Pergamon

Phytochemistry, Vol. 44, No. 7, pp. 1265-1270, 1997

0031-9422/97 $17.00+0.00

PII: S0031-9422(96)00723-6

A COMPARATIVE STUDY ON THREE CHEMO-TYPES OF THE LIVERWORT *CONOCEPHALUM CONICUM* USING VOLATILE CONSTITUENTS

Masao Toyota, Tetsuya Saito, Junko Matsunami and Yoshinori Asakawa*

Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Yamashiro-cho, Tokushima 770, Japan

(*Received* 12 *August* 1996)

Key Word Index—*Conocephalum conicum*; Conocephalaceae, Bryophytes; chemosystematics; chemo-type; volatiles; (+)-bornyl acetate; (−)-sabinene; methyl cinnamate; GC-MS; (+)-bornyl *p*-coumarate.

Abstract—GC-mass spectrometric analysis of 280 samples of *Conocephalum conicum*, collected in various places showed the presence of three chemo-types. One elaborated (−)-sabinene (type I) as the major compound the second produced (+)-bornyl acetate (type II) and the third contained methyl cinnamate (type III) as the major constituent. The structures of three new monoterpenic esters were established by chemical and spectral means. Copyright © 1997 Elsevier Science Ltd

---

INTRODUCTION

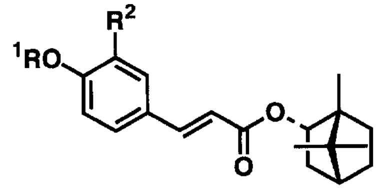
The genus *Conocephalum* (Marchantiales) comprises the thalloid *C. conicum* and *C. japonica*, which grow on wet rock or soil. *Conocephalum conicum* is one of the most widespread liverwort species in Japan and occurs in different geographical and chemical races [1, 2]. Previously, we isolated (+)-bornyl acetate [3], bornyl ferulate and bornyl 2-methoxy-4-hydroxycinnamate [4] and a sesquiterpene aldehyde [5] from the ether extracts of *C. conicum*. The GC-mass spectral analysis of 120 specimens of this species collected different places, showed the presence of three chemo-types in Japan. One of these elaborates (−)-sabinene (type I) as major compound. The other two types characteristically contain (+)-bornyl acetate (type II) and *trans*-methyl cinnamate (type III) as major constituents, respectively [6, 7]. Type-I is widely distributed while type-II grows in a coastal location in Japan. Type III is often found in mountain forests. Recently, the chemo-type III was found in southern Illinois [8].

Further investigation of this species resulted in the isolation of three new monoterpene esters (**1**, **3** and **4**) from *C. conicum* (types I and II). Here we report now the isolation of the new compounds, and the results of further investigation of the three chemo-types of *C. conicum*.

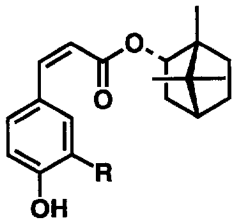
RESULTS AND DISCUSSION

Diethyl ether extracts of *C. conicum* (chemo-types I, II and III) were repeatedly chromatographed on silica gel and Sephadex LH-20, respectively. Further purification by preparative HPLC on silica gel gave two new monoterpenic esters (**3** and **4**), in addition to 11 known compounds from the type I. The ether extracts of type-II and -III of *C. conicum* were also investigated and two new monoterpenic esters (**1** and **4**), together with 12 other compounds, were isolated from the former species, and *trans*-methyl cinnamate was isolated from the latter, respectively. Compounds **2**, **6**–**15** and **17** were identified as known compounds by means of their spectral data.

