Book Chapter

Unique Polyhalogenated Peptides from the Marine Sponge *Ircinia* sp.

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Abstract

Two new bromopyrrole peptides, haloirciniamide A (**1**) and seribunamide A (**2**), have been isolated from an Indonesian marine sponge of the genus *Ircinia* collected in Thousand Islands (Indonesia). The planar structure of both compounds was assigned on the basis of extensive 1D and 2D NMR spectroscopy and mass spectrometry. The absolute configuration of the amino acid residues in **1** and **2** was determined by the application of Marfey's method. Compound **1** is the first dibromopyrrole cyclopeptide having a chlorohistidine ring while compound **2** is a rare peptide possessing a tribromopyrrole ring. Both compounds failed to show significant cytotoxicity against four human tumor cell lines and neither compound was able to inhibit the enzyme topoisomerase I or impair PD1-PDL1 interaction.

Keywords

Marine Sponge; *Ircinia* sp.; Polyhalogenated Peptides; Marfey's Analysis

Introduction

Indonesia is located at the centre of a biodiversity hotspot and around 750 structures from Indonesian waters have been published in the last 50 years [1]. The structural diversity and bioactive properties of the compounds isolated from this region encouraged us to continue to investigate this area, which still remains largely unexplored. Thus, one of the recent PharmaMar expeditions was carried out in Thousand Islands, in collaboration with Indonesian Institute of Sciences (LIPI). Thousand Islands archipelago is located about 25 miles from the coast to the northeast of Jakarta and the collection site is an area full of gentle rock and coral slopes, and is potentially a highly productive area, both in terms of the quantity and the nature of the biodiversity. In this paper we describe the isolation of two new peptides isolated from an *Ircinia* specimen from this area.

Of all the marine organisms investigated, sponges (Porifera) are the most primitive multicellular animals with ample time to evolve into more complex living organisms. In fact, marine sponges are recognized as the richest sources of MNP, contributing to nearly 30% of all marine natural products discovered so far [2]. Previous reports revealed that Marine sponges of the genus *Ircinia* are known as a rich source of varied bioactive natural products, including fatty acids [3], steroids [4], terpenes [5], macrolides [6], and peptides [7], many of which have biological activities. This structural diversity could be due to the fact that sponges harbour diverse microorganisms and in numerous cases, bacteria isolated from sponges or symbiotic bacteria are the true producers of the compounds found in their extracts [8]. Specifically, an intriguing group of *Ircinia* derived peptides are assumed to be of microbial origin due to the presence of both *D*-amino acids and unusual amino acids as illustrated by the cyclic hexapeptide waiakeamide from *Ircinia*

dendroides [9] and the cyclotheonamides E4 and E5, cyclic pentapeptides also from *Ircinia* species [10].

Undeniably, among the compounds isolated from marine sources, linear and cyclic peptides are recognized as an important class with great structural diversity and a wide range of bioactivities, and these include the antimalarial carmabin A [11], the antiproliferative jaspamides [12] and the cytotoxic patellamides [13]. Furthermore, two marine peptide-derived products have reached the market, ziconotide [14] for analgesic use and a synthetic derivative of dolastatin 10 [15] linked to an antibody for the treatment of Hodgkin´s lymphoma. PharmaMar has also developed a marine natural peptide Aplidin, originally found in the Ascidian *Aplidium albicans*, which has recently been approved for commercialization in Australia for the treatment of multiple myeloma. Recent studies suggest that Aplidin may also have antiviral properties and a clinical trial to treat patients with COVID-19 has been initiated.

In the course of our screening program to isolate novel compounds with antitumor properties from marine sources, we have isolated two unique peptides haloirciniamide A (**1**) and seribunamide A (**2**) from an *Ircinia* specimen, which was collected off the coast of Thousand Islands. It is worth mentioning that the number of known peptides with a halogenated pyrrole ring is limited, with only cyclocinamides and corticiamide A [16] as well as gunungamide A [17] having been described as possessing chlorinated pyrrole rings. Although there are dozens of dibromopyrrolecarboxamide derivatives from porifera like nagelamide [18] and carteramine [19], mainly from *Agela* and *Stylissa* species, compound **1** is the first example of a cyclopeptide containing a halogenated pyrrole ring in its structure. Indeed, haloirciniamide A represents a structurally unique depsipeptide, since it also has an unprecedented chlorohistidine moiety. Furthermore, there are only two examples of tribromopyrrole rings derived from natural sources, 2,3,4-tribromopyrrole itself, which was isolated from the marine Poychaete *Polyphysia crassa* [20] and tribromopyrrol-2-methylphenol isolated from a coralline algal-

associated *Pseudoalteromone* [21], with compound **2** being the first of its class.

Details of the isolation and structural elucidation of the new halogenated peptides **1** and **2** are provided. The results of antitumor, PD1 and TOPO I screening are also described.

Results and Discussion

Isolation and Structure Elucidation

The sponge *Ircinia* sp. was collected by hand while diving in Thousand Islands (Indonesia). The specimen was repeatedly extracted using CH_2Cl_2:MeOH (1:1 v/v). The combined concentrated extracts, after vacuum liquid chromatography (VLC) and semipreparative reverse-phase HPLC separations, led to the isolation of the two pure compounds shown in Figure 1.

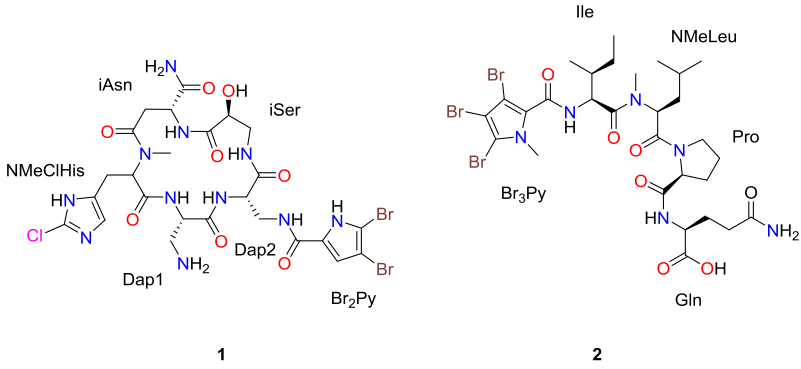

Figure 1: Chemical structures of the compounds **1** and **2** isolated from *Ircinia* sp.

Compound **1** was isolated as an amorphous white solid. The isotopic distribution observed in the (+)-LRESI mass spectrum with four protonated ion $[M+Na]^+$ peaks at *m/z* 830, 832, 834 and 836 in the ratio 3:6:3:1 respectively, showed the presence of two bromine and a chlorine atom in the molecule. The presence of these halogens in the structure was confirmed by (+)-HRESI-TOFMS analysis, with the ion peak observed at *m/z* 830.0400 $[M+Na]^+$ corresponding to the molecular formula $C_{25}H_{32}{}^{79}Br_2{}^{35}ClN_{11}O_8Na$ (calcd. 830.0383). Interpretation of the

mono NMR data (^{1}H, ^{13}C and 1D-TOCSY) compiled in **Table 1** and two-dimensional NMR spectra (gHSQC, gCOSY, gHMBC, and 2D-TOCSY) in CD_3OD, led to identification of 6 spin systems. Taking into consideration the seven carbonyl carbon resonances (δ_C 161.4 – 175.8) and the number of α-amino acid proton signals (δ_H 4.06 – 4.84), the peptide nature of compound **1** was expected. This hypothesis was confirmed by the NH signals observed in the ^{1}H NMR spectrum in CD_3OH (δ_H 7.13 – 12.68) and DMSO-d_6 (δ_H 7.37 – 12.03), with the latter solvent being chosen for full structural elucidation. The COSY correlations observed between methines at δ_H 4.23/δ_C 50.5 and δ_H 4.49/δ_C 51.5 with the diastereotopic methylenes at δ_H 2.87; 3.13/δ_C 49.2 and δ_H 3.22; 4.04/δ_C 40.5 respectively, indicated the presence of two 2,3-diaminopropionic acid units (**Figure 2**). Both amino acids were directly connected, based on the HMBC correlation of the NH signals at δ_H 9.11 for Dap1 and δ_H 7.72 for Dap2 with the same carbonyl carbon at δ_C 169.5. The next amino acid present in the peptide core was an isoserine with a methylene group at δ_H 2.75; 3.47/δ_C 42.8, a methine carbon at δ_H 4.13/δ_C 67.8 and an NH signal at δH 8.23. Isoserine was placed linked to the Dap2 by the correlation between NH at δ_H 8.23 and the carbonyl carbon of Dap2 at δ_C 170.6. In addition, an HMBC correlation of the methine carbon at δ_H 4.13 and the NH signal at δ_H 7.13, that belongs to a unit of isoasparagine (δ_H 4.58/δ_C 48.4, δ_H 2.80; 3.09/δ_C 35.0), with the carbonyl group at δ_C 170.6, allowed the sequence of amino acids to be continued. Finally, the peptide ring was closed by an NMe-histidine (δ_H 3.99/δ_C 65.6, δ_H 3.05/δ_C 25.0), whose N-methyl group showed an HMBC correlation with carbons belonging to the carbonyl group of iAsn at δ_C 173.5 and its own methine at δ_C 65.6., with an additional HMBC correlation between the α-aminoacid proton signal of NMe-histidine and the NH signal at δ_H 9.11 of Dap1 with the carbonyl carbon at δ_C 169.0 (Figure 2). NMe-histidine ring shifts at δ_C 110.5, δ_C 128.2 and δ_H 6.76/δ_C 109.5, revealed that the non-protonated carbon at δ_C 128.2 bore one of the three halogen atoms.

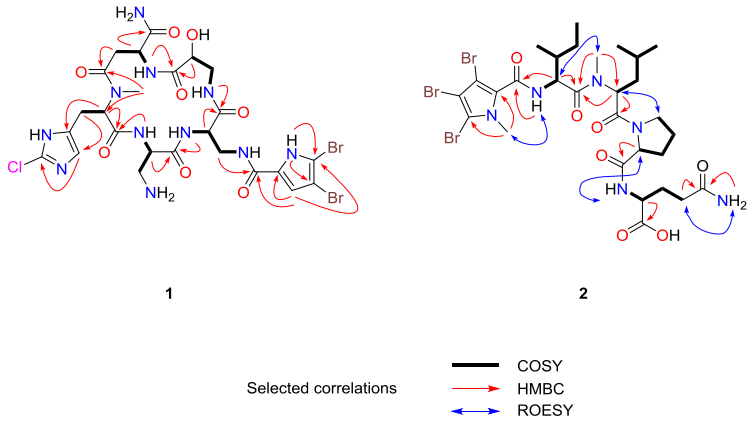

Figure 2: Selected key COSY (bold), HMBC (red) and ROESY (blue) correlations for **1** and **2**.

