Highly Oxygenated Triterpenoids from the Marine Red Alga *Laurencia mariannensis* (Rhodomelaceae)

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Two new and one known squalenoid-derived triterpenoids, namely, laurenmariannol (**1**) and (21α)-21-hydroxythyrsiferol (**2**), and the known thyrsiferol (**3**) were isolated and identified from the marine red alga *Laurencia mariannensis*, which was collected off the coast of Hainan and Weizhou Islands of China. The structures of these compounds were established by means of spectroscopic analyses, as well as by comparison with literature data. Compounds **1** and **2** displayed significant cytotoxic activity against P-388 tumor cells with IC_{50} values of 0.6 and 6.6 μg/ml, respectively.

Introduction. – Marine red algal species of the genus *Laurencia*, *i.e.*, *L. viridis*, *L. obtusa*, *L. thyrsifera*, *L. pinnatifida*, and *L. venusta* were found to be a prolific source of triterpenoids, which are characterized by a high degree of oxygenation and fused cyclic-ether systems. These triterpenoids exhibited potent cytotoxic effects against some tumor cell lines [1].

In our investigations towards the discovery of structurally new and biologically active compounds from Chinese marine red algal species of Rhodomelaceae [2–11], we examined the chemical constituents of *L. mariannensis*, which was collected off the coast of Hainan and Weizhou Islands of China. As a result, two new and one known triterpenoids, namely, laurenmariannol (**1**) and (21α)-21-hydroxythyrsiferol[1]) (**2**), and the known thyrsiferol (**3**) [12–14], were isolated and characterized. This paper reports the isolation and structure identification of these compounds and the cytotoxic activity of compounds **1** and **2**.

Results and Discussion. – The dried and powdered algal material *L. mariannensis* was extracted with $CHCl_3$/MeOH 1:1. After solvent removal, the residue was extracted with 95% EtOH. The concentrated extract was then partitioned between H_2O and AcOEt and the AcOEt-soluble fraction was separated by chromatographic procedures to yield compounds **1**–**3**. Detailed NMR and mass spectroscopic analyses established that **1**–**3** were highly oxidized squalene derivatives, of which **1** and **2** were new

[1]) Trivial atom numbering; for systematic names, see *Exper. Part.*

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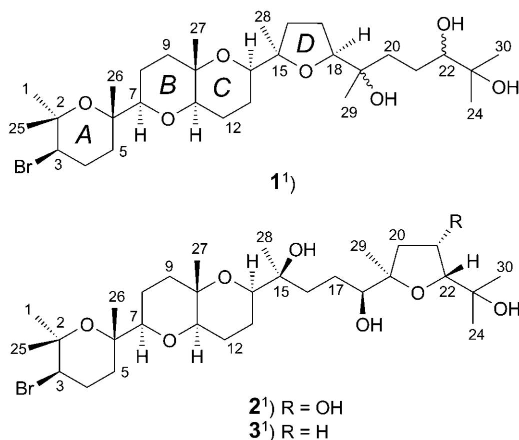

compounds, and **3** was thyrsiferol. Thyrsiferol (**3**) has previously been isolated several times from the marine algal species *L. thyrsifera* [12] and *L. obtusa* [14], and it has been synthesized by *González* and *Forsyth* [13]. However, only ^{1}H-NMR data were reported. Therefore, the ^{13}C-NMR data of **3** were recorded and assigned as listed in *Table 1*.

