

Blood–brain-barrier-crossing lipid nanoparticles for mRNA delivery to the central nervous system

In the format provided by the
authors and unedited

Blood-brain-barrier-crossing lipid nanoparticles for mRNA delivery to the central nervous system

Table of content

1. Supplementary discussion.....	3
2. Supplementary methods.....	6
3. Supplementary figures.....	23
4. Supplementary references.....	77

1. Supplementary discussion

Systemic mRNA delivery to the brain remains a formidable challenge in the field of biomaterials and RNA therapeutics.¹ This challenge primarily stems from the BBB's stringent selective permeability, which strictly regulates the transportation of biomacromolecules such as mRNA.^{2,3} Moreover, the inherent instability of mRNA and its predisposition to enzymatic degradation further complicate the delivery issues. To tackle these barriers, considerable endeavors have been devoted to the conceptualization and development of innovative nanoparticle-based delivery systems, including inorganic nanoparticles, polymeric materials, and exosomes.^{4,5} These delivery systems are typically functionalized with BBB-targeting or -penetrating ligands on the particle surface to improve the delivery of mRNA molecules to the brain. In terms of LNPs for brain delivery, researchers investigated several strategies and accomplished the delivery of small molecules, siRNA, and proteins to the brain in multiple animal models.⁶ While there have been a few explorations of mRNA-loaded LNPs for systemic brain targeting, prior studies applied external forces to disrupt BBB integrity and facilitate mRNA delivery to the brain.⁶⁻⁸ For example, microbubble-assisted focused ultrasound (FUS) has been investigated for its potential to transiently compromise the BBB and enhance the efficiency of LNP-mediated mRNA delivery to the brain.⁷ Yet, in the absence of FUS, there was a reduction in delivery efficacy. Additionally, LNPs have been formulated with mannose-conjugated PEG lipids for targeted delivery of mRNA to cerebral ischemic regions in a mouse model of ischemic stroke.⁹ This formulation, however, showed limited efficacy in delivering mRNA to the brains of healthy mice, where the BBB is intact. Besides, these approaches require additional procedures and may increase the risk of adverse effects. Consequently, researchers need to design novel strategies to address the transportation challenges of the intact BBB and effectively deliver mRNA to brain in a safe and efficient manner.

In this work, we conceived the BLNP platform, an LNP-mediated mRNA delivery strategy to cross the BBB after intravenous injection. More importantly, this is achieved through the chemical engineering of BBB-crossing molecules with ionizable lipids, which are integrated directly into the existing LNP-mRNA formulation approach. These BLNPs require no surface functionalization and BBB disruption to enable BBB crossing. The BBB-crossing molecules, which serve as the core structure in the BL lipids, would interact with the receptors or transporters on the endothelial cell layer of the BBB, thereby facilitating efficient transcytosis.

Specifically, prior studies have reported various types of small molecules with the capacity to cross the BBB. Inspired by their BBB-penetrating potential, we designed and synthesized six classes of novel ionizable lipids, termed “BBB-crossing lipids”, which are derived from the small molecules including L-DOPA (LD), D-serine (DS), temozolomide (TM), tryptamine (TD), cinnamic acid (CD), and MK-0752 (MK). Delving into the structure-activity relationship of the formulated BLNPs, we found that multiple core structures, positions of bio-responsive acetal groups in the lipid chain, as well as chain lengths would significantly affect *in vivo* mRNA brain delivery efficacy. Moreover, the molar ratios of the ionizable lipid in the formulation and its

weight ratio towards mRNA should be carefully considered in formulation optimization. Ultimately, we identified one optimal BLNP formulation, MK16, for effective systemic mRNA delivery to brain, which showed over 8-fold higher luminescence intensity than that of standard MC3 LNPs.

MK16 BLNPs can effectively deliver mRNA to various brain cells by intravenous injection. Our findings revealed that MK16 BLNPs mainly deliver mRNA to neurons, astrocytes, BCECs, and microglia, with relatively low to minimal delivery to oligodendrocytes and neural stem cells. The differential delivery preferences of MK16 BLNPs to distinct brain cell types could be attributed to the unique features of each cell type, affecting the efficiency of endocytosis and subsequent escape from endosomes for mRNA release. Additionally, each cell type may exhibit different intracellular immune responses to the exogenous mRNA, resulting in varying levels of protein expression. These variations in delivery preferences necessitate further examinations to fully understand the underlying mechanisms. Importantly, the mRNA delivery efficacy of MK16 BLNPs was found to be dose-dependent, providing an opportunity to fine-tune delivery outcomes by adjusting the dose. It is critical to understand the transcytosis mechanism for crossing the BBB. Our results indicated that the transcytosis of MK16 BLNPs is mediated by both the caveolae and γ -secretase pathways. This finding suggests that the trafficking of MK16 BLNPs across the BBB involves complex interactions and pathways. Importantly, although γ -secretase inhibitors have been extensively studied in multiple therapeutic applications, this is the first time, to our knowledge, that γ -secretase has been shown to facilitate nanomaterials crossing the BBB.

Despite incorporating MK-0752, a NOTCH inhibitor, as the head group, MK16 BLNPs do not dramatically modify the NOTCH signaling pathway post-administration as revealed genome-wide through an RNA-seq approach. Furthermore, the immunogenic responses elicited by MK16 BLNPs were found to be comparable to or milder than those induced by the clinically used MC3 LNPs at various post-injection time points. Importantly, MK16 BLNPs did not compromise hepatic or renal functions, and they did not cause detectable abnormalities in the brain or other major organs. This safety assessment underscores the tolerability of MK16 BLNPs for systemic delivery, and highlights their potential as a safe vehicle for effective mRNA delivery to the brain.

MK16 BLNPs exhibited the potent ability to deliver functional mRNAs, such as Cre recombinase mRNA, to different brain regions, including the hippocampus, thalamus and cerebral cortex. This resulted in the genetic expression of tdTomato signals in neurons and astrocytes across several brain regions of Ai14 transgenic mice. Moreover, triple injections of MK16 BLNPs substantially increased mRNA delivery efficacy in neurons, astrocytes, BCECs, and microglia compared to the one-injection group. Notably, MK16 BLNPs are well-tolerated across repeated administrations, underscoring their safety for multiple doses, which may be crucial for reaching therapeutic levels in the brain.^{10, 11} More importantly, systemic administration of Δ *Fosb* mRNA via MK16 BLNPs successfully targeted Δ FOSB expression in the NAc and enhanced drug-

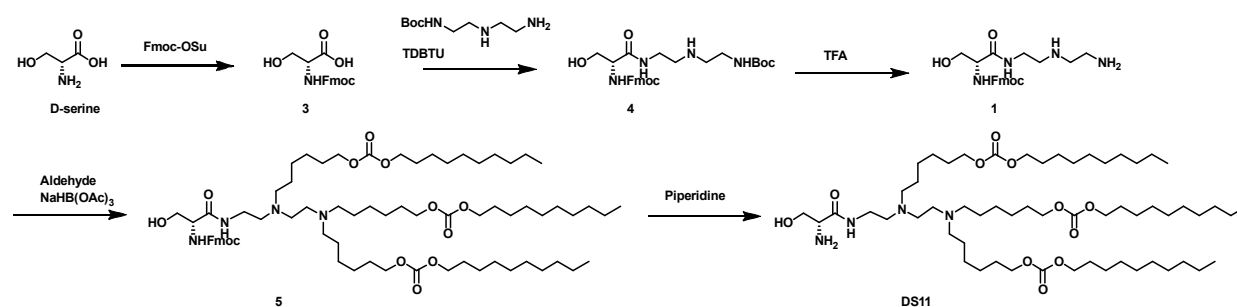
seeking behaviors in conditioned mice. This is an especially dramatic finding, since the efficacy of the MK16 BLNP approach in regulating cocaine responses roughly matches what has been observed previously upon directly targeting of Δ FOSB overexpression to NAc neurons in inducible transgenic mice and by direct intra-NAc injections of viral vectors.^{12, 13} Moreover, we show that MK16 BLNPs can efficiently deliver Δ Fosb mRNA to adult human cortical tissues *ex vivo* and induce considerable Δ FOSB expression in neurons, astrocytes, and microglia, which further highlights the potential of MK16 BLNPs for gene regulation in brain disorders. Finally, MK16 BLNP-*Pten* mRNA treatment demonstrates potent efficacy in reducing the tumor burden in an orthotopic GBM mouse model with superior therapeutic outcomes compared to the treatment of MC3 LNP-*Pten* mRNA. This treatment significantly increased the survival rates of the tumor-bearing mice compared to those treated with PBS or MK16 BLNP-control mRNA.

Intrathecal injection is a clinically used administration route. This method has also been examined for delivering mRNA via LNPs to the dorsal root ganglia.¹⁴ Although intrathecal injection enables the delivery of LNPs to the CNS with lower mRNA doses and comparable efficacy, this administration approach is technically more complex and invasive, with a risk of severe side effects like meningitis.¹⁵ Intravenous injection of BLNPs to target the brain is less invasive and far more clinically feasible for delivering therapeutic agents to the brain. However, this route necessitates that the BLNPs possess excellent stability in the bloodstream and excellent biocompatibility to minimize potential side effects. Most importantly, the BLNPs must actively interact with the BBB to facilitate efficient transcytosis and subsequently release mRNA to various brain cells. The design of our MK16 BLNPs expedites effective and well-tolerated mRNA delivery to the brain through repeated systemic administrations. Systemic administration of MK16 BLNPs resulted in certain levels of accumulation in the liver and spleen, but no significant organ toxicity was observed at the doses tested. One approach to reduce translation in non-target cells, such as hepatocytes and splenocytes, is to incorporate miRNA targeting sequences into the untranslated regions (UTR) of the mRNA molecules.¹⁶

2. Supplementary methods

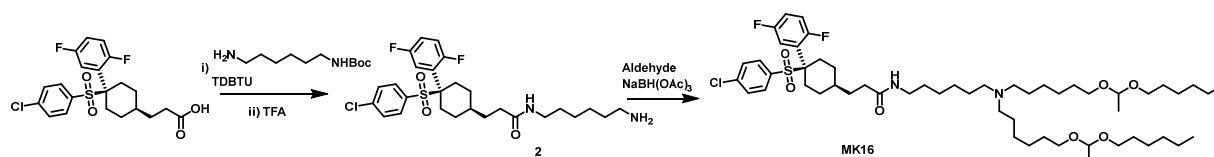
2.1 Synthesis of BBB-crossing lipids

All chemicals and solvents were purchased from Fisher Scientific unless otherwise listed. All BBB-crossing lipids were purified by column chromatography using a CombiFlash Rf system with a RediSep Gold Resolution silica column (Teledyne Isco) with gradient elution. All ^1H NMR spectra were run on Bruker Avance 300 MHz or 400 MHz instrument. Mass spectrometric measurements were performed by Acquity SQD UPLC/MS (Waters), 6210 TOF LC/MS (Agilent) or microFlex LRF MALDI-TOF mass spectrometer (Bruker).



Scheme S1. Synthesis of DS11.

Aldehydes, compound 3 were prepared as described elsewhere.¹⁷⁻¹⁹ To a solution of compound 3 (327mg, 1mmol), DMF (5 mL) and *N,N*-diisopropylethylamine (200 μL , 1.15mmol), (*N,N,N',N'*-Tetramethyl-*O*-(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl)uranium tetrafluoroborate) (420mg, 1.2mmol) was added. The resulting mixture was stirred for 20 min at room temperature. Then *N*¹-Boc-diethylenetriamine (244mg, 1.2mmol) was added, and the solution was stirred at room temperature overnight. The product mixture was added, dropwise, into 50 mL of NaHCO_3 , extracted by ethyl acetate and washed 3 times with brine (50 mL). The resulting mixture was further purified by Combiflash column chromatography with a RediSep Gold Resolution silica column with gradient elution from 100% CH_2Cl_2 to $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (90/10, v/v) to give compound 4 (230mg, 45%). Then, compound 4 was dissolved in 10 mL of CH_2Cl_2 and 1 mL of CF_3COOH and the mixture stirred for 2.5 h. After the reaction was completed, the solvent was removed under reduced pressure to afford compound 1. To a solution of compound 1 (82mg, 0.2 mmol) and triethylamine (112 μL , 0.8mmol) in THF (10 mL) was added aldehyde (300mg, 1.0 mmol) and sodium triacetoxyborohydride (253mg, 1.2 mmol). Then the resulting mixture was stirred at room temperature overnight, washed with NaHCO_3 (50ml) and brine (50ml), purified by Combiflash column chromatography with a RediSep Gold Resolution silica column (Teledyne Isco) using gradient elution from 100% CH_2Cl_2 to $\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{NH}_4\text{OH}$ (80/20/0.6, v/v/v) to afford compound 5. Fmoc was removed by adding piperidine in CH_2Cl_2 to obtain DS11 (31mg, 15%) having: ^1H NMR (400 MHz, CDCl_3) δ 4.13 (m, 12H), 3.96 – 3.78 (m, 1H), 3.69 – 3.57 (m, 1H), 3.49 – 3.44 (m, 1H), 3.38-3.33 (m, 2H), 2.52 (m, 14H), 1.85 – 1.06 (m, 75H), 0.90 (t, J = 6.8 Hz, 9H). ESI-MS for $\text{C}_{58}\text{H}_{115}\text{N}_4\text{O}_{11}$ ($[\text{M}+2\text{H}]^{2+}$) Calculated: 522.9, Found: 522.8.

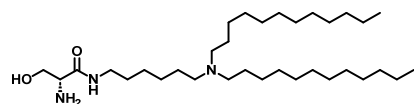


Scheme S2. Synthesis of MK16.

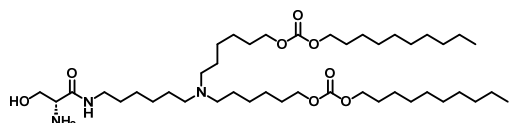
To a solution of MK-0752 (200 mg, 0.45 mmol), *N,N*-diisopropylethylamine (200 μ L, 1.15 mmol) in DMF (5 mL), O-(3,4-Dihydro-4-oxo-1,2,3-benzotriazin-3-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate (210 mg, 0.6 mmol) was added. After the resulting mixture was stirred for 20 min at room temperature, the *N*-Boc-1,6-hexanediamine (110 mg, 0.5 mmol) was added and the solution was kept stirring at room temperature overnight. The resulting mixture was added, dropwise, into 50 mL of NaHCO_3 , extracted by ethyl acetate, washed 3 times with brine (50 mL), and dried over MgSO_4 . After removing the solvents under reduced pressure, the resulting solid was further purified by Combiflash column chromatography with a RediSep Gold Resolution silica column using gradient elution from 100% CH_2Cl_2 to $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (85/15, v/v) to give a Boc protected intermediate (179 mg, 28%). This Boc protected intermediate was then dissolved in 10 mL of CH_2Cl_2 and 1 mL of trifluoroacetic acid, then the mixture was stirred for 2.5 h. After the reaction was completed monitoring by thin layer chromatography, the solvent was removed under reduced pressure to afford the deprotected amine 2. To a solution of amine 2 (50 mg, 0.1 mmol) in THF (10 mL) was added trimethylamine (112 μ L, 0.8 mmol), aldehyde (0.12 g, 0.5 mmol) and sodium triacetoxyborohydride (0.11g, 0.5mmol). The reacting mixture was stirred at room temperature overnight and evaporated under reduced pressure. The resulting product was washed with NaHCO_3 (50 mL) and brine (50 mL), purified by Combiflash column chromatography with a RediSep Gold Resolution silica column (Teledyne Isco) using gradient elution from 100% CH_2Cl_2 to and $\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{NH}_4\text{OH}$ (90/10/0.3, v/v/v) to afford product MK16 (41mg, 42%) having ^1H NMR (400 MHz, MeOD) δ 7.45 – 7.30 (m, 4H), 7.17 – 6.98 (m, 2H), 6.91 – 6.71 (m, 1H), 4.67 (q, J = 5.3 Hz, 2H), 3.68 – 3.50 (m, 4H), 3.47 – 3.28 (m, 4H), 3.24 (m, 2H), 2.56 – 2.33 (m, 12H), 2.25 – 1.08 (m, 55H), 0.89 (t, J = 6.8 Hz, 6H); HR-MS for $\text{C}_{55}\text{H}_{91}\text{ClF}_2\text{N}_2\text{O}_7\text{S}$ ($[\text{M}+\text{H}]^+$) Calculated: 997.6276, Found: 997.6257.

Synthetic procedures for DS1-DS12 and LD1-LD12 are similar to that of DS11.

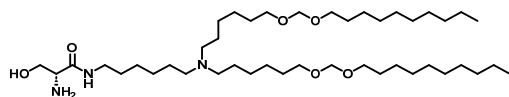
2.2 Characterization of BBB-crossing lipids



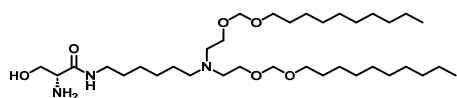
DS1 (24mg, 22%): ^1H NMR (400 MHz, CDCl_3) δ 4.25 – 3.82 (m, 2H), 3.78 – 3.60 (m, 1H), 3.28 (m, 2H), 2.50 (m, 6H), 1.74 – 1.09 (m, 46H), 0.90 (t, J = 6.7 Hz, 6H). MALDI-MS for $\text{C}_{33}\text{H}_{69}\text{N}_3\text{O}_2$ ($[\text{M}+\text{H}]^+$) Calculated: 540.5, Found: 540.5.



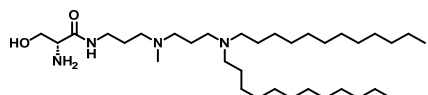
DS2 (30mg, 20%): ^1H NMR (400 MHz, CDCl_3) δ 4.14 (m, 8H), 3.97 – 3.83 (m, 1H), 3.80 – 3.61 (m, 1H), 3.33 (m, 2H), 2.66 – 2.38 (m, 6H), 1.78 – 1.10 (m, 55H), 0.90 (t, J = 6.8 Hz, 6H). ESI-MS for $\text{C}_{43}\text{H}_{85}\text{N}_3\text{O}_8$ ($[\text{M}+2\text{H}]^{2+}$) Calculated: 386.8, Found: 387.1.



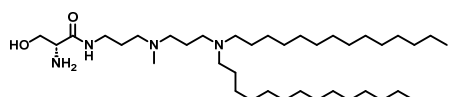
DS3 (38mg, 26%): ^1H NMR (400 MHz, CDCl_3) δ 4.68 (s, 4H), 3.88 (m, 1H), 3.78 – 3.60 (m, 1H), 3.53 (m, 8H), 3.35 (m, 2H), 2.65 – 2.33 (m, 6H), 1.83 – 1.09 (m, 57H), 0.90 (t, J = 6.8 Hz, 6H). ESI-MS for $\text{C}_{43}\text{H}_{89}\text{N}_3\text{O}_6$ ($[\text{M}+2\text{H}]^{2+}$) Calculated: 372.8, Found: 373.1.



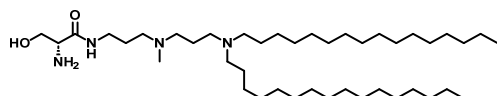
DS4 (31mg, 25%): ^1H NMR (400 MHz, CDCl_3) δ 4.68 (s, 4H), 3.94 – 3.82 (m, 1H), 3.77 – 3.68 (m, 1H), 3.67 – 3.49 (m, 8H), 3.45 – 3.16 (m, 2H), 2.81 – 2.51 (m, 6H), 1.69 – 1.07 (m, 42H), 0.90 (t, J = 6.8 Hz, 6H). ESI-MS for $\text{C}_{35}\text{H}_{73}\text{N}_3\text{O}_6$ ($[\text{M}+2\text{H}]^{2+}$) Calculated: 317.2, Found: 317.0.



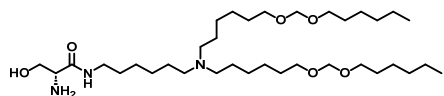
DS5 (32mg, 28%): ^1H NMR (400 MHz, MeOD) δ 3.75 (m, 2H), 3.55 (s, 1H), 3.07 – 2.92 (m, 4H), 2.62 (m, 4H), 2.39 (s, 3H), 1.98 – 1.18 (m, 59H), 0.92 (t, J = 5.9 Hz, 6H). ESI-MS for $\text{C}_{34}\text{H}_{72}\text{N}_4\text{O}_2$ ($[\text{M}+\text{H}]^+$) Calculated: 569.6, Found: 569.6.



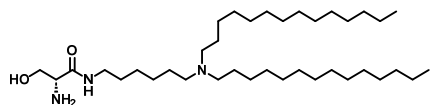
DS6 (25mg, 20%): ^1H NMR (400 MHz, MeOD) δ 4.03 – 3.78 (m, 2H), 3.38 (s, 1H), 3.27 – 3.21 (m, 1H), 3.21 – 3.07 (m, 4H), 2.98 (m, 4H), 2.68 (s, 3H), 2.26 – 1.10 (m, 49H), 0.92 (t, J = 6.0 Hz, 6H). MALDI-MS for $\text{C}_{38}\text{H}_{80}\text{N}_4\text{O}_2$ ($[\text{M}+\text{H}]^+$) Calculated: 625.6, Found: 625.4.



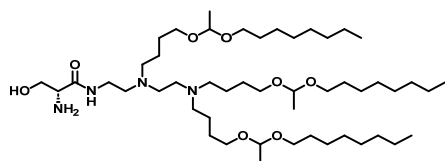
DS7 (39mg, 29%): ^1H NMR (400 MHz, MeOD) δ 4.03 – 3.78 (m, 2H), 3.38 (s, 1H), 3.27 – 3.21 (m, 2H), 3.21 – 3.07 (m, 6H), 2.98 (m, 4H), 2.68 (s, 3H), 2.26 – 1.10 (m, 61H), 0.92 (t, J = 6.0 Hz, 6H). MALDI-MS for $\text{C}_{42}\text{H}_{88}\text{N}_4\text{O}_2$ ($[\text{M}+\text{H}]^+$) Calculated: 681.7, Found: 682.0.



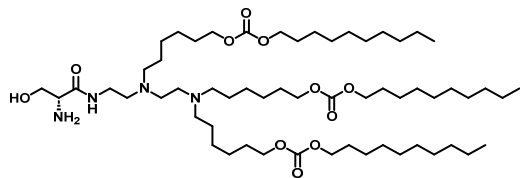
DS8 (29mg, 23%): ^1H NMR (400 MHz, MeOD) δ 4.68 (s, 4H), 3.72 – 3.60 (m, 4H), 3.56 (m, 4H), 3.41 (t, J = 5.4 Hz, 1H), 3.23 (m, 2H), 2.83 (t, J = 5.4 Hz, 4H), 2.72 – 2.39 (m, 2H), 1.78 – 1.10 (m, 40H), 0.92 (t, J = 5.8 Hz, 6H). ESI-MS for $\text{C}_{35}\text{H}_{73}\text{N}_3\text{O}_6$ ($[\text{M}+2\text{H}]^{2+}$) Calculated: 632.6, Found: 632.6.



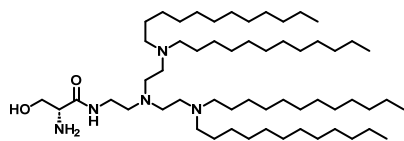
DS9 (31mg, 26%): ^1H NMR (400 MHz, MeOD) δ 3.69 (m, 1H), 3.63 (m, 1H), 3.37 (s, 1H), 3.23 (t, J = 6.9 Hz, 2H), 2.66 – 2.31 (m, 6H), 1.82 – 1.16 (m, 57H), 0.92 (t, J = 5.9 Hz, 6H). ESI-MS for $\text{C}_{37}\text{H}_{77}\text{N}_3\text{O}_2$ ($[\text{M}+2\text{H}]^{2+}$) Calculated: 596.6, Found: 596.6.



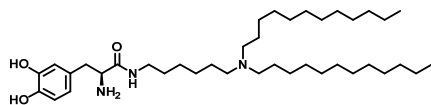
DS10 (29mg, 17%): ^1H NMR (400 MHz, CDCl_3) δ 4.71 (m, 3H), 4.39 – 3.82 (m, 1H), 3.74 – 3.44 (m, 14H), 2.79 – 2.57 (m, 14H), 2.42 – 1.11 (m, 52H), 1.03 – 0.79 (t, J = 6.7 Hz, 9H). ESI-MS for $\text{C}_{49}\text{H}_{102}\text{N}_4\text{O}_8$ ($[\text{M}+\text{H}]^+$) Calculated: 875.8, Found: 875.9.



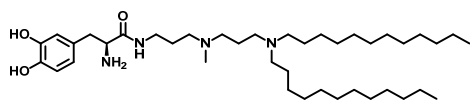
DS11 (31mg, 15%): ^1H NMR (400 MHz, CDCl_3) δ 4.13 (m, 12H), 3.96 – 3.78 (m, 1H), 3.69 – 3.57 (m, 1H), 3.49 – 3.44 (m, 1H), 3.38-3.33 (m, 2H), 2.52 (m, 14H), 1.85 – 1.06 (m, 75H), 0.90 (t, J = 6.8 Hz, 9H). ESI-MS for $\text{C}_{58}\text{H}_{114}\text{N}_4\text{O}_{11}$ ($[\text{M}+2\text{H}]^{2+}$) Calculated: 522.4, Found: 522.7.



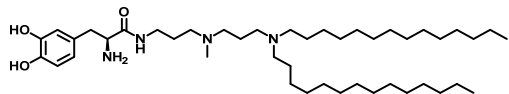
DS12 (27mg, 16%): ^1H NMR (400 MHz, MeOD) δ 4.08 – 3.78 (m, 2H), 3.53 – 3.40 (m, 1H), 3.22 (m, 8H), 2.97 (m, 4H), 2.72 (m, 2H), 1.93 – 1.14 (m, 80H), 0.92 (t, J = 5.6 Hz, 12H). MALDI-MS for $\text{C}_{57}\text{H}_{119}\text{N}_5\text{O}_2$ ($[\text{M}+\text{H}]^+$): Calculated: 906.9, Found: 907.0.



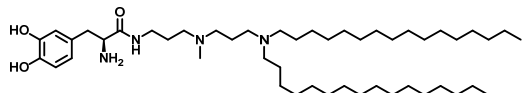
LD1: (26mg, 21%): ^1H NMR (400 MHz, CDCl_3) δ 6.71 (m, 2H), 6.50 (m, 1H), 4.19 (s, 1H), 2.95 (m, 6H), 1.47 – 1.01 (m, 35H), 0.89 (t, J = 6.8 Hz, 6H). ESI-MS for $\text{C}_{39}\text{H}_{73}\text{N}_3\text{O}_3$ ($[\text{M}+\text{H}]^+$) Calculated: 632.6, Found: 632.6.



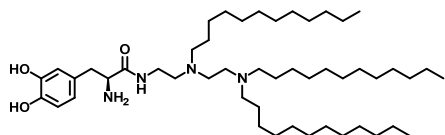
LD2 (26mg, 20%): ^1H NMR (400 MHz, CDCl_3) δ 6.82 (m, 2H), 6.63 (m, 1H), 4.10 (m, 1H), 3.33 – 2.74 (m, 15H), 2.66 (m, 4H), 2.25 – 1.02 (m, 40H), 0.87 (t, J = 6.4 Hz, 6H). ESI-MS for $\text{C}_{40}\text{H}_{76}\text{N}_4\text{O}_3$ ($[\text{M}+\text{H}]^+$) Calculated: 661.6, Found: 661.6.



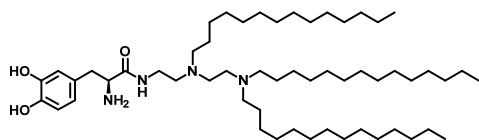
LD3 (25mg, 18%): ^1H NMR (400 MHz, MeOD) δ 6.82 (m, 2H), 6.63 (m, 1H), 4.10 (m, 1H), 3.33 – 2.74 (m, 15H), 2.66 (m, 4H), 2.25 – 1.02 (m, 45H), 0.87 (t, J = 6.4 Hz, 6H). ESI-MS for $\text{C}_{44}\text{H}_{84}\text{N}_4\text{O}_3$ ($[\text{M}+\text{H}]^+$) Calculated: 717.6, Found: 717.7.



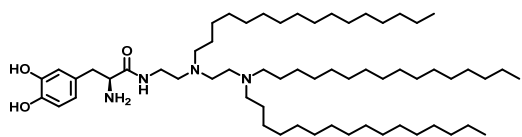
LD4 (24mg, 16%): ^1H NMR (400 MHz, MeOD) δ 6.82 (m, 2H), 6.63 (m, 1H), 4.10 (m, 1H), 3.33 – 2.74 (m, 15H), 2.66 (m, 4H), 2.25 (s, 3H), 2.20 – 1.02 (m, 54H), 0.87 (t, J = 6.4 Hz, 6H). ESI-MS for $\text{C}_{48}\text{H}_{92}\text{N}_4\text{O}_3$ ($[\text{M}+\text{H}]^+$) Calculated: 773.7, Found: 773.7.



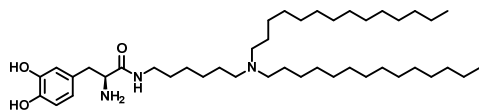
LD5 (40mg, 25%): ^1H NMR (400 MHz, CDCl_3) δ 7.07 – 6.74 (m, 2H), 6.62 (m, 1H), 3.56 (m, 1H), 3.38 – 3.23 (m, 2H), 3.17 (m, 4H), 3.08 – 2.61 (m, 12H), 2.50 (m, 4H), 1.83 – 1.05 (m, 60H), 0.90 (t, J = 6.1 Hz, 9H). ESI-MS for $\text{C}_{49}\text{H}_{94}\text{N}_4\text{O}_3$ ($[\text{M}+\text{H}]^+$) Calculated: 787.7, Found: 787.7.



