Supplementary materials

Supplementary Table S1

Histopathological findings in the lungs, thymus, ovaries and thyroid in the repeated dose study in monkeys

Organs/tissues	Microscopic findings	Main groups			Recovery groups	
		Dose (mg/kg)			Dose (mg/kg)	
		10	30	80	30	80
lungs and bronchi	Inflammation, mixed cells; Edema, multifocal, alveolar/bronchioles/capsule			± + 2+		±
Thymus	Cytopenia; Lymphocytes, Diffuse, cortex	+	± +	± +	+	+
Thyroid	Atrophic, follicle, diffuse		±	± +	±	
Ovaries	immature; Bilateral	+	±	2+	±	+

±: very slight, +: slight, 2+: moderate, 3+: marked

Supplementary Figure

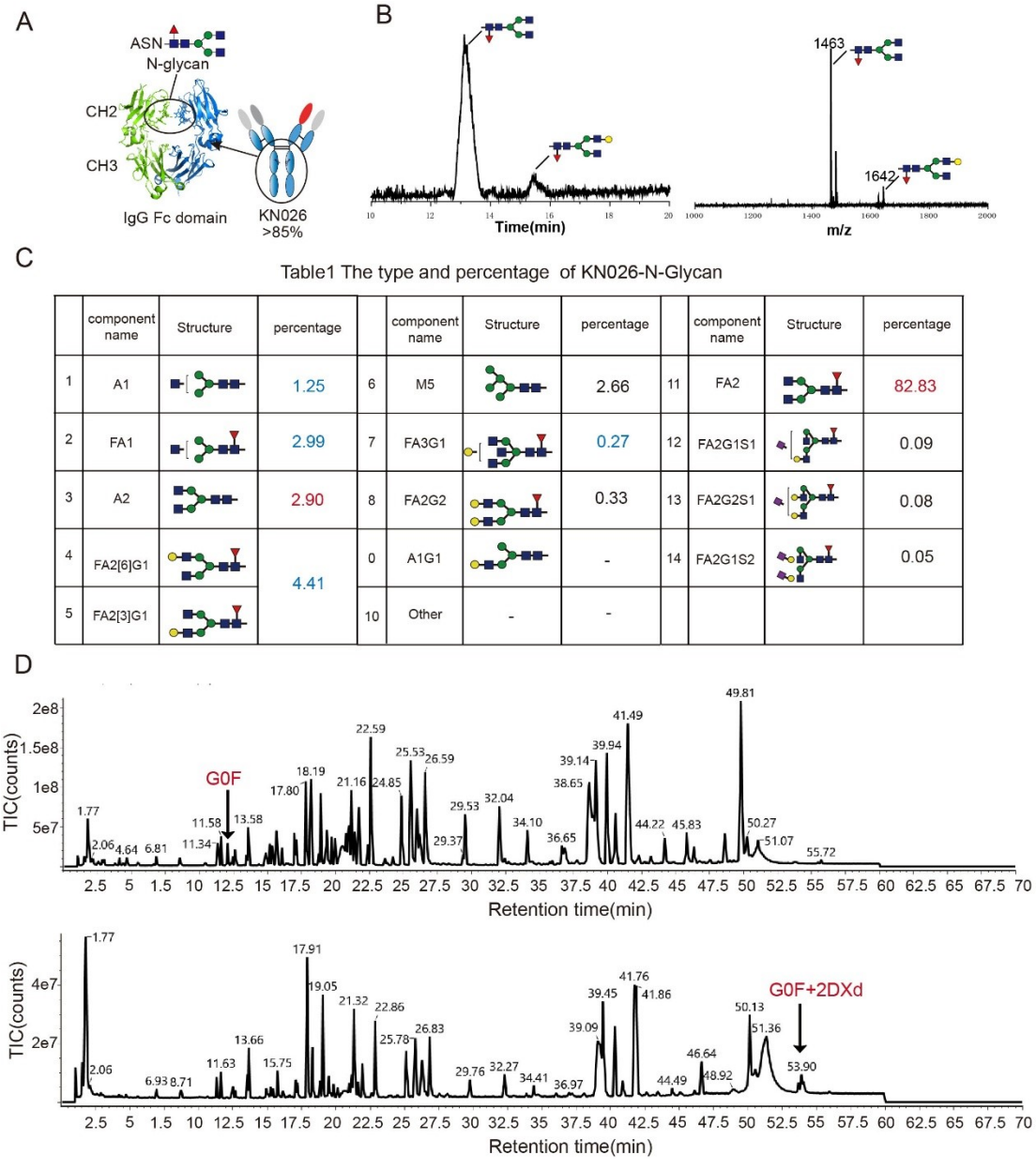


Figure S1. Glycan profile analysis of KN026 and JSKN003. (A) Schematic representation of the Fc domain of KN026. (B) LC-MS analysis of N-glycan structures enzymatically digested from KN026 (left is chromatographic trace, and right is mass spectrometry). (C) Glycan profile analysis of KN026-N-glycan. (D) Comparison of total ion chromatograms (TIC) of peptide mapping between KN026 and JSKN003.

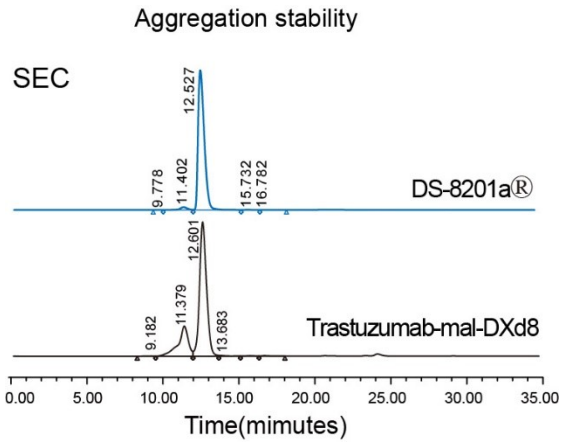


Figure S2. Size exclusion chromatography (SEC) analysis of DS-8201a and Trastuzumab-(mal-DXd)$_8$.

