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Cytotoxic *ent*-kaurane diterpenoids from *Isodon weisiensis* C. Y. Wu

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Received June 30, 2004, accepted August 3, 2004

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Pharmazie 60: 458–460 (2005)

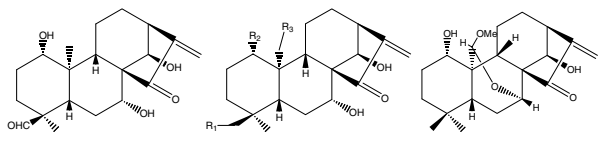
A new *ent*-kaurane diterpenoid, weisiensin B (**1**), was isolated from the leaves of *Isodon weisiensis* C. Y. Wu, along with four known ones, kamebanin (**2**), kamebacetal A (**3**), *macrocalyxin D* (**4**) and excisanin D (**5**). Their structures were determined by spectroscopic means. Compound **1**–**4** showed significant cytotoxic activity against Bel-7402 and HO-8910 cells.

1. Introduction

More than 400 *ent*-kaurane diterpenoids have been isolated from plants belonging to the genus *Isodon*, most of which have significant biologic activity (Sun et al. 2001; Hwang et al. 2001). *Isodon weisiensis* C. Y. Wu, which has been used in Chinese folk medicine to treat gastric ulcer and enteritis, is distributed in south of Gansu province and Yunnan province, P.R. China. In previous investigations, only two *ent*-kaurenoids, weisiensin A and trichorabdal A (Xu and Wu 1989) were isolated from *I. weisiensis* C. Y. Wu, which was collected in Yunnan province. Our search for bioactive diterpenoids from leaves of *I. weisiensis* C. Y. Wu, which was collected in Zang county of Gansu province, led to the isolation of a new diterpenoid, namely weisiensin B (**1**), together with four know ones, kamebanin (**2**), kamebacetal A (**3**), macrocalyxin D (**4**) and excisanin D (**5**). Compound **1**–**4** showed significant cytotoxic activity against Bel-7402 and HO-8910 cells. We report herein the isolation and structural elucidation of a new *ent*-kauranoid, as well as four known *ent*-kauranoids, from leaves of *I. weisiensis* C. Y. Wu.

2. Investigations, results and discussion

After chromatographic purification on silica gel, the EtOAc soluble portion of the Me_2CO extract give a new *ent*-kauranoid, weisiensin B (**1**), and four known *ent*-kauranoids, kamebanin (**2**), kamebacetal A (**3**), macrocalyxin D (**4**) and excisanin D (**5**).



1 **2** R_1=R_3=H, R_2=OH; **4** R_1=OXO, R_2=H, R_3=OH; **5** R_1=OAc, R_2=OH, R_3=H **3**

Weisiensin B (**1**) was isolated as colorless needles and its molecular formula was established as $C_{20}H_{28}O_5$ from its HRFABMS (m/z 349.1929 $[M+1]^+$, calcd. 349.1937). The UV spectrum (λ_{max}^{MeOH} 237 nm), IR (ν_{max} at 1726 and 1651 cm^{-1}), and NMR spectra [^{1}H NMR δ 6.28 and δ 5.30 (each 1 H, s); ^{13}C NMR δ 205.7, δ 150.2, δ 115.8] showed an *exo*-methylene group conjugated with a carbonyl group on a five-membered ring (Hou et al. 2001). The 20 carbon atoms found in the ^{13}C and DEPT NMR spectra of **1** consisted of two methyl carbons, five methylenes, six methine carbons including three oxygenated ones, three quaternary carbons, two olefinic carbins, one ketonic carbon, and one aldonic carbon, which obviously suggested a diterpene skeleton. The characterization from the spectral data and the chemotaxonomic considerations on the *Isodon* species suggested that compound **1** was C-20-non-oxygenated-*ent*-kaur-16-en-15-one, which was substituted by three hydroxyl and one aldo group.

Weisiensin B (**1**) differs from the known compound kamebanin (**2**) (Zhang and Sun 1989) by the addition of one aldo group (Table 1). The singlet at δ 9.26 was assigned to the proton of 18-CHO, which was deduced because of presence of only two methyls at δ 14.1 (Me-19) and δ 15.1 (Me-20), and the upfield shifts of C-3 (δ 32.1), C-5 (δ 44.7) and C-19 (δ 14.1) due to a γ-gauche shielding shift effect between 18-CHO and C-3, C-5, C-19, respectively (Wang et al. 2001a). The downfield shift of C-10 and the upfield shift of C-20 in NMR spectra suggested one hydroxyl group at C-1 (Wang et al. 1997 b). The peak form (dd) and the coupling constants (J = 10.0 Hz, 6.8 Hz) of H-1

