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Supporting Information

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Azumamides A-E, New HDAC Inhibitory Cyclic Tetrapeptides from the Marine Sponge *Mycale izuensis*

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Experimental Section

General Procedures: NMR spectra were recorded on a JEOL A600 NMR spectrometer operating at 600 MHz for ^{1}H and 150 MHz for ^{13}C. ^{1}H and ^{13}C NMR chemical shifts were referred to DMSO-d_6 (δ_H 2.49 and δ_C 39.5) or CD_3OH (δ_H 3.30 and δ_C 49.0). FAB mass spectra were measured on a JEOL JMX-SX102/SX102 tandem mass spectrometer using glycerol as a matrix.

Animal material: The sponge samples were collected by hand using SCUBA in the Amakusa Islands in southern Japan (32°13'00" N; 130°8'45" E) in July 1999. Samples were immediately frozen and kept at -20 °C until chemical investigation.

Isolation: Frozen samples (2.2 kg) were extracted successively with EtOH (3 x 3 L) and MeOH (1 x 2 L). The combined extracts were concentrated and partitioned between H_2O and Et_2O, and the aqueous layer was further extracted with *n*-BuOH. The *n*-BuOH extract was separated by ODS flash chromatography eluted with H_2O/MeOH (1:0, 8:2, 5:5, 3:7, 0:1). The fraction eluted with H_2O/MeOH (5:5) was gel filtered on a Sephadex LH-20 column with MeOH to obtain a brownish oily material. This was separated by reversed-phase HPLC on COSMOSIL 5C_{18}-ARII using a gradient elution from MeOH/H_2O/TFA (30:70:0.05) to MeOH/H_2O/TFA (60:40:0.05). Final purification of active substances was done by reversed-phase HPLC on COSMOSIL 5C_{18}-ARII with *n*-PrOH/H_2O/TFA (10:90:0.05) to yield azumamides A (**1**; 2.7 mg), B (**2**; 1.6 mg), C (**3**; 1.4 mg), D (**4**; 0.6 mg), and E (**5**; 0.9 mg).

Azumamide A (**1**): amorphous colorless solid; $[\alpha]^{23}_{D}$ +33° (*c* 0.1; MeOH); UV (MeOH) λ_{max} 205.5 nm (ε 6,900), 208.5 (8,300), 212.0 (8,200), 216.0 (6,600); ^{1}H and ^{13}C NMR see Tables 1 and 2; HR-FABMS *m/z* 514.3018 $[(M+H)^+$, calcd for $C_{27}H_{40}N_5O_5$, Δ -1.1 mmu].

Azumamide B (**2**): amorphous yellow solid; $[\alpha]^{23}_{D}$ +45° (*c* 0.1; MeOH); UV (MeOH) λ_{max} 204.5 (18,000), 225.5 (7,300), 256.5 (800), 278.5 (1,000); ^{1}H and ^{13}C NMR see Tables 1 and 2; HR-FABMS *m/z* 552.2802 $[(M+Na)^+$, calcd for $C_{27}H_{39}N_5O_6Na$, Δ +0.4 mmu].

Azumamide C (**3**): amorphous yellow solid; $[\alpha]^{22}_{D}$ +21° (*c* 0.1; MeOH); UV (MeOH) λ_{max} 209 (11,000), 278 (1,400); ^{1}H and ^{13}C NMR see Tables 1 and 2; HR-FABMS *m/z* 531.2811 $[(M+H)^+$, calcd for $C_{27}H_{39}N_4O_7$, Δ -0.7 mmu].

Azumamide D (**4**): amorphous yellow solid; $[\alpha]^{20}_{D}$ +25° (*c* 0.05; MeOH); UV (MeOH) λ_{max} 212 (13,000); ^{1}H and ^{13}C NMR see Tables 1 and 2; HR-FABMS *m/z* 486.2702 $[(M+H)^+$, calcd for $C_{25}H_{36}N_5O_5$, Δ -1.4 mmu].

Azumamide E (**5**): amorphous yellow solid; $[\alpha]^{21}_{D}$ +53° (*c* 0.06; MeOH); UV (MeOH) λ_{max} 212 (22,000); ^{1}H and ^{13}C NMR see Tables 1 and 2; HR-FABMS *m/z* 515.2886 $[(M+H)^+$, calcd for $C_{27}H_{39}N_4O_6$, Δ +1.7 mmu].

Marfey analysis[11] of **1** - **5**: The acid hydrolysates of peptides **1**-**5** were derivatized with FDAA, then analyzed by LC-ESIMS [COSMOSIL 5C_{18}-MS, 2 x 150 mm, Nacalai tesque, 25-50 % MeCN (linear gradient over 12 min) + 0.05 % TFA] indicating that all α-amino acids were D-forms.

Stereochemistry of the β-amino acid (Amnaa): To prevent side reactions during acid hydrolysis, **1** (1 mg) was hydrogenated over Pd-C, followed by hydrolysis with 6 M HCl at 110 °C for 12 h. The hydrolysates were esterified with CH_2N_2 and separated by reverse phase HPLC [ODS-3, 10 x 50 mm, GL Sciences, 10-50 % MeCN (linear gradient over 10 min) + 0.05 % TFA]. The fractions were monitored by ^{1}H NMR spectra and then, the

fraction which showed the doublet α-methyl signal (δ 1.23) was derivatized with (+)-MTPACl (DMAP/CH_2Cl_2). Separation by reversed-phase HPLC [ODS-3, 10 x 50 mm, 70-100 % MeOH (linear gradient over 10 min)] with the reaction mixture monitoring by ESI-MS yielded the desired MTPA ester [*m/z* 484 $(M+Na)^+$]. Comparison of the spectrum with those of the model compounds derived from four stereoisomers of 3-amino-2-methyl hexanoic acids clearly indicated its 2*S*,3*R* configuration.

HDAC inhibition assay: Inhibitory activity against histone deacetylase was determined as follows. Human HDAC was partially purified from K562 cells (human chronic myeloid leukemia cell line) by a modification of the method of Yoshida et al.[14b] In brief, cultured cells were collected and resuspended in HDA buffer (15 mM potassium phosphate pH 7.5, 5 % glycerol, 0.2 mM EDTA) and homogenized with a Dounce homogenizer. Nuclei were collected and suspended in HDA buffer containing 1 M ammonium sulfate, and the suspension was sonicated to reduce its viscosity. After collecting the supernatant by centrifugation at 500 *g* for 15 min at 4 °C, ammonium sulfate was added to the supernatant to attain a final concentration of 3.5 M. The precipitated protein was collected, dissolved in HDA buffer, and then dialyzed against the same buffer at 4 °C. The dialysate was loaded onto a Q-Sepharose FF (GE Healthcare Bio-Sciences KK) column equilibrated with HDA buffer, and eluted using a linear gradient of 0 to 1 M NaCl in HDA buffer. HDAC activity was eluted between 0.3 and 0.4 M NaCl. The fraction associated with HDAC activity was collected, dialyzed against HDA buffer, and stored at 4 °C. Biotinylated [^{3}H] acetyl-histone H4 peptide (Biotin-Gly-Ala-[^{3}H-acetyl] Lys-Arg-His-Arg-[^{3}H-acetyl] Lys-Val-amide, 90 Ci/mmol; GE Healthcare Bio-Sciences KK) was used as a substrate for HDAC.[20] Two μL of various concentrations of compounds was incubated with 25 μL of [^{3}H] acetyl-histone H4 peptide in HDA buffer at room temperature and then 25 μL of HDAC enzyme solution was added to the substrate solutions and incubated at room temperature for 2 h. Each reaction was stopped with 50 μL of 1 M HCl and the released [^{3}H] acetic acid was extracted with 1 mL of ethyl acetate. After centrifugation at 12,000 *g* for 1 min, the radioactivity in the organic layer (0.8 mL) was measured by a liquid-scintillation counter in 5 mL Aquasol-2 (PACKARD).

Western blot analysis: K562 cells were maintained in RPMI1640 (GIBCO BRL) supplemented with 10 % heat-inactivated fetal bovine serum, 50 units/mL penicillin and 50 μg/mL streptomycin at 37 °C with 5 % CO_2. K562 cells were inoculated at 1.5×10^6 cells onto 6 well plate, and then incubated with azumamide A (0.19 – 19.4 μM) and MS-275 for 6 h. For the Western blot analysis, the cells, which were recovered by centrifugation, were washed with ice-cold PBS and lysed with 50 μL of ice-cold TNE buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 % NP-40, 1 tablet Complete Mini (Roche) / 10 mL). Whole cell lysates were kept on ice for 10 min before centrifugation at 300 *g* for 1 min. The protein concentrations of the supernatants were determined using a Protein Assay Kit (Bio-Rad). Proteins were boiled in SDS sample buffer for 5 min and electrophoresed in 10 % to 20 % gradient polyacrylamide gels. After electrophoresis, proteins were transferred onto a PVDF membrane and the membrane was incubated in BlockAce blocking reagent for 1 h. The histone acetylations were detected using anti-acetylated histone H3 and H4 (Upstate Biotechnology). The signals were developed with the ECL (GE Healthcare Bio-Sciences KK).

Cytostatic Activity Test: K562 human leukemis cells or WiDr human colon cancer cells (5 x 10^3 cells/well) were seeded into individual wells of 96-well plates and incubated at 37 °C under 0.5 % CO_2. After 18 h incubation, DMSO or DMSO solutions of azumamide A were administered and incubated for further 72 h. Cell proliferation was measured with AlamarBlue™ (BIOSOURCE).

Anti-angiogenic assays using mouse ES cells: Maintenance, differentiation and culture were as described.[21] Flk1+ cells were sorted by MACS (Milteny Biotec GmbH) instead of FACS. We plated 2 x 10^4 $Flk1^+$ cells per well on collagen IV (Col. IV)-coated 24-well dishes (Becton Dickinson) in differentiation medium.[21] Single sorted cells were plated into individual wells of Col IV-coated 96-well dishes. Before three-dimensional culture, cells (5 x 10^5 cells/mL) were incubated in the differentiation medium[21] containing 50 ng/mL VEGF on uncoated petri dishes for 12 h to induce aggregation. Aggregates were resuspended in the differentiation medium and mixed with an isovolume of collagen I-A gel (3 mg/mL, Nitta Gelatin). We plated 250 μL of this mixture onto a lucent insert disc, Cell Disk (Sumitomo Bakelite), in 24-well dishes. After 15 min at 37 °C to allow polymerization, we added 750 μL of differentiation medium with VEGF (final 50 ng/mL). Human VEGF165 was purchased from R&D Systems Inc.

[20] Nare, B.; Allocco, J. J.; Kuningas, R.; Galuska, S.; Myers, R. W.; Bednarek, M. A.; Schmatz, D. M. *Anal. Biochem*. **1999**, *267*, 390-396.
[21] Nishikawa, S. I.; Nishikawa, S.; Hirashima, M.; Matsuyoshi, N.; Kodama, H. *Development* **1998**, *125*, 1747-1757.

Table S1: ^{13}C NMR data of azumamides A (**1**) – E (**5**) in CD_3OH.

	#C	**1**	**2**	**3**	**4**	**5**
Amnaa	1	176.9	176.9	176.9	177.5	177.2
/Amnda	2	43.6	43.7	44.1	43.9	44.0
	3	53.0	53.1	53.0	54.5	53.0
	4	29.5	29.8	30.0	29.7	29.7
	5	127.7	127.6	127.4	127.8	127.6
	6	131.7	131.9	131.9	131.7	131.7
	7	24.7	24.8	24.1	24.9	24.1
	8	36.3	36.3	35.0	36.4	34.9
	9	178.4	178.5	ND	179.0	177.2
	10	11.0	10.7	10.2	10.4	10.8
Phe/Tyr	11	174.0	174.2	ND	175.0	174.0
	12	60.5	60.4	59.7	59.0	60.1
	13	37.4	36.8	36.8	37.5	37.4
	14	138.5	129.0	128.9	138.6	138.5
	15	130.1	131.1	131.2	130.2	130.2
	16	129.3	116.2	116.2	129.3	129.4
	17	127.7	157.4	157.4	127.7	127.7
	18	129.3	116.2	116.2	129.3	129.4
	19	130.1	131.1	131.2	130.2	130.2
Ala	20	174.9	174.9	174.3	176.0	174.9
	21	51.6	51.9	52.3	52.5	51.9
	22	16.1	16.2	16.2	16.5	16.1
Val/Ala	23	174.6	174.5	ND	176.6	174.5
	24	65.8	65.5	65.0	53.8	65.5
	25	29.2	29.4	29.6	15.9	29.3
	26	19.6	19.7	19.6		19.7
	27	19.9	19.9	19.9		19.9

Table S2: ^{1}H NMR data of azumamides A (**1**) – E (**5**) in CD_3OH.

	#C	**1**		**2**		**3**		**4**		**5**	
Amnaa/Amna	2	2.73	dq, 4.6, 7.3	2.73	dq, 4.4, 7.5	2.74	dq, 3.1, 7.5	2.69	dq, 3.5, 7.3	2.72	dq, 4.2, 8.1
	3	4.17	dddd, 4.6, 8.1, 7.9, 7.9	4.21	dddd, 4.4, 7.7, 7.3, 7.3	4.27	dddd, 8.5, 7.7, 6.5, 3.1	4.07	dddd, 6.5, 3.5, 7.5, 7.5	4.23	m
	4a	2.69	ddd, 14.6, 7.9, 7.1	2.62	ddd, 14.6, 7.3, 7.3	2.57	ddd, 13.9, 7.2, 6.5	2.69	ddd, 15.0, 7.5, 7.5	2.62	m
	4b	2.31	m	2.31	ddd, 14.6, 7.3, 7.3	2.30	ddd, 13.9, 7.7, 7.2	2.35	dd, 15.0, 7.5, 7.5	2.32	m
	5	5.38	dt, 11.0, 7.1	5.37	dt, 11.0, 7.3	5.35	dt, 11.0, 7.2	5.40	dt, 11.0, 7.5	5.37	dt, 11.1, 7.3
	6	5.50	dt, 11.0, 7.3	5.49	dt, 11.0, 7.3	5.49	dt, 11.0, 6.9	5.48	ddd, 11.0, 7.5, 7.1	5.49	dt, 11.1, 7.3
	7a	2.47	dq, 14.6, 7.3	2.46	ddt, 14.6, 7.3, 7.3	2.40	m	2.44	ddt, 14.8, 7.1, 7.7	2.40	m
	7b	2.39	dq, 14.6, 7.3	2.39	ddt, 14.6, 7.3, 7.3			2.40	ddt, 14.8, 7.5, 7.7	2.40	m
	8	2.30	t, 7.3	2.29	t, 7.5	2.36	m	2.27	t, 7.7	2.37	m
	10	1.27	d, 7.3	1.28	d, 7.5	1.31	d, 7.5	1.29	d, 7.3	1.29	d, 8.1
Phe/Tyr	12	4.13	ddd, 10.4, 7.7, 6.7	4.15	ddd, 9.6, 8.3, 6.4	4.23	ddd, 10.4, 8.5, 6.2	4.29	ddd, 10.0, 8.5, 6.3	4.20	m
	13a	3.24	dd, 13.9, 10.4	3.10	dd, 13.8, 9.6	3.05	dd, 13.9, 10.4	3.15	dd, 13.9, 6.2	3.19	m
	13b	3.11	dd, 13.9, 6.7	3.00	dd, 13.8, 6.4	2.99	dd, 13.9, 6.2	3.11	dd, 13.9, 10.0	3.10	dd, 13.9, 5.8
	15	7.19	d, 6.9	7.02	d, 8.5	7.04	d, 8.3	7.21	d, 7.1	7.21	d, 7.3
	16	7.24	t, 7.5	6.66	d, 8.5	6.66	d, 8.3	7.24	dd, 7.5, 7.1	7.24	t, 7.3
	17	7.17	t, 6.9					7.17	t, 7.5	7.18	t, 7.3
	18	7.24	t, 7.5	6.66	d, 8.5	6.66	d, 8.3	7.24	dd, 7.5, 7.1	7.24	t, 7.3
	19	7.19	d, 6.9	7.02	d, 8.5	7.04	d, 8.3	7.21	d, 7.1	7.21	d, 7.3
Ala	21	4.29	quint., 7.6	4.28	dq, 8.0, 7.4	4.25	dq, 7.3, 7.7	4.15	dq, 6.7, 7.3	4.25	dq, 6.2, 7.7
	22	1.28	d, 6.9	1.29	d, 7.4	1.29	d, 7.7	1.24	d, 7.3	1.27	d, 7.7
Val/Ala	24	3.60	dd, 10.8, 8.1	3.67	dd, 11.2, 8.0	3.77	dd, 8.2, 8.5	4.05	dq, 6.5, 6.9	3.68	m
	25	2.33	m	2.29	dqq., 11.2, 6.5, 6.5	2.28	m	1.49	d, 6.9	2.29	m
	26	0.93	d, 6.5	0.94	d, 6.5	0.95	d, 6.9			0.94	d, 6.9
	27	0.95	d, 6.5	0.96	d, 6.5	0.97	d, 6.5			0.96	d, 6.5
NH-3		7.63	d, 8.1	7.63	d, 7.7	7.65	d, 8.5	7.28	d, 6.5	7.60	m
NH_2-9		7.58		7.60				7.59			
		6.76		6.76				6.77			
NH-12		8.15	d, 7.7	8.13	d, 8.3	8.20	d, 8.5	7.97	d, 8.5	8.03	m
NH-21		8.00	d, 8.9	8.09	d, 8.0	8.27	d, 7.3	7.94	d, 6.7	8.02	m
NH-24		7.85	d, 7.3	7.83	d, 8.0	7.80	d, 8.2	8.07	d, 6.5	7.77	m