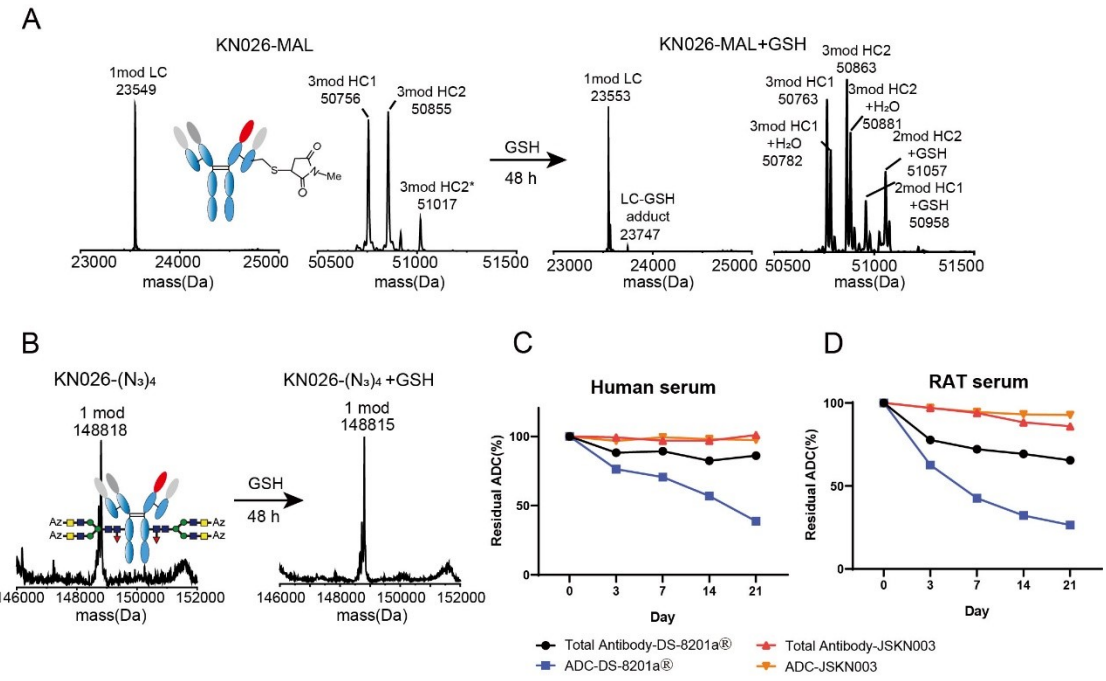


Figure S3. The chemical and serum stability of JSKN003. (A–B) LC-MS analysis of the stability of KN026-Mal and KN026-N_3 in a reducing environment following glutathione (GSH) treatment for 48 hours. (C–D) Serum stability assessment of ADCs incubated at 37°C for 0, 3, 8, 14, and 21 days. Antibody levels were quantified by

ELISA, and line charts depict temporal changes in antibody concentrations in human and rat serum.

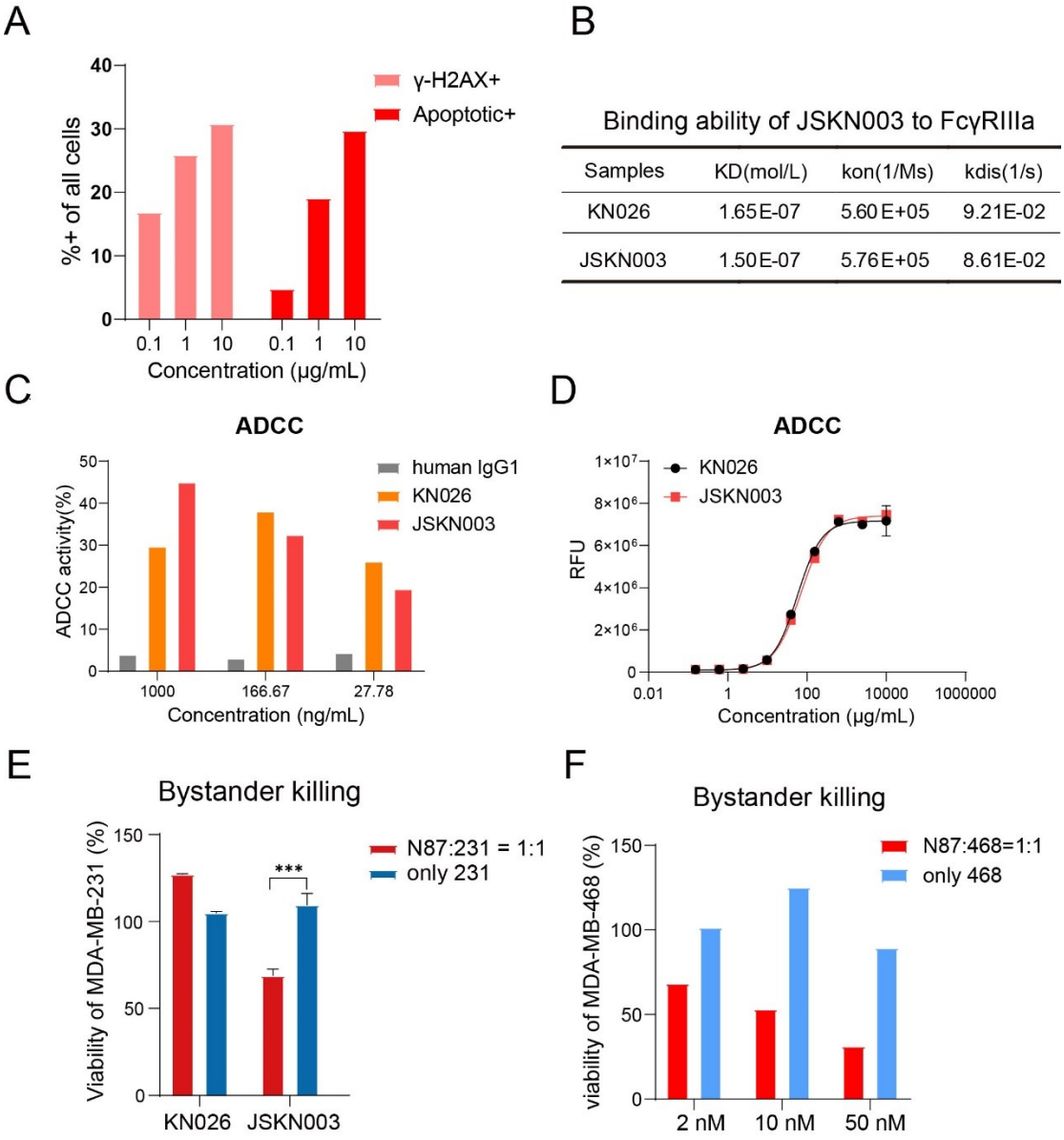


Figure S4. The mechanism of action of JSKN003. (A) Induction of DNA damage and apoptosis in NCI-N87 cells treated with increasing concentrations of JSKN003. Phosphorylation of Histone H2A.X and Annexin V/PI staining were analyzed by flow cytometry. (B) Surface plasmon resonance (SPR) analysis of KN026 and JSKN003 binding affinity to Fcγ receptors (FcγRIIIa). (C-D) Evaluation of ADCC activity of human IgG1, KN026, and JSKN003. (C) NCI-N87 cells were co-cultured with human PBMCs for 4 hours, and ADCC activity was determined by measuring LDH release. (D) NCI-N87 cells were co-cultured with Jurkat-FcγRIIIa-NFAT-luc reporter cells for

6 hours, and relative fluorescence intensity was measured to assess FcγRIIIa activation.

