SUPPLEMENTARY MATERIAL

Dendrobeaniamine A, a New Alkaloid from the Arctic Marine Bryozoan *Dendrobeania murrayana*

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Abstract

The new guanidine alkaloid Dendrobeaniamine A (**1**) was isolated from the organic extract of the Arctic marine bryozoan *Dendrobeania murrayana*. The chemical structure of **1** was elucidated by spectroscopic experiments, including 1D and 2DNMR and HRESIMS analysis. Compound **1** is a lipoamino acid, consisting of a C_{12} fatty acid anchored to the amino acid arginine. The bioactivity of **1** was evaluated using cellular and biochemical assays, but the compound did not show cytotoxic, antimicrobial, anti-inflammatory or antioxidant activities.

Key words

Arctic marine invertebrates; bryozoans; *Dendrobeania murrayana*; bioprospecting; marine secondary metabolites.

4. Experimental section

4.1 *General experimental procedure*

High-resolution mass spectra were acquired using positive electrospray ionization on a Waters Vion® IMS time-of-flight MS coupled with an Acquity I-Class UPLC (Waters, Milford, MA, USA) using MS grade solvents. The system was controlled and data was processed using UNIFI 1.8.2. Isolation of **1** was done using a HPLC autopurification system consisting of Waters 600 pump, Waters 3100 mass spectrometer, Waters 2996 photo diode array detector and Waters 2767 sample manager. The prep-HPLC system was controlled by Waters MassLynx version 4.1 and the FractionLynx application manager.

4.2 *Animal material*

Specimens of the marine bryozoan *Dendrobeania murrayana* (Johnston 1847) (Class: Gymnolaemata, Order: Cheilostomata, Family; Bugulidae) were collected by scuba diving during the springof 2012 at 30 m depth in Vesterålsfjorden, Norway (69° 26.177 N/14°, 93.773 W). The marine organism was taxonomically identified and kept at -23°C until extracted. A voucher specimen (M17034) of *D. murrayana* is deposited in Marbank, the Norwegian national marine biobank (Institute of marine research, Tromsø, Norway).

4.3. *Extraction*

Frozen specimens of *D. murrayana* (wet weight 0.55 kg) were diced and freeze dried before being extracted twice (first time 24 h, second time 30 min) using ultra-pure water (Milli-Q, Millipore, Billerica, MA, USA). After centrifugation the supernatant was removed and dried, resulting in 17.84 g aqueous extract. The sediment was freeze-dried, ground and extracted twice (first time 24 h, second time 30 min) using a 1:1 (vol:vol) mixture of dichloromethane (Merck, Darmstadt, Germany) and methanol (Sigma-Aldrich, Steinheim, Germany). The mixture was vacuum-filtered through a Whatman Ø 125 mm no.3 filter (Little Chalfont, UK). The organic extract was reduced under vacuum, resulting in 1.59 g dry organic extract, and stored at -23°C in the dark awaiting further analysis.

4.4. Preparation of the organic extract for chemical analysis

An aliquot of the organic extract (0.75 g) was dissolved in 30 mL hexane (Merck) and partitioned three times with 25 mL 90% aqueous methanol in a separating funnel. The combined methanol phases were finally reduced to dryness under reduced pressure at 40°C and redissolved in 4 mL 80% aqueous methanol

4.5. *UHPLC-DAD-HRESIMS analysis*

UHPLC-HRESIMS was used to analyse the prepared organic extract. An aliquot of 5 µL of the prepared sample was injected onto a Waters Acquity BEH C18 column (1.7 µm, 2.1× 100 mm. 40 °C). The mobile phase consisted of ultra-pure water (solvent A) and acetonitrile (solvent B) (Merck, Dermstadt, Germany), both with 0.1% formic acid (Pro-analysis, Merck, Dermstadt, Germany) delivered at a flow rate of 0.45 mL/min. The gradient started with 10% B and was linearly increased to 100% B over 12 min. The HRESIMS data was obtained in positive electrospray ionization (ESI+) mode with a data acquisition rate of 10 scans per second at a mass range of m/z 50-2000. The HRESIMS was operated under following conditions: The capillary and cone voltages were set to 0.8 kV and 40 V, respectively. The desolvation and ion source temperatures were set to 350°C and 120°C, respectively. Nitrogen gas was used for desolvation at 800 L/h and cone gas at 50 L/h. UV-data was acquired between 190 and 500 nm. Leucine-enkephalin (100 pg/µL) (Waters, Milford, MA, USA) in 50% aqueous acetonitrile with 0.1% formic acid was used for lock mass correction. ChemSpider, Dictionary of Marine Natural Products and SciFinder were used for database searches.

4.6. *Isolation of **1** using mass guided semi preparative HPLC*

Compound **1** was isolated by using a Waters preparative HPLC auto-purification system. The mass of protonated dendrobeaniamine A (1) (369.3 Da) was used as collection trigger in ESI+ mode. Aliquots of 100 µL were injected onto a Waters Atlantis® prep C18 HPLC column (10 µm, 10 mm×250 mm). The mobile phase consisted of ultrapure water (A) and acetonitrile (B) both with 0.1% formic acid, applied at a flow rate of 6 mL/min. The gradient started with 50% B and was linearly increased to 80% B over 10 min. Compound **1** eluted after 5.3 min. One percent of the flow was split to the MS. After drying and redissolving the sample, it was

injected once more onto a Waters X-Select fluorophenyl column (5 µm, 10 mm×250 mm) with the same flow rate and gradient. The final yield of **1** was 2.5 mg.

4.7. *Structure determination of **1***

Optical rotation data was obtained using an AA-10R automatic polarimeter (Optical Activity LTD). The IR spectrum was measured on a NaCl disk using a Cary 630 FT-IR spectrometer (Agilent technologies, CA, USA). NMR spectra were acquired in deuterated methanol on a Bruker Avance III HD spectrometer operating at 600 MHz for protons, equipped with an inverse TCI cryo probe enhanced for ^{1}H, ^{13}C, ^{2}H and ^{15}N. All NMR spectra were acquired at 298 K, in 3 mm solvent matched Shigemi tubes using standard pulse programs for proton, carbon, HSQC, HMBC, H2BC, DQCOSY, TOCSY and NOESY with gradient selection and adiabatic versions where applicable.

4.8 *Bioassays*

4.8.1. Cytotoxic assay (MTS assay)

The cytotoxic activity of **1** was tested at 10 µM against human melanoma A2058 cells (American Type Culture Collection (ATCC) CRL-1114, Manassas, VA, USA) and non-malignant human MRC5 lung fibroblasts (ATCC CCL-171) using by the MTS method as previously described (Hansen et al. 2017; Michael et al. 2017).

4.8.2. Antibacterial activity

The antibacterial activity of **1** was tested at ranging concentrations starting from 135.68 µM against *Staphyloccoccus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Enterococcus faecalis* (ATCC 29212) or *Streptococcus agalactiae* (gr.B) (ATCC 12386) as previously described (Michael et al. 2017).

