Five New Cycloartane-Type Triterpenoid Saponins from *Nervilia fordii*

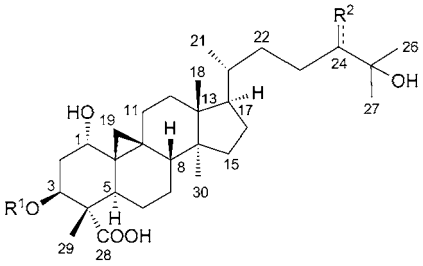
by **Liu-Bin Wei**[a]), **Hong-E Yuan**[a]), **Jin-Man Chen**[a]), **Qing-Qing Wang**[a]), **Hui Wang**[b]), **Wen-Cai Ye**[a]), and **Guang-Xiong Zhou***[a])

[a]) Institute of Traditional Chinese Medicine and Natural Products, Guangdong Province Key Laboratory of Pharmacodynamic Constituents of Traditional Chinese Medicine and New Drugs Research, College of Pharmacy, Jinan University, Guangzhou 510632, P. R. China (phone: +86-20-85221469; fax: +86-20-85221559; e-mail: guangxzh@sina.com)

[b]) School of Medicine, Jinan University, Guangzhou 510632, P. R. China

Five new cycloartane glycosides, nervisides D–H (**1**–**5**), were isolated from the AcOEt- and H_2O-soluble portions of the 90% EtOH extract of the aerial part of the plant *Nervilia fordii*. The structures of the isolated glycosides were elucidated by extensive spectroscopic analysis including HR-ESI-MS and NMR data. The isolated nervisides D–H were evaluated for the cytotoxic activity *in vitro* against human-tumor cell lines (CNE, Hep-2 and HepG2) with the MTT method.

Introduction. – The plant *Nervilia fordii* (HANCE) SCHLTR. (Orchidaceae) is an endemic source of herbal medicine in the south of China. The whole plant was used as the traditional Chinese medicine 'Qingtiankui' for the treatment of various diseases, such as tuberculosis, bronchitis, and pneumonia [1]. The study about an acetylflavonol of this plant with potent inhibitory activity against the production of nitric oxide (NO) in RAW264.7 has been reported [2]. The H_2O-soluble portion of the plant has shown antiviral activity against influenza *in vitro* [3]. The MeOH extracts of the plant with antiviral activity on *Herpes simplex* virus type 1 (HSV-1) was investigated [4]. Flavonol glycosides, steroides, and triterpenes were reported from the plant [4–6]. In this paper, five new cyclolartane glycosides, named nervisides D–H (**1**–**5**; *Fig. 1*), were isolated from the 90% EtOH extract of this plant. The structures of **1**–**5** were determined by spectroscopic analysis of their HR-ESI-MS and NMR data. The *in vitro* cytotoxic activity against human-tumor cell lines (CNE, Hep-2, and HepG2) of these compounds were measured by the MTT (=2-(4,5-dimethylthiazol-2-yl)-3,5-diphenyl-2*H*-tetrazolium bromide) method.



	R^1	R^2
1	Xyl	OH, $HOCH_2$(31)
2	Xyl	OH (24*R*)
3	Glc	OH (24*R*)
4	Xyl	O
5	Glc	O

Fig. 1. *Compounds* **1**–**5**, *isolated from* Nervilia fordii

Results and Discussion. – The 90% EtOH extract of *Nervilia fordii* was partitioned successively with petroleum ether, $CHCl_3$, and AcOEt. The AcOEt phase was subjected to column chromatography (CC; polyamide, *Sephadex LH-20*, and octadecyl silica gel (*ODS*)), and finally to prep. HPLC to give the two new glycosides **4** and **5**. The H_2O-soluble portion was subjected to CC (*D101* macroporous resin, SiO_2, *Sephadex LH-20*, and *ODS*), and finally to prep. HPLC to yield the three new glycosides **1**–**3**.

