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Supporting Information

for

New Formulation of Old Aspirin for Better Delivery

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Materials and Methods

Materials: All chemicals were used as received without further purification unless otherwise noted. Acetylsalisylic acid (aspirin), 2,2 bis(methoxy)propionic acid (Bis MPA), 2,2 dimethoxypropane, para-toluenesulfonic acid monohydrate (PTSA·H_2O), magnesium sulfate ($MgSO_4$), N, N′-dicyclohexylcarbodiimide (DCC), 6-bromohexanoic acid, triphenylphosphine, octanol, 4-dimethylaminopyridine (DMAP), pyridine, sodium carbonate (Na_2CO_3), sodium bisulfate ($NaHSO_4$), DOWEX 50W, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and oxalyl chloride were purchased from Sigma Aldrich. Ultra-pure lipopolysaccharide (LPS) was purchased from Invivogen, CA, USA. Slide-A-Lyzer mini dialysis devices with a 10 kDa MW cutoff was purchased from Thermo Scientific. Glutamine, penicillin/streptomycin trypsin-EDTA solution, HEPES buffer (1M), and sodium pyruvate were procured from Sigma Life Sciences. Fetal bovine serum (FBS) was purchased from Gibco Life Technologies. Acid terminated poly(DL-lactide-co-glycolide) (PLGA-COOH) of inherent viscosity dL/g, 0.15 to 0.25 was purchased from Durect LACTEL® Absorbable Polymers. Polyethylene glycol (HO-PEG_{3350}-OH) was procured from Sigma Aldrich. Interleukin (IL)-6, IL-10, and tumor necrosis factor alpha (TNF-*α*) cytokines were tested using BD OptEIA mouse enzyme-linked immunosorbent assay (ELISA) kits. Tween 20 was purchased from Fisher Bio-reagent. $CDCl_3$ and DMSO-d_6 were purchased from Cambridge Isotope Laboratories Inc. Regenerative cellulose membrane Amicon ultra centrifugal 100 kDa filters were purchased from Merck Millipore Ltd.

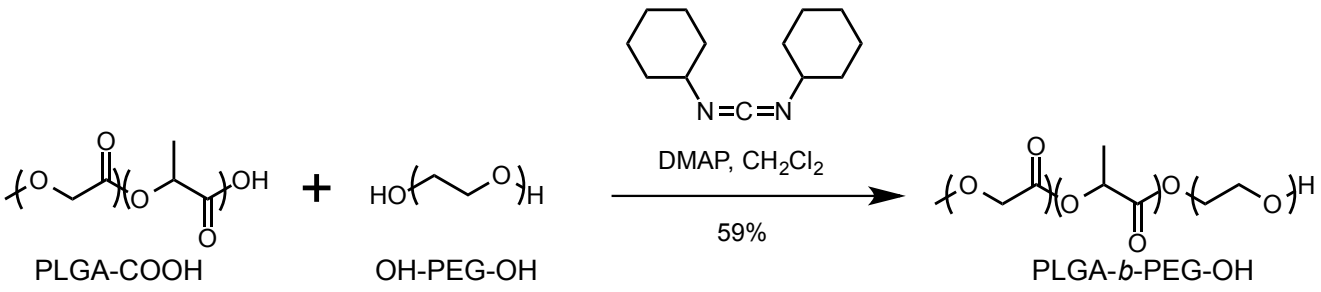
Instruments: ^1H and ^{13}C spectra were recorded on 400 MHz Varian NMR spectrometer. Electrospray ionization mass spectrometry (ESI-MS) and high-resolution mass spectrometry (HRMS)-ESI were recorded on Perkin Elmer SCIEX API 1 plus and Thermo scientific ORBITRAP ELITE instruments, respectively. Distilled water was purified by passage through a Millipore Milli-Q Biocel water purification system (18.2 MΩ) containing a 0.22 *μ*m filter. High-

performance liquid chromatography (HPLC) analyses were made on an Agilent 1200 series instrument equipped with a multi-wavelength UV-visible and a fluorescence detector. Transmission electron microscopy (TEM) images were acquired using a Philips/FEI Tecnai 20 microscope. Gel permeation chromatographic (GPC) analyses were performed on Shimadzu LC20-AD prominence liquid chromatographer equipped with a refractive index detector and Waters columns; molecular weights were calculated using a conventional calibration curve constructed from narrow polystyrene standards using tetrahydrofuran (THF) as an eluent at a temperature of 40 °C. Cells were counted using Countess® automated cell counter procured from Invitrogen life technology. Plate reader analyses were performed on a Bio-Tek Synergy HT microplate reader. Dynamic light scattering (DLS) measurements were carried out using a Malvern Zetasizer Nano ZS system.

Methods.

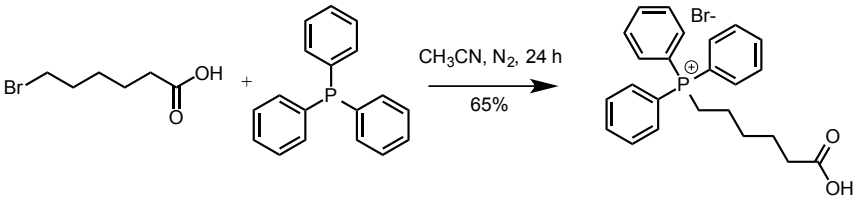
Cell Lines and Cell Culture. RAW 264.7 cell line was procured from the American type culture collection (ATCC). These macrophages were grown at 37 °C in 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% L-glutamine, 1% sodium pyruvate, 1% penicillin/streptomycin, and 10% FBS. Cells were passed every 3 to 4 days and restarted from frozen stocks after 20 passages.

Synthesis and characterization of PLGA-*b*-PEG-OH[1]: PLGA-COOH (1.0 g, 0.18 mmol; dL/g, 0.15 to 0.25), polyethylene glycol (OH-PEG_{3350}-OH) (1.53 g, 0.512 mmol), and DMAP



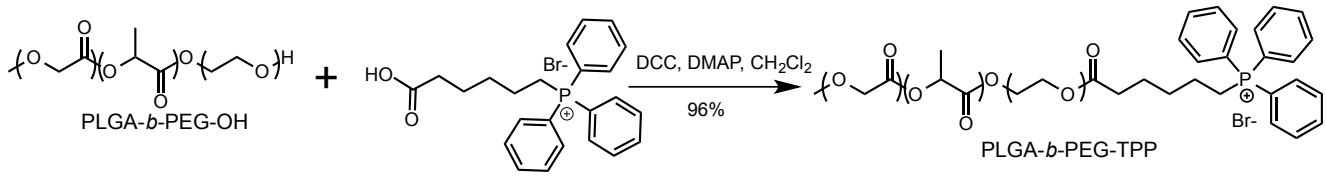
(0.02 g, 0.170 mmol) were dissolved in dry dichloromethane and stirred for 30 min at 0 °C. A solution of DCC (0.106 g, 0.512 mmol) in dichloromethane was added drop wise to the reaction mixture. The reaction mixture was stirred form 0 °C to room temperature for 18 h. Precipitated DCU by-product was filtered off and the solution was evaporated by rotavap. This residue was resuspended by sonication in ethyl acetate and remaining DCU was removed. The solvent was evaporated and the resulting residue was dissolve in 5-10 mL of dichloromethane and precipitated with 40-45 mL of 1:1 mixture of methanol:diethylether and centrifuged. This process was repeated (5x) till the supernatant becomes clear solution. The resulting residue was dried under high vacuum to get a white solid polymer. Yield 0.959 g, 59%. ^{1}H NMR (Figure S1) ($CDCl_3$, 400 MHz): δ 5.20 [m, 1H], 4.81 [m, 2H], 3.63 [s, 3H], 1.56 [s, 3H] ppm. ^{13}C NMR (Figure S2) ($CDCl_3$, 100 MHz): δ 169.22, 166.31, 70.55, 69.01, 60.79, 16.66 ppm.

Synthesis of TPP-hexanoic acid[2]: Bromohexanoic acid (0.600 g, 3.076 mmol) and triphenylphosphine (0.968 g 3.691 mmol) were dissolved in 40 mL of acetonitrile. This reaction was refluxed for 24 h under a N_2 environment. After 24 h, the solvent was evaporated to yield an oil, which was then precipitated with diethyl ether. The precipitate was filtered through a glass frit filter, and washed several times with diethyl ether to remove any impurities from the starting materials. The product was kept on vacuum for 1 h. Yield 0.760 g 65%. ^{1}H NMR (Figure S3) ($CDCl_3$, 400 MHz): δ 7.78 [m, 15H], 3.56 [m, 2H], 2.44 [m, 2H], 1.68 [m, 6H] ppm.



Synthesis of PLGA-*b*-PEG-TPP[1]: TPP-hexanoic acid (0.5 g, 0.06 mmol), PLGA-*b*-PEG-OH (0.2 mg, 0.47 mmol), and DMAP (0.02 g, 0.17 mmol) were dissolved in dry dichloromethane

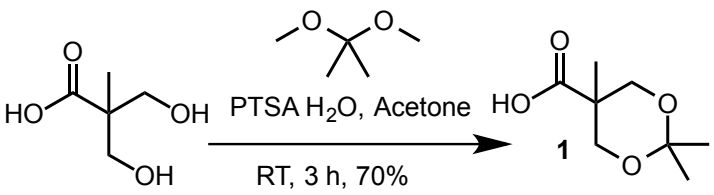
and stirred for 30 min at 0 °C. A solution of DCC (0.035 g, 0.170 mmol) in dichloromethane was added drop wise to the reaction mixture. This reaction mixture was stirred form 0 °C to room temperature for 18 h. The precipitated DCU by-product was filtered off and the solution was evaporated by rotavap to concentrate the volume to ~5 mL. The concentrated solution was then precipitated with 40-45 mL of cold diethyl ether and was centrifuged at 5000 RPM



at 4 °C for 5 min. The resulting supernatant was decanted and the pellet was dissolved in 2-3 mL of CH_2Cl_2 and 5 mL of methanol, 40 mL of diethyl ether was added to precipitate the product, and then centrifuged at the above settings. This process was repeated 3 times and the resulting pellet was lyophilized overnight to yield a white solid. Yield: 0.5 g, 96%. ^{1}H NMR (Figure S4) ($CDCl_3$, 400 MHz): δ 7.81 [m, 15H], 5.20 [m, 35H], 4.81 [m, 74H], 3.63 [s, 114H], 1.57 [m, 136H] ppm. ^{13}C NMR (Figure S5) ($CDCl_3$, 400 MHz): δ 169.23, 166.33, 134.94, 133.77, 130.38, 118.82, 117.96, 70.54, 69.00, 60.80, 16.66 ppm.

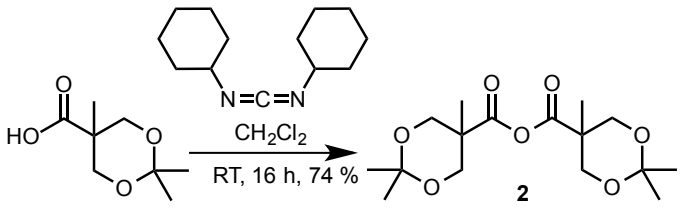
T-Asp- and NT-Asp-NP Synthesis: Aspirin encapsulated targeted and non-targeted NPs were synthesized using the nanoprecipication method. Briefly, 100 *μ*L from a 50 mg/mL CH_3CN solution of PLGA-*b*-PEG-TPP for targeted or PLGA-*b*-PEG-OH polymer for non-targeted NPs, and 100 *μ*L of a 10 mg/mL CH_3CN solution of aspirin were added to 800 *μ*L of CH_3CN. This 1 mL solution was then added drop wise to 10 mL of vigorously stirring water and was allowed to stir for 2 h. The NPs formed were then filtered using Amicon filters with a molecular weight cut off of 100 kDa, washed three times with nanopure water, and the NPs were resuspended in nanopure water at a concentration of 5 mg/mL. The size and surface charge of the NPs were characterized using dynamic light scattering method (Figure S6).

Synthesis and characterization of acetonide protected 2,2 bis(methoxy)propionic acid (bis-MPA) (1): 2,2 bis(methoxy)propionic acid (10.0 g, 0.07 mol), 2,2 dimethoxypropane

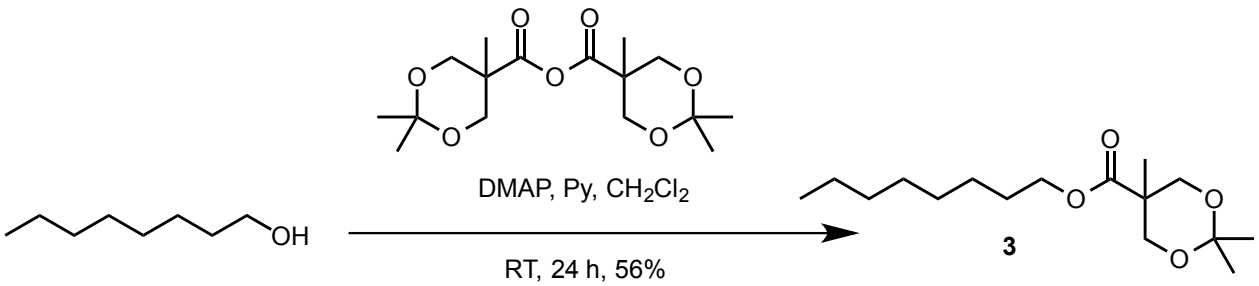


(11.6 g, 0.11 mol, 0.85 g/mL) and para-toluenesulfonic acid mono hydrate (PTSA·H_2O) (0.70 g, 0.004 mol) were dissolved in 40 mL of acetone. The reaction mixture was stirred at room temperature (RT) for 3 h. After 3 h, 3 mL of a 50:50 solution (by volume) of ammonia and ethanol was added to the reaction mixture to neutralize the PTSA. The solvent was evaporated and the resulting product was dissolved in 200 mL of CH_2Cl_2. This solution was then washed twice with 20 mL of nanopure water, followed by washing three washes with 40 mL of brine. The resulting solution was dried using magnesium sulfate ($MgSO_4$), which was then filtered out using a glass filter. The remaining CH_2Cl_2 was then evaporated and the final product was isolated as a white solid. Yield 9.0 g, (70%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 4.14 [d, 2H], 3.71 [d, 2H], 1.42 [d, 6H], 1.19 [s, 3H] ppm.

Synthesis and characterization of protected bis-MPA anhydride (2): The acetonide protected **1** (4.22 g, 0.242 mol) was dissolved in CH_2Cl_2 (25 mL) in a round bottom flask. The solution was then chilled to 0 °C using an ice bath. DCC (3.2 g, 0.016 mol) was then dissolved in a separate vial in 10 mL of CH_2Cl_2 and then added drop-wise to the CH_2Cl_2 solution of **1**. The reaction mixture was then stirred overnight at room temperature. A white precipitate of dicyclohexylurea (DCU) was formed as a byproduct. DCU was filtered out using a glass frit filter and solvent was evaporated using a rotovap to yield an oil as the final product. The final product was placed on high vacuum for drying. Yield 4.0 g (74%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 4.17 [d, 4H], 3.68 [d, 4H], 1.41 [d,12H],1.21 [s, 6H] ppm.

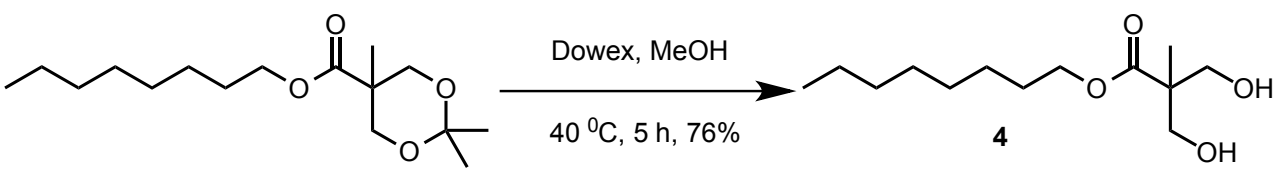


Synthesis and characterization of Oc-[G1]-An (3): Octanol (2.9 g, 0.023 mol, 0.842 g/mL), DMAP (0.42 g, 0.0034 mol), and pyridine (5.4 g, 0.07 mol, 0.98 g/mL) were dissolved in 40 mL of CH_2Cl_2 in a round bottom flask and stirred constantly. The anhydride **2** (9.0 g, 0.027



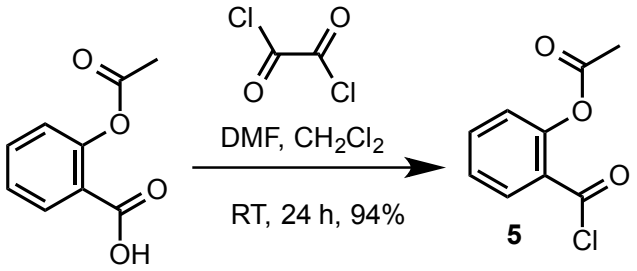
mol) was then added slowly. The reaction mixture was then stirred overnight at room temperature under nitrogen. The following day, 3 mL of nanopure water was added and stirred for 20 min, and then 200 mL of CH_2Cl_2 was added. The resulting solution was washed three times with 100 mL of 10% Na_2CO_3, three times with 100 mL of 10% $NaHSO_4$ and three times with 100 mL of brine. The resulting solution was dried over $MgSO_4$. This was then filtered using a glass filter, and remaining solvent was evaporated. The crude product was purified by silica flash chromatography (silica packed with hexanes) using ethylacetate:hexanes (5:95) solvent gradient to yield an oil as a product. Yield 3.7 g (56%). ^{1}H NMR (Figure S7) ($CDCl_3$, 400 MHz): δ 4.16 [d, 2H], 4.12 [t, 2H], 3.65 [d, 2H], 1.63 [q, 2H], 1.42 [d, 3H], 1.38 [s, 3H], 1.26 [m, 10H], 1.19 [s, 3H], 0.87 [t, 3H] ppm. ^{13}C NMR (Figure S8) ($CDCl_3$, 100 MHz): δ 174.27, 98.00, 68.79, 66.01, 64.97, 48.99, 41.74, 31.75, 30.94, 29.14, 25.80, 22.62, 17.12, 14.08 ppm. HRMS-ESI (Figure S9) (m/z): $[M+Na]^+$ Calcd. For $C_{16}H_{30}NaO_4$ 309.2042, found 309.2048.