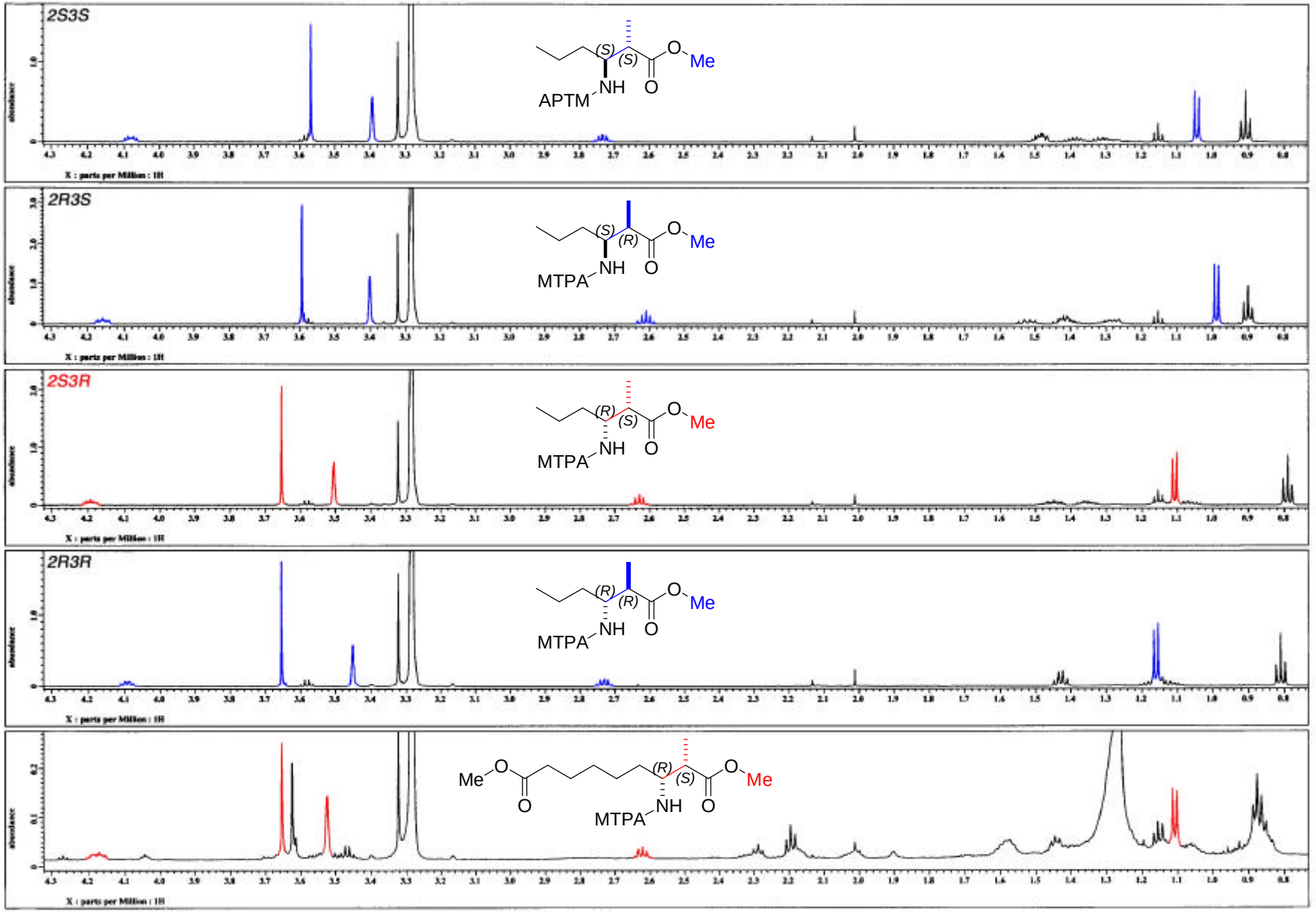

Fig. S1. Comparison of ^{1}H NMR spectra of MTPA derivatives prepared from 2*S*,3*S*- (top), 2*R*,3*S*-, 2*S*,3*R*-, 2*R*,3*R*-Amha, and azumamide A (bottom). Signals coincided in both MTPA derivatives derived from azumamide A and synthetic compounds are highlighted in red, while those not coincided in blue.

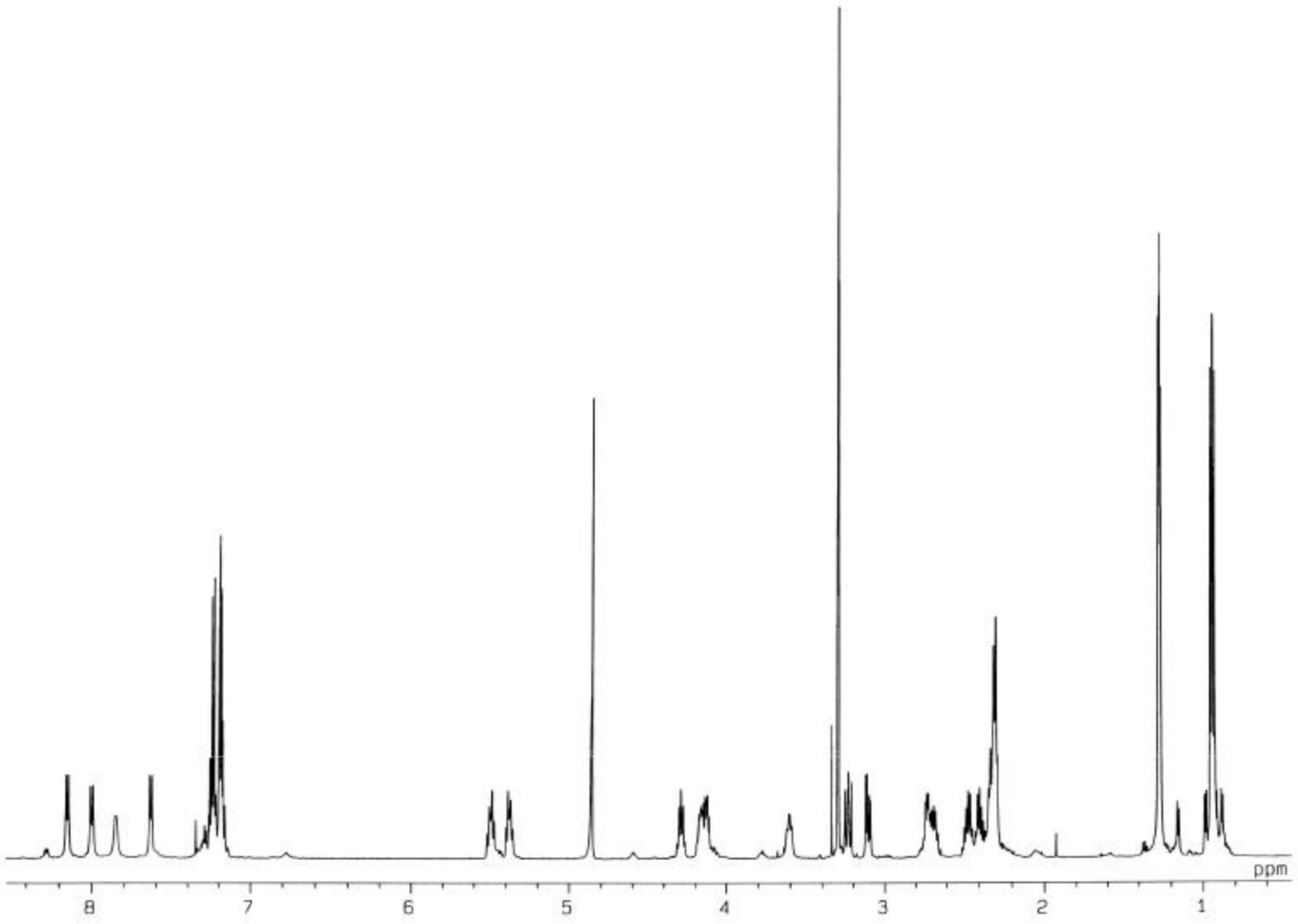

Fig. S2. ^{1}H NMR spectra of azumamide A.

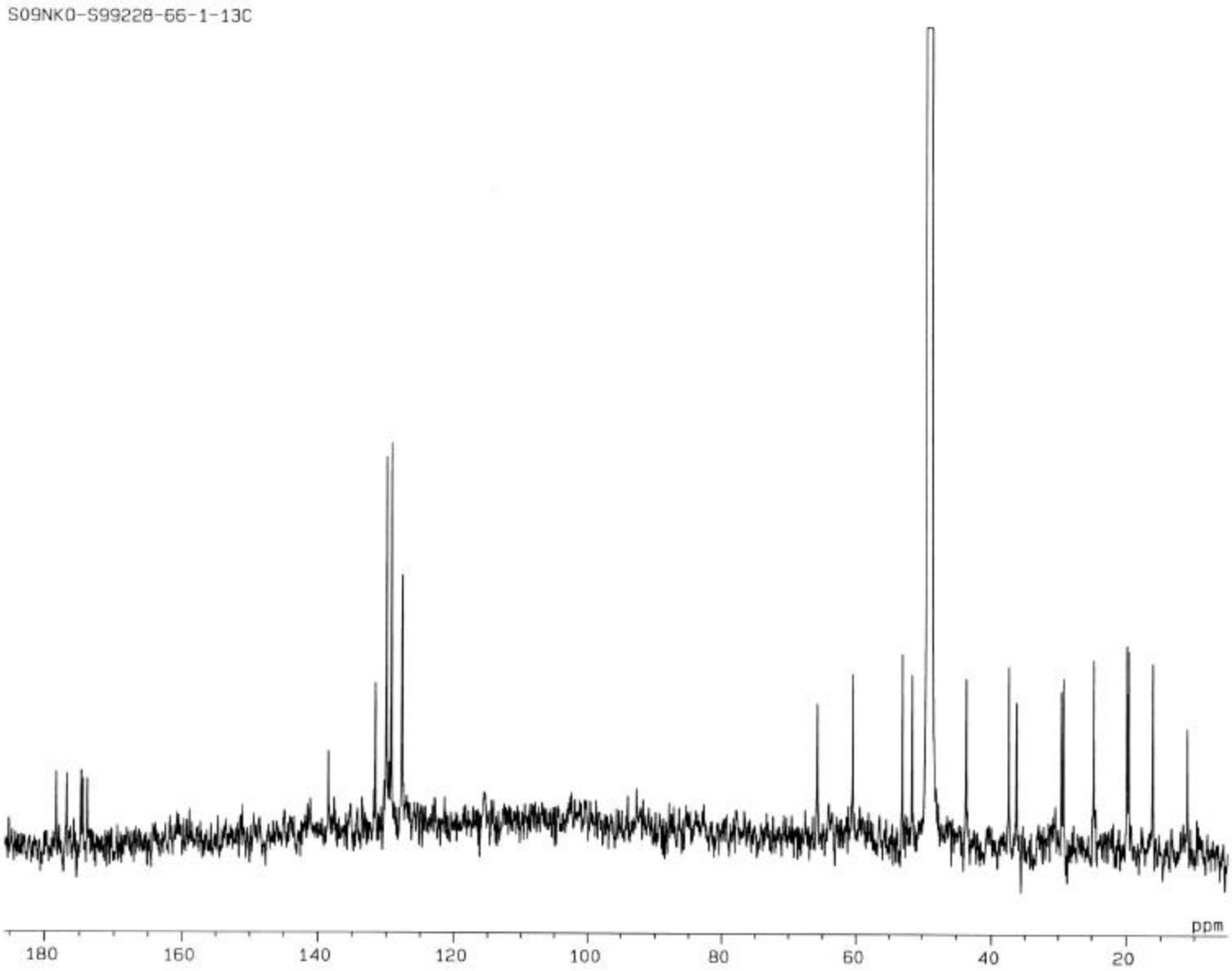

Fig. S3. ^{13}C NMR spectra of azumamide A.

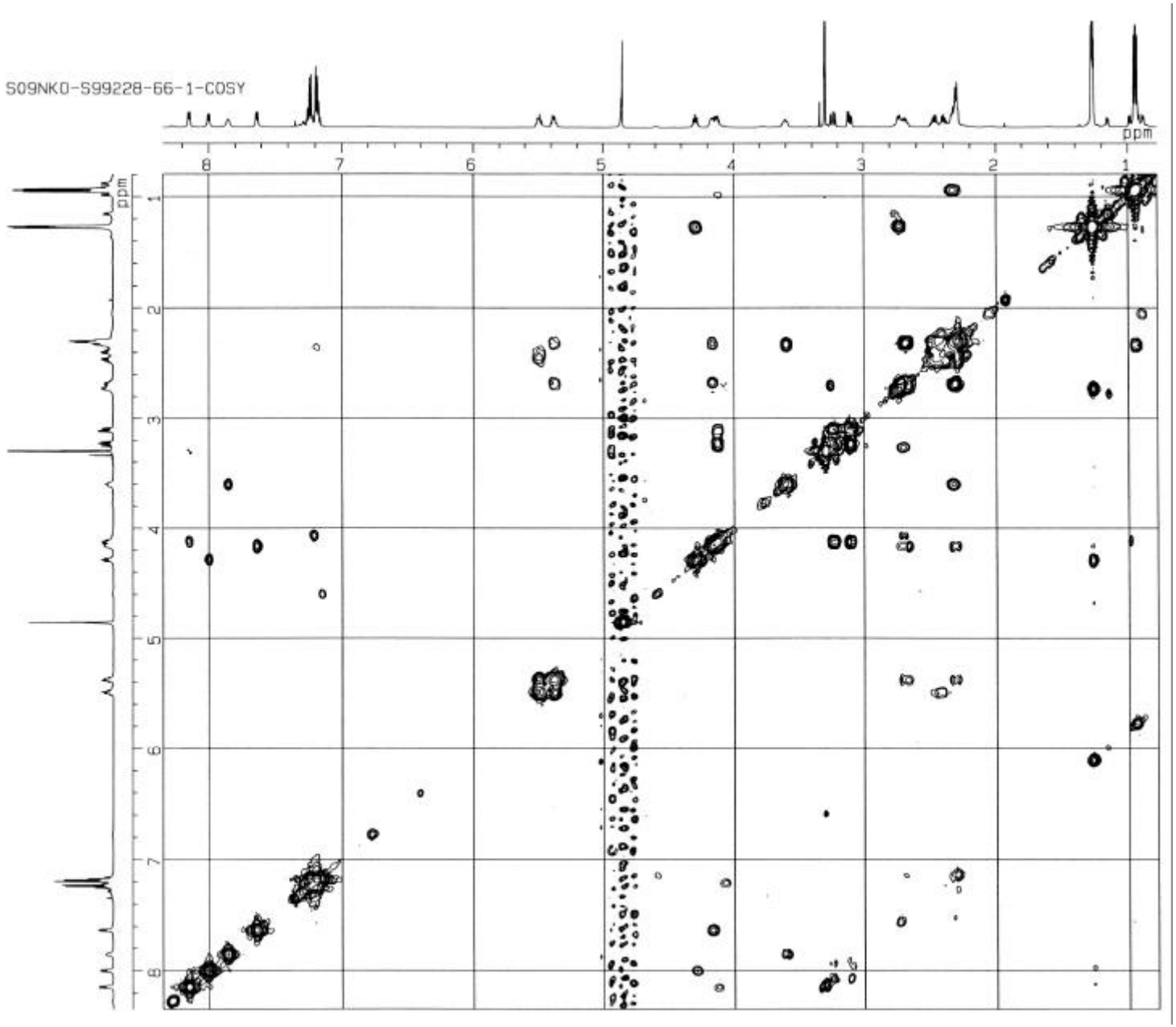

Fig. S4. COSY spectrum of azumamide A.

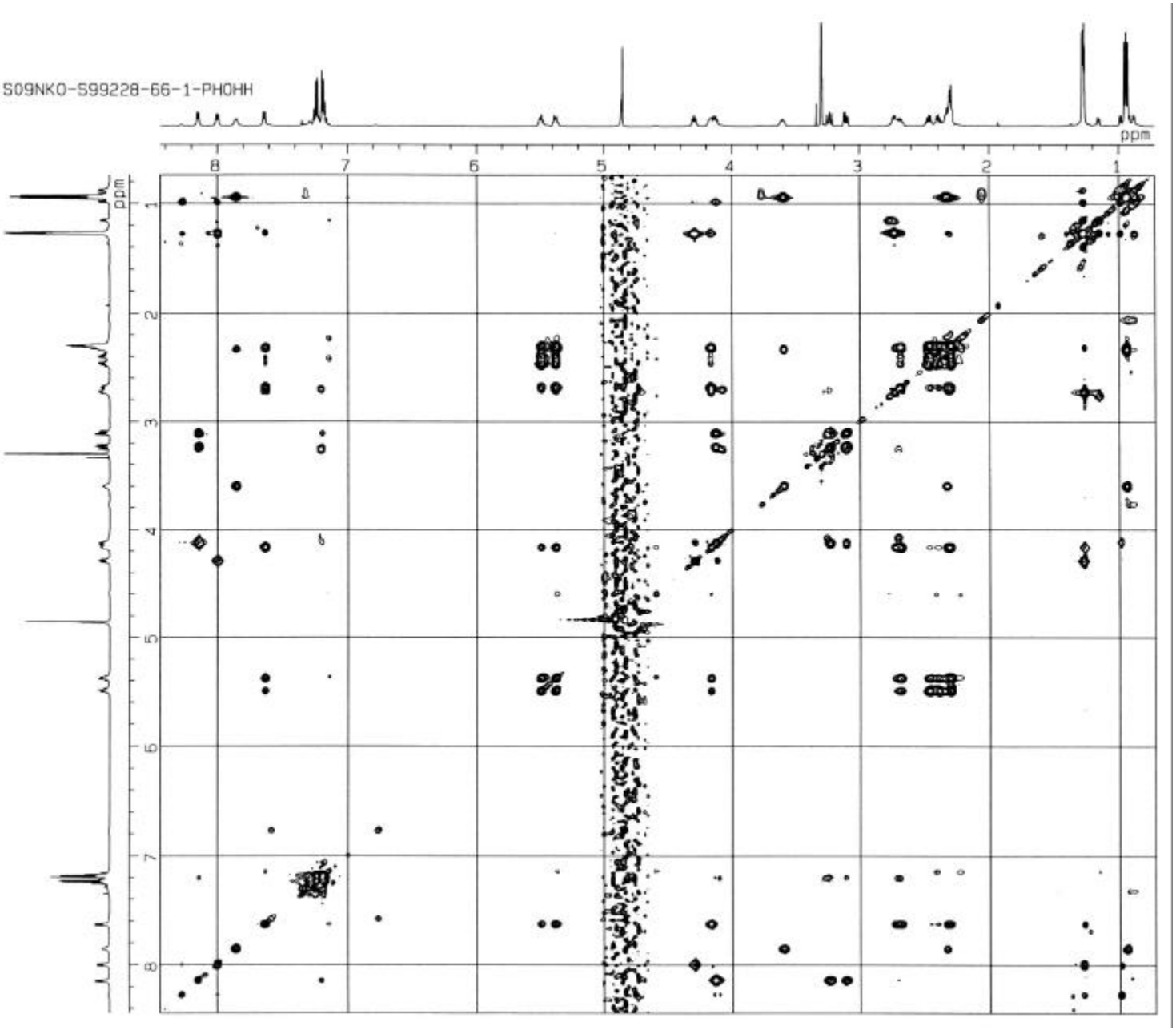

Fig. S5. HOHAHA spectrum of azumamide A.