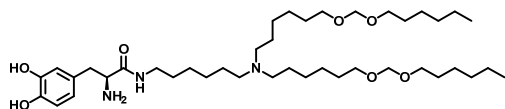
LD6 (26mg, 15%): ^1H NMR (400 MHz, CDCl_3) δ 7.07 – 6.74 (m, 2H), 6.62 (m, 1H), 4.20 (m, 1H), 3.58 – 2.50 (m, 18H), 1.83 – 1.05 (m, 90H), 0.90 (t, J = 6.1 Hz, 9H). ESI-MS for $\text{C}_{55}\text{H}_{106}\text{N}_4\text{O}_3$ ($[\text{M}+\text{H}]^+$) Calculated: 871.8, Found: 871.8.



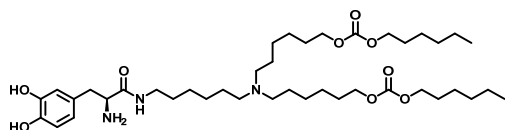
LD7 (40mg, 21%): ^1H NMR (400 MHz, MeOD) δ 7.07 – 6.74 (m, 2H), 6.62 (m, 1H), 3.56 (m, 1H), 3.38 – 3.23 (m, 4H), 3.17 (m, 4H), 3.08 – 2.61 (m, 10H), 2.50 (m, 4H), 1.83 – 1.05 (m, 86H), 0.90 (t, J = 6.1 Hz, 9H). ESI-MS for $\text{C}_{61}\text{H}_{118}\text{N}_4\text{O}_3$ ($[\text{M}+\text{H}]^+$) Calculated: 955.9, Found: 955.9.



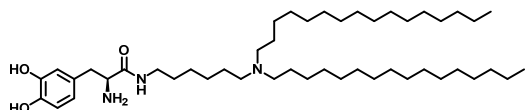
LD8 (41mg, 30%): ^1H NMR (400 MHz, MeOD) δ 6.71 – 6.60 (m, 2H), 6.53 (d, J = 7.9 Hz, 1H), 3.44 (t, J = 6.8 Hz, 1H), 3.27 – 3.09 (m, 2H), 3.03 (m, 2H), 2.88 – 2.58 (m, 7H), 1.71 – 1.08 (m, 57H), 0.92 (t, J = 5.9 Hz, 6H). ESI-MS for $\text{C}_{43}\text{H}_{81}\text{N}_3\text{O}_3$ ($[\text{M}+\text{H}]^+$) Calculated: 688.6, Found: 688.6.



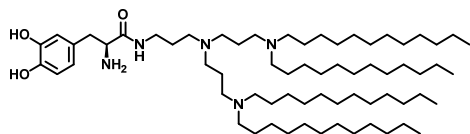
LD9 (23%, 16%): ^1H NMR (400 MHz, MeOD) δ 6.85 (m, 2H), 6.76 – 6.52 (m, 1H), 4.65 (s, 4H), 3.67 – 3.41 (m, 8H), 3.41 – 3.20 (m, 4H), 3.20 – 3.08 (m, 4H), 3.07 – 2.86 (m, 4H), 2.49 (m, 6H), 1.83 – 1.07 (m, 44H), 0.93 (t, J = 6.8 Hz, 6H). ESI-MS for $\text{C}_{41}\text{H}_{77}\text{N}_3\text{O}_7$ ($[\text{M}+\text{H}]^+$) Calculated: 724.6, Found: 724.6.



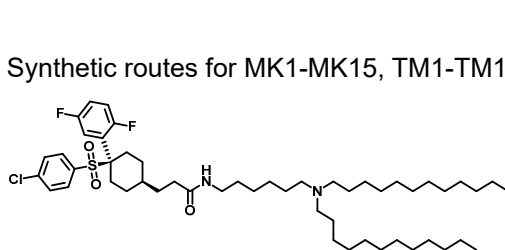
LD10 (22mg, 15%): ^1H NMR (400 MHz, MeOD) δ 7.06 – 6.73 (m, 2H), 6.67 (m, 1H), 4.12 (m, 8H), 3.67 – 3.41 (m, 1H), 3.41 – 3.20 (m, 4H), 3.20 – 3.08 (m, 4H), 3.07 – 2.46 (m, 6H), 1.83 – 1.07 (m, 42H), 0.93 (t, J = 6.4 Hz, 6H). ESI-MS for $\text{C}_{41}\text{H}_{73}\text{N}_3\text{O}_9$ ($[\text{M}+\text{H}]^+$) Calculated: 752.5, Found: 752.5.



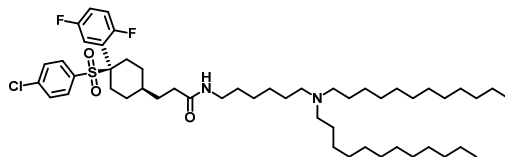
LD11 (28mg, 19%): ^1H NMR (400 MHz, MeOD) δ 6.93 (m, 2H), 6.83 (m, 1H), 3.95 (m, 1H), 3.23 (m, 1H), 3.14 (m, 6H), 2.98 (m, 8H), 2.02 – 1.08 (m, 56H), 0.89 (t, J = 5.9 Hz, 6H). ESI-MS for $\text{C}_{47}\text{H}_{89}\text{N}_3\text{O}_3$ ($[\text{M}+\text{H}]^+$) Calculated: 744.7, Found: 744.7.

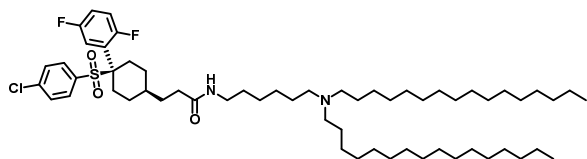


LD12 (33mg, 16%): ^1H NMR (400 MHz, CDCl_3) δ 6.93 – 6.65 (m, 2H), 6.53 (m, 1H), 4.31 – 4.03 (m, 1H), 3.45 – 2.78 (m, 20H), 2.01 – 1.43 (m, 4H), 1.28 (m, 71H), 0.90 (t, J = 6.8 Hz, 12H). ESI-MS for $\text{C}_{66}\text{H}_{129}\text{N}_5\text{O}_3$ ($[\text{M}+2\text{H}]^{2+}$) Calculated: 521.0, Found: 521.0.

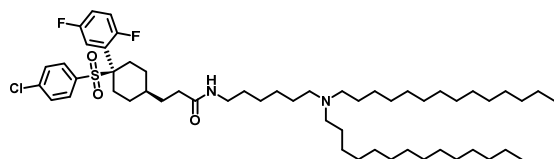


MK1 (42mg, 48%): ^1H NMR (400 MHz, CDCl_3) δ 7.42 – 7.31 (m, 4H), 7.16 – 6.96 (m, 2H), 6.92 – 6.73 (m, 1H), 3.37 – 3.14 (m, 2H), 2.43 (m, 9H), 2.23 – 2.13 (m, 2H), 1.93 – 1.10 (m, 54H), 0.89 (t, J = 6.8 Hz, 6H). ESI-MS for $\text{C}_{51}\text{H}_{83}\text{ClF}_2\text{N}_2\text{O}_3\text{S}$ ($[\text{M}+\text{H}]^+$) Calculated: 877.6, Found: 877.6.

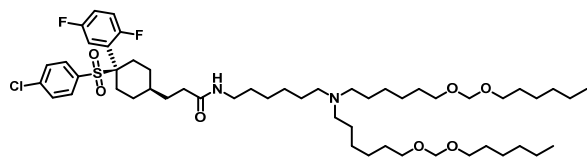




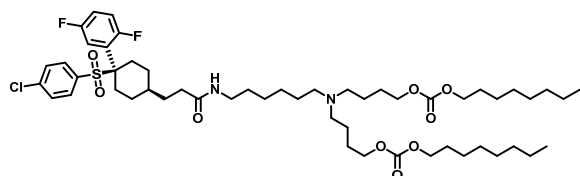
MK2 (45mg, 47%): ^1H NMR (300 MHz, CDCl_3) δ 7.46 – 7.29 (m, 4H), 7.17 – 6.94 (m, 2H), 6.96 – 6.66 (m, 1H), 3.25 (m, 2H), 2.74 (m, 4H), 2.51 – 2.27 (m, 4H), 2.31 – 2.11 (m, 2H), 1.94 – 1.00 (m, 75H), 0.89 (t, J = 6.7 Hz, 6H). ESI-MS for $\text{C}_{59}\text{H}_{99}\text{ClF}_2\text{N}_2\text{O}_3\text{S}$ ($[\text{M}+\text{H}]^+$) Calculated: 989.7, Found: 989.9.



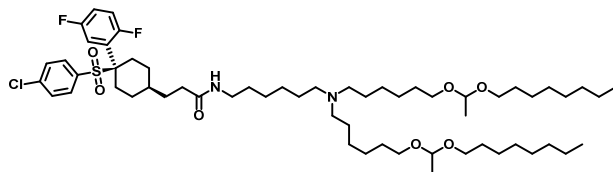
MK3 (44mg, 48%): ^1H NMR (400 MHz, CDCl_3) δ 7.34 (m, 4H), 7.11 – 6.94 (m, 2H), 6.83 (m, 1H), 3.37 – 3.11 (m, 2H), 2.57 – 2.25 (m, 10H), 2.25 – 2.08 (m, 2H), 1.95 – 1.03 (m, 71H), 0.89 (t, J = 6.8 Hz, 6H). ESI-MS for $\text{C}_{55}\text{H}_{91}\text{ClF}_2\text{N}_2\text{O}_3\text{S}$ ($[\text{M}+\text{H}]^+$) Calculated: 933.6, Found: 933.8.



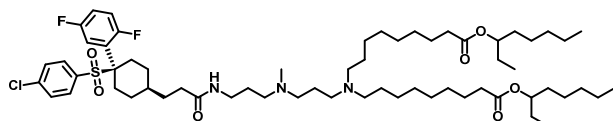
MK4 (43mg, 45%): ^1H NMR (300 MHz, CDCl_3) δ 7.44 – 7.29 (m, 4H), 7.09 – 6.95 (m, 2H), 6.83 (m, 1H), 4.66 (s, 4H), 3.51 (m, 8H), 3.36 – 3.14 (m, 2H), 2.82 – 2.11 (m, 12H), 1.91 – 1.05 (m, 51H), 0.89 (t, J = 6.8 Hz, 6H). ESI-MS for $\text{C}_{53}\text{H}_{87}\text{ClF}_2\text{N}_2\text{O}_7\text{S}$ ($[\text{M}+\text{H}]^+$) Calculated: 960.6, Found: 969.8.



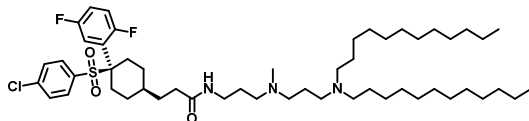
MK5 (46mg, 46%): ^1H NMR (300 MHz, CDCl_3) δ 7.42 – 7.30 (m, 4H), 7.16 – 6.96 (m, 2H), 6.92 – 6.70 (m, 1H), 4.31 – 3.93 (m, 8H), 3.36 – 3.09 (m, 2H), 2.62 – 2.08 (m, 12H), 1.94 – 1.17 (m, 47H), 0.89 (t, J = 6.7 Hz, 6H). ESI-MS for $\text{C}_{53}\text{H}_{83}\text{ClF}_2\text{N}_2\text{O}_9\text{S}$ ($[\text{M}+\text{H}]^+$) Calculated: 997.6, Found: 997.8.



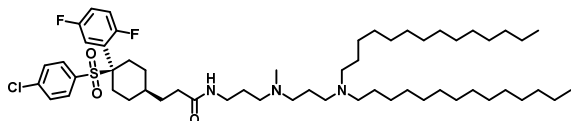
MK6 (40mg, 38%): ^1H NMR (400 MHz, CDCl_3) δ 7.47 – 7.31 (m, 4H), 7.11 – 6.95 (m, 2H), 6.92 – 6.74 (m, 1H), 4.75 – 4.57 (q, 2H), 3.68 – 3.52 (m, 4H), 3.46 – 3.31 (m, 4H), 3.31 – 3.13 (m, 2H), 2.67 – 1.98 (m, 13H), 1.87 – 1.06 (m, 58H), 0.89 (t, J = 6.8 Hz, 6H). HR-MS for $\text{C}_{59}\text{H}_{99}\text{ClF}_2\text{N}_2\text{O}_7\text{S}$ ($[\text{M}+\text{H}]^+$) Calculated: 1053.6902, Found: 1053.6880.



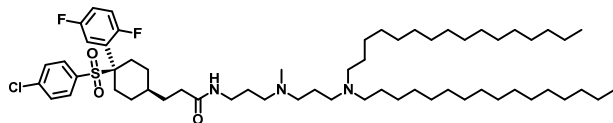
MK7 (29mg, 27%): ^1H NMR (400 MHz, CDCl_3) δ 7.43 – 7.31 (m, 4H), 7.12 – 7.01 (m, 2H), 6.89 – 6.76 (m, 1H), 4.92 – 4.69 (m, 2H), 3.45 – 3.22 (m, 2H), 2.58 – 2.46 (m, 12H), 2.42–2.30 (m, 8H), 2.24 (s, 3H), 2.17 (m, 2H), 1.88 – 1.16 (m, 56H), 0.89 (t, J = 6.8 Hz, 12H). MALDI-MS for $\text{C}_{62}\text{H}_{102}\text{ClF}_2\text{N}_3\text{O}_7\text{S}$ ($[\text{M}+\text{H}]^+$) Calculated: 1106.7, Found: 1106.7.



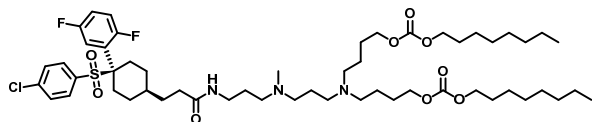
MK8 (39%, 44%): ^1H NMR (400 MHz, CDCl_3) δ 7.41 – 7.29 (m, 4H), 7.06 (m, 2H), 6.84 (m, 1H), 3.39 – 3.23 (m, 2H), 2.62 – 2.31 (m, 12H), 2.24 (s, 3H), 2.16 (m, 2H), 1.91 – 1.00 (m, 58H), 0.89 (t, J = 6.7 Hz, 6H). ESI-MS for $\text{C}_{52}\text{H}_{87}\text{ClF}_2\text{N}_3\text{O}_3\text{S}$ ($[\text{M}+2\text{H}]^{2+}$) Calculated: 454.3, Found: 454.3.



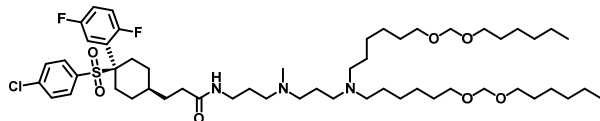
MK9 (43mg, 45%): ^1H NMR (400 MHz, CDCl_3) δ 7.48 – 7.27 (m, 4H), 7.14 – 7.00 (m, 2H), 6.88 – 6.73 (m, 1H), 3.45 – 3.27 (m, 2H), 2.63 – 2.30 (m, 12H), 2.24 (s, 3H), 2.19 (m, 2H), 1.88 – 1.09 (m, 55H), 0.90 (t, J = 6.8 Hz, 6H). ESI-MS for $\text{C}_{56}\text{H}_{94}\text{ClF}_2\text{N}_3\text{O}_3\text{S}$ ($[\text{M}+\text{H}]^+$) Calculated: 962.7, Found: 962.7.



MK10 (40mg, 39%): ^1H NMR (400 MHz, CDCl_3) δ 7.48 – 7.31 (m, 4H), 7.06 (m, 2H), 6.91 – 6.68 (m, 1H), 3.43 – 3.15 (m, 2H), 2.43 (m, 12H), 2.24 (s, 3H), 2.21 – 2.02 (m, 2H), 1.95 – 1.01 (m, 60H), 0.90 (t, J = 6.8 Hz, 6H). MALDI-MS for $\text{C}_{60}\text{H}_{102}\text{ClF}_2\text{N}_3\text{O}_3\text{S}$ ($[\text{M}+\text{H}]^+$) Calculated: 1018.7, Found: 1018.8.

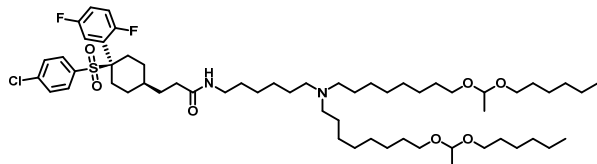


MK11 (38mg, 37%): ^1H NMR (300 MHz, CDCl_3) δ 7.49 – 7.30 (m, 4H), 7.06 (m, 2H), 6.92 – 6.74 (m, 1H), 4.14 (m, 8H), 3.44 – 3.23 (m, 2H), 2.58 – 2.43 (m, 12H), 2.26 (s, 3H), 2.21 – 2.02 (m, 2H), 1.90 – 1.06 (m, 48H), 0.90 (t, J = 6.6 Hz, 6H). ESI-MS for $\text{C}_{54}\text{H}_{87}\text{ClF}_2\text{N}_3\text{O}_9\text{S}$ ($[\text{M}+\text{H}]^+$) Calculated: 514.3, Found: 514.3.

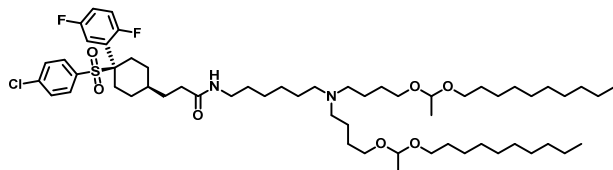


MK12 (34mg, 35%): ^1H NMR (300 MHz, CDCl_3) δ 7.46 – 7.27 (m, 4H), 7.13 – 6.96 (m, 2H), 6.89 – 6.74 (m, 1H), 4.67 (s, 4H), 3.68 – 3.45 (m, 8H), 3.32 (m, 2H), 2.63 – 2.30 (m, 14H), 2.20 (s, 3H), 2.19 (m, 2H), 1.88

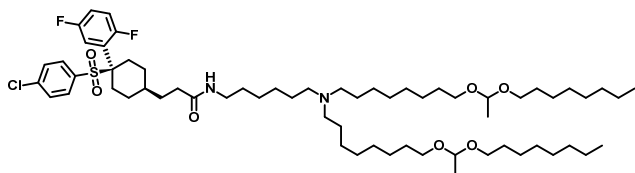
– 1.14 (m, 45H), 0.90 (t, $J = 6.8$ Hz, 6H). ESI-MS for $C_{54}H_{91}ClF_2N_3O_7S$ ($[M+2H]^{2+}$) Calculated: 500.3, Found: 500.3.



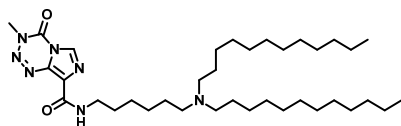
MK13 (44mg, 42%): ^1H NMR (400 MHz, $CDCl_3$) δ 7.47 – 7.31 (m, 4H), 7.12 – 6.95 (m, 2H), 6.92 – 6.74 (m, 1H), 4.68 (q, $J = 5.3$ Hz, 2H), 3.68 – 3.52 (m, 4H), 3.46 – 3.31 (m, 4H), 3.31 – 3.13 (m, 2H), 2.67 – 1.98 (m, 13H), 1.87 – 1.06 (m, 60H), 0.89 (t, $J = 6.8$ Hz, 6H). HR-MS for $C_{59}H_{99}ClF_2N_2O_7S$ ($[M+H]^+$) Calculated: 1053.6902, Found: 1053.6879.



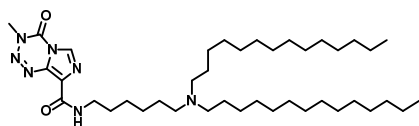
MK14 (40mg, 38%): ^1H NMR (400 MHz, $CDCl_3$) δ 7.45 – 7.33 (m, 4H), 7.11 – 6.95 (m, 2H), 6.92 – 6.74 (m, 1H), 4.67 (q, $J = 5.3$ Hz, 2H), 3.68 – 3.52 (m, 4H), 3.46 – 3.31 (m, 4H), 3.31 – 3.13 (m, 2H), 2.66 – 1.98 (m, 12H), 1.87 – 1.06 (m, 58H), 0.88 (t, $J = 6.8$ Hz, 6H). HR-MS for $C_{59}H_{99}ClF_2N_2O_7S$ ($[M+H]^+$) Calculated: 1053.6902, Found: 1053.6887.



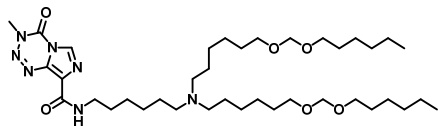
MK15 (44mg, 40%): ^1H NMR (400 MHz, $CDCl_3$) δ 7.45 – 7.32 (m, 4H), 7.10 – 6.95 (m, 2H), 6.92 – 6.74 (m, 1H), 4.67 (q, $J = 5.3$ Hz, 2H), 3.68 – 3.52 (m, 4H), 3.46 – 3.31 (m, 4H), 3.31 – 3.13 (m, 2H), 2.67 – 1.98 (m, 12H), 1.90 – 1.05 (m, 70H), 0.88 (t, $J = 6.8$ Hz, 6H). HR-MS for $C_{63}H_{107}ClF_2N_2O_7S$ ($[M+H]^+$) Calculated: 1109.7528, Found: 1109.7513.



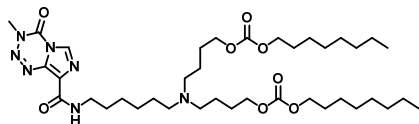
TM1 (22mg, 35%): ^1H NMR (300 MHz, $CDCl_3$) δ 8.40 (s, 1H), 4.05 (s, 3H), 3.66 – 3.38 (m, 2H), 2.68 – 2.30 (m, 6H), 1.77 – 1.12 (m, 50H), 0.88 (t, $J = 6.7$ Hz, 6H). ESI-MS for $C_{36}H_{67}N_7O_2$ ($[M+H]^+$) Calculated: 630.5, Found: 630.5.



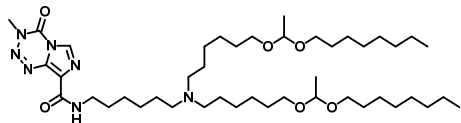
TM2 (20mg, 29%): ^1H NMR (400 MHz, CDCl_3) δ 8.40 (s, 1H), 4.05 (s, 3H), 3.66 – 3.38 (m, 2H), 2.68 – 2.30 (m, 6H), 1.77 – 1.12 (m, 60H), 0.88 (t, J = 6.7 Hz, 6H). ESI-MS for $\text{C}_{40}\text{H}_{75}\text{N}_7\text{O}_2$ ($[\text{M}+\text{H}]^+$) Calculated: 686.6, Found: 686.6.



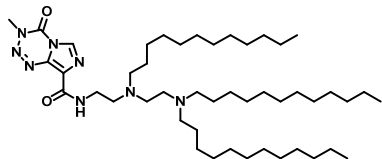
TM3 (20mg, 28%): ^1H NMR (400 MHz, CDCl_3) δ 8.39 (s, 1H), 4.67 (s, 4H), 4.04 (s, 3H), 3.66 – 3.37 (m, 8H), 2.64 – 2.18 (m, 6H), 1.81 – 1.07 (m, 40H), 0.88 (t, J = 6.8 Hz, 6H). ESI-MS for $\text{C}_{38}\text{H}_{71}\text{N}_7\text{O}_6$ ($[\text{M}+\text{H}]^+$) Calculated: 722.6, Found: 722.7.



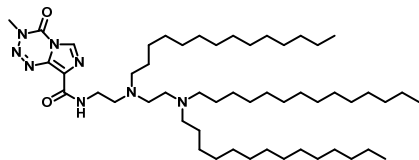
TM4 (23mg, 30%): ^1H NMR (400 MHz, CDCl_3) δ 8.39 (s, 1H), 4.37 – 4.04 (m, 8H), 4.04 (s, 3H), 3.66 – 3.37 (m, 2H), 2.64 – 2.18 (m, 6H), 1.81 – 1.07 (m, 40H), 0.88 (t, J = 6.8 Hz, 6H). ESI-MS for $\text{C}_{38}\text{H}_{67}\text{N}_7\text{O}_8$ ($[\text{M}+\text{H}]^+$) Calculated: 750.5, Found: 750.7.



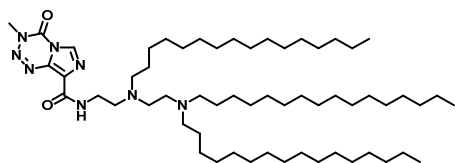
TM5 (26mg, 32%): ^1H NMR (400 MHz, CDCl_3) δ 8.40 (s, 1H), 4.63 (m, 2H), 4.15 – 3.87 (m, 4H), 3.70 – 3.26 (m, 16H), 2.72 – 2.29 (m, 8H), 1.81 – 1.02 (m, 65H), 0.89 (t, J = 6.4 Hz, 6H). ESI-MS for $\text{C}_{44}\text{H}_{83}\text{N}_7\text{O}_6$ ($[\text{M}+\text{H}]^+$) Calculated: 806.7, Found: 806.8.



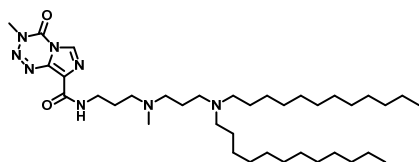
TM6 (24mg, 30%): ^1H NMR (400 MHz, CDCl_3) δ 8.40 (s, 1H), 4.05 (s, 3H), 3.66 – 3.38 (m, 2H), 2.68 – 2.30 (m, 12H), 1.77 – 1.12 (m, 65H), 0.88 (t, J = 6.7 Hz, 9H). ESI-MS for $\text{C}_{46}\text{H}_{88}\text{N}_8\text{O}_2$ ($[\text{M}+\text{H}]^+$) Calculated: 785.7, Found: 785.7.



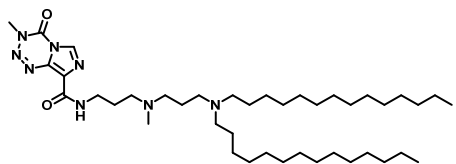
TM7 (24mg, 28%): ^1H NMR (400 MHz, CDCl_3) δ 8.40 (s, 1H), 4.05 (s, 3H), 3.66 – 3.38 (m, 2H), 2.68 – 2.30 (m, 12H), 1.77 – 1.12 (m, 74H), 0.88 (t, J = 6.7 Hz, 9H). ESI-MS for $\text{C}_{52}\text{H}_{100}\text{N}_8\text{O}_2$ ($[\text{M}+\text{H}]^+$) Calculated: 869.8, Found: 869.8.



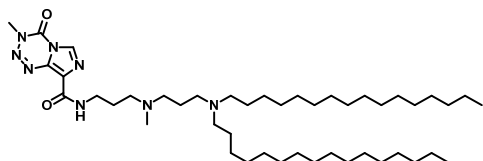
TM8 (23mg, 25%): ^1H NMR (400 MHz, CDCl_3) δ 8.40 (s, 1H), 4.05 (s, 3H), 3.66 – 3.38 (m, 2H), 2.68 – 2.30 (m, 12H), 1.77 – 1.12 (m, 86H), 0.88 (t, J = 6.7 Hz, 9H). ESI-MS for $\text{C}_{58}\text{H}_{112}\text{N}_8\text{O}_2$ ($[\text{M}+\text{H}]^+$) Calculated: 953.8, Found: 953.9.



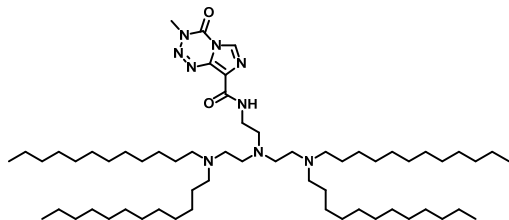
TM9 (19mg, 30%): ^1H NMR (300 MHz, CDCl_3) δ 8.46 (s, 1H), 4.10 (m, 2H), 3.19 – 3.02 (m, 4H), 2.48 – 2.30 (m, 4H), 2.18 (s, 3H), 2.07 (m, 2H), 1.93 – 1.04 (m, 44H), 0.89 (t, J = 6.5 Hz, 6H). ESI-MS for $\text{C}_{37}\text{H}_{70}\text{N}_8\text{O}_2$ ($[\text{M}+\text{H}]^+$) Calculated: 659.6, Found: 659.6.



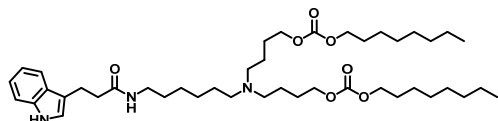
TM10 (22mg, 29%): ^1H NMR (400 MHz, CDCl_3) δ 8.36 (s, 1H), 4.67 (s, 4H), 4.17 (s, 3H), 3.60 – 3.36 (m, 18H), 2.68 – 2.31 (m, 17H), 2.31 – 2.16 (m, 4H), 1.90 – 1.01 (m, 71H), 0.90 (t, J = 6.7 Hz, 6H). ESI-MS for $\text{C}_{41}\text{H}_{78}\text{N}_8\text{O}_2$ ($[\text{M}+\text{H}]^+$) Calculated: 751.6, Found: 751.6.



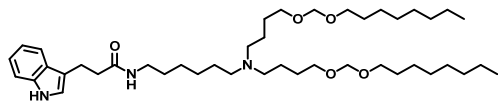
TM11 (22mg, 29%): ^1H NMR (400 MHz, CDCl_3) δ 8.13 (s, 1H), 4.81 – 4.61 (m, 2H), 3.50 (m, 6H), 2.48 – 2.30 (m, 4H), 2.17 (s, 3H), 2.07 (m, 2H), 1.89 – 1.02 (m, 56H), 0.89 (t, J = 6.8 Hz, 6H). ESI-MS for $\text{C}_{45}\text{H}_{86}\text{N}_8\text{O}_2$ ($[\text{M}+\text{H}]^+$) Calculated: 771.7, Found: 771.7.



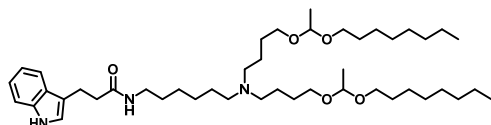
TM12 (21mg, 22%): ^1H NMR (400 MHz, CDCl_3) δ 8.39 (s, 1H), 4.09 – 3.98 (s, 3H), 3.62 – 3.51 (m, 2H), 2.76 (m, 10H), 2.50 (m, 8H), 1.62 – 1.04 (m, 84H), 0.90 (t, J = 6.8 Hz, 12H). MALDI-MS for $\text{C}_{60}\text{H}_{117}\text{N}_9\text{O}_2$ ($[\text{M}+\text{H}]^+$) Calculated: 996.9, Found: 996.9.



