Three New Diterpenoids from *Isodon nervosus*

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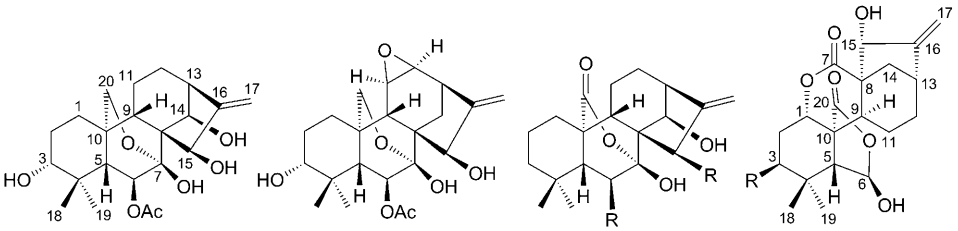
Three new *ent*-kaurane diterpenoids, rabdonervosins D–F (**1**–**3**), were isolated from the leaves and stems of *Isodon nervosus*. Their structures were elucidated on the basis of spectroscopic methods including 1D- and 2D-NMR analyses. Compounds **1**–**3** were evaluated for their cytotoxicity against HepG2, CNE2, PC-9/ZD, HeLa, MCF-7, and HCT116 cell lines. No compounds exhibited potent cytotoxicity.

Introduction. – *ent*-Kauranoids are the major secondary metabolites isolated from *Isodon* (Labiatae) plants, which are reported to have some important bioactivities, such as antibacterial and anticancer activities [1]. *Isodon nervosus* (HEMSL.) C. Y. WU et H. W. LU, a perennial herb, is distributed in Henan, Sichuan, Guangdong, Jiangxi, and some other provinces of China. Its stems and leaves have been used satisfactorily for the treatment of acute infectious hepatitis, snakebite, and skin itching [2]. Previous studies on this herb revealed the presence of three types of diterpenoids: C(20)-nonoxygenated *ent*-kauranes, C(20)-oxygenated *ent*-kauranes, and 6,7-seco-*ent*-kauranes (including enmein and compounds of the spiro-lactone type) [3–10]. In our continuing research for new bioactive natural products from this plant, we investigated the leaves and stems of *I. nervosus* collected from the Yifeng region of Jiangxi Province, P. R. China, which led to the isolation of the three new diterpenoids **1**–**3**. In this article, we present the isolation, structure elucidation, and biological evaluation of these new compounds.



Results and Discussion. – Rabdonervosin D (**1**) was obtained as colorless needles. The molecular formula $C_{22}H_{32}O_7$ was determined from the negative-mode HR-ESI-MS (*m*/*z* 407.2052 ($[M-H]^-$)), indicating seven degrees of unsaturation. On the basis of

the characteristic signals of three CH groups (δ(C) 55.8, 45.9, and 43.7), three quaternary C-atoms (δ(C) 53.2, 40.4, and 36.3), two Me groups (δ(C) 28.0 and 16.4), an O-bearing CH_2 group (δ(C) 66.1), and a hemiketal quaternary C-atom (δ(C) 98.7), we assume that **1** should be a (7α)-7,20-epoxy-*ent*-kaurane diterpenoid, similar to phyllostachysin C (**4**) [11]. Comparison with the ^{1}H- and ^{13}C-NMR data of **4** indicated that **1** (*Tables 1* and *2*) was identical to phyllostachysin C, except for the disappearance of an epoxy structure at C(11) and C(12) and an additional OH substituent at C(14). The above conclusion was further confirmed by the degrees of unsaturation and HMBC data of **1** (*Fig. 1*). The relative configuration of OH–C(3) and OH–C(15) were determined on the basis of the ROESY data (*Fig. 1*). The correlations H–C(3)/H_β–C(1) and H_β–C(5) confirmed that OH–C(3) was α-orientated, and no correlation between H–C(15) and H_β–C(9) indicated that OH–C(15) was β-orientated. The upfield shifts of C(19), C(18), and C(9) owing to the γ-steric-compression effects of OH_α–C(3) and OH_β–C(15) also supported the above described partial results. Therefore, **1** is identified as (3α,6β,7α,14*R*,15β)-7,20-epoxy-*ent*-kaur-16-ene-3,6,7,14,15-pentol 6-acetate.

