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Article

Targeted Isolation of Asperheptatides from a Coral-Derived Fungus Using LC-MS/MS-Based Molecular Networking and Antitubercular Activities of Modified Cinnamate Derivatives

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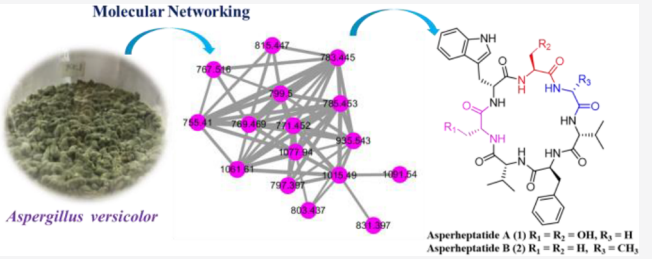
ABSTRACT: Under the guidance of MS/MS-based molecular networking, four new cycloheptapeptides, namely, asperheptatides A–D (**1**–**4**), were isolated together with three known analogues, asperversiamide A–C (**5**–**7**), from the coral-derived fungus *Aspergillus versicolor*. The planar structures of the two major compounds, asperheptatides A and B (**1** and **2**), were determined by comprehensive spectroscopic data analysis. The absolute configurations of the amino acid residues were determined by advanced Marfey's method. The two structurally related trace metabolites, asperheptatides C and D (**3** and **4**), were characterized by ESI-MS/MS fragmentation methods. A series of new derivatives (**8**–**26**) of asperversiamide A (**5**) were semisynthesized. The antitubercular activities of **1**, **2**, and **5**–**26** against *Mycobacterium tuberculosis* H37Ra were also evaluated. Compounds **9**, **13**, **23**, and **24** showed moderate activities with MIC values of 12.5 μM, representing a potential new class of antitubercular agents.



Tuberculosis (TB), a bacterial infection disease caused by *Mycobacterium tuberculosis* (Mtb), is the leading cause of death worldwide.[1,2] In recent years, the emergence of multidrug resistant tuberculosis (MDR-TB) and extensive drug resistant tuberculosis (XDR-TB) has increased the global impact of this disease.[3] WHO estimates that there are 484 000 new cases with resistance to rifampicin (the most effective first-line drug), of which 78% of them are MDR-TB.[4] On the other hand, only three new drugs, namely, delamanid, bedaquiline, and pretomanid, have been approved in the past 40 years to treat patients of MDR-TB and XDR-TB.[5] This is far from enough to deal with highly infectious and often fatal tuberculosis. Moreover, severe side effects of these drugs, such as adverse drug reaction, drug-induced liver injury, and QT interval prolongation, also have hindered their widespread use.[6−10] Therefore, the development of new antitubercular drugs has become one of the key topics in the field of pharmaceutical research on infectious diseases.

In the past few decades, marine natural products (MNPs) as a highly valuable source of new lead compounds have received considerable attention.[2] Especially, some MNPs were discovered to have significant anti-*Mycobacterium tuberculosis* activity, such as pseudopteroxazole, batzelladines L and N, callyaerins A and B, and puupehenoneand 15-cyanopuupehenone.[11−15] In the MNPs workflow, dereplication can speed up the process of discovering new compounds. Hence, molecular networking, a strategy that organizes and analyses MS/MS data based on chemical similarity, is gradually being used in natural product research.[16] It is a powerful approach that is used in natural product dereplication,[17] new compound discovery,[18] and strain screening.[19] Molecular networking can provide guidance and improve efficiency in the discovery of new bioactive substances especially for the discovery of peptides.

The investigation of new bioactive natural products from marine fungi is a major and constant research focus of our laboratory.[20−23] Under the guidance of MS/MS-based molecular networking, four new cycloheptapeptides, asperheptatides A–D (**1**–**4**), were isolated together with three known analogues, asperversiamides A–C (**5**–**7**),[24] from the coral-derived fungus *Aspergillus versicolor* (CHNSCLM-0063). Furthermore, the major metabolite asperversiamide A (**5**) was selected as a scaffold, and a series of its new derivatives (**8**–**26**) was semisynthesized. Herein, we report the discovery, structure elucidation, and antitubercular activities of these compounds.

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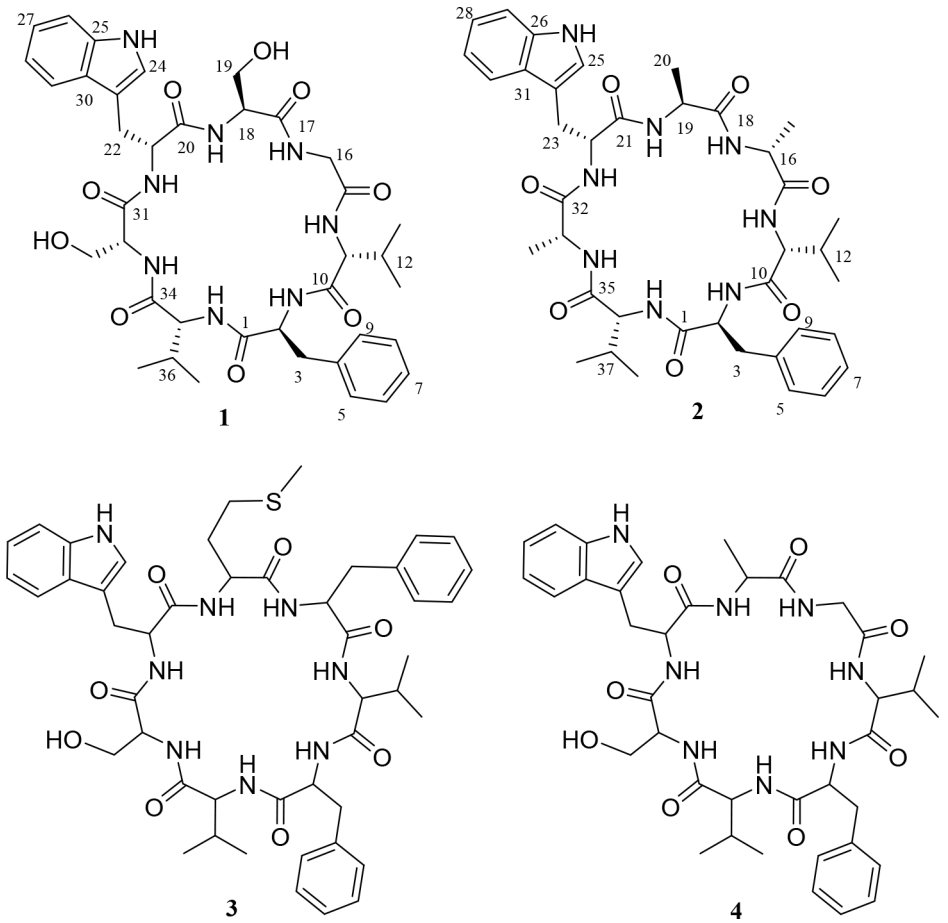

