Supporting information

Ammosamide D, An Oxidatively Ring Opened Ammosamide Analog from a Marine-derived *Streptomyces variabilis*

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I. Experimental Details

General Procedures. UV spectra were recorded on a Shimadzu UV-1601 UV–VIS spectrophotometer. ^{1}H and 2D NMR spectral data were recorded at 600 MHz in DMSO-d_6 solution on Varian System spectrometer, and chemical shifts were referenced to the corresponding residual solvent signal. ^{13}C NMR spectra were acquired at 100 MHz on a Varian System spectrometer. High resolution ESI-TOF mass spectra were provided by The Scripps Research Institute, La Jolla, CA. Low-resolution LC/ESI-MS data were measured using an Agilent 1200 series LC/MS system with a reversed-phase C18 column (Phenomenex Luna, 150 mm × 4.6 mm, 5 μm) at a flow rate of 0.7 mL/min. Preparative HPLC was performed on an Agilent 1200 series instrument with a DAD detector, using a Phenyl-Hexyl column (Phenomenex Luna, 250 ×10.0 mm, 5 μm). ODS (50 μm, Merck) and sephadex LH-20 were used for column chromatography.

Collection and phylogenetic analysis of strain SNA-020. The marine-derived bacterium, strain SNA-020, was isolated from a sediment sample collected at a depth of 1 meter near the prop roots of a mangrove tree in Sweetings Cay, Bahamas (N 26º 33'27", W 77º 51'15''). 2 g of sediment was dried over 24 h in an incubator at 35 ºC and the resulting sediment stamped onto an agar plate made with A1 media; starch (10 g/L), peptone (3 g/L), yeast extract (4 g/L), sH_2O and containing rifampicin (10 mg/L) and cycloheximide (50 mg/L). A colony of SNA-020 was selected from the plate after four weeks and re-streaked on a new A1 agar plate. Genomic DNA of strain SNA-020 was isolated using standard methods[1] and was amplified using PCR with the Universal 16S rRNA primers FC27 and RC 1492 using the method of Gontang.[1] The partial 16S rRNA sequence 1472 bp was compared to sequences in available databases using the Basic Local Alignment Search Tool and strain SNA-020 determined to be a *Streptomyces variabilis*. The 16S rRNA sequence of SNA-020 was deposited in the NCBI databank as GenBank #JQ815387.

1. Gontang, E. A.; Fenical, W.; Jensen, P. R. *Appl. Environ. Microbiol.*, **2007**, *73*, 3272.

Cultivation and extraction. Bacterium SNA-020 was cultured in 20 × 2.8 L Fernbach flasks each containing 1 L of a seawater based medium (10 g starch, 4 g yeast extract, 2 g peptone, 1 g $CaCO_3$, 40 mg $Fe_2(SO_4)_3 \cdot 4H_2O$, 100 mg KBr) and shaken at 200 rpm at 27 ^{o}C. After seven days of cultivation, sterilized XAD-7-HP resin (20 g/L) was added to adsorb the organic products, and the culture and resin were shaken at 200 rpm for 2 h. The resin was filtered through cheesecloth, washed with deionized water, and eluted with methanol. The methanol soluble fraction was dried in vacuo to yield 8 g of extract.

Isolation. The extract (8 g) was partitioned with *n*-hexane, CH_2Cl_2, and MeOH/H_2O. The MeOH/H_2O layer (4.5g) was fractionated by flash column chromatography on ODS (50 μm, 100 g), eluting with a step gradient of methanol and water with 0.1 % trifluoroacetic acid (10:90–100:0), and 14 fractions (Fr.1–Fr.14) were collected. Fraction 2 (200 mg) was fractioned by Sephadex LH-20 to afford 10 subfraction (Fr.2-I to Fr. 2-X). Fraction 2-VI (10 mg) was purified by reversed phase HPLC (Phenomenex Luna, Phenyl-Hexyl, 250 × 10.0 mm, 2.0 mL/min, 5 μm) using an isocratic solvent system 10% CH_3CN aqueous solution (0.1% trifluoroacetic acid) to afford ammosamide D (**1**, 5.0 mg, t_R = 16.7 min).

Ammosamide D (**1**, 5.0 mg) yellow solid; UV (MeOH) λ_{max} (log ε) 237 (3.8), 335 (3.3), 475 (2.7); ^{1}H NMR (400 MHz, DMSO-d_6) and ^{13}C NMR (100 MHz, DMSO-d_6) see Table 1 and Table S1. ESI-MS *m*/*z* 309.0 $[M + H]^+$, 307.0 $[M - H]^-$. HRESIMS *m*/*z* 309.0224 $[M + H]^+$ ($C_{12}H_{10}{}^{35}ClN_4O_4$, calcd 309.0391).

