New Cytotoxic Terpenoids from the Wood of *Vepris punctata* from the Madagascar Rainforest[1]

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Continuation of the chemical examination of the cytotoxic constituents of the wood of *Vepris punctata* resulted in the isolation of the two new terpenoids **1** and **2** and eight known compounds, glechomanolide (**3**), isogermafurenolide, (*E*,*E*)-germacra-1(10),4,7(11)-triene, α-amyrin, lupeol, lupeyl acetate, taraxerol, and 3-*epi*-taraxerol, in addition to the alkaloids reported reported previously. The structures of the two new compounds were established on the basis of 1D and 2D NMR spectroscopic data interpretation and chemical modifications. All the isolated compounds were tested against the A2780 human ovarian cancer cell line; the four sequiterpenoids showed moderate cytotoxic activity, while the six triterpenoids were inactive.

We recently reported the isolation of three new and four known alkaloids from the combined *n*-hexane/$CHCl_3$ fraction of a CH_2Cl_2/MeOH extract of the wood of *Vepris punctata* (I. Verd.) W. Mziray (Rutaceae).[2] In addition to the cytotoxic alkaloids found in this plant, we also noted the presence of several terpenoids. We now report the isolation and structure elucidation of two new and eight known terpenoids from this plant.

The two active fractions, D and F, previously described[2] were obtained by MCI gel chromatography of the combined *n*-hexane/$CHCl_3$ fraction of a CH_2Cl_2/MeOH extract of the wood of *V. punctata*. Reversed-phase HPLC and preparative TLC of these fractions furnished the two new terpenoids **1** and **2** and eight known compounds, which were identified as glechomanolide (**3**),[3−5] isogermafurenolide,[6] (*E*,*E*)-germacra-1(10),4,7(11)-triene,[7] α-amyrin,[8] lupeol, lupeyl acetate,[9] taraxerol, and 3-*epi*-taraxerol.[10]

Compound **1** was isolated as a colorless optically active viscous liquid, whose molecular formula was deduced as $C_{15}H_{20}O_4$ from HRFABMS and ^{13}C NMR spectral data, indicating six degrees of unsaturation. The molecular formula and the ^{13}C NMR spectrum of **1**, which showed the presence of 15 carbons, suggested its sesquiterpene nature. The absorption bands in the IR spectrum at 1748 and 1665 cm^{-1} and the UV maxima at 218.5 nm indicated the presence of an α,β-unsaturated γ-lactone moiety in **1** similar to those in glechomanolide and litseacassifolide.[11]

The ^1H NMR spectrum of **1** showed the presence of three methyl singlets at δ 1.34, 1.45, and 1.92, three oxygenated methine protons at δ 4.88 (dd, J = 11.7, 2.1 Hz), 2.72 (d, J = 8.3 Hz), and 2.68 (dd, J = 11.5, 2.5 Hz), and four methylene protons between δ 1.22 and 3.04. APT (Attached Proton Test) and HMQC spectral data showed that **1** contained two sp^2 quaternary carbons, two sp^3 quaternary carbons, three methyl groups, four sp^3 methylene groups, three sp^3 methines, and a carbonyl group (Table 1). The above spectral data (^1H and ^{13}C NMR) were similar to but not identical with those of the known compound litseacassifolide (**4**), a germacranolide diepoxide isolated earlier from a *Litsea* species.[11] In the absence of any assignable olefinic protons, the six degrees of unsaturation in the germacranolide skeleton could be satisfied by assigning two epoxide rings in **1** to C-4/C-5 and C-1/C-10, as in **4**. The ^{13}C NMR values for all the carbons were assigned on the basis of HMQC and HMBC spectral data and by comparison with the spectral data of **3**. The basic skeleton of **1** with the two epoxide rings at C-4/C-5 and C-1/C-10 was supported by COSY (H-1/H-2; H-2/H-3; H-5/H-6; H-8/H-9) and HMBC (H-1/C-2, C-9, C-10, C-14; H-5/C-3, C-4, C-6, C-7, C-15; H-8/C-7, C-9, C-10, C-11; H-13/C-7, C-11, C-12) correlations.

