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Bromoalterochromides A and A′, Unprecedented Chromopeptides from a Marine *Pseudoalteromonas maricaloris* Strain KMM 636^{T}†

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Abstract The marine strain *Pseudoalteromonas maricaloris* KMM 636^{T} was found to produce an inseparable mixture of two brominated yellow main pigments, bromoalterochromide A and A′, in a ratio of 3 : 1. Both pigments are Thr-Val-Asn-Asn-X pentapeptide lactones, where the amino group of Thr is acylated with 9-(3-bromo-4-hydroxyphenyl)-nona-2,4,6,8-tetraenoic acid, and X is *a*Ile and Leu, respectively. They possess cytotoxic effects on developing eggs of the sea urchin *Strongylocentrotus intermedius*, but no antibiotic activity.

Keywords bromoalterochromide, chromopeptide, peptide lactone, *Pseudoalteromonas maricaloris*

Introduction

The strain *Pseudoalteromonas maricaloris* KMM 636^{T} was isolated as an epibiont of the Australian sponge *Fascaplysinopsis reticulata* collected at the Great Barrier Reef. On HPLC/ESI-MS, crude extracts displayed double $[M+H]^{+}$ signals at m/z=844/846. The UV/Vis spectrum of the responsible yellow pigment was unusual, showing only an absorption maximum at λ_{max}=395 nm with a tailing into the visible region, but no absorption between 200~350 nm.

As there was no colour change neither with sodium hydroxide nor with dithionit solution, quinones were excluded. Also long polyene chains not in conjugation with carbonyl groups or aromatic systems were not likely, as the typical band structure in the absorption spectrum was missing and there was no blue or green colouration observed with concentrated sulphuric acid. Phenoxazinone chromophors as in actinomycins were excluded by direct comparison. The pigment was identified as a mixture of two isomeric chromopeptides named bromoalterochromide A (**1a**) and A′ (**1a′**) [1] (Fig. 1), respectively, together with minor amounts of further homologues and dibrominated pigments, for which we reserve the names alterochromide B/B′, *etc.* The alterochromides possessed cytotoxic effects on developing eggs of the sea urchin *Strongylocentrotus intermedius* with an MIC value of 40 μg/ml [2].

Results and Discussion

Upscaling of *Pseudoalteromonas maricaloris* KMM 636^{T} was done on agar plates, as the yellow pigments were not formed in shaken cultures. The methanol extract of 1500 agar plates gave 2.1 g of a yellow-brown powdery solid, which was defatted with cyclohexane and separated on

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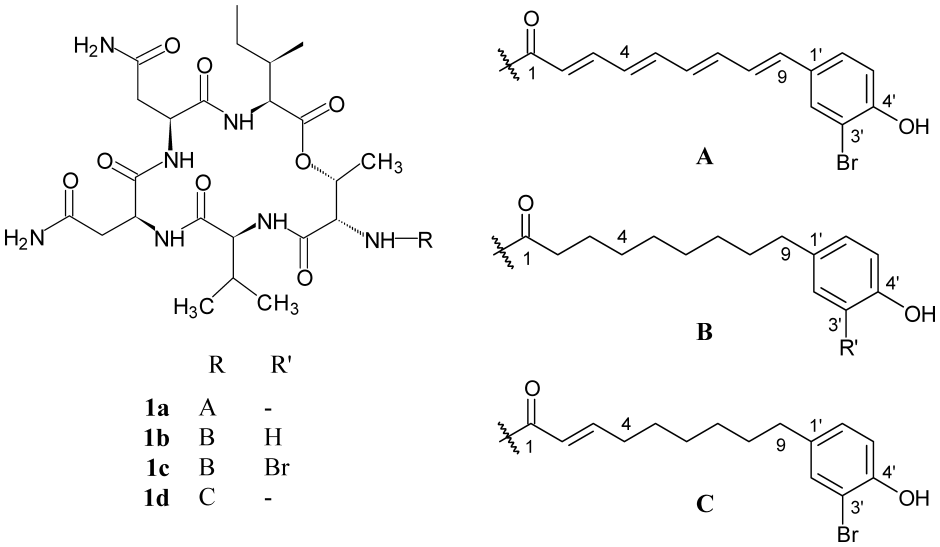


Fig. 1 Structures of bromoalterochromide A derivatives **1a**∼**1d**.