Compound **1** was obtained as colorless crystals. The broad IR absorption at 3413 cm^{-1} indicated the presence of OH groups in the molecule. The EI-MS exhibited a characteristic fragment-ion cluster at *m/z* 588 and 586 (1:1; $[M - H_2O]^+$), suggesting the presence of one Br-atom in **1**. The molecular formula $C_{30}H_{53}BrO_7$ was determined by the positive-mode HR-ESI-MS (*m/z* 607.3049 ($[M + H]^+$, $C_{30}H_{54}{}^{81}BrO_7^+$) and 605.3038 ($[M + H]^+$, $C_{30}H_{54}{}^{79}BrO_7^+$)), suggesting four degrees of unsaturation. The ^{1}H- and ^{13}C-NMR (*Table 1*), ^{1}H,^{1}H-COSY and HMBC (*Fig.*), and HSQC data and their comparison with those of thyrsiferol (**3**) [12–14] and aurilol, a cytotoxic bromotriterpenoid that was isolated from the sea hare *Dollabella auricularia* [15], established

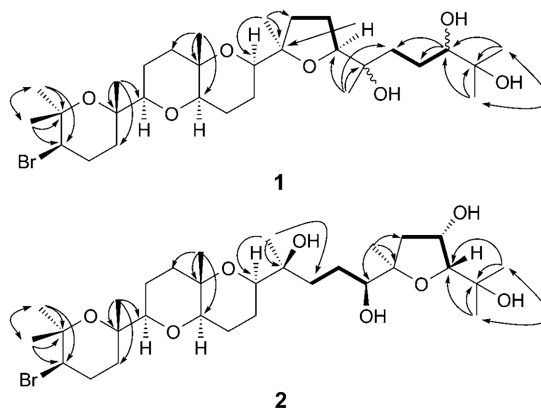

Figure. *Selected ^{1}H,^{1}H-COSY* (bold lines) *and HMBC* (arrows) *data for* **1** *and* **2**

Table 1. *^{1}H- and ^{13}C-NMR Data of* **1** *and* **2** *and ^{13}C-NMR Data of* **3**[1]). At 500 (^{1}H) and 125 MHz (^{13}C); assignments were corroborated by ^{1}H,^{1}H-COSY, HSQC, and HMBC experiments. δ in ppm, J in Hz.

