Supporting Information

The Biosynthesis of Mupirocin by *Pseudomonas fluorescens* NCIMB 10586 Involves Parallel Pathways

Shu-Shan Gao,[#] Joanne Hothersall,[§] Ji'en Wu,[#] Annabel C. Murphy,[#] Zhongshu Song,[#] Elton R. Stephens,[§] Christopher M. Thomas,[§] Matthew, P. Crump,[#] Russell J. Cox,[#] Thomas J. Simpson,*[,#] and Christine L. Willis*[,#]

[#]School of Chemistry, University of Bristol, Bristol, BS8 1TS, UK
[§]School of Biosciences, University of Birmingham, Birmingham, B15 2TT, UK

Construction of an mmpEOR in-frame deletion

A 385 aa in-frame deletion of the oxidoreductase (OR) domain of MmpE (amino acids 789 to 1173 inclusive) was constructed by suicide mutagenesis. The method has been described previously.[1] Two approximately 500 bp arms were generated by PCR using primer pairs mmpEOR1F/mmpEOR1R and mmpEOR2F/mmpEOR2R (See Table S1 for details). These were ligated into the suicide vector pAKE604 digested with *Eco*RI/*Bam*HI generating pJHmmpE01. pJHmmpE01 was transferred to wild-type *P. fluorescens* NCIMB 10586 by conjugative mating. Double cross-over events were selected and mutant *mmpE* was screened for by PCR using primer pair mmpEOR1F/mmpEOR2R. The mutant strain was called mmpEΔOR.

Construction of double mutants

An in-frame deletion and complementation of *mupF* has been described previously.[1] Double mutants of Δ*mupF* and either Δ*mupO*, *U*, *V* or *macpE* were constructed by transferring suicide plasmid pJHF01, containing the in-frame deletion of *mupF*, by conjugative mating to single mutants of Δ*mupO*, *U*, *V* or *macpE*, construction of which have been described previously.[2] Double cross-over events were selected and mutant *mupF* was screened for by PCR using primer pair mupF1F/mupF2R (See Table S1 for details).

Table S1. Primer details for in-frame deletions and complementation.

Primer	Sequence*
mmpEC1F	5'GAATTCATGACCCTGAAGACCATCCTC
mmpEC1R	5'CTCGAGGAACACATGGTGTCGAG
mmpEC2R	5'GAGCTCTCATGTACCGGTTTGTCGCTG
mmpEOR1F	5'GGATCCCCGCATCGTCGAGCTGCCGAC
mmpEOR1R	5'AAGCTTGGCAAAATCGAAGGCGTAGC
mmpEOR2F	5'AAGCTTGACGAGATCAACAATCTGGC
mmpEOR2R	5'GAATTCGGCATCGCCAGCCACTGC
mupF1F	5'GGATCCGCTGCGCGATGCATTGGC
mupF2R	5'GAATTCGTCATGCTGACCGGTGC

*Restriction sites engineered at 5' ends are underlined

Table S2. Plasmids used and constructed in this study

Plasmid	Details	Reference
pAKE604	5.9 kb, pMB1 replicon, Ap^r, *oriT*, *lacZα*, *sacB*	El-Sayed *et al* 2003[3]
pJH10	14.5 kb , IncQ replicon, Tc^r, Sm^r, *oriT*, *tac*p, $lacI^q$	El-Sayed *et al* 2003[3]
pJH10X	pJH10 + linker *Eco*RI, *Nhe*I, *Xho*I, *Kpn*I, *Cla*I, *Xba*I	This study
pJHmmpE01	pAKE604 + *Eco*RI/*Bam*HI 1018 bp PCR fragment, Δ1155 bp *mmpE* OR	This study
pJHmmpE'	pJH10X + start of *mmpE* 659 bp *Eco*RI/*Xho*I PCR fragment	This study
pJHmmpE11	pJHmmpE' + end of *mmpE* 3155 bp *Bst*BI/*Sac*I restriction fragment	This study

Complementation of *P. fluorescens* NCIMB 10586 mmpEΔOR

The start of *mmpE* was cloned *Eco*RI/*Xho*I into pJH10X as a 659 bp PCR fragment, amplified with primers mmpEC1F/mmpEC1R, generating pJHmmpE'. The whole of *mmpE* was amplified as a 3588 bp fragment using primers mmpEC1F/mmpEC2R and cloned into pGEM®T-Easy (Promega). This construct was used to clone the remainder of *mmpE* into pJHmmpE' as a 3155 bp *Bst*BI/*Sac*I restriction fragment, generating pJHmmpE11. pJHmmpE11 was transferred to *P. fluorescens* mmpEΔOR by conjugative mating, generating complementation strain NCIMB10586 mmpEΔOR (pJHmmpE11). Analysis of extracts from this strain by HPLC, as described previously,[1] showed production of a compound with the same retention time as PA-A, which was not present in mmpEΔOR (See Figure S1). This confirmed that production of PA-C in mmpEΔOR was due to inactivation of the MmpE OR domain by in-frame deletion and not due to polar effects.

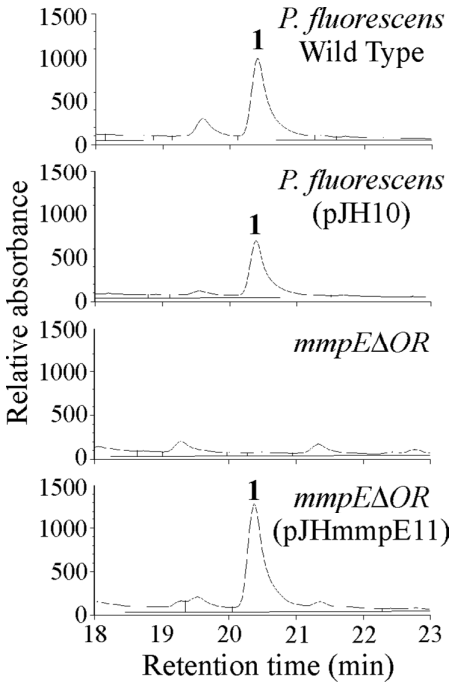


Figure S1. **HPLC analysis of PA production by *P. fluorescens* NCIMB 10586 mmpEΔOR complemented by pJHmmpE11 *in trans*.** Wild-type *P. fluorescens* NCIMB 10586 and the control *P. fluorescens* NCIMB 10586 (pJH10) both produce PA-A (**1**) which is absent in NCIMB 10586 *mmpEΔOR*. When NCIMB 10586 *mmpEΔOR* is complemented with *mmpE in trans* production of PA-A (**1**) is restored.

References:

1. J. Hothersall, J. Wu, A. S. Rahman, J. A. Shields, J. Haddock, N. Johnson, S. M. Cooper, E. R. Stephens, R. J. Cox, J. Crosby, C. L. Willis, T. J. Simpson, C. M. Thomas, *J. Biol. Chem.* **2007**, *282*, 15451–154614.
2. S.M. Cooper, W. Laosripaiboon, J. Hothersall, A. K. El-Sayed, C. Winfield, J. Crosby, R. J. Cox, T. J. Simpson and C. M. Thomas, *Chem. Biol*., 2005, **12**, 825–833.
3. A. K. El-Sayed, J. Hothersall, S. M. Cooper, E. Stephens, T. J. Simpson, C. M. Thomas, *Chem. Biol.* **2003**, *10*, 419–430.

Experimental for chemical investigations and small scale feedings

General fermentation procedure

Wild type, Δ*mupW*, Δ*mupC*, Δ*mupF*, Δ*mmpE*, *mmpE*Δ*OR*/Δ*mupC*, *mmpE*Δ*OR*/Δ*mupW*, or *mmpE*Δ*OR*/Δ*mupF* *Pseudomonas fluorescens* NCIMB 10586 was incubated on a L-agar plate at 30 ºC for 24 hours. (L-agar: bacto tryptone 10g, yeast extract 5g, sodium chloride 5g, glucose 1g , agar 18 g, and deionised water added up to 1000 mL, no antibiotic for wild type, carbenicillin(50 µg/ml) for mutants). Seed medium was inoculated in a 500 ml flask with 100 ml of L-medium by adding a single colony from the L-agar plate and incubated at 200 rpm at 25 ºC. (L-medium: bacto tryptone 10g, yeast extract 5g, sodium chloride 5g, glucose 1g, and deionised water added up to 1000 mL). 85 ml of fermentation medium was decanted into 500 ml flasks with vigorous stirring. After sterilization, 10 mLof sterilized 40% w/v glucose solution and 5 ml of seed medium were added to the fermentation medium. The culture was inoculated at 22 ºC at 250 rpm for 48 hours. (Fermentation medium is a modified L-medium: bacto tryptone 10g, yeast extract 5g, sodium chloride 5g, and deionised water added up to 850 ml). After the fermentation, the cells were separated from the medium by centrifugation (8000 rpm for 15 mins). The medium was extracted with EtOAc (0.5 v/v) 3 times. The cell pellet was lysed with acetone 3 times, and the acetone was removed *in vacuo*, and the residue extracted with EtOAc (0.5 v/v) 3 times. The combined EtOAc extracts were evaporated in *vacuo* to give a crude extract. The crude extract was filtered with cotton and then analyzed by LCMS and HPLC. Purification was performed by HPLC chromatography on different HPLC columns (250 x 4.6 mm Phenomenex Luna column, 250 x 10 mm Phenomenex Luna column and Chiralcel OD-H column). All numbers in bold correspond to the compound numbers given in schemes in the manuscript.

***P. fluorescens* NCIMB 10586 Wild Type**

A 4 L fermentation of *P. fluorescens* was carried out as per general procedure. The crude extract was subjected to HPLC chromatography on a Phenomenex Luna column (250 x 10 mm, 5µm, 40 ºC, flow 5 mL/min) eluted with isocratic wash of 45 % MeOH in water for 1 minute followed by a gradient of 45% to 85% over 20 minutes to yield 7 fractions. The fraction 1 (Rt: 12.6) was further subjected to HPLC chromatography on a Chiralceh OD-H column eluted with isocratic wash of 50 % MeOH in water for 1 minute followed by a gradient of 50% to 70% over 10 minutes to yield **6** (1.3 mg, Rt: 7.7 mins). The fractions 2 (Rt: 14.6 mins) and 3 (Rt: 15.4 mins) were **10** (1.4 mg) and **2** (14.2

mg), respectively. The fraction 4 (Rt: 16.1 mins) was further subjected to HPLC chromatography on a Chiralceh OD-H column eluted with isocratic wash of 60 % MeOH in water for 1 minute followed by a gradient of 60% to 76.5% over 11 minutes to yield **1** (125.3 mg, Rt: 9.3 mins) and **5** (1.8 mg, Rt: 10.4 mins). The fraction 5 (Rt: 17.2 mins) was further subjected to HPLC chromatography on a Chiralceh OD-H column eluted with isocratic wash of 60 % MeOH in water for 1 minute followed by a gradient of 60% to 90% over 20 minutes to yield **9** (Rt: 13.1 mins, 1.6 mg) and **11** (Rt: 16.2 mins, 1.1 mg). The fraction 6 (Rt: 18.1 mins) was sent for NMR after LC-MS analysis, and the structure was elucidated as **3** (0.8 mg). The fraction 7 (Rt: 23.9 mins) was sent for NMR after LC-MS analysis, and the structure was elucidated as **12** (8.4 mg).

9: HR-ESI-MS: *m*/*z* = 509.3092 $[M+Na]^+$ (509.3085 calcd for $C_{26}H_{44}O_9Na$). ^{1}H-NMR, δ_H (500 MHz, CD_3OD), 5.77 (1H, brs, H-2), 5.45 (1H, m, H-10), 5.41 (1H, m, H-11), 4.08 (2H, t, 6.5, H-9'), 3.99 (1H, m, H-5), 3.62 (1H, m, H-13), 3.56 (1H, dd, 8.5 and 4.3, H-6), 3.44 (1H, dd, 8.7 and 2.9, H-7), 2.55 (1H, brd, 13.7, H-4), 2.35-2.25 (4H, m, H-2', H-9, and H-4), 2.18 (3H, s, H-15), 2.14 (1H, m, H-12), 1.91 (1H, m, H-8), 1.87 (1H, m, H-9), 1.63 (2H, m, H-8'), 1.60 (2H, m, H-3'), 1.26-1.41 (8H, m, H-4', H-5', H-6' and H-7'), 1.10 (3H, d, 6.6, H-14), 0.99 (3H,d, 6.5, H-17), 0.98 (3H, d, 6.7, H-16); δ_C (observed via 2D nmr), 177.0 (C, C-1'), 167.0 (C, C-1), 158.3 (C, C-3), 133.4 (CH, C-11), 130.5 (CH, C-10), 117.3 (CH, C-2), 77.2 (CH, C-7), 74.0 (CH, C-6), 71.4 (CH, C-5), 71.1 (CH, C-13), 63.5 (CH2, C-9'), 44.1 (CH2, C-12), 42.4 (CH2, C-4), 35.2 (CH, C-8), 34.0 (CH2, C-2'), 32.8 (CH2, C-9), 28.9 (CH2, C-4', C-5', C-6' or C-7'), 28.8 (CH2, C-4', C-5', C-6' or C-7'), 28.7 (CH2, C-4', C-5', C-6' or C-7'), 28.3 (CH2, C-8'), 25.6 (CH2, C-4', C-5', C-6' or C-7'), 24.7 (CH2, C-3'), 18.8 (CH3, C-14), 17.7 (CH3, C-15), 15.6 (CH3, C-16), 15.4 (CH3, C-17).

10: HR-ESI-MS: *m*/*z* = 481.2790 $[M+Na]^+$ (481.2783 calcd for $C_{24}H_{42}O_8Na$). ^{1}H-NMR, δ_H (500 MHz, CD_3OD), 5.78 (1H, brs, H-2), 5.45 (1H, m, H-10), 5.39 (1H, m, H-11), 4.07 (2H, t, 6.5, H-9'), 3.98 (1H, m, H-5), 3.61 (1H, m, H-13), 3.56 (1H, dd, 8.7 and 4.9, H-6), 3.44 (1H, dd, 8.7 and 2.9, H-7), 2.55 (1H, brd, 13.5, H-4), 2.32-2.23 (4H, m, H-2', H-9, and H-4), 2.19 (3H, s, H-15), 2.12 (1H, m, H-12), 1.91 (1H, m, H-8), 1.87 (1H, m, H-9), 1.64 (2H, m, H-6'), 1.61 (2H, m, H-3'), 1.27-1.42 (4H, m, H-4' and H-5'), 1.11 (3H, d, 6.5, H-14), 0.99 (3H,d, 6.8, H-17), 0.97 (3H, d, 6.7, H-16); δ_C (observed via 2D nmr), 177.1 (C, C-1'), 167.6 (C, C-1), 158.3 (C, C-3), 133.3 (CH, C-11), 130.1 (CH, C-10), 117.5 (CH, C-2), 77.0 (CH, C-7), 73.8 (CH, C-6), 71.3 (CH, C-5), 71.0 (CH, C-13), 63.3 (CH2, C-7'), 44.6 (CH2, C-12), 42.4 (CH2, C-4), 35.1 (CH, C-8), 33.2 (CH2, C-2'), 32.8 (CH2, C-9), 29.2 (CH2, C-4'), 28.4 (CH2, C-6'), 25.9 (CH2, C-5'), 24.9 (CH2, C-3'), 18.9 (CH3, C-14), 17.7 (CH3, C-15), 15.9 (CH3, C-16), 15.4 (CH3, C-17).

11: HR-ESI-MS: *m*/*z* = 523.2889 $[M+Na]^+$ (509.2878 calcd for $C_{26}H_{44}O_9Na$). ^{1}H-NMR, δ_H (500 MHz, CD_3OD), 5.74 (1H, br s, H-2), 5.58 (1H, m, H-10), 5.49 (1H, m, H-11), 4.07 (2H, t, 6.5, H-9'), 3.72 (1H, br s, H-7), 3.64 (1H, m, H-5), 3.61 (1H, m, H-13), 3.43 (1H, br d, 10.9, H-16), 3.34 (1H, overlapped by solovent peak, H-6), 3.32 (1H, overlapped by solovent peak, H-16), 2.66 (1H, br d, 13.9, H-4), 2.32 (2H, d, 6.8, H-9), 2.26 (2H, t, 7.9, H-2'), 2.21-2.14 (2H, m, H-12 and H-4), 2.18 (3H, s, H-15), 1.63 (2H, m, H-8'), 1.60 (2H, m, H-3'), 1.26-1.42 (8H, m, H-4', H-5', H-6' and H-7'), 1.10 (3H, d, 6.3, H-14), 1.00 (3H, d, 6.9, H-17); δ_C (observed via 2D nmr), 177.2 (C, C-1'), 167.0 (C, C-1), 157.7 (C, C-3), 136.5 (CH, C-11), 124.4 (CH, C-10), 116.8 (CH, C-2), 73.9 (CH, C-5), 72.6 (CH, C-7), 71.2 (C, C-8), 70.7 (CH, C-13), 69.4 (CH, C-6), 68.1 (CH2, C-16), 63.5 (CH2, C-9'), 44.1 (CH2, C-12), 42.4 (CH2, C-4), 38.4 (CH2, C-9), 34.0 (CH2, C-2'), 28.9 (CH2, C-4', C-5', C-6' or C-7'), 28.8 (CH2, C-4', C-5', C-6' or C-7'), 28.7 (CH2, C-4', C-5', C-6' or C-7'), 28.3 (CH2, C-8'), 25.6 (CH2, C-4', C-5', C-6' or C-7'), 24.7 (CH2, C-3'), 19.0 (CH3, C-14), 17.9 (CH3, C-15), 15.3 (CH3, C-17).

12: HR-ESI-MS: 521.2719 $[M+Na]^+$ (521.2721 calcd for $C_{26}H_{42}O_9Na$). ^{1}H-NMR, δ_H (500 MHz, CD_3OD), 5.75 (1H, br s, H-2), 4.89 (1H, m, H-13), 4.25 (1H, m, H-9), 3.90 (1H, m, H-9'), 3.70 (1H, d, 10.6, H-16), 3.68 (1H, overlapped with H-16, H-7), 3.61 (1H, br s, H-5), 3.43 (1H, br d, 10.5, H-16), 3.34 (1H, dd, 3.0 and 8.5, H-6), 2.95 (1H, m, H-10), 2.61 (1H, dd, 8.5 and 2.1, H-11), 2.58 (1H, br d, 14.0, H-4), 2.34 (2H, m, H-2'), 2.16 (3H, s, H-15), 1.80 (1H, br d, 14.9, H-9), 1.69 (1H, dd, 14.6 and 7.8, H-9), 1.62 (2H, m, H-8'), 1.59 (2H, m, H-3'), 1.26-1.42 (8H, m, H-4', H-5', H-6' and H-7'), 1.24 (3H, d, 6.7, H-14), 0.99 (3H,d, 6.5, H-17); δ_C (125 MHz, CD_3OD), 174.0 (C, C-1'), 166.8 (C, C-1), 156.9 (C, C-3), 117.8 (CH, C-2), 74.0 (CH, C-5), 73.8 (CH, C-13), 73.1 (CH, C-7), 71.4 (C, C-8), 69.8 (CH, C-6), 67.2 (CH2, C-16), 63.2 (CH2, C-9'), 59.5 (CH, C-11), 52.0 (CH, C-10), 42.3 (CH2, C-4), 41.7 (CH2, C-12), 36.7 (CH2, C-8), 33.7 (CH2, C-2'), 28.4 (CH2, C-4', C-5', C-6' or C-7'), 28.2 (CH2, C-8'), 28.0 (CH, C-8), 29.0 (CH2, C-4', C-5', C-6' or C-7'), 27.9 (CH, C-8), 29.0 (CH2, C-4', C-5', C-6' or C-7'), 25.7 (CH2, C-4', C-5', C-6' or C-7'), 24.1 (CH2, C-3'), 17.5 (CH3, C-14), 17.3 (CH3, C-15), 12.5 (CH3, C-17).

P. fluorescens* NCIMB 10586 *mmpE*Δ*OR

A 0.8 L scale fermentation of *mmpE*Δ*OR* mutant of *P. fluorescens* NCIMB 10586 was carried out as per general procedure. The crude extract was dried and then subjected to HPLC chromatography on a Phenomenex Luna column (250 x 4.6 mm, 5µm, 40 ºC) eluted with isocratic wash of 55 % MeOH in water for 1 minute followed by a gradient of 55% to 95% over 20 minutes to yield 4 fractions. The fraction 2 (Rt: 10.7 mins) was further subjected to HPLC chromatography on a Chiralcel OD-H column eluted with isocratic wash of 62 % MeOH in water for 1 minute

followed by a gradient of 62 % to 72% over 10 minutes to yield **10** (1.9 mg, Rt: 7.5 mins). The fraction 3 (Rt: 13.8 mins) was further subjected to HPLC chromatography on a Chiralcel OD-H column eluted with isocratic wash of 65 % MeOH in water for 1 minute followed by a gradient of 65 % to 76% over 11 minutes to yield a mixture of compounds **9** and **11** (2.7 mg, Rt: 8.8 mins). The fraction 4 (Rt: 14.8 mins) was further subjected to HPLC chromatography on a Chiralcel OD-H column eluted with isocratic wash of 65 % MeOH in water for 1 minute followed by a gradient of 65 % to 78% over 13 minutes to yield **3** (2.1 mg, Rt: 11.2 mins).

P. fluorescens* NCIMB 10586 Δ*mupW

A 0.8 L scale fermentation of Δ*mupW* mutant of *P. fluorescens* NCIMB 10586 was carried out as per general procedure. The crude extract was dried and then subjected to HPLC chromatography on a Phenomenex Luna column (250 x 10 mm, 5µm, 40 ºC) eluted with isocratic wash of 45 % MeOH in water for 1 minute followed by a gradient of 45% to 85% over 20 minutes to yield 1 fractions.. The Fraction 1 (Rt: 12.7 mins, 0.7 mg) and 2 (Rt: 16.0 mins, 0.6 mg) were sent for LC-MS analysis and ^{1}H NMR and their structure are the same as **6** and **5**, respectively.

P. fluorescens* NCIMB 10586 *mmpE*Δ*OR*/Δ*mupW

A 2.4 L scale fermentation of Δ*mmpEOR*/Δ*mupW* was carried out as per general procedure. The crude extract was subjected to HPLC chromatography on a Phenomenex Luna column (250 x 10 mm, 5µm, 40 ºC, flow 5 mL/min) eluted with isocratic wash of 55 % MeOH in water for 1 minute followed by a gradient of 55% to 87% over 16 minutes to yield 2 fractions. The fraction 1 (Rt: 10.1 mins) was further subjected to HPLC chromatography on a Chiralcel OD-H column eluted with isocratic wash of 62 % MeOH in water for 1 minute followed by a gradient of 62% to 72% over 10 minutes to yield **10** (1.8 mg, Rt: 7.1 mins). The fraction 2 (Rt: 13.4 mins) was further subjected to HPLC chromatography on a Chiralcel OD-H column eluted with isocratic wash of 65 % MeOH in water for 1 minute followed by a gradient of 65% to 76% over 11 minutes to yield **9** (1.5 mg, Rt: 12.8 mins).

P. fluorescens* NCIMB 10586 Δ*mupC

A 2.4 L scale fermentation of Δ*mupC* mutant of *P. fluorescens* was carried out as per general procedure. The crude extract was subjected to HPLC chromatography on a Phenomenex Luna column (250 x 10 mm, 5µm, 40 ºC, flow 5 mL/min) eluted with isocratic wash of 45 % MeOH

in water for 1 minute followed by a gradient of 45% to 85% over 20 minutes to yield 7 fractions. The fraction 1 (Rt: 12.6 mins) was further subjected to HPLC chromatography on a Chiralcel OD-H column eluted with isocratic wash of 50 % MeOH in water for 1 minute followed by a gradient wash of 50% to 70% over 10 minutes to yield **6** (3.1 mg, Rt: 7.7 mins). The fraction 2 (Rt: 14.6 mins, 2.7 mg) and the fraction 3 (Rt: 15.4 mins, 18.2 mg) were **10** and **2**, respectively, confirmed by LC-MS analysis. The fraction 4 (Rt: 16.1 mins) was further subjected to HPLC chromatography on a Chiralcel OD-H column eluted with isocratic wash of 60 % MeOH in water for 1 minute followed by a gradient of 60% to 76.5% over 11 minutes to yield **5** (1.8 mg, Rt: 10.4 mins). The fraction 5 (Rt: 17.2 mins) was further subjected to HPLC chromatography on a Chiralcel OD-H column eluted with isocratic wash of 60 % MeOH in water for 1 minute followed by a gradient wash of 60% to 90% over 20 minutes to yield **9** (Rt: 13.4 mins, 1.8 mg), **11** (Rt: 16.0 mins, 1.6 mg), and **16** (18.7 mg, Rt: 15.7 mins). The fraction 6 (Rt: 18.1 mins) was sent for NMR after LC-MS analysis, and the structure was elucidated as **15** (20.1 mg). The Fr. 7 (Rt: 23.9 mins) was sent for NMR after LC-MS analysis, and the structure was elucidated as **12** (3.2 mg).

15: HR-ESI-MS: *m*/*z* = 519.2578 $[M+Na]^+$ (519.2565 calcd for $C_{26}H_{40}O_9Na$). ^{1}H-NMR, δ_H (500 MHz, CD_3OD), 5.76 (1H, br s, H-2), 4.47 (1H, br d, 16.5, H-16), 4.38 (1H, br d, 16.5, H-16), 4.32 (1H, d, 8.9, H-5), 4.06 (2H, t, 6.7, H-9'), 3.77 (1H, m, H-13), 2.90 (1H, dt, 6.4 and 2.3, H-10), 2.83-2.78 (2H, m, H-4 and H-11), 2.55 (1H, dd, 14.5 and 5.3, H-9), 2.50-2.42 (2H, m, H-4 and H-9), 2.27 (2H, t, 7.2, H-2'), 2.18 (3H, s, H-15), 1.63 (2H, m, H-8'), 1.60 (2H, m, H-3'), 1.41 (1H, m, H-12), 1.27-1.40 (8H, m, H-4', H-5', H-6' and H-7'), 1.19 (3H, d, 6.5, H-14), 0.92 (3H, d, 6.6, H-17); δ_C (125 MHz, CD_3OD), 191.2 (C, C-6), 176.4 (C, C-1'), 166.7 (C, C-1), 155.8 (C, C-3), 142.7 (C, C-7), 128.0 (C, C-8), 117.6 (CH, C-2), 78.2 (CH, C-5), 69.3 (CH, C-13), 66.3 (CH2, C-16), 63.5 (CH2, C-9'), 59.9 (CH, C-11), 54.0 (CH, C-10), 42.2 (CH, C-12), 40.2 (CH2, C-4), 33.6 (CH2, C-2'), 29.3 (CH2, C-9), 28.9 (CH2, C-4', C-5', C-6' or C-7'), 28.8 (CH2, C-4', C-5', C-6' or C-7'), 28.7 (CH2, C-4', C-5', C-6' or C-7'), 28.3 (CH2, C-8'), 25.6 (CH2, C-4', C-5', C-6' or C-7'), 24.7 (CH2, C-3'), 18.8 (CH3, C-14), 17.8 (CH3, C-15), 10.8 (CH3, C-17).

16: HR-ESI-MS: 519.2563 $[M+Na]^+$ (519.2565 calcd for $C_{26}H_{40}O_9Na$). δ_H (500 MHz, CD_3OD), 5.77 (1H, s, 1.2, 2-H), 4.85 (1H, m, 10-H), 4.53 (2H, m, 16-H), 4.29 (1H, dd, 9.7 and 3.2, H-5), 4.06 (1H, t, 6.6, H-9'), 4.04 (1H, qd, 6.4 and 6.1, 13-H), 3.81 (1H, dd, 8.8 and 3.2, H-11), 3.00 (1H, ddt, 18.1, 8.8, and 2.2, H-9), 2.84 (1H, ddt, 18.1, 10.8 and 2.0, H-9), 2.78 (1H, dd, 14.9 and 3.2, H-4), 2.44 (1H, ddd, 14.9, 9.7 and 1.0, H-4), 2.26 (2H, t, 7.6, H-2'), 2.16 (3H, d, 1.2, H-15), 1.65 (2H, m, H-8'), 1.64 (1H, m, H-12), 1.59 (2H, m, H-3'), 1.26-1.36 (8H, m, H-4, H-5, H-6, and H-7), 1.11 (3H, d, 6.4, H-14), 0.85 (3H, d, 7.1, H-17); δ_C (125 MHz, CD_3OD), 187.7 (C, C-6), 177.0 (C, C-1'), 166.7 (C, C-1), 155.8 (C, C-3), 146.7 (C, C-7), 135.8 (C, C-8), 117.6 (CH, C-2), 83.6 (CH, C-10), 78.8 (CH, C-5), 73.9 (CH, C-11), 68.3 (CH, C-13), 63.5 (CH, C-9'),

63.3 (CH2, C-16), 41.5 (CH, C-12), 39.6 (CH2, C-4), 33.9 (CH2, C-2'), 30.2 (CH2, C-9), 28.9 and 28.8 (C-5' and C-6'), 28.7 (CH2, C-4'), 28.3 (CH2, C-8'), 25.6 (CH2, C-7'), 24.8 (CH2, C-3'), 17.5 (CH3, C-15), 17.4 (CH3, C-14), 9.7 (CH3, C-17).

P. fluorescens* NCIMB 10586 *mmpE*Δ*OR/*Δ*mupC

A 0.8 L scale fermentation of Δ*mmpEOR/*Δ*mupC P. fluorescens* NCIMB 1058 was carried out as per general procedure. The crude extract was dried and then subjected to HPLC chromatography on a Phenomenex Luna column (250 x 4.6 mm, 5µm, 40 ºC) eluted with isocratic wash of 55 % MeOH in water for 1 minute followed by a gradient of 55% to 95% over 20 minutes to yield 4 fractions. The fraction 1 (Rt: 10.7) was further subjected to HPLC chromatography on a Chiralcel OD-H column eluted with isocratic wash of 60 % MeOH in water for 1 minute followed by a gradient of 62 % to 72% over 10 minutes to yield **10** (1.7 mg, Rt: 7.5 mins). The fraction 2 (Rt: 13.9 mins) was further subjected to HPLC chromatography on a Chiralcel OD-H column eluted with isocratic wash of 62 % MeOH in water for 1 minute followed by a gradient of 62 % to 72% over 10 minutes to yield a mixture of **9** and **11** (2.1 mg, Rt: 8.7 mins). The fraction 4 (Rt: 18.5 mins) was further subjected to HPLC chromatography on a Chiralcel OD-H column eluted with isocratic wash of 68 % MeOH in water for 1 minute followed by a gradient of 68 % to 83% over 15 minutes to yield **17** (0.4 mg, Rt: 15.4 mins).

17: HR-ESI-MS: *m*/*z* = 503.2623 $[M+Na]^+$ (503.2615 calcd for $C_{26}H_{40}O_8Na$). ^{1}H-NMR, δ_H (500 MHz, CD_3OD), 5.76 (1H, br s, H-2), 5.53 (1H, m, H-10), 5.45 (1H, m, H-11), 4.39 (1H, br d, 15.3, H-16), 4.33 (1H, br d, 15.3, H-16), 4.30 (1H, d, 9.3 H-5), 4.07 (2H, t, 6.5, H-9'), 3.60 (1H, m, H-13), 3.04 (1H, dd, 14.6 and 6.4, H-9), 2.92 (1H, dd, 14.6 and 6.4, H-9), 2.81 (1H, dd, 15.2 and 3.4, H-4), 2.45 (1H, dd, 9.6 and 15.2, H-4), 2.24 (2H, t, 6.7, H-2'), 2.17 (3H, s, H-15), 2.15 (1H, m, H-12), 1.64 (2H, m, H-8'), 1.61 (2H, m, H-3'), 1.26-1.39 (8H, m, H-4', H-5', H-6' and H-7'), 1.09 (3H, d, 6.7, H-14), 0.99 (3H,d, 6.5, H-17); δ_C (observed via 2D nmr), 191.8 (C, C-6), 176.4 (C, C-1'), 166.9 (C, C-1), 156.5 (C, C-3), 135.5 (CH, C-11), 145.5 (C, C-7), 131.2 (C, C-8), 125.1 (CH, C-10), 117.6 (CH, C-2), 78.3 (CH, C-5), 70.9 (CH, C-13), 63.6 (CH2, C-9'), 63.1 (CH2, C-16), 43.9 (CH2, C-12), 40.6 (CH2, C-4), 33.9 (CH2, C-2',), 28.9 (CH2, C-4', C-5', C-6' or C-7'), 28.8 (CH2, C-4', C-5', C-6' or C-7'), 28.7 (CH2, C-4', C-5', C-6' or C-7'), 28.3 (CH2, C-8'), 25.6 (CH2, C-4', C-5', C-6' or C-7'), 24.7 (CH2, C-3'), 19.2 (CH3, C-14), 17.6 (CH3, C-15), 15.4 (CH3, C-17).

P. fluorescens* NCIMB 10586 Δ*mupF

A 1.2 L scale fermentation of Δ*mupF P. fluorescens* NCIMB 10586was carried out as per general procedure. The crude extract was subjected to HPLC chromatography on a Phenomenex Luna column (250 x 10 mm, 5µm, 40 ºC, flow 5 mL/min) eluted with isocratic wash of 45 % MeOH in water for 1 minute followed by a gradient of 45% to 85% over 20 minutes to yield 8 fractions. The fraction 1 was a mixture of **6** and **10** confirmed by LC-MS analysis. The fraction 2 was sent for ^{1}H-NMR and the structure was elucidated as **2** (PA-B). The fraction 4 (Rt: 14.7 mins) was further subjected to HPLC chromatography on a Chiralcel OD-H column eluted with isocratic wash of 70 % MeOH in water for 1 minute followed by a gradient of 70% to 90% over 15 minutes to yield **18** (a mixture of **18a** and **18b**=1: 1, 10.1 mg, Rt: 11.2 mins) and **19** (9.8 mg, Rt: 13.1 mins). The fration 5 (Rt: 16.1 mins) was further subjected to HPLC chromatography on a Chiralcel OD-H column eluted with isocratic wash of 70 % MeOH in water for 1 minute followed by a gradient of 70% to 90% over 15 minutes to yield **9** (1.8 mg, Rt: 15.2 mins) and **11** (0.8 mg, Rt: 11.4 mins). The fraction 6 (Rt: 17.7 mins, 9.8 mg) was the same compound as **15** confirmed by ^{1}H-NMR. The fraction 7 (1.8 mg) was directly prepared for 1D NMR and the structure was the same as **12**.

P. fluorescens* NCIMB 10586 *mmpE*Δ*OR/*Δ*mupF

A 2.4 L scale fermentation of Δ*mmpE/*Δ*mupF P. fluorescens* was carried out as per general procedure. The crude extract was subjected to HPLC chromatography on a Phenomenex Luna column (250 x 4.6 mm, 5µm, 40 ºC, flow 1.5 mL/min) eluted with isocratic wash of 40 % MeOH in water for 1 minute followed by a gradient of 40% to 95% over 25 minutes to yield 7 fractions. The Fraction 1 (Rt: 14.1 mins), fraction 2 (Rt: 17.0 mins) and fraction 3 (Rt: 17.8 mins) were **10**, **9** and **11**, respectively, analyzed by LC-MS. Fraction 5 (Rt: 19.8 mins), fraction 6 (Rt: 23.6 mins), and fraction 7 (1.5 mg, Rt: 24.2 mins) was further sent for ^{1}H-NMR and Fraction 5 was **17**, while fraction 6 and fraction 7 are un known aromatic derivatives. Fraction 4 (Rt: 18.7 mins) was further subjected to HPLC chromatography on a Chiralcel OD-H column eluted with isocratic wash of 70 % MeOH in water for 1 minute followed by a gradient of 70% to 94% over 12 minutes to yield compound **20** (3.1 mg, Rt: 9.7 mins).

