Three New *ent*-Trachylobane Diterpenoids from Co-cultures of the Calli of *Trewia nudiflora* and *Fusarium* sp. WXE

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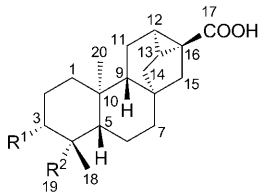
Five diterpenoids, including three new *ent*-trachylobane diterpenoids, *i.e.*, (3α)-3-hydroxy-*ent*-trachylobane-17,19-dioic acid 19-methyl ester (**1**), *ent*-trachylobane-17,19-dioic acid 19-methyl ester (**2**), *ent*-trachylobane-17,19-dioic acid (**3**), and two known atisane-type ones, *i.e.*, (16α)-16,17-dihydroxy-*ent*-atisan-19-oic acid methyl ester (**4**), and 17-hydroxy-*ent*-atisan-19-oic acid methyl ester (**5**), were isolated from the co-culture extract of the calli of *Trewia nudiflora* and its endophytic fungus *Fusarium* sp. WXE. Their structures were elucidated by spectroscopic analyses, including 1D- and 2D-NMR experiments, and HR-Q-TOF mass spectrometry. The antitumor and antibacterial properties of the new compounds were evaluated.

Introduction. – Plant endophytes are a group of microorganisms, including fungi and bacteria, which live within a plant's internal tissues or organs without causing any apparent symptoms or diseases in the host plant. The relationship between a plant endophyte and its host is a complex one, ranging from mutualistic symbiosis, as in endomycorrhizal fungi, to latent phytopathogenesis; in many cases, the exact nature of the relationship is yet unknown [1], but it is clear that plant endophytes serve as important sources of bioactive compounds [2–5].

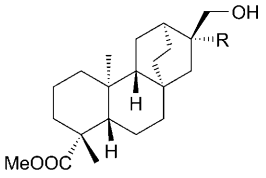
Trewia nudiflora L. is a member of the family Euphorbiaceae from which the antitumor and antileukemic macrolactam maytansinoids have been isolated [6–8]. Our previous studies showed that maytansinoids could not be detected and isolated from the callus-culture extracts of *Trewia nudiflora* [9]. To access a new source of maytansinoids and investigate the true biosynthetic origin of these compounds [10][11], a co-culture of the callus of *Trewia nudiflora* and its endophytic fungus *Fusarium* sp. WXE was assembled. Studies on the chemical constituents of the co-cultures resulted in the isolation of five compounds, including the three new *ent*-trachylobane diterpenoids **1**–**3** and two known atisane-type diterpenoids *i.e.*, (16α)-16,17-dihydroxy-*ent*-atisan-19-oic acid methyl ester (**4**) and 17-hydroxy-*ent*-atisan-19-oic acid methyl ester (**5**) [9][12]. Herein, we report the isolation and structure elucidation of these three new compounds from the dual cultures of callus and its endophytic fungus *Fusarium* sp. WXE.

Results and Discussion. – 1. *Structure Elucidation*. The calli of *T. nudiflora* were induced from the seeds on *Murashige*-and-*Skoog* (*MS*) agar media [13] as described

previously [9]. The fungal strain WXE was isolated and purified from the aseptic seeds of *T. nudiflora* by the hyphal-tip method [14] and was identified as *Fusarium sp.* WXE according to its ITS sequence of rDNA (ITS1-5.8S-ITS2). During the co-culture of the calli of *T. nudiflora* and *Fusarium* sp. WXE, the fungus slightly inhibited the growth of the calli, while the strain grew faster and developed more mycelia than left alone on *MS* agar medium. The co-cultures including plant calli, fungi, and agar media were collected together and extracted with acetone at room temperature. After the removal of solvents, the crude extract was purified by repeated column chromatography (*RP-18*, *Sephadex LH-20*, and silica gel, resp.) to afford the five diterpenoids **1**–**5**, including three new *ent*-trachylobane diterpenoids **1**–**3**[1]).