	1				**2**		**3**
	δ(C)[a])	δ(H)[a])	δ(C)[b])	δ(H)[b])	δ(C)[b])	δ(H)[b])	δ(C)[b])
Me(1)	31.4 (*q*)	1.25 (*s*)	31.0 (*q*)	1.26 (*s*)	31.0 (*q*)	1.27 (*s*)	31.0 (*q*)
C(2)	75.5 (*s*)		74.9 (*s*)		75.0 (*s*)		75.0 (*s*)
H–C(3)	60.1 (*d*)	4.02 (*dd*, J = 12.2, 4.0)	59.0 (*d*)	3.89 (*dd*, J = 12.3, 4.0)	59.0 (*d*)	3.89 (*dd*, J = 12.3, 4.0)	59.0 (*d*)
CH_2(4)	29.0 (*t*)	2.26–2.30 (*m*), 2.08–2.12 (*m*)	28.3 (*t*)	2.21–2.25 (*m*), 2.08–2.12 (*m*)	28.3 (*t*)	2.22–2.26 (*m*), 2.08–2.12 (*m*)	28.3 (*t*)
CH_2(5)	37.7 (*t*)	1.78–1.82 (*m*), 1.60–1.64 (*m*)	37.0 (*t*)	1.78–1.82 (*m*), 1.50–1.54 (*m*)	37.0 (*t*)	1.78–1.82 (*m*), 1.52–1.56 (*m*)	37.0 (*t*)
C(6)	75.3 (*s*)		74.4 (*s*)		74.4 (*s*)		74.4 (*s*)
H–C(7)	87.3 (*d*)	3.11 (*dd*, J = 11.3, 2.5)	86.6 (*d*)	3.03 (*dd*, J = 11.4, 2.2)	86.6 (*d*)	3.05 (*dd*, J = 11.4, 2.4)	86.6 (*d*)
CH_2(8)	23.8 (*t*)	1.69–1.73 (*m*), 1.43–1.47 (*m*)	23.0 (*t*)	1.68–1.72 (*m*), 1.38–1.42 (*m*)	23.0 (*t*)	1.72–1.76 (*m*), 1.40–1.44 (*m*)	23.0 (*t*)
CH_2(9)	39.6 (*t*)	1.68–1.72 (*m*), 1.51–1.55 (*m*)	38.7 (*t*)	1.70–1.74 (*m*), 1.49–1.53 (*m*)	38.6 (*t*)	1.73–1.77 (*m*), 1.52–1.56 (*m*)	38.6 (*t*)
C(10)	72.0 (*s*)		71.5 (*s*)		72.0 (*s*)		72.0 (*s*)
H–C(11)	77.5 (*d*)	3.64 (*dd*, J = 11.0, 7.0)	76.7 (*d*)	3.54 (*dd*, J = 11.1, 7.2)	76.3 (*d*)	3.57 (*dd*, J = 11.0, 7.4)	76.4 (*d*)
CH_2(12)	22.1 (*t*)	1.80–1.84 (*m*), 1.48–1.52 (*m*)	21.3 (*t*)	1.84–1.88 (*m*), 1.48–1.52 (*m*)	21.1 (*t*)	1.86–1.90 (*m*), 1.49–1.53 (*m*)	21.2 (*t*)
CH_2(13)	22.2 (*t*)	1.86–1.90 (*m*), 1.79–1.83 (*m*)	21.5 (*t*)	1.75–1.79 (*m*), 1.71–1.75 (*m*)	20.7 (*t*)	1.80–1.84 (*m*), 1.68–1.72 (*m*)	20.7 (*t*)
H–C(14)	75.8 (*d*)	3.76 (*dd*, J = 8.8, 2.6)	75.3 (*d*)	3.70 (*dd*, J = 8.3, 3.5)	76.0 (*d*)	3.71 (*dd*, J = 12.9, 2.8)	76.1 (*d*)
C(15)	84.8 (*s*)		84.3 (*s*)		73.3 (*s*)		73.2 (*s*)
CH_2(16)	36.7 (*t*)	1.98–2.02 (*m*), 1.58–1.62 (*m*)	35.7 (*t*)	1.96–2.00 (*m*), 1.63–1.67 (*m*)	33.5 (*t*)	1.82–1.86 (*m*), 1.75–1.79 (*m*)	33.6 (*t*)
CH_2(17)	26.6 (*t*)	1.81–1.85 (*m*), 1.68–1.72 (*m*)	25.9 (*t*)	1.77–1.81 (*m*), 1.64–1.68 (*m*)	25.6 (*t*)	1.58–1.62 (*m*), 1.46–1.50 (*m*)	25.5 (*t*)
H–C(18)	86.7 (*d*)	3.78 (*dd*, J = 6.3, 2.5)	86.4 (*d*)	3.72 (*dd*, J = 7.0, 3.7)	78.8 (*d*)	3.45 (br. *d*, J = 10.1)	77.7 (*d*)
C(19)	72.8 (*s*)		72.4 (*s*)		86.0 (*s*)		86.1 (*s*)
CH_2(20)	37.1 (*t*)	1.68–1.72 (*m*), 1.42–1.46 (*m*)	33.8 (*t*)	1.57–1.61 (*m*), 1.50–1.54 (*m*)	41.6 (*t*)	2.20–2.24 (*m*), 1.80–1.84 (*m*)	32.5 (*t*)
CH_2(21)	26.2 (*t*)	1.68–1.72 (*m*), 1.31–1.35 (*m*)	25.4 (*t*)	1.76–1.80 (*m*), 1.64–1.68 (*m*)	74.3 (*d*)	4.46 (br. *dd*, J = 7.0, 3.0)	26.6 (*t*)
H–C(22)	79.9 (*d*)	3.24 (br. *d*, J = 9.7)	78.5 (*d*)	3.42 (*dd*, J = 10.6, 1.9)	87.1 (*d*)	3.58 (*d*, J = 3.0)	87.5 (*d*)
C(23)	73.0 (*s*)		73.1 (*s*)		72.1 (*s*)		70.5 (*s*)
Me(24)	24.9 (*q*)	1.12 (*s*)	23.3 (*q*)	1.16 (*s*)	24.3 (*q*)	1.36 (*s*)	24.0 (*q*)
Me(25)	24.2 (*q*)	1.40 (*s*)	23.7 (*q*)	1.39 (*s*)	23.7 (*q*)	1.40 (*s*)	23.7 (*q*)
Me(26)	20.5 (*q*)	1.23 (*s*)	20.1 (*q*)	1.19 (*s*)	20.1 (*q*)	1.20 (*s*)	20.1 (*q*)
Me(27)	21.8 (*q*)	1.18 (*s*)	21.2 (*q*)	1.17 (*s*)	21.5 (*q*)	1.18 (*s*)	21.4 (*q*)
Me(28)	21.7 (*q*)	1.07 (*s*)	21.7 (*q*)	1.08 (*s*)	23.0 (*q*)	1.10 (*s*)	22.9 (*q*)
Me(29)	23.7 (*q*)	1.10 (*s*)	24.1 (*q*)	1.16 (*s*)	24.2 (*q*)	1.38 (*s*)	23.4 (*q*)
Me(30)	26.2 (*q*)	1.13 (*s*)	26.6 (*q*)	1.22 (s)	29.3 (*q*)	1.32 (*s*)	27.7 (*q*)
OH–C(15)						2.78 (*s*)	
OH–C(18)						2.99 (*d*, J = 2.2)	
OH–C(19)		2.93 (br. *s*)[c])					
OH–C(21)						4.51 (*d*, J = 2.8)	
OH–C(22)		3.30 (br. *s*)[c])					
OH–C(23)		3.58 (br. *s*)[c])				2.63 (*s*)	

