Tirucallane Triterpenoids from the Stems of *Brucea mollis*

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Three new tirucallane triterpenoids, brumollisols A–C (**1**–**3**, resp.), together with five known analogues, (23*R*,24*S*)-23,24,25-trihydroxytirucall-7-ene-3,6-dione (**4**), piscidinol A (**5**), 24-epipiscidinol A (**6**), 21α-methylmelianodiol (**7**), and 21β-methylmelianodiol (**8**), were isolated from an EtOH extract of the stems of *Brucea mollis.* Their structures were elucidated by means of spectroscopic methods including 1D- and 2D-NMR techniques and mass spectrometry. In the *in vitro* assays, compound **6** exhibited significant cytotoxic activity against A549 and BGC-823 cancer cells with IC_{50} values of 1.16 and 3.01 µM, respectively. At a concentration of 10 µM, compounds **1**–**5**, **7**, and **8** were found to inhibit NO production in mouse peritoneal macrophages with inhibitory ratios ranging from 39.8±7.7 to 68.2±4.5%.

Introduction. Plants of the *Brucea* genus (Simaroubaceae) are usually bushes or small trees mainly distributed in the tropical eastern hemisphere. The genus comprises six species, of which only two (*B. javanica* and *B. mollis*) are found in China [1]. *B. mollis*, also known as '*Da Guo Ya Dan Zi*' in Chinese, distributed in southwestern China, is being used as a remedy against malaria and other parasitic diseases. In previous phytochemical investigations, quassinoids, canthin-6-one alkaloids, and indole alkaloids were isolated from this species [2–6]; however, tirucallane triterpenoid derivatives, the likely biosynthetic precursors of quassinoids, have so far not been found in this species [7]. In a continuing effort to search for new bioactive compounds from this species, the AcOEt fraction of an EtOH extract of the dried stems of *B. mollis* was studied. Further chemical investigations resulted in the isolation of three new tirucallane triterpenoids, **1**–**3**, together with five known analogs, **4**–**8**. Herein, we describe the isolation and structure elucidation of these compounds from *B. mollis*, their NO-production-inhibitory activities induced by LPS in mouse peritoneal macrophages, as well as an evaluation of their cytotoxic activities.

Results and Discussion. – *Structure Elucidation*. Compound **1** was obtained as a white amorphous powder with a molecular formula $C_{30}H_{48}O_5$ as determined by its positive HR-ESI-MS data (*m/z* 511.3419 ([M+Na]$^+$; calc. 511.3394)), suggesting seven degrees of unsaturation. The IR spectrum displayed the characteristic

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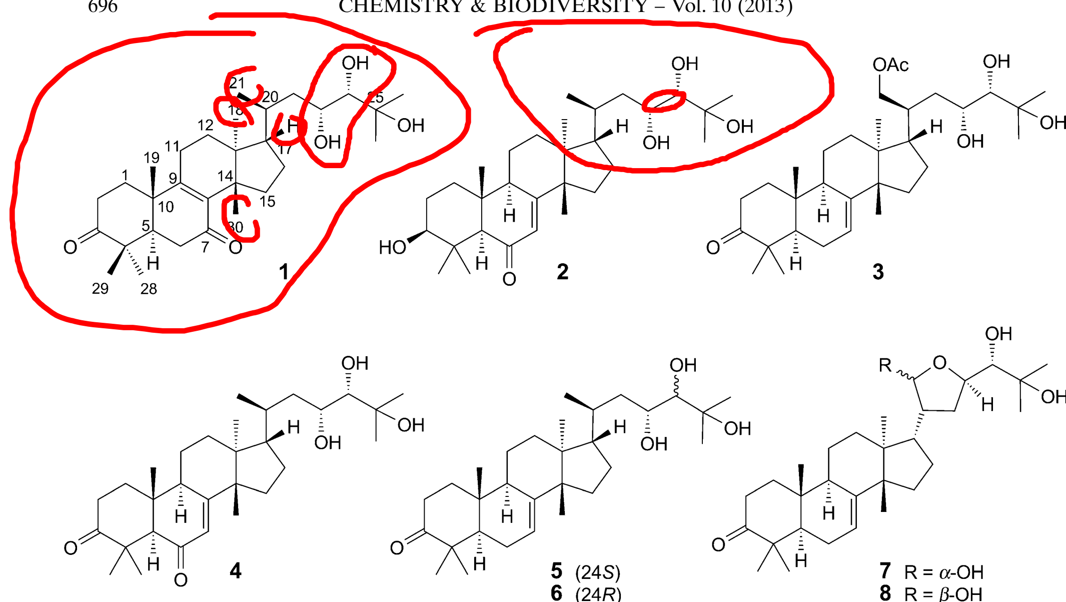

