Further Diterpenes from the Seeds of *Caesalpinia minax* HANCE

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Three new cassane-type diterpenes, neocaesalpin AF (**1**), neocaesalpin AG (**2**), and neocaesalpin AH (**3**), were isolated from the seeds of *Caesalpinia minax* HANCE. Their structures were elucidated on the basis of extensive spectroscopic analyses. The partially hydrogenated lactone unit in a tetracyclic cassane diterpene in **2** is rarely encountered in the cassane diterpenes isolated from the genus *Caesalpinia*. All compounds were evaluated for their *in vitro* cytotoxic activities against HCT-8 and MCF-7 human cancer cell lines.

Introduction. – Cassane-type diterpenes that possess tetracyclic frameworks with a fused furan ring or butenolide moiety are characteristic constituents of the genus *Caesalpinia* (Fabaceae), and they constitute a group of structurally diverse natural products [1]. The seeds of *Caesalpinia minax* HANCE, known as *kushilian* in Chinese folk medicine, have long been used for the treatment of anemopyretic colds, dysentery, skin itching, and sores [2]. Our previous phytochemical investigation on the seeds of *C. minax* resulted in the isolation of a series of new diterpenes [3]. In the continuation of our efforts to search for antitumor agents in this plant, three new cassane-type diterpenes, named neocaesalpin AF (**1**), neocaesalpin AG (**2**), and neocaesalpin AH (**3**) (*Fig. 1*), were isolated from the seeds of *C. minax*. Herein, we report their isolation, structure elucidation, and evaluation of their antiproliferative activities.

Results and Discussion. – Neocaesalpin AF (**1**) was obtained as white amorphous powder, and it gave a carmine coloration upon treatment with H_2SO_4, followed by heating, on a TLC plate. HR-ESI Mass spectrum exhibited a *quasi*-molecular ion peak at *m*/*z* 529.1856 ($[M+K]^+$; calc. 529.1840) in the positive-ion mode. In conjunction with the ^{1}H- and ^{13}C-APT spectra, the formula of **1** was deduced as $C_{26}H_{34}O_9$. Its IR spectrum indicated the presence of a OH group and of an α,β-unsaturated γ-lactone moiety (3479 and 1738 cm^{-1}, resp.). The UV absorption at 285 nm confirmed that this

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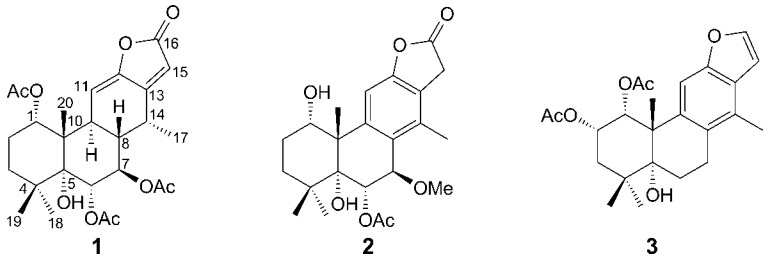

