*Electronic Supplementary information*

# Petrobactin biosynthesis: AsbB catalyzes efficient condensation of spermidine with $N^8$-citryl-spermidine or $N^1$-(3,4-dihydroxybenzoyl)-$N^8$-citryl-spermidine

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## 1 Materials, methods and procedures

### 1.1 Synthesis of $N^1$-(3,4-dihydroxybenzoyl)-spermidine (*N*-[3-(4-amino-butylamino)-propyl]-3,4-dihydroxy-benzamide) 3

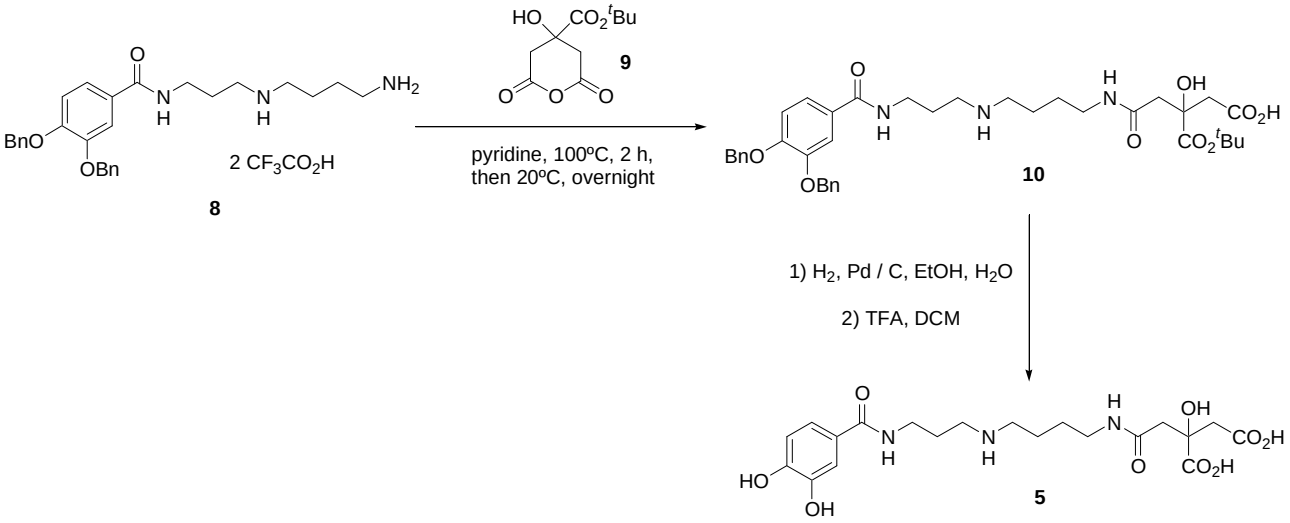
*N*-[3-(4-Amino-butylamino)-propyl]-3,4-bis-benzyloxy-benzamide trifluoroacetate salt **8**, prepared according to the procedure described by O. Phanstiel IV *et al*.,[1] (60 mg, mmol) was dissolved in a water/EtOH mixture (0.3:1) containing 30 mg of 10% Pd/C. The mixture was stirred under $H_2$ (1 atmosphere) at room temperature for 5 hours. The catalyst was removed by filtration of the mixture through celite and the solvent was removed under reduced pressure to give a solid that was purified by HPLC (Agilent eclipse XDB-C18 100 x 21 mm 5 μ, 70% water / MeCN isocratic). The spectroscopic data for the product agreed well with the published literature data for **3**.[2]

### 1.2 Synthesis of $N^8$-citryl-spermidine 4

The synthesis of $N^8$-citryl-spermidine **4** was accomplished using the methodology previously described by us.[3]

### 1.3 Synthesis of $N^1$-(3,4-dihydroxybenzoyl)-$N^8$-citryl-spermidine 5.

The synthesis of $N^1$-(3,4-dihydroxybenzoyl)-$N^8$-citryl-spermidine **5** was accomplished using the synthetic route outlined below.




**Scheme 1.** Route employed for the synthesis of $N^1$-(3,4-dihydroxybenzoyl)-$N^8$-citryl-spermidine **5**

**1.3.1 2-({4-[3-(3,4-Bis-benzyloxy-benzoylamino)-propylamino]-butylcarbamoyl}-methyl)-2-hydroxy-succinic acid 1-tert-butyl ester 10.** A solution of compound **8** (1 g, 1.45 mmol) and 4-Hydroxy-2,6-dioxo-tetrahydro-pyran-4-carboxylic acid *tert*-butyl ester **9**[4] (370 mg, 1.61 mmol) in anhydrous pyridine (30 mL) was stirred at 100ºC for 2 h and then at room temperature overnight. The solvent was removed under vacuum, and a portion of the crude material was partially purified by HPLC (Agilent-Zorbax XDB-C18 column (100 X 21 mm, 5µ)) detecting absorbance at 280 nm using the elution profile in table 1.

**Table 1.**

| Time (min) | Water (0.1% TFA) | MeCN (0.1% TFA) | Flow (mL / min) |
|---|---|---|---|
| 0 | 35 | 65 | 4.5 |
| 10 | 35 | 65 | 4.5 |
| 20 | 0 | 100 | 4.5 |
| 25 | 0 | 100 | 4.5 |

The collected fractions were analyzed by ESI-MS and those containing the compound with *m/z* 692.3 were freeze-dried.

**1.3.2 $N^1$-(3,4-dihydroxybenzoyl)-$N^8$-citryl-spermidine 5.** Partially purified **10** (300 mg) was dissolved in 60 mL of 1,4-dioxane:water (25:75). Then, 10% Pd/C (150 mg) was added, and the mixture was stirred under hydrogen (1 atm) for 5 h at room temperature. The catalyst was removed by filtration of the mixture through celite, and the solvent was removed under vacuum. The crude product thus obtained was dissolved in DCM (20 mL) and TFA (8 mL) was added dropwise. The solution was stirred at room temperature for 5 h, and then the solvent was removed under vacuum to afford 220 mg of impure **5**. 80 mg of this impure material were purified by HPLC [Agilent-Zorbax XDB-C18 column (100 X 21 mm, 5µ), retention time 6.3 min] detecting absorbance at 280 nm using the elution profile in table 2, to afford **5** as a white solid (30.1 mg). $\delta_H$ ($D_2O$, 400 MHz) 1.57 (m, 2H, C$H_2$-$CH_2$-NHCO), 1.70 (m, 2H, C$H_2$-$CH_2$-NH), 1.98 (m, 2H, C$H_2$-$CH_2$-NHCO-Ar), 2.67 (d, 1H, C*H*H-CO-NH, $^2J$ 14.3), 2.78 (d, 1H, C*H*H-CO-NH, $^2J$ 14.3), 2.80 (d, 1H, C*H*H-$CO_2$H, $^2J$ 16), 3.02-3.09 (m, 5H, 2 C$H_2$-NH + C*H*H-$CO_2$H), 3.22 (t, 2H, C$H_2$-NHCO, $^3J$ 6.5), 3.46 (2H, C$H_2$-NHCO-Ar, $^3J$ 6.5), 6.96 (d, $H_{Ar}$, $^3J$ 8.3), 7.25 (d, $H_{Ar}$, $^3J$ 8.3), 7.3 (s, $H_{Ar}$); $\delta_C$ ($D_2O$ + 1 drop $CD_3CN$, 100 MHz) 23.4, 26.0, 26.3, 37.0, 39.0, 43.8, 45.2, 45.5, 47.7, 74.3, 115.5, 116.3, 121.1, 126.0, 144.5, 148.6, 170.9, 171.7, 174.2 and 177.4; HRMS: Calculated for $C_{20}H_{30}N_3O_9^+$ $(M+H)^+$: 456.1977; found: 456.1996. Key correlations observed in COSY and HMBC spectra are summarized in Figure 1.

**Table 2.**

| Time (min) | Water (0.1% TFA) | MeCN (0.1% TFA) | Flow (mL / min) |
|---|---|---|---|
| 0 | 85 | 15 | 4.5 |
| 10 | 85 | 15 | 4.5 |
| 20 | 0 | 100 | 4.5 |
| 25 | 0 | 100 | 4.5 |

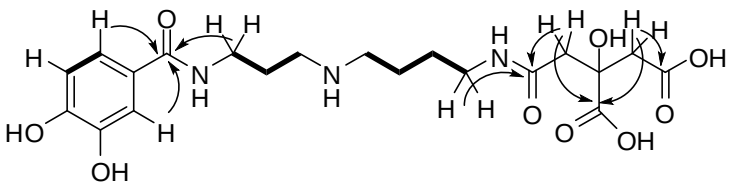



**Figure 1.** Key COSY (bold lines) and HMBC (arrows) correlations observed in NMR spectra of compound **5**.

### 1.4 Cloning and overexpression of AsbB in *E. Coli*.

**1.4.1** The coding sequence of *asbB* was amplified by polymerase chain reaction (PCR) from *B. anthracis* (Ames) genomic DNA using KOD Hot Start DNA polymerase (Novagen) and complementary gene-specific primers to which were appended sequences to facilitate ligation independent cloning (LIC)[5] (underlined) 5'-CCAGGGACCAGCAATGAGAATGGATATGTATCATACGAAAATATTG -3', 5'-GAGGAGAAGGCGCGTTAACAATTAGCATAATTTCCCCCTGTAG -3'. For LIC, the PCR amplification product was treated with LIC qualified T4 DNA polymerase (Novagen) in the presence of dATP to generate 5' single stranded overhangs at both ends of the fragment, through the enzyme's combined 3'-5' exonuclease and DNA polymerase activities. Complementary 5' single stranded overhangs were generated similarly in the cloning vector, a LIC-adapted pET-28a (Novagen) (pET-YSBLIC3C[6]) where the encoded thrombin cleavage site had previously been mutated to encode a site for HRV 3C protease cleavage. The vector pET-YSBLIC3C was digested with the restriction endonuclease *Bse*RI, purified and incubated with LIC qualified T4 DNA polymerase in the presence of dTTP to generate the required single stranded overhangs. The vector and PCR products were annealed and used to transform *E. coli* NovaBlue cells (Novagen) to kanamycin resistance. Single colonies were selected, grown overnight and plasmids isolated using a QIAprep plasmid miniprep kit (Qiagen). Incubation of the plasmids with *Nco*I and *Nde*I restriction endonucleases, followed by DNA sequencing, verified the presence of the gene insert. One correct clone named pDOC0002 was used to transform *E. coli* BL21Star (DE3) (Invitrogen).
For overexpression of *asbB*, 300 mL of LB medium supplemented with kanamycin (50μg/mL) was inoculated with a 0.5 mL overnight culture of *E. coli* BL21Star(DE3) / pDOC0002 and incubated in an orbital shaker at 190 rpm and 37°C. Incubation continued until the optical density of the culture at 600 nm reached 0.6, at which time isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM to induce expression of *asbB*. The culture was then incubated overnight at 190 rpm and 15° C.

**1.4.2 Purification of $His_6$-AsbB.** Cells were harvested by centrifugation, the pellet was resuspended in 15 mL of 20 mM Tris buffer-HCl, pH 8.0, 100 mM NaCl, 20 mM imidazole and 10 % glycerol, and lysed using a French Press (17,000 psi internal cell pressure, ThermoFinnigan). After removal of cellular debris by centrifugation (18,000 x g for 20 min, at 4°C), the supernatant was applied to a 1mL HiTrap™ HP affinity column (Nickel Sepharose High Performance, GE Healthcare) equilibrated with a solution containing 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 20 mM imidazole, and 10% glycerol. Unbound proteins were removed by washing with 10 mL of equilibration buffer and the $His_6$-AsbB fusion protein was eluted with 3 mL of elution buffer (20 mM Tris-HCl, pH8.0, 100 mM NaCl, 300 mM imidazole, 10% glycerol). Fractions were analysed by SDS-PAGE on an 8% gel and those containing $His_6$-AsbB were pooled, washed and concentrated to 1 mL using Ultrafiltration (Amicon) with a 30,000 MWCO membrane (Millipore) in buffer containing 20 mM Tris-HCl, pH 8.0, 100 mM NaCl and 10 % glycerol. The protein was further purified by gel filtration on a 110 mL superose 12 prep grade gel filtration resin poured in a XK 16/50 column (Amersham Biosciences), equilibrated with a solution containing 20 mM Tris-HCl, pH8.0, 100 mM NaCl and 10 % glycerol, at a flow rate of 0.75 mL/min. The central fractions corresponding to the $His_6$-AsbB peak were then concentrated to 0.5 mL using Amicon® Ultra filtration with a 30,000 MWCO membrane (Millipore), aliquoted and frozen at -80° C. The protein was used for all subsequent experiments without further purification. The purity of the protein at various stages of the purification was assessed by SDS-PAGE on an 8% gel (Figure 2).

