ORIGINAL ARTICLE

Structure and activity of lobophorins from a turrid mollusk-associated *Streptomyces* sp

Zhenjian Lin¹, Michael Koch², Christopher D Pond², Gaiselle Mabeza³, Romell A Seronay³,
Gisela P Concepcion³, Louis R Barrows², Baldomero M Olivera⁴ and Eric W Schmidt^{1,4}

A novel *lunun-lunun* sampling methodology was used to obtain a large diversity of micromollusks, including the new species *Lienardia totopotens*. In turn, from *L. totopotens* we cultivated a *Streptomyces* sp. strain that contained new and known spirotetronate polyketides, lobophorins (1–5). The structures were elucidated using spectroscopy, and the compounds were evaluated for cytotoxicity to human cells and activity against *Mycobacterium tuberculosis*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Compounds 2–5 showed varying degrees of activity against human cells, *M. tuberculosis* and *B. subtilis* in the low μM to mid nM range but were inactive against the other strains, while 1 lacking digitoxose was inactive. Very slight structural changes in 2–5 led to varying antibacterial:cytotoxicity ratios, providing a possible basis to synthesize more selective derivatives.

The Journal of Antibiotics (2014) 67, 121–126; doi:10.1038/ja.2013.115; published online 13 November 2013

Keywords: cone snail; *Mycobacterium tuberculosis*; *Streptomyces*

INTRODUCTION

There is an urgent need for new antibiotics to combat the rise of resistant strains.¹ As an important example, *Mycobacterium tuberculosis* infects 1/3 of the global population and kills 1.4 million people annually.² The rate of *M. tuberculosis* drug resistance is steadily rising, progressing from multiple drug-resistant forms to extremely drug-resistant and finally totally drug-resistant forms.³ Natural products represent a major group of successful antituberculosis drugs, including the first-line therapy, rifampicin. Natural products also continue to provide leads for antimycobacterial therapy,⁴ indicating that the natural world should still be examined as a source for new antituberculosis drugs. In addition, further therapies are needed to combat difficult infections, such as drug-resistant *Pseudomonas*.⁵

A problem with natural product-based antibiotic discovery programs is that the rediscovery rate can be high.⁴ It is straightforward to screen for antibiotic activity, and such screens have been ongoing for about 80 years. Several novel strategies have been suggested to overcome this problem. One approach is to seek novel habitats for bacteria that have not been previously well explored, with the as-yet unproven expectation that new compounds are more likely to be discovered. For this reason, we have been examining bacterial associates of gastropod mollusks, especially from the Family Conidae, which have yielded many novel isolates with novel chemistry.^{6–10} Many of the bacteria cultivated from gastropods probably reflect the microbiology of the surrounding environment,¹¹

but at least some of these bacteria are long-term associates that produce compounds important to the mollusks.⁹ Thus, cultivation of mollusk-associated bacteria leads to a library of diverse bacteria, some of which produce new, bioactive natural products.

Symbiotic bacteria from animals provide a good and relatively untapped source of new natural products, but obtaining diverse, novel animals can be time consuming and expensive. We have employed an approach to easily access a large diversity of mollusks, *lunun-lunun*.¹² This method, invented and widely used by fishermen in the central Visayan region of the Philippines, involves placing bundles of nets (*lunun-lunun*) in the sea for 1–6 months. When the nets are harvested, they are found to contain a large abundance and diversity of micromollusks, which are valuable to collectors. In addition, the obtained micromollusks are often novel species, previously unknown to science.^{13–15} Conoidean mollusks, the turrids, are related to cone snails and are overrepresented in these nets.¹⁶ Turrids are the most speciose of the cone-related mollusks. Ongoing research shows that, like cone snails, each turrid contains a wealth of novel venom peptides that are neuroactive and potentially have other activities.¹²

