SUPPLEMENTARY MATERIAL

Isolation and Structural Characterization of a Non-Diketopiperazine Phytotoxin from a Potato Pathogenic *Streptomyces* Strain

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Abstract

Two *Streptomyces* spp. strains responsible for potato common scab infections in Uruguay which do not produce diketopiperazines were identified through whole-genome sequencing, and the virulence factor produced by one of them was isolated and characterized. Phylogenetic analysis showed that both pathogenic strains can be identified as *S. niveiscabiei*, and the structure of the phytotoxin was elucidated as that of the polyketide desmethylmensacarcin using MS and NMR methods. The metabolite is produced in yields of ~200 mg/L of culture media, induces deep necrotic lesions on potato tubers, stuns root and shoot growth in radish seedlings, and is comparatively more aggressive than thaxtomin A. This is the first time that desmethylmensacarcin, a member of a class of compounds known for their antitumor and antibiotic activity, is associated with phytotoxicity. More importantly, it represents the discovery of a new virulence factor related to potato common scab, an economically-important disease affecting potato production worldwide.

Keywords: Desmethylmensacarcin; Phytotoxin; Potato common scab; *Streptomyces* spp.

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Experimental section

General experimental procedures

HPLC was performed on a Shimadzu Prominence LC-20AT equipped with an SPD-M20A diode-array detector and fitted with an Agilent ZORBAX Eclipse XDB-C18 column (4.6 x 250 mm, 5 μm particle size). Preparative TLC was carried out on silica gel 60G F_{254} plates. Melting points were determined on an Electrothermal IA9100 melting point apparatus and are uncorrected. IR spectra were obtained on a Shimadzu IRAffinity-1 FT-IR spectrophotometer fitted with a PIKE MIRacle™ single reflection ATR accessory. Optical rotations were recorded on a Bellingham+Stanley ADP440+ polarimeter equipped with a 5 × 50 mm stainless steel cell. NMR experiments were carried out on Bruker AVANCE III 500 and 400 spectrometers operating at ^{1}H frequencies of 500.13 and 400.13 MHz, and ^{13}C frequencies of 125.76 and 100.62 MHz, respectively. Gradient-enhanced COSY, HSQC, and HMBC spectra were acquired using standard pulse sequences provided with the instruments. HR-ESIMS and $ESIMS^2$ data were collected on Bruker Daltonik Apex VI FT-ICR and AB SCIEX 4000 QTrap mass spectrometers, respectively.

Bacterial strains

The pathogenic strains used in this study were *Streptomyces acidiscabies* ST105, *S. scabiei* ST129, *S. europaeiscabiei* ST1229, *Streptomyces* sp. ST1015, and *Streptomyces* sp. ST1020. These five strains were isolated from potatoes with common scab lesions in Uruguay and identified in previous work (Lapaz et al. 2017). *S. acidiscabies* strain DSM41668 from the DSMZ culture collection (Braunschweig, Germany) was used as reference. Isolates were routinely grown on yeast-malt extract (YME) agar at 28 °C (Shirling & Gottlieb 1966), and maintained as spore suspensions in 20% glycerol at -70 °C.

Phylogenomic analysis

Genomic DNA from *Streptomyces* isolates was extracted according to Sambrook and Russell (2001). Whole-genome sequencing of strains ST105, ST129, and ST1229 was performed with the Illumina MiSeq platform. Around 20 million 100 bp paired-end reads for each genome were obtained, producing approximately 100X coverage. Reads were assembled using SPAdes 3.5 (Bankevich et al. 2012). Resulting contigs were annotated using the Prokka annotation pipeline (Seemann 2014). The draft genome sequences have been deposited in the NCBI Whole Genome Shotgun database with accession numbers PCGS00000000, PCGT00000000, and PCGU00000000, respectively. The phylogenetic

analysis was constructed with the genomic sequences of the three strains mentioned above, ST1015 and ST1020 (Lapaz et al. 2017), and 40 other pathogenic and non-pathogenic *Streptomyces* genome sequences obtained from GenBank. Parsnp (Treangen et al. 2014) was used to obtain core SNPs from the whole aligned genome sequences. The phylogenetic tree was reconstructed from the core SNPs using the maximum likelihood method implemented in RAxML (Stamatakis 2006) with the GTR gamma substitution model. The confidence of the tree was estimated using 1000 bootstrap replicates.

Extraction and detection of thaxtomin A

Streptomyces spp. strains were cultured in 5 mL of tryptic soy broth (TSB) medium from 48 to 72 h. Cells were pelleted by centrifugation at 10000 rpm, washed twice with sterile water, and resuspended in enough sterile water to obtain an OD_{600} of ~1.0. An aliquot of the cell suspension (200 μL) was inoculated into 3 × 50 mL of oat bran broth (OBB) medium (Johnson et al., 2007). Cultures were aerated and incubated at 28 °C in an orbital shaker at 150 rpm for 7 days. Extraction and detection of compound thaxtomin A was carried out following the protocol described by Johnson and coworkers (2007) with minor modifications. Briefly, OBB supernatants were aliquoted in 15 mL tubes and frozen at -20 °C overnight before further processing. The aliquots were then thawed, and cells and particulates were removed by centrifugation at 4 °C and 4000 rpm for 5 min. 1 mL of the resulting supernatants were applied to Discovery® C18 SPS cartridges that had been preconditioned by passing CH_3OH (1 mL) and deionized water (1 mL). Once loaded, the cartridges were washed with deionized water (1 mL) and a CH_3OH/water solution (25:75, 1 mL), and then eluted with CH_3OH (1 mL). These extracts were loaded onto the HPLC column and eluted with an isocratic mobile phase consisting of 40% acetonitrile and 60% water at a constant flow rate of 1.0 mL/min and monitored at 380 nm. Thaxtomin A was identified by comparison of its retention time to that of a standard (see Figure S2).

Isolation and characterization of desmethylmensacarcin

Cultures of *Streptomyces* ST1015 were grown in OBB medium as described previously for the production of thaxtomin A. The cells were pelleted by centrifugation at 10000 rpm for 15 min, and the supernatant was filter-sterilized using 0.45 μm syringe filters and lyophilized. 20 mg of the lyophilized material were dissolved in a small quantity of absolute CH_3OH and subjected to preparative TLC using EtOAc/CH_2Cl_2 (75:25) as eluant to afford compound **1** in 17.5% yield (3.5 mg), which corresponds to 201.3 mg/L of culture media.

*Desmethylmensacarcin (**1**).* Pale yellow crystalline solid. Rf: 0.50 (EtOAc/CH_2Cl_2, 75:25). M.p.: 134.0 °C (dec.). IR (ATR) ν_{max}/cm^{-1}: 3447, 2926, 1723, 1688, 1597, 1456, 1438, 1259, 1117, 1072, 946, 756 (see Figure S4). $[\alpha]_D^{22}$: -67.0 (c = 0.18, CH_3OH). ^1H and ^{13}C NMR: see Table S1, Table S2, and Figures S5-S10. HR-ESIMS *m*/*z*: 407.1324 $[M+H]^+$ (calcd. for $C_{20}H_{23}O_9$: 407.1337), 429.1143 $[M+Na]^+$ (calcd. for $C_{20}H_{22}O_9Na$: 429.1156), 835.2403 $[2M+Na]^+$ (calcd. for $C_{40}H_{44}O_{18}Na$: 835.2420) (see Figure S11). $ESIMS^2$ (407→): *m*/*z* 425, 389, 371, 353 (see Figure S12).

Pathogenicity and phytotoxicity assays

The pathogenicity of the different *Streptomyces* spp. strains was tested on potato disks and radish seedlings as described earlier (Lapaz et al. 2017). The phytotoxicity of metabolites secreted by the strains was screened as recently reported by Fyans and coworkers (2016). The strains were cultured in OBB medium as outlined above, the cells were pelleted by centrifugation at 10000 rpm, and the supernatants were filter-sterilized using 0.45 μm syringe filters and assayed on potato tuber slices and radish seedlings (see Figure S1). In addition, the phytotoxicity of compound **1** at a concentration of 1 mg/mL was evaluated on potato tuber slices and radish seedlings in CH_3OH and aqueous solutions, respectively. To compare the phytotoxicities of thaxtomin A and **1** they were evaluated on potato discs analogously, using in this case CH_3OH solutions of both compounds in concentrations ranging from 0.01 to 10.0 mg/mL (see Figure S3).

