Click and Count: Specific Detection of Acid Ceramidase Activity in Live Cells.

Mireia Casasampere, Eduardo Izquierdo, Josefina Casas, José Luís Abad, Xiao Liu, Ruijuan Xu, Cungui Mao, Young-Tae Chang, Antonio Delgado, Gemma Fabrias

SUPPLEMENTARY MATERIALS

Supplementary discussion

Intriguingly, the intracellular staining results found in this article are in discrepancy with the data reported by Alecu et al.[1]. Using an ω-alkyne derivative of deoxysphinganine and a sulfo-BODIPY substituted azide reagent for copper-catalyzed click reaction, these authors clearly showed that their system did not localize in the lysosome, but in the mitochondria, and nicely correlated this localization with the mitochondrial disfunction provoked by deoxysphingolipids in diabetic neuropathy and HSAN1. However, in all the conditions tested, the RBM5-019/CO-1 labeling system studied here exhibited exclusive localization in the lysosome with no mitochondria staining as found in co-localization studies with Mitotracker (ESI Fig. S5) (Pearson= 0.298; M1= 0.179; M2=0.070 on average). The reasons for the discordance between our results and those of Alecu et al. are so far unknown. Both systems are based on the use of a labelable deoxysphinganine probe in combination with a suitable BODIPY-based reagent. However, the bioothogonal labelling schemes are different. In the method reported by Alecu *et al.* an alkyne-tagged deoxysphinganine probe is reacted with an azide-tagged BODIPY reagent through a Cu(I)-catalyzed alkyne-azide cycloaddition in fixed cells, whereas in the present work, the azido-tagged DOXSa probe RBM5-019 undergoes strain-promoted alkyne-azide cycloaddition with the bicyclo[6.1.0]nonyne-tagged BODIPY reagent CO-1 in live cells. Differences in the cell lines used may also account for the different localization. Interestingly, part of the RBM5-177/CO-1 extra-lysosomal staining observed in AC-overexpressing cells (see Fig. 4) was found to localize significantly with mitotracker (Mander's coefficient M2= 0.697) (Fig. S5), although most of the labelling was found in the lysosome (Pearson's coefficient with mitotracker was 0.355). As expected, mitochondria labelling was precluded by independent treatments with FB1 and SOCLAC, proving that it was both AC and CerS-dependent. Localization studies with the acylated derivative of their alkynyl deoxysphinganine were not reported.

Experimental procedures

CHEMISTRY

Reagents and general methods

All chemicals were purchased from commercial sources and used as received unless otherwise noted. Dry solvents (THF, DMF and DCM), obtained from a PureSolv dispenser and subsequently degassed with inert gas, were used in most reactions. Synthesis grade (Hexane, EtOAc, Et2O and DCM) or HPLC–grade (MeOH) solvents were used for extractions and purifications. Unless otherwise specified all reactions were performed under Ar inert atmosphere. Progression of the reactions was controlled by thin layer chromatography (TLC),

using ALUGRAM® SIL G/UV254 (Macherey–Nagel) silica gel pre–coated aluminum sheets (Layer: 0.2 mm, silica gel 60). Compounds were detected by using UV light (λ=254 nm) and a stain solution of phosphomolibdic acid (5.7% in EtOH). Compounds were purified by flash column chromatography, using silica gel (Chromatogel 60 Å, 35–75 μm) as stationary phase. Mobile phases and gradients are specified in each case in the following section. ^{1}H and ^{13}C Nuclear Magnetic Resonance spectra were recorded on a Varian – Mercury 400 (^{1}H NMR at 400 MHz and ^{13}C NMR at 100.6 MHz) spectrometer using $CDCl_3$ or CD_3OD as solvent. Chemical shifts of deuterated solvents were used as internal standards. Chemical shifts are given in parts per million (ppm) and coupling constants (*J*) in Hertz (Hz). Splitting patterns have been described as singlet (s), broad singlet (br s), doublet (d), triplet (t), quartet (q), quintuplet (p), multiplet (m), apparent (app) or combinations of these descriptive names. Fourier transform infrared spectra were recorded on a FTIR Avatar 360 spectrophotometer. Samples were recorded neat using an attenuated total reflectance (ATR) accessory, or as thin films on NaCl plates (specified in each case in the following section). IR frequencies are reported in wave number (cm^{-1}). Specific optical rotations were recorded on a digital Perkin–Elmer 34 polarimeter at 25 ºC in a 1-dm 1-mL cell, using a sodium light lamp (λ=589 nm). Specific optical rotation values ([α]D) are expressed in $deg^{-1}{\cdot}cm^{3}{\cdot}g^{-1}$, and concentrations (c) are reported in g/100 mL of solvent. High–resolution mass spectra were recorded Waters Aquity UPLC system connected to a Waters LCT Premier Orthogonal Accelerated Time of Flight Mass Spectrometer (Waters,Milford, MA, USA) operated in positive electrospray ionisation mode. Samples were analyzed by FIA (Flow Injection Analysis), using ACN/water (70:30) as mobile phase. Samples were analyzed using a 10 μL volume injection. M/z ratios are reported as values in atomic mass units. The synthesis of compounds RBM2-031, RBM2-037 and CO-1 has been previously reported in the literature.[2,3]

***tert*-Butyl(dodec-11-yn-1-yloxy)diphenylsilane (RBM5-013).** Neat tert-butyldiphenylsilyl chloride (7.0 mL, 26.4 mmol) was added dropwise to an ice–cooled suspension of dodec-11-yn-ol[4] (4.0 g, 22.0 mmol) and imidazole (3.0 g, 43.9 mmol) in anhydrous CH2Cl2 (80 mL). After stirring for 2 h at rt, the reaction was quenched by the addition of water (50 mL), and the resulting mixture was extracted with CH2Cl2 (3 x 50 mL). The combined organic extracts were washed with brine, dried over anhydrous MgSO4, and concentrated under reduced pressure. The crude product was purified by flash column chromatography (from 0 to 2 % Et2O in hexanes) to give compound RBM5-013 (7.8 g, 84 %) as a colourless oil. Rf: 0.76 (Hexanes–EtOAc 8:2). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.69 – 7.65 (m, 4H), 7.44 – 7.35 (m, 6H), 3.65 (t, J = 6.5 Hz, 2H), 2.18 (td, J = 7.1, 2.6 Hz, 2H), 1.94 (t, J = 2.6 Hz, 1H), 1.61 – 1.46 (m, 4H), 1.36 (dt, J = 21.3, 7.6 Hz, 4H), 1.26 (s, 8H), 1.05 (s, 9H).^{13}C NMR (101 MHz, CDCl3) δ 135.7, 134.3, 129.6, 127.7, 84.9, 68.2, 64.1, 32.7, 29.7, 29.6, 29.5, 29.2, 28.9, 28.6, 27.0, 25.9, 19.4, 18.5. ATR–FTIR (neat) (ν, cm^{-1}): 3311, 3071, 2930, 2856, 1471, 1428, 1389, 1361, 1111, 823, 739, 701, 687, 613.

***tert*-Butyl ((2S,E)-15-((tert-butyldiphenylsilyl)oxy)-3-hydroxypentadec-4-en-2-yl)carbamate (RBM5-014).** Neat RBM5-013 (1.8 g, 4.2 mmol) was added to an ice–cooled suspension of Cp2Zr(H)Cl (1.3 g, 5.1 mmol) in anhydrous CH2Cl2 (5 mL) under argon. After stirring at rt for 40 min, the resulting orange solution was cooled to –40 ºC and then treated with Et2Zn (1.0 M in hexanes, 5.7 mL, 5.7 mmol), followed by the dropwise addition of a solution of N-Boc L-alaninal.[5](700 mg, 4.04 mmol) in anhydrous CH2Cl2 (3 mL). After stirring for 2 h at 0 ºC, the reaction mixture was poured onto ice-water and extracted with CH2Cl2 (3 x 50 mL). The combined organic extracts were washed with brine, dried over anhydrous MgSO4, filtered and concentrated in vacuo. Purification of the crude product by flash column chromatography (from 0 to 30 % EtOAc in hexanes) furnished RBM5-014 (450 mg, 19 %, inseparable 5:1 mixture

of syn/anti diastereomers) as a light-yellow thick oil. Rf: 0.62 (Hexanes–EtOAc 6:4). 1H NMR (400 MHz, CDCl3) (syn diastereomer) δ 7.69 – 7.65 (m, 4H), 7.44 – 7.35 (m, 6H), 5.71 (dtd, J = 15.4, 6.7, 1.0 Hz, 1H), 5.47 (ddt, J = 15.4, 7.1, 1.5 Hz, 1H), 4.63 (br s, 1H), 3.96 – 3.91 (m, 1H), 3.65 (app t, J = 6.5 Hz, 3H), 2.26 (s, 1H), 2.08 – 2.00 (m, 2H), 1.60 – 1.51 (m, 2H), 1.45 (s, 9H), 1.40 – 1.31 (m, 4H), 1.25 (t, J = 3.4 Hz, 10H), 1.14 (d, J = 6.8 Hz, 3H), 1.05 (s, 9H). 13C NMR (101 MHz, CDCl3) (syn diastereomer) δ 156.2, 135.6, 134.2, 133.8, 129.6, 129.5, 127.6, 79.3, 75.8, 64.0, 51.0, 32.6, 32.4, 29.7, 29.6, 29.5, 29.4, 29.3, 29.2, 28.5, 26.9, 25.8, 19.2, 17.6. FTIR (NaCl) (ν, cm^{-1}): 3439, 3071, 2929, 2855, 1693, 1502, 1428, 1390, 1366, 1171, 1111, 1050, 702, 603. HRMS calcd. for C36H58NO4Si ([M+H]+): 596.4130, found: 596.4130.

***tert*-Butyl ((2S)-15-((tert-butyldiphenylsilyl)oxy)-3-hydroxypentadecan-2-yl)carbamate (RBM5-015).** Compound RBM5-015 (light yellow oil, 260 mg, 86 %, inseparable 5:1 mixture of syn/anti diastereomers) was obtained from alkene RBM5-014 (300 mg, 0.50 mmol), and Rh/Al2O3 (30 mg) in degassed EtOAc (10 mL) by hydrogenation at 1 atm. After stirring for 3 h, the catalyst was removed by filtration through a Celite® pad, and the solids were rinsed with EtOAc (3 x 10 mL). The combined filtrates were concentrated in vacuo, and the resulting residue was subjected to flash chromatography on silica gel. The title compound was purified by flash chromatography (from 0 to 18 % EtOAc in hexanes). Rf: 0.68 (Hexanes–EtOAc 6:4). 1H NMR (400 MHz, CDCl3) (syn diastereomer) δ 7.69 – 7.65 (m, 4H), 7.44 – 7.35 (m, 6H), 4.66 (br s, 1H), 3.65 (app t, J = 6.5 Hz, 3H), 3.51 – 3.44 (m, 1H), 1.59 – 1.51 (m, 2H), 1.48 – 1.40 (m, 11H), 1.36 – 1.22 (m, 18H), 1.17 (d, J = 6.8 Hz, 3H), 1.05 (s, 9H). 13C NMR (101 MHz, CDCl3) (syn diastereomer) δ 156.3, 135.7, 134.3, 129.6, 127.7, 79.4, 75.0, 64.1, 50.3, 34.3, 33.6, 32.7, 29.8, 29.8, 29.8, 29.7, 29.7, 29.5, 28.5, 28.5, 27.0, 26.2, 25.9, 25.8, 19.3, 18.5. FTIR (NaCl) (ν, cm^{-1}): 3438, 3071, 3051, 2927, 2854, 1715, 1687, 1501, 1365, 1167, 1105, 700. HRMS calcd. for C36H60NO4Si ([M+H]+): 598.4286, found: 598.4270.

(4S,5R)-5-(12-((tert-Butyldiphenylsilyl)oxy)dodecyl)-4-methyloxazolidin-2-one (RBM5-016). Neat methanesulfonyl chloride (109 µL, 1.4 mmol) was added to an ice–cooled stirred solution of RBM5-015 (560 mg, 0.9 mmol) in anhydrous CH2Cl2 (20 mL) containing Et3N (260 µL, 1.9 mmol). After stirring for at rt for 2 h, the reaction mixture was quenched by adding water (15 mL) and extracted with CH2Cl2 (3 x 25 mL). The combined organic extracts were washed with brine (25 mL), dried over anhydrous MgSO4, and evaporated to dryness. The resulting solid residue was taken up in 1,2–DCE (20 mL), treated with Et3N (653 µL, 4.7 mmol) and heated to reflux. After stirring overnight, the reaction was cooled to rt, water was added (15 mL), and the resulting mixture was extracted with CH2Cl2 (3 x 25 mL). The combined organic extracts were washed with brine (25 mL), dried over anhydrous MgSO4, filtered and concentrated under reduced pressure. Flash column chromatography of the residue (from 0 to 30 % EtOAc in hexanes) yielded RBM5-016 (300 mg, major (4S,5R)-anti-isomer, 61 %), and the minor (4S,5S)-syn-isomer (80 mg, 16 %) as light-yellow oils. The configuration of compound RBM5-016 was assigned by nOe experiments, similarly as reported by us in a previous related work.[6] Rf: 0.23 (Hexanes–EtOAc 1:1). [α]20D = +6.5 (c = 1, CHCl3). 1H NMR (400 MHz, CDCl3) δ 7.69 – 7.65 (m, 4H), 7.44 – 7.34 (m, 6H), 4.88 (br s, 1H), 4.56 (ddd, J = 9.0, 7.1, 3.8 Hz, 1H), 3.88 (app p, J = 6.7 Hz, 1H), 3.65 (t, J = 6.5 Hz, 2H), 1.78 – 1.69 (m, 1H), 1.60 – 1.46 (m, 5H), 1.39 – 1.21 (m, 16H), 1.16 (d, J = 6.5 Hz, 3H), 1.04 (s, 9H). 13C NMR (101 MHz, CDCl3) δ 160.0, 135.6, 134.3, 129.5, 127.6, 80.3, 64.1, 51.2, 32.7, 29.7, 29.7, 29.7, 29.6, 29.5, 29.5, 29.5, 29.2, 27.0, 26.0, 25.9, 19.3, 15.9. FTIR (NaCl) (ν, cm–1): 3583, 3283, 3071, 2928, 2855, 1754, 1428, 1110, 702. HRMS calcd. for C32H50NO3Si ([M+H]+): 524.3554, found: 524.3544.

(4S,5R)-5-(12-Azidododecyl)-4-methyloxazolidin-2-one (RBM5-018). Step 1: A solution of RBM5-016 (220 mg, 0.4 mmol) in anhydrous THF (10 mL) was treated with TBAF (1.0 M in THF, 840 µL, 0.8 mmol). After stirring for 2 h at 0 ºC, the reaction was quenched by adding saturated aqueous NH4Cl (10 mL) and extracted with Et2O (3 x 25 mL). The combined organic extracts were washed with brine (2 x 10 mL), dried over anhydrous MgSO4, concentrated under reduced pressure, and purified by flash column chromatography (from 0 to 100 % EtOAc in hexanes) to give the deprotected alcohol RBM5–017 (110 mg, 92 %) as an off-white solid. Rf: 0.16 (Hexanes–EtOAc 3:7). [α]20D = +12.3 (c = 1, CHCl3). 1H NMR (400 MHz, CDCl3) δ 6.09 (br s, 1H), 4.53 (td, J = 9.3, 8.4, 3.5 Hz, 1H), 3.87 (app p, J = 6.7 Hz, 1H), 3.61 (t, J = 6.7 Hz, 2H), 1.87 (br s, 1H), 1.77 – 1.64 (m, 1H), 1.59 – 1.43 (m, 3H), 1.37 – 1.19 (m, 18H), 1.13 (d, J = 6.5 Hz, 3H). 13C NMR (101 MHz, CDCl3) δ 159.9, 80.4, 63.1, 51.2, 32.9, 29.7, 29.6, 29.6, 29.5, 29.5, 29.5, 29.5, 29.2, 25.9, 25.8, 16.0. ATR–FTIR (neat) (ν, cm–1): 3421, 3282, 2980, 2920, 2849, 1732, 1698, 1392, 1235, 1105. HRMS calcd. for C16H32NO3 ([M+H]+): 286.2377, found: 286.2360.

Step 2: A solution of NBS (49 mg, 0.2 mmol) in DMF (1 mL) was added dropwise to an ice-cooled solution of RBM5-017 (65 mg, 0.2 mmol) and triphenylphosphine (66 mg, 0.3 mmol) in DMF (2 mL). After stirring at rt for 2 h, NaN3 (44 mg, 0.7 mmol) was added in one portion and the mixture was heated to 75 ºC and stirred for additional 5 h. After completion (TLC), the reaction was diluted with brine (10 mL) and the resulting suspension was extracted with Et2O (3 x 20 mL). The combined organic extracts were thoroughly washed with brine (3 x 5 mL), dried over anhydrous MgSO4, concentrated to dryness and the residue was purified by flash column chromatography (from 0 to 34 % EtOAc in hexanes) to afford RBM5-018 (50 mg, 71 %) as an off-white solid. Rf: 0.42 (Hexanes–EtOAc 3:7). [α]20D = +12.3 (c = 1, CHCl3). 1H NMR (400 MHz, CDCl3) δ 5.89 (br s, 1H), 4.54 (ddd, J = 9.5, 7.4, 3.9 Hz, 1H), 3.88 (app p, J = 6.7 Hz, 1H), 3.24 (t, J = 7.0 Hz, 2H), 1.76 – 1.67 (m, 1H), 1.62 – 1.54 (m, 2H), 1.54 – 1.44 (m, 2H), 1.39 – 1.21 (m, 17H), 1.14 (d, J = 6.5 Hz, 3H). 13C NMR (101 MHz, CDCl3) δ 159.9, 80.3, 51.6, 51.2, 29.6, 29.6, 29.6, 29.5, 29.5, 29.5, 29.2, 29.2, 28.9, 26.8, 26.0, 16.0. ATR–FTIR (neat) (ν, cm–1): 3273, 2981, 2917, 2850, 2093, 1742, 1712, 1470, 1398, 1253. HRMS calcd. for C16H31N4O2 ([M+H]+): 311.2442, found: 311.2432.

(2S,3R)-2-Amino-15-azidopentadecan-3-ol (RBM5-019). Compound RBM5-018 (55 mg, 0.18 mmol) was dissolved in EtOH-2.0 N aq. NaOH (1:1) (6 mL) and the resulting solution was heated to reflux temperature. After stirring for 4 h, the reaction mixture was concentrated to dryness and the residue was diluted with water (5 mL) and extracted with EtOAc (3 x 20 mL). The combined organic extracts were dried over anhydrous MgSO4, evaporated, and the crude product was purified by flash column chromatography (from 0 to 20 % MeOH in CH2Cl2) to furnish RBM5-019 (40 mg, 79 %) as an off-white waxy solid. Rf: 0.09 (CH2Cl2–MeOH 9:1). [α]20D = +5.2 (c = 1, CHCl3). 1H NMR (400 MHz, CDCl3) δ 3.42 (ddd, J = 8.0, 4.7, 3.5 Hz, 1H), 3.24 (t, J = 7.0 Hz, 2H), 2.96 (qd, J = 6.5, 3.5 Hz, 1H), 1.76 – 1.64 (m, 3H), 1.63 – 1.55 (m, 2H), 1.39 – 1.22 (m, 20H), 1.00 (d, J = 6.6 Hz, 2H). 13C NMR (101 MHz, CDCl3) δ 74.9, 51.6, 50.5, 32.6, 29.9, 29.7, 29.7, 29.7, 29.7, 29.6, 29.3, 29.0, 26.9, 26.4, 17.0. ATR–FTIR (neat) (ν, cm–1): 3336, 2919, 2850, 2094, 1581, 1467, 1371, 1292. HRMS calcd. for C15H33N4O ([M+H]+): 285.2649, found: 285.2640.

N-((2S,3R)-15-azido-3-hydroxypentadecan-2-yl)octanamide (RBM5-177). To a solution of EDC·HCl (14 mg, 70 µmol) and HOBt (7 mg, 52 µmol) in anhydrous CH2Cl2 (2.5 mL) was added octanoic acid (7 mg, 48 µmol) under argon atmosphere. The resulting mixture was vigorously stirred at rt for 10 min, and next added dropwise to a solution of RBM5-019 (14 mg, 44 µmol) and Et3N (30 µL, 0.2 mmol) in anhydrous CH2Cl2 (2.5 mL). The reaction mixture was stirred at rt for 2 h under argon atmosphere. Subsequently, the mixture was diluted by addition of

CH_2Cl_2 (10 mL) and washed with brine (2 x 5 mL). The organic layer was dried over anhydrous $MgSO_4$, filtered and the solvent was removed under reduced pressure. Purification of the crude by flash chromatography (from 0 to 5 % MeOH in CH_2Cl_2) gave the pure amide RBM5-177 (16 mg, 89 %) as an off-white waxy solid. Rf: 0.66 (CH_2Cl_2–MeOH 95:5). 1H NMR (400 MHz, $CDCl_3$) δ 5.86 (s, 1H), 4.01 (s, 1H), 3.62 (s, 1H), 3.25 (t, J = 6.9 Hz, 2H), 2.31 (s, 1H), 2.17 (t, J = 7.0 Hz, 2H), 1.67 – 1.54 (m, 4H), 1.43 – 1.21 (m, 28H), 1.09 (d, J = 6.6 Hz, 3H), 0.91 – 0.82 (m, 3H). 13C NMR (101 MHz, $CDCl_3$) δ 173.5, 74.5, 51.6, 49.7, 37.0, 33.7, 31.8, 29.8, 29.7, 29.7, 29.6, 29.6, 29.4, 29.3, 29.1, 29.0, 26.8, 26.1, 26.0, 22.7, 14.2, 14.2. HRMS calcd. for $C_{23}H_{47}N_4O_2$ ([M+H]+): 411.3694, found: 411.3699.