1[1]) R^1 = OH, R^2 = COOMe
2[1]) R^1 = H, R^2 = COOMe
3[1]) R^1 = H, R^2 = COOH



4 R = OH
5 R = H

Compound **1** was obtained as colorless needles. HR-Q-TOF-MS established the molecular formula $C_{21}H_{30}O_5$ (*m/z* 385.2759 ($[M+Na]^+$)), indicating seven degrees of unsaturation in the molecule. The IR spectra of **1** revealed the presence of an OH group (3577 cm^{-1}), a carboxylic acid ester (1729 cm^{-1}), and a COOH group (1698 cm^{-1}). The COOH and ester signals were also present in the ^{13}C-NMR spectrum at δ 176.1 (*s*, 2 C). The remaining five degrees of unsaturation indicated that **1** was a pentacyclic diterpene. Further spectral data and their comparison with those of known analogues [15–17] established that **1** is an *ent*-trachylobane-type diterpene. The ^{1}H-NMR spectrum (*Table 1*) of **1** suggested the presence of two Me signals at δ 1.23 and 0.71, and one MeO signal at δ 3.56. The ^{13}C-NMR and DEPT spectra (*Table 2*) showed 21 signals for three Me groups, seven CH_2, five CH groups, thereof one O-bearing, and six quaternary C-atoms, including two C=O C-atoms. The structure of fragment **1a** (*Fig. 1*) was determined based on the HMBCs from H-atoms of the three Me groups to their corresponding C-atoms, *i.e.*, Me(18)/C(3), C(4), C(5), and C(19), Me(20)/C(1), C(5), C(9), and C(10), and Me(21)/C(19), along with the ^{1}H,^{1}H-COSYs H_α–C(1)/H_α–C(2) and H_α–C(2)/H–C(3). The HMBCs H_α–C(7)/C(6), C(8), C(9), and C(15), and H_β–C(14)/C(8), C(9), and C(13) defined the fragment **1b** (*Fig. 1*). Furthermore, the HMBC spectra showed the correlations H–C(12)/C(11), C(13), and C(17), and H–C(13)/C(12), C(16), and C(17), determining the fragment **1c** (*Fig. 1*). The connection of fragments **1a** and **1b** was provided by the HMBCs from the H-atoms at δ 0.93 (H–C(5)) in the fragment **1a** to the C-atoms at δ 21.6 (C(6)) and 38.2 (C(7)) in fragment **1b**, and the ^{1}H,^{1}H-COSY H–C(5)/H–C(6). Fragments **1b** and **1c** were connected on the basis of the HMBCs from the H-atom at δ 1.67 (H–C(11)) to C(9), from H–C(12) (δ 1.57) to C(15), and from H_β–C(15) (δ 1.34–1.41) to C(16), along

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

Table 1. *^{1}H-NMR Data* (600 MHz, (D_6)DMSO) *of* **1**–**3**[1]). δ in ppm, J in Hz.

	1	**2**	**3**
CH_2(1)	0.89 (*dt*, J = 13.5, 4.1, H_β), 1.47–1.51 (*m*, H_α)	0.80 (*dt*, J = 13.1, 3.8, H_β), 1.42–1.48 (*m*, H_α)	0.79 (*dt*, J = 13.1, 3.8, H_β), 1.43–1.46 (*m*, H_α)
CH_2(2)	1.47–1.51 (*m*, H_α), 2.09 (*dq*, J = 13.1, 3.7, H_β)	1.27–1.35 (*m*, H_α), 1.65–1.75 (*m*, H_β)	1.27–1.34 (*m*, H_α), 1.65–1.75 (*m*, H_β)
H–C(3) or CH_2(3)	3.03 (*dt*, J = 12.5, 4.3, H_β)	0.99 (*dd*, J = 13.4, 4.1, H_β), 2.01 (*m*, H_α)	0.85–0.89 (*m*, H_β), 1.97–1.99 (*m*, H_α)
H–C(5)	0.93 (br. *d*, J = 11.8)	1.03 (*dd*, J = 10.0, 1.9)	0.98 (*dd*, J = 9.2, 1.9)
CH_2(6)	1.38 (br. *d*, J = 11.8, H_α), 1.68–1.70 (*m*, H_β)	1.40 (br. *d*, J = 11.8, H_α), 1.69–1.71 (*m*, H_β)	1.40 (br. *d*, J = 11.7, H_α), 1.69–1.71 (*m*, H_β)
CH_2(7)	1.30–1.34 (*m*, H_β), 1.45 (br. *d*, J = 12.3, H_α)	1.33–1.35 (*m*, H_β), 1.46 (br. *d*, J = 13.1, H_α)	1.32–1.34 (*m*, H_β), 1.45–1.46 (*m*, H_α)
H–C(9)	1.08 (*dd*, J = 10.9, 6.6)	1.10–1.12 (*m*)	1.10–1.13 (*m*)
CH_2(11)	1.67 (*dd*, J = 6.6, 2.2, H_α), 1.92 (*ddd*, J = 17.4, 11.8, 2.8, H_β)	1.67 (*dd*, J = 6.6, 2.2, H_α), 1.92 (*ddd*, J = 17.6, 11.6, 3.0, H_β)	1.67 (*dd*, J = 6.6, 2.2, H_α), 1.92 (*ddd*, J = 17.8, 11.6, 3.1, H_β)
H_β–C(12)	1.57 (br. *d*, J = 8.0)	1.57 (br. *d*, J = 8.6)	1.58 (br. *d*, J = 8.3)
H–C(13)	1.68–1.70 (*m*)	1.69–1.70 (*m*)	1.69–1.70 (*m*)
CH_2(14)	1.19 (*d*, J = 10.5, H_a), 2.00 (*d*, J = 12.2, H_b)	1.22 (br. *d*, J = 12.1, H_a), 2.02 (*d*, J = 12.2, H_b)	1.22 (br. *d*, J = 9.7, H_a), 2.04 (*d*, J = 12.1, H_b)
CH_2(15)	1.34–1.41 (*m*, H_β), 1.66–1.70 (*m*, H_α)	1.56–1.57 (*m*, H_β), 1.69–1.72 (*m*, H_α)	1.61–1.64 (*m*, H_β), 1.69–1.72 (*m*, H_α)
Me(18)	1.23 (*s*)	1.09 (*s*)	1.09 (*s*)
Me(20)	0.71 (*s*)	0.69 (*s*)	0.81 (*s*)
MeO–C(19)	3.56 (*s*)	3.56 (*s*)	–
OH–C(3)	4.09 (*d*, J = 8.7)		

