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Supporting Information

Identification of Isonitrile-Containing Natural Products in Complex Biological Matrices through Ligation with Chlorooximes

Rebecca J. B. Schäfer, Kayla Wilson, Maurice Biedermann, Bradley S. Moore,* Simon Sieber,* and Helma Wennemers*

Table of content

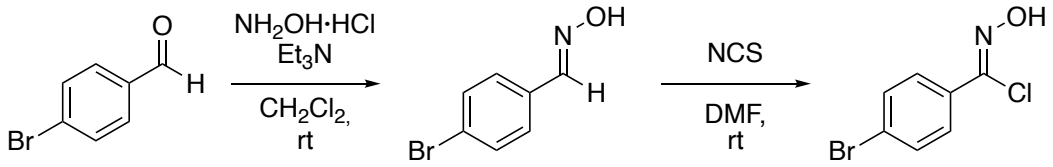
1. General aspects and materials

All chemicals were purchased from Fisher Scientific, Alfa Aesar, or MilliporeSigma and used without further purification. All solvents were of HPLC grade or higher. Preparative flash column chromatography was carried out on a Teledyne ISCO Combiflash® Rf+ Lumen™ system using liquid loading and silica gel (EMD, 40-63μm) for the stationary phase. Normal phase semi-preparative HPLC purification was carried out using a Shimadzu SCL-10A system and a Shimadzu SPD-M10A UV/Vis detector (Shimadzu Corp., Kyoto Japan). Preparative TLC purifications were carried out using TLC Silica gel 60 F254 glass plates (20x20cm, MilliporeSigma™ Supelco™).

NMR spectroscopic data were obtained either on a 500 MHz JEOL NMR spectrometer with a 3.0 mm probe, a 600 MHz Bruker NMR spectrometer with a 1.7 mm cryoprobe, or a 500 MHz Varian NMR spectrometer with a 5 mm, ^{13}C optimized cold probe. The values of the chemical shifts are listed in ppm and coupling constants are reported in Hz. NMR chemical shifts were referenced to the residual solvent peaks (d_H 7.26 and d_C 77.16).

High performance liquid chromatography high resolution mass spectrometry (HPLC-HRMS) analysis was conducted on an Agilent 6530 Accurate-Mass Q-TOF MS (MassHunter software, Agilent) equipped with a dual electrospray ionization (ESI) source and an Agilent 1260 LC system (ChemStation software, Agilent) with a diode array detector. Q-TOF MS settings and LC gradients are indicated in the respective experimental sections of the supplementary information.

2. Synthesis and analytical data of probe 1



S1 **S2** **1**

Scheme S1. Synthesis of chlorooxime probe **1**.

Oxime S2: 4-bromobenzaldehyde (**S1)** (1.0 g, 5.4 mmol, 1.0 equiv.) was dissolved in CH_2Cl_2 (30 mL). Hydroxylamine hydrochloride (751 mg, 10.8 mmol, 2.0 equiv.) was added, followed by NEt_3 (1.51 mL, 10.8 mmol, 2.0 equiv.). The reaction mixture was stirred at room temperature until complete consumption of starting material was observed by TLC. The crude reaction mixture was diluted with one reaction volume of water. Phases were separated and the aqueous phase was extracted three times with CH_2Cl_2. The combined organic extracts were washed with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude product was purified by automated normal phase column chromatography (24 g cartridge, 0-45% EtOAc over 15 min). Oxime **S2** was obtained in 90% yield (980 mg, 4.9 mmol) as a white powder. The analytical data correspond to the reported values in the literature.[1]

^{1}H-NMR (500 MHz, $CDCl_3$) δ [ppm] = 8.09 (s, 1H), 7.91 (s, 1H), 7.54–7.50 (m, 2H), 7.45–7.43 (m, 2H). **^{13}C-NMR (121 MHz, $CDCl_3$) δ [ppm]** = 149.6, 132.2, 130.9, 128.6, 124.5.

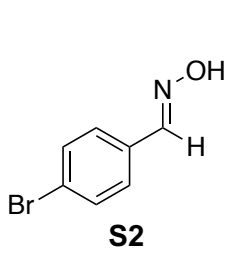
TLC: Hex:EtOAc (4:1), R_F = 0.5 and 0.25 (*E* and *Z* isomers, respectively)

Chlorooxime 1: 4-bromobenzaldoxime (**S2**) (400 mg, 2.0 mmol, 1.0 equiv.) was dissolved in DMF (1.5 mL). *N*-chlorosuccinimide (280 mg, 2.1 mmol, 1.05 equiv.) was added in small portions over 2 min at room temperature. The reaction mixture was stirred at room temperature until complete consumption of starting material was observed by TLC. The crude reaction mixture was diluted with EtOAc (6 mL) and H_2O (8 mL). Phases were separated and the aqueous phase was extracted three times with EtOAc (3 mL). The combined organic extracts were then washed five times with 5% $LiCl_{aq}$ solution (10 mL each). The organic phase was washed with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure to complete dryness. Product **1** was obtained as a white crystalline powder in 89% yield (420 mg, 1.79 mmol). The analytical data correspond to the reported values in the literature.[2]

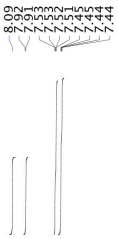
^{1}H-NMR (500 MHz, $CDCl_3$) δ [ppm] = 8.04 (s, 1H), 7.74–7.69 (m, 2H), 7.56-7.52 (m, 2H).
^{13}C-NMR (121 MHz, $CDCl_3$) δ [ppm] = 139.5, 131.9, 131.4, 128.8, 125.5.

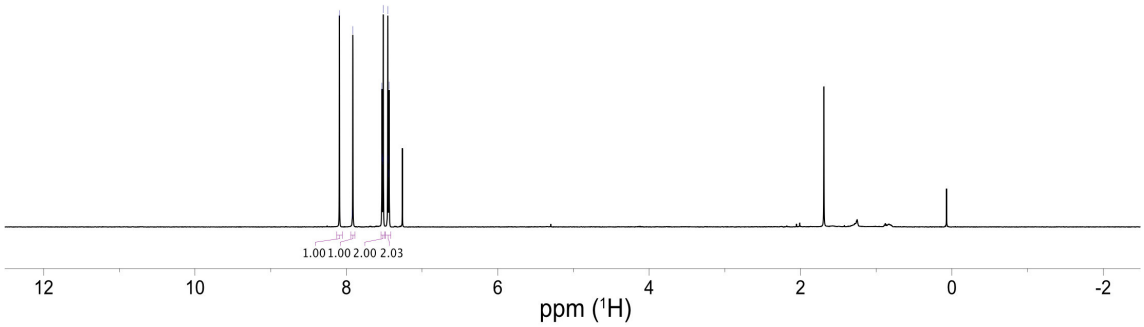
TLC: Hex:EtOAc (4:1) R_F = 0.71

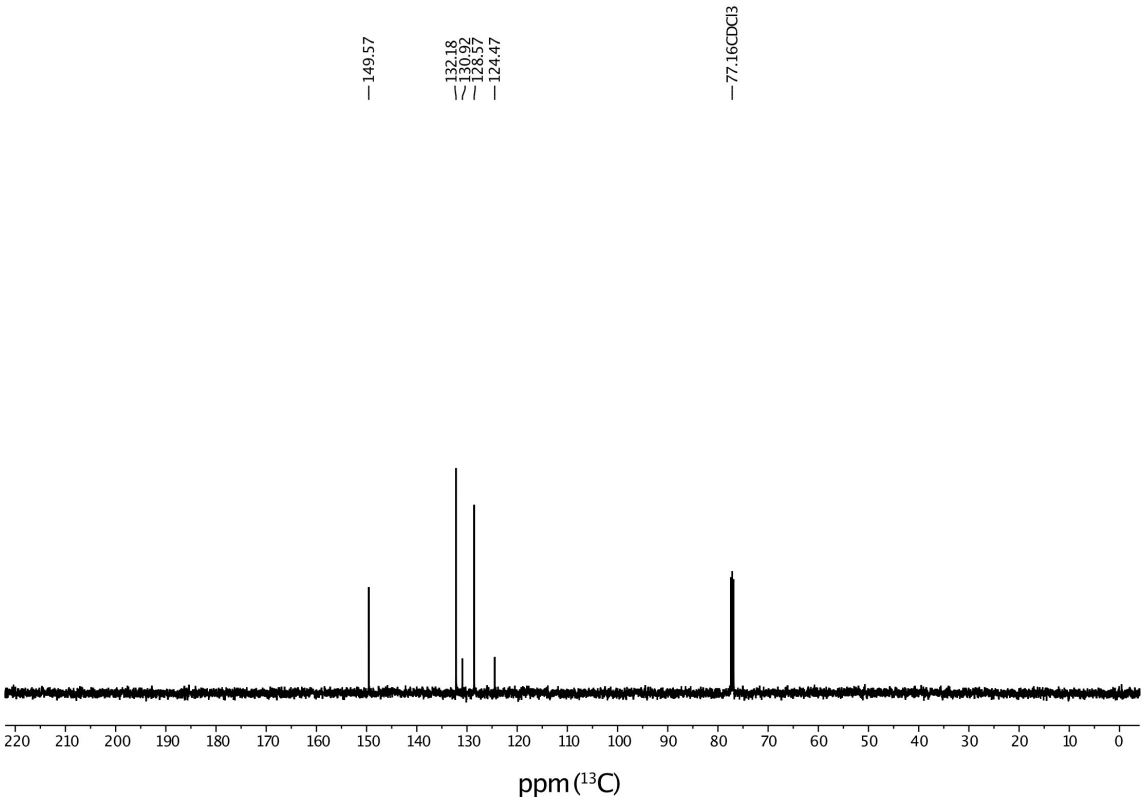
^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 121 MHz) spectra of oxime S2



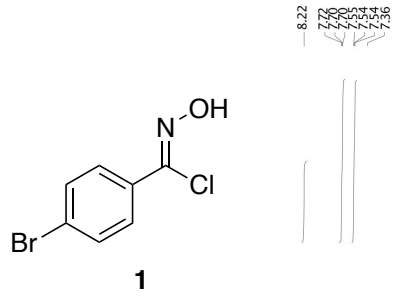
S2



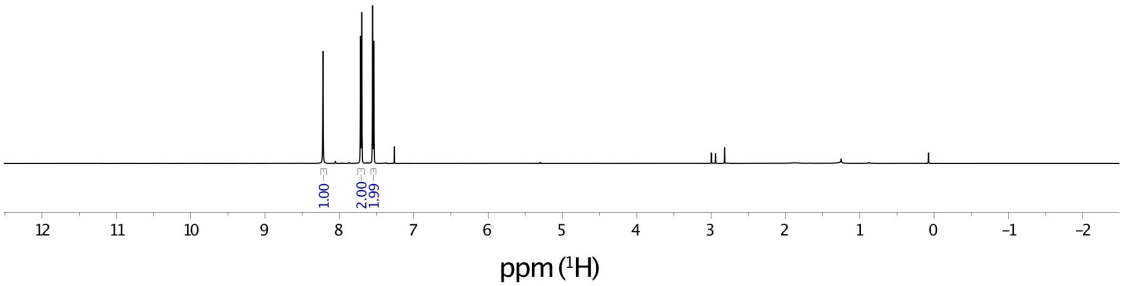


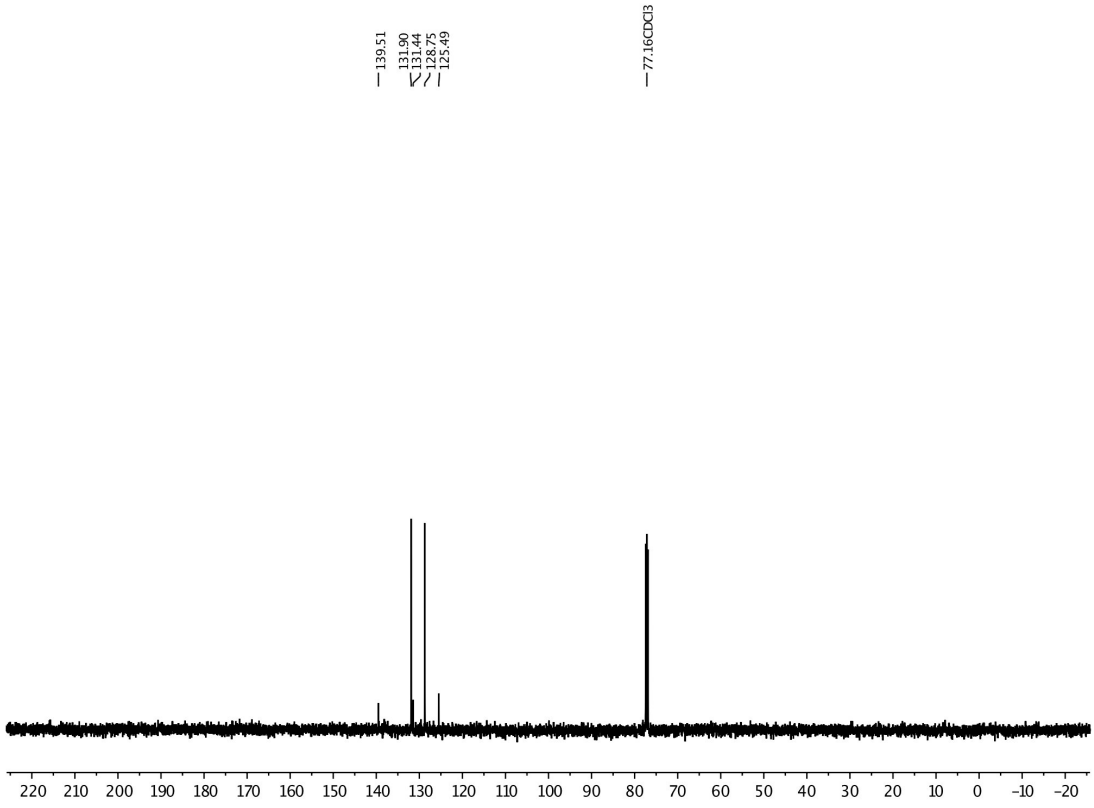


H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 121 MHz) spectra of probe 1



1





3. Isonitrile natural product detection in cyanobacterium *Fischerella ambigua* UTEX 1903

Cultivation and extraction of F. ambigua

The cyanobacteria strain *Fischerella ambigua* UTEX 1903 was cultivated using a modified BG11 medium (without addition of $NaNO_3$ salt) for approximately 8 weeks. The culture (20 mL) was centrifuged (30 min, 4500 g) and the supernatant separated from the biomass. The biomass was extracted with MeOH (50 mL), the mixture was sonicated (3 x 3 min), centrifuged (30 min, 4500 g), the solvent was removed by decantation, and the process was repeated twice. The methanol extracts were combined, and the solvent was removed at a rotary evaporator at 40 °C. The crude extract (70 mg) was dissolved in MeOH (1 mL), the mixture was centrifuged (10 min, 10000 g), and the solution was analyzed by UHPLC-MS (1 µL).

Labeling protocol of cyanobacterial extract

All reactions were performed under stirring conditions in HPLC vials. In the first reaction, the chemoselective probe **1** (10 µL in THF, conc.: 1 mg/100 µL) was mixed with the cyanobacteria crude extract (40 µL, 70 mg/mL), and H_2O (50 µL) was added. The second reaction (control 1) contained only probe **1** (10 µL in THF, conc.: 1 mg/100 µL) and was mixed with THF (40 µL) and H_2O (50 µL). A second negative control (control 2) contained only the cyanobacteria crude extract (40 µL, 70 mg/mL) with THF (10 µL) and H_2O (50 µL). All reactions were stirred for 20 min at room temperature. The solvent was removed under a gentle nitrogen stream, and the residue was dissolved in MeOH (50 µL) and centrifuged (5 min, 10000 g). The samples were analyzed by UHPLC/HRMS. All reactions were performed in triplicates.

UHPLC-MS, HR-ESI-MS and HPLC analysis

HR-ESI-MS data were collected on an Ultimate 3000 UHPLC instrument (Thermo Fisher Scientific, Switzerland) coupled to a QExactive instrument (Thermo Fisher Scientific, Switzerland). A BEH C_{18} column (150 x 2.1 mm, 1.7 µm, Phenomenex, USA) was used for the separation, the column oven was kept at 40 °C, the flow was set to 0.4 mL/min, and the volume of injection was 1 µL. The solvent system was composed of A (H_2O + 0.1 % FA), B (MeCN + 0.1 % FA) and the gradient starting from 3 % B. The gradient increased to 100% B in 12 min and the column was washed for 3 min.

MS Spectra and Extracted Ion Chromatograms

The following LCMS traces are extracted ion chromatograms (EICs) for either the natural product mass or the natural product probe conjugate mass of isonitriles from *Fischerella ambigua* UTEX 1903 followed by the corresponding HRMS spectrum of the highlighted signal.[3] The boxes highlight the chemical structure and the corresponding EIC in each figure. To ensure that the detected masses derive from the conjugated products, traces of the control reactions were also extracted for the same mass. The absence of any signal in the respective control reaction corroborates ligation of probe **1** with the isonitrile natural product.