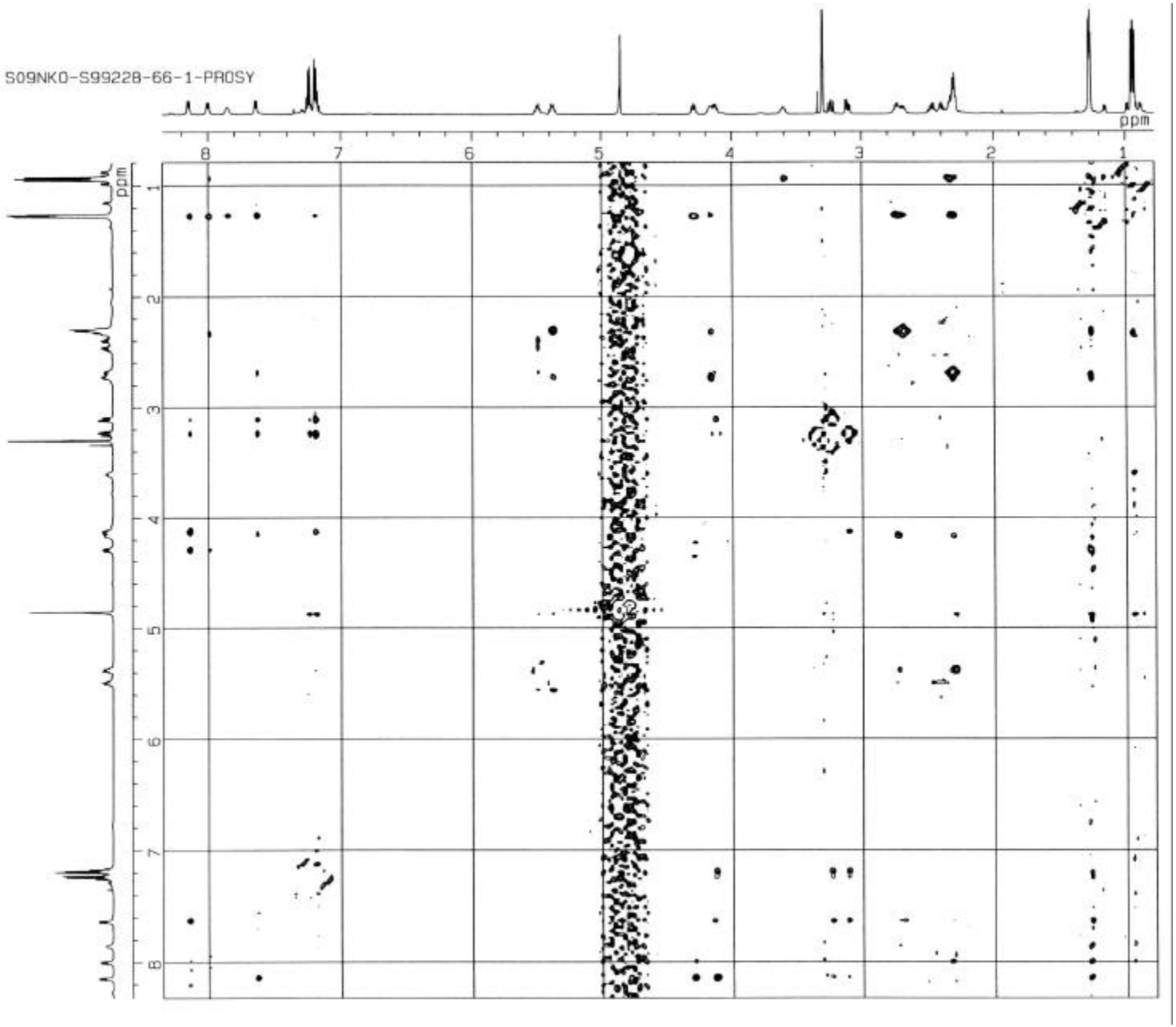

Fig. S6. ROESY spectrum of azumamide A.

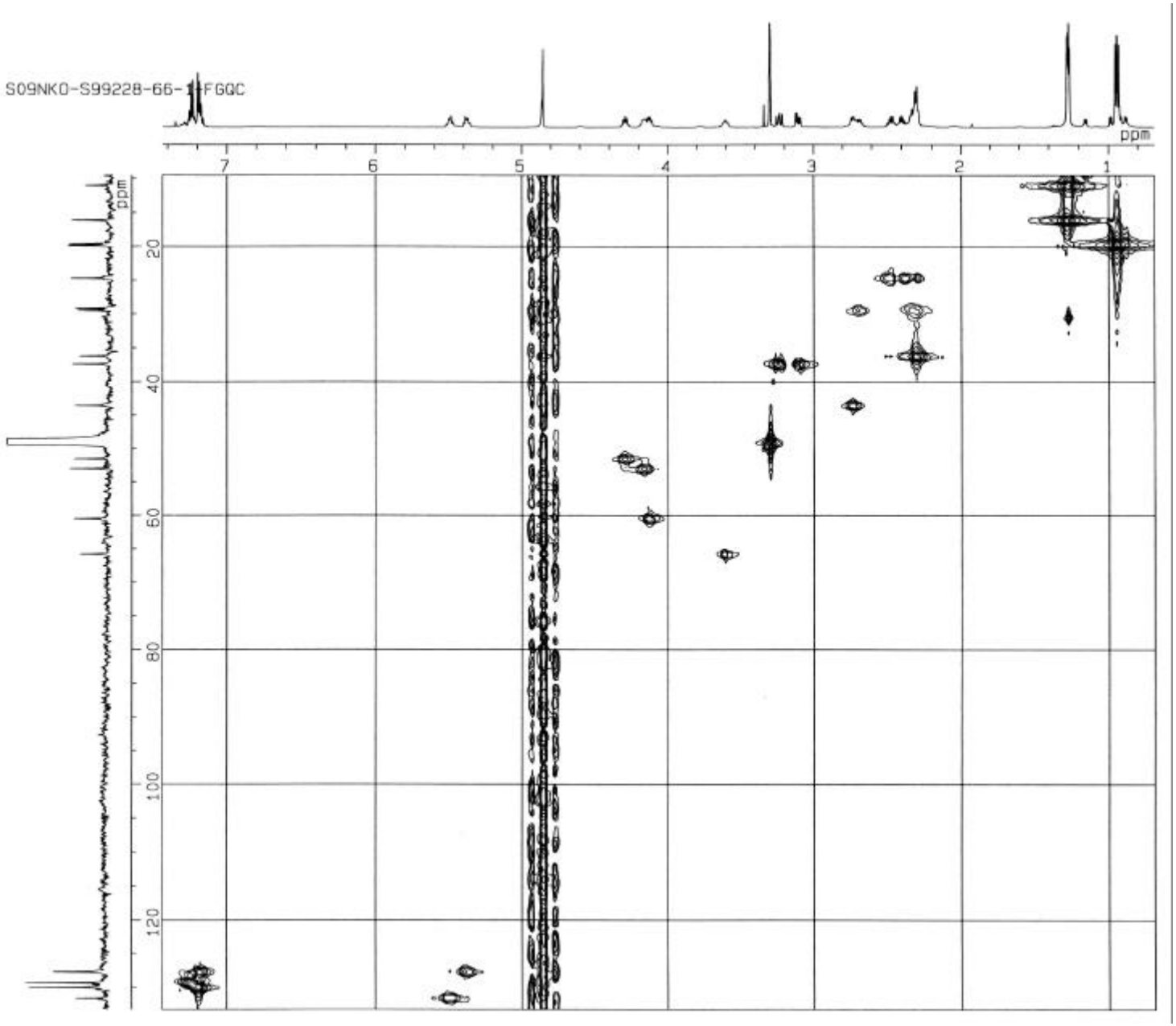

Fig. S7. HMQC spectrum of azumamide A.

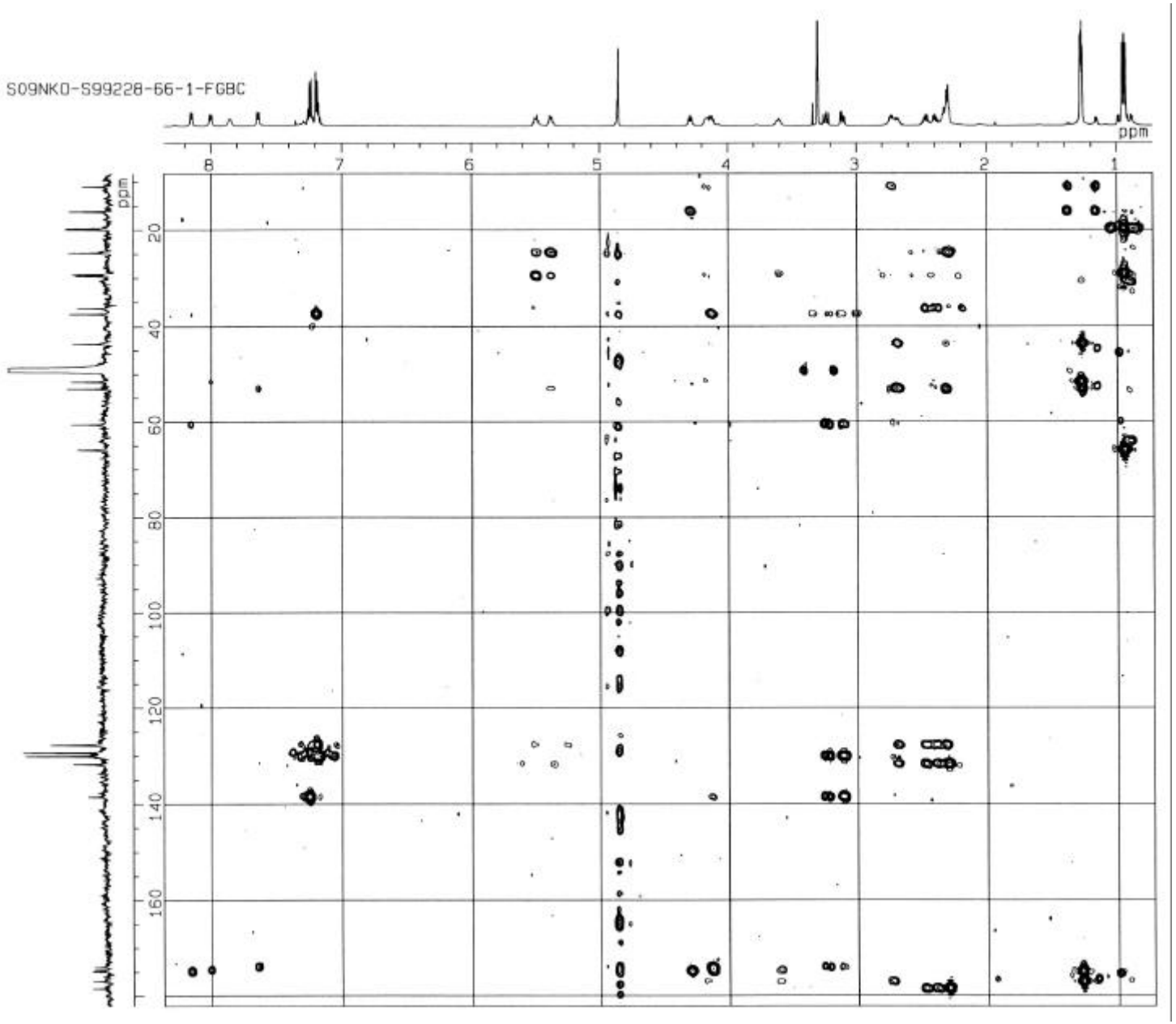

Fig. S8. HMBC spectrum of azumamide A.

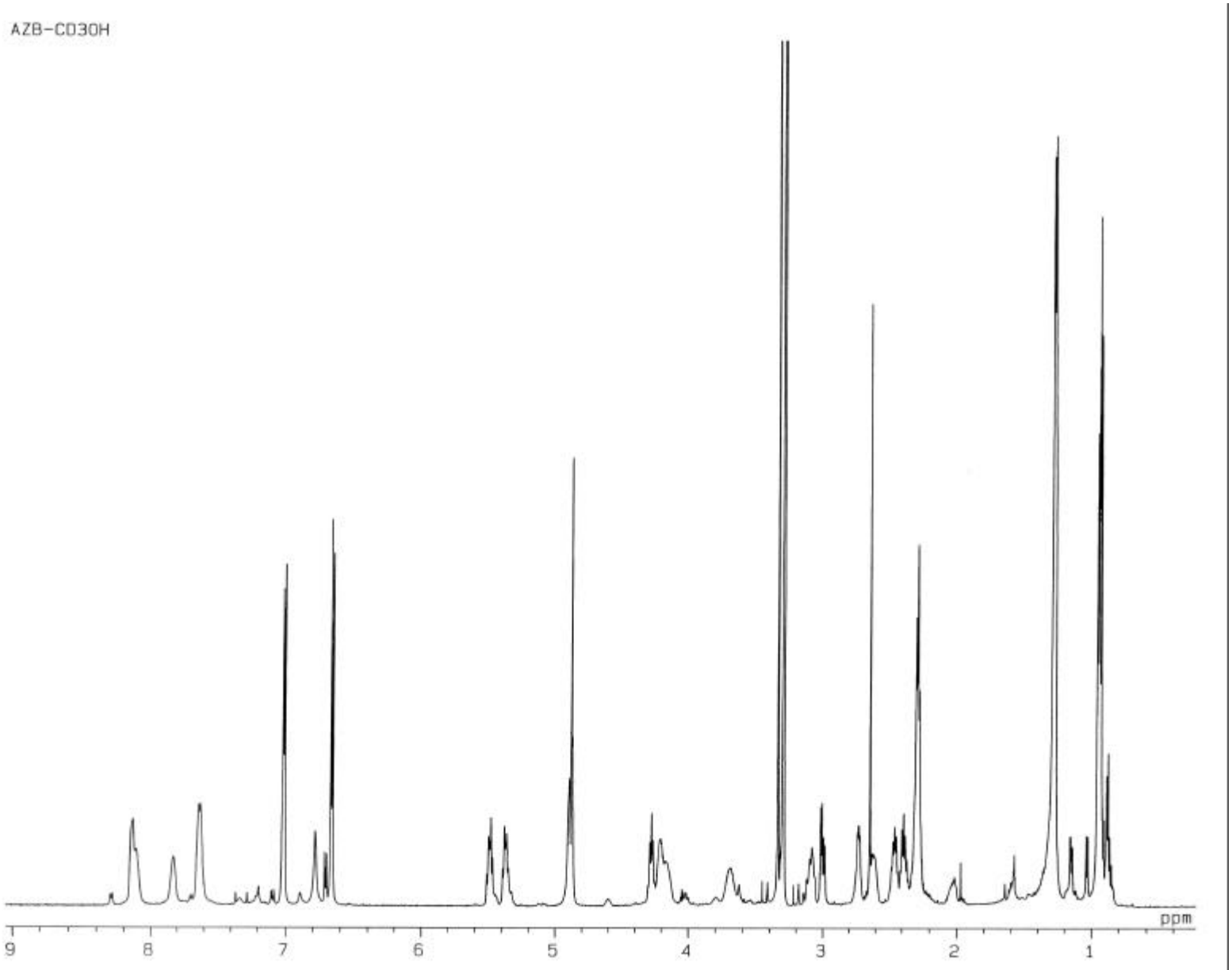

Fig. S9. ^{1}H NMR spectrum of azumamide B.

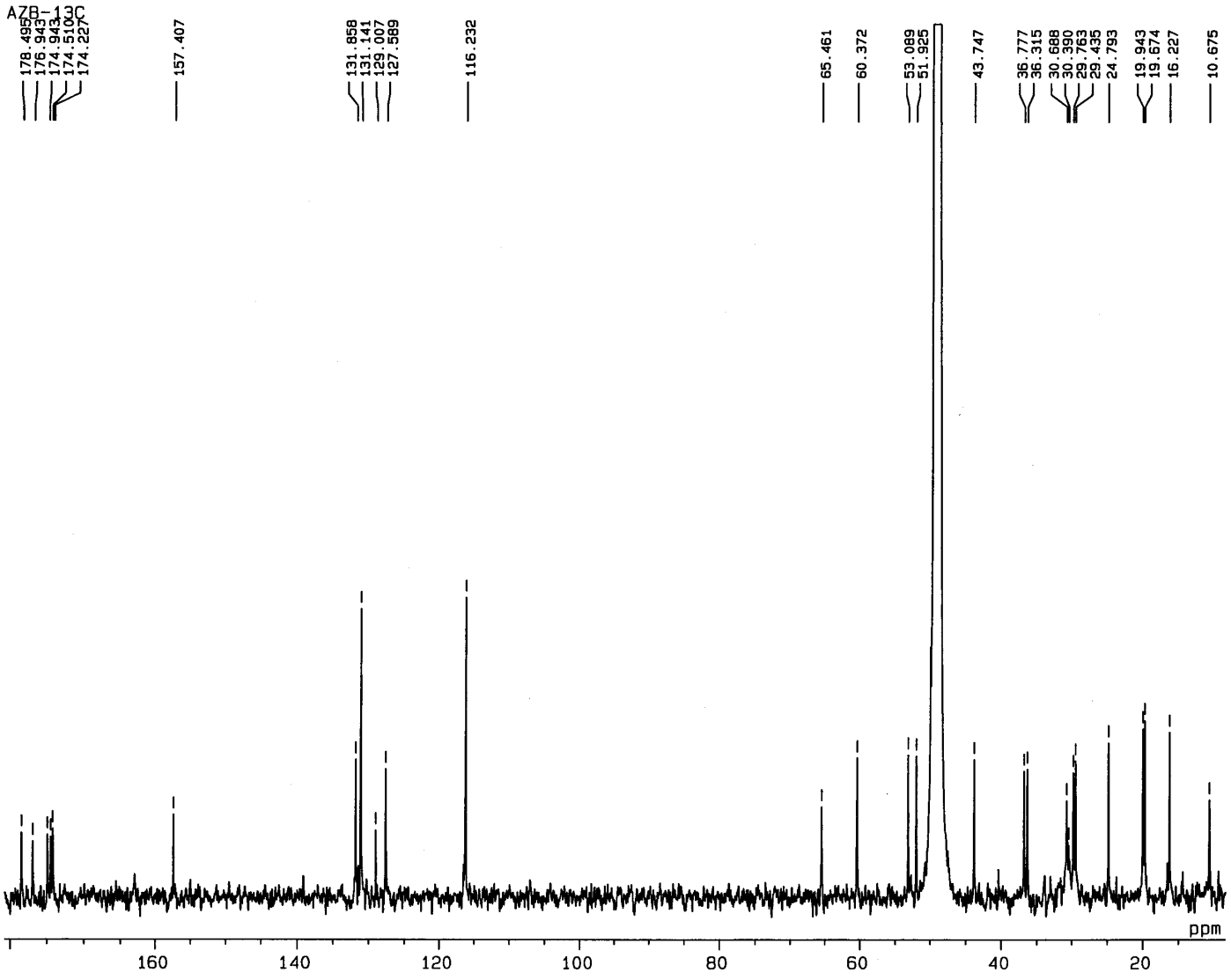

Fig. S10. ^{13}C NMR spectrum of azumamide B.

