New Triterpene Aldehydes, Lucialdehydes A—C, from *Ganoderma lucidum* and Their Cytotoxicity against Murine and Human Tumor Cells

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Three new lanostante-type triterpene aldehydes, named lucialdehydes A—C (1—3), were isolated from the fruiting bodies of *Ganoderma lucidum*, together with ganodermanonol (4), ganodermadiol (5), ganodermanondiol (6), ganodermanontriol (7), ganoderic acid A (8), ganoderic acid B8 (9), and ganoderic acid C1 (10). The structures of the new triterpenes were determined as (24*E*)-3β-hydroxy-5α-lanosta-7,9(11),24-trien-26-al (1), (24*E*)-3,7-dioxo-5α-lanosta-8,24-dien-26-al (2), and (24*E*)-3β-hydroxy-7-oxo-5α-lanosta-8,24-dien-26-al (3), respectively, by spectroscopic means. The cytotoxicity of the compounds isolated from the ganoderma mushroom was tested *in vitro* against Lewis lung carcinoma (LLC), T-47D, Sarcoma 180, and Meth-A tumor cell lines. Lucialdehydes B, C (2, 3), ganodermanonol (4) and ganodermanondiol (6) showed cytotoxic effects on tested tumor cells. Of the compounds, lucialdehyde C (3) exhibited the most potent cytotoxicity against LLC, T-47D, Sarcoma 180, and Meth-A tumor cells with ED_{50} values of 10.7, 4.7, 7.1, and 3.8 μg/ml, respectively.

Key words lucialdehyde A—C; lanostane-type triterpene aldehyde; *Ganoderma lucidum*; cytotoxicity

The fruiting bodies of *Ganoderma* (*G.*) *lucidum* KARST (Polyporaceae) are a well known Chinese crude drug component which has been used clinically in China, Japan and Korea for a long time as a home remedy. It is considered to promote longevity and maintain the vitality of humans.[1)] Nowadays, in China, this mushroom is used for leukopenia.[2)] More than 130 highly oxygenated and pharmacological active triterpenoids have been isolated from the fruiting bodies, mycelia, and spores of *G. lucidum*. Some of them have been shown to have cytotoxicity against hepatoma cells (ganoderic acids U—Y), and hepatoma PLC/PRF/5 and KB cells (ganoderic aldehyde A) *in vitro*,[3,4)] an inhibitory effect on angiotensin converting enzyme (ganoderic acid F),[5)] inhibitory activity against farnesyl protein transferase (ganoderic acid A and methyl ganoderate A),[6)] β-glucosidase-inhibitory activity and hepatoprotective effect (ganoderic acid A),[7)] inhibitory effects on eukaryotic DNA polymerases (lucidenic acid O and lucidenic lactone),[8)] and antinociceptive activity (ganoderic acids A, B, G, H and compound C6).[9)] In our previous paper, we reported the isolation of new triterpenoids, ganoderic acids α—θ, lucidumols A and B, and lucidenic acid SP1, along with several known triterpenes from the spores and fruiting bodies of *G. lucidum*.[10—13)] Of the triterpenes purified, ganoderiol F and ganodermanontriol were found to have anti-human immunodeficiency virus (anti-HIV-1) and anticomplement activity; lucidumol A showed cytotoxicity against Lewis lung carcinoma (LLC) cells; ganodermanondiol had a cytotoxic effect on Meth-A (sarcoma, murine) cells; and ganoderic acid β, lucidumol B and ganolucidic acid A exhibited an inhibitory effect on human immunodeficiency virus (HIV)-1 protease.

This paper describes the structural determination of three new triterpenoid compounds, named lucialdehydes A—C (**1**—**3**), from the fruiting bodies of *G. lucidum*, as well as their cytotoxic activity against LLC, Meth-A, Sarcoma-180, and T-47D (human breast cancer) cells.

