Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.
This journal is © The Royal Society of Chemistry 2024

Supporting Information

Non-enzymatic Synthesis of *C*-Methylated Fluostatins: Discovery and Reaction Mechanism

Bidhan Chandra De *et al*

Content

Materials and Methods

1.1. General information

Column chromatography (CC) materials included silica gel (100−200 mesh; 300−400 mesh; Jiangyou Silica gel development, Inc.), Sephadex LH-20 (40−70 μm; Amersham Pharmacia Biotech AB), and YMC*GEL ODS-A (12 nm S-50 μm; YMC Company Ltd.). Deuterated NMR solvents were purchased from Cambridge Isotopes (Andover, MA). Thin-Layer-Chromatography (TLC, 0.1−0.2 mm or 0.3−0.4 mm) was conducted with pre-coated glass plates (silica gel GF254, 10−40 nm, Jiangyou Silica gel development, Inc.). Medium pressure liquid chromatography (MPLC) was performed on automatic flash chromatography (Cheetahtmmp 200, Bonna-Agela Technologies Co., Ltd.) at a wavelength of 304 nm. Semi-preparative HPLC was carried out on a Hitachi HPLC station (Hitachi-L2130) with Diode Array Detector (Hitachi L-2455) using a Agilent Eclipse XDB-C_{18} (250 mm × 9.4 mm, 5 μm; Agilent technology Ltd., USA) or Phenomenex ODS column (250 mm × 10.0 mm, 5 μm; Phenomenex, USA), with a flow rate of 2.5 mL min^{-1}. HR-ESI-MS data were collected using a MaXis 4G UHR-TOFMS spectrometer (Bruker Daltonics Inc.). The optical rotations were recorded on a 341 Polarmeter (PerkinElme, Inc.). CD spectra were measured on a Chirascan circular dichroism spectrometer (Applied Photophysics, Ltd.). ^{1}H, ^{13}C, and 2D NMR spectra were recorded on Bruker AVANCE III HD 700 MHz NMR spectrometer (Bruker Biospin GmbH), with tetramethylsilane (TMS) as an internal standard. Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Sweden).

1.2. General analytical HPLC methods

HPLC analysis of reactions was generally carried out on an Agilent 1260 Infinity series instrument (Agilent Technologies Inc., USA) using a reversed phase C_{18} column (Kinetex® 5μm C18 100 Å, LC Column 150 × 4.6 mm, Phenomenex, USA) or a polar column (Comixsep®, P/N FMG-BPF5EONU, Polar BiPFP 5u, 250 × 4.6 mm, China) with UV detection at 256 or 304 nm under the following program: solvent A, 10% acetonitrile (MeCN) in water supplementing with 0.1% formic acid; solvent B, 90% MeCN in water; 5% B to 80% B (0 to 20 min), 80% B to 100% B (20 to 21 min), 100% B (21 to 24 min), 100% B to 5% B (24 to 25 min), 5% B (25 to 30 min); flow rate at 1 mL min^{-1}.

1.3. Bacterial strains, compounds, and reagents

The strain *Micromonospora rosaria* SCSIO N160 was used for the isolation of FST C (**9**). Chemicals, reagents were purchased from standard commercial sources and used according

to the recommendations of the manufacturer.

1.4. Fermentation and isolation

The wild type of strain *Micromonospora. rosaria* SCSIO N160 were used for the isolation of FST C (**9**).[1, 2] The strain *M. rosaria* SCSIO N160 were regularly propagated on Gauze's Medium No. 1, containing 3% sea salts. A single colony of *M. rosaria* SCSIO N160 was inoculated into liquid production media in a 250 mL Erlenmeyer flask containing 50 mL media in a rotary shaker (200 rpm) at 28 ℃ for 3 days. This seed culture was used to inoculate a 24 liter-scale fermentation using the production media supplemented with polystyrene resin (Amberlite XAD-16; 5% vol, 200 mL) at 28 ℃ for 7 days.[1] The fermentation cultures were centrifuged at 3900 rpm. The mycelia and XAD-16 resins were separated from the liquid portion by filtration through a metal sieve (40 mesh). The resins were washed, transferred to a glass column, and extracted with 6 L acetone. The acetone fractions were concentrated, and the aqueous residue was extracted four times with 2 L butanone. The mycelia cake was sonicated and extracted with 6 L butanone. After drying under vacuum, the crude extracts (20 g) were obtained. The crude extracts were dissolved in 20 mL of CH_3Cl/MeOH (8:2, v/v) and subjected to normal phase silica column (100-200 mesh) chromatography. The elution was performed by four gradient flow of CH_3Cl/MeOH (0:100; 95:5; 90:10 and 0:100) to yield four fractions (Fr.1-4). Fr. 1 was collected and further purified by semi-preparative C_{18} reverse phase Medium Pressure Liquid Chromatography (MPLC) (YMC*GEL ODS-A, 12 nm S-50 μm, 30 × 2.5 cm I.D.), eluting with a linear gradient (CH_3CN/H_2O, 20%-100%, 20 mL min^{-1}, 230 min). Fr.1-2 was further purified by semi-preparative HPLC using Phenomenex C_{18} column (5 μm, 250 mm × 10 mm, 2.5 mL min^{-1}). An isocratic elution gradient of 70% A (H_2O with 0.8% formic acid) /30% B (CH_3CN) with a flow rate of 2.5 mL min^{-1} was applied to afford FST C (**9**) (100 mg). The identity and purity of isolated compounds were verified by comparing the HPLC retention time, UV spectrum, and LC-MS data with those of standard samples.

1.5. Nonenzymatic reactions with FAD/NADH

Substrate FST C (**9**) was dissolved in DMSO before the reaction. A typical reaction mixture, with a total volume 100 μL, included 100 μM of **9**, 100 μM FAD, and 10 mM NADH in 50 mM PBS buffer (pH 7.0) with incubation at 30 ℃ for 2 h. General, reactions were terminated by adding 100 μL of ice-cold MeOH and were analyzed by HPLC using the general analytical HPLC method or subjected to LC-MS analysis.

1.6. Nonenzymatic transformation of **3**

The dry powder of substrate **3** was dissolved in DMSO prior to start the experiment. The standard reaction was set up in 100 μM **3** in 50 mM buffer with pH 7.0 and pH 9 of total volume of 100 μL. The buffers were prepared as following, PBS buffer (50 mM, pH 7); borax/NaOH buffer (50 mM, pH 9). The reaction mixtures were incubated 2 hrs at 30 ℃ for 2 h. Finally, the reactions were quenched by adding 100 μL ice-cold CH_3OH, samples are centrifuged at maximum speed. The supernatants were applied for LC-ESIMS analysis using the general analytical HPLC method. For analytical scale of isotope labeling reactions with DMSO-d6. The substrate **3** dissolved in DMSO-d_6 followed by reaction was set up in a total volume of 100 μL in buffers (pH 7.0 and 9.0). The reaction mixtures were incubated at 30 °C for 2 hrs. The reactions were quenched after addition 100 μL ice cold CH_3OH. The assay supernatants were applied for LC-ESIMS analysis using the general analytical HPLC method.

1.7. Preparation and isolation of **1** and **2**

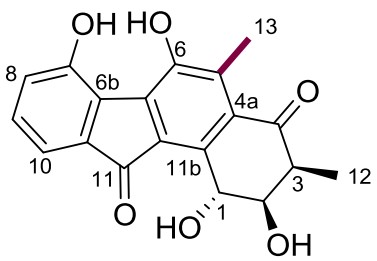
A scaled-up reaction of FST C (**9**) was resuspended in DMSO. A total volume of 5 L was performed in 50 mM PBS buffer (pH 7.0) containing 100 μM 9, 10 mM NADH and 100 μM FAD at 30 °C for 2 h with occasional shaking. The reaction was quenched by the addition of an equal volume of ice-cold butanone and centrifuged at 4000 rpm for 20 min at 4 ℃. The reaction mixtures were then extracted with an equal volume of butanone three times and the solvents were removed under vacuum on an ice bath. The crude extracts were dissolved in 1.5 mL MeOH and subjected to semi-preparative HPLC using an Agilent Eclipse XDB-C_{18} column (250 mm × 9.4 mm, 5 μm; Agilent technology Ltd., USA) with an isocratic elution gradient of 70 % A (H_2O with 0.8% formic acid) and 30% B (MeCN) at a flow rate of 2.5 mL min^{-1}. In this way, compounds **1** (2.5 mg), **2** (2.8mg).

Table S1. The ^{1}H and ^{13}C NMR data of **1** and **2** in DMSO-d_6.