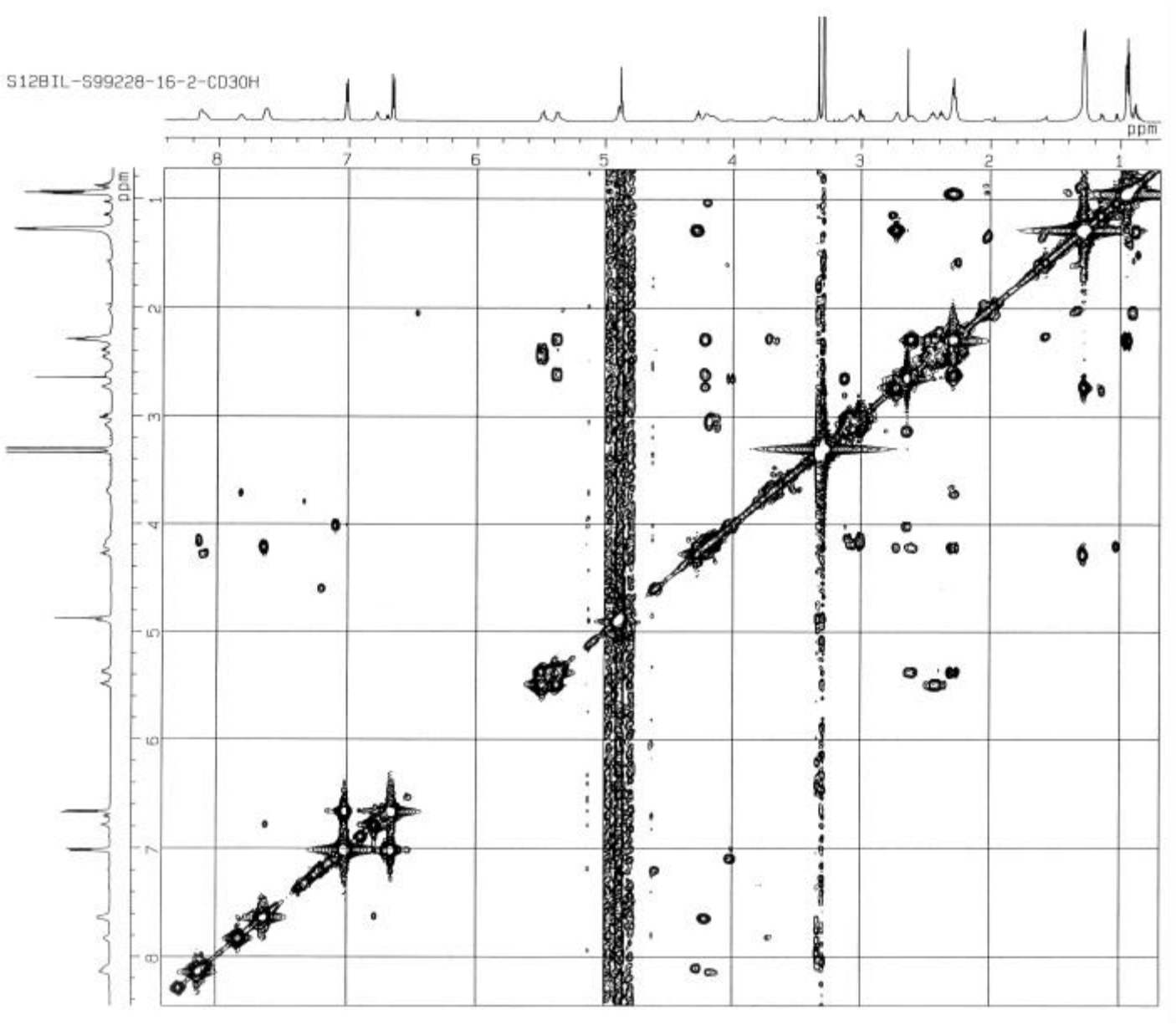

Fig. S11. COSY spectrum of azumamide B.

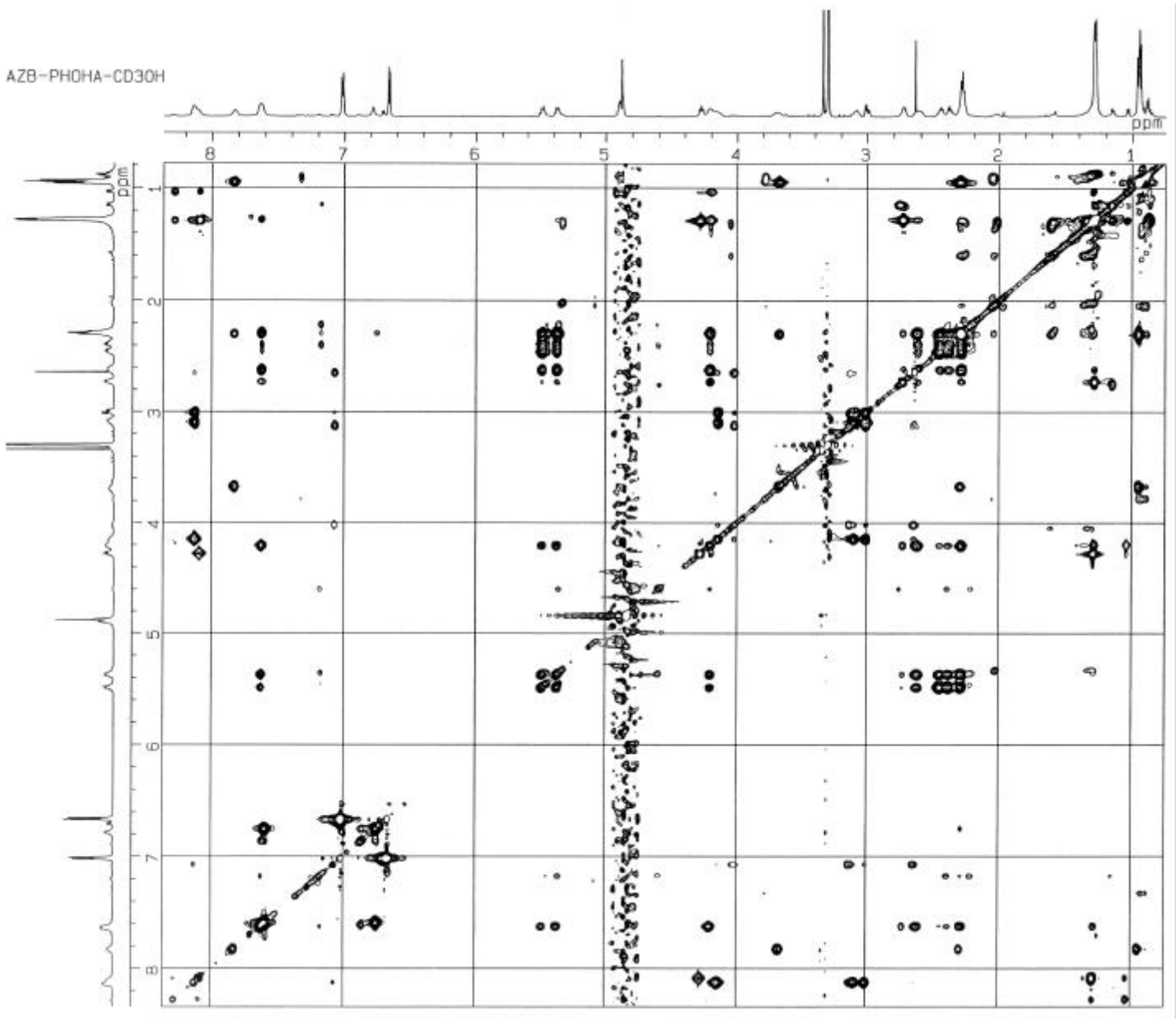

Fig. S12. HOHAHA spectrum of azumamide B.

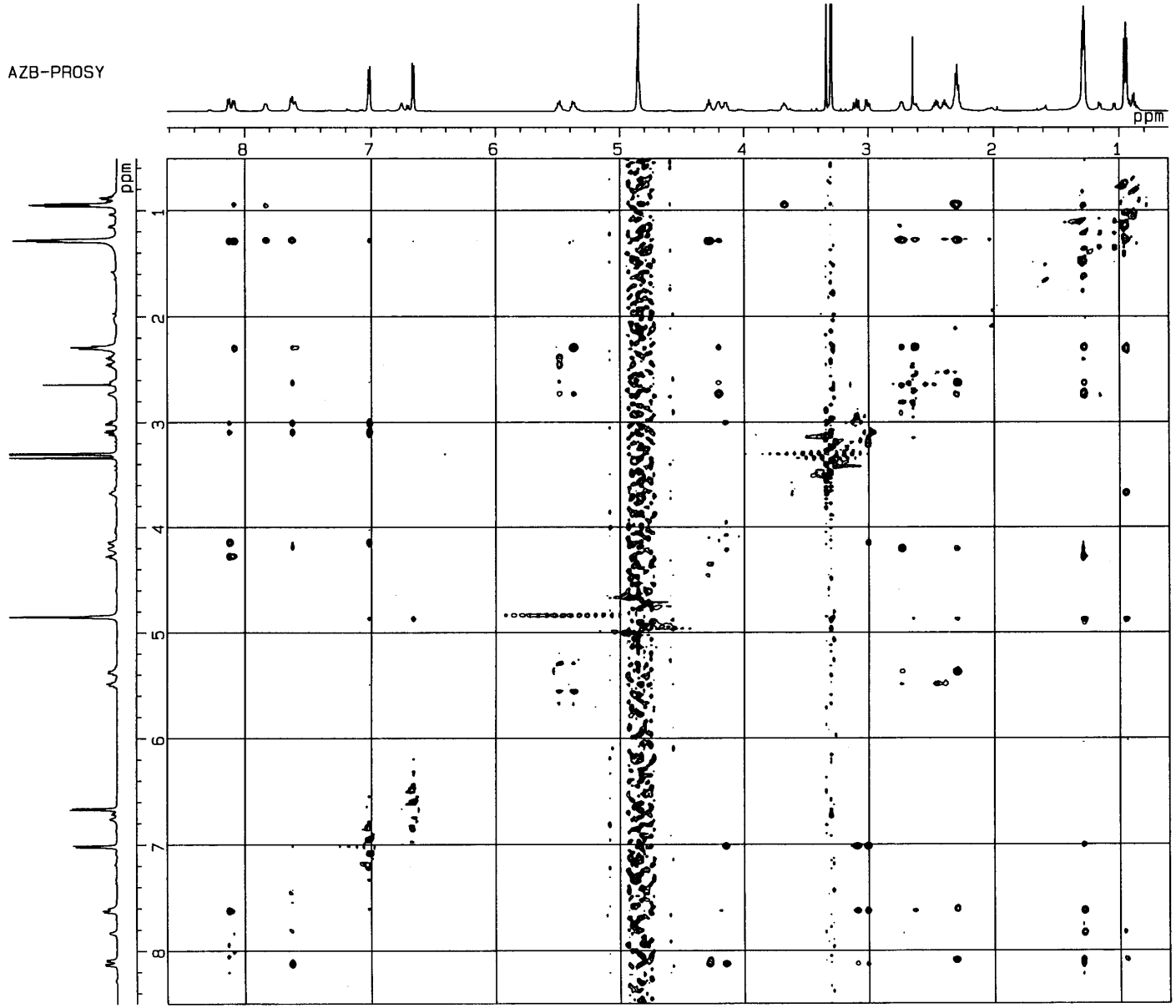

Fig. S13. ROESY spectrum of azumamide B.

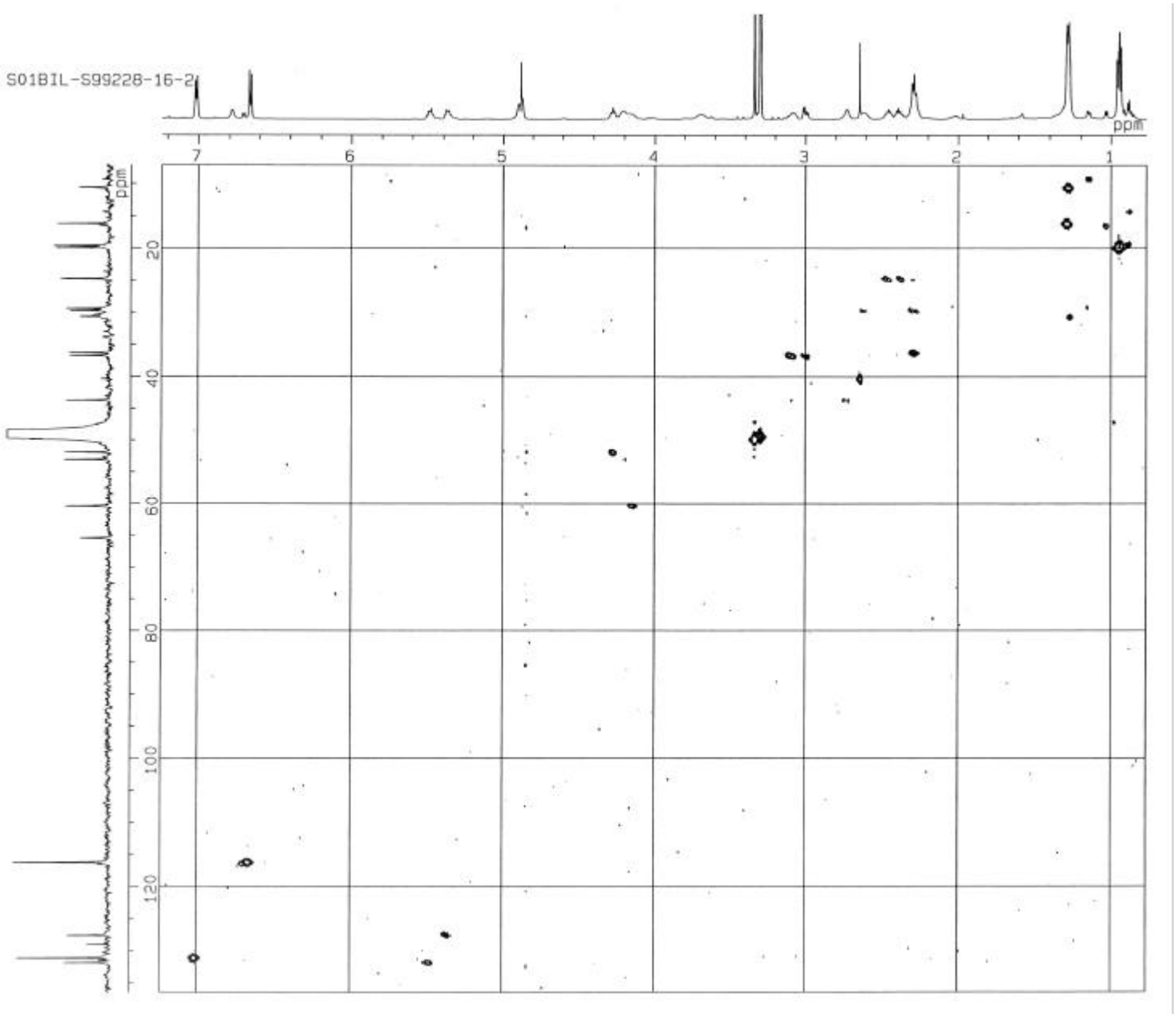

Fig. S14. HMQC spectrum of azumamide B.

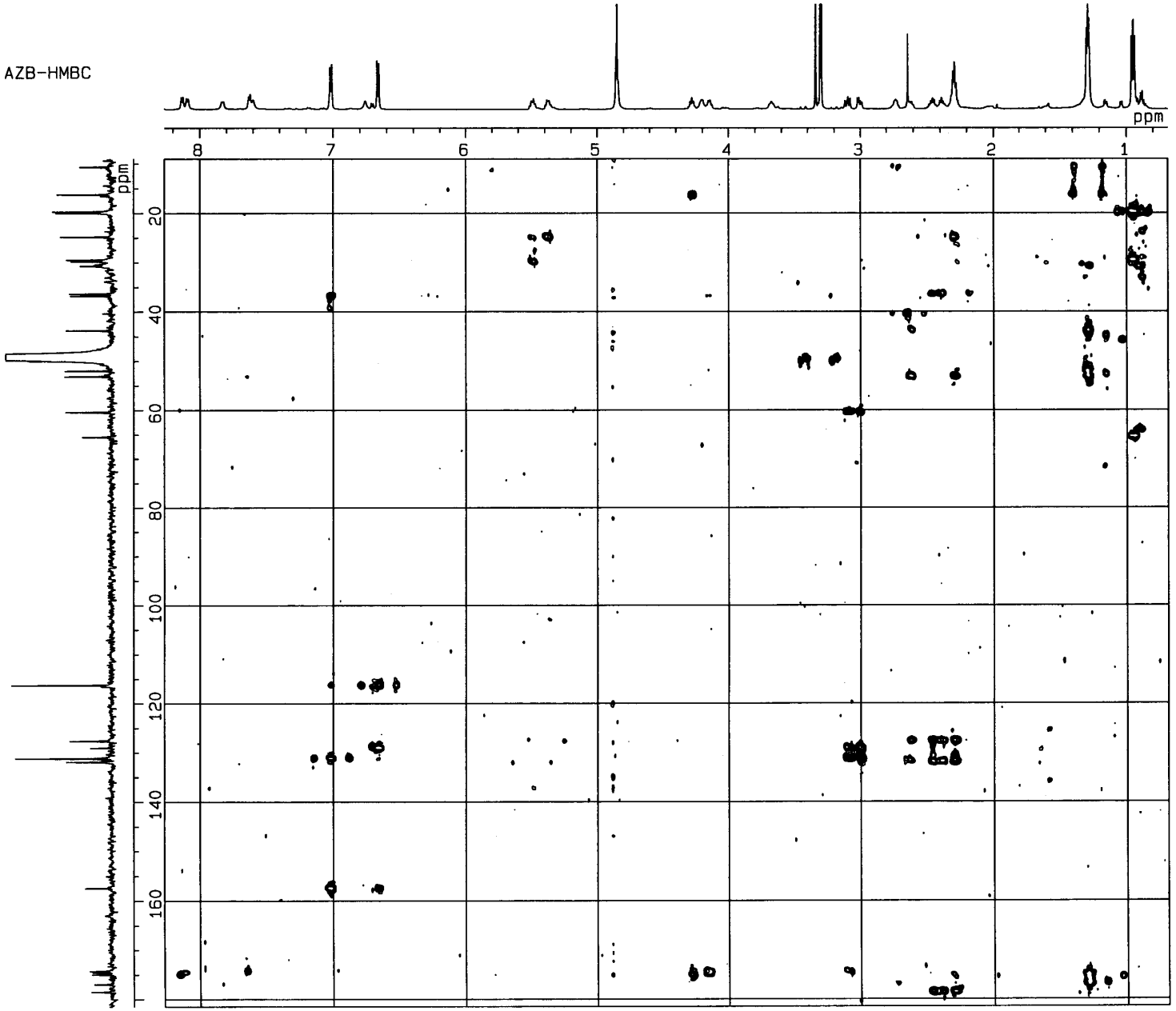

Fig. 15. HMBC spectrum of azumamide B.