with the ^{1}H,^{1}H-COSY H–C(9)/H_β–C(11). The relative configuration was deduced from a ROESY experiment (*Fig. 2*). Particularly, C(17)OOH of **1** was determined to be β-oriented, which was consistent with the literature for the structurally similar, known compounds methyl *ent*-trachyloban-19-oate [17], (3α)-*ent*-trachylobane-3,19-diol [16], and (3β)-*ent*-trachylobane-3-ol [15]. Indeed, comparison of the ^{1}H- and ^{13}C-NMR spectra of **1** with those of methyl *ent*-trachyloban-19-oate [17] revealed that they were very similar, but the Me(17) was oxidized to a COOH group and the CH_2(3) is hydroxylated in **1**. Thus, the structure of **1** was elucidated as (3α)-3-hydroxy-*ent*-trachylobane-17,19-dioic acid 19-methyl ester[1]).

Compound **2** was isolated as lilac needles and determined to have the molecular formula $C_{21}H_{30}O_4$ based on the HR-Q-TOF-MS (*m*/*z* 369.2852 ($[M + Na]^+$)) and NMR data. The IR spectra of **2** revealed the presence of a carboxylic acid ester (1726 cm^{-1}) and a carboxylic acid (1678 cm^{-1}). In the ^{13}C-NMR spectra and DEPT, 21 resonances for six quaternary C-atoms, five CH, seven CH_2, and three Me groups were observed. The ^{1}H- and ^{13}C-NMR data showed that the spectral parameters of **2** were similar to those of **1**, and were consistent with the presence of a trachyloban skeleton. The only difference between **1** and **2** was that **2** is lacking the OH group at C(3). The relative configuration of **2** was determined based on the similar NOE correlations as in **1** and as in methyl *ent*-trachyloban-19-oate [17][18]. Therefore, compound **2** was determined to be *ent*-trachylobane-17,19-dioic acid 19-methyl ester[1]).

Table 2. *^{13}C- NMR Data* (150 MHz, (D_6)DMSO) *of* **1**–**3**. δ in ppm.

