Supplementary Information

Mutational Biosynthesis to Generate Novel Analogs of Nosiheptide Featuring Fluorinated Indolic Acid Moiety

E Zhang, [a] Heng Guo, [b] Dandan Chen, [b] Qian Yang, [b] Yafei Fan, [a] Yu Yin, [e] Wengui Wang, [a, c] Daijie Chen, [e] Shoufeng Wang,*[a, c] Wen Liu*[b, d]

[a] School of Chemistry and Chemical Engineering, University of Jinan, 336 West Road of Nan Xinzhuang, Jinan, 250022, China; E-mail: chm_wangsf@ujn.edu.cn.

[b] State Key Laboratory of Bioorganic and Natural Products Chemistry, Center for Excellence on Molecular Synthesis, Shanghai Institute of Organic Chemistry, University of Chinese Academy of Sciences, 345 Lingling Road, Shanghai 200032, China; E-mail: wliu@mail.sioc.ac.cn.

[c] Shandong Provincial Key Laboratory of Fluorine Chemistry and Chemical materials, 336 West Road of Nan Xinzhuang, Jinan, 250022, China.

[d] Huzhou Center of Bio-Synthetic Innovation, 1366 Hongfeng Road, Huzhou 313000, China.

[e] School of Pharmacy, Shanghai Jiaotong University, Shanghai 200240, China.

Correspondence to: Prof. Shoufeng Wang (E-mail: chm_wangsf@ujn.edu.cn) and Prof. Wen Liu (E-mail: wliu@mail.sioc.ac.cn)

Table of Contents

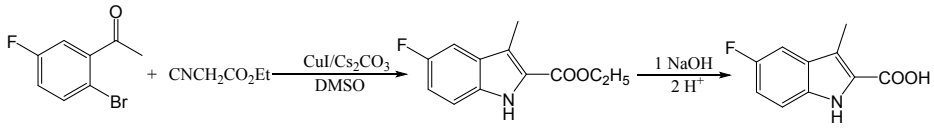
1. Supplementary Methods

1.1 General materials and methods

Materials and bacterial strains. The bacterial strains were purchased from American Type Culture Collection (ATCC). Biochemical and media were purchased from Sinopharm Chemical Reagent Co., Ltd. (China), Oxoid Ltd. (U.K.) or Sigma-Aldrich Co. LLC. (USA). Chemical reagents were purchased from standard commercial sources.

General chemical analysis. High performance liquid chromatography (HPLC) analysis was carried out on an Agilent 1260 HPLC system (Agilent Technologies Inc., USA) equipped with a DAD detector. Semi-preparative HPLC was performed on an Agilent 1100 system. HPLC-electrospray ionization-mass spectrometry (HPLC-ESI-MS) and ESI-MS/MS were performed on a Thermo Fisher LTQ Fleet ESI-MS spectrometer (Thermo Fisher Scientific Inc., USA), and the data were analyzed using Thermo Xcalibur software. ESI-high resolution MS (ESI-HR-MS) analysis was carried out on an instrument consisting of a 1260 HPLC system or a 6538 UHD quadrupole time of flight (QTOF) high resolution mass spectrometry (Agilent Technologies, Santa Clara, USA). NMR data were recorded on a Bruker AV-600 (Bruker Co. Ltd, Germany).

1.2 Chemical synthesis of 6-fluoro-MIA



1-(2-bromo-4-fluorophenyl)ethan-1-one (10.0 mmol), Cs_2CO_3 (20.0 mmol) , CuI (1.0 mmol) and DMSO (10 mL) were added together into a reacting tube under nitrogen atmosphere. Then ethyl isocyanoacetate (11.0 mmol) was slowly added and the mixtures were stirred at 50℃ temperature. After the reactions were finished, ethyl acetate (100 mL) and water (40 mL) were added. The organic phase was separated, dried over sodium sulphate and evaporated in vacuum. The residues were loaded on silica gel column and purified to get ethyl 6-fluoro-3-methyl-1H-indole-2-carboxylate[1] (1.99 g) in 90% yield。^{1}H NMR ($CDCl_3$, 600 MHz) δ 8.85 (br, 1H), 7.58 (dd, J = 8.8, 5.3Hz, 1H), 7.02 (dd, J = 9.4, 2.2 Hz, 1H), 6.90 (td, J = 9.2, 2.2 Hz,

1H), 4.42 (q, J = 7.1 Hz, 2H), 2.59 (s,3H), 1.43 (t, J = 7.1 Hz, 3H); ESI-MS m/z 222.0 $(M+H)^+$.

Ethyl 6-fluoro-3-methyl-1H-indole-2-carboxylate (5 mmol), NaOH (50 mmol) and EtOH (20 mL) were added together into a round-bottom flask and the mixture was refluxed overnight. After the reaction was finished, the solvent was evaporated in vacuum and the residue was redissolved in water (20 mL). Ethyl acetate (40 mL) was added and the aqueous phase was separated and acidification to pH 3.5 by using 1M HCl. Ethyl acetate (40 mL) was added and the organic phase was separated, dried over sodium sulphate and evaporated in vacuum to get 6'-F-MIA (0.85 g) in 88% yield. ^{1}H NMR (CH_3OH-d_4, 600 MHz) δ7.58 (dd, J = 8.8, 5.4Hz, 1H), 7.06 (dd, J = 9.9, 2.2 Hz, 1H), 6.84 (td, J = 9.2, 2.3 Hz, 1H), 4.94 (br, 1H), 2.57 (s, 3H); ^{13}C NMR (CH_3OH-d_4, 150 MHz) δ164.09, 161.79 (d, J = 239.5 Hz), 136.45 (d, J = 13.1 Hz), 125.03, 124.34, 121.30 (d, J = 10.6 Hz), 119.40, 108.17 (d, J = 25.7 Hz), 96.95 (d, J = 26.2 Hz), 8.57; HRMS (ESI) calculated for $C_{10}H_9FNO_2$ ($M + H^+$) m/z 194.0612; found: 194.0610.

1.3 Fermentation and chemical feeding

Fermentation. For sporulation, the *Streptomyces actuosus* strains were cultured on MS agar plates (mannitol 2.0%, soybean cake meal 2.0%, and agar 2.0%) at 30°C for 3 days. The *S. actuosus* spores were inoculated into a 500-mL flask containing 100 mL of primary fermentation medium (sucrose 2.0%, corn steep liquor 3.0%, peptone 0.5% and $CaCO_3$ 0.5%, pH 7.2-7.6) and incubated at 30℃ and 220 rpm for 30 hrs.

Chemical Feeding. 0.2 mL synthetic 6-fluoro-MIA (1mM dissolved in DMSO) was fed to 100 mL fermentation broth of *S. actuosus* SL4005, which would be incubated at 30℃ and 220 rpm for 48 hrs.

1.4 Compound analysis and isolation

Compound analysis. The fermentation broth was centrifuged, and the supernatant was discarded. The mycelia cake was soaked with acetone and sonicated for 30 min. The acetone sample was then centrifuged for HPLC analysis on an Agilent Zorbax column. The column was eluted with solvents A (H_2O+1mM HCOOH) and B (CH_3CN+1mM HCOOH) at a flow rate of 1 mL min^{-1} as follows: T = 0 min, 15% B;

T = 3 min, 15% B; T = 6 min, 45% B; T = 12 min, 45% B; T = 19 min, 55% B； T = 22 min, 85% B; T = 28 min, 85% B; T = 30 min, 15% B. UV absorbance was monitored at 330 nm.

Compound isolation. A total of 8 L fermentation broth was treated by the above method. The crude product was dissolved in tap water, and then extracted twice with an equal volume of n-butanol, and the organic phase was concentrated and purified by silica gel chromatography eluting with 100% CH_2Cl_2 followed by CH_2Cl_2-MeOH (100:1 to 100:10). The component 6'-fluoro-NOS and 6'-fluoro-NOSint were then collected separately, and the organic solvent was evaporated to obtain a crude extract. The 6'-fluoro-NOS crude extract was further purified by semi-preparative HPLC perf ormed on an Agilent 1100 with a Zorbax SB-C18 column (9.4 mm × 25 cm) via gradient elution of solvent A (H_2O + 10 mM HCOOH) and solvent B (CH_3CN) at a flow rate of 3 mL min^{-1} as follows: T = 0 min, 45% B; T = 5 min, 45% B; T =6 min, 50% B; T = 22 min, 50% B; T = 24 min, 45% B; T=35 min, 45% B. The 6'-fluoro-NOSint crude extract was further purified by semi-preparative HPLC perf ormed on an Agilent 1100 with a Zorbax SB-C18 column (9.4 mm × 25 cm) via gradient elution of solvent A (H_2O + 10 mM HCOOH) and solvent B (CH_3CN) at a flow rate of 3 mL min^{-1} as follows: T = 0 min, 15% B; T = 3 min, 15% B; T =6 min, 45% B; T = 12 min, 45% B; T = 19 min, 55% B; T=22 min, 75% B; T = 35 min, 85% B; T=37 min, 15% B.

1.5 *In vitro* anti-infective assays

Determination of minimum inhibitory concentrations (MICs)[2]. Drug-resistant Gram-positive pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC43300 and vancomycin-resistant *Enterococcus* (VRE) ATCC29212/51299/51559 were used to determine the antibacterial activities of NOS, 6'-fluoro-NOSint and 6'-fluoro-NOS. For bacteria culture, brain-heart infusion broth (BHI; Difco Laboratories, Detroit, MI, USA) was used and the bacteria were incubated at 37℃ and 220 rpm for 16 hrs. Each tested sample was dissolved in DMSO to produce a stock solution (128 μg mL^{-1}), which was serially diluted according to the corresponding strain in a 96-well microtiter plate to a final concentration ranging from 0.256 to 0 μg mL^{-1} in two-fold serial. Next, 200 μL of the testing strain (diluted to a final concentration of 1×10^5 CFU mL^{-1}) was added into each well of the microtiter plate, followed by 20 hrs of incubation period at 37℃. Vancomycin was chosen as the control drug. The MIC

value was defined as the lowest concentration that inhibited visible bacterial growth. All tests were carried out three times.

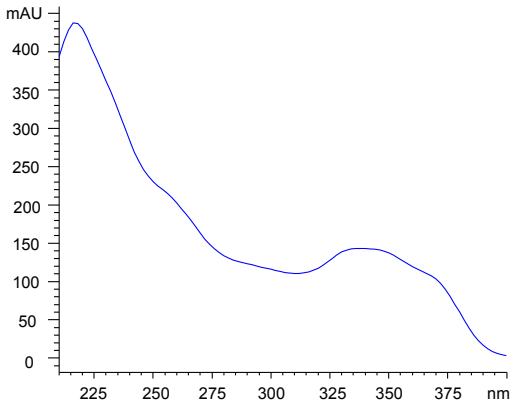
1.6 Compound solubility tests

Determination of compound water solubility[3]. To determine the water solubility of NOS and 6'-fluoro-NOS, 3 mg of solid was added to 1 mL of water in a vial. The vial was gently stirred over a 24-hr period at 30 °C to ensure the solution was at equilibrium. A 0.22-μm centrifugal filter unit (Millipore Ultrafree-MC, USA) was used for filtration to remove the excess solid. The saturated solution was then subjected to HPLC analysis to determine the concentration of the sample based on the established calibration curve. To establish the calibration curve, a set of solutions of varying concentrations of NOS and 6'-fluoro-NOS were analyzed independently by HPLC. The peak areas as a function of concentration were fitted to a regression equation, resulting in a linear standard curve for estimating the concentrations of the unknowns. The solubility of each compound was measured three times.