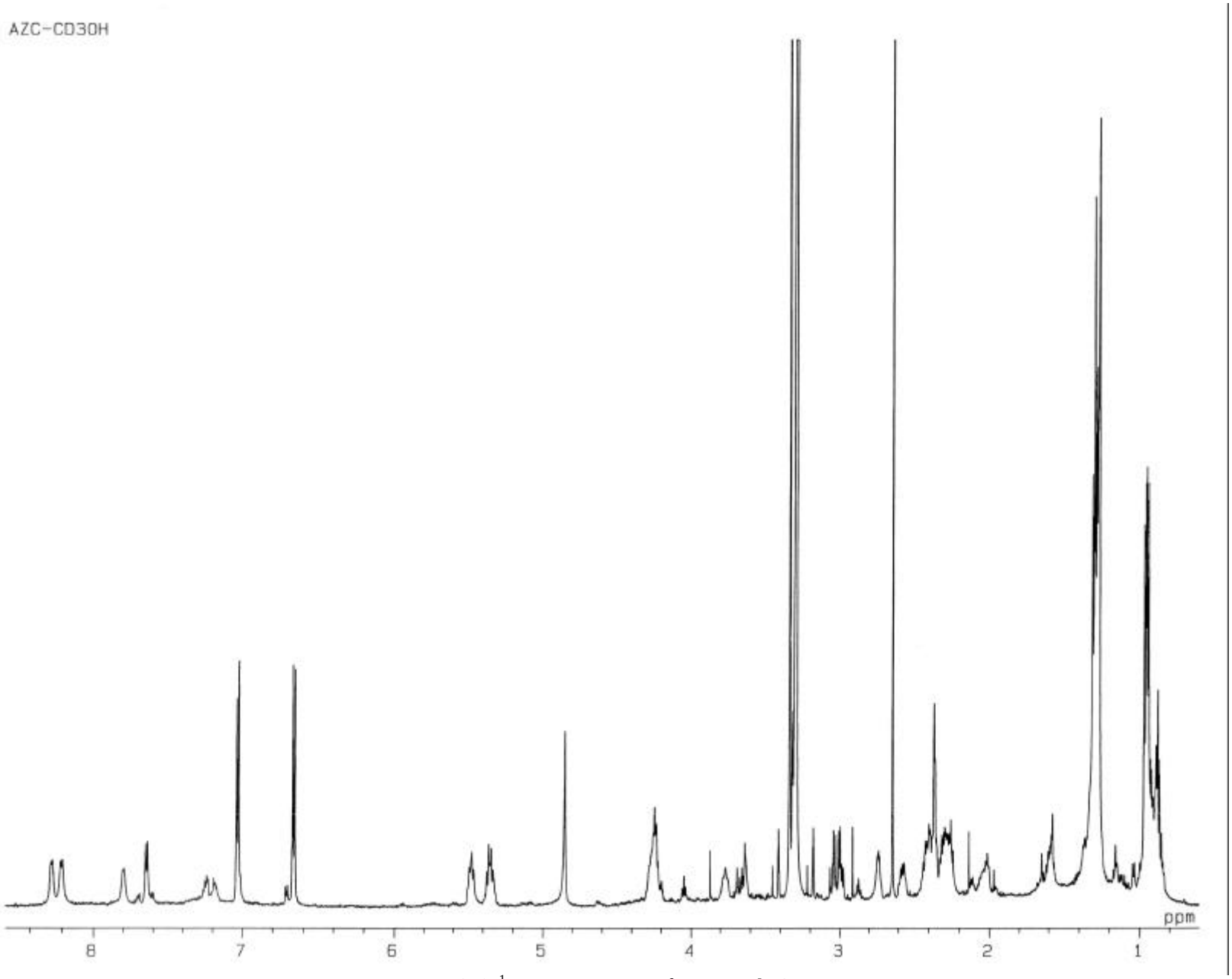

Fig. S16. ^{1}H NMR spectrum of azumamide C.

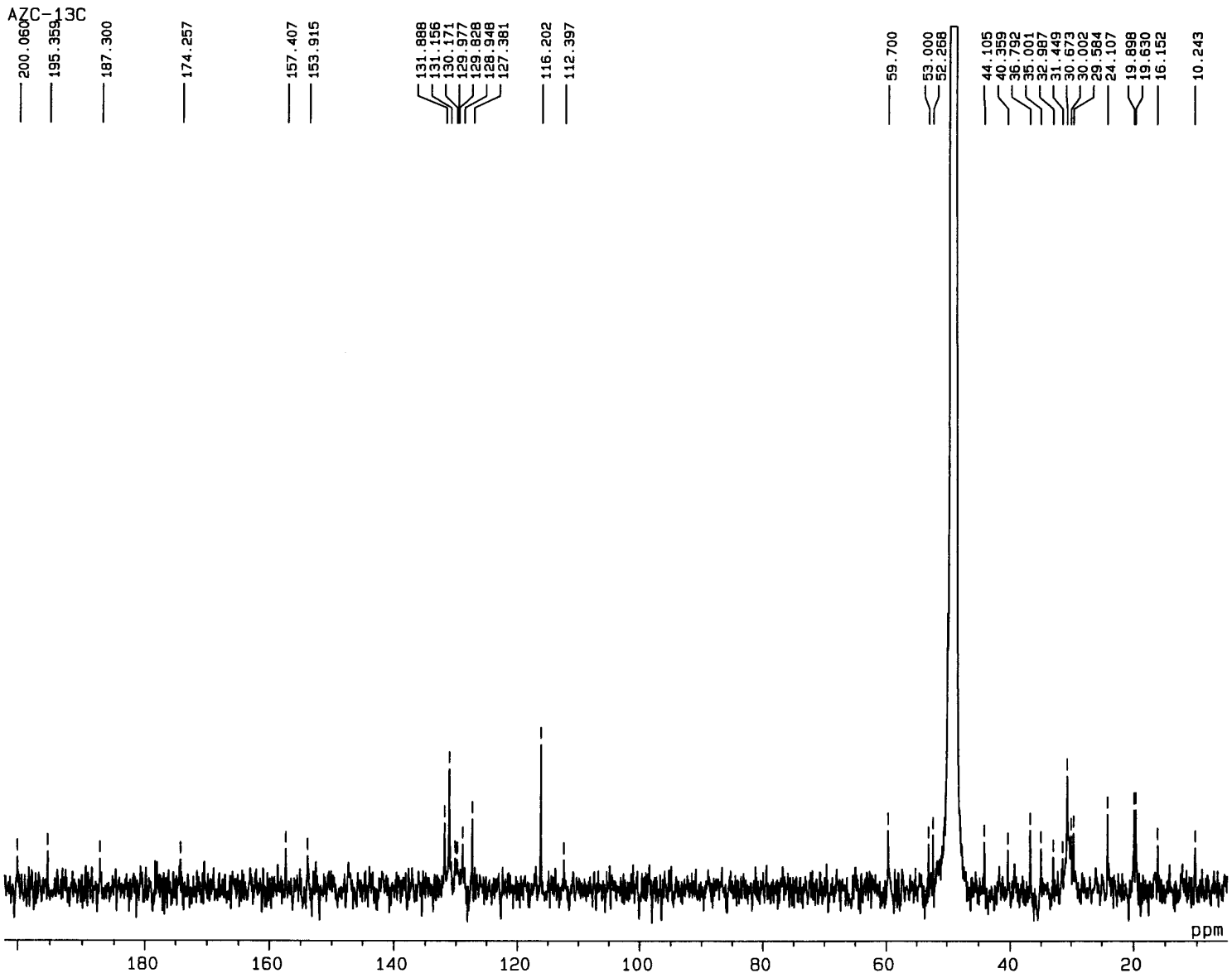

Fig. S17. ^{13}CNMR spectrum of azumamide C.

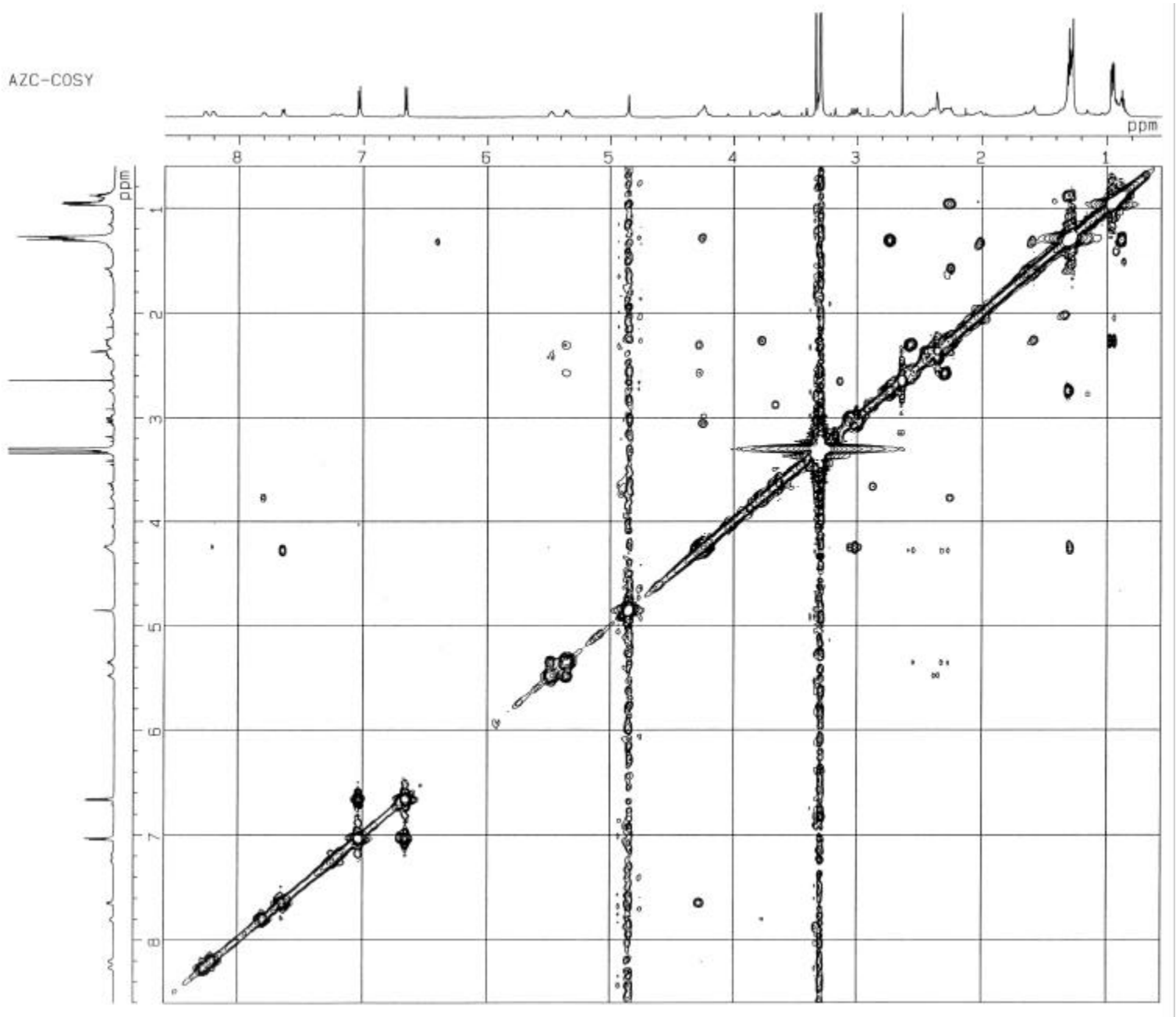

Fig. S18. COSY spectrum of azumamide C.

Fig. S19. ROESY spectrum of azumamide C.

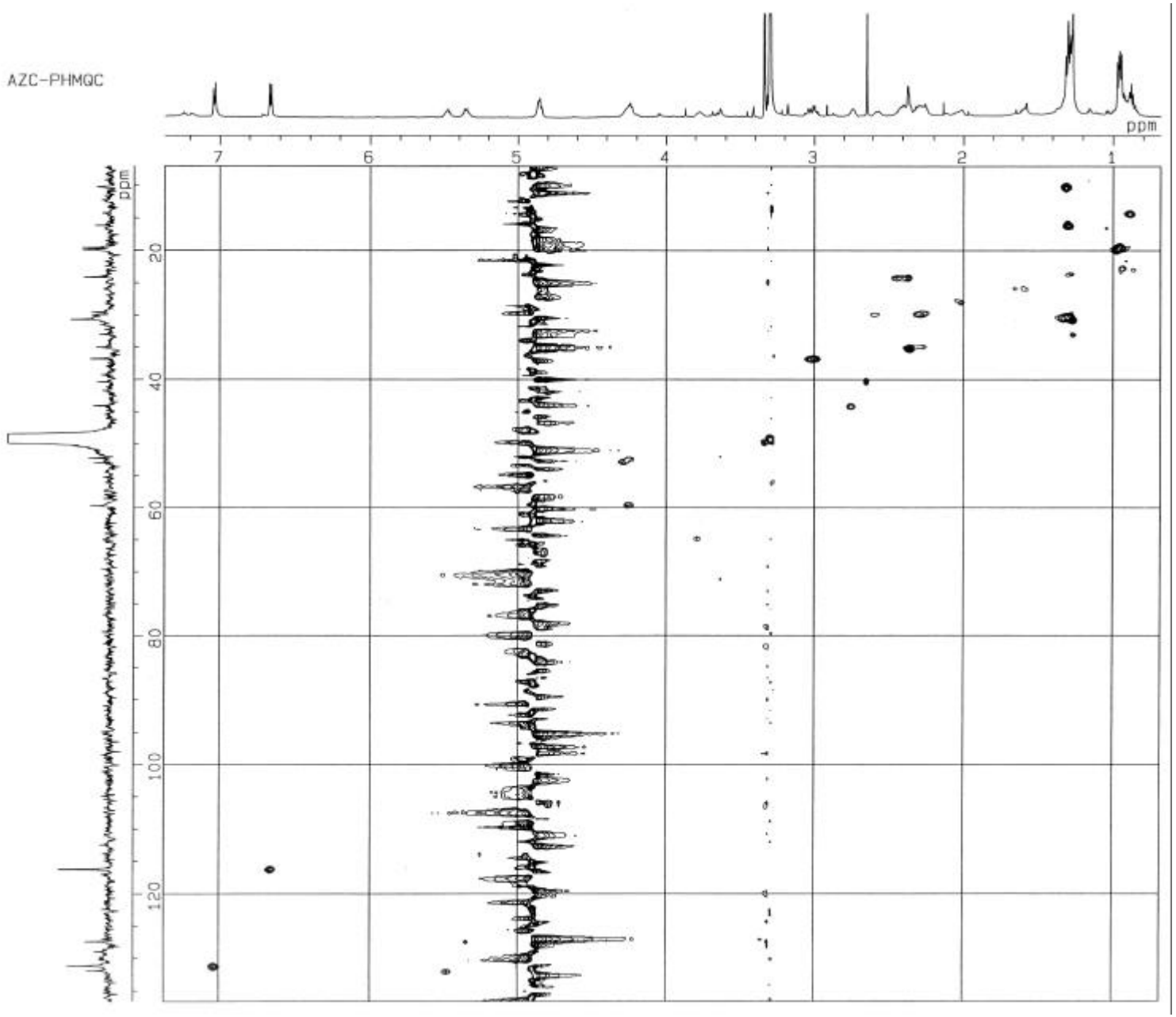

Fig. S20. HMQC spectrum of azumamide C.