Nerviside D (**1**) was obtained as white powder. The molecular formula was established to be $C_{36}H_{60}O_{11}$ from a quasimolecular ion at *m/z* 691.4026 ($[M+Na]^+$) in the HR-ESI-MS. The ^{1}H-NMR spectrum (*Table 1*) displayed the signals of a CH_2 group at δ(H) 0.48 and 0.77 (2*d*, $J = 4.3$ Hz, 1 H each), of five tertiary Me groups at δ(H) 0.95, 1.00, 1.64, 1.65 and 1.66 (5*s*), of a secondary Me group at δ(H) 0.99 (*d*, $J = 6.4$ Hz), and of an anomeric H-atom at δ(H) 5.03 (*d*, $J = 7.0$ Hz). The ^{13}C-NMR spectrum (*Table 2*) exhibited signals of 36 C-atoms, of which 31 accounted for the aglycone moiety. The remaining signals were in good accordance with the presence of a xylose unit at δ(C) 106.6 (*d*, C(1′)), 75.5 (*d*, C(2′)), 78.1 (*d*, C(3′)), 71.1 (*d*, C(4′)), and 67.0 (*t*, C(5′)) [7]. The resonances assigned to the aglycone moiety arose from six Me groups, eleven CH_2 groups, six CH groups, seven quaternary C-atoms, and one COOH group. The signals at δ(C) 72.3 (*d*), 81.3 (*d*), 76.5 (*s*), 76.7 (*s*), and 65.6 (*t*) showed five O-bearing C-atoms. All of the above evidences suggested that **1** was a highly oxygenated cycloartane (= 9,19-cyclolanostane) triterpene monoglycoside [8–10]. The anomeric center of the xylopyranosyl moiety was determined to have β-configuration from the large ^3J(1,2) value. A long-range correlation between an anomeric H-atom at δ(H) 5.03 (*d*, $J =$ 7.0 Hz, H–C(1′)) and δ(C) 81.3 (*d*, C(3)) in the HMBC spectrum demonstrated that the sugar moiety was bound to C(3) (*Fig. 2*). The β-orientation of the sugar moiety at C(3) was determined from the coupling pattern and constant of H–C(3) (*dd*, $J = 12.1$ and 4.3 Hz). Furthermore, the HMBCs H–C(1)/C(3) and C(5), H–C(3)/C(1), C(28), and C(29), and Me(29)/C(3), C(4), C(5), and C(28) allowed to place a COOH group at C(4) and an OH group at C(1) of ring *A* (*Fig. 2*). In addition, the HMBCs CH_2(31)/C(23), C(24), and C(25) suggested the location of a CH_2OH group at C(24) of the side chain. The structure of the aglycone moiety was confirmed by the ^{1}H,^{1}H-COSY connectivities H–C(1)/H–C(2)/H–C(3), H–C(5)/CH_2(6)/CH_2(7)/H–C(8), CH_2(11)/CH_2(12), CH_2(15)/CH_2(16)/H–C(17)/H–C(20)/CH_2(22)/CH_2(23) and H–C(20)/Me(21) (*Fig. 2*). The relative configurations α and β of OH–(1) and XylO–C(3), respectively, were confirmed by the key ROESY correlations H–C(1)/H_{ax}–C(2) and

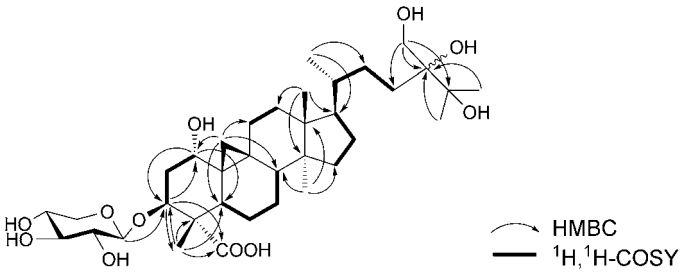


Fig. 2. *Major HMBC and ^{1}H,^{1}H-COSY features of* **1**

Table 1. *^{1}H-NMR Data* (C_5D_5N, 400 MHz) *of Compounds* **1**–**5**[a]). δ in ppm, J in Hz.

