JOURNAL OF NATURAL PRODUCTS

Note

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Thiasporines A–C, Thiazine and Thiazole Derivatives from a Marine-Derived *Actinomycetospora chlora*

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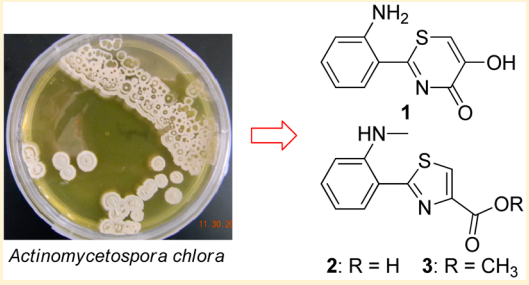
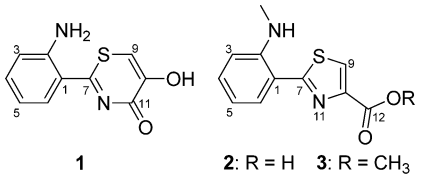
Supporting Information

ABSTRACT: Thiasporine A (**1**), the first natural product with a 5-hydroxy-4*H*-1,3-thiazin-4-one moiety, along with two new thiazole derivatives, thiasporines B and C (**2** and **3**), were isolated from the marine-derived *Actinomycetospora chlora* SNC-032. The structures of **1**–**3** were established on the basis of comprehensive spectroscopic analysis and chemical methods. Thiasporine A showed cytotoxicity against the non-small-cell lung cancer cell line H2122 with an IC_{50} value of 5.4 μM.



Marine actinomycetes are prolific producers of biologically active natural products. This unique habitat has led to the abundant chemical diversity of metabolites, which provides a foundation for the discovery of promising drug lead compounds. Among all known marine microbial secondary metabolites, over half were produced by actinomycetes.[1] From this resource, more than 400 new active secondary metabolites have been isolated.[2] Some of them, represented by abyssomycin C,[3] diazepinomicin,[4] salinosporamide A,[5] and the marinomycins,[6] are potent antibiotics and possess novel structures. Many biologists and chemists have focused on exploiting biologically and structurally interesting compounds from marine microbes. As part of our ongoing research on structurally novel and bioactive compounds from marine-derived actinomycetes, 6500 microbial natural product fractions were screened against a panel of 17 comprehensively annotated non-small-cell lung cancer cell lines.[7] The results showed that a series of natural product fractions demonstrated selective activity against a subset of these lines at 5 μg/mL. Analysis of an active fraction from the strain *Actinomycetospora* sp. SNC-032 by LC-UV-MS revealed it contained metabolites with similar UV absorptions at 230, 284, and 385 nm. This fraction showed selective cytotoxicity against the HCC44 cell line, while not showing activity against any of the other 16 cell lines. Bioassay-guided chemical investigation resulted in the isolation of a new thiazine and two new thiazole derivatives, which we have named thiasporines A–C (**1**–**3**).[8] A known dithiopyrrolone, thiolutin, presenting antibacterial, antifungal, and anticancer properties, was also isolated.[9] Thiasporine A (**1**) possessed a unique 5-hydroxy-2-phenyl-4*H*-1,3-thiazin-4-one core, which is the first example of a naturally occurring compound possessing this skeleton. Thiasporine A exhibited moderate cytotoxicity against non-small-cell lung cancer cell line H2122 with an IC_{50} value of 5.4 μM, but no activity against HCC366, A549, and HCC44 cell lines.

Marine-derived bacterium SNC-032 was isolated from a sediment sample collected from a mangrove swamp in Vava'u, Tonga (18°36′45″ S, 173°59′29″ W), and was isolated on a media prepared from rabbit manure. Analysis of its 16S rRNA sequence revealed SNC-032 to have greater than 99% identity to *Actinomycetospora chlora* TT071-57^T.[10] After identification of an active fraction in the library, a large-scale (10 L) shake fermentation was carried out to obtain sufficient material for full chemical and biological analysis of the metabolites. The excreted metabolites were collected using XAD-7-HP resin, and the resulting extract was separated by a combination of solvent/solvent extraction and reversed-phase flash chromatography to give fractions that showed cytotoxicity. Final purification by Sephadex LH-20 and gradient reversed-phase HPLC gave thiasporines A (**1**, 0.8 mg), B (**2**, 3.2 mg), and C (**3**, 1.8 mg).



