Phomoxanthones A and B, Novel Xanthone Dimers from the Endophytic Fungus *Phomopsis* Species

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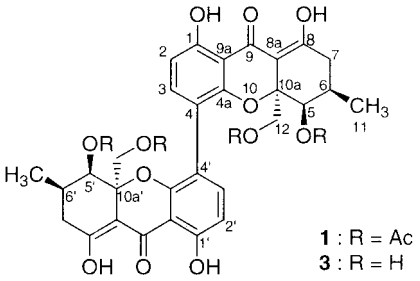
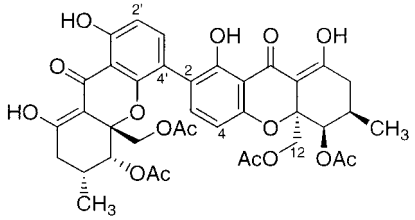
Phomoxanthones A (**1**) and B (**2**), two novel xanthone dimers, were isolated from the endophytic fungus *Phomopsis* sp. BCC 1323. Structures of **1** and **2** were elucidated by spectroscopic methods. These compounds exhibited significant in vitro antimalarial and antitubercular activities and cytotoxicity.

The genus *Phomopsis* has been known to be a rich source of bioactive secondary metabolites of diverse structures, including, for example, phomopsichalasin[1] and several other cytochalasins[2] from a *Phomopsis* spp., phomopsin A (a hexapeptide mycotoxin)[3] from *P. leptostromiformis*, convolvulanic acids and other phytotoxic isobenzofuranones[4] from *P. convolvulus*, and oblongolide and phomopsolides (phytotoxic sesquiterpene γ-lactones)[5,6] from *P. oblogna*.

In an ongoing effort to search for bioactive fungal metabolites, we discovered antimalarial activity in the extract from the fungus *Phomopsis* sp. BCC 1323, a teak endophyte collected from northern Thailand and deposited at the BIOTEC Culture Collection. Due to our interest in the biological activity as well as the diverse metabolite production of this genus, an investigation of the chemical constituents of this strain was undertaken. Activity-guided fractionation of the crude extract led to the isolation and identification of two novel xanthone dimers, **1** and **2**, which are named phomoxanthones A and B, respectively. We report herein the isolation, structure elucidation, and biological activities of these compounds.





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Results and Discussion

Phomoxanthones A (**1**) and B (**2**) were isolated as yellow powders from the MeOH extract of mycelia from flask cultures of *Phomopsis* sp. BCC 1323. Phomoxanthone A, the major metabolite, has a molecular formula of $C_{38}H_{38}O_{16}$, as established by HRMS. The presence of only 19 carbon signals in the ^{13}C NMR spectrum indicated the symmetric, homodimer structure of this compound. Analyses of ^{1}H and ^{13}C NMR, DEPTs, COSY, and HMQC spectra revealed that half of the molecule, $C_{19}H_{19}O_6$, possesses one conjugated ketone carbonyl (δ_C 187.7 ppm), two acetoxy groups, and two downfield signals for hydroxyl protons at δ_H 11.52 and 14.09 ppm. The IR spectrum of phomonone A supported the presence of the carbonyl groups, showing strong absorptions at ν 1744 and 1615 cm^{-1}. Analysis of HMBC correlations suggested the presence of a tetrahydroxanthone unit as shown in Figure 1. Although no long-range correlation to the ketone carbonyl carbon at δ_C 187.7 ppm was observed, the formula of $C_{19}H_{19}O_8$ necessitated the ketone linkage of the two terminal rings to construct a tetrahydroxanthone ring. The keto−enol structure is consistent with the low-field chemical shift of the corresponding proton at δ_H 14.09 ppm. The relative configuration at C-6, C-5, and C-10a depicted in **1** was deduced from ^{1}H NMR and NOESY spectral data. The ring fusion necessitates the pseudoaxial orientation of the acetoxymethyl group adjacent to C-10a. In the NOESY spectrum of phomoxanthone A, a correlation between one of the C-12 methylene protons at δ_H 4.17 ppm and H-6 was observed. This supported the presence of an acetoxymethyl group and H-6 on the same side of the six-membered ring and the pseudoaxial orientation of H-6 in the pseudochair conformation. In the ^{1}H NMR spectrum, H-5 appears as a sharp singlet, which rules out the pseudoaxial orientation of H-5 (no large $J_{5,6}$ value). NOESY correlation of H-5 with both H-6 and methyl protons (H-11) supported the pseudoequatorial orientation of H-5. Thus, the acetoxy group on C-5 must occupy the pseudoaxial position, and, consequently, the methyl group on C-6 should be placed in the pseudoequatorial position. Unfortunately, detailed NMR analysis of the relationship between the three protons, H-6 and

