

## Supporting Information

### Napyradiomycins CNQ525.510B and A80915C target the Hsp90 paralogue Grp94

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**A. General experimental methods:** Chemical reagents were purchased from Acros, Fluka, Sigma–Aldrich, or TCI. Deuterated NMR solvents were purchased from Cambridge Isotope Laboratories. All reactions were conducted with rigorously dried anhydrous solvents that were obtained by passing through a solvent column composed of activated A1 alumina. *N,N*-diisopropylethylamine (Et<sub>3</sub>NiPr<sub>2</sub>) was distilled from ninhydrin, then from KOH. All reactions conducted in oven–dried glassware sealed with septa, with stirring from a Teflon coated stir bars. Solutions were heated in a silicon oil bath. Analytical Thin Layer Chromatography (TLC) was performed on Silica Gel 60 F<sub>254</sub> precoated glass plates (EM Sciences). Visualization was achieved with UV light or stained with ceric ammonium molybdate. Flash chromatography was carried out Geduran Silica Gel 60 (40–63 mesh) from EM Biosciences. Yields and characterization data correspond to isolated, chromatographically and spectroscopically homogeneous materials. NMR spectra were collected on a Varian Inova 300, a Varian Inova 500, or a Bruker Biospin 600 equipped with a 1.7 mm probe. All <sup>13</sup>C NMR spectra were recorded with complete proton decoupling. Chemical shifts were referenced to the residual solvent for <sup>1</sup>H spectra, or to the <sup>13</sup>C signal from the deuterated solvent. Chemical shift  $\delta$  values for <sup>1</sup>H and <sup>13</sup>C spectra are reported in parts per million (ppm) relative to these referenced values, and multiplicities are abbreviated as s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. FID files were processed using Mestranova 8.0.2. (MestreLab Research). Electrospray (ESI) mass spectrometric analyses were performed using a ThermoFinnigan LCQ Deca spectrometer, and high–resolution analyses were conducted using a ThermoFinnigan MAT900XL mass spectrometer with electron impact (EI) ionization. A Thermo Scientific LTQ Orbitrap XL mass spectrometer was used for high–resolution electrospray ionization mass spectrometry analysis (HR–ESI–MS).

**B. Synthesis of probe mixture 9a/9b.** Construction of probe **9a/9b** was accomplished through a three-step process. CNQ525.510B (**1**) (4.0 mg, 7.8  $\mu$ mol) was dissolved in anhydrous DMF (0.4 mL). K<sub>2</sub>CO<sub>3</sub> (8.0 mg, 57.8  $\mu$ mol) as added followed by *tert*-butyl 2-bromoacetate (5.0  $\mu$ L, 33.9  $\mu$ mol) dissolved in DMF (100  $\mu$ L). After stirring at rt for 1h, the sample was dried with by airflow. A ~1:1 mixture of esters C15-labeled **6a** and C17-labeled **6b** (3.5 mg, 72%) was obtained by purification using Silica-gel HPLC eluting with a gradient of isoctane/EtOAc. A copy of a <sup>1</sup>H-NMR spectrum from the mixture of **6a/6b** is provided in Fig. S2. Esters **6a/6b** (3.5 mg, 5.6  $\mu$ mol) were dissolved in CH<sub>2</sub>Cl<sub>2</sub> (1 mL) and trifluoroacetic acid (150  $\mu$ L) was added. After stirring at rt for 2h, the sample was dried by airflow. HATU (15 mg, 39.4  $\mu$ mol) was added to a mixture of the crude acids **7a/7b** and IAF tag **8a** (8.0 mg, 27.6  $\mu$ mol) dissolved in Et<sub>3</sub>NiPr<sub>2</sub> (50  $\mu$ L) and DMF (400  $\mu$ L). After 12 h at rt, the sample was dried by airflow. The reaction product was dissolved in CH<sub>3</sub>CN and purified by reversed phase C18 HPLC to yield 3.3 mg (47%) of an ~1:1 mixture of probes **9a/9b**. Repetitive HPLC or pTLC purification was able to enrich the isomeric ratios providing up to 1:2 or 2:1 mixtures of **9a/9b**. Samples containing a 1:2 mixture of **9a/9b** were used for the biological studies.

**Probe mixture 9a/9b:** <sup>1</sup>H NMR (CDCl<sub>3</sub>, 600 MHz)  $\delta$  12.35 (s, 1H), 12.22 (s, 1H), 7.52 (d, *J* = 8.9 Hz, 1H), 7.32 (s, 1H), 7.29 (s, 1H), 7.24 (m, 1H), 6.67 (d, *J* = 7.5 Hz, 1H), 6.56 (s, 1H), 6.08 (s, 1H), 5.99 (bs, 1H), 4.91(d, *J* = 12.9 Hz, 1H), 4.75-4.71 (m, 4H), 4.51-4.46 (m, 3H), 4.36 (d, *J* = 2.6 Hz, 1H), 4.23 (d, *J* = 13.7 Hz, 1H), 3.73 (d, *J* = 4.9 Hz, 1H), 3.63 (s, 1H), 3.36 (m, 2H), 3.22 (m, 2H), 3.11 (s, 2H), 3.07 (s, 6H), 3.03 (s, 2H), 2.42-2.23 (m, 5H), 2.34 (s, 3H), 2.32 (s, 3H), 2.00 (t, *J* = 3.0 Hz, 1H), 1.98 (t, *J* = 3.0 Hz, 1H), 1.84 (td, *J* = 2.8, 5.7 Hz, 1H), 1.81 (td, *J* = 2.8, 5.4 Hz, 1H), 1.61-1.46 (m, 4H), 1.51 (s, 3H), 1.50 (s, 3H), 1.45 (m, 1H), 1.39 (m, 1H), 1.21 (m, 1H), 1.18 (s, 3H), 1.18 (s, 3H), 1.16-1.11 (m, 2H), 1.06 (s, 3H), 1.04 (s, 3H), 0.90 (s, 3H), 0.89

(s, 3H), 0.30 (s, 3H), 0.28 (s, 3H);  $^{13}\text{C}$ -NMR ( $\text{CDCl}_3$ , 150 MHz)  $\delta$  196.9, 195.1, 193.2, 184.1, 167.6, 166.7, 162.9, 162.2, 158.6, 138.7, 137.1, 130.5, 126.0, 122.6, 120.0, 118.7, 116.8, 111.2, 109.9, 106.3, 105.5, 99.2, 89.5, 88.6, 87.8, 86.6, 85.2, 86.6, 82.5, 79.3, 78.0, 77.2, 72.5, 65.7, 65.0, 59.1, 59.1, 52.4, 52.9, 51.7, 49.4, 48.1, 41.5, 40.8, 40.8, 36.7, 36.3, 35.6, 32.1, 31.5, 29.3, 28.1, 27.3, 26.6, 25.9, 23.9, 22.6, 21.4, 21.2, 21.0, 20.8, 10.3; ESI-MS  $m/z$  840.24  $[\text{M}+\text{H}]^+$ ; HR-ESI-MS  $m/z$  calcd. for  $\text{C}_{43}\text{H}_{52}\text{Cl}_2\text{N}_3\text{O}_{10}$   $[\text{M}+\text{H}]^+$ : 840.2952, found 840.2132. Copies of  $^1\text{H}$ -NMR (Fig. S3),  $^1\text{H}, ^1\text{H}$ -gCOSY (Fig. S4),  $^1\text{H}, ^1\text{H}$ -ROESY (Fig. S5),  $^1\text{H}, ^{13}\text{C}$ -HSQC (Fig. S6) and  $^1\text{H}, ^{13}\text{C}$ -HMBC (Fig. S7) are provided at the end of this document. NMR characterization was complicated due to pH effects adjusting the spectral data due to the presence of amine and phenolic groups. Due to this minor peaks were observed for several of the residues.

**C. Synthesis of probe mixture 12a/12b.** The synthesis of probe **12** from A80915C (**2**) was achieved through a comparable three-step process. A80915C (**2**) (15.0 mg, 27.4  $\mu\text{mol}$ ) was dissolved in anhydrous DMF (0.4 mL).  $\text{K}_2\text{CO}_3$  (30.0 mg, 217.1  $\mu\text{mol}$ ) as added followed by *tert*-butyl 2-bromoacetate (15.0  $\mu\text{L}$ , 101.6  $\mu\text{mol}$ ) dissolved in DMF (200  $\mu\text{L}$ ). After stirring at rt for 1h, the sample was dried by airflow. Copies of  $^1\text{H}$ -NMR spectra from **2** and ester **10** are provided in Fig. S8 and Fig. S9, respectively. Ester **10** (11.8 mg, 65%) was obtained by purification using Silica-gel HPLC eluting with a gradient of isooctane/EtOAc. Directly after purification, ester **10** (11.8 mg, 17.8  $\mu\text{mol}$ ) was dissolved in  $\text{CH}_2\text{Cl}_2$  (1 mL) and trifluoroacetic acid (150  $\mu\text{L}$ ) was added. After stirring at rt for 2h, the sample was dried by airflow. HATU (30 mg, 78.9  $\mu\text{mol}$ ) was added to a mixture of the crude acid **11** and IAF tag **8a** (15.0 mg, 51.8  $\mu\text{mol}$ ) dissolved in  $\text{Et}_3\text{N}$  (50  $\mu\text{L}$ ) and DMF (400  $\mu\text{L}$ ). After 12 h at rt, the sample was dried by airflow. The reaction product was dissolved in  $\text{CH}_3\text{CN}$  and purified by reversed phase C18 HPLC to yield 8.3 mg (53% from **10** or 34% overall from **2**) of probe mixture **12**.