Although many turrids are found in a single net, we carefully first examined a small subset of these turrids to explore their potential for production of novel microbial natural products. In particular, a new species, *Lienardia totopotens*,¹³ was found in a *lunun-lunun* net, dissected and used to obtain novel venoms with potential in drug

¹Department of Medicinal Chemistry, L.S. Skaggs Pharmacy Institute, University of Utah, Salt Lake City, UT, USA; ²Department of Pharmacology and Toxicology, University of Utah, Salt Lake City, UT, USA; ³Marine Science Institute, University of the Philippines Diliman, Quezon City, Philippines and ⁴Department of Biology, University of Utah, Salt Lake City, UT, USA

Correspondence: Professor EW Schmidt, Department of Medicinal Chemistry, The University of Utah, 30 South 2000 East, Room 307, Salt Lake City, UT 84112, USA.
E-mail: ews1@utah.edu

We are grateful to Professor Christopher Walsh for his inspiring studies of natural products and for his profound impact on the development of our field.

Received 29 July 2013; revised 7 October 2013; accepted 11 October 2013; published online 13 November 2013

discovery. Tissues were obtained, crushed and plated on microbial media. We previously reported a structurally relatively novel compound from one of these bacterial strains, but unfortunately we could not determine the activity of the compound.⁸ Here, we report a series of less novel compounds, which are in fact relatively trivial new derivatives of the lobophorin series.^{17–20} These compounds were inhibitory to *Bacillus subtilis*, *M. tuberculosis* and human CEM-TART cell lines, but not to *Pseudomonas aeruginosa* or *Burkholderia cepacia*.

RESULTS

Strain *Streptomyces* sp. 1053U.I.1a.3b was cultivated from dissected hepatopancreas tissues of *L. totopotens*, a new species of conoidean mollusk, which was found in a *lumun-lumun* net in the Philippines. Crude extracts of 1053U.I.1a.3b were active in an antibacterial assay against *B. subtilis*. High-performance liquid chromatography (HPLC) analysis showed that the strain produced a series of related families of metabolites, which were further purified by bioassay-guided fractionation to yield compounds 1–5 (Figure 1). Using NMR and mass spectrometry, three compounds were shown to be identical to the previously reported lobophorins F (3),¹⁹ B (4)¹⁷ and C (5),¹⁸ while the remaining two compounds had not been previously described and were named as lobophorin H (1) and I (2).

The molecular formula of 1 was established as $C_{42}H_{60}N_2O_{10}$ by high-resolution electrospray ionization mass spectrometry (HRESIMS) (m/z 753.4344 for $[M+H]^+$). Interpretation of the 1H and ^{13}C NMR spectra (Table 1 and Supplementary Figures S1–S6) showed that 1 has structural features that are almost identical to those of 5 (Supplementary Table S1), including a sugar and a complex, highly oxygenated aglycone. 1D and 2D NMR data of 1 supported a lack of three sugar units (A–C) in comparison with 5 (Table 1). Indeed, only one hemiacetal group (δ_H 4.75, H-D1, δ_C 96.7, C-D1) was observed and assigned as the anomeric carbon of a sugar unit. Moreover, the 1H – 1H COSY correlations between H-D6/H-D5 and H-D1/H-D2,

together with the heteronuclear multiple-bond correlation spectroscopy (HMBC) correlations from H-D7 to C-D2, D3, D4, and from both H-D9 and H-D4 to a carbonyl at C-D8 confirm the presence of sugar unit D. This sugar has 1H and ^{13}C chemical shifts that are identical to those of the same sugar found in 5. The C-9 ^{13}C chemical shift (δ_C 74.9) in 1 indicated a free secondary alcohol group instead of the *O*-glucosidic bond that is found in 5. A comprehensive interpretation of HSQC, COSY and HMBC correlations (Figure 2) performed on 1 led to the identification of the key features of the aglycone of 5. The only exceptions were three quaternary carbons (C-1, δ_C 168.4; C-2, δ_C 103.1; and C-26, δ_C 197.6), which were observed in the ^{13}C NMR spectrum of 1 but lacked 2D NMR correlations. These were assigned to the tetronic acid core of the aglycone based upon similarity to the chemical shifts found in related compounds. On the basis of these data, the structure of 1 was identified as the aglycone of 5.

