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STYLISSAMIDE I, A NEW CYCLIC HEPTAPEPTIDE FROM AN OKINAWAN MARINE SPONGE *STYLISSA* sp.

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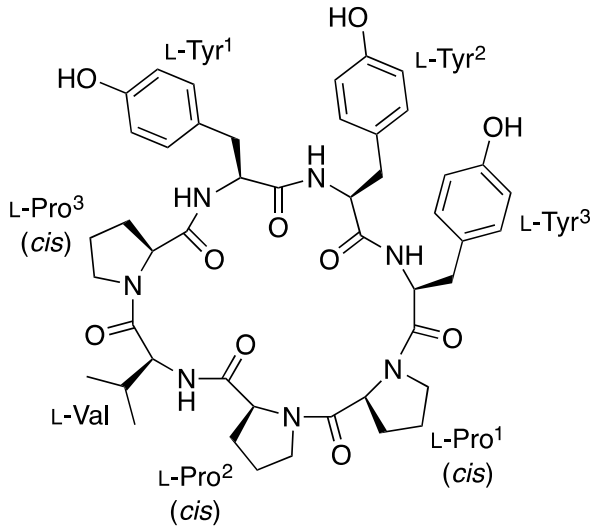
Abstract – A new cyclic heptapeptide, stylissamide I (**1**), was isolated from an Okinawan marine sponge *Stylissa* sp. The structure of stylissamide I (**1**) was elucidated to be *cyclo*-(L-Tyr^1-L-Tyr^2-L-Tyr^3-L-Pro^1-L-Pro^2-L-Val-L-Pro^3) by extensive spectral analyses and Marfey's method. Stylissamide I (**1**) showed antifungal activity against *Aspergillus niger*.

INTRODUCTION

Marine sponges were known as a rich source of variety of bioactive natural products, which were recognized as promising seeds for lead compounds of new drugs.[1] Among them, a number of proline-rich cyclic peptides have been isolated from sponges belonging to the genus *Axinella*,[2] *Hymeniacidon*,[3] *Phakellia*,[4] *Stylissa*,[5] and so on. In our continuing search for bioactive natural products from marine organisms, we previously isolated cyclic heptapeptides[3b-3e] and octapeptides[3f] from Okinawan marine sponges of genus *Hymeniacidon*. Recently, we have investigated the extract of an Okinawan marine sponge *Stylissa* sp. and isolated a new cyclic heptapeptide, stylissamide I (**1**). Here we describe the isolation and structure elucidation of **1** (Figure 1).



1

Fugure 1. Structure of stylissamide I (**1**)

