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# Trichomycins A and B: Antibacterial Triterpenes from the New Species *Tricholoma* sp. AU1

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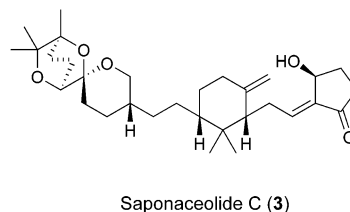
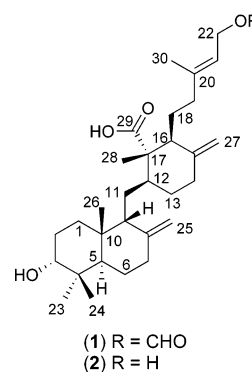
To new triterpenes, trichomycins A (**1**) and B (**2**), were purified from the new species *Tricholoma* sp. AU1 by activity-guided fractionation following their antibacterial activity. The two compounds were found to have a hitherto unreported triterpenoid skeleton. The structures and relative stereochemistry of **1** and **2** were determined through extensive 2D NMR spectroscopy, while the inhibitory activity of **1** and **2** against two Gram-positive and two Gram-negative bacteria and a mammalian cell line was determined.

Basidiomycetes of the genus *Tricholoma* occur ubiquitously, with several species highly valued due to their medicinal properties<sup>1</sup> or as culinary delicacies.<sup>2</sup> The genus is known to produce a multitude of different secondary metabolites derived from the polyketide,<sup>3</sup> fatty acid,<sup>4</sup> alkaloid,<sup>5</sup> and terpenoid<sup>6</sup> biosynthetic pathways.

In the course of a high-throughput screening (HTS) campaign of a library of natural product extracts to identify novel antibacterial agents, the crude MeOH extract of a *Tricholoma* species (Tricholomataceae), collected in North West Victoria, Australia, exhibited notable antibacterial activity. The source organism was subsequently identified as a new species of the genus *Tricholoma* and is referred to as *Tricholoma* sp. AU1. Bioassay-guided fractionation of the crude MeOH extract led to the isolation of two new triterpenes, trichomycins A (**1**) and B (**2**). Reported below in detail is the structure elucidation, determination of relative stereochemistry, and biological profiles of **1** and **2**.

Trichomycin A (**1**) was isolated as a colorless oil and yielded a molecular ion in the positive HRESIMS spectrum at  $m/z$  523.3389 ( $[M + Na]^+$ ), indicative of the molecular formula  $C_{31}H_{48}O_5$  ( $\Delta +1.1$  mmu) and equating to eight double-bond equivalents. Immediately identifiable from the NMR data for **1** (Table 1) were resonances consistent with one trisubstituted double bond [ $^{13}C$ : 144.6, 120.0 ppm;  $^1H$ :  $\delta$  5.30], as well as two 1,1-disubstituted double bonds [ $^{13}C$ : 152.3, 114.6, 110.1, 108.6 ppm;  $^1H$ :  $\delta$  4.91, 4.63 ( $\times 3$ )], a carboxy [ $^{13}C$ : 181.6 ppm; IR: 1720  $cm^{-1}$ ], and a formate carbonyl [ $^{13}C$ : 163.4 ppm;  $^1H$ :  $\delta$  8.07; IR: 1699  $cm^{-1}$ ]. In the absence of any other  $sp$  or  $sp^2$  carbons, the gross structure of **1** must be tricyclic. Also evident were resonances consistent with one olefinic [ $^{13}C$ : 16.8 ppm;  $^1H$ :  $\delta$  1.69] and four tertiary methyls [ $^{13}C$ : 29.5, 23.5, 23.4, 11.4 ppm;  $^1H$ :  $\delta$  0.92, 0.90, 0.83, 0.80], a hydroxy [ $^{13}C$ : 77.7 ppm;  $^1H$ :  $\delta$  3.35], and an oxy methine [ $^{13}C$ : 61.9 ppm;  $^1H$ :  $\delta$  4.65], as well as nine methylenes, five methines, and three quaternary carbons (Table 1).

