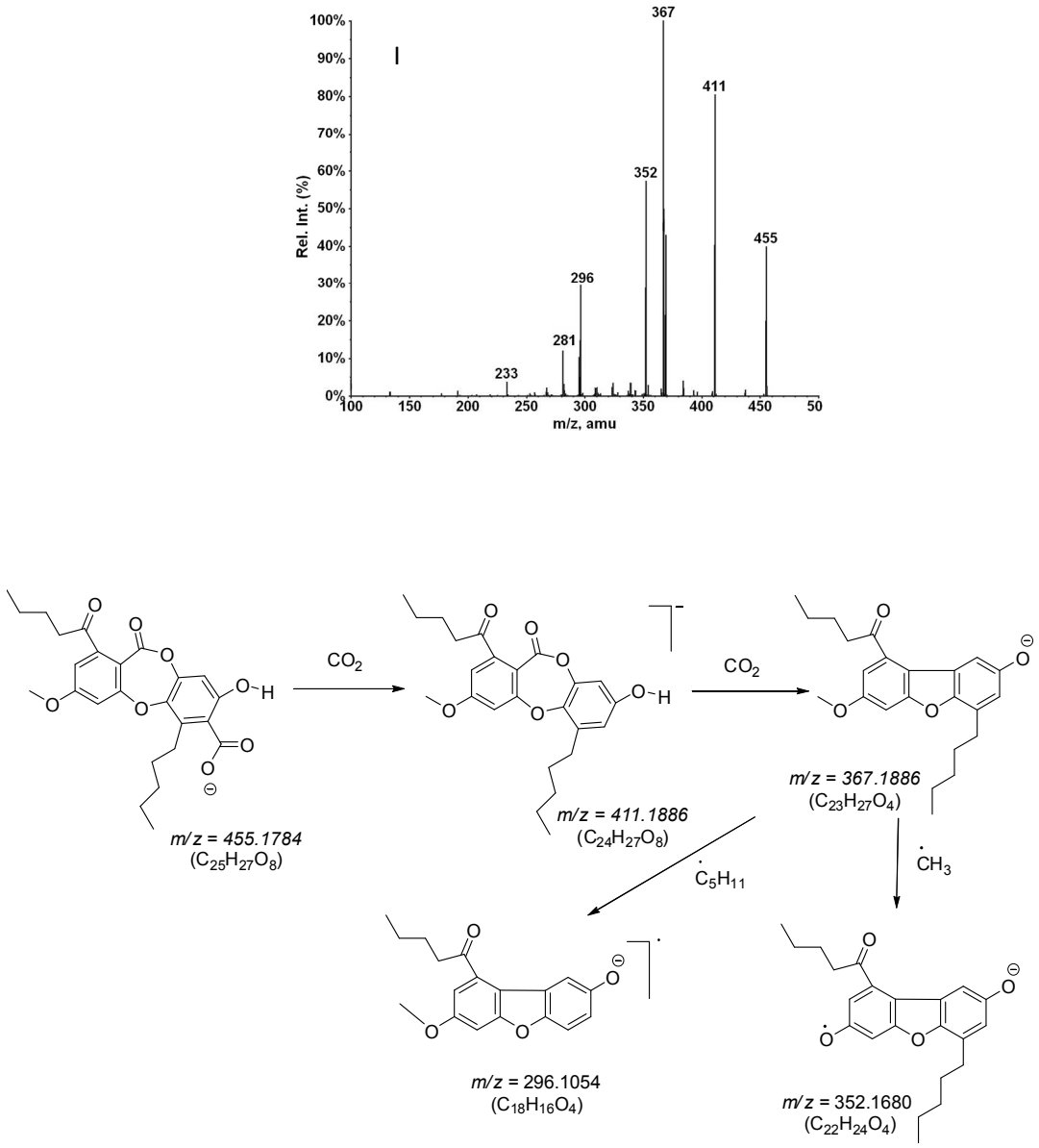


Figure 6. CID-MS/MS Spectra of $[M-H]^-$ and proposed fragmentation pathway of lobaric acid (**9**, I).