Table 1. *^{1}H-NMR Data* (400 MHz, C_5D_5N) *of Compounds* **1**–**3**. δ in ppm, *J* in Hz.

	1	**2**	**3**
H_α–C(1)	1.10–1.20[a])	1.46–1.50[a])	–
H_β–C(1)	1.25–1.30 (*m*)	2.20–2.25[a])	4.78 (*dd*, *J* = 6.8, 11.5)
H_α–C(2)	1.68–1.74 (*m*)	1.35–1.40[a])	1.96–1.98[a])
H_β–C(2)	1.64–1.68[a])	1.19–1.23 (*m*)	1.96–1.98[a])
H_α–C(3)	–	1.35–1.40[a])	1.30–1.32[a])
H_β–C(3)	3.48 (br. *d*, *J* = 8.3)	1.15–1.18 (*m*)	1.30–1.32[a])
H_β–C(5)	1.76 (*d*, *J* = 5.9)	1.88 (*d*, *J* = 7.0)	2.41 (*s*)
H_α–C(6)	5.97 (*d*, *J* = 6.0)	5.89 (*d*, *J* = 7.0)	5.96 (*s*)
H_α–C(9)	–	–	3.73 (*dd*, *J* = 3.0, 12.0)
H_β–C(9)	2.68 (*dd*, *J* = 6.1,12.6)	2.76–2.79[a])	–
H_α–C(11)	1.40–1.44[a])	1.84–1.87 (*m*)	1.55–1.58 (*m*)
H_β–C(11)	1.10–1.20[a])	1.46–1.50[a])	2.26 (*s*)
H_α–C(12)	2.31 (*ddd*, *J* = 9.0, 9.0, 13.4)	2.30 (*ddd*, *J* = 8.9, 8.9, 13.6)	1.64–1.69 (*m*)
H_β–C(12)	1.64–1.68[a])	0.98–1.01[a])	2.17–2.20 (*m*)
H_α–C(13)	2.83 (br. *d*, *J* = 9.2)	2.76–2.79[a])	–
H_β–C(13)	–	–	2.69–2.73 (*m*)
H_α–C(14)	4.94 (br. *s*)	4.88 (*s*)	1.77 (*dd*, *J* = 4.8, 11.6)
H_β–C(14)	–	–	2.06 (*d*, *J* = 11.6)
H_α–C(15)	5.53 (br. *s*)	6.72 (br.*s*)	–
H_β–C(15)	–	–	5.61 (br. *s*)
CH_2(17)	5.67 (*s*), 5.37 (*s*)	5.43 (br. *s*), 5.29 (*dd*, *J* = 1.1, 2.4)	5.49 (*s*), 5.15 (br. *s*)
Me–C(18)	1.24 (*s*)	0.9 (*s*)	1.03 (*s*)
Me–C(19)	1.42 (*s*)	1.0 (*s*)	0.92 (*s*)
CH_2(20)	4.27 (*d*, *J* = 9.7), 4.02 (*d*, *J* = 9.7)	–	–
AcO	2.20 (*s*)	2.37 (*s*)	–
AcO	–	2.20 (*s*)	–

[a]) Overlapped.

Table 2. *^{13}C-NMR Data* (100 MHz, C_5D_5N) *of Compounds* **1**–**3**. δ in ppm.

	1	**2**	**3**
C(1)	29.5 (*t*)	31.8 (*t*)	74.1 (*d*)
C(2)	28.2 (*t*)	17.1 (*t*)	25.7 (*t*)
C(3)	77.1 (*d*)	41.0 (*t*)	37.1 (*t*)
C(4)	40.4 (*s*)	34.3 (*s*)	31.7 (*s*)
C(5)	55.8 (*d*)	51.2 (*d*)	52.5 (*d*)
C(6)	74.1 (*d*)	71.7 (*d*)	98.5 (*d*)
C(7)	98.7 (*s*)	106.0 (*s*)	175.0 (*s*)
C(8)	53.2 (*s*)	52.4 (*s*)	52.3 (*s*)
C(9)	43.7 (*d*)	45.4 (*d*)	37.1 (*d*)
C(10)	36.3 (*s*)	44.9 (*s*)	47.3 (*s*)
C(11)	15.2 (*t*)	19.2 (*t*)	18.1 (*t*)
C(12)	32.3 (*t*)	30.0 (*t*)	32.8 (*t*)
C(13)	45.9 (*d*)	45.2 (*d*)	37.2 (*d*)
C(14)	76.2 (*d*)	74.9 (*d*)	34.3 (*t*)
C(15)	73.1 (*d*)	73.6 (*d*)	78.1 (*d*)
C(16)	160.9 (*s*)	157.3 (*s*)	159.6 (*s*)
C(17)	110.4 (*t*)	112.2 (*t*)	109.2 (*t*)
C(18)	28.0 (*q*)	31.1 (*q*)	31.2 (*q*)
C(19)	16.4 (*q*)	21.1 (*q*)	22.6 (*q*)
C(20)	66.1 (*t*)	174.6 (*s*)	176.3 (*s*)
AcO	169.2 (*s*), 21.6 (*q*)	171.2 (*s*), 22.1 (*q*)	
AcO		170.6 (*s*), 21.0 (*q*)	

