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Bioactivity guided isolation of antifungal compounds from the liverwort *Bazzania trilobata* (L.) S.F. Gray

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Abstract

A dichloromethane and a methanol extract of the liverwort *Bazzania trilobata* showed antifungal activity against the phytopathogenic fungi *Botrytis cinerea*, *Cladosporium cucumerinum*, *Phythophthora infestans*, *Pyricularia oryzae* and *Septoria tritici*. Bioautography on thin-layer chromatograms was used to isolate six antifungal sesquiterpenes: 5- and 7-hydroxycalamenene, drimenol, drimenal, viridiflorol, gymnomitrol and three bisbibenzyls: 6′,8′-dichloroisoplagiochin C, isoplagiochin D and 6′-chloroisoplagiochin D. Furthermore we report the isolation of gymnomitr-8(12)-en-4-one and the new coumarin 7,8-dihydroxycoumarin-7-*O*-β-D-glucuronide. Their structures have been elucidated based on extensive NMR spectral evidence.
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Keywords: *Bazzania trilobata*; Hepaticae; Liverwort; Antifungal; Bioautography

1. Introduction

Bryophytes are known to possess various rare and novel natural products. Many of them exhibit antimicrobial effects against fungi and bacteria (Frahm and Kirchhoff, 2002). The known antifungal compounds from bryophytes belong to terpenes (Asakawa, 1981, 1984, 1990; Becker, 2001), bibenzyls (Lorimer et al., 1993), bisbibenzyls (Asakawa et al., 2000; Kámory et al., 1995), derivatives of fatty acids (Borel et al., 1993; Ichikawa et al., 1984) and acetophenones (Lorimer and Perry, 1994). *Bazzania trilobata* (L.) S. F. Gray (Lepidoziaceae) represents one of the four European species of the genus *Bazzania* that grows in dense, widespread pads on forest ground, boggy soil and trunks (Müller, 1954).

The dichloromethane and methanol extract of *B. trilobata* excerted antifungal activity against the plant pathogenic fungus *Cladosporium cucumerinum* by means of direct bioautography (Homans and Fuchs, 1970). Bioautographic assays are ideally suited for activity-guided fractionation, due to the fact that they permit a localization of active compounds within a complex matrix and thereby facilitate their efficient isolation from a crude extract (Rahalison et al., 1993). With the aid of *C. cucumerinum*, as indicator organism we isolated six sesquiterpenes and three bisbibenzyls (Fig. 1). The antifungal activity of the isolated compounds was quantified in collaboration with BASF (Limburgerhof) against the phytopathogenic fungi *Botrytis cinerea*, *C. cucumerinum*, *Pyricularia oryzae*, *Phythophthora infestans* and *Septoria tritici* (Table 1). One of the most active compounds 7-hydroxycalamenene was also tested in vivo for the protection of plants against fungal infection. Furthermore, the present report describes the isolation and structure elucidation of gymnomitr-8(12)-en-4-one and the new coumarin 7,8-dihydroxycoumarin-7-*O*-β-D-glucuronide.

2. Results and discussion

2.1. Antifungal compounds from B. trilobata

The air-dried plant material was first extracted with dichloromethane and then with methanol. The methanol

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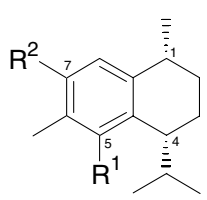
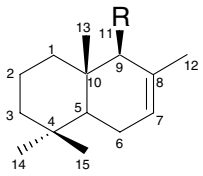
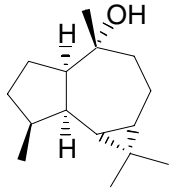


1 $R_1 = OH, R_2 = H$
2 $R_1 = H, R_2 = OH$



3 $R = CH_2OH$
4 $R = CHO$



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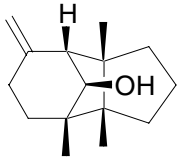