Synthesis and characterization of Oc-[G1]-$(OH)_2$ (4): Compound **3** (3.7 g, 0.012 mol) was dissolved in 50 mL of methanol in a round bottom flask and heated to 40 °C. To this mixture,



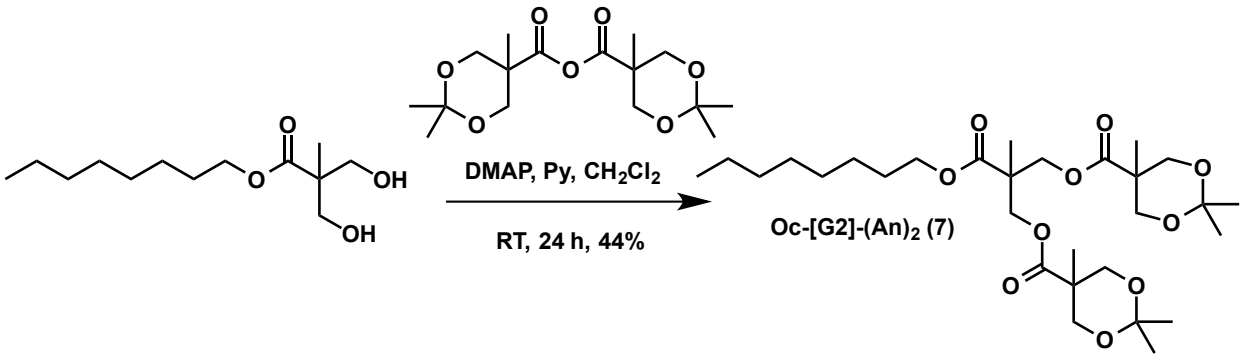
Dowex resin (3.5 g) was slowly added and the solution was stirred for 5 h at 40 °C. The final solution was filtered through a glass frit. The methanol was then completely evaporated using a rotavap. The resulting oil was dissolved in CH_2Cl_2. This solution was filtered through $MgSO_4$ and the remaining solvent was evaporated using a rotovap to yield **4** as the final product. The final product was placed on high vacuum for further drying. Yield 2.4 g, 76%. ^{1}H NMR (Figure S10) ($CDCl_3$, 400 MHz): δ 4.15 [t, 2H], 3.89 [d, 2H], 3.72 [d, 2H], 2.25 [s, 2H] 1.65 [t, 2H], 1.26 [m, 10H], 1.05 [s, 3H], 0.87 [t, 3H] ppm. ^{13}C NMR (Figure S11) ($CDCl_3$, 100 MHz): δ 175.96, 67.40, 65.14, 49.15, 31.71, 29.11, 28.45, 25.80, 22.58, 17.13, 14.03 ppm. HRMS-ESI (Figure S12) (m/z): $[M+H]^+$ Calcd. for $C_{13}H_{27}O_4$ 247.1909; found 247.1902.

Synthesis and characterization of aspirin acid chloride (5): Acetylsalicylic acid (2.2 g, 0.0122 mol) and oxalyl chloride (3.1 g, 0.0244 mol, 1.5 g/mL) were dissolved in 50 mL of CH_2Cl_2 in a 100 mL round bottom flask. Few drops of DMF were added to catalyze the reaction. The reaction mixture was then stirred overnight at room temperature. The solvent was then evaporated to yield **5** as a yellow oil. Yield 2.2 g, 94%. ^{1}H NMR (Figure S13) ($CDCl_3$, 400 MHz): δ 8.23 [d, 1H], 7.69 [t, 1H], 7.42 [t, 1H], 7.18 [d, 1H], 2.36 [s, 3H] ppm. ^{13}C NMR (Figure S14) ($CDCl_3$, 100 MHz): δ 169.18, 164.64, 150.33, 136.06, 134.39, 132.48, 126.47, 124.24, 20.87 ppm. HRMS-ESI (Figure S15) (m/z): $[M-H]^-$ calcd. for $C_9H_6ClO_3$ 197.5940; found, 197.8038.



Synthesis and characterization of Oc-[G1]-(Asp)$_2$ (6): Compound **4** (0.5 g, 0.00204 mol), DMAP (7.5 mg, 0.00006 mol), and pyridine (1.3 g, 0.016 mol, 0.98 g/mL) were dissolved in 50 mL of CH_2Cl_2. Compound **5** (1.6 g, 0.008 mol) was added drop-wise. The reaction mixture was stirred overnight at room temperature under N_2 flow. The following day, 3 mL of nanopure water was added, followed by the addition of 40 mL of CH_2Cl_2. The solution was

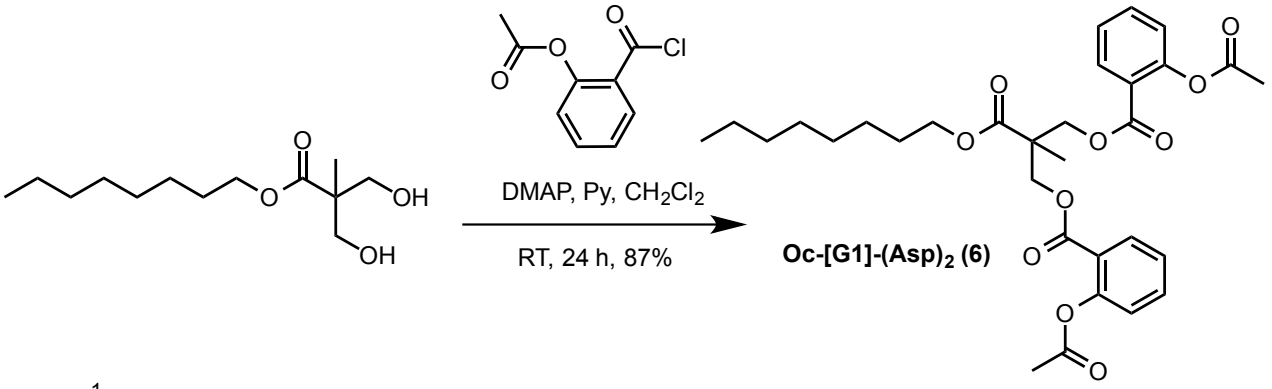
then washed three times with 50 mL of 1 M $NaHCO_3$, three times with 50 mL of 10% $NaHSO_4$, and three times with 50 mL of brine. The final solution was dried over $MgSO_4$ to remove any remaining water. The solvent was then evaporated to yield an oil. The crude product was then purified using silica flash column chromatography (silica packed with



hexanes) using ethylacetate:hexane (10:90) and then ethylacetate:hexane (15:85), and the product was isolated as a pale yellow oil. Yield: 947 mg, 87%. ^{1}H NMR (Figure S16) ($CDCl_3$, 400 MHz): δ 7.92 [d, 2H], 7.56 [t, 2H], 7.28 [t, 2H], 7.11 [d, 2H], 4.49 [s, 3H], 4.13 [t, 2H], 2.32 [s, 5H], 1.58 [q, 1H], 1.37 [s, 2H], 1.18 [m, 10H], 0.85 [t, 3H] ppm. ^{13}C NMR (Figure S17) ($CDCl_3$, 100 MHz): δ 1172.61, 69.62, 163.51, 151.01, 134.09, 131.45, 126.01, 123.90,122.55, 66.15, 65.57, 46.60, 31.74, 29.11, 29.05, 28.50, 25.80, 22.59, 21.02, 20.98, 17.97, 14.07 ppm. HRMS-ESI (Figure S18) (m/z): $[M+Na]^+$ calcd. for $C_{31}H_{39}NaO_{10}$ 593.2363; found 593.2354.

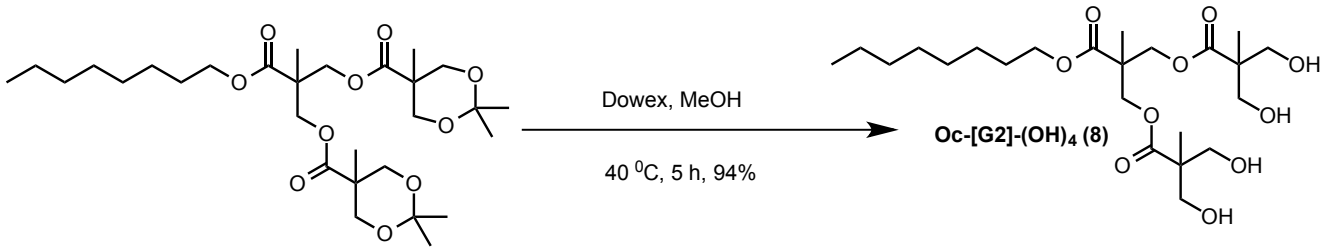
Synthesis and Characterization of Oc-[G2]-(An)$_2$ (7): Compound **4** (1.8 g, 0.007 mol), DMAP (0.269 g, 0.0022 mol) and pyridine (4.7 g, 0.06 mol, 0.98 g/mL) were dissolved in 60 mL of CH_2Cl_2 in a round bottom flask and stirred constantly. Compound **2** (6.3 g, 0.02 mol) was added drop-wise into the stirring solution. This reaction mixture was stirred over night at room temperature under constant N_2 flow. The following day, 3 mL of nanopure water was added; followed by the addition of 200 mL of CH_2Cl_2. The solution was then washed three times with 50 mL of 1 M $NaHCO_3$, three times with 50 mL of 10% $NaHSO_4$ and three times with 50 mL of brine. The final solution was dried over $MgSO_4$ to remove any remaining water.

The solvent was then evaporated to yield an oil. The crude product was then purified using silica flash column chromatography (silica packed with hexanes) using ethylacetate:hexane (10:90), then ethylacetate:hexane (20:80), and the product was isolated as an oil. Yield: 1.83



g, 44%. ^{1}H NMR (Figure S19) ($CDCl_3$, 400 MHz): δ 4.31 [s, 4H], 4.13 [d, 4H], 4.10 [t, 2H], 3.63 [d, 4H], 1.62 [q, 2H], 1.41 [s, 6H], 1.35 [s, 6H], 1.27 [m, 12H], 1.15 [s, 6H], 0.87 [t, 3H] ppm. ^{13}C NMR (Figure S20) ($CDCl_3$, 100 MHz): δ 173.51, 172.59, 98.07, 65.95, 65.90, 65.46, 65.33, 46.68, 41.99, 31.75, 29.16, 29.13, 28.50, 25.85, 24.80, 22.61, 22.37, 18.54, 17.75, 14.06 ppm. HRMS-ESI (Figure S21) (m/z): $[M+Na]^+$ calc. for $C_{29}H_{50}NaO_{10}$ 581.3302; found 581.3293.

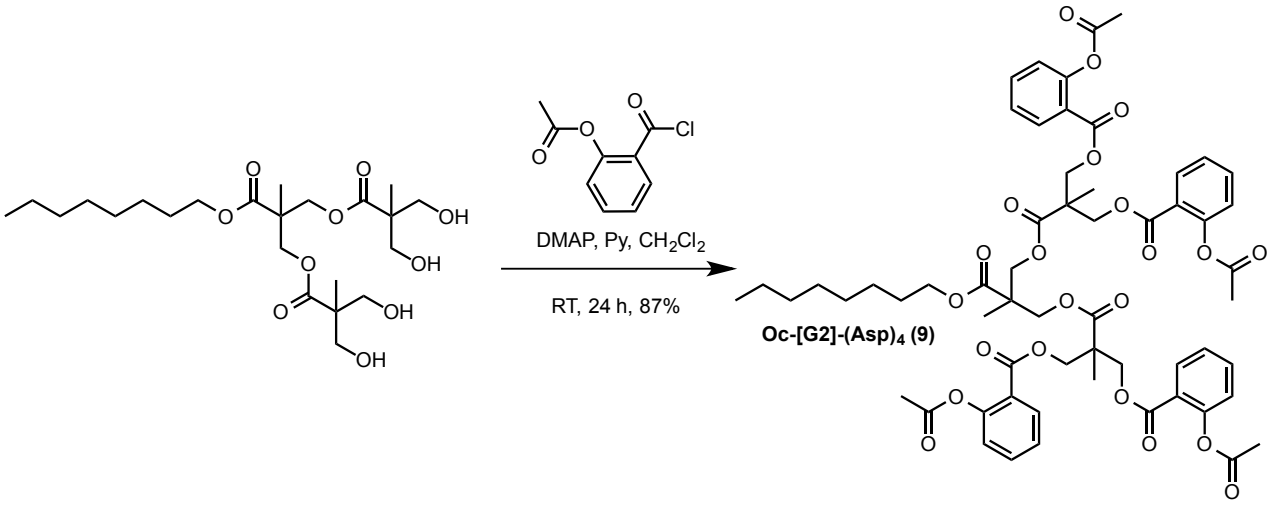
Synthesis and characterization of Oc-[G2]-(OH)$_4$ (8): Compound **7** (1.83 g, 0.003 mol) was dissolved in 70 mL of methanol in a round bottom flask and the reaction mixture was heated to 40 °C. Dowex resin (8 g) was slowly added to the mixture. This solution was stirred for 5 h



at 40 °C. The final solution was then filtered through a glass frit, and the solvent was evaporated completely to yield a red oil. This oil was then dissolved in 40 mL of CH_2Cl_2 and filtered through $MgSO_4$. The remaining solvent was then evaporated to yield a light orange solid. The final product was placed on high vacuum for drying. Yield: 1.5 g, 94%. ^{1}H NMR

(Figure S22) ($CDCl_3$, 400 MHz): δ 4.43 [d, 2H], 4.28 [d, 2H], 4.13 [t, 2H], 3.82 [t, 4H], 3.70 [t, 4H], 3.20 [s, 4H], 1.62 [q, 2H], 1.30 [s, 3H], 1.26 [m, 10H], 1.03 [s, 6H], 0.87 [t, 3H] ppm. ^{13}C NMR (Figure S23) ($CDCl_3$, 100 MHz): 175.13, 173.01, 68.19, 65.68, 64.84, 49.62, 46.31, 31.74, 29.13, 28.47, 25.82, 22.61, 18.16, 17.09, 14.07 ppm. HRMS-ESI (Figure S24) (m/z): [M+H] calc. for $C_{23}H_{43}O_{10}$ 479.2856; found 479.2862.

Synthesis and Characterization of Oc-[G2]-(Asp)$_4$ (9): Compound **8** (1 g, 0.002 mol), DMAP (153 mg, 0.0013 mol), and pyridine (2.7 g, 0.034 mol, 0.98 g/mL) were dissolved in 80



mL of CH_2Cl_2 and stirred at room temperature. Compound **5** (2.4 g, 0.011 mol) was then added drop-wise to the stirring solution. The reaction mixture was stirred overnight under N_2 flow. The next day, 10 mL of CH_2Cl_2 was added. 10 min later, 3 mL of nanopure water was added, and the resulting solution was washed three times with 50 mL of 1 M $NaHCO_3$, three times with 50 mL of 10% $NaHSO_4$ and three times with 50 mL of brine. The solution was then dried over $MgSO_4$, and the solvent was evaporated to yield a dark brown oil. The crude product was then purified using silica flash column chromatography (silica packed with hexanes) using ethylacetate:hexane (5:95) followed by ethylacetate:hexane (10:90), ethylacetate:hexane (15:85), ethylacetate:hexane (20:80), ethylacetate:hexane (25:75) and finally ethylacetate:hexane (30:70). The separate fractions were concentrated and the purity

of the concentrated product was checked by thin layer chromatography (TLC). The product showed two spots on TLC. This crude product was then purified using silica flash column (packed with CH_2Cl_2) initially with methanol:dichloromethane (0.5:99.5) and then methanol:dichloromethane (1:99). The solvent was evaporated to yield a pale yellow oil. Yield 1.2 g, 87%. ^{1}H NMR (Figure S25) ($CDCl_3$, 400 MHz): δ 7.90 [d, 4H], 7.54 [t, 4H], 7.27 [t, 4H], 7.09 [d, 4H], 4.44 [m, 8H], 4.26 [m, 4H], 3.67 [t, 2H], 2.30 [s, 12H], 1.51 [q, 2H], 1.30 [s, 6H], 1.22 [m, 10H], 1.15 [m, 3H], 0.85 [t, 3H] ppm. ^{13}C NMR (Figure S26) ($CDCl_3$, 400 MHz): δ 172.07, 171.82, 169.57, 163.40, 150.97, 134.13, 131.44, 126.06, 123.88, 122.46, 70.55, 65.79, 53.42, 46.66, 46.51, 31.74, 29.12, 28.41, 25.73, 22.60, 20.97, 17.84, 17.47, 14.08 ppm. HRMS-ESI (Figure S27) (m/z): [M+Na] calc. for $C_{59}H_{66}NaO_{22}$ 1149.3943; found 1149.3945.

T-(Asp)$_4$- and NT-(Asp)$_4$-NP Synthesis: T-(Asp)$_4$- and NT-(Asp)$_4$-NPs were synthesized using the nanoprecipication method. Briefly, 100 *μ*L from a 50 mg/mL CH_3CN solution of PLGA-*b*-PEG-OH for NT-NPs or PLGA-*b*-PEG-TPP for T-NPs was mixed with 100 *μ*L of a 10 mg/mL solution of Oc-[G2]-(Asp)$_4$ in CH_3CN and this solution was then diluted to 1 mL using CH_3CN. This 1 mL solution containing the polymer and aspirin analogues was added drop wise to 10 mL of vigorously stirring water and was allowed to stir for 2 h. These solutions were then filtered using Amicon filters with a molecular weight cut off of 100 kDa, washed three times with nanopure water, and the NPs were resuspended in nanopure water at a concentration of 5 mg/mL. Diameter ($Z_{average}$) and surface charge of the NPs were determined using 0.5 mg/mL NP suspension in water on a Malvern Zetasizer. Characterization of NPs by TEM was conducted on samples by mixing 50 *μ*g/mL NP suspension with 2% weight/volume uranyl acetate in nanopure water and depositing this mixture on a carbon coated copper grid (Cat. No. 71150, CF300-Cu, Electron Microscopy Science, Hatfield, PA). After drop casting, water evaporated by drying the grid overnight at room temperature.

T-$(Asp)_2$- and NT-$(Asp)_2$-NP Synthesis: These NPs were synthesized following methods mentioned above for T/NT-$(Asp)_4$-NPs using Oc-[G1]-$(Asp)_2$ instead Oc-[G2]-$(Asp)_4$.