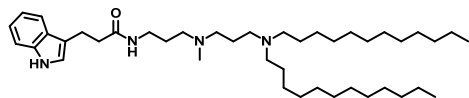
TD1 (29mg, 39%): ^1H NMR (400 MHz, CDCl_3) δ 7.62 (d, $J = 7.8$ Hz, 1H), 7.38 (d, $J = 8.1$ Hz, 1H), 7.17 (m, 2H), 7.04 (d, 1H), 4.36 – 3.94 (m, 8H), 3.30 – 2.99 (m, 4H), 2.68 – 2.26 (m, 8H), 1.87 – 1.08 (m, 45H), 0.90 (t, $J = 6.7$ Hz, 6H). ESI-MS for $\text{C}_{43}\text{H}_{73}\text{N}_3\text{O}_7$ ($[\text{M}+\text{H}]^+$) Calculated: 744.6, Found: 744.7.



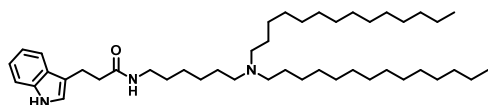
TD2 (27mg, 38%): ^1H NMR (400 MHz, CDCl_3) δ 7.69 – 7.52 (d, 1H), 7.37 (d, $J = 12.3$ Hz, 1H), 7.25 – 7.06 (m, 2H), 7.02 (d, $J = 8.4$ Hz, 1H), 4.68 (s, 4H), 3.54 (m, 8H), 3.28 – 3.03 (m, 4H), 2.66 – 2.35 (m, 8H), 1.70 – 1.03 (m, 42H), 0.90 (t, $J = 6.8$ Hz, 6H). ESI-MS for $\text{C}_{43}\text{H}_{77}\text{N}_3\text{O}_5$ ($[\text{M}+\text{H}]^+$) Calculated: 716.6, Found: 716.7.



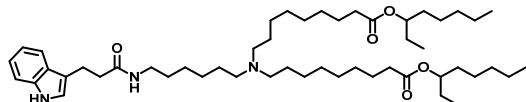
TD3 (28mg, 38%): ^1H NMR (400 MHz, CDCl_3) δ 7.69 – 7.49 (m, 1H), 7.35 (d, $J = 8.0$ Hz, 1H), 7.22 – 7.04 (m, 2H), 7.01 (d, $J = 14.0$ Hz, 1H), 4.80 – 4.54 (m, 2H), 3.69 – 3.52 (m, 4H), 3.48 – 3.33 (m, 4H), 3.24 – 3.03 (m, 4H), 2.68 – 2.30 (m, 8H), 1.68 – 1.04 (m, 52H), 0.89 (t, $J = 6.7$ Hz, 6H). ESI-MS for $\text{C}_{45}\text{H}_{81}\text{N}_3\text{O}_5$ ($[\text{M}+\text{H}]^+$) Calculated: 744.6, Found: 744.8.



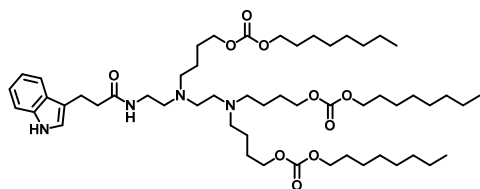
TD4 (27mg, 42%): ^1H NMR (400 MHz, CDCl_3) δ 7.62 (d, $J = 7.8$ Hz, 1H), 7.49 – 7.31 (m, 1H), 7.16 (m, 2H), 7.03 (d, $J = 13.9$ Hz, 1H), 3.41 – 3.21 (m, 2H), 3.06 (m, 2H), 2.71 – 2.41 (m, 8H), 2.38 – 2.19 (m, 4H), 2.19 (s, 3H), 1.77 – 1.09 (m, 45H), 0.90 (t, $J = 6.8$ Hz, 6H). ESI-MS for $\text{C}_{42}\text{H}_{76}\text{N}_4\text{O}$ ($[\text{M}+\text{H}]^+$) Calculated: 653.6, Found: 653.7.



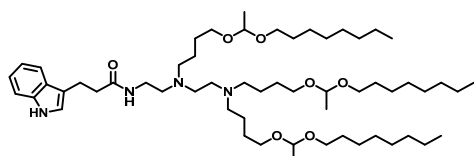
TD5 (30mg, 44%): ^1H NMR (400 MHz, CDCl_3) δ 7.58 (d, 7.6 Hz, 1H), 7.35 (d, $J = 8.1$ Hz, 1H), 7.21 – 7.06 (m, 2H), 7.02 (d, 1H), 3.21 – 3.06 (m, 4H), 2.77 – 2.27 (m, 8H), 1.82 – 1.04 (m, 60H), 0.90 (t, $J = 6.8$ Hz, 6H). ESI-MS for $\text{C}_{45}\text{H}_{81}\text{N}_3\text{O}$ ($[\text{M}+\text{H}]^+$) Calculated: 680.6, Found: 680.7.



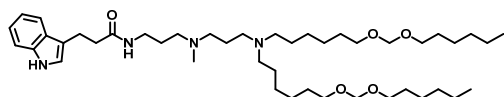
TD6 (23mg, 28%): ^1H NMR (400 MHz, CDCl_3) δ 7.60 (d, $J = 13.8$ Hz, 1H), 7.37 (d, $J = 8.1$ Hz, 1H), 7.24 – 7.09 (m, 2H), 7.04 (d, 1H), 4.95 – 4.67 (m, 2H), 3.29 – 3.01 (m, 4H), 2.69 – 2.51 (m, 3H), 2.51 – 2.20 (m, 10H), 1.80 – 1.04 (m, 60H), 1.02 – 0.79 (t, $J = 6.8$ Hz, 12H). ESI-MS for $\text{C}_{51}\text{H}_{89}\text{N}_3\text{O}_5$ ($[\text{M}+\text{H}]^+$) Calculated: 824.7, Found: 824.8.



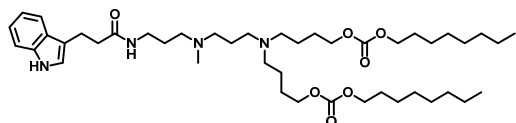
TD7 (31mg, 32%): ^1H NMR (400 MHz, CDCl_3) δ 7.59 (d, J = 14.0 Hz, 1H), 7.34 (d, J = 9.8 Hz, 1H), 7.13 (m, 2H), 7.04 (d, 1H), 4.12 (m, 12H), 3.21 (m, 4H), 2.70 – 2.21 (m, 14H), 1.82 – 1.11 (m, 48H), 0.89 (t, J = 6.7 Hz, 9H). ESI-MS for $\text{C}_{54}\text{H}_{95}\text{N}_4\text{O}_{10}$ ($[\text{M}+2\text{H}]^{2+}$) Calculated: 480.9, Found: 480.7.



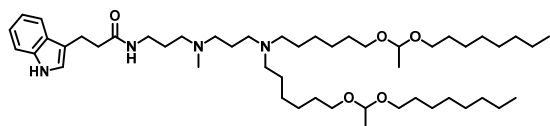
TD8 (29mg, 35%): ^1H NMR (400 MHz, CDCl_3) δ 7.57 (d, J = 14.0 Hz, 1H), 7.37 (d, J = 10.2 Hz, 1H), 7.23 – 6.96 (m, 3H), 4.66 (m, 3H), 3.72 – 3.30 (m, 12H), 3.30 – 3.07 (m, 4H), 2.72 – 2.23 (m, 14H), 1.97 – 1.01 (m, 58H), 0.89 (t, J = 6.7 Hz, 9H). ESI-MS for $\text{C}_{57}\text{H}_{106}\text{N}_4\text{O}_7$ ($[\text{M}+\text{H}]^+$) Calculated: 959.8, Found: 959.9.



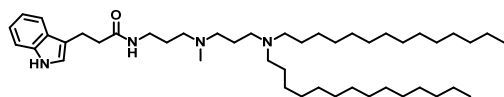
TD9 (25mg, 33%): ^1H NMR (400 MHz, CDCl_3) δ 7.59 (d, 1H), 7.47 – 7.29 (d, 1H), 7.13 (m, 2H), 7.04 (d, J = 2.0 Hz, 1H), 4.68 (s, 4H), 3.67 – 3.44 (m, 8H), 3.23 (m, 4H), 2.67 – 2.25 (m, 12H), 2.11 (s, 3H), 1.79 – 1.16 (m, 36H), 0.90 (t, J = 6.8 Hz, 6H). ESI-MS for $\text{C}_{44}\text{H}_{80}\text{N}_4\text{O}_5$ ($[\text{M}+\text{H}]^+$) Calculated: 745.6, Found: 745.7.



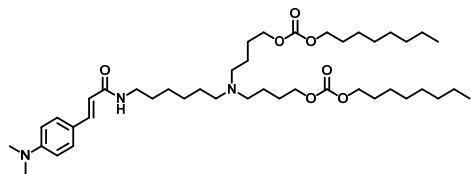
TD10 (27mg, 35%): ^1H NMR (300 MHz, CDCl_3) δ 7.60 (d, J = 7.7 Hz, 1H), 7.34 (d, J = 10.5 Hz, 1H), 7.24 – 7.04 (m, 2H), 7.03 (d, J = 1.8 Hz, 1H), 6.94 – 6.67 (m, 1H), 4.26 – 4.00 (m, 8H), 3.28 (m, 2H), 3.12 (m, 3H), 2.68 – 2.51 (m, 2H), 2.50 – 2.20 (m, 10H), 2.12 (s, 3H), 1.75 – 1.08 (m, 40H), 0.89 (t, J = 6.6 Hz, 6H). ESI-MS for $\text{C}_{44}\text{H}_{76}\text{N}_4\text{O}_7$ ($[\text{M}+\text{H}]^+$) Calculated: 773.6, Found: 773.7.



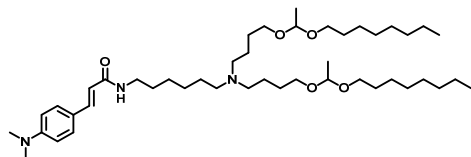
TD11 (32mg, 39%): ^1H NMR (300 MHz, CDCl_3) δ 7.58 (d, J = 7.7 Hz, 1H), 7.34 (d, J = 10.4 Hz, 1H), 7.15 (m, 2H), 7.04 (d, 1H), 4.75 – 4.55 (m, 2H), 3.70 – 3.01 (m, 12H), 2.67 – 2.15 (m, 14H), 2.14 – 1.99 (m, 4H), 1.79 – 1.12 (m, 52H), 0.89 (t, J = 6.7 Hz, 6H). ESI-MS for $\text{C}_{50}\text{H}_{92}\text{N}_4\text{O}_5$ ($[\text{M}+\text{H}]^+$) Calculated: 829.7, Found: 829.9.



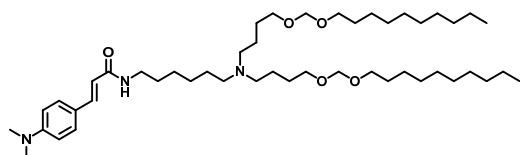
TD12 (32mg, 40%) ^1H NMR (300 MHz, CDCl_3) δ 7.62 (d, $J = 7.7$ Hz, 1H), 7.43 – 7.32 (m, 1H), 7.15 (m, 2H), 7.04 (d, $J = 2.1$ Hz, 1H), 3.39 – 3.18 (m, 2H), 3.13 (m, 2H), 2.71 – 2.36 (m, 8H), 2.36 – 2.17 (m, 4H), 2.08 (s, 3H), 1.78 – 1.08 (m, 60H), 0.93 – 0.84 (t, $J = 6.7$ Hz, 6H). ESI-MS for $\text{C}_{46}\text{H}_{84}\text{N}_4\text{O}$ ($[\text{M}+\text{H}]^+$) Calculated: 709.7, Found: 709.8.



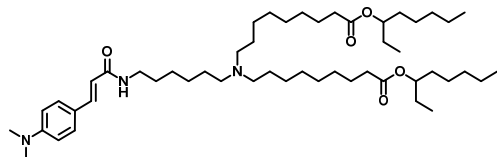
CD1 (30mg, 40%): ^1H NMR (300 MHz, CDCl_3) δ 7.53 (d, $J = 15.5$ Hz, 1H), 7.37 (d, $J = 8.8$ Hz, 2H), 6.71 – 6.51 (m, 2H), 6.23 (d, $J = 15.5$ Hz, 1H), 4.23 – 3.99 (m, 8H), 3.44 – 3.23 (m, 2H), 2.97 (s, 6H), 2.67 – 2.37 (m, 4H), 1.76 – 1.09 (m, 41H), 0.86 (t, $J = 6.6$ Hz, 6H). ESI-MS for $\text{C}_{43}\text{H}_{75}\text{N}_3\text{O}_7$ ($[\text{M}+\text{H}]^+$) Calculated: 746.6, Found: 746.7.



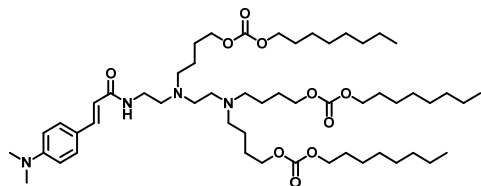
CD2 (31mg, 42%): ^1H NMR (400 MHz, CDCl_3) δ 7.53 (d, 1H), 7.41 (d, $J = 8.8$ Hz, 2H), 6.70 (m, 2H), 6.21 (d, $J = 15.5$ Hz, 1H), 4.68 (m, 2H), 3.64 – 3.54 (m, 4H), 3.46 – 3.35 (m, 6H), 3.02 (s, 6H), 2.84 (m, 4H), 1.75 – 1.07 (m, 45H), 0.89 (t, $J = 6.7$ Hz, 6H). ESI-MS for $\text{C}_{45}\text{H}_{83}\text{N}_3\text{O}_5$ ($[\text{M}+\text{H}]^+$) Calculated: 746.6, Found: 746.6.



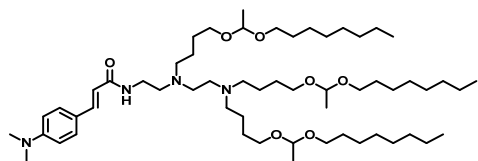
CD3 (30mg, 39%): ^1H NMR (400 MHz, CDCl_3) δ 7.56 (d, $J = 15.5$ Hz, 1H), 7.40 (d, 2H), 6.77 – 6.55 (m, 2H), 6.19 (d, 1H), 4.66 (s, 4H), 3.62 – 3.45 (m, 8H), 3.38 (m, 2H), 3.01 (s, 6H), 2.42 (m, 6H), 1.71 – 1.04 (m, 47H), 0.89 (t, $J = 6.5$ Hz, 6H). ESI-MS for $\text{C}_{47}\text{H}_{87}\text{N}_3\text{O}_5$ ($[\text{M}+\text{H}]^+$) Calculated: 744.7, Found: 774.8.



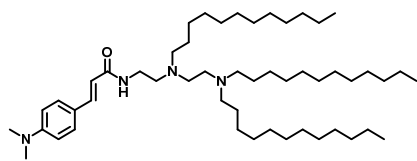
CD4 (25mg, 31%): ^1H NMR (400 MHz, CDCl_3) δ 7.53 (d, 1H), 7.39 (d, $J = 12.7$ Hz, 2H), 6.73 – 6.56 (d, 2H), 6.22 (d, $J = 15.5$ Hz, 1H), 4.90 – 4.75 (m, 2H), 3.35 (m, 2H), 3.01 (s, 6H), 2.40 – 2.20 (m, 4H), 1.80 – 1.11 (m, 52H), 0.90 (t, $J = 6.7$ Hz, 12H). ESI-MS for $\text{C}_{51}\text{H}_{91}\text{N}_3\text{O}_5$ ($[\text{M}+\text{H}]^+$) Calculated: 826.7, Found: 826.7.



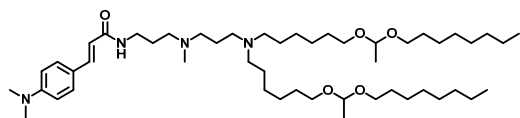
CD5 (31mg, 32%): ^1H NMR (400 MHz, CDCl_3) δ 7.55 (d, J = 15.5 Hz, 1H), 7.42 (d, J = 8.5 Hz, 2H), 6.64 (m, 2H), 6.23 (d, J = 15.3 Hz, 1H), 4.14 (m, 12H), 3.40 (m, 2H), 3.02 (s, 6H), 2.53 (m, 10H), 1.83 – 1.06 (m, 49H), 0.89 (t, J = 6.5 Hz, 9H). ESI-MS for $\text{C}_{54}\text{H}_{96}\text{N}_4\text{O}_{10}$ ($[\text{M}+2\text{H}]^{2+}$) Calculated: 481.4, Found: 481.7.



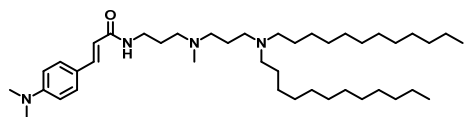
CD6 (35mg, 36%): ^1H NMR (400 MHz, CDCl_3) δ 7.55 (d, J = 7.5 Hz, 1H), 7.42 (d, J = 7.9 Hz, 2H), 6.70 (d, J = 11.3 Hz, 2H), 6.21 (d, J = 15.3 Hz, 1H), 4.74 – 4.53 (m, 3H), 3.67 – 3.26 (m, 14H), 3.03 (s, 6H), 2.54 (m, 8H), 1.74 – 1.08 (m, 56H), 0.90 (t, J = 6.6 Hz, 9H). MALDI-MS for $\text{C}_{57}\text{H}_{108}\text{N}_4\text{O}_7$ ($[\text{M}+\text{H}]^+$) Calculated: 961.8, Found: 961.8.



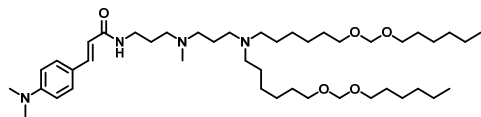
CD7 (28mg, 36%): ^1H NMR (400 MHz, CDCl_3) δ 7.65 – 7.45 (m, 1H), 7.41 (d, J = 8.7 Hz, 2H), 6.75 – 6.54 (m, 2H), 6.25 (d, J = 15.6 Hz, 1H), 3.42 (m, 2H), 3.12 – 2.93 (s, 6H), 2.68 – 2.31 (m, 14H), 1.64 – 1.05 (m, 59H), 0.97 – 0.75 (t, J = 6.7 Hz, 9H). MALDI-MS for $\text{C}_{51}\text{H}_{96}\text{N}_4\text{O}$ ($[\text{M}+\text{H}]^+$) Calculated: 781.8, Found: 781.7.



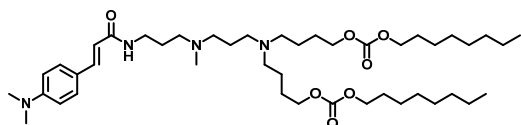
CD8 (34mg, 41%): ^1H NMR (400 MHz, CDCl_3) δ 7.58 – 7.45 (d, 1H), 7.39 (d, J = 8.7 Hz, 2H), 6.68 (d, 2H), 6.29 – 6.10 (d, 1H), 4.76 – 4.53 (m, 2H), 3.68 – 3.52 (m, 4H), 3.47 – 3.29 (m, 6H), 3.00 (s, 6H), 2.61 – 2.30 (m, 12H), 2.25 (s, 3H), 1.84 – 1.15 (m, 55H), 0.88 (t, J = 6.8 Hz, 6H). ESI-MS for $\text{C}_{50}\text{H}_{94}\text{N}_4\text{O}_5$ ($[\text{M}+\text{H}]^+$) Calculated: 831.7, Found: 831.8.



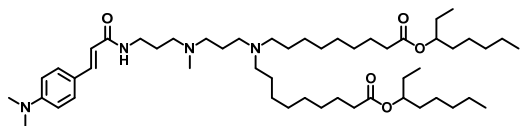
CD9 (29mg, 45%): ^1H NMR (400 MHz, CDCl_3) δ 7.63 – 7.47 (d, 1H), 7.39 (d, J = 14.1 Hz, 2H), 6.70 (d, 2H), 6.22 (d, J = 15.6 Hz, 1H), 3.58 – 3.35 (m, 2H), 3.01 (s, 6H), 2.62 – 2.34 (m, 10H), 2.24 (s, 3H), 1.84 – 1.11 (m, 46H), 0.90 (t, J = 6.8 Hz, 6H). ESI-MS for $\text{C}_{42}\text{H}_{78}\text{N}_4\text{O}$ ($[\text{M}+\text{H}]^+$) Calculated: 655.6, Found: 655.8.



CD10 (31mg, 42%): ^1H NMR (400 MHz, CDCl_3) δ 7.48 (d, J = 14.3 Hz, 1H), 7.39 (d, J = 8.8 Hz, 2H), 6.76 – 6.55 (d, 2H), 6.20 (d, J = 15.6 Hz, 1H), 4.66 (s, 4H), 3.59 – 3.36 (m, 10H), 2.97 (s, 6H), 2.61 – 2.30 (m, 13H), 2.30 – 2.12 (s, 3H), 1.82 – 1.13 (m, 36H), 0.89 (t, J = 6.8 Hz, 6H). ESI-MS for $\text{C}_{44}\text{H}_{82}\text{N}_4\text{O}_5$ ($[\text{M}+\text{H}]^+$) Calculated: 747.6, Found: 747.8.



CD11 (29mg, 38%): ^1H NMR (400 MHz, CDCl_3) δ 7.57 – 7.44 (d, 1H), 7.41 (d, 2H), 6.75 – 6.62 (d, 2H), 6.20 (d, J = 15.6 Hz, 1H), 4.28 – 3.99 (m, 8H), 3.60 – 3.39 (m, 2H), 2.99 (s, 6H), 2.58 – 2.38 (m, 9H), 2.29 (s, 3H), 1.80 – 1.17 (m, 37H), 0.89 (t, J = 6.8 Hz, 6H). ESI-MS for $\text{C}_{44}\text{H}_{78}\text{N}_4\text{O}_7$ ($[\text{M}+\text{H}]^+$) Calculated: 775.6, Found: 775.7.



CD12 (28mg, 33%): ^1H NMR (400 MHz, CDCl_3) δ 7.53 (d, J = 15.5 Hz, 1H), 7.40 (d, J = 12.8 Hz, 2H), 6.70 (d, J = 8.8 Hz, 2H), 6.21 (d, J = 14.5 Hz, 1H), 4.92 – 4.72 (m, 2H), 4.14 (m, 2H), 3.61 – 3.34 (m, 4H), 3.02 (s, 6H), 2.68 – 2.16 (m, 17H), 2.07 (s, 3H), 1.84 – 1.08 (m, 52H), 0.89 (t, J = 6.8 Hz, 12H). ESI-MS for $\text{C}_{52}\text{H}_{94}\text{N}_4\text{O}_5$ ($[\text{M}+\text{H}]^+$) Calculated: 855.7, Found: 855.8.

2.3 BBB crossing mechanism study

The bEnd.3 cells were seeded in the upper chamber of a 24-well transwell (Corning, $1 \times 10^5/\text{well}$) with a pore diameter of 0.4 μm to construct an *in vitro* BBB model. The cells were cultured for 7-9 days with medium changed every other day. The integrity of the BBB model was verified by measuring transendothelial electrical resistance (TEER), with a value over 60 $\Omega \cdot \text{cm}^2$. The fluorescein isothiocyanate (FITC)-dextran trans-epithelial permeability assay was further used to confirm the integrity of the trans-endothelial cell monolayer.²⁰ The bEnd.3 cells in the upper chamber were pre-treated with EIPA (10 μM), M β CD (4.5mM), CPZ (5 $\mu\text{g}/\text{mL}$) and MK-0752 (10nM) before the addition of MK16 BLNPs encapsulating Alexa Fluor 647 RNA. The fluorescence spectrophotometer was used to detect the fluorescence intensity of the lower chamber after 12 h. To evaluate whether MK16 BLNPs could deliver mRNA to cells after BBB crossing, N2a cells ($1 \times 10^5/\text{well}$) were seeded in the lower chamber of the transwell assay one day before BLNP treatment. The bEnd.3 cells in the upper chamber were pre-treated with M β CD (4.5mM) and nirogacestat (10nM) before the addition of MK16 BLNP-FLuc mRNA. After 18h, the cells in both chambers were harvested by trypsin and treated with Bright-Glo luciferase substrate. After a 5-minute incubation, the relative luminescence intensity was quantified using the BioTek Cytation 5 Cell Imaging Multimode Reader. To evaluate BBB crossing of MK16 BLNPs *in vivo*, the mice were treated with M β CD (100 mg/kg) or NGST (30 mg/kg) 1h before administration of MK16 BLNPs encapsulating Alexa Fluor 647 RNA. After 1h, the brains were dissected from mice and immediately imaged using a Xenogen IVIS imaging system. The signals in the brain were quantified using regions of interest (ROIs).

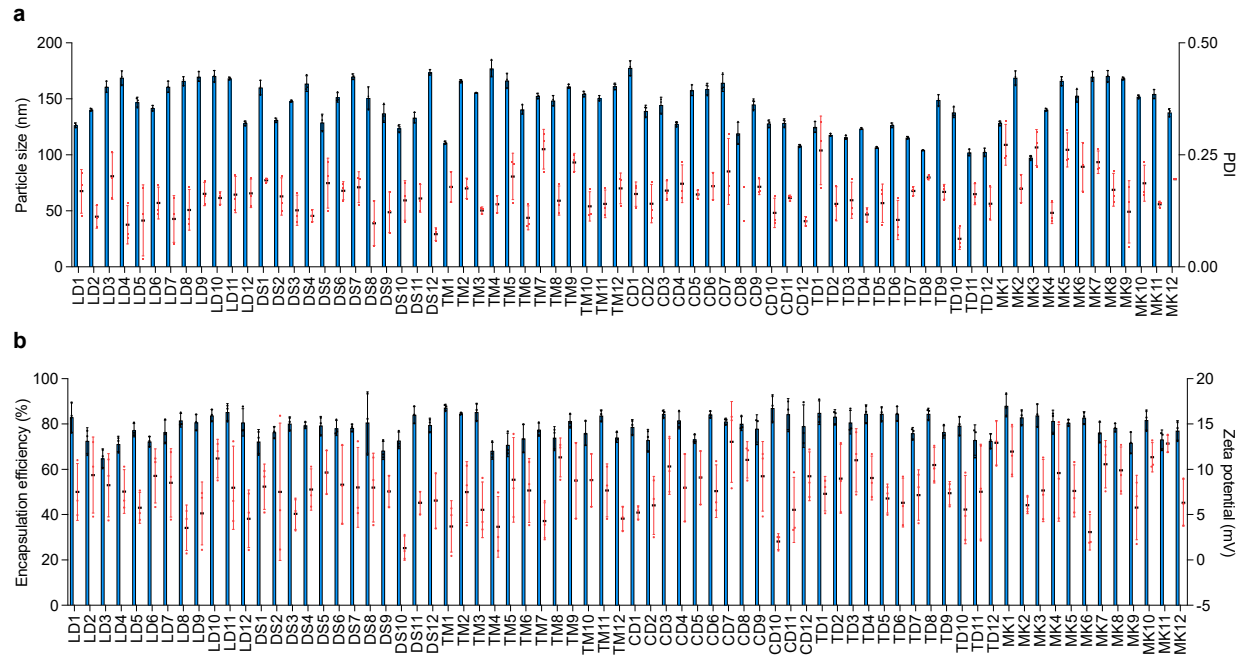
2.4 *In vivo* biocompatibility evaluation

Mouse plasma and major organs were collected after 6h, 24h and 48h post MK16 BLNPs or MC3 LNPs administration at the FLuc mRNA dose of 1 mg/kg. For Ai14 mouse model, mouse plasma and major organs were collected after 5 days post-final administrations. The whole blood was collected in tubes with sodium citrate and centrifugated to collect plasma. Mouse cytokines and chemokines were evaluated by mouse cytokine/chemokine 32-plex discovery assay® (MD32, Eve Technologies). The level of alanine aminotransferase, aspartate aminotransferase, and blood urea nitrogen levels were quantified using enzyme-linked immunosorbent assay (ELISA) kit. To examine potential systemic or local toxicity, tissue sections were subjected to hematoxylin and eosin (H&E) staining followed by examination using an optical microscope.

