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Amino Acid Conjugated Anthraquinones from the Marine-Derived Fungus *Penicillium* sp. SCSIO sof101

Minghe Luo,[†,‡] Zhaomeng Cui,[§] Hongbo Huang,[†] Xianqin Song,[‡] Aijun Sun,[†] Yongjun Dang,[§] Laichun Lu,[*,‡] and Jianhua Ju[*,†,⊥]

[†]CAS Key Laboratory of Tropical Marine Bio-resources and Ecology, Guangdong Key Laboratory of Marine Materia Medica, RNAM Center for Marine Microbiology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, 164 Xingang Road West, Guangzhou 510301, People's Republic of China

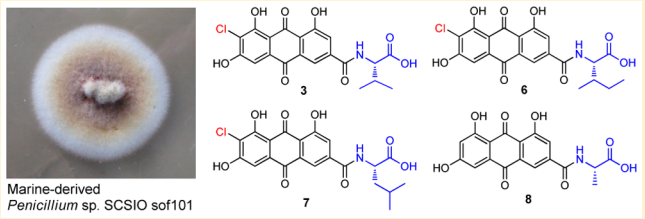
[‡]Department of Pharmacy, Institute of Surgery Research, Daping Hospital, and College of Pharmacy, Third Military Medical University, 10 Changjiang Branch Road, Chongqing 400042, People's Republic of China

[§]Key Laboratory of Molecular Medicine, Ministry of Education, and Department of Biochemistry and Molecular Biology, Fudan University Shanghai Medical College, 130 Dong An Road, Shanghai 200032, People's Republic of China

[⊥]University of Chinese Academy of Sciences, 19 Yuquan Road, Beijing 10049, People's Republic of China

Supporting Information

ABSTRACT: Emodacidamides A–H (**1**–**8**), natural products featuring anthraquinone–amino acid conjugates, have been isolated from a marine-derived fungus, *Penicillium* sp. SCSIO sof101, together with known anthraquinones **9** and **10**. The planar structures of **1**–**8** were elucidated using a combination of NMR spectroscopy and mass spectrometry. The absolute configurations of the amino acid residues were confirmed using Marfey's method and chiral-phase HPLC analyses. Additionally, isolates were evaluated for possible immunomodulatory and cytotoxic activities. Emodacidamides A (**1**), C (**3**), D (**4**), and E (**5**) inhibited interleukin-2 secretion from Jurkat cells with IC_{50} values of 4.1, 5.1, 12, and 5.4 μM, respectively.



Anthraquinones represent a large group of type II PKS-derived natural products usually found in higher plants and fungi, especially in fungal members of the genera *Aspergillus*, *Pyrenochaeta*, and *Pestalotiopsis*.[1] Anthraquinone derivatives possess various biological activities and are widely used as anticancer, antimalarial, and laxative agents. For example, mitoxantrone is a clinically relevant chemotherapy drug,[2] and emodin and rhein are used as cathartic agents.[3] A typical anthraquinone isolated from rhubarb, rhein (also known as cassic acid) has served as an important lead compound from which amino acid conjugates with radiosensitizing activities in H460 human lung cancer cells (*in vitro*) have been generated and studied.[4] Despite the clinical potential of such anthraquinone–amino acid conjugates, such species have never been identified from natural sources.

Marine-derived fungi now serve as important and manageable sources of bioactive natural products; anticancer, antibacterial, antiplasmodial, anti-inflammatory, and antiviral activities are but a handful of the activities associated with such natural products.[5−8] During recent screenings for new secondary metabolites from fungi originating from the South China Sea, we discovered halogenated anthraquinones from *Aspergillus* sp. SCSIO F063,[9] as well as cytotoxic cycloheptapeptides and cytochalasins.[10,11] Recently, metabolomics analysis and subsequent chemical investigations of *Penicillium* sp. SCSIO sof101 enabled the isolation of 10 anthraquinone compounds, including eight new anthraquinones representing rhein–amino acid conjugates herein regarded as emodacidamides A–H (**1**–**8**), together with two known anthraquinones (**9** and **10**). We report herein the production, isolation, structure elucidation, and biological activities of these marine-derived fungal compounds.

RESULTS AND DISCUSSION

The fungus *Penicillium* sp. SCSIO sof101 was fermented in rice solid medium supplemented with 0.2% yeast extract and 3% sea salt on a 6 kg scale. The culture was extracted three times with MeOH, affording, after solvent removal *in vacuo*, a residue that was subjected to silica gel column chromatography (CC), followed by Sephadex LH-20 CC and semipreparative HPLC, to yield emodacidamides A–H (**1**–**8**) and two previously reported compounds, 2-chloro-1,3,8-trihydroxy-6-(hydroxymethyl)anthracene-9,10-dione (**9**)[12] and emodic acid (**10**).[13] Anthraquinones **9** and **10** were identified on the basis of spectroscopic data comparisons to previously reported data sets.