Aspirin Quantification in NPs: The amount of aspirin encapsulated in the synthesized NPs was quantified using HPLC. To create the standard curves for aspirin, Oc-[G1]-$(Asp)_2$, or Oc-[G2]-$(Asp)_4$, 800 μL of a 1 mg/mL solution was created, and then serial dilutions were done to create 400 μL solutions with concentration of 1000 μg/mL, 500 μg/mL, 250 μg/mL, 125 μg/mL, 62.5 μg/mL, 31.25 μg/mL, 15.625 μg/mL, and 7.8125 μg/mL. To these solutions, 100 μL of 0.1 M NaOH solution and 100 μL of water were added for a total volume of 600 μL. To prepare the samples for analysis, 400 μL of CH_3CN, 100 μL of 0.1 M NaOH, and 100 μL of the synthesized NPs were added. These solutions were then incubated at 37 °C, for 24 h, and then analyzed using an Agilent 1260 Infinity series HPLC system. The mobile phase used was 50:50 CH_3CN:water. Aspirin was converted to salicylic acid, which produces a peak at approximately 12.5 min at 295 nm wavelength. The peak areas of the samples were obtained and using the prepared standard curve the aspirin concentration in the NPs were calculated.

Release of Aspirin from T-$(Asp)_4$- and NT-$(Asp)_4$-NPs: To assess the release of aspirin from the NPs, 800 μL of each 5 mg/mL NP solution was diluted to 2.4 mL with nanopure water. Then, 100 μL of this solution was added to 24 Thermo Scientific Slide-A-Lyzer MINI dialysis tubes. These tubes were then floated in a bath of 1x PBS with gentle shaking at 37 °C for 120 h. For the first 12 h of incubation, the PBS bath was changed every 3 hours. After that point, the bath was changed every 12 hours. For each nanoparticle type, two of these tubes were collected from the bath at time points of 0, 1, 2, 4, 6, 8, 12, 24, 48, 72, 96, and 120 h after the beginning of incubation. The solution in the tubes was collected in 1.5 mL microcentrifuge tubes, diluted to 500 μL to make volumes uniform, and stored at 4 °C. Once all tubes were collected in this way, 100 μL of the collected solutions was added to 400 μL of

CH_3CN and 100 μL of 0.1 M NaOH. These were incubated at 37 °C, along with standard samples prepared as in the aspirin Quantification procedure, and then analyzed with the Agilent 1260 Infinity series HPLC system. The concentrations of aspirin determined through this analysis were then used to calculate the percent of the aspirin mass that had been released at each time point.

Cytotoxicity Assay in RAW 264.7 Cells: Toxicity of T/NT-$(Asp)_2$/$(Asp)_4$-NPs was studied in RAW 264.7 macrophages by using the well known MTT-based colorimetric assay. RAW 264.7 cells (3000 cells/well/100 μL) were seeded on a 96 well plate and allowed to grow 24 h at 37 °C in 5% CO_2. Next day, media in each well was removed and replenished with 100 μL of fresh media. All four types of NPs were added in increasing concentrations and each concentration had three replicates. After 24 h of incubation with the NPs, media was again changed and fresh media was added to each well. Following 48 h of further incubation, MTT was added (5 mg/mL, 20 μL/well), and the plates were incubated for 5 h for conversion of MTT to formazan by cellular oxidoreductase enzymes. The media was removed and lysis was carried out using 100 μL of DMSO, followed by homogenization of formazan with gentle shaking for 5 min at room temperature. The absorbance of the resultant solution in each well was read at 550 nm with a background reading at 800 nm. Cytotoxicity was expressed as mean percentage increase relative to the untreated control ± standard deviation. Control values were set at 0% cytotoxicity or 100% cell viability. Cytotoxicity data (where appropriate) was fitted to a sigmoidal curve and a three parameters logistic model used to calculate the inhibitory concentration-50 (IC_{50}) that is the concentration of test article under investigation showing 50% inhibition in comparison to untreated controls. These analyses were performed with GraphPad Prism (San Diego, U.S.A).

***In Vivo* Inflammation Studies:** Anti-inflammatory properties of T/NT-$(Asp)_4$-NPs and aspirin were evaluated in LPS stimulated mouse model. C57BL//6 types of male mice of 12 week age or BALB/c white albino male mice of 8 week age were first injected with 20 mg/kg of T/NT-$(Asp)_4$-NPs (these concentrations are with respect to aspirin) or 20 mg/kg of aspirin by tail vein injection. In control experiments, T-Empty-NPs or NT-Empty-NPs (200 mg/kg) was injected by tail vein injection. After 12 h, 100 μg of LPS/animal was administered by intraperitoneal injection to investigate whether this new aspirin analogue in NP formulation can prevent the animals from LPS induced inflammatory responses. Either 1.5 h or 3 h after LPS injection, blood samples were collected and serum was isolated by centrifugation (2400 rpm, 30 min) for analyses of pro-inflammatory IL-6 and TNF-α cytokines and IL-10 as anti-inflammatory cytokine. ELISA was carried out on the serum samples for the cytokines IL-6, TNF-α, IL-10 according to the methods reported by us[3-5] by performing blocking of antibody coated plates using 10% FBS in PBS for 1 h at room temperature followed by 3 washes. The serum samples (20 μL) or standard were incubated on the plates for 2 h at room temperature followed by several washing steps and serial incubations with the cytokine-biotin conjugate and streptavidin working solution. ELISA was finally followed by using a colorimetric assay by adding the substrate reagent containing 3,3',5,5'-tetramethylbenzidine (100 μL) to each well and incubation got 15 min, the reaction was then stopped by using 50 μL H_2SO_4 (2N). The absorbance of the product formed was recorded at 450 nm using a BioTek Synergy HT well plate reader.

Table S1. Characterization of Oc-[G1]-$(Asp)_2$ loaded NPs						
	T-$(Asp)_2$-NP			**NT-$(Asp)_2$-NP**		
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
$Z_{average}$ (nm)	202.0±1.2	249.2±6.6	240.0±2.9	173.6±2.2	188.9±1.1	161.2±2.5
Zeta Potential (mV)	69.7±0.9	71.8±0.2	70.2±0.8	-27.1±0.5	-31.7±0.6	-31.7±0.2
PDI	0.250	0.310	0.280	0.279	0.268	0.292
%Oc-[G1]-$(Asp)_2$ Loading	10	19	8	12	13	12

Table S2. Characterization of Oc-[G1]-$(Asp)_4$ loaded NPs						
	T-$(Asp)_4$-NP			**NT-$(Asp)_4$-NP**		
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
$Z_{average}$ (nm)	64.4±0.5	70.9±0.4	64.6±1.7	56.5±0.2	60.7±0.7	54.2±0.6
Zeta Potential (mV)	64.1±0.7	57.6±6.6	67.4±0.8	-17.4±0.5	-21.7±6.6	-18.9±0.8
PDI	0.255	0.287	0.291	0.116	0.115	0.096
%Oc-[G1]-$(Asp)_4$ Loading	16	17	16	19	16	15

Table S3. Characterization of Oc-[G1]-$(Asp)_4$ loaded NPs used in inflammatory studies in mice		
	T-$(Asp)_4$-NP	**NT-$(Asp)_4$-NP**
$Z_{average}$ (nm)	66.4±0.7	95.3±1.6
Zeta Potential (mV)	53.5±1.0	-34.0±1.6
PDI	0.216±0.011	0.159±0.011

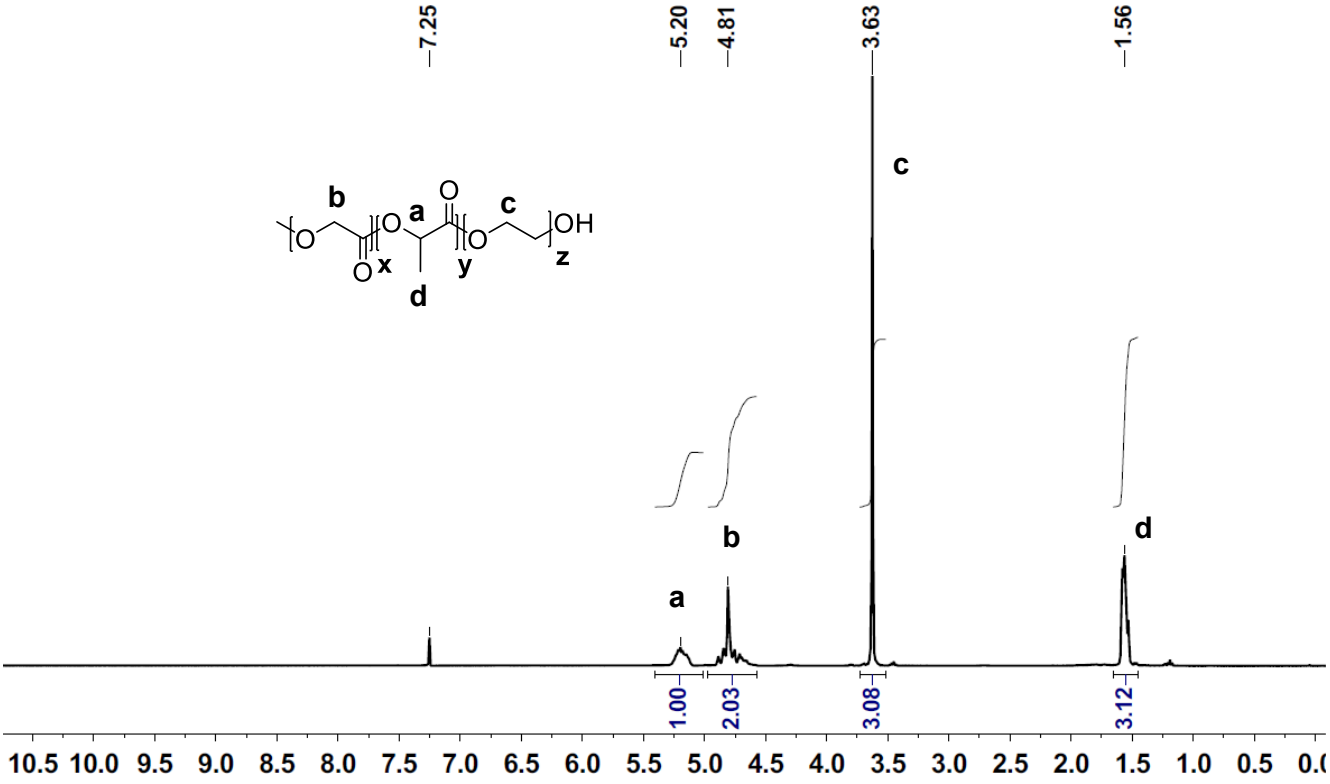


Figure S1. ^{1}H NMR of PLGA-*b*-PEG-OH in $CDCl_3$.

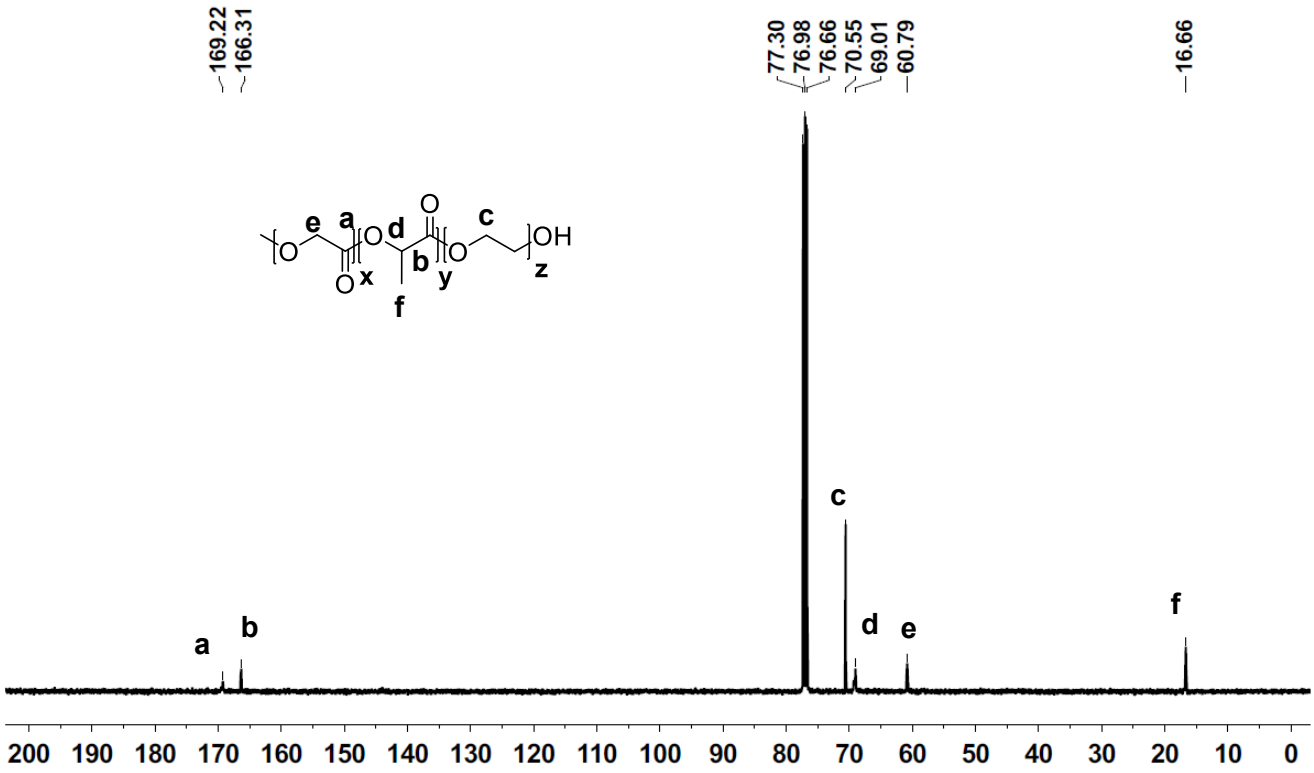


Figure S2. ^{13}C NMR of PLGA-*b*-PEG-OH in $CDCl_3$.

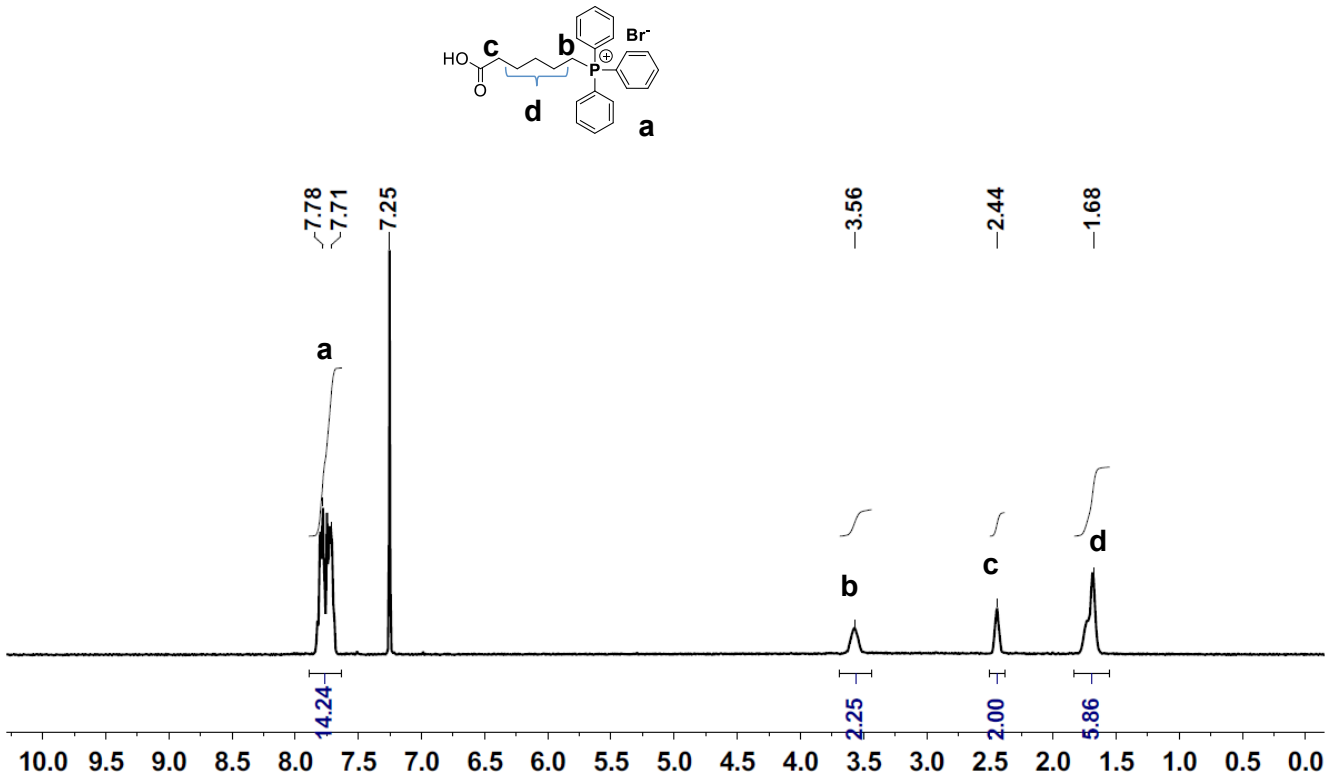


Figure S3. ^{1}H NMR of TPP-hexanoic acid in $CDCl_3$.

