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New cyclic tetrapeptides and asteltoxins from gorgonian-derived fungus *Aspergillus* sp. SCSGAF 0076

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ABSTRACT

Three new cyclic tetrapeptides, aspergillipeptides A–C (**1**–**3**), and one new asteltoxin B (**4**) were isolated from a culture broth of gorgonian-derived fungal strain *Aspergillus* sp. SCSGAF 0076. Their structures were elucidated by spectroscopic analysis, and the absolute configurations of aspergillipeptides A–C were determined by Marfey's method. Aspergillide C (**3**) showed strong antifouling activity against *Bugula neritina* larvae settlement with $LC_{50}/EC_{50}>25$.

1. Introduction

Marine surface-associated microorganisms have proven to be a rich source of novel bioactive metabolites due to the necessity to evolve allelochemicals capable of protecting the producers from the fierce competition that exists between microorganisms on the surfaces of marine eukaryotes.[1] As an important part of these microbes, in recent years, coral-associated fungi have attracted many attentions, which led to the isolation of a series of new bioactive compounds.[2–5] During the course of our ongoing pursuit for novel and bioactive compounds from coral-associated fungi, we found a culture broth of the fungal strain *Aspergillus* sp. 0076 that was isolated from the South China Sea gorgonian *Melitodes squamata*, exhibiting strong antifouling activity against *Bugula neritina* larvae settlement. Continuous chemical study on the culture broth of this fungal strain led to the isolation of three new cyclic tetrapeptides aspergillipeptides A–C (**1**–**3**), one new asteltoxin B (**4**), and a known asteltoxin (**5**).[6] Antifouling activities of compounds **1**–**3** were evaluated in settlement inhibition assays with laboratory-reared *B. neritina* larvae, and compounds **1**–**5** were also tested for antibacterial and enzyme-inhibitory activities. In this paper, we describe the isolation, structure elucidation, and bioactivities of compounds **1**–**5**.

2. Results and discussion

Marine-derived fungal strain SCSGAF 0076 was isolated from the South China Sea gorgonian *Melitodes squamata*, and it was identified as *Aspergillus* sp. by ITS gene sequences analysis. The fungal strain was inoculated in liquid medium under static condition and finally harvested and extracted with EtOAc. The extract was fractionated by chromatographic techniques including silica gel column, Sephadex LH-20, and semipreparative HPLC to yield compounds **1**–**5**.

Compound **1** was isolated as a pale yellow solid. Its molecular formula of $C_{26}H_{36}N_4O_7$ was determined by HRESIMS (*m*/*z* 517.2643 $[M+H]^+$, calcd for 517.2657), ^1H and ^{13}C NMR spectra. The presence of five downfield carbon signals for amide or ester carbonyl groups at δ_C 172.0, 171.81, 171.76, 170.9 and 165.9 in the ^{13}C NMR spectrum (Table 1), and four amide proton signals at δ_H 8.81, 8.78, 8.08 and 7.35 in the ^1H NMR spectrum (Table 1), revealed that **1** should be a peptide. HSQC, HMBC and ^1H–^1H COSY spectral data (Fig. 1) displayed that the ^{13}C resonances at δ_C 165.9 (s, C-18), 118.2 (d, C-19), 139.7 (d, C-20), 125.8 (s, C-21), 129.2 (d, C-22), 115.7 (d, C-23), 158.9 (s, C-24), as well as ^1H resonances at δ_H 7.45 (2H, d, J=8.5 Hz, H-22), 6.82 (2H, d, J=8.5 Hz, H-23), 6.87 (1H, d, J=16 Hz, H-19), 7.40 (1H, d, J=16 Hz, H-20), 9.90 (1H, s, OH) were assigned to a 3-(4′-hydroxylphenyl)-2-propenamide (HPPA) residue (Fig. 1). The coupling constant of $J_{H\text{-}19/H\text{-}20}$ (16 Hz) revealed *E*-configuration of the double bond C_{19}=C_{20}. More detailed analysis of HSQC, HMBC and ^1H-^1H COSY data (Fig. 1)