Reaction of RBM5-177 and RBM5-019 with CO-1

To prove that the reactions between CO-1 and either RBM5-019 or RBM5-177 lead to similar fluorescence intensities, CO-1 (1 uM) was reacted with 5 and 10 uM of either lipid (so that no free CO-1 remains). As shown in Fig. S7, similar fluorescence values were obtained. That no free CO-1 remained unreacted and that both click reactions occurred to similar extenses was demonstrated by UPLC-HRMS.

BIOLOGY

Cells

The A375 cell line stably overexpressing ASAH1 under the control of a doxycycline-responsive promoter was kindly provided by Dr. Carmen Bedia and Prof. Thierry Levade.[7] The antibiotic selection of this cell line was performed with blasticidin (3 µg/mL) and hygromycin B (250 µg/mL). Ectopic expression of AC was induced with doxycycline at 1 µg/mL for 24 h before use. Farber disease fibroblasts FD and FD/AC cell lines were a kind gift from Prof. Thierry Levade (Laboratoire de Biochimie Métabolique, CHU Toulouse, France). HeLa T-Rex ACER1-TET-ON and HeLa T-Rex ACER2-TET-ON cells stably overexpress ACER1 or ACER2 were obtained as reported.[8] The antibiotic selection of HeLa T-Rex TET-ON cells was performed with zeocin (25 µg/ml) and blasticidin (5 µg/ml). Expression of ACER1 or ACER2 was induced with tetracycline 0.05 µM for 24 h before use. MEF5 was a kind gift of Prof. Richard Proia (Genetics of Development and Disease Branch, NIDDK, National Institutes of Health, Bethesda, MD, USA). It was chosen to study ACER3 because was defective in the NC gene and has high levels of ACER3 transcript. All cells were grown in a humidified 5% CO_2 atmosphere at 37 °C in DMEM medium supplemented with 10% fetal bovine serum.

Cell lysates

Cells were suspended in the appropriate volume of a 0.25 M saccharose solution with the proteases inhibitors aprotinin (1 mg/ml), leupeptin (1 mg/mml) and PMSF (100 mM). The suspension was submitted to three cycles of a 5 s sonication (probe) at 10 watts/5 s resting on ice. The cell lysate was centrifuged at 600 g for 5 min. The supernatant was collected and protein concentration was determined as specified below.

Microsomal preparations

Cell lysates obtained from HeLa T-Rex ACER1-TET-ON, HeLa T-Rex ACER2-TET-ON and MEF5 overexpressing ACER3 cells were transferred to ultracentrifuge tubes and were spun at 100000 g for 1 h at 4°C. Pellets were resuspended in a 0.25 M saccharose solution and protein concentration was determined as specified below.

Determination of protein concentration

Protein concentrations were determined with BSA as a standard using a BCA protein determination kit (Thermo Scientific) according to the manufacturer's instructions.

Cell viability

A375 cells were seeded at 2.5×10^4 cells/mL in 96-well plates (0.1 ml/well) and grown for 24 h. Overexpression of AC was induced with doxycycline at 1 µg/mL for 24 h. Cell viability was examined in triplicate samples by MTT method after treatment with the test compounds at different concentrations or with the corresponding percentage of vehicle for 4 h (DM120) or 24 h (RBM5-019 and RBM5-177).

Optimal probe concentration

In order to assess whether the fluorescent probe self-quenches at high concentrations, we analyzed by flow cytometry the concentration response curve for the probe (RBM5-177). Cells were treated (4 h) with different concentrations of compound and further analyzed by flow cytometry after labeling with CO-1 under the standard reaction conditions. As shown in Figure S8, the response is linear from 1.25 to 5 µM, which is the concentration used in the assays. Therefore, the assay is quantitative and useful for genotypic screening in the reported conditions.

Fluorogenic AC activity in intact cells

To determine AC activity in intact cells, A375 cells were seeded at 2.5×10^4 cells/mL in 96-well plates (0.1 ml/well) and grown for 24 h. Overexpression of AC was induced with doxycycline at 1 µg/mL for 24 h. Medium was replaced by DM120 at different concentrations and substrate RBM14C12 at a final concentration of 40 µM in DMEM. The plate was incubated for 4 h at 37°C in 5% CO_2. The reaction was stopped with 25 µL of MeOH and then 100 µL of $NaIO_4$ (2.5 mg/mL in glycine-NaOH buffer, pH 10.6) was added. After incubation at 37°C for 1 h, 100 µL of 0.1 M glycine-NaOH buffer (pH 10.6) was added and fluorescence was measured spectrophotometrically at excitation and emission wavelengths of 355 and 460 nm, respectively. The same reaction mixture without cells was used as blank.

UPLC-HRMS

Cells were seeded at 5×10^5 cells/mL into 6 well plates (1 mL/well) and were allowed to adhere for 24 h. Medium was replaced with fresh medium containing RBM5-177, RBM5-019 or RBM2-37 (5 µM final concentration). After 30 min, medium was removed; cells were washed with 400 µL PBS and harvested with 400 µL Trypsin-EDTA and 600 µL of medium. NC recombinant enzyme, lysates from A375-AC overexpressing cells, ASAH2-null mouse embryonic fibroblasts or microsomes from HeLa T-Rex ACER1-TET-ON and HeLa T-Rex ACER2-TET-ON cells stably overexpressing ACER1 or ACER2 respectively were incubated with RBM5-177 or RBM5-019 (10 µM final concentration) for 3 h in the corresponding ceramidase activity buffer. Cell pellets were resuspended with 100 µL of H_2O and mixed with 750 µL of methanol: chloroform, 2:1 fortified with internal standards (*N*-dodecanoylsphingosine, *N*-dodecanoyl-glucosylsphingosine, *N*-dodecanoylsphingosyl-phosphorylcoline and C17-sphinganine 0.2 nmol each). Samples were heated at 48°C overnight and next day, 75 µL of 1 M KOH in methanol were added, followed by 2 h incubation at 37°C. Afterwards, the saponification was neutralised with 75 µL of 1 M acetic acid and solvent was removed using a Speed Vac Savant SPD131DDA (Thermo Scientific). Sphingolipid extracts were solubilised in 150 µL of methanol.

Samples were then centrifuged at 9,300 g for 3 min and 130 µL of the supernatant were injected to a Waters Aquity UPLC system connected to a Waters LCT Premier Orthogonal Accelerated Time of Flight Mass Spectrometer (Waters,Milford, MA, USA) operated in positive electrospray ionisation mode. Full scan spectra from 50 to 1500 Da were acquired and individual spectra were summed to produce data points each 0.2 s. Mass accuracy and reproducibility were maintained by using an independent reference spray by the LockSpray interference. The analytical column was a 100 mm x2.1 mm i.d., 1.7 µm C8 Acquity UPLC BEH (Waters). The two mobile phases were phase A: methanol/water/formic acid (74/25/1 v/v/v); phase B: methanol/formic acid (99/1 v/v), both also contained 5 mM ammonium formate. A linear gradient was programmed—0.0 min: 80 % B; 3 min: 90 % B; 6 min: 90 % B; 15 min: 99 % B; 18 min: 99 % B; 20 min: 80 % B. The flow rate was 0.3 mL min−1.

Statistical analysis

All experiments were performed in duplicate or triplicate and data from at least two independent experiments are given as means ± SD. For comparison between means, data were analysed by one-way ANOVA followed by Dunnett's (pairwise comparisons of multiple groups with a single control group) or Bonferroni's (comparisons between all data groups) multiple comparison post-tests. Differences with $P < 0.05$ were considered significant. Dose response curves were adjusted with sigmoidal dose-response curve with variable slope equation. All test were run with the GraphPad software. The confocal images are representative of at least two independent experiments with a similar pattern.

References

1 Alecu I, Tedeschi A, Behler N, Wunderling K, Lamberz C, Lauterbach MAR, Gaebler A, Ernst D, Van Veldhoven PP, Al-Amoudi A, Latz E, Othman A, Kuerschner L, Hornemann T, Bradke F, Thiele C, Penno A. Localization of 1-deoxysphingolipids to mitochondria induces mitochondrial dysfunction. *J Lipid Res* 2017; **58**: 42–59.

2 Garrido M, Abad JL, Fabrias G, Casas J, Delgado A. Azide-Tagged Sphingolipids: New Tools for Metabolic Flux Analysis. *ChemBioChem* 2015; **16**: 641–650.

3 Alamudi SH, Satapathy R, Kim J, Su D, Ren H, Das R, Hu L, Alvarado-Martínez E, Lee JY, Hoppmann C, Peña-Cabrera E, Ha H-H, Park H-S, Wang L, Chang Y-T. Development of background-free tame fluorescent probes for intracellular live cell imaging. *Nat Commun* 2016; **7**: 11964.

4 Xue Y, Zimmt MB. Patterned monolayer self-assembly programmed by side chain shape: Four-component gratings. *J Am Chem Soc* 2012; **134**: 4513–4516.

5 Ocejo M, Vicario JL, Badía D, Carrillo L, Reyes E. A Direct and Efficient Stereoconservative Procedure for the Selective Oxidation of N-Protected β-Amino Alcohols. *Synlett* 2005; **2005**: 2110–2112.

6 Abad JL, Nieves I, Rayo P, Casas J, Fabrias G, Delgado A. Straightforward access to spisulosine and 4,5-dehydrospisulosine stereoisomers: probes for profiling ceramide synthase activities in intact cells. *J Org Chem* 2013; **78**: 5858–66.

7 Bedia C, Casas J, Andrieu-Abadie N, Fabriàs G, Levade T. Acid ceramidase expression modulates the sensitivity of A375 melanoma cells to dacarbazine. *J Biol Chem* 2011; **286**: 28200–28209.

8 Sun W, Hu W, Xu R, Jin J, Szulc ZM, Zhang G, Galadari SH, Obeid LM, Mao C. Alkaline ceramidase 2 regulates beta1 integrin maturation and cell adhesion. *FASEB J* 2009; **23**: 656–66.

SUPPLEMENTARY FIGURES

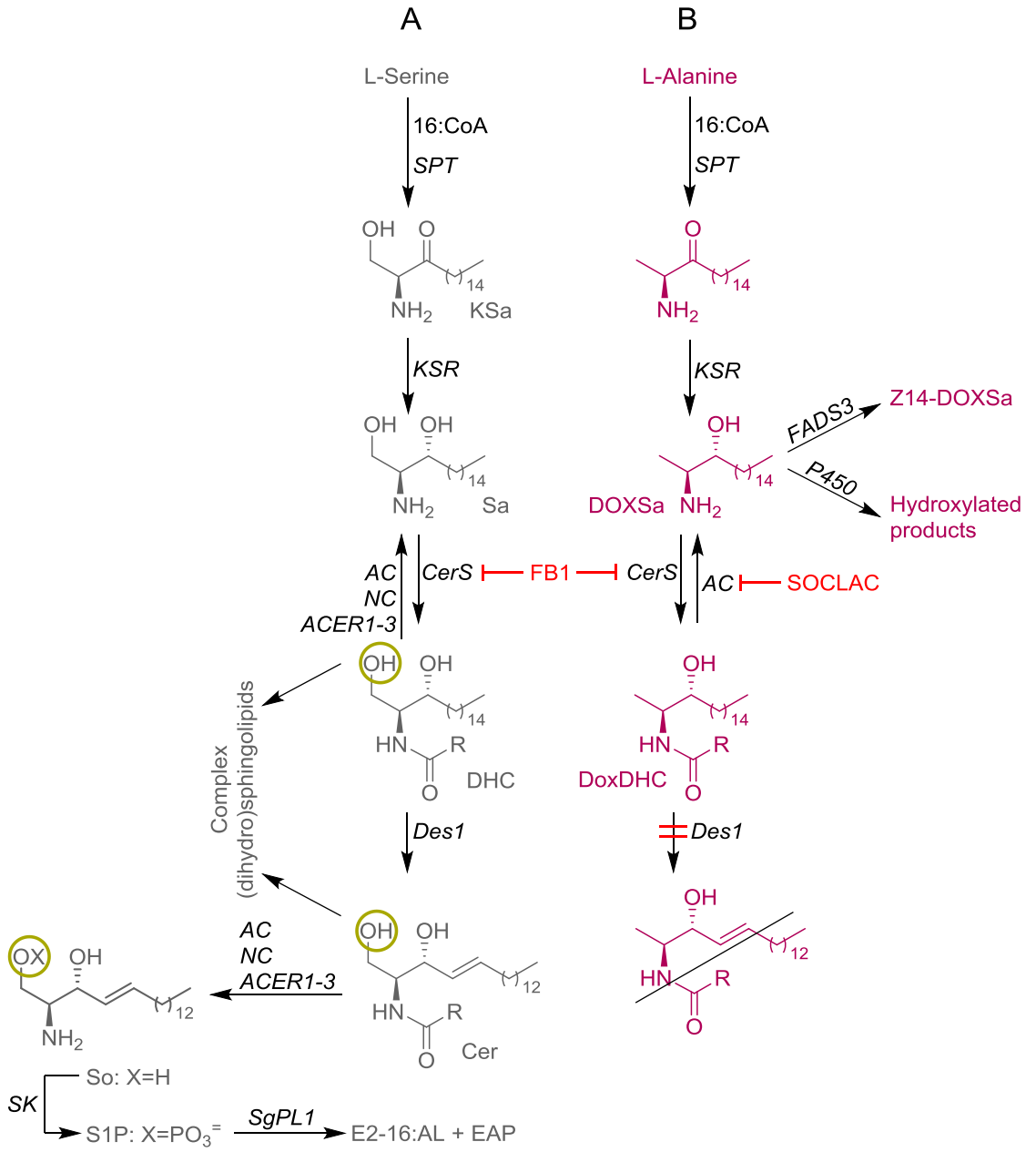


Figure. S1. Metabolism of sphinganine (A) and deoxysphinganine (B). 16:CoA, palmitoyl CoA; AC, acid ceramidase; ACER, alkaline ceramidase; Cer, ceramides.; CerS, ceramides synthase; Des1, dihydroceramide desaturase 1; DHC, dihydroceramide; DOXDHC, 1-deoxydihydroceramide; DOXSa, 1-deoxysphinganine; E2-16:AL, (E)-2-hexadecenal; EAP, ethanolamine phosphate; FADS3, fatty acid desaturase 3; FB1, fumonisin B1; KSa, 3-ketosphinganine; KSR, 3-ketosphinganine reductase; NC, neutral ceramidase; P450, cytochrome P450; S1P, sphingosine 1-phosphate; Sa, sphinganine; Sgpl1, sphingosine 1-phosphate lyase; SK, sphingosine kinase; So, sphingosine; SPT, serine palmitoyltransferase; Z14-DOXSa, (*Z*)-14-deoxysphinganine.

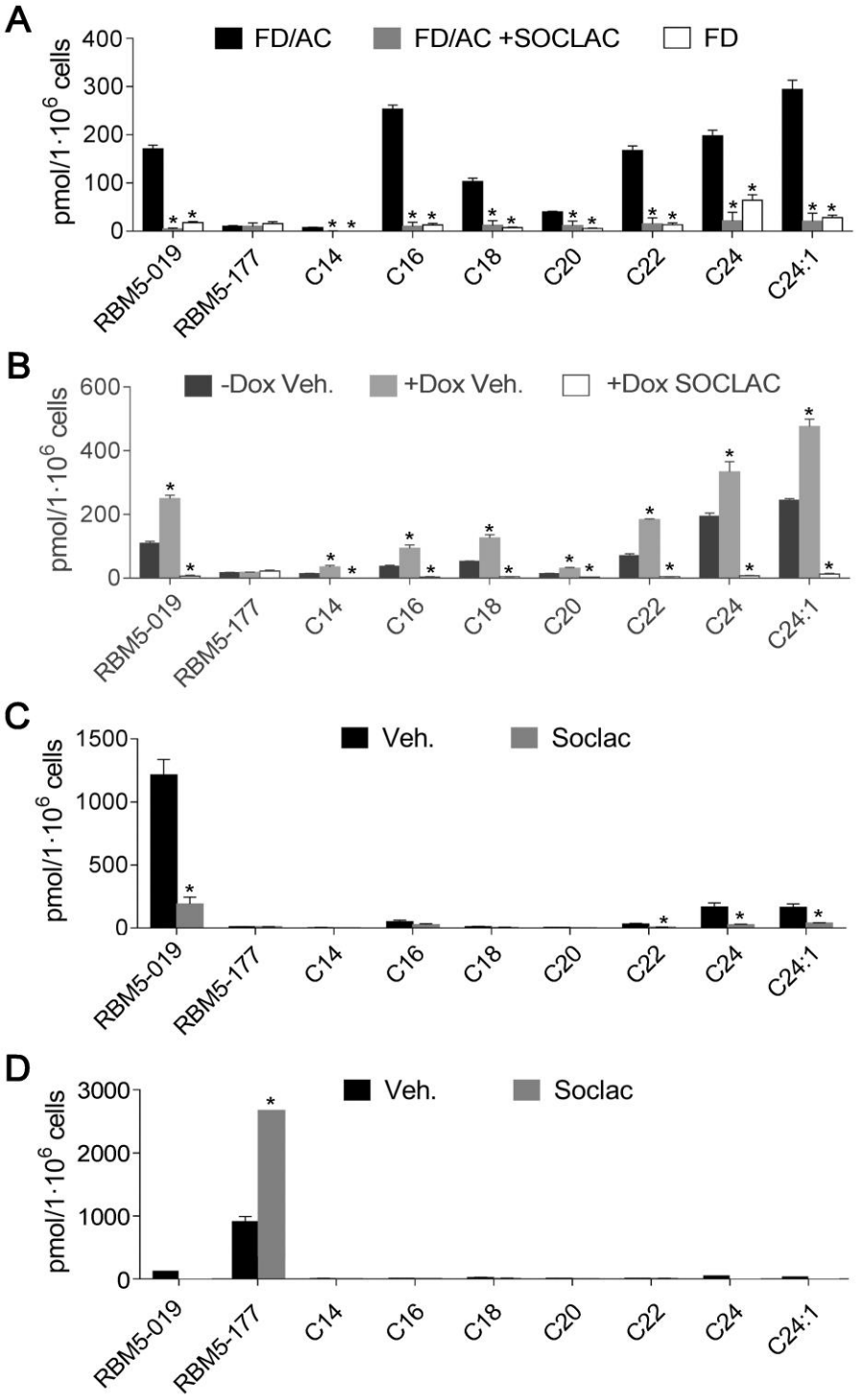


Figure S2. AC activity-dependence of RBM5-177 metabolization. FD and FD/AC cells (A) and A549 cells (C,D) were incubated with RBM5-177 (5 μM, 4 h), prior treatment with SOCLAC (5 μM, 1 h) or vehicle (Veh.) and the lipid extracts from either cells (A-C) or medium (D) were analyzed by UPLC-HRMS. A375/+Dox or A375/-Dox (B) were incubated with RBM5-177 (5 μM, 20 min), prior treatment with SOCLAC (5 μM, 1 h) or vehicle (Veh.) and the lipid extracts were analyzed by UPLC-HRMS. Data (mean ± SD) were obtained from three different experiments with triplicates. Data were analysed by two-way ANOVA test followed by Dunnett's multiple comparison test if ANOVA $P < 0.05$. (*, $P < 0.001$ from vehicle or FD cells). The acyl chain of reacylated RBM5-019 are abbreviated as C14, tetradecanoyl, etc.