**1.4.3 Confirmation of $His_6$-AsbB identity.** Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard.[7] The identity of purified $His_6$-AsbB was confirmed by peptide mass fingerprinting of tryptic digests of the protein (The Biological Mass

Spectrometry and Proteomics Facility in the Department of Biological Sciences, University of Warwick). 10 predicted tryptic fragments of AsbB with a coverage of 18.6% were identified by this analysis.

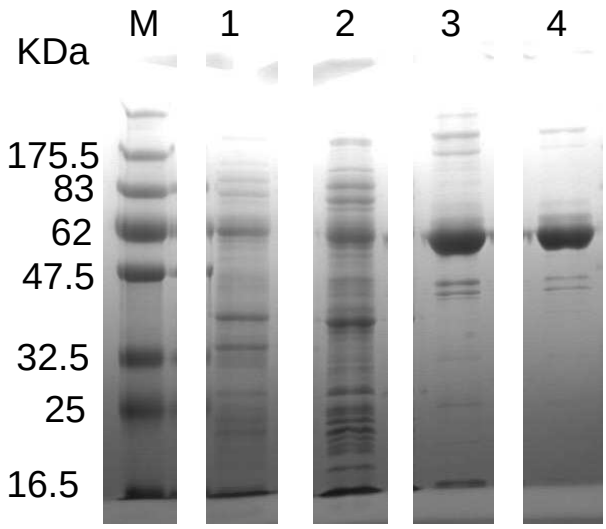



**Figure 2:** 8% SDS-PAGE analysis of $His_6$-AsbB overproduction and purification. Lane M = molecular weight standards, Lane 1 = total protein after lysis, lane 2 = soluble protein fraction after lysis, lane 3 = protein eluted from Ni-NTA column with 300mM imidazole, lane 4 = protein obtained after gel filtration.

**1.5 Incubation of $His_6$-AsbB with different combinations of 2, 3, 4 and 5.** 1.5 mM $N^8$-citryl-spermidine **4** or $N^1$-(3,4-dihydroxybenzoyl)-$N^8$-citryl-spermidine **5**, 3 mM ATP, 7.5 mM $MgCl_2$, 2 mM nucleophile spermidine **2** or $N^1$-(3,4-dihydroxybenzoyl)-spermidine **3**, 100 mM Tris-HCl, 3 μM $His_6$-AsbB (after Ni-NTA purification, gel filtration and concentration) in a final volume of 150 μL were incubated for 90 minutes at 37 °C. The reactions were initiated by addition of the enzyme and were stopped by addition of 75 μL of a 5% trichlororoacetic acid solution. Reaction mixtures were passed through a 0.45μ filter prior to analysis. The corresponding controls were carried out in the same way using:

a) Denatured $His_6$-AsbB (heated at 100 °C for 20 minutes prior to addition to the incubation mixture).
b) Using GTP, TTP or CTP in place of ATP.
c) Without adding $MgCl_2$ to the incubation mixture.

LC-MS analysis of the reaction mixtures were carried out using a reverse phase column (Eclipse XDB-C18, 150 X 4.6 mm, 5μ, Agilent) connected to an Agilent 1100 HPLC instrument. The outflow was routed via a splitter (10% to mass spectrometer, 90% to waste) to a Bruker HCT+ spectrometer fitted with an electrospray source operating in positive ion mode. The column was eluted using the profile in table 3. Retention times of the products were:

a) 5.25 min (*m*/*z* 447.3, corresponding to $N^8$,$N'^8$-citryl-bis(spermidine) **6**), for the incubation containing $N^8$-citryl-spermidine **4** and spermidine **2**.

b) 14.15 min (*m*/*z* 583.3, corresponding to $N^1$-(3,4-dihydroxybenzoyl)-$N^8$,$N'^8$-citryl-bis(spermidine) **7**), for the incubations of $N^8$-citryl-spermidine **4** with $N^1$-(3,4-dihydroxybenzoyl)-spermidine **3**, and $N^1$-(3,4-dihydroxybenzoyl)-$N^8$-citryl-spermidine **5** with spermidine **2**.

c) 14.75 min (*m*/*z* 719.3, corresponding to petrobactin **1**), for the incubation containing $N^1$-(3,4-dihydroxybenzoyl)-$N^8$-citryl-spermidine **5** and $N^1$-(3,4-dihydroxybenzoyl)-spermidine **3**.

**Table 3.**

| Time (min) | Water (0.1% TFA) | MeCN (0.1% TFA) | Flow (mL / min) |
|---|---|---|---|
| 0 | 90 | 10 | 1 |
| 5 | 90 | 10 | 1 |
| 25 | 0 | 100 | 1 |
| 30 | 0 | 100 | 1 |

**1.6 Scaled-up incubation of $His_6$-AsbB with $N^8$-citryl-spermidine 4 and spermidine 2. Purification and characterization of $N^8$, $N'^8$-citryl-bis(spermidine) 6.** 1.5 mM $N^8$-citryl-spermidine **4**, 3 mM ATP, 7.5 mM $MgCl_2$, 5 mM spermidine **2**, 2.88 μM $His_6$-AsbB (after Ni-NTA purification and concentration) in a final volume of 3 mL were incubated for 16 hours at 37 °C. The crude mixture was then centrifuged at 4000 rpm for 10 min. The supernatant was passed through a 0.45μ filter and purified on a reverse phase column (Zorbax XDB-C18 column, 100 X 21 mm, 5μ, Agilent) attached to an Agilent 1100 HPLC instrument fitted with a quaternary pump and VWD detector, detecting absorption at 210 nm. The elution profile in table 4 was used.

**Table 4.**

| Time (min) | Water (0.1% TFA) | MeCN (0.1% TFA) | Flow (mL / min) |
|---|---|---|---|
| 0 | 95 | 5 | 4.5 |
| 10 | 95 | 5 | 4.5 |
| 20 | 0 | 100 | 4.5 |
| 25 | 0 | 100 | 4.5 |

The collected fractions were analysed by ESI-MS and those containing the compound with *m/z* 447.3 were freeze dried (retention time 10 min).
The $N^8$,$N'^8$-citryl-bis(spermidine) **6** thus obtained was analysed by ESI-MS/MS (Bruker HCT+ spectrometer equipped with an electrospray source in positive ion mode), ESI-TOF-MS (Bruker MicroTof) and NMR spectroscopy ($^1$H, $^{13}$C, COSY, HMQC and HMBC; Bruker Avance 700 spectrometer equipped with a TCI cryoprobe). $\delta_H$ ($D_2O$, 700 MHz) 1.58 (m, 4H, 2x C$H_2$-$CH_2$-NH-CO), 1.71 (m, 4H, 2x C$H_2$-$CH_2$-NH), 2.09 (m, 4H, 2x C$H_2$-$CH_2$-$NH_2$), 2.64 (d, 2H, 2x C*H*H-CO-NH, $^2J$ 14.6), 2.80 (d, 2H, 2x C*H*H-CO-NH, $^2J$ 14.6), 3.10 (m, 8H, 2x C$H_2$-NH + 2x C$H_2$-$NH_2$), 3.16 (t, 4H, 2x C$H_2$-NH, $^3J$ 7.9), 3.23 (m, 4H, 2x C$H_2$-NH-CO); $\delta_C$ ($D_2O$ containing 1 drop of $CD_3CN$, 175 MHz) 23.4, 24.2, 26.0, 37.1, 38.8, 44.9, 45.3, 47.8, 75.3, 172.3 and 178.3; HRMS: Calculated for $C_{20}H_{43}N_6O_5^+$ $(M+H)^+$: 447.3289; found: 447.3306. Key correlations observed in COSY and HMBC spectra are showed in Figure 3.

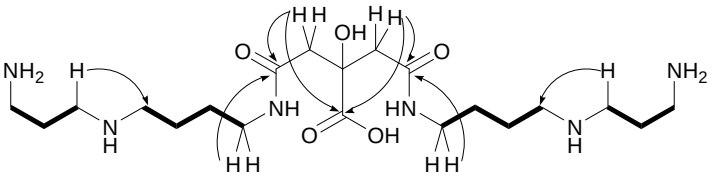



**Figure 3.** Key COSY (bold lines) and HMBC (arrows) correlations observed in the NMR spectra of **6**.

**1.7 Scaled-up incubation of $His_6$-AsbB with $N^1$-(3,4-dihydroxybenzoyl)-$N^8$-citryl-spermidine 5 and spermidine 2. Purification and characterization of $N^1$-(3,4-dihydroxybenzoyl)-$N^8$,$N'^8$-citryl-bis(spermidine) 7.** 1.5 mM $N^1$-(3,4-dihydroxybenzoyl)-$N^8$-citryl-spermidine **5**, 5 mM ATP, 7.5 mM $MgCl_2$, 5 mM spermidine **2** and an initial concentration of

7.2 μM $His_6$-AsbB (after Ni-NTA purification and concentration) in a final volume of 4 mL were incubated for 22 hours at 37 °C. Successive additions of $His_6$-AsbB (0.012 μmoles in each) were made 1.5, 4.5 and 6 h after the incubation was started. The crude mixture was centrifuged at 4000 rpm for 10 min. The supernatant was passed through a 0.45μ filter and purified on a reverse phase column (Zorbax XDB-C18 column, 100 X 21 mm, 5μ, Agilent) attached to an Agilent 1100 HPLC instrument fitted with a quaternary pump and VWD detector, detecting absorption at 250 nm. The elution profile in table 5 was used.

**Table 5.**

| Time (min) | Water (0.1% TFA) | MeCN (0.1% TFA) | Flow (mL / min) |
|---|---|---|---|
| 0 | 90 | 10 | 4.5 |
| 10 | 90 | 10 | 4.5 |
| 20 | 0 | 100 | 4.5 |
| 25 | 0 | 100 | 4.5 |

The collected fractions were analysed by ESI-MS and those containing the compound with *m/z* 583.3 were freeze dried (retention time 18 min).
The semi-purified material was purified on a reverse phase column (Synergi fusion-RB 80, 250 x 10 mm, 4 micron, Phenomenex) attached to an Agilent 1100 HPLC instrument fitted with a quaternary pump and VWD detector, detecting absorption at 250 nm. The elution profile in table 6 was used.

**Table 6**.

| Time (min) | Water (0.1% TFA) | MeCN (0.1% TFA) | Flow (mL / min) |
|---|---|---|---|
| 0 | 90 | 10 | 1.5 |
| 10 | 90 | 10 | 1.5 |
| 20 | 0 | 100 | 1.5 |
| 25 | 0 | 100 | 1.5 |

The collected fractions were analysed by ESI-MS and those containing the compound with *m/z* 583.3 were freeze dried (retention time 11.4 min).

The $N^1$-(3,4-dihydroxybenzoyl)-$N^8$,$N'^8$-citryl-bis(spermidine) **7** thus obtained was analysed by ESI-MS/MS (Bruker HCT+ spectrometer equipped with an electrospray source in positive ion mode), ESI-TOF-MS (Bruker MicroTof) and NMR spectroscopy ($^1$H, $^{13}$C, COSY, HMQC and HMBC; Bruker Avance 700 spectrometer equipped with a TCI cryoprobe). $\delta_H$ ($D_2O$, 700 MHz) 1.58 (m, 4H, 2x C$H_2$-$CH_2$-NH-CO), 1.69 (m, 4H, 2x C$H_2$-$CH_2$-NH), 2.00 (m, 2H, C$H_2$-$CH_2$-NH-CO-Ar), 2.09 (m, 2H, C$H_2$-$CH_2$-$NH_2$), 2.61 (d, 1H, C*H*H-C(OH), $^2J$ 14.5), 2.62 (d, 1H, C*H*H-C(OH), $^2J$ 14.5 2.75 (d, 1H, C*H*H-C(OH), $^2J$ 14.5), 2.77 (d, 1H, C*H*H-C(OH), $^2J$ 14.5), 3.05-3.15 (m, 10H, 4 x C$H_2$-NH + C$H_2$-$NH_2$), 3.23 (m, 4H, 2x C$H_2$-NH-CO), 3.49 (t, 2H, C$H_2$-NH-CO-Ar, $^3J$ 6.6), 7.00 (d, $H_{Ar}$, $^3J$ 8.4), 7.29 (d, $H_{Ar}$, $^3J$ 8.4), 7.34 (s, $H_{Ar}$); $\delta_C$ ($D_2O$ containing 1 drop of $CD_3CN$, 175 MHz) 23.3, 23.4, 24.2, 26.0, 26.0, 26.3, 37.0, 37.1, 38.8, 38.9, 44.9, 45.1, 45.4, 45.5, 47.7, 47.8, 75.4, 115.6, 116.4, 121.1, 126.1, 144.5, 148.6, 171.0, 172.4, 172.5 and 178.4 (two non-equivalent carbons with the same chemical shift of 26.0 ppm were observed in the HMQC spectrum); HRMS: Calculated for $C_{27}H_{46}N_6O_8^+$ $(M+H)^+$: 583.3432; found: 583.3450. Key correlations observed in COSY and HMBC spectra are showed in Figure 4.