Fig. 1. *Structures of compounds* **1**–**3**

compound had a β-butenolide ring conjugated with one additional C=C bond [4]. This was also supported by the ^{1}H-NMR (δ(H) 5.72 (H–C(11)), 6.06 (H–C(15)) and ^{13}C-APT-NMR spectrum (APT, attached proton test; δ(C) 110.2 (C(11)), 150.1 (C(12)), 158.3 (C(13)), 111.9 (C(15)), 169.2 (C(16))) (*Table 1*). These data combined with four Me signals (δ(H) 1.11, 1.14, 1.22, 1.28; δ(C) 14.3, 19.0, 24.0, 30.1) in the NMR spectra indicated that basic structure of **1** was of the cassane lactone type, similar to the known compound neocaesalpin V [5]. All C-atom-bound H-atoms were assigned on the basis of the HSQC spectrum. The only differences between compound **1** and neocasalpin V were the appearance of two extra AcO Me signals (δ(H) 2.04, 2.11; δ(C) 21.4, 21.9) at C(6) and C(7) in the spectra of **1**, which were supported by HMBC and ^{1}H,^{1}H-COSY 2D-NMR spectra (*Fig. 2*). In the HMBC spectrum, the correlations between the signals at δ(H) 2.04 and δ(C) 75.5, 170.4, and those at δ(H) 2.11 and δ(C) 72.2, 170.8 suggested that the two AcO groups were located at C(6) and C(7) respectively. The relative configuration of compound **1** was determined on the basis of coupling constants and a NOESY spectrum. The NOE correlations H–C(1)/H–C(6) to Me(20), H–C(7)/Me(17) to H–C(9) and Me(18) indicated that H–C(1) and H–C(6) were β-oriented, whereas H–C(7) and Me(17) were α-oriented. The ^3J coupling constant (7.8 Hz) also supported the antiperiplanar relationship between H–C(6) and H–C(7) [6]. Therefore, the structure of compound **1** was established as 1α,6α,7β-triacetoxy-5α-hydroxy-14α-methylcassa-11(12),13(15)-dien-16,12-olide and named neocaesalpin AF.

Neocaesalpin AG (**2**) was isolated as white amorphous powder. HR-ESI Mass spectrum displayed a *quasi*-molecular ion peak at *m*/*z* 441.1867 ($[M+\mathrm{Na}]^+$; $C_{23}H_{30}O_7Na$; calc. 441.1889) in the positive-ion mode. The IR absorptions revealed the presence of OH group (3426 cm^{-1}), and unsaturated C=O (1723 cm^{-1}) and aromatic (3012 and 1607 cm^{-1}) functionalities. The ^{1}H-NMR spectrum (*Table 1*) exhibited four Me signals at δ(H) 1.31, 1.36, 1.42 and 2.24, one olefinic H-atom signal at

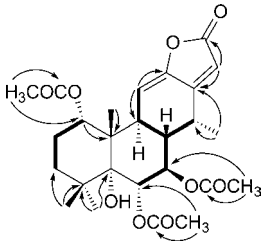

Fig. 2. *Key* ^{1}*H,*^{1}*H-COSY* (—) *and HMB* (H → C) *correlations of compound* **1**

Table 1. *^{1}H- and ^{13}C-NMR Data* (600 and 150 MHz, resp.; $CDCl_3$) of *Compounds* **1**–**3**. Atom numbering as indicated in *Fig. 1*; δ in ppm, J in Hz.

Position	**1**		**2**		**3**	
	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)
1	5.11 (t, J = 1.8)	74.3	4.43 (s)	75.4	6.03 (d, J = 1.8)	74.8
2	1.78–1.80 (m), 2.05–2.07 (m)	22.6	1.90–1.98 (m), 1.72–1.75 (m)	29.8	5.54 (m)	68.1
3	1.13–1.15 (m), 1.80–1.82 (m)	32.5	1.75–1.80 (m) 1.96–2.05 (m)	32.6	1.35–1.40 (m), 1.90–1.97 (m)	36.2
4		38.8		38.5		40.2
5		79.1		77.4		75.9
6	5.54 (d, J = 7.8)	75.5	5.51 (d, J = 7.8)	76.0	2.04–2.10 (m), 2.14–2.18 (m)	24.1
7	5.72 (t, J = 7.8)	72.2	4.41 (s)	85.1	2.70–2.74 (m), 2.77–2.83 (m)	23.9
8	2.08–2.10 (m)	48.3		128.1		129.1
9	3.25 (m)	36.1		142.6		140.5
10		44.5		41.7		46.8
11	5.72 (s)	110.2	6.32 (s)	102.2	7.12 (s)	104.5
12		150.1		141.4		154.2
13		158.3		123.2		125.2
14	3.72 (qd, J = 7.2, 4.8)	36.1		133.5		128.5
15	6.06 (s)	111.9	3.41 (s)	35.5	6.74 (d, J = 2.4)	105.0
16		169.2		178.3	7.54 (d, J = 2.4)	144.6
17	1.28 (d, J = 7.2)	14.3	2.24 (s)	15.9	2.40 (s)	16.1
18	1.11 (s)	30.1	1.31 (s)	27.7	1.22 (s)	28.2
19	1.14 (s)	24.0	1.36 (s)	25.8	1.27 (s)	25.9
20	1.22 (s)	19.0	1.42 (s)	30.6	1.49 (s)	30.7
1-AcO	2.13 (s)	169.2, 21.7			2.07 (s)	169.0, 21.1
2-AcO					1.98 (s)	170.2, 21.2
6-AcO	2.04 (s)	170.4, 21.4	2.00 (s)	171.3, 21.7		
7-AcO	2.11 (s)	170.8, 21.9				
7-MeO			3.62 (s)	57.5		
5-OH	3.03 (br. s)					

