Asperelines A−F, Peptaibols from the Marine-Derived Fungus *Trichoderma asperellum*

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Fermentation of the marine-derived fungus *Trichoderma asperellum*, collected from the sediment of the Antarctic Penguin Island, resulted in the isolation of six new peptaibols named asperelines A−F (**1**−**6**), which are characterized by an acetylated *N*-terminus and a *C*-terminus containing an uncommon prolinol residue. Structures were determined by extensive 1D and 2D NMR (^{1}H−^{1}H COSY, HMQC, HMBC, NOESY) spectroscopic data analysis combined with ESIMS/MS fragmentation. The absolute configurations of the amino acid residues possessing a chiral α-carbon and of the prolinol residue were determined to be L and *S*, respectively, using a new method of ^{1}H NMR spectroscopic comparison of complexes formed between the chiral reagent Ru(D$_4$-Por*)CO and amino acids derived from the peptaibols with those formed with reference standards.

Peptaibols are a class of linear or cyclic peptides characterized by chain lengths of 4−21 amino acid residues, which are usually classified according to their chain length, as long-chain (17−21 residues), medium-chain (11−16 residues), and short-chain (4−10 residues) sequences.[1−3] Their structural patterns feature an abundance of α-aminoisobutyric acid residues (Aib), an *N*-acyl terminus such as an acetyl group, and a *C*-terminus containing an amino alcohol residue (phenylalaninol, leucinol, valinol, and others). Generally, the trivial name of peptaibols represents a group of peptides containing a number of Aib residues and 2-amino alcohols. In addition, the unique α-dialkyl amino acid residues of peptaibols such as Acc, Lva, and EtNva are also included in the peptaibol family.[1,4] Naturally occurring peptaibols are mixtures of isoforms, and more than 850 sequences have been isolated from at least 23 genera of ascomycetous fungi or their anamorphs.[1−8] These peptides are regarded to be synthesized nonribosomally by large multidomain enzymes.[9,10] They possess diverse bioactivities, as antibacterials, antifungals, and antiparasitics,[11−13] and are used as biocontrol agents against fungal phytopathogens.[14−16]

Trichoderma species (Hyphomycetes) have been studied extensively as potential sources of biocontrol agents, enzymes, and diverse bioactive peptaibols.[17−21] *T. asperellum* is widely distributed in soil and is a mycoparasitic fungus used for antiphytopathogens.[22] It is highly effective against the rice diseases caused by seedborne pathogens.[23] Additionally, it is a rich source of peptaibols, and previous chemical examination led to the isolation of two trichotoxins, 1704E and 1717A,[24] and identification of seven peptaibols by means of "peptaibiomics" using a HPLC-MS^n method,[25] which are characterized by a valinol linked to the *C*-terminus. Trichotoxins A-50[26] and A-40[27] are also produced by a strain of *T. asperellum*, which was originally misidentified as *T. viride*. Lieckfeldt et al.[28] assumed that the *T. viride* biocontrol strains reported to have high-temperature optima are actually *T. asperellum* and that no true *T. viride* strain has been found to produce antibiotics. Thus, all trichotoxins reported to date are considered to be produced by strains of *T. asperellum*. In the course of investigation of new metabolites and their biofunctions from extremophilic microorganisms, a *T. asperellum* strain was isolated from the sediment of the Antarctic Penguin Island. Chemical investigation of its fermentation broth led to the isolation of six new peptaibols (**1**−**6**), all of them featuring a structurally unique prolinol residue at the *C*-terminus and an acetylated *N*-terminus.

Results and Discussion

The chromatographic separation of the fermentation broth (80 L) using Amberlite-XAD, Si gel, and a Sephadex LH-20 column in combination with semipreparative HPLC resulted in the isolation of six pure peptaibols (**1**−**6**).

Aspereline A (**1**) was isolated as colorless crystals. The molecular formula was determined to be $C_{45}H_{80}N_{10}O_{11}$ on the basis of the HRESIMS (*m*/*z* 937.6088 [M + H]$^+$) and NMR data, indicating 11 degrees of unsaturation. The ^{1}H NMR spectrum in DMSO-d_6 displayed nine resonances in the downfield range between δ_H 7.10 and 8.59, which were assignable to the NH protons of amides. The ^{13}C NMR spectrum contained 10 carbonyl signals between δ_C 177.4 and 171.3, confirming **1** to be a peptide. Chemical shift assignments for each amino acid residue were based on the interpretation of ^{1}H, ^{13}C NMR, DEPT, and 2D NMR data including ^{1}H−^{1}H COSY, HMQC, and HMBC, which revealed the presence of nine amino acid residues, involving an isoleucine (Ile), a valine (Val), an alanine (Ala), and six 2-aminoisobutyric acids (Aib-1 to Aib-6), along with acetyl and prolinol residues. For instance, the spin systems of the α-H and NH resonances of Val (δ_H 3.51/δ_H 7.76), Ile (δ_H 3.62/δ_H 7.10), and Ala (δ_H 4.00/δ_H 7.37) and the correlations from the α-methine resonances to the side chains of the individual amino acid residues were clearly observed in the COSY spectrum. Six NH singlets were attributed to the Aib residues, which were proven by the HMBC correlation between NH resonances and the quaternary carbons resonating around δ_C 56, characteristic of the α-C of Aib residues. In addition to an acetyl group represented by a methyl singlet at δ_H 1.93 (3H, s) and its HMBC correlation to a carbonyl carbon at δ_C 171.3 (s), a prolinol ring was recognized from the COSY couplings of H$_2$-5 (δ_H 3.38, 3.40)/H-1 (δ_H 3.95), H-1/H$_2$-2 (δ_H 1.69, 1.75), H$_2$-2/H$_2$-3 (δ_H 1.60, 1.75), and H$_2$-3/H$_2$-4 (δ_H 3.30, 3.59). The carbons corresponding to the protons in the molecule were assigned by a HMQC spectrum. The sequence of amino acid residues was established through the HMBC correlations of the NH and α-H resonances to the amide carbonyl carbons through $^2J_{CH}$ and $^3J_{CH}$ couplings (Table 1). The HMBC correlations of the acetyl methyl protons at δ_H 1.93 (3H, s, H-1) and the NH at

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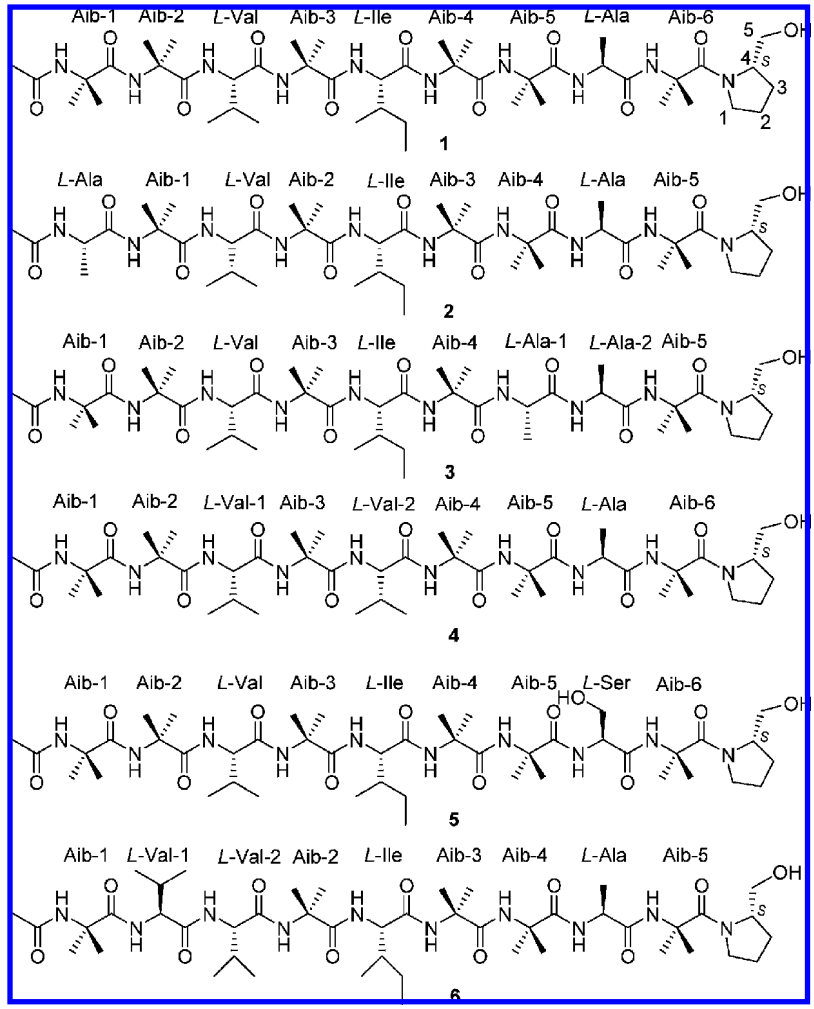