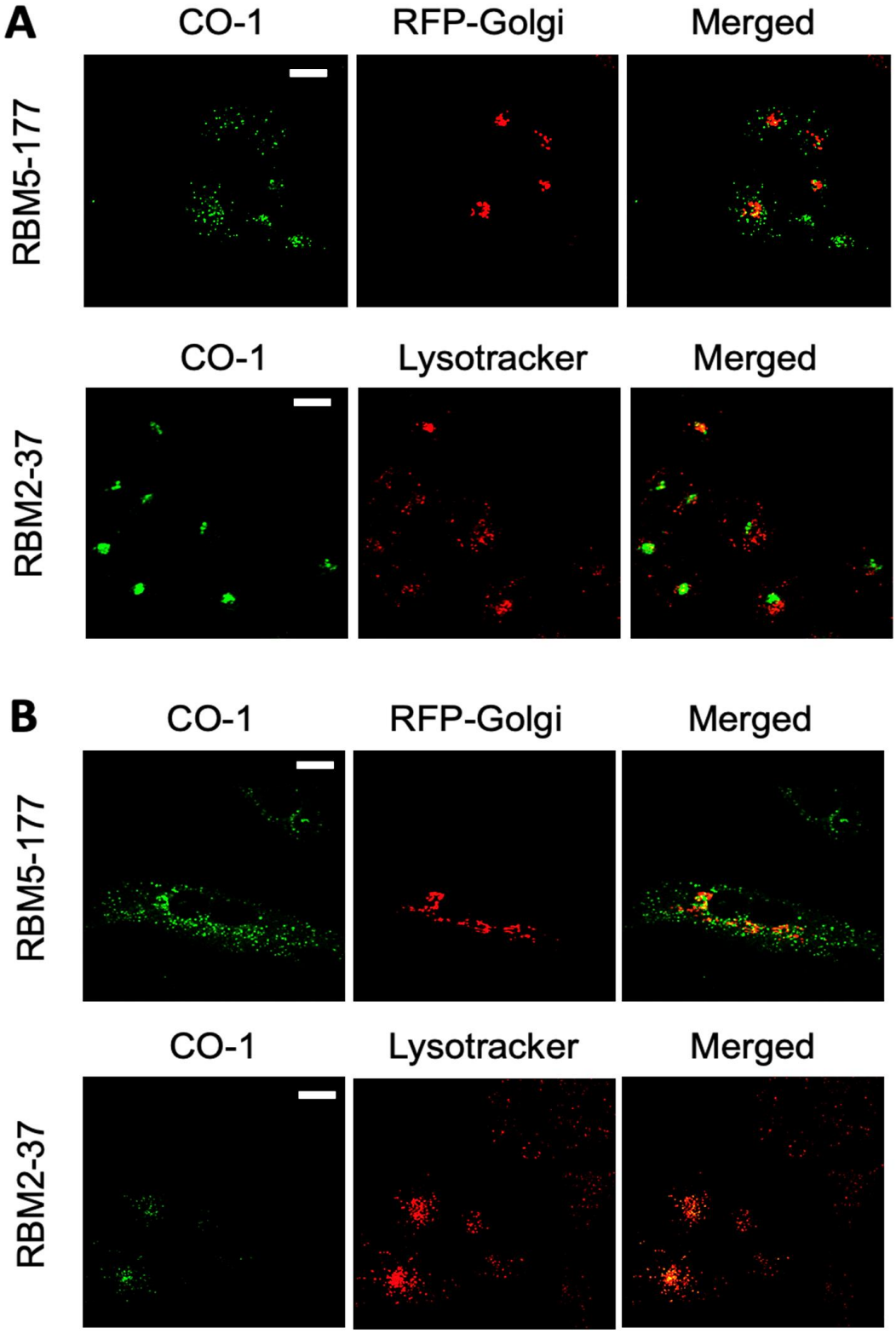


Figure S3. Co-localization studies of the indicated probes in A549 cells. A) A549 cells incubated or not with RFP-Golgi were treated with the indicated probe (5 μM, 20 min), washed an incubated with CO-1 (1 μM, 10 min). B) After the probe treatment, cells were washed and incubated with medium for 5 h, washed and incubated with CO-1 (1 μM, 10 min). Lysotracker (75 nM) was added 30 minutes before fluorescence confocal microscopy (Scale bar: 10 μm). Co-localization coefficients were measured on the different stacks of images (at least n=4) with each stack containing 3-10 cells.

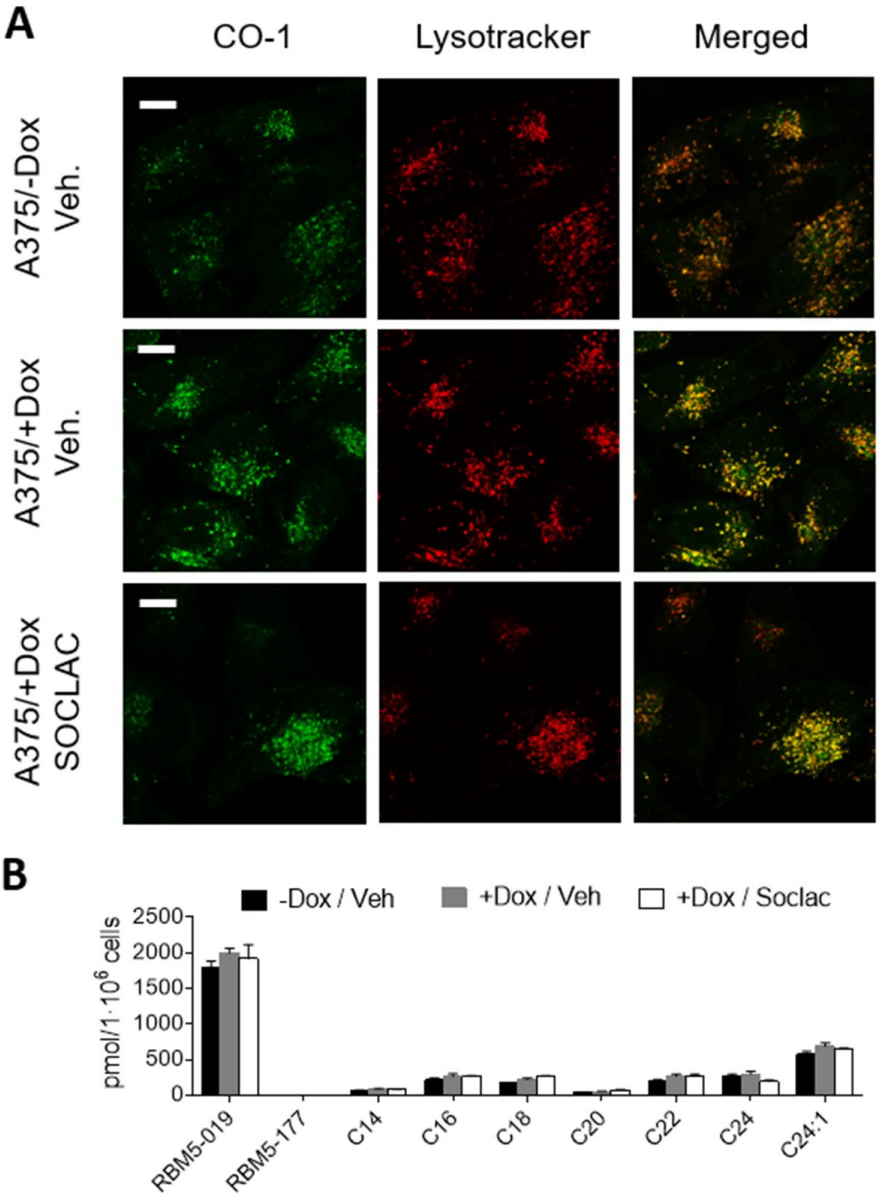


Figure S4. AC activity-independence of lysosome staining with RBM5-019. A375/+Dox or A375/-Dox were incubated with RBM5-019 (5 μM, 20 min) prior treatment with SOCLAC (5 μM, 1 h) or vehicle (Veh.). The click reaction was carried out by incubation with CO-1 (10 min, 1 μM) and cells were analysed by fluorescence confocal microscopy (scale bar: 10 μm) (A) or the lipid extracts were analysed by UPLC-HRMS (B). Lysotracker (75 nM, 30 min) was used to stain lysosomes. Co-localization coefficients were measured on the different stacks of images (at least n=4) with each stack containing 3-10 cells. Pearson's (P) and Manders' (M1 and M2) coefficients: RBM5-019-Lysotracker in A375/-Dox with vehicle (P=0.845; M1=0.829; M2=0.733), RBM5-019-Lysotracker in A375/+Dox with vehicle (P=0.869; M1=0.836; M2=0.730) and RBM5-019-Lysotracker in A375/+Dox with SOCLAC (P=0.863; M1=0.810; M2=0.795). Data (mean ± SD) were obtained from three different experiments with triplicates. Data were analysed by two-way ANOVA test followed by Dunnett's multiple comparison post-test if ANOVA $P < 0.05$. The right panel in B corresponds to the acyl chain of reacylated RBM5-019 (C14, tetradecanoyl, etc.).

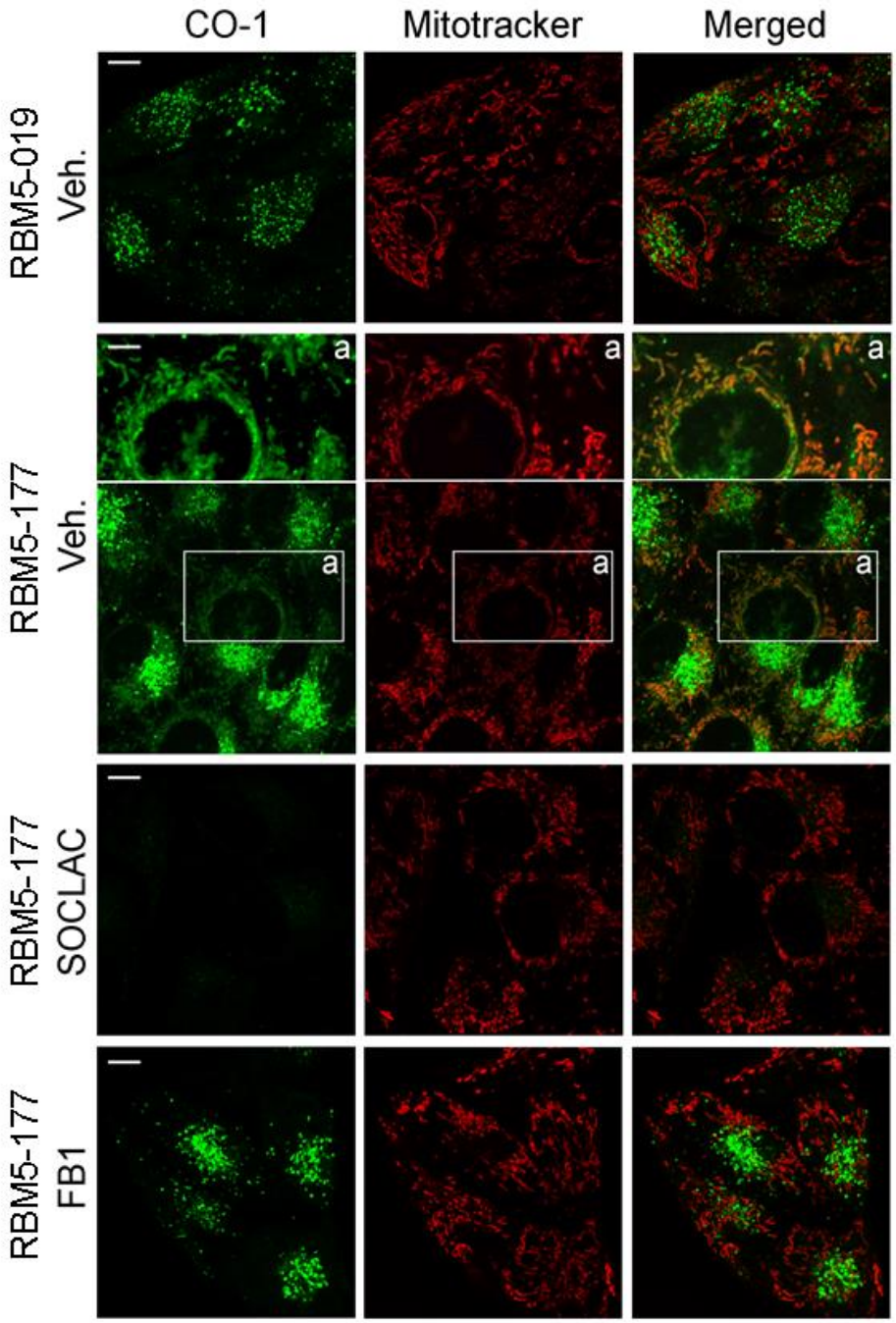


Figure S5. CerS activity-dependence of mitochondria staining with RBM5-177. AC overexpressing A375 cells(A375/+Dox) were incubated with the indicated probes (5 μM, 4 h) prior treatment with Soclac (5 μM, 1 h), FB1 (100 μM, 24 h) or vehicle (Veh.). The click reaction was carried out by incubation with CO-1 1 μM for 10 min. Mitotracker (75 nM) was used to stain mitochondria. Cells were analyzed by fluorescence confocal microscopy (scale bar: 10 μm). Mitochondria labelling is shown in the amplification of the selected area (a). Co-localization coefficients were measured on the different stacks of images (at least n=4) with each stack containing 3-10 cells. Pearson's (P) and Manders' (M1 and M2) coefficients: RBM5-019-Mitotracker in A375/+Dox with vehicle (P=0.298; M1=0.179; M2=0.070), RBM5-177-Mitotracker in A375/+Dox with vehicle (P=0.355; M1=0.194; M2=0.697) and RBM5-177-Mitotracker in A375/+Dox with FB1 (P=0.224; M1=0.059; M2=0.043).

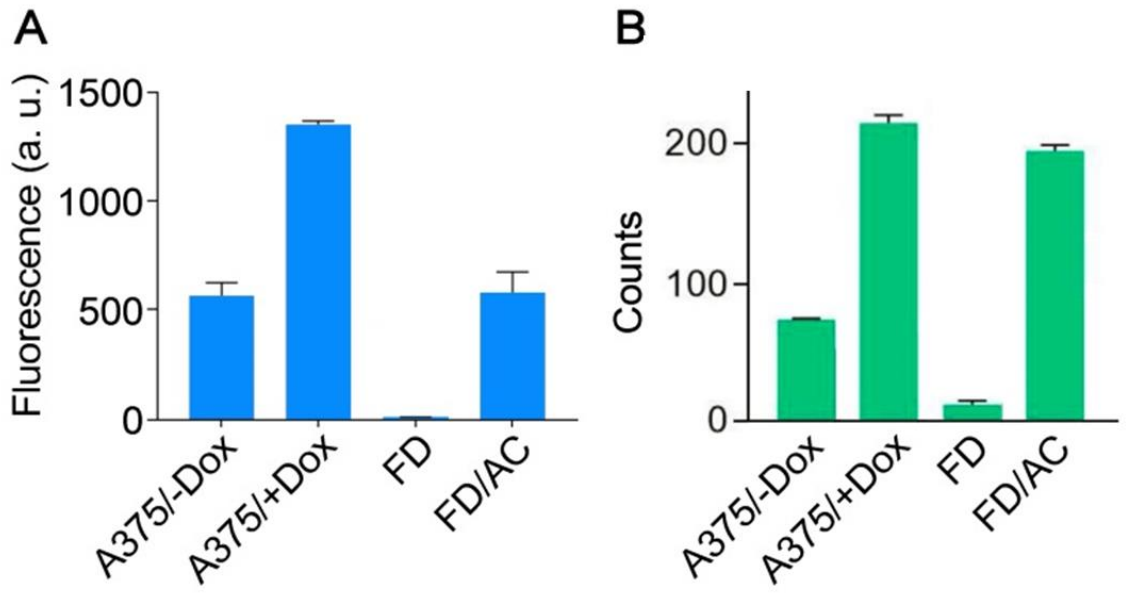


Figure S6. AC enzyme assay using the fluorogenic assay (Bedia et al., J. Lipid Res. 2010, 51, 3542-3547) (A) and the flow cytometry assay reported in this article (B). A375 cells stimulated (+Dox) or not (-Dox) with doxycycline for AC overexpression, or FD and FD/AC cells were incubated with RBM14C12 (A) (10 μM, 3 h) or RBM5-177 (B) (5 μM, 4 h). In A the amount of umbelliferone released from RBM14C12 was measured. In B cells were incubated with CO-1 (1 μM, 10 min) and analyzed by flow cytometry measuring green fluorescence. Data (mean ± SD) were obtained from two experiments with triplicates.

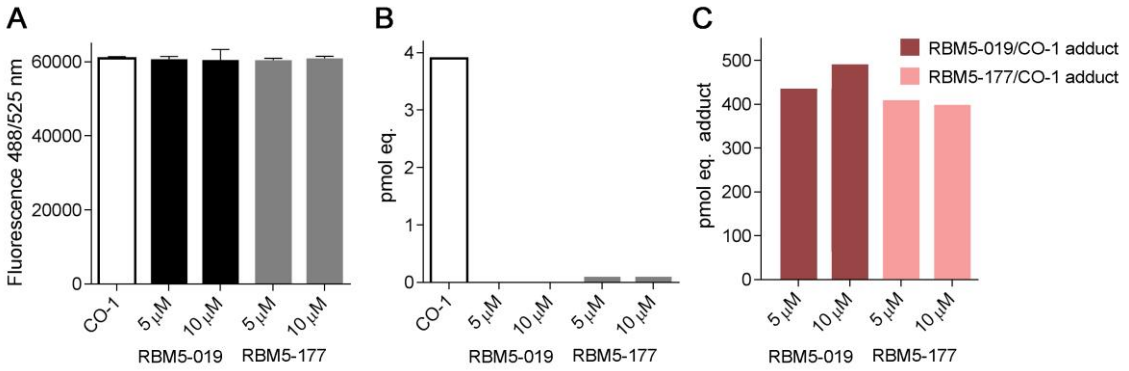


Figure S7. Compounds RBM5-019 and RBM5-177 (5 and 10 uM) were reacted with CO-1 (1 uM) for 1 h. Fluorescence was then measured in a microtiter plate reader at 488 and 525 excitation and emission wavelengths, respectively (A). The replicates for each condition were combined and analysed by UPLC-HRMS (B and C). The ions at *m/z* 496.2588, 285.2654, 411.3699, 780.5167 and 906.6213, corresponding to CO-1, RBM1-019, RBM1-177 and their adducts were selected and their areas were referred to an internal standard of C17-sphinganine (for CO-1, RBM5-019 and the adduct) and C12-ceramide (for RBM5-177 and its CO-1 adduct).

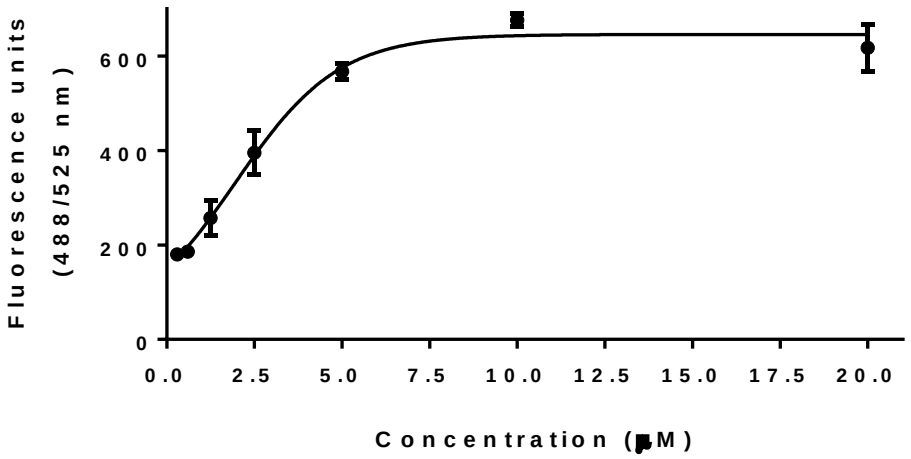


Figure S8. Concentration-response curves for RBM5-177/CO-1 by flow cytometry.. A375 cells stimulated (+Dox) with doxycycline for AC overexpression were incubated for 4 h with different concentrations of RBM5-177, and then with CO-1 (1 μM, 10 min). Cells were analyzed by flow cytometry measuring green fluorescence. Fluorescence at excitation /emission 488/525 nm. Data (mean ± SD) were obtained from two experiments with triplicates.