RESULTS AND DISCUSSION

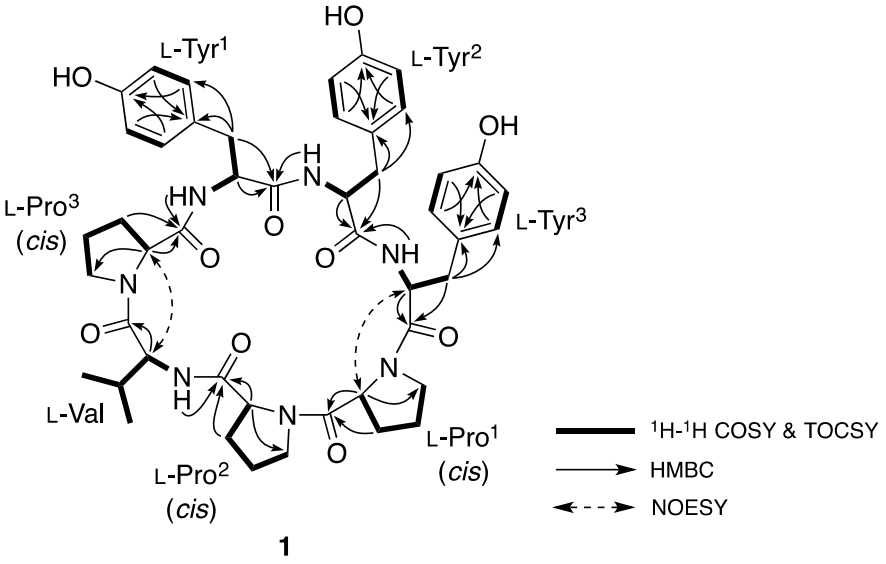
The sponge *Stylissa* sp. collected off Unten Port, Okinawa was extracted with MeOH, and the extract was partitioned between EtOAc and H_2O. EtOAc-soluble materials were separated by a Sephadex LH-20 column, a C_{18} flash column, and C_{18} HPLC to obtain stylissamide I (**1**, 0.000047% wet weight). Stylissamide I (**1**) was obtained as an optically active colorless amorphous solid. The UV absorption at 283 nm (ε 4870) indicated the presence of aromatic rings, while the IR absorptions (3379, 3248 and 1676 cm^{-1}) were attributed to hydroxy group and amide linkage. The molecular formula of **1** was established to be $C_{47}H_{57}N_7O_{10}$ by HRESIMS data [*m/z* 902.40697 $(M+Na)^+$ (calcd for $C_{47}H_{57}N_7O_{10}Na_1$, 902.40646)]. Inspection of the HSQC spectrum with the ^1H and ^{13}C NMR spectra of **1** disclosed the existence of forty-seven carbons consisting of seven amide carbonyl carbons (δ_C 171.6, 171.2, 170.2, 169.4, 169.3, 169.5, and 168.5), six sp^2 quaternary carbons (δ_C 156.4, 156.3, 155.5, 129.1, 126.3, and 125.6), twelve sp^2 methine carbons (δ_C 130.3 (2C), 130.1 (2C), 129.7 (2C), 115.3 (2C), 115.1 (2C), and 114.5 (2C)), eight sp^3 methine carbons (δ_C 61.1, 60.0, 57.8, 54.8, 54.4, 54.2, 50.9, and 32.2), twelve sp^3 methylene carbons (δ_C 46.6, 45.9 (2C), 38.7, 35.9, 35.4, 31.1, 30.5, 30.0, 22.0, 21.1, and 20.9), and two sp^3 methyl carbons (δ_C 20.4 and 17.1) (Table 1). Among them, seven sp^3 methine carbons (δ_C 61.1, 60.0, 57.8, 54.8, 54.4, 54.2, and 50.9) were ascribed to α-carbons of amino acid residues. These data implied that **1** was a cyclic heptapeptide.

Table 1. ^{1}H and ^{13}C NMR data of stylissamide I (**1**) in DMSO-d_6

position	δ_H[a]	multi (*J* in Hz)	δ_C[b]	position	δ_H[a]	multi (*J* in Hz)	δ_C[b]
Tyr^1				Pro^1			
α	4.11	nd[c]	54.2	α	3.42	nd[c]	61.1
β	2.94	nd[c]	35.9	β	1.68	nd[c]	31.1
	2.75	nd[c]			1.45	m	
1			125.6	γ	1.69[d]	nd[c]	22.0
2,6	6.99	d (8.0)	130.3	δ	3.48	nd[c]	45.9
3,5	6.67	d (8.0)	115.1		3.25	nd[c]	
4			156.4	CO			170.2
4-OH	9.33[e]	br s					
CO			169.3	Pro^2			
NH	8.84	s		α	2.96	nd[c]	60.0
				β	1.67	nd[c]	30.0
Tyr^2					0.87	nd[c]	
α	4.15	nd[c]	54.4	γ	1.35	m	20.9
β	2.68	dd (13.2, 3.0)	35.4		0.53	m	
	2.58	dd (13.2, 13.2)		δ	3.11	m	45.9
1			129.1		2.93	nd[c]	
2,6	7.00	d (7.8)	129.7	CO			169.5
3,5	6.68	d (7.8)	114.5				
4			155.5	Val			
4-OH	9.33[e]	br s		α	4.18	dd (5.3, 2.2)	54.8
CO			169.4	β	1.98	nd[c]	32.2
NH	8.95	d (8.5)		Me	0.90	d (6.8)	20.4
				Me	0.72	d (6.7)	17.1
Tyr^3				CO			168.5
α	4.58	nd[c]	50.9	NH	6.61	d (5.3)	
β	2.77	nd[c]	38.7				
	2.63	dd (12.6, 5.6)		Pro^3			
1			126.3	α	4.59	nd[c]	57.8
2,6	6.96	d (8.2)	130.1	β	2.16	m	30.5
3,5	6.69	d (8.2)	115.3		2.00	nd[c]	
4			156.3	γ	1.93	nd[c]	21.1
4-OH	9.06[e]	br s			1.87	m	
CO			171.6	δ	3.53	nd[c]	46.6
NH	8.44	d (8.9)			3.30	nd[c]	
				CO			171.2