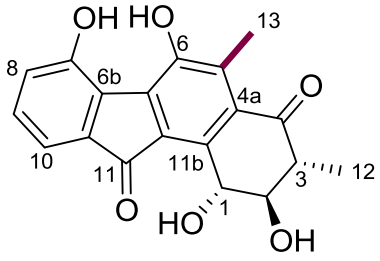
.

No	**1**		**2**	
	δ_H, mult. (J in H_Z)	δ_C, type	δ_H, mult. (J in H_Z)	δ_C, type
1	5.33, br s	65.3, CH	5.06, d (5.9)	69.5, CH
2	3.89, br s	76.1, CH	3.56, dd (7.6, 5.8)	75.2, CH
3	3.11, dq (6.8, 2.1)	43.5, CH	2.50, overlapping	49.8, CH
4		201.1, C		201.8, C
4a		131.2, C		131.5, C
5		135.4, C		135.2, C
6		157.2, C		157.5, C
6a		130.9, C		n
6b		130.3, C		130.2, C
7		160.5, C		160.4, C
8	6.48, d (8.12)	125.4, CH	6.52, br d (8.1)	126.1, CH
9	6.84, dd (7.9, 7.0)	129.9, CH	6.85, dd (8.1, 7.1)	130.1, CH
10	6.57, d (7.0)	110.3, CH	6.62, dd (7.0, 0.7)	111.1, CH
10a		135.6, C		135.6, C
11		190.6, C		197.7, C
11a		135.2, C		132.3, C
11b		128.4, C		128.0, C
12	1.07, d (6.9)	12.5, CH_3	1.15, d (5.74)	14.0, CH_3
13	2.29, s	15.1, CH_3	2.24, s	14.7, CH_3
1-OH	4.92, br s			
2-OH	5.02, br s			

Data were recorded on a Bruker Avance 700 MHz NMR spectrometer in DMSO-d_6 with TMS as an internal standard. n no signal.



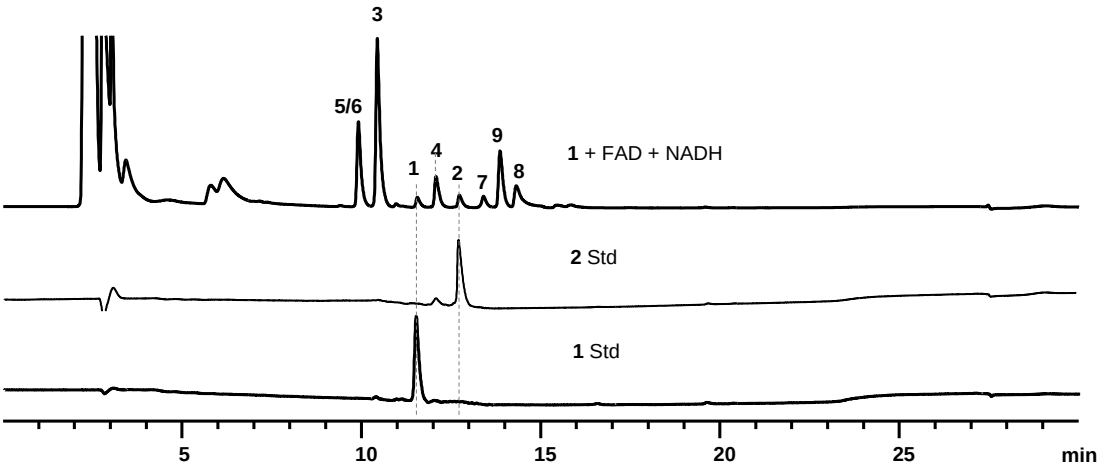
1



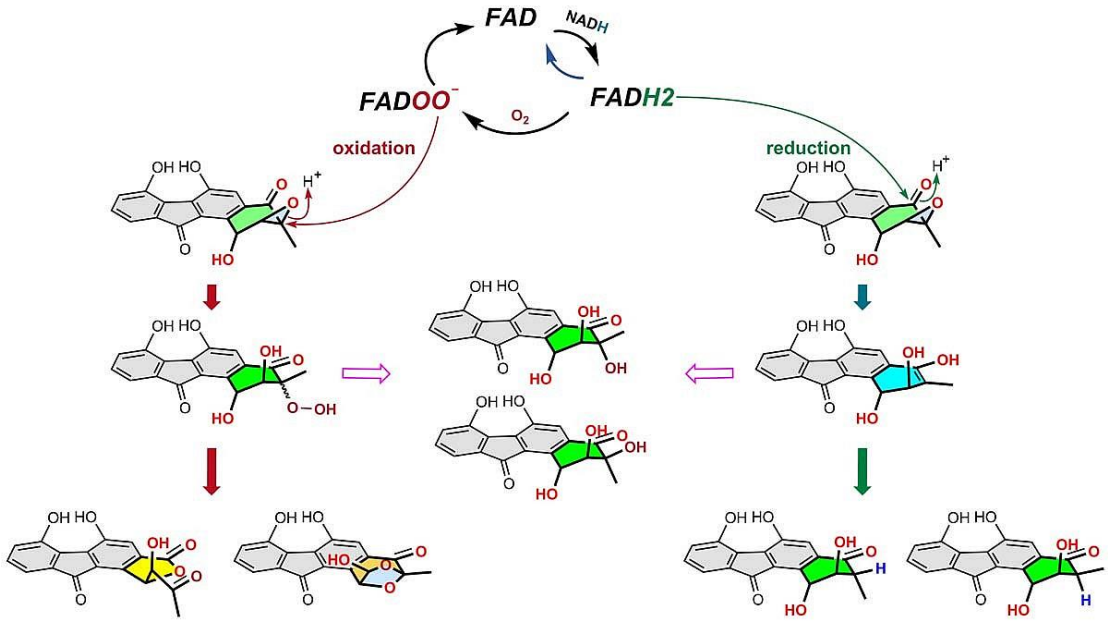
2

Fig. S1. HPLC analysis of reactions with FST C (**9**) and proposed oxidative epoxide ring opening reactions mechanism.[3]

(A)



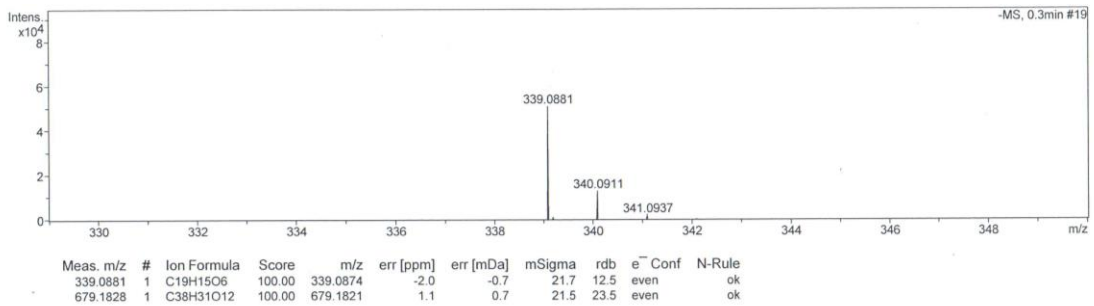
(B)



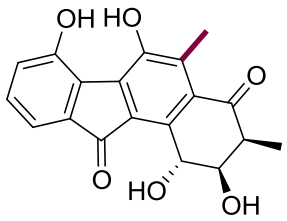
(A) HPLC analysis of reactions of 100 μM **9**, and 10 mM NADH in 50 mM PBS buffer (pH 7) for 2 h at 30 °C. (i) Complete reaction; (ii) **2** standard; (iii) **1** standard; (B) Proposed oxidative epoxide ring opening reactions mechanism.

Fig. S2. Spectroscopic data for FST B3 (**1)**.

(A) HRESIMS.



Meas. m/z	#	Ion Formula	Score	m/z	err [ppm]	err [mDa]	mSigma	rdb	e¯ Conf	N-Rule
339.0881	1	C19H15O6	100.00	339.0874	-2.0	-0.7	21.7	12.5	even	ok
679.1828	1	C38H31O12	100.00	679.1821	1.1	0.7	21.5	23.5	even	ok



1

Fig. S2. Spectroscopic data for FST B3 (**1**).

(B) The ^{1}H NMR spectrum of **1** in DMSO-d_6.