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Table S1. ^{1}H (500 MHz) and ^{13}C NMR (100 MHz) data for desmethylmensacarcin (**1**) in $CDCl_3$ (δ in ppm, *J* in Hz).

Position	**δ_H**	**δ_C**	**HMBC**
1	4.57 (d, *J* = 6.7, 1H)	70.9	C-10, C-10a, C-4a
2	-	83.1	-
3	2.33 (dq, *J* = 7.2, *J* = 3.4, 1H)	32.1	C-15
4	4.74 (dd, *J* = 4.0, *J* = 3.4, 1H)	68.4	C-4a, C-2
4a	-	63.4	-
5	-	192.8	-
5a	-	128.4	-
6	7.71 (dd, *J* = 7.9, *J* = 1.1, 1H)	120.3	C-8, C-5, C-5a
7	7.48 (dd, *J* = 8.3, *J* = 7.9, 1H)	129.6	C-9, C-9a, C-8, C-6
8	7.24 (dd, *J* = 8.3, *J* = 1.1, 1H)	116.6	C-9, C-6, C-5a
9	-	157.6	-
9a	-	129.4	-
10	5.82 (d, *J* = 1.8, 1H)	63.7	C-9, C-5a
10a	-	65.3	-
11	-	208.2	-
12	3.96 (d, *J* = 1.9, 1H)	58.0	C-13, C-11
13	3.10 (dq, *J* = 5.1, *J* = 1.9, 1H)	56.8	C-14
14	1.49 (d, *J* = 5.1, 3H)	17.8	C-13
15	1.13 (d, *J* = 7.2, 3H)	10.9	C-4, C-3, C-2
1-OH	3.64 (d, *J* = 6.7, 1H)	-	C-2
2-OH	4.86 (s, 1H)	-	C-11, C-3, C-2
4-OH	3.75 (d, *J* = 4.0, 1H)	-	C-3
10-OH	4.68 (d, *J* = 1.8, 1H)	-	C-10a
9-OMe	4.01 (s, 3H)	56.2	C-9

Table S2. ^{1}H (500 MHz) and ^{13}C NMR (100 MHz) data for desmethylmensacarcin (**1**) in CD_3OD (δ in ppm, *J* in Hz).

Position	δ_H	δ_C	**HMBC**
1	4.35 (s, 1H)	70.8	C-11, C-10a, C-5, C-4a
2	-	83.8	-
3	2.16 (dq, *J* = 7.1, *J* = 3.1, 1H)	31.8	C-15
4	4.67 (d, *J* = 3.1, 1H)	67.1	C-10a, C-4a, C-2
4a	-	64.1	-
5	-	189.9	-
5a	-	129.3	-
6	7.68 (dd, *J* = 7.9, *J* = 0.9, 1H)	118.5	C-8, C-5, C5a
7	7.48 (dd, *J* = 8.1, *J* = 7.9, 1H)	129.0	C-9, C-5a
8	7.36 (dd, *J* = 8.1, *J* = 0.9, 1H)	116.2	C-9, C-8, C-6
9	-	158.5	-
9a	-	129.6	-
10	5.87 (s, 1H)	60.1	C-9, C5a
10a	-	66.5	-
11	-	210.6	-
12	4.08 (d, *J* = 1.9, 1H)	58.2	C-13, C-11
13	3.02 (dq, *J* = 5.1, *J* = 1.9, 1H)	56.5	C-14
14	1.44 (d, *J* = 5.1, 3H)	16.5	C-13, C-12
15	1.06 (d, *J* = 7.1, 3H)	10.2	C-4, C-3, C-2
9-OMe	3.98 (s, 3H)	55.1	C-9

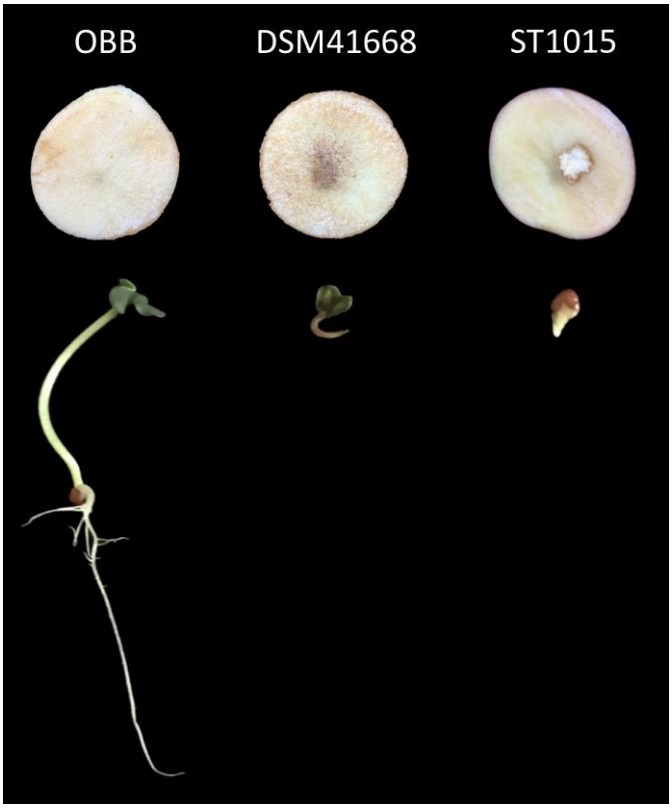

Figure S1. Phytotoxic activity of culture supernatants from *S. acidiscabies* DSM41668 and *S. niveiscabiei* ST1015 on potato tuber slices (top) and radish seedlings (bottom). Sterile and uninoculated OBB medium was included as a negative control.