[a]) Measured in (D_6)acetone. [b]) Measured in $CDCl_3$. [c]) Data interchangeable.

the constitution of **1**. The relative configurations of **1** at C(3), C(6), C(7), C(10), C(11), and C(14) were shown to be the same as those of thyrsiferol (**3**) by analysis of a NOESY experiment and detailed NMR data comparison (*Table 1*) [12–14][16]. The Me groups at C(15) and H–C(18) were *cis*-positioned as confirmed by a NOESY experiment and detailed ^{13}C-NMR-data comparison with those of aurilol [15]. However, the relative configurations at C(19) and C(22) remain unknown. Compound **1** was named laurenmariannol.

In accordance with the molecular formula, 30 C-atom signals were observed in the ^{13}C-NMR spectrum of **1**, which were further classified by DEPT experiments into the categories of eight Me, ten CH_2, six CH, and six quaternary C-atoms. The presence of 11 C-atom signals between δ(C) 70 and 90 suggested that **1** was highly oxygenated. The ^{1}H-NMR spectrum ((D_6)acetone; *Table 1*) exhibited the presence of eight Me *s* at δ(H) 1.07 (Me(28)), 1.10 (Me(29)), 1.12 (Me(24)), 1.13 (Me(30)), 1.18 (Me(27)), 1.23 (Me(26)), 1.25 (Me(1)), and 1.40 (Me(25)) as well as six oxygenated and/or halogenated CH at δ(H) 3.11 (*dd*, J = 11.3, 2.5 Hz, H–C(7)), 3.24 (br. *d*, J = 9.7 Hz, H–C(22)), 3.64 (*dd*, J = 11.0, 7.0 Hz, H–C(11)), 3.76 (*dd*, J = 8.8, 2.6 Hz, H–C(14)), 3.78 (*dd*, J = 6.3, 2.5 Hz, H–C(18)), and 4.02 (*dd*, J = 12.2, 4.0 Hz, H–C(3)). Three resonances at δ(H) 2.93, 3.30, and 3.58, which had no correlation with any C-atom signals in the HSQC plot, were attributable to the protons of OH groups. Since no signal for an unsaturated functional group was observed in the NMR spectra, **1** was deduced to be tetracyclic. A comprehensive analysis of the ^{1}H- and ^{13}C-NMR data as well as 2D-NMR spectra indicated that the six-membered-ring moieties *A*, *B*, and *C* in **1** were the same as those of thyrsiferol (**3**; *Table 1*) [12–14]. However, the side chain (from C(15) to C(24)) was different. The ether linkage between C(15) and C(18) in the side chain of **1** was established by the observed HMBC cross-peak of H–C(18) at δ(H) 3.78 and C(15) at δ(C) (84.8, *s*). Because only four degrees of unsaturation are present in **1**, there should be no further cyclic linkage in the side chain. The other functional groups and their linkage within the side chain were connected by the observed spin systems as indicated by the ^{1}H,^{1}H-COSY data (*Fig.*) as well as by the ^{1}H,^{13}C-HMBC cross-peaks Me(29)/C(18), C(19), and C(20), Me(24)/C(22), C(23), and C(30), Me(30)/C(22), C(23), and C(24), and H–C(22)/C(20) and C(21) (*Fig.*). The NMR data of this side chain were also in agreement with those of aurilol [15]. In addition, the observed ^{1}H,^{13}C-HMBC cross-peaks Me(1)/C(2), C(3), and C(25), Me(25)/C(1), C(2), and C(3), Me(26)/C(5), C(6), and C(7), Me(27)/C(9), C(10), and C(11), and Me(28)/C(14), C(15), and C(16) further confirmed the suggested constitutional formula of **1** (*Fig.*).

