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Identification of a new minor iridoid glycoside in *Symplocos glauca* by thermospray liquid chromatography–mass spectrometry

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ABSTRACT

Iridoid glycosides in *Symplocos glauca* were identified by thermospray liquid chromatography–mass spectrometry. The conditions for optimization of the separation of iridoid glycosides were investigated. The mass spectra obtained gave qualitative information concerning both the aglycone and sugar moieties and also the molecular weights. Methanol extracts from the leaves of *S. glauca* were analysed for iridoid glycosides. Not only major but also minor components could be separated and identified from the mass spectra and mass chromatograms. A new minor component in *S. glauca* was tentatively identified as 6-dihydroverbenalin.

INTRODUCTION

Ethnopharmacological studies have revealed a wide variety of naturally occurring substances of plant origin which are used in Chinese and other folk remedies. These folk preparations are often effective antipyretics, anti-inflammatories, tranquillizers and laxatives, and contain a number of iridoid glycosides which appear to play an important role in their efficacy. The identification of iridoid glyosides has been attempted using several analytical methods, and some molecular weight determinations based on mass spectrometry have been reported. However, the successful isolation and identification of these glycosides has been hampered by their highly polar and thermally labile nature. These characteristics have limited MS applications to identifications that rely mostly on field desorption (FD) and fast atom bombardment (FAB) techniques[1–5]. With both techniques, isolation and purification of target components is a prerequisite.

Liquid chromatography–mass spectrometry (LC–MS) has been applied to the separation and MS characterization of known glycosides using moving-belt[6] and frit

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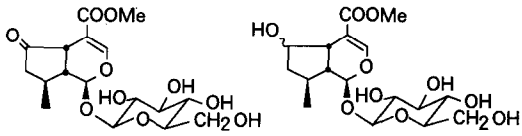

Fig. 1. Structural formulae of verbenalin (left) and 6-dihydroverbenalin (right). Me = Methyl.

FAB[7] techniques. However, these methods do not appear suitable for the identification of unknown glycosides when present in trace amounts.

We have been investigating iridoid glyosides in various medicinal plants using thermospray (TSP) LC–MS. Spectra obtained from extracts of *Symplocos glauca* suggested the presence of a minor glycoside which is closely related to the known substance verbenalin (Fig. 1). LC separation conditions were examined in order to provide a clearer separation of this unknown trace component, which was subsequently tentatively identified as 6-dihydroverbenalin (Fig. 1).

EXPERIMENTAL

Materials

Fresh chopped leaves of *S. glauca* (10 g) were extracted by shaking with hot methanol (3 × 20 ml) and the combined extracts were evaporated to dryness *in vacuo*. The residue was taken up in water and insoluble substances were removed by filtration through a layer of Celite. The filtrate was evaporated to dryness *in vacuo* and residue was taken up in methanol just prior to measurement.

Verbenalin standard was isolated and purified in the following way. Fresh leaves of *S. glauca* (11.87 g) were extracted by shaking with hot methanol (5 × 18 ml) and the combined extracts were concentrated to 30 ml *in vacuo*. After filtration through Celite, the filtrate was saturated with water and concentrated to 11.1 ml *in vacuo*, then filtered through Celite to remove precipitates. Dry yeast (55.5 mg) was suspended in the filtrate, which was subsequently allowed to stand overnight at 30°C to decompose sugars. After filtration of the reaction mixture through Celite, the solvent was removed *in vacuo*. The residue (51.66 mg) was recrystallized from ethanol to afford pure verbenalin (25 mg). The purity of the verbenalin obtained was confirmed by melting point, elemental analysis, refractive index and NMR measurements.

Chemicals and reagents

Ammoniom acetate (special grade) and acetic acid (special grade) were purchased from Wako (Osaka, Japan) and was used as received. Pure water obtained with a Toraypure system (Toray, Tokyo, Japan) was used in the mobile phase. All other chemicals and reagents were of analytical-reagent or chromatographic grade.

