Supramolecular Azasugar Clusters based on an Amphiphilic Fatty-Acid-Deoxynojirimycin Derivative as Multivalent Glycosidase Inhibitors

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1. Materials and Methods

1.1 General Instrumentation

^{1}H NMR and ^{13}C NMR spectra were recorded on a 600 MHz spectrometers using tetramethylsilane (TMS) as the internal standard. The chemical shifts are expressed in part per million (ppm) relative to TMS (δ = 0 ppm). HRMS analysis was performed on a FT-MALDI MS (Bruker Company) or an ABI 4700 Proteomics Analyzer (Applied Biosystems) in positive ion reflection mode or positive ion liner mode. The Dynamic Light Scattering (DLS) result was performed on a light scattering spectrometer (Brookhaven BI-APDV, USA) equipped with a He−Ne laser working at 4 mW (λ = 633 nm). Transmission Electron Microscope (TEM) image was recorded on Tecnai G^2 F20 (FEI). The blood glucose levels were measured with a glucometer (Roche, Germany). The glycosidases of α-mannosidase (*jack bean*, pH = 5.5, Product number: M7257-5MG), β-mannosidase (*helix pomatia*, pH = 5.5, Product number: M9400-5UN), α-galactosidase (*green coffee beans*, pH = 6.8, Product number: G8507-25UN) and β-galactosidase (*E. coli*, pH = 7.3, Product number: G8511-5MG) were all purchased from Sigma-Aldrich. And the corresponding reaction substrates of 4-nitrophenyl α-D-mannopyranoside (Product number: N2127-1G), 4-nitrophenyl β-D-mannopyranoside (Product number: N1268-100MG), 4-nitrophenyl α-D-galactopyranoside (Product number: N0877-1G) and 2-nitrophenyl β-D-galactopyranoside (Product number: N1127-1G) were all purchased from Sigma-Aldrich.

1.2 Methods

Surface tension experiment. Surface tension was measured with the pendant drop method at room temperature by a contact angle meter (Dataphysics OCA15EC Micro, Germany). The determination of the critical micelle concentration (*CMC*) of FA-DNJ was achieved with the method as described previously. Briefly, the surface tension method was applied to determine the *CMC* of FA-DNJ with its concentration varied from 1×10^{-5} mol/mL to 5×10^{-4} M. Data acquisition was made using the pendant drop method via a contact angle meter at room temperature.

Dynamic Light Scattering (DLS) and ζ-potential values experiments. The size distribution and ζ-potential values of the self-assembly formed in different pH values in citric acid-phosphate buffer at the concentration of 1.0×10^{-4} M were measured with the DLS technique employed a light scattering spectrometer (Brookhaven BI-APDV，USA) equipped with a He−Ne laser working at 4 mW (λ= 633 nm). The sample solutions were heating in water bath in 10 minutes and standing in 10 h at room temperature. Then solutions were filtered through a 0.45 μm syringe filter (Triton-

free). All measurements were performed at 25 ± 0.1°C. Each sample was performed at 8 runs. The size distribution of the aggregates formed in citric acid-phosphate buffer with pH 2.0, 3.0 4.0, 5.0, 6.0 and 7.0 were measured on same method with above.

Transmission Electron Microscopy (TEM). 0.1 mM of **FA-DNJ** solution was dropped on a carbon-coated copper grid, and which was sir-dried in temperature.

Determination of the standard curve of p-nitrophenol (PNP) in the citric acid-phosphate buffer (pH 5.5, 0.1 mmol), and in the citric acid-phosphate buffer (pH 6.8, 0.1 mmol).

p-Nitrophenol (PNP) (13.9 mg, 0.1 mmol) was dissolved in 5 mL of the citric acid-phosphate buffer (pH 5.5, 0.1 mmol) or the PBS buffer (pH 6.8, 0.1 mmol), the final concentration was 20 mmol/L, and then which was diluted to the final concentrations of 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, 32.0, 64.0, 128.0 and 256.0 μmol/L by the buffer solution, respectively. 100 μL of the above standard solutions (0.5, 1.0, 2.0, 4.0, 8.0, 16.0, 32.0, 64.0, 128.0 and 256.0 μmol/L) was plated in 96-well microassay culture plates, and then 100 μL of Na_2CO_3 solution was added, the plates were mixed for 3 min. The optical density of each well was then measured on a microplate spectrophotometer at a wavelength of 405 nm. The reaction dynamics of the standard curve of p-nitrophenol (PNP) or o-Nitrophenol (ONP) were fitted by line formula (y = Ax + B), the slopes (K_{slope}) were obtained.

Glycosidase Inhibitory Activities of FA-DNJ

Inhibition assay was determined by spectrophotometrically measuring the residual hydrolytic activities of the different glycosidases against the respective p-nitro-phenyl-α-glycopyranoside, or β-D-glycopyranoside, or o-nitro-phenyl-α- glycopyranoside, in the presence of the assemblies of **FA-DNJ** or the control drug. The glycosidase inhibitory activities of **FA-DNJ** were performed in 96-well microassay culture plates with blank group, control group, experimental blank group and experimental group, 3 parallel experiments for per group.

Firstly, 20 μL of the citric acid-phosphate buffer (pH 5.5, 0.1 mmol) was added to the 96-well plates, then 10 μL of α-mannosidase (0.02U/mL) was added to per well. After that, various concentrations of **FA-DNJ** (20 μL of 5, 10, 20, 40, 80 and 160 μmol/L) were added the 96-well plates for the final concentrations of 0, 0.5, 1.0, 2.0, 4.0, 8.0 and 16.0 μmol/L. The mixture was well mixed for 3 min, and incubated at 37 °C in a 5% CO_2 incubator for 10 min. Then, reaction substrates of 4-nitrophenyl α-D-mannopyranoside (50 μL of 0.5, 1.0, 2.0, 4.0, 8.0 and 16.0 mmol/L) were added, mixed for 3 min, and incubated at 37 °C in a 5% CO_2 incubator for 20 min. At last, 100 μL of Na_2CO_3 (1 mol/L) was added, mixed for 3 min. The absorbance of the reaction mixture was determined at 405 nm with a microplate spectrophotometer.

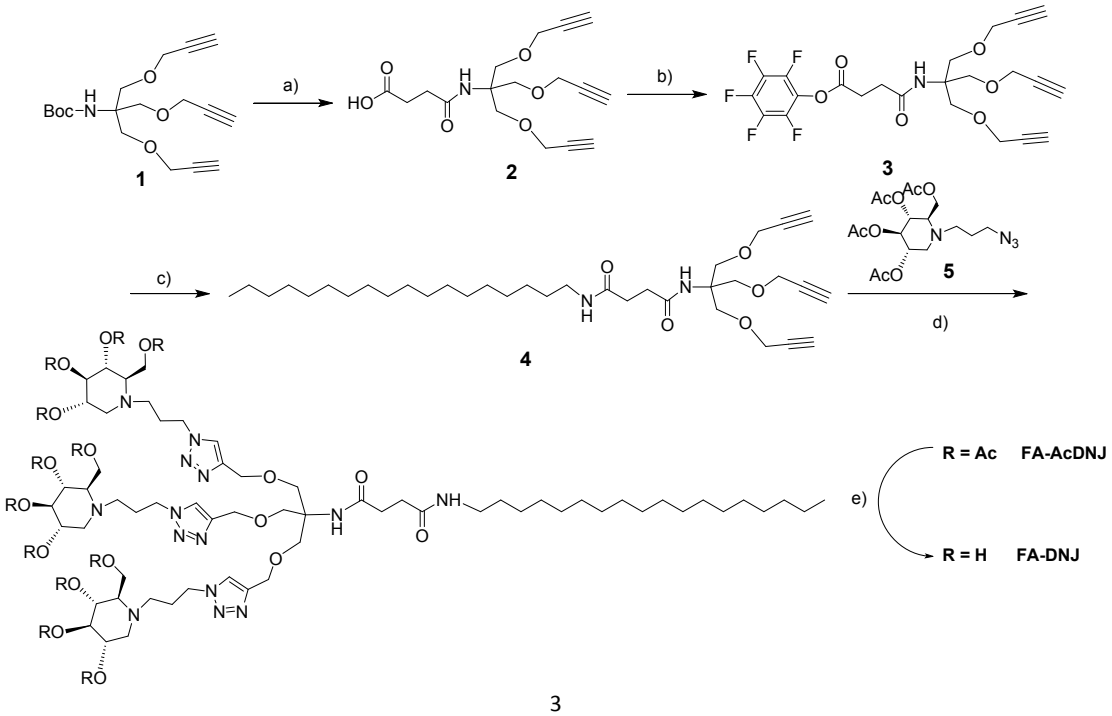
According to the standard curves of p-nitrophenol (PNP) in the citric acid-phosphate buffer (pH 5.5, 0.1 mmol), the inhibition concentration of PNP was obtained by coupling of the K_{slope} with the changes of the absorption intensity for the experimental blank group and experimental group, then the reaction speed of PNP with α-mannosidase was obtained by against the reaction time. The reaction speed (V) was obtained by the concentration of PNP against the reaction time (20 min). Then plot of 1/V against 1/S, the slopes of various concentrations of **FA-DNJ** were obtained. The V was the reaction speed of PNP with α-mannosidase in presence of various concentrations of **FA-DNJ**. The S was the concentrations of PNP added in the 96-well plates. After that, the K_i value of **FA-DNJ** with α-mannosidase was obtained by plot of the concentrations of **FA-DNJ** against the slopes obtained by the above results by the Lineweaver-Burk method. The K_i values and enzyme inhibition mode were obtained from the slope of Linewaver-Burk plots and double reciprocal analysis using Origin 8.0. The K_i values of **FA-DNJ** against the other glycosidases were obtained

by the similar method.