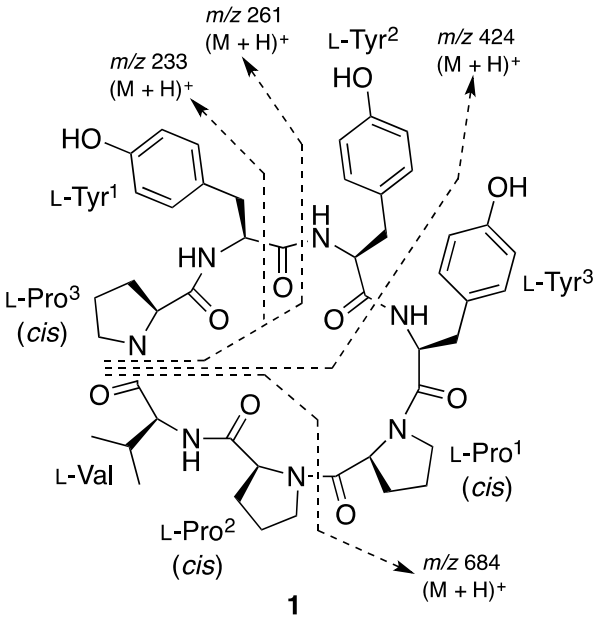
[a]600 MHz. [b]150 MHz. [c]nd: *J*-values were not determined because of overlapping with other signals. [d]2H. [e]Signals may be interchangeable.

The correlations observed in the ^{1}H-^{1}H COSY, TOCSY, and HMBC spectra revealed that **1** consisted of three proline residues, three tyrosine residues and a valine residue (Figure 2). The amino acid sequence of **1** was derived from information of the HMBC and NOESY spectra. The HMBC correlations for Tyr^1-NH/Pro^3-CO, Tyr^2-NH/Tyr^1-CO, Tyr^3-NH/Tyr^2-CO, and Val-NH/Pro^2-CO indicated the amide linkages between Pro^3 and Tyr^1, Tyr^1 and Tyr^2, Tyr^2 and Tyr^3, and Pro^2 and Val, respectively. While, the connections of Tyr^3 and Pro^1 and Val and Pro^3 were suggested by the NOESY correlations for Pro^1-Hα/Tyr^3-Hα and Pro^3-Hα/Val-Hα, respectively (Figure 2). Considering the molecular formula of **1**, the forming of a cyclic heptapeptide by an amide linkage between Pro^1-CO and Pro^2-N was assumed.



Fugure 2. Selected 2D NMR correlations for stylissamide I (**1**)

The fragmentation peaks observed in the FABMS spectrum supported the sequence of amino acid residues of **1** (Figure 3). Thus, the gross structure of **1** was elucidated to be *cyclo*-(Tyr^1-Tyr^2-Tyr^3-Pro^1-Pro^2-Val- Pro^3).



Fugure 3. Fragmentation patterns observed in positive ion FABMS of stylissamide I (**1**)

The geometries for amide bonds of Tyr^3-Pro^1, Pro^1-Pro^2, and Val-Pro^3 of **1** were assigned as all *cis* on the basis of $\delta_{C\gamma}$ and $\Delta\delta_{C\beta\text{-}C\gamma}$ values of prolines (Pro^1 $\delta_{C\gamma}$ 22.0 ppm, $\Delta\delta_{C\beta\text{-}C\gamma}$ 9.13 ppm; Pro^2 $\delta_{C\gamma}$ 20.9 ppm, $\Delta\delta_{C\beta\text{-}C\gamma}$ 9.12 ppm; Pro^3 $\delta_{C\gamma}$ 21.1 ppm, $\Delta\delta_{C\beta\text{-}C\gamma}$ 9.42 ppm).[6-8] Though the NOESY correlation for Pro^1-Hα/Pro^2-Hα was not observed because of overlapping with signal of H_2O, the NOESY correlations

for Tyr^3-Hα/Pro^1-Hα and Val-Hα/Pro^3-Hα supported the *cis* geometries of Tyr^3-Pro^1 and Val-Pro^3, respectively (Figure 2). The absolute configuration of each amino acid in **1** was assigned as all L by HPLC analysis of 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (FDAA) derivatives of the acid hydrolysate of **1**.[9] Therefore, the structure of **1** was elucidated to be *cyclo*-(L-Tyr^1-L-Tyr^2-L-Tyr^3-L-Pro^1-L-Pro^2-L-Val-L-Pro^3).

