Antituberculosis Agents and an Inhibitor of the *para*-Aminobenzoic Acid Biosynthetic Pathway from *Hydnocarpus anthelminthica* Seeds

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Investigation on the extracts of *Hydnocarpus anthelminthica* seeds led to the isolation of three new compounds, anthelminthicins A–C (**1**–**3**, resp.), and two known ones, namely chaulmoogric acid (**4**) and ethyl chaulmoograte (**5**). Their structures were determined mainly by using spectroscopic techniques. The absolute configuration at the cyclopentenyl moiety of compound **2** was rationalized by quantum calculations. Base hydrolysis, followed by optical-rotation comparison, allowed assignment of the configuration of chaulmoogric-acid moiety of compounds **3** and **5**. Biological assays revealed that compounds **1**–**5** significantly inhibit *Mycobacterium tuberculosis* (MTB) growth with *MIC* values of 5.54, 16.70, 4.38, 9.82, and 16.80 μM, respectively. Compound **3** was found to inhibit the pathway between chorismate and *para*-aminobenzoic acid (*p*Aba) with a *MIC* value of 11.3 μM, representing a new example of *p*Aba inhibitor isolated from a natural source. All compounds were not toxic to *Candida albicans* SC5314 at a concentration up to 100 μM.

Introduction. – Antibiotic resistance challenges the treatment of all infectious diseases [1]. Multidrug-resistant tuberculosis (MDR-TB) has become a great threat to global health, it was reported that two million deaths due to TB infection each year, and three million people are becoming infected with both HIV and TB. Therefore, search for new effective drugs against MDR-TB has never been more intensive [2][3]. Among biosynthetic pathways, chorismate is an important and central biosynthetic metabolite that branches off to the biosynthesis of *p*-aminobenzoic acid (*p*Aba). The biosynthesis of *p*Aba is known to take place in plants, fungi, prokaryotes, and parasites but not in humans; therefore, this pathway is expected to be an ideal target for the development of novel antibiotics [4][5]. Medicinal plants have been used for years to combat infection diseases, and several antimicrobial agents have been successfully identified [6]. Therefore, medicinal plants will be a potential source for the discovery of new antibiotic drugs.

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Hydnocarpus anthelminthica PIERRE ex LANESS (Flacourtiaceae) is a tall evergreen tree which mainly grows in Southeast Asia [7]. Its seeds have been used as a well-known folk medicine against leprosy, tinea, gonorrhea, and tuberculosis [8][9]. Previous studies on the other *Hydnocarpus* plants led to the isolation of flavonolignans, flavones, and chaulmoogric acid analogues [10–12]. Among these, flavonolignans were found to have antitumor and hypoglycemic activities [13]. In our search for anti-infective metabolites from medicinal plants, we investigated the seeds of this plant and isolated five compounds from the nonpolar extracts, three of which are new (*Fig. 1*). Here, we describe the isolation, structural identification, and anti-infective activities of these isolates.

