

Article

Spiculisporic Acids B–D, Three New γ -Butenolide Derivatives from a Sea Urchin-Derived Fungus *Aspergillus* sp. HDf2

Rong Wang [†], Tian-Mi Liu [†], Ming-Hui Shen, Ming-Qiu Yang, Quan-Ying Feng, Xian-Ming Tang and Xiang-Min Li ^{*}

Hainan Provincial Fisheries Research Institute, Haikou 570203, Hainan, China

[†] These authors contributed equally to this work.

^{*} Author to whom correspondence should be addressed; E-Mail: hn.lxm@163.com; Tel.: +86-898-6651-7579; Fax: +86-898-6652-7571.

Received: 18 October 2012; in revised form: 30 October 2012 / Accepted: 31 October 2012 /

Published: 5 November 2012

Abstract: Three new γ -butenolide derivatives **1–3**, named spiculisporic acids B–D, were isolated from the culture of *Aspergillus* sp. HDf2, a marine-derived fungus that resides in the sea urchin, *Anthocidaris crassispina*. The structures of **1–3** were elucidated on the basis of spectroscopic methods, including MS and 2D NMR techniques. Their *in vitro* cytotoxic activities against two cell lines (SGC-7901, human gastric adenocarcinoma and SPC-A-1, human lung adenocarcinoma) and inhibitory activities against *Staphylococcus aureus* ATCC 51650 were investigated.

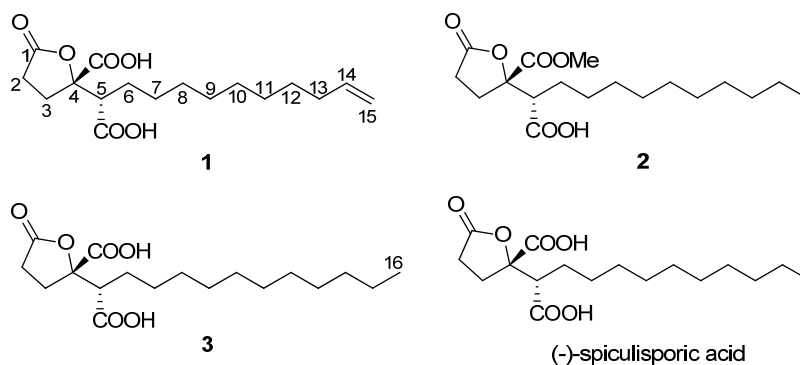
Keywords: γ -butenolide derivatives; spiculisporic acids B–D; *Aspergillus* sp.; marine fungus

1. Introduction

Microorganisms of marine origin have proven to be a rich source of novel and/or biologically active natural products with promising pharmacological properties [1–4]. In related studies, the Tsukamoto laboratory reported a new antimicrobial anthraquinone (monodictyquinone A) from a sea urchin-derived fungus *Monodictys* sp. [5]. In our search for new natural products from marine-derived microorganisms, the fungus *Aspergillus* sp. HDf2 was isolated from the sea urchin *Anthocidaris crassispina*, collected from the seashore of Qionghai, Hainan, China. The genus *Aspergillus* (Trichocomaceae) is one of the most prolific fungi that produce a variety of secondary metabolites

with novel structures and interesting bioactivities [6]. Subsequent chemical study on the fermentation broth of the fungus *Aspergillus* sp. HDF2 led to the isolation of three new γ -butenolide derivatives, the structures of which were similar to that of spiculisporic acid [7,8], and these compounds were thus named as spiculisporic acids B–D (compounds 1–3, Figure 1). Their structures were elucidated by comprehensive spectroscopic analyses. Herein we report the isolation, structural determination, bioactivities of these new natural products.

Figure 1. Structures of compounds 1–3.



