A Designer Bleomycin with Significantly Improved DNA Cleavage Activity

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Supporting Online Materials (SOM)

General experimental procedures

Optical rotations were measured in water on a Perkin-Elmer 241 polarimeter at the sodium D line (589 nm). ^{1}H and ^{13}C NMR spectra were recorded at 25 °C on a Varian Unity Inova 500 instrument operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C nuclei. ^{1}H-^{1}H gCOSY (mixing time = 80 ms), gHSQC and gHMQC ($^{1}J_{CH}$ = 140 Hz), gHMBC ($^{2\text{-}3}J_{XH}$ = 8.0 Hz), and HSQC-TOCSY spectra were performed using standard VARIAN pulse sequences. High resolution mass spectral analyses were acquired on an IonSpec HiResMALDI FT–Mass spectrometer with a 7 tesla superconducting magnet. HPLC-MS (Microsorb-MV C18 column, 250 × 4.6 mm, 5 μm) analysis was conducted on an Agilent Technologies 1100 series LC/MSD equipped with a DAD, an electrospray ion source and a quadrupole mass analyzer. Analytical and semipreparative HPLC was performed on a Varian liquid chromatograph system with an Altima-C18, 4.6 mm × 25 cm and 10 mm × 25 cm columns, respectively. Column chromatographies were performed on Sephadex LH-20 (GE Healthcare, Glies, UK), Amberlite® IRC50 resin (Sigma-Aldrich), and Diaion HP-20 resin (Supelco, PA), respectively. DNA sequencing was performed at the University of Wisconsin-Madison Biotechnology Center facility using the dideoxy chain-termination method.

Plasmids and biochemical

Vector pStreptoBAC V was used for the BAC library construction (S1). Plasmids pGEM-3Zf (Promega, Madison, WI), Litmus 28 (New England Biolabs, Beverly, MA), and pSPORT1 (Invitrogen, Carlsbad, CA), were from commercial sources. Plasmids pOJ260 (S2), pSET 151 (S2), pSET152 (S2), pWHM1250 (S3), pBS6 (S4), pBS9 (S4), pBS9010 (S5), pBS9019 (S5), and pBS9005 (S5) have been described previously. Common biochemicals, chemicals, media, restriction enzymes, and other molecular biology reagents were from standard commercial sources. Antibiotics were added when necessary at the following concentrations: ampicillin (150 μg/mL), apramycin (50 μg/mL), kanamycin (50 μg/mL), thiostrepton (50 μg/mL), and nalidixic acid (50 μg/mL).

Bacterial strains and culture conditions

E. coli strains DH5α and DH10B (Invitrogen) were used for routine cloning. *E. coli* ET12567/pUZ8002 and *E. coli* S17-1 were used as the donor strains in intergeneric conjugation with *Streptomyces* strains (S2). All the mutants and recombinant strains generated in this paper were routinely grown at 28 °C in TSB liquid medium (S5). Modified ISP4 medium (IWL4) supplemented with 0.05% yeast extract, 0.1% tryptone, and $MgCl_2$ to a final concentration of 20 mM was used to prepare *S. flavoviridis* SB9001 spores and to plate out conjugation mixtures. Previously described fermentation conditions for *S. flavoviridis* SB9001 were used for the production of ZBM and other secondary metabolites (S5, S6). For preparing intact genomic DNA in plugs, *Streptomyces* strains were cultured in TSB medium containing 0.5% glycine (S1). All *Streptomyces* strains were cultured at 28 °C with 250 rpm (liquid culture) (S2). *E. coli* strains were cultured in LB at 37 °C with 250 rpm (liquid culture) (S7).

DNA manipulation and PCR

DNA manipulation was carried out using standard methods (S7). Unless specifically noted, restriction enzymes and other molecular biology reagents were purchased from standard commercial sources and used as per manufacturer instructions. PCR amplification was carried out on a GeneAmp® PCR system 2400 (Applied Biosystems, Foster City, CA) with *Takara LA Taq*™ (Takara, Otsu, Shiga, Japan) with supplied GC buffer II. Southern blotting was performed according to the standard protocols using the DIG-system (Roche, Palo Alto, CA) (S7). Appropriate DNA fragments were labeled with DIG-labeled dUTP and used as probes. BAC DNA was introduced into heterologous hosts by known conjugation methods (S7).

Cross-complementation of *ΔzbmL* mutant strain SB9003 with *blmGF, blmGFE*, and *blmG*

Inactivation of *zbmL* in *S. flavoviridis* SB9001 to afford the *S. flavoviridis ΔzbmL* mutant strain SB9003 and complementation of SB9003 with the *zbmL* expression plasmid pBS9019 have been described previously (S5). Cross-complementation plasmids that express *blmGF, blmGFE*, or *blmG*, respectively, under the control of *ErmE** were constructed as follows:

For the *blmGF* expression construct pBS57, a 6.6-kb *Sna*BI-*Spe*I fragment containing *blmV*, *blmG* and *blmF* was isolated from pBS6 and cloned into the same sites of pSPORT1 to form pBS56. The 4.3-kb *Bam*HI-*Spe*I fragment containing *blmG* and *blmF* isolated from pBS56 and the 0.3-kb *Eco*RI-*Bam*HI fragment containing the *ErmE** promoter from pWHM1250 were inserted into the *Eco*RI-*Xba*I sites of pBS9010 via a three piece ligation reaction to afford the *blmGF* expression plasmid pBS57.

For the *blmGFE* expression construct pBS61, the *blmE* gene was isolated as a 1.5-kb *Bst*BI-*Nco*I fragment from pBS9 and cloned into the same sites of Litmus 28 to yield pBS58. The *blmE* gene was then isolated from pBS58 as a *Spe*I-*Sst*I fragment and added to the end of the insert in pBS56 at the same sites to create pBS59. The 5.8-kb *Bam*HI-*Xba*I fragment was isolated from pBS59 and transferred into the same sites of pWHM1250 behind the *ErmE** promoter, creating pBS60. The entire insert containing *ErmE** and *blmGFE* was then moved as a 6.1-kb *Eco*RI-*Xba*I fragment into the same sites of pBS9010 to afford the *blmGFE* expression plasmid pBS61.

For the *blmG* expression construct pBS63, a 3.8-kb *Eco*RI-*Xmn*I fragment containing the *ErmE** promoter and *blmG* was isolated from pBS57 and cloned into the same sites of pGEM-3Zf to yield pBS62. The entire insert was then moved as a 3.8-kb *Eco*RI-*Xba*I fragment into the same sites of pBS9010 to afford the expression plasmid pBS63.

Plasmids pBS57, pBS61 and pBS63 were introduced into the *S. flavoviridis* SB9003 mutant strain by intergeneric conjugation, and selection with thiostrepton afforded the Δ*zbmL*-cross complemented recombinant strain SB9011 (SB9003/pBS57), SB9012 (SB9003/pBS61), and SB9013 (SB9003/pBS63). Maintenance of the plasmids was verified by colony PCR analysis using oligonucleotides blmGFf (5'-GCACCCGTCTGACGCTGG-3') and blmGFr (5'-CCACGATGCCGCACACG-3') for pBS57, blmGFf and blmEr (5'-CGAGCGAGGAGTCGTTGC-3') for pBS61, and blmGFf and blmGr (5'-GCTCGATCCACGGGTAGG-3') for pBS63. The cross-complementation strains were cultured and investigated for production of ZBM or ZBM analogues with SB9001 (the ZBM high producer), SB9003 (the Δ*zbmL* mutant strain), and SB9009 (Δ*zbmL* complementation strain of SB9003/pBS9019) as controls.

Construction of the *ΔzbmVIII* in-frame deletion mutant strain SB9025 and confirmation of its genotype by Southern analysis

To inactivate *zbmVIII* by in-frame deletion in SB9001, a 10-kb *Bgl*II DNA fragment from cosmid pBS9005 (S5) containing *zbmVIII* was cloned into *Bam*HI-*Bgl*II sites of Litmus28 to afford pBS9034. This construct was cut with *Sbf*I to remove a 3.7-kb fragment internal to *zbmVIII* and self-ligated to afford pBS9035, which contained an in-frame deletion within *zbmVIII*. The *ΔzbmVIII* gene was first moved as a 6.3-kb *Spe*I-*Hind*III fragment from pBS9035 into the *Xba*I-*Hind*III sites of pSET151 to afford pBS9036 and subsequently moved as a 6.3-kb *Eco*RI-*Hind*III fragment from pBS9036 into the same sites of pOJ260 to afford pBS9037. pBS9037 was introduced into the *S. flavoviridis* SB9001 by conjugation mediated by *E. coli* S17-1 (S2), and single crossover mutants were generated by homologous recombination and selected for on IWL4 plates with apramycin. Apramycin resistant

exconjugants were checked via PCR with a pair of primers (5'-GCAGTCCGTTCTTCGTCACG-3' and 5'-GGAGGTGTACTGGAGGAATGC-3'). Single crossovers showing both the 6.6-kb wild type band and the 2.9-kb mutant band were then grown nonselectively for several rounds on IWL4 plates, and single colonies were then checked for the loss of apramycin resistance. Apramycin sensitive colonies were then isolated as the desired Δ*zbmVIII* mutant strain SB9025.

The Δ*zbmVIII* genotype in the SB9025 mutant strain was confirmed by PCR with the same two primers mentioned above (Fig. S5A) and unambiguously established by Southern analysis using the 790-bp *Sst*I-*Kpn*I fragment from pBS9034 as a probe (Fig. S5B).

Construction of a BAC library of *S. verticillus* to isolate pBS54 that harbors the entire BLM cluster

The construction of *S. verticillus* ATCC15003 BAC library was achieved by following the protocol described (S1). The insert size varies from 20-kb to 110-kb, with 80-kb in average, as determined by restriction digestion of 36 randomly BAC clones. Approximately 2000 clones were collected to provide approximately16-fold genome coverage of the 10 Mb genome on average for *Streptomyces* species. The BAC clones were picked from the plates and placed into freezing medium in 96-well microplates, cultured overnight, and stored at -80 °C. The *S. verticillus* BAC library clones were transferred onto Hybond-N+ membranes (Amersham Pharmacia, Piscataway, NJ) and fixed for hybridization. The membranes were hybridized with a *blmA* probe (S8, S9), which was amplified from *S. verticillus* using primers BlmAF (5'-CATATGGTGAAATTCTTGGGTGCCG-3') and BlmAR (5'-AAGCCTCTCCCCCGCGGTGAAGTG-3'). The positive clones were then screened by PCR with *blmorf32* primers C-PHNA-F1 (5'-GCAGCGTCATGAACAGGGTG-3') and C-PHNA-R1 (5'-CCGGACCATCATGTAGCGAC-3') (S8, S9) under the following conditions: 94 °C for 2 min; 35 cycles each of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min; 72 °C for 7 min. Two positive clones, pBS54 and pBS55, were identified, containing a 120-kb and an 80-kb insert, respectively. End-sequencing of both clones confirmed that only pBS54 harbored the entire intact BLM cluster (S8, S9).

Introduction of pBS54 into heterologous *Streptomyces* hosts for production of BLM, ZBM, and analogues

pBS54 was introduced into various *Streptomyces* heterologous hosts, including *S. albus* J1074 (S10), *S. lividans* K4-114 (S11), *S. coelicolor* M512 (S12), *S. flavoviridis* SB9001 (S12), and the *S. flavoviridis ΔzbmVIII* mutant SB9025 strains via conjugation mediated by *E. coli* ET12567/pUZ8002 (S2). Exconjugants were selected on IWL4 plates with apramycin. The desired transformants, SB9026 (SB9025/pBS54), SB9027 (SB9001/pBS54), SB9028 (*S. albus* J1074/pBS54), SB9029 (*S. lividans* K4-114/pBS54), and SB9030 (*S. coelicolor* M512/pBS54), were isolated, and the genotypes were confirmed by PCR using C-PHNA-F1 and C-PHNA-R1 primers.

Fermentation, production, isolation, and HPLC analyses of ZBM, 6'-hydroxy-ZBM, BLM Z, and 6'-deoxy-BLM Z

S. flavoviridis SB9001 (ZBM high producer), SB9003 (*ΔzbmL* mutant), SB9009 (*ΔzbmL* complemented by *zbmL*), SB9011 (*ΔzbmL* complemented by *blmGF*), SB9012 (*ΔzbmL* complemented by *blmGFE*), SB9013 (*ΔzbmL* complemented by *blmG*), SB9026 (SB9025/pBS54), and SB9027 (SB9001/pBS54), and SB9027 (SB9001/pBS54), as well as *S. albus* SB9028 (*S. albus* J1074/pBS54), *S. lividans* SB9029 (*S. lividans* K4-114/pBS54), and *S. coelicolor* SB9030 (*S. coelicolor*/pBS54) were cultured in 250 mL baffled flasks containing 50 mL of the seed medium (1.5% glycerol, 1.5% pharmamedia, 0.3% NaCl, 0.2% asparagine, with the pH adjusted to 7.0). After growth at 28 °C and 250 rpm for 2 days, 5 mL of the seed cultures were inoculated into 250-mL flasks containing 50 mL of the production medium (6.4%

millet jelly, 0.5% glucose, 3.5% soy meal, 0.75% corn steep solid, 0.2% $NaNO_3$, 0.3% NaCl, 0.1% K_2HPO_4, 0.01% $CuSO_4 \cdot 5H_2O$, 0.05% $ZnSO_4 \cdot 7H_2O$, pH 7.0). The resulting cultures were fermented at 28 °C and 250 rpm for 12 days.

