Diterpenoids from Aerial Parts of *Clerodendranthus spicatus* and Their Cytotoxic Activity

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A chemical investigation of AcOEt-soluble portion of the 95% EtOH extract of the aerial parts of *Clerodendranthus spicatus* led to the isolation and characterization of a new diterpenoid, named clerospicasin J (**1**). Structure elucidation of **1** was achieved by spectroscopic analyses, including 2D-NMR. The diterpenoid **1** exhibited significant inhibitory activity on the proliferation of the SKOV3 cell lines. Moreover, the cytotoxicity of **1** against SKOV3 cells was related to apoptotis as confirmed by DAPI (=2-(4-amidinophenyl)-1*H*-indole-6-carboxamidine) nuclear staining and flow cytometry.

Introduction. – *Clerodendranthus spicatus* (THUNB.) C. Y. WU ex H. W. LI (syn.: *Orthosiphon stamineus*, *O. grandiflorus*, *O. spicatus*, *O. aristatus*), which is a perennial herb of the family Lamiaceae [1], has been used as one of the popular traditional folk medicines in Southeast Asia for the treatment of renal inflammation, kidney stones, dysuria, diabetes, hypertension, rheumatism, tonsillitis, and menstrual disorder [2]. A series of highly oxygenated isopimarane- and staminane-type diterpenes, as well as flavonoids, rosmarinic acid, triterpenes, hexoses, organic acids, and saponins has been isolated from this plant [3–6]. In the course of our investigation on the anticancer metabolites of Chinese medicinal plants, the aerial parts of *C. spicatus* were investigated, and a new diterpene, namely clerospicasin J (**1**), and six known ones were isolated. The cytotoxicities of the isolated terpenoids against SKOV3 cancer cell line were also evaluated, and the mechanism of compound **1** in killing SKOV3 cells was preliminarily investigated.

Results and Discussion. – From an AcOEt extract of *C. spicatus*, the new compound **1** was isolated by repeated column chromatography on silica gel and *Sephadex LH-20*, followed by semipreparative high-performance liquid chromatography (HPLC), along with six known diterpenoids, *i.e.*, orthosiphols A and B (**2** and **3**, resp.) [7], clerospicasin D (**4**) [6], secoorthosiphol B (**5**) [6], and neoorthosiphol A and B (**6** and **7**, resp.) [8] by comparison of their spectroscopic data with those reported in the literature (*Fig. 1*).

Structure Elucidation. Clerospicasin J (**1**) was obtained as a white amorphous solid ($[\alpha]_D^{25} = -98.9$, MeOH). The molecular formula was established as $C_{30}H_{38}O_{10}$ by ESI-MS (*m*/*z* 557.4 ($[M-H]^-$; $C_{30}H_{37}O_{10}$; calc. 557.6)). The IR spectrum showed absorption bands of OH (3439 cm^{-1}) and ester C=O (1716 cm^{-1}) groups, and of a Ph ring (1602 and 1451 cm^{-1}).