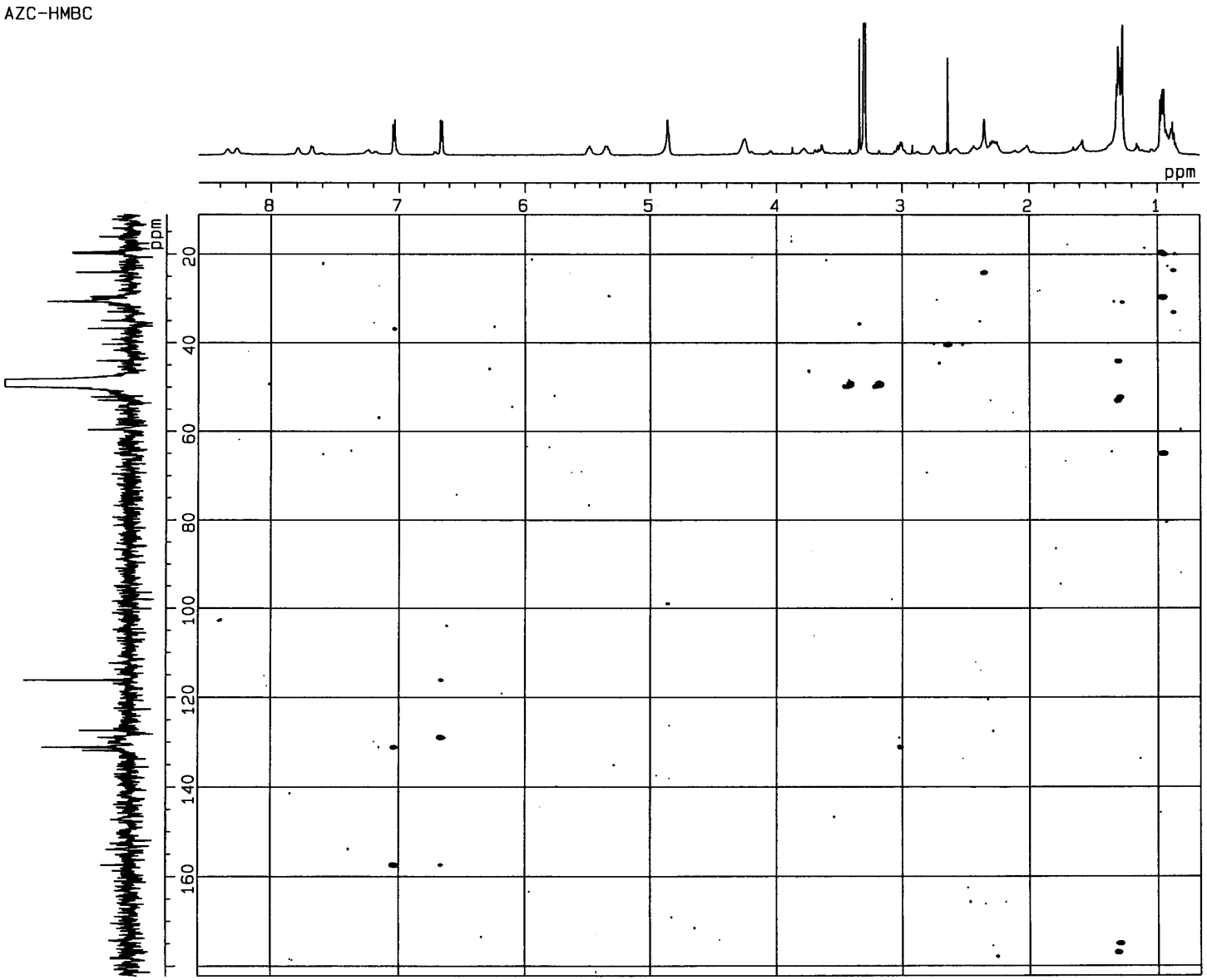

Fig. S21. HMBC spectrum of azumamide C.

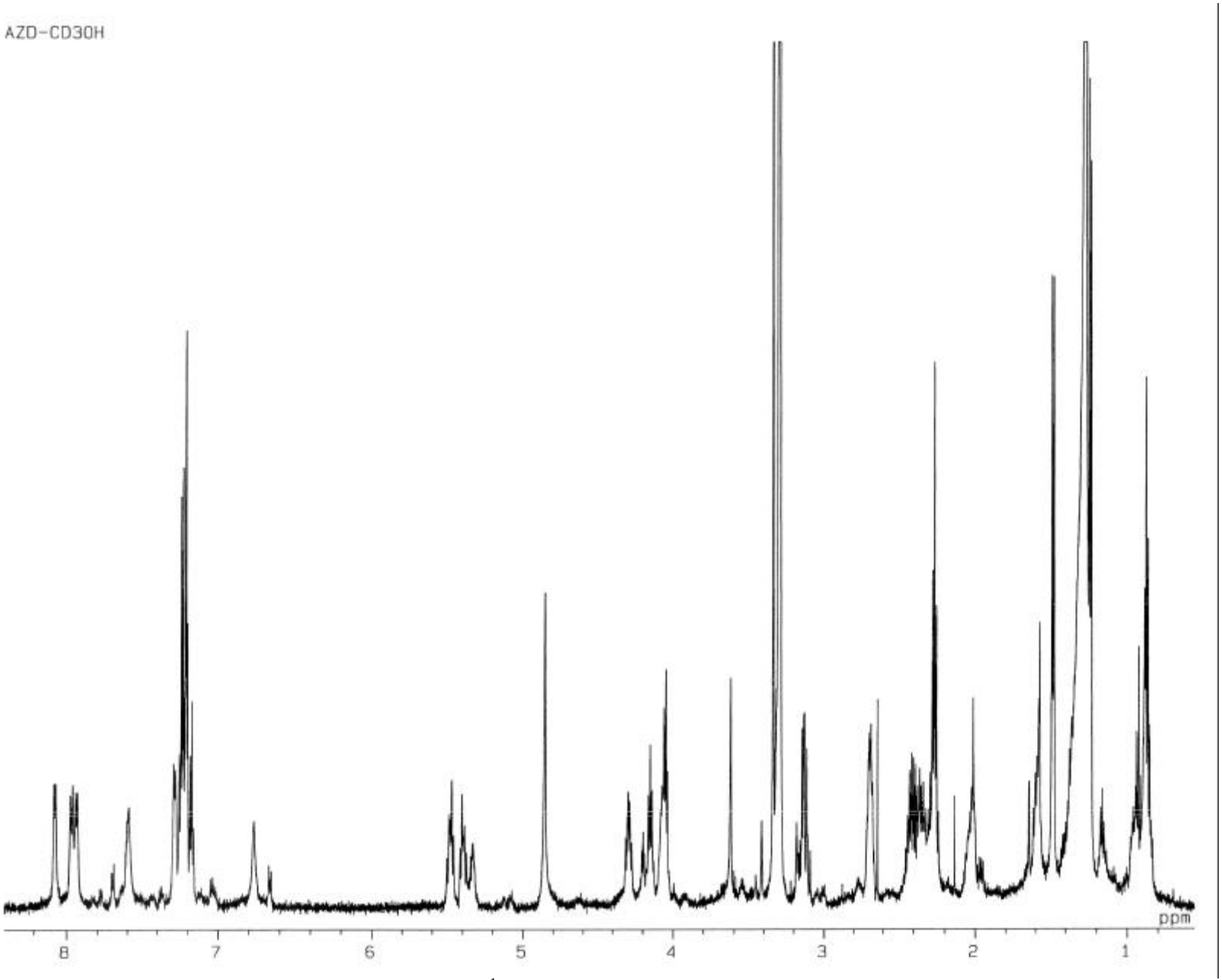

Fig. S22. ^{1}H NMR spectrum of azumamide D.

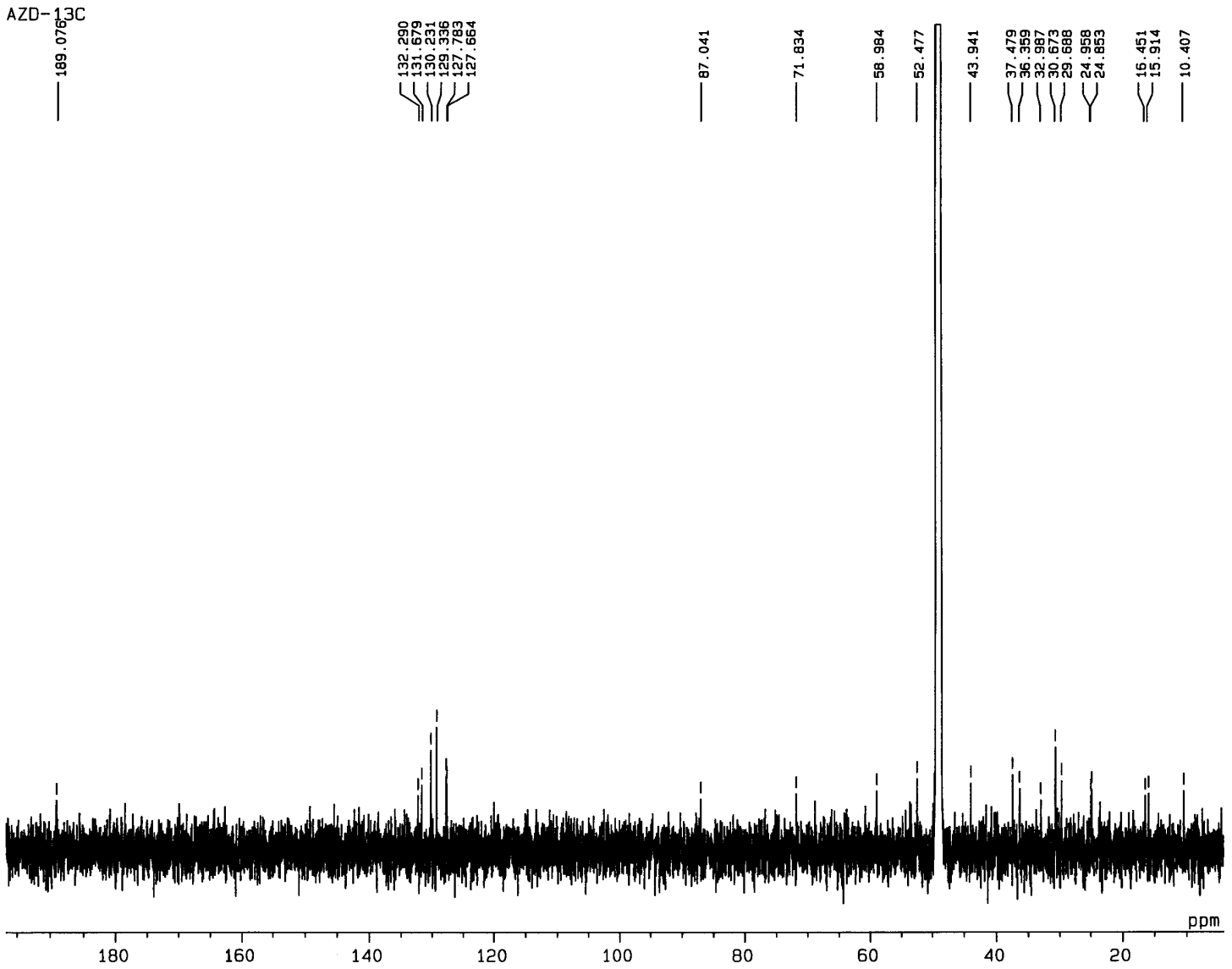

Fig. S23. ^{13}CNMR spectrum of azumamide D.

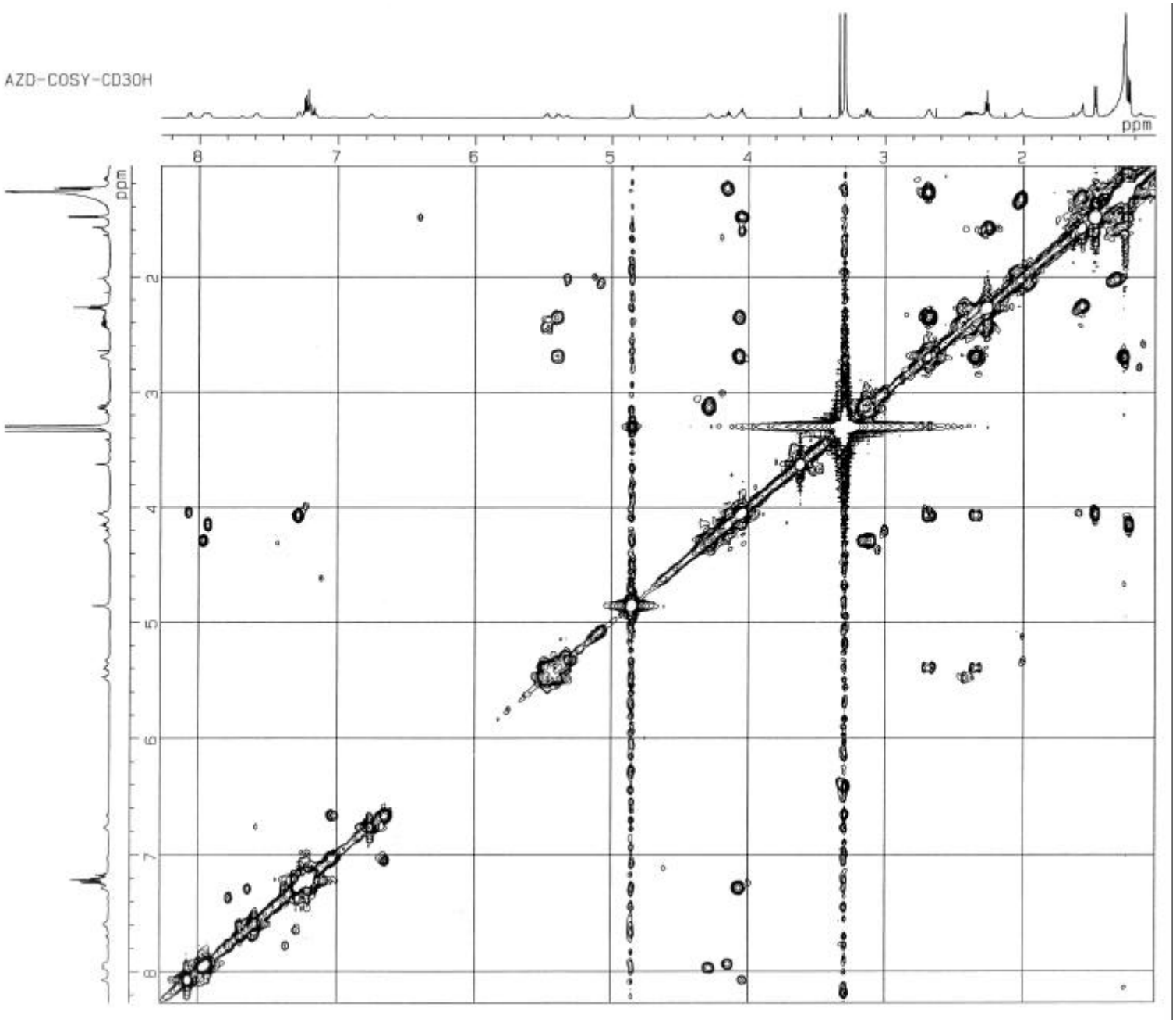

Fig. S24. COSY spectrum of azumamide D.

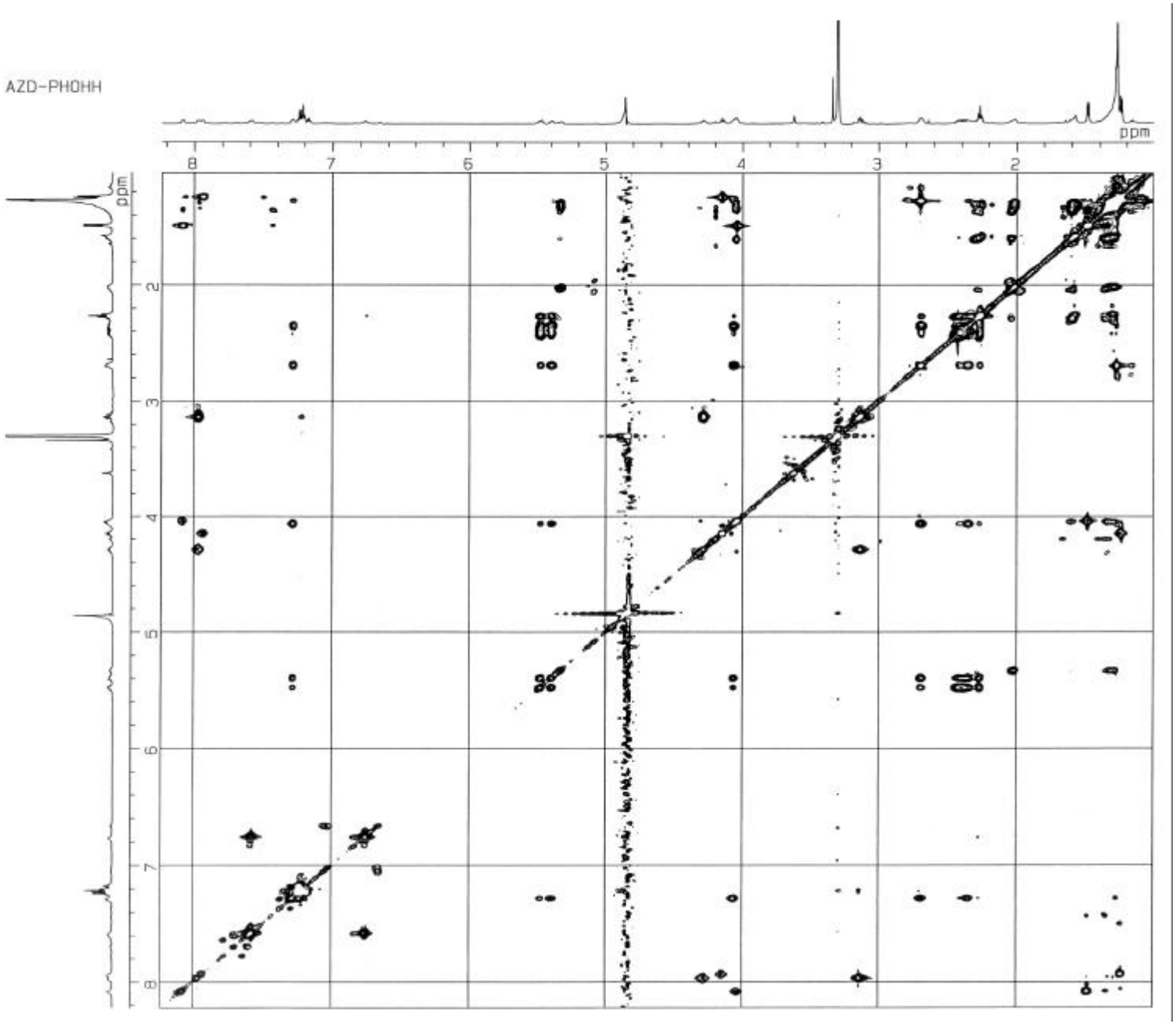

Fig. S25. HOHAHA spectrum of azumamide D.

