Electronic Supplementary Materials

Stereoisomerism metabolites found in rats after oral administration of timosaponin B-II using HPLC-Q-TOF-MS and NMR method

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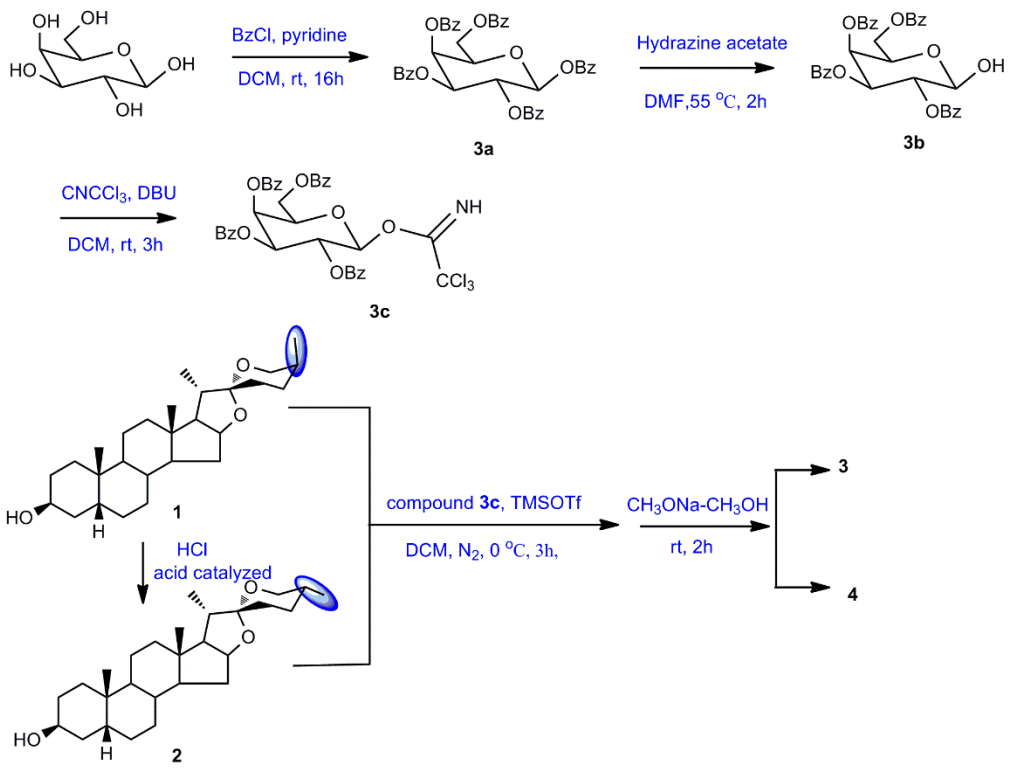


Scheme 1. Synthetic pathway for obtaining deglycosylated spirostanol metabolites: compounds **1-4**.

1. Synthesis of some derivates

1.1 Sarsasapogenin (1)

The dried and powdered extract of Rhizoma anemarrhenae (100 g) was dissolved in 95% EtOH (800 mL) and concentrated HCl (200 mL), the resulting solution was stirred and refluxed at 100 °C for 2 h. Then, the reaction solution was poured into cooled water (1 L) and filtered to obtain the precipitate. The deposit was washed with demineralized water, and dried under vacuum to obtain the crude product (4.0 g), which was recrystallized from acetone two times to yield sarsasapogenin (1.0 g).

White crystalline needle; ^{1}H-NMR (400 MHz, chloroform-*d*), δ_H (ppm): 4.40 (td, J = 7.9, 7.3, 6.2 Hz, 1H, H-16), 4.10 (brs, 1H, H-3), 3.94 (dd, J = 11.0, 2.8 Hz, 1H, H-26), 3.29 (d, J = 10.9 Hz, 1H, H-26), 1.07 (d, J = 7.1 Hz, 3H, H-21), 0.98 (d, J = 6.4 Hz, 3H, H-27), 0.97 (s, 3H, H-19), 0.75 (s, 3H, H-

18); ^{13}C-NMR (100 MHz, chloroform-*d*), δ_C (ppm): 109.9 (C-22), 81.2 (C-16), 67.2 (C-3), 65.3 (C-26), 62.2 (C-17), 56.6 (C-14), 42.3 (C-20), 40.8 (C-13), 40.5 (C-12), 40.0 (C-9), 36.7 (C-5), 35.4 (C-8), 35.4 (C-10), 33.7 (C-4), 31.9 (C-15), 30.1 (C-1), 28.0 (C-23), 27.2 (C-25), 26.7 (C-7), 26.7 (C-6), 26.1 (C-2), 25.9 (C-24), 24.1 (C-19), 21.0 (C-11), 16.6 (C-27), 16.2 (C-18), 14.5 (C-21); positive-ion HR-ESI/MS *m/z*: 417.3365 for $[M+H]^+$ (calcd. for $C_{27}H_{45}O_3^+$, 417.3363).

1.2 Isosarsasapogenin (2)

To a boiling solution of 5 g of sarsasapogenin in 500 mL of 95% ethanol was added a mixture of 300 mL of 95% EtOH and 150 mL of concentrated hydrochloric acid. After refluxing for 40 h on the oil-bathing, 100 mL concentrated hydrochloric acid was added and the refluxing continued for an additional 10 h. The solution was then poured into water and the resulting mixture was extracted with ether. The ether extract was washed with water, evaporated and removed. The residue was crystallized from acetone to give white needle crystals.

White crystalline needle; ^{1}H-NMR (400 MHz, chloroform-*d*), δ_H (ppm): 4.37 (td, J = 8.2, 7.5, 6.4 Hz, 1H, H-16), 4.08 (brs, 1H, H-3), 3.45 (dd, J = 11.0, 2.0 Hz, 1H, H-26), 3.35 (d, J = 10.8, 1H, H-26), 0.96 (s, 3H, H-19), 0.94 (d, J = 6.9 Hz, 3H, H-21), 0.77 (d, J = 6.2 Hz, 3H, H-27), 0.74 (s, 3H, H-18); ^{13}C-NMR (100 MHz, chloroform-*d*), δ_C (ppm): 109.4 (C-22), 81.0 (C-16), 67.2 (C-3), 67.0 (C-26), 62.4 (C-17), 56.6 (C-14), 41.7 (C-20), 40.8 (C-13), 40.4 (C-12), 40.0 (C-9), 36.6 (C-5), 35.4 (C-8), 35.4 (C-10), 33.6 (C-4), 31.9 (C-15), 31.5 (C-23), 30.4 (C-25), 30.1 (C-1), 28.9 (C-24), 27.9 (C-2), 26.7 (C-7), 26.7 (C-6), 24.0 (C-19), 21.0 (C-11), 17.2 (C-27), 16.6 (C-18), 14.6 (C-21); positive-ion HR-ESI/MS *m/z*: 417.3366 $[M+H]^+$ (calcd. for $C_{27}H_{45}O_3^+$, 417.3363).

1.3 Galactosyl trichloroacetimidate (3c)

To a magnetically stirred solution of galactose (5.0 g, 27.8 mmol) in anhydrous pyridine (100 mL) and

DMF (200 mL), benzoyl chloride (17.8 mL, 152.4 mmol) was added dropwise under ice bath. The reaction continued for 16 h after naturally rising to room temperature. Then the solution was extracted with EtOAc (150 mL*3) following water (50 mL) was added into the mixture to quench the excess benzoyl chloride. The organic layer was merged and washed with water, diluted hydrochloric acid, water, saturated $NaHCO_3$ solution and water in sequence. Afterwards, it was concentrated to dryness under reduced pressure and used as the material for next step without purification, compound **3a**.

Compound **3a** (15.0 g, 21.5 mmol) was dissolved in dry DMF (100 mL), and then the hydrazine acetate (2.5 g 27 mmol) was added. After stirred at 55 °C for 1.5 h, the flask was cooled to room temperature with running water. The solution was extracted with EtOAc and washed with saline solution, and then the organic layer was separated out and concentrated to dryness under reduced pressure. Finally, the residue was purified by column chromatography over the silica gel with petroleum ether-ethyl acetate (5:1) to yield the white power, compound **3b** (9.6 g, yield 75%).