Interpretation of the  $^1H$ – $^1H$  COSY and gHMBC data readily identified the three partial structures for **1** as shown in Figure 1. The observation of a gHMBC correlation



from H-16 to C-19 allowed for the connection of partial structures A and B, while additional gHMBC correlations from H-12 to C-9 and C-11, and from H<sub>b</sub>-11 to C-8, C-9, C-10, C-12, and C-13, allowed for the connection of partial structures B and C. This accounted for all atoms except for one exchangeable proton. The presence of a carboxylic acid accounted for the remaining proton and hence the gross structure of **1** as shown.

The relative stereochemistry of **1** was determined through 2D NOESY analysis and proton coupling constants. The observation of a NOESY correlation from H<sub>2</sub>-22 to H<sub>3</sub>-30 established an *E* configuration for  $\Delta^{20,21}$ , while NOESY correlations from H-3 to H<sub>3</sub>-23 and H<sub>3</sub>-24 suggested that H-3 was equatorial. Further NOESY correlations from H<sub>3</sub>-24 to H<sub>a</sub>-2, H<sub>a</sub>-6, and H<sub>3</sub>-26, and from H<sub>3</sub>-26 to H<sub>a</sub>-2, H<sub>a</sub>-6, and H-9, placed H-9, H<sub>3</sub>-24, and H<sub>3</sub>-26 on the same face of the molecule. Similarly, a NOESY correlation from H-5 to H<sub>3</sub>-23 placed H-5 on the opposite face of the molecule from H<sub>3</sub>-26, suggesting an axial orientation for H-5 and hence a trans ring junction. These observations allowed for the determination of the relative stereochemistry of the lab-dane portion of **1** as shown in Figure 2. For the cyclohexane

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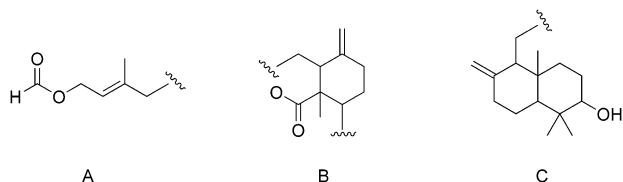
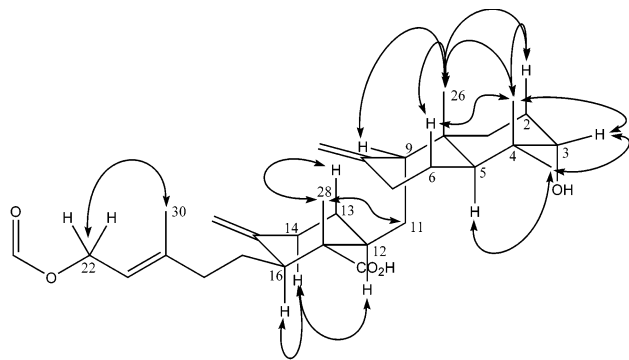
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**Table 1.** One- and Two-Dimensional NMR Data for Trichomycin A (1)