Cytotoxicity *in Vitro*. Compound **FA-DNJ** was dissolved in PBS buffer and diluted to the required concentration with culture medium. The cytotoxicity was studied by a MTT assay. Briefly, cells were plated in 96-well microassay culture plates (7.0×10^3 cells per well) and grown overnight at 37 °C in a 5% CO_2 incubator. Compound **FA-DNJ** was then added to the wells to achieve final concentrations ranging from 10^{-7} to 10^{-4} M. Wells containing culture medium without cells were used as control blanks; wells containing culture medium was used as positive control. The plates were incubated at 37 °C in a 5% CO_2 incubator for 48 h. Upon completion of the incubation, stock MTT dye solution (20 μL, 5 mg/μL) was added to each well. After 4 h incubation, Dimethyl Sulfoxide (DMSO, 150 μL) was added to solubilize the MTT formazan. The optical density of each well was then measured on a microplate spectrophotometer at a wavelength of 490 nm.

Oral Sugar Tolerance Test. Ethics statement Animal procedures were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health. The animal study was approved by the Institutional Animal Care and Use Committees of Hebei University (Permit Number: IACUC-2017002). Kunming mice (18-22 g, 4-5 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China, Certificate NO. 11400700275568). The animals were provided a standard rodent diet and free access to water and were maintained at a temperature of 20-22 °C. The oral sugar tolerance test was studied according to a previous method. Mice were randomly divided into six groups (five mice per group). The mice were maintained for two days. Then maltose (2 $g \cdot kg^{-1}$ of body weight) was administered by gavage as the control experiment. For the test group, maltose (2.0 $g \cdot kg^{-1}$ of body weight) along with **FA-DNJ** (0.1, 0.2 and 0.5 $mg \cdot kg^{-1}$ of the body weight) or miglitol (0.1, 0.2 and 0.5 $mg \cdot kg^{-1}$ of the body weight) were performed. The blood samples were collected from the tail vein at the time of 0, 0.5, 1.0, 2.0 and 3.0 h, and the blood glucose levels were measured with a glucometer. The inhibition blood glucose level was evaluated by comparing the blood glucose level of **FA-DNJ** group and miglitol group with control group.

1.3 Synthesis procedures and analytical data



Scheme S1. a) TFA, CH_2Cl_2; succinic anhydride, THF, reflux, 10 h; b) Penta-fluorophenol, EDC, CH_2Cl_2, r.t., 8 h; c) Octadecylamine, DIPEA, CH_2Cl_2, r.t., 10 h; d) $CuSO_4$, soium ascorbate, THF/H_2O (1/1), 55 ºC, 10 h; e) NaOMe, MeOH.

Compound 2: Compound **1** (5.00 g, 15 mmol) was dissolved in a solution of TFA/CH_2Cl_2 (1/1, 20 mL). The mixture was stirred for 5 h at room temperature under nitrogen atmosphere. Then the solution was concentrated, and the residue was dissolved by ethyl acetate (200 mL), washed with saturated $NaHCO_3$ water, saturated NaCl, and dried by anhydrous Na_2SO_4. The solvent was concentrated under reduced pressure. The residue was dried overnight in a vacuum dryer. After that, the residue and butanedioic anhydride (1.50 g, 15 mmol) were dissolved in THF solution (80 mL), the mixture was refluxing for 12 h. Then, the solvent was removed, and the residue was purified using a silica gel chromatography (CH_2Cl_2/MeOH, 20/1) to afford compound **2** as a pale yellow solid (4.8 g, 95%). m.p. 85.9-86.7 ℃;^{1}H NMR (600MHz, $CDCl_3$, ppm): δ 5.93 (NH-), 4.14(d, J = 2.4 Hz, 6H), 3.83 (s, 6H), 2.66 (t, J = 6.6 Hz, 2H), 2.51 (t, J = 7.2 Hz, 2H), 2.45 (t, J = 2.4 Hz, 3H); ^{13}C NMR (150 MHz,$CDCl_3$, ppm): δ 176.83, 172.19, 79.50, 74.82, 68.42, 59.49, 58.66, 31.34, 29.64; HRMS (MALDI-TOF): calcd. for $C_{17}H_{22}NO_6$, 336.1447, found 336.1446.

Compound 3: Compound **2** (1.00 g, 2.98mmol) and pentafluorophenol (0.66 g, 3.58 mmol) were dissolved in anhydrous CH_2Cl_2 (20 mL). The mixture was stirred at -10 ℃ for 10 min, then a solution of EDC (0.71 g 3.70 mmol) in anhydrous CH_2Cl_2 (15 mL) was added, and the mixture was stirred for another 5 h at room temperature. The solvent was removed and the residue was purified using a silica gel chromatography (AcOEt/petroleum ether, 1/5) to give compound **3** as a white solid (1.38 g, 92%). m.p. 110.5-112.5 ℃; ^{1}H NMR (600MHz, $CDCl_3$, ppm): δ 5.82 (NH-), 4.15 (d, J = 1.8 Hz, 6H), 3.85 (s, 6H), 2.99 (t, J = 6.9 Hz, 2H), 2.63 (t, J = 6.9 Hz, 2H), 2.44 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$, ppm): δ 170.21, 168.84, 141.98, 140.34, 138.62, 137.01, 79.47, 74.66, 68.43, 59.43, 58.65, 31.11, 28.64; HRMS (MALDI-TOF): calcd. for $C_{23}H_{20}F_5NO_6$, 502.1289, found 502.1290.

Compound 4: A solution of compound **3** (1.5 g, 2.99 mmol) in anhydrous CH_2Cl_2 (15 mL) was stirred at -10℃ for 1 0min, then a mixture of DIPEA (1.89 mL) and stearylamine (672mg, 2.49 mmol) in anhydrous CH_2Cl_2 (15 mL) was added dropwise. The reaction mixture was stirred for 8 h at room temperature under nitrogen atmosphere. After completion of reaction, the reaction mixture was concentrated and purified by a silica gel chromatography (AcOEt/petroleum ether, 1/4) to afford compound **4** as a pale yellow solid (1.07 g, 80 %). m.p. 78.1-79.9 ℃; ^{1}H NMR (600 MHz, $CDCl_3$, ppm): δ 5.97 (s, 1H, NH-), 5.93 (s, 1H, NH-), 4.14 (d, J = 2.4 Hz, 6H), 3.82 (s, 6H), 3.20 (m, 2H), 2.51 (t, J = 6.6 Hz, 2H), 2.45 (d, J = 7.2 Hz, 2H), 2.43 (t, J = 2.4 Hz, 3H), 1.47 (m, 2H), 1.24 (m, 30 H), 0.82 (t, J = 6.9 Hz, 3H); ^{13}C NMR (150 MHz,$CDCl_3$, ppm): δ 173.34, 171.94, 79.54, 76.76, 68.53, 58.68, 59.35, 39.66, 32.60, 31.92, 29.32, 29.57, 26.95, 22.68, 14.10; MS (MALDI-TOF): 609.42 $[M+Na]^+$.

Compound FA-AcDNJ: To a solution of compound **4** (102 mg, 0.174 mmol) and compound **5** (323 mg, 0.79 mmol) in THF (15 mL), a solution of $CuSO_4 \cdot 5H_2O$ (78.3 mg, 0.31 mmol) and sodium ascorbate (59.7 mg, 0.31 mmol) in H_2O (1 mL) was added. The reaction solution was stirred at 55 ℃ for 10 h. The solvent was concentrated and purified by a silica gel chromatography (CH_2Cl_2/MeOH, 20/1) to afford compound **FA-AcDNJ** as a pale yellow solid (186 mg, 60 %). m.p. 115.3-126.4 ℃; ^{1}H NMR (600 MHz, $CDCl_3$, ppm): δ 7.63 (s, 3H, triazole-H), 6.38 (s, 1H, NH-), 6.21 (s, 1H, NH-), 5.04 (m, 6H), 4.93 (m, 3H), 4.57 (s, 6H), 4.39 (m, 6H), 4.12 (s, 6H), 3.76 (s, 6H),

3.20 (s, 3H), 3.15 (t, J = 6.6 Hz, 2H), 2.88 (m, 3H), 2.64 (m, 3H), 2.55 (m, 3H), 2.46 (t, J = 6.3 Hz, 2H), 2.40 (t, J = 6.3 Hz, 2H), 2.28 (t, J = 11.1 Hz, 3H), 2.08 (m, 6H), 2.05 (s, 9 H, CH_3-), 2.01 (s, 18 H, CH_3-), 2.00 (s, 9 H, CH_3-), 1.82 (m, 2H), 1.45 (m, 2H), 1.23 (m, 28 H), 0.86 (t, J = 6.9 Hz, 3H); ^{13}C NMR (150 MHz, DMSO-d_6, ppm): δ 171.92, 171.52, 172.00, 171.44, 144.08, 123.85, 79.05, 70.64, 69.31, 67.96, 64.30, 59.64, 56.86, 48.98, 47.90, 31.94, 38.58, 31.40, 30.94, 29.19, 28.90, 28.80, 26.54, 25.99, 22.21, 14.66; HRMS (MALDI-TOF): calcd. for $C_{86}H_{136}N_{14}O_{29}Na$, 1851.96, found 1851.97.