To complete the structure elucidation, the two remaining doublets with a small coupling constant value of 2.7 Hz were assigned to a sp^2 methine at δ_H 6.30/δ_C 110.4 and a significant downfield NH signal at δ_H 12.68. An HMBC correlation of these two protons with three non-protonated sp^2 carbons (δ_C 96.9, 118.1 and 123.2), demonstrated the existence of a trisubstituted pyrrol moiety, with two of these three positions bearing halogens. The placement of this heterocycle was established by the HMBC correlation of the methylene group of Dap2 with a carbonyl group at δ_C 158.6. To confirm the direct connection between the pyrrol moiety and this carbonyl group, a new gHMBC experiment with J = 3 Hz was conducted. The position of the sp^2 methine in the pyrrol unit and the bond to the ring with the cyclopeptide, was settled by the HMBC correlation between the methine proton at δ_H 6.30 and the carbonyl group at δ_C 158.6. Although the chlorine and two bromine atoms were undoubtedly located on the three free positions of the heterocycle ring and the carbon shifts (δ_C 96.9 and 123.2) suggested that both bromine atoms were on the pyrrol moiety, this evidence was insufficient to fully confirm this proposal. Fortunately, this could be resolved by detailed study of the peptide structure by (+)-HRESI-TOFMS and QTOF (Figure 3), which showed significant cluster ions at *m/z* 158.0487/160.0460 in a 3:1 ratio corresponding to the iAsn moiety. These *m/z* values, the mass error observed and the

isotopic distribution clearly confirmed the presence of a chlorine atom on the NMeHis amino acid.

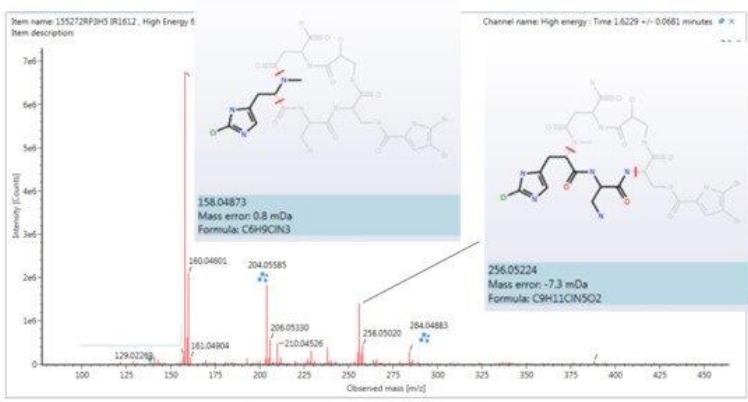

Figure 3: Fragment found for **1** by QTOF.

The absolute stereochemistry of compound **1** was established on the basis of Marfey's analysis with the 1-fluoro-2,4-di-nitrophenyl-5-*L*-alanine amide (*L*-FDAA) [22]. Compound **1** was hydrolyzed in strong acid conditions and derivatization of the free aminoacids with *L*-FDAA allowed an exhaustive analysis by HPLC-MS. A comparison of the retention times of the derivatized amino acids present in **1** and the suitably derivatized pure amino acid standards, unambiguously demonstrated the absolute configuration as *L*-Dap, *L*-iSer and *D*-Asn. The absolute configuration of NMeClHis could not be determined due to the absence of the standard amino acid.

Table 1: NMR spectroscopy data for **1** (^{1}H NMR MHz, ^{13}C NMR 125 MHz).

	Pos	**δ_C, mult[a]**	**δ_H, mult (*J* in Hz)[a]**	**δ_C, mult[b]**	**δ_H, mult (*J* in Hz)[b]**
Dap1	1	169.5, C		172.3, C	
	2	50.3, CH	4.23 ddd (6.2, 6.2, 2.8)	52.2, CH	4.54 dd (5.6, 2.9)
	3	49.2, CH_2	2.87 d (13.9, 6.2)	50.5, CH_2	3.17 dd (14.6, 5.6)
			3.13 d (13.9, 2.8)		3.54 d (14.2)
	NH		9.11 d (6.2)		8.97 d (6.6)*

	NH_2				
NMeClHis	1	169.0, CO		170.9, CO	
	2	65.6, CH	3.99 dd (10.6, 3.9)	67.0, CH	4.06 dd (10.1, 4.5)
	3	24.9, CH_2	3.05 m	25.9, CH_2	3.19 dd (15.2, 10.1)
					3.30 dd (15.2, 4.5)
	4	110.5, C		135.6, C	
	5	109.5, CH	6.76 s	120.8, CH	6.96 s
	6	128.2, C		131.0, C	
	NMe	39.6, CH_3	2.84 s	40.4, CH_3	3.02 s
iAsn	1	173.5, C		175.8, C	
	2	35.0, CH_2	2.80 dd (16.6, 2.7)	36.2, CH_2	3.02 m
			3.09 dd (16.6, 5.8)		3.27 m
	3	48.4, CH	4.58 ddd (8.0, 5.8, 2.7)	50.3, CH	4.84 m
	4	172.2, CO		173.8, CO	
	NH		7.13 d (8.0)		7.61 d (6.3)*
	NH_2				
iSer	1	171.9, C		173.3, C	
	2	67.8, CH	4.13 dd (9.0, 4.2)	69.5, CH	4.40 dd (9.5, 4.0)
	3	42.8, CH_2	2.75 ddd (9.0, 9.4, 5.4)	44.2, CH_2	3.00 m
			3.47 m		3.75 dd (13.2, 4.0)
	NH		8.23 t (5.4)		8.25 s*
Dap2	1	170.5, C		172.8, C	
	2	51.5, CH	4.49 ddd (9.2, 9.2, 6.3)	53.4, CH	4.84 m
	3	40.1, CH_2	3.22 m	41.6, CH_2	3.57 dd (13.8, 8.6)
			4.04 ddd (12.9, 6.3, 6.3)		4.22 dd (13.8, 5.4)
	NH-1		7.72 d (9.2)		7.98 d (9.5)*
	NH-2		7.20 t (6.3, 6.3)		7.37 t (6.0)*
Br_2Py	1	158.6, CO		161.4, CO	
	2	118.0, C		120.2, C	
	3	110.4, CH	6.30 d (2.7)	112.0, CH	6.15 s
	4	96.9, C		99.4, C	
	5	123.2, C		124.2, C	
	NH		12.68 d (2.7)		12.03 s

[a] In DMSO-d_6. [b] In CD_3OD (*CD_3OH).

Compound **2** was isolated as an amorphous white solid. Its (+)-LRESI showed a m/z=825 $[M+H]^+$ with a characteristic cluster corresponding to the presence of three bromine atoms. The molecular formula $C_{29}H_{43}{}^{79}Br_3N_6O_7$ was established by (+)-HRESI-TOFMS analysis of the $[M+H]^+$ at m/z 825.0806 (calcd. 825. 0816). The peptide nature of **2** was evident from its ^1H and ^{13}C NMR spectra (Table 2). ^1H NMR in DMSO showed the characteristic α-proton resonances of four α-amino acids in the range δ_H 5.35 to 4.10 ppm, five interchangeable protons at δ_H 12.53, 8.56, 8.08, 7.19 and 6.77 ppm and two NMe signals at δ_H 3.61 and 3.04 ppm. ^{13}C NMR data displayed six carbonyl signals between δ_C 173.5 and 159.1 ppm, four adjacent methine carbons in the range δ_C 59.1-51.5 ppm, and two NMe signals at δ_C 35.7 and 30.5 ppm. Extensive 2D NMR analysis, including COSY, TOCSY, HSQC and HMBC was used to determine the identity of the four amino acids and to assign the NMR signals. As a result of these studies, the amino acids were found to be one Ile, one NMe-Leu, one Pro and one Asn unit. A long-range correlation between protons at δ_H 7.19/6.77 and 2.15/2.11 ppm with the carbonyl group at δ_C 173.5 ppm and the observation of ROESY cross-peaks between protons at δ_H 7.19/6.77 ppm and the CH_2 of position 4 at δ_H 2.15/2.11 ppm established the presence of a Gln. A N-methyl-2,3,4-bromopyrrol unit was inferred by the presence of four aromatic non-protonated carbons at δ_C 128.5, 107.9, 100.7 and 93.4 ppm with chemical shifts similar to those described for bromopseudoceratines [23].

Table 2: NMR spectroscopy data for **2** (^1H NMR 500 MHz, ^{13}C NMR 125 MHz).

	Pos	**δ_H, mult (*J* in Hz)[a]**	**δ_C, mult[a]**	**δ_H, mult (*J* in Hz)[b]**	**δ_C, mult[b]**
Br_3Py	1	-	161.8, CO	-	159.1, CO
	2	-	128.6, C	-	128.5, C
	3	-	102.7, C	-	100.7, C
	4	-	101.3, C	-	93.4, C
	5	-	110.8, C	-	107.9, C
	NMe	3.76, s	36.7, CH_3	3.61, s	35.7, CH_3
Ile	1	-	174.3, CO	-	171.4, CO
	2	4.84, m	55.7, CH	4.65, dd, 8.35, 8.5	53.7, CH

	3	1.96, m	38.0, CH	1.87, m	35.8, CH
	4	1.73, m, 1.22, m	25.9, CH_2	1.57, m; 1.21, m	24.2, CH_2
	5	0.95, t, 7.4	11.2, CH_3	0.83, t, 7.4	10.7, CH_3
	6	0.99, d, 6.8	15.7, CH_3	0.85, d, 6.9	15.1, CH_3
	NH	8.24, d, 8.1	-	8.56, d, 8.2	-
NMeLeu	1	-	171.8, CO	-	168.8, CO
	2	5.51, dd, 10.3, 4.7	54.3, CH	5.35, dd, 10.1, 4.3	51.7, CH
	3	1.77, m; 1.61, m	38.0, CH_2	1.59, m; 1.42, m	36.7, CH_2
	4	1.56, m	25.7, CH	1.43, m	23.9, CH
	5	0.97, d, 6.2	23.6, CH_3	0.87, d, 6.2	23.1, CH_3
	6	0.93, d, 6.1	22.3, CH_3	0.83, d, 6.2	21.8, CH_3
	NMe	3.21, s	31.8, CH_3	3.04, s	30.5, CH_3
Pro	1	-	174.5, CO	-	171.4, CO
	2	4.41, m	61.6, CH	4.31, dd, 8.3, 4.1	59.1, CH
	3	2.23, m; 2.00, m	30.5, CH_2	2.02, m; 1.80, m	28.9, CH_2
	4	2.08, m; 1.92, m	26.0, CH_2	1.90, m; 1.77, m	24.4, CH_2
	5	3.75, m; 3.69, m	48.8, CH_2	3.53, m; 3.50; m	46.7, CH_2
Gln	1	-	174.7, CO_2H	12.53, brs	173.3, CO_2H
	2	4.41, m	52.8, CH	4.10, ddd, 8.6, 8.5, 5.2	51.5, CH
	3	2.27, m; 1.92, m	30.5, CH_2	1.93, m; 1.74, m	27.0, CH_2
	4	2.41, m; 2.32, m	32.6, CH_2	2.15, m; 2.11, m	31.3, CH_2
	5	-	177.9, $CONH_2$	7.19, s; 6.77, s	173.5, $CONH_2$
	NH	-	-	8.08, brs	-

[a] In CD_3OD. [b] In DMSO-d_6.

The sequencing for compound **2** was carried out using a combination of HMBC and ROESY data. Long-range correlations from α-protons, NH and NMe to carbonyl carbons

of adjacent amino acids plus ROESY correlations between α-protons, NH and NMe protons of adjacent amino acids (see Figure 2) allowed us to establish the sequence as Br_3Py-Ile-NMeLeu-Pro-Gln.