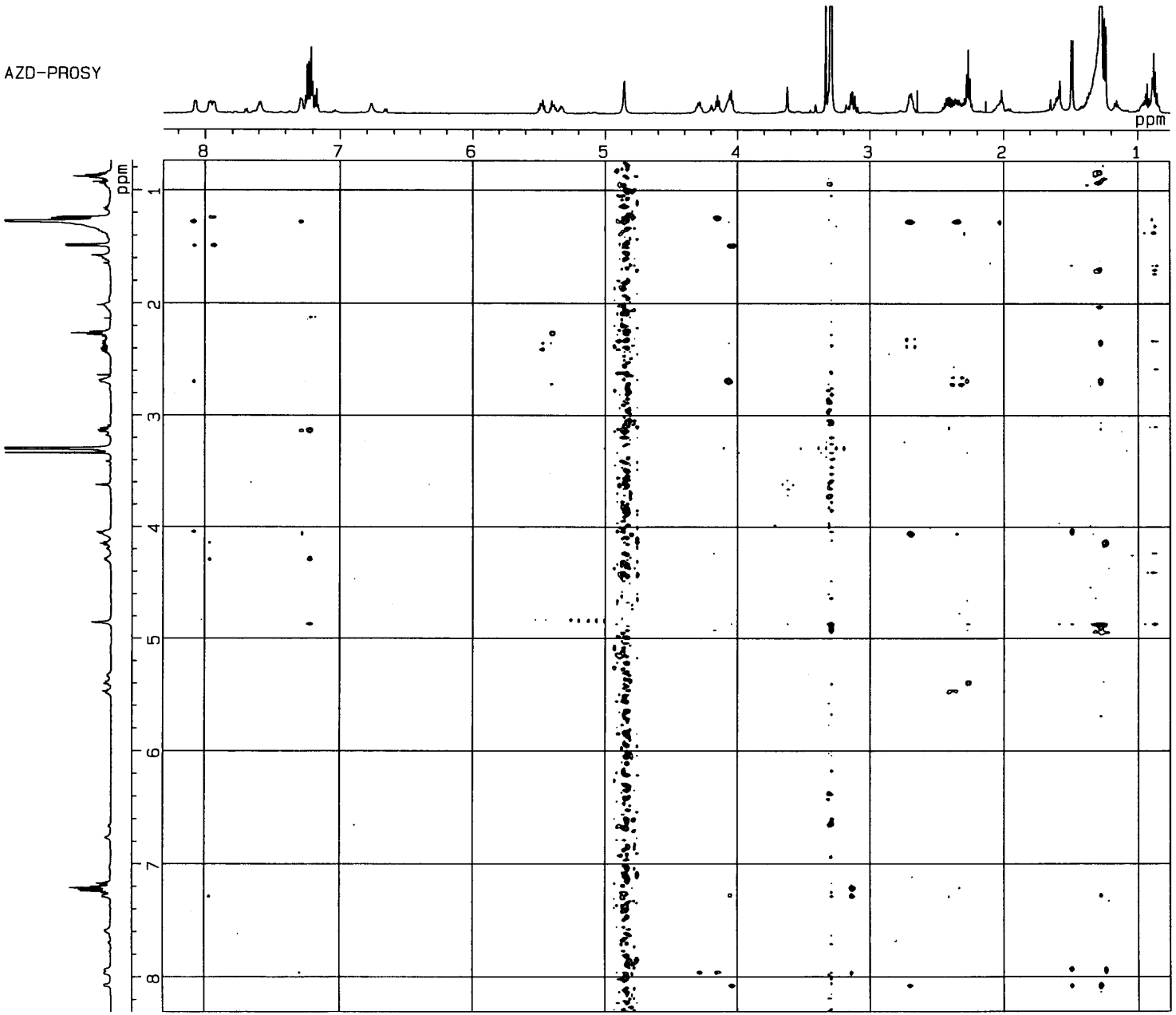

Fig. S26. ROESY spectrum of azumamide D.

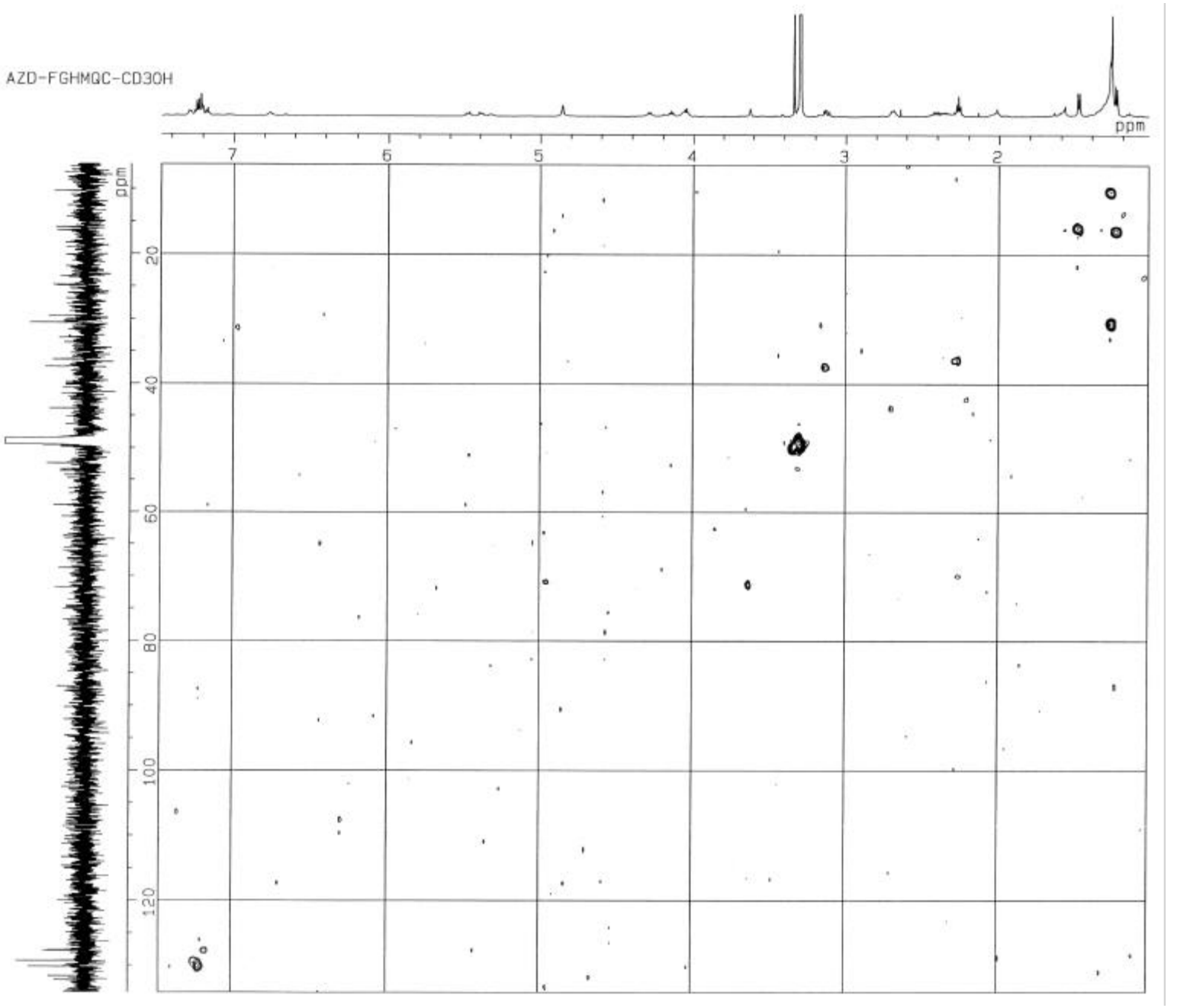

Fig. S27. HMQC spectrum of azumamide D.

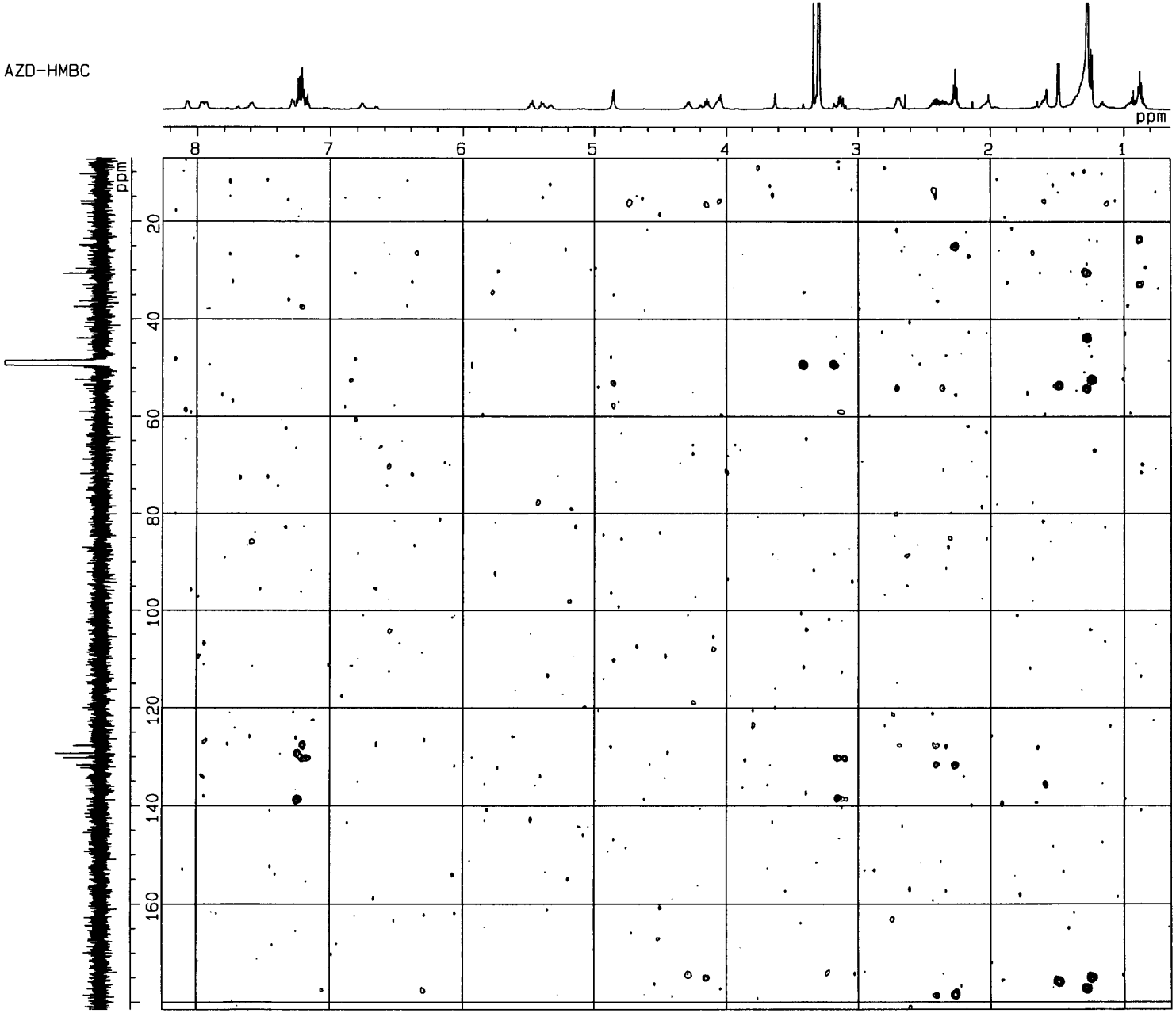

Fig. S28. HMBC spectrum of azumamide D.

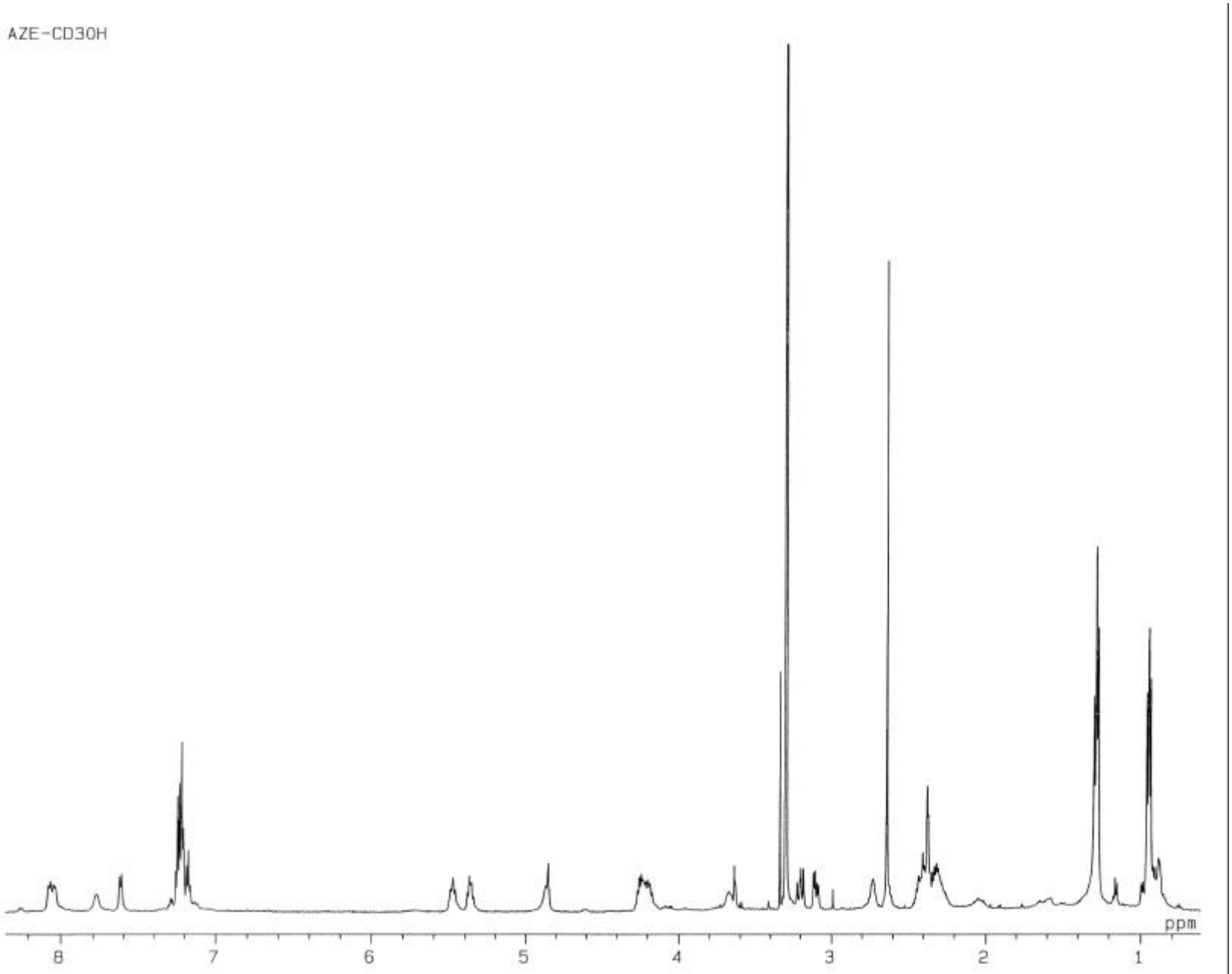

Fig. S29. ^{1}H NMR spectrum of azumamide E.

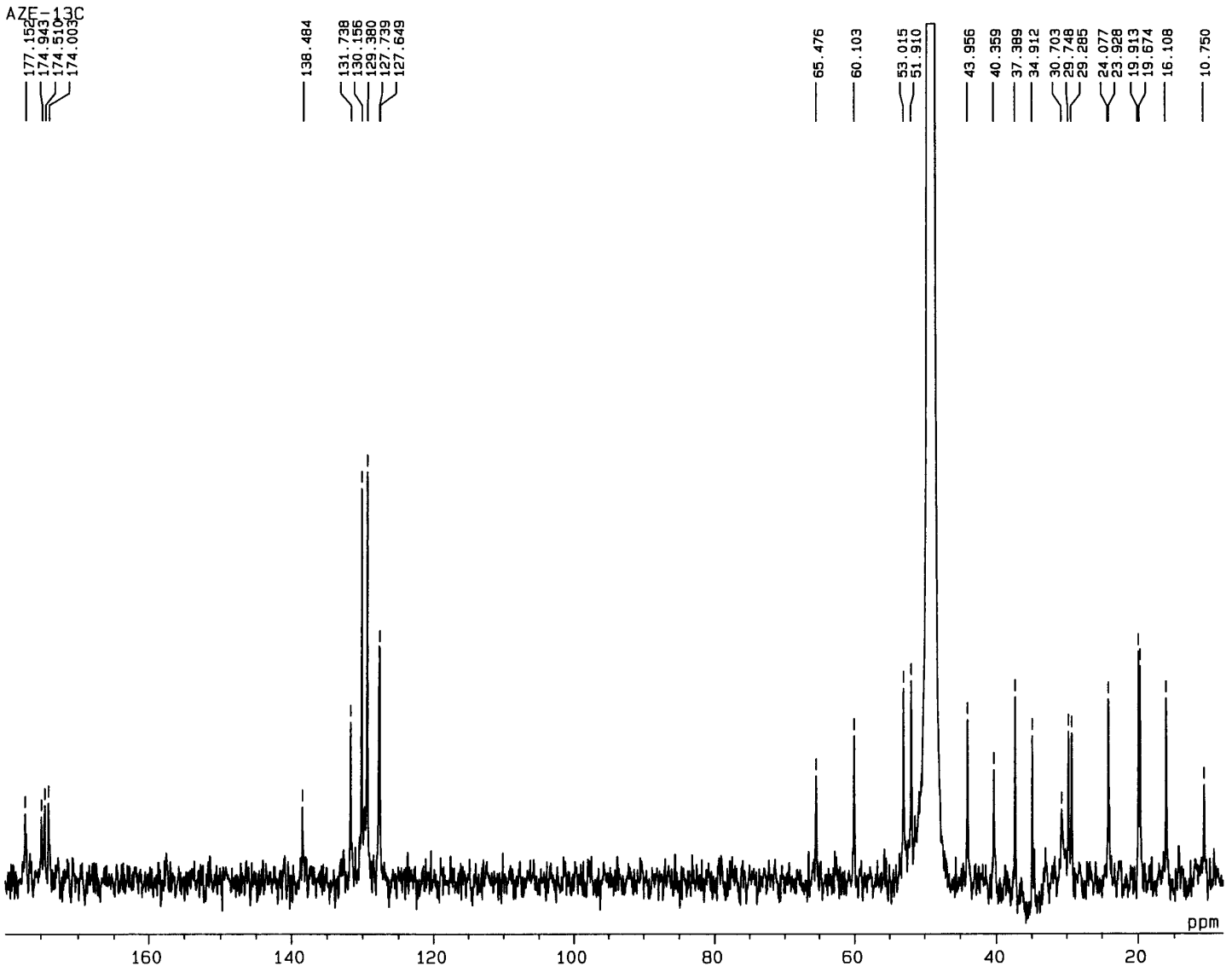

Fig. S30. ^{13}CNMR spectrum of azumamide E.