The DBU (0.55 mL, 5.8 mmol) was added to the solution of compound **3b** (5.0 g, 8.3 mmol) and $CNCCl_3$ (5 mL, 50.7 mmol) in anhydrous CH_2Cl_2 (70 mL) at room temperature. Then, the mixture was continued to stir until the compound **3b** was not observed by TLC. The crude products were obtained by removing the solvent, which were purified by column chromatography over the silica gel with petroleum ether-ethyl acetate (10:1, 1% Et_3N) to yield the white power, compound **3c** (4.8 g, yield 78%).

1.4 Timosaponin AI (3)

To a stirring solution of **3c** (0.74 g, 1.0 mmol) in 15 mL of dry CH_2Cl_2 was added 4 Å molecular sieves (0.5-1.0 mm, 2 g) followed by sarsasapogenin (0.5 g, 1.2 mmol) as a solid powder. It was stirred for 0.5 h under ice-bath, and then TMSOTf (60 μL, 0.3 mmol) was added under nitrogen (N_2) protection. The

mixture was continuously stirred at 0 °C for 1 h and room temperature for another 0.5 h, and then neutralized with Et_3N. The yellow solution was diluted with CH_2Cl_2 and filtered to remove the molecular sieves. The organic layer was concentrated under reduced pressure to give brownish syrup, which was purified by column chromatography with petroleum ether-ethyl acetate (10:1) to afford compound **3d** (0.9 g, yield 90.5%) as a white power.

Compound **3d** (0.7 g, 0.7 mmol) was dissolved in MeOH-CH_2Cl_2 (1:1, 40mL), and then 40 mg CH_3ONa was added. After stirring at room temperature for 3 h, the solution was neutralized with ion-exchange resin (H^+), and then filtered and concentrated. The white residue was purified by column chromatography with ethyl acetate-methanol (20:1) to afford the white solid, compound **3** (0.28 g, yield 96.2%).

White crystalline needle; ^{1}H-NMR (400 MHz, DMSO-d_6) , δ_H (ppm): 4.67 (m, 1H, H-16), 4.64 (d, J = 7.0 Hz, 1H, H-1'), 4.53 (m, 1H, H-2'), 4.30 (m, 2H, H-6'), 4.09 (m, 1H, H-4'), 3.89 (o, 2H, H-26, H-5'), 3.64 (m, 1H, H-3'), 3.29 (o, H, H-26), 1.00 (d, J = 7.1 Hz, 3H, H-21), 0.92 (d, J = 6.8 Hz, 3H, H-27), 0.90 (s, 3H, H-19), 0.70 (s, 3H, H-18); ^{13}C-NMR (100 MHz, DMSO-d_6), δ_C (ppm): 108.8 (C-22), 101.8 (C-1′), 80.4 (C-16), 75.0 (C-3), 73.6 (C-5′), 72.7 (C-3′), 70.7 (C-2′), 68.1 (C-4′), 64.3 (C-26), 61.9 (C-17), 60.4 (C-6′), 55.7 (C-14), 41.6 (C-20), 40.2 (C-13), 39.7 (C-12), 39.4 (C-9), 35.9 (C-5), 34.9 (C-8), 34.6 (C-10), 31.4 (C-4), 30.1 (C-15), 29.5 (C-1), 26.5 (C-25), 26.4 (C-2), 26.2 (C-7), 26.0 (C-6), 25.6 (C-23), 25.4 (C-24), 23.6 (C-19), 20.5 (C-11), 16.2 (C-27), 16.0 (C-18), 14.5 (C-21); positive-ion HR-ESI/MS *m/z*: 579.3899 $[M+H]^+$ (calcd. for $C_{33}H_{55}O_8^+$, 579.3891).

1.5 Isotimosaponin AI (4)

The similar procedure for the preparation of compound **3** was adopted, and thus isosarsasapogenin (0.5 g, 1.2 mmol) produced compound **4** (0.26 g, yield 89.3%) as a white solid.

White crystalline needle; ^{1}H-NMR (400 MHz, DMSO-d_6), δ_H (ppm):4.66 (m, 1H, H-16), 4.62 (d, J = 7.2 Hz, 1H, H-1'), 4.51 (m, 1H, H-2'), 4.29 (m, 2H, H-6'), 4.09 (m, 1H, H-4'), 3.89 (o, H, H-5'), 3.61 (m, 1H, H-3'), 3.25 (o, 2H, H-26), 0.91 (d, J = 19.7 Hz, 3H, H-21), 0.90 (s, 3H, H-19), 0.73 (d, J = 6.2 Hz, 3H, H-27), 0.71 (s, 3H, H-18).; ^{13}C-NMR (100 MHz, DMSO-d_6), δ_C (ppm): 108.4 (C-22), 101.8 (C-1′), 80.3 (C-16), 75.0 (C-3), 73.6 (C-5′), 72.7 (C-3′), 70.7 (C-2′), 68.1 (C-4′), 65.9 (C-26), 62.0 (C-17), 60.3 (C-6′), 55.7 (C-14), 41.4 (C-20), 40.2 (C-13), 39.7 (C-12), 39.4 (C-9), 35.9 (C-5), 34.9 (C-8), 34.6 (C-10), 31.4 (C-4), 31.0 (C-23), 30.1 (C-15), 29.8 (C-25), 29.5 (C-1), 28.5 (C-24), 26.4 (C-2), 26.2 (C-7), 26.0 (C-6), 23.6 (C-19), 20.5 (C-11), 17.1 (C-27), 16.2 (C-18), 14.7 (C-21); positive-ion HR-ESI/MS *m/z*: 579.3897 $[M+H]^+$ (calcd. for $C_{33}H_{55}O_8^+$, 579.3891). It was determined to be (25*R*)-5*β*-spirostan-3-*O*-*β*-D-galactopyranoside, and simply named isotimosaponin AI.

1.6 Timosaponin BIII-d (5)

A solution of timosaponin BII (2.0 g) and weak hydrochloric acid (approximately, 0.25 mol·L^{-1}) was refluxed at 100 °C for 3 h. The reaction solution was partitioned with *n*-butanol and the *n*-butanol soluble portion was evaporated to dryness and the residue was separated by chromatography on preparative HPLC (λ=218 nm) eluting with 30% acetonitrile to afford the white powder. And it was regarded as the novel compound that had not been reported from natural sources or synthesis previous.

White, amorphous powder, the detail ^{13}C-NMR (pyridine-d_5, 100MHz) and ^{1}H-NMR (pyridine-d_5, 400MHz) information see the next section Table 1; positive-ion HR-ESI/MS *m/z*: 741.4422 $[M+H]^+$ (calcd. for $C_{39}H_{65}O_{13}^+$, 741.4420). It was determined to be (25*S*)-26-*O*-*β*-D-glucopyranosyl -20(22)-ene-5*β*-furost-3-*O*-*β*-D-galactopyranoside.