Thiasporine A (**1**) was obtained as a white, amorphous powder. Its molecular formula was assigned as $C_{10}H_8N_2O_2S$ from the $[M + H]^+$ peak at m/z 221.0380 in the HRESIMS spectrum, which requires 8 degrees of unsaturation. The ^{13}C NMR resolved 10 carbon signals, which were classified by HMQC spectra as five olefinic or aromatic methine carbons and five quaternary carbons (Table 1). The proton signals at δ_H 7.51 (1H, d, J = 7.9), 7.10 (1H, t, J = 8.1), 6.79 (1H, d, J = 8.1), and 6.56 (1H, t, J = 7.8), along with their contiguous COSY

Special Issue: Special Issue in Honor of William Fenical

Received: November 20, 2014
Published: January 13, 2015

DOI: 10.1021/np500929z
J. Nat. Prod. 2015, 78, 548–551

Table 1. 1D and 2D NMR Data for Compound 1 in DMSO-d_6[a]

no.	δ_C	δ_H, mult. (J in Hz)	COSY	HMBC
1	114.4, C			
2	146.5, C			
3	116.1, CH	6.79, d (8.1)	4	1, 5
4	130.2, CH	7.10, t (8.1)	3, 5	2, 6
5	115.2, CH	6.56, t (7.8)	4, 6	1, 3
6	128.4, CH	7.51, d (7.9)	5	2, 4, 7
7	166.4, C			
9	118.7, CH	7.66, s		7, 11
10	164.2, C			
11	157.7, C			
2-NH_2		7.20, brs		1, 3

[a]Spectra were recorded at 600 MHz for ^{1}H and 100 MHz for ^{13}C using the corresponding solvent residual signal as internal standard.

correlations (Figure 1), indicated the presence of a 1,2-disubstituted phenyl nucleus. The HMBC correlations from

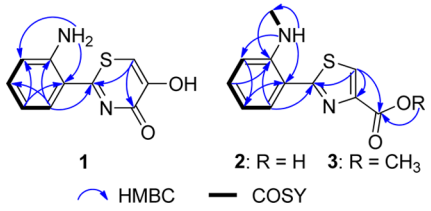
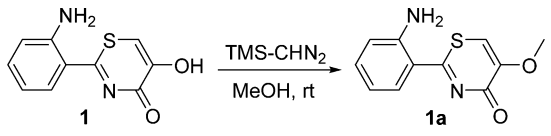


Figure 1. Key correlations for the structural assignment of **1**–**3**.

exchangeable proton (δ_H 7.20, 2H, brs) to C-1 and C-3 (Figure 1) indicated C-2 might be substituted by $-NH_2$. The ^{13}C shift of C-2 at δ_C 146.5 confirmed that C-2 was connected to the nitrogen of an amino group. In addition to the signals of the aniline unit, signals of one olefinic methine ($\delta_{C/H}$ 118.7/7.66) and three quaternary carbons (δ_C 166.4, 164.2, 157.7) were also observed. Otherwise, analysis of the HMBC spectrum revealed the correlations from H-9 to C-7 and C-11. The remaining structural assignment of **1** required $C_4H_2NO_2S$ and four degrees of unsaturation. On the basis of the remaining molecular formula and HMBC correlations, we deduced **1** to contain only two possible fragments, 5-hydroxy-4*H*-1,3-thiazin-4-one or thiazole-4-carboxylic acid. Careful comparison of its ^{13}C NMR with those of previously reported thiazole-4-carboxylic acids (Figure S2) revealed that the chemical shift values of **1** were not consistent with thiazole-4-carboxylic acid.[11] As the signal of 10-OH was not observed in the ^{1}H NMR spectra, methylation of **1** was carried out using TMS–CHN_2 to yield compound **1a** (Scheme 1). The LC-MS analysis showed the presence of a new methyl group, while NMR analysis gave ^{1}H chemical shifts of a methoxy group at δ_H 3.94 (10-OCH_3, s) and an HMBC correlation from 10-OCH_3 to C-10 (Figure S1), which confirmed the C-10 –OH substitution. The HMBC correlation from H-6 to C-7 suggested that the thiazine moiety connected aniline to thiasporine A (**1**) via a

Scheme 1. Methylation of 1 with TMS–CHN_2



single bond between C-1 and C-7. There is a CAS registry entry for a synthetic compound containing the 5-hydroxy-2-phenyl-4*H*-1,3-thiazin-4-one core (# 1135682-81-3); however, there are no references associated with this entry.