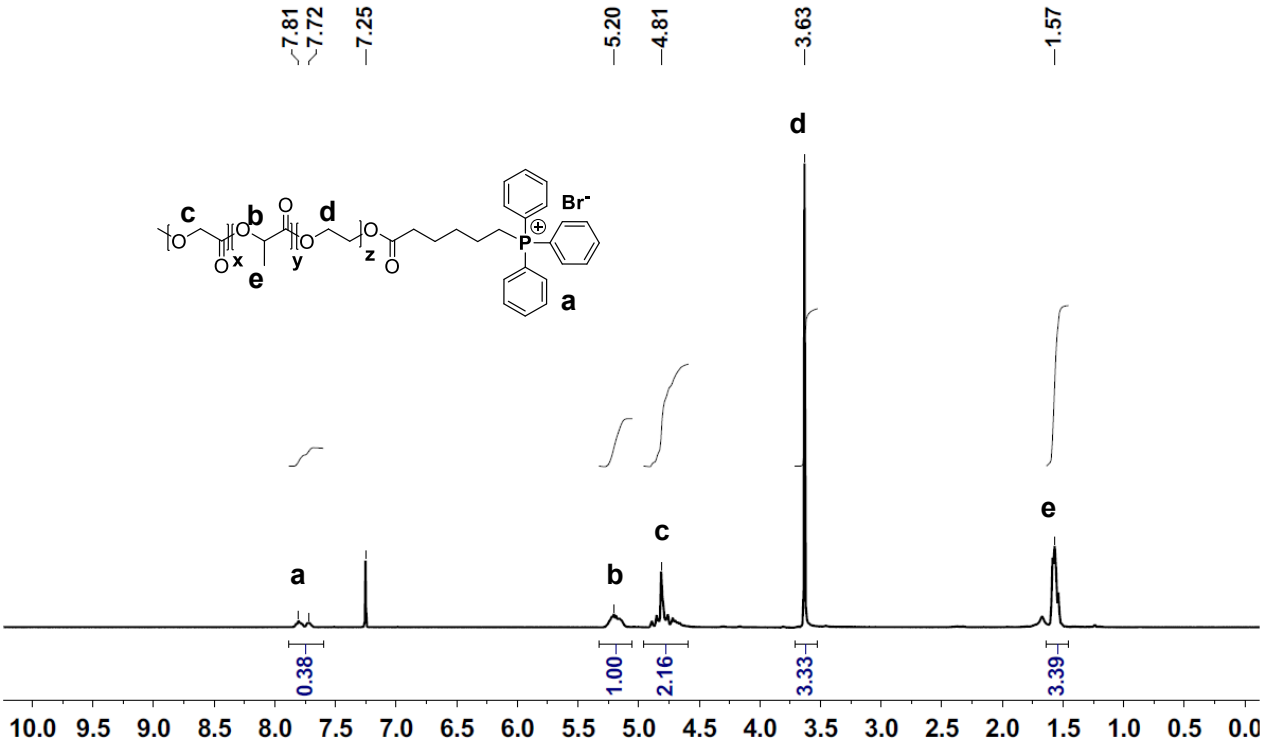


Figure S4. ^{1}H NMR of PLGA-*b*-PEG-TPP in $CDCl_3$.

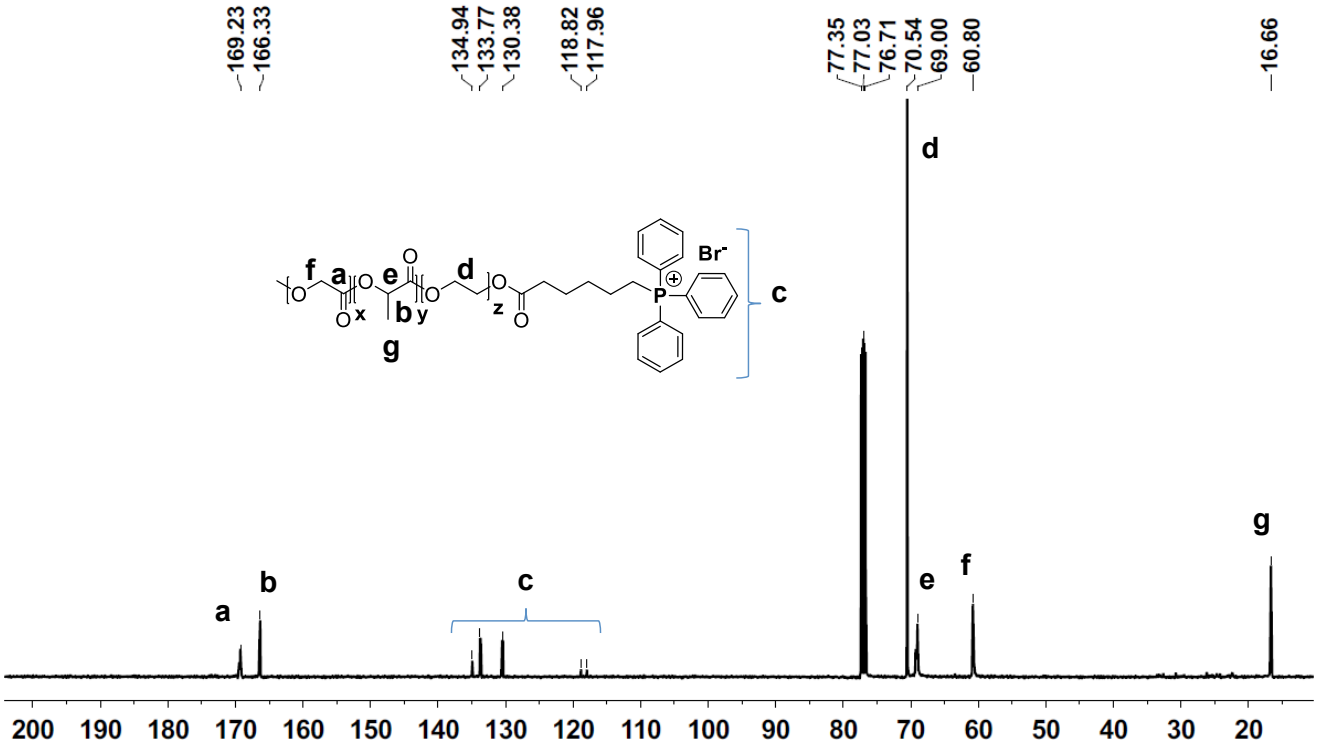
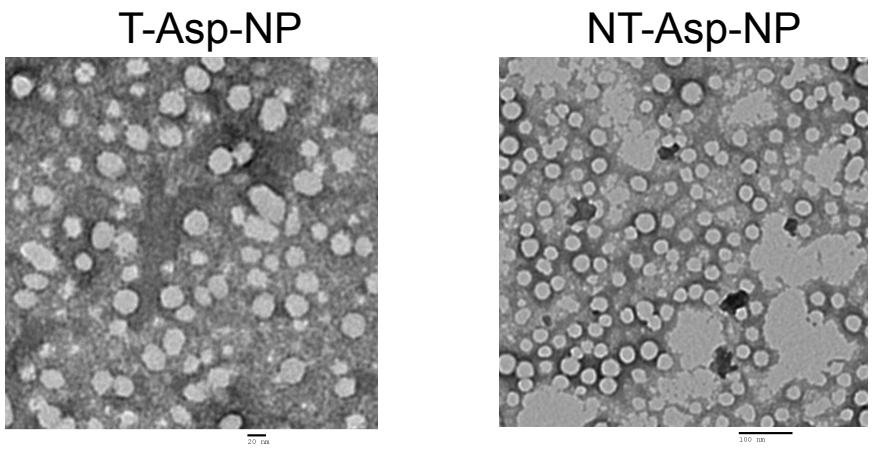


Figure S5. ^{13}C NMR of PLGA-*b*-PEG-TPP in $CDCl_3$.

(A)

	T-Asp-NP			NT-Asp-NP		
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
$Z_{average}$ (nm)	57.29±1.10	61.98±1.39	62.47±2.61	46.92±0.44	48.00±0.65	47.65±0.66
Zeta Potential (mV)	46.7±2.5	43.9±2.4	59.1±1.6	-8.15±1.52	-11.20±0.60	-17.00±6.60
PDI	0.267	0.420	0.282	0.065	0.092	0.095

(B)



(C)

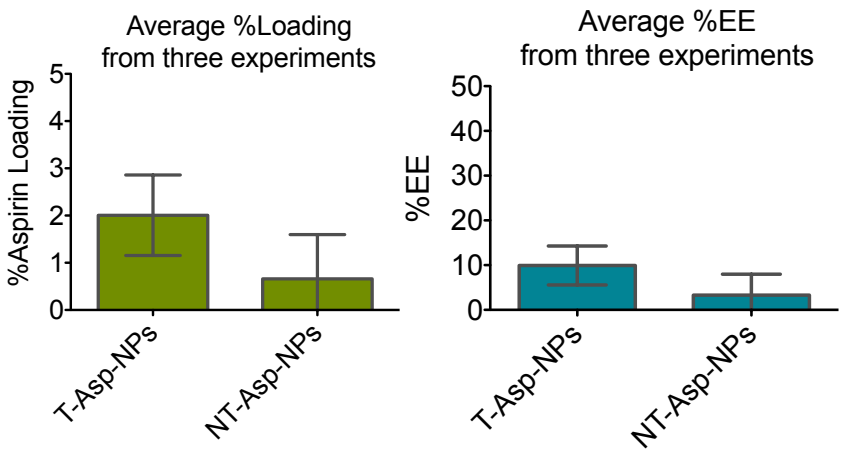


Figure S6. (A) Diameter, zeta potential, and polydispersity index (PDI) as of T/NT-Asp-NPs as determined by DLS. (B) Morphology, dispersity, and diameter determined by TEM. (C) Percent loading and percent encapsulation efficiency (EE) of aspirin in PLGA-*b*-PEG-TPP and PLGA-*b*-PEG-OH NPs as determined by HPLC.

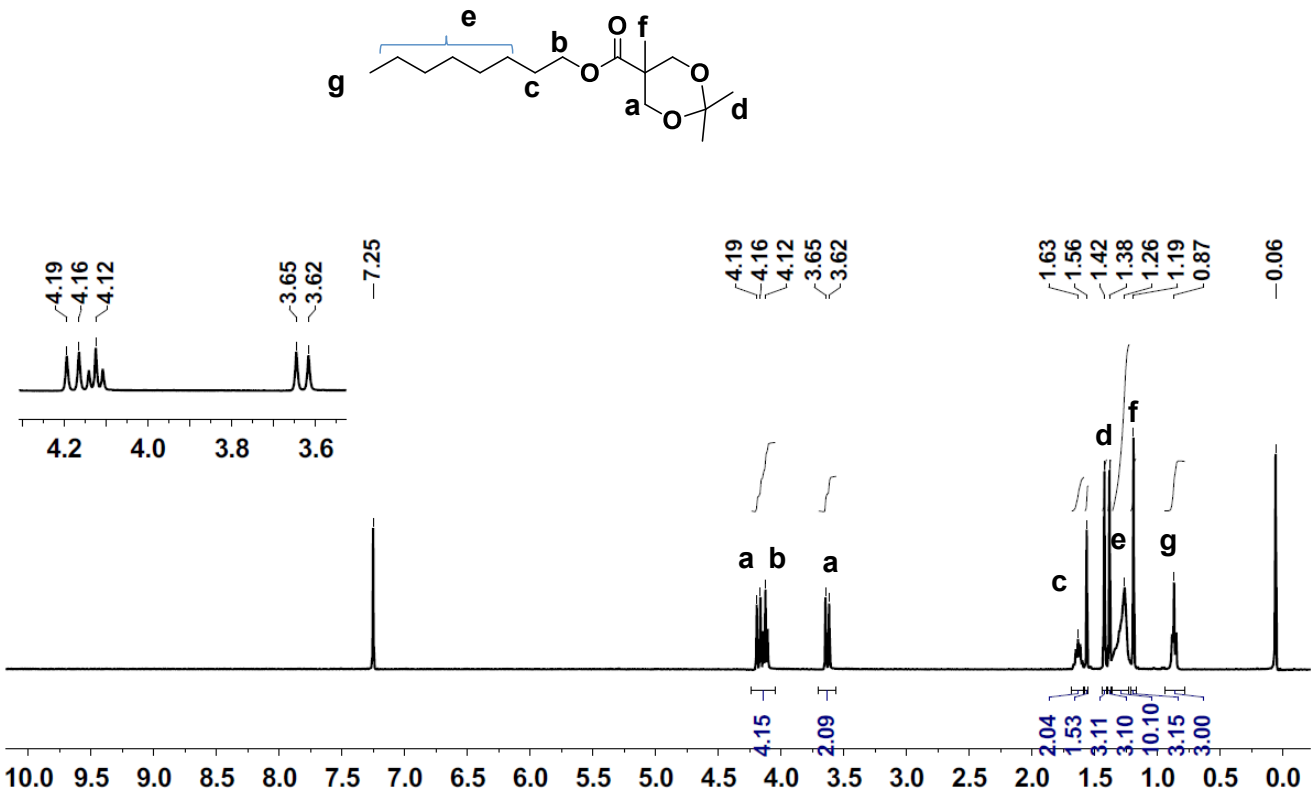


Figure S7. ^{1}H NMR of Oc-[G1]-An (**3**) in $CDCl_3$.

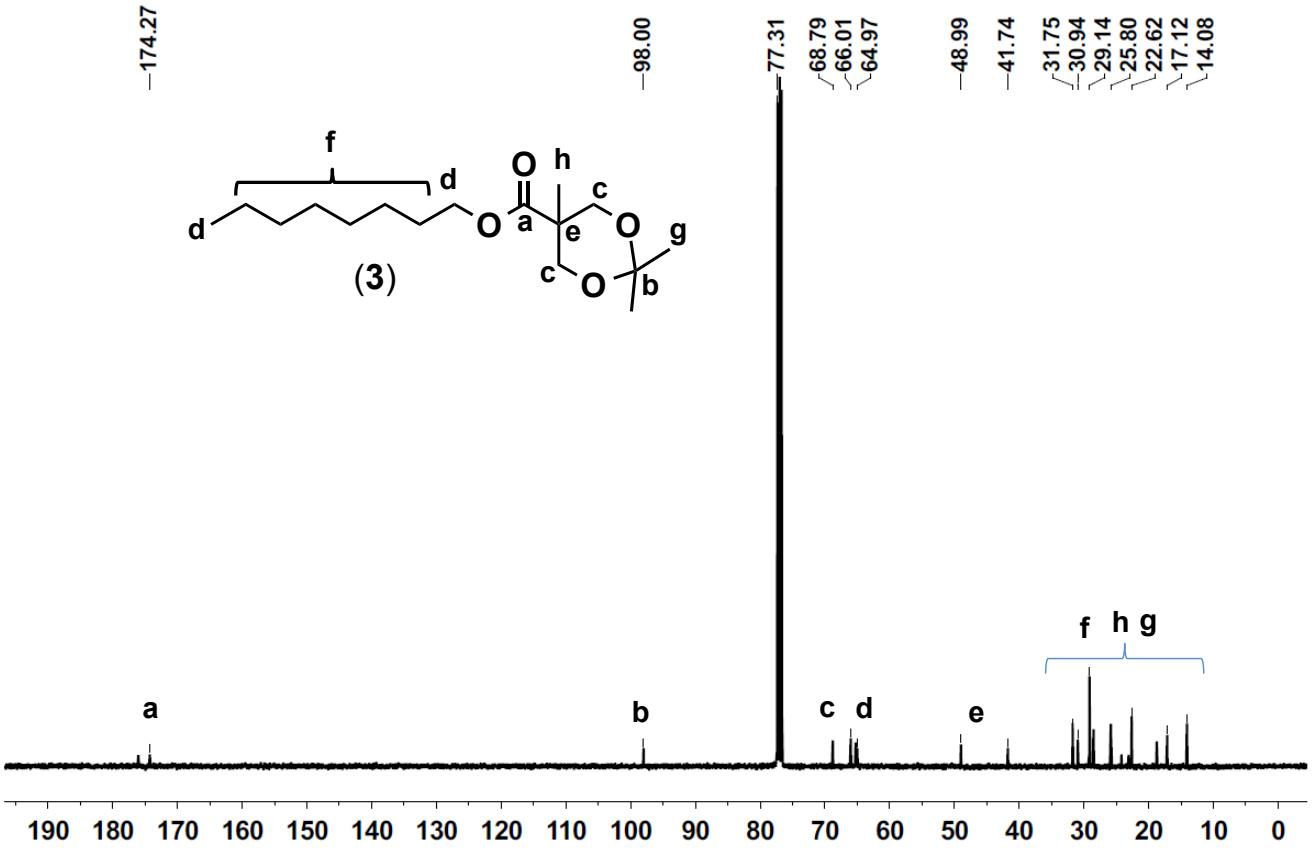


Figure S8. ^{13}C NMR of Oc-[G1]-An (**3**) in $CDCl_3$.

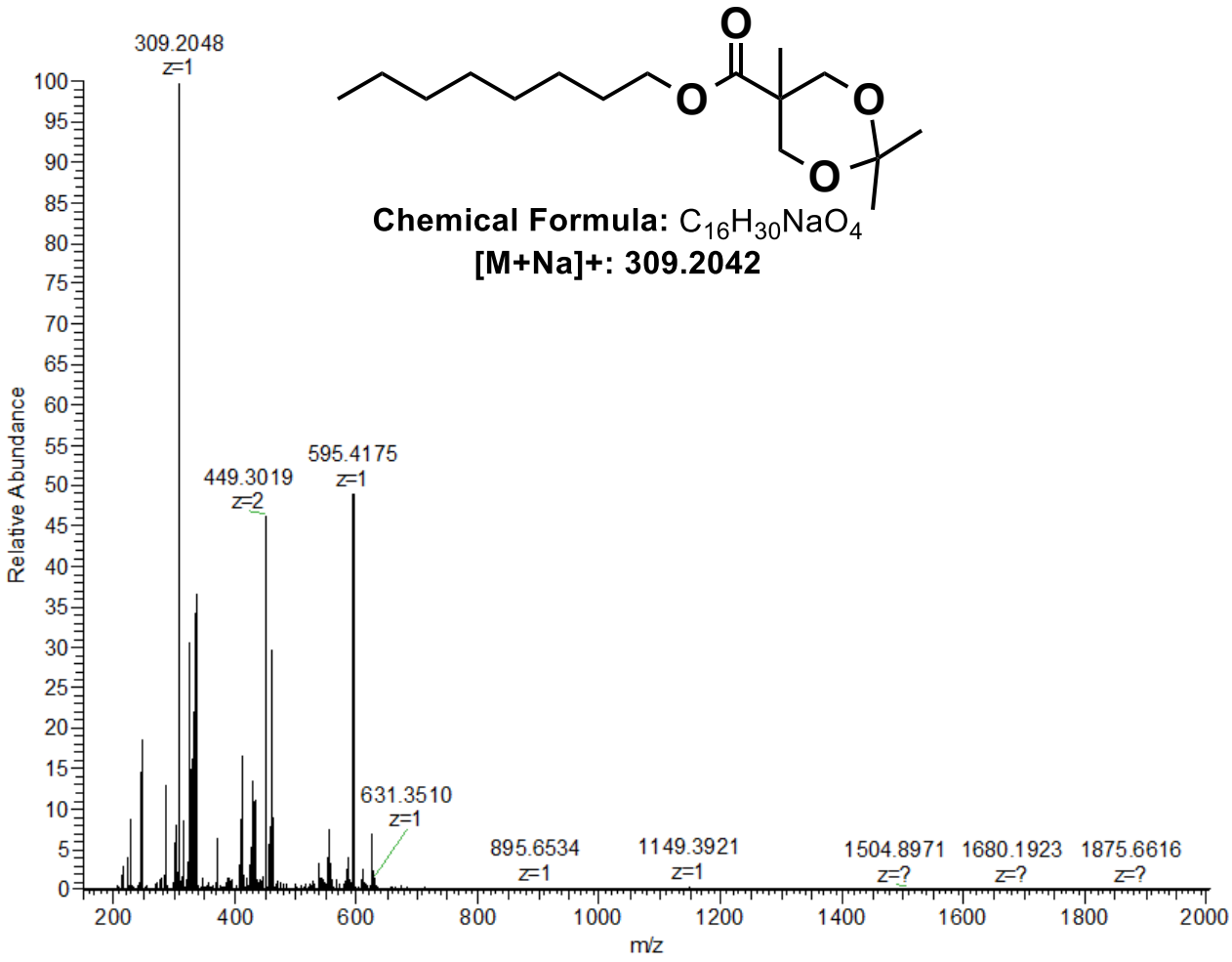


Figure S9. ESI-HRMS of compound **3** in positive ion mode.