Crystal data for 1: Triclinic, 7.4256(10) Å, 9.7072(13) Å 10.5790(14) Å, β = 94.560(2)°, V = 757.13(18) Å3, T = 100 (2) K, space group P1, Z = 4, 7430 reflections measured, 3696 independent reflections (Rint = 0.0414). R1 = 0.0394 (I > 2σ(I)), wR(F2) = 0.1122 (all data). CCDC 873753 contains the supplementary crystallographic data for **1**. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Attempts at conversion of ammosamide B (3) to ammosamide D (1):

Conversion of 3 to 1 by stirring in air: Compound **3** (0.5 mg) was dissolved in 1mL methanol/H_2O (1:1) and stirred under air atmosphere at room temperature for 10 days. 0.2 mL of reaction solution was taken and analyzed by LC-MS, looking for conversion to **1**. LC-MS data indicated no compound **1** was formed under this reaction condition.

Conversion of 3 to 1 under at atmosphere of O_2: Compound **3** (0.5 mg) in methanol/H_2O (1:1, 0.5 mL) was stirred under a balloon of O_2. The temperature of the solution was increased to 50 ºC and stirred for 3 days. 0.2 mL of sample was taken and analyzed by LC-MS, looking for conversion to **1**. Compound **1** was not seen by LC-MS.

Conversion of 3 to 1 under fermentation conditions: Compound **3** (1.5 mg) was dissolved in DMSO (0.5 mL) to make 10mM stock solution of **3**. 50 µL of the solution was added into fermentation media (A1) and shaken at 200 rpm for 7 days at 30 ºC.. The reaction mixture was dried in vacuo and dissolved in 1mL methanol/H_2O (1:1) solution. 0.2 mL of the solution was taken and analyzed by LC-MS. No conversion of **3** to **1** was detected.

Conversion of 3 to 1 with IBX: Compound **3** (0.5 mg) was dissolved in DMSO (0.5 mL) and treated with 2-iodoxybenzoic acid (0.95 mg, 2.0 eq.) and stirred at room temperature for 24 hours. Aliquots (0.02 mL) were taken every four hours and analyzed by LC-MS, looking for conversion to **1**. After 24 hours, by LC-MS analysis the presence of **3** was undetectable with 75% conversion to **1** (with an identical retention time, UV and MS as authentic **1**) and 35% conversion to a compound with a molecular weight consistent with **6** (ESI-MS $[M + H]^+$ 308.05). Purification by C18 HPLC (Phenomenex Luna C18, 10 x 250 mm, gradient from 90:10 H_2O:CH_3CN to 50:50 H_2O:CH_3CN (0.05% TFA) over 25 minutes gave only **1** (0.23 mg, 46% yield).

Cytotoxicity assays: Cell lines were cultured in 10 cm dishes (Corning, Inc.) in NSCLC cell-culture medium: RPMI/L-glutamine medium (Invitrogen, Inc.), 1000 U/ml penicillin (Invitrogen, Inc.), 1 mg/ml streptomycin (Invitrogen, Inc.), and 5% fetal bovine serum (Atlanta Biologicals, Inc.). Cell lines were grown in a humidified environment in the presence of 5% CO_2 at 37 ^{o}C. For cell viability assays, MIA PaCa-2 cells (60 µL) were plated individually at a density of 750 and

500 cells/well, respectively, in 384 well microtiter assay plates (Bio-one; Greiner, Inc.). After incubating the assay plates overnight under the growth conditions described above, purified compounds were dissolved and diluted in DMSO and subsequently added to each plate with final compound concentrations ranging from 40 μM to 1 nM and a final DMSO concentration of 0.5%. After an incubation of 96 h under growth conditions, Cell Titer GloTM reagent (Promega, Inc.) was added to each well and mixed. Plates were incubated for 10 min at room temperature and luminescence was determined for each well using an Envision multi-modal plate reader (Perkin-Elmer, Inc.). Relative luminescence units were normalized to the untreated control wells (cells plus DMSO only).

II.