2. Results and Discussion

Spiculisporic acid B (**1**) was isolated as a white solid, with the molecular formula $C_{17}H_{26}O_6$ (five degrees of unsaturation) as derived from ESI high-resolution mass spectrometry ($[M-H]^-$ at m/z 325.1664, calculated 325.1657) and 1H - and ^{13}C -NMR spectral data (Tables 1 and 2). The ^{13}C -NMR showed three carbonyl carbons at δ_C 178.5, 175.5, and 174.2, one olefinic methine carbon at δ_C 140.2, one olefinic methylene carbon at δ_C 114.8, one oxygen bearing quaternary carbon at δ_C 88.2, one methine carbon at δ_C 52.5, and ten aliphatic carbons in the upfield (δ_C 35.0 to 28.9) region. The 1H -NMR spectrum displayed signals of one terminal vinyl group at δ_H 5.81 (ddt, $J = 16.1, 10.2, 6.8$ Hz, H-14), 4.98 (br d, $J = 16.1$ Hz, H-15a), and 4.91 (br d, $J = 10.2$ Hz, H-15b), and 21 aliphatic protons. Together, these data indicate that compound **1** has one double bond and three carbonyls, which account for 4 out of the 5 degrees of unsaturation required by the molecular formula, so spiculisporic acid B must contain a ring. The structural information for **1** was determined from a series of 2D NMR analyses, including HSQC, 1H - 1H COSY, and HMBC spectra (Figure 2). The 1H - 1H COSY experiment revealed a correlation between H-2 (δ_H 2.60) and H-3 (δ_H 2.49), and a separate spin system, $H_2C=CH-CH_2-(CH_2)_6-CH_2-CH-$. The methine proton H-14 was coupled with the methylene protons H-13 (δ_H 2.04) and H-15, and the methylene proton H-13 was coupled with the methylene proton at H-12 (δ_H 1.37). The correlations between the methylene proton H-6a (δ_H 1.85) and H-5 [δ_H 3.01 (br d, $J = 9.2$ Hz)] and H-7 (δ_H 1.25–1.37) were observed in the 1H - 1H COSY spectrum. HMBC correlations from H-5 to C-4 (δ_C 88.2) and two carbonyl carbons (δ_C 175.5 and 174.2), from H-3 to C-1 (δ_C 178.5), C-4, C-5 (δ_C 52.5), and one of the carbonyl carbons (δ_C 174.2), and from H-2 to C-1 and C-4, were observed. These observations allowed the structure of **1** to be determined as shown in Figure 1.

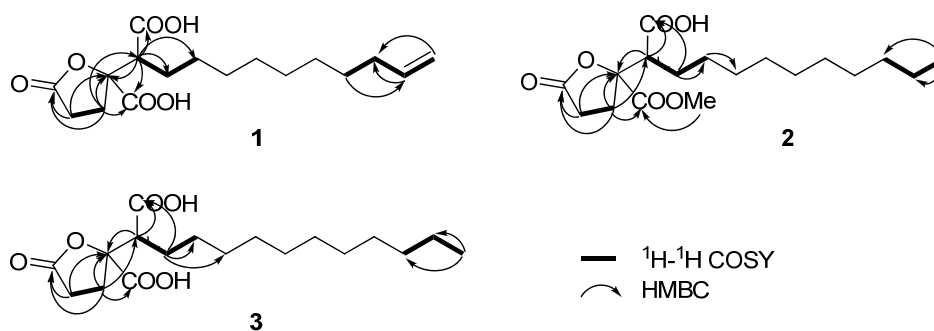
Table 1. ¹H-NMR spectral data (500 MHz, CD₃OD) of compounds 1–3.

Position	1	2	3
2	2.60 (m)	2.59 (m)	2.59 (m)
3	2.49 (m)	2.48 (m)	2.46 (m)
4-COOMe		3.80 (s)	
5	3.01 (br d, $J = 9.2$)	2.97 (dd, $J = 10.8, 2.5$)	3.03 (dd, $J = 11.0, 3.0$)
6	1.85 (m); 1.50 (m)	1.82 (m); 1.51 (m)	1.85 (m); 1.53 (m)
7	1.25–1.37 (m) ^a	1.43 (m); 1.32 (m)	1.42 (m); 1.32 (m)
8	1.25–1.37 (m) ^a	1.25–1.37 (m) ^b	1.25–1.38 (m) ^c
9	1.25–1.37 (m) ^a	1.25–1.37 (m) ^b	1.25–1.38 (m) ^c
10	1.25–1.37 (m) ^a	1.25–1.37 (m) ^b	1.25–1.38 (m) ^c
11	1.25–1.37 (m) ^a	1.25–1.37 (m) ^b	1.25–1.38 (m) ^c
12	1.37 (m)	1.25–1.37 (m) ^b	1.25–1.38 (m) ^c
13	2.04 (m)	1.25–1.37 (m) ^b	1.25–1.38 (m) ^c
14	5.81 (ddt, $J = 16.1, 10.2, 6.8$)	1.25–1.37 (m) ^b	1.25–1.38 (m) ^c
15	4.98 (br d, $J = 16.1$) 4.91 (br d, $J = 10.2$)	0.90 (t, $J = 6.8$)	1.25–1.38 (m) ^c
16			0.90 (t, $J = 7.0$)

^{a–c} Overlapping signals.**Table 2.** ¹³C-NMR spectral data (125 MHz, CD₃OD) of compounds 1–3.