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Table 1
^{1}H and ^{13}C NMR spectroscopic data for **1**–**3** (500 and 125 MHz, respectively, in DMSO-d_6, δ ppm)

Residue	Position	Aspergillipeptide A (**1**)		Aspergillipeptide B (**2**)		Aspergillipeptide C (**3**)	
		δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
AMPA	1	171.8, s		171.8, s		171.8, s	
	2	39.8, d	2.67 (m)	39.8, d	2.67 (m)	39.8, d	2.70 (m)
	3	15.0, q	1.04 (d, 7.0)	14.9, q	1.02 (d, 7.0)	15.0, q	1.04 (d, 7.0)
	4	41.3, t	3.27 (m)	41.1, t	3.23 (m)	41.2, t	3.27 (m)
	NH		7.35 (t, 6.0)		7.30 (t, 6.0)		7.42 (t, 6.0)
Ala	5	172.0, s		171.9, s		172.0, s	
	6	49.3, d	3.97 (m)	49.2, d	3.96 (m)	49.2, d	3.94 (t, 6.0)
	7	16.5, q	1.23 (d, 7.5)	16.5, q	1.23 (d, 7.5)	16.5, q	1.23 (d, 6.5)
	NH		8.81 (d, 7.0)		8.81 (d, 6.5)		8.80 (br s)
Leu	8	171.8, s		171.8, s		171.7, s	
	9	52.1, d	4.22 (dd, 7.5, 14)	52.0, d	4.22 (m)	52.1, d	4.20 (br s)
	10	38.4, t	1.49 (m)	38.3, t	1.52 (t, 7.0)	38.3, t	1.48 (m)
	11	24.1, d	1.58 (m)	24.1, d	1.56 (m)	24.1, d	1.57 (m)
	12	22.4, q	0.93 (d, 6.5)	22.3, q	0.94 (d, 6.5)	22.4, q	0.93 (d, 6.5)
	13	22.0, q	0.86 (d, 6.5)	22.0, q	0.87 (d, 6.5)	22.0, q	0.86 (d, 6.5)
	NH		8.78 (d, 6.5)		8.77 (d, 6.0)		8.77 (br s)
Thr	14	170.9, s		170.8, s		170.9, s	
	15	54.1, d	4.82 (d, 9.5)	54.1, d	4.73 (d, 9.0)	54.1, d	4.81 (d, 8.5)
	16	71.5, d	5.08 (m)	71.2, d	5.08 (m)	71.5, d	5.06 (m)
	17	16.0, q	1.09 (d, 6.5)	15.9, q	1.10 (d, 6.0)	16.0, q	1.09 (d, 6.5)
	NH		8.08 (d, 9.5)		8.16 (d, 9.5)		8.10 (d, 9.0)
HPPA	18	165.9, s		166.2, s		165.9, s	
	19	118.2, d	6.87 (d, 16)	120.0, d	6.10 (d, 13)	118.0, d	6.79 (d, 15.5)
	20	139.7,d	7.40 (d, 15.5)	138.1, d	6.65(d, 13)	140.0,d	7.30 (d, 15.5)
	21	125.8, s		126.1, s		126.3, s	
	22	129.2, d	7.45 (d, 8.5)	132.1, d	7.69 (d, 8.5)	120.4, d	6.89 (dd, 1.5, 8.5)
	23	115.7, d	6.82 (d, 8.5)	114.6, d	6.70 (d, 8.5)	115.6, d	6.76 (d, 8.0)
	24	158.9, s		157.9, s		145.5, s	
	25	115.7, d	6.82 (d, 8.5)	114.6, d	6.70 (d, 8.5)	147.4, s	
	26	129.2, d	7.45 (d, 8.5)	132.1, d	7.69 (d, 8.5)	114.1, d	7.00 (s)
	24-OH		9.90 (s)		9.75 (s)		
	25-OH		—		—		

