JOURNAL OF NATURAL PRODUCTS

Volume 71, Number 8 August 2008

Full Papers

Epoxydons and a Pyrone from the Marine-Derived Fungus *Nigrospora* sp. PSU-F5

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Received April 26, 2008

Nigrospoxydons A−C (**1**−**3**) and nigrosporapyrone (**4**), four new metabolites, were isolated from the marine-derived fungus *Nigrospora* sp. PSU-F5 together with nine known compounds. Their structures were elucidated by spectroscopic methods, mainly 1D and 2D NMR spectroscopic techniques. The antibacterial activity against the standard *Staphylococcus aureus* ATCC 25923 and methicillin-resistant *S. aureus* was evaluated.

Fungi belonging to the genus *Nigrospora* have been a rich source of bioactive secondary metabolites, such as plant growth-inhibiting nigrosporolide[1] and phomalactone,[2] phytotoxic and antibacterial nigrosporins,[3] and phytotoxic lactones.[4] In our ongoing search for bioactive metabolites from marine-derived fungi, the crude extract of *Nigrospora* sp. PSU-F5 exhibited antibacterial activity against *Staphylococcus aureus* ATCC 25923 (SA) and a clinical isolate of methicillin-resistant *S. aureus* (MRSA). This paper reports the isolation, structure elucidation, and antibacterial activity of three new epoxydons, nigrospoxydons A (**1**), B (**2**), and C (**3**), one new pyrone, nigrosporapyrone (**4**), and nine known compounds, (+)-epoxydon (**5**),[5] (+)-deoxyabscisic acid (**6**),[6] (+)-abscisic acid (**7**),[7,8] (+)-phaseic acid (**8**),[9] pestalopyrone (**9**),[10] hydroxypestalopyrone (**10**),[10] 3,4-dihydro-3,4,8-trihydroxy-1[2*H*]-naphthalenone (**11**),[11] clavatol (**12**),[12] and 3-hydroxymethylphenol.[13] Their structures were assigned by spectroscopic methods and comparison of ^{1}H and ^{13}C NMR data with those reported in the literature. We also report herein their antibacterial activity against SA and MRSA.

Results and Discussion

The fungus PSU-F5 was isolated from a sea fan collected near Similan Island, Thailand. The sea fan was identified as *Annella* sp. As neither conidia nor spores were observed, the fungus PSU-F5 was identified on the basis of sequence analyses of the ribosomal internal transcribed spacer (ITS) and partial large subunit (LSU) rDNA. A search of partial LSU rDNA sequences for the closest match in GenBank indicated that this fungus has affinity with the Apiosporaceae, without any closely related taxa. However, few LSU sequences for members of the Apiosporaceae are available in GenBank for meaningful comparisons. Therefore, phylogenetic analysis of the ITS sequence was performed. The results showed that the fungus PSU-F5 can be placed in the *Nigrospora* clade with good statistical support. The *Nigrospora* clade always clusters next to the Apiosporaceae, Sordariomycetes Incertae Sedis,[14] and this is supported by a bootstrap value of 100%. The most closely related fungi were *Nigrospora* sp. AM262341, *N. oryzae* DQ219433, and *Nigrospora* sp. EF589888, with nucleotide similarity of 97%, 95.4%, and 95.2%, respectively. Therefore, the isolate was identified as *Nigrospora* sp. PSU-F5 (GenBank accession numbers: EF564154, EF564155). The fungus PSU-F5 is the first example of a marine-derived *Nigrospora* sp., as all *Nigrospora* species in the *Nigrospora* clade have been isolated as plant endophytes.

Nigrospoxydon A (**1**), with the molecular formula $C_{22}H_{28}O_8$ from HREIMS, was obtained as a colorless solid. The IR spectrum revealed absorption bands at 3442 (hydroxyl group), 1716 (carbonyl group of a conjugated ester), and 1699 and 1685 (carbonyl groups of α,β-unsaturated ketone units) cm^{-1}. The ^{1}H and ^{13}C NMR spectra (Table 1) consisted of signals similar to those of (+)-epoxydon (**5**). The major differences were the downfield shifts of H-6 (δ 5.32