The absolute configurations of the aminoacids were determined by comparing the hydrolysis products of **2** (6 N HCl, 110 °C, 18 h) after derivatization with Marfey's reagent (N-(3-fluoro-4,6-dinitrophenyl)-*L*-alaninamide, *L*-FDAA), with appropriate amino acid standards using HPLC-MS chromatography. As a result, all the amino acids were determined to be *L*.

Materials and Methods

General Experimental Procedures

Optical rotations were determined using a Jasco P-1020 polarimeter. UV spectra were performed using an Agilent 8453 UV−vis spectrometer. IR spectra were obtained with a Perkin-Elmer Spectrum 100 FT-IR spectrometer with ATR sampling. NMR spectra were recorded on a Varian "Unity 500" spectrometer at 500/125 MHz (^{1}H/^{13}C). Chemical shifts were reported in ppm using residual CD_3OH (δ 3.31 ppm for ^{1}H and 49.0 ppm for ^{13}C) and DMSO-d_6 (δ 2.50 ppm for ^{1}H and 39.5 ppm for ^{13}C) as an internal reference. HRESITOFMS was performed on an Agilent 6230 TOF LC/MS chromatograph spectrometer. (+)-ESIMS were recorded using an Agilent 1100 Series LC/MSD spectrometer. HRESITOFMS was performed on an Agilent 6230 TOF LC/MS chromatograph spectrometer. ESI(+) and MS^e were performed on an Waters UHPLC-QTOF Acquity I-Class + Xevo G2-XS.

Biological Material

The sponge *Ircinia* sp. (158 g) was collected by hand using a diving rebreather system in Thousand Islands (Indonesia). The sponge was immediately frozen and kept under these conditions until extraction. The specimen was identified by María Jesús Uriz at CEAB, Blanes, Spain. A voucher specimen (ORMA155272) is deposited at PharmaMar facilities (Madrid, Spain).

Extraction and Isolation

The sponge *Ircinia* sp. (158 g) was triturated and exhaustively extracted with MeOH:DCM (1:1, 3 × 500 mL). The combined extracts were concentrated to yield a crude mass of 7.9 g. The crude product was subjected to VLC on Lichroprep RP-18 with a stepped gradient from H_2O to MeOH to CH_2Cl_2. The fractions eluting with H_2O:MeOH (3:1, 606 mg) and H_2O:MeOH (1:1, 89.6 mg) were subjected to semipreparative HPLC (Symmetry Prep C_{18} 5 µm, 10 × 150 mm; 3 min isocratic H_2O + 0.04 % TFA: CH_3CN + 0.04 % TFA 95:5 and then gradient from 5% to 68% CH_3CN + 0.04 % TFA in 25 min, flow 3 mL/min, UV detection) to obtain 4.3 mg of compound **1**. The fraction eluting with H_2O:MeOH (1:3, 43.9 mg) was subjected to semipreparative HPLC (Symmetry Prep C_{18} 5 µm, 10 × 150 mm; 3 min. isocratic H_2O + 0.04 % TFA: CH_3CN + 0.04 % TFA 90:10 and then gradient from 10% to 75% CH_3CN + 0.04 % TFA in 25 min, flow 3 mL/min, UV detection) to obtain 3.0 mg of compound **2**.

Haloirciniamide A (**1**): amorphous white solid; $[\alpha]^{25}_D$ -62.7º (c 0.1, MeOH); IR υmax 3314, 2920, 2850, 1644, 1523, 1416, 1311, 1239, 1199, 1041 cm^{-1}; UV (MeOH) λ_{max} 198, 268 nm. ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) see Table 1; (+)-HREI-TOFMS *m/z* 830.0400 [M+Na]+ (calcd for $C_{25}H_{32}{}^{79}Br_2N_{11}O_8Na$ *m/z* 830.0383).

Seribunamide A (**2**): amorphous white solid; $[\alpha]^{25}_D$ -38.4º (c 0.2, MeOH); IR υmax 3352, 2932, 2850, 1658, 1515, 1320, 1236, 1035 cm^{-1}; UV (MeOH) λ_{max} 197, 266 nm. ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) see Table 2; (+)-HREI-TOFMS *m/z* 825.0806 [M+H]+ (calcd for $C_{29}H_{44}{}^{79}Br_3N_6O_7$ *m/z* 825.0816).

Absolute Configuration

Absolute Configuration of **1**: 0.5 mg of haloirciniamide A was hydrolyzed in 0.5 mL of 6 N HCl at 110 °C for 15 h. The excess aqueous HCl was removed under a N_2 stream, and a solution of 700 µg of *L*-FDAA (N-(3-fluoro-4,6-dinitrophenyl)-*L*-alanine-amide) in acetone (160 µL), H_2O (100 µL) and $NaHCO_3$ 1N (50

µL) was added to the dry hydrolysate. The resulting mixture was heated at 40 ºC for 1 h, before being cooled to 23 ºC, quenched by addition of 2N HCl (20 µL), dried, and dissolved in H_2O (800 µL). The resultant aqueous solution was subjected to reversed-phase LC/MS (column: Waters Symmetry 4.6x150 mm, 3.5 µm, flow rate 0.8 mL/min) in three different gradient.

Gradient 1 for iSer (mobile phase CH_3CN + 0.04% formic acid /H_2O + 0.04% formic acid, using a linear gradient from 5 to 20% CH_3CN in 10 min and them from 20% to 35% CH_3CN in 25 min): the retention time was 23.9 min for *L*-iSer.

Gradient 2 for Asp (mobile phase CH_3CN + 0.04% formic acid/H_2O + 0.04% formic acid, using a linear gradient from 5 to 30% CH_3CN in 10 min and them from 30% to 50% CH_3CN in 30 min): the retention time was 17.0 min for *D*-Asp.

Gradient 3 for Dap: mobile phase CH_3CN + 0.04% formic acid/H_2O + 0.04% formic acid, using a linear gradient from 5 to 10% CH_3CN in 5 min and them from 10% to 35% CH_3CN in 25 min): the retention times was 15.5 min for *L*-Dap.

Retention times for the derivatized amino acids standards were as follows: gradient 1 (23.2 min for *D*-iSer and 23.8 min for the *L*-iSer); gradient 2 (16.2 min for *L*-Asp and 17.0 min for *D*-Asp) and gradient 3 (15.5 min for *L*-Dap and 16.6 min for the *D*-Dap).

Absolute Configuration of **2**: 0.3 mg of seribunamide A was hydrolyzed in 0.4 mL of 6 N HCl, 110 °C for 15 h. The excess aqueous HCl was removed under a N_2 stream, and a solution of 400 µg of *L*-FDAA (N-(3-fluoro-4,6-dinitrophenyl)-*L*-alanine-amide) in acetone (160 µL), H_2O (100 µL) and $NaHCO_3$ 1N (50 µL) was added. The vial was heated at 40 ºC for 1 h, and the contents neutralized with 2N HCl (20 µL) after cooling to room temperature. The resulting solution was dried in vacuum and reconstituted in H_2O (600 µL) before being analyzed by HPLC-MS using two different methods.

Ile was analyzed using: Lux Cellulose-4, 5 µm, flow 1 mL/min, H_2O/AcN + 0.04%TFA isocratic 65:35 in 60 min. The retention

time of the *L*-FDAA amino acid in the hydrolysate of **2** were established as *L*-Ile 36.1 min. Retention times for the derivatized amino acids standards were as follows: *L*-allo-Ile 31.7 min, *L*-Ile 36.1 min, *D-allo*-Ile 38.1 min and *D*-Ile 51.4 min.

Pro, NMeLeu and Glu were analyzed using: Symmetry 4.6x150 mm, 3.5 µm, flow 0.8 mL/min, H_2O+ 0.04%TFA/ CH_3CN + 0.04%TFA from 20 to 50% in 30 min. The retention time of the *L*-FDAA amino acids in the hydrolysate of **2** were established as *L*-Glu 14.0 min, *L*-Pro 16.8 min and NMe-*L*-Leu 28.5 min. Retention times for the derivatized amino acids standards were as follows: *L*-Glu 14.1 min, *D*-Glu 15.2 min, *L*-Pro 16.8 min, *D*-Pro 14.01 min, NMe-*L*-Leu 28.5 min and NMe-*D*-Leu 30.1 min.

Biological Activity

The cytotoxic activity of **1** and **2** was tested against four human tumour cell lines, lung (A-549), colon (HT-29), breast (MDA-MB-231) and pancreas PSN-1 and both compounds displayed an GI_{50} >1.2 E-5 M. Compound **1** was further tested for the capacity to inhibit the enzyme topoisomerase I, but showed no inhibition of the enzyme at 1.0E-5 M, and, was therefore not considered active in inhibiting this enzyme. Likewise, compounds **1** and **2** were unable to impair the interaction between the programmed cell death protein PD-1 and its natural ligand PD-L1 as demonstrated by their lack of effect in a cell-based assay whose final readout was dependent on the interaction between the two proteins (Table 3).

Table 3: % PD-1 and TOPO-I Inhibition for compounds **1** and **2.**

Compound	Target	%Inhibition at 1 E-5 M
1	Top-I	3
1	PD-1	0.3
2	PD-1	-3.5

Conclusions

In summary, two new peptides bearing unprecedented halogenated moieties, haloirciniamide A (**1**) and **seribunamide**

A (**2**) were isolated from a marine sponge belonging to the *Irnicia* genus which was selected for further studies. The sample was collected around Thousand Islands (Indonesia) by the Pharmamar expedition team in collaboration with the Research Center for Oceanography, Indonesian Institute of Sciences (RCO-LIPI). The planar structures of the novel compounds were determined by a combination of extensive NMR and HPLC-MS experiments. The absolute configuration was achieved by Marfey's analysis after acid hydrolysis. Cytotoxic activity in the four cancer cell lines tested was not observed for **1** and **2**. In addition, neither compound was able to impair PD1-PDL1 interaction, and compound **1** failed to inhibit the enzyme topoisomerase I. This work is the first example of the isolation and structural elucidation of novel compounds with unique structural features from an *Ircinia* sponge, which highlights this gender and its microbiota as a distinctive source of novel structures.

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Supplementary Materials

Figure S1. Picture of the fresh sponge.

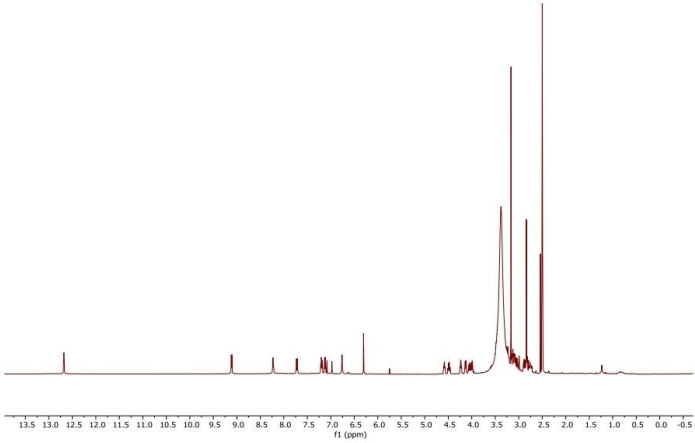


Figure S2 ^{1}H NMR spectrum of Haloircinamide A (**1**) 500 MHz, DMSO.