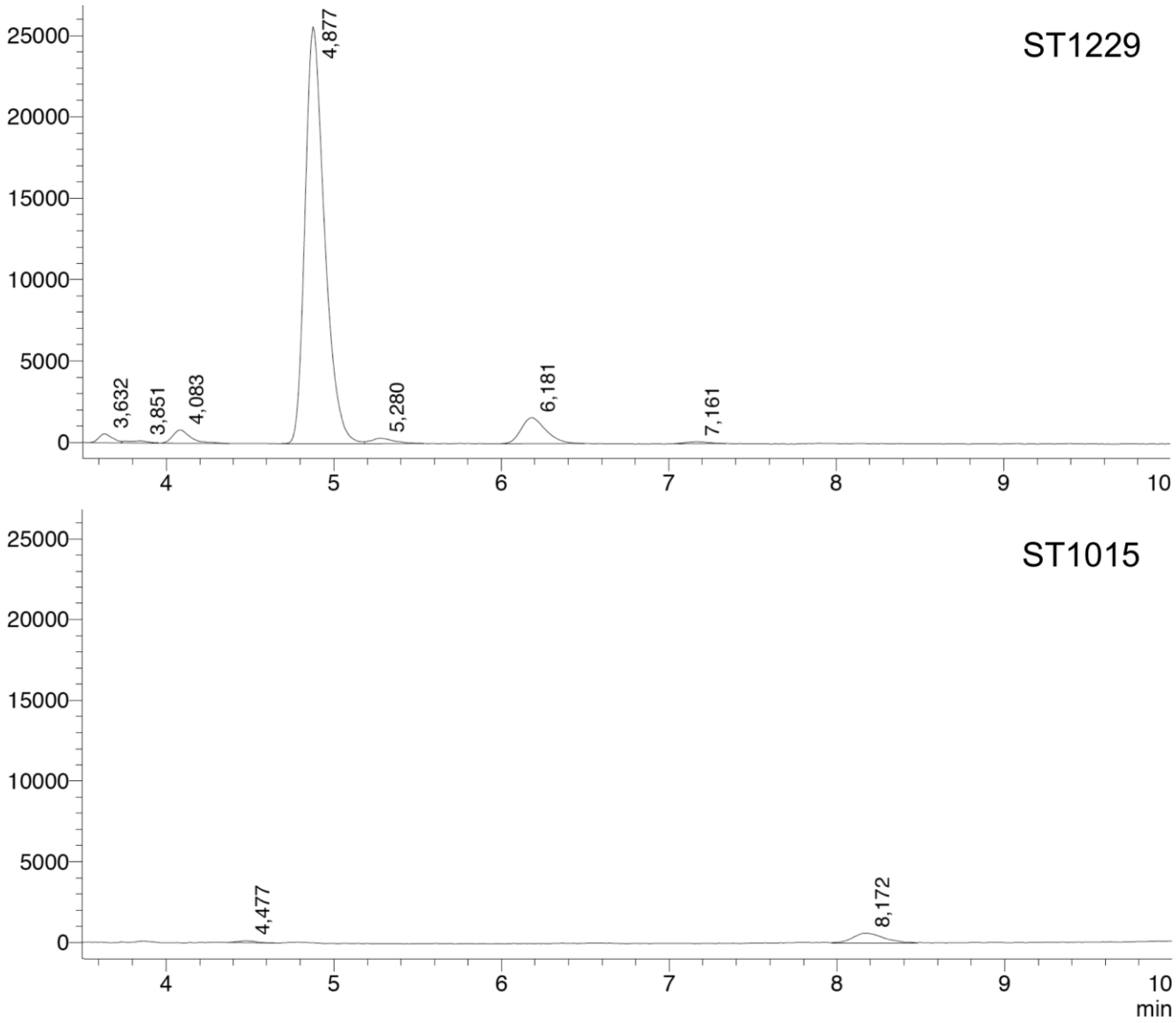

Figure S2. HPLC traces of culture medium extracts from strains ST1229 and ST1015 monitored at 380 nm. The peak at a retention time of 4.88 min corresponding to thaxtomin A is clearly visible in the chromatogram corresponding to strain ST1229.

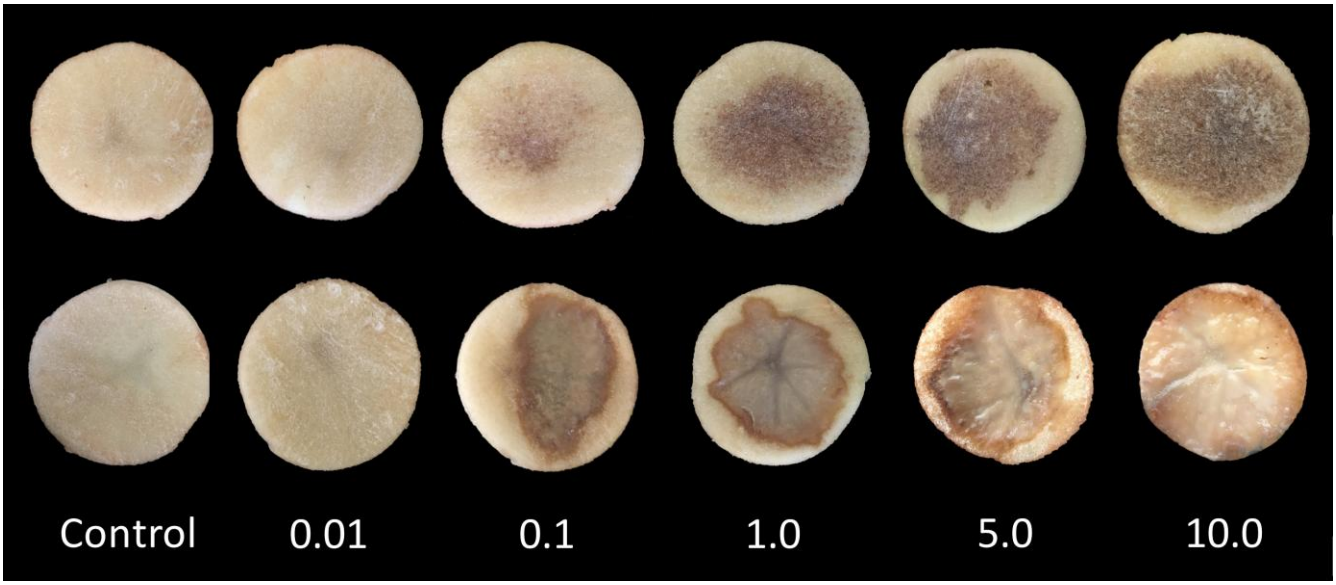

Figure S3. Comparison of necrotic lesions on potato tuber slices produced by thaxtomin A (top) and desmethylmensacarcin (bottom) as a function of concentration (mg/mL). Pure CH_3OH was employed as a negative control.

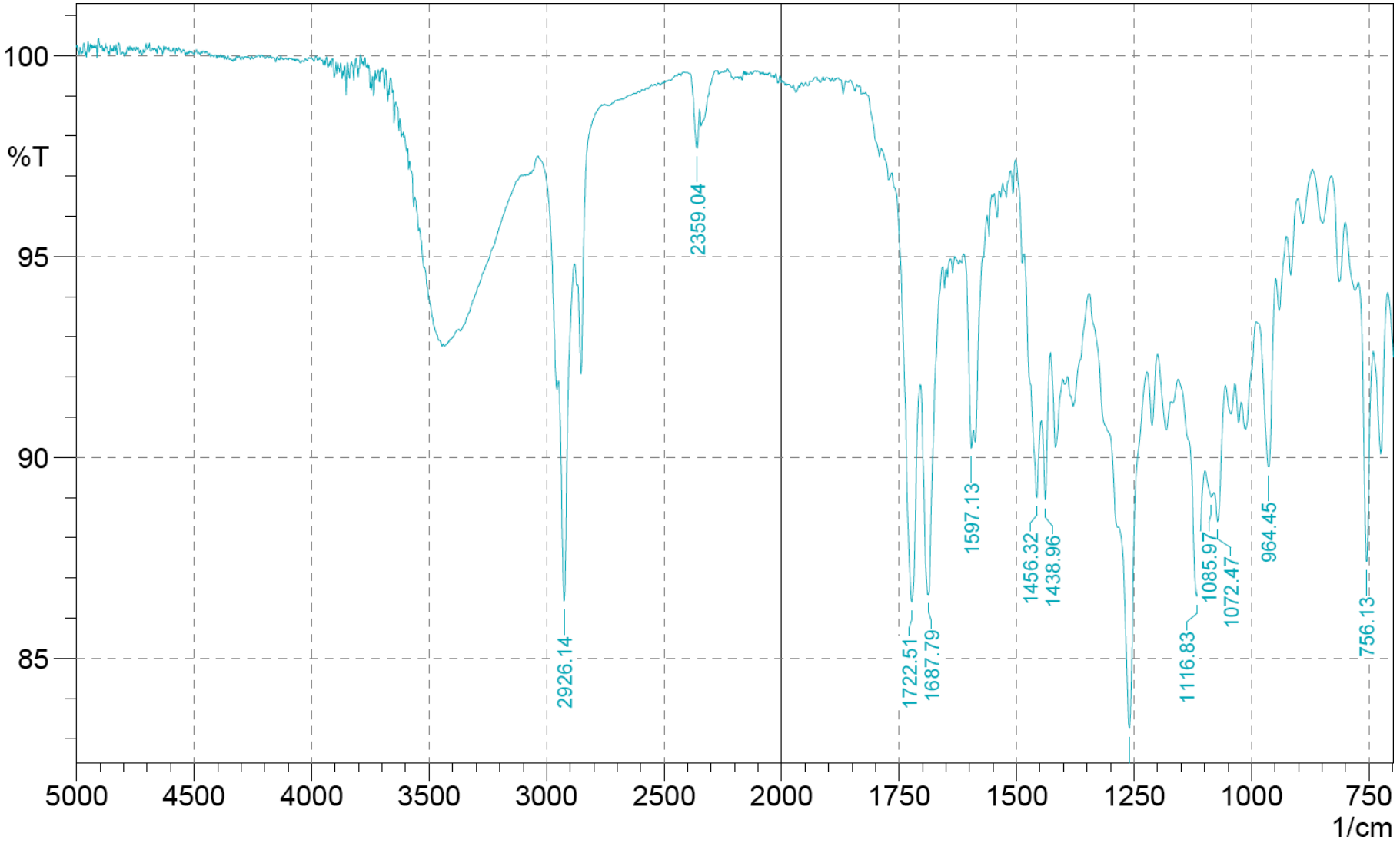

Figure S4. IR (ATR) spectrum of desmethylmensacarcin (**1**).