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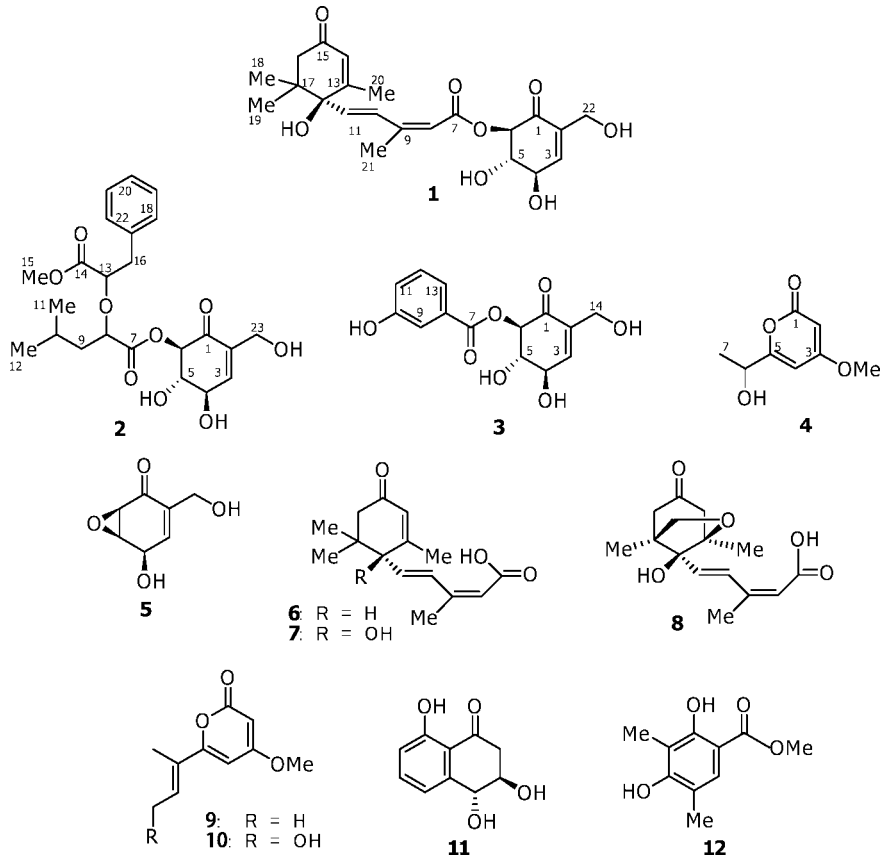
10.1021/np8002595 CCC: $40.75
Published on Web 07/23/2008

Table 1. NMR Data for Compounds **1** and **2** in Acetone-d_6

	1		**2**	
position	δ_H, mult. (*J* in Hz)	δ_C, mult.	δ_H, mult. (*J* in Hz)	δ_C, mult.
1		192.2, qC		191.7, qC
2		137.2, qC		138.1, qC
3	6.94, d (2.0)	145.6, CH	6.93, s	145.7, CH
4	4.54, m	71.6, CH	4.56, d (8.4)	71.4, CH
5	3.90, ddd (11.5, 8.0, 4.5)	76.1, CH	3.87, dd (11.1, 8.4)	75.9, CH
6	5.32, d (11.5)	77.1, CH	5.25, d (11.1)	77.6, CH
7		164.5, qC		174.4, qC
8	5.84, s	117.2, CH	3.33, dd (9.0, 5.4)	58.6, CH
9		150.9, qC	a: 1.59, ddd (13.5, 8.1, 5.4) b: 1.45, ddd (13.5, 9.0, 6.0)	43.0, CH_2
10	7.78, d (16.0)	127.9, CH	1.88, m	24.3, CH
11	6.43, d (16.0)	138.1, CH	0.87, d (6.6)	22.5, CH_3
12		79.3, qC	0.81, d (6.6)	21.4, CH_3
13		162.3, qC	3.62, dd (6.9, 6.0)	61.3, CH
14	5.83, q (1.0)	126.5, CH		173.7, qC
15		196.7, qC	3.60, s	51.0, CH_3
16	a: 2.57, d (17.0) b: 2.16, d (17.0)	49.5, CH_2	2.93, m	39.3, CH_2
17		41.4, qC		138.1, qC
18	1.08, s	22.7, CH_3	7.25, m	129.5, CH
19	1.05, s	23.8, CH_3	7.25, m	128.0, CH
20	1.91, d (1.0)	18.4, CH_3	7.25, m	126.2, CH
21	2.10, d (1.0)	20.4, CH_3	7.25, m	128.0, CH
22	4.21, m	58.0, CH_2	7.25, m	129.5, CH
23			a: 4.26, d (13.6) b: 4.15, d (13.6)	57.9, CH_2
4-OH	4.96, d (4.5)			
5-OH	5.06, d (4.5)			
12-OH	4.56, s			