δ_H 8.59 (s) to the carbonyl carbon at δ_C 171.3 (s) indicated that the *N*-terminus of **1** was acetylated. Subsequently, the interactions of NH (δ_H 8.59, s) with δ_C 175.4 (C=O, Aib-1), NH (δ_H 8.41, s) with C=O (Aib-1) and δ_C 177.4 (C=O, Aib-2), NH (δ_H 7.76, d) and α-H (δ_H 3.51, m, Val) with C=O (Aib-2) and δ_C 172.8 (C=O, Val), NH (δ_H 7.53, s) with C=O (Val) and δ_C 175.4 (C=O, Aib-3), NH (δ_H 7.10, d) and α-H (δ_H 3.62, m, Ile) with C=O (Aib-3) and δ_C 173.4 (C=O, Ile), NH (δ_H 7.84, s) with C=O (Ile) and δ_C 174.1 (C=O, Aib-4), NH (δ_H 7.80, s) with C=O (Aib-4) and δ_C 175.4 (C=O, Aib-5), NH (δ_H 7.37, d) and α-H (δ_H 4.00, m, Ala) with C=O (Aib-5) and δ_C 172.3 (C=O, Ala), and NH (δ_H 7.53, s) with C=O (Ala) and δ_C 171.3 (C=O, Aib-6) established the sequence of Ac-Aib-Aib-Val-Aib-Ile-Aib-Aib-Ala-Aib. The prolinol residue was deduced to form an amide at the *C*-terminus on the basis of HMBC correlations from the methylene protons at δ_H 3.30 (1H, m) and 3.59 (1H, m) and the NH (Aib-6) at δ_H 7.53 (s) to the carbonyl carbon at δ_C 171.3. Additionally, the negative ion ESIMS displayed the deprotonated molecule at *m/z* 935 $[M - H]^-$, which was selected as a precursor ion for ESIMS/MS experiments. Analysis of the resulting product ion spectrum indicated that the MS cleavage process followed B-type fragmentation and that fragment ions resulting from sequential loss of the *N*-terminal amino acid residues were observed. For example, ESIMS^2 of the precursor ion produced B-type ions at *m/z* 893 $[M - Ac]^-$, 808 $[M - Ac - Aib]^-$, 723 $[M - Ac - 2Aib]^-$, 624 $[M - Ac - 2Aib - Val]^-$, 539 $[M - Ac - 3Aib - Val]^-$, 426 $[M - Ac - 3Aib - Val - Ile]^-$, 341 $[M - Ac - 4Aib - Val - Ile]^-$, and 256 $[M - Ac - 5Aib - Val - Ile]^-$ (Figure 1). This result further supported the sequence assignment by NMR spectroscopic data analysis. In order to determine the absolute configurations of the individual amino acid residues in the parent peptide, a new method for the ^{1}H NMR spectroscopic comparison of complexes formed between the chiral reagent Ru(D$_4$-Por*)CO[30,31] and amino acids derived from the peptaibols with those formed with reference standards is established. Acidic hydrolysis[29] of **1** followed by derivatization with a chiral reagent Ru(D$_4$-Por*)CO was performed (Figure 2). The configuration of the individual amino acid residue was determined through the comparison of the ^{1}H NMR data of the complex between the L/D standard amino acids and the corresponding residue derived from **1**, analyzed as a mixture. For instance, the complex with L-Ala exhibited resonances at δ −3.00 (α-H), −4.50 (NHa), and −5.69 (NHb), whereas the corresponding protons for the D-Ala complex are at δ −2.83 (α-H), −4.43 (NHa), and −5.70 (NHb). Thus, the ^{1}H NMR signals observed for the Ala complex in the mixture of amino acids being hydrolyzed from **1** (Figure 3) were consistent with the L-form. On the basis of the same protocol, the configuration of Ile in **1** was determined to be the L-form (Figure 3). In addition, the analytical result revealed the L-configuration for Val residue, whereas prolinol possesses the *S*-configuration (see Supporting Information for additional figures).

The molecular formula of aspereline B (**2**) was established as $C_{44}H_{78}N_{10}O_{11}$, based on the HRESIMS (*m/z* 945.5744 $[M + Na]^+$) and NMR data. The molecular weight of **2** was thus 14 amu lower than that of **1**. Analysis of 2D NMR (^{1}H−^{1}H COSY, HMQC, and HMBC) spectroscopic data revealed that the structure of **2** contained spin systems for five Aib, two Ala, one Val, and one Ile, as well as an acetyl group and a prolinol subunit. The sequence of the linear peptide was established on the basis of HMBC cross-peaks from NH and α-H resonances to amide carbonyl carbons through $^2J_{CH}$ and $^3J_{CH}$ correlations (Figure 4), which indicated that the larger part of the linear sequence was the same as that of **1**. The only difference was found at the *N*-terminus, where the Aib residue of

Table 1. ^{1}H and ^{13}C NMR Data and COSY and HMBC Correlations of **1** (in DMSO-d_6)[a]

