Supporting information

Enantioselective desymmetrisation of citric acid catalysed by the substrate-tolerant petrobactin biosynthetic enzyme AsbA

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1 Materials, methods and procedures

1.1 Synthesis of racemic N^8-citryl-spermidine 2

The synthesis of unlabelled N^8-citryl-spermidine **2** was accomplished using the methodology previously described by us.[1]

1.2 Cloning and overexpression of *asbA* in *Escherichia coli.*

The cloning, overproduction and purification of AsbA was carried out using the methodology previously described by us.[1]

1.3 Incubation of [1, 2-$^{13}C_2$]acetic acid, coenzyme A, ATP, oxaloacetic acid, spermidine and Mg^{2+} with acetyl-CoA synthetase, *si*-citrate synthase and purified recombinant His_6-AsbA

The experiment was carried out in a sequential manner, starting with the synthesis of 3*S*-[1, 2-$^{13}C_2$]citric acid. Thus, in a total volume of 1 mL, [1, 2-$^{13}C_2$]acetic acid (0.005 mmol), CoASH (0.0075 mmol), ATP (0.02 mmol), $MgCl_2$ (0.0225 mmol), Tris-HCl (pH 8.0, 0.02 mmol) and Acetyl-CoA synthetase (2.6 units) were incubated at 22 °C for 30 minutes. The mixture was centrifuged (3000 rpm, 10 minutes) and oxaloacetic acid (0.005 mmol) and *si*-citrate synthase (44 units) were added to the supernatant. The resulting mixture was incubated for 30 minutes at 22 °C, followed by centrifugation (3000 rpm, 10 minutes). To the supernatant spermidine (0.015 mmol), ATP (0.01 mmol), Tris-HCl (pH 8.0, 0.04 mmol), His_6-AsbA (0.01 μmol) were added and the total volume was made up to 3 mL with distilled water. The solution was incubated at 22 °C for 5 hours. The sample was centrifugated (3000 rpm, 10 minutes), the supernatant was passed through a 0.45 micron filter, diluted 4-fold with water, and partially-purified by reverse phase HPLC (Agilent-Zorbax XDB-C18 column, 100 X 21 mm, 5 micron, retention time ~6 min] detecting absorbance at 210 nm using the elution profile in table 1.

Table 1

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 analyzed by ESI-MS and those containing the compound with *m/z* 322.2 were freeze-dried. The semi-purified ^{13}C-labeled N^8-citryl-spermidine thus obtained was further purified by reverse phase HPLC (Phenomenex Synergi fusion-RB 80, 250 x 10 mm, 4 micron, retention time ~10 min) detecting absorbance at 210 nm using the elution profile in table 2.

Table 2

Time (min)	Water (0.1% TFA)	MeCN (0.1% TFA)	Flow (mL / min)
0	90	10	1.3
10	90	10	1.3
20	0	100	1.3
25	0	100	1.3

The collected fractions were analyzed by ESI-MS and those containing the compound with *m/z* 322.2 were freeze-dried. The purified ^{13}C-labeled N^8-citryl-spermidine obtained was analyzed by ESI-TOF-MS (Bruker MicroTof) and NMR spectroscopy (D_2O + 1 drop of CD_3CN for calibration purposes; ^{1}H, ^{13}C, COSY, HSQC and HMBC; Bruker Avance 700 spectrometer equipped with a TCI cryoprobe). Its identity was confirmed by comparison of the NMR data with that of unlabeled N^8-citryl-spermidine.[1]

Analysis of the HMBC and HSQC spectra indicated that **2a** and not **2b**, was the product of the AsbA-catalyzed reaction. To further confirm this conclusion, a 700 MHz ^{13}C-NMR spectrum of a mixture of a mixture of the ^{13}C-labeled N^8-citryl-spermidine and chemically-synthesised racemic N^8-citryl-spermidine was recorded. This spectrum showed unequivocally that compound **2a** was the product of the AsbA-catalyzed reaction.

1.4 Incubation of His_6-AsbA with citric acid and spermidine analogues

2.0 mM citric acid, 4 mM ATP, 7.5 mM $MgCl_2$, 100 mM Tris-HCl (pH 8.0), 0.85 μM His_6-AsbA (after Ni-NTA purification) and 2 mM amine (1,3-propanediamine, 1,4-butanediamine, 1,5-pentanediamine, 1,7-heptanediamine, 1,8-octanediamine, nor-spermidine, 3,4-dihydroxybenzoyl-spermidine,) 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 0.45 micorn filter prior to analysis. The corresponding controls were carried out in the same way using denatured His_6-AsbB (heated at 100 °C for 20 minutes prior to addition to the incubation mixture).
LC-MS analysis of the reaction mixtures were carried out using a reverse phase column (Agilent Eclipse XDB-C18, 150 X 4.6 mm, 5 micron) 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 eluting profile shown in table 3 was used.

Table 3

Time (min)	Water (0.1% TFA)	MeCN (0.1% TFA)	Flow (mL / min)
0	90	10	1
10	90	10	1
25	0	100	1
30	0	100	1
40	90	10	1
55	90	10	1

1.4.1 Scaled-up incubation of His_6-AsbA with citric acid and 1,5-pentanediamine

The enzymatic incubation using citric acid and 1,5-pentanediamine was scaled-up (total volume of 3 mL) and the corresponding product was partially-purified using reverse phase HPLC (Agilent

Zorbax XDB-C18 column, 150 X 21 mm, 5 micron), detecting absorbance at 210 nm and with the elution profile shown in table 4 (retention time 2.1 min)

Table 4

Time (min)	Water (0.1% TFA)	MeCN (0.1% TFA)	Flow (mL / min)
0	90	10	20
5	90	10	20
15	0	100	20

The identity of compound was confirmed as **8** by ESI-TOF-MS (Bruker MicroTof, calculated for $C_{11}H_{21}N_2O_6$ 277.1394; found 277.1404) and ESI-MS/MS (Bruker HCT+).

1.4.2 Scaled-up incubation of His_6-AsbA with citric acid and 1,7-heptanediamine

The enzymatic incubation using citric acid and 1,7-pentanediamine was scaled-up (total volume of 3 mL) and the corresponding product was partially purified using reverse phase HPLC (Agilent Zorbax XDB-C18 column, 150 X 21 mm, 5 micron), detecting absorbance at 210 nm and with the elution profile shown in table 5 (retention time 18.5 min).