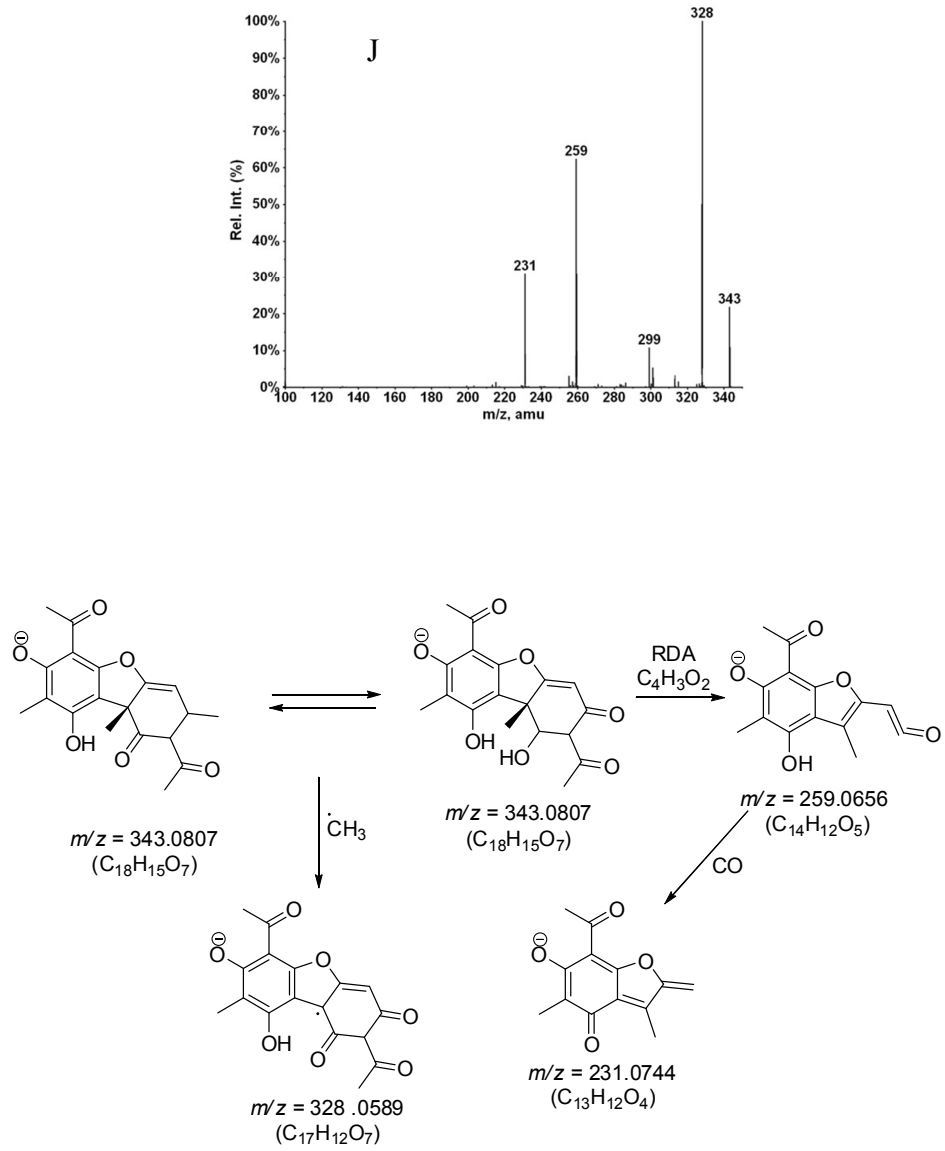


Figure 7. CID-MS/MS Spectra of $[M-H]^-$ and proposed fragmentation pathway of usnic acid (**10**, J).