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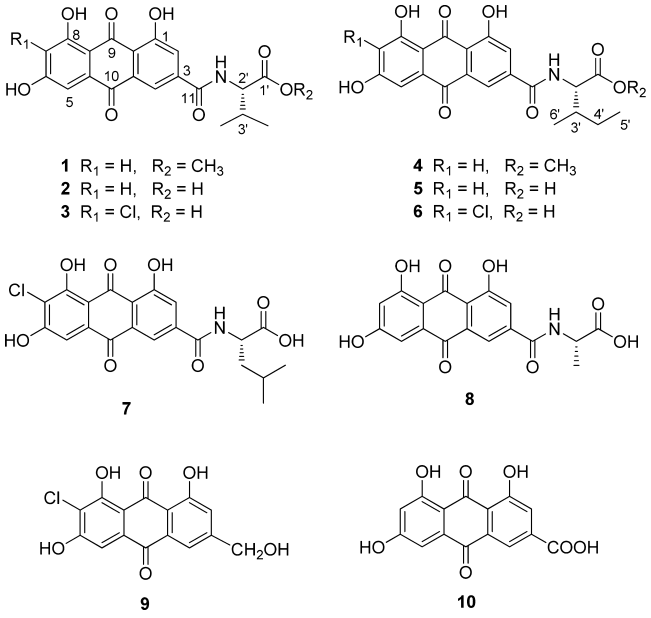

Compound **1** was obtained as a yellow powder, and its molecular formula established as $C_{21}H_{19}NO_8$ on the basis of (+)-HRESIMS, which showed a protonated molecule peak at *m/z* 414.1173 ($[M + H]^+$) and a sodium adduct ion peak at *m/z* 436.0990 ($[M + Na]^+$), indicating 13 degrees of unsaturation. The ^{1}H NMR spectrum of **1** (Table 1) was characterized by resonances consistent with two hydrogen-bonded phenol moieties at δ_H 12.09 (br s, OH-1) and 12.01 (br s, OH-8), one NH proton at δ_H 9.09 (1H, d, J = 8.0 Hz), two pairs of *meta*-coupled aromatic protons at δ_H 7.79 and 8.11 (d, J = 1.6 Hz, H-2 and H-4) and 7.17 and 6.63 (d, J = 2.2 Hz, H-5 and H-7), one nitrogen- or oxygen-bearing methine proton at δ_H 4.32 (t, J = 7.6 Hz, H-2′), one methine proton at δ_H 2.21 (m, H-3′), one methoxy resonance at δ_H 3.67 (s, OMe), and two doublet methyl protons at δ_H 0.94 (d, J = 6.8 Hz, H-5′) and 0.99 (d, J = 6.8 Hz, H-4′). The ^{13}C NMR spectroscopic data (Table 2) unveiled signals corresponding to two carbonyls (δ_C 189.5, 181.1, C-9, 10), four aromatic methine carbons, and eight aromatic nonprotonated carbons, including three oxygen-bearing aromatic nonprotonated carbons at δ_C 165.9 (C-6), 164.7 (C-8), and 161.0 (C-1) with chemical shifts attributable to a highly substituted anthraquinone scaffold, as well as two ester or amide carbonyls at δ_C 171.9 (C-1′) and 165.0 (C-11), two methine carbons at δ_C 58.9 (C-2′) and 29.5 (C-3′), one methoxy carbon at δ_C 51.8, and two methyl carbons at δ_C 19.1 (C-4′, 5′). The two phenolic hydroxy groups at δ_H 12.09 and 12.01 were postulated to associate with the carbonyl (C-9) via intramolecular H-bonding on the basis of their chemical shifts, suggesting the presence of a 1,8-dihydroxyanthraquinone moiety.[9] In the HMBC spectrum, correlations from the aromatic protons H-4 and H-5 to the carbonyl (C-10) localized the two protons to C-4 and C-5, respectively. Subsequent HMBC correlations of H-2 to C-1, C-4, H-4 to C-2, C-9a, H-5 to C-6, C-7, C-8a, and H-7 to C-5, C-6, C-8a established a 3-substituted-1,6,8-trihydroxyanthraquinone moiety (Figure 1). The carbonyl at δ_C 165.0 (C-11) was linked to C-3 on the basis of HMBC correlations for H-2/C-11 and H-4/C-11. A valine residue was elucidated by the HMBC correlations of H-2′/C-1′, C-3′, H-3′/C-2′, C-4′, C-5′, H-4′/C-2′, C-3′, C-5′, H-5′/C-2′, C-3′, C-4′, and NH/C-1′ (Figure 1). The important HMBC correlations from an NH proton and H-2′ to C-11 confirmed the presence of an amide bond between C-2′ and C-11. The methoxy group was connected to the carbonyl (C-1′) of the Val residue, as revealed by an HMBC correlation of $-OCH_3$ and C-1′.

The absolute configuration of the Val residue was determined using Marfey's method. Hydrolysis of compound **1** followed by HPLC analysis of both the hydrolysates and Val standards (after derivatization with FDAA) revealed that an L-Val was, in fact, present in **1** (Figure S41). Compound **1** was consequently named emodacidamide A.

Compound **2** was isolated as a yellow powder, and its molecular formula was found to be $C_{20}H_{17}NO_8$ on the basis of HRESIMS; the formula of **2** was thus 14 mass units less that of **1**. The ^{1}H and ^{13}C NMR spectroscopic data of **2** were very similar to those of **1** (Tables 1 and 2), although the methoxy signals were missing in **2**, suggesting that the methoxy group of **1** was replaced in **2** by an OH moiety. On the basis of Marfey's method, the amino acid residue was assigned as L-Val (Figure S41). Compound **2** was correspondingly named emodacidamide B.