(E) Bystander killing effect of JSKN003 compared with KN026. Antibody/ADC treatments were administered at a final concentration of 2 μg/mL in the co-cultured group and 5 μg/mL in the MDA-MB-231-only group. (F) Evaluation of the bystander killing effect of JSKN003. ADC treatments were administered at final concentrations of 2, 20, and 50 nM in NCI-N87/MDA-MB-468 co-cultured cells and the MDA-MB-468-only group. Data are presented as mean ± SEM.; n = 3. Student's t test. n.s, not significant; *P < 0.05; **P < 0.01; ***P < 0.001.

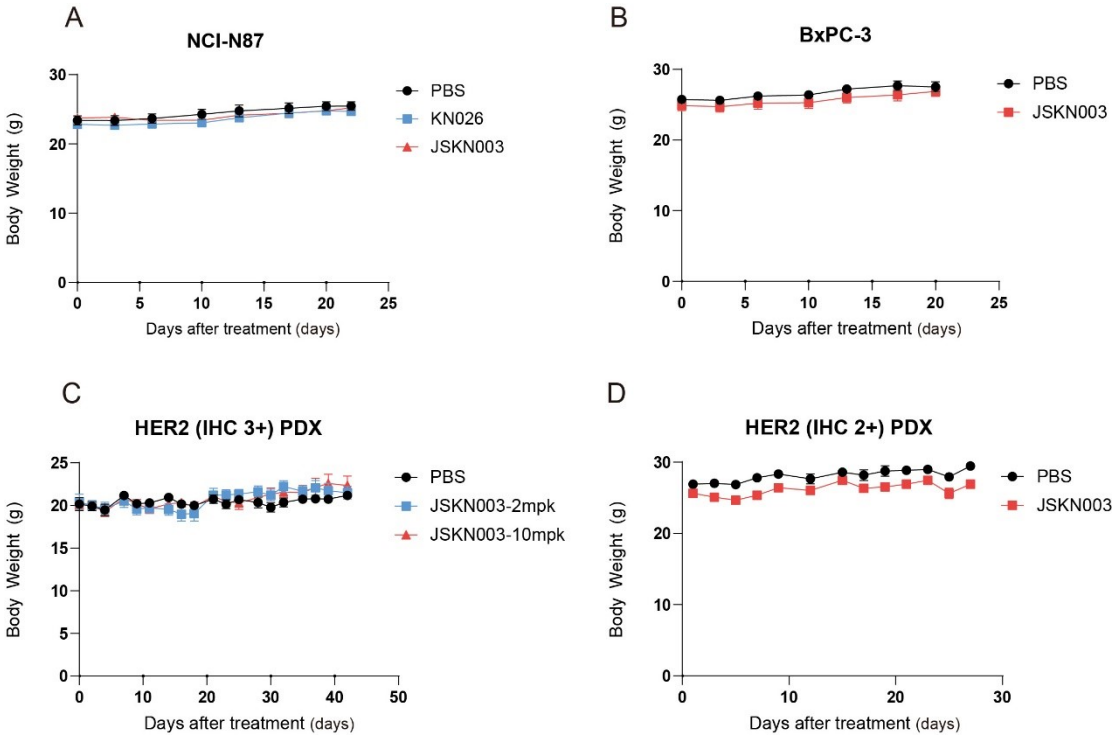
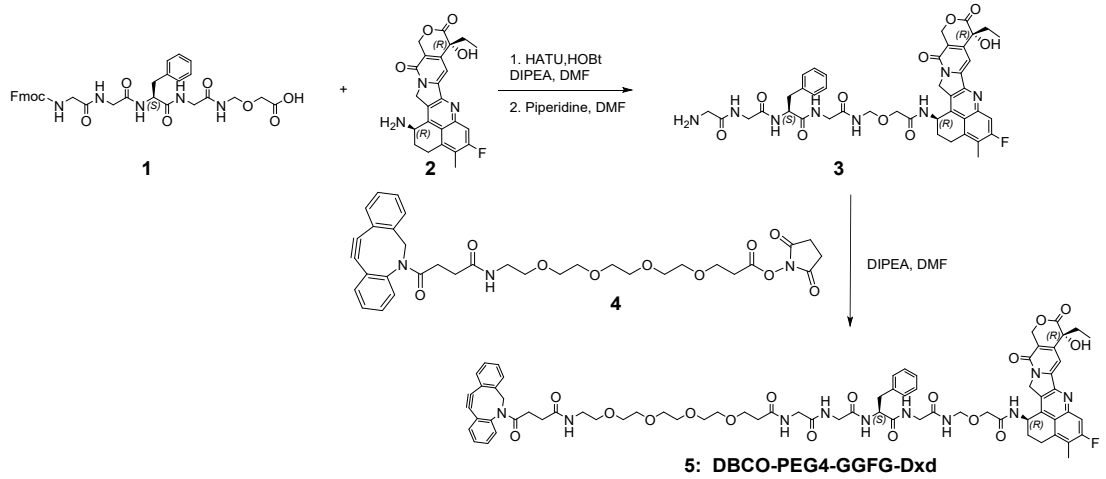


Figure S5. Body weight changes in xenograft and patient-derived xenograft (PDX) models. (A–B) Body weight measurements of mice bearing NCI-N87 (A) and BxPC-3 (B) xenograft tumors. (C–D) Body weight changes in HER2-high (C) and HER2-low (D) patient-derived xenograft (PDX) models.

Chemical synthesis



Synthesis of 3

To a mixture of 1 (10.0 mg, 1.0 eq) and 2 (4.7 mg, 1.0 eq) in DMF (2 mL), were added HATU (8.8 mg, 1.5 eq) 、HOBt (3.1 mg, 1.5 eq) and DIPEA (6 mg, 3 eq). The solution was stirred at rt for 4 h until the reaction was finished as indicated by TLC. The solution was diluted with CH_2Cl_2/H_2O (10 mL/2 mL), and the organic layer was washed with brine (3 times), dried over $MgSO_4$, concentrated to give crude product.

