Targeting tubulin C-terminal tail by charged small molecules

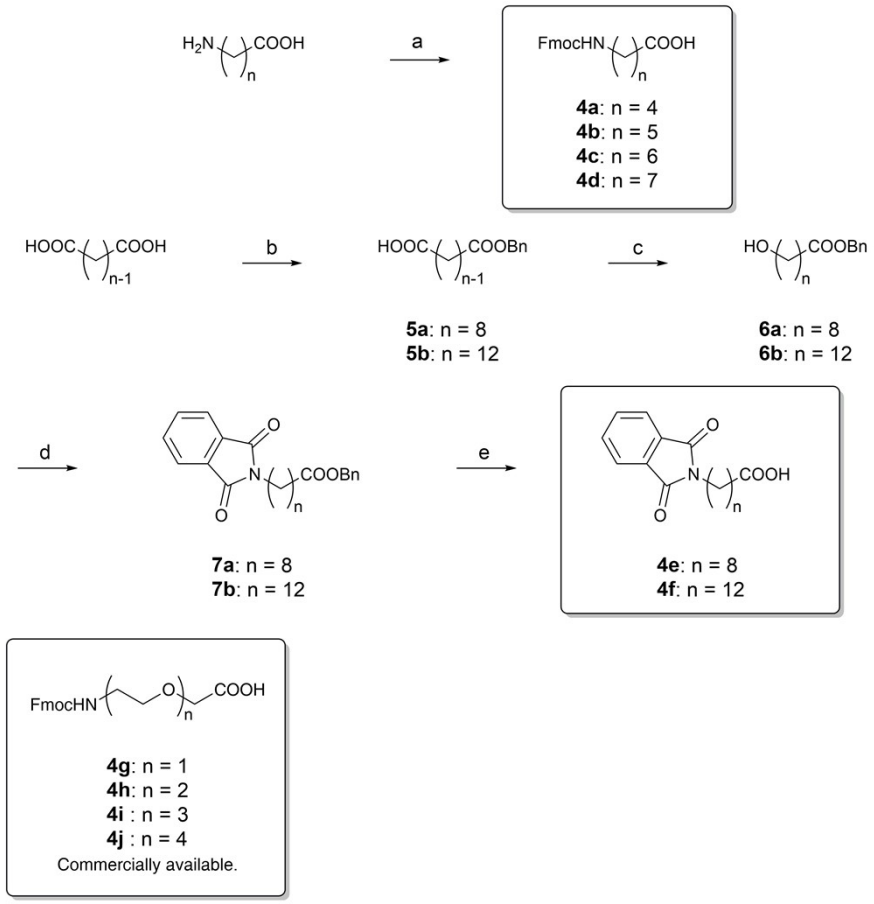
Supporting information

Shuo Li, Mattia Mori, Mingyan Yang, Soumia Elfazazi, Rafael Hortigüela, Peter Chan, Xinyue Feng, April Risinger, Zhiyou Yang, María Ángela Oliva, J. Fernando Díaz, Wei-Shuo Fang

Chemistry.

1. General experimental procedures.

The ^{1}H and ^{13}C NMR spectra were recorded by JEOL ECZ-400S spectrometer (400 MHz), WNMR-I 500 spectrometer (500 MHz), Bruker AVANCE III 400 spectrometer (400 MHz) and Varian INOVA-500 spectrometer (500 MHz). The mass spectra were recorded by Thermo Exactive Plus series mass spectrometer. Reagents were purchased from Beijing Tongguang Chemicals Co. Ltd., Beijing Innochem Chemicals Co. Ltd., Beijing J&K Chemicals Co. Ltd. and Tianjin Heowns Chemicals Co. Ltd. Specifically, resins were purchsed from Beijing J&K Chemicals Co. Ltd. All reagents were directly used without further purification. Reactions were monitored by thin-layer chromatography (silica gel, GF254). Most compounds were purified by flash chromatography on Biotage Isolera One purification system. The final products were purified on Shimadzu LC-6AD preparative HPLC.



Scheme S1 Preparation of the linkers.

Reagents and conditions: (a) FmocOSu, $NaHCO_3$, THF, H_2O, r.t., 47-89%; (b) BnBr, DBU, DCM, 0 ºC to r.t., 47-64%; (c) BH_3-THF (1 M), THF, 0 ºC, 84-88%; (d) PhthNH, PPh_3, DEAD, THF, 0 ºC to

r.t., 82-87%; (e) 10% Pd/C, H_2, MeOH, r.t., 70-73%.

2. Synthesis of the alkyl linkers **4a**-**4f**.

5-((Fluorenylmethyloxycarbonyl)amino)pentanoic acid (**4a**).

To a stirred solution of 5-aminopentanoic acid (3 mmol, 0.35 g) in water (15 mL) was added $NaHCO_3$ (7.5 mmol, 0.63 g) and FmocOSu (3 mmol, 1.01 g) dissolved in THF (15 mL). The reaction mixture was stirred at room temperature for 5 h, acidified to pH = 1 by concentrated HCl and extracted with ethyl acetate. The organic phase was dried by Na_2SO_4 and concentrated. The residue was purified by flash chromatography (petroleum ether-ethyl acetate, 90: 10 for one column volume, 90: 10 to 60: 40 for ten column volumes and 60: 40 for the rest procedure). White solid (0.70 g, 69%).

^{1}H NMR (400 MHz, DMSO-d_6) δ 12.00 (s, 1H, COOH), 7.88 (d, J = 7.6 Hz, 2H, Ar), 7.68 (d, J = 7.6 Hz, 2H, Ar), 7.41 (t, J = 7.6 Hz, 2H, Ar), 7.32 (td, J = 7.6 Hz, 1.2 Hz, 2H, Ar), 7.28 (t, J = 5.6 Hz, 1H, NH), 4.29 (d, J = 7.2 Hz, 2H, OCH_2), 4.20 (t, J = 6.8 Hz, 1H, CH), 2.97 (q, J = 6.4 Hz, 2H, NCH_2), 2.20 (t, J = 7.2 Hz, 2H, $COCH_2$), 1.51-1.33 (m, 4H, CH_2).

6-((Fluorenylmethyloxycarbonyl)amino)hexanoic acid (**4b**).

To a stirred solution of 6-aminohexanoic acid (6 mmol, 0.79 g) in water (30 mL) was added $NaHCO_3$ (15 mmol, 1.26 g) and FmocOSu (6 mmol, 2.02 g) dissolved in THF (30 mL). The reaction mixture was stirred at room temperature for 4 h, acidified to pH = 1 by concentrated HCl and extracted with ethyl acetate. The organic phase was dried by Na_2SO_4 and concentrated. The residue was purified by flash chromatography (petroleum ether-ethyl acetate, 90: 10 for one column volume, 90: 10 to 60: 40 for ten column volumes and 60: 40 for the rest procedure). White solid (1.89 g, 89%).

^{1}H NMR (400 MHz, DMSO-d_6) δ 11.98 (s, 1H, COOH), 7.88(d, J = 7.6 Hz, 2H, Ar), 7.68 (d, J = 7.2 Hz, 2H, Ar), 7.41 (t, J = 7.2 Hz, 2H, Ar), 7.33 (td, J = 7.6 Hz, 1.2 Hz, 2H, Ar), 7.25 (t, J = 5.6 Hz, 1H, NH), 4.29 (d, J = 6.8 Hz, 2H, OCH_2), 4.20 (t, J = 6.8 Hz, 1H, CH), 2.96 (q, J = 6.8 Hz, 2H, NCH_2), 2.19 (t, J = 7.2 Hz, 2H, $COCH_2$), 1.52-1.35 (m, 4H, CH_2), 1.28-1.20 (m, 2H, CH_2).

7-((Fluorenylmethyloxycarbonyl)amino)heptanoic acid (**4c**).

To a stirred solution of 7-aminoheptanoic acid (3 mmol, 0.44 g) in water (15 mL) was added $NaHCO_3$ (7.5 mmol, 0.63 g) and FmocOSu (3 mmol, 1.01 g) dissolved in THF (15

mL). The reaction mixture was stirred at room temperature for 5 h, acidified to pH = 1 by concentrated HCl and extracted with ethyl acetate. The organic phase was dried by Na_2SO_4 and concentrated. The residue was purified by flash chromatography (petroleum ether-ethyl acetate, 90: 10 for one column volume, 90: 10 to 60: 40 for ten column volumes and 60: 40 for the rest procedure). White solid (0.51 g, 47%).

^{1}H NMR (500 MHz, CD_3OD) δ 7.79 (d, J = 7.0 Hz, 2H, Ar), 7.64 (d, J = 7.0 Hz, 2H, Ar), 7.38 (t, J = 7.0 Hz, 2H, Ar), 7.31 (t, J = 6.5 Hz, 2H, Ar), 4.53 (s, 1H, NH), 4.35 (d, J = 6.0 Hz, 2H, OCH_2), 4.19 (m, 1H, CH), 3.09 (m, 2H, NCH_2), 2.28 (t, J = 6.5 Hz, 2H, $COCH_2$), 1.65-1.43 (m, 4H, CH_2), 1.40-1.27 (m, 4H, CH_2).

8-((Fluorenylmethyloxycarbonyl)amino)octanoic acid (**4d**).

To a stirred solution of 8-aminooctanoic acid (6 mmol, 0.96 g) in water (30 mL) was added $NaHCO_3$ (15 mmol, 1.26 g) and FmocOSu (6 mmol, 2.02 g) dissolved in THF (30 mL). The reaction mixture was stirred at room temperature for 4 h, acidified to pH = 1 by concentrated HCl and extracted with ethyl acetate. The organic phase was dried by Na_2SO_4 and concentrated. The residue was purified by flash chromatography (petroleum ether-ethyl acetate, 90: 10 for one column volume, 90: 10 to 60: 40 for ten column volumes and 60: 40 for the rest procedure). White solid (1.57 g, 69%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76 (d, J = 7.5 Hz, 2H, Ar), 7.59 (d, J = 7.5 Hz, 2H, Ar), 7.40 (t, J = 7.5 Hz, 2H, Ar), 7.31 (t, J = 7.0 Hz, 2H, Ar), 4.75 (s, 1H, NH), 4.40 (d, J = 6.5 Hz, 2H, OCH_2), 4.22 (t, J = 6.5 Hz, 1H, CH), 3.18 (q, J = 6.5 Hz, 2H, NCH_2), 2.35 (t, J = 7.5 Hz, 2H, $COCH_2$), 1.70-1.44 (m, 4H, CH_2), 1.40-1.27 (m, 6H, CH_2).

9-Benzyloxy-9-oxononanoic acid (**5a**).

To a stirred solution of nonanedioic acid (20 mmol, 3.76 g) in THF (25 mL) was added DBU (20 mmol, 3 mL) and BnBr (10 mmol, 1.2 mL) at 0 ºC. The reaction mixture was stirred at room temperature for 24 h, diluted with water and extracted with DCM. The organic phase was dried by Na_2SO_4 and concentrated. The residue was purified by flash chromatography (petroleum ether-ethyl acetate, 100: 0 for one column volumes, 100: 0 to 85: 15 for ten column volume and 85: 15 for the rest procedure). Colorless oil (1.31 g, 47%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39-7.30 (m, 5H, Ar), 5.12 (s, 2H, Bn CH_2), 2.34 (q, J = 7.6 Hz, 4H, $COCH_2$), 1.68-1.58 (m, 4H, CH_2), 1.38-1.25 (m, 6H, CH_2).

13-Benzyloxy-13-oxotridecanoic acid (**5b**).

To a stirred solution of tridecanedioic acid (20 mmol, 4.89 g) in THF (25 mL) was added DBU (20 mmol, 3 mL) and BnBr (10 mmol, 1.2 mL) at 0 ºC. The reaction mixture was stirred at room temperature for 24 h, diluted with water and extracted with DCM. The organic phase was dried by Na_2SO_4 and concentrated. The residue was purified by flash chromatography (petroleum ether-ethyl acetate, 100: 0 for one column volume, 100: 0 to 85: 15 for ten column volumes and 85: 15 for the rest procedure). White solid (2.16 g, 64%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38-7.30 (m, 5H, Ar), 5.12 (s, 2H, Bn CH_2), 2.37-2.31 (m, 4H, $COCH_2$), 1.69-1.57 (m, 4H, CH_2), 1.38-1.21 (brs, 14H, CH_2).

Benzyl 9-hydroxynonanoate (**6a**).

To a stirred solution of **5a** (4.65 mmol, 1.29 g) in THF (25 mL) was added 1 M BH_3-THF (6.98 mmol, 7 mL) at 0 ºC. The reaction mixture was stirred at 0 ºC for 1 h and 1 M BH_3-THF (2.33 mmol, 2.3 mL) was added again. The reaction mixture was stirred at 0 ºC for another 1 h, diluted with brine and extracted with ethyl acetate. The organic phase was dried by Na_2SO_4 and concentrated. The residue was purified by flash chromatography (petroleum ether-ethyl acetate, 90: 10 for one column volume, 90: 10 to 84: 16 for ten column volumes and 84: 16 for the rest procedure). Colorless oil (1.04 g, 84%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39-7.29 (m, 5H, Ar), 5.11 (s, 2H, Bn CH_2), 3.62 (t, J = 6.4 Hz, 2H, OCH_2), 2.35 (t, J = 7.2 Hz, 2H, $COCH_2$), 1.67-1.50 (m, 4H, CH_2), 1.35-1.27 (brs, 8H, CH_2).

Benzyl 13-hydroxytridecanoate (**6b**).

To a stirred solution of **5b** (6.43 mmol, 2.15 g) in THF (30 mL) was added 1 M BH_3-THF (9.64 mmol, 9.6 mL) at 0 ºC. The reaction mixture was stirred at 0 ºC for 1 h and 1 M BH_3-THF (3.21 mmol, 3.2 mL) was added again. The reaction mixture was stirred at 0 ºC for another 1 h, diluted with brine and extracted with ethyl acetate. The organic phase was dried by Na_2SO_4 and concentrated. The residue was purified by flash chromatography (petroleum ether-ethyl acetate, 90: 10 for one column volume, 90: 10 to 84: 16 for ten column volumes and 84: 16 for the rest procedure). White solid (1.81 g, 88%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38-7.29 (m, 5H, Ar), 5.11 (s, 2H, Bn CH_2), 3.63 (t, J = 6.4 Hz, 2H, OCH_2), 2.35 (t, J = 7.2 Hz, 2H, $COCH_2$), 1.67-1.52 (m, 4H, CH_2), 1.38-1.23 (brs, 16H, CH_2).

Benzyl 9-(1,3-dioxoisoindolin-2-yl)nonanoate (**7a**).

To a stirred solution of **6a** (3.90 mmol, 1.03 g) in THF (40 mL) was added phthalimide (4.68 mmol, 0.69 g), PPh_3 (4.68 mmol, 1.23 g) and DEAD (4.68 mmol, 680 μL) at 0 ºC. The reaction mixture was stirred at room temperature for 12 h and concentrated. The residue was purified by flash chromatography (petroleum ether-ethyl acetate, 100: 0 for one column volume, 100: 0 to 90: 10 for ten column volumes and 90: 10 for the rest procedure). Colorless oil (1.33 g, 87%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.85-7.80 (m, 4H, Ar), 7.72-7.67 (m, 4H, Ar), 7.39-7.28 (m, 5H, Bn Ar), 5.10 (s, 2H, Bn CH_2), 3.66 (t, J = 7.2 Hz, 2H, NCH_2), 2.34 (t, J = 7.6 Hz, 2H, $COCH_2$), 1.68-1.59 (m, 4H, CH_2), 1.35-1.25 (m, 8H, CH_2); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.7, 168.6 (2C), 136.3, 133.9 (2C), 132.3, 128.6 (2C), 128.3 (4C), 123.3 (2C), 66.2, 38.1, 34.4, 29.2, 29.1 (2C), 28.7, 26.9, 25.0; ESI-MS *m/z* $[M + H]^+$ 394.2, $[M + Na]^+$ 416.2.

Benzyl 13-(1,3-dioxoisoindolin-2-yl)tridecanoate (**7b**).

To a stirred solution of **6b** (5.65 mmol, 1.87 g) in THF (55 mL) was added phthalimide (6.78 mmol, 1.00 g), PPh_3 (6.78 mmol, 1.78 g) and DEAD (6.78 mmol, 980 μL) at 0 ºC. The reaction mixture was stirred at room temperature for 12 h and concentrated. The residue was purified by flash chromatography (petroleum ether-ethyl acetate, 100: 0 for one column volume, 100: 0 to 90: 10 for ten column volumes and 90: 10 for the rest procedure). White solid (2.07 g, 82%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.86-7.81 (m, 4H, Ar), 7.72-7.67 (m, 4H, Ar), 7.38-7.28 (m, 5H, Bn Ar), 5.11 (s, 2H, Bn CH_2), 3.67 (t, J = 7.2 Hz, 2H, NCH_2), 2.34 (t, J = 7.6 Hz, 2H, $COCH_2$), 1.70-1.59 (m, 4H, CH_2), 1.36-1.20 (m, 16H, CH_2); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.8, 168.6 (2C), 136.3, 134.0 (2C), 132.3, 128.7 (2C), 128.3 (4C), 123.3 (2C), 66.2, 38.2, 34.5, 29.6 (3C), 29.5, 29.4, 29.3 (2C), 28.7, 27.0, 25.1; ESI-MS *m/z* $[M + Na]^+$ 472.2, $[M + K]^+$ 488.2.

9-(1,3-Dioxoisoindolin-2-yl)nonanoic acid (**4e**).

To a stirred solution of **7a** (3.38 mmol, 1.33 g) in methanol (34 mL) was added 10% Pd/C (130 mg). The reaction mixture was stirred at room temperature for 16 h under H_2 atmosphere, filtered, washed with methanol and DCM, and concentrated. The residue was purified by flash chromatography (petroleum ether-ethyl acetate, 70: 30). White solid (0.72

g, 70%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.86-7.81 (m, 2H, Ar), 7.73-7.68 (m, 2H, Ar), 3.67 (t, J = 7.2 Hz, 2H, NCH_2), 2.33 (t, J = 7.6 Hz, 2H, $COCH_2$), 1.70-1.58 (m, 4H, CH_2), 1.37-1.25 (m, 8H, CH_2); ^{13}C NMR (100 MHz, $CDCl_3$) δ 179.1, 168.6 (2C), 134.0 (2C), 132.3 (2C), 123.3 (2C), 38.2, 34.0, 29.2, 29.1 (2C), 28.7, 26.9, 24.7; ESI-MS *m/z* $[M + H]^+$ 304.2.

13-(1,3-Dioxoisoindolin-2-yl)tridecanoate acid (**4f**).

To a stirred solution of **7b** (4.60 mmol, 2.07 g) in methanol (46 mL) was added 10% Pd/C (210 mg). The reaction mixture was stirred at room temperature for 16 h under H_2 atmosphere, filtered, washed with methanol and DCM, and concentrated. The residue was purified by flash chromatography (petroleum ether-ethyl acetate, 70: 30). White solid (1.20 g, 73%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.86-7.81 (m, 2H, Ar), 7.72-7.68 (m, 2H, Ar), 3.67 (t, J = 7.2 Hz, 2H, NCH_2), 2.34 (t, J = 7.6 Hz, 2H, $COCH_2$), 1.70-1.58 (m, 4H, CH_2), 1.39-1.22 (m, 16H, CH_2); ^{13}C NMR (100 MHz, $CDCl_3$) δ 179.0, 168.7 (2C), 134.0 (2C), 132.3 (2C), 123.3 (2C), 38.2, 34.0, 29.6 (3C), 29.5, 29.3 (2C), 29.2, 28.7, 27.0, 24.8; ESI-MS *m/z* $[M + H]^+$ 360.2.

3. Synthesis of linear and cyclic peptides containing RRR sequence.

General procedure A (Elongation of the peptide sequence). 2-CTC resin (0.12 mmol, 100 mg) was added to the solid phase synthetic tube and was swollen by DMF (5 mL) for 30 min. To the resin was added protected amino acid (0.5 mmol) dissolved in DMF (2 mL) and DIEA (1 mmol, 165 μL) and the reaction mixture was bubbled for 3 h. To the resin was subsequently added methanol (3 mL) and DIEA (1 mmol, 165 μL) after the solvent was removed and the reaction mixture was bubbled for another 30 min. For any coupling step, to a solution of protected amino acid (0.5 mmol) in DMF (5 mL) was added HBTU (0.5 mmol, 186 mg), HOBt (0.5 mmol, 68 mg) and DIEA (1 mmol, 165 μL). The solution was subsequently added to the resin and the reaction mixture was bubbled for 1 h. For any Fmoc removal step, to the resin was added 20% piperidine/DMF (5 mL) and the reaction mixture was bubbled for 30 min. For any Phth removal step, to the resin was added 60% hydrazine hydrate/DMF and the reaction mixture was bubbled for 2 h. The resin was washed with DMF after solvent removal for each step. At the end of the solid phase procedure, the resin was washed with DCM.