FA-DNJ: A solution of compound **FA-AcDNJ** (156 mg, 0.085 mmol), sodium methoxide (27.6 mg, 0.51 mmol) and anhydrous methanol (10 mL) was stirred at room temperature for 5 h. And then the solvent was removed. The residue was dissolved with 5 mL of water, dialyzed with a dialysis bag (retained molecular weight: 1000) for 10 h, and then which was freeze-dried. The **FA-DNJ** was obtained as a pale yellow solid (102 mg, 90%). m.p.: 115.3-126.4 ℃; ^{1}H NMR (600 MHz, DMSO-d_6, ppm): δ 8.04 (s, 3H), 4.45(s, 6H), 4.32 (m, 6H), 3.68 (d, J = 10.8 Hz, 3H), 3.61 (s, 6H), 3.48-3.52 (m, 9 H), 3.21 (m, 3H), 3.04 (t, J = 8.7 Hz, 3H), 2.97 (t, J = 6.9 Hz, 2H), 2.92 (t, J = 8.7 Hz, 3H), 2.78 (m, 6H), 2.37 (m, 3H), 2.29 (t, J = 7.8 Hz, 2H), 2.22 (t, J = 7.8 Hz, 2H), 1.93 (m, 6H), 1.34 (m, 2H), 1.22 (m, 30 H), 0.84 (t, J = 6.9 Hz, 3H); ^{13}C NMR (150 MHz, DMSO-d_6, ppm): δ 171.92, 171.43, 144.07, 123.84, 79.04, 70.63, 69.30, 67.95, 64.29, 59.63, 56.85, 48.97, 47.89, 38.57, 31.51, 31.39, 29.12, 28.89, 28.79, 26.53(C-16), 25.98,14.06, 22.20; HRMS (MALDI-TOF): calcd. for $C_{62}H_{112}N_{14}O_{17}Na$, 1347.83, found 1347.84.

2. Additional figures and table

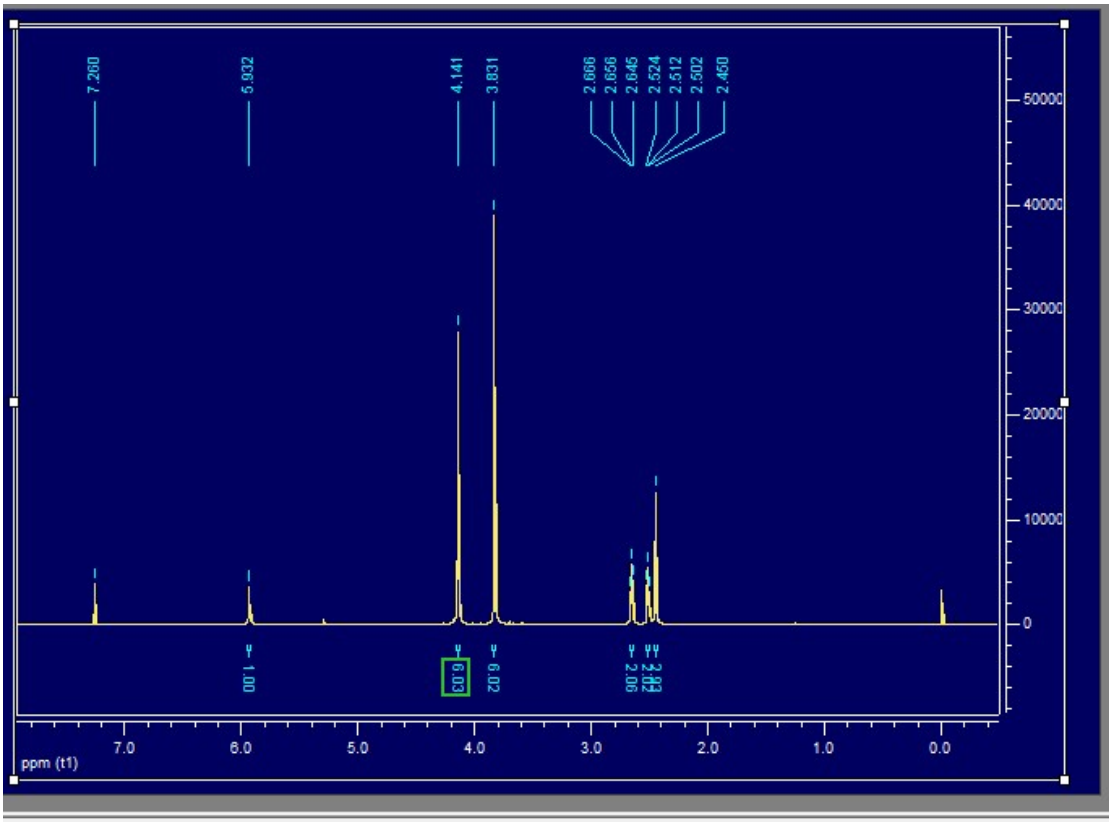


Fig. S1 ^{1}H NMR ($CDCl_3$, 600 MHz) of compound **2**.

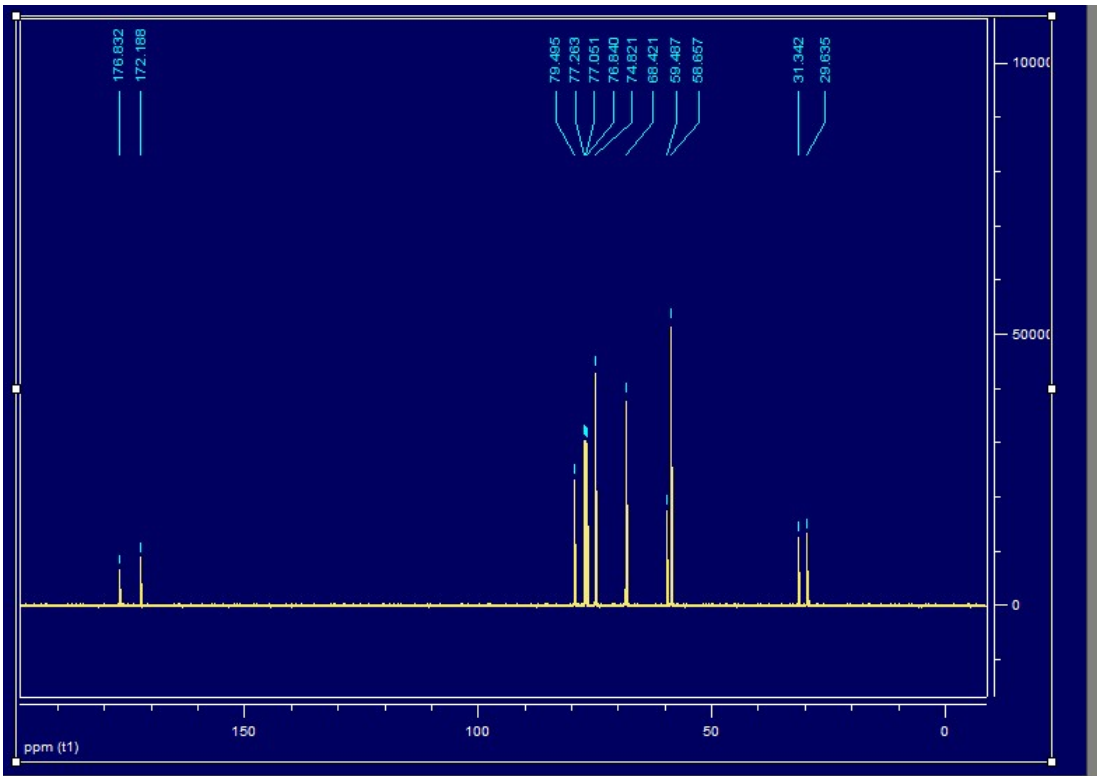


Fig. S2 ^{13}C NMR ($CDCl_3$, 150 MHz) of compound **2**.