To the crude product were added piperidine (20%) in DMF. The solution was stirred at rt for 30 min until the reaction was finished as indicated by TLC. The solution was diluted with CH_2Cl_2/H_2O (10 mL/2 mL), and the organic layer was washed with brine (3 times), dried over $MgSO_4$, concentrated and purified with CombiFlash to give 3 (8.8 mg, 68%). **^{1}H NMR (600 MHz, DMSO-d6):** δ 8.69 (t, *J* = 6.7 Hz, 1H,NH), 8.55 -8.45 (m, 2H, NH), 8.39-8.29 (m, 2H,NH), 7.96 (s, 1H, NH), 7.79 (d, *J* = 10.8 Hz, 1H, H_{Ph}), 7.33 (s, 1H, H_{Ph}), 7.28-7.21 (m, 3H, H_{Ph}), 7.20-7.15 (m, 1H, H_{Ph}), 6.53 (s, 1H, CH), 5.60 (ddd, *J* = 8.7, 6.5, 4.6 Hz, 1H, CH), 5.49-5.38 (m, 2H, CH_2), 5.21 (d, *J* = 2.5 Hz, 2H, CH_2), 4.65 (d, *J* = 6.6 Hz, 2H, CH2), 4.55 (ddd, *J* = 9.7, 8.1, 4.6 Hz, 1H, CH), 4.03 (s, 2H, CH_2), 3.87 (dd, *J* = 16.8, 5.7 Hz, 2H, CH_2), 3.74 (qd, *J* = 16.8, 5.6 Hz, 2H, CH_2), 3.58 (d, *J* = 3.9 Hz, 2H, CH_2), 3.24-3.10 (m, 2H, CH_2), 3.04 (dd, *J* = 13.8, 4.5 Hz, 1H, CH_2), 2.75 (dd, *J* = 13.8, 9.7 Hz, 1H, CH_2), 2.40 (d, *J* = 1.8 Hz, 3H, CH_3), 2.19 (ddt, *J* = 16.6, 13.1, 7.1 Hz, 2H, CH_2), 1.87 (dtt, *J* = 21.4, 14.4, 7.3 Hz, 2H, CH_2), 0.88 (t, *J* = 7.4 Hz, 3H, CH_3); **^{13}C NMR (151 MHz, DMSO-d6):** δ 172.89, 171.82, 170.57, 169.66,

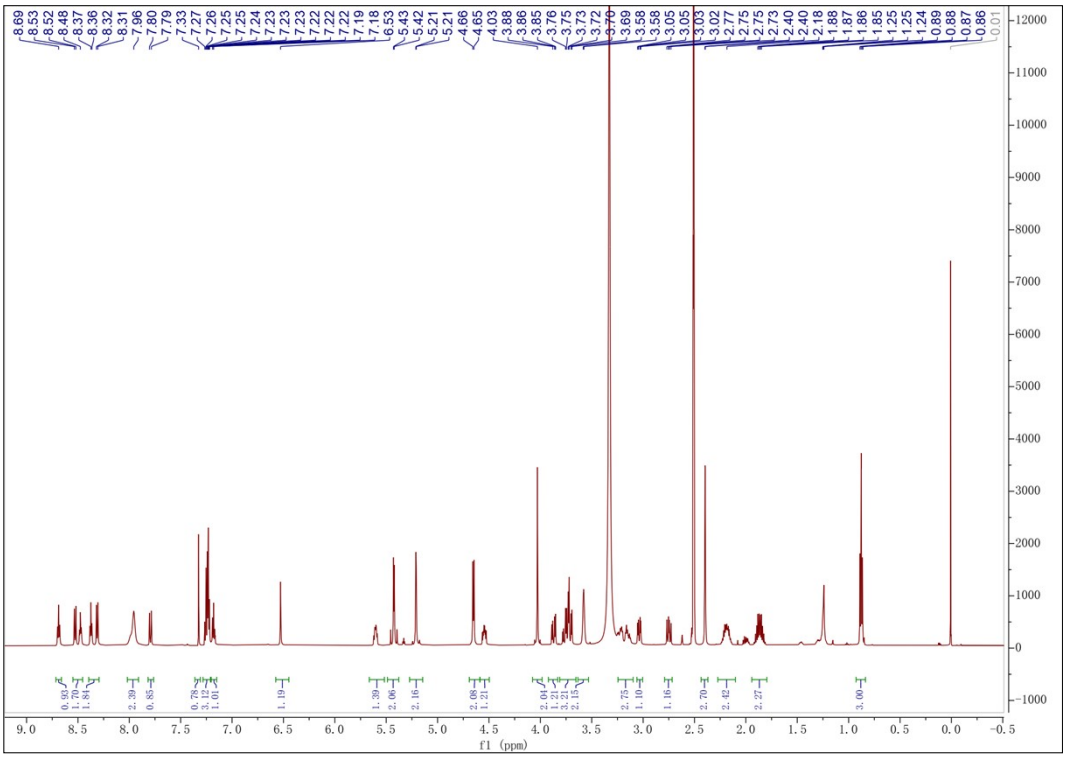
168.59, 166.57 (CO); 162.97 (CF), 161.32 (CO), 157.21, 152.90, 150.56, 148.48, 148.39, 145.71, 141.01, 138.22, 136.94 (C_q); 130.12, 129.61, 128.52, 126.76 (CH); 126.01, 124.24, 124.11, 122.23, 119.57 (C_q); 110.43 (CH), 110.28 (CH), 97.23(CH), 72.83 (C-OH), 70.22, 67.48, 65.67 (CH_2); 54.52 (CH), 50.18 (CH_2), 45.00 (CH), 42.47, 42.12, 40.54, 38.03, 30.74, 29.04, 24.06 (CH_2); 11.46, 8.22 (CH_3). **HRMS m/z (ESI):** Calcd for $C_{42}H_{46}FN_8O_{10}$ $[M+H]^+$: 841.3321, found 841.3320; Calcd for $C_{42}H_{45}FN_8O_{10}Na$ $[M+Na]^+$: 863.3140, found 863.3137.