The bromoalterochromide A′ derivatives (**1a**′∼**1d**′) are containing Leu instead of *a*lle.

Sephadex LH-20. By analytical HPLC, MS and NMR spectroscopy, the yellow main fraction turned out to be a mixture of one main and three minor isomers, which probably differed in their double bond configuration. As equilibration occurred within a few hours at room temperature, a separation was not achieved on a preparative scale.

The mass differences of $\Delta m=2$ of the molecular ions and their intensities of ∼1 : 1 pointed to monobrominated compounds, and high resolution MS confirmed indeed a composition of $C_{38}H_{50}BrN_7O_{10}$ for the main pigment.

The proton NMR spectrum (Fig. 2) showed signals at $\delta=4.8\sim3.9$ and D_2O-exchangeable signals at $\delta=8.7\sim7.1$, typical for α-CH protons and NH groups of peptides, and after acidic hydrolysis, Asx, Thr, Val, Leu, and the non-proteinogenic amino acid *allo*-Ile were found in a ratio of 2 : 1 : 1 : 0.25 : 0.75. In the ^{13}C NMR spectrum, beside 8 carbonyl signals, 14 sp^2 carbon signals were visible for the main component, indicating a benzene ring and four double bonds. Further NMR analysis was difficult due to strong signal overlapping and the presence of minor isomers.

Catalytic hydrogenation yielded a mixture of three colourless, but still UV absorbing products **1b**∼**1d** and **1b**′∼**1d**′, respectively. As these compounds were more stable than the parent pigment and their NMR signals were better separated, further investigation was carried out on the hydrogenation products.

The polar main component **1b**/**1b**′ was obtained by HPLC as a white solid. The missing bromine isotope pattern in the mass spectrum and $m/z=773$ (ESI MS) indicated, that bromine was lost and four double bonds had been hydrogenated.

The ^{13}C NMR spectrum of **1b**/**1b**′ showed still four aromatic sp^2 C atoms, two of them with double intensity, thus indicating a *para*-disubstituted benzene. This was confirmed by the proton spectrum with two 2H AB doublets at $\delta=6.93$ and 6.63. The absence of this AB pattern in the ^1H NMR spectrum of the parent compound **1a**/**1a**′ indicated that bromine had been removed from a ring position.

The HMBC couplings and a complete set of all expected H,H COSY signals confirmed the carbon skeletons of Thr, Val, Leu, *a*lle, and Asx fragments, and the proton spectrum of **1b**/**1b**′ allowed also to differentiate between asparagine and aspartic acid: Two pairs of broadened NH proton singlets at $\delta=7.27/6.85$ and 7.25/6.75, respectively, showed cross couplings in the H,H COSY spectrum and were exchanged much quicker by D_2O than the other NH signals thus indicating two asparagine moieties [3].

As the intensities of the *a*lle and Leu signals in the ^1H NMR spectrum of **1b**/**1b**′ showed the same intensity ratio as in the amino acid analysis of the native pigment, it was obvious that both the native pigment and the hydrogenation product were 3 : 1 mixtures of two peptides, which differed by their *a*lle and Leu content, respectively, but were not separated by HPLC.

The sequence of the amino acids was unambiguously derived from COSY and HMBC spectra of **1b**/**1b**′, as it is exemplified for the Thr-Val-Asn substructure: The Val_{NH} signal at δ 8.03 and the valine carbonyl group (δ 171.9) were clearly assigned by their 2D couplings with the α-H (δ 3.98), which itself coupled with the isopropyl

Table 1 NMR Data of Bromoalterochromides in [D_6]DMSO at 500 MHz (^{1}H) and 125 MHz (^{13}C); data indicate shift values (δ), signal patterns, and coupling constants in [Hz]