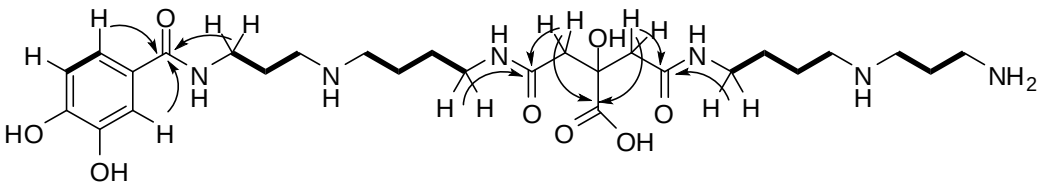


**Figure 4.** Key COSY (bold lines) and HMBC (arrows) correlations observed in the NMR spectra of **7**.

**1.8 Analysis of AMP and ADP formation in incubation mixtures**. For analysis of AMP formation, reactions containing 25 mM Tris-HCl buffer (pH 8.0), 9 mM ATP, 15 mM $MgCl_2$, 2 mM phosphoenolpyruvate, 0.4 mM NADH, 1 μM $His_6$-AsbB (after Ni-NTA purification, gel filtration and concentration), 12.6 units of lactate dehydrogenase, 8.4 units of pyruvate kinase, 4 units of myokinase, 2 mM $N^8$-citryl-spermidine **4** or $N^1$-(3,4-dihydroxybenzoyl)-$N^8$-citryl-spermidine **5**, and 2 mM spermidine **2** or $N^1$-(3,4-dihydroxybenzoyl)-spermidine **3** in a total volume of 140 μL were incubated at 37 °C for 20 min in a quartz cuvette in a Varian Cary 1 UV-Vis spectrometer. The decrease in absorbance at 340 nm with time due to oxidation of NADH was monitored. Control reactions were carried out using either boiled $His_6$-AsbB, or without **2** / **3** in the incubation mixture. For the ADP assay, the same procedure was used except that myokinase was omitted from the reaction.

This assay was also used to measure the initial rates of AMP formation, which can be correlated with the initial rates of amide bond formation for the different condensation reactions. For this purpose, concentrations of 0.5, 1, 2 and 4 mM $N^8$-citryl-spermidine **4** or $N^1$-(3,4-dihydroxybenzoyl)-$N^8$-citryl-spermidine **5** with 2 mM spermidine **2** or $N^1$-(3,4-dihydroxybenzoyl)-spermidine **3**, and 0.5, 1, 2 and 4 mM spermidine **2** or $N^1$-(3,4-dihydroxybenzoyl)-spermidine **3** with 2 mM $N^8$-citryl-spermidine **4** or $N^1$-(3,4-dihydroxybenzoyl)-$N^8$-citryl-spermidine **5** were used, in order to ensure that saturating conditions were being employed for both substrates. Figures 5-8 show the results obtained when using 2 mM **4** + **2**, **4** + **3**, **5** + **2** and **5** + **3**.

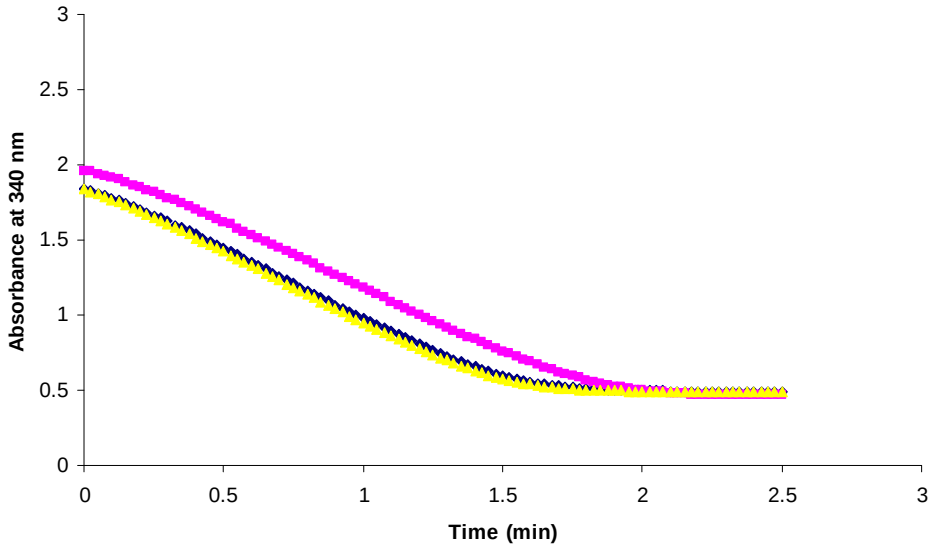



**Figure 5:** Three independent AMP formation assays using 2 mM $N^8$-citryl-spermidine **4** with 2 mM spermidine **2**.

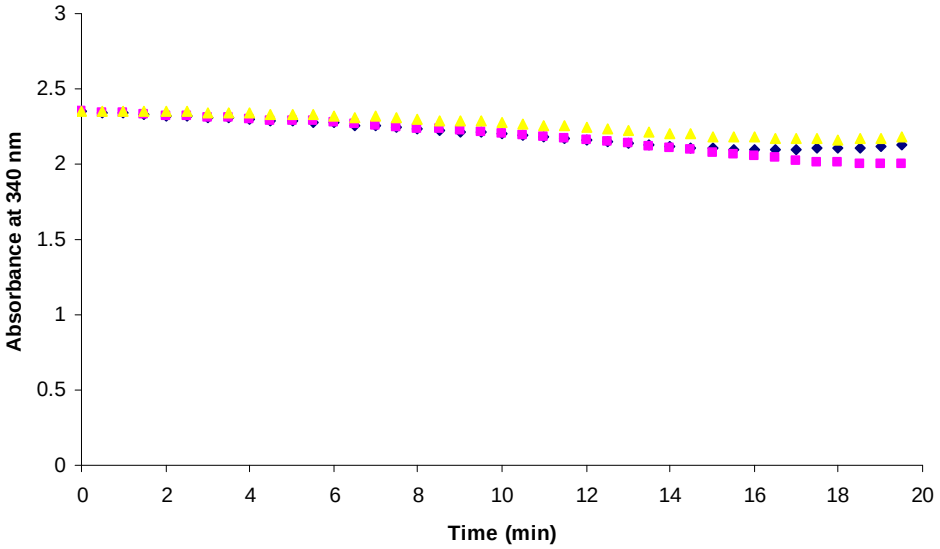



**Figure 6:** Three independent AMP formation assays using 2 mM $N^8$-citryl-spermidine **4** with 2 mM $N^1$-(3,4-dihydroxybenzoyl)-spermidine **3**.

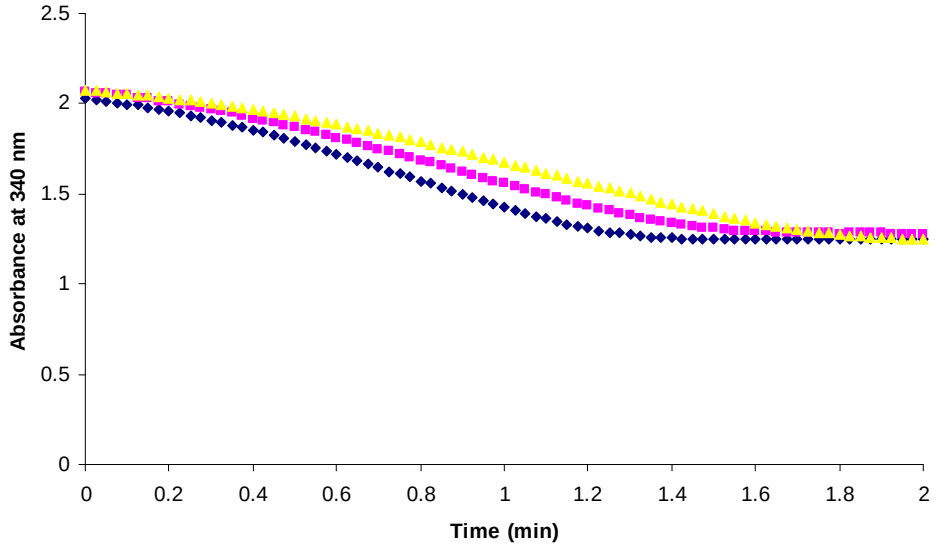



**Figure 7:** Three independent AMP formation assays using 2 mM $N^1$-(3,4-dihydroxybenzoyl)-$N^8$-citryl-spermidine **5** with 2 mM spermidine **2**.

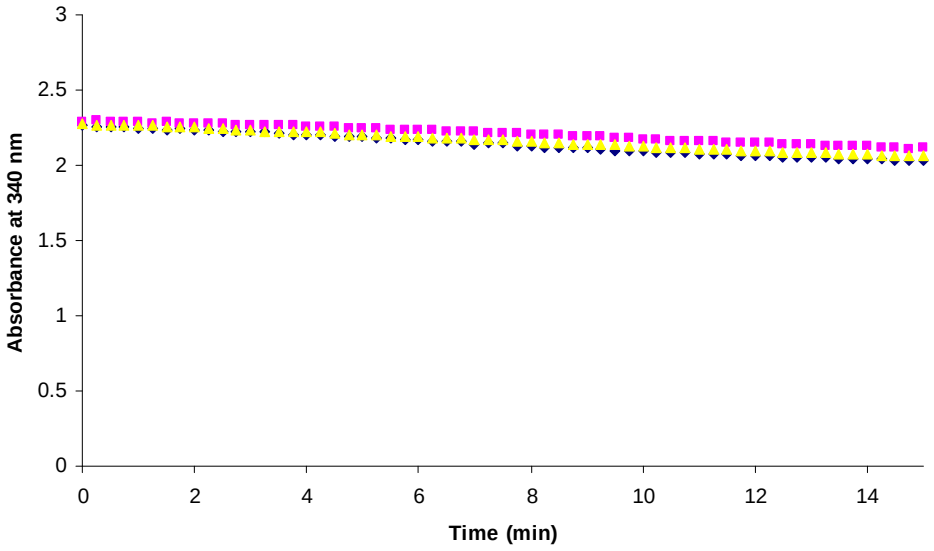



**Figure 8:** Three independent AMP formation assays using 2 mM $N^1$-(3,4-dihydroxybenzoyl)-$N^8$-citryl-spermidine **5** with 2 mM $N^1$-(3,4-dihydroxybenzoyl)-spermidine **3**.

**1.9 Comparison of the relative rates of AMP formation:** In the linear region of each plot in Figures 5-8 (0.5-1 min for Figure 5, 8-14 min for Figure 6, 0.4-0.9 min for Figure 7 and 5-10 min for Figure 8), a linear regression analysis was performed. A minimum of 13 points were used for the linear regressions, with the $r^2$ values obtained ranging from 0.983 to 0.999. The slope of each linear regression was determined, and the mean and standard deviation for three separate experiments for each of the 4 different reactions was calculated. These values are given in table 1 of the manuscript along with the corresponding values determined in the same way for control reactions lacking **2** or **3**, but containing **4** or **5**.