The fermentation broths (50 mL) of SB9001 and the recombinant strains were each harvested and treated separately as follows: The culture was centrifuged and the supernatant was adjusted to pH 7.0 with 5.0 N HCl and loaded onto an Amberlite® IRC50 column (NH_4^+ type). After washing the column with 10 bed volumes of H_2O, the resin was eluted with 50 ml of 20% NH_4OAc. The resulting eluate was mixed with 3 g Diaion HP-20 resin and incubated at room temperature under gentle agitation for about 45 min. The Diaion HP-20 resin was then packed into a column, washed with 10 bed volumes of H_2O, and drained of excess water. The column was then eluted with five bed volumes of methanol, and the methanol eluate was combined and concentrated in vacuum to give the crude extract. The crude extracts were subjected to HPLC analyses to check the production of ZBM, BLM, and analogues. Analytical HPLC was carried out on an Altima C18 column (5 μm, 4.6 mm × 25 cm, Alltech Associates, Inc., Deerfield, IL). The column was equilibrated with 100% solvent A (H_2O with 0.1% HOAc) and 0% solvent B (100% methanol with 0.1% HOAc) and developed with a linear gradient (0 to 15 min, from 100% A and 0% B to 40% A and 60% B; 15 to 20 min, from 40% A and 60% B to 0% A and 100% B) at a flow rate of 0.8 mL/min and with UV detection at 300 nm.

To isolate 6'-hydroxy-ZBM, the fermentation culture of SB9013 (30 L) was centrifuged, and the supernatant (22 L) was collected, adjusted to pH 7.0 with 5.0 N HCl, and loaded onto an Amberlite® IRC50 column (NH_4^+ type, 2.2 L). After washing the column with 10 bed volumes of H_2O, the resin was eluted with 6.6 L of 20% NH_4OAc. The resulting Amberlite® IRC50 eluate was mixed with 500 g Diaion HP-20 resin and incubated at room temperature under gentle agitation for 45 min. The Diaion HP-20 resin was then packed into a column, washed with 10 bed volumes of H_2O, and drained of excess water. The column was then eluted with eight bed volumes of 80% methanol, and the fractions containing 6'-hydroxy-ZBM were combined and concentrated in vacuum to 6 mL. Final purification of 6'-hydroxy-ZBM was achieved by semipreparative HPLC on an Altima C18 column (5 μm, 10 mm × 25 cm, Alltech Associates, Inc., Deerfield, IL). HPLC isolation was carried out under the following conditions. The column was equilibrated with 100% solvent A (water with 0.1% HOAc) and 0% solvent B (methanol with 0.1% HOAc) and developed with a linear gradient (0 to 20 min, from 100%A and 0% B to 36% A and 64% B) at a flow rate of 3 mL/min and with UV detection at 300 nm. The methanol was removed from the eluate by evaporation at 40 °C. Pure 6'-hydroxy-ZBM was finally obtained by freeze-drying as a blue 6'-hydroxy-ZBM●Cu^{2+} complex (10.0 mg). Cu^{2+}-free 6'-hydroxy-ZBM was obtained by treating the 6'-hydroxy-ZBM●Cu^{2+} complex with 0.5 M EDTA-Na (pH 7.3). After the final HPLC purification, Cu^{2+}-free 6'-hydroxy-ZBM (3.6 mg) was obtained as a white powder.

To isolate BLM Z and 6'-deoxy-BLM Z, the fermentation broth (12 L) of strain SB9026 was centrifuged and the supernatant was adjusted to pH 7.0 with 5.0 N HCl and loaded onto an Amberlite® IRC50 column (NH_4^+ type, 1.6 L). After washing the column with 10 bed volumes of H_2O, the resin was eluted with 4 liters of 20% NH_4OAc. The resulting eluate was mixed with 300 g Diaion HP-20 resin and incubated at room temperature under gentle agitation for about 45 min. The Diaion HP-20 resin was then packed into a column, washed with 10 bed volumes of H_2O, and drained of excess water. The column was then eluted with five bed volumes of methanol, and the methanol eluates were combined and concentrated in vacuum to give the crude extract. The crude extract was loaded onto a Sephadex LH-20 column and eluted with pure methanol to yield 10 fractions. Fractions containing BLM Z and 6'-deoxy-BLM Z were combined and evaporated to dryness. Final purification of BLM Z and 6'-deoxy-BLM Z was achieved by semipreparative HPLC on an Altima C18 column (5 μm, 10 mm × 25 cm, Alltech Associates, Inc., Deerfield, IL). HPLC isolation was carried out under the following conditions: The column was equilibrated with 100% buffer A (H_2O containing 0.1% HOAc) and 0% buffer B (100% CH_3OH containing 0.1% HOAc) and developed with a linear gradient (0 to 20 min, from 100%A and

0%B to 36% A and 64% B) at a flow rate of 3 mL/min and with UV detection at 300 nm. The methanol was removed by evaporation at 40 °C. Pure BLM Z and 6'-deoxy-BLM Z were finally obtained as a blue BLM Z●Cu^{2+} complex (8.3 mg) and a blue 6'-deoxy-BLM Z●Cu^{2+} complex (9.7 mg), respectively, by freeze drying. Cu^{2+}-free BLM Z and 6'-deoxy-BLM Z were obtained by treating the corresponding Cu^{2+}●complex with 0.5 M EDTA-Na (pH 7.3). After the final HPLC purification, Cu^{2+}-free BLM Z (2.1 mg) and 6'-deoxy-BLM Z (2.3 mg) were obtained as white powders.

Physicochemical properties of 6'-hydroxy-ZBM, BLM Z, and 6'-deoxy-BLM Z

6'-Hydroxy-ZBM: $[\alpha]_D^{23}$ +60.5 (*c* 0.02, H_2O); LC-MS (positive ion) *m*/*z* 745.5 $[M+Cu]^{2+}$; HR-MALDI-MS (positive ion) *m*/*z* 1428.5634 (calculated for $C_{55}H_{86}N_{19}O_{22}S_2$ $[M+H]^+$, 1428.5631). ^1H and ^{13}C NMR data (in D_2O), see Figures S3 and S8-S12 and Tables S1 and S2.

BLM Z: $[\alpha]_D^{23}$ +26.5 (*c* 0.05, H_2O); LC-MS (positive ion) *m*/*z* 722.3 $[M+Cu]^{2+}$; HR-MALDI-MS (positive ion) *m*/*z* 1382.52121 (calculated for $C_{53}H_{80}N_{19}O_{21}S_2$ $[M+H]^+$, 1382.52745). ^1H and ^{13}C NMR data (in D_2O), see Figures S6 and S13-S18 and Tables S1 and S2.

6'-Deoxy-BLM Z: $[\alpha]_D^{23}$ +18.1 (*c* 0.05, H_2O); LC-MS (positive ion) *m*/*z* 714.4 $[M+Cu]^{2+}$; HR-MALDI-MS (positive ion) *m*/*z* 1366.51983 (calculated for $C_{53}H_{80}N_{19}O_{20}S_2$ $[M+H]^+$, 1366.51983). ^1H and ^{13}C NMR data (in D_2O), see Figures S6 and S19-S23 and Tables S1 and S2.

DNA cleavage assay

The DNA cleavage assays were performed in 10 µL (total volume) of 25 mM pH 7.5 Tris-HCl buffer, containing 0.65 µg of pBluescript SK II(+) plasmid DNA, 10 µM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (freshly prepared solution in 1 mM H_2SO_4) and the varying concentrations of BLM A2, ZBM, 6'-hydroxy-ZBM, BLM Z, and 6'-deoxy-BLM Z. The reactions were incubated at 37 °C for 30 min and stopped by the addition of 5 mM EDTA and 5 µl of 6X loading dye (30% glycerol containing 0.25% (w/v) bromphenol blue). The samples were applied to a 0.8% agarose gel containing 1 µg/mL ethidium bromide, and gel electrophoresis was carried out in 40 mM Tris acetate buffer, pH 8.0, containing 1 mM disodium EDTA at 90 V for 1h (S8, S13).

The relative DNA cleavage activity was determined by comparing the EC_{50} values of 6'-hydroxy-ZBM, BLM Z, and 6'-deoxy-BLM Z. with that of ZBM and BLM A2 (Fig. S7). Varying concentrations of BLM A2, ZBM, 6'-hydroxy-ZBM, BLM Z, and 6'-deoxy-BLM Z. were added to the 10 µL reactions as mentioned above. The samples were applied to a 0.8% agarose gel containing 1 µg/mL ethidium bromide, and gel electrophoresis was carried out in 40 mM Tris acetate buffer, pH 8.0, containing 1 mM disodium EDTA at 66 V for 2h. The DNA bands (forms I, II, and III) and quantitation were evaluated using a Bio-RAD ChemiDOCTM imaging system (software version: image lab 3.0) under UV light. The DNA cleavage efficiency of compounds on supercoiled pBluescript SK II(+) plasmid DNA was expressed as an EC_{50} value (the drug concentration needed to attain 50% cleavage of the total DNA, calculated from the corresponding linear-dose cleavage line) and was estimated by analysis of dose-response curves.

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Table S1. ^{1}H NMR data of BLM Z, 6'-deoxy-BLM Z, 6'-hydroxy-ZBM, and ZBM in D_2O (500 MHz, δ in ppm, J in Hz) [a]

No.	BLM Z	6'-deoxy-BLM Z	6'-hydroxy-ZBM	ZBM	No.	BLM Z	6'-deoxy-BLM Z	6'-hydroxy-ZBM	ZBM
2	3.56 [b]	4.06 t (7.0)	4.10 [b]	4.07 [b]	32			5.91 dd (9.0,7.0)	5.89 dd (9.0,7.0)
3a	2.66 [b]	2.99 2H, m	3.06 2H, m	3.00 2H, m	34	8.22 s	8.24 s	8.17 s	8.16 s
3b	2.76 m				37	3.82 2H, m	3.83 2H, m	3.78 2H, m	3.81 2H, m [b]
5a	2.68 [b]	2.72 dd (15.2, 4.9)	2.80 [b]	2.78 [b]	38	2.83 2H, t (6.8)	2.84 2H, t (6.5)	2.81 m [b]	2.80 m [b]
5b	2.68 [b]	2.64 dd (15.2, 9.0)	2.70 dd (15.0, 9.0)	2.69 dd (15.0, 9.0)	40	1.12 d (6.5)	1.12 d (6.5)	1.20 s	1.19 s
6	3.95 [b]	3.99 dd (9.0, 5.5)	4.08 [b]	4.05 [b]	41			1.22 s	1.22 s
12	2.04 (s)	1.99 s	2.20 s	2.16 s	42			3.50 2H, m	3.50 2H, m [b]
14	5.07 d (6.0)	5.09 d (6.5)	5.13 d (6.5)	5.09 d (6.5)	1'	5.27 d (3.5)	5.24 d (3.5)	5.33 d (3.5)	5.25 d (3.5)
15	5.29 d (6.0)	5.20 d (6.5)	5.39 d (6.5)	5.28 d (8.0)	2'	4.05 [b]	3.96 [b]	3.99 [b]	3.99 [b]
17	7.30 brs	7.29 brs	7.34 brs	7.30 brs	3'	4.13 m	4.08 [b]	4.04 [b]	4.12 [b]
18	7.82 brs	7.85 brs	7.95 brs	7.91 brs	4'	3.88 brd (3.0)	3.66 [b]	4.08 [b]	3.64 [b]
19	1.15 d (6.5)	1.18 d (6.5)	1.60 m	1.70 m	5'	4.04 [b]	4.10 [b]	3.89 [b]	4.20 [b]
20	3.91 m	3.94 [b]	3.80 [b]	3.82 m [b]	6'a	3.57 [b]	0.90 d (6.5)	3.58 [b]	0.92 d (8.0)
21	3.74 tlike (5.8)	3.76 tlike (6.2)	3.72 [b]	3.74 m [b]	6'b	3.42 dd (11.8, 5.5)		3.41 [b]	
22	2.51 m	2.52 m	2.48 m	2.36 m	1"	5.03 d (1.8)	5.03 d (1.5)	5.03 brs	5.02 brs
24	1.13 d (7.0)	1.15 d (7.0)	1.09 d (7.0)	1.07 d (7.0)	2"	4.08 m	4.09 [b]	3.84 [b]	4.06 [b]
25	4.12 [b]	4.11 [b]			3"	3.85 [b]	3.84 [b]	4.62 [b]	4.65 [b]
26	4.25 d (4.8)	4.26 d (5.0)	4.29 s	4.29 s	4"	3.82 [b]	3.82 m	3.80 [b]	3.82 [b]
28	3.64 2H, m	3.66 2H, m	3.56 [b]	3.58 m [b]	5"	4.74 m	4.75 [b]	3.82 [b]	3.84 [b]
29	3.27 2H, m	3.28 2H, m	2.92 m	2.92 m	6"a	3.96 [b]	3.96 [b]	3.92 [b]	3.94 [b]
31a	8.02 s	8.05 s	3.70 [b]	3.82 2H, m	6"b	3.81 [b]	3.84 [b]	3.80 [b]	3.80 [b]
31b			3.88 [b]						

[a] Assignments were confirmed by ^{1}H-^{1}H gCOSY, gHSQC, and gHMBC spectra. [b] Overlapping signals

Table S2. ^{13}C NMR data of BLM Z, 6'-deoxy-BLM Z, 6'-hydroxy-ZBM, and ZBM in D_2O (125 MHz, δ in ppm) [a]