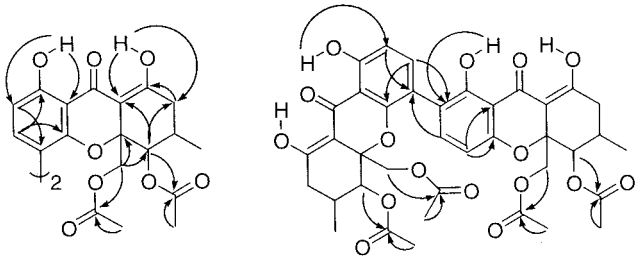

Figure 1. Selected HMBC correlations for **1** and **2**.

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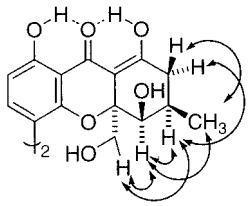

Figure 2. NOESY correlations of **3**.

methylene protons (H-7), could not be achieved due to the close chemical shifts of these protons.

The proposed relative stereochemistry of **1** was confirmed by NMR analyses of the deacetyl derivative **3**, which was obtained by treatment of **1** with concentrated H_2SO_4/MeOH. In the ^{1}H NMR spectrum of **3**, the signals for H-6 and the methylene protons (H-7) do not overlap, appearing at δ_H 2.14 as a multiplet and 2.48 (dd, J = 19.2, 11.4 Hz) and 2.35 (dd, J = 19.2, 6.1 Hz) ppm, respectively. Due to the large vicinal coupling constant of $J_{6,7}$ = 11.4 Hz, the δ_H 2.48 signal was assigned as the pseudoaxial H-7, and the δ_H 2.35 signal ($J_{6,7}$ = 6.1 Hz) as the pseudoequatorial H-7. Thus, based on the $J_{6,7}$-coupling, H-6 must occupy the pseudoaxial orientation. NOESY correlations between H-6 and one of the H-12 methylene signals at δ_H 3.42 indicate that the hydroxymethyl group on C-10a and H-6 are on the same side of the six-membered ring (Figure 2). The NOESY correlation between the δ_H 3.42 signal (H-12) and H-5 was consistent with the established relative configuration, where the hydroxymethyl group and H-5 are on the same side of the pseudochair cyclohexene ring, hence, the pseudoequatorial orientation of H-5.

The symmetry observed in the ^{1}H and ^{13}C spectra of phomoxanthone A indicated that this molecule is a homodimer with the xanthone ring, having identical absolute configurations. Due to the fact that phomoxanthone A was isolated from the culture broth of the strain BCC 1323 as a single stereoisomer of the C-4−C-4′ dimer, the absolute configuration of this compound must be either 5*R*,6*R*,10a*R*,5′*R*,6′*R*,10a′*R* or 5*S*,6*S*,10a*S*,5′*S*,6′*S*,10a′*S*.

Phomoxanthone B (**2**) has the same molecular formula as phomoxanthone A, $C_{38}H_{38}O_{16}$, as determined by HRMS. However, phomoxanthone B is not symmetrical and shows 38 peaks in its ^{13}C NMR spectrum. The IR and UV spectra of phomoxanthone B were similar to those of phomoxanthone A. Analyses of 1D and 2D NMR spectra, particularly HMBC, revealed that this compound is also a dimer of the same xanthone unit as in **1**, but with the dimer formation between C-2 and C-4′, as determined from the long-range C−H correlations of H-3 to C-4′ (q) and H-3′ to C-2 (q). The signal for the methyl proton of one of the acetoxy groups, on either C-12 or C-12′, was shifted upfield (δ_H 1.79 ppm, 3H, s), while the other resonates at a typical chemical shift (δ_H 2.11). This upfield shift should be due to the shielding effect by the aromatic ring of the other half of the dimer. Thus, the δ_H 1.79 signal was assigned to the acetoxy group on C-12′, and the signal at δ_H 2.11 to the acetoxy group on C-12. The NMR assignments of protons and carbons of phomoxanthone B were established on the basis of the connectivity network achieved by the analyses of COSY, NOESY, HMQC, and HMBC correlations (Table 1). Also, on the basis of the analysis of J values and NOESY correlations in the same manner as described for compounds **1** and **3**, phomoxanthone B was proposed to possess either the 5*R*,6*R*,10a*R*,5′*R*,6′*R*,10a′*R* configuration shown as **2** or its mirror image.