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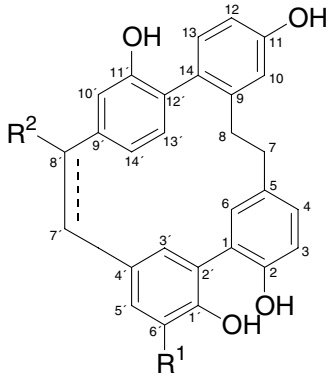

	R^1	R^2	$\Delta^{7',8'}$
7	Cl	Cl	+
8	H	H	-
9	Cl	H	-

Fig. 1. Antifungal compounds from *B. trilobata*.

extract was diluted in water and partitioned with ethyl acetate and *n*-butanol. The ethyl acetate-soluble fraction and the dichloromethane extract showed antifungal activity against the indicator organism *C. cucumerinum*. Bioautographic TLC assay-guided fractionation of the dichloromethane and the ethyl acetate extract led to the isolation of six antifungal sesquiterpenes: 5- and 7-hydroxycalamenene (**1** and **2**), drimenol (**3**), drimenal (**4**), viridiflorol (**5**), gymnomitrol (**6**) and three antifungal bisbibenzyls: 6′,8′-dichloroisoplagiochin C (bazzanin B) (**7**), isoplagiochin D (**8**) and 6′-chloroisoplagiochin D (bazzanin S) (**9**). Apart from 7-hydroxycalamenene (Burden and Kemp, 1983) and viridiflorol (Gijsen et al., 1992) the antifungal activities of the isolated compounds have not been described before.

5- and 7-Hydroxycalamenene (**1** and **2**) have already been isolated from *B. trilobata* (Nagashima et al., 1996). Both compounds gave large areas of inhibition zones when bioassayed against *C. cucumerinum* on TLC plates. Furthermore, 5-hydroxycalamenene showed strong inhibition activity against *P. oryzae* (IC50's see Table 1) in the microtiter plate tests, whereas 7-hydroxycalamenene exhibited strong antifungal activities against *B. cinerea*, *C. cucumerinum*, *P. infestans*, *P. oryzae* and *S. tritici*. Furthermore 7-hydroxycalamene was tested for its in vivo activity towards plant pathogenic fungi under glasshouse conditions. A significant reduction in the disease development, compared with the untreated control, was seen at a test concentration of 250 ppm against *P. viticola* on grape vine leaves, whereby the disease was reduced from 100% in the control to 30% in the treated plants. No report about the antifungal activity of 5-hydroxycalamenene is published so far, whereas 7-hydroxycalamenene is known as a phytoalexin from *Tilia europaea* (Burden and Kemp, 1983).

Drimenol **3** was obtained as white powder. The sesquiterpene alcohol was first identified by Huneck (1967) in *B. trilobata* by GC-MS. Drimenol was considerably less active than the hydroxylated calamenenes. The strongest activity was observed against *C. cucumerinum* and *S. tritici* (Table 1).

Compound **4** was obtained as yellow oil. Its mass spectrum ($m/z = 220$) suggested $C_{15}H_{24}O$ as molecular formula. The NMR data of compound **4** were almost identical to **3**, except for the presence of an aldehyde group (δ_H 9.68, *d*, $J = 5.0$ Hz, H-11, δ_C 206.6, C-11) and the absence of the primary alcohol group. The location of the aldehyde group (C-9) as well as the whole structure of **4** was established by HSQC, HMBC, H,H-COSY and NOESY experiments. Drimenal was previously only detected by GC-MS in the essential oil from *Drimys winteri* (Barrero et al., 2000). We could not find published NMR data for **4**, therefore we want to introduce its NMR data measured in $CDCl_3$. The double bond isomers of compound **4** has been isolated from the liverwort *Diplophyllum serrulata* (Toyota et al., 1994). Drimenal showed moderate antifungal activity against *B. cinerea*, *P. oryzae* and strong activity against *S. tritici* and *P. infestans*. The closely related polygodial with an additional aldehyde function at C-8 is a known antifungal compound from the liverwort *Porella vernicosa* (Asakawa, 1981).