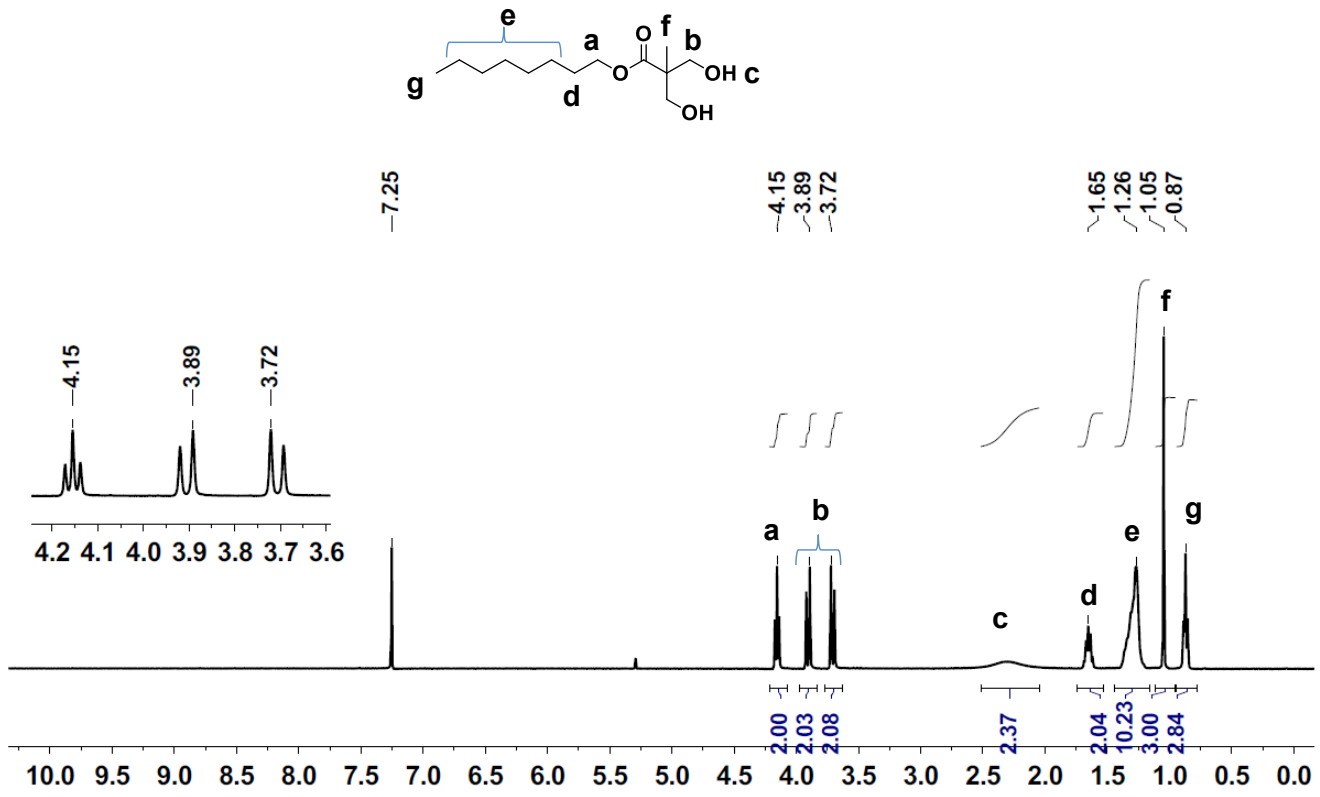


Figure S10. ^{1}H NMR of compound **4** in $CDCl_3$.

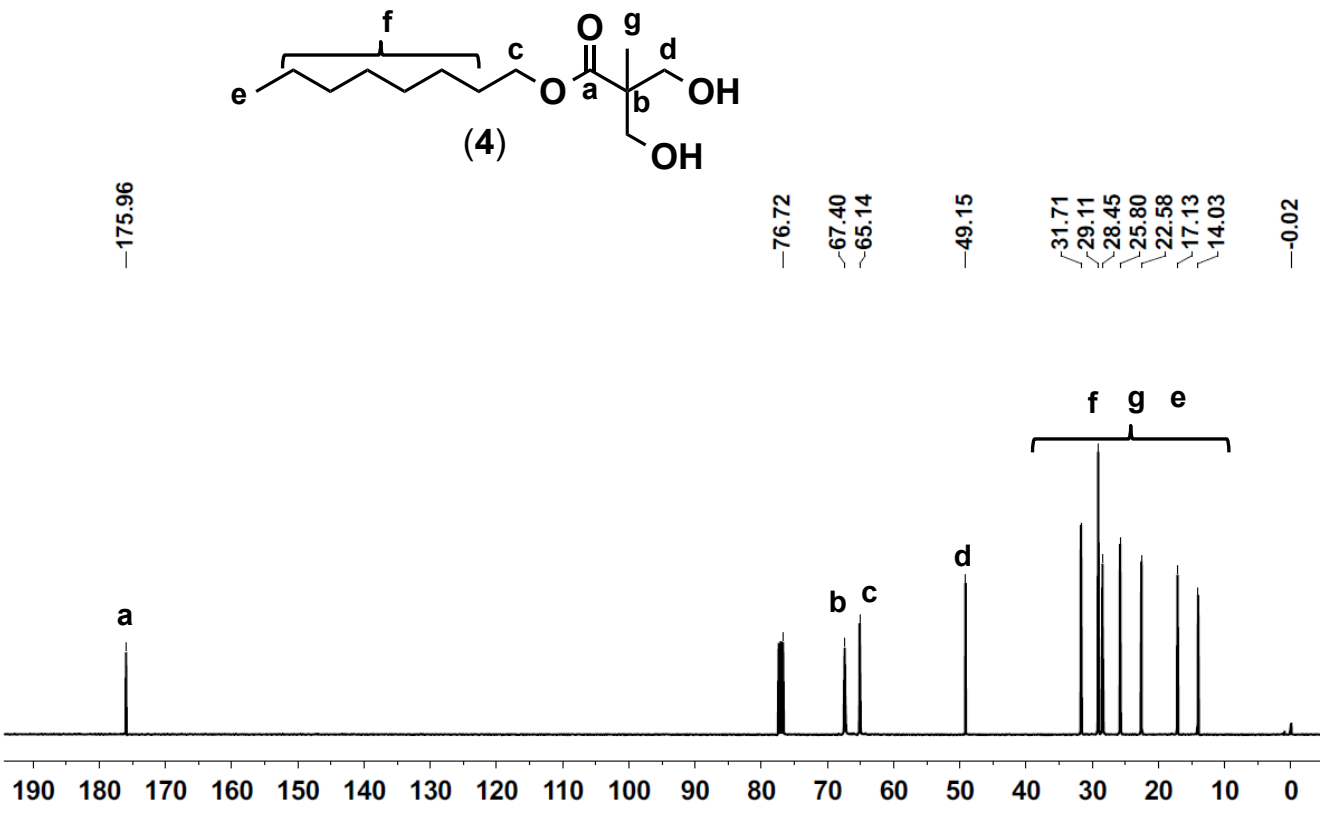


Figure S11. ^{13}C NMR of compound **4** in $CDCl_3$.

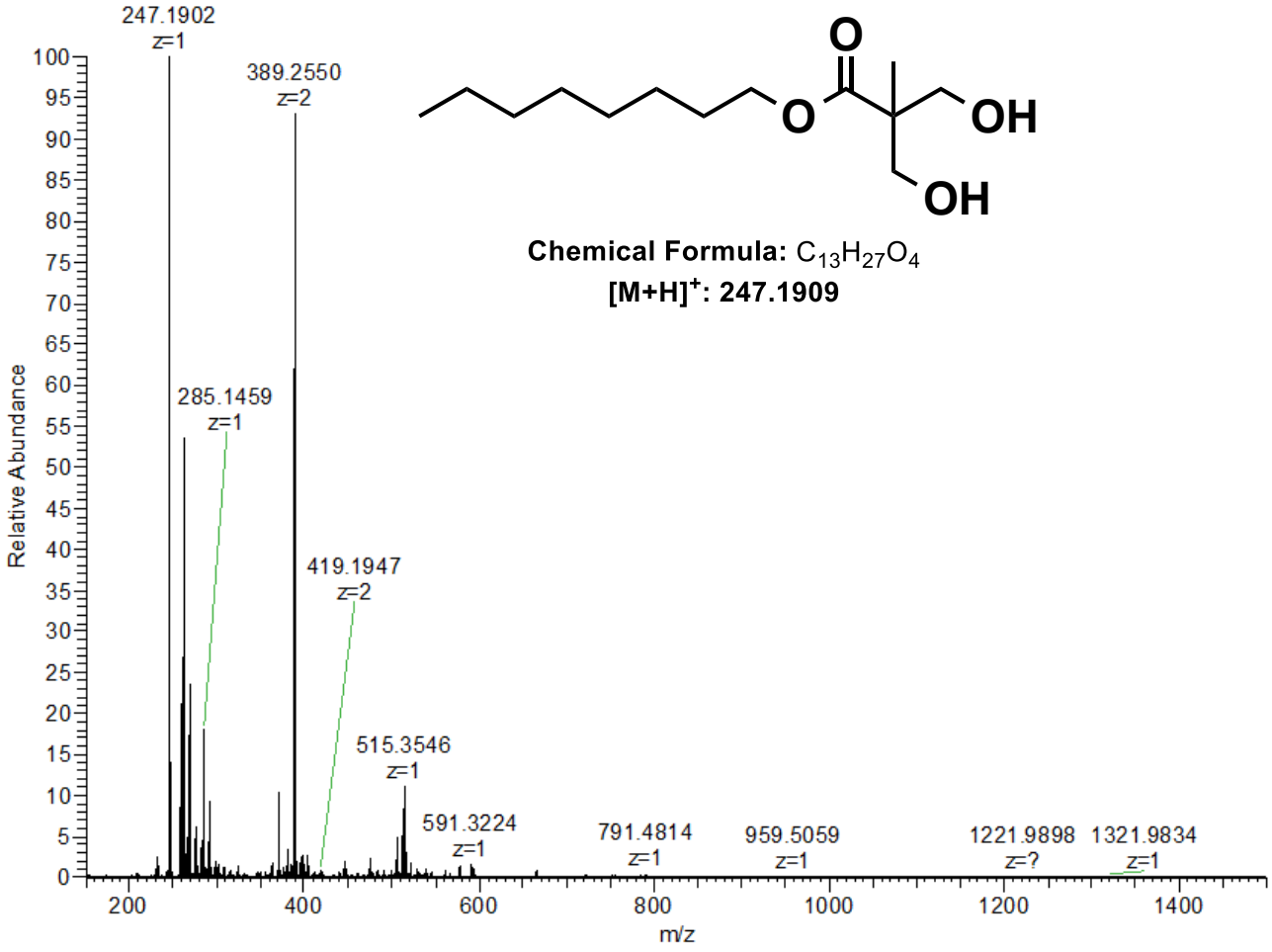


Figure S12. ESI-HRMS of compound **4** in positive ion mode.

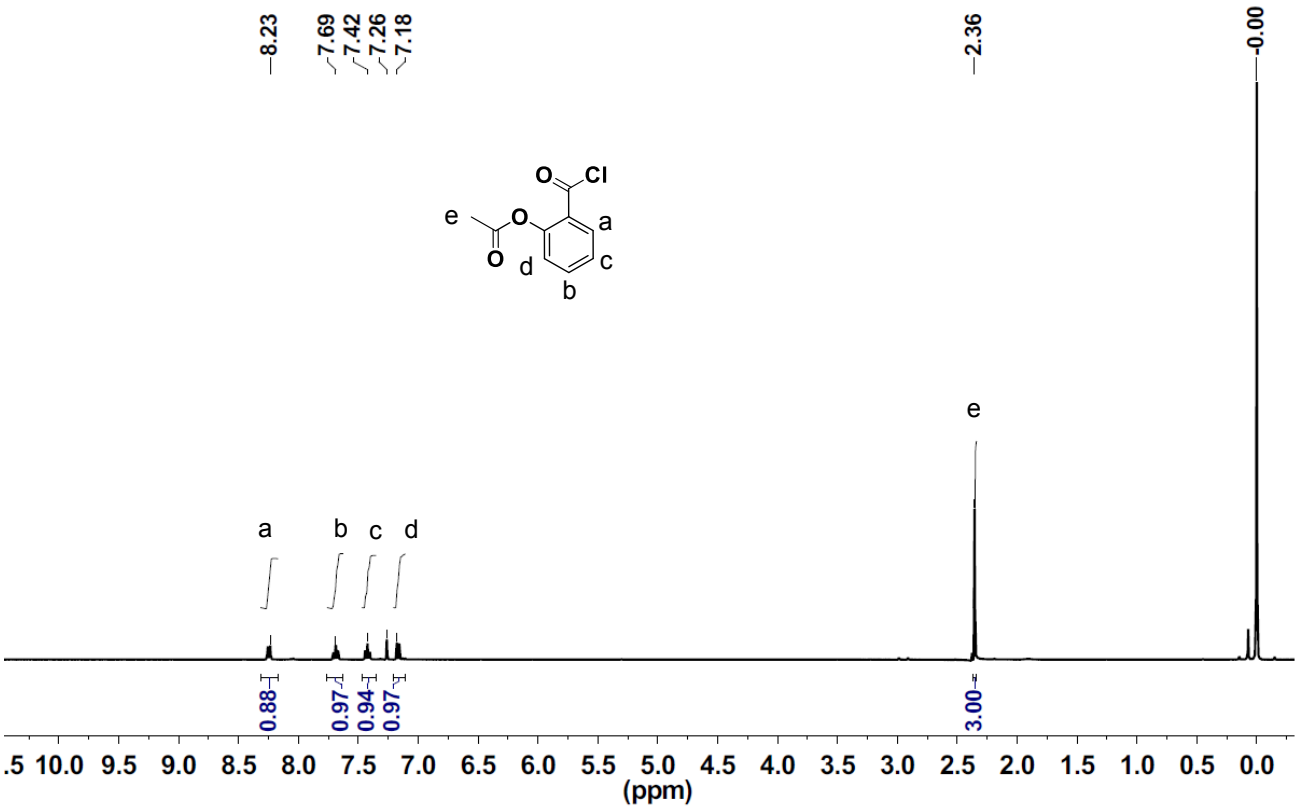


Figure S13. ^{1}H NMR of compound **5** in $CDCl_3$.

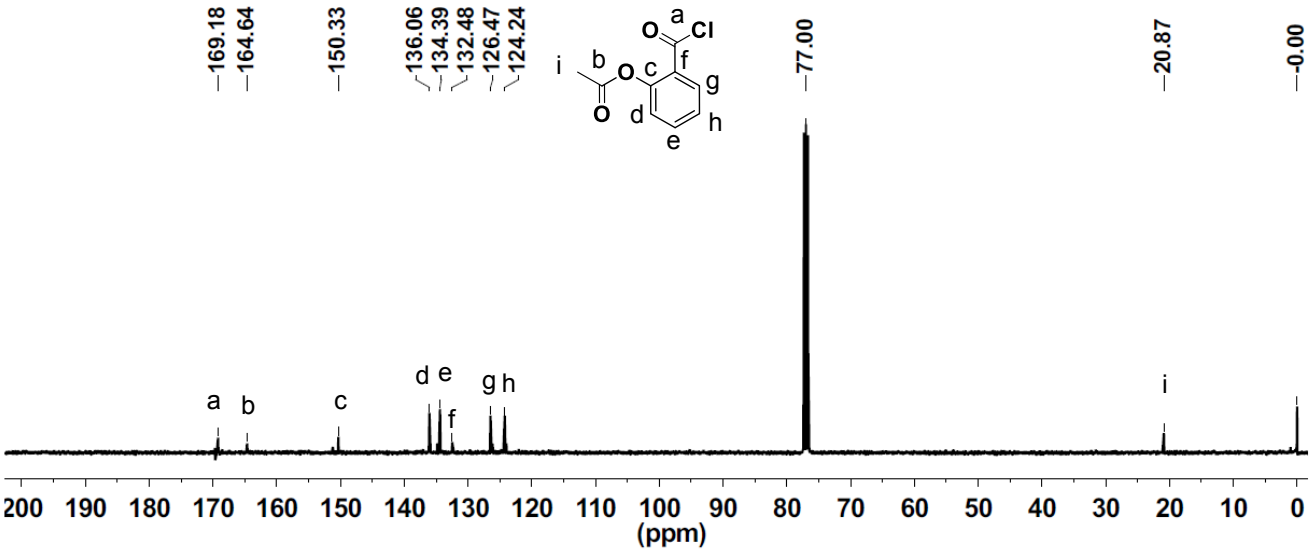


Figure S14. ^{13}C NMR of compound **5** in $CDCl_3$.