no.	$^{13}\text{C}$ ( $\delta$ , m)	$^1\text{H}$ [ $\delta$ , m, $J$ (Hz)]	COSY	gHMBC $^1\text{H}$ to $^{13}\text{C}$	NOESY
1	30.5 (t)	2.04 (m)	H <sub>a</sub> -2		
2	27.2 (t)	1.93 (m)	H <sub>2</sub> -1		H <sub>3</sub> -24, H <sub>3</sub> -26
		1.56 (m)	H-3		
3	77.7 (d)	3.35 (br s)	H <sub>b</sub> -2	C-1, C-2, C-4, C-5, C-23, C-24	H <sub>3</sub> -23, H <sub>3</sub> -24
4	38.6 (s)				
5	41.1 (d)	1.64 (m)	H <sub>b</sub> -6		H <sub>3</sub> -23
6	24.9 (t)	1.54 (m)	H-7	C-8	H <sub>3</sub> -24, H <sub>3</sub> -26
		1.34 (ddd, 12.4, 5.6, 5.2)	H-5, H-7		
7	33.6 (t)	2.10 (m)	H <sub>2</sub> -6	C-8, C-25	
8	152.3 (s)				
9	60.5 (d)	1.55 (m)	H <sub>b</sub> -11	C-8, C-25	H <sub>3</sub> -26
10	39.7 (s)				
11	30.9 (t)	1.64 (m)	H <sub>b</sub> -11		H <sub>3</sub> -28
		1.00 (dt, 14.2, 8.3)	H-9, H <sub>a</sub> -11	C-8, C-9, C-10, C-12, C-13	
12	46.9 (d)	1.90 (m)	H <sub>b</sub> -13	C-9, C-11, C-16, C-28	H <sub>a</sub> -14
13	32.4 (t)	1.72 (m)	H <sub>b</sub> -13		H <sub>3</sub> -28
		1.12 (m)	H-12, H <sub>a</sub> -13, H <sub>a</sub> -14		
14	38.9 (t)	2.26 (m)	H <sub>b</sub> -13, H <sub>b</sub> -14	C-13, C-15, C-16, C-27	H-12, H-16
		1.94 (m)	H <sub>a</sub> -14	C-13, C-15, C-16, C-27	
15	148.6 (s)				
16	50.7 (d)	2.37 (d, 10.8)	H <sub>a</sub> -18	C-12, C-14, C-15, C-17, C-18, C-19, C-27, C-28, C-29	H <sub>a</sub> -14
17	55.7 (s)				
18	27.4 (t)	1.64 (m)	H-16, H <sub>a</sub> -19		
		1.18 (m)	H <sub>a</sub> -19, H <sub>b</sub> -19		
19	39.6 (t)	2.13 (m)	H <sub>a</sub> -18, H <sub>b</sub> -18		
		1.93 (m)	H <sub>b</sub> -18		
20	144.6 (s)				
21	120.0 (d)	5.30 (t, 6.8)	H <sub>3</sub> -20, H-22	C-19, C-22, C-30	
22	61.9 (t)	4.65 (d, 6.8)	H-21	C-20, C-31	H <sub>3</sub> -30
23	29.5 (q)	0.92 (s)		C-3, C-4, C-5, C-24	H-5, H-3
24	23.5 (q)	0.80 (s)		C-3, C-4, C-5, C-23	H <sub>a</sub> -2, H-3, H <sub>a</sub> -6, H <sub>3</sub> -26
25	110.1 (t)	4.63 (br s)		C-8, C-9	
26	23.4 (q)	0.90 (s)		C-1, C-5, C-9, C-10	H <sub>a</sub> -2, H <sub>a</sub> -6, H-9, H <sub>3</sub> -24
27	108.6 (t)	4.91 (br s)	H <sub>b</sub> -27	C-14, C-15, C-16	
		4.63 (br s)	H <sub>a</sub> -27	C-14, C-15, C-16	
28	11.4 (q)	0.83 (s)		C-12, C-16, C-17, C-29	H <sub>2</sub> -11, H <sub>a</sub> -13
29	181.6 (s)				
30	16.8 (q)	1.69 (s)	H-21	C-19, C-20, C-21	H <sub>2</sub> -22
31	163.4 (d)	8.07 (s)		C-22	

**Figure 1.** Generated partial structures for trichomycin A (1).**Figure 2.** Observed NOESY correlation for trichomycin A (1). For clarity only protons where NOESY correlations were observed are shown.

moiety of **1**, the observation of NOESY correlations from H<sub>a</sub>-14 to H-12 and H-16 place them on the same face of the molecule. In addition, the large coupling constant for H-16 ( $J = 10.8$  Hz) suggested that these protons were all

axial (Figure 2). Finally, NOESY correlations from H<sub>3</sub>-28 to H<sub>a</sub>-13 and H<sub>2</sub>-11 were suggestive of H<sub>3</sub>-28 and H<sub>a</sub>-13 axial, and H<sub>2</sub>-11 equatorial (Figure 2). Hence the relative stereochemistry of **1** is proposed as shown. The absolute stereochemistry of **1** remains unassigned at this time.