No.	BLM Z	6'-deoxy-BLM Z	6'-hydroxy-ZBM	ZBM	No.	BLM Z	6'-deoxy-BLM Z	6'-hydroxy-ZBM	ZBM
1	173.9 (s)	171.5 (s)	173.9 (s)	173.8 (s)	29	32.3 (t)	32.3 (t)	42.1 (t)	42.1 (t)
2	53.9 (d)	52.7 (d)	55.4 (d)	55.2 (d)	30	171.0 (s)	171.0 (s)	180.5 (s)	180.3 (s)
3	50.2 (t)	47.3 (t)	49.8 (t)	49.8 (t)	31	119.4 (d)	119.4 (d)	41.5 (t)	41.4 (t)
4	176.4 (s)	176.5 (s)	179.2 (s)	179.0 (s)	32	147.2 (s)	147.2 (s)	78.5 (d)	78.4 (d)
5	40.7 (t)	40.5 (t)	43.1 (t)	43.1 (t)	33	163.1 (s)	163.1 (s)	175.2 (s)	175.0 (s)
6	60.4 (d)	59.9 (d)	62.6 (d)	62.5 (d)	34	125.6 (d)	125.6 (d)	128.4 (d)	128.3 (d)
7	165.9 (s)	165.6 (s)	168.2 (s)	168.2 (s)	35	148.9 (s)	148.9 (s)	151.1 (s)	151.0 (s)
8	164.8 (s)	164.8 (s)	167.8 (s)	167.6 (s)	36	163.7 (s)	163.7 (s)	166.4 (s)	165.9 (s)
9	112.4 (s)	112.2 (s)	115.8 (s)	115.5 (s)	37	36.9 (t)	36.9 (t)	39.5 (t)	39.4 (t)
10	152.5 (s)	152.8 (s)	154.7 (s)	154.8 (s)	38	33.0 (t)	33.0 (t)	35.6 (t)	35.5 (t)
11	168.0 (s)	167.9 (s)	170.9 (s)	170.8 (s)	39	169.2 (s)	169.3 (s)	171.9 (s)	171.8 (s)
12	11.1 (q)	11.0 (q)	13.9 (q)	13.8 (q)	40	19.1 (q)	19.1 (q)	28.4 (q)	28.3 (q) [b]
13	169.4 (s)	169.4 (s)	172.3 (s)	172.2 (s)	41			28.7 (q)	28.6 (q) [b]
14	57.4 (d)	57.3 (d)	60.0 (d)	60.4 (d)	42			61.4 (t)	61.4 (t)
15	73.7 (d)	74.1 (d)	74.9 (d)	75.6 (d)	1'	97.8 (d)	98.7 (d)	99.9 (d)	100.8 (d)
16	135.2 (s)	135.3 (s)	137.3 (s)	137.6 (s)	2'	70.6 (d)	70.9 (d)	74.5 (d) [c]	73.4 (d)
17	118.1 (d)	117.8 (d)	120.4 (d)	120.0 (d)	3'	68.2 (d)	68.7 (d)	71.2 (d)	70.9 (d)
18	137.2 (d)	137.1 (d)	139.7 (d)	139.6 (d)	4'	69.3 (d)	71.9 (d)	72.9 (d)	74.4 (d)
19	14.9 (q)	15.0 (q)	34.5 (t)	34.5 (t)	5'	67.3 (d)	63.2 (d)	70.5 (d)	66.0 (d)
20	47.8 (d)	47.9 (d)	51.8 (d)	51.8 (d)	6'	60.5 (t)	14.7 (q)	63.4 (t)	17.3 (q)
21	74.6 (d) [c]	74.6 (d) [c]	76.69 (d) [b]	76.5 (d) [c]	1''	98.5 (d)	99.1 (d)	100.8 (d)	101.3 (d)
22	42.9 (d)	42.9 (d)	45.4 (d)	45.2 (d)	2''	68.6 (d)	68.8 (d)	72.0 (d)	71.2 (d)
23	177.8 (s)	177.8 (s)	179.8 (s)	179.6 (s)	3''	73.8 (d)	73.8 (d)	77.3 (d)	77.1 (d)
24	12.2 (q)	12.2 (q)	14.5 (q)	14.3 (q)	4''	65.0 (d)	65.2 (d)	67.5 (d)	67.4 (d)
25	67.3 (d)	67.3 (d)	74.5 (s) [c]	74.3 (s)	5''	74.6 (d) [c]	74.6 (d) [c]	76.61 (d) [b]	76.5 (d) [c]
26	59.4 (d)	59.4 (d)	63.7 (d)	63.6 (d)	6''	61.2 (t)	61.3 (t)	63.8 (t)	63.8 (t)
27	172.2 (s)	172.3 (s)	174.3 (s)	174.2 (s)	7''	158.3 (s)	158.3 (s)	160.8 (s)	160.8 (s)
28	39.3 (t)	39.3 (t)	40.3 (t)	40.1 (t)					

[a] Assignments were confirmed by ^1H-^1H gCOSY, gHSQC, and gHMBC spectra. [b] Interchangeable signal. [c] Overlapping signals.

Figure S1. Comparison of the organization of the BLM, TLM, and ZBM biosynthetic gene clusters. Proposed functions for individual ORFs have been reported previously. This figure was adopted from reference S14.

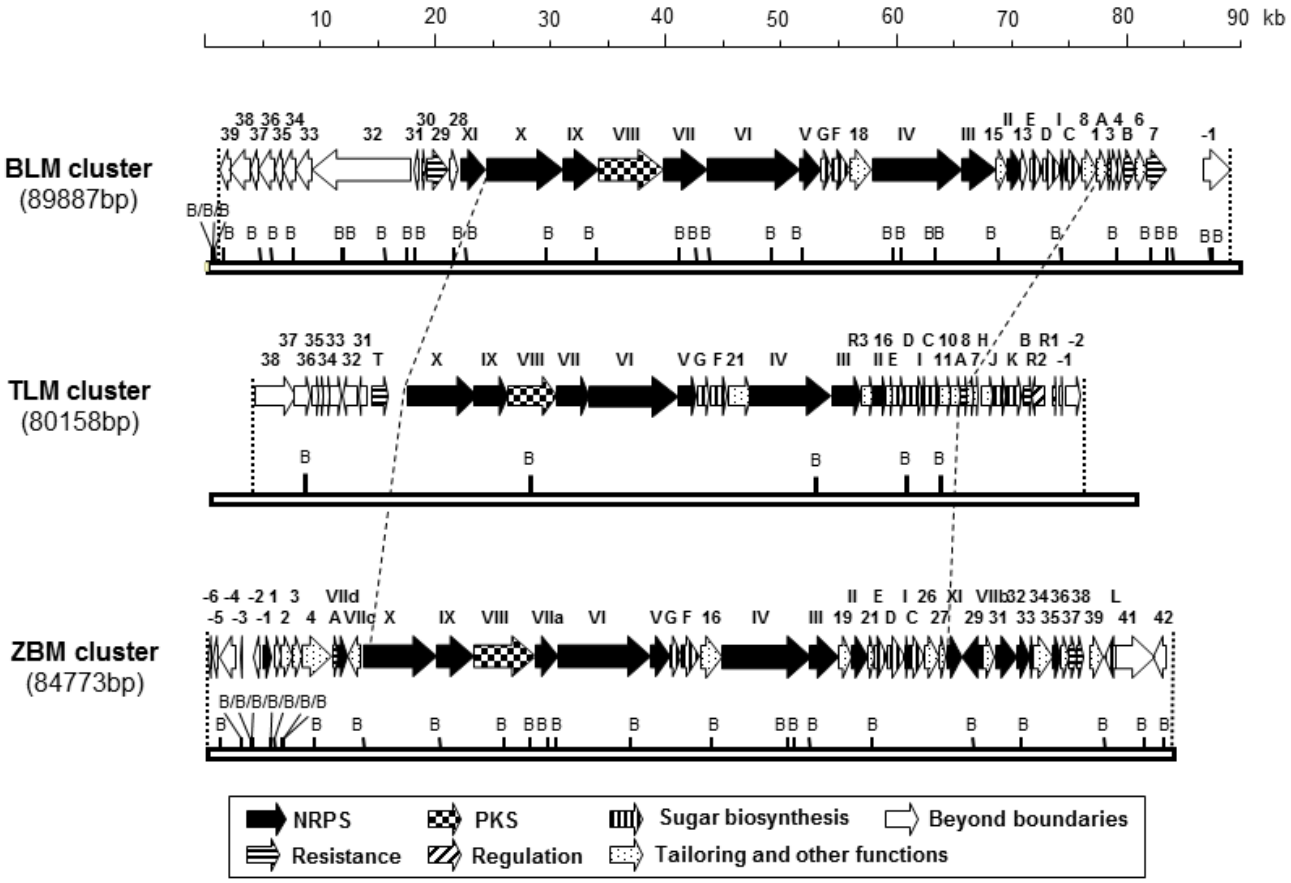


Figure S2. Proposed pathways for BLM, TLM, and ZBM sugar biosynthesis and attachment to their respective aglycones. This figure was adopted from reference S14.

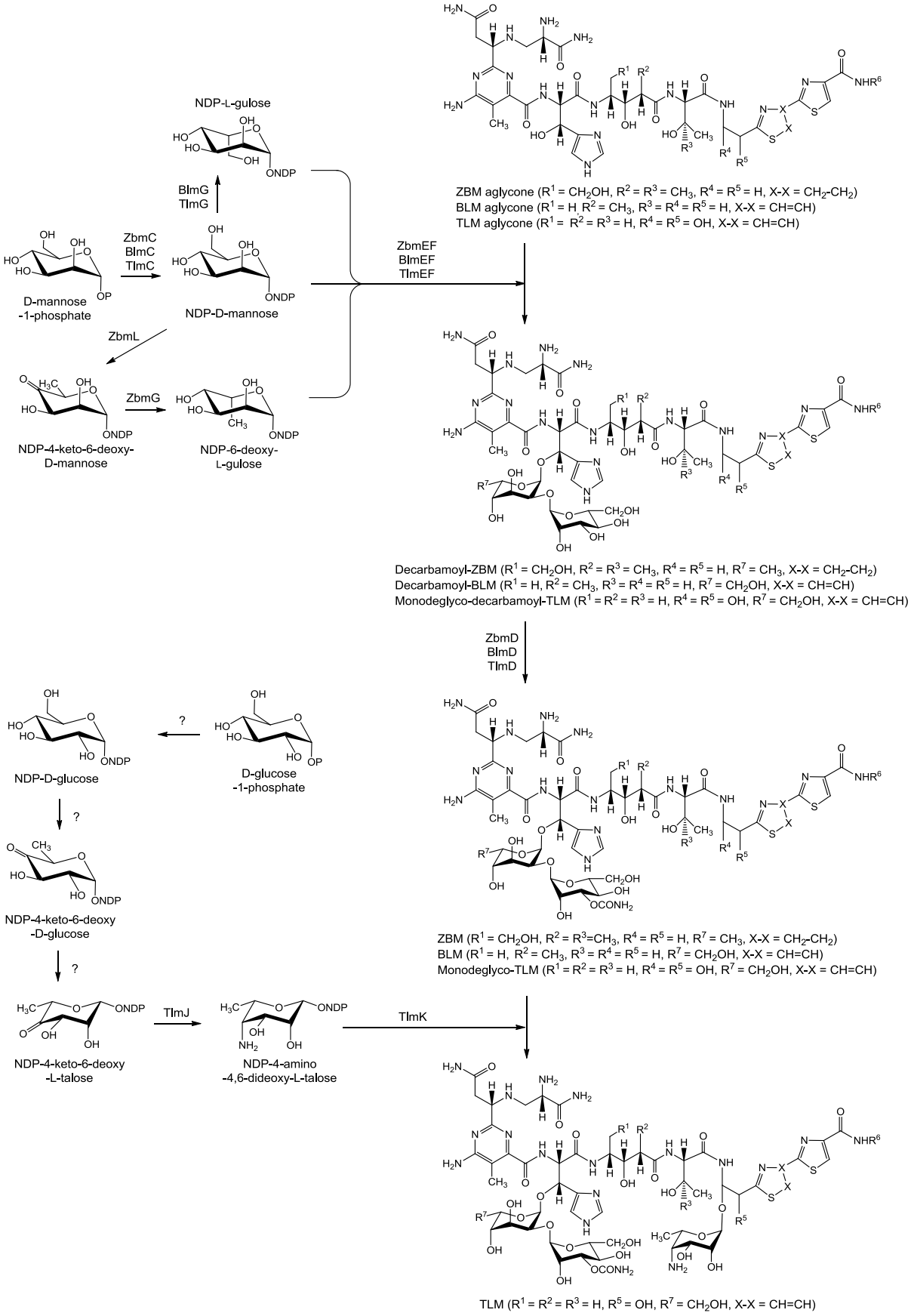


Figure S3. The ^{1}H-^{1}H gCOSY and selected HMBC correlations of ZBM and 6'-hydroxy-ZBM

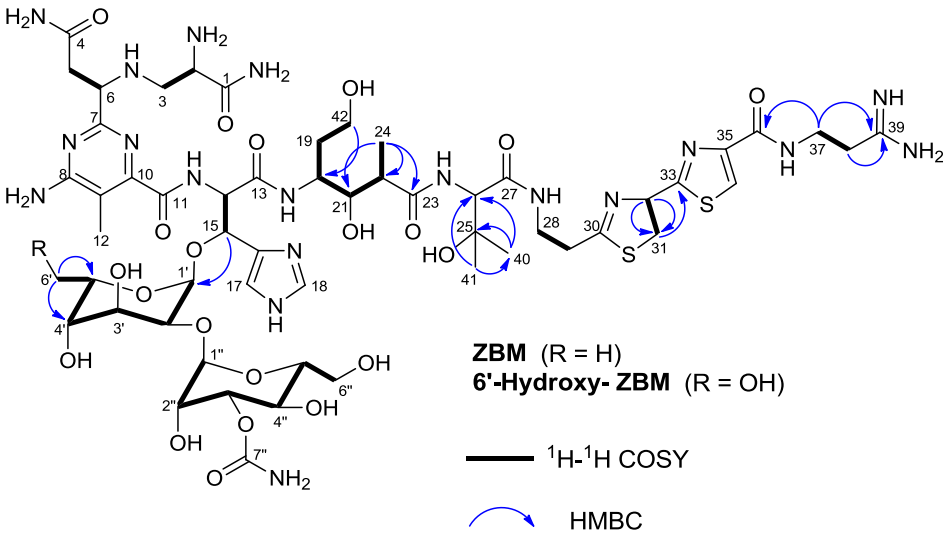


Figure S4. HPLC analysis for BLM production in SB9028, SB9029, and SB9030 with *S. verticillus* ATCC15003, *S. albus* J1074, *S. lividans* K4-114, and *S. coelicolor* M512 as controls: (I) *S. verticillus* ATCC15003 (BLM producing); (II) *S. albus* J1074 (host); (III) SB9028 (*S. albus* J1074/pBS54); (IV) *S. lividans* K4-114 (host); (V) SB9029 (*S. lividans* k4-114/pBS54); (VI) *S. coelicolor* M512 (host); (VII) SB9030 (*S. coelicolor* M512/pBS54).

