Untargeted analysis of sesquiterpene pyridine alkaloids from the dried roots of *Tripterygium wilfordii* using high-performance liquid chromatography/electrospray ionization tandem mass spectrometry

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**RATIONAL**E: Sesquiterpene pyridine alkaloids are a large group of highly oxygenated sesquiterpenoids that have attracted attention in the fields of medicine because of their significant biological activities.

**METHODS:** Reference compounds including 14 sesquiterpene pyridine alkaloids and one dihydroagarofuran ester were analyzed by collision-induced dissociation tandem mass spectrometry (CID-MS/MS). A high-performance liquid chromatography/electrospray ionization (HPLC/ESI)-MS/MS method at two collision energies was adopted to investigate the botanical extracts of *Tripterygium wilfordii*.

**RESULTS:** For 15 reference compounds, in the high mass range, the product ions were formed by the loss of side chains or H2O. In the low mass range, the high-abundance product ions at m/z 206, 204, or 194 were the characteristic ions of the pyridine moiety. The characteristic product ion at m/z 310 was formed through an ion–neutral complex intermediate. Fifty-four sesquiterpenoid derivatives, including 50 sesquiterpene pyridine alkaloids, were identified or tentatively characterized in botanical extracts of *T. wilfordii* based on their elemental constituents, characteristic fragmentation patterns, and the major product ion profiles of the reference compounds ascertained with HPLC/ESI-MS/MS at two collision energies. It seems that isocratic energy was appropriate for the untargeted analysis of compounds with molecular weights exceeding 800 Da, whereas a linear gradient energy vs molecular weight was suitable for those compounds with molecular weights below 800 Da.

**CONCLUSIONS:** The HPLC/ESI-MS/MS method, combining characteristic fragmentation patterns and the profiles of the product ions generated at different collision energies, is an effective technique for characterizing untargeted compounds. Copyright © 2015 John Wiley & Sons, Ltd.
In our study, a botanical extract of the dried roots of *Tripterygium wilfordii* was analyzed using HPLC/electrospray ionization quadrupole time-of-flight-type tandem mass spectrometry (ESI-QTOF)-MS/MS in positive-ion mode. Members of a series of unknown sesquiterpene pyridine alkaloids were identified or tentatively characterized based on the exact masses, and the fragmentation patterns of reference compounds, and their MS/MS profiles at two collision energies. Although energy-resolved experiments are often performed to analyze isomers in pure samples,[25,26] the amount of data increase sharply when complex multi-component samples are analyzed, such as biological fluids. Here, isocratic energy and a linear gradient energy vs molecular weight were used for the untargeted analysis of the botanical extracts.

**EXPERIMENTAL**

**Chemicals**

HPLC-grade acetonitrile, thrice deionized water, and a mass calibration standard for use in the analyses were obtained from Fisher Scientific (Pittsburgh, PA, USA), Thermo Scientific (Palo Alto, CA, USA), Haihong (Chengdu, China), Guoyao (Chengdu, China), and Agilent Technologies (Palo Alto, CA, USA), respectively. The air-dried roots of *T. wilfordii* (product no. 9005107) were purchased from Sichuan Neautus Traditional Chinese Medicine Co. Ltd. (Chengdu, China). A voucher specimen (973–06) was identified by Prof. Fading Fu at the Chengdu Institute of Biology of the Chinese Academy of Sciences (CIBAS) and was deposited in the Herbarium of CIBAS. 

The CHCl₃-soluble extract was separated by with column chromatography (silica gel, 160–200 mesh) eluted with CHCl₃/acetone (10:1, v/v) to produce six fractions.[14] The air-dried and powdered roots of *Tripterygium wilfordii* were soaked in 70% ethanol. The mixture was heated and boiled for 1 h and cooled to room temperature. The resulting dry extract was suspended in water and extracted with CHCl₃. The CHCl₃-insoluble extract was separated by column chromatography (silica gel, 160–200 mesh) eluted with CHCl₃/acetonitrile (10:1, v/v) to produce six fractions.[14]

**RESULTS AND DISCUSSION**

**ESI-MS/MS analysis of reference compounds 1–15**

References compounds 1–15 were analyzed by HPLC/ESI-MS and ESI-MS/MS in positive-ion mode. The precursor [M + H]+ ions for all the compounds were selected and the product ions were recorded by ESI-QTOF-MS/MS. Accurate mass data were obtained using an external standard. These compounds were classified into eight groups (compounds 1–2, compound 3–6, compounds 7, compounds 8–10, compounds 11–13, compound 14, and compound 15, for structures, see Fig. 1) based on their chemical structures, dominant fragmentation pathways, and the profiles of their major product ions.