RESULTS AND DISCUSSION

This study applied a molecular networking strategy to accelerate the discovery of new molecules. First, the fungus *A. versicolor* (CHNSCLM-0063) was cultured on a rice solid medium prepared with seawater at room temperature for 50 days. Following extraction and concentration, the EtOAc extract was subjected to untargeted HPLC-MS/MS analysis, and a visualized network was constructed with the converted MS/MS data. The production of the cycloheptapeptide asperversiamides as a strong marker of this strain showed a similar pattern to that in previous studies.[24] After the cluster illustrated in Figure 1 was analyzed, the metabolites with $[M + Na]^+$ m/z 799.50 could be proposed to be asperversiamide A ($C_{39}H_{52}N_8O_9$), and the m/z 783.45 ion represented asperversimides B and C ($C_{39}H_{52}N_8O_8$),[24] which were confirmed by the isolation and identification of these compounds by ^{1}H NMR analysis. The cycloheptapeptide family in this network consists of 16 nodes. Four new asperheptatides A, B, C, and D could be correlated to four of the 16 nodes visualized in the cluster shown in Figure 1, including asperheptatide A ($[M + Na]^+$ m/z 785.45), asperheptatide B ($[M + Na]^+$ m/z 767.52), asperheptatide C ($[M + K]^+$ m/z 935.54), and asperheptatide D ($[M + Na]^+$ m/z 769.47). Thus, efforts were concentrated on this network cluster.

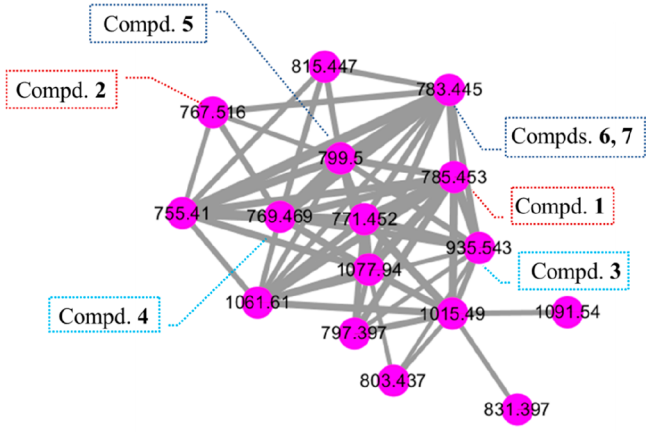

Figure 1. Cluster of nodes from *A. versicolor* with compounds **1**–**7**.

Efficient targeted isolation of these compounds was performed by transferring the analytical profiling conditions based on molecular networking. Four new asperheptatides A–D (**1**–**4**) were purified in addition to the known asperversiamides A–C (**5**–**7**) from the same fraction.

The molecular formula of asperheptatide A (**1**) was determined to be $C_{38}H_{50}N_8O_9$ (18 degrees of unsaturation) on the basis of the observed positive HRESIMS ion at m/z 763.3789 $[M + H]^+$. The ^{1}H NMR spectrum in pyridine-d_5 (Table 1) displayed the characteristics of a typical peptide, illustrating seven amide (NH) signals as well as seven α-amino proton signals in the region of δ_H 5.14–4.44. In its ^{13}C NMR spectrum, seven carbonyl signals were observed (Table 1). Detailed analysis of the 1D and 2D NMR spectra allowed seven amino acid residues to be established, one phenylalanine (Phe), one tryptophan (Trp), two valines (Val), two serines (Ser), and one glycine (Gly). These signals accounted for 17 of

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Table 1. ^{1}H and ^{13}C NMR Assignments for Asperheptatide A (1) in Pyridine-d_5

no.	δ_C, type[b]	δ_H[a] (J in Hz)	HMBC
1	173.3, C		
2	57.3, CH	5.10, m	
3a	38.1, CH_2	3.16, dd (13.2, 7.2)	1
3b		3.41, dd (13.2, 9.2)	
4	138.2, C		
5/9	130.2, CH	7.30, m	
6/8	129.4, CH	7.30, m	
7	127.6, CH	7.25, m	4
2-NH		9.33, s	
10	173.8, C		
11	60.2, CH	4.57, m	10
12	29.7, CH	2.47, m	
13	20.0, CH_3	0.91, d (6.4)	11, 14
14	19.8, CH_3	1.06, d (6.4)	
11-NH		8.05, d (8.3)	15
15	172.3, C		
16	44.2, CH_2	4.44, m	
16-NH		8.80, t (5.6)	15, 17
17	171.7, C		
18	56.8, CH	5.14, m	
19a	62.5, CH_2	4.00, d (10.5)	
19b		4.34, m	
18-NH		9.69, d (7.2)	20
20	174.5, C		
21	58.3, CH	5.10, m	
22a	28.3, CH_2	3.60, dd (14.0, 7.2)	20, 24, 30
22b		3.68, dd (14.0, 7.4)	
23	110.9, C		
24	125.2, CH	7.51, s	
NH		11.97, s	23, 30
25	137.9, C		
26	112.5, CH	7.52, d (8.0)	
27	122.3, CH	7.24, overlapped	
28	119.7, CH	7.10, t (7.6)	26, 30
39	119.5, CH	7.73, d (7.6)	25, 27
30	128.9, C		
21-NH		9.37, d (6.7)	31
31	174.2, C		
32	57.0, CH	5.14, m	
33a	63.2, CH_2	4.44, m	
33b		4.57, m	
32-NH		8.59, d (7.5)	31, 34
34	172.8, C		
35	59.5, CH	4.86, dd (8.6, 5.4)	34
36	29.6, CH	2.61, m	
37	18.1, CH_3	0.79, m	35, 38
38	20.4, CH_3	0.79, m	
35-NH		9.17, d (8.6)	1

