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Note

Rapid and micro identification of biologically active diterpenes in *Rabdosia umbrosus* var. *excisinflexus* (Labiatae) by reversed-phase high-performance liquid chromatography

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The genus *Rabdosia* previously recognized as *Isodon* belongs to the family Labiatae[1]. The interest in *Rabdosia* diterpenes is growing since they possess interesting biological activity[2]. As part of our continuing studies on biologically active substances from *Rabdosia* plants, we have examined *R. umbrosus* var. *excisinflexus* whose chemical constituent has not been reported previously. Since the presence of an α-methylenecyclopentanone moiety is essential for biological activity, the compound which has this chromophore in the molecule has been investigated.

In a previous paper, we reported that high-performance liquid chromatography (HPLC) is an efficient method for identification of these diterpenoids[3]. This paper describes the application of this HPLC technique for the crude methanol extract prepared from only one dried leaf of *R. umbrosus* var. *excisinflexus* as the simplest example. The mass spectral data which support the identification of three major diterpenoids in this plant are also discussed.

MATERIALS AND METHODS

The separations were performed on a DuPont, Model 850 liquid chromatograph. A prepacked DuPont Zorbax ODS C_{18} (particle size 5–6 μm) stainless-steel column (25 cm × 4.6 mm I.D.) equipped with Whatman stainless-steel guard column (7 cm × 2.1 mm I.D.) packed with pellicular Co:Pell ODS was used. The compounds were monitored by ultraviolet (UV) absorption at 230, 265 and 280 nm using a DuPont variable-wavelength ultraviolet spectrophotometer detector. UV spectra were recorded on a Hitachi UV-100-80 spectrophotometer with 10-mm cells. Mass spectra were obtained with a Hitachi Perkin-Elmer, Model RMU-6D, single-focusing spectrometer operating at 14 and 70 eV. The dried leaf of *R. umbrosus* var. *excisinflexus* was obtained from Aburahi Laboratories (Shionogi Research Laboratories, Sendai, Japan). The extract was prepared by homogenizing one dried leaf (45 mg) with 2 ml methanol, then passing it through 5-cm silica gel packed in a column

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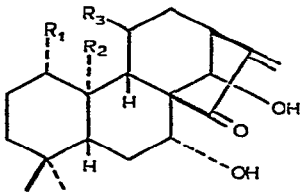
(0.5 cm O.D.) followed by 2 ml methanol as washing solution. Ten microlitres of the methanolic extract were injected into the chromatograph. Methanol solutions of the analytically pure *Rabdosia* diterpenes, from our previous studies[2], were co-injected with the plant extract using a Rheodyne rotary valve 7120 syringe-loading sample injector.

RESULTS AND DISCUSSION

During the course of our systematic studies on biologically active diterpenoids from *Rabdosia* plants, we found that the presence of an α-methylenecyclopentanone moiety is essential for their biological activity[2]. This chromophore is responsible for their UV absorption around 230 nm. Therefore, the presence of small amounts of the natural products bearing this functional group can easily be detected by monitoring the effluent at 230 nm. Our previous work indicated that the best separations were obtained on the reversed-phase C_{18} column, using methanol–water mixtures as mobile phase. Since many interfering substances are present in crude methanol extracts, a reversed-phase C_{18} pre-column was employed in order to avoid column contamination.



(1) R_1 = OH, R_2 = CH_3, R_3 = H
(2) R_1 = OH, R_2 = CH_2OH, R_3 = H
(3) R_1 = H, R_2 = CH_2OH, R_3 = OH

The presence of *ent*-kaurene diterpenoids, kamebanin (1), kamebakaurin (2) and kamebakaurinin (3), has been detected in the crude methanol extract of the leaf of *R. umbrosus* var. *excisinflexus* as three major constituents as shown in Fig. 1a. The methanol–water mixture (45:55 v/v) was the best mobile phase and the retention times under these chromatographic conditions were 16.5 min for kamebanin, 12.4 min for kamebakaurin and 6.4 min for kamebakaurinin. The backgrounds of the chromatograms were almost free of interference. These diterpenes were previously isolated from other *Rabdosia* plants[4–6] as antitumor, antibacterial and insect growth inhibitory principles. These compounds are all bitter to the taste[7]. The analytically pure samples of these bitter diterpenes were applied as internal references. The presence of these *ent*-kaurene diterpenoids in the examined extract was also checked by an increase in the sensitivity while monitoring at 230 nm, compared with measurements at 265 and 280 nm as shown in Fig. 1b and 1c, respectively. The retention time is governed by the number and stereochemical configuration of the hydroxyl groups in the tetracyclic structure; *e.g.*, compare compound 1 with 2 or 3 and diterpene 2 with 3, respectively. The lowest detection limit for these diterpenes in the crude extract was 0.1–0.2 μg of an individual compound.