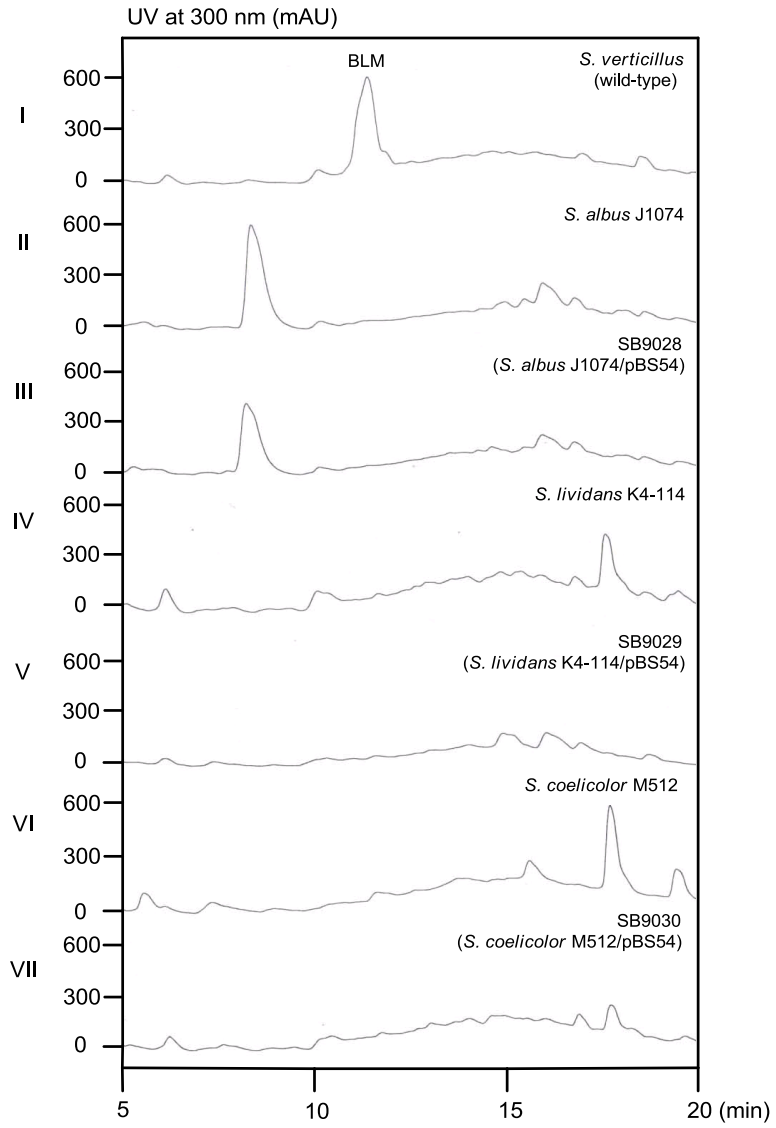
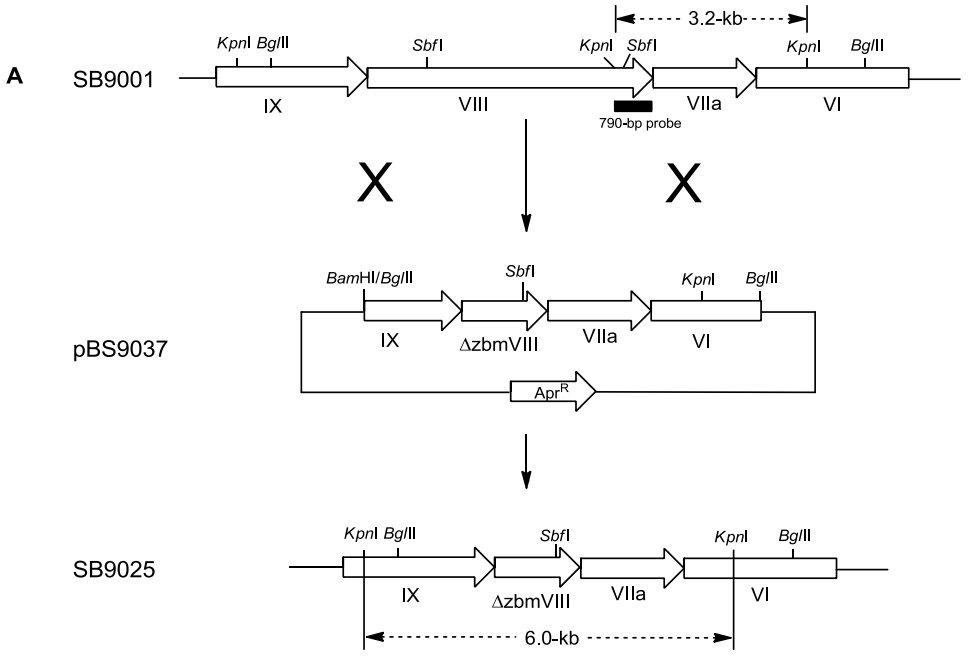


Figure S5. Construction of the *ΔzbmVIII* mutant strain SB9025 and confirmation of its genotype by Southern analysis. (A) Construction of the *ΔzbmVIII* in-frame deletion mutant strain SB9025 and restriction maps of the wild-type SB9001 and the *ΔzbmVIII* mutant SB9025 strains showing predicted fragment sizes upon *Kpn*I digestion. Apr^R, apramycin resistance. (B) Southern analysis of the SB9001 (lane 2) and SB9025 (lane 3) genomic DNAs digested with *Kpn*I using the 790-bp *Sst*I-*Kpn*I fragment from plasmid pBS9034 as a probe. Lane 1, molecular weight standard.



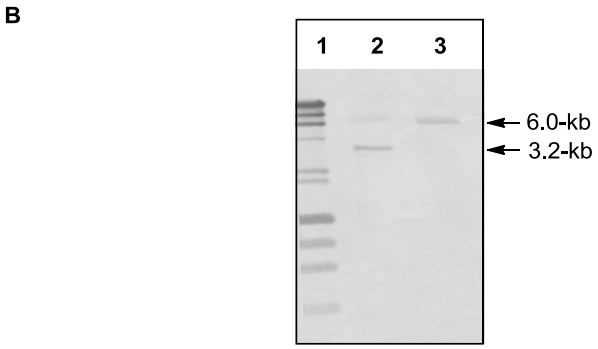


Figure S6. The ^{1}H-^{1}H gCOSY and selected HMBC correlations of BLM Z and 6'-deoxy-BLM Z

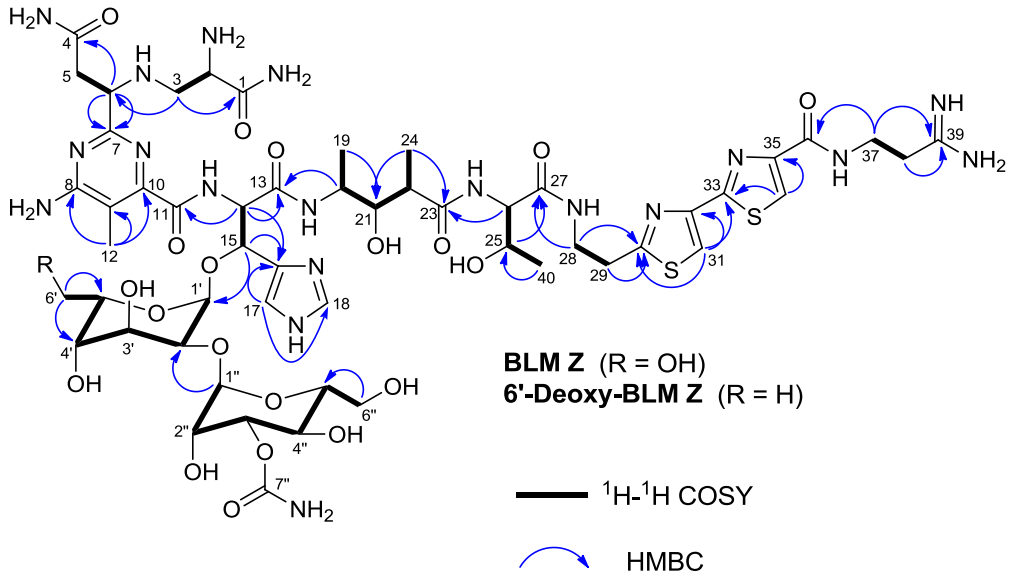


Figure S7. DNA cleavage activities of 6'-hydroxy-ZBM, BLM Z, and 6'-deoxy-BLM Z in comparison with BLM A2 and ZBM: (A) ZBM varying concentration from 0.0 to 2.0 μM, (B) 6'-hydroxy-ZBM varying concentration from 0.0 to 3.0 μM, (C) BLM A2 varying concentration from 0.0 to 1.0 μM, (D) BLM Z varying concentration from 0.0 to 1.0 μM, and (E) 6'-deoxy-BLM Z varying concentration from 0.0 to 1.0 μM. EC_{50} values (μM) (the drug concentrations needed to attain 50% cleavage of the total DNA) were estimated by analysis of the dose-response curves. Form I, supercoiled plasmid DNA; form II, open circular plasmid DNA; form III, linearized plasmid DNA.