Table 1. Summary of ^{1}H (500 MHz) NMR Spectroscopic Data for Compounds 1−8

	1[a]	2[a]	3[b]	4[a]	5[a]	6[b]	7[b]	8[a]
position	δ_H mult. (J in Hz)	δ_H mult. (J in Hz)	δ_H mult. (J in Hz)	δ_H mult. (J in Hz)	δ_H mult. (J in Hz)	δ_H mult. (J in Hz)	δ_H mult. (J in Hz)	δ_H mult. (J in Hz)
1-OH	12.09, br s	11.99, br s		12.13, br s	12.10, br s			12.14, br s
2	7.79, d (1.6)	7.74, d (1.5)	7.59, s	7.78, d (1.5)	7.77, s	7.37, s	7.49, s	7.78, s
4	8.11, d (1.6)	8.05, d (1.5)	7.97, s	8.11, d (1.5)	8.09, s	7.68, s	7.85, s	8.13, s
5	7.17, d (2.2)	7.08, d (2.5)	7.14, s	7.17, d (2.5)	7.14, s	6.79, s	6.93, s	7.15, s
6-OH	11.51, br s	9.53, br s		11.70, br s	11.49, br s			11.64, br s
7	6.63, d (2.2)	6.52, d (2.5)		6.62, d (2.5)	6.57, s			6.60, s
8-OH	12.01, br s	11.95, br s		12.04, br s	12.10, br s			12.05, br s
2′	4.32, t (7.6)	4.30, t (7.5)	4.52, d (6.0)	4.38, t (7.5)	4.34, t (7.5)	4.55, d (5.5)	4.68, br s	4.44, m
3′	2.21, m	2.22, m	2.33, m	1.99, m	1.97, m	2.07, m	1.78, m; 1.88, m	1.42, d (7.0)
4′	0.99, d (6.8)	1.02, d (6.8)	1.10, d (7.0)	1.28, m; 1.51, m	1.27, m; 1.52, m	1.39, m; 1.68, m	1.84, m	
5′	0.94, d (6.8)	0.99, d (6.8)	1.09, d (7.0)	0.88, t (7.5)	0.88, t (7.0)	1.01, t (7.5)	1.02, d (6.0)	
6′				0.91, d (7.5)	0.94, d (6.5)	1.06, d (7.0)	1.04, d (6.0)	
NH	9.09, d (8.0)	8.88, d (8.0)		9.08, d (7.5)	8.90, d (7.5)			9.11, d (7.0)
OMe	3.67, s			3.67, s				

[a]Recorded in DMSO-d_6. [b]Recorded in CD_3OD.

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Table 2. Summary of ^{13}C (125 MHz) NMR Spectroscopic Data for Compounds 1−8

	1[a]	2[a]	3[b]	4[a]	5[a]	6[b]	7[b]	8[a]
position	δ_C	δ_C	δ_C	δ_C	δ_C	δ_C	δ_C	δ_C
1	161.0, C	161.1, C	163.2, C	160.9, C	161.0, C	162.9, C	163.1, C	161.1, C
2	123.0, CH	123.0, CH	124.3, CH	122.8, CH	122.9, CH	124.3, CH	124.3, CH	122.7, CH
3	140.5, C	140.8, C	142.9, C	133.3, C	135.1, C	142.6, C	142.6, C	140.5, C
4	118.1, CH	118.2, CH	119.2, CH	118.0, CH	118.0, CH	119.0, CH	119.2, CH	117.6, CH
4a	133.4, C	133.2, C	134.7, C	135.1, C	133.3, C	134.1, C	134.4, C	133.5, C
5	109.0, CH	109.1, CH	109.7, CH	109.1, CH	108.8, CH	109.5, CH	109.7, CH	109.1, CH
6	165.9, C	166.1, C	163.2, C	166.1, C	166.9, C	162.8, C	163.1, C	166.6, C
7	108.1, CH	108.0, CH	115.3, C	108.0, CH	108.0, CH	115.3, C	115.4, C	108.2, CH
8	164.7, C	164.7, C	161.7, C	164.6, C	164.8, C	161.4, C	161.6, C	164.8, C
8a	109.4, C	109.2, C	110.6, C	109.2, C	109.7, C	110.1, C	110.3, C	109.5, C
9	189.5, C	189.3, C	191.4, C	189.3, C	188.9, C	190.7, C	190.9, C	189.2, C[c]
9a	117.5, C	117.3, C	118.4, C	117.5, C	117.5, C	117.8, C	118.2, C	117.8, C
10	181.1, C	180.9, C	181.5, C	181.0, C	181.2, C	180.8, C	181.1, C	181.2, C
11	165.0, C	164.9, C	168.1, C	164.8, C	164.8, C	167.6, C	167.7, C	164.1, C
10a	135.2, C	135.1, C	133.5, C	140.4, C	140.7, C	132.8, C	133.2, C	135.2, C
1′	171.9, C	172.8, C	174.9, C[c]	171.8, C	172.8, C	174.9, C	176.5, C[c]	173.9, C
2′	58.9, CH	58.7, CH	60.3, CH	57.5, CH	57.6, CH	59.2, CH	53.3, CH	48.5, CH
3′	29.5, CH	29.5, CH	31.8, CH	35.5, CH	35.7, CH	38.3, CH	41.5, CH_2	16.7, CH_3
4′	19.1, CH_3	19.3, CH_3	19.8, CH_3	25.2, CH_2	25.2, CH_2	26.7, CH_2	26.4, CH	
5′	19.1, CH_3	18.9, CH_3	19.1, CH_3	10.8, CH_3	11.1, CH_3	11.9, CH_3	23.7, CH_3	
6′				15.4, CH_3	15.6, CH_3	16.2, CH_3	22.1, CH_3	
OMe	51.8, CH_3			51.6, CH_3				

