Cytotoxic Germacranolide Sesquiterpene from *Inula cappa*

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A new germacranolide, inulacappolide (1), was isolated from the EtOH extract of the whole plant of *Inula cappa* along with 16 known compounds. The structure of inulacappolide was a rare 1(10)-saturated type of germacran-6,12-olide, identified as 2α-acetoxy-3β-hydroxy-9β-angeloyloxygermacra-4-en-6α,12-olide by spectral analysis (IR, HR-ESI/MS, ^{1}H-NMR, ^{13}C-NMR, HMQC, HMBC, NOESY). *In vitro*, it showed antiproliferative effects against human cervical cancer HeLa, human leukemia K562 and human nasopharyngeal carcinoma KB cell lines with IC_{50} values of 1.2 μM, 3.8 μM and 5.3 μM, respectively.

Key words *Inula cappa*; germacranolide sesquiterpene; inulacappolide; cytotoxicity

Inula cappa DC is a widespread plant growing in the south of China. The whole plant and the roots have traditionally been used for treating rheumatoid arthritis, malaria, dysentery and hepatitis.[1)] The characteristic constituents of the genus *Inula* are sesquiterpene lactones,[2)] especially eudesmanolides, guaianolides, and germacranolides. Earlier investigators reported the isolation of sesquiterpene lactones[3)] and inositol derivatives[4)] from *I. cappa* DC. As one of the components in Ya-Jiao-Ha-Dun San,[5)] a famous formula of medicines of Dai nationality, the active constituents of *I. cappa* DC are not clear. This paper reports the isolation of a new germacranolide (**1**) along with 16 known compounds from the whole plant of *I. cappa* DC. The structures of the known compounds were determined to be friedelin (**2**),[6)] epifriedelanol (**3**),[7)] α-amyrin (**4**),[8)] β-amyrin (**5**),[9)] oleanolic acid (**6**), ursolic acid (**7**), stigmast-4-en-3-one (**8**),[10)] stigmasta-4,22-dien-3-one (**9**),[11)] β-sitosterol (**10**), stigmasterol (**11**),[12)] 7-oxo-β-sitosterol (**12**),[13)] stigmast-5-ene-3β,7β-diol (**13**),[14)] stigmasta-5,22-diene-3β,7β-diol (**14**), stigmast-5-ene-3β,7α-diol (**15**), stigmasta-5,22-diene-3β,7α-diol (**16**), and daucosterol (**17**) by analysis of physical and spectroscopic evidence, and confirmed by comparison of their spectral data with values reported in the literature or those of authentic samples. In addition, the cytotoxic activities of **1** were determined *in vitro* against human cervical cancer HeLa, human leukemia K562, and human nasopharyngeal carcinoma KB cell lines.

The ethanol extract from the whole plant of *I. cappa* DC gave a new germacranolide, inulacappolide (**1**). The molecular formula of **1** was determined to be $C_{22}H_{30}O_7$ by HR-ESI-MS ($[M+Na]^+$, 429.1927, Calcd 429.1889). The IR spectrum shows absorption bands of hydroxyl (3475 cm^{-1}), double bonds (3050, 1680, 1650, 981 cm^{-1}), an α-methylene-γ-lactone moiety (1774 cm^{-1}), a saturated ester (1737 cm^{-1}), and an α,β-unsaturated ester (1712 cm^{-1}).

The methyl singlet at δ 2.13 in the ^{1}H-NMR spectrum and the signals at δ 171.62 and 21.34 in the ^{13}C-NMR spectrum were attributed to the signals of an acetoxy group. The presence of an angeloyl group in **1** was suggested by the signals for protons at δ 6.09 (qq, J=7.0, 1.5 Hz, H-3′), 1.86 (3H, quintet, J=1.5 Hz, H-4′), and 1.97 (3H, dq, J=7.0, 1.5 Hz, H-5′) in the ^{1}H-NMR spectrum and the signals for carbons at δ 167.37, 138.79, 127.68, 20.62, and 15.93 in the ^{13}C-NMR spectrum. The fragment ion peaks at m/z 83 and 55 in EI-MS of **1** also supported this conclusion.