Table 1: ^{13}C NMR data of compound 1 and compound 2 [100 MHz, in C_5D_5N]

C	1 (δ_C)	2 (δ_C)	C	1 (δ_C)	2 (δ_C)
1	79.1	79.1	11	20.3	19.8
2	30.2	29.0	12	31.6	31.5
3	32.1	38.8	13	47.2	46.9
4	49.7	32.6	14	75.8	74.6
5	44.7	51.0	15	208.1	207.6
6	28.0	29.1	16	150.2	149.0
7	73.5	73.3	17	115.8	115.6
8	62.6	61.2	18	205.7	32.9
9	56.0	56.0	19	14.1	21.3
10	44.6	44.5	20	15.1	14.5

Table 2: ^{1}H-^{1}H COSY, HMBC and NOSEY results of compound 1 [in C_5D_5N]

H	δ_H (J, Hz)	^{1}H-^{1}H COSY ((H)	NOSEY (H)	HMBC (C)
H-1β	3.51 dd (10.0, 6.8)	2β	H-5β, H-9β	9, 20
H-2β	1.16	2α	H-1β	1, 3, 4, 18, 19
H-2α	1.50 ddd (13.6, 13.6, 4.0)	2β, 9β	H-19, H-20	
H-3β	1.68 t (4.0)	2β	H-18	1, 4, 5, 18, 19
H-3α	1.70 t (4.4)	2β	H-18, H-19	1, 4, 5, 18, 19
H-5β	1.61 m	6β, 7β	H-1β, H-6β, H-7β, H-9β	4, 7, 9, 10
H-6β	1.93 m	5β, 19 (Me)	H-5β, H-7β, H-18, H-19	5
H-6α	1.83 ddd (6.8, 6.4, 2.0)	19 (Me)		5, 7, 8
H-7β	4.83 dd (12.0, 3.6)	5β	H-5β, H-6β, H-9β	8, 9, 14
H-9β	1.96 s	1β, 12β	H-1β, H-5β, H-7β,	5, 8, 10, 11, 12, 14, 20
H-11β	1.65 m	11α, 12α	H-5β, H-9β	8, 9, 10, 12, 13
H-11α	3.59 dd (13.6, 4.4)	11β, 12β	H-1α, H-12α, H-20	9
H-12β	2.23 m	7β, 11α		11, 13, 14, 16
H-12α	2.13 ddd (7.2, 5.6, 2.0)	11β	H-13α, H-14α	8, 10
H-13α	3.23 br s	11β, 12β, 14α, 17a	H-12α, H-14α, H-17a	12, 14, 15
H-14α	5.23 br s	13α	H-12α, H-13α, H-20	8, 15, 16, 17
H-17a	6.28 s	17b, 13α	H-17b, H-13α	
H-17b	5.30 s	17a, 13α	H-17a	
H-18	9.26 s			4, 19
Me-19	1.14 s		H-2α, H-20	3, 4, 5, 18
Me-20	1.42 s		H-19, H-14α, H-11α, H-2α	1, 9, 10

resulted from the α-orientation of 1-OH (Sun et al. 2001). The chemical shift values for C-13 and H-14, δ 47.2 and δ 5.23 (br, s), respectively, suggested that there was also an oxygen substituent at the 14β-position (Wang et al. 1997b). Furthermore ^{1}H-^{1}H COSY, HMQC, and HMBC techniques disclosed that three hydroxyl groups and one aldo group were located at C-1, C-7, C-14 and C-18, respectively (Table **2**), and NOESY spectra exhibited correlations for H-1β with H-5β and H-9β, H-7β with H-5β and H-9β, H-14α with H-12α and Me-20. Thus, the structure of weisiensin B was elucidated to be 1α, 7α, 14β-trihydroxy-18-formyl-*ent*-kaur-16-en-15-one.

Four know diterpenoids were also isolated from the same plant, which were identified by comparison of their spectral data with those in the literature (Wang et al. 1985; Xu and Wu 1989; Sun et al. 2001), as kamebanin (**2**), kamebacetal A (**3**), macrocalyxin D (**4**) and excisanin D (**5**).

Compounds **1**–**4** were examined for their cytotoxicity against two kinds of human tumor cells. As shown in Table **3**, these compounds exihibited significant inhibitory effects on human tumor Bel-7402 and HO-8910 cells with IC_{50} values lower than 30.45 μmol/ml in the SRB assay.

3. Experimental

3.1. Equipment

M.p.: Kofler-microscope (Reichert) uncorr. Optical rotation: Polarimeter 241 (Perkin Elmer) solvent C_5H_5N or MeOH. IR-spectra were recorded on an IFS-120H IR spectrometer. ^{1}H and ^{13}C NMR spectra were recorded with an INOVA-400 (Varian), solvent C_5D_5N and DMSO-d_6, using TMS as internal standard. EIMS and FABMS were determined on a ZAB-HS mass spectrometer.