	1	**2**	**3**
CH_2(1)	37.7 (*t*)	39.1 (*t*)	39.3 (*t*)
CH_2(2)	27.7 (*t*)	18.7 (*t*)	18.7 (*t*)
CH(3)	77.1 (*d*)	37.9 (*t*)	38.0 (*t*)
C(4)	49.3 (*s*)	43.5 (*s*)	43.6 (*s*)
CH(5)	55.4 (*d*)	56.1 (*d*)	56.1 (*d*)
CH_2(6)	21.6 (*t*)	21.8 (*t*)	21.9 (*t*)
CH_2(7)	38.2 (*t*)	38.5 (*t*)	38.6 (*t*)
C(8)	40.0 (*s*)	40.0 (*s*)	40.0 (*s*)
CH(9)	51.4 (*d*)	51.5 (*d*)	51.5 (*d*)
C(10)	38.3 (*s*)	38.6 (*s*)	38.8 (*s*)
CH_2(11)	19.4 (*t*)	19.3 (*t*)	19.3 (*t*)
CH(12)	23.7 (*d*)	23.7 (*d*)	23.8 (*d*)
CH(13)	29.7 (*d*)	29.6 (*d*)	29.6 (*d*)
CH_2(14)	32.1 (*t*)	32.0 (*t*)	32.1 (*t*)
CH_2(15)	43.5 (*t*)	43.5 (*t*)	43.2 (*t*)
C(16)	29.8 (*s*)	29.8 (*s*)	29.9 (*s*)
C(17)	176.1 (*s*)	177.3 (*s*)	178.0 (*s*)
Me(18)	24.2 (*q*)	28.6 (*q*)	28.9 (*q*)
C(19)	176.1 (*s*)	176.1 (*s*)	178.0 (*s*)
Me(20)	12.7 (*q*)	12.5 (*q*)	12.8 (*q*)
MeO	51.3 (*q*)	51.4 (*q*)	–

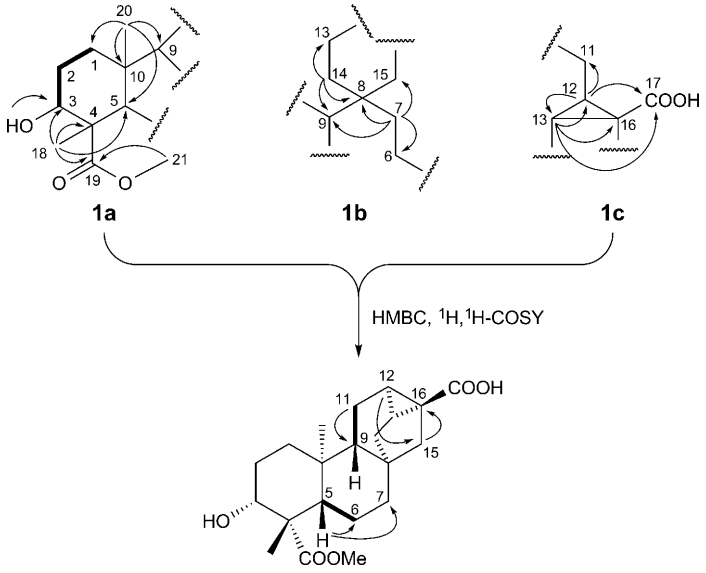


Fig. 1. *Fragments* **1a**, **1b**, *and* **1c** *of* **1** *and selected HMBC* (H → C) *and ^{1}H,^{1}H-COSY data* (—)

Compound **3** was isolated as colorless needles and has the molecular formula $C_{20}H_{28}O_4$ based on the HR-Q-TOF-MS (*m*/*z* 355.2664 ([*M* + Na]$^+$)) and NMR data.

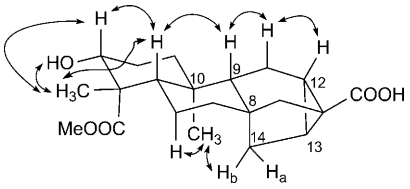


Fig. 2. *Selected ROESY correlations for* **1**

The ^{13}C-NMR (DEPT) data showed 20 resonances for two Me, seven CH_2, five CH, and six quaternary C-atoms including two COOH signals at δ 178.0. Compared with the NMR data of **2**, the structure of **3** was revealed to be *ent*-trachylobane-17,19-dioic acid[1]). The relative configuration of **3** was determined based on the similar NOE correlations as in **1** and as in *ent*-trachyloban-19-oic acid [16][17].

2. *Biological Studies.* Compounds **1**–**5** exhibited no evident antifungal activities against *Candida albicans* or antibacterial assays against *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis* at 50 μg/ml. The cytotoxicity of compounds **1**–**3** against HeLa and HepG2 cell lines were analyzed by the MTT method [19], but none of them was found to be cytotoxic at 20 μg/ml, which was surprising in contrast to that (3β)-*ent*-trachyloban-3-ol which has an IC_{50} of 7.3 μg/ml against HeLa cells [15].

