Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.

Supporting Information

Amphetamine Decorated Cationic Lipid Nanoparticles Cross Blood-Brain Barrier: Therapeutic Promise for Combating Glioblastoma

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Synthetic procedure

The procedure used for the synthesis of 14-BACL, 16-BACL, and 18-BACL is shown in Scheme 1 and described below.

Steps i-iv: Preparation of intermediates Ia/Ib/Ic: The intermediate Ia (or Ib or Ic) was prepared from ethylene diamine using alkyl halide RBr (R = n-$C_{14}H_{29}$, R = n-$C_{16}H_{33}$ and R = n-$C_{18}H_{37}$). The procedure is reported earlier[36]. Intermediate Ia (or Ib or Ic) (1.25 g, 0.002 mol) was treated with 2 mL TFA: DCM (1:2, v/v) at 0 °C for 3 h. The resulting solution was diluted with 5 mL chloroform & TFA was removed by washing with sat. $NaHCO_3$ (50 mL×3) in a separatory funnel. The collected organic layer was dried over anhydrous Na_2SO_4 and concentrated using rotary evaporator (1.04 g, yield 99%). The concentrated residue (1.04 g, 0.002 mol) was dissolved in 5 mL dry dioxane and added to the ice-cold solution of succinic anhydride (1.26 g/0.0756 mol in dioxane) with vigorous stirring. The Reaction was incubated for 8 h at rt. Upon completion dioxane was removed at 60 °C by rotary evaporator. The mixture was suspended in chloroform and filtered using whatman filter paper. The filtered solution was washed with brine (3 × 50 mL) and organic layer was collected. It was then dried over anhydrous Na_2SO_4 & concentrated. Flash column chromatography (60-120 mesh silica gel) was used to separate the intermediate IIa (or IIb or IIc) from the crude residue. The pure compound was eluted at 10-14% v/v, MeOH-$CHCl_3$, (0.813 g, yield 65%).

^{1}H NMR of IIa (300 MHz, CDCl3): □/ppm = 0.88 (t, 6H), 1.25 (m, 48H), 2.43 (m, 4H), 2.88 (t, 4H), 3.04 (t, 2H), 3.55 (t, 2H)

^{1}H NMR of IIb (300 MHz, CDCl3): □/ppm = 0.88 (t, 6H), 1.25 (m, 56H), 2.48-2.5 (m, 4H), 2.88 (t, 4H), 3.04 (t, 2H), 3.52 (t, 2H)

^{1}H NMR of IIc (300 MHz, CDCl3): □/ppm = 0.88 (t, 6H), 1.25 (m, 64H), 2.42-2.5 (m, 4H), 2.88 (t, 4H), 3.04 (t, 2H), 3.6 (t, 2H)

Step v: Preparation of 2-((tert-butoxycarbonyl)amino)-3-phenylpropanoic acid (III): 5 g of (0.03 mol) *(S)-2-amino-3-phenylpropanoic acid* (*s-phenylalanine)*, was dissolved in 20 mL of aqueous

4N NaOH. After that BOC_2O (13.21 g, 1.210 mol), dissolved in tertiary butanol (10 mL), was introduced in the reaction media and the reaction was carried out for 8 h at rt. Tertiary butanol was evaporated and the residue was dissolved in water (100 mL) and neutralized with sat.$KHSO_4$. The aqueous part was extracted with $CHCl_3$ (3 × 100 mL). The collected organic layer was dried over anhydrous Na_2SO_4 and concentrated to obtain BOC-protected intermediate III (7.62 g, yield ~94%).

^{1}H NMR of III (300 MHz, CDCl3): □/ppm = 1.42 (s, 9H), 2.84 (d, 2H), 3.56 (m, 1H), 7.2-7.32 (m, 5H)

Step vi: Preparation of tert-butyl (1-hydroxy-3-phenylpropane-2-yl) carbamate (IV): $LiAlH_4$ (1.14 g, 0.062 mol) was suspended in 5 mL dry THF and cooled to 0 °C. To it, intermediate III (4 g, 0.015 mol) dissolved in dry THF (20 mL) was added drop wise over a 15 min time period under stirring condition. The reaction mixture was incubated for 2 h, quenched with few drops of sat.Na_2SO_4 and stirred until a white precipitated formed. The residue was separated using G4 sintered funnel and washed thoroughly with ethyl acetate. The filtered solution was concentrated and intermediate IV was separated by column chromatography (60-120 mesh silica gel). The pure compound (intermediate IV) was eluted at 15-18% EtOAc-Hexane (2.34 g, yield 62%).

^{1}H NMR of IV (300 MHz, CDCl3): □/ppm = 1.42 (s, 9H), 2.85 (d, 2H), 3.56 (m, 1H), 3.68 (d, 2H) 7.2-7.32 (m, 5H)

ESI-MS of IV: 252 [M+H]+, 274 [M+Na]+ for $C_{14}H_{21}NO_3$

Step vii: Preparation of 2-((tert-butoxycarbonyl)amino)-3-phenylpropyl 4-methylbenzenesulfonate (V): Intermediate IV (1.464 g, 0.005 mol) and 1.2 mL of Et_3N (0.88 g, 0.013 mol) were taken in 15 mL of dry DCM and allowed to cool at 0 °C in an ice bath. After that, TsCl (1.66 g, 0.013 mol) dissolved in dry DCM (10 mL) was added slowly. The reaction was monitored for 4 h at rt. The DCM was evaporated from the reaction mixture and purified by column chromatography (60-120 mesh silica gel). The pure compound (intermediate V) was eluted at 3-5% EtOAc-Hexane. (1.7 g, yield 76%).

^{1}H NMR of V (300 MHz, CDCl3): □/ppm = 1.39 (s, 9H), 2.46 (s, 3H), 2.74-2.89 (m, 2H), 3.87-4.00 (m, 3H) 7.08 (m, 3H), 7.21 (m, 2H) 7.35 (d, 2H), 7.77 (d, 2H)

ESI-MS of V: 306 [M-100]+, 350 [M-56]+, 406 [M]+, 428 [M+Na]+ for $C_{21}H_{27}NO_5S$

Step viii: Preparation of tert-butyl (1-phenylpropane-2-yl)carbamate (VI): $LiAlH_4$ (0.5 g, 0.039 mol) suspended in 5 mL dry THF was cooled to 0 °C in an ice bath. After 15 min, intermediate V (1.7 g, 0.004 mol) dissolved in dry THF (10 mL) was added drop wise over a 15 min time period under stirring condition. The reaction was left for 0.5 h at 0 °C. After that the reaction mixture was quenched with a few drops of sat.Na_2SO_4 and stirred until a white precipitated observed. The residue was filtered using G4 sintered funnel and washed several times with ethyl acetate. The filtered ethyl acetate extract was collected, concentrated and subjected to column chromatography (60-120 mesh silica gel). The pure compound (intermediate VI) was eluted at 3% EtOAc-Hexane. (0.66 g, yield 66%)

^1H NMR of VI (300 MHz, $CDCl_3$): □/ppm = 1.09 (d, 3H), 1.42 (s, 9H), 2.66-2.80 (m, 2H), 3.91 (m, 1H), 7.17-7.30 (m, 5H)

ESI-MS of VI: 236 $[M+1]^+$, 258 $[M+Na]^+$ for $C_{14}H_{21}NO_2$

Step ix and x: In step ix, the BOC group was removed from the intermediate VI (0.1 g, 0.43 mmol) by treating with 2 mL TFA: DCM (1:2, v/v) mixture at 0 °C for 2 h of stirring. TFA was eliminated by the nitrogen gas flashing then the reaction mixture was diluted with $CHCl_3$ and washed with sat.$NaHCO_3$ (3 × 10 mL) followed by brine (3 × 10 mL). The organic layer was collected, dried over anhy.Na_2SO_4 and evaporated to dryness on rotary evaporator. The concentrated amine intermediate VII (0.056 g, yield 99%) was immediately used in further step x. Intermediate IIa (or IIb or IIc) was (0.308 g, 0.6 mmol) solubilized in 2mL DCM and allowed to cool at 0 °C. 0.192 g (0.6 mmol) of HBTU was added to it. After 15 min of stirring, intermediate VII (0.056 g, 0.42 mmol) obtained in step ix (dissolved in 2 mL DCM) was added slowly into the above reaction at 0 °C. 72 µL DIPEA (0.065 g, 0.6 mmol) was also added to the reaction mixture drop wise to make the solution basic to litmus paper (~pH 9). The reaction was left stirring for 12 h at rt under N_2 atmosphere. After that the reaction was diluted with 20 mL of $CHCl_3$ & washed sequentially with 1N HCl (3 × 50 mL), sat.aqueous $NaHCO_3$ (3 × 50 mL) and saturated aqueous NaCl solution (3 × 50 mL). The collected organic fraction was dried using anhy.Na_2SO_4 & concentrated. Column chromatography (60-120 mesh silica gel) was used to purify the resultant product. The pure

compound intermediate VIIIa (or VIIIb or VIIIc) was eluted at 8% EtOAc-Hexane (v/v). (0.125 g, yield 41%).

Step xi and xii: The intermediate VIIIa (or VIIIb or VIIIc) (0.125 g, 0.00017 mol) was solubilized in 0.5 mL DCM and 5 equivalents of CH_3I (0.054 mL, 1.90 mmol), K_2CO_3 (0.047 g, 0.68 mmol) were added. The reaction was continued for 48 h at rt. The reaction mixture was concentrated and the residue was dissolved in 3 mL of chloroform. Until a white precipitate separated n-Pentane was added to the mixture. The white precipitate was collected, and dried. Amberlyst IR-26 Cl^- resin was used in the chloride ion exchange chromatography for getting pure **14-BACL (**or **16-BACL** or **18-BACL)** (0.124 g, yield 93%).