absorptions for OH (3308 cm^{-1}), unconjugated C=O (1709 cm^{-1}), and α,β-conjugated C=O (1648 cm^{-1}) groups. In accordance with the molecular formula, 30 C-atom resonances were resolved in the ^{13}C-NMR and DEPT spectra (*Tables 1* and *2*), and they were further classified by HSQC experiments as corresponding to eight Me, eight CH_2, five CH groups, and nine quaternary C-atoms (containing two C=O and two olefinic C-atoms). The latter C-atoms account for three degrees of unsaturation. The remaining five degrees of unsaturation suggested that the molecule of **1** contains five rings. The ^{1}H-NMR spectrum of **1** displayed signals of seven tertiary Me groups, two of which (δ(H)1.12 and 1.08) mutually correlated by HMBC and showed cross-peaks with the ketone C=O C-atom (δ(C) 214.1), and with a CH group C-atom (δ(C) 47.0). These data suggested a tetracylic core for **1**, represented by a 3-oxotirucallane triterpenoid [8]. A conjugated α,β-unsaturatured cyclohexanone was evidenced by the observed maximum absorption at 252 nm (log ε 4.07) in the UV spectrum. The key HMBCs from CH_2(6) (δ(H) 2.44–2.50 and 2.38–2.41) to C(7) (δ(C) 197.0) and C(8) (δ(C) 139.0), from CH_2(11) (δ(H) 2.36–2.38 and 2.25–2.30) to C(8) (δ(C) 139.0) and C(9) (δ(C) 163.4), and from Me(19) (δ(H) 1.27) to C(9) (δ(C) 163.4) confirmed the presence of the C(7)=O group and the C(8)=C(9) bond. The 23-, 24-, and 25-OH substituents of the side chain were established by the ^{1}H,^{1}H-COSY cross-peaks CH_2(22)/H–C(23) and H–C(23)/H–C(24), and HMBC cross-peaks from H–C(24) (δ(H) 3.16) to C(22) (δ(C) 40.4), C(23) (δ(C) 69.5), and C(25) (δ(C) 74.0). Thus, the constitutional formula of **1** was determined as shown in *Fig. 1*.

The NOESY experiment of **1** provided the relative configuration of the tetracyclic core as shown (*Fig. 1*). The NOESY correlations CH_3(18)/H–C(20) and H_α–C(12)/CH_3(21) were observed, which were possible only when **1** had (*S*)-configuration at C(20) (tirucallol skeleton) according to a molecular model [9]. Due to the free rotation

Table 1. *^{1}H-NMR Data of* **1**–**3**. Recorded at 500 MHz in $CDCl_3$; δ in ppm, J in Hz.