Conclusions. – We found the differences in the product compositions of pure cultures of endophytic fungi, and the co-cultures with plant cells in this study and previous work [3]; the exact reasons for and the courses of these changes are presently being investigated. However, our here reported data suggest that co-culturing of plant cells and endophytic fungi may offer an alternative approach to producing new secondary metabolites, and that *ent*-trachyloban diterpenes may play an undiscovered, yet pivotal role in the interaction between the host plant and *Fusarium* sp. WXE.

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Experimental Part

General. MPLC = Medium-pressure liquid chromatography. Column chromatography (CC): silica gel (SiO_2; 200–300 or 80–100 mesh; *Qingdao Marine Chemical Factory*, Qingdao, P. R. China), SiO_2 *GF254* (*Merck*), *RP-18* gel (*Merck*), and *Sephadex LH-20* gel (*Amersham Biosciences*). Thin-layer chromatography (TLC): precoated SiO_2 *GF254* plates (0.20–0.25 mm; *Qingdao*). Optical rotations: *Perkin-Elmer-341* polarimeter; in $CHCl_3$. UV Spectra: *Thermo-spectronic-genesys-2* UV/VIS spectrophotometer; in $CHCl_3$; λ_{max} in nm. IR Spectra: *Nicolet-FT-IR-360* apparatus; KBr matrix; in cm^{-1}. ^{1}H- and ^{13}C-NMR Spectra: *Bruker-DRX-600* spectrometer; at 600 and 150 MHz, resp.; in (D_6)DMSO; δ in ppm rel. to Me_4Si, *J* in Hz. HR-Q-TOF-MS: *API-QStar-Pulsar* LC-Q-TOF mass spectrometer; in *m*/*z*.

Plant Material and Callus Induction. The seeds of *T. nudiflora* were collected in Xishuangbanna, Yunnan Province, P. R. China. Seeds were washed with flowing water for 30 min, then sterilized for 1 min with 75% EtOH and for 8 min with 0.1% $HgCl_2$, rinsed three times with sterilized water and cut into small pieces of 5 mm length. The callus tissues were induced by a 1 month incubation. The best formation and growth of calli were observed on *MS* agar medium containing (2,4-dichlorophenoxy)acetic acid (1 mg/l of medium), naphthalene-1-acetic acid (1 mg/l), and kinetin (0.2 mg/l). The callus were

subcultured at 1 month intervals at 26° in the dark on *MS* agar medium containing the above plant hormones.

Fungal Material. The fungus was isolated from the aseptic seedling of *T. nudiflora*. The aseptic seedlings were induced on agar medium containing gibberellin (4 mg/l) at 26° in the dark. When the aseptic seedlings grew up to 5–6 cm, they were cut into 5 mm fragments and placed onto the surface of melted potato dextrose agar (PDA; 15 ml) as medium in *Petri* dishes, cultured at 28°. And the fungus was purified by the hyphal-tip method [14]. Sequencing was performed according to both traditional morphology and internal transcribed spaces (ITS), which established that the fungus belongs to *Fusarium* species.

Co-culture of Callus and Fungus. Callus grew on *MS*-completed media supplemented with 1 mg/l of (2,4-dichlorophenoxy)acetic acid, 1 mg/l of naphthalene-1-acetic acid, and 0.2 mg/l of kinetin. After the calli grew under dark at 26° for 20 d, the strain *Fusarium sp.* WXE was inoculated on the calli and co-cultured for 10 d. The co-culture was performed continually until 10 l of *MS*-completed media were collected.