Viridiflorol (**5**) was isolated as colourless oil. The weak antifungal activity of viridiflorol against *C. cucumerinum* is described in literature (Gijsen et al., 1992). Furthermore we detected weak activity against *P. oryzae* (Table 1).

Table 1
Assay results for **1**–**9**[a]

Compound	*B. cinerea*	*C. cucumerinum*	*P. infestans*	*P. oryzae*	*S. tritici*
1	>125	97.0	–[b]	1.7	53.0
2	14.2	11.8	0.9	4.1	10.0
3	>125	6.6	–[b]	>125	80.1
4	81.8	>125	<0.03	61.6	17.6
5	>125	>125	–[b]	105.2	>125
6	103.2	80.5	0.1	59.4	29.0
7	18.9	17.5	–[b]	3.9	23.5
8	7.6	13.0	–[b]	4.0	15.9
9	50.6	30.8	29.2	2.6	4.5
Folicur®	0.08	0.3	–[b]	0.8	0.1
Rovral®	<0.03	0.8	–[b]	0.3	4.0
Amistar®	104.5	0.3	–[b]	0.1	0.04

Folicur® (tebuconazole), Rovral® (iprodione), Amistar® (azoxystrobin).
[a] Averages of three seperate assays, IC_{50} in μg/ml.
[b] Not tested.

Gymnomitrol (**6**), for the first time isolated from *Gymnomitrion obtusum* (Connolly et al., 1972) showed moderate antifungal activity against *C. cucumerinum*, *P. oryzae* and *S. tritici* and strong activity against *P. infestans* (Table 1). Warmers and König (1999) reported the presence of viridiflorol and gymnomitrol in *B. trilobata*.

The known bisbibenzyls 6′,8′-dichloroisoplagiochin C (bazzanin B) (**7**) (Martini et al., 1997), isoplagiochin D (**8**) (Hashimoto et al., 1996) and 6′-chloroisoplagiochin D (bazzanin S) (**9**) (Scher et al., 2004) were also isolated by bioautographic TLC assay-guided fractionation. They gave large areas of inhibition zones on TLC and showed strong antifungal activities in the microtiter plate test (Table 1). Furthermore, we compared the antifungal activity of **9** with its permethylated derivative by bioautography against *C. cucumerinum*. Only **9** with free OH groups gave a large inhibition zone. This result, though limited, shows that free OH groups seem to be necessary for biological activity as has been found in other studies for bibenzyls (Lorimer et al., 1993). However, the antifungal activity of these bisbibenzyls has not been described in literature before. A wide range of biological activities is reported for other bisbibenzyls (Asakawa et al., 2000). The antifungal activity against *Trichophyton mentagrophytes* is published for marchantin A (Asakawa, 1984) as well as neomarchantin A and B (Scher et al., 2002). Bibenzyls, the biogenetic precursors of the bisbibenzyls are also known for their strong antifungal activities (Lorimer et al., 1993).

2.2. Other compounds from B. trilobata

Gymnomitr-8(12)-en-4-one (**10**) was obtained as yellow oil. Mass spectroscopy ($m/z = 218$) established the molecular formula $C_{15}H_{22}O$, whilst ^1H and ^{13}C NMR spectroscopy revealed the presence of an exomethylene group (δ_H 4.63 and 4.64 both *t*, $J = 2.5$ Hz, H-12a and H-12b and δ_C 109.9, *t*, C-12 and δ_C 149.5, *s*, C-8), a ketone (δ_C 221.5, *s*, C-4) and three tertiary methyl groups (δ_H 0.90, 1.06, 1.18, each *s*; δ_C 24.0, 24.8, 27.9) which, together with five methylenes (δ_C 28.2, 37.9, 43.4, 48.9, 50.1) one methine (δ_C 57.2), and three quaternary carbon atoms (δ_C 44.9, 49.8, 50.7), constitute a tricarbocyclic system. These data suggest that **10** is a gymnomitrane-type sesquiterpenoid with a 8,12-double bond and a ketone group. The location of the ketone at C-4 was established by the HMBC. Gymnomitr-8(12)-en-4-one was first isolated from the liverwort *Reboulia hemisphaerica* (Toyota et al., 1999) and now for the first time from *B. trilobata*.