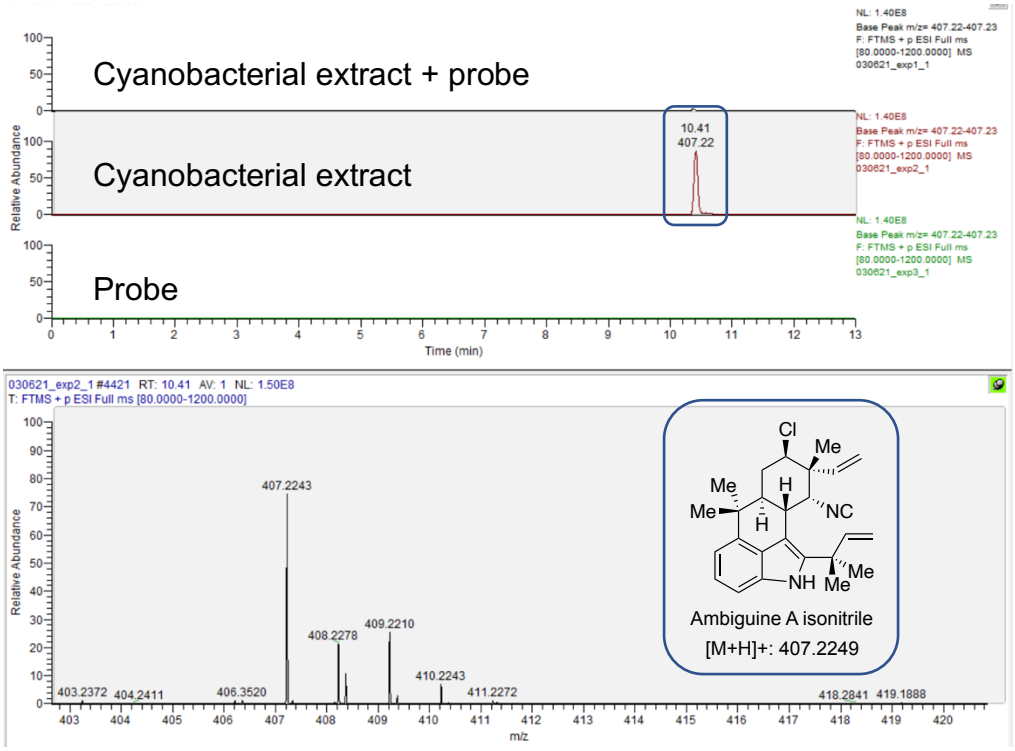


Figure S1a. Ambiguine A isonitrile (EIC m/z 407.22 – 407.23)

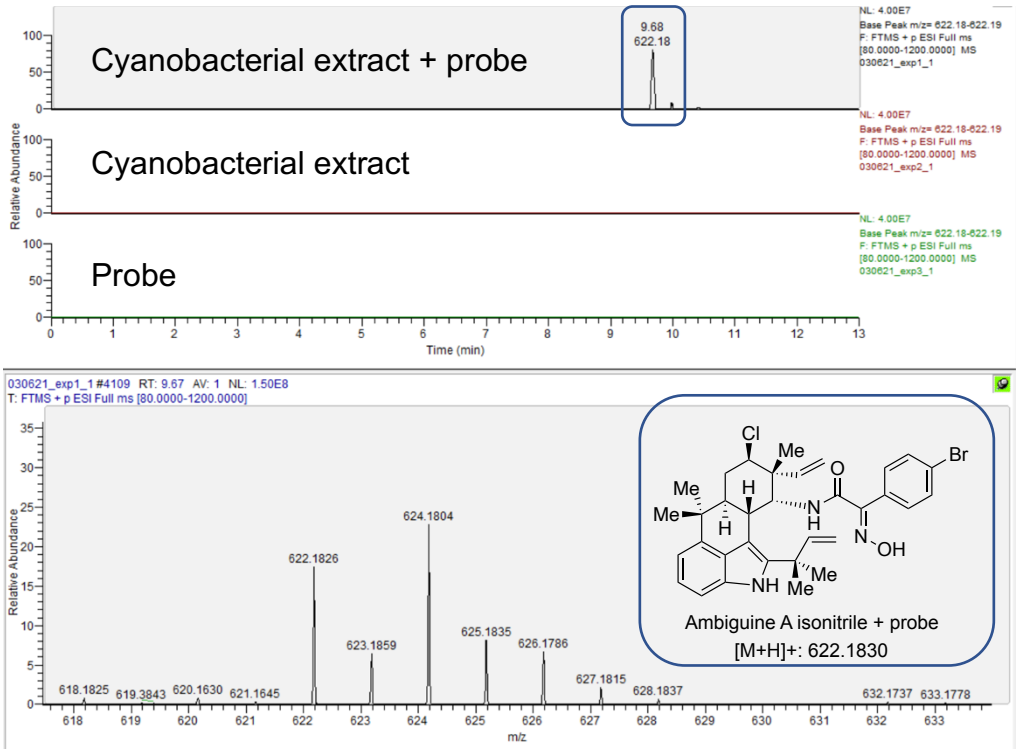


Figure S1b. Ambiguine A–probe **1** conjugate (EIC m/z 622.18 – 622.19)

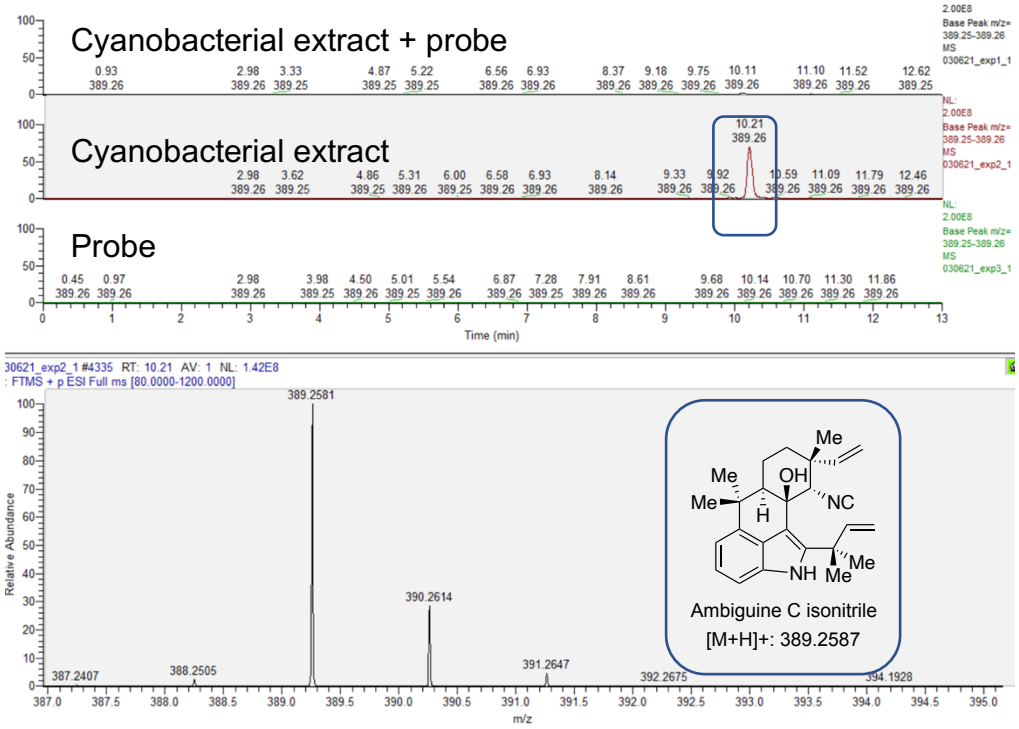


Figure S2a. Ambiguine C (EIC m/z 389.25 – 389.26)

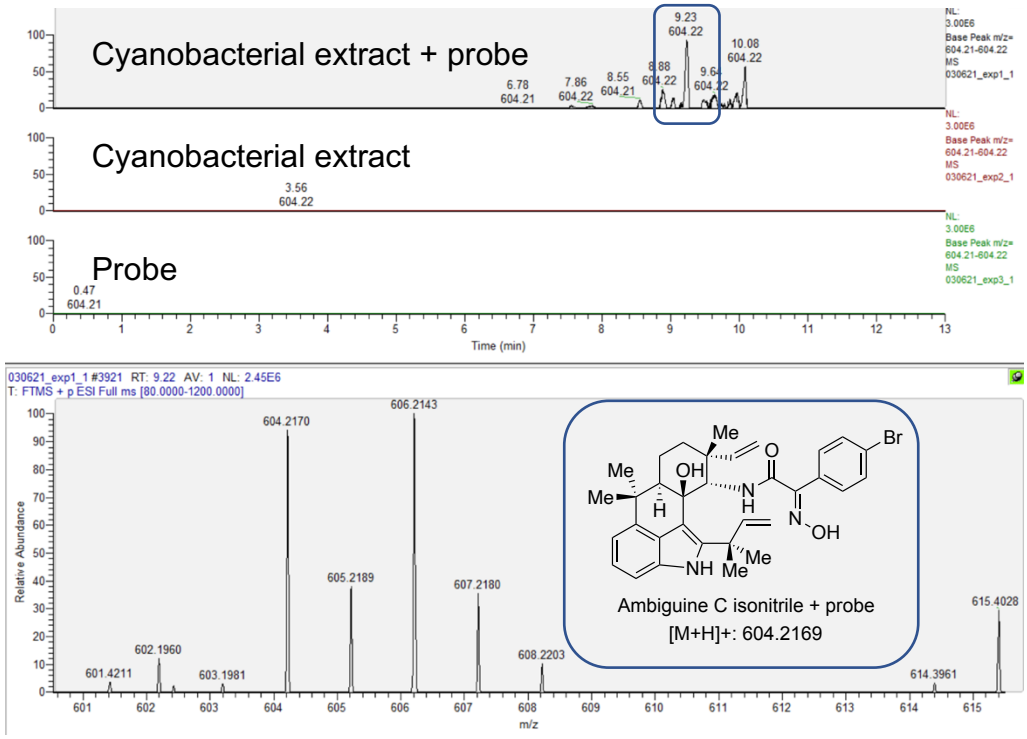


Figure S2b. Ambiguine C–probe **1** conjugate (EIC m/z 604.21 – 604.22)

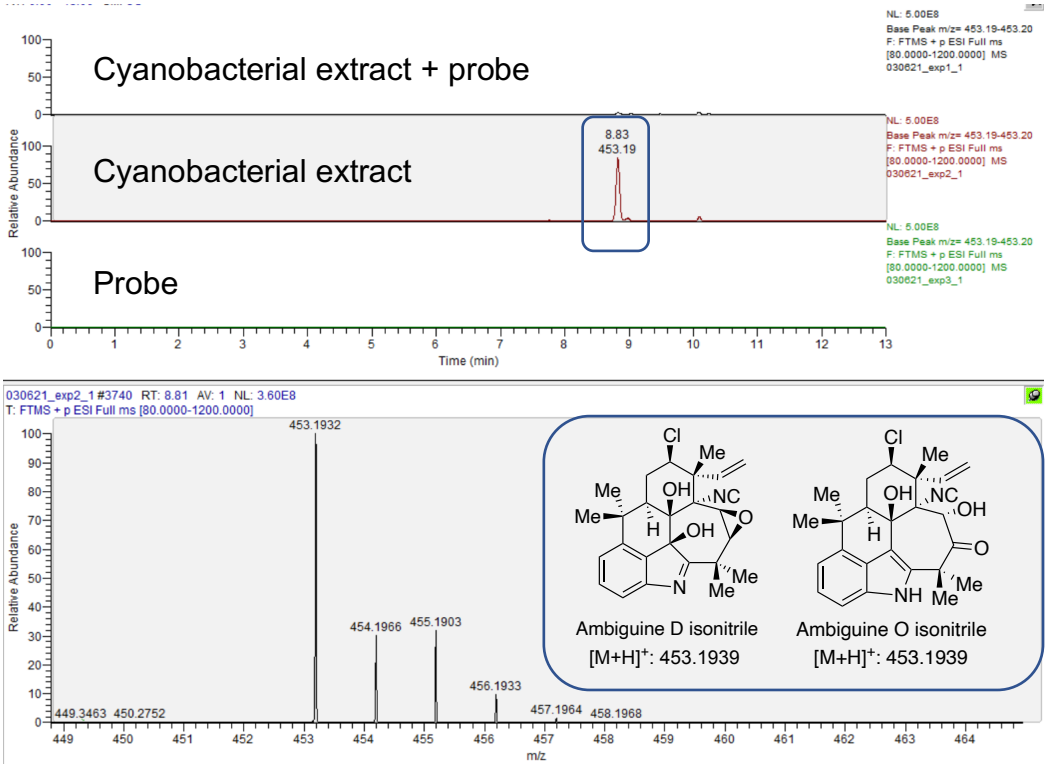


Figure S3a. Ambiguine D or O (EIC m/z 453.19 – 453.20)

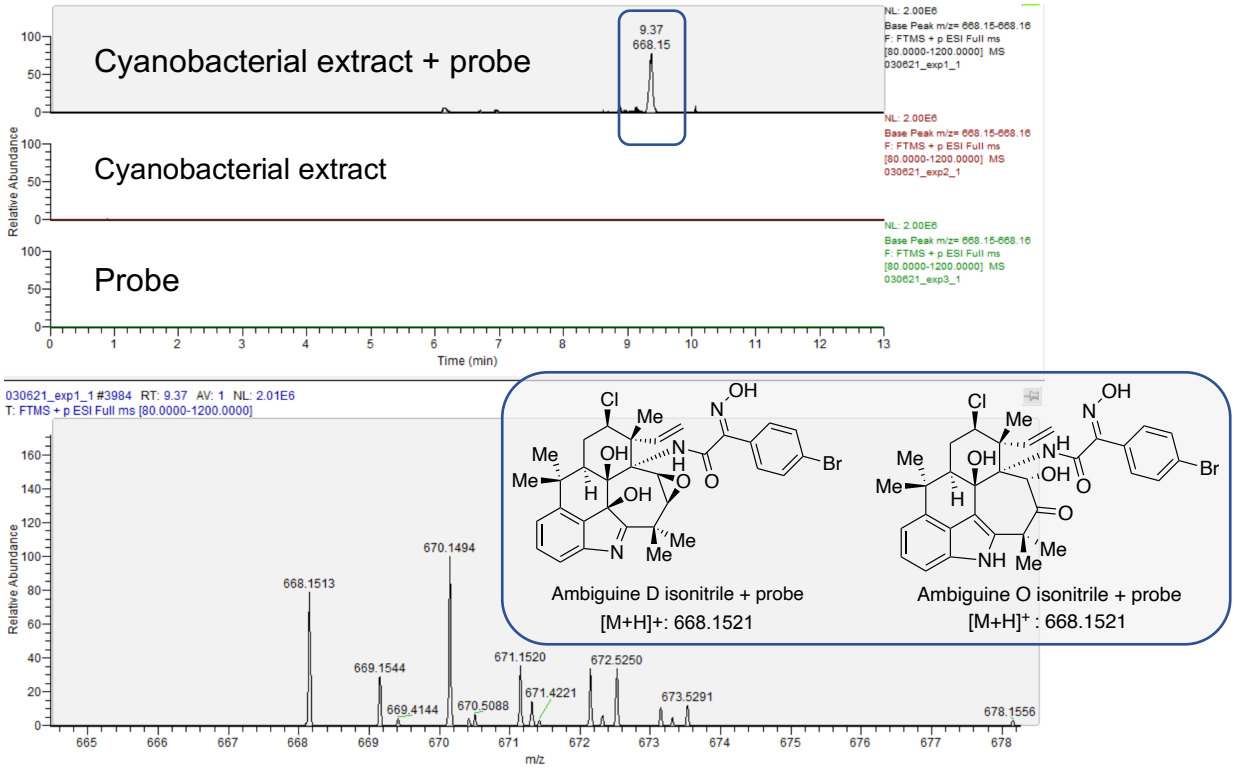


Figure S3b. Ambiguine D– or O– probe **1** conjugate (EIC m/z 668.15 – 668.16)

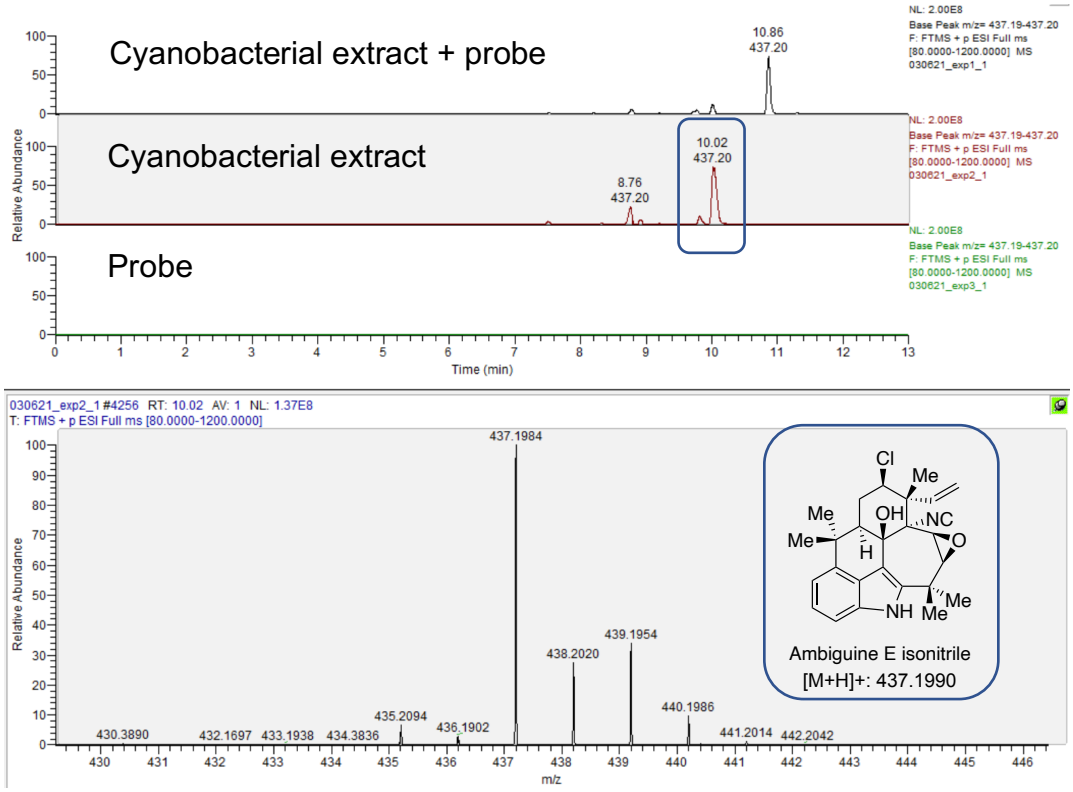


Figure S4a. Ambiguine E (EIC m/z 437.19 – 437.20)

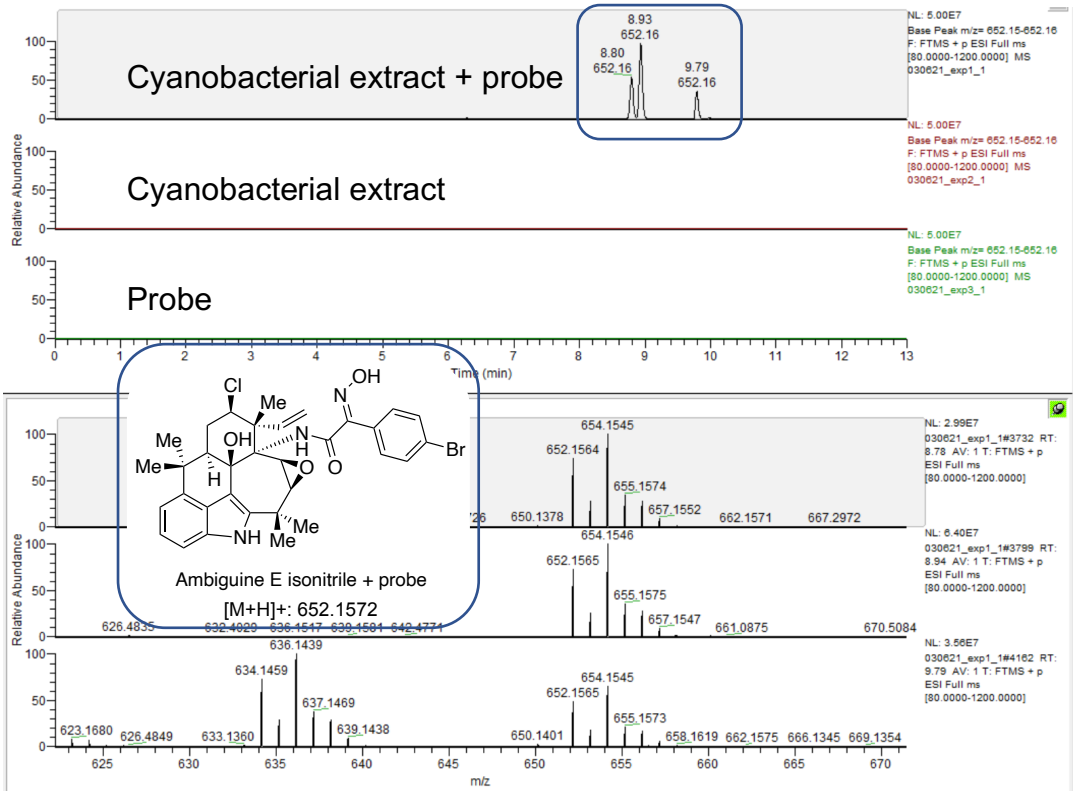


Figure S4b. Ambiguine E–probe **1** conjugate (EIC m/z 652.15 – 652.16)