H-Atom	**1**	**2**	**3**	**4**	**5**
H–C(1)	3.88 (br. *s*)	3.88 (br. *s*)	3.81 (br. *s*)	3.87 (br. *s*)	3.80 (br. *s*)
CH_2(2)	2.25–2.31, 2.75–2.80 (2*m*)	2.26–2.32, 2.77–2.82 (2*m*)	2.18–2.23, 2.82–2.88 (2*m*)	2.24–2.32, 2.75–2.82 (2*m*)	2.18–2.23, 2.82–2.87 (2*m*)
H–C(3)	5.50 (*dd*, *J* = 12.1, 4.3)	5.49 (*dd*, *J* = 11.9, 4.1)	5.51 (*dd*, *J* = 12.1, 4.0)	5.51 (*dd*, *J* = 11.2, 4.3)	5.53 (*dd*, *J* = 12.0, 4.1)
H–C(5)	3.40 (*dd*, *J* = 10.8, 3.2)	3.39 (*dd*, *J* = 12.3, 4.2)	3.38 (*dd*, *J* = 11.5, 3.8)	3.40 (*dd*, *J* = 11.2, 3.4)	3.40 (*dd*, *J* = 10.8, 4.0)
CH_2(6)	1.20[b]), 1.75[b])	1.22[b]), 1.75[b])	1.23[b]), 1.75[b])	1.25[b]), 1.75[b])	1.24[b]), 1.75[b])
CH_2(7)	1.24[b]), 1.40[b])	1.26[b]), 1.39[b])	1.20[b]), 1.37[b])	1.27[b]), 1.39[b])	1.23[b]), 1.35[b])
H–C(8)	1.53[b])	1.58[b])	1.55[b])	1.55[b])	1.53[b])
CH_2(11)	1.39[b]), 2.67–2.72 (*m*)	1.41[b]), 2.68–2.73 (*m*)	1.39[b]), 2.66–2.71 (*m*)	1.33[b]), 2.64–2.71 (*m*)	1.35[b]), 2.66–2.72 (*m*)
CH_2(12)	1.55[b]), 1.67[b])	1.50[b]), 1.92[b])	1.44[b]), 1.92[b])	1.53[b]), 1.97[b])	1.45[b]), 1.97[b])
CH_2(15)	1.14–1.20 (*m*), 1.28[b])	1.15–1.19 (*m*), 1.28[b])	1.12–1.18 (*m*), 1.25[b])	1.14–1.21 (*m*), 1.29[b])	1.15–1.19 (*m*), 1.30[b])
CH_2(16)	1.24[b]), 1.87[b])	1.31[b]), 1.92[b])	1.26[b]), 1.91[b])	1.32[b]), 1.87[b])	1.30[b]), 1.88[b])
H–C(17)	1.59[b])	1.63[b])	1.68[b])	1.58[b])	1.57[b])
Me(18)	1.00 (*s*)	1.03 (*s*)	1.02 (*s*)	0.98 (*s*)	0.97 (*s*)
CH_2(19)	0.77, 0.48 (2*d*, *J* = 4.3)	0.76, 0.48 (2*d*, *J* = 3.9)	0.76, 0.44 (2*d*, *J* = 3.7)	0.74, 0.46 (2*d*, *J* = 4.0)	0.73, 0.42 (2*d*, *J* = 4.3)
H–C(20)	1.42[b])	1.53[b])	1.42[b])	1.44[b])	1.45[b])
Me(21)	0.99 (*d*, *J* = 6.4)	0.98 (*d*, *J* = 6.4)	0.98 (*d*, *J* = 6.4)	0.88 (*d*, *J* = 6.4)	0.89 (*d*, *J* = 6.4)
CH_2(22)	1.32[b]), 1.79[b])	1.51[b]), 1.79[b])	1.50[b]), 1.77[b])	1.43[b]), 1.95–2.01 (*m*)	1.43[b]), 1.96–2.00 (*m*)
CH_2(23)	1.80[b]), 2.17[b])	1.70[b]), 1.95[b])	1.64[b]), 1.94[b])	1.68[b]), 2.95–3.01 (*m*)	1.63[b]), 2.94–3.03 (*m*)
H–C(24)		3.73–3.78 (*m*)	3.73–3.79 (*m*)		
Me(26)	1.65 (*s*)	1.52 (*s*)	1.53 (*s*)	1.56[b]) (br. *s*)	1.56[b]) (br. *s*)
Me(27)	1.64 (*s*)	1.50 (*s*)	1.51 (*s*)	1.56[b]) (br. *s*)	1.56[b]) (br. *s*)
Me(29)	1.66 (*s*)	1.64 (*s*)	1.65 (*s*)	1.65 (*s*)	1.66 (*s*)
Me(30)	0.95 (*s*)	0.95 (*s*)	0.95 (*s*)	0.92 (*s*)	0.91 (*s*)
CH_2(31)	4.23 (br. *s*)				
H–C(1′)	5.03 (*d*, *J* = 7.0)	5.02 (*d*, *J* = 6.9)	5.06 (*d*, *J* = 7.6)	5.03 (*d*, *J* = 7.0)	5.08 (*d*, *J* = 7.5)
H–C(2′)	3.92–3.96 (*m*)	3.92–3.97 (*m*)	3.90–3.98 (*m*)	3.93–3.98 (*m*)	3.92–3.99 (*m*)
H–C(3′)	3.96–3.99 (*m*)	3.97–4.02 (*m*)	4.06 (*t*, *J* = 8.9)	3.98–4.01 (*m*)	4.05–4.11 (*m*)
H–C(4′)	4.11–4.15 (*m*)	4.14–4.16 (*m*)	4.14–4.21 (*m*)	4.11–4.16 (*m*)	4.17–4.25 (*m*)
CH_2(5′) or H–C(5′)	3.47 (*t*, *J* = 10.0), 4.17 (*dd*, *J* = 10.0, 5.6)	3.46 (*t*, *J* = 10.0), 4.18 (*dd*, *J* = 10.0, 5.2)	3.66–3.73 (*m*)	3.46 (*t*, *J* = 10.8), 4.18 (*dd*, *J* = 10.8, 5.2)	3.65–3.71 (*m*)
CH_2(6′)			4.28 (*dd*, *J* = 11.7, 4.8), 4.38 (*dd*, *J* = 11.7, 2.2)		4.30 (*dd*, *J* = 11.4, 4.4), 4.36 (*dd*, *J* = 11.4, 1.0)

[a]) Assignments were performed by means of HSQC, HMBC, and COSY experiments. [b]) Overlapped signals.