H-Atom	**1**	**2**	**3**
H_α–C(1)	1.79–1.82 (*m*)	1.07–1.12 (*m*)	1.40–1.47 (*m*)
H_β–C(1)	2.15–2.18 (*m*)	1.65–1.68 (*m*)	1.94–2.20 (*m*)
H_α–C(2)	2.39–2.42 (*m*)	1.62–1.66 (*m*)	2.21–2.24 (*m*)
H_β–C(2)	2.73–2.79 (*m*)	1.71–1.76 (*m*)	2.74 (*ddd*, J = 14.5, 5.5, 5.5)
H_α–C(3)		3.22 (*dd*, J = 12.0, 4.0)	
H_α–C(5)	2.11–2.16 (*m*)	2.11 (*s*)	1.68–1.71 (*m*)
H_α–C(6)	2.44–2.50 (*m*)		
H_β–C(6)	2.38–2.41 (*m*)		
H–C(7)		5.69 (*d*, J = 2.5)	5.30 (*d*, J = 2.5)
H_α–C(11)	2.36–2.38 (*m*)	1.68–1.72 (*m*)	1.71–1.76 (*m*)
H_β–C(11)	2.25–2.30 (*m*)	1.66–1.71 (*m*)	1.53–1.58 (*m*)
H_α–C(12)	1.80–1.82 (*m*)	1.72–1.76 (*m*)	1.79–1.83 (*m*)
H_β–C(12)	2.24–2.26 (*m*)	1.88–1.91 (*m*)	1.94–1.99 (*m*)
H_α–C(15)	1.47–1.53(*m*)	1.49–1.53 (*m*)	1.48–1.53 (*m*)
H_β–C(15)	1.74–1.78 (*m*)	1.76–1.78 (*m*)	1.76–1.79 (*m*)
H_α–C(16)	1.35–1.41 (*m*)	1.35–1.38 (*m*)	1.26–1.31 (*m*)
H_β–C(16)	2.06–2.12 (*m*)	2.02–2.08 (*m*)	2.00–2.04 (*m*)
H_β–C(17)	1.52–1.57 (*m*)	1.54–1.60 (*m*)	1.84–1.91 (*m*)
H_3–C(18)	0.72 (*s*)	0.82 (*s*)	0.83 (*s*)
Me(19)	1.27 (*s*)	0.85 (*s*)	0.98 (*s*)
H–C(20)	1.41–1.46 (*m*)	1.40–1.43 (*m*)	1.65–1.70 (*m*)
Me(21) or CH_2(21)	0.97 (*d*, J = 6.5)	0.94 (*d*, J = 6.5)	4.27 (*dd*, J = 11.5, 3.0, H_a), 3.90 (*dd*, J = 11.5, 5.0, H_b)
H_a–C(22)	1.85–1.90 (*m*)	1.85–1.88 (*m*)	1.71–1.76 (*m*)
H_b–C(22)	1.19–1.24 (*m*)	1.16–1.22 (*m*)	1.58–1.62 (*m*)
H–C(23)	4.12 (*dd*, J = 8.5, 5.0)	4.12 (*dd*, J = 8.5, 5.0)	4.08 (*dd*, J = 6.5, 6.0)
H–C(24)	3.16 (br. *s*)	3.15 (br. *s*)	3.10 (br. *s*)
Me(26)	1.31 (*s*)	1.31 (*s*)	1.28 (*s*)
Me(27)	1.30 (*s*)	1.30 (*s*)	1.28 (*s*)
Me(28)	1.08 (*s*)	1.30 (*s*)	1.03 (*s*)
Me(29)	1.12 (*s*)	1.12 (*s*)	1.09 (*s*)
Me(30)	0.98 (*s*)	1.05 (*s*)	1.01 (*s*)
21-OAc			2.06 (*s*)

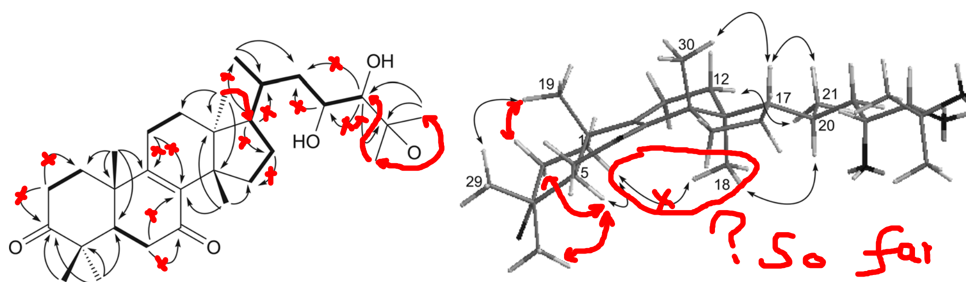

Fig. 1. *Key HMBC* (H → C), *^{1}H,^{1}H-COSY* (—), *and NOESY* (H ↔ H) *features of brumollisol A* (**1**)

Table 2. *^{13}C-NMR Data of* **1**–**3**. Recorded at 125 MHz in $CDCl_3$; δ in ppm, J in Hz.