Atom number	^{1}H NMR				^{13}C NMR			
	1a	**1b**	**1c**	**1d**	**1a**	**1b/1b′**	**1c**	**1d**
Thr-1	—	—	—	—	168.4	168.5	168.4	168.5
2	4.79 m	4.62 dd, 9, 8	4.62 dd, 9, 8	4.62 dd, 9, 8	55.1	54.9	54.9	54.9
2-NH	8.28∼8.05	7.92 d, 10	8.10 m	8.10 m	—	—	—	—
3	4.79 m	4.72 m	4.72 m	4.72 m	72.3	72.0	72.3	72.1*
4	1.36 d, 7	1.32 d, 7	1.32 d, 7	1.32 d, 7	16.7	16.6	16.6	16.6
Val-1	—	—	—	—	171.9	171.9	171.9	171.9
2	3.96 dd, 7, 7	3.96 dd, 7, 7	3.96 dd, 7, 7	3.96 dd, 7, 7	59.3	59.1	59.3	59.2*
2-NH	8.28∼8.05	8.03 d, 6	8.10 m	8.10 m	—	—	—	—
3	1.86 m	1.86 m	1.86 m	1.86 m	29.3	29.3	29.3	29.3
3-Me	0.91 d, 7	0.91 d, 7	0.91 d, 7	0.91 d, 7	18.8	18.8	18.8	18.8
4	0.86 m	0.86 m	0.86 m	0.86 m	19.0	18.9	18.9	18.9
Asn_a 1	—	—	—	—	171.4	171.3	171.3	171.3
2	4.35 m	4.35 m	4.35 m	4.35 m	51.4	51.4	51.4	54.4
2-NH	8.62 m	8.59 d, 8	8.59 m	8.59 m	—	—	—	—
3	2.41 m	2.41 m	2.41 m	2.41 m	36.2	36.2	36.2	36.2
4	—	—	—	—	170.6	170.6	170.7	170.7
4-NH_2	8.28∼8.05, 6.85∼6.10	7.25 s 6.85 s	7.26 s 6.85 s	7.26 s 6.85 s	—	—	—	—
Asn_b 1	—	—	—	—	169.9	169.9	169.9	169.9
2	4.35 m	4.35 m	4.35 m	4.35 m	50.8	50.8	50.8	50.8
2-NH	8.28∼8.05	8.09 d, 9	8.10 m	8.10 m	—	—	—	—
3	2.73 m	2.73 m	2.73 m	2.73 m	35.3	34.9	34.9	34.9
4	—	—	—	—	172.2	172.1	172.2	172.2
4-NH_2	7.18 d, 8 6.85∼6.10	7.27 s 6.75 s	7.26 s 6.75 s	7.26 s 6.75 s	—	—	—	—
*a*lle-1/Leu-1*	—	—	—	—	169.2	169.1/171.9	169.1	169.1
2	4.35 m	4.35 m	4.35 m	4.35 m	56.2	56.2/50.0	56.2	56.2
2-NH	7.36 d, 8	7.14 d, 10	7.14 d, 10	7.14 d, 10	—	—	—	—
3	1.95 m	1.95 m	1.95 m	1.95 m	36.7	36.6/40.9	36.7	36.7
3-Me/4-Me	0.81 d, 7	0.81 d, 7	0.81 d, 7	0.81 d, 7	14.4	14.4/21.3	14.4	14.4
4	1.36 m, 1.04 m	1.32 m, 1.04 m	1.32 m, 1.04 m	1.32 m, 1.04 m	25.7	25.7/23.9	25.7	25.7
5	0.86 m	0.86 m	0.86 m	0.86 m	11.2	11.1/23.0	11.1	11.1
acyl-1	—	—	—	—	164.8	172.0	172.1	164.6
2	6.85∼6.10	2.11 m	2.11 m	6.05 d, 16	126.9*	34.9	34.9	123.9
3		1.46 m	1.46 m	6.63 dt, 16, 8	139.8*	28.5*	28.5*	143.6
4		1.21 m	1.21 m	2.11 m	127.3*	25.2*	25.2*	31.9
5		1.21 m	1.21 m	1.46 m	139.3*	28.5*	28.5*	28.5*
6		1.21 m	1.21 m	1.21 m	130.7*	28.7*	28.7*	28.7*
7		1.21 m	1.21 m	1.21 m	136.4*	28.7*	28.7*	28.7*
8		1.46 m	1.46 m	1.46 m	130.1*	31.2	31.2*	31.2*
9		2.41 m	2.41 m	2.41 m	131.9*	34.2	34.2	34.2
1′	—	—	—	—	124.0	132.2	134.5	134.5
2′	7.62 s br	6.93 d, 8	7.26 s	7.26 s	132.6	128.9	132.1	132.1
3′	—	6.63 d, 8	—	—	109.8	114.9	108.9	108.9
4′	—	—	—	—	153.9	155.1	151.8	151.8
4′-OH	10.41 s br	9.03 s	9.83 s	9.83 s	—	—	—	—
5′	6.92 d, 8	6.63 d, 8	6.83 d, 8	6.83 d, 8	116.5	114.9	116.2	116.2
6′	7.28 m	6.93 d, 8	6.96 d, 8	6.96 d, 8	129.9	128.9	128.3	128.3

* Tentatively assigned.