Compound **2** was obtained as colorless crystals. The broad IR absorption at 3391 cm^{-1} indicated the presence of OH groups in the molecule. The EI-MS exhibited a characteristic fragment-ion cluster at *m/z* 604 and 602 (1:1; $[M - H_2O]^+$), suggesting the presence of one Br-atom in **2**. The positive-mode HR-ESI-MS exhibited a characteristic quasi-molecular-ion-peak cluster at *m/z* 645.2791 ($[M+Na]^+$, $C_{30}H_{53}{}^{81}BrNaO_8^+$) and at *m/z* 643.2819 ($[M+Na]^+$, $C_{30}H_{53}{}^{79}BrNaO_8^+$), corresponding to the molecular formula $C_{30}H_{53}BrO_8$ and suggesting four degrees of unsaturation. The ^{1}H- and ^{13}C-NMR (*Table 1*), ^{1}H,^{1}H-COSY and HMBC (*Fig.*), and HSQC data and their comparison with those of thyrsiferol (**3**) [12–14] established the constitution of **2**. The relative configurations at C(3), C(6), C(7), C(10), C(11), C(14), C(15), C(18), C(19), and C(22) were shown to be the same as those of thyrsiferol (**3**) by detailed NMR-data comparison [12–14] and by analysis of NOESY correlations. Based on these evidences, the structure of compound **2** was established, which was named (21α)-21-hydroxythyrsiferol.

The ^{1}H-NMR spectrum of **2** exhibited the presence of eight Me, seven oxygenated and/or halogenated CH, and four OH groups. The ^{13}C-NMR and DEPT experiments revealed the presence of 30

C-atoms including eight Me, nine CH_2, seven CH, and six quaternary C-atoms. Since no signal for an unsaturated functional group was observed in the NMR spectra, **2** was also deduced to be tetracyclic. A detailed NMR-data comparison revealed that the structure of **2** was very similar to thyrsiferol (**3**) [12–14]. However, an additional OH group was present at C(21) of **2**. This was evidenced by the analysis of the 1D- and 2D-NMR data. The oxygenated −CH signal that resonated at δ(H) 4.46 (*dd*, J = 7.0, 3.0 Hz, H−C(21)) correlated with C(21) at δ(C) 74.3 in the HSQC plot of **2**. In addition, the ^{1}H,^{1}H-COSY cross-peaks CH_2(20)/H−C(21), H−C(21)/H−C(22), and OH−C(21)/H−C(21) supported the attachment of an OH group at C(21) (*Fig.*). The other observed ^{1}H,^{13}C-HMBC cross-peaks Me(1)/C(2), C(3), and C(25), Me(25)/C(1), C(2), and C(3), Me(26)/C(5), C(6), and C(7), Me(27)/C(9), C(10), and C(11), Me(28)/C(14), C(15), and C(16), Me(29)/C(18), C(19), and C(20), Me(24)/C(22), C(23), and C(30), and Me(30)/C(22), C(23), and C(24) established the constitution of **2** (*Fig.*). The coupling constant J(H−C(21),H−C(22)) = 3.0 Hz and the observed NOESY correlation H−C(21)/H−C(22) as well as the observed long-range *W*-type coupling (J = 2.8 Hz) between OH−C(21) and H−C(22), indicated a *cis*-orientation for H−C(21) and H−C(22).