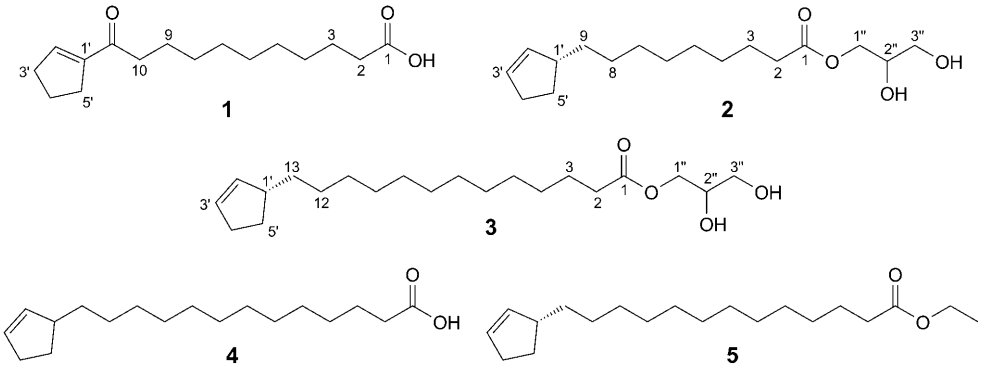

Fig. 1. *The structures of compounds* **1**–**5**

Results and Discussion. – 1. *Structure Elucidation*. Compound **1** was obtained as a white powder. Its molecular formula was established as $C_{16}H_{26}O_3$ from its negative-ion HR-ESI-MS (*m/z* 265.1796 ($[M-H]^-$, $C_{16}H_{25}O_3^-$; calc. 265.1803)), indicating four degrees of unsaturation. ^{1}H,^{1}H-COSY (H–C(2′)/H–C(3′)/H–C(4′)/H–C(5′)) and HMBC interactions of H–C(2′), H–C(5′), H–C(9), and H–C(10) with a C=O group (δ(C) 183.0) suggested an α,β-unsaturated cyclopentenyl ketone moiety. HMBCs (*Fig. 2*) of H–C(2) and H–C(3) with a CO group (δ(C) 176.1) established the connection between C(1)–C(3). The other overlapped signals were assigned as five CH_2 groups, *i.e.*, CH_2(4)–CH_2(8) by a ^{13}C-DEPT spectrum. The structure of **1** was thus identified as 11-(cyclopent-1-en-1-yl)-11-oxoundecanoic acid, and was given the trivial name anthelminthicin A.

Compound **2** was obtained as a yellowish syrup. Its molecular formula was established as $C_{17}H_{30}O_4$ from its HR-ESI-MS (positive-ion mode; *m/z* 321.2041 ($[M+Na]^+$, $C_{17}H_{30}NaO_4^+$; calc. 321.2041)), indicating three degrees of unsaturation. The upfield NMR signals of **2** were similar to those of **1**, indicating that they are closely related. The ^{1}H,^{1}H-COSY spectrum showed the spin system H–C(2′)/H–C(3′)/H–C(4′)/H–C(5′), and the HMBC spectrum displayed cross-peaks of H–C(2′) and H–C(5′), both correlating with C(1′) and C(9), indicating the presence of a cyclopentenyl moiety. Additional ^{1}H,^{1}H-COSY peaks H–C(1″)/H–C(2″)/H–C(3″) and typical chemical shifts for C(1″)–C(3″) revealed the presence of a glycerol residue,

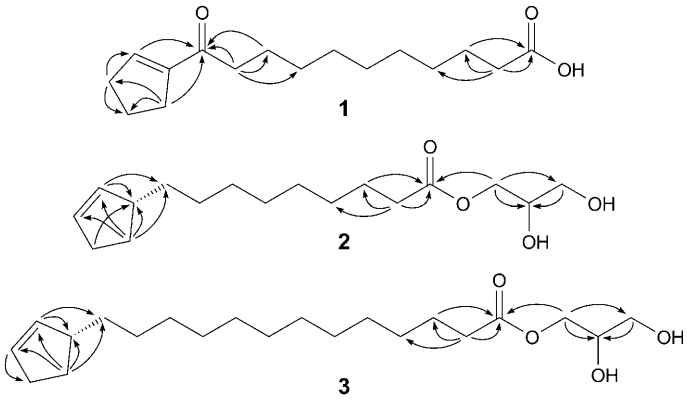

Fig. 2. *Important HMBCs for compounds* **1**–**3**

linked to a CO group, based on the observed HMBC H–C(1″)/C(1) (δ(C) 174.4). Considering the molecular composition, the remaining five overlapping CH_2 groups were assigned as $CH_2(4)-CH_2(8)$. The absolute configuration at C(1′) of **2** was assigned by quantum calculations. In general, the optical-rotation (OR) computations for compounds with such long side chains are very difficult. However, based on the results of *Wipf* and co-workers [14], chiral compounds with a long chain may be cut off into several different small parts, the major effect from the substituents that are connected to the stereogenic center are exerted by the atoms that are close to the stereogenic center. On the other hand, with the increase of chain length, the OR values will decrease [15]. Thus, we selected three models as described below to compute their OR values to determine the OR-change tendency. It was found that the OR values decreased from 144.4 to 128.0, when the substituent changed from Pr to Bu (*i.e.*, **B**; *Fig. 3*). Increasing the chain length by one C-atom resulted in a decrease by 16.4 degrees. When the last C-atom in **A** was changed to an O-atom (**C**), the OR decreased from 144.4 degree to 116.7 degrees, the difference being 27.7 degrees. These results are consistent with the reported observations [15]. When the C-atom chain length is increased to seven, or eight, or nine, the OR values will decrease too much. Indeed, the recorded OR for the hydrolysis product of **5** was *ca.* +57 degrees. Thus, the absolute configuration for compound **2** was predicted as (1′*R*). Accordingly, the structure of **2** was determined as 2,3-dihydroxypropyl 9-[(*R*)-cyclopent-2-en-1-yl]nonanoate, with the trivial name anthelminthicin B. The relative configuration at C(2″) remained to be established.