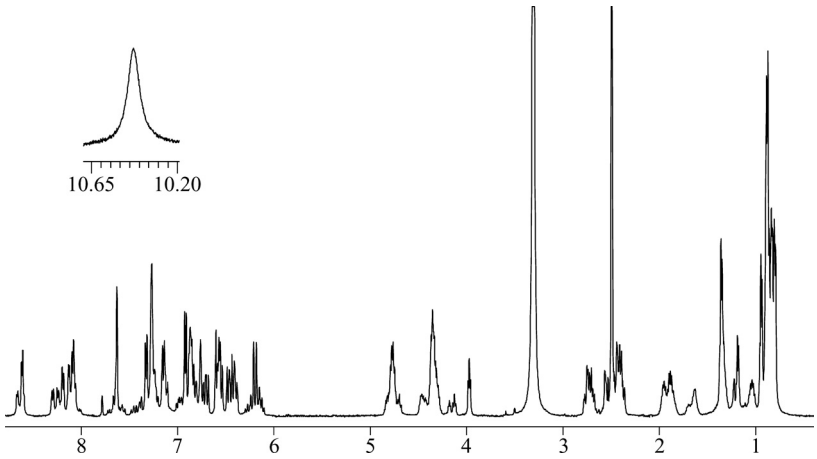


Fig. 2 ^{1}H NMR spectrum (500 MHz) of bromoalterochromides A/A′ (**1a**/**1a**′) in [D_6]DMSO.

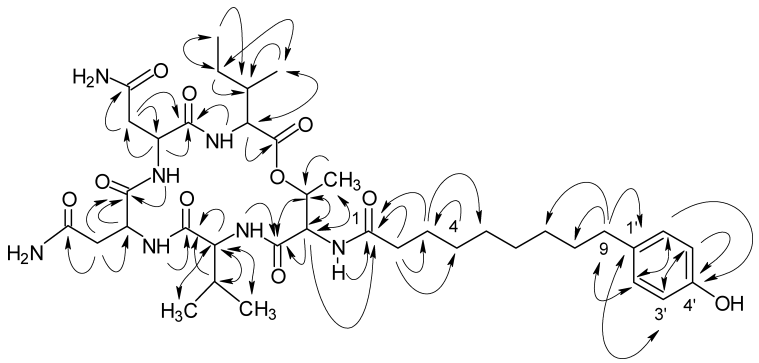
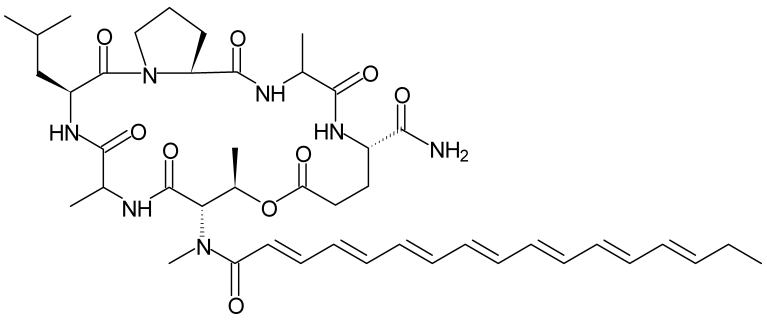


Fig. 3 HMBC couplings of **1b** in [D_6]DMSO.
→ couplings from H to C, ↔ H, C couplings in both directions.



