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Supporting Information

Discovery of a New Asymmetric Dimer Nenestatin B and Implications of Dimerizing Enzyme in a Deep Sea Actinomycete

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Content

Experimental procedures	S3
General materials.....	S3
DNA isolation, manipulation and sequencing.....	S3
HPLC analysis.....	S3
Isolation of 2 from <i>M. echinospora</i> SCSIO 04089.....	S3
Construction of the $\Delta nes18$ mutant.....	S4
Fermentation and compound isolation of $\Delta nes18$ mutant.....	S4
Acid hydrolysis of 2	S5
Cytotoxic activity assay.....	S5
Evaluation of antibacterial activities.....	S6
Biotransformation of Nes C (3) in $\Delta nes53$ mutant.....	S6
Table S1. The NMR data of 4- <i>O</i> -methyl-L-angolosamine units in 2	S7
Table S2. Strains and plasmids used and generated in this study.....	S8
Table S3. Primers used in this study.....	S8
Fig. S1. The spectroscopic data of NEN B (2).....	S9
Fig. S2. Construction of the $\Delta nes18$ mutant.....	S20
Fig. S3. The spectroscopic data of NEN C (3).....	S21
Fig. S4. The spectroscopic data of NEN D (4).....	S28
Fig. S5. Feeding experiments in the $\Delta nes53$ mutant.....	S36
References	S37

Experimental procedures

General materials

Chemicals, enzymes, and other molecular biological reagents were purchased from standard commercial sources and used according to the manufacturers' recommendations. Bacterial strains, plasmids, and primers used and constructed in this study are listed in Table S2† and Table S3†.

DNA isolation, manipulation and sequencing

DNA isolation and manipulation in *E. coli* and *M. echinospora* SCSIO 04089 were carried out according to standard procedures.^{1, 2} Primers used in this study (Table S3†) were synthesized at the Shanghai Invitrogen Biotech Co., Ltd. PCR amplifications were carried out on an Authorized Thermal Cycler (Eppendorf AG). DNA sequencing was performed at the Invitrogen Biotech Co., Ltd. (Guangzhou), and Chinese National Genome Center (Shanghai).

HPLC analysis

For analyzing the metabolites of *M. echinospora* SCSIO 04089 and mutant $\Delta nes18$, the HPLC was carried out on the Agilent 1260 Infinity series instrument (Agilent Technologies Inc., USA) using a reversed phase column (Phenomenex kinetex, C18, 5 μm , 250 mm \times 4.6 mm) with UV detection at 342 nm under the following program: solvent system (solvent A, 10% CH_3CN in water supplemented with 0.08% formic acid; solvent B, 90% CH_3CN in water); 5%B to 80% B (0–20 min), 80% B to 100% B (20–21 min), 100% B (21–25 min), 100% B to 5%B (25–26 min), 5% B (26–30 min), flow rate at 1 mL min^{-1} .

Isolation of 2 from *M. echinospora* SCSIO 04089

The strain *M. echinospora* SCSIO 04089 was resurrected on an ATCC172 (soluble starch 20.0 g L^{-1} , glucose 10.0 g L^{-1} , yeast extract 5.0 g L^{-1} , Aobox casein 5.0 g L^{-1} , CaCO_3 19.0 g L^{-1} , artificial sea salt 10.0 g L^{-1} , pH 7.0) agar plate at 28 °C for 7 days, and then was transferred to 250 mL Erlenmeyer flask containing 50 mL of A1 medium (soluble starch 10.0 g L^{-1} , yeast extract 4.0 g L^{-1} , bacterial peptone 2.0 g L^{-1} , artificial sea salt 10.0 g L^{-1} , pH 7.0) for incubation at 28 °C, 200 rpm for 3 days. The seed cultures were then transferred to 1000 mL Erlenmeyer flask containing 200 mL of the N4 medium (soluble starch 15.0 g L^{-1} , fish peptone 8.0 g L^{-1} , bacterial peptone 5.0

g L⁻¹, glycerol 7.5 g L⁻¹, CaCO₃ 2.0 g L⁻¹, KBr 0.2 g L⁻¹, artificial sea salt 30.0 g L⁻¹, pH 7.0). A total of 20 L cultures was prepared and incubated at 28 °C, 200 rpm for 7 days. The secondary metabolites were absorbed by HP20 resin, and the resin was then washed with 4 L CH₃CN for several times to yield the extract (10 g). The extract was subjected to column chromatography (CC) with normal phase silica gel (100–200 mesh), and eluted with a gradient of CHCl₃/CH₃OH (100:0–0:100, v/v) to give five fractions (Fr.1–Fr.5). Fr.4 was purified by silica gel thin-layer chromatography, developed with CHCl₃/CH₃OH (3:1, v/v) to give 3 fractions (Fr.4.1–Fr.4.3). Fr.4.2 was further purified by semi-preparative HPLC on Hitachi workstation (Hitachi-L2130, Tokyo, Japan) using a reversed phase column (Phenomenex kinetex, C18, 5 μm, 250 mm × 10.0 mm) with UV detection at 342 nm to afford compound **2** (14 mg).

Construction of the $\Delta nes18$ mutant

The lambda-RED-mediated gene replacement was performed as standard procedure.² Briefly, the gene cassette comprising *aac(3)IV* and *oriT* was amplified from plasmid pIJ773 with primers *nes18-tarF* and *nes18-tarR* (Table S3⁺), and the purified PCR product was introduced into *E. coli* BW25113/pIJ790 containing the cosmid pCSG4102 by electroporation. The positive clones that the targeted gene was replaced by the *aac(3)IV* and *oriT* cassette were selected with apramycin resistance to afford the cosmid pCSG4115 (Table S2⁺). The conjugation between *E. coli* ET12567/pUZ8002/pCSG4115 and *M. echinospora* SCSIO 04089 was carried out in a similar way as previously described (Fig. S2⁺).³ The exconjugants were selected after 1–2 weeks and the double-crossover mutant strains were identified by diagnostic PCR with primers *nes18* test F and *nes18* test R (Table S3⁺) to afford the $\Delta nes18$ (NES004) (Table S2⁺). Three independent clones from the double-crossover mutant were randomly chosen for small scale fermentation and the extract was subjected to HPLC analysis.

Fermentation and compound isolation of $\Delta nes18$ mutant

The $\Delta nes18$ mutant strain was resurrected on an ATCC172 agar plate at 28 °C for 5 days, and then was transferred to 250 mL Erlenmeyer flask containing 50 mL of the N4 medium for incubation at 28 °C, 200 rpm for 3 days. Next, 5 mL of cultures were

extracted with equal volume of ethyl acetate, which was dried under vacuum condition and re-dissolved in 0.2 mL methanol for HPLC analysis.

For isolating compound **3**, a total of 16 L culture was prepared by adding pre-washed HP20 resin in the media (5%, v/v). After incubated at 28 °C, 200 rpm for 7 days, the secondary metabolites were absorbed by HP20 resin, changing the color of the resin from white to pink. The pink-colored HP20 resin was collected by filtration and washed with 4 L CH₃CN for several times to yield extract (12 g). The extract was subjected to column chromatography (CC) with normal phase silica gel (100–200 mesh), and eluted with a gradient of CHCl₃/CH₃OH (100:0–0:100, v/v) to give five fractions (Fr.1–Fr.5). Fr.4 was separated by Sephadex LH-20, and eluted with CHCl₃/CH₃OH (1:1, v/v) to give 7 fractions (Fr.4.1–Fr.4.7). Fr.4.6 was purified by semi-preparative HPLC to get **3** (10.5 mg).

Acid hydrolysis of 2

Compound **2** (14 mg) was heated in 1 N HCl (2 mL) at 95 °C for 2 h. The acidic aqueous mixture was extracted with 4 mL of ethyl acetate for three times. The extract was dried under vacuum condition, and the residue was re-dissolved and purified by semi-preparative HPLC to yield **4** (1.5 mg).

Cytotoxic activity assay

Compounds **1–4** were assayed for their cytotoxic activities against SF-268, MCF-7, A549, and HepG2 cell lines with the SRB method.⁴ Cells (180 µL) with a density of 3×10^4 cells·mL⁻¹ of media were seeded onto 96-well plates and incubated for 24 h at 37 °C, 5% CO₂. Then, 20 µL of various concentrations (final concentration are at 1.0, 10.0 and 100.0 µM) of compounds were added to the plate wells and the plates were further incubated for 72 h at 37 °C. After incubation, cell monolayers were fixed with 50% (wt/v) trichloroacetic acid (50 µL) and stained for 30 min with 0.4% (wt/v) SRB dissolved in 1% acetic acid. Unbound dye was removed by washing repeatedly with 1% acetic acid. The protein-bound dye was dissolved in 10 mM Tris-base solution (200 µL) for the determination of OD (optical density) at 570 nm using a microplate reader. The cytotoxic compound cisplatin was used as a positive control. All data were obtained in triplicate and presented as means ± S.D.

Evaluation of antibacterial activities

Minimal Inhibition Concentration (MIC) values for compounds **1–4** were evaluated against seven bacterial strains *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Micrococcus. Luteus* SCSIO ML01, *Enterococcus faecalis* ATCC29212, *Acinetobacter baumannii* ATCC 19606, methicillin-resistant *S. aureus* ATCC 43300 and *Vibrio alginolyticus* XSBZ14 (a coral pathogenic bacterium⁵) by previously described methods based on three independent parallel experiments.

Feeding experiments in the $\Delta nes53$ mutant

The $\Delta nes53$ ⁶ mutant strain was incubated into 10 mL of N4 medium supplemented with **3** at a final concentration of 60 μ M. After incubation at 28 °C for 48 h, 3 mL of cultures were separated to supernatant and cell sediments by centrifugation at 4000 rpm for 20 min. The supernatant was extracted with 3 mL ethyl acetate, and the cell sediments were washed with 3 mL of distilled water and extracted with 1.5 mL of acetone. After drying under vacuum condition, the extracts were redissolved in 50 μ L of DMSO and subjected to HPLC analysis.

Table S1. The NMR data of 4-*O*-methyl-L-angolosamine units in **2**.

4- <i>O</i> -methyl-L-angolosamine in 2			4- <i>O</i> -methyl-L-angolosamine in 2		
No.	(Unit A)		No.	(Unit B)	
	δ_C , type	δ_H , mult (<i>J</i>)		δ_C , type	δ_H , mult (<i>J</i>)
1 _S	90.8, CH	6.01, m	1' _S	91.1 CH	6.01, m
2 _S	30.7, CH ₂	2.08, m	2' _S	30.7, CH ₂	2.08, m
3 _S	62.7, CH	3.44, m	3' _S	62.7, CH	3.48, m
4 _S	79.2, CH	3.24, m	4' _S	79.5, CH	3.15, m
5 _S	67.9, CH	3.75, m	5' _S	67.5, CH	4.13, m
6 _S	18.2 CH ₃	1.23, d (6.3)	6' _S	18.4 CH ₃	1.29, d (6.3)
7 _S	59.5 CH ₃	3.44, s	7' _S	59.2 CH ₃	3.48, s
8 _S	39.5 CH ₃	2.52, s	8' _S	39.5 CH ₃	2.52, s
9 _S	39.5 CH ₃	2.52, s	9' _S	39.5 CH ₃	2.52, s

700 MHz for ¹H, 175 MHz for ¹³C, DMSO-*d*₆, tetramethylsilane (TMS) as an internal standard.

Table S2. Strains and plasmids used and generated in this study.

Strain/plasmid	Characteristic(s)	Sources
<i>E. coli</i>		
BW25113	Host strain for PCR targeting	3
ET12567	Host strain for PCR targeting	7
<i>Micromonospora</i>		
<i>M. echinospora</i> SCSIO 04089	Wild type strain, nenestatin A producer	6
NES001	The <i>nes53</i> gene disrupted mutant of SCSIO 04089	6
NES004	The <i>nes18</i> gene disrupted mutant of SCSIO 04089	This study
Plasmid		
pIJ773	Apr ^r , source of <i>aac(3)IV</i>	3
pUZ8002	<i>Tra</i> , <i>neo</i>	8
pCSG4102	Strain SCSIO 04089 genomic library cosmid	6
pCSG4115	<i>nes18</i> was disrupted by <i>aac(3)IV</i> in pCSG4102	This study

Table S3. Primers used in this study.

For PCR targeting	sequences
Nes18 Tar F	GTGGCGGTAGATGGGCCGATTCTGGTGCTGGGCGGTACcattccggggat ccgtcgacc
Nes18 Tar R	TCAGGCCGCCACAGGGGCGGTCCAGCTGGTACGCAGGAAtgtaggctgg agctgcttc
Nes18 test F	CCTGGCGGAGGAAGGCGTCG
Nes18 test R	GTCGTACCTGATCCGCGCG

Fig. S1. The spectroscopic data of NEN B (**2**).
(A) The HRESIMS spectrum of NEN B (**2**).

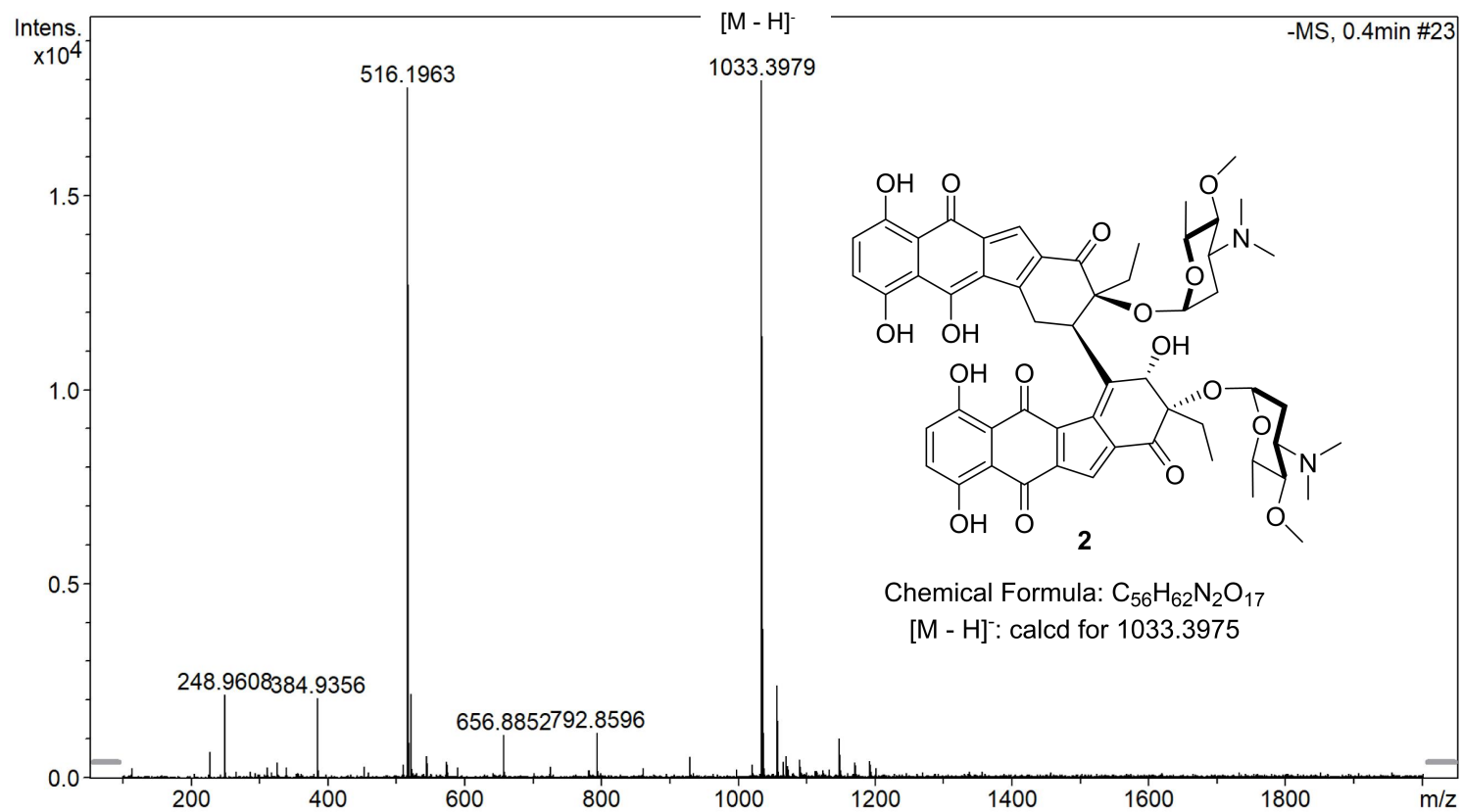


Fig. S1. The spectroscopic data of NEN B (**2**).