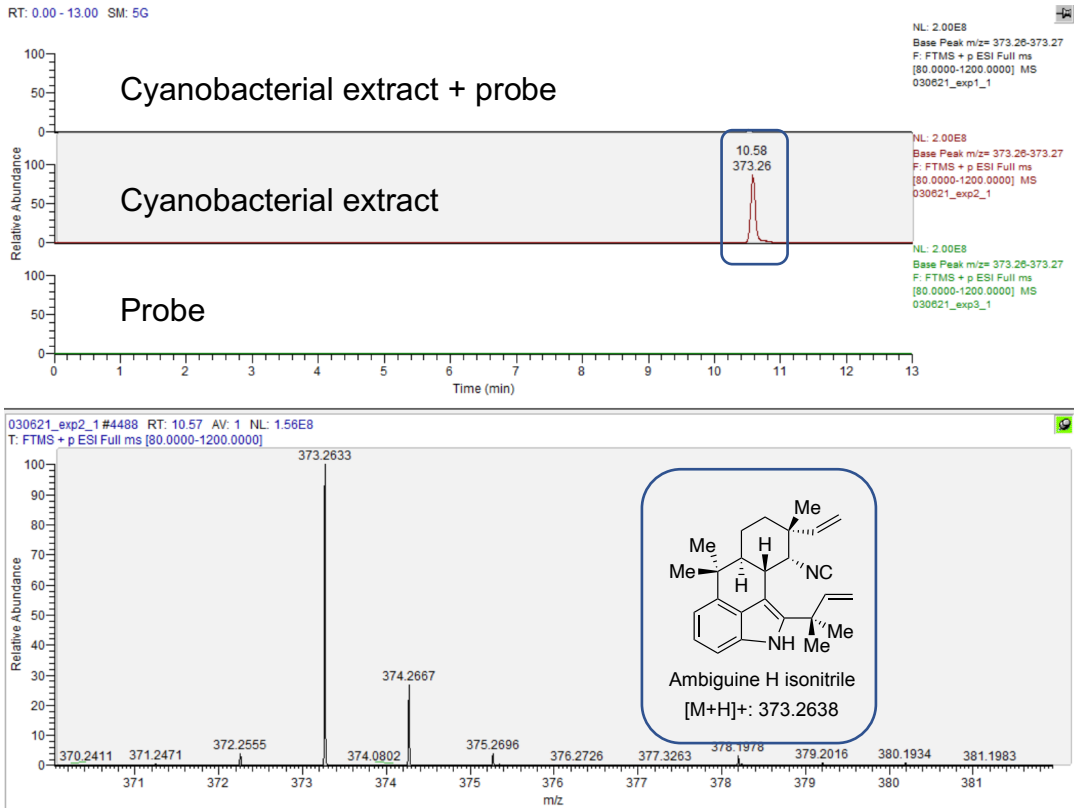


Figure S5a. **.**Ambiguine H (EIC m/z 373.26 – 373.27)

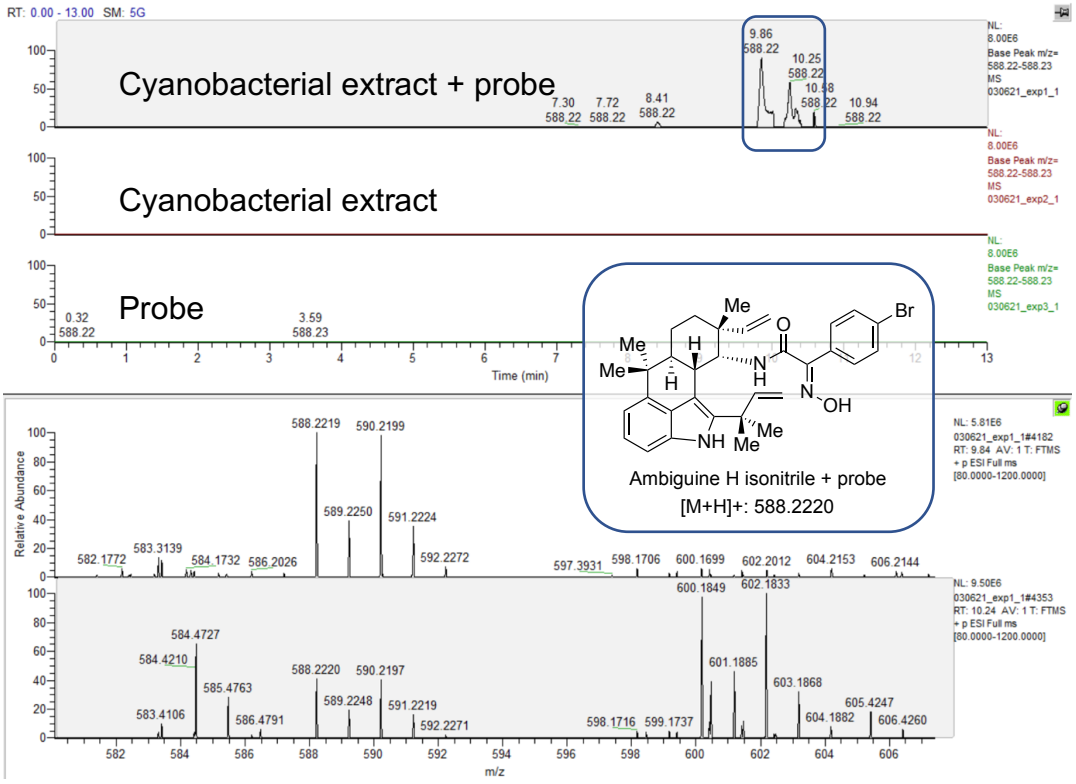


Figure S5b. Ambiguine H–probe **1** conjugate (EIC m/z 588.22 – 588.23)

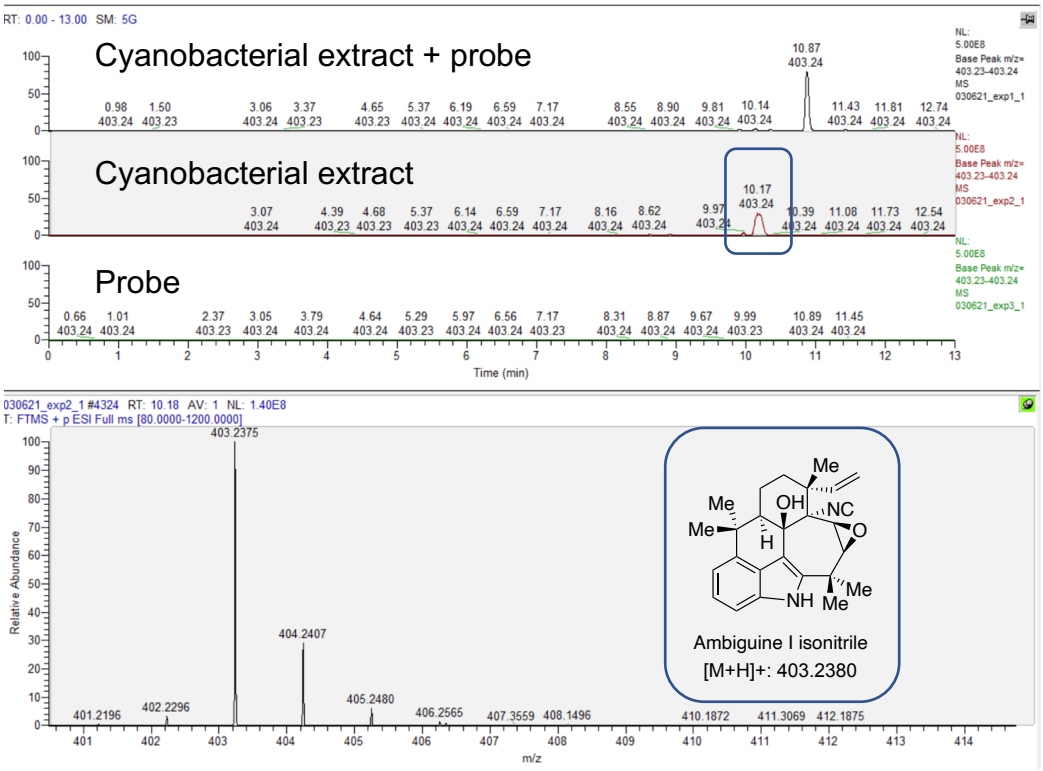


Figure S6a. Ambiguine I (EIC m/z 403.23 – 403.24)

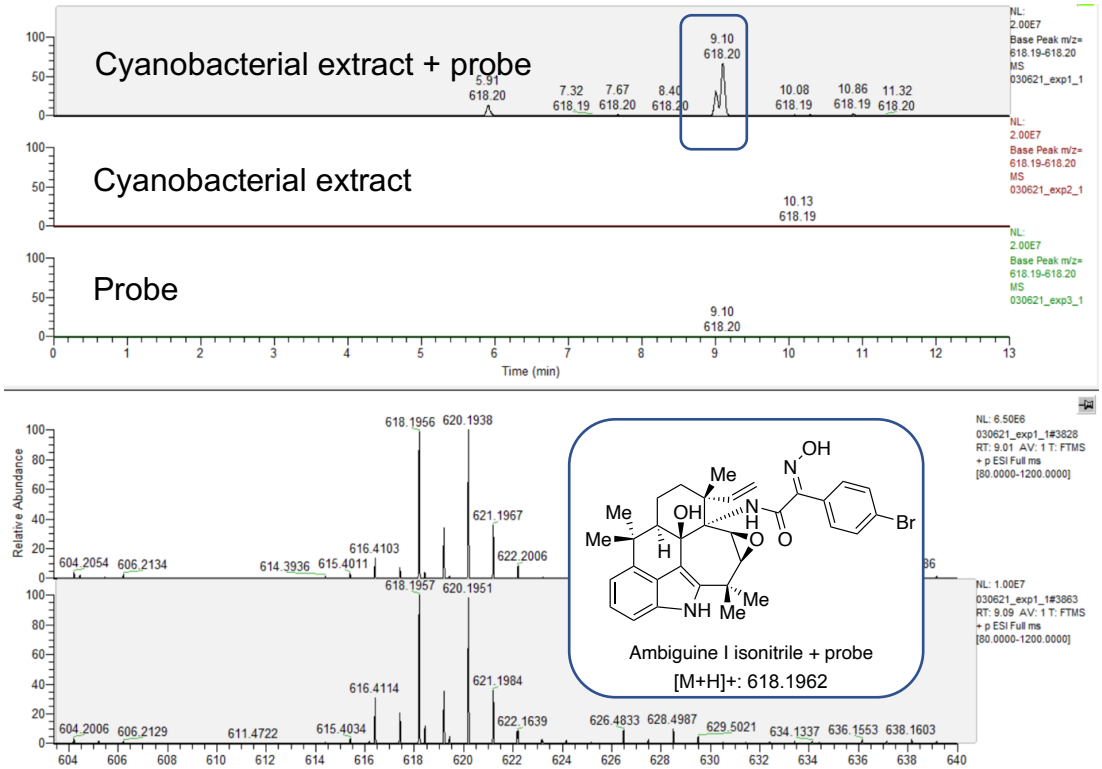


Figure S6b. Ambiguine I–probe **1** conjugate (EIC m/z 618.19 – 618.20)

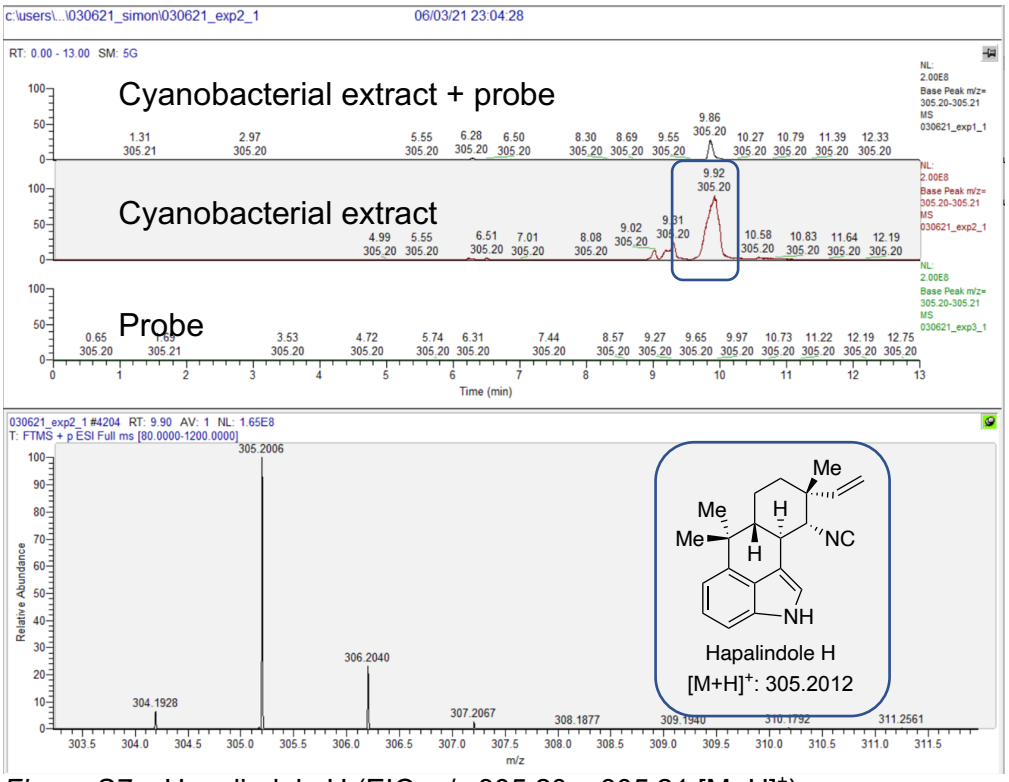


Figure S7a. Hapalindole H (EIC m/z 305.20 – 305.21 $[M+H]^+$)

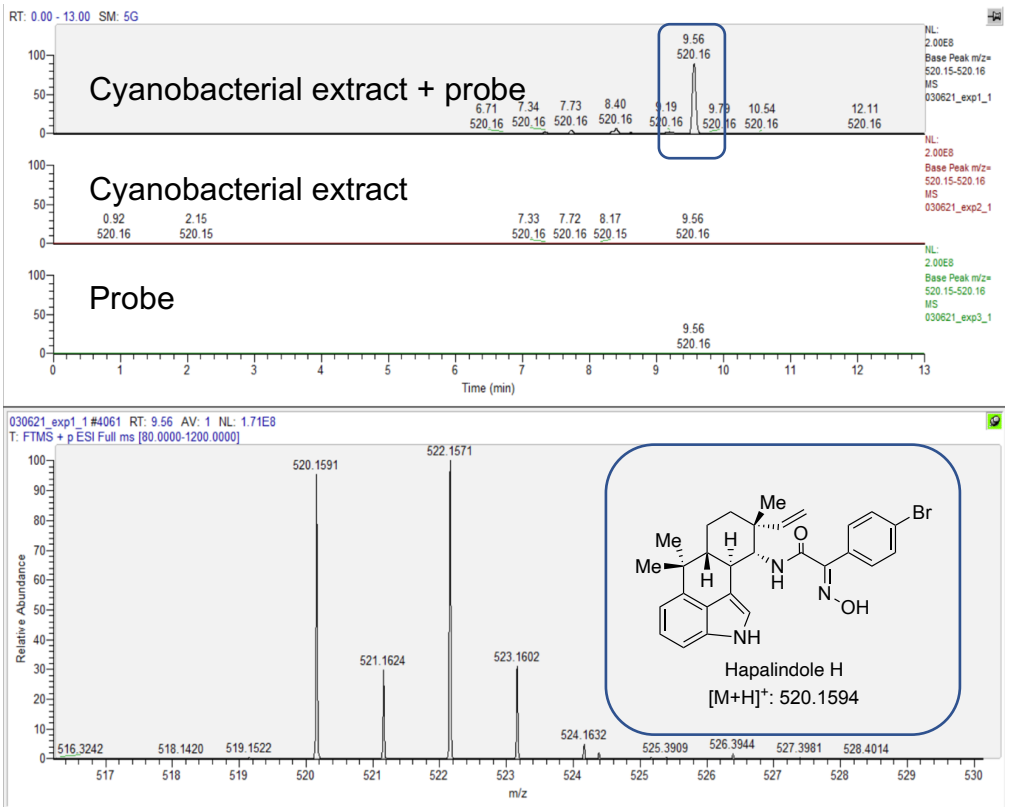


Figure S7b. Hapalindole H–probe **1** conjugate (EIC m/z 520.15 – 520.16)

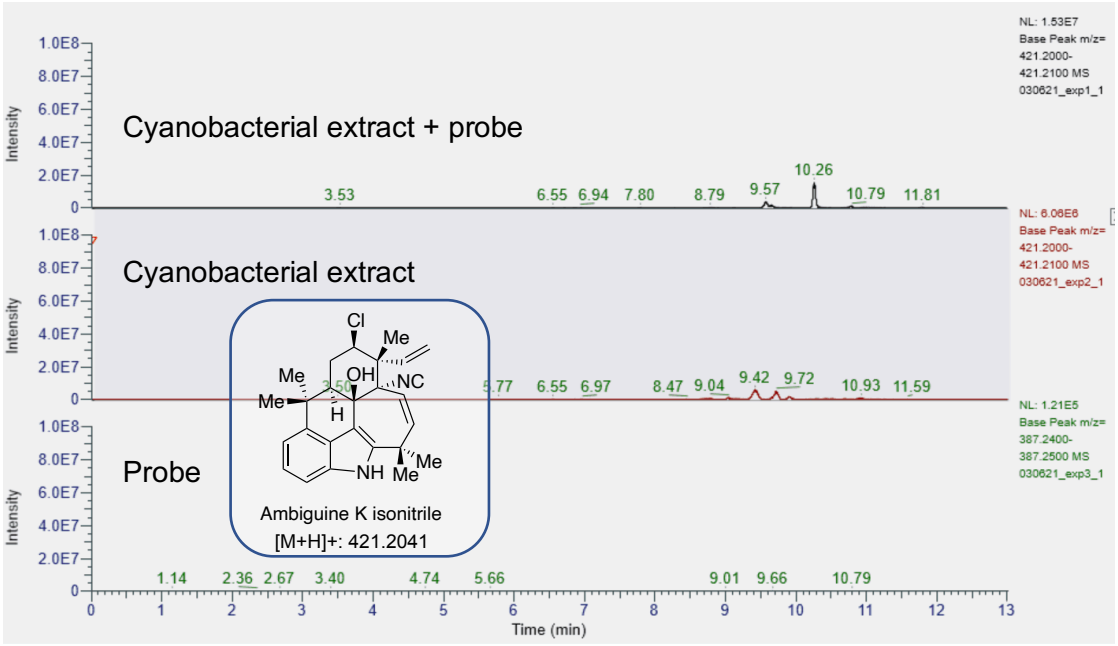


Figure S8a. Ambiguine K (EIC m/z 421.20 – 421.21)