4.8.3. Antifungal activity assay

The antifungal activity of **1** was tested at 135.68 µM against *Candida albicans* (ATCC 90028) using a broth dilution assay. *C. albicans* was inoculated on a potato dextrose broth containing dextrose (20.0 g), potato extract (4.0 g), agar (15.0 g) and pH 5.6±0.2) and incubated at 37^{o}C for 24 hrs. From the subculture, 5-8 colonies were picked and resuspended in 5 mL of sterile 0.9% NaCl (Sigma),vortexed for 15 seconds and adjusted to a density of 0.5 McFarland standard. Thereafter, the inoculum was further diluted in the sterile assay medium to achieve the final concentration at 0.5-2.5 x 10^6 CFU/mL. The assay medium contained Roswell Park Memorial Institute (RPMI) powder without bicarbonate (Sigma-Aldrich) (10.4 g), L-glutamine (Biochrom, Berlin, Germany) (10.25 mL), MOPS buffer (Sigma-Aldrich) (34.53 g), pH 7 and distilled water (900 mL). The fungal cell suspension of 100 µL was transferred to a 96 microtitre plate (Nunclon^{TM} Delta Surface, Thermo Fisher Scientific, Denmark) followed by the addition of 100 µL of **1** in triplicate. Prior to incubation at 37°C, the optical density was measured immediately at 600 nm (OD_{600}) by using Victor^3 plate reader with WorkOut 2.0 software. After 48 hrs incubation, the OD_{600} was measured once more. Amphotericin B (A2942 Sigma) (8 µg/mL) was used as a reference control, culture with sterile MilliQ was used as positive control and assay medium with sterile Milli-Q water was used as negative control. OD_{600} readings < 0.05 was considered as active.

4.8.4. Biofilm inhibition assay

The inhibition of biofilm formation by **1** was tested at 135.68 µM against *Staphylococcus epidermidis* (ATCC-35984 RP62A-42-77). The assay was performed as previously described (Michael et al. 2017).

4.8.5. Anti-inflammatory assay

THP-1, a human acute leukemia monocytic cell line (ATCC TIB-202, Manassas, VA, USA) was used to study an inhibition of lipopolysaccharide (LPS) induced cytokines secretion of tumor necrosis factor (TNF-α) by **1** at 10 µM. THP-1 cells were cultured and assayed in low endotoxin RPMI–1640 (Biochrome GmbH, Germany), supplemented with fetal bovine serum (10%) (Ultralow endotoxin, Biowest), gentamycin (10 mg/mL) and L-alanyl-L-glutamine (200 mM) (Biochrome). Briefly, 1×10^5cells/well were seeded in a flat bottom NuncTM 96 microtitre plate (Thermo Fisher Scientific, Denmark) and the cells were immediately treated

with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma) for monocyte to macrophage differentiation. Following incubation at 5% CO_2 and 37°C for 48 hrs, cells were washed with endotoxin free tris buffered saline (TBS- 0.05% Tween-20; pH 7.4) and replaced with 100 µL of new RPMI growth medium before 24 hrs incubation. The old medium was replaced with 80 µL fresh RPMI growth medium and the cells were treated with 10 µL of test **1** in triplicate. Cell control wells were incubated with growth medium alone. After 1 hr incubation, the cells were stimulated with 10 µL of 10 ng/mL LPS to all wells except the negative control. Positive control were cells treated with LPS in growth medium. The plate was incubated for another 6 hrs at 37°C. Cytokine production was stopped by freezing the plates at -80°C immediately after incubation. TNF-α concentration in the collected supernatants was measured by sandwich Enzyme-linked immunosorbent assay (ELISA). One day prior to the ELISA test, a 96-well flat-bottom Nunc Maxisorp plate was coated with 2 µg/mL capture antibody (eBioscience, San Diego, CA, USA) in TBS and kept at 4°C for overnight. The coating solution was washed twice with TBS at room temperature. A blocking buffer (TBS; 2% BSA) 200 µL was added to each well and incubated at room temperature for an hr with gentle shaking. A TNF-α serial dilution (1000, 500, 250, 125, 62, 7, 31,5, 15, 6 and 0 pg/mLwas used as standard curve. After TBS washing, 50 µL of biotin conjugated antibody (3 µg/mL) (eBiosciences) (diluted in assay diluent - TBS with 1% BSA) was added to each well and incubated at 37°C for one hr. The substrate reagent ExtrAvidin ® - Alkaline phosphatase (Sigma-Aldrich) was added after washing the wells with TBS. After 30 min incubation, each well was washed thoroughly with TBS (5 times wash in 30 sec interval). Simultaneously, 100 µL of substrate pNPP (1 mg/mL in 1 M diethanolamin buffer pH 9.8, Sigma-Aldrich) was added to each well and incubated at 37°C for 45 min. After color development, the absorbance was read at 405 nm by using an ELISA plate reader. Inhibition of inflammatory activity below 50 % compared to LPS positive control was considered active.

4.8.6. Antioxidant assay (Oxygen Radical Absorbance Capacity (ORAC) assay)

The ORAC assay was carried out in a black polystyrene 96 well microtiter plate (FluoroNunc ™ MaxiSorp Surface, TX, USA). The antioxidant activity was measured against a peroxyl radical generated by thermal decomposition of AAPH (2,2'-Azobis(2-amidinopropane)

dihydrochloride) at 37°C in the presence of fluorescein. Fluorescence was measured at 485 nm excitation and 528 nm emission on a Victor^3 plate reader with WorkOut 2.5 software. Results were calculated from the area under the fluorescence decay curve (AUC)substracted AUC_{blank} values. Working solution AAPH (44 mM), fluorescein (52 nM) and Trolox® (6-hydroxy-2,5,7,8-tetrametmethylchroman-2-carboxylic acid) (0-25 µM) were prepared in a phosphate buffer (75 mM, pH 7.4) Compound **1** was diluted in 75 mM phosphate buffer and the final test concentration of **1** was 135.68 µM. All reagents were purchased from Sigma-Aldrich. A standard curve was derived from a Trolox titration (0-25 µM). Phosphate buffer (75 mM) was used as blank. Compound **1** was transferred to the wells in parallel followed by addition of 125 µL of flurorescein to all wells including controls. The plate was allow to equilibrate by incubating at 37°C for 15 min. The reactions were initiated by adding 60 µL of AAPH to the preincubated microtitre plate. The fluorescence readings were recorded every 70 sec25 times. Antioxidant activity was expressed as trolox equivalents.

Table S1. NMR spectroscopic data recorded for dendrobeniamine A (**1**)

Position	δ_C/δ_N, type	δ_H (*J* in Hz)	COSY	HMBC	H2BC	NOESY
1	14.4, CH_3	0.89, t (6.8)	2	2		2
2	23.6, CH_2	1.29[o]	1	1, 3		1
3	33.0, CH_2	1.26[o]		1		
4	30.8, CH_2	1.32[o]				
5	30.7, CH_2	1.29[o]				
6	30.6, CH_2	1.29[o]				
7	30.4, CH_2	1.29[o]	8	8[o], 9		9
8	29.4, CH_2	1.46, p (6.2)	9,7	7, 9	7, 9	9, 11
9	33.9, CH_2	2.60, m[b]	8	7, 8, 11, 12	8	7, 8, 11
10	155.9, C			8, 9, 11, 12		
11	24.8, CH_3	1.84, s	12	9, 12	12	8, 9, 12
12	119.8, CH	5.74, s	11	9, 11		11, 14
13	168.7, C			11, 12		
14	127.7, NH	7.40, s[b]	15			12
15	53.1, CH	4.32, s[b]	17a, 17b, 14			
16	169.6, C					
17a	31.2, CH_2	1.87, m[b]	15	18, 19	18	
17b		1.72, m[b]	15			
18	26.1, CH_2	1.63, m[b]	17a, 17b, 19	17, 19	19	19
19	42.2, CH_2	3.21, m	18, 20	17, 18	18, 20	18
20	84.1, NH	7.55, s[b]	19	18[*]		
21	158.7, C			19		

[o]Overlapped, [b]Broadened, [*]in ^{15}N HMBC spectrum

Figure S1. ESI+ base peak intensity chromatogram of the organic extract of *D. murrayana* analyzed by UHPLC-HRESIMS. The arrow indicates the prominent peak at Rt 5.7 min.