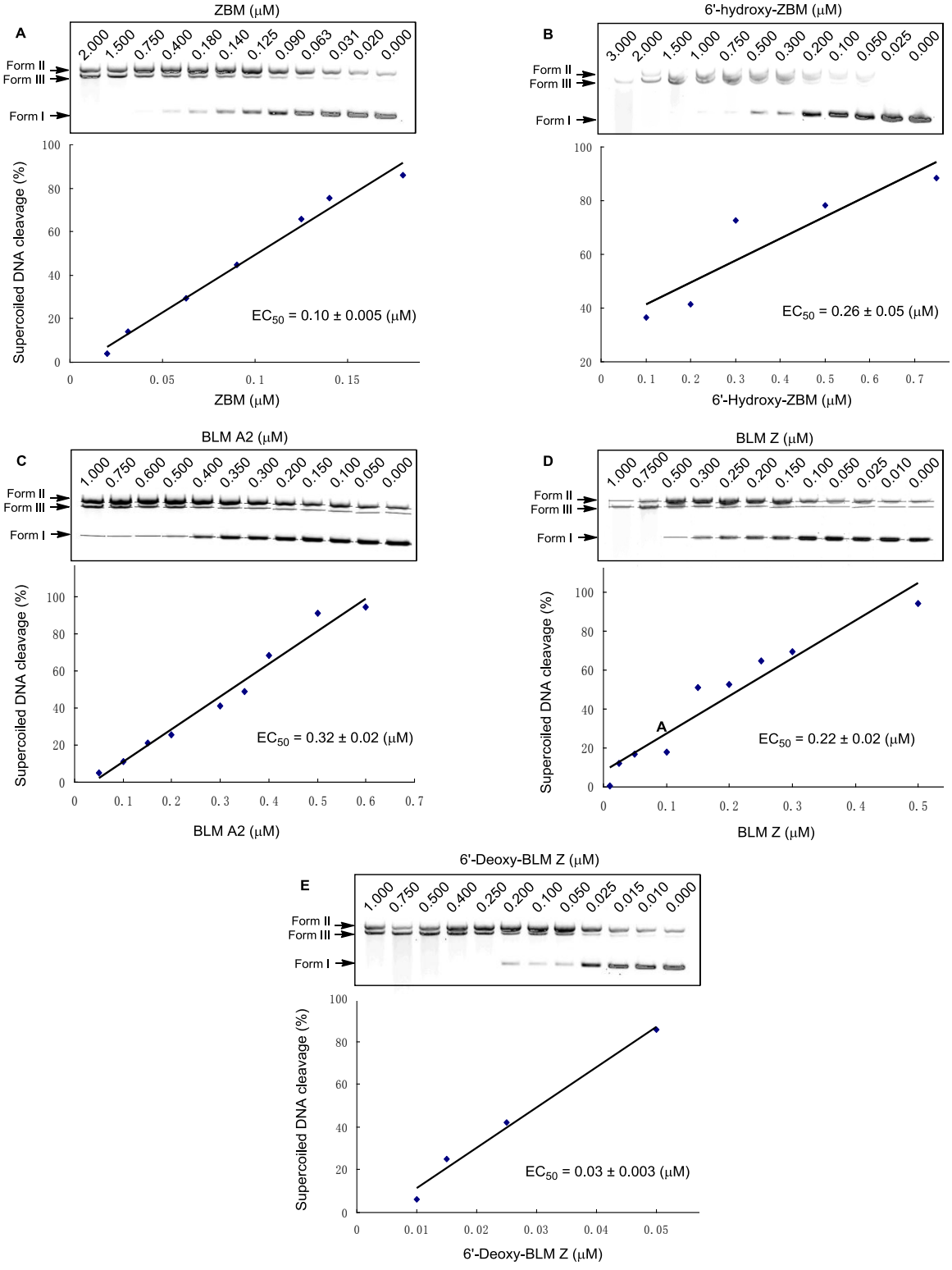


Figure S8. ^{1}H NMR spectrum of 6'-hydroxy-ZBM in D_2O

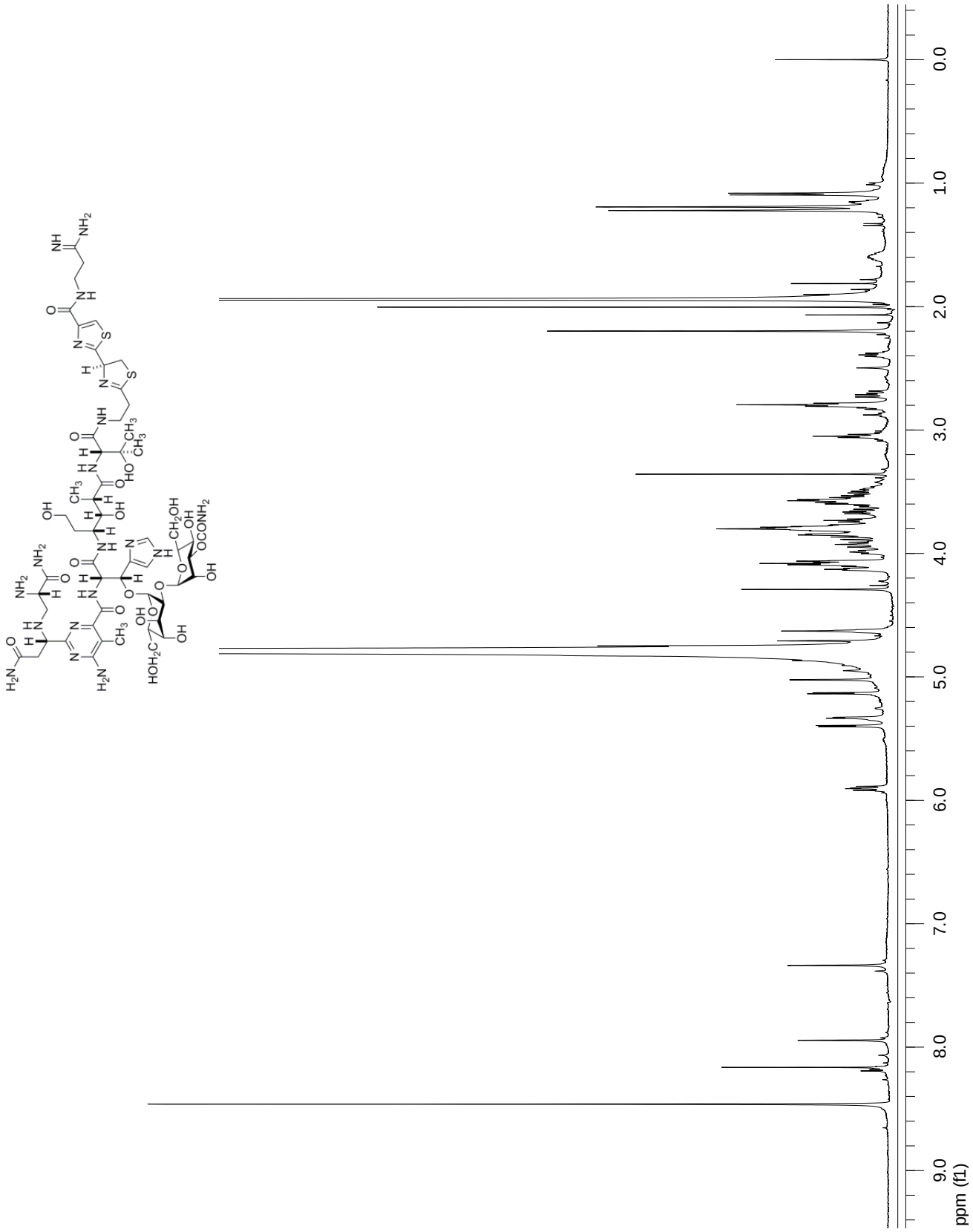


Figure S9. ^{13}C NMR spectrum of 6'-hydroxy-ZBM in D_2O

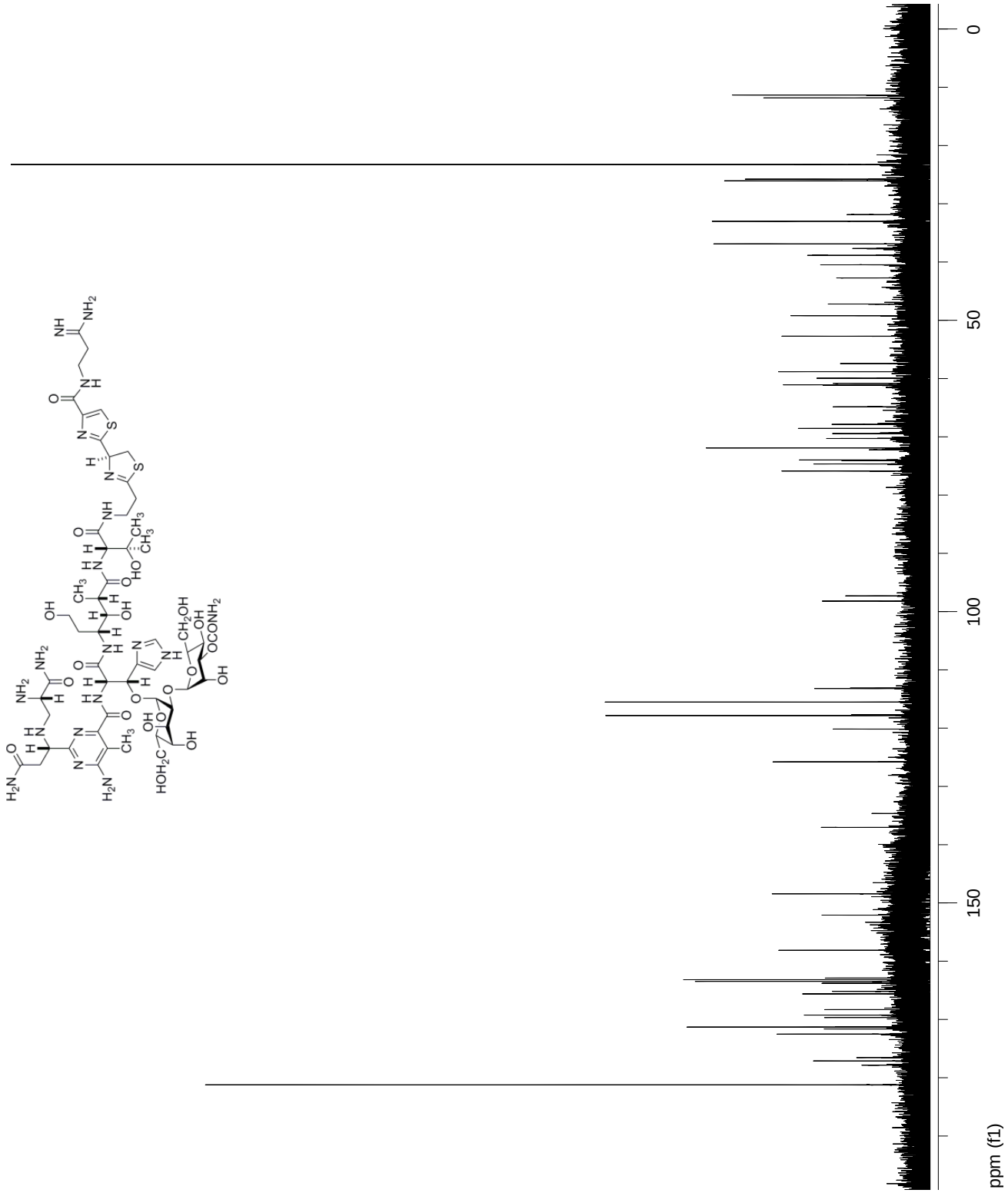


Figure S10. ^{1}H-^{1}H gCOSY NMR spectrum of 6'-hydroxy-ZBM in D_2O

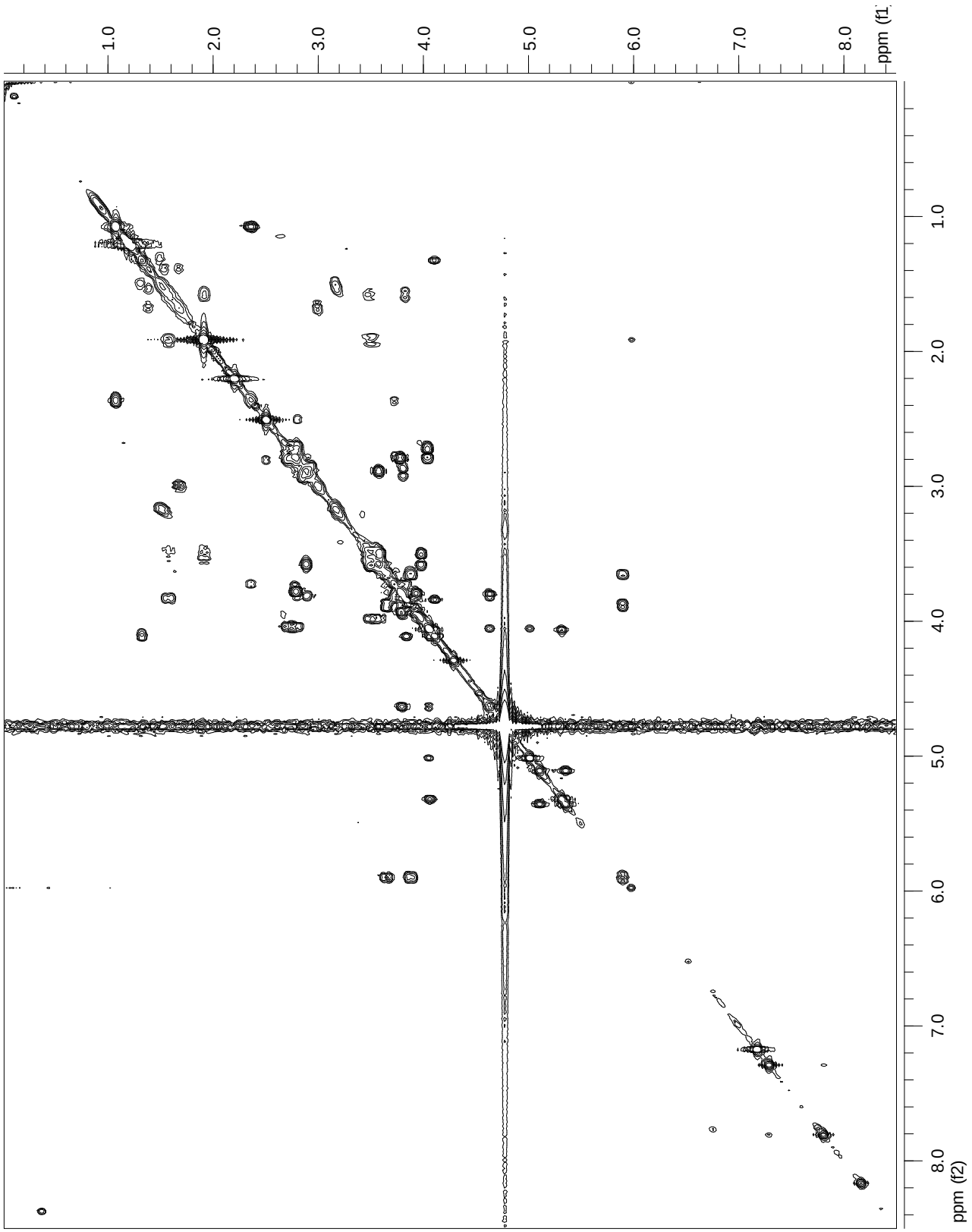


Figure S11. gHMBC NMR spectrum of 6'-hydroxy-ZBM in D_2O