General procedure B (Cleavage of the side chain-protected peptides from resin). To

the resin was added 20% HFIP/DCM (5 mL) and the reaction mixture was bubbled for 2 h, filtered and concentrated. The residue was purified by flash chromatography (ethyl acetate-methanol, 100: 0 for ten column volumes, 100: 0 to 50: 50 for ten column volumes and 50: 50 for the rest procedure).

General procedure C (Cyclization). To a stirred solution of HATU (1 mmol, 380 mg) and DIEA (1 mmol, 165 μL) in DMF (25 mL) was added dropwise the cyclization precursor dissolved in DMF (25 mL) at 0 ºC. The reaction mixture was stirred at 0 ºC or room temperature for 1 h, diluted with ethyl acetate and washed with brine. The organic phase was dried by Na_2SO_4 and concentrated. The residue was purified by flash chromatography (ethyl acetate-methanol, 100: 0 for ten column volumes, 100: 0 to 84: 16 for ten column volumes and 84: 16 for the rest procedure).

General procedure D (Side chain deprotection of the cyclized intermediates). To the cyclized intermediate was added cleavage cocktail (TFA: TIS: H_2O = 95: 2.5: 2.5, 3 mL) at 0 ºC. The reaction mixture was stirred at 0 ºC for 2 h and titrated into cold ether. The precipitate was collected by centrifugation (3000 rpm, 5 min), washed with ether and dried *in vacuo*. The residue was dissolved in water-acetonitrile (1: 1), filtered through a millipore filter and purified by preparative HPLC. Column: Greenherb GH0525010 C18AQ (250 mm × 10 mm, 5 μm). Mobile phase A: water (0.1% TFA). Mobile phase B: acetonitrile (0.1% TFA). Flow rate: 3 mL/min. Detector wave length: 220 nm. The purification method was t = 0, B 0%; t = 30 min, B 50%; t = 60 min, B 50%.

General procedure E (Cleavage and side chain deprotection of the linear peptides). To the resin was added cleavage cocktail (TFA: TIS: H_2O = 95: 2.5: 2.5, 3 mL) at 0 ºC. The reaction mixture was stirred at 0 ºC for 2 h and titrated into cold ether. The precipitate was collected by centrifugation (3000 rpm, 5 min), washed with ether and dried *in vacuo*. The residue was dissolved in water-acetonitrile (1: 1), filtered through a millipore filter and purified by preparative HPLC. Column: Greenherb GH0525010 C18AQ (250 mm × 10 mm, 5 μm). Mobile phase A: water (0.1% TFA). Mobile phase B: acetonitrile (0.1% TFA). Flow rate: 3 mL/min. Detector wave length: 220 nm. The purification method for each compound is shown below.

cyclo[-Arg-5-aminopentanoyl-Arg-Arg-] (**1a**).

The compound was synthesized following general procedure A, B, C and D. In general procedure A, **4a** was used as the building block of the linker. In general procedure D, the eluent at 14.259 min was collected during the purification procedure. Acetonitrile

was removed under reduced pressure and the aqueous phase was lyophilized. White solid (45.4 mg, 42% when calculated as ×3 TFA salt).

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.16 (d, J = 7.6 Hz, 1H, amide NH), 8.09 (d, J = 8.0 Hz, 1H, amide NH), 7.97 (d, J = 8.4 Hz, 1H, amide NH), 7.55-6.68 (m, 13H, amide NH, guanidine H), 4.12 (dd, J = 14.8 Hz, 8.4 Hz, 1H, Arg αH), 4.03-3.94 (m, 2H, Arg αH), 3.38-3.25 (m, 1H, NCH_2), 3.17-3.02 (m, 6H, Arg NCH_2), 2.86-2.76 (m, 1H, NCH_2), 2.29-2.19 (m, 1H, $COCH_2$), 1.98-1.79 (m, 2H, $COCH_2$, CH_2), 1.78-1.33 (m, 15H, CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.2, 172.4, 171.1, 170.3, 156.8 (3C), 54.2, 54.0, 53.0, 40.4 (2C), 40.2, 37.6, 34.8, 28.7, 27.9, 27.7 (2C), 25.4 (2C), 25.3, 22.8; HR-MS *m/z* calculated for $[M + H]^+$ 568.3795, found 568.3789.

cyclo[-Arg-6-aminohexanoyl-Arg-Arg-] (**1b**).

The compound was synthesized following general procedure A, B, C and D. In general procedure A, **4b** was used as the building block of the linker. In general procedure D, the eluent at 18.149 min was collected during the purification procedure. Acetonitrile was removed under reduced pressure and the aqueous phase was lyophilized. White solid (39.7 mg, 35% when calculated as ×3 TFA salt).

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.31 (d, J = 7.2 Hz, 1H, amide NH), 8.10-8.03 (m, 2H, amide NH), 7.86 (dd, J = 6.8 Hz, 4.0 Hz, 1H, amide NH), 7.59-6.75 (m, 12H, guanidine H), 4.10-3.98 (m, 2H, Arg αH), 3.95-3.85 (m, 1H, Arg αH), 3.45-3.33 (m, 1H, NCH_2), 3.17-3.00 (m, 6H, Arg NCH_2), 2.87-2.77 (m, 1H, NCH_2), 2.24-2.04 (m, 2H, $COCH_2$), 1.80-1.04 (m, 18H, CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.5, 172.3, 170.9, 170.0, 156.8 (2C), 156.7, 54.4, 53.9, 53.6, 40.4, 40.2 (2C), 38.1, 34.6, 28.9, 28.7, 28.4, 28.0, 25.6, 25.4, 25.3, 25.2, 24.6; HR-MS *m/z* calculated for $[M + H]^+$ 582.3952, found 582.3953.

cyclo[-Arg-7-aminoheptanoyl-Arg-Arg-] (**1c**).

The compound was synthesized following general procedure A, B, C and D. In general procedure A, **4c** was used as the building block of the linker. In general procedure D, the eluent at 16.665 min was collected during the purification procedure. Acetonitrile was removed under reduced pressure and the aqueous phase was lyophilized. White solid (48.9 mg, 44% when calculated as ×3 TFA salt).

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.19 (d, J = 7.2 Hz, 1H, amide NH), 8.12 (d, J = 7.2 Hz, 1H, amide NH), 7.98 (d, J = 7.2 Hz, 1H, amide NH), 7.70-7.66 (m, 1H, amide NH), 7.58-6.71 (m, 12H, guanidine H), 4.11-3.98 (m, 2H, Arg αH), 3.84 (dd, J = 14.8 Hz,

7.2 Hz, 1H, Arg αH), 3.34-3.22 (m, 1H, NCH_2), 3.17-3.00 (m, 6H, Arg NCH_2), 2.87-2.77 (m, 1H, NCH_2), 2.26-2.09 (m, 2H, $COCH_2$), 1.89-1.12 (m, 20H, CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.1, 172.0, 170.9, 170.7, 156.8 (2C), 156.7, 54.1, 53.5, 53.3, 40.4, 40.3 (2C), 37.9, 34.3, 28.3, 28.0, 27.9, 27.3 (2C), 25.5, 25.4, 25.2, 24.9, 24.7; HR-MS *m/z* calculated for $[M + H]^+$ 596.4108, found 596.4085.

cyclo[-Arg-8-aminooctanoyl-Arg-Arg-] (**1d**).

The compound was synthesized following general procedure A, B, C and D. In general procedure A, **4d** was used as the building block of the linker. In general procedure D, the eluent at 18.046 min was collected during the purification procedure. Acetonitrile was removed under reduced pressure and the aqueous phase was lyophilized. White solid (47.6 mg, 42% when calculated as ×3 TFA salt).

^1H NMR (400 MHz, DMSO-d_6) δ 8.22 (d, J = 8.4 Hz, 1H, amide NH), 8.15 (d, J = 5.2 Hz, 1H, amide NH), 7.96-7.87 (m, 1H, amide NH), 7.59-6.73 (m, 13H, amide NH, guanidine H), 4.29-4.20 (m, 1H, Arg αH), 4.20-4.12 (m, 1H, Arg αH), 3.92-3.84 (m, 1H, Arg αH), 3.48-3.35 (m, 1H, NCH_2), 3.18-2.98 (m, 6H, Arg NCH_2), 2.81-2.70 (m, 1H, NCH_2), 2.25-2.14 (m, 1H, $COCH_2$), 2.13-2.02 (m, 1H, $COCH_2$), 1.93-1.78 (m, 2H, CH_2), 1.75-1.06 (m, 20H, CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.0, 171.4, 170.9, 170.5, 156.9, 156.8 (2C), 54.4, 52.3, 51.3, 40.6, 40.4, 40.3, 37.9, 35.6, 29.1, 28.8 (2C), 28.4, 27.6, 27.4, 25.5, 25.3 (2C), 24.9, 23.6; HR-MS *m/z* calculated for $[M + H]^+$ 610.4265, found 610.4266.

cyclo[-Arg-9-aminononanoyl-Arg-Arg-] (**1e**).

The compound was synthesized following general procedure A, B, C and D. In general procedure A, **4e** was used as the building block of the linker. In general procedure D, the eluent at 19.240 min was collected during the purification procedure. Acetonitrile was removed under reduced pressure and the aqueous phase was lyophilized. White solid (41.0 mg, 35% when calculated as ×3 TFA salt).

^1H NMR (400 MHz, DMSO-d_6) δ 8.28-8.18 (m, 2H, amide NH), 7.90-7.83 (m, 1H, amide NH), 7.56-6.75 (m, 13H, amide NH, guanidine H), 4.26 (dd, J = 11.6 Hz, 6.4 Hz, 1H, Arg αH), 4.13-4.04 (m, 1H, Arg αH), 3.93 (q, J = 6.4 Hz, 1H, Arg αH), 3.38-3.27 (m, 1H, NCH_2), 3.16-2.99 (m, 6H, Arg NCH_2), 2.86-2.77 (m, 1H, NCH_2), 2.29-2.18 (m, 1H, $COCH_2$), 2.17-2.05 (m, 1H, $COCH_2$), 1.91-1.73 (m, 2H, CH_2), 1.73-1.12 (m, 22H, CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.9, 171.3, 171.1, 170.7, 156.9, 156.8 (2C), 53.9,

52.6, 51.2, 40.6, 40.4, 40.3, 38.5, 35.2, 29.4, 29.2, 28.8 (2C), 27.6, 27.4, 27.1, 25.6, 25.4 (2C), 25.2, 23.8; HR-MS *m/z* calculated for $[M + H]^+$ 624.4421, found 624.4412.

cyclo[-Arg-13-aminotridecanoyl-Arg-Arg-] (**1f**).

The compound was synthesized following general procedure A, B, C and D. In general procedure A, **4f** was used as the building block of the linker. In general procedure D, the eluent at 25.302 min was collected during the purification procedure. Acetonitrile was removed under reduced pressure and the aqueous phase was lyophilized. White solid (53.8 mg, 44% when calculated as ×3 TFA salt).

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.26 (d, J = 7.6 Hz, 1H, amide NH), 8.09 (d, J = 7.6 Hz, 1H, amide NH), 7.74-7.69 (m, 1H, amide NH), 7.58-6.77 (m, 13H, amide NH, guanidine H), 4.32-4.24 (m, 1H, Arg αH), 4.19-4.08 (m, 2H, Arg αH), 3.36-3.24 (m, 1H, NCH_2), 3.17-3.00 (m, 6H, Arg NCH_2), 2.87-2.76 (m, 1H, NCH_2), 2.24-2.06 (m, 2H, $COCH_2$), 1.84-1.12 (m, 32H, CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.5, 171.5, 171.2, 170.8, 156.8 (3C), 52.7, 52.6, 51.8, 40.4 (2C), 40.3, 38.3, 35.1, 29.7, 28.7, 28.4, 28.1 (3C), 27.9, 27.8, 27.7, 27.6, 27.5, 25.5, 25.3 (2C), 24.9, 24.5; HR-MS *m/z* calculated for $[M + H]^+$ 680.5047, found 680.5020.

5-Aminopentanoyl-Arg-Arg-Arg-COOH (**2a**).

The compound was synthesized following general procedure A and E. In general procedure A, **4a** was used as the building block of the linker. In general procedure E, the purification method was t = 0, B 0%; t = 30 min, B 30%; t = 60 min, B 30%. The eluent at 18.032 min was collected during the purification procedure. Acetonitrile was removed under reduced pressure and the aqueous phase was lyophilized. White solid (20.2 mg, 39% when calculated as ×3 TFA salt).

^{1}H NMR (400 MHz, DMSO-d_6) δ 12.72 (s, 1H, COOH), 8.20 (d, J = 6.8 Hz, 1H, amide NH), 8.05 (d, J = 7.6 Hz, 1H, amide NH), 8.00 (d, J = 7.2 Hz, 1H, amide NH), 7.76 (brs, 1H, amide NH), 7.67-6.84 (m, 12H, guanidine H), 4.34-4.22 (m, 2H, Arg αH), 4.19-4.07 (m, 1H, Arg αH), 3.09 (brs, 6H, Arg NCH_2), 2.77 (brs, 2H, NCH_2), 2.24-2.08 (m, 2H, $COCH_2$), 1.81-1.38 (m, 16H, CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.3, 172.1, 171.6, 171.4, 156.9 (3C), 52.1, 51.9, 51.7, 40.5, 40.3, 38.6, 34.4, 29.3, 29.2, 28.1, 26.7 (2C), 25.2, 25.0, 24.9, 22.1; HR-MS *m/z* calculated for $[M + H]^+$ 586.3901, found 586.3887.

6-Aminohexanoyl-Arg-Arg-Arg-COOH (**2b**).

The compound was synthesized following general procedure A and E. In general procedure A, **4b** was used as the building block of the linker. In general procedure E, the purification method was t = 0, B 0%; t = 30 min, B 30%; t = 60 min, B 30%. The eluent at 19.509 min was collected during the purification procedure. Acetonitrile was removed under reduced pressure and the aqueous phase was lyophilized. White solid (21.8 mg, 41% when calculated as ×3 TFA salt).

^{1}H NMR (400 MHz, DMSO-d_6) δ 12.74 (s, 1H, COOH), 8.21 (d, J = 7.2 Hz, 1H, amide NH), 8.01 (d, J = 7.6 Hz, 1H, amide NH), 7.96 (d, J = 7.6 Hz, 1H, amide NH), 7.73 (t, J = 5.6 Hz, 1H, amide NH), 7.63-6.88 (m, 12H, guanidine H), 4.34-4.21 (m, 2H, Arg αH), 4.20-4.11 (m, 1H, Arg αH), 3.17-3.00 (m, 6H, Arg NCH_2), 2.82-2.70 (m, 2H, NCH_2), 2.21-2.06 (m, 2H, $COCH_2$), 1.80-1.37 (m, 16H, CH_2), 1.34-1.21 (m, 2H, CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.2, 172.4, 171.6, 171.4, 156.9 (3C), 52.1, 51.9, 51.7, 40.4, 40.3, 38.7, 34.9, 29.3, 29.1, 28.1, 26.8 (2C), 25.5, 25.2, 25.1, 24.9, 24.8; HR-MS *m/z* calculated for $[M + H]^+$ 600.4058, found 600.4030.

7-Aminoheptanoyl-Arg-Arg-Arg-COOH (**2c**).

The compound was synthesized following general procedure A and E. In general procedure A, **4c** was used as the building block of the linker. In general procedure E, the purification method was t = 0, B 0%; t = 30 min, B 30%; t = 60 min, B 30%. The eluent at 23.308 min was collected during the purification procedure. Acetonitrile was removed under reduced pressure and the aqueous phase was lyophilized. White solid (26.8 mg, 50% when calculated as ×3 TFA salt).

^{1}H NMR (400 MHz, DMSO-d_6) δ 12.76 (s, 1H, COOH), 8.17 (d, J = 7.2 Hz, 1H, amide NH), 8.04-7.94 (m, 2H, amide NH), 7.76 (t, J = 5.6 Hz, 1H, amide NH), 7.66-6.93 (m, 12H, guanidine H), 4.33-4.20 (m, 2H, Arg αH), 4.18-4.08 (m, 1H, Arg αH), 3.09 (brs, 6H, Arg NCH_2), 2.76 (brs, 2H, NCH_2), 2.20-2.05 (m, 2H, $COCH_2$), 1.80-1.39 (m, 16H, CH_2), 1.34-1.17 (m, 4H, CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.3, 172.6, 171.6, 171.4, 156.9 (3C), 52.1, 51.9, 51.8, 40.5, 40.3, 38.8, 35.0, 29.3, 29.1, 28.2 (2C), 26.9 (2C), 25.6, 25.2, 25.0 (2C), 24.9; HR-MS *m/z* calculated for $[M + H]^+$ 614.4214, found 614.4190.

8-Aminooctanoyl-Arg-Arg-Arg-COOH (**2d**).

The compound was synthesized following general procedure A and E. In general procedure A, **4d** was used as the building block of the linker. In general procedure E, the purification method was t = 0, B 0%; t = 30 min, B 30%; t = 60 min, B 30%. The eluent at

23.664 min was collected during the purification procedure. Acetonitrile was removed under reduced pressure and the aqueous phase was lyophilized. White solid (20.9 mg, 39% when calculated as ×3 TFA salt).

^{1}H NMR (400 MHz, DMSO-d_6) δ 12.73 (s, 1H, COOH), 8.18 (d, J = 7.2 Hz, 1H, amide NH), 8.03-7.93 (m, 2H, amide NH), 7.75-7.68 (m, 1H, amide NH), 7.64-6.87 (m, 12H, guanidine H), 4.34-4.21 (m, 2H, Arg αH), 4.18-4.08 (m, 1H, Arg αH), 3.18-3.00 (m, 6H, Arg NCH_2), 2.82-2.70 (m, 2H, NCH_2), 2.20-2.04 (m, 2H, $COCH_2$), 1.81-1.38 (m, 16H, CH_2), 1.33-1.17 (m, 6H, CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.3, 172.6, 171.6, 171.4, 156.9 (2C), 156.8, 52.1, 51.9, 51.7, 40.5, 40.3, 38.8, 35.1, 29.3, 29.0, 28.5, 28.3, 28.1, 27.0 (2C), 25.7, 25.2 (2C), 25.0, 24.9; HR-MS *m/z* calculated for $[M + H]^+$ 628.4371, found 628.4346.

9-Aminononanoyl-Arg-Arg-Arg-COOH (**2e**).

The compound was synthesized following general procedure A and E. In general procedure A, **4e** was used as the building block of the linker. In general procedure E, the purification method was t = 0, B 0%; t = 30 min, B 35%; t = 60 min, B 35%. The eluent at 24.758 min was collected during the purification procedure. Acetonitrile was removed under reduced pressure and the aqueous phase was lyophilized. White solid (20.8 mg, 38% when calculated as ×3 TFA salt).

^{1}H NMR (400 MHz, DMSO-d_6) δ 12.75 (s, 1H, COOH), 8.23-7.93 (m, 3H, amide NH), 7.72 (t, J = 5.6 Hz, 1H, amide NH), 7.62-6.85 (m, 12H, guanidine H), 4.32-4.19 (m, 2H, Arg αH), 4.14-4.02 (m, 1H, Arg αH), 3.17-2.99 (m, 6H, Arg NCH_2), 2.76 (brs, 2H, NCH_2), 2.20-2.04 (m, 2H, $COCH_2$), 1.81-1.38 (m, 16H, CH_2), 1.34-1.15 (m, 8H, CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.5, 172.6, 171.7, 171.3, 156.9 (2C), 156.8, 52.2, 52.0, 40.5, 40.4 (2C), 38.8, 35.1, 29.2 (2C), 28.6 (2C), 28.5 (2C), 27.0 (2C), 25.8, 25.3, 25.2, 25.0 (2C); HR-MS *m/z* calculated for $[M + H]^+$ 642.4527, found 642.4509.

13-Aminotridecanoyl-Arg-Arg-Arg-COOH (**2f**).

The compound was synthesized following general procedure A and E. In general procedure A, **4f** was used as the building block of the linker. In general procedure E, the purification method was t = 0, B 0%; t = 30 min, B 35%; t = 60 min, B 35%. The eluent at 34.006 min was collected during the purification procedure. Acetonitrile was removed under reduced pressure and the aqueous phase was lyophilized. White solid (10.0 mg, 17% for overall yield).

^{1}H NMR (400 MHz, DMSO-d_6) δ 12.73 (s, 1H, COOH), 8.32-7.87 (m, 3H, amide NH), 7.63 (t, J = 5.6 Hz, 1H, amide NH), 7.57-6.69 (m, 12H, guanidine H), 4.32-4.15 (m, 2H, Arg αH), 4.04 (brs, 1H, Arg αH), 3.18-2.96 (m, 6H, Arg NCH_2), 2.76 (brs, 2H, NCH_2), 2.20-2.02 (m, 2H, $COCH_2$), 1.85-1.64 (m, 4H, CH_2), 1.63-1.36 (m, 12H, CH_2), 1.34-1.12 (m, 16H, CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.0, 172.6, 171.8, 171.2, 156.9, 156.8 (2C), 52.4, 52.2, 40.7, 40.6, 40.4, 38.8, 35.1, 29.3, 29.1, 29.0 (2C), 28.9 (2C), 28.8, 28.6 (2C), 27.0 (2C), 25.8, 25.3, 25.1, 25.0 (2C); HR-MS *m/z* calculated for $[M + H]^+$ 698.5153, found 698.5159.

cyclo[-Arg-2-(2-aminoethoxy)acetyl-Arg-Arg-] (**3a**).