A literature survey revealed that the biological assay of squalenoid triterpenoids was mainly focused on evaluating their cytotoxic property with cultured cell lines of P-388 (murine lymphoid neoplasm), A-549 (human-lung carcinoma), HT-29 (human-colon carcinoma), and MEL-28 (human melanoma), and revealed that this type of compounds possess potent and selective activity against P-388 cells [1]. The new compounds **1** and **2** were, therefore, evaluated for their cytotoxic activity against P-388 tumor cells by using a reported procedure [17]. The results (*Table 2*) show that **1** and **2** were cytotoxic against P-388 cells with IC_{50} values of 0.60 and 6.6 µg/ml, respectively, compared to the positive control, VP-16 (etoposide), which had an IC_{50} of 0.30 µg/ml.

Table 2. *Inhibitory Activities of* **1**, **2**, *and VP-16 Against Cell Line P-388*

Concentration [µmol/l]	Inhibition ratio [%]		
	1	**2**	VP-16
0.01	12.7	5.5	11.5
0.1	20.9	5.6	34.0
1.0	62.2	12.9	73.6
10.0	80.0	61.0	81.5
100.0	81.3	77.8	82.2

A variety of unique squalenoid-derived triterpenoids has been isolated and reported from the marine red algae of the genus *Laurencia*. However, to the best of our knowledge, this is the first time that triterpenoids were discovered in *Laurencia mariannensis*. The isolation of **1** and **2** constitutes a new addition to the molecular diversity of squalenoid triterpenoids. Data obtained with compounds **1** and **2** confirmed their *in vitro* cytotoxic activity against the P-388 tumor cells. Squalenoid-derived natural products have been reported to possess various activities including cytotoxicity and inhibition of protein serine/threonine phosphatase 2A [13]. Our results provide new data, which might be useful for the future study of structure–activity relationship of squalenoid triterpenoids.

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Experimental Part

General. Column chromatography (CC): commercial silica gel (*Qingdao Haiyang Chemical Group Co.*; 200–300 mesh) or *Sephadex LH-20* (*Sigma*). TLC: precoated silica-gel plates *GF-254* (*Qingdao Haiyang*). Melting points: *SGW X-4* micro melting-point apparatus; uncorrected. Optical rotation: *Atago-Polax-L* polarimeter. IR Spectra: *Nicolet Nexus-470* spectrophotometer; in cm^{-1}. NMR Spectra: *Bruker Avance-500* spectrometer; at 500 (^{1}H) and 125 MHz (^{13}C); δ in ppm, J in Hz. EI-MS and HR-ESI-MS: *VG Autospec-3000* spectrometer; in *m/z* (rel. %).

Algal Material. The marine red alga *Laurencia mariannensis* YAMADA was collected off the coast of Hainan and Weizhou Islands, P. R. China, in March and April, 2006, resp., and identified by one of the authors (*L.-P. D.*). A voucher specimen (HZ06 M04b) was deposited with the Key Laboratory of Experimental Marine Biology of the Institute of Oceanology, Chinese Academy of Sciences.

Extraction and Isolation. The dried and powdered alga *L. mariannensis* (0.5 kg) was extracted with $CHCl_3$/MeOH 1:1. After solvent removal, the residue was further extracted with 95% EtOH. The concentrated extracts were combined and partitioned between H_2O and AcOEt. The AcOEt-soluble fraction was subjected to CC (silica gel, step gradient AcOEt/petroleum ether 0 → 100%): *Fractions I–VI*. The polar *Fr. V* was further purified by CC (silica gel and *Sephadex LH-20*), and by prep. TLC: **1** (4.4 mg), **2** (6.1 mg), and **3** (4.3 mg).