Table 5

Time (min)	Water (0.1% TFA)	MeCN (0.1% TFA)	Flow (mL / min)
0	95	5	5
10	95	5	5
20	0	100	5
25	0	100	5
30	95	5	5
40	95	5	5

The identity of the compound was confirmed as **7** by ESI-TOF-MS (calculated for $C_{13}H_{25}N_2O_6$ 305.1707; found 305.1713) and ESI-MS/MS.

1.4.3 Scaled-up incubation of His_6-AsbA with citric acid and 1,8-heptanediamine

The enzymatic incubation using citric acid and 1,7-pentanediamine was scaled-up (total volume of 3 mL) and the corresponding product was partially purified using reverse phase HPLC (Agilent Zorbax XDB-C18 column, 150 X 21 mm, 5 micron), detecting absorbance at 210 nm and with the elution profile shown in table 5 (above, retention time 19.8 min). The identity of the compound was confirmed as **6** by ESI-TOF-MS (calculated for $C_{14}H_{27}N_2O_6$ 319.1864; found 319.1867) and ESI-MS/MS.

1.4.4 Scaled-up incubation of His_6-AsbA with citric acid and norspermidine

The enzymatic incubation using citric acid and norspermidine was scaled-up (total volume of 3 mL) and the corresponding product was partially purified using reverse phase HPLC (Agilent Zorbax XDB-C18 column, 150 X 21 mm, 5 micron), detecting absorbance at 210 nm and with the elution profile shown in table 5 (above, retention time 20 min). The identity of the compound was confirmed as **5** by ESI-TOF-MS (calculated for $C_{12}H_{24}N_3O_6$ 306.1660; found 306.1645) and ESI-MS/MS.

1.5 Incubation of His_6-AsbA with spermidine and citric acid analogues

2.0 mM spermidine, 4 mM ATP, 7.5 mM $MgCl_2$, 100 mM Tris-HCl (pH 8.0), 0.85 μM His_6-AsbA (after Ni-NTA purification) and 2 mM citric acid analogue (2-ketoglutaric acid, 3-ketoglutaric acid, *D*- glutamic acid, *L*-glutamic acid, *DL*-isocitric acid, glutaric acid and tricarballylic acid) 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 0.45 micron filter prior to analysis. The corresponding controls were carried out in the same way using denatured His_6-AsbB (heated at 100 °C for 20 minutes prior to addition to the incubation mixture).
LC-MS analyses of the reaction mixtures were carried as described in section 1.4 above.

1.5.1 Scaled-up incubation of His_6-AsbA with tricarballylic acid and spermidine

The enzymatic incubation using tricarballylic acid and spermidine was scaled-up (total volume of 3 mL) and the corresponding product was partially purified using reverse phase HPLC (Agilent Zorbax XDB-C18 column, 150 X 21 mm, 5 micron), detecting absorbance at 210 nm and using the elution profile shown in table 6 (retention time 7.9 min).

Table 6

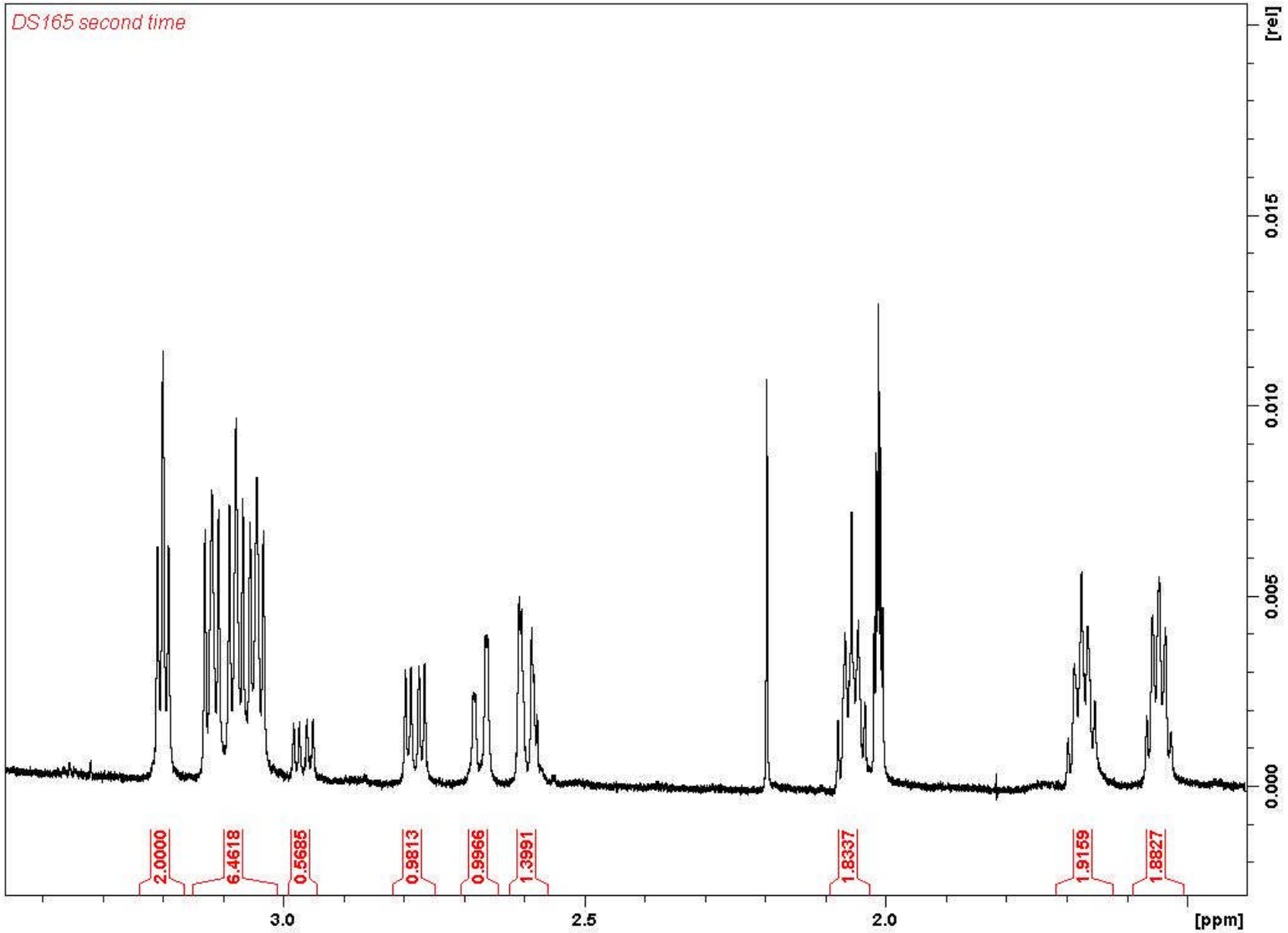
Time (min)	Water (0.1% TFA)	MeCN (0.1% TFA)	Flow (mL / min)
0	90	10	5
10	90	10	5
20	0	100	5
25	0	100	5
30	10	90	5
40	10	90	5