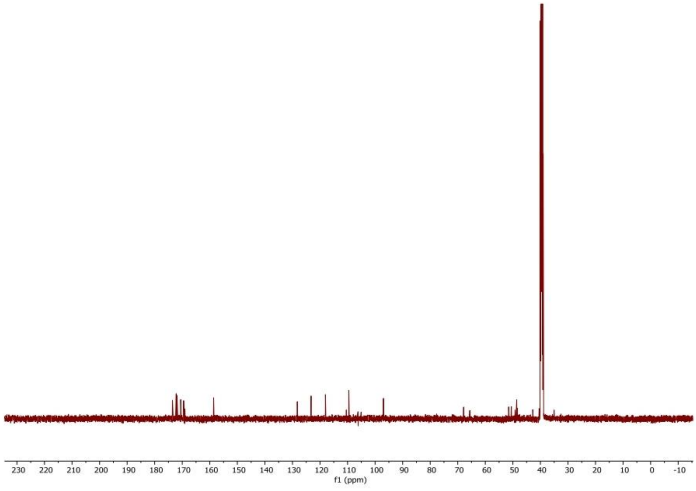


Figure S3 ^{13}C NMR spectrum of Haloircinamide A (**1**) 125 MHz, DMSO.

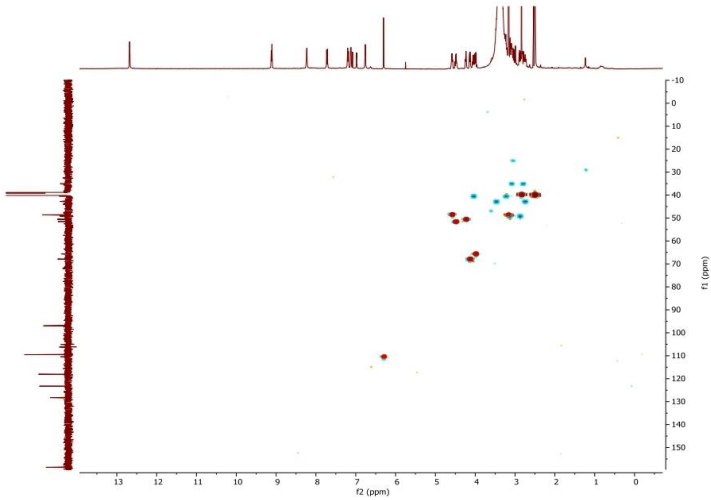


Figure S4 *g*-HSQC spectrum of Haloircinamide A (**1**) 500 MHz, DMSO

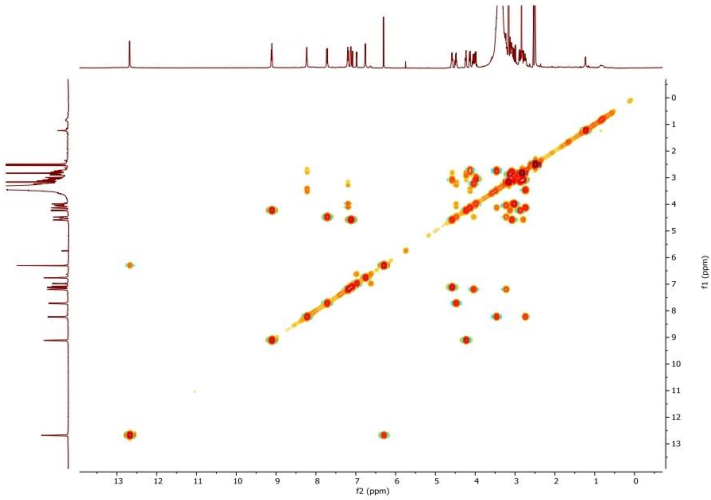


Figure S5 *g*-COSY spectrum of Haloircinamide A (**1**) 500 MHz, DMSO

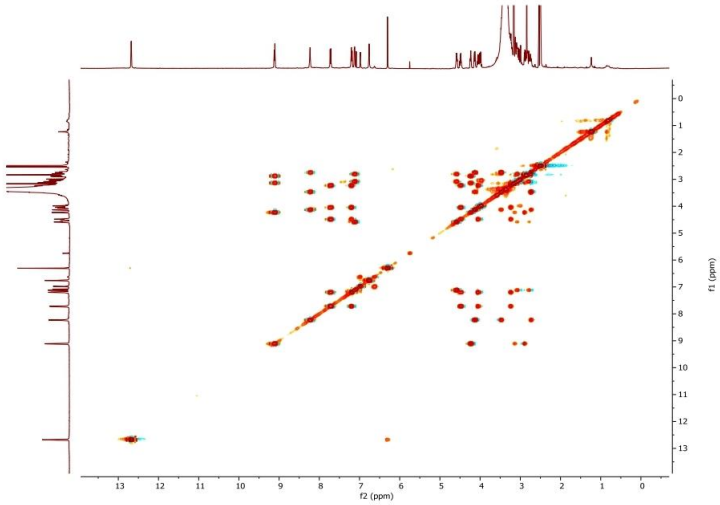


Figure S6 TOCSY spectrum of Haloircinamide A (**1**) 500 MHz, DMSO

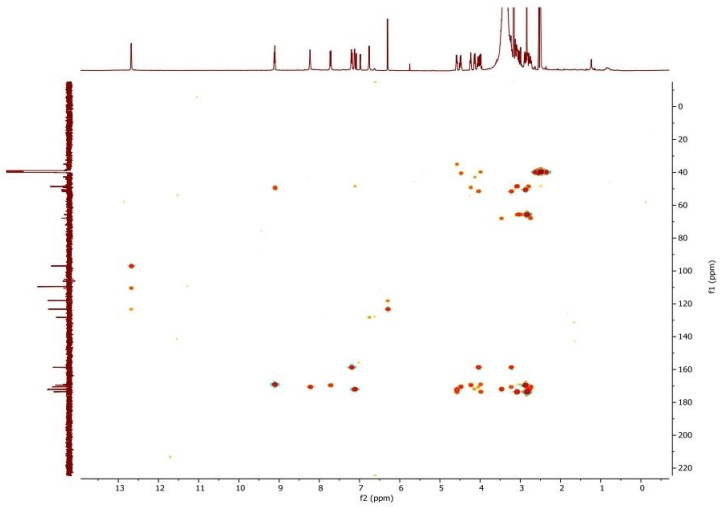


Figure S7 *g*-HMBC spectrum of Haloircinamide A (**1**) 500 MHz, DMSO

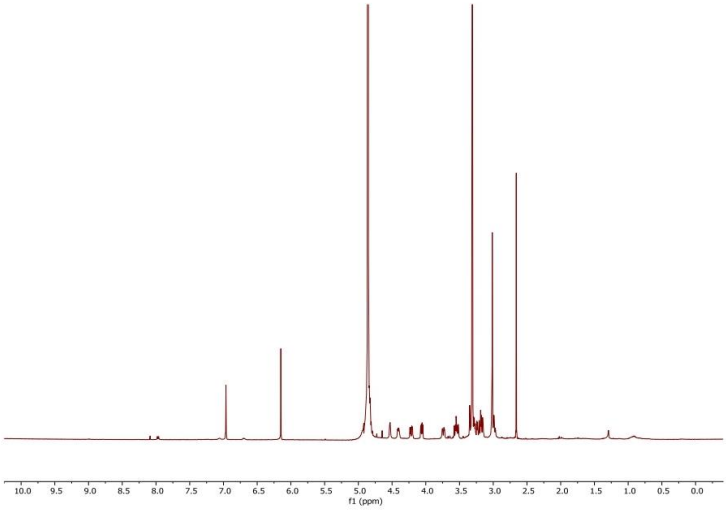


Figure S8 ^{1}H NMR spectrum of Haloircinamide A (**1**) 500 MHz, CD_3OD.

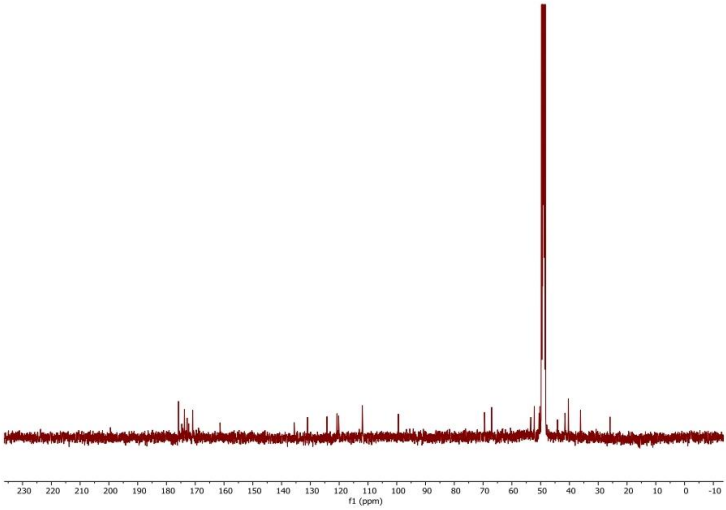


Figure S9 ^{13}C NMR spectrum of Haloircinamide A (**1**) 125 MHz, CD_3OD.