The fragmentation pathways of the precursor [M + H]+ ions for compounds 1–2 were very similar. Compound 1 is used as a representative compound in the following discussion and the corresponding fragmentation pathways are shown in Scheme 1(a). In the high mass range, major product ions were detected at *m/z* 850, 808, 790, and 748 (Fig. 2(a) and Table 1). The high-abundance product ion at *m/z* 850 was formed from the precursor [M + H]+ ion at *m/z* 868 by the loss of H₂O and further yielded the high-abundance product ion at *m/z* 790 with the loss of AcOH. Sequential losses of AcOH from *m/z* 868 produced the product ions at *m/z* 808 and 748. In the low mass range, the product ions at *m/z* 105 and 206 are the characteristic ions of the benzoic acid group linked to C1 and the pyridine part, respectively. The product ion at *m/z* 178 resulted from *m/z* 206 with the loss of CO. An interesting product ion at *m/z* 310 was detected and corresponding processes involve an intermediate formed by the McLafferty-type rearrangement and an ion-neutral complex intermediate (Scheme 1(a)).[27–31] An interesting product ion was also detected at *m/z* 310 in the MS/MS spectrum of compound 2. The major fragmentation pathways of compound 3 are similar to those of compounds 1 and 2. However, no product ion formed via an ion-neutral complex intermediate was observed.
The structures of compounds 4–7 are similar. Compound 5 is an isomer of 1. The loss of AcOH or benzoic acid or both are major fragmentation pathways in the high mass range of compound 5 (Scheme 1(b) and Table 1). In the low mass range, the characteristic ion at m/z 310 was not detected in the MS/MS spectrum of compound 5 (Fig. 2(b)). The fragmentation pathways and profiles of the corresponding product ions of compounds 4 and 6 are very similar to those of compound 5. Although the structure of compound 7 is similar to those of compounds 4–6, except for the 2-OH group (Fig. 1), which is not esterified, their MS/MS spectra differ. Few product ions were detected in the high mass range of the MS/MS spectrum of compound 7. It seems that the esterified side chain on C2 is readily lost, compared to the loss of H2O in compound 7.

In compound 8, C3 is linked to a hydroxyl group (Fig. 1). A high-abundance ion at m/z 846 was detected, resulting from the precursor ion at m/z 874 after the loss of CO (Fig. 2(c), Scheme 1(c)). The product ions at m/z 856 and 828 were produced from m/z 874 and 846, respectively, with the loss of H2O. The product ion at m/z 846 yielded m/z 674 by the sequential losses of furan-3-carboxylic acid and AcOH. Unlike compounds 1–7, the product ions at m/z 204, 194, and 176 are the main ions in the low mass range of compound 8. The product ion at m/z 194 is formed from m/z 846 (Scheme 1(c)). The product ion at m/z 95 is the characteristic ion of furan-3-carboxylic acid. The fragmentation pathways of compounds 9–10 are similar to those of 8.

The 3-OH group in compound 8 is esterified to yield compound 11. The fragmentation pathways between compounds 8 and 11 vary greatly. The base peak at m/z 804 resulted from the precursor ion at m/z 916 after the loss of furan-3-carboxylic acid (Fig. 2(d) and Scheme 1(d)). The product ion at m/z 888 resulted from m/z 916 after the loss of CO. The ion at m/z 804 produced the product ions at m/z 786, 744, and 684 by the loss of a H2O, an AcOH or two AcOH molecules, respectively (Scheme 1(d)). In the low mass range, the product ions at m/z 204, 186, and 176 are the main product ions of compound 11. The fragmentation pathways of compounds 11–13 are similar.