Table S1. NMR data for ammosamide D (**1**) and ammosamide A (**2**) in DMSO-d_6

No.	**1**				**2**[a]	
	δ_H, mult, (J in Hz)	δ_C	COSY	HMBC	δ_H	δ_C
1	8.18 q (4.7)		H1a	C1a,C2		
1a	2.77 d (4.7)	25.9	H1	C2	4.03 s	33.3
2	-	166.3				177.2
2a	-	146.8				
3	8.00 s	122.7		C2,C2a,C4a,C5b	8.47 s	116.5
4	-	152.0				144.6
4a	-	163.7				166.0
5a	-	146.7				
5b	-	125.4				119.7
6	-	151.7				
7	-	106.9				103.1
8	-	168.9				
8a	-	178.3				110.5
CONH$_2$-a	9.17 s;	-	CONH$_2$-b	C4a	8.92 (br s)	
CONH$_2$-b	8.02 s		CONH$_2$-a	C4	7.68 (br s)	
NH_2(6)-a	9.35 s;	-	NH_2(6)-b	C5a, C7,C8	7.16 (br s)	
NH_2(6)-b	8.55 s		NH_2(6)-a	C5a,C5b,C6,C7	6.63 (s)	
NH_2(8)					6.89 (s)	

[a]C-2a, C-5a, C-6, and C-8 (δ_C = 132.6, 134.7, 136.8, 142.7 ppm) could not be unambiguously assigned.[2]

2. Hughes, C. C.; MacMillan, J. B.; Gaudencio, S. P.; Jensen, P. R.; Fenical, W. *Angew. Chem. Int. Ed.* **2009**, *48*, 725.

III.