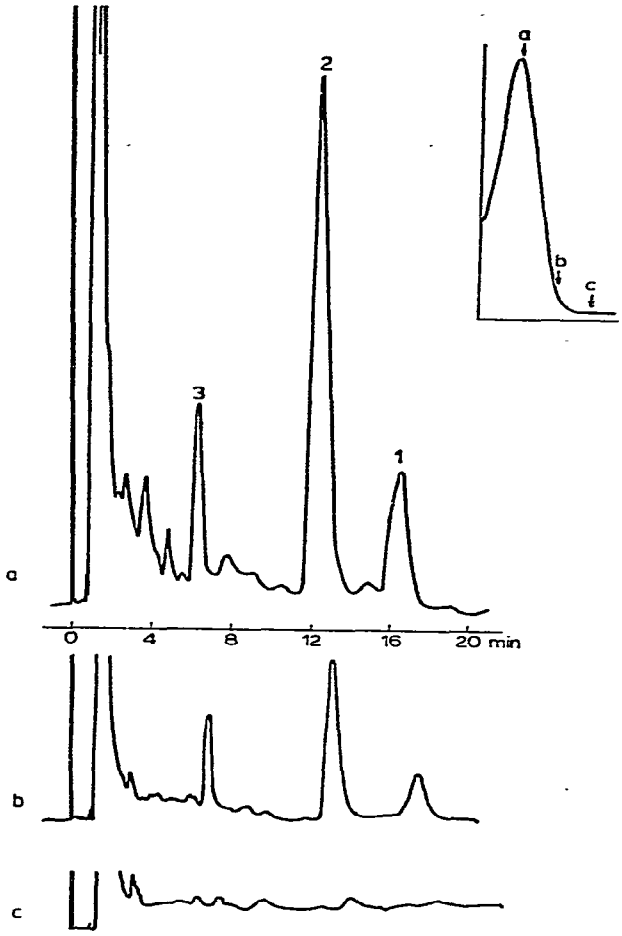
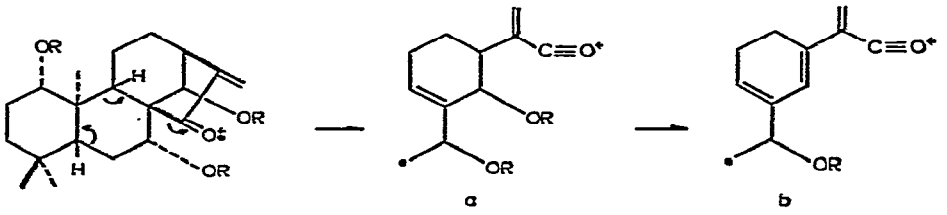


Fig. 1. Reversed-phase HPLC separation of the methanol extract of *Rabdosia umbrosus* var. *excisinflexus* constituents. Mobile phase: methanol–water (45:55 v/v); flow-rate, 2 ml/min. Detection at different wavelengths: (a) 230 nm, (b) 265 nm and (c) 280 nm.

The identification of these *ent*-kaurene diterpenoids was readily supported by mass spectral data[8]; the detailed data on kamebanin are described as an example. The high-resolution electron-impact mass spectrum of 1 shows the peaks at *m/e* 194 and 176 which were observed at *m/e* 196 and 177 in the deuterated compound. These fragments were formed by cleavage of the B-ring as shown in Scheme 1. This cleavage occurred in preference to the loss of water when the mass spectrum was taken at low energy (14 eV). Basically the same cleavage was observed in the two other diterpene compounds 2 and 3.

For isolation and characterization of *Rabdosia* diterpenes the laborious open-column chromatography has been used as a standard procedure. These results prove the usefulness of the reversed-phase HPLC technique as a rapid micro-screening procedure for identification of the biologically active diterpenoids. Within the range of the solvent system and with appropriate choice of the UV detector wavelength



	m/e	m/e	m/e
R=H	334	194	176
R=D	337	196	177

Scheme 1. High-resolution mass-spectral fragmentation of kamebanin. The intensity of fragment ion a at 70 eV was 45% while at lower energy, 14 eV, it appeared as 100%.

the possible presence of other members of these *ent*-kaurene diterpenoids could easily be detected in the extract of unknown *Rabdosia* plants. The taxon of *Rabdosia* plants has been confused; this rapid micro identification technique may offer an easy means for taxonomic marking of *Rabdosia* plants.

CONCLUSION

The identification of a variety of *ent*-kaurene diterpenes in extremely small amounts of plant material is the great advantage of this process. The saving of time and solvent by the direct injection of the crude extract is another important benefit. Similar techniques can be extended to many natural products.

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