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group. The Val_{NH} signal coupled also with the carbonyl group of the adjacent Thr residue at δ 168.5, and the Val-CO group had a cross signal with α-H of one of the Asn residues. Further couplings (see Fig. 3) delivered the sequence Acyl-Val-Asn-Asn-Leu/*a*Ile-lactone. For the acyl side chain, the 2D NMR experiments resulted finally in a 9-(*p*-hydroxyphenyl)-nonanoic acid fragment, which is connected through an amide bond with the Thr nitrogen of the peptide lactone. The final structure **1b**/**1b**′ was consistent with all analytical results.

To further confirm the structure, ESI daughter ions were investigated. It is known that the highly unsaturated lipopeptide lacton myxochromide A (**2**) is cleaved first at the ester bond between Thr and Gln by a McLafferty

rearrangement, forming a double bond [3]. In analogy, **1b**/**1b**′ gave a daughter ion at 667 which may be explained by a loss of Leu or *a*Ile. Unfortunately, fragmentation by a sequential loss of further amino acids was not observed, and only smaller fragments were obtained.

According to the (+)-FAB mass spectrum with masses of *m*/*z* 854, 852 and 850, the more lipophilic hydrogenation product was a mixture of isomers of octahydro- and hexahydroalterochromides A/A′ (**1c**/**1c**′, **1d**/**1d**′). The pattern of three aromatic ^{1}H NMR signals indicated protons in 1,2,4-position of a benzene ring. On comparison with **1b**/**1b**′, the third substituent must be bromine, whose position at C-3′ of the benzene ring was further confirmed by HMBC spectra and by comparison with reference data of aplysillin A [4] and bastadin-1 [5]. The expected olefin signals of **1d**/**1d**′ appeared at δ=6.83 and 6.02, which is explained best by a double bond in α-position to the carbonyl group. The ^{13}C NMR spectrum showed a signal at δ=172.1 for C-1 of **1c**/**1c**′, while the respective signal of **1d**/**1d**′ was found at 164.6, as expected for a conjugated amide. The latter carbonyl C atom showed a significant correlation with the α-CH proton of Thr, thus providing further evidence for the connection site of the chromophore to the peptide cycle.

According to the NMR and mass spectra of the native and the hydrogenated products, the novel *Pseudoalteromonas* main pigments **1a** and **1a**′ are acyl-peptide lactones containing *a*Ile and Leu, respectively, with 9-(3-bromo-4-hydroxyphenyl)-nona-2,4,6,8-tetraenoic acid as chromogenic residue [1]. The halogen-free isomeric chromopeptides were named alterochromides A and A′, so that **1a** and **1a**′ are bromoalterochromides A and A′. The minor fractions contained also dibrominated chromopeptides (dibromoalterochromides), as ESI HRMS indicated. Further measurements were, however, not possible due to decomposition. Also the chirality determination is preliminary for the same reason: GC analysis of *N*-trifluoroacetyl amino acid isopropyl esters obtained from 1 mg hydolysate on a chiral Permabond®-L-Chirasil-Val column gave signals corresponding to L-amino acids.

Cyclic peptides with a related chromophore are not known; the closest similarity shows whose cyclopeptide consists of six amino acids, but carries a polyene chain without an aromatic ring.

Experimental

Materials and Methods

^{1}H NMR spectra: Varian INOVA 500 (499.8 MHz). Coupling constants (*J*) in Hz; s=singlet, d=doublet, dd=double doublet, t=triplet, q=quartet, m=multiplet, br=broad. ^{13}C NMR spectra: Varian INOVA 500 (125.7 MHz). Chemical shifts are δ values with TMS as internal standard.–Mass spectra: FAB-MS with 3-nitrobenzylalcohol; ESI MS LCQ mass spectrometer (Finnigan) with ion trap and nano-ESI-API-Ion source. APEX IV (7 Tesla-Fourier Transform Ion Cyclotron Resonance (FTICR) mass spectrometer, Bruker).–CD spectra: Jasco J-600. HPLC: All eluents were filtered through membrane filters and for 10 minutes degassed by ultrasonic irradiation; Analytical separations: Jasco Multiwavelength Detector MD-910, two pumps Type Jasco Intelligent Prep. pump PU-987 with high pressure mixer, degasser VDS Degasys DG-1310, and Reodyne valve with 20 μl injection loop. Software: Borwin HPLC software. Columns: 1) Vertex 4×250 mm with 4×4 mm pre-column. Stationary phase: Eurochrom Eurospher RP 60-10 C18 60 Å 7~12 μm, Merck Lichrosorb RP C18 7 μm or ODS-AQ/303; preparative separations on Eurochrom Europrep RP 60-10 C18 60 Å 7~12 μm with acetonitril/water aceotrope (83.7% acetonitril/16.3% water, bp. 78.5°C). GC-MS: Varian 3400 GC, capillary column DB 5, 20 m×0.25 mm. GC for amino acid analysis: Siemens Sichromat 1, split/splitless, gas/liquid injector i.d. 0.3 cm, FID. Column: Permabond®-L-Chirasil-Val (Macherey-Nagel, Dueren, Germany) 25 m×0.32 mm, eluent gas: helium, gas for FID: artificial air/hydrogen. Amino acid analyser: Beckman System 6300; detection with ninhydrine. Thin layer chromatography (TLC): Polygram SIL G/UV$_{254}$ (Macherey-Nagel & Co., Germany). Column chromatography (CC): MN silica gel 60: 0.05~0.2 mm, 70~270 mesh (Macherey-Nagel & Co.); Sephadex LH-20 (Pharmacia).

