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Pteratides I−IV, New Cytotoxic Cyclodepsipeptides from the Malaysian Basidiomycete *Pterula* sp.

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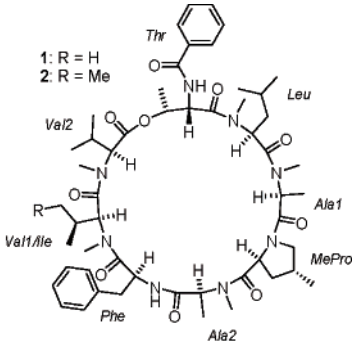

Four new cyclodepsipeptides, pteratides I−IV (**1**−**4**), have been isolated from the extract of a *Pterula* species collected from a Malaysian tropical forest. Homonuclear and heteronuclear 2D NMR techniques as well as MS fragmentation experiments, in combination with methanolysis, determined the gross structures of the peptides and showed that pteratides I and II each contained the nonproteinogenic amino acid 4-methylproline. The absolute configurations of the amino acids in pteratides I−IV were established using Marfey's method. Pteratides I and II are each potently cytotoxic against the P388 murine leukemia cell line (IC_{50} values of 41 and 40 nM, respectively). Pteratides III and IV show weaker, but still notable, activity with IC_{50} values of 7.4 and 2.9 μM, respectively.

Introduction

Many natural cyclodepsipeptides exhibit potent biological activities, for example, the cytotoxic and antiviral sansalvamide,[1] the fusaricidins,[2,3] which are antimicrobial against Gram-positive bacteria, and the antitumor agent kahalalide F,[4] which is currently in clinical trials against prostate cancer. We now report the isolation of a new group of highly cytotoxic cyclodepsipeptides, the pteratides, from the fruiting bodies of the coral-shaped Malaysian basidiomycete, identified as a *Pterula* species. Despite the wide geographical distribution, the secondary metabolites of this genus have not been extensively studied. The *Pterula* sp. most studied to date has been a cultivated strain, *Pterula* sp. 82168, which was found to produce the five previously unknown bioactive natural products hydroxystrobilurin A,[5] pterulones A and B,[6,7] pterulinic acid,[6] and noroudemansin A.[8] The *Pterula* sp. described here has also been the source of the pterulamides, six highly *N*-methylated linear peptides with unusual N- and C-end groups.[9]

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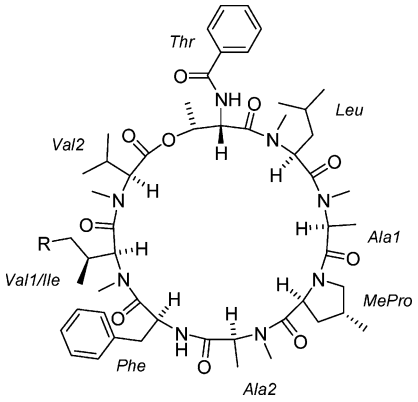
Results and Discussion

The MeOH extracts of three separate *Pterula* fruiting bodies (A−C) each exhibited potent cytotoxicity against P388 murine leukaemia cells (IC_{50} < 1 μg/mL). Despite morphological similarity, the extracts from the three fruiting bodies differed in their chemical profile as assessed by HPLC−UV−MS.[10] Bioactivity-guided isolation of the cytotoxic principles yielded four new cyclodepsipeptides: pteratides I (**1**; from A and C), II and III (**2** and **3**; from B), and IV (**4**; from C), along with the less cytotoxic linear pterulamides.[9] None of the previously described *Pterula* metabolites were found in these extracts. [5−8]



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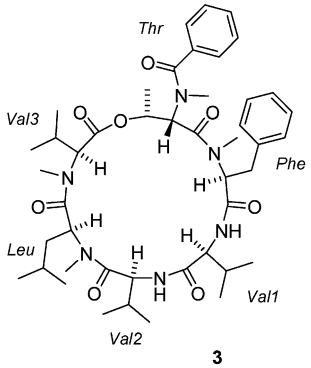

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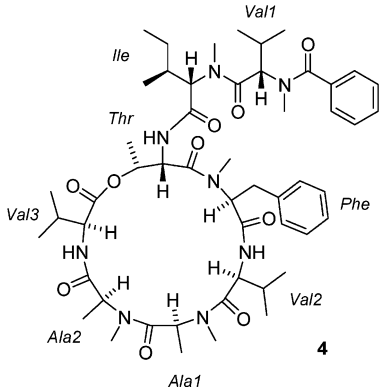