Synthesis of 5

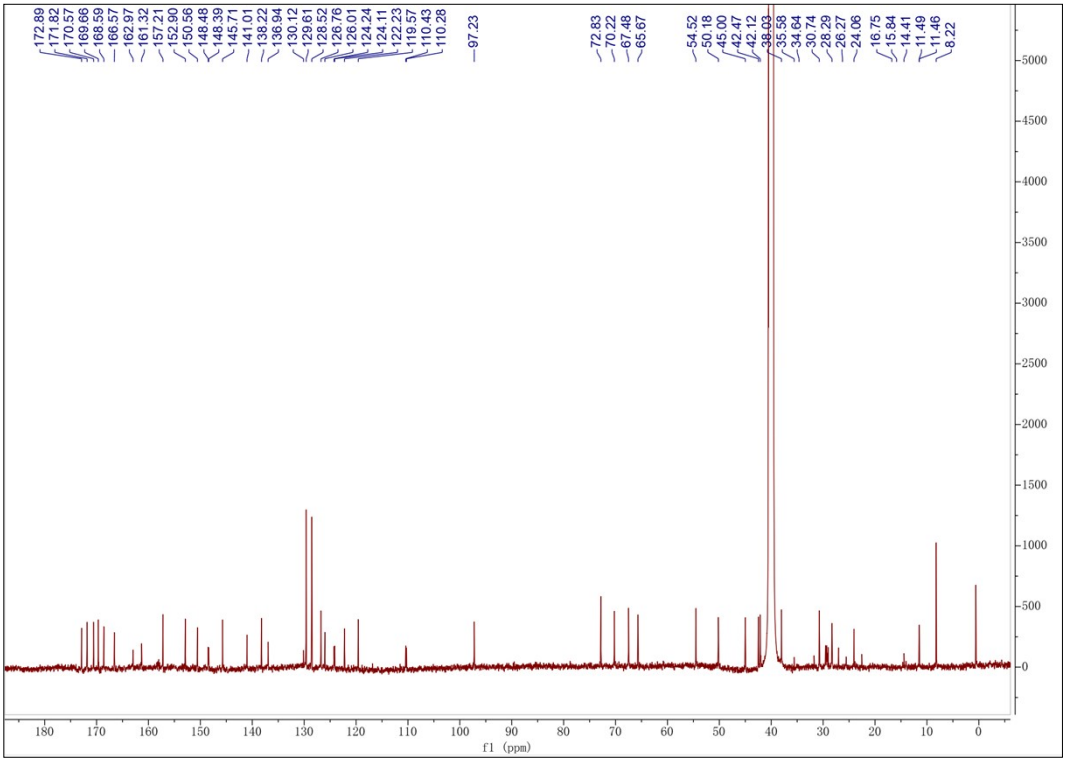
To a mixture of 3 (5 mg, 1.0 eq) and 4 (3.9 mg, 1.0 eq) in DMF (2 mL), were added DIPEA (2.3 mg, 3 eq). The solution was stirred at rt for 4 h until the reaction was finished as indicated by TLC. The solution was diluted with CH_2Cl_2/H_2O (10 mL/2 mL), and the organic layer was washed with brine (3 times), dried over $MgSO_4$, concentrated and purified with CombiFlash to give 5 (6.5 mg, 79%). **^{1}H NMR (600 MHz, DMSO-d6):** δ 8.63 (t, *J* = 6.7 Hz, 1H, NH), 8.49 (d, *J* = 8.9 Hz, 1H, NH), 8.29 (t, *J* = 5.9 Hz, 1H, NH), 8.15 (t, *J* = 5.7 Hz, 1H, NH), 8.11 (d, *J* = 8.0 Hz, 1H, NH), 8.00 (t, *J* = 5.8 Hz, 1H, NH), 7.74 (t, *J* = 5.7 Hz, 1H, H_{Ph}), 7.65 (m, 1H, H_{Ph}), 7.61 (d, *J* = 7.5 Hz, 1H, H_{Ph}), 7.51-7.40 (m, 2H, H_{Ph}), 7.40-7.14 (m, 8H, H_{Ph}), 6.51 (s, 1H, NH), 5.75 (s, 1H, CH_2), 5.59 (dt, *J* = 8.6, 6.1 Hz, 1H, CH), 5.46-5.37 (m, 2H, CH_2), 5.20-5.12 (m, 2H, CH_2), 5.02 (d, *J* = 14.1 Hz, 1H, CH_2), 4.65 (d, *J* = 6.5 Hz, 2H, CH_2), 4.48 (ddd, *J* = 9.6, 8.0, 4.6 Hz, 1H, CH_2), 4.03 (s, 2H, CH_2), 3.78-3.71 (m, 2H, CH_2), 3.69-3.57 (m, 3H, CH_2), 3.64-3.56 (m, 5H, CH_2), 3.49-3.41 (m, 10H, CH_2), 3.30-3.28 (m, 2H, CH_2), 3.23-3.18 (m, 1H, CH_2), 3.14-3.11 (m, 1H, CH_2), 3.10-3.01 (m, 3H, CH_2), 2.78 (dd, *J* = 13.8, 9.6 Hz, 1H, CH_2), 2.57 (dt, *J* = 16.0, 7.7 Hz, 1H, CH_2), 2.41-2.35 (m, 5H, CH_3+CH_2), 2.26-2.14 (m, 3H, CH_2), 2.00 (ddd, *J* = 15.4, 8.2, 5.7 Hz, 1H, CH_2), 1.89-1.73 (m, 2H, CH_2), 0.87 (t, *J* = 7.4 Hz, 3H, CH_3). **^{13}C NMR (151 MHz, DMSO-d6):** δ 172.89, 171.83, 171.58, 171.50, 171.20, 170.60, 169.77, 169.66, 169.32 (CO); 162.91 (CF), 161.26 (CO), 157.16, 152.83, 152.05, 150.49, 148.88, 148.43, 148.33, 145.65, 140.98, 138.24, 136.86 (C_q); 132.86, 130.06, 129.58, 129.37, 128.57, 128.53, 128.43, 128.11, 127.23, 126.73 (CH); 125.89 (C_q), 125.59 (CH), 124.19, 124.06,

122.98, 122.16, 121.86, 119.55, 114.67 (C_q); 110.37, 110.22 (CH); 108.60 (C_q), 97.18 (CH), 72.82 (C-OH), 70.21, 70.13, 70.09, 69.99, 69.45, 67.46, 67.11, 65.69, 55.34 (CH_2); 54.66 (CH), 50.12 (CH_2), 45.03 (CH), 42.57, 42.29 ,38.95, 37.74, 36.32, 30.80, 30.17, 28.28, 24.09 (CH_2); 11.41, 8.21 (CH_3). **HRMS m/z (ESI):** Calcd for $C_{72}H_{80}FN_{10}O_{17}$ $[M+H]^+$: 1375.5687, found 1375.5642; Calcd for $C_{72}H_{79}FN_{10}O_{17}Na$ $[M+Na]^+$: 1397.5506, found 1398.5540.