δ(H) 6.32 (H–C(11)), and CH_2 H-atom at δ(H) 3.41 (CH_2(15)). The ^{13}C-APT-NMR spectrum (*Table 1*) displayed 23 C-atom signals including those of six olefinic C-atoms (δ(C) 102.2, 123.2, 128.1, 133.5, 141.4, and 142.6) and two C=O groups (δ(C) 171.3 and 178.3). These suggested that the basic skeleton of **2** was an aromatic tetracyclic cassane diterpene with a partially hydrogenated lactone ring [7]. All C-atom-bound H-atoms were assigned on the basis of the HSQC spectrum. The presence of an aromatic ring was confirmed by the HMBC features from H–C(11) (δ(H) 6.32 (*s*)) to C(9) (δ(C) 142.6), C(12) (δ(C) 141.4), and from Me(17) (δ(H) 2.24 (*s*) to C(8) (δ(C) 128.1), C(13) (δ(C) 123.2), C(14) (δ(C) 133.5), and C(15) (δ(C) 35.5). One AcO group was located at C(6) on the basis of the correlations from the signal at δ(H) 2.00 (6-AcO) to those at δ(C) 76.0 (C(6)) and 171.3 (6-AcO). A MeO group is located at C(7) on the basis of the correlation from the signal at δ(H) 3.62 (7-MeO) to that at δ(C) 85.1 (C(7)). Furthermore, the correlations from the signal at δ(H) 1.42 (Me(20)) to that at δ(C) 85.1 (C(1)), together with the molecular composition ($C_{23}H_{30}O_7$), indicated that one OH group was located at C(1). In the NOESY spectrum, the NOE between Me(20), and H–C(1) and H–C(6); and between Me(18) and H–C(7) indicated that 1-OH and 6-AcO were α-oriented, and 7-MeO was β-oriented. Thus, the structure of compound **2** was assigned as 6α-acetoxy-1α,5α-dihydroxy-7β-methoxycassa-8,11,13(15)-trien-16,12-olide and named neocaesalpin AG.

Neocaesalpin AH (**3**), which was also obtained as white amorphous powder, was assigned the molecular formula $C_{24}H_{30}O_6$ on the basis of its positive-ion mode HR-ESI-MS (*m*/*z* 437.1933 ($[M+Na]^+$)). The ^{1}H-NMR spectrum (*Table 1*) exhibited four Me signals at δ(H) 1.22, 1.27, 1.49, and 2.40, two CH–O signals at δ(H) 6.03 and 5.54. Two coupled olefinic H-atom signals at δ(H) 6.74 (*d*, $J = 2.4$, H–C(15)) and 7.54 (*d*, $J = 2.4$, H–C(16)) suggested the presence of a fused furan ring [8]. The ^{13}C-APT-NMR data (*Table 1*) showed 24 C-atom signals, including those of four Me C-atoms (δ(C) 16.1, 25.9, 28.2, and 30.7), eight olefinic C-atoms (δ(C) 104.5, 105.0, 125.2, 128.5, 129.1, 140.5, 144.6, and 154.2), and two C=O signals (δ(C) 169.0 and 170.2). These data suggested that **3** was a cassane furanoditerpene [8]. The conjugation of the benzene ring with the fused furan ring was confirmed by the HMBC features from the signal at δ(H) 7.12 (*s*, H–C(11)) to those at δ(C) 140.5 (C(9)) and 154.2 (C(12)); from the signal at δ(H) 7.54 (*d*, $J = 2.4$, H–C(16)) to those at δ(C) 105.0 (C(15)), 125.2 (C(13)), and 128.5 (C(14)); from the signal at δ(H) 2.40 (*s*, Me(17)) to those at δ(C) 129.1 (C(8)) and 128.5 (C(14)). The locations of substituents of compound **3** were confirmed by analysis of the HMBC spectrum. In the HMBC spectrum, the correlations between the signals at δ(H) 2.07 (1-AcO), and at δ(C) 74.8 (C(1)), 169.0 (1-AcO); the signals at δ(H) 1.98 (2-AcO) and at δ(C) 68.1 (C(2)) and 170.2 (2-AcO) suggested that the AcO groups were located at C(1) and C(2). Combined with the NOESY spectrum, the structure of compound **3** was determined as 1α,2α-diacetoxy-5α-hydroxy-14-methylvoucapa-8(14),9(11)-diene and named neocaesalpin AH.