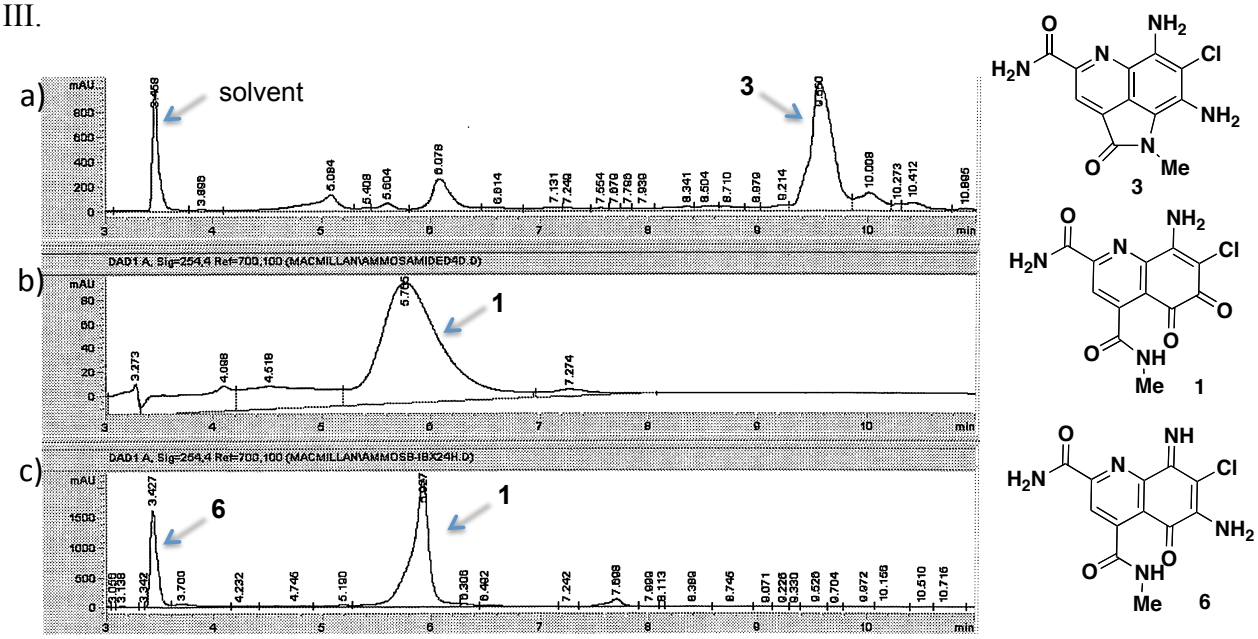


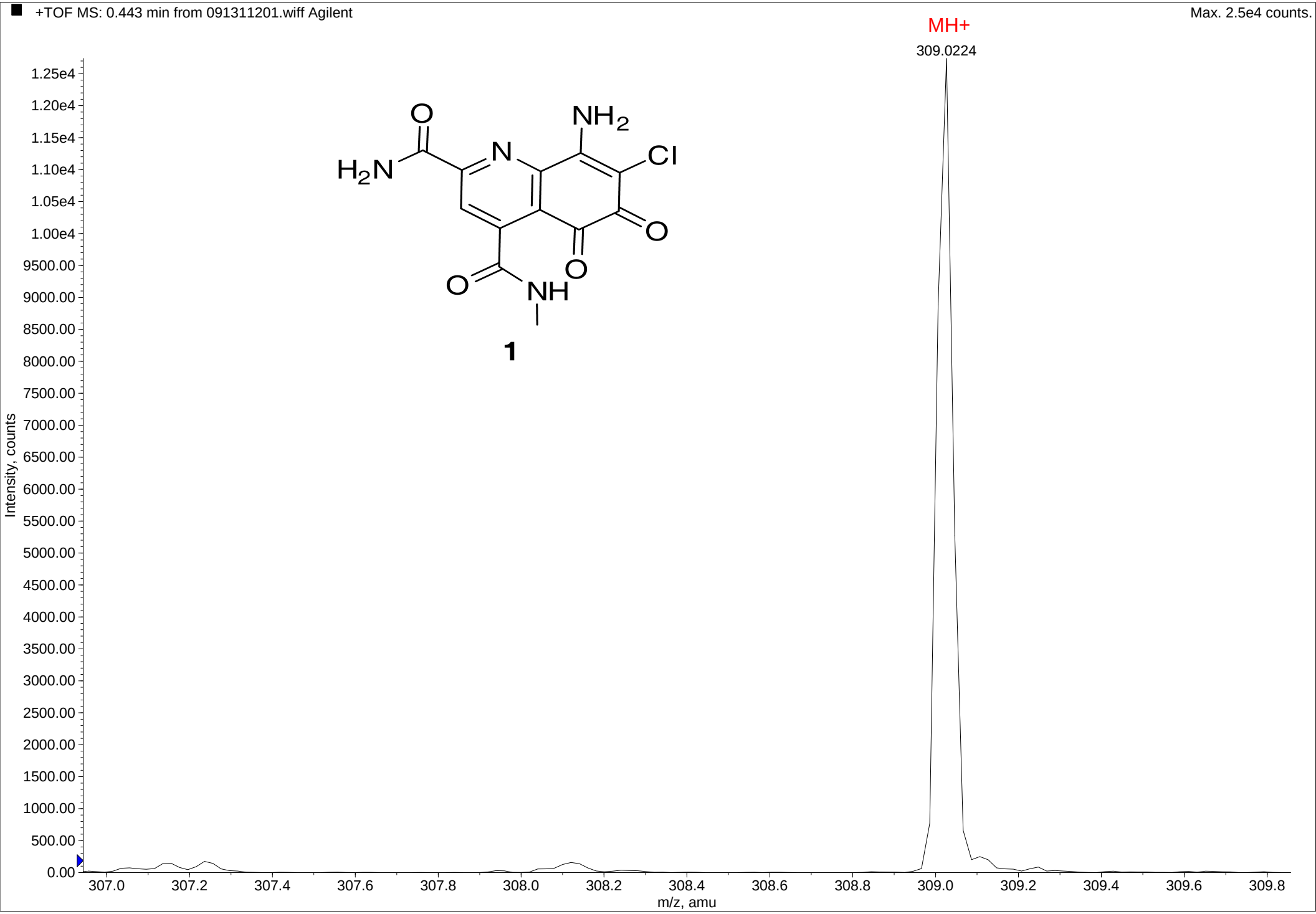
Figure S1. LC-MS traces at 254 nm for a) ammosamide B (**3**); b) ammosamide D (**1**); c) conversion of **3** to **1** with IBX after 24 hours. Data acquired on a Phenomenex Luna C18(2) analytical column, 4.6 x 100 mm, with a gradient from 95:5 H_2O:CH_3CN (0.05% formic acid) to 60:40 H_2O:CH_3CN (0.05% formic acid) over 12 minutes. The signal for **6** overlaps with a solvent signal. The conditions used for isolation of the ammosamides can cause dram atic broadening of signals in chromatography.