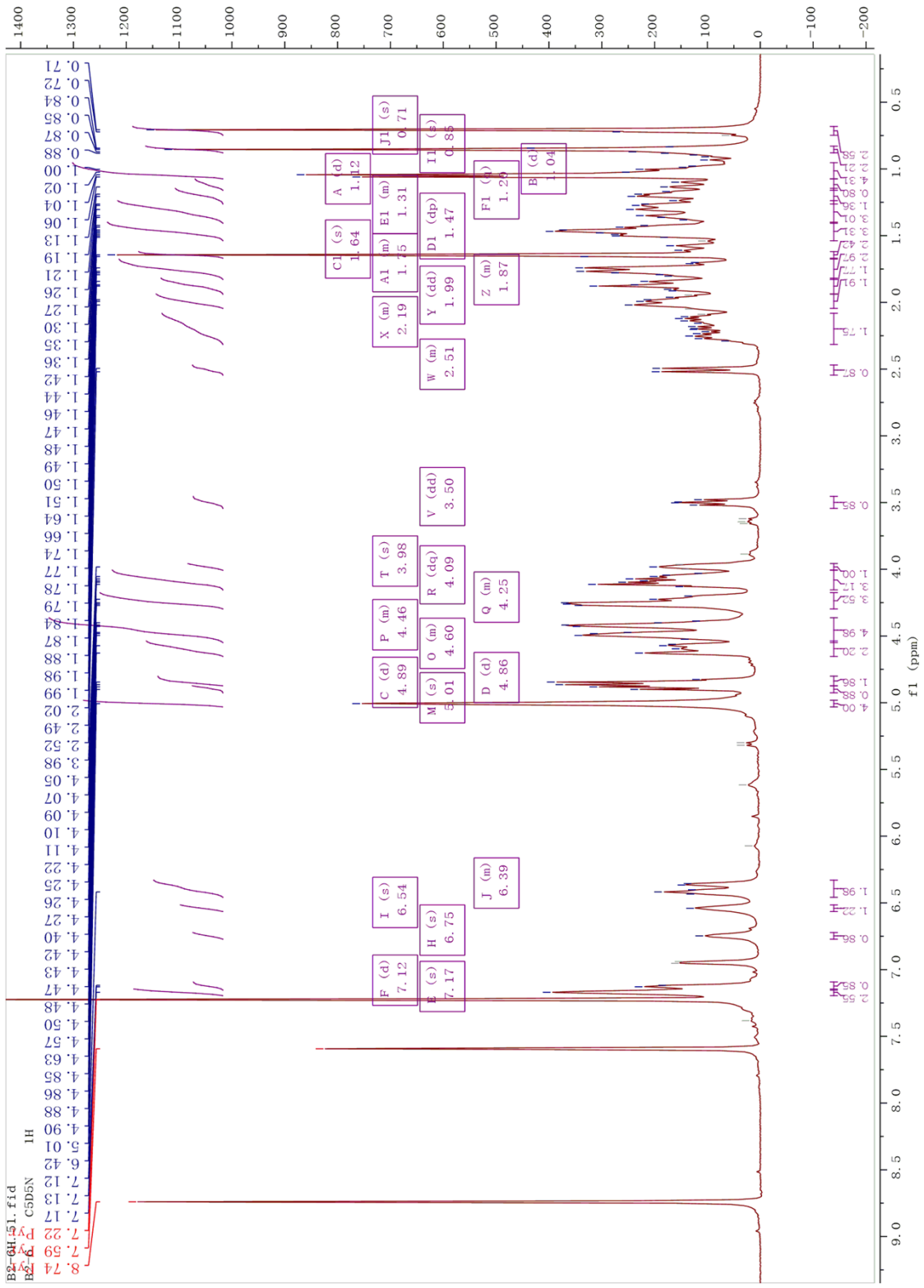


Fig. S1 The ^{1}H-NMR spectra of timosaponin BIII-d (400 MHz, in pyridine-d_5)

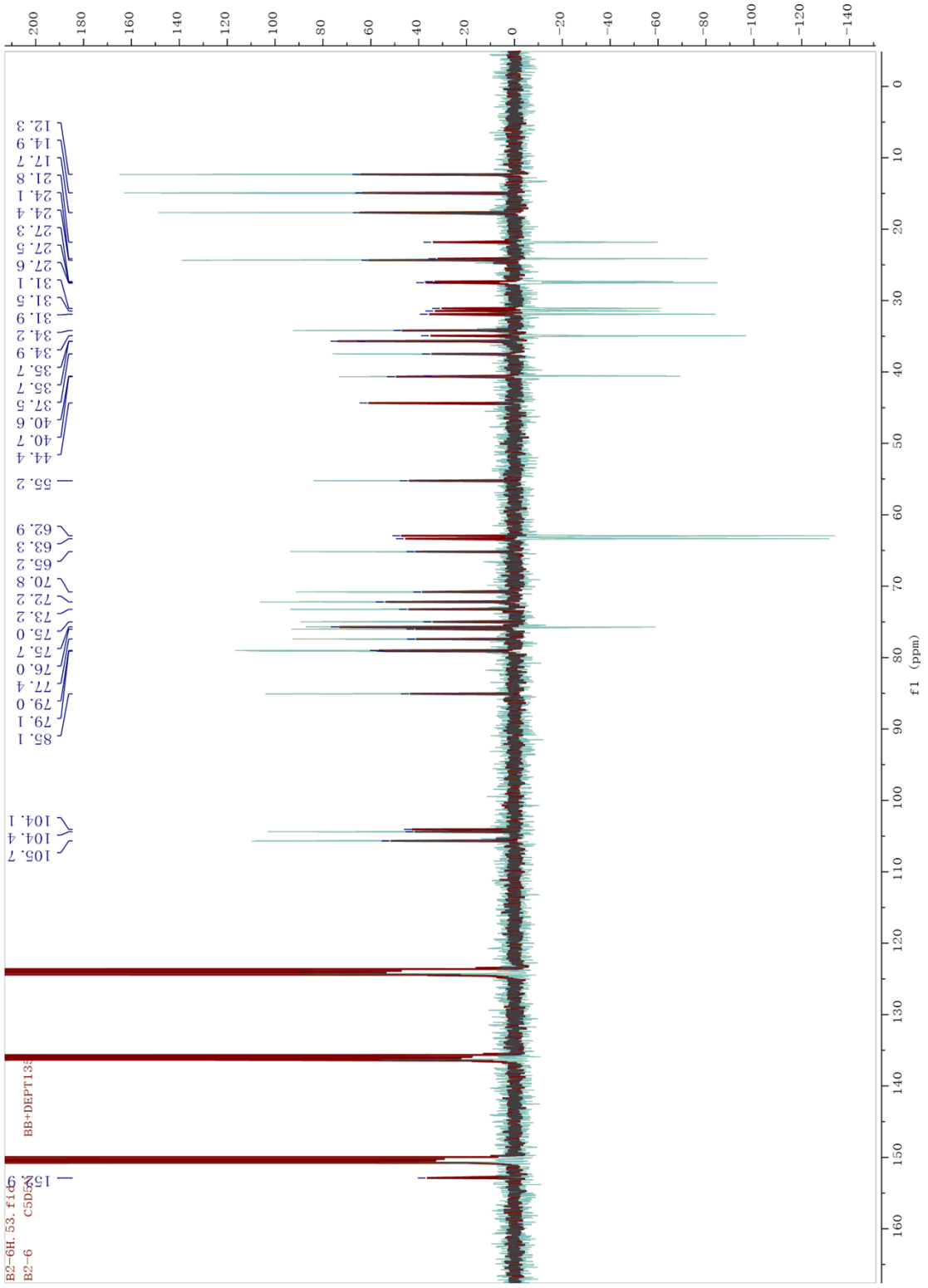


Fig. S2 The superposition of ^{13}C-NMR spectra of timosaponin BIII-d (100 MHz, in pyridine-d_5): brownish for BB and blue for DEPT-135.