**Probe 12:**  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 500 MHz)  $\delta$  12.13 (s, 1H), 7.43 (d,  $J = 9.0$  Hz, 1H), 7.15 (s, 1H), 7.04 (bs, 1H), 6.61, (dd,  $J = 8.9, 2.6$  Hz, 1H), 6.51 (d,  $J = 2.5$  Hz, 1H), 6.39 (bs, 1H), 6.03 (s, 1H), 4.61 (d,  $J = 14.7$  Hz, 1H), 4.55 (d,  $J = 13.6$  Hz, 1H), 4.53 (dd,  $J = 4.4, 11.5$  Hz, 1H), 3.67 (m, 1H), 3.62 (s, 1H), 3.55-3.42 (m, 4H), 3.07 (s, 3H), 2.65 (m, 1H), 2.62 (s, 1H), 2.57 (dd,  $J = 4.1, 14.2$  Hz, 1H), 2.45 (dd,  $J = 11.9, 14.2$  Hz), 2.29 (s, 3H), 2.01 (s, 2H), 1.96 (ddd,  $J = 3.2, 7.4, 14.0$  Hz, 1H), 1.86 (dd,  $J = 3.4, 3.4, 13.0$  Hz, 1H), 1.76 (m, 1H), 1.62-1.50 (m, 6H), 1.55 (s, 3H), 1.53 (s, 3H), 1.32 (s, 3H), 1.20 (s, 3H), 0.66 (s, 3H), 0.28 (s, 3H);  $^{13}\text{C}$ -NMR ( $\text{CDCl}_3$ , 150 MHz)  $\delta$  195.5, 190.2, 167.4, 162.8, 157.9, 156.4, 153.5, 132.6, 130.9, 125.4, 123.5, 120.8, 118.5, 110.0, 109.7, 109.0, 103.3, 97.9, 84.2, 81.6, 79.5, 78.4, 71.5, 71.3, 70.8, 67.5, 65.7, 59.9, 58.5, 50.1, 49.7, 46.8, 41.8, 41.3, 40.9, 40.1, 39.8, 38.9, 30.6, 29.1, 28.7, 28.5, 28.4, 28.2, 26.8, 24.6, 24.0, 22.9, 22.4, 21.5, 20.7, 20.2, 16.8, 16.2, 8.6; ESI-MS  $m/z$  876.25  $[\text{M}+\text{H}]^+$ ; HR-ESI-MS  $m/z$  calcd. for  $\text{C}_{43}\text{H}_{53}\text{Cl}_3\text{N}_3\text{O}_{10}$   $[\text{M}+\text{H}]^+$ : 876.2718, found 876.2226. Copies of  $^1\text{H}$ -NMR (Fig. S10 and Fig. S11),  $^1\text{H}, ^1\text{H}$ -gCOSY (Fig. S12),  $^1\text{H}, ^{13}\text{C}$ -HSQC (Fig. S13) and  $^1\text{H}, ^{13}\text{C}$ -HMBC (Fig. S14) are provided at the end of this document. The NMR data showed two sets of peaks for probe 12 with a major and minor ratio of ~4:1 likely due to the formation of zwitterionic states between the amine and phenolic groups.

**D. Tissue culture protocols.** HeLa and HCT116 cells were cultured in DMEM/Glutamax media (Life Technologies) supplemented with 10% fetal bovine serum (FBS). Cells were cultured in T<sub>75</sub> flasks at 37 °C with a 5% CO<sub>2</sub> atmosphere. Cells for imaging were grown uncoated 35 mm glass bottom microwell dishes (MatTek Cultureware #P35G-010-C).

**E. Apoptosis assays.** Cells were incubated in six well plates with a given concentration of compound for 24 h. Cells were detached from plates using trypsin digestion and collected by centrifugation followed by washing twice with Dulbecco's Phosphate-Buffered Saline (DPBS, Life Technologies). Apoptotic analysis was performed using YO-PRO-1 iodide - propidium iodide cocktail (Life Technologies) according to manufacturer's instructions. Briefly, cells were incubated on ice in a final concentration of 0.1 µM Yo-Pro1 and 4.5 mM propidium iodide for 30 min prior to flow cytometric analysis. Flow cytometric analysis was performed on a Becton-Dickinson LSR1 flow cytometer. Cell gating was performed by analysis of both a negative control (DMSO) and a positive control treated for 24 h with 10 µM camptothecin. Cells were gated to exclude debris. For each sample, 10,000 events were recorded.

**G. Subcellular localization studies.** Cells were cultured on uncoated 35mm glass bottom microwell dishes (MatTek Cultureware #P35G-010-C). Live cell staining was conducted using conventional methods. Briefly, HCT-116 and Hela cells were incubated for 24 h in 1 mL of 20 nM probe mixture **9a/9b** or **12** in DMEM/Glutamax media (Life Sciences) supplemented with 10% FBS. Subsequently, an equal volume of 5 µM BODIPY FL ceramide (Life Sciences) and 1 µM ER-tracker Red (BODIPY TR glibenclamide, Life Sciences) in Hanks Balanced Salt Solution (HBSS, Life Sciences) was added, and cells were incubated for 45 min at 37 °C. Cells were washed once in DPBS (1 mL) and then imaged live. Imaging was performed on a CARVIL spinning disk confocal microscope (Becton Dickinson Biosciences).

**H. Immunoprecipitation protocol.** HeLa or HCT-116 cell lysate was prepared by collecting cells with 2 volumes of DPBS with cell scraper (BD Falcon). The cells were frozen at -80 °C prior to use. The cells were re-suspended in DPBS containing 5 mM EDTA, 10 mM dithiothreitol (DTT), 1% NP-40. A mammalian protease inhibitor cocktail (Sigma-Aldrich) was added according to the manufacturer's protocols. The cell suspension was then passed through 27.5 gauge needle at least five times with syringe. The crude cell lysate was filtered through a 0.45 µm membrane to remove unlysed cells and nucleic acids. The resulting material was concentrated via centrifugation on a 3 kDa spin Ultra concentrator (Amicon) until the net concentration was 1 mg/mL in total protein content using the bicinchoninic acid assay (BCA) assay (ThermoScientific Pierce) according to manufacturer's instructions. Cell lysates were prepared fresh for all IP experiments and kept on ice or at 4 °C during the entire procedure. Probe mixture **9a/9b** or probe **12** was prepared as 20x stocks in DPBS containing 10% DMSO (10 mL) was added to the lysate (200 µL). After gentle shaking at 4 °C for 4 h, a 25-35 µL aliquot Affigel-10 resin containing ~4 mg/mL of anti-IAF XRI-TF35 antibody was added. The resulting slurry was incubated at 4 °C with shaking by gentle inversion using a Rotisserie Hybridization Rotator (Labquake). After 12 h, the mother liquor was removed and the resin was rapidly washed twice with ice-cold wash buffer (phosphate buffered saline (PBS), pH 7.2, 5 mM

EDTA, 1% NP-40, and 0.1% sodium dodecylsulfate (SDS)). The bound materials were eluted by incubation by addition of 35  $\mu$ L of 100  $\mu$ M 7-dimethylamino-4-coumarinacetic acid in PBS at rt for 30 min followed by centrifugation at 15,000 RPM for 5 min. The resulting solution was removed, diluted with 4x SDS-PAGE gel loading buffer (200 mM Tris-Cl pH 6.8, 400 mM DTT, 8% SDS, 0.4% bromophenol blue and 40% glycerol) and evaluated on a 4-14% gradient Bis-Tris SDS PAGE gel (Life Technologies).

**I. Trypsin Digest LC-MS/MS analyses.** The following procedures were used for the In Gel Digestion and LC-MS/MS of the bands presented in Fig. 3 from the bands identified in Fig. 2a. Protein ID analyses were conducted at UC San Diego's Biomolecular and Proteomics Mass Spectrometry Facility.

*In Gel Digest:* The gel slices were cut to 1 mm  $\times$  1 mm cubes and destained three times by first washing with 100  $\mu$ L of 100 mM  $\text{NH}_4\text{HCO}_3$  for 15 min, followed by addition of the same volume of  $\text{CH}_3\text{CN}$  for 15 min. The supernatant wash and samples were dried in on a Speedvac (Thermo Scientific). Samples were then reduced by mixing with 200  $\mu$ l of 100 mM  $\text{NH}_4\text{HCO}_3$  containing 10 mM DTT and incubated at 56  $^\circ\text{C}$  for 30 min. The liquid was removed and 200  $\mu$ L of 100 mM  $\text{NH}_4\text{HCO}_3$  containing 55 mM iodoacetamide was added to gel pieces and incubated at rt in the dark for 20 min. After the removal of the supernatant and one wash with 100 mM  $\text{NH}_4\text{HCO}_3$  for 15 min, the same volume of  $\text{CH}_3\text{CN}$  was added to dehydrate the gel pieces. The solution was then removed and samples were dried on a Speedvac (ThermoScientific). For digestion, enough solution of ice-cold trypsin (0.01  $\mu\text{g}/\mu\text{l}$ ) in 50 mM  $\text{NH}_4\text{HCO}_3$  was added to cover the gel pieces and set on ice for 30 min. After complete rehydration, the excess trypsin solution was removed, replaced with fresh 50 mM  $\text{NH}_4\text{HCO}_3$ , and left overnight at 37  $^\circ\text{C}$ . The peptides were extracted twice by the addition of 50  $\mu$ l of 0.2% formic acid and 5%  $\text{CH}_3\text{CN}$  and vortex mixing at rt for 30 min. The supernatant was removed and saved. A total of 50  $\mu$ l of 50%  $\text{CH}_3\text{CN}$  containing 0.2% formic acid was added to the sample, and the sample was vortexed at rt for 30 min. The supernatant was removed and combined with the supernatant from the first extraction. The combined extractions are analyzed directly by liquid chromatography (LC) in combination with tandem MS/MS mass spectroscopy using electrospray ionization.