The identity of the compound as **12** was confirmed by ESI-TOF-MS (calculated for $C_{13}H_{26}N_3O_5$ 304.1867; found 304.1862) and ESI-MS/MS analyses.

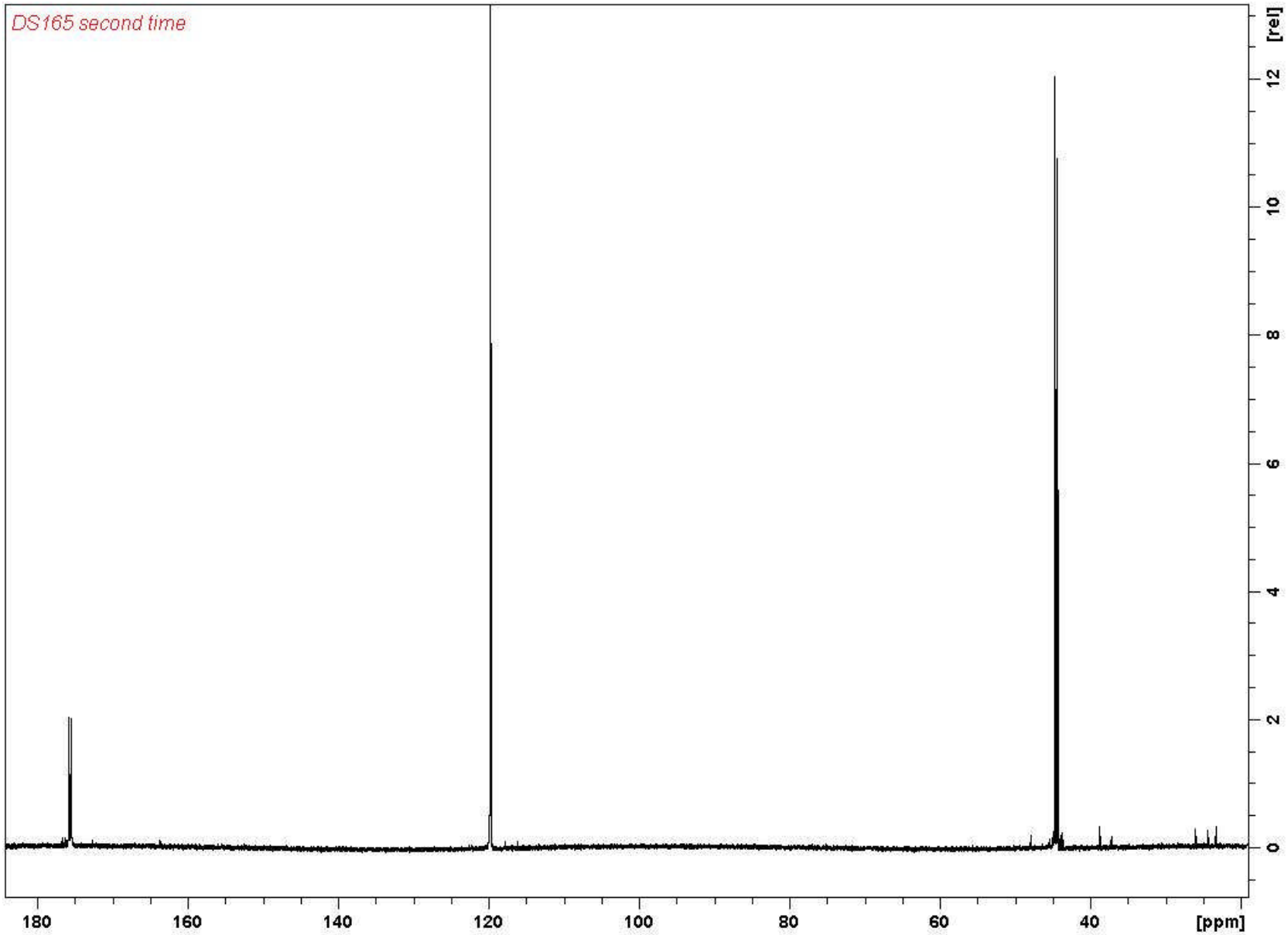
1.6 References

1. D. Oves-Costales, N. Kadi, M. J. Fogg, L. Song, K. S. Wilson and G. L. Challis, *J. Am. Chem. Soc.* 2007, **129**, 8416-8417.