[a]Measured at 500 MHz. [b]Measured at 125 MHz.

18 degrees of unsaturation, indicating the final degree of unsaturation arises from the cyclic feature of **1**.

The HMBC correlations from the NHs to its respective adjacent carbonyl established the connectivity and the sequence of the amino acids in **1**. The key HMBC (Figure 2) correlations were observed between Val_2−NH/Phe−CO, Phe−NH/Val_1−CO, Val_1−NH/Gly−CO, Gly−NH/Ser_1−CO, Ser_1−NH/Trp−CO, Trp−NH/Ser_2−CO, and Ser_2−NH/Val_2−CO. Furthermore, the key ESI-MS/MS fragment ions at m/z 646, 499, 400, and 343 revealed the neutral losses of [Val_2], [Val_2−Phe], [Val_2−Phe−Val_1], and [Val_2−Phe−Val_1−Gly] (Figure 3). Thus, the cyclic planar structure of **1** was established as *cyclo*-[(NH)Ser_2−Trp−Ser_1−Gly−Val_1−Phe−Val_2(CO)].

The ^{1}H and ^{13}C NMR spectroscopic features (Table 2) suggested that asperheptatide B (**2**) was very similar to asperheptatide A (**1**). The only significant differences of **2** in comparison with those of **1** were three methyl groups [C-17, δ_H 1.34 (3H), δ_C17.1 (CH_3); C-20, δ_H 1.61 (3H), δ_C18.8 (CH_3); C-34 δ_H 1.74 (3H), δ_C18.1 (CH_3)] in **2** instead of three methylene units [δ_H 4.44 (2H), δ_C44.2 (CH_2); δ_H 4.00 (1H), δ_H 4.34 (1H), δ_C62.5 (CH_2); δ_H 4.44 (1H), δ_H 4.57 (1H), δ_C63.2 (CH_2)] in **1**. Additionally, its mass was 18 Da less than that of asperheptatide A (**1**), indicating that two Sers and one Gly residues in **1** were replaced by three Alas in **2**. The key HMBC (Figure 2) correlations and the ESI-MS/MS (Figure 3) experimental results confirmed the connections of these residues as *cyclo*-[(NH)Ala_2−Trp−Ala_1−Ala_3−Val_1−Phe−Val_2(CO)].

The absolute configurations of the amino acid residues of **1** and **2** were established by HPLC analysis of their acid hydrolysates derivatized with Marfey's reagent (N_α-(2,4-dinitro-5-fluorophenyl)-L-alalinamide, L-FDAA). All derivatives were identified by their retention times on HPLC analysis by comparison to standards, confirming L-Phe, D-Trp, D-Val, D-Ser, and L-Ser in **1** and D-Ala, L-Ala, L-Phe, D-Trp, and D-Val in **2**. On the basis of the absolute configuration of asperversiamides A−C (**5**−**7**) and a shared biogenesis in the same fungus, the locations of L and D-Ser in **1** and L and D-Ala in **2** were assigned. Finally, the chemical structures of asperheptatides A and B (**1** and **2**) were elucidated as *cyclo*-[(NH)D-Ser_2−D-Trp−L-Ser_1−Gly−D-Val_1−L-Phe−D-Val_2(CO)] and *cyclo*-[(NH)D-Ala_2−D-Trp−L-Ala_1−D-Ala_3−D-Val_1−L-Phe−D-Val_2(CO)].

In addition, two trace analogues of asperheptatide A (**1**), namely, asperheptatides C (**3**) and D (**4**), were also tentatively identified from this extract under the guidance of molecular networking. Therefore, their planar structures could be characterized only by the use of ESI-MS/MS fragmentation experiments. The HRESIMS ion at m/z 897.4328 $[M + H]^+$ indicated that molecular weight of **3** was 896. The peaks at m/z 780, 633, 534, and 386 suggested that compound **3** lost Val, Phe, Val, and Phe fragments in turn (Figure 4). Similarly, ions at m/z 648, 483, 443, and 345 implied that compound **4** dropped Val, Phe, Gly, and Val in turn (Figure 4) as well as ions at m/z 561 and 490 intimated the presence of Ser and Val (Figures S26 and S27). Thus, their planar structures were proposed as *cyclo*-[(NH)Ser−Trp−Met−Phe−Val−Phe−Val−(CO)] and *cyclo*-[(NH)Ser−Trp−Ala−Gly−Val−Phe−Val−(CO)].

Cinnamic acid as an important natural substance has shown potential for anti-TB drug discovery.[25] In our previous research, asperversiamide A (**5**) showed moderate activity against *Mycobacterium marinum* with the MIC value of 23.4 μM.[24] Thus, we chose asperversiamide A (**5**) as a scaffold, and a series of its new derivatives (**8**−**26**) were semisynthesized as shown in Scheme 1. Compound **5** was dissolved in CH_2Cl_2 at 50 °C. Then, DMAP, EDC·HCl, and cinnamic acid derivatives were added sequentially and reacted for 2−7 h. The solvents were removed under reduced pressure, and the residues obtained after workup were purified by HPLC with MeCN−H_2O to give derivatives **8**−**26** (Table 3).