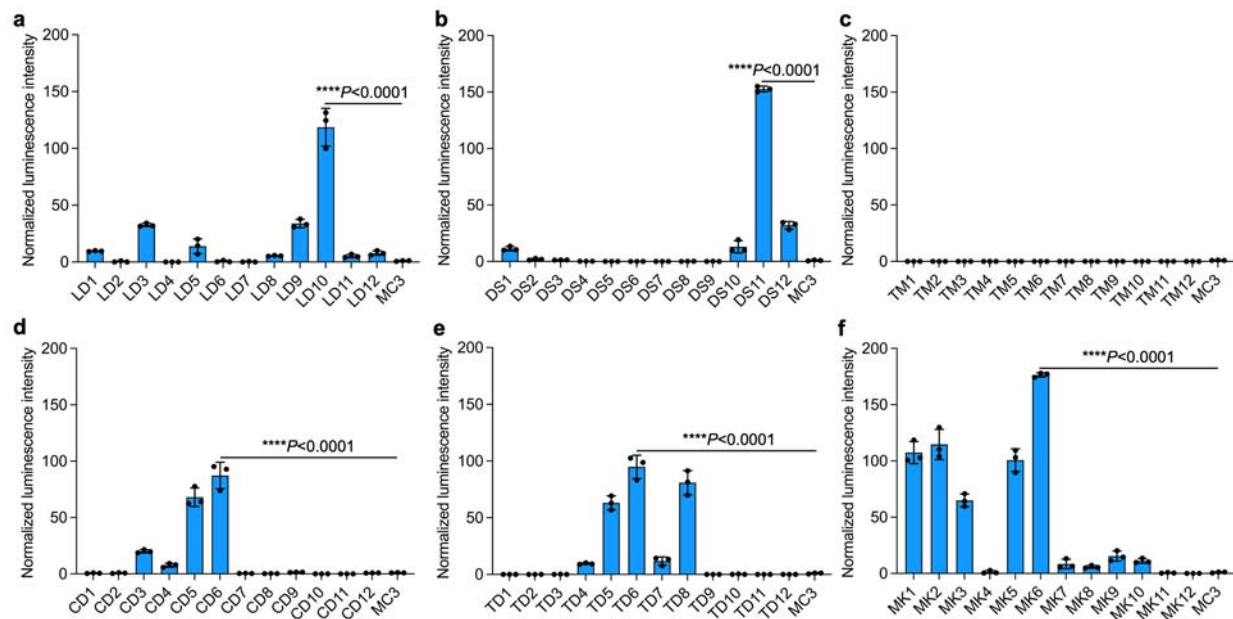
For the bulk RNA sequencing study, the mice were intravenously injected with MK16 BLNP-FLuc mRNA (1 mg/kg) or orally administered with MK-0752 (50 mg/kg). After 24h, the brain, liver and spleen tissues of the treated mice were harvested and stored in RNA later solution (Thermo Fisher Scientific) at -20 °C until RNA extraction. mRNA in brain, liver and spleen tissues were extracted using RNeasy Mini kit (QIAGEN) following the manufacturer's instructions. The quantification of the extracted total RNA was performed utilizing a Nanodrop One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific), and the integrity of the RNA was assessed via an Agilent 2100 Bioanalyzer (Agilent Technologies). The construction of the RNA-seq library commenced with the enrichment of mRNA through oligo(dT) beads, followed by its random fragmentation. Complementary DNA (cDNA) was synthesized using random hexamers primers. Subsequent processes included terminal repair, adaptor ligation, size selection, and PCR enrichment to finalize the double-stranded cDNA library preparation (Illumina). The prepared cDNA libraries were then sequenced on an Illumina NovaSeq 6000 system, enabling deep sequencing.

Adapter sequences from the bulk RNA-seq raw reads were removed using fastp software.²¹ The quality of the FASTQ files was assessed both before and after adapter trimming using FASTQC software to ensure data integrity. The cleaned reads were then aligned to the mouse reference genome assembly GRCm38.99 using HISAT2, version 2.2.1, for precise mapping.²² The alignment outputs, in SAM format, were converted to compressed BAM files, which were subsequently sorted and indexed using SAMtools for efficient data handling.²³ Feature identification and quantification of total counts per feature were conducted using featureCounts, version 1.6.4, based on annotations from the GENCODE GTF file.²⁴ Data normalization, dispersion estimation, and differential expression analysis were performed with the DESeq2 R package, identifying genes with significant expression differences at a false discovery rate (FDR) < 0.05.²⁵ The DESeq2 package also facilitated the adjustment of p-values for multiple testing through the Benjamini and Hochberg method. Normalized counts were used to generate the heatmap.

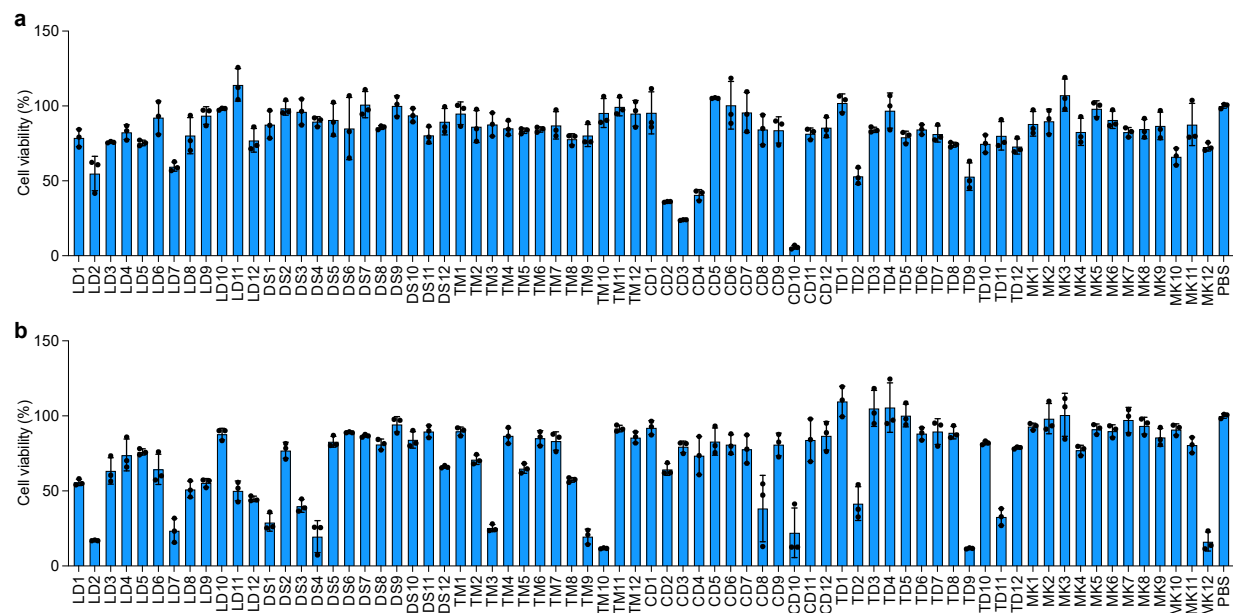
3. Supplementary figures



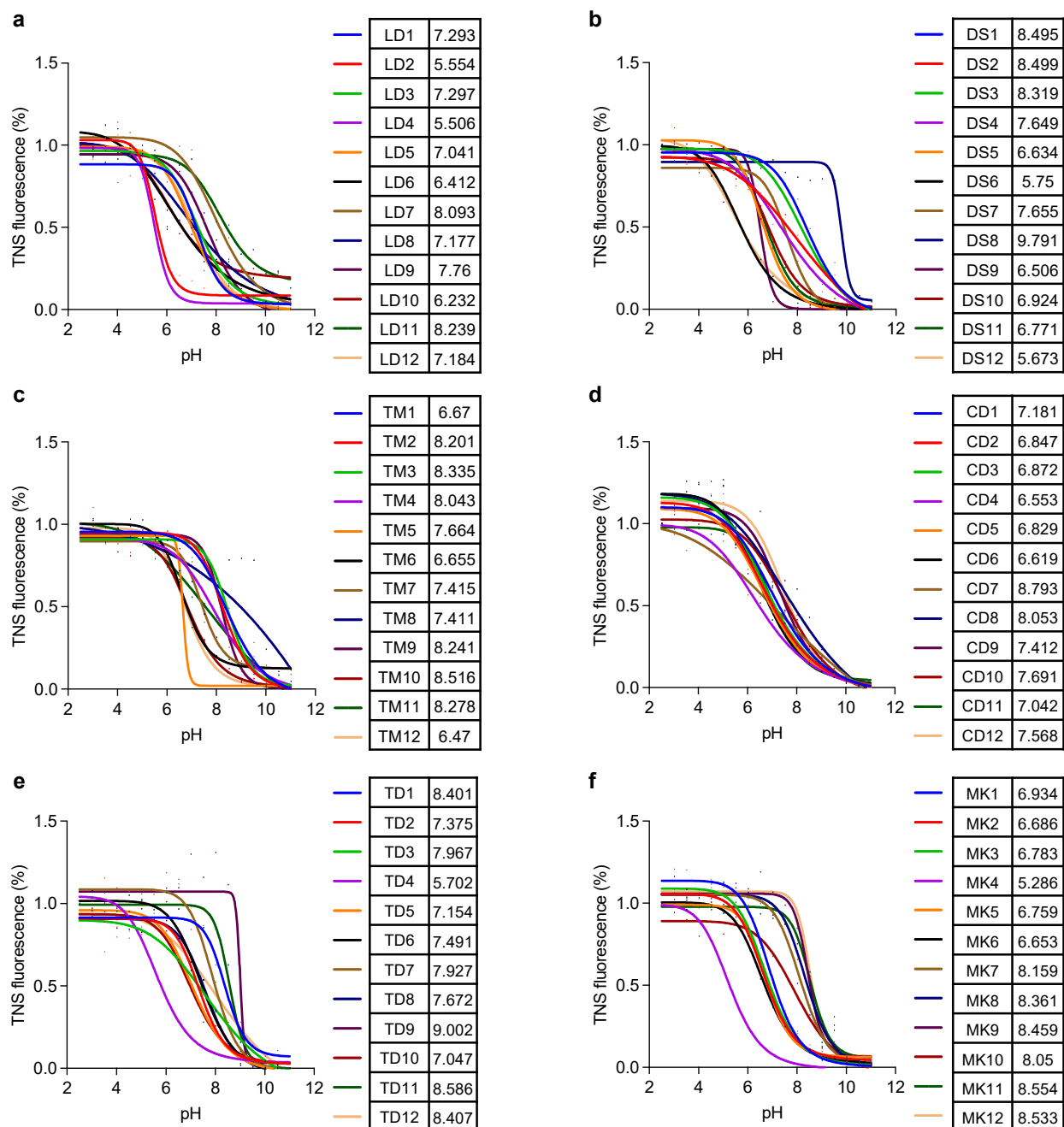
Supplementary Fig. 1. Characterizations of BLNP-FLuc mRNA. (a) Particle size and PDI of BLNP-FLuc mRNA. (b) Encapsulation efficiency and Zeta potential of BLNP-FLuc mRNA. Data in **a** and **b** are from $n = 3$ biologically independent samples. Data are presented as mean $\pm$ SD.



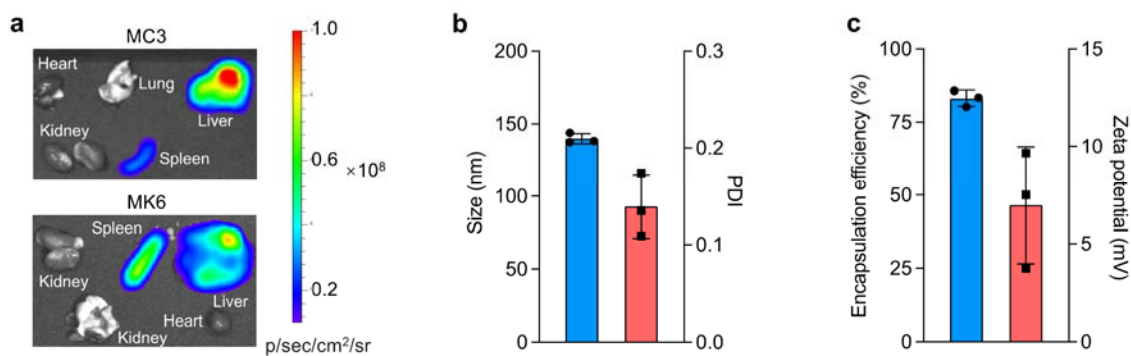
Supplementary Fig. 2. BLNP-FLuc mRNA delivery in bEnd.3 cells. (a-f) The luminescence intensity of BLNP-FLuc mRNA treated bEnd.3 cells. The intensity was normalized to the MC3 LNPs group. Data in a-f are from $n = 3$ biologically independent samples. Data are presented as mean $\pm$ SD. Statistical significance was determined by one-way ANOVA followed by Dunnett's multiple comparison test. **** $P < 0.0001$.



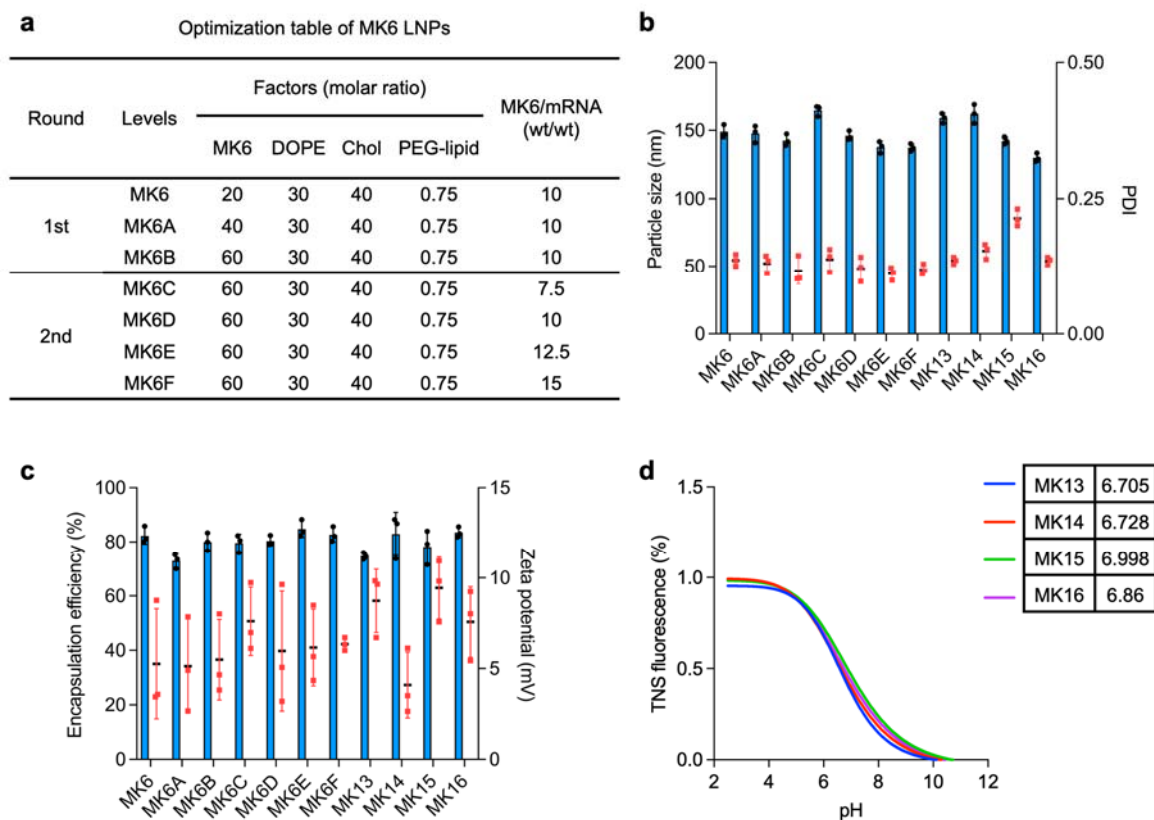
Supplementary Fig. 3. Cell viability of BLNPs in N2a cells and bEnd.3 cells. **(a)** Cell viability of BLNPs in N2a cells. The viability was normalized to the PBS group. **(b)** Cell viability of BLNPs in bEnd.3 cells. Data in **a-b** are from $n = 3$ biologically independent samples. Data are presented as mean $\pm$ SD.



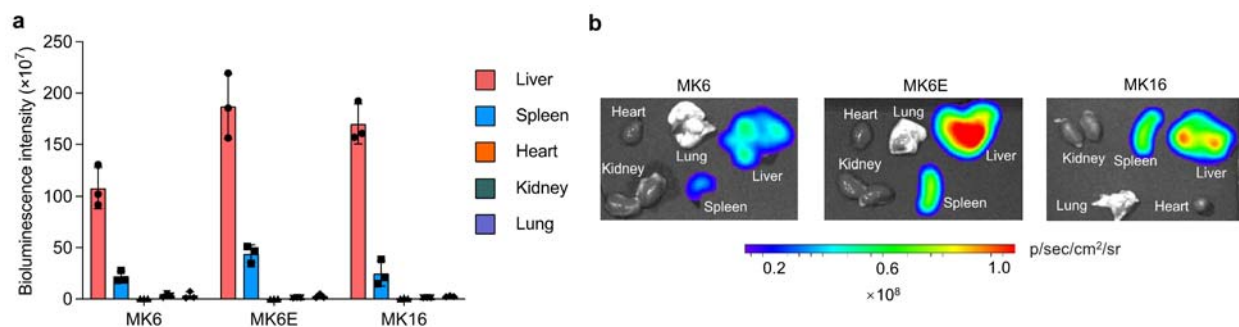
Supplementary Fig. 4. Representative TNS assay curves for determining the apparent pKa of BLNPs. **(a)** The apparent pKa of LD BLNPs; **(b)** The apparent pKa of DS BLNPs; **(c)** The apparent pKa of TM BLNPs; **(d)** The apparent pKa of CD BLNPs; **(e)** The apparent pKa of TD BLNPs; **(f)** The apparent pKa of MK BLNPs; n = 3 biologically independent samples.



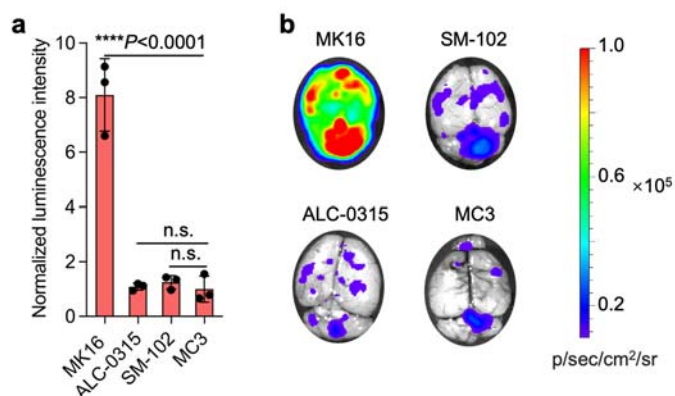
Supplementary Fig. 5. Characterizations of MK6 BLNPs. **(a)** Representative luminescence intensity in major organs of mice treated with MK6 BLNPs or MC3 LNPs. **(b)** Size and PDI of MK6 BLNPs. **(c)** Encapsulation efficiency and zeta potential of MK6 BLNPs. Data in **a-c** are from $n = 3$ biologically independent samples. Data are presented as mean $\pm$ SD.



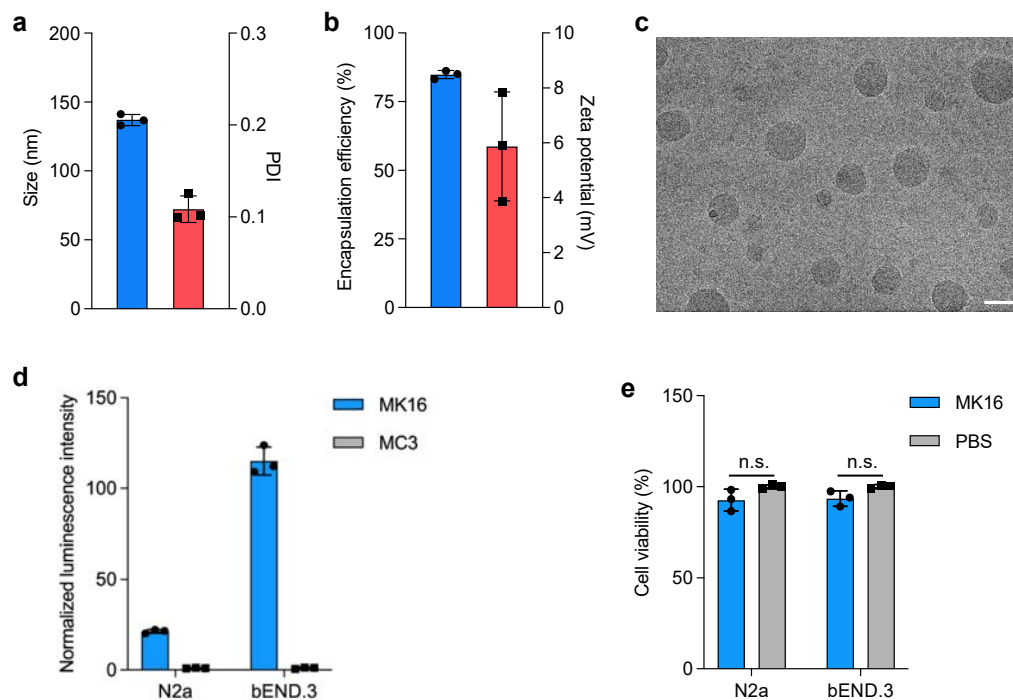
Supplementary Fig. 6. Optimization of MK6 BLNPs. **(a)** Optimization table of MK6 BLNP series. **(b)** Particle size and PDI of MK6 BLNP series. **(c)** Encapsulation efficiency and zeta potential of MK6 BLNP series. **(d)** Representative TNS assay curves for determining the apparent pKa of MK13, MK14, MK15 and MK16 BLNPs. Data in **b-d** are from $n = 3$ biologically independent samples. Data are presented as mean $\pm$ SD.



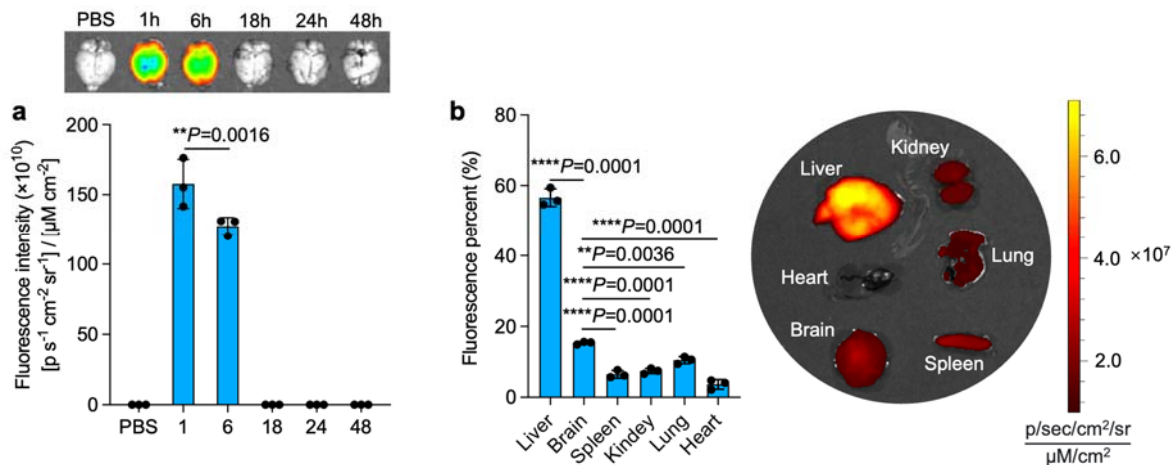
Supplementary Fig. 7. Biodistribution of MK16 BLNPs. (a) Bioluminescence quantification of the major organs from MK6 BLNP-treated mice. (b) Representative luminescence intensity in major organs of mice treated with MK6 BLNPs, MK6E BLNPs, or MK16 BLNPs. Data in **a-b** are from $n = 3$ biologically independent samples and are presented as mean $\pm$ SD.



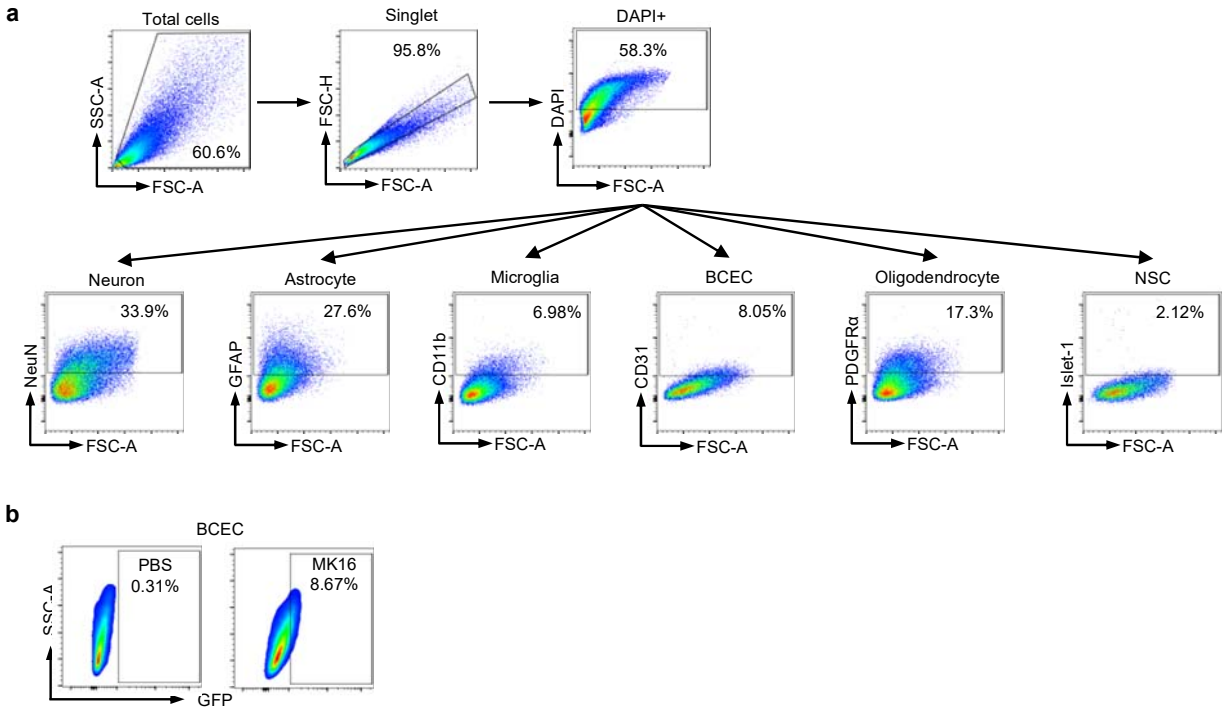
Supplementary Fig. 8. Comparison of mRNA delivery efficiency to the brain between MK16 BLNPs and clinically approved LNP formulations. **(a)** The luminescence intensity of brain tissues from the mice after systemic delivery of MK16 BLNPs or the clinically approved LNP formulations. The intensity was normalized to the MC3 LNPs group. **(b)** Representative images of brains from the mice *i.v.* treated with MK16 BLNP, ALC-0315 LNP, SM-102 LNP, and MC3 LNP. Data in **a-b** are from $n = 3$ biologically independent samples and are presented as mean $\pm$ SD. Statistical significance and P values were determined by one-way ANOVA followed by Dunnett's multiple comparison test. n.s., not significant, $P > 0.05$, **** $P < 0.0001$.



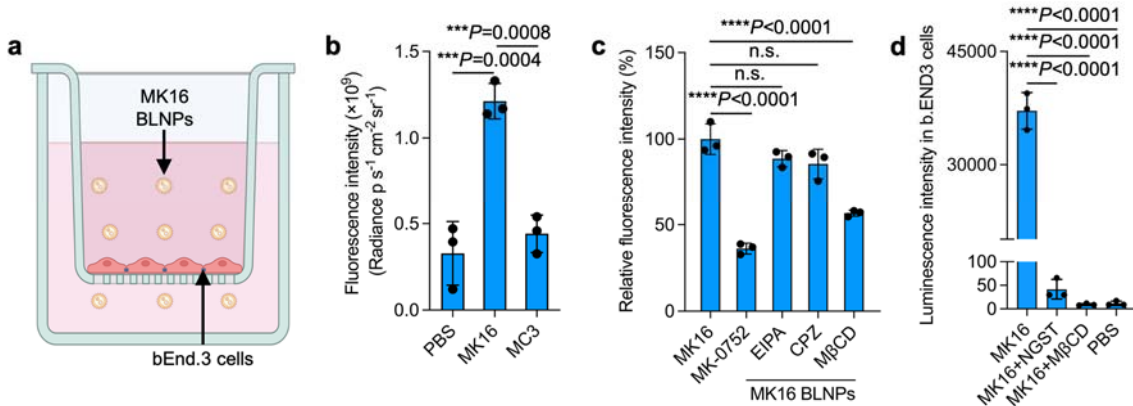
Supplementary Fig. 9. Characterizations of MK16 BLNPs. **(a)** Size and PDI of MK16 BLNPs. **(b)** Encapsulation efficiency and zeta potential of MK16 BLNPs. **(c)** Cryo-EM image of MK16 BLNPs. Scale bar = 100 nm. **(d)** Normalized luminescence intensity of MK16 BLNP in N2a and bEnd.3 cells. The intensity was normalized to MC3 LNP. **(e)** Cell viability of MK16 BLNPs in N2a cells and bEnd.3 cells. Data in **a-e** are from $n = 3$ biologically independent samples. Data are presented as mean $\pm$ SD. Statistical significance and P values were determined by the two-tailed Student's t -test. n.s., not significant, $P > 0.05$.



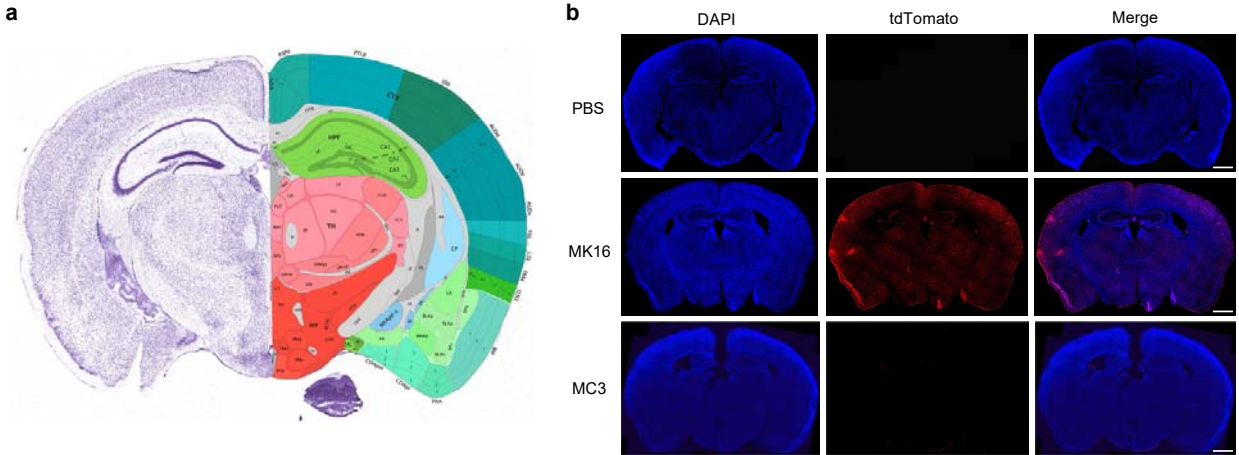
Supplementary Fig. 10. Kinetics and biodistribution of MK16 BLNPs after systemic administration. **(a)** Dynamic fluorescence intensity of brain tissues from the mice i.v. injected with MK16 BLNP loaded with Alexa-Fluor 647 labeled RNAs. **(b)** Fluorescence intensity of major tissues from the mice i.v. injected with MK16 BLNP loaded with Alexa-Fluor 647 labeled RNAs. Data in **a** and **b** are from $n = 3$ biologically independent samples and are presented as mean $\pm$ SD. Statistical significance was determined by one-way ANOVA followed by Dunnett's multiple comparison test. $**P < 0.01$, $***P < 0.001$.