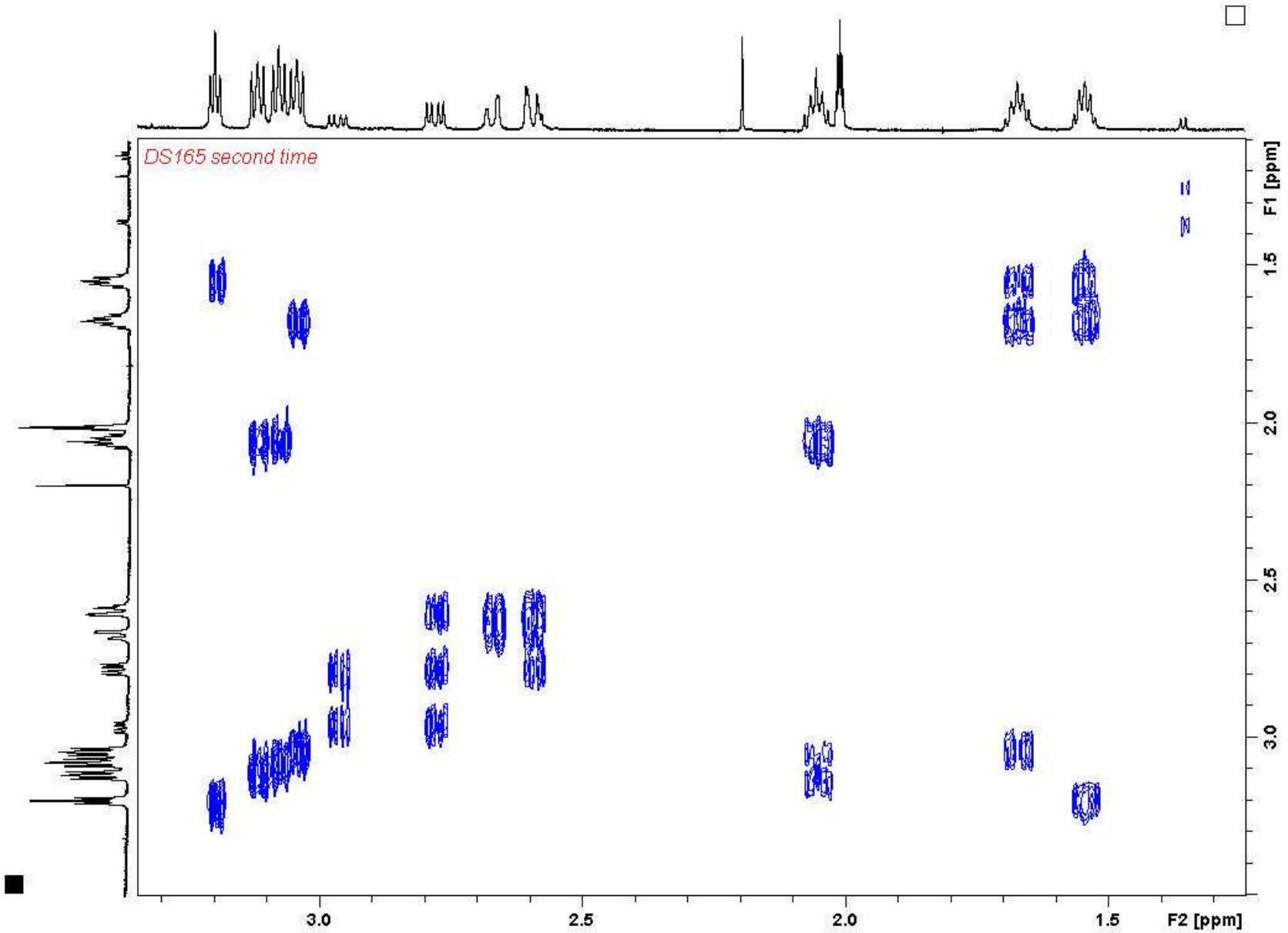
2 Spectroscopic data



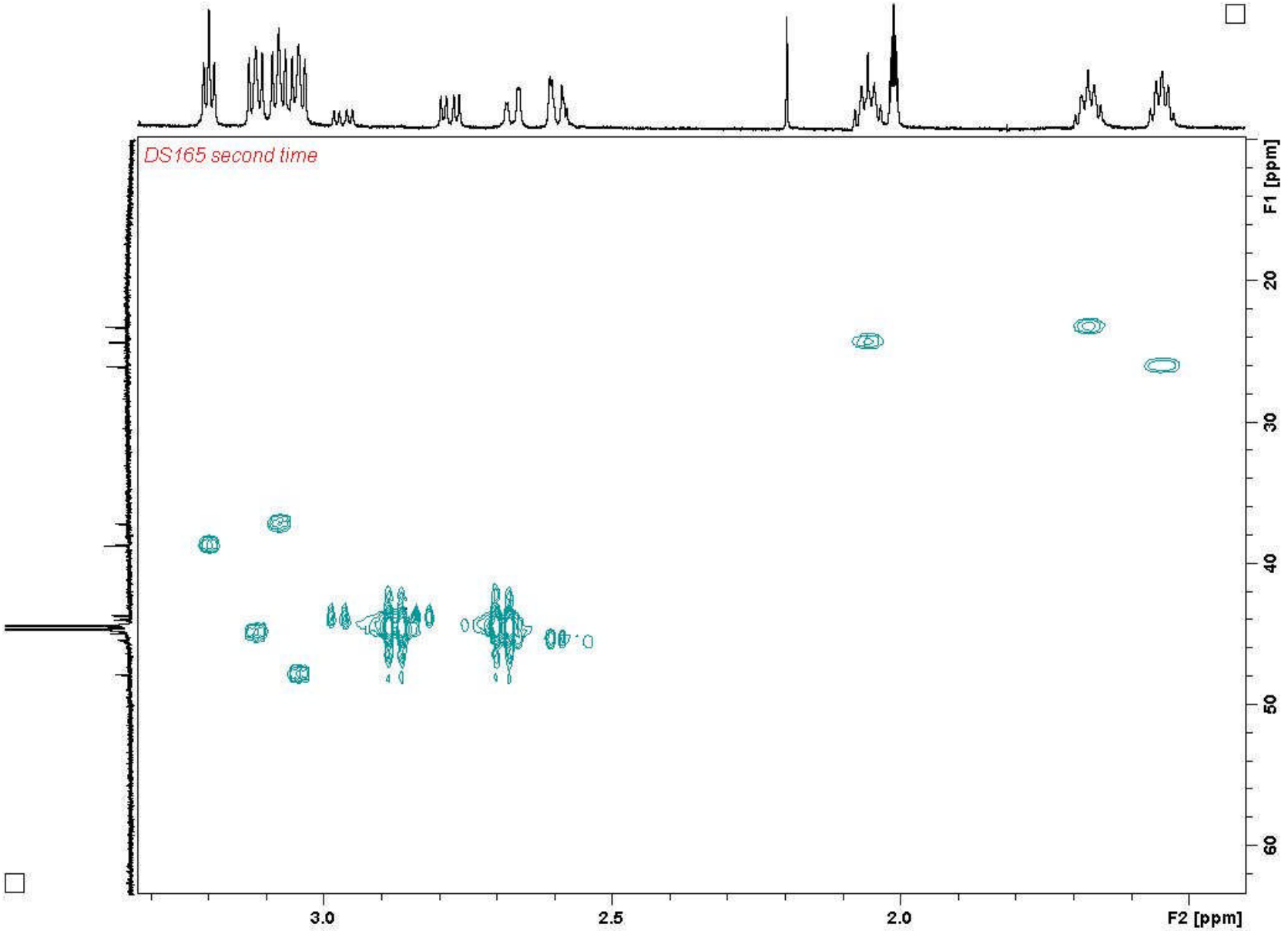
^{1}H-NMR spectrum of ^{13}C-labeled N^{8}-citryl-spermidine **2a**