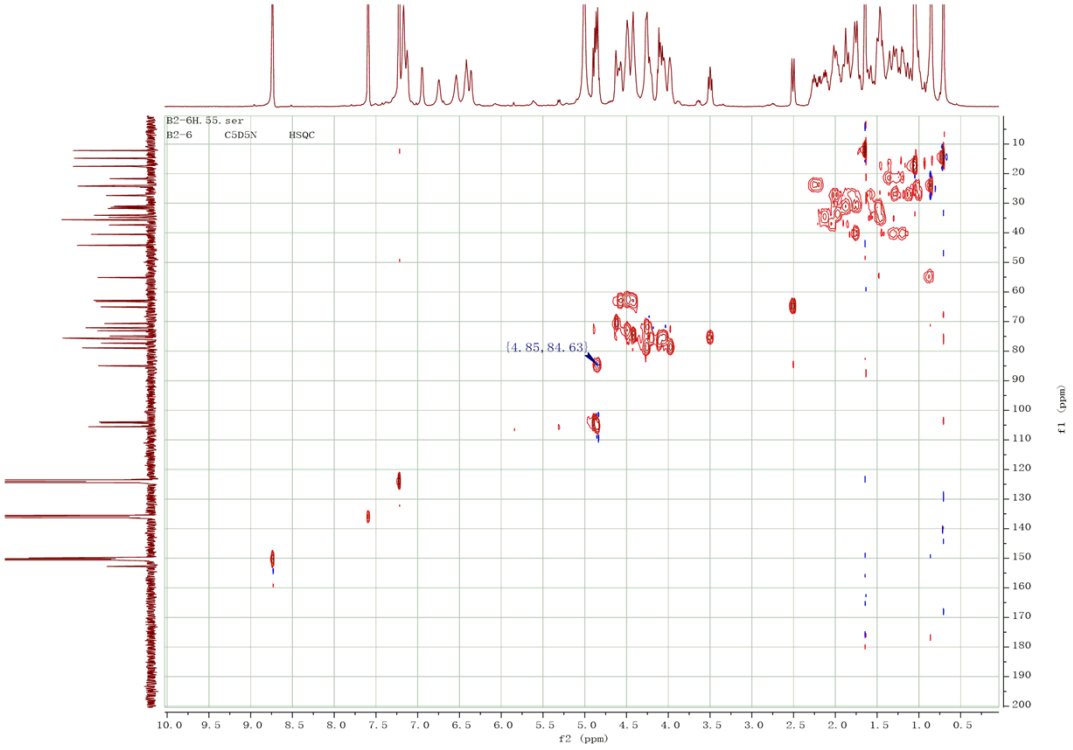


Fig. S3 The HSQC spectra of timosaponin BIII-d

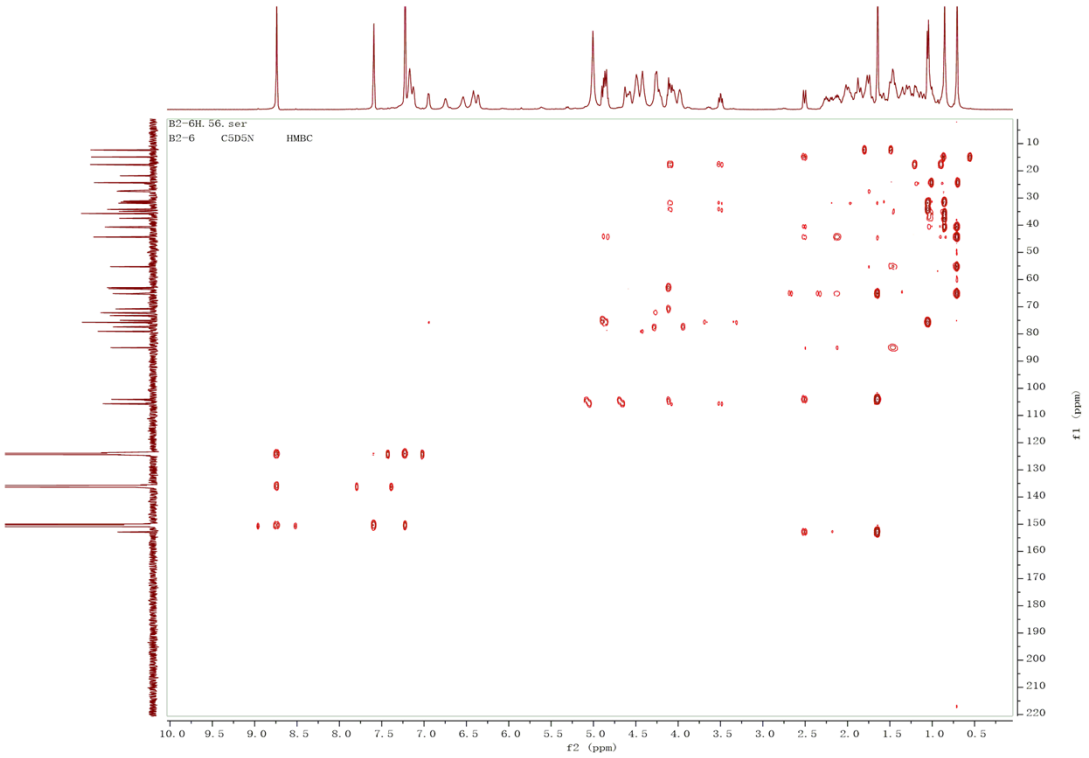


Fig. S4 The HMBC spectra of timosaponin BIII-d

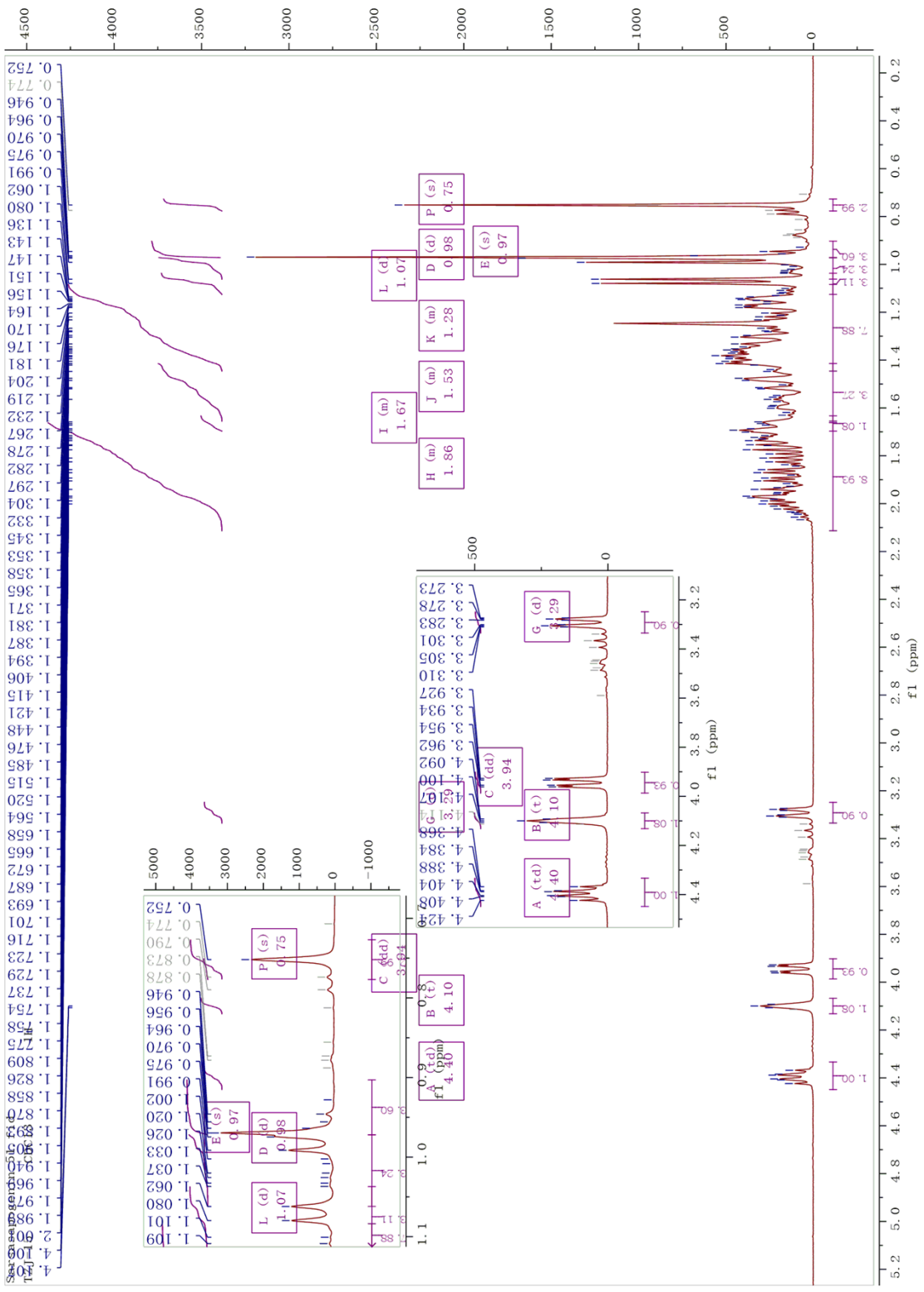


Fig. S5 The [1]H-NMR spectra of Sarsasapogenin (400 MHz, in chloroform-*d*)