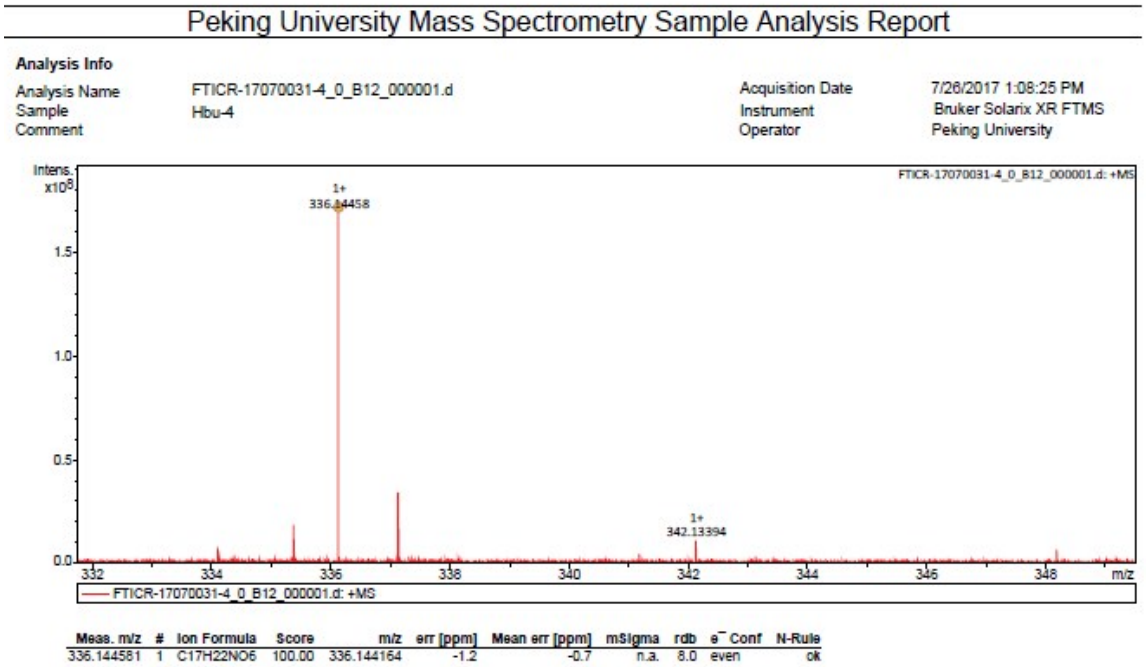


Fig. S3 HRMS (MALDI-TOF) of compound **2.**

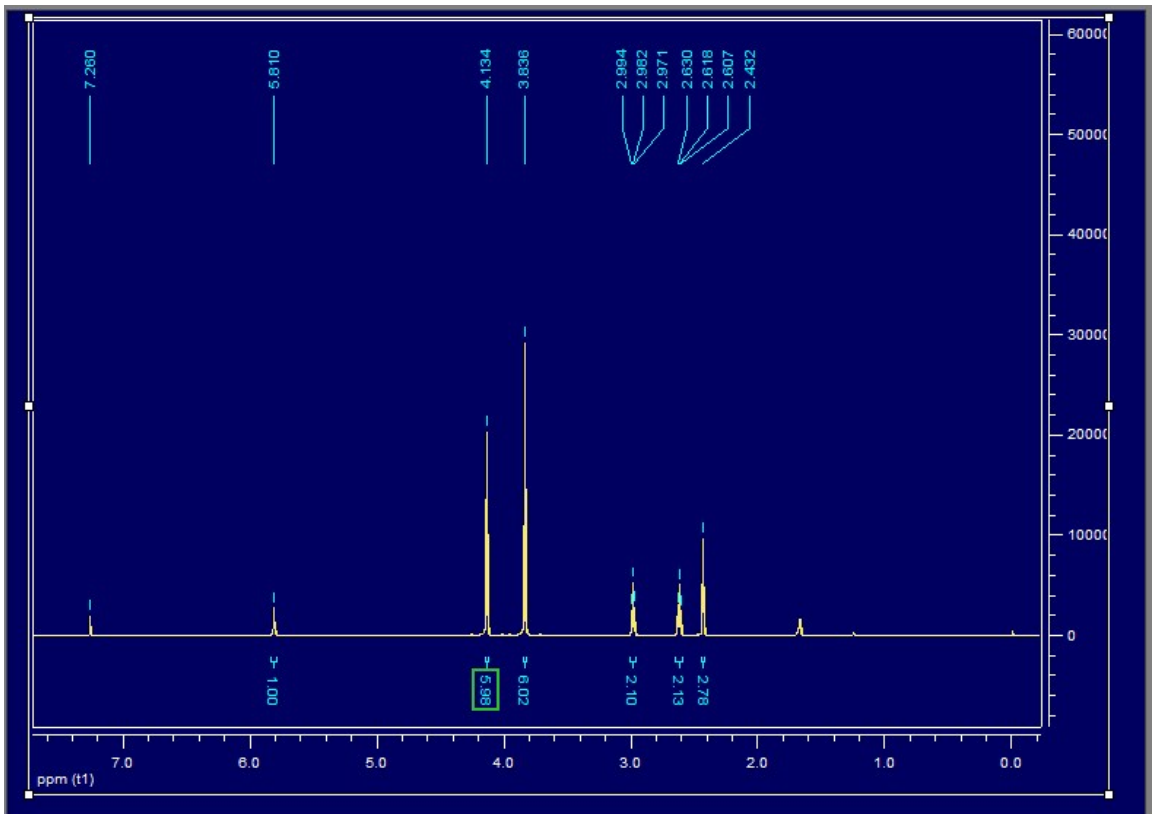


Fig. S4 ^{1}H NMR ($CDCl_3$, 600 MHz) of compound **3**.

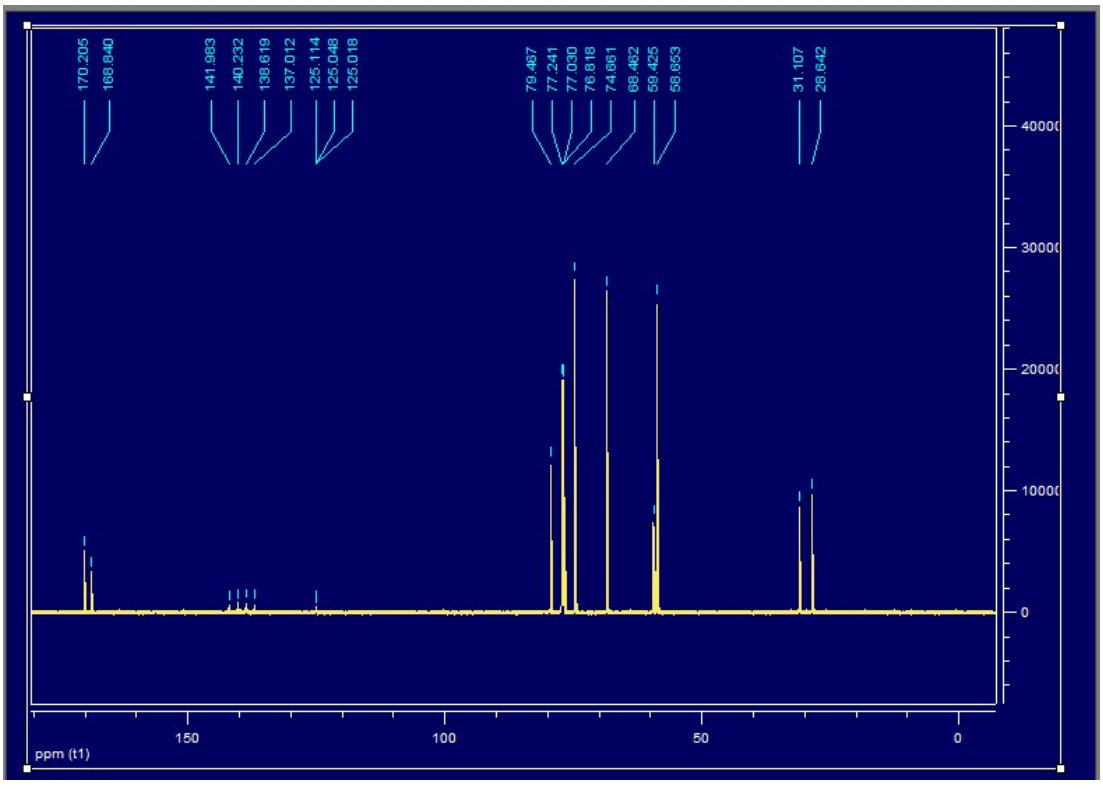


Fig. S5 ^{13}C NMR ($CDCl_3$, 150 MHz) of compound **3**.