Table 1. NMR Data for Compounds **1** and **5** ($CDCl_3$)[a]

	1		**5**	
position	^1H	^{13}C	^1H	^{13}C
1	2.68 dd 11.5, 2.5	67.8	4.95 dd 12.1, 5.1	130.4
2a	1.45 m	25.6	2.13 m	37.0
2b	2.27 td 13.5, 3.5		2.34 m	
3a	1.30 m	35.3	2.37 m	24.9
3b	2.15 dq 14.0, 2.0		1.63 m	
4		57.6		61.3
5	2.72 d 8.3	60.9	2.55 d 8.3	63.4
6a	2.35 dd 15.2, 8.0	26.7	2.35 m	25.9
6b	3.04 d 15.2		2.93 d 14.6	
7		128.4		128.3
8	4.88 dd 11.7, 2.1	81.3	5.14 dd 11.3, 1.8	82.9
9a	1.22 m	44.1	2.16 m	47.4
9b	2.93 dd 14.0, 4.2		3.05 dd 13.6, 3.8	
10		61.1		128.6
11		158.0		159.8
12		172.8		173.2
13	1.92 s	9.1	1.91 s	9.0
14	1.34 s	24.7	1.79 s	17.1
15	1.45 s	16.9	1.27 s	16.8

[a] Assignments made on the basis of COSY, HMQC, and HMBC spectra and comparison with literature data.[3,11]

The structure and stereochemistry of **1** were established by correlation with the known sesquiterpene glechomanolide (**3**). Epoxidation of **3** with *m*-CPBA furnished a mixture of oxidized products, which were purified to yield two purified compounds and two inseparable mixtures. One of the purified compounds was identical with **1**, and the second was identified as the monoepoxide **5**. This result confirms the basic skeleton of **1** and the relative β stereochemistry of the oxymethine hydrogen at the C-8 position. The ^1H NMR spectrum of one of the mixtures had peaks

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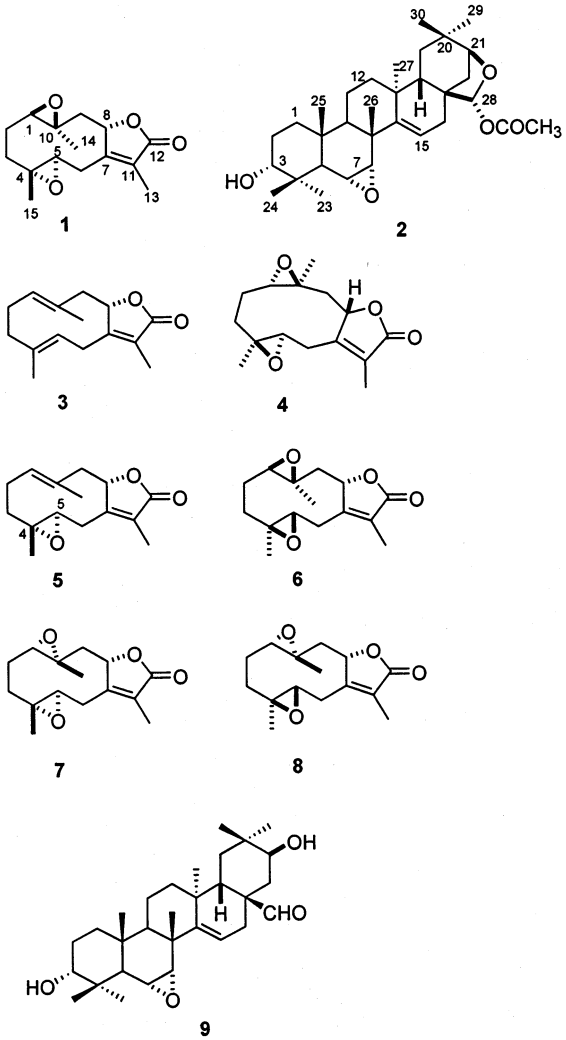
for the major component that were similar to but not identical with those of litseacassifolide (**4**), suggesting that the major component was a stereoisomer of **4**.