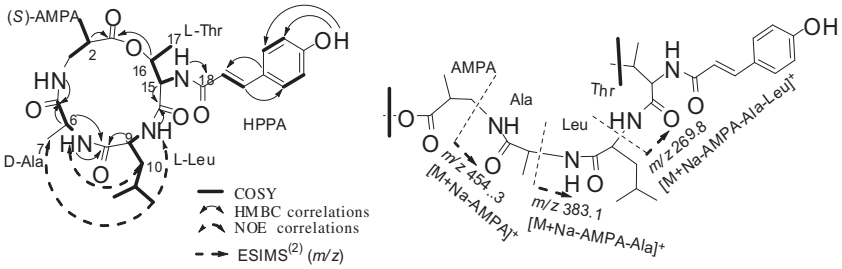

Fig. 1. Key ^{1}H–^{1}H COSY, HMBC, NOESY correlations and (+)-ESIMS$^{(2)}$ fragmentations of **1**.

led to the routine identification of leucine (Leu), threonine (Thr), alanine (Ala) and 3-amino-2-methylpropanoic acid (AMPA) residues (Fig. 1, Table 1), and established the two substructures of Leu-Thr-HPPA and Ala-AMPA. The connection of Leu with Ala was established by HMBC correlation from Ala-NH to C-8 and NOE correlations from Me-7 to Leu-NH, and from H-10 to Ala-NH (Fig. 1), and connection of AMPA with Thr by an ester group was inferred from HMBC correlation of H-16 with C-1 (Fig. 1). These data established the cycle tetrapeptide skeleton of (HPPA-Thr)-Leu-Ala-AMPA in **1**, which was further proved by the key ESIMS$^{(2)}$ fragmentations of **1** (Fig. 1).

Hydrolysis of **1** in 6 N HCl followed by Marfey's analysis established the configurations of amino acids in **1** as D-Ala, L-Leu, and L-Thr. Regretfully, the Marfey derivatives of standard (*R*) and (*S*)-AMPA had identical retention times, and (*R*) and (*S*)-AMPA also could not be identified by MCI GEL™ CRS10W packed column. The configuration of AMPA residue was finally determined by purifying the hydrolysate of **1** by ion-exchange resin column to offer pure AMPA and testing its optical rotation value. The tested $[\alpha]_D^{20}$ value of AMPA (+8, *c*, 0.1, H_2O) was compared with reported data,[7] which suggested that the AMPA residue was (*S*)-AMPA. Therefore, the structure of **1** was determined as shown and named aspergillipeptide A.

Compound **2** was obtained as a pale yellow solid. It had the same molecular formula of $C_{26}H_{36}N_4O_7$ as **1** inferred from HRESIMS (*m*/*z* 517.2651 $[M+H]^+$, calcd for 517.2657), ^{1}H and ^{13}C NMR spectra. The ^{1}H and ^{13}C NMR data of **2** (Table 1) showed great similarity to those of **1** with the only obvious difference of high-field shift of chemical shifts of H-19 [δ_H 6.10 (d, J=13 Hz) in **2** and δ_H 6.87 (d, J=16 Hz) in **1**] and H-20 [δ_H 6.65 (d, J=13 Hz) in **2** and δ_H 7.40 (d, J=16 Hz) in **1**]. These data indicated that **1** and **2** were a pair of geometric isomer of the double bond C_{19}=C_{20}. The coupling constant of $J_{H\text{-}19/H\text{-}20}$ (13 Hz) revealed *Z*-configuration of C_{19}=C_{20} in **2**[8,9] instead of *E*-configuration ($J_{H\text{-}19/H\text{-}20}$=13 Hz in **1**). The high-field chemical shifts of H-19 and H-20 was due to the shielding effect of 18-carbonyl.[10,11] The sequences and absolute configurations of amino acid units in **2** were confirmed as **1** by 2D NMR spectral data including HSQC, HMBC, ^{1}H–^{1}H COSY and NOESY spectra, chemical degradation, and Marfey's method. Therefore, the structure of **2** was determined as shown and named aspergillipeptide B.