The molecular formula of 2 was determined as $C_{61}H_{88}N_2O_{21}$ by HRESIMS (m/z 1185.5962 for $[M+H]^+$) and 1H and ^{13}C NMR data (Table 1 and Supplementary Figures S7–S11). As with 1, a complete set of 2D NMR data were used to elucidate the structure of 2 and to confirm its relationship to compound 1 (Figure 2). In comparison with 1, compound 2 has three more sugar units, which were deduced by the observation of three more hemiacetal groups (δ_H 4.75, H-A1, δ_C 100.3, C-A1; δ_H 5.18, H-B1, δ_C 93.97, C-B1; and δ_H 4.94, H-C1, δ_C 101.4, C-C1). The carbon chemical shift of C-9 at 87.2 p.p.m. in 2 indicated an *O*-glucosidic bond. The NMR data of 2 were largely identical to previously reported lobophorin C (4) (Supplementary Table S2). In fact, the only major difference was the presence of an aldehyde residue (δ_H 9.43 and δ_C 196.4) in place of a CH_2OH group. The C-21 and C-22 ^{13}C resonances of 2 were shifted 34.3 and 3.5 p.p.m. downfield in comparison with those of 4, indicating conjugation between the aldehyde and double bond C-21–C-22. The HMBC correlations from H-32 to C-32 and from H-21 to C-32 confirmed the partial structure of the conjugated aldehyde group.

Figure 1 Structures of lobophorin compounds 1–5.