Chart 1



in **1** and δ 3.41 in **5**), C-5 (δ 76.1 in **1** and δ 58.0 in **5**), and C-6 (δ 77.1 in **1** and δ 53.2 in **5**). The chemical shifts of these carbon signals indicated the presence of two oxysubstituents at C-5 and C-6 in **1**, rather than an epoxide ring as in **5**. Irradiation of H-6 in a NOEDIFF experiment enhanced only the signal intensity of H-4, indicating that H-4 and H-6 were *cis*, but *trans* to H-5. Since H-5 was coupled with both H-4 and H-6 with large coupling constants of 8.0 and 11.5 Hz, respectively, all protons were assigned to pseudoaxial positions. In addition, the ^1H and ^{13}C NMR spectra of **1** exhibited signals almost identical to those of (+)-abscisic acid

(**7**). The presence of such a unit was confirmed by the following HMBC correlations: H-8/C-7, C-10, and C-21; H-10/C-8, C-12, and C-21; 12-OH/C-12, C-13, and C-17; H-14/C-12, C-16, and C-20; H_{ab}-16/C-12, C-14, C-15, and C-17; H_3-18 and H_3-19/C-12, C-16, and C-17. The configurations of C8−C9 and C13−C14 double bonds were both determined to be *Z* on the basis of the NOE enhancement of H_3-21 (δ 2.10) and H_3-20 (δ 1.91) observed upon irradiation of H-8 (δ 5.84) and H-14 (δ 5.83), respectively. A HMBC correlation of H-6 of the hydroxyepoxydon unit with the ester carbonyl carbon of the abscisic acid unit established an ester linkage between these units. Consequently, nigrospoxydon A was identified as **1**, which could be obtained by condensation of **7** with the hydroxyepoxydon moiety. The absolute configurations in **1** were proposed to be 4*R*, 5*S*, 6*R*, and 12*S* on the basis of the known absolute configurations of all chiral carbons in **5**[5] and **7**[15] as well as their co-occurrence with **1**.

Nigrospoxydon B (**2**) was obtained as a colorless solid whose molecular formula was assigned as $C_{23}H_{30}O_9$ by HREIMS. The IR spectrum showed absorption bands for a hydroxyl group (3370 cm^{-1}), ester carbonyl groups (1749 and 1734 cm^{-1}), and a conjugated ketone carbonyl group (1698 cm^{-1}). Three carbonyl carbon resonances at δ 191.7, 174.4, and 173.7 suggested the presence of one conjugated ketone carbonyl and two ester carbonyl carbons. The ^1H NMR spectrum (Table 1) showed characteristic signals for a monosubstituted benzene, one 1-substituted-3-methylbutoxyl group, one oxymethine proton, one methoxyl group, and one isolated methylene unit. $^1H-^1H$ COSY data were fully consistent with the presence of the 1-substituted-3-methylbutoxyl unit. Furthermore, H_{ab}-9 (δ 1.59 and 1.45) and H-8 (δ 3.33) showed HMBC cross-peaks with one of the ester carbonyl carbons (δ 174.4, C-7), thus joining C-8 (δ 58.6) of the 1-substituted-3-methylbutoxyl unit with C-7 to form a 2-oxy-4-methylpentanoyl fragment. The oxymethine proton, H-13 (δ 3.62), showed a cross-peak in the $^1H-^1H$ COSY spectrum with H_2-16 (δ 2.93). In addition, H-13 showed HMBC cross-peaks with C-8 of the pentanoyl unit, C-17 (δ 138.1) of the monosubstituted benzene, and the other ester carbonyl carbon, C-14 (δ 173.7). This carbonyl carbon further gave a HMBC correlation with the methoxy protons (δ 3.60). These data led to construction of a methyl 2-oxy-3-phenylpropanoate moiety, of which C-13 (δ 61.3) formed an ether linkage with C-8 of the pentanoyl moiety. Irradiation of H-8 enhanced the signal intensity of H-13, thus indicating that they were located at the same side of the molecule. The remaininig ^1H and ^{13}C NMR data were characteristic of a hydroxyepoxydon fragment identical to that of **1**. The ester linkage between the pentanoyl and hydroxyepoxydon fragments was established by a HMBC correlation of H-6 (δ 5.25) with C-7. Consequently, nigrospoxydon B had the structure **2**. However, the relative configuration at C-8 and C-13 could not be assigned on the basis of NOEDIFF results due to a lack of correlations between protons of the ester moiety and those of the hydroxyepoxydon fragment.