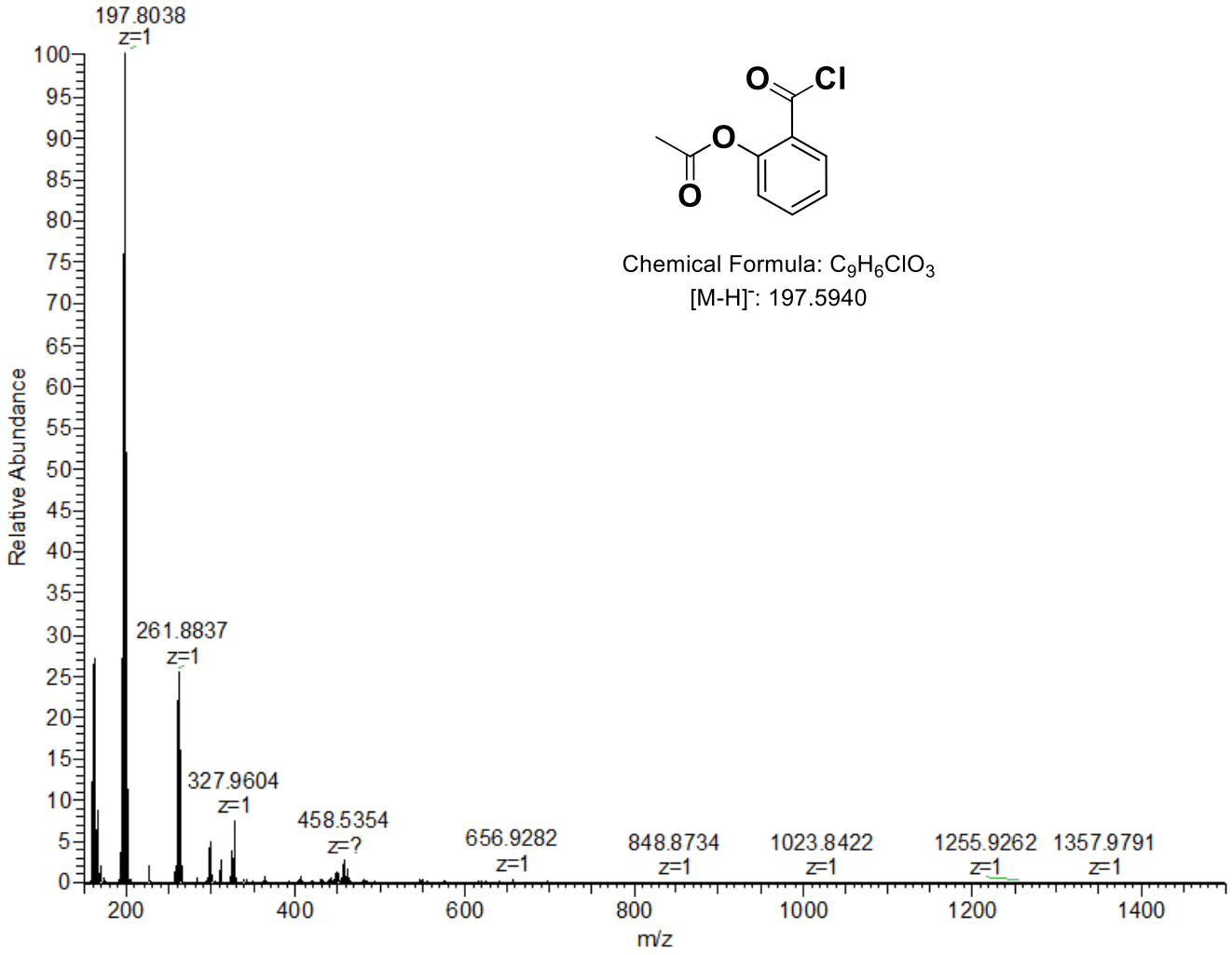


Figure S15. ESI-HRMS of compound **5** in negative ion mode.

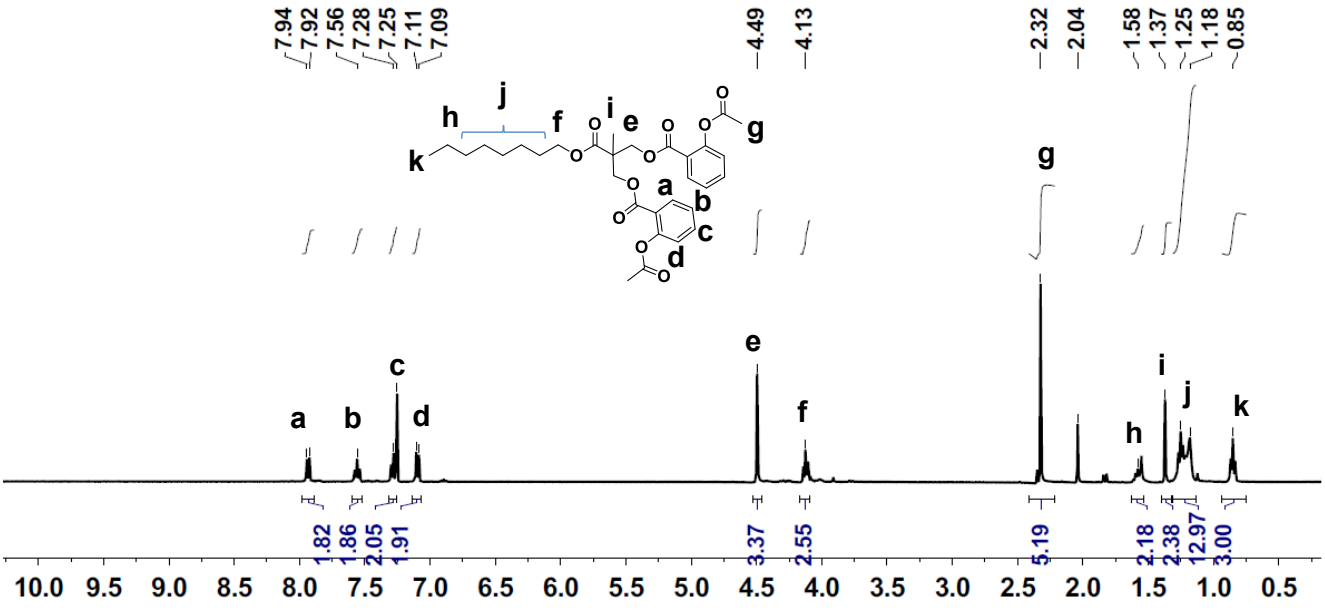


Figure S16. ^{1}H NMR of Oc-[G1]-$(Asp)_2$ (**6**) in $CDCl_3$.

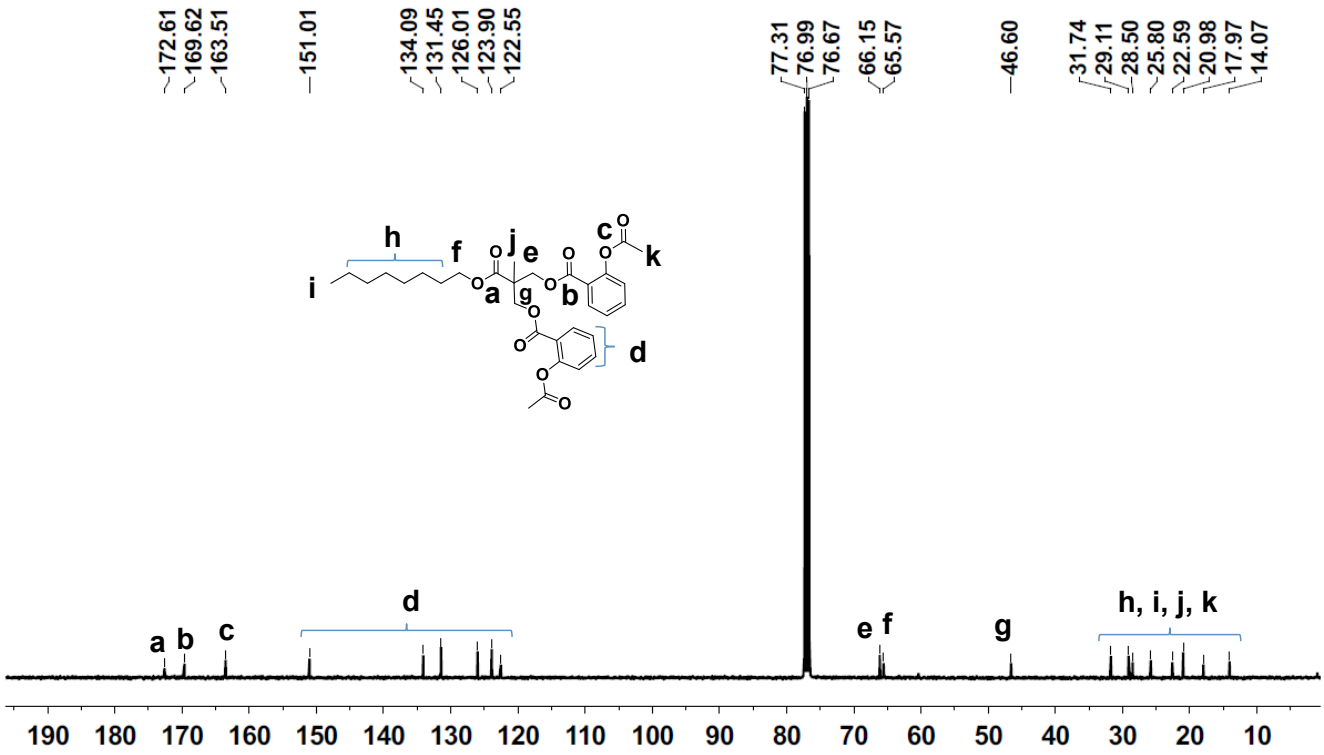


Figure S17. ^{13}C NMR of Oc-[G1]-$(Asp)_2$ (**6**) in $CDCl_3$.

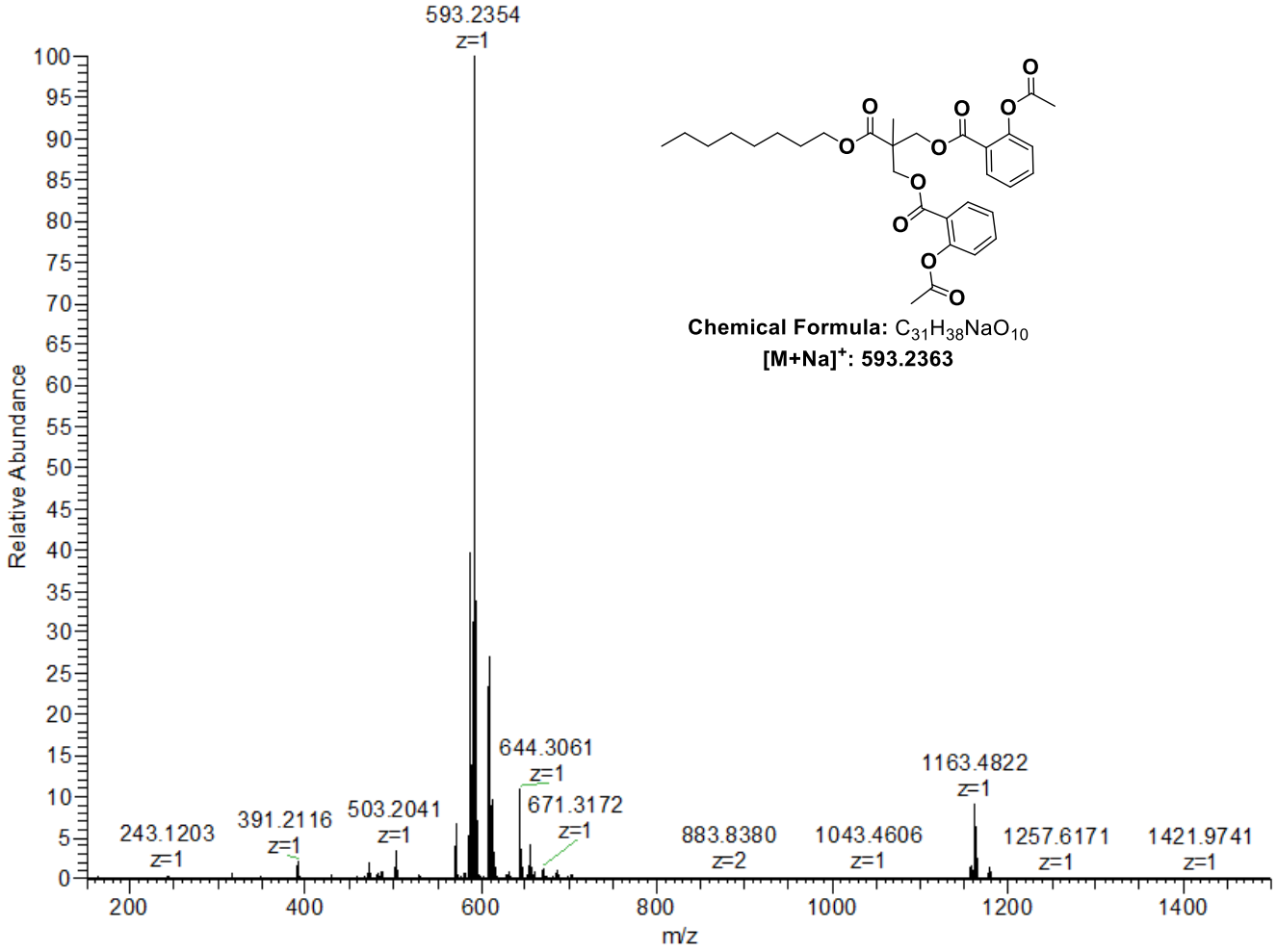


Figure 18. ESI-HRMS of Oc-[G1]-$(Asp)_2$ (**6**) in the positive mode.

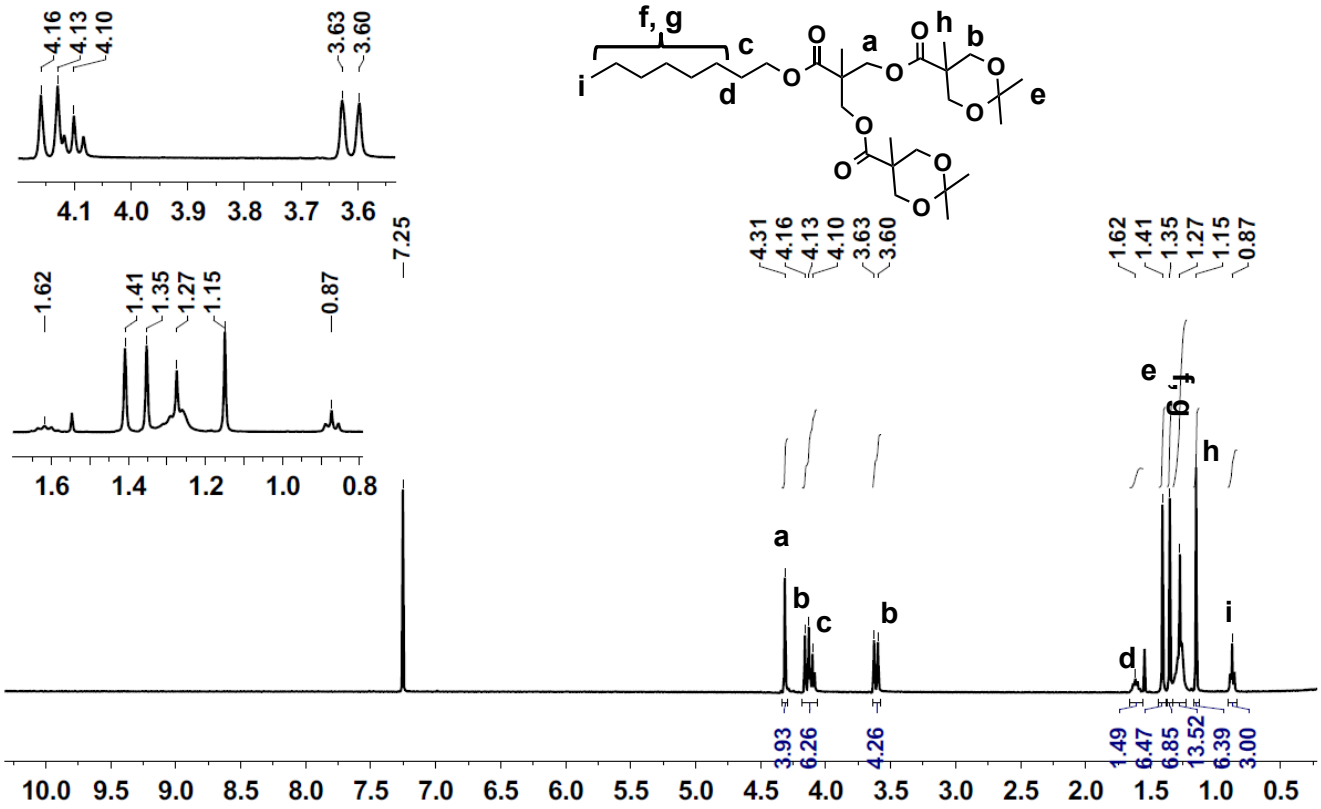


Figure S19. ^{1}H NMR of Oc-[G2]-$(An)_2$ (**7**) in $CDCl_3$.

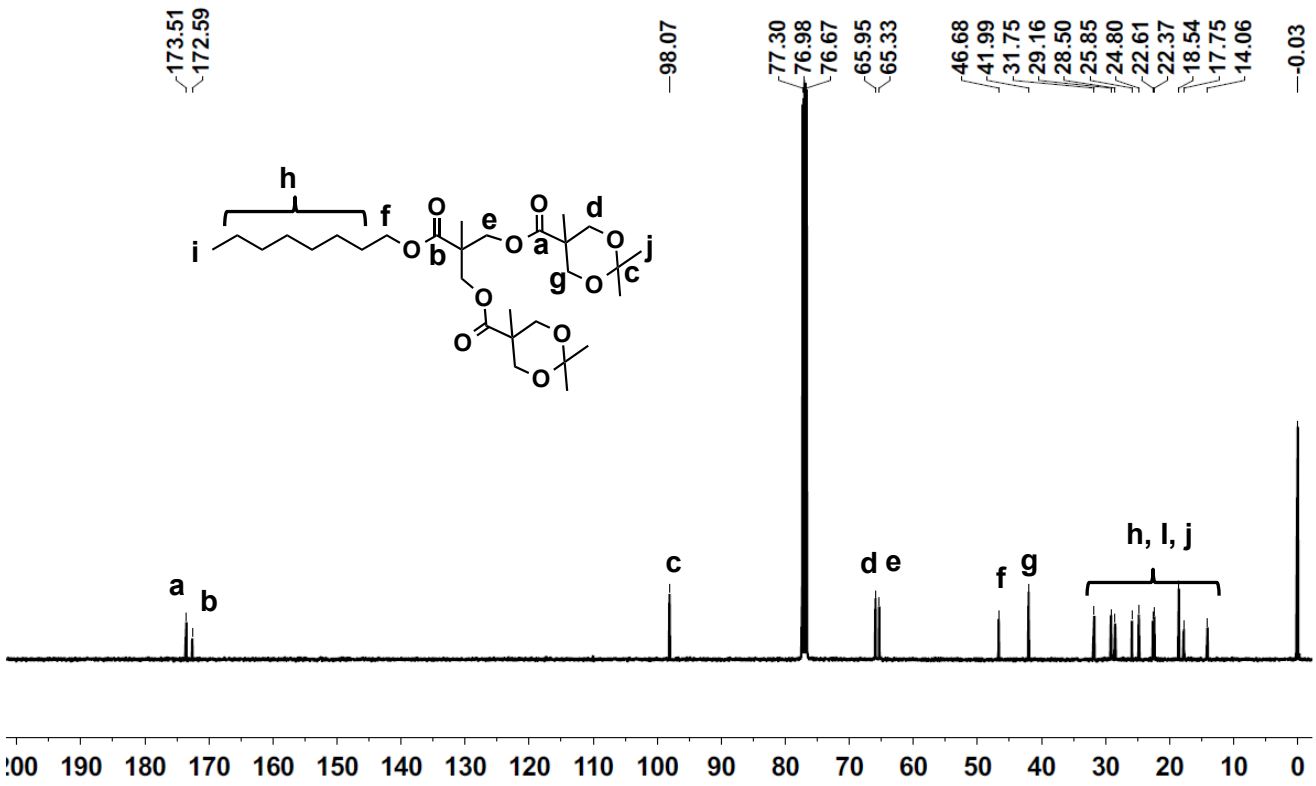


Figure S20. ^{13}C NMR of Oc-[G2]-$(An)_2$ (**7**) in $CDCl_3$.