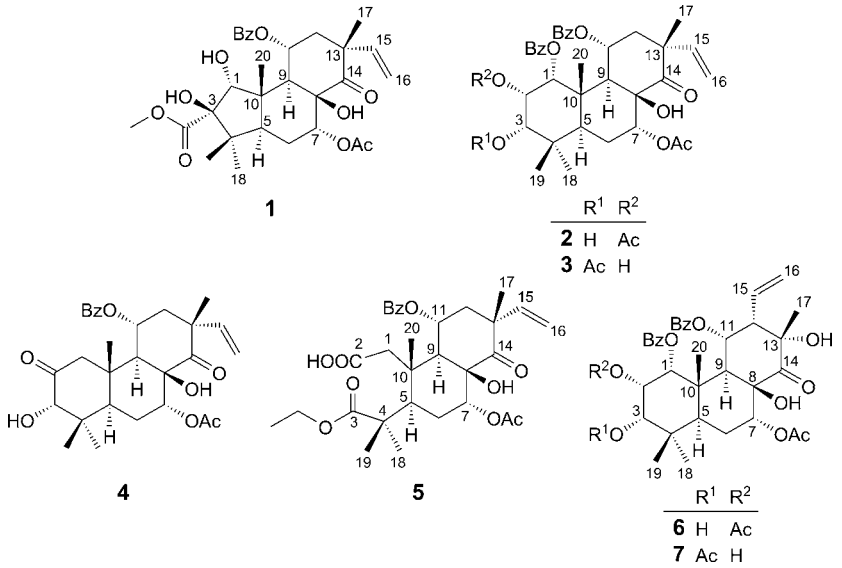

Fig. 1. *Structures of compounds* **1**–**7**

The ^{1}H-NMR spectrum (*Table 1*) revealed the presence of four tertiary Me groups (δ(H) 0.87, 0.98, 1.23, and 1.32), a vinyl group (δ(H) 6.09 (*dd*, J = 17.6, 10.8), 5.24 (*d*, J = 17.6); and 5.12 (*d*, J = 10.8), three O-bearing CH groups (δ(H) 4.09 (*d*, J = 2.2), 5.29 (*t*, J = 3.3); and 5.97 (*ddd*, J = 9.4, 6.5, 2.8), two aliphatic CH groups (δ(H) 3.54 (*d*, J = 9.4), and 3.10 (*dd*, J = 15.0, 1.8), as well as two Me and one benzoyl group. The ^{13}C-NMR spectrum (*Table 1*) displayed signals for 30 C-atoms, which included those of a ketone CO group (δ(C) 209.7 ppm), three ester CO groups (δ(C) 165.6, 169.1, and 174.1), one Ph ring, six Me groups (including one O-bearing Me group (δ(C) 52.2)), three CH_2 groups (including one sp^2 C-atom), six CH groups (including three O-bearing CH groups (δ(C) 71.7, 80.2, and 68.4 ppm) and one sp^2 C-atom), and four quaternary C-atoms (including two O-bearing quaternary C-atoms (δ(C) 77.8 and 85.9)). The gross structure of **1** was deduced from the ^{13}C-NMR data, indicating a diterpene containing a benzoyl moiety which was closely similar to clerospicasin D [6]. The significant differences in the chemical shifts of **1** consisted in the absence of resonances of a hexatomic ring attached at C(5) and C(10). Instead, a five-membered ring was combined with C(5) and C(10) in **1**, which was supported by the HMBC from H–C(1) (δ(H) 4.09) to C(3) and C(4); H–C(5) (δ(H) 5.49) to C(3) and C(1); MeO to C(2). The HMBC from H–C(1) (δ(H) 4.09) to C(2) and C(3) indicated that C(2) (δ(C) 174.1) was attached to C(3) (δ(C) 85.9). Then C(2) in the five-membered ring was substituted by an acetyl unit and by an OH group. The structure of **1** is as shown in *Fig. 1*.

The configuration was assigned on the basis of a NOESY experiment and analysis of ^{1}H,^{1}H coupling constants. The NOE correlations (*Fig. 2*) H–C(1)/Me(19), H–C(1)/Me(20), Me(19)/Me(20), and H–C(5)/H–C(9) indicated that H–C(1) was β-oriented (*Fig. 2*). The NOE correlations MeO/Me(18) indicated that HO–C(3) was β-oriented.

Table 1. *^{1}H- and ^{13}C-NMR* (600 and 125 MHz, resp., $CDCl_3$) *Data of* **1**. δ in ppm, J in Hz.

Position	δ(H)	δ(C)
1	4.09 (*d*, *J* = 2.2)	80.2
2	–	174.1
3	–	85.9
4	–	45.8
5	3.54 (*d*, *J* = 2.5)	35.1
6	2.02 (*td*, *J* = 15.0, 1.8), 1.93 (*dt*, *J* = 15.0, 1.8)	21.1
7	5.29 (*t*, *J* = 2.5)	71.7
8	–	77.8
9	3.10 (*d*, *J* = 9.4)	44.0
10	–	49.0
11	5.97 (*ddd*, *J*= 9.4, 6.5, 2.8)	68.4
12	2.44 (*dd*, *J* = 15.4, 6.5), 2.15 (*dd*, *J* = 15.4, 2.8)	39.7
13	–	47.4
14	–	209.7
15	6.09 (*dd*, *J* = 17.6, 10.8)	140.9
16	5.24 (*d*, *J* = 17.6), 5.12 (*d*, *J* = 10.8)	115.1
17	1.23 (*s*)	15.3
18	0.87 (*s*)	21.8
19	0.98 (*s*)	26.0
20	1.32 (*s*)	26.6
*Me*COO–C(7)	2.00 (*s*)	21.1
Me*C*OO–C(7)		169.1
1′	–	130.1
2′,6′	8.10 (*d*, *J* = 7.8)	129.5
3′,5′	7.50 (*t*, *J* = 7.8)	128.8
4′	7.62 (*t*, *J* = 7.8)	133.4
BnO*C*O	–	165.6
MeO	3.71 (*s*)	52.2