Trichomycin B (**2**) was isolated and gave a molecular ion at  $m/z$  471.3472 in the negative HRESIMS ( $[\text{M} - \text{H}]^-$ ) spectrum, indicative of the molecular formula  $\text{C}_{30}\text{H}_{48}\text{O}_4$  ( $\Delta +0.2$  mmu), 28 amu less than the molecular ion measured for trichomycin A (**1**) and equating to seven double-bond equivalents. Analysis of the NMR data for **2** showed that it was remarkably similar to that for **1**, with the exception of the  $^{13}\text{C}$  NMR data of C-20 [**2** (139.4 ppm) vs **1** (144.6 ppm)] and C-21 [**2** (124.5 ppm) vs **1** (120.0 ppm)], the  $^{13}\text{C}$  and  $^1\text{H}$  NMR data for C-22 [**2** ( $^{13}\text{C}$ : 59.0 ppm;  $^1\text{H}$ :  $\delta$  4.05) vs **1** ( $^{13}\text{C}$ : 61.9 ppm;  $^1\text{H}$ :  $\delta$  4.65)], and the absence of a formate proton at  $\sim\delta$  8.00. Further analysis of the  $^1\text{H}$ - $^1\text{H}$  COSY and gHMBC showed that indeed the only difference between **2** and **1** was the absence of the formate group. Hence the gross structure for **2** is as shown. The proposed relative stereochemistry of **2** has been assigned the same as **1** on the basis of  $^{13}\text{C}$  chemical shifts. Interestingly, when **2** was first purified, it had a molecular weight 28 amu higher than **1**, as well as an additional formate resonance in the  $^1\text{H}$  NMR spectrum. It appears that over time, compound **2** has undergone a di-deformylation. It is possible that the formate substitution observed in **1** and initially present in **2** are artifacts of the isolation process, as formic acid was used as a solvent modifier. The pos-

**Table 2.** Antibacterial and Cytotoxic Activities for Trichomycins A (1) and B (2) against *K. pneumoniae*, *P. aeruginosa*, *S. aureus*, *S. pneumoniae*, and THP-1 Cells (IC<sub>50</sub>'s in  $\mu$ M)

	<i>K. pneumoniae</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>S. pneumoniae</i>	THP-1
1	>500	>500	12	5	>1000
2	>473	>473	12	6	640

sibility of artifact formation could not be addressed any further, because no further dried fungal material was available to repeat the isolation under acid-free conditions.

There have been 12 reports<sup>6–17</sup> in the literature concerning the isolation of terpenes from species of *Tricholoma*, four of which describe the isolation of steroids,<sup>6,8–10</sup> and a further four describing the isolation of diterpenes.<sup>7,11–13</sup> Furthermore, an additional four publications concern the unusual oxygenated triterpenes saponaceolides A (3), B, C, and D, which were reported from *T. saponaceum*<sup>14–16</sup> and *T. terreum*.<sup>17</sup> These compounds show some resemblance to trichomycins A (1) and B (2) by sharing similar partial structures A and B (Figure 2). To the best of our knowledge, this is the first report of ring-opened triterpenes from a *Tricholoma* species. Furthermore, to the best of our knowledge, the triterpene skeleton as exemplified by 1 and 2 has not previously been reported.

The antibacterial activity for 1 and 2 was determined against two Gram-positive and two Gram-negative bacterial strains. In addition, cytotoxicity and selective antibacterial activity was assessed by exposure to a human leukemic cell line (THP-1). The results are summarized in Table 2. Both 1 and 2 were found to be selective against Gram-positive bacteria (*Staphylococcus aureus* and *Streptococcus pneumoniae*), with no activity at the highest concentration detected against Gram-negative bacteria (*Klebsiella pneumoniae* and *Pseudomonas aeruginosa*). Very weak activity at the highest concentration was noted for 2 against the mammalian cell line (THP-1), indicating a selectivity index toward the antibacterial activity of almost 100-fold.

## Experimental Section

**General Experimental Procedures.** SPE was performed using Varian Megabond Elute C18 SPE cartridges (10 g, 50  $\mu$ m). HPLC was performed on either a Waters Delta Prep 4000 chromatography system equipped with a Waters 2487 dual wavelength UV detector, a Waters prep LC system controller and a Waters fraction collector, or a system equipped with a Waters 600 controller, a Waters 996 photodiode array detector, a Waters 717 plus autosampler, and a Waters fraction collector II. All data generated from these chromatographic systems were collected using Waters Millennium<sup>32</sup> data collection package.

All NMR spectra were collected on a Varian Unity Inova 400 MHz spectrometer in the solvents indicated, with spectra referenced to residual <sup>1</sup>H in the deuterated NMR solvents.