Table 2. *^{13}C-NMR Data* (C_5D_5N, 100 MHz) *of Compounds* **1**–**5**

C-Atom	**1**	**2**	**3**	**4**	**5**
C(1)	72.3 (*d*)	72.4 (*d*)	72.3 (*d*)	72.4 (*d*)	72.3 (*d*)
C(2)	37.5 (*t*)	37.6 (*t*)	37.4 (*t*)	37.6 (*t*)	37.4 (*t*)
C(3)	81.3 (*d*)	81.4 (*d*)	81.9 (*d*)	81.4 (*d*)	81.9 (*d*)
C(4)	54.7 (*s*)	54.8 (*s*)	54.7 (*s*)	54.7 (*s*)	54.6 (*s*)
C(5)	37.9 (*d*)	37.9 (*d*)	37.9 (*d*)	37.9 (*d*)	37.9 (*d*)
C(6)	23.1 (*t*)	23.1 (*t*)	23.2 (*t*)	23.1 (*t*)	23.1 (*t*)
C(7)	25.8 (*t*)	25.9 (*t*)	25.8 (*t*)	25.8 (*t*)	25.8 (*t*)
C(8)	48.2 (*d*)	48.3 (*d*)	48.2 (*d*)	48.2 (*d*)	48.2 (*d*)
C(9)	20.9 (*s*)	21.0 (*s*)	20.9 (*s*)	20.9 (*s*)	20.9 (*s*)
C(10)	30.1 (*s*)	30.2 (*s*)	30.2 (*s*)	30.1 (*s*)	30.2 (*s*)
C(11)	26.2 (*t*)	26.2 (*t*)	26.0 (*t*)	26.1 (*t*)	26.1 (*t*)
C(12)	33.3 (*t*)	33.3 (*t*)	33.4 (*t*)	33.2 (*t*)	33.2 (*t*)
C(13)	45.6 (*s*)	45.7 (*s*)	45.7 (*s*)	45.6 (*s*)	45.6 (*s*)
C(14)	49.1 (*s*)	49.2 (*s*)	49.2 (*s*)	49.2 (*s*)	49.2 (*s*)
C(15)	35.9 (*t*)	35.9 (*t*)	35.9 (*t*)	35.8 (*t*)	35.8 (*t*)
C(16)	28.4 (*t*)	28.4 (*t*)	28.5 (*t*)	28.3 (*t*)	28.3 (*t*)
C(17)	53.1 (*d*)	52.9 (*d*)	53.0 (*d*)	52.6 (*d*)	52.6 (*d*)
C(18)	18.5 (*q*)	18.5 (*q*)	18.5 (*q*)	18.4 (*q*)	18.4 (*q*)
C(19)	29.7 (*t*)	29.7 (*t*)	29.8 (*t*)	29.6 (*t*)	29.6 (*t*)
C(20)	37.6 (*d*)	36.4 (*d*)	37.7 (*d*)	36.2 (*d*)	36.1 (*d*)
C(21)	18.8 (*q*)	18.6 (*q*)	18.7 (*q*)	18.5 (*q*)	18.3 (*q*)
C(22)	30.3 (*t*)	34.2 (*t*)	34.2 (*t*)	30.6 (*t*)	30.7 (*t*)
C(23)	30.7 (*t*)	29.0 (*t*)	29.0 (*t*)	33.5 (*t*)	33.5 (*t*)
C(24)	76.5 (*s*)	79.2 (*d*)	79.1 (*d*)	216.6 (*s*)	216.6 (s)
C(25)	76.7 (*s*)	72.9 (*s*)	72.8 (*s*)	76.9 (*s*)	76.9 (*s*)
C(26)	26.0 (*q*)	26.0 (*q*)	26.3 (*q*)	27.3 (*q*)	27.3 (*q*)
C(27)	26.1 (*q*)	26.3 (*q*)	26.5 (*q*)	27.4 (*q*)	27.4 (*q*)
C(28)	180.0 (*s*)	180.1 (*s*)	180.2 (*s*)	180.0 (*s*)	180.2 (*s*)
C(29)	10.4 (*q*)	10.4 (*q*)	10.5 (*q*)	10.4 (*q*)	10.5 (*q*)
C(30)	19.5 (*q*)	19.6 (*q*)	19.6 (*q*)	19.5 (*q*)	19.5 (*q*)
C(31)	65.6 (*t*)				
C(1′)	106.6 (*d*)	106.6 (*d*)	105.9 (*d*)	106.6 (*d*)	105.9 (*d*)
C(2′)	75.5 (*d*)	75.5 (*d*)	75.7 (*d*)	75.5 (*d*)	75.7 (*d*)
C(3′)	78.1 (*d*)	78.1 (*d*)	78.3 (*d*)	78.1 (*d*)	78.3 (*d*)
C(4′)	71.1 (*d*)	71.1 (*d*)	71.5 (*d*)	71.1 (*d*)	71.4 (*d*)
C(5′)	67.0 (*t*)	67.0 (*t*)	78.1 (*d*)	67.0 (*t*)	78.1 (*d*)
C(6′)			62.8 (*t*)		62.7 (*t*)

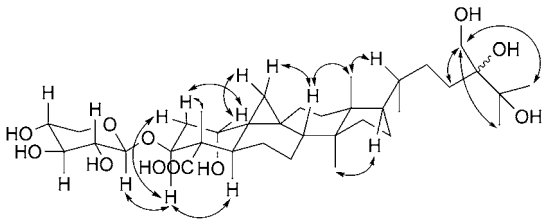


Fig. 3. *Key ROESY correlations* (H ↔ H) *of* **1**

H_a–C(19), and H–C(3)/H–C(1′), H–C(5), and H_{eq}–C(2) (*Fig. 3*). On acid hydrolysis, **1** afforded D-xylose, the structure of which was confirmed by HPLC analysis according to the method of *Tanaka*, *Kouno*, and co-workers [11]. Therefore, the structure of **1** was deduced to be (1α,3β,24ζ)-1,3,24,25-tetrahydroxy-24-(hydroxymethyl)cycloartan-28-oic acid 3-(β-D-xylopyranoside).