Table 1 ^1H and ^{13}C NMR data for compounds 1 and 2

No	1^a		2^b	
	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}
1	—	168.4 C	—	ND
2	—	103.1 C	—	ND
3	—	204.0 C	—	200.5 C
4	—	51.8 C	—	53.2 C
5	1.89 m	43.4 CH	2.11 dd (10.0, 8.5)	46.1 CH
6	1.47 m	31.5 CH	1.51 m	33.2 CH
7	1.44 m	42.2 CH ₂	1.49 m, 1.58 m	43.9 CH ₂
8	2.07 m	35.4 CH	2.20 m	36.6 CH
9	3.40 m	74.9 CH	3.40 dd (10.5, 5.1)	87.2 CH
10	1.90 m	39.4 CH	2.02 brdd (12.0, 11.0)	40.7 CH
11	6.02 d (10.0)	128.0 CH	5.72 brd (10.2)	127.5 CH
12	5.28 m	125.8 CH	5.36 ddd (10.0, 5.3, 2.7)	130.0 CH
13	3.39 m	52.8 CH	3.78 brd (4.9)	53.3 CH
14	—	136.3 C	—	139.2 C
15	5.04 d (9.4)	123.9 CH	5.23 brd (8.5)	123.6 CH
16	2.30 m, 2.18 m	31.8 CH ₂	2.44 ddd (15.2, 9.6, 2.4), 2.20 m	32.8 CH ₂
17	4.12 brs	79.7 CH	4.24 brs	81.2 CH
18	—	138.6 C	—	139.3 C
19	4.97 d (10.0)	119.1 CH	5.25 brd (9.3)	119.7 CH
20	3.48 dd (10.0, 2.5)	40.4 CH	3.63 brd (9.3)	43.8 CH
21	5.34 brs	120.9 CH	6.66 brs	155.2 CH
22	—	142.5 C	—	146.0 C
23	2.55 m	27.7 CH	2.84 dd (9.2, 2.7)	27.2 CH
24	2.24 m, 1.75 m	35.4 CH ₂	2.27 dd (14.5, 7.5), 1.68 m	36.2 CH ₂
25	—	83.6 C	—	85.2 C
26	—	197.6 C	—	ND
27	1.44 s	15.6 CH ₃	1.47 s	16.0 CH ₃
28	0.55 brs	23.0 CH ₃	0.65 d (6.7)	23.7 CH ₃
29	0.92 d (6.8)	14.0 CH ₃	1.12 d (6.5)	15.3 CH ₃
30	1.29 s	14.4 CH ₃	1.37 s	15.2 CH ₃
31	1.33 s	15.4 CH ₃	1.42 s	16.0 CH ₃
32	4.00 d (13.4), 3.93 d (13.4)	63.8 CH ₂	9.43 s	196.4 CH
33	1.14 d (7.0)	20.4 CH ₃	1.31 d (7.3)	21.1 CH ₃
A1	—	—	4.75 brd (4.4)	100.3 CH
A2	—	—	2.37 dd (14.5, 3.4), 1.72 m	32.3 CH ₂
A3	—	—	4.01 dd (6.0, 3.0)	70.0 CH
A4	—	—	3.26 dd (9.6, 3.3)	74.0 CH
A5	—	—	4.10 dq (9.6, 6.3)	66.5 CH
A6	—	—	1.19 d (6.3)	18.6 CH ₃
B1	—	—	5.18 brd (3.4)	93.9 CH
B2	—	—	2.09 m, 1.99 ddd (15.0, 3.8, 3.6)	36.5 CH ₂
B3	—	—	4.17 dd (6.3, 3.6)	68.8 CH
B4	—	—	3.28 dd (9.9, 3.6)	84.0 CH
B5	—	—	4.04 dq (9.4, 6.6)	64.4 CH
B6	—	—	1.19 d (6.5)	18.6 CH ₃
C1	—	—	4.94 brd (9.7)	101.4 CH
C2	—	—	2.04 m, 1.72 m	39.3 CH ₂
C3	—	—	4.29 dd (6.0, 3.1)	64.9 CH
C4	—	—	2.84 dd (9.2, 2.7)	84.3 CH
C5	—	—	3.82 m	70.5 CH
C6	—	—	1.22 d (6.5)	19.1 CH ₃
C7	—	—	3.69 s	53.6 CH ₃
D1	4.75 d (9.8)	97.6 CH	4.52 brd (9.6)	99.5 CH
D2	1.90 m, 1.73 m	37.0 CH ₂	2.71 brd (15.0), 1.79 dd (15.0 m 9.4)	37.0 CH ₂
D3	—	57.0 C	—	92.9 C
D4	3.55 m	54.0 CH	4.35 brs	55.8 CH
D5	3.99 m	67.9 CH	3.52 q	70.7 CH
D6	1.00 d (6.2)	17.6 CH ₃	1.13 d (6.5)	18.1 CH ₃
D7	1.18 s	24.2 CH ₃	1.52 s	26.3 CH ₃
D8	—	158.5 C	—	160.7 C
D9	3.55 s	52.5 CH ₃	3.68 s	53.6 CH ₃

^aIn DMSO-*d*₆.^bIn CD₃OD-*d*₄.

Figure 2 Key HMBC, ^1H - ^1H COSY and ROESY correlations for compounds **1** and **2**.

Table 2 Antimycobacterial activity (MIC_{90}) and CEM-TART cytotoxicity (LD_{50}) for compounds **1**–**5**

	1	2	3	4	5
<i>M. tuberculosis</i>	>100	2.6	7.8	1.3	1.4
<i>P. aeruginosa</i>	>100	>100	>100	>100	>100
<i>B. cepacia</i>	>100	>100	>100	>100	>100
<i>B. subtilis</i>	>100	10.6	24.4	1.3	21.6
CEM-TART	>100	8.6	0.3	1.6	1.7

Compounds **1**–**5** were tested for their inhibitory activity against a panel of bacteria for which further drugs are needed. All compounds except **1** showed strong inhibitory activity to *M. tuberculosis* and *B. subtilis* with MIC values ranging from 1.3 to $24\ \mu\text{M}$ (Table 2). In contrast, none of the compounds showed inhibitory activity to *P. aeruginosa* or *B. cepacia*. To examine the potential to develop lobophorins to treat infections, we also tested the cytotoxicity of these compounds against human CEM-TART cancer cell line. Unfortunately, all the antimycobacterial active compounds also showed strong cytotoxicity with potencies similar to those of their antimycobacterial activities (Table 2).