The compound was synthesized following general procedure A, B, C and D. In general procedure A, **4g** was used as the building block of the linker. In general procedure D, the eluent at 13.933 min was collected during the purification procedure. Acetonitrile was removed under reduced pressure and the aqueous phase was lyophilized. White solid (26.4 mg, 24% when calculated as ×3 TFA salt).

^{1}H NMR (400 MHz, MeOD) δ 4.47-4.38 (m, 2H, Arg αH), 4.27 (t, J = 8.0 Hz, 1H, Arg αH), 4.15, 3.85 (ABq, J = 15.2 Hz, 2H, linker $COCH_2O$), 3.78-3.71 (m, 1H, linker OCH_2), 3.57-3.49 (m, 1H, linker OCH_2), 3.46-3.34 (m, 2H, linker NCH_2), 3.28-3.16 (m, 6H, Arg NCH_2), 2.01-1.54 (m, 12H, Arg CH_2); ^{13}C NMR (100 MHz, MeOD) δ 174.2, 174.1, 173.7, 173.3, 158.7 (3C), 71.2, 70.8, 56.9, 55.2, 54.7, 41.8 (2C), 41.7, 40.6, 30.2, 29.4, 28.7, 26.7, 26.5 (2C); HR-MS *m/z* calculated for $[M + H]^+$ 570.3588, found 570.3578.

cyclo[-Arg-2-(2-(2-aminoethoxy)ethoxy)acetyl-Arg-Arg-] (**3b**).

The compound was synthesized following general procedure A, B, C and D. In general procedure A, **4h** was used as the building block of the linker. In general procedure D, the eluent at 14.738 min was collected during the purification procedure. Acetonitrile was removed under reduced pressure and the aqueous phase was lyophilized. White solid (37.9 mg, 33% when calculated as ×3 TFA salt).

^{1}H NMR (400 MHz, MeOD) δ 4.52 (q, J = 4.0 Hz, 1H, Arg αH), 4.17-4.05 (m, 2H, Arg αH, linker $COCH_2O$), 4.00-3.95 (m, 2H, Arg αH, linker $COCH_2O$), 3.83-3.76 (m, 1H, linker OCH_2), 3.71-3.49 (m, 6H, linker OCH_2, linker NCH_2), 3.39-3.32 (m, 1H, linker NCH_2), 3.29-3.15 (m, 6H, Arg NCH_2), 2.13-1.52 (m, 12H, Arg CH_2); ^{13}C NMR (100 MHz, MeOD) δ 174.5, 174.3, 173.4, 173.2, 158.7 (2C), 158.6, 72.3, 71.4, 71.0, 70.6, 57.2, 55.2, 53.1, 41.9 (2C), 41.8, 40.9, 30.9, 28.3, 28.2, 26.9, 26.7, 26.1; HR-MS *m/z* calculated for

$[M + H]^+$ 614.3850, found 614.3829.

cyclo[-Arg-2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)acetyl-Arg-Arg-] (**3c**).

The compound was synthesized following general procedure A, B, C and D. In general procedure A, **4i** was used as the building block of the linker. In general procedure D, the eluent at 15.424 min was collected during the purification procedure. Acetonitrile was removed under reduced pressure and the aqueous phase was lyophilized. White solid (16.9 mg, 14% when calculated as ×3 TFA salt).

^{1}H NMR (400 MHz, MeOD) δ 4.52 (q, J = 4.4 Hz, 1H, Arg αH), 4.22-4.12 (m, 2H, Arg αH), 4.04 (s, 2H, linker $COCH_2O$), 3.84-3.47 (m, 12H, linker OCH_2, linker NCH_2), 3.28-3.16 (m, 6H, Arg NCH_2), 2.07-1.55 (m, 12H, Arg CH_2); ^{13}C NMR (100 MHz, MeOD) δ 174.3 (2C), 173.6, 173.3, 158.7 (3C), 72.1, 71.9, 71.3 (2C), 70.9, 70.7, 55.9, 55.0, 53.5, 41.9 (2C), 41.8, 40.8, 30.6, 29.3, 29.0, 26.6, 26.4 (2C); HR-MS *m/z* calculated for $[M + H]^+$ 658.4112, found 658.4090.

cyclo[-Arg-2-(2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethoxy)acetyl-Arg-Arg-] (**3d**).

The compound was synthesized following general procedure A, B, C and D. In general procedure A, **4j** was used as the building block of the linker. In general procedure D, the eluent at 16.097 min was collected during the purification procedure. Acetonitrile was removed under reduced pressure and the aqueous phase was lyophilized. White solid (14.7 mg, 12% when calculated as ×3 TFA salt).

^{1}H NMR (400 MHz, MeOD) δ 4.46 (q, J = 4.8 Hz, 1H, αH), 4.34-4.26 (m, 2H, αH), 4.08, 4.01 (ABq, J = 13.2 Hz, 2H, linker $COCH_2O$), 3.82-3.48 (m, 15H, linker OCH_2, linker NCH_2), 3.34-3.09 (m, 7H, Arg NCH_2, linker NCH_2), 2.05-1.53 (m, 12H, Arg CH_2); ^{13}C NMR (100 MHz, MeOD) δ 174.2, 173.9, 173.7, 173.3, 158.7 (3C), 72.1, 71.6 (2C), 71.5, 71.4, 71.1, 70.9, 70.5, 55.0, 54.4, 54.1, 41.9 (2C), 41.8, 40.5, 30.4, 30.1, 29.8, 26.5, 26.3 (2C); HR-MS *m/z* calculated for $[M + H]^+$ 702.4375, found 702.4370.

Bioassays.

1. Proteins and chemicals.

Purified calf brain tubulin and chemicals were obtained as previously described.[S1] Crosslinked stabilized microtubules (MTX) in which the LP site is preserved were prepared as described,[S2] drop frozen and stored in liquid nitrogen. The concentration of sites in the preserved microtubules was calculated using the stoichiometry of Flutax-2

binding.[S3]

Tubulin with the deleted C-terminal tail (STubulin A) was prepared by differential C-terminal cleaveage of 50 mM purified tubulin with 0.025 mg/mL Carlsberg Subtilisin (Sigma-Aldrich) in 50 mM MES, 1 mM $MgCl_2$, 1 mM GTP, pH 6.5 (Buffer A) at 25 ºC for 20 min.[S4] The digestion was stopped by the addition of 1 mM PMSF. Active protein was selected by assembly of the mixture induced at 37 ºC for 30 min. Active STubulin A was collected by centrifugation in a Beckman Ultracentrífuga Optima™ TLX using a Beckman TLA100.2 rotor warmed at 37 ºC (30000 *g* for 20 min). The pellet was resuspended in buffer A, and disassembled at 4 ºC for 30 min, centrifuged again at 4 ºC (30000 *g* for 20 min) and used. Concentration was measured by the Bradford method[S5] using tubulin as standard. Digestion of the C-terminal fragment was checked by SDS-PAGE in a 12% gel.

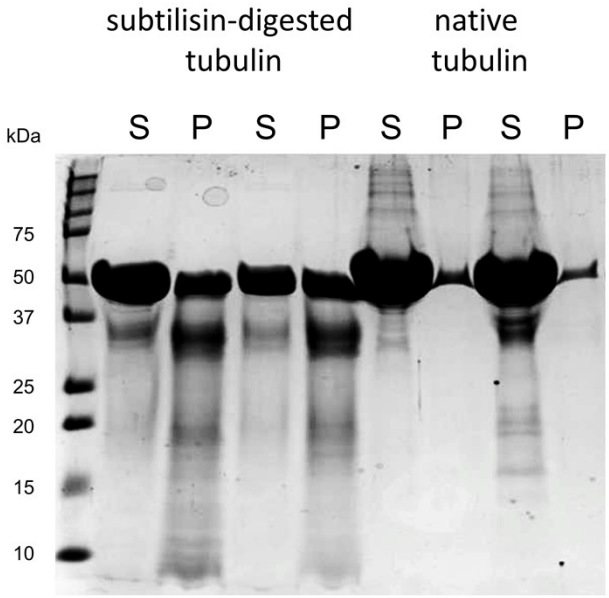


Figure S1 SDS-PAGE analysis of subtilisin-digested tubulin and native tubulin.

The protein weight is indicated by the marker in the left-most column. S, supernatant; P, precipitate.

2. Tubulin polymerization assay.

All compounds were dissolved in DMSO and diluted to a concentration that exactly 1 μL of the solution would be neccessary for each well. Tubulin was diluted with buffer B (3.4 M glycerol, 10 mM phosphate buffer, 1 mM EGTA, 6 mM $MgCl_2$, 1 mM GTP, pH = 6.5) to 20 μM. To each well of a precooled transparent 96-well microplate was rapidly added 1 μL of the compound solution and 100 μL of the tubulin solution. Each compound was added to at least three wells. The measurement was performed on TECAN Infinite

F200 plate reader which was prewarmed to 37 ºC. The absorbance at 340 nm of each well was recorded once per minute for 120 min at 37 ºC. The background absorbance of each compound was measured in buffer B without tubulin by the same method. The normalized absorbance was then obtained from the two data sets and was plotted for each compound.

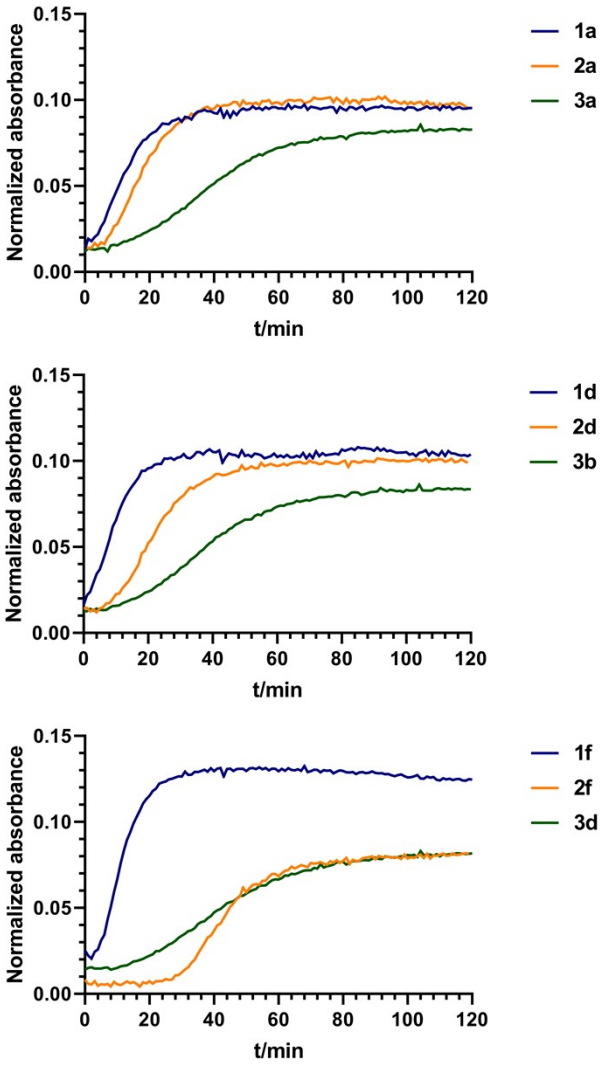


Figure S2 Tubulin polymerization assay viewed by linker length.

The curves were directly retrieved from Figure 2.

3. Negative stained electron microscopy.

Tubulin 5 μM and **1f** 1 mM were incubated in 80 mM Pipes pH 6.8, 1 mM EGTA, 1 mM $MgCl_2$, 10% glycerol, 1 mM GTP for 30 min at 37 ºC. The sample was centrifugated at 17000 RFC for 15 min at room temperature and polymers were resuspended in 50 μL of 80 mM Pipes pH 6.8, 1 mM EGTA, 1 mM $MgCl_2$, 0.05% Nonidet P4, 2 mM GTP. Then 10 μL of sample was deposited on carbon-coated 400 mesh grid for 1 min, washed with

water and stained using 2% (w/v) uranyl acetate for 30 s. Images were taken at 40000X magnification in a JEOL JEM-1230 transmission electron microscope operated at 100 KeV and a 16-megapixel CMOS TVIPS TemCam-F416 CCD camera.

4. Co-sedimentation assay.

50 μM of tubulin in buffer GAB (10 mM Sodium Phosphate,1 mM EGTA, 6 mM $MgCl_2$, 0.1 mM GTP, 3.4 M glycerol, pH 6.5) was assembled into MTs at 37 ºC for 45 min. Then 50 μM of **1f** was added and the sample was incubated at 37 ºC for another 30 min. The mixture was centrifuged in a Beckman Ultracentrífuga Optima™ TLX using a Beckman TLA100.2 rotor warmed at 37 ºC (96000 *g* for 20 min), the supernatant was discarded and the pellet was resuspended in cold buffer GAB for SDS-PAGE analysis. Alternatively, 50 μM (in tubulin concentration) of crosslinked stabilized MTs in buffer GAB were incubated with 50 μM of **1f** at 37 ºC for 30 min and the sample processed as described above. Finally, 50 μM of STubulin A in buffer A was assembled at 37 ºC for 45 min. Then 50 μM of **1f** was added and the sample was incubated at 37 ºC for another 30 min and the sample processed as described above except for the buffer employed to resuspend the pellet was buffer A.

The samples prepared (pellet and supernatants) were mixed 1:1 with loading buffer (0.1 M Tris-HCl pH6.8, 3.5 M glycerol, 0.3 M SDS, 0.02% coomassie blue G-250, 0.2 M DTT), run in a 12% Tris-Tricine gel, Coomassie stained, and quantified using Quantity One 4.6 (Bio-Rad, CA, USA).

5. Primary cortical neuronal culture and axonal density measurement.

The cortical neurons were dissociated from 14 days of gestational embryos in ICR mice (Guangdong medical laboratory animal center, Guangzhou, China), as described previously.[S6] In brief, the cerebral cortices were dissected and the dura mater was removed. The neurons were isolated from minced cortices after trypsinization with 0.05% trypsin-EDTA (Gibco, 25300054) and treatment with DNase I/Trypsin inhibitor (Gibco, 18047-019; Gibco, 17075-011), and were cultured in Neurobasal medium (Gibco, 21103-049) containing 0.6% D-glucose, 2 mM L-glutamine, and 2% B27 after washing with CMF-HBSS (Gibco, 14185-052) containing 4.17 mM $NaHCO_3$. The cells were plated in a 48-well dish at a density of 2.2×10^4 cells/cm^2.

To test the effect of compounds on Aβ25-35-induced axonal atrophy, 3 days after neuronal culture, the cortical neurons were cultured for 3 days, pretreated with Aβ25-35

(10 μM) for 0.5 h, and then cocultured with compounds (10 μM) for 3 days. The neurons were fixed with 4% paraformaldehyde (PFA; Solarbio, Beijing, China) for 1 h, and immunostained with phosphorylated neurofilament H (pNF-H, 1:500; Covance, SMI-35R, CA, United States) and microtubule-2 associated protein 2 (MAP2, 1:2000; Abcam, ab32454) overnight. Secondary antibodies Alexa Fluor 594-conjugated goat anti-mouse IgG (1:400, Abcam, ab150116) and Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:400, Abcam, ab150081) were applied to cells after washing 1st antibodies. 4',6-diamidino-2-phenylindole (DAPI) (1 μg/mL, Biomol, Hamburg, Germany) was used for nuclear counterstaining. A fluorescent microscope system (ECHO RVL-100-M, United States) was used for image capture at size of 259 × 346 μm. The images of each group were captured and analyzed by ImageJ (NIH), with the Neurite Tracer plugins. The average lengths of pNF-H positive axons per neuron (i.e. axonal density) were measured.

Molecular modeling.

The interaction between the compounds and the isolated βI-tubulin CTT peptide was initially studied. MD simulations were performed with AMBER14.[S7, 8] The isolated N-capped βI-tubulin CTT peptide (Ac-EEEEDFGEEAEEEA) and each compound were placed at a non-binding distance (>40 Å) in a box of TIP3P type water molecules buffering 12 Å from the molecular system. The ff99SB-ildn force field was used to parametrize βI-tubulin CTT peptide and arginine residues in the small molecules,[S9] while the general amber force field (GAFF) was used to parametrize non-aminoacidic components of RRR macrocycles such as the carbon linkers.[S10] Point charges for linker atoms were obtained at the am1-bcc level of theory. The total charge of the systems was neutralized by the addition of Na^+ counter-ions to the solvent box. In agreement with recent works, each system was relaxed as follows: i) energy minimization of the solvent for 500 steps with the steepest descent algorithm (SD) followed by 1500 steps with the conjugate gradient algorithm (CG); ii) energy minimization of the solvated solute for 1000 steps with the SD and 3500 steps with the CG; iii) heating from 0 to 300 K at constant volume with the Langevin thermostat for 100 ps; iv) density equilibration at constant pressure with the Berendsen barostat for 100 ps; v) preliminary equilibration at constant pressure for 50 ns; vi) trajectory production for 200 ns at constant pressure without positional restraints.[S11, 12] In all MD simulations, the time-step was set to 2 fs and the SHAKE algorithm was used. The representative binding conformation of each compound was selected by a cluster analysis performed on MD simulation by CPPTRAJ[S13] and corresponded to the most populated

cluster. The delta energy of binding of of the compounds to the βI-tubulin CTT peptide was estimated by the MM/GBSA and MM/PBSA approaches, as implemented in AMBER14.[S14]

The interaction between the compounds and tubulin CTT peptide in the context of MT lattice was subsequently observed. The MT lattice was built by applying symmetry operations to PDB: 3E22 as reported previously,[S15-17] whereas the human full length α- and βI-tubulin were generated by homology modeling based on the templates PDB: 3E22 and PDB: 5LXT,[S18] which were selected based on sequence similarity and structural integrity. Due to the lack of structural information from the literature or databases, the C-terminal tail of each subunit was first modeled in an elongated conformation exposed to the solvent and perpendicular to the plane of the MT lattice that corresponds ideally to the MT surface. Nucleotides and magnesium ions were applied to each subunit. The system was the included in a box of TIP3P type water molecules and the overall charge was neutralized by the addition of Na^+ counter-ions. The system was parametrized as described above, while parameters for nucleosides were retrieved from Carlson et al.[S19] MD simulations on the MT lattice were conducted as described above although the final production of trajectories was extended to 500 ns. A representative frame of the MT lattice was extrapolated by cluster analysis with CPPTRAJ, and used for further investigations *in silico*. The recognition and interaction between the MT lattice and the compounds was investigated by MD simulations. To this aim, four molecules of each compound were positioned randomly within the "outer" side of the MT lattice at a non-binding distance (>40 Å) from the representative MD structure of the MT lattice. The MD protocol described above was used to run MD simulations lasting 500 ns. The delta energy of binding of the compounds to the MT lattice was estimated by the MM/GBSA and MM/PBSA approaches, as implemented in AMBER14.[S14]

References.

S1 J. F. Díaz*, et al.*, *Biochemistry*, 1993, **32**, 10067-10077.

S2 J. F. Diaz*, et al.*, *J. Biol. Chem.*, 2003, **278**, 8407-8419.

S3 J. F. Díaz*, et al.*, *J. Biol. Chem.*, 2000, **275**, 26265-26276.

S4 S. De la Vina*, et al.*, *Biochemistry*, 1988, **27**, 5352-5365.

S5 M. M. Bradford, *Anal. Biochem.*, 1976, **72**, 248-254.

S6 J. Deng*, et al.*, *Food Res. Int.*, 2022, **158**, 111576.

S7 D. A. Case*, et al.*, AMBER 14, University of California, San Francisco, 2014.

S8 R. Salomon-Ferrer*, et al.*, *WIREs Comput. Mol. Sci.*, 2013, **3**, 198-210.

S9 K. Lindorff-Larsen*, et al.*, *Proteins Struct. Funct. Bioinf.*, 2010, **78**, 1950-1958.

S10 J. Wang*, et al.*, *J. Comput. Chem.*, 2004, **25**, 1157-1174.

S11 F. Picarazzi*, et al.*, *J. Mol. Graphics Modell.*, 2022, **110**, 108076.

S12 C. Platella*, et al.*, *Pharmaceutics*, 2021, **13**, 1611.

S13 D. R. Roe and T. E. Cheatham, *J. Chem. Theory Comput.*, 2013, **9**, 3084-3095.

S14 B. R. Miller*, et al.*, *J. Chem. Theory Comput.*, 2012, **8**, 3314-3321.

S15 A. Cormier*, et al.*, *EMBO Rep.*, 2008, **9**, 1101-1106.

S16 I. Barasoain*, et al.*, *Chem. Biol.*, 2010, **17**, 243-253.