Optical rotations were performed on a Jasco Dip-1000 digital polarimeter, while infrared spectra were acquired on a Bio-Rad FTS-165 Fourier transform infrared spectrometer.

Low-resolution mass spectral data were collected on a ThermoFinnigan LCQ ion trap mass spectrometer, with an ESI probe. High-resolution mass measurements were collected on a Bruker BioApex FT mass spectrometer.

**Fungal Material.** Fruiting bodies of *Tricholoma* sp. AU1 (*Tricholomataceae*) were collected in a river flood plain near the Wimmera River at the border of the Little Desert National Park, 2.1 km south of Horseshoe Bend camping ground (36°30.86' S, 142°01.58' E), in *Eucalyptus microcarpa* woodland, Victoria, in June 1998. Description: Fruit-body robust, lamellate, growing on soil. Pileus large, to 9 cm diameter, white with slight yellow stain. Stipe central, white, without annulus.

Spores 5–6  $\times$  3–4  $\mu$ m, broadly ellipsoid, hyaline, smooth, inamyloid. Pileipellis composed of repent, cylindrical, hyaline hyphae, looser at surface. Clamp connections absent. All characteristics are typical for *Tricholoma*, but do not match those of known Australian species. A voucher specimen (MEL2049115) was deposited with the National Herbarium of Victoria, Australia.

**Extraction and Isolation.** Dried and ground fungal material (10 g) was extracted twice with MeOH (500 mL) over 36 h. The combined MeOH was concentrated in vacuo and subjected to C18 SPE (10% gradient elution from 20% MeOH/H<sub>2</sub>O to 80% MeOH/H<sub>2</sub>O, and a flush with 100% MeOH), generating eight fractions, with activity localized in the 80% MeOH/H<sub>2</sub>O fraction. This fraction was further purified on C18 preparative HPLC [16 mL/min, gradient elution from 3:7 (MeCN/H<sub>2</sub>O + 0.1% formic acid) to 9:1 (MeCN/H<sub>2</sub>O + 0.1% formic acid) over 25 min through a Varian C18 250  $\times$  50 mm, 5  $\mu$ m, preparative HPLC axial compression column] and C18 semipreparative HPLC [4 mL/min, gradient elution from 1:1 (MeCN/H<sub>2</sub>O + 0.1% formic acid) to MeCN (+0.1% formic acid) over 18 min through a Waters C18 Xterra 100  $\times$  7.8 mm, 5  $\mu$ m, HPLC column] to yield trichomycins A (1) (5.4 mg, 0.054%) and B (2) (0.4 mg, 0.004%) as the compounds responsible for the activity of the extract.

**Trichomycin A (1):** colorless oil; [ $\alpha$ ]<sub>D</sub> 16.6° (c 0.27, CHCl<sub>3</sub>); IR (film)  $\nu_{\max}$  3406, 2937, 2879, 1720, 1699 cm<sup>-1</sup>; <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD), see Table 1; <sup>13</sup>C NMR data (100 MHz, CD<sub>3</sub>OD), see Table 1; HRESIMS *m/z* 523.3389 (calcd for C<sub>31</sub>H<sub>48</sub>O<sub>5</sub>-Na, 523.3400).