Results and Discussion

A $CHCl_3$–MeOH extract of the fruiting bodies of *G. lucidum* was subjected to repeated chromatography on silica gel and Florisil, followed by preparative high performance liquid chromatography (HPLC) to give ten compounds (**1**—**10**). Of these, seven compounds were identified as ganodermanonol (**4**),[14)] ganodermadiol (**5**),[14)] ganodermanondiol (**6**),[15)] ganodermanontriol (**7**),[15)] ganoderic acid A (**8**),[16)] ganoderic acid B9 (**9**),[17)] and ganoderic acid C1 (**10**)[17)] by comparison of their spectroscopic data with those reported. The other three were new compounds having an aldehyde group in the side chain of lanostan-type triterpens. The structures of these compounds (named lucialdehydes A, B and C) were determined as follows:

Lucialdehyde A (**1**) was obtained as an amorphous powder (MeOH–H_2O) with a positive optical rotation, $[\alpha]_D$ +32° ($CHCl_3$). The ultraviolet (UV) absorptions at 232, 243, and 252 nm (log ε 4.03, 4.10, 3.96) suggested the presence of a heteroannular diene, when compared to that of ganoderiol A.[5)] The presence of a conjugated carbonyl group (1685 cm^{-1}) was suggested by IR spectrometry. The high-resolution electron impact mass (HR-EIMS) spectrum revealed the molecular formular of **1** to be $C_{30}H_{46}O_2$. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of **1** analyzed by the aid of ^{1}H–^{1}H shift correlated spectroscopy (COSY) and ^{1}H-detected multiple quantum coherence spectroscopy (HMQC) experiments showed signals for seven methyls (including one vinyl methyl at δ 1.75), a methine proton at δ 3.25 (dd, J=11.2, 4.5 Hz), and three olefinic protons at δ 5.32 (d, J=6.3 Hz), 5.48 (br s) and 6.49 (td, J=7.6, 1.2 Hz). In addition, a singlet at δ 9.40 for an aldehyde proton was also observed (Table 1). The carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum demonstrated signals characteristic for seven methyls, six olefinic carbons, a hydroxyl-bearing methine carbon at δ 78.9, and an aldehyde carbon at δ 195.4 (Table 2). These data suggested a highly oxygenated $\Delta^{7,9(11)}$-lanostane-type triterpene close to the structure of ganodermadiol (**5**). However, the chemical shift of C-26 (δ

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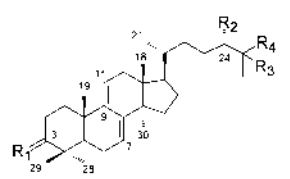

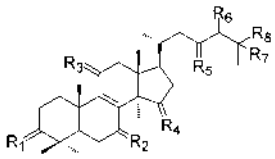

	R_1	R_2	R_3	R_4
1	OH (H)	$\Delta^{24(25)}$		CHO
4	O	$\Delta^{24(25)}$		CH_2OH
5	OH (H)	$\Delta^{24(25)}$		CH_2OH
6	O	OH	OH	CH_3
7	O	OH	OH	CH_2OH

	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8
2	O	O	H_2	H_2	H_2	$\Delta^{24(25)}$		CHO
3	OH (H)	O	H_2	H_2	H_2	$\Delta^{24(25)}$		CHO
8	O	OH (H)	O	OH (H)	O	H_2	H	COOH
9	O	OH (H)	O	OH (H)	O	H_2	H	COOH
10	O	OH (H)	O	O	O	H_2	H	COOH

Chart 1. Structures of Compounds Isolated from the Fruiting Bodies of *Ganoderma lucidum*

Table 1. ^{1}H-NMR Spectral Data of Compounds **1**—**3** (400 MHz, $CDCl_3$)

	1	**2**	**3**
1-H (α)	1.45 m	1.80 m	1.40 m
1-H (β)	2.00 dt (13.0, 3.5)	2.08 m	1.85 dt (12.8, 3.5)
2-H (α)	1.63 m	2.47 ddd (15.8, 6.1, 3.4)	1.62 m
2-H (β)	1.72 m	2.71 ddd (15.8, 12.2, 6.8)	1.70 m
3-H	3.25 dd (11.2, 4.5)		3.28 dd (10.8, 4.5)
5-H (α)	1.10 dd (10.5, 5.2)	2.51 dd (14.4, 3.4)	1.64 m
6-H (α)	2.08 m	2.35 dd (16.0, 3.4)	2.43 m
6-H (β)	2.08 m	2.55 dd (16.0, 14.4)	
7-H	5.48 br s		
11-H	5.32 d (6.3)	2.32 m	2.31 m
12-H (α)	2.22 m	1.82 m	1.79 m
12-H (β)	2.01 m		
15-H (α)	1.41 m	1.97—2.02 m	1.73 m
15-H (β)	1.64 m		
16-H (α)	1.28 m	2.10 m	2.09 m
16-H (β)	1.73 m		
17-H	1.61 m	1.46 m	1.46 m
18-H_3	0.58 s	0.69 s	0.67 s
19-H_3	0.99 s	1.34 s	1.17 s
20-H	1.41 m	1.48 m	1.48 m
21-H_3	0.96 d (6.5)	0.98 d (5.8)	0.98 d (5.8)
22-H	1.20 m, 1.67 m	1.26 m, 1.63 m	1.26 m, 1.67 m
23-H	2.28 m, 2.40 m	2.27 m, 2.39 m	2.27 m, 2.39 m
24-H	6.49 td (7.6, 1.2)	6.49 td (6.8, 1.2)	6.49 t (7.2)
26-H	9.40 s	9.40 s	9.40 s
27-H_3	1.75 d (1.2)	1.75 br s	1.75 br s
28-H_3	1.01 s	1.10 s	1.00 s
29-H_3	0.89 s	1.12 s	0.89 s
30-H_3	0.89 s	0.95 s	0.93 s