Nigrospoxydon C (**3**) was obtained as a colorless gum with the molecular formula $C_{14}H_{14}O_7$ on the basis of HREIMS data. The IR spectrum exhibited absorption bands at 3360 (hydroxyl group), 1717 (conjugated ester carbonyl), and 1696 (conjugated ketone carbonyl) cm^{-1}. The ^1H and ^{13}C NMR spectra showed characteristic signals for a 1,3-disubstituted benzene moiety and a hydroxyepoxydon unit analogous to those present in **1** and **2**. The substituent at C-10 of the 1,3-disubstituted benzene was identified as a hydroxyl group on the basis of the chemical shift of C-10 (δ 157.5). ^3J HMBC correlations of the aromatic protons H-9 (δ 7.55) and H-13 (δ 7.56) and the oxymethine proton H-6 (δ 5.50) of the hydroxyepoxydon unit with the ester carbonyl carbon (δ 165.3, C-7) enabled assignment of an ester linkage between C-8 (δ 131.4) of the 1,3-disubstituted benzene unit and C-6 (δ 78.2) of the hydroxyepoxydon unit. Therefore, nigrospoxydon C was characterized as **3**, which could be a condensation product of the hydroxyepoxydon unit and 3-hydroxybenzoic acid.

Nigrosporapyrone (**4**), with the molecular formula $C_8H_{10}O_4$ from HREIMS, was obtained as a colorless solid. Its UV spectrum was similar to that of **9**, while the IR spectrum showed an additional absorption band for a hydroxyl group at 3365 cm^{-1}. Its ^1H NMR spectrum was similar to that of **9** except for the replacement of the 2-substituted-2-butenyl signals in the NMR data for **9** with signals for a 1-hydroxyethyl unit. The attachment of this fragment at C-5 (δ 164.6) was confirmed by a HMBC correlation of the methine proton, H-6 (δ 3.04), with C-5. Consequently, nigrosporapyrone was identified as a new pyrone derivative (**4**).

The crude ethyl acetate extract obtained from the culture broth showed antibacterial activity against SA and MRSA with minimum inhibitory concentration (MIC) values of 64 and 128 μg/mL, respectively. The isolated metabolites, except for **4**, **8**, **11**, and **12**, for which insufficient material was available, were tested for antibacterial activity against both strains. Only compounds **1** and **5** showed activity against both strains, while the remaining compounds were inactive. Compound **1** was more active than **5** against SA (MIC 64 μg/mL), but was less active against MRSA (MIC >128 μg/mL). Compound **5** gave the MIC value of 128 μg/mL against both strains.

Experimental Section

General Experimental Procedures. Melting points were determined on an Electrothermal 9100 melting point apparatus. Optical rotations were measured on a JASCO P-1020 polarimeter. Ultraviolet (UV) absorption spectra were recorded in MeOH on a Shimadzu UV-160A spectrophotometer. Infrared spectra (IR) were obtained on a Perkin-Elmer 783 FTS 165 FT-IR spectrometer. ^1H and ^{13}C NMR spectra were recorded on a 500 MHz Bruker FTNMR Ultra Shield spectrometer. Chemical shifts are expressed in δ (ppm) referring to the TMS peak. Mass spectra were obtained on a MAT 95 XL mass spectrometer (Thermofinnigan). TLC was performed on precoated silica gel GF_{254} (Merck) plates (PTLC). Column chromatography (CC) was performed on silica gel (Merck) type 100 (70−230 mesh ASTM) eluting with a gradient of MeOH−CH_2Cl_2, on Sephadex LH-20 eluting with MeOH, or on reversed-phase silica gel C-18 eluting with a gradient of MeOH−H_2O. Light petroleum had a bp of 40−60 °C.