Position	**1**	**2**	**3**
1	35.2	36.8	38.3
2	34.2	26.5	34.9
3	214.1	79.1	216.9
4	38.8	37.9	48.6
5	47.0	65.1	52.2
6	35.9	199.9	24.3
7	197.0	124.9	118.2
8	139.0	170.3	171.3
9	163.4	50.3	48.2
10	38.7	43.7	34.8
11	23.6	17.6	18.1
12	29.6	32.8	32.3
13	44.3	43.0	43.2
14	47.3	52.2	51.2
15	31.0	32.8	33.8
16	28.6	27.8	28.1
17	49.2	53.2	48.7
18	15.2	21.9	22.1
19	17.8	14.2	12.7
20	33.7	33.5	38.3
21	19.0	18.9	66.2
22	40.4	40.3	35.8
23	69.5	69.5	70.4
24	74.8	74.9	76.1
25	74.0	74.3	74.0
26	27.1	26.2	26.3
27	25.9	27.4	27.3
28	24.2	28.3	24.4
29	21.1	14.7	21.6
30	24.0	24.8	27.4
21-OAc			171.3, 27.4

of the triol side chain, the configurations at C(23) and C(24) was difficult to establish from the NOESY spectrum. However, H–C(23) and H–C(24) displayed a coupling constant J of 0 Hz, indicating a *syn-gauche* conformation [10]. This implied that the configurations of the side chain in **1** are (23*R*,24*S*) or (23*S*,24*R*). By comparison of the ^{1}H- and ^{13}C-NMR chemical shifts of the side chain of **1** (δ(H) 4.12 (*dd*, $J = 8.5$, 5.0, H–C(23)), 3.16 (br. *s*, H–C(24)); δ(C) 69.5 (C(23)), 74.8 (C(24))) with those of the (23*R*,24*S*)-isomer of piscidinol A (**5**) [11] (δ(H) 4.11 (*dd*, $J = 8.5$, 5.0, H–C(23)), 3.16 (br. *s*, H–C(24)); δ(C) 69.6 (C(23)), 74.9 (C(24))) and of the (23*S*,24*R*)-isomer of alisol A [12] (δ(H) 3.77 (*dd*, $J = 9.3$, 3.5, H–C(23)), 3.00 (br. *s*, H–C(24)); δ(C) 69.5 (C(23)), 77.6 (C(24))) allowed assignment of (23*R*,24*S*)-configuration for **1**. Thus, the structure of **1**, named brumollisol A, was elucidated as (23*R*,24*S*)-23,24,25-trihydroxytirucall-8-ene-3,7-dione.

Compound **2** was obtained as a white amorphous powder with a molecular formula $C_{30}H_{50}O_5$ as determined by its positive-ion HR-ESI-MS data (*m/z* 513.3558 ([*M*+Na]$^+$;

calc. 513.3550); two mass units more than **1**). Its IR absorptions indicated the presence of OH (3419 cm^{-1}) and *α,β*-conjugated C=O (1657 cm^{-1}) groups. The ^{1}H- and ^{13}C-NMR spectra of **2** were similar to those of **4**, except for the replacement of the C(3)=O group by an oxygenated H–C(3) group in **2** (*δ*(H) 3.22 (*dd*, *J* = 12.0, 4.0); *δ*(C) 79.1) in **2**. The HMBCs from H–C(3) to C(29) (*δ*(C) 14.7), C(28) (*δ*(C) 28.3), C(2) (*δ*(C) 26.5), and C(1) (*δ*(C) 36.8) indicated the presence of a OH group at C(3). The signal at *δ*(H) 3.22 (*dd*, H–C(3)) with coupling constants *J* of 12.0 and 4.0 Hz indicated a *β*-orientation of the 3-OH group, which was confirmed by NOESY correlations from H–C(3) to H–C(5) and CH_3(28). The configurations at C(23) and C(24) of **2** were identical with those in **1** owing to the similarity of their side-chain NMR signals. Therefore, the structure of **2**, named brumollisol B, was elucidated as (3*β*,23*R*,24*S*)-3,23,24,25-tetrahydroxytirucall-7-en-6-one.