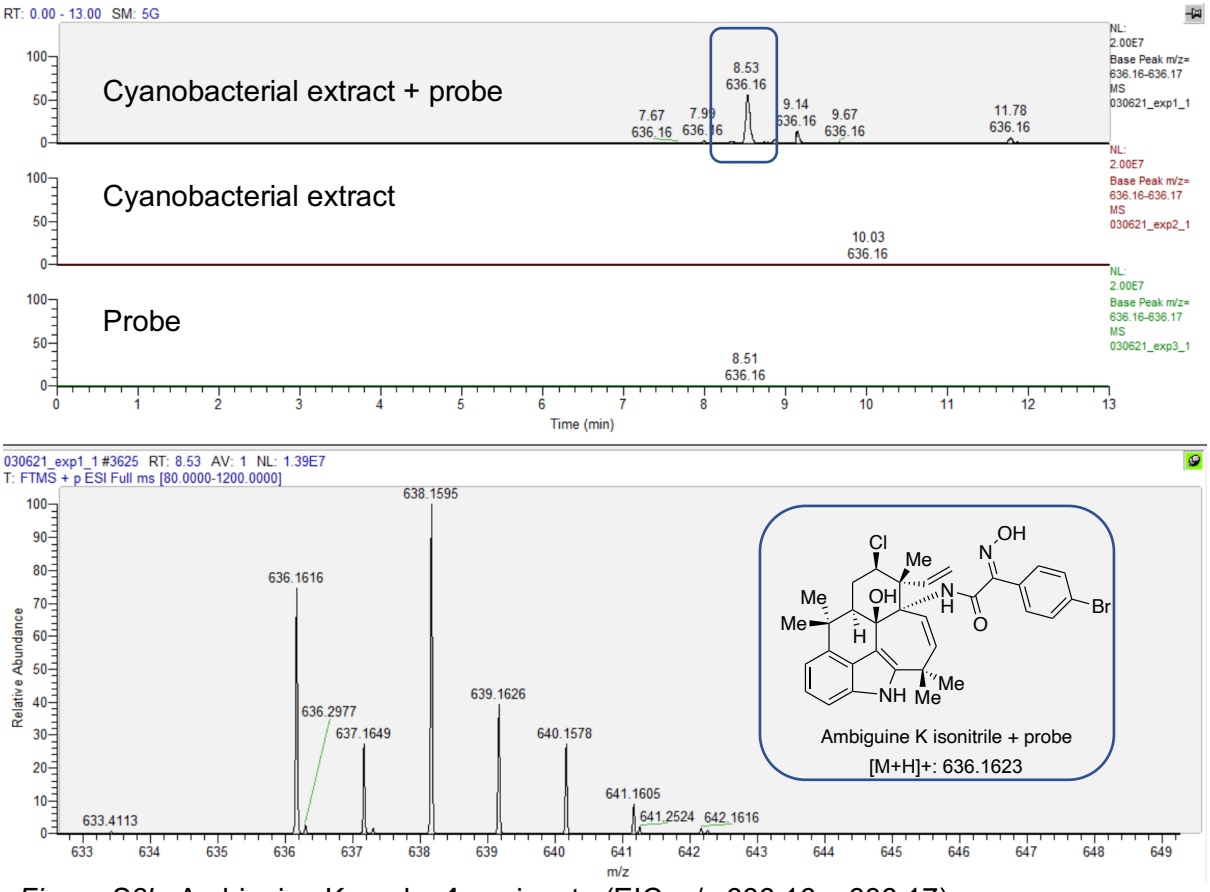


Figure S8b. Ambiguine K–probe **1** conjugate (EIC m/z 636.16 – 636.17)

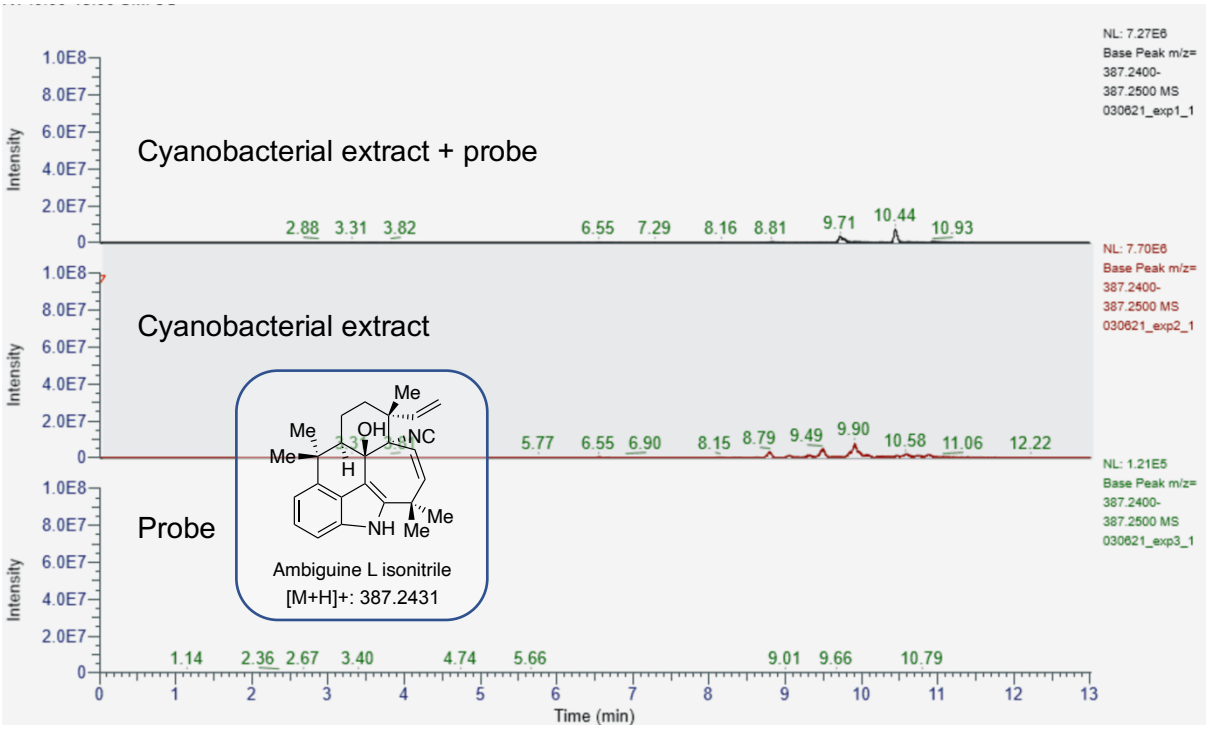


Figure S9a. Ambiguine L (EIC m/z 387.24 – 387.25)

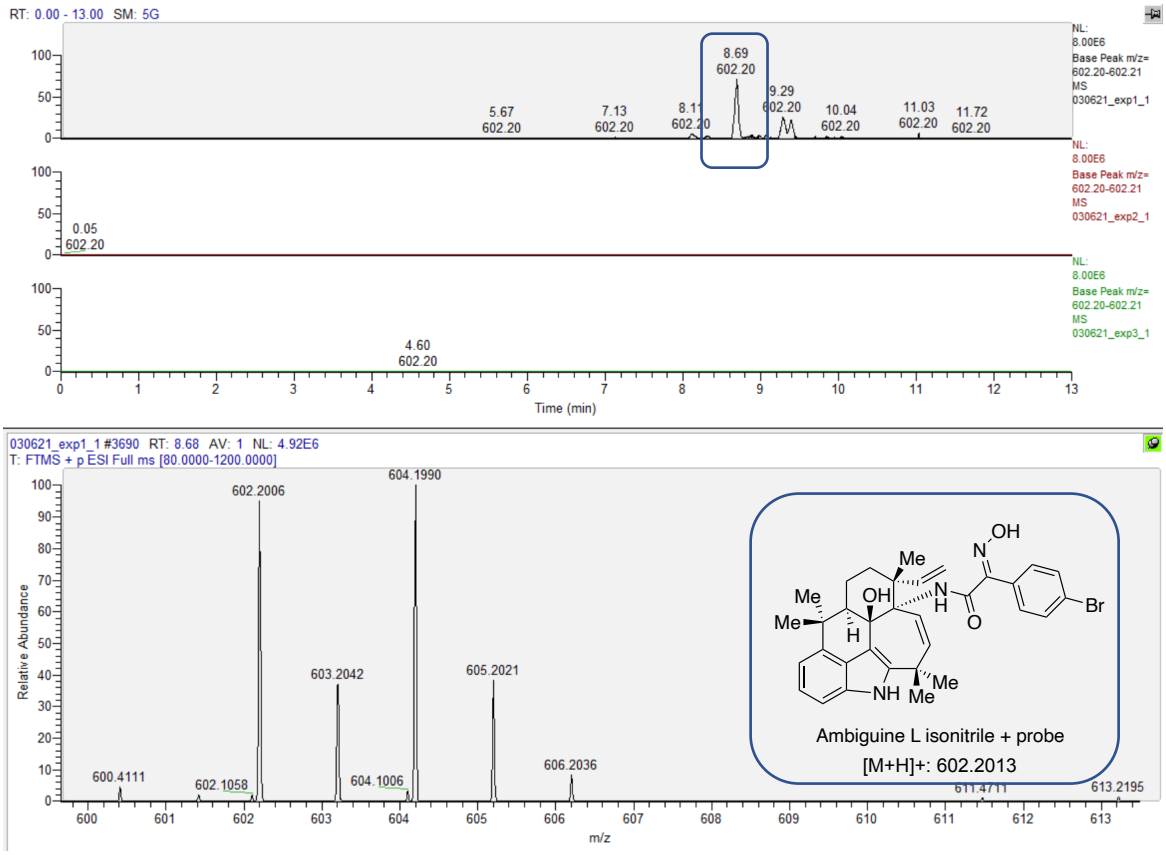


Figure S9b. Ambiguine L–probe **1** conjugate (EIC m/z 602.20 – 602.21)

4. Isonitrile natural product detection in marine sponge *Axinella sp.*

Sponge collection and extraction

Approximately 20 g (wet weight) of fresh *Axinella* sponge was collected by SCUBA from San Diego waters at approximately 15 meters depth in January 2019. The sponge was homogenized with liquid nitrogen and mortar and pestle. The resulting sponge powder was then extracted with CH_2Cl_2 (225 mL), vacuum filtered and carefully concentrated at 30 °C using a rotary evaporator to a final volume of 20 mL. The sponge extract was stored at –20°C.

GCMS analysis of sponge Axinella sp. extract

10 µL of the CH_2Cl_2 sponge extract was diluted in hexanes (40 µL) and 1 µL was analyzed on an Agilent 7890A gas chromatograph with Agilent 5975C mass spectrometer, using a HP-5MS 30 m × 0.25 mm, 0.25 µ column. Oven program was as follows: hold 70 °C for 3 min, 10 °C/min to 325 °C, hold at 325 °C for 3 min. Flow rate: 0.44 mL/min, Injection volume: 1 µL, Splitless injection, Inlet temperature: 200 °C, MS transfer line temperature: 250 °C, MS source temperature 230 °C, MS quad temperature: 150 °C

NIST mass spectral library (https://chemdata.nist.gov) was used as a resource to assist compound identification of the sponge extract by comparing our raw data with reference mass spectra and GC retention times deposited in the NIST mass database (Structures in Figure S10)

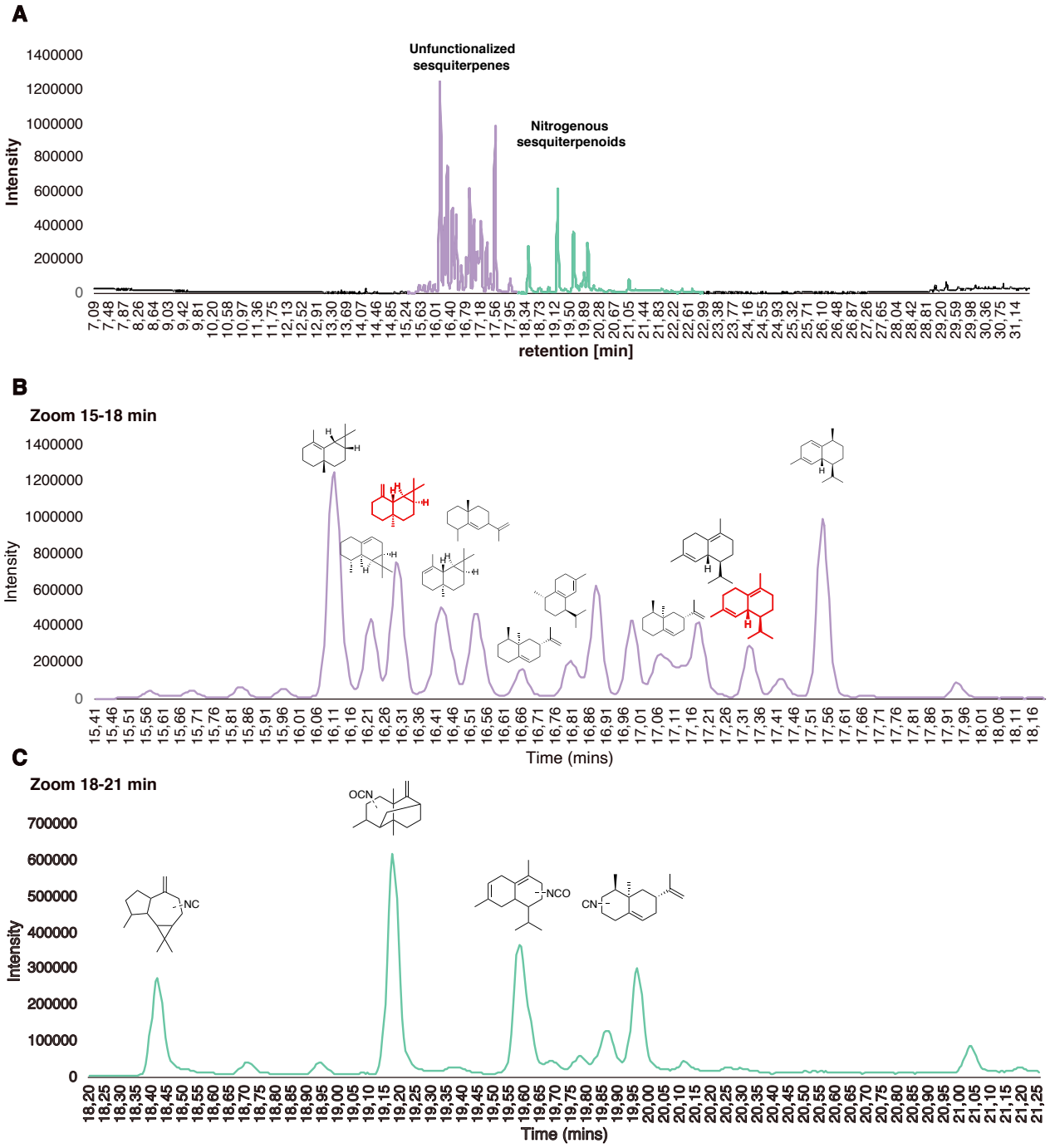


Figure S10. A) Full GC-MS chromatogram of the CH_2Cl_2-extract of *Axinella* sp. with the region of unfunctionalized sesquiterpenes highlighted in purple and nitrogenous sesquiterpenoids highlighted in green. B) Zoom of unfunctionalized sesquiterpene region (t = 15–18 min). Chemical structures are putative annotations based on NIST GCMS library hits. C) Zoom of nitrogenous sesquiterpene region (t = 18–21 min). Chemical structures are putative annotations based on NIST GCMS library hits.

Labeling protocol of sponge crude extract for MS analysis

Preparation of stock solutions:
Sponge extract: 100 µL of CH_2Cl_2 sponge extract was carefully concentrated to dryness under reduced pressure and redissolved in THF (40 µL).
Probe: 1 mg of **1** was dissolved in THF (100 µL).
Labeling reaction with extract: 20 µL of the sponge extract stock solution was transferred to an HPLC vial and diluted with THF (20 µL). Then citrate buffer (50 µL, 55 mM, pH 5) was added, followed by 10 µL of a stock solution of **1**. The reaction was left standing at room temperature for 1 h.
Control 1 – only sponge extract: For control 1, 20 µL of the sponge extract solution were transferred to an HPLC vial and diluted with THF (30 µL). Then citrate buffer (50 µL, 55 mM, pH 5) was added. The reaction was left standing at room temperature for 1 h.
Control 2 – only probe **1**: For control 2, 20 µL of stock solution of **1** was transferred into an HPLC vial and diluted with THF (30 µL). Then citrate buffer (50 µL, 55 mM, pH 5) was added. The reaction was left standing at room temperature for 1 h.

Of note, the labeling reaction can be performed in THF:H_2O (1:1) (pH 6.5) or under buffered conditions in THF:citrate buffer (1:1) at pH 5 without noticeable differences in labeling efficiency. We observed a higher amount of hydrolyzed probe product under buffered conditions, which did, however, not impact the labeling reaction. In case of thiol-rich natural product extracts, we recommend buffered conditions, as thiols can react with the chlorooxime probe as competitive nucleophiles at neutral pH.[5] Any undesired labeling with thiols can be easily differentiated through the absence of the diagnostic MS2 fragment **A**.

HPLC-MS analysis
HPLC samples were prepared by diluting 1µL of each crude reaction mixture with acetonitrile (99 µL).
LC-MS analysis was performed with an Agilent Technologies 1260 Infinity series HPLC equipped with a degasser, binary pump, autosampler, and diode array detector coupled to an Agilent Technologies 6530 Accurate-Mass Q-TOF LC/MS. The instrument was calibrated using the Agilent Reference Calibration Mix®. Separations were performed with a Kinetex™ 5 µm C18 100 Å, 150 x 4.6 mm column. The solvent system contained water + 0.1% formic acid as solvent A, and acetonitrile + 0.1% formic acid as solvent B. Solvents were of mass spectrometry-grade. Data were collected and analyzed using MassHunter Workstation Software version B.05.01.

HPLC-HRMS method for MS1
0.75 mL min^{-1} flow rate; gradient: 0-3 min 5% B, 3-23 min 5-100% B, 23-30 min 100% B, 30-37 min 5% B. DAD 190-600 nm. Dual ESI ion source, positive polarity. T = 300°C; Drying gas: 11L/min;
V_{cap}: 3000 V. Acquisition mass range: 100-1700 m/z; rate: 2 spectra/sec, 500 ms/spectrum. Sample was prepared in acetonitrile; V_{inj}: 10 µL.

Degradation products of probe 1

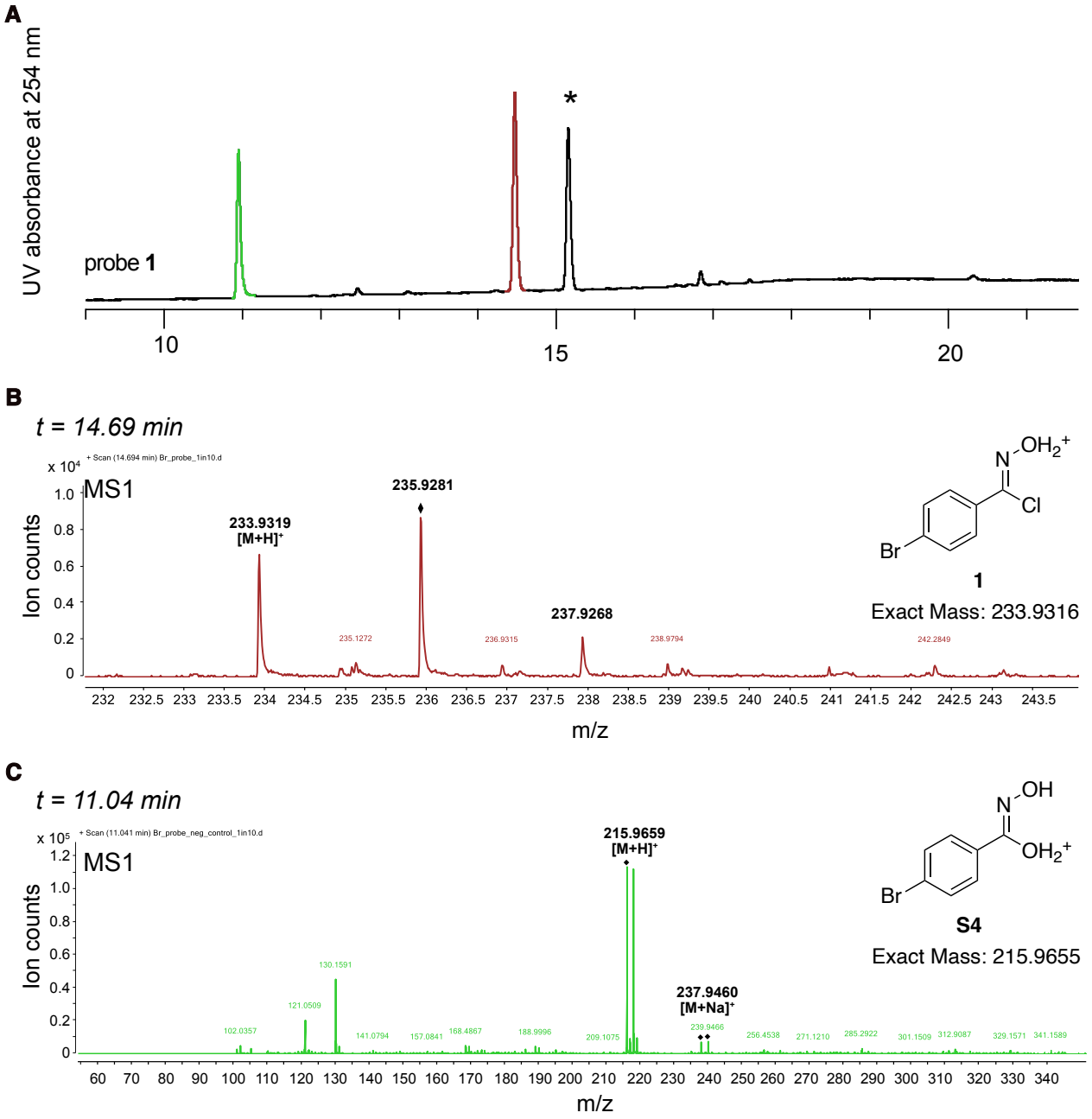


Figure S11. A) LC-UV chromatogram of negative control with probe **1** in THF:H_2O (1:1) shows three UV signals. Middle: MS spectrum derived from UV signal highlighted in red in LC-UV chromatogram shows a mass peak of m/z 233.9319 which corresponds to the mass of the proton-adduct of **1** $(M+H]_{calc}$ 233.9316). C) MS spectrum derived from the UV signal highlighted in green in LC-UV chromatogram corresponds to the degradation product **S4** formed through hydrolysis of probe **1**. The origin of the signal indicated by an asterisk is not known.