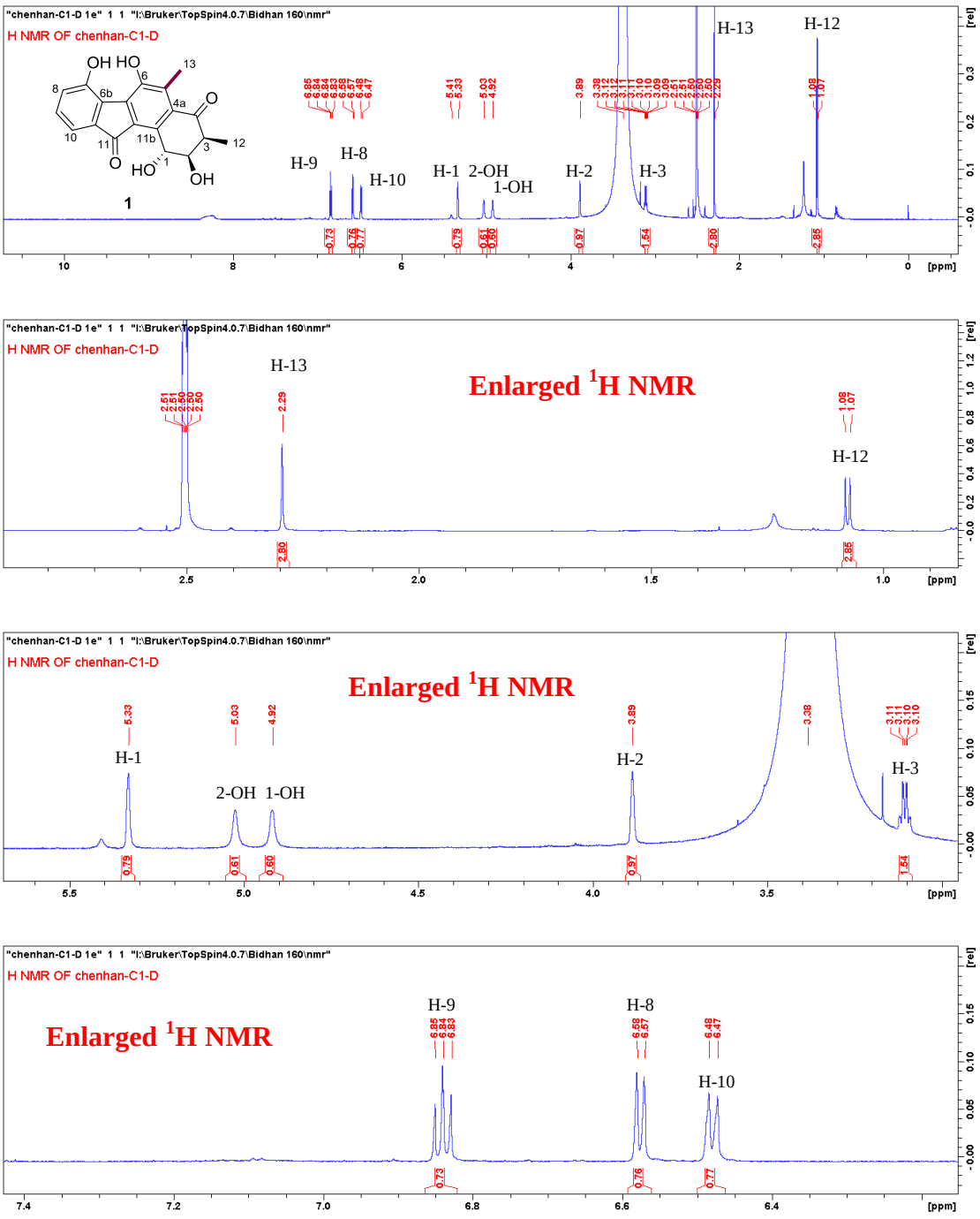


Fig. S2. Spectroscopic data for FST B3 (**1**).

(C) The ^{13}C NMR and DEPT 135 spectrum of **1** in DMSO-d_6.

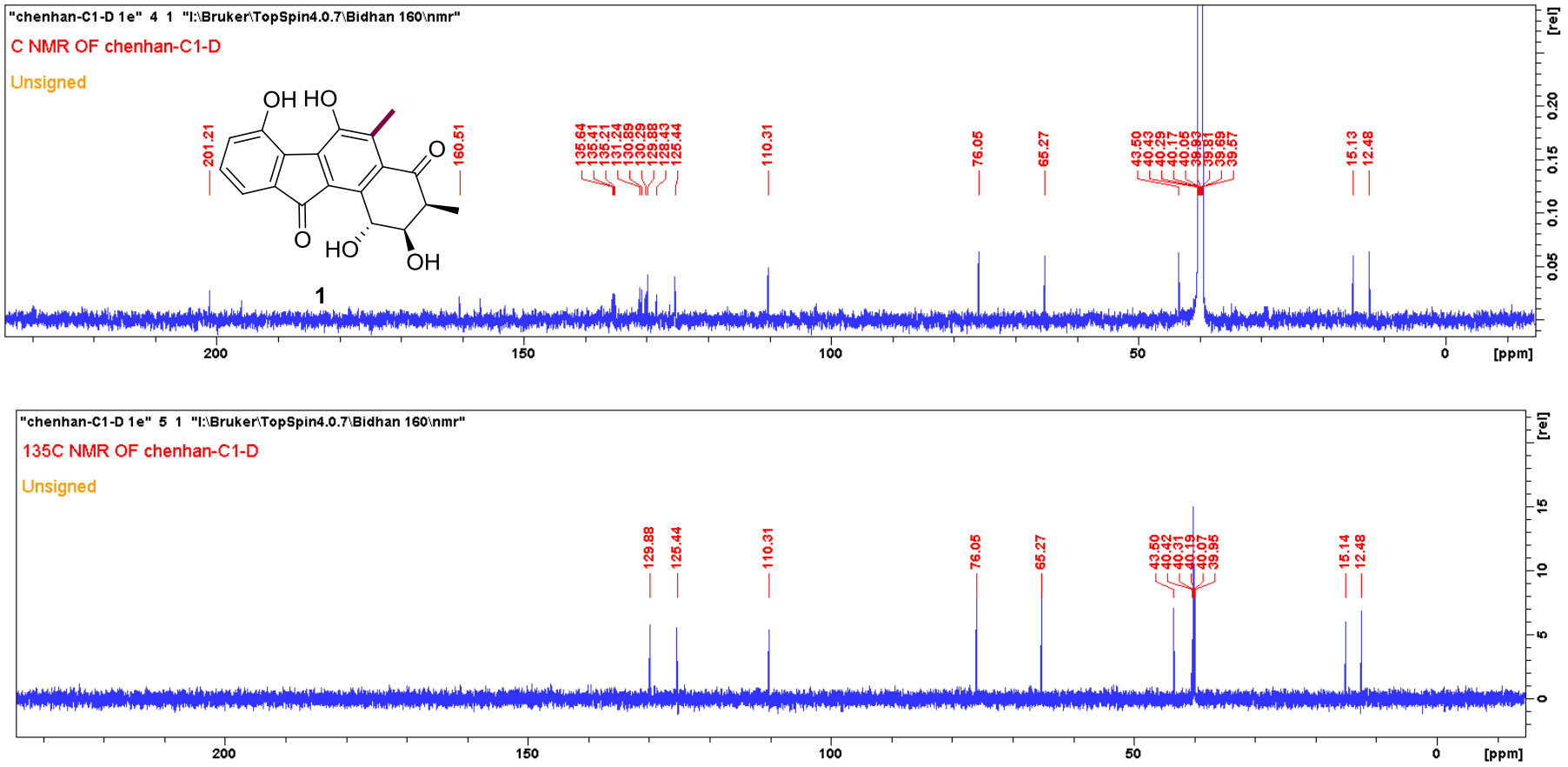


Fig. S2. Spectroscopic data for FST B3 (**1**).

(D) The HSQC spectrum of **1** in DMSO-d_6.