Position	1	2	3
1	178.5 (s)	178.0 (s)	178.7 (s)
2	28.9 (t) ^a	28.7 (t)	29.0 (t) ^e
3	30.3 (t)	30.5 (t)	30.6 (t) ^d
4	88.2 (s)	88.2 (s)	88.7 (s)
4-COOH	174.2 (s)		174.9 (s)
4-CO		172.8 (s)	
OMe		53.6 (q)	
5	52.5 (d)	52.9 (d)	52.7 (d)
5-COOH	175.5 (s)	175.2 (s)	175.8 (s)
6	29.2 (t)	28.9 (t) ^b	29.1 (t)
7	28.9 (t) ^a	28.9 (t) ^b	29.0 (t) ^e
8	30.7 (t) ^f	30.5 (t) ^{c,g}	30.6 (t) ^d
9	30.4 (t) ^f	30.4 (t) ^g	30.6 (t) ^{d,h}
10	30.5 (t) ^f	30.5 (t) ^{c,g}	30.8 (t) ^h
11	30.5 (t) ^f	30.8 (t) ^g	30.9 (t) ^h
12	30.2 (t)	30.7 (t) ^g	30.7 (t) ^h
13	35.0 (t)	33.1 (t)	30.5 (t)
14	140.2 (d)	23.8 (t)	33.2 (t)
15	114.8 (t)	14.5 (q)	23.8 (t)
16			14.5 (q)

^{a–e} Overlapping signals. ^{f–h} Interchangeable signals.

Figure 2. ^1H - ^1H COSY and selected HMBC correlations of compounds 1–3.

To confirm the absolute configuration of **1**, through comparison of its chemical shifts for C-4 (δ_{C} 88.2) and C-5 (δ_{C} 52.5) with those of the known compounds (–)-spiculisporic acid (δ_{C} 88.1, 52.5) and (–)-epispiculisporic acid (δ_{C} 88.0, 51.9) [7,8], we tentatively propose the absolute configuration at C-4 and C-5 in **1** to be 4*S* and 5*S* as (–)-spiculisporic acid. Thus, the structure was identified as (4*S*,5*S*)-4-(5-carboxyl-undecyl-14-enyl)-1-oxo-tetrahydrofuran-4-carboxyl acid, named spiculisporic acid B.

Spiculisporic acid C (**2**) was obtained as a waxy solid that analyzed for the molecular formula $\text{C}_{18}\text{H}_{30}\text{O}_6$ by HR-ESI-MS data ($[\text{M}+\text{Na}]^+$ m/z 365.1927), and by comprehensive analysis of NMR data. This formula differed by the addition of CH_2 to the molecular formula of spiculisporic acid [7,8], suggesting an additional methylene or methyl group had been added to the structure. The ^1H and ^{13}C -NMR data for **2** were almost identical to those of spiculisporic acid, except for the presence of a new oxygenated methyl group (δ_{C} 53.6, δ_{H} 3.80). The HMBC spectrum showed a strong correlation between the oxygenated methyl protons and carbonyl carbon at δ_{C} 172.8, which was correlated with H-3 (δ_{H} 2.48), thus indicating the position of the methoxyl group. Based on the HSQC, ^1H - ^1H COSY, and HMBC analyses of **2** (Figure 2), and the good comparison of NMR data of C-4 (δ_{C} 88.2) and C-5 (δ_{C} 52.9) in **2** to those from (–)-spiculisporic acid, we proposed the structure of **2** to be (4*S*,5*S*)-4-(5-carboxyl-undecyl)-1-oxo-tetrahydrofuran-4-carboxyl acid methyl ester, named spiculisporic acid C. It was possible that the methoxyl group in **2** was a result of a reaction with methanol in the procedure of isolation.