^{1}H NMR of **14-BACL** (300 MHz, CDCl3): □/ppm = 0.88 (t, 6H), 1.08 (d, 3H), 1.25 (m, 44H), 1.67 (m, 4H) 2.50-2.90(m, 6H), 3.24 (s, 3H), 3.35-3.5 (m, 4H), 3.64-3.71 (m, 4H), 4.15 (m, 1H) 7.19-7.28 (m, 5H)

ESI-MS of **14-BACL**: 685 [M]+ for $C_{44}H_{82}N_3O_2$+

^{1}H NMR of **16-BACL** (300 MHz, $CDCl_3$): □/ppm = 0.89 (t, 6H), 1.1 (d, 3H), 1.25-1.40 (m, 52H), 1.70 (m, 4H) 2.52-2.84 (m, 6H), 3.26 (s, 3H), 3.35 (m, 4H), 3.56-3.74 (m, 4H), 4.20 (m, 1H) 7.19-7.30 (m, 5H).

ESI-MS of **16-BACL**: 741 [M]+ for $C_{48}H_{90}N_3O_2^+$

1H NMR of **18-BACL** (300 MHz, CDCl3): □/ppm = 0.89 (t, 6H), 1.09 (d, 3H), 1.25-1.4 (m, 60H), 1.69 (m, 4H) 2.51-2.89 (m, 6H), 3.25 (s, 3H), 3.36 (m, 4H), 3.66-3.73 (m, 4H), 4.18 (m, 1H) 7.18-7.28 (m, 5H)