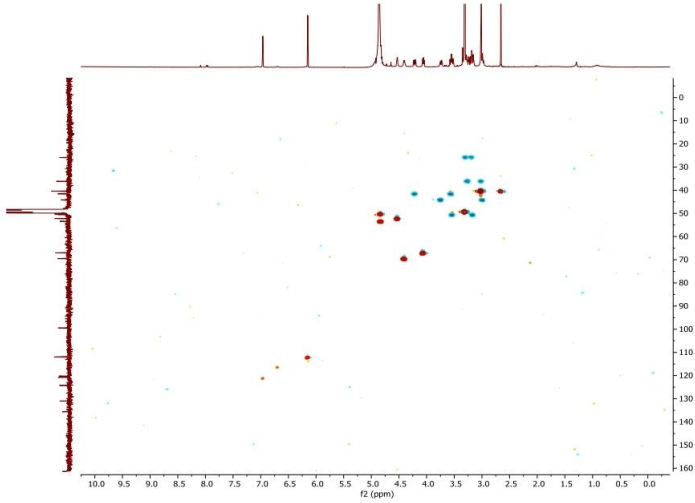


Figure S10 g-HSQC spectrum of Haloircinamide A (**1**) 500 MHz, CD_3OD

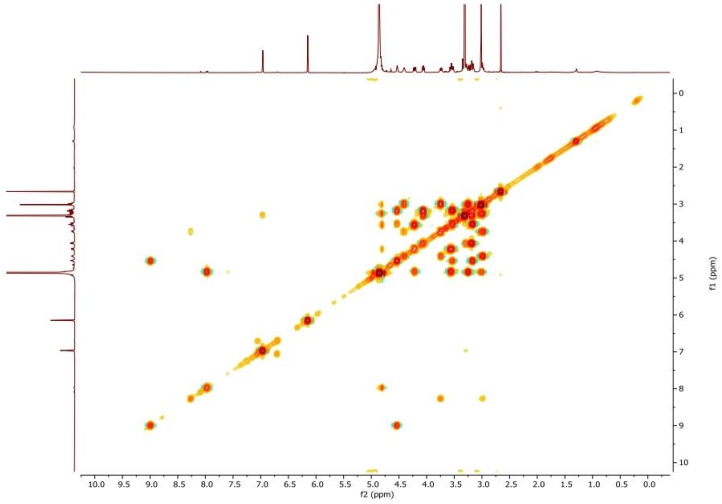


Figure S11 g-COSY spectrum of Haloircinamide A (**1**) 500 MHz, CD_3OD

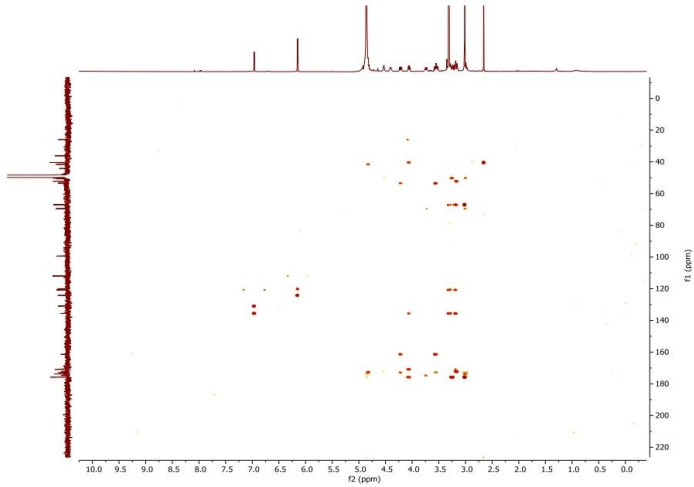


Figure S12 g-HMBC spectrum of Haloircinamide A (**1**) 500 MHz, CD_3OD

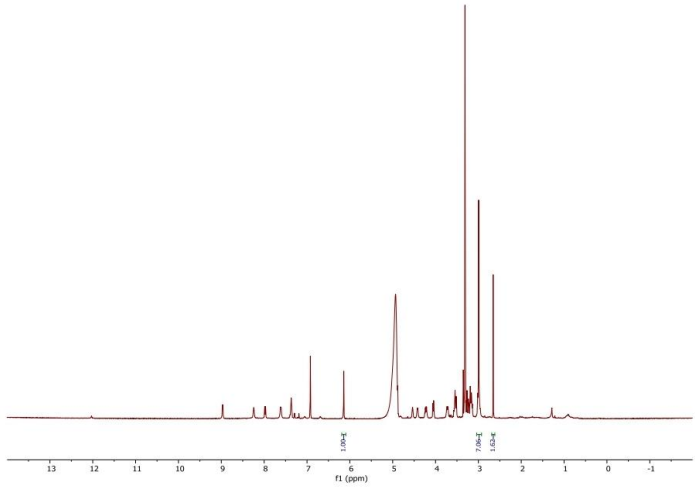


Figure S13 ^1H NMR spectrum of Haloircinamide A (**1**) 500 MHz, CD_3OH.