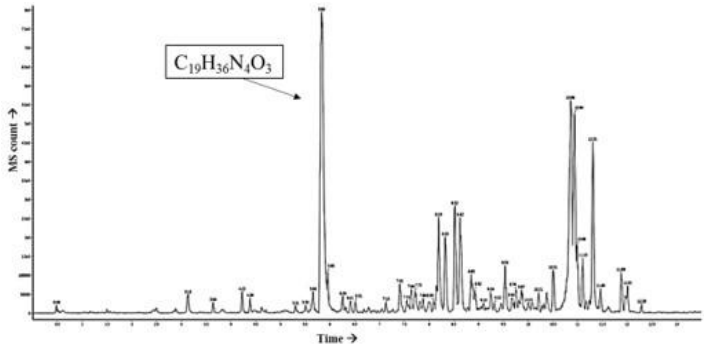


Figure S2. Structure of **1** was elucidated as new alkaloid dendrobeaniamine A. Highlighted is key COSY (bold), HMBC (black arrows) and H2BC (green arrows) correlations for 1.

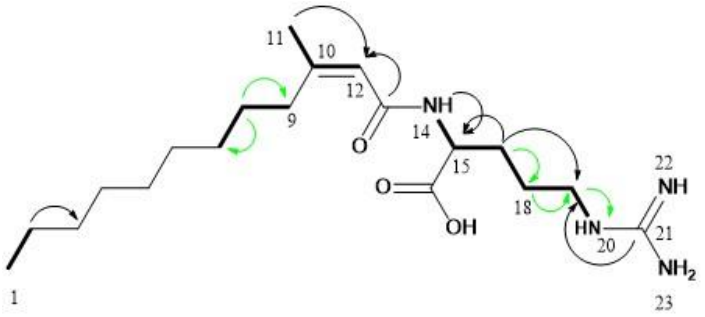


Figure S3. The structures of **1** (green), lauric arginate (**2**, pink) and spermidine 1 (**3**, orange) with highlighted key structural features. Charge: + and -, oxygen: red, nitrogen: blue, hydrogen: white, double bond: =.

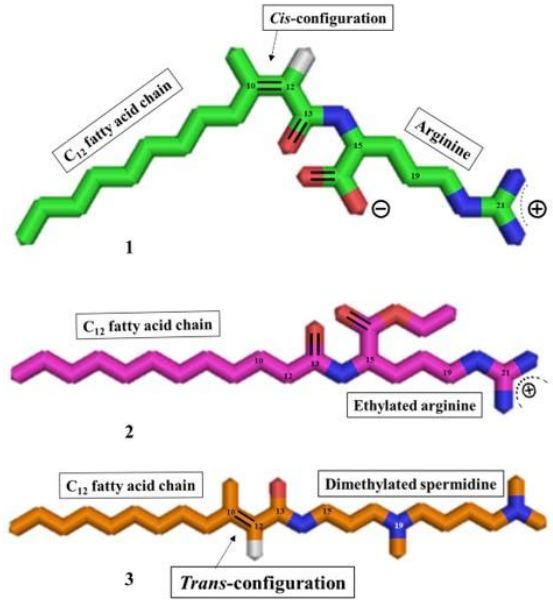


Figure S4. ^{1}H NMR (600 MHz, CD_3OD) spectrum of Dendrobeaniamine A (**1**)

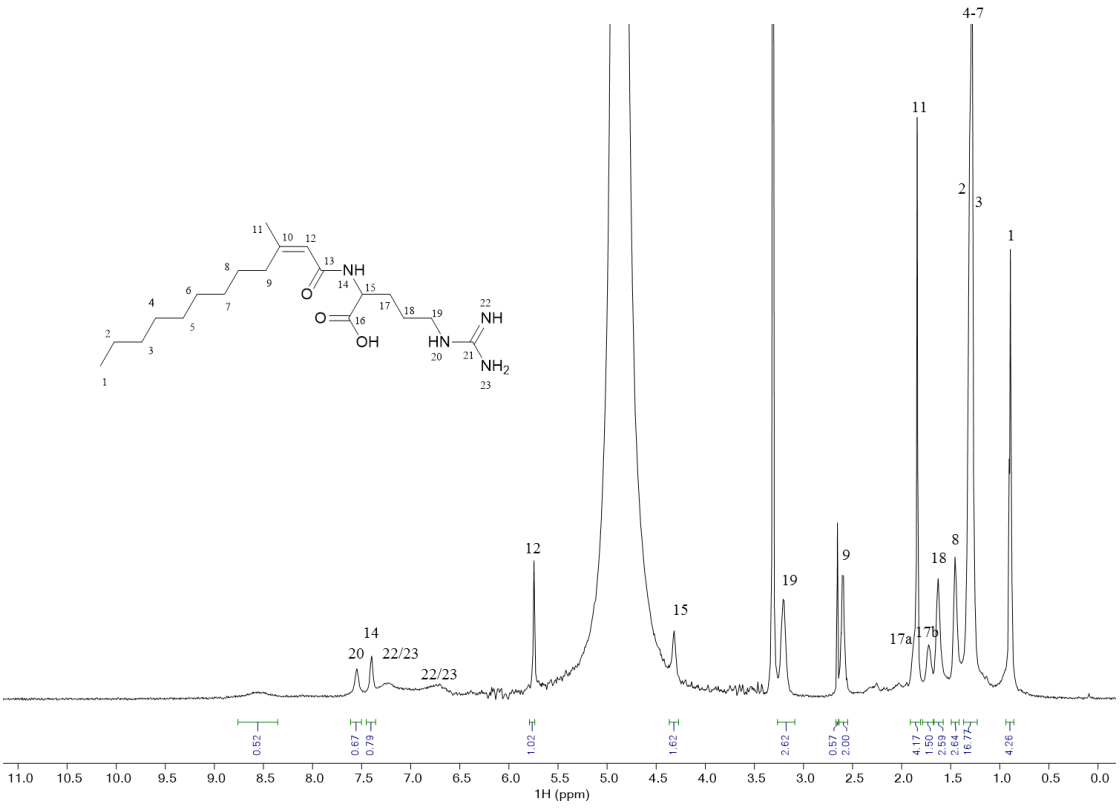


Figure S5. ^{13}C (151 MHz, CD_3OD) spectrum of Dendrobeaniamine A (**1**)