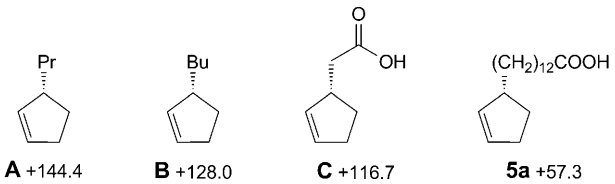

Fig. 3. *The optical-rotation computations for compounds* **A**–**C** *and* **5a**

Compound **3** was obtained as a yellowish syrup. The molecular formula of **3** was established as $C_{21}H_{38}O_4$ from its HR-ESI-MS (positive-ion mode; *m/z* 377.2660 ($[M+Na]^+$, $C_{21}H_{38}NaO_4^+$; calc. 377.2667), indicating three degrees of unsaturation. The IR absorptions and NMR data of **3** (*Table 1*) were very similar to those of **2**. The main difference was that the number of aliphatic CH_2 groups in **3** was four more than that in **2**, which was confirmed by HR-ESI-MS and 2D-NMR (*Fig. 2*). Base hydrolysis of **3**, followed by partition with AcOEt after neutralization, afforded chaulmoogric acid, of which the absolute configuration was determined to be (*R*) by comparison its optical-rotation value with literature data [16]. Actually, compound **3** has been previously synthesized [9]. However, as a new natural product, it was isolated from this species for the first time. As a result, the structure of **3** was deduced as 2,3-dihydroxypropyl 13-[(*R*)-cyclopent-2-en-1-yl]tridecanoate, with the trivial name anthelminthicin C.

The two known compounds were identified as chaulmoogric acid (**4**) [16] and ethyl chaulmoograte (**5**) [17] by comparison of their spectroscopic data with literature values. The optical rotation of compound **4** was determined to be $[\alpha]_D^{25.5} = +25.51$ ($c = 0.48$, $CHCl_3$), indicating that **4** is a partially mixture of isomers with the (1′*R*)-configuration dominating. The absolute configuration of **5** at C(1′) was assigned as (*R*) by measuring optical rotation for the hydrolysis product of **5** ($[\alpha]_D^{28.0} = +57.27$ ($c = 0.55$,

Table 1. *NMR Data for Compounds* **1**–**3**. δ in ppm, *J* in Hz.

Position	**1**[a])		**2**[b])		**3**[b])	
	δ(H)[c])	δ(C)	δ(H)[c])	δ(C)	δ(H)[d])	δ(C)
1		176.1		174.4		175.8
2	2.52 (*t*, *J* = 7.6)	34.9	2.35 (*t*, *J* = 7.6)	34.1	2.32 (*t*, *J* = 8.0)	35.6
3	1.79–1.81 (*m*)	25.7	1.60–1.64 (*m*)	24.9	1.60–1.61 (*m*)	26.3
4	1.19–1.26 (*m*)	29.7–29.8	1.25–1.28 (*m*)	29.1–29.4	1.24–1.28 (*m*)	29.4–31.3
5	1.19–1.26 (*m*)	29.7–29.8	1.25–1.28 (*m*)	29.1–29.4	1.24–1.28 (*m*)	29.4–31.3
6	1.19–1.26 (*m*)	29.7–29.8	1.25–1.28 (*m*)	29.1–29.4	1.24–1.28 (*m*)	29.4–31.3
7	1.19–1.26 (*m*)	29.7–29.8	1.25–1.28 (*m*)	29.1–29.4	1.24–1.28 (*m*)	29.4–31.3
8	1.19–1.26 (*m*)	29.7–29.8	1.25–1.28 (*m*)	29.1–29.4	1.24–1.28 (*m*)	29.4–31.3
9	1.40–1.41 (*m*)	27.2	2.27–2.29 (*m*)	32.0	1.24–1.28 (*m*)	29.4–31.3
10	2.18 (*t*, *J* = 7.6)	33.5			1.24–1.28 (*m*)	29.4–31.3
11		183.0			1.24–1.28 (*m*)	29.4–31.3
12					1.24–1.28 (*m*)	29.4–31.3
13					2.29, 2.23 (*m*)	33.4
1′		n.d.[e])	2.60–2.63 (*m*)	45.6	2.58–2.62 (*m*)	47.0
2′	6.03 (*d*, *J* = 1.2)	129.5	5.67 (*dd*, *J* = 5.6, 1.6)	135.4	5.65 (*dd*, *J* = 6.0, 1.5)	136.9
3′	2.35–2.36 (*m*)	31.6	5.70 (*dd*, *J* = 5.6, 2.0)	130.0	5.68 (*dd*, 6.0, 2.0)	131.4
4′	1.36–1.38 (*m*)	29.6	1.34–1.35 (*m*)	36.1	1.35–1.36 (*m*)	37.6
5′	2.33–2.35 (*m*)	35.6	2.01–2.03 (*m*)	29.8	2.01–2.03 (*m*)	31.3
1″			4.20 (*dd*, *J* = 11.6, 4.8), 4.15 (*dd*, *J* = 11.6, 6.0)	65.1	4.15 (*dd*, 11.5, 5.0), 4.11 (*dd*, 11.5, 6.0)	66.5
2″			3.93–3.94 (*m*)	70.2	3.89–3.90 (*m*)	71.7
3″			3.70 (*dd*, *J* = 11.2, 4.0), 3.60 (*dd*, *J* = 11.2, 5.6)	63.3	3.67 (*dd*, *J* = 11.5, 3.5), 3.56 (*dd*, *J* = 11.5, 6.0)	64.8