Since **1** was formed by the bis-epoxidation of glechomanolide, it must have one of the four stereoisomeric structures **1**, **6**, **7**, or **8**. A distinction between these structures was achieved by a combination of NOESY and molecular modeling studies. The Spartan '02 1.0.5 molecular modeling program was used to calculate the conformer distribution for each possible structure; key interproton distances were then measured on the lowest energy conformer. These distances are given in Table 2, together with selected observed NOESY correlations. From Table 2 it is evident that the observed NOESY correlations are consistent only with structure **1**. Thus NOESY correlations were observed for H-5/H-14 and H-8/H-15, and structure **1** is the only structure for which both these internuclear distances are calculated to be less than 4 Å. Conversely NOESY correlations were not observed for H-1/H-5 or H-8/H-14; such correlations would have been expected for one or more of the structures **6**−**8**.[12] On the basis of the above spectral and chemical evidence, the structure of **1** was assigned as 1β,10β:4α,5α-diepoxy-7(11)-enegermacr-8α,12-olide.



The monoepoxide **5** was characterized as 4α,5α-epoxy-1(10),7(11)-dienegermacr-8α,12-olide on the basis of 1D and 2D NMR as well as HRFABMS studies; its stereochemistry was assigned on the basis of NOE and NOESY studies. Epoxidation of **5** with *m*-CPBA furnished **1**, confirming the orientation of the C-15 methyl group.

Table 2. Energy and Bond Distances (Å) of Selected NOESY Correlations for Compounds **1** and **6**−**8**[a]

compound	energy (kcal/mol)	H-1/H-5	H-5/H-14	H-8/H-14	H-8/H-15
1	36.8	3.5	2.3	4.6	3.6
6	30.6	2.2	4.3	4.1	5.3
7	29.7	2.4	4.0	2.4	4.4
8	35.2	3.6	2.3	2.3	5.5
NOE obsd		no	yes	no	yes

[a] Calculated using the Spartan '02 v1.0.5 program.

Table 3. NMR Data for Compounds **2** and **9** ($CDCl_3$)[a]

	2		**9**	
position	^{1}H	^{13}C	^{1}H	^{13}C
1	1.84 m	32.6	1.92 m	32.6
	1.36 m		1.42 m	
2	1.86 m	25.1	2.04 m	25.4
	1.47 m		1.38 m	
3	3.40 br s	76.2	3.40 br s	76.1
4		40.5		40.2
5	1.38 m	52.6	1.44 m	52.4
6	3.91 br s	66.8	3.91 br s	67.3
7	2.66 d 7.6	73.0	2.81 d 7.3	73.6
8		44.3		42.7
9	1.88 m	46.8	1.79 m	47.6
10		37.1		36.0
11	1.28 m	19.4	1.25 m	19.8
12	1.64 m	31.5	1.50 m	31.6
13		37.8		37.8
14		162.3		160.1
15	5.46 t 2.8	119.3	5.38 t 3.3	119.9
16	2.02 m	35.2	2.04 m	32.3
	1.64 m		1.58 m	
17		57.2		58.4
18	2.35 m	44.5	2.41 m	44.8
19	1.82 m	41.5	1.72 m	41.2
	1.48 m		1.62 m	
20		25.0		27.9
21	3.91 br s	79.8	3.91 br s	75.4
22	1.58 m	28.2	1.48 m	30.8
23	0.95 s	28.0	0.96 s	28.0
24	0.84 s	16.4	0.85 s	16.9
25	1.29 s	15.2	1.28 s	15.3
26	1.32 s	23.8	1.30 s	24.4
27	1.04 s	19.6	1.05 s	19.8
28	6.24 d 4.0	96.8	ND[b]	205.8
29	1.02 s	22.2	1.02 s	22.8
30	0.88 s	31.5	0.89 s	29.2
O*C*OCH$_3$		170.1		
OCO*C*H$_3$	2.05 s	21.6		

[a] Assignments made on the basis of COSY, HMQC, and HMBC spectra and on comparison with literature data.[8,10] [b] ND: The corresponding proton signal was not detected in the ^{1}H NMR spectrum.