Table 2. ^{13}C-NMR Spectral Data of Compounds **1**—**3** (100 MHz, $CDCl_3$)

C	**1**	**2**	**3**
1	35.7	35.4	34.8
2	28.0	34.3	28.8
3	78.9	214.6	78.0
4	38.7	47.2	39.8
5	49.1	50.4	50.7
6	23.0	37.1	36.7
7	120.4	198.0	199.0
8	142.5	139.8	138.9
9	146.0	162.6	164.7
10	37.4	39.4	38.9
11	116.1	23.8	23.6
12	37.8	30.1	30.2
13	43.8	45.0	45.0
14	50.3	47.8	49.0
15	31.5	28.7	27.5
16	27.8	31.8	32.0
17	50.9	49.0	49.9
18	15.7	15.9	15.3
19	22.7	17.9	18.4
20	36.2	36.2	36.3
21	18.3	18.6	18.6
22	34.7	34.7	34.8
23	26.1	26.0	26.0
24	155.4	155.2	155.3
25	139.1	139.2	139.2
26	195.4	195.3	195.3
27	9.2	9.2	9.2
28	28.1	25.4	27.5
29	15.8	21.4	15.8
30	25.6	24.9	25.0

195.4) indicated the presence of an aldehyde in the side chain, when compared to that of **5** (δ 69.1). This was further supported by a proton signal at δ 9.40 in the ^{1}H-NMR spectrum.

The precise connectivities of **1** were established by interpretation of the significant heteronuclear multiple bond correlation (HMBC) spectrum (Fig. 1). Long-range correlations between signals of H-5 and C-7; H-11 and C-8; H_3-30 and C-8; H-7 and C-9; H_3-19 and C-9; and H_2-12 and C-11 confirmed the presence of a heteroannular diene at C-7 (8) and C-9 (11). Correlations between signals of H_2-22 and C-24 (an olefinic carbon at δ_C 155.4), and between signals of H-24 and C-27 (an aldehyde carbon at δ 195.4), as well as those of H-24 and C-26 revealed a conjugated aldehyde at C-24—C-26 (Fig. 1). Since signals of H_3-28 and H_3-29 were shift-correlated with that of C-3, a hydroxy group was concluded to be at C-3.

An equatorial hydroxyl group at C-3 (β-orientation) was deduced from the multiplicities of an H-3 signal (δ 3.25, dd, J=11.2, 4.5 Hz), which was also supported with correlations observed between H-3 and H-5, as well as H-3 and H_3-28 in the nuclear Overhauser effect spectroscopy (NOESY) spectrum. As regards the stereochemistry at C-24, the configuration was suggested to be *E* with respect to an aldehyde group, when compared to that of ganoderal A showing the same NMR spectral data.[5] Consequently, the structure of **1** was determined to be (24*E*)-3β-hydroxy-5α-lanosta-7,9(11),24-trien-26-al.