*LC-MS/MS analysis:* Trypsin-digested peptides extracted from SDS-PAGE as described above were analyzed by liquid chromatography LC-MS/MS with electrospray ionization. All nanospray ionization experiments were performed by using a QSTAR-Elite hybrid mass spectrometer (AB/MDS Sciex) interfaced to a nanoscale reversed-phase high-pressure liquid chromatograph (Tempo) using a 10 cm 180 ID glass capillary packed with 5  $\mu\text{m}$  C18 Zorbax<sup>TM</sup> beads (Agilent). The buffer compositions were as follows. Buffer A was composed of 98%  $\text{H}_2\text{O}$ , 2%  $\text{CH}_3\text{CN}$ , 0.2% formic acid, and 0.005% trifluoroacetic acid; Buffer B was composed of 100%  $\text{CH}_3\text{CN}$ , 0.2% formic acid, and 0.005% trifluoroacetic acid. Peptides were eluted from the 18 column into the mass spectrometer using a linear gradient of 5–60% Buffer B over 60 min at 400  $\mu\text{L}/\text{min}$ . LC-MS/MS data were acquired in a data-dependent fashion by selecting the 4 most intense peaks with charge state of 2 to 4 that exceeds 20 counts, with exclusion of former target ions set to "360 seconds" and the mass tolerance for exclusion set to 100 ppm. Time-of-flight MS were acquired at  $m/z$  400 to 1600 Da for 1 s with 12 time bins to sum. MS/MS data were acquired from  $m/z$  50 to 2,000 Da by using "enhance all" and 24 time bins to sum, dynamic background subtract, automatic collision energy, and automatic MS/MS accumulation with the

fragment intensity multiplier set to 6 and maximum accumulation set to 2 s before returning to the survey scan. Peptide identifications were made using paragon algorithm executed in Protein Pilot 2.0 (Life Technologies).

**J. Western blot analyses.** Samples of the immunoprecipitated fractions obtained from the immunoprecipitation assay were evaluated for their protein content using bicinchoninic acid assay (BCA) assay 23235 (ThermoScientific Pierce) according to manufacturer's instructions. Protein (20 µg) was loaded per well on a 10% tris PAG polyacrylamide gel. Gels were wet transferred onto a polyvinylidene fluoride (PVDF) membrane and blocked in 5% dry milk. After blocking, the membrane was incubated overnight at 4° C with primary antibody mouse anti-GRP94 (G4545, Sigma Aldrich) at 1:500 in Tris-buffered saline containing 0.01% tween and 5% dry milk (TBST). The membrane was washed four times with an equal volume of TBST. Blot was incubated for 1 h in TBST with anti-mouse HRP antibody (1:5000). Detection was performed using Super Signal West Pico Chemiluminescent Substrate 34078 (ThermoScientific Pierce) according to manufacturer's instructions.

**K. Cloning and expression of hGrp94.** hGrp94 cloned from a cDNA library (OriGene) into pET28b vector containing 819 amino acids (22-803 aa) and an *N*-terminal 10 tag. The resulting hGrp94a5 vector was transfected into *Escherichia coli* One Shot BL-21 Star cells (Life Technologies). Protein (was obtained from bacterial pelleted from a 6 L production using standard IPTG induction procedures. Cells were lysed by French Press and the recombinant hGrp94 was purified via sequential Ni-NTA agarose resin, followed by anion exchange and gel filtration as described for the purification of canine Grp94 by Gewirth.<sup>1-3</sup> A total of 18.2 mg as determined by Bradford analysis was obtained from this growth. Protein was stored at 1 mg/mL in 20mM Tris-HCl, pH 8, 1mM EDTA, 0.1M NaCl, 1mM DTT and 10% glycerol. The His<sub>10</sub> tag was not removed for binding studies.

**L. Binding studies.** The hGrp94 protein was dialyzed from storage buffer into 20mM Tris-HCl, 0.1M NaCl, pH 8, and spin concentrated to afford a 20 µM stock as determined by Bradford analysis. This protein was used immediately after preparation (no storage or reuse). Equilibrium microdialysis was conducted on 5 kDa DispoEquilibrium Dialyzer (Harvard Apparatus) by loading 50 µL of 20 µM hGrp94 and 50 µL of analyte solution on opposite sides of the membrane. Seventeen different analyte concentrations (1, 2, 3, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, and 250 µM) were evaluated for each compound. Three repeats were conducted at each concentration. After addition, each device was stored at 4 °C. After 12 h, the analyte layer was removed and evaluated for the concentration of the analyte using LC-MS analysis equipped with a UV-Vis detector at 380 nm, a wavelength that only compounds **1** and **2** showed significant absorption. Concentrations of **1** and **2** were determined by comparison with analytical standards prepared at concentrations from 0.01 µM to 250 µM.

## Method A

Column: 4.5 x 250 mm Beckman Ultrasphere C18 (5  $\mu$ m) ion pairing (#235335).

Injection volume: 10  $\mu$ L

Temperature: 23°C

Flow rate: 1.2 ml/min.

Buffer A= aqueous 0.25% v/v formic acid adjusted to pH 6.0 with NaOH.

Buffer B= HPLC grade methanol

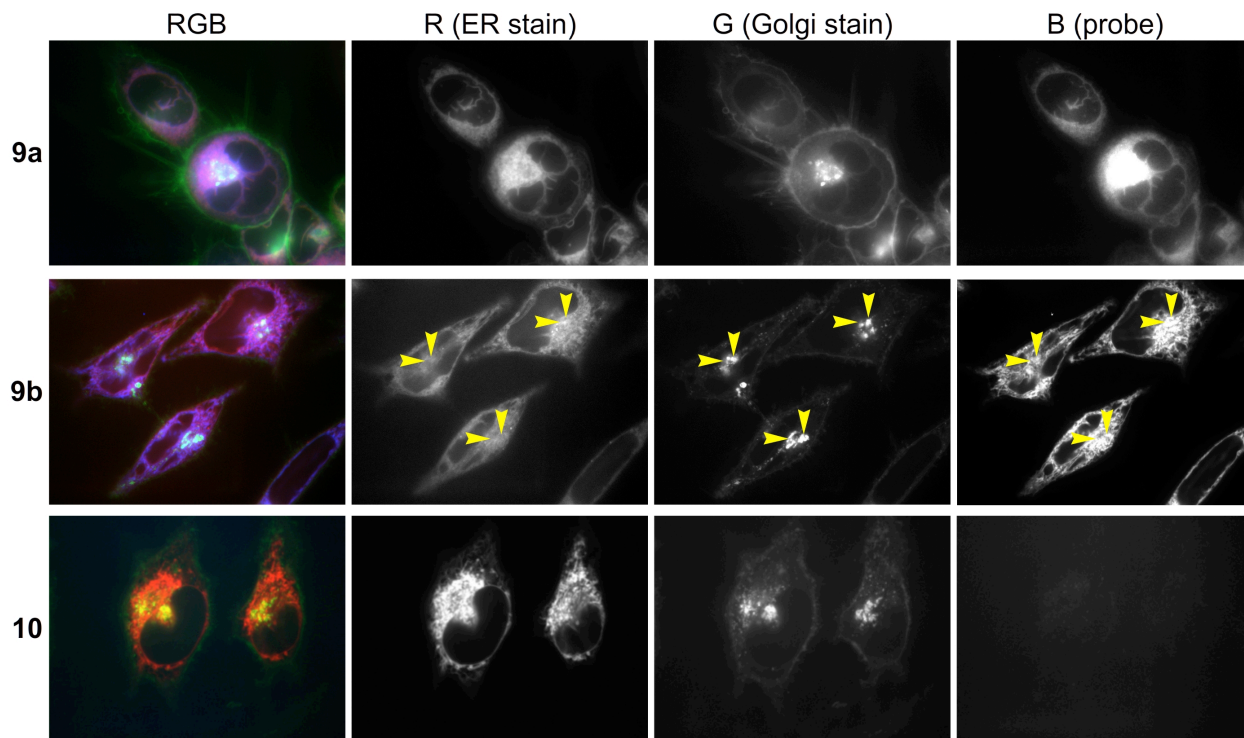
Binary linear gradient:

Time	%B
0	10
5	10
15	20
30	30
32	100
34	10
45	reinjection

Analytical reversed-phase HPLC chromatography was performed on a Waters model 600E liquid chromatograph with a C18 column (Vydac, 218TP1022, 10 x 250 mm), Waters Wisp 712 autoinjector, a Waters 486 tunable absorbance detector and a St. Johns 2001A Fluoro-Tec filter fluorometer. Compounds were evaluated using Method A (below). The retention times of 18.2 $\pm$ 0.3 min was observed for **1** and 22.5 $\pm$ 0.3 min for **2**. HPLC analyses were conducted in triplicate for each and the average was reported all studies within 3% deviation. Data were reported using the UV absorbance detected at 380 nM as given by peak area. Concentrations were determined by comparison with a series of standards of **1** and **2** with concentrations of 100 nM, 500 nM, 1  $\mu$ M, 2  $\mu$ M, 5  $\mu$ M, 10  $\mu$ M, 15  $\mu$ M, 25  $\mu$ M and 50  $\mu$ M. Each experiment was conducted in triplicate and error bars were reported depicting deviations from these runs. For each experiment, an average of three HPLC readings was collected. Data was processed and plotted using Prism 6 (Graphpad).