20: HR-ESI-MS: *m*/*z* = 505.2780 $[M+Na]^{+}$ (505.2771 calcd for $C_{26}H_{42}O_8Na$). ^{1}H-NMR, δ_H (500 MHz, CD3OD), 5.78 (1H, br s, H-2), 5.51 (1H, dd, 15.4 and 7.9, H-11), 5.35 (1H, ddt, 14.4, 7.1 and 1.1, H-10), 4.07 (2H, t, 9.3 H-5), 4.03 (1H, br d, 12.0, H-16), 4.00 (1H, d, 9.8, H-6), 3.67 (1H, dd, 12.5 and 2.8, H-16), 3.59 (1H, m, H-13), 3.47 (1H, dt, 9.4 and 2.6, H-5), 2.75 (1H, br d, 15.5, H-4), 2.51 (1H, m, H-8), 2.41-2.49 (3H, m, H-4 and H-9), 2.27 (2H, t, 6.6, H-2'), 2.20 (3H, d, 1.3, H-15), 2.13 (1H, m, H-12), 1.64 (2H, m, H-8'), 1.59 (2H, m, H-3'), 1.32-1.40 (8H, m,

H-4', H-5', H-6' and H-7'), 1.08 (3H, d, 6.7, H-14), 0.96 (3H, d, 6.5, H-17); δ_C (125 MHz, CD_3OD), 209.4 (C, C-7), 176.6 (C, C-1'), 167.1 (C, C-1), 156.6 (C, C-3), 135.8 (CH, C-11), 126.2 (CH, C-10), 117.5 (CH, C-2), 82.2 (CH, C-5), 75.0 (CH, C-6), 70.6 (CH, C-13), 69.6 (CH2, C-16), 63.5 (CH2, C-9'), 52.2 (CH, C-8), 43.9 (CH2, C-12), 43.3 (CH2, C-4), 33.6 (CH2, C-2',), 33.1 (CH2, C-9), 28.3 (CH2, C-8'), 24.6 (CH2, C-3'), 28.9 (CH2, C-4', C-5', C-6' or C-7'), 28.8 (CH2, C-4', C-5', C-6' or C-7'), 28.7 (CH2, C-4', C-5', C-6' or C-7'), 28.3 (CH2, C-8'), 25.6 (CH2, C-4', C-5', C-6' or C-7'), 18.8 (CH3, C-14), 17.9 (CH3, C-15), 15.2 (CH3, C-17).

General isolation and fermentation procedures for Δ*KR6*+PJH2 and Δ*mupO/W/I+I*

Mutants of *Pseudomonas fluorescens* NCIMB 10586 were grown at 25 ºC on L agar (L broth with 1.5% agar) for 24 hours. Single colonies were used to inoculate in L broth (50 ml in a 250 ml conical flask) supplemented with carbonicillin (100 μg/ml) and cultured at 25 ºC for overnight to prepare seed culture. 1000 ml secondary stage medium (20 g soya flour, 6.25 g $CaCO_3$, 5.0 g $(NH_4)_2SO_4$, 2.5 g spray dried corn liquor, 1.5 g KH_2PO_4, 1.0 g Na_2HPO_4, 1.0 g KCl, 0.5 g $MgSO_4.7H_2O$ and 40 g glucose) in 10 × 500 ml conical flasks, was inoculated with 5% seed culture and grown at 22 ºC, 250 rpm for 50 hours. Cells were removed by centrifugation at 7500 rpm for 30 minutes. The supernatant was acidified to pH 4.5 by diluted HCl and extracted by ethyl acetate (0.6 v/v) twice. After the ethyl acetate had been removed by rotary evaporation, the residue was subjected to gel filtration chromatography on Sephadex LH-20 eluted with MeOH. Fractions were analyzed by NMR and mupirocin derivatives were found in the earlier fractions. Further purification was carried on by gradient flash chromatography on normal-phase silica gel and eluted by MeOH in $CHCl_3$ from (0:100 to 15:85) or by gradient HPLC (CH_3CN in H_2O) on a reverse phase column (Phenomenex Luna 5 μ C_{18} 250 × 4.60 mm) and the peaks were monitored by UV detector with λ = 222 and 233 nm. Gradient program (CH_3CN in H_2O): 5% (0-5 minutes), 5%-75% (5-35 minutes), 75%-95% (35-36 minutes), 95% (36- 40 minutes), 95%-5% (40-41 minutes), 5% (41-45 minutes). L broth (pH: 7.0): 0.1% glucose, 0.5% NaCl, 0.5% yeast extract and 1% tryptone.

***P. fluorescens* NCIMB 10586 Δ*KR6*+PJH2**

2 litres culture of KR6+pJH2 mutant was prepared as described in general experimental part. About 250 mg residue was subjected to Sephadex column (25 × 1.5 cm) and colourful fractions were collected by 7 ml vials. Fraction 3 from gel filtration was selected for further purification. Totally 45 mg residue was purified by HPLC and 1.2 mg **21** (Rt: 32.1 mins) was isolated together with <1.0 mg **22** (Rt: 28.0 mins).

18: $[\alpha]_D$ +16 ($CHCl_3$, *c* 2.5), HR-ESI-MS: *m*/*z* = 507.2922 $[M+Na]^+$ (507.2928 calcd for $C_{26}H_{44}O_8Na$). δ_H (CD_3OD, 400 MHz), 5.76 (1H, d, *J* 1.0, 2-H), 5.41 (2H, m, 10-H and 11-H), 4.10 (1H, d, *J* 5.9, 6-H), 4.08 (2H, t, *J* 6.6, 9'-H_2), 4.00 (1H, ddd *J* 9.5, 5.9, 3.4), 3.59 (1H, m, 13-H), 3.08 (1H, m, 8-H), 2.45 (1H, m, 9-*H*H), 2.40 (1H, m, 4-*H*H), 2.26 (2H, t, *J* 7.6, 2'-H_2), 2.26 (1H, m, 4-H*H*), 2.16 (3H, d, *J* 1.0, 15-H_3), 2.11 (1H, m 12-H), 2.06 (1H, m, 9-H*H*), 1.63 (4H, m, 3'-H_2 and 8'-H_2), 1.36 (8H, m, 4', 5', 6'and 7'-H_2), 1.09 (3H, d, *J* 6.3, 14-H_3), 1.06 (3H, d, *J* 7.1, 16-H_3), 0.98 (3H, d, *J* 6.9, 17-H_3); δ_C (CD_3OD, 100 MHz), 216.5 (C-7), 177.2 (C-1'), 167.7 (C-1), 157.8 (C-3), 135.2 (C-11), 128.4 (C-10), 118.3 (C-2), 79.3 (C-6), 71.4 (C-5), 71.2 (C-13), 64.2 (C-9'), 44.5 (C-12), 44.4 (C-8), 43.5 (C-4), 36.1 (C-9), 34.3 (C-2'), 29.6/29.54 (C-5'/6'), 29.48 (C-4'), 29.1 (C-8'), 26.4 (C-7'), 25.4 (C-3'), 19.7 (C-14), 18.5 (C-15), 15.9 (C-16), 15.6 (C-17).

19: HR-ESI-MS: *m*/*z* = 479.2608 $[M+Na]^+$ (479.2615 calcd for $C_{24}H_{40}O_8Na$). δ_H (CD_3OD, 400 MHz), 5.76 (1H, d, *J* 1.0, 2-H), 5.41 (2H, m, 10-H and 11-H), 4.10 (1H, d, *J* 5.9, 6-H), 4.08 (2H, t, *J* 6.6, 9'-H_2), 4.00 (1H, ddd *J* 9.5, 5.9, 3.4), 3.59 (1H, m, 13-H), 3.08 (1H, m, 8-H), 2.45 (1H, m, 9-HH), 2.40 (1H, m, 4-HH), 2.26 (2H, t, *J* 7.6, 2'-H_2), 2.26 (1H, m, 4-HH), 2.16 (3H, d, *J* 1.0, 15-H_3)2.11 (1H, m 12-H), 2.06 (1H, m, 9-HH), 1.63 (4H, m, 3'-H_2 and 8'-H_2), 1.36 (4H, m, 4', 5', 6'and 7'-H_2), 1.09 (3H, d, *J* 6.3, 14-H_3), 1.06 (3H, d, *J* 7.1, 16-H_3), 0.98 (3H, d, *J* 6.9, 17-H_3).

P. fluorescens* NCIMB Δ*mupO/W/I+I

1 litre culture of mupO/W/I+I mutant was prepared as described in general experimental part. About 150 mg residue was subjected to Sephadex column (25 × 1.5 cm) and colourful fractions were collected by 7 ml vials. Fraction 3 from gel filtration was selected for further purification. Totally 30 mg residue was purified by HPLC and 2.0 mg **5** (Rt: 27.2 mins), 2.2 mg **6** (Rt: 24.5 mins), <0.5 mg **7** (Rt: 21.1 mins), and <1.0 mg **8** (Rt: 17.5 mins) were isolated.

5: HR-ESI-MS: *m*/*z* = 497.2721 $[M+Na]^+$ (497.2721calcd for $C_{24}H_{42}O_9Na$). ν_{max}/cm^{-1} 3379, 2936, 1706, 1647, 1360, 1142, 1027, 922. δ_H (400 MHz, methanol-d_4) 0.84 (3H, d, *J* 7.1, 17-H_3), 1.11 (3H, d, *J* 6.4, 14-H_3), 1.17 (3H, d, *J* 6.4, 16-H_3), 1.37 (2H, m, 5'-H_2), 1.39 (2H, m, 4'-H_2), 1.56 (1H, m, 12-H), 1.58 (1H, m, 8-H), 1.60 (2H, m, 3'-H_2), 1.65 (2H, m, 6'-H_2), 2.06 (1H, ddd, *J* 12.0, 7.1, 5.6, 9-H*H*), 2.20 (3H, d, *J* 1.2, 15-H_3), 2.24 (1H, dd, *J* 14.2, 10.3, 4-H*H*), 2.29 (2H, t, *J* 7.3, 2'-H_2), 2.33 (1H, m, 9-*H*H), 2.60 (1H, bd, *J* 14.2, 4-*H*H), 3.50 (1H, dd, *J* 6.1, 6.1, 6-H), 3.62 (1H, dd, *J* 6.8, 6.1, 7-H), 3.63 (1H, dd, *J* 6.4, 4.4, 11-H), 3.87 (1H, ddd, *J* 10.3, 6.1, 2.7, 5-H), 3.98 (1H, qd, *J* 6.4, 6.4, 13-H), 4.07 (2H, t, *J* 6.6, 7'-H), 4.10 (1H, dd, *J* 5.6, 4.4, 10-H), 5.77 (1H, bs, 2-H); δ_C (100 MHz, CD_3OD) 11.3 (C-17), 19.0 (C-15), 19.3 (C-14), 19.3

(C-16), 26.1 (C-3'), 26.9 (C-5'), 29.8 (C-6'), 29.9 (C-4'), 34.9 (C-2'), 36.7 (C-9), 38.6 (C-8), 44.4 (C-12), 45.0 (C-4), 64.8 (C-7'), 70.4 (C-13), 72.2 (C-5), 76.0 (C-11), 78.1 (C-6), 81.5 (C-10), 87.3 (C-7), 118.6 (C-2), 159.6 (C-3), 168.5 (C-1), 177.7 (C-1').

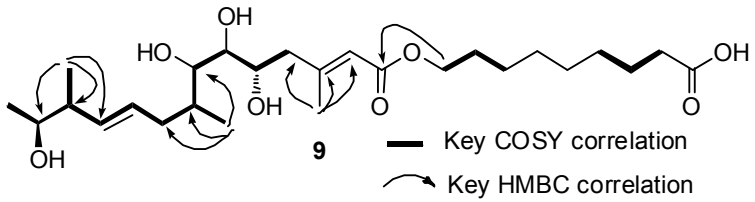
6: HR-ESI-MS: *m*/*z* = 469.2425 $[M+Na]^+$ (469.2408 calcd for $C_{22}H_{38}O_9Na$). δ_H (400 MHz, CD_3OD) 0.85 (3H, d, *J* 7.1, 17-H_3), 1.12 (3H, d, *J* 6.4, 14-H_3), 1.18 (3H, d, *J* 6.6, 16-H_3), 1.30-1.35 (CH_2), 1.56 - 1.65 (CH_2), 2.06 (1H, 9-H*H*), 2.20 (3H, 15-H_3), 2.20 (2H, 2'-H_2), 2.60 (1H, bd, *J* 14.9, 4-*H*H), 3.51 (1H, 6-H), 3.62 - 3.65 (CH), 3.87 (1H, 5-H), 4.00 (1H, qd, *J* 6.4, 6.4, 13-H), 4.10 (2H, 7'-H), 4.10 (1H, 10-H), 5.78 (1H, bs, 2-H).

7: HR-ESI-MS: *m*/*z* = 369.1886 $[M+Na]^+$ (369.1884 calcd for $C_{17}H_{30}O_7Na$). δ_H (400 MHz, CD_3OD) 0.85 (3H, d, *J* 7.1, 17-H_3), 1.12 (3H, d, *J* 6.4, 14-H_3), 1.18 (3H, d, *J* 6.4, 16-H_3), 1.56 (1H, m, 12-H), 1.58 (1H, m, 8-H), 2.03 (3H, d, *J* 1.2, 15-H_3), 2.06 (1H, 9-H*H*), 2.12 (1H, dd, *J* 13.2, 9.5, 4-H*H*), 2.33 (1H, m, 9-*H*H), 2.54 (1H, bd, *J* 13.2, 4-*H*H), 3.53 (1H, 6-H), 3.65 (1H, dd, *J* 7.3, 4.1, 7-H), 3.68 (1H, dd, *J* 7.8, 5.4, 11-H), 3.85 (1H, ddd, *J* 9.7, 6.1, 2.8, 5-H), 4.00 (1H, qd, *J* 6.4, 6.4, 13-H), 4.10 (1H, ddd, *J* 9.8, 5.4, 4.4, 10-H), 5.78 (1H, bs, 2-H)

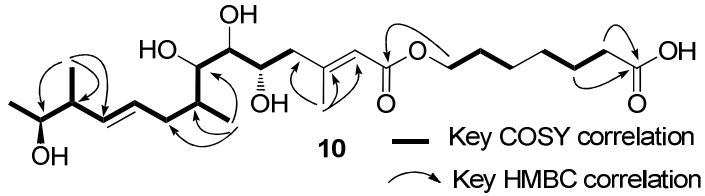
General experimental procedures for small scale feedings

P. fluorescens mutant strains were incubated on an L-agar plate at 30 ºC for 24 hours. Using a toothpick, 25 ml of a modified L-medium in a 100 ml flask was inoculated with as much bacteria as possible and grown at 22 ºC, 200 rpm for 60 hours. The compounds isolated from mutant strains were added to the flasks immediately after inoculation. The cells were separated from the medium by centrifugation (9000 rpm for 10 mins). The medium was extracted with EtOAc (0.5 v/v) 2 times. The crude extract obtained from the medium was filtered with cotton and then analyzed by LCMS and HPLC (HPLC column: Phenomenex Luna column, 250 x 3.0 mm, 5µm, 40 ºC, and flow: 0.7 mL/min).

Structural elucidation of new pseudomonic acids isolated from Wild Type and mutants

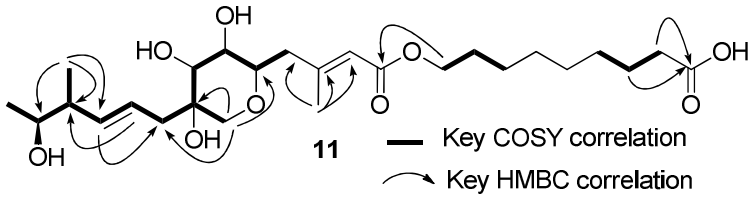


Compound **9** was obtained as colorless oil. The molecular formula was determined as $C_{26}H_{44}O_9$ by positive HRESIMS data. The general features of its ^{1}H and ^{13}C NMR data closely resembled those of PA-C (**3**), a pesudomonic acid derivative with a 10,11-alkene. However the oxygenated 16-methlene signal for **3** disappeared in the ^{1}H spectrum of **8**. Instead an additional methyl signal at δ_H 0.97 in ^{1}H spectrum was observed. The evidences indicated that the tetrahydropyran ring in **3** opened at C-16 in the structure of **8**. The observed ^{1}H–^{1}H COSY correlations from CH_3-16 to H-8 as well as the ^3J-HMBC correlations from CH_3-16 to C-7 and C-9 confirmed the above deduction. Thus the structure of **9** was determined and named as mupirocin W4.

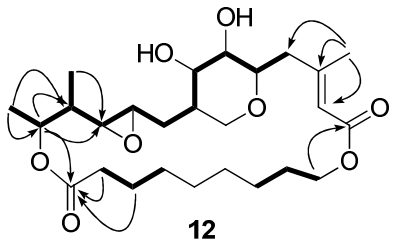


Compound **10** was obtained as colorless oil. The structure elucidation of **10** was straightforward due to its close relationship with **9**. Compound **10** was assigned the molecular formula $C_{24}H_{42}O_8$, having 28 units less than **9**, on the basis of positive HRESIMS. The ^{1}H NMR data of monic acid unit for **10** was identical to that of **9**. All the obove eviedences implied that **10** was the 7-hydroxyheptanoic acid (7-HN) homologue of **9**. The ^{1}H and 2D NMR data of confirmed that a 7-hydroxyheptanoic acid existing in the structure. Thus compound **10** was

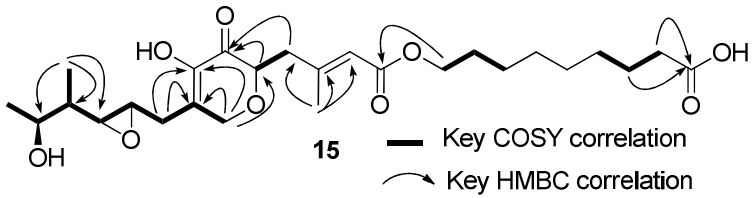
assigned and named as mupirocin W5.



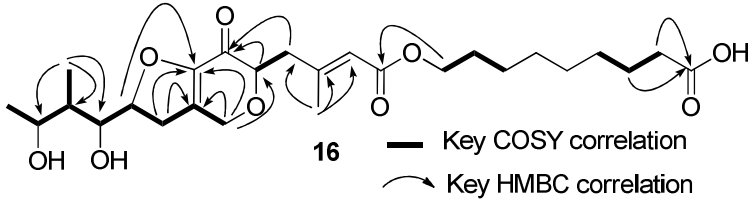
The molecular formula of **11** was determined to be $C_{26}H_{44}O_9$, two units less than PA-B (**2**), based on the positive HRESIMS data. The only structural difference between **2** and **11** observed is with regard to the 10,11-epoxide in **2**, which was replaced by a 10,11-alkene in **11**. The ^{1}H–^{1}H COSY correlations from the olfenic signal H-10 to H-11 and H-9 and from the olfenic signal H-11 to H-10 and H-12 confirmed the deduction. The structure of **11** was thus assigned and named as desepoxy-PA-B.



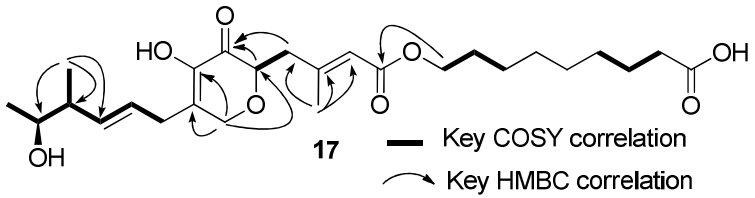
Compound **12** was obtained as colorless oil. Its molecular formula was determined as $C_{26}H_{42}O_9$, having 18 units less than PA-B, based on HRESIMS. The molecular formula as well as the HMBC correlation from H-13 to C-1’ revealed that **12** was the marocyclic lactone formed by dehydration betweenC-13 and C-1’ of PA-B. Thus **12** was assigned as PA-B macrolactone.



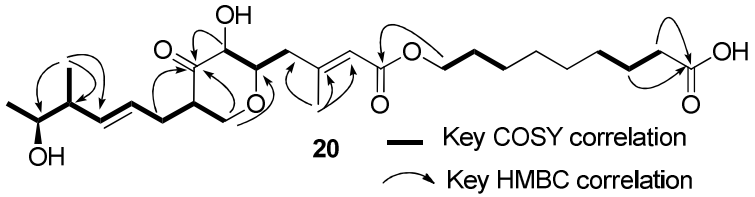
Compound **15** was isolated as colorless oil compounds, possessing a molecular formula of $C_{26}H_{40}O_9$, 4 units less than PA-A. Detailed analysis of NMR data of **15** peak revealed that its structure are very similar to PA-A, except two additional ketonic groups were present in ^{13}C NMR spectrum. 2D NMR indicated that these two ketones were located at C-6 and C-7, respectively. So this structure was assigned as metabolite mupirocin C1.



Compound **16** was isolated as colorless oil compounds, possessing a same molecular formula of $C_{26}H_{40}O_9$ as **16**, based on HRESIMS data. Further examination of 1D and 2D NMR data of **16** revealed that the most striking differences compared with **15** were the lack of 10,11-epoxide resonances. Instead two additional oxygenated methine were observed from ^{13}C NMR and ^{1}H NMR. The ^{1}H–^{1}H COSY correlations from the two oxygenated methane protons to H-12 and H-9, respectively, indicated the two oxygenated methine carbons were located at C-10 and C-11, respectively, while the HMBC correlation from H-10 to C-7 indicated the existence of a furan ring. Thus the structure of **16** was assigned and named as mupirocin C2.



Compound **17** was obtained as colorless oil. Its molecular formula was determined as $C_{26}H_{40}O_8$, two units less than mupirocin C1 (**15**), based on HRESIMS. Its ^{1}H NMR and ^{13}C NMR closely resembled those of mupirocin C1 (**15**), except that the 10,11-epoxide was replaced by a 10,11-alkene in **17**. This deduction was confirmed by the COSY correlations from H-10 to H-9 and from H-11 to H-12. Thus the structure of **17** was assigned as desepoxy-mupirocin C1.



Compound **20** was obtained as colorless oil. Its molecular formula was determined as $C_{26}H_{42}O_8$, two units less than mupirocin F1 (**18a**), based on HRESIMS. Its ^{1}H NMR and ^{13}C NMR were very simmlar to those of mupirocin F1 (**18a**), except the disappearance of 10, 11-epoxide in **20**, which was replaced by a 10,11-alkene. This deduction was confirmed by the COSY correlations from H-10 to H-9 and from H-11 to H-12. Thus the structure of 16 was assigned as desepoxy-mupirocin F1.

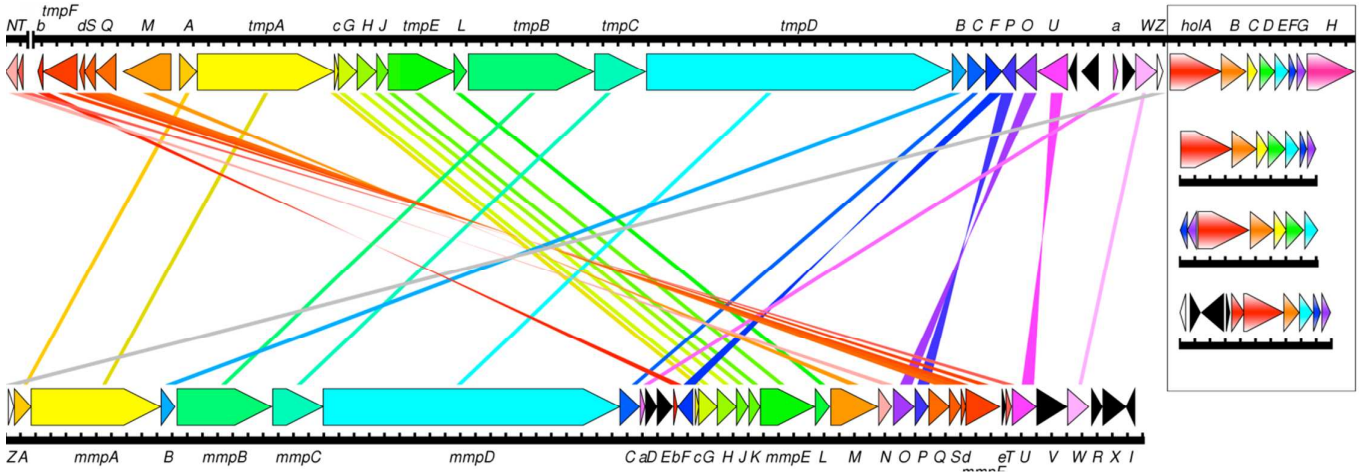


Figure S2. Comparison of the organisation of the thiomarinol gene cluster (upper line) with the mupirocin biosynthesis gene cluster from *Pseudomonas fluorescens* (lower line) and (boxed, on right) with related NRPS clusters. The HCS cassette comprises *mACPc*, *mupG,H, J* and *K*. In the thiomarinol cluster, *tmlK* is fused to *tmpE*.

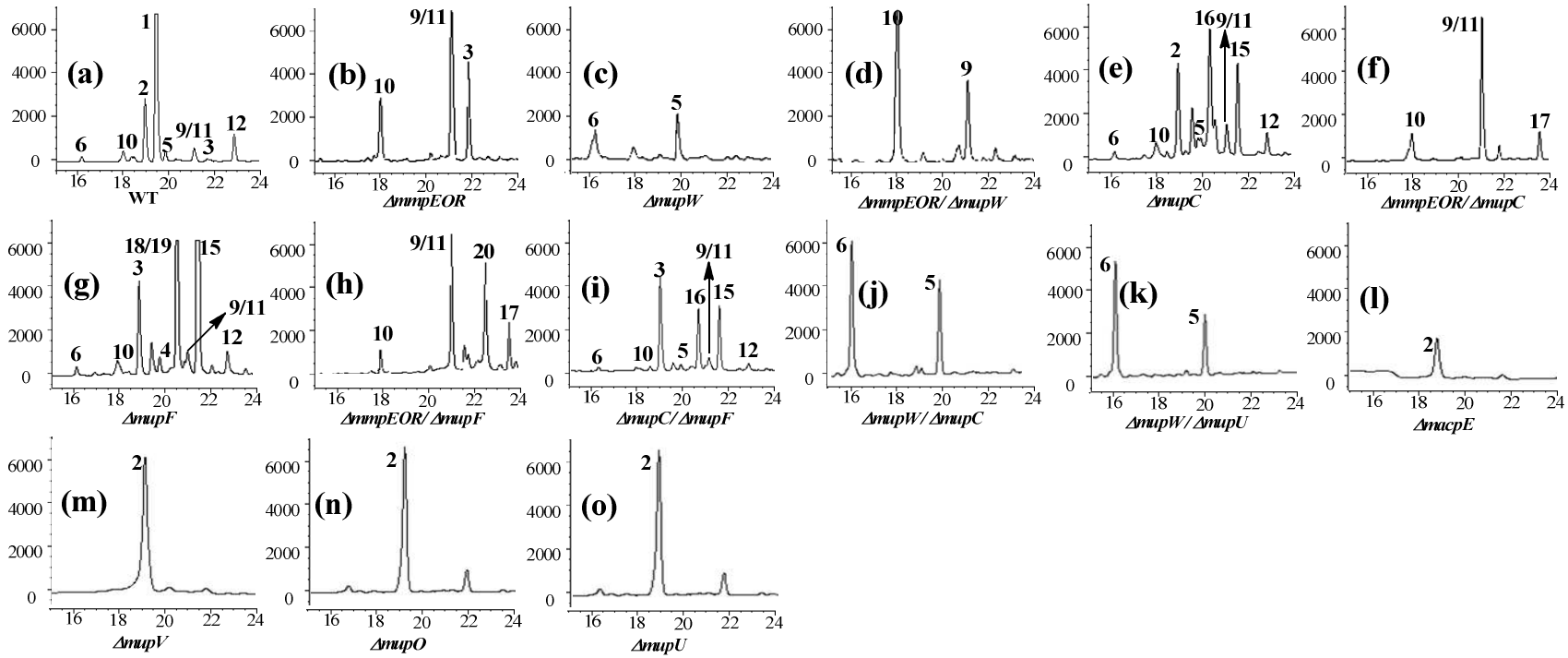


Figure S3. HPLC diode array traces of extracts from WT and mutant strains of *P. fluorescens* NCIMB 10586. The crude extract was subjected to HPLC chromatography on a Phenomenex Luna column (250 x 4.6 mm, 5µm, 40 ºC, flow 1.5 mL/min) eluted with isocratic wash of 40 % MeOH in water for 5 minute followed by a gradient of 40% to 95% over 25 minutes. (a) WT; (b) Δ*mmpEOR*; (c) Δ*mupW*; (d) Δ*mmpEOR* /Δ*mupW*; (e) Δ*mupC*; (f) Δ*mmpEOR* /Δ*mupC*; (g) Δ*mupF*; (h) Δ*mmpEOR* /Δ*mupF*; (i) Δ*mupC* /Δ*mupF*; (j) Δ*mupW* /Δ*mupC*; (k) Δ*mupW*/Δ*mupU*; (l) Δ*macpE*; (m) Δ*mupV*; (n) Δ*mupO*; (o) Δ*mupU*. **1**: PA-A; **2**: PA-B; **3**: PA-C; **5**: mupirocin W1; **6**: mupirocin W2; **9**: mupirocin W4; **10**: mupirocin W5; **11**: desepoxy-PA-B; **12**: PA-B macrolactone; **15**: mupirocin C1; **16**: mupirocin C2; **17**: desepoxy-mupirocin C1; **18**: mupirocin F1; **19**: mupirocin F2; **20**: desepoxy-mupirocin F1.

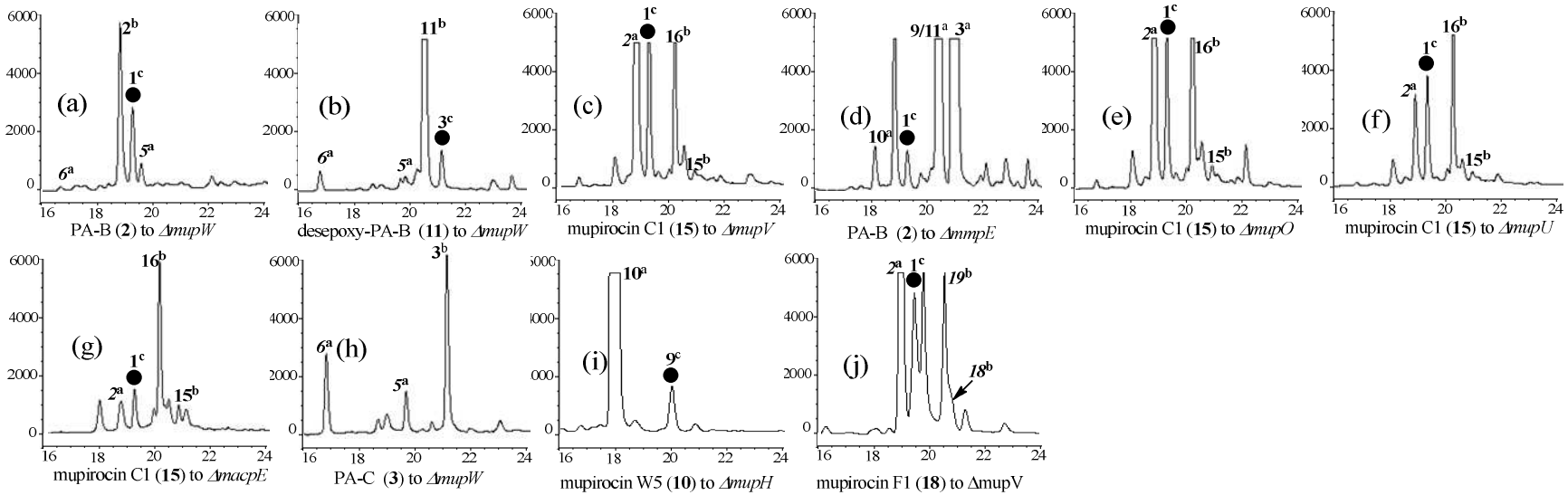
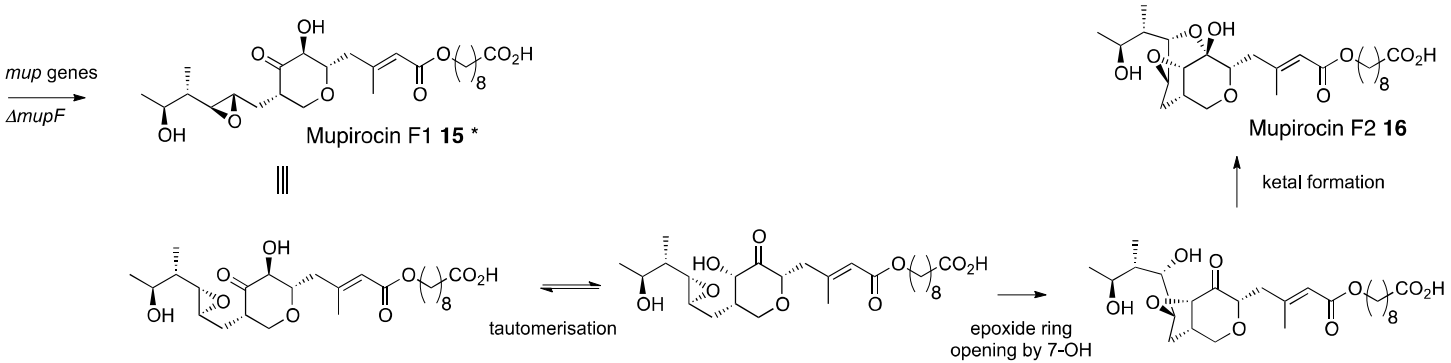


Figure S4. HPLC diode array traces of extracts from small scale feedings to *P. fluorescens* mutants. The crude extract was subjected to HPLC chromatography on a Phenomenex Luna column (250 x 3.2 mm, 5µm, 40 ºC, flow 0.7 mL/min) eluted with isocratic wash of 40 % MeOH in water for 4 minutes followed by a gradient of 40% to 100% over 20 minutes. (a) PA-B (**2**) fed to *ΔmupW*; (b) desepoxy-PA-B (**11**) fed to *ΔmupW*; (c) mupirocin C1 (**15**) fed to *ΔmupV*; (d) PA-B (**2**) fed to *ΔmmpE*; (e) mupirocin C1 (**15**) fed to *ΔmupO*; (f) mupirocin C1 (**15**) fed to *ΔmupU*; (g) mupirocin C1 (**15**) fed to *ΔmacpE*; (h) PA-C (**3**) fed to *ΔmupW*; (i) mupirocin W5 (**10**) fed to *ΔmupH*; (j) mupirocin F1 (**18**) fed to *ΔmupV*; [a]PAs produced by the mutants; [b]PA substrates or their rearrangement products; [c]PAs generated from incorporated intermediates(●).



Scheme S1. Epoxide mediated rearrangement of mupirocin F1 to mupirocin F2

Figure S5. ^{1}H NMR spectrum of pseudomonic acid A (PA-A, **1**) measured in CD_3OD at 500 MHz.

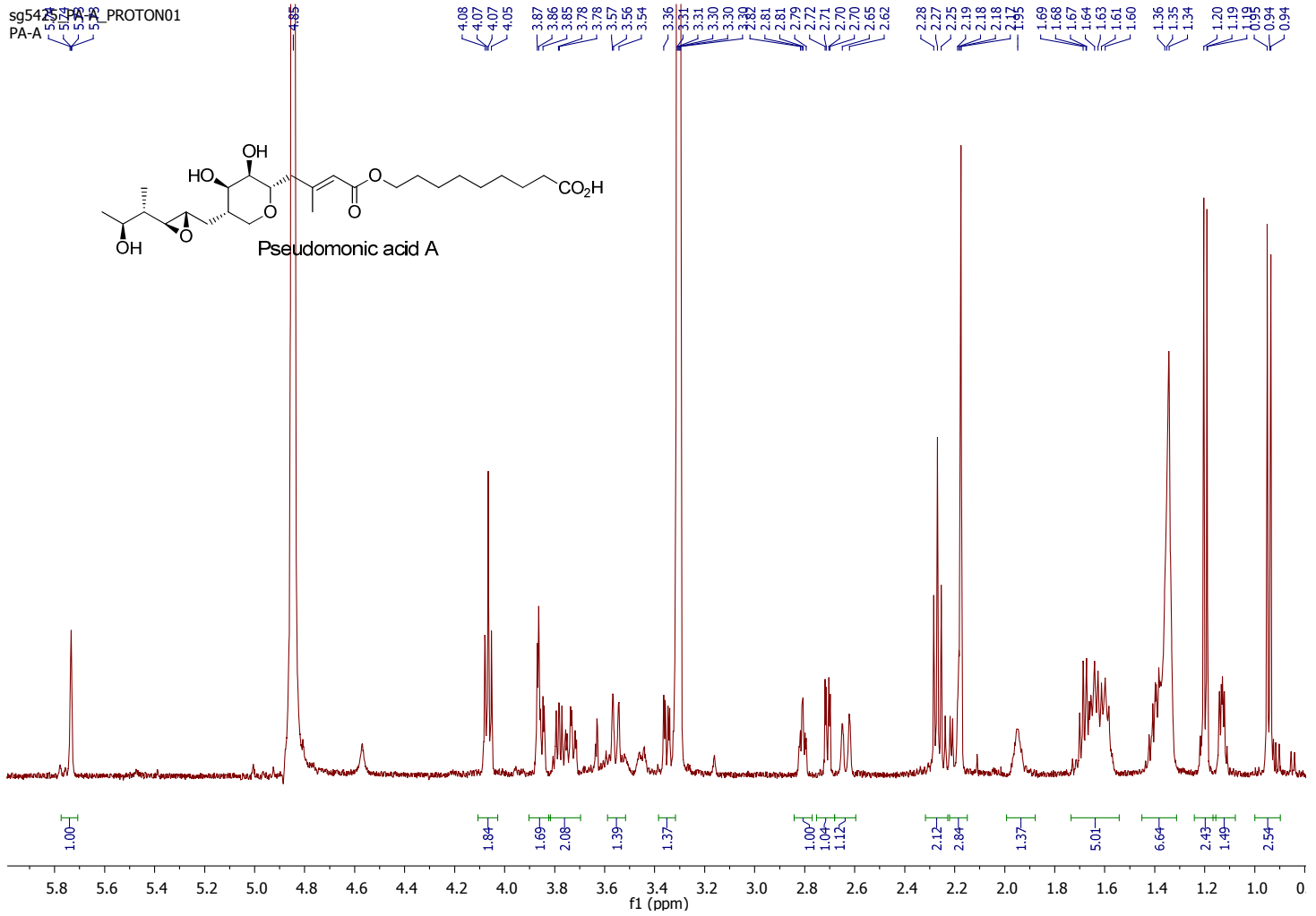


Figure S6. ^{1}H NMR spectrum of pseudomonic acid B (PA-B, **2**) measured in CD_3OD at 500 MHz.