Determination of the Cytotoxic Activity

Eggs and sperm from the sea urchin *Strongylocentrotus intermedius*, collected in the Troitza bay of Japan of Sea (Russia) was used. The cleavage rate of blastomers was determined according to Biyity *et al.* [2].

Eggs were rinsed, filtered and diluted by natural seawater (NSW) to a concentration of 2000 eggs per ml. Sperm was collected “dry” and shortly before use the semen was diluted (1 : 50) in NSW. Fertilisation was obtained by adding 10 μl of sperm to 1 ml of eggs suspension. The mixture of pigments was used in ethanol solution; the maximal ethanol concentration of 1% used in the experiments did not affect cell division. The percentage of divided cells was determined under the light microscope. MIC, defined as lowest concentration pigments stopping the cleavage of the eggs on zygote stage was examined.

Taxonomy

Pseudoalteromonas maricaloris KMM 636^T is a marine, Gram-negative, aerobic bacterium and was isolated from the Australian sponge *Fascaplysinopsis reticulata* Hentschel. The phenotype, genotype and phylotype place the organism in the genus *Pseudoalteromonas* [6].

Fermentation, Isolation and Purification

The strain was cultivated for 5 days at 25~28°C on M_2/50% sea water agar plates (see page 36, footnote). The cell material of 1500 Petri dishes was suspended in 100 ml of CH_3OH, sonificated for 2 minutes and filtered. The cell residue was extracted twice with each 200 ml CH_3OH in the same manner. On evaporation, the extracts gave 2.1 g of a brownish-yellow, powdery residue.

This crude extract was suspended in 70 ml CH_3OH, and 120 mg insoluble material was filtered off. The soluble part was separated by CC on Sephadex LH-20 (30×600 mm, CH_3OH), giving four fractions: I, colorless, Rf=0.85~0.53, 750 mg; II: yellow, Rf <0.05, 50 mg; III: yellow, Rf <0.05, 60 mg; IV: orange, Rf <0.05, 40 mg.

Fraction I contained uracil (identified by HPLC/MS), fraction III consisted mainly of fats and was discarded. Fraction II contained **1a**/**1a′**, fraction IV decomposed on storage.

Fraction II delivered by HPLC (linear gradient, CH_3CN-H_2O aceotrope/H_2O: starting with 40/60; 5 minutes, 40/60; 25 minutes, 100/0; 35 minutes, 100/0; 40 minutes 40/60; 17.5 ml/minute) four yellow fractions with t_{ret} 21.2, 22.5, 24.3, and 25.6 minutes. The constituent of the main fraction (t_{ret} 21.2) equilibrated after separation, yielding again the composition of the starting mixture. The other II-fractions showed the same behaviour.