[a]Recorded in DMSO-d_6. [b]Recorded in CD_3OD. [c]Assigned on the basis of HMBC correlation.

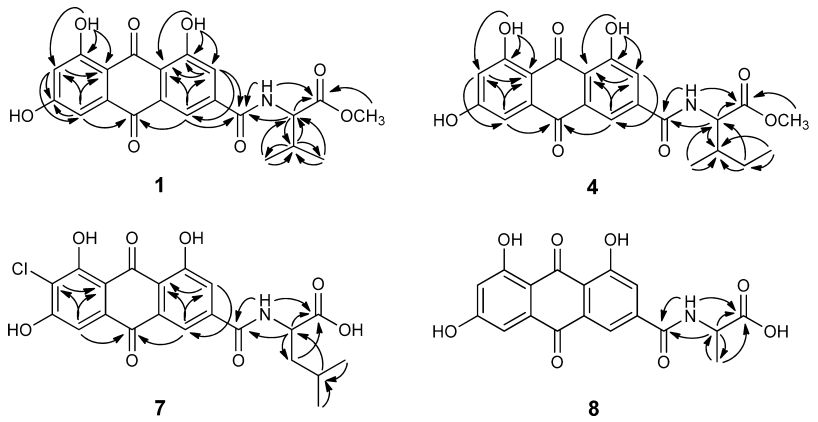

Figure 1. Key HMBC correlations for compounds 1, 4, 7, and 8.

Compound 3 was acquired as a yellow powder with a molecular formula of $C_{20}H_{16}ClNO_8$ as determined by HRESIMS. The presence of a chlorine in 3 was based on the ca. 3:1 ratio of isotopic peak intensities for the deprotonated molecule peaks at m/z 432.0489 $[M - H]^-$ and at m/z 434.0471 $[M - H + 2]^-$ identified in the HRESIMS spectrum. The ^1H NMR spectroscopic data for 3 were almost identical to those of 2 (Tables 1 and 2) with the exception of the absence of an H-7 signal. Moreover, the ^{13}C NMR resonance of C-7 was shifted from δ_C 108.0 in 2 to δ_C 115.3 in 3, suggesting replacement of H-7 in 2 with a chloride in 3. The HMBC correlations from H-5 to C-6, C-7 confirmed the presence of the C-7 chloro substitution. Again, application of Marfey's method (Figure S41) revealed that the amino acid in 3 is L-Val. Compound 3 was named emodacidamide C.

The molecular formula $C_{22}H_{21}NO_8$ of 4, determined by HRESIMS, is 14 mass units greater in mass than 1. On comparing the ^1H and ^{13}C NMR spectroscopic data of 4 with those of 1 (Tables 1 and 2), additional signals for a methene at δ_H 1.28, 1.51 (H-4′) and δ_C 25.2 (C-4′) were noted for 4. HMBC correlations for H-6′/C-3′, C-5′, H-5′/C-3′, C-4′, C-6′, H-4′/C-2′, C-3′, C-5′, and H-2′/C-1′, C-3′, C-4′, C-5′ confirmed that the Val in 1 was replaced by an Ile residue in compound 4 (Figure 1). Subsequent analyses using Marfey's method and chiral-phase HPLC detection revealed that L-Ile constitutes the amino acid fragment (Figures S41, S42) in 4, which was subsequently referred to as emodacidamide D.

Compound 5 was isolated as a yellow powder; its molecular formula $C_{21}H_{19}NO_8$, determined by HRESIMS, indicated a species 14 amu lighter than 4. ^1H and ^{13}C NMR spectroscopic data for 5 closely resembled those of 4 (Tables 1 and 2) with the exception of signals at δ_H 3.67 and δ_C 51.6 absent in 5. These data implied that the OMe within 4 is replaced by an OH moiety in 5. The presence of L-Ile within compound 5 was determined on the basis of Marfey's method and chiral-phase

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HPLC analyses (Figures S41, S42). Compound **5** was named emodacidamide E.

Compound **6**, with its molecular formula $C_{21}H_{18}ClNO_8$ as determined using HRESIMS, was found to be chlorinated using the same methods applied to the study of **3**. The ^1H and ^{13}C NMR spectroscopic data of **6** were found to be slightly different from those of **5** (Tables 1 and 2). The ^1H NMR signal at δ_H 6.57 (H-7) was missing for **6** and the ^{13}C NMR chemical shift for C-7 was changed from δ_C 108.0 in **5** to δ_C 115.3 in **6**, confirming the presence of a C-7 chloro substitution in **6**. Employing the same derivatization/analysis approach applied to **1**−**5**, it was found that **6** contains L-Ile as its amino acid fragment. Compound **6** was named emodacidamide F.