The small coupling constant observed for H–C(7) (*t*, $J = 2.5$) evidenced β-equatorial orientation. As for ring *C*, the NOE correlations H–C(1)/H–C(11), Me(20)/H–C(11), and H–C(11)/Me(17) indicated β-axial orientation of H–C(11). Thus, the structure of **1** was determined as depicted and named clerospicasin J.

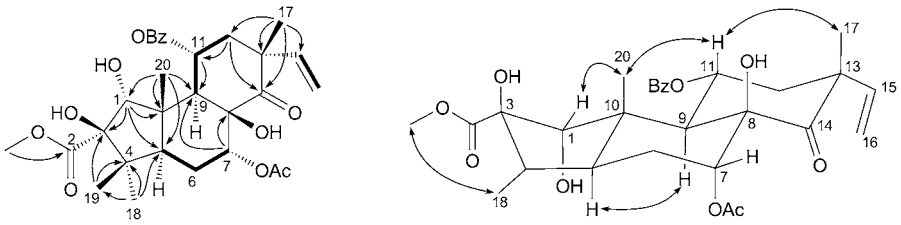

Fig. 2. *Key HMBCs* (H → C), *and ^{1}H,^{1}H-COSY* (—) *and NOE* (H ↔ H) *correlations of* **1**

Bioactivities. – The diterpenoids **1**–**7** were evaluated for their inhibitory activities for cell proliferation using SKOV3 ovarian carcinoma cell lines. Among the tested compounds, **1** showed significant cytotoxic activity with an IC_{50} value of 6.9 μM for SKOV3, but **2**–**7** showed only weak inhibitory activities against these cells (*Table 2*). Doxorubicin, with an IC_{50} value of 1.4 μM against SKOV3, used as a positive control.

Table 2. *Cytotoxicities of Compounds* **1**–**7** *Evaluated by the MTT Assay* (IC_{50} [μM])

Compound	SKOV3	Compound	SKOV3
1	6.9	**5**	41.5
2	21.9	**6**	30.8
3	25.5	**7**	39.4
4	30.3	Doxorubicin[a])	1.4

[a]) Positive control.

We carried out further tests to evaluate whether the cytotoxic effect of **1** was related to apoptosis. Apoptotic characteristics were observed by DAPI (4′,6-diamidino-2-phenylindole = 2-(4-amidinophenyl)-1*H*-indole-6-carboxamidine) staining in SKOV3 cells treated with compound **1**. In addition, compound **1** induced marked apoptotic morphologic alterations, including cell shrinkage, granular apoptotic bodies, and nuclear and cytoplasmic condensation (*Fig. 3*), compared with the control. The quantitative analysis of apoptosis of SKOV3 cells was monitored using the annexin V-FITC and propidium iodide (PI) double staining through flow cytometry. Annexin V staining in cells enabled detection of the expression of phosphatidylserine to the plasma membrane surface, which is a characteristic related to apoptosis. Flow cytometry revealed that the proportion of cells stained with annexin V increased in compound **1**-treated cells in a dose-dependent manner. The percentages of annexin V-positive cells were 9.8, 23.2, 31.3, and 69.9 for HeLa cells treated with 0, 1, 2, and 4 μM for 24 h, respectively. These results suggested that **1**-induced cytotoxicity of Hela cells arises from the induction of apoptosis.