Nerviside E (**2**) was also obtained as white powder. It had the molecular formula $C_{35}H_{58}O_{10}$ on the basis of a quasimolecular ion at *m/z* 637.3954 ($[M-H]^-$) in the HR-ESI-MS (negative-ion mode). The ^{1}H- and ^{13}C-NMR data (*Tables 1* and *2*) showed good coincidence with those of **1**, except for the side chain, which suggested that **2** was also a cycloartane triterpene monoglycoside. The HMBC between an anomeric H-atom at δ(H) 5.02 (*d*, $J = 6.9$ Hz, H–C(1′)) and δ(C) 81.4 (*d*, C(3)) demonstrated that the sugar moiety was attached to C(3). Furthermore, the HMBCs H–C(24)/C(22), C(26), and C(27), Me(26)/C(24), C(25), and C(27), Me(27)/C(24), C(25), and C(26)) suggested the absence of a CH_2OH group at C(24). Additionally, the other key HMBCs were similar to those of **1** confirming the same substitutions at the cycloartane ring system as in **1**. The key ROESY correlations were identical to that of **1** showing the same relative configurations in ring *A*. The configuration at the OH-substituted C(24) was deduced to be (24*R*) by comparison with the chemical-shift values of cyclounifolioside C ((24*R*); C(24) at δ(C) 80.3), cyclocantogenin ((24*S*); C(24) at δ(C) 77.0), oleifoliosides A and B ((24*S*); C(24) at δ(C) 77.1) [12][13]. On acid hydrolysis, **2** afforded D-xylose. Thus, the structure of **2** was elucidated as (1α,3β,24*R*)-1,3,24,25-tetrahydroxycycloartan-28-oic acid 3-(β-D-xylopyranoside).

Nerviside F (**3**) was also obtained as white powder. The molecular formula was determined to be $C_{36}H_{60}O_{11}$ from a quasimolecular ion at *m/z* 667.4063 ($[M-H]^-$) in the HR-ESI-MS (negative-ion mode). A comparison of the ^{1}H- and ^{13}C-NMR data (*Tables 1* and *2*) of **3** with those of **2** showed that, structurally, **3** closely resembled **2**, except for the presence of a glucose instead of a xylose moiety. Therefore, **3** was also a cycloartane triterpene monoglycoside. The signals at δ(C) 105.9 (*d*, C(1′)), 75.7 (*d*, C(2′)), 78.3 (*d*, C(3′)), 71.5 (*d*, C(4′)), 78.1(*d*, C(5′)), and 62.8 (*t*, C(6′)) belonged to the glucose unit [9]. The HMBC between an anomeric H-atom at δ(H) 5.06 (*d*, $J = 7.6$ Hz, H–C(1′)) and δ(C) 81.9 (*d*, C(3)) established that the sugar moiety was connected to C(3). The key ROESY correlations were identical to those of **2**, indicating the same relative configurations in the cycloartane ring system. The configuration at the OH-substituted at C(24) was deduced to be (24*R*) by comparison with the chemical-shift values of **2**. On acid hydrolysis, **3** afforded D-glucose. From the above evidences, the structure of **3** was identified to be (1α,3β,24*R*)-1,3,24,25-tetrahydroxycycloartan-28-oic acid 3-(β-D-glucopyranoside).

Nerviside G (**4**) was isolated as white powder. The molecular formula was calculated as $C_{35}H_{56}O_{10}$ from a quasimolecular ion at *m/z* 659.3762 ($[M+Na]^+$) in the HR-ESI-MS. The ^{1}H- and ^{13}C-NMR data (*Tables 1*and *2*) were similar to those of **2**, except for the side chain, which showed that **4** was a cycloartane triterpene monoglycoside. In the ^{13}C-NMR spectrum of **4**, a highly deshielded signal at δ(C) 216.6 (*s*) was expected to represent an isolated C=O group. The HMBCs Me(26)/C(24), C(25), and C(27), and Me(27)/C(24), C(25), and C(26) suggested the location of the C=O group at C(24). In addition, the sugar moiety was attached to C(3) as shown by the HMBC between an anomeric H-atom at δ(H) 5.03 (*d*, $J = 7.0$ Hz,

H–C(1′)) and δ(C) 81.4 (*d*, C(3)). The major ROESY correlations were similar to those of **2**, indicating the same relative configurations the in cycloartane ring system. On acid hydrolysis, **4** afforded D-xylose. Therefore, the structure of **4** was established as (1α,3β)-1,3,25-trihydroxy-24-oxocycloartan-28-oic acid 3-(β-D-xylopyranoside).