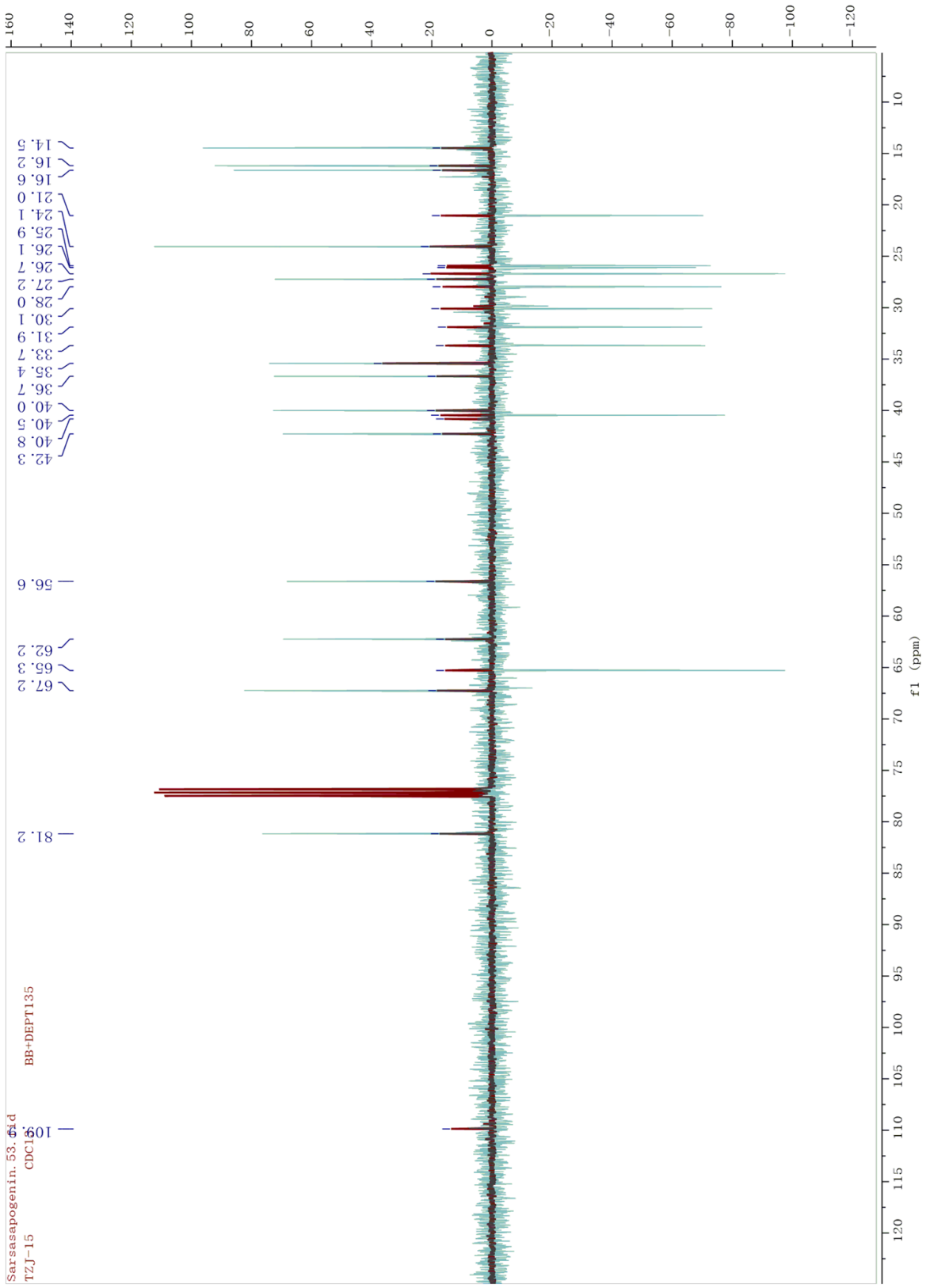


Fig. S6 The superposition of ^{13}C-NMR spectra of Sarsasapogenin (100 MHz, in chloroform-*d*): brownish for BB and blue for DEPT-135.

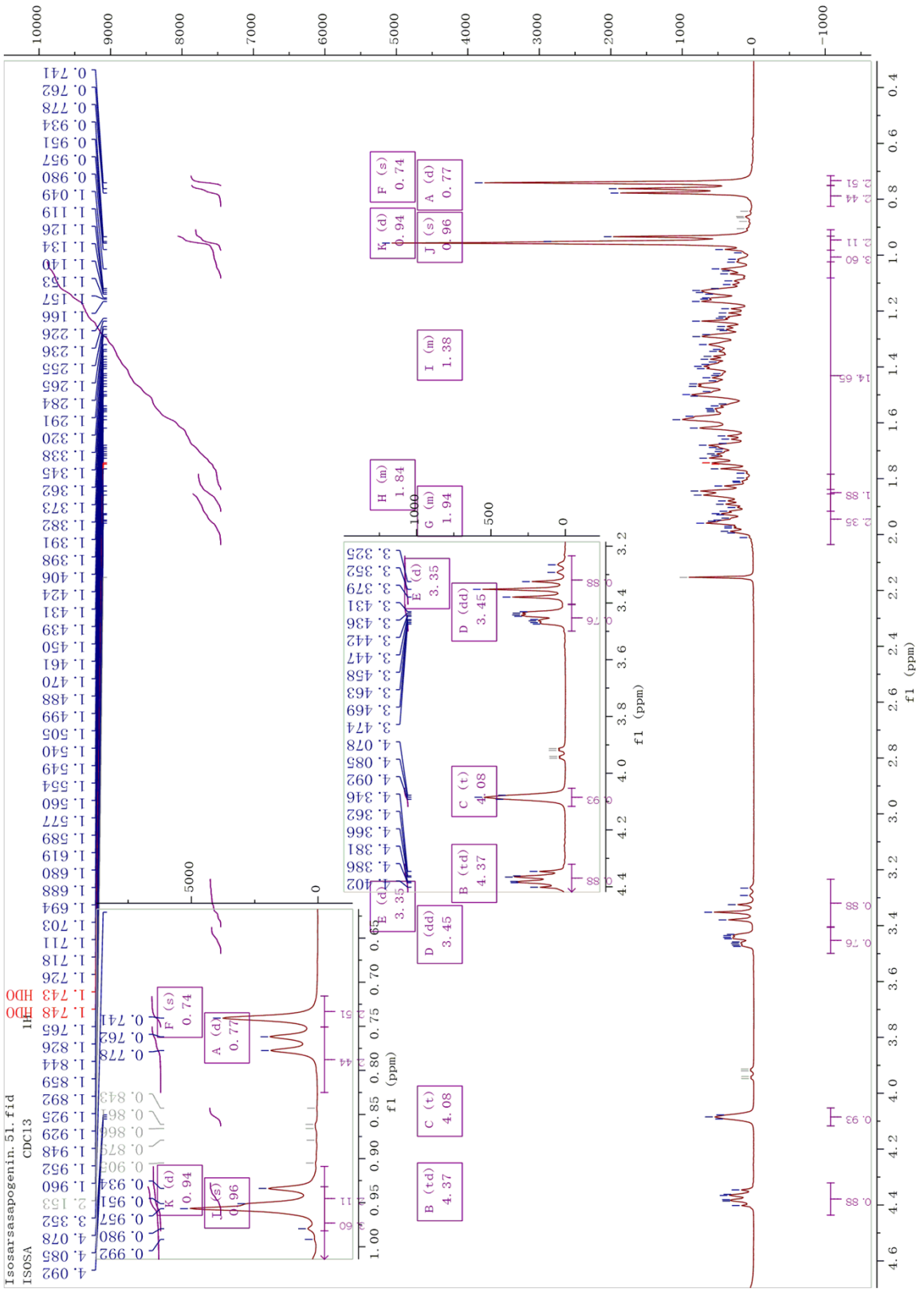


Fig. S7 The ^{1}H-NMR spectra of Isosarsasapogenin (400 MHz, in chloroform-*d*)