NMR SPECTRA:

Compound RBM5-013

Compound RBM5-014

Compound RBM5-015

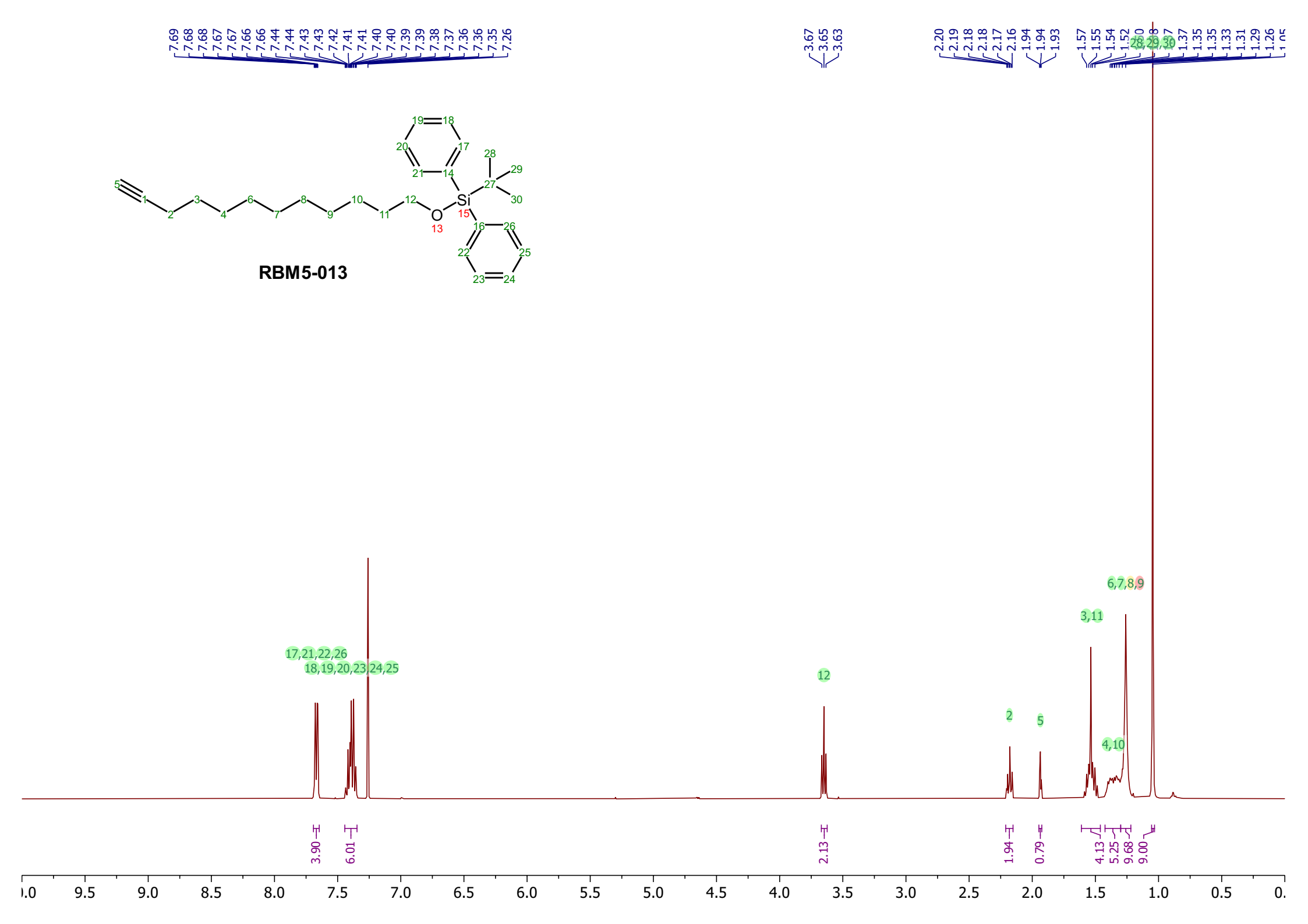
Compound RBM5-016

Compound RBM5-017

Compound RBM5-018

Compound RBM5-019

Compound RBM5-177


RBM5-013
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3.67 3.65 3.63
2.20 2.19 2.18 2.18 2.17 2.16 1.94 1.94 1.93
1.57 1.55 1.54 1.52 1.37 1.35 1.35 1.33 1.31 1.29 1.26
17,21,22,26
18,19,20,23,24,25
12
2
5
3,11
6,7,8,9
4,10
3.90
6.01
2.13
1.94
0.79
4.13
5.25
9.68
9.00
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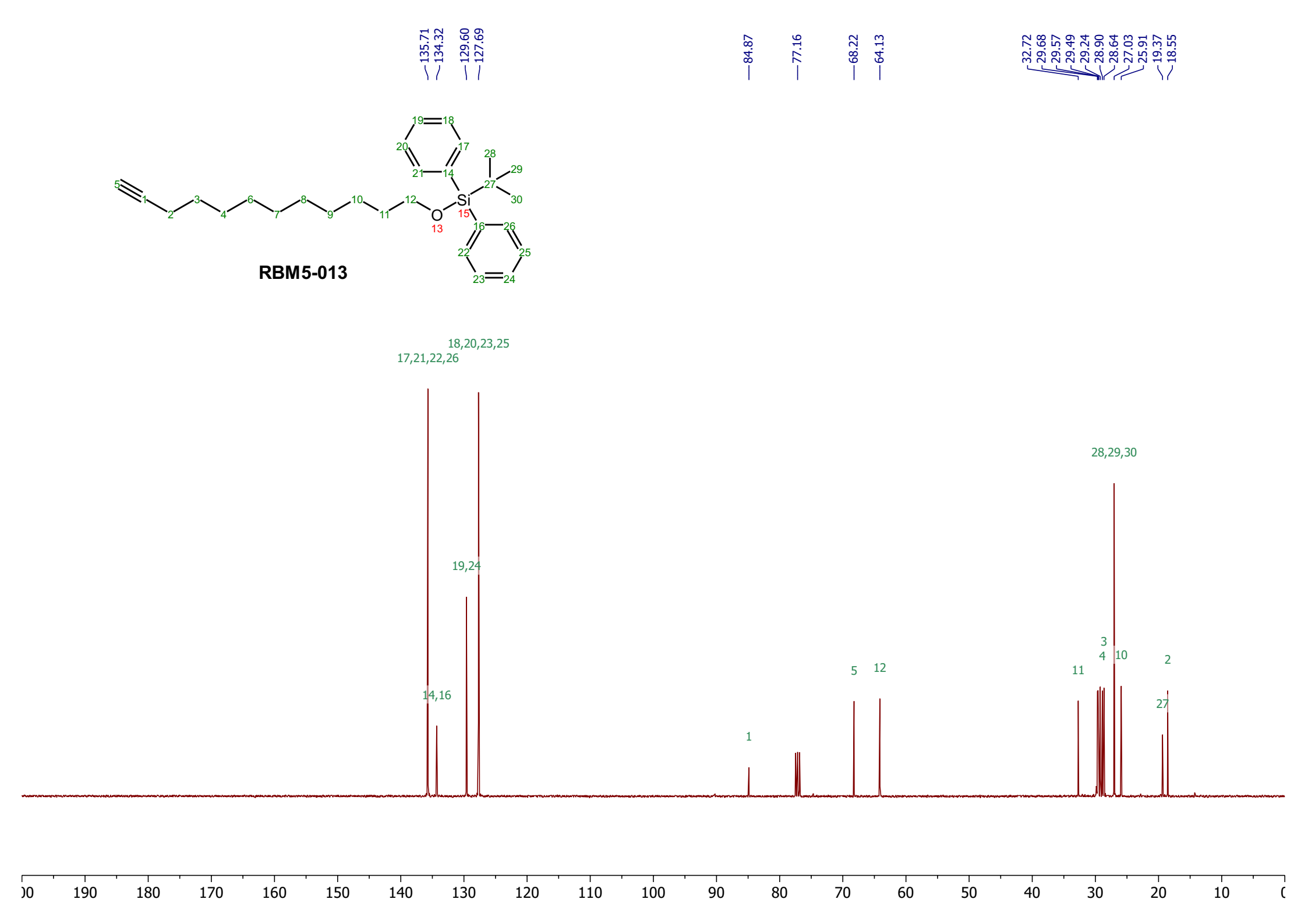
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28.90
28.64
27.03
25.91
19.37
18.55
RBM5-013
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19,24
14,16
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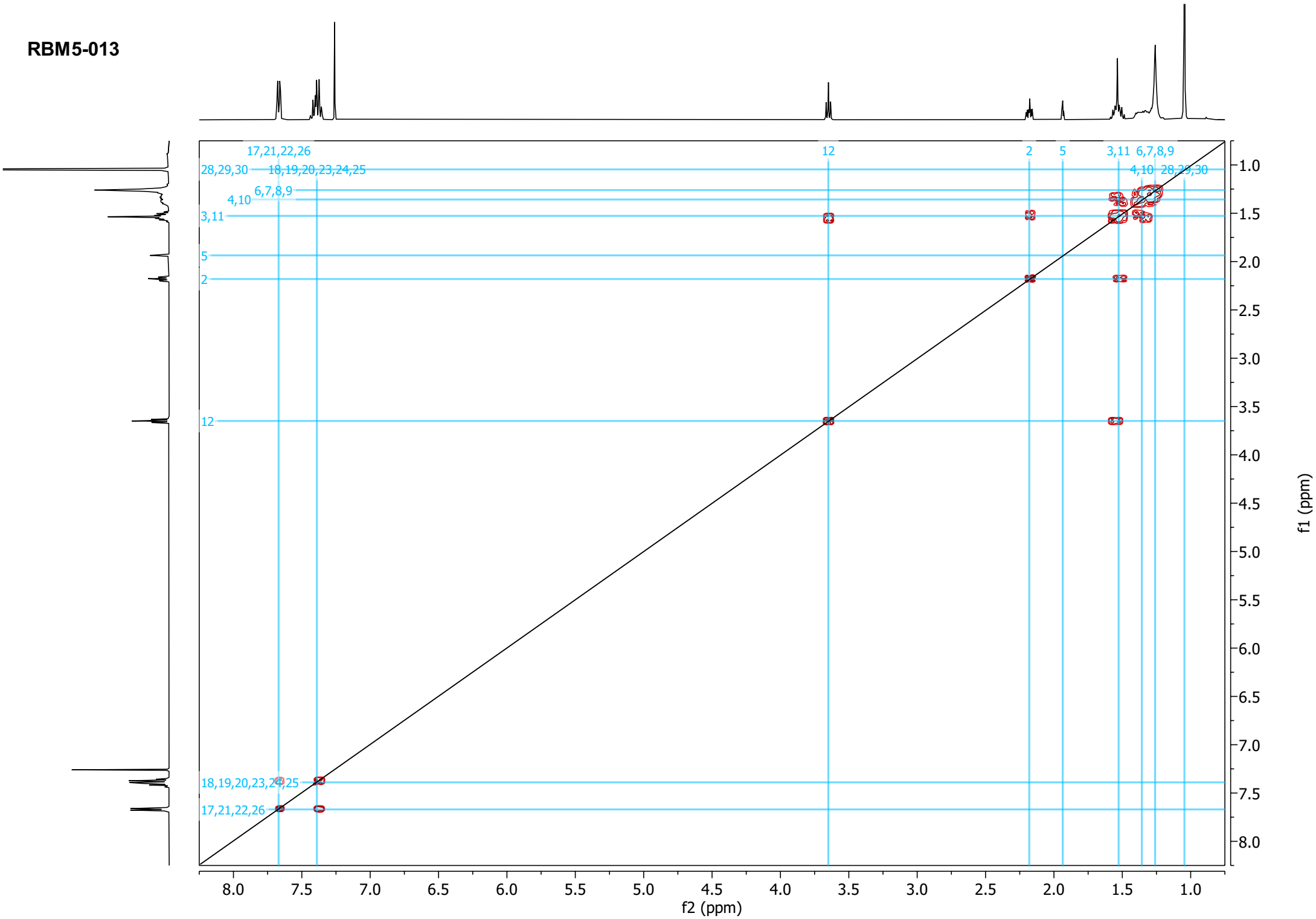
RBM5-013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17,21,22,26
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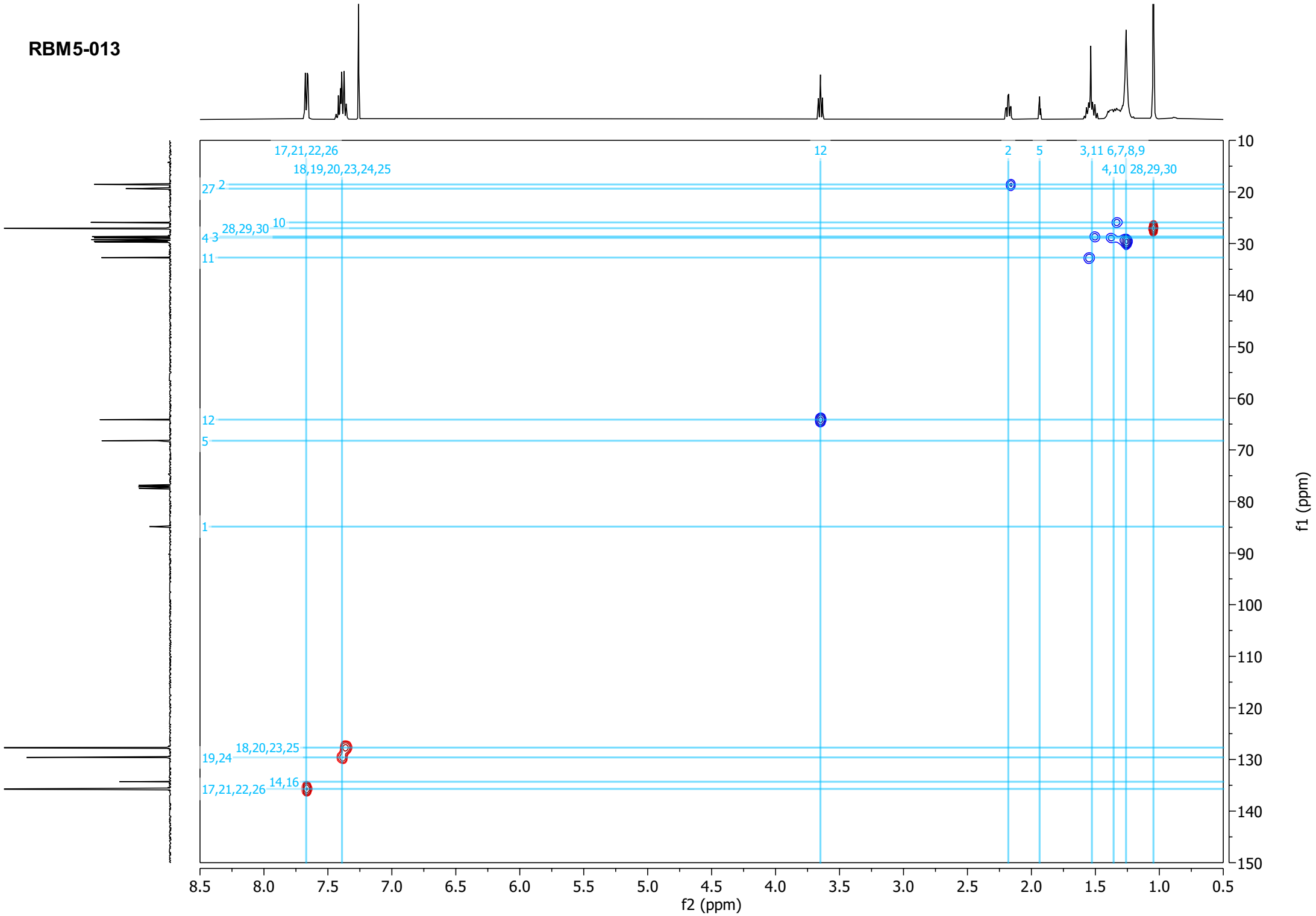
f1 (ppm)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4
3
11
12
5
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19,24
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6.5
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4.0
3.5
3.0
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1.0
0.5
f2 (ppm)
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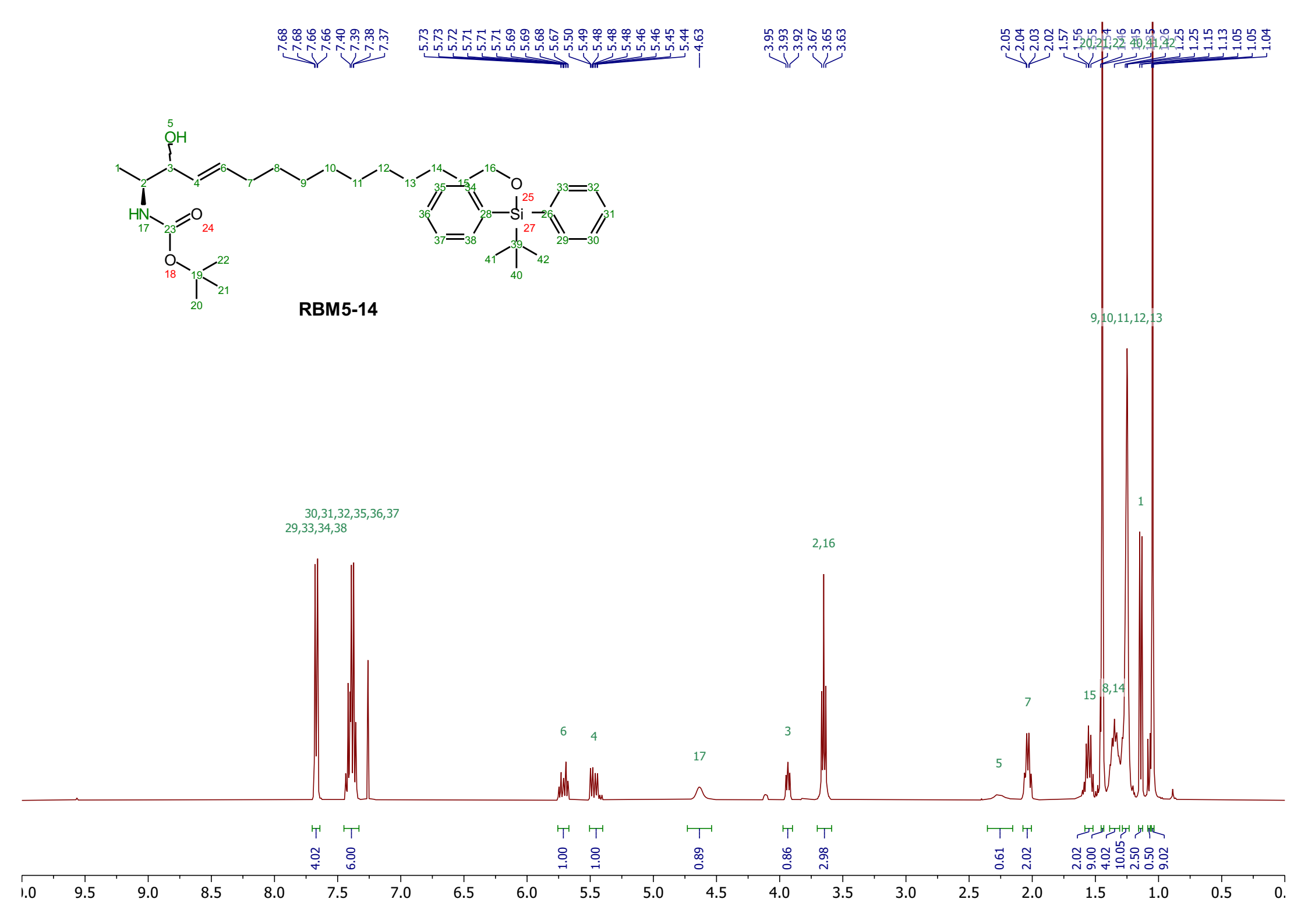
f1 (ppm)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3
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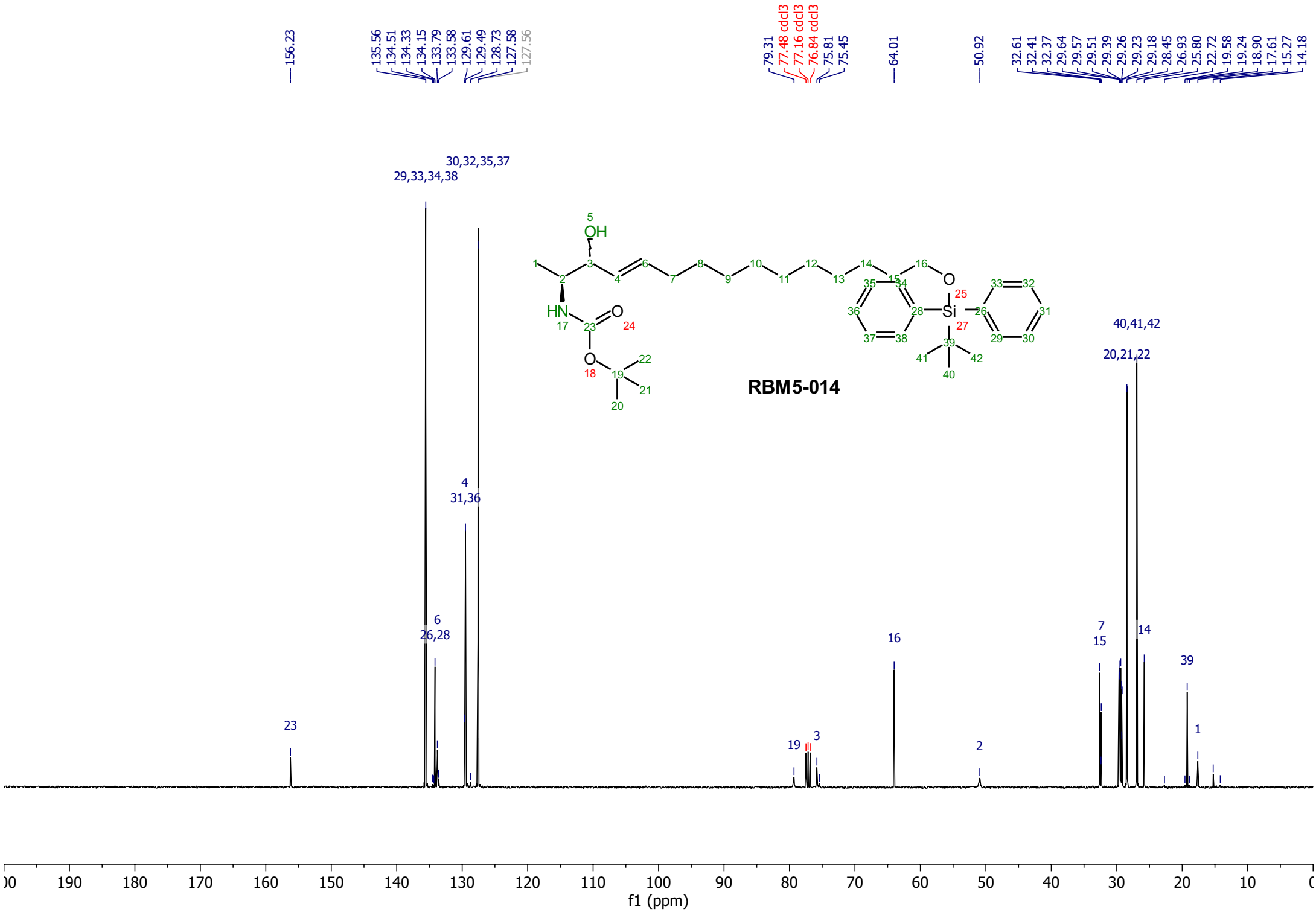
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31,36
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26,28
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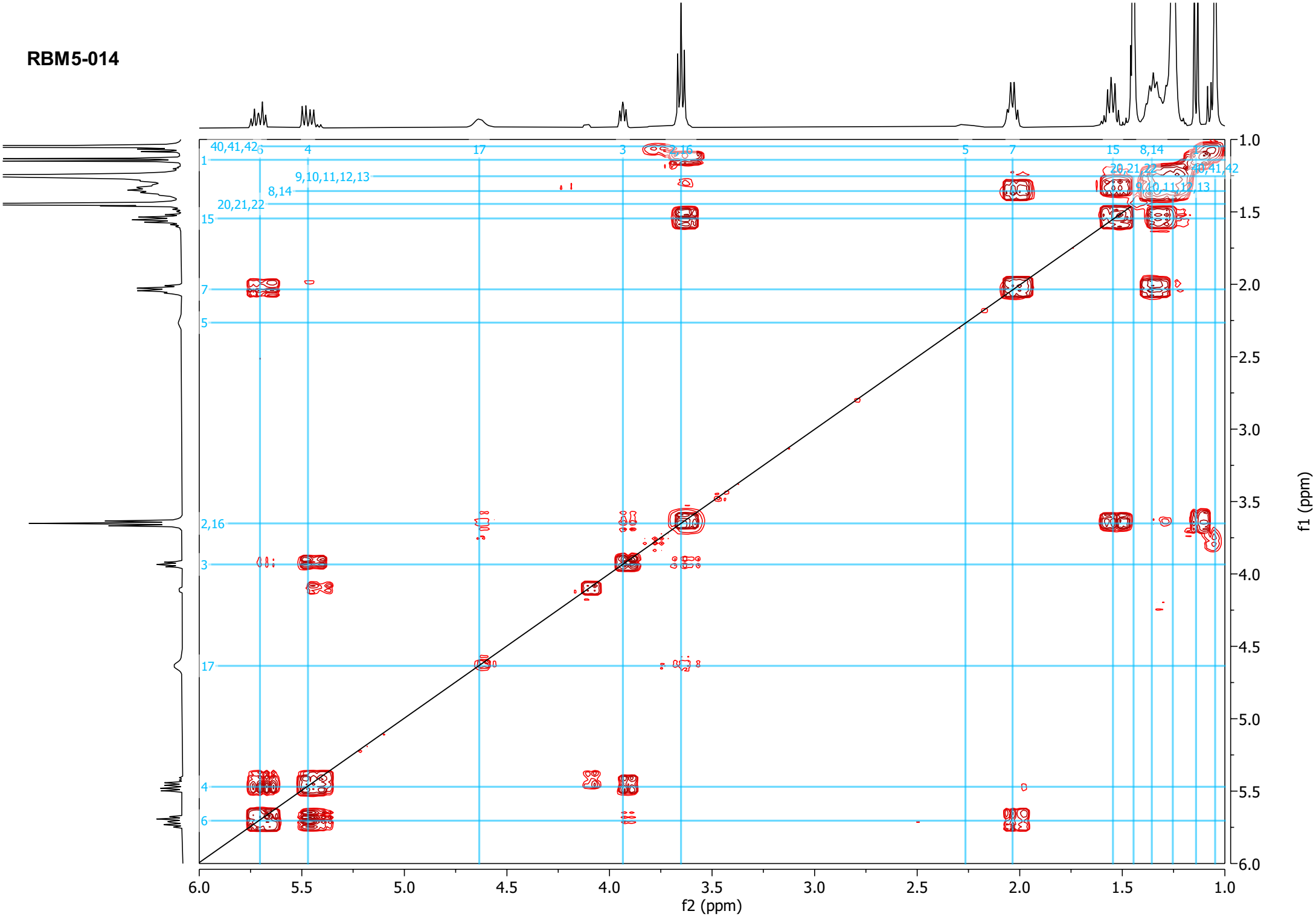
RBM5-014
f1 (ppm)