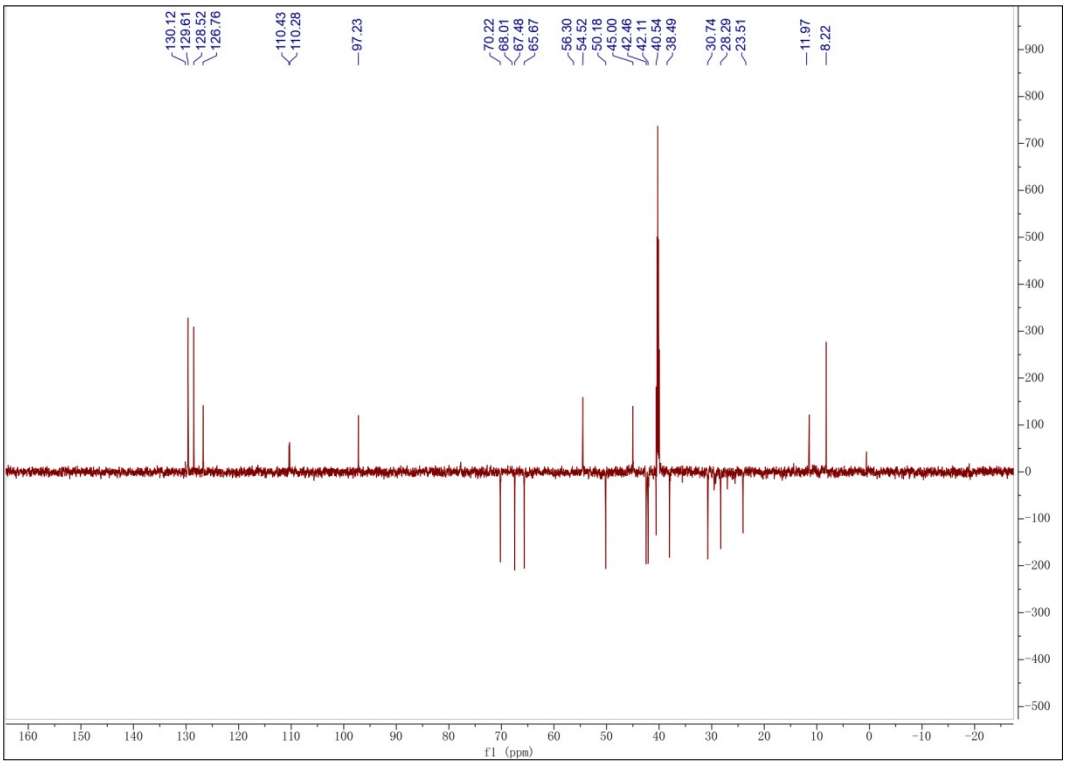
^{1}H NMR of 3 in DMSO-d6 at 600 MHz



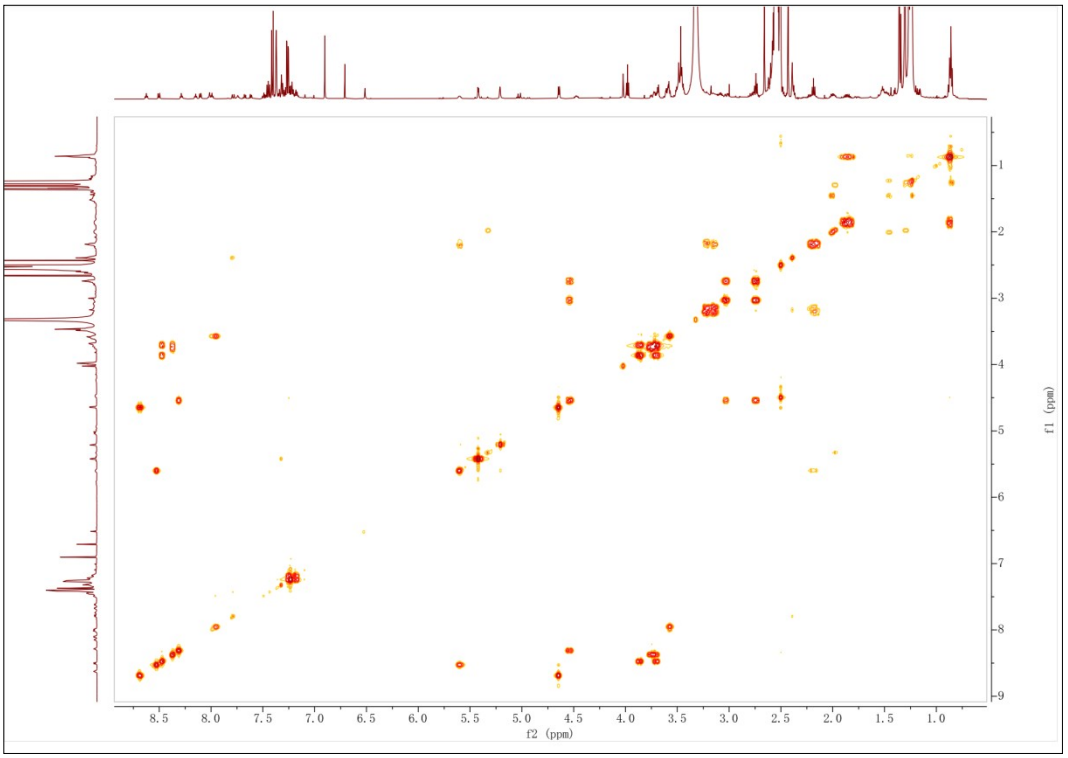
^{13}C NMR of 3 in DMSO-d6 at 151 MHz



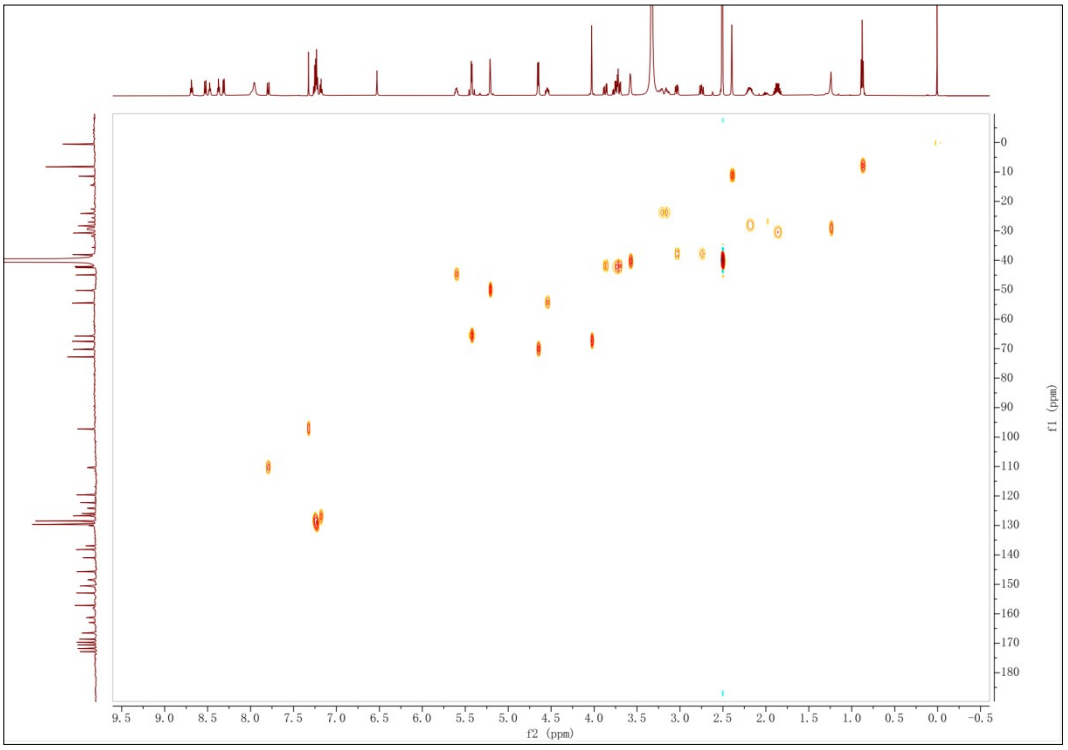
DEPT NMR of 3 in DMSO-d6 at 151 MHz



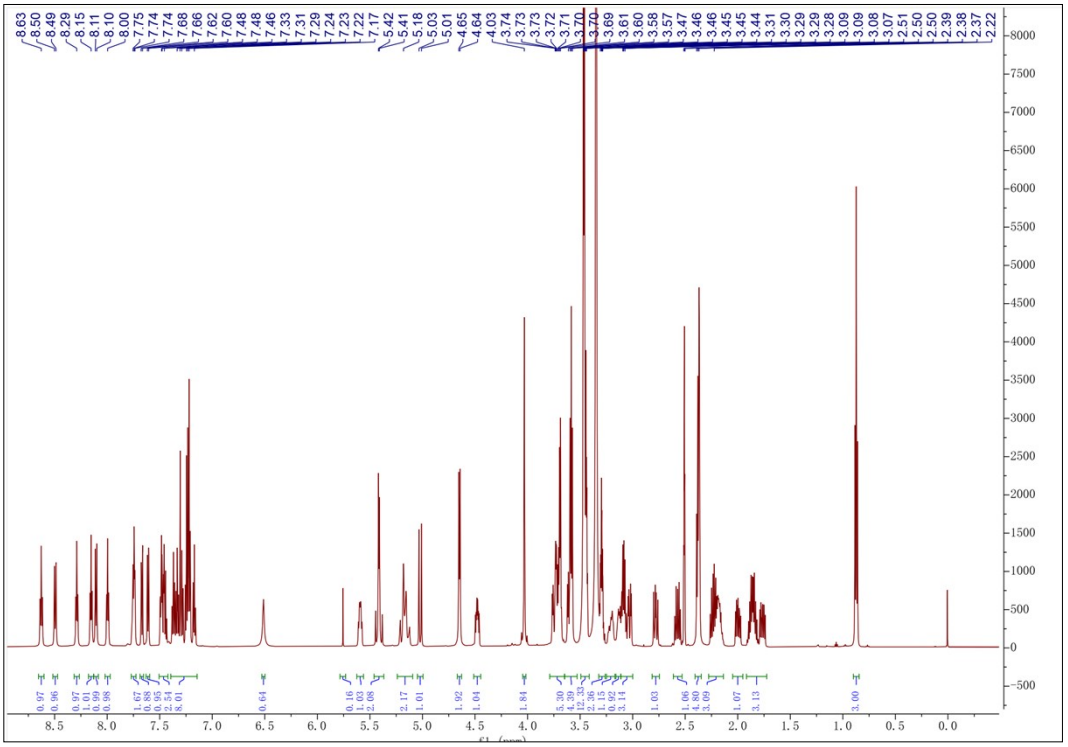