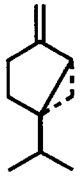
Compound **1** $[\alpha]_D$ +15.6 ($CHCl_3$, *c* 3.6), was isolated from the species of type II. The structure of **1** was deduced by comparing its spectral data with those of (+)-bornyl ferulate (**2**), isolated from the present species [4]. The EI mass spectrum of **1** showed a molecular ion peak at m/z 300 and a base peak at m/z 147. The presence of an α, β-unsaturated carbonyl, an aromatic ring and a hydroxyl group was apparent from UV absorption at 228 and 315 nm, and an intense absorption at 3343 and 1682 cm^{-1} in its IR spectrum. The methylation with diazomethane gave a monomethyl ether whose ^1H NMR spectrum exhibited a signal due to a methoxyl group at δ 3.80. This supported the presence of a phenolic hydroxyl group of **1**. The carbonyl group was shown to arise from an ester moiety, since the ^{13}C NMR spectrum of **1** had a signal at δ 168.1. The ^1H and ^{13}C NMR spectra of **1** were similar to those of **2**. Whereas the signal for a methoxyl group was missing in the ^1H NMR spectrum of **1**, the presence of a *p*-coumaroyl moiety [δ 6.86 and 7.44 (each 2H, *d*, $J = 8.8$ Hz) and δ 6.34 and 7.62



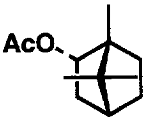
1 R^1=R^2=H
2 R^1=H, R^2=OMe
5 R^1=Me, R^2=H



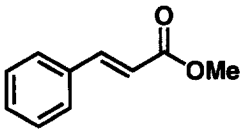
3 R=H
4 R=OMe



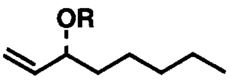
6



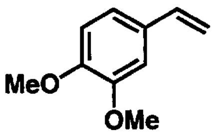
7



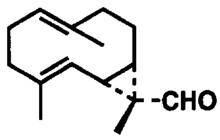
8



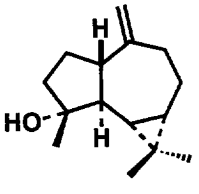
9 R=H
10 R=Ac



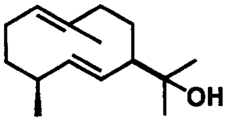
11



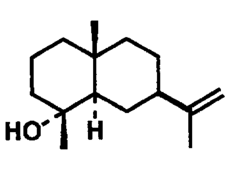
12



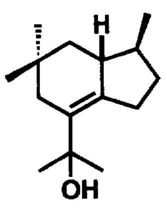
13



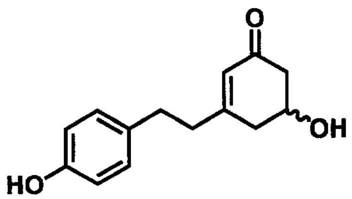
14



15



16



17

(each 1H, *d*, $J = 16$ Hz)] was confirmed. The alkaline hydrolysis of monomethyl ether **5** yielded (+)-borneol and *p*-coumaric acid methyl ether. This provided further evidence for the structure of **1**. Accordingly, the structure of **1** was established as (+)-bornyl *p*-coumarate.

Compound **3** was isolated from the plants of type I. The EI mass spectrum of **3** exhibited a molecular ion peak at m/z 300 at the same M_r as **1**. The base peak at m/z 147 corresponds to the loss of a *p*-coumaroyl radical. The presence of a *cis*-*p*-coumaroyl moiety was confirmed by the J value (13 Hz) of the doublet signals at δ 5.87 and 6.84 in the ^{1}H NMR spectrum of **3**. Thus, the structure of **3** was elucidated as bornyl *cis*-4-hydroxycinnamate.

Compound **4** $[\alpha]_D$ +18.2 (CHCl$_3$, c 0.43), was isolated from the collections of types I and II. The EI mass spectrum of **4** showed a molecular ion peak at m/z 330 and base peak at m/z 177. The presence of a carbonyl and a hydroxyl group was apparent from the IR absorption at 3383 and 1709 cm^{-1}. The ^{1}H NMR spectrum of **4** was similar to that of **2**, except for the replacement of *trans*-olefinic protons ($J = 16$ Hz) of **2** by signals at δ 5.86 and 6.79 (each 1H, *d*, $J = 13$ Hz). Since the structure of **4** was apparent from the above mentioned features, it was shown to be (+)-bornyl *cis*-4-hydroxy-3-methoxycinnamate. Prelunularin (**17**) [9] was isolated from chemo-type I.