Compound **7** was found to have the same molecular formula as **6** ($C_{21}H_{18}ClNO_8$) as determined by HRESIMS. Not surprisingly, the ^1H and ^{13}C NMR spectroscopic data for **7** closely paralleled those of **6** (Tables 1 and 2), with the exception that the triplet and doublet signals for methyl protons at δ_H 1.01 and 1.06 in **6** were replaced by two doublet signals at δ_H 1.02 and 1.04 (d, J = 6.0 Hz) in **7**. Moreover, the ^{13}C NMR signals for two methyl groups in **7** were observed at δ_C 23.7 (C-5′) and 22.1 (C-6′). These data suggested that the Ile in **6** was substituted by a Leu residue in **7**. HMBC correlations of H-6′/C-3′, C-4′, C-5′, H-5′/C-3′, C-4′, C-6′, H-4′/C-2′, C-5′, C-6′, H-3′/C-1′, C-5′, C-6′, and H-2′/C-1′, C-4′ (Figure 1), combined with the results of Marfey-based analyses, confirmed the presence of an L-Leu residue in **7** (Figure S41), herein named emodacidamide G.

Compound **8** was isolated as a yellow powder, and its molecular formula determined to be $C_{18}H_{13}NO_8$ on the basis of HRESIMS. In comparing ^1H and ^{13}C NMR spectroscopic data for **8** with those of compounds **1**, **2**, **4**, and **5** (Tables 1 and 2), we found a great deal of similarity among the aromatic signals. However, when considering the aliphatic regions of the relevant spectra, only resonances consistent with a methine group and a methyl group were apparent in **8**, as reflected by signals at δ_H 4.44 (m) and δ_C 48.5 (CH-2′) and δ_H 1.42 (d, J = 7.0 Hz) and δ_C 16.7 (CH_3-3′), respectively. HMBC correlations of H-2′/C-1′, C-3′ and of H-3′/C-1′, C-2′ were consistent with the presence of an Ala residue in **8**. Application of Marfey's method revealed the amino acid in **8** to be L-Ala, and **8** was named emodacidamide F

Although anthraquinones are common in nature, reports of amino acid conjugates of such species have not yet appeared. In this study, new emodacidamides A−H (**1**−**8**), together with two known compounds, **9** and **10**, were evaluated for their abilities to inhibit interleukin 2 (IL-2) secretion by Jurkat cells. Cytotoxic activities of **1**−**10** also were first examined using previously established methods.[14] We found that compounds **1**−**10** were all inactive (IC_{50} > 10 μM) against human T lymphocyte Jurkat cells, the chronic myelogenous leukemia cell line K562, and HeLa cervical cancer cells. Emodacidamides A−H (**1**−**8**) were all more effective at inhibiting IL-2 secretion than emodic acid (**10**) at a concentration of 20 μM, revealing that amino acid appendages clearly enhance cellular IL-2 retention in the Jurkat model. Compounds **1**, **3**, **4**, and **5** inhibited IL-2 secretion with IC_{50} values of 4.1, 5.1, 12, and 5.4 μM, respectively (Table 3, Figure S43).

Table 3. Inhibiting Effects (IC_{50}, μM) of Compounds 1, 3, 4, and 5 on IL-2 Secretion

Tested compounds	IC_{50} (μM)
emodacidamide A (**1**)	4.1
emodacidamide C (**3**)	5.1
emodacidamide D (**4**)	12.0
emodacidamide E (**5**)	5.4
FK506[a]	5.8

[a]Positive control.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were obtained with an MCP 500 polarimeter (Anton Paar). UV spectra were recorded with a U-2910 spectrometer (Hitachi). IR spectra were recorded on an IRAffinity-1 spectrometer (Shimadzu). NMR spectra were recorded with an Avance-500 spectrometer (Bruker) at 500 MHz for ^1H nuclei and 125 MHz for ^{13}C nuclei. Chemical shifts (δ) are given with reference to tetramethylsilane. Mass spectra were obtained on an Amazon SL ion trap instrument and a Maxis quadrupole-time-of-flight mass spectrometer (Bruker). Medium-pressure liquid chromatography (MPLC) was performed on a Cheetah 100 automatic flash chromatograph (Bonna-Agela). Semipreparative HPLC was operated with two 210 solvent delivery modules with a 335 PDA detector (Varian) and an ODS-A column (10 × 250 mm, 5 μm, YMC). Column chromatography was performed using silica gel (100−200 mesh, Qingdao Marine Chemical Corporation, China) and Sephadex LH-20 (Amersham Pharmacia). Natural sea salt is a commercial product (Guangdong Province Salt Industry Group Co., Ltd.). The amino acid standards were purchased from Sigma Chemical Co. All chemicals and solvents were of analytical or chromatographic grade.

Fungal Material. Strain SCSIO sof101 was isolated from a marine sediment sample collected in the South China Sea (112°124′ E, 18°0.541′ N) at a depth of 2448 m in 2011. The fungus was identified using a molecular biological protocol calling for DNA amplification and sequencing of the ITS-5.8S r DNA region.[15] The BLAST sequenced data have been deposited at GenBank (accession no. KM115654). This fungus was preserved at the RNAM Center for Marine Microbiology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, in Guangzhou, China.