Spiculisporic acid D (**3**), isolated as a pale white solid, gave a $[\text{M}+\text{Na}]^+$ ion peak at m/z 365.1940 in its positive-mode HR-ESI-MS, indicating its molecular formula to be $\text{C}_{18}\text{H}_{30}\text{O}_6$, which was the same as that of **2**. Through detailed analyses of the ^1H - and ^{13}C -NMR spectra of **3**, the major difference between **3** and spiculisporic acid was the presence of a new methylene group in the aliphatic chain. Unambiguous assignments of ^1H - and ^{13}C -NMR data were obtained by interpretation of HSQC, ^1H - ^1H COSY, and HMBC data (Figure 2), confirming the structure for **3** as shown. The absolute configuration of **3**, determined by comparison of NMR data of C-4 (δ_{C} 88.7) and C-5 (δ_{C} 52.7) in **3** with those of (–)-spiculisporic acid, was elucidated as (4*S*,5*S*)-4-(5-carboxyl-dodecyl)-1-oxo-tetrahydrofuran-4-carboxyl acid, named spiculisporic acid D.

Compounds **1**–**3** were subjected to cytotoxic activity tests against two cell lines, SGC-7901 and SPC-A-1 by MTT methods [9]. However, none of these compounds were active with $\text{IC}_{50} > 50 \mu\text{g/mL}$. Compounds **1**–**3** showed antibacterial activities against *Staphylococcus aureus* ATCC 51650 with inhibition zone of 9.6, 11.6, and 11.5 mm at 20 mg/mL, while the diameter of inhibition zone of the positive control was 23.6 mm. Spiculisporic acid, a fermentation adduct from the culture broth of *Penicillium spiculisporum* has found potential use as new controlled release carriers of active

chemicals [7], and commercial application as a biosurfactant for metal decontamination processes to remove hard, large metal cations from water [10]. These interesting properties of spiculisporic acids B–D are currently under investigation.

3. Experimental

3.1. General Experimental Procedures

Optical rotations were taken on a Rudolph Autopol III. UV spectra were measured on a Hitachi U-3000 spectrophotometer, and IR spectra (KBr) were obtained on a Nicolet 380 FT-IR spectrometer. NMR spectra were recorded on a Bruker AVIII-500 spectrometer at 500 MHz for ^1H -NMR and at 125 MHz for ^{13}C -NMR. Chemical shifts are given in δ (ppm) and referenced to the solvent signal (methanol- d_4 , δ_{H} 3.31, δ_{C} 49.1) as the internal standard, and coupling constants (J) are reported in Hz. HR-ESI-MS spectra were recorded on a Agilent 6210 TOF LC/MS mass spectrometer. Silica gel (200–300 mesh) for column chromatography (CC) and silica GF₂₅₄ (10–20 mm) for TLC were obtained from Qingdao Marine Chemical Factory (Qingdao, China). YMC ODS gel (50 μm) was purchased from Shanghai HANKING Instrument & Equipment Co., Ltd. (Shanghai, China) Sephadex LH-20 for chromatography was purchased from Merck (Darmstadt, Germany). Semipreparative HPLC was performed on a Hitachi L-7110 pump, and UV detector L-7400 equipped with a Waters ODS column (5 μm , 250 \times 4.6 mm).

3.2. Fungal Material and Cultivation

The fungus *Aspergillus* sp. HDf2 was isolated and identified by one of the authors (R.W.) from the gut of a healthy sea urchin *Anthocidaris crassispina* collected from the seashore of Qionghai, Hainan, China, in October 2009. A voucher specimen with the code HNF-HD02 is deposited in the Hainan Provincial Fisheries Research Institute. The fungus was cultivated on MEA solid medium composed of 20 g/L malt extract, 20 g/L sucrose, 1 g/L peptone, 20 g/L agar and deionized water for 5 days at 28 °C. Agar plugs were used to inoculate in 1000-mL Erlenmeyer flasks, each containing 300 mL of ME liquid media. Fermentation was carried out on a rotary shaker (140 rpm) at 26 °C for 12 days in 40 \times 1,000 mL Erlenmeyer flasks.