Compound **2** was found to have the molecular formula $C_{32}H_{48}O_5$ by HRFABMS, indicating nine degrees of unsaturation. The IR spectrum showed the presence of hydroxyl (3440 cm^{-1}) and carbonyl (1725 cm^{-1}) functional groups in its structure. It gave a positive Lieberman-Burchard test for triterpenoids. The ^{1}H NMR spectrum showed the presence of seven methyl singlets at δ 0.84, 0.88, 0.95, 1.02, 1.04, 1.29, and 1.32, an olefinic proton at δ 5.46 (t, J = 2.8 Hz), a broad singlet for a methine group connected to a secondary hydroxyl group at δ 3.40, four methine protons on oxygenated carbons at δ 2.66 (1H, d, J = 7.6 Hz), 3.91 (2H, br s), and 6.24 (1H, d, J = 4.0 Hz), and a singlet at δ 2.05 for an acetate methyl group. The ^{13}C NMR values for all 32 carbons (Table 3) were determined by APT, and HMQC and HMBC studies suggested the presence of one sp^2 and six sp^3 quaternary carbons, one sp^2 and eight sp^3 methines, seven sp^3 methylene groups, eight methyl groups, and a carbonyl group. The above spectral data (^{1}H and ^{13}C

NMR) suggested a 3-*epi*-taraxerol structure for **2**, with an additional acetyloxy group and three oxygenated methine protons in its structure. The basic skeleton of a 3-*epi*-taraxerol[10] triterpene for **2** was supported by key HMBC correlations (H-3/C-1, C-2, C-4; H-5/C-4, C-9, C-10; H-9/C-8, C-11, C-14; H-15/C-13, C-14, C-16, C-17; H-18/C-13, C-17, C-19, C-20).

Between the acetate group and the 3-*epi*-taraxerol skeleton in **2**, seven degrees of unsaturation are accounted for, leaving two double-bond equivalents to be identified. The lack of additional olefinic and carbonyl signals in the ^{13}C NMR spectrum of **2** indicated the existence of two additional rings to account for the remaining two degrees of unsaturation. The presence of a hemiacetal linkage between C-21 and C-28 and an epoxide ring between C-6 and C-7 was supported by key HMBC correlations: H-15/C-13, C-14, C-17; H-18/C-13, C-16, C-17, C-19, C-20, C-28; H-21/C-17, C-20, C-22; H-28/C-17, C-18, O*CO*CH$_3$, OCO*CH$_3$*. The presence of a hemiacetal acetate in **2** was further supported by the chemical shift of C-28 at δ 96.8. The NOESY spectrum of **2** showed correlations between H-3/H-24, H-24/H-6, and H-6/H-7, suggesting the β orientation of the oxymethine proton at C-3 and the two epoxide protons at C-6 and C-7, and thus the α orientation of the epoxide. The acetal bridge was shown to be β on the basis of a NOESY correlation between the C-21 proton and the C-29 methyl group, and by 1D NOESY studies, in which irradiation of the hemiacetal methine group at C-28 increases the intensities of the methine proton at C-18 and the methyl group at C-30.

The hemiacetal ring of **2** was opened by the hydrolysis with 2 M HCl as reported for a similar compound,[13] and the product obtained (**9**) was shown to have the molecular formula $C_{30}H_{46}O_4$ by HRFABMS. The ^1H and ^{13}C NMR values of **9** were assigned on the basis of HMQC and HMBC spectral data and are also given in Table 3. A close comparison of the NMR (^1H and ^{13}C) values of compounds **2** and **9** suggested their identical triterpene nature in rings A−D. Further, the presence of an aldehyde (δ_C 205.8) and an oxymethine (δ_C 75.4) and the absence of the hemiacetal group in **9** confirmed the cleavage of the hemiacetal ring. The presence of aldehyde and oxymethine groups at the C-28 and C-21 positions was supported by the key HMBC correlations: H-18/C-13, C-17, C-19, C-28 and H-21/C-17, C-20, C-22, C-29, C-30. The NOESY spectrum of **9**, which showed cross-peaks H-3/H-24, H-24/H-6, and H-6/H-7, suggests the relative β orientations of the oxymethine proton at C-3 and the two epoxide protons at C-6 and C-7, similar to **2**. The NOESY spectrum of **9** also showed correlations between the oxymethine proton at C-21 and the methyl group at C-29, suggesting the relative β orientation of the hydroxyl group at the C-21 position. On the basis of the above spectral and chemical data, the structure of **2** was established as 28α-acetyloxy-6α,7α:21β,-28-diepoxytaraxer-3α-ol.