The structures of phomoxanthones are related to secalonic acids, also referred to as ergochromes,[7,8] which are C-2−C-2′ dimers of similar tetrahydroxanthones. Seven isomers of secalonic acids, A−G, with different configurations at C-5, C-6, C-10a, C-5′, C-6′, and C-10a′, have been isolated from several fungi such as *Claviceps purpurea*,[9] *Aspergillus ochraceus*,[10] *A. aculeatus*,[11] *Pyrenochaeta terrestris*,[12] and *Penicillium oxalicum*.[13] Interestingly, secalonic acids A−D were initially reported as C-4−C-4′ dimers, but later revised to the correct C-2−C-2′ linkage.[14,15] Secalonic acid A isomers having C-2−C-4′ and C-4−C-4′ linkage have previously been prepared,[16] but phomoxanthones A and B are the first examples of naturally occurring C-4−C-4′ and C-2−C-4′ tetrahydroxanthone dimers of this emodin-derived xanthone. It should also be pointed out that the phomoxanthones bear acetoxymethyl substituents on C-10a and C-10a′, while all the secalonic acids are substituted with methoxycarbonyl groups on these positions.

Phomoxanthones A (**1**) and B (**2**) exhibited significant activity against *Plasmodium falciparum* (K1, multi drug-resistant strain) and against *Mycobacterium tuberculosis* (H37Ra strain), although weaker than standard drugs (Table 2). However, these compounds are also cytotoxic to two cancer cell lines (KB, BC-1) and to Vero cells. It is interesting to note that compound **3**, the deacetyl analogue of **1**, was inactive in all the assays. This may due to the low lipophilicity of **3** compared to those of **1** and **2**. The related secalonic acids are reported to exhibit various biological activities such as cytostatic activity (mouse leukemia L1210 cells),[17] phlogistic activity,[18] inhibition of protein kinase C and cyclic AMP-dependent protein kinase,[19] and toxicity to mice.[20]

Experimental Section

General Experimental Procedures. Melting points were measured with a Electrothermal IA9100 digital melting point apparatus and are uncorrected. Optical rotations were measured in $CHCl_3$ with a JASCO DIP-370 digital polarimeter. UV spectra were recorded in MeOH on a Varian CARY 1E UV−visible spectrophotometer. FT-IR spectra were taken as KBr pellets on a Perkin-Elmer system 2000 spectrometer. ESI (electron spray ionization)−TOF mass spectra were measured with a Micromass LCT mass spectrometer. ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz), DEPTs, and 2D NMR spectra (COSY, NOESY, HMQC, and HMBC) were taken in $CDCl_3$ on a Bruker DRX400 spectrometer.

Fungal Material. The endophyte, *Phomopsis* sp. BCC 1323, was isolated from a teak leaf, *Tectona grandis* L., at Mee Rim district, Chaingmai Province, Northern Thailand, by Dr. Sureewan Mekkamol. The strain is deposited at the BIOTEC Culture Collection as BCC 1323.

Production and Isolation of Phomoxanthones. An isolated culture of the strain BCC 1323 was grown on potato dextrose agar (PDA) at 22 °C for 5 days, before inoculation into 20 × 1 L Erlenmeyer flasks each containing 250 mL of bacto-malt extract broth (MEB). The cultures were incubated at 22 °C for 20 days. The mycelia were extracted with MeOH (1 L, 2 days), and to the extract was added H_2O (100 mL). The mixture was then washed with hexane (800 mL). The aqueous MeOH solution was dried under reduced pressure, and the residual oil was dissolved in EtOAc (1 L). The EtOAc solution was washed with H_2O (500 mL) and concentrated to obtain a brown solid (0.34 g). The crude solid was passed through a Sephadex LH-20 chromatography column (3.5 × 25 cm) with MeOH as eluent. A yellow pigment was eluted after ca. 200 mL elution with MeOH. This process was repeated twice to yield a yellow powder (116 mg), which after triturating twice with MeOH (5 mL, room temperature, 12 h) gave a pure compound **1** (64 mg). The combined filtrate, containing a mixture of **1** and **2**, was subjected to separation by repeated preparative HPLC using a reversed-phase column (NovaPak