Compound **11** was obtained as yellow brown amorphous powder. Its mass spectrum ($[M + H]^+$ $m/z = 355$) was in agreement with a molecular formula of $C_{15}H_{14}O_{10}$. The carbon spectrum displayed 15 carbon resonances, assigned by DEPT to six quaternary and nine tertiary carbons which accounted together with four hydroxyl groups and one carboxylic group for the expected 14 protons. The ^1H NMR spectrum showed the signal due to an anomeric proton at δ_H 5.05 (H1′, *d*, $J = 7.5$ Hz) and the ^{13}C NMR spectrum showed six carbon signals (δ_C 72.9, C-4′; 74.5, C-2′; 76.7, C-5′; 76.9, C-3′; 103.5, C-1′; 172.2, C-6′) which suggested the presence of a glucuronic acid subunit. The remaining nine signals (five quarternary and four tertiary) must be assigned to the aglycone. The ^1H NMR spectrum shows the expected signals of the glucuronic acid subunit together with the doublets of two *cis*-coupled olefinic protons ($J = 9.5$ Hz) at δ_H 6.28 (H-3) and 7.83 (H-4) and two *ortho*-coupled aromatic protons at δ_H 7.06 and 7.14 (both *d*, $J = 8.5$ Hz, H-5 and H-6). Considering the 2D NMR data, 7,8-dihydroxycoumarin is the only plausible structure for the aglycone. The HMBC (Fig. 2) and HMQC spectra confirmed the structure of **11** as 7,8-dihydroxy-7-*O*-β-D-glucuronide. Coumarins are rare compounds in liverworts. The only previous described coumarin from a liverwort is the structurally closely

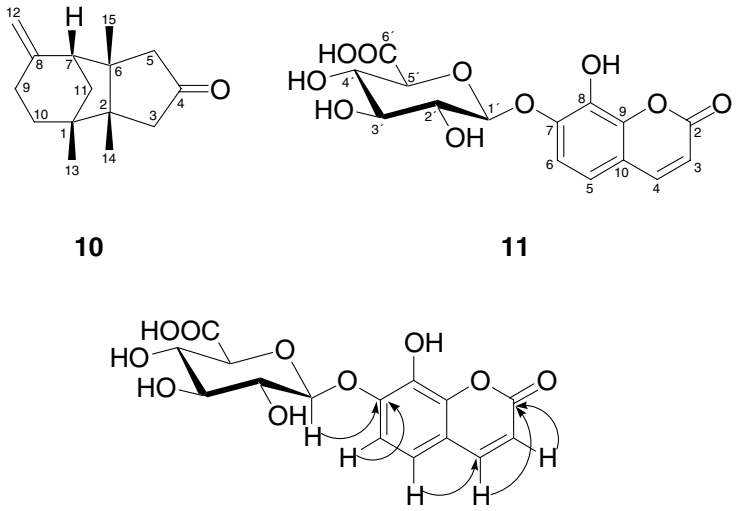

Fig. 2. Key HMBC-couplings of **11**.

related daphnin (7,8-dihydroxy-7-*O*-β-D-glucopyranoside) (Martini, 1996; Sanders, 1996; Schuld, 1997).