Liquid chromatography–mass spectrometry

The mobile phase established by examination of LC separations consisted of solution A [0.1 *M* ammonium acetate–acetic acid buffer (pH 4.7)] and solution B [0.2 *M* ammonium acetate–acetic acid buffer (pH 4.7)–acetonitrile (1:1, v/v)]. Linear binary gradient elution was used (0 to 50% solution B in 20 min, then held for 10 min) at a total flow-rate of 1.0 ml/min through the column [Shimadzu Shim-pack

CLC-ODS(M), 150 mm × 4.6 mm I.D.]. Solutions A and B were degassed prior to use and were delivered by a pair of Shimadzu LC-6A pumps. A 10-μl volume of the extract from *S. glauca* was injected with a Rheodyne Model 7124 injector. The column oven temperature was 45°C. A UV detector (Shimadzu SPD-6AV) equipped with a high-pressure-resistant UV cell (maximum pressure 400 kg/cm^2) was fitted in series between the column and the mass spectrometer and UV absorbance was detected together with mass spectrometric acquisition. The UV absorbance of the verbenalin standard was measured with a Shimadzu UV-1600 instrument and the liquid chromatograph's UV detector was set at 240 nm (λ_{max} of verbenalin).

The LC eluate was introduced into a Shimadzu LCMS-QP1000 mass spectrometer equipped with a Vestec thermospray interface. The thermospray ionization mode was used for ionization. The ion source block temperature was 240°C according to previous results[8]. The measurement mass range was *m/z* 170–800.

RESULTS AND DISCUSSION

Only verbenalin has been reported as an iridoid glycoside in *S glauca*. The verbenalin standard and the extract from *S. glauca* were analysed using the mobile phase composition 0.1 *M* ammonium acetate–acetic acid buffer (pH 4.7)–acetonitrile (70:30, v/v). On the mass spectrum of the verbenalin standard (Fig. 2a), the $[M + H]^+$ ion, the proton adduct ion of the aglycone ($[A + H]^+$, A = aglycone) and the ammonium adduct ion of the aglycone ($[A + NH_4]^+$) were observed at *m/z* 389, 227 and 244, respectively. An ion cleaving at the glycoside bond appeared at *m/z* 209 and an ion derived from the sugar moiety was observed at *m/z* 180. Closer examination of the mass spectra of the verbenalin standard and the major peak corresponding to verbenalin in the extract (Fig. 2b) revealed stronger relative intensities of *m/z* 229 (the isotopic ion of $[A + H]^+$ 227), *m/z* 391 (the isotopic ion of $[M + H]^+$ 389) and *m/z* 211 (the isotopic ion of *m/z* 209) for the latter than those of the standard, while the relative intensities of *m/z* 228 ($[A + H]^+$ 227 + 1 u), *m/z* 390 ($[M + H]^+$ 389 + 1 u) and *m/z* 210 (*m/z* 209 + 1 u) were almost the same on the two mass spectra. These results

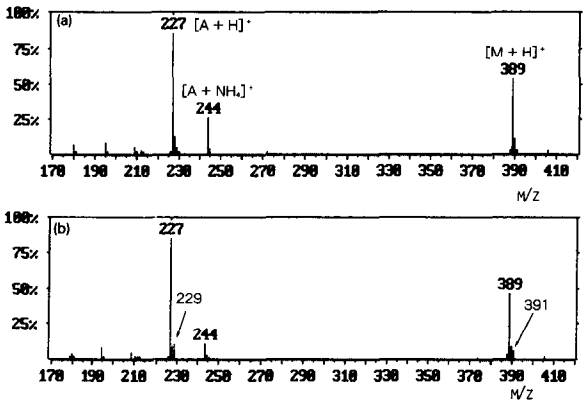

Fig. 2. Mass spectra of (a) the verbenalin standard and (b) the major peak corresponding to verbenalin in the extract. Mobile phase: 0.1 *M* ammonium acetate–acetic acid buffer (pH 4.7)–acetonitrile (70:30, v/v).

suggest that some other compounds which have a 2 a.m.u. higher mass number than verbenalin co-eluted with verbenalin in the major peak of the extract. In order to verify the existence of these unknowns, the LC separation conditions were investigated. Considering the previous study of *S. glauca* and the mass spectrum of the extract, the amount of the unknowns was expected to be small. As there is a possibility that minor components may not be detected under isocratic conditions, gradient elution was investigated.