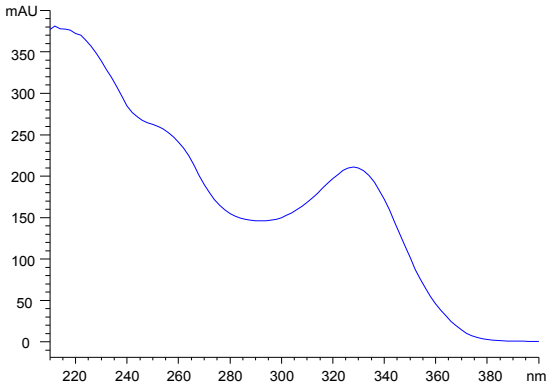
2. Supplementary Results

Fig. S1. UV spectra of 6'-fluoro-NOS, 6'-fluoro-NOSint, and NOS. A, UV spectrum of 6'-fluoro-NOS; B, UV spectrum of 6'-fluoro-NOSint; C, UV spectrum of NOS.

A



B



C

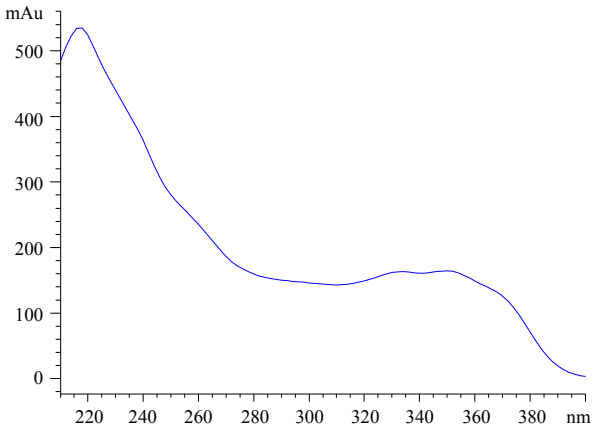
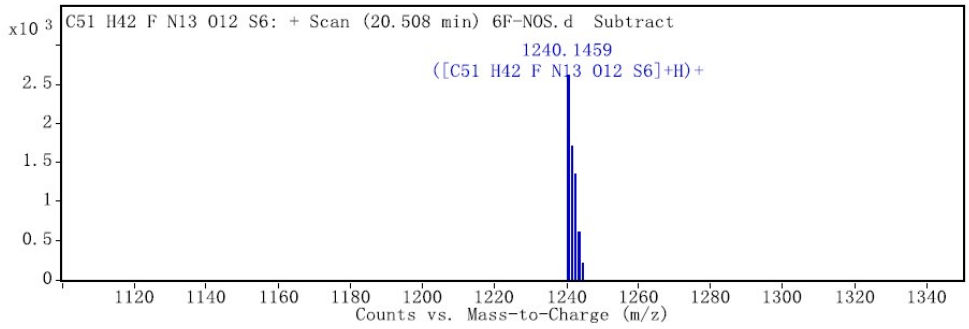


Fig. S2. HR-ESI-MS analysis of 6'-fluoro-NOS.

User Spectra

Fragmentor Voltage	Collision Energy	Ionization Mode
75	0	ESI



Peak List

m/z	z	Abund	Formula	Ion
1240.1459	1	2623.36	C51 H42 F N13 O12 S6	(M+H)+
1241.1436	1	1724.85	C51 H42 F N13 O12 S6	(M+H)+
1242.1424	1	1358.68	C51 H42 F N13 O12 S6	(M+H)+
1243.1444	1	634.09	C51 H42 F N13 O12 S6	(M+H)+
1244.1576	1	232.93	C51 H42 F N13 O12 S6	(M+H)+

Formula Calculator Element Limits

Element	Min	Max
C	3	60
H	1	120
O	10	18
N	10	15
F	1	1
S	6	6

Formula Calculator Results

Ion Formula	m/z	m/z (Calc)	Diff (ppm)	DBE	Score (MFG)
C53 H45 F N10 O13 S6	1240.1459	1240.1470	0.92	36.5	99.24
C51 H43 F N13 O12 S6	1240.1459	1240.1457	-0.17	37	99.97
C48 H45 F N12 O15 S6	1240.1459	1240.1430	-2.33	32.5	95.28
C41 H49 F N12 O20 S6	1240.1459	1240.1489	2.41	23.5	94.97
C39 H47 F N15 O19 S6	1240.1459	1240.1475	1.33	24	98.42

--- End Of Report ---

Fig. S3. LC-MS / MS analysis of 6’-fluoro-NOS.

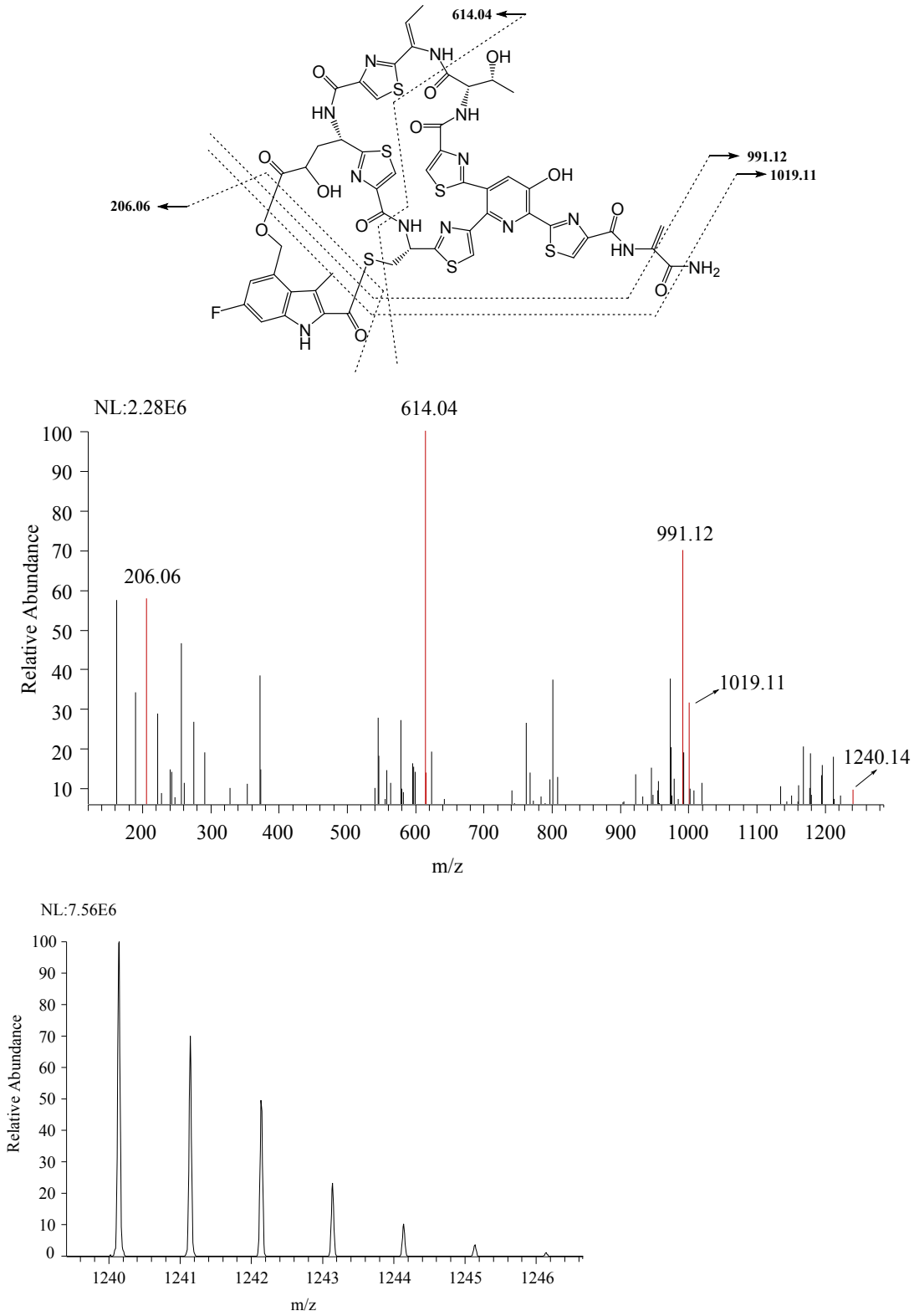
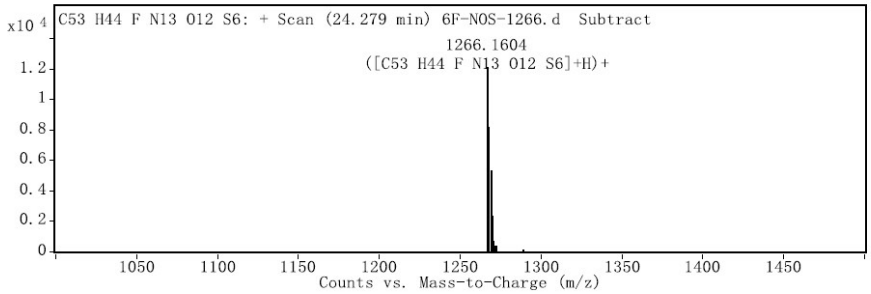


Fig. S4. HR-ESI-MS analysis of 6'-fluoro-NOSint.

User Spectra

Fragmentor Voltage	Collision Energy	Ionization Mode
75	0	ESI



Peak List

m/z	z	Abund	Formula	Ion
1266.1604	1	12139.31	C53 H44 F N13 O12 S6	(M+H)+
1267.1604	1	8195.56	C53 H44 F N13 O12 S6	(M+H)+
1268.1581	1	5377.91	C53 H44 F N13 O12 S6	(M+H)+
1269.159	1	2369.93	C53 H44 F N13 O12 S6	(M+H)+
1270.154	1	816.4	C53 H44 F N13 O12 S6	(M+H)+

Formula Calculator Element Limits

Element	Min	Max
C	3	60
H	1	120
O	10	15
N	10	15
S	6	6
F	1	1

Formula Calculator Results

Ion Formula	m/z	m/z (Calc)	Diff (ppm)	DBE	Score (MFG)
C55 H47 F N10 O13 S6	1266.1604	1266.1627	1.81	37.5	97.08
C53 H45 F N13 O12 S6	1266.1604	1266.1613	0.75	38	99.49
C50 H47 F N12 O15 S6	1266.1604	1266.1587	-1.37	33.5	98.29

--- End Of Report ---

Fig. S5. LC-MS/MS analysis of 6'-fluoro-NOSint.