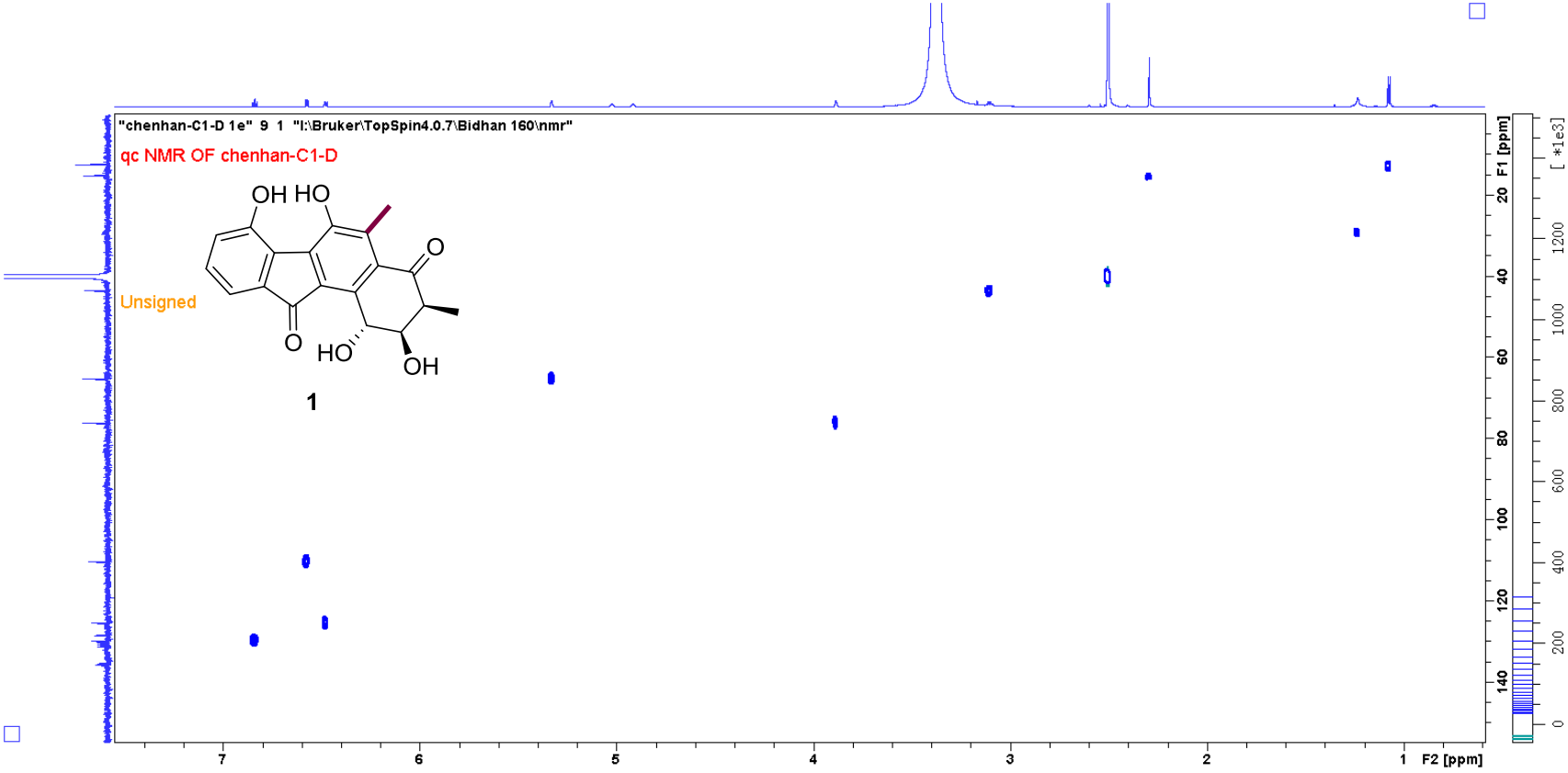


Fig. S2. Spectroscopic data for FST B3 (**1**).

(E) The COSY spectrum of **1** in DMSO-d_6.

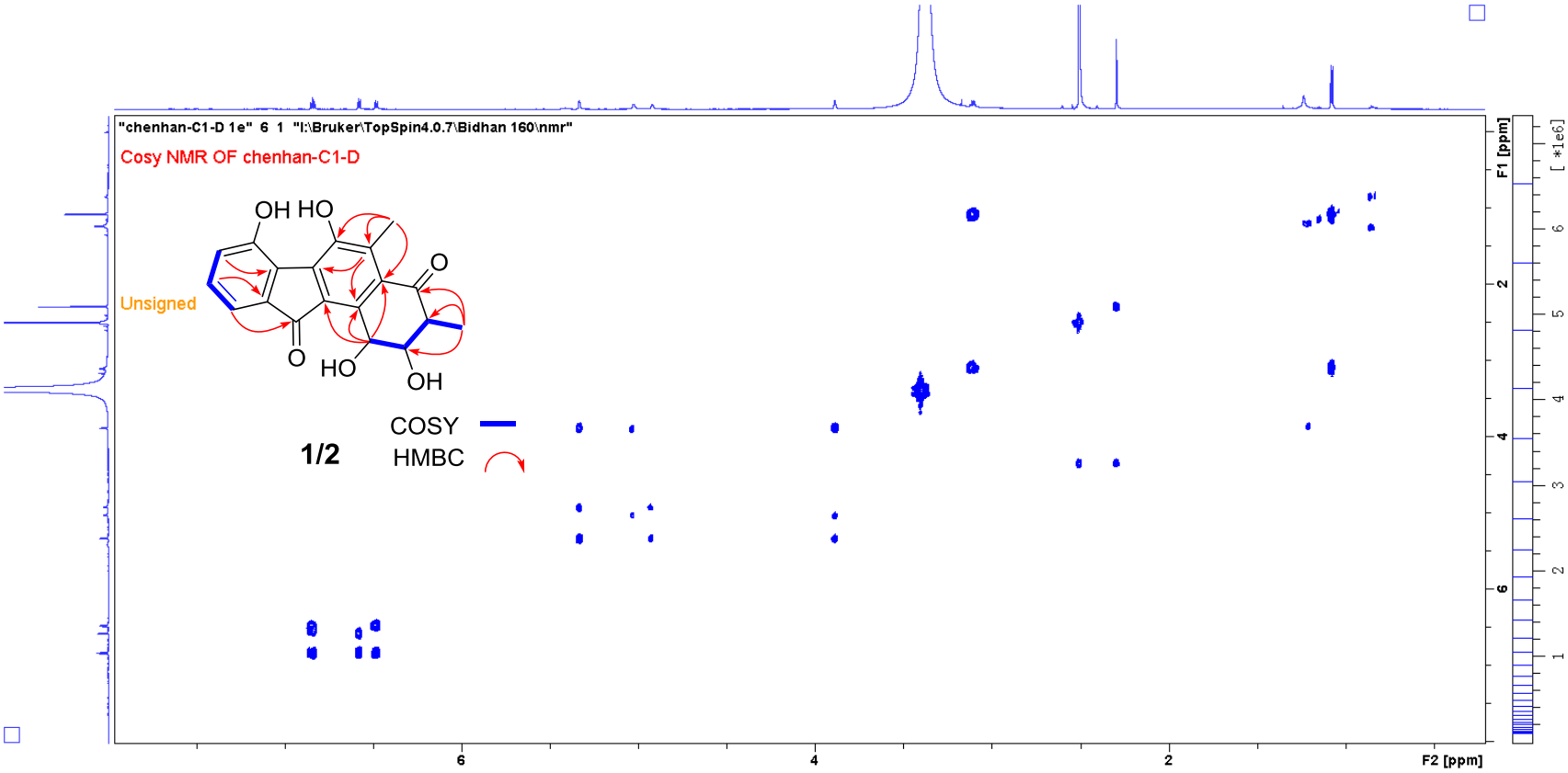


Fig. S2. Spectroscopic data for FST B3 (**1**).

(F) The HMBC spectrum of **1** in DMSO-d_6.