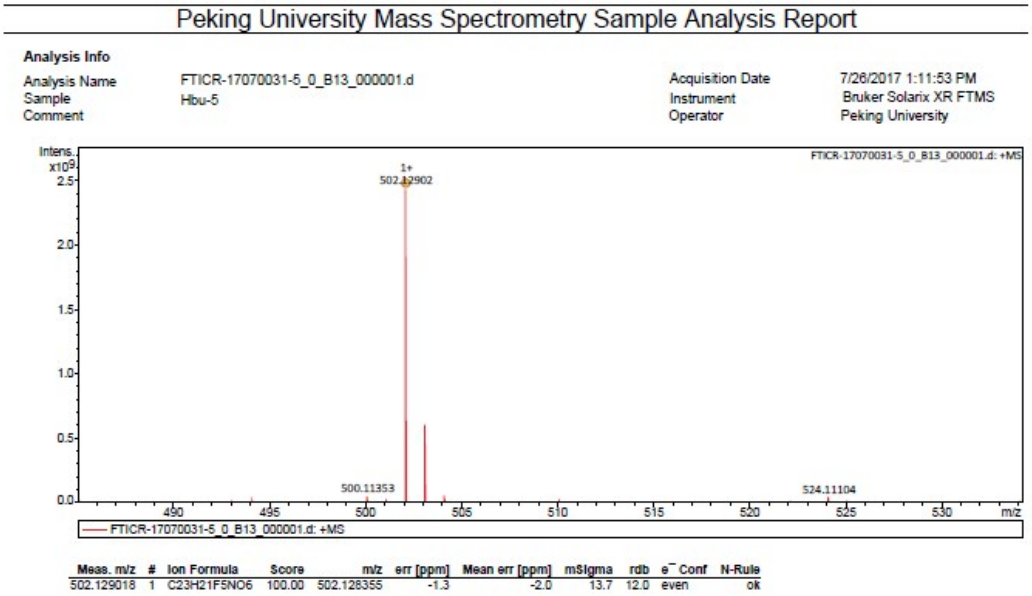


Fig. S6. HRMS (MALDI-TOF) of compound **3**.

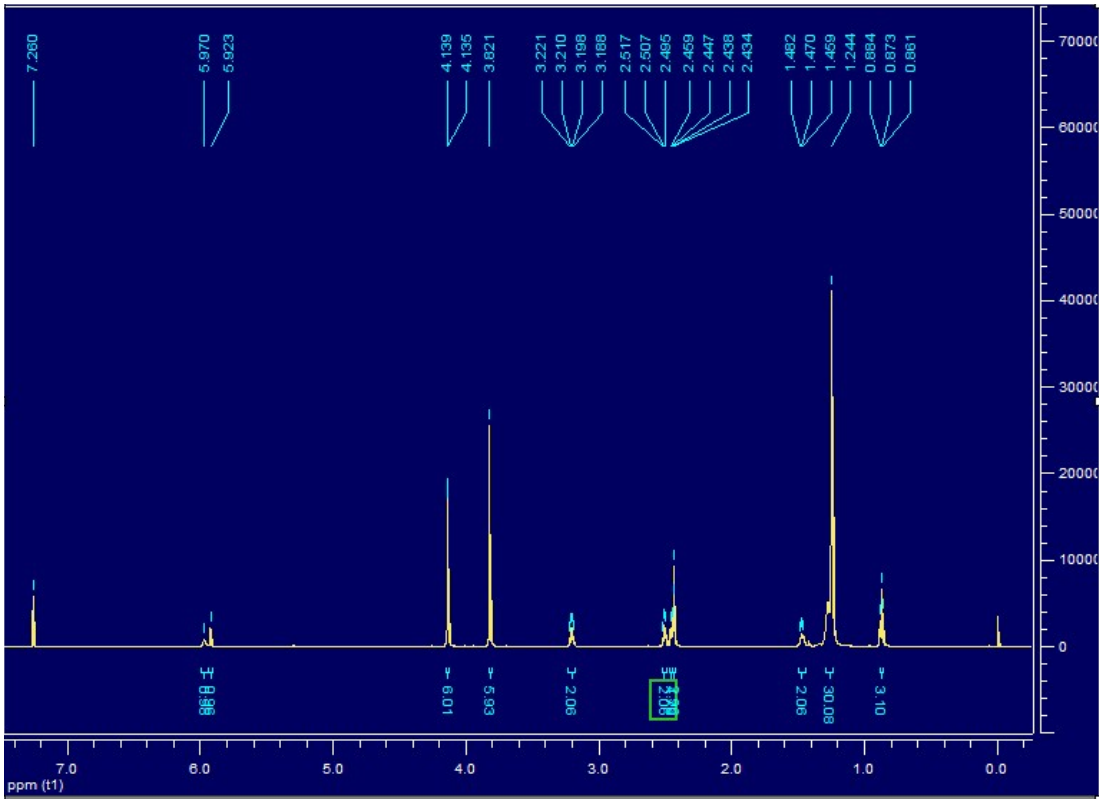


Fig. S7 ^{1}H NMR ($CDCl_3$, 600 MHz) of compound **4**.

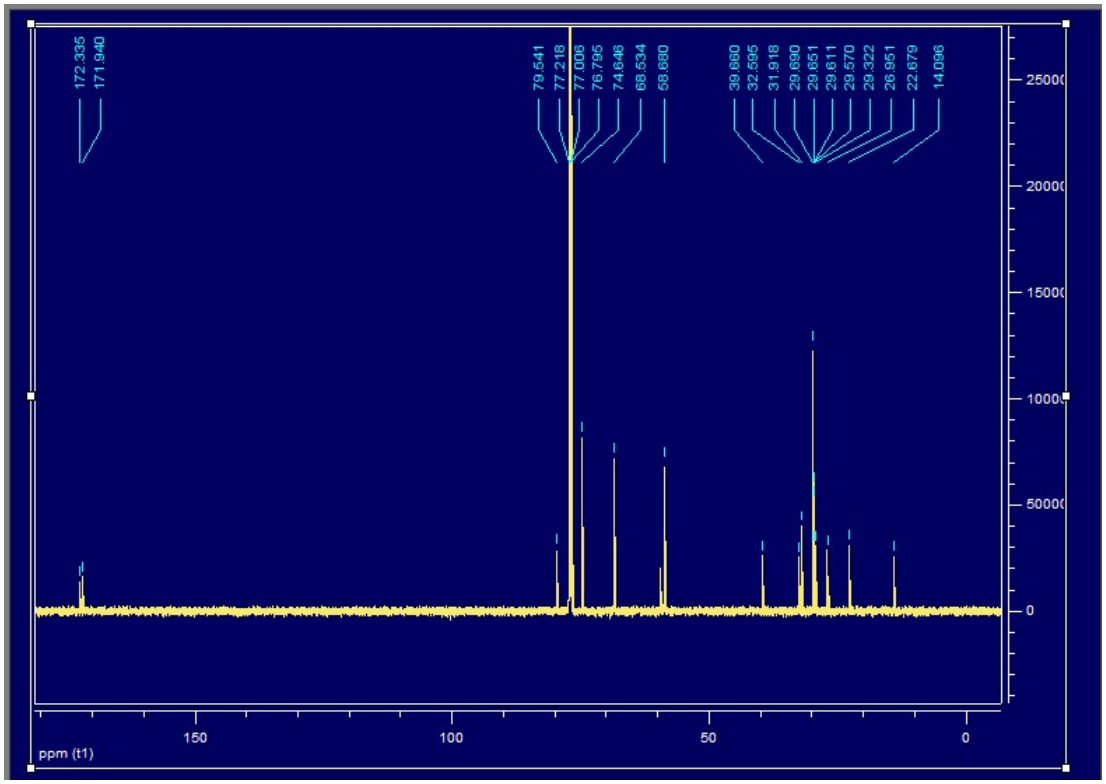


Fig. S8 ^{13}C NMR ($CDCl_3$, 150 MHz) of compound **4**.

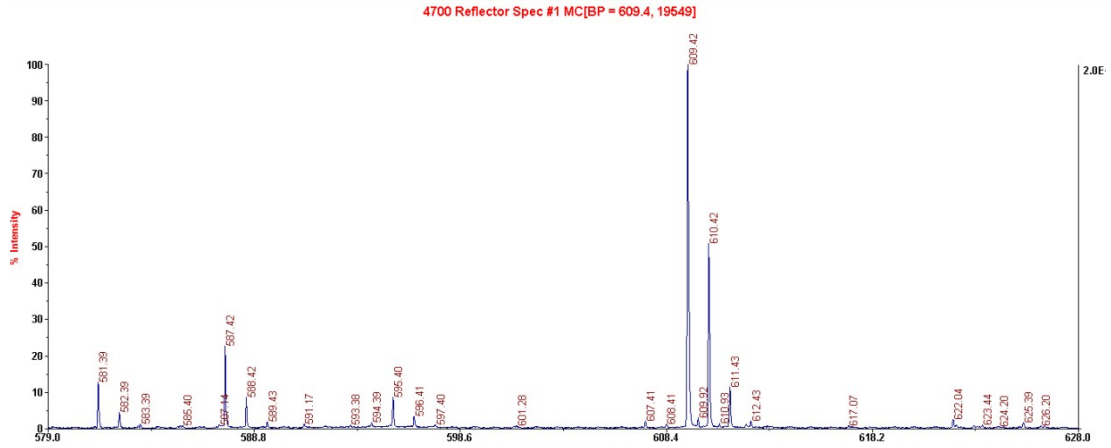


Fig. S9 HRMS (MALDI-TOF) of compound **4**.