RBM5-014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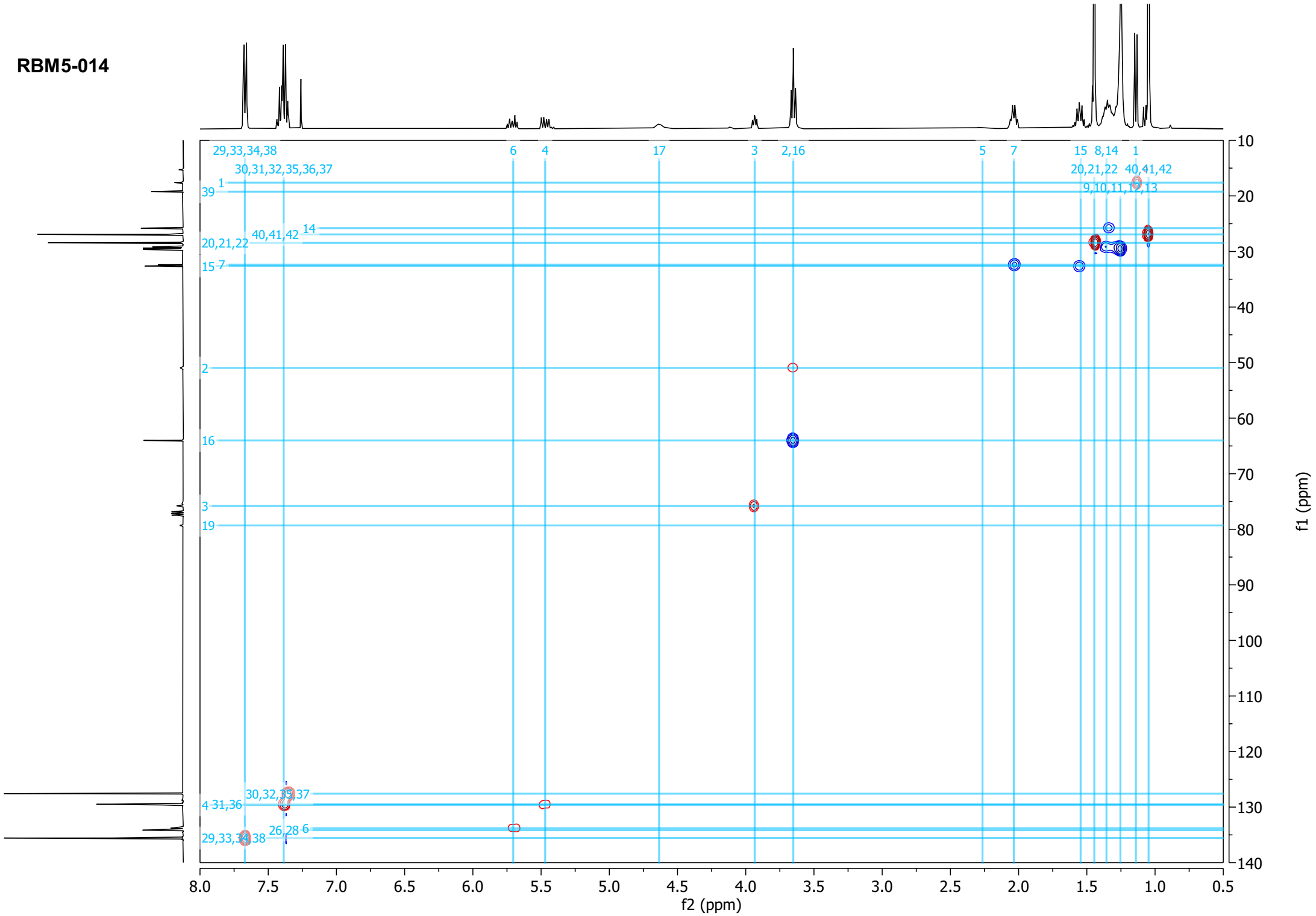
f1 (ppm)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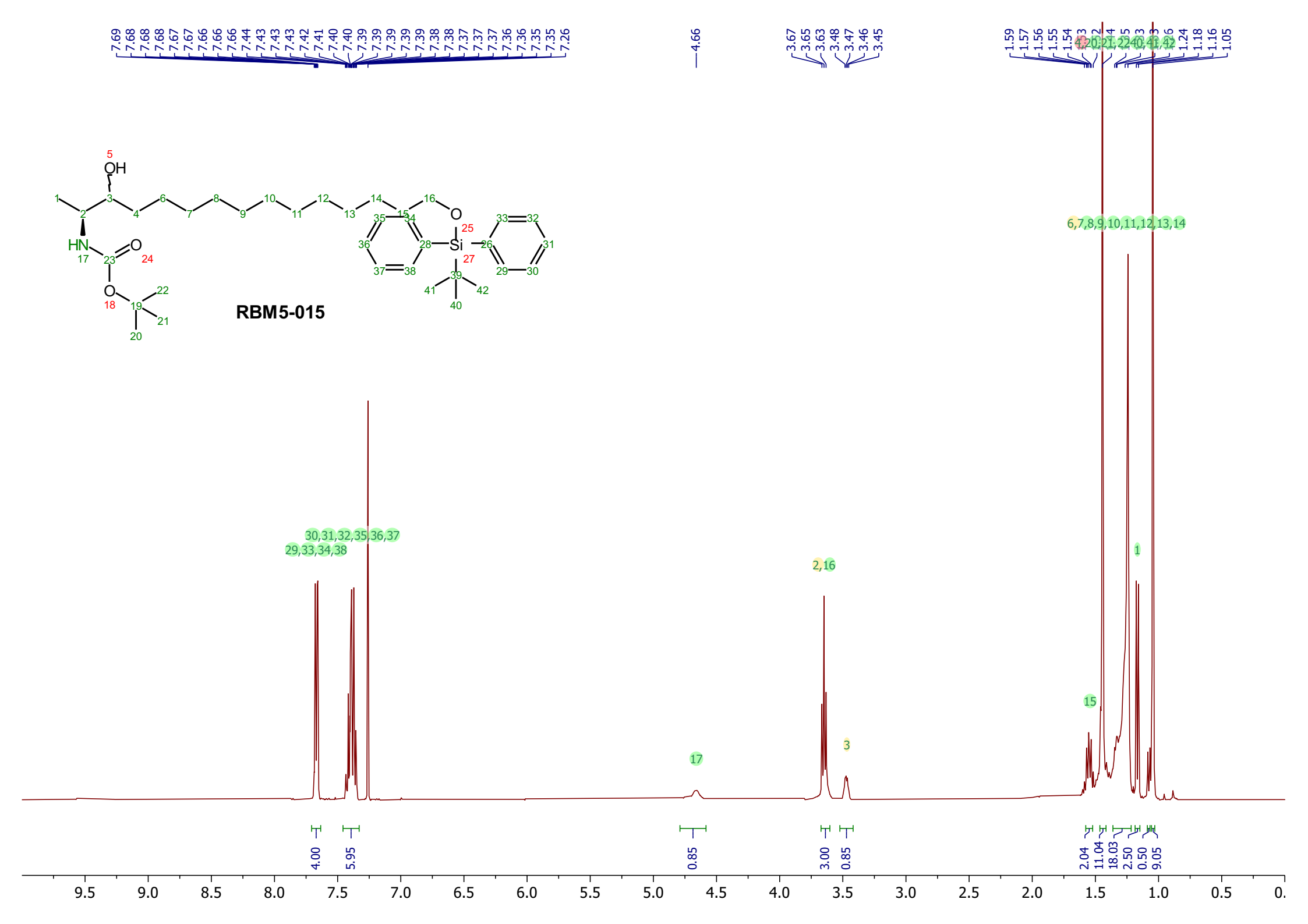
f1 (ppm)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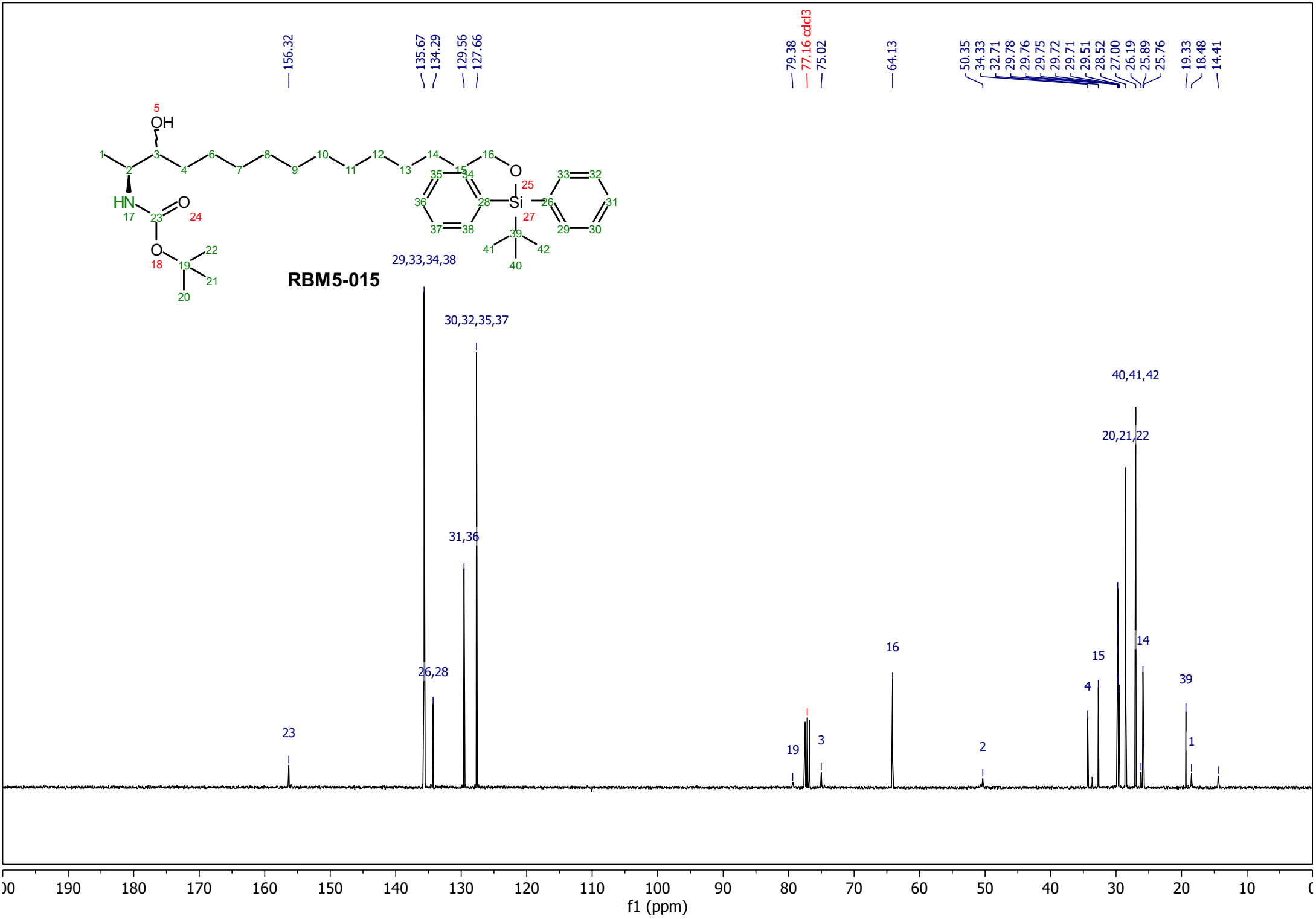
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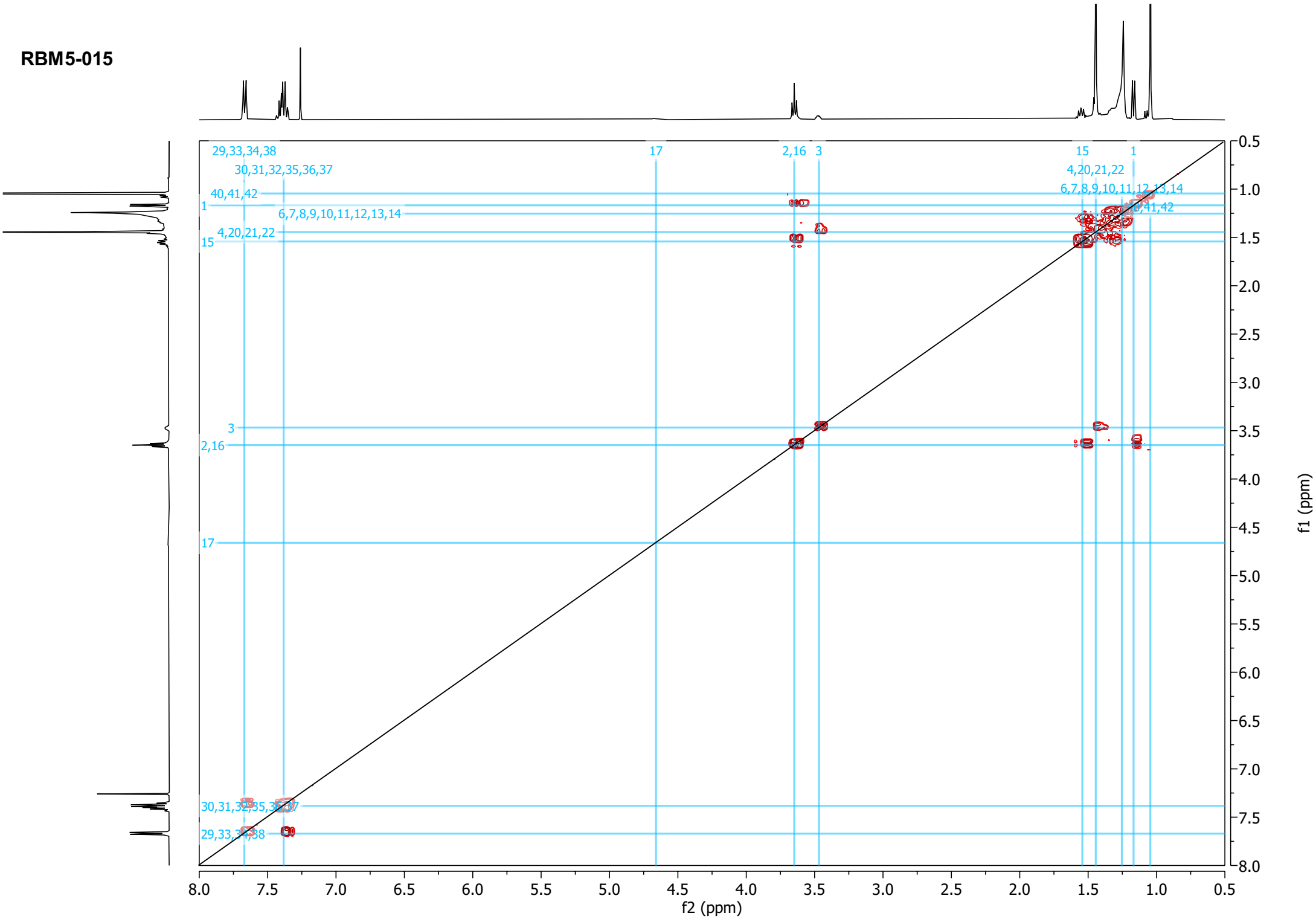
f1 (ppm)