Antimicrobial activities of stylissamide I (**1**) against bacteria (*Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Micrococcus luteus*) and fungi (*Aspergillus niger*, *Trichophyton mentagrophytes*, *Candida albicans*, and *Cryptococcus neoformans*) were tested. Stylissamide I (**1**) showed antifungal activity against *A. niger* (IC_{50} 4 μg/mL), while **1** did not show antimicrobial activities against other bacteria (MIC >32 μg/mL) and fungi (IC_{50} > 32 μg/mL).

EXPERIMENTAL

General Methods. Optical rotations were recorded on a JASCO P-2200 polarimeter. UV spectra were recorded on a JASCO V-630BIO spectrophotometer. IR spectra were recorded on a HORIBA FT710 spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance II 600 MHz NMR spectrometer equipped with a cryoplatform using 3.0 mm micro cells (Shigemi Co., Ltd.) for DMSO-d_6. The 2.49 ppm resonance of residual DMSO-d_6 and 39.5 ppm resonance of DMSO-d_6 were used as internal references for ^{1}H and ^{13}C NMR spectra, respectively. MS spectra were recorded on a JMS-T100LP spectrometer. Flash column chromatography was performed with a Biotage Isolera flash purification system.

Sponge Description. The sponge (SS-611; order Halichondrida, family Dictyonellidae, *Stylissa* sp.) was collected off Unten Port, Okinawa, and kept frozen until used. The sponge was fibrous and coarse. The sponge had some large canals (~ 5 mm wide) and the interior was porous. The surface was smooth and collagenous and the color was light brown to ochre. The texture was soft, compressible, and springy. Plumoreticulate fibre skeleton was centrally cored with styles. Spicules were styles with large size range, long, slender, curved (580 x 9 μm). The voucher specimen was deposited at Showa Pharmaceutical University.

Extraction and Isolation. The sponge (SS-611, 1.5 kg, wet wt.) was extracted with MeOH (2.0 L x 3), and the extract (64.85 g) was partitioned between EtOAc (600 mL x 3) and H_2O (600 mL) to give EtOAc-soluble materials (9.72 g). The aqueous layer was extracted with BuOH (600 mL x 3) to give BuOH-soluble materials (14.72 g). The EtOAc-soluble materials were fractionated by a silica gel column (Silica gel 60N (spherical, neutral, 40-50 μm), Kanto Chemical Co., Inc.; 50 x 350 mm; eluent, $CHCl_3$/MeOH, 100:0 to 0:100), and a fraction eluted with around $CHCl_3$/MeOH = 85:15 was separated by a flash C_{18} column chromatography (Isolera SNAP Ultra C18 12 g, Biotage; eluent, MeOH/H_2O, 0:100 to

100:0) and C_{18} HPLC (Cosmosil 5C_{18}-PAQ 10 x 250 mm, Nakarai Tesque Inc.; eluent, MeCN/H_2O/TFA, 50:50:0.1; flow rate, 2.0 mL/min; UV detection at 220 nm to yield stylissamide I (**1**, 0.7 mg, 0.000047% wet weight, t_R 31.5 min). Three known related cyclic peptides, hymenamides E[3c] and G[3e] and phakellistatin 18[4j], were isolated in the purification process of **1**. Two known related cyclic peptides, hymenamides A[3b] and C[3c], were isolated from the BuOH-soluble materials.