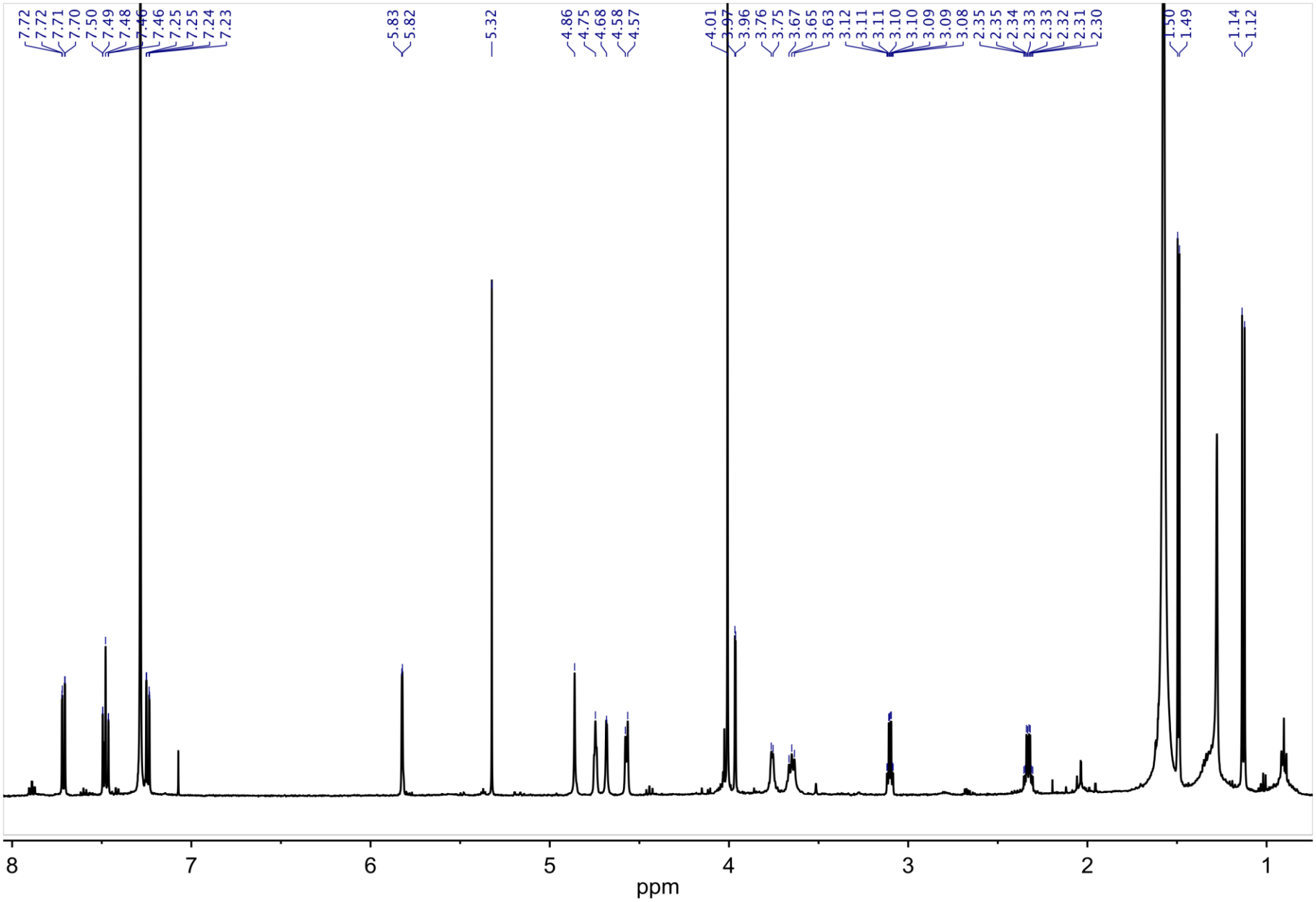

Figure S5. ^{1}H NMR (500 MHz) spectrum of desmethylmensacarcin (**1**) in $CDCl_3$.

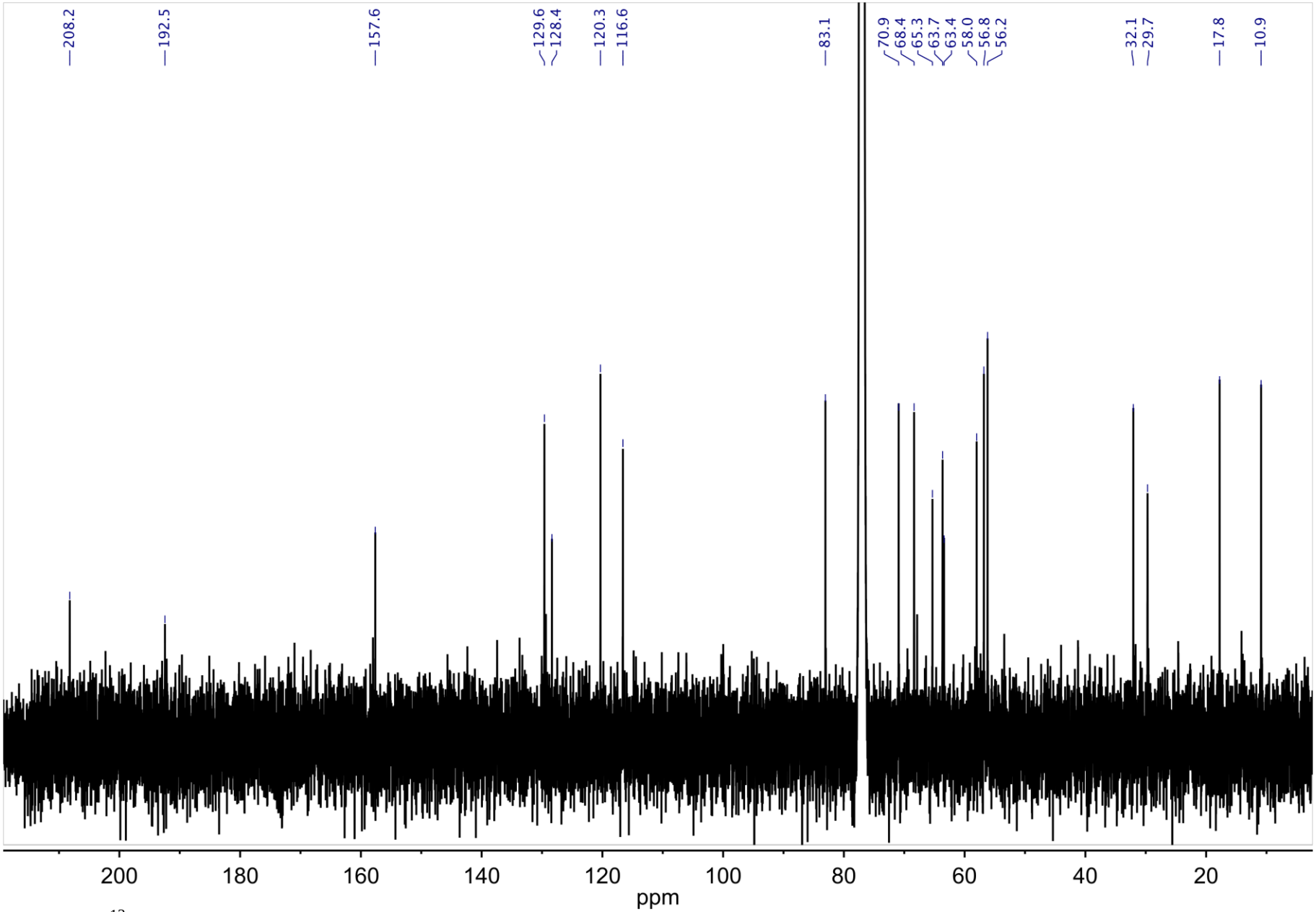

Figure S6. ^{13}C NMR (100 MHz) spectrum of desmethylmensacarcin (**1**) in $CDCl_3$.