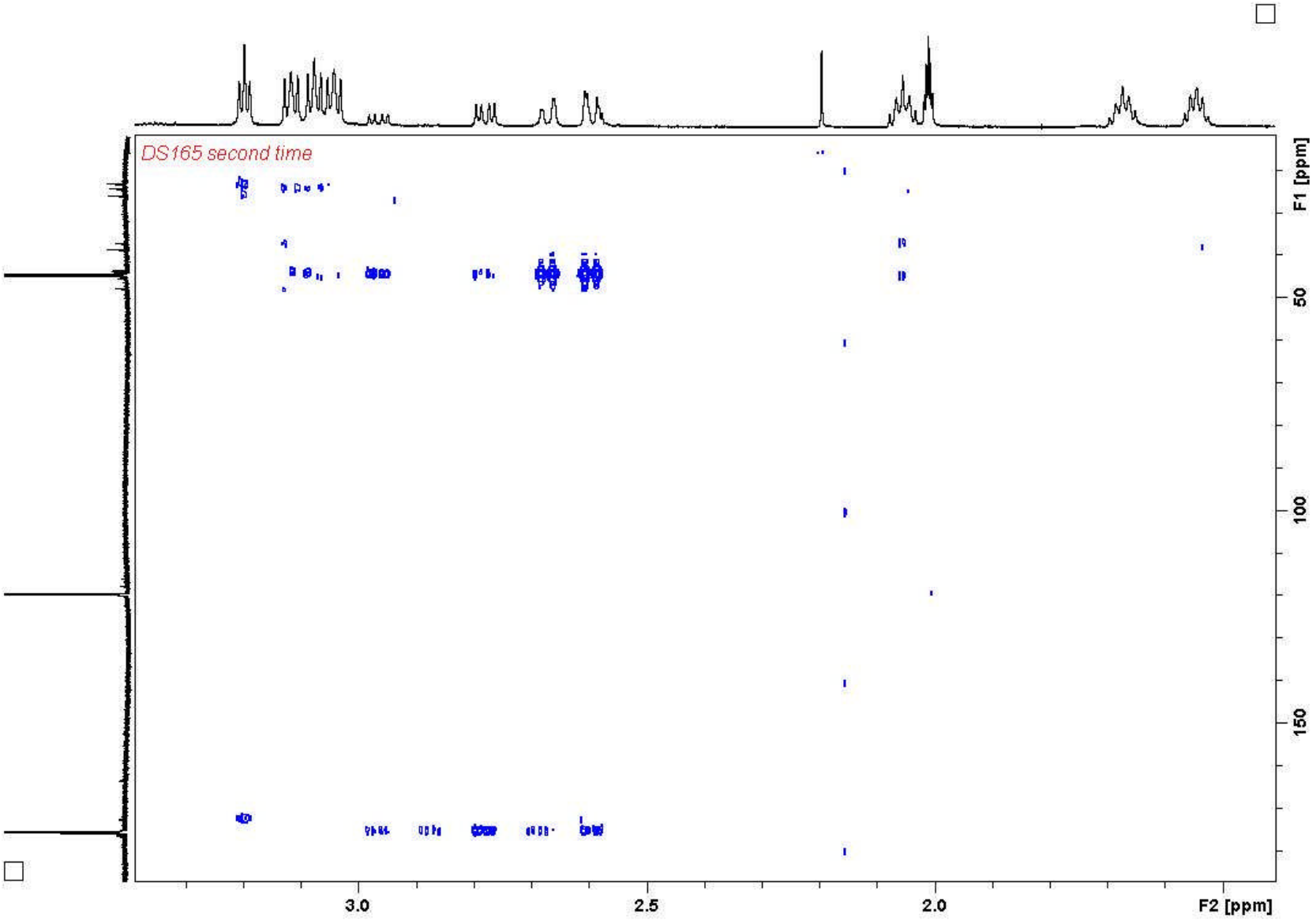
^{13}C-NMR spectrum of ^{13}C-labeled N^{8}-citryl-spermidine **2a**