Stylissamide I (1): a colorless amorphous solid; $[\alpha]_D^{24}$ +22 (*c* 0.20, MeOH); UV (MeOH) λ_{max} 229 (ε 15600) and 283 (ε 4870) nm; IR (KBr plate) ν_{max} 3379, 3248, 2962, 2881, 1676, 1637, 1516, 1448, 1350, 1238, 1207, 1186, and 1137 cm^{-1}; ^1H and ^{13}C NMR data (Table 1); HRESIMS (pos) *m/z* 902.40697 $(M+Na)^+$ (calcd for $C_{47}H_{57}N_7O_{10}Na_1$, 902.40646).

Elucidation of Absolute Stereochemistry of Amino Acid Residue. Stylissamide I (**1**, 0.1 mg) was dissolved in 6 M hydrochloric acid (500 μL). After heating at 110 °C for 24 h, the excess hydrochloric acid was removed by nitrogen blowing. The hydrolysate was dissolved in H_2O (25 μL) and treated with sat. $NaHCO_3$ aq. (40 μL) and 1% FDAA/acetone (50 μL). After heating at 40 °C for 1 h, the reaction mixture was neutralized with 1 M hydrochloric acid (30 μL). The standard amino acids were treated by the same procedure as described above. The FDAA derivatives of the hydrolysate and standard amino acids were subjected to C_{18} HPLC analyses [Cosmosil 5C_{18}-AR-II 4.6 x 250 mm, Nakarai Tesque Inc.; eluent, MeCN/H_2O/TFA, 30:70:0.1; flow rate, 1.0 mL/min; UV detection at 340 nm]. The retention time (min) of each FDAA derivatives of authentic L and D amino acids was appeared as follows; L-Pro (12.6), D-Pro (14.1), L-Tyr (16.8), D-Tyr (20.4), L-Val (24.8), D-Val (44.0). The retention time (min) of FDAA derivatives of amino acids in the hydrolysate of **1** were found as follows: L-Pro (12.7), L-Tyr (16.9), L-Val (24.8).

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REFERENCES

1. J. W. Blunt, B. R. Copp, R. A. Keyzers, M. H. G. Munro, and M. R. Prinsep, *Nat. Prod. Rep.*, 2016, **36**, 382, and references therein.
2. (a) G. R. Pettit, C. L. Herald, M. R. Boyd, J. E. Leet, C. Dufresne, D. L. Doubek, J. M. Schmidt, R. L. Cerny, J. N. A. Hooper, and K. C. Rutzler, *J. Med. Chem.*, 1991, **34**, 3339; (b) G. R. Pettit, F. Gao, and R. Cerny, *Heterocycles*, 1993, **35**, 711; (c) G. R. Pettit, F. Guo, R. Cerry, D. L. Doubek, L. P.

Tackett, J. M. Shmidt, and J. C. Chapuis, *J. Med. Chem.*, 1994, **37**, 1165; (d) G. R. Pettit, F. Guo, J. M. Schmidt, J. C. Chapuis, and R. L. Cerny, *Bioorg. Med. Chem. Lett.*, 1994, **4**, 2935; (e) A. Randazzo, F. Dal Piaz, S. Orru, C. Debitus, C. Roussakis, P. Pucci, and L. Gomez-Paloma, *Eur. J. Org. Chem.*, 1998, **1998**, 2659; (f) D. J. Milanowski, M. A. Rashid, K. R. Gustafson, B. R. O'Keefe, J. P. Nawrocki, L. K. Pannell, and R. Michael, *J. Nat. Prod.*, 2004, **67**, 441.