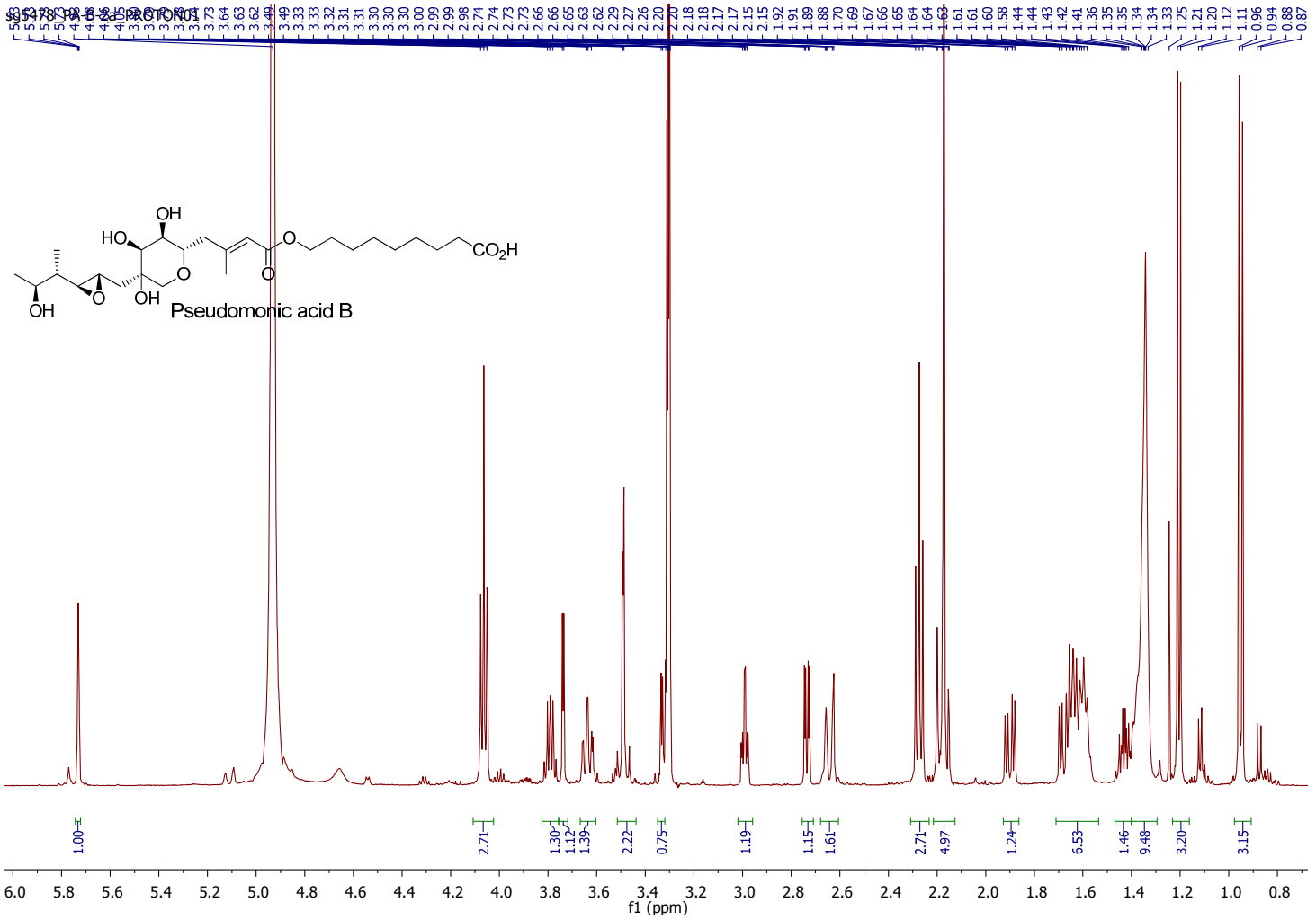


Figure S7. ^{1}H NMR spectrum of pseudomonic acid C (PA-C, **3**) measured in CD_3OD at 500 MHz.

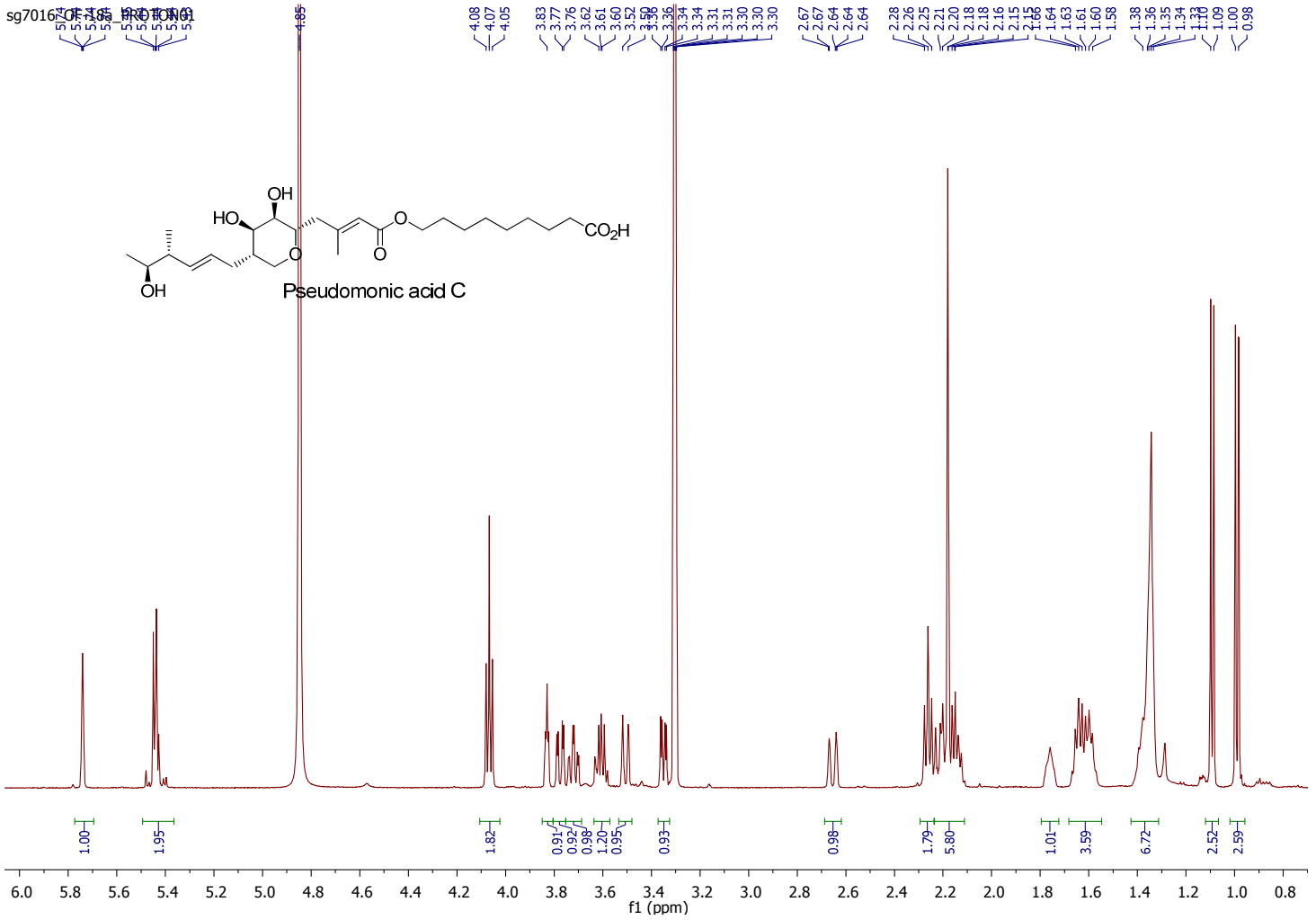


Figure S8. ^{1}H-^{1}H COSY spectrum of pseudomonic acid C (PA-C, **3**) measured in CD_3OD at 500 MHz.

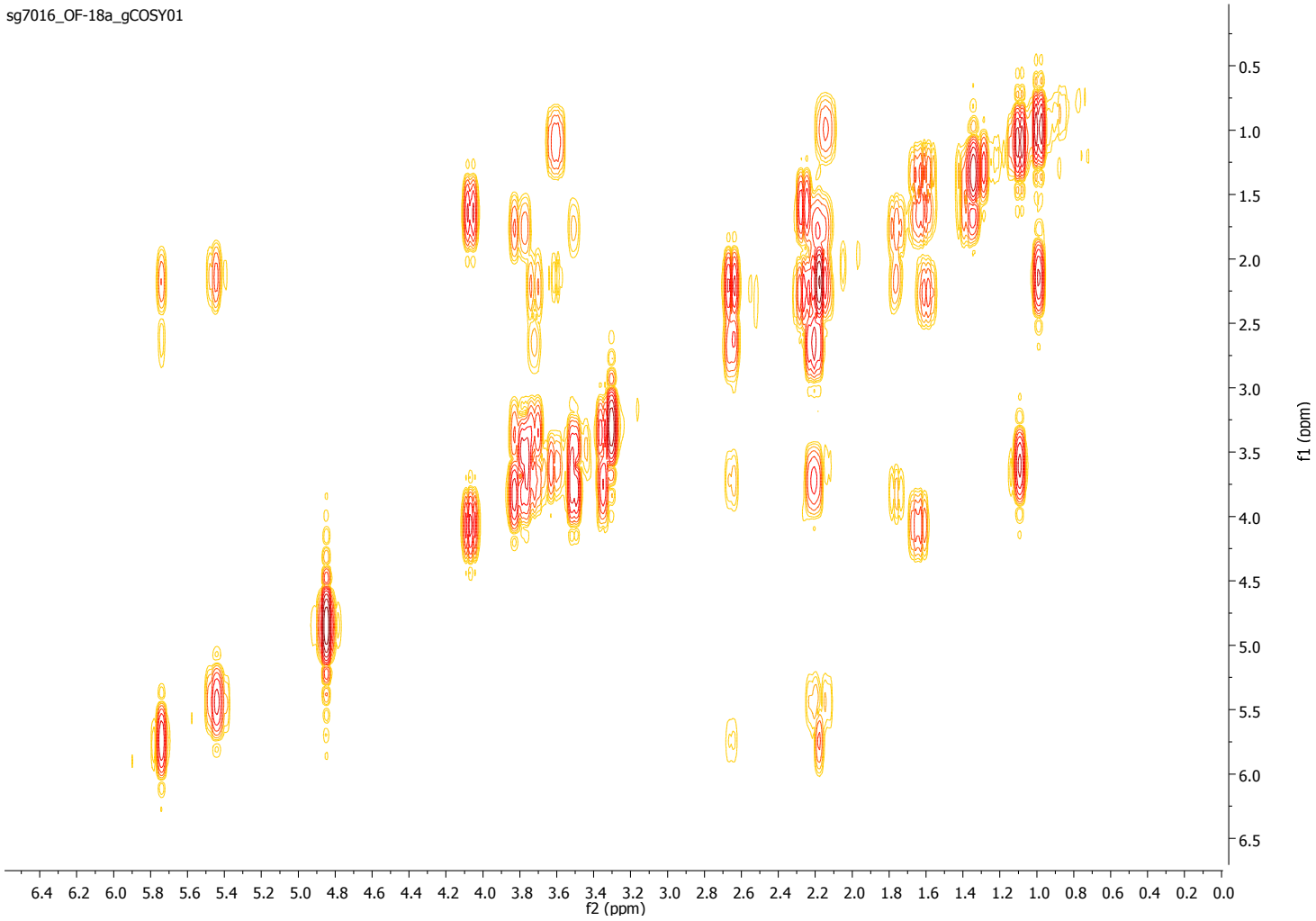


Figure S9. HSQC spectrum of pseudomonic acid C (PA-C, **3**) measured in CD_3OD at 500 MHz.

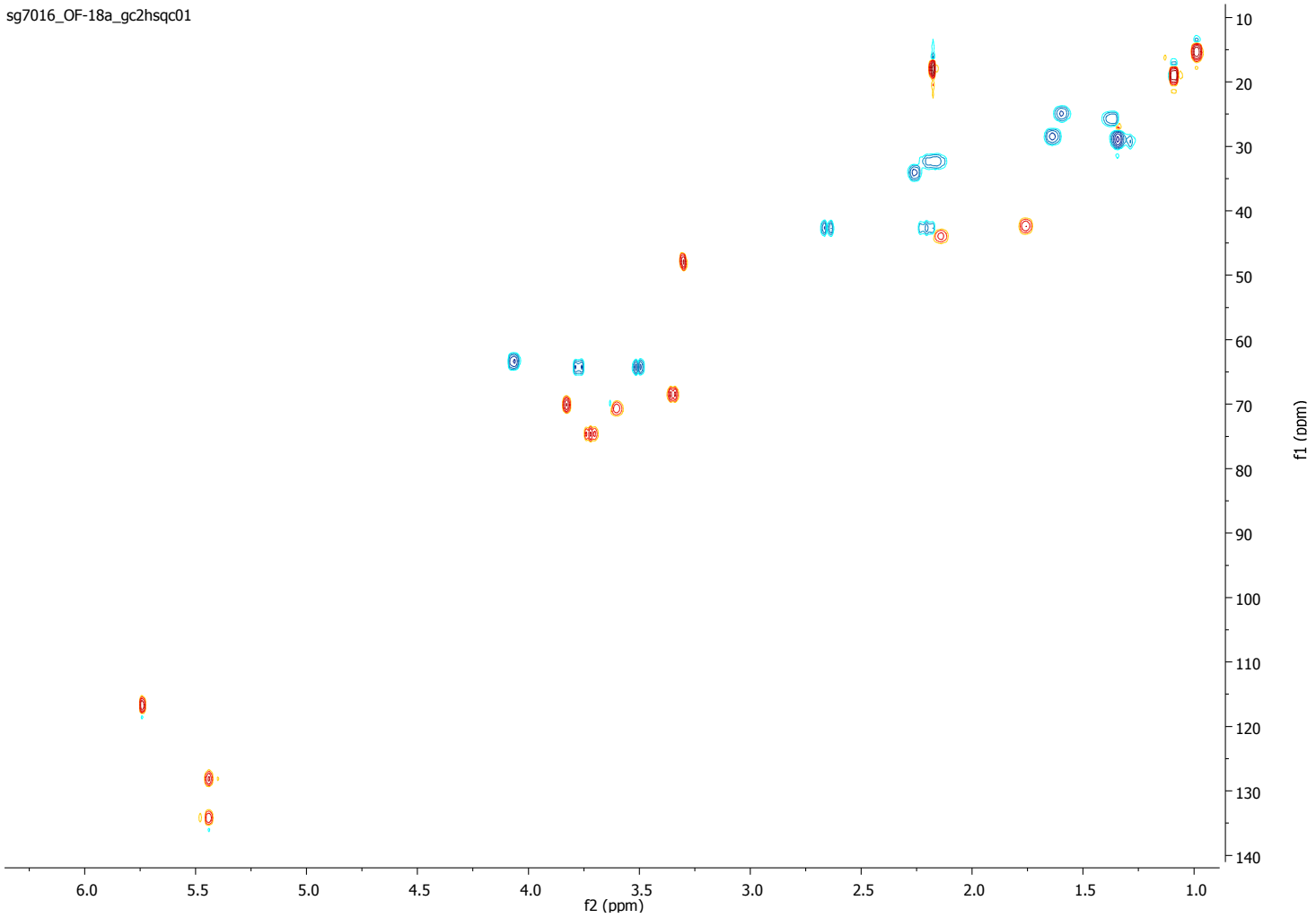


Figure S10. HMBC spectrum of pseudomonic acid C (PA-C, **3**) measured in CD_3OD at 500 MHz.

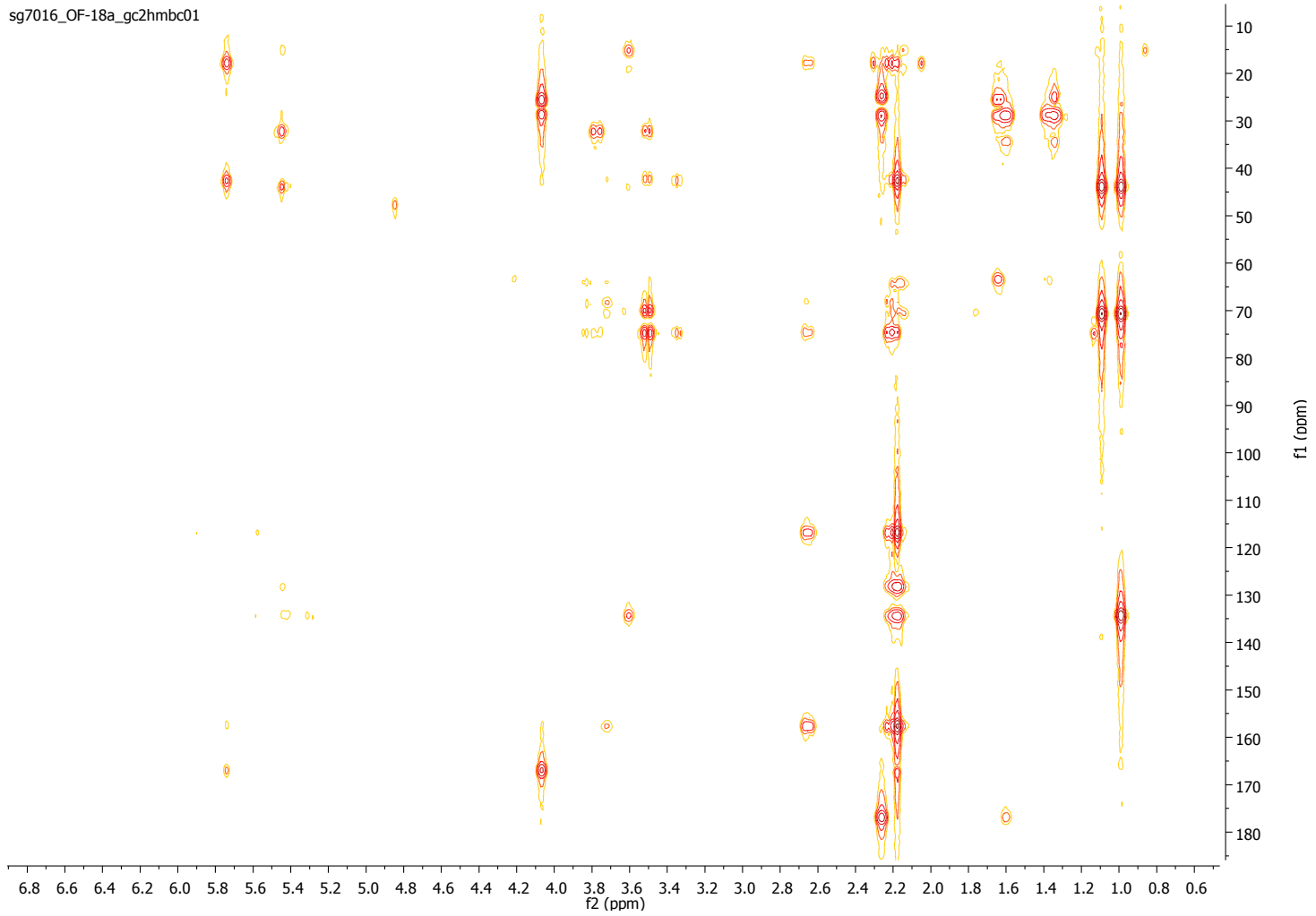


Figure S11. ^{1}H-NMR spectrum of mupirocin W1 (**5**) measured in CD_3OD at 500 MHz.

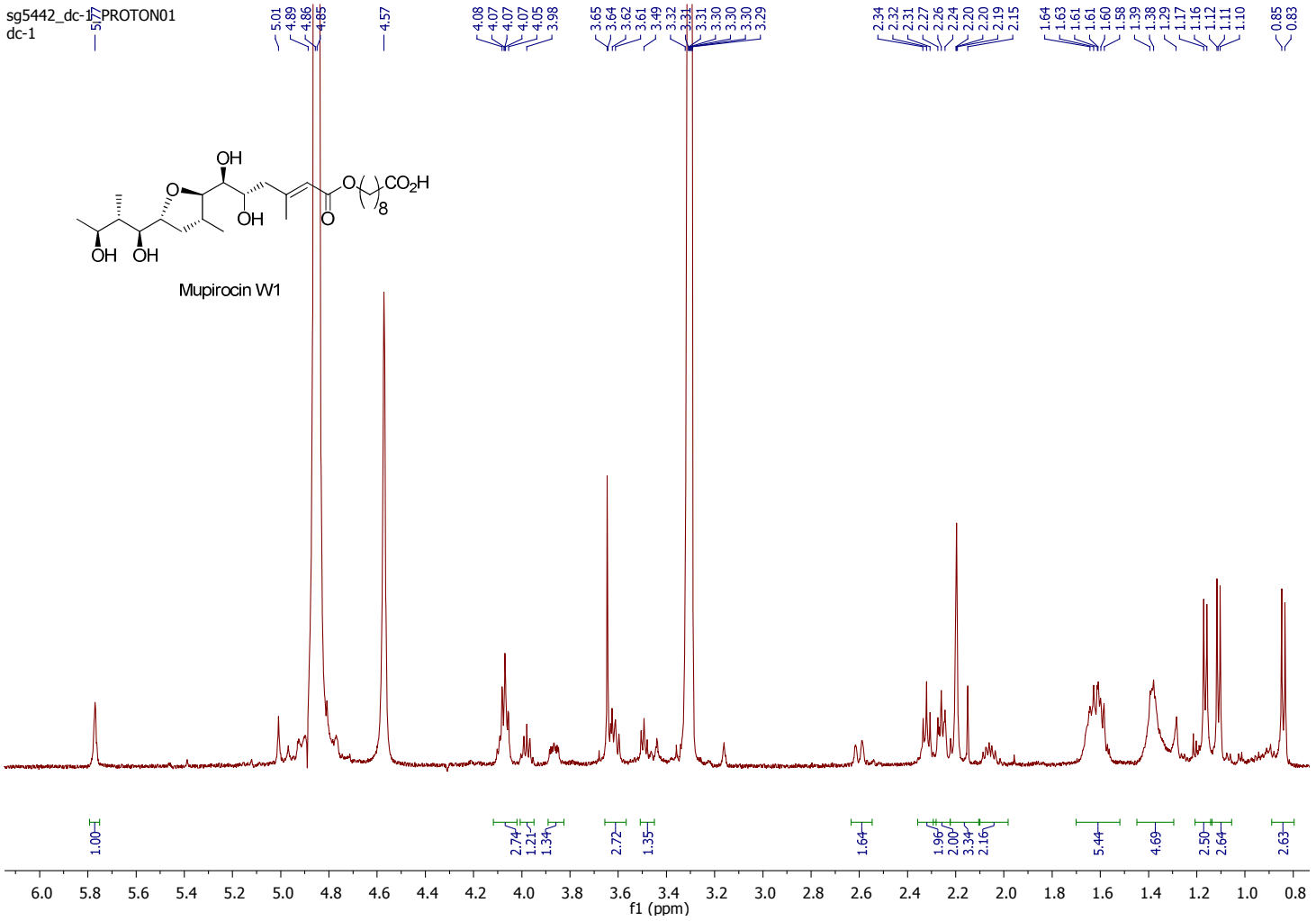


Figure S12. ^{1}H NMR spectrum of mupirocin W2 (**6**) measured in CD_3OD at 400 MHz.

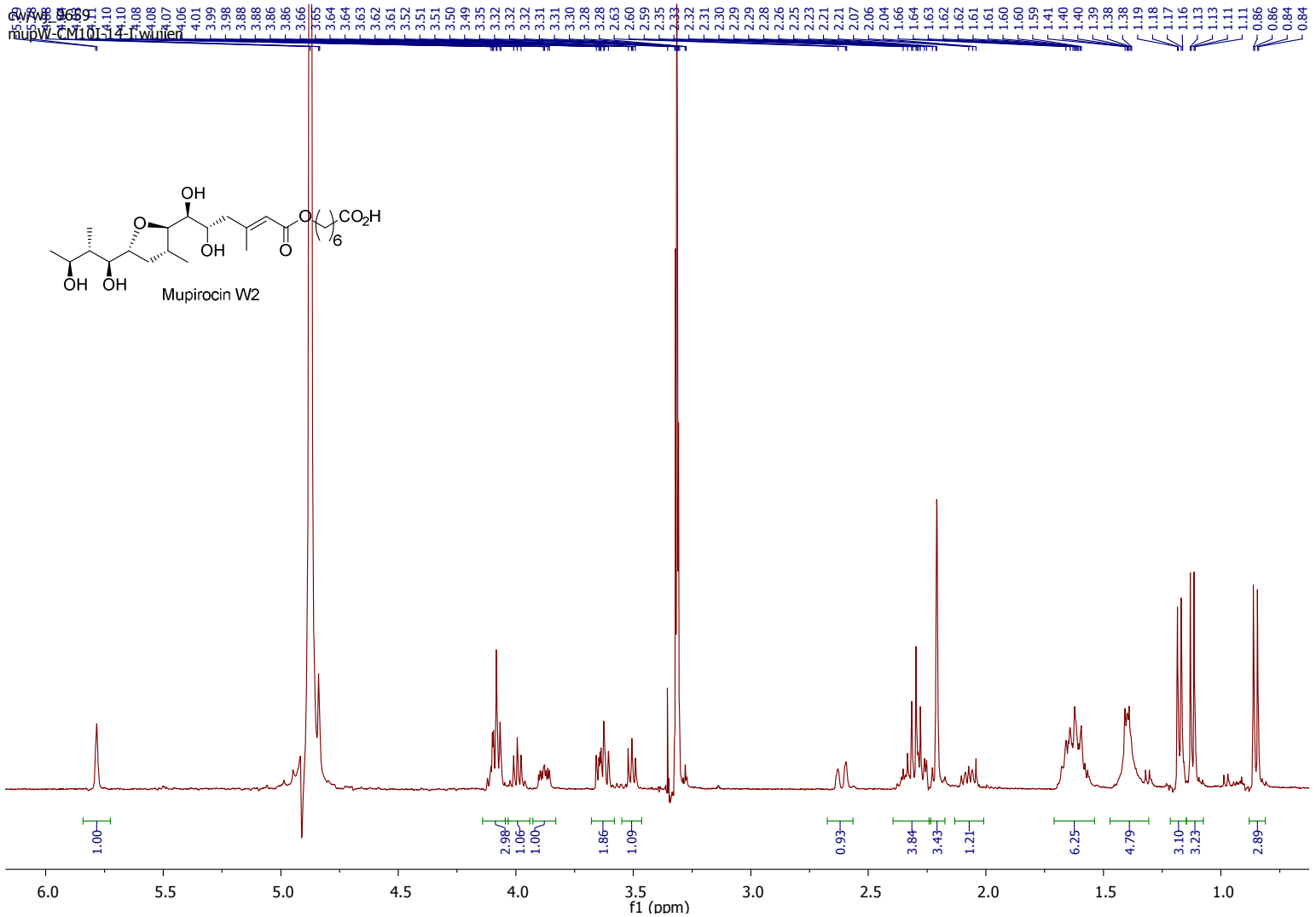


Figure S13. ^{13}C-NMR spectrum of mupirocin W2 (**6**) measured in CD_3OD at 100 MHz.

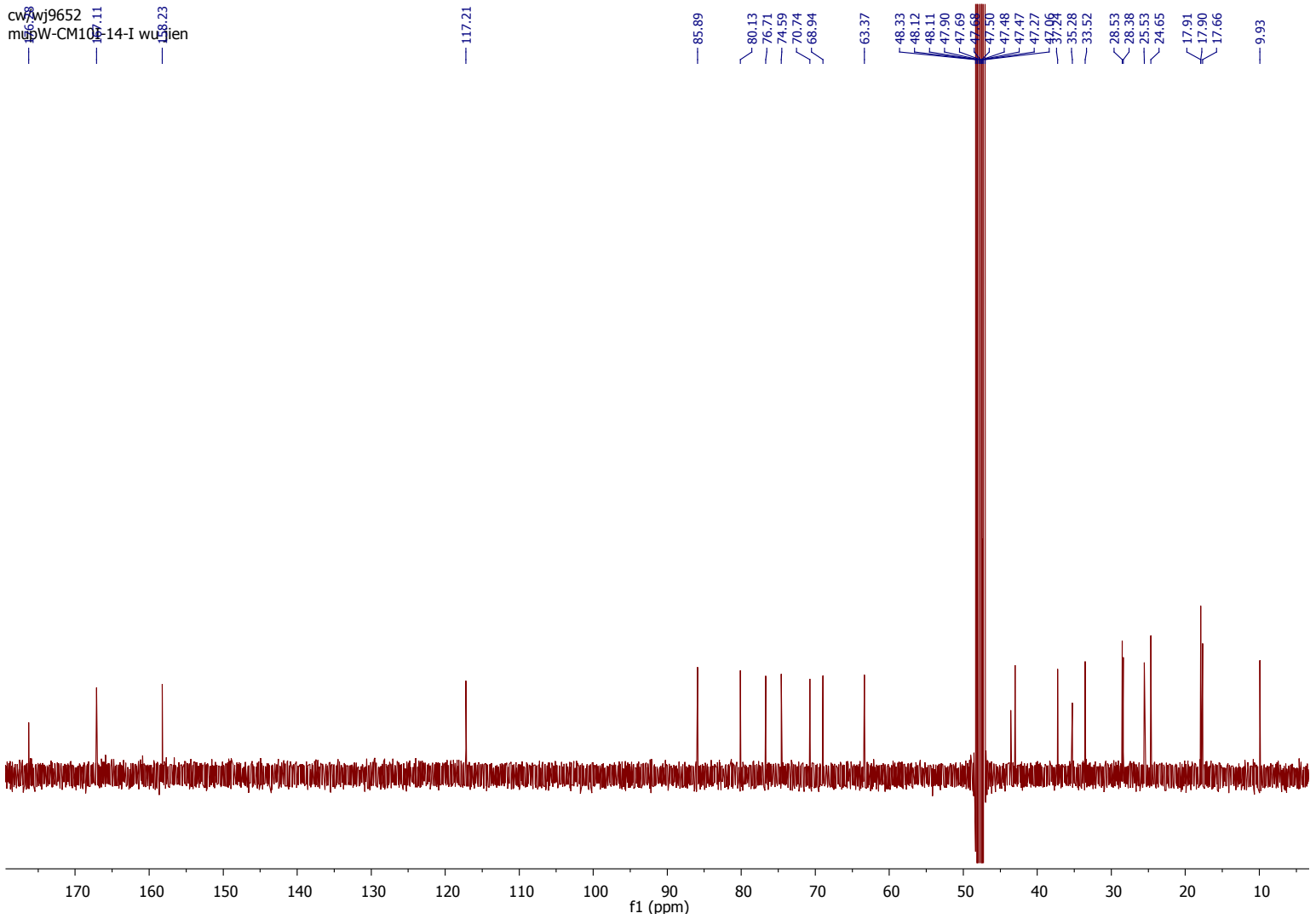


Figure S14. HMBC spectrum of mupirocin W2 (**6**) measured in CD_3OD at 400 MHz.

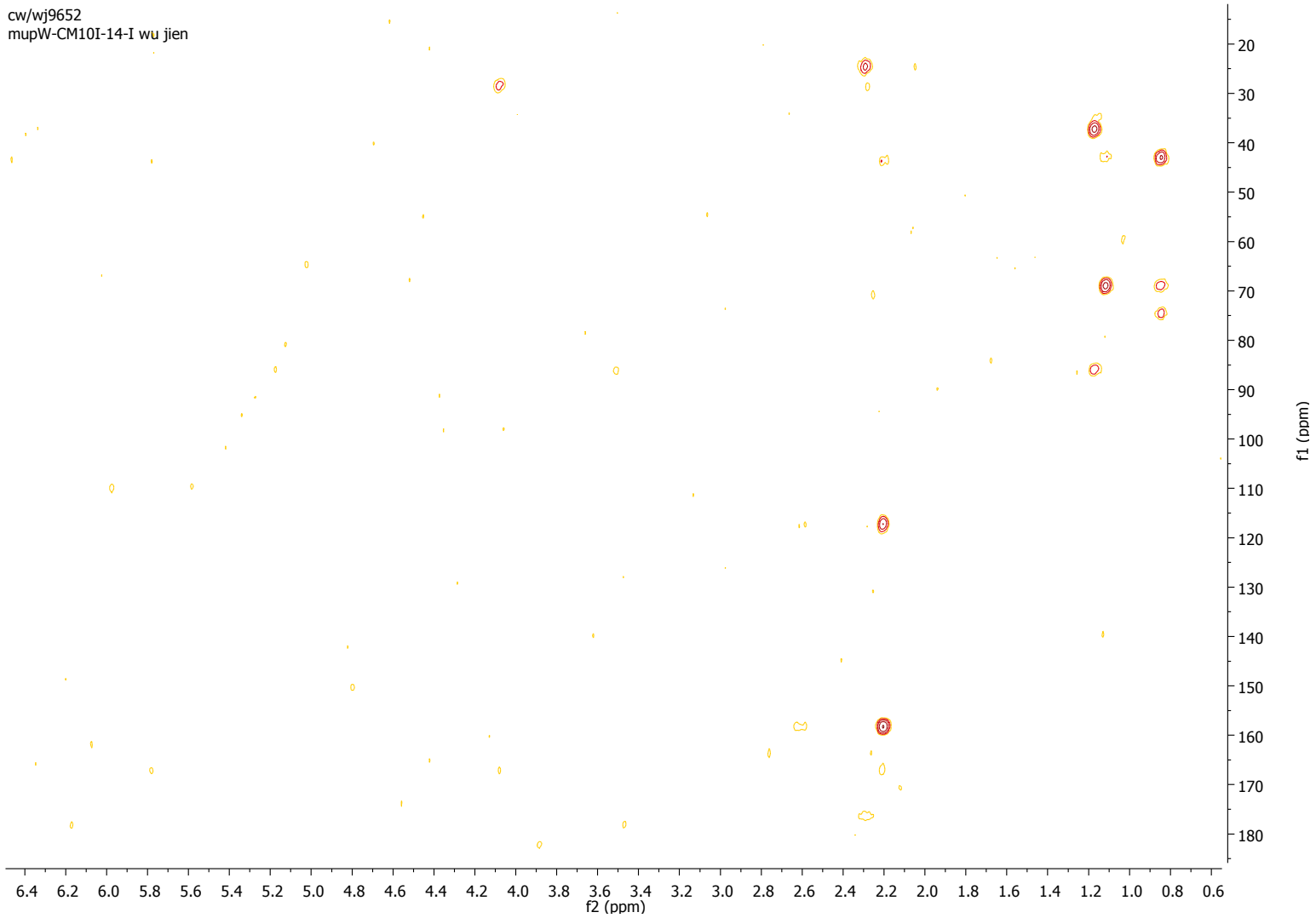


Figure S15. ^{1}H NMR spectrum of mupirocin W3 (**7**) measured in CD_3OD at 400 MHz.