The ^{13}C-NMR spectrum of **1** showed 15 carbons excluding the signals of an acetoxy ester group and an angeloyl group, which suggests a sesquiterpene skeleton. The presence of the α-methylene-γ-lactone moiety[15)] was deduced from the carbon signals at δ 138.20, 120.05, and 169.63 in the ^{13}C-NMR spectrum of **1**. Consequently, in the ^{1}H-NMR spectrum two characteristic doublets appeared at δ 6.28 (d, J=3.5 Hz, H-13a) and 5.68 (d, J=3.5 Hz, H-13b), both coupled to H-7 at δ 2.41 (m, $W_{1/2}$=22 Hz) in the H–H COSY spectrum. The H-7 also coupled with H-8α at δ 2.11 (m) and H-6 at δ 4.62 (t, J=10.5 Hz). The coupling constants between H-6 and H-7 ($J_{6,7}$=10.5 Hz) indicated a *trans* fused lactone ring,[16)] which was bound to a germacraenolide system. The ^{1}H-NMR spectrum showed an oliefinic proton at δ 5.55 (d, J=10.5 Hz, H-5) and a methyl doublet at δ 1.92 (3H, d, J=1.0 Hz, H-15), indicating the presence of a tri-substituted C_4–C_5 double bond. The corresponding signals of olefinic carbons appeared at δ 140.24 and 127.00 in the ^{13}C-NMR spectrum. A triplet at δ 4.62 (J=10.5 Hz) was coupled with H-7 and H-5 in the ^{1}H–^{1}H COSY spectrum, which indicated a *trans*-axial relationship between H-5, H-6, and H-7, *i.e.* H-5α, H-6β, and H-7α oriented.[15,16)] These assignments were based on the fact that H-7 has been assumed to be α-oriented as in all other naturally occurring germacranolides.[17)] Furthermore, the observed NOEs between H-5/H-7 were in agreement with the *syn*-α-disposition of H-5/H-7. No NOESY correlation between H-5 and 15-CH_3 indicated an *E*-configuration of the 4,5 double bond.

Four signals of the oxygenated carbons were observed at δ 78.05, 80.38, 78.68, and 80.08, which coupled with the protons at δ 5.15 (ddd, J=7.0, 3.0, 3.0 Hz, H-2), 4.41 (d, J=7.0 Hz, H-3), 4.74 (dd, J=11.5, 3.0 Hz, H-9), and 4.62 (t, J=10.5 Hz, H-6), respectively, in the HMQC spectrum. The ^{1}H–^{1}H COSY spectrum of **1** displayed cross peaks between H-9 and H-8β at δ 1.45 (ddd, J=15.0, 11.5, 3.0 Hz), H-8β and H-8α at δ 2.11 (m), H-8α and H-7, H-2 and H-3, and H-2 and H-1. The correlation between δ_H 4.74 and δ_C 167.37 in the HMBC spectrum (Fig. 1, Table 1) indicated that the angeloyl group was located on C-9. However, no long-range correlation between the acetoxyl carbon and protons was observed. The downfield shift of the H-2 signal required the acetoxyl group at C-2.[15)] To elucidate the stereochemistry of compound **1**, the 3D model of its structure was established and the most stable conformation was obtained by the energy

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Table 1. The ^{1}H- (500 MHz) and ^{13}C-NMR (125 MHz) Data of Inulacappolide (**1**) ($CDC1_3$)

	C	H	HMBC	NOESY
1	30.14	1.56 (ddd, J=16.0, 10.0, 3.0 Hz, 1β)	H-9, 10, 14	H-2, 1α, 14
		1.72 (dd, J=16.0, 3.0 Hz, 1α)		H-2, 1β, 8β
2	78.05	5.15 (ddd, J=7.0, 3.0, 3.0 Hz)	H-1α, 3, 10	H-1β, 1α, 3, 15
3	80.38	4.41 (d, J=7.0 Hz)	H-5, 15	H-5
4	140.24	—	H-3, 6, 15	
5	127.00	5.55 (d, J=10.5 Hz)	H-3, 15	H-7, 3
6	80.08	4.62 (t, J=10.5 Hz)	H-8β	H-8β, 15
7	45.52	2.41 (ddd, J=10.5, 7.0, 3.5 Hz)	H-8β, 5, 13a, 13b, 15	H-5, 9
8	28.56	1.45 (ddd, J=15.0, 11.5, 3.0 Hz, 8β)	H-6, 10	H-1α, 6, 8α
		2.11 (m, 8α)		H-13b
9	78.68	4.74 (dd, J=11.5, 3.0 Hz)	H-8β, 14	H-7
10	28.96	2.13 (m)	H-1α, 1β, 8β, 14	
11	138.20	—		
12	169.63	—		
13	120.05	6.28 (d, J=3.5 Hz, 13a)		H-13b
		5.68 (d, J=3.5 Hz, 13b)		H-13a, 8α
14	20.52	1.10 (3H, d, J=6.5 Hz)	H-1α, 8α, 10	H-9, 1β
15	12.49	1.92 (3H, d, J=1.0 Hz)	H-3, 5	H-1α, 2, 6
1′	167.37	—	H-4′	
2′	127.68	—	H-4′, 5′	
3′	138.79	6.09 (qq, J=7.0, 1.5 Hz)	H-4′, 5′	H-4′, 5′
4′	20.62	1.86 (3H, quintet, J=1.5 Hz)	H-3′, 5′	H-3′
5′	15.93	1.97 (3H, dq, J=7.0, 1.5 Hz)		H-3′
1″	171.62	—	H-2″	
2″	21.34	2.13 (3H, s)	H-14	