DISCUSSION

Lobophorins belong to a family of bacterial spirotetronates, which comprise a large class of antibiotics and antitumor agents. In addition to lobophorins,^{17–19} the family includes antlermicins,^{21,22} chlorothricin,²³ kijanimicin,²⁴ decatromicins,²⁵ saccharocarins,²⁶ tetrocarins²⁷ and versipelostatins.²⁸ We tested the new isolated lobophorin derivatives against a panel of bacteria. Of note, we

found that the lobophorins are relatively potent against mycobacteria: compounds **4** and **5** were about $4 \times$ less potent than rifampicin control. Very recently, other members of this series were also tested for their activity on *M. tuberculosis*.²⁰ We show that the antimycobacterial compounds also exhibit toxicity to human cells, limiting their potential utility.

Some interesting structure–activity relationships were observed, which might be useful in designing more selective antimycobacterial agents. Derivative **1** lacks the three digitoxose sugar moieties, and concomitantly also lacks activity against *M. tuberculosis* and human cells. Loss of one of the digitoxose units, as in compound **3**, led to a >25-fold selective toxicity to human vs *M. tuberculosis* cells. Compounds **4** and **5** have nearly equal potency to both cell types. By contrast, new compound **2**, which contains an α , β -unsaturated aldehyde, provided a >3-fold selectivity for antitubercular activity vs cytotoxicity. This finding suggests that simple modifications of the lobophorin scaffold may provide derivatives that are selective antimicrobial agents. Recently, biosynthetic gene clusters for lobophorins and its polyketide relatives have been described, providing a platform for engineering to produce desired derivatives.^{29–31} Interestingly, elucidation of the glycosylation steps shows definitively that compounds similar to **1** are precursors to other compounds in the series.

Many novel metabolites have been discovered in marine actinomycetes,³² although arguably most of the metabolites so far discovered fall into structural categories that are very similar to those of their terrestrial relatives. Even so, discovery of related derivatives have been very useful in the development of therapeutic agents. Examples include salinisporamide,^{33,34} which is structurally related to omuralide³⁵ but that has features making it highly novel in its mode of action. Lomaiviticin³⁶ provides another example where a

play on a biosynthetic theme has led to a series of quite different molecules. A novel, but structurally trivially new bottromycin³⁷ derivative was found in an ascidian-associated *Streptomyces* strain; the bottromycin biosynthetic gene cluster was relatively divergent (about 85% DNA sequence identity) in comparison with those found in several terrestrial strains, which has some biotechnological application in the creation of derivatives. However, not all marine strains differ from terrestrial relatives genetically, and even those that have specific associations with marine invertebrates may be nearly identical to terrestrial strains. For example, it was found that a *Nocardiopsis* strain living in close association with a cone snail is genomically nearly identical to a strain that lives with bees.⁹ Therefore, accessing actinomycetes from the marine environment, including marine animals, has demonstrated utility in finding both novel strains and novel metabolites, including some in clinical trials.³⁴ Those strains and metabolites are related to those found on land, and are sometimes even identical, but nonetheless novel derivatives have been extremely useful in drug discovery.