Compounds 14, 6, and 3 are isomers. Although the fragmentation patterns of compounds 6 and 3 are similar, the relative abundances of their product ions resulting from the same pathways vary in the high mass range. Interestingly, the structure of compound 14 is very similar to that of 6, but their MS/MS spectra are quite different. The product ion of the sesquiterpenoid moiety at m/z 421 formed by the loss of the pyridine part can be detected in the spectrum of compound 14, but not in the MS/MS spectrum of 6. This may be attributable to a difference in the protonation sites of compounds 6 and 14. Compound 15 is a dihydroagarofuran ester. The product ions at m/z 233, 131, and 124 are the characteristic ions of the sesquiterpenoid moiety, cinnamic acid, and nicotinic acid, respectively.

Comparing the fragmentation pathways of compounds 1–14, we conclude that: (1) the ion (oxo(phenyl)methyl) formed from the side chain on C1 can react with the pyridine part of the molecule and further provides the characteristic product ion at m/z 310; (2) the substituent groups on C2 are readily lost; and (3) the hydroxyl or ester linked to C3 can greatly influence the fragmentation pathways.
HPLC/ESI-MS/MS analysis of sesquiterpene pyridine alkaloids from the dried roots of *T. wilfordii*

In total, 54 sesquiterpenoid derivatives (see Supporting Information), including 50 sesquiterpene pyridine alkaloids, in botanical extracts of *T. wilfordii* were identified or tentatively characterized based on their elemental constituents, characteristic fragmentation patterns, and the major product ion profiles of the reference compounds determined with HPLC/ESI-MS/MS at two collision energies.

**Scheme 1.** Major fragmentation patterns of [M + H]^+ for compounds (a) 1, (b) 5, (c) 8, and (d) 11.

Structural analysis of compounds 16–18

The protonated ions for compounds 16–18 were detected in positive-ion mode. The profiles of the major product ions are similar to those of compounds 1–2. We assumed that the skeletons of these compounds (1–2 and 16–18), including the positions of substituted groups, would be the same. Compound 16 was selected as the representative compound for the structural analysis. The molecular formula of compound 16 was confirmed as...
from its accurate mass value, relative isotopic abundance measurements, and MS/MS spectrum. In the high mass range, the major product ions were detected at m/z 902, 860, 842, and 800, and were formed by the sequential losses of H₂O, AcOH, or both, similar to the fragmentation patterns of compound 1 (Scheme 1). In the

\[
\begin{align*}
\text{C}_{46}\text{H}_{49}\text{NO}_{19} & \text{ from its accurate mass value, relative isotopic abundance measurements, and MS/MS spectrum.}^{[32]} \text{ In the high mass range, the major product ions were detected at m/z 902, 860, 842, and 800, and were formed by the sequential losses of H}_2\text{O, AcOH, or both, similar to the fragmentation patterns of compound 1 (Scheme 1). In the}\n\end{align*}
\]

\[
\begin{align*}
\text{Figure 2. Product ion scan of the selected precursor [M+H]⁺ ion (a) at m/z 868 (collision energy at 25 eV) for cangorinine E-1 (1), (b) at m/z 868 (collision energy at 25 eV) for wilforine (5), (c) at m/z 874 (collision energy at 25 eV) for wilfortrine (8), and (d) at m/z 916 (collision energy at 25 eV) for 9'-O-acetylwilfortrine (11).}\n\end{align*}
\]

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few product ions were detected in the high mass range for high-abundance product ion at is the characteristic of these compounds, together with the loss of the side chain groups linked to C2 occurs readily. This

Structural analysis of compounds

unknown compounds.

Linear gradient collision energies vs molecular weights were selected for the analysis of compounds

have the same skeleton. Notably, their collision energies in the collision-induced dissociation (CID) experiments differed.

The product ion at m/z 804 was formed from m/z 916 in 11 with the loss of furan-3-carboxylic acid, and m/z 804 was formed from m/z 926 in 39 with the loss of benzoic acid. This implies that when the 3-furanoyl group in compound 11 is substituted with a benzoyl group, compound 39 is produced.