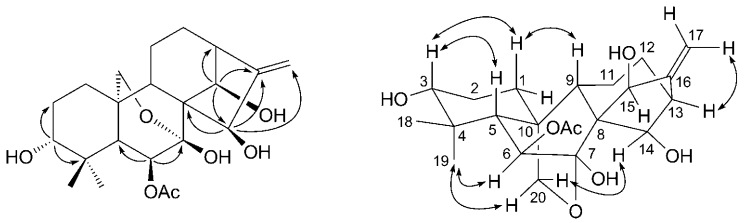


Fig. 1. *Selected HMBC* (H → C) *and key ROESY* (H ↔ H) *features of* **1**

Rabdonervosin E (**2**) was obtained as colorless crystals. The molecular formula of **2** was determined to be $C_{24}H_{32}O_8$ by the HR-ESI-MS (*m*/*z* 447.2022 ($[M-H]^-$)). The IR spectrum of **2** showed characteristic absorption bands at 3422, 1755, and 1210 cm^{-1} corresponding to OH and lactone groups, respectively. The ^{1}H-, ^{13}C- and DEPT-NMR spectra of **2** showed signals of 24 C-atoms including four C-atoms of two AcO groups (δ(C) 171.2, 170.6, 22.1, and 21.0) the other 20 C-atoms being attributed to a lactone C=O group, five quaternary C-atoms (including an olefinic one), six CH groups including three O-bearing ones, six CH_2 groups including an olefinic one, and two Me groups. Futhermore, the following typical ^{13}C-NMR signals (*Table 2*), including those of a hemiketal quaternary C-atom (δ(C) 106.0) and of a lactone C=O group (δ(C) 174.6), were characteristics of a (7α)-7,20-epoxy-*ent*-kauranoid similar to rabdoternin A (=(6β,7α,14*R*,15β)-6,7,7,14,15-pentahydroxy-*ent*-kaur-16-en-20-oic acid 20,7-lac-

tone; **5**) [12]. Comparison of the spectroscopic NMR data with those of rabdoternin A suggested that the only difference between them was that OH–C(6) and OH–C(15) in **2** were both acetylated, which was confirmed by the HMBCs H–C(6)/AcO (δ(C) 170.6) and H–C(15)/AcO (δ(C) 171.2) (*Fig. 2*). NOE Interactions of H–C(6) at δ(H) 5.89 (*d*, J = 7.0) with Me(19) at δ(H) 1.00 (*s*) was observed, which indicated the α-orientation of H–C(6), and no correlation between H–C(15) and H_α–C(9) suggested that AcO–C(15) was β-oriented (*Fig. 2*). Therefore, the structure of **2** was determined to be (6β,7α,14*R*,15β)-6,7,7,14,15-pentahydroxy-*ent*-kaur-16-en-20-oic acid 6,15-diacetate 20,7-lactone.