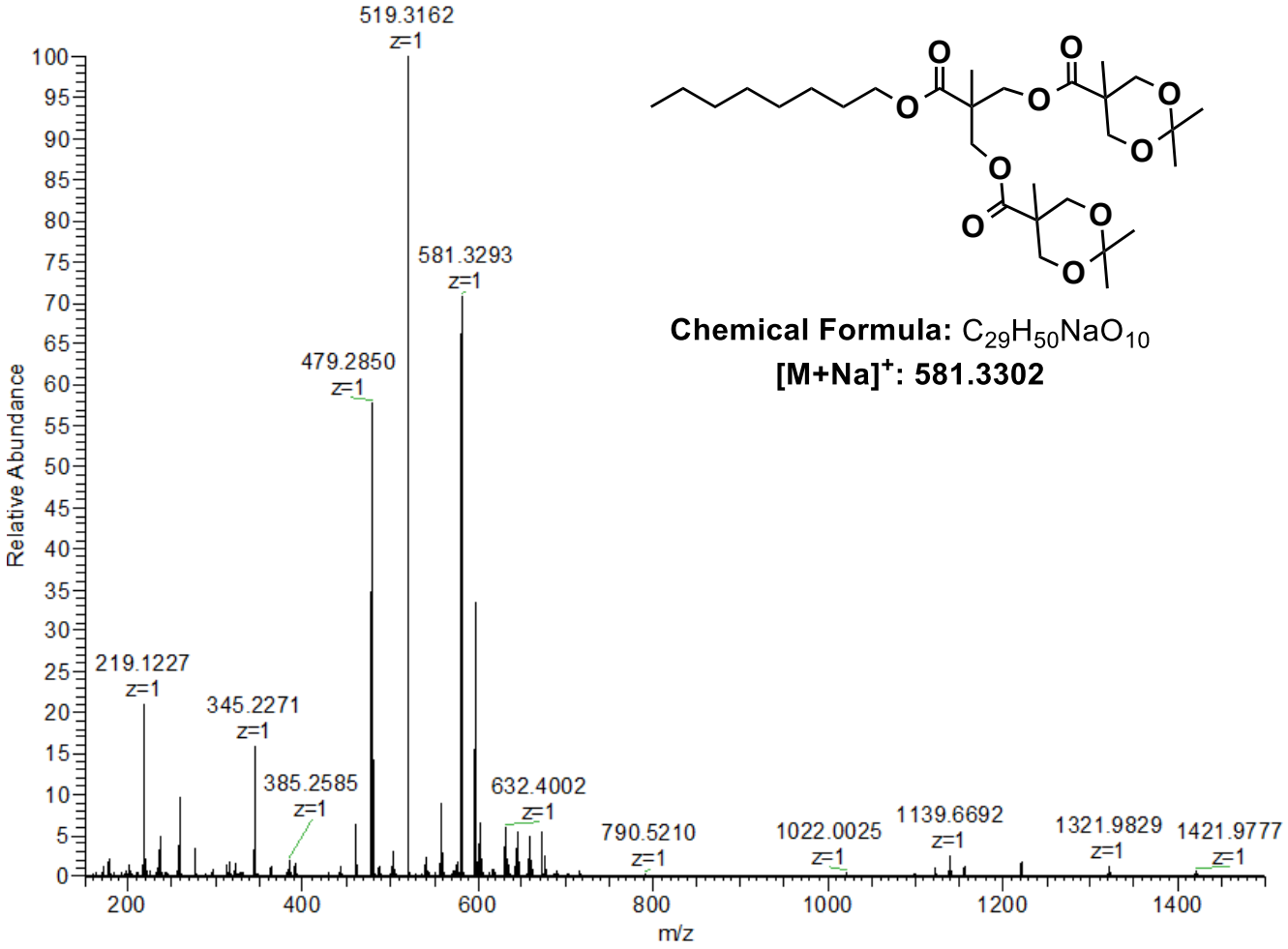


Figure S21. ESI-HRMS of Oc-[G2]-$(An)_2$ (**7**) in the positive ion mode.

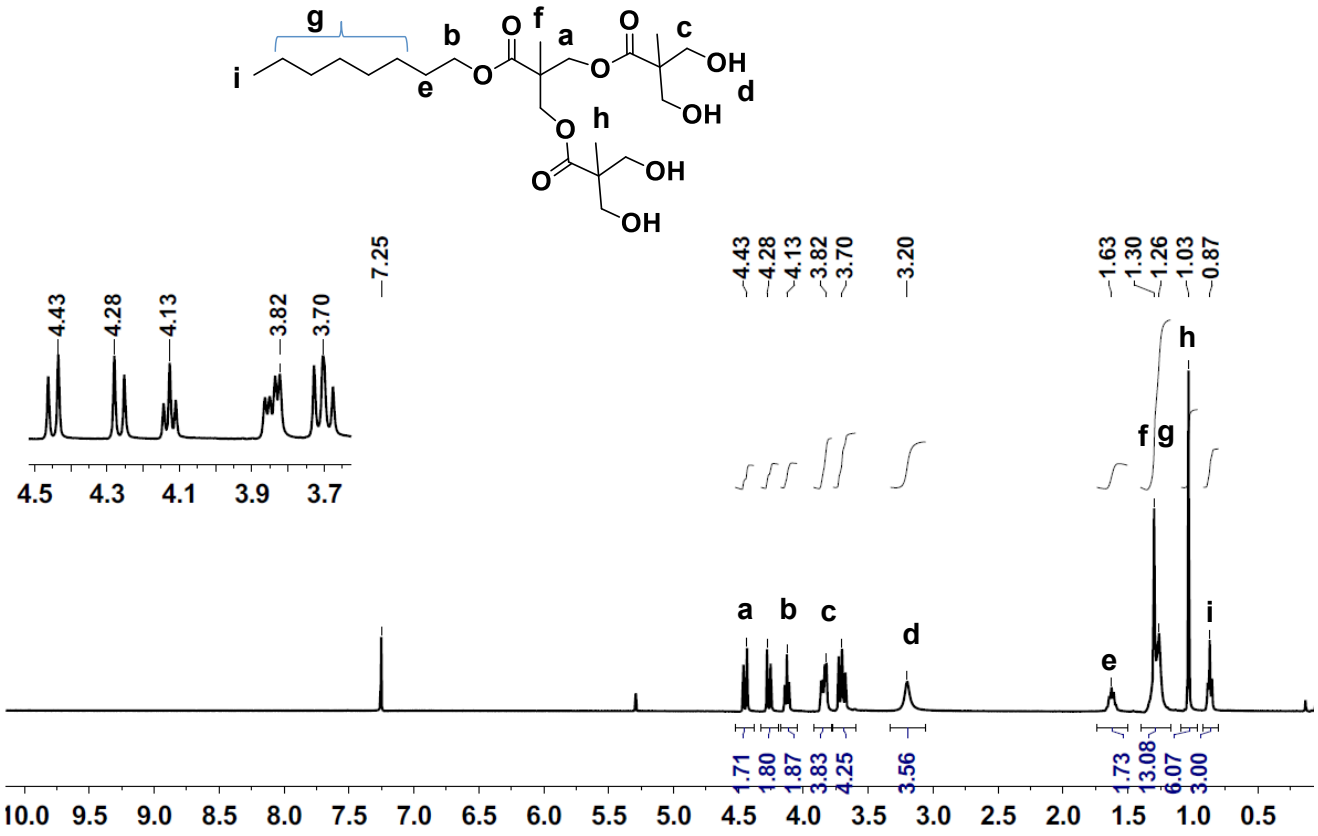


Figure S22. ^{1}H NMR of Oc-[G2]-$(OH)_4$ (**8**) in $CDCl_3$.

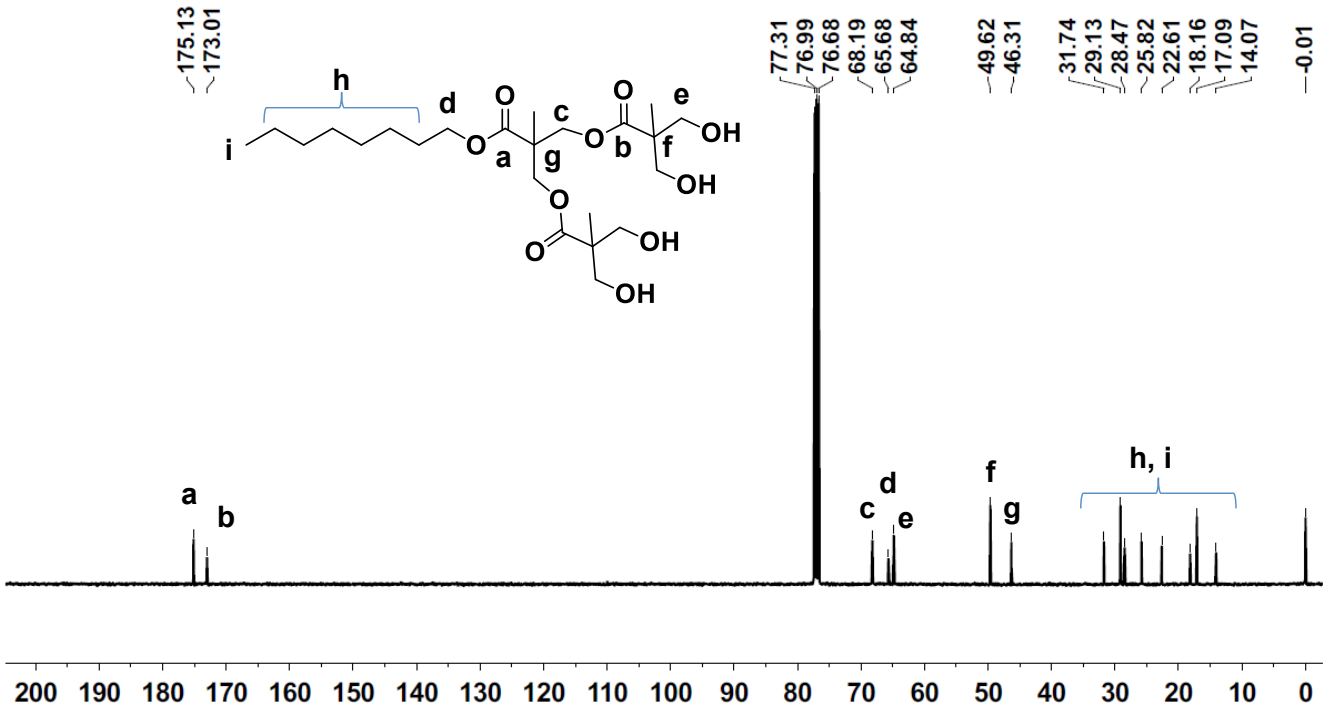


Figure S23. ^{13}C NMR of Oc-[G2]-$(OH)_4$ (**8**) in $CDCl_3$.

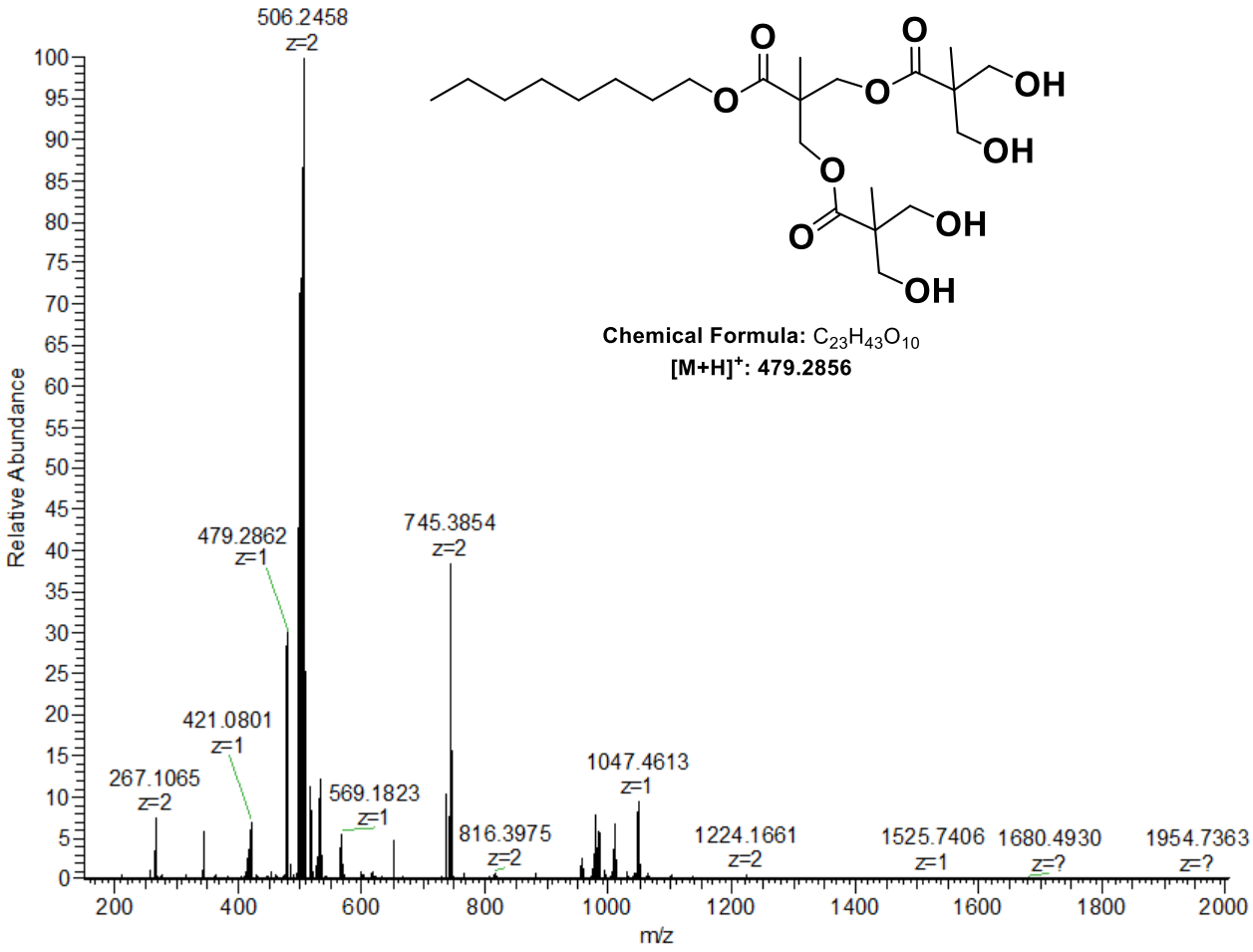


Figure S24. ESI-HRMS of Oc-[G2]-$(OH)_4$ (**8**) in the positive ion mode.

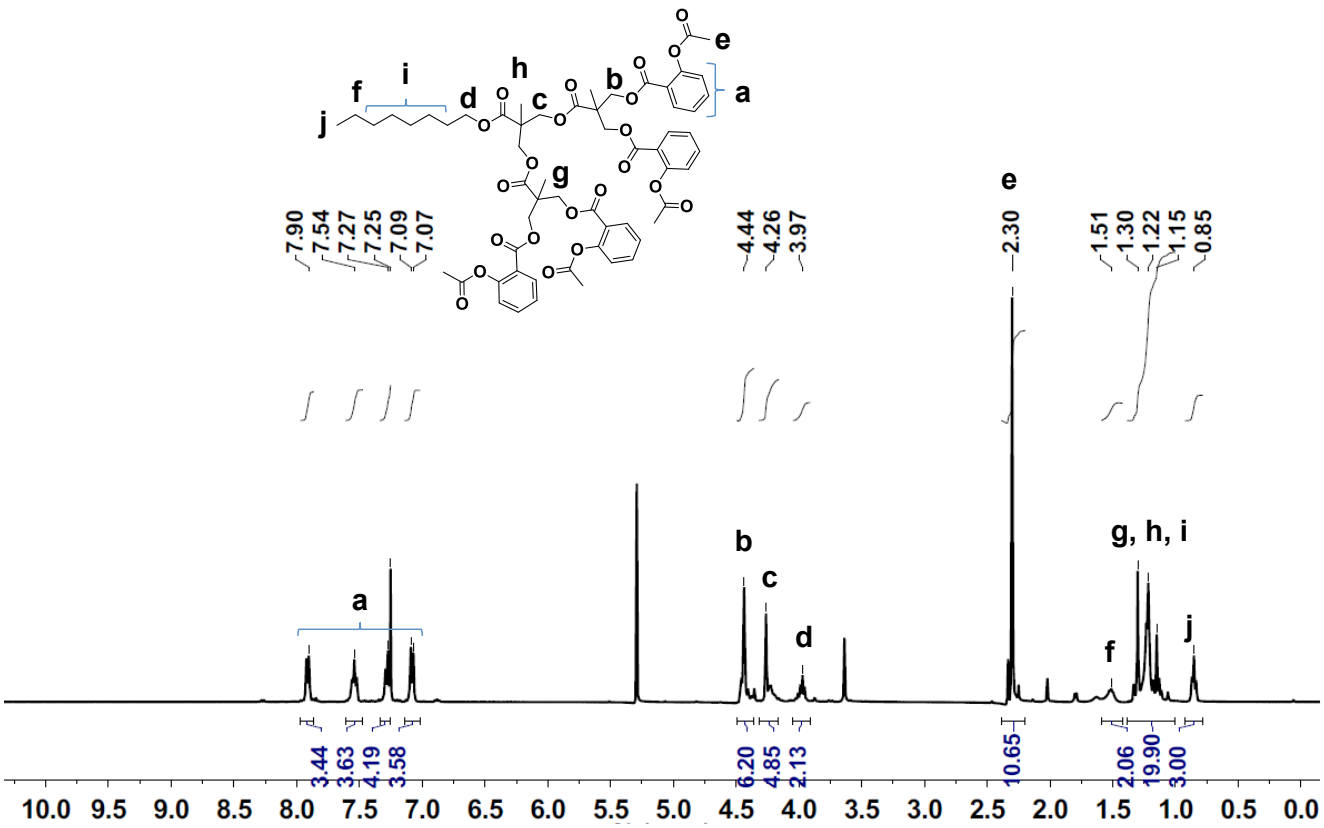


Figure S25. ^{1}H NMR of Oc-[G2]-$(Asp)_4$ (**9**) in $CDCl_3$.