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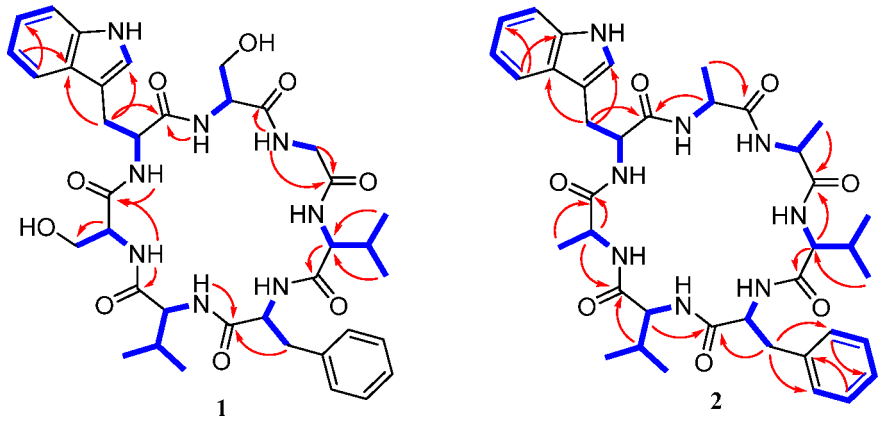

Figure 2. Key COSY, TOCSY (bold), and HMBC (arrows) correlations of **1** and **2**.

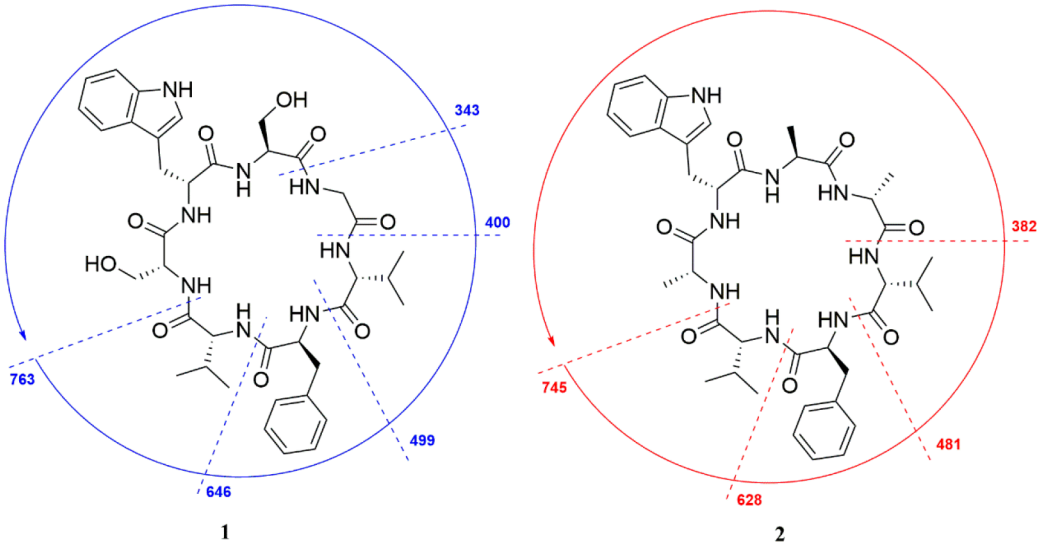

Figure 3. ESI-MS/MS analysis of **1** and **2**.

The structures of **8**–**26** were confirmed by analysis of their ^{1}H NMR, ^{13}C NMR, and HRESIMS data. In the ^{1}H NMR spectrum, the respective chemical shifts of the proton signals were deshielded compared with the data of parental **5** after esterification (Table S1 and Figures S89 and S90). At the same time, obvious double bond hydrogen signals of cinnamic acid were observed (Table S1 and Figure S91). Furthermore, HRESIMS data analysis confirmed the molecular formula of this derivative. In addition, the position of esterification for the monoesterified compound **25** was determined by 1D and 2D NMR data (Figures S79–S84 and S88).

Compounds **1**, **2**, and **5**–**26** were tested for their antitubercular activities against *Mycobacterium tuberculosis* HR37a (Table 4). Compared with the natural product **5**, the assay results suggested that 11 derivatives exhibited increased activities with MIC values ranging from 12.5 to 50 μM. Among them, **9**, **13**, **23**, and **24** displayed moderate antitubercular activities with MIC values of 12.5 μM, approximately 10-fold higher than that of their parent compound. Thus, preliminary structure–activity relationship based on 24 analogues revealed that esterification of 20- and 34-OH simultaneously was effective for improving the antitubercular activity, and the introduction of modified cinnamic acid groups was also important to the activity.

In conclusion, four new cycloheptapeptides, asperheptatides A–D (**1**–**4**), were isolated from *Aspergillus versicolor* under the guidance of molecular networking. A series of new derivatives (**8**–**26**) were semisynthesized, and some of them (**9**, **13**, **23**, and **24**) showed moderate antitubercular activities with MIC values of 12.5 μM. Further investigations are focused on the semisynthesis of these cycloheptapeptides and structure–activity relationship studies.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a JASCO P-1020 digital polarimeter. UV spectra were recorded on a Beckman DU 640 spectrophotometer. IR spectra were recorded on a Nicolet-Nexus-470 spectrometer using KBr pellets. NMR spectra were recorded on a JEOL JEM-ECP NMR spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C), using TMS as an internal standard. HRESIMS and ESI-MS spectra were obtained from a Micromass Q-TOF spectrometer (Waters Ltd.) and a Thermo Scientific LTQ Orbitrap XL spectrometer. HPLC-MS was performed on an Agilent series 1290 Infinity HPLC instrument, coupled with a Q-TOF Mass spectrometer (Agilent, Technologies), with a YMC C18 column [(YMC Co.) YMC-Park, ODS-A, 250 × 2.1 mm, S-5 μm, 12 nm, 0.5 mL/min]. HPLC analysis was performed on a Hitachi L-2000 system (Hitachi Ltd.) using a C18 column [(YMC Co., Ltd.) YMC-Park, ODS-A, 250 × 4.6 mm, S-5 μm, 12 nm, 1.0 mL/min]. Semipreparative HPLC was performed on a Hitachi L-2000 system