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HRESIMS on pteratide I (**1**) established the mass of the pseudomolecular ion $[M + H]^+$ as 987.5918 Da, which corresponded to a molecular formula for **1** of $C_{53}H_{78}N_8O_{10}$. The ^{1}H NMR spectrum of **1** clearly revealed its peptidic nature; two amide resonances (δ 9.31 and δ 7.24), signals for eight α-protons in the range between 4.6 and 5.5 ppm, and five *N*-methyl groups between 2.5 and 3.2 ppm were observed. TOCSY, HSQC, and HMBC spectra were used to identify the constituent amino acids in **1**. Two valine, two alanine, one leucine, one phenylalanine, and one threonine unit could be readily identified. The signals of an additional aromatic group were attributed to a benzoyl residue.

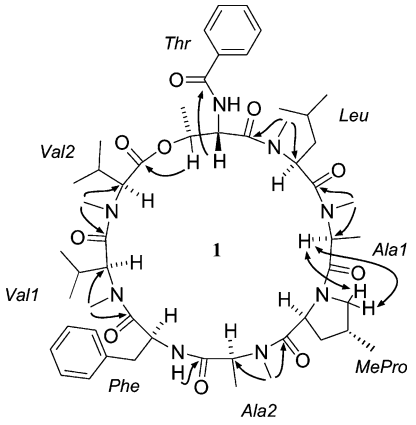

FIGURE 1. HMBC (single arrows) and NOESY (double arrows) correlations establishing the amino acid sequence of **1**.

The amino acid with the α-proton at δ_H 4.90 did not belong to the set of standard proteinogenic amino acids. In addition to this α-proton, there were also signals for two sets of diastereotopic methylene groups (δ_H 1.79 and 2.06, 2.93 and 3.89), a methine proton (δ_H 2.75), and a methyl group (δ_H 1.03). COSY, HMBC correlations and chemical shift arguments established the relationships between these groups leading to the identification of the amino acid as 4-methylproline (4-MePro). A ROESY correlation between the H-2 and the 4-methyl group and the absence of such a correlation between the H-2 and H-4 established a *trans*-relationship, i.e., (2*S**,4*R**)-configuration. The locations of the five *N*-methyl groups were determined using HMBC correlations to the α-carbons of the amino acid residues. By this approach the two valine, the two alanine, and the leucine residues were each found to be *N*-methylated. Long-range H,C-couplings from the amide proton at δ_H 7.24 were observed to the carbonyl carbons of the benzoyl group and to the threonine residue indicating *N*-benzoylation of this amino acid. HMBC correlations between the *N*-methyl groups, or the amide protons to the carbonyl carbon of the next respective amino acid, provided the necessary information for establishing the amino acid sequence of **1**. Two partial sequences were identified: NBz-Thr-NMe-Leu-NMe-Ala1 and 4-MePro-NMe-Ala2-NH-Phe-NMe-Val1-NMe-Val2 (Figure 1). An ester linkage between the β-hydroxyl group of the threonine residue and Val2 was evident from the long-range coupling of the threonine β-proton (δ_H 5.12) to the carbonyl carbon of Val2 (δ_C 168.7) and was supported by the low-field chemical shift of this β-proton. The remaining ring-closing connection between Ala1 and the 4-methylproline was confirmed by NOESY correlations between the α-proton of Ala1 and H-5a as well as H-5b of 4-MePro (Figure 1).

To confirm this sequence, pteratide I (**1**) was ring-opened by methanolysis, and the resultant methyl ester was analyzed by ESI MS/MS. The fragmentation of the sodiated molecular ion of the methyl ester of **1** (*m*/*z* 1041.7) gave y_5, y_6 and y_7

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fragment ions due to sequential losses of NBz-Thr, NMe-Leu, and NMe-Ala, respectively (see the Supporting Information). The a_6 and b_6 fragment ions were also observed in the MS/MS spectrum of **1** methyl ester confirming the presence of the fragment NBz-Thr-NMe-Leu-NMe-Ala-MePro-NMe-Ala-Phe.