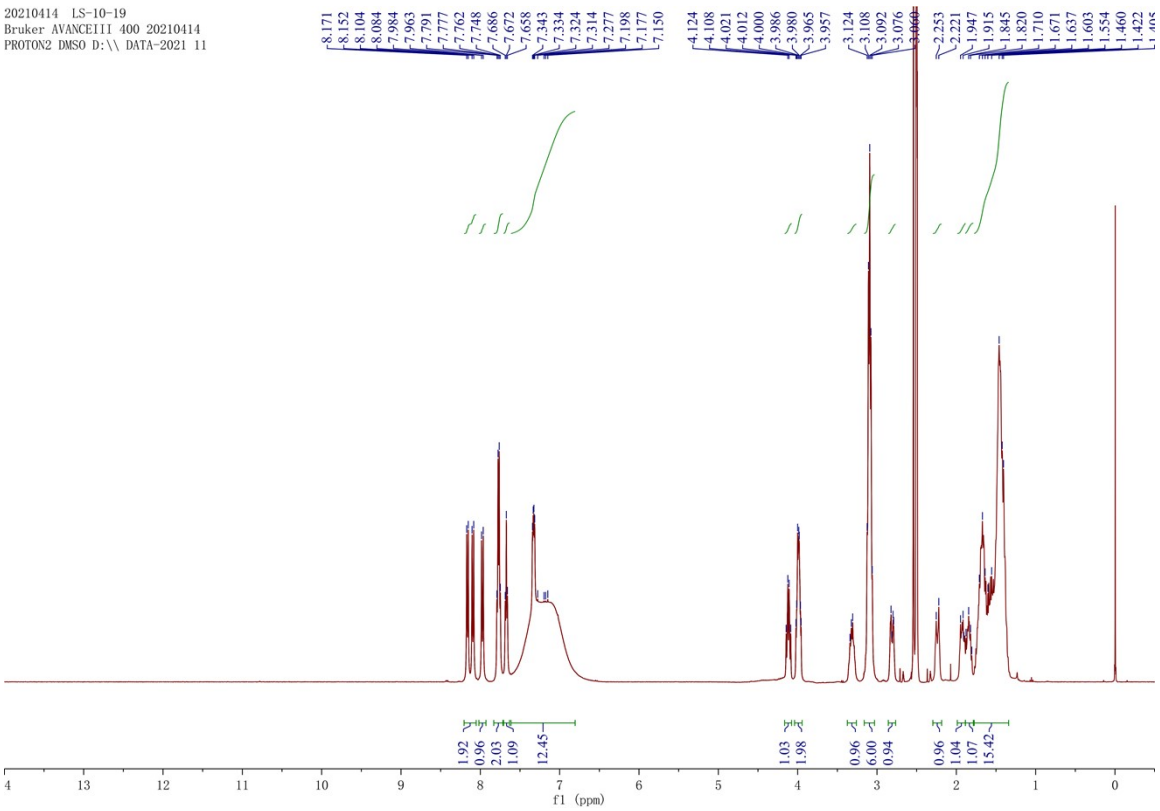
S17 G. Maccari*, et al.*, *J. Chem. Theory Comput.*, 2013, **9**, 698-706.

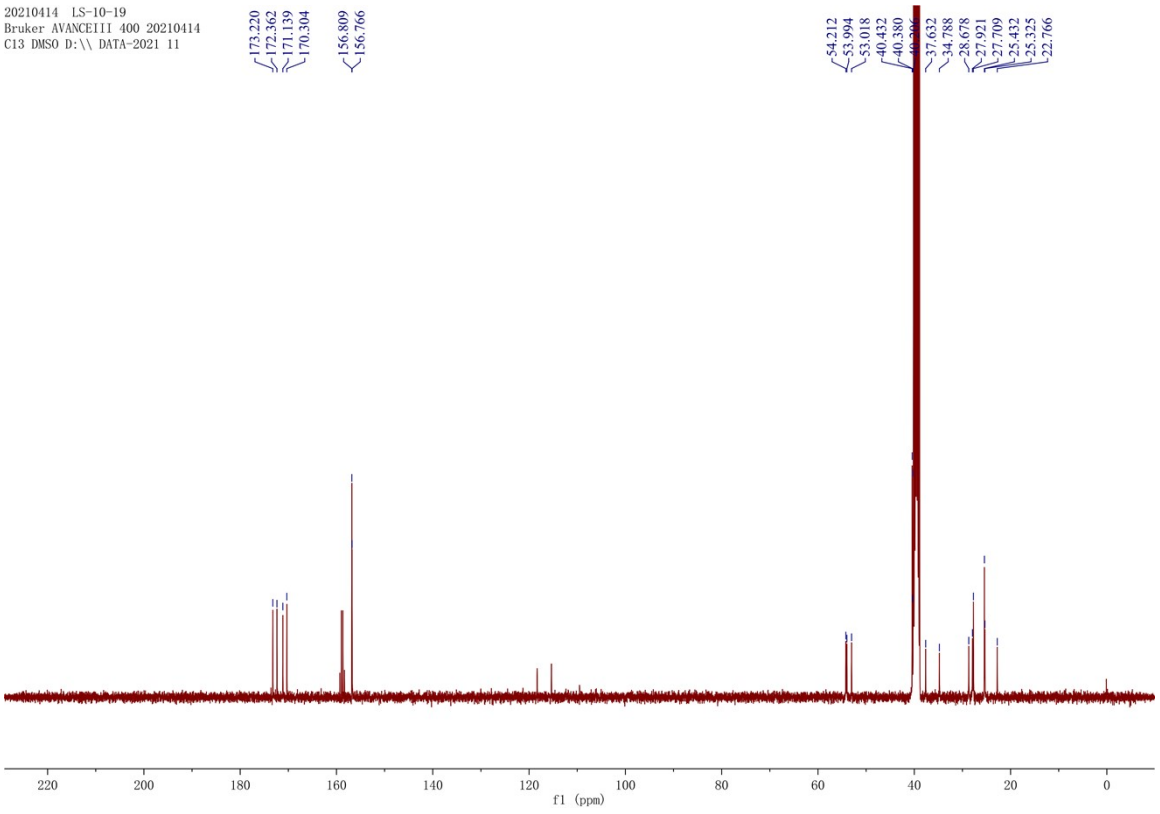
S18 A. E. Prota*, et al.*, *ChemBioChem*, 2017, **18**, 905-909.

S19 K. L. Meagher*, et al.*, *J. Comput. Chem.*, 2003, **24**, 1016-1025.

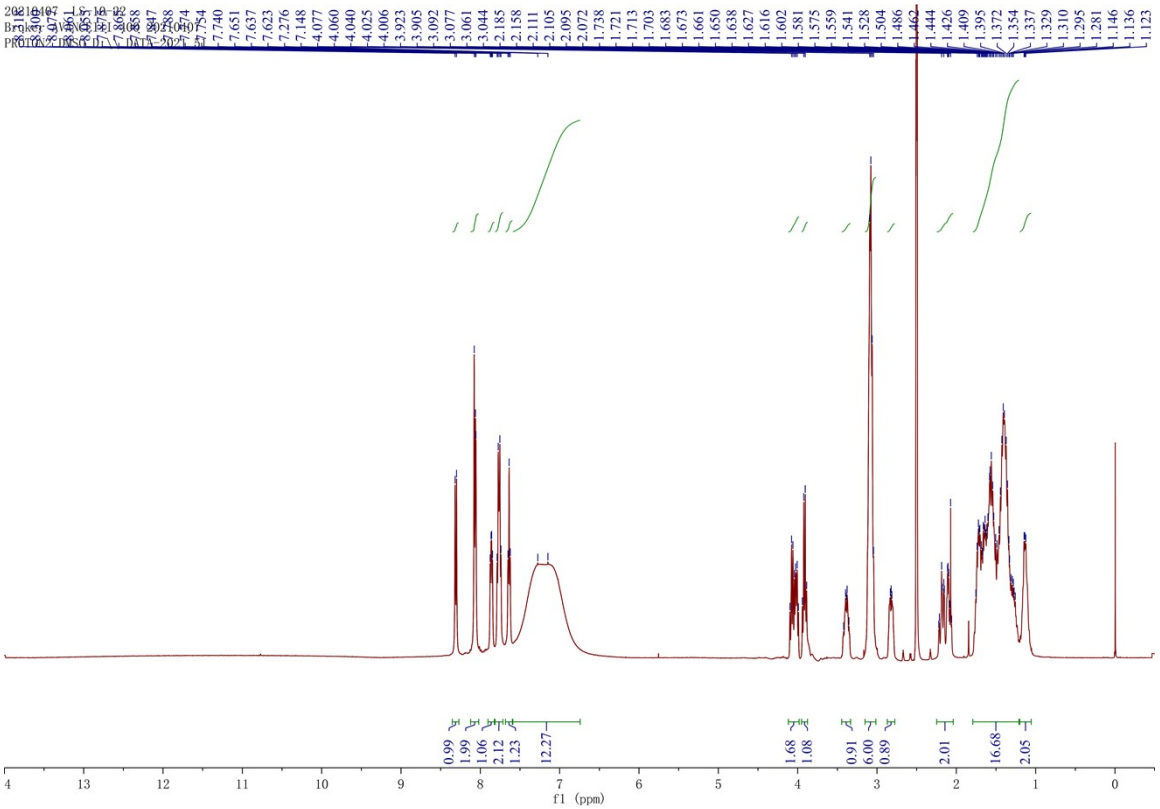
Spectra.