All the isolated compounds were tested against the A2780 human ovarian cancer cell line, and the results are shown in Table 4. The six triterpenoids were inactive, with IC_{50} values > 20 μg/mL, while the four sesquiterpenoids showed weak cytotoxic activity, with IC_{50} values in the range 3.8−6.4 μg/mL.

Table 4. Cytotoxicities of Isolated Compounds from *V. punctata*[a]

compound	IC_{50} (μg/mL)
1	3.8
2	21.5
3	5.3
isogermafurenolide	4.5
(*E*,*E*)-germacr-1(10),4,7(11)-triene	6.4
α-amyrin	20.6
lupeol	26.4
lupeyl acetate	22.6
taraxerol	21.8
3-*epi*-taraxerol	24.4

[a] Concentration of each compound that inhibited 50% (IC_{50}) of the growth of the A2780 mammalian cell line according to the procedure described,[1,14] with actinomycin D (IC_{50} 1−3 ng/mL) as the positive control.

Experimental Section

General Experimental Procedures. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter. IR and UV spectra were measured on MIDAC M-series FTIR and Shimadzu UV-1201 spectrophotometers, respectively. NMR spectra were obtained in $CDCl_3$ on a JEOL Eclipse 500 spectrometer. Chemical shifts are given in δ (ppm) with TMS (tetramethylsilane) as internal reference, and coupling constants are reported in Hz. Sephadex LH-20 and MCI gel (CHP20P) were used for column chromatography. Mass spectra were obtained on a JEOL HX-110 instrument. Reversed-phase preparative TLC was performed on Baker Si-C_{18}F plates. HPLC was performed on a Shimadzu LC-10AT instrument with an ODS A323 column (250 × 10 mm).

Molecular Modeling. Molecular modeling of structures **1** and **6**−**8** was carried out using the Spartan '02 v1.0.5 program. The conformer distribution for each compound was calculated by molecular mechanics using the MMFF force field, and internuclear distances were measured on the lowest energy conformer of each isomer. The energies of each lowest energy conformer and the relevant distances are reported in Table 2.

Cytotoxicity Bioassays. The A2780 ovarian cancer cell line assay was performed at Virginia Polytechnic Institute and State University as previously reported.[1,14]

Plant Material and Extract Preparation. The wood sample of *V. punctata* was collected and extracted as previously described.[2]

Isolation of Compounds. Fractionation of the combined *n*-hexane- and $CHCl_3$-soluble portions of the extract to give fractions D and F has been reported.[2] Fraction D on column chromatography over MCI gel using MeOH/H_2O (70:30 to 100:0) yielded two almost equally active fractions, D_1 and D_2. Fraction D_1 on reversed-phase HPLC with the mobile phase CH_3CN/H_2O (80:20) yielded the new sequiterpenoid **1** (1.4 mg) in addition to the two known compounds glechomanolide (**3**, 5.1 mg) and isogermafurenolide (1.6 mg). Fraction D_2 on reversed-phase HPLC with the mobile phase CH_3CN/H_2O (80:20) yielded the known compound (*E*,*E*)-germacra-1(10),4,7(11)-triene (1.7 mg). Fraction F on column chromatography over MCI gel using MeOH/H_2O (80:20) furnished three active fractions, F_1, F_2, and F_3. Fraction F_1 on reversed-phase HPLC with the mobile phase CH_3CN/H_2O (75:25) furnished the new triterpenoid **2** (2.6 mg). Fraction F_2 on reversed-phase preparative TLC (MeOH/H_2O, 85:15) yielded the five known compounds (*E*,*E*)-germacra-1(10),4,7(11)-triene (3.2 mg), lupeol (2.4 mg), lupeyl acetate (2.1 mg), taraxerol (2.2 mg), and 3-*epi*-taraxerol (3.2 mg). The structures of the known compounds were identified by comparison of their spectral data with literature values.[3−10]

1β,10β:4α,5α-Diepoxy-7(11)-enegermacr-8α,12-olide (1): colorless liquid; $[\alpha]_D$ −42.4° (*c* 0.5, $CHCl_3$); UV (MeOH) λ_{max} 218.5 nm (ϵ 11 400); IR ν_{max} 2940, 2730, 1748, 1665, 1445, 1150, 1050, 850 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HRFABMS *m*/*z* 265.1441 $[M + H]^+$ (calcd for $C_{15}H_{21}O_4$, 265.1440).