The HRESIMS of pteratide II (**2**) showed the pseudomolecular ion $[M + Na]^+$ at *m*/*z* 1023.5902, indicating a molecular formula of $C_{54}H_{80}N_8O_{10}$ for this compound. The structural similarity to pteratide I (**1**) was immediately apparent from the ^{1}H NMR spectrum. Detailed analysis of the 2D NMR spectra revealed that the constituent amino acids were one valine, two alanine, one leucine, one isoleucine, one phenylalanine, and one *N*-benzoylated threonine. In addition to these amino acids, a *trans*-4-methylproline unit was again identified. Using the correlation data from the HMBC NMR experiment, it was found that the five *N*-methyl groups observable in the ^{1}H NMR spectrum were associated with the two alanine, valine, leucine, and the isoleucine residue. The sequence was established using HMBC correlations as NBz-Thr-NMe-Leu-NMe-Ala1-4-MePro-NMe-Ala2-NH-Phe-NMe-Ile-NMe-Val. As in **1**, the ring-closing ester bond was evident from the long-range coupling between the β-proton of threonine (δ_H 5.20) and the carbonyl carbon of the *C*-terminal valine (δ_C 168.8). Pteratide II (**2**) therefore is a homologue of pteratide I (**1**), with a valine substituted by isoleucine. The suggested sequence was again confirmed by ESI MS/MS analysis of the ring-opened methanolysis product of **2**.

Pteratide III (**3**), isolated from the same extract as **2**, had a pseudomolecular ion $[M + H]^+$ at *m*/*z* 819.5000, corresponding to a molecular formula for **3** of $C_{45}H_{66}N_6O_8$. The ^{1}H NMR spectrum again suggested a peptidic nature for this compound. As for the spectra of **1** and **2**, signals for amide protons, aromatic protons, α-protons of amino acids, *N*-methyl groups, and several overlapping methyl signals were observed. Analysis of the separate spin systems revealed that **3** contained six amino acid residues: three valine units, one leucine, one phenylalanine, and one threonine. The phenylalanine accounted for only half of the aromatic protons observed. The other aromatic protons belonged to a benzoyl group that was found to be attached to the amino group of threonine, as shown by a HMBC correlation of the α-proton of threonine (δ_H 5.74) to the carbonyl carbon of the benzoyl group (δ_C 174.4). The four *N*-methyl groups were attached to valine-3, leucine, phenylalanine, and threonine, which was thus both *N*-benzoylated and *N*-methylated. The sequence of the amino acids (HMBC correlation data) was NBz-NMe-Thr-NMe-Phe-NH-Val1-NH-Val2-NMe-Leu-NMe-Val3. Again, a long-range H,C-coupling from the β-proton of threonine (δ_H 5.68) to the carbonyl carbon of the *C*-terminal valine unit (δ_C 170.2) indicated the ring-closing ester bond between these two amino acids.

The molecular formula of pteratide IV (**4**) was found to be $C_{52}H_{78}N_8O_{10}$ by evaluation of HRESIMS and ^{13}C NMR data. Using TOCSY, HSQC, and HMBC data, this peptide was found to contain eight amino acids, five of them *N*-methylated, and also one benzoyl group. The sequence of these amino acids was elucidated using long-range H,C-couplings detected with the IMPRESS NMR technique, which provides higher resolution in the F1 dimension.[11] The sequence was determined as NBz-NMe-Val1-NMe-Ile-NH-Thr-NMe-Phe-NH-Val2-NMe-Ala1-NMe-Ala2-NH-Val3 with a ring-closing ester bond between Val3 and Thr.

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Using Marfey's method,[12] all amino acids in **1**−**4** were found to be of (*S*)-configuration. By comparison against standards the isoleucines found in **2** and **4**, the threonines in **1**, **2**, and **4** and the *N*-methylthreonine in **3** could be assigned as (2*S*,3*S*), (2*S*,3*R*), and (2*S*,3*R*), respectively. The 4-methylproline[13,14] in **1** and **2** was found in both cases to be (2*S*,4*R*).

Pteratides I (**1**) and II (**2**) exhibited potent cytotoxicity against P388 murine leukaemia cells with IC_{50} values of 41 nM (0.039 μg/mL) and 40 nM (0.039 μg/mL), respectively. Pteratides III (**3**) and IV (**4**) were 2 orders of magnitude less active than **1** and **2** with IC_{50} values of 7.4 μM (6.1 μg/mL) and 2.9 μM (2.9 μg/mL), respectively.