Fermentation and Isolation. The fungus *Penicillium* sp. SCSIO sof101, which was maintained on potato dextrose agar supplemented with 3% sea salt, was inoculated into 250 mL Erlenmeyer flasks, each containing 25 mL of potato dextrose broth supplemented with 3% sea salt. Flask cultures were incubated at 28 °C on a rotary shaker at 200 rpm for 2 days as seed cultures. Each of the seed cultures (50 mL) was transferred into 2 L Erlenmeyer flasks containing 400 g of rice solid medium supplemented with 0.2% yeast extract and 3% sea salt. These flasks were incubated at room temperature (rt) for 30 days. The harvested whole culture was extracted three times with MeOH to afford a residue after solvent evaporation. The extract was subjected to silica gel CC using gradient elution with a mixture of $CHCl_3$/MeOH (100:0, 98:2, 96:4, 94:6, 92:8, 90:10, 85:15, 80:20, 50:50, v/v) to give nine fractions, Fr.A1−Fr.A9, respectively. Fr.A(2−5) were combined and isolated by silica gel CC eluting with petroleum ether (PE) and EtOAc mixtures (100:0, 90:10, 80:20, 70:30, 60:40, 40:60, 20:80, 0:100 v/v) to afford Fr.B1−Fr.B8. Fr.B3 and Fr.B4 were isolated by MPLC with an ODS column, eluting with CH_3CN/H_2O from 5/95 to 100/0 (v/v) over 90 min at 18 mL/min to give subfractions Fr.C1−Fr.C10. Fr.C(9−10) were combined and purified by semipreparative HPLC with an ODS column, eluting with CH_3CN/H_2O from 60/40 to 95/5 (v/v) over 20 min (2.5 mL/min) to obtain compounds **1** (15.6 mg) and **4** (12.4 mg). Fr.A(6−8) were suspended in MeOH to get a soluble component and a residue. The MeOH-soluble substance was subjected to silica gel CC using gradient elution with a mixture of $CHCl_3$/MeOH (100:0, 98:2, 96:4, 94:6, 92:8, 90:10, v/v) to give Fr.D1−Fr.D6. Fr.D(3−5) were isolated by MPLC with an ODS column, eluting with CH_3CN/H_2O from 5/95 to 100/0 (v/v) over 90 min at 18 mL/min, to afford Fr.E1−Fr.E7. Fr.E2 was isolated by silica gel CC using gradient elution with a mixture of $CHCl_3$/MeOH (100:0, 99:1, 98:2, 97:3, 96:4, 95:5, 93:7, 90:10, v/v) to give **10** (30.1 mg) and Fr.F2−Fr.F8. Fr.F(5−6) were further purified by semi-

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preparative HPLC with an ODS column using an elution system consisting of solvent A (0.1% HOAc/15% CH_3CN in H_2O) and solvent B (0.1% HOAc/85% CH_3CN in H_2O), eluting with a linear gradient from 30% to 65% solvent B over 20 min (2.5 mL/min) to yield **8** (6.3 mg). Fr.E4 was isolated by semipreparative HPLC with the same system, eluted with a linear gradient of 40% to 80% solvent B over 20 min, to give **2** (16.2 mg) and **9** (18.3 mg). Fr.E6 was applied to silica gel CC eluting with a mixture of EtOAc/MeOH (100:0, 99:1, 98:2, 97:3, 96:4, 95:5, v/v) to give Fr.G1−Fr.G6. Fr.G(1−2) were isolated by semipreparative HPLC using the same system, eluting with a linear gradient of 40% to 80% solvent B over 20 min, to give **3** (20.1 mg) and **5** (19.5 mg). Fr.E7 was purified by semipreparative HPLC with the same system, eluting with a linear gradient of 40% to 80% solvent B over 20 min, to give compounds **6** (14.6 mg) and **7** (17.1 mg).

*Emodacidamide A (**1**):* yellow powder; $[\alpha]^{25}_D$ +120 (*c* 0.04, MeOH); UV (MeOH) λ_{max} (log ε) 219 (4.15), 252 (3.98), 438 (3.69) nm; IR (ATR) ν_{max} 3302, 2970, 1743, 1628, 1533, 1269, 1215 cm^{-1}; ^1H and ^{13}C NMR spectroscopic data, Tables 1 and 2; (+)HRESIMS *m*/*z* 414.1173 $[M + H]^+$ (calcd for $C_{21}H_{20}NO_8$, 414.1183).

*Emodacidamide B (**2**):* yellow powder; $[\alpha]^{25}_D$ +43 (*c* 0.16, MeOH); UV (MeOH) λ_{max} (log ε) 219 (4.08), 250 (3.88), 438 (3.68) nm; IR (ATR) ν_{max} 3300, 2972, 1725, 1628, 1534, 1273, 1217 cm^{-1}; ^1H and ^{13}C NMR spectroscopic data, Tables 1 and 2; (+)HRESIMS *m*/*z* 400.1011 $[M + H]^+$ (calcd for $C_{20}H_{18}NO_8$, 400.1027).