**Trichomycin B (2):** colorless oil; [ $\alpha$ ]<sub>D</sub> 69.5° (c 0.04, CHCl<sub>3</sub>); IR (film)  $\nu_{\max}$  3406, 2931, 2874, 1720 cm<sup>-1</sup>; <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD)  $\delta$  5.29 (1H, t, *J* = 6.6 Hz, H-21), 4.90 (1H, br s, H<sub>a</sub>-27), 4.64 (1H, br s, H<sub>b</sub>-25), 4.63 (1H, br s, H<sub>a</sub>-25), 4.60 (1H, br s, H<sub>b</sub>-27), 4.05 (2H, d, *J* = 6.6 Hz, H<sub>2</sub>-22), 3.34 (1H, br s, H-3), 2.37 (1H, d, *J* = 10.5 Hz, H-16), 2.25 (1H, d, *J* = 12.1 Hz, H<sub>a</sub>-19), 2.15 (2H, m, H<sub>2</sub>-7), 2.13 (1H, m, H<sub>a</sub>-14), 2.04 (2H, m, H<sub>2</sub>-1), 1.94 (1H, m, H<sub>b</sub>-19), 1.93 (1H, m, H<sub>a</sub>-2), 1.90 (1H, m, H<sub>b</sub>-14), 1.88 (1H, m, H-12), 1.75 (1H, m, H<sub>a</sub>-13), 1.66 (1H, m, H<sub>a</sub>-11), 1.65 (1H, m, H-5), 1.64 (1H, m, H<sub>a</sub>-18), 1.63 (3H, s, H<sub>3</sub>-30), 1.56 (2H, m, H<sub>b</sub>-2, H<sub>a</sub>-6), 1.55 (1H, m, H-9), 1.25 (1H, m, H<sub>b</sub>-6), 1.13 (2H, m, H<sub>b</sub>-13, H<sub>b</sub>-18), 1.00 (1H, m, H<sub>b</sub>-11), 0.92 (3H, s, H<sub>3</sub>-23), 0.90 (3H, s, H<sub>3</sub>-26), 0.82 (3H, s, H<sub>3</sub>-28), 0.80 (3H, s, H<sub>3</sub>-24); <sup>13</sup>C NMR (100 MHz, CD<sub>3</sub>OD)  $\delta$  181.1 (s, C-29), 151.4 (s, C-8), 147.9 (s, C-15), 139.4 (s, C-20), 124.5 (d, C-21), 109.4 (t, C-25), 107.9 (t, C-27), 76.9 (d, C-3), 59.7 (d, C-9), 59.0 (t, C-22), 55.1 (s, C-17), 50.2 (d, C-16), 46.4 (d, C-12), 41.4 (s, C-10), 40.0 (d, C-5), 39.1 (s, C-4), 38.6 (t, C-14), 37.8 (t, C-19), 32.8 (t, C-7), 31.6 (t, C-13), 29.8 (t, C-11), 29.6 (t, C-1), 28.8 (t, C-2), 28.6 (q, C-23), 26.9 (t, C-18), 26.5 (t, C-6), 22.5 (q, C-24), 22.4 (q, C-26), 15.9 (q, C-30), 10.8 (q, C-28); HRESIMS *m/z* 471.3472 (calcd for C<sub>30</sub>H<sub>47</sub>O<sub>4</sub>, 471.3474).

**Antibacterial Assays.** Strains of *K. pneumoniae*, *P. aeruginosa*, *S. aureus*, and *S. pneumoniae* were initially grown on Mueller-Hinton agar, then subcultured into Mueller-Hinton broth supplemented with CaCl<sub>2</sub>/MgCl<sub>2</sub> and grown at 37 °C for 18 h. Serial dilutions were made (*K. pneumoniae* and *P. aeruginosa* diluted to 10<sup>-6</sup> cfu/mL, *S. aureus* to 10<sup>-8</sup> cfu/mL, and *S. pneumoniae* to 10<sup>-4</sup> cfu/mL) and all broths left to equilibrate for 30 min at 37 °C. Extracts were added to a 96-well microtiter plate, and 200  $\mu$ L of each culture was dispensed into each well. Negative control wells contained the respective bacterial strain without inhibitors, while positive control wells contained bacterial strains with 50  $\mu$ g/mL of streptomycin. The plates were incubated and shaken at 37 °C and 60% humidity for 18 h, after which optical density at 650 nm was measured. Test wells showing little or no growth/turbidity were indicative of antibacterial activity.

**Mammalian Cytotoxicity Assay.** Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 5 units/mL penicillin, 5 mg/mL streptomycin, and 10  $\mu$ M 2-mercaptoethanol at 37 °C, 5% CO<sub>2</sub>. The assay was conducted in a 96-well microtiter plate at a cell density of 2.5  $\times$  10<sup>4</sup> cells per well, in a total volume of 200  $\mu$ L. Natural product samples in each well were tested at 250  $\mu$ g/mL final concentration. Positive control wells contained 10  $\mu$ M camp-

tothecin, while negative control wells contained 0.1% DMSO. Plates were incubated for 72 h at 37 °C, 5% CO<sub>2</sub>, prior to the addition of WST-1 reagent (20 µL) and further incubated for 1–2 h. Absorbance at 450 nm was measured and activity compared to positive controls.

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