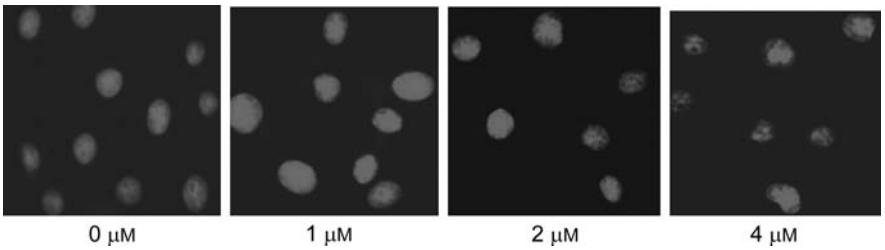

Fig. 3. *Compound* **1**-*induced apoptosis in SKOV3 cells, evaluated by DAPI staining*

Conclusions. – Seven diterpenes, *i.e.*, one new diterpene, clerospicasin J (**1**), and six known ones, were isolated from the aerial parts of the herb *C. spicatus*. Compounds **1**

showed a significant inhibitory activity against the proliferation of the SKOV3 cell lines, by inducing apoptosis.

Experimental Part

General. Column chromatography (CC): silica gel (SiO_2; 200–300 mesh; *Qingdao Marine Chemical Industry*), *Sephadex LH-20* gel (*Pharmacia Biotek*), and *MCI* gel (*CHP20P*, 75–150 μm, *Mitsubishi Chemical Industries Ltd.*). TLC: GF_{254} Plates (*Qingdao Marine Chemical Industry*); detection by using UV (254 nm) light and heating silica-gel plates sprayed with 10% H_2SO_4/EtOH. HPLC: *Agilent 1100-G1310A* isopump equipped with a *G1322A* degasser, a *G1314A VWD* detector (210 nm), and a *YMCPack ODS-A* column (250 × 20 mm, *S-10* μm, 12 nm). Optical rotations: *Perkin-Elmer 241 MC* polarimeter; in MeOH. CD Spectra: *Chirascan* spectrophotometer. UV Spectra: *Shimadzu UV-2450* spectrophotometer; λ_{max} (log ε) in nm. IR Spectra: *Thermo-Nicolet 670* spectrophotometer; KBr disks; $\tilde{\nu}_{max}$ in cm^{-1}. NMR Spectra: *Bruker Avance-DRX-600* spectrometer; at 600 (^1H) or 150 MHz (^{13}C); δ in ppm rel. to Me_4Si as internal standard; *J* in Hz. MS: *API-4000* Triple-stage quadrupole instrument for electrospray ionization (ESI) and *Finnigan LC-Q^{DECA}* mass spectrometer for HR-ESI; in *m*/*z* (rel. %).

Plant Material. The aerial parts of *Clerodendranthus spicatus* were collected from Xishuangbanna County, Yunnan Province, P. R. China, in September 2010. The plant material was identified by Dr. *Lingchuan Xu*, Shandong University of Traditional Chinese Medicine. A voucher specimen (CS01-2010-09) was deposited with the Shandong University of Traditional Chinese Medicine, P. R. China.

Extraction and Isolation. The air-dried and powdered plant material (6.0 kg) was extracted with 95% EtOH (4 × 20 l; each for 5 d) at r.t. The combined extracts were concentrated under reduced pressure to afford a dark gum (600 g), which was suspended in H_2O and partitioned successively with petroleum ether (PE; 5 × 1 l), AcOEt (5 × 1 l), and BuOH (5 × 1 l). The AcOEt-soluble fraction (100 g) was subjected to CC (SiO_2 PE/acetone 20 : 1 → 1 : 1) to yield 14 fractions, *Frs. 1–14*. *Fr. 7* (8.0 g) was subjected to CC (*MCI* gel; EtOH/H_2O 30 : 70 → 90 : 10) to yield five subfractions, *Subfrs. 7.1–7.5*. *Subfr. 7.3* (1.1 g) was further separated by CC (*Sephadex LH-20*; MeOH) to furnish five fractions, *Subfrs. 7.3.1–7.3.5*. *Subfr. 7.3.1* (70 mg) was purified by CC (reversed-phase (RP) C_{18}; MeOH/H_2O 30 : 70 → 90 : 10) to afford **12** (20.2 mg). *Subfr. 7.3.2* (70 mg) was further purified by prep. HPLC (MeOH/H_2O 75 : 25; 1.5 ml/min) to afford **1** (t_R 12.02 min; 1.5 mg), **4** (t_R 18.08 min; 5.0 mg), and **5** (t_R 25.20 min; 1.5 mg). *Fr. 8* (14.6 g) was further separated by CC (*MCI* gel; MeOH/H_2O 30 : 70 → 90 : 10) to give eight subfractions, *Subfrs. 8.1–8.8*. *Subfr. 8.6* (2.0 g) was first separated by CC (SiO_2; CH_2Cl_2/MeOH 100 : 0 → 15 : 1) to obtain five major fractions, *Subfrs. 8.6.1–8.6.5*. *Subfr. 8.6.3* (450.1 mg, 0.0075%) was purified by prep. HPLC (MeOH/H_2O 83 : 17; 1.3 ml/min) to give **2** (t_R 22.66 min; 220.0 mg). *Subfr. 8.6.5* (450.1 mg) was separated by prep. HPLC (MeOH/H_2O 80 : 20; 1.5 ml/min) to afford **3** (t_R 15.56 min; 200 mg). *Fr. 9* (32.8 g) was subjected to CC (*MCI* gel; MeOH/H_2O 30 : 70 → 90 : 10) to yield five subfractions, *Subfrs. 9.1–9.5*. *Subfr. 9.4* (2.8 g) was first separated by CC (*Sephadex LH-20*; MeOH) and then purified by prep. HPLC (MeOH/H_2O 78 : 22, 1.5 ml/min) to furnish **7** (t_R 18.08 min; 5.0 mg), and **6** (t_R 19.08 min; 5.0 mg).