Thiasporine B (**2**) was isolated as a white, amorphous powder and assigned the molecular formula $C_{11}H_{10}N_2O_2S$ on the basis of the HRESIMS peak at m/z 235.0542 $[M + H]^+$. The ^{1}H NMR signals at δ_H 7.66 (1H, dd, J = 7.9, 1.4), 7.33 (1H, td, J = 8.5, 1.3), 6.79 (1H, d, J = 8.3), and 6.67 (1H, td, J = 8.0, 1.0) and their COSY correlations indicated that a 1,2-disubstituted phenyl nucleus was present in compound **2** (Table 2). The C-2 $-NHCH_3$ substitution was determined by

Table 2. ^{1}H and ^{13}C NMR Data for Compounds 2 and 3 in DMSO-d_6[a]

	2		3	
no.	δ_C	δ_H, mult. (J in Hz)	δ_C	δ_H, mult. (J in Hz)
1	113.8, C		113.6, C	
2	147.0, C		147.0, C	
3	111.0, CH	6.79, d (8.3)	111.1, CH	6.81, d (8.3)
4	131.9, CH	7.33, td (8.5, 1.3)	132.1, CH	7.34, td (8.4, 1.3)
5	115.1, CH	6.67, td (8.0, 1.0)	115.3, CH	6.68, td (8.0, 1.0)
6	129.2, CH	7.66, dd (7.9, 1.4)	129.3, CH	7.68, dd (7.8, 1.4)
7	168.8, C		169.2, C	
9	125.8, CH	8.35, s	127.1, CH	8.51, s
10	147.6, C		145.2, C	
12	162.0, C		160.9, C	
2-NH		8.47, q (4.8)		8.39, q (4.9)
2-$NHCH_3$	29.5, CH_3	2.92, d (4.8)	29.5, CH_3	2.92, d (5.0)
12-OCH_3			52.2, CH_3	3.88, s

[a]Spectra were recorded at 600 MHz for ^{1}H and 100 MHz for ^{13}C using the corresponding solvent residual signal as internal standard.

the COSY correlation of 2-$NHCH_3$ (δ_H 2.92, 3H, d, J = 4.8) to 2-NH (δ_H 8.47, 1H, q, J = 4.8) and the key HMBC correlations from 2-$NHCH_3$ (δ_H 2.92, 3H, d, J = 4.8) to C-2 and from 2-NH (δ_H 8.47, 1H, q, J = 4.8) to C-1 and C-3 (Figure 1). The remaining ^{1}H and ^{13}C signals ($\delta_{H/C}$ 8.35/125.8, δ_C 168.8, 162.0, 147.6) and the rest of the elements ($C_4H_2NO_2S$) based on the molecular formula were consistent with the thiazole-4-carboxylic acid moiety (Figure S2).[11] Moreover, the HMBC correlations from H-9 to C-7, C-10, and C-12 further confirmed this unit. The HMBC correlation from H-6 to C-7 indicated that C-1 was connected with C-7.

The molecular formula of thiasporine C (**3**) was assigned to be $C_{12}H_{12}N_2O_2S$ based on the HRESIMS data, which was 14 mass units (one CH_2) more than that of **2**. Analysis of the ^{1}H and ^{13}C NMR spectra revealed that the methoxy signals at $\delta_{H/C}$ 3.88/52.2 appeared in compound **3**. Thus, compound **3** was determined to be the methyl ester analogue of **2**. Furthermore, the COSY and HMBC correlations confirmed this structure (Figure 1).