COSY NMR of 3 in DMSO-d6 at 600 MHz



HMQC NMR of 3 in DMSO-d6



^{1}H NMR of 5 in DMSO-d6 at 600 MHz



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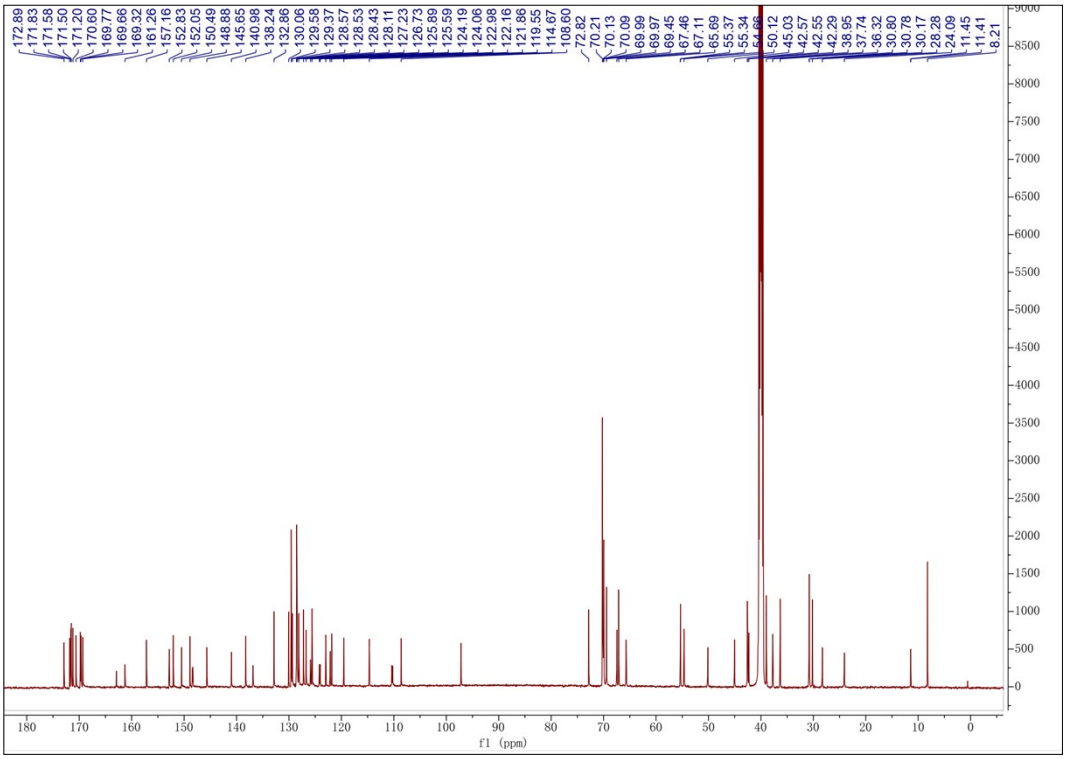
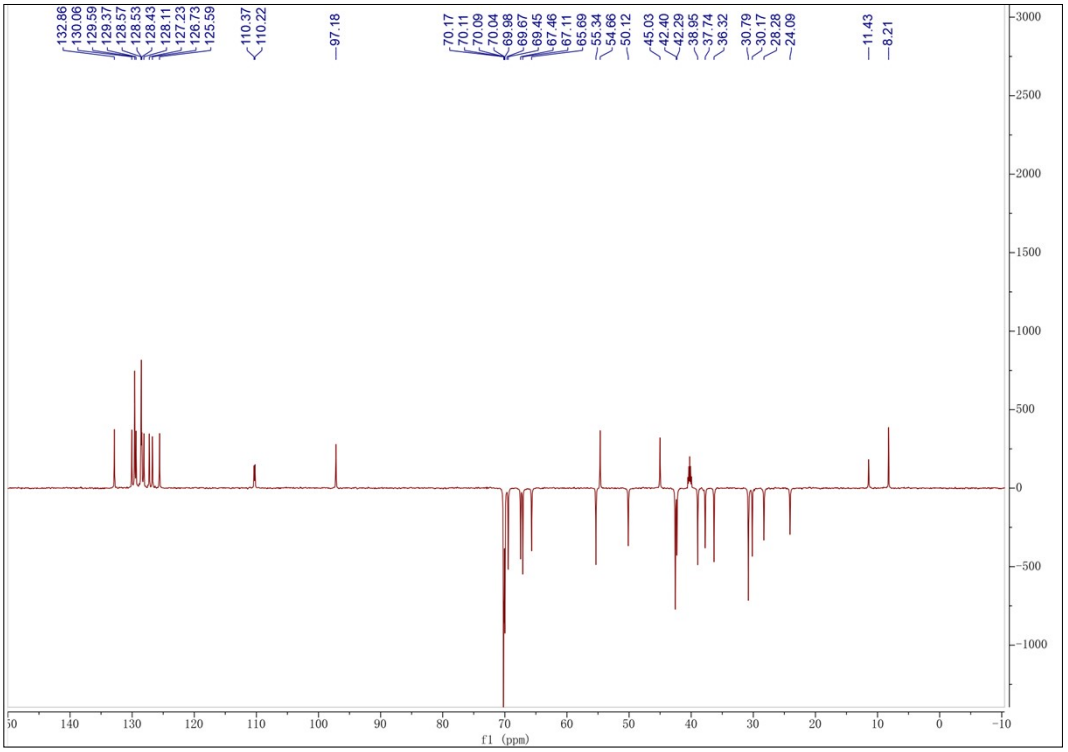