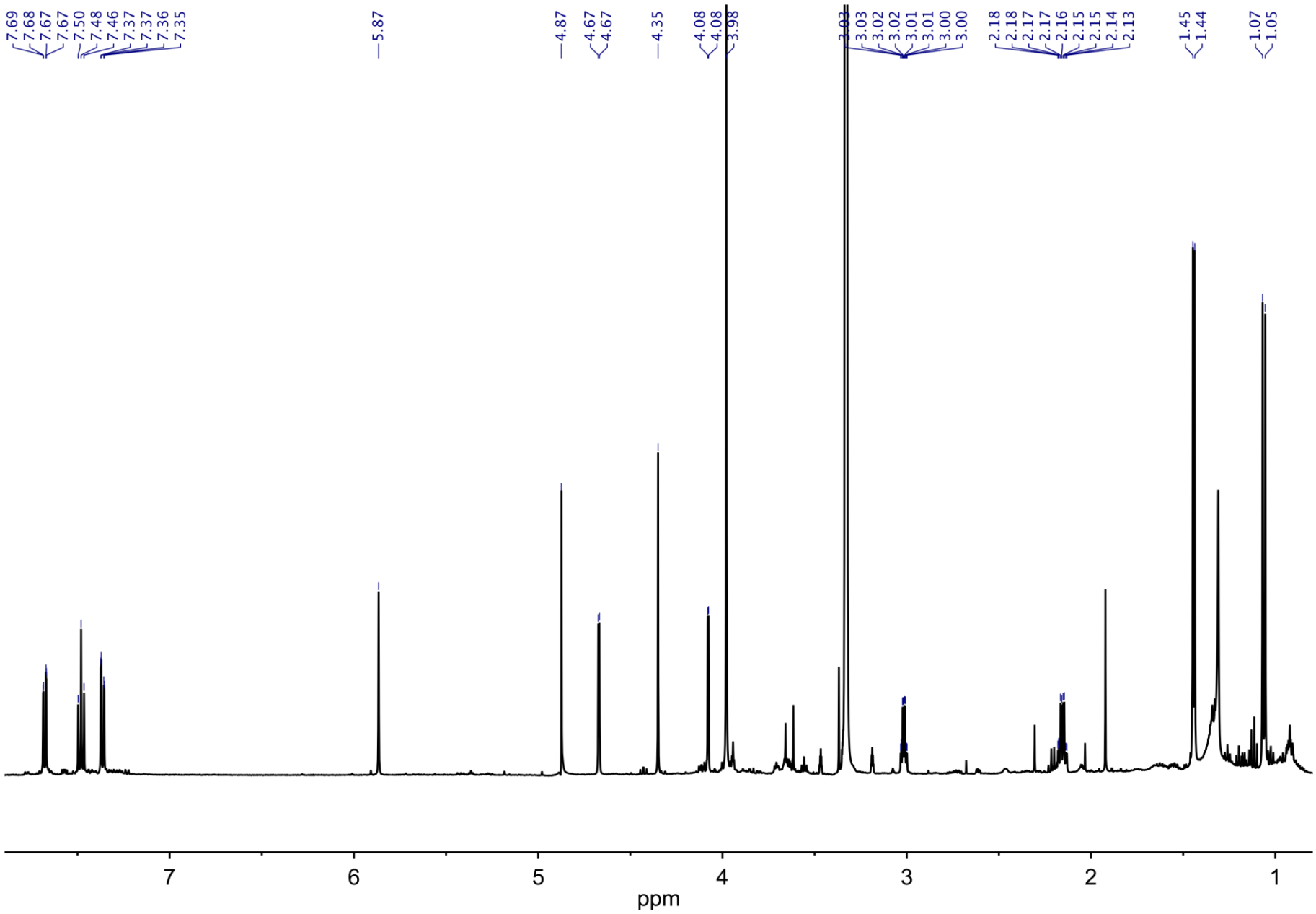

Figure S7. ^{1}H NMR (500 MHz) spectrum of desmethylmensacarcin (**1**) in CD_3OD.

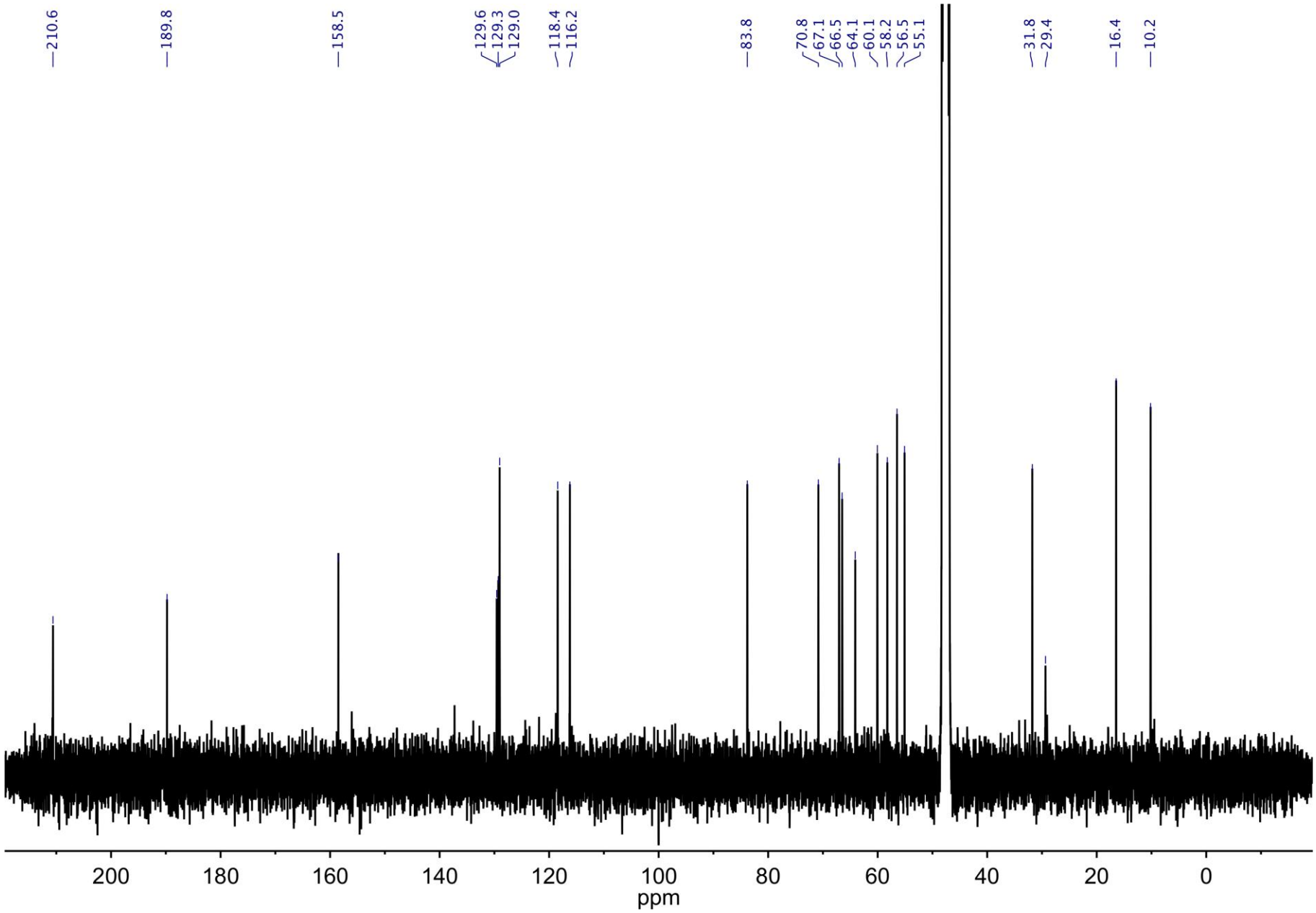

Figure S8. ^{13}C NMR (100 MHz) spectrum of desmethylmensacarcin (**1**) in CD_3OD.