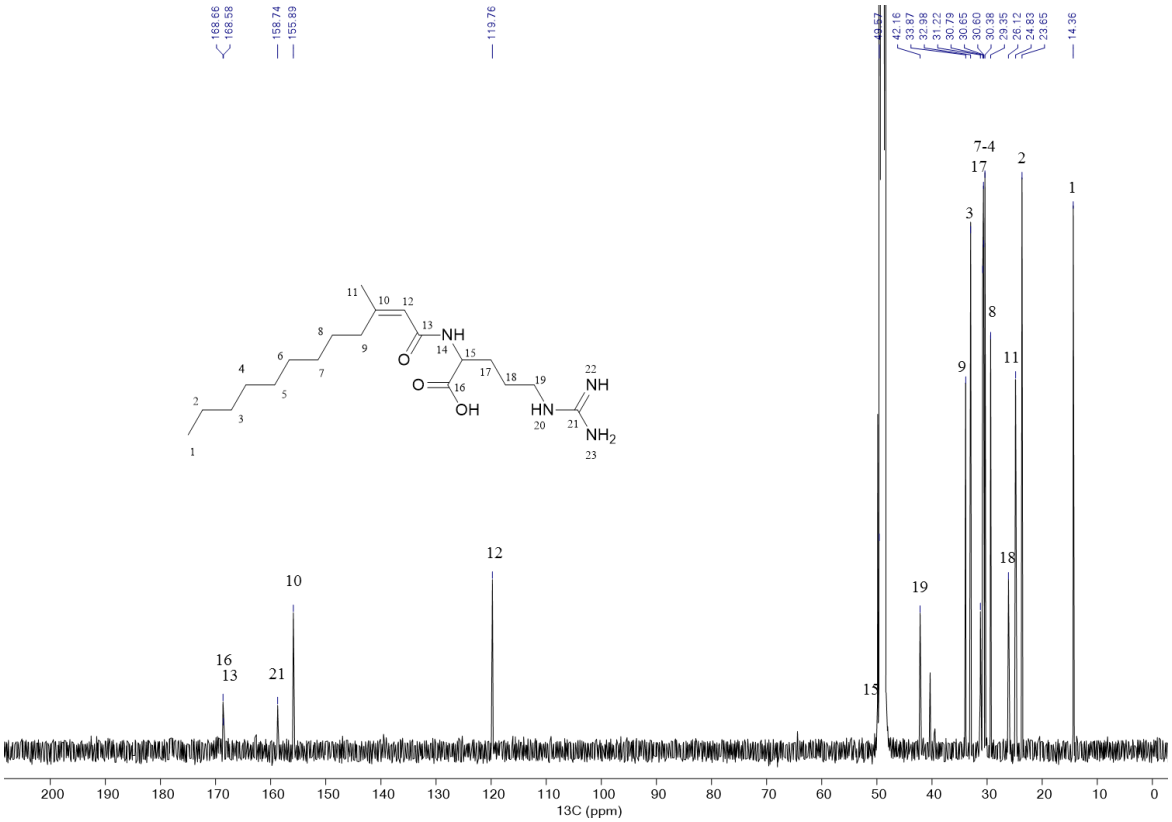


Figure S6. HSQC + HMBC (600 MHz, CD_3OD) spectrum of Dendrobeaniamine A (**1**). The inlaid figure is the $^{1}J_{C15H15}$ HMQC that is not detected in the HSQC.

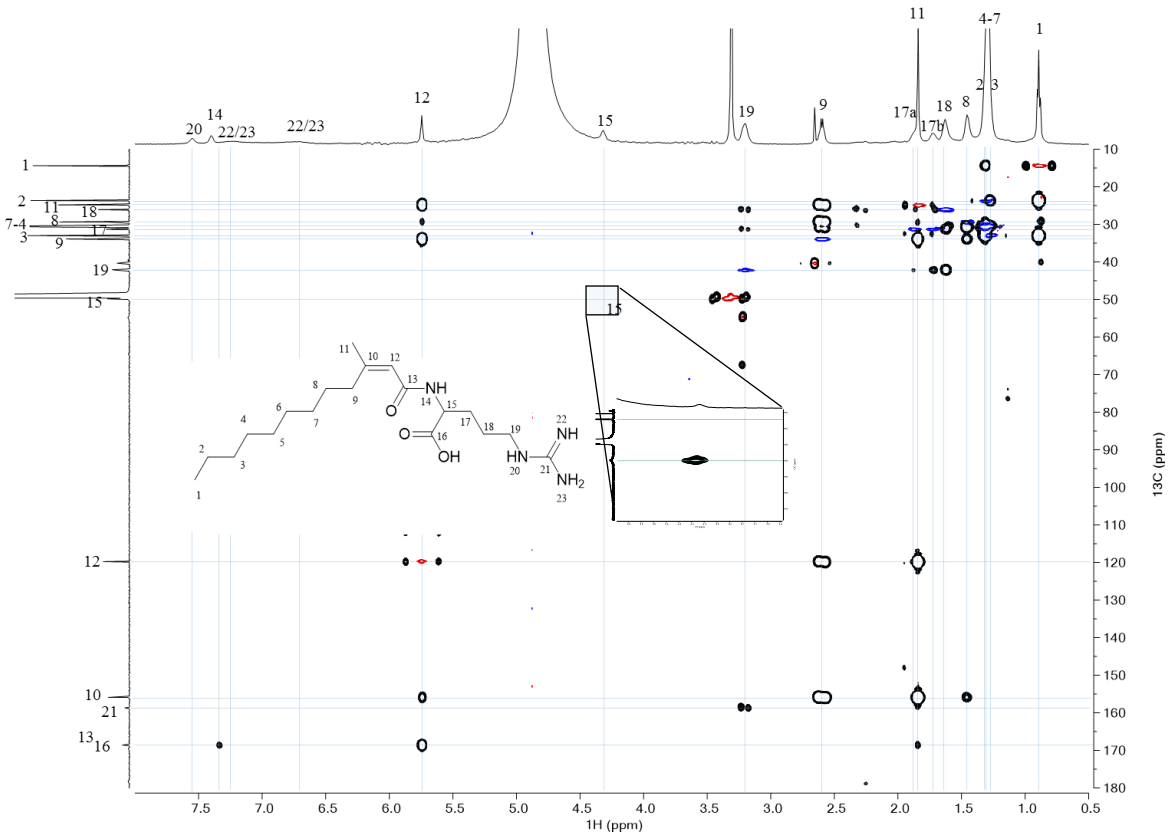


Figure S7. H2BC (600 MHz, CD_3OD) spectrum of Dendrobeaniamine A (**1**)