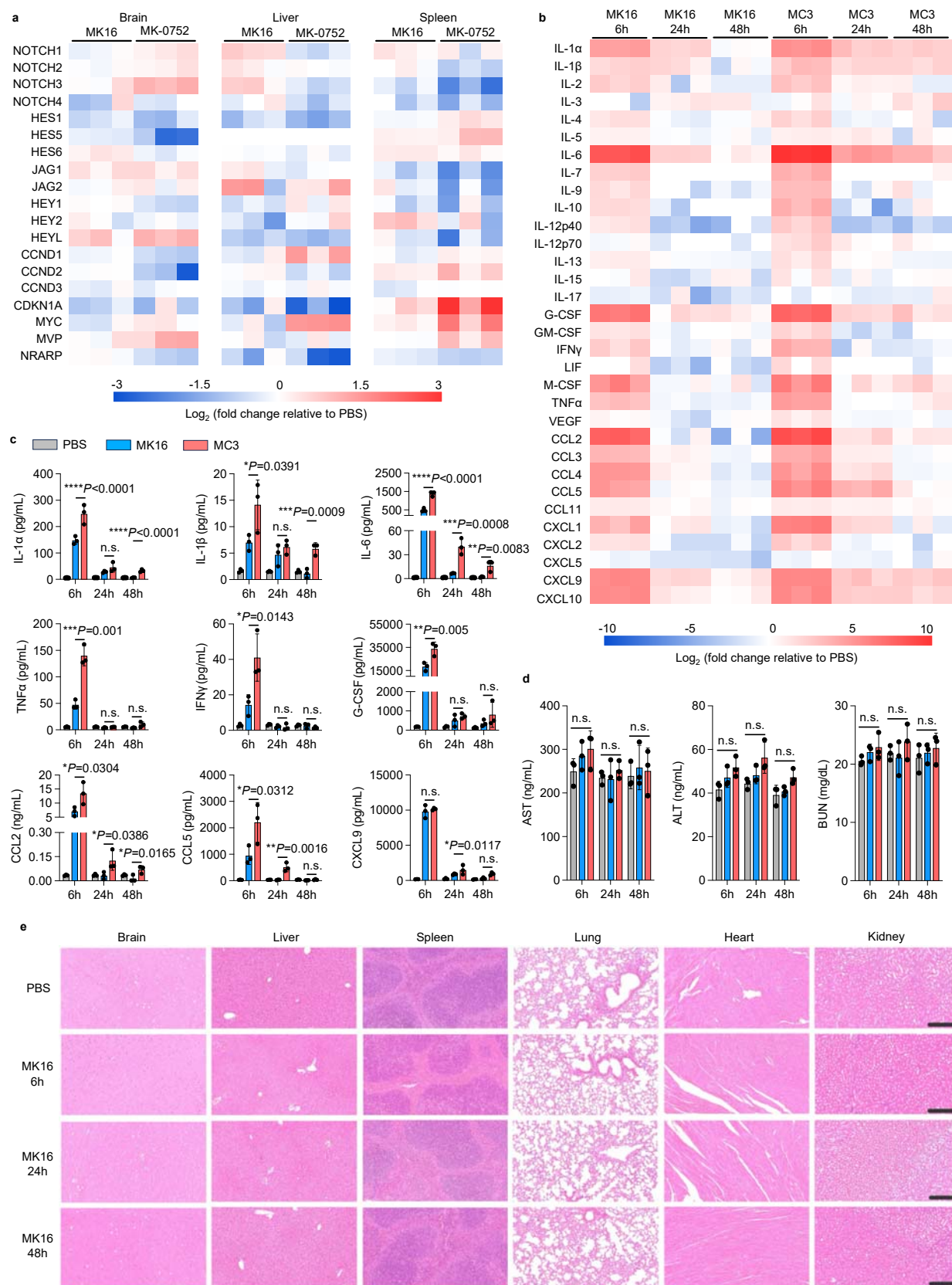
Supplementary Fig. 11. Gating scheme for immunofluorescence flow cytometry analysis of brain cells. **(a)** The forward and side scatter properties were used to determine the 'Cell' gating and confirmed by positive labeling with nuclear DAPI staining. The cell markers used for subsequent gating were: NeuN for neurons, GFAP for astrocytes, CD11b for microglia, CD31 for brain capillary endothelial cell (BCEC), Platelet-derived growth factor receptor alpha (PDGFR α) for oligodendrocyte and Islet-1 for neural stem cell (NSC). **(b)** Representative histograms of GFP expression in BCEC after intravenous injection of MK16 BLNP-GFP mRNA (1.0 mg/kg).



Supplementary Fig. 12. BBB transportation mechanism of MK16 BLNPs. **(a)** Illustration of a bEND.3 transwell cell assay for BBB penetration assessment. **(b)** Fluorescence intensity of MK16 BLNPs loaded with Alexa Fluor 647 labeled RNAs in transwell medium. **(c)** Relative fluorescence intensity of MK16 BLNP-Alexa Fluor 647-RNA in transwell medium when bEnd.3 cells were pre-treated with various endocytic inhibitors or MK-0752 compounds. The intensity was normalized to the MK16 BLNP group. **(d)** The luminescence intensity of bEND.3 cells in the upper compartment after MK16 BLNP-FLuc mRNA treatment to the upper compartment. The bEND.3 cells were pre-treated with either M β CD or NGST. Data in **b-d** are from $n = 3$ biologically independent samples and are presented as mean $\pm$ SD. Statistical significance and P values were determined by one-way ANOVA followed by Dunnett's multiple comparison test. n.s., not significant, $P > 0.05$, *** $P < 0.001$, **** $P < 0.0001$.

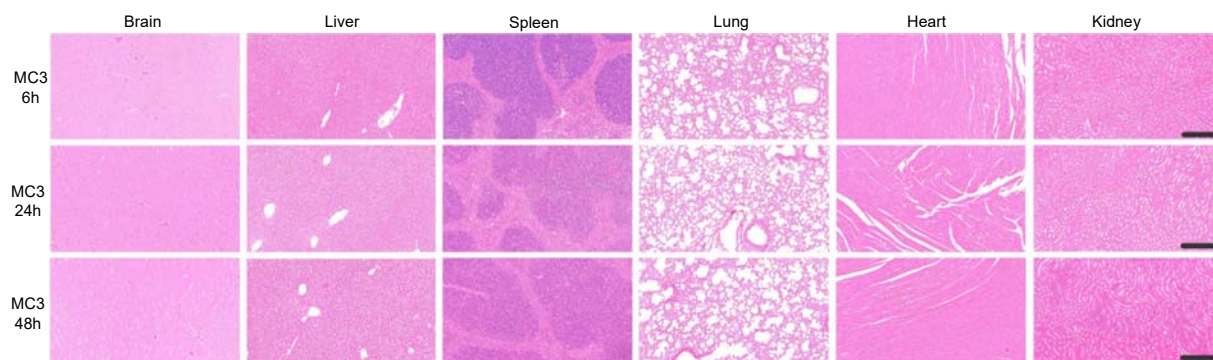


Supplementary Fig. 13. Delivery distribution of MK16-Cre mRNA BLNPs in mouse brain. **(a)** Coronal sections of the mouse brain.²⁶ **(b)** Representative brain images of the Ai14 mice intravenously injected with PBS, MK16 BLNP-Cre mRNA and MC3 LNP-Cre mRNA. n = 3 biologically independent samples. Scale bar: 1 mm. The “Merge” image is also shown in Figure 4b in the main figure section.

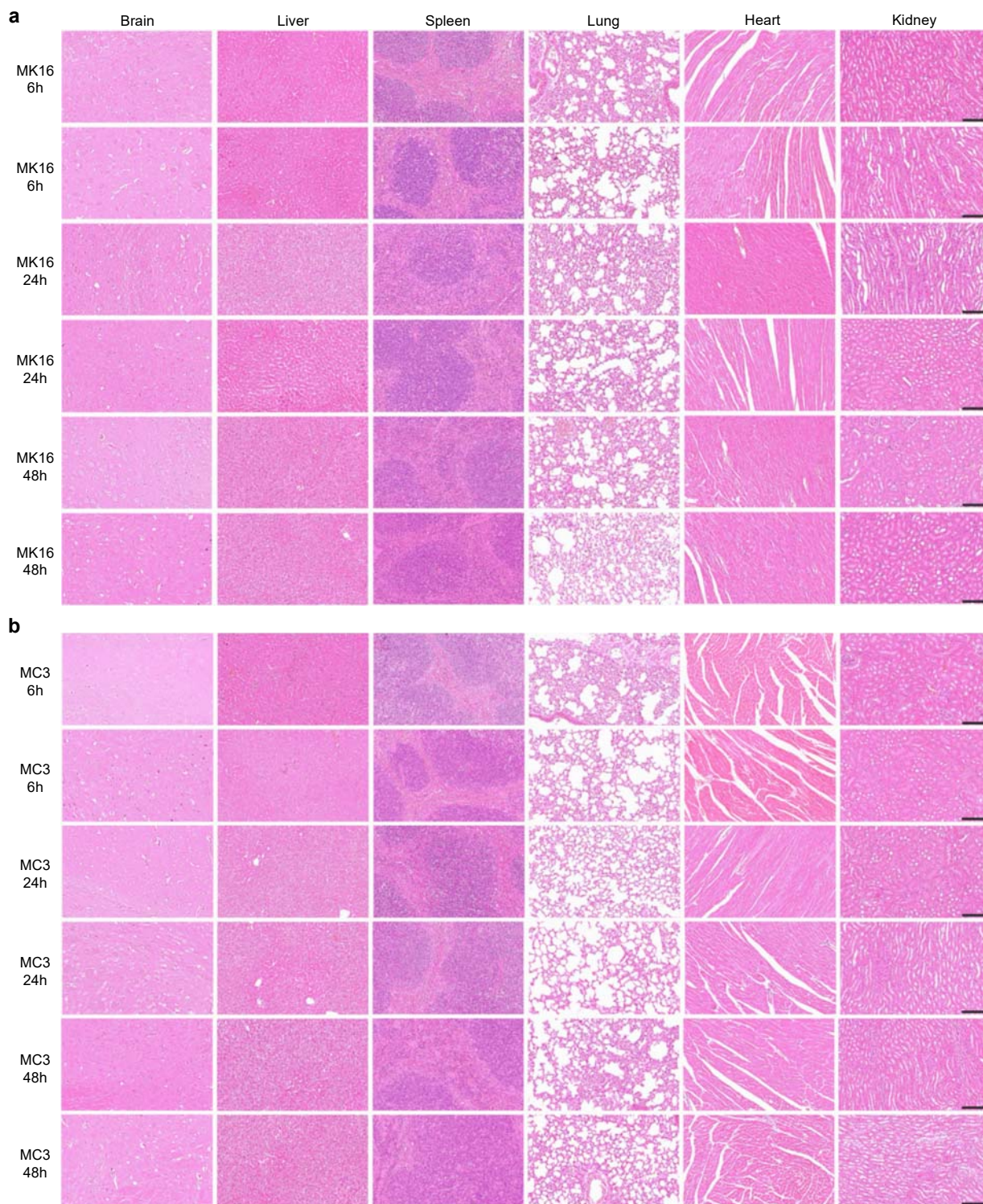


Supplementary Fig. 14. Safety profiles of MK16 BLNPs for systemic delivery. (a) Heatmaps displaying

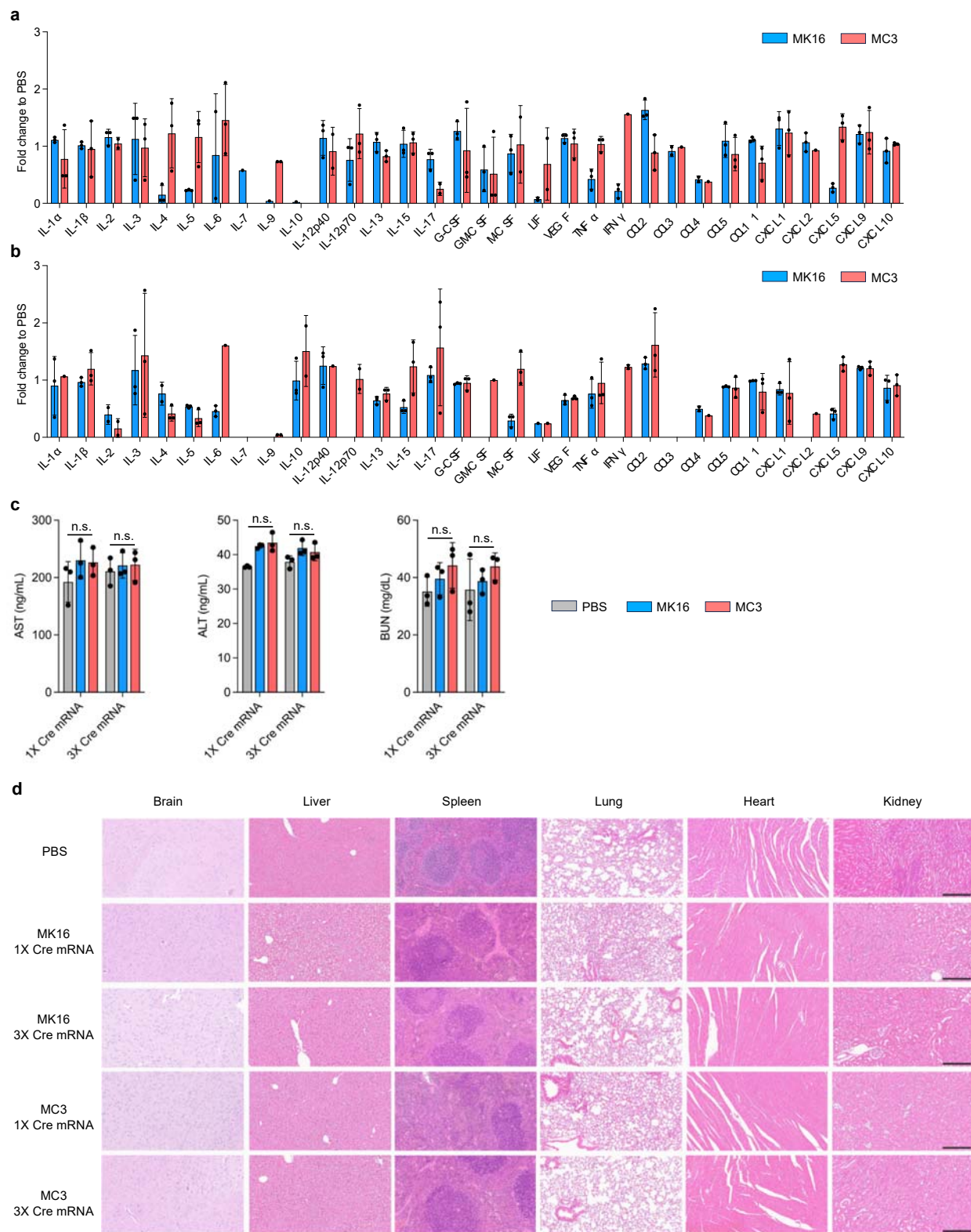
relative NOTCH-related gene expression in brain, liver and spleen of the mice treated with MK16 BLNPs or MK-0752 as compared to PBS, and measured by RNA-sequencing. Results are calculated based on normalized counts and indicate three biologically independent samples. **(b)** Heatmaps displaying dynamic expression of cytokines and chemokines in blood of the mice after systemic administration of MK16 BLNPs or MC3 LNPs. Results indicate three replicates. **(c)** Dynamic expression level of certain critical cytokines and chemokines from **b**. **(d)** Dynamic hepatic and renal functional evaluations of the mice after systemic administration of MK16 BLNPs or MC3 LNPs. ALT, alanine transaminase; AST, aspartate aminotransferase; BUN, blood urea nitrogen. **(e)** Representative histopathological images from mice after systemic administration of MK16 BLNPs. Scale bars, 250 μ m. Data in **a-e** are from $n = 3$ biologically independent samples and are presented as the mean $\pm$ SD. Statistical significance and P values were determined by one-way ANOVA followed by Dunnett's multiple comparison test. n.s., not significant, $P > 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.



Supplementary Fig. 15. Representative histopathological images from mice after systemic administration of MC3 LNPs at different time intervals. n = 3 biologically independent samples. Scale bars, 250 μm.

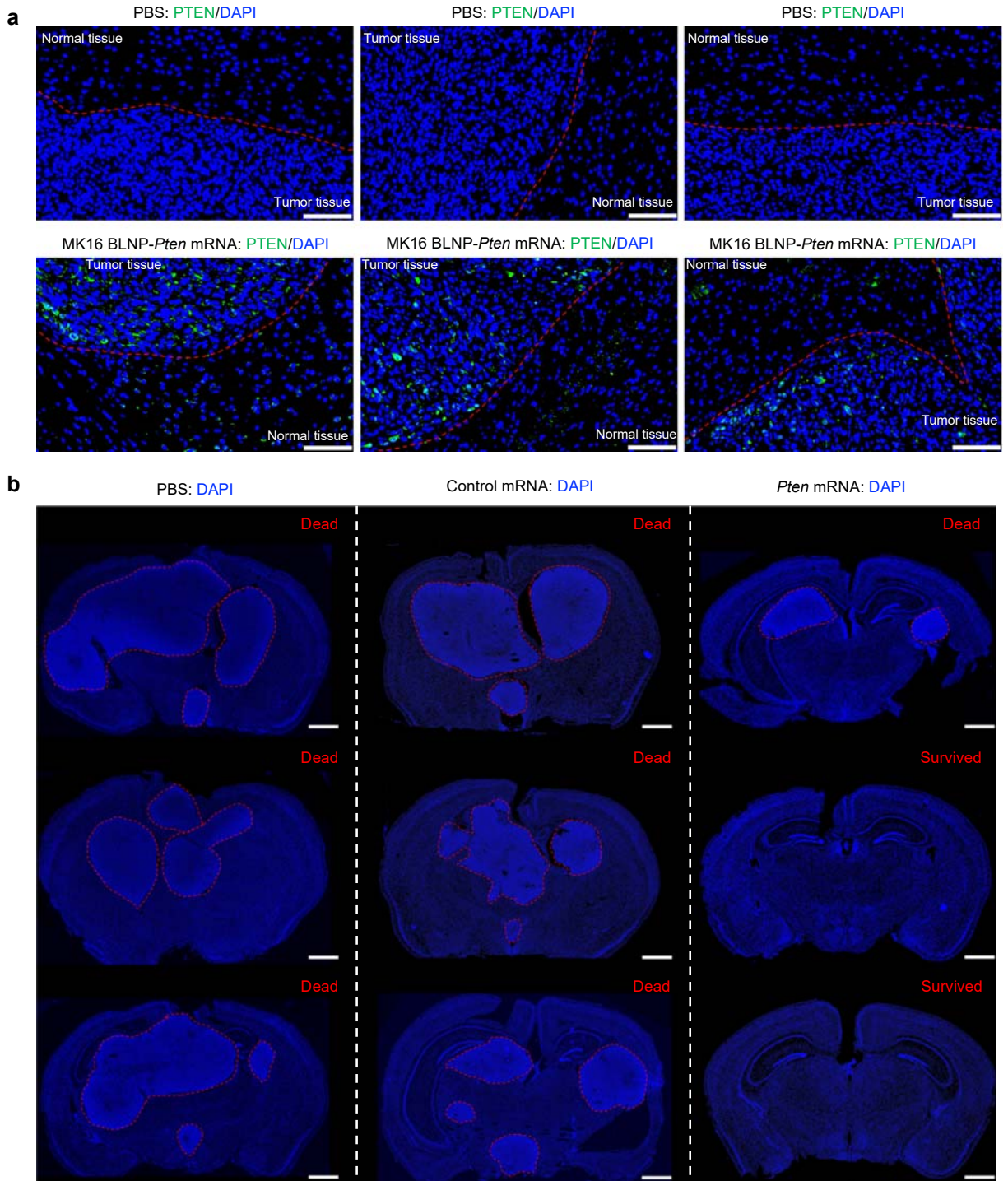


Supplementary Fig. 16. Histopathological images from mice after systemic administration of MK16 (**a**) or MC3 LNPs (**b**) at different time intervals. n = 3 biologically independent samples for **a** and **b**. These samples were from the remaining subjects in the same safety experiment depicted in **Supplementary Fig.14e** and **Supplementary Fig.15**. Scale bars, 125 μ m.

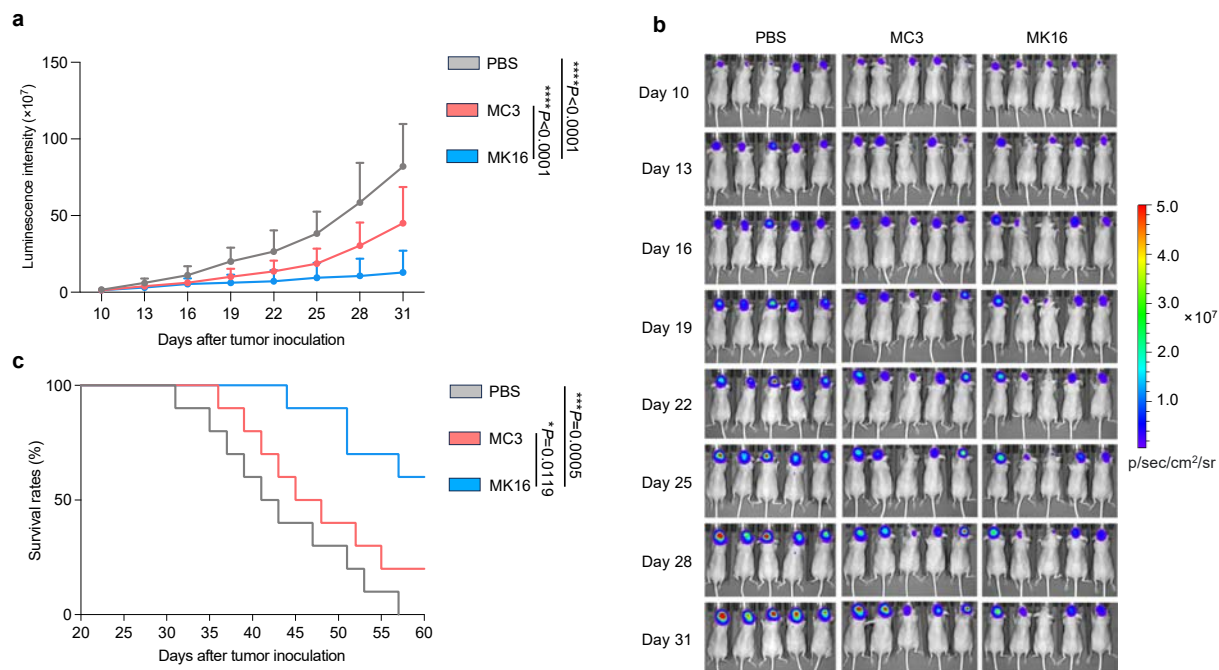


Supplementary Fig. 17. Safety profiles of MK16 BLNPs for repeated injections. Expression of cytokines and chemokines in blood of the mice after single (a) or triple (b) systemic administrations of MK16 BLNPs or MC3 LNPs. (c) Hepatic and renal functions of the mice after single or triple systemic administration of

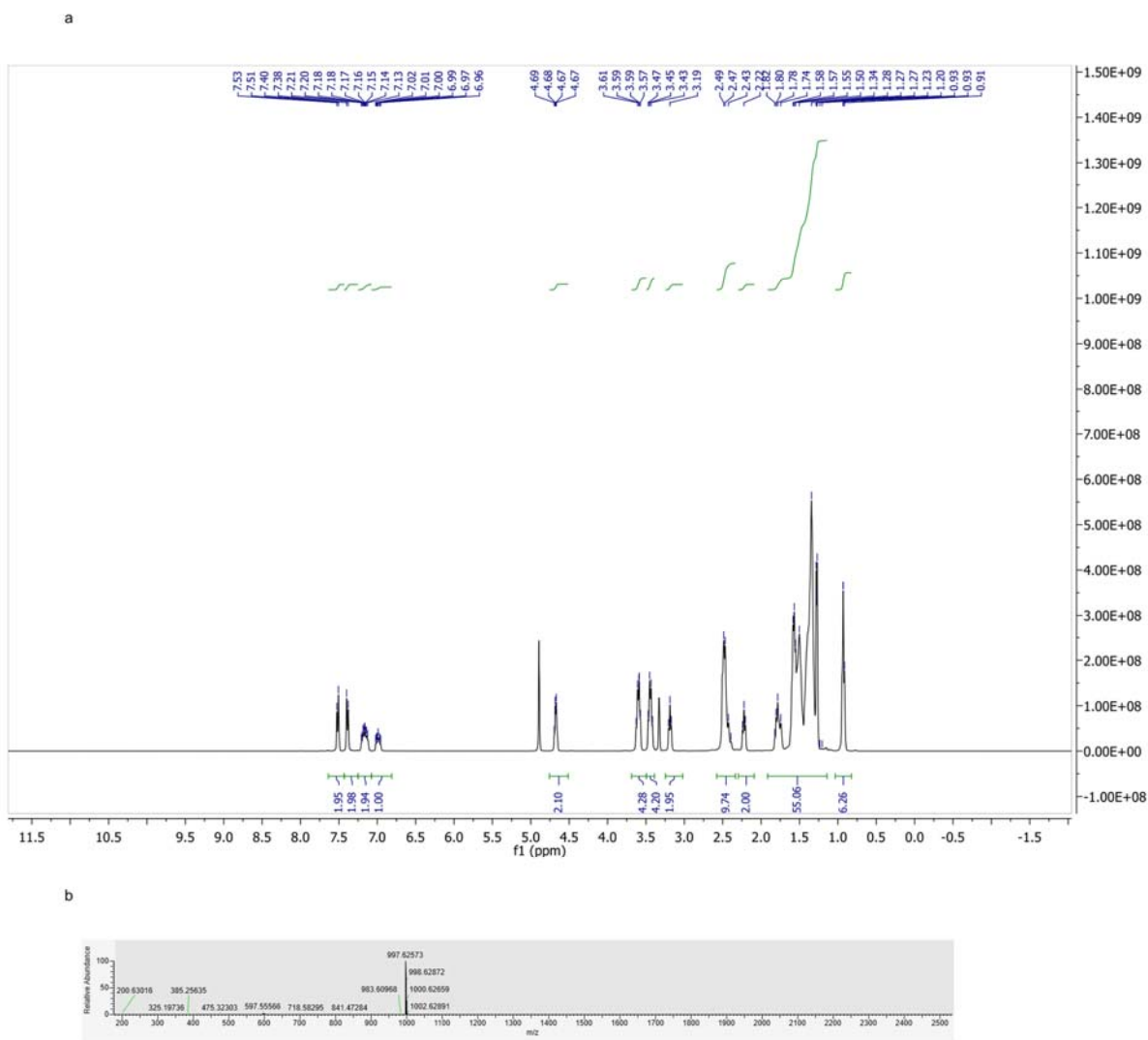
MK16 BLNPs or MC3 LNPs. ALT, alanine transaminase; AST, aspartate aminotransferase; BUN, blood urea nitrogen. **(d)** Histopathological images from mice after single or triple systemic administration of MK16 BLNPs or MC3 LNPs. Scale bars, 250 μ m. Data in **a-d** are from n = 3 biologically independent samples and are presented as the mean $\pm$ SD. Statistical significance and P values were determined by one-way ANOVA followed by Dunnett's multiple comparison test. n.s., not significant. P>0.05.



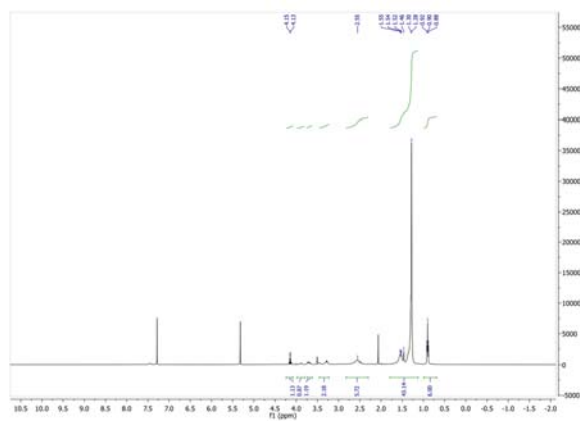
Supplementary Fig. 18. Delivery of MK16 BLNP-*Pten* mRNA in GBM regions and the size of tumor residues after treatment. **a**, Expression of PTEN in the GBM region of the mouse brain after intravenous administration of MK16 BLNP-*Pten* mRNA. Scale bar: 100 μ m. $n = 3$ biologically independent samples. **b**, GBM tumor residues with different treatments. Scale bar: 1 mm.