Compounds **1**–**3** were tested for the antiproliferative activities against HCT-8 (colorectal cancer) and MCF-7 (breast cancer) human cancer cell lines. It was found that compound **3** showed moderate antiproliferative activity with an IC_{50} value of 15.3 μg/ml against MCF-7. Compounds **1** and **2** did not show any significant antiproliferative activity, and both had IC_{50} values greater than 20 μg/ml (*Table 2*). It was previously reported that some cassane-type diterpenes with structures similar to

Table 2. In vitro *Antiproliferative Activities of Compounds* **1**–**3**.

Compounds	IC_{50} (μg/ml)	
	HCT-8	MCF-7
1	> 50	34.7
2	> 50	> 50
3	41.4	15.3
Cisplatin[a])	2.5	10.1

[a]) Positive control.

the isolated compounds showed moderate cytotoxicities against several cancer cell lines [9].

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Experimental Part

General. All solvents used were of anal. grade (*Beijing Chemical Works*). Column chromatography (CC): *C18* reversed-phase (RP) silica gel (SiO_2, 40–63 μm; *Merck*, DE-Darmstadt), *Sephadex LH-20* (*Pharmacia*, SE-Uppsala), *MCI* gel (CHP 20P, 75–150 μm, *Mitsubishi Chemical Corporation*, Tokyo, Japan), and silica gel (SiO_2, 100–200 and 300–400 mesh; *Qingdao Haiyang Chem. Ind. Ltd.*, P. R. China). TLC: Precoated silica gel *GF254* plates (*Zhi Fu Huang Wu Pilot Plant of Silica Gel Development*, Yantai, P. R. China). Optical rotations: *Perkin-Elmer 341* digital polarimeter. UV Spectra: *Shimadzu UV2550*. IR Spectra: *FTIR-8400S* spectrometer; KBr discs; $\tilde{\nu}$ in cm^{-1}. ^1H- and ^{13}C-NMR Spectra: *Bruker-AV-600* instrument; at 600 (^1H) and 150 MHz (^{13}C); δ in ppm rel. to Me_4Si as internal standard, *J* in Hz. HR-ESI-MS spectra: *LTQ-Obitrap XL* spectrometer; in *m*/*z*.

Plant Material. The seeds of *C. minax* were collected in September 2008, in Nanning, Guangxi Province, China, and identified by Prof. *Jingquan Yuan*, Department of Pharmaceutical Chemistry, Guangxi Botanical Garden of Medical Plants. A voucher specimen (NO. 21648) was deposited with the Guangxi Botanical Garden of Medical Plants.