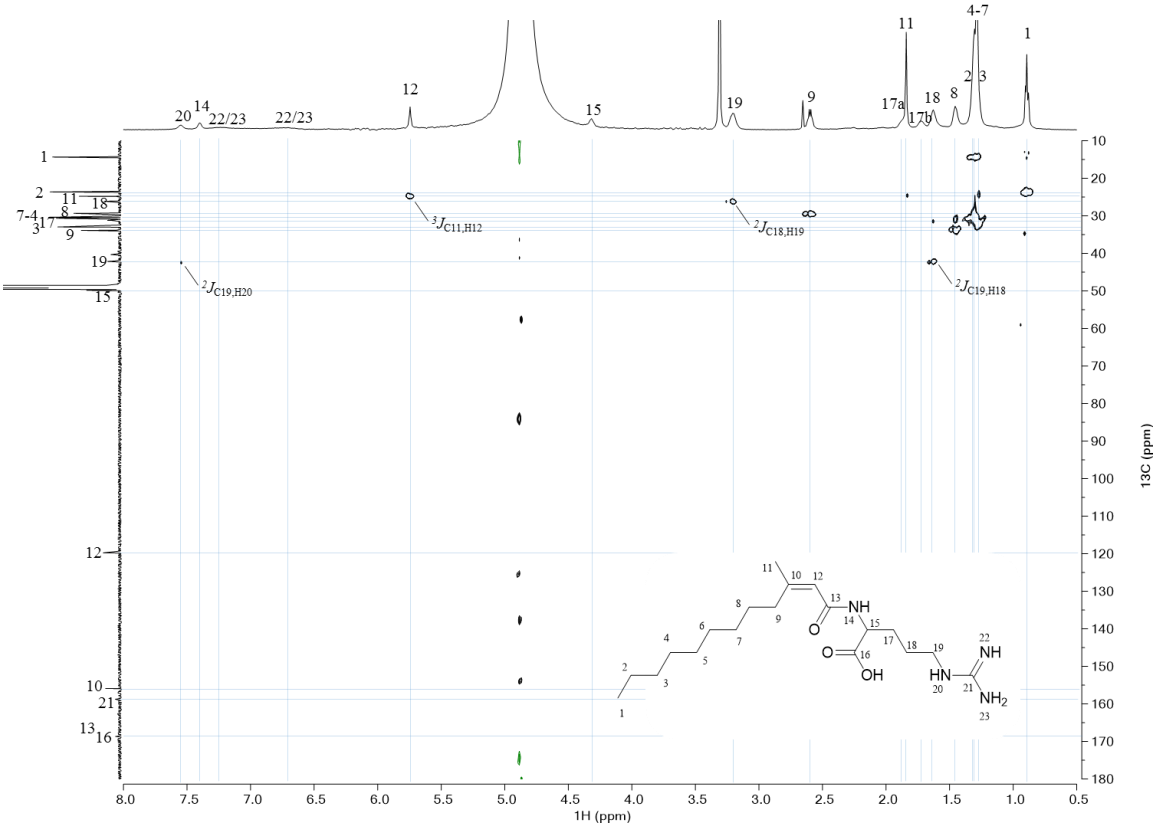


Figure S8. COSY (600 MHz, CD_3OD) spectrum of Dendrobeaniamine A (**1**)

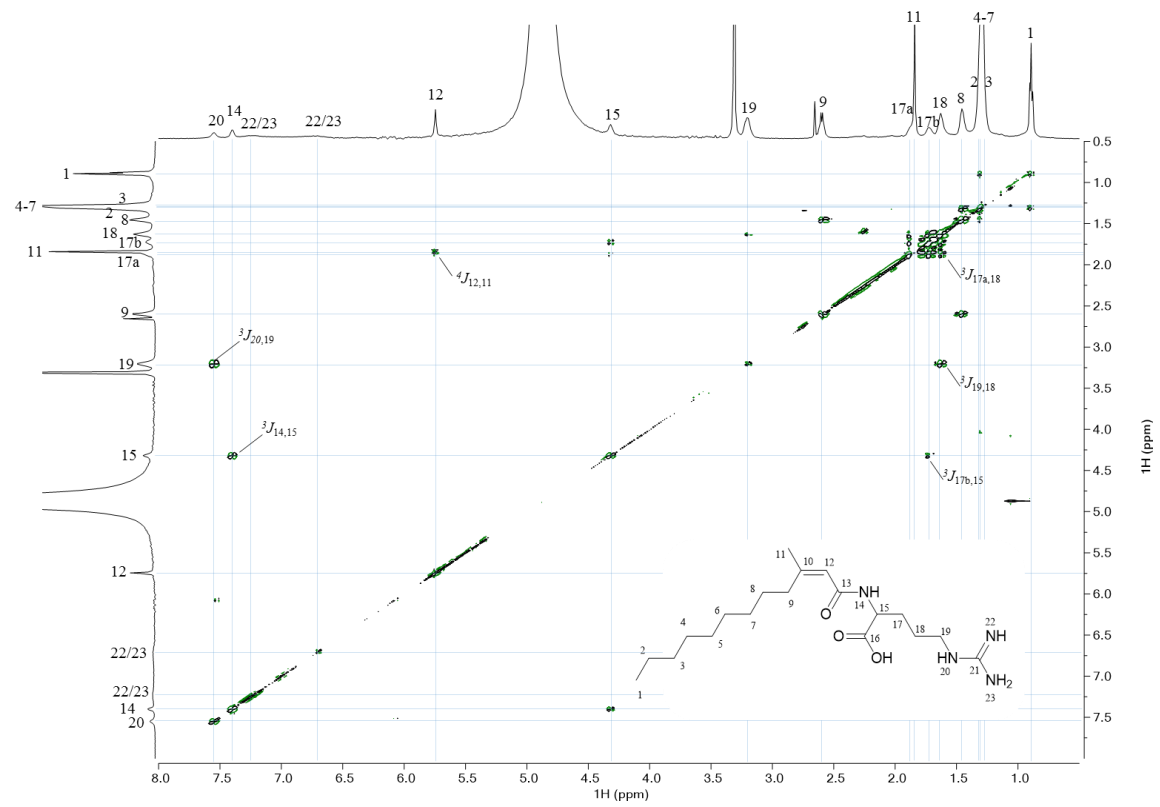


Figure S9. TOCSY (600 MHz, CD_3OD) spectrum of Dendrobeaniamine A (**1**)

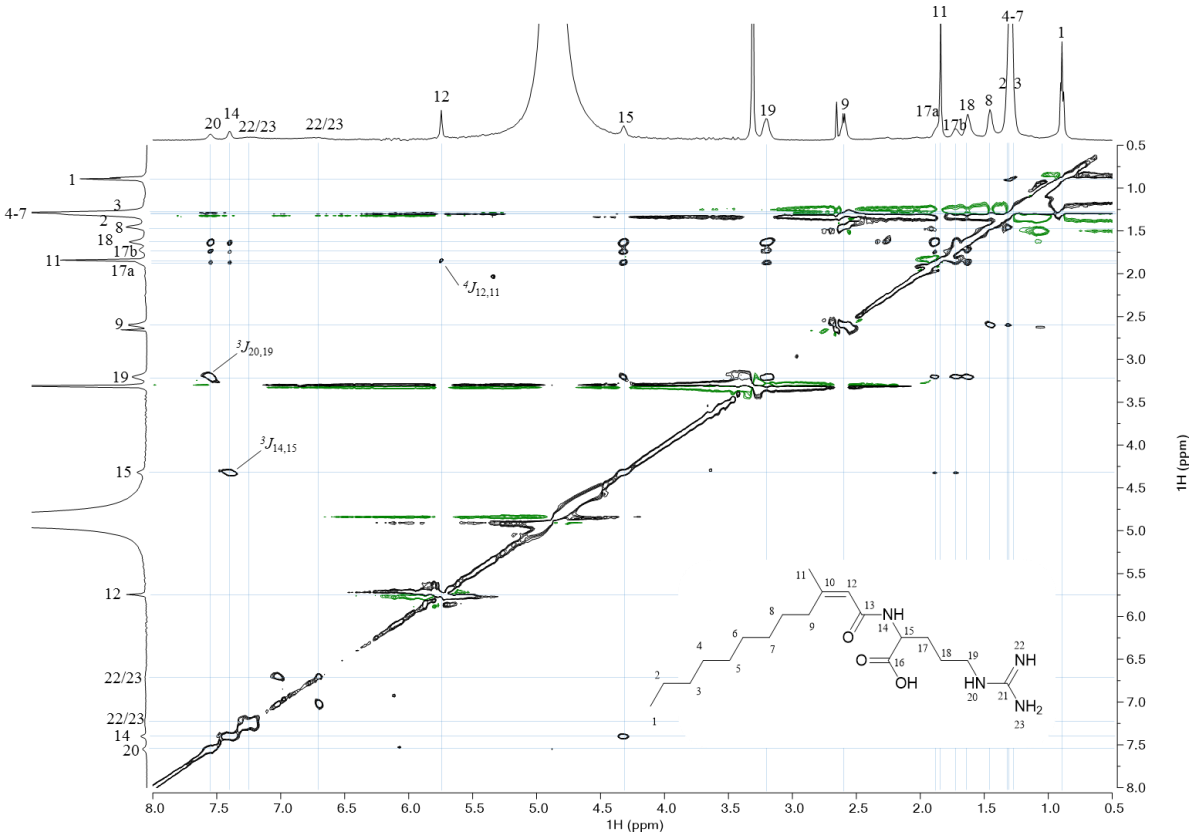


Figure S10. NOESY (600 MHz, CD_3OD) spectrum of Dendrobeaniamine A (**1**)