Labeling protocol for isolation of labeled isonitrile natural products

4 mL of CH_2Cl_2 extract was concentrated to dryness under reduced pressure to give 12.9 mg of crude dried extract. The residual solid was resuspended in THF (940 µL) in a 10 mL screwcap vial. Citrate buffer (1 mL, 50 mM, pH 5) was added to the extract, followed by 180 µL of a freshly prepared stock solution of **1** (10 mg/mL THF). The labeling reaction was followed by TLC (4:1 Hex:EtOAc, R_F (**1**) = 0.71) by monitoring consumption of **1** and formation of new UV active spots. More stock solution was added in 15 min intervals until the probe was not consumed anymore, indicating complete labeling of putative isonitriles in the extract (approx. 1–2h). After completion of the reaction, $NaCl_{sat}$ (2 mL) was added and the two phases were separated. The aqueous layer was extracted three times with EtOAc (3x2mL). The organic phases were combined and dried over $MgSO_4$, filtered and concentrated to dryness under reduced pressure.
The crude extract was purified by automated normal phase column chromatography (4 g cartridge, 0–60% EtOAc in hexanes, 12 min). Two major peaks were collected (compound **2** and compound **4**). Compound **4** required further purification by normal phase semi-preparative HPLC (Luna® 5 µm Silica (2) 100 Å, LC column 100 x 10 mm; gradient: 0–60% EtOAc/hexanes, 20 min, elution time = 10.2 min). Compound **2** was used as is for NMR and HRMS analysis.

HRMS analysis of **2**: calc. 447.1647; found: 447.1626 Δ m/z: 4.7 ppm
HRMS analysis of **4**: calc. 447.1647; found: 447.1669 Δ m/z: 4.9 ppm

Difference in MS-detectability of non-conjugated isonitrile terpenoids and isonitrile terpenoids ligated with probe 1

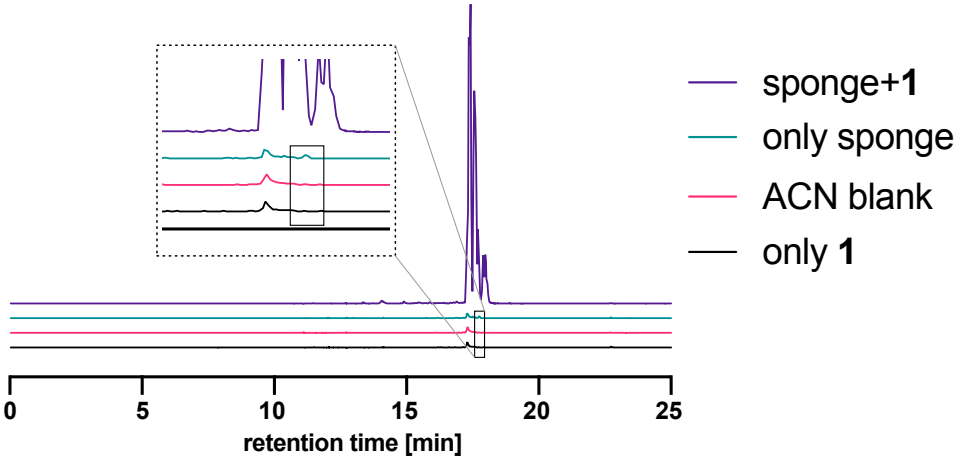


Figure S12. Extracted ion chromatograms of sponge extract + **1** (purple), only sponge extract (green), acetonitrile blank (red), and only probe **1** (black). All samples were extracted for the exact masses of sesquiterpene-probe conjugate **2** (m/z 447.165) and isonitrile sequiterpenoid **3** (m/z 232.206), The MS ion count signal corresponding to the non-ligated terpene is highlighted in a grey box (green graph). The detectability of the isonitrile terpene natural product after ligation with probe **1** increased by approximately 200-fold.

NMR spectroscopic analysis of sponge-derived sesquiterpene-conjugate 2

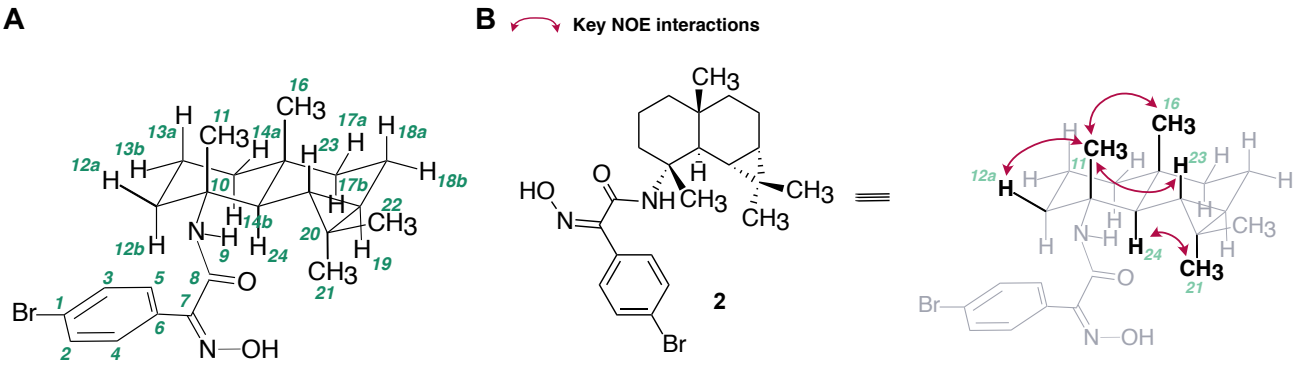


Figure S13. Left: Structure of **2** with numbered atoms. Right: Structure of **2** with relative stereochemistry, the absolute stereochemistry is not known. Red arrows indicate key NOEs.

Table S1. NMR spectroscopic data for sesquiterpenoid-conjugate **2** in $CDCl_3$ (500MHz)

Position	δ_C	δ_H (mult, *J* in Hz)	NOESY	COSY	HMBC
1	131.5	–	–	–	4, 5
2	132.3	7.55 (m)	–	4	1, 3, 6
3	132.3	7.55 (m)	–	5	1, 2, 6
4	129.8	7.43 (m)	–	2	5, 6, 7
5	129.8	7.43 (m)	–	3	4, 6, 7
6	124.6	–	–	–	2, 3, 4, 5
7	150.7	–	–	–	2, 3, 4, 5
8	161.9	–	–	–	9
9	–	5.86 (bs)	11, 21, 23, 24	–	8
10	59.4	–	–	–	11, 12a, 23, 24
11	19.2	1.52 (s)	12a, 16, 18a	–	12, 24, 10
12a	38.7	2.70 (m)	–	12b	11, 10, 24, 14
12b	38.7	1.62 (m)	–	12a, 14b	10, 11
13a	19.1	1.59 (m)	12a	–	12
13b	19.1	1.59 (m)	–	–	12
14a	39.4	1.37 (m)	–	14b	16, 17a, 17b
14b	39.4	1.01 (m)	–	13, 14a	16, 17a, 17b
15	32.9	–	–	–	16, 18, 24
16	19.5	0.93 (s)	11, 14, 18a	–	14, 15, 17, 24
17a	41.7	1.12 (dd, 13.4, 8.4)	14, 16	17b, 18a	15, 18, 19, 24
17b	41.7	0.73 (below 21)	–	17a, 18a, 18b	16, 18b, 18a, 19, 24
18a	15.5	1.79 (m)	16	17a, 17b, 18, 19	20, 23
18b	15.5	1.49 (m)	–	17b, 18a	15, 17, 20, 23
19	19.5	0.63 (dd, 8.9, 8.7)	–	18a, 23	17, 18, 22
20	17.7	–	–	–	18, 21, 22, 24
21	15.3	0.73 (s)	9, 18b, 24	–	19, 20, 22
22	28.7	0.78 (s)	9	–	20, 21, 23
23	19.8	0.37 (dd, 9.3, 6.6)	9, 11	19, 24	10, 22
24	47.0	1.04 (d, 6.6)	9, 21	12b, 14b, 23	10, 15, 17, 19, 20

NMR spectroscopic analysis of sponge-derived sesquiterpene-conjugate 4

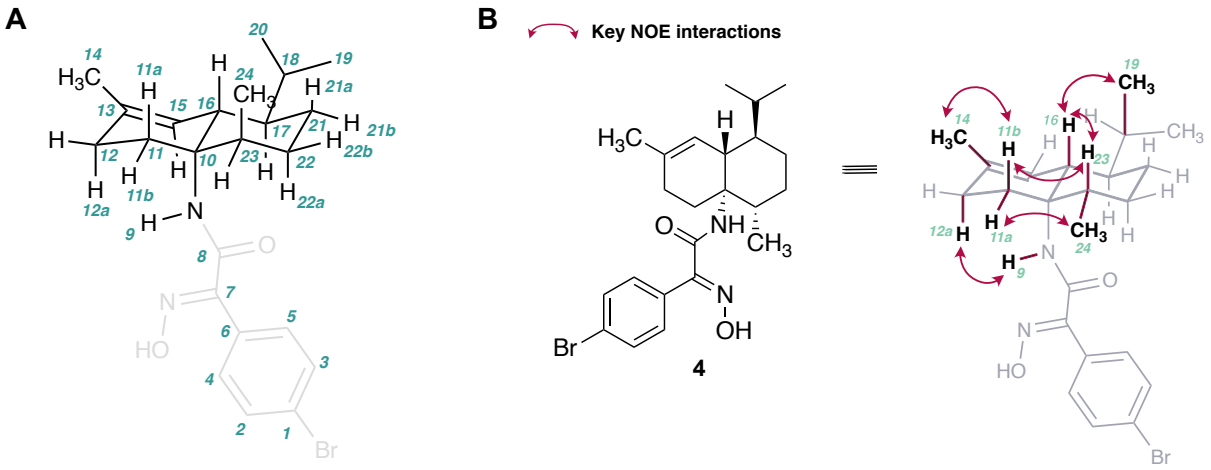


Figure S14. Left: Structure of **4** with numbered atoms. Right: Structure of **2** with relative stereochemistry, the absolute stereochemistry is not known. Red arrows indicate key NOEs.

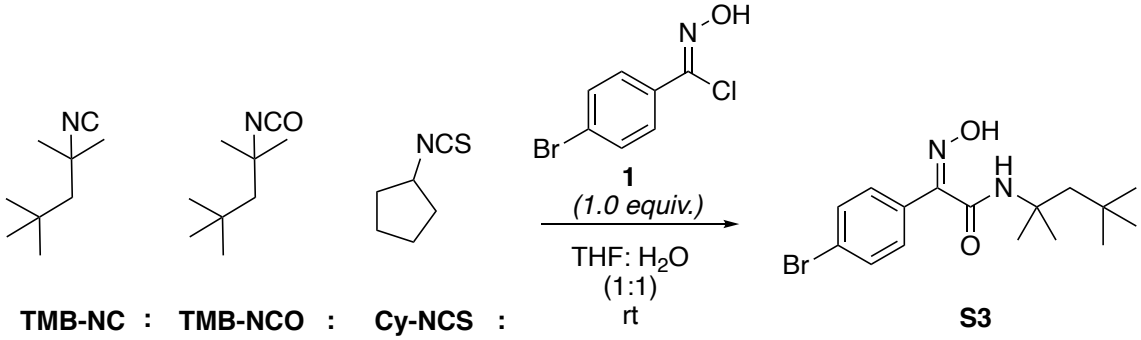
Table S2. NMR spectroscopic data for sesquiterpenoid-conjugate **4** in $CDCl_3$ (500 MHz).

Position	δ_C	δ_H (mult, *J* in Hz)	NOESY	COSY	H2BC	HMBC
1	128.6	–	–	–	2, 3	4, 5
2	131.2	7.51 (m)	–	4	4	3, 5
3	131.2	7.51 (m)	–	5	5	2, 4
4	128.1	7.40 (m)	–	2	2	5, 1, 7
5	128.1	7.40 (m)	–	3	3	4, 1, 7
6	124.2	–	–	–	–	–
7	151.4	–	–	–	–	–
8	not found	–	–	–	–	–
9	–	5.55 (bs)	12a	–	16	21, 12, 16, 10
10	60.5	–	–	–	–	9, 12, 14, 24
11a	19.7	1.69 (m)	–	11b, 16	12	10, 12, 16
11b	19.7	1.92 (ddd, 13.9, 10.7, 7.4)	–	12b, 11a	12	12
12a	25.9	1.77 (m)	–	12b, 11b	11	11, 13
12b	25.9	2.04 (dd, 18.3, 7.3)	–	12a, 11b, 11a	11	10, 13
13	131.6	–	–	–	–	12a, 12b, 14, 16
14	22.6	1.68 (s)	11b	15	12	12, 13, 15
15	123.0	5.56 (m)	18	14, 16	16, 14	11, 12a, 12b, 16
16	40.7	2.86 (dd, 11.1, 5.9)	19, 23, 11b	14, 15, 17	17	11, 17, 10, 13, 15
17	47.2	1.27 (overlapping grease)	20	21b, 18, 16	16, 18	14, 18, 19, 20, 21a, 22b
18	26.3	1.98 (m)	–	19, 20, 16	19, 20	19, 20
19	14.6	0.85 (d, 6.9)	16	18	18	20
20	20.9	0.89 (d, 6.9)	–	18	18	19
21a	22.9	1.16 (m)	–	21b, 17	23, 17	18
21b	22.9	1.56 (dt, 9.4, 3.4, 3.4)	19	21a, 17	–	18
22a	29.9	1.17 (m)	–	23, 21a	17, 23	24
22b	29.9	1.63 (dt, 9.9, 3.9, 3.9)	–	22a, 23	23	24
23	36.3	2.59 (m)	16	22b, 21a, 24	22, 24	24, 21b, 22a, 22b
24	15.2	0.96 (d, 6.9)	11a	23	23	10, 22, 23

5. Competition experiments between isonitriles, isocyanates, isothiocyanates for probe 1

Isonitrile **TMB-NC** and either isothiocyanate **Cy-NCS** or isocyanate **TMB-NCO** (1.0 equiv.) were dissolved in THF (480µL). Then water (500 µL) was added. Lastly, 10 µL (2.0 equiv.) of a stock solution of **1** in THF (26 mg/100 µL THF) was added and the reaction mixture was stirred for 1 h at room temperature (reactions 1–6, Scheme S2). Brine was added and the two phases were separated. The organic phase was dried over $MgSO_4$, filtered, and concentrated under reduced pressure.
Comparison of all reactions by HPLC-UV analysis revealed that only reactions containing isonitrile **TMB-NC** and probe **1** (reaction (1), (2) and (4)) showed a new signal (Figure S14, t = 20 min). No new signal was observed in the control reactions and competition reactions with **TMB-NCO** and **Cy-NCS** (reactions (2), (3), (5) and (6)). Additionally, HRMS analysis confirmed formation of isonitrile-probe conjugate **S3** in reactions (1), (2) and (3) (Figure S15).

S3: **^{1}H-NMR (500 MHz, $CDCl_3$) δ [ppm]** : 10.28 (bs, OH, 1H), 7.55-7.52 (m, 2H), 7.46-7.44 (m, 2H), 5.82 (s, NH, 1H), 1.75 (s, 2H), 1.51 (s, 6H), 0.99 (s, 3H). **^{13}C-NMR (121 MHz, $CDCl_3$) δ [ppm]**: 162.01, 151.65, 132.16, 131.12, 129.26, 124.64, 56.97, 52.83, 31.81, 31.62, 28.84.



	TMB-NC :	TMB-NCO :	Cy-NCS :
(1)	1.0 equiv.	–	–
(2)	1.0 equiv.	–	1.0 equiv.
(3)	–	–	1.0 equiv.
(4)	1.0 equiv.	1.0 equiv.	–
(5)	–	1.0 equiv.	–
(6)	1.0 equiv.	–	–

Scheme S2. Competition experiments between isonitrile **TMB-NC**, isocyanate **TMB-NCO** and isothiocyanate **Cy-NCS** for reaction with probe **1**.

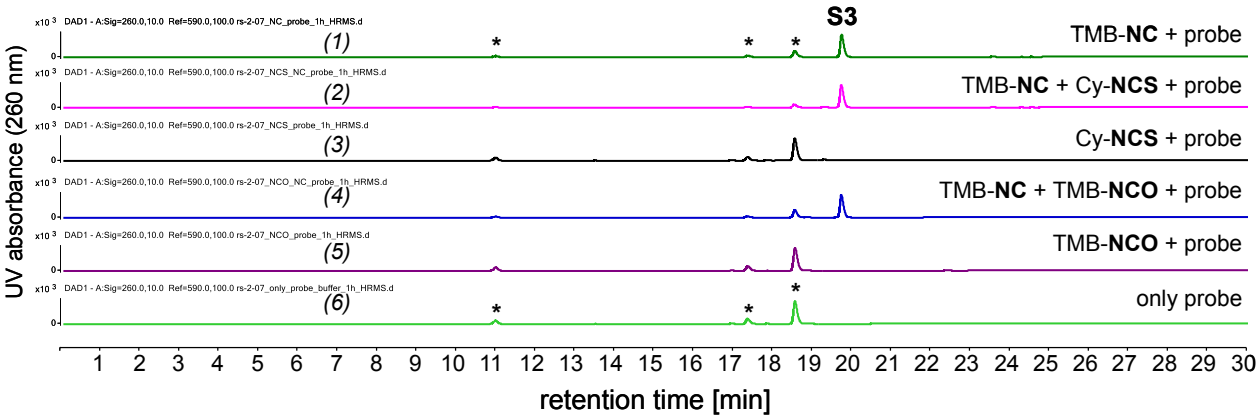


Figure S15. LC-UV chromatograms of reactions *(1)* to *(6)* with absorbance measured at 260 nm. **S3** indicated the desired conjugated product at t = 20 min. Stars indicate UV signals derived from LC-degradation products of probe **1** at t = 18.7, 17.4 min and 11.0 min, as discussed previously.