(B) The $^1\text{H-NMR}$ spectrum of NEN B (**2**). (700 MHz for ^1H NMR in $\text{DMSO-}d_6$).

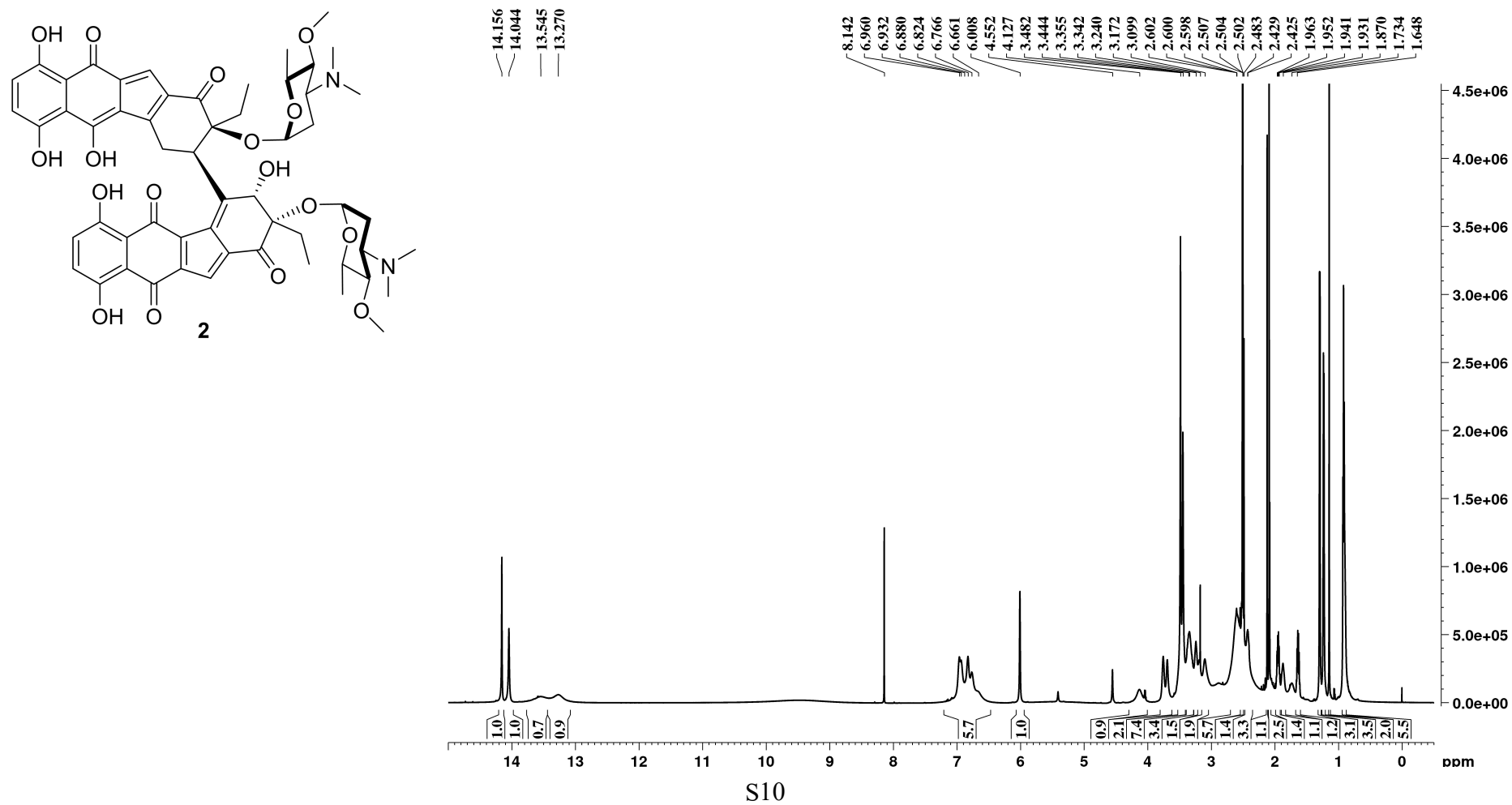


Fig. S1. The spectroscopic data of NEN B (**2**).

(C) The ^{13}C -NMR spectrum of NEN B (**2**). (175 MHz for ^{13}C NMR in $\text{DMSO-}d_6$).

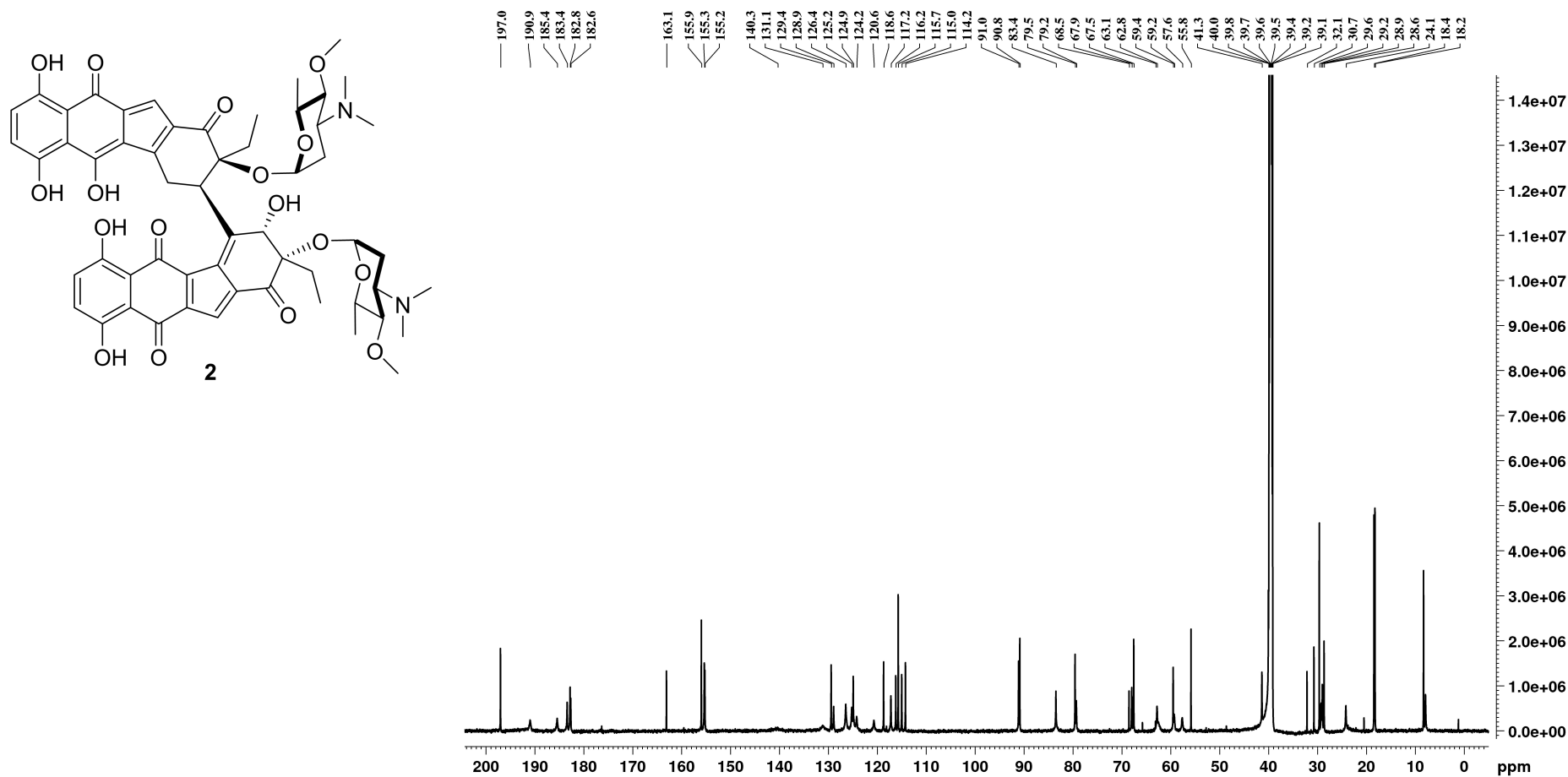


Fig. S1. The spectroscopic data of NEN B (**2**).

(D) The DEPT135 spectrum of NEN B (**2**). (175 MHz for ^{13}C NMR in $\text{DMSO-}d_6$).

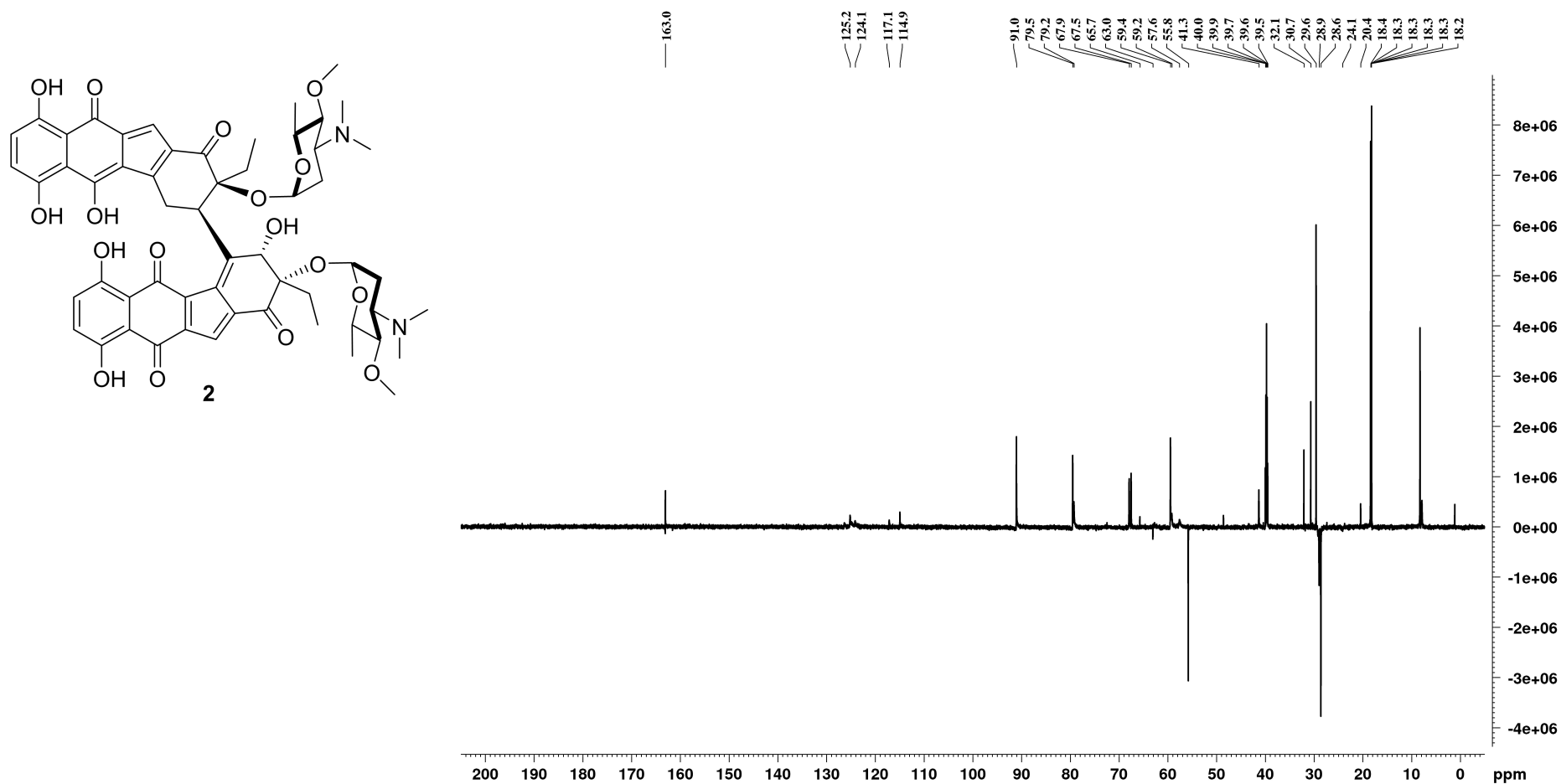


Fig. S1. The spectroscopic data of NEN B (**2**).
(E) The HSQC spectrum of NEN B (**2**).