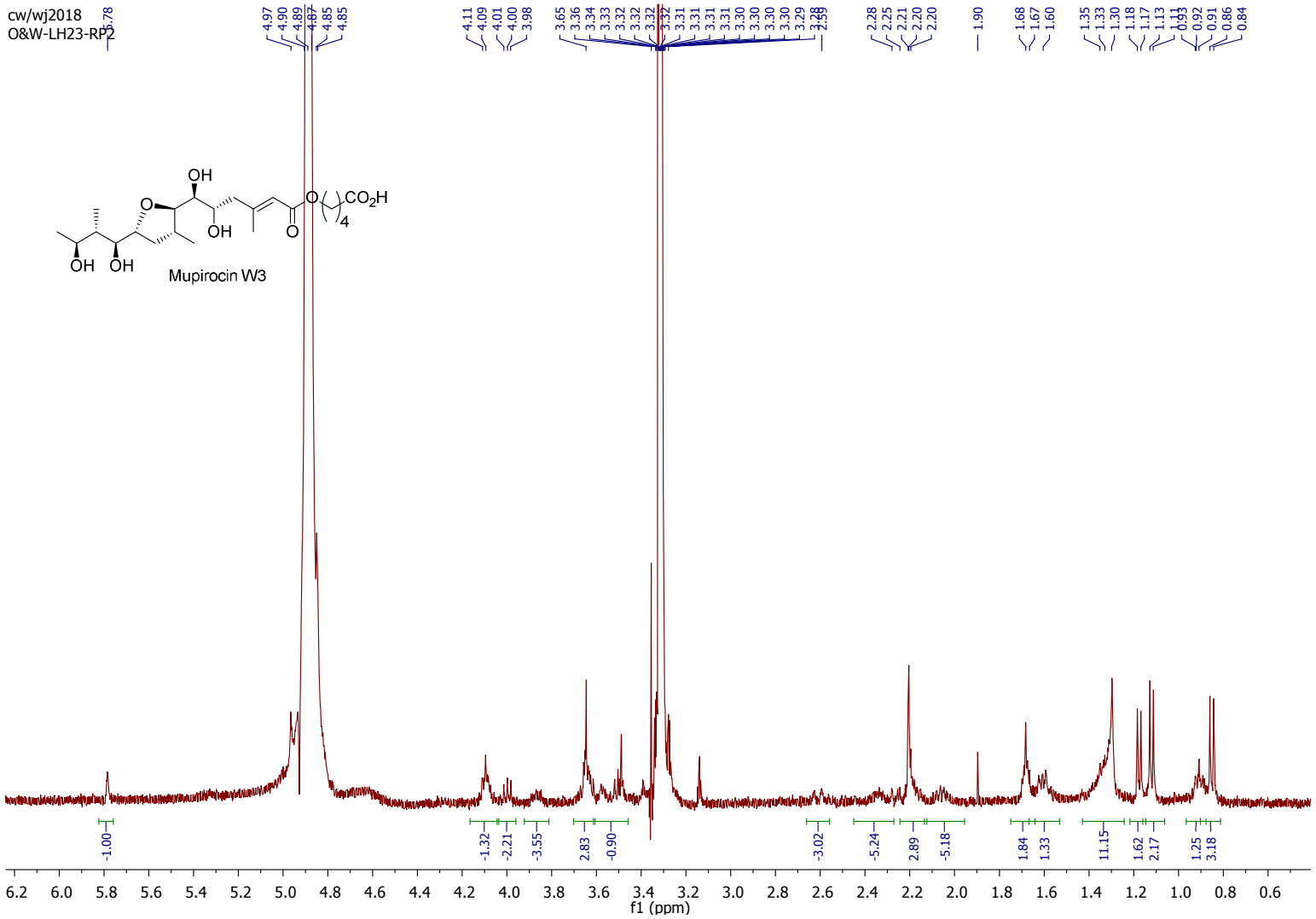


Figure S16. ^{1}H NMR spectrum of mupirocin W6 (**8**) measured in CD_3OD at 400 MHz.

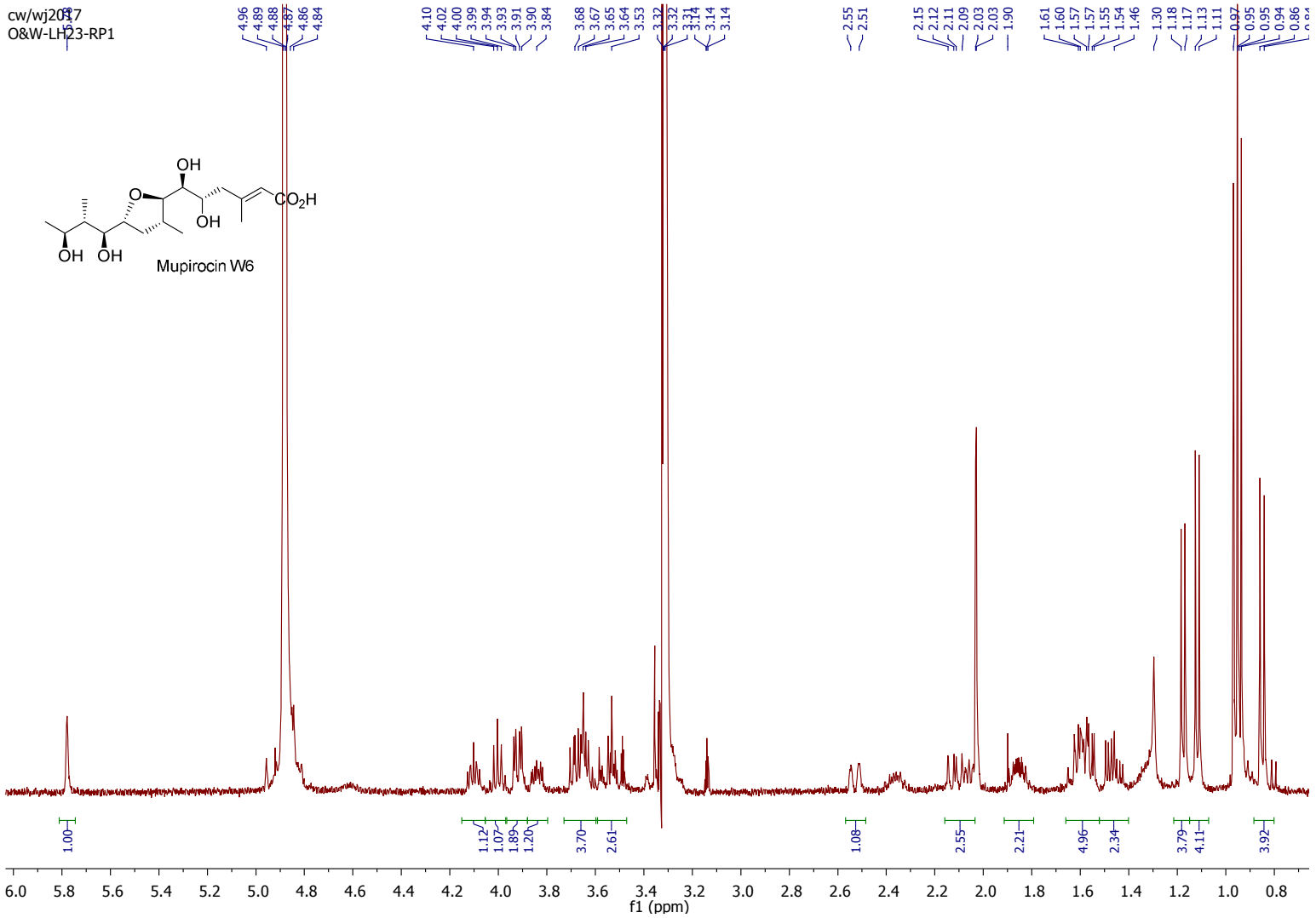


Figure S17. ^{1}H-^{1}H COSY spectrum of mupirocin W6 (**8**) measured in CD_3OD at 400 MHz.

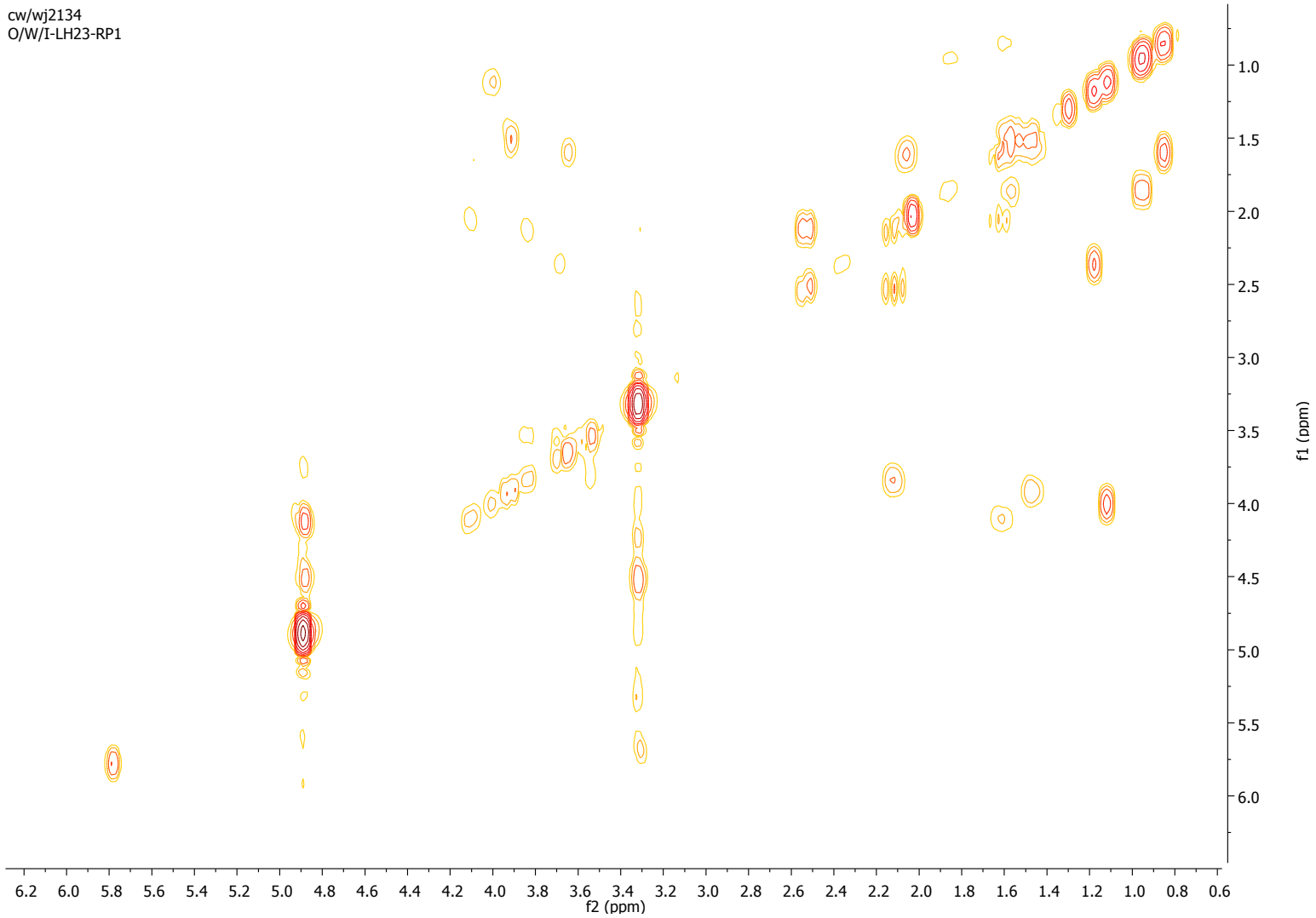


Figure S18. HMBC spectrum of mupirocin W6 (**8**) measured in CD_3OD at 400 MHz.

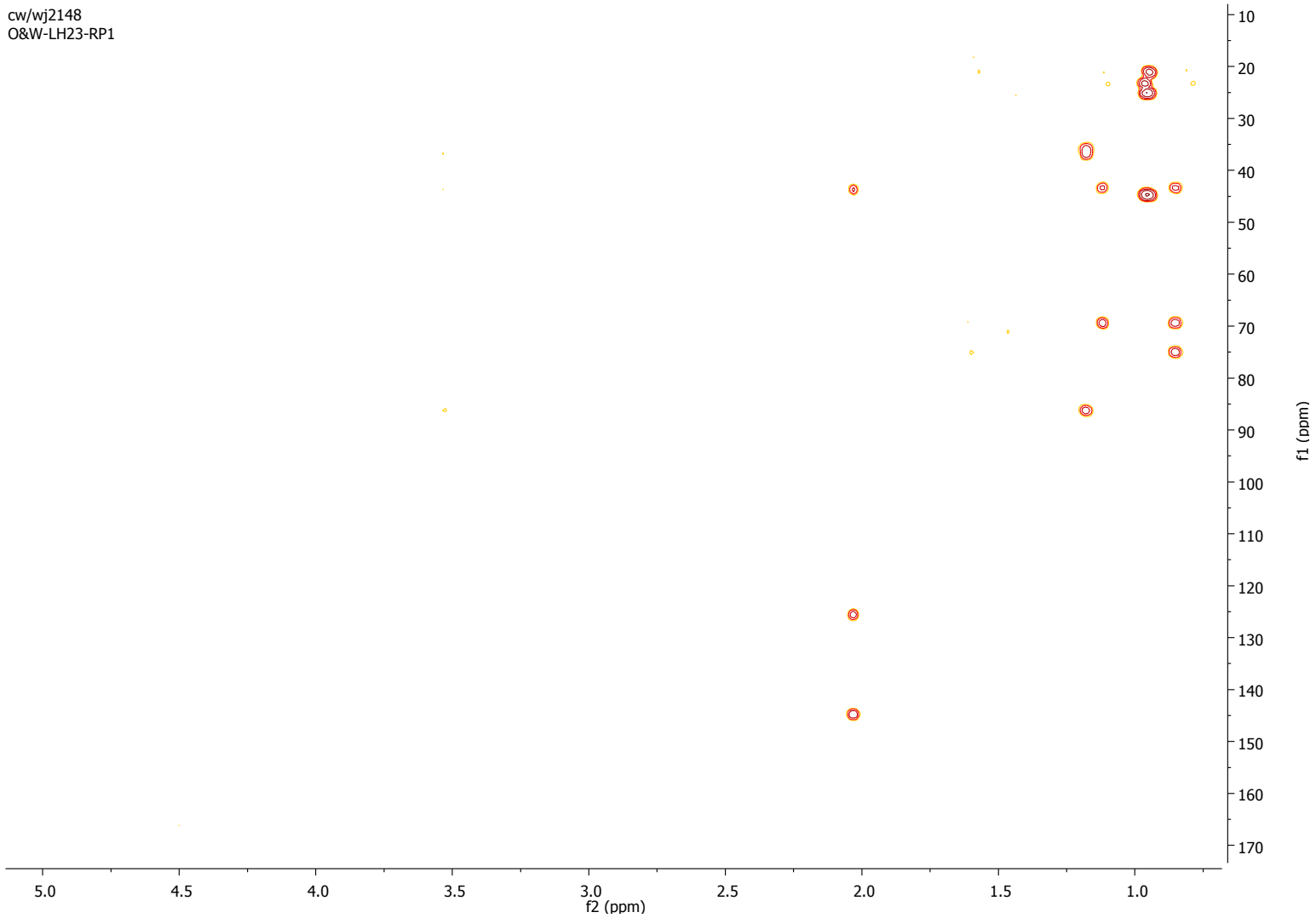


Figure S19. ^{1}H NMR spectrum of mupirocin W4 (**9**) measured in CD_3OD at 500 MHz.

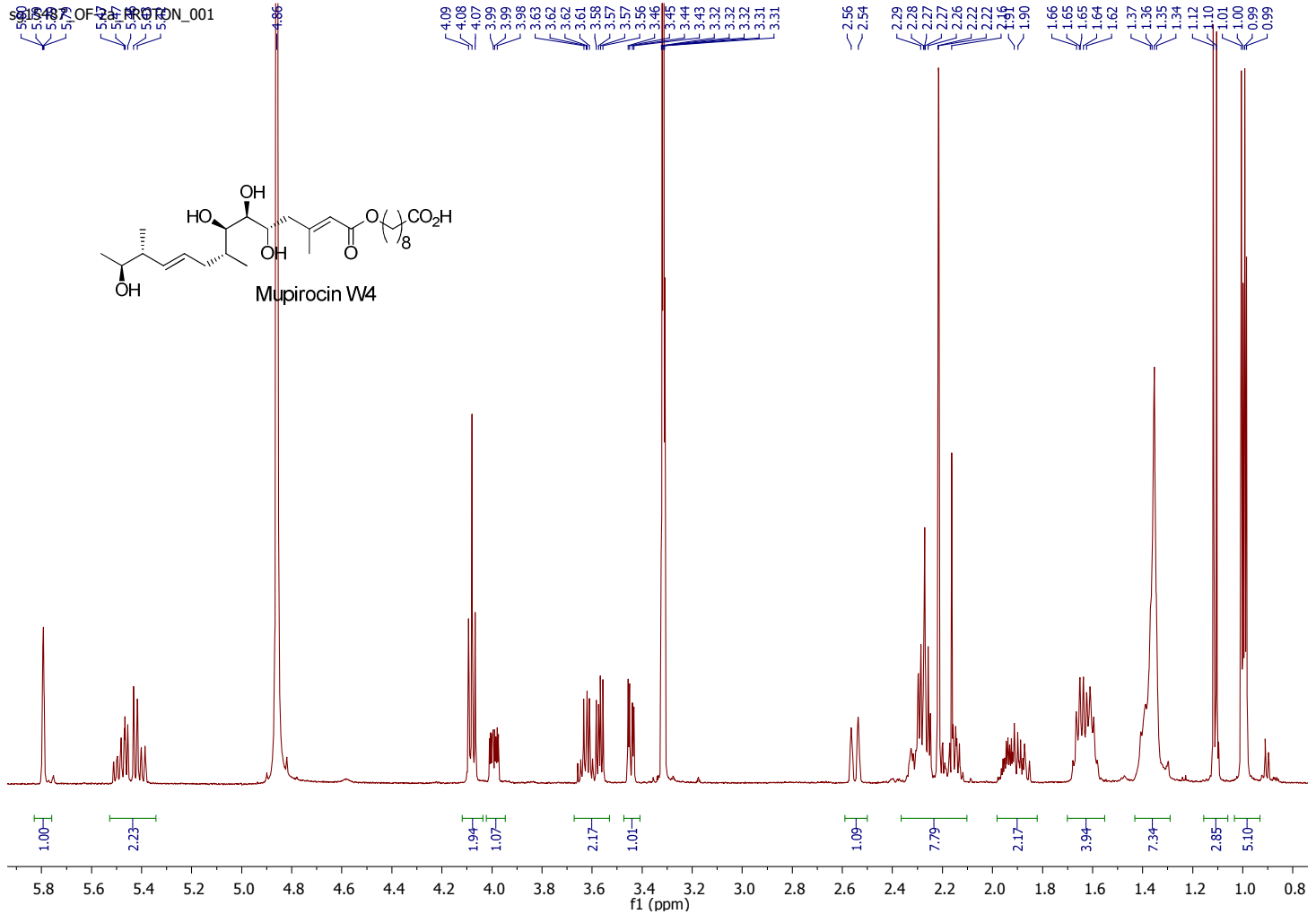


Figure S20. ^{1}H-^{1}H COSY spectrum of mupirocin W4 (**9**) measured in CD_3OD at 500 MHz.

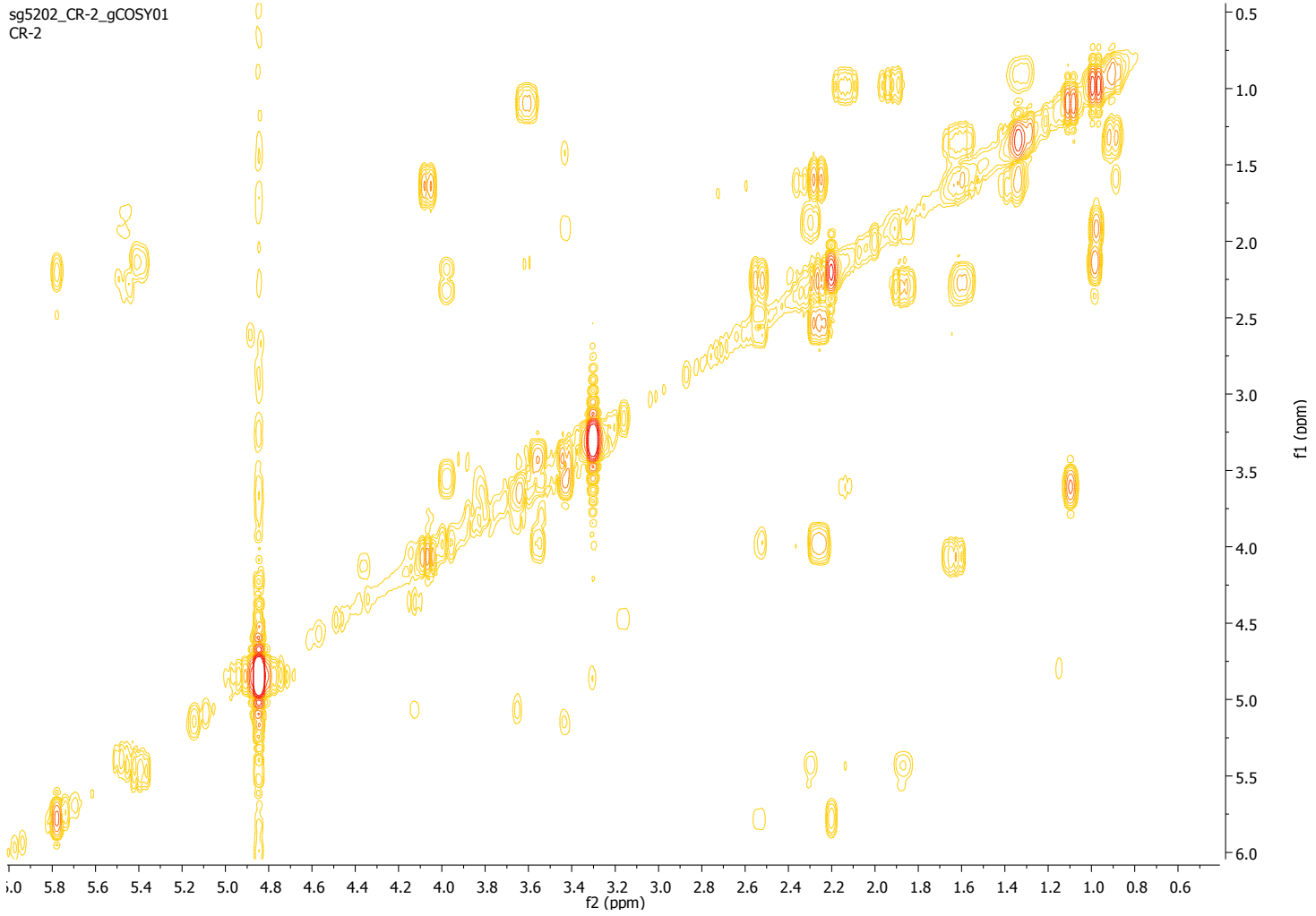
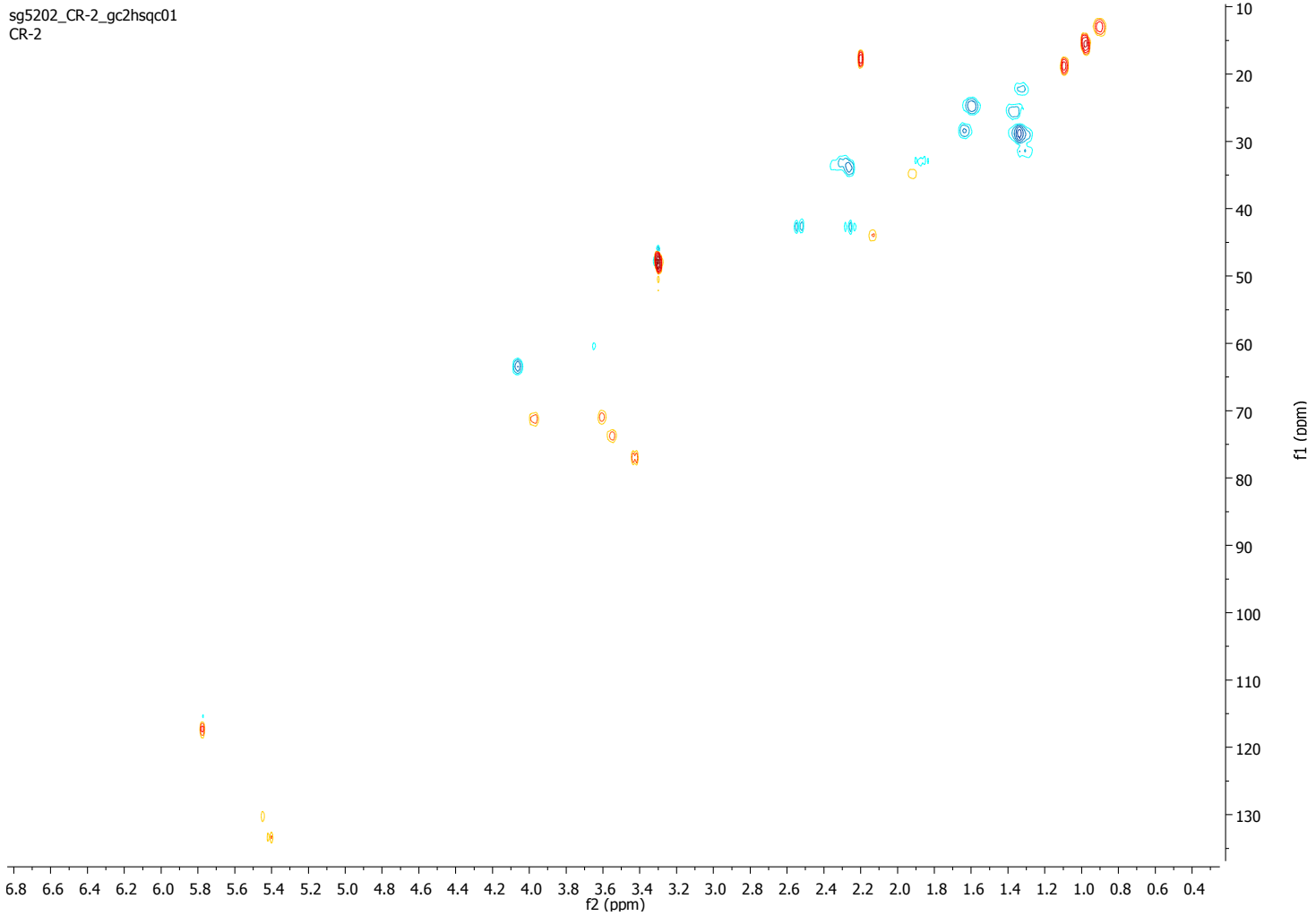


Figure S21. HSQC spectrum of mupirocin W4 (**9**) measured in CD_3OD at 500 MHz.



FigureS22. HMBC spectrum of mupirocin W4 (**9**) measured in CD_3OD at 500 MHz.

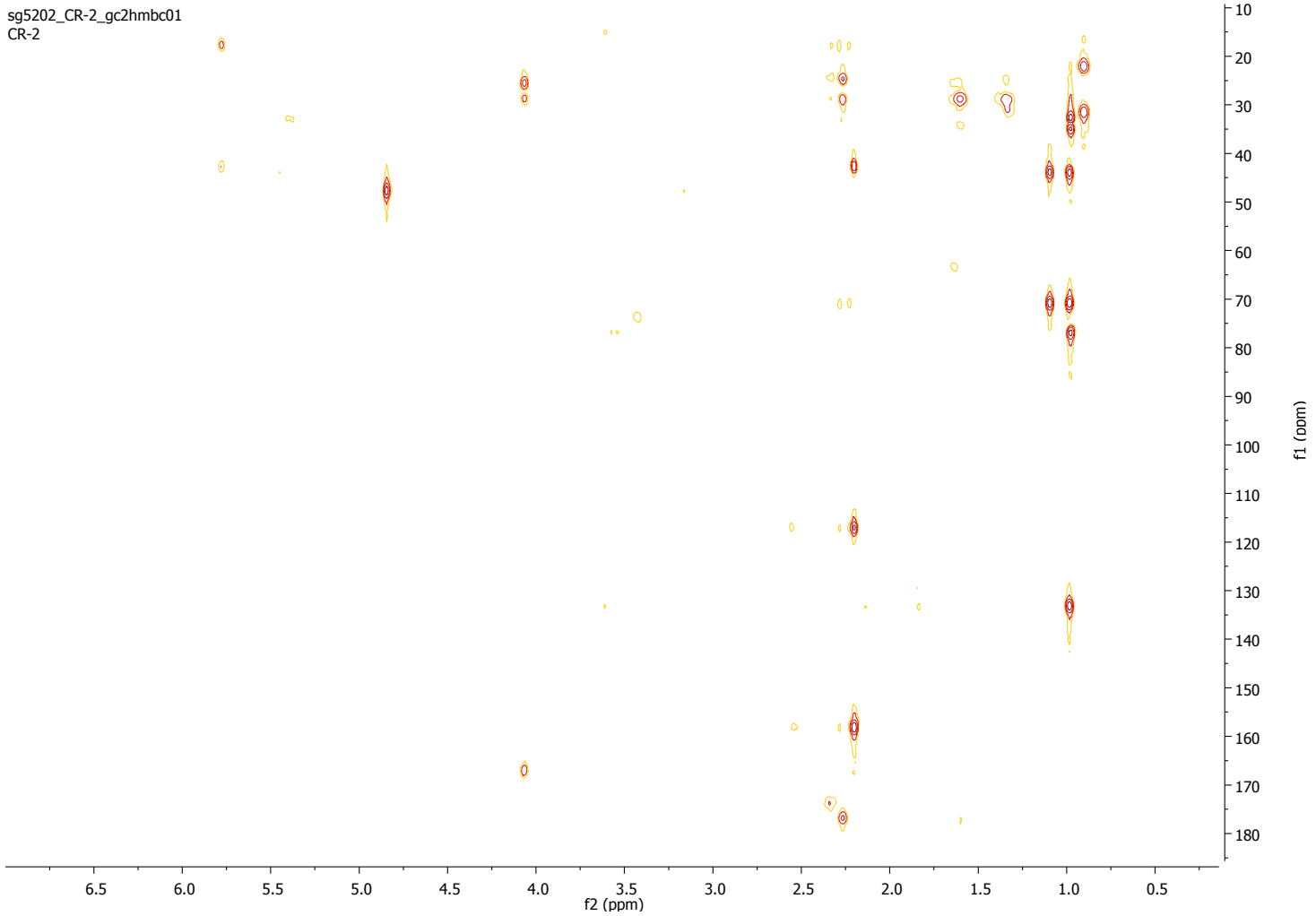


Figure S23. ^{1}H-NMR spectrum of mupirocin W5 (**10**) measured in CD_3OD at 500 MHz.

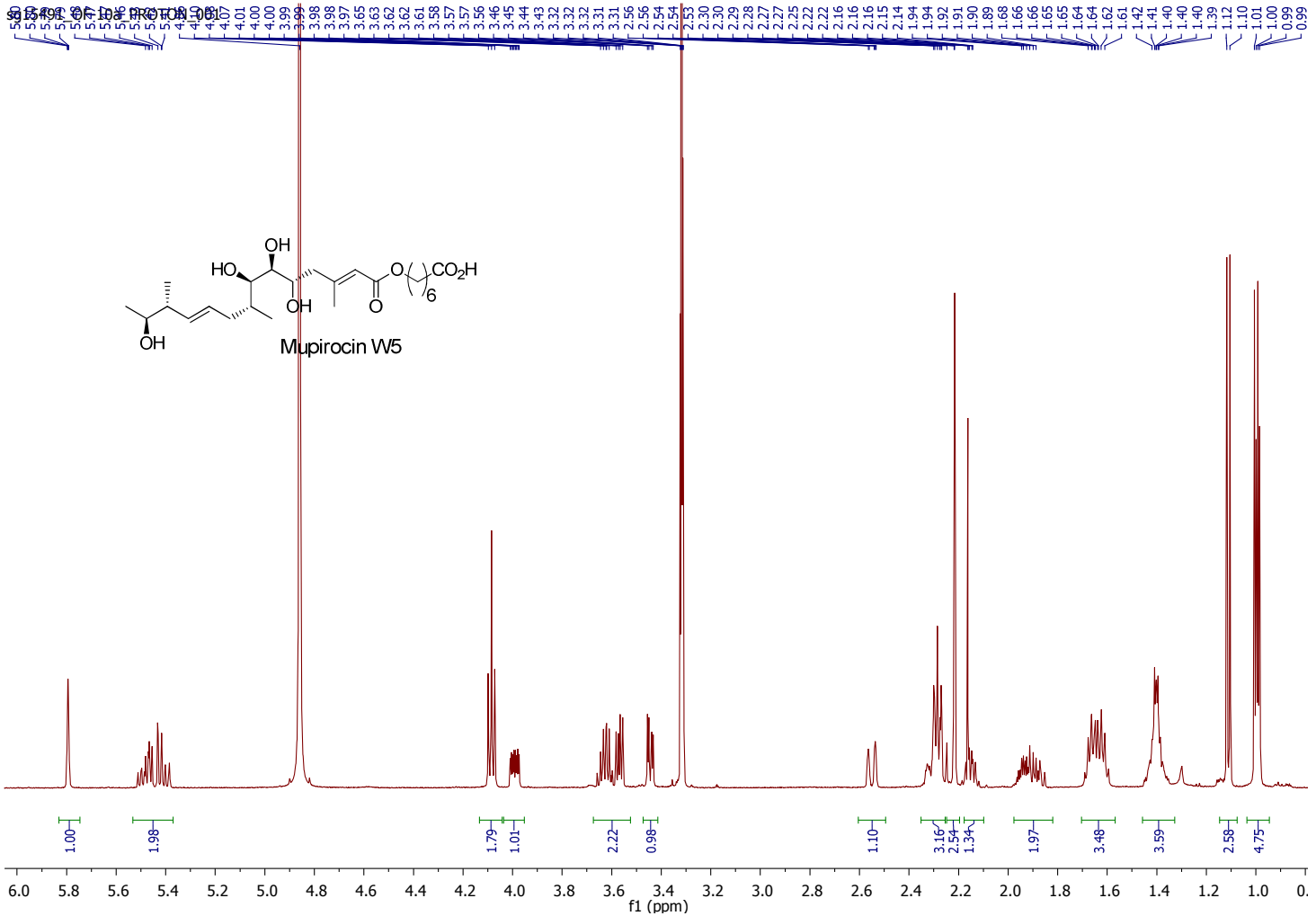


Figure S24. ^{1}H-^{1}H COSY spectrum of mupirocin W5 (**10**) measured in CD_3OD at 500 MHz.