residue	position	δ_C, mult.	δ_H (J in Hz)	COSY correlation	HMBC correlation
Ac	C=O	171.3, qC			
	CH_3	23.1, CH_3	1.93, s		C=O
Aib-1	C=O	175.4, qC			
	1	56.1, qC			
	2	26.2, CH_3	1.35, s		C-3, C-1, C=O (A-1)
	3	23.2, CH_3	1.37, s		C-1, C-2, C=O (A-1)
	NH		8.59, s		C=O (Ac), C=O (A-1), C-1
Aib-2	C=O	177.4, qC			
	1	56.2, qC			
	2	27.8, CH_3	1.35, s		C-3, C-1, C=O (A-2)
	3	24.4, CH_3	1.34, s		C-1, C-2, C=O (A-2)
	NH		8.41, s		C=O (A-1), C=O (A-2), C-1
Val	C=O	172.8, qC			
	1	62.8, CH	3.51, m	NH, H-2	C=O (A-2), C=O (V-1), C-2, C-3, C-4
	2	29.2, CH	2.17, m	H-1, H-3, H-4	C=O (V-1), C-3, C-4, C-1
	3	20.0, CH_3	0.98, d (6.5)	H-2	C-4, C-2, C-1
	4	19.1, CH_3	0.91, d (6.5)	H-2	C-3, C-2, C-1
	NH		7.76, d (6.0)	H-1	C=O (V-1), C=O (A-2), C-1
Aib-3	C=O	175.4, qC			
	1	56.3, qC			
	2	27.1, CH_3	1.35, s		C-3, C-1, C=O (A-3)
	3	23.4, CH_3	1.37, s		C-1, C-2, C=O (A-3)
	NH		7.53, s		C=O (A-3), C=O (V-1), C-1
Ile	C=O	173.4, qC			
	1	61.1, CH	3.62, m	NH, H-2	C=O (A-3), C=O(I), C-2, C-5, C-3
	2	35.1, CH	1.75, m	H-1, H-3, H-5	
	3	26.0, CH_2	1.15, m	H-2, H-4	
			1.55, m		
	4	10.4, CH_3	0.76, t (7.0)	H-3	C-2, C-3
	5	15.2, CH_3	0.82, d (6.5)	H-2	C-1, C-2, C-3
	NH		7.10, d (6.5)	H-1	C-1, C=O(I), C=O (A-3)
Aib-4	C=O	174.1, qC			
	1	56.4, qC			
	2	26.0, CH_3	1.35, s		C-3, C-1, C=O (A-4)
	3	22.9, CH_3	1.37, s		C-1, C-2, C=O (A-4)
	NH		7.84, s		C=O (A-4), C=O (I), C-1
Aib-5	C=O	175.4, qC			
	1	56.4, qC			
	2	27.5, CH_3	1.35, s		C-3, C-1, C=O (A-5)
	3	23.5, CH_3	1.33, s		C-1, C-2, C=O (A-5)
	NH		7.80, s		C=O (A-5), C=O (A-4), C-1
Ala	C=O	172.3, qC			
	1	49.6, CH	4.00, m	NH, H-2	C=O (A-5), C=O(Al), C-2
	2	17.6, CH_3	1.33, m	H-1	C=O (Al), C-1
	NH		7.37, d (7.5)	H-1	C=O (A-5), C=O(Al), C-1, C-2
Aib-6	C=O	171.3, qC			
	1	56.3, qC			
	2	25.1, CH_3	1.37, s		C-3, C-1, C=O (A-6)
	3	22.9, CH_3	1.37, s		C-1, C-2, C=O (A-6)
	NH		7.53, s		C=O (A-5), C=O (A-6), C-1
Prolinol	1	60.2, CH_2	3.95, m	H-5, H-2	C=O(A-6), C-5, C-2
	2	26.3, CH_2	1.69, m	H-1, H-3	
			1.75, m	H-1, H-3	
	3	25.2, CH_2	1.60, m	H-2, H-4	
			1.75, m	H-2, H-4	
	4	48.0, CH	3.30, m	H-3, H-4b	C-1, C=O(A-6), C-3, C-2
			3.59, m	H-3, H-4a	C-1, C=O(A-6), C-3, C-2
	5	61.5, CH_2	3.38, m	H-1, H-5b	C-1, C-2
			3.40, dd (3.0, 10.5)	H-1, H-5a	C-1, C-2

[a] A = Aib, I = Ile, V = Val, Al = Ala.

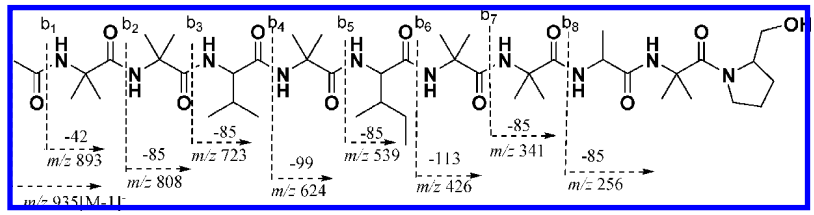

Figure 1. (−) ESIMS/MS fragmentation of **1** by ionization of $[M - H]^-$.

1 was replaced by an alanine in **2**, as evident from the $^{1}H-^{1}H$ COSY correlation of δ_H 4.01 (1H, m, α-H) with the methyl protons at δ_H 1.32 (3H, d) and 8.66 (1H, d, J = 7.5 Hz, NH) and the HMBC interaction of the NH and the acetyl protons at δ_H 1.92 (3H, s) to

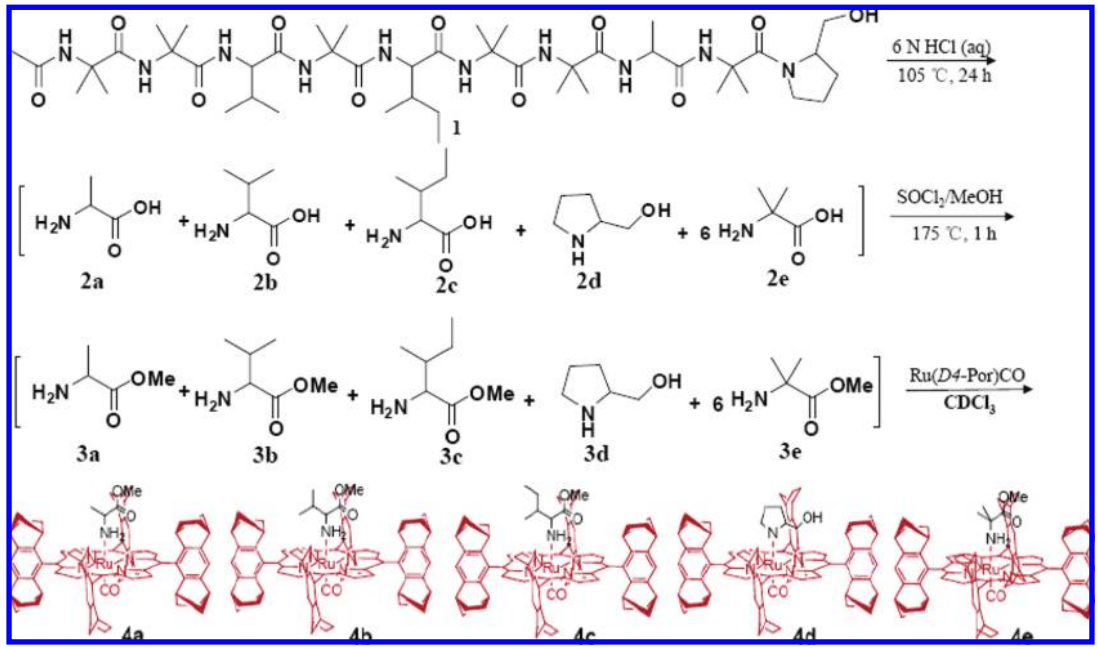

Figure 2. Route to prepare Ru(D_4-Por)CO complexes of the amino acids derived from **1**.

the carbonyl carbon at δ_C 171.5 (s). The assignment of the amino acid sequence was further corroborated by ESIMS/MS fragmentation. The configurations of the amino acid residues and of the prolinol unit were the same as for **1**.

Aspereline C (**3**) had the same molecular formula as **2**, as determined by HRESIMS data. A comparison of the NMR spectroscopic data (Table 2) revealed that **3** possesses the same amino acid residues as **2**. In addition, the signals of an acetyl group and a prolinol residue were also observed from its ^{1}H and ^{13}C NMR spectra. 2D NMR data (Figure 4) indicated that the amino acid residues corresponding to both the *N*- and *C*-termini were Aib residues as in the case of **1**, which were connected to the acetyl and prolinol residues, respectively. The NH resonance at δ_H 7.38 (1H, s, NH) belonging to the Aib adjacent to the *C*-terminus interacted with the α-H of an alanine at δ_H 4.07 (1H, m, Ala-2) in the NOESY spectrum and clarified the location of the alanine near the *C*-terminus. The COSY correlations of δ_H 3.93 (1H, m, α-H)/δ_H 7.79 (1H, d, J = 4.5 Hz, NH) and α-H/Me (δ_H 1.31, d) confirmed the presence of a second Ala residue (Ala-1). The HMBC correlation of the protons at δ_H 4.07, 3.93, and 7.42 (d, NH-Ala-2) to the amide carbonyl carbon at δ_C 172.6 (s) allowed the determination of an Ala−Ala dipeptide linkage. The remaining sequence of the residues was identical to that of **1** on the basis of the 2D NMR data analysis and the comparison of the NMR data with those reported for **1**. The ESIMS/MS spectra of protonated and deprotonated **3** (Figure 5) exhibited the successive fragment ions as generated by B-type fragmentation from the *N*-terminus and X-type fragmentation from the *C*-terminus, respectively. These findings provided additional evidence for assigning the amino acid sequence as Aib-Aib-Val-Aib-Ile-Aib-Ala-Ala-Aib. The configurations of the amino acid residues were in agreement with those of **1**.