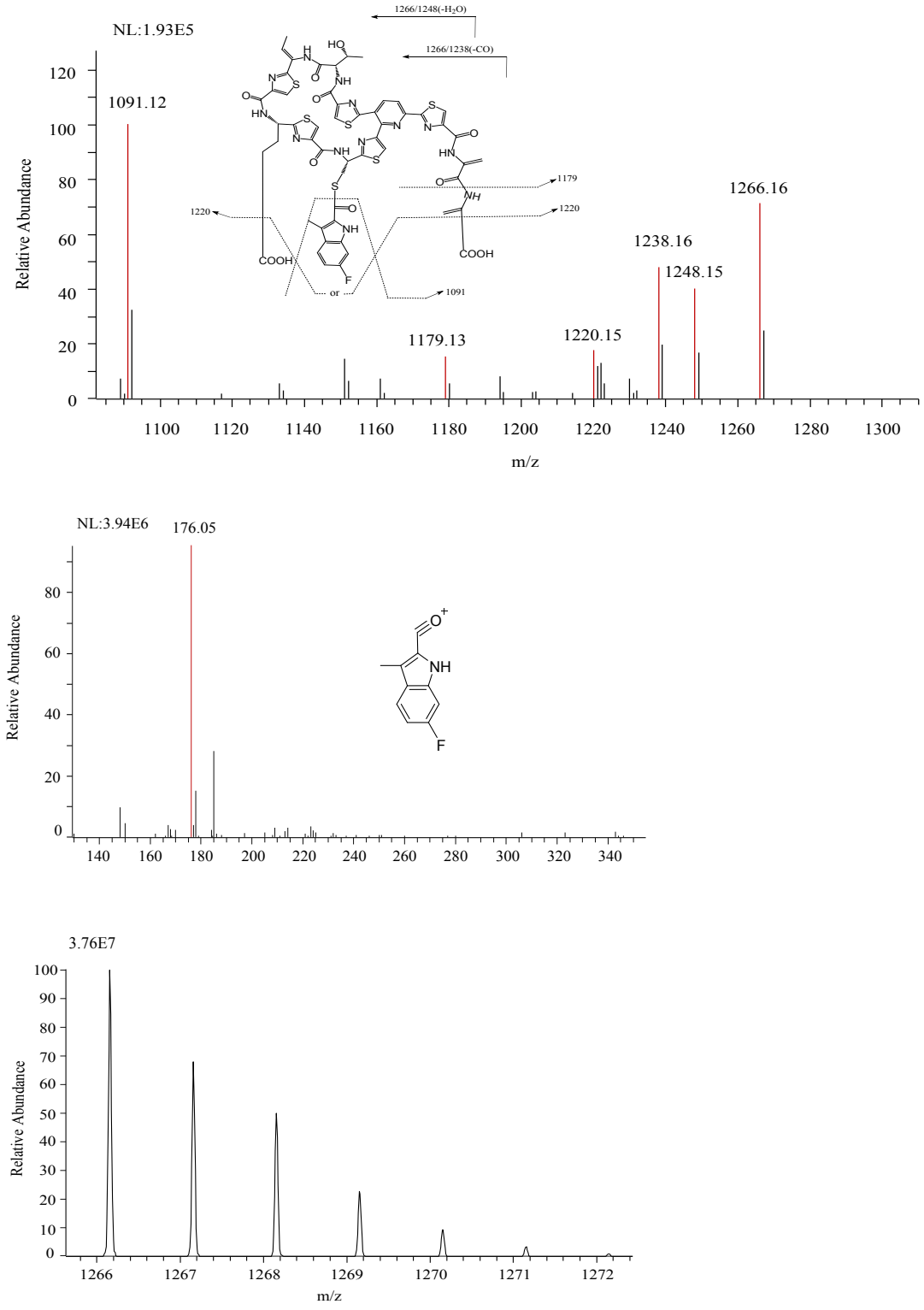
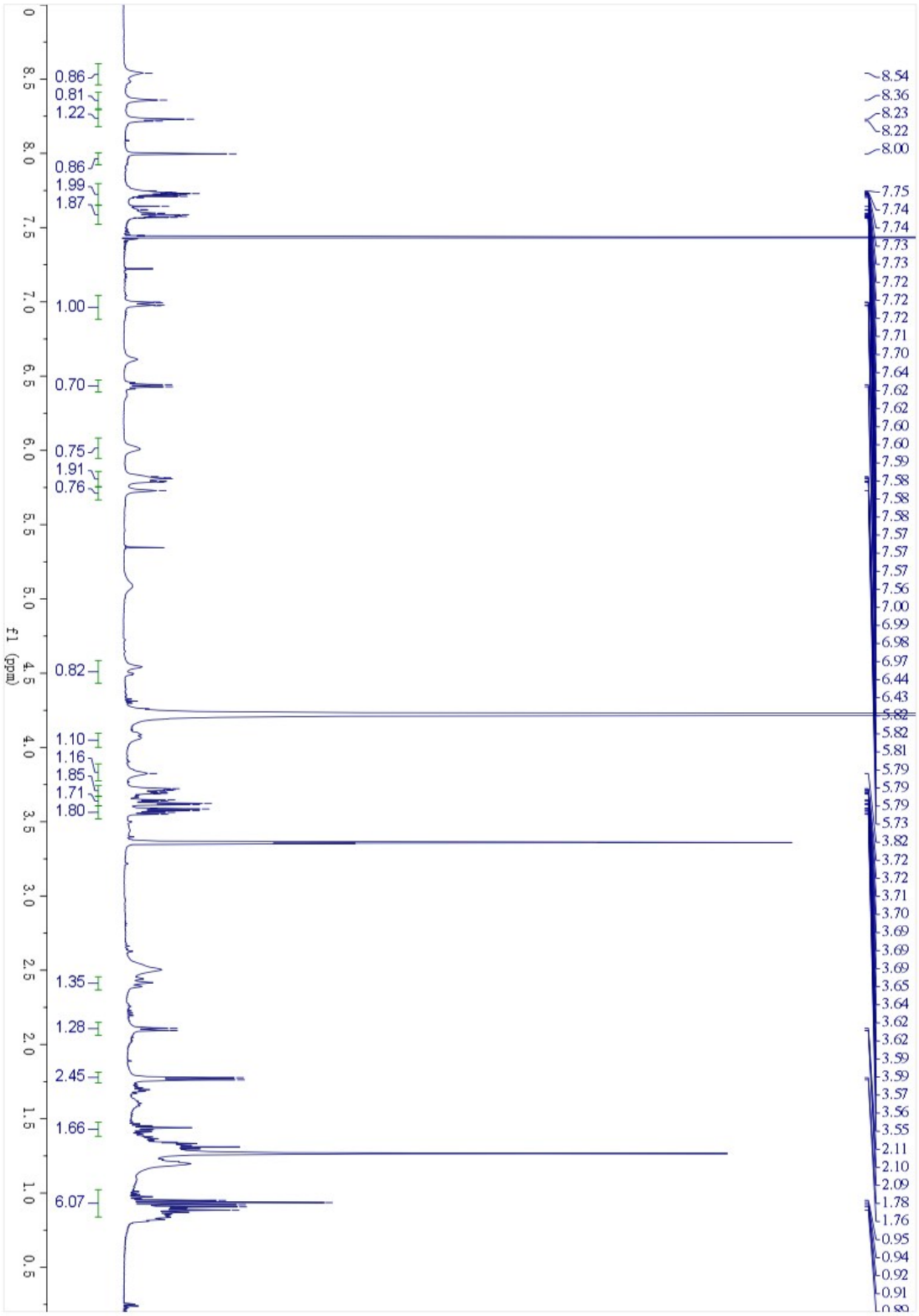