During this investigation, 280 samples of *C. conicum*, collected at different places were further analysed by GC-mass spectrometry. This resulted in the recognition of three chemo-types in Tokushima, Japan. The samples, belonging to the type I, elaborate (−)-sabinene (**6**) as a major constituent, and type II contains a large amount of (+)-bornyl acetate (**7**). Methyl cinnamate (**8**) was detected in the species of type III as a major volatile constituent (Figs. 1 and 2). Since the species of type III emits the potent mushroom (*Tricholoma matsutake*) odour when the gametophyte is crushed, it is very easy to find the species of type III in mountains. The GC-mass spectral analysis of four irregular species which were collected in northern Japan, detected limonene, camphene or 3,4-dimethoxystyrene (**11**) as a major volatile component. 1-Octen-3-yl acetate (**10**) has been found in the all of types I, II and III, but also often encountered in many other species of liverworts. In contrast with the type I being especially widely distributed in Japan, type II grows in coastal locations. The habitat segregation between types II and III is clearly observed, although type I often associates with II or III in a coastal place or in forest (Fig. 2).

Szweykowski and Odrzyloski [10] distinguished the sibling species of European *C. conicum* using enzymic markers. Sibling species of the *C. conicum* complex can also be recognized by the naked eye in specimens growing side by side in the glasshouse. The constituents of European *C. conicum* and Japanese species of type I are very similar, except for the presence of a large proportion of a sesquiterpene alcohol, conocephalenol (**16**), in the former chemo-type. There are two chemo-types in European species and the remaining type is the same as the Japanese type I, so far examined.

The ether extract constituents of type I are very similar to those of type II whereas the GC-mass spectral analysis of both types showed that type I is clearly different from type II (Fig. 1). On the other hand, the chemical constituents of type III is not only clearly different from the former two types, but also morphologically different. We propose that Japanese *C. conicum* should be separated into at least two species.

EXPERIMENTAL

General. TLC was carried out on silica gel precoated glass plates with *n*-hexane–EtOAc (1 : 1 and 4 : 1). Detection was with Godin reagent [11]. For normal phase CC, silica gel 60 (40–63 μm) was used. CH_2Cl_2–MeOH (1 : 1) was used as solvent for CC on Sephadex LH-20. For normal phase prep. HPLC a column (250 × 10 mm i.d.) of silica gel (3.5–6.5 μm) was used.

Spectral data. NMR spectra were recorded at 100 MHz (^{13}C) and 400 MHz (^{1}H). EIMS were measured at 70 eV. Analysis of the Et_2O extracts was carried out using a gas chromatograph coupled with a mass selective detector at 70 eV and a fused silica column coated with DB-17 (30 m × 0.25 mm i.d., film thickness 0.25 μm) using He as carrier gas (1 ml min^{-1}). The temp. programming of GC-MS analysis was from 50° isothermal for 3 min, then 50–250° at 5° min^{-1}, and finally isothermal at 250° for 15 min. Injection temp. was 250°.

Preparation of the samples for GC-MS analysis. The samples of *C. conicum* (L.) Underw. collected in various places, were washed with H_2O, impurities removed and the plant materials ground in a mortar with Et_2O, then filtered through a short column (20 × 5 mm i.d.) on celite. Concn of the filtrate was done under a stream of air.

Plant material. Conocephalum conicum (L.) Underw. (type I; CC145 and CC255) (913 and 720 g) were collected in August 1994 at Kamikatsu-cho, Katsuura-gun, and in April 1996 at Katsuura-cho, Katsuura-gun, Tokushima, Japan, respectively. The samples of type II (CC248; 210 g) were collected in December 1995 at Tosashimizu, Kochi, Japan. Samples of type III (CC256; 15.2 g) were collected in April 1996 at Katsuura, Katsuura-gun, Tokushima, Japan. Voucher specimens (CC145, 248, 255 and 256) are deposited at the Faculty of Pharmaceutical Sciences, Tokushima Bunri University.