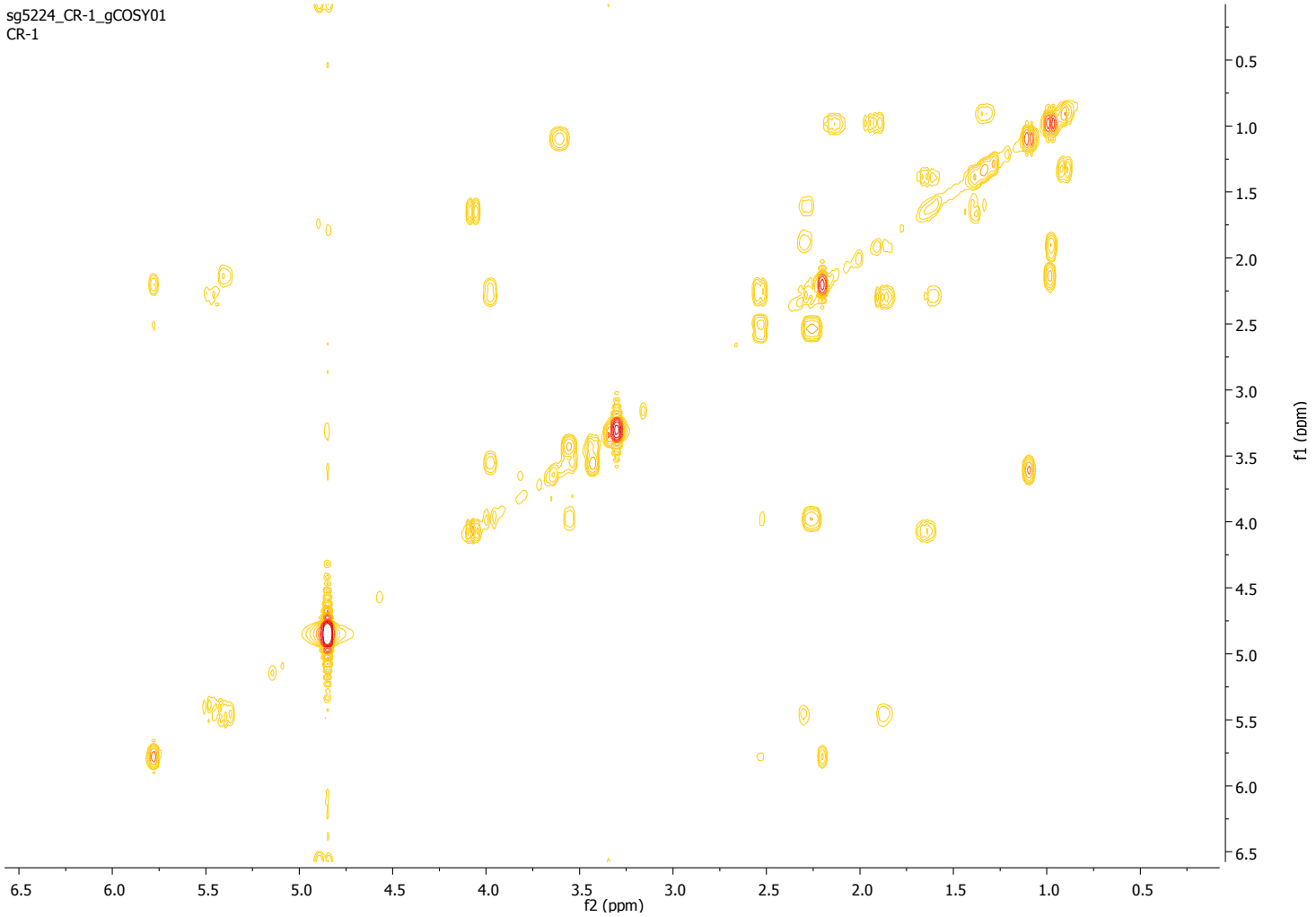


Figure S25. HSQC spectrum of mupirocin W5 (**10**) measured in CD_3OD at 500 MHz.

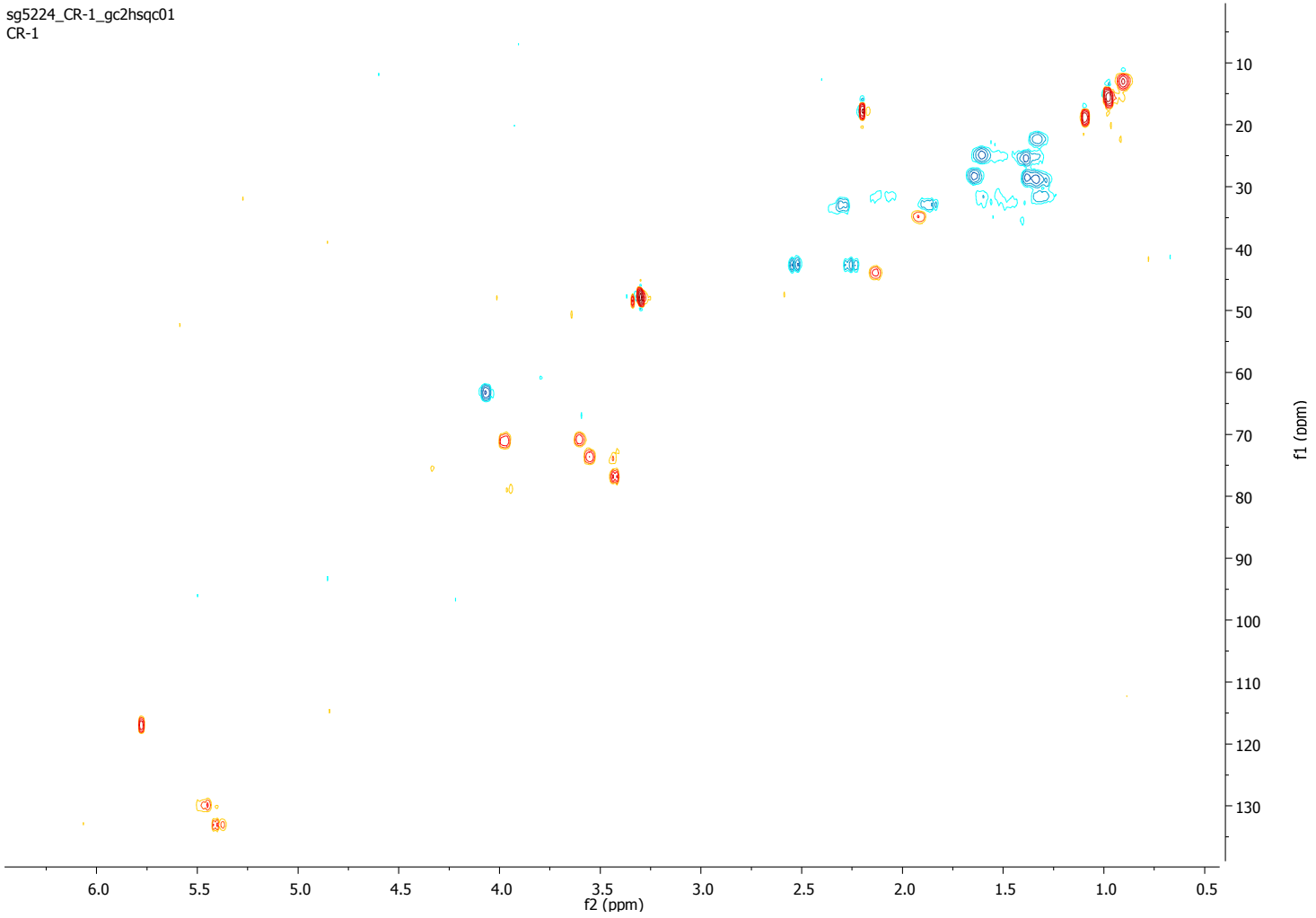


Figure S26. HMBC spectrum of mupirocin W5 (**10**) measured in CD_3OD at 500 MHz.

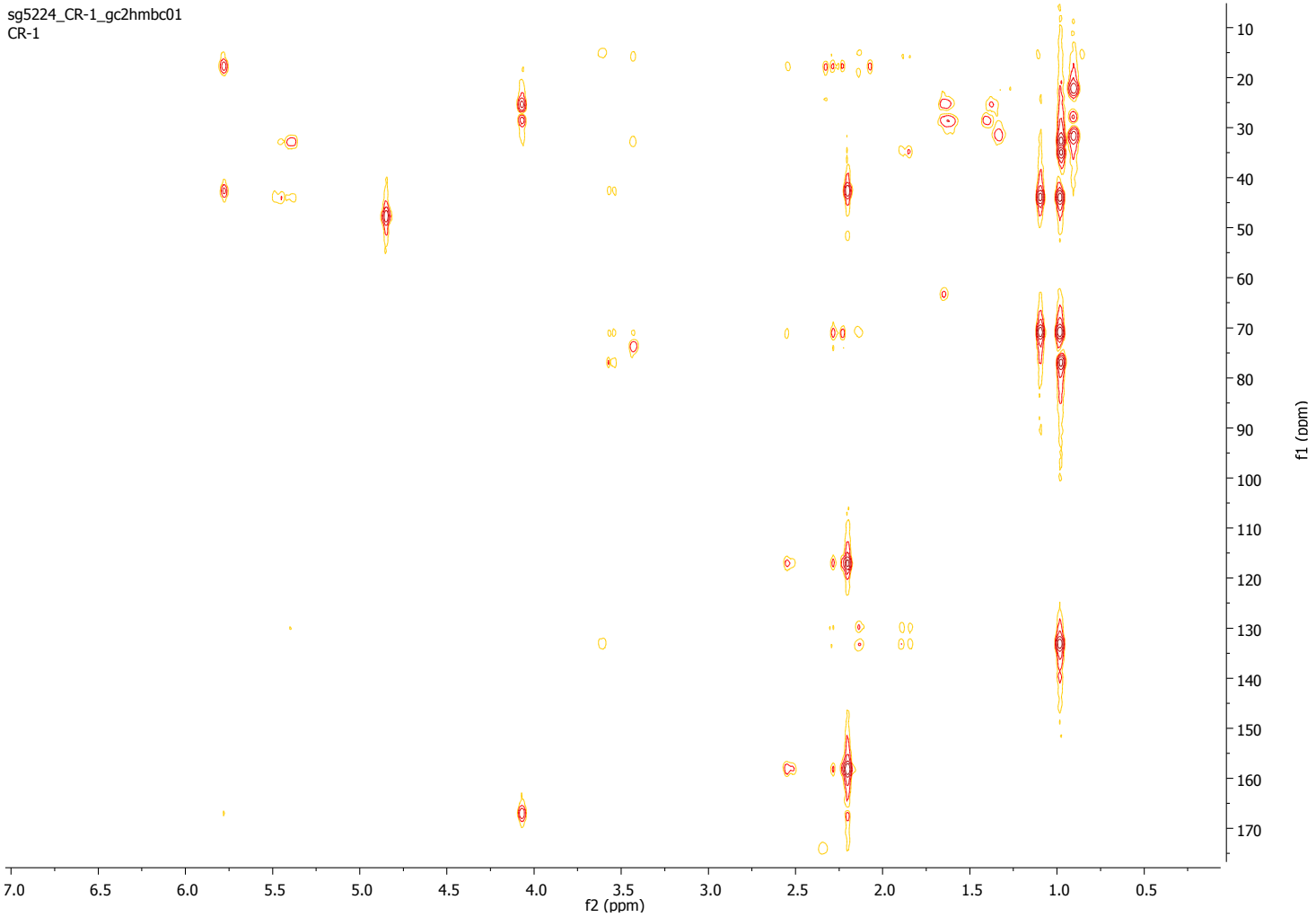


Figure S27. ^{1}H-NMR spectrum of desepoxy-PA-B (**11**) measured in CD_3OD at 500 MHz.

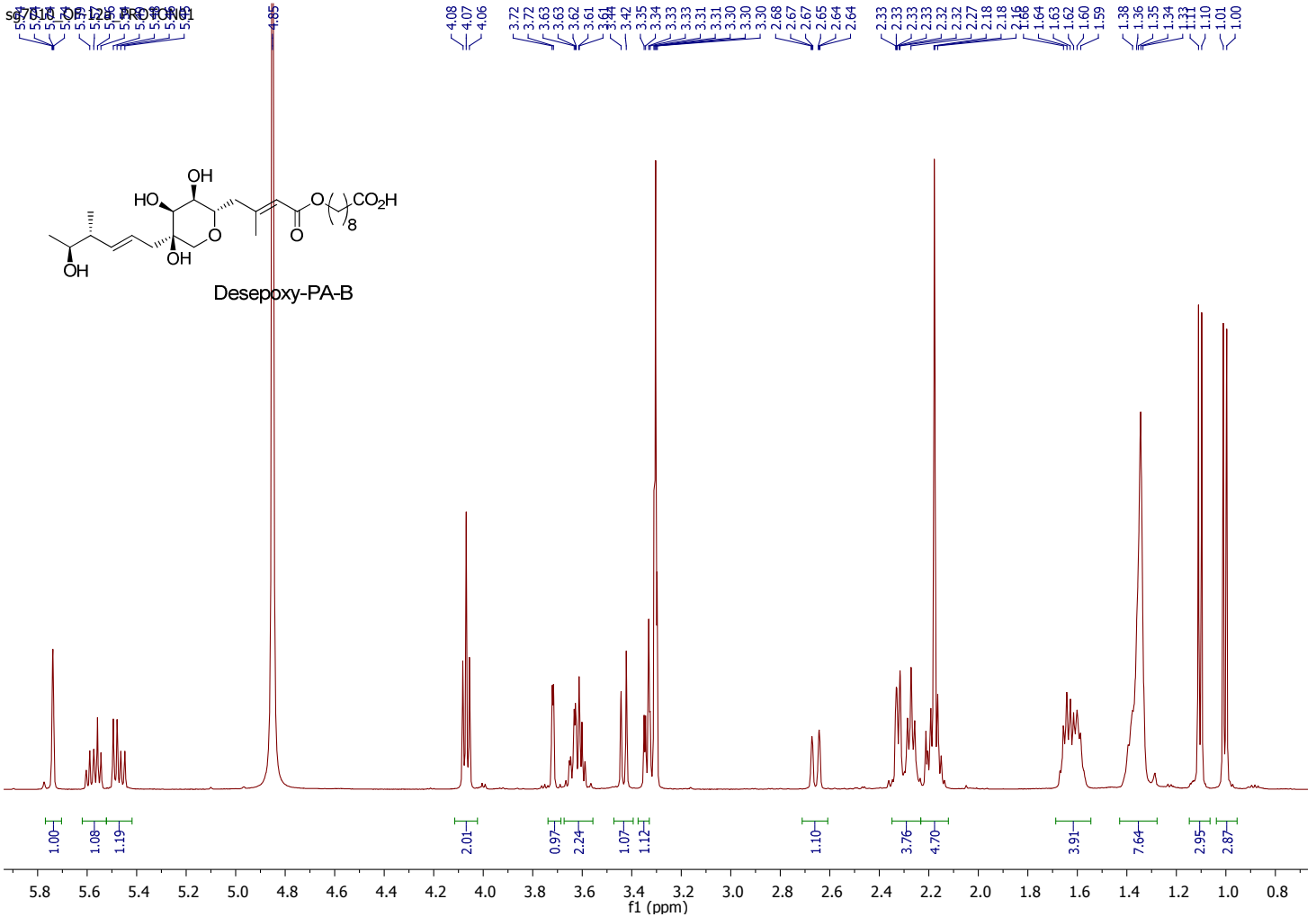


Figure S28. ^{1}H-^{1}H COSY spectrum of desepoxy-PA-B (**11**) measured in CD_3OD at 500 MHz.

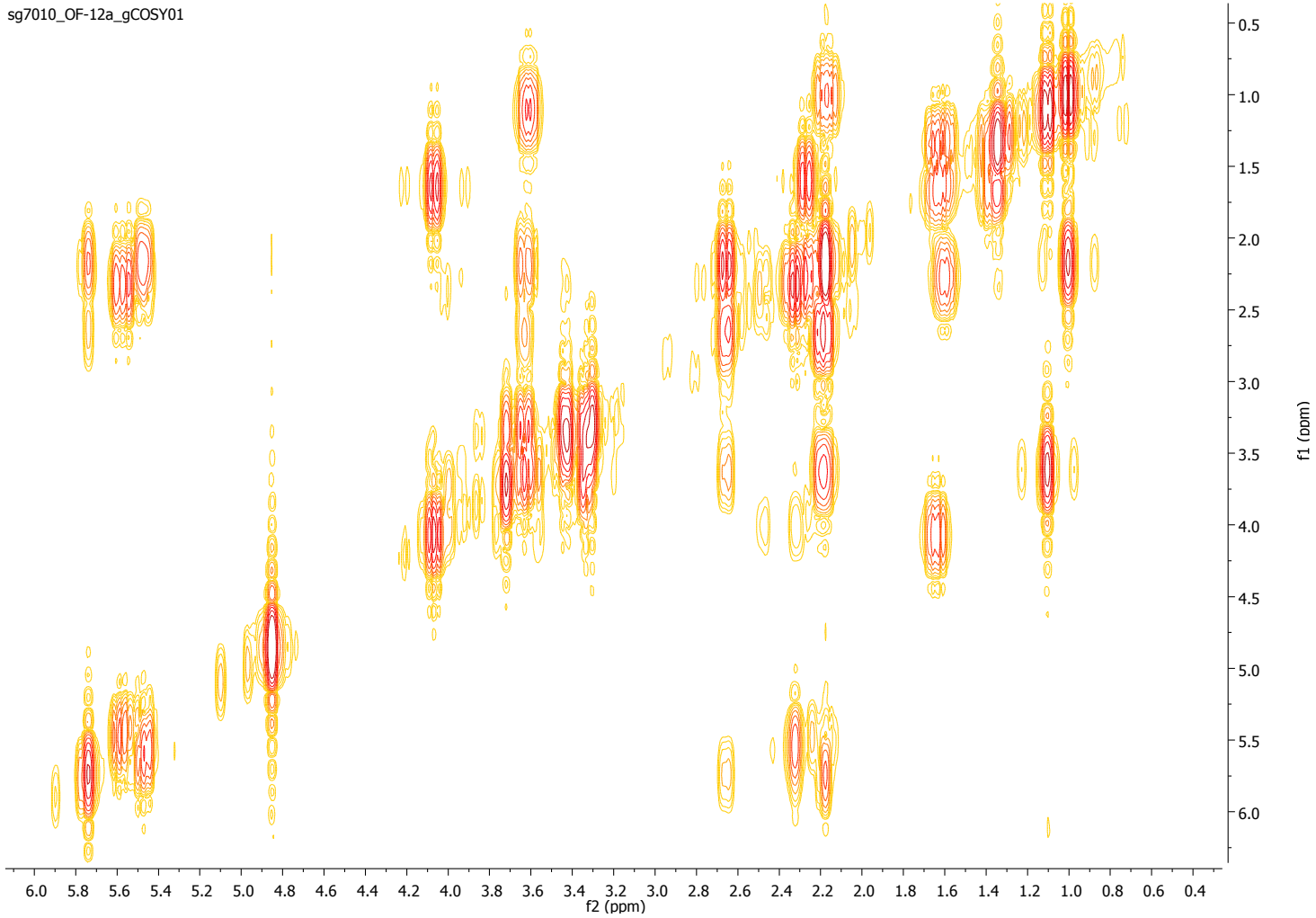


Figure S29. HSQC spectrum of desepoxy-PA-B (**11**) measured in CD_3OD at 500 MHz.

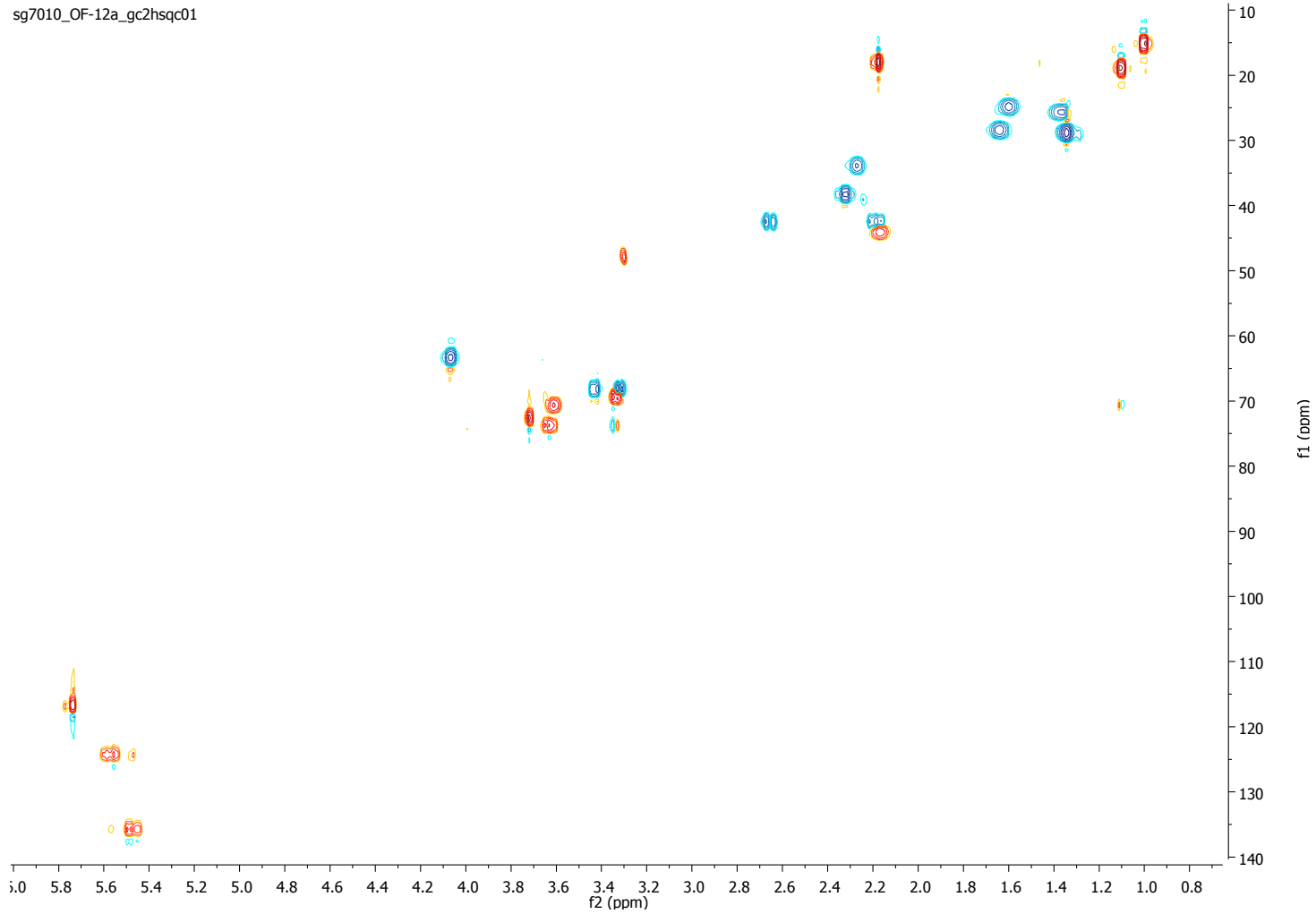


Figure S30. HMBC spectrum of desepoxy-PA-B (**11**) measured in CD_3OD at 500 MHz.

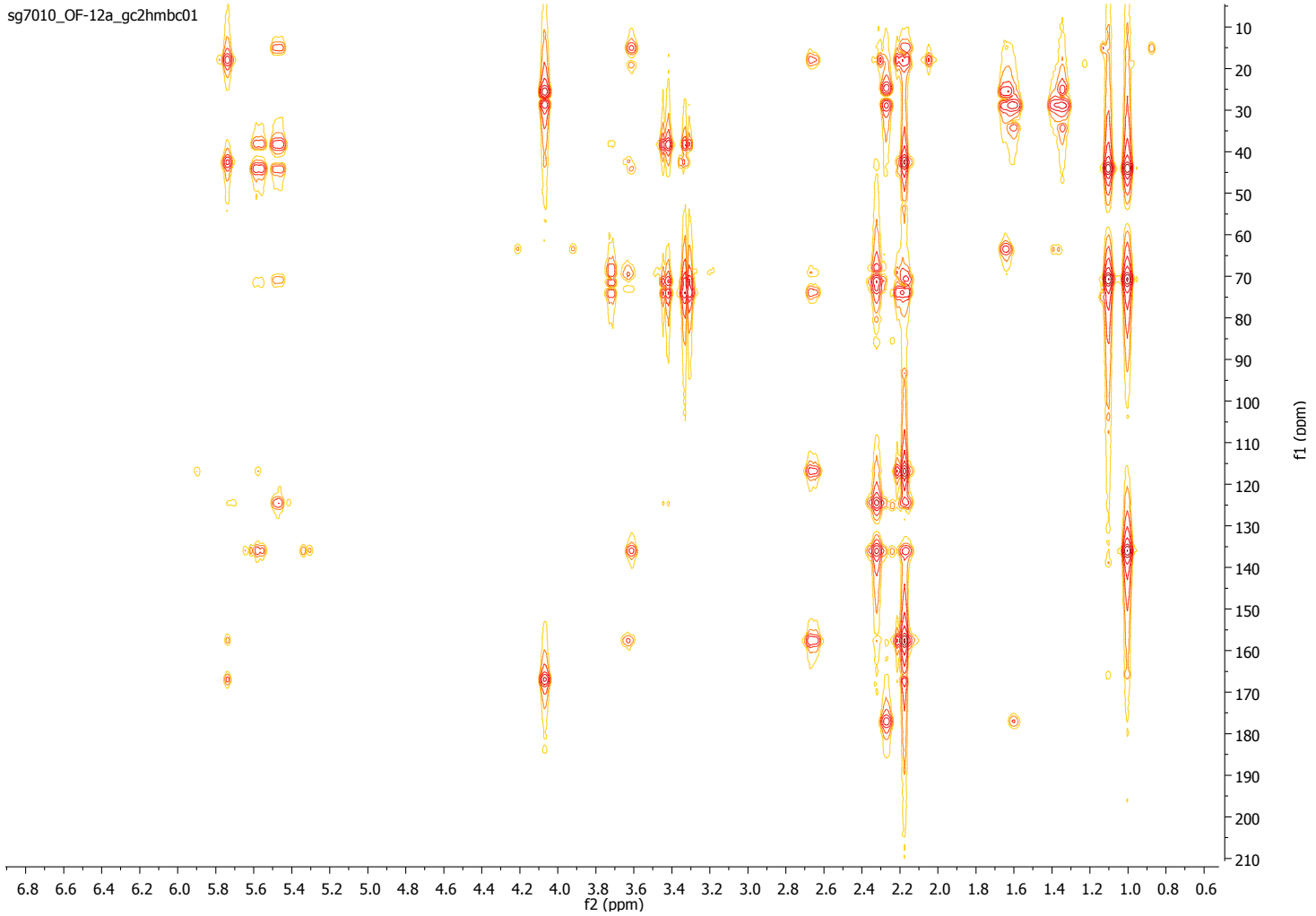


Figure S31. ^{1}H-NMR spectrum of PA-B macrolactone (**12**) measured in CD_3OD at 500 MHz.

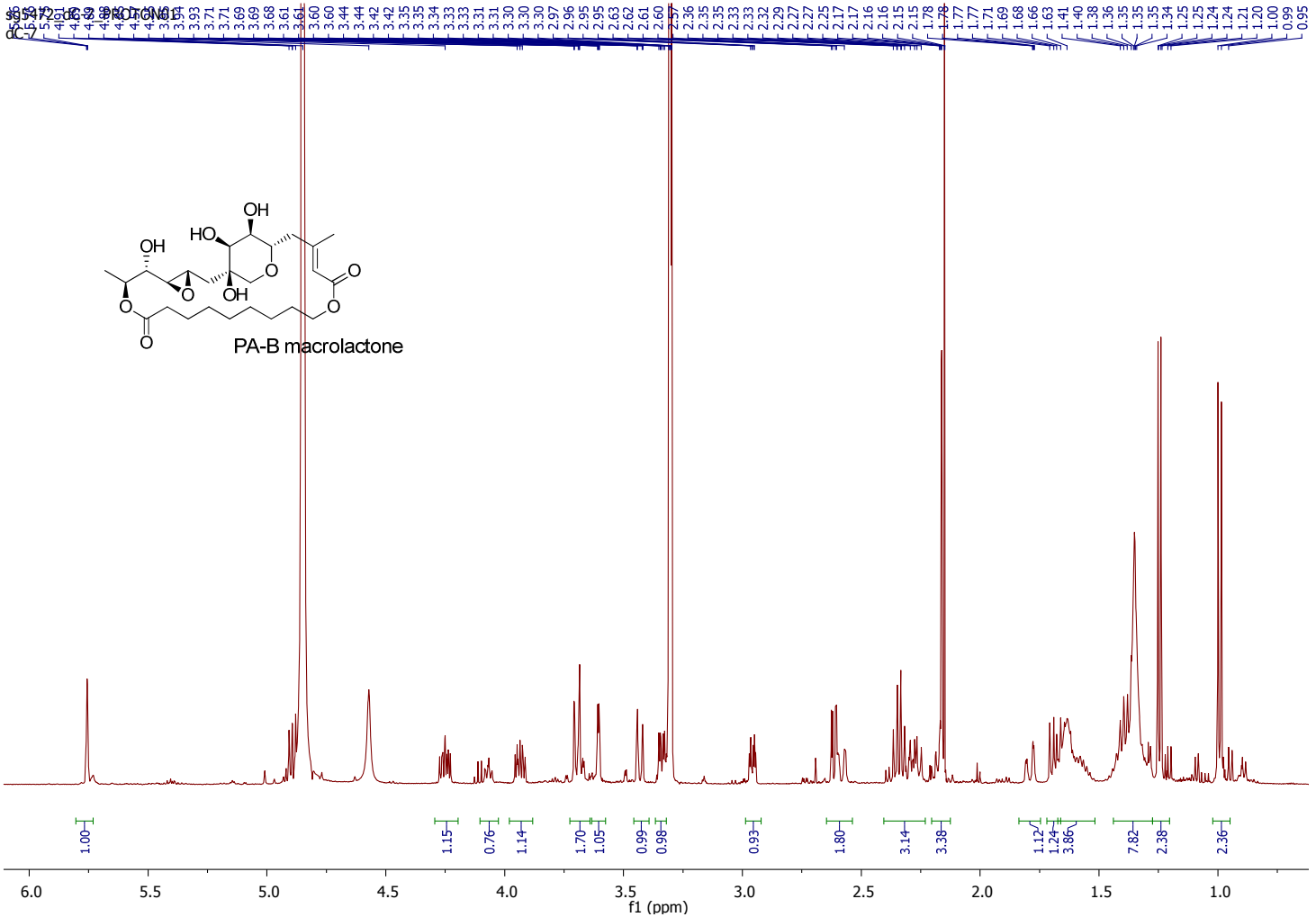


Figure S32. ^{13}C-NMR spectrum of PA-B macrolactone (**12**) measured in CD_3OD at 125 MHz.

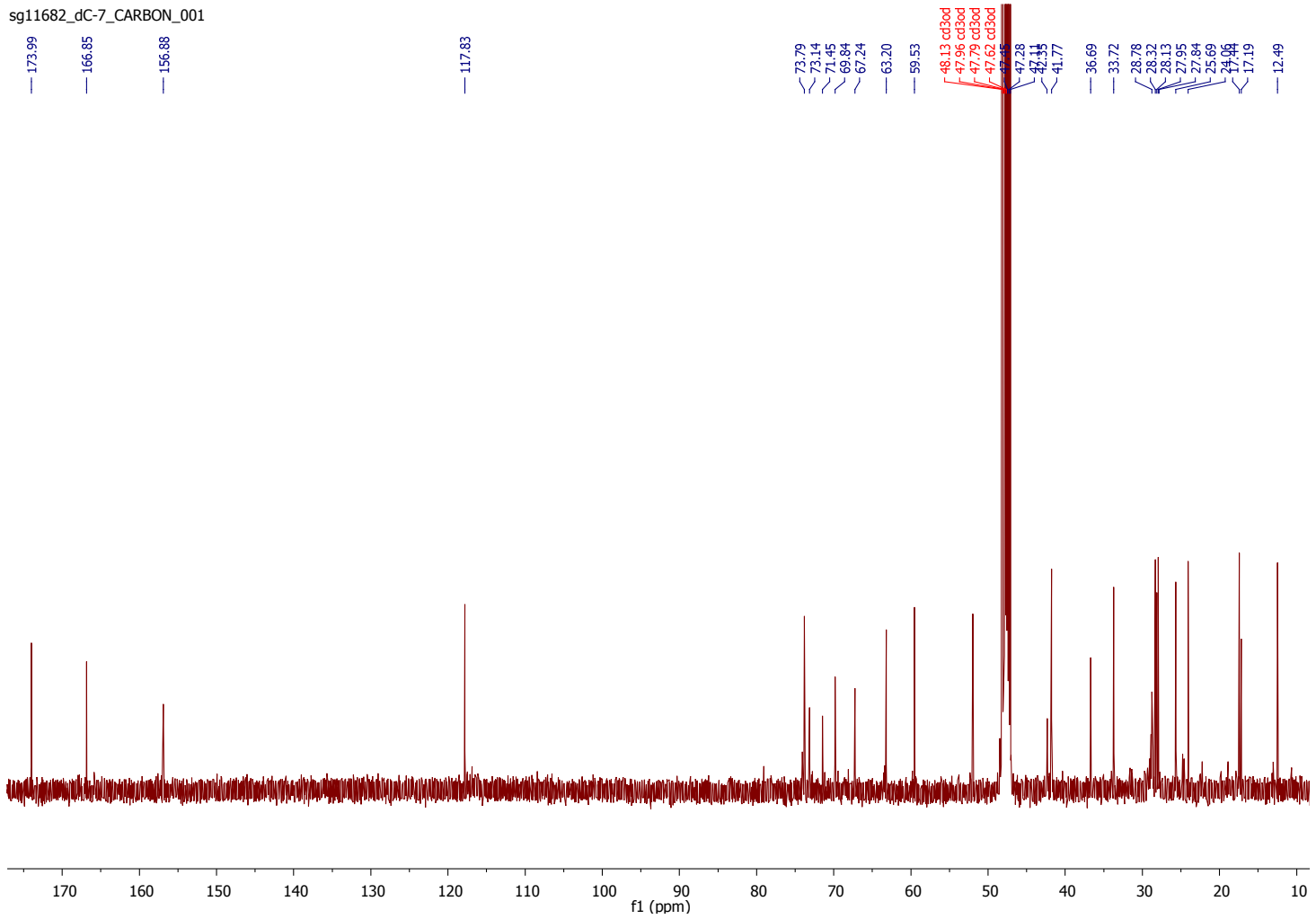


Figure S33. ^{1}H-^{1}H COSY spectrum of PA-B Macrolactone (**12**) measured in CD_3OD at 500 MHz.

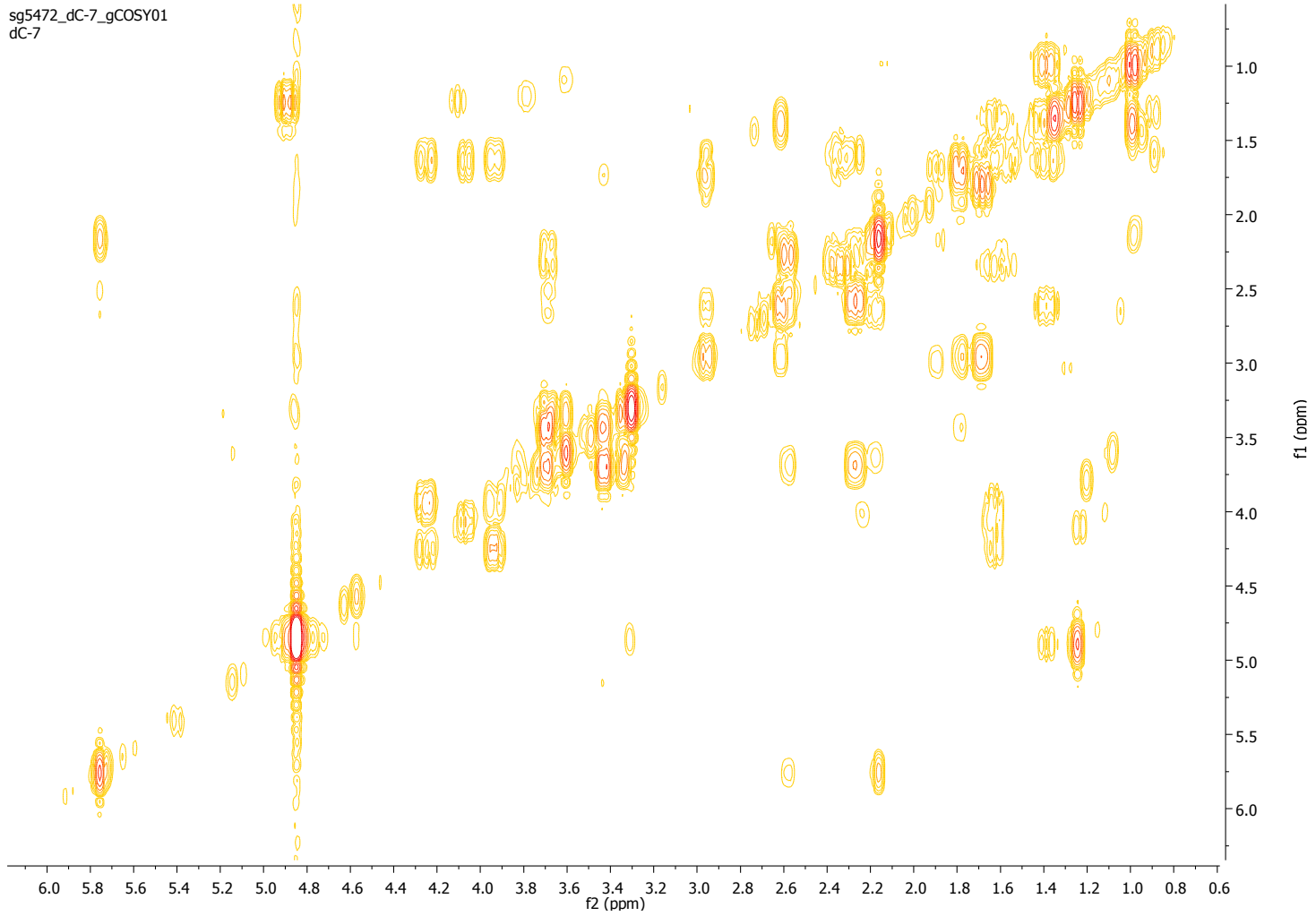


Figure S34. HSQC spectrum of PA-B macrolactone (**12**) measured in CD_3OD at 125 MHz.