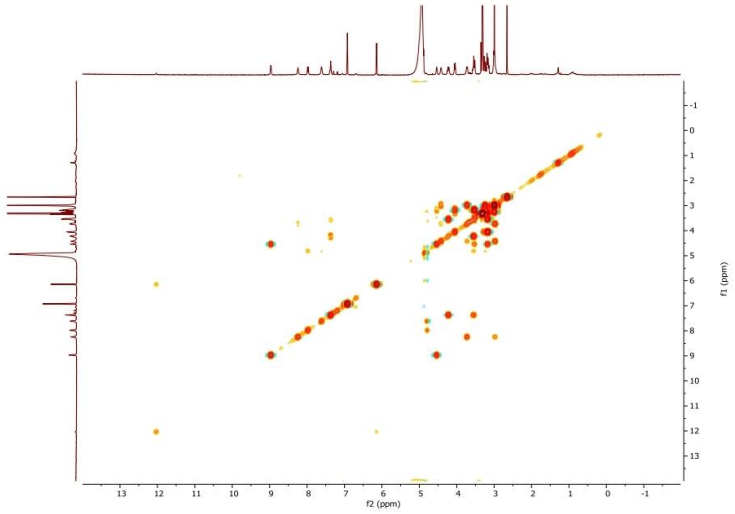


Figure S14 g-COSY spectrum of Haloircinamide A (**1**) 500 MHz, CD_3OH

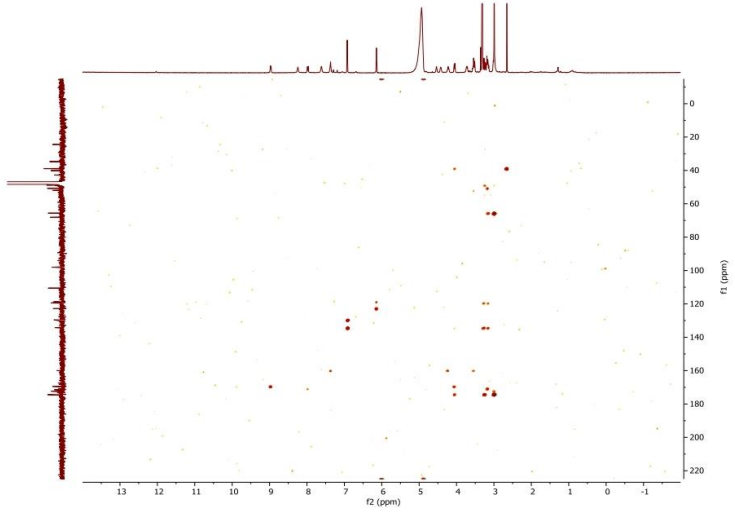


Figure S15 g-HMBC spectrum of Haloircinamide A (**1**) 500 MHz, CD_3OH

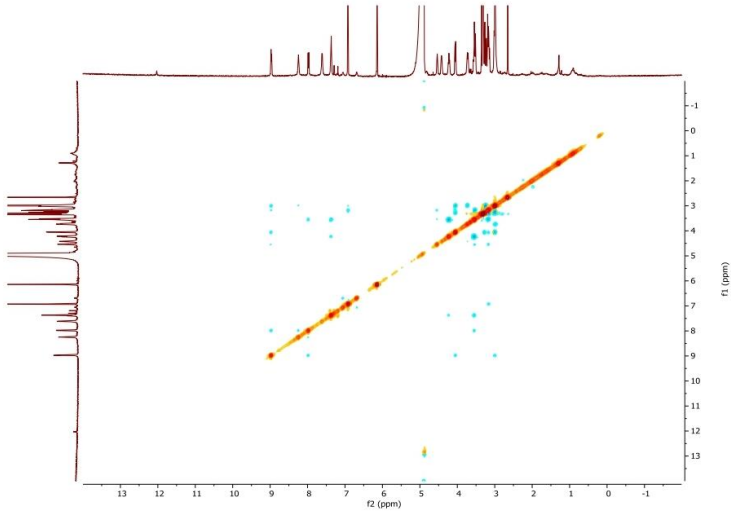


Figure S16 ROESY spectrum of Haloircinamide A (**1**) 500 MHz, CD_3OH

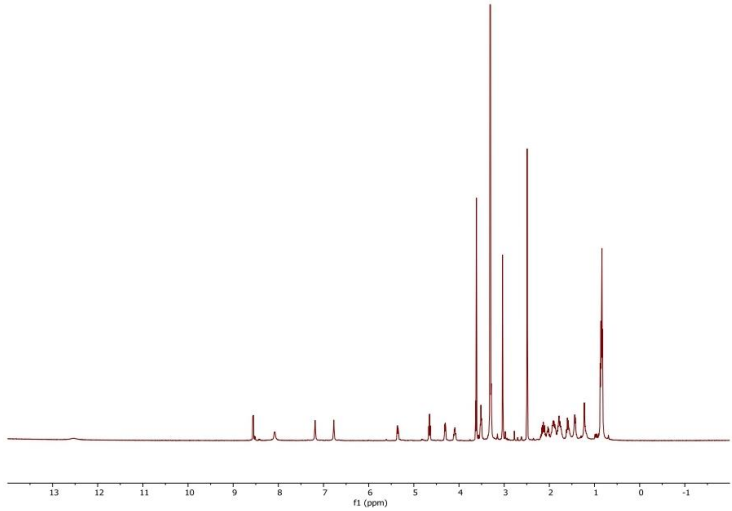


Figure S17 ^{1}H NMR spectrum of Seribunamide A (**2**), 500 MHz, DMSO

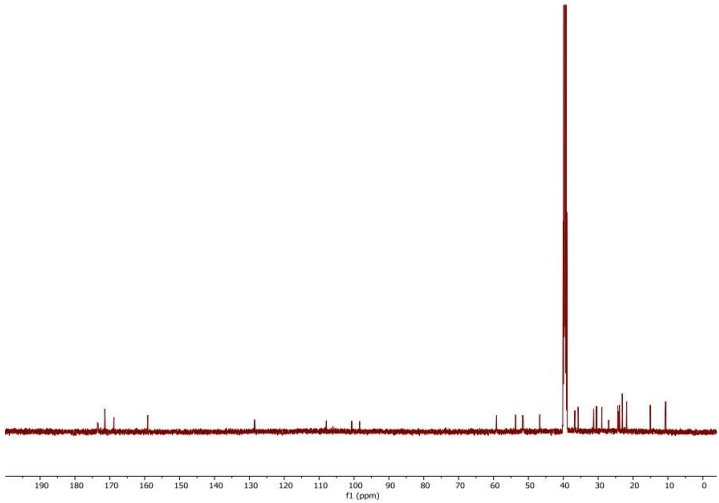


Figure S18 ^{13}C NMR spectrum of Seribunamide A (**2**), 125 MHz, DMSO

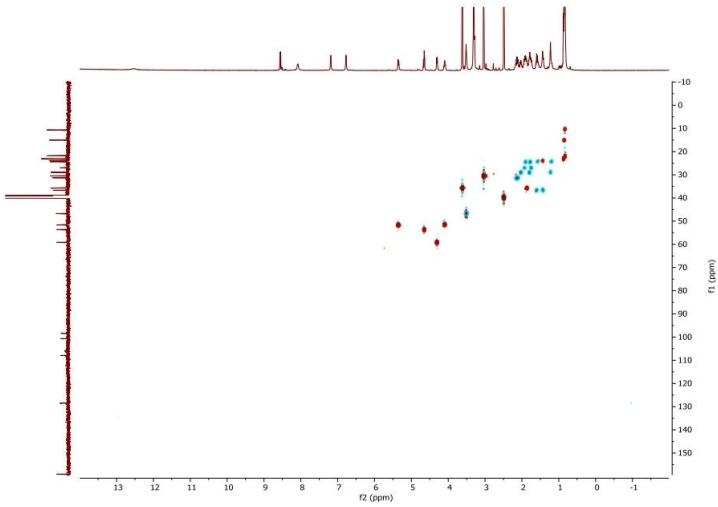


Figure S19 gHSQC spectrum of Seribunamide A (**2**), 500 MHz, DMSO

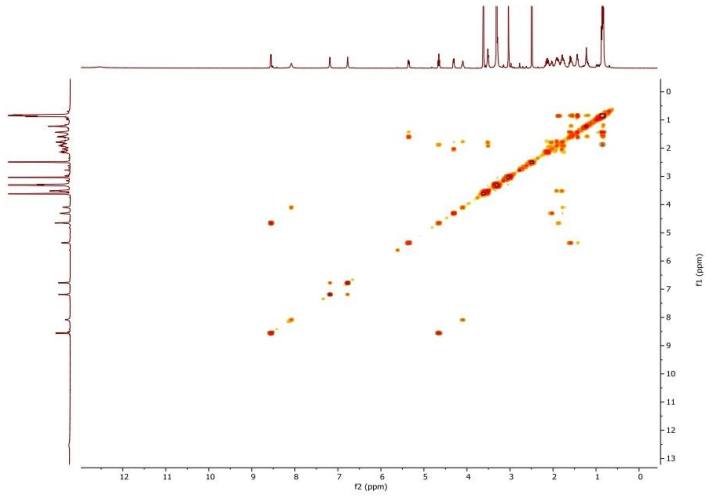


Figure S20 gCOSY spectrum of Seribunamide A (**2**), 500 MHz, DMSO

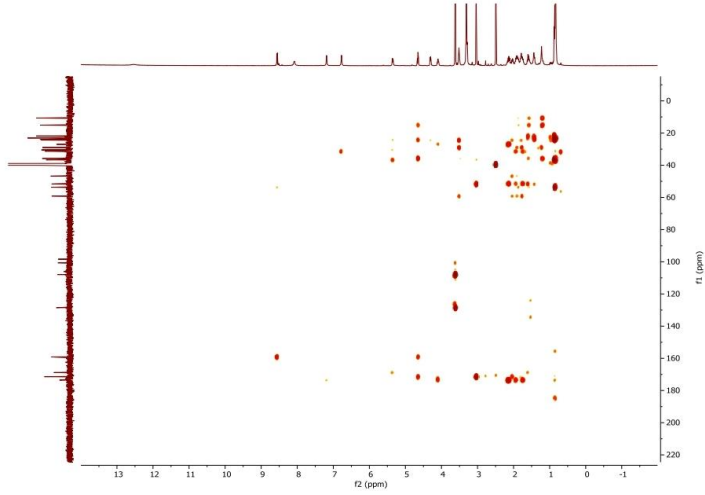


Figure S21 gHMBC spectrum of Seribunamide A (**2**), 500 MHz, DMSO

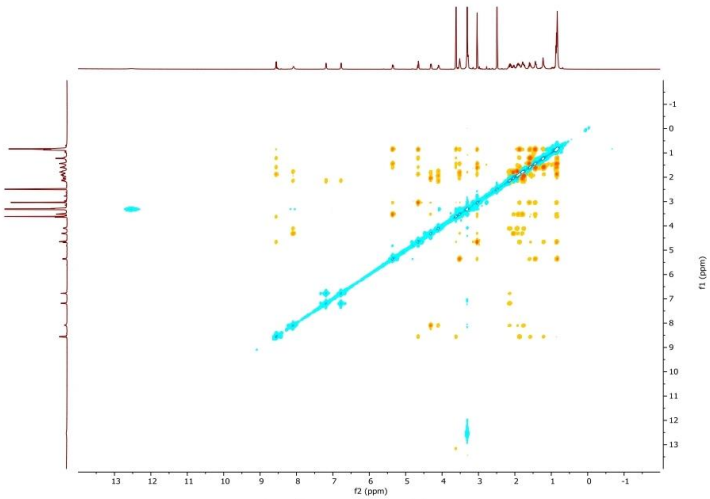


Figure S22 ROESY spectrum of Seribunamide A (**2**), 500 MHz, DMSO

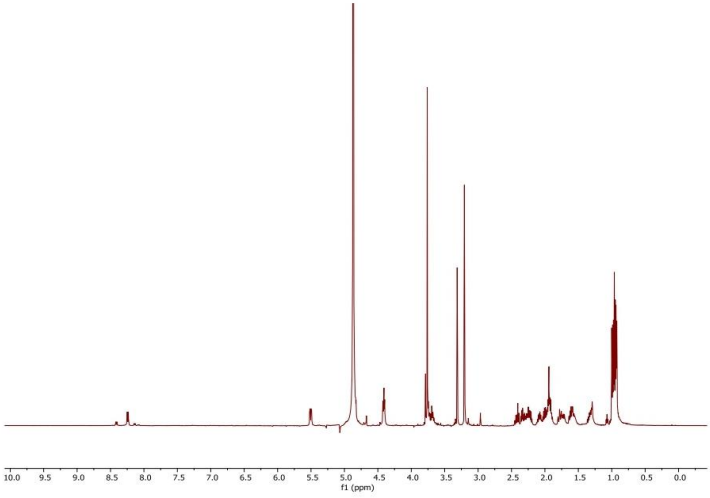


Figure S23 ^{1}H NMR spectrum of Seribunamide A (**2**), 500 MHz, CD_3OD

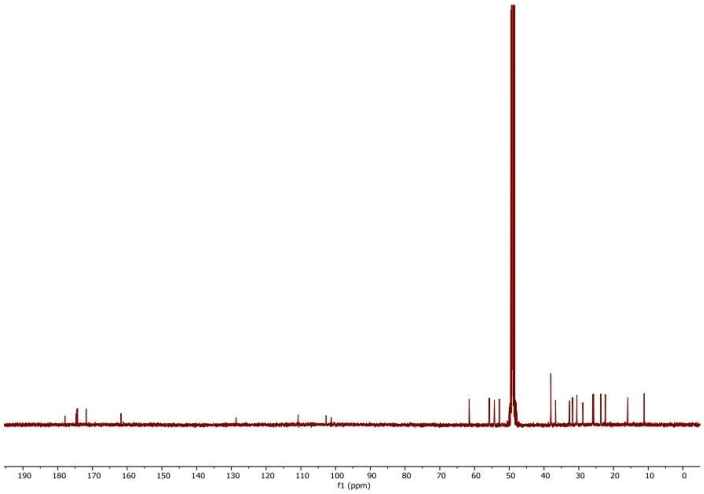


Figure S24 ^{13}C NMR spectrum of Seribunamide A (**2**), 125 MHz, CD_3OD

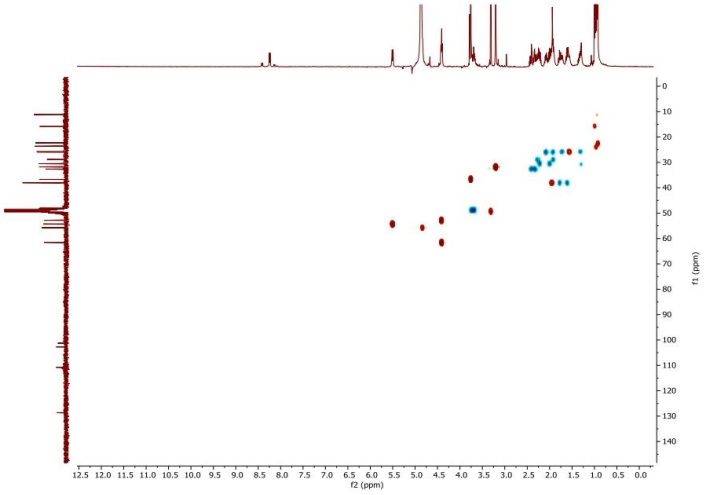


Figure S25 gHSQC spectrum of Seribunamide A (**2**), 500 MHz, CD_3OD

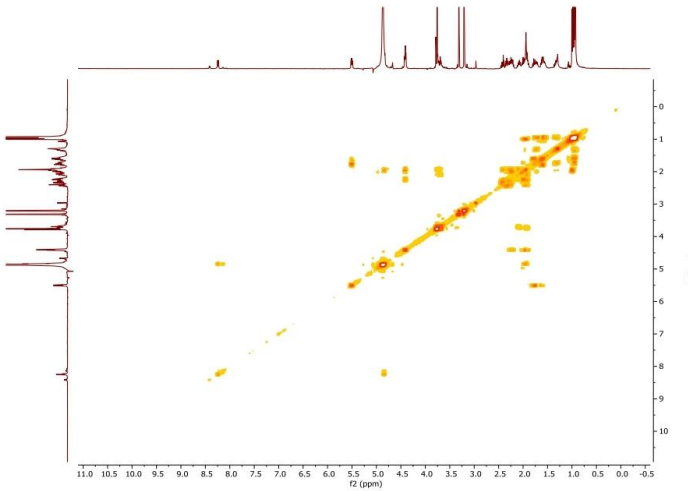


Figure S26 gCOSY spectrum of Seribunamide A (**2**), 500 MHz, CD_3OD

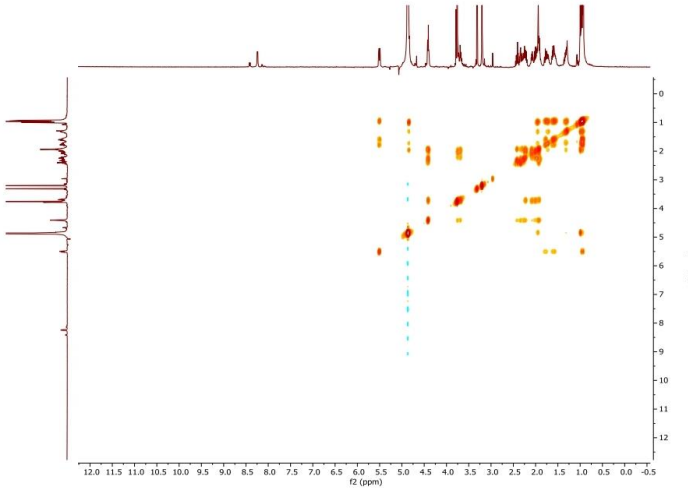


Figure S27 TOCSY spectrum of Seribunamide A (**2**), 500 MHz, CD_3OD

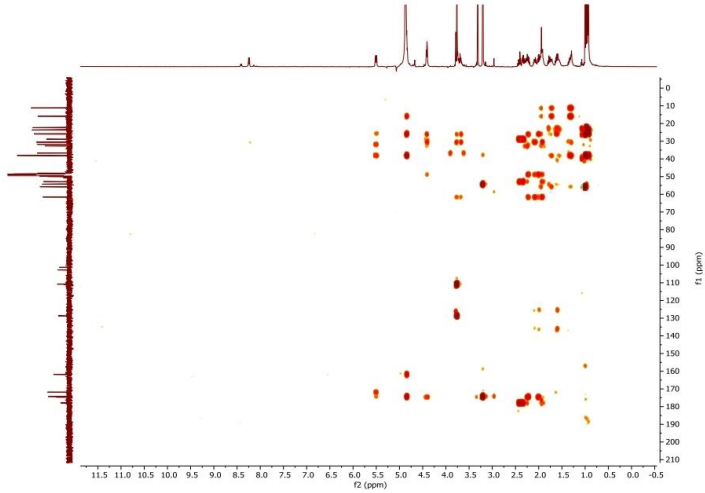


Figure S28 gHMBC spectrum of Seribunamide A (**2**), 500 MHz, CD_3OD

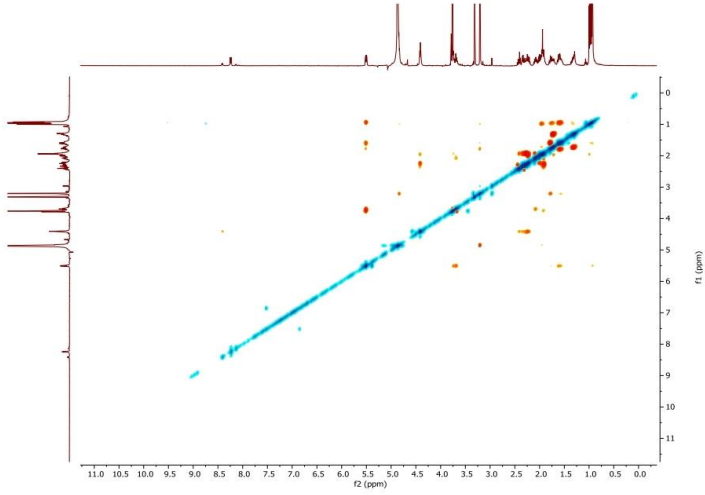
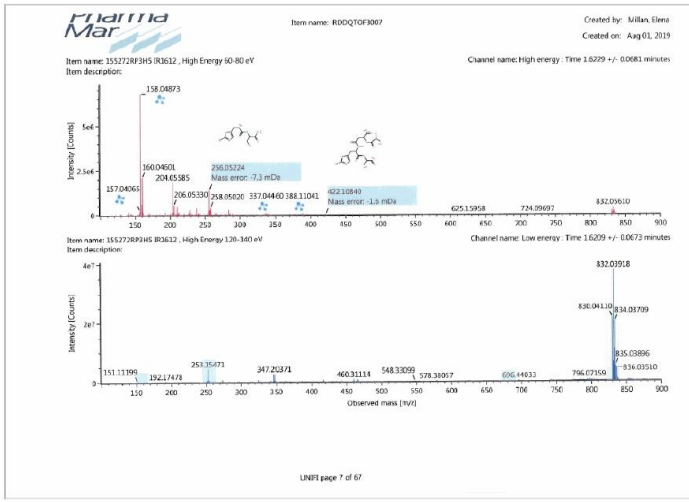