Extraction and isolation. Liverworts were dried for 1 day, impurities removed, ground mechanically and then extracted with Et_2O for 10 days. Type I (CC-145) was further extracted with MeOH for 10 days.

Type-I. The Et_2O extract (12.4 g) of *C. conicum* (CC145) was subjected to CC on silica gel using a *n*-hexane–EtOAc gradient, giving 8 frs (I–VIII). Fr. III (1.31 g) was rechromatographed on Sephadex LH-20 to give a fragrant mixt. Further purification of the mixt. by prep. HPLC on silica gel using EtOAc–*n*-hexane (1 : 9) gave **2** (203.4 mg; 1.64% of total extract), **3** (1 mg; 0.01%), **4** (19.8 mg; 0.16%), **9** (8.1 mg; 0.07%), **13** (7.32 mg; 0.06%) and **14** (12.4 mg; 0.1%). Fr. II (1.84 g) was rechromatographed on Sephadex LH-20 and on silica gel, affording **7** (13.6 mg; 0.11%) and **12** (49.2 mg; 0.40%). Fr. IV (3.06 g) was repeatedly chromatographed on Sephadex LH-20 and on silica gel, then subjected to prep. HPLC on silica gel to give **15** (2.6 mg; 0.02%). The Et_2O extract (16.9 g) of *C. conicum* (CC255; type I) was repeatedly chromatographed on Sephadex LH-20 and on silica gel, then purified by prep. HPLC using EtOAc–*n*-hexane to give **2** (80 mg; 0.47%), **4** (47.9 mg; 0.28%), **7** (14.5 mg; 0.09%), **8** (3.6 mg; 0.02%), **9** (17.1 mg; 0.10%), **10** (34.3 mg; 0.20%), **12** (76.8 mg; 0.46%), **14** (35.8 mg; 0.21%) and **17** (16.4 mg; 0.10%).

Type II. The Et_2O extract (3.2 g) of *C. conicum* (CC248) was repeatedly subjected to CC on Sephadex LH-20 and on silica gel, then to prep. HPLC or prep. TLC using EtOAc–*n*-hexane to give **1** (10.9 mg; 0.34%), **2** (24.6 mg; 0.77%), **3** (4.4 mg; 0.13%), **4** (7.04 mg; 0.22%), **7** (71.18 mg; 2.22%), **10** (14.0 mg; 0.44%), **12** (30.8 mg; 0.96%), **13** (0.7 mg; 0.02%) and **14** (4.4 mg; 0.13%).

Type III. The Et_2O extract (187.6 mg) of *C. conicum* (CC256) was repeatedly chromatographed on silica