[a]) Recorded in (D_5)pyridine. [b]) Recorded in $CDCl_3$. [c]) Recorded at 400 MHz. [d]) Recorded at 500 MHz. [e]) n.d. = Not determined.

$CHCl_3$); [16]: $[\alpha]_D^{25.0} = +47.90$ ($c = 0.30$, $CHCl_3$)) [16]. Furthermore, compounds **4** and **5** were both isolated from this plant for the first time.

2. *Bioassay.* The seeds of *H. anthelminthica* have been used for the treatment of leprosy, acariasis, gonorrhea, and tuberculosis; therefore, all isolates from the seeds were evaluated for their anti-infectious potential. In this study, Bacille *Calmette–Guerin* (BCG) and *M. tuberculosis* were used in a whole cell screen assay, and the data were summarized in *Table 2*. The results showed that all isolates exhibit inhibitory activity towards BCG and *M. tuberculosis.* The *MIC* values of compounds **1**–**5** against *M. tuberculosis* were 5.54, 16.70, 4.38, 9.82, and 16.80 μM, respectively. In an antifungal assay, however, all the compounds did not show toxicity against *C. albicans* SC5314 at the concentration up to 100 μM (data not shown). These results not only confirmed the traditional use of this plant, but also provided potential leads for tuberculosis therapy.

Table 2. *Biological Evaluations of Compounds* **1**–**5** (*MIC* [μM])

	1	**2**	**3**	**4**	**5**	Positive control
BCG[a])	13.8	0.52	11.2	2.36	0.26	Hygromycin B: 0.68
M.TB[b])	5.54	16.7	4.38	9.82	16.8	Isoniazid: 2.04
*p*Aba[c])	>187.1	>168.2	11.3	>178.6	>162.3	Abyssomycin C: 8.3

[a]) BCG: Mycobacterial *bovis* BCG *Pasteur* expressing GFP assay. [b]) M.TB: *M. tuberculosis* H37Rv expressing GFP assay. [c]) *p*Aba: *para*-Aminobenzoic acid assay.

*p*Aba was synthesized *via* the shikimate biosynthesis pathway, which mediates the production of folic acid. The *p*Aba pathway is involved in plants, algae, bacteria, fungi, and parasites, but absent in mammals; therefore, inhibitors of the *p*Aba pathway are attractive for the development of selective antimicrobial agents. Nevertheless, natural products targeting this pathway are still rare; to the best of our knowledge, so far only abyssomycin C (*MIC* value of 8.3 μM) has been reported to be an inhibitor of *p*Aba biosynthesis [18]. We examined the activity of the isolates on *p*Aba biosynthesis, and found that compound **3** could inhibit this pathway with a *MIC* value of 11.3 μM, comparable to that of abyssomycin C, representing another example of *p*Aba inhibitor from nature.