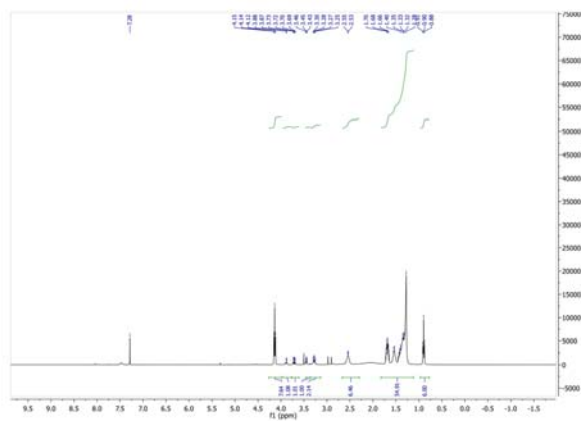
Supplementary Fig. 19. MK16 BLNP-*Pten* mRNA outperforms MC3 LNP-*Pten* mRNA in treating orthotopic glioblastoma. **a**, Luminescence intensity of orthotopic GBM tumor tissues in the mice *i.v.* treated with PBS, MC3 LNP-*Pten* mRNA and MK16 BLNP-*Pten* mRNA at the mRNA dose of 1.0 mg/kg, respectively. **b**, Representative IVIS images of the tumor-bearing mice. **c**, Mouse survival over time. $n=10$ mice for all groups. Data in **a** are presented as the mean $\pm$ SD. Statistical significance and P values in **a** were determined by two-way ANOVA with Fisher's LSD. Statistical significance and P values in **c** were analyzed by the log-rank (Mantel-Cox) test. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$.



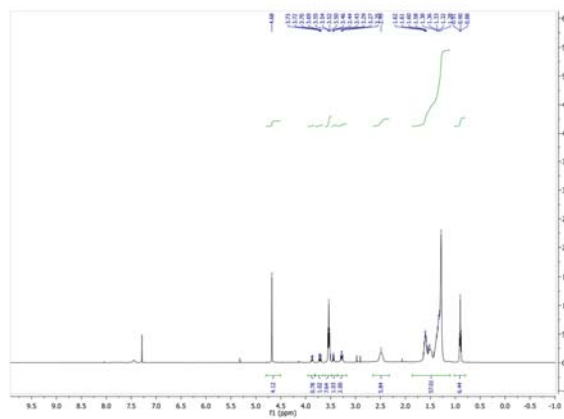
Supplementary Fig. 20. (a) ^1H NMR spectra of MK16 in CD_3OD . (b) HRMS of MK16.



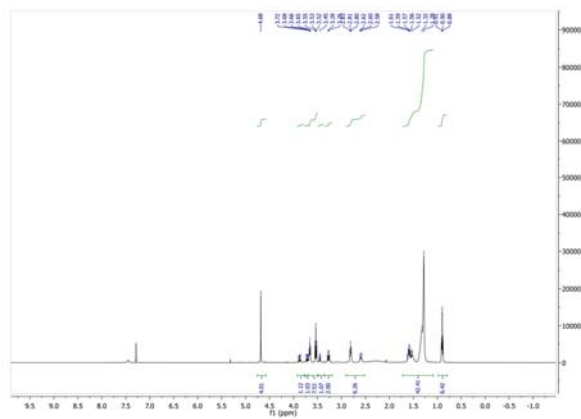
DS1



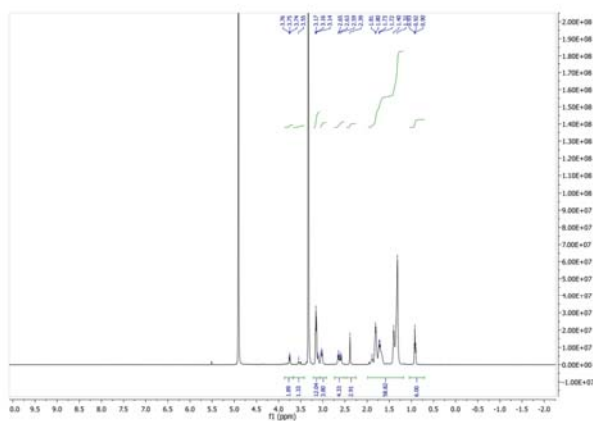
DS2



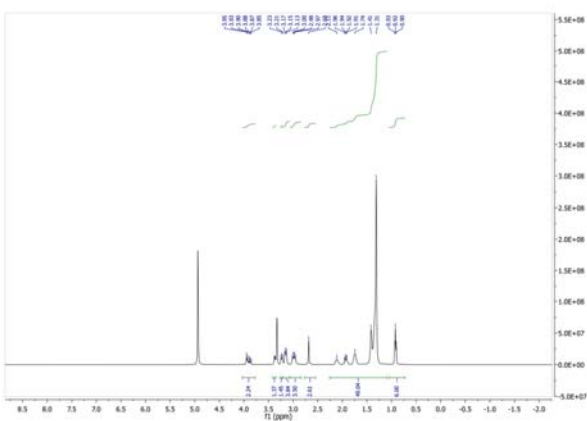
DS3



DS4

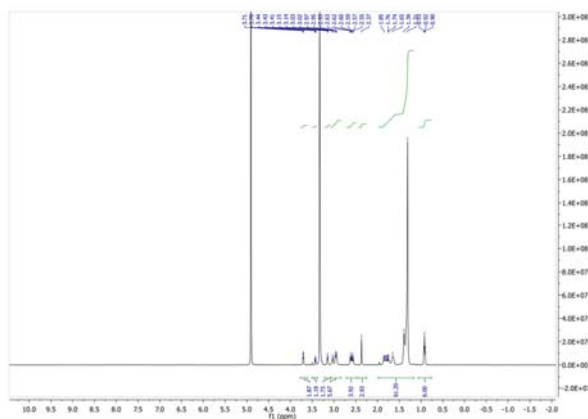


DS5

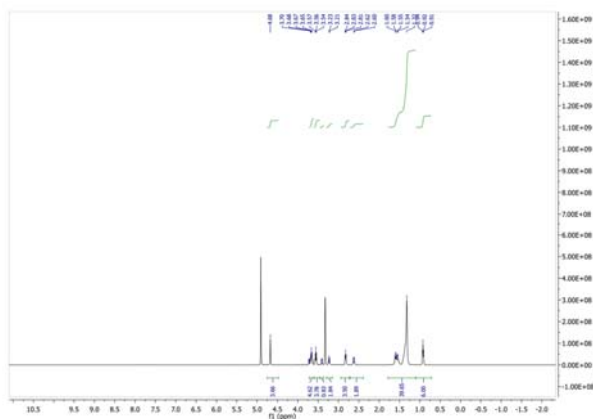


DS6

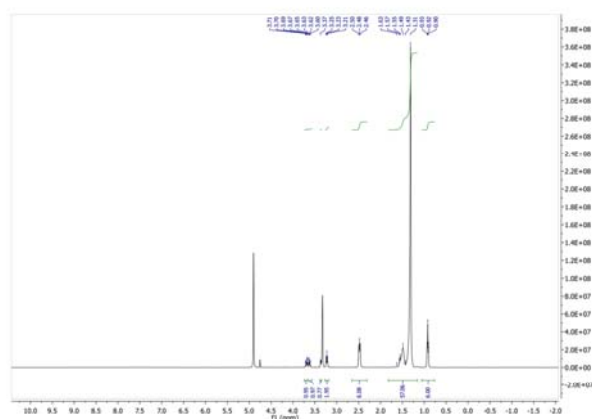
Supplementary Fig. 21. ^1H NMR spectra of DS1-DS6.



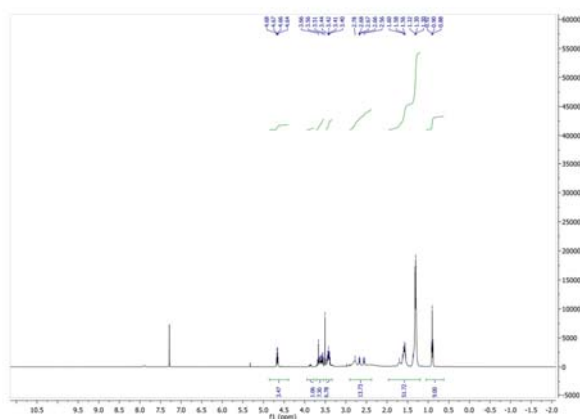
DS7



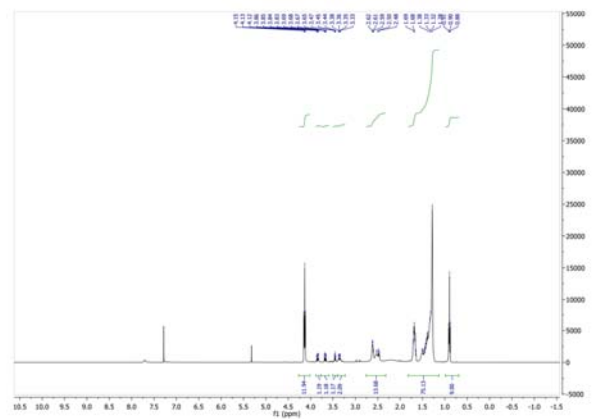
DS8



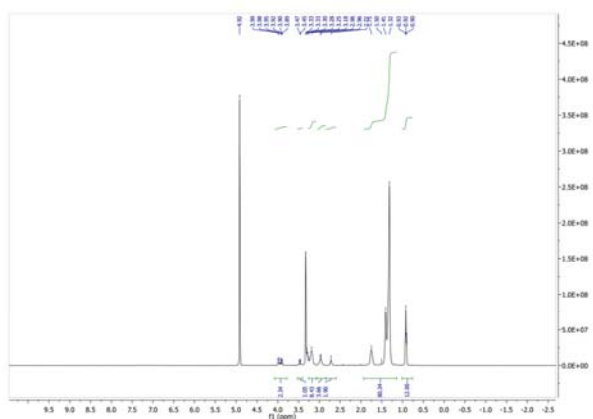
DS9



DS10

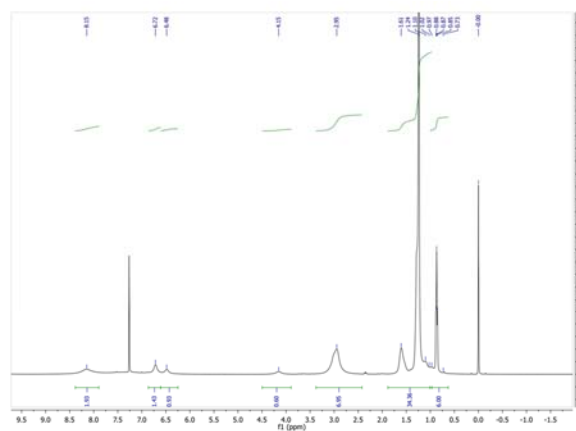


DS11

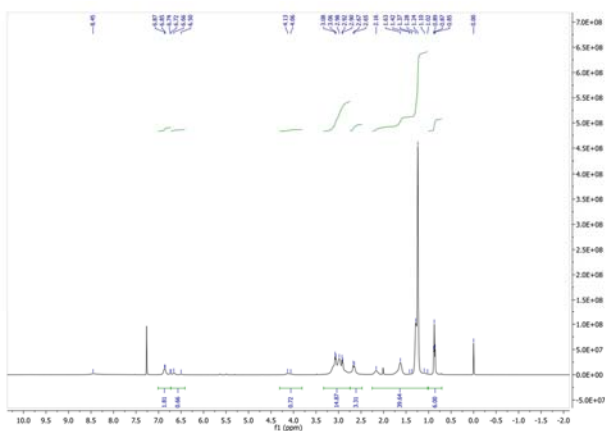


DS12

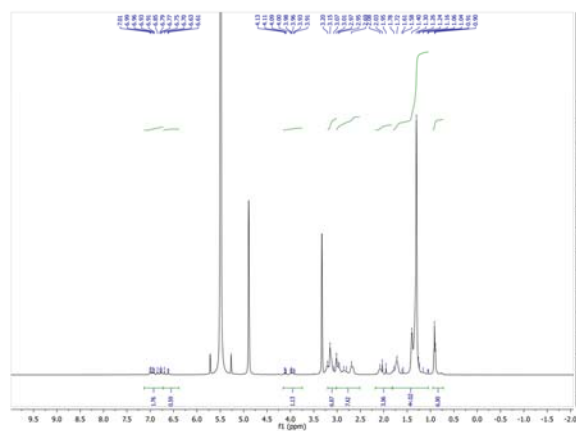
Supplementary Fig. 22. ^1H NMR spectra of DS7-DS12.



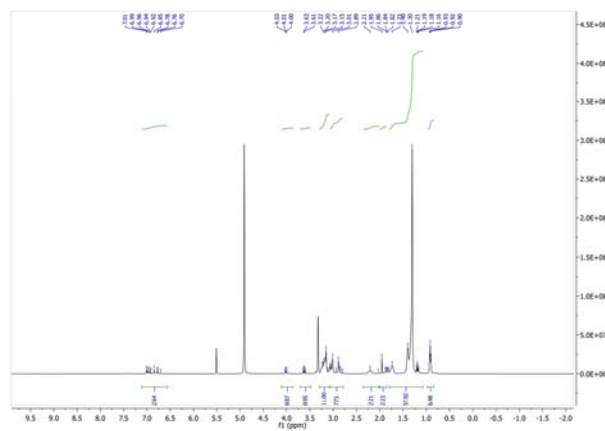
LD1



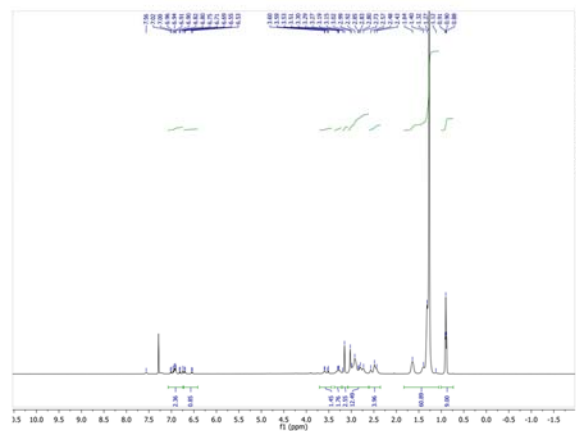
LD2



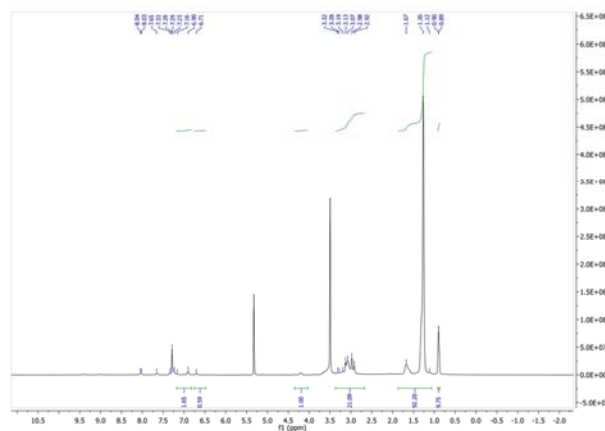
LD3



LD4

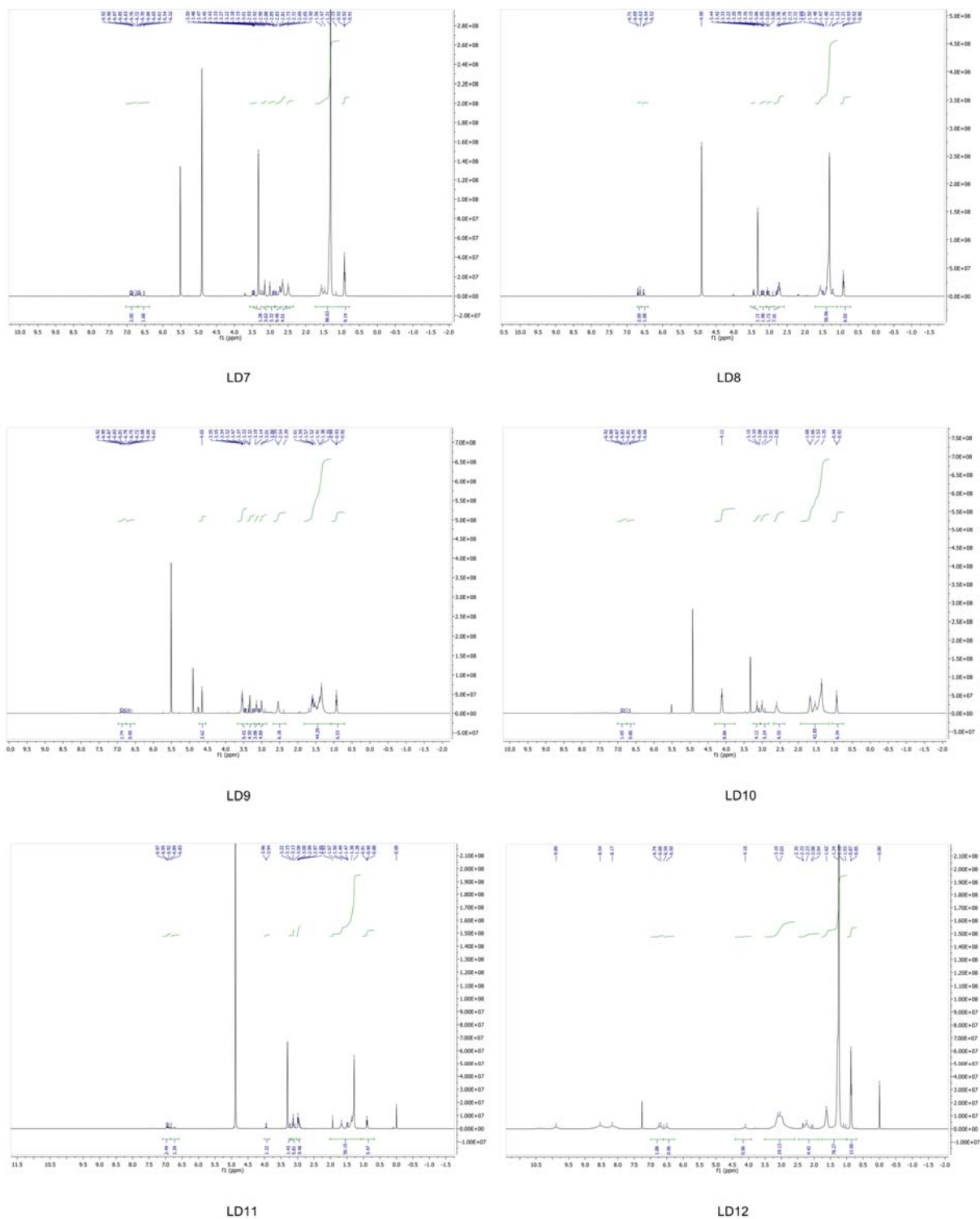


LD5

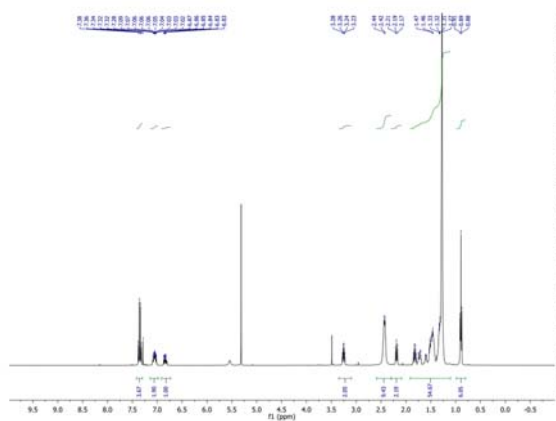


LD6

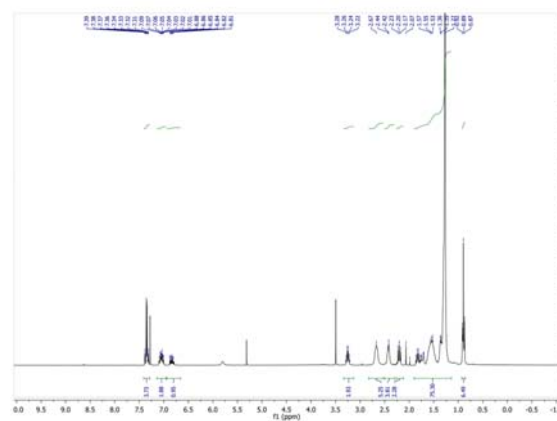
Supplementary Fig. 23. ^1H NMR spectra of LD1-LD6.



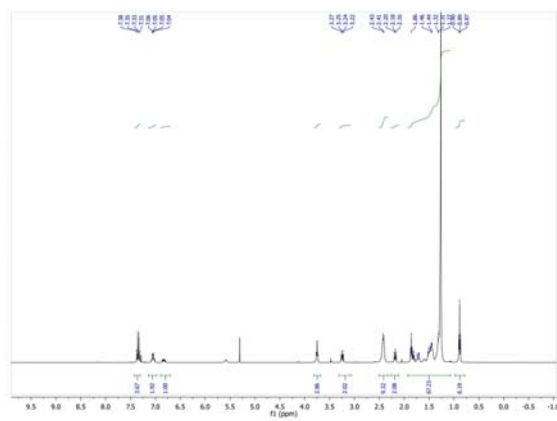
Supplementary Fig. 24. ^1H NMR spectra of LD7-LD12.



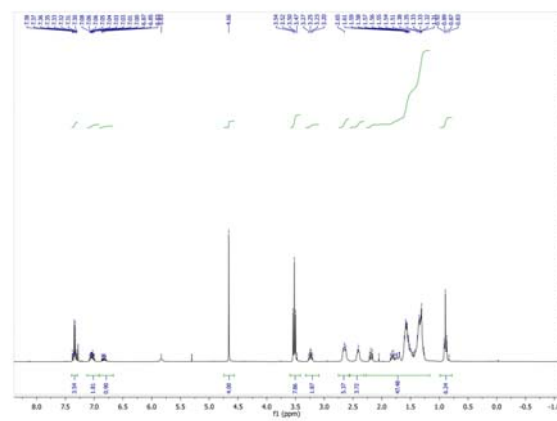
MK1



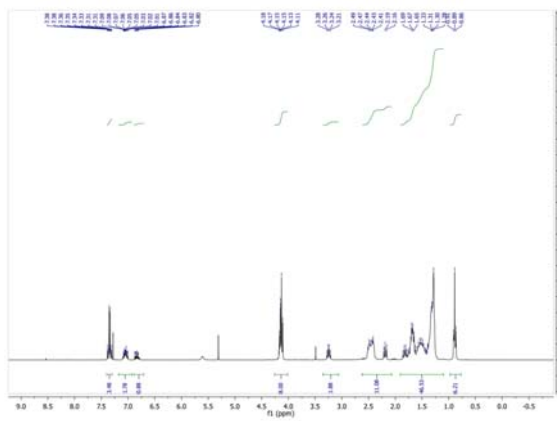
MK2



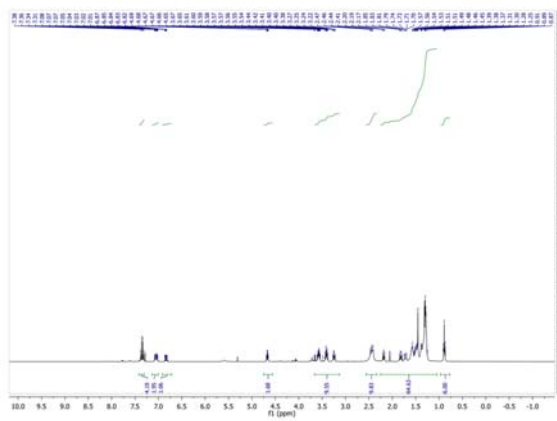
MK3



MK4

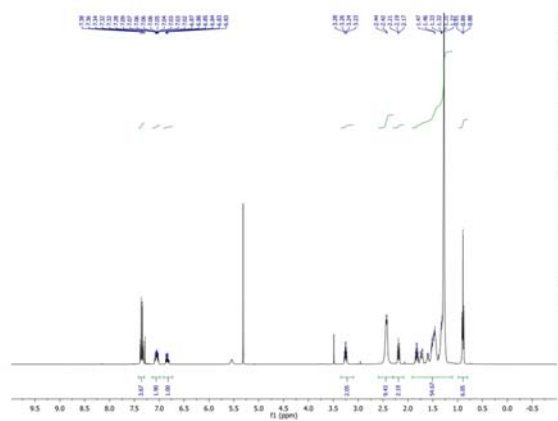


MK5

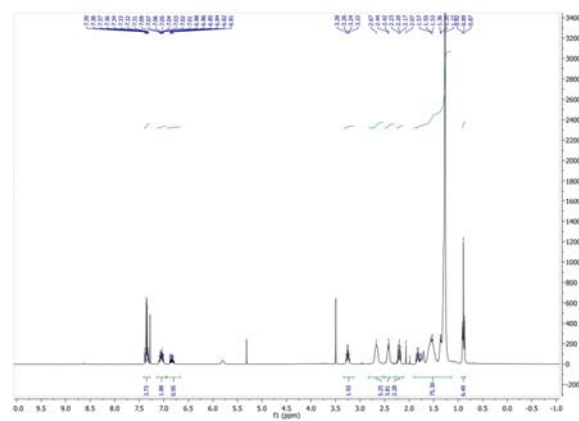


MK6

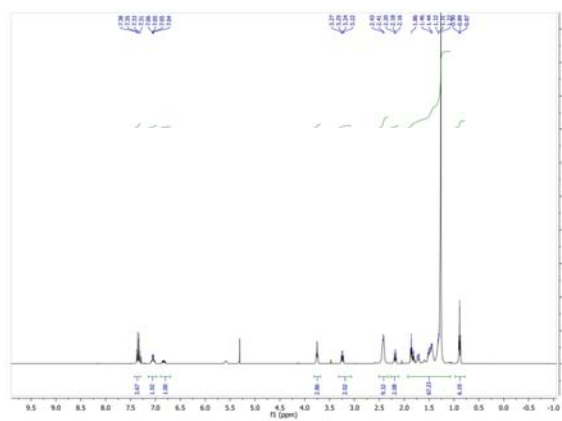
Supplementary Fig. 25. ^1H NMR spectra of MK1-MK6.



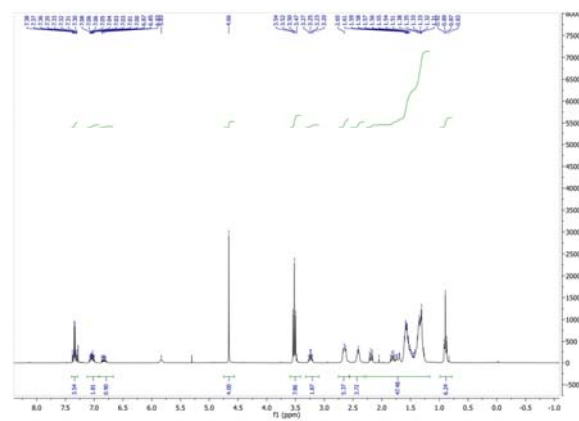
MK1



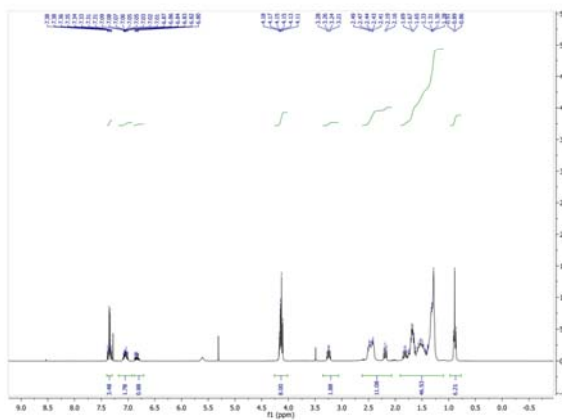
MK2



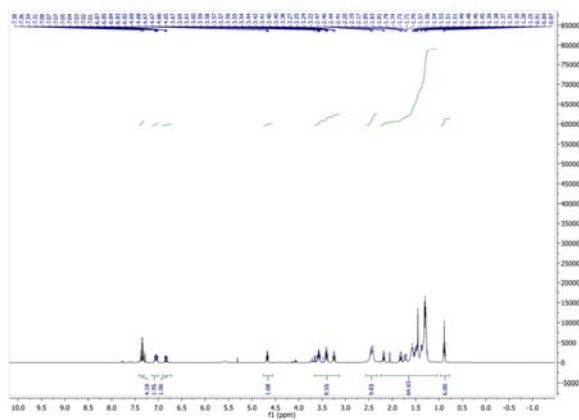
MK3



MK4

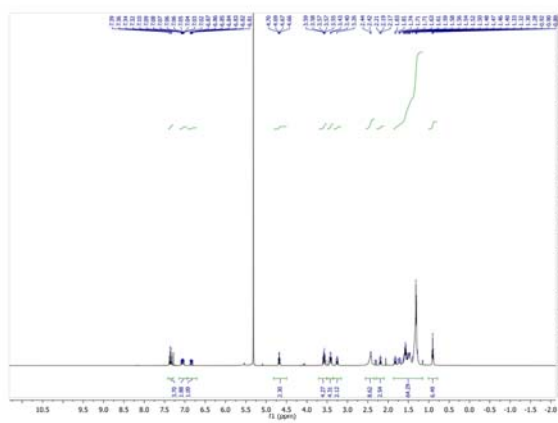


MK5

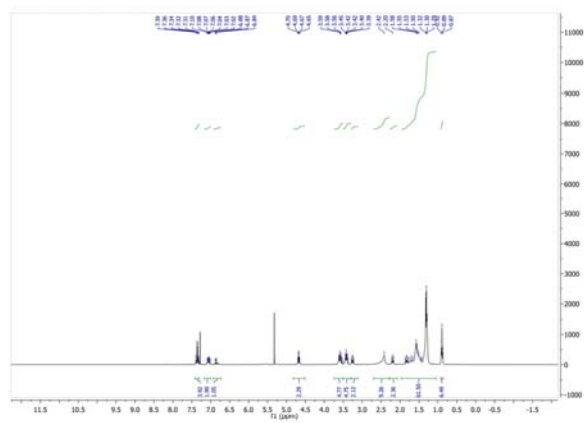


MK6

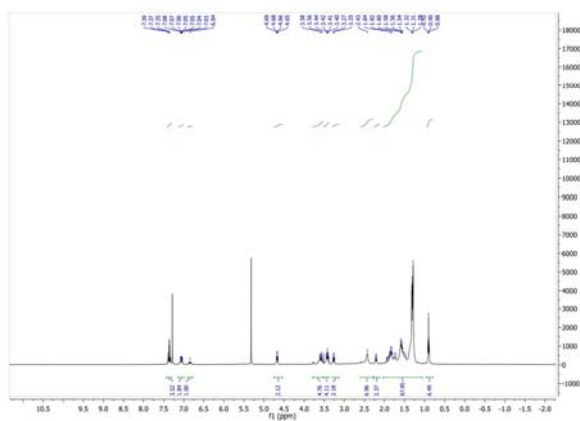
Supplementary Fig. 26. ^1H NMR spectra of MK7-MK12.