## References

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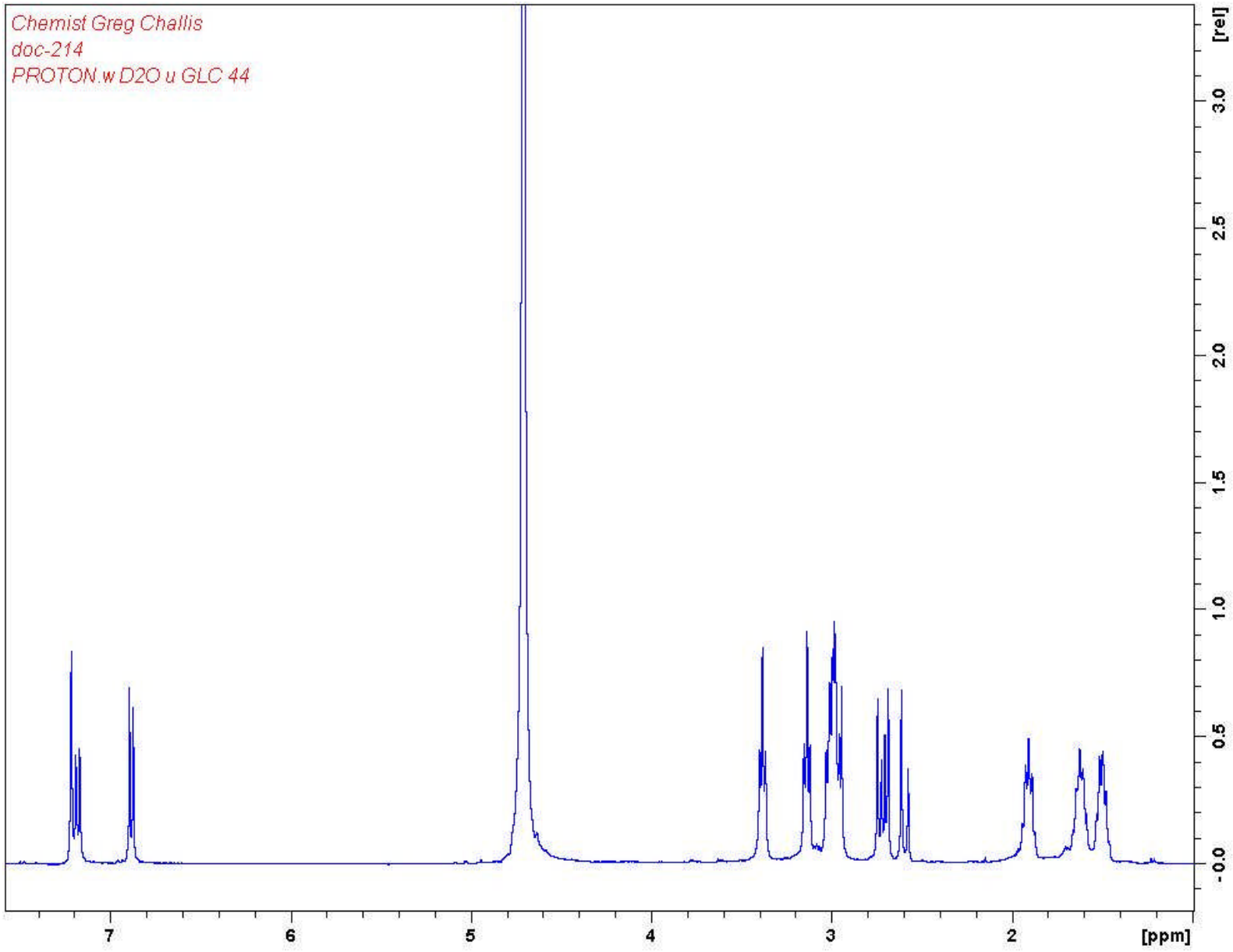
## 2 Spectroscopic data

• $^1$H, $^{13}$C, COSY, HMBC and HMQC spectra for $N^1$-(3,4-dihydroxybenzoyl)-$N^8$-citryl-spermidine **5** (S11-S15).

• $^{1}H$, $^{13}C$, COSY, HMBC and HMQC spectra for $N^{8}$,$N'^{8}$-citryl-bis(spermidine) **6** (S16-S20).

• $^{1}H$, $^{13}C$, COSY, HMBC and HMQC spectra for $N^{1}$-(3,4-dihydroxybenzoyl)-$N^{8}$,$N'^{8}$-citryl-bis(spermidine) **7** (S21-S25).