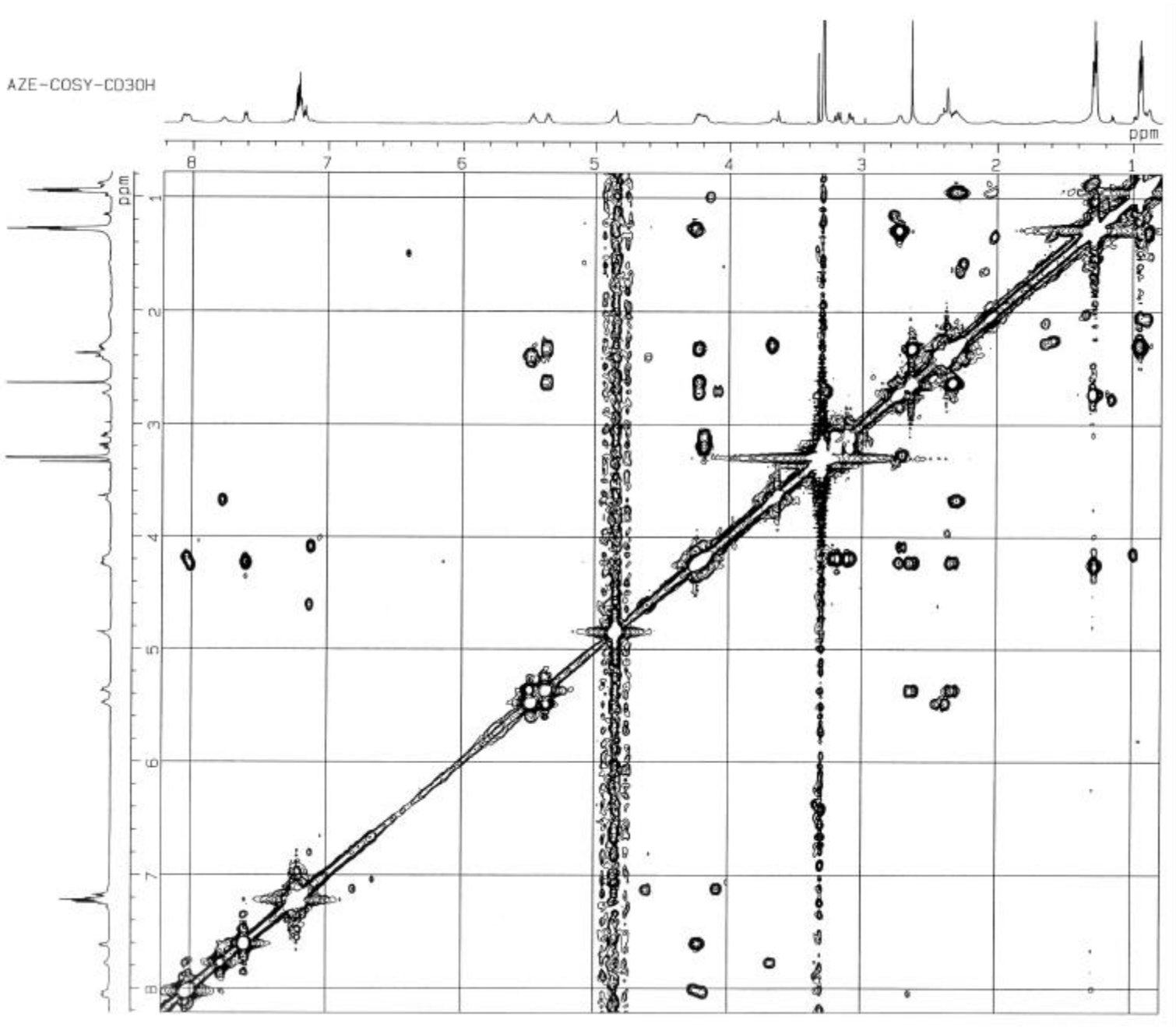

Fig. S31. COSY spectrum of azumamide E.

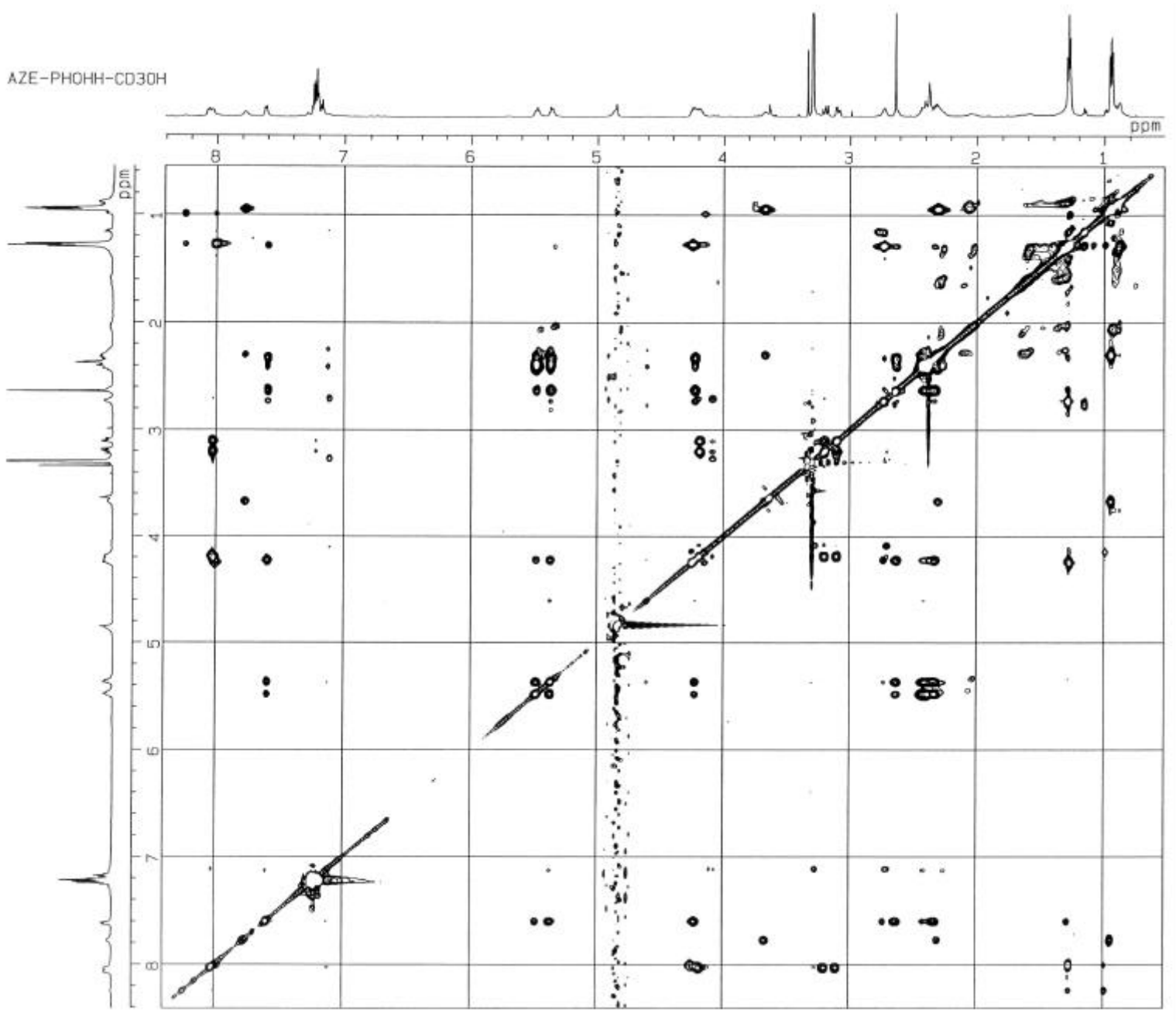

Fig. S32. HOHAHA spectrum of azumamide E.

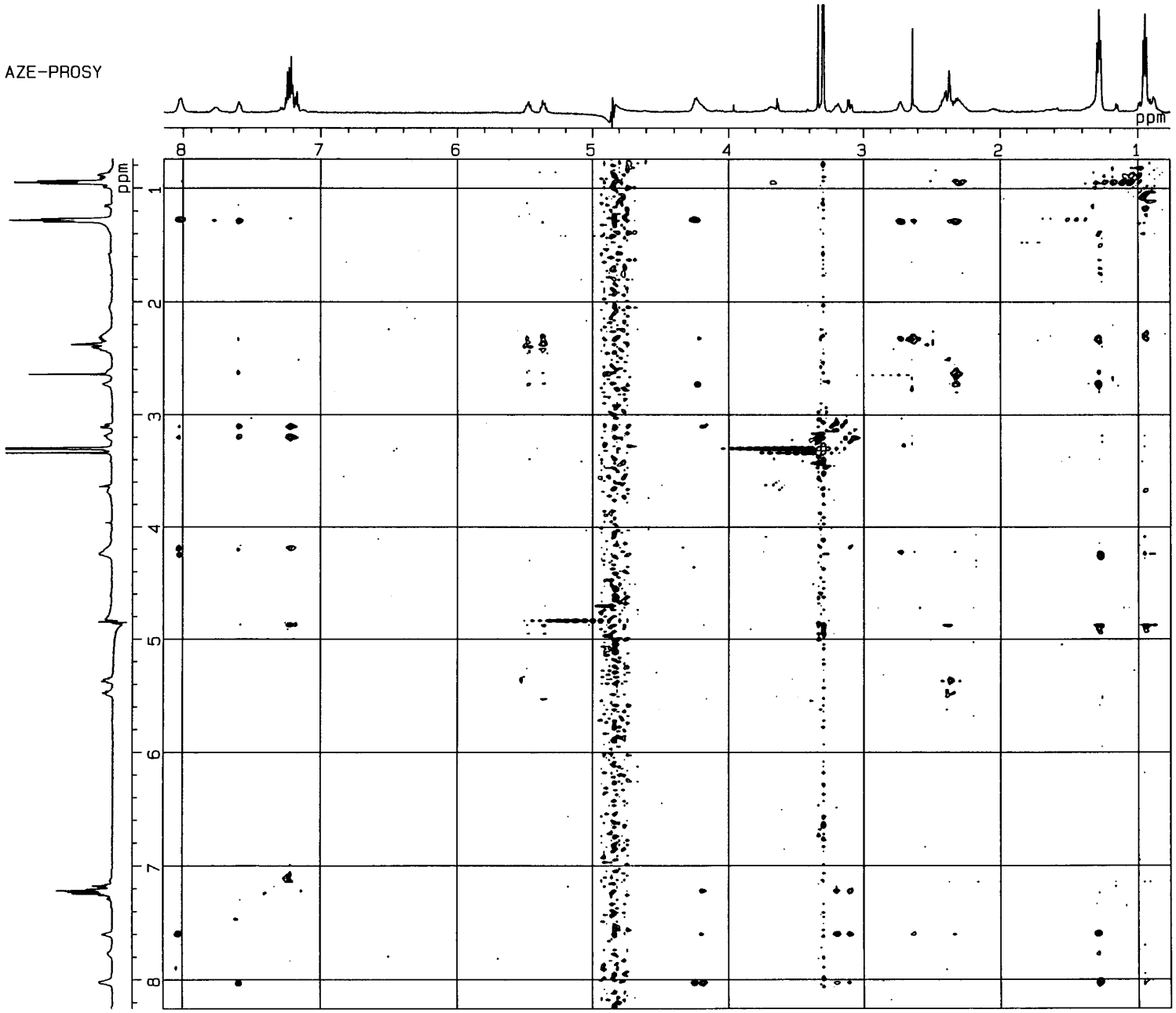

Fig. S33 ROESY spectrum of azumamide E.

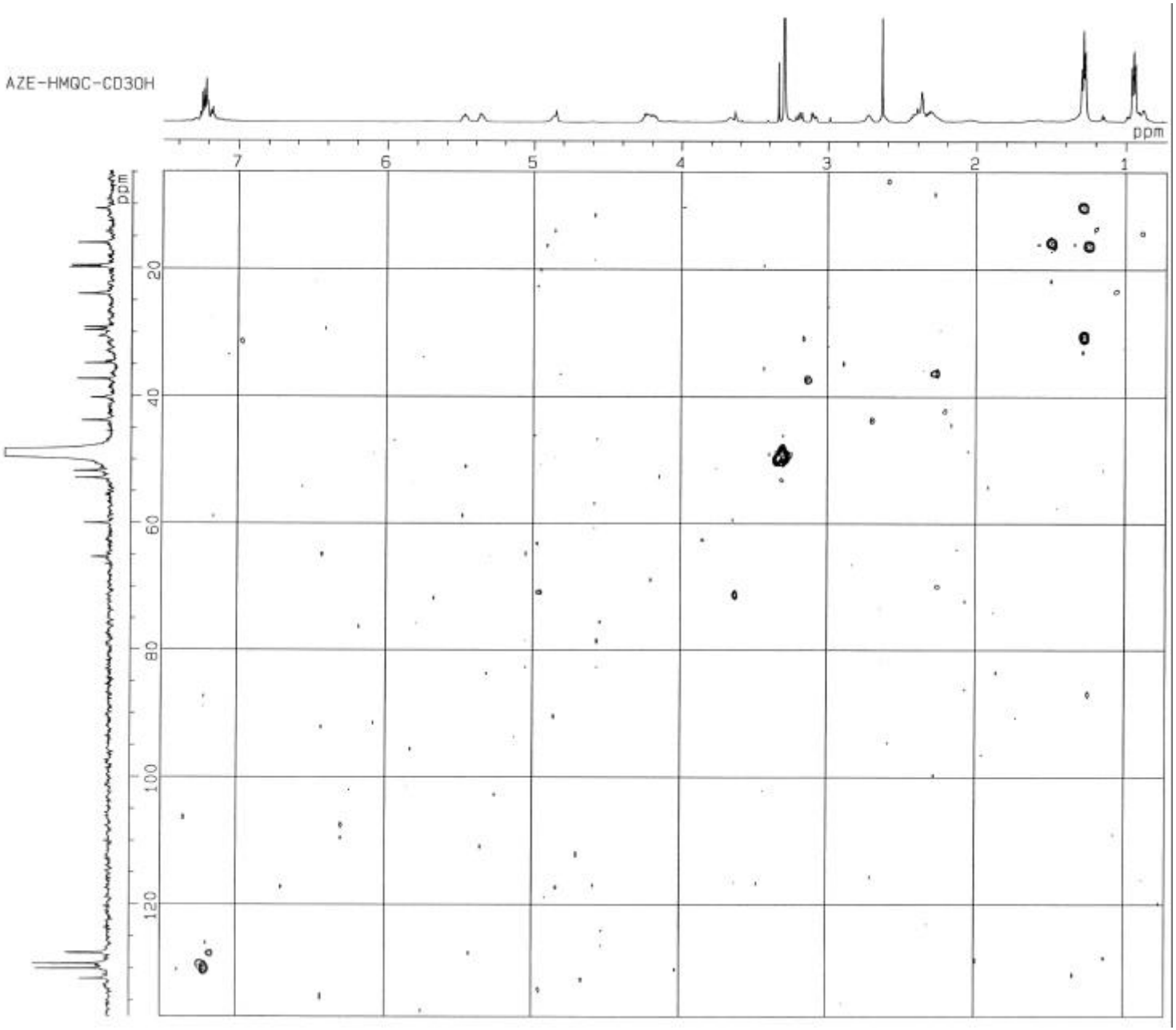

Fig. S34. HMQC spectrum of azumamide E.

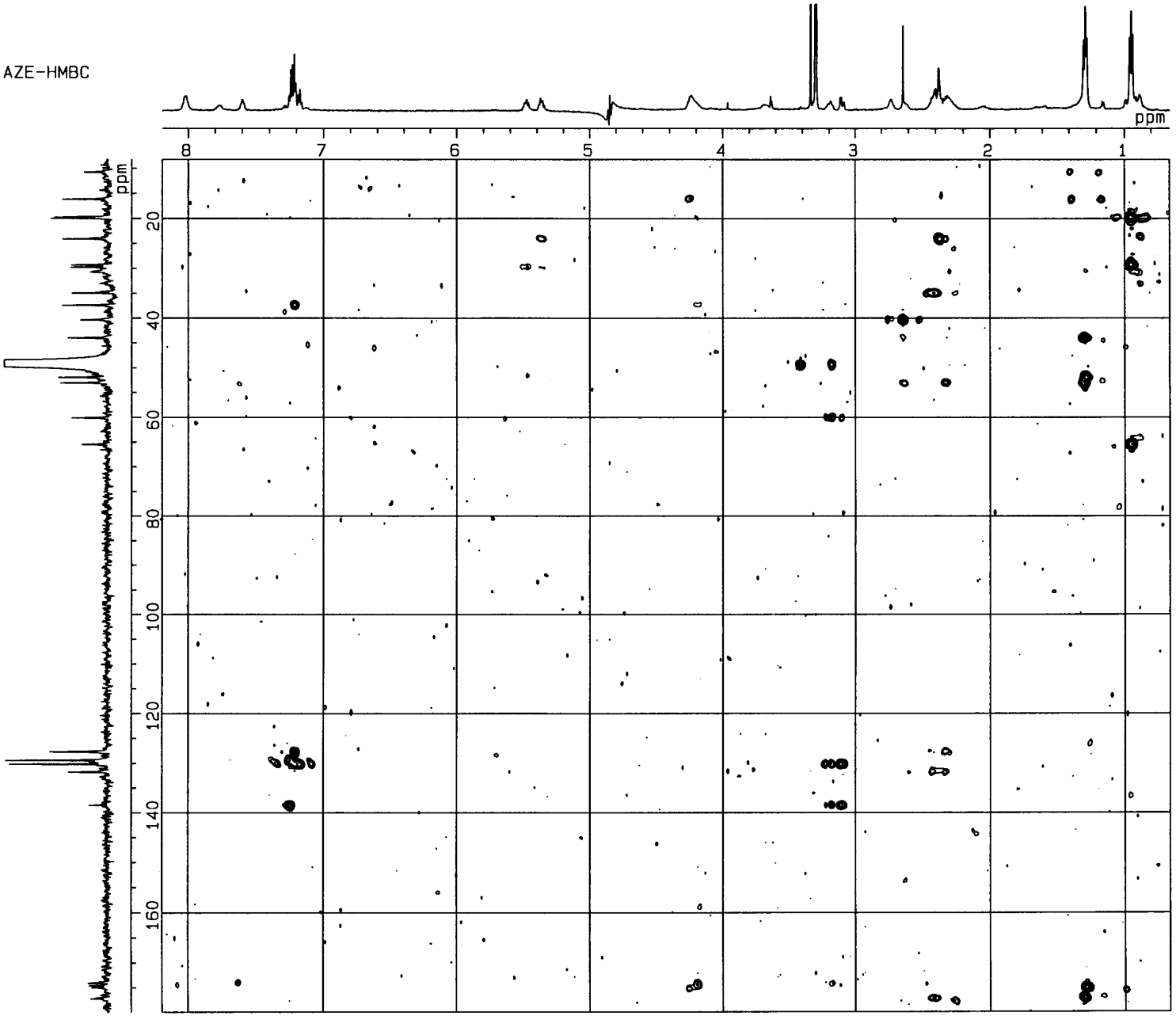

Fig. S35. HMBC spectrum of azumamide E.