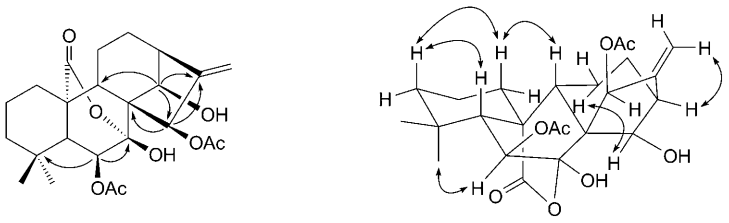


Fig. 2. *Selected HMBC* (H → C) *and key ROESY* (H ↔ H) *features of* **2**

Rabdonervosin F (**3**), obtained as colorless block crystals, was determined to possess the molecular formula $C_{20}H_{26}O_6$ by the HR-ESI-MS (*m*/*z* 361.1639 ([*M* – H]$^-$)). The IR spectrum exhibited the presence of lactone C=O (1741 cm^{-1}) and OH groups (3395 cm^{-1}). The ^{13}C-NMR and DEPT-NMR spectra of **3** (*Table 2*) showed signals of 20 C-atoms comprising two lactone C=O groups, four quaternary C-atoms (including an olefinic one), six CH groups including three O-bearing ones, six CH_2 groups including an olefinic one, and two Me groups, which suggested that **3** is an *ent*-kauranoid, which was also suggested by the consideration of a similar diterpenoid previously isolated from this plant [8][9]. The absence of HMBC cross-peaks between H–C(5) and H–C(6) and C(7) indicated a 6,7-seco structure, and a lactone group involving C(7) and C(1) was supported by an H_β–C(1)/C(7) correlation in the HMBC spectrum; therefore, compound **3** was determined to possess an enmein-type skeleton. Futhermore, the second lactone C=O group and the absence of CH_2(20) indicated that C(20) was oxidized, which was unambiguously established by the correlation between H_α–C(6) at δ(H) 5.96 (*s*) and C(20) (δ(C) 176.3) in the HMBC spectrum (*Fig. 3*). Comparison of the structure of compound **3** with ememogin (=(1α)-1-deoxo-1-hydroxy-8-oxoenmein; **6**) [13] revealed that **3** was 3-dehydroxyememogin, which was confirmed by its ^{1}H- and ^{13}C-NMR data (*Tables 1 and 2*). In its ROESY plot, H–C(15) at δ(H) 5.61 (br. *s*) showed correlation with H_α–C(14) (δ(H) 1.77 (*dd*, J = 4.8, 11.6 Hz)) but no correlation with H_α–C(9) (δ(H) 3.73 (*dd*, J = 3.0, 12.0 Hz)), and H–C(6) (δ(H) 5.96 (*s*)) showed correlation with Me(19) (δ(H) 0.92 (*s*)) (*Fig. 3*). It showed that H–C(6) was in α-orientation, while H–C(15) was in β-orientation. Therefore, the structure of **3** is determined to be (1α,6*R*,15α)-1,6,6,15-tetrahydroxy-6,7-seco-*ent*-kaur-16-ene-7,20-dioic acid 7,1 : 20,6-dilactone (=(1α)-1-dehydroxy-1-deoxo-1-hydroxy-8-oxoenmein).

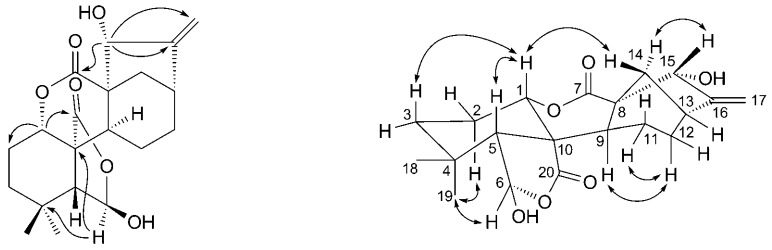


Fig. 3. *Selected HMBC* (H → C) *and key ROESY* (H ↔ H) *features of* **3**

The new compounds **1**–**3** were evaluated for their cytotoxic activities against HepG2 (human hepatoma), CNE2 (nasopharyngeal carcinoma), PC-9/ZD (lung cancer), HeLa (cervical cancer), MCF-7 (breast cancer), and HCT116 (colon cancer) cell lines, by using cisplatin as positive control. None of the compounds showed any potent cytotoxicity against the cell lines, the IC_{50} values being above 50 μM.

The technical support in the cytotoxic-activity screening tests from the State Key Laboratory of Oncology in South China, Cancer Center, Sun Yat-sen University, is gratefully acknowledged.