Extraction and Isolation. The air-dried and powered seeds of *C. minax* HANCE (8.0 kg), were extracted three times with MeOH. Removal of the MeOH under reduced pressure yielded a MeOH extract (2020 g). The residue was subjected to CC (SiO_2; hexane, $CHCl_3$, AcOEt, acetone, and MeOH, resp.). The $CHCl_3$ fraction (325 g) was subjected to CC (SiO_2 (100–200 mesh); a petroleum ether (PE)/AcOEt from 1:0 to 1:1) to yield twelve fractions, *Fr. A*–*L*. *Fr. I* (9.4 g) was separated by CC (*Sephadex LH-20* and *MCI*), and four fractions, *Fr. I1*–*I4*, were obtained. Then, *Fr. I1* (1.5 g) were purified by CC (SiO_2 (300–400 mesh); PE/$CHCl_3$ 10:1; 6:1; 4:1; 0:1, followed by $CHCl_3$/MeOH 80:1; 60:1; 40:1; 20:1) to yield **1** (4.3 mg). *Fr. I2* (2.1 g) were submitted to CC (SiO_2 (300–400 mesh); PE/AcOEt 50:1; 20:1; 8:1; 4:1; 1:1; 0:1) to yield **2** (1.5 mg). *Fr. I3* (2.5 g) were subjected to CC (SiO_2 (300–400 mesh); PE/AcOEt 80:1; 40:1; 20:1; 10:1; 5:1; 1:1; 0:1) and then purified by semi-prep. liquid chromatography (*YMC RP-18* column; MeOH/H_2O 68:32) to afford **3** (t_R 24.5 min; 1.7 mg).

Neocaesalpin AF (=*1α,6α,7β-Triacetoxy-5α-hydroxy-14α-methylcassa-11(12),13(15)-diene-16,12-olide* = rel-*(1S,4aR,5S,6R,6aS,7R,11aR,11bS)-1,5,6-tris(acetyloxy)-2,3,4,4a,5,6,6a,7,11a,11b-decahydro-*

*4a-hydroxy-4,4,7,11b-tetramethylphenanthro[3,2-*b*]furan-9(1*H*)-one*; **1**) White amorphous powder. $[\alpha]_D^{20} = +0.5$ ($c = 0.05$, MeOH). UV (MeOH): 285 (3.04). IR (KBr) 3479, 1738. ^{1}H- and ^{13}C-APT ($CDCl_3$): see *Table 1*. HR-ESI-MS: 529.1856 ($[M+K]^+$; calc. 529.1840).

Neocaesalpin AG (=6α-Acetoxy-1α,5α-dihydroxy-7β-methoxycassa-8,11,13(15)-trien-16,12-olide = rel-*(1*S*,4a*R*,5*S*,6*R*,11b*S*)-5-(acetyloxy)-2,3,4,4a,5,6,8,11b-octahydro-1,4a-dihydroxy-6-methoxy-4,4,7,11b-tetramethylphenanthro[3,2-*b*]furan-9(1*H*)-one*; **2**) White amorphous powder. $[\alpha]_D^{20} = +0.6$ ($c = 0.05$, MeOH). UV (MeOH): 249 (3.18). IR (KBr) 3426, 3012, 1723, 1606. ^{1}H- and ^{13}C-APT ($CDCl_3$): see *Table 1*. HR-ESI-MS: 441.1867 ($[M+Na]^+$; calc. 441.1889).

Neocaesalpin AH (=1α,2α-Diacetoxy-5α-hydroxy-14-methylvoucapa-8(14),9(11)-diene = rel-*(1*R*,2*S*,4a*R*,11b*S*)-1,2-Bis(acetyloxy)-1,3,4,5,6,11b-hexahydro-4,4,7,11b-tetramethylphenanthro[3,2-*b*]furan-4a(2*H*)-ol*; **3**) White amorphous powder. $[\alpha]_D^{20} = -0.25$ ($c = 0.1$, MeOH). UV (MeOH): 249 (4.15). IR (KBr) 3451, 2953, 1738, 1634, 1463. ^{1}H- and ^{13}C-APT ($CDCl_3$): see *Table 1*. HR-ESI-MS: 437.1933 ($[M+Na]^+$; calc. 437.1940).