Compound **3** was isolated as a white amorphous powder. Its HR-ESI-MS showed a peak at *m/z* 555.3669 ($[M+Na]^+$), indicating the molecular formula $C_{32}H_{52}O_6$. The IR spectrum evidenced the presence of OH (3450 cm^{-1}), AcO (1737, 1242 cm^{-1}), and unconjugated C=O groups (1708 cm^{-1}). Analysis the ^{1}H- and ^{13}C-NMR spectra of **3** suggested that its structure was closely related to that of piscidinol A (**5**), with the only difference being the presence of an AcO group at C(21). This was confirmed by the HMBCs from CH_2(21) (*δ*(H) 4.27, 3.90) to C(20) (*δ*(C) 38.3), C(22) (*δ*(C) 35.8), and AcO (*δ*(C) 171.3), and $^{1}H,^{1}H$-COSY correlations from H–C(20) to CH_2(21) and CH_2(22). Similar NMR signals were observed for the side chains of both **3** and **5**. Hence, the structure of **3**, named brumollisol C, was elucidated as (23*R*,24*S*)-23,24,25-trihydroxy-3-oxotricucall-7-en-21-yl acetate.

The configuration of **4** [8], a known compound reported with the unassigned configuration within the side chain, could be assigned as (23*R*,24*S*) by comparing the corresponding ^{1}H- and ^{13}C-NMR data with those of **1**–**3**. The other known compounds, piscidinol A (**5**) [11], 24-epipiscidinol A (**6**) [11], 21*α*-methylmelianodiol (**7**) [13], and 21*β*-methylmelianodiol (**8**) [13], were identified by comparison of their spectroscopic data with those in the literature.

Compounds **1**–**8** were examined for their inhibitory effects on NO production induced by LPS in macrophages *in vitro*. Dexamethasone (Dex) was used as a positive control, which showed a value of 93.5 ± 0.6%. Cell viability was also determined by the MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) method to find whether inhibition of NO production was due to cytotoxicity of the test compounds (see *Fig. 2*). All compounds, except **6**, inhibited LPS-stimulated NO expression to various degrees at a concentration of 10 μM. Compounds **2**, **3**, **5**, **7**, and **8** exhibited conspicuous inhibitory activity, and the inhibitory ratios were 53.8 ± 6.1, 52.5 ± 4.2, 68.2 ± 4.5, 62.7 ± 2.9, and 61.9 ± 9.2%, respectively. Compounds **1** and **4** showed moderate inhibitory effects with inhibitory ratios of 39.8 ± 7.7 and 44.7 ± 5.7%, respectively. It should be noted that compound **5** with the (*S*)-configuration at C(24) showed a stronger inhibitory activity than **6** with (*R*)-configuration at C(24). This finding indicated that the (*S*)-configuration at C(24) might contribute to the inhibitory activity. The C(8)=C(9) bond in **1**, C=O group at C(6) in **4**, and AcO group at C(21) in **3** reduced inhibitory activities compared to other analogs. In addition, compound **2**, with an OH group at C(3), displayed better activity than **4**. The NO-inhibitory activities of compounds **7** and **8** have already been reported by *Zhou et al.* [11].