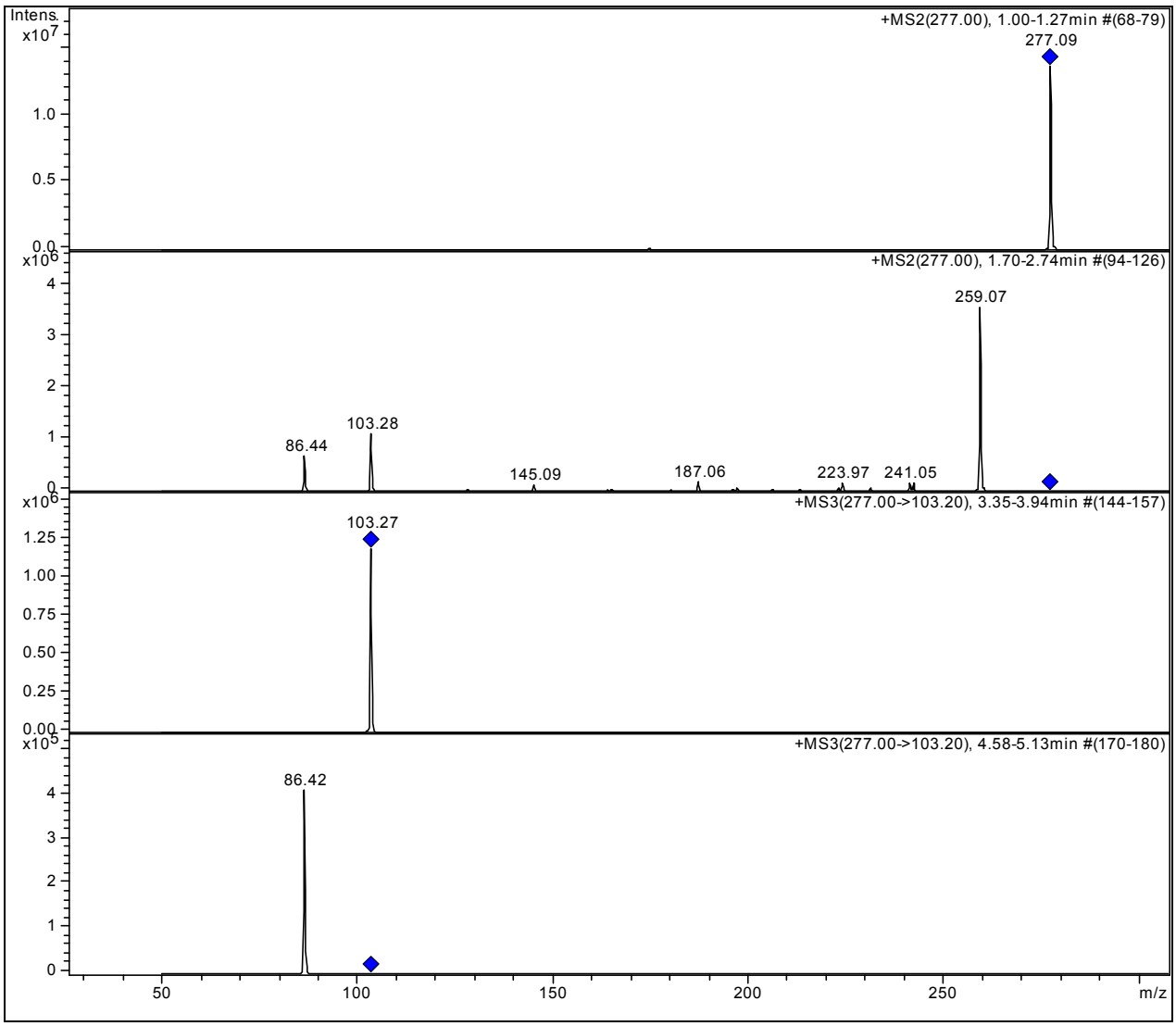
COSY spectrum of ^{13}C-labeled N^8-citryl-spermidine **2a**



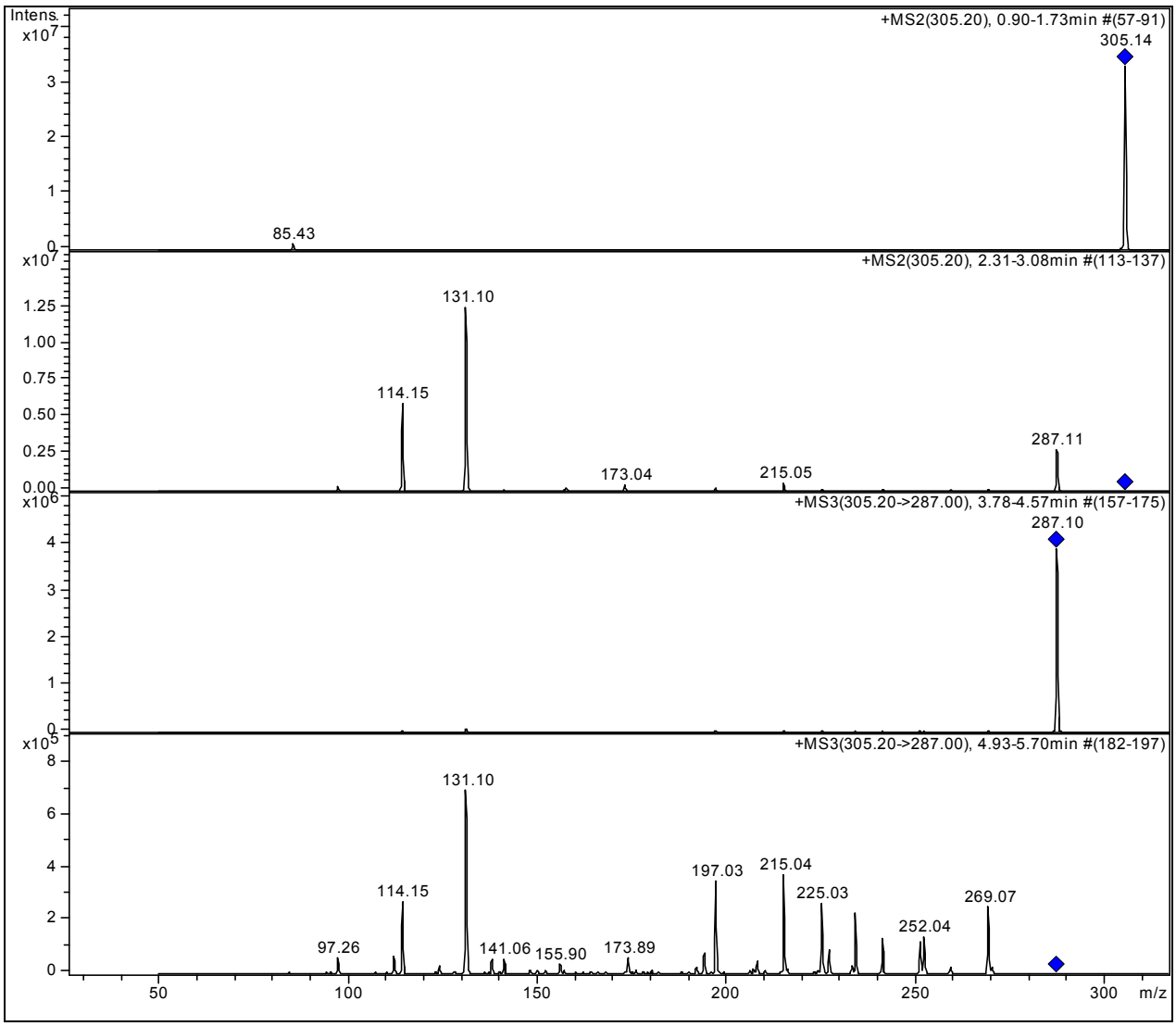
HSQC spectrum of ^{13}C-labeled N^8-citryl-spermidine **2a**