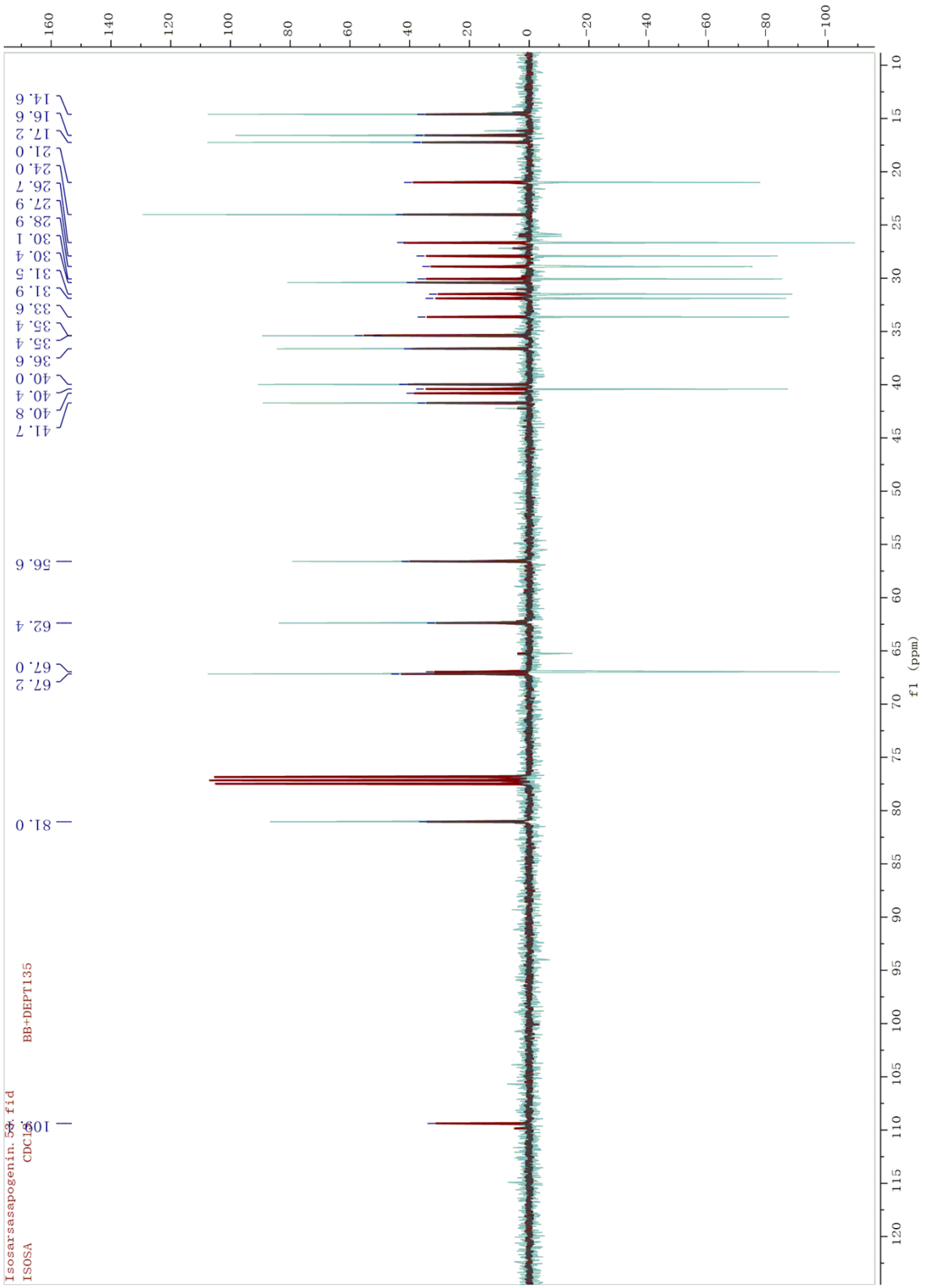


Fig. S8 The superposition of ^{13}C-NMR spectra of Isosarsasapogenin (100 MHz, in chloroform-*d*): brownish for BB and blue for DEPT-135.

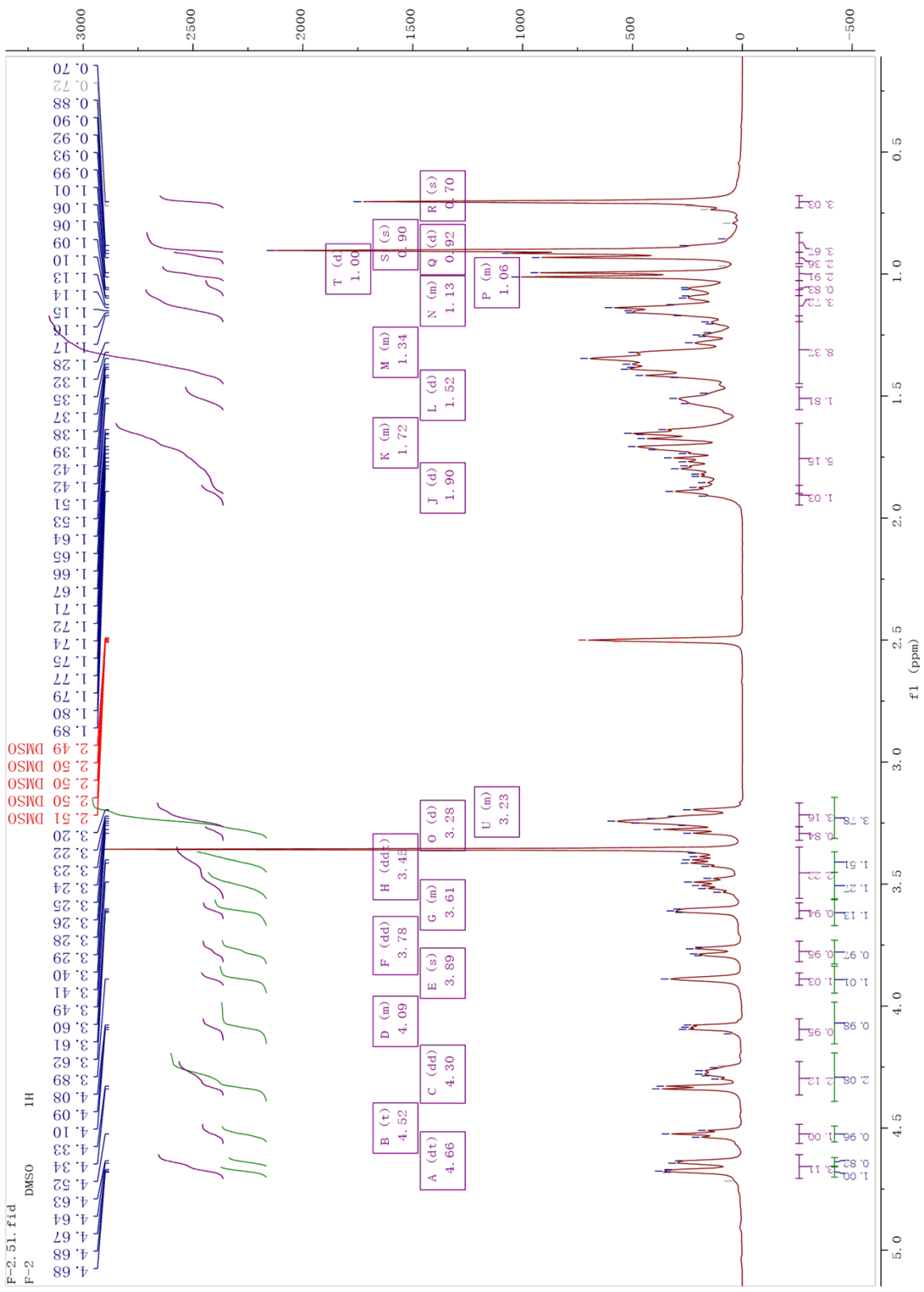


Fig. S9 The ^{1}H-NMR spectra of Timosaponin A-I (400 MHz, in DMSO-d_6)