• MS-MS spectra of $N^{8}$,$N'^{8}$-citryl-bis(spermidine) **6** (S26) and $N^{1}$-(3,4-dihydroxybenzoyl)-$N^{8}$,$N'^{8}$-citryl-bis(spermidine) **7** (S27). It is important to notice that the fragmentation pattern obtained from the ion with m/z = 429.3 is identical in both spectra. The m/z = 429.3 corresponds to the ion resulting from loss of water in the case of $N^{8}$,$N'^{8}$-citryl-bis(spermidine) **6**, and loss of water and the benzoyl group from $N^{1}$-(3,4-dihydroxybenzoyl)-$N^{8}$,$N'^{8}$-citryl-bis(spermidine) **7**.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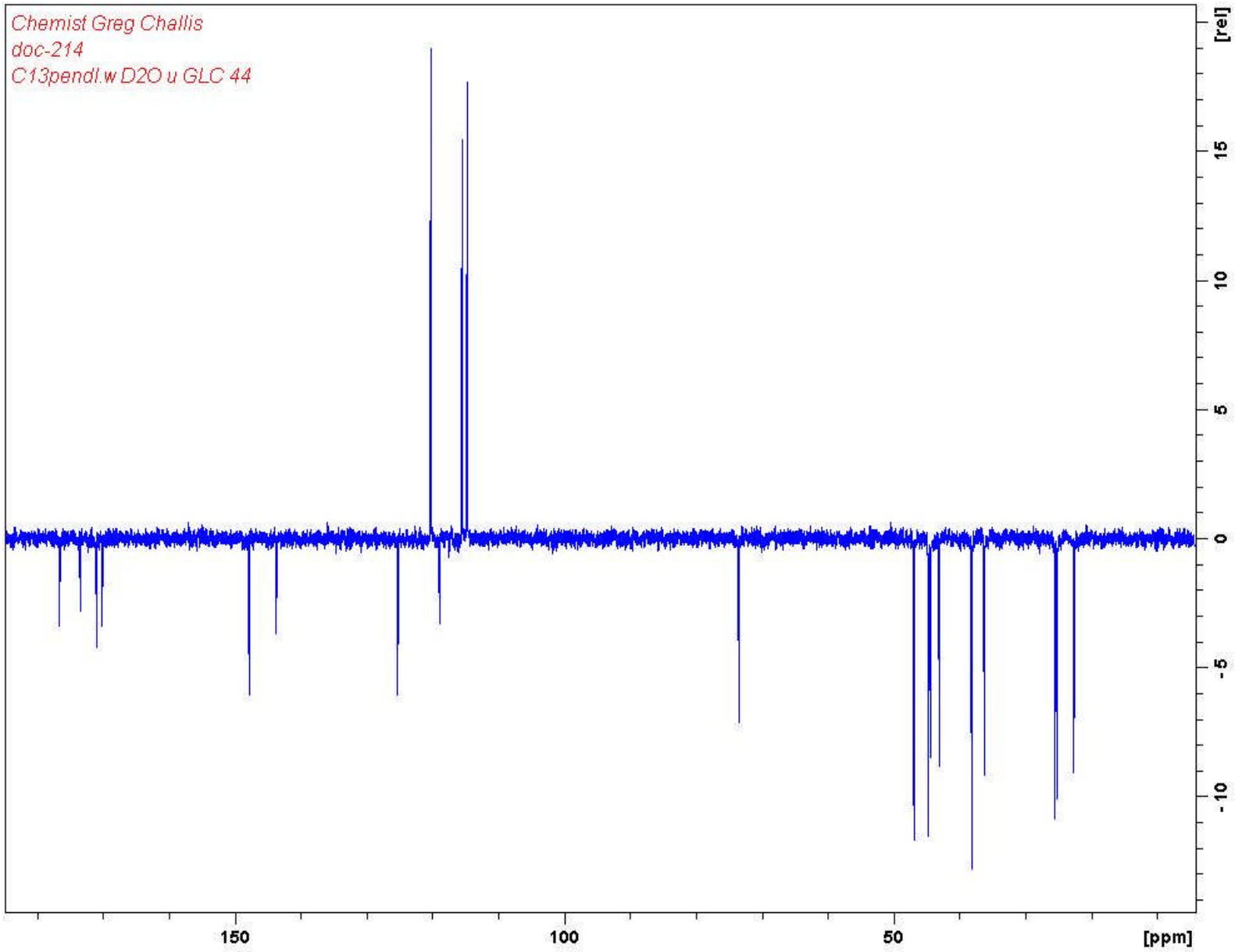
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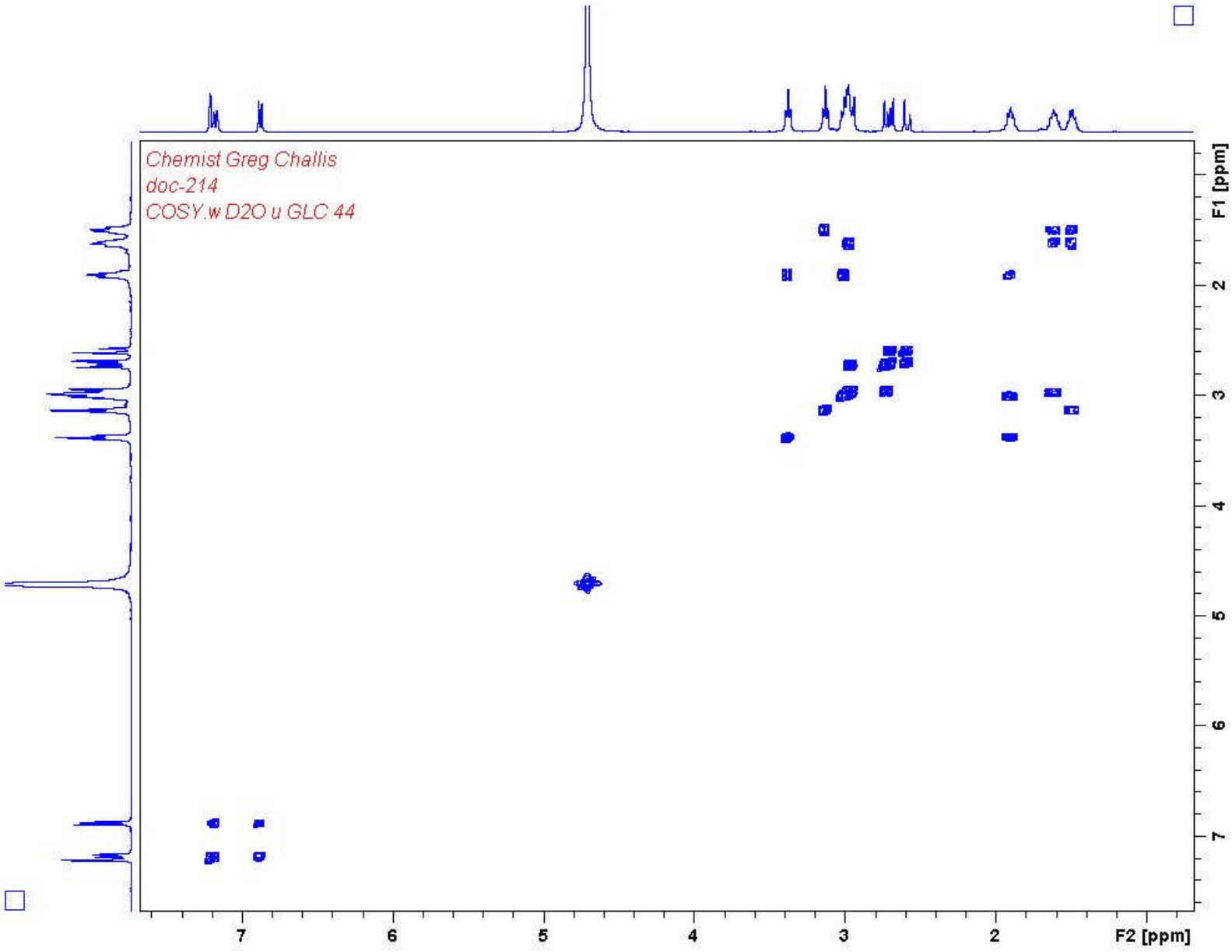
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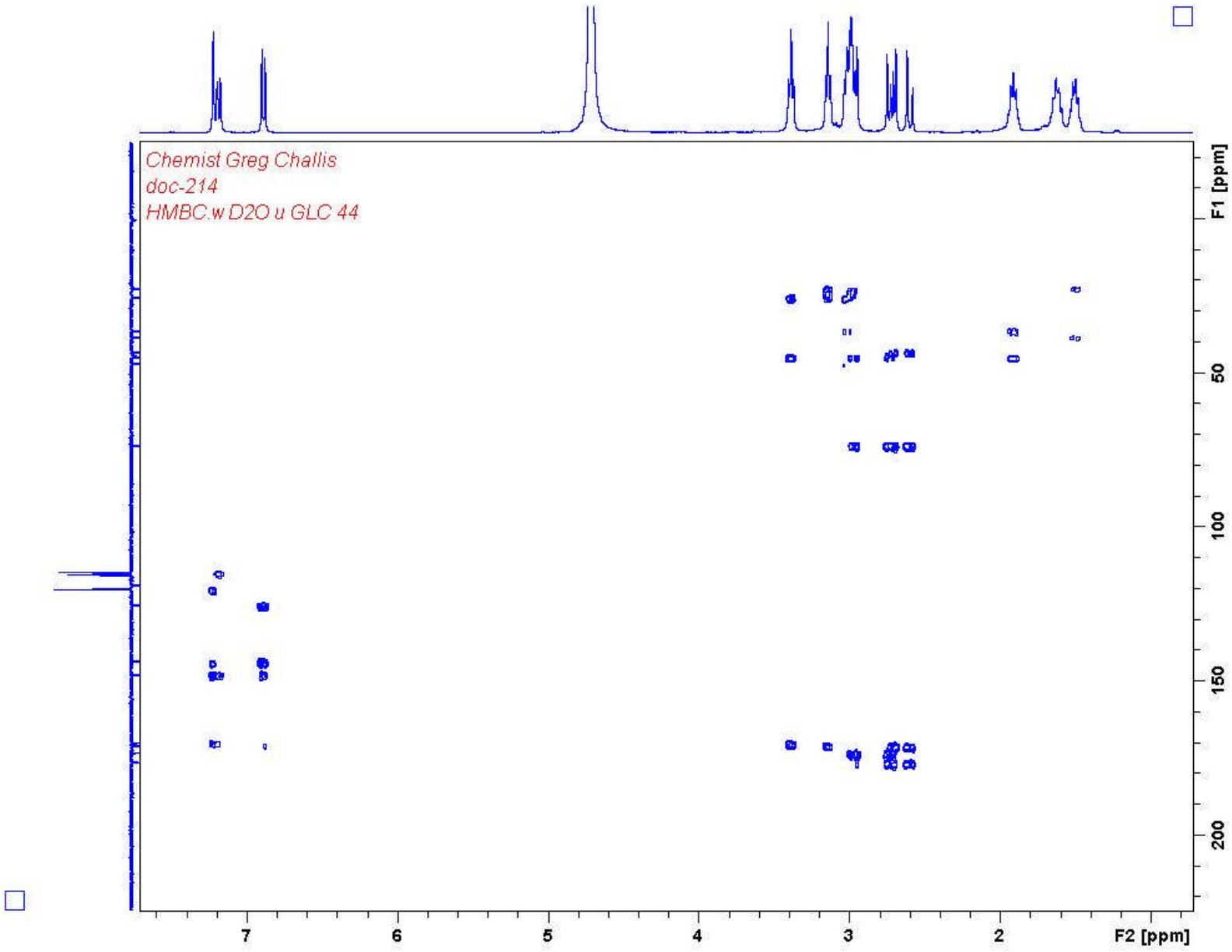
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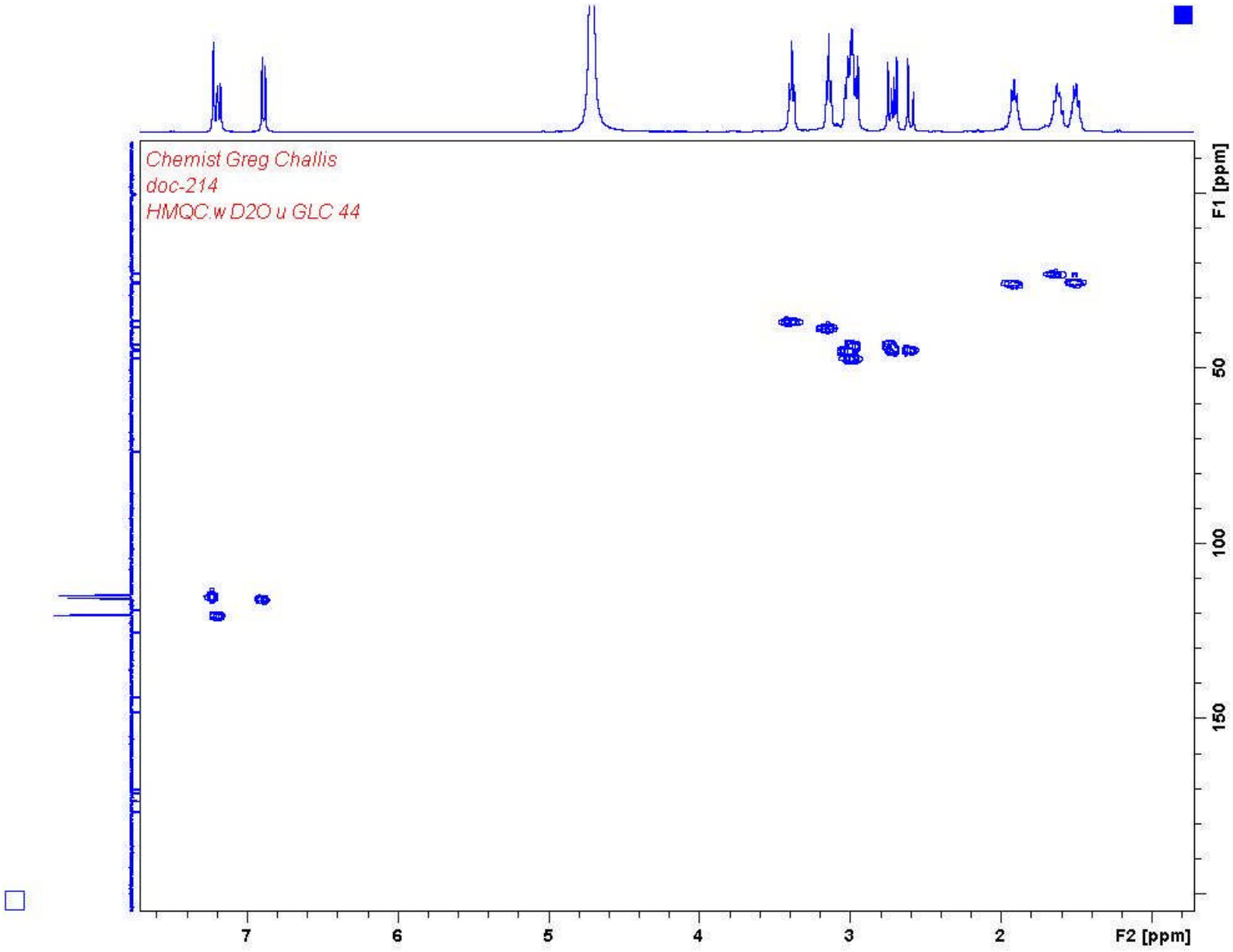
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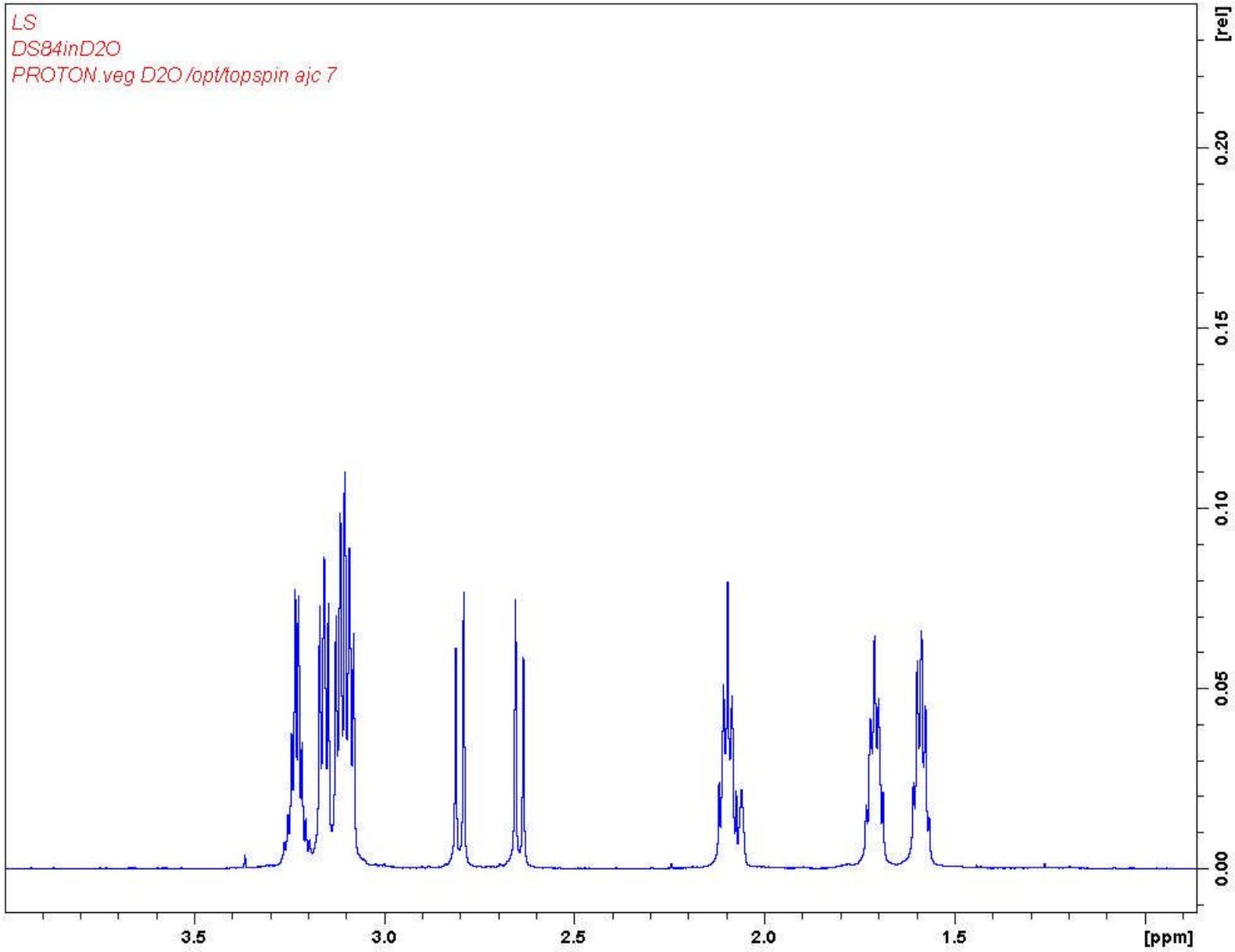
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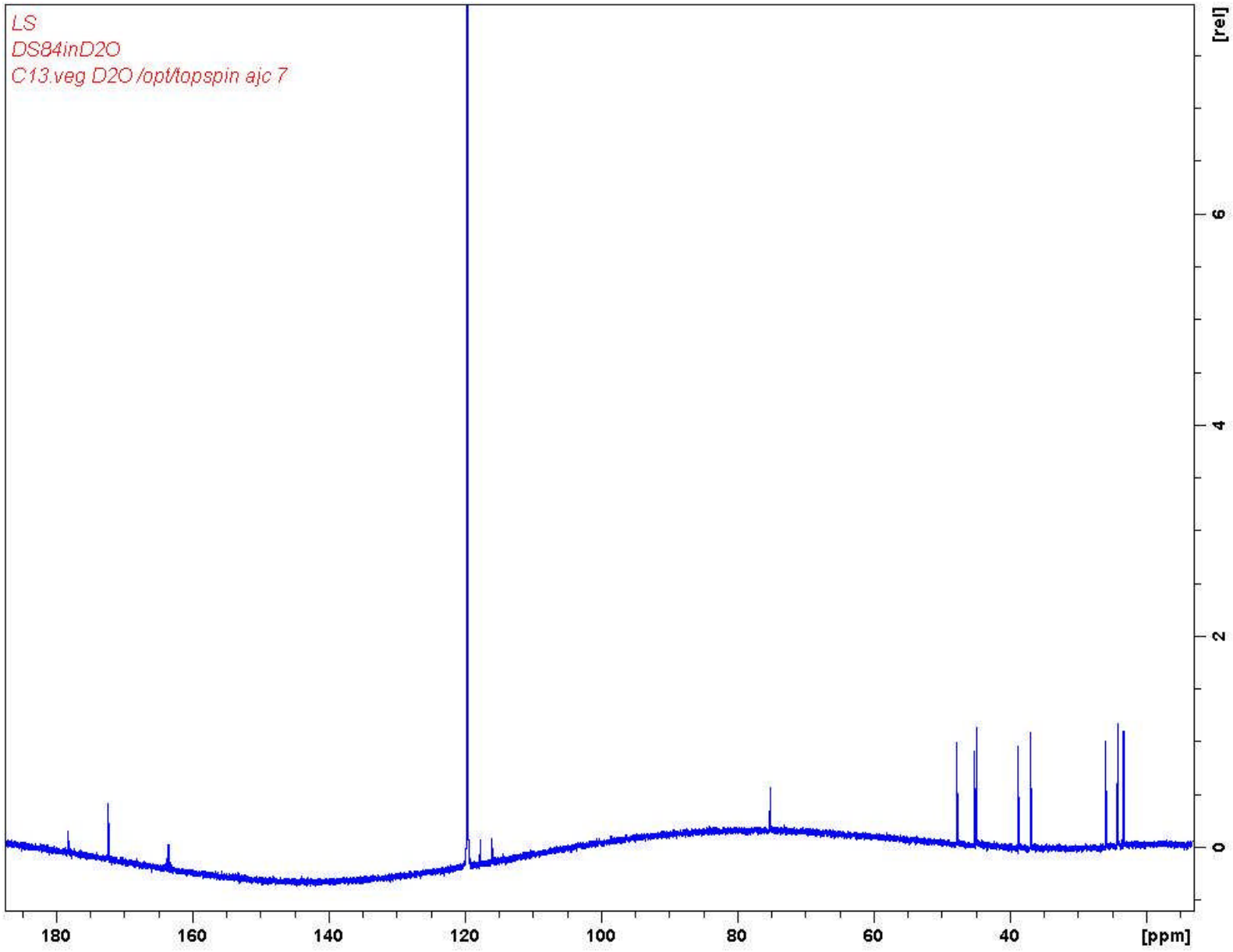