Fig. S6. NMR spec

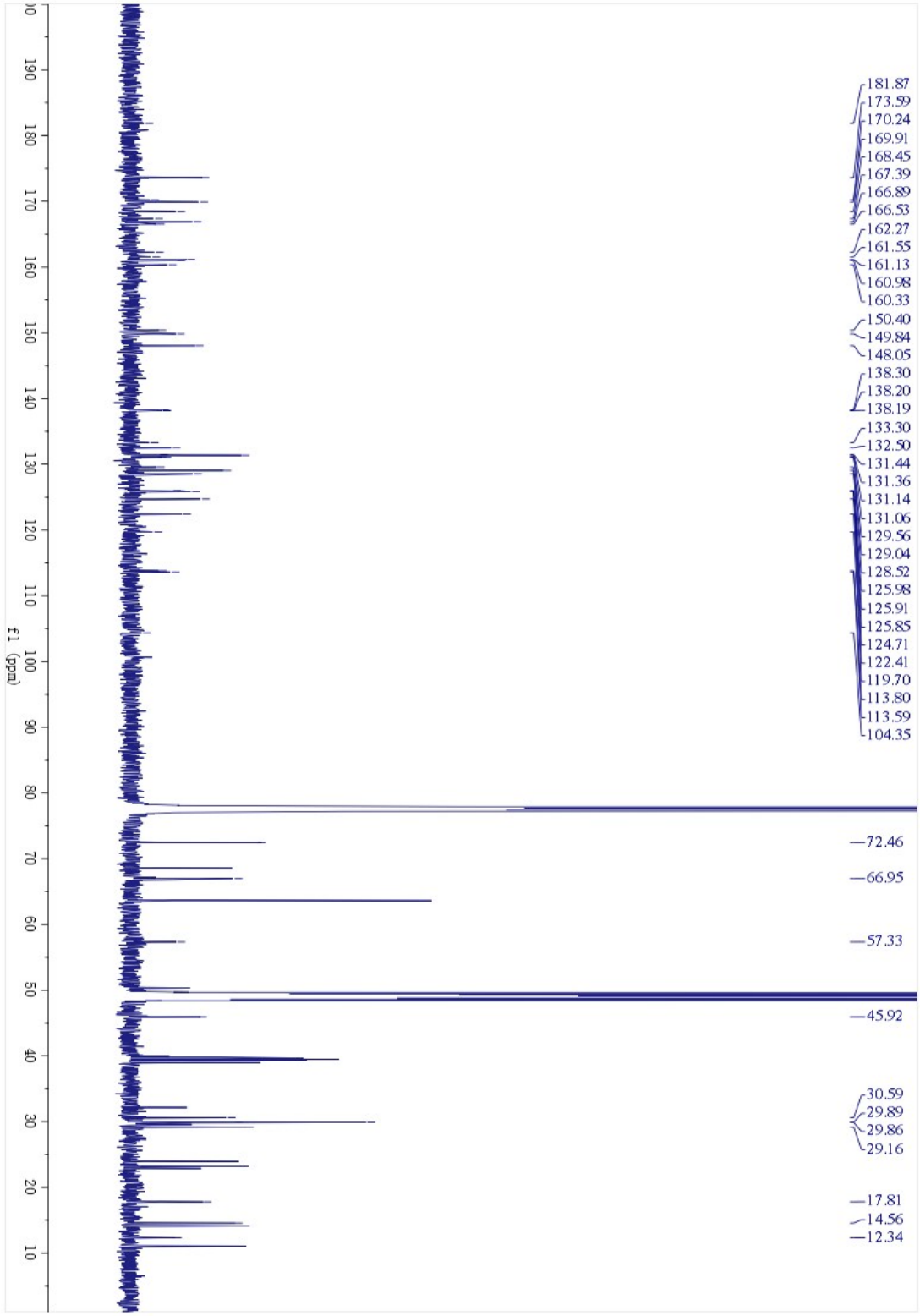
B, ^{13}C NMR(CDC

F, ROESY; G, ^{19}F

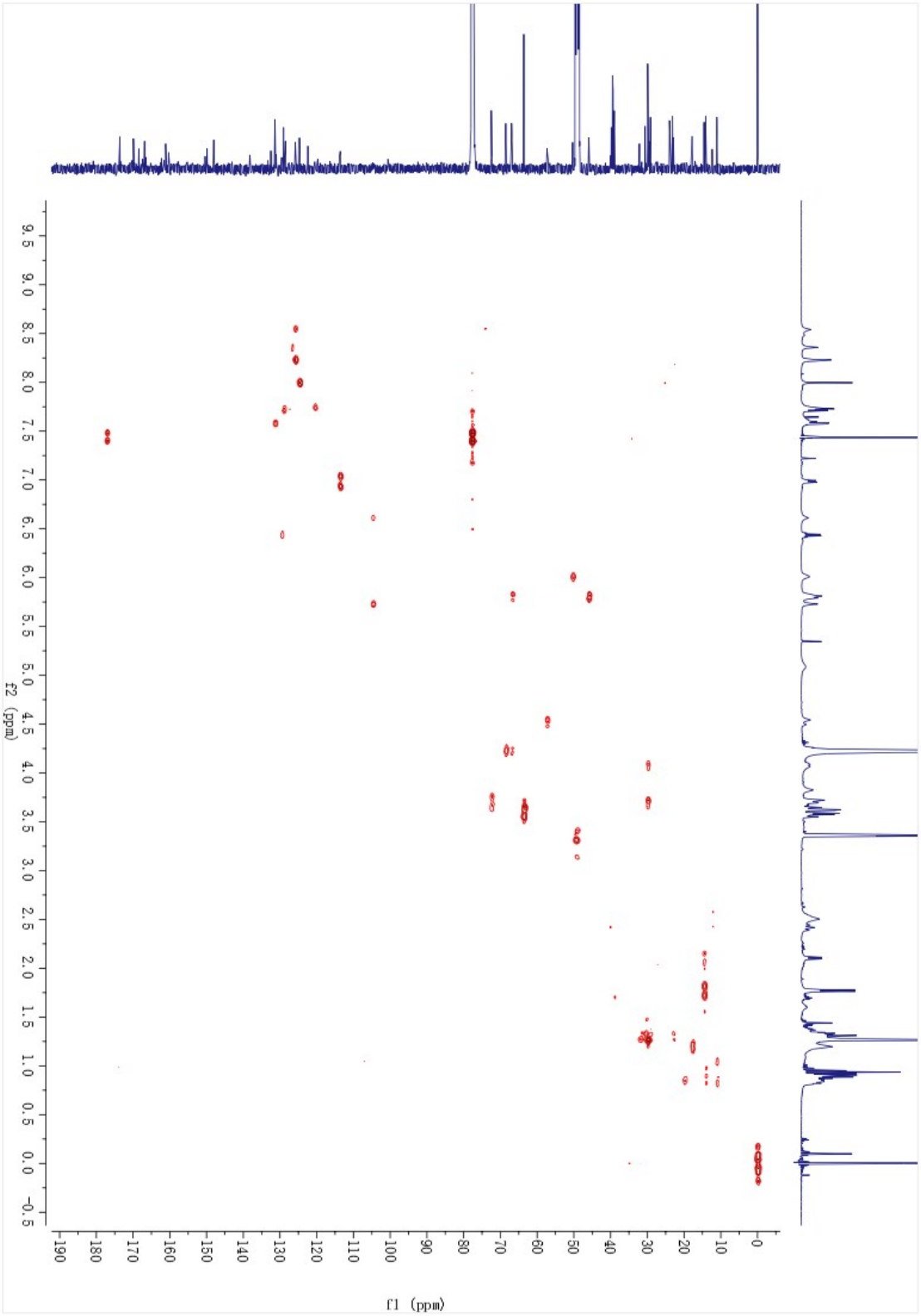
A ^{1}H NMR (CDCl



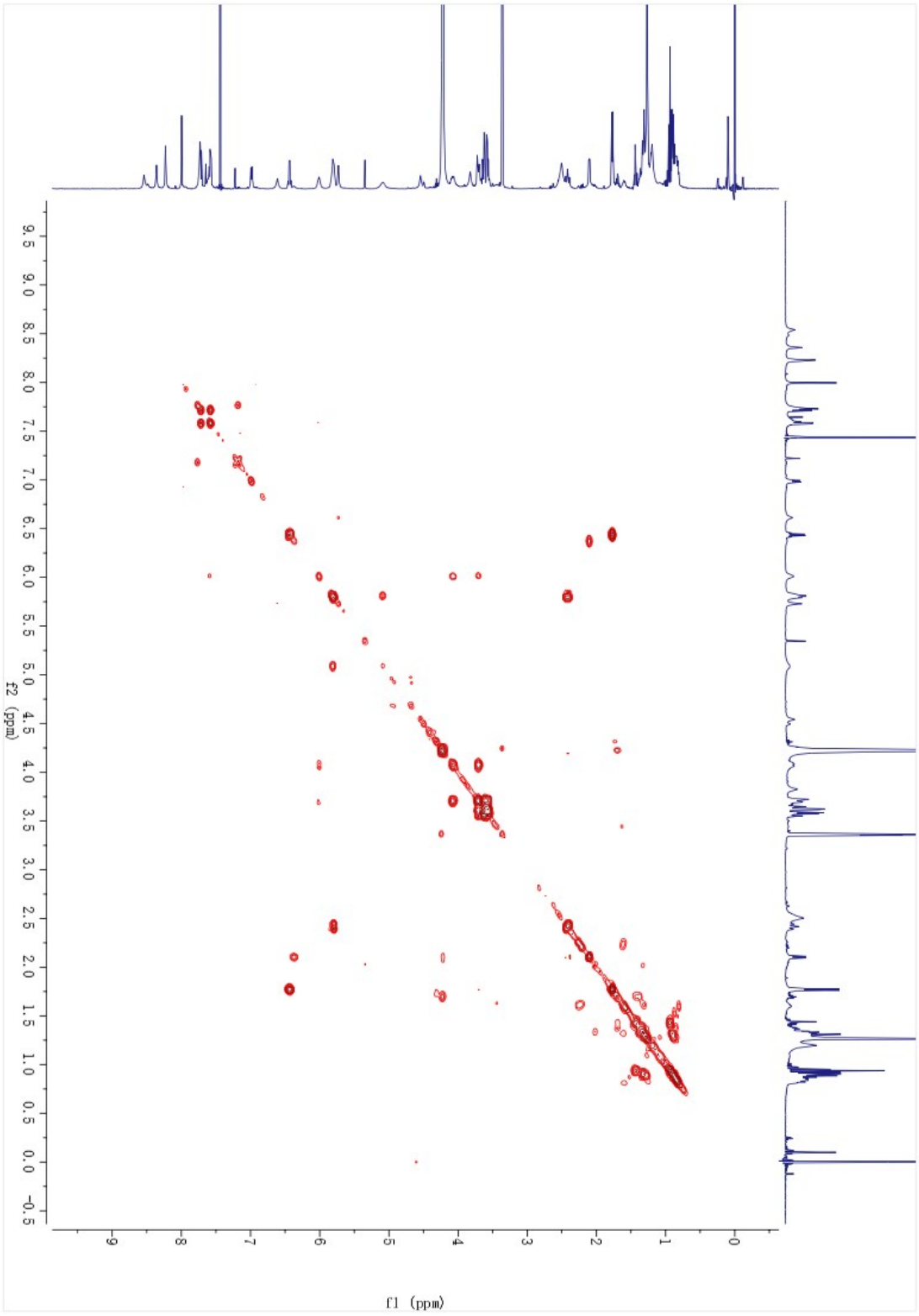
B ^{13}C NMR(CDCl



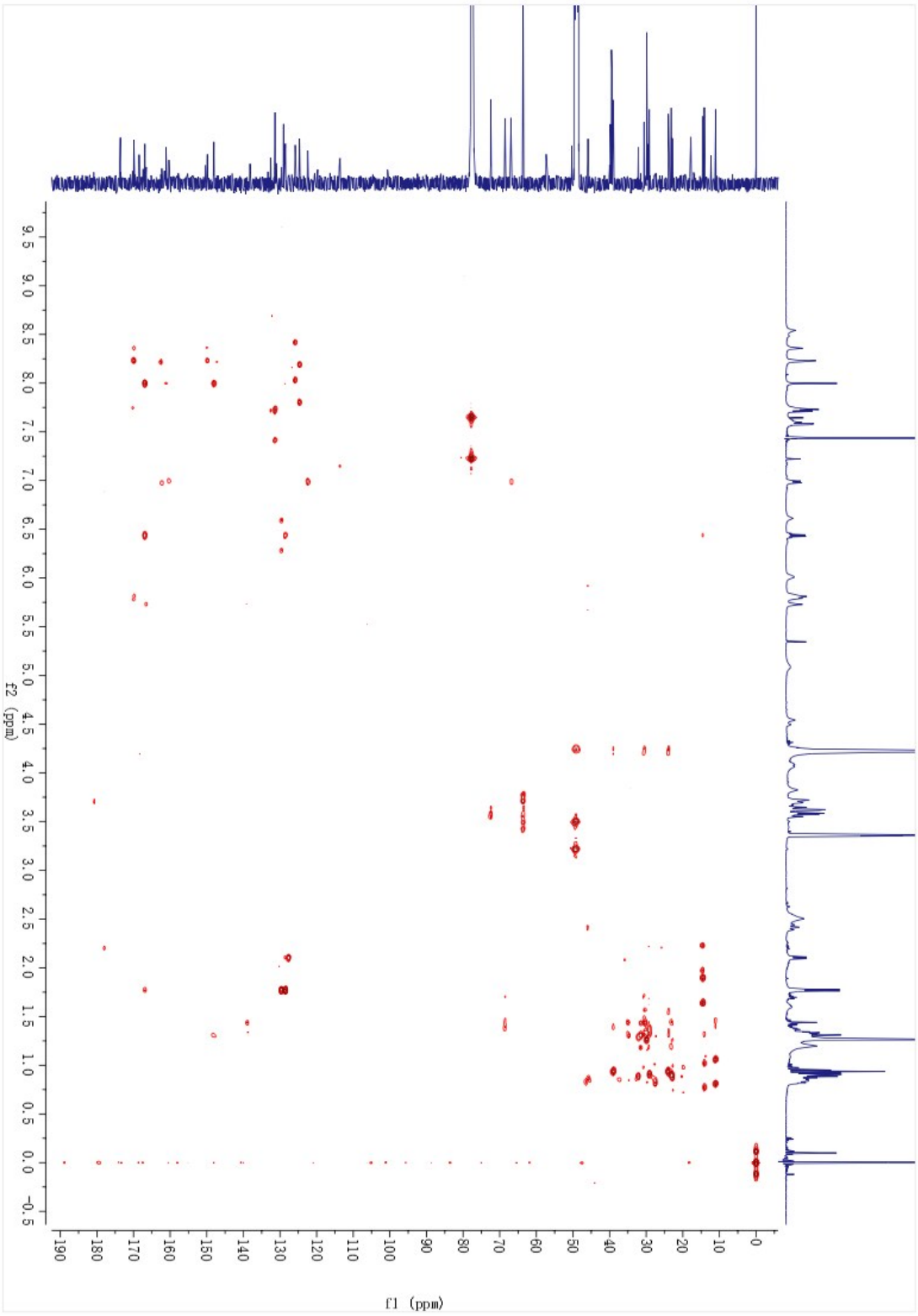
C HMQC



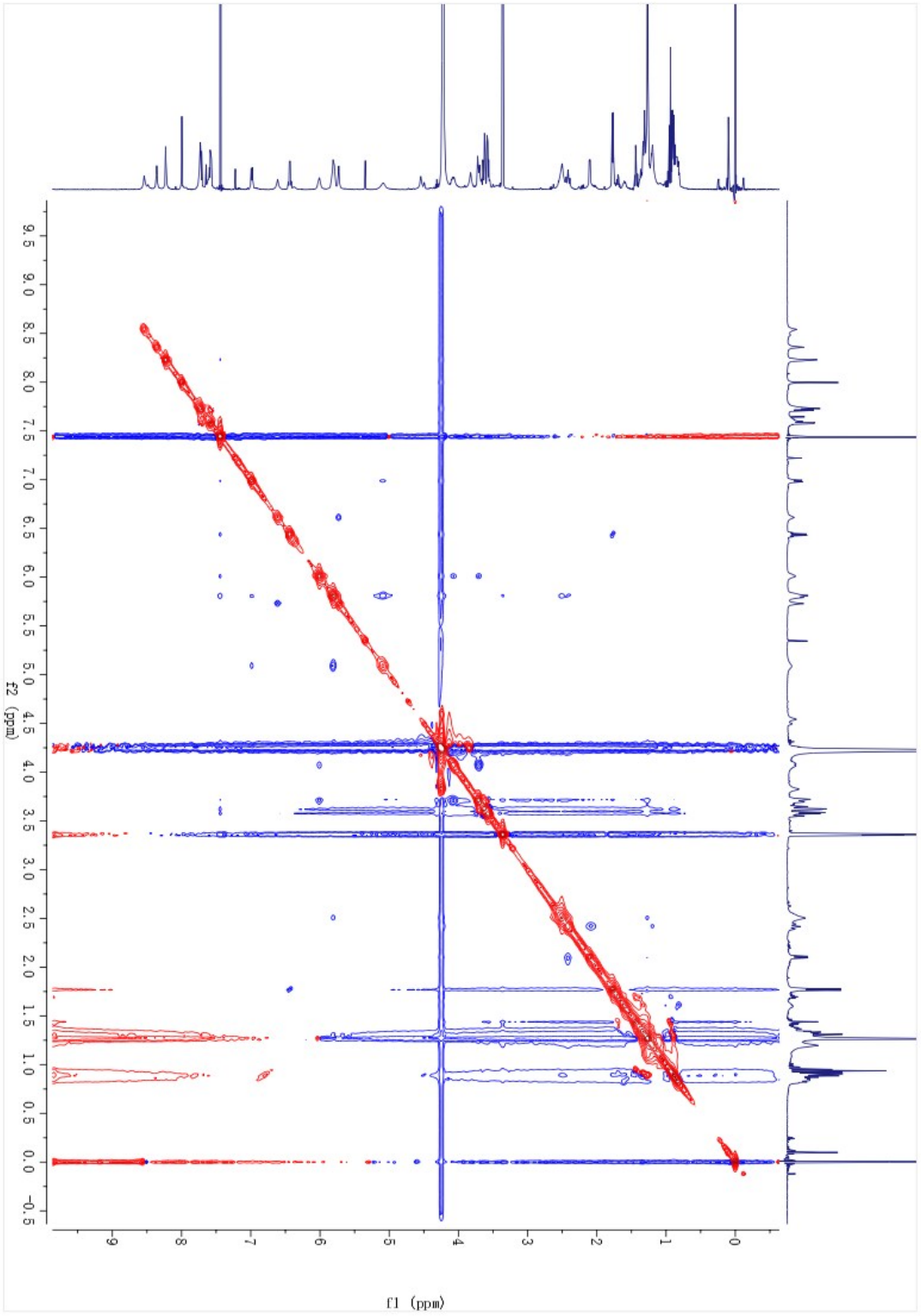
D ^{1}H-^{1}HCOSY



E HMBC



F ROESY



G ^{19}F NMR.