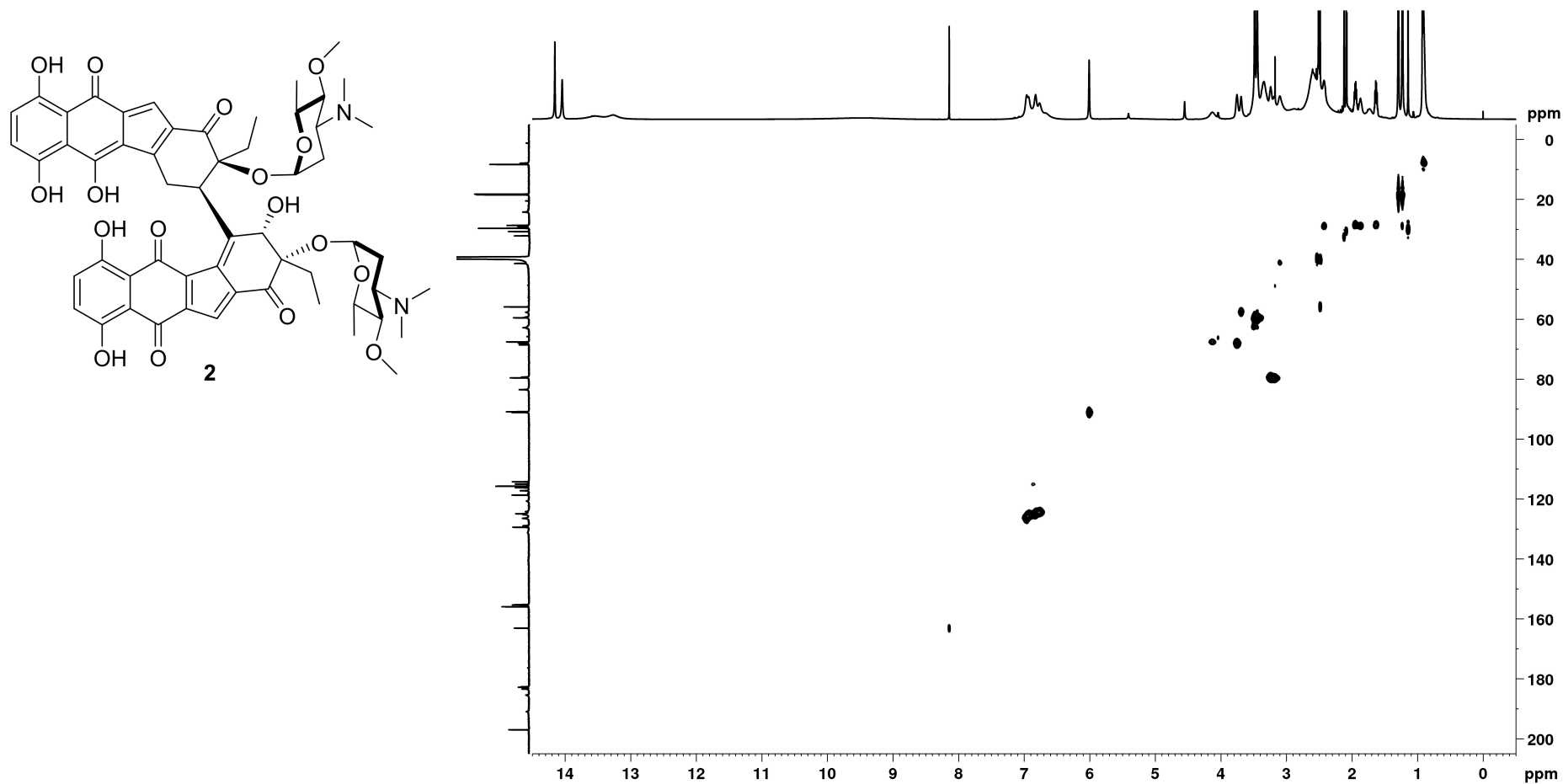


Fig. S1. The spectroscopic data of NEN B (**2**).
(F) The COSY spectrum of NEN B (**2**).

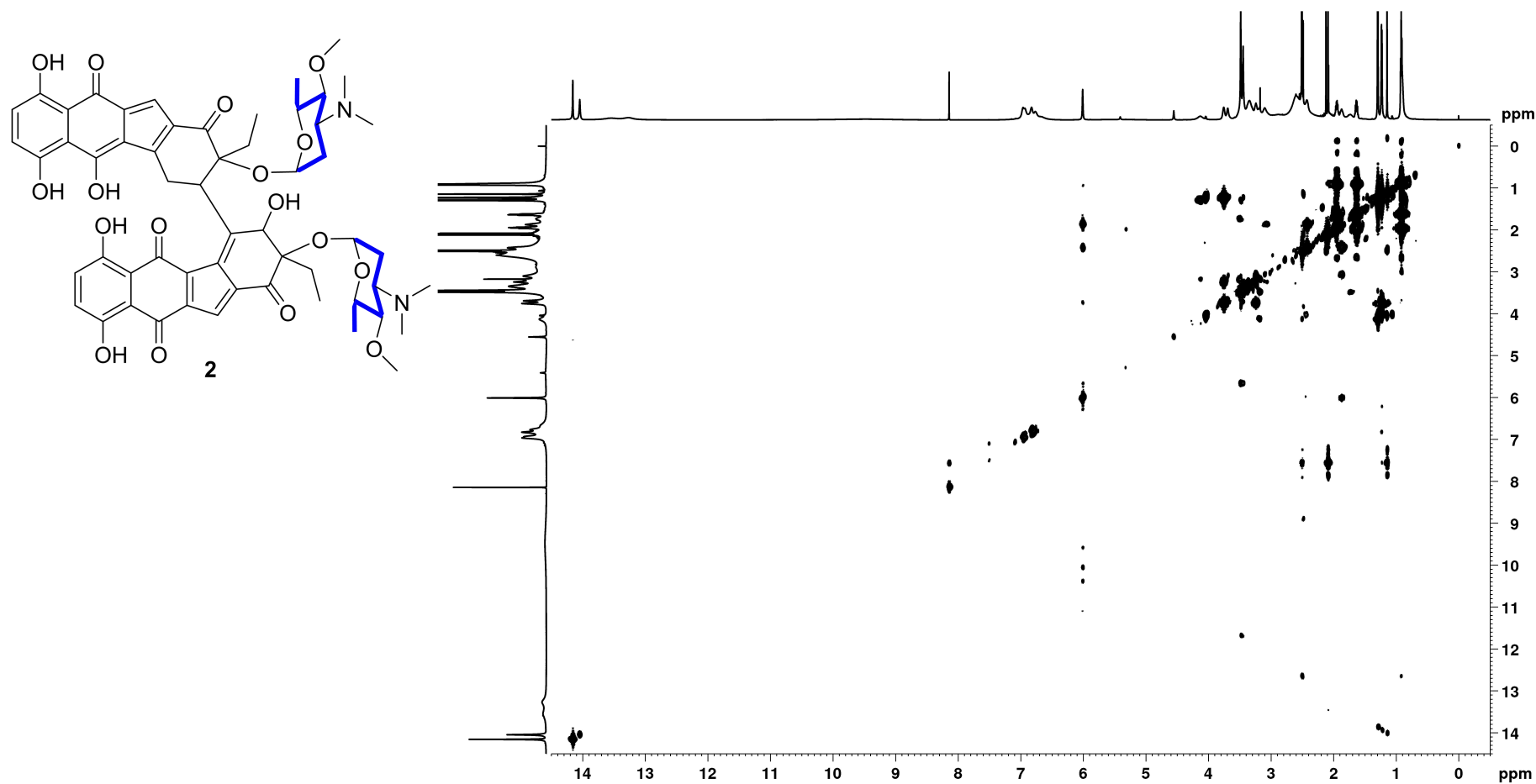


Fig. S1. The spectroscopic data of NEN B (**2**).
(G) The HMBC spectrum of NEN B (**2**).

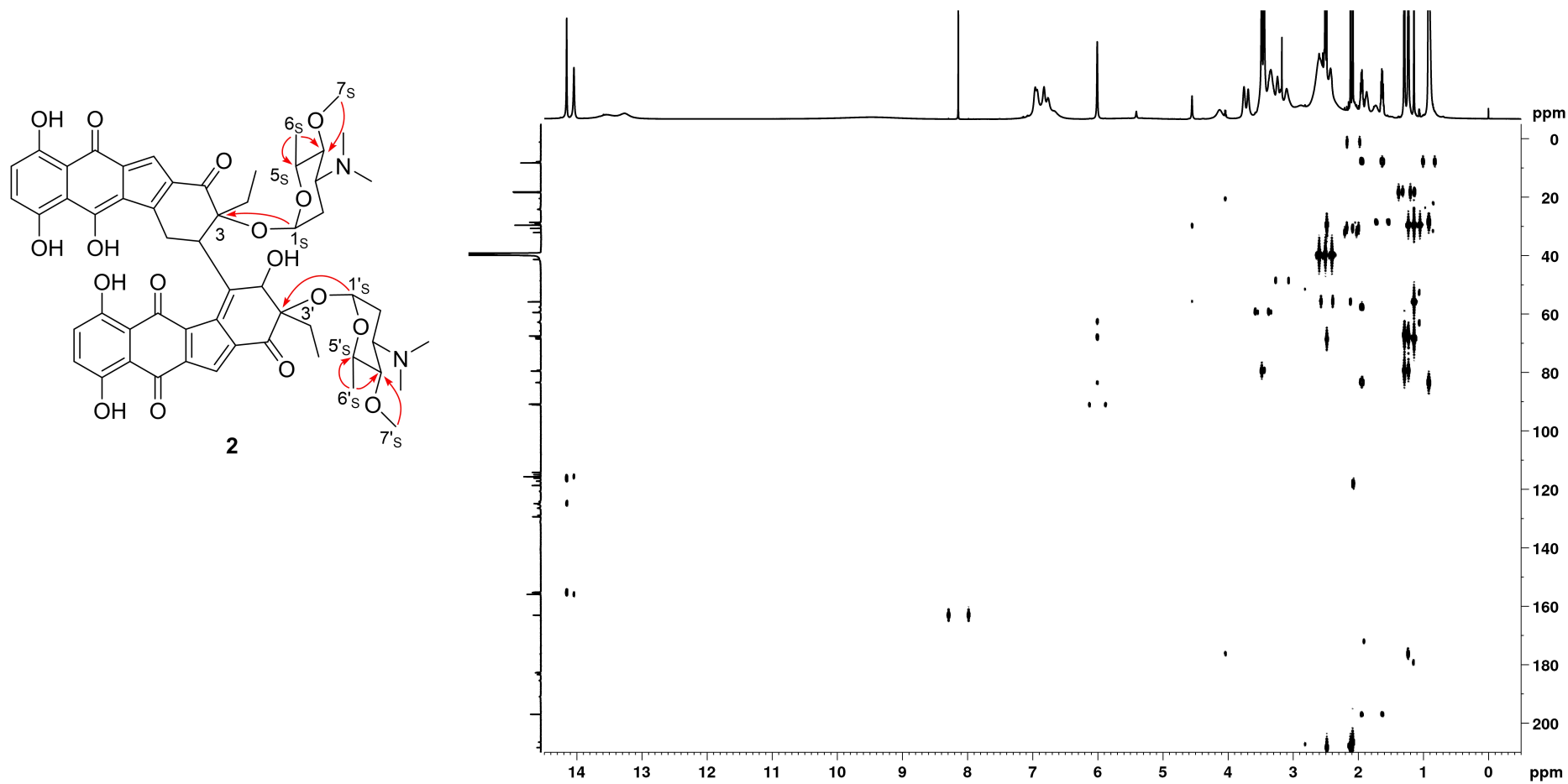


Fig. S1. The spectroscopic data of NEN B (**2**).
(H) The NOSEY spectrum of NEN B (**2**).

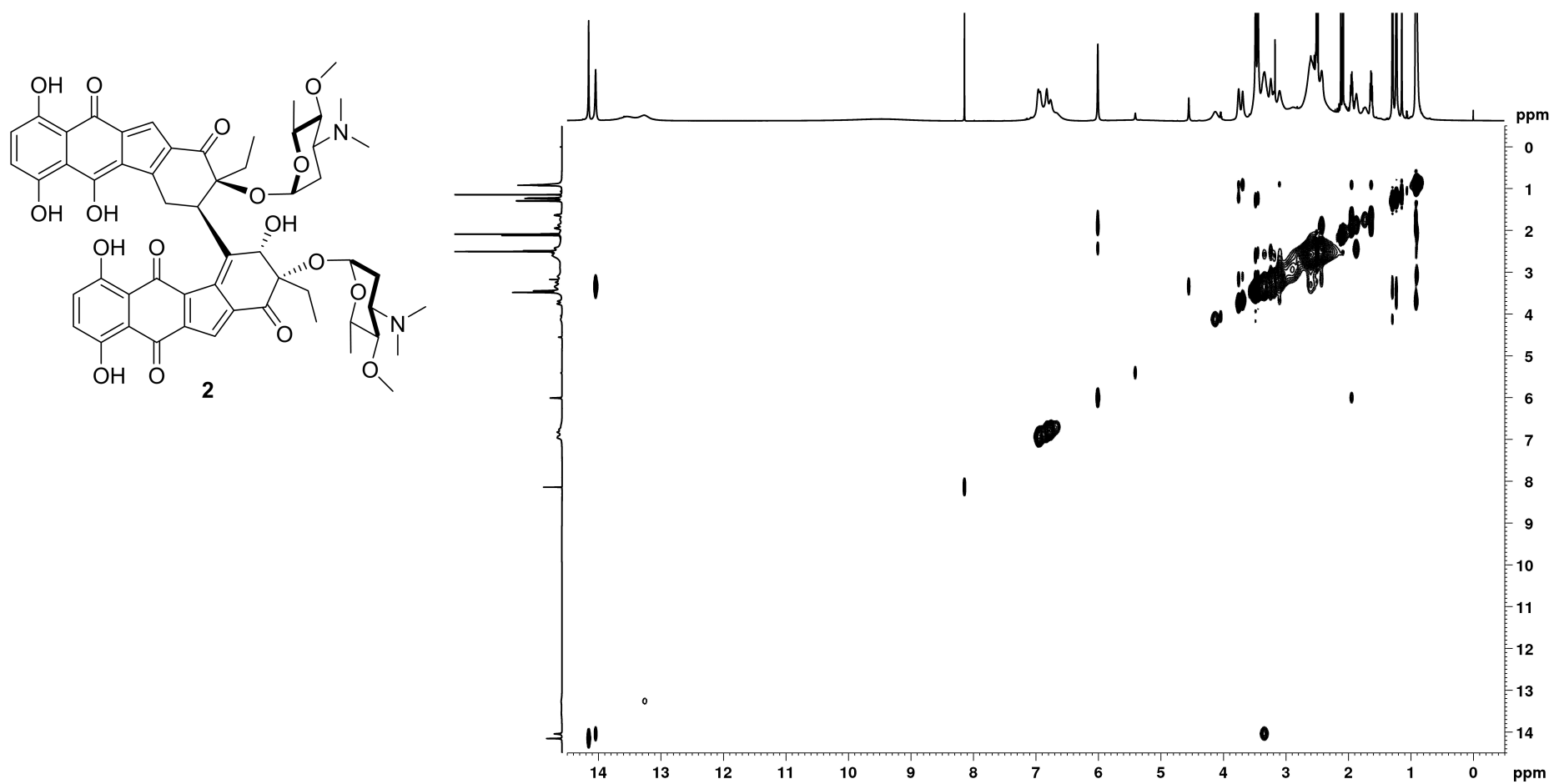


Fig. S1. The spectroscopic data of NEN B (**2**).

(I) The $^1\text{H-NMR}$ spectrum of NEN B (**2**). (700 MHz for ^1H NMR in acetone- d_6).