RBM5-015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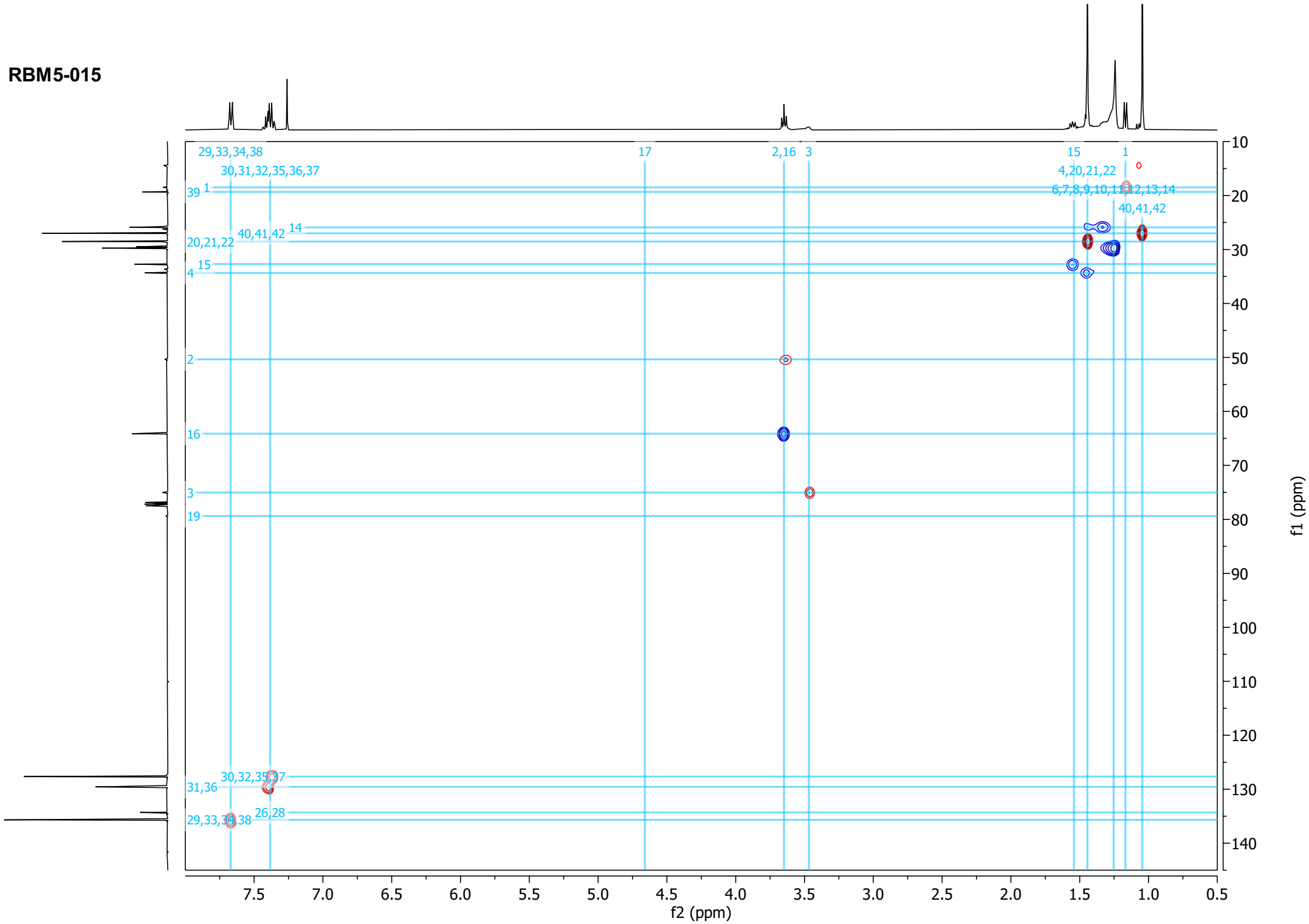
f1 (ppm)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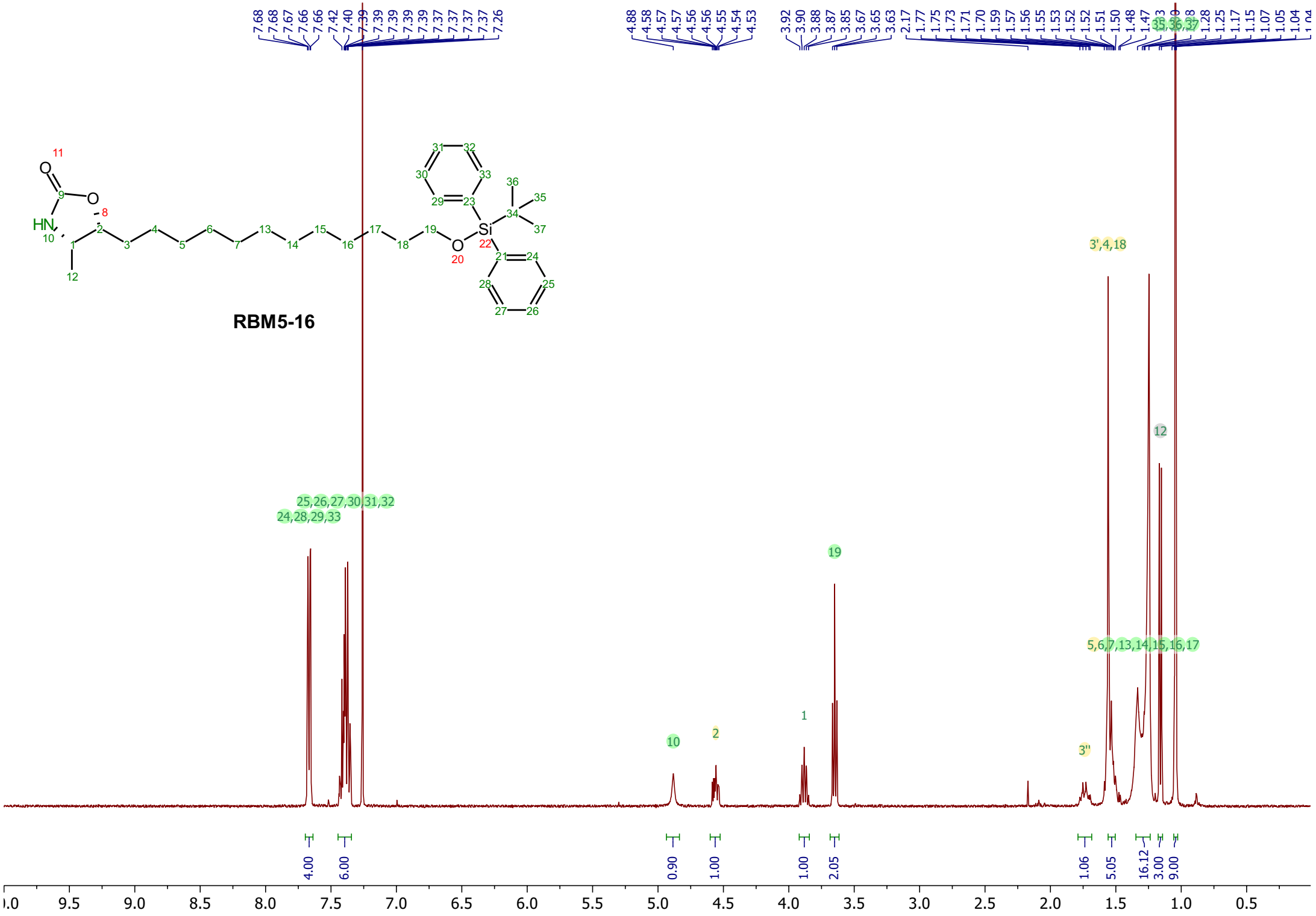
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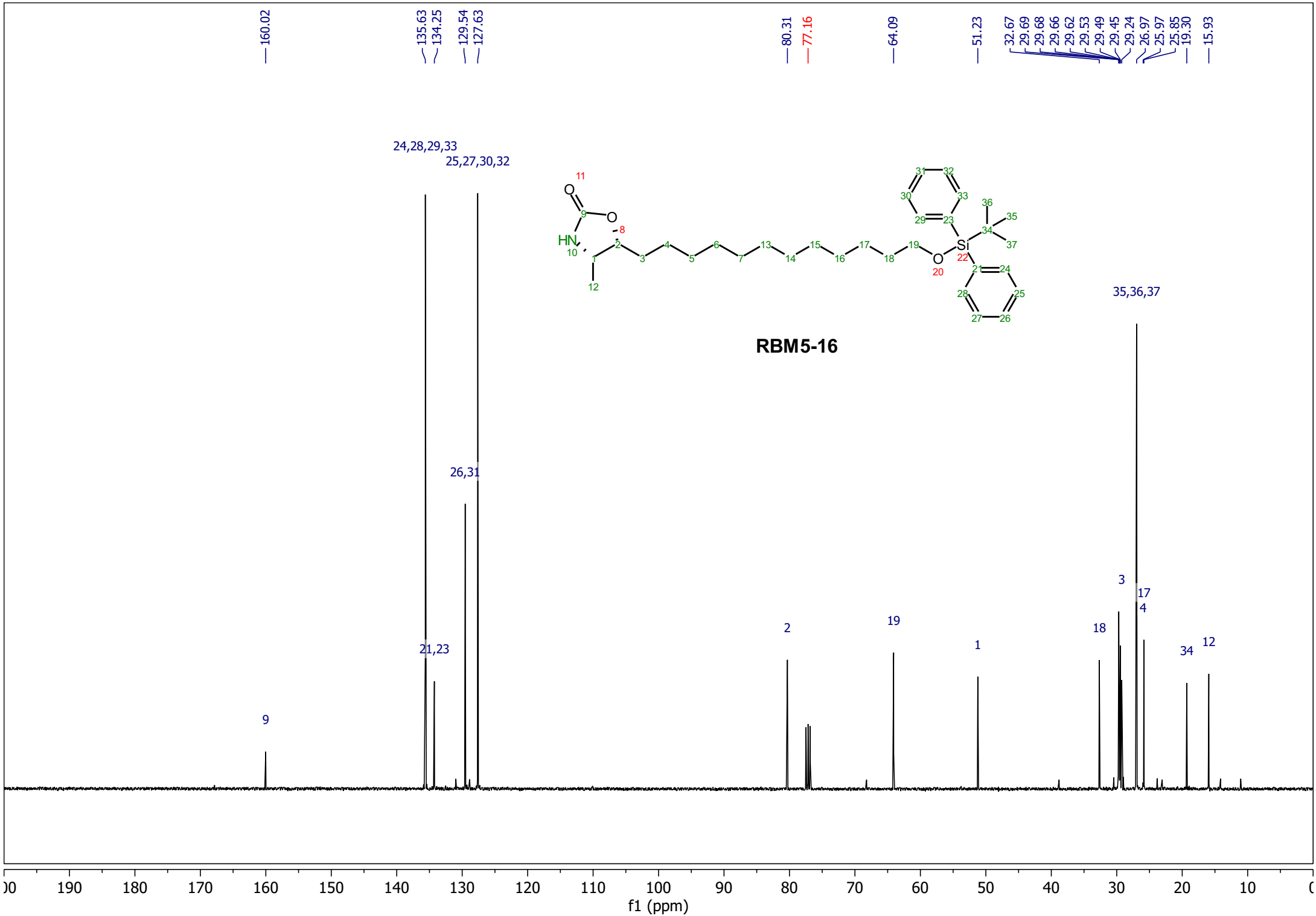
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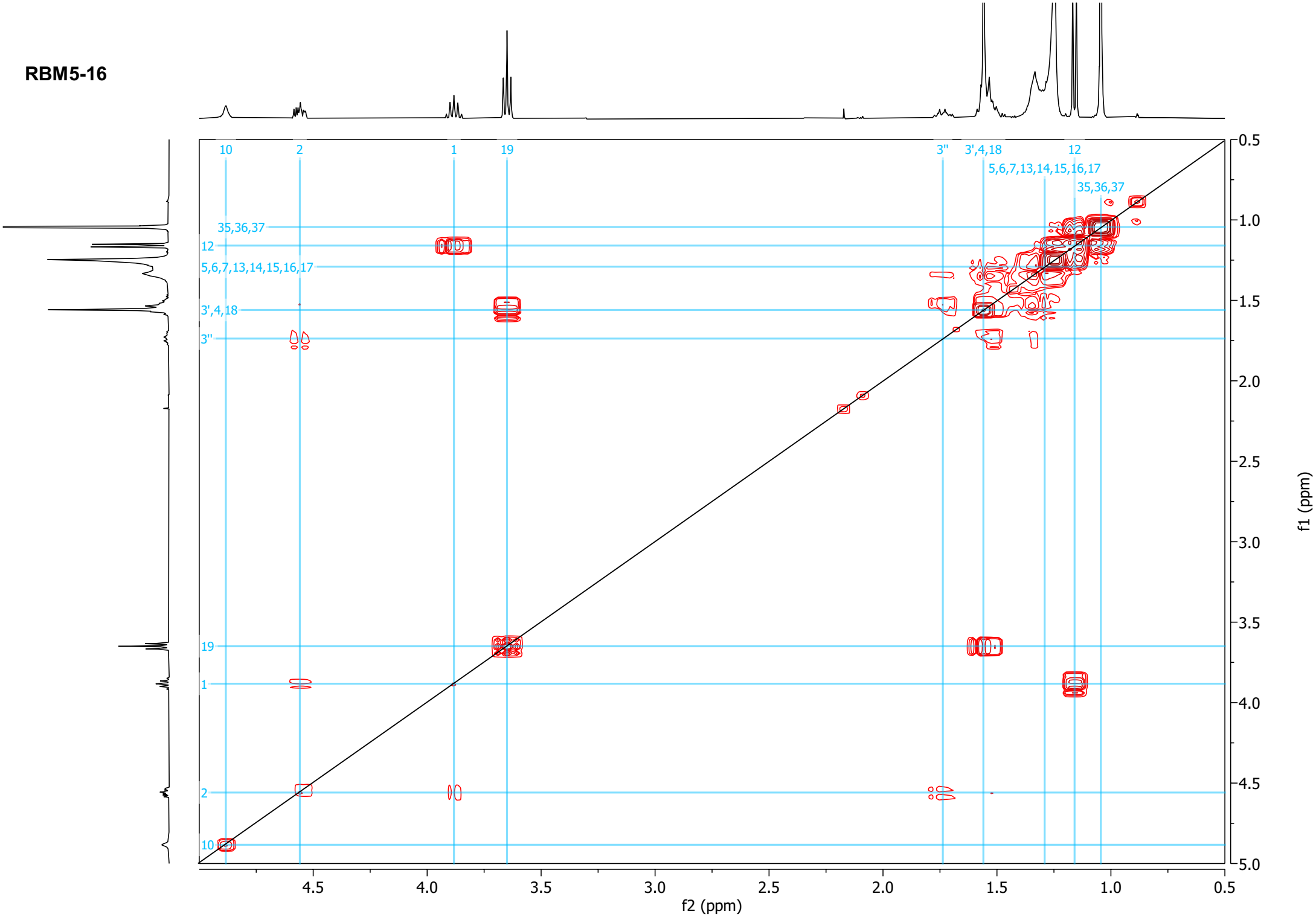
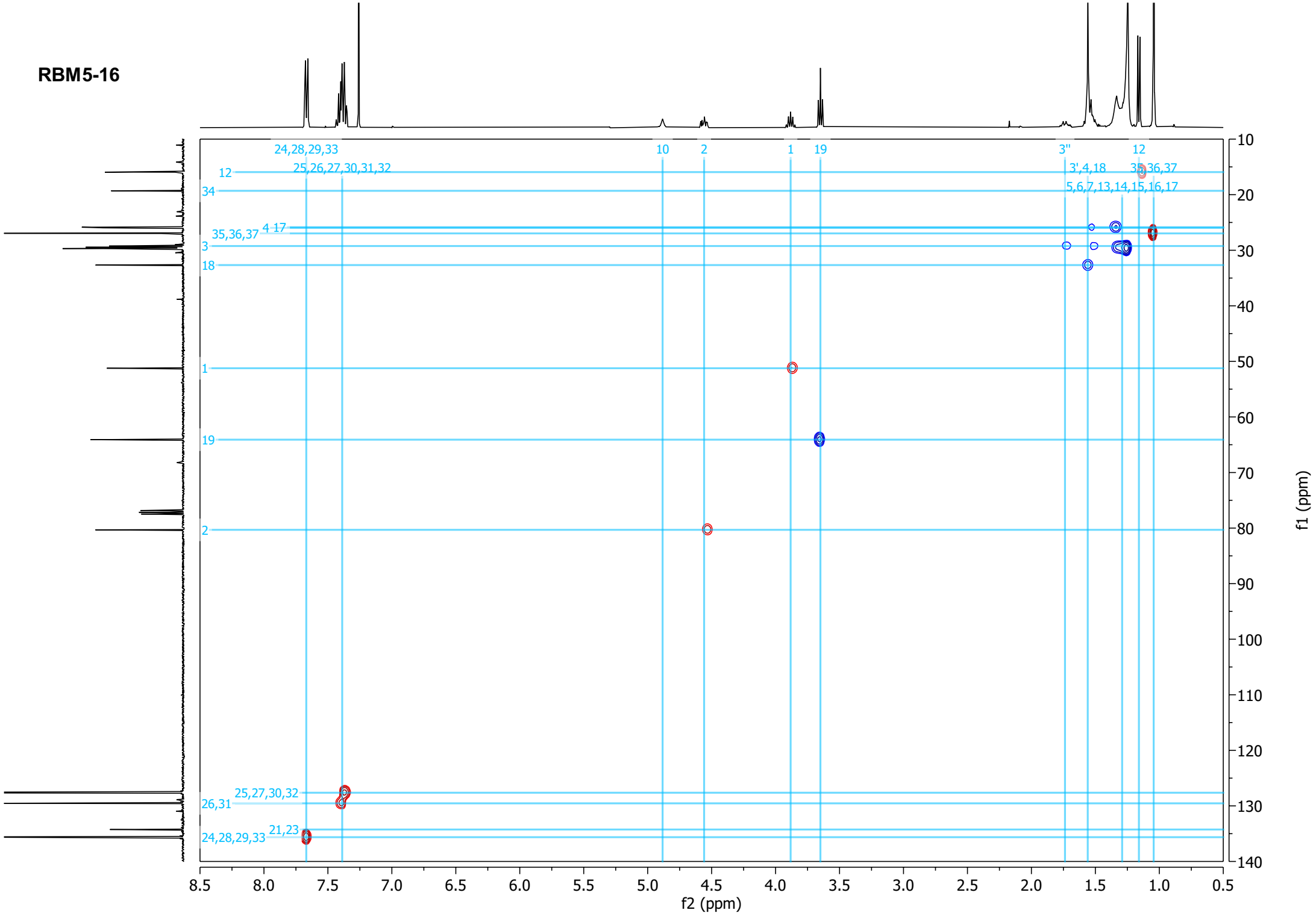
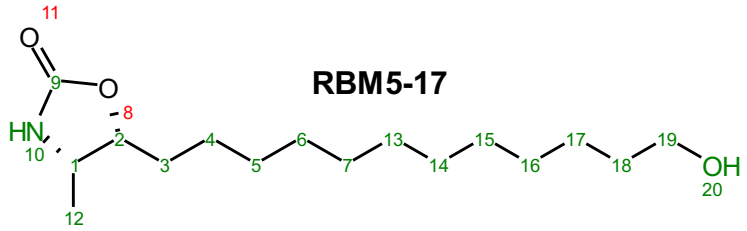
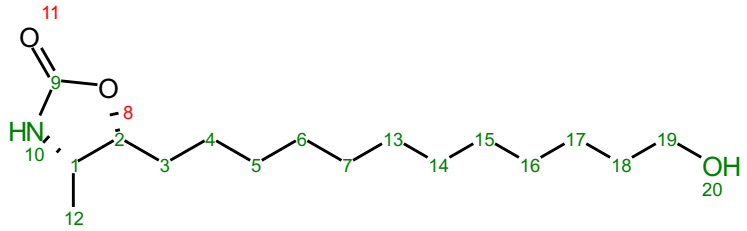
1
18
3
17
4
34
12
35,36,37
RBM5-16
f1 (ppm)

RBM5-16


10
2
1
19
3''
3',4,18
12
5,6,7,13,14,15,16,17
35,36,37
f2 (ppm)
f1 (ppm)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5


RBM5-16
24,28,29,33
25,26,27,30,31,32
10
2
1
19
3"
12
3',4,18
35,36,37
5,6,7,13,14,15,16,17
12
34
35,36,37
4 17
3
18
1
19
2
25,27,30,32
26,31
24,28,29,33
21,23
f2 (ppm)
f1 (ppm)