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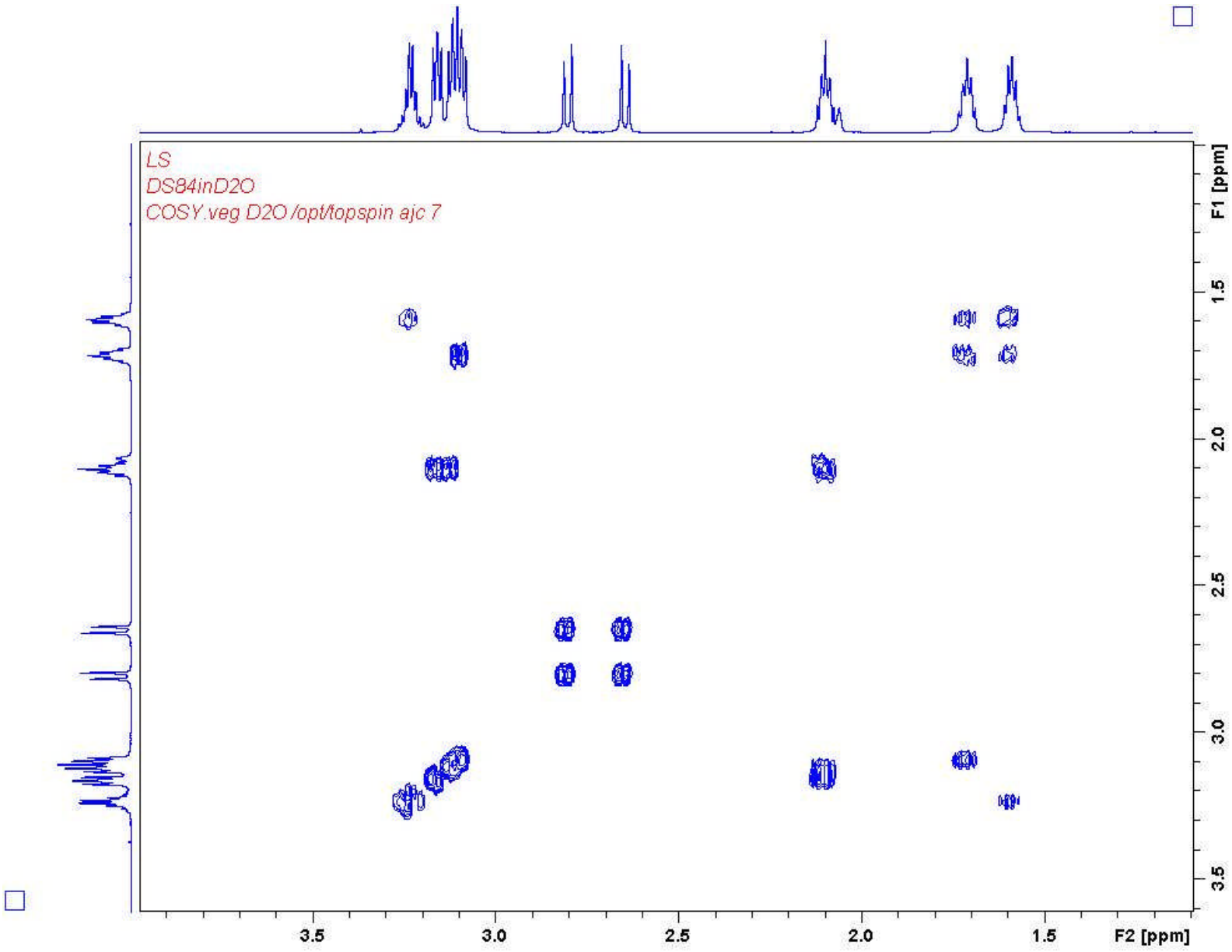
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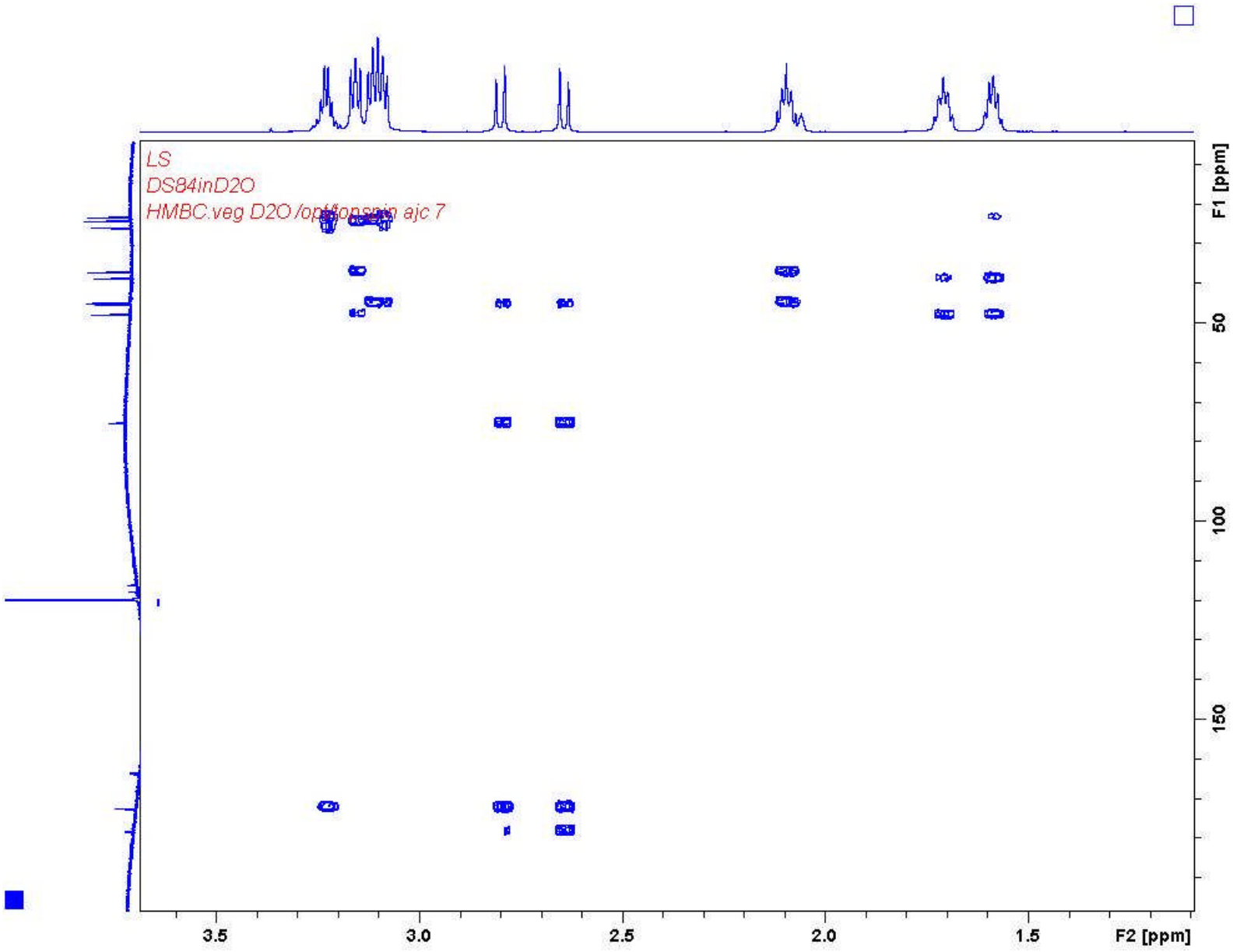
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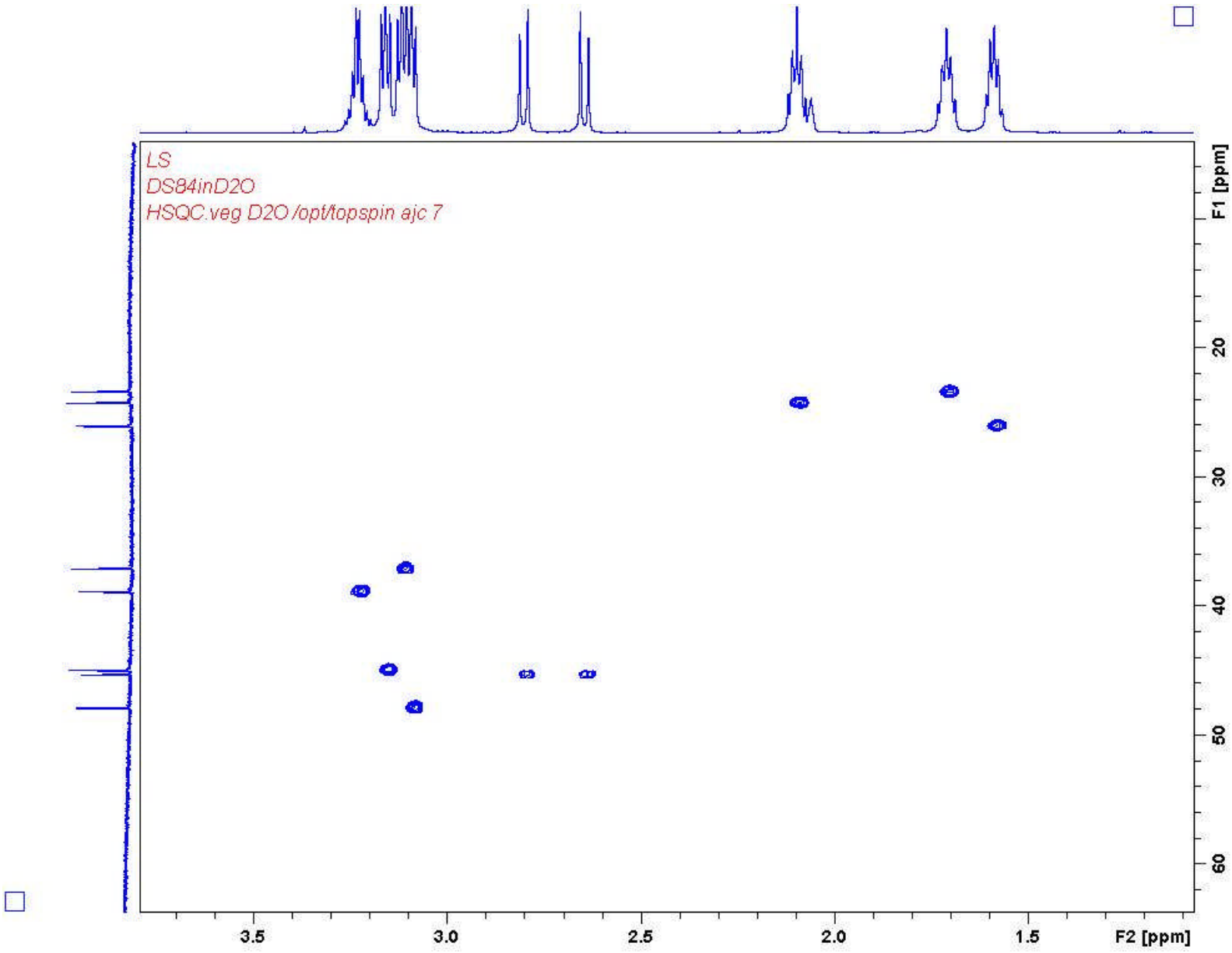
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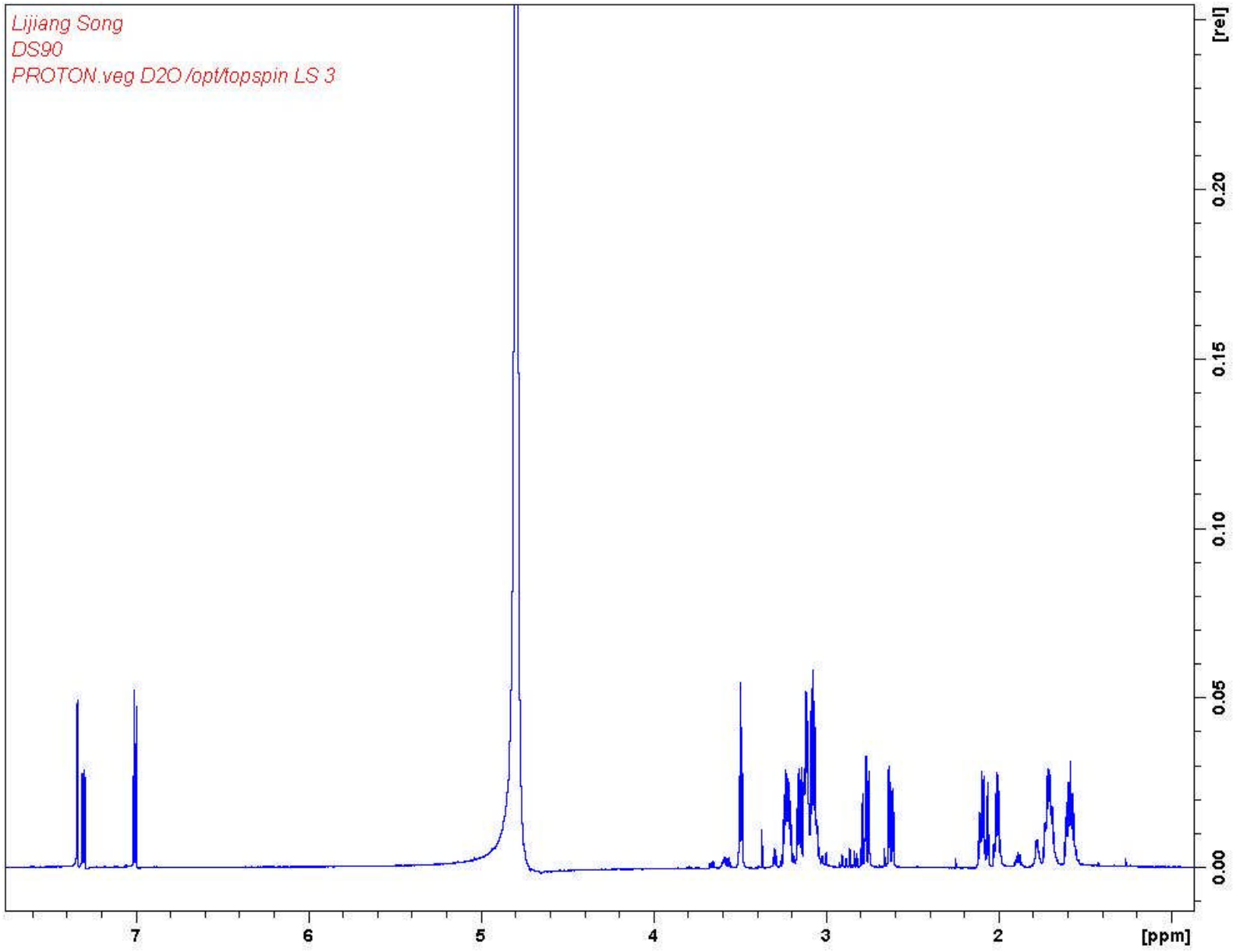
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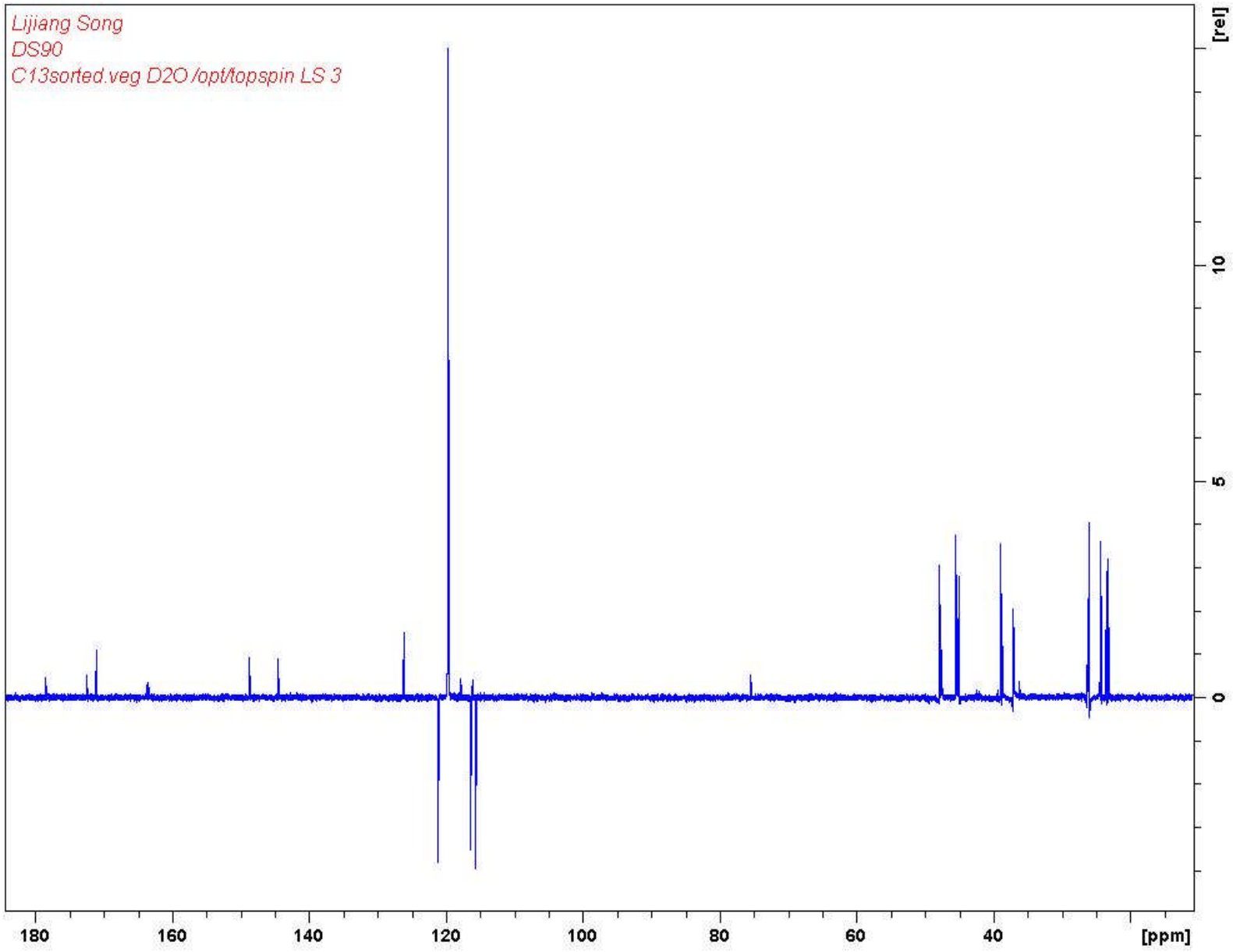
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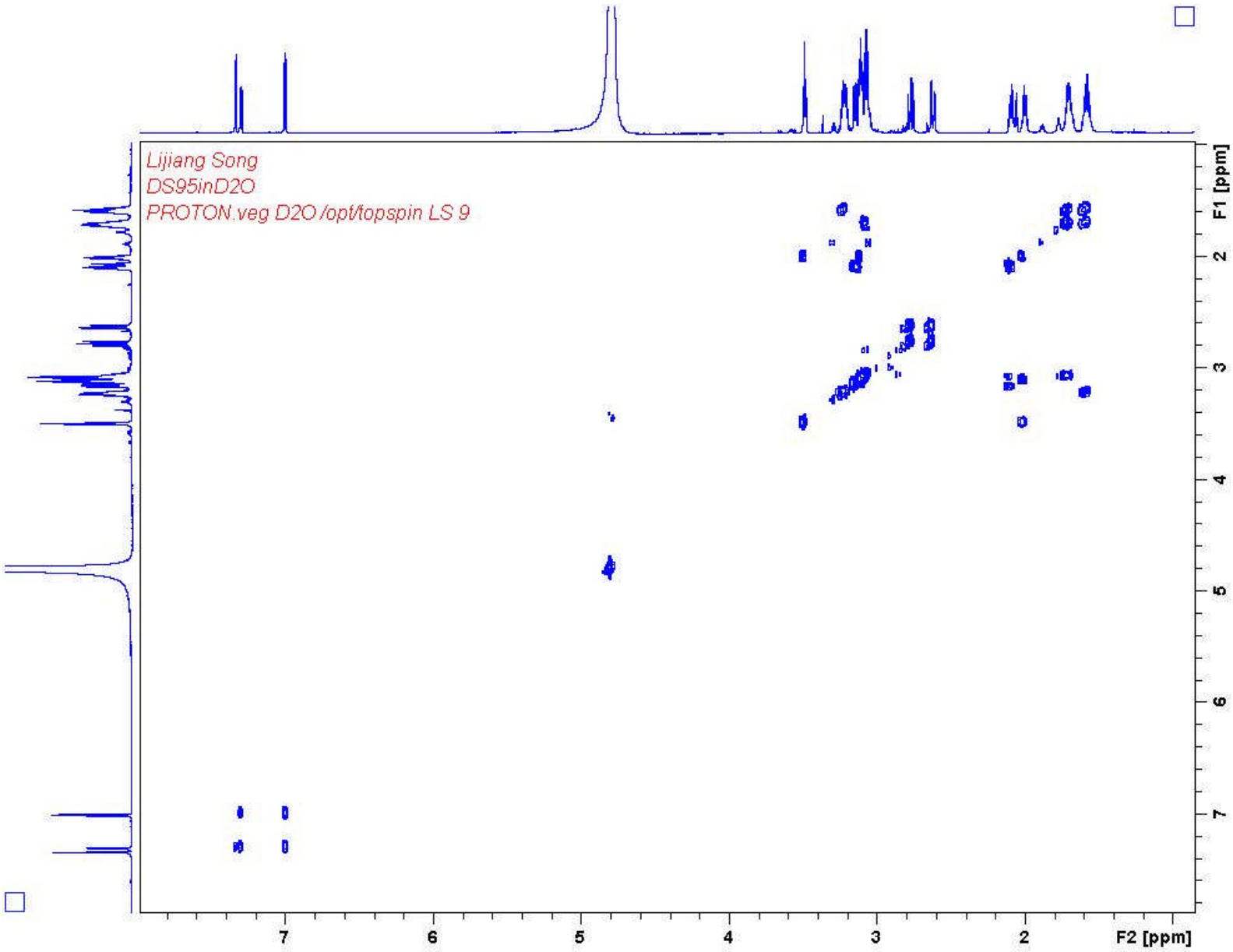