Cytotoxicity Assay. Compounds **1**–**3** were assessed by MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) method using HCT-8 and MCF-7 human cancer cell lines. Each cell was seeded onto 96-well microtiter plates at a density of 6×10^4 cells/ml (100 µl) per well. Cells were preincubated for 24 h at 37° in 5% CO_2 for 24 h. Then, five different concentrations of each compound dissolved in DMSO were added to each well. Each concentration was tested in triplicate. After 48 h, 10 µl of MTT (4 mg/ml) was added to each well and incubated for another 4 h. Then, the liquid in the well was removed, and DMSO (200 µl) was added to each well. The absorbance was recorded on a microplate reader at a wavelength of 570 nm [10][11].

REFERENCES

[1] R. W. Jiang, S. C. Ma, P. P. H. But, T. C. W. Mak. *J. Nat. Prod.* **2001**, *64,* 1266; M. J. Huang, Y. D. Chen, D. Z. Wei, *Mod. Chin. Med.* **2010**, *12*, 11; D. M. Li, L. Ma, G. M. Liu, L. H. Hu, *Chem. Biodiversity* **2006**, *3*, 1260; Z. H. Wu, Y. Y. Wang, J. Huang, B. H. Sun, L. J. Wu, *Asian J. Trad. Med.* **2007**, *2*, 135; Z. H. Wu, J. Huang, W. D. Li, L. J. Wu, H. Y. Gao, *J. Asian Nat. Prod. Res.* **2010**, *12*, 781; R. A. Dickson, P. J. Houghton, P. J. Hylands, *Phytochemistry* **2007**, *68*,1436; S. K. Kalauni, S. Awale, Y. Tezuka, A. H. Banskota, T. Z. Linn, S. Kadota, *Chem. Pharm. Bull*. **2005**, *53*, 1300.

[2] Z. H. Wu, L. B. Wang, H. Y. Gao, B. H. Sun, J. Huang, L. J. Wu. *China J. Chin. Mat. Med.* **2008**, *10*, 1145.

[3] G. X. Ma, X. D. Xu, L. Cao, J. Q. Yuan, J. S. Yang, L. Y. Ma, *Planta Med*. **2012**, *78*, 1363; G. X. Ma, J. Q. Yuan, L. Cao, J. S. Yang, X. D. Xu, *Chem. Pharm. Bull.* **2012**, *60*, 759.

[4] T. Kinoshita, *Chem. Pharm. Bull*. **2000**, *48*, 1375.

[5] Y. Matsuno, J. Deguchi, Y. Hirasawa, K. Ohyama, H. Toyoda, C. Hirobe, W. Ekasari, A. Widyawaruyanti, N. C. Zaini, H. Morita, *Bioorg. Med. Chem. Lett*. **2008**, *18*, 3774.

[6] Y. Matsuno, J. Deguchi, T. Hosoya, Y. Hirasawa, C. Hirobe, M. Shiro, H. Morita, *J. Nat. Prod.* **2009**, *72*, 976.

[7] S. Cheenpracha, R. Srisuwan, C. Karalai, C. Ponglimanont, S. Chantrapromma, K. Chantrapromma, H. K. Fun, S. Anjum, A. U. Rahman, *Tetrahedron* **2005**, *61*, 8656.

[8] A. N. Jadhav, N. Kaur, K. K. Bhutani, *Phytochem. Anal*. **2003**, *14*, 315.

[9] P. P. Yadav, R. Maurya, J. Sarkar, A. Arora, S. Kanojiya, S. Sinha, M. N. Srivastava, R. Raghubir, *Phytochemistry* **2009**, *70*, 256.

[10] W. W. Lü, Y. J. Gao, M. Z. Su, Z. Luo, W. Zhang, G. B. Shi, Q. C. Zhao, *Helv. Chim. Acta* **2013**, *96*, 109.

[11] Y. S. Sun, Z. Zhao, Q. S. Feng, Q. Q. Xu, L. X. Liu, J. K. Liu, L. Zhang, B. Wu, Y. Q. Li, *Helv. Chim. Acta* **2013**, *96*, 76.

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