ESI-MS of **18-BACL**: 797 [M]+ for $C_{52}H_{98}N_3O_2^+$

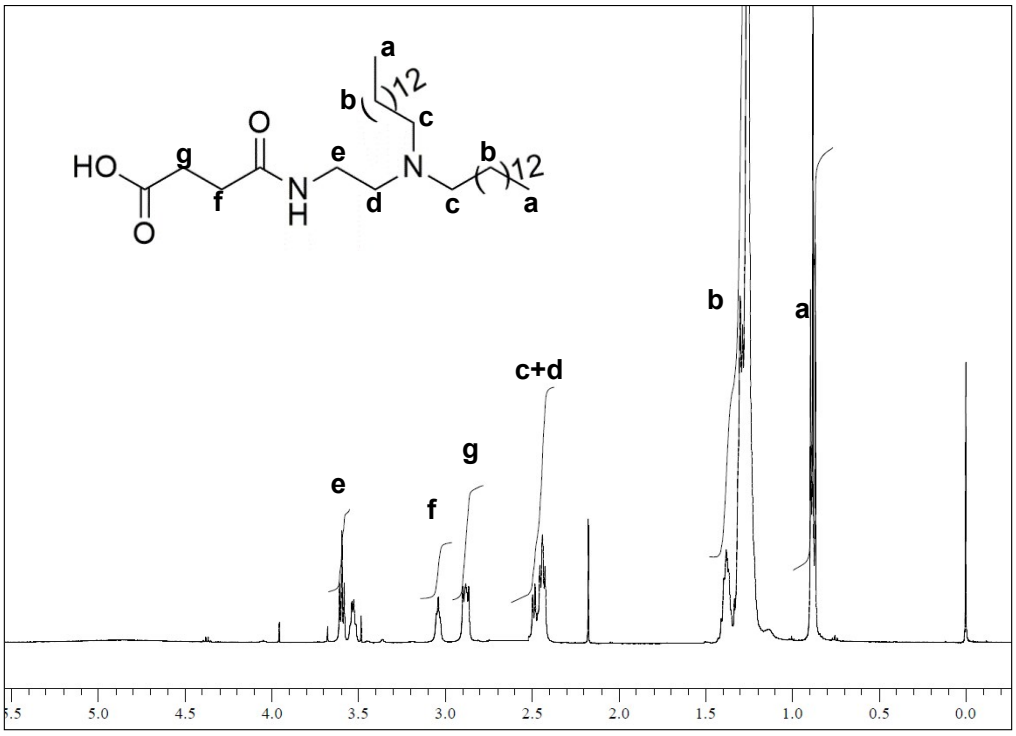


Fig. S1. ^{1}H NMR of intermediate IIa

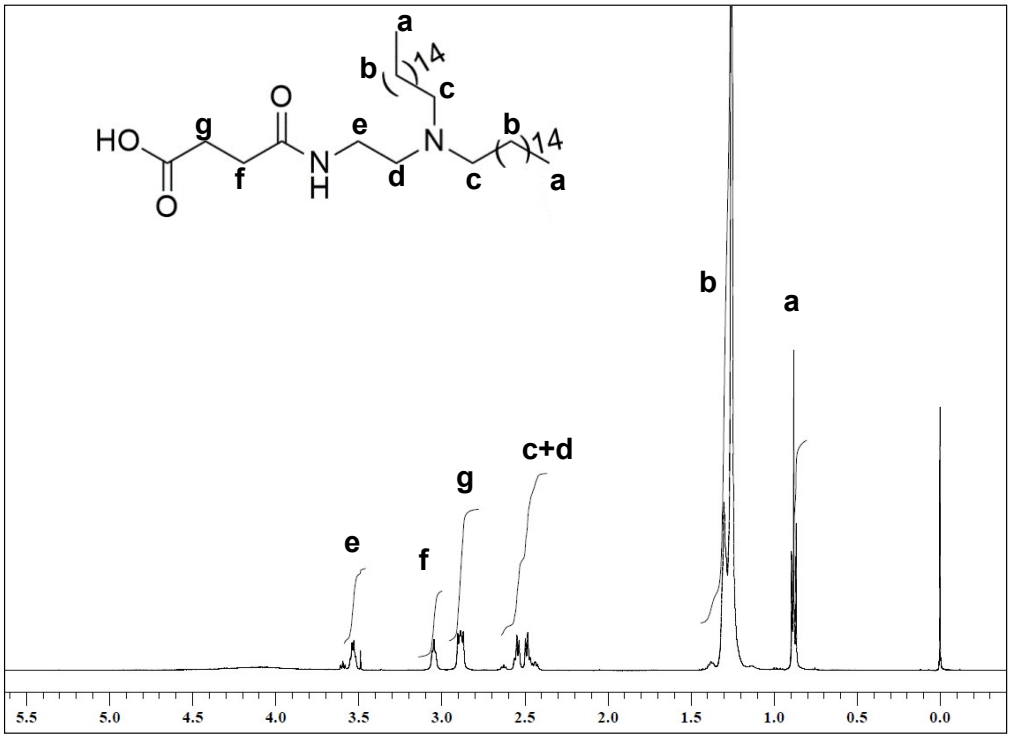


Fig. S2. ^{1}H NMR of intermediate IIb

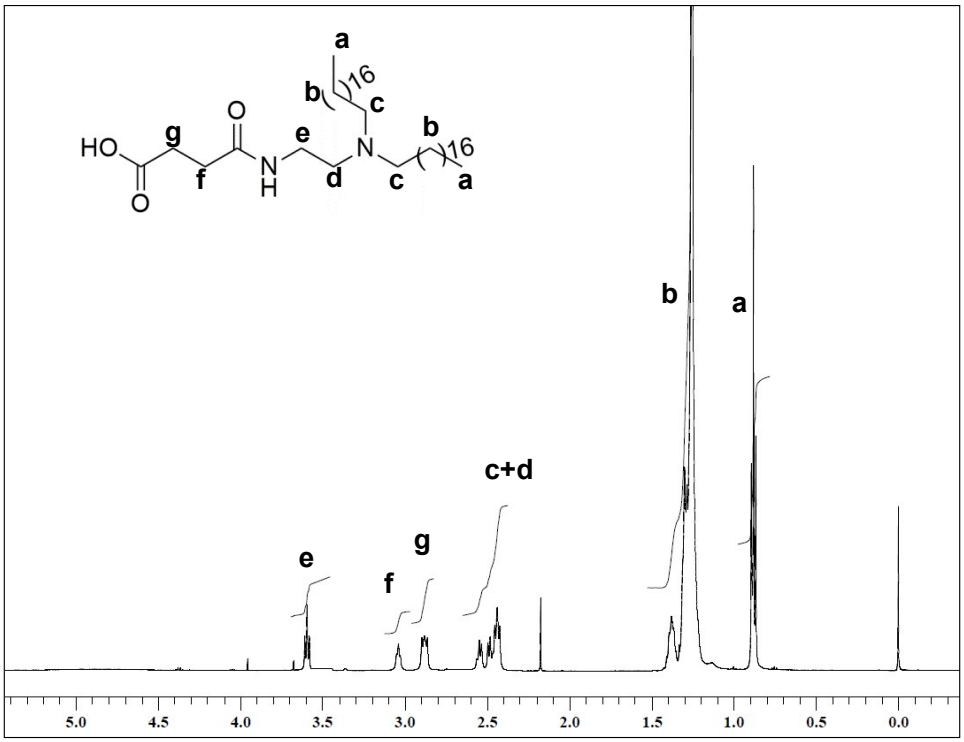


Fig. S3. ^{1}H NMR of intermediate IIc

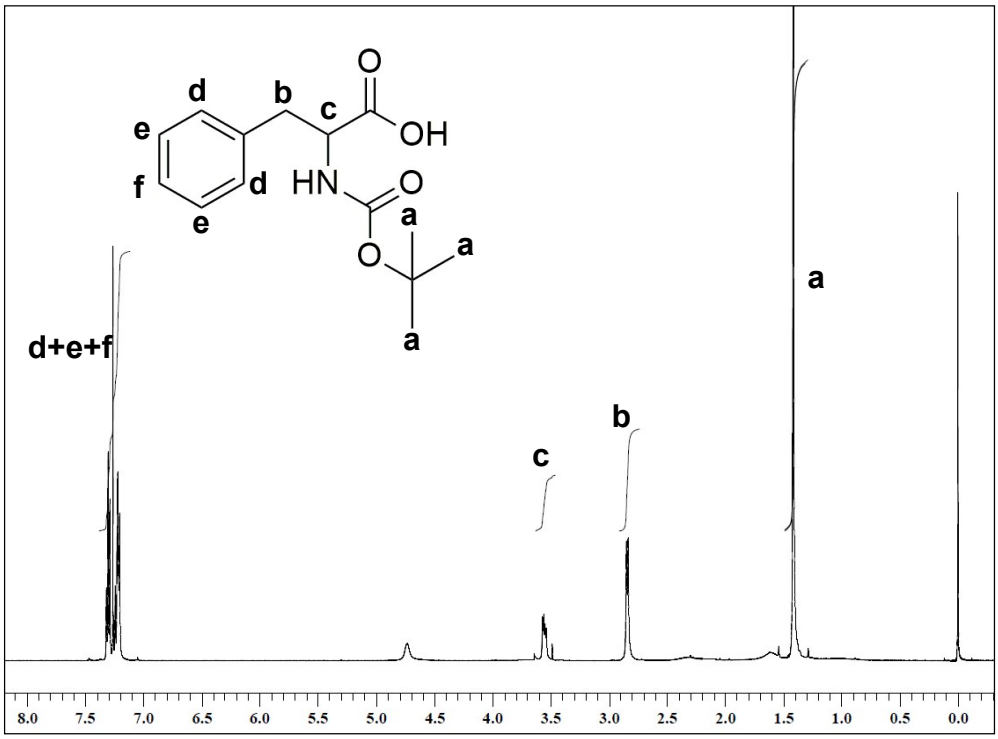


Fig. S4. ^{1}H NMR of intermediate III

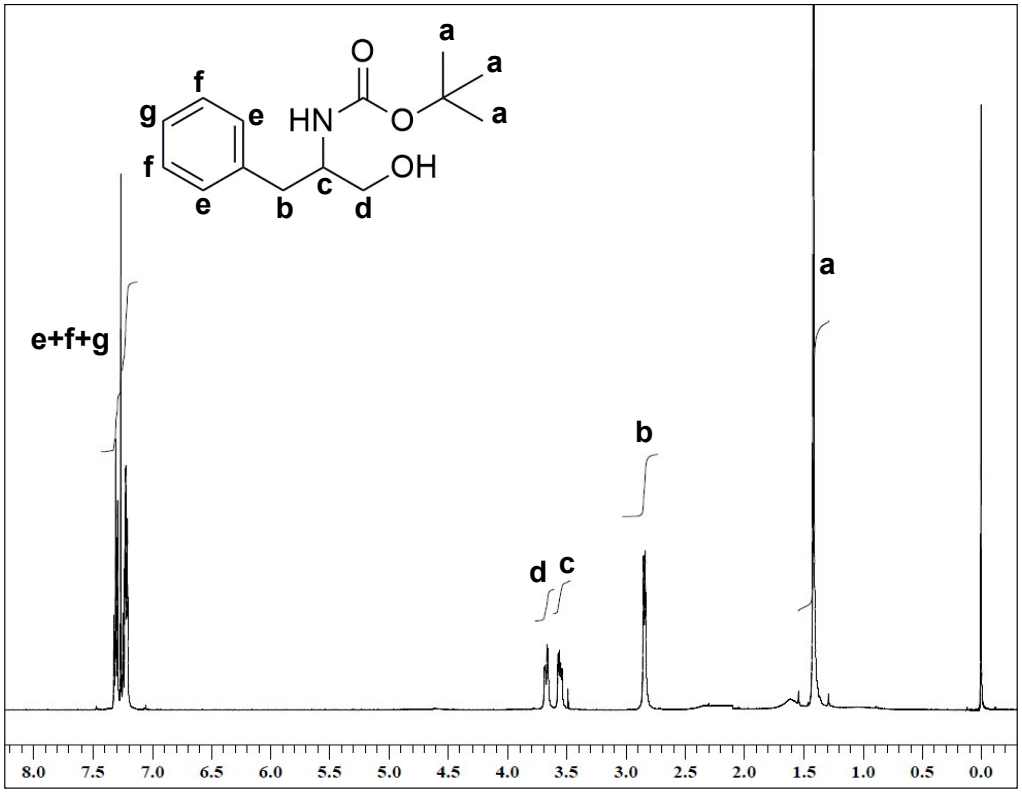


Fig. S5. ^{1}H NMR of intermediate IV