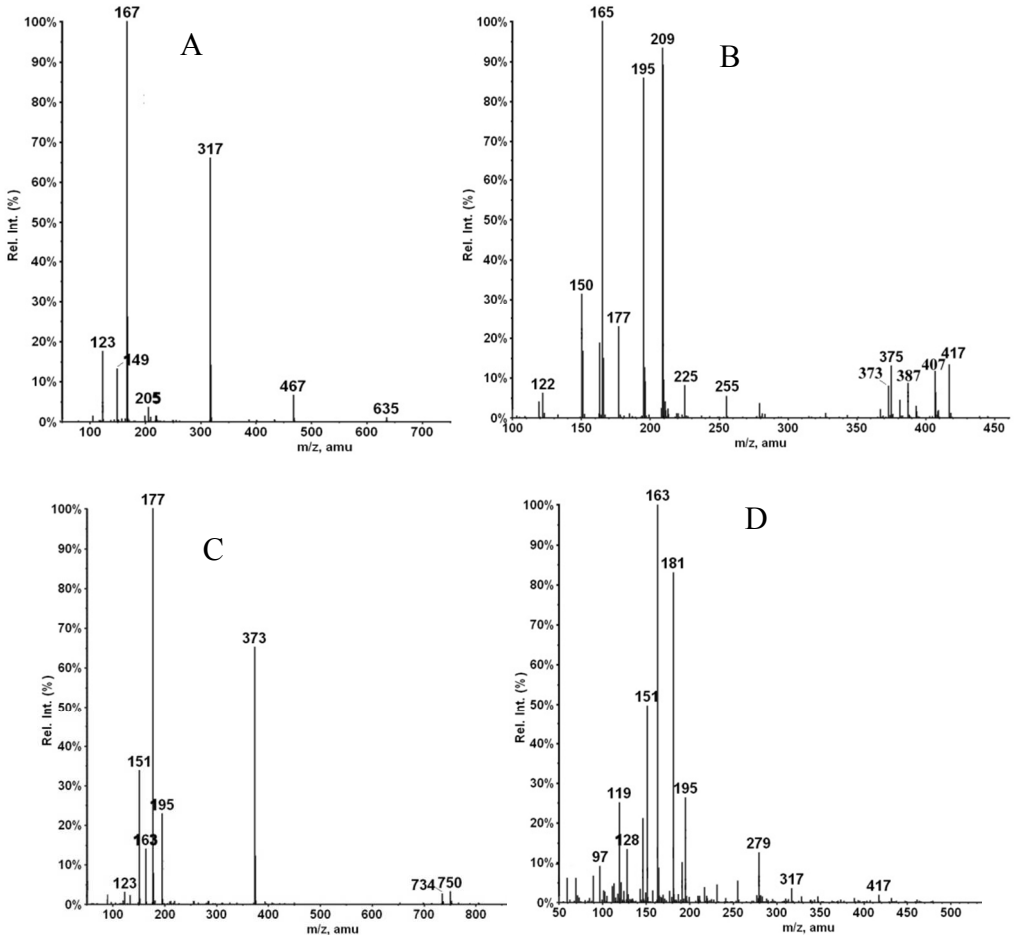


Figure 8. Full scan (-) ESI-QqTOF mass spectrum of secondary metabolites of (A) dichloromethane extract, (B) methanolic extract of *Parmotrema grayana*, (C) dichloromethane extract, and (D) methanolic extract of *Heterdermia obscurata*.

Table 1. HR-ESI-MS data of secondary metabolites isolated from lichens.

Name of compound	Chemical Formulae $[M-H]^-$	Calculated Exact mass (*m/z*)	Measured Exact mass (*m/z*)	Error (ppm)
Orsellinic acid	$C_8H_7O_4$	167.0344	167.0349	-1.0921
Methylorsellinate	$C_9H_9O_4$	181.0506	181.0498	-4.5981
Ethylhaematomate	$C_{11}H_{11}O_5$	223.0606	223.0610	1.5751
Methyl –β-orcinolcarboxylate	$C_{10}H_{11}O_4$	195.0662	195.0657	-2.9865
Atronorin	$C_{19}H_{17}O_8$	373.0928	373.0947	4.8476
Lecanoric acid	$C_{16}H_{13}O_7$	317.0661	317.0670	2.7502
Erythrin	$C_{20}H_{21}O_{10}$	421.1147	421.1134	2.915
Sekikaic acid	$C_{22}H_{25}O_8$	417.1554	417.1565	2.417
Usnic acid	$C_{18}H_{15}O_7$	343.0817	343.0807	-3.1423
Lobaric acid	$C_{25}H_{27}O_8$	455.1711	455.1691	-3.2805