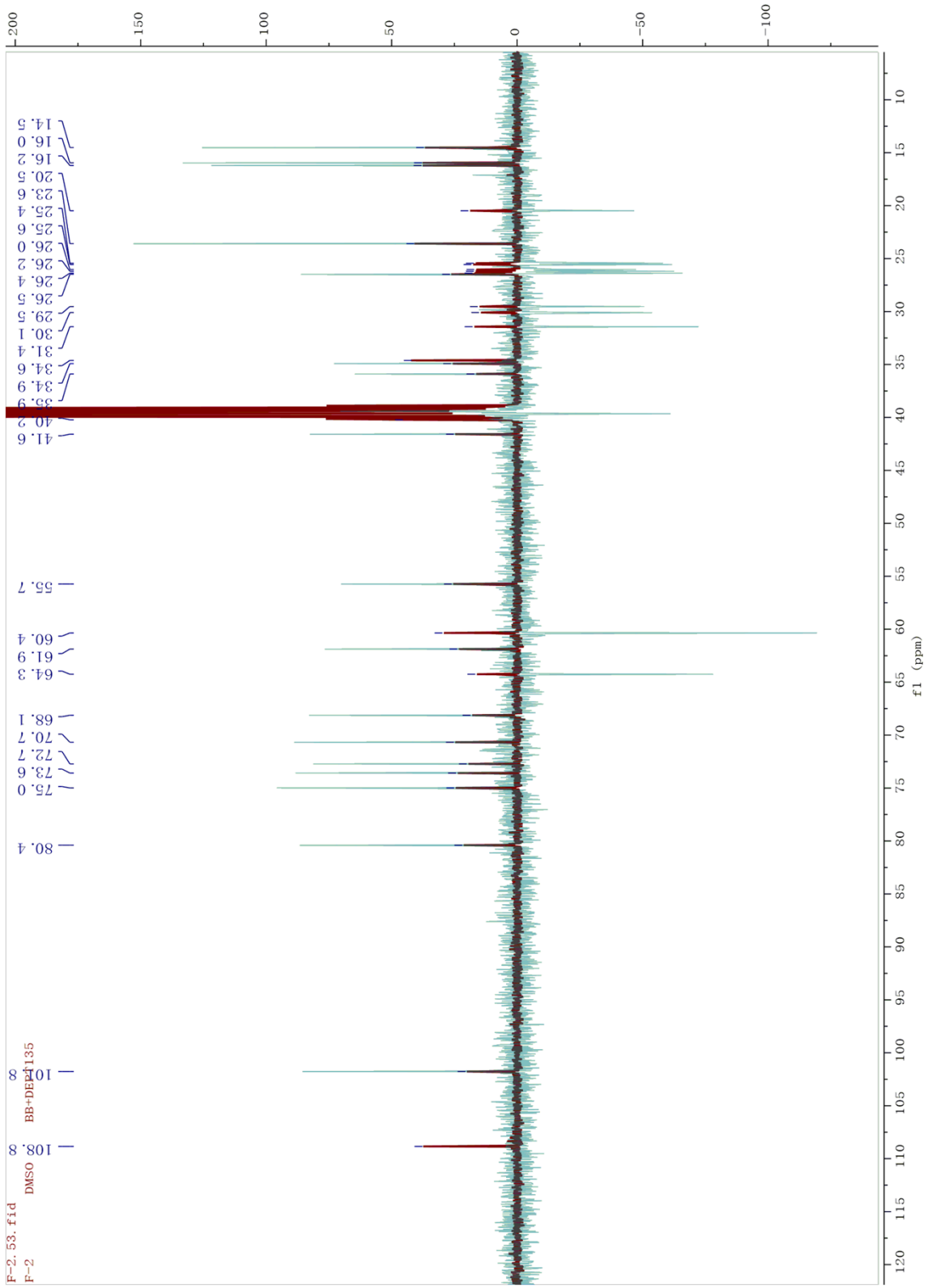


Fig. S10 The superposition of ^{13}C-NMR spectra of Timosaponin A-I (100 MHz, in DMSO-d_6): brownish for BB and blue for DEPT-135.

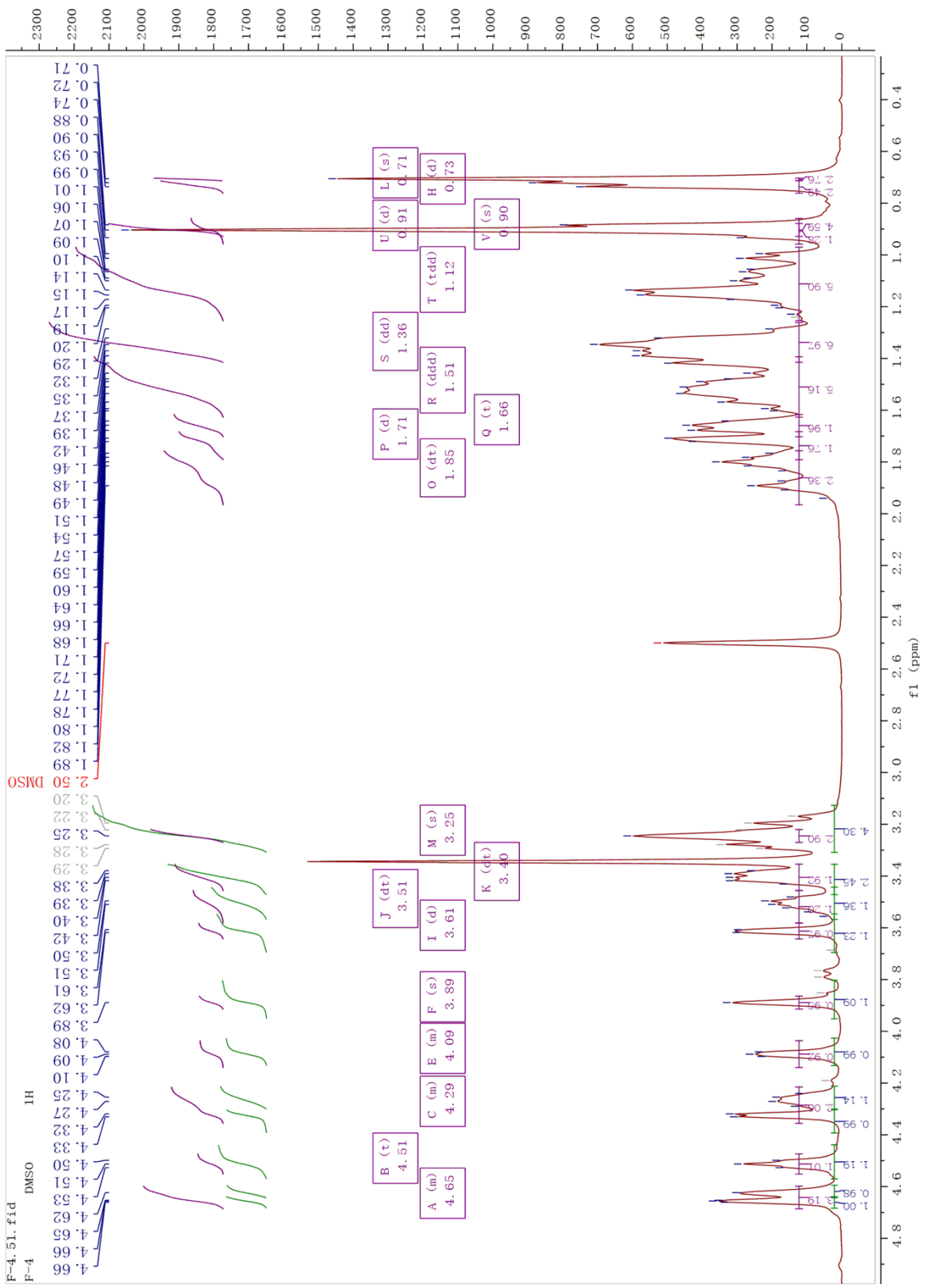
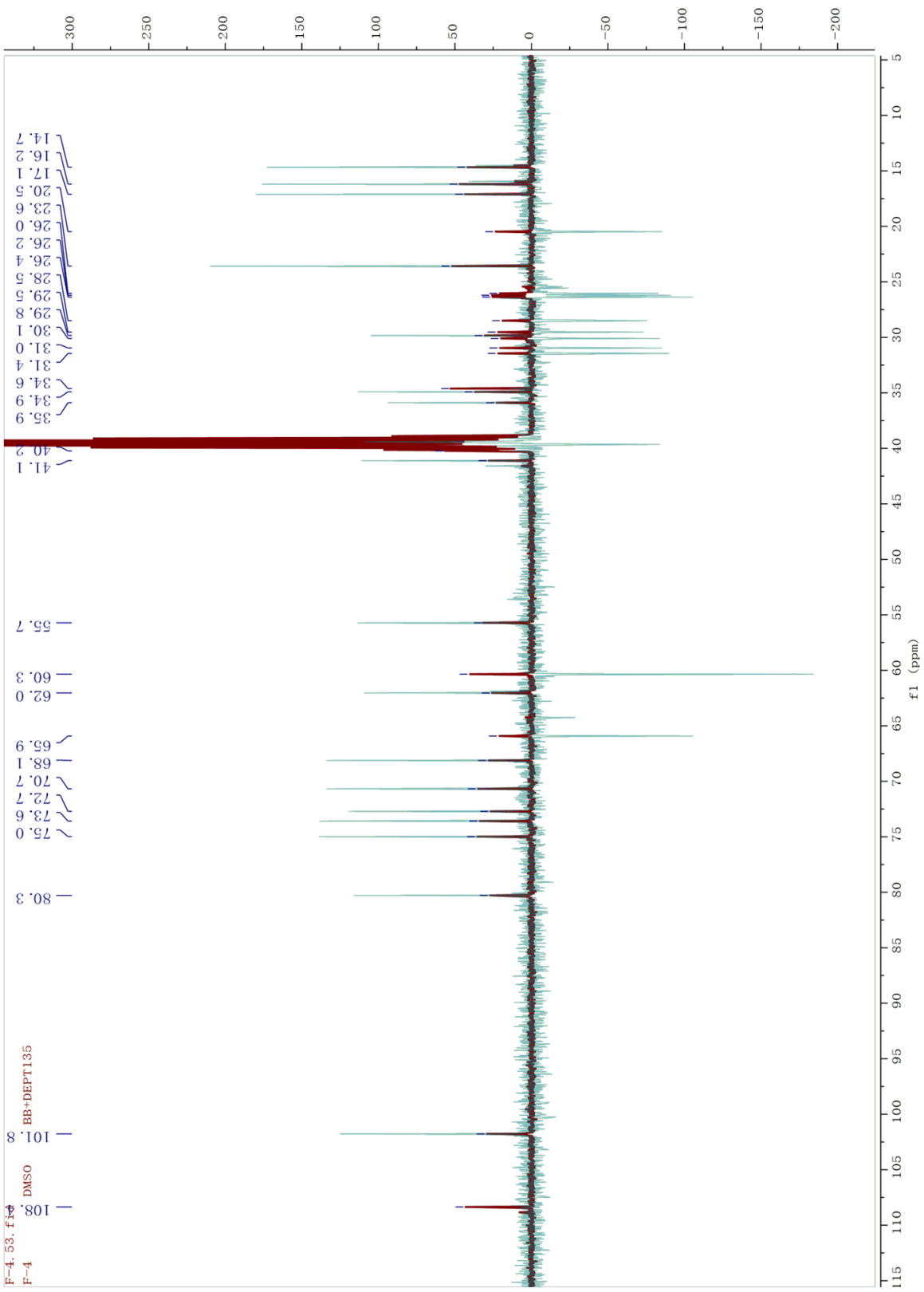