*Emodacidamide C (**3**):* yellow powder; $[\alpha]^{25}_D$ +57 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 218 (4.08), 274 (3.91), 327 (3.39), 435 (3.55) nm; IR (ATR) ν_{max} 3406, 2973, 1720, 1626, 1558, 1396, 1261, 1217 cm^{-1}; ^1H and ^{13}C NMR spectroscopic data, Tables 1 and 2; (−)HRESIMS *m*/*z* 432.0489 $[M - H]^-$ (calcd for $C_{20}H_{15}ClNO_8$, 432.0492).

*Emodacidamide D (**4**):* yellow powder; $[\alpha]^{25}_D$ −337 (*c* 0.12, MeOH); UV (MeOH) λ_{max} (log ε) 220 (4.23), 251 (4.05), 267 (4.00), 438 (3.78) nm; IR (ATR) ν_{max} 3360, 2959, 1732, 1628, 1556, 1396, 1272, 1215 cm^{-1}; ^1H and ^{13}C NMR spectroscopic data, Tables 1 and 2; (+)HRESIMS *m*/*z* 428.1335 $[M + H]^+$ (calcd for $C_{22}H_{22}NO_8$, 428.1340).

*Emodacidamide E (**5**):* yellow powder; $[\alpha]^{25}_D$ −95 (*c* 0.11, MeOH); UV (MeOH) λ_{max} (log ε) 221 (4.50), 250 (4.32), 437 (4.09) nm; IR (ATR) ν_{max} 3354, 2969, 1716, 1628, 1556, 1404, 1269, 1217 cm^{-1}; ^1H and ^{13}C NMR spectroscopic data, Tables 1 and 2; (−)HRESIMS *m*/*z* 412.1011 $[M - H]^-$ (calcd. for $C_{21}H_{18}NO_8$, 412.1038).

*Emodacidamide F (**6**):* red powder; $[\alpha]^{25}_D$ −109 (*c* 0.14, MeOH); UV (MeOH) λ_{max} (log ε) 218 (4.46). 275 (4.33), 434 (4.02) nm; IR (ATR) ν_{max} 3360, 2966, 1723, 1624, 1550, 1390, 1259, 1219 cm^{-1}; ^1H and ^{13}C NMR spectroscopic data, Tables 1 and 2; (+)HRESIMS *m*/*z* 448.0798 $[M + H]^+$ (calcd for $C_{21}H_{19}ClNO_8$, 448.0794).

*Emodacidamide G (**7**):* red powder; $[\alpha]^{25}_D$ −321 (*c* 0.12, MeOH); UV (MeOH) λ_{max} (log ε) 218 (4.38), 274 (4.25), 434 (3.93) nm; IR (ATR) ν_{max} 3310, 2958, 1732, 1626, 1556, 1396, 1259, 1217 cm^{-1}; ^1H and ^{13}C NMR spectroscopic data, Tables 1 and 2; (+)HRESIMS *m*/*z* 448.0791 $[M + H]^+$ (calcd for $C_{21}H_{19}ClNO_8$, 448.0794).

*Emodacidamide H (**8**):* yellow powder; $[\alpha]^{25}_D$ +11 (*c* 0.07, MeOH); UV (MeOH) λ_{max} (log ε) 219 (4.18), 249 (3.94), 268 (3.89), 437 (3.67) nm; IR (ATR) ν_{max} 3330, 2949, 1627, 1553, 1394, 1271, 1222 cm^{-1}; ^1H and ^{13}C NMR spectroscopic data, Tables 1 and 2; (−)HRESIMS *m*/*z* 370.0571 $[M - H]^-$ (calcd for $C_{18}H_{12}NO_8$, 370.0568).

Marfey's Analysis. Compounds **1** (0.18 mg), **2** (0.54 mg), **3** (0.33 mg), **7** (0.46 mg), and **8** (0.80 mg) were each dissolved in 200 μL of 1 M NaOH solution and heated at 70 °C for 5 h, and 235 μL of 1 mol/L HCl solution was then added to each reaction to effect reaction quenching and neutralization. Compounds **4** (0.47 mg), **5** (0.42 mg), and **6** (0.55 mg) were each dissolved in 200 μL of 2 M NaOH solution and heated at 90 °C for 5 h. Reactions were then quenched and neutralized via addition of 235 μL of 2 mol/L HCl. After cooling to rt, hydrolysates were each extracted with EtOAc to remove anthraquinone, and the remaining aqueous fractions were dried under reduced pressure and dissolved in 20 μL of H_2O. Aqueous amino acid-containing samples were then treated with 5 μL of 1% (w/v) 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (FDAA) in acetone and 10 μL of 1 M $NaHCO_3$. Mixtures were then heated at 40 °C for 1.5 h, cooled to rt, and neutralized via addition of 15 μL of 1 M HCl. For each sample, the solvent was then removed under reduced pressure and the remaining residue dissolved into MeOH (500 μL). Aliquots (50 μL) of each sample were then analyzed by HPLC with an ODS column (Alltima C18, 4.6 × 250 mm, 5 μm) using an elution system consisting of solvent A (0.1% TFA/10% CH_3CN in H_2O) and solvent B (0.1% TFA/90% CH_3CN in H_2O). Samples were eluted using a linear gradient of 5% to 30% over the course of 30 min, then 30% to 50% over 10 min, and finally 50% to 100% over the course of 5 min; flow rates were kept constant at 1 mL/min, and UV detection employed 254 nm. Amino acid standards (10 μmol/L) were prepared by dissolving amino acids in 20 μL of H_2O followed by addition of 5 μL of 1% FDAA and 10 μL of 1 M $NaHCO_3$. Reaction solutions were placed into a water bath at 40 °C for 1.5 h and neutralized with 15 μL of 1 mol/L HCl. Mixtures were then processed for HPLC in a fashion similar to that used for sample hydrolysate analyses; the retention times for FDAA derivatives of the D-Val, L-Val, D-Ile, L-Ile, D-Leu, L-Leu, D-Ala, and L-Ala were 24.4, 21.1, 33.5, 28.0, 34.2, 29.0, 20.6, and 17.7 min, respectively. The Val fragments in **1**−**3** were all assigned as L-Val (t_R 21.1 min), the Ile fragments in **4**−**6** were all assigned as L-Ile (t_R 28.1 min), the Leu in **7** was assigned as L-Leu (t_R 29.0 min), and the Ala moiety in **8** was assigned as L-Ala (t_R 17.7 min) (Figure S41).