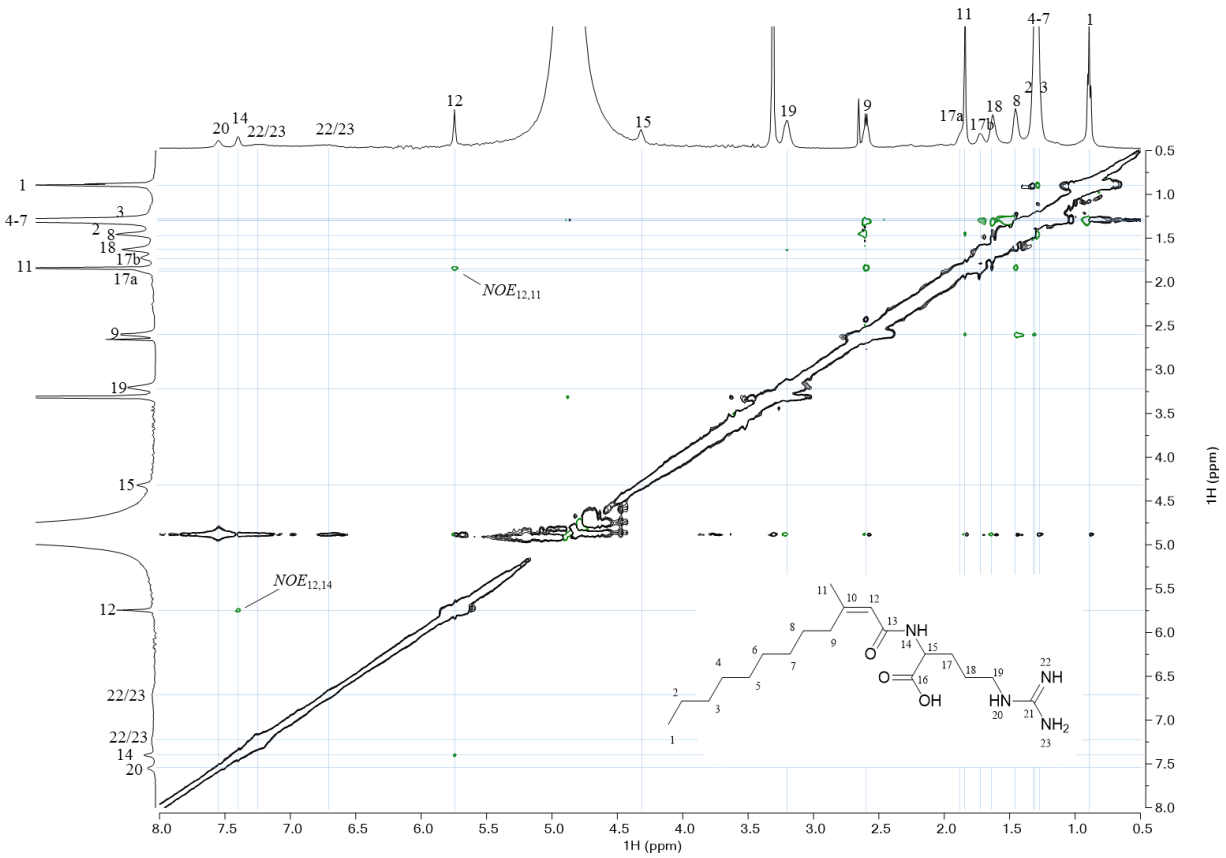


Figure S11. CLIP-sel HSQMBC (600 MHz, CD_3OD) spectrum of H-12 of Dendrobeaniamine A (**1**)

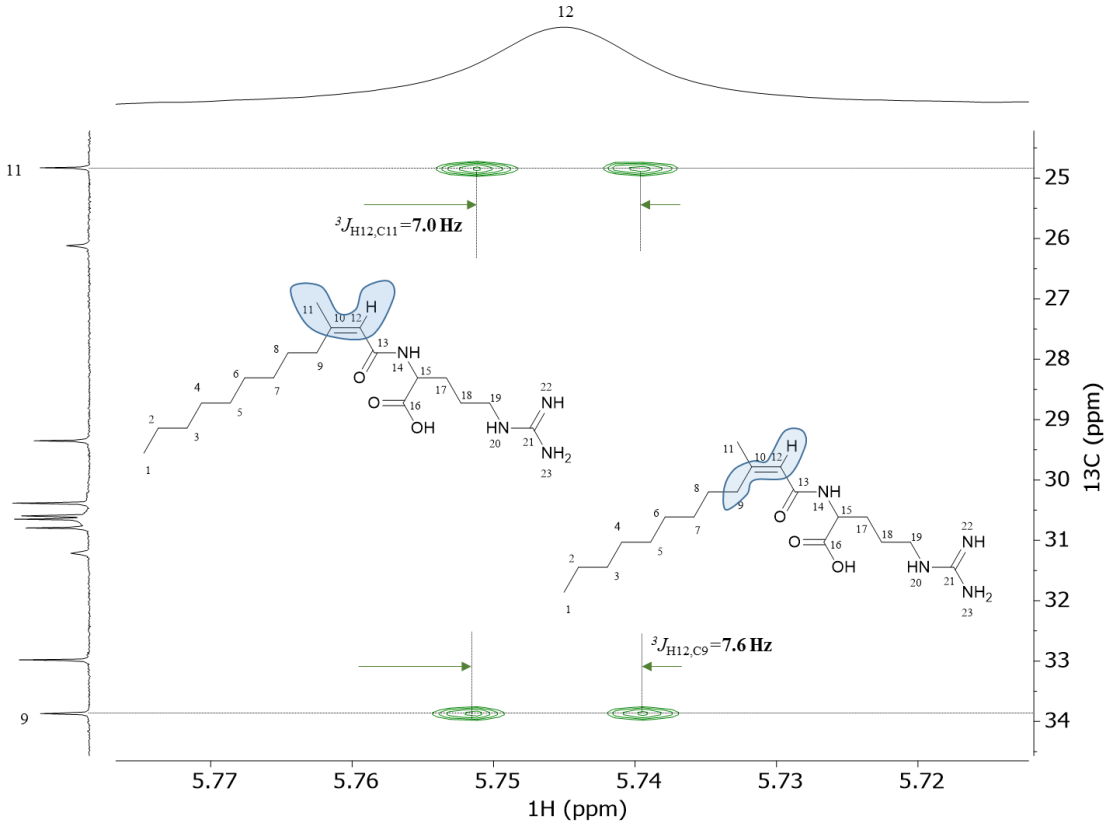


Figure S12. J-resolved SJS-BIRD spectrum (600 MHz, CD_3OD) (Sel. Inv: H-12) of Dendrobeaniamine A (**1**)