HRESIMS data indicated that aspereline D (**4**) had the same molecular formula as **2** and **3**. Analysis of 1D and 2D NMR spectroscopic data disclosed the presence of six Aib, two Val, and one Ala, along with an acetyl group and a prolinol residue. The sequence of the amino acid residues was established by 2D NMR (NOESY and HMBC) and ESIMS/MS techniques, which revealed the structure of **4** to be closely related to that of **1**. The only difference found was the presence of an additional Val residue of **4** instead of the Ile residue of **1**, which was evident from the COSY coupling system of the proton signals at δ_H 2.16 (1H, m, H-2), 0.87 (3H, d, J = 6.5 Hz, H-3), 0.86 (3H, d, J = 6.5 Hz, H-4), 3.36 (1H, m, α-H), and 7.06 (1H, d, J = 7.0 Hz, NH). Detailed HMBC data analysis (Figure 4) confirmed that the second Val of **4** replaced the Ile in **1**. The negative ion ESIMS/MS revealed B-type fragments, which confirmed the entire sequence of **4** to be Ac-Aib-Aib-Val-Aib-Val-Aib-Aib-Ala-Aib-prolinol.

The HRESIMS data provided the molecular formula of aspereline E (**5**) as $C_{45}H_{80}N_{10}O_{12}$, according to the protonated molecular ion at m/z 953.6031 $[M + H]^+$ and NMR data. The ^{1}H and ^{13}C NMR data of **5** (Table 2) were closely related to those of **1**, except for the presence of a serine residue noted by the ^{1}H−^{1}H COSY correlations from δ_H 4.05 (1H, ddd) to δ_H 3.69 (1H, dd) and 3.81 (1H, dd) and also the NH at δ_H 7.43 (1H, d, J = 8.0 Hz). This residue was deduced to be connected to the Aib at the *C*-terminus, replacing an Ala of **1**, on the basis of the HMBC correlations from δ_H 4.05 and 7.49 (1H, s, NH-Aib) to δ_C 169.5 (s) and from the latter NH to δ_C 56.5 (s, α-C, Aib) and 171.3 (s, Aib). The negative ESIMS/MS spectrum revealed that an additional early fragmentation was caused by the loss of 31 amu, which was generated from cleavage of the CH_2OH unit of Ser, while the remaining fragments originated by a cleavage mechanism similar to that for other linear peptides. Moreover, the positive ion ESIMS/MS spectrum followed the regular cleavage pathway through X-type fragmentation. The configurations of the amino acid residues were determined using the same protocol described for **1**, which resulted in the L-configuration for Val, Ala, Ile, and Ser.

Aspereline F (**6**) had the molecular formula $C_{46}H_{82}N_{10}O_{11}$, as determined by the HRESIMS and NMR data. The ^{1}H and ^{13}C NMR data (Table 2) indicated that it is a linear peptide, closely related to the other asperelines. The ^{1}H NMR spectrum presented four α-H protons at δ 3.73 (1H, dd, J = 6.0, 7.0 Hz), 3.51 (1H, dd, J = 6.5, 7.0 Hz), 3.60 (1H, m), and 4.00 (1H, m), which were ascribed to two Val, one Ile, and one Ala, as proven by the COSY and HMBC correlations. The remaining residues were Aib units. Thus, this peptide contained nine amino acids apart from an acetyl and a prolinol residue. The amino acid sequence was unambiguously established through HMBC correlations from the NH and α-H protons to the carbonyl carbons, which defined the sequence of **6** to be Aib-Val-Val-Aib-Ile-Aib-Aib-Ala-Aib. The *N*- and *C*-termini are substituted by acetyl and prolinol residues, respectively, as in the case of **1**−**5**. The negative ion ESIMS/MS data supported the structure assignment. The configurations of the chiral amino acid

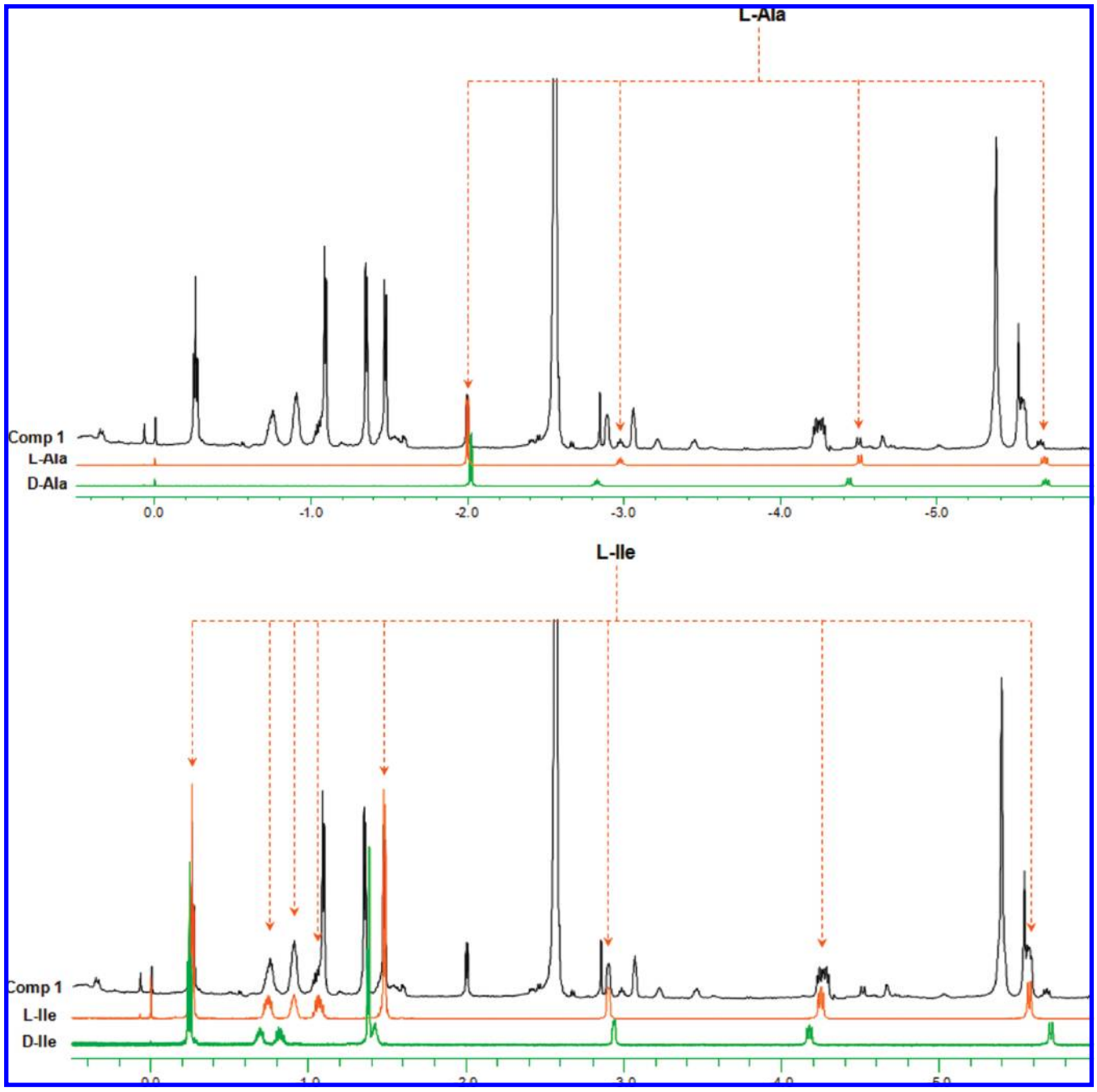

Figure 3. ^{1}H NMR spectroscopic comparison of the Ru(D$_4$-Por*)CO complexes between L-Ala and L-Ile in the mixture derived from **1** and the reference standards.

residues and prolinol were assigned as L and *S* using the same method as described for **1**.