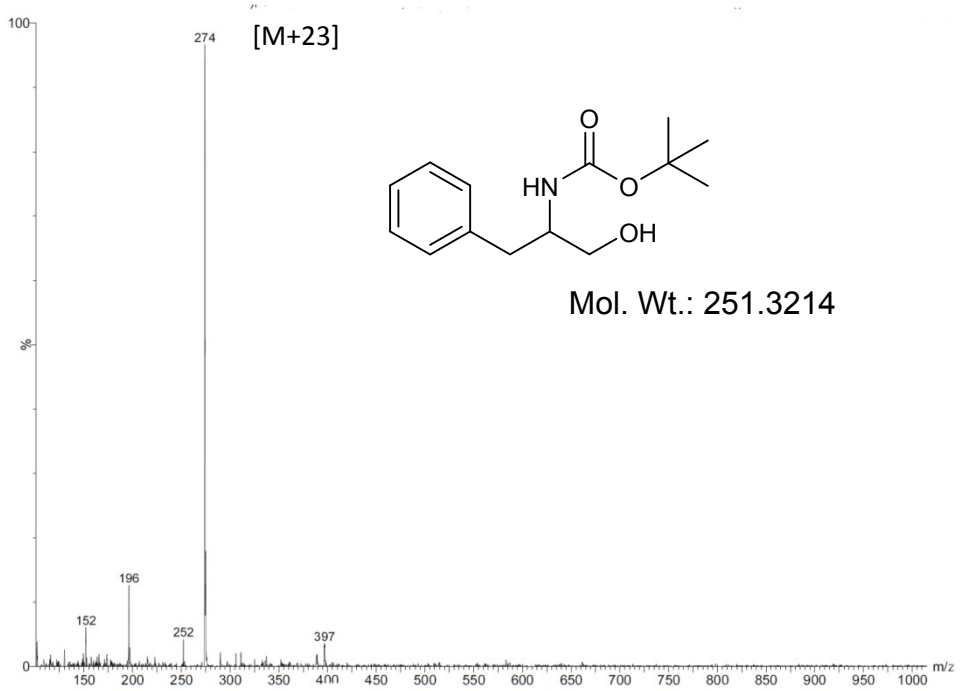


Fig. S6. ESI-MS of intermediate IV

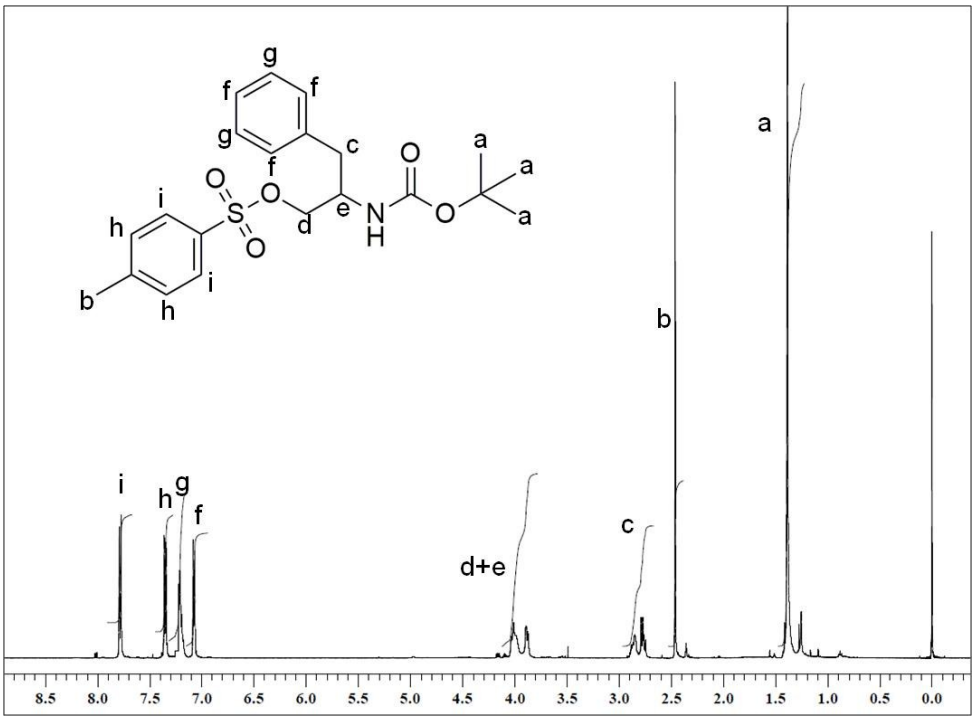


Fig. S7. ^{1}H NMR of intermediate V

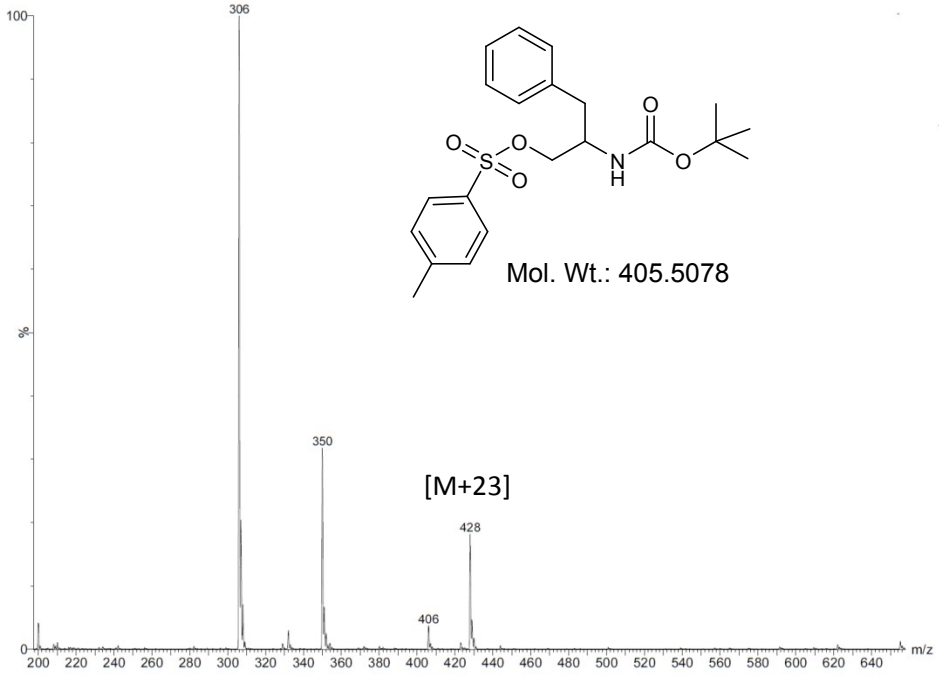


Fig. S8. ESI-MS of intermediate V

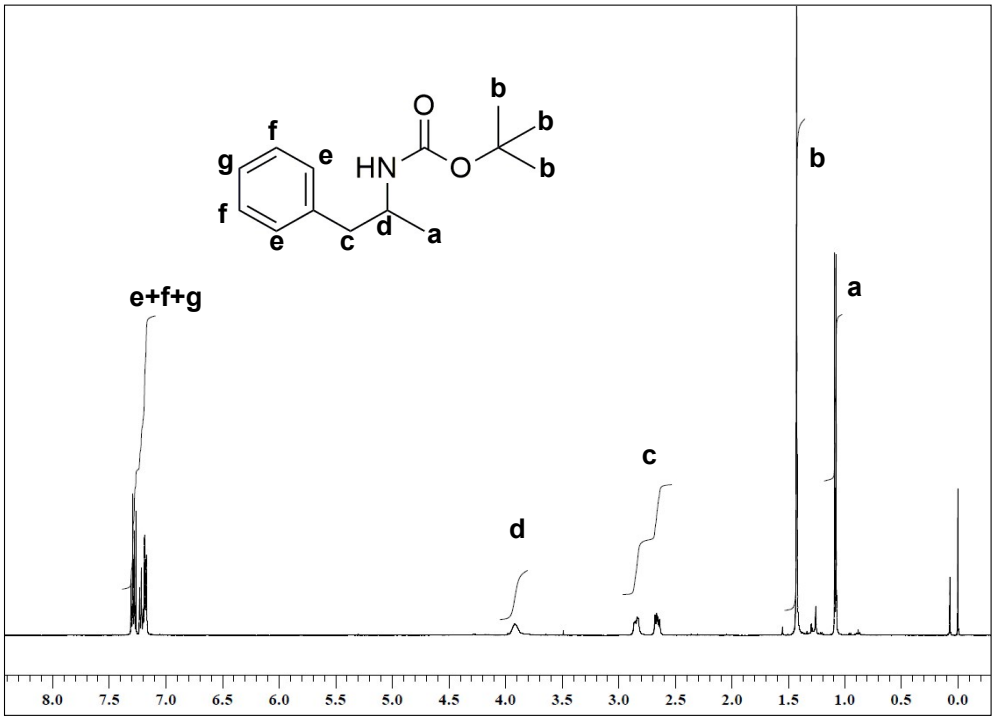


Fig. S9. ^{1}H NMR of intermediate VI

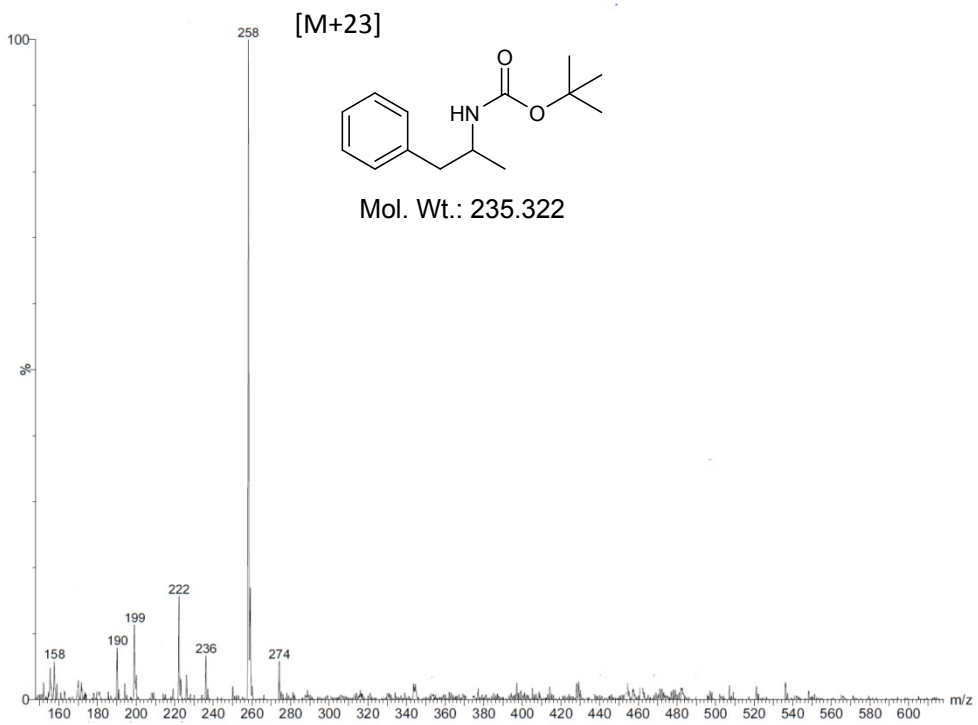


Fig. S10. ESI-MS of intermediate VI

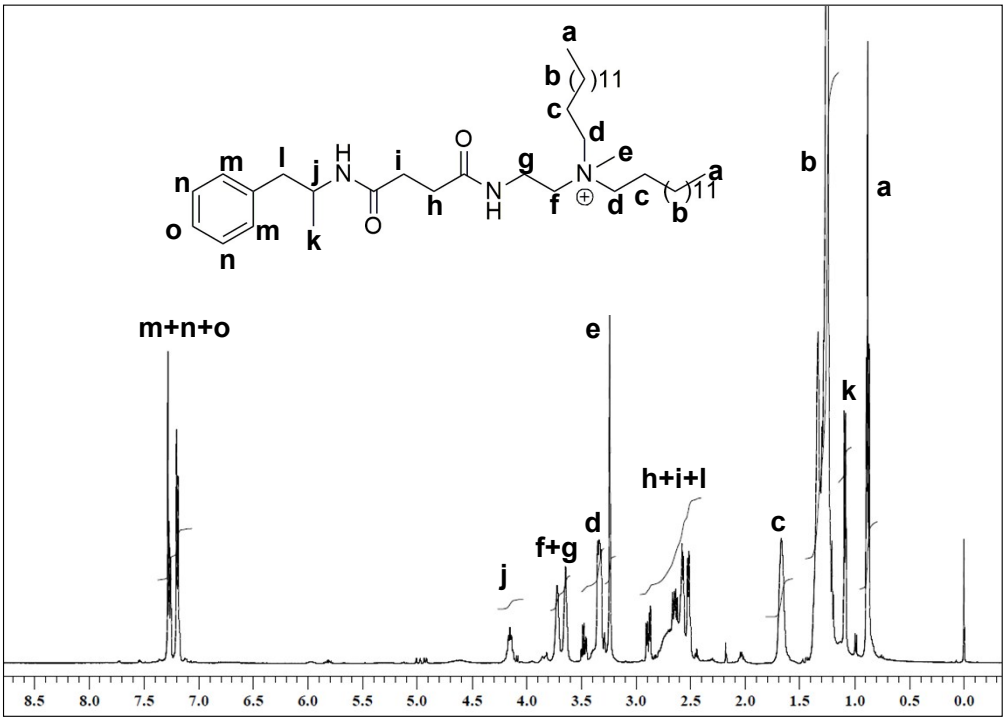


Fig. S11. ^{1}H NMR of intermediate 14-BACL