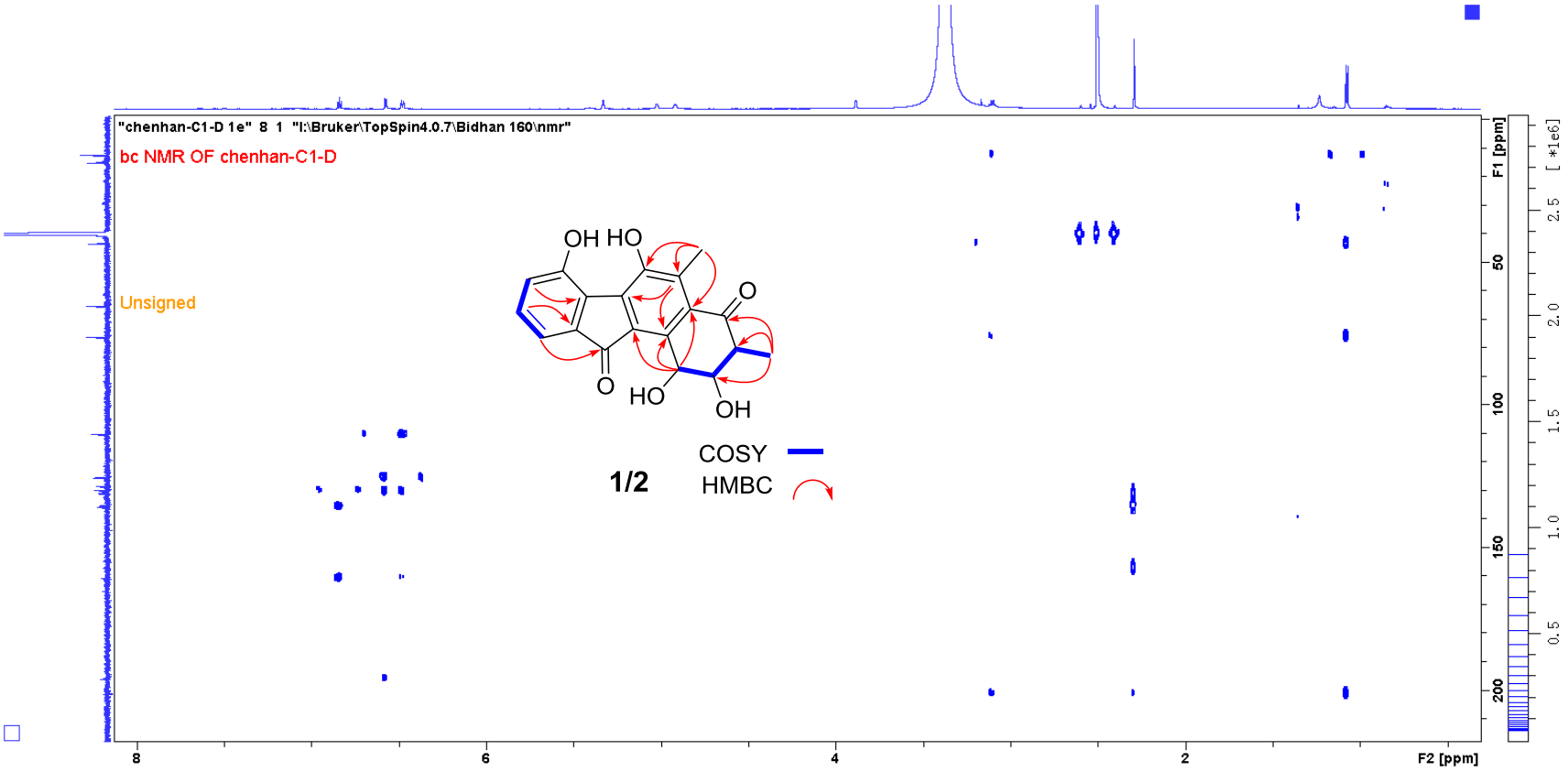


Fig. S2. Spectroscopic data for FST B3 (**1**).

(G) The NOESY spectrum of **1** in DMSO-d_6.

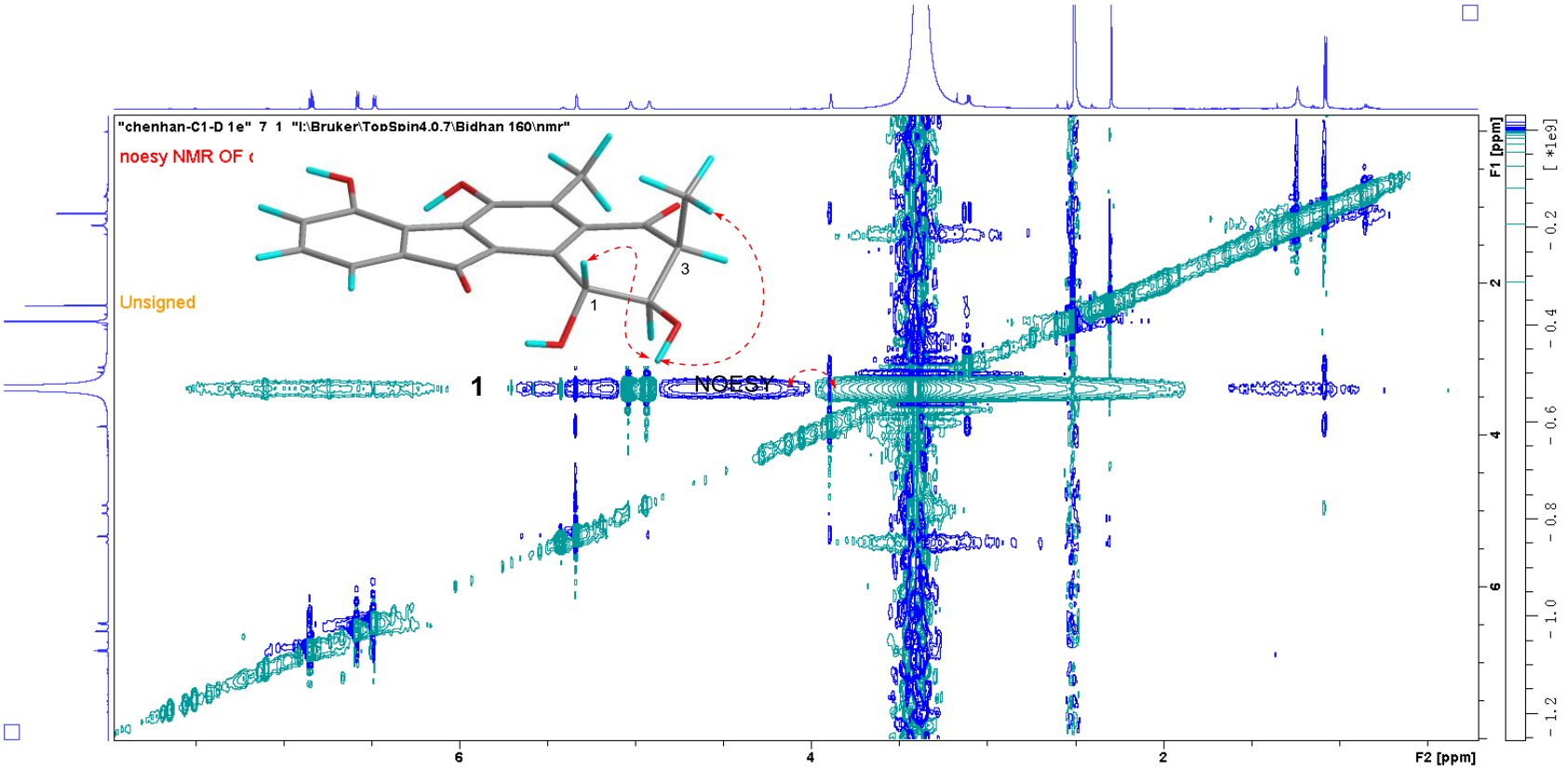
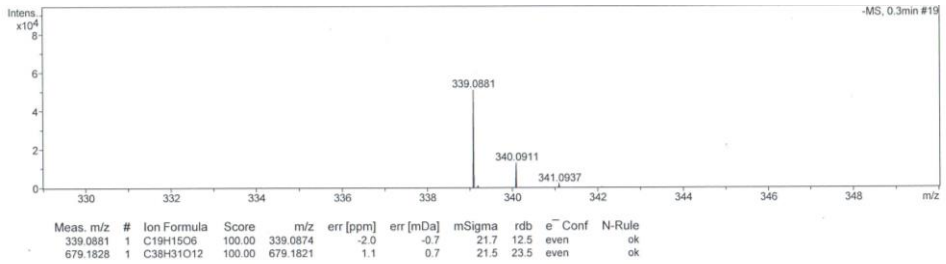
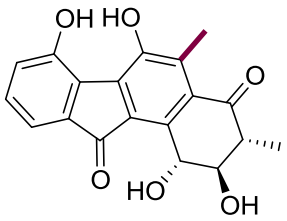


Fig. S3. Spectroscopic data for FST B4 (**2**).

(A) HRESIMS.



Meas. m/z	#	Ion Formula	Score	m/z	err [ppm]	err [mDa]	mSigma	rdb	e⁻ Conf	N-Rule
339.0881	1	C19H15O6	100.00	339.0874	-2.0	-0.7	21.7	12.5	even	ok
679.1828	1	C38H31O12	100.00	679.1821	1.1	0.7	21.5	23.5	even	ok



2

Fig. S3. Spectroscopic data for FST B4 (**2**).

(B) The ^{1}H NMR spectrum of **2** in DMSO-d_6.