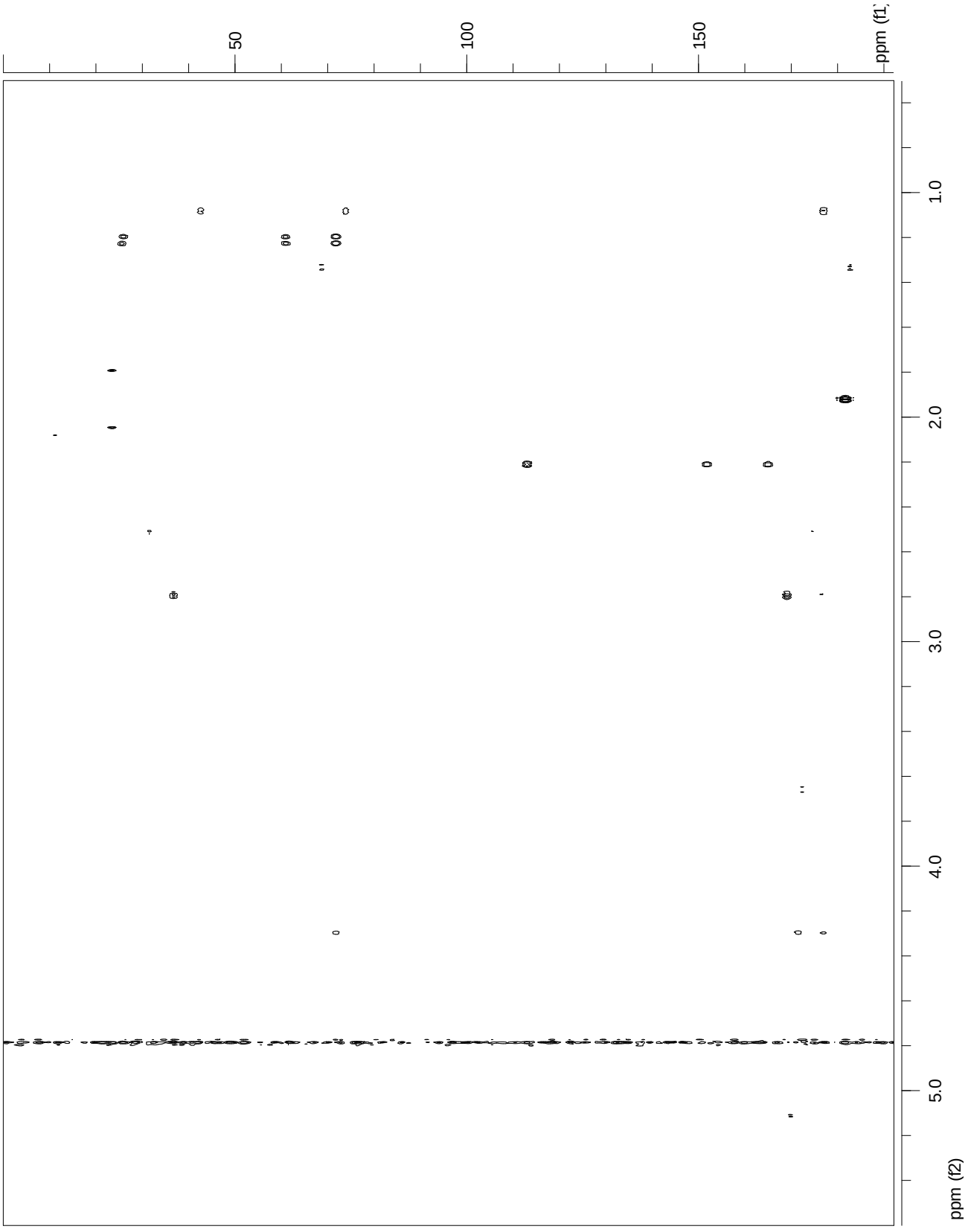


Figure S12. gHMQC NMR spectrum of 6'-hydroxy-ZBM in D_2O

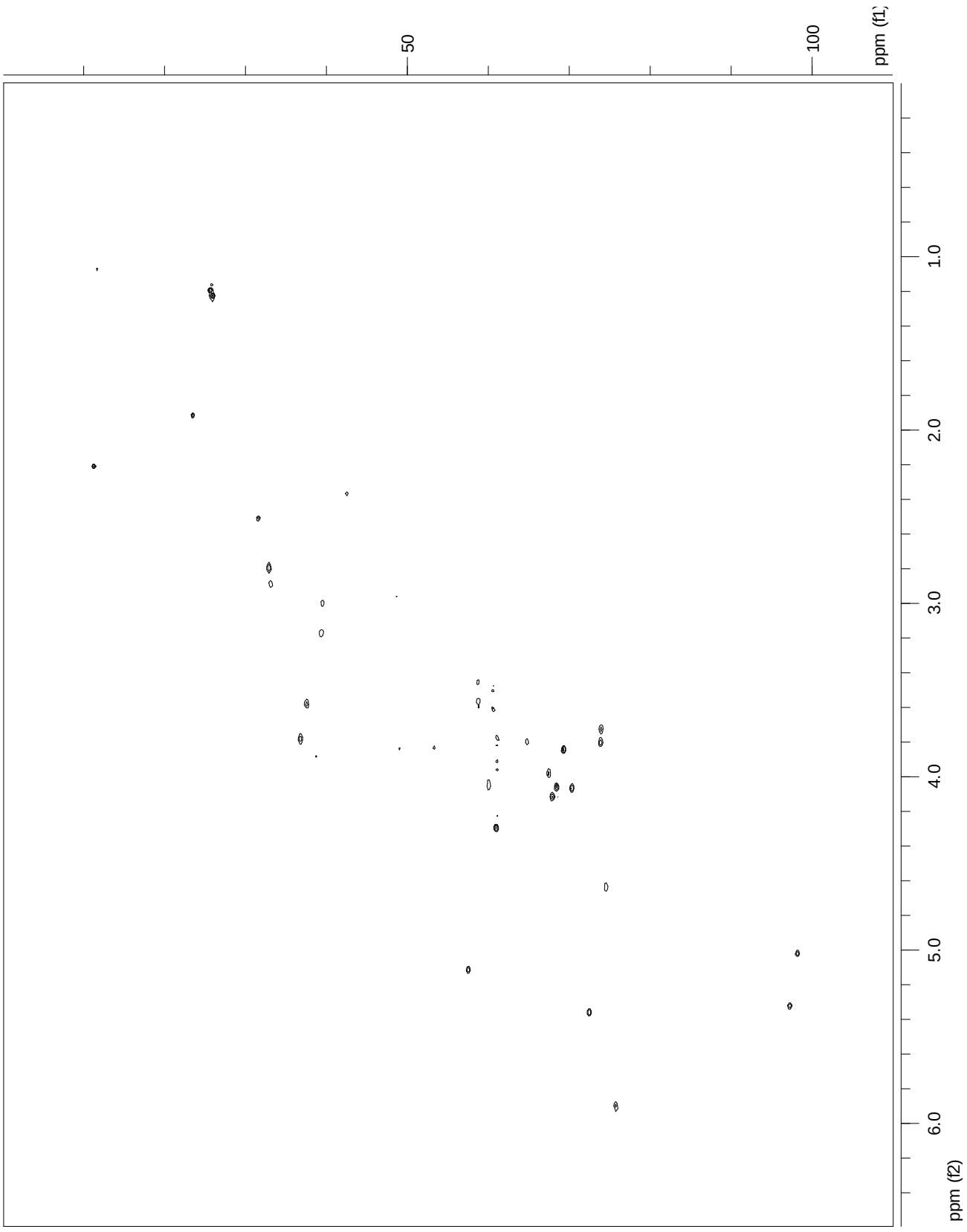


Figure S13. ^{1}H NMR spectrum of BLM Z in D_2O

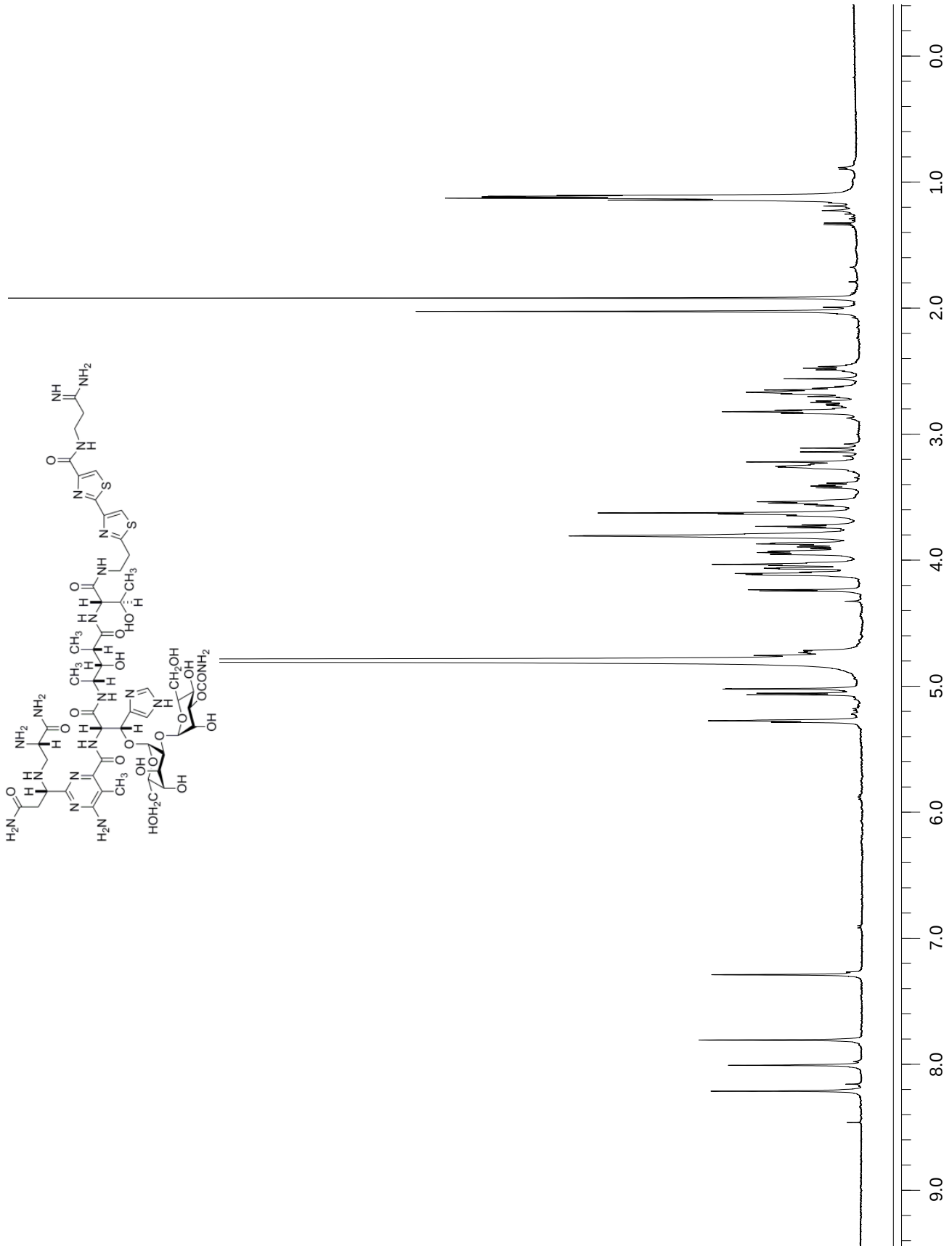


Figure S14. ^{13}C NMR spectrum of BLM Z in D_2O

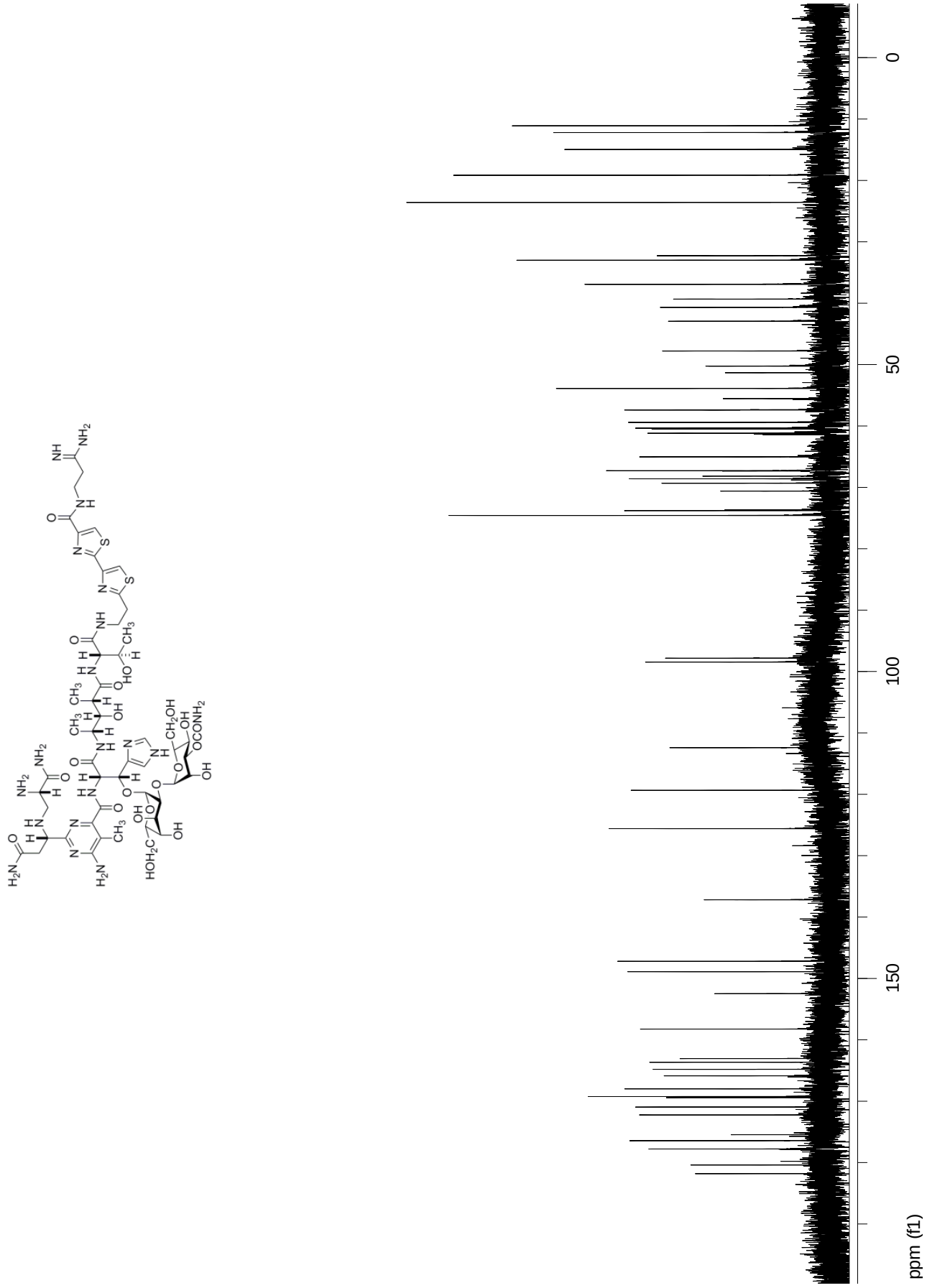


Figure S15. ^{1}H-^{1}H gCOSY NMR spectrum of BLM Z in D_2O