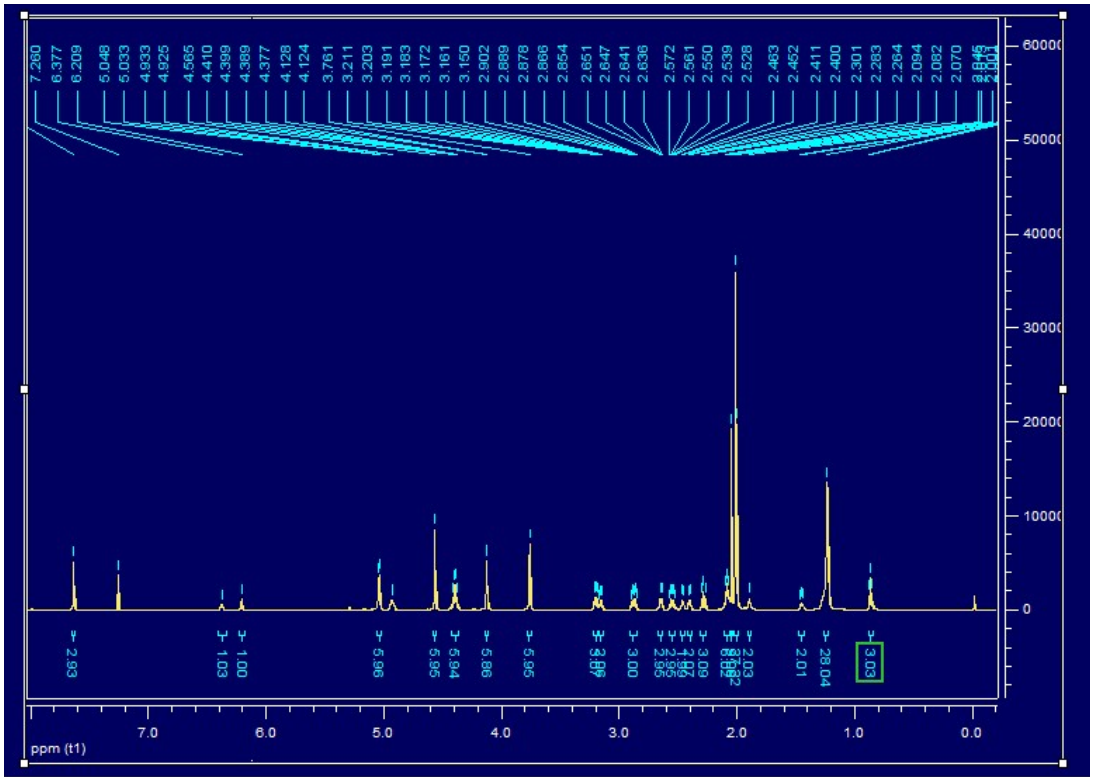


Fig. S10 ^{1}H NMR ($CDCl_3$, 600 MHz) of compound **FA-AcDNJ**.

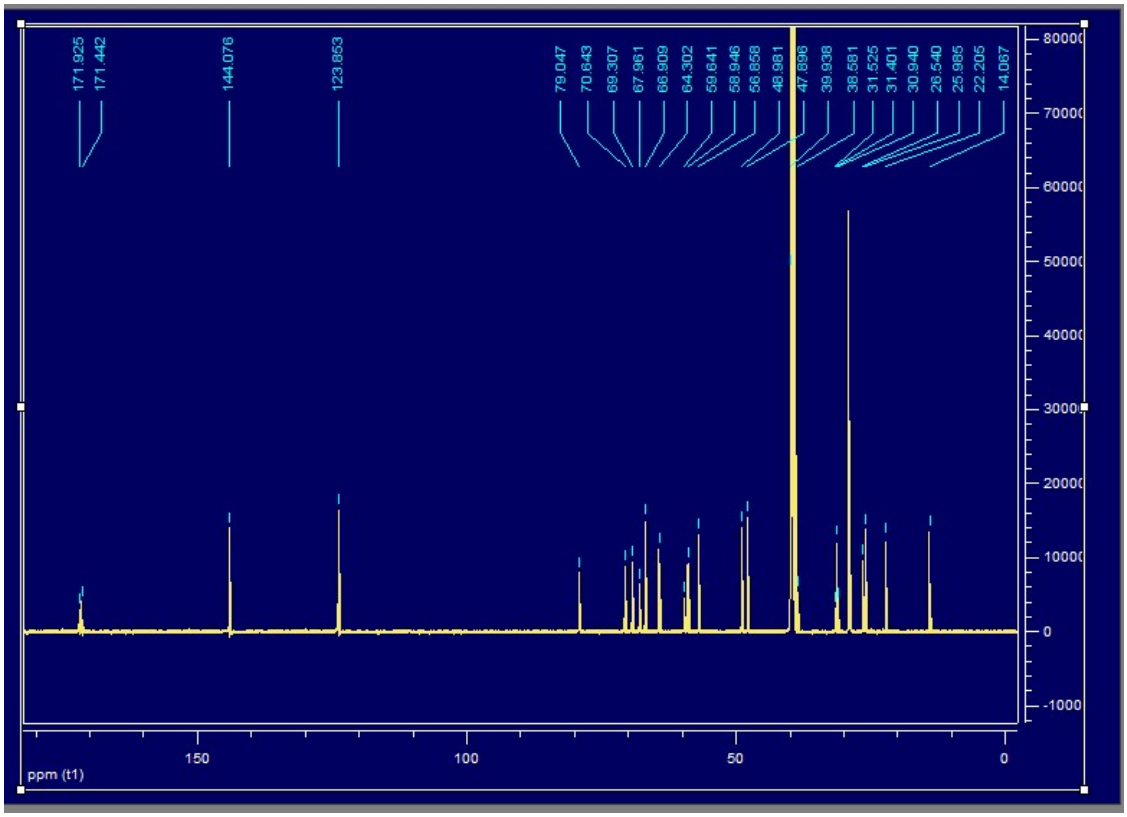


Fig. S11 ^{13}C NMR ($CDCl_3$, 150 MHz) of compound **FA-AcDNJ**.

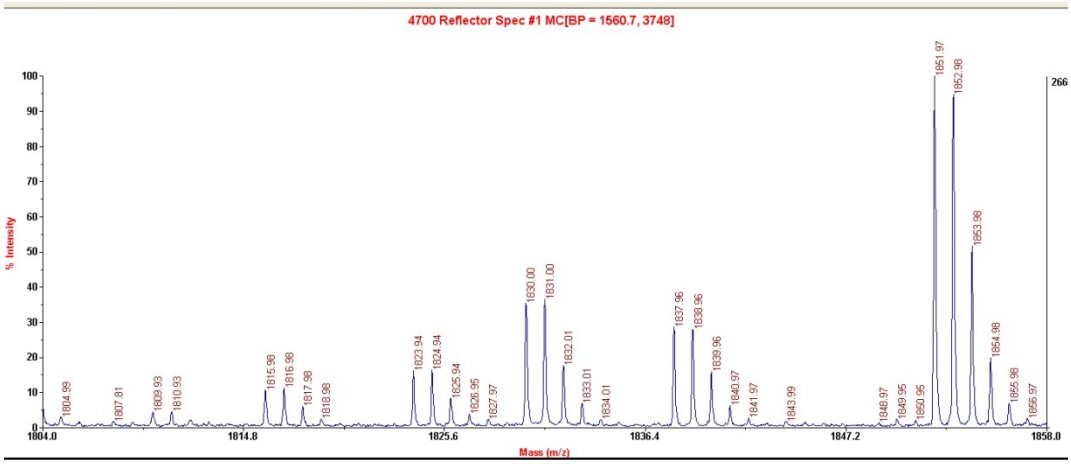


Fig. S12 HRMS (MALDI-TOF) of compound **FA-AcDNJ**.

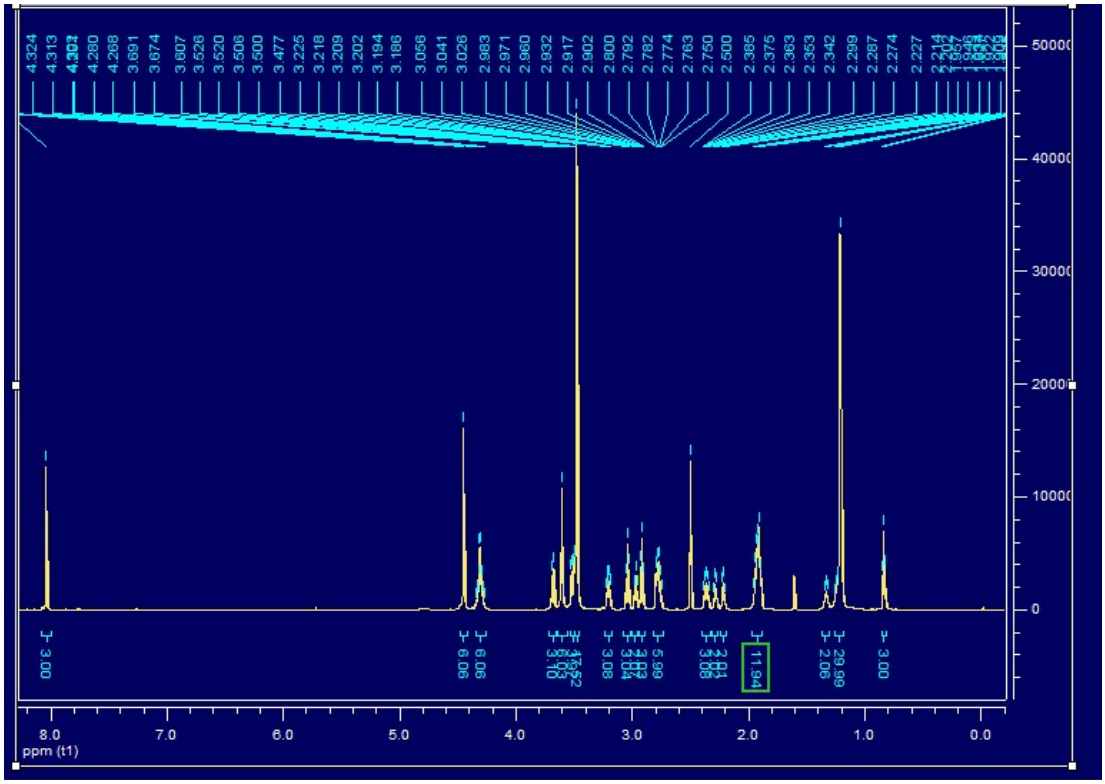


Fig. S13 ^{1}H NMR (DMSO-d_6+D_2O, 600 MHz) of compound **FA-DNJ**.