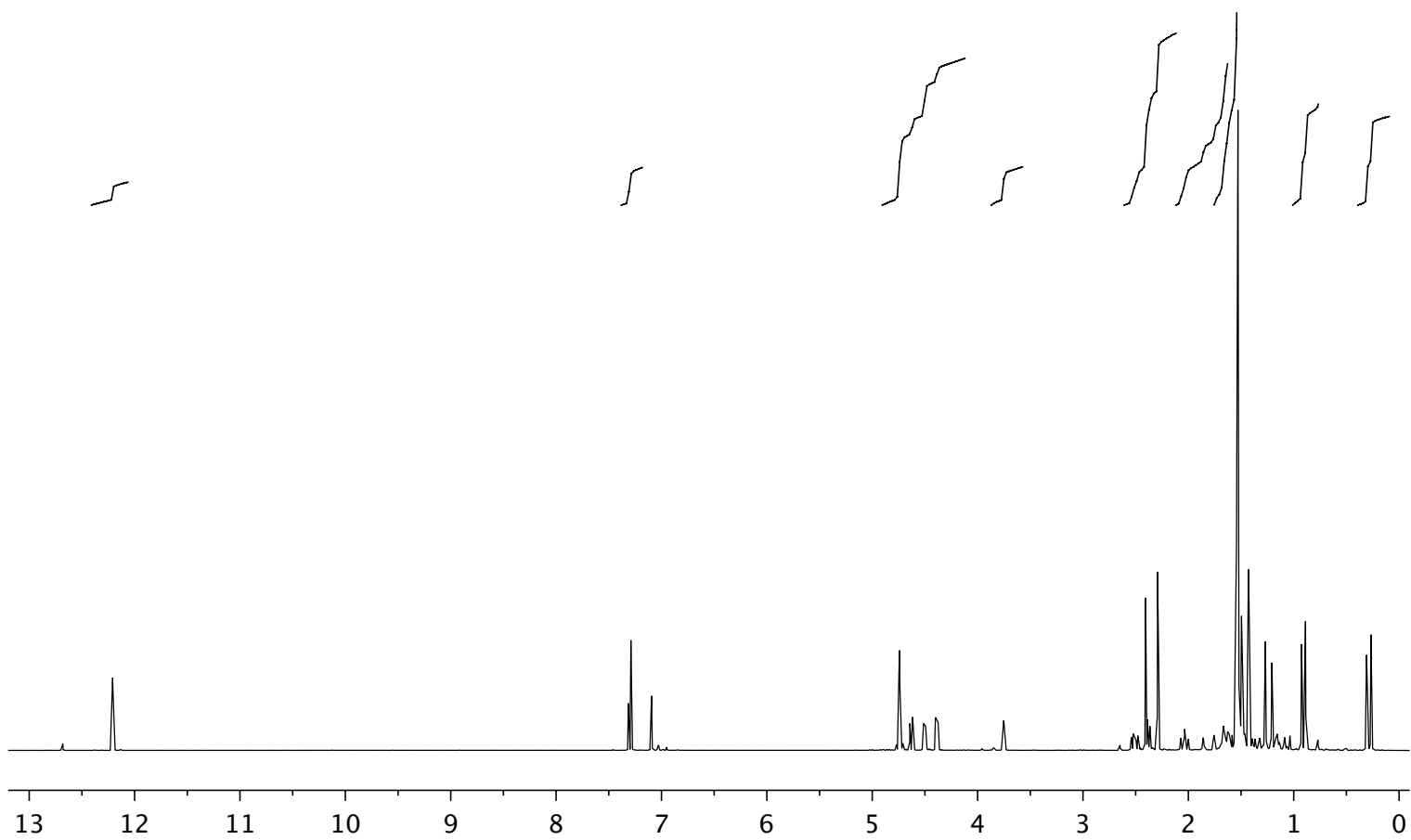
## References

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2. R. M. Immormino, D. E. Dollins, P. L. Shaffer, K. L. Soldano, M. A. Walker, and D. T. Gewirth, *J. Biol. Chem.* 2004, **279**, 46162
3. K. L. Soldano, A. Jivan, C. V. Nicchitta, and D. T. Gewirth, *J. Biol. Chem.* 2003, **278**, 48330

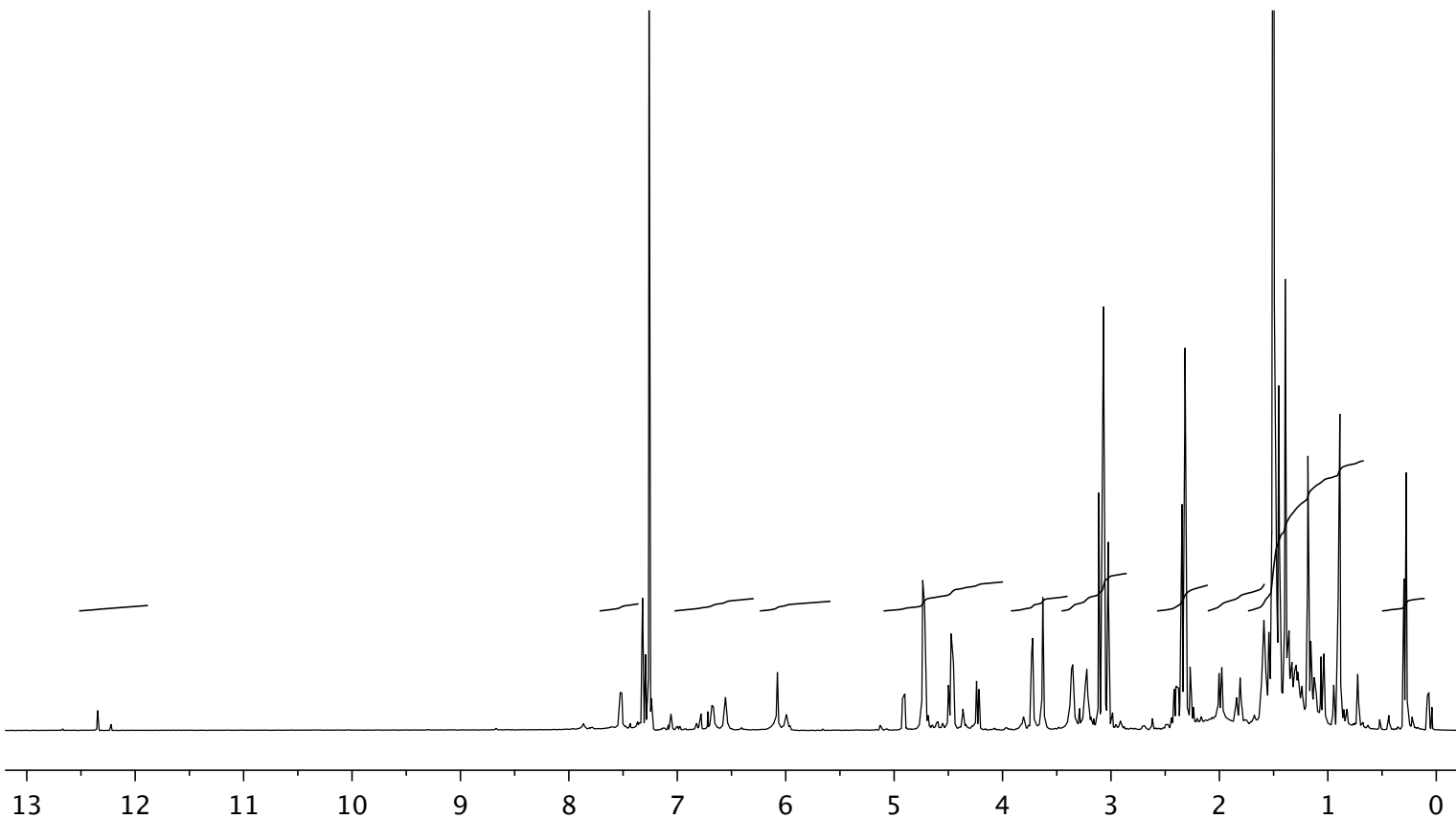


**Figure S1.** Expansion and single color-channel display of the images provided in Fig. 4. Images particularly of that shown for probe **12** show the selective localization of the blue fluorescence from the probe (right) overlapping the red fluorescence from the ER stain (left), as given by the yellow arrows.