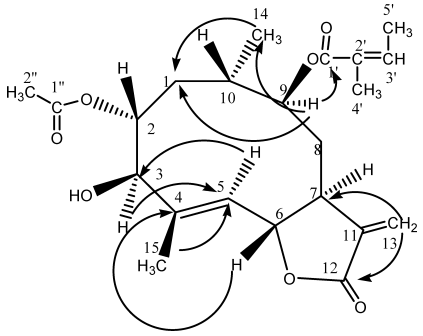

Fig. 1. The Key HMBC of Compound **1**

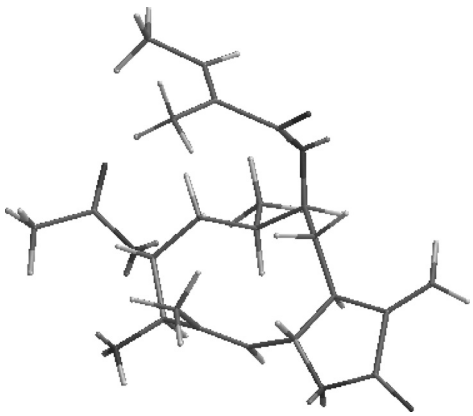

Fig. 2. The Minimized Energy Conformation of Compound **1**

minimization using MM2 computations in Chem3D (Fig. 2). The dihedral angle between H-2 and H-3 is about 160°, which is in agreement with the experimental coupling constant between H-2 and H-3. Also, this conformation explains the observed NOEs. The cross peaks between H-5 and H-3, and 15-CH_3 and H-2 in the NOESY experiment indicated the H-3 is α-oriented and H-2 β-oriented. The interactions between H-9 and H-7, and 14-CH_3 in the NOESY experiment suggested the β-orientation of the angeloyl group at C-9 and α-orientation of 14-CH_3 at C-10. The established most stable conformation supported the above conclusions. Thus, compound **1** was identified as 2α-acetoxy-3β-hydroxy-9β-angeloyloxygermacra-4-en-6α,12-olide and named inulacappolide.

Most of the germacran-6,12-olides have a double bond or epoxide at the 1(10) and 4(5) positions. Compound **1** represents a rare 1(10)-saturated type[3] and is the first one reported with cytotoxic activity. The cytotoxicity assay showed that **1** has antiproliferative effects against human cervical cancer (HeLa), human leukemia (K562), and human nasopharyngeal carcinoma (KB) cell lines, with IC_{50} values of 1.2 μM, 3.8 μM, and 5.3 μM, respectively.

Experimental

General Melting points are uncorrected. Optical rotations were recorded on a Perkin-Elmer 241 automatic recording spectropolarimeter; IR spectra were measured on a IMPACT-400 instrument; ^{1}H-NMR (500 MHz), ^{13}C-NMR (125 MHz) and 2D NMR spectra were recorded on a Bruker AM 500 spectrometer. ESI-MS were recorded on Esquire-LC 00054, and EI-MS on a Zabspec E mass spectrometer; Silica gel (100—200 mesh) used for column chromatography and silica gel GF254 used for TLC were purchased from Qingdao Marine Chemical Factory, China. Sephadex LH-20 was the product of Pharmacia.