Experimental Part

General. Column chromatography (CC): silica gel (SiO_2; 100–200 or 200–300 mesh; *Qingdao Marine Chemical Factory*, Qingdao, P. R. China); *RP-18* gel (75 μm, *YMC Co., Ltd.*, Japan). TLC: visualization by spraying with 10% H_2SO_4/EtOH, followed by heating. M.p.: *XT-4A* micro melting-point apparatus; uncorrected. Optical rotations: *Perkin–Elmer-341* polarimeter. UV Spectra: *Agilent-8453E* spectrophotometer; λ_{max} (log ε) in nm. IR Spectra: *Thermo-Nicolet-Nexus-300* FT-IR spectrophotometer; KBr pellets; $\tilde{\nu}$ in cm^{-1}. 1D- and 2D-NMR Spectra: *Bruker-Avance-400* spectrometer; δ in ppm rel. to Me_4Si as internal standard, *J* in Hz. HR-ESI-MS: *Shimadzu LC-MS-IT-TOF* mass spectrometer; in *m*/*z*.

Plant Material. The leaves and stems of *I. nervosus* were collected in Yifeng County, Jiangxi Province, P. R. China, in July 2007. Voucher specimens (2007-XCC-1) have been deposited with the Laboratory of Phytochemistry, Department of Phytochemistry, Guangzhou University of Chinese Medicine, and were identified by Prof. *Xue-Wen Lai*, Jiangxi University of Chinese Medicine.

Extraction and Isolation. The air-dried and milled plant (10 kg) was extracted with 90% EtOH (3 × 20 l, each 2 h) in a *DTQ-100L* multi-function extractor, and the extract was filtered. The filtrate was concentrated to give a residue (1.2 kg), which was suspended in diatomite (1.2 kg) and then extracted successively with petroleum ether (b.p. 60–90°; 6 l), AcOEt (10 l), acetone (8 l), and MeOH (6 l) by means of a *Soxhlet* extractor. The AcOEt extract (600 g) was dissolved in MeOH and filtrated and the filtrate decolorated 3 × by adding 4‰ (*w*/*v*) of activated carbon and filtrated and then the filtrate concentrated to yield a yellowish gum (320 g). Part of the gum (200 g) was subjected to CC (SiO_2 (100–200 mesh; 1.2 kg), $CHCl_3$/acetone 1:0 → 0:1): *Fractions A–F. Fr. F* (37 g) was applied to CC (SiO_2 (200–300 mesh; 400 g), $CHCl_3$/acetone 30:1 → 1:1): *Frs. F1–F4. Fr. F3* (9.2 g) was purified by CC (SiO_2, petroleum ether/acetone 9:1 → 1:1): **2** (42 mg). *Fr. D* (18.0 g) was subjected to CC (SiO_2, petroleum ether/acetone 8:1 → 1:1): *Fr. D1* (7 g) and *D2* (5 g). Compound **3** (31 mg) was isolated from *Fr. D2* by CC (SiO_2, $CHCl_3$/MeOH 15:1 → 1:1). *Fr. E* (25.5 g) was subjected to CC (SiO_2, $CHCl_3$/acetone 12:1 → 1:1): *Frs. E1–E3. Fr. E3* (4 g) was subjected to CC (*RP-18* gel, MeOH/H_2O 50 → 90%): **1** (27 mg).

Rabdonervosin D (=*(3α,6β,7α,14*R,*15β)-7,20-Epoxy*-ent-*kaur-16-ene-3,6,7,14,15-pentol 6-Acetate*; **1**): Colorless needles (MeOH). M.p. 249–250°. $[\alpha]_D^{25} = -4.0$ ($c = 0.10$, MeOH). UV (MeOH): 203

(3.05). IR (KBr): 3506, 3422, 2925, 1755, 1631, 1384, 1210, 1057. ^{1}H- and ^{13}C-NMR: see *Tables 1* and *2*. HR-ESI-MS: 407.2052 ($[M-H]^-$, $C_{22}H_{31}O_7^-$; calc. 407.2070).

Rabdonervosin E (=*(6β,7α,14*R,*15β)-6,7,7,14,15-Pentahydroxy*-ent-*kaur-16-en-20-oic Acid 6,15-Diacetate 20,7-Lactone*; **2**). Colorless crystal (MeOH). M.p. 244–246°. $[\alpha]_D^{25} = -100.0$ ($c = 0.16$, MeOH). UV (MeOH): 203 (2.94). IR (KBr): 3486, 3277, 2945, 1751, 1718, 1380, 1265, 1228, 1207. ^{1}H- and ^{13}C-NMR: see *Tables 1* and *2*. HR-ESI-MS: 447.2022 ($[M-H]^-$, $C_{24}H_{31}O_8^-$; calc. 447.2019).