MK13

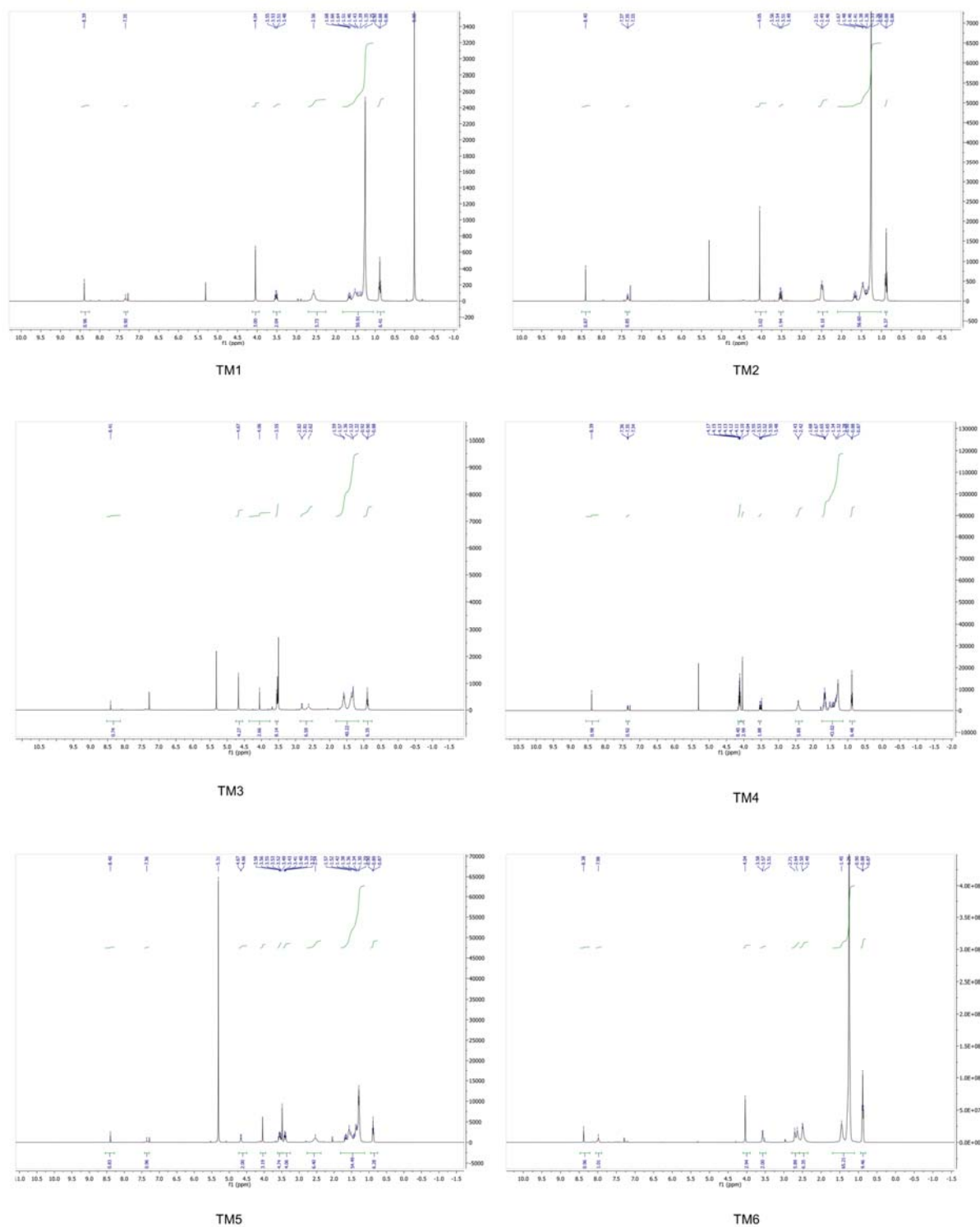


MK14

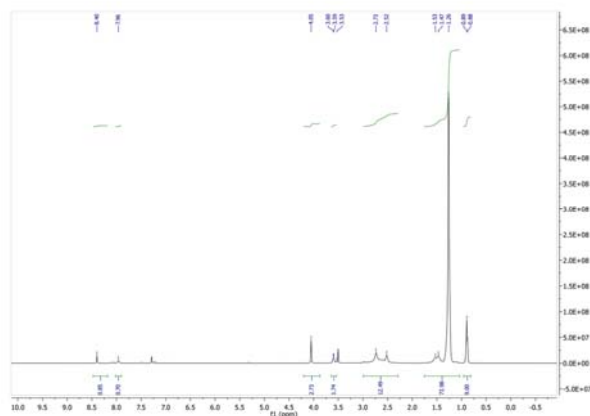


MK15

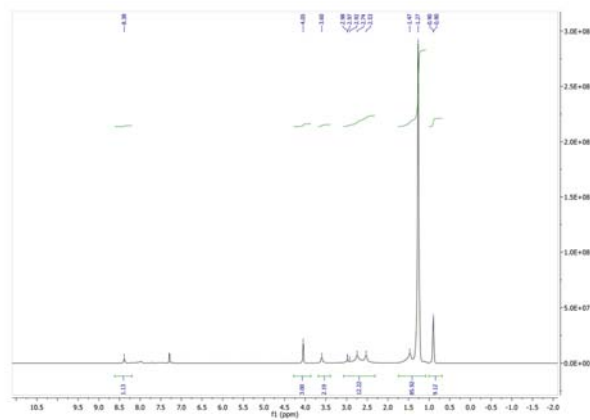
Supplementary Fig. 27. ^1H NMR spectra of MK13-MK15.



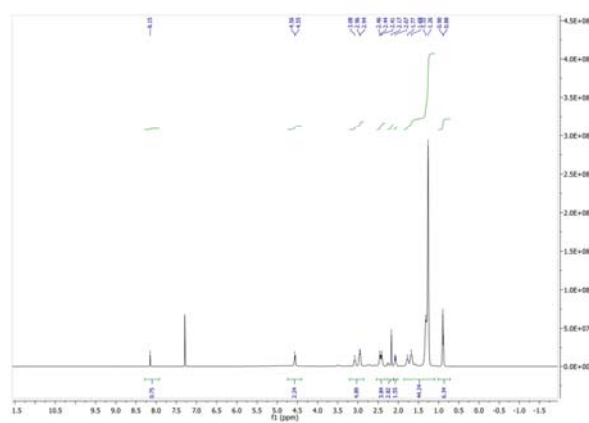
Supplementary Fig. 28. ^1H NMR spectra of TM1-TM6.



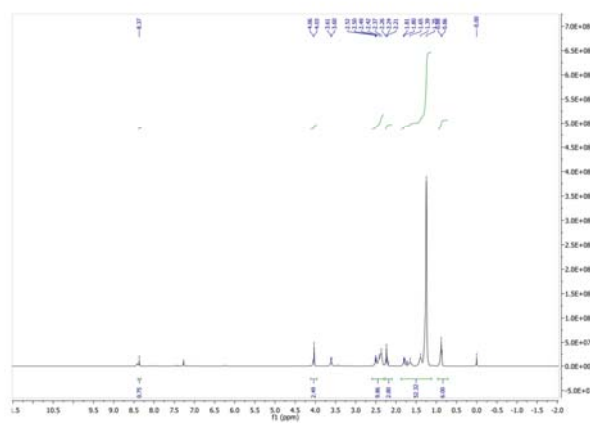
TM7



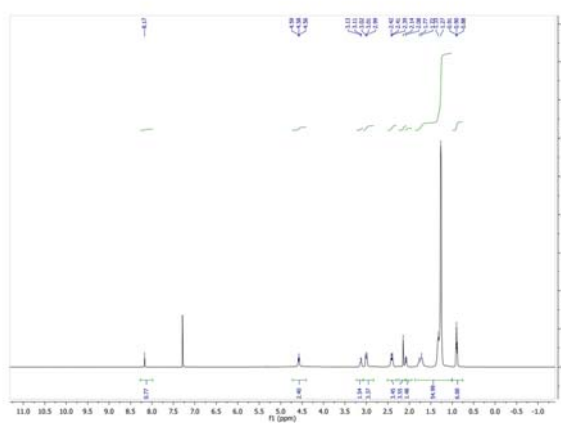
TM8



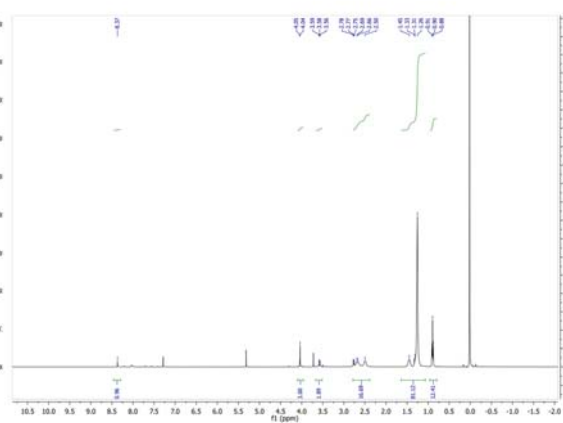
TM9



TM10

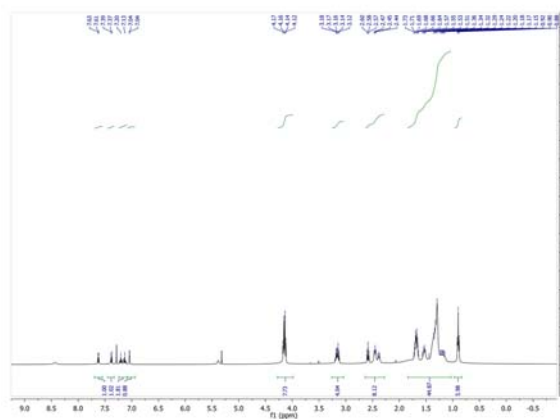


TM10

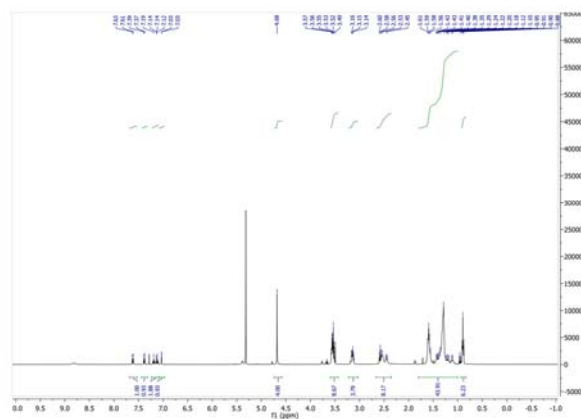


TM12

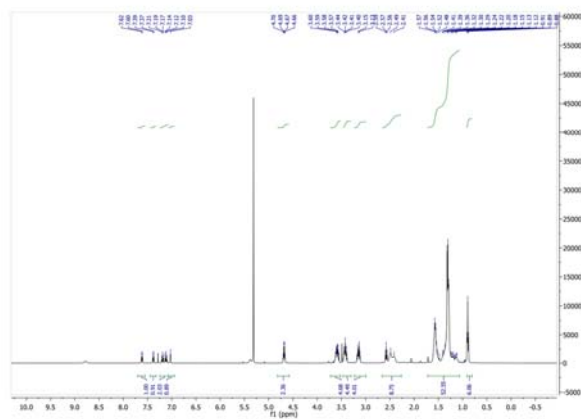
Supplementary Fig. 29. ^1H NMR spectra of TM7-TM12.



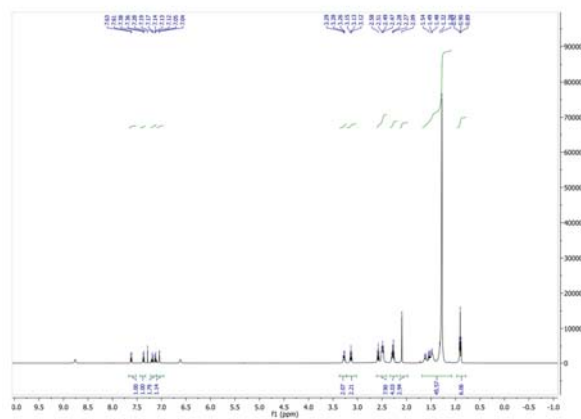
TD1



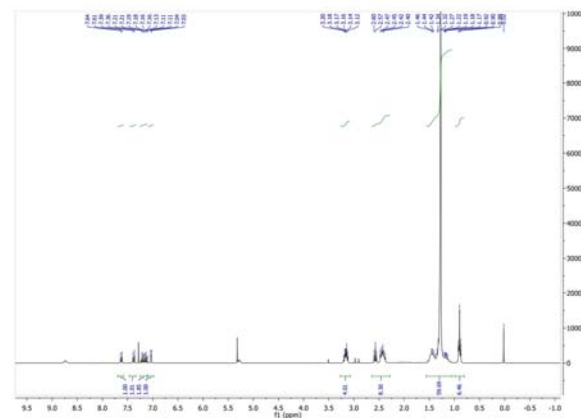
TD2



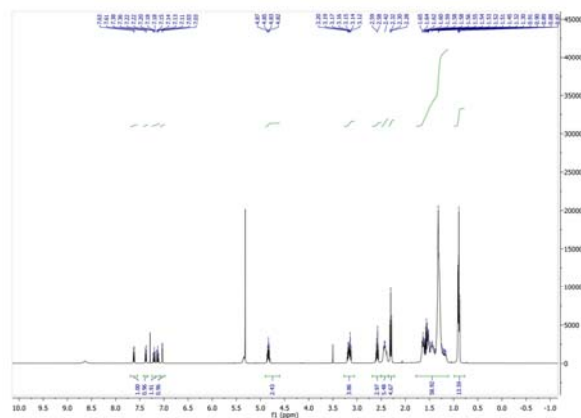
TD3



TD4

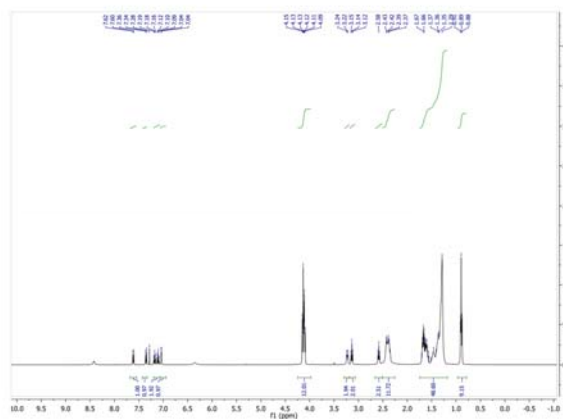


TD5

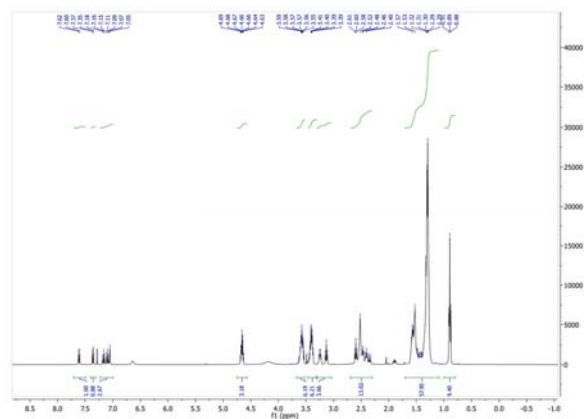


TD6

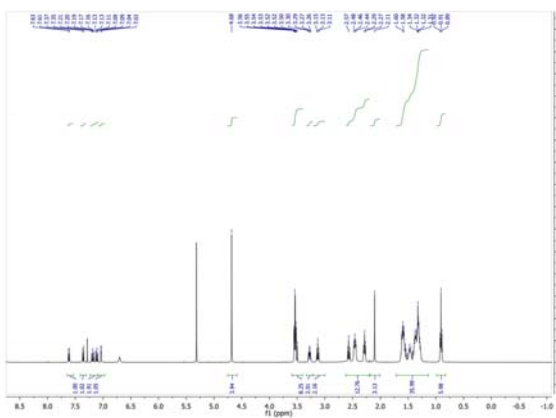
Supplementary Fig. 30. ^1H NMR spectra of TD1-TD6.



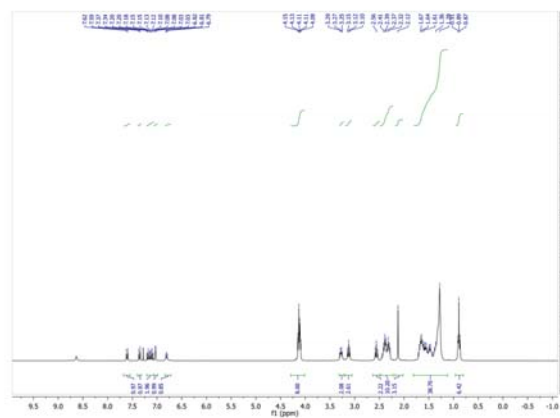
TD7



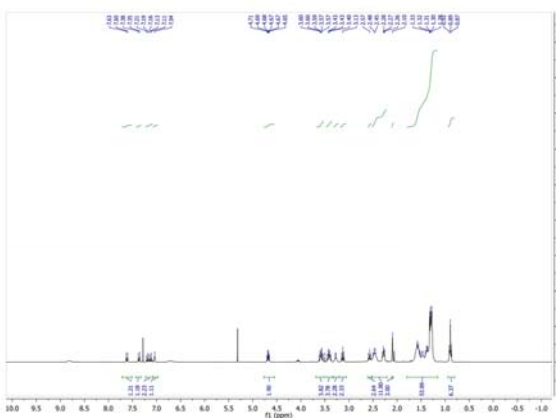
TD8



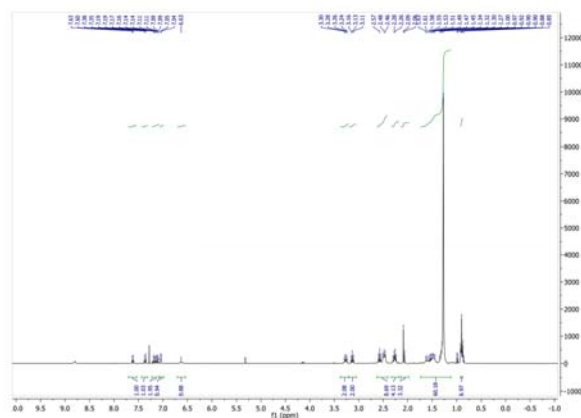
TD9



TD10

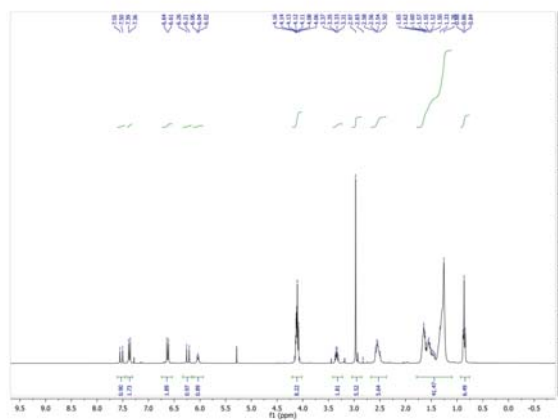


TD11

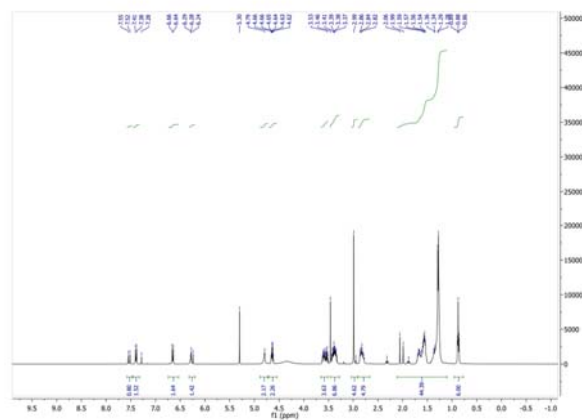


TD12

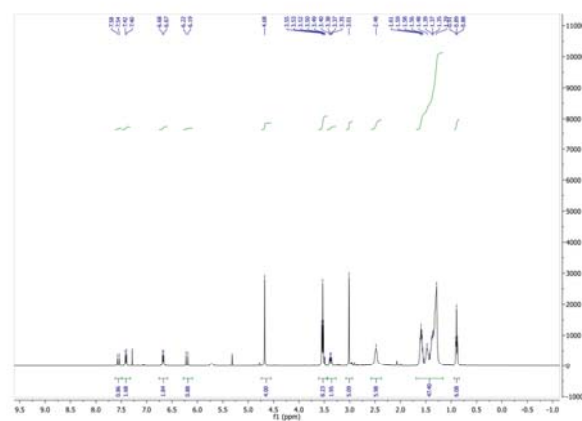
Supplementary Fig. 31. ^1H NMR spectra of TD7-TD12.



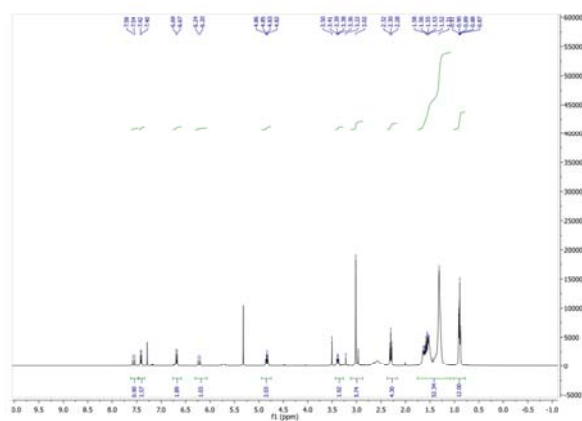
CD1



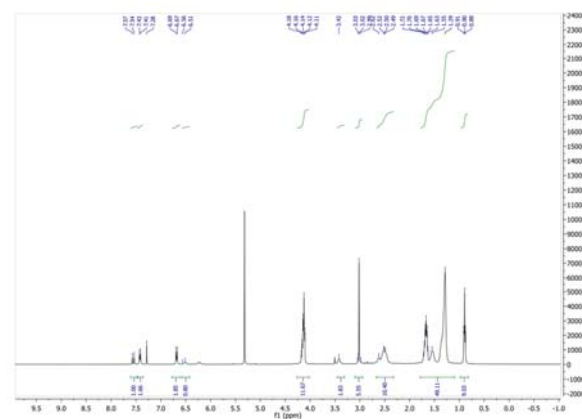
CD2



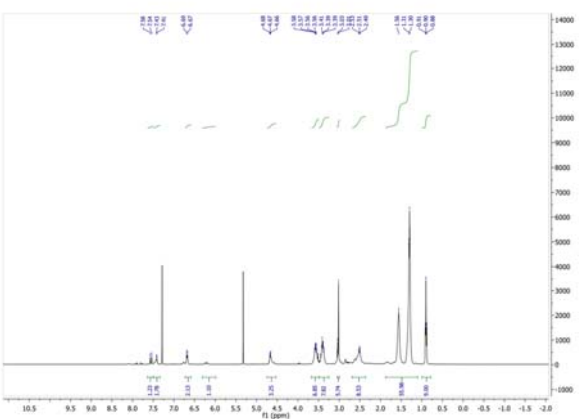
CD3



CD4

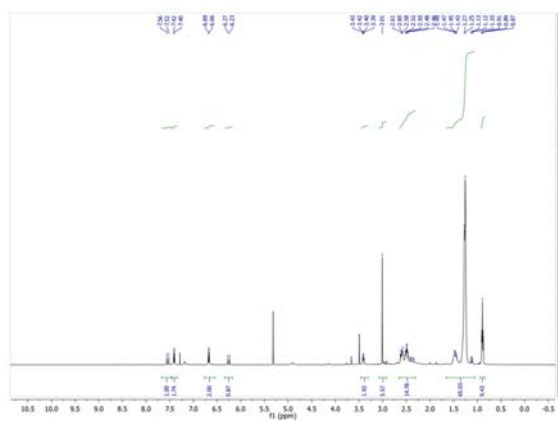


CD5

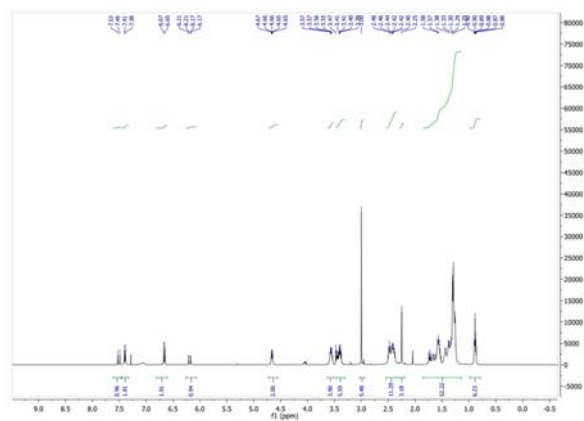


CD6

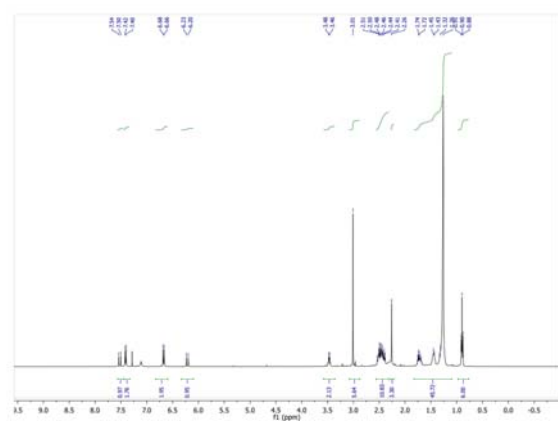
Supplementary Fig. 32. ^1H NMR spectra of CD1-CD6.



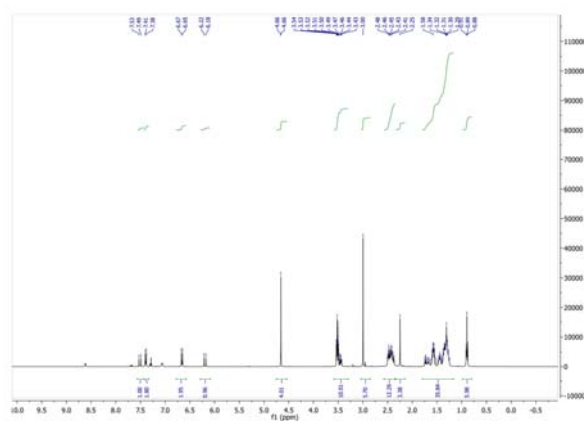
CD7



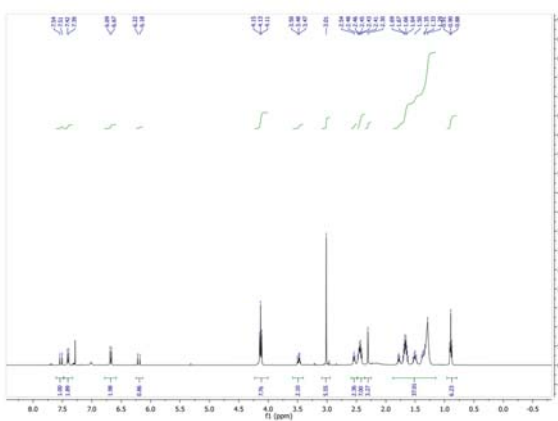
CD8



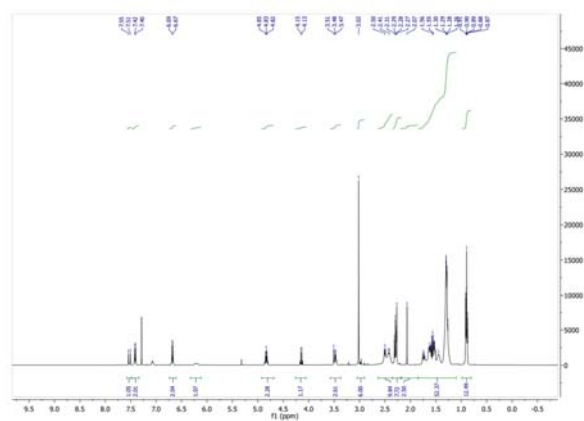
CD9



CD10



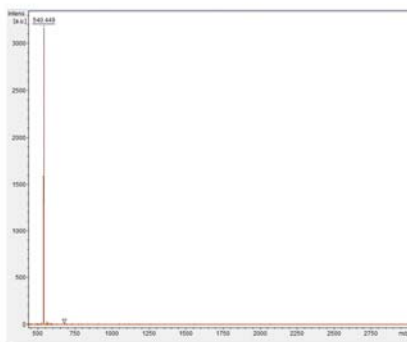
CD11



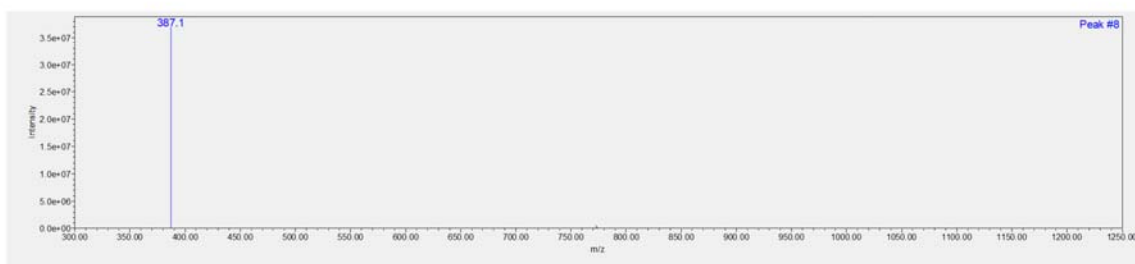
CD12

Supplementary Fig. 33. ^1H NMR spectra of CD7-CD12.