RBM5-17
11
O
9
O
8
HN
10
2
1
3
4
5
6
7
13
14
15
16
17
18
19
OH
20
12

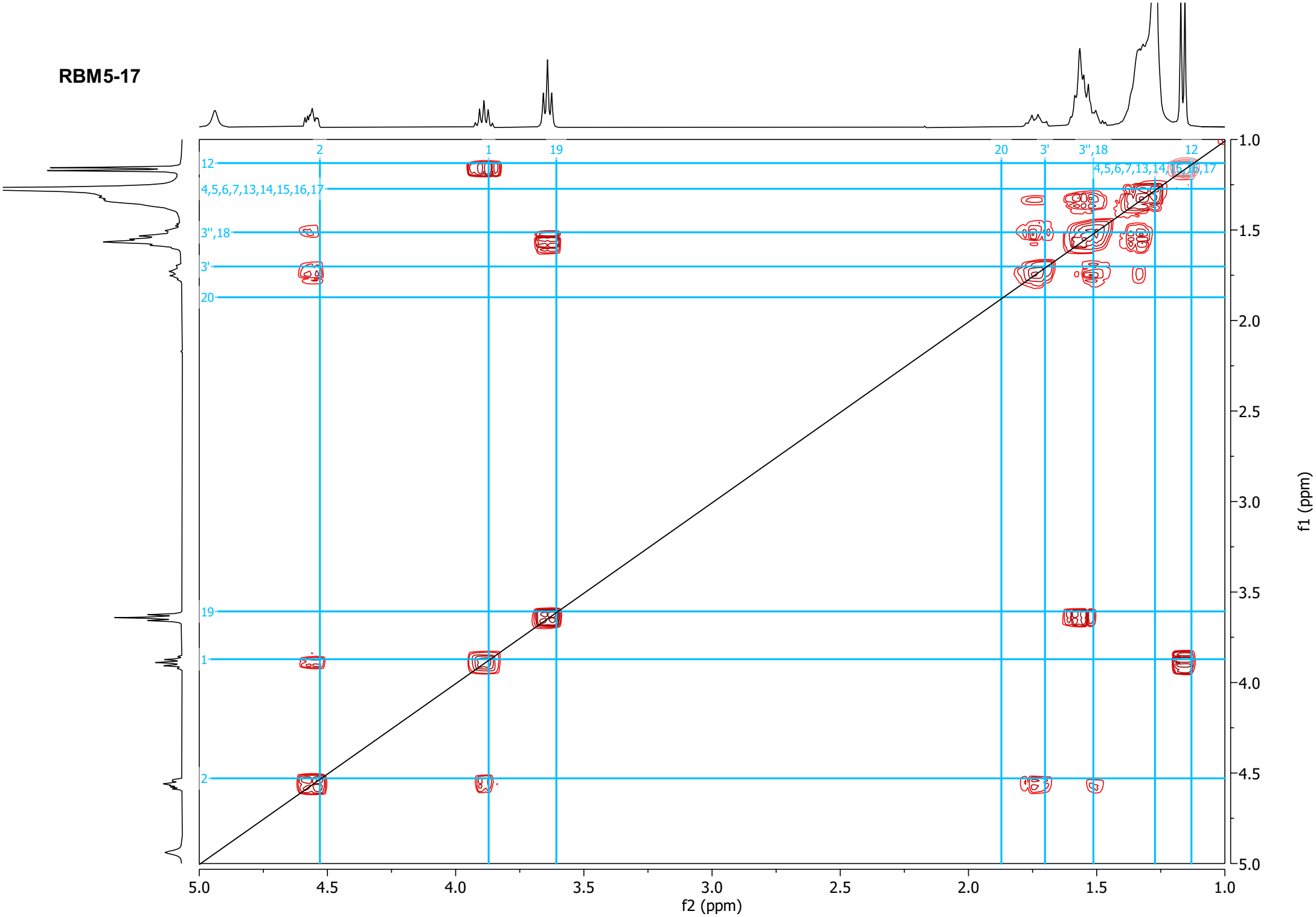


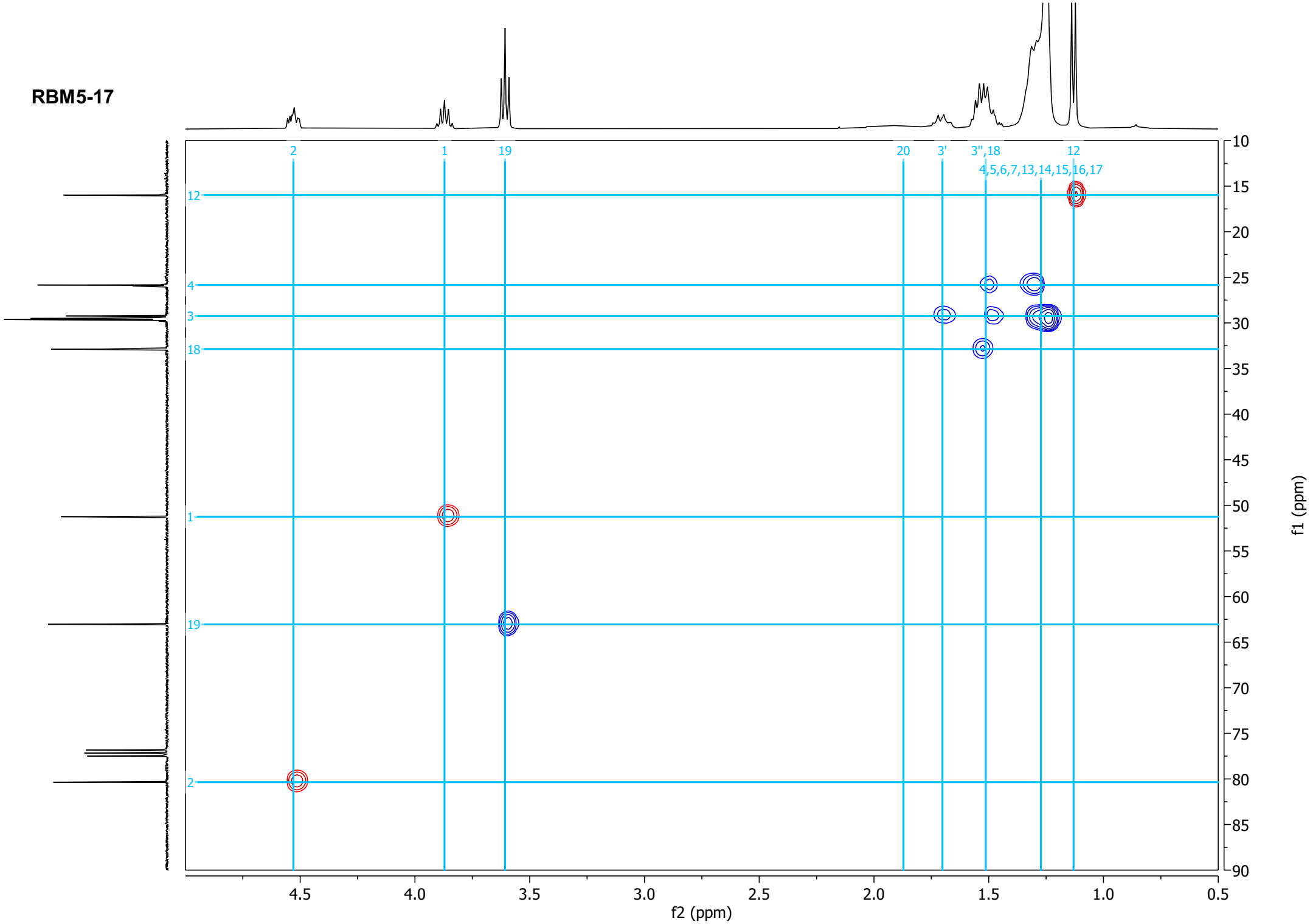
RBM5-17

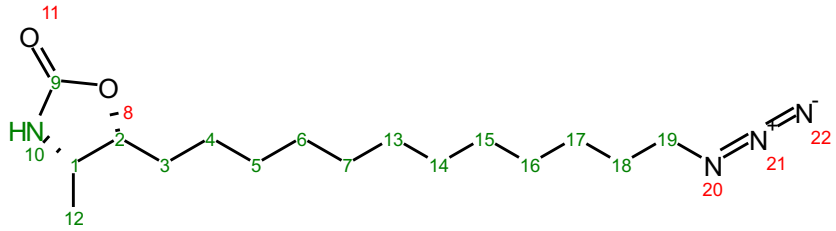
159.92
80.35
77.16
63.05
51.24
32.87
29.66
29.63
29.62
29.55
29.51
29.49
29.45
29.24
25.94
25.84
15.98

9
2
19
1
18
3
4
12

f1 (ppm)

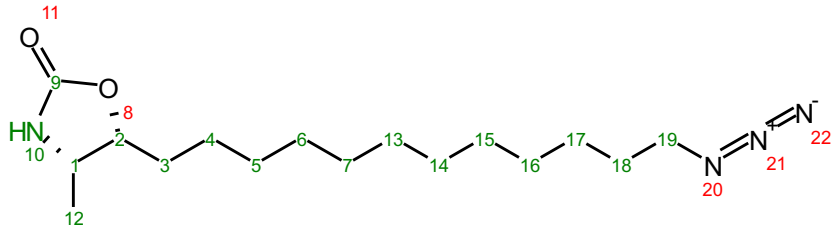

RBM5-17
2
1
19
20
3'
3'',18
12
4,5,6,7,13,14,15,16,17
12
4,5,6,7,13,14,15,16,17
3'',18
3'
20
19
1
2
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
f1 (ppm)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
f2 (ppm)


RBM5-17
2
1
19
20
3'
3",18
12
4,5,6,7,13,14,15,16,17
12
4
3
18
1
19
2
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
f2 (ppm)
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
f1 (ppm)


11
O
9
O
8
HN
10
2
1
12
3
4
5
6
7
13
14
15
16
17
18
19
N
20
N
21
N
22

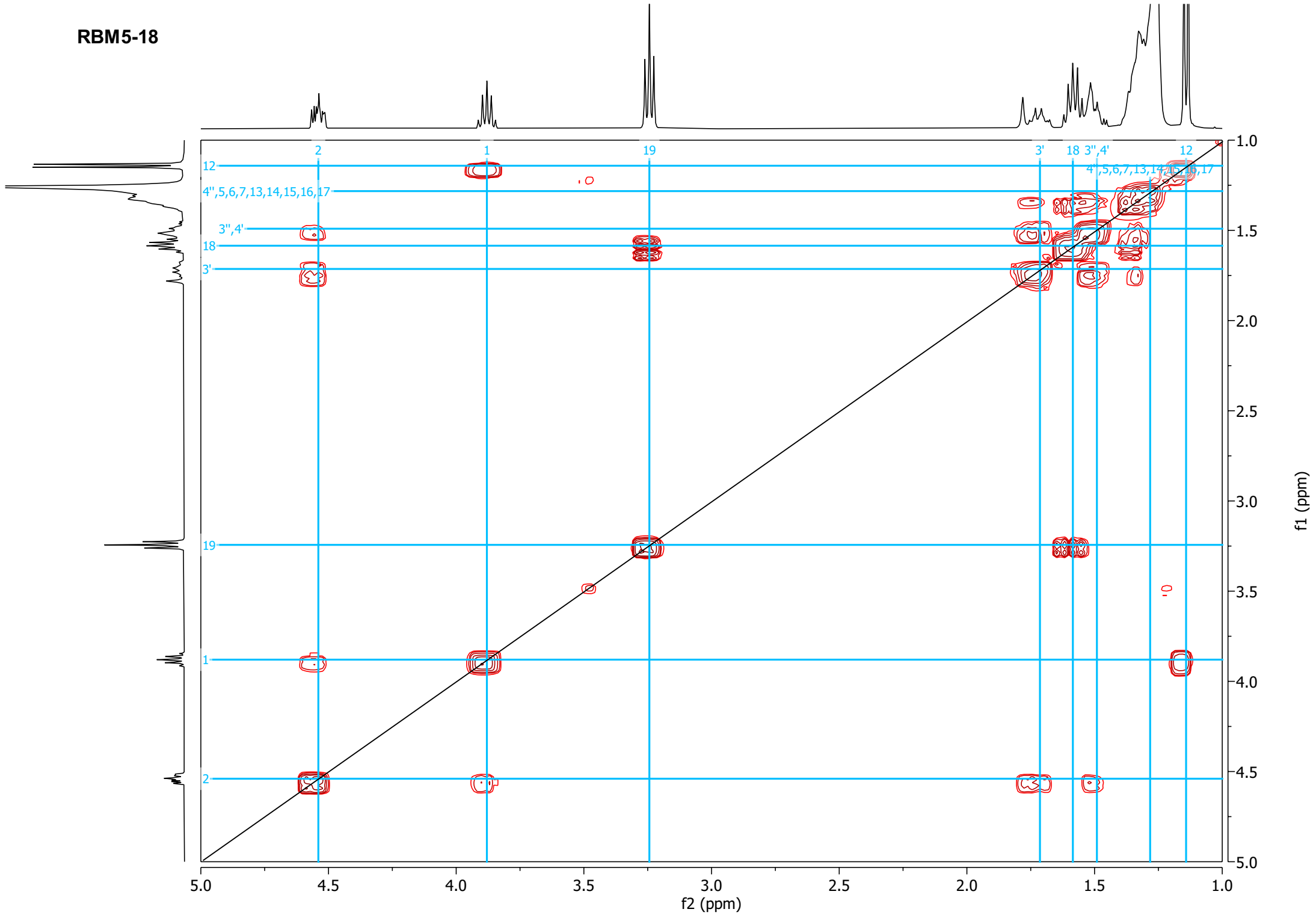
RBM5-18

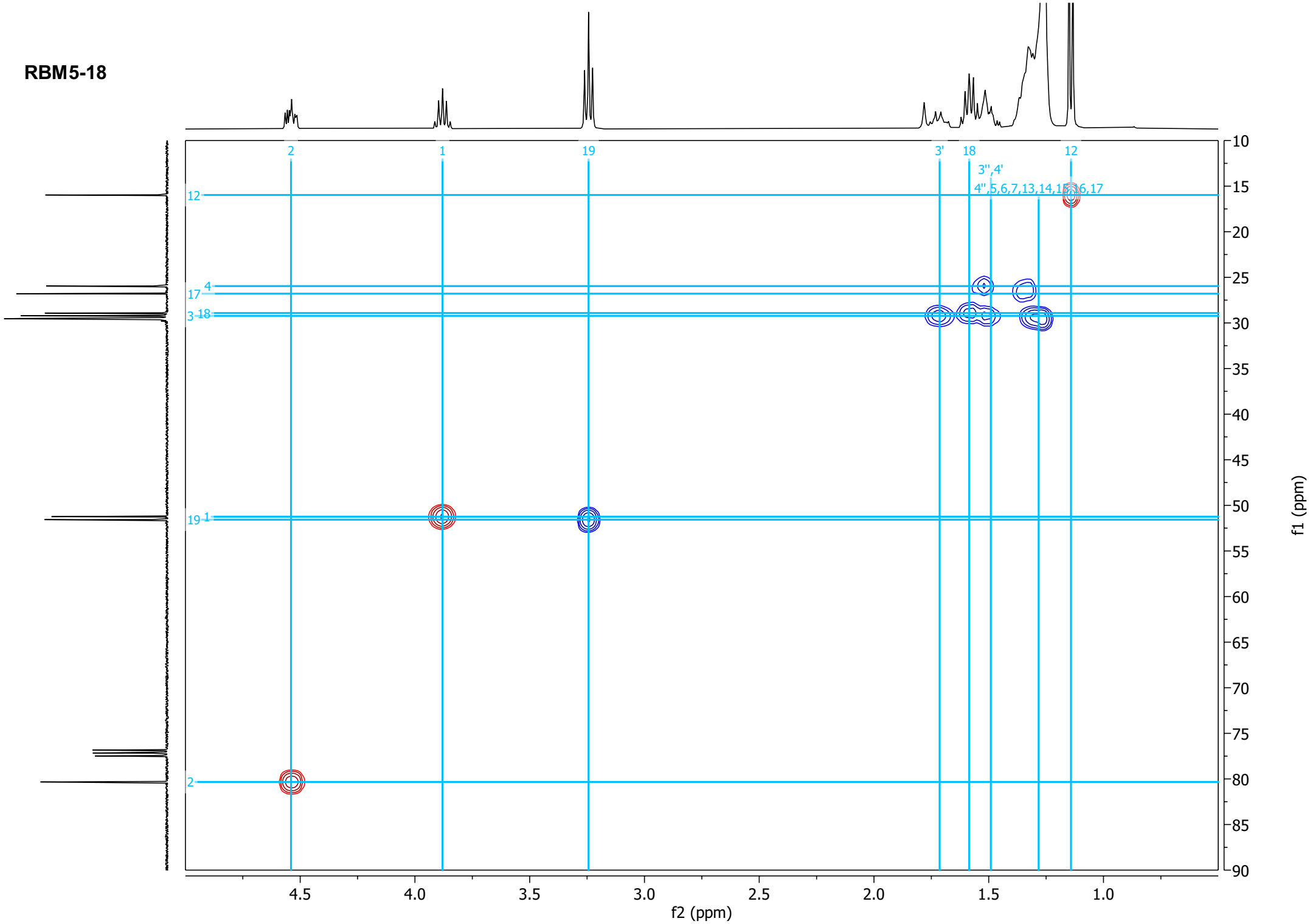
4",5,6,7,13,14,15,16,17
12
18
3",4'
3'
7.26
10
3.26
3.24
3.23
0.96
1.00
1.01
2.00
1.02
2.01
2.01
17.02
3.01

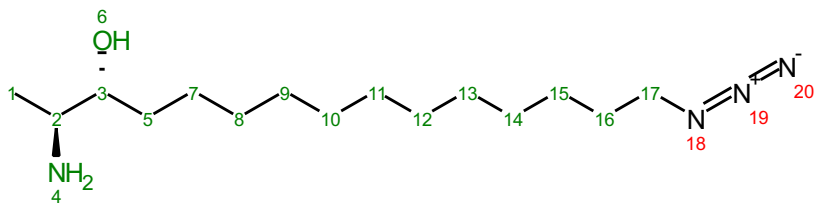

11
O
9
O
8
HN
10
2
1
12
3
4
5
6
7
13
14
15
16
17
18
19
N
20
N
21
N
22

RBM5-18

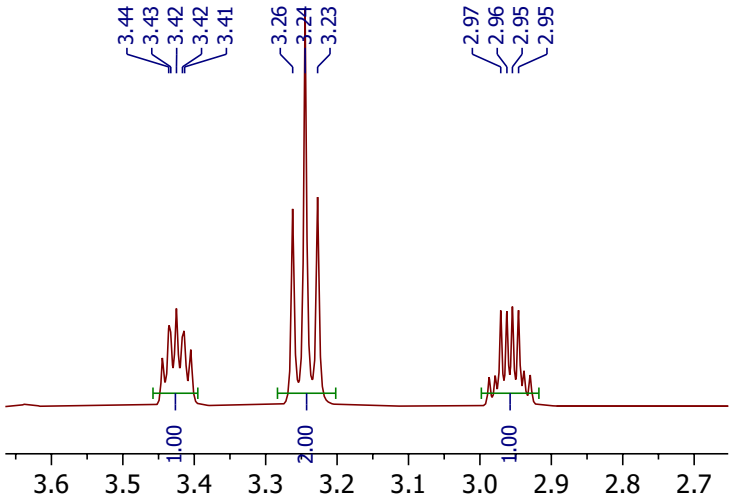
159.92
80.33
77.16
51.57
51.24
29.62
29.58
29.57
29.54
29.51
29.47
29.24
29.22
28.92
26.79
25.96
15.97
3
18
17
2
1
19
4
12
9
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10


RBM5-18
2
1
19
3'
18
3",4'
12
4",5,6,7,13,14,15,16,17
12
4",5,6,7,13,14,15,16,17
3",4'
18
3'
19
1
2
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
f2 (ppm)
f1 (ppm)

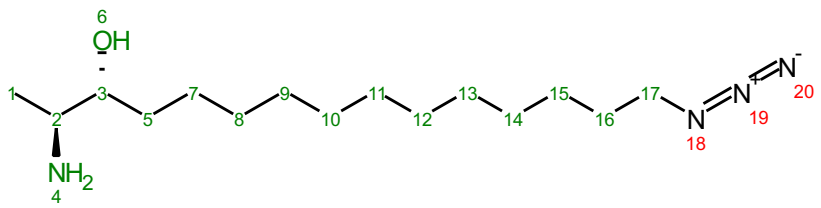

RBM5-18
2
1
19
3'
18
12
3'',4'
4'',5,6,7,13,14,15,16,17
12
17
4
3
18
19
1
2
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
f2 (ppm)
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
f1 (ppm)