A current area of research interest is the determination of whether many of these actinomycetes are specifically found in the marine environment and whether they are associated with animals. A wealth of data shows that *Salinispora* spp. are marine-specific strains.³⁸ Much less data are available concerning potential interactions of marine actinomycetes with marine animals. In at least the case of the cone snails described above, one strain seems to live specifically with animals, although it is identical to a terrestrial strain.⁹ *Lumun-lumun* collection provides rapid access to diverse micromollusks, including many new species. Because many related species are conveniently obtained from the same habitat in a single net, *lumun-lumun* provides a method to answer questions about the specific interactions of marine bacteria, including actinomycetes, with host animals. This method was also shown to be of utility in providing rapid access to diverse, novel and bioactive venom peptides that might find therapeutic application.¹² Here, and in a recent report on structurally novel compounds, we show that this method leads to discovery of both highly novel compounds and a series of trivial derivatives, the lobophorins, which has led to an interesting structure–activity relationship series.

MATERIALS AND METHODS

General experimental procedures

UV spectra were obtained using a Perkin-Elmer Lambda2 UV/vis spectrometer. The NMR data were collected using either a Varian (Palo Alto, CA, USA) INOVA 500 (¹H 500 MHz, ¹³C 125 MHz) NMR spectrometer with a 3-mm Nalorac MDBG probe or a Varian INOVA 600 (¹H 600 MHz, ¹³C 150 MHz) NMR spectrometer equipped with a 5-mm [¹H/¹³C/¹⁵N] triple resonance cold probe with a z-axis gradient, utilizing residual solvent signals for referencing. High-resolution mass spectra (HRESIMS) were obtained using a Bruker (Billerica, MA, USA) APEXII FTICR mass spectrometer equipped with an actively shielded 9.4-T superconducting magnet (Magnex Scientific Ltd., Oxford, UK), an external Bruker APOLLO ESI source, and a Synrad (Mukilteo, WA, USA) 50 W CO₂ CW laser. Supelco (St Louis, MO, USA) Discover HS (4.6 × 150 mm) and semipreparative (10 × 150 mm) C₁₈ (5 μm) columns were used for analytical and semipreparative HPLC, respectively, as conducted on a Hitachi (Dallas, TX, USA) Elite Lachrom System equipped with a Diode Array L-2455 detector. Antibacterial and cytotoxicity assay plates were evaluated by measuring A₅₈₀ on a Multiskan FC plate reader (Fisher Scientific, Waltham, MA, USA). Chemicals were sourced from Sigma-Aldrich (St Louis, MO, USA) or Fisher Scientific with the exception of the following: fetal bovine serum was obtained from Atlanta Biologicals (Lawrenceville, GA, USA), ADC supplement from Remel (Lenexa, KS, USA), and antimycotic/antibacterial supplement from MP Biomedicals (Solon, OH, USA).

Bacterial material

Bacteria were cultivated from *L. totopotens* obtained by professional collectors near Mactan Island, Cebu, Philippines as previously described.¹¹ Relevant permission from local and national authorities in the Philippines was obtained before beginning this study. *Streptomyces* sp. 1053U.I.1a.3b was obtained from dissected hepatopancreas tissue, purified into an axenic culture, and stored as a glycerol stock at –80 °C. The strain was subsequently recovered from a glycerol stock and used for further chemical analysis. The 16S gene was cloned using primers 8-27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGA CTT-3') and submitted to GenBank (accession number KF137580).

Fermentation and extraction

For the first-stage seed, an agar grown culture of *Streptomyces* sp. 1053U.I.1a.3b was inoculated into 150 ml of ISP2 medium (0.4% yeast extract, 1% malt extract, 0.4% glucose, 2% NaCl). After 3 days incubation at 22 °C with shaking, the first stage (100 ml) was used to inoculate in a New Brunswick BioFlo110 fermenter containing 10 l of the medium ISP2 at 30 °C. After 8 days, the broth was centrifuged and the supernatant was extracted with Diaion HP-20 resin for 4 h. The resin was filtered through cheesecloth and washed with H₂O to remove salts. The filtered resin was eluted with MeOH to yield a primary extract.