Organism Collection and Identification. The fungus *Nigrospora* sp. PSU-F5 was isolated from a sea fan that was collected at 8°39′9″ N, 97°38′27″ E, 60 feet deep near Similan Island, Thailand, in 2005. The sea fan was identified as *Annella* sp. by C. Benzies and S. Plathong from the Coral Reef and Benthos Research Unit, Center for Biodiversity of Peninsular Thailand (CBIPT), Department of Biology, Prince of Songkla University, Thailand. The sample was deposited at CBIPT.

This fungus was deposited as PSU-F5 (GenBank accession numbers EF564154 and EF564155) at the Department of Microbiology, Faculty of Science, Prince of Songkla University. The fungus PSU-F5 was identified on the basis of analyses of partial LSU rDNA and ITS. Genomic DNA was extracted from fungal mycelia grown with potato dextrose agar (PDA) using the CTAB (cetyl trimethyl ammonium bromide) method.[16] Primers ITS5 (GGAAGTAAAAGTCGTAACAAGG), ITS4 (TCCTCCGCTTATTGATATGC), LROR (ACCCGCTGAACTTAAGC), and LR7 (TACTACCACCAAGATCT) were used.[17,18] The PCR reaction was performed using a DNA Engine DYAD ALD 1244 thermocycler (MJ Research, Inc.) with the following cycles: (1) 94 °C for 2 min; (2) 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, and (3) 72 °C for 10 min.[18] The amplified DNA fragment was purified with a NucleoSpin Extract DNA purification kit, cat. no. 740 609.50 (Macherey-Nagel), and was sequenced by Macrogen Inc. using the same primers as for amplification.

Fermentation and Isolation. The marine-derived fungus *Nigrospora* sp. PSU-F5 was grown on PDA at 25 °C for 5 days. Three pieces (0.5 × 0.5 cm^2) of mycelial agar plugs were inoculated into 500 mL Erlenmeyer flasks containing 300 mL of potato dextrose broth and incubated at room temperature for 4 weeks. The cultures (10 L) were filtered to separate filtrate from wet mycelia. The filtrate was extracted twice with EtOAc (2 × 300 mL). The combined EtOAc solution was dried over anhydrous Na_2SO_4 and evaporated to dryness to yield a dark brown gum (800 mg). The EtOAc extract was fractionated by CC over

Sephadex LH-20 to afford five fractions (A−E). Fraction B (307 mg) was further purified by silica gel CC to give seven fractions (B1−B7). Fraction B1 (15.6 mg, eluted with 5% MeOH−CH_2Cl_2) was then separated by PTLC with 80% EtOAc−light petroleum to give **4** (1.5 mg) and **9** (5.5 mg). Fraction B3 (25.4 mg, eluted with 5% MeOH−CH_2Cl_2) was purified by PTLC using 20% EtOAc−light petroleum as a mobile phase to afford **6** (4.0 mg) and **10** (3.5 mg). Compound **7** (3.8 mg) was obtained from fraction B4 (15.4 mg, eluted with 5% MeOH−CH_2Cl_2) after CC over Sephadex LH-20 followed by PTLC with 30% acetone−light petroleum. Fraction B6 (71.3 mg, eluted with 7−10% MeOH−CH_2Cl_2) was purified by CC over reversed-phase silica gel to give **1** (4.2 mg), **2** (6.2 mg), **5** (4.5 mg), and **8** (1.2 mg). Fraction D (181.3 mg), upon silica gel CC, yielded **3** (18.3 mg), **11** (1.9 mg), **12** (1.8 mg), and **13** (3.4 mg).

Nigrospoxydon A (1): colorless solid, mp 173.6−173.8 °C; $[\alpha]_D^{29}$ +10 (*c* 0.06, EtOH); UV (MeOH) λ_{max} (log ε) 281 (3.41) nm; IR (neat) ν_{max} 3442, 1716, 1699, 1685, 1650 cm^{-1}; ^1H NMR (acetone-d_6, 500 MHz), see Table 1; ^{13}C NMR (acetone-d_6, 125 MHz), see Table 1; HREIMS *m*/*z* $[M]^+$ 420.1771 (calcd for $C_{22}H_{28}O_8$, 420.1784).