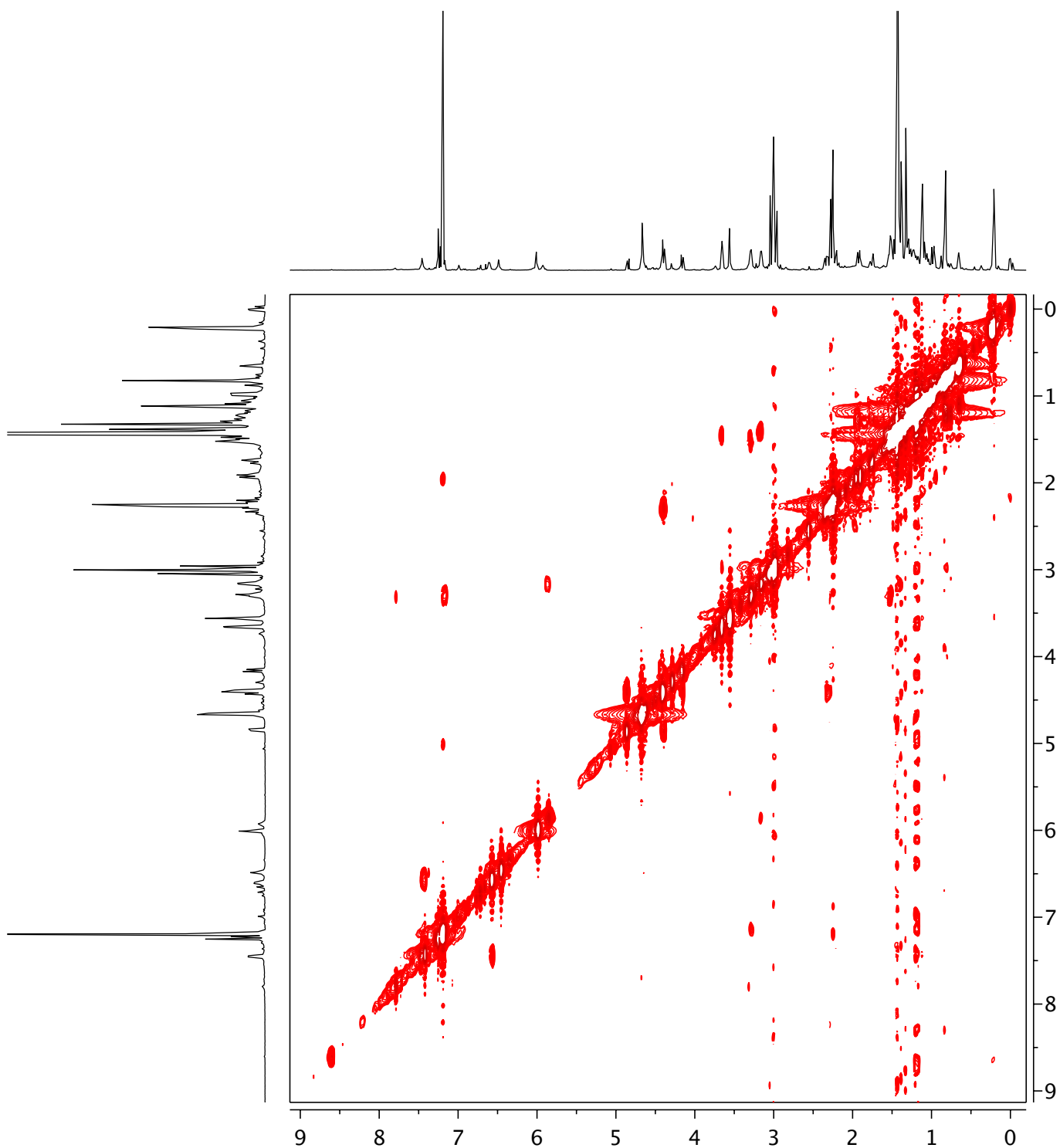




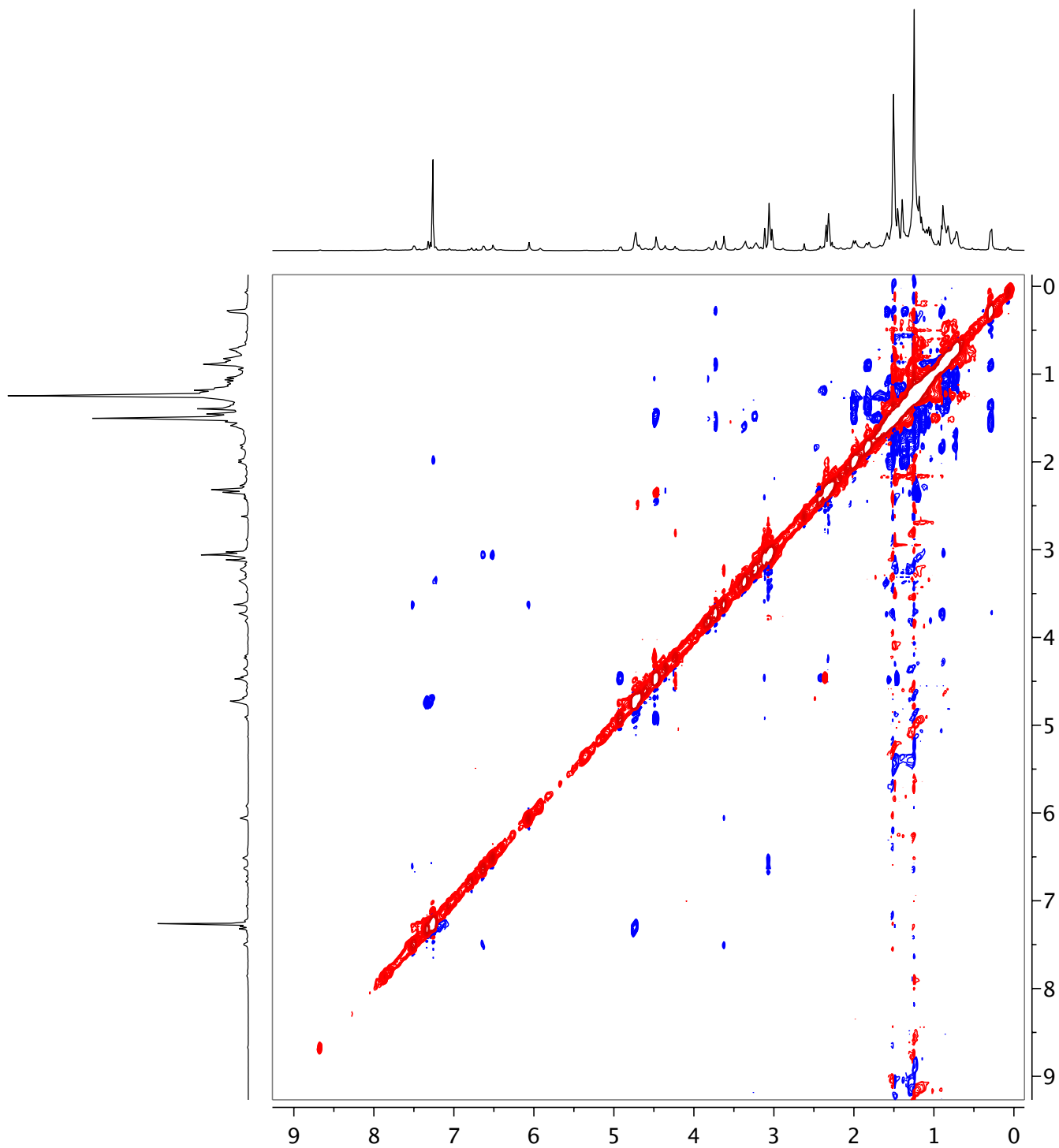
**Figure S2.**  $^1\text{H-NMR}$  spectrum (600 MHz) of a 1:1 mixture of intermediates **6a/6b** in  $\text{CDCl}_3$



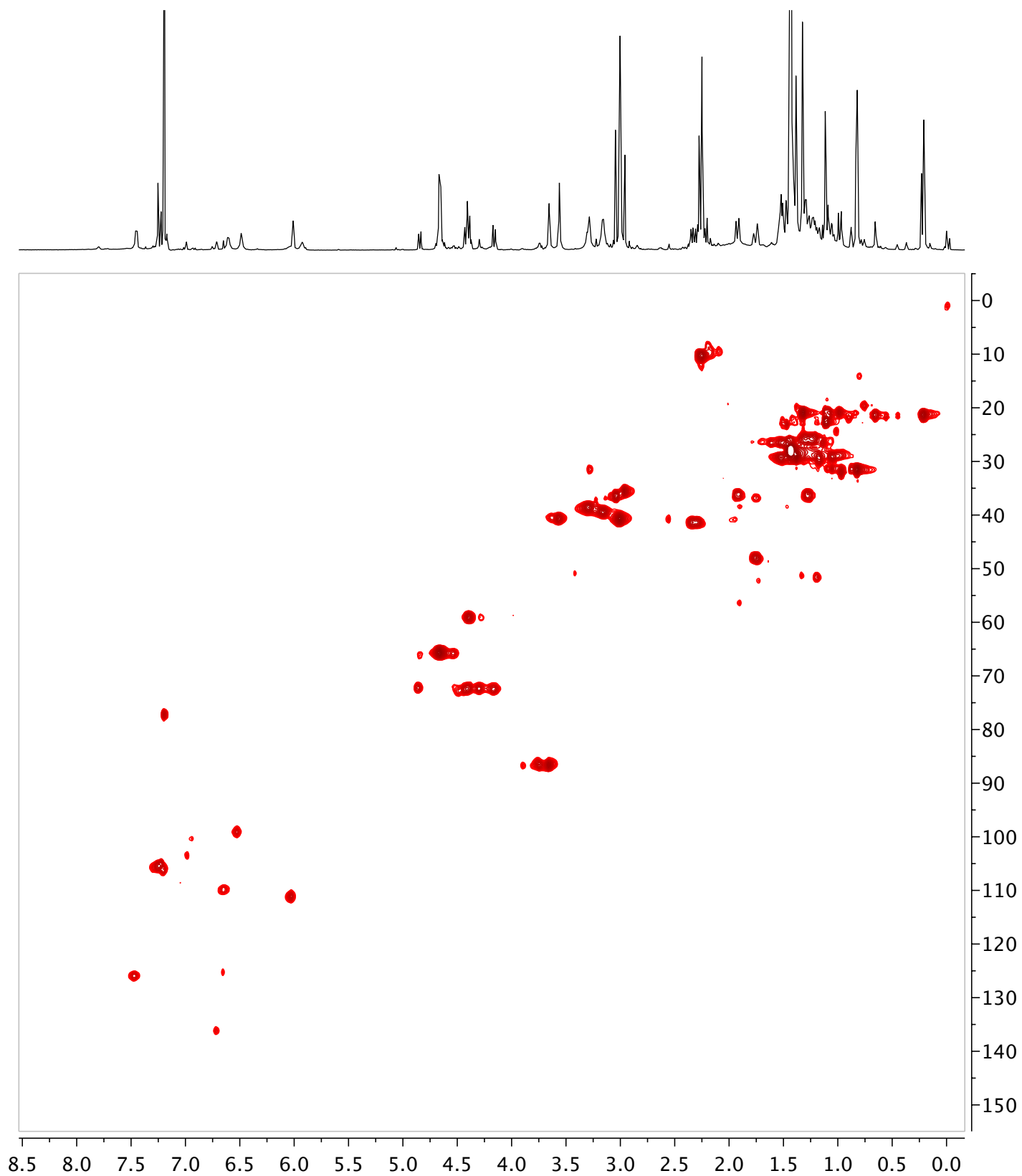
**Figure S3.**  $^1\text{H-NMR}$  (600 MHz) spectra of probes **9a/9b** in  $\text{CDCl}_3$