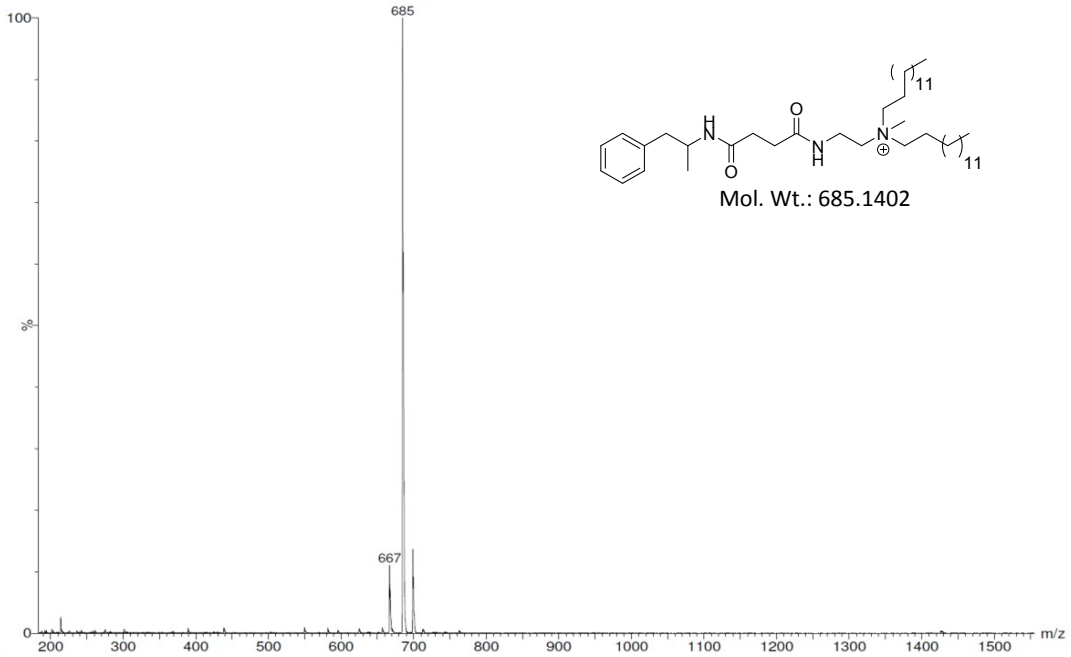
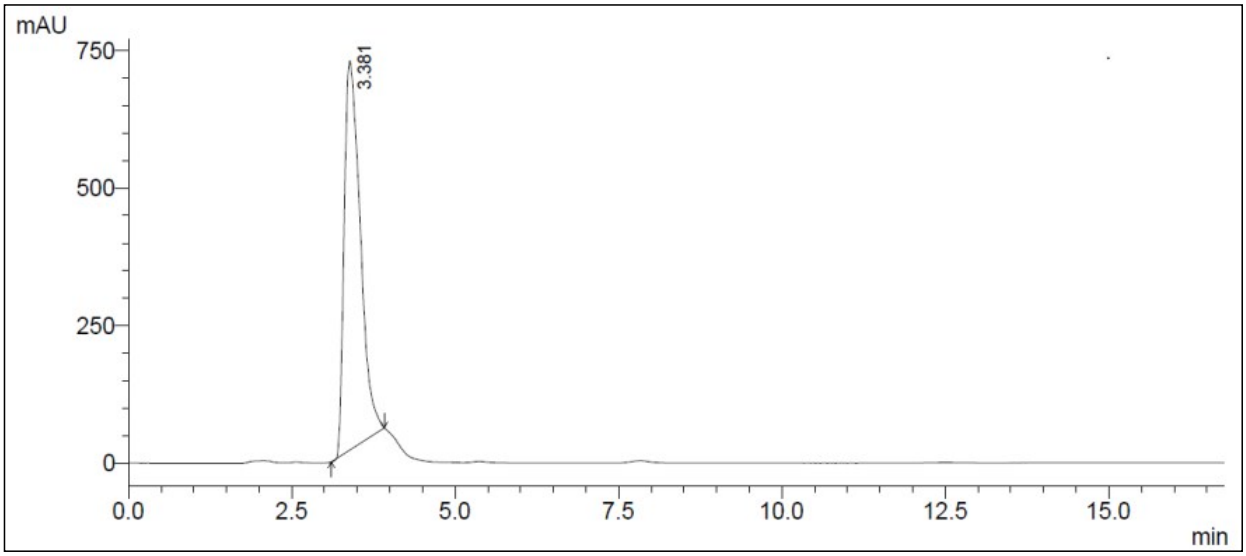


Fig. S12. ESI-MS of intermediate 14-BACL

A



B

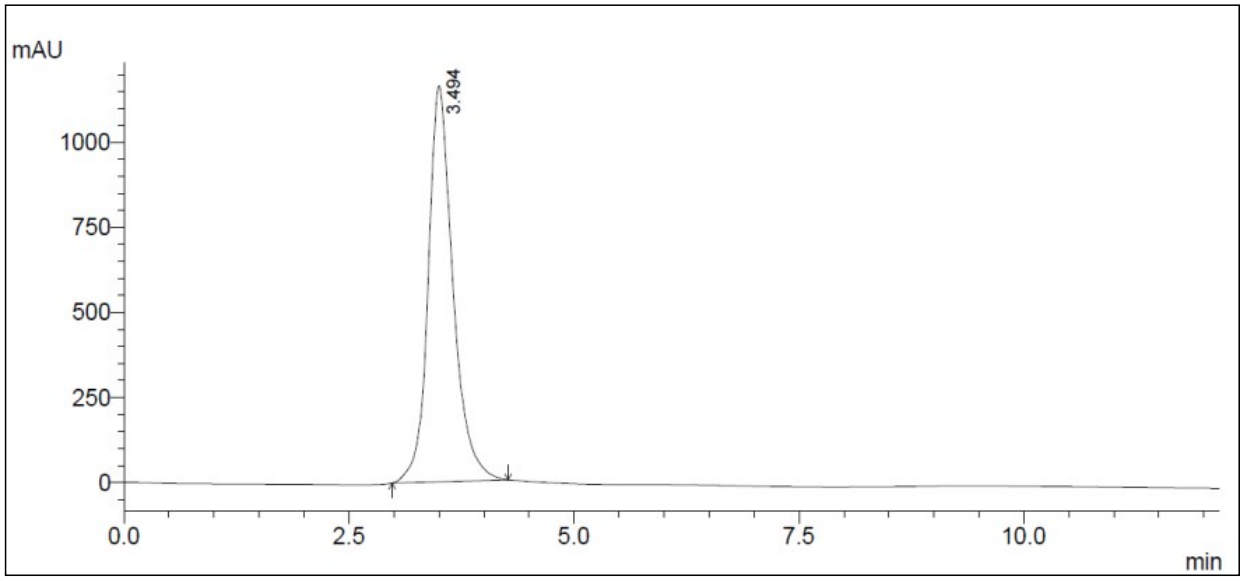


Fig. S13. HPLC chromatogram of 14-BACL in methanol (A) and 95:5, (v/v) water:methanol (B).

HPLC Conditions:

System: Varian Prostar series

Column: Lichrospher® 100, RP-18e (5 µm)

Mobile Phase: Methanol (A); Methanol:Water, 95:5, v/v, (B). Flow Rate: 1.0 mL/min, Typical Column Pressure: 60-65 Bars, Detection: UV at 210 nm