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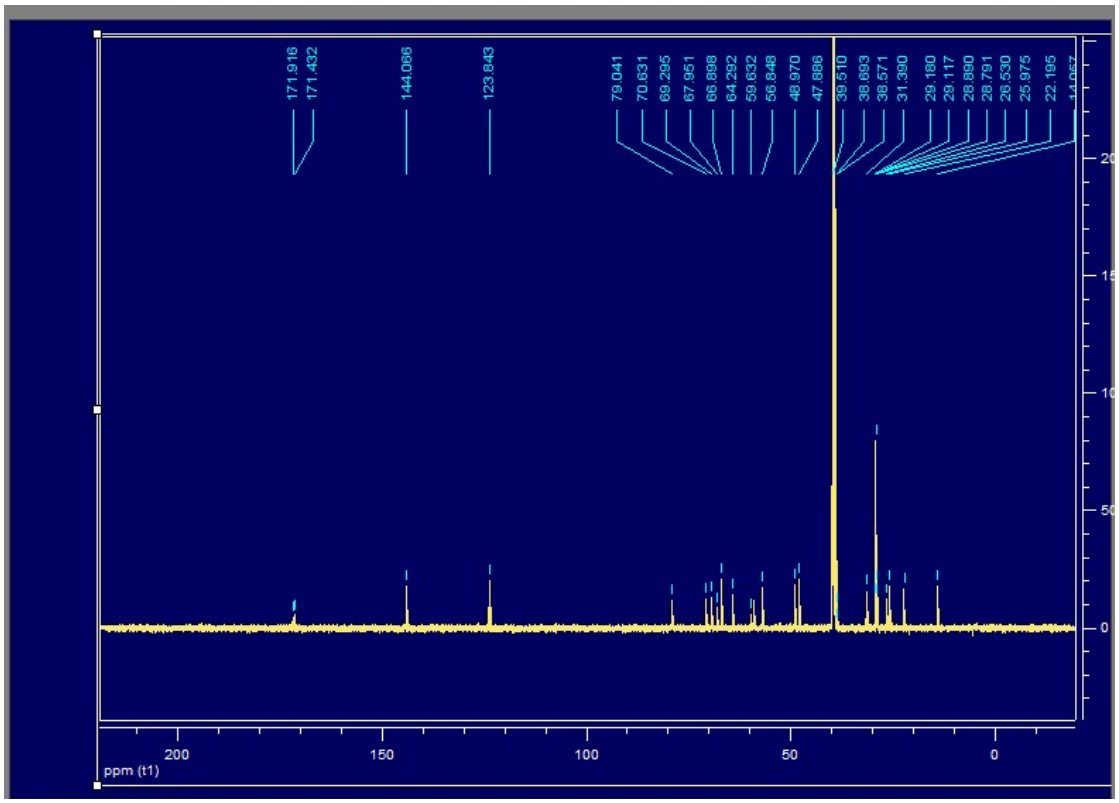

Fig. S14 ^{13}C NMR (DMSO-d_6+D_2O, 150 MHz) of compound **FA-DNJ**.

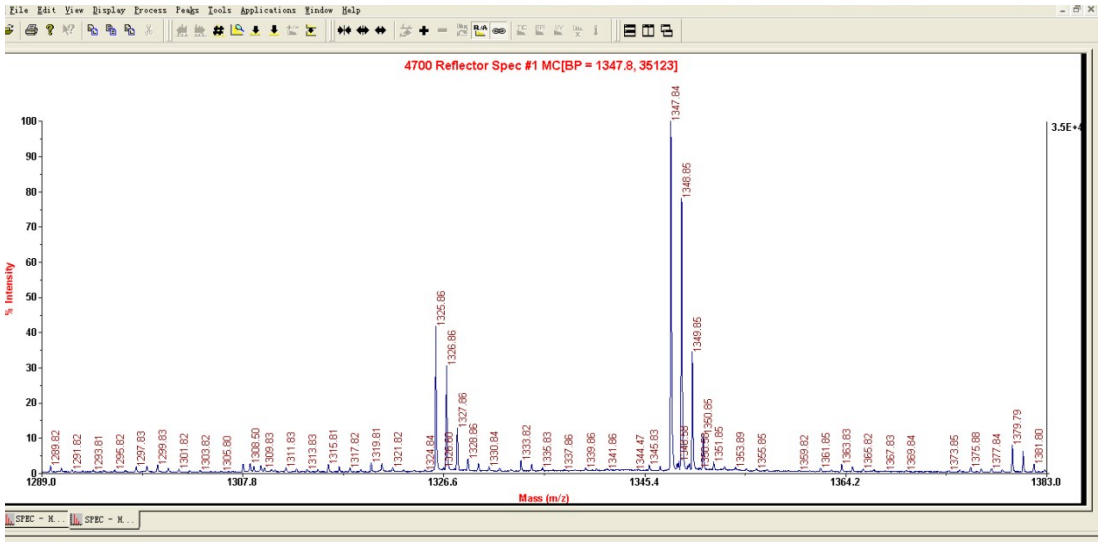

Fig. S15 HRMS (MALDI-TOF) of compound **FA-DNJ**.

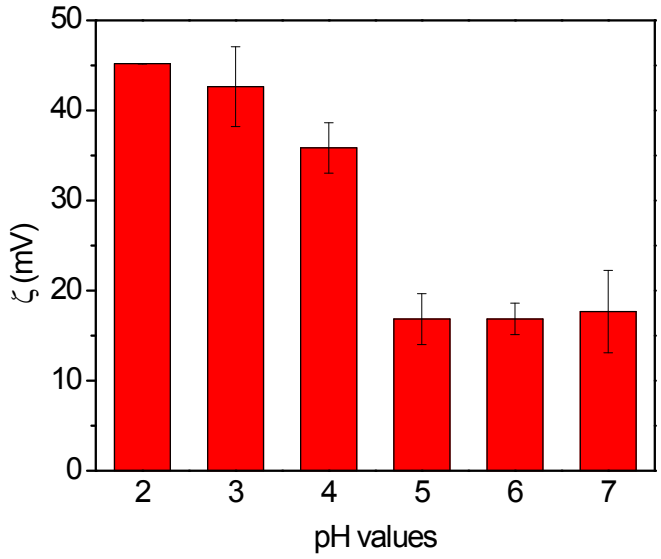

Fig. S16 The ζ-potential values of the self-assemblies of **FA-DNJ** at different pH values (pH 2.0~7.0) in citric acid-phosphate buffer at the concentration of 1.0×10^{-4} M.