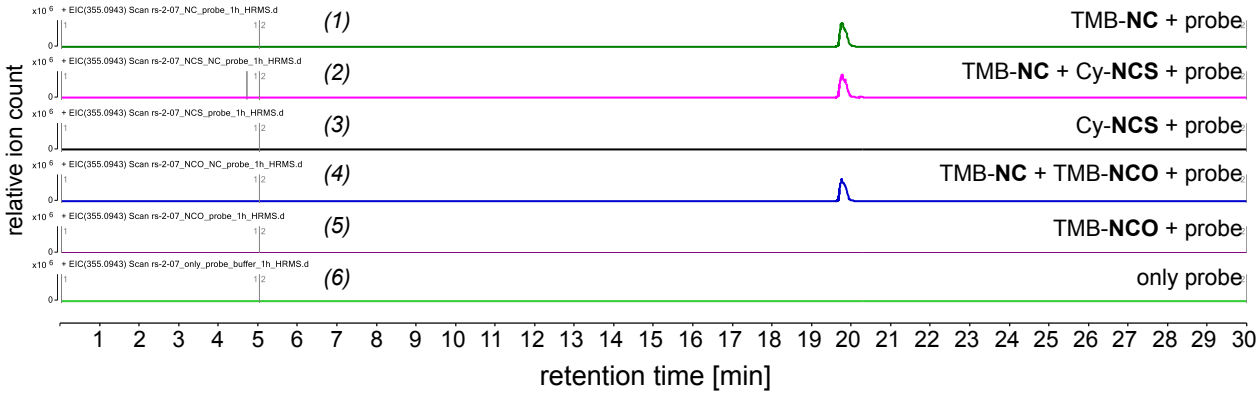


Figure S16. Extracted ion chromatograms (EICs) of reactions *(1)* to *(6)* extracted for the mass of conjugated product **S3** (m/z 355.0943 $[M+H]^+$).

6. MS/MS fragmentation of hydroxyimino amide products

HPLC samples were prepared, and LC-MS analysis was performed with the same equipment and solvents as described above (page S18).

HPLC-HRMS method for MS2
0.75 mL min^{-1} flow rate; gradient: 0-3 min 5% B, 3-23 min 5-100% B, 23-30 min 100% B, 30-37 min 5% B. DAD 190-600 nm. Dual ESI ion source, positive polarity. T = 300°C; Drying gas: 11L/min; V_{cap}: 3000 V.
Acquisition for MS1: mass range: 100-1700 m/z, rate: 10 spectra/s, 100 ms/spectrum
Acquisition for MS2: mass range 50-1700 m/z, rate:4 spectra/s, 250 ms/spectrum, E_{coll} = 5 V.
Injection volume, V_{inj}: 5 µL.

The MS/MS fragmentation patterns of the parent ions m/z 511.14, 447.16, 447.16 identified as the source of UV signals I, II, III contain a MS/MS signal with a mass-over-charge ratio of 242.98 [M+H] (Figure S16).
We hypothesized that this fragment could be a diagnostic for isonitriles that reacted with probe **1**. To test this hypothesis, we synthesized probe conjugate **S3** (page S23) from commercially available 1,1,4,4-tetramethylbutyl isocyanide (TMB-NC) and probe **1** and compared its MS2 fragmentation pattern with that of the sponge natural product probe conjugates.
S3 fragments into 4 major fragments: 1 (m/z 242.98), 2 (m/z 225.95), 3 (m/z 197.95), and 4 (m/z 181.96) (Figure S17), thus confirming that fragment **A** is a diagnostic MS2 fragment for isonitrile-probe conjugates. Scheme S3 shows a proposed mechanism for the MS/MS fragmentation of a conjugate between an isonitrile and probe **1**.
This diagnostic fragment, in combination with the UV absorbance and the bromine isotope pattern, allows for an unambiguous identification of isonitrile functionalized natural products.

MS/MS analysis of conjugated sponge natural products

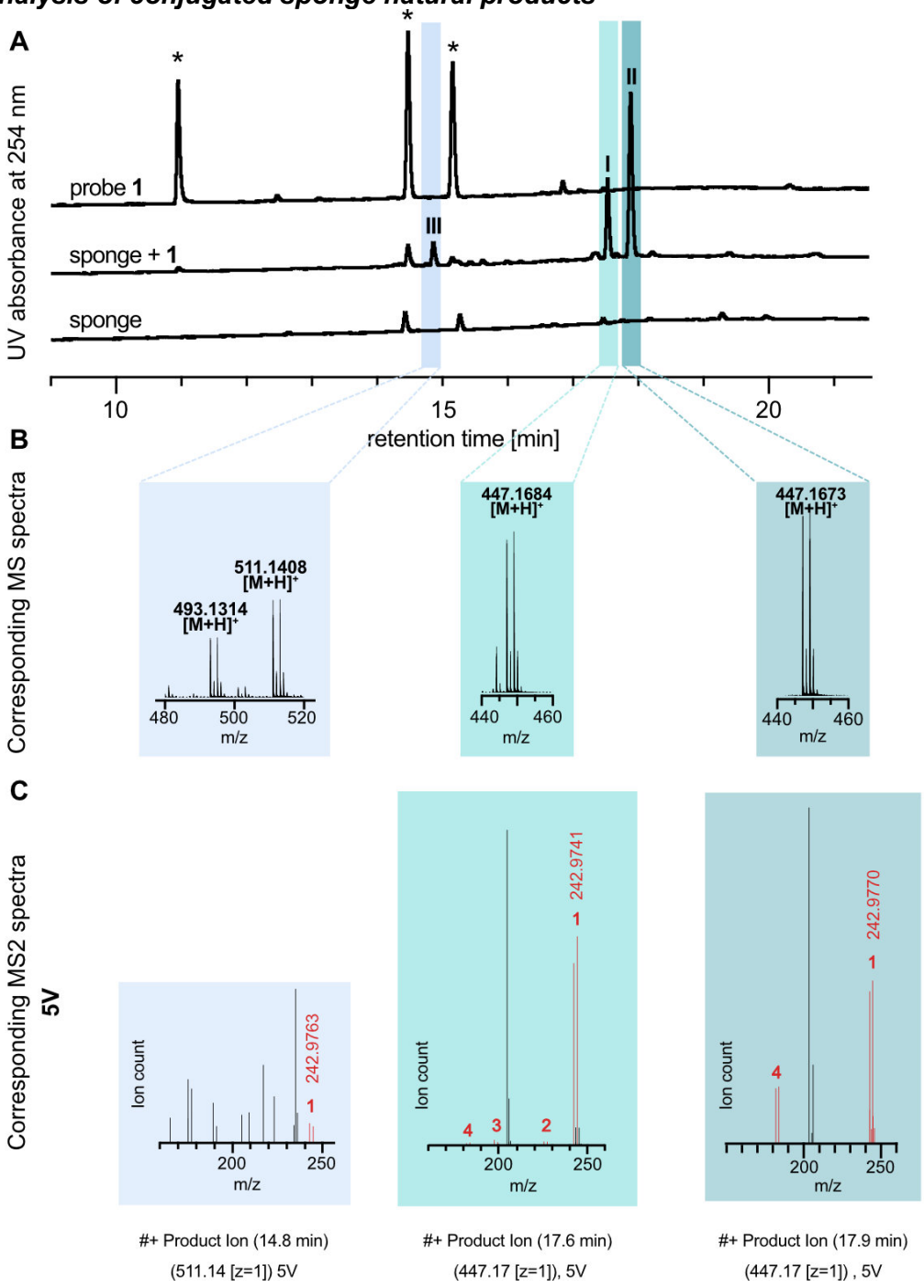


Figure S17. A) LC-UV analysis of only probe control (top), labeling reaction of sponge crude extract with probe **1** (middle), only sponge crude extract (bottom). Boxes highlight new UV signals **I**, **II** and **III** that are only observed in the labeling reaction. Asterisk indicate signals derived from probe **1**. B) MS spectra corresponding to newly formed UV signals show the anticipated bromine isotope pattern. C) MS/MS fragmentation of the identified brominated product ions at a collision energy of 5V all produce diagnostic fragment **A** (indicated as 1). MS2 spectrum of **II** (light blue box) additionally produces fragment 2, 3 and 4. MS2 spectrum of **I** (dark blue box) additionally produces fragment 4.

MS/MS analysis of probe conjugate S3 and proposed fragmentation mechanism

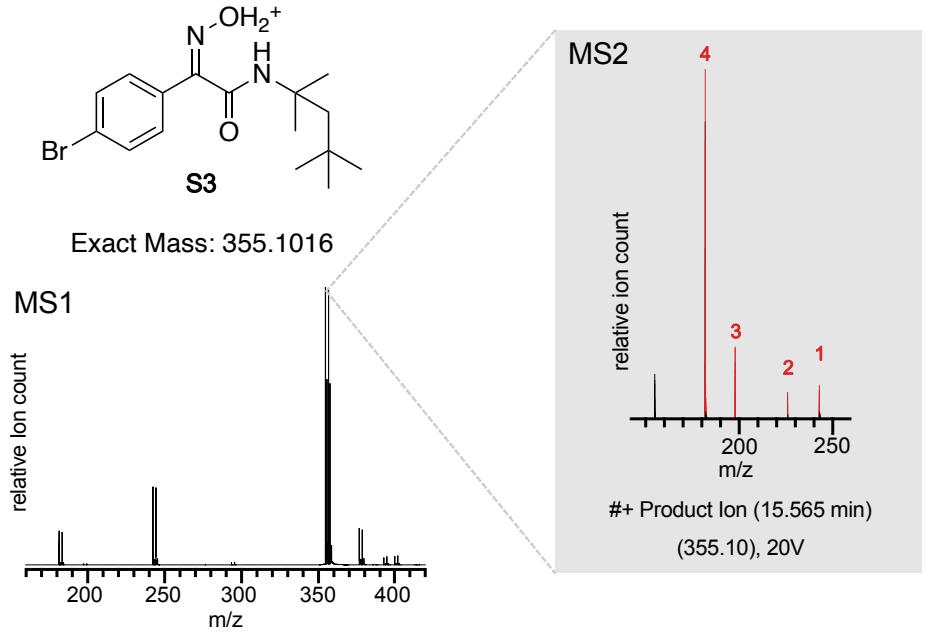
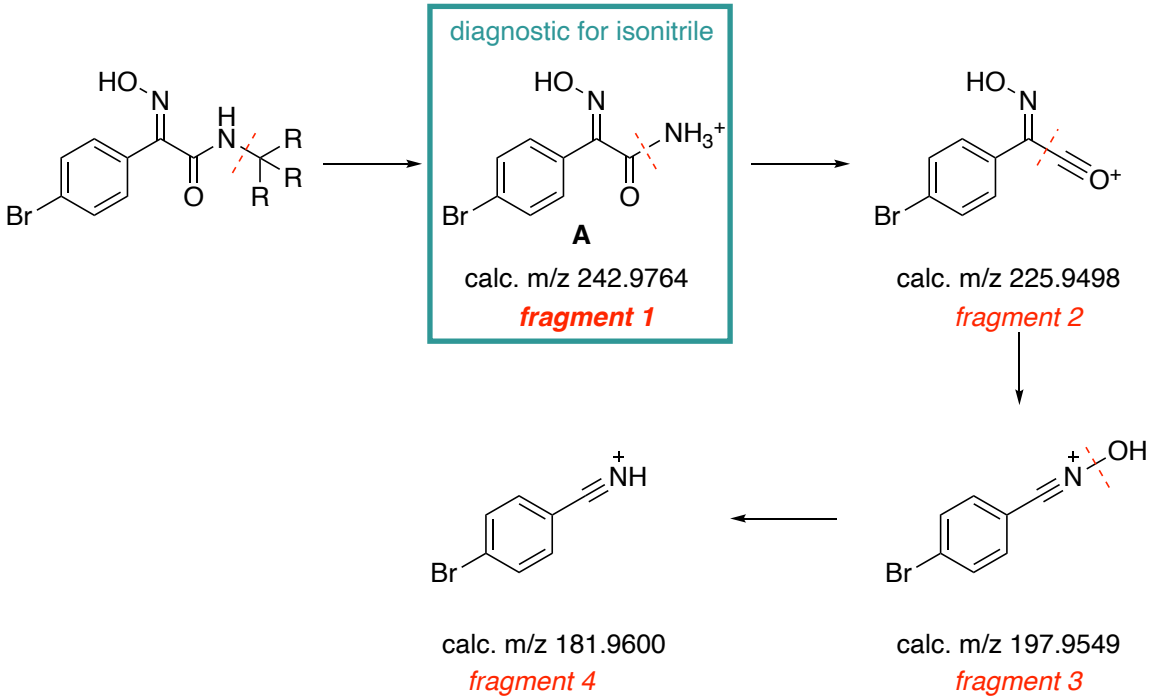


Figure S18. Left: Structure of **S3,** below extract of the MS1 spectrum of synthetic probe conjugate **S3** with the expected product mass peak at m/z 355.10. Right: MS/MS analysis of product ion m/z 355.10 generates MS fragments 1-4 with measured mass-to-charge ratios of 1 (m/z 242.9768), 2 (m/z 225.9504), 3 (m/z 197.9537) and 4 (m/z 181.9608).

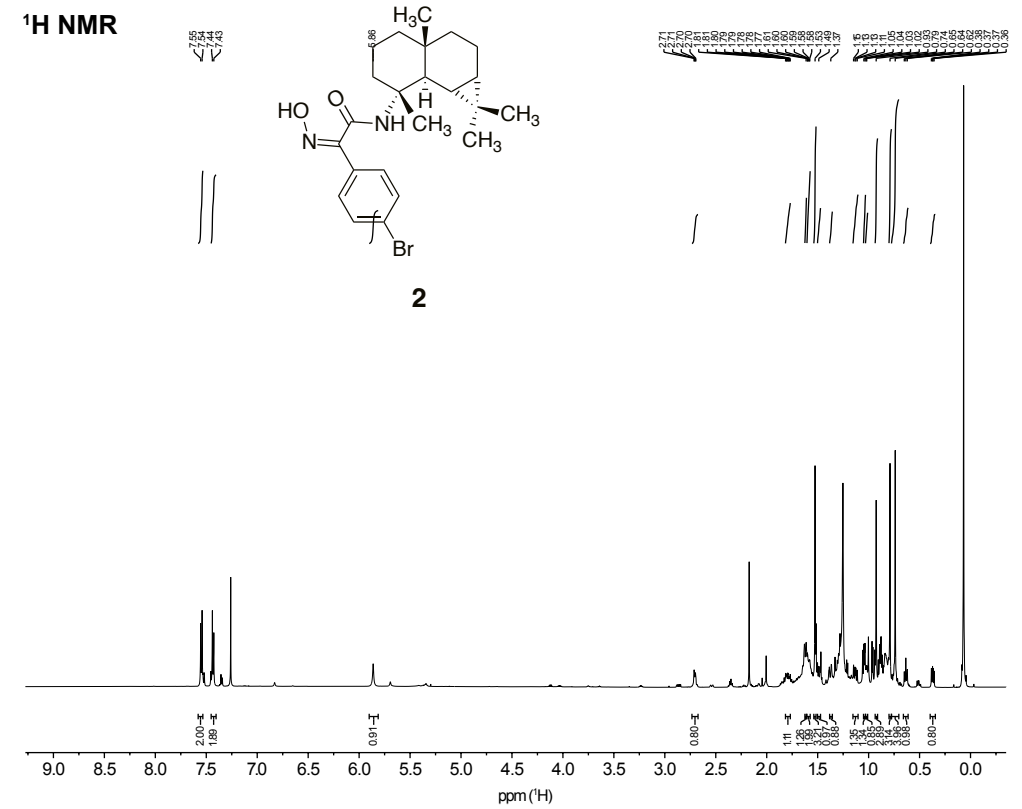


Scheme S3. Proposed fragmentation mechanism of isonitrile-probe conjugates with the calculated expected exact mass-over-charge- ratios (m/z) for each fragment. Formation of **A** (fragment 1, blue box) is diagnostic for the reaction of chlorooxime probe **1** with isonitriles.

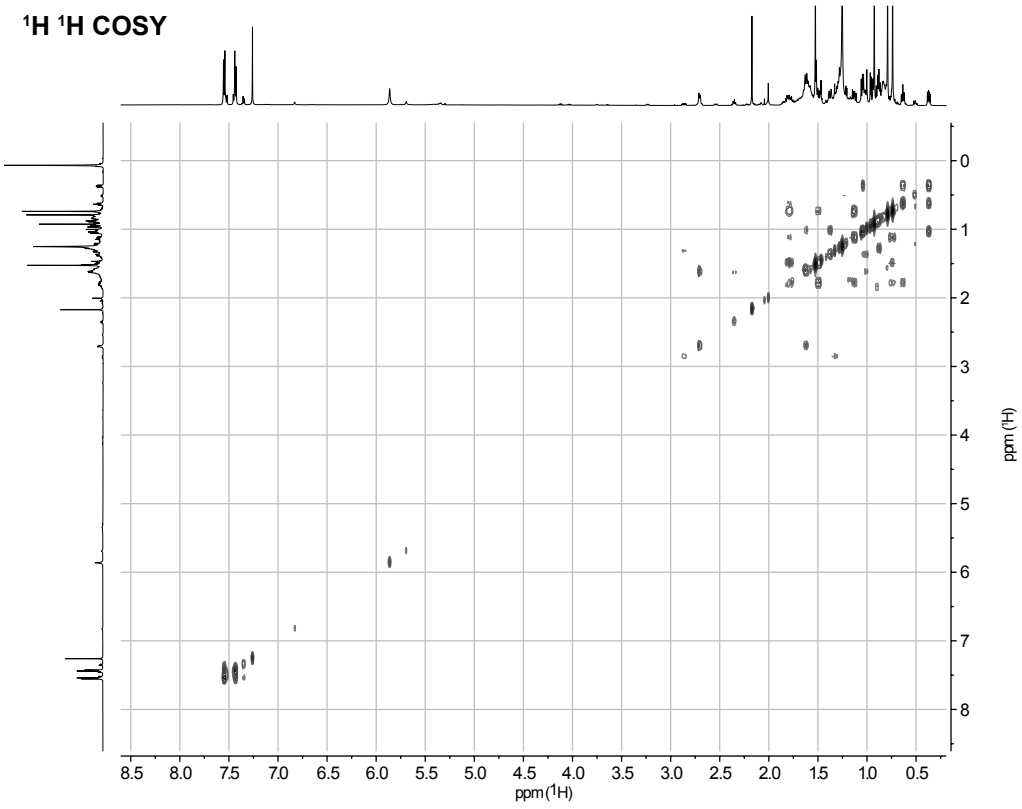
7. NMR spectra of natural product probe conjugates and compound S3