A plausible biogenetic pathway for thiasporines A–C (**1**–**3**) (Scheme 2) begins by amide bond formation, which is followed by an intramolecular condensation of the anthranilic acid moiety and cysteine to yield a hydroxy-thiazolidinecarboxylic acid ring system (intermediate **a**), which can undergo dehydration to produce the 4,5-dihydro-5-thiazolecarboxylic acid system (ring **b**). Oxidation of intermediate **b** and

DOI: 10.1021/np500929z
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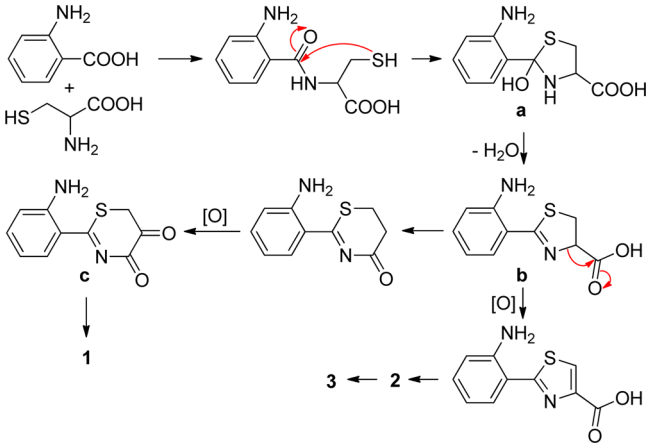
Scheme 2. Plausible Biosynthetic Pathway of 1−3



subsequent *N*-methylation of the aniline would give **2**. More interesting is the biosynthetic conversion to **1**. In order to go from intermediate **b** to the final 5-hydroxy-4*H*-1,3-thiazin-4-one ring system in **1** requires a ring expansion and oxidation to give intermediate **c**. Finally, enolization would yield **1**.

The active fraction showed selective cytotoxicity against the non-small-cell lung cancer cell line HCC44, so compounds **1**−**3** were evaluated for their cytotoxicity against the HCC44 cell line and another three non-small-cell lung cancer cell lines (HCC366, A549, and H2122). Compound **1** showed modest cytotoxicity against the cell line H2122 with an IC_{50} value of 5.4 μM. **1** showed no activity up to 24 μM against HCC366, A549, and HCC44. Because **1** showed only modest cytotoxicity against HCC44, we did not pursue testing the compound against the full panel of non-small-cell lung cancer cell lines. Compounds **2** and **3** did not show significant cytotoxicity against any of the four cell lines tested. In addition to the cytotoxicity of **1**, the co-isolated molecule thiolutin is known to be cytotoxic.

EXPERIMENTAL SECTION

General Experimental Procedures. UV spectra were recorded on a Shimadzu UV-1601 UV−vis spectrophotometer. ^{1}H and 2D NMR spectroscopic data were recorded at 600 MHz in CD_3OD or DMSO-d_6 solution on a Varian System spectrometer. ^{13}C NMR spectra were acquired at 100 MHz on a Varian System spectrometer. Chemical shifts were referenced to the corresponding solvent residual signal (3.31/49.00 in CD_3OD, 2.50/39.52 in DMSO-d_6). High-resolution ESI-TOF mass spectra were provided by The Scripps Research Institute, La Jolla, CA, USA. Low-resolution LC/ESIMS data were measured using an Agilent 1200 series LC/MS system with a reversed-phase C_{18} column (Phenomenex Luna, 150 mm × 4.6 mm, 5 μm) at a flow rate of 0.7 mL/min. Preparative HPLC was performed on an Agilent 1200 series instrument with a DAD detector, using a C_{18} column (Phenomenex Luna, 250 × 10.0 mm, 5 μm). Sephadex LH-20 (GE Healthcare, Sweden) and ODS resin (50 mm, Merck) were used for column chromatography. Artificial seawater was used in microbial fermentations as described in a previous reference.[12]

Collection and Phylogenetic Analysis of Strain SNC-032. The actinomycete SNC-032 was isolated from a sediment sample collected from a mangrove swamp in Vava'u, Tonga (18°36′45″ S, 173°59′29″ W). The sediment samples were collected under permits from the Tonga Ministry of Agriculture and Food, Forests and Fisheries, in conjunction with Dr. Peter Northcote (Victoria University, Wellington, NZ). The isolation media was prepared by boiling 100 g of rabbit manure in 1 L of seawater for 1 h and filtering, and the subsequent supernatant was added to 15 g of agar. Analysis of the 16S rRNA sequence of SNC-032 revealed 99% identity to *Actinomycetospora chlora*. The sequence was deposited in GenBank under accession no. KJ754374.