Bromoalterochromides A and A′ (1a/1a′)

UV/vis (MeOH): λ_{max}=385, 470 nm. CD (MeOH): λ_{ext} ($[\theta]_{\%}^{25}$)=470 (−80), 360 (+80), 280 (−85), 237 nm (+100). (+)-FAB MS: *m/z* (%)=846 ($[M+H]^+$, 100), 844 ($[M+H]^+$, 100). (−)-FAB-MS: *m/z* (%)=844 ($[M-H]^-$, 100), 842 ($[M-H]^-$, 100). (+)-ESI-MS: *m/z* (%)=868 ($[M+Na]^+$, 100), 866 ($[M+Na]^+$, 100). (−)-ESI-MS: *m/z* (%)=880 ($[M+Cl]^-$, 100), 878 ($[M+Cl]^-$, 100), 844 ($[M-H]^-$, 43), 842 ($[M-H]^-$, 43). (+)-ESI HRMS: *m/z*=844.28750 (calcd. 844.28754 for $[M+H]^+$, $C_{38}H_{51}BrN_7O_{10}$).

Hydrolysis of Bromoalterochromides A/A′ (1a/1a′)

A solution of ~1 mg **1a**/**1a′** in 1 ml 6 M HCl was kept under N_2 for 24 hours at 110°C. On evaporation, the residue gave on amino acid analysis 2.13 nM Asn, 0.98 nM Thr, 0.90 nM Val, 0.76 nM *a*Ile, and 0.27 nM Leu, corresponding to a ratio of 2 : 1 : 1 : 0.75 : 0.25.

Hydrogenation of Bromoalterochromides A/A′ (1a/1a′)

A suspension of 20 mg **1a**/**1a′** and 50 mg Pd/C in 50 ml CH_3OH was hydrogenated for 16 hours under hydrogen at room temperature and normal pressure. After filtration and evaporation, the residue gave two main fractions on HPLC (flow rate 17.5 ml/minute; solvent A: CH_3CN/H_2O aceotrope; solvent B: H_2O; 10 minutes 20% A; linear to 100% A in 60 minutes, then 10 minutes 100% A; before next cycle, 15 minutes equilibrating with 20% A) with t_{ret}=39.3 minutes (2.3 mg **1b**/**1b′**) and 43.9 minutes (3.6 mg mixture of **1c**/**1c′** and **1d**/**1d′**).

1b/**1b′**: (+)-ESI MS: *m/z* (%)=1569 ($[2M+Na]^+$, 36), 796 ($[M+Na]^+$, 100), 774 ($[M+H]^+$, 8). (+)-ESI HRMS: *m/z*=774.43970 (calcd. 774.43964 for $[M+H]^+$, $C_{38}H_{60}BrN_7O_{10}$).

1c/**1c′** (as mixture with **1d**/**1d′**): (+)-FAB MS: *m/z* (%)=854 ($[M_{\mathbf{1c}}+H]^+$, 60), 852 ($[M_{\mathbf{1c,1d}}+H]^+$, 100), 850 ($[M_{\mathbf{1d}}+H]^+$, 48). (−)-FAB MS: *m/z* (%)=852 ($[M-H]^-$, 50), 850 ($[M-H]^-$, 100), 848 ($[M-H]^-$, 40). (+)-ESI-MS: *m/z* (%)=876 ($[M+Na]^+$, 58), 874 ($[M+Na]^+$, 100), 872 ($[M+Na]^+$, 59), 854 ($[M_{\mathbf{1c}}+H]^+$, 8), 852 ($[M_{\mathbf{1c,1d}}+H]^+$, 10), 850 ($[M_{\mathbf{1d}}+H]^+$, 8). (+)-ESI HRMS: *m/z*=852.35010 (calcd. 852.35016 for $[M+H]^+$, $C_{38}H_{59}BrN_7O_{10}$); (+)-ESI HRMS: *m/z*=850.33450 (calcd. 850.33447 for $[M+H]^+$, $C_{38}H_{57}BrN_7O_{10}$).

Determination of Chirality

Reference amino acids (3~5 mg) and a hydrolysed peptide sample (1~2 mg, see above) were esterified by boiling with 5 ml of 2-propanol under reflux for 2 hours while the solution was saturated with HCl gas. The sample was evaporated *in vacuo* and the residue dissolved in 0.5 ml of dichloromethane. For trifluoracetylation, 0.2 ml of trifluoro acetanhydride were added and the mixture stirred for 3 hours at room temperature. After careful evaporation to dryness, the sample was again dissolved in 2 ml dichloromethane and subjected to GC on Permabond®-L-Chirasil-Val. By comparison with reference samples, the hydrolysate was tentatively assigned as mixture of L-amino acids.

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