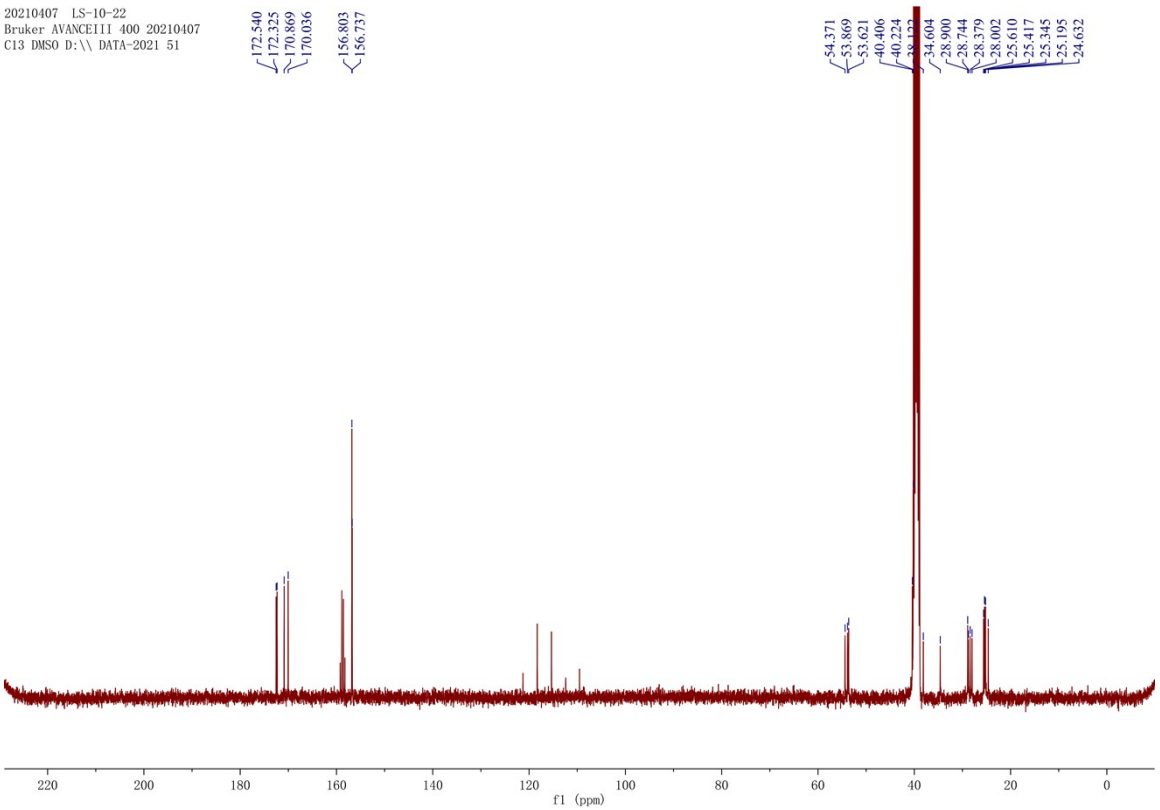
1a


20210414 LS-10-19
Bruker AVANCEIII 400 20210414
PROTON2 DMSO D:\\ DATA-2021 11
f1 (ppm)

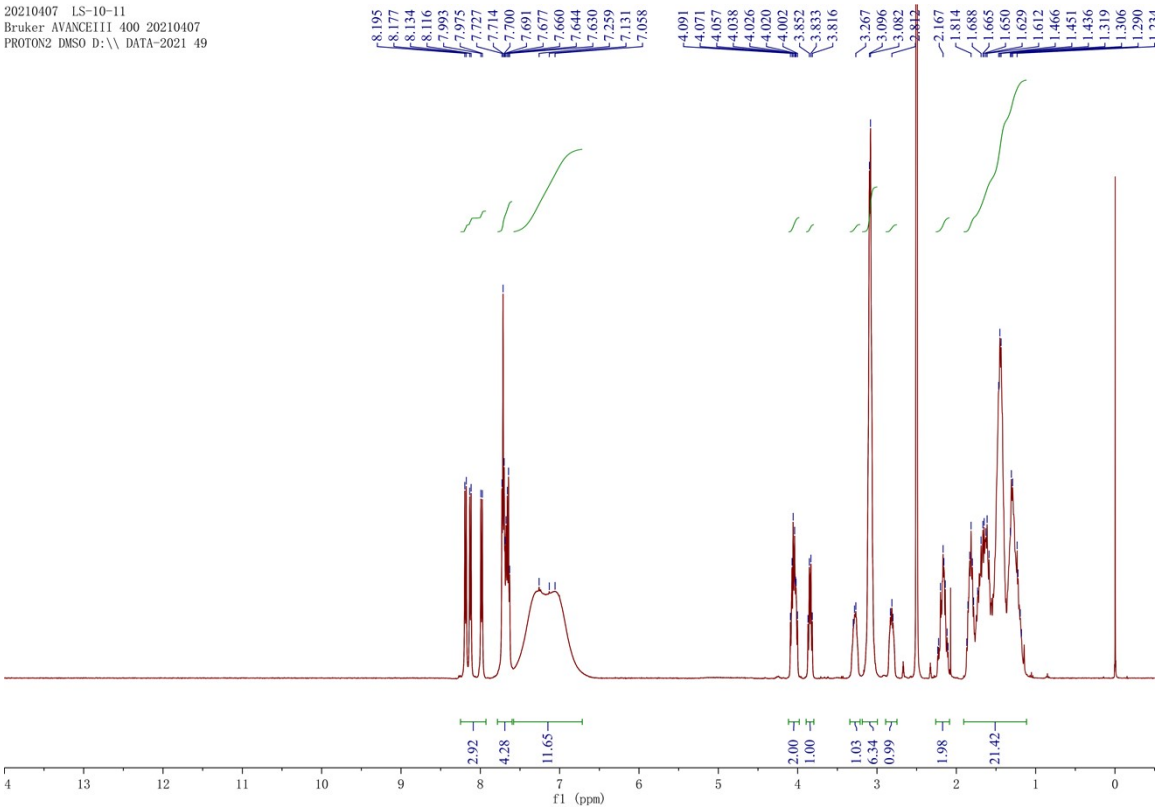

20210414 LS-10-19
Bruker AVANCEIII 400 20210414
C13 DMSO D:\\ DATA-2021 11
f1 (ppm)

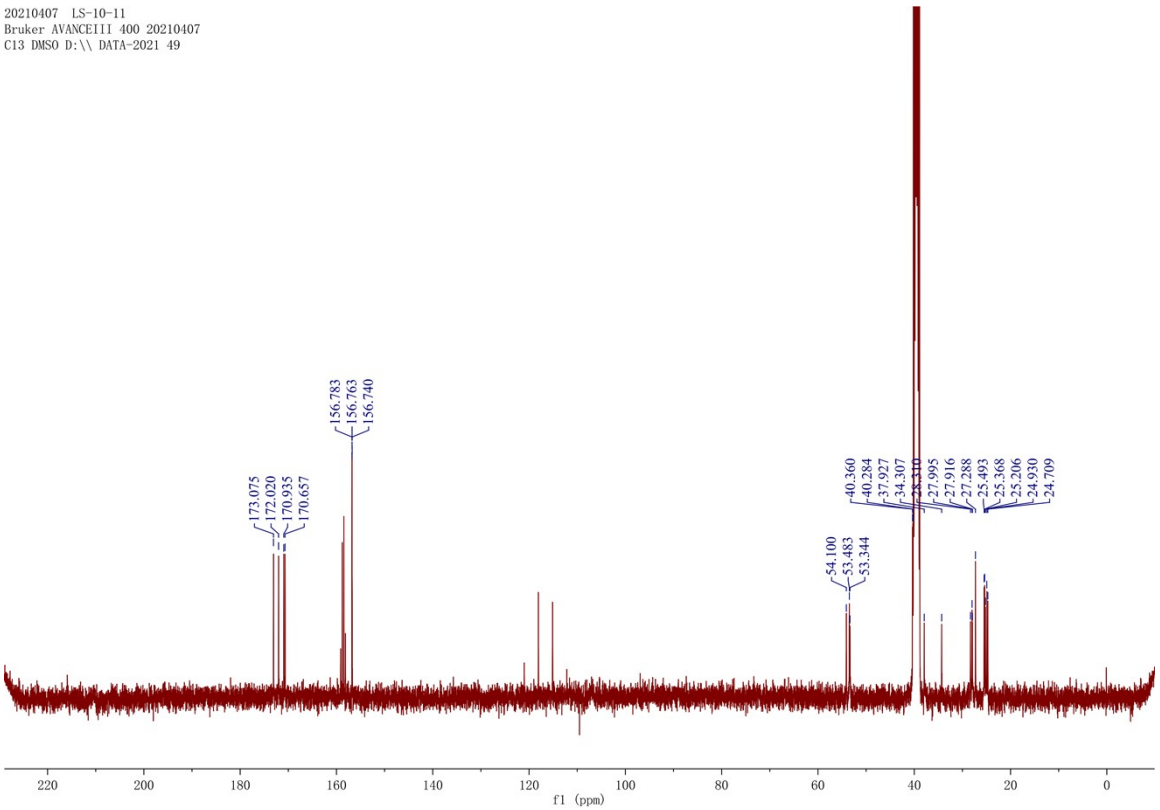
1b



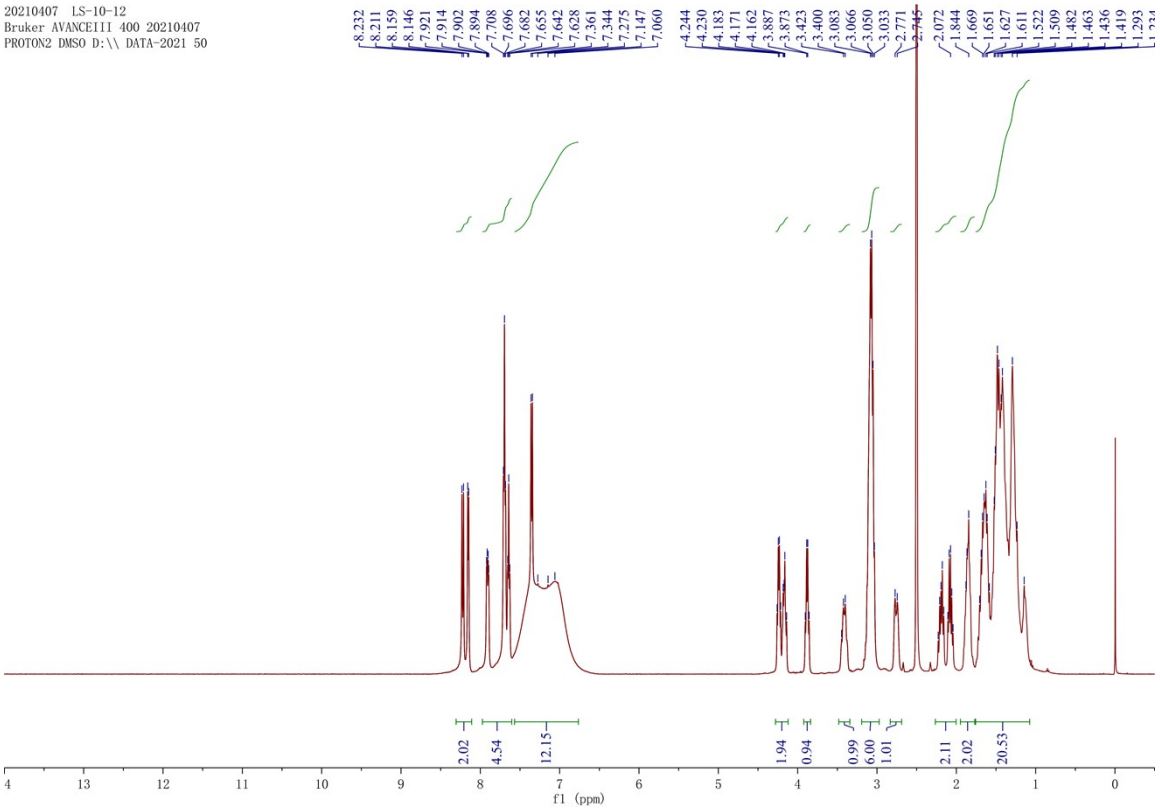


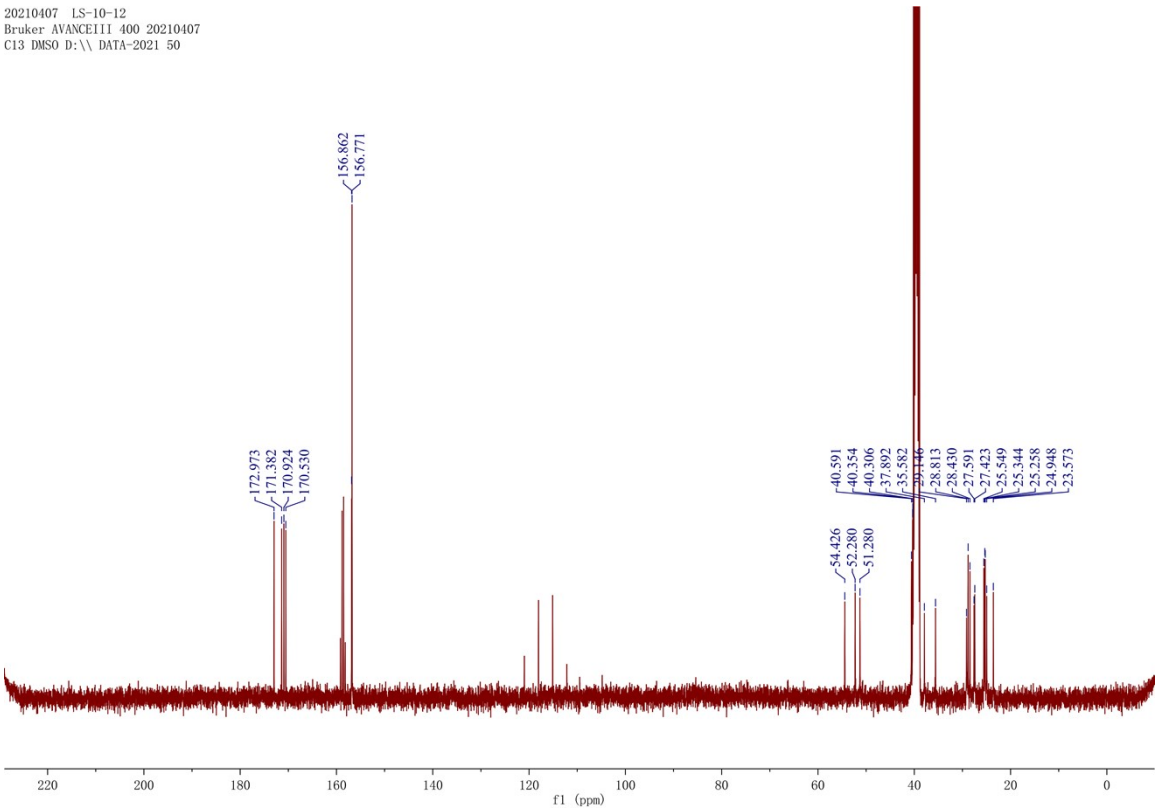
1c


20210407 LS-10-11
Bruker AVANCEIII 400 20210407
PROTON2 DMSO D:\\ DATA-2021 49
f1 (ppm)

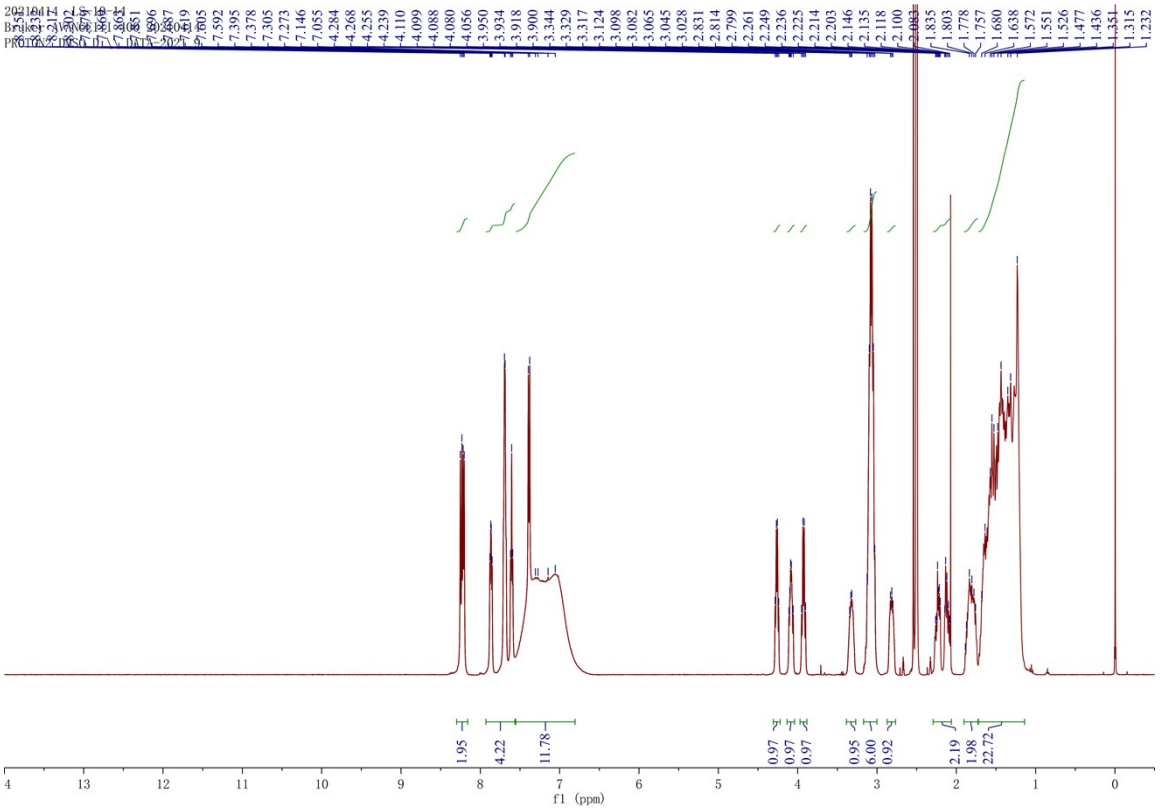

20210407 LS-10-11
Bruker AVANCEIII 400 20210407
C13 DMSO D:\\ DATA-2021 49
f1 (ppm)

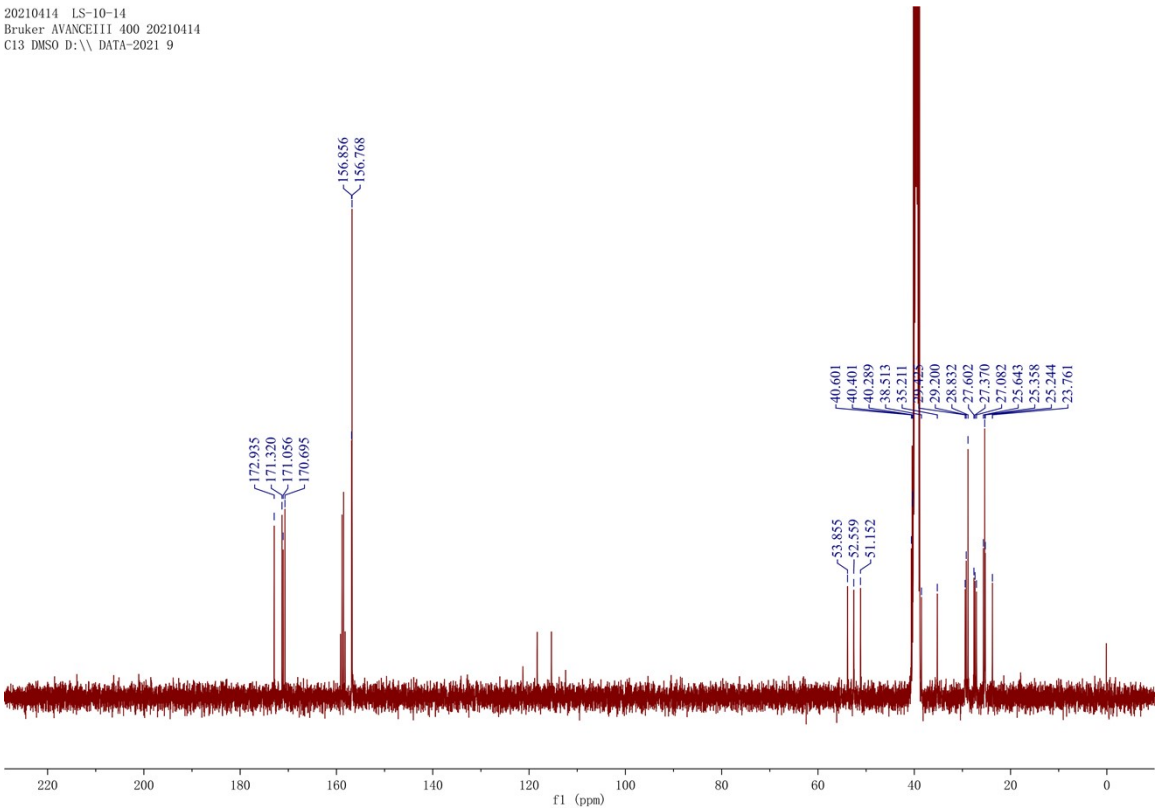
1d


20210407 LS-10-12
Bruker AVANCEIII 400 20210407
PROTON2 DMSO D:\\ DATA-2021 50
f1 (ppm)

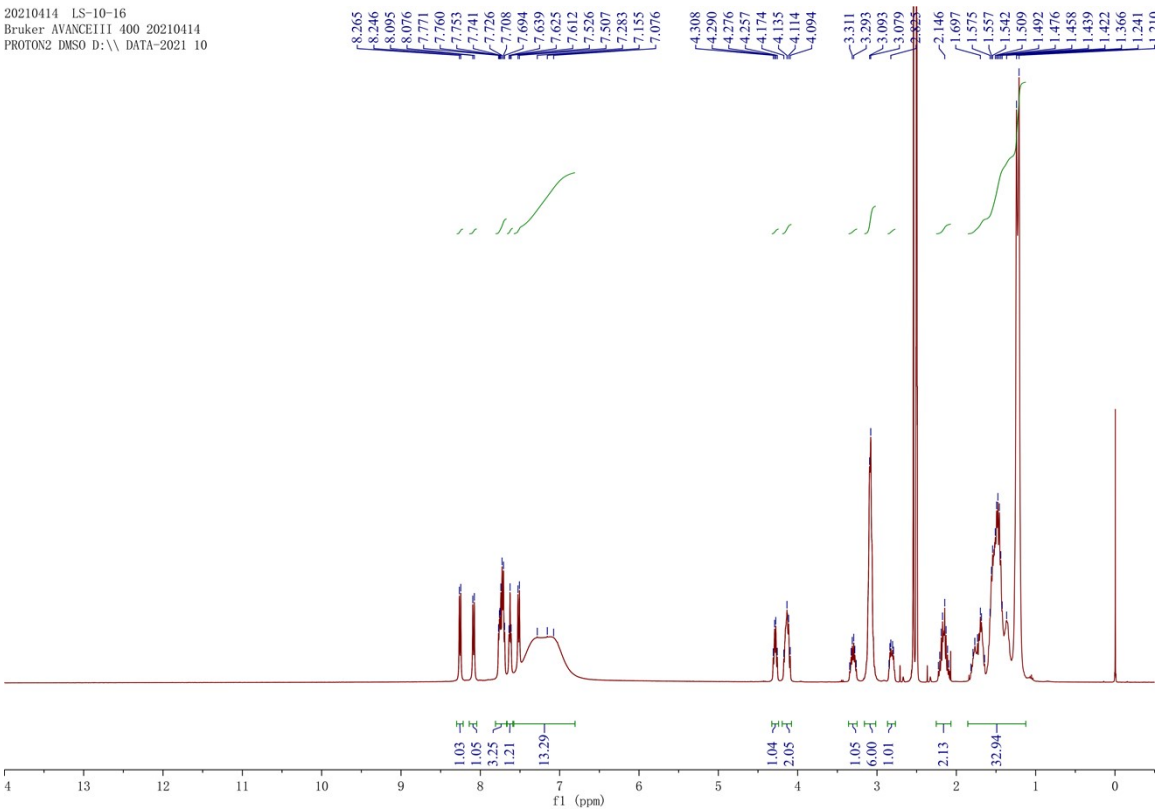

20210407 LS-10-12
Bruker AVANCEIII 400 20210407
C13 DMSO D:\\ DATA-2021 50
f1 (ppm)

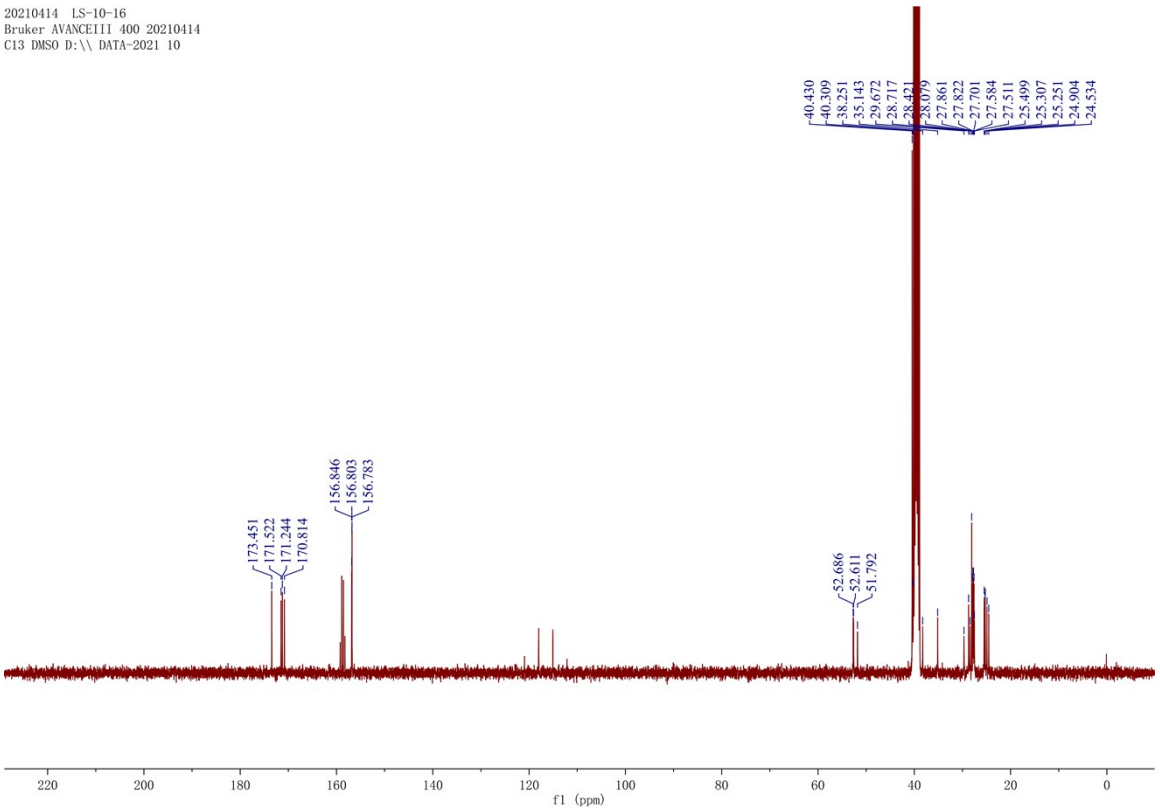
1e



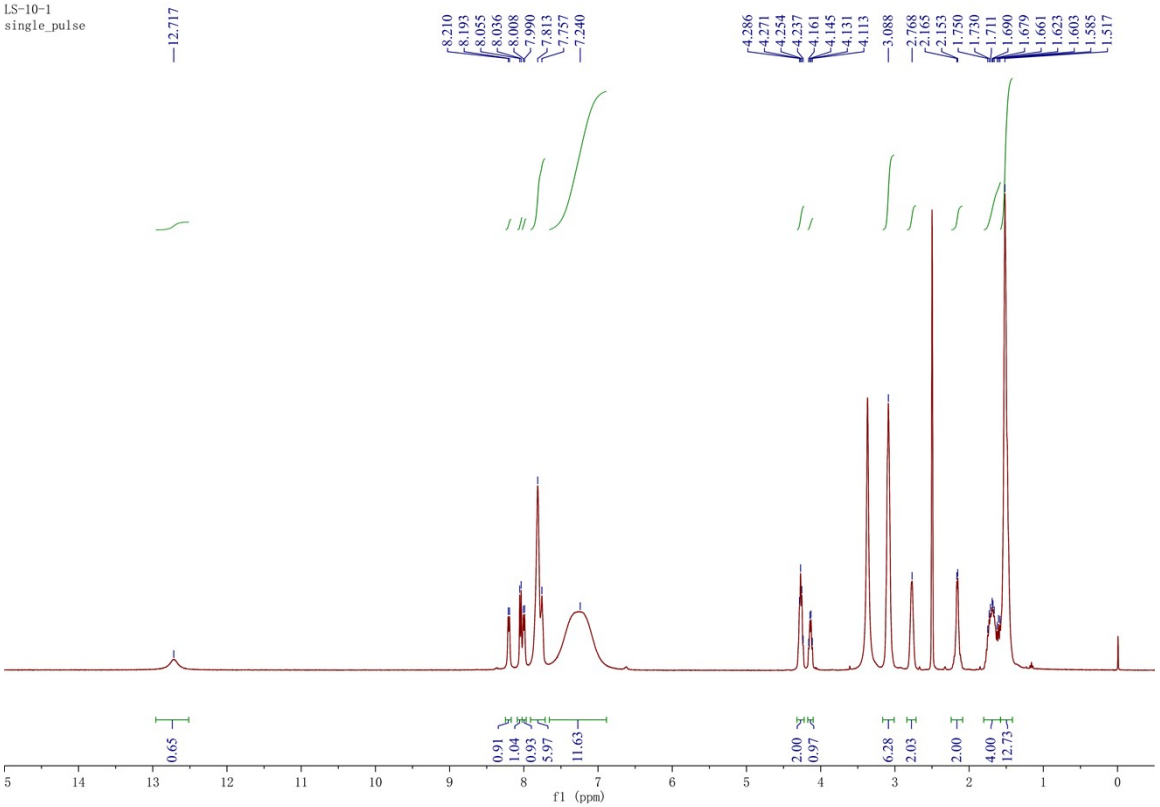

20210414 LS-10-14
Bruker AVANCEIII 400 20210414
C13 DMSO D:\\ DATA-2021 9
156.856
156.768
172.935
171.320
171.056
170.695
40.601
40.401
40.289
38.513
35.211
29.425
29.200
28.832
27.602
27.370
27.082
25.643
25.358
25.244
23.761
53.855
52.559
51.152
f1 (ppm)