In the examination of the gradient conditions for TSP LC–MS, attention was paid to maintaining the concentration of the electrolytes in the mobile phase constant while increasing the ratio of the aqueous solution. When using TSP LC–MS, electrolytes such as ammonium acetate are added to the mobile phase in order to promote the ion–molecular reaction, which is the main ionization mechanism in TSP LC–MS[9,10]. As the concentration of the electrolytes influences the ionization efficacy, it should be kept constant during measurements. On the other hand, a mobile phase that includes a low proportion of organic solvent and a high proportion of aqueous solution is preferable for ionization efficacy. From this viewpoint, of methanol and acetonitrile, which are commonly used in reversed-phase chromatography as organic modifiers, the latter is more suitable because it has a greater eluting ability. Considering that ammonium acetate dissolves slightly in acetonitrile, solution A [0.1 *M* ammonium acetate–acetic acid buffer (pH 4.7)] and solution B [0.2 *M* ammonium acetate–acetic acid buffer (pH 4.7)–acetonitrile (1:1, v/v)] were used. By changing the concentration of solution B from 0 to 50% linearly over 20 min, and then holding for 10 min, the major component (verbenalin) and other components could be separated satisfactorily.

The chromatogram of the extract from *S. glauca* detected by measuring the UV absorbance under the conditions mentioned above is shown in Fig. 3a and the total ion chromatogram obtained is shown in Fig. 3b. The amount of sample injected was equivalant to 0.11 mg of the leaf. The mass spectra of peaks 4 and 6 are shown in Fig. 4a

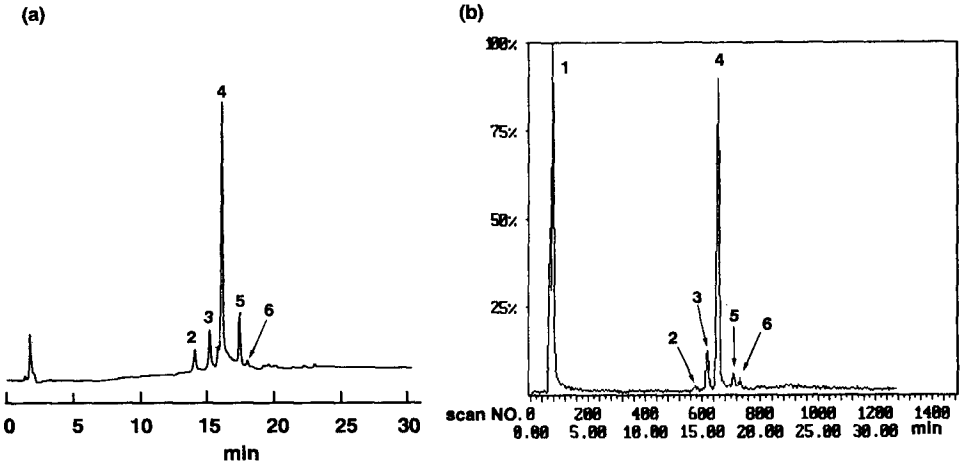

Fig. 3. (a) Chromatogram of the extract from the leaf of *S. glauca* (UV detection at 240 nm). (b) Total ion chromatogram of the extract from the leaf of *S. glauca*.