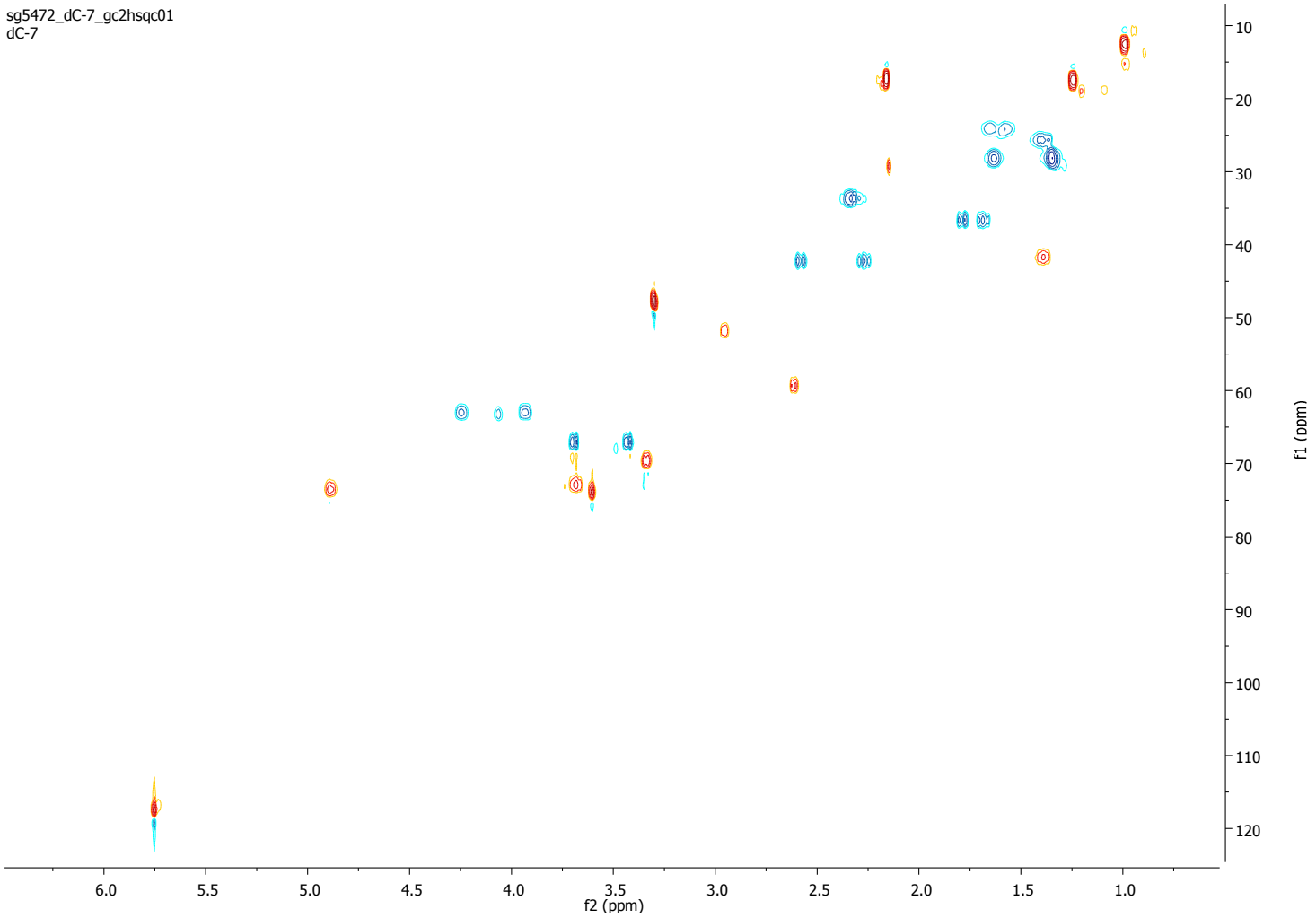


Figure S35. HMBC spectrum of PA-B macrolactone (**12**) measured in CD_3OD at 125 MHz.

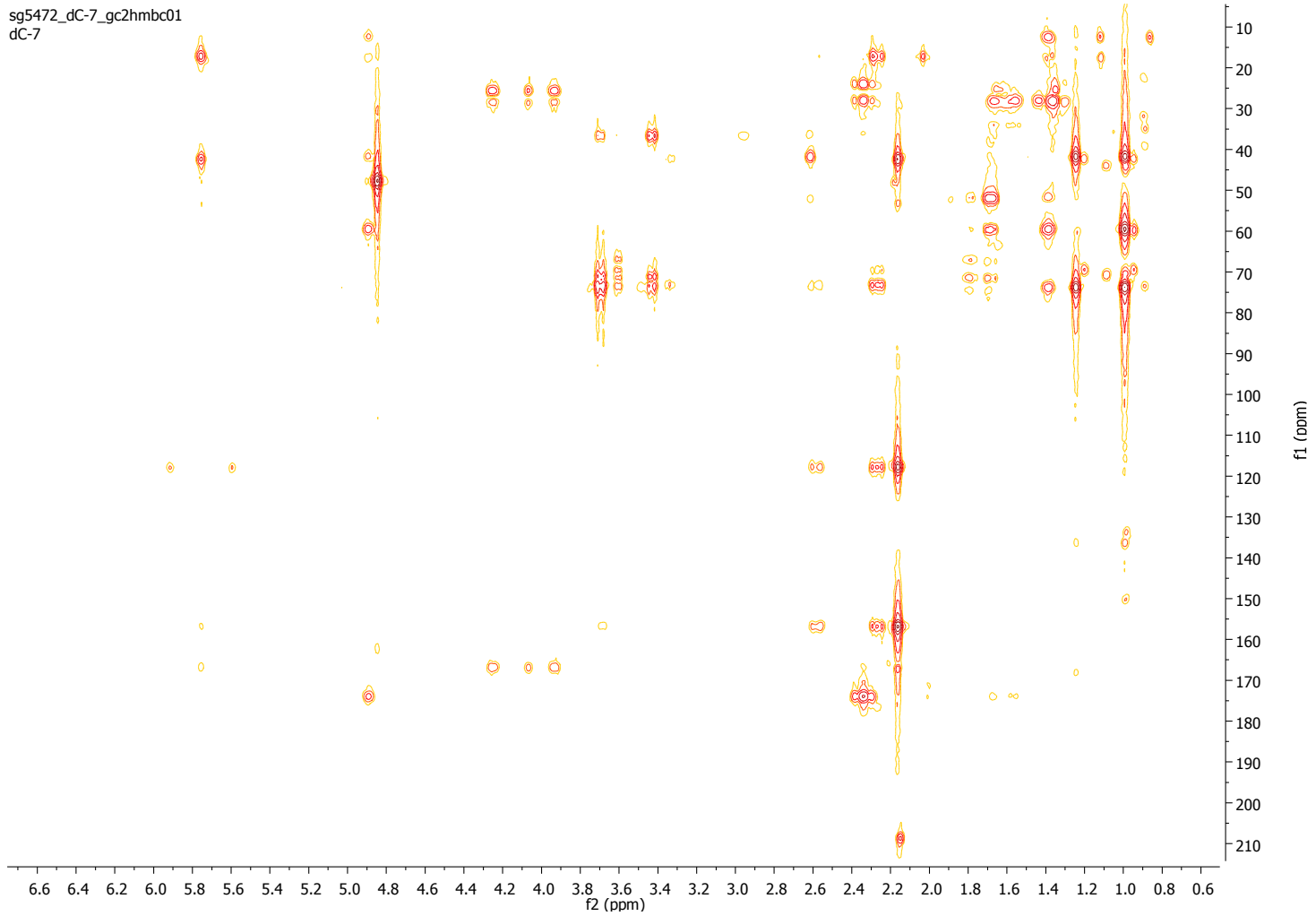


Figure S36. ^{1}H-NMR spectrum of mupirocin C1 (**15**) measured in CD_3OD at 500 MHz.

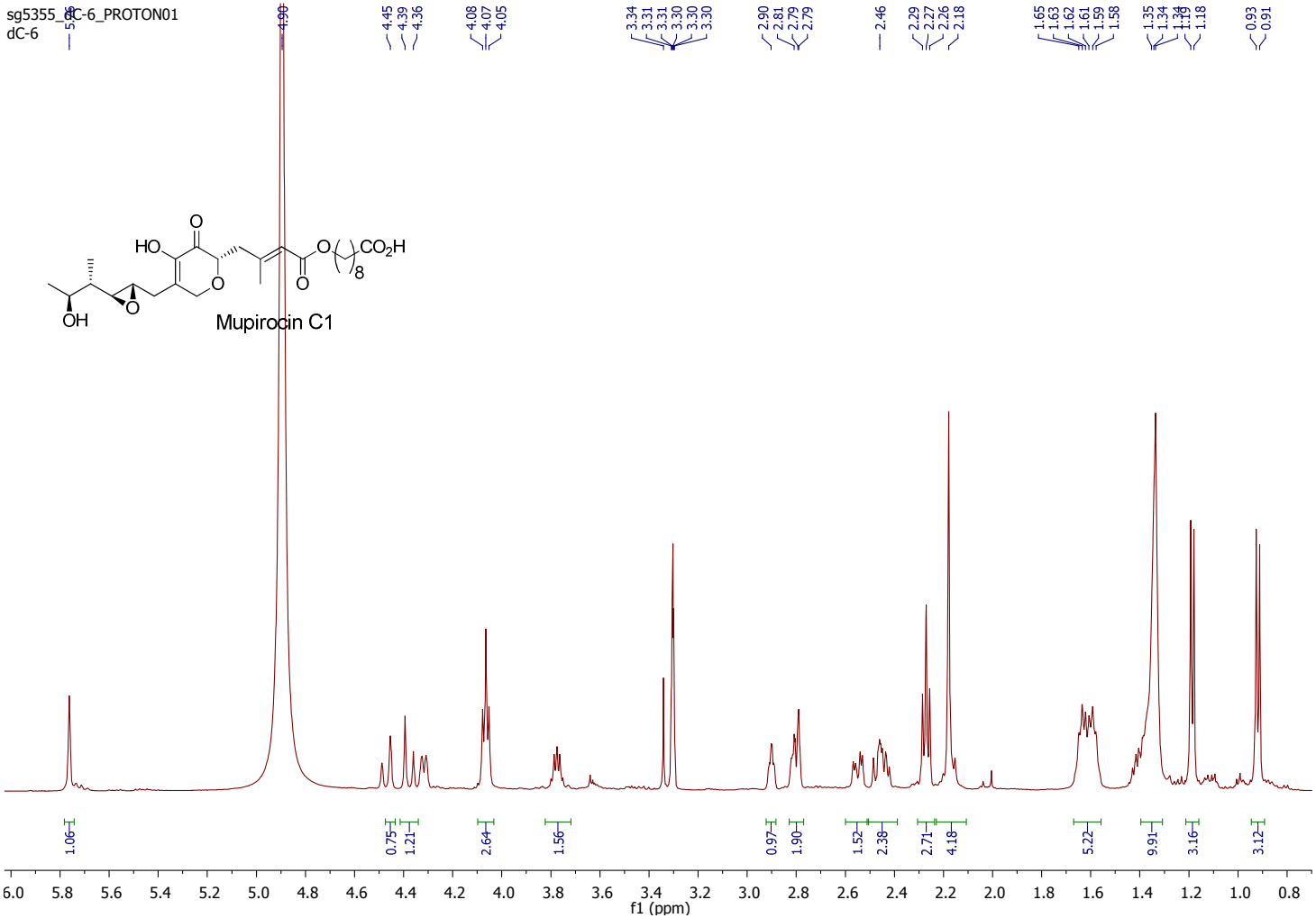


Figure S37. ^{13}C-NMR spectrum of mupirocin C1 (**15**) measured in CD_3OD at 125 MHz.

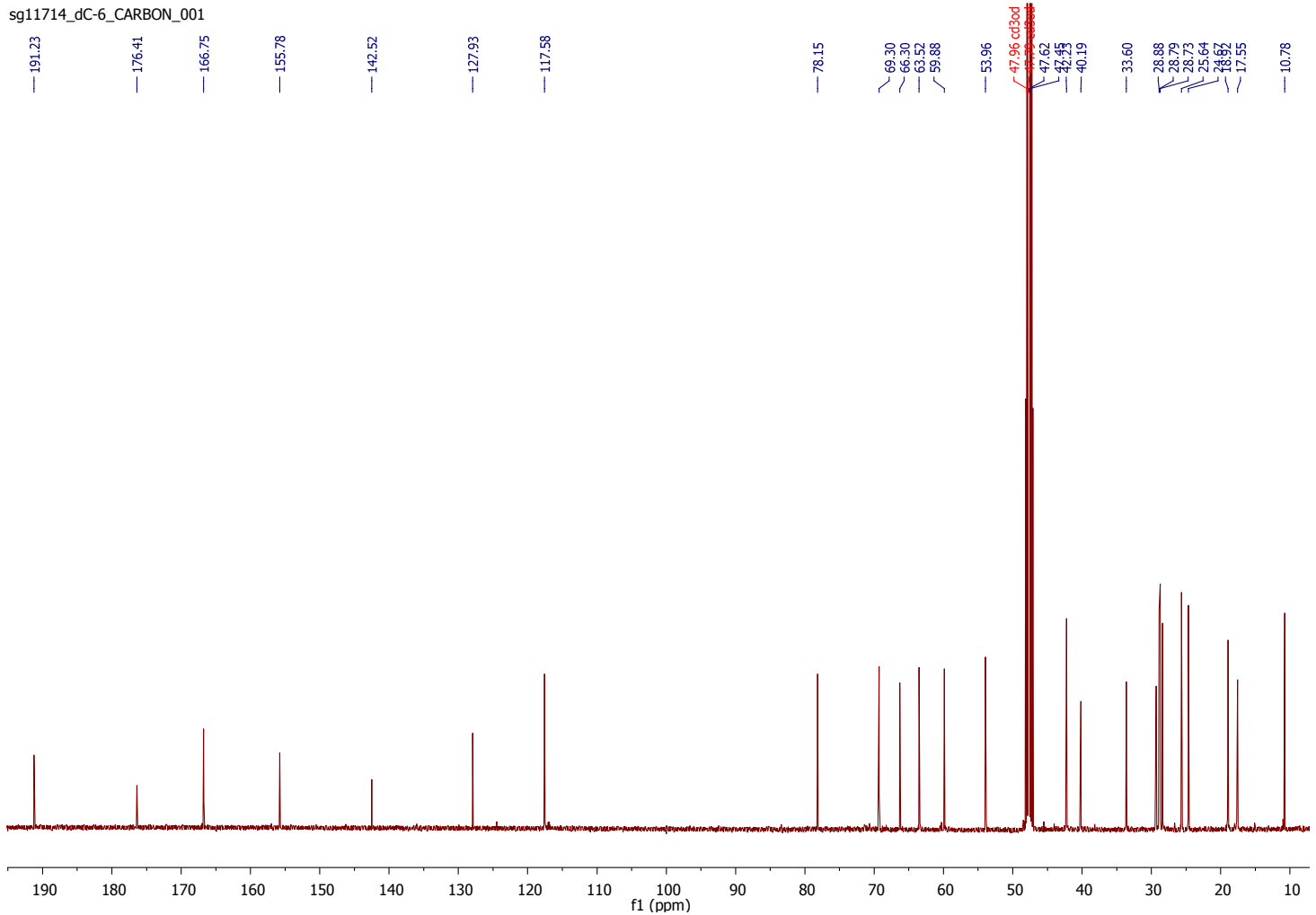


Figure S38. ^{1}H-^{1}H COSY spectrum of mupirocin C1 (**15**) measured in CD_3OD at 500 MHz.

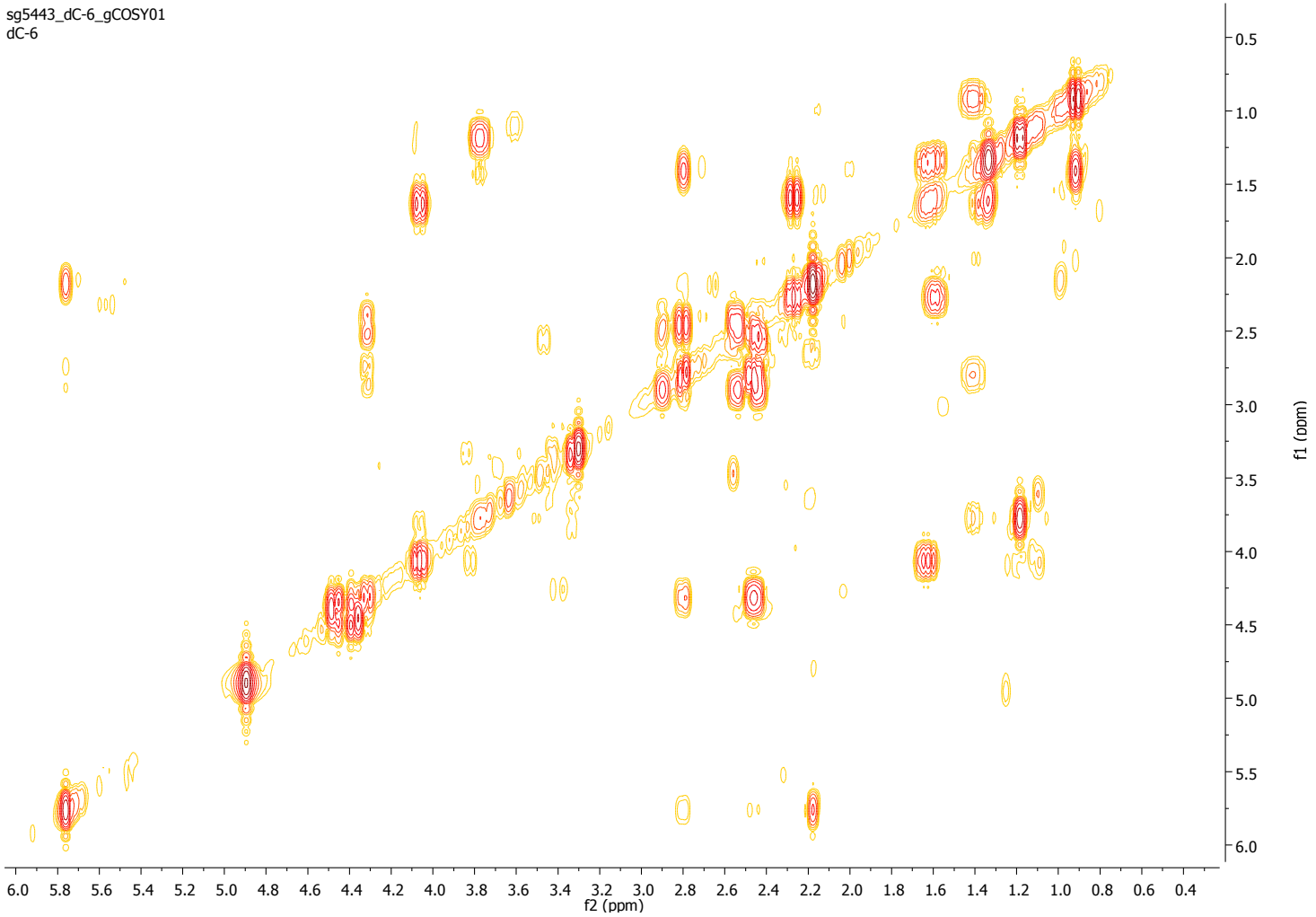


Figure S39. HSQC spectrum of mupirocin C1 (**15**) measured in CD_3OD at 500 MHz.

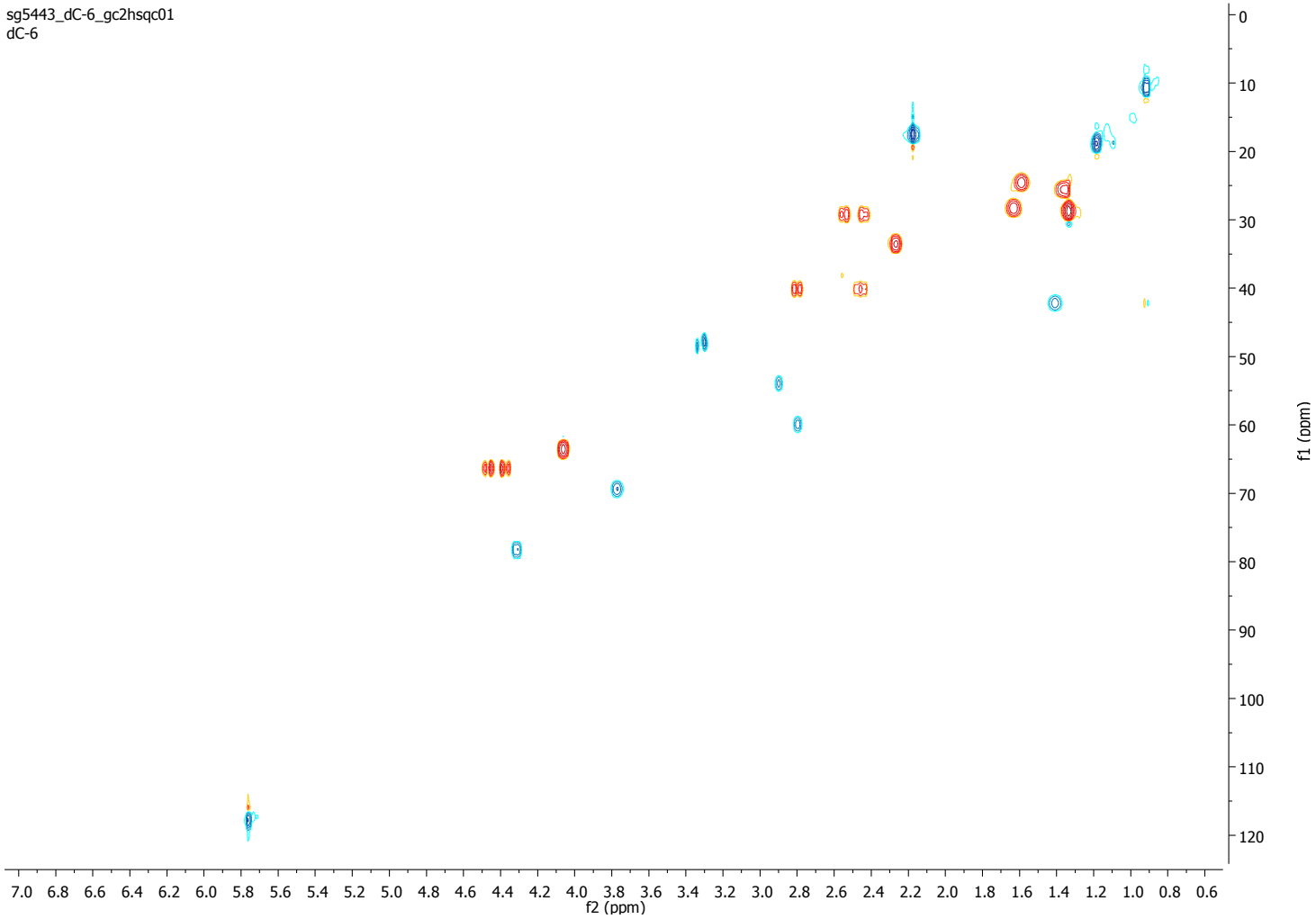


Figure S40. HMBC spectrum of mupirocin C1 (**15**) measured in CD_3OD at 500 MHz.

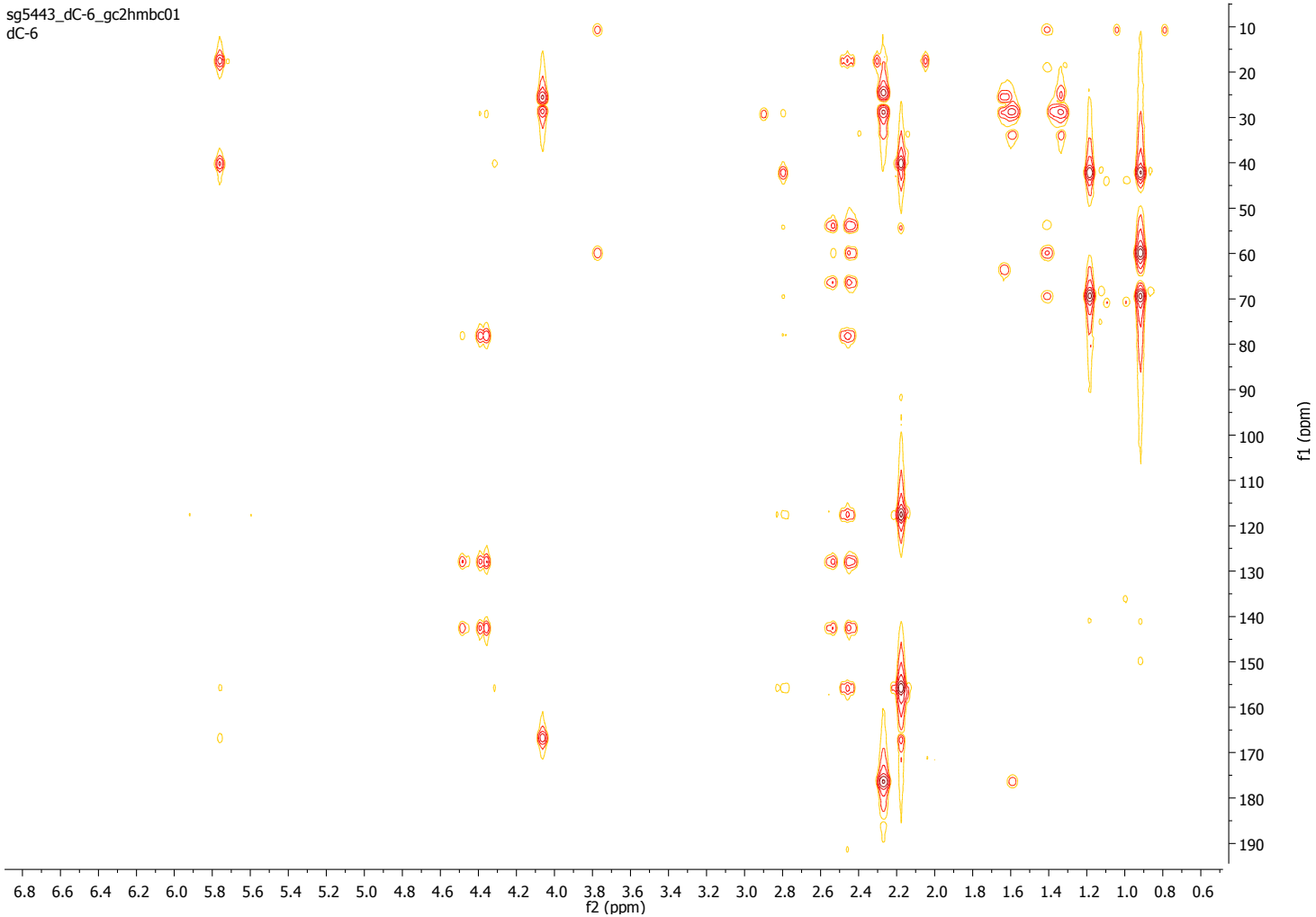


Figure S41. [1]H-NMR spectrum of mupirocin C2 (**16**) measured in CD_3OD at 500 MHz.

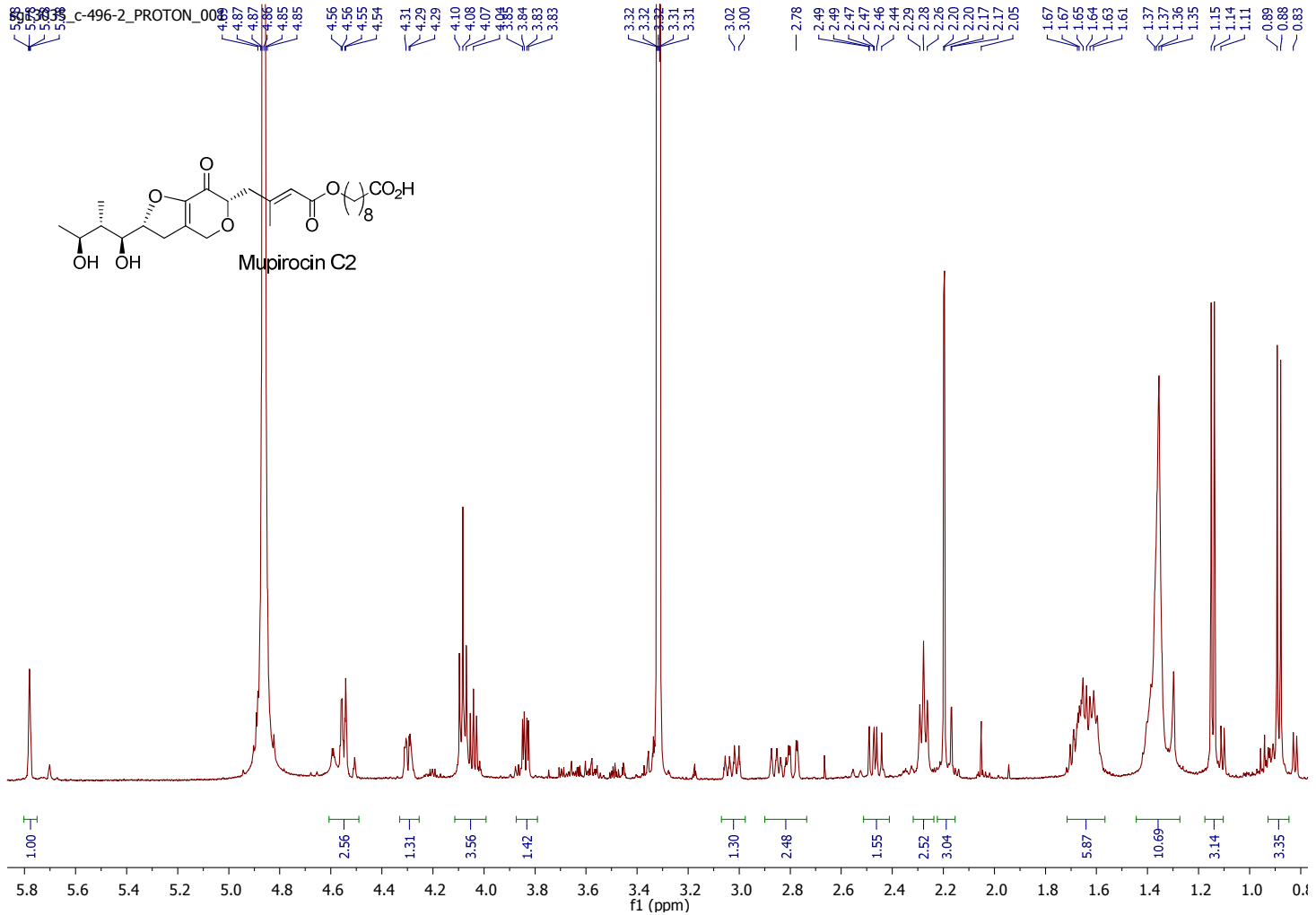


Figure S42. ^{13}C-NMR spectrum of mupirocin C2 (**16**) measured in CD_3OD at 125 MHz.

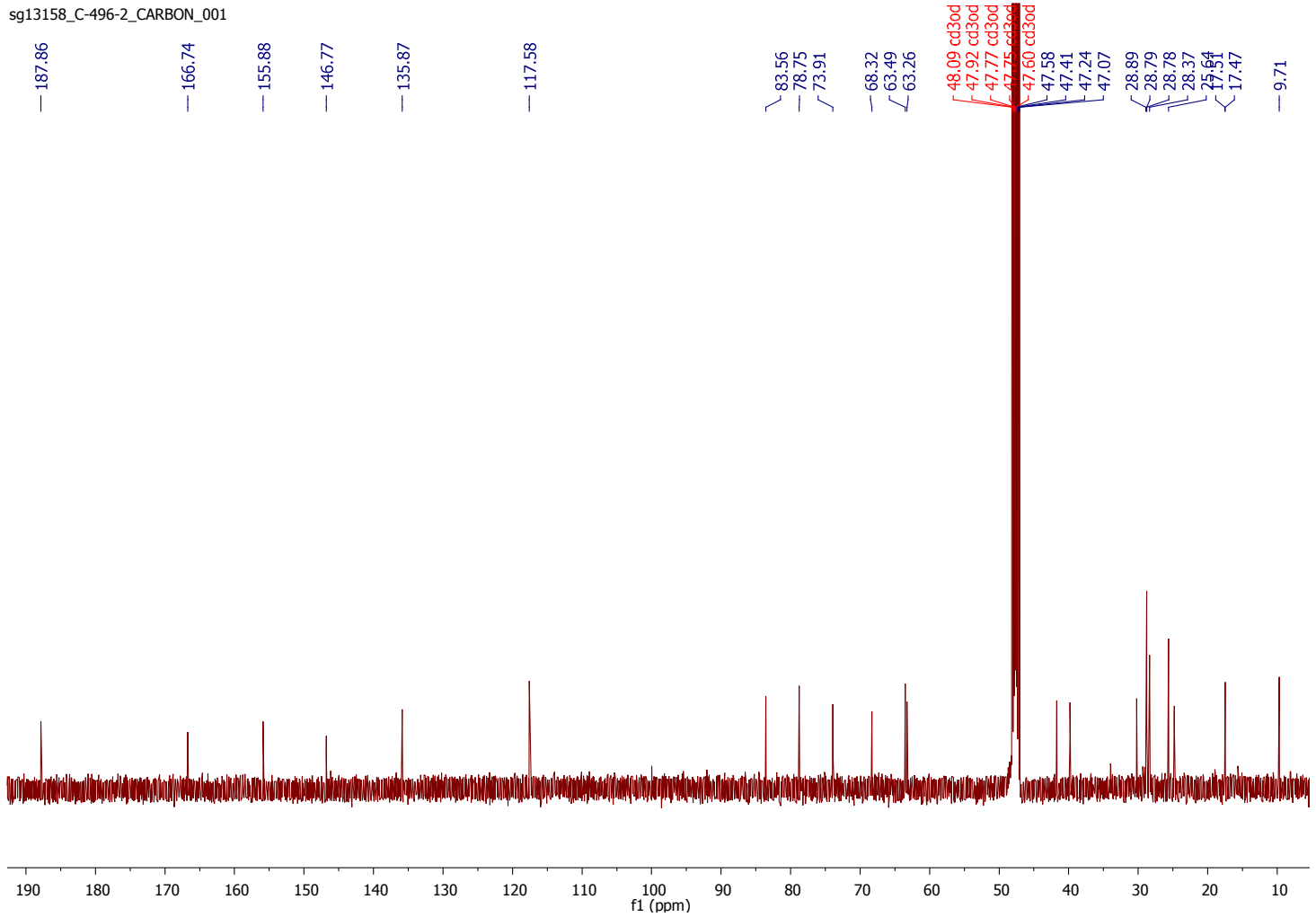


Figure S43. ^{1}H-^{1}H COSY spectrum of mupirocin C2 (**16**) measured in CD_3OD at 500 MHz.