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Experimental Part

General. Column chromatography (CC): silica gel (SiO_2, 200–300 mesh; *Qingdao Marine Chemical Inc.*, P. R. China), C_{18} reversed-phase (RP) silica gel (40–60 μM; *Daiso Co.*, Japan), and *Sephadex LH-20* (*Amersham Pharmacia*, Sweden). Semi-prep. HPLC: *Agilent 1100* liquid chromatography with a *Zorbax SB-C_{18}* column (9.4 × 250 mm, i.d.). Optical rotations: *Horiba SEPA-300* polarimeter. UV Spectra:

Shimadzu UV-2401PC spectrometer, λ_{max} in nm. IR Spectrum: *Tensor 27* spectrometer, with KBr pellets; in cm^{-1}. NMR Spectra: *Bruker AV-400* or *DRX-500* spectrometer, with TMS as internal standard. FAB-MS: *VG Autospec-3000* spectrometer. ESI-MS and HR-ESI-MS: *API QSTAR Pulsar 1* spectrometer.

Plant Material. The dried seeds of *H*. anthelminthica were purchased from *Yunnan Company of Materia Medica* (YCMM), Yunnan Province, P. R. China, in December 2007, and identified by Mr. *H. Y. Sun* at YCMM. A voucher specimen (CHYX-0321) was deposited with the State Key Laboratory of Photochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, P. R. China.

Extraction and Isolation. The air-dried and milled seeds of *H. anthelminthica* (26 kg) were extracted with 95% EtOH under reflux (4 × 30 l). The combined extracts were concentrated and suspended in H_2O, followed by successive partition with petroleum ether (PE; 4 × 3 l), AcOEt (4 × 3 l), and BuOH (4 × 3 l). After removal of the solvent, 120 g of the PE extract were subjected to CC (SiO_2, 10.5 × 60 cm, 200–300 mesh, 1.8 kg, gradient of PE/AcOEt): *Frs. A–D. Fr. B* (5 g) was separated on a C_{18} eluted with a gradient ^{i}PrOH/H_2O: *Frs. BI–BIII*. Compound **1** (48 mg) was readily obtained from *Fr. BI* (76 mg) as a white powder. *Fr. BIII* (1.6 g) was separated by CC (SiO_2; PE/Me_2CO 70 : 10): compounds **2** (47 mg), **3** (130 mg), and **5** (360 mg). *Fr. C* (4 g) was divided into *Frs. CI–CIV* by CC (SiO_2; PE/Me_2CO 70 : 10). *Fr. CII* (1.2 g) was further separated by CC (SiO_2; PE/AcOEt 70 : 30): **4** (350 mg).

11-(Cyclopent-1-en-1-yl)-11-oxoundecanoic Acid (**1**). White powder. R_f (SiO_2 GF_{254}; $CHCl_3$/MeOH 12 : 1) 0.78. IR (KBr): 3432, 2924, 2849, 1724, 1655, 1601, 1470, 1413, 1336, 1284, 1231, 1202, 1175, 980, 904. ^{1}H- (400 MHz) and ^{13}C-NMR (100 MHz): see *Table 1*. FAB-MS (neg.): 265 ($[M-H]^-$). HR-ESI-MS (neg.): 265.1796 ($[M-H]^-$, $C_{16}H_{25}O_3^-$; calc. 265.1803).

*2,3-Dihydroxypropyl 9-[(1*R*)-Cyclopent-2-en-1-yl]nonanoate* (**2**). Yellowish syrup. R_f (SiO_2 GF_{254}; PE/AcOEt 1 : 1) 0.36. $[\alpha]^{20}_D = +30.84$ ($c = 0.23$, $CHCl_3$). IR (KBr): 3421, 2924, 2853, 1738, 1462, 1383, 1178, 1118, 1054, 936, 870, 720. ^{1}H- (400 MHz) and ^{13}C-NMR (100 MHz): see *Table 1*. ESI-MS (pos.): 321 ($[M+Na]^+$). HR-ESI-MS (pos.): 321.2041 ($[M+Na]^+$, $C_{17}H_{30}NaO_4^+$; calc. 321.2041).