Compound **3** was acquired as a brown-yellow solid, having a molecular formula of $C_{26}H_{36}N_4O_8$ deduced from HRESIMS (*m*/*z* 533.2607 $[M+H]^+$, calcd for 533.2611), ^{1}H and ^{13}C NMR spectra. The ^{1}H and ^{13}C NMR data of **3** (Table 1) also showed great similarity to those of **1**, and the only obvious difference between them was the

presence of one 3,4-dihydroxy-1,3,4-trisubstituted phenyl ring [δ_H 6.76 (1H, d, J=8.0 Hz), 6.89 (1H, dd, J=1.5, 8.5 Hz), δ 7.00 (1H, s) and δ_C 114.1 (d), 115.6 (s), 120.4 (d), 126.3 (s), 145.5 (s), 147.4 (s)] in **3** instead of one 4-hydroxy-1,4-disubstituted phenyl ring in **1**, which was supported by HMBC and ^{1}H–^{1}H COSY spectra of **3**. Based on all the 2D NMR data including HSQC, HMBC, ^{1}H–^{1}H COSY and NOESY spectra, chemical degradation, and Marfey's method, the structure of **3** was determined as shown and named aspergillipeptide C.

Compound **4** was isolated as a yellow solid with molecular formula of $C_{23}H_{30}O_8$ deduced from HRESIMS (m/z 435.2030 $[M+H]^+$, calcd for 435.2013), ^{1}H and ^{13}C NMR spectra. The ^{1}H and ^{13}C data of **4** showed great similarity to those of asteltoxin (**5**)[6] and the only obvious difference between them was the presence of two additional oxymethines [δ_C 77.2 (d), 83.4 (d), δ_H 3.91 (1H, br s), 4.38 (1H, t, J=7.0 Hz)] in **4** instead of two olefinic methines in **5**. Considering that the molecular mass of **4** was larger 16 than **5**, these data suggested that one 1,2-disubstituted double bond was oxygenated to a ternary oxygen ring. ^{1}H–^{1}H COSY spectrum of **4** (Fig. 2) showed correlations of H-8 (δ_H 4.81) with H-9 (δ_H 3.91), H-9 with H-10 (δ_H 4.38) and H-10 with H-11 (δ_H 5.98), which indicated that the ternary oxygen ring was established by C-9, C-10 and oxygen atom. Consistent with those observations, detailed analysis of ^{1}H–^{1}H COSY, HSQC and HMBC data (Fig. 2) established the plane structure of **4**. Relative configuration of **4** was further determined by NOESY spectrum. NOE correlations of H-3 (δ_H 4.10) with H-20 (δ_H 1.39), H-20 with H-7 (δ_H 4.27), H-21 (δ_H1.11) with H-7/H-6 (δ_H 5.47)/H-8 (δ_H 4.81), H-8 with H-9 and H-3 with H-10 indicated that H-3, H-6, H-7, H-8, H-9, H-10, Me-20 and Me-21 were α-configuration. Correspondingly, the ethyl at C-3 and hydroxyl at C-4 were β-configuration, which was different from those of **5**. Therefore, the structure of **4** was determined as shown and named asteltoxin B.

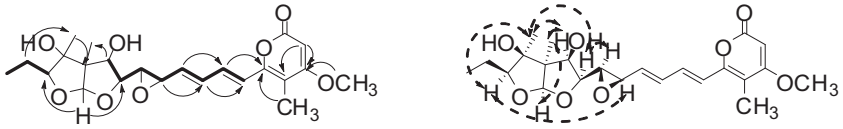

Fig. 2. Key ^{1}H–^{1}H COSY, HMBC and NOESY correlations of compound **4**.

Antifouling activities of compounds **1**–**3** were tested in larvae settlement inhibition assays with *B. neritina* larvae. Compound **3** showed strong antifouling activity against *B. neritina* larvae settlement with EC_{50} value of 11 μg/ml and LC_{50}>300 μg/ml. Usually, the standard requirement of an efficacy EC_{50} level for natural antifoulant is 25 μg/ml, and a compound with LC_{50}/EC_{50}>15 is often considered as a non-toxic antifouling compound.[12] This indicated that **3** was a promising candidate for natural antifoulant. It was also an evidence to explain the hypothesis that coral-associated microbes are a possible first line of chemical defense for corals, possibly through production of bioactive substances.[13]

Compounds **1**–**5** were also tested for antibacterial activity towards bacteria *Bacillus subtilis* and *Escherichia coli*, and enzyme-inhibitory activities towards enzymes of cathepsin B, human monoacylglycerol lipase, human leukocyte elastase, inosine monophosphate dehydrogenase, proteasome, protein tyrosine phosphatase 1B, and Src homology 2 (SH2) domain containing phosphotyrosinephosphatase 2 (SHP2). Unfortunately, all these compound showed no or weak activity.