Figure S29 ROESY spectrum of Seribunamide A (**2**), 500 MHz, CD_3OD



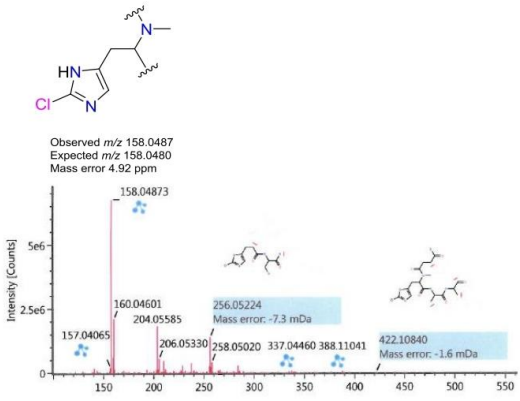


Figure S30 QTOFMS spectrum of Haloirciamide A (**1**) and fragments found

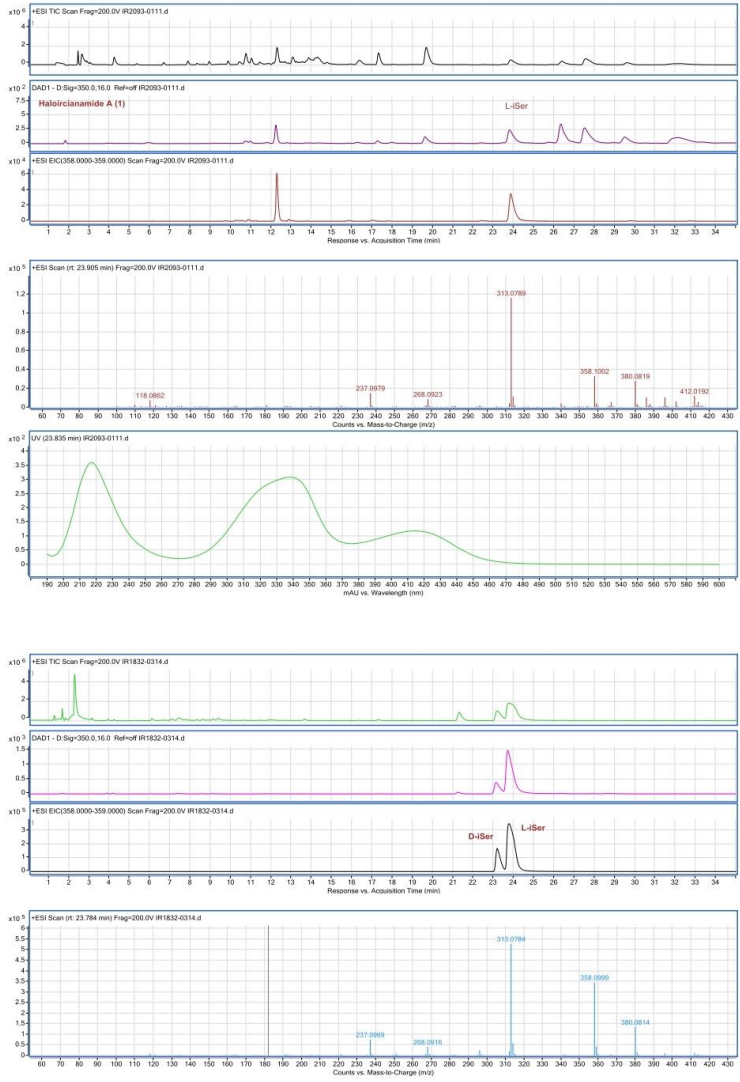
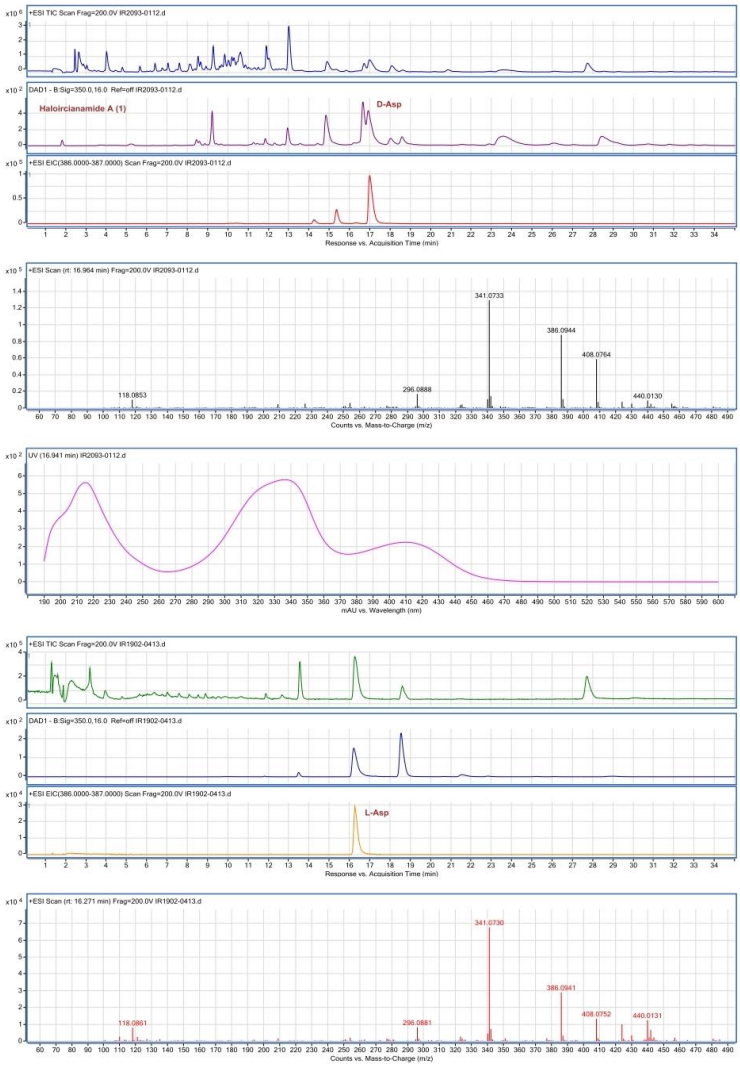


Figure S31 Marfey of Haloirciamide A (**1**) and iSer standards


+ESI TIC Scan Frag=200.0V IR2093-0112.d
DAD1 - B,Sig=350,0,16,0 Ref=off IR2093-0112.d
Haloircianamide A (1)
D-Asp
+ESI EIC(386.0000-387.0000) Scan Frag=200.0V IR2093-0112.d
Response vs. Acquisition Time (min)
+ESI Scan (rt: 16.964 min) Frag=200.0V IR2093-0112.d
341.0733
386.0944
408.0764
296.0888
118.0853
440.0130
Counts vs. Mass-to-Charge (m/z)
UV (16.941 min) IR2093-0112.d
mAU vs. Wavelength (nm)
+ESI TIC Scan Frag=200.0V IR1902-0413.d
DAD1 - B,Sig=350,0,16,0 Ref=off IR1902-0413.d
+ESI EIC(386.0000-387.0000) Scan Frag=200.0V IR1902-0413.d
L-Asp
Response vs. Acquisition Time (min)
+ESI Scan (rt: 16.271 min) Frag=200.0V IR1902-0413.d
341.0730
386.0941
408.0752
440.0131
118.0861
296.0881
Counts vs. Mass-to-Charge (m/z)

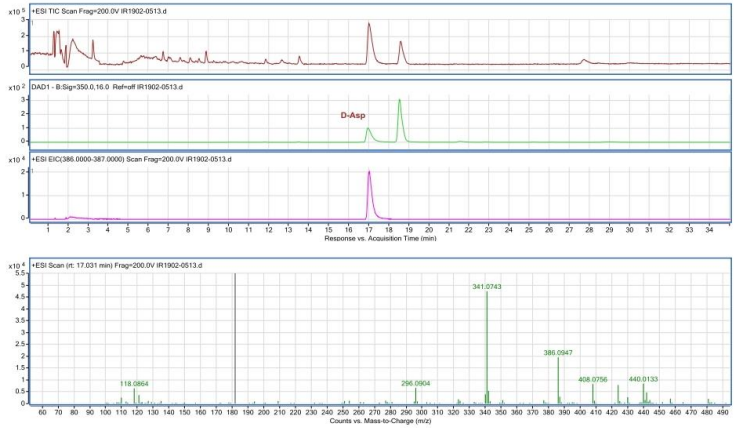
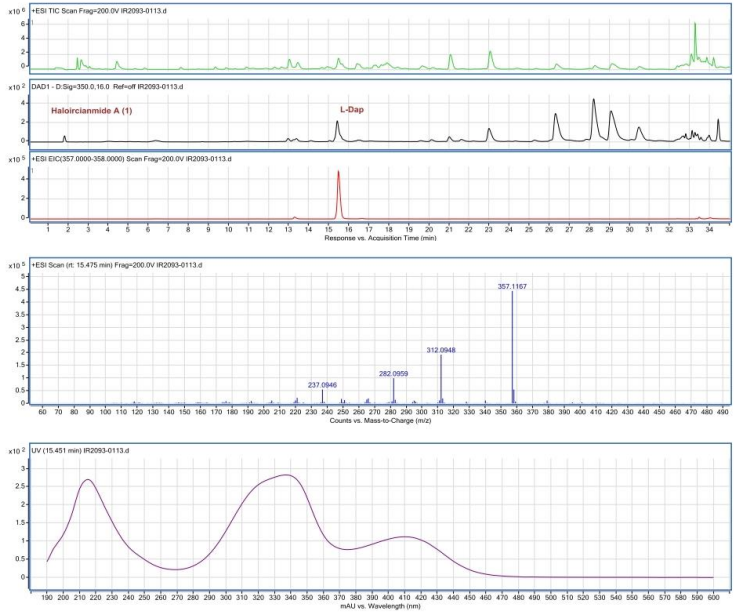