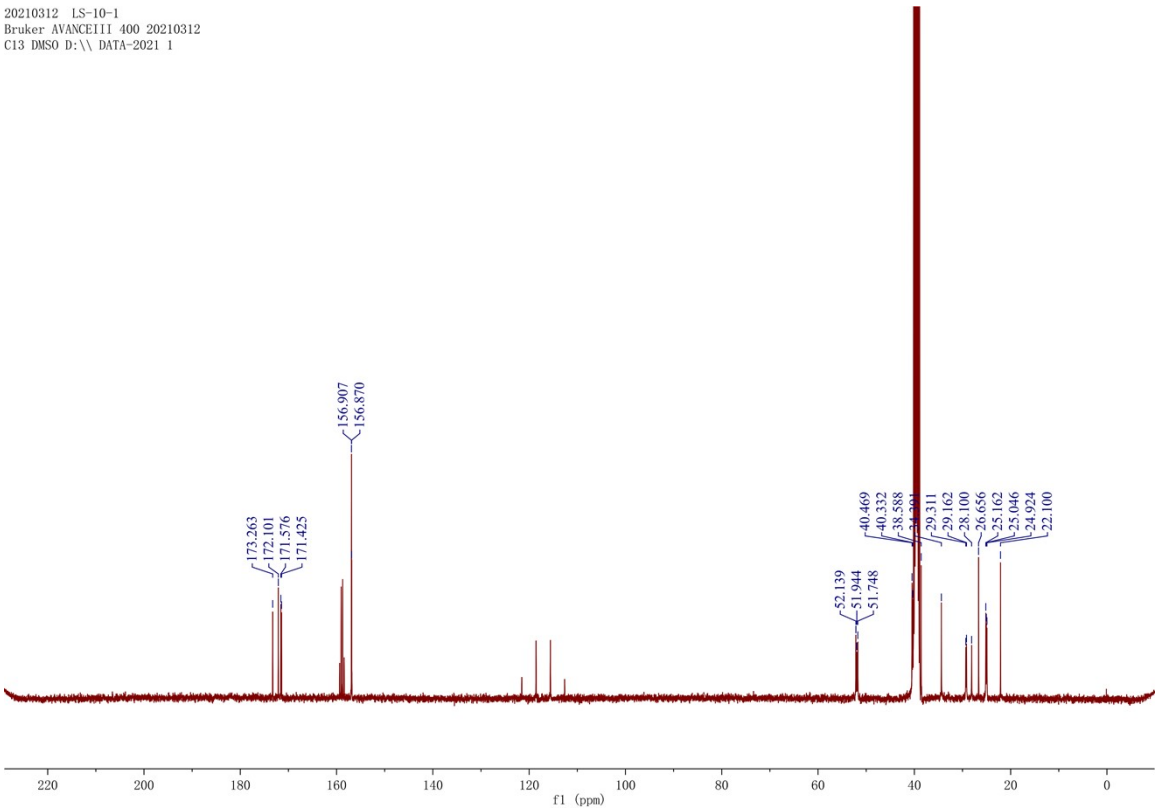
1f


20210414 LS-10-16
Bruker AVANCEIII 400 20210414
PROTON2 DMSO D:\\ DATA-2021 10
f1 (ppm)

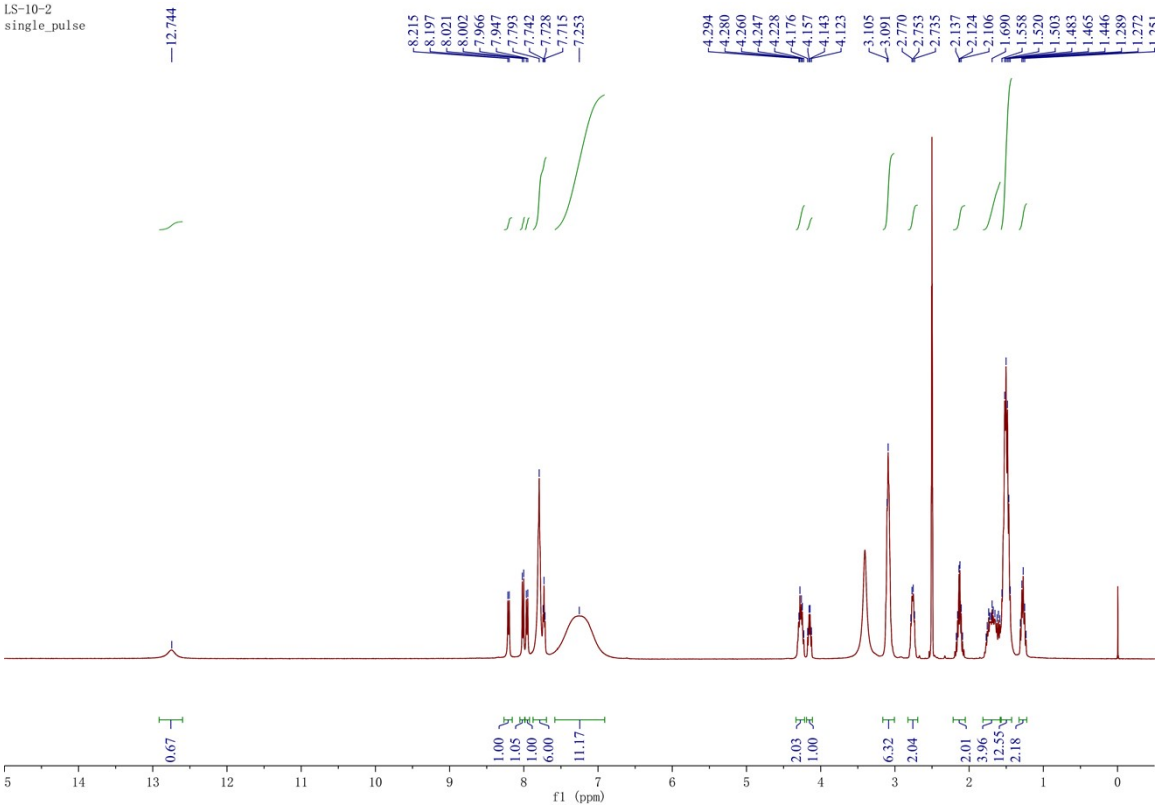

20210414 LS-10-16
Bruker AVANCEIII 400 20210414
C13 DMSO D:\\ DATA-2021 10
f1 (ppm)

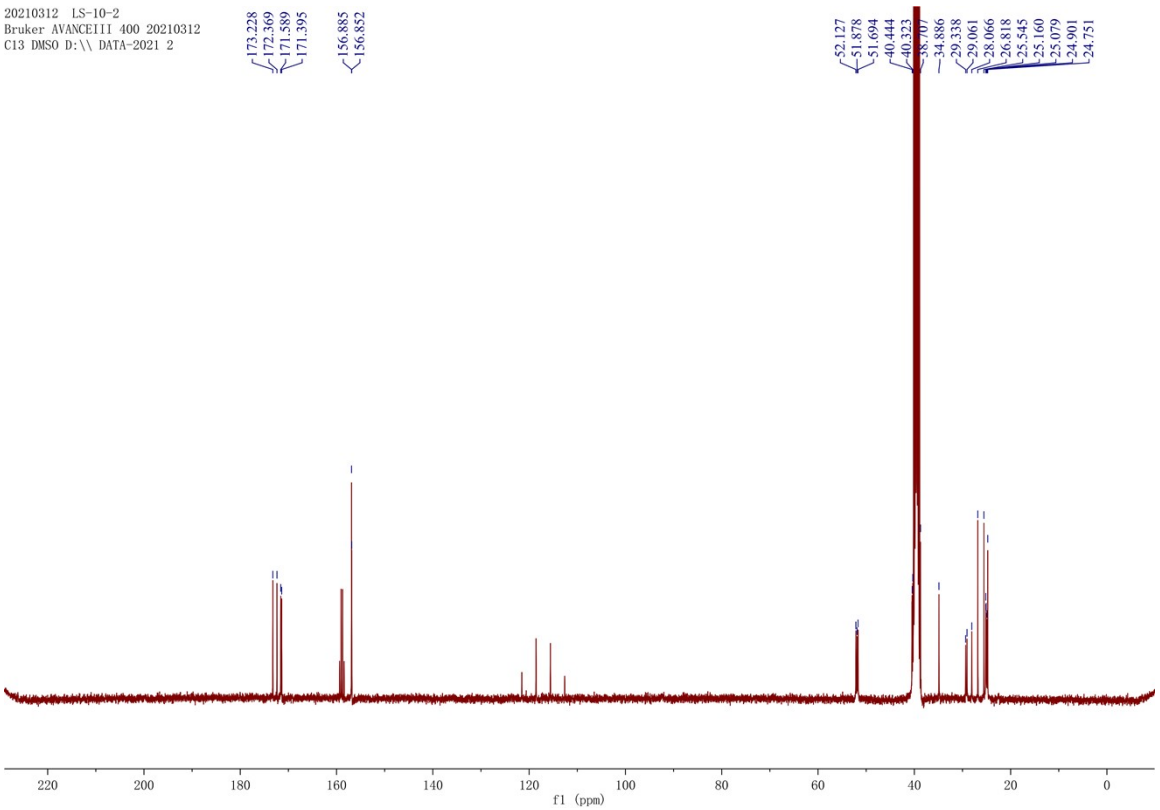
2a


LS-10-1
single_pulse
12.717
8.210
8.193
8.055
8.036
8.008
7.990
7.813
7.757
7.240
4.286
4.271
4.254
4.237
4.161
4.145
4.131
4.113
3.088
2.768
2.165
2.153
1.750
1.730
1.711
1.690
1.679
1.661
1.623
1.603
1.585
1.517
0.65
0.91
1.04
0.93
5.97
11.63
2.00
0.97
6.28
2.03
2.00
4.00
12.73
f1 (ppm)

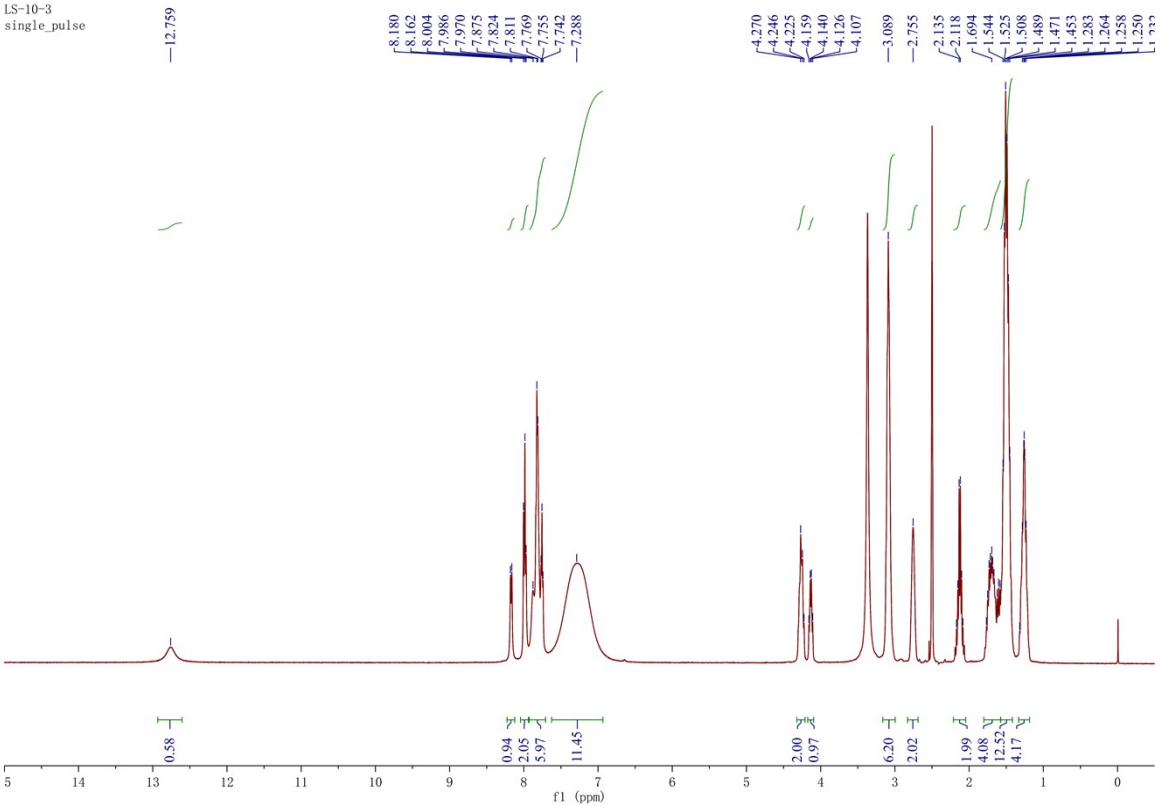

20210312 LS-10-1
Bruker AVANCEIII 400 20210312
C13 DMSO D:\\ DATA-2021 1
156.907
156.870
173.263
172.101
171.576
171.425
40.469
40.332
38.588
29.311
29.162
28.100
26.656
25.162
25.046
24.924
22.100
52.139
51.944
51.748
f1 (ppm)

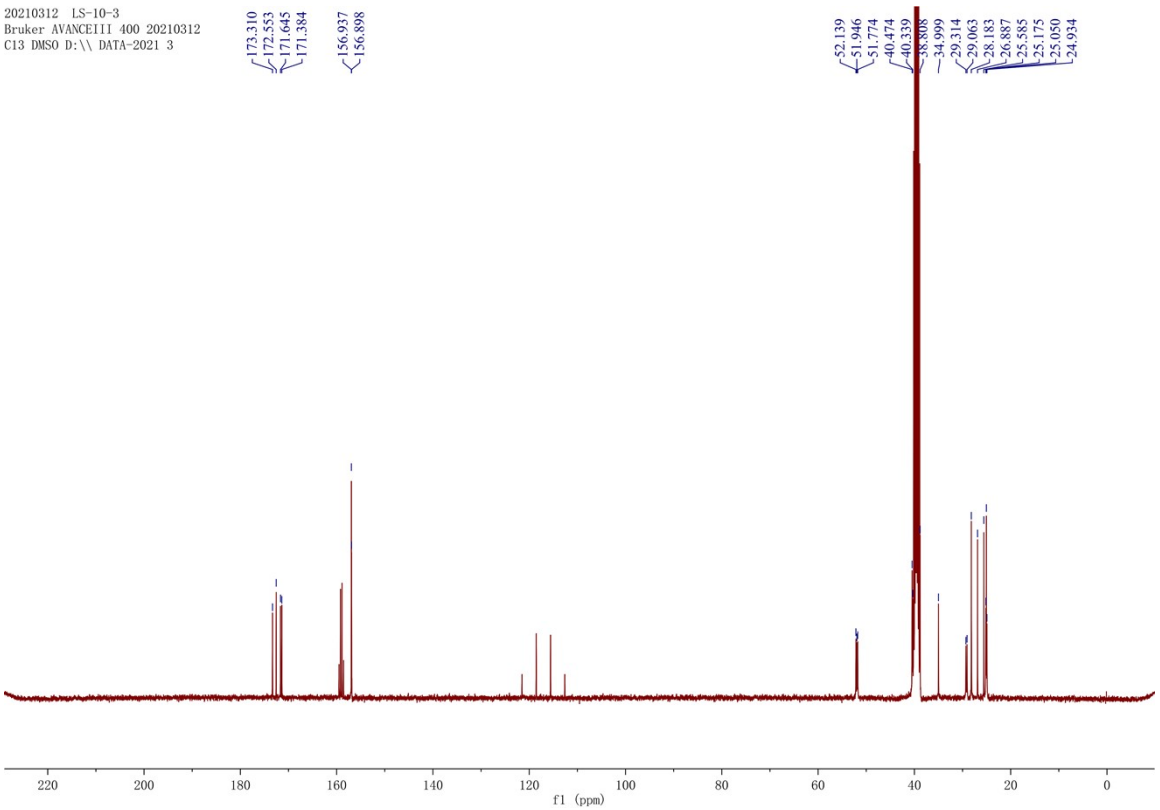
2b


LS-10-2
single_pulse
12.744
8.215
8.197
8.021
8.002
7.966
7.947
7.793
7.742
7.728
7.715
7.253
4.294
4.280
4.260
4.247
4.228
4.176
4.157
4.143
4.123
3.105
3.091
2.770
2.753
2.735
2.137
2.124
2.106
1.690
1.558
1.520
1.503
1.483
1.465
1.446
1.289
1.272
0.67
1.00
1.05
1.00
6.00
11.17
2.03
1.00
6.32
2.04
2.01
3.96
12.55
2.18
f1 (ppm)

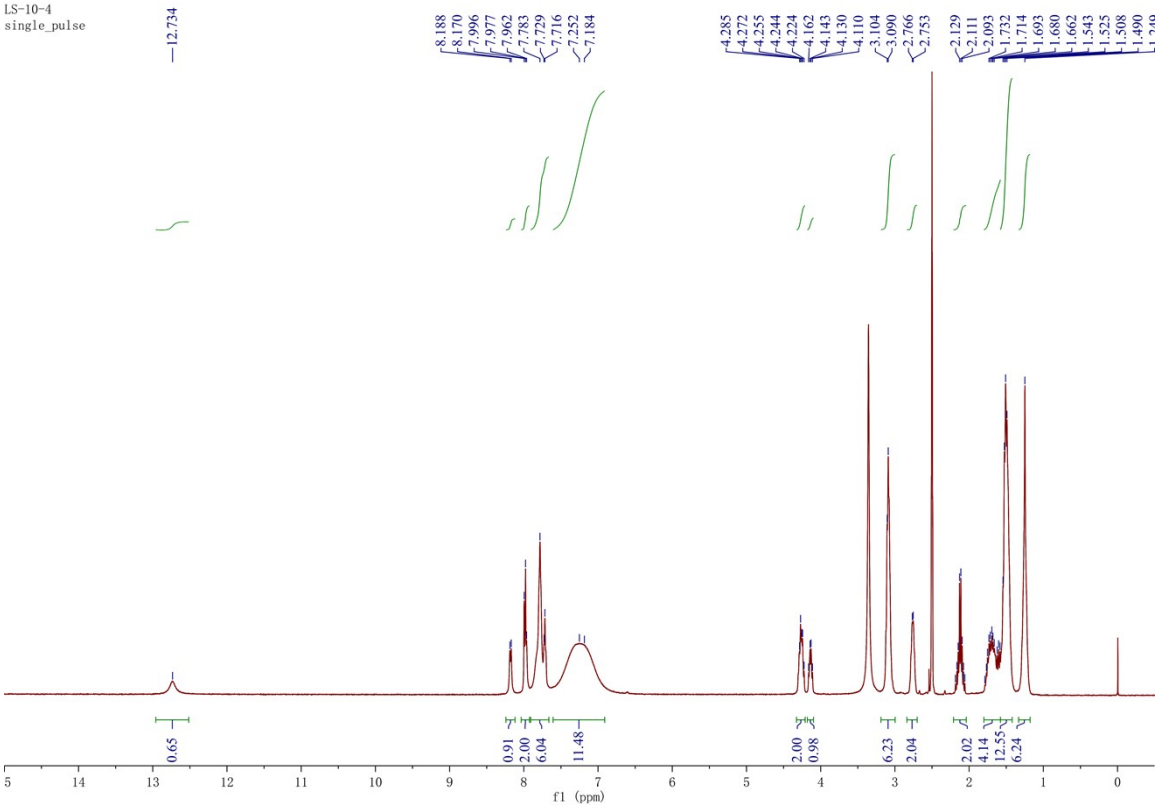

20210312 LS-10-2
Bruker AVANCEIII 400 20210312
C13 DMSO D:\\ DATA-2021 2
173.228
172.369
171.589
171.395
156.885
156.852
52.127
51.878
51.694
40.444
40.323
38.707
34.886
29.338
29.061
28.066
26.818
25.545
25.160
25.079
24.901
24.751
f1 (ppm)

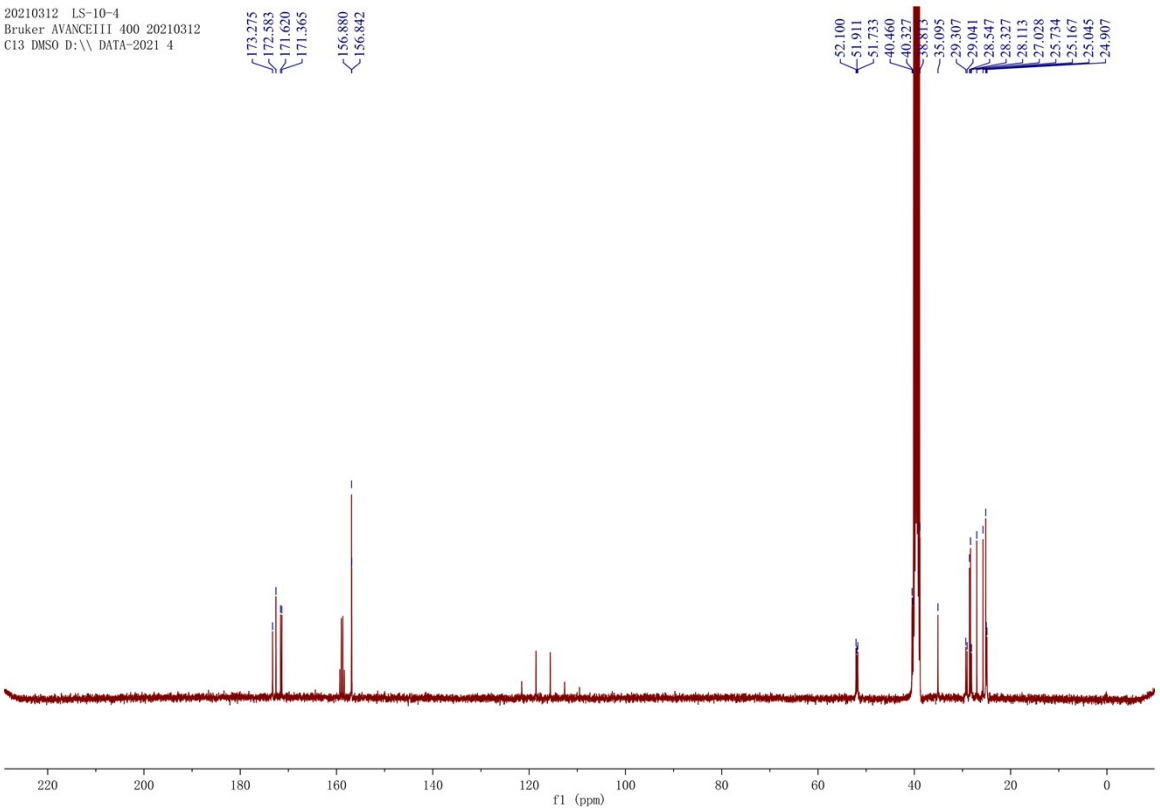
2c


LS-10-3
single_pulse
12.759
8.180
8.162
8.004
7.986
7.970
7.875
7.824
7.811
7.769
7.755
7.742
7.288
4.270
4.246
4.225
4.159
4.140
4.126
4.107
3.089
2.755
2.135
2.118
1.694
1.544
1.525
1.508
1.489
1.471
1.453
1.283
1.264
1.258
1.250
0.58
0.94
2.05
5.97
11.45
2.00
0.97
6.20
2.02
1.99
4.08
12.52
4.17
f1 (ppm)