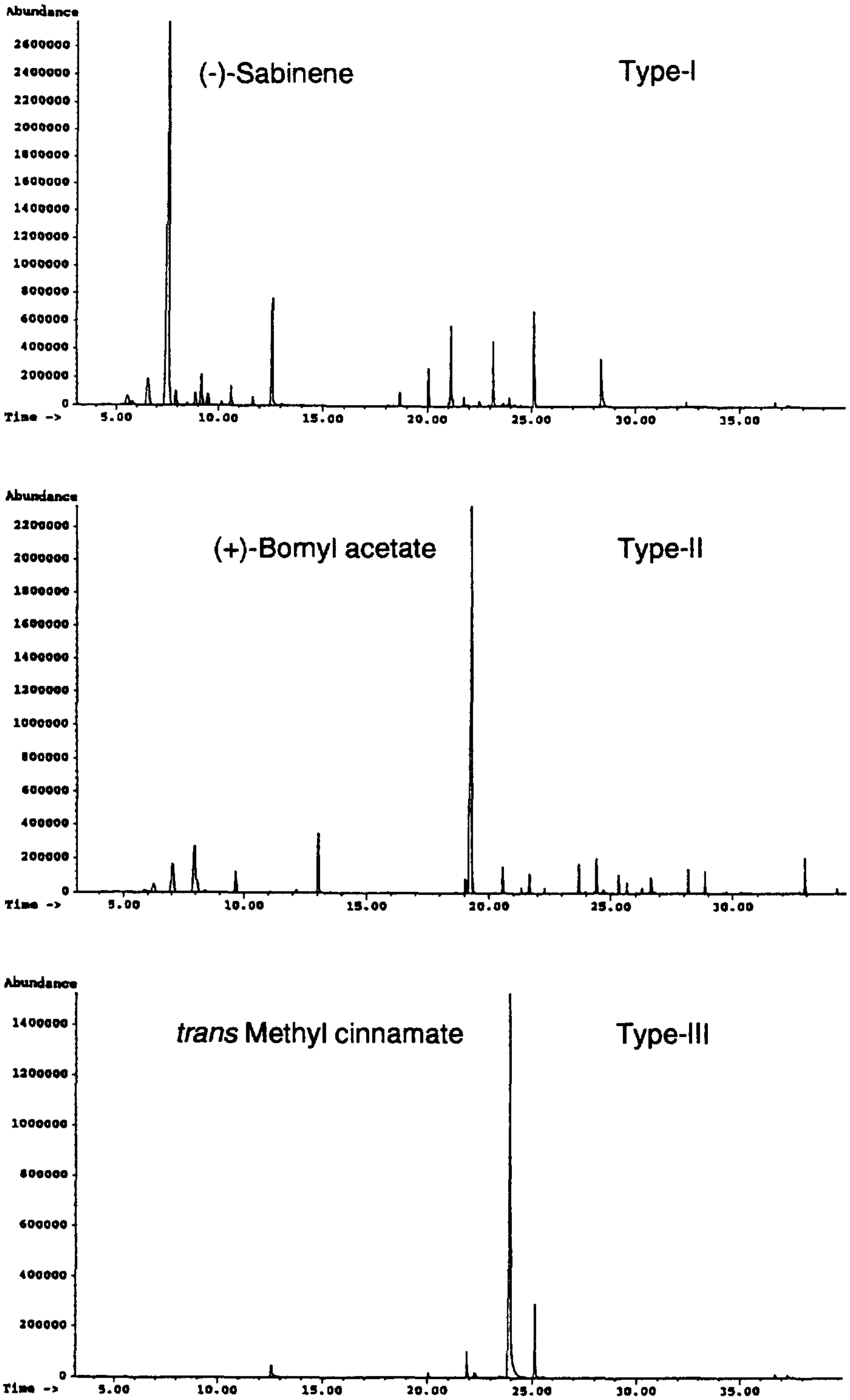


Fig. 1. TIC of the ether extracts of chemo-types I, II and III of the liverwort *Conocephalum conicum*.

gel and on Sephadex LH-20 to give **8** (8.54 mg; 4.55%).

(+)-*Bornyl* p-*coumarate* (**1**). $[\alpha]_D$ +15.6 ($CHCl_3$; *c* 3.6); FT-IR ν_{max}^{film} cm^{-1}: 3343, 1682, 1605, 1516, 1171; UV λ_{max}^{EtOH} nm (log ε): 315 (4.37), 228 (4.09), 212 (4.07); EIMS *m/z* (rel. int.): 300 $[M]^+$(15), 164 (4), 147 (100), 136 (3), 119 (7), 91 (6), 69 (2). ^1H NMR: δ 0.88, 0.90, 0.94 (each 3H, *s*), 5.01 (1H, *ddd*, *J* = 10, 4, 2 Hz), 5.96