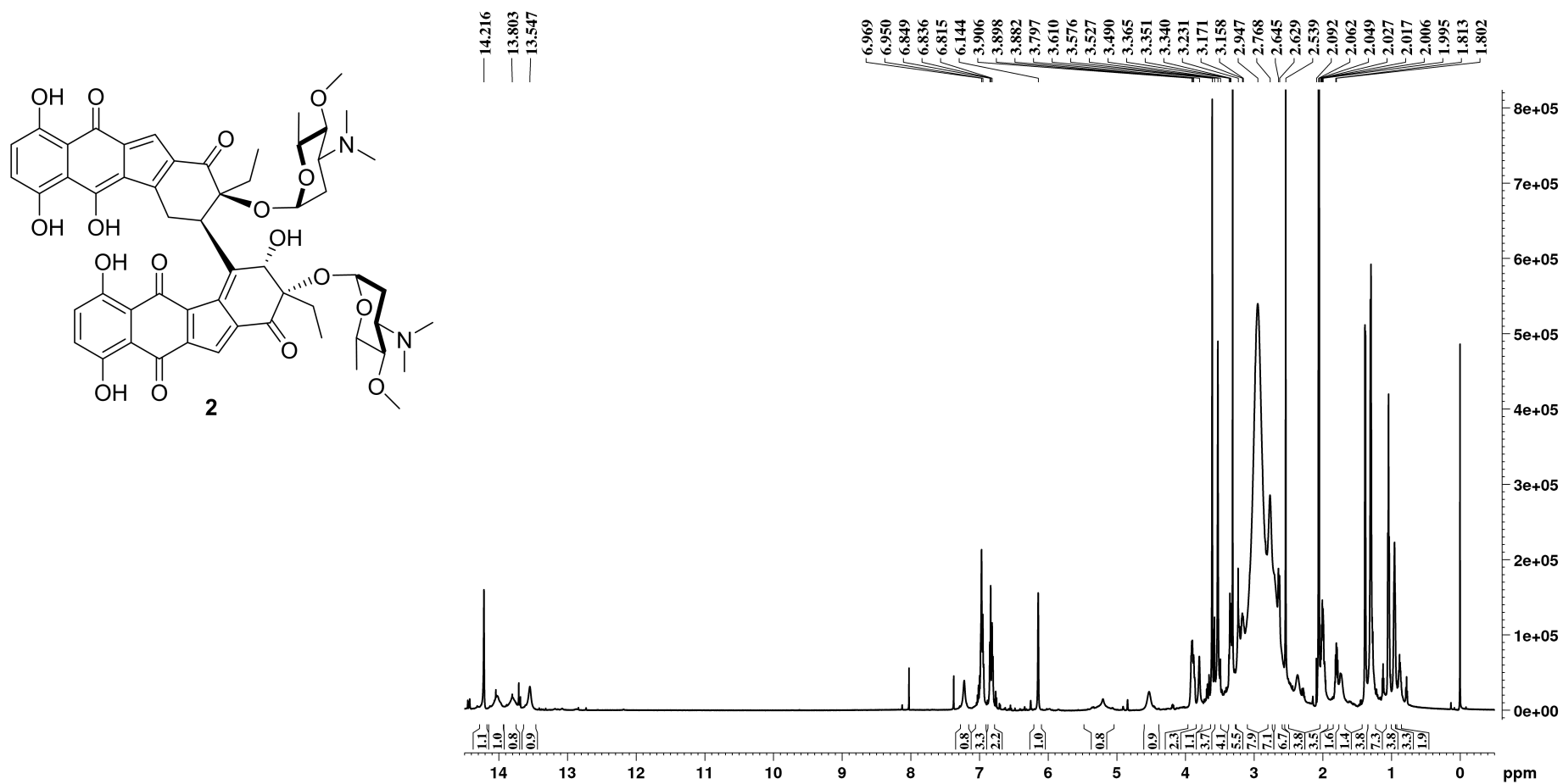


Fig. S1. The spectroscopic data of NEN B (**2**).

(J) The ^{13}C -NMR spectrum of NEN B (**2**). (175 MHz for ^{13}C NMR in acetone- d_6).

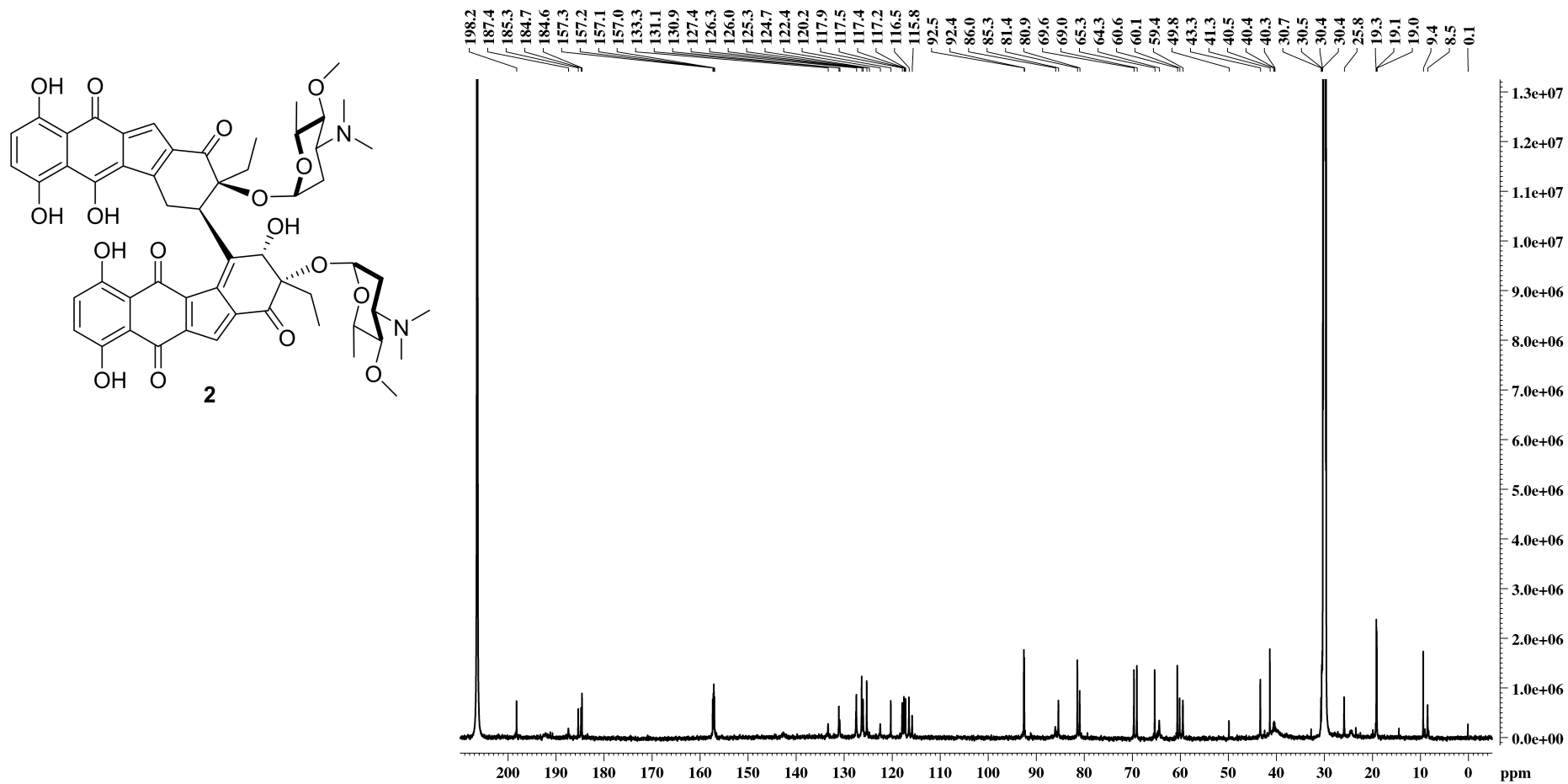


Fig. S1. The spectroscopic data of NEN B (**2**).

(K) The $^1\text{H-NMR}$ spectrum of NEN B (**2**). (700 MHz for ^1H NMR in methanol- d_4).

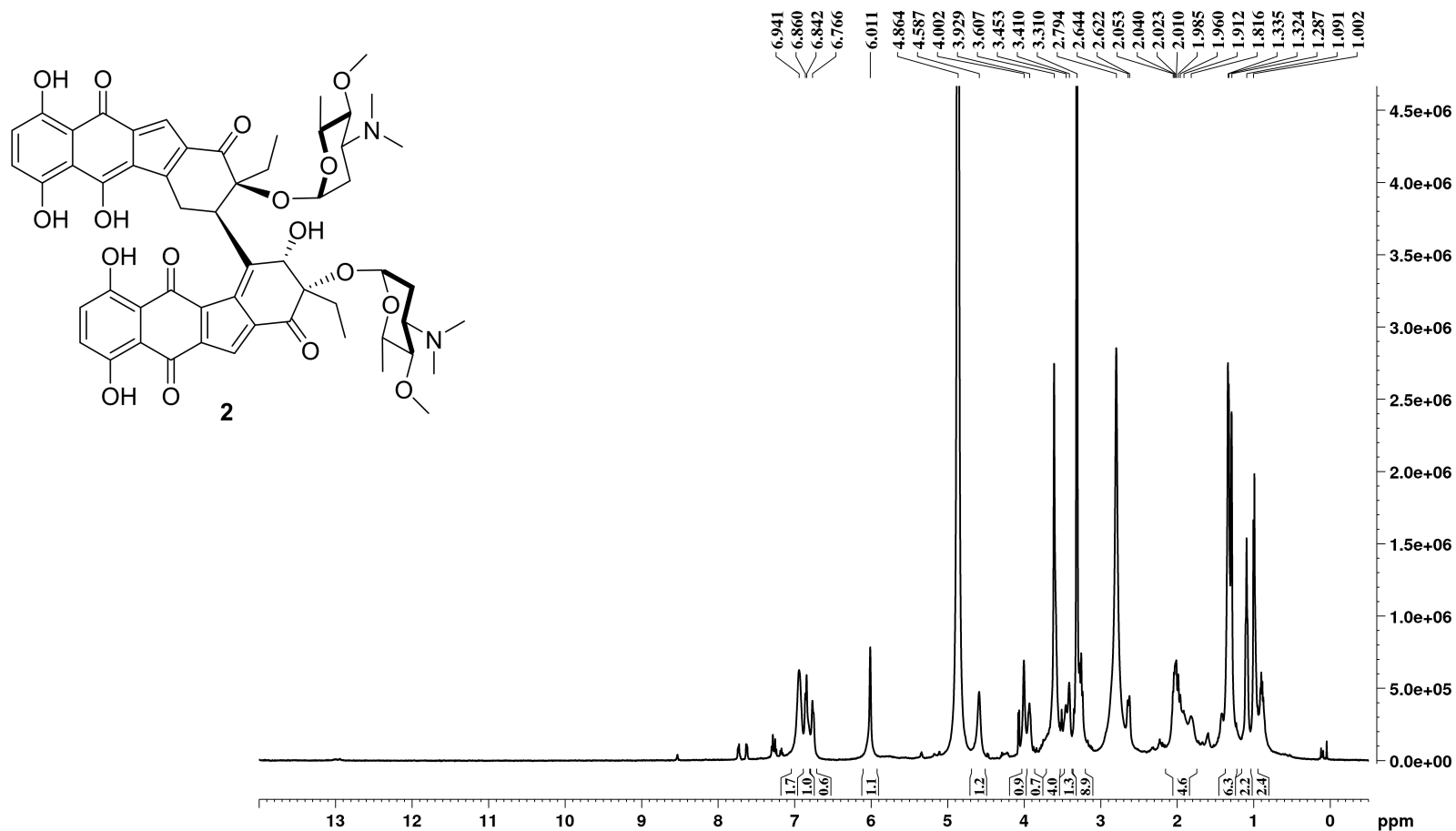
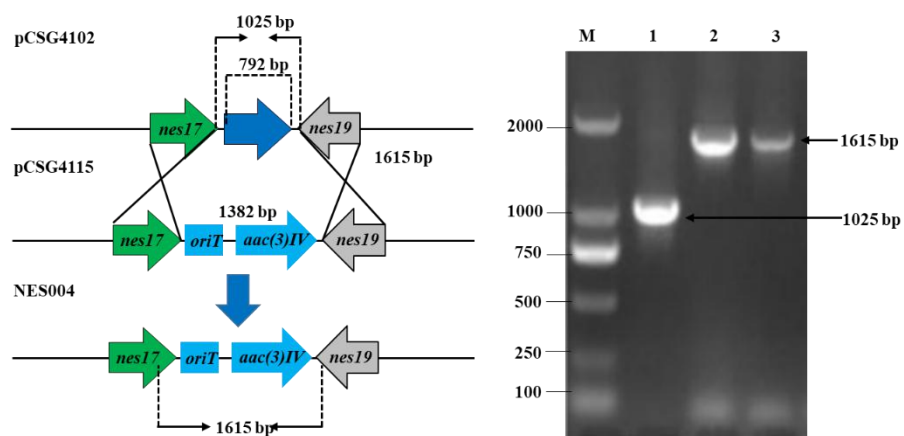
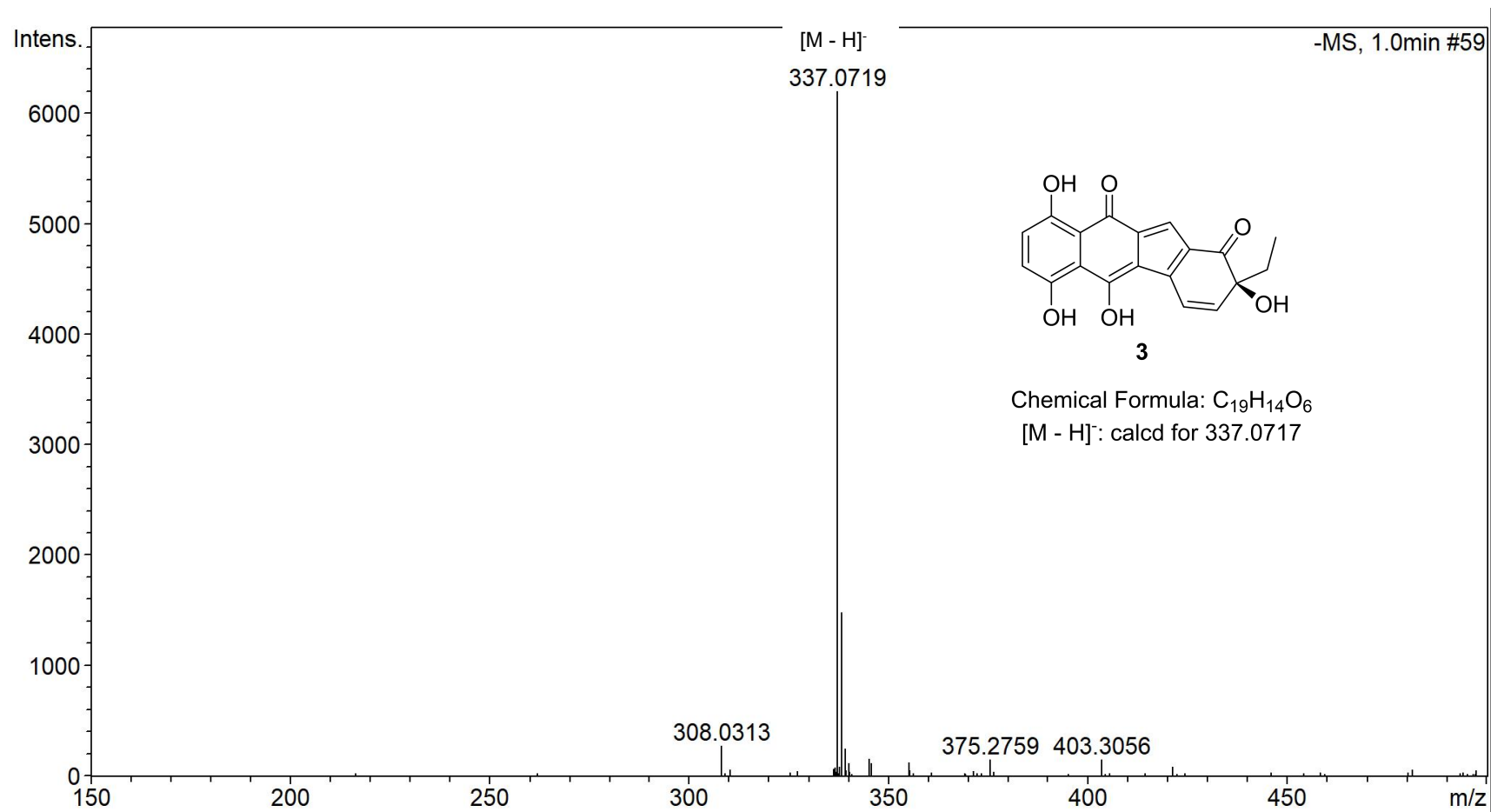


Fig. S2. Construction of the $\Delta nes18$ mutant



(A) Schematic representation for insertion of the $\Delta nes18$ mutant. (B) Gel electrophoresis of PCR products. DNA templates were from $\Delta nes18$ mutant (lane 3), pCSG4115 (lane 2, positive control), pCSG4102 (lane 1, negative control), and DL 2000 plus DNA marker (Takara, lane M).