Nerviside H (**5**) was also isolated as white powder. The molecular formula was assigned to be $C_{36}H_{58}O_{11}$ from a quasimolecular ion at *m*/*z* 689.3866 ($[M+Na]^+$) in the HR-ESI-MS. The ^{1}H- and ^{13}C-NMR data (*Tables 1* and *2*) showed good coincidence with those of **4**, except for the sugar moiety, which suggested that **5** was a cycloartane triterpene monoglycoside. The signals at δ(C) 105.9 (*d*, C(1′)), 75.7 (*d*, C(2′)), 78.3 (*d*, C(3′)), 71.4 (*d*, C(4′)), 78.1(*d*, C(5′)), and 62.7 (*t*, C(6′)) belonged to the glucose unit [9]. The HMBC between an anomeric H-atom at δ(H) 5.08 (*d*, J = 7.5 Hz, H–C(1′)) and δ(C) 81.9 (*d*, C(3)) indicated that the sugar moiety was bound to C(3). In addition, the other key HMBCs of **5** were similar to those of **4**, confirming the same substitutions at the cycloartane moiety as in **4**. The relative configuration was determined to be α for OH–C(1) and β for the 3-(glucosyloxy) substituent at ring *A* by the key ROESY correlations H–C(1)/H_{ax}–C(2) and H–C(19a), and H–C(3)/H–C(1′) and H–C(5). On acid hydrolysis, **5** afforded D-glucose. Accordingly, the structure of **5** was assigned to be (1α,3β)-1,3,25-trihydroxy-24-oxocycloartan-28-oic acid 3-(β-D-glucopyranoside).

Nervisides D – H (**1** – **5**) were all found to be inactive (test concentration 100 μg/ml) in the evaluation for the cytotoxic activity against CNE, Hep-2 and HepG2 cell lines by the MTT assay method [14].

This research was financially supported by the *Foundation of Guangdong Natural Sciences Committee* (No. 8351063201000003). The authors are grateful to Ms. *Chun-Chi Feng* and Ms. *Xiao-Lin Feng*, for the measurement of all NMR spectra and Ms. *Hui-Nan Zhao* for the recording of the HR-ESI-MS (College of Pharmacy, Jinan University, Guangzhou, P. R. China).

Experimental Part

General. Column chromatography (CC): polyamide (80 – 100 mesh; *Taizhou Luqiao Plaschem Co., Ltd.*, Taizhou, P. R. China), silica gel (SiO_2, 200 – 300 mesh; *Qingdao Marine Chemical Co. Ltd.*, Qingdao, P. R. China), *D101* macroporous resin (*Tianjin Agricultural Pesticide Co.*, Tianjin, P. R. China), *ODS* (50 μm; *YMC*, Tokyo, Japan); *Sephadex LH-20* (*Phamacia Co.* Sweden). TLC: precoated aluminium silica gel plates (*Yantai Chemical Industry Research Institute*, Yantai, P. R. China). Anal. HPLC: *Agilent 1200* instrument, equipped with a quaternary pump, a multiple-wavelength detector, an autosampler, and an *Ultimate™-XB-C18* column (5 μm, 250 × 4.6 mm; *Welch*, USA); t_R in min. Prep. HPLC: UV detectors from *Varian* (Palo Alto, CA, USA); *Ultimate™-XB-C18* column (5 μm, 21.2 × 250 mm; *Welch*, USA). Optical rotations: *Jasco P-1030* automatic digital polarimeter. M.p.: *X-5* micro-melting point apparatus; uncorrected. UV Spectra: *Jasco V-550* UV/VIS spectrometer; λ_{max} (log ε) in nm. IR Spectra: *Jasco FT/IR-480 Plus* spectrometer; KBr pellets; $\tilde{\nu}$ in cm^{-1}. NMR Spectra: *Bruker-AV-400* instrument; chemical shifts δ in ppm rel. to Me_4Si as internal standard, *J* in Hz. HR-ESI-MS: *Agilent-6210-LC/MSD-TOF* mass spectrometer; in *m*/*z*.

Plant Material. The aerial part of the plant *N. fordii* as the experimental material was purchased at the Qingping market for Chinese Medicinal Material of Guangzhou, P. R. China, in January 2011. The material was collected in Guangxi and traded to Guangzhou. The original plant of the aerial material was authenticated as *N. fordii* (HANCE) SCHLTR. by Prof. *G.-X. Zhou*, College of Pharmacy, Jinan University. A specimen (No. 110113) has been deposited with the Department of Pharmacognosy in the college of Pharmacy, Jinan University, Guangzhou, P. R. China.