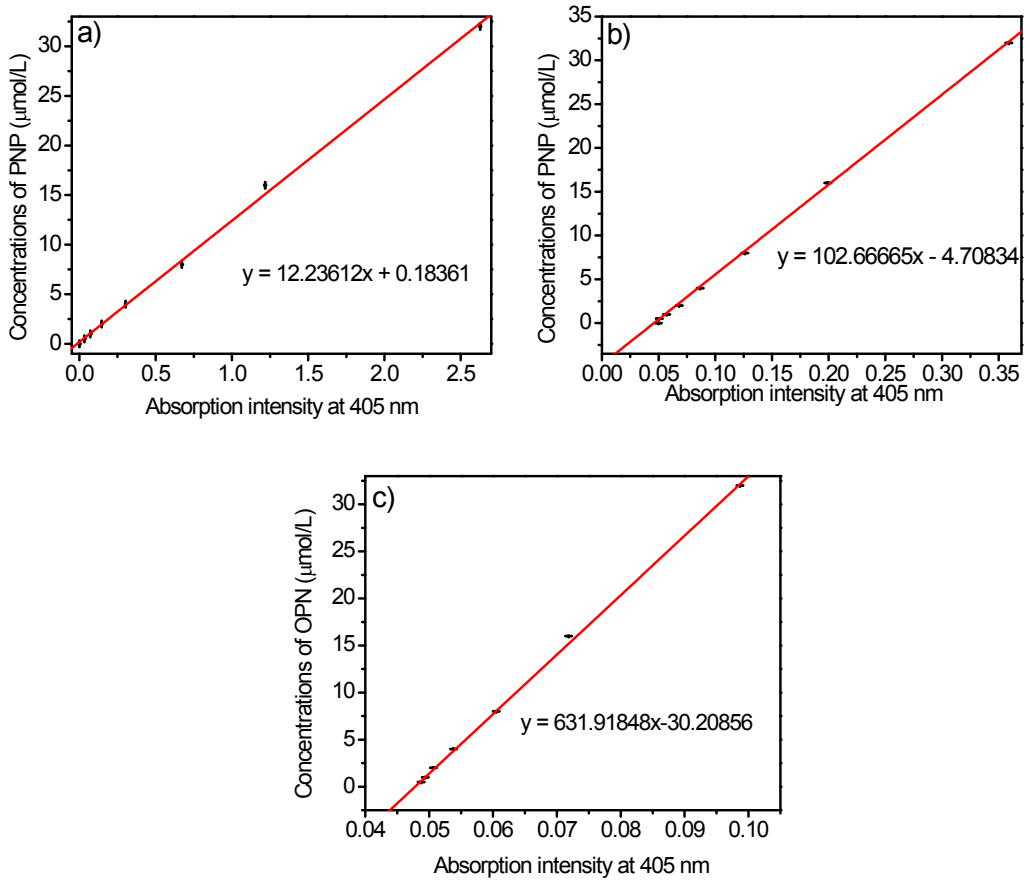

Fig. S17 The standard curves of p-nitrophenol (PNP) in the citric acid-phosphate buffer (pH 5.5, 0.1 mmol) (a), in the PBS buffer (pH 6.8, 0.1 mmol) (b) and (c) the standard curve of o-nitrophenol (ONP) in the PBS buffer (pH 7.3, 0.1 mmol).

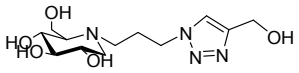

Fig. S18. The structure of monomer DNJ molecule (**DNJ-1**).

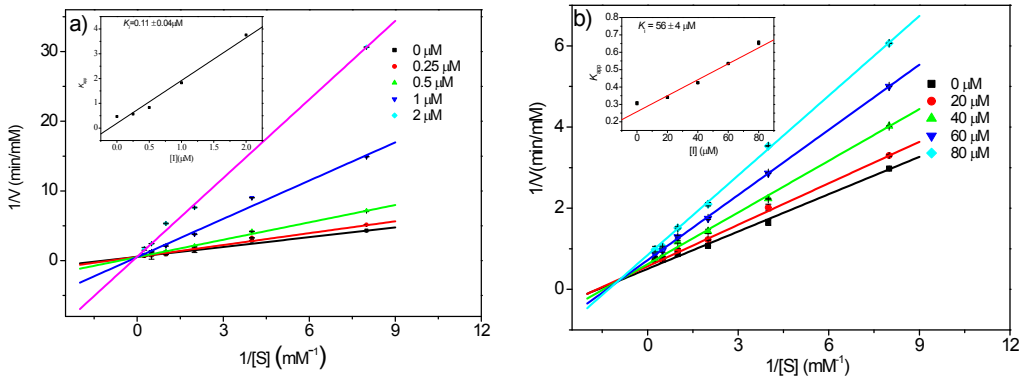

Fig. S19 Lineweaver-Burk Plot for K_i determination of **FA-DNJ** against □*α*-mannosidase (*Jack Bean*, pH = 5.5, a) and *α*-galactosidase (*green coffee beans*, pH = 6.8, b).

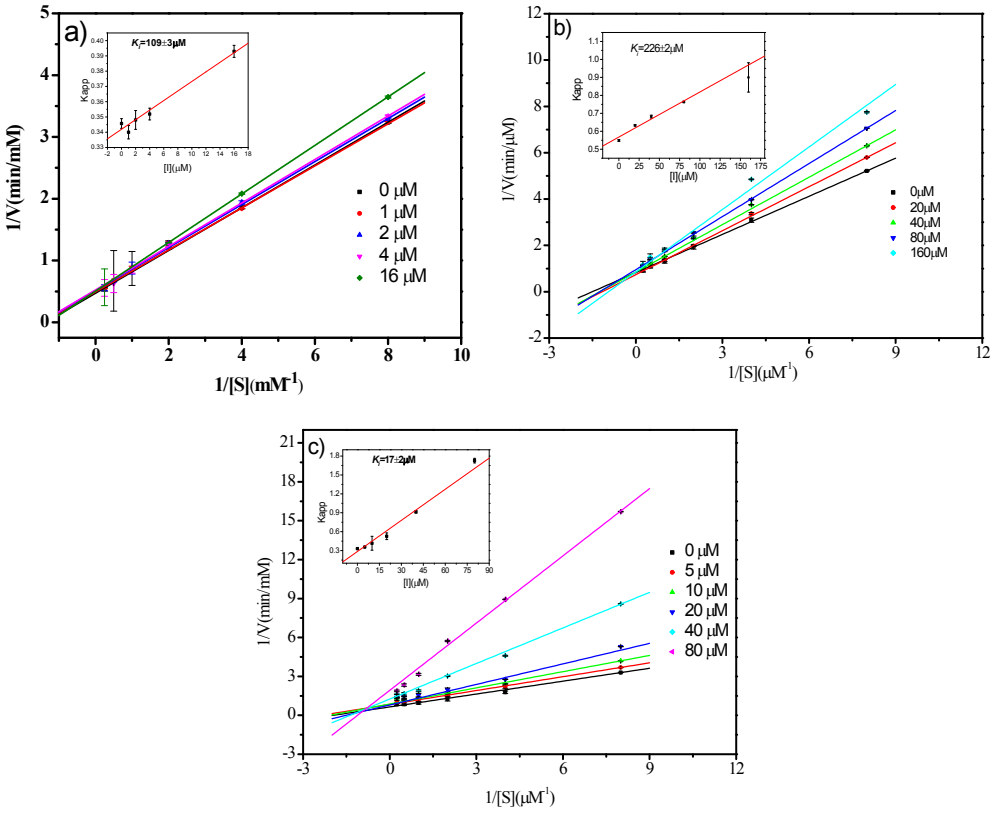

Fig. S20 (a) Lineweaver-Burk plot for K_i determination of miglitol against *α*-mannosidase (*Jack bean*), and Lineweaver-Burk plot for K_i determination of **DNJ-1** against *α*-mannosidase (*Jack bean*, b) and *α*-galactosidase (*green coffee beans*, c).

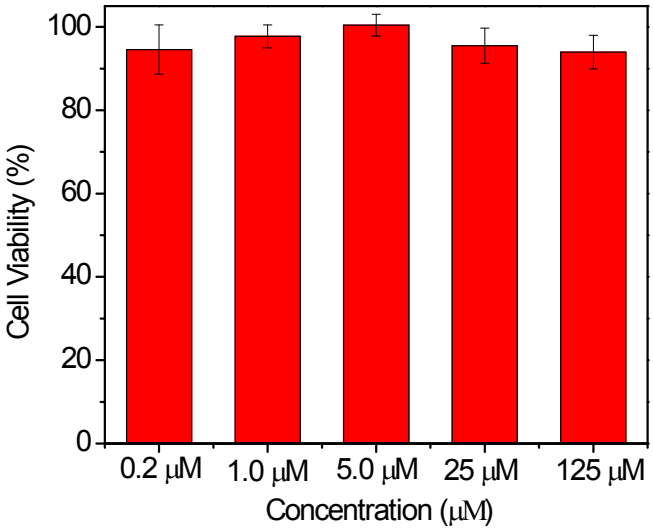

Fig. S21 Cell survival rate of **FA-DNJ** against HKC 60 cells at different concentrations.

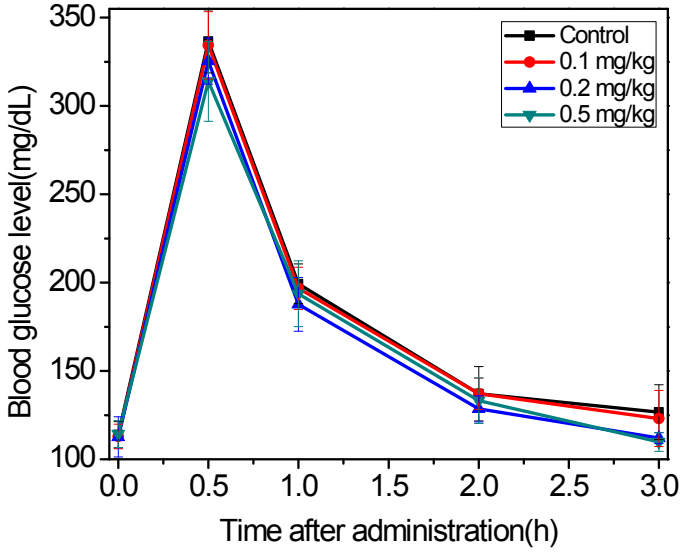

Fig. S22 The hypoglycaemic effects of miglitol on blood glucose level in mice, maltose (2 g·kg^{-1} of body weight) was orally administered to mice along with miglitol (0.1, 0.2 and 0.5 mg·kg^{-1} of body weight). Each value represents the mean ± SEM (n = 5).