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Table 2. ^{1}H and ^{13}C NMR Assignments for Asperheptatide B (2) in Pyridine-d_5

no.	δ_C, type[b]	δ_H[a] (J in Hz)	HMBC
1	173.1, C		
2	56.1, CH	5.25, m	
3a	37.9, CH_2	3.15, dd (13.5, 9.0)	1, 5, 9
3b		3.61, m	
4	138.2, C		
5/9	129.8, CH	7.35, m	
6/8	128.9, CH	7.31, m	4
7	127.0, CH	7.25, m	5, 9
2-NH		9.78, br s	
10	172.5, C		
11	60.7, CH	4.50, t (6.6)	10, 13, 15
12	29.9, CH	2.34, br s	
13	19.0, CH_3	0.88, br s	
14	19.6, CH_3	0.94, m	
11-NH		8.85, br s	14, 15
15	172.7, C		
16	50.2, CH	4.88, dt (13.8, 6.8)	
17	17.1, CH_3	1.34, d (6.4)	15
16-NH		9.49, br s	
18	173.2, C		
19	49.8, CH	5.16, m	21
20	18.8, CH_3	1.61, d (6.8)	18
19-NH		8.70, br s	
21	172.8, C		
22	57.3, CH	4.80, br s	
23a	27.4, CH_2	3.59, m	21, 25, 31
23b		3.80, dd (13.8, 8.5)	
24	110.9, C		
25	124.6, CH	7.40, s	
NH		11.97, s	24
26	138.0, C		
27	112.0, CH	7.63, d (8.0)	
28	121.8, CH	7.31, m	
29	119.3, CH	7.11, t (7.6)	27, 31
30	119.2, CH	7.76, d (7.6)	24, 26, 28
31	128.4, C		
22-NH		9.78, br s	
32	174.5, C		
33	49.8, CH	4.97, m	32, 35
34	18.1, CH_3	1.73, d (6.8)	32
33-NH		8.84, d (6.5)	
35	172.1, C		
36	60.6, CH	4.79, br s	38, 39
37	30.6, CH	2.54, m	35
38	18.4, CH_3	0.93, d (6.8)	
39	19.7, CH_3	0.98, d (6.5)	
36-NH		9.25, br s	

[a]Measured at 500 MHz. [b]Measured at 125 MHz.

(Hitachi Ltd.) using a C18 column [(Eka Ltd.) Kromasil 250 × 10 mm, 5 μm, 2.0 mL/min]. Silica gel (Qingdao Haiyang Chemical Group Co.; 200–300 mesh), octadecylsilyl silica gel (YMC Co., Ltd.; 45–60 μm), and Sephadex LH-20 (GE Ltd.) were used for column chromatography. Precoated silica gel plates (Yantai Zhifu Chemical Group Co.; G60, F-254) were used for thin layer chromatography.

Fungal Material. The fungal strain CHNSCLM-0063 was isolated from the gorgonian coral *Rumphella aggregata* collected from Nansha Islands in the South China Sea in April 2015. The strain was identified as *Aspergillus versicolor* according to morphologic traits and molecular identification. Its 617 base pair ITS sequence had 100% sequence identity to that of *A. versicolor* (AY373882). The sequence data have been submitted to GenBank with the accession number MG736310. The strain was deposited at the Key Laboratory of Marine Drugs, the Ministry of Education of China, School of Medicine and Pharmacy, Ocean University of China, Qingdao, China.

Fermentation and Extraction. The fungus was cultured at room temperature on rice solid medium hydrated with seawater (30 g of natural sea salt (Yangkou saltern), 1L H_2O) for 50 days (500 Erlenmeyer flasks (1000 mL), each containing 50 g of rice and 50 mL of seawater). The fermented rice substrate was extracted three times with 200 mL of EtOAc per flask to give an organic extract (100 g).

LC-MS/MS and Molecular Networking Analysis. LC-MS-MS was performed on an Agilent series 1290 Infinity HPLC instrument, coupled with a Q-TOF Mass spectrometer (Agilent Technologies), with a YMC C18 column [(YMC Co., Ltd.) YMC-Park, ODS-A, 250 × 2.1 mm, S-5 μm, 12 nm, 0.5 mL/min]. Fraction (Fr.) 5-4 (0.5 mg/mL, 10 μL) was analyzed by LC-MS with a gradient program of MeCN–H_2O (0.1% formic acid) [0–2 min 10%, 2–17 min 10–100%, 17–19 min 100%; 0.5 mL/min; MS scan 150–2000 Da] and then with an automated full dependent MS-MS scan. Differentiation of the protonated molecules, adducts, and fragment ions was done by identification of the $[M + H]^+$ ion. All MS/MS data were converted to .mzXML format files using MSConver software. The molecular networking was performed using the GNPS data analysis workflow using spectral clustering algorithm. The spectral networks were imported into Cytoscape 3.7.1 and visualized using the force-directed layout.