Lucialdehyde B (**2**) was obtained as an amorphous powder (MeOH–H_2O) with a positive optical rotation, $[\alpha]_D$ +31° ($CHCl_3$). The UV absorption at 245 nm (log ε 4.17) and the IR band at 1655 cm^{-1} suggested the presence of a conjugated carbonyl group. The molecular formula was determined to be $C_{30}H_{44}O_3$ by HR-EIMS. The ^{1}H-NMR spectrum showed signals for seven methyls including a doublet at δ 0.98 (J=5.8 Hz) and a vinyl methyl at δ 1.75, an olefinic proton at δ 6.49 (td, J=6.8, 1.2 Hz), and an aldehyde proton at δ 9.40 (Table 1). The ^{13}C-NMR spectrum showed signals characteristic of seven methyls, four olefinic carbons, two ketone carbons at δ 198.0 and 214.6, and an aldehyde carbon at δ 195.3 (Table 2). These data suggested a highly oxygenated Δ^8-lanostane-type triterpene close to the structure of ganoderal B.[18] However, the chemical shift of C-7 (δ 198.0) indicated the presence of a ketone group instead of a hydroxyl group, when compared to that of ganoderal B. This was further supported by HMBC correlations observed between signals of H-5 and C-7 (Fig. 1). Furthermore, correlations between signals of H-5/H-28/H-29 and C-3 confirmed the presence of another carbonyl group at C-3 in the HMBC spectrum. The configuration of C-24 was assigned as 24*E* on the basis of the proton and carbon signals of the side chain moiety (C-20—27), which were quite similar to those of **1**. Consequently, the structure of **2** was determined to be (24*E*)-3,7-dioxo-5α-lanosta-8,24-dien-26-al.

Lucialdehyde C (**3**) was isolated as an amorphous powder (MeOH–H_2O) with a positive optical rotation, $[\alpha]_D$ +18° ($CHCl_3$). The UV absorption at 242 nm (log ε 4.15) and the IR band at 1655 cm^{-1} suggested the presence of an α,β-unsaturated carbonyl group. The molecular formula, $C_{30}H_{46}O_3$, was determined by HR-EIMS. The ^{1}H- and ^{13}C-NMR spectra of **3** were similar to those of **2**. However, new methine signals at δ_H 3.28 and δ_C 78.0 were observed in the spectral data of **3**, instead of a carbonyl signal at δ_C 214.6 of **2**. This indicated that **3** had a hydroxyl group at C-3. The connectivity of the hydroxyl group was confirmed by HMBC correlations observed between signals of H-28/H-29 and C-3 (Fig. 1). The configuration of a hydroxyl group at C-3 was assigned as β on the basis of multiplicity (δ 3.28, dd, J=10.8, 4.5 Hz). This was further supported by NOE correlations observed between H-3 and H-5/H-28 in the NOESY spectrum (Fig. 1). The configuration of C-24 was assigned as 24*E* on the basis of NMR data, which were similar to those of **1**. Lucialdehyde C (**3**) was accordingly determined to be (24*E*)-3β-hydroxy-7-oxo-5α-lanosta-8,24-dien-26-al.

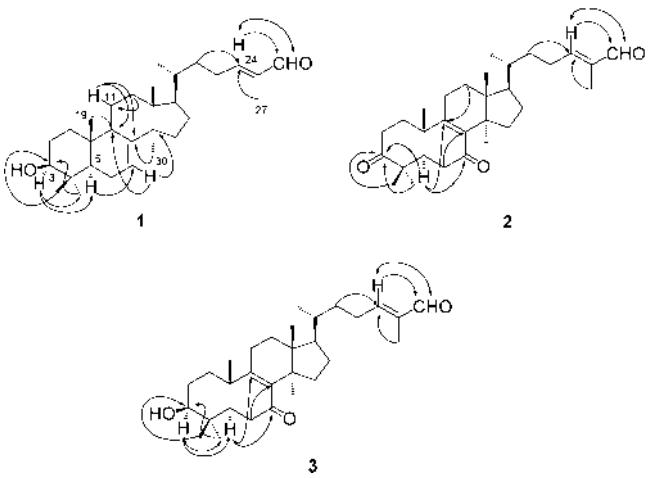

Fig. 1. Long-Range and NOE Correlations Observed in the HMBC and NOESY Spectra of **1**—**3** (HMBC →, NOE ↔)

Since aldehydes of the lanostane-type triterpene have been reported to be cytotoxic against human hepatoma PLC/PRF/5 and KB cells,[4] we tested the new compounds **1**—**3** together with **4**—**10** for their cytotoxic activity against LLC, T-47D, S-180, and Meth-A cell lines *in vitro* (Table 3). Lucialdehyde C (**3**) and ganodermanondiol (**6**) showed cytotoxic effects on all the tumor cell lines, and ganodermanonol (**4**) exhibited significant cytotoxicity against Meth-A cells (murine sarcoma). Of the compounds tested, lucialdehyde C (**3**) showed the most appreciable toxicity against LLC, T-47D, S-180, and Meth-A cell lines with ED_{50} values of 10.7, 4.7, 7.1, and 3.8 μg/ml, respectively. Among the cell lines examined, Meth-A cells exhibited high sensitivity for these lanostane-type compounds.