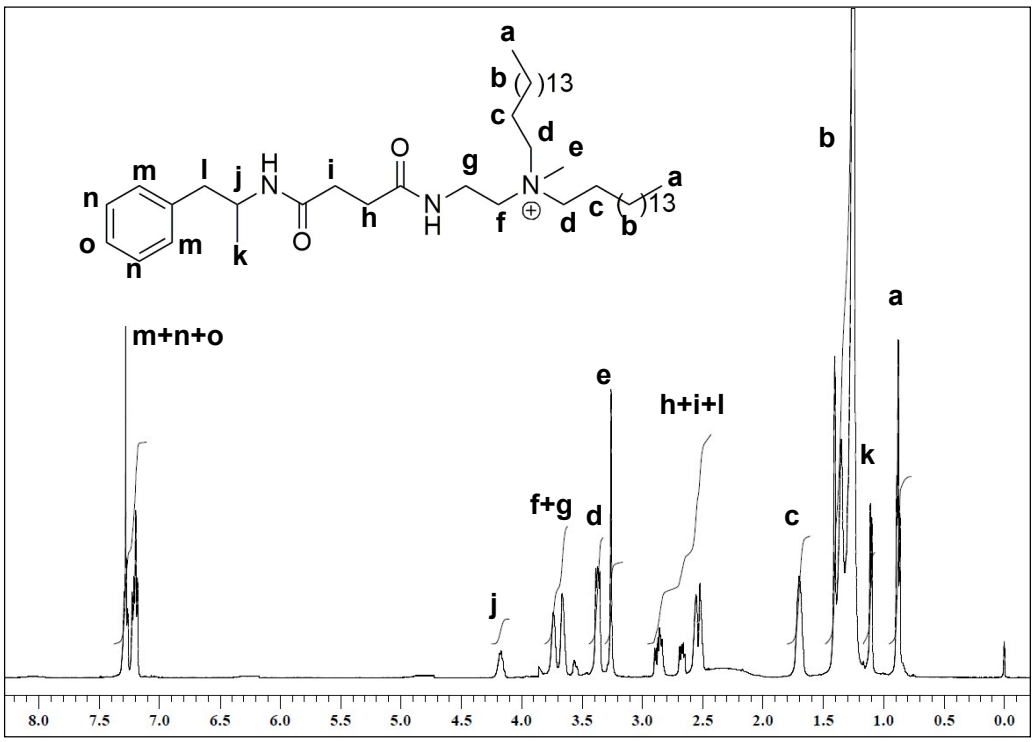


Fig. S14. ^{1}H NMR of intermediate 16-BACL

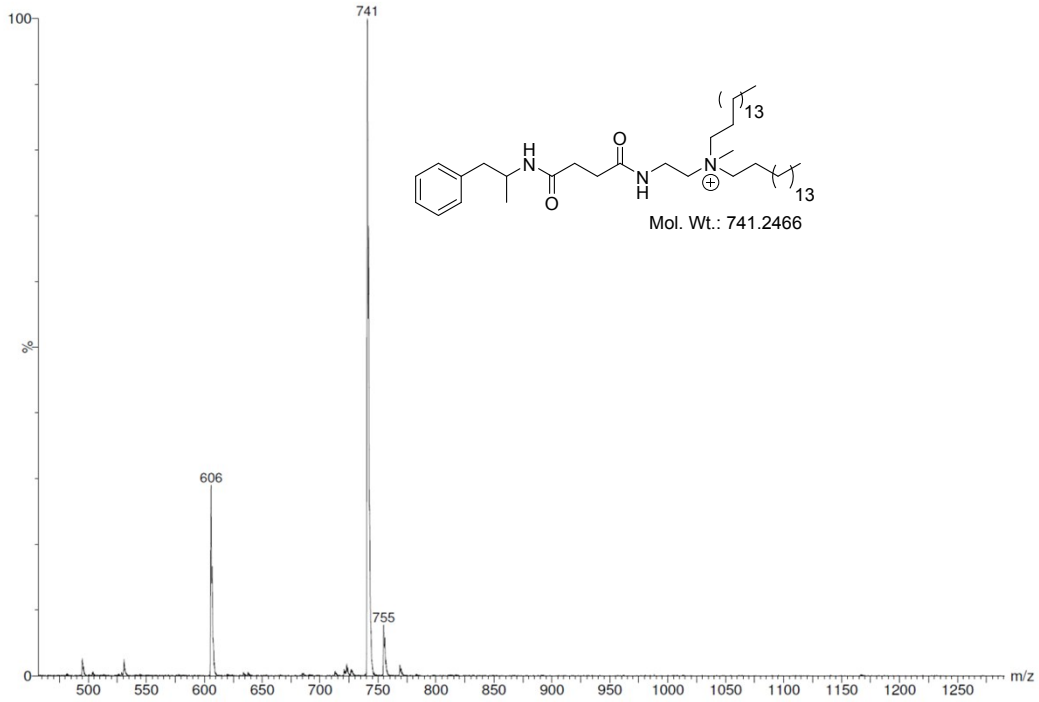
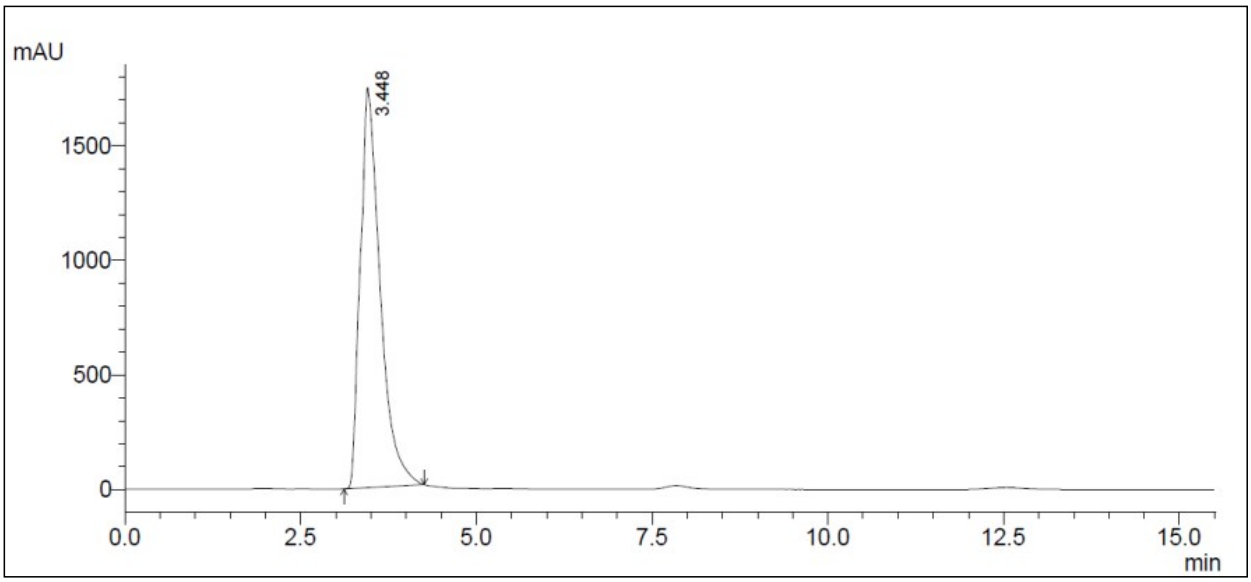


Fig. S15. ESI-MS of intermediate 16-BACL

A



B

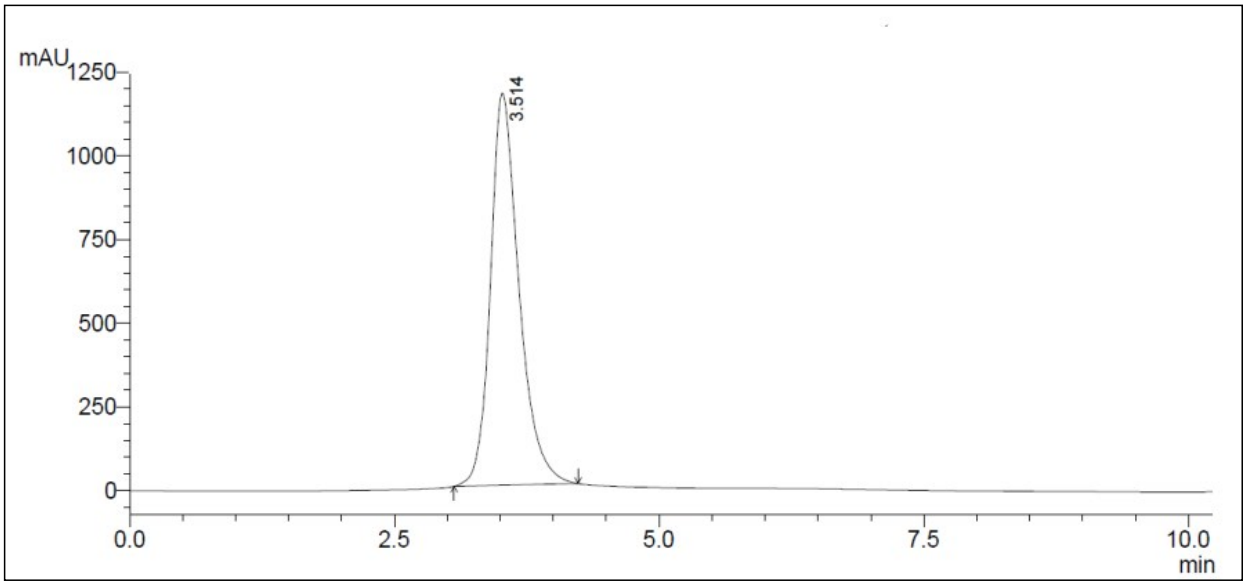


Fig. S16. HPLC chromatogram of 16-BACL in methanol (A) and 95:5, (v/v) water:methanol (B).

HPLC Conditions:

System: Varian Prostar series, Column: Lichrospher® 100, RP-18e (5 µm), Mobile Phase: Methanol (A); Methanol:Water, 95:5, v/v, (B). Flow Rate: 1.0 mL/min, Typical Column Pressure: 60-65 Bars, Detection: UV at 210 nm