The pteratides have intriguing structures characterized by the high degree of *N*-methylation and, in the case of pteratides I−III (**1**-**3**), by *N*-benzoylation of the ring-closing threonine residues. Additionally, the rare nonproteinogenic amino acid (2*S*,4*R*)-4-methylproline is found in pteratides I and II (**1**, **2**). The (2*S*,4*S*) diastereoisomer of this unusual amino acid is known from the peptides of cyanobacteria,[15,16] while 4-methylproline from cyclopeptides of the sponge *Theonella* sp.[17] and an imperfect fungus[18] have undetermined stereochemistry. Beyond doubt, the most remarkable feature of pteratides is their potent cytotoxicity: the mechanism of action remains to be examined.

Experimental Section

Fungal Material. Fruiting bodies of an unidentified *Pterula* species were collected in the Sungkai Wildlife Forest, Perak, Malaysia (collections A and B) and at Krau, Pahang, Malaysia (collection C). Voucher specimens have been deposited in the collection at the School of Chemical Sciences and Food Technology, Universiti Kebangsaan Malaysia (collection A, UKM-F3384A; collection B, UKM-F3384B; collection C, UKM-F4794). Identification of the fungal material was made by one of the authors (A.L.J.C.) based on the characteristic ageotropic, multifid, brown fruit bodies growing on dead sticks. Fungal thalli from the three separate collections were cut into pieces and separately extracted with MeOH.

Isolation of Pteratides I−IV (1−4). The compounds were isolated by standard chromatographic methods (see the Supporting Information).

Pteratide I (1): amorphous white solid; $[\alpha]^{20}_D$ −128 (*c* 0.1, MeOH); for ^{1}H and ^{13}C NMR data see Table 1, for TOCSY, HMBC, and NOESY data see the Supporting Information; HRESIMS *m*/*z* 987.5918 $[M + H]^+$ (calcd for $C_{53}H_{79}N_8O_{10}$, 987.5919).

Pteratide II (2): amorphous white solid; $[\alpha]^{20}_D$ −40 (*c* 0.1, MeOH); for ^{1}H and ^{13}C NMR data see Table 1, for TOCSY, HMBC, and NOESY data see the Supporting Information; HRESIMS *m*/*z* 1023.5902 $[M + Na]^+$ (calcd for $C_{54}H_{80}N_8O_{10}Na$, 1023.5895).

Pteratide III (3): amorphous white solid; $[\alpha]^{20}_D$ −47 (*c* 0.1, MeOH); for ^{1}H and ^{13}C NMR data see Table 2, for TOCSY,

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TABLE 1. NMR Data for Pteratide I (1) and II (2)

		1[a]		**2**[b]				**1**[a]		**2**[b]	
position		^{13}C	^{1}H	^{13}C	^{1}H	position		^{13}C	^{1}H	^{13}C	^{1}H
Thr	NH		7.24		7.85	Ala2	NMe	29.0	2.50	28.2	2.32
	1	168.9		169.5			1	170.7		171.8	
	2	52.2	5.33	53.0	5.22		2	56.2	4.63	55.9	4.91
	3	73.0	5.12	72.5	5.20		3	15.0	2.50	13.5	1.22
	4	18.1	1.39	16.9	1.34	Phe	NH		9.31		9.31
	Bz-1	167.7		169.4			1	172.3		172.8	
	Bz-2	133.8		132.0			2	54.5	4.60	54.7	4.53
	Bz-3/7	127.5	7.85	127.1	7.77		3	35.0	3.05	34.0	3.00
	Bz-4/6	132.5	7.50	128.6	7.43				3.37		3.29
	Bz-5	129.1	7.58	132.1	7.51		4	139.0		138.5	
Leu	NMe	30.6	2.91	29.5	2.89		5/9	129.5	7.41	128.8	7.36
	1	169.2		169.7			6/8	128.7	7.26	128.2	7.16
	2	53.6	5.28	53.2	5.27		7	126.9	7.19	126.8	7.10
	3	37.5	1.34	37.0	1.29	Val1	NMe	29.9	3.24		
			1.97		1.83		1	171.1			
	4	25.0	1.50	24.7	1.43		2	57.7	5.10		
	5	22.5	0.91	21.4	0.83		3	27.6	2.45		
	6	23.2	0.93	21.9	0.84		4	18.5	0.92		
Ala1	NMe	29.3	2.58	29.0	2.54		5	20.2	0.88		
	1	168.6		168.4		Ile	NMe			29.2	3.14
	2	52.0	5.48	51.7	5.33		1			171.4	
	3	14.4	1.25	12.9	1.15		2			56.2	5.14
MePro	1	171.8		173.2			3			33.2	2.17
	2	56.4	4.90	55.7	5.24		3-Me			18.8	0.77
	3	36.0	1.79	35.3	1.81		4			23.9	0.93
			2.06		1.88						1.43
	4	33.5	2.75	33.2	2.53		5			15.0	0.78
	5	54.0	2.93	53.5	2.95	Val2	NMe	29.7	2.71	29.0	2.65
			3.89		3.72		1	168.7		168.8	
	4-Me	17.9	1.03	16.9	0.97		2	66.2	4.88	66.5	4.87
							3	27.8	2.31	27.0	2.42
							4	20.0	1.18	19.6	1.07
							5	19.9	0.98	19.1	0.88