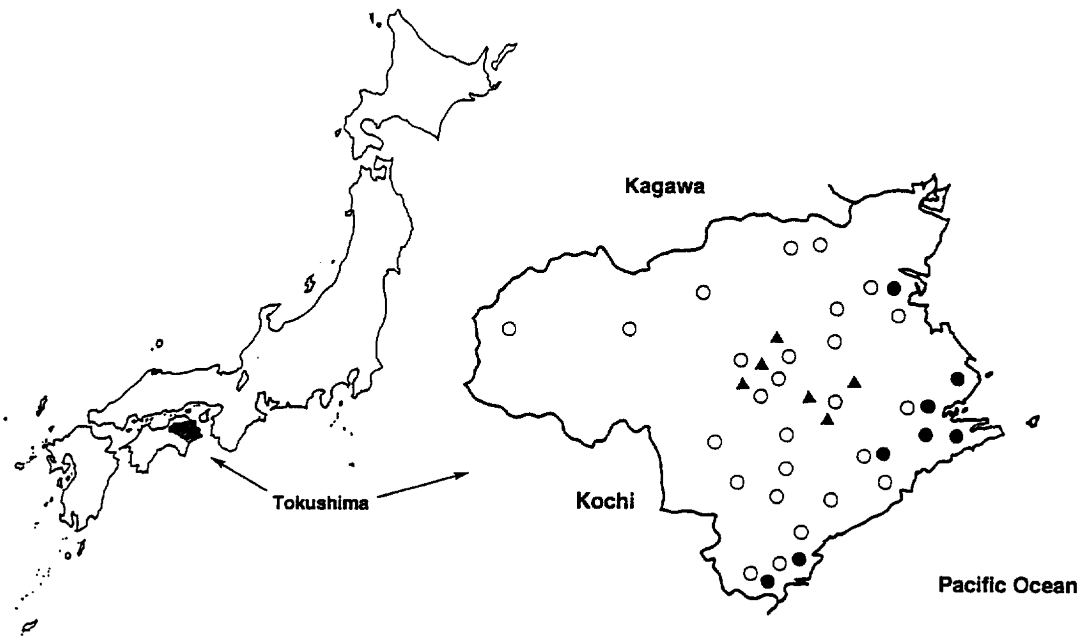


Fig. 2. The distribution of each type of *C. conicum* is shown with one symbol in the case of more than two of the same type collected within 1 km of each other. Symbols used: (○) type I, (●) type II and (▲) type III.

(1H, *s*), 6.34 (1H, *d*, *J* = 16 Hz), 6.86 (2H, *d*, *J* = 8.8 Hz), 7.44 (2H, *d*, *J* = 8.8 Hz), 7.62 (1H, *d*, *J* = 16 Hz); ^{13}C NMR (100 MHz): δ 13.6, 18.9, 19.7, 27.2, 28.1, 36.9, 45.0, 47.9, 49.0, 80.1, 115.9 (×2), 116.0, 127.2, 130.0 (×2), 144.2, 157.9, 168.1.

Bornyl cis-4-*hydroxycinnamate* (**3**). FT-IR ν_{max}^{film} cm^{-1}: 3376, 1688, 1605, 1512, 1157; UV λ_{max}^{EtOH} nm (log ε): 310 (4.54), 226 (4.38); EIMS *m/z* (rel. int.): 300 $[M]^+$(10), 177 (10), 164 (5), 147 (100), 136 (3), 119 (7), 91 (5), 81 (4), 69 (3), 55 (2); ^1H NMR: δ 0.83, 0.87, 0.91 (each 3H, *s*), 4.94 (1H, *ddd*, *J* = 10, 4, 2 Hz), 5.24 (1H, *s*), 5.87 (1H, *d*, *J* = 13 Hz), 6.80 (2H, *d*, *J* = 8.8 Hz), 6.84 (1H, *d*, *J* = 13 Hz), 7.63 (2H, *d*, *J* = 8.8 Hz).