Fig. S3. The spectroscopic data of NEN C (**3**).
(A) The HRESIMS spectrum of NEN C (**3**).



S21

Fig. S3. The spectroscopic data of NEN C (**3**).

(B) The ^1H -NMR spectrum of NEN C (**3**). (700 MHz for ^1H NMR in $\text{DMSO-}d_6$).

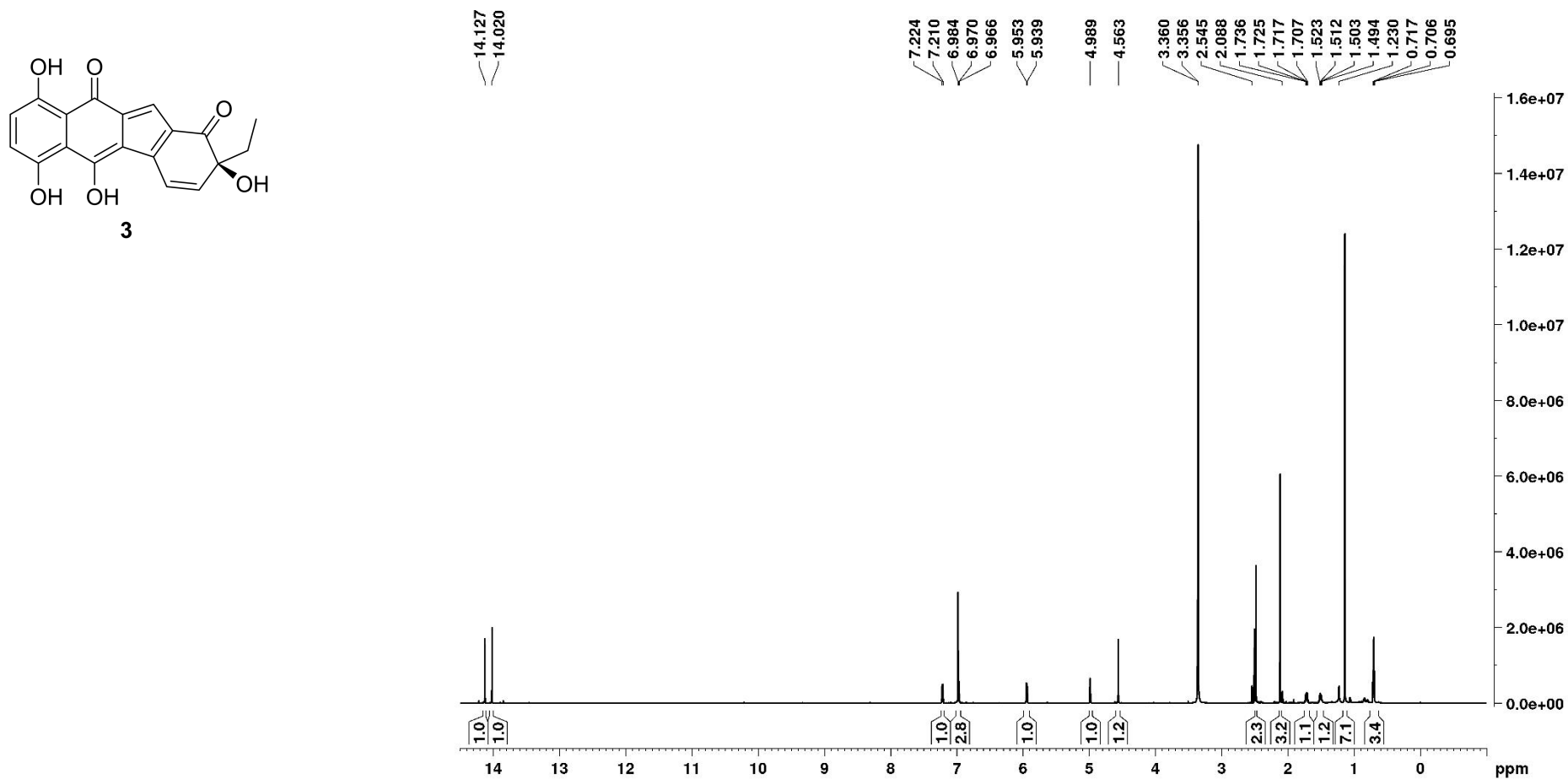


Fig. S3. The spectroscopic data of NEN C (**3**).

(C) The ^{13}C -NMR spectrum of NEN C (**3**). (175 MHz for ^{13}C NMR in $\text{DMSO-}d_6$).

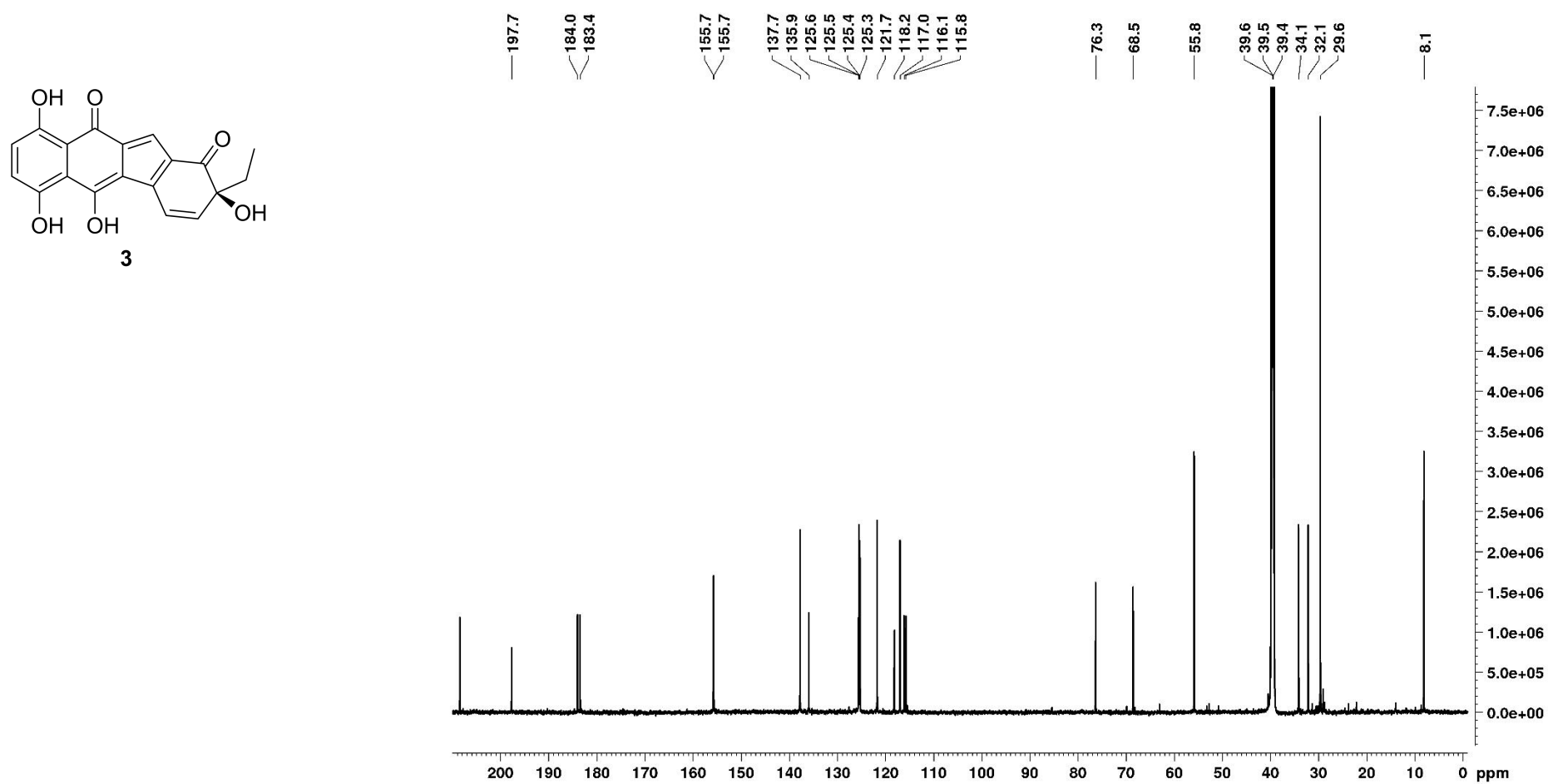


Fig. S3. The spectroscopic data of NEN C (**3**).

(D) The DEPT135 spectrum of NEN C (**3**). (175 MHz for ^{13}C NMR in $\text{DMSO-}d_6$).

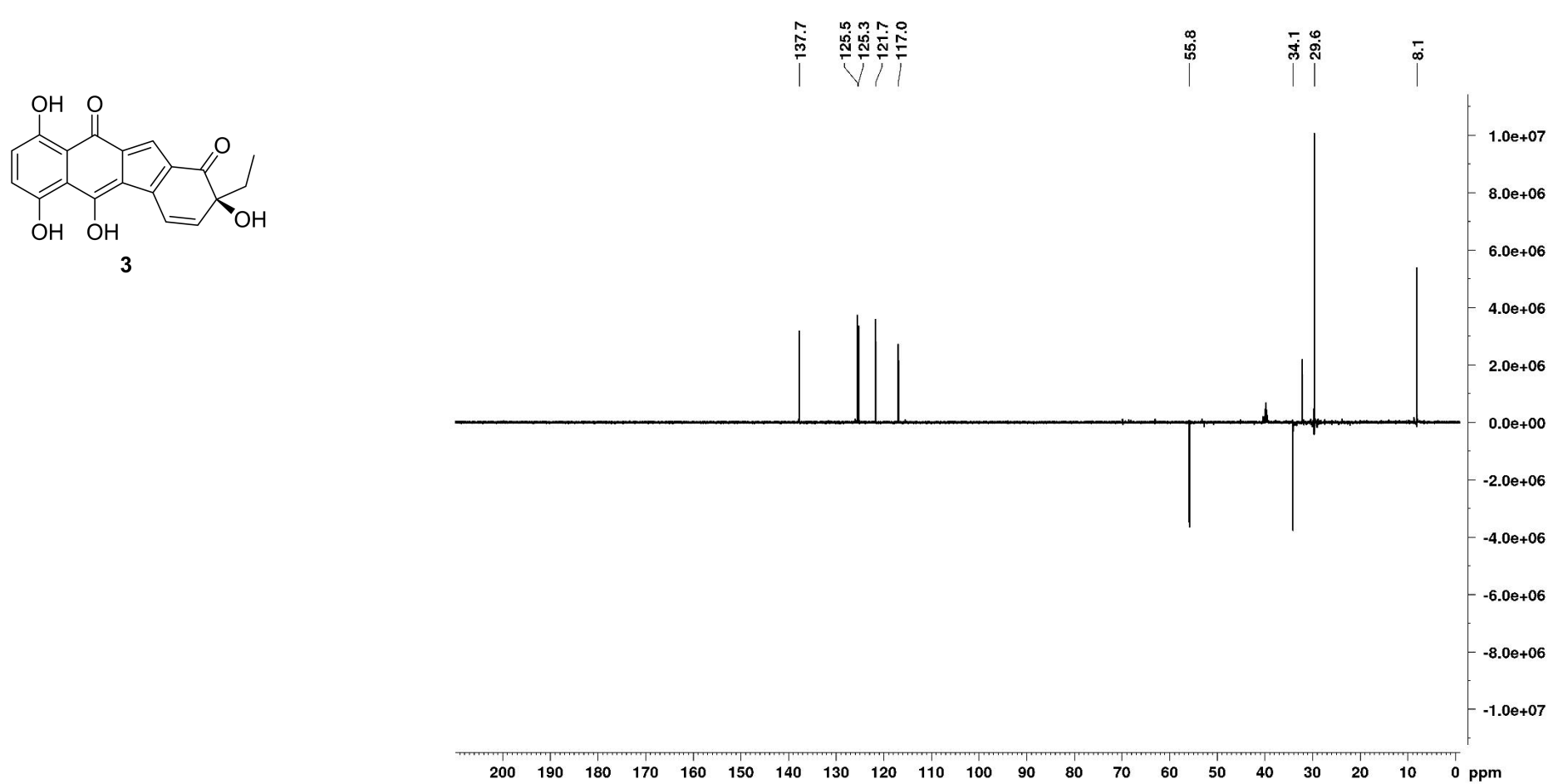


Fig. S3. The spectroscopic data of NEN C (**3**).
(E) The HSQC spectrum of NEN C (**3**).