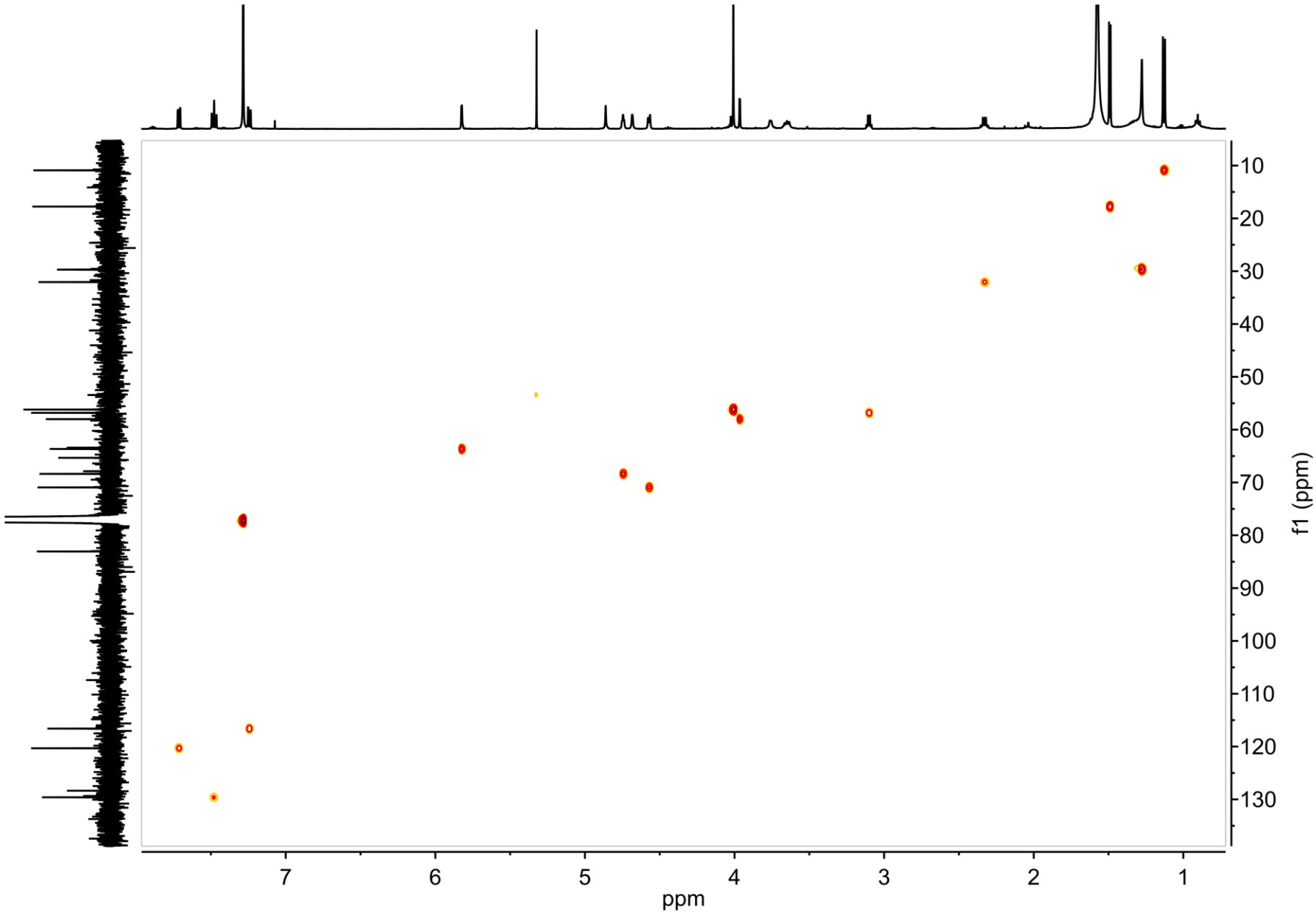

Figure S9. HSQC (500 MHz) spectrum of desmethylmensacarcin (**1**) in $CDCl_3$.

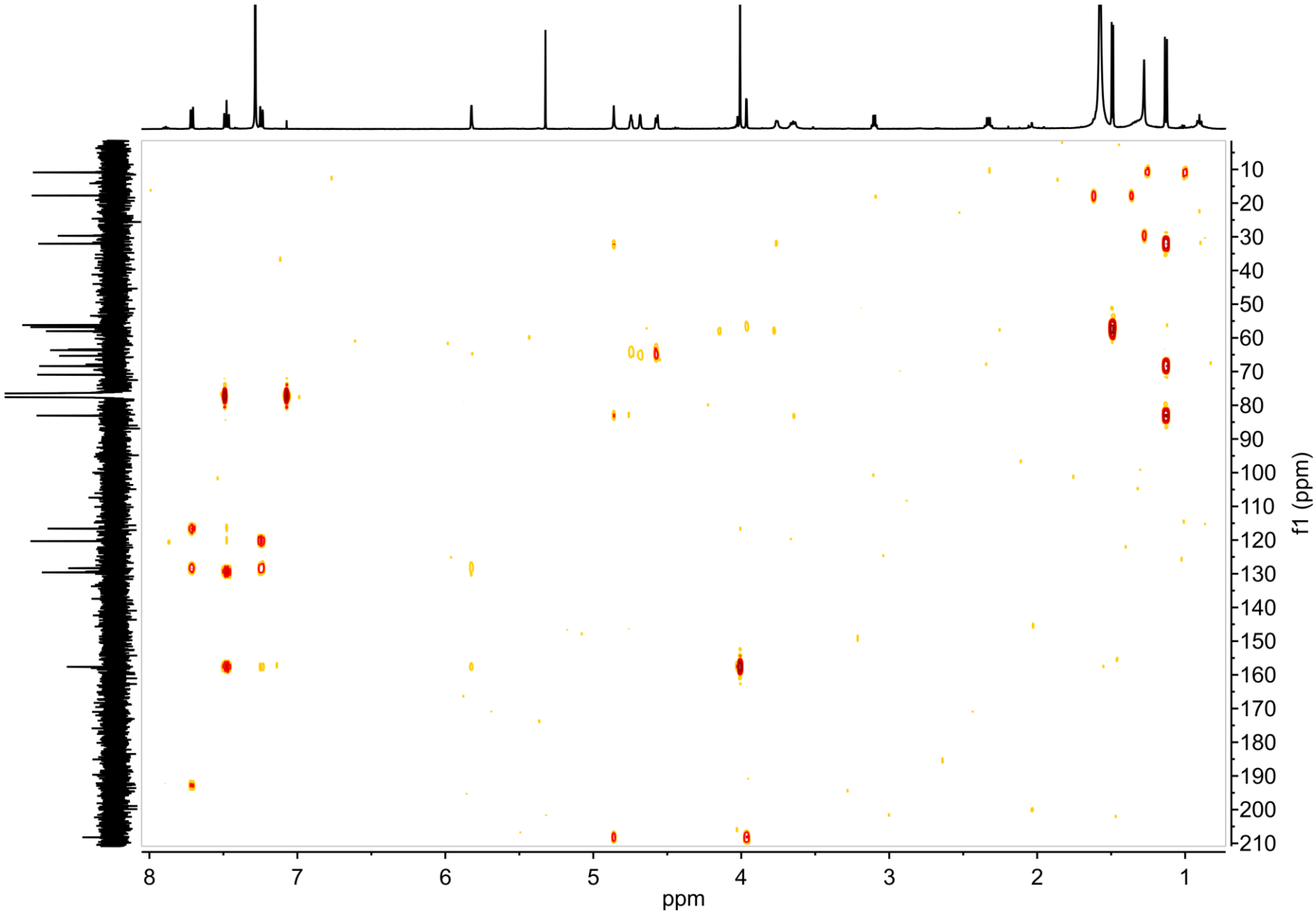

Figure S10. HMBC (500 MHz) spectrum of desmethylmensacarcin (**1**) in $CDCl_3$.