Isolation. Based on TLC analysis, the EtOAc extract was subjected to silica gel vacuum liquid chromatography (VLC) to afford five fractions (Fr. 1–Fr. 5) by a gradient of petroleum ether (PE)–EtOAc (PE, 100%–0), EtOAc–MeOH (v:v, 9:1), and MeOH. Fr. 5 was purified on Sephadex LH-20 column chromatography (CC) and eluted with a mixture of CH_2Cl_2–MeOH (v:v, 1:1) to obtain four subfractions (Fr. 5-1–Fr. 5-4). Fr. 5-4 was then repeatedly separated by reversed-phase C18 CC and then purified by HPLC (MeCN–H_2O, 20–80%) to afford compounds **1** (5.0 mg), **2** (5.0 mg), **5** (100 mg), **6** (5.0 mg), and **7** (5.0 mg), together with trace amounts of **3** and **4**.

Asperheptatide A(1). This compound is a white powder; $[\alpha]^{25}_D$ +33 (*c* 0.4, MeOH); UV (MeOH) λ_{max} (log ε) 210 (3.60), 213 (3.63), 281 (3.65), 290 (3.59) nm; IR (KBr) ν_{max} 3296, 1653, 1539 cm^{-1}; ^{1}H and ^{13}C NMR, Table 1; ESI-MS/MS *m/z* 646.30 $[M + H - H_2O - Val]^+$, *m/z* 499.23 $[M + H - H_2O - Val - Phe]^+$, *m/z* 400.16 $[M + H - H_2O - Val - Phe - Val]^+$, *m/z* 343.18 $[M + H - H_2O - Val - Phe - Val - Gly]^+$; HRESIMS *m/z* 763.3789 $[M + H]^+$(calcd for $C_{38}H_{51}N_8O_9^+$, 763.3774).

Asperheptatide B(2). This compound is a white powder; $[\alpha]^{25}_D$ +150 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 205 (3.53), 214 (3.64), 281 (3.55), 290 (3.49), 365 (2.81) nm; IR (KBr) ν_{max} 3304, 1659, 1525 cm^{-1}; ^{1}H and ^{13}C NMR, Table 2; ESI-MS/MS *m/z* 628.33 $[M + H - H_2O - Val]^+$, *m/z* 481.26 $[M + H - H_2O - Val - Phe]^+$, *m/z* 382.23 $[M + H - H_2O - Val - Phe - Val]^+$; HRESIMS *m/z* 745.4043 $[M + H]^+$ (calcd for $C_{39}H_{53}N_8O_7^+$, 745.4032).

Asperheptatide C (3). This compound is a white powder; UV (MeOH) λ_{max} (log ε) 213 (3.62), 281 (3.65), 290 (3.57) nm; ESI-MS/MS *m/z* 879.30 $[M + H - H_2O]^+$, 780.12 $[M + H - H_2O - Val]^+$, *m/z* 711.19 $[M + H - Val - Ser]^+$, *m/z* 633.00 $[M + H - H_2O - Val - Phe]^+$, *m/z* 534.00 $[M + H - H_2O - Val - Phe - Val]^+$, *m/z* 386.84 $[M - H_2O - Val - Phe - Val - Phe]^+$. HRESIMS *m/z* 897.4310 $[M + H]^+$ (calcd for $C_{47}H_{60}N_8O_8S^+$, 897.4328).

Asperheptatide D (4). This compound is a white powder; UV (MeOH) λ_{max} (log ε) 210 (3.64), 281 (3.60), 290 (3.52) nm; ESI-MS/MS *m/z* 729.22 $[M + H - H_2O]^+$, 648.13 $[M + H - Val]^+$, *m/z* 630.12 $[M + H - H_2O - Val]^+$, *m/z* 561.09 $[M + H - Val - Ser]^+$, *m/z* 483.00 $[M + H - H_2O - Val - Phe]^+$, *m/z* 383.93 $[M + H - H_2O - Val - Phe - Val]^+$, *m/z* 345.334 $[M + H - Val - Phe - Val - Gly]^+$. HRESIMS *m/z* 747.3824 $[M + H]^+$ (calcd for $C_{38}H_{50}N_8O_8^+$, 747.3824).

Marfey's Analysis of 1–2. A solution of **1** (0.1 mg) and HCl (6 M, 1 mL) was heated at 70 °C for 10 h. The hydrolyzed solution was

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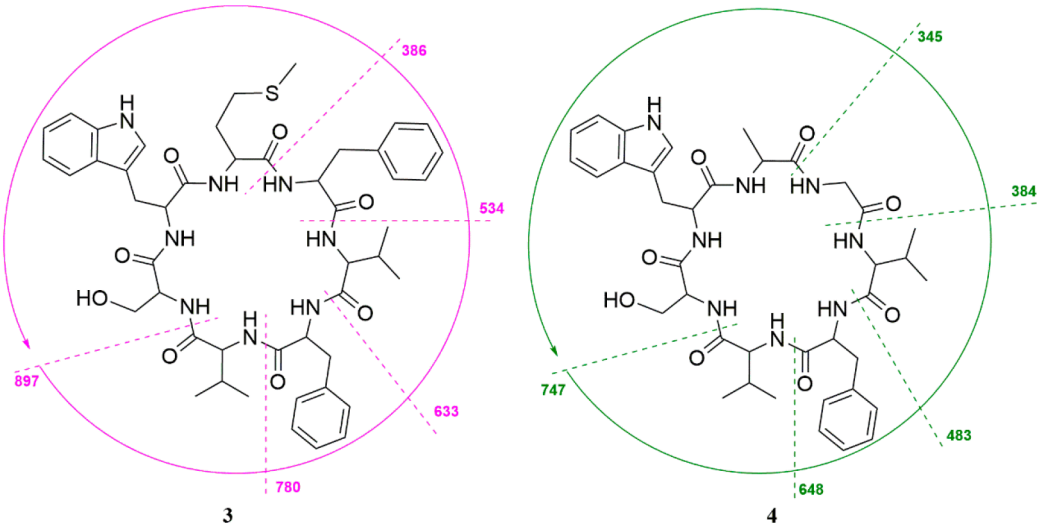
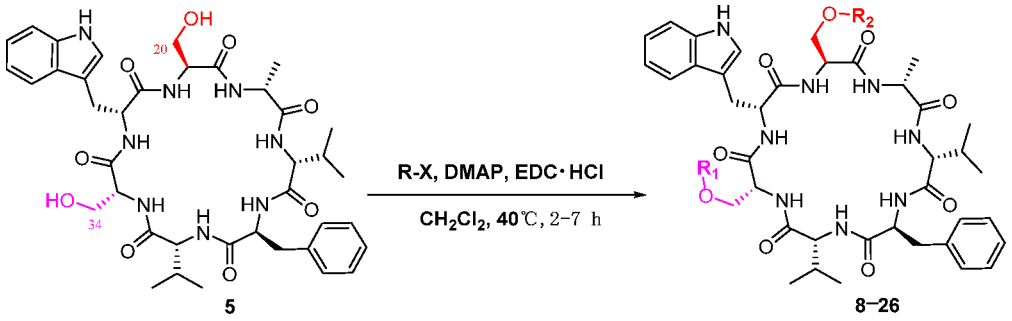