3. Experimental section

3.1. General experimental procedures

UV spectra were obtained using a Shimadzu UV-1750 spectrophotometer. ^{1}H, ^{13}C NMR and 2D NMR spectra were recorded on a Bruker AV-500 MHz NMR spectrometer with TMS as internal standard. IR spectra were got on a Shimadzu IR Affinity-1. Optical rotations were measured with a Perkin Elmer 341 polarimeter. HRESIMS spectra were measured on a Bruker microTOF-QII mass spectrometer. semipreparative HPLC was operated on a Shimadzu LC-20AT pump with a Shimadzu SPD-M20A Photodiode Array Detector using an YMC-Pack ODS-A column (250×10 mm, 5 μm). Amino acid was analysed on a MCI GELTM CRS10W packed column (4.6×50 mm). Column chromatography (CC) was performed on Silica gel (200–300 mesh, Qingdao Marine Chemical), Sephadex LH-20 or Rp-18 (Pharmacia Co. ODS).

3.2. Fungal material

The fungus SCSGAF 0076 strain was isolated from gorgonian *Melitodes squamata* collected from the South China Sea, Sanya (18°11′N, 109°25′E), Hainan Province, China. It was identified as *Aspergillus* sp. based on a molecular biological protocol calling for DNA amplification and ITS region sequence comparison with the GenBank database and shared a similarity of 100% with *Aspergillus* sp. OY10607 (JN851015). It was deposited in RNAM center, South China Sea Institute of Oceanology, Chinese Academy of Sciences.

3.3. Fermentation and extraction

Aspergillus sp. SCSGAF 0076 was maintained on PDA agar medium. A spore suspension and mycelium were inoculated into 500 ml flask containing 150 ml liquid medium consisting of 2.0% sorbic alcohol, 0.3% yeast extract, 2.0% Maltose, 1.0% monosodium glutamate, 0.05% tryptophan, 0.03%, $MgSO_4 \cdot 7H_2O$, 0.05% KH_2PO_4 and 3.0% marine salt (pH 6.5). The flasks were incubated on a rotary shaker (200 rpm) at 28 °C for 3 days. Then this seed culture (10 ml) was transferred into 1000 ml flask containing 300 ml liquid medium as above. The flasks were grown statically at 28 °C for 30 days.

The total 40 L fermentation broth was harvested and filtered through cheesecloth to separate the broth supernatant and mycelia. The broth supernatant was extracted with ethyl acetate, while the mycelia were extracted with 80% acetone. The acetone extract was evaporated under reduced pressure to afford an aqueous solution, and then extracted with ethyl acetate. The two organic extracts were combined to give a crude gum (14 g).

3.4. Isolation and purification

The crude extract was subjected to Rp-18 reverse-phase column chromatography eluting with H_2O/MeOH (v/v 95:5–0:100) to give ten fractions (Fr.1–Fr.10). Fr.3 obtained from H_2O/MeOH (v/v 60:40) as elution, was chromatographed on a Sephadex LH-20 column eluting with $CHCl_3$/MeOH (v/v 1:1) to obtain four subfractions. Fr.3–2 (41 mg) was further purified by semipreparative HPLC with MeOH/H_2O (v/v 47:53, 3 ml/min) to yield **3** (t_R=26.5 min, 10 mg). Fr.4 obtained from H_2O/MeOH(v/v 50:50) as elution, was subjected to silica gel column chromatography eluting with $CHCl_3$/MeOH (v/v 100:0–50:50) to give five subfractions. Fr.4–5 (120 mg) was further purified by Semipreparative HPLC eluting with MeOH/H_2O (v/v 50:50, 3 ml/min) to afford **1** (t_R=16.0 min, 40 mg) and **2** (t_R=20.0 min, 15 mg). Fr.4–3 (65 mg) was applied to preparation TLC plate to give Fr.4–3–1, and then was further purified by

semipreparative HPLC with eluting CH_3CN/H_2O (v/v 35:65, 3 ml/min) to obtain **4** (t_R=25.0 min, 3.5 mg) and **5** (t_R=28.5 min, 2.5 mg).