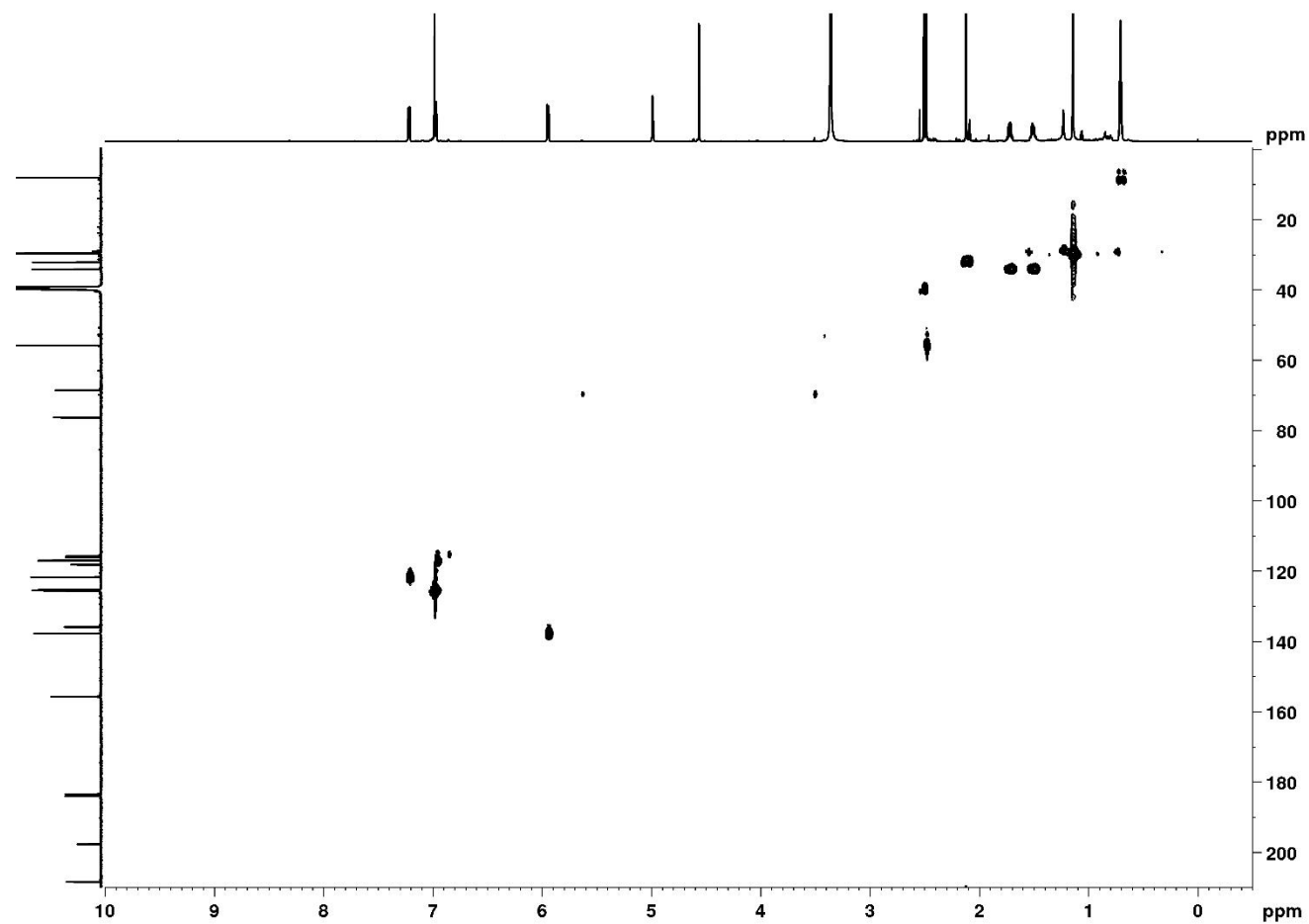
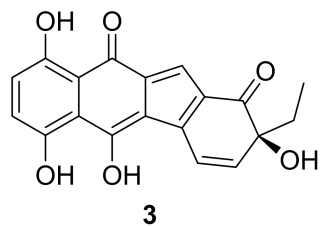


Fig. S3. The spectroscopic data of NEN C (**3**).
(F) The COSY spectrum of NEN C (**3**).

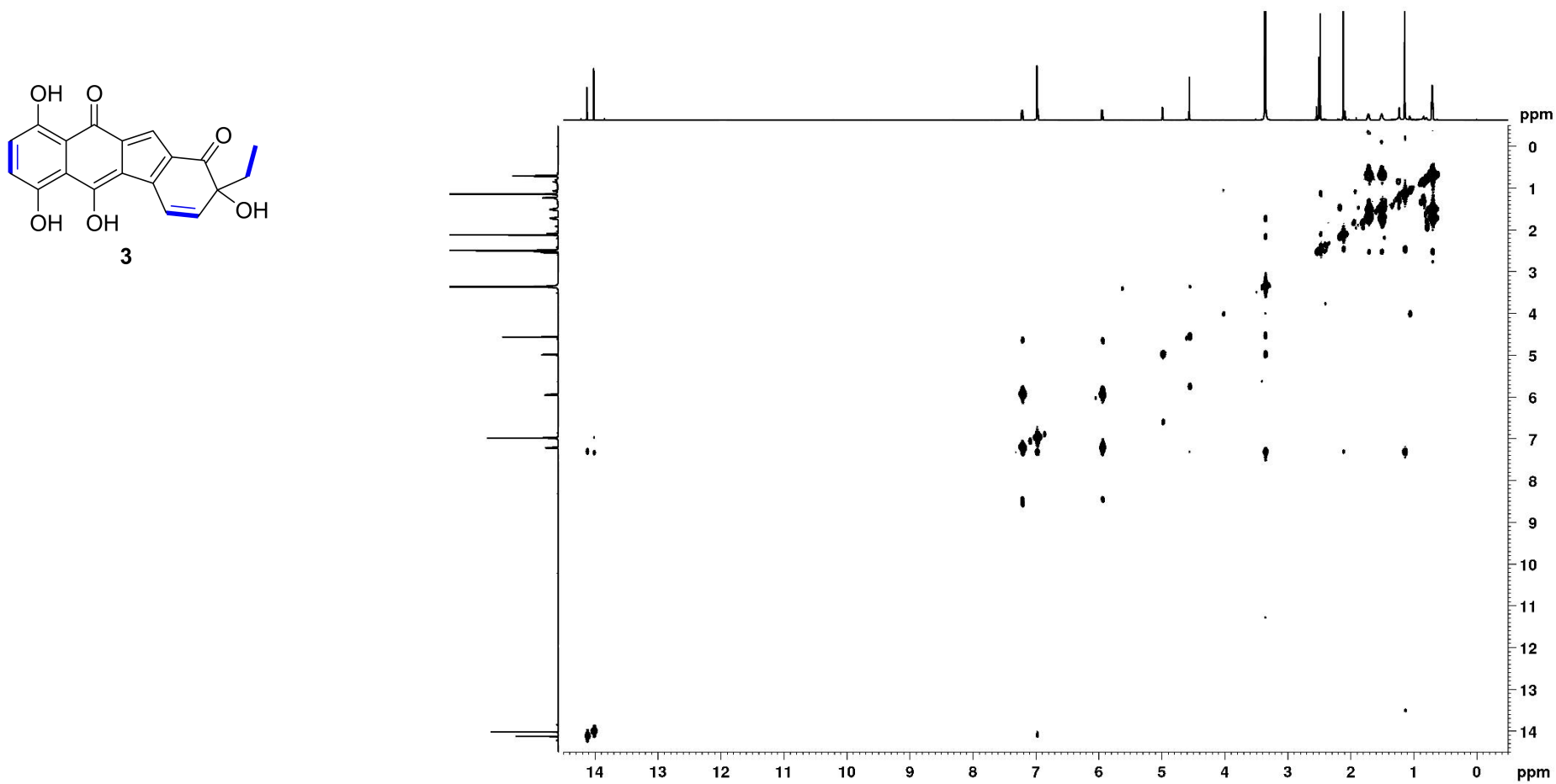


Fig. S3. The spectroscopic data of NEN C (**3**).
(G) The HMBC spectrum of NEN C (**3**).

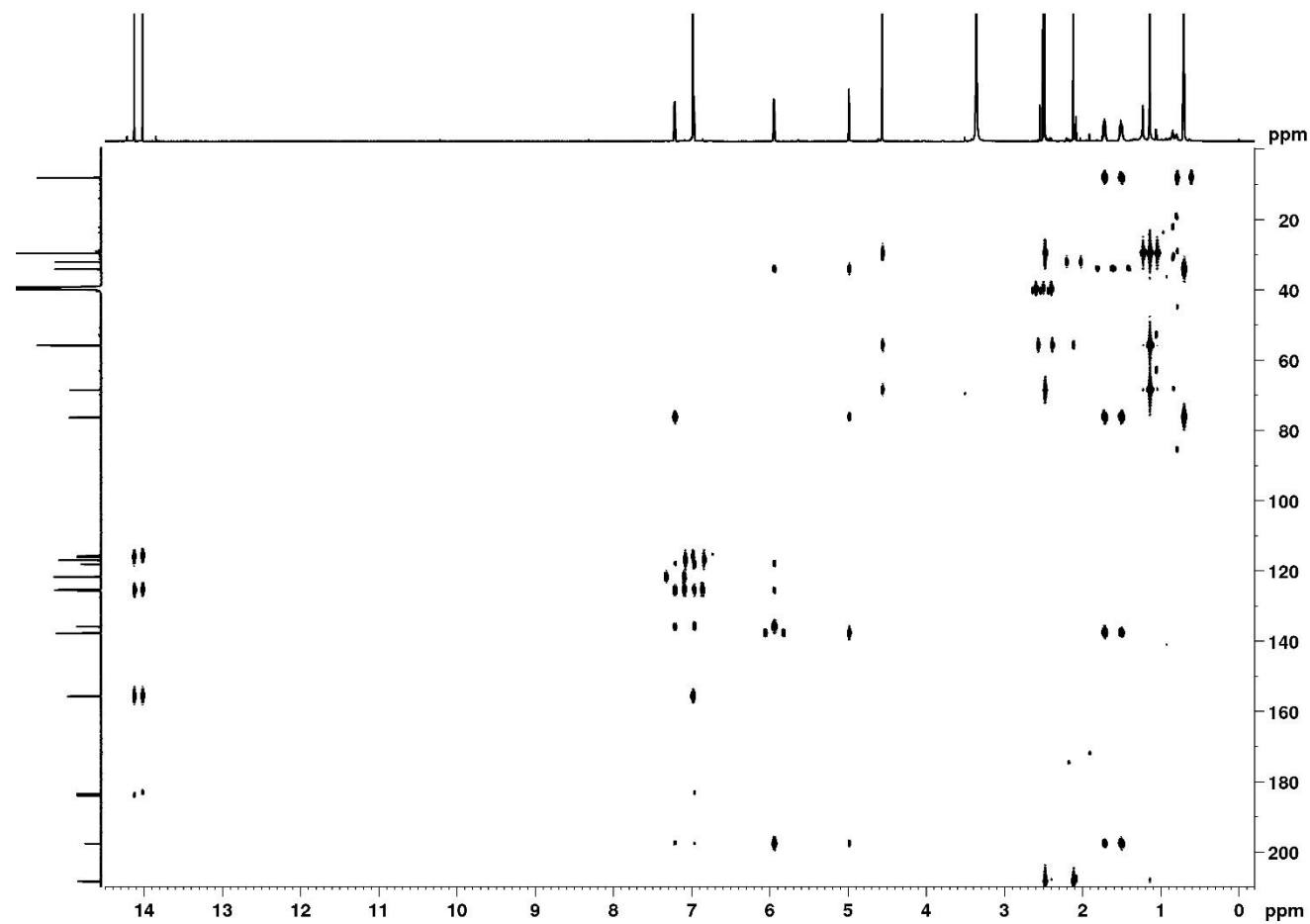
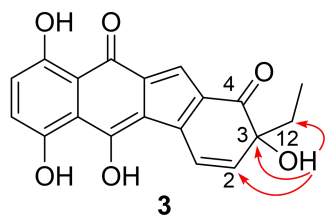


Fig. S4. The spectroscopic data of NEN D (**4**).
(A) The HRESIMS spectrum of NEN D (**4**).

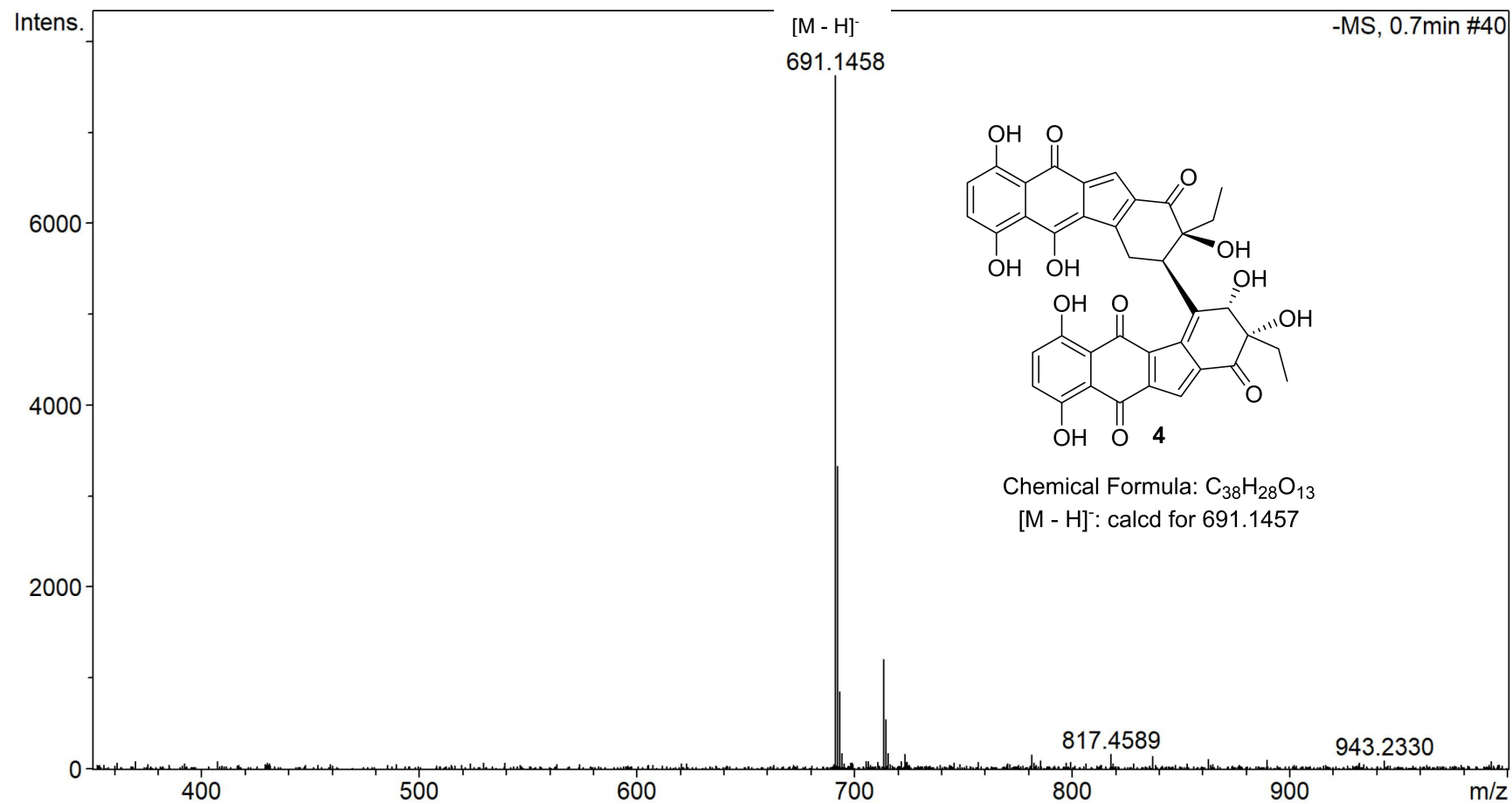


Fig. S4. The spectroscopic data of NEN D (**4**).