3.3. Extraction and Isolation

The filtrate (12 L) of the fermented culture broth was extracted three times with EtOAc (12 L \times 4) at room temperature, and the organic solvent was evaporated to dryness under reduced pressure to afford a yellow crude extract (4.1 g), which was subjected to silica gel (41 g, 200–300 mesh) CC (4 \times 75 cm) eluted with a gradient of CHCl_3 –MeOH (v/v 100:0, 100:1, 100:2, 100:4, 100:8, 100:16 and 0:100, each 600 mL) to give seven fractions. The CHCl_3 –MeOH (100:4) fraction (710.3 mg) was further purified by Sephadex LH-20 CC (1.5 \times 30 cm) eluting with MeOH (500 mL) and then by ODS CC (2.5 \times 40 cm) with a gradient of MeOH– H_2O (v/v 50:50, 65:35, 80:20, 100:0, each 400 mL) to afford a fraction (110.5 mg) (MeOH– H_2O , 80:20) containing **1**–**3**, which were purified by semipreparative reversed-phase HPLC [2 mL/min; MeOH-0.1% TFA in H_2O (78:22)] (**1**, 10.3 mg, t_{R} = 16.0 min; **2**, 16.2 mg, t_{R} = 27.8 min; **3**, 22.6 mg, t_{R} = 29.6 min). All these compounds were stored at 4 °C.

Spiculisporic acid B (1): (4*S*,5*S*)-4-(5-Carboxylundecyl-14-enyl)-1-oxo-tetrahydrofuran-4-carboxylic acid. White solid; $[\alpha]_D^{30} = -4.8$ ($c = 0.028$, EtOH); UV (MeOH) λ_{\max} ($\log \epsilon$): 199 (3.07), 215 (3.89) nm; IR (KBr) ν_{\max} : 2912, 2853, 1711, 1688, 1415, 1272, 1175, 932 cm^{-1} ; ^1H and ^{13}C -NMR spectral data are listed in Tables 1 and 2; HR-ESI-MS: m/z 325.1664 $[\text{M}-\text{H}]^-$ (calculated for $\text{C}_{17}\text{H}_{25}\text{O}_6$, 325.1657).

Spiculisporic acid C (2): (4*S*,5*S*)-4-(5-Carboxylundecyl)-1-oxo-tetrahydrofuran-4-carboxylic acid methyl ester. Waxy solid; $[\alpha]_D^{30} = -24.7$ ($c = 0.078$, EtOH); UV (MeOH) λ_{\max} ($\log \epsilon$): 214 (3.18) nm; IR (KBr) ν_{\max} : 2921, 2855, 1716, 1663, 1412, 1274, 1183, 952 cm^{-1} ; ^1H and ^{13}C -NMR spectral data are listed in Tables 1 and 2; HR-ESI-MS: m/z 365.1927 $[\text{M}+\text{Na}]^+$ (calculated for $\text{C}_{18}\text{H}_{30}\text{O}_6\text{Na}$, 365.1935).

Spiculisporic acid D (3): (4*S*,5*S*)-4-(5-Carboxyl-dodecyl)-1-oxo-tetrahydrofuran-4-carboxylic acid. Pale white solid; $[\alpha]_D^{30} = -11.8$ ($c = 0.028$, EtOH); UV (MeOH) λ_{\max} ($\log \epsilon$): 212 (3.22) nm; IR (KBr) ν_{\max} : 2918, 2860, 1721, 1657, 1423, 1268, 1175, 944 cm^{-1} ; ^1H and ^{13}C -NMR spectral data are listed in Tables 1 and 2; HR-ESI-MS: m/z 365.1940 $[\text{M}+\text{Na}]^+$ (calculated for $\text{C}_{18}\text{H}_{30}\text{O}_6\text{Na}$, 365.1935).

3.4. In Vitro Cytotoxicity Test

The cytotoxic activities for compounds **1–3** were tested *in vitro* against two cell lines, SGC-7901 (human gastric adenocarcinoma) and SPC-A-1 (human lung adenocarcinoma), which were purchased from the Jiangsu Provincial Center for Disease Prevention and Control. The purity of the tested compounds and doxorubicin-HCl was determined to be over 95% by using the HPLC-DAD method. The cytotoxic *in vitro* effects on these tested cell were assessed by the IC_{50} values, and determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method [8]. Each set of test was conducted three times to confirm reproducibility of the results. The compounds were dissolved in DMSO (dimethyl sulfoxide). Doxorubicin-HCl was used as a positive control, and the medium without test compound as a negative control in the bioassay.