Glechomanolide (3): ^1H NMR identical with the literature data[3] except that the three-proton singlet for H-15 was observed at 1.61 ppm instead of the literature value of 1.69 ppm.[15] ^{13}C NMR: δ 8.9, 16.5, 16.8, 25.7, 27.5, 38.5, 47.2, 82.8, 123.8, 126.1, 130.7, 132.5, 132.9, 162.8, 173.8.

Epoxidation of Glechomanolide (3). To a suspension of **3** (3.8 mg) and K_2CO_3 (5 mg) in $CHCl_3$ (3 mL) was added *m*-CPBA (8 mg), and the mixture was stirred at room tem-

perature for 6 h. The reaction was then diluted with 10 mL of $CHCl_3$ and washed with 5% $NaHCO_3$ (3 × 10 mL) followed by water (2 × 10 mL). The $CHCl_3$ layer was dried over anhydrous Na_2SO_4, and the solvent was removed under vacuum. The crude product (3.2 mg) showed the presence of four compounds. Purification by reversed-phase HPLC with the mobile phase CH_3CN-H_2O (80:20) furnished two pure compounds in addition to two inseparable mixtures, A (0.6 mg) and B (0.6 mg). One of the two pure compounds was identified as **1** (0.4 mg) based on its spectral data (^{1}H NMR and EIMS). The second pure compound was a colorless viscous oil characterized as 4α,5α-epoxy-1(10),7(11)-dienegermacr-8α,12-olide (**5**): UV (MeOH) λ_{max} 221.5 nm (ϵ 12 680); IR ν_{max} 2950, 1742, 1653, 1440, 1165, 1045, 860 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; HRFABMS *m/z* 249.1492 $[M + H]^+$ (calcd for $C_{15}H_{21}O_3$, 249.1491). Attempts to purify the mixtures A and B were unsuccessful, but their ^{1}H NMR spectrum resembled those of **1** and **4**, and their HRFABMS indicated that they were mixtures of diepoxide products of glechomanolide, presumably with alternative epoxide stereochemistries.

28-Acetyloxy-6α,7α:21*β*,28-diepoxytaraxer-3α-ol (2): colorless solid; $[\alpha]_D$ +21.4° (*c* 0.25, $CHCl_3$); IR ν_{max} 3440, 2935, 2450, 1725, 1170, 1040, 925, 825 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 3; HRFABMS *m/z* 513.3586 $[M + H]^+$ (calcd for $C_{32}H_{49}O_5$, 513.3578).

Acid Hydrolysis of 2. To a solution of compound **2** (1.4 mg) in dioxane (2 mL) was added 2 M HCl (1 mL), the solution was stirred at 60 °C, and the reaction was monitored by TLC. After 2 h the starting material was found to be absent, the mixture was dried under vacuum, and the crude product obtained was purified over reversed-phase HPLC (CH_3CN-H_2O, 8:2), furnishing a product (0.8 mg) that was characterized as 6α,7α-epoxy-3α,21*β*-dihydroxytaraxer-28-al (**9**) as a white amorphous solid: $[\alpha]_D$ +56.6° (*c* 0.42, $CHCl_3$); IR ν_{max} 3420, 2955, 2455, 1160, 1030, 940, 820 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 3; HRFABMS *m/z* 471.3469 $[M + H]^+$ (calcd for $C_{30}H_{47}O_4$, 471.3474).

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Supporting Information Available: ^{1}H NMR spectra for compounds **1** and **2** and structures of all isolated compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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(15) The original paper reporting the structure **4** does not record the chemical shift of these protons, but a careful measurement of the spectrum reproduced in the paper indicates that this peak occurred at 1.62 ppm. This was confirmed by correspondence with Dr. Morikawa of Dr. Yoshikawa's group, who confirmed that this resonance in their sample of glechomanolide (**5**) occurred at 1.63 ppm.

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