Table 1. ^{13}C and ^{1}H NMR Data for Compounds **1** and **2**

	phomoxanthone A (**1**)		phomoxanthone B (**2**)	
position	δ_C (mult.)	δ_H (mult., *J* in Hz)	δ_C (mult.)	δ_H (mult., *J* in Hz)
1	161.6 (s)		159.8 (s)	
2	109.5 (d)	6.58 (d, 8.8)	118.4 (s)	
3	141.1 (d)	7.38 (d, 8.8)	139.2 (d)	7.18 (d, 8.3)
4	115.3 (s)		107.6 (d)	6.43 (d, 8.0)
4a	153.8 (s)		157.1 (s)	
5	70.3 (d)	5.40 (s)	70.2 (d)	5.53 (s)
6	27.5 (d)	2.37 (m)	27.7 (d)	2.40 (m)
7	33.2 (t)	2.46 (m), 2.33 (m)	33.2 (t)	2.45−2.37 (m)
8	177.6 (s)		177.7 (s)	
8a	100.1 (s)		100.8 (s)	
9	187.7 (s)		188.0 (s)[b]	
9a	106.2 (s)		106.3 (s)[c]	
10a	80.3 (s)		80.5 (s)	
11	17.5 (q)	1.01 (d, 5.8)	17.6 (q)	1.06 (d, 5.4)
12	64.5 (t)	4.28 (d, 12.8), 4.17 (d, 12.8)	64.4 (t)	4.55 (d, 13.2), 4.30 (d, 13.1)
5-O*C*(O)CH_3	169.6 (s)		170.5 (s)	
5-OC(O)*C*H_3	20.6 (q)	2.07 (s)	20.9 (q)[d]	2.10 (s)[e]
12-O*C*(O)CH_3	170.0 (s)		170.3 (s)	
12-OC(O)*C*H_3	20.4 (q)	1.89 (s)	20.7 (q)[d]	2.11 (s)[e]
1-O*H*		11.52 (s)		11.61 (s)
8-O*H*		14.09 (s)		14.08 (s)
1′			161.6 (s)	
2′			109.9 (d)	6.57 (d, 8.5)
3′			139.4 (d)	7.32 (d, 8.5)
4′			117.1 (s)	
4a′			154.7 (s)	
5′			69.3 (d)	5.35 (d, 1.3)
6′			27.6 (d)	2.28 (m)
7′			33.3 (t)	2.45−2.37 (m)
8′			177.6 (s)	
8a′			100.4 (s)	
9′			188.1 (s)[b]	
9a′			106.2 (s)[c]	
10a′			80.2 (s)	
11′			17.3 (q)	0.99 (d, 6.3)
12′			64.1 (t)	4.54 (d, 12.7), 3.86 (d, 12.8)
5′-O*C*(O)CH_3			169.7 (s)	
5′-OC(O)*C*H_3			20.9 (q)	2.07 (s)
12′-O*C*(O)CH_3			169.8 (s)	
12′-OC(O)*C*H_3			20.2 (q)	1.79 (s)
1′-O*H*				11.38 (s)
8′-O*H*				14.01 (s)

[a] δ values are expressed in ppm. [b−e] Assignments may be exchangeable.

Table 2. Antimalarial and Antitubercular Activity and Cytotoxicity of Compounds **1**, **2**, and **3**

	antimalarial activity (IC_{50}, μg/mL)[a]	antitubercular activity (MIC, μg/mL)[b]	cytotoxicity (IC_{50}, μg/mL)[c]		
compound	*P. falciparum* K1	*M. tuberculosis* H37Ra	KB cells	BC-1 cells	Vero cells
phomoxanthone A (**1**)	0.11	0.50	0.99	0.51	1.4
phomoxanthone B (**2**)	0.33	6.25	4.1	0.70	1.8
deacetylphomoxanthone A (**3**)	>20	>200	>20	>20	>50

[a] The IC_{50} values of the standard antimalarial compounds, chloroquine diphosphate and artemisinin, are 0.16 and 0.0011 μg/mL, respectively. [b] The MIC values of the antituberculosis drugs, isoniazide and kanamycin sulfate, are 0.050 and 2.5 μg/mL, respectively. [c] The IC_{50} values of the standard compound, ellipticine, are 0.46 μg/mL for KB cells and 0.60 μg/mL for BC-1 cells.