Compound ***2***

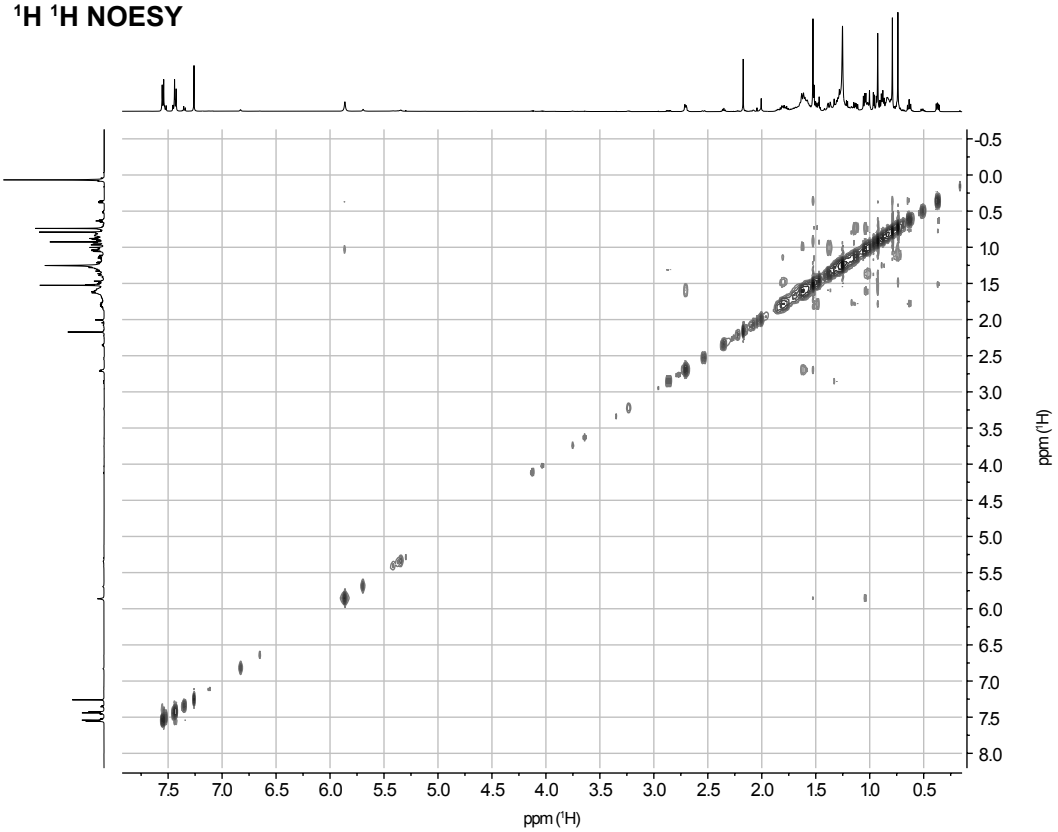
^{1}H NMR



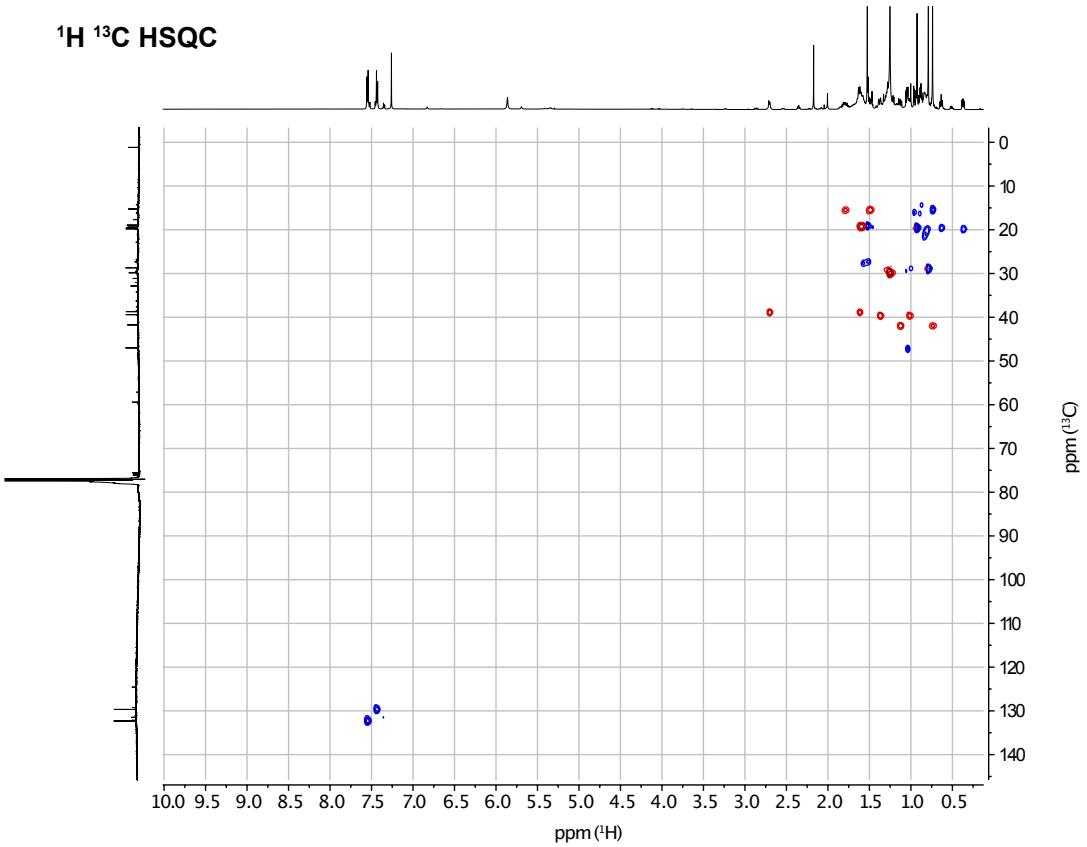
^{1}H ^{1}H COSY



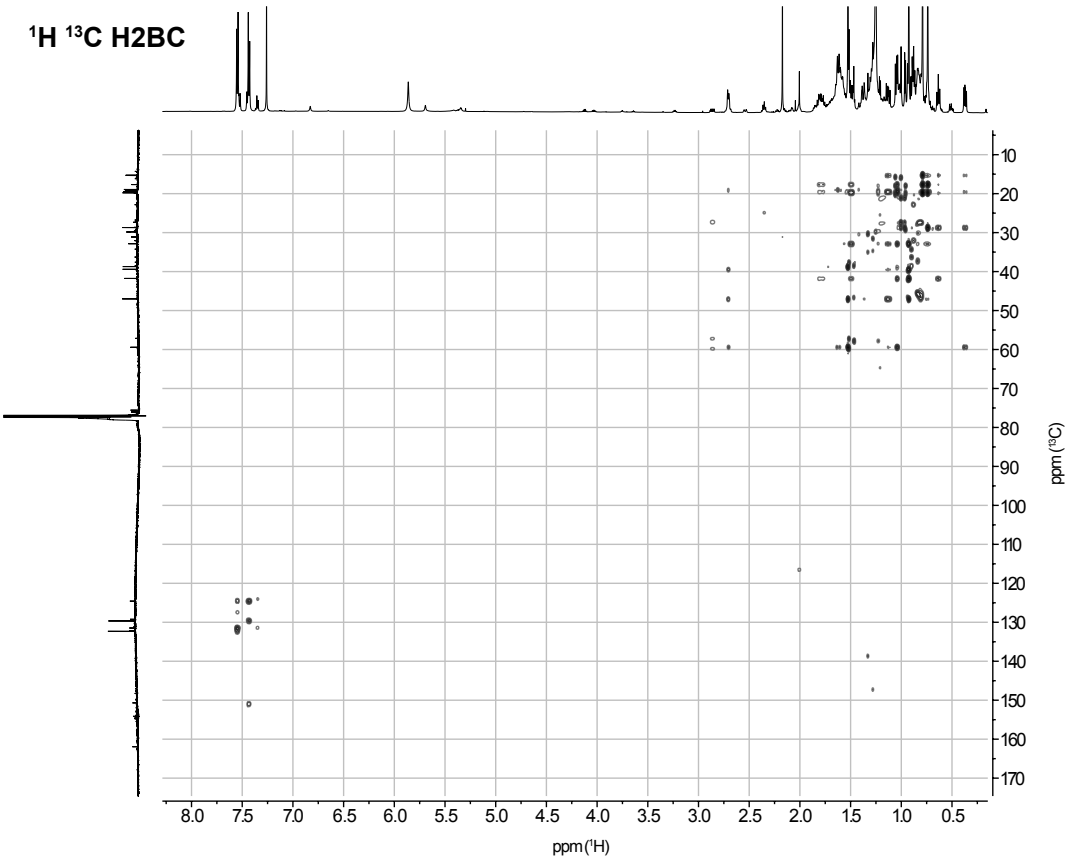
^{1}H ^{1}H NOESY



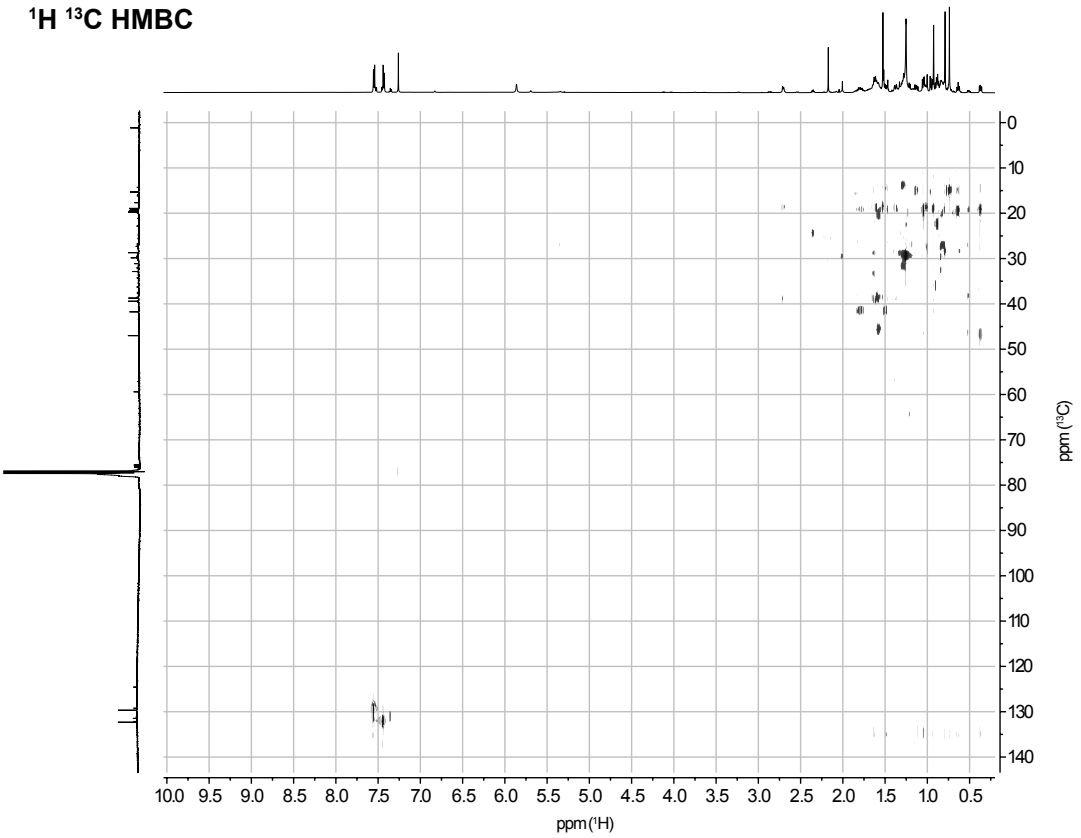
^{1}H ^{13}C HSQC



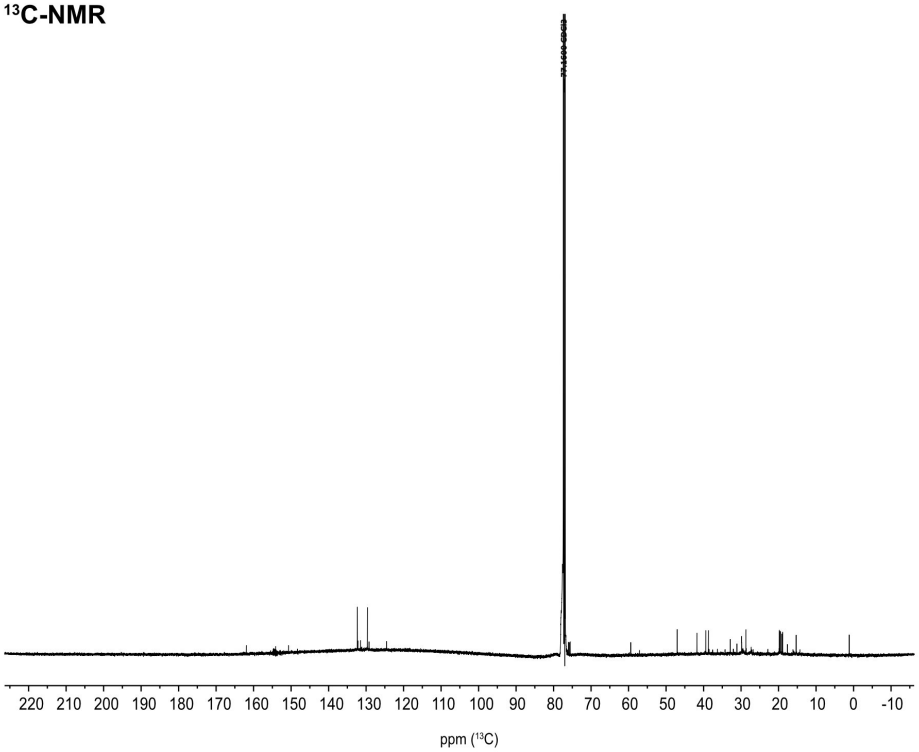
^{1}H ^{13}C H2BC



^{1}H ^{13}C HMBC

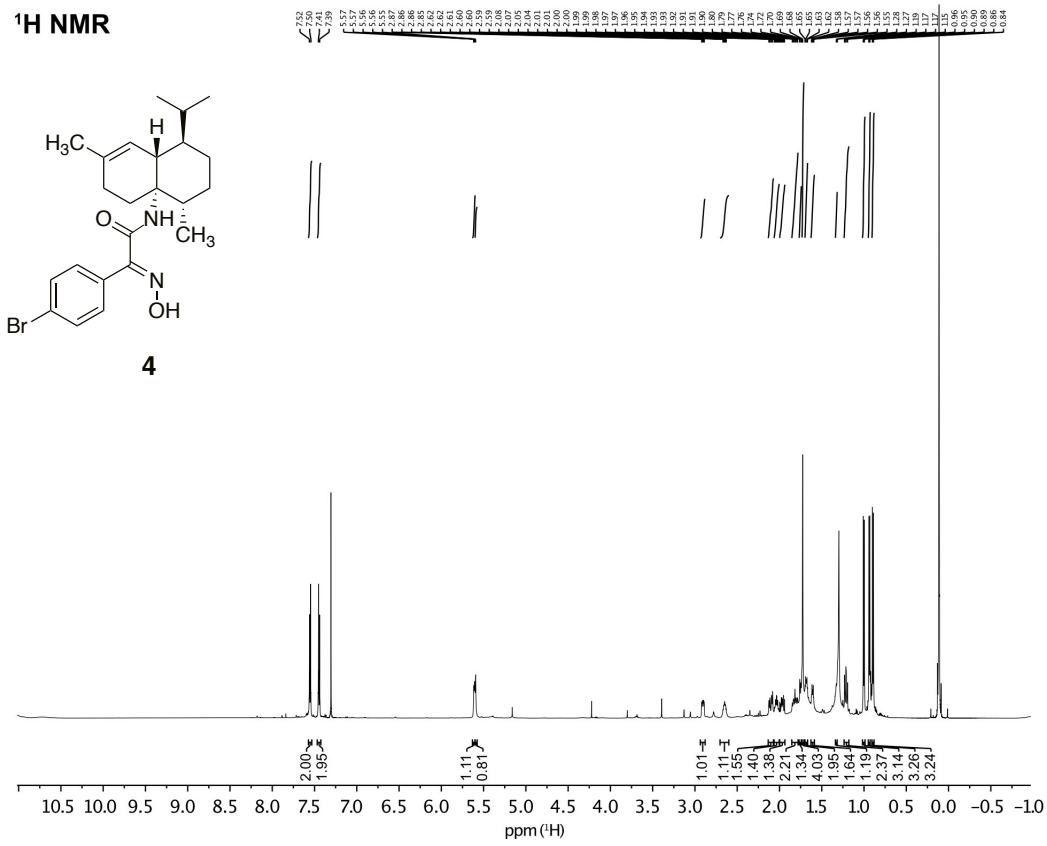


^{13}C-NMR



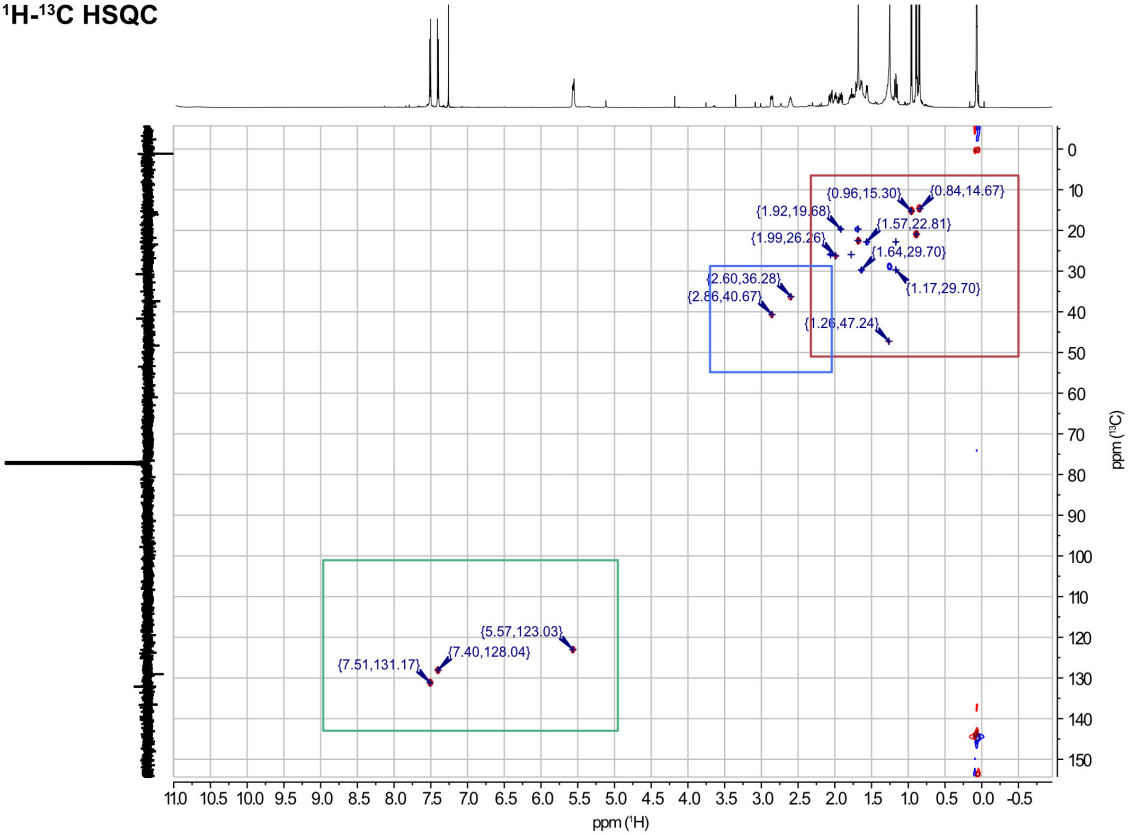
*Compound **4***

^{1}H NMR

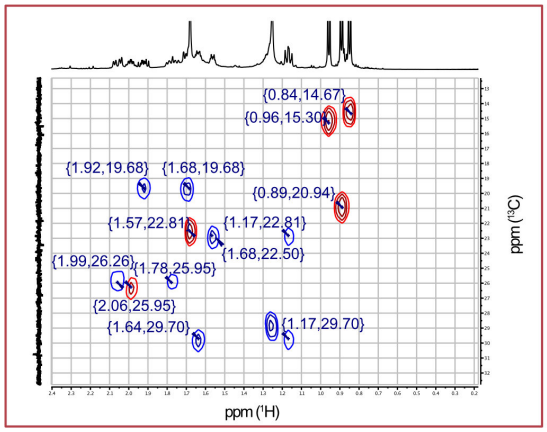


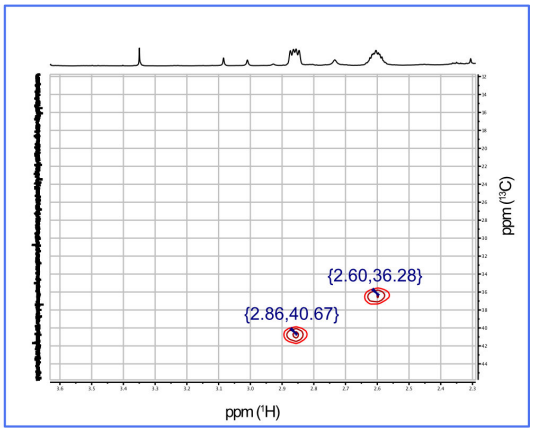
4

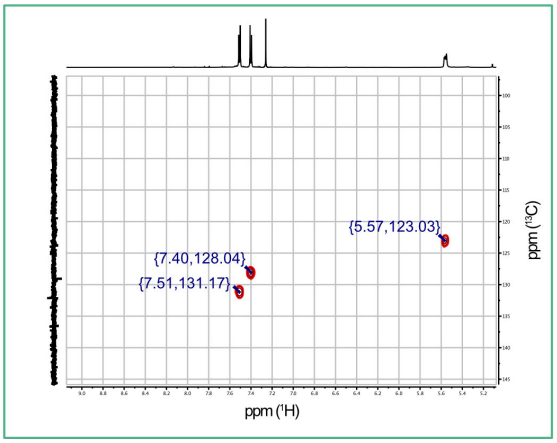
^{1}H-^{13}C HSQC



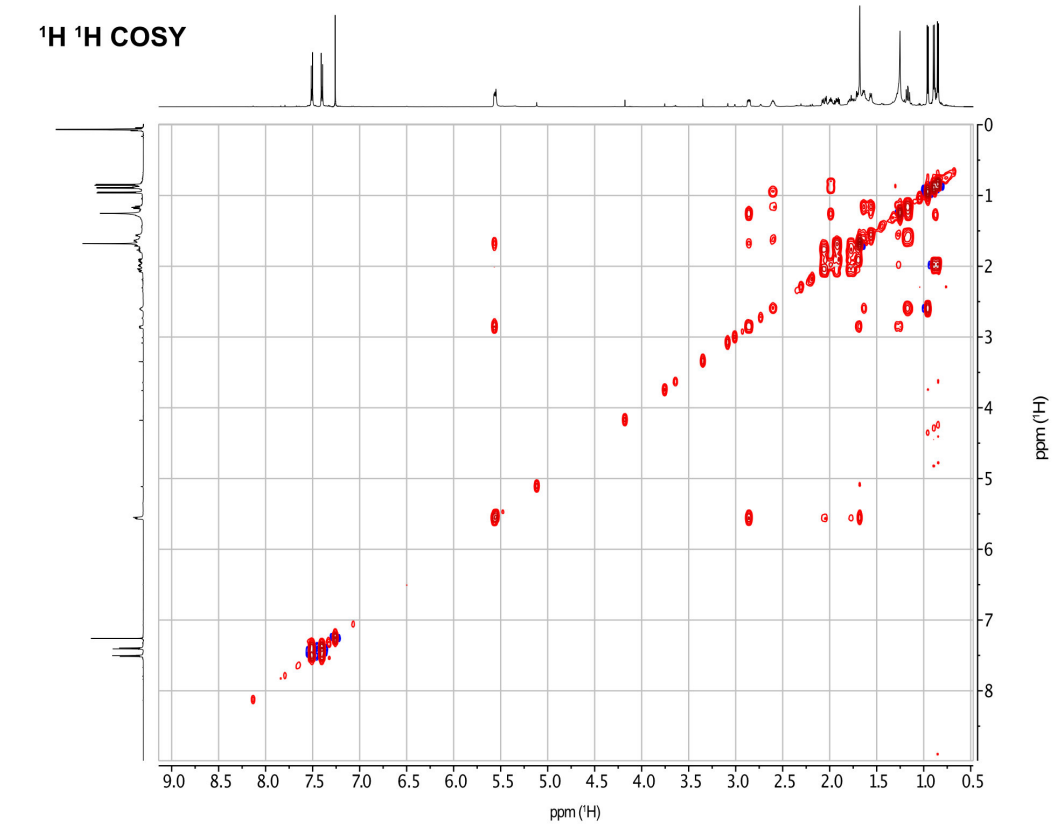