Chiral-Phase HPLC Analysis. To determine the absolute configurations of Ile in **4**−**6**, chiral-phase HPLC analyses of the alkaline hydrolysates were conducted. Compounds **4** (0.44 mg), **5** (0.56 mg), and **6** (0.72 mg) were each dissolved in 2 M NaOH (200 μL) and heated at 90 °C for 5 h; reactions were then quenched and neutralized via addition of 2 mol/L HCl (235 μL). After cooling to rt, hydrolysates were each extracted with EtOAc to remove cleaved anthraquinones. The dried hydrolysates were purified by Sephadex LH-20 CC, eluting with 80% MeOH. The purified hydrolysates were each dissolved in 80 μL of H_2O, and 15 μL aliquots of each sample were analyzed by chiral-phase HPLC with a chiral-phase column (MCI GEL CRS10W, 4.6 × 50 mm, Mitsubishi Chemical Corporation) using a 2 mM $CuSO_4/H_2O$ solution as the mobile phase at a flow rate of 1 mL/min with UV detection at 254 nm. Alongside these analyses, samples of D-Ile, D-*allo*-Ile, L-Ile, and L-*allo*-Ile were employed as standards against which to compare amino acids derived from the natural products. The retention times for L-*allo*-Ile and L-Ile were 17.8 and 25.2 min, respectively. Hence, Ile residues in **4**−**6** were reliably assigned as L-Ile (25.2 min) (Figure S42)

Interleukin 2 Secretion Assay. Emodacidamides A−H (**1**−**8**), together with known compounds **9** and **10**, were evaluated for their ability to inhibit IL-2 secretion of Jurkat T cells.[14] Cells (1×10^5 cells/well) were seeded into a 96-well plate and incubated with compounds as indicated; DMSO served as the negative control and FK506 (100 nM) as a positive control. After 20 min, phorbol 12-myristate 13-acetate (PMA, 40 nM) and ionomycin (1 μM) were added to each well and incubated at 37 °C for 12 h. IL-2 concentrations were performed as per the manufacturer's instructions (BD OptEIA, Human IL-2 ELISA Set, Cat:555190). Briefly, 100 μL of diluted capture antibody in coating buffer (0.1 M sodium carbonate, pH 9.5, 1:500) was coated onto each well of a 96-well plate overnight (12 h) at 4 °C. After washing with 300 μL of washing buffer (phosphate-buffered saline (PBS) with 0.05% Tween-20) three times, antibody-coated wells were each doped with 50 μL of assay solution (PBS with 10% fetal bovine serum) and incubated with 100 μL of supernatant from Jurkat cells at rt for 2 h. After washing five times, 100 μL of diluted working detector (SAv-conjugated streptavidin 1:500 in assay diluent) was added into each well and incubated at rt for 1 h. After washing seven times, to each flushed well was then added 100 μL of IL-TMB substrate and incubated (protected from light) at 37 °C for 30 min. Reactions were then quenched via addition of 50 μL of stopping solution, and IL-2 concentrations were immediately determined on the basis of absorbances registered at λ = 450 nm.

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Cytotoxicity Assay. Compounds **1**–**10** were tested for their cytotoxic activities against Jurkat, K562, and HeLa cell lines according to the previously reported methods.[14] Cultured cells (2×10^4 cells/well) were seeded into 96-well plates and then incubated with compounds as indicated; DMSO served as the negative control, and doxorubicin was used as the positive control. After 72 h, AlamarBlue dye (1:10 dilution) was added to each well and incubated at 37 °C for 4 h. Cell effects (resulting from cytotoxicity) were then assessed on the basis of measured absorbances at 590 nm.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.7b00269.

HRESIMS and NMR spectra of compounds **1**–**8** (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*Tel/Fax: +86-20-89023028. Tel: +86-23-68757092. E-mail: lulaicq@163.com (L. Lu).
*E-mail: jju@scsio.ac.cn (J. Ju).

ORCID

Hongbo Huang: 0000-0002-5235-739X
Jianhua Ju: 0000-0001-7712-8027

Notes

The authors declare no competing financial interest.

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