Figure S32 Marfey of Haloircianmide A (**1**) and Asp standards



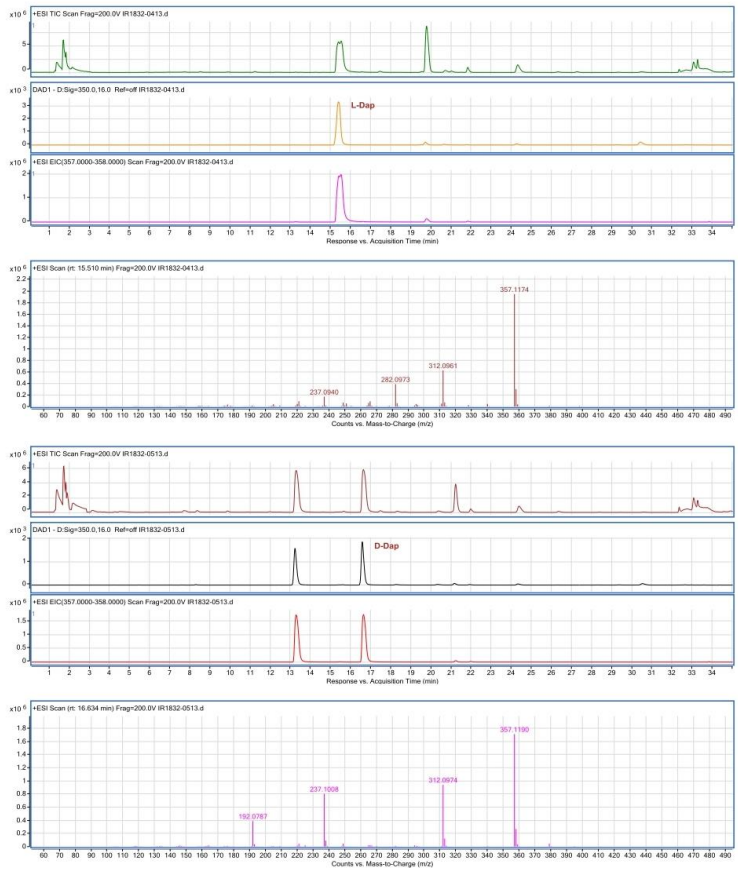


Figure S33 Marfey of Haloirciamide A (**1**) and Dap standards

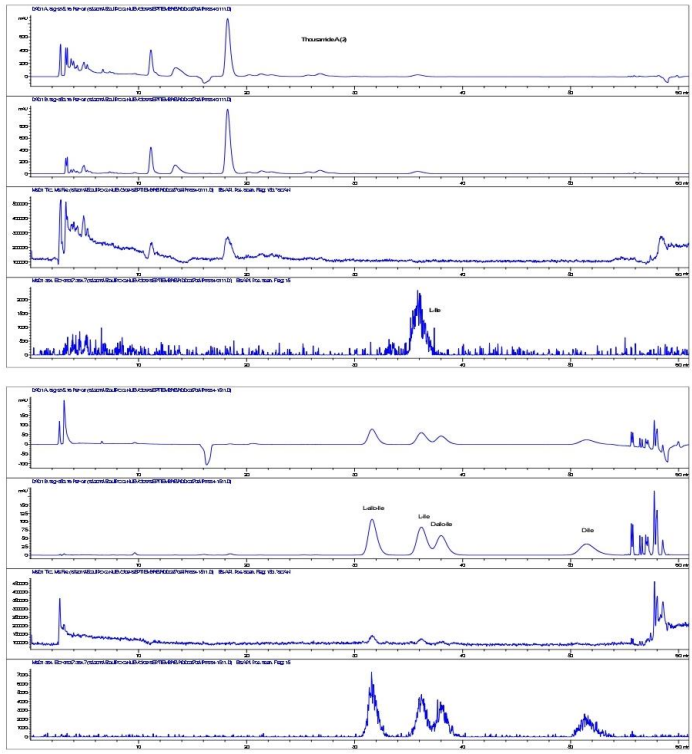


Figure S34 Marfey of Seribunamide A (**2**) and Ile standards

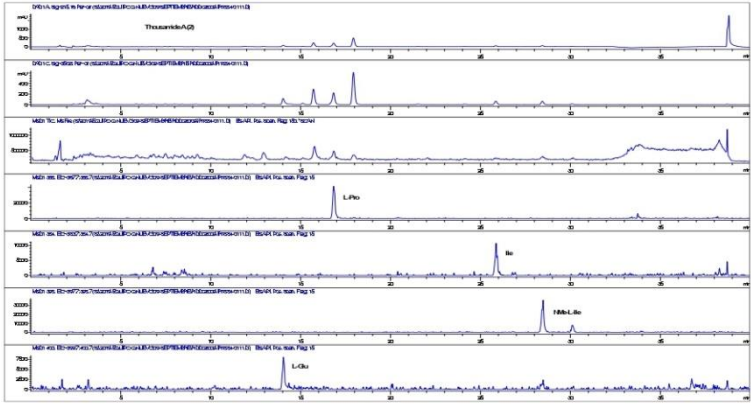


Figure S35 Marfey of Seribunamide A (**2**)

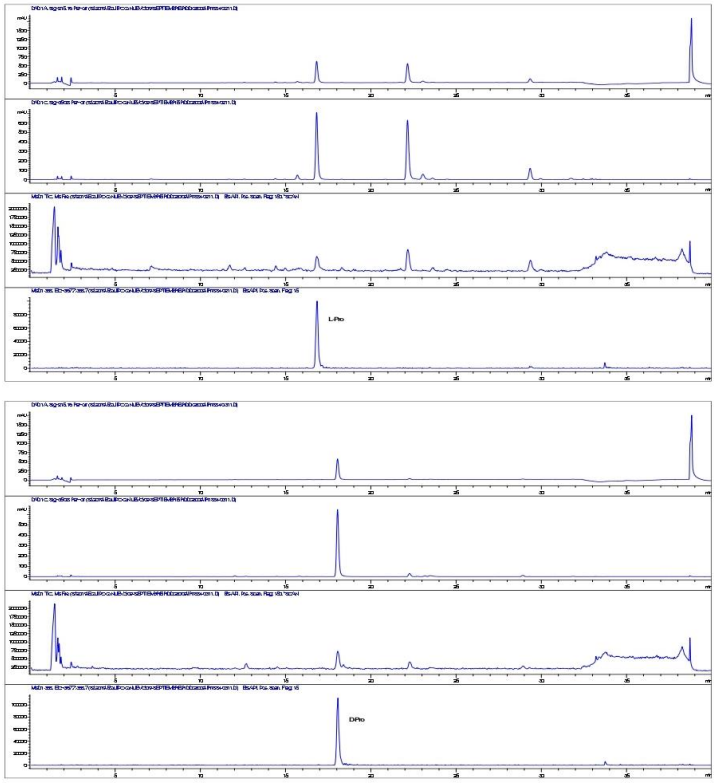


Figure S36 Marfey of Seribunamide A (**2**). Pro standards

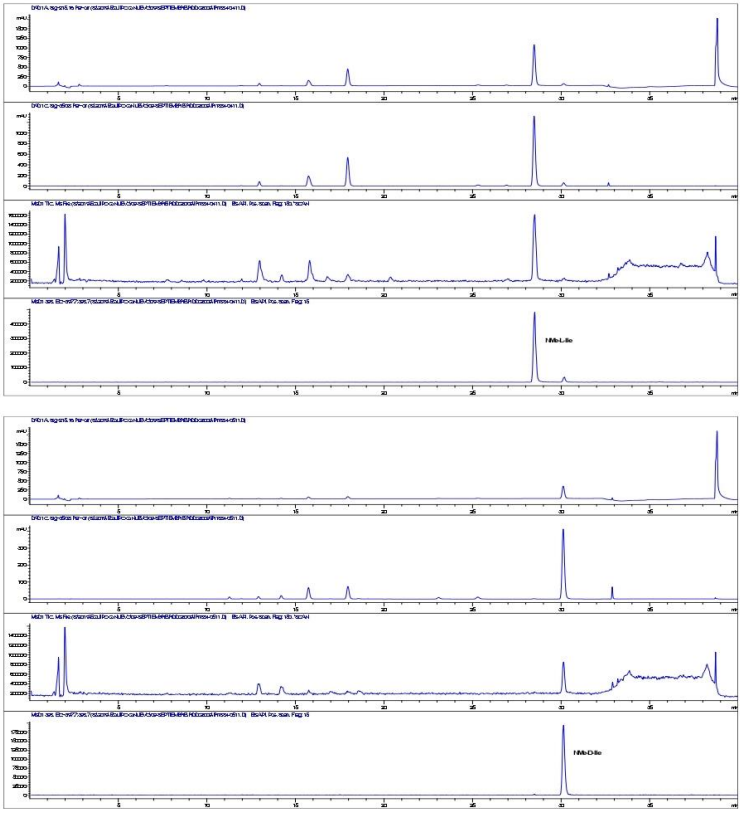


Figure S37 Marfey of Seribunamide A (**2**). NMeLeu standards

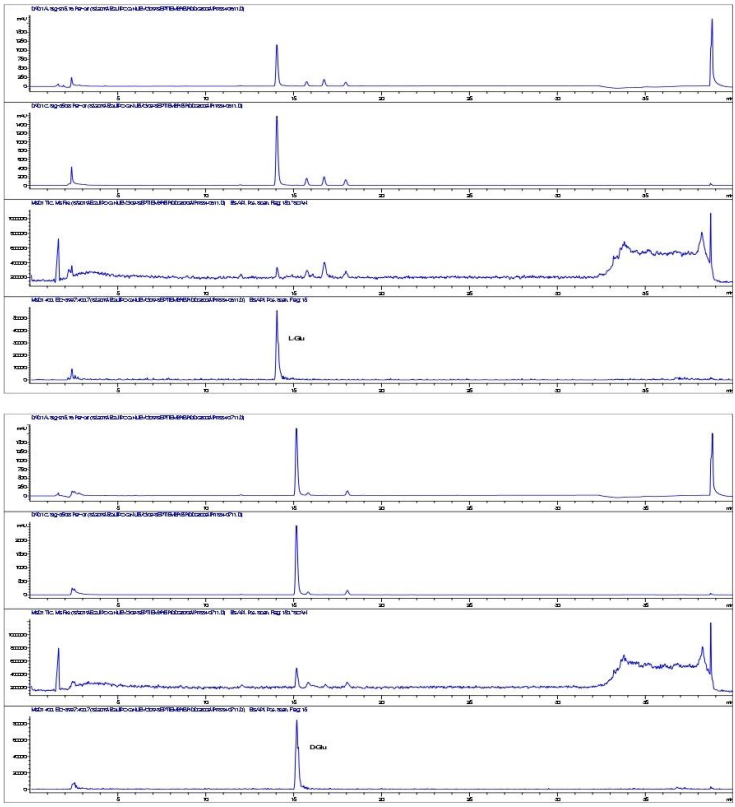


Figure S38 Marfey of Seribunamide A (**2**). Glu standards