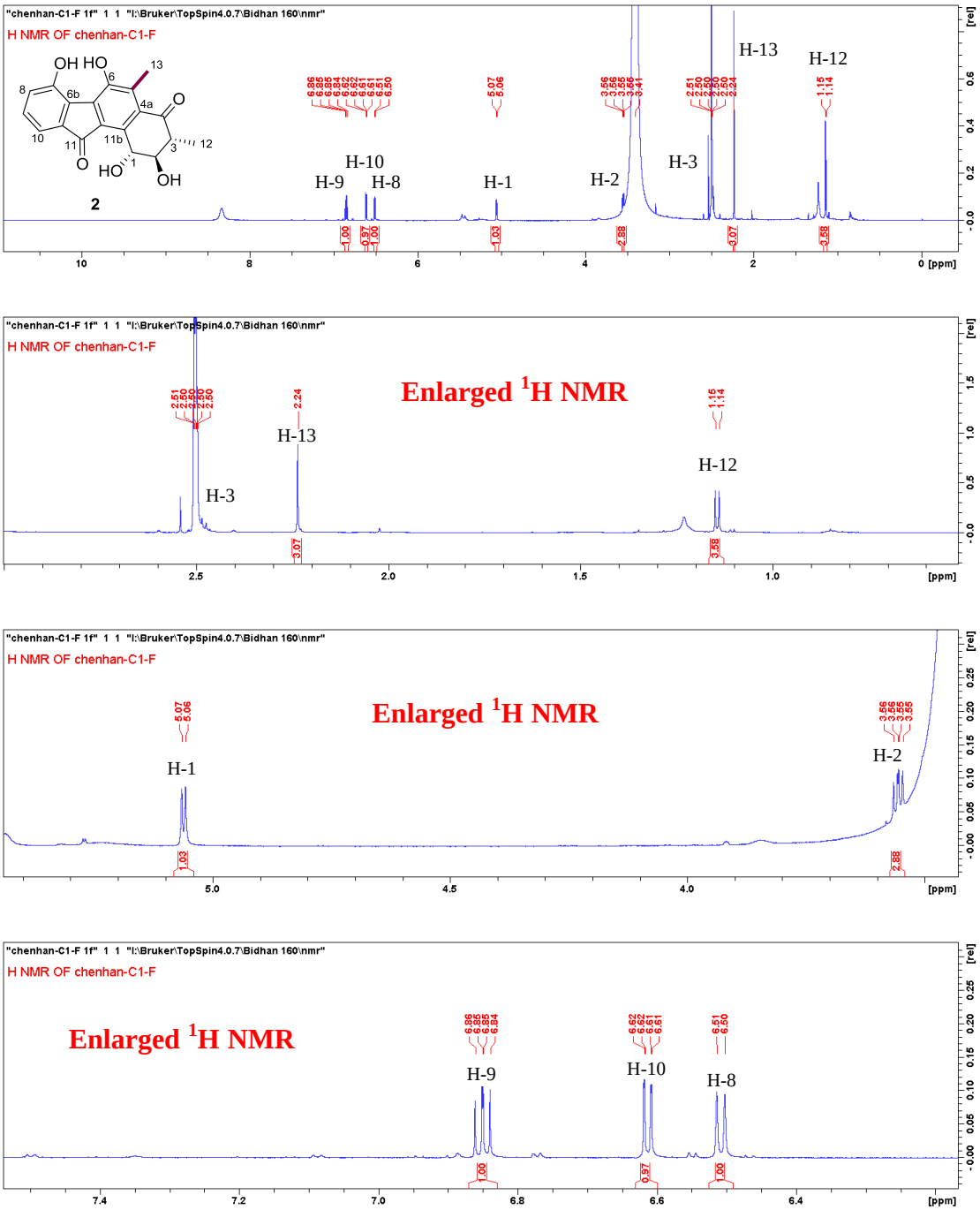


Fig. S3. Spectroscopic data for FST B4 (**2**).

(C) The ^{13}C NMR spectrum of **2** in DMSO-d_6.

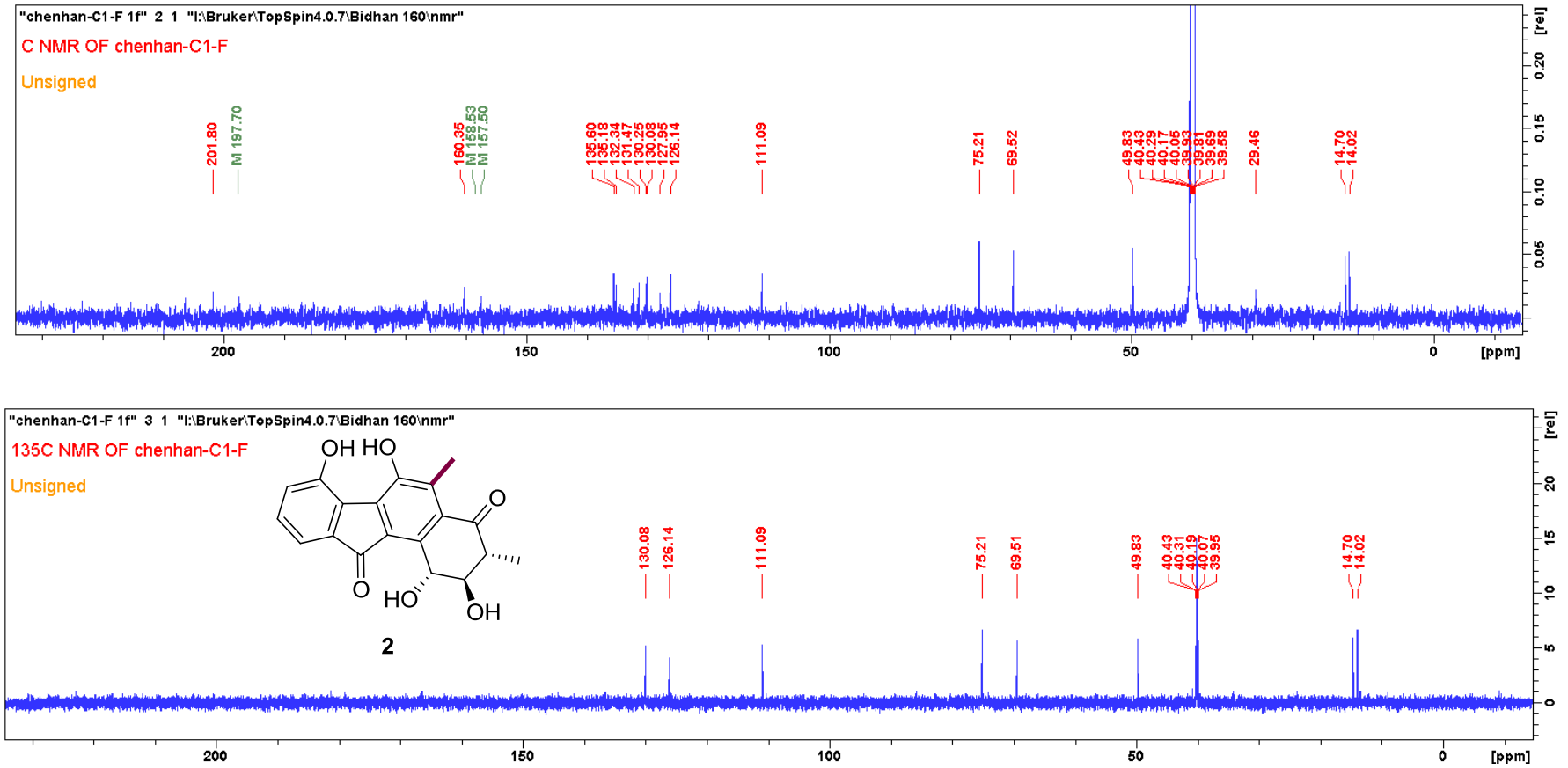


Fig. S3. Spectroscopic data for FST B4 (**2**).

(D) The HSQC spectrum of **2** in DMSO-d_6.