(+)-*Bornyl* cis-4-*hydroxy*-3-*methoxycinnamate* (**4**). $[\alpha]_D$ +18.2 ($CHCl_3$; *c* 0.43); FT-IR ν_{max}^{film} cm^{-1}: 3383, 1709, 1595, 1514, 1454, 1368, 1279, 1177, 1034, 866, 829; UV λ_{max}^{EtOH} nm (log ε): 326 (4.09), 263 (3.53); EIMS *m/z* (rel. int.): 330$[M]^+$(17), 194 (8), 177 (100), 145 (15), 117 (3), 93 (3), 81 (3), 69 (2); ^1H NMR: δ 0.83, 0.87, 0.92 (each 3H, *s*), 3.92 (3H, *s*), 4.94 (1H, *ddd*, *J* = 10, 4, 2 Hz), 5.83 (1H, *s*), 5.86 (1H, *d*, *J* = 13 Hz), 6.79 (1H, *d*, *J* = 13 Hz), 6.88 (1H, *d*, *J* = 8 Hz), 7.12 (1H, *dd*, *J* = 8, 1.5 Hz), 7.67 (1H, *d*, *J* = 1.5 Hz); ^{13}C NMR (100 MHz): δ 13.5, 18.9, 19.7, 27.1, 28.0, 36.8, 44.9, 47.8, 48.8, 56.0, 79.7, 112.8, 113.9, 117.7, 125.3, 127.4, 143.2, 145.9, 146.9, 166.9.

Methylation of **1** *with* CH_2N_2. To a soln. of **1** (11 mg) in Et_2O (1 ml), excess of CH_2N_2 in Et_2O was added. The mixt. was left overnight and monomethyl ether **5** (1 mg) was purified by HPLC. ^1H NMR: δ 0.88, 0.90, 0.95, 3.84 (each 3H, *s*), 5.01 (1H, *ddd*, *J* = 9.5, 3, 1.5 Hz), 6.34, 7.63 (each 1H, *d*, *J* = 16 Hz), 6.91, 7.49 (each 2H, *d*, *J* = 8.8 Hz). EIMS *m/z* (rel. int.): 314$[M]^+$(15), 162 (13), 161 (100), 133 (7).

Alkaline hydrolysis of **5**. Compound **5** (1 mg) was dissolved in 5% KOH–MeOH (1 ml) and refluxed for 5 min. After evapn, H_2O (2 ml) was added and the mix. was extracted with Et_2O. The Et_2O layer was washed with H_2O, dried over Na_2SO_4 and borneol was identified by GC-MS. The aq. layer of the reaction mixt. was acidified with 5% HCl and extracted with Et_2O. The extract was treated with bis(trimethylsilyl)acetamide to yield a TMSi ester whose R_t and mass spectrum in the GC-MS analysis were identical to those of an authentic sample.

Acknowledgements—We thank Dr M. Mizutani (Hattori Botanical Laboratory, Nichinan, Miyazaki, Japan) for the identification of the species. This work was supported in part by a Grant-in-Aid for Scientific Research (B) (No. 08459026) from the Ministry of Education, Japan.

REFERENCES

1. Markham, K. R., Porter, L. J., Mues, R., Zinsmeister, H. D. and Brehm, B. G., *Phytochemistry*, 1976, **15**, 147.
2. Porter, L. J., *Taxon*, 1981, **30**, 739.
3. Asakawa, Y., Toyota, M. and Aratani, T., *Phytochemistry*, 1976, **15**, 2025.
4. Suire, C., Asakawa, Y., Toyota, M. and Takemoto, T., *Phytochemistry*, 1982, **21**, 349.

5. Toyota, M., Nagashima, F., Fukuyama, Y. and Asakawa, Y., *Phytochemistry*, 1988, **27**, 3317.
6. Asakawa, Y., in *Progress in the Chemistry of Organic Natural Products*, Vol. 65, eds W. Herz, G. W. Kirby, R. E. Moore, W. Steglich and C. Tamm. Springer, Vienna, 1995, pp. 1–562.
7. Toyota, M., *Journal of the Hattori Botanical Laboratory*, 1994, **76**, 273.
8. Wood, W. F., Lancaster, W. C., Fisher, C. O. and Stotler, R. E., *Phytochemistry*, 1996, **42**, 241.
9. Kunz, S. and Becker, H., *Phytochemistry*, 1994, **36**, 675.
10. Szweykowski, J. and Odrzykoski, I., in *Proceedings of the Phytochemical Society of Europe*, Vol. 29, *Bryophytes, Their Chemistry and Chemical Taxonomy*, eds H. D. Zinsmeister and R. Mues. Clarendon Press, Oxford, U.K., 1990, pp. 437.
11. Godin, P., *Nature*, 1954, **174**, 134.