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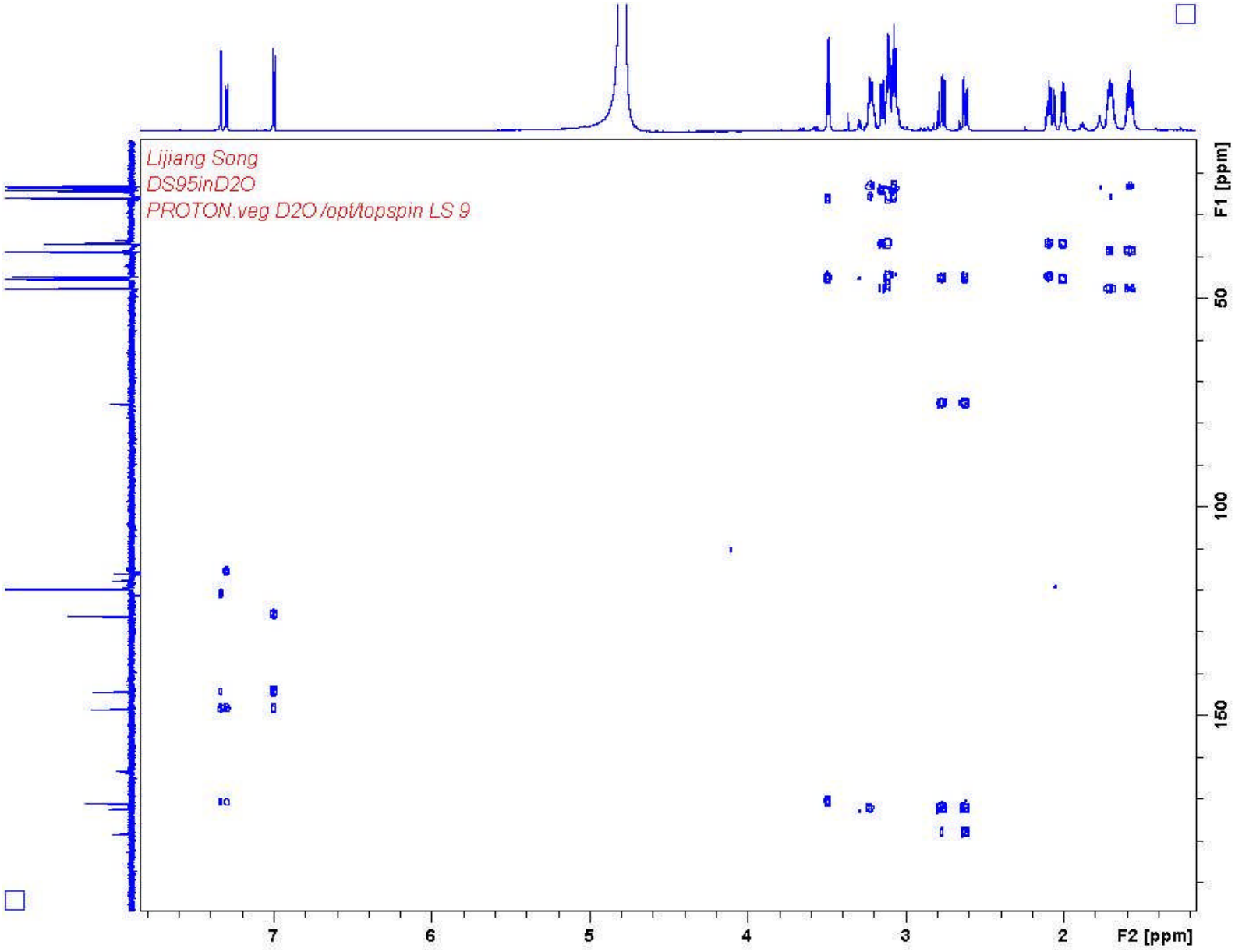
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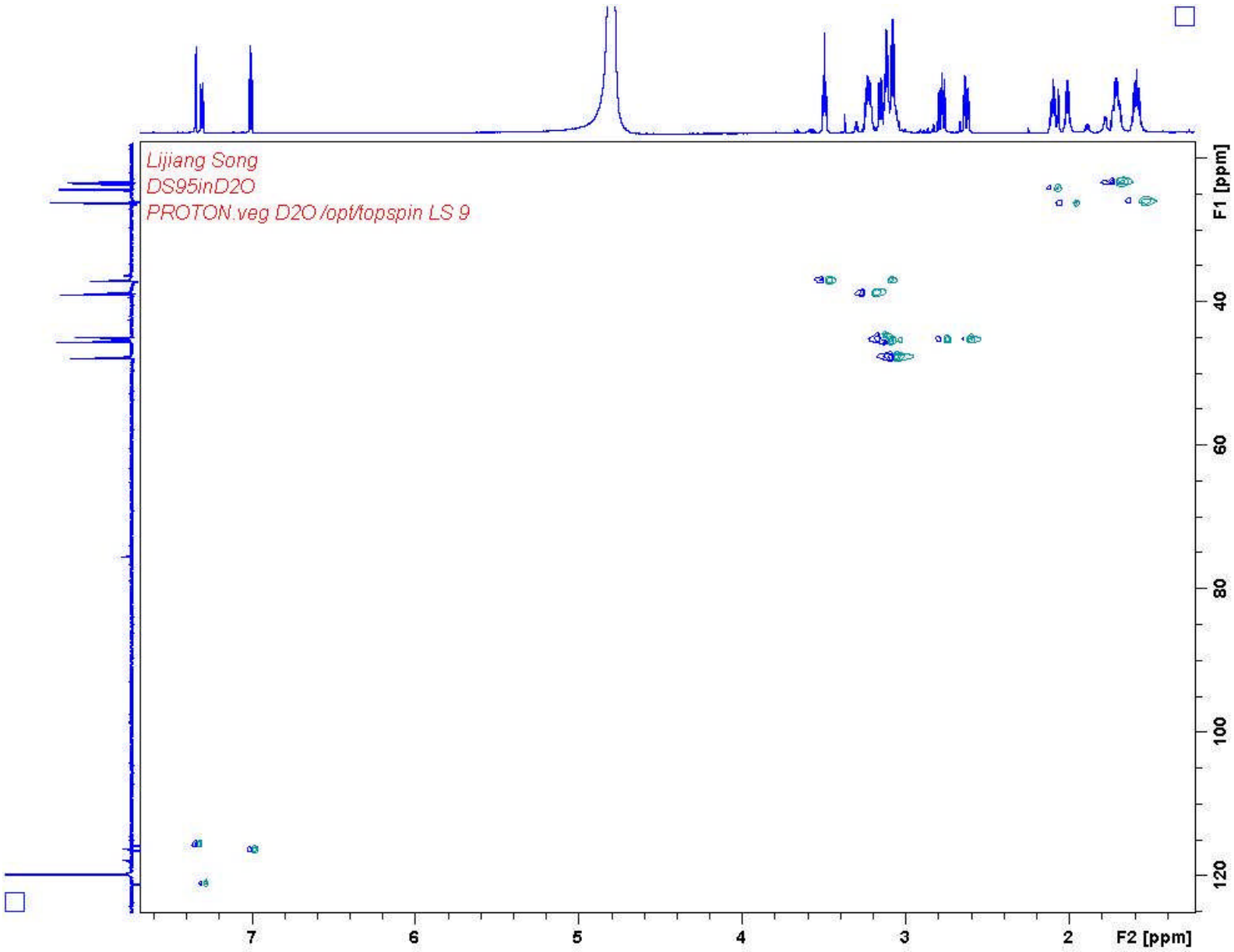
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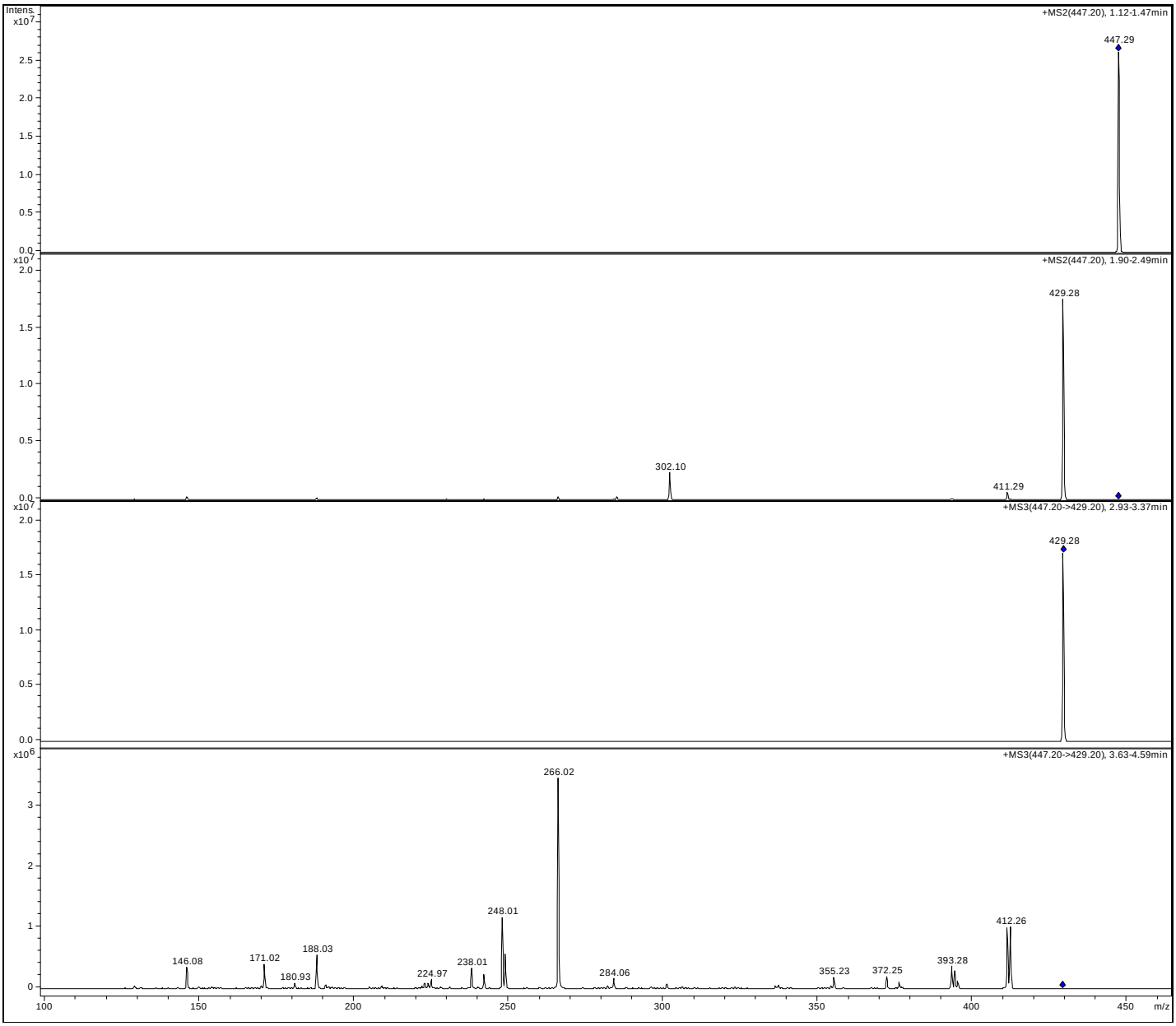
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+MS3(447.20->429.20), 3.63-4.59min
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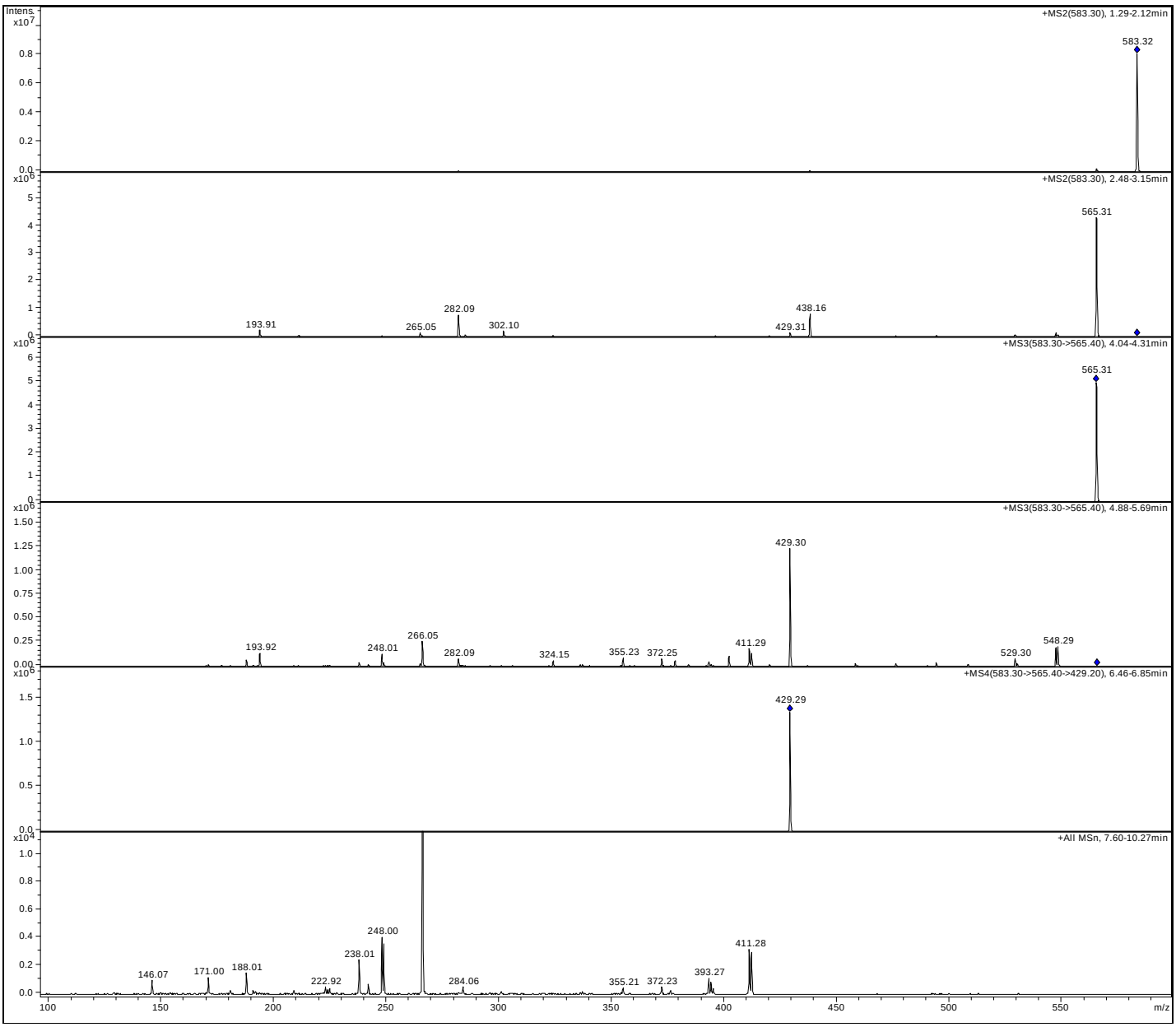
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