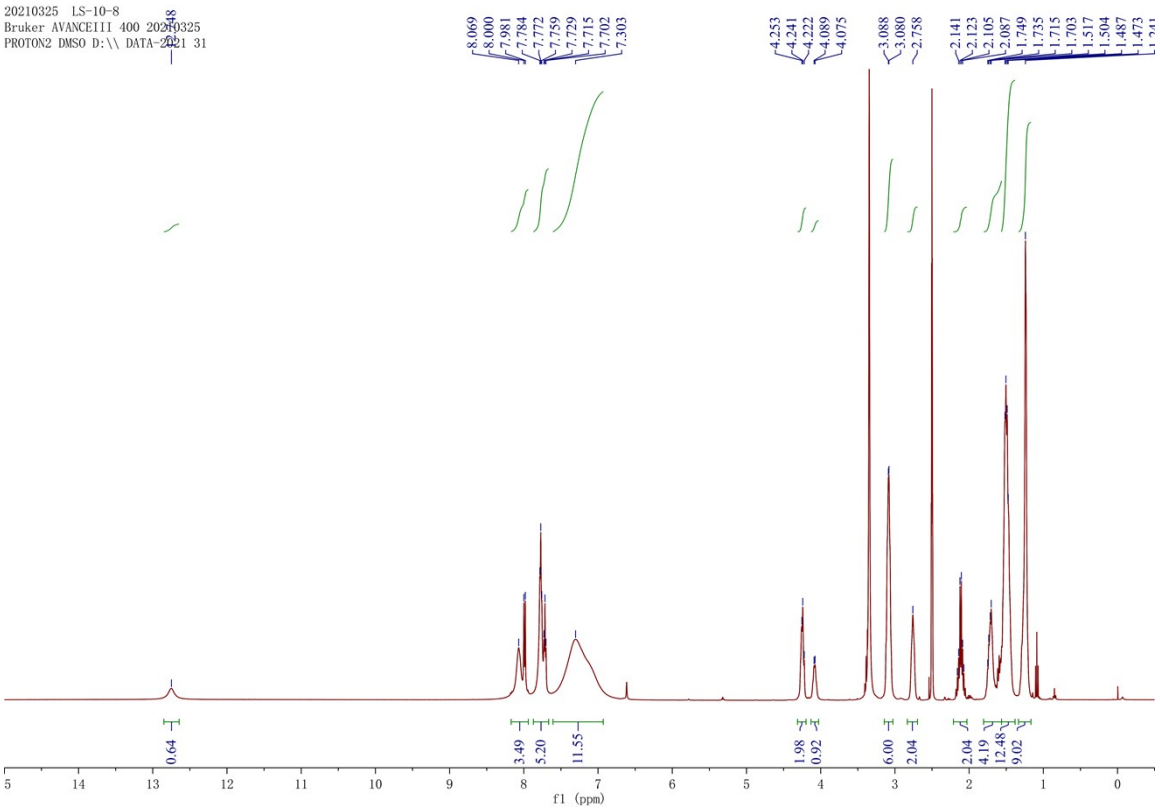

20210312 LS-10-3
Bruker AVANCEIII 400 20210312
C13 DMSO D:\\ DATA-2021 3
173.310
172.553
171.645
171.384
156.937
156.898
52.139
51.946
51.774
40.474
40.339
38.808
34.999
29.314
29.063
28.183
26.887
25.585
25.175
25.050
24.934
f1 (ppm)

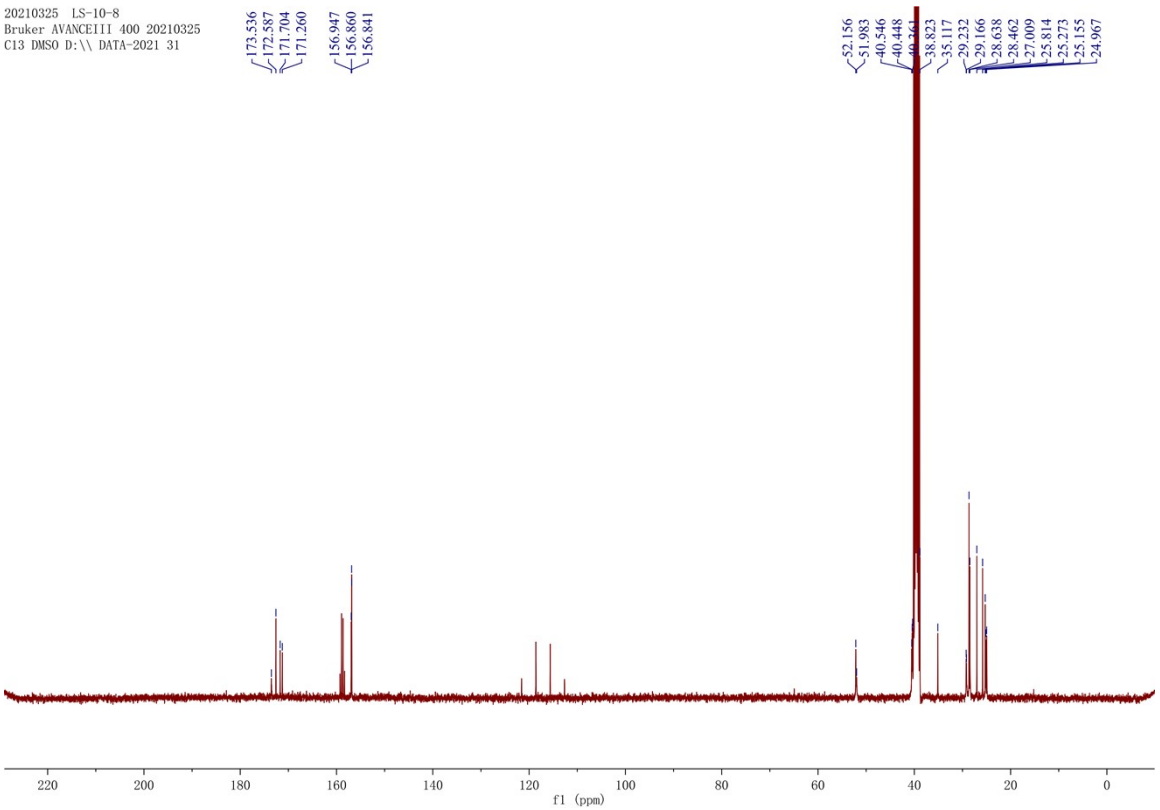
2d


LS-10-4
single_pulse
12.734
8.188
8.170
7.996
7.977
7.962
7.783
7.729
7.716
7.252
7.184
4.285
4.272
4.255
4.244
4.224
4.162
4.143
4.130
4.110
3.104
3.090
2.766
2.753
2.129
2.111
2.093
1.732
1.714
1.693
1.680
1.662
1.543
1.525
1.508
1.490
0.65
0.91
2.00
6.04
11.48
2.00
0.98
6.23
2.04
2.02
4.14
12.55
6.24
f1 (ppm)

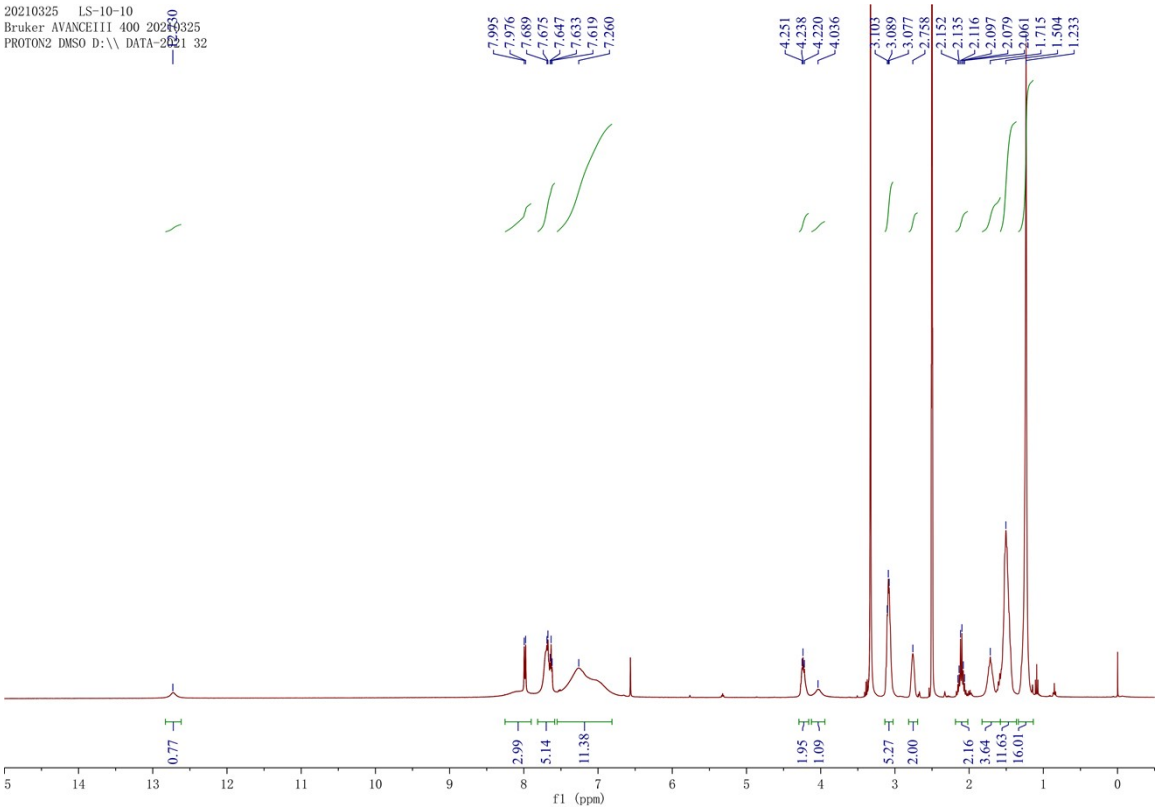

20210312 LS-10-4
Bruker AVANCEIII 400 20210312
C13 DMSO D:\\ DATA-2021 4
173.275
172.583
171.620
171.365
156.880
156.842
52.100
51.911
51.733
40.460
40.327
38.813
35.095
29.307
29.041
28.547
28.327
28.113
27.028
25.734
25.167
25.045
24.907
f1 (ppm)

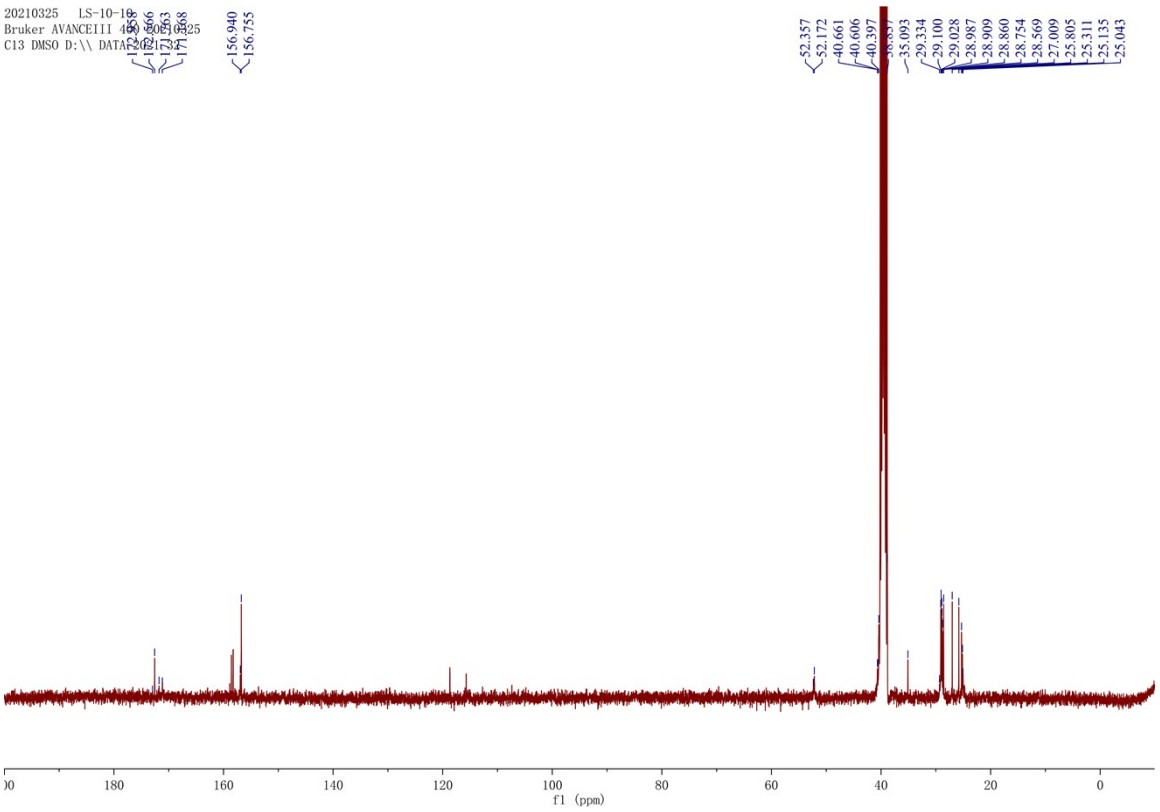
2e


20210325 LS-10-8
Bruker AVANCEIII 400 20210325
PROTON2 DMSO D:\\ DATA-2021 31
f1 (ppm)

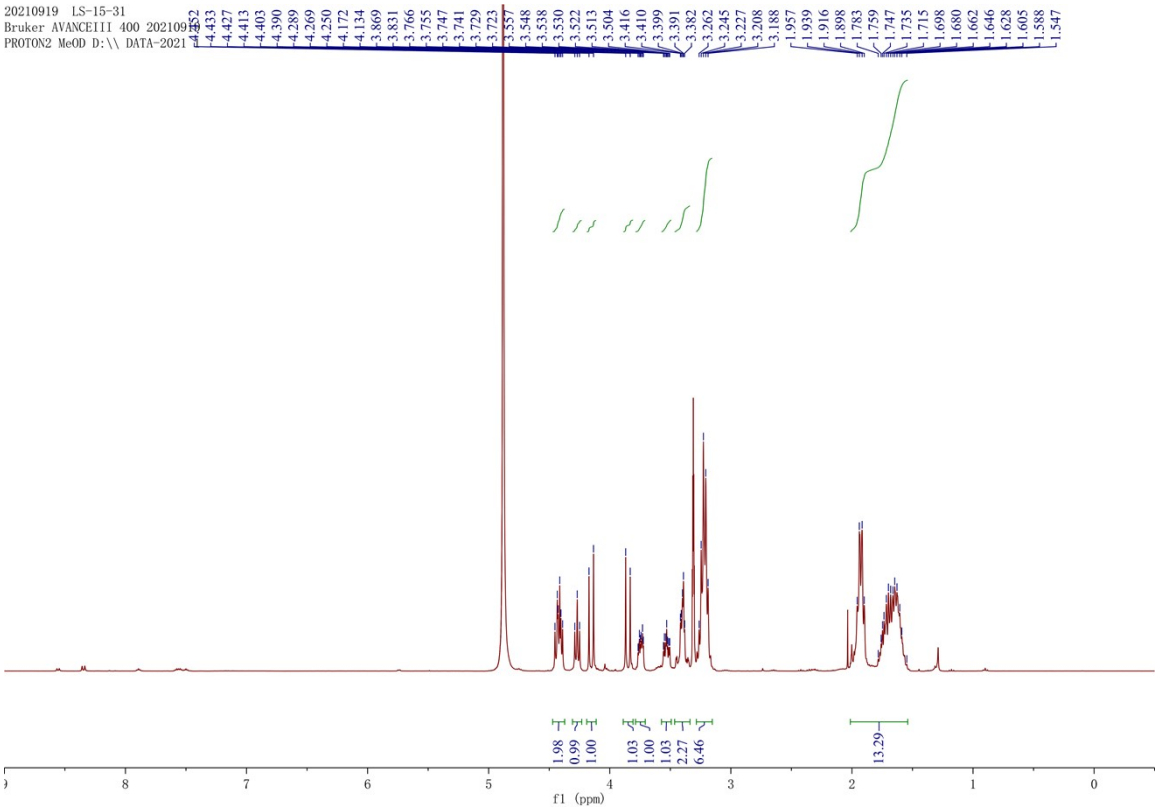

20210325 LS-10-8
Bruker AVANCEIII 400 20210325
C13 DMSO D:\\ DATA-2021 31
f1 (ppm)

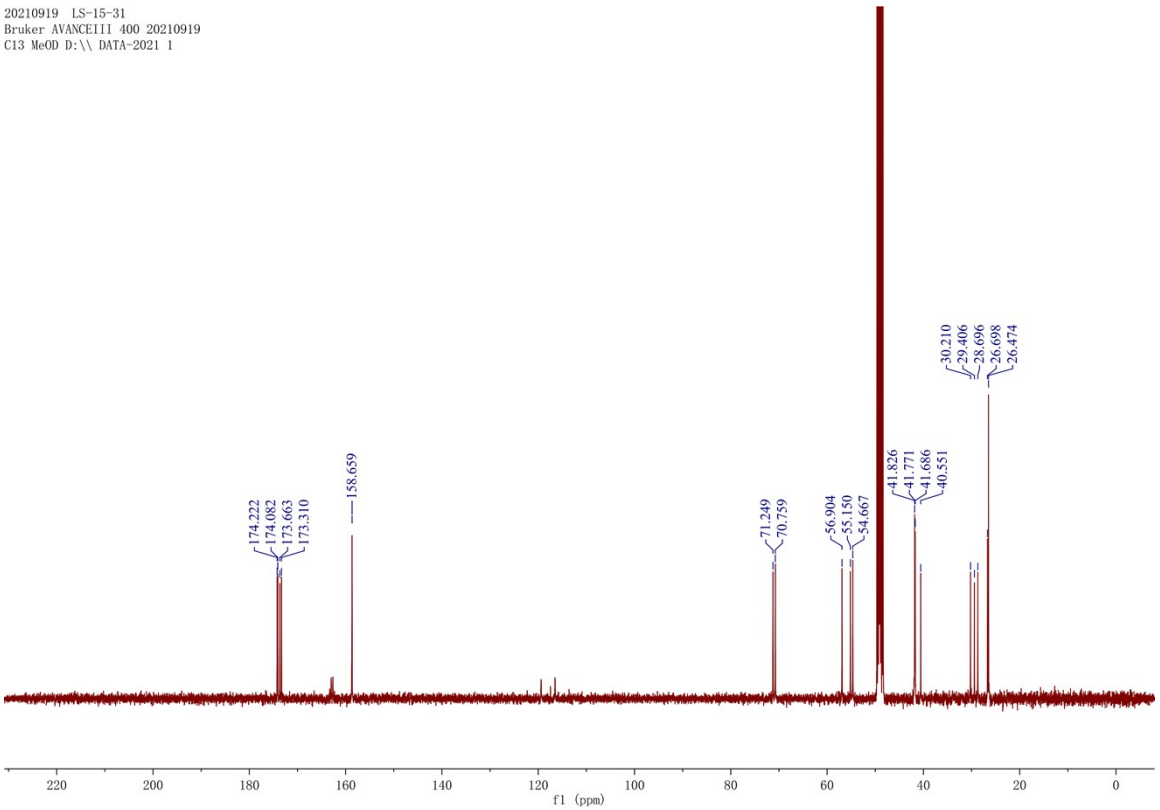
2f


20210325 LS-10-10
Bruker AVANCEIII 400 20210325
PROTON2 DMSO D:\\ DATA-2021 32
12.830
7.995
7.976
7.689
7.675
7.647
7.633
7.619
7.260
4.251
4.238
4.220
4.036
3.103
3.089
3.077
2.758
2.152
2.135
2.116
2.097
2.079
2.061
1.715
1.504
1.233
0.77
2.99
5.14
11.38
1.95
1.09
5.27
2.00
2.16
3.64
11.63
16.01
f1 (ppm)