Nigrospoxydon B (2): colorless solid, mp 366.2−366.7 °C; $[\alpha]_D^{29}$ +39 (*c* 0.31, EtOH); UV (MeOH) λ_{max} (log ε) 282 (2.89) nm; IR (neat) ν_{max} 3370, 1749, 1734, 1698, 1652 cm^{-1}; ^1H NMR (acetone-d_6, 500 MHz), see Table 1; ^{13}C NMR (acetone-d_6, 125 MHz), see Table 1; HREIMS *m*/*z* $[M]^+$ 450.1897 (calcd for $C_{23}H_{30}O_9$, 450.1890).

Nigrospoxydon C (3): colorless gum, $[\alpha]_D^{29}$ +24 (*c* 0.25, EtOH); UV (MeOH) λ_{max} (log ε) 210 (4.55), 232 (4.32), 270 (4.06) nm; IR (neat) ν_{max} 3360, 1717, 1696, 1652 cm^{-1}; ^1H NMR (acetone-d_6, 500 MHz) δ 7.56 (1H, dt, J = 8.1 and 1.5 Hz, H-13), 7.55 (1H, dd, J = 2.7 and 1.5 Hz, H-9), 7.35 (1H, t, J = 8.1 Hz, H-12), 7.12 (1H, ddd, J = 8.1, 2.7 and 1.5 Hz, H-11), 6.99 (1H, q, J = 1.8 Hz, H-3), 5.50 (1H, d, J = 11.1 Hz, H-6), 4.64 (1H, brd, J = 8.1 Hz, H-4), 4.28 (1H, d, J = 15.3 Hz, H_a-14), 4.23 (1H, d, J = 15.3 Hz, H_b-14), 4.08 (1H, dd, J = 11.1 and 8.1 Hz, H-5); ^{13}C NMR (acetone-d_6, 125 MHz) δ 191.9 (C, C-1), 165.3 (C, C-7), 157.5 (C, C-10), 145.7 (CH, C-3), 137.2 (C, C-2), 131.4 (C, C-8), 129.5 (CH, C-12), 120.8 (CH, C-13), 120.2 (CH, C-11), 116.3 (CH, C-9), 78.2 (CH, C-6), 76.1 (CH, C-5), 71.5 (CH, C-4), 58.0 (CH_2, C-14); HREIMS *m*/*z* $[M]^+$ 294.0740 (calcd for $C_{14}H_{14}O_7$, 294.0740).

Nigrosporapyrone (4): colorless solid, mp 196.6−196.8 °C; $[\alpha]_D^{29}$ −17 (*c* 0.52, MeOH); UV (MeOH) λ_{max} (log ε) 206 (3.64), 273 (3.64) nm; IR (neat) ν_{max} 3365, 1697 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 5.95 (1H, d, J = 2.5 Hz, H-2), 5.36 (1H, d, J = 2.5 Hz, H-4), 3.73 (3H, s, H-8), 3.04 (1H, q, J = 5.0 Hz, H-6), 1.32 (3H, d, J = 5.0 Hz, H-7); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 170.4 (C, C-3), 164.6 (C, C-5), 163.1 (C, C-1), 96.5 (CH, C-2), 87.4 (CH, C-4), 60.7 (CH, C-6), 55.0 (CH_3, C-8), 12.9 (CH_3, C-7); HREIMS *m*/*z* $[M]^+$ 170.0572 (calcd for $C_8H_{10}O_4$, 170.0579).

Antibacterial Assay. MICs were determined by the agar microdilution method.[19] The test substances were dissolved in DMSO (Merck, Germany). Serial 2-fold dilutions of the test substances were mixed with melted Mueller-Hinton agar (Difco) in the ratio of 1:100 in microtiter plates with flat-bottomed wells (Nunc, Germany). Final concentration of the test substances in agar ranged from 0.03 to128 μg/mL. *S. aureus* ATCC 25923 and MRSA SK1 were used as test strains. Inoculum suspensions (10 μL) were spotted on agar-filled wells. The plates were incubated at 35 °C for 18 h. MICs were recorded by reading the lowest substance concentration that inhibited visible growth. Vancomycin, a positive control drug, exhibited an MIC value of 1 μg/mL. Growth controls were performed on agar containing DMSO.

Acknowledgment. K.T. thanks the Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program for a scholarship. The Center for Innovation in Chemistry: Postgraduate Education and Research Program in Chemistry (PERCH-CIC), the Commission on Higher Education (CHE-RG), and Prince of Songkla University are gratefully acknowledged for partial support. Finally, V.R. thanks Prince of Songkla University for a research grant.

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NP8002595