Cultivation and Extraction of SNC-032. Bacterium SNC-032 was cultivated in 10 2.8 L Fernbach flasks each containing 1 L of a seawater-based medium (10 g starch, 4 g yeast extract, 2 g peptone, 1 g $CaCO_3$, 40 mg $Fe_2(SO_4)_3{\cdot}4H_2O$, 100 mg KBr) and shaken at 200 rpm at 27 °C. After 7 days of cultivation, sterilized XAD-7-HP resin (20 g/L) was added to adsorb the organic products, and the culture and resin were shaken at 200 rpm for 2 h. The resin was filtered through cheesecloth, washed with deionized water, and eluted with acetone. The acetone-soluble fraction was dried in vacuo to yield 4.2 g of extract.

Purification. The extract of strain SNC-032 (4.2 g) was partitioned with EtOAc, *n*-butyl alcohol, and H_2O. The EtOAc extract (710 mg) was fractionated by flash column chromatography on ODS (50 μm, 30 g), eluting with a step gradient of MeOH and H_2O (10:90−100:0), and eight fractions were collected. Fraction 3 (84.9 mg) was separated by Sephadex LH-20, eluting with MeOH, to give 10 fractions. Subfraction 3-9 (8.6 mg) was purified by reversed-phase HPLC (Phenomenex Luna, C_{18}, 250 × 10.0 mm, 2.5 mL/min, 5 μm) using a gradient solvent system from 20% to 100% CH_3CN (0.1% formic acid) over 15 min to yield compound **1** (0.8 mg, t_R = 13.7 min). Fractions 4 (83.9 mg) and 5 (75.0 mg) were combined and then recrystallized from MeOH to give thiolutin (69.7 mg). The mother liquor of the recrystallization was separated by Sephadex LH-20, eluting with MeOH, to give nine fractions. Subfraction 4-2 (11.9 mg) was purified by reversed-phase HPLC (Phenomenex Luna, C_{18}, 250 × 10.0 mm, 2.5 mL/min, 5 μm) using a gradient solvent system from 20% to 100% CH_3CN (0.1% formic acid) over 20 min to yield compound **2** (3.2 mg, t_R = 17.5 min). Fractions 6 (29.1 mg) and 7 (37.0 mg) were combined and then separated by Sephadex LH-20, eluting with MeOH, to give eight fractions. Subfraction 6-5 (9.4 mg) was purified by reversed-phase HPLC (Phenomenex Luna, C_{18}, 250 × 10.0 mm, 2.5 mL/min, 5 μm) using a gradient solvent system from 30% to 100% CH_3CN (0.1% formic acid) over 25 min to afford compound **3** (1.8 mg, t_R = 21.5 min).

*Thiasporine A (**1**):* white, amorphous powder; UV (MeOH) λ_{max} (log ε) 232 (4.08), 282 (3.62), 359 (3.58) nm; ^{1}H and ^{13}C NMR, see Table 1; HRESIMS m/z 221.0380 $[M + H]^+$ (calcd for $C_{10}H_9N_2O_2S$, 221.0379).

*Thiasporine B (**2**):* white, amorphous powder; UV (MeOH) λ_{max} (log ε) 227 (4.04), 284 (3.70), 384 (3.68) nm; ^{1}H and ^{13}C NMR, see Table S1; HRESIMS m/z 235.0542 $[M + H]^+$ (calcd for $C_{11}H_{11}N_2O_2S$, 235.0536).

*Thiasporine C (**3**):* white, amorphous powder; UV (MeOH) λ_{max} (log ε) 231 (4.11), 284 (3.71), 385 (3.66) nm; ^{1}H and ^{13}C NMR, see Table S1; HRESIMS m/z 249.0696 $[M + H]^+$ (calcd for $C_{12}H_{13}N_2O_2S$, 249.0692).