[a] Recorded in $CDCl_3$ (500 MHz). [b] Recorded in MeOH-d_4 (500 MHz).

TABLE 2. NMR Data for Pteratide III (3) in MeOH-d_4

position		^{13}C[a]	^{1}H	position		^{13}C	^{1}H	position		^{13}C[a]	^{1}H
Thr	NMe	36.5	3.27	Phe	5/9	130.0	7.35	Leu	NMe	30.0	3.11
	1	172.7			6/8	128.9	7.31		1	174.1	
	2	61.8	5.74		7	127.2	7.25		2	50.5	5.48
	3	68.5	5.68	Val1	NH		7.66		3	37.1	1.34
	4	16.1	0.58		1	173.7					2.02
	Bz-1	174.4			2	54.1	4.51		4	25.1	1.59
	Bz-2	135.2			3	29.7	1.53		5	22.5	1.03
	Bz-3/7	128.3	7.47		4	16.1	0.16		6	21.3	1.07
	Bz-4/6	128.4	7.50		5	18.1	0.63	Val3	NMe	29.5	2.98
	Bz-5	131.2	7.50	Val2	NH		8.41		1	170.2	
Phe	NMe	30.7	3.04		1	174.2			2	65.4	4.39
	1	170.6			2	64.1	3.74		3	30.1	2.45
	2	63.3	5.05		3	29.5	2.29		4	19.7	1.05
	3	35.1	3.13		4	18.5	1.00		5	19.4	1.13
			3.68		5	19.4	1.09				
	4	137.8									

[a] The ^{13}C chemical shifts were determined from HSQC-DEPT and HMBC-CIGAR experiments.

HMBC, and NOESY data see the Supporting Information; HRES-IMS *m*/*z* 819.5000 $[M + H]^+$ (calcd for $C_{45}H_{67}N_6O_8$, 819.5020).

Pteratide IV (4): amorphous white solid; $[\alpha]^{20}_D$ −85 (*c* 0.33, MeOH); for ^{1}H and ^{13}C NMR data see Table 3, for TOCSY, HMBC, and NOESY data see the Supporting Information; HRES-IMS *m*/*z* 975.5940 $[M + H]^+$ (calcd for $C_{52}H_{79}N_8O_{10}$, 975.5919).

Methanolysis of Pteratides I and II (1, 2). The peptides **1** and **2** (20 μg each) were dissolved in dry MeOH (100 μL), and NaOMe solution (4%, 100 μL) was added. The mixtures were stirred for 3 h at 30 °C. After cooling, the solutions were evaporated to dryness and redissolved in ice H_2O−EtOAc (1:1) (1 mL). After solvent/solvent partitioning, the EtOAc layer for each compound was evaporated to dryness and analyzed by the ESI MS/MS.

Methyl Ester of 1: ESI MS/MS *m*/*z* 1041.7 (80) $[MCOOMe + Na]^+$, 997.8 (100) $[1041.7 - C_2H_4O]^+$, 836.8 (40) $[1041.7 - (NBz\text{-}Thr)]^+$, 783.5 (6) [(NBz-Thr-NMe-Leu-NMe-Ala-MePro-NMe-Ala-Phe) + $Na]^+$, 755.7 (20) $[783.5\text{-}CO]^+$, 709.7 (20) $[836.8 - (NMe\text{-}Leu)]^+$, 624.7 (10) $[709.7 - (NMeAla)]^+$.