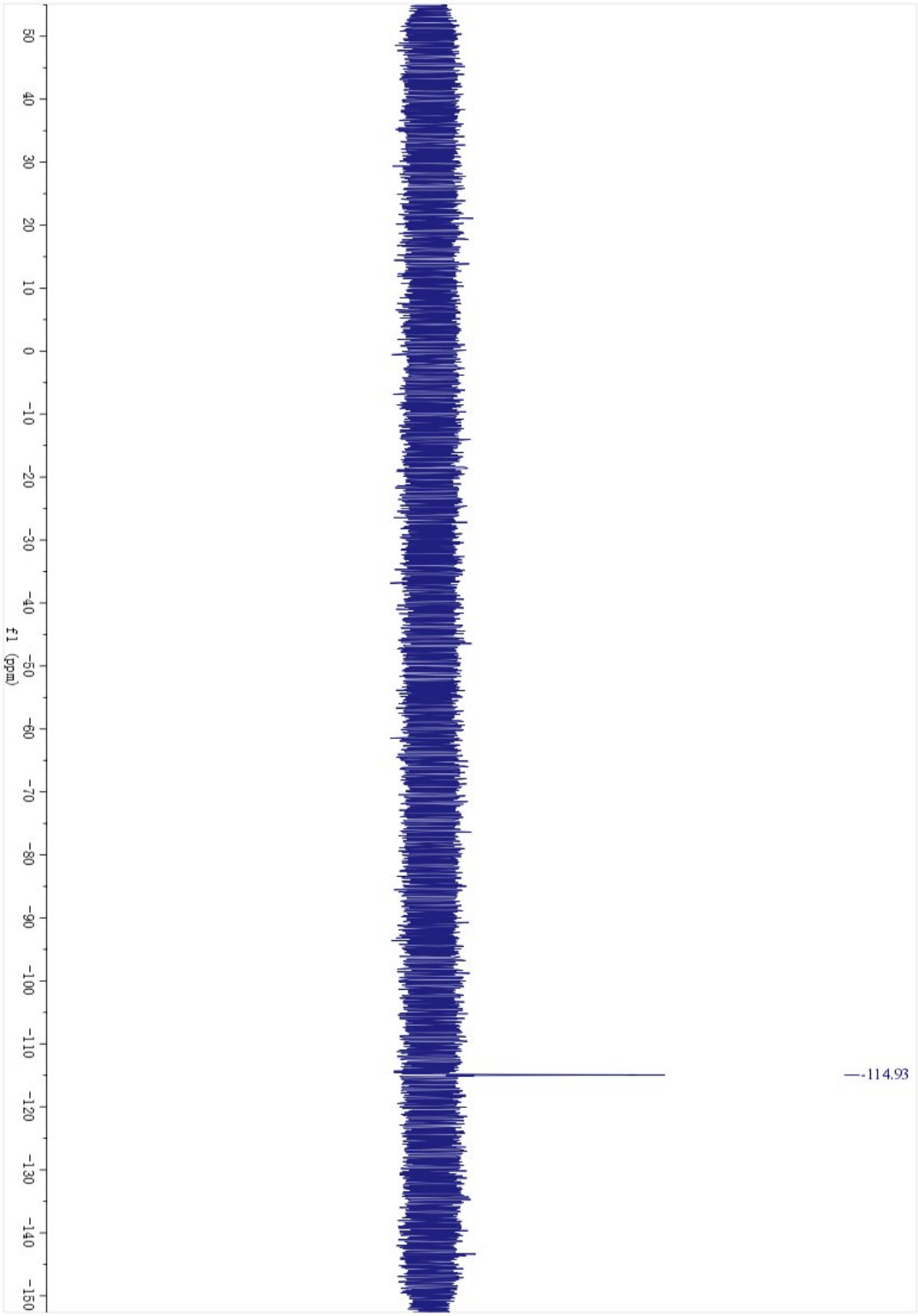

50
40
30
20
10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
-110
-120
-130
-140
-150
f1 (ppm)
-114.93

Fig. S7. Chemical structure of 6'-fluoro-NOS.

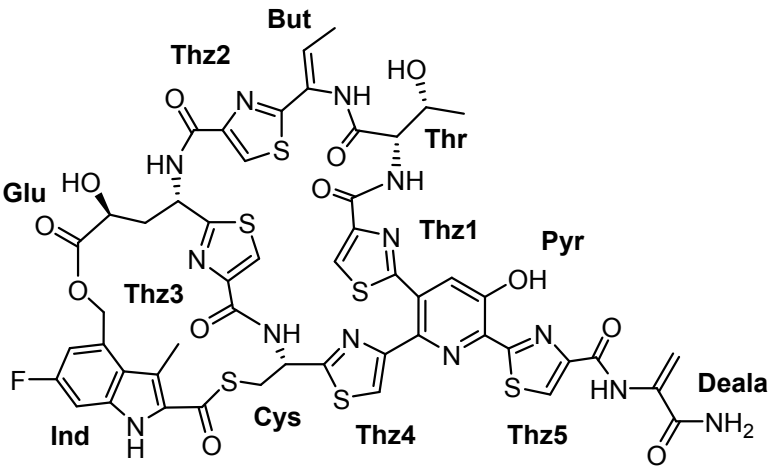


Fig. S8. The key of 2D NMR correlations of 6'-fluoro-NOS.

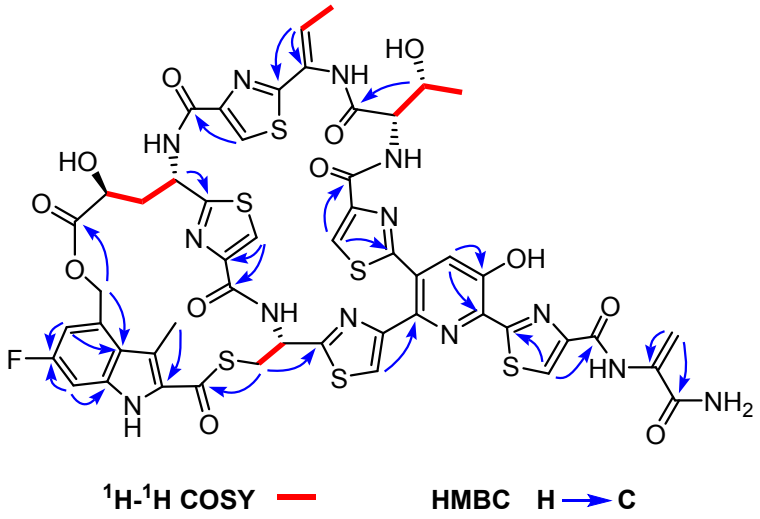
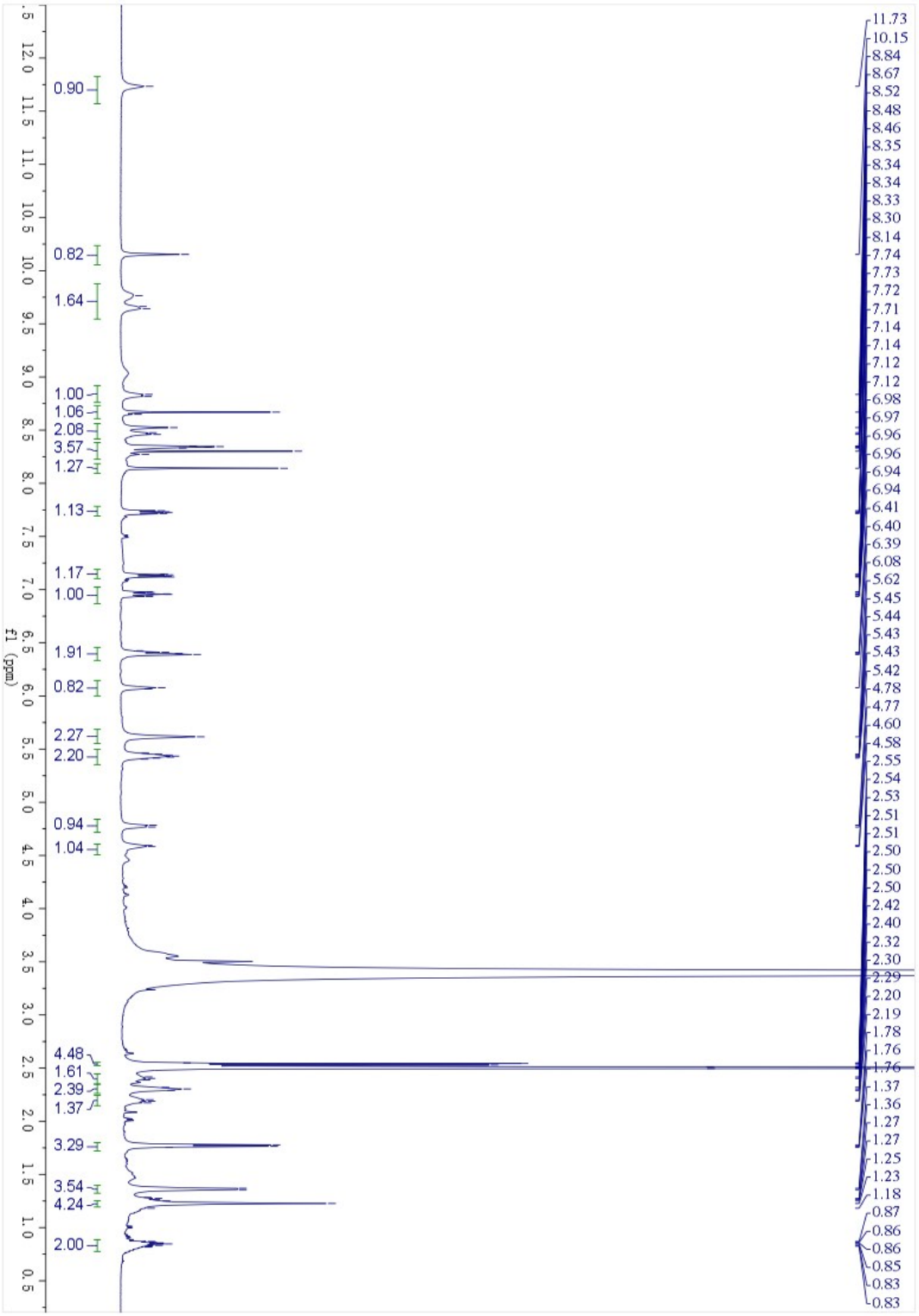