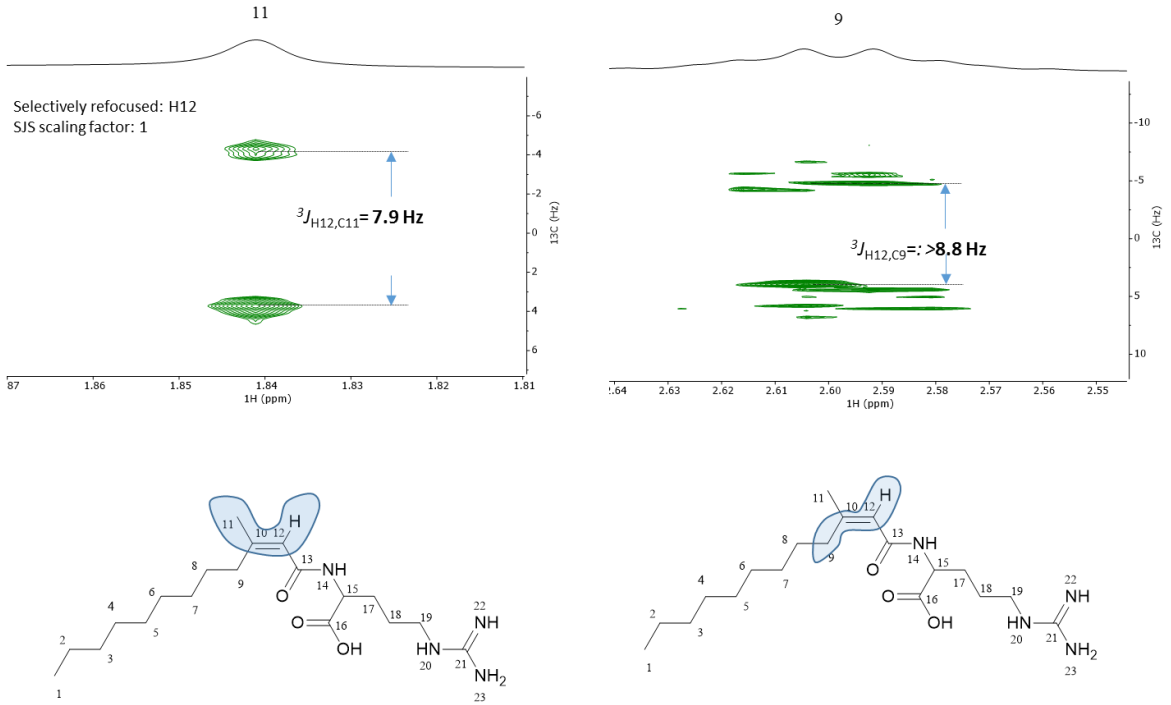


Figure S13. ^{15}N HSQC (600 MHz, CD_3OD) spectrum of Dendrobeaniamine A (**1**)

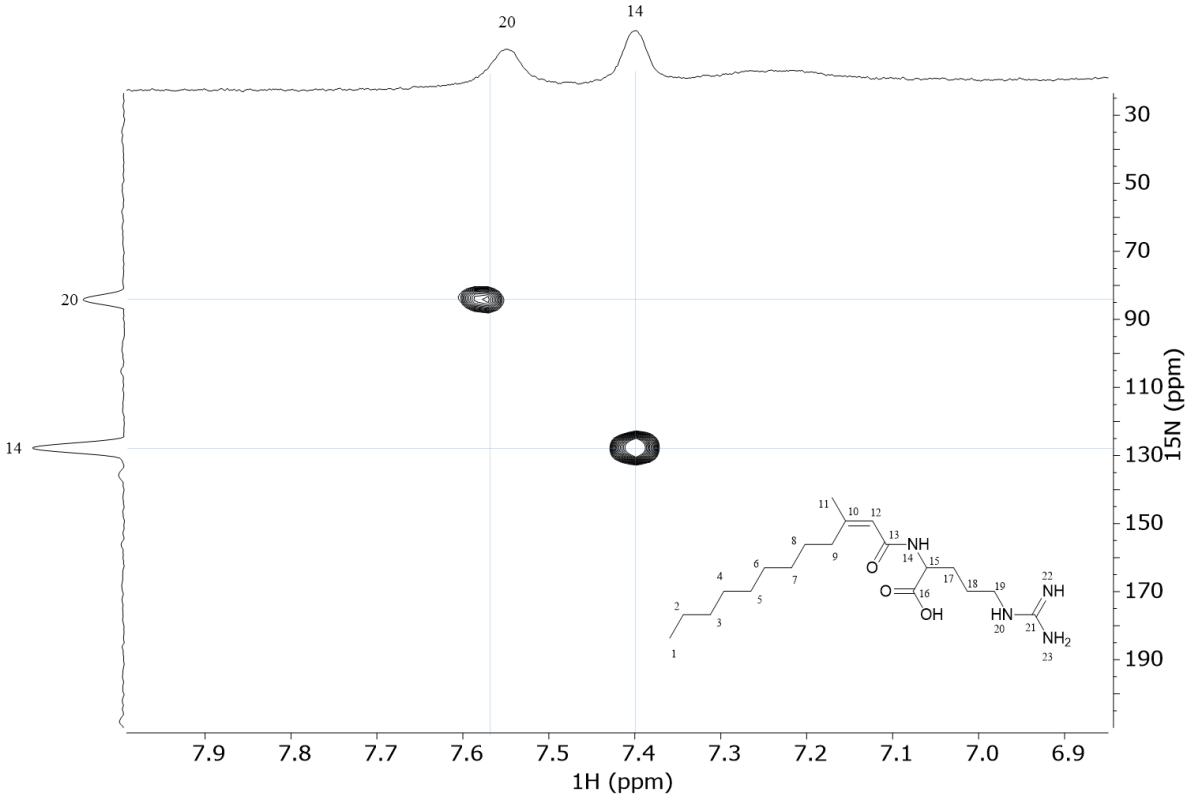


Figure S14. ^{15}N HMBC (600 MHz, CD_3OD) spectrum of Dendrobeaniamine A (**1**)

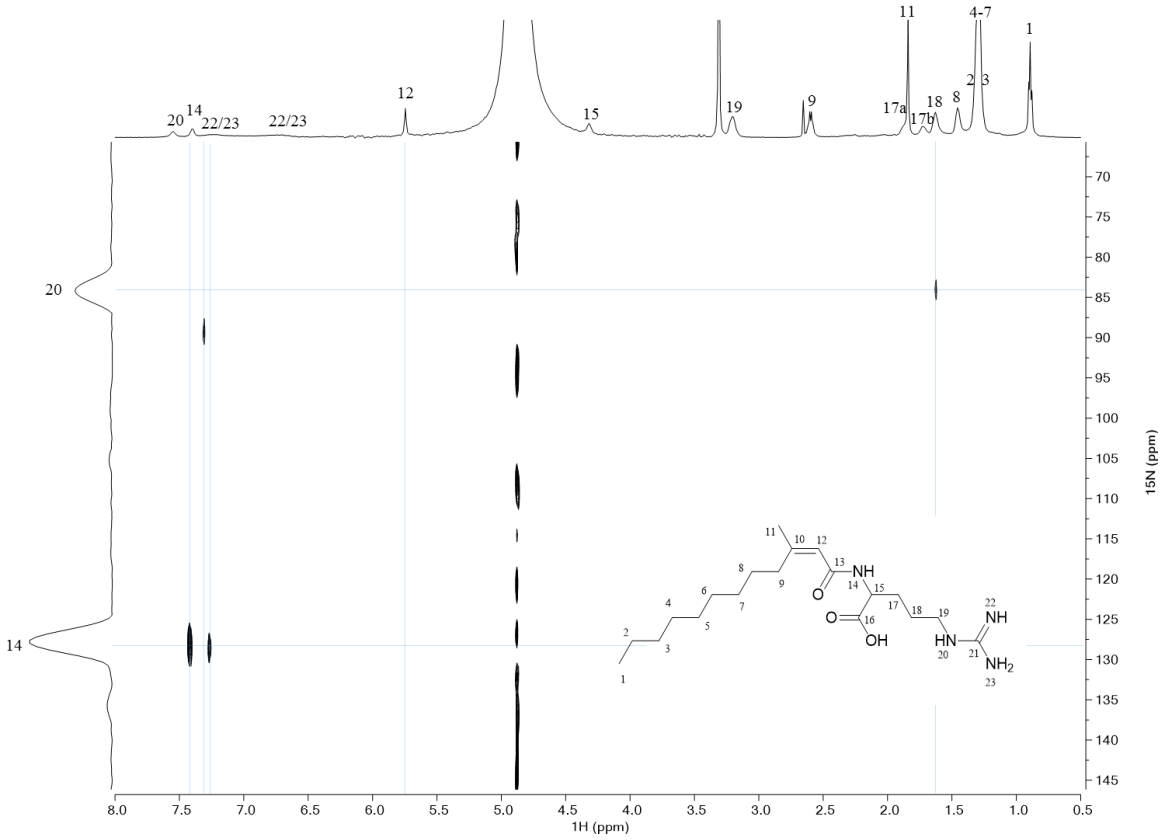


Figure S15. ^{15}N prediction vs experimental shifts of Dendrobeaniamine A (**1**)