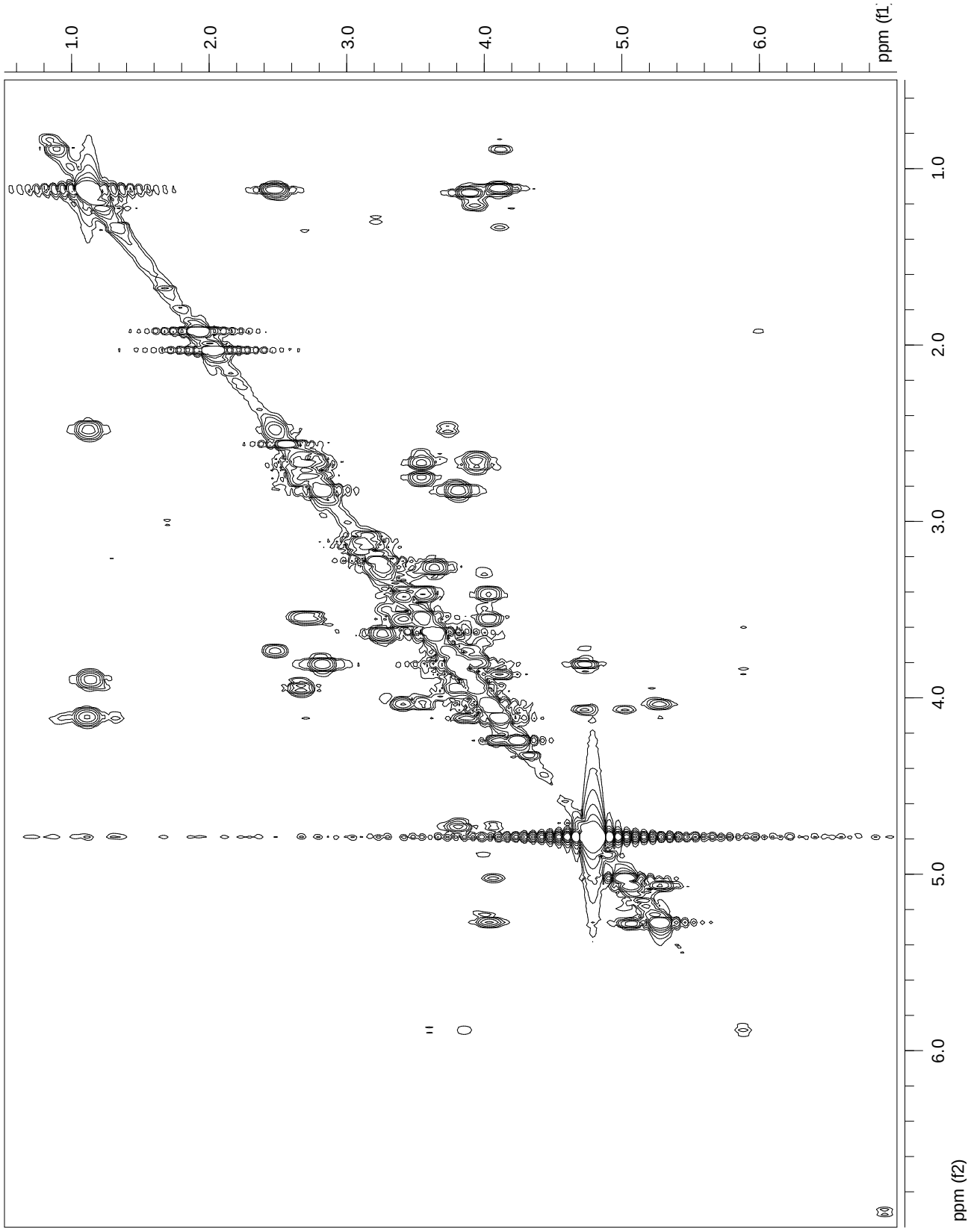


Figure S16. gHMBC NMR spectrum of BLM Z in D_2O

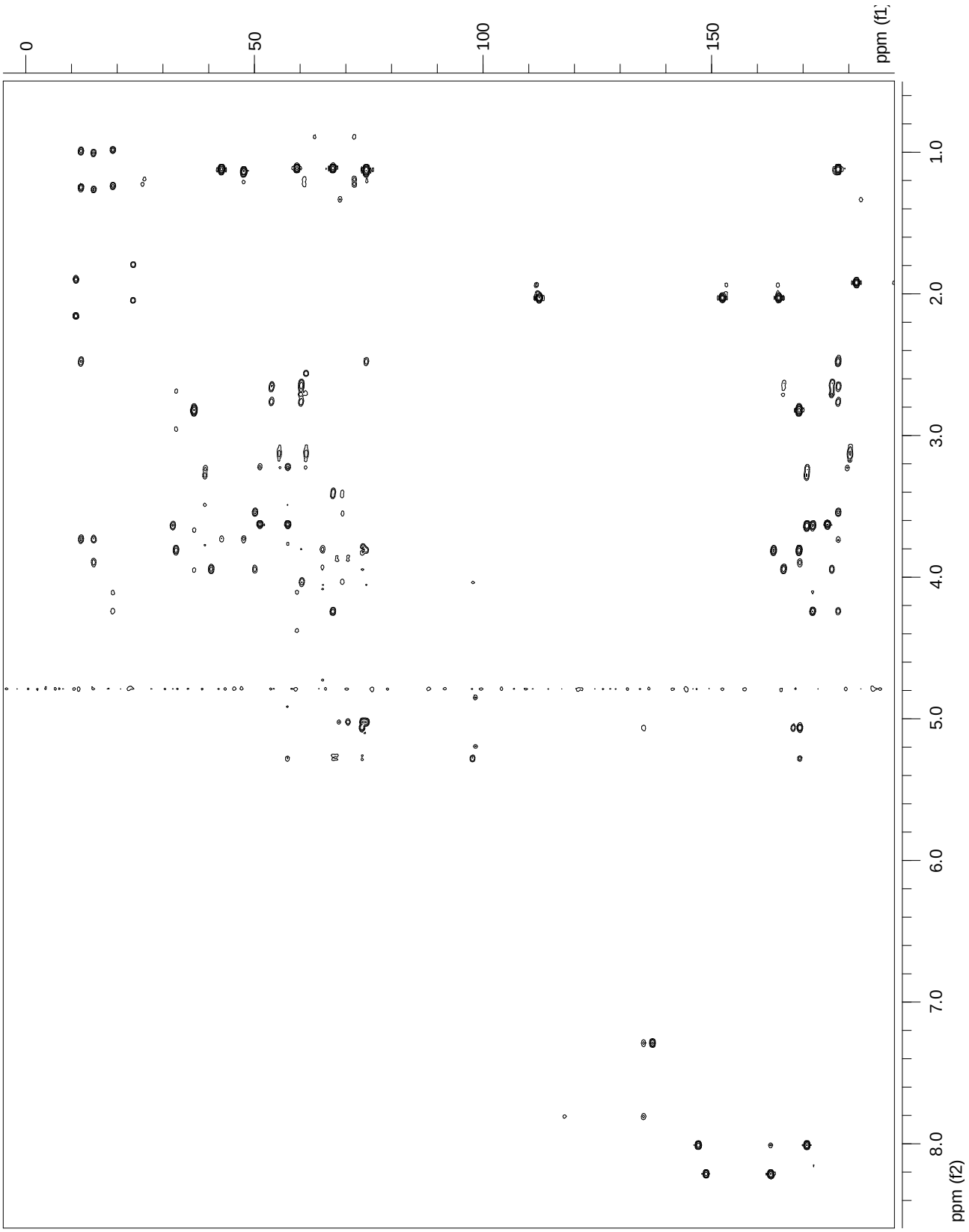


Figure S17. gHSQC NMR spectrum of BLM Z in D_2O

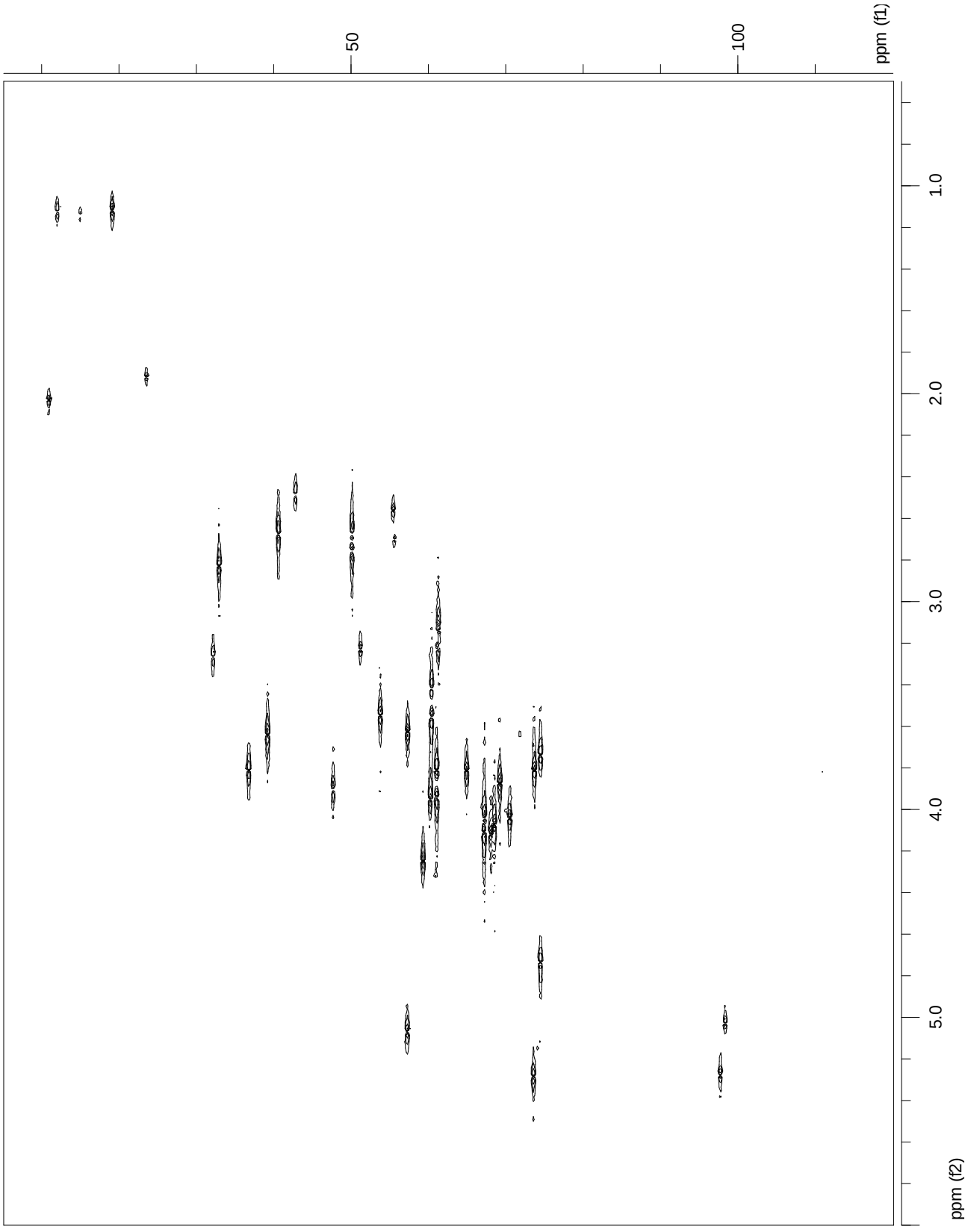


Figure S18. HSQC-TOCSY NMR spectrum of BLM Z in D_2O

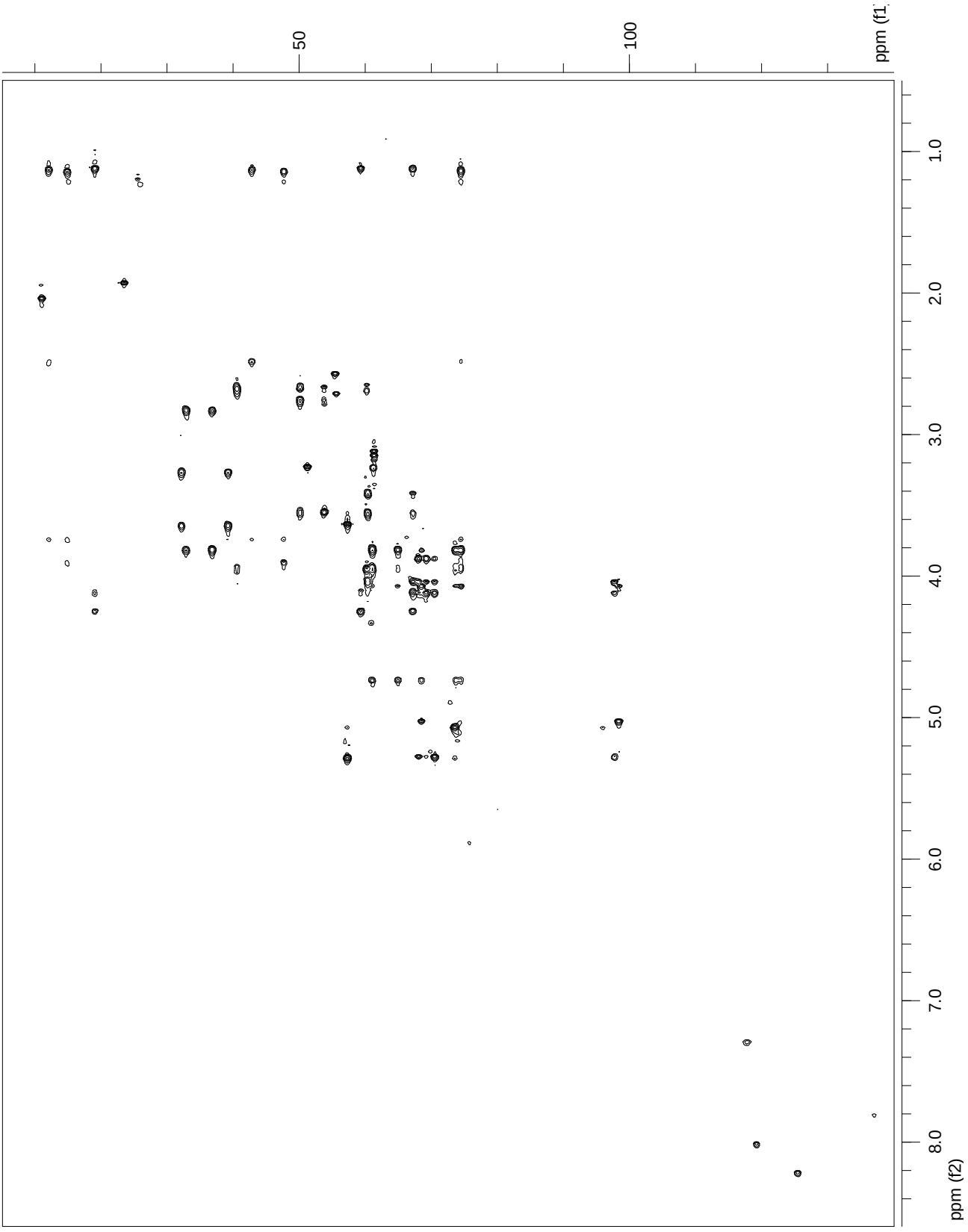


Figure S19. ^{1}H NMR spectrum of 6'-deoxy-BLM Z in D_2O