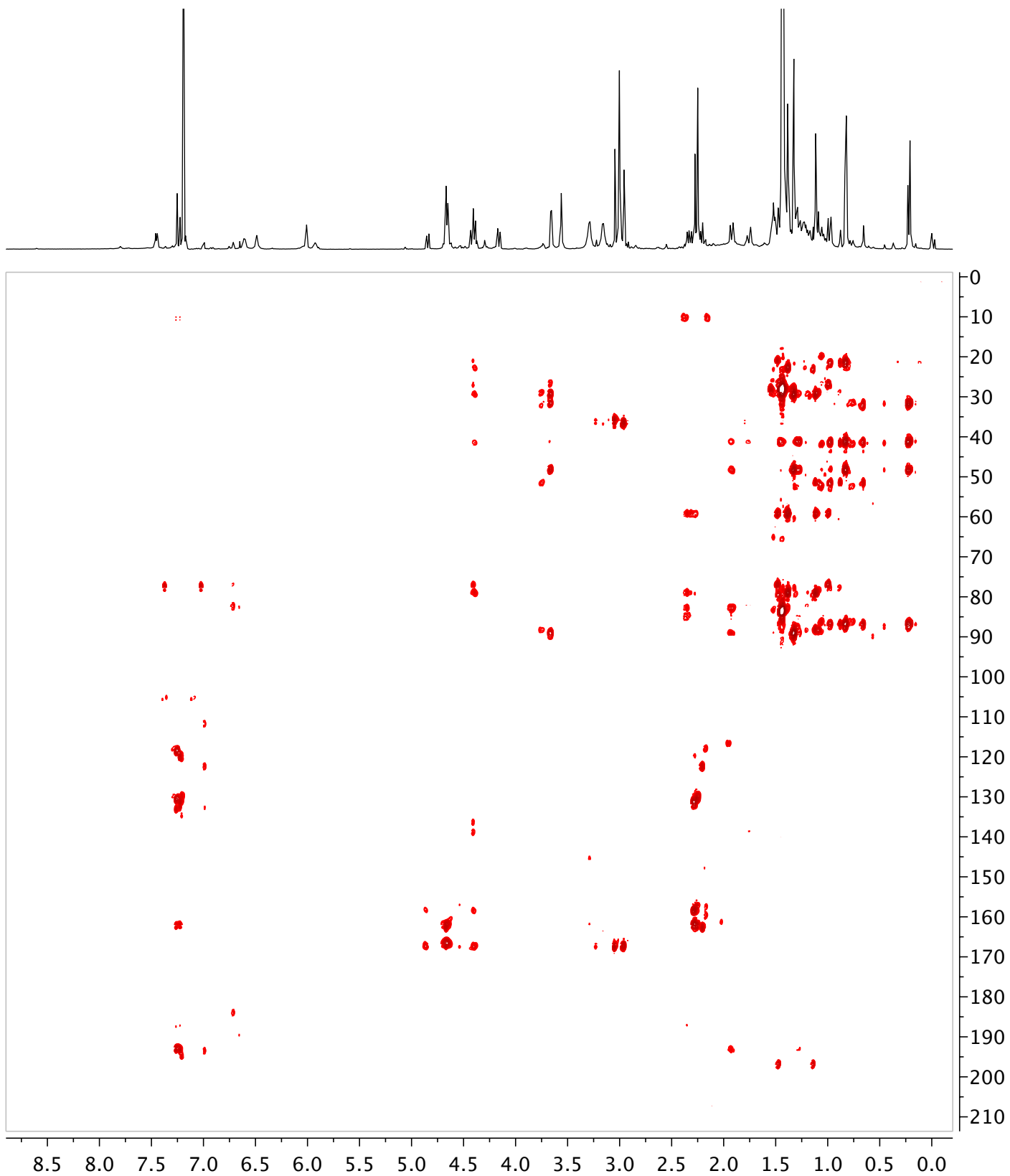
**Figure S4.**  $^1\text{H}, ^1\text{H}$ -gCOSY (600 MHz) spectra of probes **9a/9b** in  $\text{CDCl}_3$



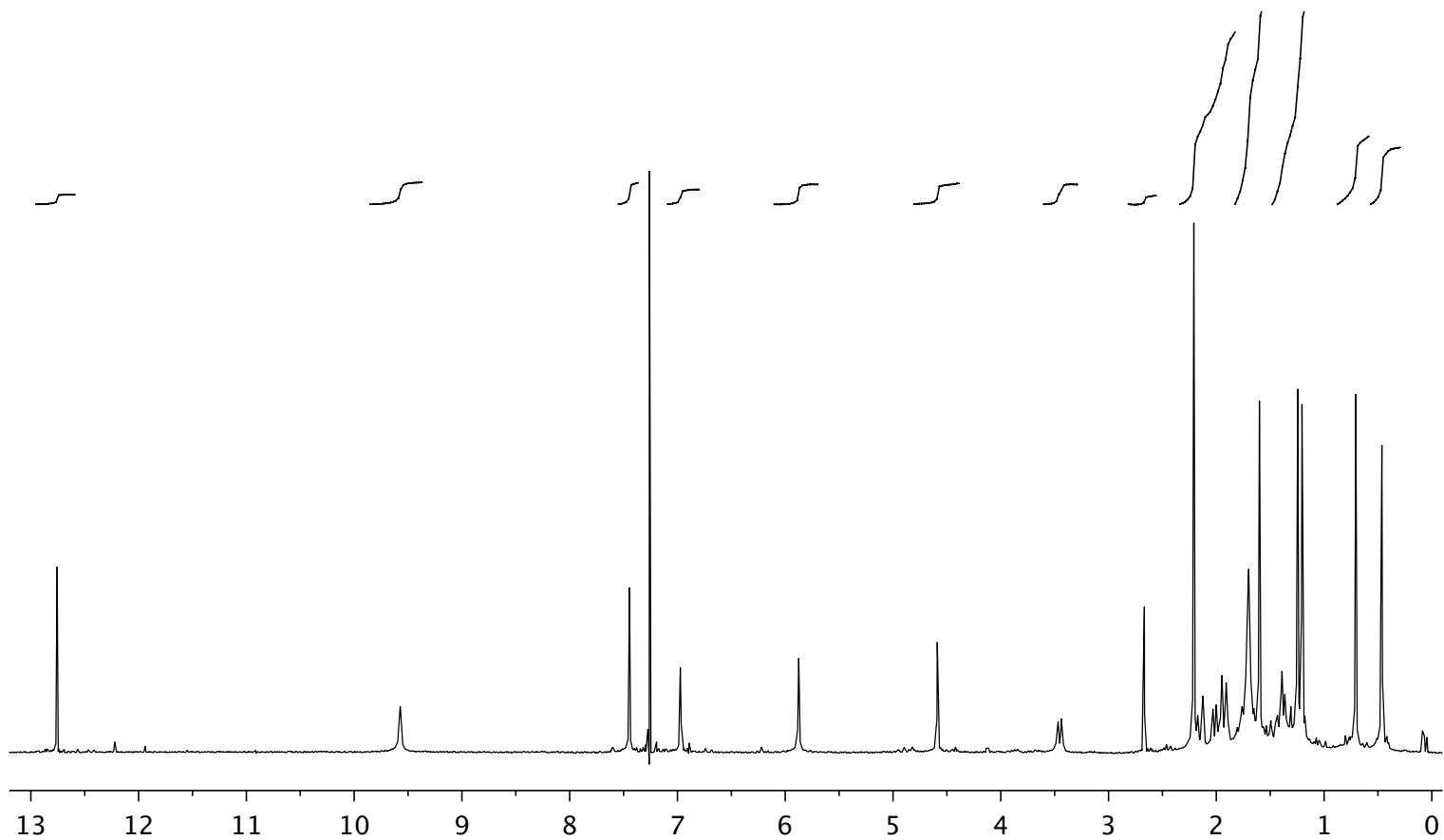
**Figure S5.**  $^1\text{H}$ ,  $^1\text{H}$ -ROESY spectrum (600 MHz) of probes **9a/9b** in  $\text{CDCl}_3$



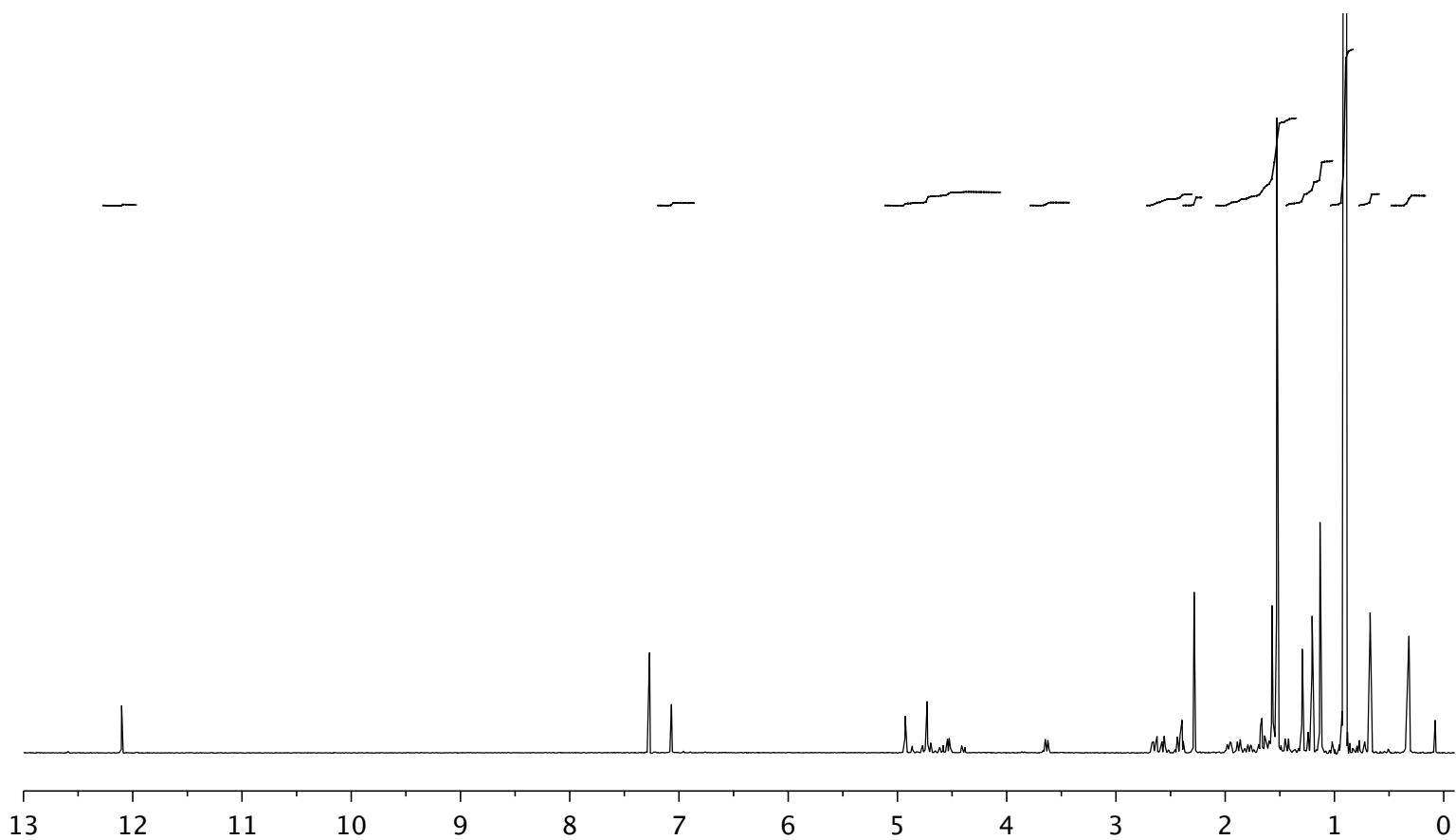
**Figure S6.**  $^1\text{H}$ ,  $^{13}\text{C}$ -HSQC spectrum of probes **9a/9b** in  $\text{CDCl}_3$