3.5. Antibacterial Test

Compounds **1–3** were tested for *in vitro* antimicrobial activity against *Staphylococcus aureus* ATCC51650 by the filter paper disc agar diffusion method. The NA medium was mixed with 2 mL of suspension containing $1 \times 10^5 \sim 1 \times 10^7$ cfu/mL of *Staphylococcus aureus*, and then poured into petri-plates. 2 μL 20 mg/mL of the isolated compounds dissolved in DMSO were impregnated on sterile filter paper discs (6 mm diameter) and then were applied on the surface of the solidified agar plates. Every sample was tested in triplicate. Streptomycin sulfate (2 μL , 20 mg/mL) was used as positive control. The test plates were incubated at 37 °C for 24 h. Then the diameters of the inhibition zones including the 6 mm disc diameter were measured.

4. Conclusions

In our screening for new secondary metabolites from marine-derived fungi associated with marine animals from the coast of Hainan Island, three new secondary metabolites **1–3**, named spiculisporic

acids B–D were isolated from a sea urchin (*Anthocidaris crassispinga*)-associated fungus *Aspergillus* sp. HDf2 for the first time and characterized. Their structures were elucidated by NMR spectroscopic methods, and the absolute configurations were determined by comparing the chemical shifts of their chiral carbons with those of related known analogues. The compounds displayed no cytotoxic activity against human gastric adenocarcinoma cell line SGC-7901 and human lung adenocarcinoma cell line SPC-A-1 with $IC_{50} > 50 \mu\text{g/mL}$. Compounds 1–3 showed weak antibacterial activities against *Staphylococcus aureus* ATCC 51650 at 20 mg/mL. Further research into their new activities is in progress.

Supplementary Materials

Supplementary materials can be accessed at: <http://www.mdpi.com/1420-3049/17/11/13175/s1>.

Acknowledgments

This work was supported by funds for the Science and Technology Program of Hainan Province to Promote the Research and Development of Marine Resources, and the Fundamental Research Foundation for Youth, Hainan Provincial Fisheries Research Institute (No.20091006).

References

1. Li, D.; Xu, Y.; Shao, C.L.; Yang, R.Y.; Zheng, C.J.; Chen, Y.Y.; Fu, X.M.; Qian, P.Y.; She, Z.G.; de Voogd, N.J.; *et al.* Antibacterial bisabolane-type sesquiterpenoids from the sponge-derived fungus *Aspergillus* sp. *Mar. Drugs* **2012**, *10*, 234–241.
2. Gao, S.S.; Li, X.M.; Du, F.Y.; Li, C.S.; Proksch, P.; Wang, B.G. Secondary metabolites from a marine-derived endophytic fungus *Penicillium chrysogenum* QEN-24S. *Mar. Drugs* **2011**, *9*, 59–70.
3. Blunt, J.W.; Copp, B.R.; Munro, M.H.G.; Northcote, P.T.; Prinsep, M.R. Marine natural products. *Nat. Prod. Rep.* **2011**, *28*, 196–268.
4. Kwon, H.C.; Kauffman, C.A.; Jensen, P.R.; Fenical, W. Marinisporolides, Polyene-polyol macrolides from a marine actinomycete of the new genus “*Marinispora*”. *J. Org. Chem.* **2009**, *74*, 675–684.
5. El-Beih, A.A.; Kawabata, T.; Koimaru, K.; Ohta, T.; Tsukamoto, S. Monodictyquinone A: A new antimicrobial anthraquinone from a sea urchin-derived fungus *Monodictys* sp. *Chem. Pharm. Bull.* **2007**, *55*, 1097–1098.
6. Keller, N.P.; Turner, G.; Bennett, J.W. Fungal secondary metabolism—From biochemistry to genomics. *Nat. Rev. Microbiol.* **2005**, *3*, 937–947.
7. Brown, S.P.; Goodwin, N.C.; MacMillan, D.W.C. The first enantioselective organocatalytic Mukaiyama-Michael reaction: A direct method for the synthesis of enantioenriched γ -Butenolide Architecture. *J. Am. Chem. Soc.* **2003**, *125*, 1192–1194.
8. Asano, M.; Kameda, S. Spiculisporic acid, a metabolic product of *Penicillium*. *Rept. Jpn. Assoc. Adv. Sci.* **1942**, *17*, 60–64.
9. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immun. Methods* **1983**, *65*, 55–63.

10. Pekdemir, T.; Tokunaga, S.; Ishigami, Y.; Hong, H.-J. Removal of cadmium or lead from polluted water by biological amphiphiles. *J. Surfactants Deterg.* **2000**, *3*, 43–46.

Samples Availability: Samples are available from the authors.

© 2012 by the authors; licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution license (<http://creativecommons.org/licenses/by/3.0/>).