6
OH
1
2
3
5
7
8
9
10
11
12
13
14
15
16
17
NH2
4
18
19
20

RBM5-19

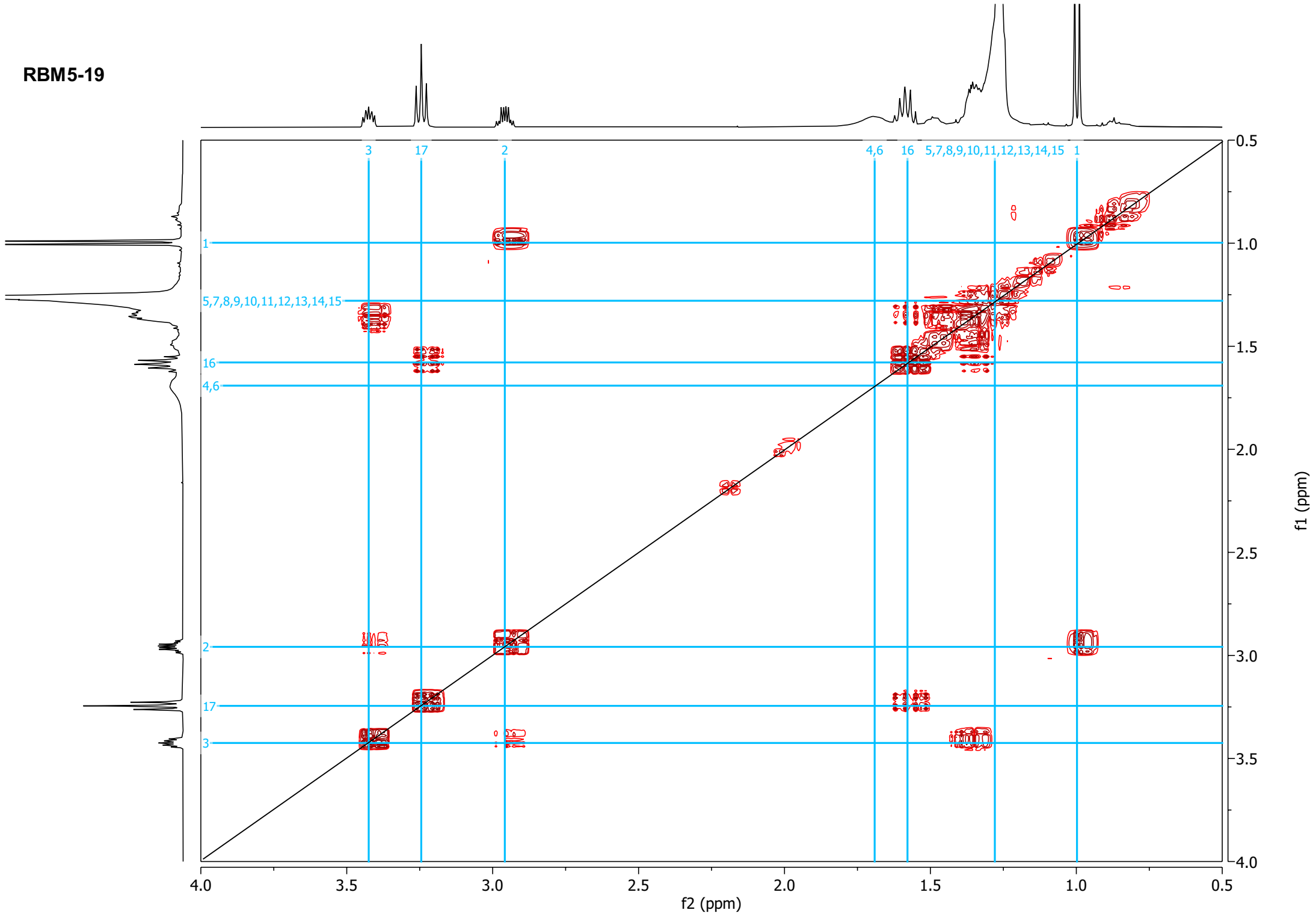

3.44
3.43
3.42
3.42
3.41
3.26
3.24
3.23
2.97
2.96
2.95
2.95
1.00
2.00
1.00
3.6
3.5
3.4
3.3
3.2
3.1
3.0
2.9
2.8
2.7

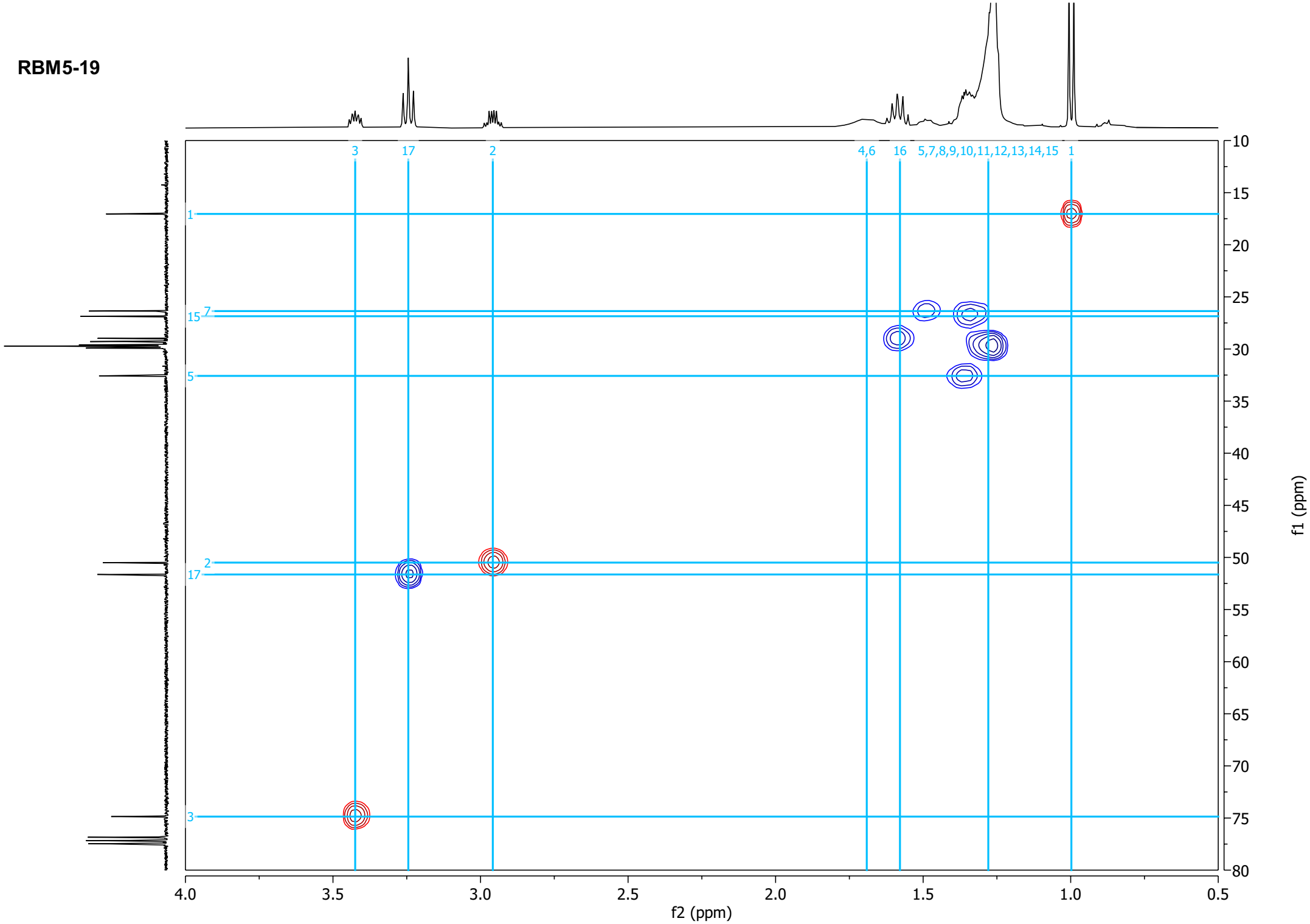
3.44
3.43
3.42
3.42
3.41
3.26
3.24
3.23
2.97
2.96
2.95
2.95
1.69
1.61
1.55
1.53
1.51
1.29
1.27
1.25
1.07
0.99
17
16
3
2
4,6
1.00
2.00
1.00
2.08
2.06
0.97
20.12
3.01
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0

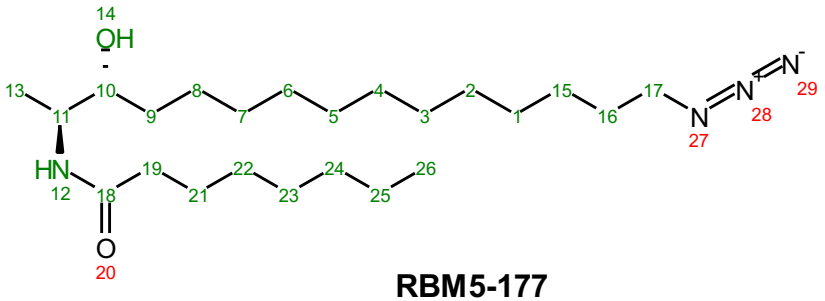

6
OH
1
2
3
5
7
8
9
10
11
12
13
14
15
16
17
N
N
N
20
19
18
NH2
4

RBM5-19

77.16
74.86
51.63
50.49
32.58
29.92
29.74
29.74
29.73
29.66
29.61
29.29
28.98
26.86
26.36
17.04
3
2
17
5
7
15
1
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10

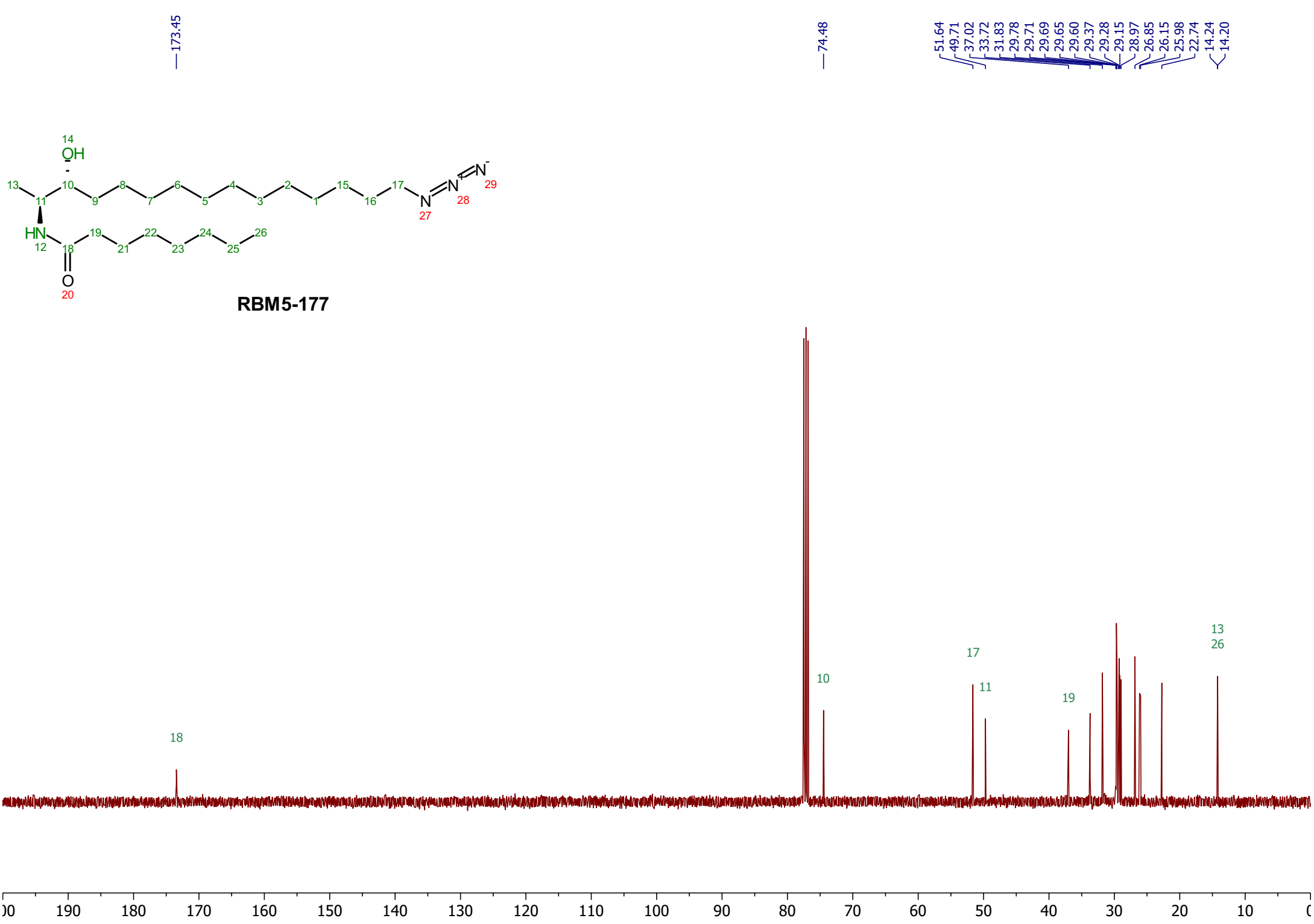

RBM5-19
3
17
2
4,6
16
5,7,8,9,10,11,12,13,14,15
1
5,7,8,9,10,11,12,13,14,15
16
4,6
2
17
3
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
f2 (ppm)
f1 (ppm)

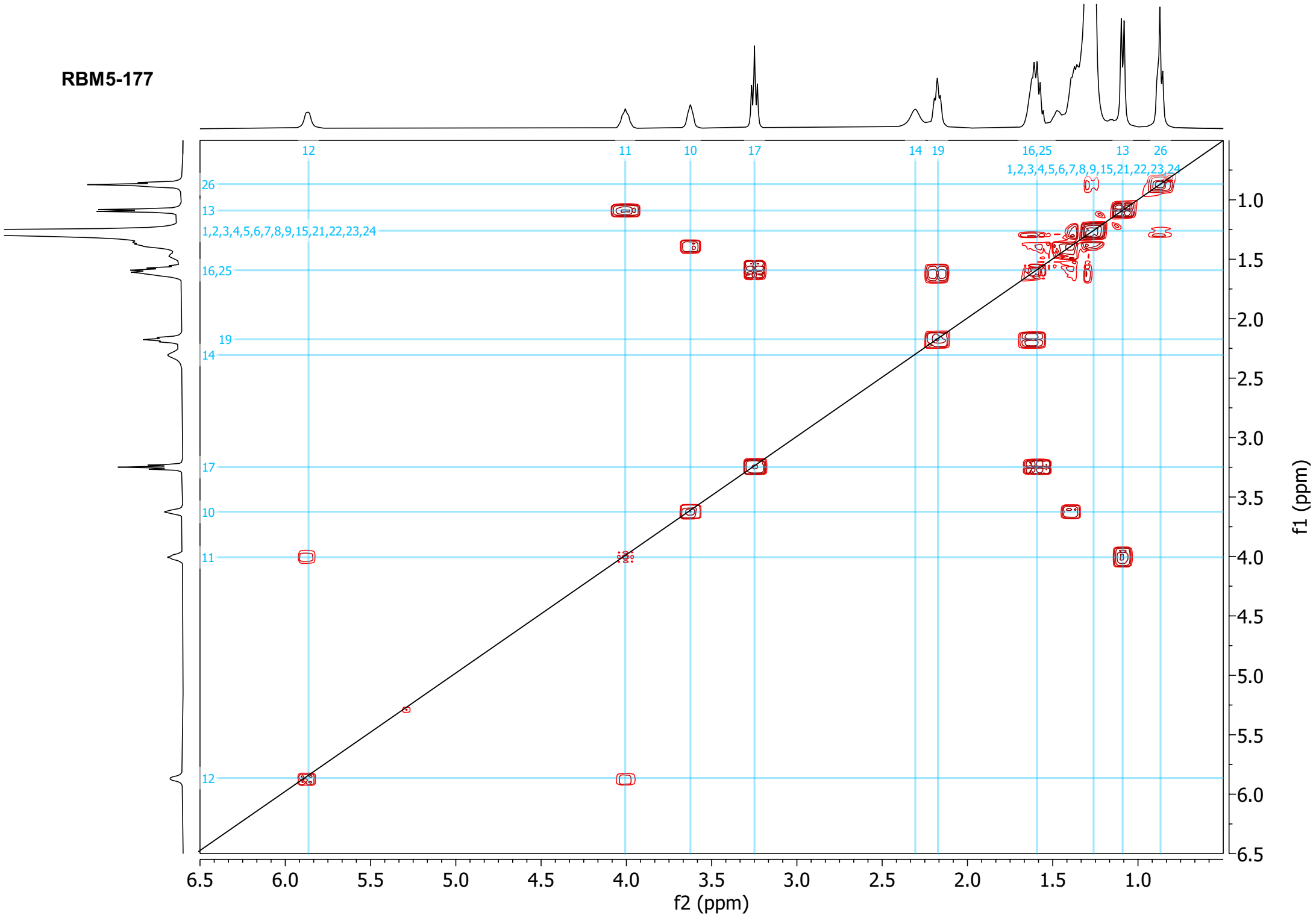

RBM5-19
3
17
2
4,6
16
5,7,8,9,10,11,12,13,14,15
1
1
15
7
5
2
17
3
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
f2 (ppm)
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
f1 (ppm)

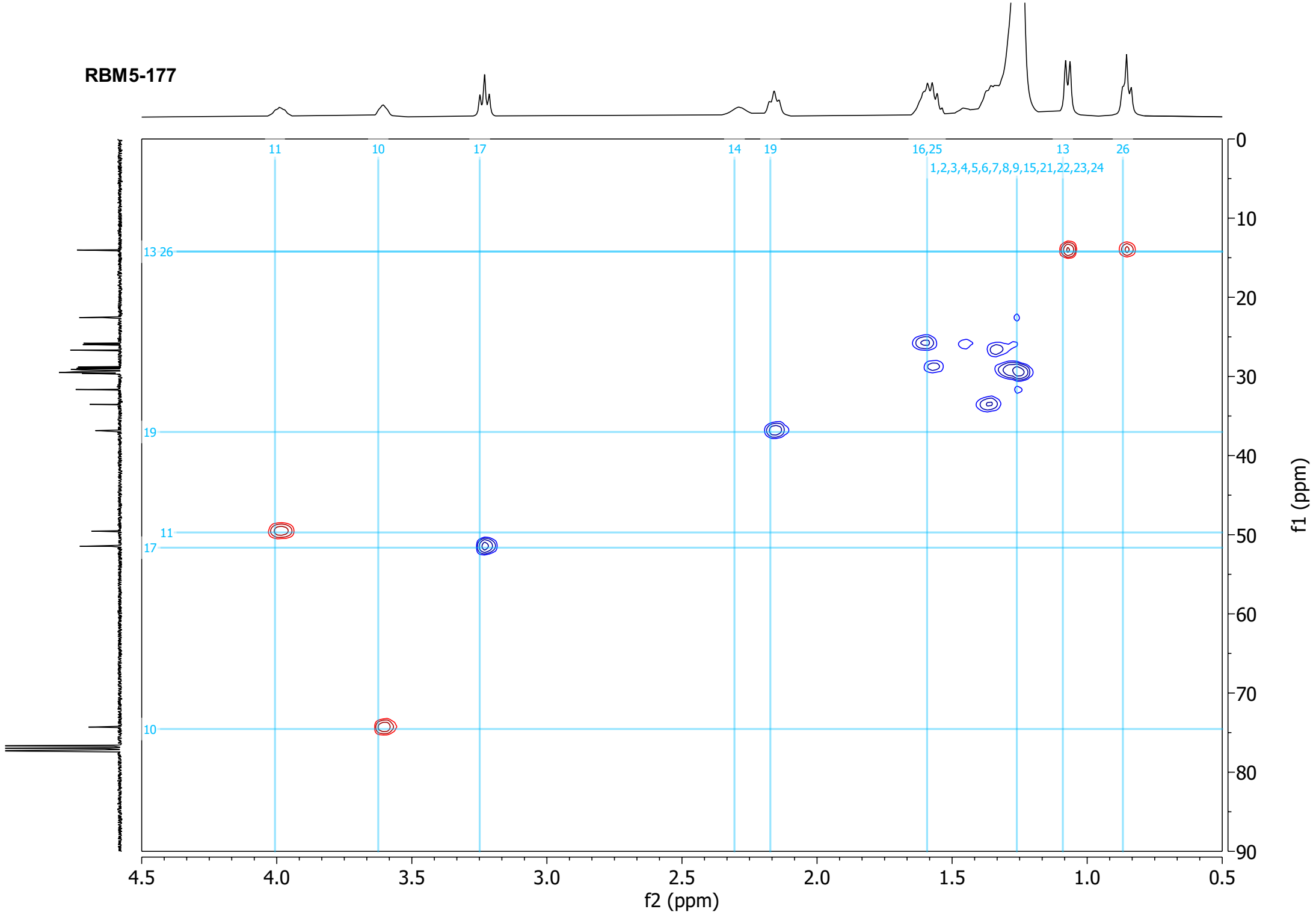

14
OH
13
10
9
8
7
6
5
4
3
2
1
15
16
17
N
N
29
28
27
11
HN
12
18
19
21
22
23
24
25
26
O
20

RBM5-177

5.9
4.0
3.6
3.3
3.2
3.2
2.3
2.2
2.2
2.2
1.6
1.6
1.6
1.6
1.4
1.4
1.3
1.1
1.1
0.9
0.9
1,2,3,4,5,6,7,8,9,15,21,22,23,24
12
11
10
17
14
19
16,25
13
26
0.80
0.94
0.92
1.92
1.02
1.96
3.99
28.00
3.06
3.08
.4
13
12
11
10
9
8
7
6
5
4
3
2
1
0
-1


173.45
74.48
51.64
49.71
37.02
33.72
31.83
29.78
29.71
29.69
29.65
29.60
29.37
29.28
29.15
28.97
26.85
26.15
25.98
22.74
14.24
14.20
RBM5-177
18
10
17
11
19
13
26


RBM5-177
12
11
10
17
14
19
16,25
13
26
1,2,3,4,5,6,7,8,9,15,21,22,23,24
f2 (ppm)
f1 (ppm)


RBM5-177
11
10
17
14
19
16,25
1,2,3,4,5,6,7,8,9,15,21,22,23,24
13
26
13 26
19
11
17
10
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
f2 (ppm)
0
10
20
30
40
50
60
70
80
90
f1 (ppm)