Fig. S9. NMR spe

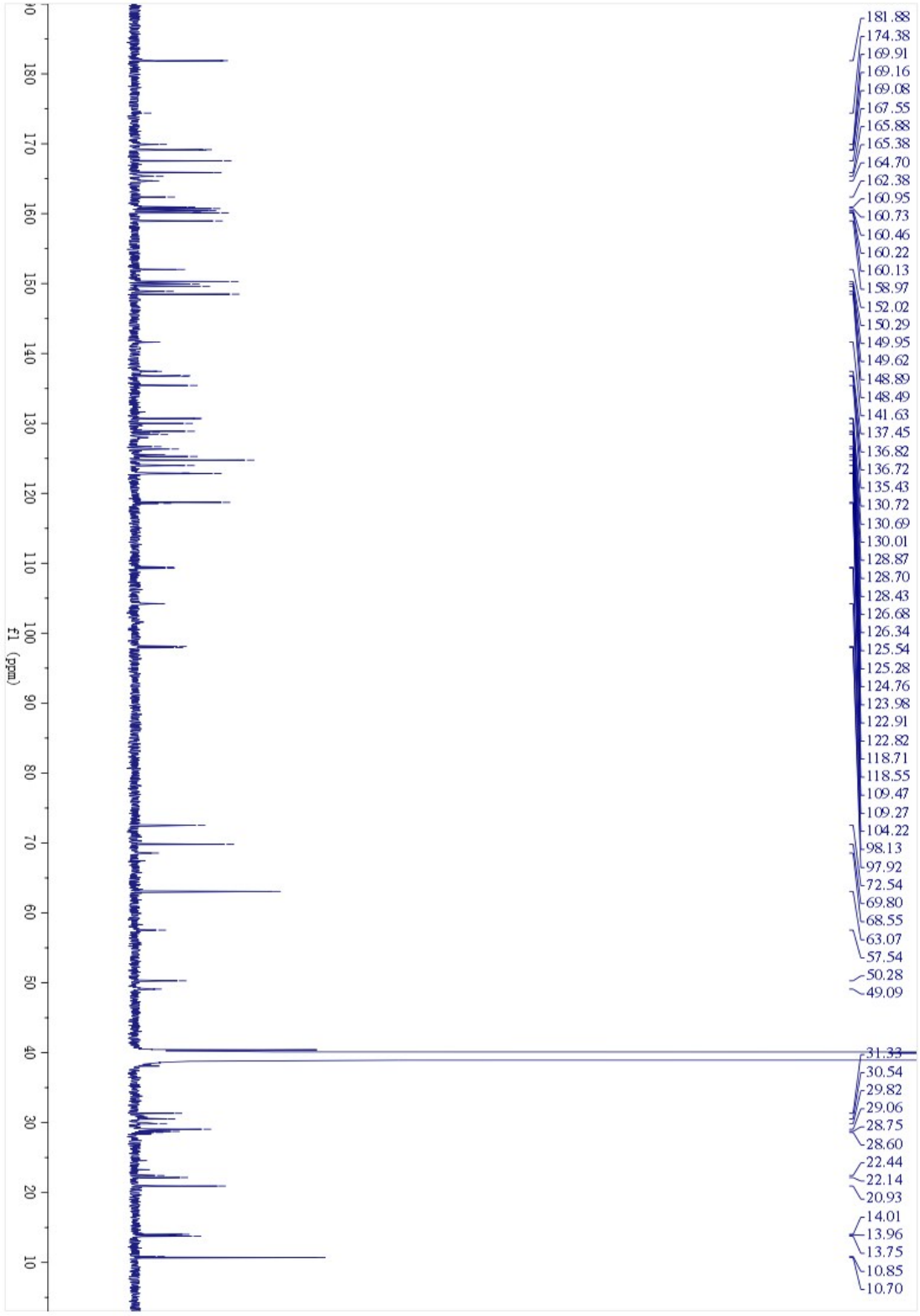
NMR(DMSO-d_6,1

^{19}F NMR.