Methylation of 1 with TMS−CHN_2. To a solution of **1** (0.4 mg) in MeOH (anhydrous, 0.4 mL) was added 150 μL of TMS−CHN_2 (2.0 M in Et_2O) until a yellow color persisted upon addition. After allowing it to stir for 2 h, the solvent was removed via a stream of N_2, and the reaction mixture was analyzed via LC/MS. The reaction product was purified by reversed-phase HPLC (Phenomenex Luna, C_{18}, 250 × 10.0 mm, 2.5 mL/min, 5 μm) using a gradient solvent system (solvents: A H_2O + 0.1% formic acid; B CH_3CN + 0.1% formic acid; gradient: 0 min, 30% B; 15 min, 100% B; 25 min, 100% B) to yield compound **1a** (0.2 mg, t_R = 14.9 min, 47% yield). Compound **1a**: white powder; ^{1}H NMR (600 MHz, CD_3OD) δ 8.28 (s, 1H, H-9), 7.61 (dd, J = 7.9, 1.4 Hz, 1H, H-6), 7.18 (ddd, J = 8.4, 8.4, 1.4 Hz, 1H, H-4), 6.85 (dd, J = 8.4, 1.0 Hz, 1H, H-3), 6.67 (ddd, J = 8.1, 8.1, 1.1 Hz, 1H, H-5), 3.94 (s, 3H, 10-OCH_3); ESIMS m/z 257.0 $[M + Na]^+$.

Cytotoxicity Assays. Cell lines were cultivated in 10 cm dishes (Corning, Inc.) in NSCLC cell-culture medium: RPMI/L-glutamine medium (Invitrogen, Inc.), 1000 U/mL penicillin (Invitrogen, Inc.), 1 mg/mL streptomycin (Invitrogen, Inc.), and 5% fetal bovine serum (Atlanta Biologicals, Inc.). Cell lines were grown in a humidified environment in the presence of 5% CO_2 at 37 °C. For cell viability assays, HCC366, A549, HCC44, and H2122 cells (60 μL) were plated individually at a density of 1200, 750, and 500 cells/well, respectively,

DOI: 10.1021/np500929z
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in 384-well microtiter assay plates (Bio-one; Greiner, Inc.). After incubating the assay plates overnight under the growth conditions described above, purified compounds were dissolved and diluted in DMSO and subsequently added to each plate with final compound concentrations ranging from 50 μM to 1 nM and a final DMSO concentration of 0.5%. After an incubation of 96 h under growth conditions, Cell Titer Glo reagent (Promega, Inc.) was added to each well (10 mL of a 1:2 dilution in NSCLC culture medium) and mixed. Plates were incubated for 10 min at room temperature, and luminescence was determined for each well using an Envision multimodal plate reader (PerkinElmer, Inc.). Relative luminescence units were normalized to the untreated control wells (cells plus DMSO only). Data were analyzed using the Assay Analyzer and Condoseo modules of the Screener Software Suite (GeneData, Inc.) as described previously.[13]

■ ASSOCIATED CONTENT

Supporting Information

HRESIMS and NMR spectra for compounds **1**−**3** and **1a**. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We acknowledge the following grants for funding this project: NIH R01 CA149833, U01 CA176284, and the Welch Foundation I-1689. J.B.M. is a Chilton/Bell Foundation Endowed Scholar. We are also grateful for the support of the UT Southwestern High Throughput Screening Core Facility and the grants that support it (NCI Cancer Center Support Grant P30CA142543 and the CPRIT MIRA Grant RP110708-C2).

■ DEDICATION

Dedicated to Dr. William Fenical of Scripps Institution of Oceanography, University of California−San Diego, for his pioneering work on bioactive natural products

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(7) The natural product fraction library was created from bacterial extracts of 5 L fermentations of bacterial strains. Fractions were generated using reversed-phase C_{18} chromatography on an ISCO medium-pressure automatic purification system (gradient from 90:10 H_2O/CH_3CN to 0:100 H_2O/CH_3CN over 25 min) to generate 10−20 fractions.

(8) Compounds **2** and **3** have been reported in a paper that was published in *Journal of Natural Products* when our manuscript was reviewed: Kim, H.; Yang, I.; Patil, R. S.; Kang, S.; Lee, J.; Choi, H.; Kim, M.; Nam, S.; Kang, H. *J. Nat. Prod.* **2014**, *77*, 2716−2719.

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DOI: 10.1021/np500929z
J. Nat. Prod. 2015, 78, 548−551