Compounds **1**−**6** were tested against fungi and bacteria, but they showed only weak inhibitory activity toward the early blight pathogen *Alternaria solani*, the rice blast *Pyricularia oryzae*, and the bacteria *Staphylococcus aureus* and *Escherichia coli* with IC_{50} > 100 μg/mL and IC_{90} > 500 μg/mL.

Herein, a new group of peptaibols displaying nine amino acid residues is described, which enriches the diverse peptaibol family. The (*S*)-prolinol residue is reported here as a novel, *C*-terminal constituent of peptaibiotics for the first time. In addition, this is the first report on the production of peptaibols by a psychrophilic fungus isolated from an Antarctic habitat. Recently, Brückner and his co-workers[1] hypothesized that extremophilic fungi, including those from Antarctic habitats, are capable of producing peptaibiotics. The results presented here serve as a first proof for this hypothesis.

In addition to the 1D and 2D NMR spectra, ESIMS/MS effectively elucidated the peptaibol sequences. The experimental results indicated that the negative ion ESIMS/MS of the deprotonated precursor ion always produces sequential fragments from the *N*-terminus via the B-type pathway, whereas the amino acid residues are fragmented sequentially from the *C*-terminus through an X-type route in positive ion ESIMS/MS (Figure 6). In the case of the peptaibol containing the Ser residue, prior cleavage occurred at Ser by loss of a CH_2OH residue in the negative ion ESIMS/MS. Thus, the combination of the ESIMS/MS spectra, recorded by deprotonated and protonated ionization, allowed the determination of the entire sequences of the peptaibols. This method also can be used to determine the individual peptides from a peptide-containing mixture through online HPLC-ESIMS/MS techniques.

In the ^{1}H NMR spectra, peptaibols are easily recognized by the broad signals integrated around δ_H 1.34−1.37 arising from the

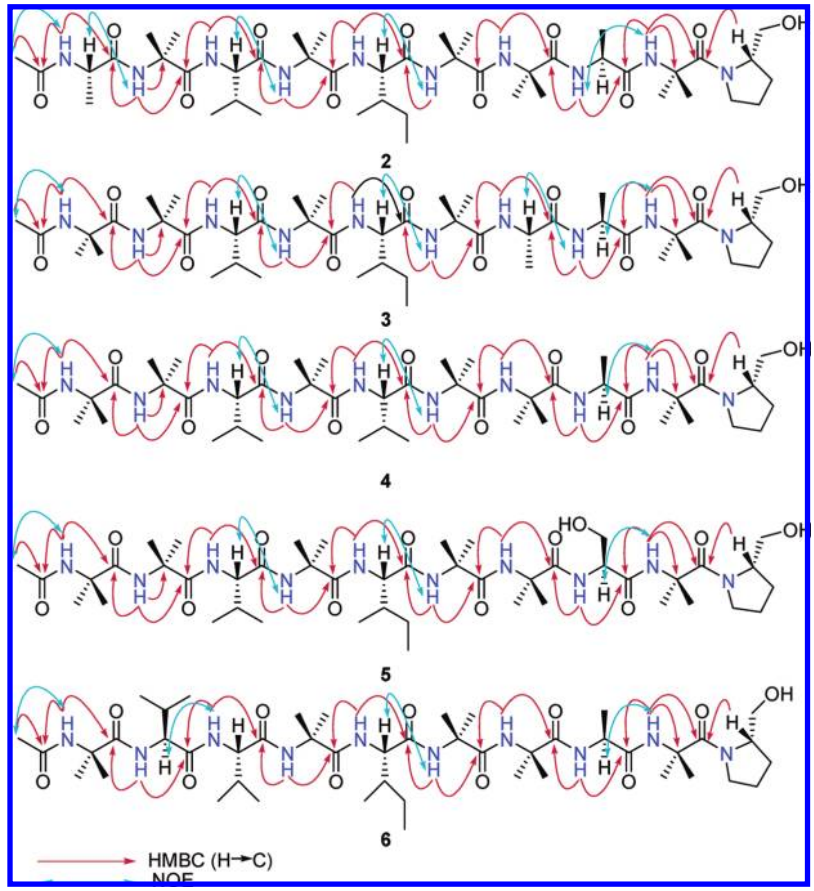

Figure 4. HMBC and NOESY correlations of asperelines B−F (**2**−**6**).

multiple methyl groups of Aib's, which distinguish these compounds from other peptides not having Aib residues.

The organometallic compound Ru(D4-Por*)CO served as a new type of sensitive chiral NMR shift reagent to determine the L/D configurations of amino acids at the microgram level. It is achieved by observing the resonances of amino acids exclusively below 0 ppm upon binding the amino group to the ruthenium porphyrin in an axial orientation to form a stable complex. The significant upfield shifts of the NH_2 and α-H resonances are induced by the ring-current effect generated by the Ru(D4-Por*)CO planar geometry. The resonances of the protons of L- and D-amino acids are shifted upfield to different extents. The chiral reagent Ru(D4-Por*)CO induced a greater upfield shift for the α-H than for the alkyl side chain protons, and the α-H resonances of L- and D-amino acids are baseline-resolved. Thus, the chemical shift differences of the α-H in association with other signals within the amino acid residue are employed to distinguish the L- and D-configurations. The advantage of this method is that it can discriminate the configurations of constituent amino acids in a peptide in a mixture of hydrolyzed products without chromatographic separation.

In recent years, large numbers of peptaibol sequences have been elucidated, due to the development of modern spectroscopic techniques, including various 2D NMR methods and ESIMS/MS spectrometric techniques, which have been frequently used for the complete sequencing of peptaibols. The optimization of the production of peptides, which are important for biotechnological and pharmacological studies, requires further investigation.

Experimental Section

General Experimental Procedures. Melting points were measured on a XT4A microscopic melting-point detector. Optical rotations were recorded on a Perkin-Elmer 341 LC polarimeter. UV spectra were measured on a Shimadzu UV-210A spectrophotometer. ^1H and ^{13}C NMR and 2D NMR spectra were recorded on Bruker Avance-500 and Varian INOVA-500 NMR spectrometers using TMS as an internal standard. The chemical shifts are given in δ (ppm) and coupling constants in Hz. ESIMS spectra were measured on a Quttra Premier XE tandem mass spectrometer (Micromass, UK), while HRESIMS spectra were measured on a LTQ Obitrap X1 Thermo Scientific and a FT-MS-Bruker APEX IV (7.0T). Silica gel (GF254) for TLC and H-Sil gel (200−300 mesh) for column chromatography (CC) were purchased from Qingdao Marine Chemical Company, Qingdao, China. Semi-preparative HPLC was performed on an Alltech-HPLC (USA) using a Kromasil column (ODS, 10 μm, 10 × 250 mm). The chemical reagents used for chromatography were purchased from Beijing Chemical Works Co. Ltd. (Beijing).

Fungal Strain and Identification. *Trichoderma asperellum* was collected from the sediment of the Antarctic Penguin Island. The strain (Y19-07, accession number HTTM-Z00002) was isolated and identified by Prof. T. Li of the First Institute of Oceanography, SOA, and was deposited in SOA and the State Key Laboratory of Natural and Biomimetic Drugs, Peking University. The strain sequence is accessed under GenBank DQ767600.