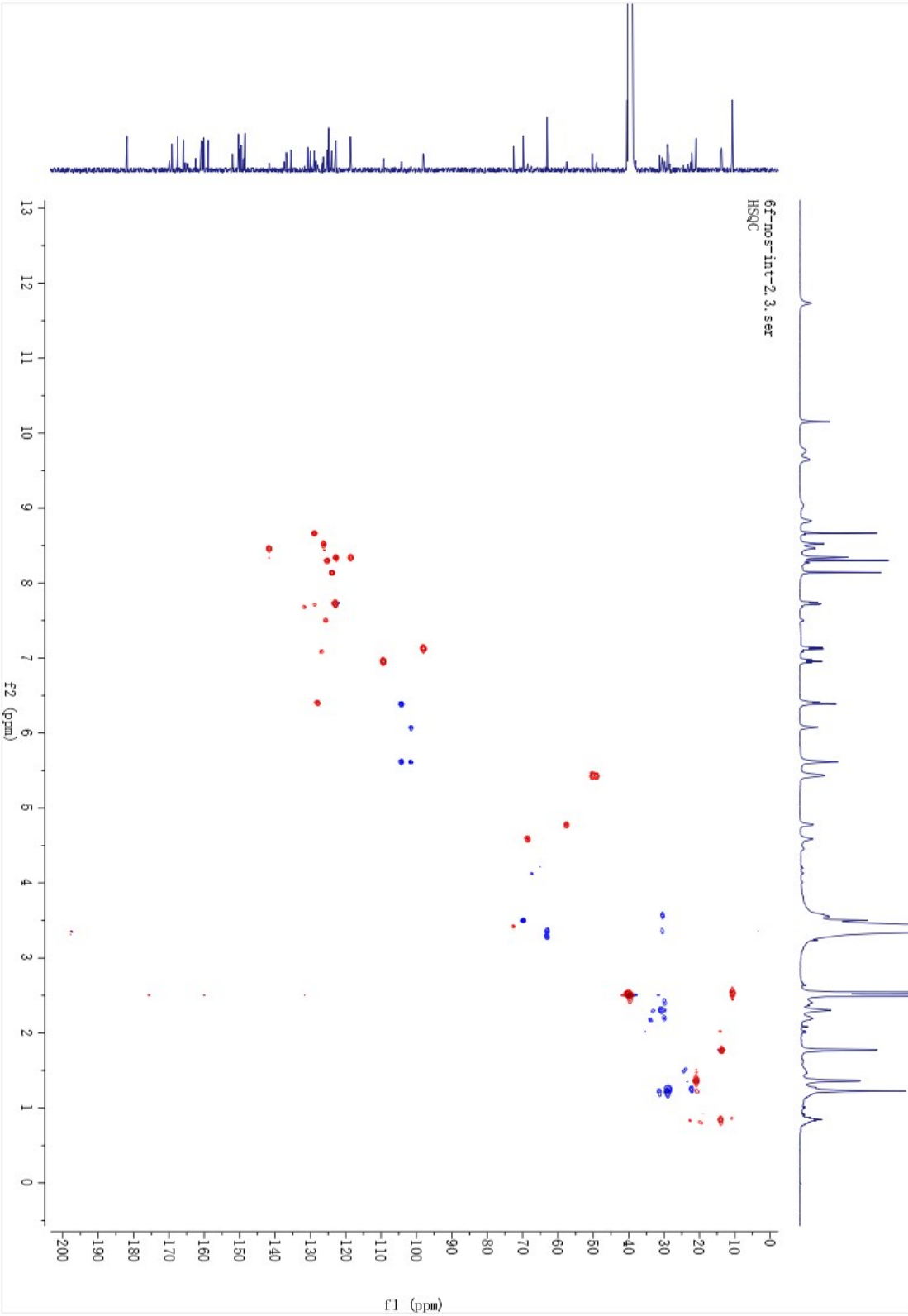
A ^{1}H NMR (DMS



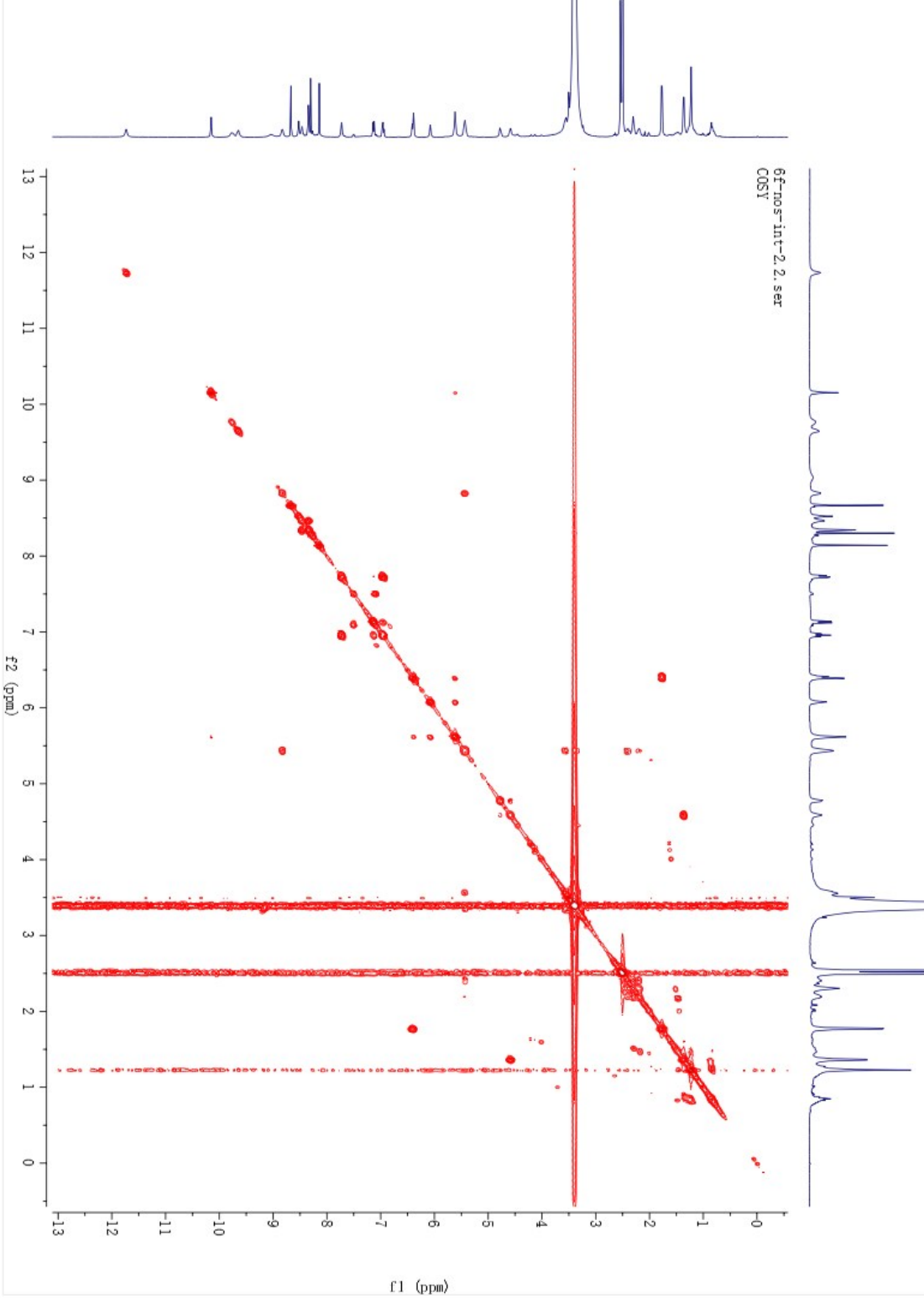
B ^{13}C NMR(DMS


f1 (ppm)
181.88
174.38
169.91
169.16
169.08
167.55
165.88
165.38
164.70
162.38
160.95
160.73
160.46
160.22
160.13
158.97
152.02
150.29
149.95
149.62
148.89
148.49
141.63
137.45
136.82
136.72
135.43
130.72
130.69
130.01
128.87
128.70
128.43
126.68
126.34
125.54
125.28
124.76
123.98
122.91
122.82
118.71
118.55
109.47
109.27
104.22
98.13
97.92
72.54
69.80
68.55
63.07
57.54
50.28
49.09
31.33
30.54
29.82
29.06
28.75
28.60
22.44
22.14
20.93
14.01
13.96
13.75
10.85
10.70