Structural analysis of compounds 23–29

The structures of compounds 23–29 might be similar to those of compounds 4–6 according to their MS/MS spectra. The loss of the side chain groups linked to C2 occurs readily. This is the characteristic of these compounds, together with the high-abundance product ion at m/z 206. Like compound 7, few product ions were detected in the high mass range for compounds 26–29. This is probably attributable to the nonesterified hydroxyl linked to C2, and the other side chain groups are not readily lost.

Structural analysis of compounds 30–45

The characteristic product ions at m/z 204, 194, and 176 in the low mass range and the loss of CO from the precursor ions indicate that the structures of compounds 30–34 are similar to those of compounds 8–10. For example, in the MS/MS spectrum of compound 34, the product ion at m/z 630 resulted from m/z 812 with the sequential losses of C7H6O2 and AcOH. This suggests that benzoic acid is linked to C2. Compounds 32 and 34 might be alatusinine and alatamine, respectively.[33,34] The structures of compounds 35–45 are clearly similar to those of compounds 11–13, when their MS/MS spectra are compared. Compound 39 was selected for the following analysis. The product ions for compounds 11 and 39 are the same, except for the product ions derived by the loss of CO from the precursor ions. The product ion at m/z 804 was formed from m/z 916 in 11 with the loss of furan-3-carboxylic acid, and m/z 804 was formed from m/z 926 in 39 with the loss of benzoic acid. This implies that when the 3-furanoyl group in compound 11 is substituted with a benzoyl group, compound 39 is produced.

Structural analysis of compounds 46–49

The product ion at m/z 421 in the MS/MS spectrum of compound 46 is the characteristic ion of sesquiterpenoid moieties, which indicates that the structure of compound 46

Table 1. Characterizations of sesquiterpene pyridine alkaloid and dihydroagarofuran ester references (1–15) using ESI-MS/MS (collision energy: 25 eV)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Formula [M + H]+</th>
<th>Calculated</th>
<th>Observed</th>
<th>Error (ppm)</th>
<th>Product ion (relative abundance %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C43H50NO18</td>
<td>868.3022</td>
<td>868.3049</td>
<td>–3.1</td>
<td>868 (100), 850 (55), 808 (11), 790 (25), 748 (10), 310 (8), 206 (66), 178 (5), 105 (3)</td>
</tr>
<tr>
<td>2</td>
<td>C48H52NO18</td>
<td>930.3179</td>
<td>930.3210</td>
<td>–3.3</td>
<td>930 (100), 912 (62), 870 (11), 852 (24), 810 (8), 310 (8), 206 (67), 178 (5), 105 (5)</td>
</tr>
<tr>
<td>3</td>
<td>C46H50NO18</td>
<td>806.2866</td>
<td>806.2861</td>
<td>0.6</td>
<td>858 (98), 840 (24), 788 (13), 746 (17), 686 (26), 206 (100), 178 (20)</td>
</tr>
<tr>
<td>4</td>
<td>C47H52NO9</td>
<td>858.2815</td>
<td>858.2808</td>
<td>0.8</td>
<td>858 (100), 850 (25), 808 (15), 746 (23), 686 (28), 206 (80), 178 (10), 105 (8)</td>
</tr>
<tr>
<td>5</td>
<td>C43H50NO18</td>
<td>868.3022</td>
<td>868.3022</td>
<td>0.0</td>
<td>806 (85), 788 (24), 746 (27), 728 (11), 686 (30), 206 (100), 178 (18)</td>
</tr>
<tr>
<td>6</td>
<td>C39H48NO18</td>
<td>806.2866</td>
<td>806.2864</td>
<td>0.2</td>
<td>806 (85), 788 (24), 746 (27), 728 (11), 686 (30), 206 (100), 178 (18)</td>
</tr>
<tr>
<td>7</td>
<td>C41H48NO17</td>
<td>826.2917</td>
<td>826.2910</td>
<td>0.9</td>
<td>826 (8), 206 (100), 178 (13), 105 (1)</td>
</tr>
<tr>
<td>8</td>
<td>C36H46NO18</td>
<td>780.2709</td>
<td>780.2705</td>
<td>0.5</td>
<td>874 (8), 856 (19), 846 (100), 828 (10), 674 (13), 204 (10), 194 (26), 176 (15)</td>
</tr>
<tr>
<td>9</td>
<td>C36H50NO19</td>
<td>884.2972</td>
<td>884.3000</td>
<td>–3.3</td>
<td>884 (11), 866 (19), 856 (100), 838 (12), 674 (15), 204 (11), 194 (26), 176 (15)</td>
</tr>
<tr>
<td>10</td>
<td>C36H46NO18</td>
<td>780.2709</td>
<td>780.2705</td>
<td>0.5</td>
<td>874 (8), 856 (19), 846 (100), 828 (10), 674 (13), 204 (10), 194 (26), 176 (15)</td>
</tr>
<tr>
<td>11</td>
<td>C37H48NO17</td>
<td>916.2870</td>
<td>916.2892</td>
<td>–2.4</td>
<td>916 (10), 888 (7), 804 (100), 786 (9), 744 (9), 684 (8), 204 (9), 186 (4), 176 (2)</td>
</tr>
<tr>
<td>12</td>
<td>C41H48NO19</td>
<td>978.3026</td>
<td>978.3008</td>
<td>1.9</td>
<td>978 (14), 950 (6), 856 (100), 838 (8), 804 (4), 744 (6), 684 (8), 204 (12), 186 (5), 176 (2)</td>
</tr>
<tr>
<td>13</td>
<td>C41H46NO20</td>
<td>872.2608</td>
<td>872.2619</td>
<td>–1.0</td>
<td>872 (6), 844 (5), 760 (100), 718 (12), 686 (13), 204 (15), 186 (8)</td>
</tr>
<tr>
<td>14</td>
<td>C38H48NO18</td>
<td>806.2866</td>
<td>806.2876</td>
<td>–1.3</td>
<td>806 (100), 788 (74), 746 (93), 704 (36), 686 (31), 644 (28), 421 (18), 361 (21), 259 (27), 206 (85), 178 (9)</td>
</tr>
<tr>
<td>15</td>
<td>C37H40NO8</td>
<td>626.2748</td>
<td>626.2741</td>
<td>1.2</td>
<td>626 (6), 233 (15), 215 (14), 173 (8), 131 (18), 124 (100)</td>
</tr>
</tbody>
</table>
is similar to that of compound 14. One hydroxyl in 14 was not esterified, producing compound 46. Like compound 15, compounds 47–49 are dihydroagarofuran esters. For example, when the cinnamoyl group in compound 15 was substituted with a benzoyl group, compound 48 was generated. Compound 49 might be wilforcidine.\(^{[35,36]}\)