DNA Extractions. The strain Y19-07 was cultured in liquid medium (potato broth in seawater 20%, peptone 0.2%, yeast extract 0.1%, glucose 3%, NaCl 2%, $MgCl_2 \cdot 6H_2O$ 0.15%, KCl 0.02%, $FePO_4$ 0.001%, pH 6.5) at 15 °C for 2 days. The mycelium, DNA extraction buffer (500 μL), NaCl (5M, 25 μL), and glass beads (250−300 μL) were mixed thoroughly in a microcentrifuge tube. The suspension was centrifuged for 2 min at 10 000 rpm. The supernatant was then transferred into a new microcentrifuge tube, and a phenol−chloroform−isoamyl alcohol (25:24:1, 500 μL) mixture was added. The suspension was centrifuged for 5 min at 10 000 rpm. To a microcentrifuge tube with the supernatant, the 2-propanol (*i*-PrOH) (100 μL, 70%) was added at −20 °C for 1 h. After centrifuging for 3 min (10 000 rpm), the pellet was resuspended in 70% EtOH (800 μL) to centrifuge for 5 min at 10 000 rpm. The air-dried supernatant was suspended in 500 μL of TE buffer (10 mM Tris-Cl, pH 7.5; 1 mM EDTA) and mixed with EDTA-Sark (50 μL) and proteinase K (20 mg/mL, 2.5 μL) to incubate for 30

Table 2. ^{1}H and ^{13}C NMR Data of **2**−**6** in DMSO-d_6

	2		**3**		**4**		**5**		**6**	
no.	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
Ac										
C═O	171.5		171.4		171.6		171.6		171.3	
CH_3	22.9	1.92, s	23. 5	1.93, s	23.4	1.93, s	23.7	1.93, s	23.6	1.94, s
	Ala-1		Aib-1		Aib-1		Aib-1		Aib-1	
C═O	174.4		175.6		175.9		173.8		176.2	
1	51.2	4.01, m	56.2		56.3		56.2		56.3	
2	17.6	1.32, d (7.0)	26.3	1.33, s	26.2	1.34, s	26.2	1.36, s	26.8	1.38, s
3			23.4	1.35, s	23.0	1.34, s	26.3	1.36, s	23.1	1.35, s
NH		8.66, d (7.5)		8.87, s		9.25, s		9.18, s		8.58, s
	Aib-1		Aib-2		Aib-2		Aib-2		Val-1	
	176.9		177.4		177.6		177.6		172.6	
1	56.2		56.2		56.3		56.2		61.8	3.73, dd (6.0,7.0)
2	27..5	1.40, s	27.8	1.40, s	27.1	1.33, s	27.1	1.36, s	29.0	2.14, m
3	23.2	1.35, s	24.4	1.36, s	23.4	1.33, s	27.7	1.36, s	19.2	0.91, d (6.5)
4									19.7	0.96, d (6.5)
NH		7.87, s		8.60, s		8.84, s		8.79, brs		8.05, d (6.0)
	Val		Val		Val-1		Val		Val-2	
C═O	172.4		173.0		172.9		172.9		173.9	
1	62.5	3.55, dd (6.5,7.0)	62.9	3.48, m	62.9	3.46, m	62.9	3.47, dd (6.0,7.0)	62.9	3.51, dd (6.5,7.0)
2	29.2	2.10, m	29.2	2.17, m	29.4	2.16, m	29.2	2.18, m	29.2	2.12, m
3	19.1	0.89, d (7.0)	19.2	0.98, d (6.5)	19.2	0.99, d (6.5)	20.1	0.99, d (6.5)	19.5	0.92, d (6.5)
4	20.1	0.96, d (7.0)	20.1	0.91, d (6.5)	20.1	0.98, d (6.5)	19.1	0.91, d (6.5)	20.0	0.97, d (6.5)
NH		7.67, d (6.5)		7.73, d (7.5)		7.78, d (8.0)		7.75, d (6.0)		7.62, d (6.5)
	Aib-2		Aib-3		Aib-3		Aib-3		Aib-2	
C═O	175.5		175.9		175.5		176.1		175.3	
1	56.3		56.2		56.3		56.4		56.2	
2	26.9	1.41, s	27.1	1.43, s	27.8	1.34, s	27.8	1.44, s	27.5	1.36, s
3	23.6	1.35, s	23.4	1.40, s	25.1	1.34, s	26.3	1.36, s	25.1	1.35, s
NH		7.77, s		7.54, s		7.55, s		7.59, s		8.07, s
	Ileu		Ileu		Val-2		Ileu		Ileu	
C═O	173.5		173.5		173.4		174.7		173.3	
1	61.2	3.60, dd (7.0,8.5)	61.4	3.61, dd (6.5,7.2)	62.9	3.36, m	61.3	3.60, dd (6.5,7.0)	61.3	3.60, m
2	35.2	1.80, m	34.9	1.87, m	29.1	2.16, m	34.9	1.88, m	35.7	1.85, m
3	26.3	1.15, m 1.55, m	26.1	1.16, m 1.55, m	20.2	0.87, d (6.5)	26.0	1.15, m 1.59, m	26.2	1.33, m 1.14, m
4	10.7	0.78, t (7.0)	10.4	0.77, t (7.0)	19.1	0.86, d (6.5)	10.4	0.78, t (7.5)	11.2	0.80, t (6.5)
5	15.3	0.82, d (6.5)	15.2	0.83, d (7.2)			15.1	0.82, d (6.5)	15.5	0.85, d (7.0)
NH		7.40, d (8.5)		7.21, d (6.5)		7.06, d (7.0)		7.27, d (6.5)		7.14, d (6.5)
	Aib-3		Aib-4		Aib-4		Aib-4		Aib-3	
C═O	175.5		175.9		175.4		175.8		175.5	
1	62.5		56.2		56.3		56.4		56.3	
2	25.0	1.36, s	26.5	1.45, s	23.2	1.34, s	25.2	1.36, s	26.9	1.36, s
3	23.2	1.34, s	22.9	1.35, s	27.5	1.33, s	26.0	1.32, s	23.2	1.35, s
NH		7.79, s		7.86, s		7.87, s		7.99, s		7.60, s
	Aib-4		Ala-1		Aib-5		Aib-5		Aib-4	
C═O	174.2		172.6		174.1		175.3		174.2	
1	56.3		51.1	3.93, m	56.3		56.7		56.2	
2	23.1	1.35, s	17.0	1.31, d (7.0)	26.3	1.34, s	27.7	1.43, s	26.4	1.36, s
3	26.3	1.32, s			24.3	1.33, s	27.1	1.43, s	24.4	1.35, s
NH		7.86, s		7.79, d (4.5)		7.83, s		7.91, s		7.72, s
	Ala-2		Ala-2		Ala		Ser		Ala	
C═O	172.3		172.0		172.2		169.5		172.3	
1	49.6	4.00, m	49.6	4.07, m	49.6	4.00, m	57.2	4.05, ddd (3.0,8.0,8.5)	49.6	4.00, m
2	17.0	1.32, d (7.0)	17.6	1.32, s	17.6	1.34, d (7.0)	62.1	3.69, dd (3.0,11.5) 3.81, dd (8.5,11.5)	17.6	1.32, m
NH		7.37, d (7.0)		7.42, d (9.0)		7.38, d (7.5)		7.43, d (8.0)		7.42, d (7.5)
	Aib-5		Aib-5		Aib-6		Aib-6		Aib-5	
C═O	171.3		171.2		171.3		171.3		171.2	
1	56.2		56.2		56.3		56.5		56.4	
2	26.8	1.36, s	25.9	1.35, s	25.9	1.37, s	25.2	1.38, s	25.9	1.36, s
3	23.6	1.34, s	22.8	1.35, s	22.7	1.37, s	25.9	1.38, s	23.4	1.35, s
NH		7.54, s		7.38, s		7.53, s		7.49, s		7.52, s
prolinol										
1	60.2	3.96, m	60.2	3.94, m	60.2	3.49, m	60.2	3.94, m	60.2	3.96, m
2	26.3	1.58, m	26.3	1.69, m	26.2	1.44, m	26.3	1.67, m	26.3	1.67, m
		1.74, m		1.75, m		1.72, m		1.74, m		1.74, m
3	25.2	1.41, m	25.2	1.60, m	25.1	1.52, m	25.6	1.60, m	25.2	1.60, m
		1.75, m		1.75, m		1.77, m		1.75, m		1.74, m
4	48.0	3.28, m	48.0	3.26, m	48.0	3.58, m	48.0	3.27, m	48.0	3.27, m
		3.59, m		3.57, m		3.25, m		3.59, m		3.59, m
5	61.4	3.35, dd (6.5,10.5)	61.4	3.35, dd (5.0,10.5)	61.4	3.35, m	61.4	3.35, dd (7.5,10.5)	61.5	3.39, dd (7.5,10.5)
		3.40, dd (3.0,10.5)		3.40, dd (3.4,10.5)		3.40, m		3.40, dd (3.0,10.5)		3.52, dd (3.0,10.5)

min at 37 °C. The incubated solution was diluted by NaCl (5 M, 17 μL) and then centrifuged for 5 min (10 000 rpm). Afterward, EtOH (70%, 800 μL) was added gently to centrifuge for 5 min at 10 000 rpm. The supernatant was air-dried and finally resuspended in TE buffer (40 μL).