(B) The $^1\text{H-NMR}$ spectrum of NEN D (**4**). (700 MHz for ^1H NMR in acetone- d_6).

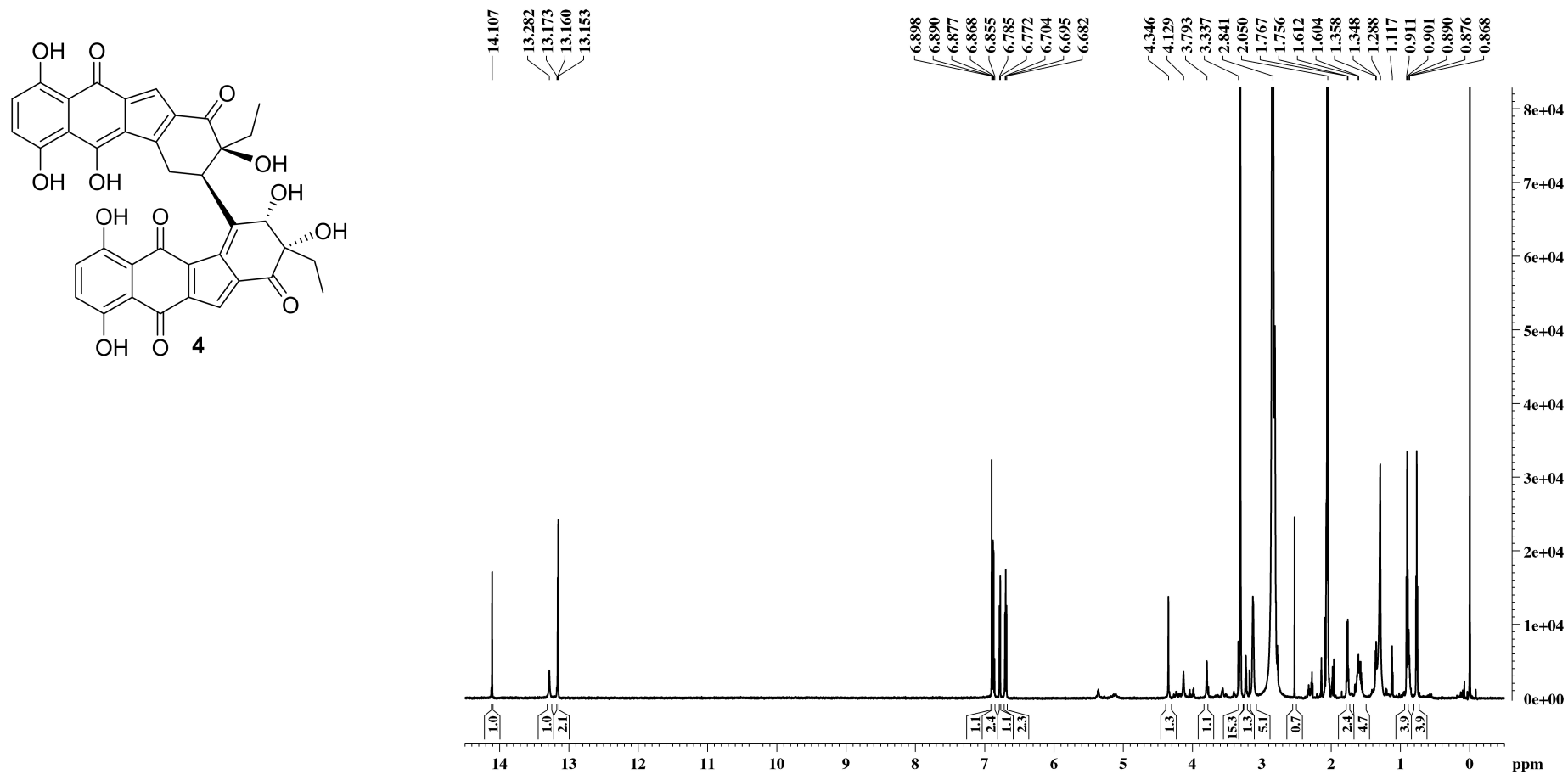


Fig. S4. The spectroscopic data of NEN D (**4**).

(C) The ^{13}C -NMR spectrum of NEN D (**4**). (175 MHz for ^{13}C NMR in acetone- d_6).

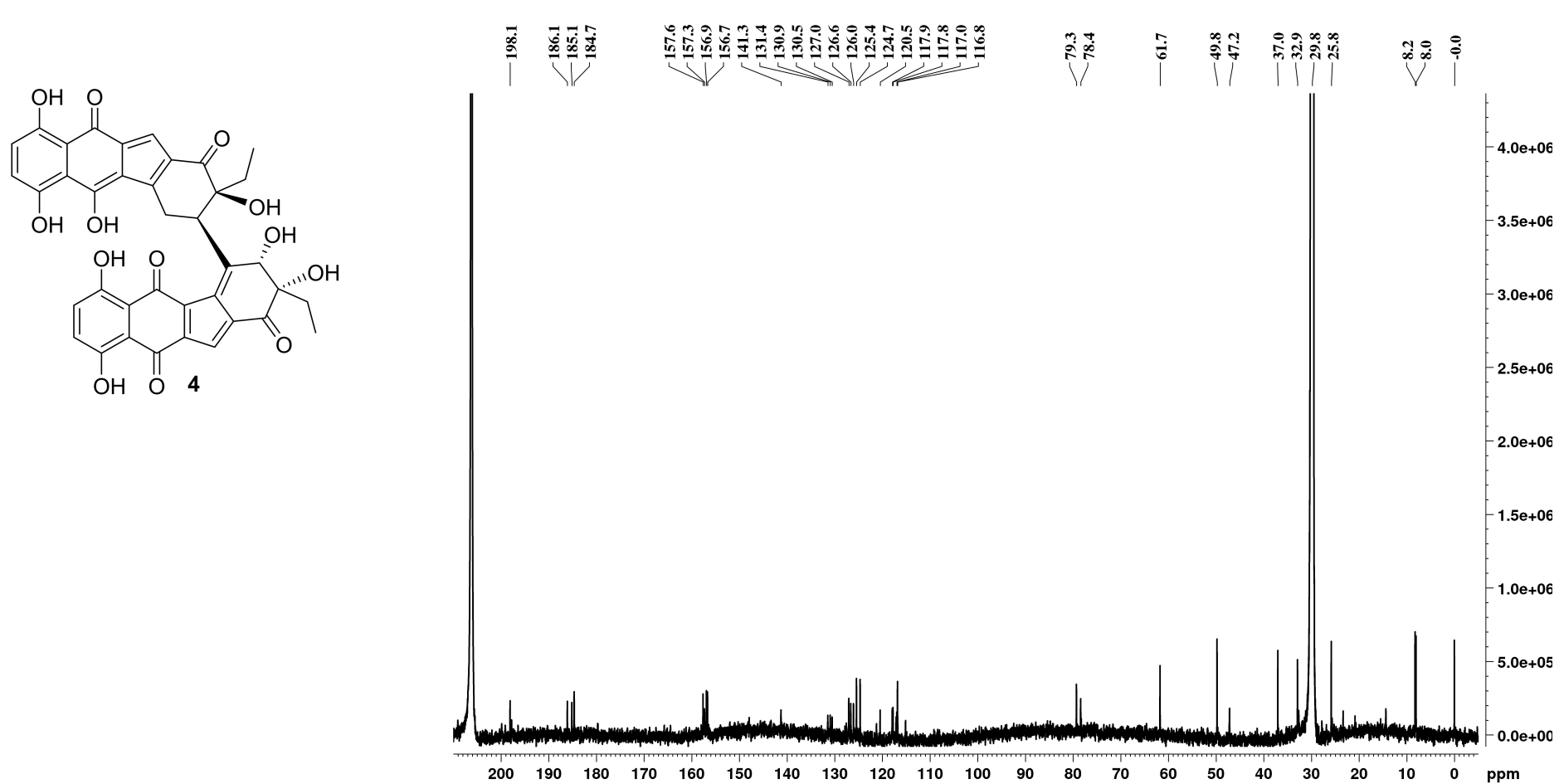


Fig. S4. The spectroscopic data of NEN D (**4**).

(D) The DEPT135 spectrum of NEN D (**4**). (175 MHz for ^{13}C NMR in acetone- d_6)

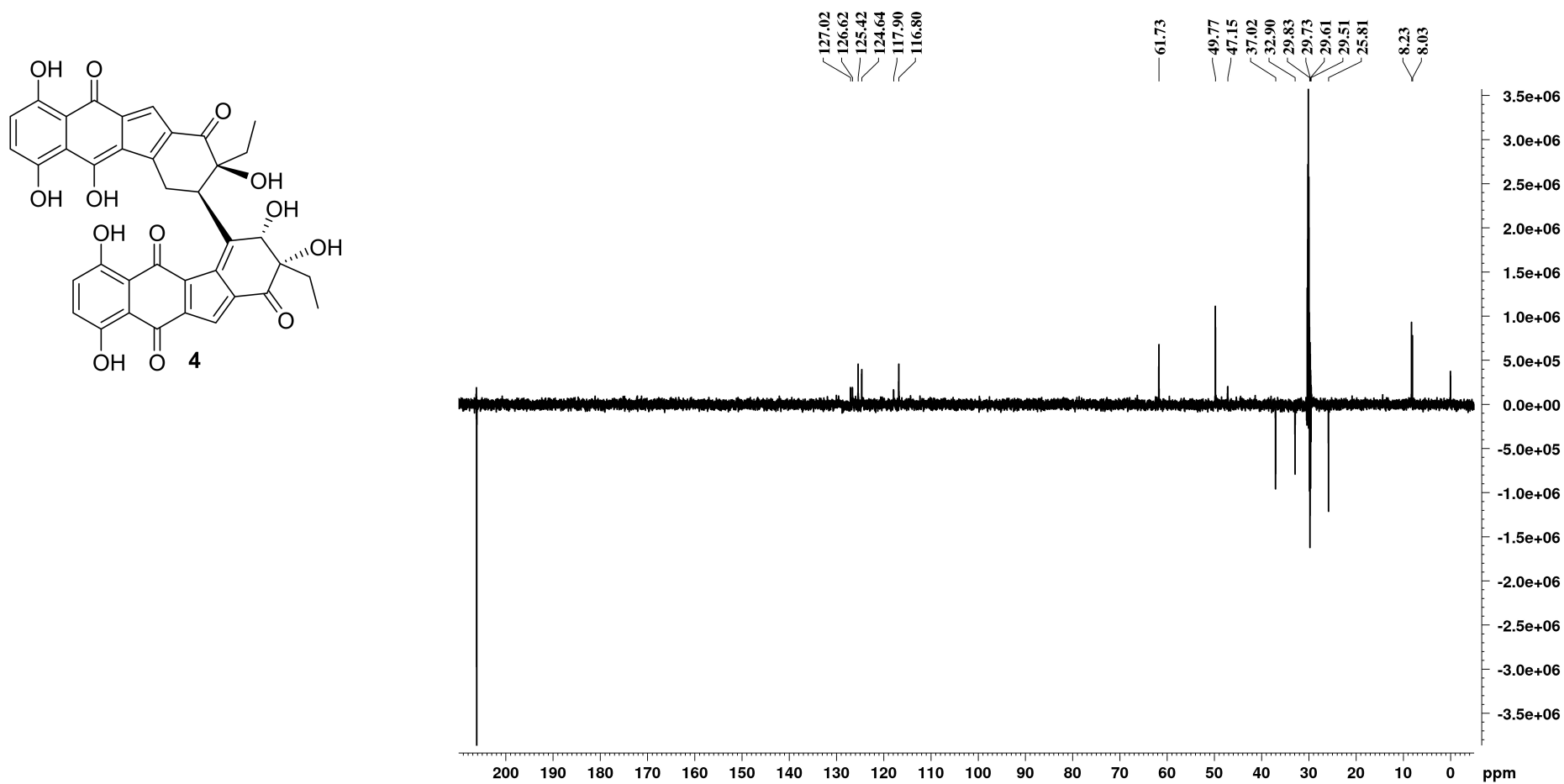


Fig. S4. The spectroscopic data of NEN D (**4**).
(E) The HSQC spectrum of NEN D (**4**).

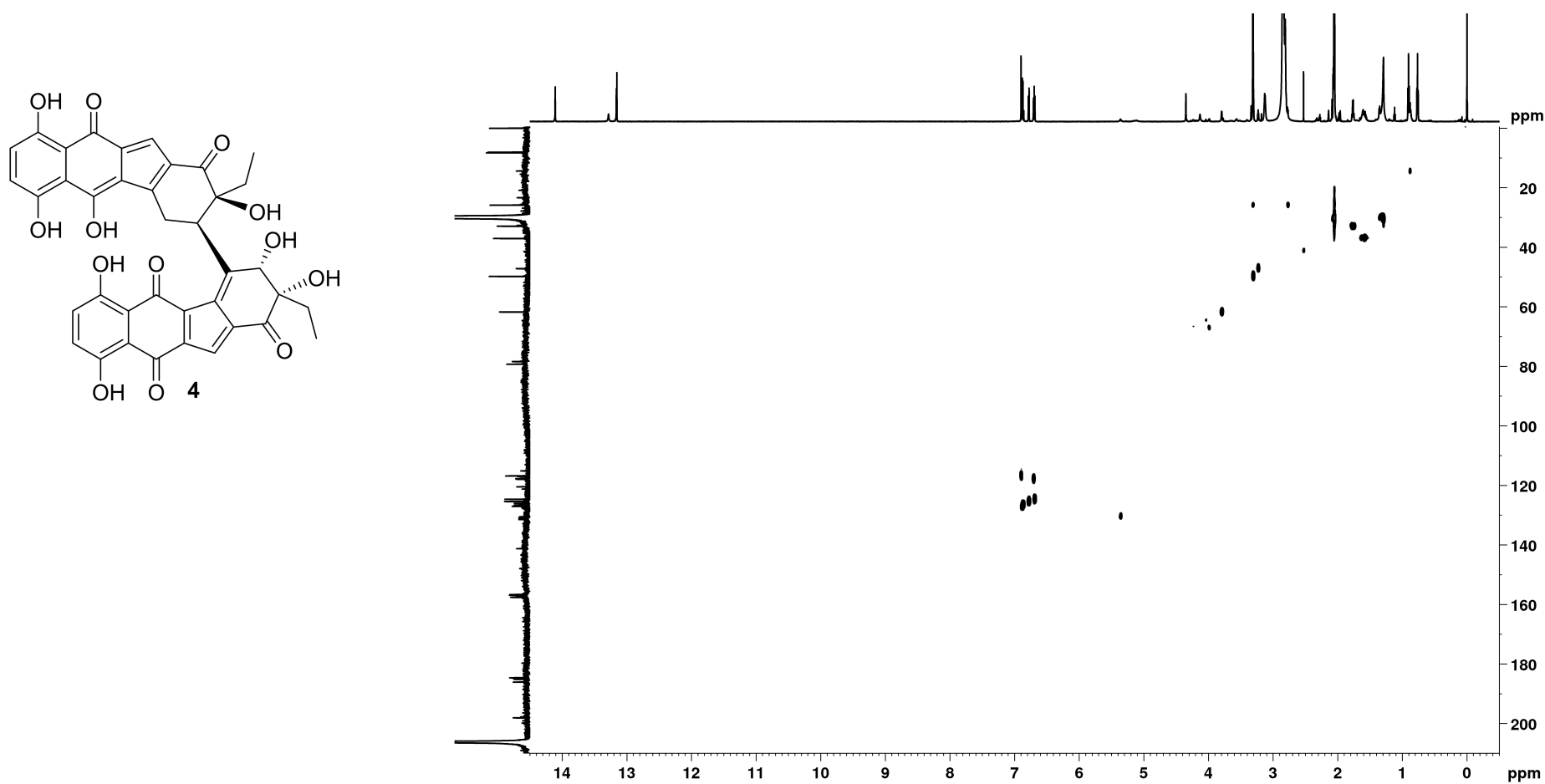


Fig. S4. The spectroscopic data of NEN D (**4**).
(F) The COSY spectrum of NEN D (**4**).