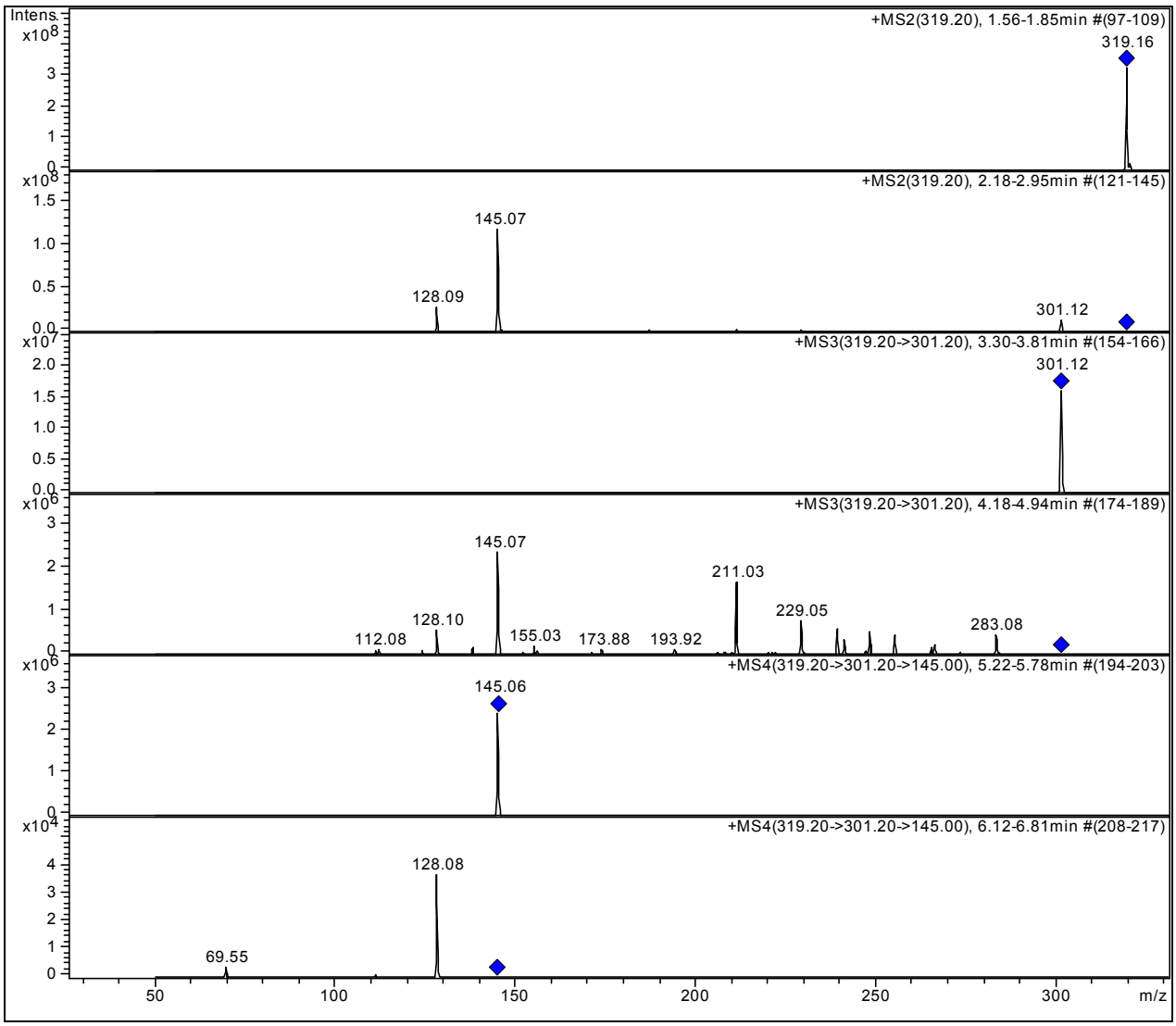
HMBC spectrum of ^{13}C-labeled N^8-citryl-spermidine **2a**