Table 3: Cytotoxicity of compounds 1–4

		IC_{50} (μmol/ml)	
Test substance	MW	Bel-7402	HO-8910
1	348	15.42 ± 2.06	21.60 ± 2.60
2	334	9.86 ± 1.32	14.54 ± 3.31
3	362	30.45 ± 3.25	28.65 ± 1.64
4	348	9.77 ± 1.03	9.26 ± 1.25

3.2. Plant material

The leaves of *Isodon weisiensis* C. Y. Wu were collected in Zang county of Gansu province, People's Republic of China, June 2003, and were identified by Prof. Sun Kun and a voucher specimen (XCC-03-6-15) was deposited in College of Life Sciences, Northwest Normal University.

3.3. Extraction and isolation

The dried leaves of *I. weisiensis* C. Y. Wu were extracted with 70% Me_2CO and filtered. The filtrate was concentrated and extracted with EtOAc successively. EtOAc extract (80 g) was applied to a silica gel column eluting with $CHCl_3$–Me_2CO gradient system to yield fractions I–IV. All fractions were collected and combined by monitoring with TLC. Each fraction was further purified by recrystallization obtaining weisiensin B (**1**), kamebanin (**2**), kamebacetal A (**3**), macrocalyxin D (**4**) and excisanin D (**5**).

3.3.1. Weisiensin B (**1**)

White needles (MeOH); m.p. 208–210 °C; $[\alpha]_D^{20}$ −68° (c 0.2, C_5H_5N); UV λ_{max}^{MeOH} 237 nm; IR ν_{max}^{KBr} cm^{-1}: 3466, 3278, 2933, 2873, 1726, 1651, 1406, 963; FAB-MS m/z: 371 $[M + Na]^+$, 349 $[M + H]^+$, 331 $[M + H{-}H_2O]^+$, 313 $[M + H\text{-}2 \times H_2O]^+$, 285, 176, 89, 57; HRFABMS m/z: 349.1929 $[M + 1]^+$ (calculated for $C_{20}H_{28}O_5$, 349.1937).
^{1}H NMR (400 MHz C_5D_5N) and ^{13}C NMR (100 MHz C_5D_5N) see Table 1.

3.3.2. Kamebanin (**2**)

Colorless needles (MeOH); m.p. 238–240 °C; $[\alpha]_D^{20}$ −86° (c 0.5, C_5H_5N); UV λ_{max}^{MeOH} nm: 235; IR ν_{max}^{KBr} cm^{-1}: 3350, 1742, 1636, 1080, 1045; EIMS m/z: 334 $[M]^+$, 316 $[M{-}H_2O]^+$, 298 $[M{-}2\,H_2O]^+$, 283, 195, 176, 121; ^{1}H NMR (400 MHz C_5D_5N) δ: 6.19 and 5.25 (each 1 H, br s, H_2-17), 5.95 (1 H, br s, H-14α), 4.66 (1 H, m, H-7β), 4.41 (1 H, dd, J = 11.2 Hz, 6.8 Hz, H-1β), 3.16 (1 H, m, H-13α), 1.36 (3 H, s, Me-20), 0.83 (3 H, s, Me-18), 0.80 (3 H, s, Me-19). ^{13}C NMR (100 MHz C_5D_5N) see Table 1.

3.3.3. Kamebacetal B (**3**)

Colorless needles (MeOH); m.p. 214–216 °C; $[\alpha]_D^{20}$ −43° (c 0.2, MeOH); UV λ_{max}^{MeOH} nm: 234; IR ν_{max}^{KBr} cm^{-1}: 3523, 3510, 1718, 1640, 1230, 1018, 980; EIMS m/z: 362 $[M]^+$ (27), 331 $[M{-}OCH_3]^+$ (11), 313 $[M{-}OCH_3{-}H_2O]^+$ (36), 294 (6), 269 (35), 230 (100). ^{13}C NMR (100 MHz DMSO-d_6) δ: 74.8 (C-1), 29.8 (C-2), 39.0 (C-3), 33.5 (C-4), 47.7 (C-5), 24.7 (C-6), 65.2 (C-7), 57.1 (C-8), 50.0 (C-9), 42.3 (C-10), 22.6 (C-11), 31.6 (C-12), 42.9 (C-13), 69.4 (C-14), 205.6 (C-15), 153.1 (C-16), 115.4 (C-17), 31.2 (C-18), 20.3 (C-19), 101.0 (C-20), 54.7 (OMe). ^{1}H NMR (400 MHz DMSO-d_6) δ: 5.74 and 5.22 (each 1 H, br s, H_2-17), 5.31 (1 H, d, J = 2.0 Hz, H-20), 5.11 (1 H, d, J = 3.2 Hz, H-14α), 4.10 (1 H, d, J = 2.0 Hz, H-7β), 3.37 (3 H, s, OMe-20), 2.88 (1 H, d, J = 10.0 Hz, H-13α), 0.91 and 0.78 (each 3 H, s, 2 × Me).