Extraction and Isolation. The calli, fungi, and media were all extracted 5 times with acetone. The org. soln. was filtrated and concentrated *in vacuo* at 40° to yield a crude syrup (4 g). The extract was subjected to MPLC (*RP-18* (140 g), H_2O, then acetone/H_2O 1:2, 1:1, and 2:1, and neat acetone): *Fractions A–F. Fr. A* (1.50 g) was subjected to CC (*Sephadex LH-20* (120 g), MeOH). All fractions were analyzed by TLC ($CHCl_3$/MeOH 20:1) and pooled accordingly into three fractions: *Frs. A.1–A.3. Fr. A.1* (440 mg) was subjected to MPLC (*RP-18* (30 g; acetone/H_2O 40:60 and 50:50): *Frs. A.1.a* and *A.1.b. Fr. A.1.a* (43 mg) was subjected to CC (*Sephadex LH-20* (40 g), acetone): *Frs. A.1.a$_1$–A.1.a$_3$. Fr. A.1.a$_2$* (21 mg) was subjected to CC (SiO_2, petroleum ether/acetone 40:1): **4** (2 mg). *Fr. A.1.b* (40 mg) was subjected to CC (SiO_2, petroleum ether/$CHCl_3$ 5:1): **5** (2 mg). *Fr. A.2* (272 mg) was subjected to MPLC (*RP-18* (30 g), acetone/H_2O 38:62): *Frs. A.2.a–A.2.d. Fr. A.2.b* (86 mg) was subjected to CC (*Sephadex LH-20* (40 g), acetone): *Frs. A.2.b$_1$–A.2.b$_3$. Fr. A.2.b$_2$* (43 mg) was subjected to CC (SiO_2, petroleum ether/acetone 50:1): **1** (8.1 mg). *Fr. A.3* (110 mg) was subjected to CC (*Sephadex LH-20* (40 g), acetone): *Frs. A.3.a–A.3.d. Fr. A.3.b* (16 mg) was subjected to MPLC (*RP-18* (30 g), acetone/H_2O 47:53): *Frs. A.3.b$_1$* and *A.3.b$_2$. Fr. A.3.b$_1$* (10 mg) was subjected to CC (SiO_2, petroleum ether/AcOEt 5:1): **3** (2.0 mg). *Fr. C* (132 mg) was subjected to CC (*Sephadex LH-20* (120 g), MeOH). All fractions were analyzed by TLC ($CHCl_3$/MeOH 20:1) and pooled into two fractions: *Frs. C.1* and *C.2. Fr. C.1* (14 mg) was subjected to CC (SiO_2, petroleum ether/acetone 40:1): **2** (8.4 mg).

(3α)-3-Hydroxy-ent-*trachylobane-17,19-dioic Acid 19-Methyl Ester* (rel-(*3*R,*4*S,*4a*S,*6a*S,*7a*S,*8*-S,*8a*R,*9a*R,*9b*S)*-Dodecahydro-3-hydroxy-4,9b-dimethyl-6,8-methano-6a*H*-cyclopropa[*b*]phenanthene-4,7a(7*H*)-dicarboxylic Acid 4-Methyl Ester* **1**): White needles. $[\alpha]_D^{20} = -48.0$ ($c = 0.5$, $CHCl_3$). UV ($CHCl_3$): 241.0, 235.0, 226.0. IR (KBr): 3577, 2925, 1729, 1698, 1436, 1261, 1194, 1126, 1046. ^{1}H- and ^{13}C-NMR: *Tables 1* and *2*, resp. HR-Q-TOF-MS: 385.2759 ($[M+Na]^+$, $C_{21}H_{30}NaO_5^+$; calc. 385.1991).

ent-*Trachylobane-17,19-dioic Acid 19-Methyl Ester* (= rel-*(4*R,*4a*S,*6a*S,*7a*S,*8*S,*8a*R,*9a*R,*9b*S*)-Dodecahydro-4,9b-dimethyl-6,8-methano-6a*H*-cyclopropa[*b*]phenanthrene-4,7a(7*H*)-dicarboxylic Acid 4-Methyl Ester* **2**): Lilac needles. $[\alpha]_D^{20} = -39.2$ ($c = 0.5$, $CHCl_3$). UV ($CHCl_3$): 244.0, 235.0, 217.0. IR (KBr): 2930, 1726, 1678, 1434, 1262, 1154, 1093. ^{1}H and ^{13}C-NMR: *Tables 1* and *2*, resp. HR-Q-TOF-MS: 369.2852 ($[M+Na]^+$, $C_{21}H_{30}NaO_4^+$; calc. 369.2042).

ent-*Trachylobane-17,19-dioic Acid* (**3**): White needles. $[\alpha]_D^{20} = -40.8$ ($c = 0.1$, $CHCl_3$). UV ($CHCl_3$): 244.0, 235.0, 226.0, 220.0. IR (KBr): 3444, 2930, 1674, 1651, 1384, 1091. ^{1}H and ^{13}C-NMR: *Tables 1* and *2*, resp. HR-Q-TOF-MS: 355.2664 ($[M+Na]^+$, $C_{20}H_{28}NaO_4^+$; calc. 355.1885).

Biological Studies. The cytotoxicities of **1**–**3** were investigated by means of the MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay [19] and the human cancer HepG2 and HeLa cell lines, cisplatin being used as pos. control. Further, the antibacterial activities of the new isolates were tested against three bacteria (*Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis*) and one yeast (*Candida albicans*) by diffusion assay on agar plate as described [20]. Three replicates were performed for each compound at a concentration of 50 μg/disc.

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