Rabdonervosin F (=*(1α,6*R,*15α)-1,6,6,15-Tetrahydroxy-6,7-seco*-ent-*kaur-16-ene-7,20-dioic Acid 7,1:20,6-Dilactone*=*(1α)-13-Dehydroxy-1-deoxo-1-hydroxy-8-oxoenmein*; **3**). Colorless block crystal (MeOH). M.p. 262–264°. $[\alpha]_D^{25} = -140$ ($c = 0.10$, MeOH). UV (MeOH): 204 (2.77). IR (KBr): 3568, 3395, 2927, 1741, 1691, 1384, 1201, 1125, 1068, 915. ^{1}H- and ^{13}C-NMR: see *Tables 1* and *2*. HR-ESI-MS: 361.1639 ($[M-H]^-$, $C_{20}H_{25}O_6^-$; calc. 361.1651).

Cytotoxicity Assay. MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay was performed to evaluate the activity of **1**–**3**. The HepG2 (human hepatoma), CNE2 (nasopharyngeal carcinoma), PC-9/ZD (lung cancer), HeLa (cervical cancer), MCF-7 (breast cancer) and HCT116 (colon cancer) cell lines were seeded in a 96-well plate at a density of 3000 to 8000 cells per well in 195 µl of medium, treated with 5 µl of different concentrations of the compound studied in medium for the indicated times, and then cultivated for 72 h at 37°. Then 10 µl of a 5 mg/ml MTT soln. in medium was added into each well before the termination of the experiment. The plates were incubated in an incubator (37°, 5% CO_2) for 4 h. The medium was removed. DMSO (100 µl) was added into each well to dissolve the dark blue crystal. Absorbance values with a test wavelength of 570 nm were recorded. The rates of cell-growth inhibition were calculated. The IC_{50} were calculated by the *Bliss* method. Cisplatin was used as a positive control.

REFERENCES

[1] H.-D. Sun, S.-X. Huang, Q.-B. Han, *Nat. Prod. Rep.* **2006**, *23*, 673.
[2] Jiangsu New Medical College, in 'A Dictionary of Chinese Herb', Shanghai Science and Technology Press, Shanghai, 1986, p. 159.
[3] Y.-H. Gao, Z.-X. Wan, X.-W. Lai, Y. Zhu, G.-Y. Li, S.-H. Wu, *Zhongguo Zhongyao Zazhi* **1994**, *19*, 295.
[4] Y.-H. Gao, Z.-X. Wan, R.-C. Xu, Y. Zhu, G.-Y. Li, S.-H. Wu, K.-F. Yu, G.-S. Liu, *Zhongcaoyao* **1994**, *17*, 28.
[5] Y.-H. Gao, S.-H. Wu, R.-J. Zhong, G.-Y. Li, *Zhongcaoyao* **1996**, *27*, 579.
[6] Y.-H. Gao, Y. Cheng, S.-H. Wu, *Zhongcaoyao* **1999**, *30*, 408.
[7] Y.-H. Gao, Y. Cheng, H.-C. Ye, *Zhongcaoyao* **2000**, *31*, 646.
[8] F.-L.Yan, L.-Q. Guo, J.-X. Zhang, S.-P. Bai, H.-D. Sun, *Chin. Chem. Lett.* **2008**, *19*, 441.
[9] F.-L.Yan, L.-Q. Guo, S.-P. Bai, H.-D. Sun, *J. Chin. Chem. Soc.* **2008**, *55*, 933.
[10] L.-M. Li, G.-Y. Li, L.-S. Ding, L.-B. Yang, Y. Zhao, J.-X. Pu, W.-L. Xiao, Q.-B. Han, H.-D. Sun, *J. Nat. Prod*. **2008**, *71*, 684.
[11] A.-J. Hou, H. Yang, B. Jiang, Q.-S. Zhao, Z.-W. Lin, H.-D. Sun, *Fitoterapia* **2000**, *71*, 418.
[12] Y. Takeda, K. Takeda, T. Fujita, H.-D. Sun, Y Minami, *Chem. Pharm. Bull*. **1990**, *38*, 441.
[13] Y. Takeda, K. Takeda, T. Fujita, *J. Chem. Soc., Perkin Trans. 1* **1986**, 690.

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