3. (a) G. R. Pettit, P. J. Clewlow, C. Dufresne, D. L. Doubek, R. L. Cerny, and K. Rutzler, *Can. J. Chem.*, 1990, **68**, 708; (b) J. Kobayashi, F. Kanda, M. Ishibashi, and H. Shigemori, *J. Org. Chem.*, 1991, **56**, 4574; (c) J. Kobayashi, M. Tsuda, T. Nakamura, Y. Mikami, and H. Shigemori, *Tetrahedron*, 1993, **49**, 2391; (d) M. Tsuda, H. Shigemori, Y. Mikami, and J. Kobayashi, *Tetrahedron*, 1993, **49**, 6785; (e) M. Tsuda, T. Sasaki, and J. Kobayashi, *Tetrahedron*, 1994, **50**, 4667; (f) J. Kobayashi, T. Nakamura, and M. Tsuda, *Tetrahedron*, 1996, **52**, 6355.
4. (a) G. R. Pettit, Z. Cichacz, J. Barkoczy, A. C. Dorsaz, D. L. Herald, M. D. Williams, D. L. Doubek, J. M. Schmidt, L. P. Tackett, D. C. Brune, R. L. Cerny, J. N. A. Hooper, and G. J. Bakus, *J. Nat. Prod.*, 1993, **56**, 260; (b) G. R. Pettit, R. Tan, M. D. Williams, L. Tackett, J. M. Schmidt, R. L. Cerny, and J. N. A. Hooper, *Bioorg. Med. Chem. Lett.*, 1993, **3**, 2869; (c) G. R. Pettit, J. P. Xu, Z. A. Cichacz, M. D. Williams, A. C. Dorsaz, D. C. Brune, M. R. Boyd, and R. L. Cerny, *Bioorg. Med. Chem. Lett.*, 1994, **4**, 2091; (d) G. R. Pettit, J. P. Xu, Z. A. Cichacz, M. D. Williams, J. C. Chapuis, and R. L. Cerny, *Bioorg. Med. Chem. Lett.*, 1994, **4**, 2677; (e) G. R. Pettit, J.-p. Xu, Z. Cichacz, J. M. Schmidt, A.-C. Dorsaz, M. R. Boyd, and R. L. Cerny, *Heterocycles*, 1995, **40**, 501; (f) G. R. Pettit, R. Tan, Y. Ichihara, M. D. Williams, D. L. Doubek, L. Tackett, J. M. Schmidt, R. L. Cerny, M. R. Boyd, and J. N. Hooper, *J. Nat. Prod.*, 1995, **58**, 961; (g) G. R. Pettit, J. P. Xu, A. C. Dorsaz, and M. D. Williams, *Bioorg. Med. Chem. Lett.*, 1995, **5**, 1339; (h) W. L. Li, Y. H. Yi, H. M. Wu, Q. Z. Xu, H. F. Tang, D. Z. Zhou, H. W. Lin, and Z. H. Wang, *J. Nat. Prod.*, 2003, **66**, 146; (i) G. R. Pettit and R. Tan, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 685; (j) G. R. Pettit and R. Tan, *J. Nat. Prod.*, 2005, **68**, 60; (k) H. J. Zhang, Y. H. Yi, G. J. Yang, M. Y. Hu, G. D. Cao, F. Yang, and H. W. Lin, *J. Nat. Prod.*, 2010, **73**, 650.
5. (a) R. Mohammed, J. Peng, M. Kelly, and M. T. Hamann, *J. Nat. Prod.*, 2006, **69**, 1739; (b) G. Schmidt, A. Grube, and M. Köck, *Eur. J. Org. Chem.*, 2007, 4103; (c) C. Cychon and M. Köck, *J. Nat. Prod.*, 2010, **73**, 738; (d) M. Arai, Y. Yamano, M. Fujita, A. Setiawan, and M. Kobayashi, *Bioorg. Med. Chem. Lett.*, 2012, **22**, 1818; (e) M. Kita, B. Gise, A. Kawahara, and H. Kigoshi, *Tetrahedron Lett.*, 2013, **54**, 6826; (f) X. Wang, B. I. Morinaka, and T. F. Molinski, *J. Nat. Prod.*, 2014, **77**, 625; (g) A. H. Afifi, A. H. El-Desoky, H. Kato, R. E. P. Mangindaan, N. J. de Voogd, N. M. Ammar, M. S. Hifnawy, and S. Tsukamoto, *Tetrahedron Lett.*, 2016, **57**, 1285.
6. D. E. Dorman and F. A. Borvey, *J. Org. Chem.*, 1973, **38**, 1719.

7. D. E. Dorman and F. A. Borvey, *J. Org. Chem.*, 1973, **38**, 2379.
8. I. Z. Siemion, T. Wieland, and K. H. Pook, *Angew. Chem., Int. Ed. Engl.*, 1975, **14**, 702.
9. P. Marfey, *Carlsberg Res. Commun.*, 1984, **49**, 591.
10. H.-J. Zhang, Y.-H. Yi, G.-J. Yang, M.-Y. Hu, G.-D. Cao, F. Yang, and H.-W. Lin, *J. Nat. Prod.*, 2010, **73**, 650.