Plant Material The whole plant of *I. cappa* DC was collected in August 2000 from Xishuangbanna, Yunnan province, China, and identified by Prof. Zai-Lin Li, Yunnan Branch, IMPLAD, China. A voucher specimen (YN2000A) is deposited in the Herbarium of IMPLAD.

Extraction and Isolation Air-dried and powdered plant material (7.2 kg) was extracted with 95% EtOH at room temperature and successively extracted with 70% EtOH with reflux. The ethanol extracts (425 g) was suspended in water and partitioned with $CHCl_3$. The $CHCl_3$-soluble fraction was concentrated, suspended in 90% EtOH and partitioned with petroleum ether.

The petroleum ether portion (71 g) was subjected to column chromatogra-

phy over silica gel eluted with petroleum ether (60—90 °C)–EtOAc (10 : 0—0 : 10) to yield nine fractions. Fr. 2 was purified on a silica gel column eluted with petroleum ether (60—90 °C)–EtOAc (100 : 1) to yield **2** (1 g). Successive silica gel column chromatography of fr. 4 using a petroleum ether (60—90 °C)–EtOAc (gradient, 98 : 2—95 : 5), and petroleum ether (60—90 °C)–Me_2CO (97 : 3) afforded **4** and **5** (500 mg), and **8** and **9** (80 mg), respectively. Fr. 5 was repeatedly chromatographed on silica gel eluted with petroleum ether (60—90 °C)–EtOAc to yield **10** (3 g), **6**, and **7** (20 mg), successively. Fr. 6 was separated on preparative TLC [petroleum ether (60—90 °C)–Me_2CO 8 : 2] to give **11** (2 mg) and **12** (8 mg).

The 90% EtOH portion (60 g) was subjected to column chromatography over silica gel eluted with $CHCl_3$–MeOH (10 : 0—0 : 10) and four fractions were obtained. Fr. 1 was chromatographed over Sephadex LH-20 ($CHCl_3$: MeOH 1 : 1) and then silica gel eluted with petroleum ether (60—90 °C)–EtOAc (10 : 1) to give **3** (5 mg); **1** (30 mg) was obtained from fr. 2 by repeated silica gel and Sephadex LH-20 column chromatography. Fr. 3 was subjected to column chromatography over silica gel eluting with $CHCl_3$–MeOH (10 : 0—0 : 10) to give **13** and **14** (8 mg), **15** and **16** (10 mg), and **17** (10 mg), respectively.

Compound **1**: White powder, mp 99—101 °C, $[\alpha]_D$ +115° (c=0.112, MeOH); HR-ESI-MS m/z: 429.1927 $[M+Na]^+$ ($C_{22}H_{30}O_7Na$, Calcd 429.1889); IR ν_{max} cm^{-1}: 3475, 3050, 2798, 2795, 1774, 1737, 1712, 1680, 1650, 1456, 1379, 981; (+)ESI-MS m/z: 429 $[M+Na]^+$; EI-MS m/z (%): 306 (1), 246 (3), 218 (2), 83 (100), 55 (50), 43 (40); CD (MeOH, 0.05 mg/ml) $[\theta]_{260}$ −11.98 (neg max). ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) data: see Table 1.

Cytotoxicity Assay The cytotoxic activities of compound **1** were evaluated against HeLa, K562, and KB cell lines. HeLa, K562, and KB cells were maintained in RPMI-1640 (Gibco) containing 10% FBS (Gibco), 2 mg/ml of sodium bicarbonate, 100 μg/ml of penicillin sodium salt, and 100 μg/ml of streptomycin sulfate. Cells were grown to 70% confluence, trypsinized with 0.05% trypsin-EDTA 2 mM, and plated for experimental use. In all experiments, cells were grown in RPMI-1640 medium with 10% FBS for 24 h prior to treatment. All compounds were dissolved in DMSO at a concentration of 100 mM, then diluted in tissue culture medium and filtered before use. HeLa, K562, and KB cells 1.0×10^4 were seeded in 96-well tissue culture plates and treated with the test compounds or vehicle (0.1% DMSO) at various concentrations and incubated for 48 h, followed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay at 570 nm. Briefly, IC_{50} values of the test compounds in different cell lines were obtained from the concentration-effect curves. Each experiment was repeated at least three times and the combined data were compared using Student's paired t-test.

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