PCR Amplifications. Each PCR mixture (50 μL) contained $MgC1_2$ (3 μL, 25 mmol/L), DNA polymerase buffer (5 μL, 10 × Taq), dNTP (1.0 μL, 5 mM/L), primer (1.0 μL, each 10 μM), Taq DNA polymerase (0.25 unit), diluted genomic DNA (0.5 μL), and deionized water (38 μL). Primers used for PCR amplifications were ITS1 and ITS4 (5-

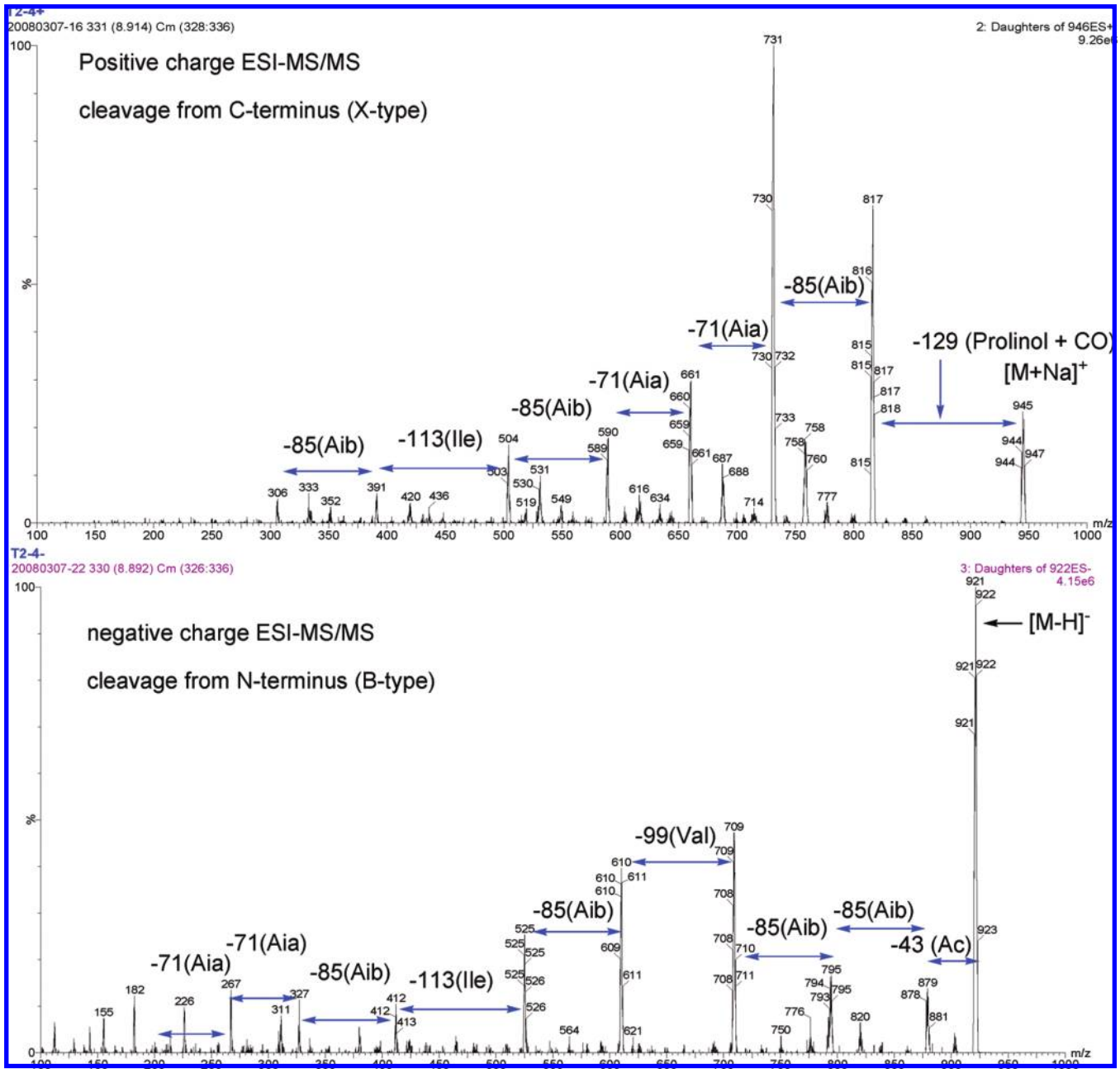

Figure 5. (+) and (−) ESIMS/MS fragmentation of **3**.

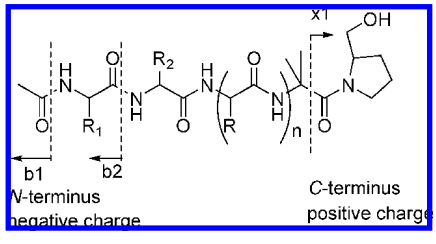

Figure 6. Cleavage pathway of (+)- and (−)-ESIMS/MS fragmentation of peptaibols.

TCCGTAGGTGAACCTGCGG-3/5-TCCTCCGCTTATTGATATGC-3). Amplifications were performed using the following parameters: a 5 min step at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 51 °C, and 50 s at 72 °C, and then extended to a final 10 min at 72 °C. The genomic DNA was verified by 1% agarose gel electrophoresis.

Culture Conditions. The initial cultures were maintained on the artificial seawater agar by dissolving beef extract (10 g) and peptone (10 g) by heating in tap water (250 mL) to adjust pH to 7.8 and boiling for 10 min, readjusting pH to 7.3, adding agar (20 g), autoclaving at 121 °C for 20 min, cooling to 50 °C, and adding warm (50 °C) sterile artificial seawater (ASW) (750 mL). ASW consists of NaCl (28.13 g), KCl (0.77 g), $CaCl_2 \cdot 2H_2O$ (1.60 g), $MgCl_2 \cdot 6H_2O$ (4.80 g), $NaHCO_3$ (0.11 g), $MgSO_4 \cdot 7H_2O$ (3.50 g), and distilled water (1000 mL). Pieces of mycelium were cut into small segments and aseptically transferred to a 250 mL Erlenmeyer flask containing 100 mL of culture media (potato broth in seawater 20%, peptone 0.2%, yeast extract 0.1%, glucose 3%, NaCl 2%, $MgCl_2 \cdot 6H_2O$ 0.15%, KCl 0.02%, $FePO_4$ 0.001%, pH 6.5). The flasks were incubated at 15 °C on a rotary shaker, at 150 rpm, for 12 days.

Extraction and Isolation. The culture broth (80 L) was centrifuged at 4000 rpm. The supernatant was adsorbed onto Amberlite-XAD (macroporous resin), then the column was eluted with H_2O, 5% EtOH, and 95% EtOH, successively. The 95% EtOH fraction was collected and evaporated in vacuo to give a residue, which was partitioned between H_2O and EtOAc. The organic layer was concentrated in vacuo to give the EtOAc fraction. The EtOAc fraction (14.4 g) was subjected to vacuum liquid chromatography (160−200 mesh Si gel) and eluted with a gradient of petroleum ether (PE)−acetone (5:1, 3:1, 2:1, 1:1) to obtain seven fractions (FA−FG). FC (1.9 g) was fractionated on a Sephadex LH-20 column

eluting with MeOH to yield four subfractions (SF1−SF4). SF1 (300 mg) mainly contained peptides as determined by ^{1}H NMR spectroscopy, and it was subsequently separated on semipreparative HPLC (YMC, 10 μm, 10 × 250 mm) with a mobile phase of MeOH−H_2O (70:30) to obtain **1** (79 mg, t_R 73.9 min), **6** (15 mg, t_R 85.2 min), a mixture of **2** and **3** (43 mg), and a mixture of **4** and **5** (32 mg). The mixture of **2** and **3** was then separated by semipreparative HPLC (YMC, 10 μm, 10 × 250 mm) using CH_3CN−H_2O (4:6) as the mobile phase to obtain **2** (7.0 mg, t_R 31.5 min) and **3** (8.0 mg, t_R 46.5 min), while the mixture of **4** and **5** was separated by the same protocol as for compounds **2** and **3** using CH_3CN−H_2O (38:62) as the mobile phase to yield **4** (12 mg, t_R 71.5 min) and **5** (15 mg, t_R 87.5 min).