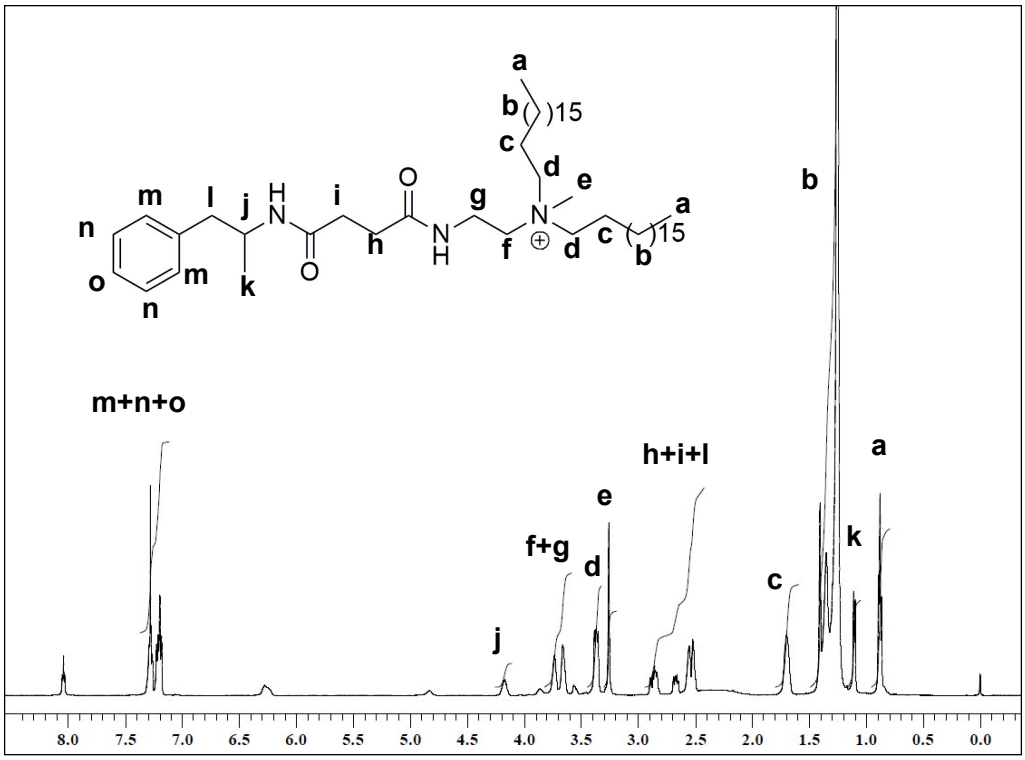


Fig. S17. ^{1}H NMR of intermediate 18-BACL

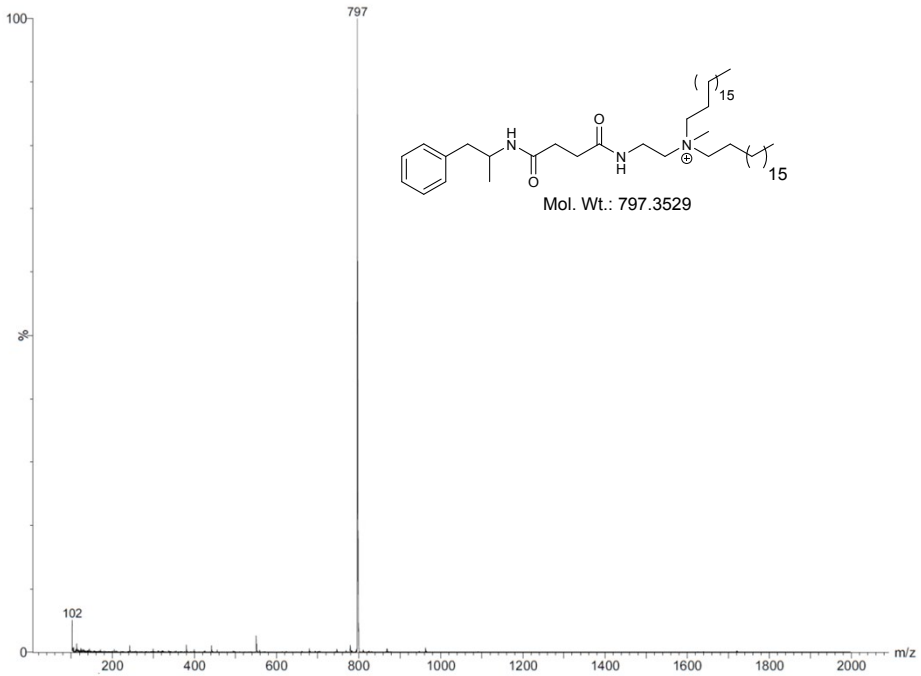
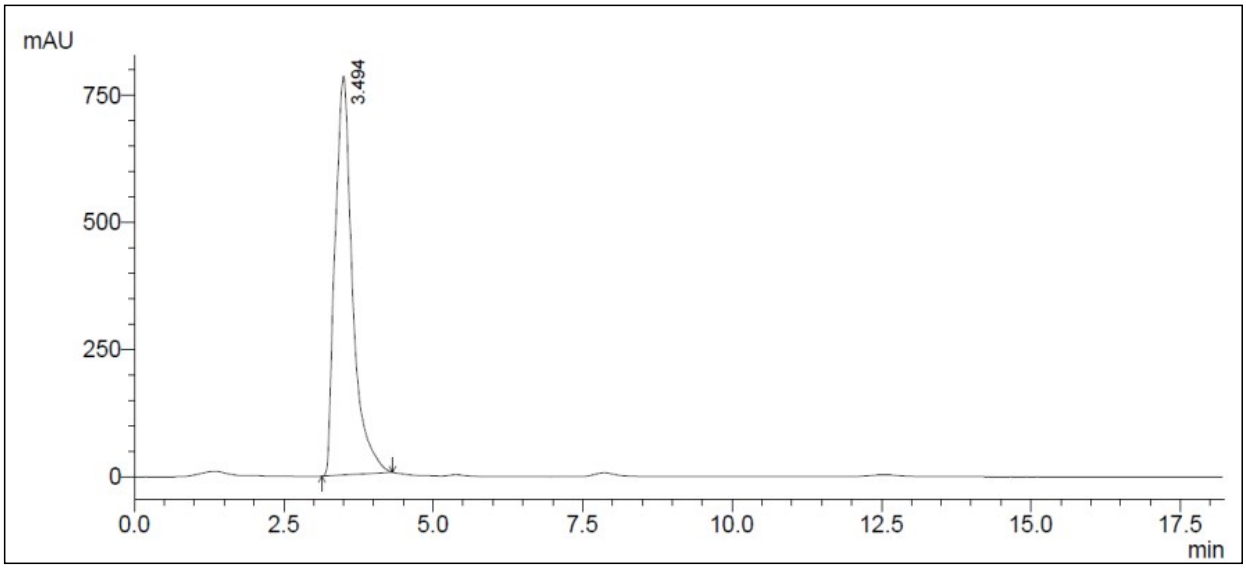


Fig. S18. ESI-MS of intermediate 18-BACL

A



B

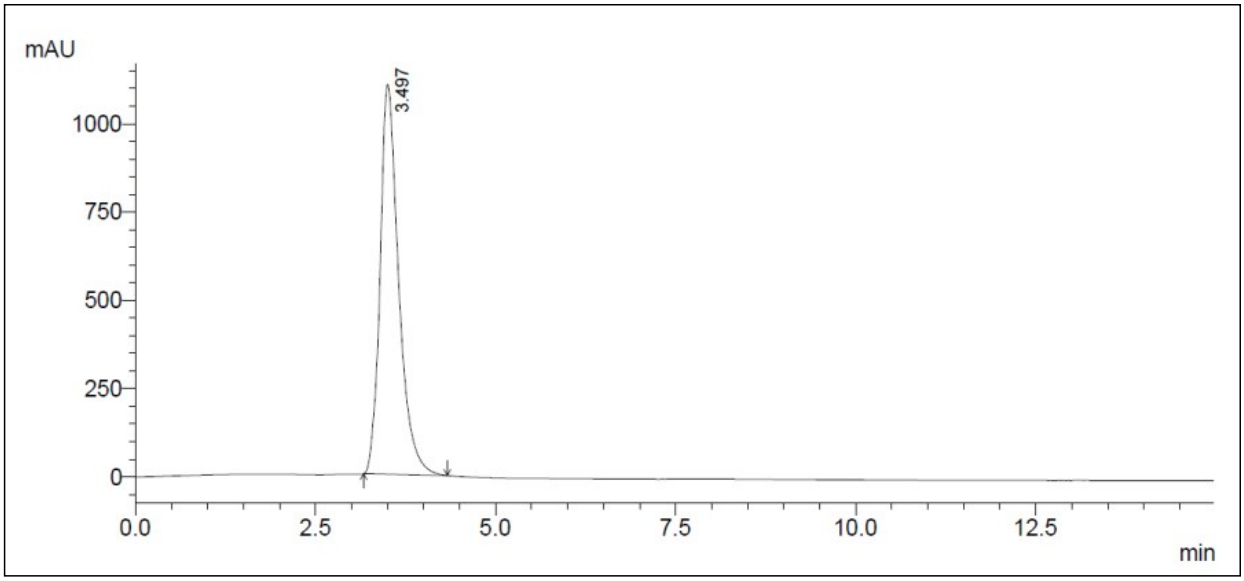


Fig. S19. HPLC chromatogram of 18-BACL in methanol (A) and 95:5, (v/v) water:methanol (B).

HPLC Conditions:

System: Varian Prostar series

Column: Lichrospher® 100, RP-18e (5 µm), Mobile Phase: Methanol (A); Methanol:Water, 95:5, v/v, (B). Flow Rate: 1.0 mL/min, Typical Column Pressure: 60-65 Bars, Detection: UV at 210 nm

Table S1. Physicochemical properties (size and zeta potentials) of the lipid nanoparticles.

Lipid nano-formulations	Size (nm)	Zeta potential (mV)
14-BACL	67.1 ± 6.3	8.7 ± 4.3
16-BACL	60.2 ± 7	13 ± 4.4
18-BACL	64.6 ± 4.2	14.6 ± 2.3

Table S2. Physicochemical properties of the optimized lipid nanoparticles.

Lipid nano-formulations	Size(nm)	Polydispersity index (%)	Zeta potential (mV)
Empty lipid nanoparticle of 16-BACL	176.5 ± 6.6	17.4 ± 1.6	18.7 ± 1.3
PTX-loaded lipid nanoparticle of 16-BACL	179.1 ± 3.3	13.3 ± 0.4	14.2 ± 1.1
FAM-siRNA-loaded lipid nanoparticle of 16-BACL	182 ± 6.7	23.1 ± 0.8	9.7 ± 1.1
PTX & FAM-siRNA co-loaded lipid nanoparticle of 16-BACL	187.6 ± 5.4	19.6 ± 2.4	6.4 ± 2.9
Empty lipid nanoparticle of NT lipids	185.1 ± 3.6	22.1 ± 1.3	18 ± 1.5
PTX-loaded lipid nanoparticle of NT lipids	189.9 ± 3.1	15.6 ± 1.2	14.2 ± 0.4
FAM-siRNA-loaded lipid nanoparticle of NT lipids	187.4 ± 4.8	17.8 ± 0.6	11.8 ± 0.7
PTX & FAM-siRNA co-loaded lipid nanoparticle of NT lipids	193.6 ± 5.2	22.1 ± 1.4	6.6 ± 3.2

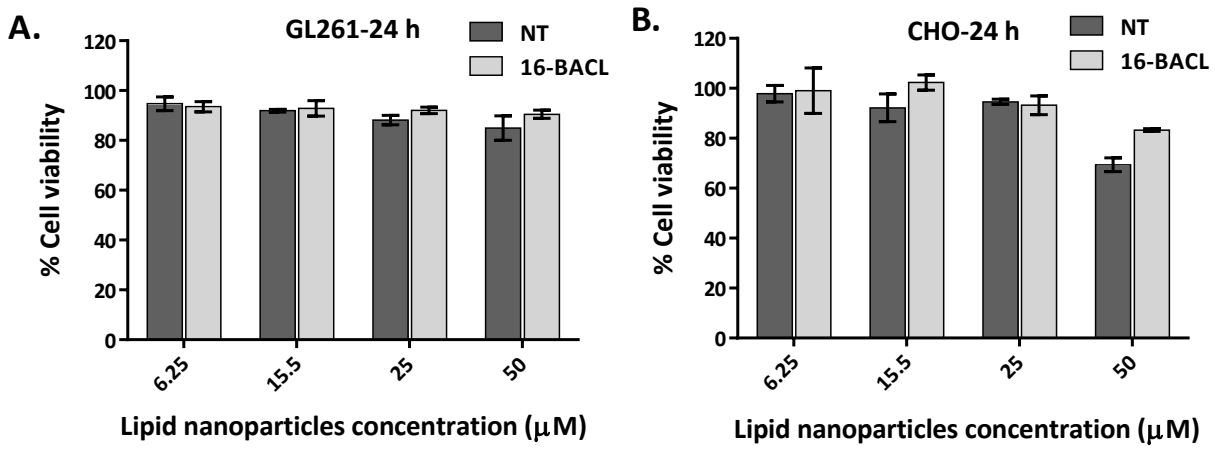


Figure S20. Cytotoxicity of various empty lipid nanoparticle formulations as measured in non-cancerous Chinese Hamster Ovary (CHO, A) and glioblastoma (GL261, B) cells. Cells were treated with increasing concentrations (6.25-50 μM) of empty lipid nanoparticles of 16-BACL and non-targeting (NT) control lipid nanoparticles for 24 h. Percentages of cell viabilities were measured by MTT assay. The percent cell viabilities shown are the average values for triplicate treatments performed on the same day.