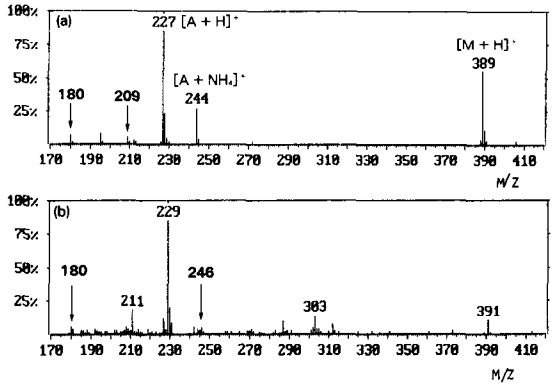

Fig. 4. Mass spectra of (a) peak 4 (verbenalin) and (b) peak 6 in Fig. 2b. A = Aglycone.

and b, respectively. Fig. 4a was in agreement with the mass spectrum of the verbenalin standard. In Fig. 4b, the peak of *m/z* 229, which is identical with the 2 a.m.u. shifted ion of the base peak of verbenalin (*m/z* 227), appeared as the base peak ion.

Further, peaks at *m/z* 391 and 211, which are identical with the 2 a.m.u. shifted ion of the $[M + H]^+$ ion and the diagnostic fragment ion, respectively, are observed. The 2 a.m.u. shifted ion from $[A + NH_4]^+$ (*m/z* 244) of verbenalin is also observed at *m/z* 246, although the intensity is weak. Whereas the quasimolecular ion and fragment ions from the aglycone moiety in Fig. 4b are shifted 2 a.m.u. from the corresponding ions in Fig. 4a, the ion from the sugar moiety appeared at *m/z* 180 in both Fig. 4a and b and is the characteristic ion of a monosaccharide.

Fig. 5 shows mass chromatograms produced by quasimolecular ions and fragment ions from the aglycone moieties of peaks 4 and 6. It was therefore confirmed that *m/z* 229, 391, 246 and 211 are all derived from peak 6. The compound shows UV absorption at 240 nm and the retention time is close to that of verbenalin. Based on these facts and considering the biosynthetic pathway of the congener, *i.e.*, verbenalin,

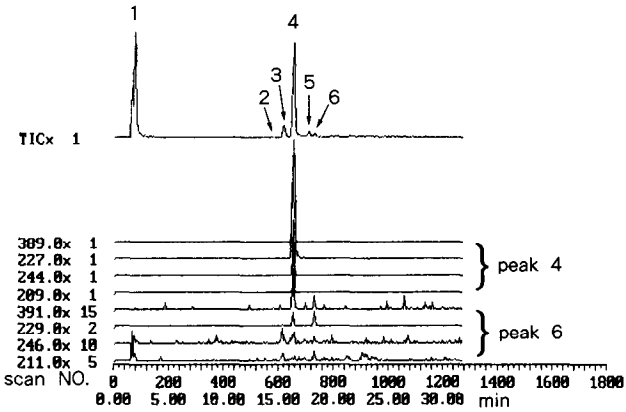

Fig. 5. Mass chromatograms produced by characteristic ions of peaks 4 and 6. × 1, etc. indicates intensity scale magnification factor.

peak 6 was presumed to be 6-dihydroverbenalin. In the structure of this compound, the ketone group of verbenalin is reduced to hydroxyl groups. As there is no possibility of reduction during the extraction procedure, this compound can be regarded as present in *S. glauca*. 6-Dihydroverbenalin is a new iridoid glycoside which has not been found in any plants hitherto. Verification of this trace component by other methods will be investigated in further work. It was recognized from the mass spectrum that peak 1, which was not detected by UV measurement but appeared in Fig. 2b, is a saccharide. Judging from their mass spectra, peaks 2, 3 and 5 are not iridoid glycosides.

CONCLUSION

TSP LC–MS has been applied satisfactorily to highly polar and thermally labile compounds such as iridoid glycosides. This method gives information about both molecular weight and structure. By optimization of the separation conditions, it was possible to identify a new component even though it was present in only trace amounts in a complex plant extract sample.

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