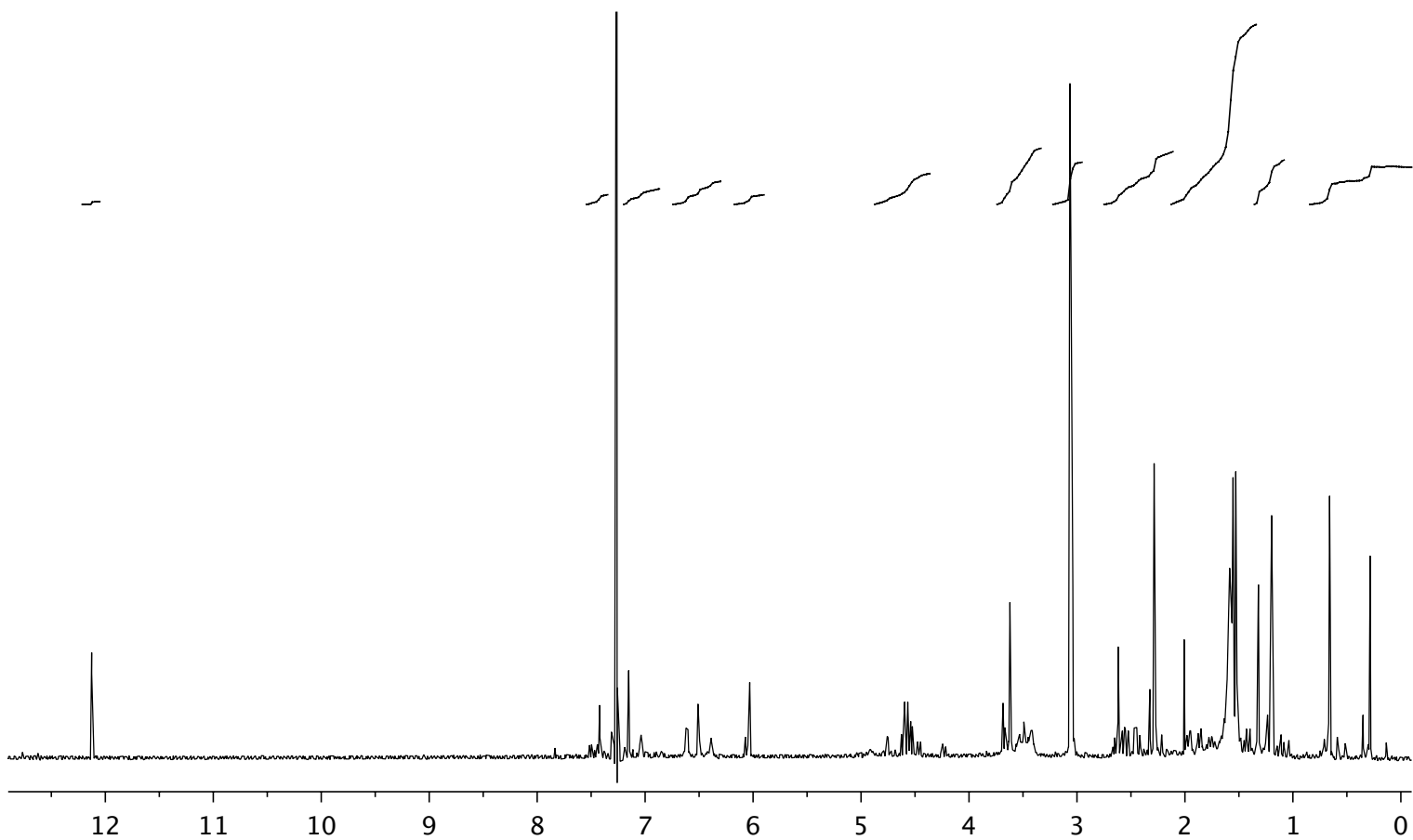
**Figure S7.**  $^1\text{H}$ ,  $^{13}\text{C}$ -HMBC spectrum of probes **9a/9b** in  $\text{CDCl}_3$



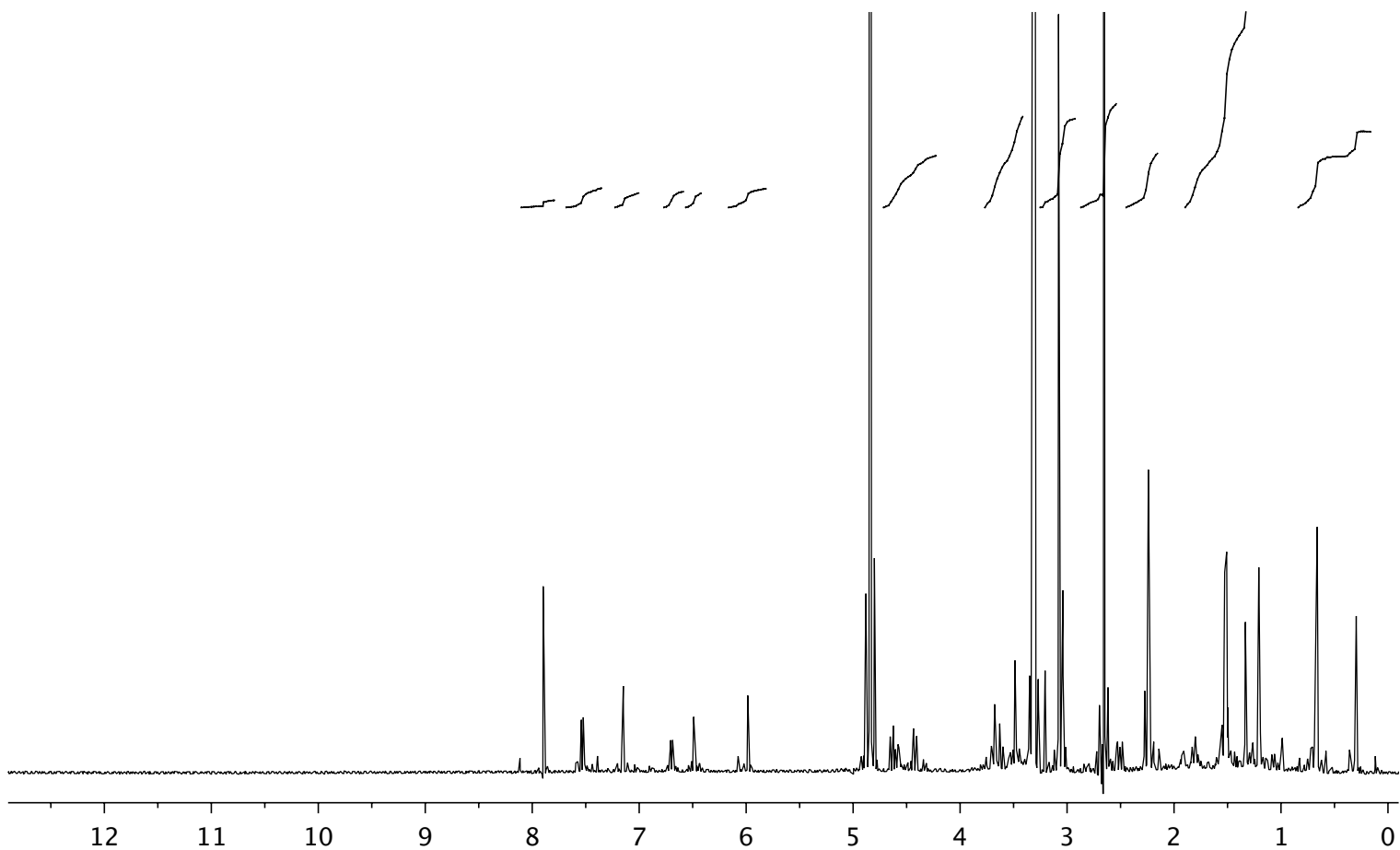
**Figure S8.** <sup>1</sup>H-NMR spectrum (300 MHz) of A80915 (**2**) in CDCl<sub>3</sub>



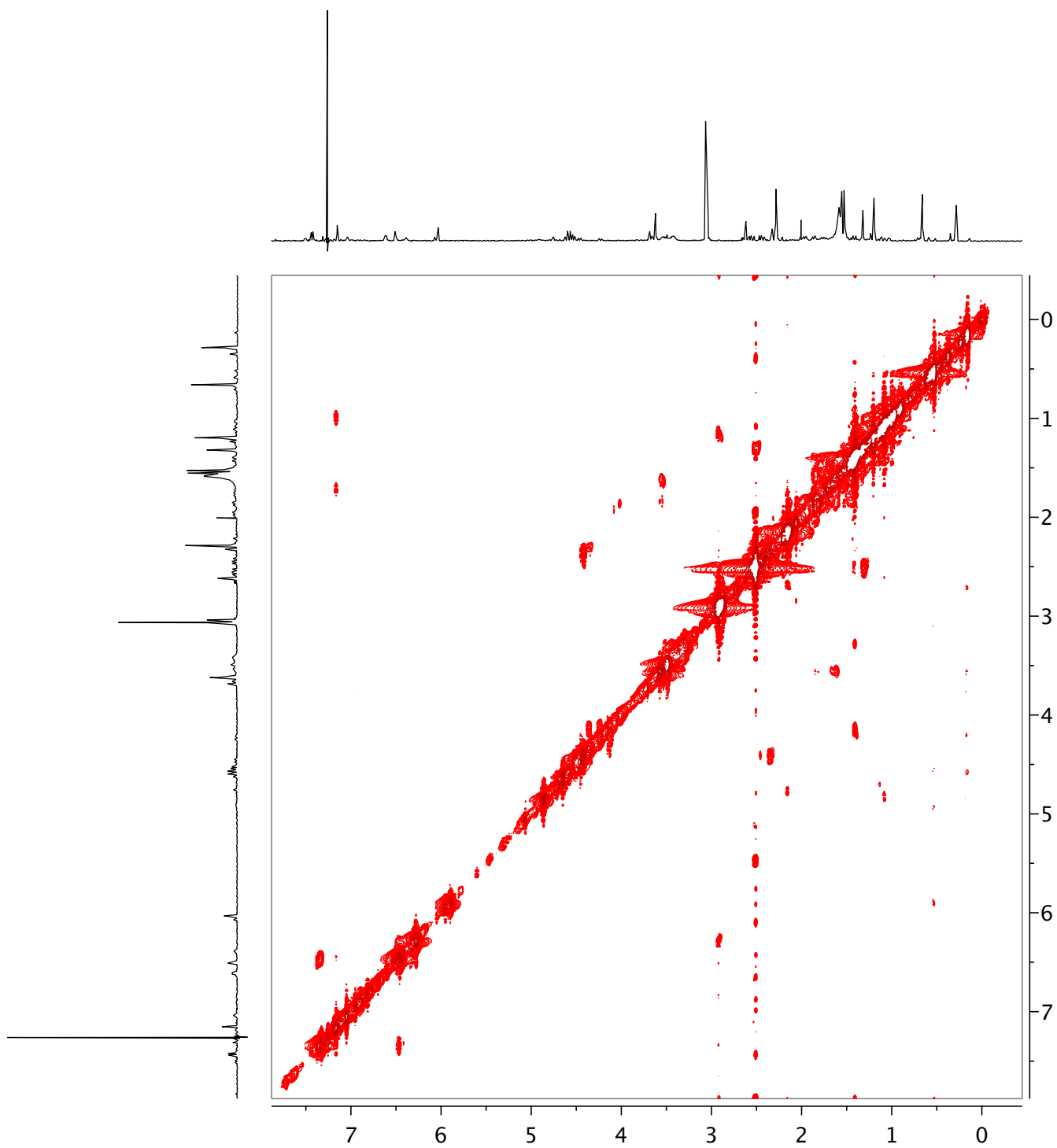
**Figure S9.** <sup>1</sup>H-NMR spectrum (500 MHz) of intermediate **10** in CDCl<sub>3</sub>