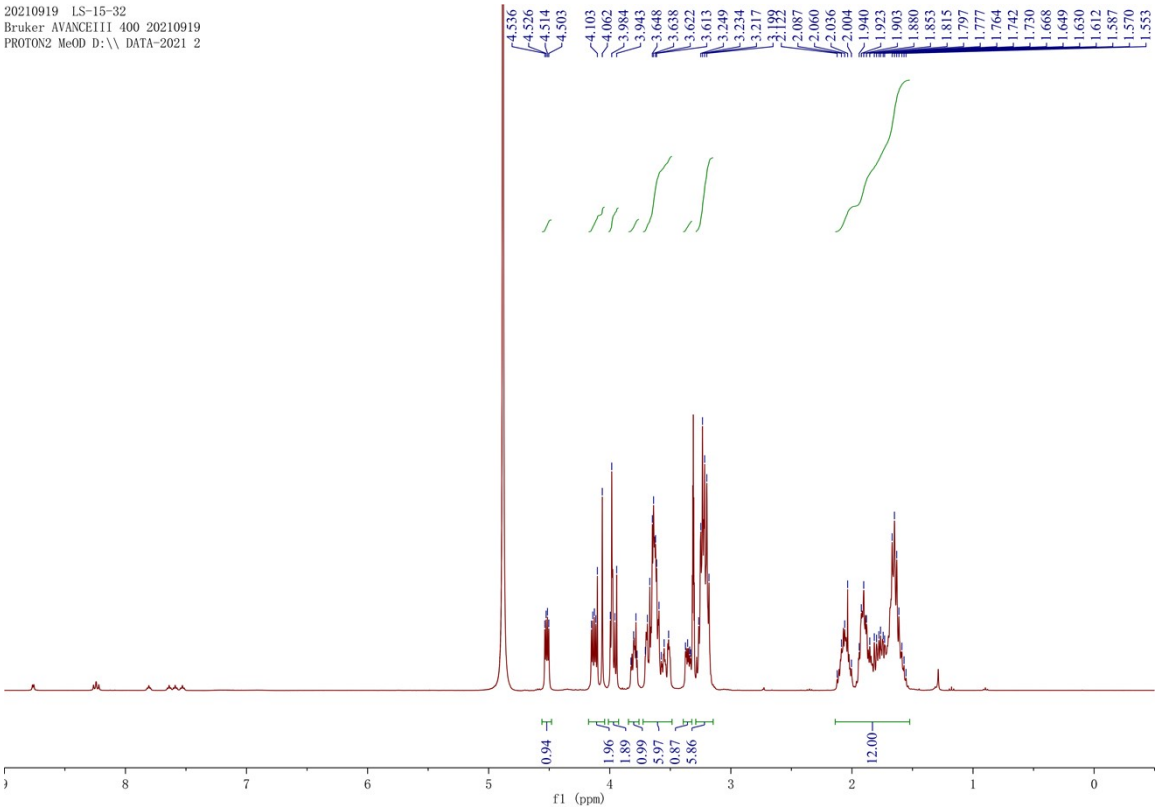

20210325 LS-10-10
Bruker AVANCEIII 400 20210325
C13 DMSO D:\\ DATA-2021
156.940
156.755
52.357
52.172
40.661
40.606
40.397
35.093
29.334
29.100
29.028
28.987
28.909
28.860
28.754
28.569
27.009
25.805
25.311
25.135
25.043
f1 (ppm)

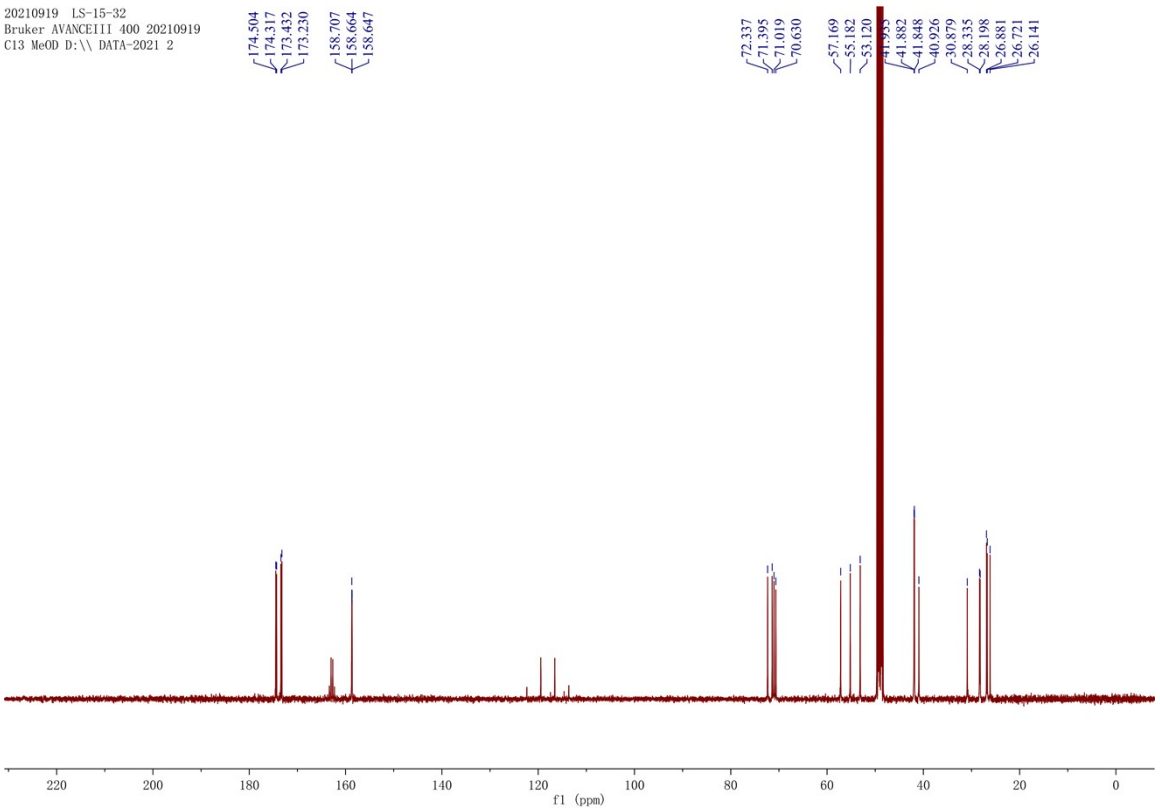
3a


20210919 LS-15-31
Bruker AVANCEIII 400 20210919
PROTON2 MeOD D:\\ DATA-2021
4.433
4.427
4.413
4.403
4.390
4.289
4.269
4.250
4.172
4.134
3.869
3.831
3.766
3.755
3.747
3.741
3.729
3.723
3.557
3.548
3.538
3.530
3.522
3.513
3.504
3.416
3.410
3.399
3.391
3.382
3.262
3.245
3.227
3.208
3.188
1.957
1.939
1.916
1.898
1.783
1.759
1.747
1.735
1.715
1.698
1.680
1.662
1.646
1.628
1.605
1.588
1.547
1.98
0.99
1.00
1.03
1.00
1.03
2.27
6.46
13.29
f1 (ppm)

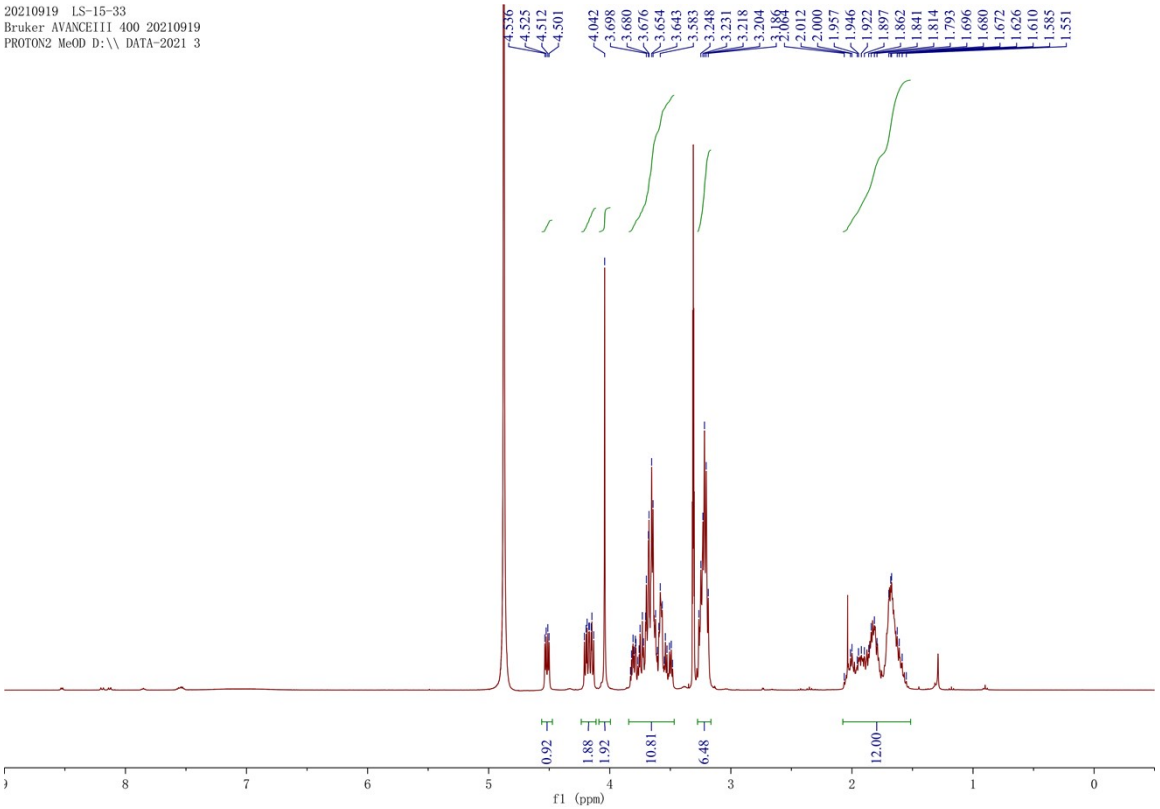

20210919 LS-15-31
Bruker AVANCEIII 400 20210919
C13 MeOD D:\\ DATA-2021 1
174.222
174.082
173.663
173.310
158.659
71.249
70.759
56.904
55.150
54.667
41.826
41.771
41.686
40.551
30.210
29.406
28.696
26.698
26.474
f1 (ppm)

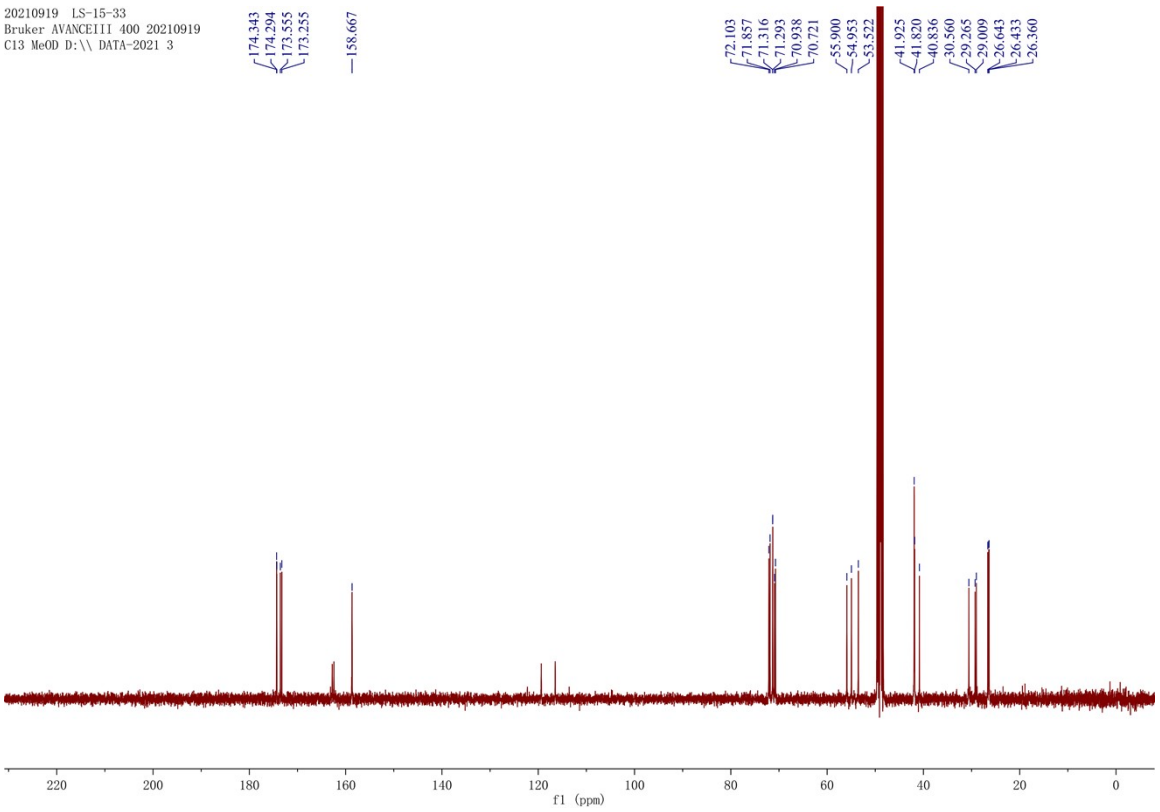
3b


20210919 LS-15-32
Bruker AVANCEIII 400 20210919
PROTON2 MeOD D:\\ DATA-2021 2
4.536
4.526
4.514
4.503
4.103
4.062
3.984
3.943
3.648
3.638
3.622
3.613
3.249
3.234
3.217
2.087
2.060
2.036
2.004
1.940
1.923
1.903
1.880
1.853
1.815
1.797
1.777
1.764
1.742
1.730
1.668
1.649
1.630
1.612
1.587
1.570
1.553
0.94
1.96
1.89
0.99
5.97
0.87
5.86
12.00
f1 (ppm)

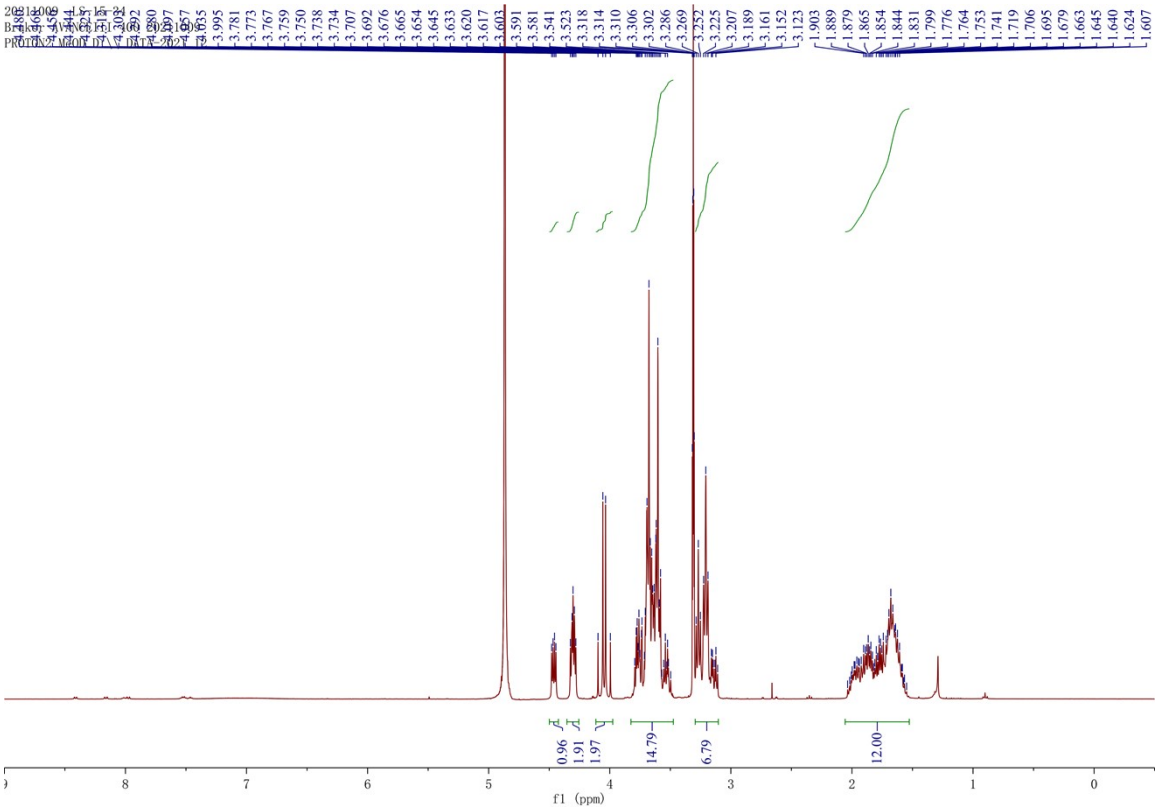

20210919 LS-15-32
Bruker AVANCEIII 400 20210919
C13 MeOD D:\\ DATA-2021 2
174.504
174.317
173.432
173.230
158.707
158.664
158.647
72.337
71.395
71.019
70.630
57.169
55.182
53.120
41.882
41.848
40.926
30.879
28.335
28.198
26.881
26.721
26.141
f1 (ppm)

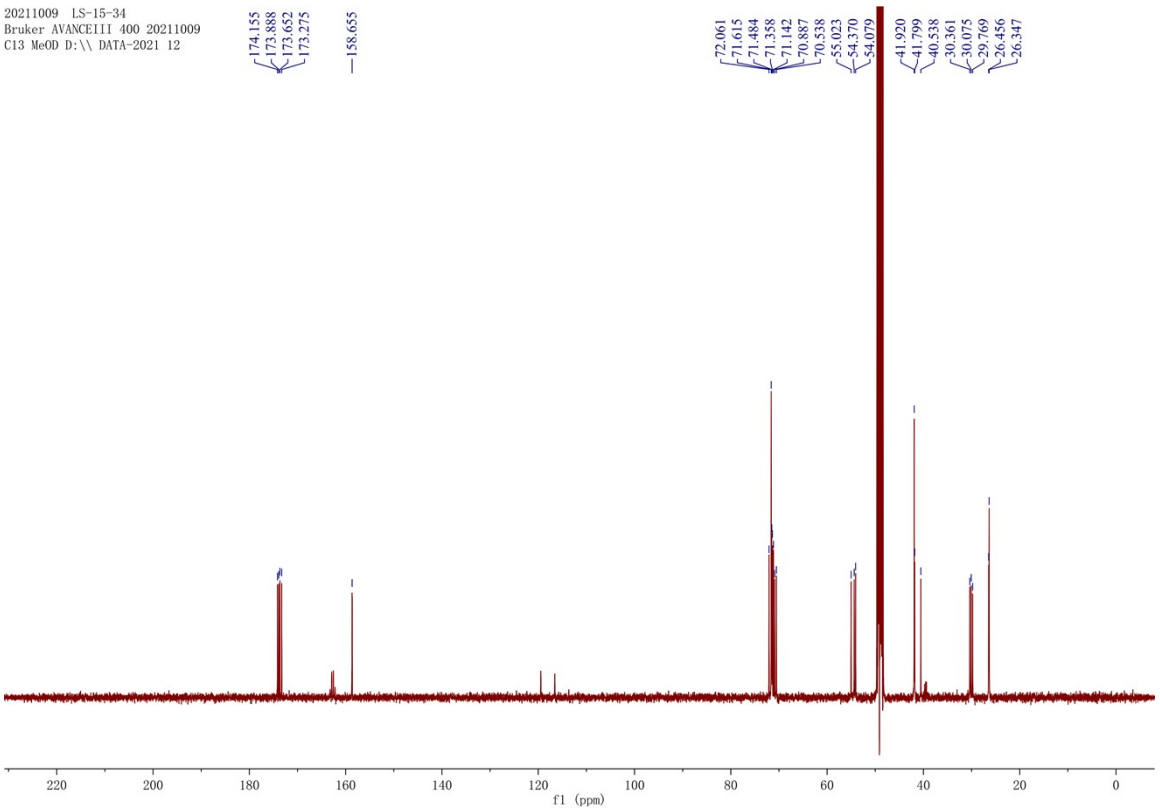
3c


20210919 LS-15-33
Bruker AVANCEIII 400 20210919
PROTON2 MeOD D:\\ DATA-2021 3
4.536
4.525
4.512
4.501
4.042
3.698
3.680
3.676
3.654
3.643
3.583
3.248
3.231
3.218
3.204
2.012
2.000
1.957
1.946
1.922
1.897
1.862
1.841
1.814
1.793
1.696
1.680
1.672
1.626
1.610
1.585
1.551
0.92
1.88
1.92
10.81
6.48
12.00
f1 (ppm)


20210919 LS-15-33
Bruker AVANCEIII 400 20210919
C13 MeOD D:\\ DATA-2021 3
174.343
174.294
173.555
173.255
158.667
72.103
71.857
71.316
71.293
70.938
70.721
55.900
54.953
53.522
41.925
41.820
40.836
30.560
29.265
29.009
26.643
26.433
26.360
f1 (ppm)

3d


0.96
1.91
1.97
14.79
6.79
12.00
f1 (ppm)


20211009 LS-15-34
Bruker AVANCEIII 400 20211009
C13 MeOD D:\\ DATA-2021 12
f1 (ppm)