3.5. Characteristics of compounds

*3.5.1. Aspergillide A (**1**).* Pale yellow solid; $[\alpha]_D^{20}$ +49 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε): 224 (3.87), 300 (4.00), 310 (4.01) nm; IR (KBr) ν_{max}: 3306, 3060, 2959, 2940, 1728, 1653, 1640, 1517, 1457, 1382, 1272, 1215, 1104, 1079 cm^{-1}; ^1H and ^{13}C NMR spectral data, see Table 1; (+)-HRESIMS *m*/*z* 517.2643 $[M+H]^+$ (calcd for $C_{26}H_{37}N_4O_7$, 517.2657).

*3.5.2. Aspergillide B (**2**).* Pale yellow solid; $[\alpha]_D^{20}$ +18 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε): 224 (3.87), 296 (3.99), 306 (4.01) nm; IR ν_{max}: 3277, 3047, 2958, 2939, 1728, 1647, 1604, 1514, 1456, 1381, 1271, 1232, 1215, 1172, 1078; ^1H and ^{13}C NMR spectral data, see Table 1; (+)-HRESIMS *m*/*z* 517.2651 $[M+H]^+$ (calcd for $C_{26}H_{37}N_4O_7$, 517.2657).

*3.5.3. Aspergillide C (**3**).* Brown solid; $[\alpha]_D^{20}$ +63 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε): 216 (3.86), 293 (3.99), 323 (4.03) nm; IR ν_{max}: 3323, 3016, 2958, 2941, 1732, 1651, 1604, 1527, 1456, 1384, 1269, 1215, 1026; ^1H and ^{13}C NMR spectral data, see Table 1; (+)-HRESIMS *m*/*z* 533.2607 $[M+H]^+$ (calcd for $C_{26}H_{37}N_4O_8$, 533.2611).

*3.5.4. Asteltoxin B (**4**).* Yellow solid;$[\alpha]_D^{20}$ +76 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 223 (3.87), 288 (3.98), 330 (4.04) nm; IR ν_{max} 3390, 3271, 2929, 1722, 1691, 1678, 1631, 1408, 1253, 1060 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ_H: 1.06 (3H, t, *J*=7.43 Hz, H-1), 1.58 (2H, m, H-2), 4.10 (1H, dd, *J*=3.7, 9.05 Hz, H-3), 5.47 (1H, s, H-6), 4.27 (1H, d, *J*=4.05 Hz, H-7), 4.81 (1H, t, *J*=4.4 Hz, H-8), 3.91 (1H, br s, H-9), 4.38 (1H, t, *J*=7.0 Hz, H-10), 5.98 (1H, dd, *J*=6.5, 15.2 Hz, H-11), 6.47 (1H, dd, *J*=11.1, 15.2 Hz, H-12), 7.17 (1H, dd, *J*=11.1, 15.1 Hz, H-13), 6.39 (1H, d, *J*=15.2 Hz, H-14), 5.51(1H, s, H-18), 1.39 (3H, s, H-20), 1.11 (3H, s, H-21), 1.96 (3H, s, H-22), 3.83 (3H, s, H-23); ^{13}C NMR ($CDCl_3$, 125 MHz) δ_C : 11.5 (q, C-1), 22.4 (t, C-2), 90.8 (d, C-3), 80.6 (s, C-4), 62.7 (s, C-5), 113.9 (d, C-6), 87.4 (d, C-7), 86.3 (d, C-8), 77.2 (d, C-9), 83.4 (d, C-10), 136.2 (d, C-11), 131.2 (d, C-12), 134.8 (d, C-13), 120.5 (d, C-14), 154.1 (s, C-15), 108.6 (s, C-16), 170.7 (s, C-17), 89.3 (d, C-18), 163.8 (s, C-19), 19.1 (q, C-20), 15.1 (q, C-21), 9.1 (q, C-22), 56.4 (q, C-23); (+)-HRESIMS *m*/*z* 435.2030 $[M+H]^+$ (calcd for $C_{23}H_{31}O_8$, 435.2013).