**Figure S10.**  $^1\text{H-NMR}$  spectrum (500 MHz) of probe **12** in  $\text{CDCl}_3$

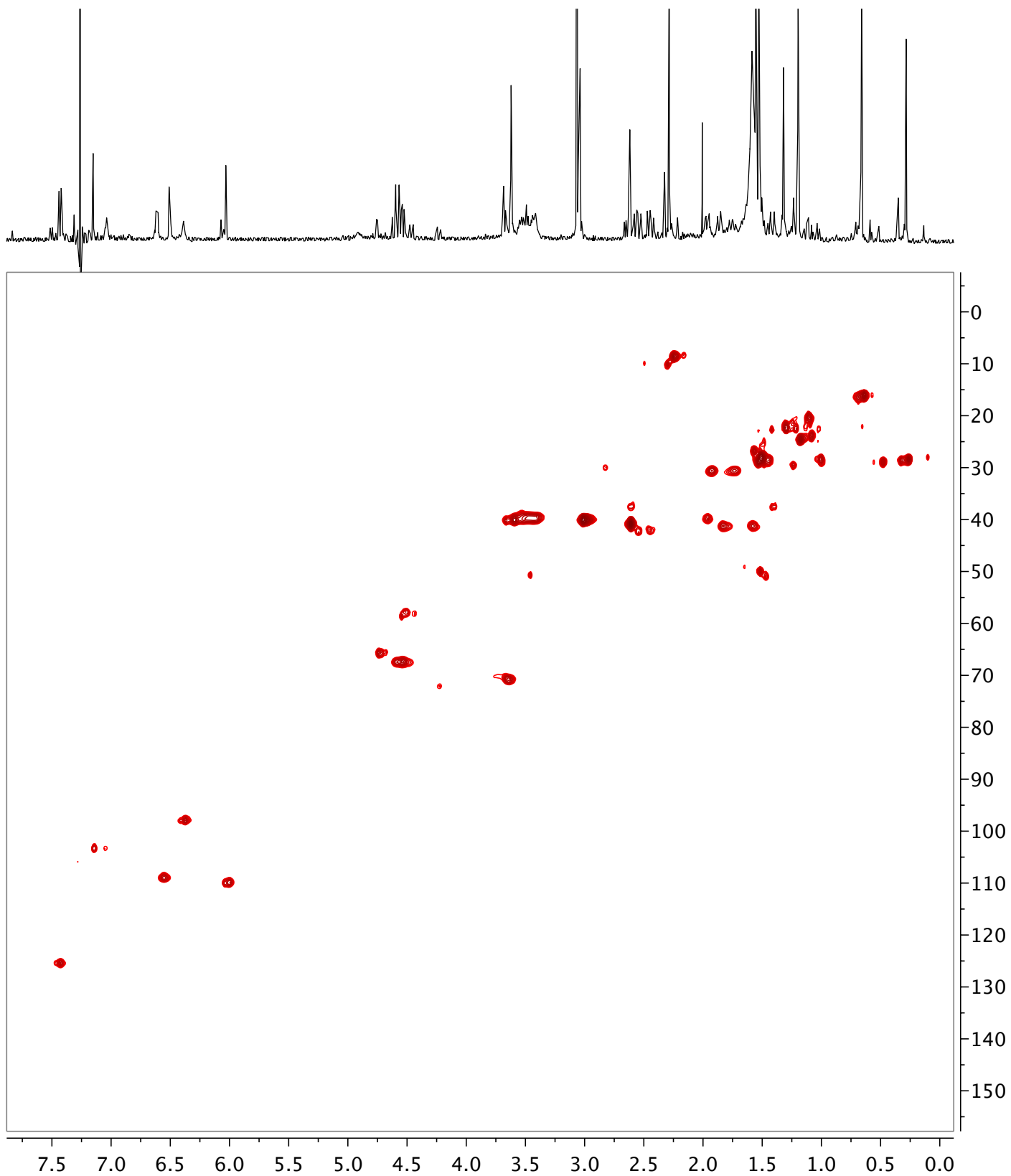


**Figure S11.**  $^1\text{H-NMR}$  spectrum (500 MHz) of probe **12** in  $\text{CD}_3\text{OD}$

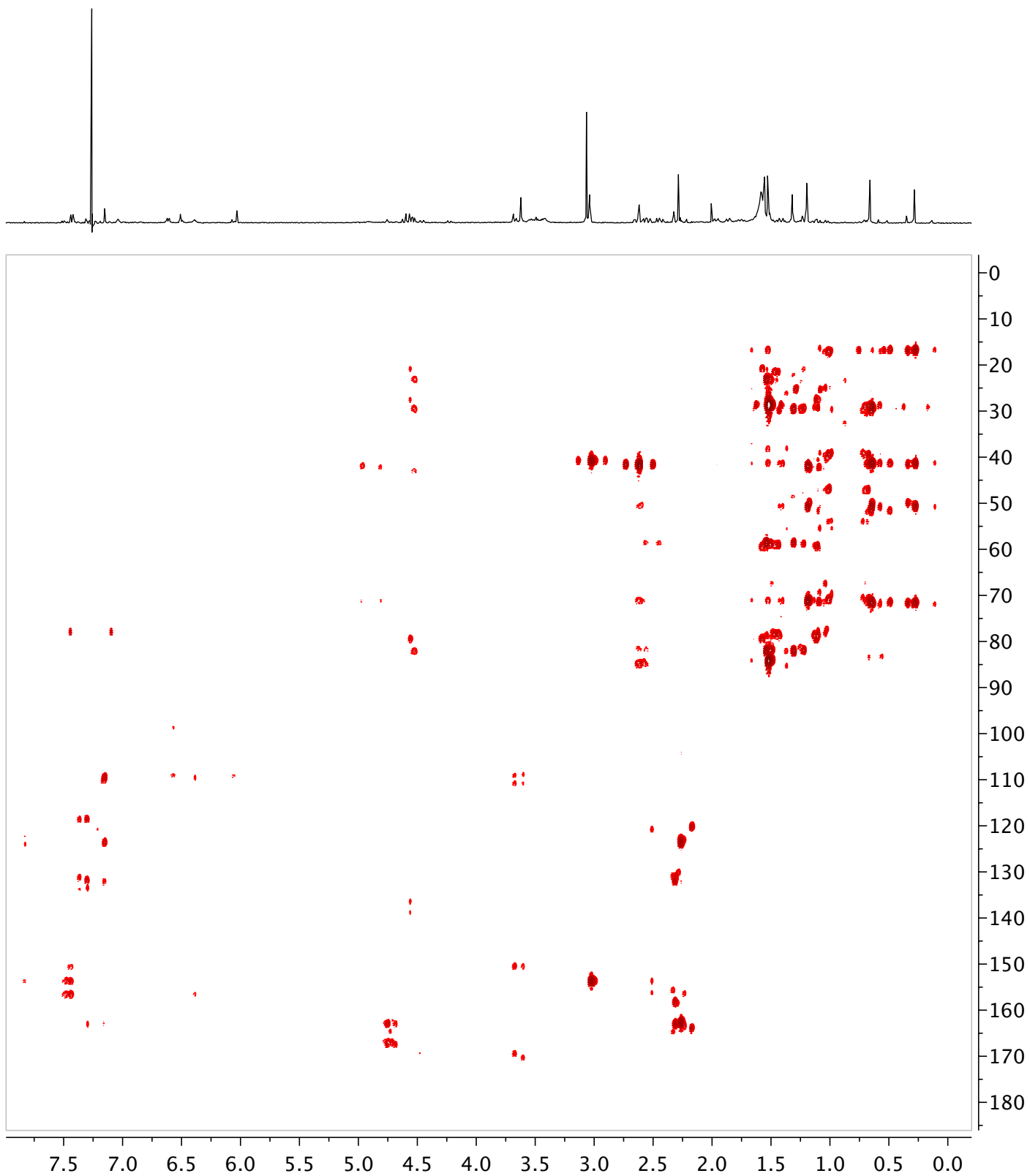


**Figure S12.**  $^1\text{H},^1\text{H}$ -gCOSY spectrum (600 MHz) of probe **12** in  $\text{CDCl}_3$





**Figure S13.**  $^1\text{H}$ ,  $^{13}\text{C}$ -HMBC spectrum of probe **12** in  $\text{CDCl}_3$



**Figure S14.**  $^1\text{H}$ ,  $^{13}\text{C}$ -HMBC spectrum of probe **12** in  $\text{CDCl}_3$