Aspereline A (1): colorless needles (MeOH); mp 296−298 °C; $[\alpha]^{20}_D$ −42 (*c* 0.1, MeOH); ^{1}H and ^{13}C NMR data, see Table 1; (−)ESIMS/MS *m/z* 935 $[M - H]^-$, 893, 808, 723, 624, 539, 426; (+)ESIMS/MD *m/z* 937 $[M + H]^+$, 835, 751, 680, 595, 510, 397, 312; HRESIMS *m/z* 937.6088 $[M + H]^+$(calcd for $C_{45}H_{81}N_{10}O_{11}$, 937.6081).

Aspereline B (2): colorless needles (MeOH); mp 273−275 °C; $[\alpha]^{20}_D$ −5.0 (*c* 0.1, MeOH); ^{1}H and ^{13}C NMR data, see Table 2; (−)ESIMS *m/z* 921 $[M - H]^-$, 879, 808, 723, 624, 539, 426; (+)ESIMS *m/z* 945 $[M + Na]^+$, 815, 731, 660, 575, 490, 377, 292; HRESIMS *m/z* 945.5744 $[M + Na]^+$ (calcd for $C_{44}H_{78}N_{10}O_{11}Na$, 945.5743).

Aspereline C (3): colorless needles (MeOH); mp 284−285 °C; $[\alpha]^{20}_D$ −22 (*c* 0.1, MeOH); ^{1}H and ^{13}C NMR data, see Table 2; (−)ESIMS/MS *m/z* 921 $[M - H]^-$, 879, 794, 709, 610, 525, 412, 327; (+)ESIMS/MS *m/z* 945 $[M + Na]^+$, 815, 731, 660, 589, 504, 391, 306; HRESIMS *m/z* 945.5746 $[M + Na]^+$ (calcd. for $C_{44}H_{78}N_{10}O_{11}Na$, 945.5743).

Aspereline D (4): colorless needles (MeOH); mp 301−303 °C; $[\alpha]^{20}_D$ −10.5 (*c* 0.1, MeOH); ^{1}H and ^{13}C NMR data, see Table 2; (−)ESIMS/MS *m/z* 921 $[M - H]^-$, 879, 794, 709, 610, 525, 426; (+)ESIMS/MS *m/z* 945 $[M + Na]^+$, 815, 731, 660, 575, 490, 391, 306, 211; HRESIMS *m/z* 945.5740 $[M + Na]^+$ (calcd for $C_{44}H_{78}N_{10}O_{11}Na$, 945.5743).

Aspereline E (5): colorless needles (MeOH); mp 295−297 °C; $[\alpha]^{20}_D$ −12 (*c* 0.1, MeOH); ^{1}H and ^{13}C NMR data, see Table 2; (−)ESIMS/MS *m/z* 951, 922, 880, 794, 709, 610, 525, 412; (+)ESIMS/MS *m/z* 976 $[M + Na]^+$, 847, 761, 674, 589, 504, 391, 306; HRESIMS *m/z* 953.6031 $[M + H]^+$(calcd for $C_{45}H_{81}N_{10}O_{12}$, 953.6035).

Aspereline F (6): colorless needles (MeOH); mp 281−283 °C; $[\alpha]^{20}_D$ −12 (*c* 0.1, MeOH); ^{1}H and ^{13}C NMR data, see Table 2; (−)ESIMS/MS *m/z* 949, 907, 822, 723, 624, 539, 426; (+)ESIMS/MS *m/z* 973 $[M + Na]^+$, 844, 759, 688, 603, 518, 405, 321; HRESIMS *m/z* 973.6047 $[M + Na]^+$ (calcd for $C_{46}H_{82}N_{10}O_{11}Na$, 973.6042).

Ru((+)-D_4-Por*)CO preparation. The chiral reagent Ru((+)-D_4-Por*)CO was synthesized as reported.[29,30]

Configuration Determination. Compound **1** (0.2 mg) was dissolved in 6 N HCl (2 mL) and heated at 105 °C for 24 h in a sealed ampule. The resulting solution was cooled to rt and concentrated in vacuo to remove H_2O and HCl. Then, 500 μL of $SOCl_2$−CH_3OH (v/v = 1:10) was added and heated at 75 °C in a H_2O bath for 1 h. The dried sample was mixed with 40 μL of saturated Na_2CO_3 to basify the aqueous solution. Then a 0.6 mL solution of 2.0 mg of Ru((+)-D_4-Por*)CO in $CDCl_3$ was added to extract the amino acids. The $CDCl_3$ layer was quickly passed through a 2 × 5 mm silica gel column and then collected in a NMR tube. A ^{1}H NMR spectrum was recorded at 600 MHz. The standards L/D Ala, L/D Ile, L/D Val, L/D Ser, and *S*/*R* prolinols were purchased from Sigma, and the same protocol was used as for **1** to record the ^{1}H NMR spectrum of the individual complexed amino acids. The complexed amino acid residues from **2**−**6** were treated similarly to **1**.

L-Ala-Ru((+)-D_4-Por*)CO: ^{1}H NMR δ ($CDCl_3$) −2.00 (3H, d, Me), −3.00 (1H, m, α-H), −4.46 (1H, m, NHa), −5.66 (1H, m, NHb).

L-Ile-Ru((+)-D_4-Por*)CO: ^{1}H NMR δ ($CDCl_3$) −0.21 (3H, t, J = 7.0 Hz, H-4), −0.75 (1H, m, H-3a), −1.10 (1H, m, H-3b), −0.90 (1H, m, H-2), −1.50 (3H, d, J = 6.5 Hz, H-5), −2.92 (1H, m, α-H), −4.30 (1H, m, NHa), −5.60 (1H, m, NHb).

L-Val-Ru((+)-D_4-Por*)CO: ^{1}H NMR δ ($CDCl_3$) −0.90 (1H, m, H-2), −1.10 (3H, d, J = 6.5 Hz, H-3), −1.36 (3H, d, J = 6.5 Hz, H-4), −3.07 (1H, m, α-H), −4.30 (1H, m, NHa), −5.61 (1H, m, NHb).

L-Ser- Ru((+)-D_4-Por*)CO: ^{1}H NMR δ ($CDCl_3$) −0.87 (1H, m, H-2b), −0.81 (1H, m, H-2b), −2.63 (1H, m, α-H), −4.02 (1H, m, NHa), −4.18 (1H, m, NHb).

***S*-Prolinol-Ru((+)-D_4-Por*)CO.** ^{1}H NMR δ ($CDCl_3$) −0.77 (2H, m, H_2-2), −1.42 to −1.52 (2H, m, H_2-3), −1.52 to −1.55 (2H, m, H_5-5), −1.60 (1H, m, H-4a), −3.44 (1H, m, H-4b), −3.22 (1H, m, α-H), −4.70 (1H, m, NH).

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Supporting Information Available: 1D and 2D NMR (COSY, HMQC, HMBC, NOESY) and ESIMS/MS spectra of compounds **1**−**6** and the ^{1}H NMR spectra of Ru(D_4-Por*)CO complexes of reference standards and the amino acids derived from the peptaibols. This material is available free of charge via the Internet at http://pubs.acs.org.

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