Clerospicasin A (= *Methyl (1*R,*2*R,*3a*R,*5*R,*5a*R,*7*R,*9*R,*9a*S,*9b*S*)-5-(Acetyloxy)-9-(benzoyloxy)-7-ethenyldodecahydro-1,2,5a-trihydroxy-3,3,7,9b-tetramethyl-6-oxo-1*H*-cyclopenta[a]naphthalene-2-carboxylate*; **1**). White amorphous solid (1.5 mg). $[\alpha]_D^{25} = -98.9$ ($c = 0.09$, MeOH). UV (MeOH): 228 (4.25), 272 (3.17). IR: 3439, 2967, 2933, 1716, 1602, 1451, 1371, 1315, 1279, 1114, 1071. ^1H- and ^{13}C-NMR: see *Table 1*. ESI-MS: 557.4 ($[M-H]^-$).

Cytotoxicity Assay. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay was used to determine cell viability [9]. The cancer cells were all cultured under standard culture conditions. The test compounds or vehicle control (DMSO) were added to appropriate wells, and the cells were incubated for 72 h. Then MTT was added into the assay plates (final concentration, 0.5 mg/ml). After shaking for 10 s, plates were returned to incubator and kept for 4 h. The supernatants were removed carefully, followed by the addition of 100 μl of DMSO to each well to dissolve the precipitate. Then, the absorbance was measured at 570 nm with a model *680* microplate reader (*Bio-Rad*, USA). The cell viability was expressed as absorbance in the presence of test compound as a percentage of that in the vehicle control. Doxorubicin and DMSO were used as positive and negative controls, resp.

DAPI Staining. Cells were seeded on 12-mm round-glass cover slips in 24-well plates. Twenty-four h after treatment with **1**, cells were washed in phosphate buffered saline (PBS), followed by fixation with cold MeOH/acetone 1:1 for 5 min. The fixed cells were washed three times in PBS for 5 min, followed by staining with 4 mg/ml DAPI (=4′,6-diamidino-2-phenylindole) for 15 min at r.t. The cover slips containing cells were then mounted on a microscope slide using mounting medium and analyzed by fluorescence microscopy [10].

Analysis of Apoptosis by Flow Cytometry. To determine the effect of compound **1** on the cell cycle, SKOV3 cells were treated with 5, 10, and 15 μM **1** at 24 h, resp., washed, and fixed with 70% EtOH. After incubation overnight at −20°, cells were washed with PBS and then suspended in staining buffer (propidium iodide, 10 mg/ml; *Tween-20*, 0.5%; RNase, 0.1% in PBS). Cell cycle was analyzed by flow cytometry (*FACSAria*, Becton Dickinson, USA), and the percentage of cells in various phases of cell cycle was calculated by WinMDI 2.9 software. Gating was set to exclude cell debris, cell doublets, and cell clumps.

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Received March 22, 2014