Different zooms of ^{1}H ^{13}C HSQC



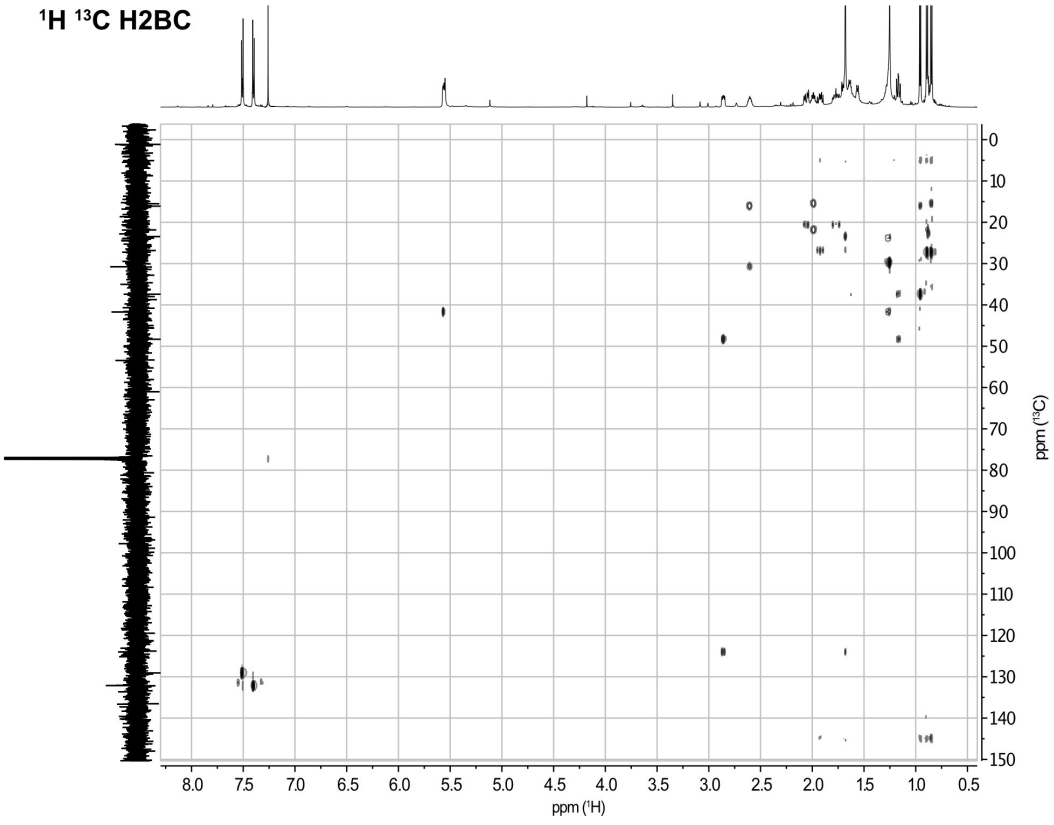


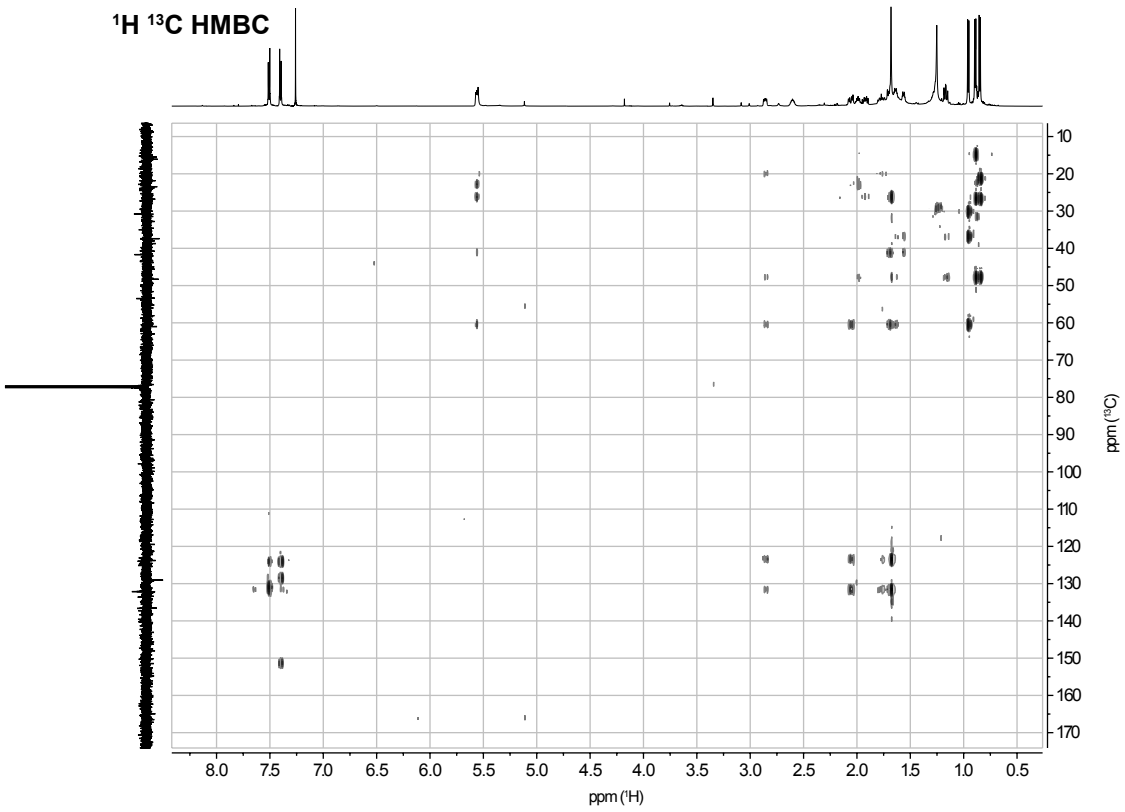


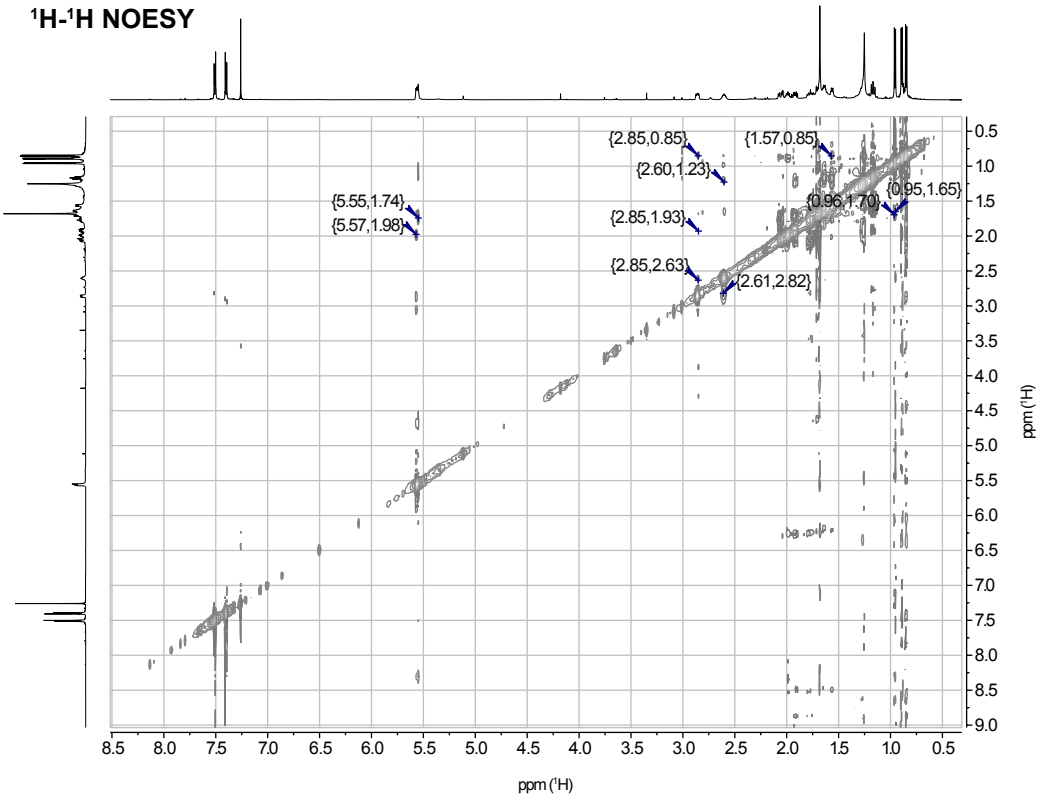
^{1}H ^{1}H COSY



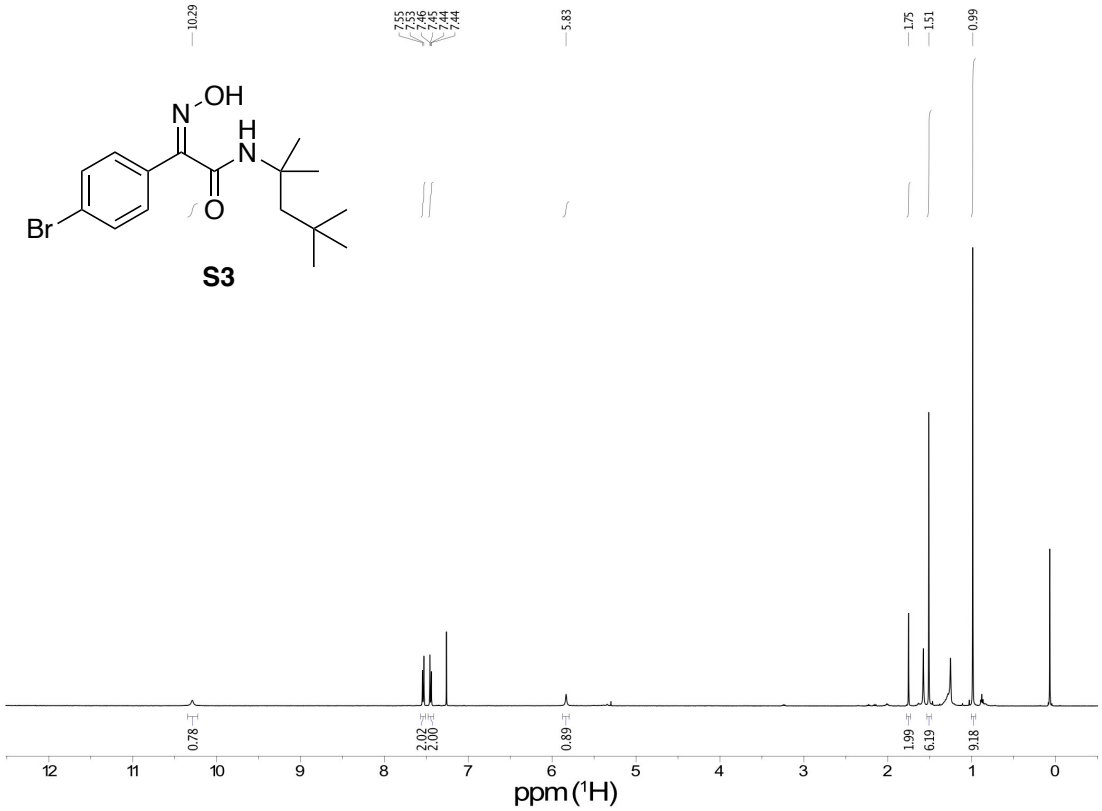
^{1}H ^{13}C H2BC

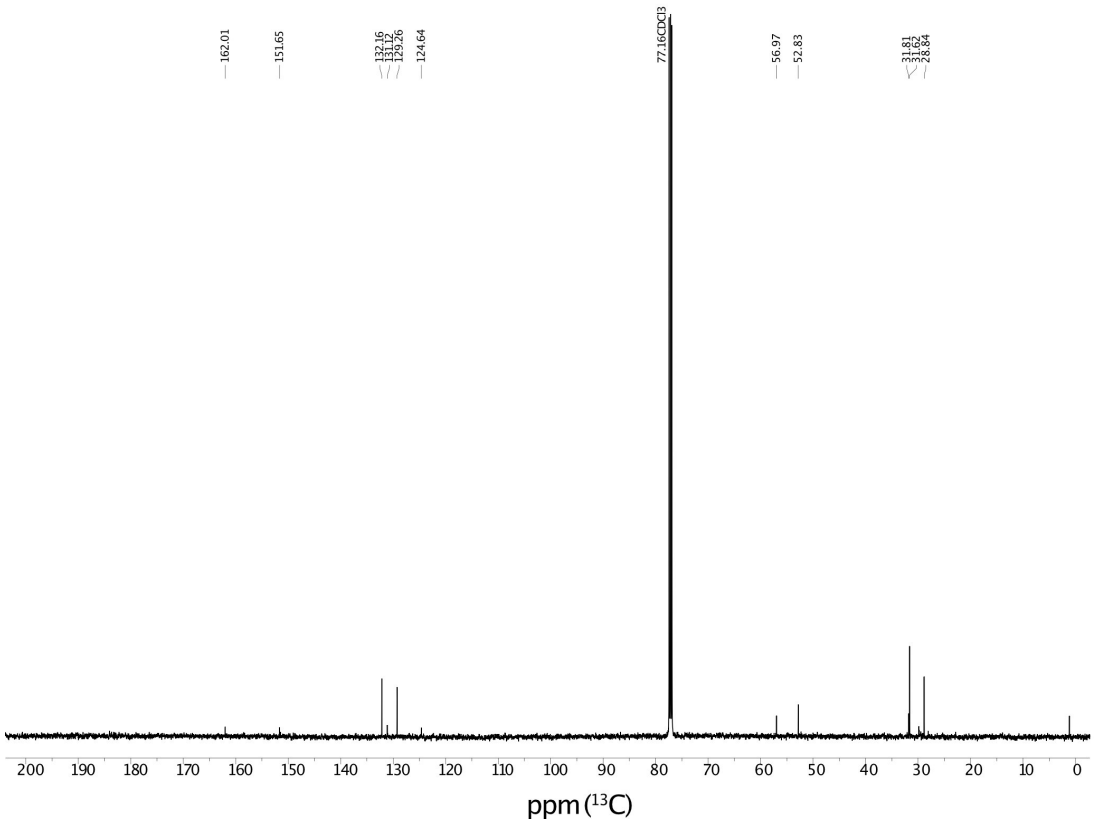



^{1}H ^{13}C HMBC
ppm (^{1}H)
ppm (^{13}C)


^{1}H-^{1}H NOESY
{2.85,0.85}
{1.57,0.85}
{2.60,1.23}
{0.95,1.65}
{0.96,1.70}
{5.55,1.74}
{5.57,1.98}
{2.85,1.93}
{2.85,2.63}
{2.61,2.82}
ppm (^{1}H)
ppm (^{1}H)

^{1}H NMR ($CDCl_3$) and ^{13}C NMR ($CDCl_3$) spectra of compound **S3**





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