3.6. Determination of absolute configuration of 1–3 (Marfey's method[14])

Compounds **1**–**3** (0.5 mg, each) were separately dissolved in 6 N HCl (1 ml) in a sealed glass bottle and incubates at 115 °C for 17 h. After cooling, the resultant hydrolysate was dried to remove the remaining HCl and dissolved in 100 μl of H_2O. Then 1 M $NaHCO_3$ (20 μl) and 1% FDAA (Marfey's reagent, 100 μl) were added and the mixture was incubated at 40 °C for 1 h. The reaction was quenched by adding 2 N HCl (10 μl). The mixture was dissolved in MeOH (500 μl) and analysed by HPLC (YMC-Pack ODS-A column, 250×10 mm) with gradient elution CH_3CN/H_2O with 0.04% TFA (from 15% to 45% acetonitrile during 45 min, flow rate 1.0 ml/min, at 340 nm). The standard amino acids D-Ala, L-Ala, D-Leu, L-Leu, D-Thr, L-Thr, D/L-AMPA were treated as the above process. FDAA derivates of **1**–**3** and standard amino acids were compared with retention times.

Retention times in minutes for the FDAA derivates of standard amino acids were as follows: L-Ala 22.6, D-Ala 26.4, L-Leu 37.4, D-Leu 42.7, L-Thr 17.2, D-Thr 21.5, D/L-AMPA 25.8 and amino acid residues from compounds **1**–**3** were deduced to be D-Ala, L-Leu, L-Thr. In order to further confirm the difference between L-Thr and L-allo-Thr, another analysis method by HPLC with gradient elution CH_3CN/H_2O with 0.04% TFA (from 15% to 20% acetonitrile during 45 min, flow rate 1.0 ml/min, at 340 nm) was applied, and the Thr residue was finally determined to be L-Thr instead of L-allo-Thr.

The configuration of AMPA residue was determined by purifying the hydrolysate of **1** to offer pure AMPA and testing its optical rotation value. Firstly, compound **1** (5.0 mg) was dissolved in 6 N HCl (2 ml) in a sealed glass bottle and incubated at 115 °C for 17 h. After cooling, the resultant hydrolysate was dried to remove the remaining HCl and dissolved in 1 ml of H_2O. Then, it was subjected to strong acid styrene type cation exchange resin column, eluting with pH 4.3 citric acid–NaOH–HCl buffer to offer the mixture of D-Ala, L-Leu, L-Thr and HPPA, and then eluting with 2 N $NH_3 \cdot H_2O$ to offer AMPA. The tested $[\alpha]_D^{20}$ value of AMPA (+8, *c*, 0.1, H_2O) suggested that the AMPA residue was (*S*)-AMPA.[7]

3.7. Larval settlement bioassays

Antifouling activity of compounds **1**–**3** was evaluated in settlement inhibition assays with laboratory-reared *B. neritina* larvae. Larval settlement bioassays were performed using sterile 24-well polystyrene plates as previously reported.[15] Briefly, the stock solution of tested samples in DMSO was diluted with autoclaved filtered sea water (FSW) to concentrations ranging from 5 to 300 ppm. Then the EC_{50} and LC_{50} values of active compounds were calculated. In this way, about 20 competent larvae were added to each well in 1 ml of the test solution. Wells containing only FSW with DMSO served as the controls. Three replicates of each treatment were used. The plates were incubated at 27 °C for 1 h. The percentage of larval settlement was determined by counting the settled, live individuals under a dissecting microscope and expressing the result as a proportion of the total number of larvae in the well. EC_{50} (inhibits 50% of settlement of *B. neritina* larvae in comparison with the control) and LC_{50} (refers to the concentration that kills 50% of the test organisms in comparison with the control) levels of active compounds were calculated by using the Excel software program.

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Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tet.2013.01.021.

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