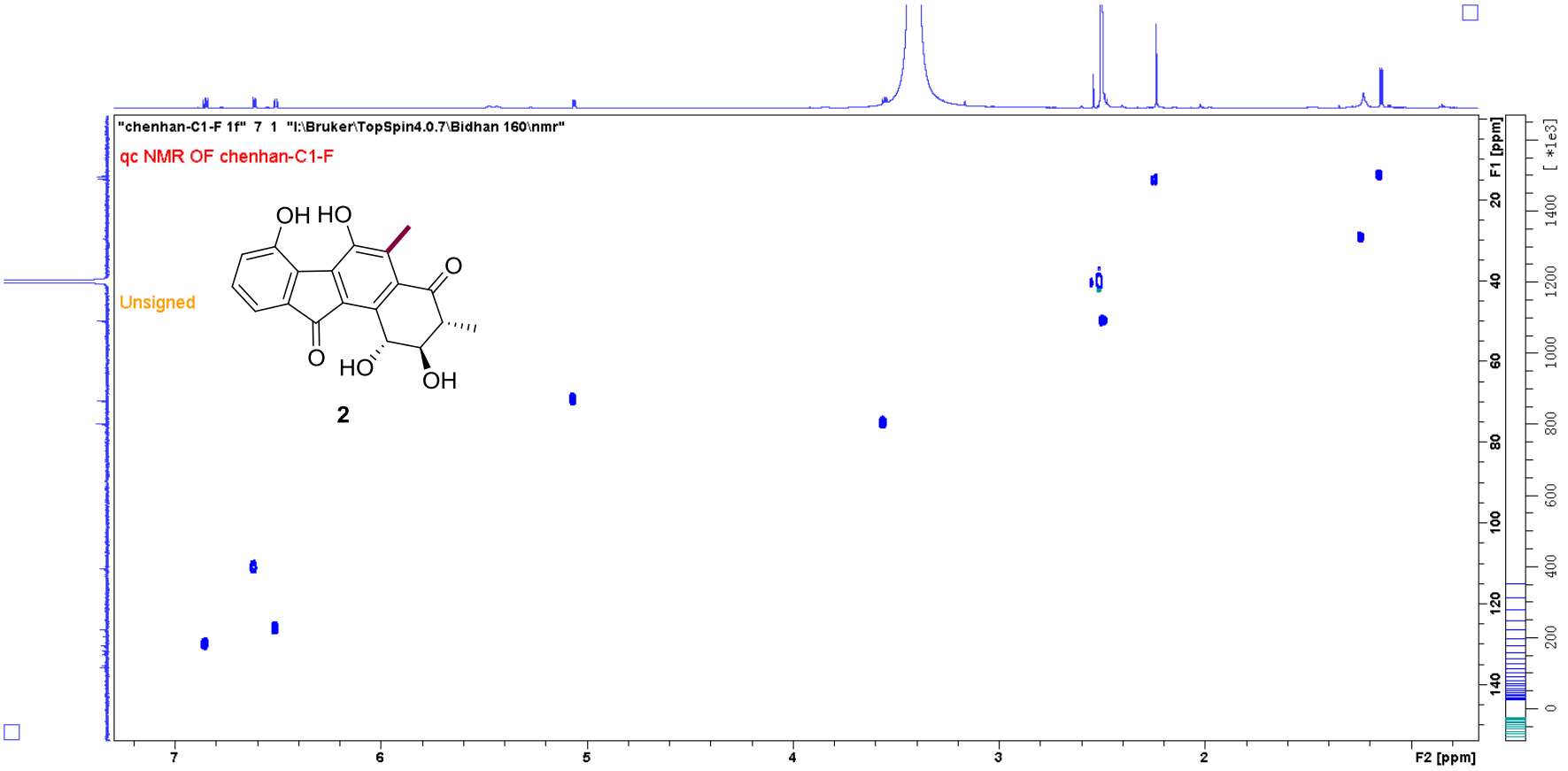


Fig. S3. Spectroscopic data for FST B4 (**2**).

(E) The COSY spectrum of **2** in DMSO-d_6.

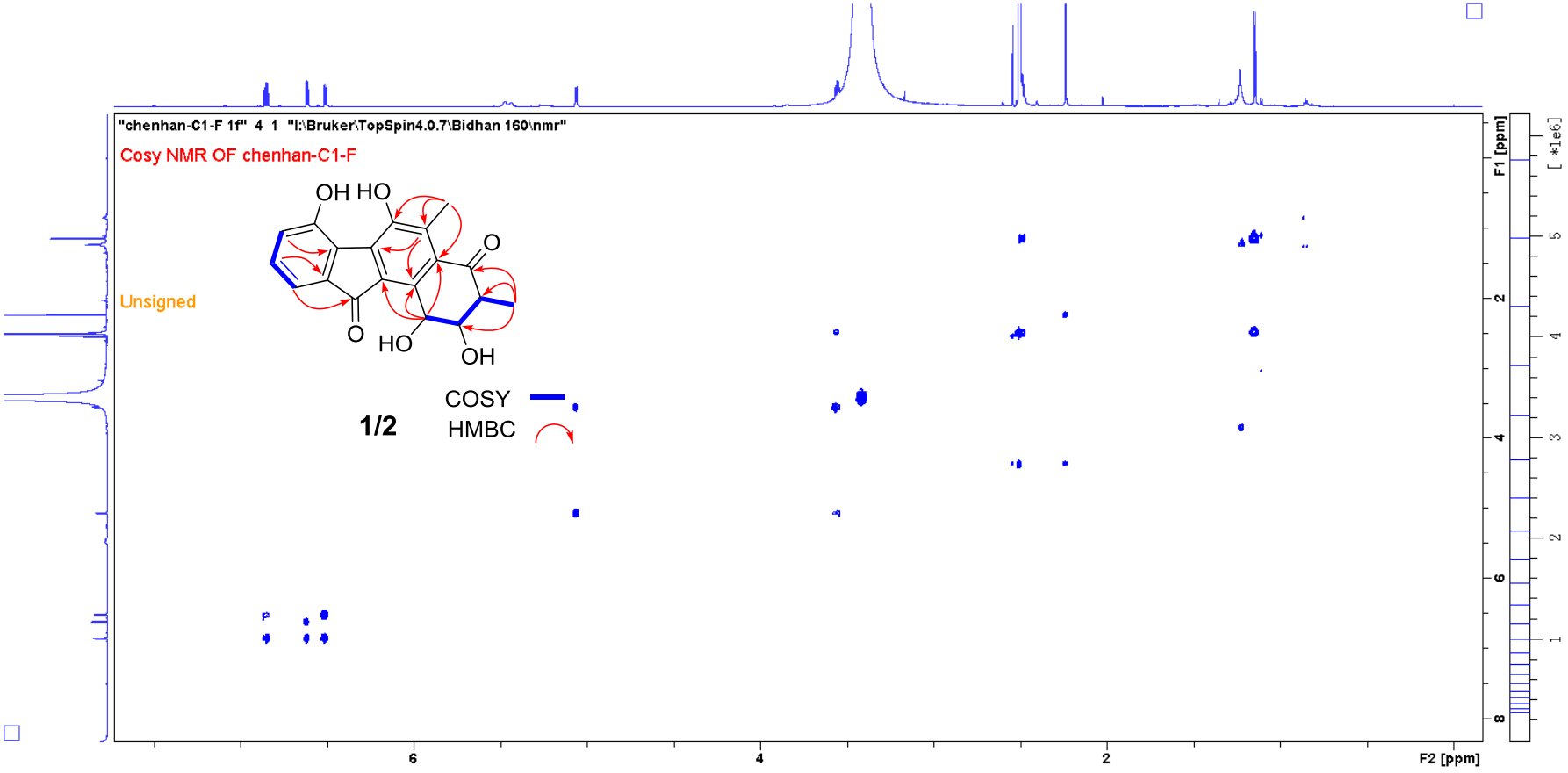


Fig. S3. Spectroscopic data for FST B4 (**2**).

(F) The HMBC spectrum of **2** in DMSO-d_6.