*2,3-Dihydroxypropyl 13-[(1*R*)-Cyclopent-2-en-1-yl]tridecanoate* (**3**). Yellowish syrup. R_f (SiO_2 GF_{254}; PE/AcOEt 1 : 1) 0.42. $[\alpha]^{20}_D = +32.55$ ($c = 1.37$, $CHCl_3$). IR (KBr): 3318, 2919, 1731, 1419, 1393, 1201, 1102, 1061, 1045, 991, 943, 914, 850, 829, 720. ^{1}H- (500 MHz) and ^{13}C-NMR (125 MHz): see *Table 1*. ESI-MS (pos.): 377 ($[M+Na]^+$). HR-ESI-MS (pos.): 377.2660 ($[M+Na]^+$, $C_{21}H_{38}NaO_4^+$; calc. 377.2667).

Acid Hydrolysis. Compound **3** (6 mg) or **5** (8 mg) was dissolved in anh. EtOH (3 ml) and 2M NaOH (8 ml), and then heated in a H_2O bath at 65° for 3 h. After cooling, the mixture was neutralized to a pH value of 1–2, and extracted with AcOEt, followed by *Sephadex LH-20* purification to yield chaulmoogric acid: 4.4 mg from **3** and 5.6 mg from **5**; their optical rotations were determined as $[\alpha]^{28.0}_D = +58.24$ ($c = 4.8$, $CHCl_3$) and $[\alpha]^{28.0}_D = +57.27$ ($c = 0.55$, $CHCl_3$), resp.

Assay for Anti-BCG and TB (H37Rv) *in Logarithmic Phase of Growth.* Bacille *Calmette–Guerin* (BCG) and *Myobacterium tuberculosis* (MTB) assays were carried out utilizing a constitutive Green Fluorescence Protein (GFP) expression vector direct readout of fluorescence as a measure of bacterial growth [19]. The production of GFP by replicating cells as a marker for live cells has been shown to result in the same *MIC* value as measured by direct enumeration of colony forming units (CFUs) for all the front-line antituberculosis agents [20]. MTB H37Rv and BCG *Pasteur* with a constitutive GFP plasmid (pSC301, kindly provided by Dr. *Yossef Av-Gay*, University of British Columbia, Canada) were used as test strains. All strains were precultured at 37° in Middlebrook 7 H9 broth (*Difco*) supplemented with 0.05% *Tween 80* for 14 d. Compounds were prepared at 100× stocks in DMSO. Serial dilutions of the compounds were prepared in the same solvent and added to the wells in a 2 µl volume. Hygromycin B (purchased from *Ameresco*, ultrapure grade) was used as a pos. control for BCG assay, and isoniazid (purchased from *Sigma*) for MTB assay. The assay was modified from that described by *L. Collins* and *S. G. Franzblau* using black, clear-bottom, 384-well microtiter plates. Forty µl of H37Rv-GFP bacterial suspension diluted to 2×10^6 CFU/ml (based on GFP fluorescence assessment and a reference curve) were then added to the diluted compound resulting in a final volume of 50 µl containing 1% DMSO. Plates were incubated at 37° for 7 d. Mycobacterial growth was determined by measuring GFP-fluorescence using an envision *2103* multilabel reader (*Perkin-Elmer Life Sciences*) with excitation at 485 nm and emission at 508 nm.

Assay for Inhibiting the Biosynthetic Pathway from Chorismate to pAba. Bacillus subtilis ATCC 66333 was grown in an agar plate using a MM minimal medium consisting of glucose, 0.5%; trisodium citrate·2 H_2O, 0.05%; KH_2PO_4, 0.3%; K_2HPO_4 0.7%; $MgSO_4 \cdot 7\ H_2O$, 0.01%; $(NH_4)_2SO_4$, 0.1% in deionized H_2O. The *B. subtilis* strain was also grown in the MP medium (MM medium supplemented with 5 mM *p*Aba). The assay was carried out in flat-bottom, 96-well microtiter plates (*Greiner*, Germany) according to the method described in [18] with some modification. Two µl of the compounds dissolved in DMSO were transferred to each well. The strains diluted by MM and MP medium with the final concentration of 1×10^5 CFU/ml were added to 80 µl medium. The hits showed a selective antibacterial activity against the test organism grown on MM medium, but no activity was observed on MP medium. Identified hits were picked and screened in dose–response tested in 8 twofold dilutions 90 µM to 0.7 µM. The assay plate was incubated at 37° overnight and then the OD value was determined at 595 nm using envision 2103 multilabel reader (*Perkin-Elmer Life Sciences*). Abyssomycin C (kind gift from Prof. *M. Goodfellow* at School of Biology, Newcastle University, and purified by HPLC) was used as a pos. control.

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