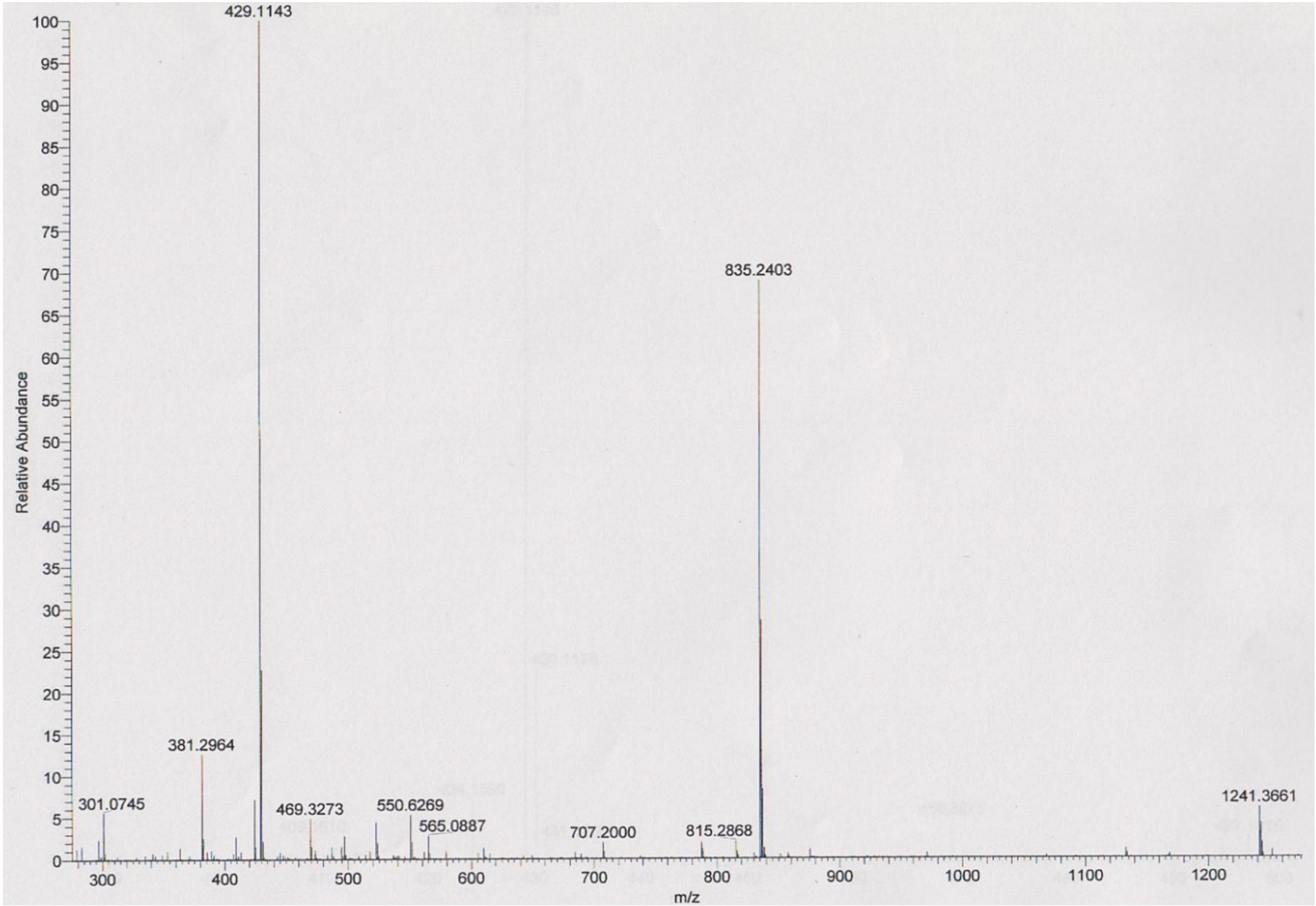

Figure S11. HR-ESIMS spectra of desmethylmensacarcin (**1**)

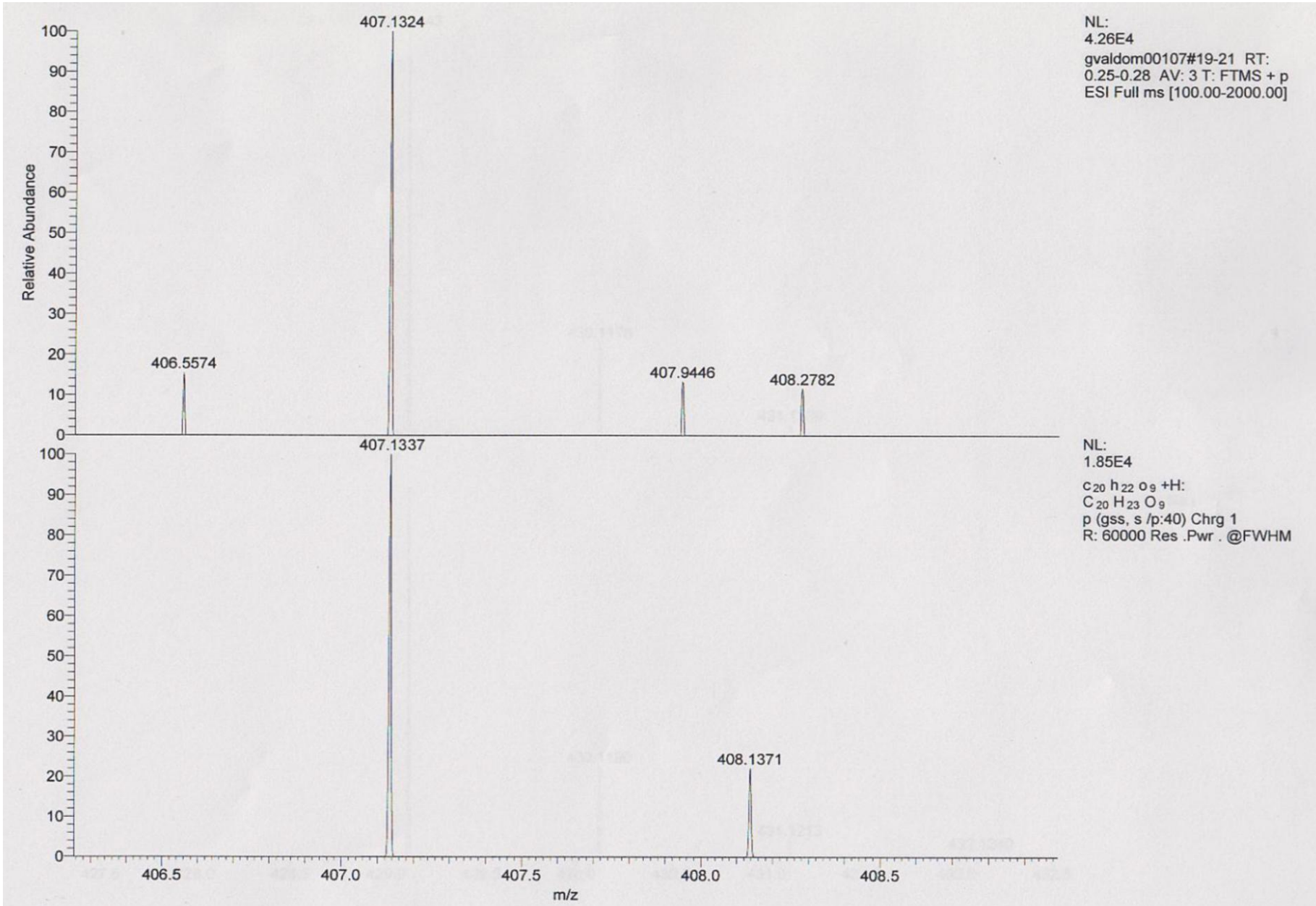

Figure S11 (continued). HR-ESIMS spectra of desmethylmensacarcin (**1**).