Laurenmariannol (=*6-{(2S,5R)-5-{(2R,4aR,6R,8aS)-6-[(2S,5R)-5-Bromotetrahydro-2,6,6-trimethyl-2H-pyran-2-yl]octahydro-8a-methylpyrano[3,2-b]pyran-2-yl}tetrahydro-5-methylfuran-2-yl}-2-methylheptane-1,2,6-triol*[2]); **1**): Colorless crystals. M.p. 159–160°. $[\alpha]^{18}_{D} = -15.7$ ($c = 0.41$, $CHCl_3$). IR (KBr): 3413, 2973, 2866, 1461, 1373, 1131, 1102, 1062. ^{1}H- and ^{13}C-NMR: *Table 1*. EI-MS: 588 (0.3, $[M - H_2O]^+$), 586 (0.3, $[M - H_2O]^+$), 573 (2), 571 (2), 570 (1), 568 (1), 555 (2), 553 (2), 524 (4), 245 (18), 227 (84), 209 (100), 143 (34), 125 (35), 85 (32). HR-ESI-MS: 607.3049 ($[M + H]^+$, $C_{30}H_{54}{}^{81}BrO_7^+$; calc. 607.3032), 605.3038 ($[M + H]^+$, $C_{30}H_{54}{}^{79}BrO_7^+$; calc. 605.3053).

(21α)-21-Hydroxythyrsiferol (=*(α²S,2R,4S,5R)-α²-{(3S)-3-{(2R,4aR,6R,8aS)-6-[(2S,5R)-5-Bromotetrahydro-2,6,6-trimethyl-2H-pyran-2-yl]octahydro-8a-methylpyrano[3,2-b]pyran-2-yl}-3-hydroxybutyl}tetrahydro-4-hydroxy-α⁵,α⁵,2-trimethylfuran-2,5-dimethanol*[2]); **2**). Colorless crystals. M.p. 185–186°. $[\alpha]^{18}_{D} = +4.0$ ($c = 0.44$, $CHCl_3$). IR (KBr): 3391, 2975, 2933, 2867, 1458, 1377, 1123, 1101, 1061. ^{1}H- and ^{13}C-NMR: *Table 1*. EI-MS: 604 (3, $[M - H_2O]^+$), 602 (3, $[M - H_2O]^+$), 586 (2), 584 (2), 571 (2), 569 (2), 568 (1), 566 (1), 540 (10), 445 (9), 443 (8), 363 (16), 243 (100), 225 (100), 207 (60), 159 (78), 141 (56), 125 (60), 83 (58). HR-ESI-MS: 645.2791 ($[M + Na]^+$, $C_{30}H_{53}{}^{81}BrNaO_8^+$; calc. 645.2801), 643.2819 ($[M + Na]^+$, $C_{30}H_{53}{}^{79}BrNaO_8^+$; calc. 643.2821).

Cytotoxic Assay. The cytotoxic activities of the compounds on the proliferation of P-388 cells were determined by the MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay [17]. Briefly, P-388 cells (10000–15000) in 100 μl of culture medium per well were seeded in 96-well plates, treated in triplicate with gradient concentrations of the studied compounds, and incubated at 37° for 72 h. Five mg/ml of MTT (20 μl/well) was added to each well, and cells were incubated for additional 4 h at 37°, then 'triplex solution' (10% SDS, 5% ^{i}BuOH, and 12 mM HCl) was added to dissolve the formazan crystals overnight. Absorbance (A_{570}) was measured at 570 nm by an enzyme immunoassay instrument (*SpectraMax*, *Molecular Devices*, USA). The inhibition rate was calculated as $[1 - (A_{570\,treated}/A_{570\,control})] \cdot 100\%$.

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