DEPT NMR of 5 in DMSO-d6 at 151 MHz



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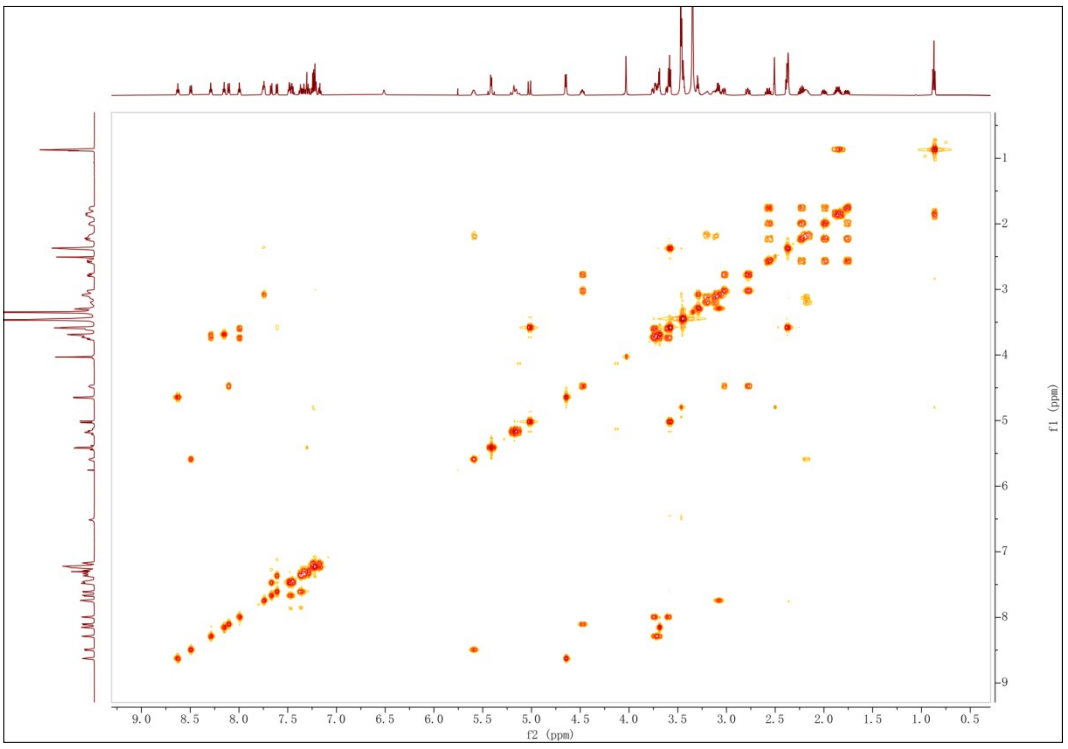

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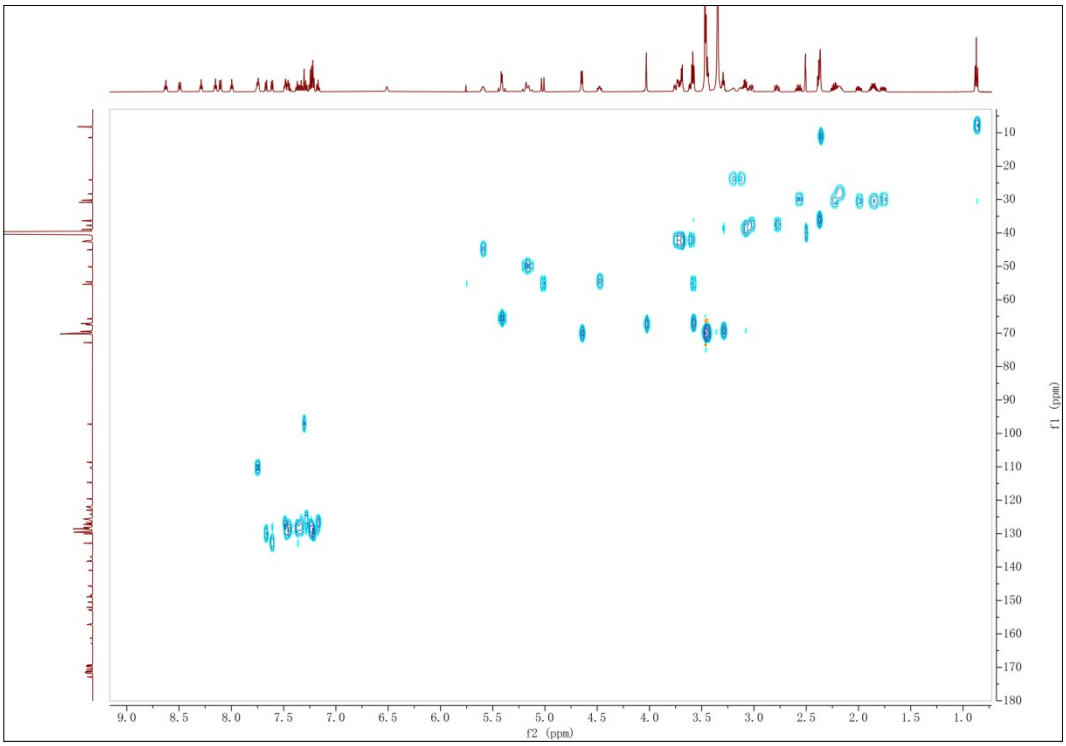
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