3. Experimental

3.1. General

All solvents were distilled before use. They were removed from the extracts by rotary evaporation under reduced pressure at temperatures up to 40 °C. Silica gel (LiChroprep, 40–63 μm, Merck), LiChroprep, 15 μm and diol modified silica gel (LiChroprep Diol, 40–63 μm LiChroprep Diol, 15 μm), were used for vacuum liquid chromatography (VLC), respectively, while thin layer chromatography was performed on silica gel (Kieselgel 60 F254, Merck), reversed phase C18 (HPTLC-Fertigplatten RP18, F254, Merck), diol and cyano modified silica (HPTLC-Fertigplatten RP18, F254, Merck). GC-MS was performed with a Hewlett Packard G1800A GCD system using helium (60 kPa, 1 ml/min) as the carrier gas. Samples were analysed on a HP-5 column (15 m × 0.25 mm ID, 0.25 μm film); CI-MS, DCI-MS data were recorded on a Finnigan MAT 90 spectrometer. The optical rotations were recorded on a Perkin–Elmer 241 polarimeter in MeOH. IR spectra were measured on a Perkin–Elmer 257 spectrometer and on a Bio Rad FTS 3000 MX excalibur FT-IR spectrometer. UV spectra were recorded on a Perkin–Elmer Lambda 2. ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) data were measured on a Bruker AM 400. Two dimensional NMR spectra were performed on a Bruker DRX 500. NMR spectra were recorded in $CDCl_3$ and CD_3OD. Chemical shifts are given in parts per million (ppm) on the δ scale relative to the solvent peaks $CHCl_3$ at δ_H 7.25, $CDCl_3$ at δ_C 77.0 and CH_3OH at δ_H 3.30, CD_3OD at δ_C 49.0. ^{13}C multiplicities were determined using the DEPT pulse sequence. Two dimensional spectra were recorded as H,H-COSY, HSQC, HMBC and NOESY experiments.

3.2. Plant material

B. trilobata (L.) S.F. Gray was collected in Rinnthal, Rheinland-Pfalz, Germany during June 1999. A voucher specimen is deposited at Herbarium SAAR, Saarbrücken (No. 6329).

3.3. Extraction and isolation

The extraction scheme followed the standard procedure of our group (Adam et al., 1998). Air dried, powdered plant material (900 g) of *B. trilobata* was sequentially extracted with CH_2Cl_2 and MeOH. The MeOH extract was evaporated in vacuo and distributed between EtOAc and H_2O. The H_2O layer was evaporated in vacuo and distributed between H_2O and *n*-butanol. The EtOAc phase and the CH_2Cl_2 extract showed antifungal activity in the direct bioautography with *C. cucumerinum*. The CH_2Cl_2 extract was evaporated in vacuo (37.61 g) and chromatographed on Sephadex LH-20 using MeOH–CH_2Cl_2 (1:1) as eluent to yield eight fractions (frs.). Frs. 4–8, which showed antifungal activity, were combined (18.02 g) and chromatographed on silica gel via VLC using a *n*-hexane–EtOAc gradient yielding nine fractions. (frs. 1–9). Fr. 4 (2.88 g) was chromatographed on silica gel via VLC using a *n*-hexane–EtOAc gradient yielding six fractions. (frs. 4.1–4.6). Fraction 4.2 yielded pure **1** (460 mg). Further separation of fr. 4.3 by HPLC (LiChropher 100 Si, 5 μm, *n*-hexane–EtOAc, 96.5:3.5) afforded **4** (6 mg) and **2** (20 mg). Further HPLC separation (LiChropher 100 Si, 5 μm, *n*-hexane--EtOAc, 95:5) of fraction 4.4 afforded **6** (12 mg) and **10** (15 mg). Fr. 5 (4.29 g) was chromatographed on silica gel via VLC using a *n*-hexane–EtOAc gradient

yielding three fractions (frs. 5.1-5.3). Fr. 5.1 yielded **5** (840 mg). Separation of fr. 5.2 using HPLC (LiChrospher 100 diol, 5 μm, *n*-hexane–EtOAc, 99:1), yielded **5** (1.60 g) and **3** (0.98 g). Fr. 9 (830 mg) was chromatographed on diol modified silica gel via VLC using a *n*-hexane–EtOAc gradient yielding four fractions (frs. 9.1–9.4). Further purification of fr. 9.4 using HPLC (LiChropher 100 Si, 5 μm, *n*-hexane–EtOAc, 60:40) yielded 4 mg of **7**.