Table 2. Calculated energies for deprotonated representive secondary metabolites at basis set 6-31G*

Secondary metabolites	anion	Deprotonation at position	E (Hartree)	Energy difference from A of desire compound
Methyl-*β*-orcinolcarboxylate	A	C-2	-649.30786	0.00
	B	C-4	-649.30787	0.00001
Atronorin	A	C-2	-1336.15630	0.00
	B	C-4	-1336.15449	0.00191
	C	C-2'	-1336.12775	0.02855
Lobaric acid	A	C-7'	-1570.80727	0.00
	B	C-2'	-1570.80313	0.00414
Usnic acid	A	C-6	-1221.57046	0.000
	B	C-8	-1221.56900	0.00146

Table 3. Compounds identified by ESI-QqTOF-MS/MS analyses of *Parmotrema grayana* and *Heterdermia obscurata*

No.	Molecular Formula	Exact mass	Observed Mass	Error (ppm)	MS/MS fragments	Identified compound
I[a,c]	$C_7H_7O_2$	123.0446	123.0444	-1.66	79, 81	Orcinol
II[a]	$C_8H_5O_3$	149.0248	149.0248	2.5653	121,93	4-hydroxy-1,3-benzenedicarboxaldehyde
III[a]	$C_8H_7O_4$	167.0344	167.0336	-4.992	123,79,81	Orsellinic acid
IV[a,d]	$C_{16}H_{13}O_7$	317.0661	317.0653	-2.6114	167,149,123	Lecanoric acid
V[a]	$C_{24}H_{19}O_{10}$	467.0978	467.0987	1.879	317,167,149,123	Gyrophoric acid
VI[b]	$C_9H_{10}O_2$	150.068	150.0669	-7.861	122,135,108	unknown
VII[b]	$C_{11}H_{13}O_4$	209.0813	209.0824	4.8589	165,150,122	Divaricatinic acid
VIII[b]	$C_{10}H_{13}O_2$	165.0921	165.0918	-1.8376	150,122	3-methoxy-5-propylphenol
IX[b,c,d]	$C_{10}H_{11}O_4$	195.0657	195.0651	-3.2501	163,119	Methyl-*β*-orcinolcarboxylate
X[b]	$C_{11}H_{13}O_5$	225.0762	225.0739	-10.6574	-	unknown
XI[b]	$C_{16}H_{31}O_2$	255.2324	255.2304	-7.8576	-	unknown
XII[b,c]	$C_{19}H_{17}O_8$	373.0923	373. 0917	-0.6507	195,177,163	Atranorin
XIII[b]	$C_{19}H_{19}O_8$	375.1079	375.1067	-3.4466	343,299,259, 231, 83	Placodiolic acid/ pseudoplacodiolic acid
XIV[b]	$C_{21}H_{23}O_7$	387.1443	387.1459	3.9303	209,195,177,151	Divaricatic acid
XV[b]	$C_{19}H_{16}O_8Cl$	407.0533	407.0515	-4.592	331,255,211,195,163	Chloroatranorin
XVI[b,d]	$C_{22}H_{25}O_8$	417.1549	417.1553	0.8559	225,209,165	Sekikaic acid
XVII[b,c]	$C_9H_5O_4$	177.0817	177.017	-10.0767	133	unknown
XVIII[c,d]	$C_8H_7O_3$	151.0395	151.0389	-4.0996	123,79,81	Atranol
XIX[c,d]	$C_9H_7O_3$	163.0395	163.0375	-12.3848	119	unknown
XX[d]	$C_9H_9O_4$	181.05	181.0509	4.5074	163,149	Methylorsellinate
XXI[d]	$C_{14}H_{31}O_5$	279.2180	279.2171	3.0462	127,97,79	unknown

[a] Dichloromethane extract of *Parmotrema grayana* ,[b] methanolic extract of *Parmotrema grayana*, [c]dichloromethane extract of *Heterdermia obscurata* and [d] methanolic extract of *Heterdermia obscura*