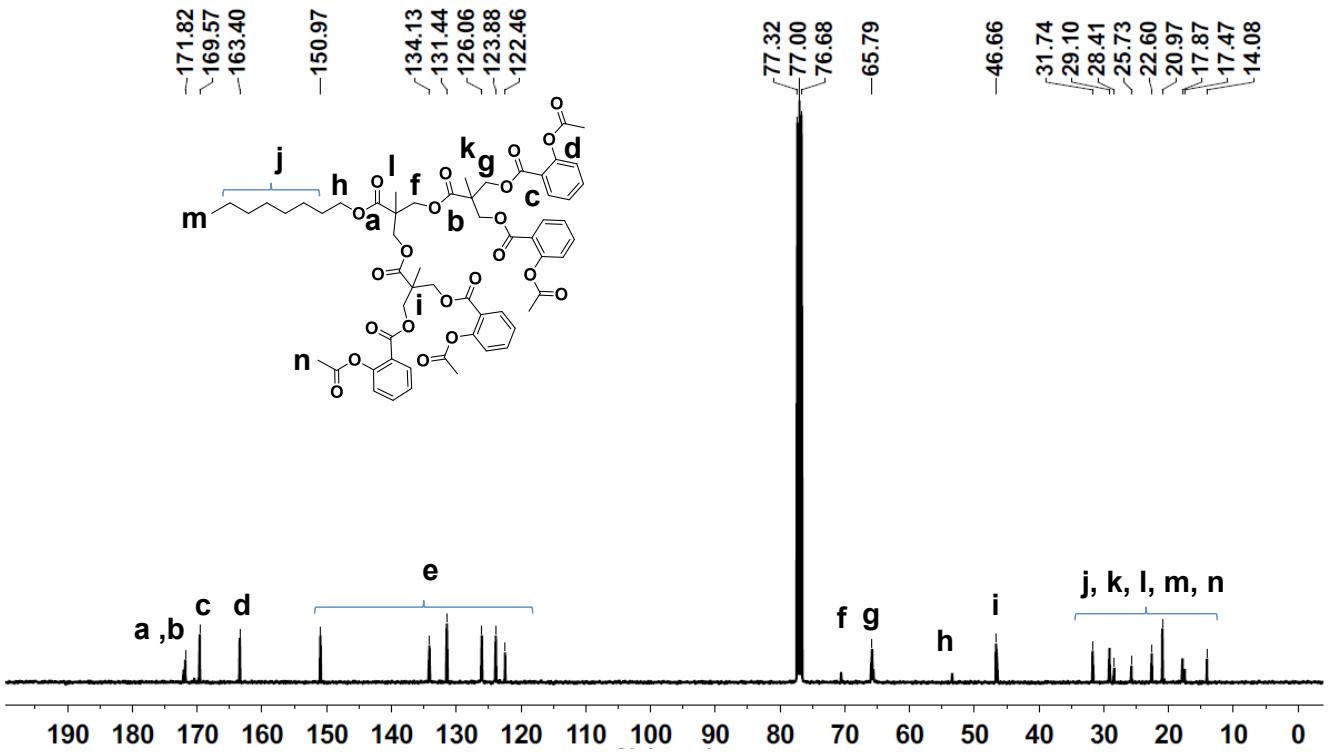


Figure S26. ^{13}C NMR of Oc-[G2]-$(Asp)_4$ (**9**) in $CDCl_3$.

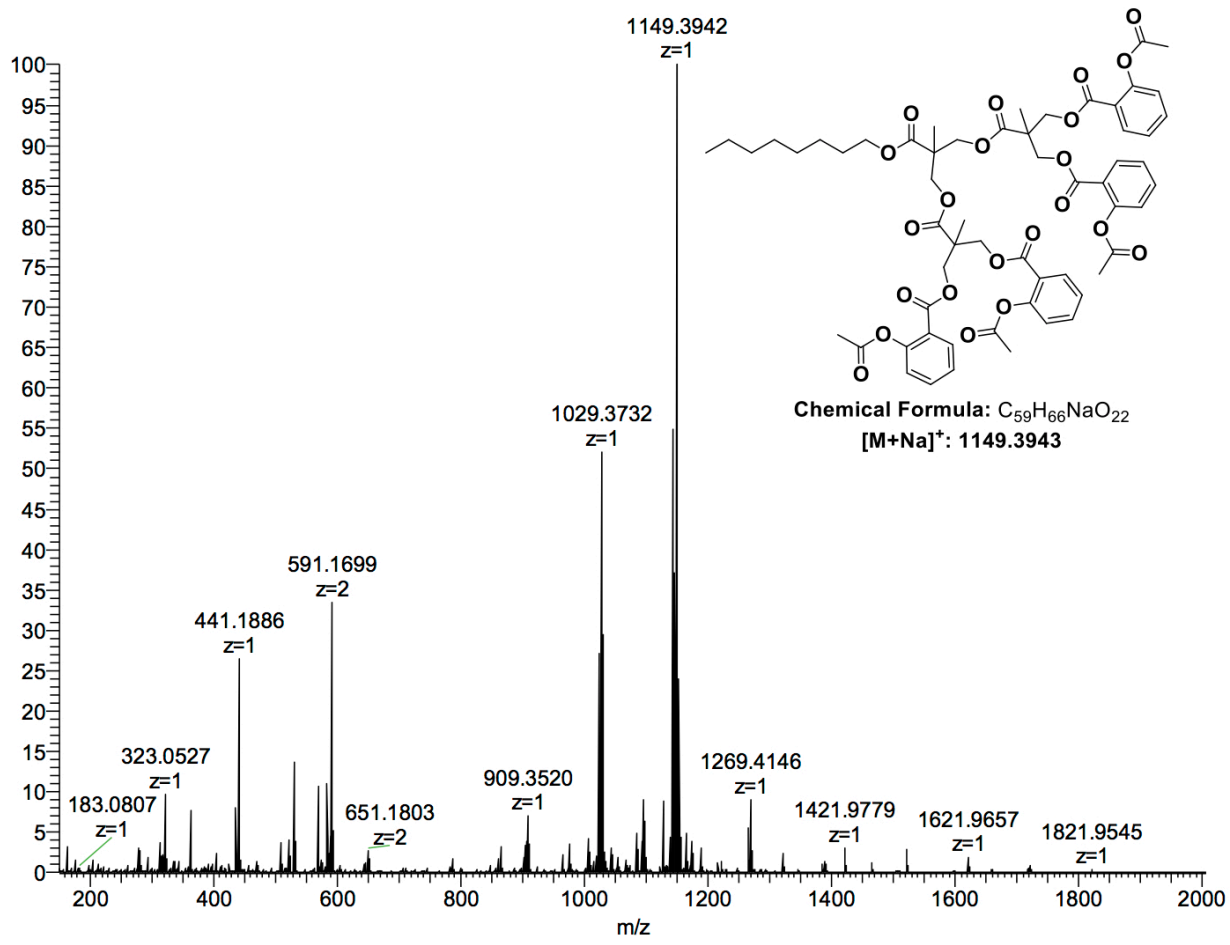


Figure S27. ESI-HRMS of Oc-[G2]-$(Asp)_4$ (**9**) in the positive ion mode.

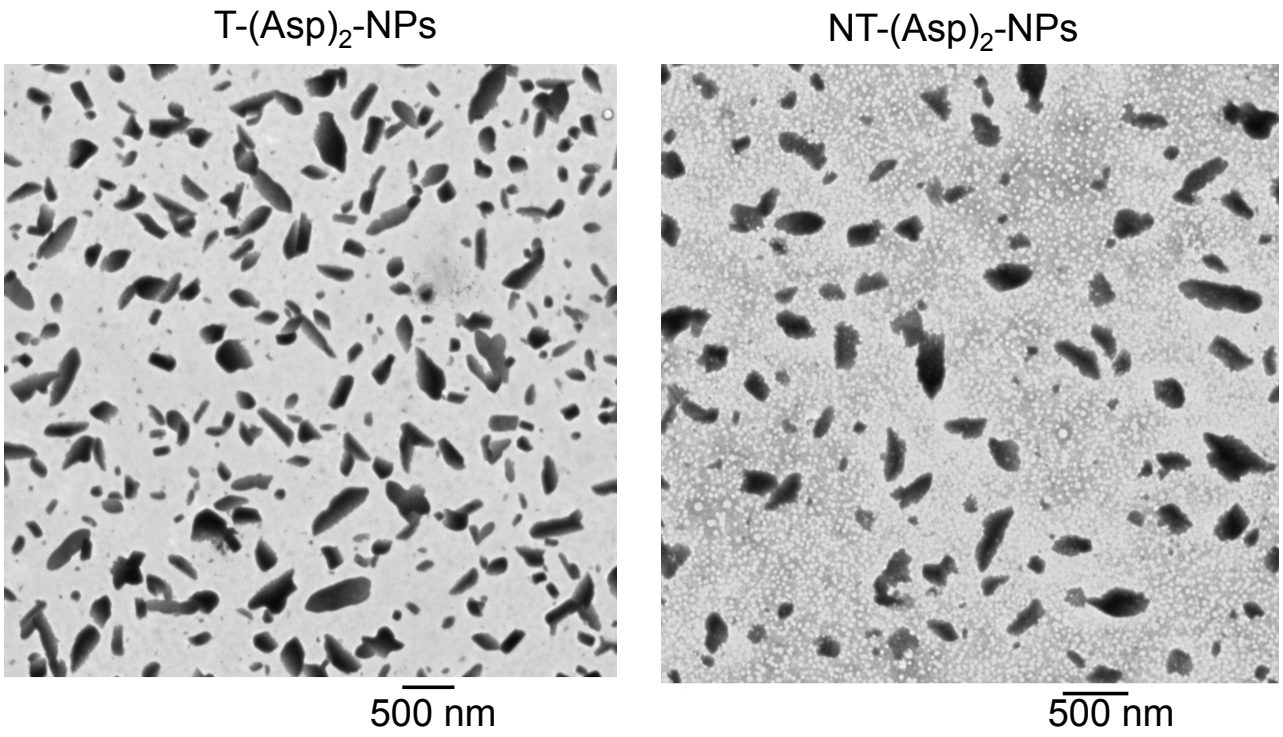


Figure S28. TEM of T-$(Asp)_2$-NPs and NT-$(Asp)_2$-NPs.

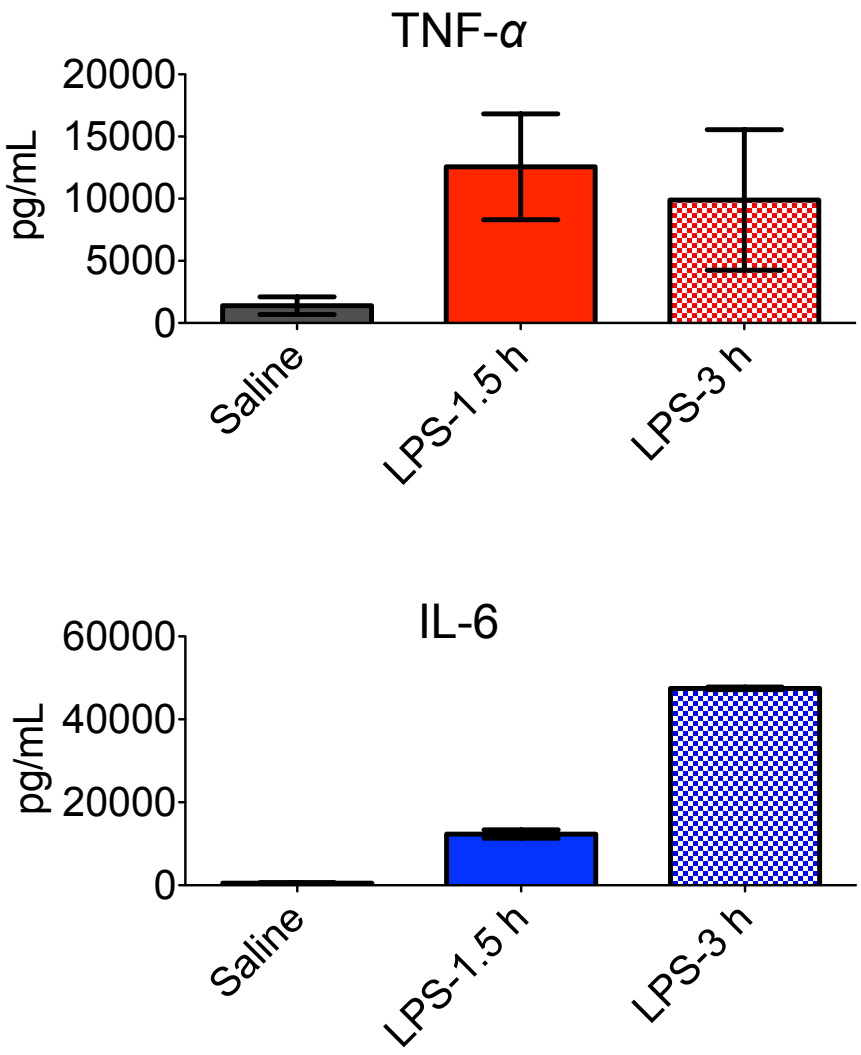


Figure S29. TNF-α (top) and IL-6 (bottom) levels in the serum samples of C57BL/6 mice treated with intraperitoneally administered LPS (100 μg/animal).

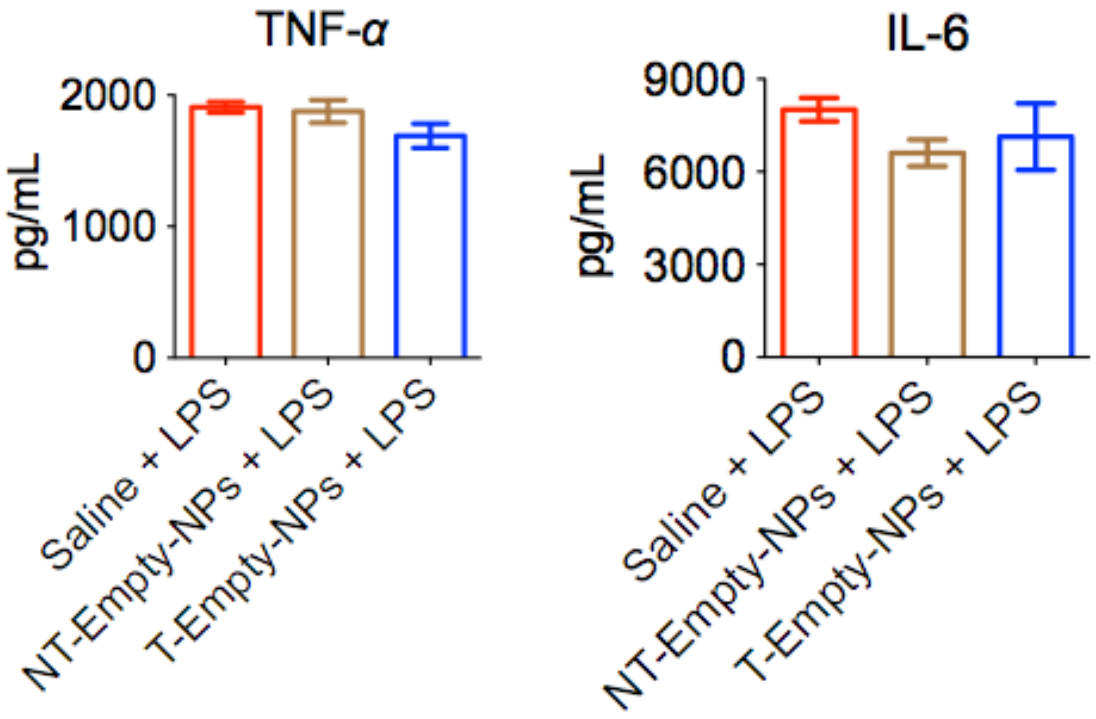


Figure S30. TNF-α and IL-6 levels in the serum samples of BALB/c Albino mice treated with T/NT-Empty-NPs and LPS. [NP]: 200 mg/kg; [LPS]: 100 μg/animal; treatment time with NPs: 12 h; LPS treatment time: 1.5 h.

References:

1. S. Marrache, R. Pathak and S. Dhar, in *Mitochondrial Medicine*, eds. V. Weissig and M. Edeas, Springer New York, 2015, vol. 1265, ch. 8, pp. 103-112.
2. R. K. Pathak, S. Marrache, D. A. Harn and S. Dhar, *ACS Chemical Biology*, 2014, 9, 1178-1187.
3. S. Marrache, J. H. Choi, S. Tundup, D. Zaver, D. A. Harn and S. Dhar, *Integr. Biol.*, 2013, 5, 215-223.
4. S. Marrache, S. Tundup, D. A. Harn and S. Dhar, *ACS Nano*, 2013, DOI: 10.1021/nn403158n, 7392-7402.
5. R. K. Pathak, S. Marrache, J. H. Choi, T. B. Berding and S. Dhar, *Angew. Chem. Int. Ed. Engl.*, 2014, 53, 1963-1967.