Experimental

Optical rotations were measured with a DIP-360 automatic polarimeter (JASCO). UV spectra were measured with a UV-2200 UV-VIS recording spectrophotometer (Shimadzu). IR spectra were measured with an FT/IR-230 infrared spectrometer (JASCO). ^{1}H- and ^{13}C-NMR spectra were measured with a JNA-LAA 400 WB-FT (^{1}H, 400 MHz; ^{13}C, 100 MHz; JEOL) spectrophotometer, the chemical shifts being represented as ppm with tetramethylsilane as an internal standard. HR-EIMS were measured with a JMX-AX 505 HAD mass spectrophotometer (JEOL). Preparative HPLC was carried out on a Gilson HPLC system; pump: model 305 and 306, detector: 119 UV/VIS detector. Column chromatography was carried out on silica-gel (Kieselgel 60, 70—230 mesh, Merck). Thin layer chromatography (TLC) was carried out on pre-coated Silica-gel 60 F_{254} plates (0.25 mm, Merck) and RP-18 F_{254} S (0.25 mm, Merck), and spots were detected under a UV light and by spraying with 10% H_2SO_4, followed by heating.

Table 3. Cytotoxicity of Compounds Isolated from the Fruiting Bodies of *Ganoderma lucidum*

	LLC (μg/ml)	T-47D (μg/ml)	S-180 (μg/ml)	Meth-A (μg/ml)
Lucialdehyde A (**1**)	>20	>20	>20	10.4
Lucialdehyde B (**2**)	14.3	15.0	>20	4.0
Lucialdehyde C (**3**)	10.7	4.7	7.1	3.8
Ganodermnonol (**4**)	>20	4.8	10.0	2.8
Ganodermadiol (**5**)	>20	>20	>20	10.3
Ganodermanondiol (**6**)	14.0	4.7	11.0	9.2
Ganodermanontriol (**7**)	>20	>20	>20	>20
Ganoderic acid A (**8**)	>20	>20	>20	>20
Compound B8 (**9**)	>20	>20	>20	>20
Methyl ganoderate C1 (**10**)	>20	>20	>20	>20
Adriamycin	0.06	0.02	0.11	0.13

Plant Materials The fruiting bodies of *G. lucidum* were kindly provided by Toyoseyaku Co., Ltd. (Tokyo). The voucher specimen is deposited in our laboratory.

Cells Meth-A cells (mouse sarcoma) and LLC (mouse lung carcinoma) were purchased from RIKEN Cell Line Bank (Tsukuba, Japan). Sarcoma 180 (mouse sarcoma) and T-47D (human carcinoma) were obtained from Dainippon-Pharmaceutical Co. (Osaka, Japan). The cells were maintained as monolayer cultures in RPMI 1640 medium supplemented with 5% fetal bovine serum, sodium bicarbonate (2 g), penicillin G (100000 units), and streptomycin (100 mg).