Polarity/Scan Type: Positive **Sample Name: u6jm4560** **Acq. File: 091311201.wiff**

Acq. Date: Tuesday, September 13,

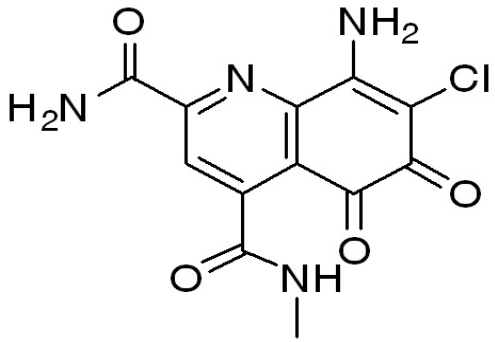
+TOF MS: 0.443 min from 091311201.wiff Agilent

Max. 2.5e4 counts.



1

1H NMR spectrum of Ammosamide D



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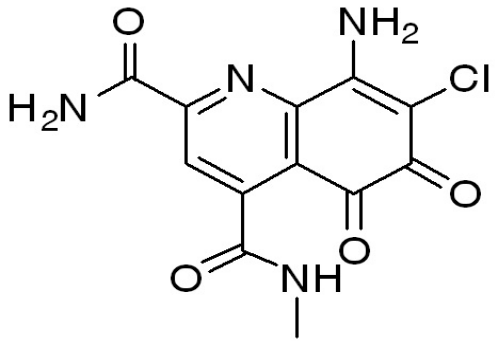
13C NMR spectrum of Ammosamide D



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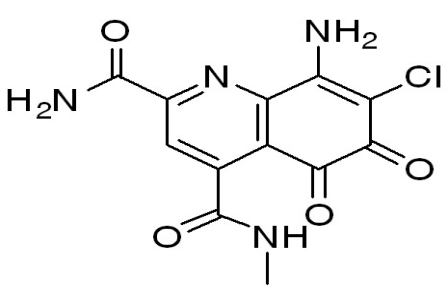

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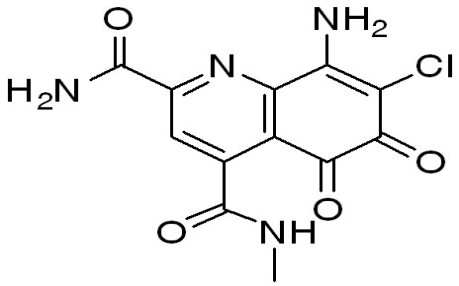
HSQC spectrum of Ammosamide D



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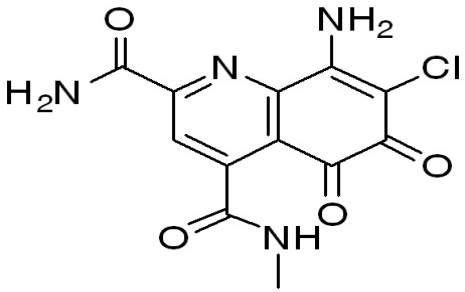
HMBC spectrum of Ammosamide D



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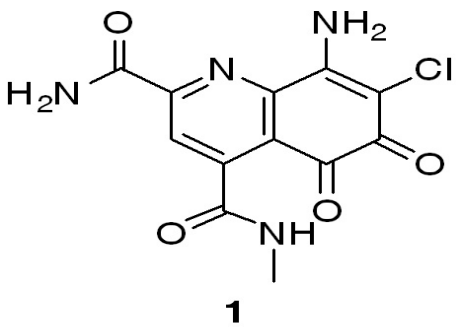
HMBC spectrum of Ammosamide D

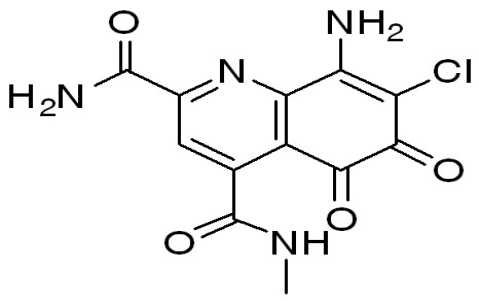


1

COSY of Ammosamide D

f2 (ppm)

f1 (ppm)