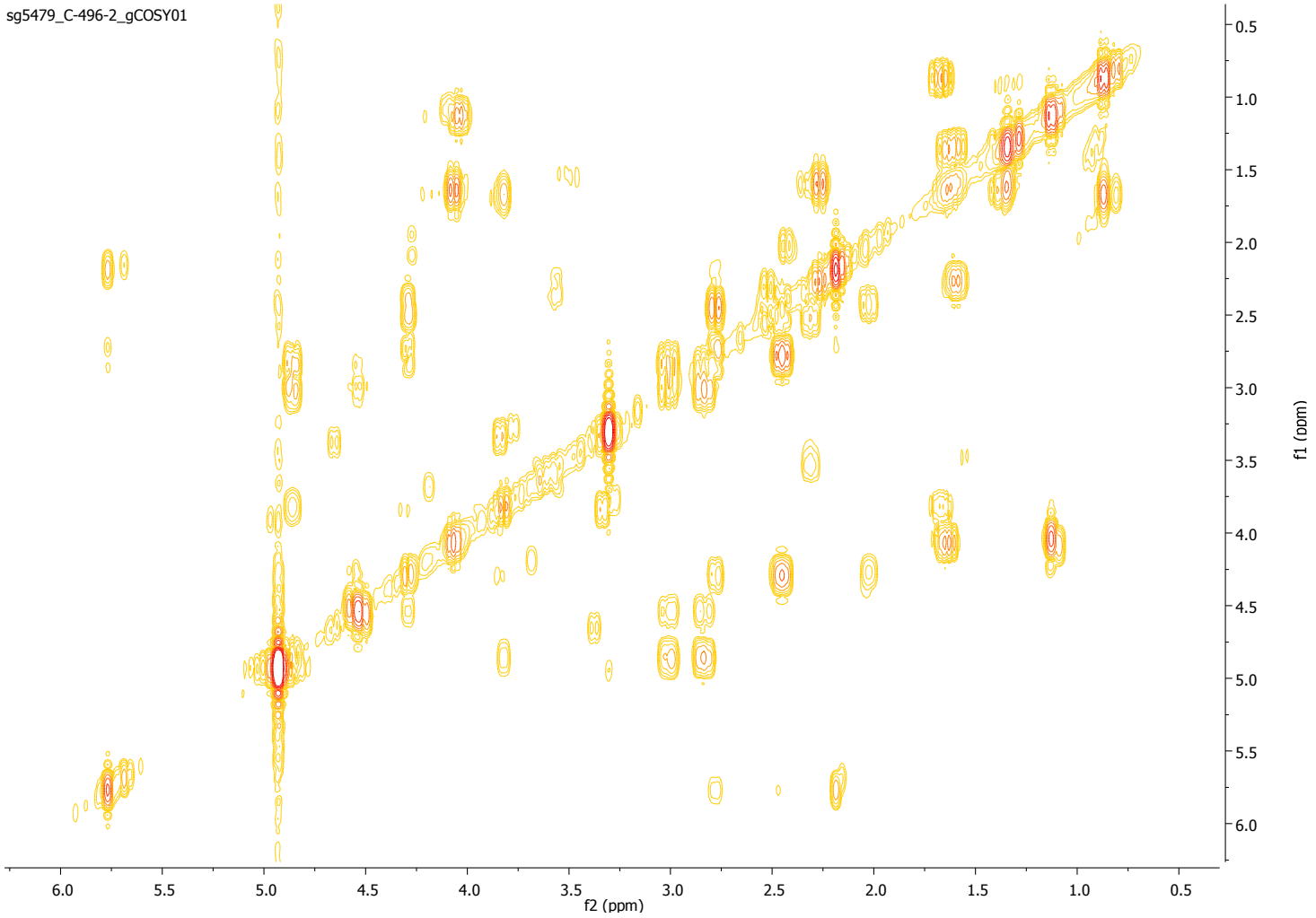


Figure S44. HSQC spectrum of mupirocin C2 (**16**) measured in CD_3OD at 500 MHz.

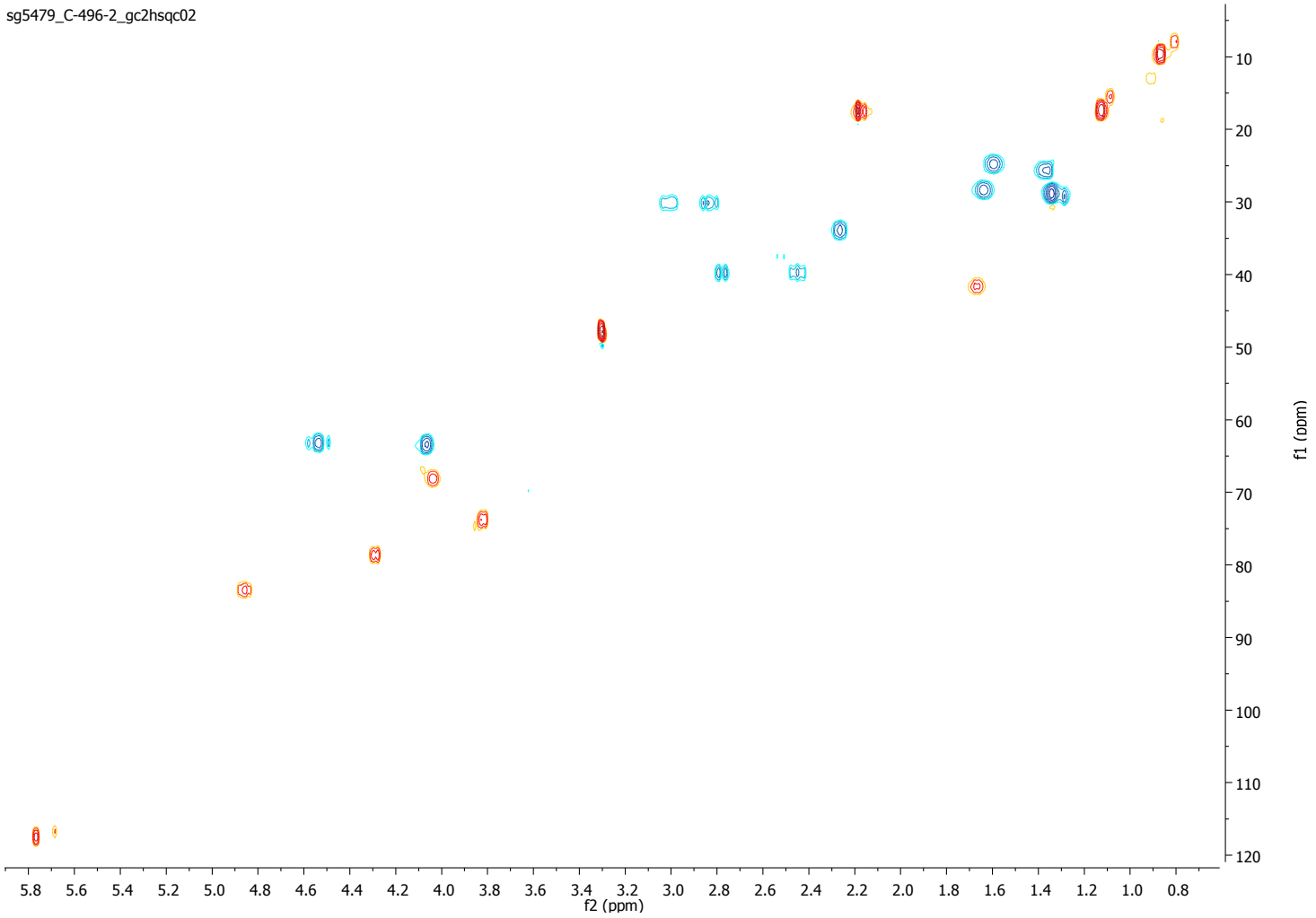


Figure S45. HMBC spectrum of mupirocin C2 (**16**) measured in CD_3OD at 500 MHz.

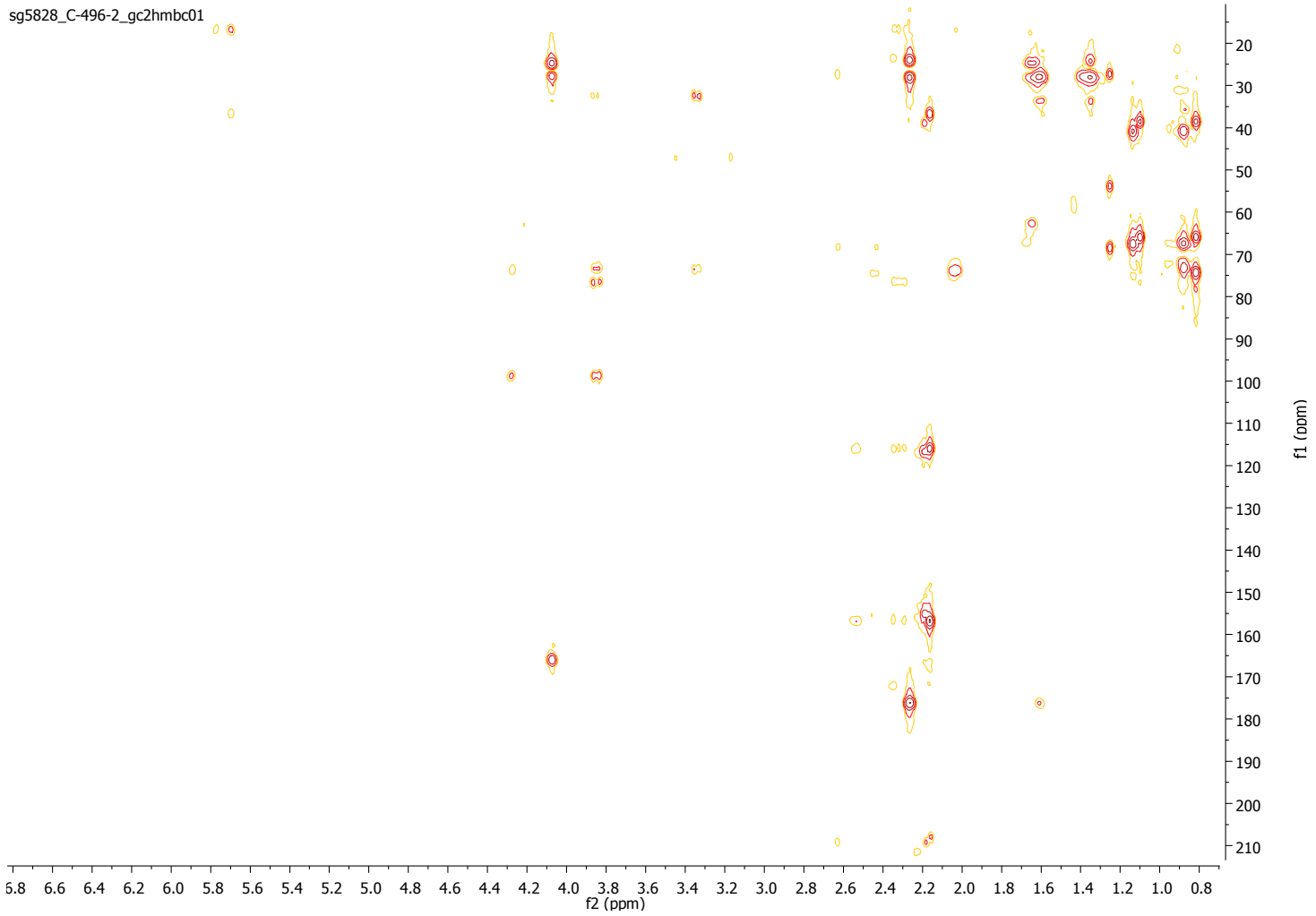


Figure S46. ^{1}H-NMR spectrum of mupirocin C3 (**17**) measured in CD_3OD at 500 MHz.

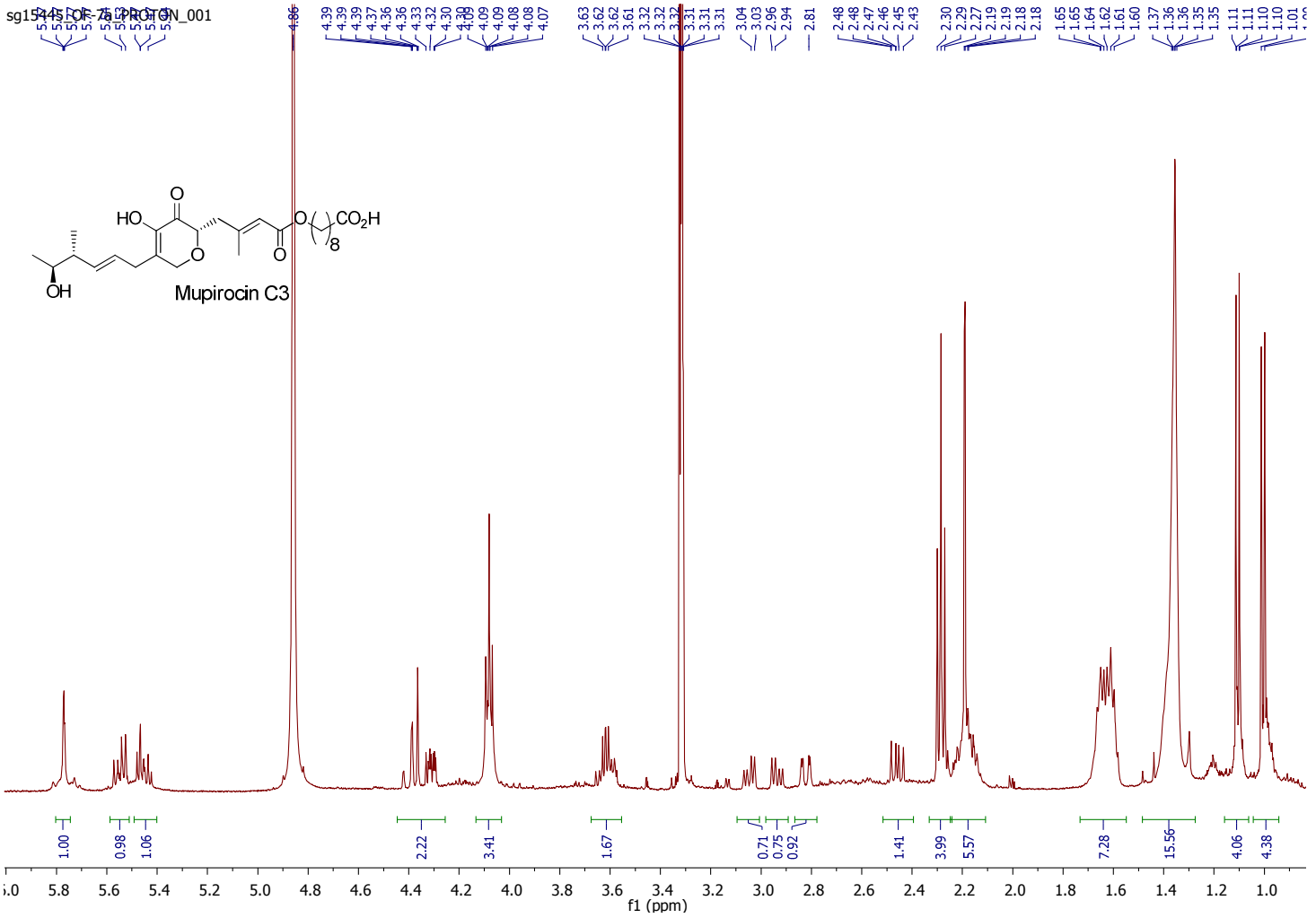


Figure S47. ^{1}H-^{1}H COSY spectrum of mupirocin C3 (**17**) measured in CD_3OD at 500 MHz.

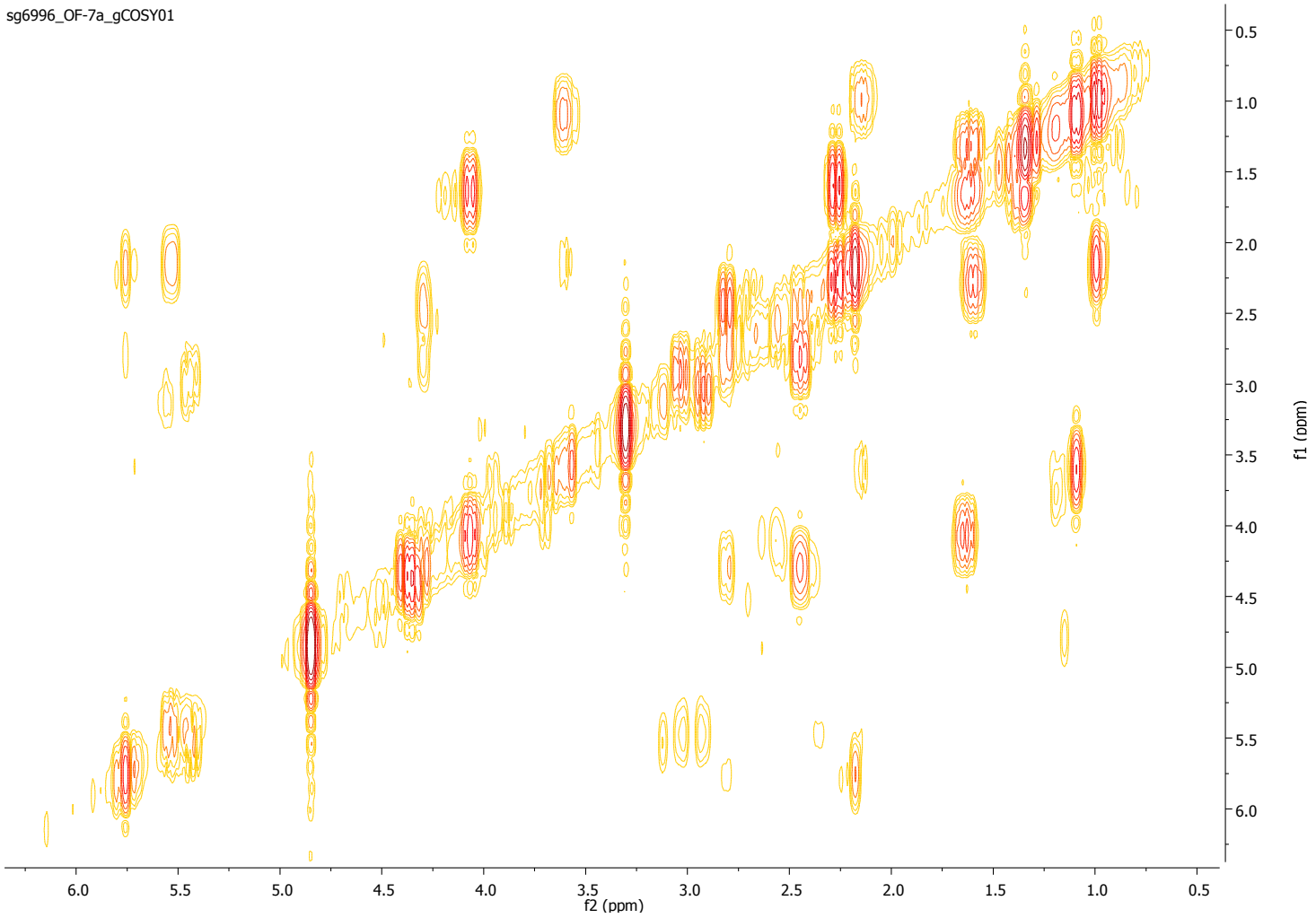


Figure S48. HSQC spectrum of mupirocin C3 (**17**) measured in CD_3OD at 500 MHz.

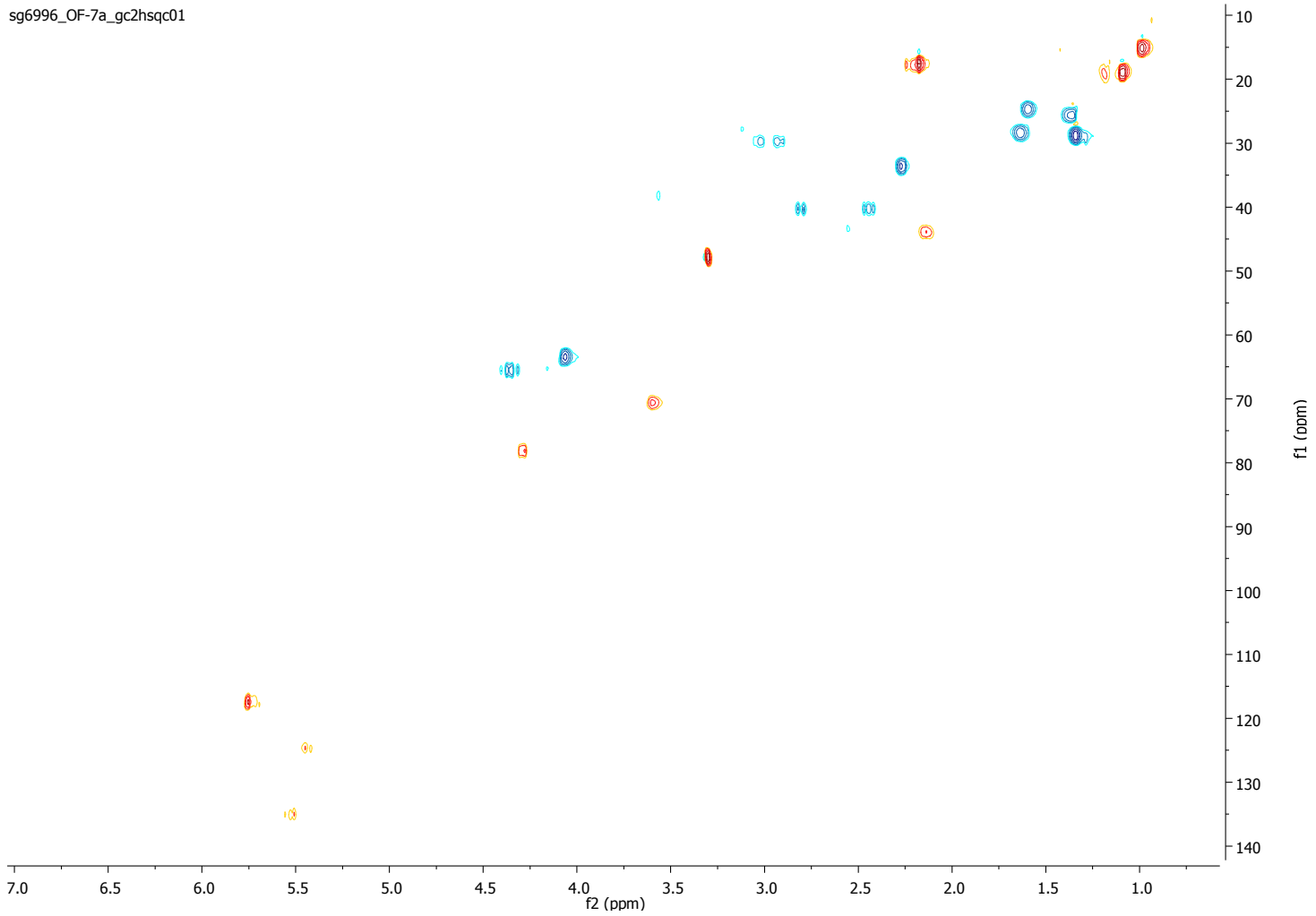


Figure S49. HMBC spectrum of mupirocin C3 (**17**) measured in CD_3OD at 500 MHz.

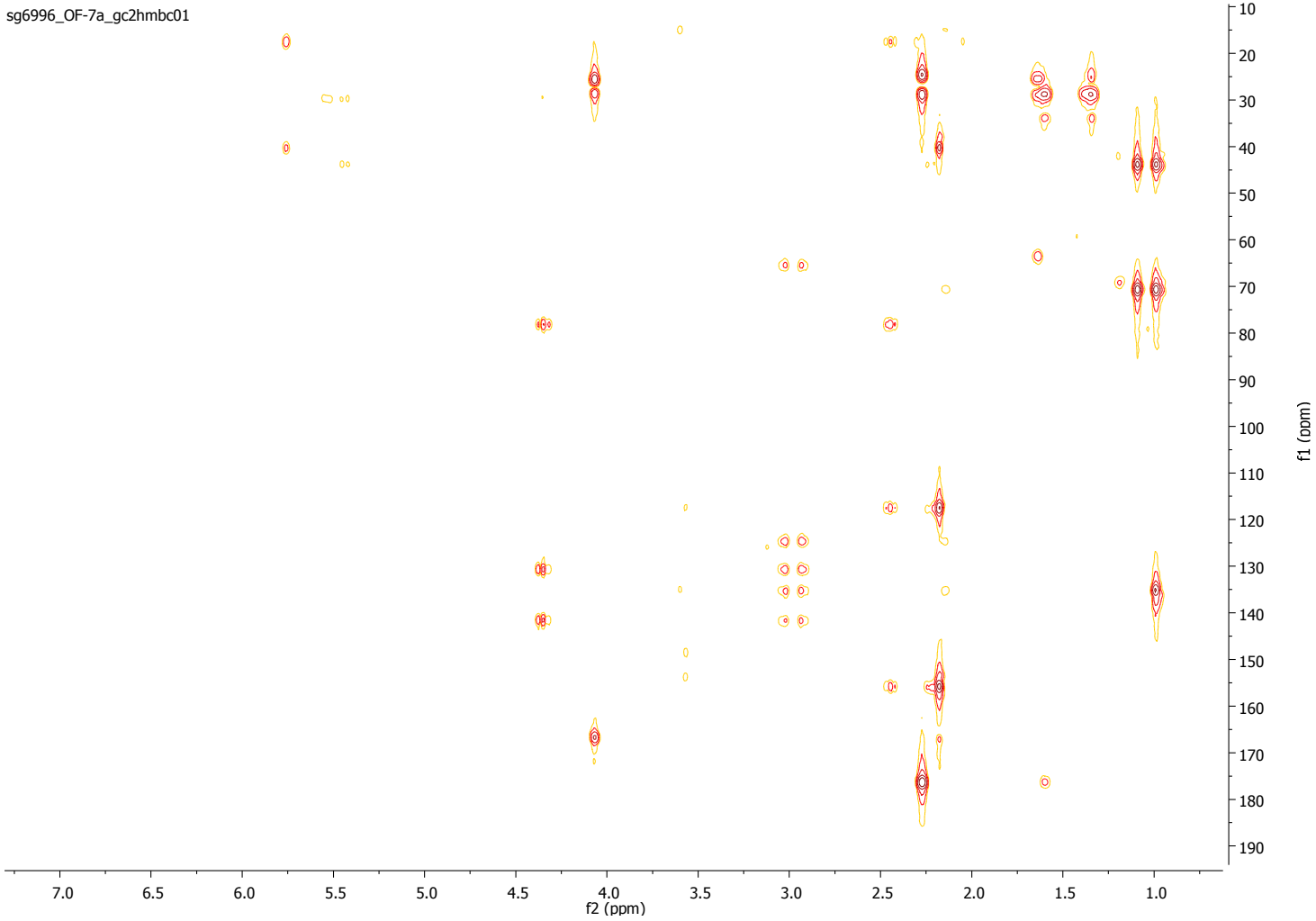


Figure S50. ^{1}H-NMR spectrum of mupirocin F1 (**18a** and **18b**) measured in CD_3OD at 500 MHz.

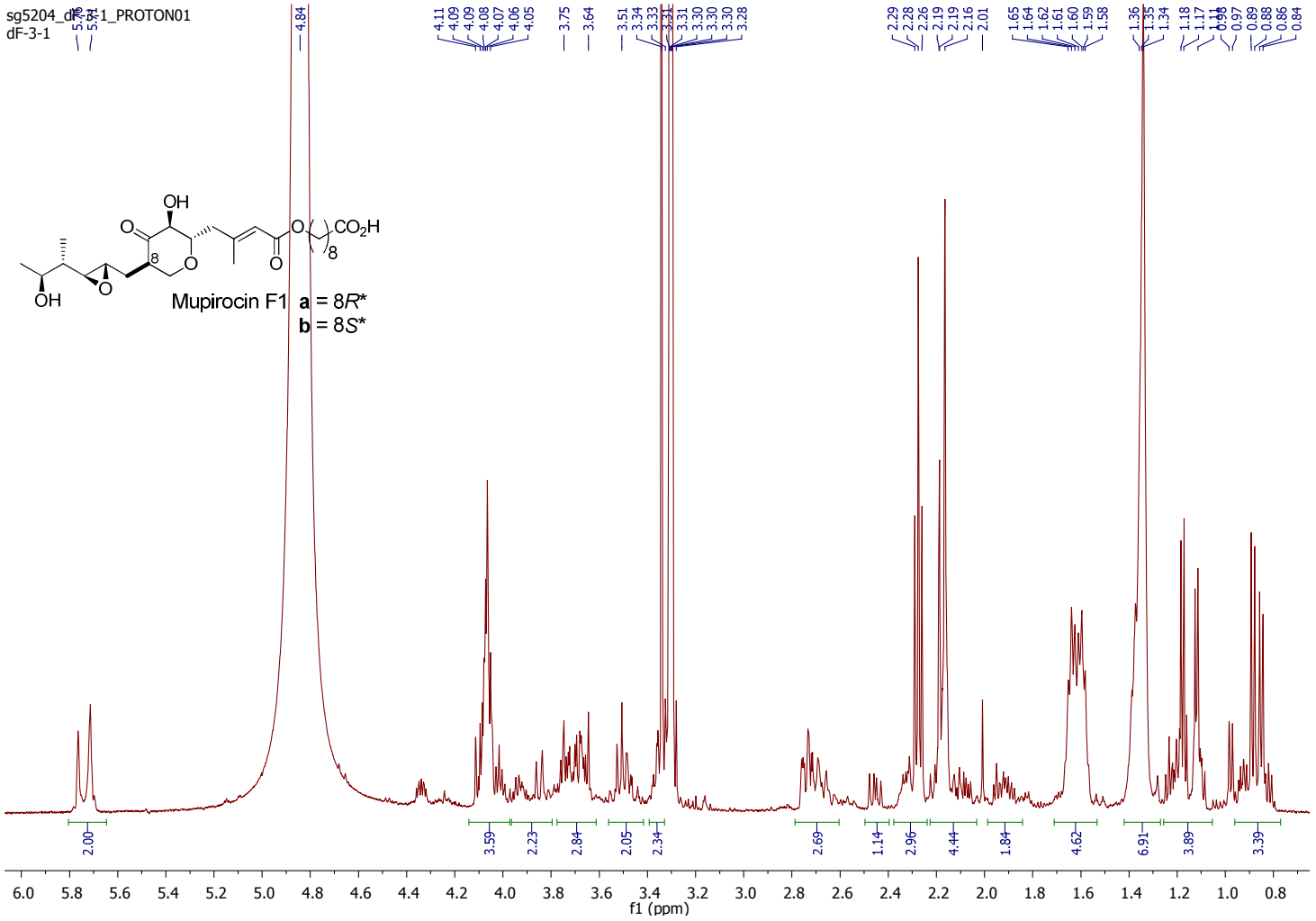


Figure S51. ^{1}H-NMR spectrum of mupirocin F2 (**19**) measured in CD_3OD at 500 MHz.

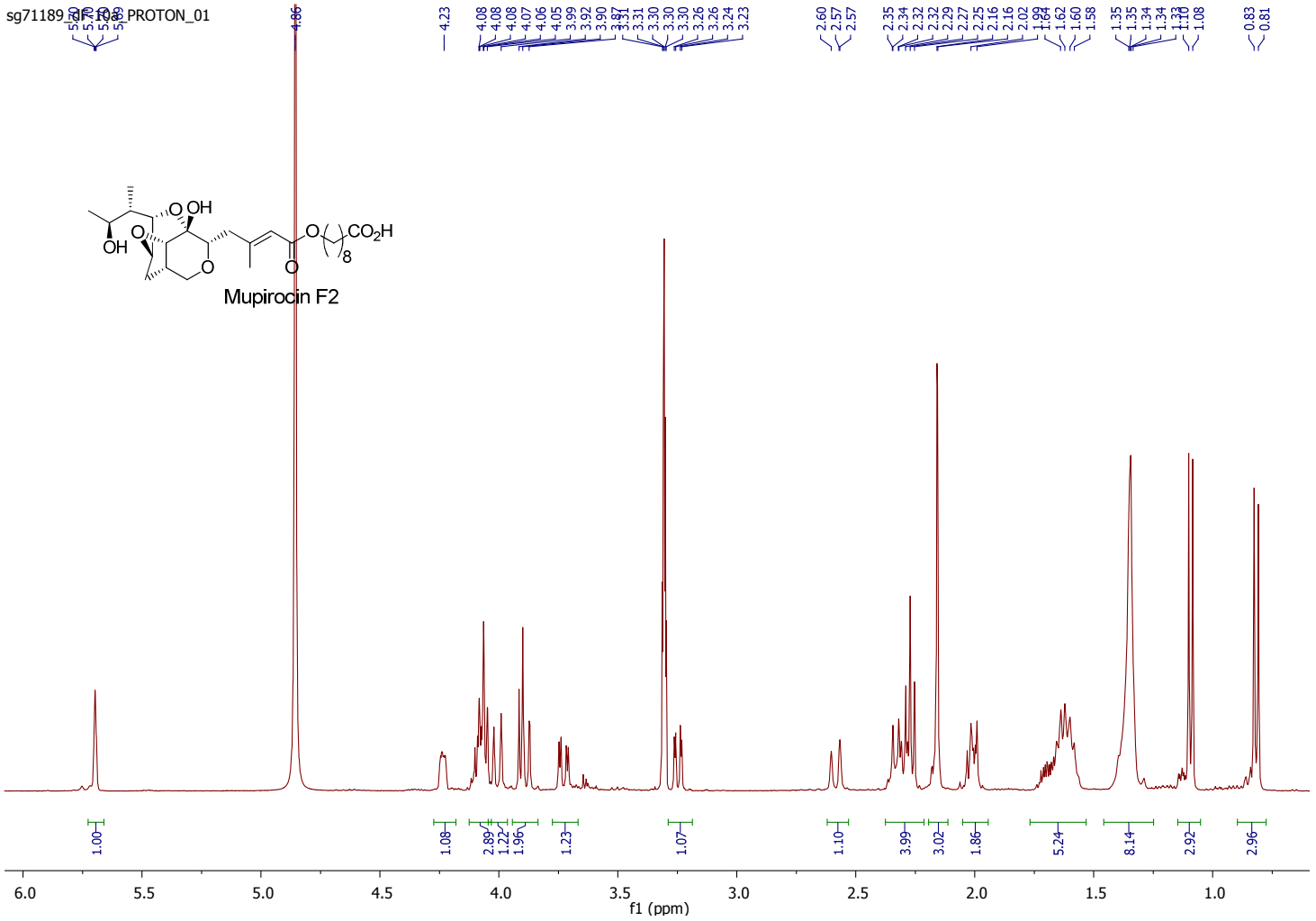


Figure S52. ^{1}H-NMR spectrum of desepoxy-mupirocin F1 (**20**) measured in CD_3OD at 500 MHz.

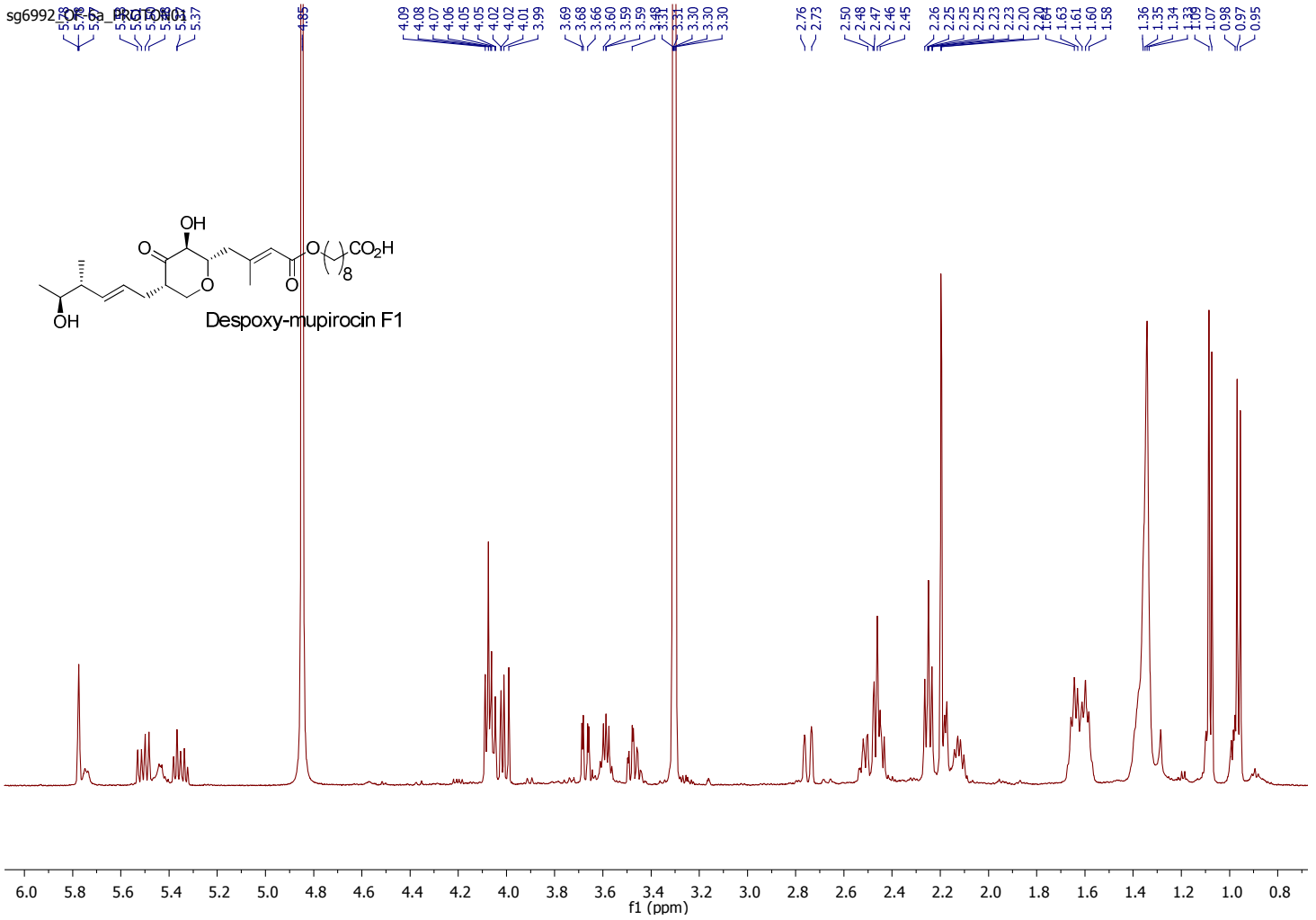


Figure S53. ^{1}H-^{1}H COSY spectrum of desepoxy-mupirocin F1 (**20**) measured in CD_3OD at 500 MHz.