3.3.4. Macrocalyxin D (**4**)

White powder (MeOH); m.p. 218–220 °C; $[\alpha]_D^{20}$ −78° (c 0.37,C_5H_5N); UV λ_{max}^{MeOH} nm: 235; IR ν_{max}^{KBr} cm^{-1}: 3445, 3350, 3311, 2910, 1714, 1640,

1210; FABMS m/z: 371 $[M + Na]^+$, 349 $[M + H]^+$, 331, 313, 285, 177, 89, 57. ^{13}C NMR (100 MHz C_5D_5N) δ: 34.2 (C-1), 17.5 (C-2), 32.1 (C-3), 49.5 (C-4), 45.7 (C-5), 32.3 (C-6), 73.9 (C-7), 61.9 (C-8), 54.9 (C-9), 41.9 (C-10), 18.5 (C-11), 30.8 (C-12), 47.4 (C-13), 76.5 (C-14), 208.2 (C-15), 150.3 (C-16), 115.9 (C-17), 205.6 (C-18), 14.9 (C-19), 60.4 (C-20). ^1H NMR (400 MHz C_5D_5N) δ: 9.26 (1 H, s, CHO), 6.34 and 5.65 (each 1 H, br s, H_2-17), 5.38 (1 H, s, H-14α), 4.97 (1 H, dd, J = 12.0 Hz, 4.8 Hz, H-7β), 4.28 and 4.16 (each 1 H, ABd, J = 12.0 Hz, H_2-20), 3.29 (1 H, br s, H-13α), 1.21 (3 H, s, Me-19).

3.3.5. Excisanin D (**5**)

White powder (MeOH); m.p. 144–146°C; $[\alpha]_D^{20}$ −52° (c 0.46, C_5H_5N); UV λ_{max}^{MeOH} nm: 232; IR $\nu_{max}^{KBr}cm^{-1}$: 3566, 3345, 1728, 1710, 1645, 1438; FABMS m/z: 415 $[M + Na]^+$, 393 $[M + H]^+$, 375, 357, 315, 297, 43. ^{13}C NMR (100 MHz C_5D_5N) δ: 79.8 (C-1), 29.6 (C-2), 33.5 (C-3), 37.2 (C-4), 46.6 (C-5), 29.5 (C-6), 74.0 (C-7), 62.5 (C-8), 56.7 (C-9), 45.5 (C-10), 20.4 (C-11), 32.0 (C-12), 47.4 (C-13), 76.1 (C-14), 208.8 (C-15), 150.7 (C-16), 115.6 (C-17), 72.5 (C-18), 17.8 (C-19), 15.6 (C-20). ^1H NMR (400 MHz C_5D_5N) δ: 6.29 and 5.36 (each 1 H, br s, H_2-17), 5.25 (1 H, s, H-14α), 4.83 (1 H, dd, J = 12.0 Hz, 4.2 Hz, H-7β), 4.12 and 3.85 (each 1 H, ABd, J = 11.8 Hz, H_2-18), 3.68 (1 H, m, H-1β), 3.52 (1 H, br s, H-13α), 1.90 (3 H, s, OAc), 1.38 (3 H, s, Me-20), 1.21 (3 H, s, Me-19).

3.4. Cytotoxicity against human tumor Bel-7402 and HO-8910 cells

The SRB assay was performed according to the method of Skehan et al. (Paoazisis et al. 1997), with minor modifications. Culture medium was aspirated prior to fixation of the cells by the addition of 200 µl 10% cold trichloroacetic acid. After 1 h incubation at 4 °C, cells were washed five times with deionised water. Then the cells were stained with 200 µl 0.1% SRB dissolved in 1% acetic acid for at least 15 min and subsequently washed four times with 1% acetic acid to remove unbound stain. The plates were left to dry at room temperature and bound protein stain was solubilized with 200 µl 10 mM unbuffered TRIS base and transferred to 96-well plates for reading the optical density (OD) at 540 nm (Biorad 550 microplate reader, Nazareth, Belgium).

Acknowledgements: The authors are grateful to Professor Sun Kun (College of Life Science, Northwest Normal University, PR China) for his help in identification of the plant material, and to Professor Xin-Pin Yang (National Laboratory of Applied Organic Chemistry and Analytic Center, Lanzhou University) for measurement of EIMS and FABMS spectra. This work was supported by the Innovation project Council of Northwest Normal University (Project No. 02).

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