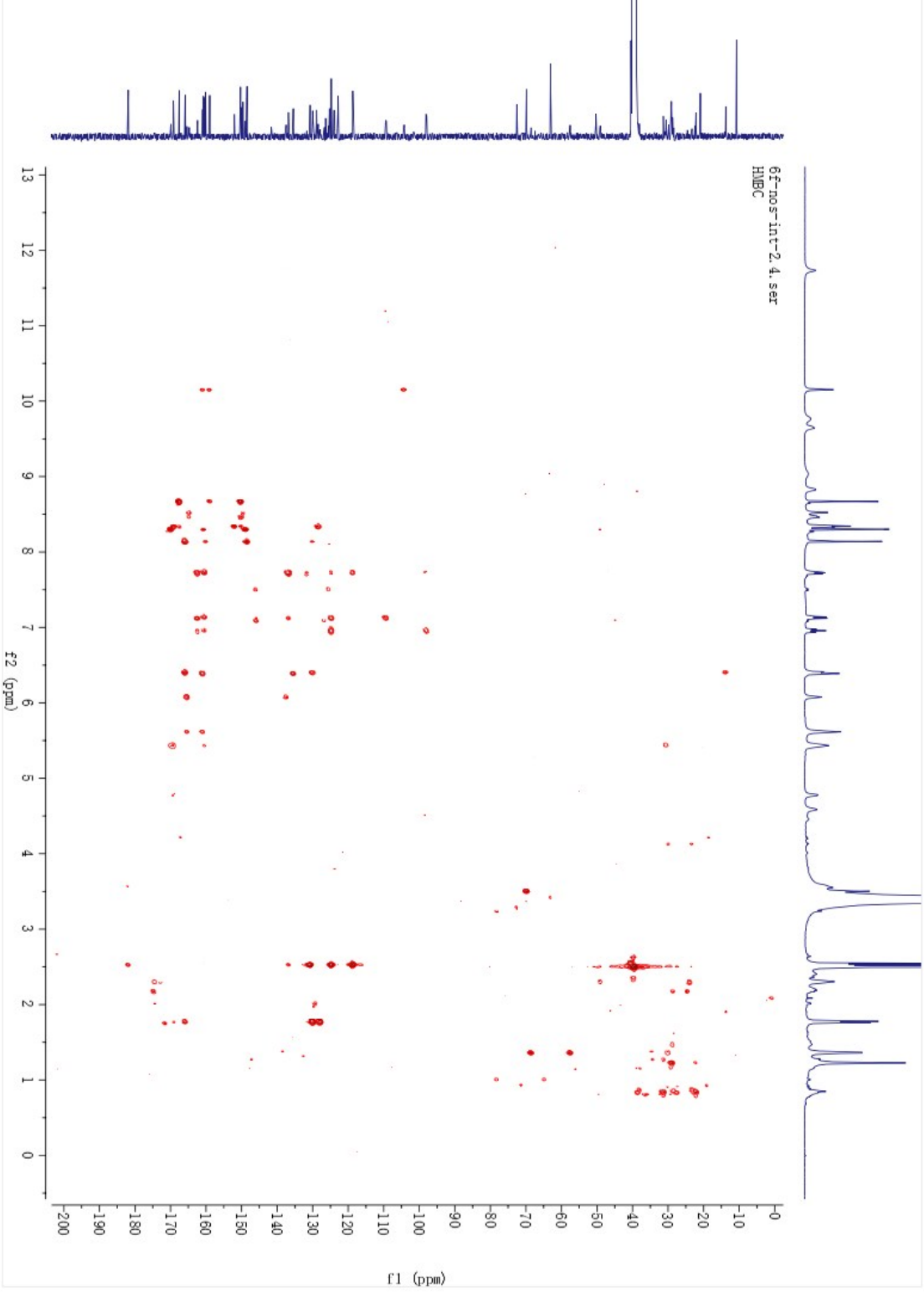
C HSQC



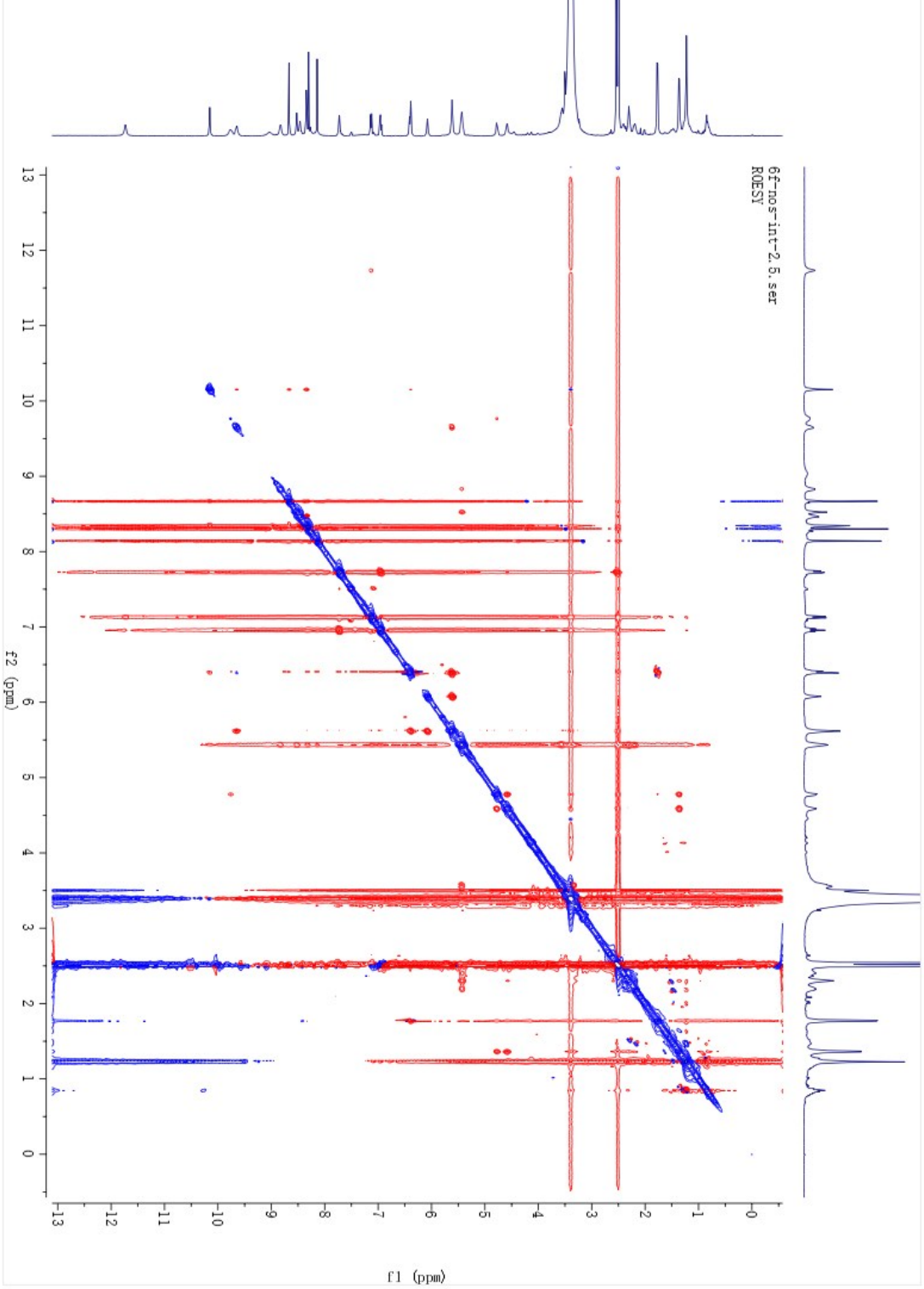
D. ^{1}H-^{1}H COSY



E. HMBC



F. ROESY



G. ^{19}F NMR

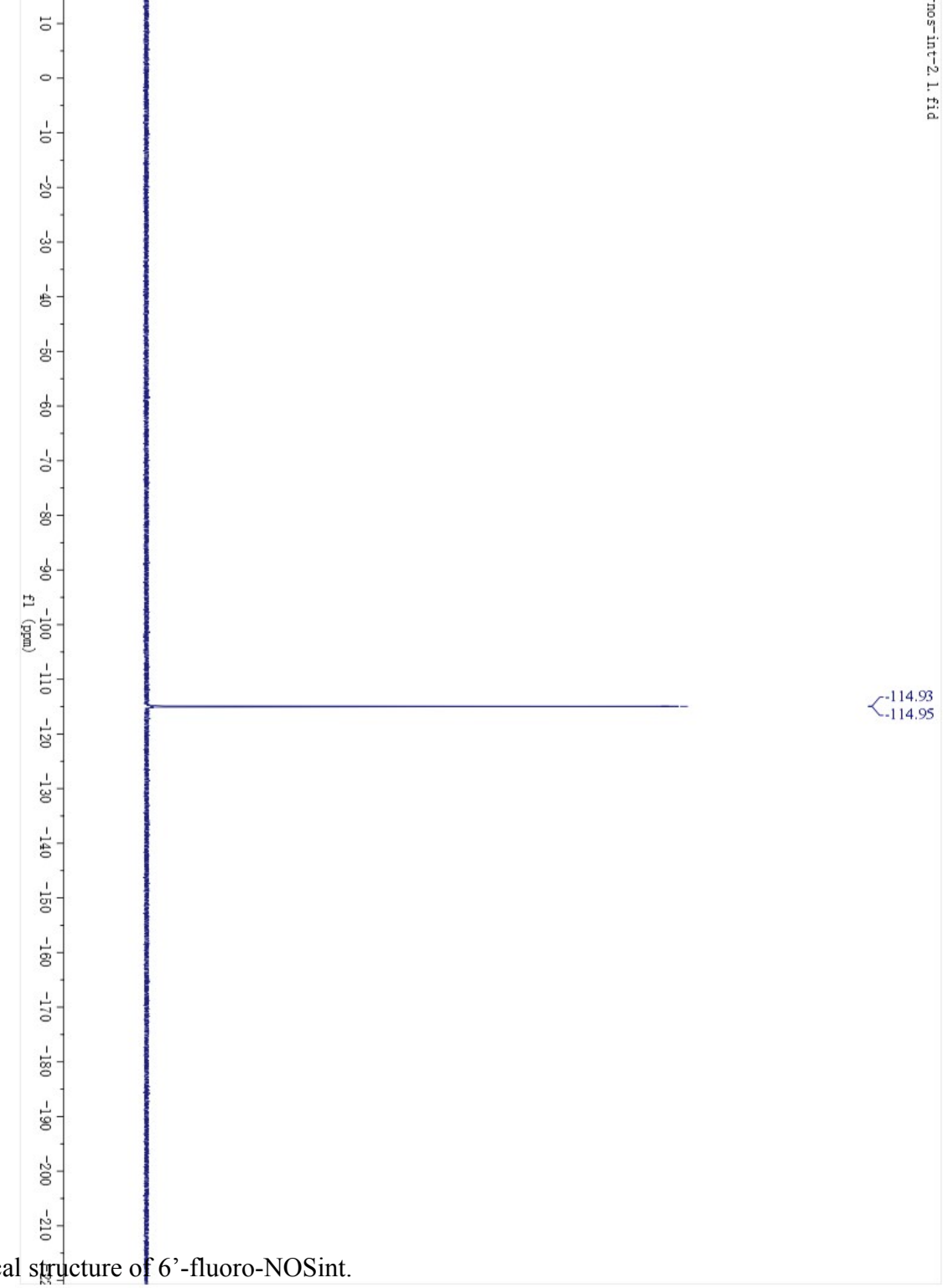


Fig. S10. Chemical structure of 6’-fluoro-NOSint.

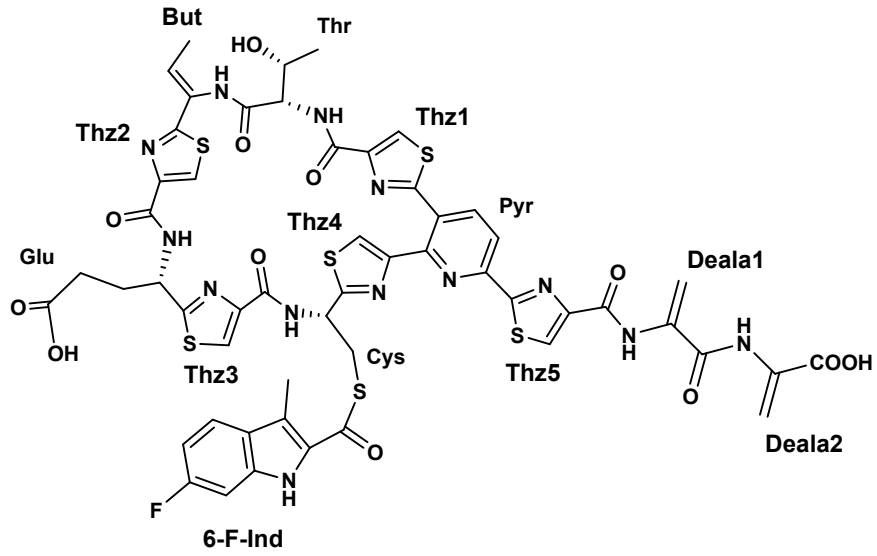
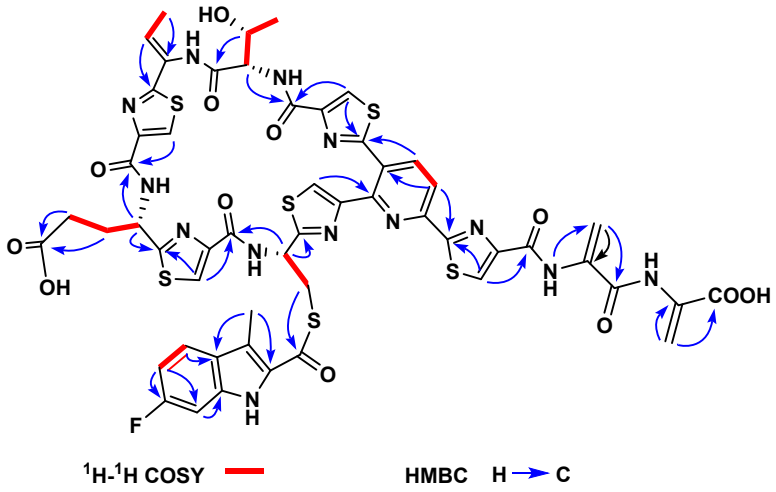


Fig. S11. The key of 2D NMR correlations of 6'-fluoro-NOSint.



^{1}H-^{1}H COSY HMBC H → C

Table S1. NMR data for 6'-fluoro-NOS in $CDCl_3$-CD_3OD (3:1) (δ in ppm, *J* in Hz).

Substructure (No.)	**δ_C ppm**	**δ_H (mult., *J*, Hz)**	**Substructure (No.)**	**δ_C ppm**	**δ_H (mult., *J*, Hz)**
Pyr (2)	149.8		**Thz4 (4)**	149.9	
Pyr (3)	150.4		**Thz4 (5)**	126.6	8.36 (s)
Pyr (4)	128.5	7.71 (br s, overlap)	**Thz5 (2)**	167.4	
Pyr (5)	125.8		**Thz5 (4)**	150.4	
Pyr (6)	138.5		**Thz5 (5)**	126.0	8.54 (s)
Thz1 (2)	162.3		**Thr5 (C=O)**	167.4	
Thz1 (4)	148.1		**Deala (1)**	166.5	
Thz1 (5)	129.0	7.71 (br s, overlap)	**Deala (2)**	133.3	
Thr1 (C=O)	161.1		**Deala (3)**	104.4	6.62 (br s) 5.73 (br s)
Thr (C=O)	169.9		**Glu (1)**	173.6	
Thr (2)	57.3	4.50 (br s)	**Glu (2)**	72.5	3.71 (m, overlap)
Thr (3)	66.8	4.23 (m)	**Glu (3)**	29.9	
Thr (4)	17.8	1.20 (br s)	**Glu (4)**	50.3	6.00 (br s)
But (2)	128.5		**Ind (2)**	132.5	
But (3)	129.6	6.44 (q, 6.9)	**Ind (3)**	119.6	
But (4)	14.6	1.76 (d, 7.0)	**Ind (3a)**	122.4	
Thz2 (2)	166.9		**Ind (4)**	138.2	
Thz2 (4)	148.1		**Ind (5)**	113.6	6.99 (m, overlap)
Thz2 (5)	124.7	8.00 (s)	**Ind (6)**	160.5	
Thr2 (C=O)	168.5		**Ind (7)**	113.8	6.99 (m, overlap)
Thz3 (2)	169.9		**Ind (7a)**	138.3	
Thz3 (4)	149.8		**Ind (3')**	12.3	2.52 (br s)
Thz3 (5)	125.9	8.23 (s)	**Ind (4')**	66.8	5.80 (m, overlap) 5.08 (br s)
Thr3 (C=O)	161.6		**Cys (2)**	30.6	4.07 (m) 3.71 (m, overlap)
Thz4 (2)	169.9		**Cys (3)**	46.0	5.80 (m, overlap)
			-S-C=O	181.9	

(^{1}H NMR spectrum recorded in 600 MHz and ^{13}C NMR spectrum recorded in 150 MHz)

^{19}F NMR (δ_F ppm)	-114.9

(^{19}F NMR spectrum recorded in 564 MHz)

NOTE: All HO- and NH- groups were not observed due to H/D exchange.

Table S2. NMR data for 6'-fluoro-NOSint in DMSO-d_6(δ in ppm, *J* in Hz).

Substructure (No.)	**δ_C ppm**	**δ_H (mult., *J*, Hz)**	**Substructure (No.)**	**δ_C ppm**	**δ_H (mult., *J*, Hz)**
Pyr (2)	149.9		**Thz5 (2)**	167.6	
Pyr (3)	118.6	8.34 m (overlap)	**Thz5 (4)**	150.3	
Pyr (4)	141.6	8.47 d (8.1)	**Thz5 (5)**	128.9	8.67 s
Pyr (5)	128.7		**Thz5 (C=O)**	159.0	
Pyr (6)	150.3		**Deala1 (1)**	161.0	
Thz1 (2)	170.0		**Deala1 (2)**	135.4	
Thz1 (4)	148.9		**Deala1 (3)**	104.2	6.40 m (overlap) 5.61 m (overlap)
Thz1 (5)	125.3	8.30 s	**Deala2 (1)**	165.4	
Thz1 (C=O)	160.7		**Deala2 (2)**	137.5	
Thr (C=O)	169.2		**Deala2 (3)**	101.6	6.07 d (6.5) 5.61 m (overlap)
Thr (2)	57.5	4.77 d (8.6)	**Glu (1)**	174.4	
Thr (3)	68.6	4.59 brs	**Glu (2)**	20.9	1.47 m 1.23 m
Thr (4)	20.9	1.37 d (6.3)	**Glu (3)**	29.8	2.20 m 2.41 m
But (2)	130.0		**Glu (4)**	50.3	5.43 m
But (3)	128.0	6.40 m (overlap)	**6-F-Ind (2)**	130.7	
But (4)	13.8	1.77 d (6.9)	**6-F-Ind (3)**	118.7	
Thz2 (2)	165.9		**6-F-Ind (3a)**	124.8	
Thz2 (4)	148.5		**6-F-Ind (4)**	122.9	7.73 dd (8.9, 5.4)
Thz2 (5)	124.0	8.14 s	**6-F-Ind (5)**	109.5	6.96 td (8.9, 2.3)
Thz2 (C=O)	160.1		**6-F-Ind (6)**	160.5	
Thz3 (2)	169.2		**6-F-Ind (7)**	98.2	7.14 dd (9.8, 2.3)
Thz3 (4)	152.0		**6-F-Ind (7a)**	136.8	
Thz3 (5)	122.8	8.34 s	**6-F-Ind (3')**	10.7	2.53 s
Thz3 (C=O)	167.6		**Cys (2)**	30.5	3.55 m 3.36 m
Thz4 (2)	164.7		**Cys (3)**	49.1	5.44 m
Thz4 (4)	149.6		**-S-C=O**	181.2	
Thz4 (5)	126.3	8.52 s			

Thz5-NH		10.15 s	**Deala2-NH**		9.65 brs
6F-Ind-NH		11.73 s			

(^{1}H NMR spectrum recorded in 500 MHz and ^{13}C NMR spectrum recorded in 125 MHz)

^{19}F NMR (δ_F ppm)	-114.9

(^{19}F NMR spectrum recorded in 470 MHz)

3. Supplementary References

1. Q. Cai, Z. Li, J. Wei, C. Ha, D. Pei and K. Ding, *Chem. Commun.,* 2009, **(48)**:7581.

2. CLSI. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement. CLSI document M100-S21. Wayne, PA: Clinical and Laboratory Standards Institute. 2011.

3. R. Liao and W. Liu, *J. Am. Chem. Soc.,* 2011, **133**, 2852.