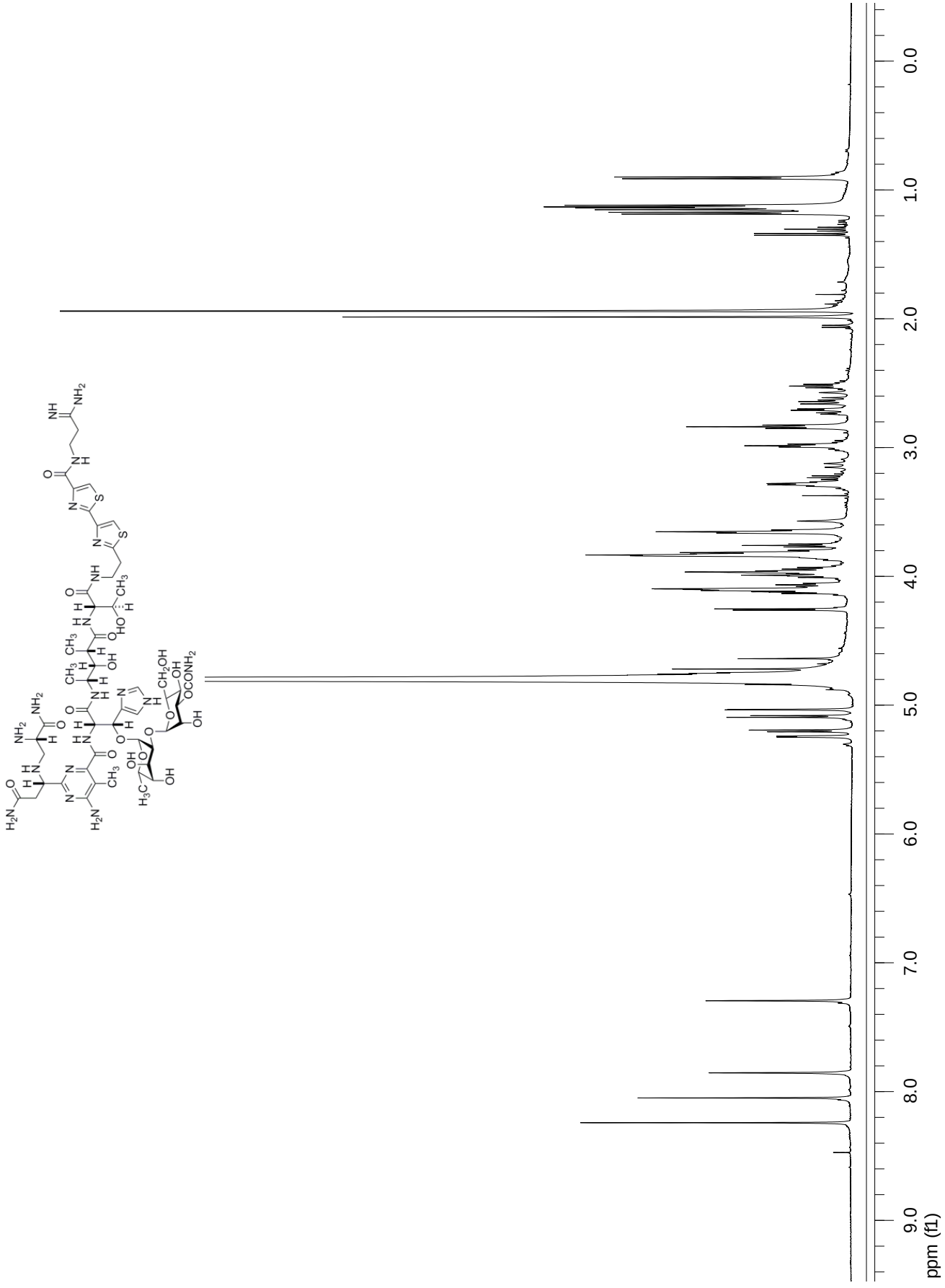


Figure S20. ^{13}C NMR spectrum of 6'-deoxy-BLM Z in D_2O

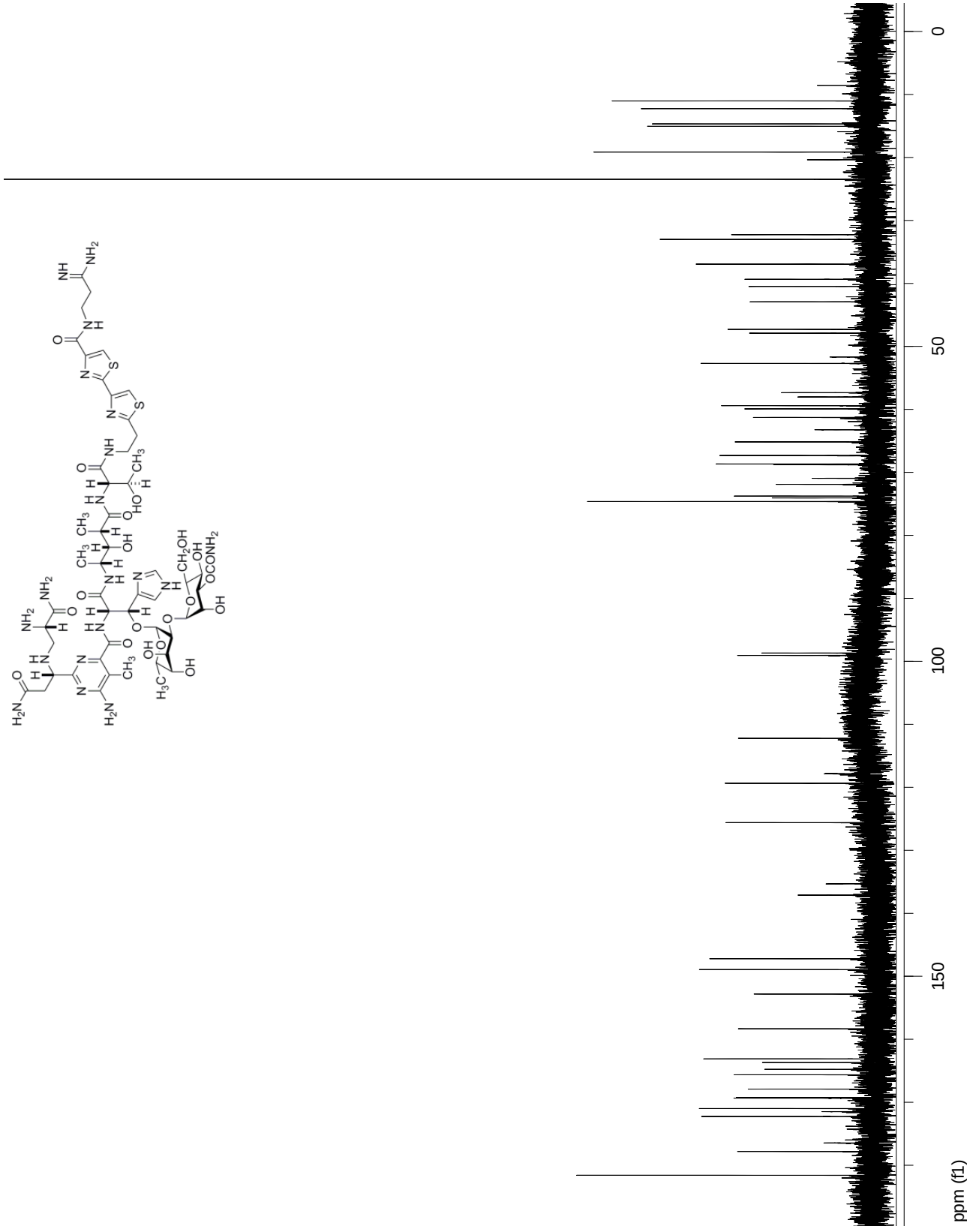


Figure S21. ^{1}H-^{1}H gCOSY NMR spectrum of 6'-deoxy-BLM Z in D_2O

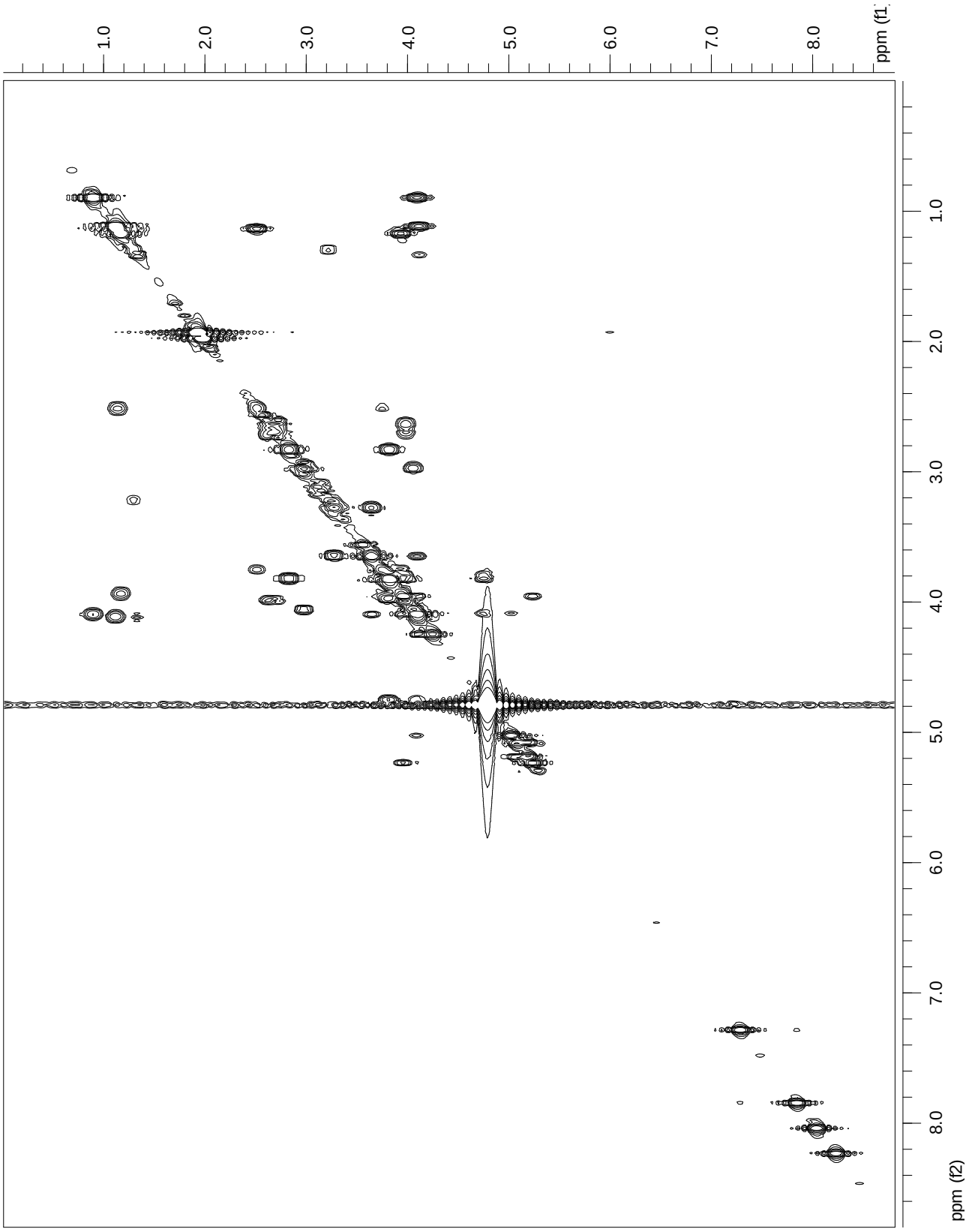


Figure S22. gHMBC NMR spectrum of 6'-deoxy-BLM Z in D_2O

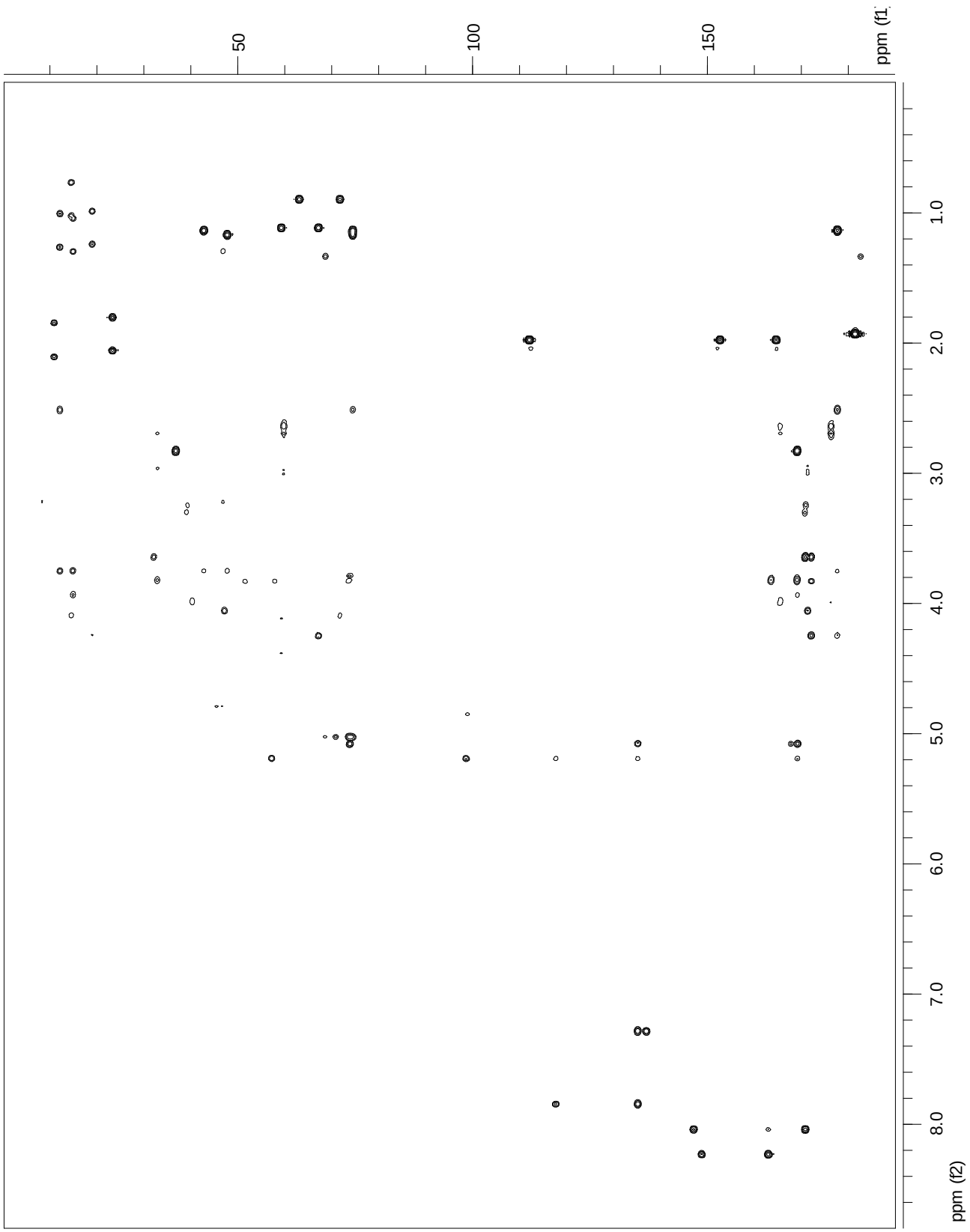


Figure S23. gHSQC NMR spectrum of 6'-deoxy-BLM Z in D_2O