**Structural analysis of other sesquiterpene pyridine alkaloids**

Many other sesquiterpene pyridine alkaloids were detected in the extract. However, their skeletons could not be identified because of a lack of reference compounds. Interestingly, the characteristic product ion at \(m/z\) 316 or 326 in the MS/MS spectra of compounds 50–54 might be formed by an addition reaction of the product ion at \(m/z\) 204 and the neutral loss of furan-3-carboxylic acid (112 Da) or benzoic acid (122 Da). This characteristic fragmentation pattern implies that the corresponding substituted groups were linked to C1.

**CONCLUSIONS**

Reference compounds including 14 sesquiterpene pyridine alkaloids and one dihydroagarofuran ester were analyzed by CID-MS/MS, and classified into eight groups according to their chemical structures, characteristic fragmentation patterns, and the profiles of their major product ions. An interesting fragmentation pattern forming \(m/z\) 310 was proposed. The characteristic fragmentation pattern is related to the position of the side chain. Fifty-four sesquiterpenoid derivatives, including 50 sesquiterpene pyridine alkaloids, were identified or tentatively characterized in botanical extracts of *T. wilfordii* based on their elemental constituents, characteristic fragmentation patterns, and the profiles of the major product ions of the reference compounds using HPLC/ESI-MS/MS at two collision energies. MS/MS spectra determined at two collision energies can improve the structural identification of unknown compounds and simultaneously avoid the production of complex data, like that generated in energy-resolved experiments, and can be applied to high-throughput analyses. Many isomers can be distinguished. Although complete determination of unknown compounds needs more standard compounds, the combination of characteristic fragmentation patterns and the profiles of the product ions at different collision energies is an effective technique for characterizing untargeted sesquiterpene pyridine alkaloids from botanical extracts.

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**REFERENCES**


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