Purification

The primary extract (300 mg) was dissolved in 2 ml MeOH and then saturated with RP18 silica gel (2 g). The RP18 silica gel with extract was dried under vacuum and load on a glass column with RP18 silica gel (5 g). The column was first flushed with 50 ml H₂O, and followed by four more washes using step-gradient elution of MeOH in H₂O (40, 60, 70 and 80%; 100 ml each), leading to four fractions (Fr1–Fr4). Fr3 eluted in 70% MeOH and was further fractionated by C₁₈ HPLC using 68% MeOH in H₂O with 0.05% TFA to obtain pure compounds **3** (1.5 mg), **4** (2.0 mg), and a fraction Fr3.2 containing compounds **2** and **5**. Fr3.2 was further purified by C₁₈ HPLC using 65% MeOH in H₂O with 0.05% TFA to obtain compounds **2** (1.1 mg) and **5** (0.9 mg). Fr4 eluted in 80% MeOH and was further purified by C₁₈ HPLC using 75% MeOH in H₂O with 0.05% TFA to obtain compound **1** (1.3 mg).

Lobophorin H (1)

Colorless solid; UV (MeOH) λ_{max} 204, 241, 267 nm; ¹H and ¹³C NMR (see Table 1); HRESIMS *m/z* 753.4344 [M+H]⁺ (calcd for C₄₂H₆₁N₂O₁₀, 753.4326).

Lobophorin I (2)

Colorless solid; UV (MeOH) λ_{max} 204, 241, 267 nm; ¹H and ¹³C NMR (see Table 1); HRESIMS *m/z* 1185.5962 [M+H]⁺ (calcd for C₆₁H₈₉N₂O₂₁, 1185.5958).

B. subtilis disk assay

B. subtilis ATCC 6633 was plated on Mueller-Hinton agar media. Compounds and controls were added to paper disks at varying concentrations, and zones of inhibition were detected by eye.

Antimicrobial broth dilution assays

The assay was performed as previously described,³⁹ with the exception that four wells per compound were used for each dilution for MIC determination. DMSO served as a negative control. Bacterial strains used, along with the positive control antibiotic for each strain, were *M. tuberculosis* H37Ra (rifampicin); *P. aeruginosa* ATCC 27853 (gentamicin); *B. subtilis* ATCC 6633 (gentamicin); and *B. cepacia* ATCC 25608 (chloramphenicol).

Cytotoxicity assay

Cytotoxicity testing was performed using CEM-TART lymphoblastoid cells⁴⁰ cultured in RPMI-1640 with 20% fetal bovine serum and antibiotic/antimycotic supplement at 37 °C with 5% CO₂ in moisture saturated atmosphere in 96-well culture clusters. Test compounds dissolved in 1 μl

DMSO, negative controls (DMSO alone) or positive controls (doxorubicin in DMSO) were added in triplicate wells together with 50 000 cells in 200 μ l of fresh media and incubated for 72 h. Each well then received 11 μ l of 5 mg ml⁻¹ MTT (Sigma, St Louis, MO, USA) followed by 2 h incubation after which the formazan precipitate was pelleted by centrifugation. The spent media was aspirated and the formazan crystals solubilized in DMSO. Absorbance at 580 nm was quantified on a Multiskan FC (Fisher Scientific) plate reader and inhibition determined as described in the antimycobacterial assay procedure.

ACKNOWLEDGEMENTS

Collection, cultivation, isolation and chemical characterization were funded by ICBG grant U01TW008163 from Fogarty (NIH). We thank the government of the Philippines and the community of Mactan Island for permission to conduct this study. Bioassay work was funded by ICBG grant U01T006671 from Fogarty (NIH). CEM-TART cells were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH—Drs Herbert Chen, Terence Boyle, Michael Malim, Bryan Cullen, and H. Kim Lyerly.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (<http://www.nature.com/ja>)