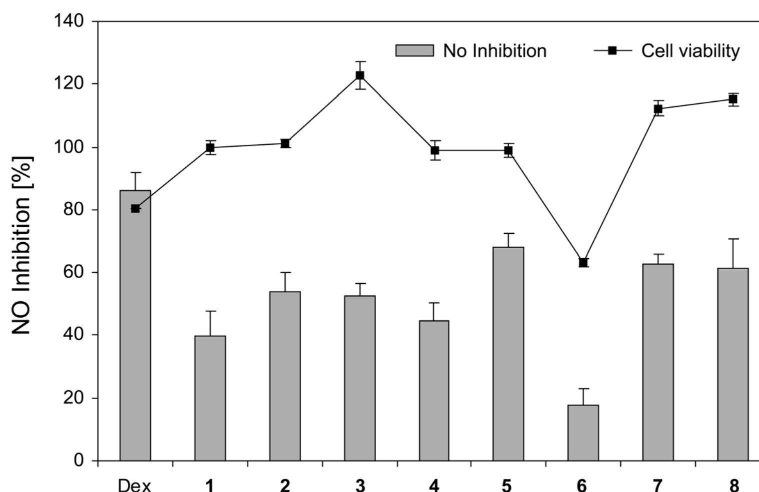

Fig. 2. *NO-Inhibition ratio and cell viability of compounds* **1**–**8** *in murine macrophage stimulated by LPS at 10 μM*. Dexamethasone (Dex) used as a positive control.

All compounds isolated were also evaluated for their cytotoxicity *in vitro* against two human cancer cell lines, A549 and BGC-823, using the MTT method. Compound **6** exhibited significant activities with IC_{50} values of 1.16 (A549) and 3.01 (BGC-823) μM, respectively, while the other compounds were inactive ($IC_{50} > 10$ μM). Adriamycin was used as a positive control, with IC_{50} values of 0.98 (BGC-823) and 0.72 (A549) μM, respectively.

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Experimental Part

General. All solvents were of anal. or chromatographic grade, and were purchased from *Beijing Chemical Company* (Beijing, China). Column chromatography (CC): silica gel (200–300 mesh) TLC: silica gel GF_{254} (*Qingdao Marine Chemical Factory*, China); visualization by spraying with 98% H_2SO_4/EtOH 5:95, followed by heating at 110°; *Sephadex LH-20* (*Pharmacia*), and *ODS* gel (50 μm; *Merck*). HPLC: *Shimadzu LC-6AD* equipped with an *SPD-10A* detector, with reversed-phase (RP) C_{18} column (*YMC-Pack, ODS-A;* 20 × 250 mm, 5 μm). Optical rotations: *Perkin-Elmer-241* digital polarimeter. IR Spectra: *Nicolet 5700* (FT-IR) apparatus; in cm^{-1}. ^1H- and ^{13}C-NMR spectra: at 500 and 125 MHz, resp.; δ in ppm, J in Hz. HSQC and HMBC spectra: *Varian INOVA-500* spectrometers. ESI-MS: *Agilent-1100* LC/MSD trap mass spectrometer. HR-ESI-MS: *Agilent Technologies 6250 Accurate-Mass* Q-TOF LC/MS spectrometer; in *m*/*z*.

Plant Material. The stems of *B. mollis* were collected from Guangxi Province, P. R. China, in July 2009. This plant was identified by Prof. *Wei Songji*, Guangxi College of Traditional Chinese Medicine. A voucher specimen (ID-21977) was deposited with the Herbarium of the Department of Medicinal Plants, Institute of Materia Medica, Chinese Academy of Medical Sciences.

Extraction and Isolation. Air-dried, powdered stems of *B. mollis* (6.5 kg) were macerated for 3 h with 20 l of 95% EtOH and further refluxed for 9 h (3 × 20 l). The filtrate was concentrated under reduced

pressure, and the residue (320 g) was suspended in H_2O and then successively partitioned with AcOEt and BuOH. The AcOEt extract (80 g) was subjected to CC (silica gel (200–300 mesh; 650 g); CH_2Cl_2/MeOH 70:1, 50:1, 30:1, 20:1, 10:1, 5:1, 1:1 (*v*/*v*)) to yield seven fractions, *Frs. A–G*. *Fr. A* (7.50 g) was subjected to CC (petroleum ether (PE)/AcOEt 3:1, 2:1, 1:1) to afford five subfractions; *Frs.* A_1–A_5. Compound **3** (12 mg), **5** (34 mg), and **6** (20 mg) were precipitated from *Frs.* A_2, A_4, and A_5, resp. *Fr.* A_3 was separated by prep. HPLC (MeCN/H_2O 60:40) to yield **7** (7 mg) and **8** (11 mg). *Fr. B* (10.2 g) was subjected to CC (*ODS* (45–70 μm, 400 g), MeOH/H_2O from 15:85 to 90:10) to give six subfractions, *Frs.* B_1–B_6). *Fr.* B_2 (750 mg) was submitted to CC (*Sephadex LH-20*; MeOH) and further purified by prep. HPLC (MeCN/H_2O 55:45) to yield **1** (12 mg) and **2** (6 mg). *Fr.* B_3 (220 mg) was separated by prep. HPLC (MeCN/H_2O 60:40) to yield **4** (25 mg).