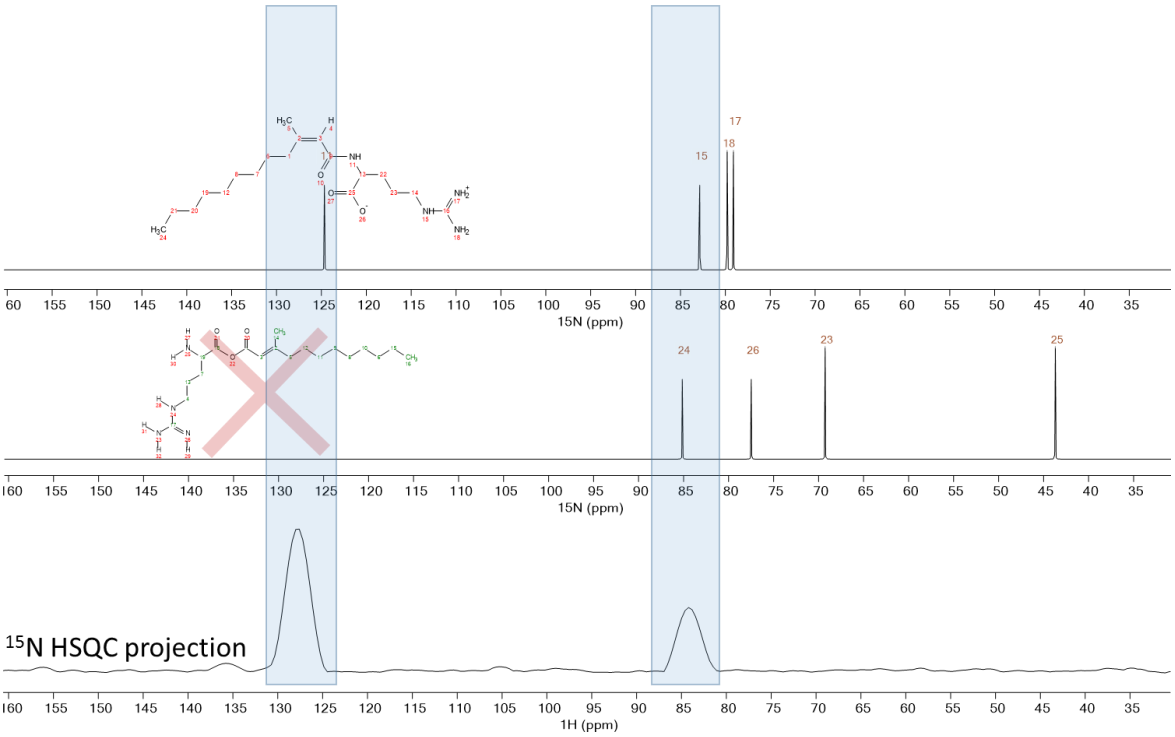
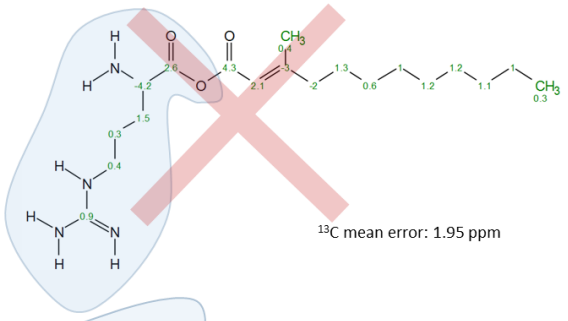
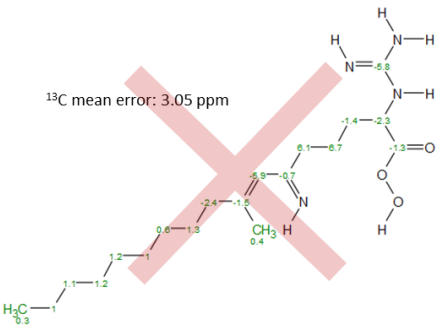
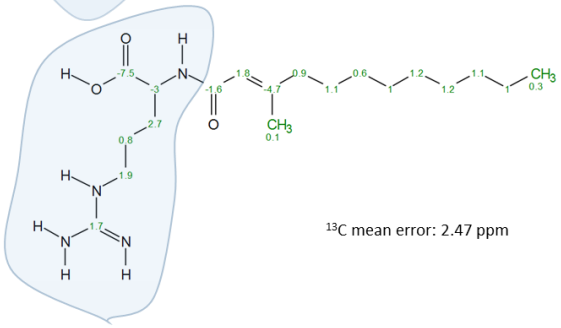
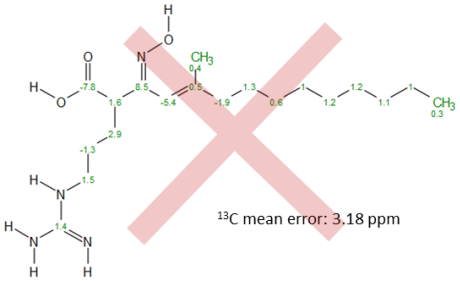


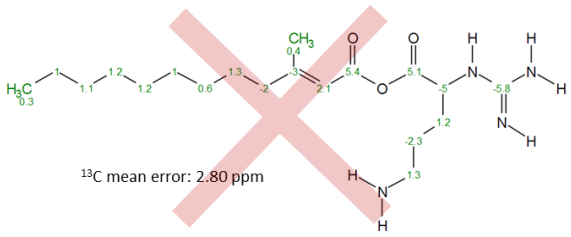
Figure S16. ^{13}C prediction vs experimental shifts of potentially possible structures of Dendrobeaniamine A (**1**)

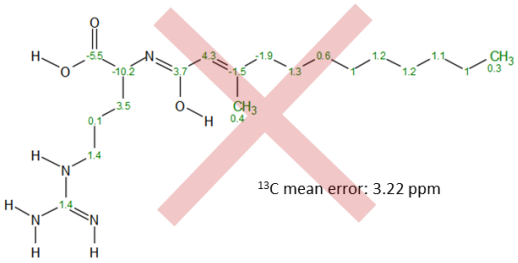












Reference:

Hansen KO, Isaksson J, Bayer A, Johansen JA, Andersen JH, Hansen E. 2017. Securamine derivatives from the Arctic bryozoan *Securiflustra securifrons*. J Nat Prod. 80(12):3276-3283.
Michael P, Hansen KO, Isaksson J, Andersen JH, Hansen E. 2017. A novel brominated alkaloid securidine A, isolated from the marine bryozoan *Securiflustra securifrons*. Molecules. 22(7).