HR C_{18}, 6 μM, 40 × 100 mm) with MeCN/H_2O = 65:35 as an eluent at a flow rate of 20 mL/min to obtain **1** (t_R 16 min) and **2** (3.7 mg, t_R 21 min).

Phomoxanthone A (1): yellow powder; mp 214−216 °C; $[\alpha]^{25}_D$ +99° (*c* 0.40, $CHCl_3$); UV λ_{max} (log ϵ) 212 (4.48), 258 (4.26), 336 (4.54) nm; IR (KBr) ν_{max} 1744, 1615, 1586, 1470, 1366, 1223, 1044, 897, 830 cm^{-1}; MS (ESI-TOF) *m/z* 773 $[M + Na]^+$ (22), 751 $[M + H]^+$ (100), 691 (13), 416 (10), 376 (9); HRMS (ESI-TOF) *m/z* $[M + H]^+$ 751.2241 (calcd for $C_{38}H_{39}O_{16}$, 751.2238).

Phomoxanthone B (2): yellow amorphous solid; mp 119−122 °C; $[\alpha]^{26}_D$ −120° (*c* 0.15, $CHCl_3$); UV λ_{max} (log ϵ) 210 (sh) (4.42), 240 (4.19), 335 (4.49) nm; IR (KBr) ν_{max} 1748, 1611, 1439, 1368, 1220, 1045, 733 cm^{-1}; MS (ESI-TOF) *m/z* 773 $[M + Na]^+$ (34), 751 $[M + H]^+$ (100), 662 (9), 416 (65); HRMS (ESI-TOF) *m/z* $[M + H]^+$ 751.2232 (calcd for $C_{38}H_{39}O_{16}$, 751.2238).

Deacetylation of 1. To a suspension of **1** (9.0 mg) in dry MeOH (2 mL) was added concentrated H_2SO_4 (0.2 mL), and the mixture was stirred for 45 h. The resulting homogeneous solution was partially concentrated under reduced pressure, and the residue was diluted with EtOAc (30 mL). The EtOAc solution was washed with H_2O (3 × 10 mL) and concentrated in vacuo to yield a yellow powder (7.9 mg). Purification by preparative HPLC (MeCN/H_2O = 40:60; 20 mL/min; t_R 14 min) gave compound **3** (5.2 mg, 74% yield).

Deacetylphomoxanthone A (3): yellow powder; mp 208−210 °C; $[\alpha]^{25}_D$ +14° (*c* 0.087, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 218 (4.46), 250 (4.27), 340 (4.50) nm; IR (KBr) ν_{max} 3503, 1610,

1586, 1559, 1471, 1226, 1046 cm^{-1}; MS (ESI-TOF) *m/z* 605 $[M + Na]^+$ (18), 583 $[M + H]^+$ (73), 565 (8), 352 (44), 343 (55), 208 (100); HRMS (ESI-TOF) *m/z* $[M + H]^+$ 583.1823 (calcd for $C_{30}H_{31}O_{12}$, 583.1815); ^1H NMR (400 MHz, $CDCl_3$) differed in respect to **1** only in the following signals, δ 4.07 (2H, s, H-5), 3.85 (2H, d, J = 13.1 Hz, H-12a), 3.42 (2H, d, J = 13.1 Hz, H-12b), 2.48 (2H, dd, J = 19.2, 11.4 Hz, H-7a), 2.42 (2H, brs, −O*H*), 2.35 (2H, dd, J = 19.2, 6.1 Hz, H-7b), 2.14 (2H, m, H-6).