Figure 4. ESI-MS/MS analysis of **3** and **4**.

Scheme 1. General Strategy for Semisynthesis of Asperversiamide A (5) Derivatives



evaporated to dryness and then redissolved in 250 μL of H_2O. The acid hydrolysate (50 μL) was treated with 1% L-FDAA (20 μL) acetone solution and then with $NaHCO_3$ (1 M, 10 μL) solution. The mixture was heated at 60 °C for 1 h. The reaction was stopped with HCl (2 M, 5 μL). Amino acid standards L-Ser, D/L-Ser, L-Val, D/L-Val, L-Trp, D/L-Trp, L-Phe, and D/L-Phe were derivatized with L-FDAA in the same manner. All L-DAA derivatives were analyzed and detected by HPLC eluting with a gradient of MeCN (A) and H_2O (B) (0−30 min, 23.5% A, 30−45 min, 23.5−30% A, 45−126 min, 30−40% A, 1 mL/min, UV 340 nm). The retention times of the L-DAA-derivatized amino acid standards were as follows: D/L-Phe (125.8 min, 105.2 min), L-Phe (105.2 min), D/L-Trp (111.7 min, 99.2 min), L-Trp (99.2 min), D/L-Val (102.7 min, 78.4 min), L-Val (78.4 min), D/L-Ala (63.4 min, 54.3 min), L-Ala (54.3 min), D/L-Ser (34.2 min, 32.1 min), L-Ser (32.1 min). The retention times of the acid hydrolysate derivatives of **1** and **2** were as follows: D/L-Ser (34.2 min, 32.1 min), D-Val (102.7 min), D-Trp (111.7 min), L-Phe (105.2 min), D/L-Ala (63.4 min, 54.3 min).

Preparation of Compound 5 Derivatives. Asperversiamide A (**5**) (1 equiv), the corresponding cinnamic acid derivative (1−4 equiv), and an appropriate amount of DMAP (2 equiv) and EDA·HCl (2 equiv) were added in CH_2Cl_2 (20 mL), and then the reaction mixture was stirred at 40 °C for 2−7 h and then quenched with saturated $NaHCO_3$ solution and diluted with EtOAc. The EtOAc layer was separated and dried under vacuum. Then, the crude residue was purified through silica gel column chromatography followed by semipreparative HPLC to yield compounds **8**−**26**.

Derivative ***8***. This compound is a white powder; ^1H and ^{13}C NMR, Supporting Information; HRESIMS *m*/*z* 1073.4583 $[M + H]^+$ (calcd for $C_{57}H_{63}F_2N_8O_{11}^+$, 1073.4579).

Derivative ***9***. This compound is a white powder; ^1H and ^{13}C NMR, Supporting Information; HRESIMS *m*/*z* 1109.4395 $[M + H]^+$ (calcd for $C_{57}H_{61}F_4N_8O_{11}^+$, 1109.4390).

Derivative ***10***. This compound is a white powder; ^1H and ^{13}C NMR, Supporting Information; HRESIMS *m*/*z* 1173.4519 $[M + H]^+$ (calcd for $C_{59}H_{63}F_6N_8O_{11}^+$, 1173.4515).

Derivative ***11***. This compound is a yellow powder; ^1H and ^{13}C NMR, Supporting Information; HRESIMS *m*/*z* 950.4766 $[M + H]^+$ (calcd for $C_{50}H_{64}N_9O_{10}^+$, 950.4771).

Derivative ***12***. This compound is a yellow powder; ^1H and ^{13}C NMR, Supporting Information; HRESIMS *m*/*z* 1123.5597 $[M + H]^+$ (calcd for $C_{61}H_{75}N_{10}O_{11}^+$, 1123.5611).

Derivative ***13***. This compound is a white powder; ^1H and ^{13}C NMR, Supporting Information; HRESIMS *m*/*z* 937.4453 $[M + H]^+$ (calcd for $C_{49}H_{61}N_8O_{11}^+$, 937.4454).

Derivative ***14***. This compound is a white powder; ^1H and ^{13}C NMR, Supporting Information; HRESIMS *m*/*z* 1097.4982 $[M + H]^+$ (calcd for $C_{59}H_{69}N_8O_{13}^+$, 1097.4979).

Derivative ***15***. This compound is a white powder; ^1H and ^{13}C NMR, Supporting Information; HRESIMS *m*/*z* 1157.5193 $[M + H]^+$ (calcd for $C_{61}H_{73}N_8O_{15}^+$, 1157.5190).

Derivative ***16***. This compound is a white powder; ^1H and ^{13}C NMR, Supporting Information; HRESIMS *m*/*z* 1045.3667 $[M + H]^+$ (calcd for $C_{50}H_{62}BrN_8O_{12}^+$, 1045.3665).