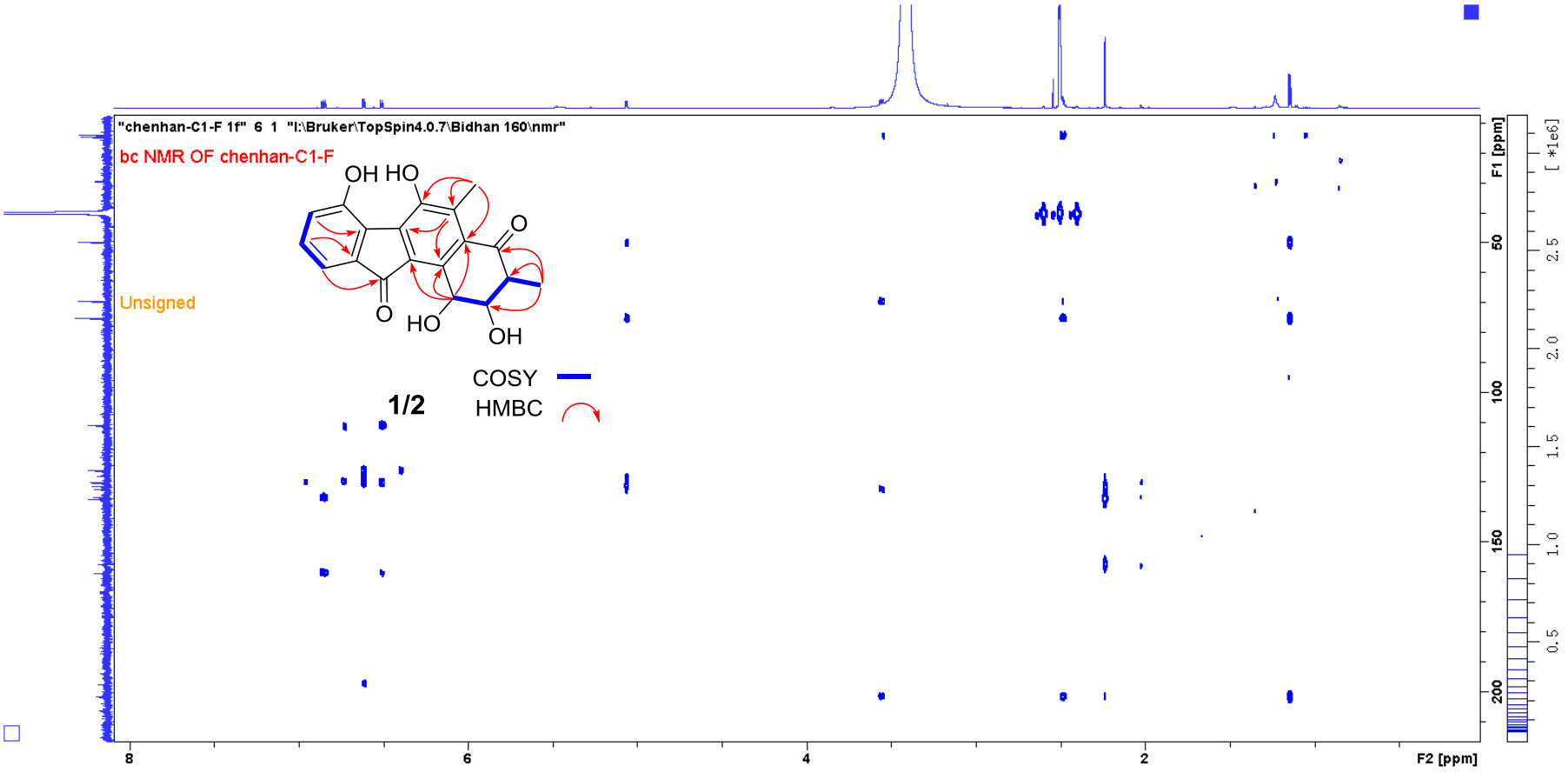


Fig. S3. Spectroscopic data for FST B4 (**2**).

(G) The NOESY spectrum of **2** in DMSO-d_6.

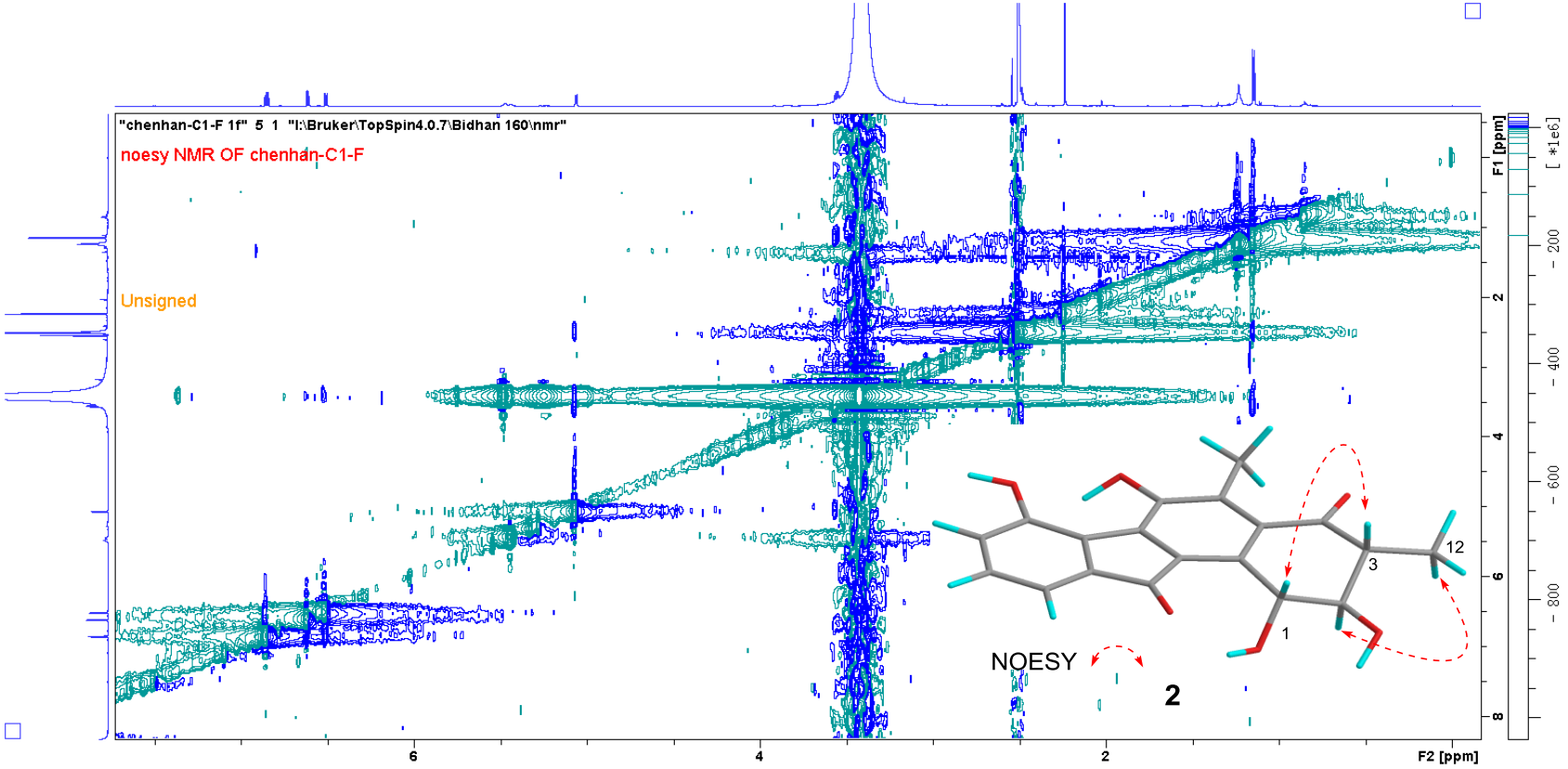
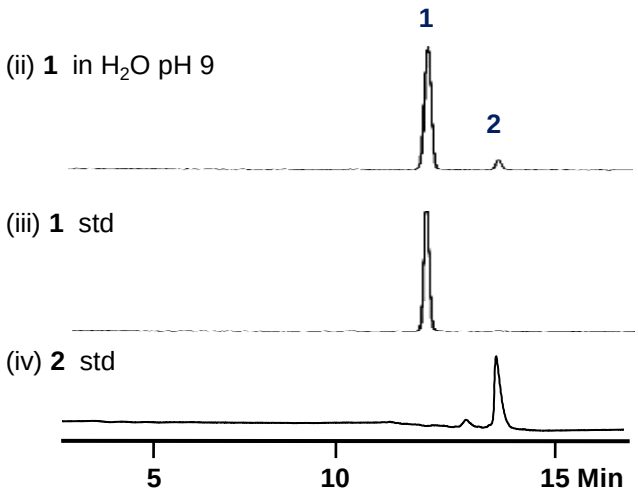


Fig. S4. HPLC analysis of reactions involving **1** converting to **2**.



HPLC analysis of reactions involving **1** converting to **2**. (i) **1** in H_2O at pH 9; (ii) **1** std; (iii) **2** std; at 30 ℃ for 30 min. HPLC analysis was run with UV detection at 304 nm using a reversed phase C_{18} column.

References

1. W. Zhang, Z. Liu, S. Li, Y. Lu, Y. Chen, H. Zhang, G. Zhang, Y. Zhu, G. Zhang, W. Zhang, J. Liu and C. Zhang, *J. Nat. Prod.*, 2012, **75**, 1937-1943.
2. W. Zhang, C. Yang, C. Huang, L. Zhang, H. Zhang, Q. Zhang, C. S. Yuan, Y. Zhu and C. Zhang, *Org. Lett.*, 2017, **19**, 592-595.
3. B. C. De, W. Zhang, C. Yang, A. Mándi, C. Huang, L. Zhang, W. Liu, M. W. Ruszczycky, Y. Zhu, M. Ma, G. Bashiri, T. Kurtán, H.-w. Liu and C. Zhang, *Nat. Commun.*, 2022, **13**, 4896.