Biological Assay. Assay for activity against *P. falciparum* (K1, multi-drug-resistant strain) was performed using the protocol previously reported,[21] which follows the microculture radioisotope technique described by Desjardins.[22] IC_{50} represents the concentration that causes 50% reduction of parasite growth as indicated by the in vitro uptake of [^{3}H]-hypoxanthine by *P. falciparum*. Growth inhibitory activity against *Mycobacterium tuberculosis* H37Ra was performed using the Microplate Alamar Blue Assay (MABA).[23] Cytotoxicity of the purified compounds against human epidermoid carcinoma (KB), human breast cancer cells (BC-1), and African monkey kidney fibroblast (Vero) cells was evaluated using the colorimetric method.[24]

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References and Notes

(1) Horn, W. S.; Simmonds, M. S. J.; Schwartz, R. E.; Blaney, W. M. *Tetrahedron* **1995**, *51*, 3969−78.
(2) Izawa, Y.; Hirose, T.; Shimizu, T.; Koyama, K.; Natori, S. *Tetrahedron* **1989**, *45*, 2323−35.
(3) Culvenor, C. C. J.; Edgar, J. A.; Marasas, W. F. O.; Mackay, M. F.; Gorst-Allman, C. P.; Steyn, P. S.; Vleggaar, R.; Wessels, P. L. *Tetrahedron* **1989**, *45*, 2351−72.
(4) Tsantrizos, Y. S.; Ogilvie, K. K.; Watson, A. K. *Can. J. Chem*. **1992**, *70*, 2276−84.
(5) Beglev, M. J.; Grove, J. F. *J. Chem. Soc., Perkin Trans. 1* **1985**, 861−3.
(6) Grove, J. F. *J. Chem. Soc., Perkin Trans. 1* **1985**, 865−9.
(7) Cole, R. J.; Cox, R. H. *Handbook of Toxic Fungal Metabolites*; Academic Press: New York, 1981; Chapter 13, pp 646−69.
(8) Franck, B.; Flasch, H. *Forschr. Chem. Org. Chem*. **1973**, *30*, 151−206.
(9) Franck, B.; Gottschalk, E. M.; Ohnsorge, U.; Hüper, F. *Chem. Ber.* **1966**, *99*, 3842−62.
(10) Yamazaki, M.; Maebayashi, Y.; Miyaki, K. *Chem. Pharm. Bull*. **1971**, *19*, 199−201.
(11) Andersen, R.; Büchi, G.; Kobbe, B.; Demain, A. L. *J. Org. Chem*. **1977**, *42*, 352−3.
(12) Kurobane, I.; Vining, L. C. McInnes, A, G. *J. Antibiot*. **1979**, *32*, 1256−66.
(13) Steyn, P. S. *Tetrahedron* **1970**, *26*, 51−7.
(14) Hopper, J. W.; Marlow, W.; Whalley, W. B.; Borthwick, A. D. *Chem. Commun*. **1971**, 111−2.
(15) Hooper, J. W.; Marlow, W.; Whalley, W. B.; Borthwick, A. D.; Bowden, R. *J. Chem. Soc. (C)* **1971**, 3580−90.
(16) Kurobane, I.; Vining, L. C.; McInnes, A. G. *Tetrahedron Lett*. **1978**, 4633−6.
(17) Kurobane, I.; Iwahashi, S.; Fukuda, A. *Drugs Exp. Clin. Res*. **1987**, *13*, 339−44.
(18) Harada, M.; Yano, S.; Watanabe, H.; Yamazaki, M.; Miyaki, K. *Chem. Pharm. Bull*. **1974**, *22*, 1600−6.
(19) Wang, B. H.; Polya G. M. *Planta Med*. **1996**, *62*, 111−4.
(20) Reddy, C. S.; Hayes, A. W.; Williams, W. L.; Ciegler, A. *J. Toxicol. Environ. Health* **1979**, *5*, 1159−69.
(21) Isaka, M.; Punya, J.; Lertwerawat, Y.; Tanticharoen, M.; Thebtaranonth, Y. *J. Nat. Prod*. **1999**, *62*, 329−31.
(22) Desjardins, R. E.; Canfield, C. J.; Chulay, J. D. *Antimicrob. Agents Chemother*. **1979**, *16*, 710−8.
(23) Collins, L.; Franzblau, S. G. *Antimicrob. Agents Chemother*. **1997**, *41*, 1004−9.
(24) Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. *J. Natl. Cancer Inst*. **1990**, *82*, 1107−12.

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