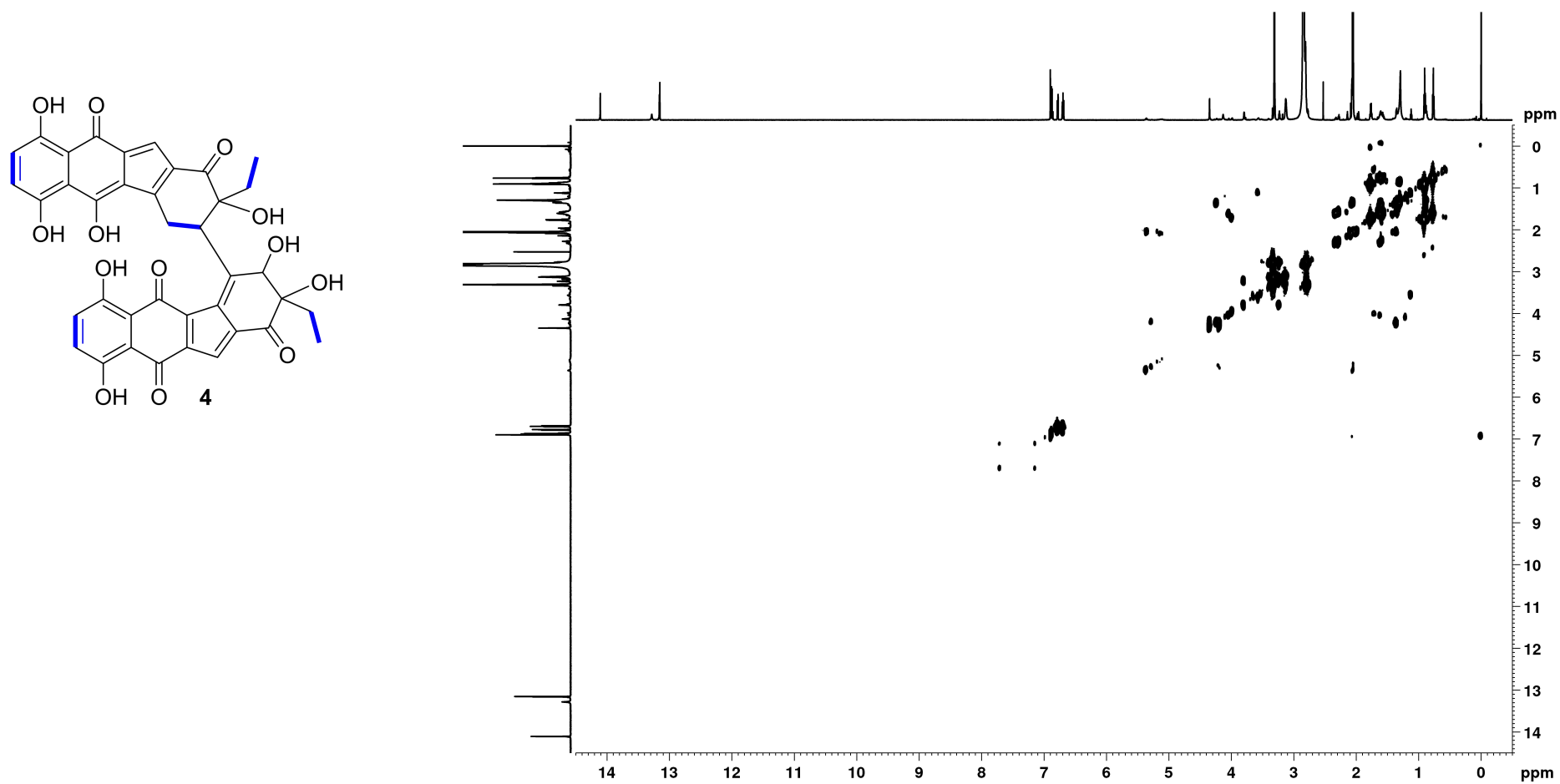


Fig. S4. The spectroscopic data of NEN D (**4**).
(G) The HMBC spectrum of NEN D (**4**).

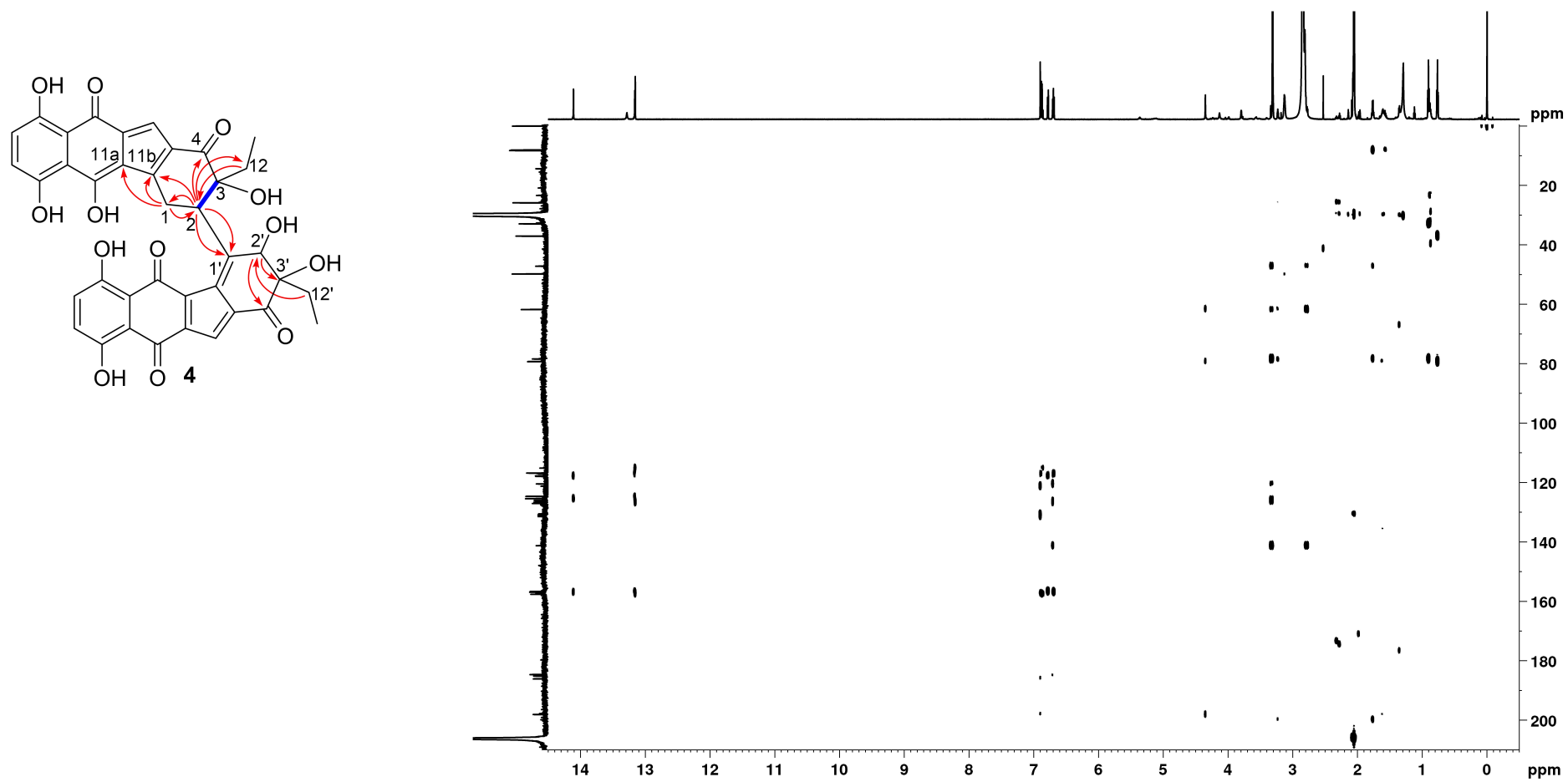


Fig. S4. The spectroscopic data of NEN D (**4**).
(H) The NOESY spectrum of NEN D (**4**).

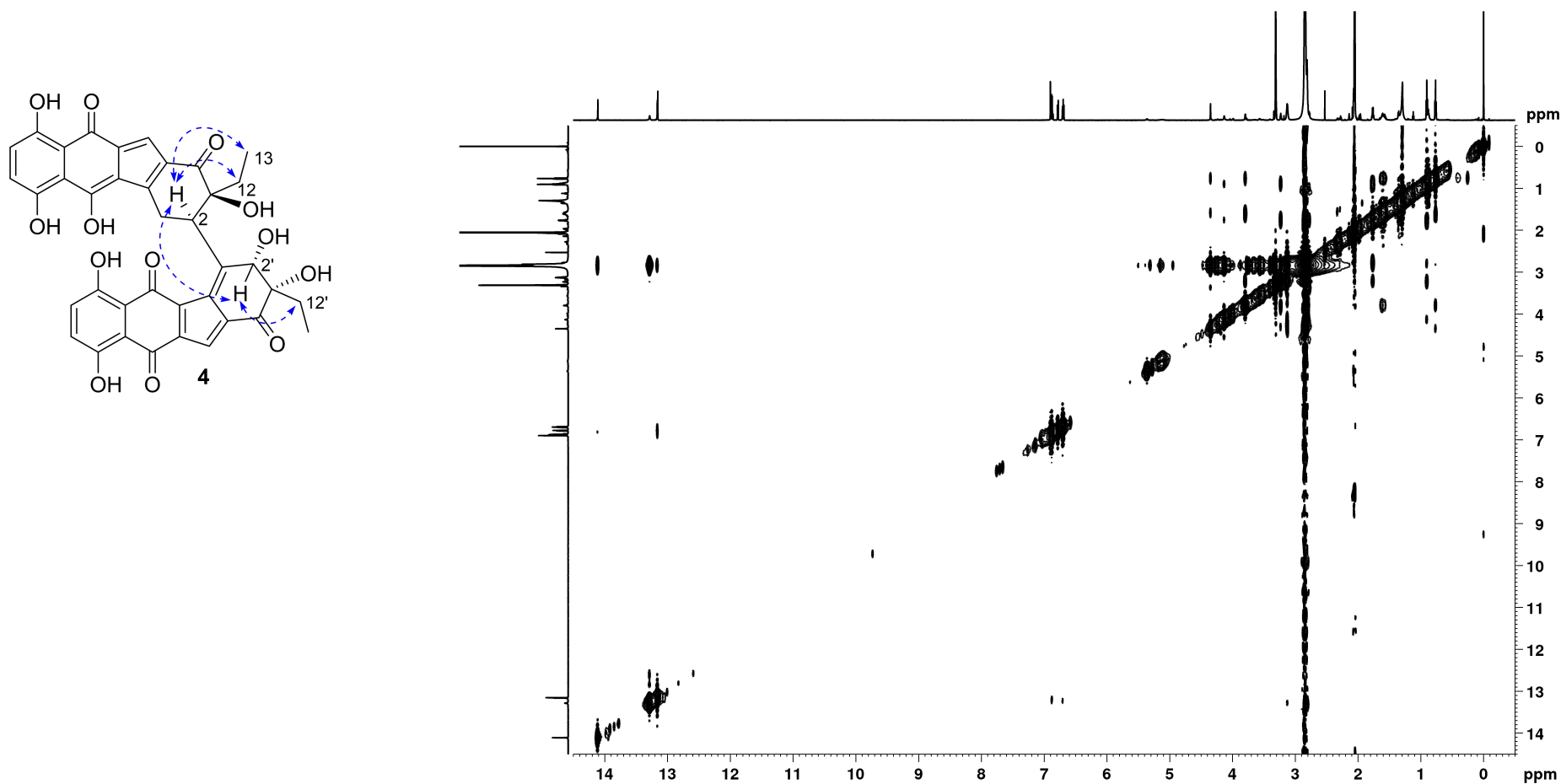
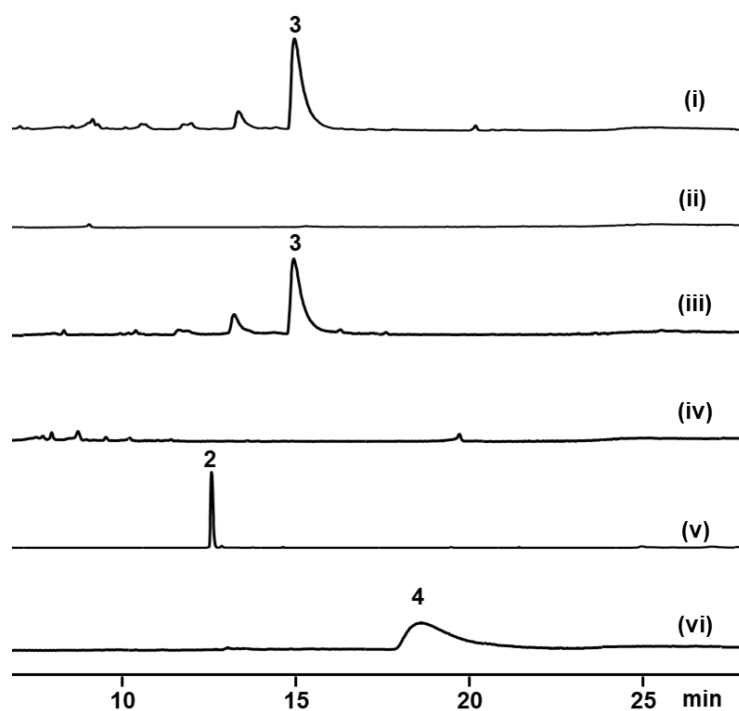


Fig. S5. Feeding experiments in the $\Delta nes53$ mutant.



HPLC analyses of metabolite profile of the $\Delta nes53$ mutant fermented in N4 media supplemented with **3** (final concentration 60 μ M) for 48 h. (i) the supernatants ; (ii) cell sediments; (iii) **3** in N4 medium for 48 h; (iv) the $\Delta nes53$ mutant without supplement of **3**; (v) NEN B (**2**) standard; (vi) NEN D (**4**) standard.

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