The EtOAc phase was evaporated in vacuo (7.29 g) and chromatographed on Sephadex LH-20 using MeOH–CH_2Cl_2 (4:1) as eluent to yield six fractions (frs. 1–6). Fr. 3 (998 mg) was chromatographed on diol modified silica gel via VLC using a *n*-hexane–EtOAc gradient yielding three fractions (frs. 3.1–3.3). Further separation of fr. 3.2 by HPLC (Spherisorb CN, 5 μm, *n*-hexane–EtOAc, 60:40) afforded **8** (10 mg) and **9** (21 mg).

The butanol phase (15.3 g) was chromatographed on Sephadex LH-20 using MeOH as eluent to yield two fractions. (frs. 1–2). Fr. 1 (13.9 g) was chromatographed on RP 18 silica gel via VLC using a methanol–H_2O gradient yielding four fractions (frs. 1.1–1.4). Further purification of fr. 1.2 by RP 18 HPLC (Phenomenex Aqua, 5 μm, H_2O–acetonitrile–HCOOH, 84:15:1) afforded 20 mg of **11**.

3.4. Antifungal bioassays

Direct bioautography with *C. cucumerinum*: Crude extracts were dissolved to a concentration of 10 mg/ml in the solvent of extraction and pure compounds at a concentration of 1–2 mg/ml in $CHCl_3$ or MeOH. Ten microlitre of these solutions were applied on silica gel TLC plates (DC-Fertigplatten, Kieselgel 60, F254, Merck). TLC plates were developed in appropriate solvent systems and thoroughly dried for complete removal of the solvents. Afterwards, the chromatograms were sprayed with a spore suspension of *C. cucumerinum* in a nutritive medium and incubated for two to three days at room temperature in a moisture chamber. Clear inhibition zones appeared against a dark greenish background. Ketoconazole was used as positive control in the bioautography.

Antifungal microtiter plate test: To examine the antifungal activity of compounds **1**–**9**, a stock dilution of 10,000 ppm in appropriate solvents was prepared. Fifty microlitre were plated in triplicates in a 96-well microtiter plate with or without addition of 50 μl serial dilution of each compound. Negative control wells received 50 μl appropriate solvent without compound; positive control wells received 50 μl solutions containing Folicur® (tebuconazole), Rovral® (iprodione) and Amistar® (azoxystrobin). The necessary spore suspensions of the pathogens in a nutritive medium was prepared and 50 μl spore suspension was added. Growth after 168 h (18 °C) was measured as increased absorbance at 405 nm.

Antifungal glasshouse test: The following host plant/pathogen combinations were investigated: tomato/*Alternaria solani*, bell pepper/*B. cinerea*, cucumber/*Erysiphe cichoracearum*, wheat/*Blumeria graminis*, *Puccinia triticina*, tomato/*Phytophthora infestans*, grape vine/*Plasmopara viticola*, wheat and rice/*P. oryzae*. Young plants were sprayed to run-off with solutions of the compound at 250, 63 and 16 ppm. After treatment, the plants were allowed to dry for 24 h and then inoculated with spore suspensions of the pathogens; untreated plants served as controls. The inoculated plants were then placed under glasshouse conditions ideally suited for the development of the disease; disease development was assessed up to seven days after inoculation, the incubation period being dependant on the pathogen.