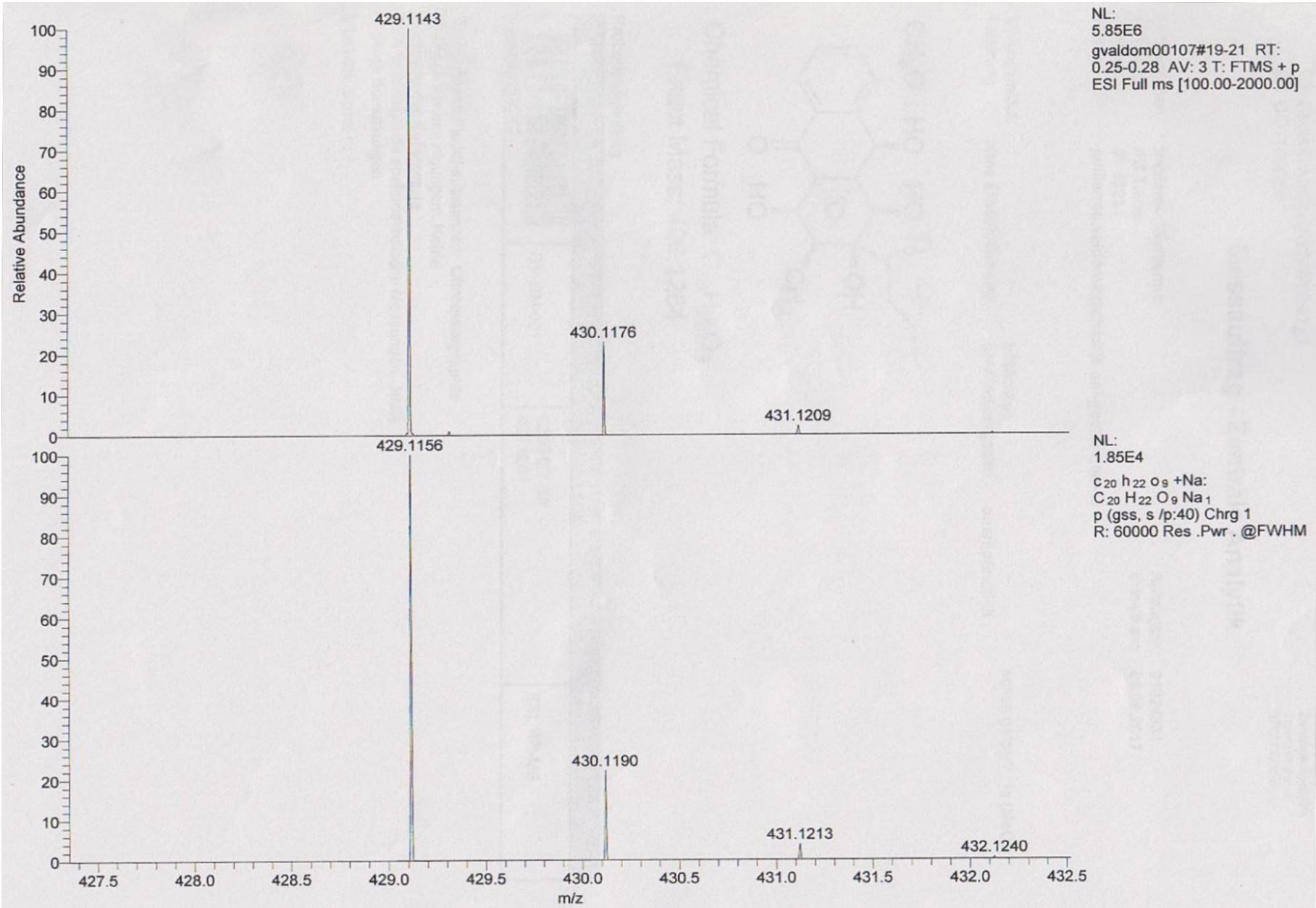

Figure S11 (continued). HR-ESIMS spectra of desmethylmensacarcin (**1**).

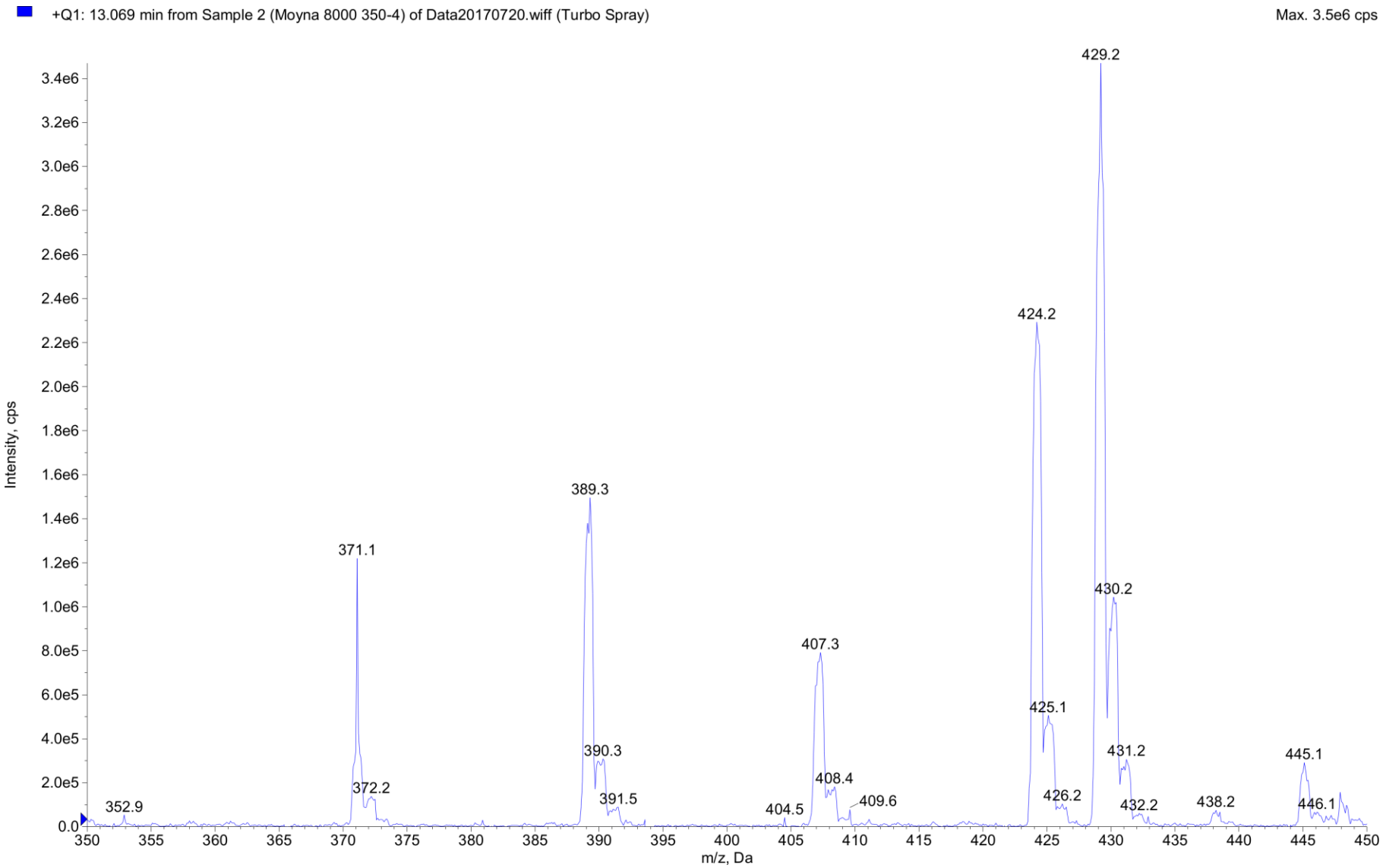

Figure S12. ESIMS^2 spectrum of desmethylmensacarcin (**1**) obtained from the $[M+H]^+$ ion at *m*/*z* 407.