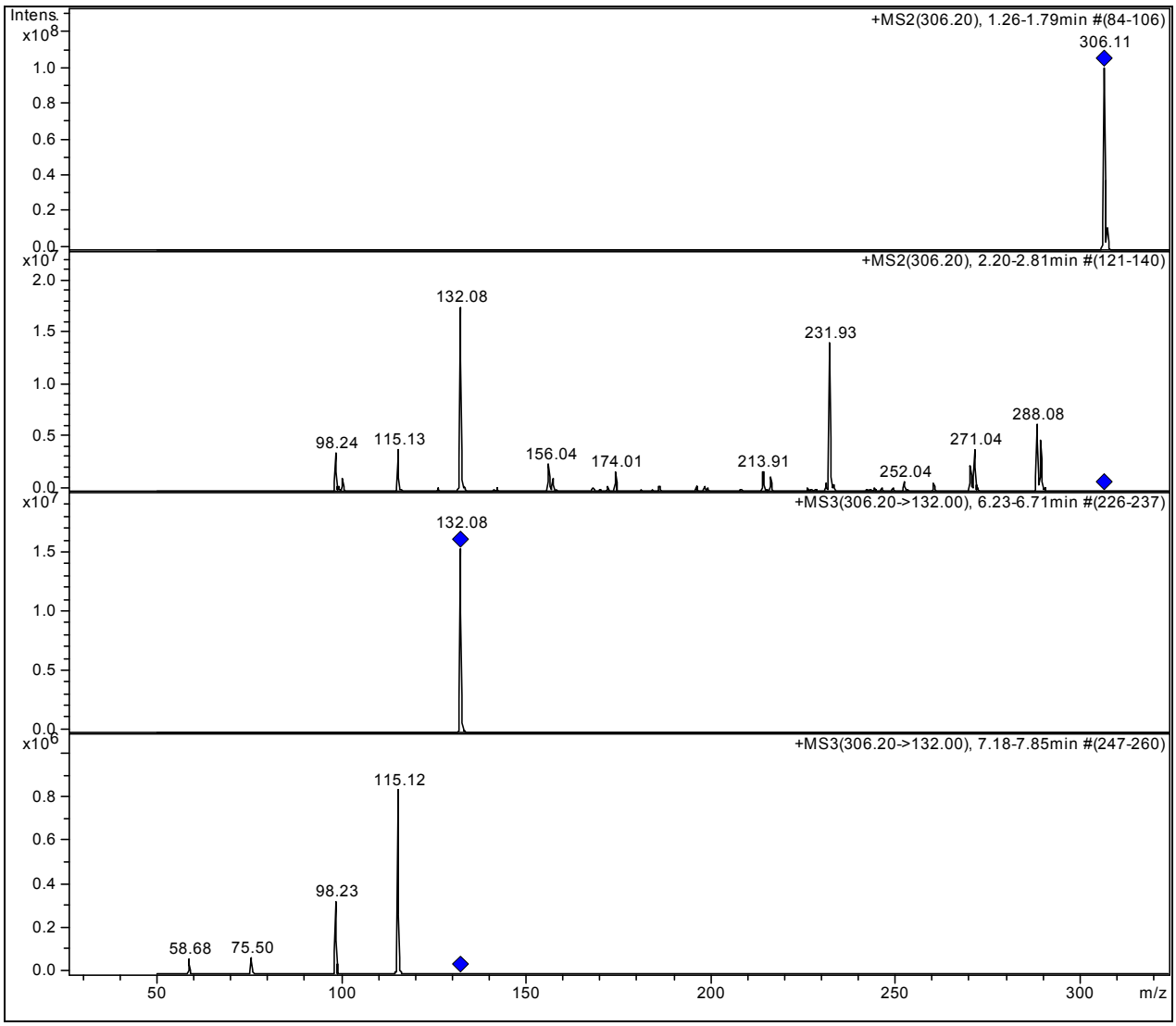
ESI-MS/MS spectrum of *N*-citryl-pentane-1,5-diamine **8**



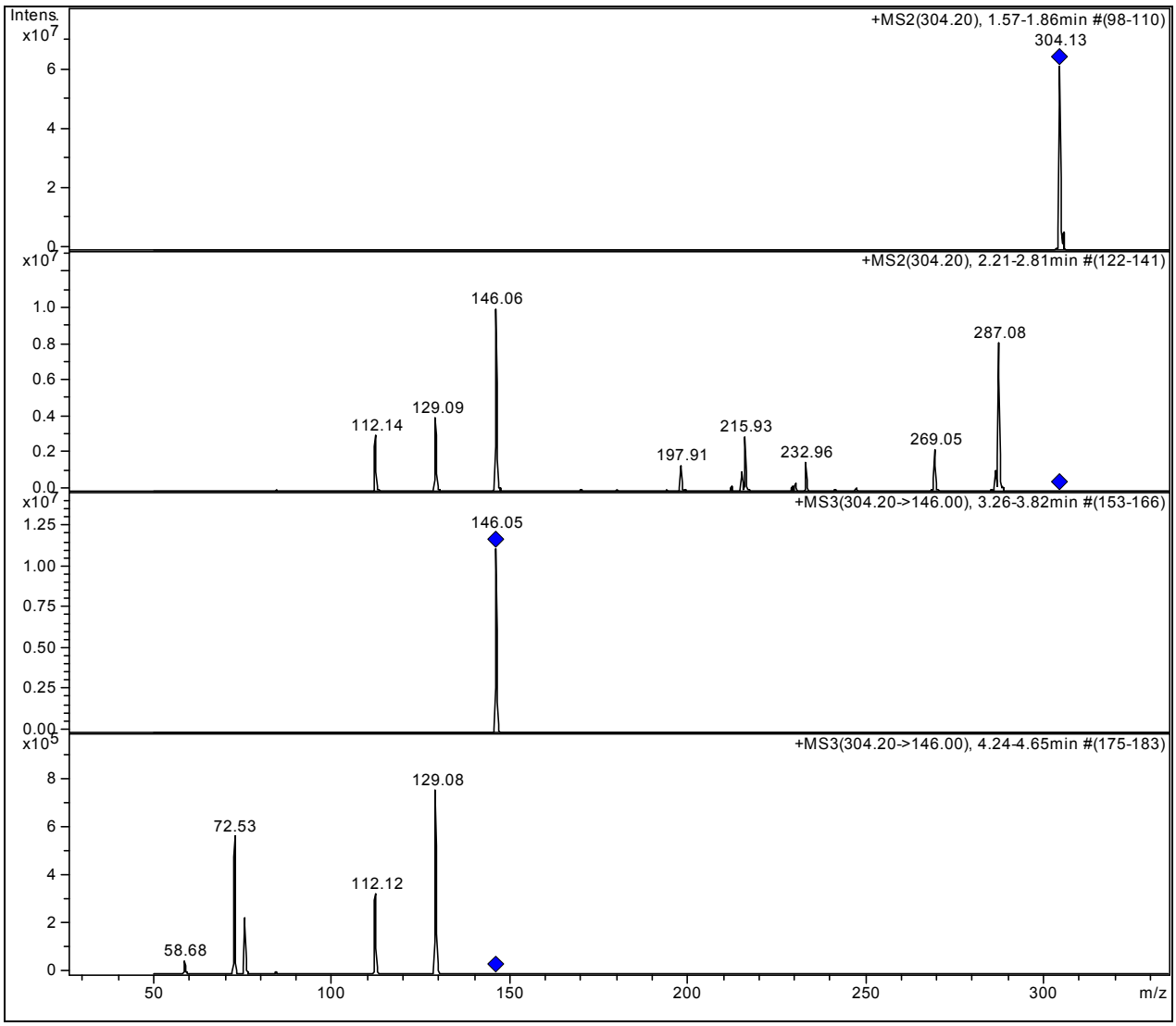
ESI-MS/MS spectrum of *N*-citryl-heptane-1,7-diamine **7**



ESI-MS/MS spectrum of *N*-citryl-octane-1, 8-diamine **6**



ESI-MS/MS spectrum of N^1-citryl-norspermidine **5**



ESI-MS/MS spectrum of N^8-tricarballyl-spermidine **12**