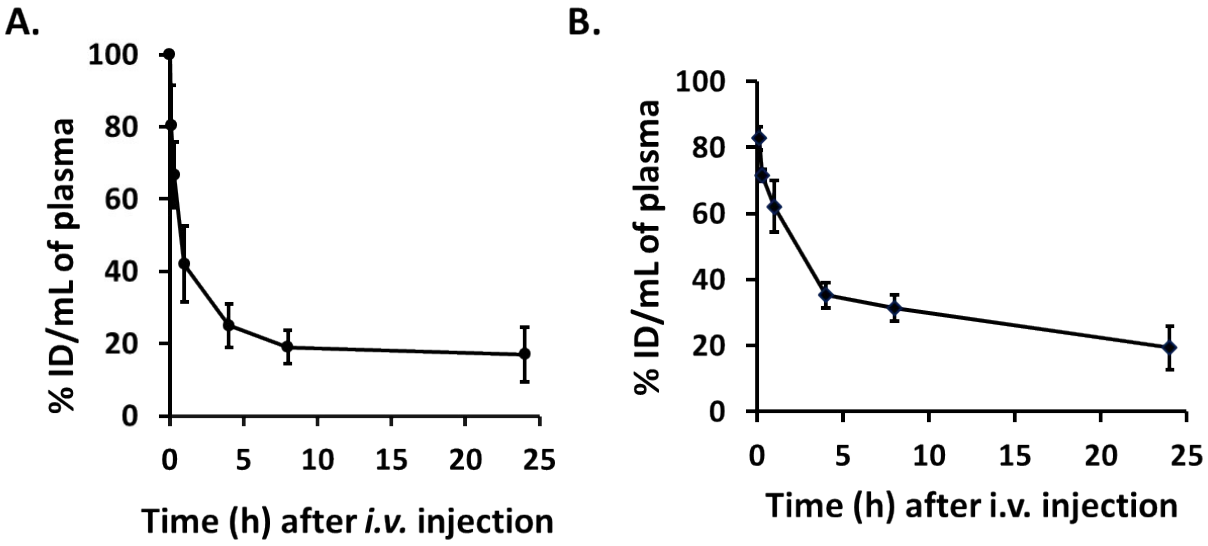


Figure S21. In vivo siRNA and paclitaxel release study. In vivo Plasma stability profile of FAM-siRNA-loaded (A) or paclitaxel (B) loaded lipid nanoparticles of 16-BACL i.v. administered into mice (n=3) were monitored by measuring fluorescence intensities (for FAM siRNA) or LC-MS (for PTX) of blood samples collected by retro orbital puncture from tumor bearing mice after 0.10, 0.30, 1, 4, 8 and 24 h post i.v. Injections.

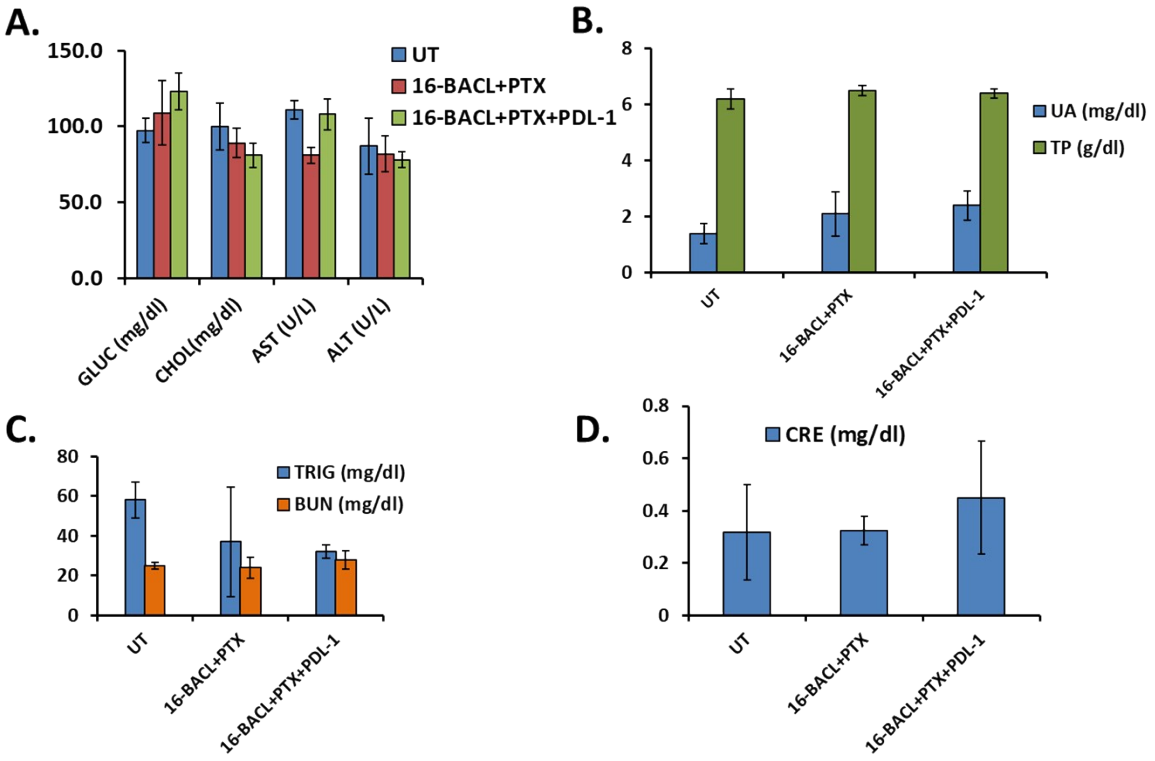


Figure S22. *In vivo* toxicity (biochemical parameters) of paclitaxel loaded and paclitaxel & PDL-1siRNA co-loaded lipid nanoparticles of 16-BACL. Serum analysis were conducted to measure total glucose (GLUC), cholesterol(CHOL), aspartate aminotransferase(AST), alanine amino transferase(ALT), uric acid (UA), total protein (TP), triglycerides (TRIG), blood urea nitrogen (BUN). creatinine (CRE). Data were presented as mean ± SDEV.

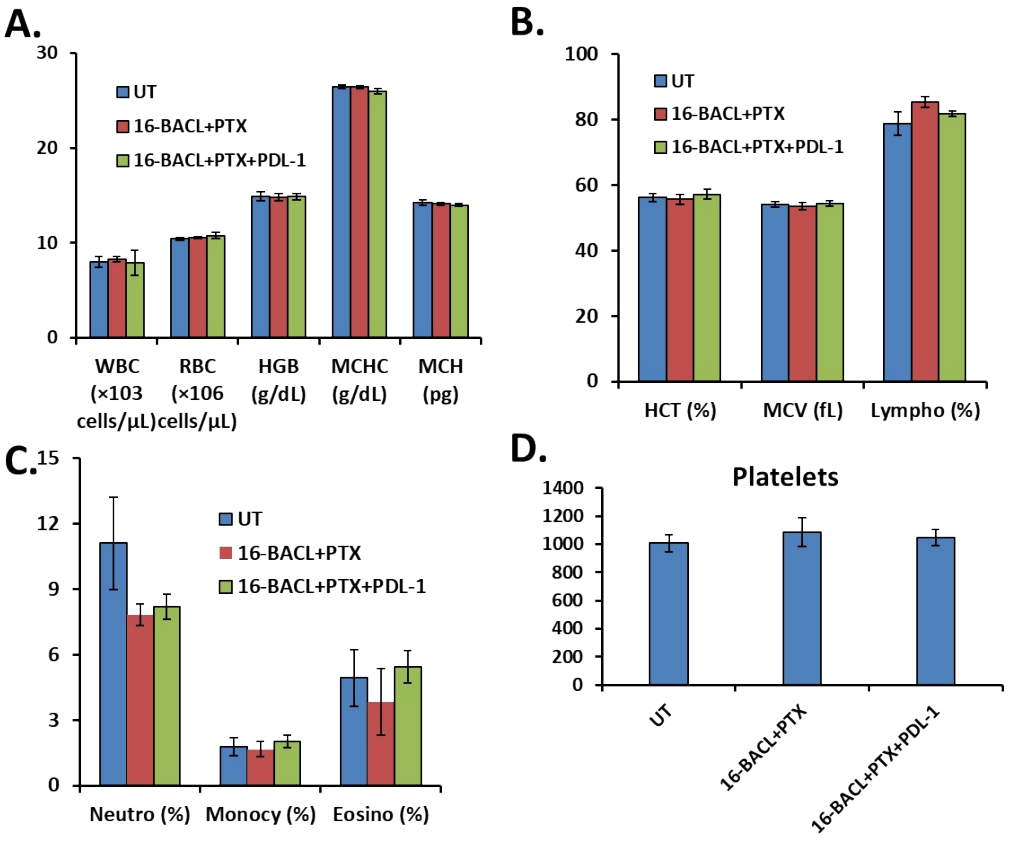


Figure S23. *In vivo* toxicity (hematological parameters) of paclitaxel loaded and paclitaxel & PDL-1siRNA co-loaded lipid nanoparticles of 16-BACL. WBC, white blood cell; RBC, red blood cell; HGB, hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCH, mean corpuscular hemoglobin; HCT, Hematocrit ; MCV, mean corpuscular volume; Lymphocytes, Neutrophils, Monocytes, Eosinophils, and Platelets. Data were presented as mean ± SDEV.