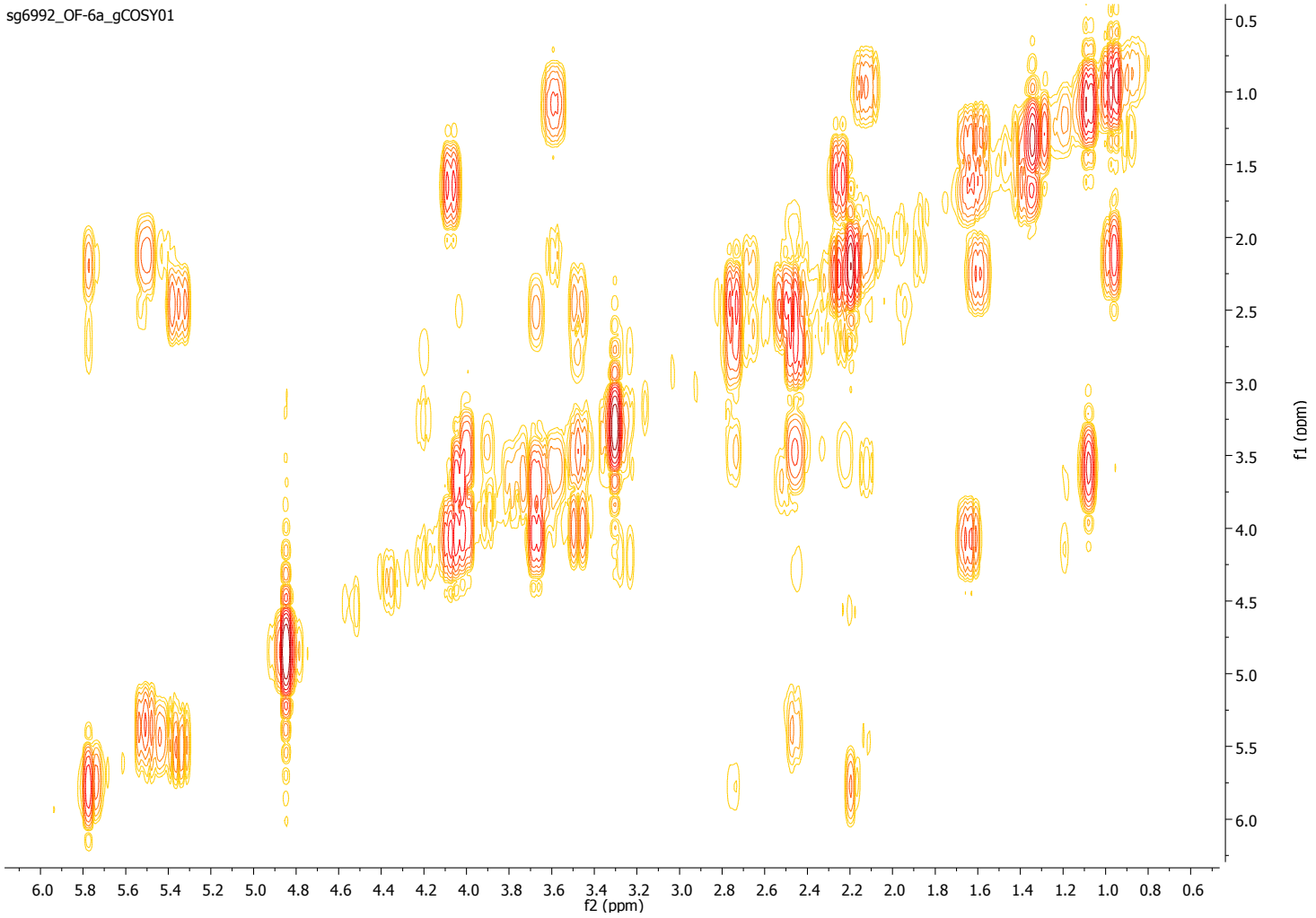


Figure S54. HSQC spectrum of desepoxy-mupirocin F1 (**20**) measured in CD_3OD at 500 MHz.

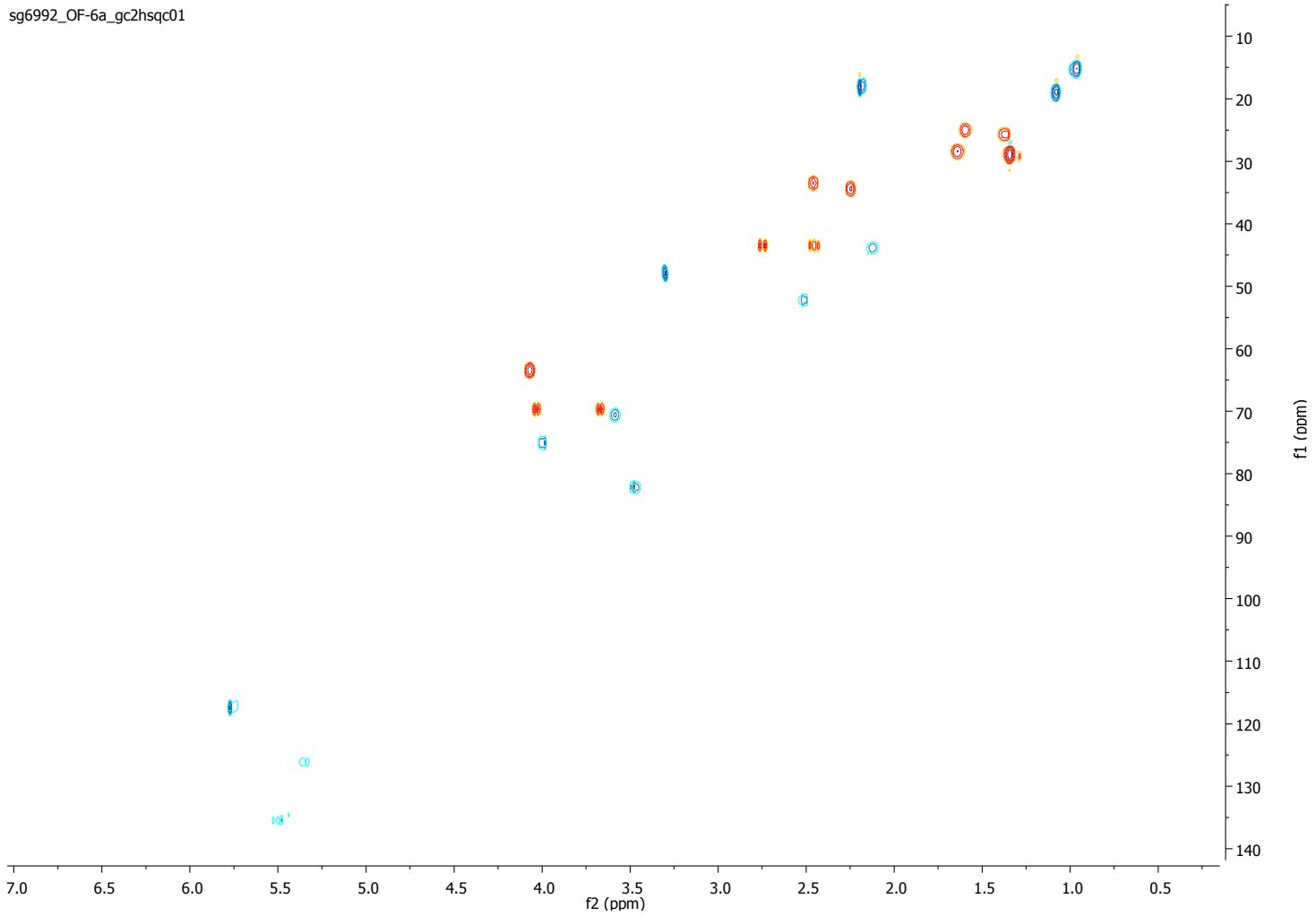


Figure S55. HMBC spectrum of desepoxy-mupirocin F1 (**20**) measured in CD_3OD at 500 MHz.

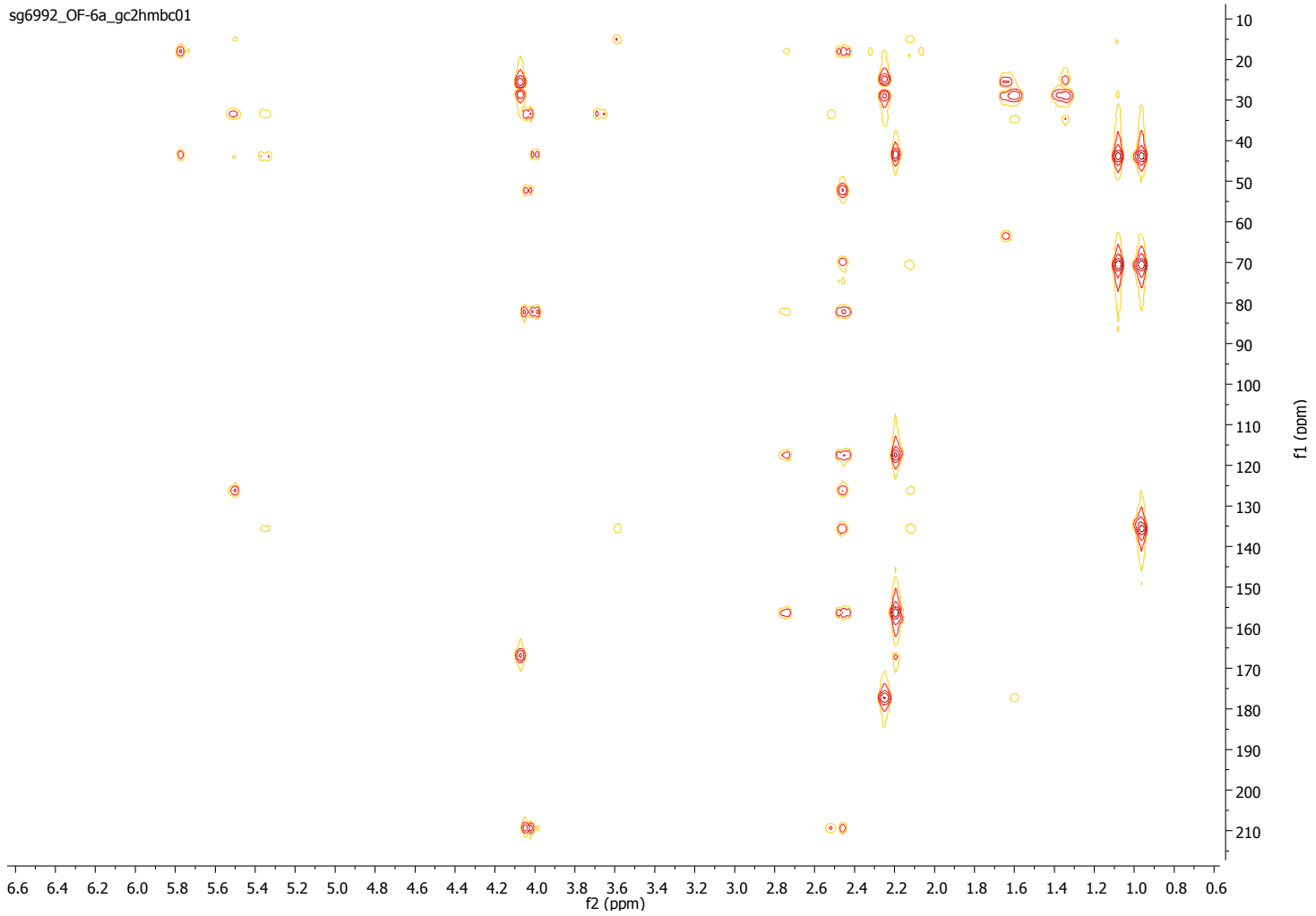


Figure S56. ^{1}H-NMR spectrum of 7-keto-mupirocin W4 (**21**) measured in CD_3OD at 400 MHz.

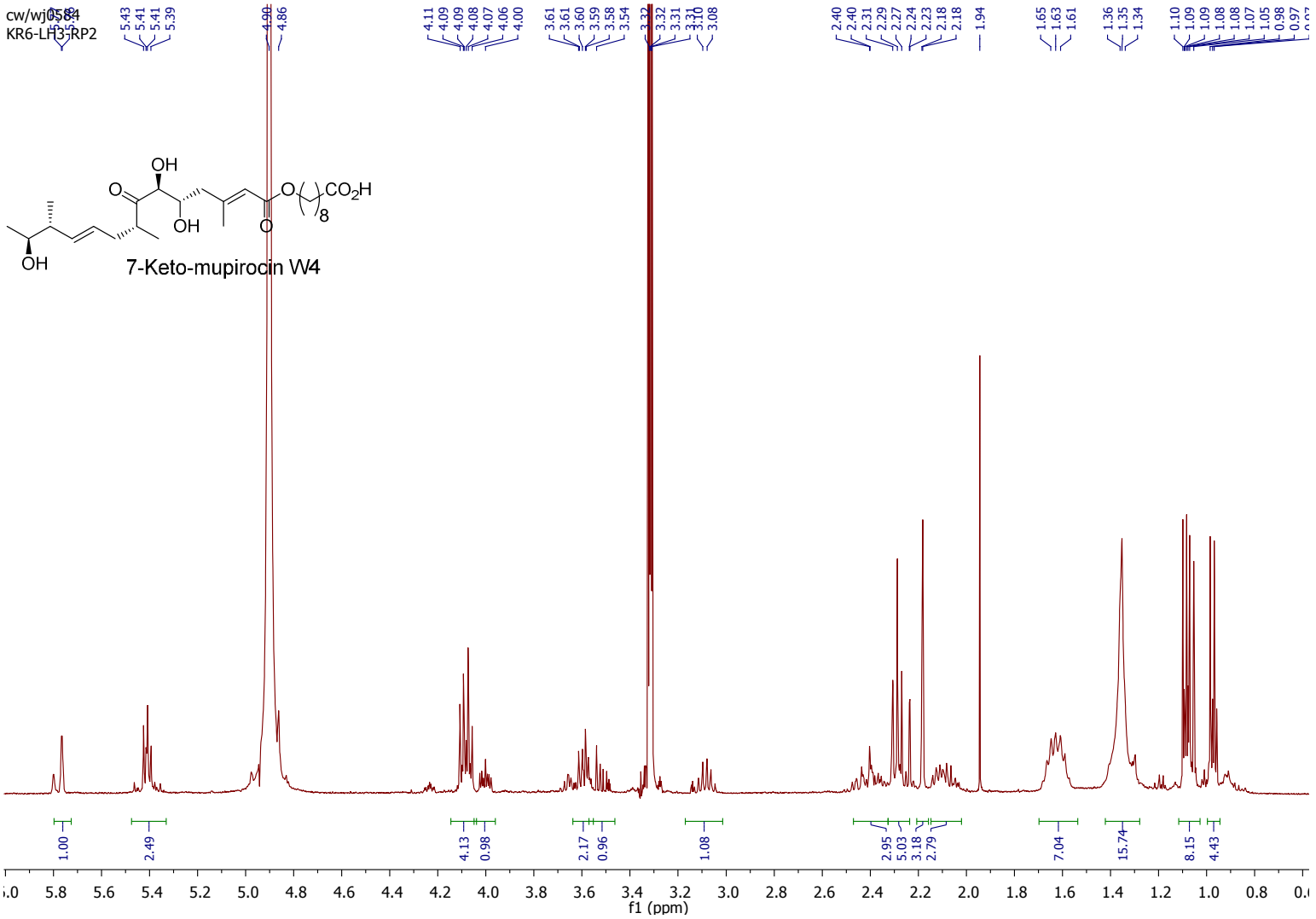


Figure S57. ^{13}C-NMR spectrum of 7-keto-mupirocin W4 (**21**) measured in CD_3OD at 100 MHz.

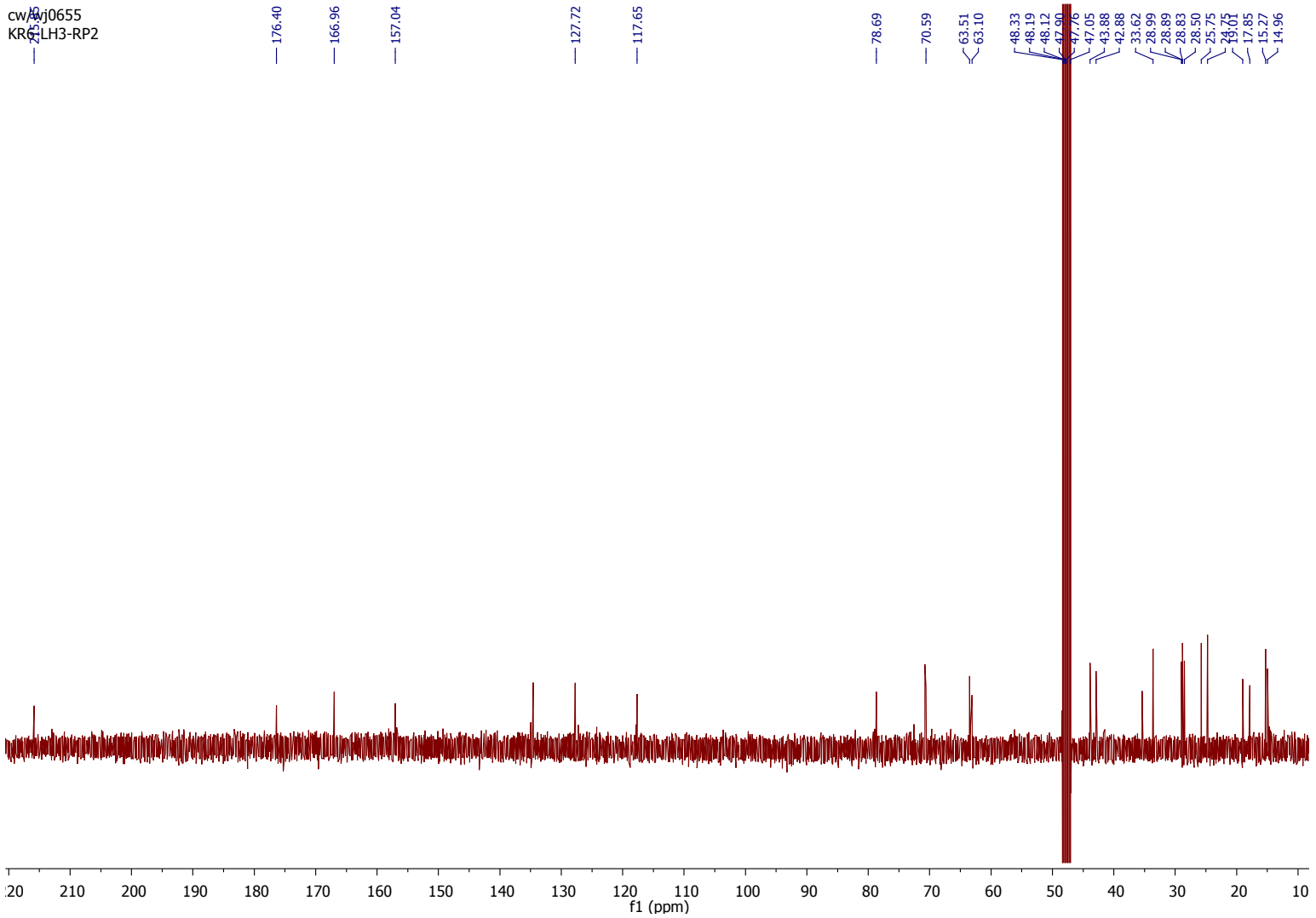


Figure S58. ^{1}H-^{1}H COSY spectrum of 7-keto-mupirocin W4 (**21**) measured in CD_3OD at 400 MHz.

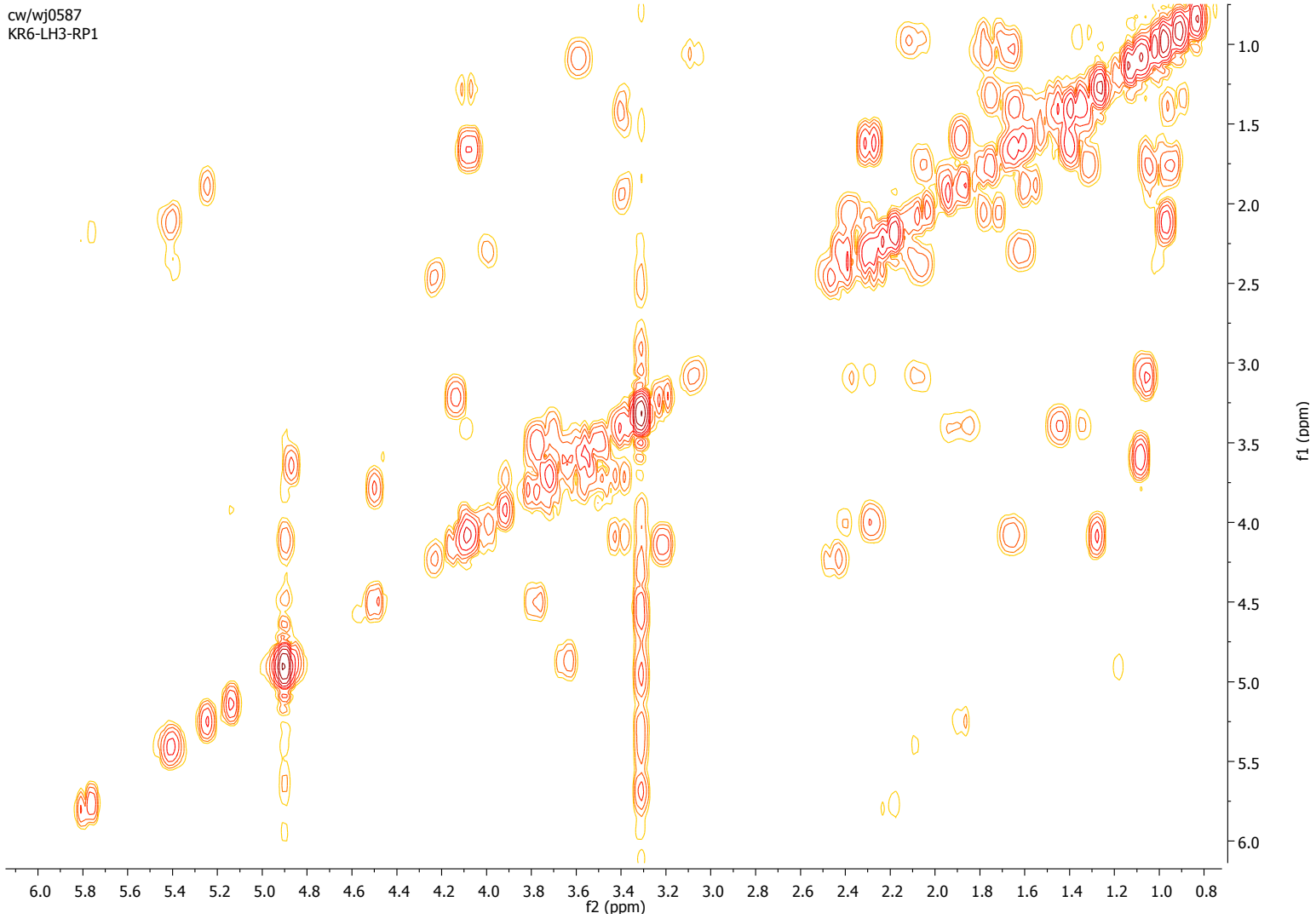


Figure S59. HSQC spectrum of 7-keto-mupirocin W4 (**21**) measured in CD_3OD at 400 MHz.

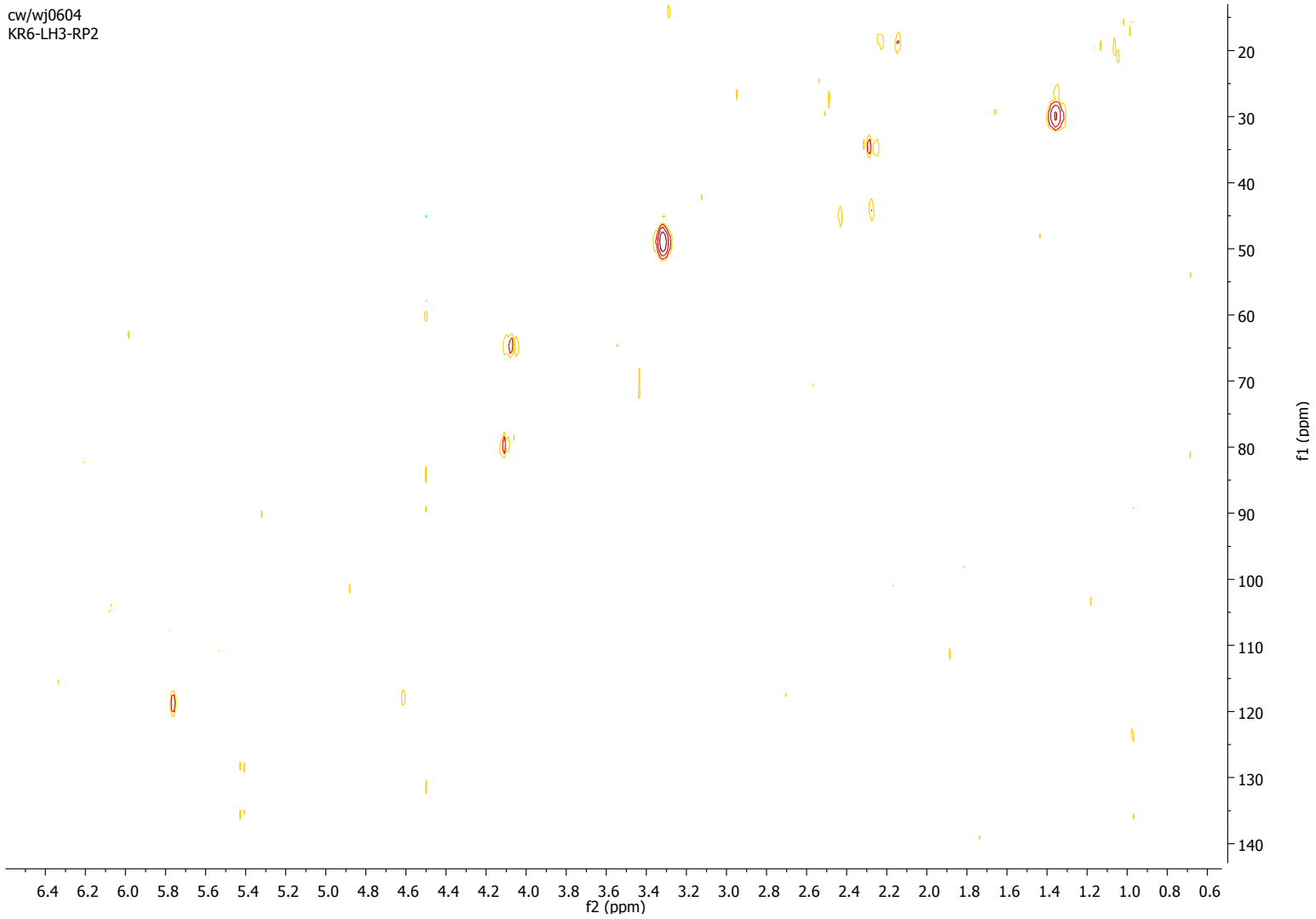


Figure S60. HMBC spectrum of 7-keto-mupirocin W4 (**21**) measured in CD_3OD at 400 MHz.

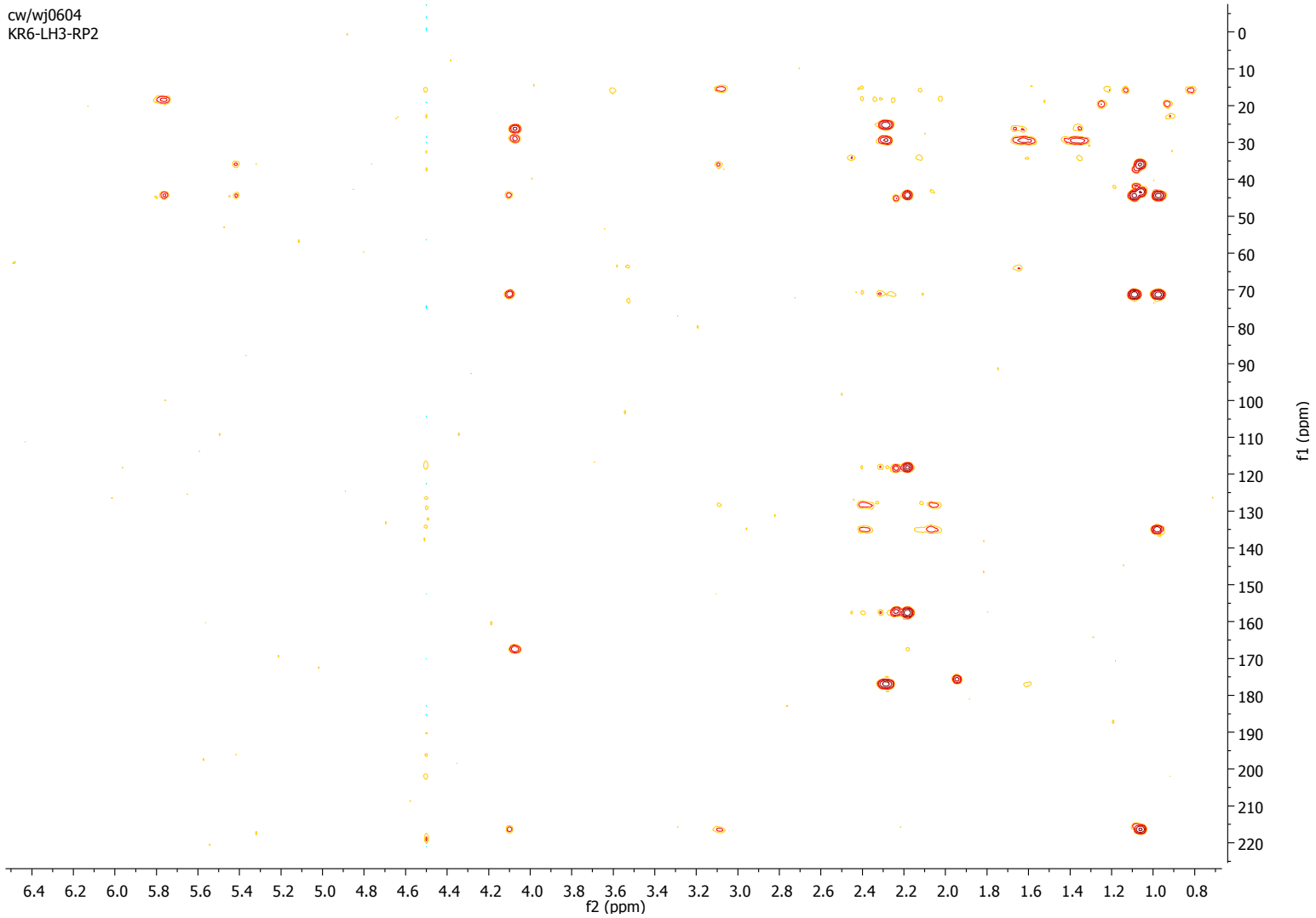


Figure S61. ^{1}H-NMR spectrum of 7-keto-mupirocin W5 (**22**) measured in CD_3OD at 400 MHz.

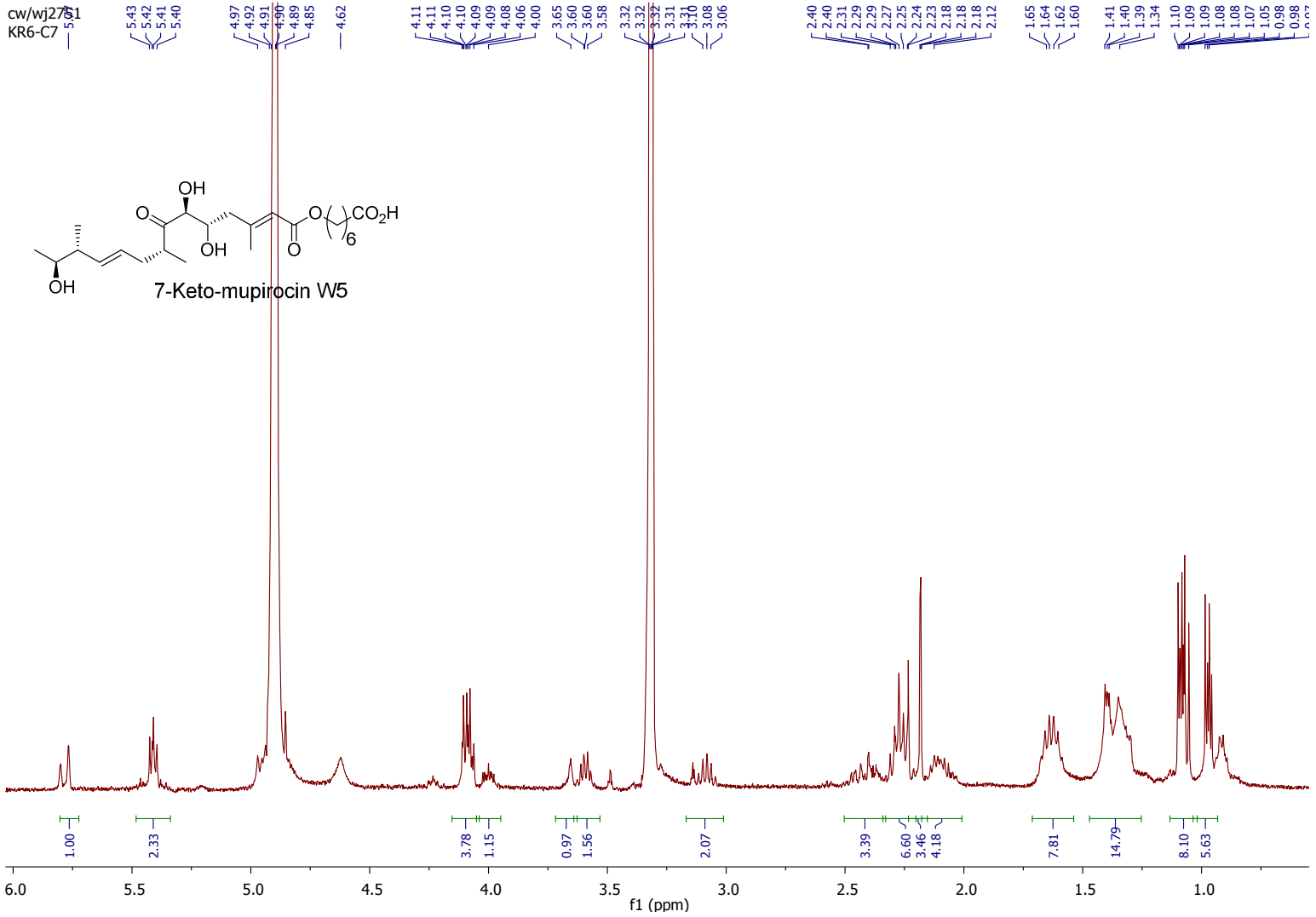


Figure S62. ^{1}H-^{1}H COSY spectrum of 7-keto-mupirocin W5 (**22**) measured in CD_3OD at 400 MHz.