Extraction and Isolation. The aerial part of the plant *N. fordii* (5.0 kg) was extracted with 90% EtOH under reflux (3 × 20 l, each 2 h). The combined extracts were concentrated to *ca.* 2.5 l and then

partitioned successively with petroleum ether, $CHCl_3$, and AcOEt, to afford 330.5 g, 45.6 g, and 18.6 g of extracts, resp., and 555.0 g of residue from the H_2O layer. The AcOEt (18.6 g) fraction was subjected to CC (polyamide, MeOH/H_2O 1:4 → 1:0): *Fractions 1–10*. *Fr. 3* (2.3 g) was fractionated by CC (*Sephadex LH-20*, $CHCl_3$/MeOH 1:1): *Frs. H_1–H_8*. *Fr. H_5* (1.2 g) was subjected to CC (*ODS*, MeOH/H_2O 1:5 → 1:0): *Frs. H_5-1–H_5-7*. *Fr. H_5-3* (126 mg) was finally purified by prep. HPLC (MeCN/H_2O 1:1 containing 0.05% of CF_3COOH): **4** (16 mg) and **5** (9 mg). The H_2O-soluble portion (500.0 g) was separated by CC (*D101* macroporous resin, EtOH/H_2O 0:1 → 95:5): *Frs. D_1–D_4*. *Fr. D_2* (15.6 g) was subjected to CC (SiO_2, $CHCl_3$/MeOH/H_2O 8:2:0.2 → 6:4:0.5): *Frs. D_2-a–D_2-h*. *Fr. D_2-e* (1.8 g) was fractionated by CC (*Sephadex LH-20*, $CHCl_3$/MeOH 1:1): *Frs. D_2-e-1–D_2-e-9*. *Fr. D_2-e-4* (860 mg) was subjected to CC (*ODS*, MeOH/H_2O 6:4 → 9:1): *Frs. D_2-e-4.1–D_2-e-4.15*. *Fr. D_2-e-4.6* (156 mg) was finally purified by prep. HPLC (MeCN/H_2O 45:55 containing of CF_3COOH): **2** (13 mg) and **3** (10 mg). *Fr. D_2-e-4.2* (125 mg) was purified by prep. HPLC (MeCN/H_2O 4:6 containing 0.05% of CF_3COOH): **1** (18 mg).

Acid Hydrolysis and Identification of the Sugar Moieties of Compounds **1**–**5** *and Determination of Their Absolute Configuration*. The absolute configuration of the sugar moieties in the structures was determined by the method of *Tanaka*, *Kouno*, and co-workers [11]. Compound **1** (1 mg) was hydrolyzed with 1N HCl (2 ml) for 2 h at 90°. The mixture was concentrated the residue dissolved in pyridine (1 ml) containing L-cysteine methyl ester (1 mg; *Adamas-beta*, China), and the mixture heated at 60° for 1 h. Then, *o*-tolyl isothiocyanate (5 µl; *Sigma*, USA) was added, and the mixture was heated at 60° for 1 h. Then the mixture was directly analyzed by anal. reversed-phase HPLC (30°, isocratic elution with 25% MeCN containing 0.08% of formic acid for 40 min, then washing of the column with 90% MeCN; flow rate 0.8 ml/min. UV detection at 250 nm): derivative of **1** at t_R 25.442 (D-Xyl). Standard sugars (*Sigma*, USA) such as D-glycose, L-glycose, D-xylose, and L-xylose were subjected to the same method and their derivatives recorded: t_R 22.291 (D-Glc), 20.298 (L-Glc), 25.536 (D-Xyl), and 23.828 (L-Xyl). Following the above procedure, the derivatives of **2**–**5** gave peaks at t_R 25.391 (D-Xyl), 22.329 (D-Glc), 25.661 (D-Xyl) and 22.309 (D-Glc), resp.

Nerviside D (=*(1α,3β,24ζ)-1,3,24,25-Tetrahydroxy-24-(hydroxymethyl)cycloartan-28-oic Acid 3-(β-D-Xylopyranoside)* = rel-*(1α,3β,24ζ)-1,24,25-Trihydroxy-24-(hydroxymethyl)-3-(β-D-xylopyranosyl-oxy)-9,19-cyclolanostan-28-oic Acid*; **1**). White powder. M.p. 267–268°. $[\alpha]_D^{25} = +40.5$ ($c = 0.60$, MeOH). UV (MeOH): 203. IR (KBr): 3421, 2943, 2870, 1703. ^{1}H- and ^{13}C-NMR: *Tables 1* and *2*. HR-ESI-MS: 691.4026 ($[M+Na]^+$, $C_{36}H_{60}NaO_{11}^+$; calc. 691.4028).

Nerviside E (=*(1α,3β,24*R*)-1,3,24,25-Tetrahydroxycycloartan-28-oic Acid 3-(β-D-Xylopyrano-side)* = rel-*(1α,3β,24*R*)-1,24,25-Trihydroxy-3-(β-D-xylopyranosyloxy)-9,19-cyclolanostan-28-oic Acid*; **2**). White powder. M.p. 245–246°. $[\alpha]_D^{25} = +42.8$ ($c = 0.69$, MeOH). UV (MeOH): 202. IR (KBr): 3403, 2943, 2861, 1714. ^{1}H- and ^{13}C-NMR: *Tables 1* and *2*. HR-ESI-MS: 637.3954 ($[M-H]^-$, $C_{35}H_{57}O_{10}^-$; calc. 637.3957).