Derivative ***17***. This compound is a white powder; ^1H and ^{13}C NMR, Supporting Information; HRESIMS *m*/*z* 1313.3402 $[M + H]^+$ (calcd for $C_{61}H_{71}Br_2N_8O_{15}^+$, 1313.3400).

Derivative ***18***. This compound is a white powder; ^1H and ^{13}C NMR, Supporting Information; HRESIMS *m*/*z* 1043.4878 $[M + H]^+$ (calcd for $C_{56}H_{67}N_8O_{12}^+$, 1043.4873).

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Table 3. Derivatives 8−26 of Asperversiamide A (5)

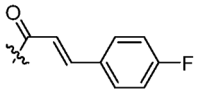
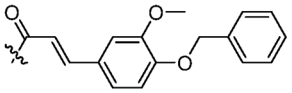
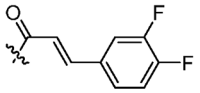
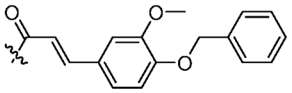
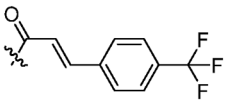
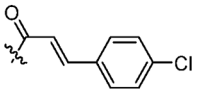
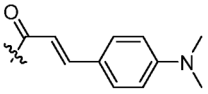
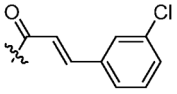
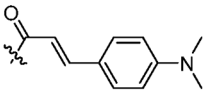
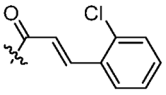
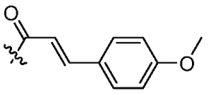
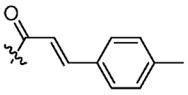
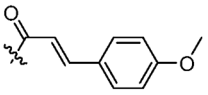
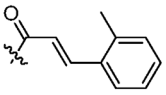
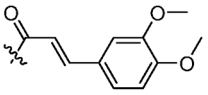
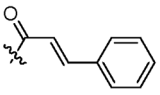
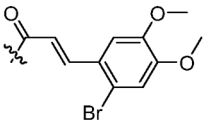
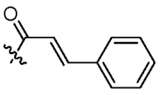
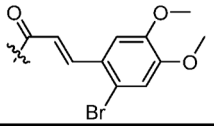
No.	R1	R2	No.	R1	R2
8		R1	18		H
9		R1	19		R1
10		R1	20		R1
11		H	21		R1
12		R1	22		R1
13		H	23		R1
14		R1	24		R1
15		R1	25		H
16		H	26		R1
17		R1			

Table 4. Antitubercular Activity of 1, 2, and 5−26 against *Mycobacterium tuberculosis* H37Ra

no.	MICs (μM)	no.	MICs (μM)
1	100	16	50
2	100	17	100
5	>100	18	50
6	100	19	100
7	100	20	50
8	50	21	100
9	12.5	22	50
10	100	23	12.5
11	50	24	12.5
12	50	25	>100
13	12.5	26	>100
14	100	rifampin	0.00625
15	100	isoniazid	0.0125

Derivative ***19***. This compound is a white powder; ^{1}H and ^{13}C NMR, Supporting Information; HRESIMS m/z 1309.5826 $[M + H]^+$ (calcd for $C_{73}H_{81}N_8O_{15}^+$, 1309.5816).

Derivative ***20***. This compound is a white powder; ^{1}H and ^{13}C NMR, Supporting Information; HRESIMS m/z 1105.3994 $[M + H]^+$ (calcd for $C_{57}H_{63}Cl_2N_8O_{11}^+$, 1105.3988).

Derivative ***21***. This compound is a white powder; ^{1}H and ^{13}C NMR, Supporting Information; HRESIMS m/z 1105.3995 $[M + H]^+$ (calcd for $C_{57}H_{63}Cl_2N_8O_{11}^+$, 1105.3988).

Derivative ***22***. This compound is a white powder; ^{1}H and ^{13}C NMR, Supporting Information; HRESIMS m/z 1105.3995 $[M + H]^+$ (calcd for $C_{57}H_{63}Cl_2N_8O_{11}^+$, 1105.3988).

Derivative ***23***. This compound is a white powder; ^{1}H and ^{13}C NMR, Supporting Information; HRESIMS m/z 1065.5081 $[M + H]^+$ (calcd for $C_{59}H_{69}N_8O_{11}^+$, 1065.5080).

Derivative ***24***. This compound is a white powder; ^{1}H and ^{13}C NMR, Supporting Information; HRESIMS m/z 1065.5070 $[M + H]^+$ (calcd for $C_{59}H_{69}N_8O_{11}^+$, 1065.5080).

Derivative ***25***. This compound is a white powder; ^{1}H and ^{13}C NMR, Supporting Information; HRESIMS m/z 907.4368 $[M + H]^+$ (calcd for $C_{48}H_{59}N_8O_{10}^+$, 907.4349).

Derivative ***26***. This compound is a white powder; ^{1}H and ^{13}C NMR, Supporting Information; HRESIMS m/z 1037.4770 $[M + H]^+$ (calcd for $C_{57}H_{65}N_8O_{11}^+$, 1037.4767).

Antitubercular Assay. Antimycobacterial activity was determined against *Mycobacterium tuberculosis* H37Ra (ATCC 25177) in a microplate Alamar Blue assay system as described previously.[26] The antitubercular drugs rifampin and isoniazid were used as positive controls.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jnatprod.0c00804.

LC-MS/MS-derived molecular networking of extracts, HPLC analyses, HRESIMS of compounds, ^{1}H NMR spectra, ^{13}C NMR spectra, HSQC spectra, ^{1}H-^{1}H COSY spectra, HMBC spectra, TOCSY spectra, ESI-MS/MS of compounds, and ^{13}C spectra (PDF)

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[1]These authors contributed equally to this work.

Notes

The authors declare no competing financial interest.

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