3.5. Drimenal (*4*)

Colourless oil; UV (*n*-hexane) λ_{max} 230.0 nm; IR (KBr) ν_{max} 2923, 2851, 2360, 2336, 1714, 1460, 1389, 1260, 1182, 1107, 1015, 807 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) $\delta = 9.68$ (^1H, *d*, $J = 5.0$ Hz, H-11), 5.68 (^1H, *brs*, H-7), 2.57 (^1H, *m*, H-9), 2.04 (^1H, *m*, H-6), 1.98 (^1H, *m*, H-6), 1.65 (^1H, *brddd*, H-1), 1.60 (3H, *brs*, H-12), 1.51(1H, *s*, H-2), 1.43 (1H, *brdd*, H-2), 1.43 (1H, *brdd*, H-3), 1.27 (1H, *dd*, $J = 13.2$, 3.5 Hz, H-1), 1.18 (1H, *dd*, $J = 13.3$, 3.1 Hz, H-3), 1.12 (1H, *dd*, $J = 12.0$, 4.9, H-5), 1.05 (3H, *s*, H-13), 0.90 (3H, *s*, H-15), 0.85 (3H, *s*, H-14); ^{13}C NMR ($CDCl_3$, 125 MHz) $\delta = 206.6$ (*d*, C-11), 127.8 (*s*, C-8), 125.5 (*d*, C-7), 67.6 (*d*, C-9), 49.1 (*d*, C-5), 42.0 (*d*, C-3), 40.4 (*d*, C-1), 37.0 (*s*, C-10), 33.3 (*t*, C-14), 33.0 (*s*, C-4), 23.7 (*t*, C-6), 22.0 (*t*, C-15), 21.6 (*t*, C-12), 18.3 (*d*, C-2), 15.7 (*t*, C-13); GC-MS *m*/*z* (rel. int.) $[M]^+$ 220 (1.0), 163 (27), 109 (58), 83 (100), 55 (30).

3.6. 7,8-Dihydroxycoumarin-7-*O*-β-D-glucuronide (*11*)

Brown solid; $[\alpha]_D^{20}$-75.11° (c 1.35, MeOH); UV (MeOH) λ_{max} 253.5, 305.5; IR (KBr) ν_{max} 3300, 2900, 1700, 1610, 1240, 1030 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz) $\delta = 7.84$ (1H, *d*, $J = 9.5$ Hz, H-4), 7.14 (1H, *d*, $J = 8.5$ Hz, H-6), 7.06 (1H, *d*, $J = 8.5$ Hz, H-5), 6.28 (1H, *d*, $J = 9.5$ Hz, H-3), 5.04 (1H, *d*, $J = 7.5$ Hz, H-1′), 4.00 (1H, *d*, $J = 10.0$ Hz, H-5′), 3.63 (1H, *dd*, $J = 10.0$, 9.5 Hz, H-4′), 3.60 (1H, *brd*, $J = 9.0$, 7.5 Hz, H-2′), 3.54 (1H, *dd*, $J = 9.5$, 9.0 Hz, H-3′); ^{13}C NMR (CD_3OD, 125 MHz) $\delta = 172.2$ (*s*, C-6′), 162.8 (*s*, C-2), 149.3 (*s*, C-7), 146.0 (*d*, C-4), 144.5 (*s*, C-9), 136.1 (*s*, C-8), 119.6 (*d*, C-5), 116.7 (*s*, C-10), 114.7 (*d*, C-3), 114.5 (*d*, C-6), 103.5 (*d*, C-1′), 76.9 (*d*, C-3′), 76.7 (*d*, C-5′), 74.5 (*d*, C-2′), 72.9 (*d*, C-4′); FAB-MS *m*/*z* (rel. int.) $[M]^+$ 354.5 (24.7), 340.5 (20.6), 280.7 (63.0), 221 (100), 207.1 (60.6).

The NMR data of all other compounds match literature: 5-hydroxycalamenene (**1**) (Huneck et al., 1984), 7-hydroxycalamenene (**2**) (Burden and Kemp, 1983), drimenol (**3**) (Toyota et al., 1981), viridiflorol (**5**)

(Warmers and König, 1999), gymnomitrol (**6**) (Connolly et al., 1972; Konecny et al., 1985), 6′,8′-dichloroisoplagiochin C (**7**) (Martini et al., 1997), isoplagiochin D (**8**) (Hashimoto et al., 1996), 6′-chloroisoplagiochin D (**9**) (Scher et al., 2004), gymnomitr-8(12)en-4-one (**10**) (Toyota et al., 1999).

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