Methyl Ester of 2: ESI MS/MS *m*/*z* 1055.7 (64) $[MCOOMe + Na]^+$, 1011.7 (100) $[1055.7 - C_2H_4O]^+$, 850.8 (12) [1055.7 −

TABLE 3. NMR Data for Pteratide IV (4) in $CDCl_3$

position		^{13}C	^{1}H	position		^{13}C	^{1}H	position		^{13}C	^{1}H
Val1	NMe	32.3	2.74	Thr	NH		7.15	Val2	3	29.7	2.26
	1	170.9			1	168.8			4	20.2	1.02
	2	58.6	5.28		2	54.85	4.46		5	15.8	0.66
	3	26.9	2.21		3	71.6	5.71	Ala1	NMe	31.3	3.27
	4	19.5	0.77		4	16.0	0.93		1	173.4	
	5	17.5	0.95	Phe	NMe	31.8	2.82		2	49.8	6.41
	Bz-1	171.5			1	168.52			3	15.0	1.29
	Bz-2	135.6			2	62.8	4.63	Ala2	NMe	28.7	2.72
	Bz-3/7	126.3	7.27		3	34.0	2.85		1	169.2	
	Bz-4/6	128.5	7.42				3.62		2	54.9	5.52
	Bz-5	129.9	7.33		4	136.9			3	14.7	1.36
Ile	NMe	30.3	3.09		5/9	128.8	7.28	Val3	NH		7.52
	1	169.6			6/8	128.7	7.30		1	168.46	
	2	60.4	4.45		7	127.1	7.30		2	58.2	4.57
	3	30.9	2.20	Val2	NH		7.15		3	32.3	1.89
	3-Me	16.1	0.70		1	172.6			4	18.1	0.77
	4	24.1	1.32		2	54.3	4.88		5	19.4	0.91
	5	9.8	0.84								

(NBz-Thr)]$^+$, 783.5 (6) [NBz-Thr-NMe-Leu-NMe-Ala-MePro-NMe-Ala-Phe + Na]$^+$, 755.7 (20) [783.5 − CO]$^+$, 723.7 (16) [850.8 − (NMeLeu)]$^+$, 638.7 (10) [723.7 − (NMeAla)]$^+$.

Stereochemical Analysis. The peptides **1**−**4** (0.5 mg each) were each hydrolyzed by heating (110 °C for 24 h) in HCl (6 M; 1 mL). After cooling, the solutions were evaporated to dryness and redissolved in H_2O (100 μL). A 1% (w/v) solution (100 μL) of FDAA (Marfey's reagent, N^{α}-(2,4-dinitro-5-fluorophenyl)-L-alaninamide)[12] in acetone was added to an aliquot (50 μL) of each acid hydrolysate solution (or to 50 μL of a 50 mM solution of the respective amino acid). After addition of $NaHCO_3$ solution (1 M; 20 μL), the mixture was incubated (1 h at 40 °C). The reaction was stopped by addition of HCl (2 M; 10 μL), the solvents were evaporated to dryness, and the residue was redissolved in MeOH−H_2O (1:1; 1 mL). An aliquot of each of these solutions (10 μL) was analyzed by HPLC (Prodigy C18, 250 × 4.6, 5 μm; solvents: A: water + 0.05% TFA, B: MeCN; linear gradient: 0 min 35% B, 30 min 45% B; 25 °C; 1 mL min^{-1}; detection at 330 nm). In all HPLC analyses of the FDAA derivatives the same column and flow rate were used and the compounds were detected at 330 nm. For the separation of the Thr, NMe-Thr, NMe-Ala, and 4-MePro FDAA derivatives, an alternative linear gradient was used (A: water + 0.05% TFA, B: MeOH; 0 min 45% B, 30 min 65% B). The FDAA derivatives of 4-MePro were analyzed by a third linear gradient (A: water + 0.05% HCOOH, B: MeOH; 0 min 45% B, 30 min 65% B). Retention times (min) of the FDAA amino acid derivatives used as standards together with the data for the observed peaks in the HPLC trace of the FDAA-derivatized hydrolysis products of **1**−**4** are included in the Supporting Information (Table T5).

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Supporting Information Available: General experimental procedures, 1D and 2D NMR spectra of pteratides (**1**−**4**), tables with 1D and 2D NMR data for **1**−**4**, and a table with data for HPLC analysis of the FDAA-derivatized hydrolysate of **1**−**4** and amino acid standards. This material is available free of charge via the Internet at http://pubs.acs.org.

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