Fig. S11 The ^{1}H-NMR spectra of Isotimosaponin A-I (400 MHz, in DMSO-d_6)



147

148 **Fig. S12** The superposition of ^{13}C-NMR spectra of Isotimosaponin A-I (100 MHz, in DMSO-d_6):
149 brownish for BB and blue for DEPT-135.

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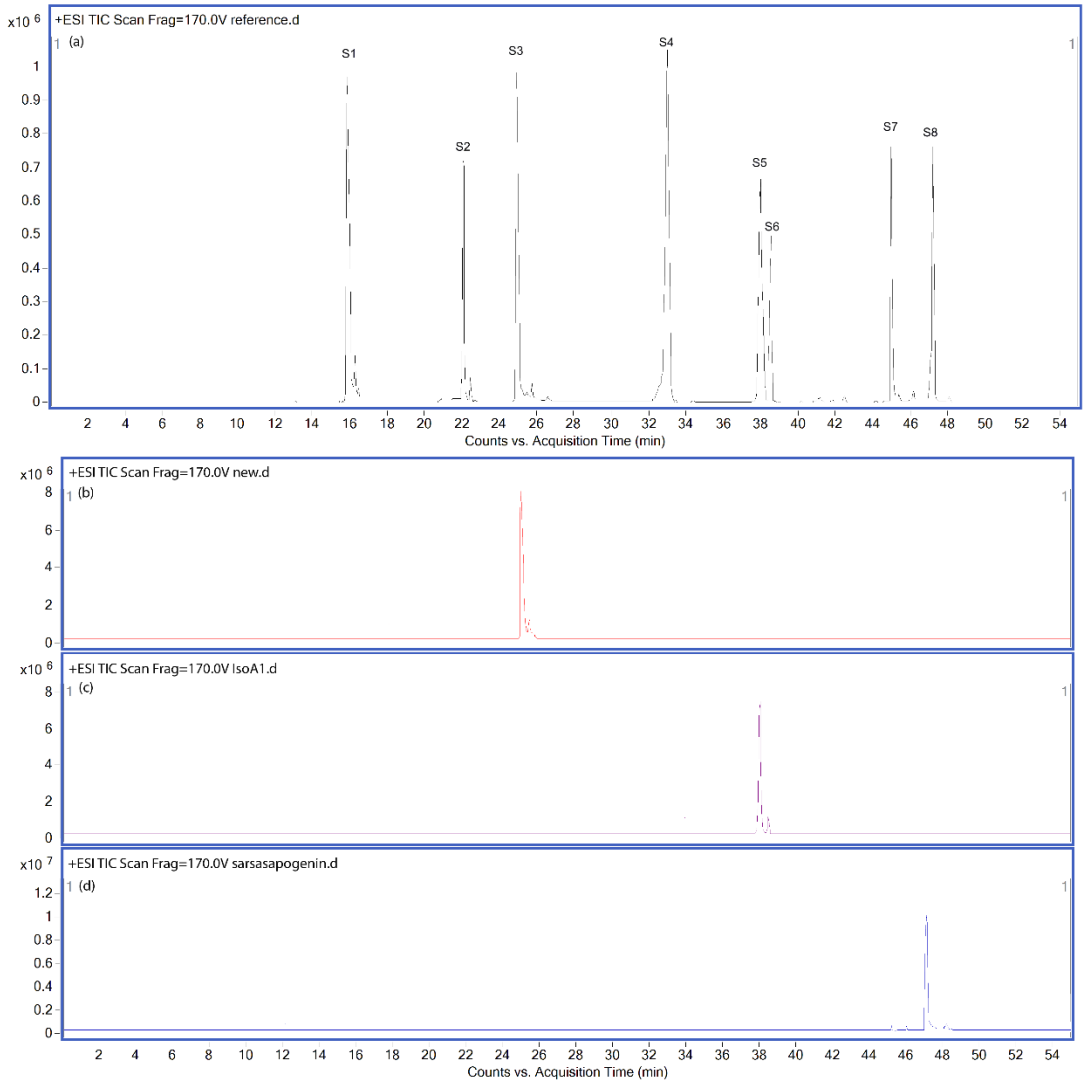

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153 **Fig. S13** Total ion chromatograms for eight reference compounds (a), the single injection analysis

154 of timosaponin BIII-d (b), isotimosaponin A-I (c), and sarsasapogenin (d) in positive mode.

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