Nerviside F (=*(1α,3β,24*R*)-1,3,24,25-Tetrahydroxycycloartan-28-oic Acid 3-(β-D-Glucopyrano-side)* = rel-*(1α,3β,24*R*)-3-(β-D-Glucopyranosyloxy)-1,24,25-Trihydroxy-9,19-cyclolanostan-28-oic Acid*; **3**). White powder. M.p. 261–262°. $[\alpha]_D^{25} = +51.9$ ($c = 0.87$, MeOH). UV (MeOH): 203. IR (KBr): 3417, 2943, 2870, 1703. ^{1}H- and ^{13}C-NMR: *Tables 1* and *2*. HR-ESI-MS: 667.4063 ($[M-H]^-$, $C_{36}H_{59}O_{11}^-$; calc. 667.4063).

Nerviside G (=*(1α,3β)-1,3,25-Trihydroxy-24-oxocycloartan-28-oic Acid 3-(β-D-Xylopyranoside)* = rel-*(1α,3β)-1,25-Dihydroxy-24-oxo-3-(β-D-xylopyranosyloxy)-9,19-cyclolanostan-28-oic Acid*; **4**). White powder. M.p. 258–259°. $[\alpha]_D^{25} = +27.8$ ($c = 0.73$, MeOH). UV (MeOH): 203. IR (KBr): 3421, 2942, 2873, 1708, 1696. ^{1}H- and ^{13}C-NMR: *Tables 1* and *2*. HR-ESI-MS: 659.3762 ($[M+Na]^+$, $C_{35}H_{56}NaO_{10}^+$; calc. 659.3766).

Nerviside H (=*(1α,3β)-1,3,25-Trihydroxy-24-oxocycloartan-28-oic Acid 3-(β-D-Glucopyranoside)* = rel-*(1α,3β)-3-(β-D-glucopyranosyloxy)-1,25-dihydroxy-24-oxo-9,19-cyclolanostan-28-oic Acid*; **5**). White powder. M.p. 250–251°. $[\alpha]_D^{25} = +37.6$ ($c = 0.73$, MeOH). UV (MeOH): 204. IR (KBr): 3418, 2939, 2875, 1709, 1682. ^{1}H- and ^{13}C-NMR: *Tables 1* and *2*. HR-ESI-MS: 689.3866 ($[M+Na]^+$, $C_{36}H_{58}NaO_{11}^+$; calc. 689.3871).

REFERENCES

[1] H.-H. Xu, J.-M. Liu, 'Dictionary of Zhong Cao Yao Tu Pu', Guangdong Scientific and Technological Press, Guangzhou, 2007, p. 264.
[2] G.-X. Zhou, C.-L. Lu, H.-S. Wang, X.-S. Yao, *J. Asian Nat. Prod. Res.* **2009**, *11*, 498.
[3] Z.-H. Wang, Q. Du, F.-X. Zhang, *Shizhen Guo Yi GuoYao* **2007**, *18*, 2940.
[4] L.-W. Tian, Y. Pei, Y.-J. Zhang, Y.-F. Wang, C.-R. Yang, *J. Nat. Prod.* **2009**, *72*, 1057.
[5] J. Y. Zhao, *Int. J. Appl. Chem.* **2006**, *2*, 201.
[6] H.-S. Zhen, Y.-Y. Zhou, Y.-F. Yuan, H.-H. Mo, Z.-G. Zhong, C.-Y. Liang, *Zhong Yao Cai* **2007**, *30*, 942.
[7] J.-H. Ju, D. Liu, G. Lin, Y.-M. Zhang, J.-S. Yang, Y. Lu, N.-B. Gong, Q.-T. Zhang, *J. Nat. Prod.* **2002**, *65*, 147.
[8] A. Yokosuka, K. Sato, Y. Mimaki, *Phytochemistry* **2010**, *71*, 2174.
[9] Z. Zhao, K. Matsunami, H. Otsuka, T. Shinzato, Y. Takeda, *Chem. Pharm. Bull.* **2008**, *56*, 1153.
[10] R. Kasai, A. Sasaki, T. Hashimoto, T. Kaneko, K. Ohtani, K. Yamasaki, *Phytochemistry* **1999**, *51*, 803.
[11] T. Tanaka, T. Nakashima, T. Ueda, K. Tomii, I. Kouno, *Chem. Pharm. Bull.* **2007**, *55*, 899.
[12] K. D. Kucherbaev, K. K. Uteniyazov, V. V. Kachala, Z. Saatov, A. S. Shashkov, *Chem. Nat. Compd.* **2002**, *38*, 447.
[13] M. Özipek, A. A. Dönmez, İhsan Çalış, R. Brun, P. Rüedi, D. Tasdemir, *Phytochemistry* **2005**, *66*, 1168.
[14] T. Mosmann, *J. Immunol. Methods* **1983**, *65*, 55.

Received March 21, 2012