Isolation Procedure Powder of the fruiting bodies of *G. lucidum* KARST (3.1 kg) was extracted with a mixed solvent of $CHCl_3$–MeOH (80 : 20 v/v, 6000 ml×3) at room temperature, and the combined solutions were evaporated *in vacuo* to give a residue (523.4 g). A portion of the residue (500 g) was suspended in 90% MeOH (1000 ml) and extracted with hexane (500 ml×2). The residual MeOH solution was concentrated *in vacuo* to give a residue (491.1 g). The residue (480 g) was applied to a column of silica gel. Elution was started with $CHCl_3$, and then $CHCl_3$–MeOH (9 : 1 and 8 : 1) to yield 4 fractions (frac. A—D; 263.1, 122.3, 54.9, and 27.1 g, respectively). Column chromatography of frac. A on silica gel (hexane–acetone, 3 : 2→2 : 3) yielded four subfractions (subfrac. A1—A4; 17.8, 108.5, 101.9, and 24.8 g, respectively). Repeated column chromatography of subfrac. A1 on silica gel (hexane–acetone, 5 : 1) and Florisil (hexane–acetone, 3 : 2), followed by preparative HPLC [a linear gradient of CH_3CN (75%→95%) in 2% AcOH], afforded **4** (24.8 mg, t_R 58.1 min), **5** (36.8 mg, t_R 63.5 min), **1** (2.6 mg, t_R 68.9 min), **2** (3.9 mg, t_R 74.4 min), **6** (4.6 mg, t_R 79.4 min) and **3** (2.8 mg, t_R 85.7 min). A portion of subfrac. A2 (40 g) was chromatographed on silica gel (hexane–acetone, 4 : 1) and Florisil (hexane–acetone, 3 : 2), followed by preparative HPLC [a linear gradient of CH_3CN (70%→90%) in 2% AcOH] afforded **7** (44.7 mg, t_R 77.0 min), **10** (12.0 mg, t_R 83.1 min), **8** (16.7 mg, t_R 89.0 min) and **9** (32.0 mg, t_R 98.6 min).

(24*E*)-3β-Hydroxy-5α-lanosta-7,9(11),24-trien-26-al (**1**, Lucialdehyde A): Amorphous powder (MeOH–H_2O). $[\alpha]_D$ +32° (c=0.097, $CHCl_3$). UV λ_{max} nm (log ε): 232 (4.03), 243 (4.10), 252 (3.96). IR ν_{max} cm^{-1}: 3450 (OH), 2853, 1685 (C=O), 1459, 1377. ^{1}H- and ^{13}C-NMR data: see Tables 1 and 2. HR-EIMS *m*/*z*: 438.3470 (M^+, Calcd for $C_{30}H_{46}O_2$: 438.3499).

(24*E*)-3,7-Dioxo-5α-lanosta-8,24-dien-26-al (**2**, Lucialdehyde B): Amorphous powder (MeOH–H_2O). $[\alpha]_D$ +31° (c=0.105, $CHCl_3$). UV λ_{max} nm (log ε): 245 (4.17). IR ν_{max} cm^{-1}: 2855, 2372, 1655 (C=O), 1459. ^{1}H- and ^{13}C-NMR data: see Tables 1 and 2. HR-EIMS *m*/*z*: 452.3255 (M^+, Calcd for $C_{30}H_{44}O_3$: 452.3292).

(24*E*)-3β-Hydroxy-7-oxo-5α-lanosta-8,24-dien-26-al (**3**, Lucialdehyde C): Amorphous powder (MeOH–H_2O). $[\alpha]_D$ +18° (c=0.092, $CHCl_3$). UV λ_{max} nm (log ε): 242 (4.15). IR ν_{max} cm^{-1}: 3450 (OH), 2925, 2853, 1655 (C=O), 1459. ^{1}H- and ^{13}C-NMR data: see Tables 1 and 2. HR-EIMS *m*/*z*: 454.3419 (M^+, Calcd for $C_{30}H_{44}O_3$: 454.3449).

Cytotoxicity Assay Tumor cells were cultured with a RPMI 1640 medium containing 5% fetal bovine serum (FBS). For a sulforhodamine B (SRB) assay,[19] the cells were cultured in RPMI 1640 medium containing 5% FBS. One hundred microliters of the cell suspension (4—50000 cells/ml) in the culture medium were inoculated to each well of 96-well microtiter plates. One day after plating, a time zero control plate was made. Compounds were directly treated, and the cells were incubated for another 48 h in a humidified 5% CO_2 atmosphere at 37 °C. The cells were fixed with 50 μl of 50% trichloroacetic acid (TCA) solution for 1 h at 4 °C and the plates were washed 5 times with tap water and air-dried. Fifty microliter of SRB solution (0.4% in 1% acetic acid) were added, and staining was done at room temperature for 30 min. The residual dye was washed out with 1% acetic acid and the plates were air-dried. To each well, one hundred microliters of Tris buffer solution (10 mM, pH 10.5) were added. The optical density (OD) of each well was measured with a microtiter plate reader at 540 nm. The growth inhibition was calculated as follows:

$$\text{inhibition (\%)} = (OD_{compound} - OD_{blank})/(OD_{control} - OD_{blank}) \times 100$$

The 50% growth inhibition (ED_{50}) was calculated by the Probit method.[20]

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