Brumollisol A (=*(23*R*,24*S*)-23,24,25-Trihydroxytirucall-8-ene-3,7-dione*; **1**). White amorphous powder. $[\alpha]_D^{20} = -20.4$ ($c = 0.05$, $CHCl_3$). UV (MeOH): 252 (4.07). IR (KBr): 3308, 2991, 1709, 1648, 1586, 1372. ^1H- and ^{13}C-NMR: see *Tables 1* and *2*, resp. HR-ESI-MS: 511.3419 ($[M+Na]^+$, $C_{30}H_{48}NaO_5^+$; calc. 511.3394).

Brumollisol B (=*(3β,23*R*,24*S*)-3,23,24,25-Tetrahydroxytirucall-7-en-6-one*; **2**). White amorphous powder. $[\alpha]_D^{20} = -31.6$ ($c = 0.05$, $CHCl_3$). UV (MeOH): 241 (4.20). IR (KBr): 3419, 2960, 2935, 2877, 1657, 1466, 1383, 1241, 1160, 1048, 732. ^1H- and ^{13}C-NMR: see *Tables 1* and *2*, resp. HR-ESI-MS: 513.3558 $[M+Na]^+$, $C_{30}H_{50}NaO_5^+$; calc. 513.3550).

Brumollisol C (=*(23*R*,24*S*)-23,24,25-Trihydroxy-3-oxotirucall-7-en-21-yl Acetate*; **3**). White amorphous powder. $[\alpha]_D^{20} = -73.0$ ($c = 0.05$, $CHCl_3$). UV (MeOH): 239 (4.11). IR (KBr): 3450, 2966, 1737, 1708, 1386, 1242, 1027, 730. ^1H- and ^{13}C-NMR: see *Tables 1* and *2*, resp. HR-ESI-MS: 555.3669 $[M+Na]^+$, $C_{32}H_{52}NaO_6^+$; calc. 555.3656).

Inhibition of NO Production Assay. Compounds **1–8** were assessed by measuring the inhibitory effects on NO production induced by LPS in murine macrophage as described in [14]. Mouse peritoneal macrophages (*PEM*™) were treated with carrier control (DMSO only), LPS, or test compunds (LPS + compounds of 1×10^{-6} M final concentration), or DEX (1×10^{-6} M final concentration). All incubation procedures were performed with 5% CO_2 in humidified air at 37°. NO Production was determined by detection the accumulation of nitrite in the culture medium by *Griess* reagent. Briefly, 100 μl of the supernatant of culture medium was mixed with an equal volume of *Griess* reagent (0.1% *N*-[1-naphthyl]ethylenediamine and 1% sulfanilamide in 5% H_3PO_4). Cell viability was examined by the MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) colorimetric assay after 24 h incubation with test compounds. The results are representative of at least three independent experiments. Statistical differences were evaluated using a *Student*'s *t*-test and considered significant at $P \leq 0.05$.

Cytotoxicity Asssay. All the isolates were tested against BGC-823 (human gastric cancer) and A549 (human lung epithelia cancer) cancer cell lines according to established colorimetric MTT assay protocols [15]. BGC-823 and A549 cell lines were cultured in *RPMI-1640* containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate. Cells were maintained at 37° in a 5% CO_2 air. Human cancer cells were seeded at the initial density of 1.5×10^3 cells/ml in 96-well tissue culture plates. After incubation for 24 h at 37°, test compounds were dissolved in small amounts of DMSO and diluted in the appropriate culture medium (final concentration of DMSO < 0.1%). After removal of pre-incubated culture medium, media (100 μl) containing various concentrations of test compound were added and further incubated for 96 h. Cell viability was determined by MTT colorimetric assay. The IC_{50} values (concentration in μM required to inhibit cell viability by 50%) were calculated using *Microsoft Excel* software.

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