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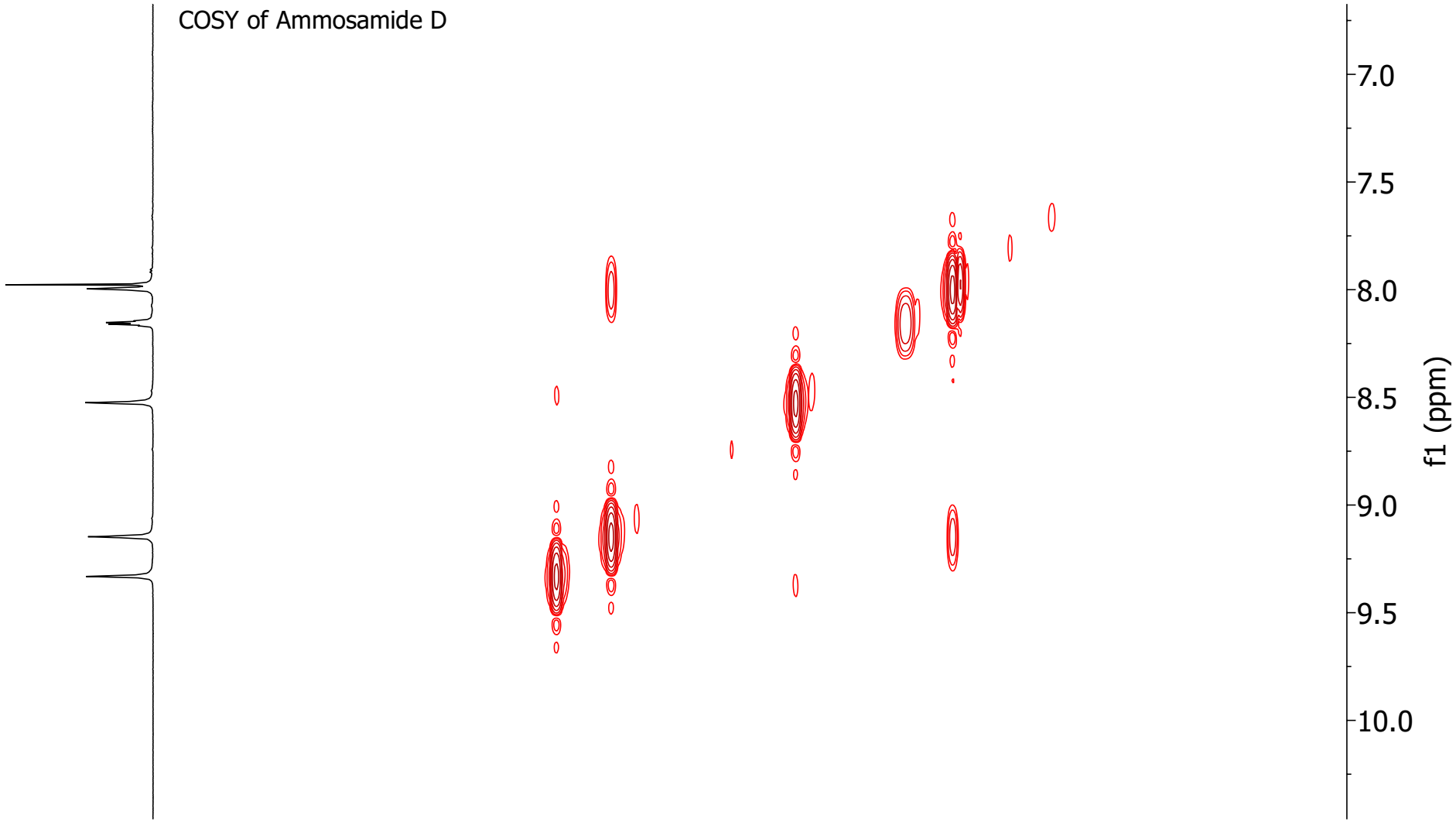

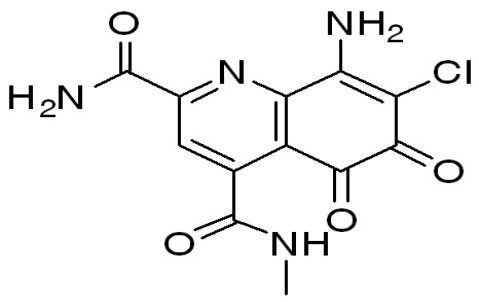

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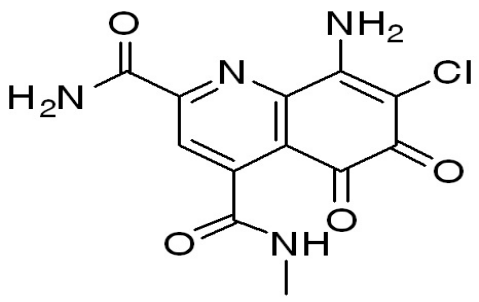
NOESY spectrum of Ammosamide D

f2 (ppm)

f1 (ppm)



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NOESY spectrum of Ammosamide D