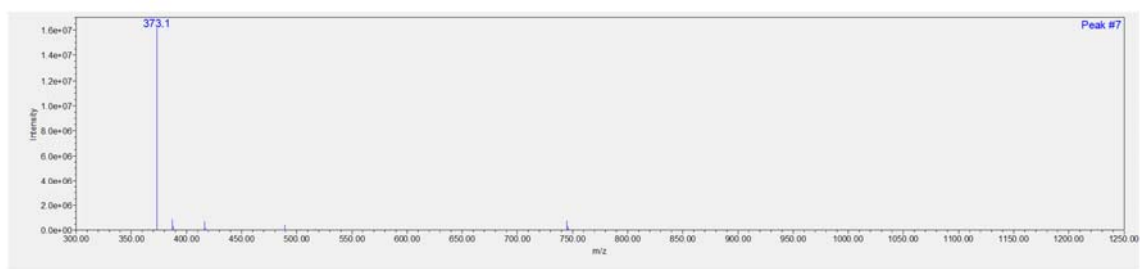
DS1



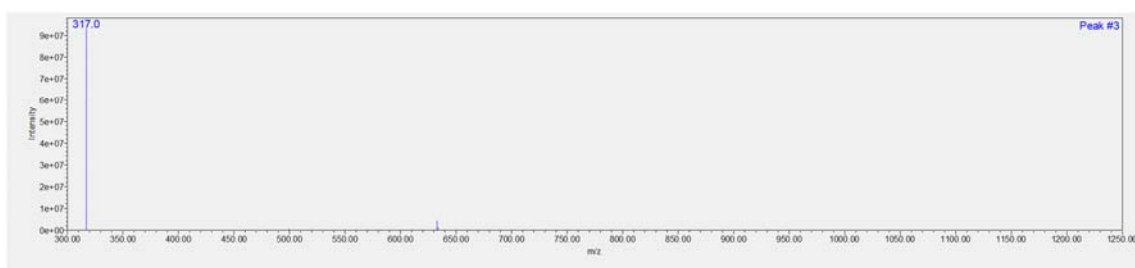
DS2



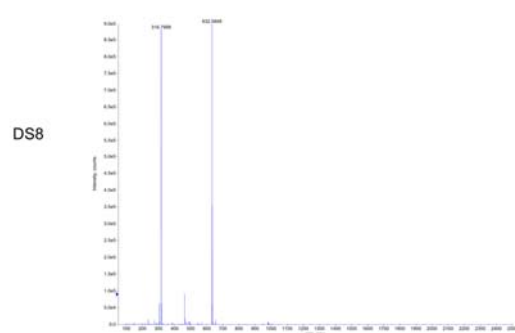
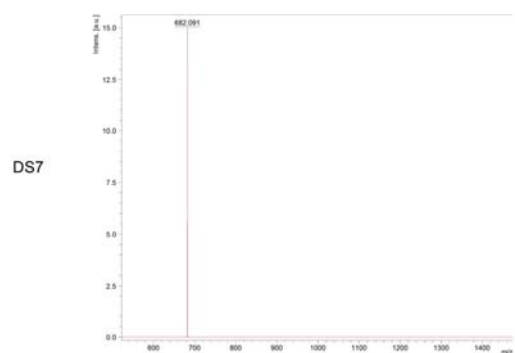
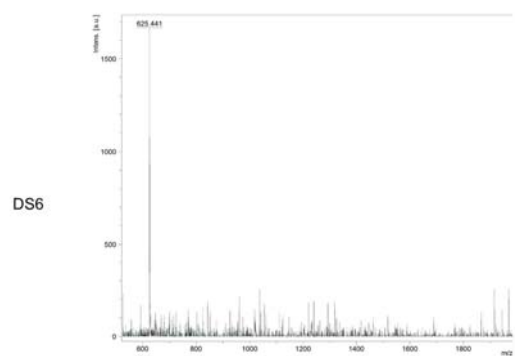
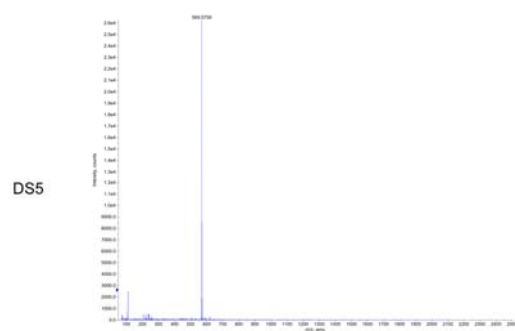
DS3



DS4

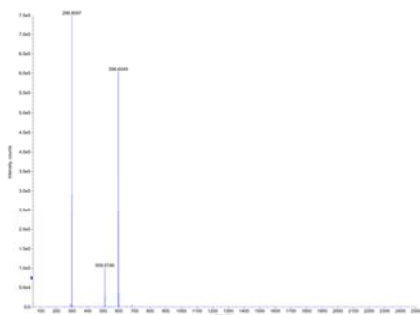


Supplementary Fig. 34. MS of DS1-DS4.

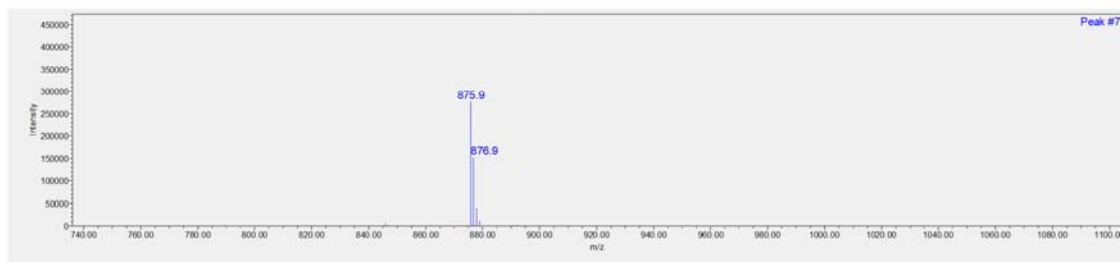


Supplementary Fig. 35. MS of DS5-DS8.

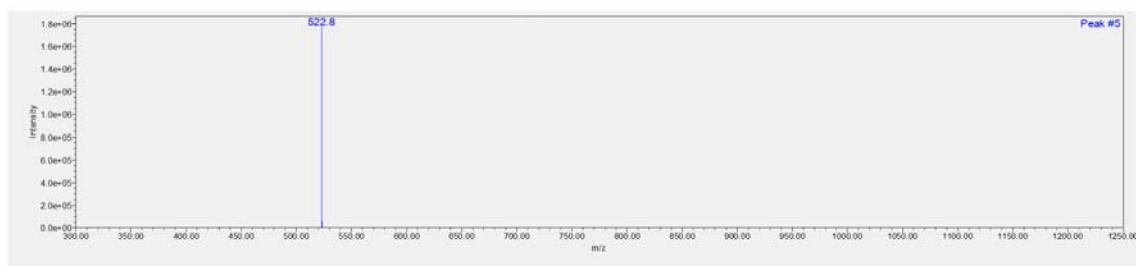
DS9



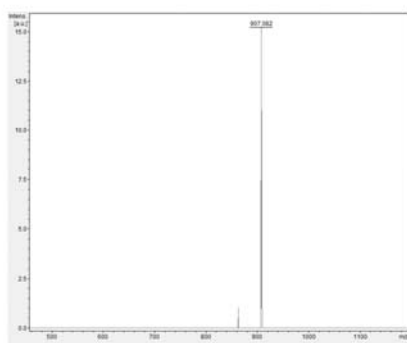
DS10



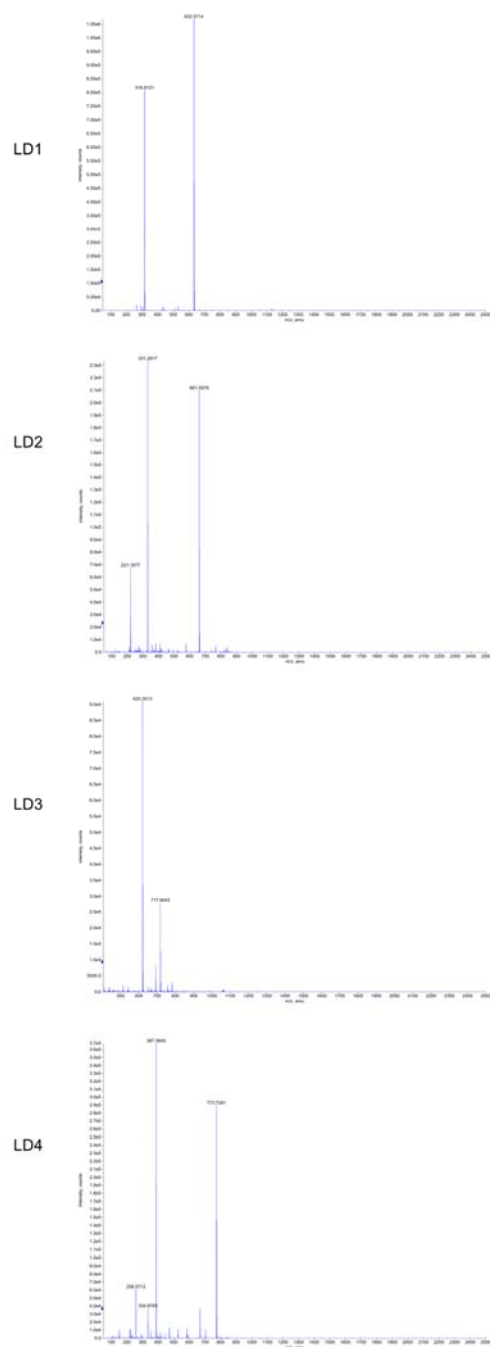
DS11



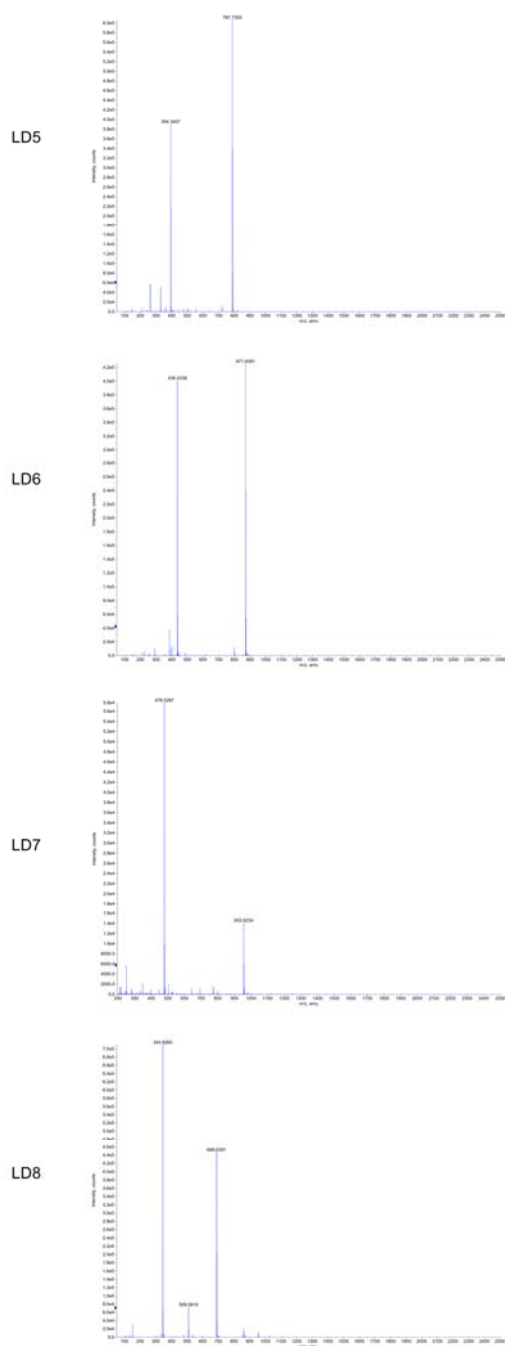
DS12



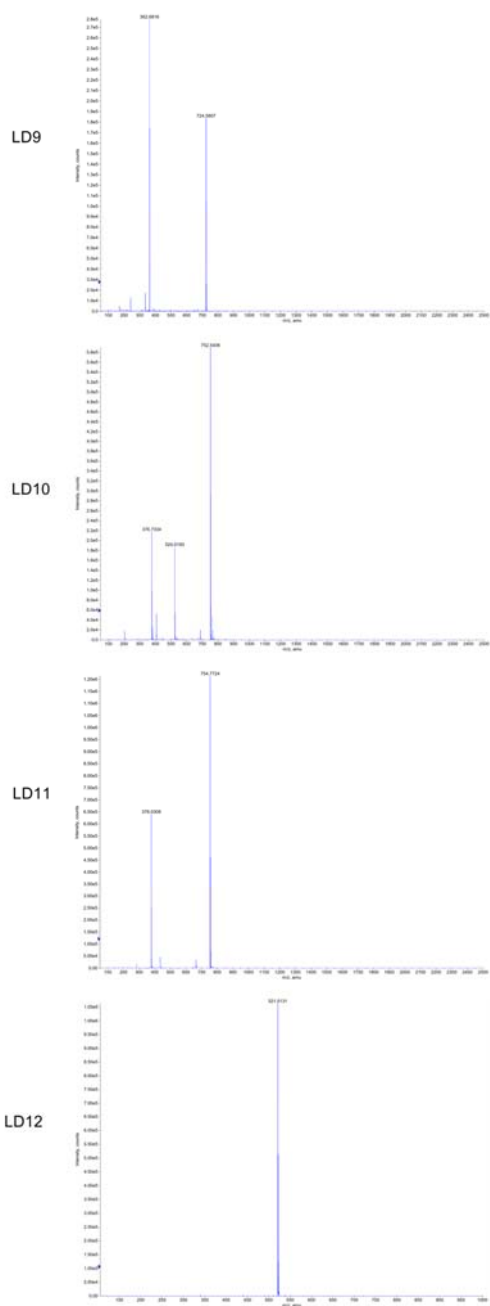
Supplementary Fig. 36. MS of DS9-DS12.



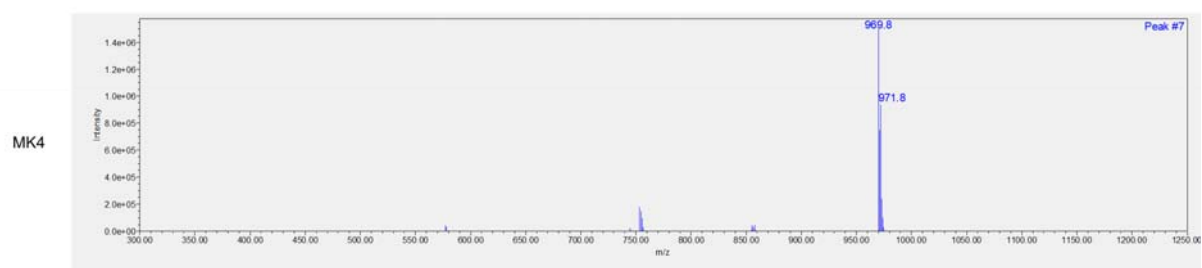
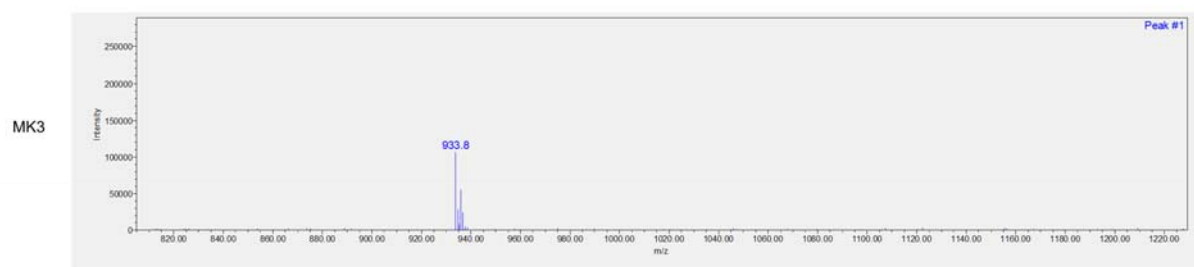
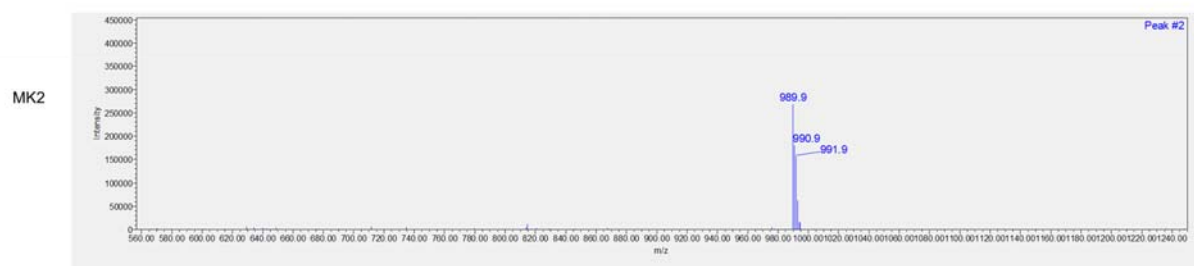
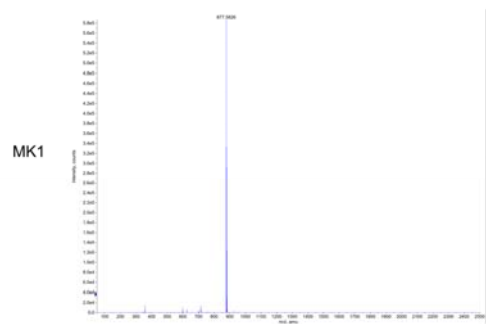
Supplementary Fig. 37. MS of LD1-LD4.



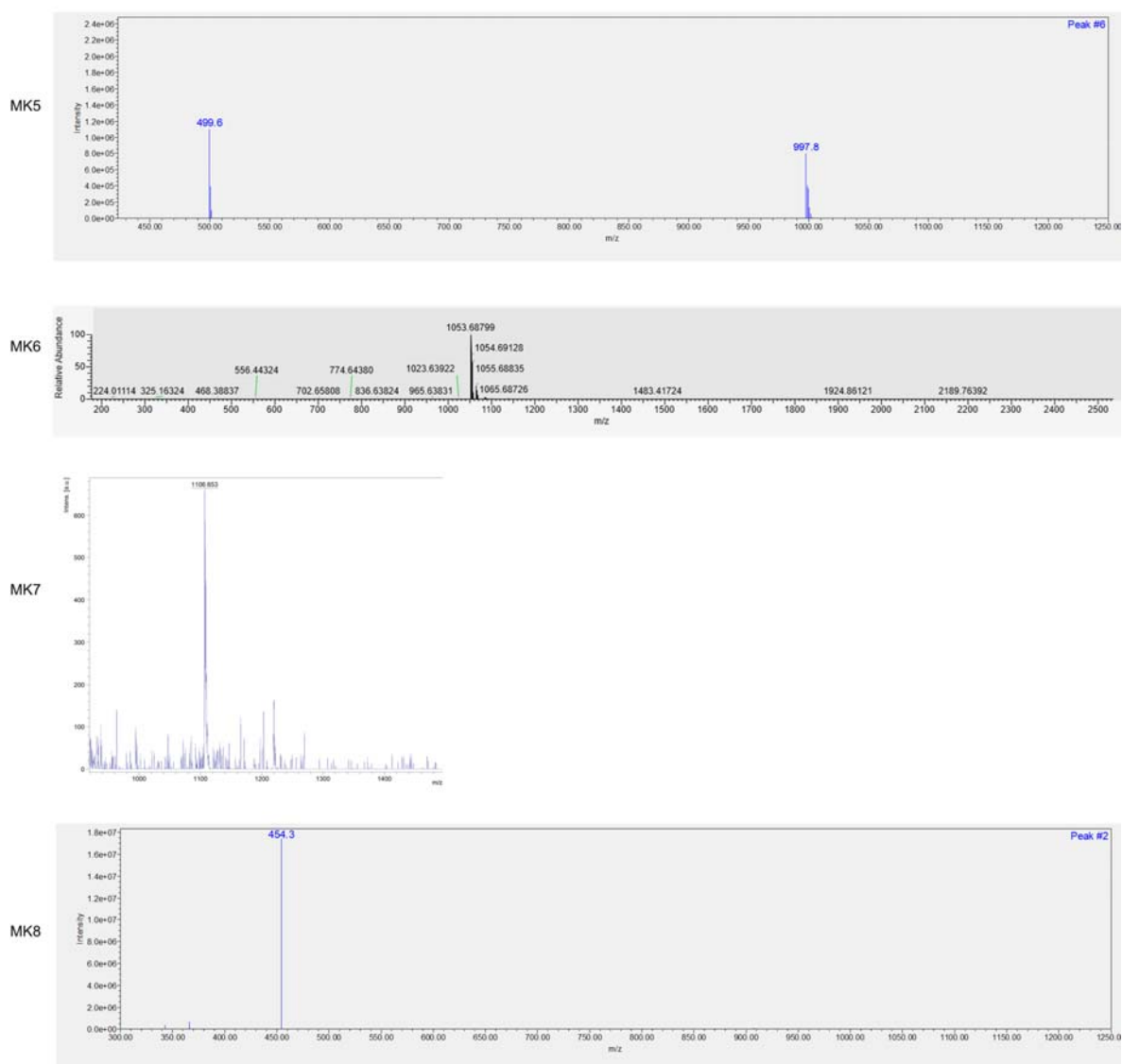
Supplementary Fig. 38. MS of LD5-LD8.



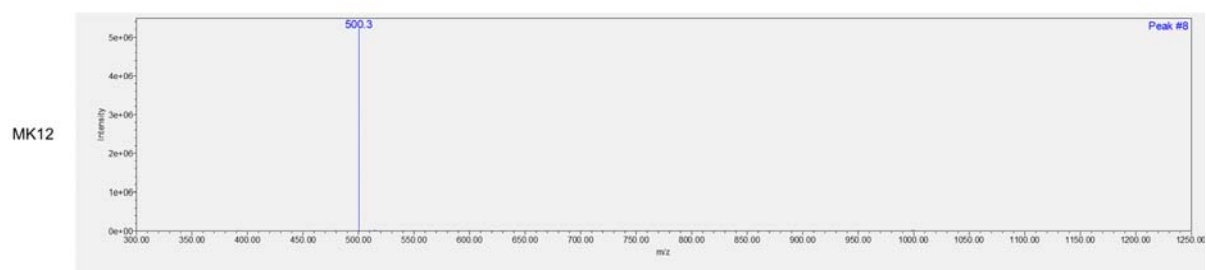
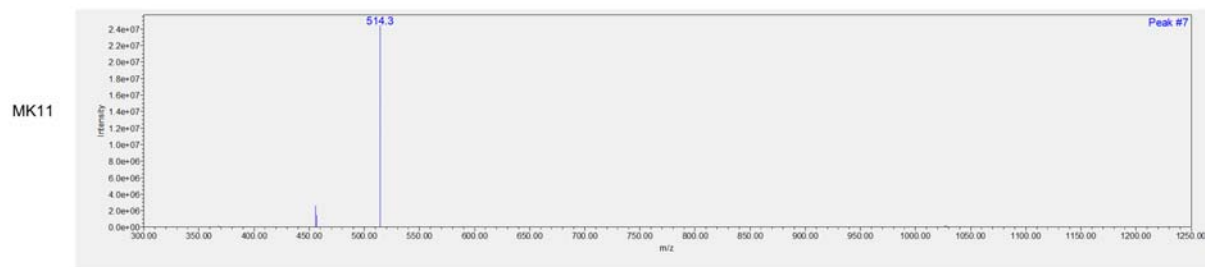
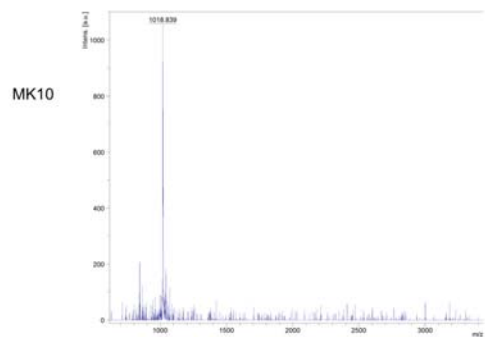
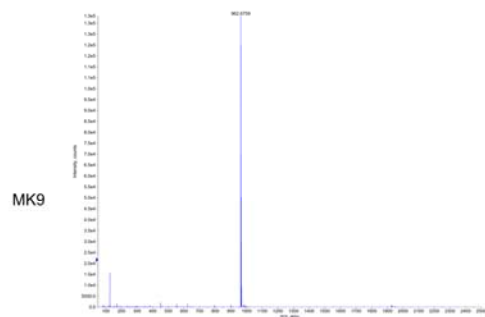
Supplementary Fig. 39. MS of LD9-LD12.



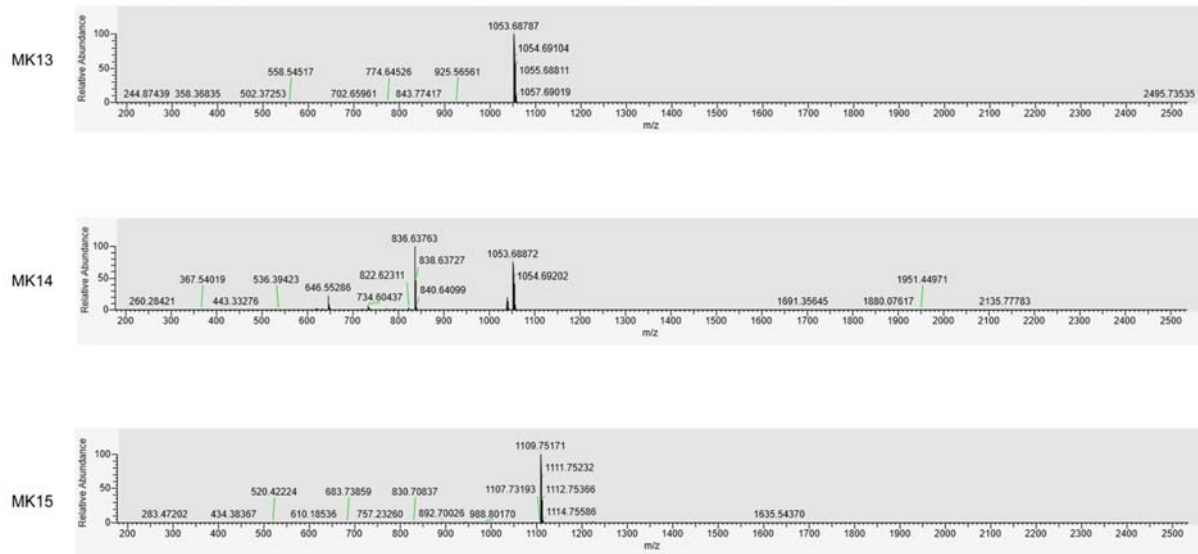
Supplementary Fig. 40. MS of MK1-MK4.



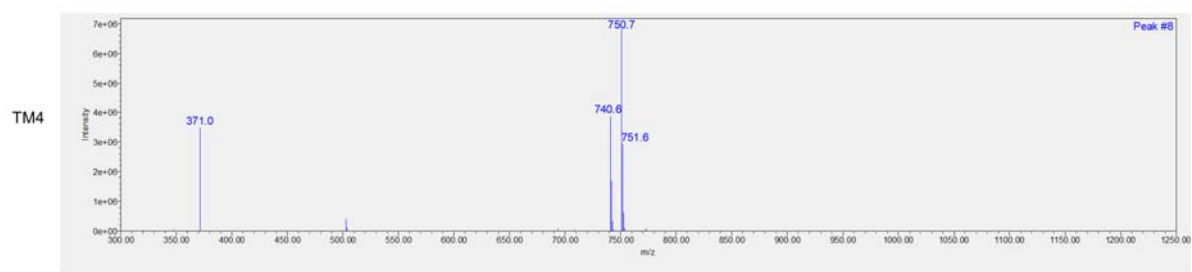
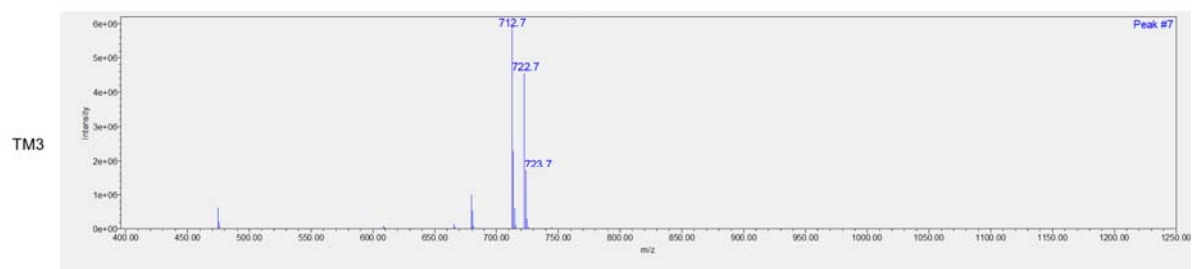
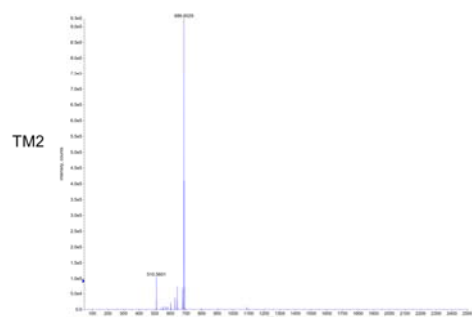
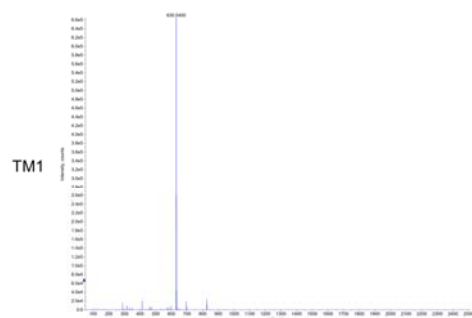
Supplementary Fig. 41. MS of MK5-MK8.



Supplementary Fig. 42. MS of MK9-MK12.

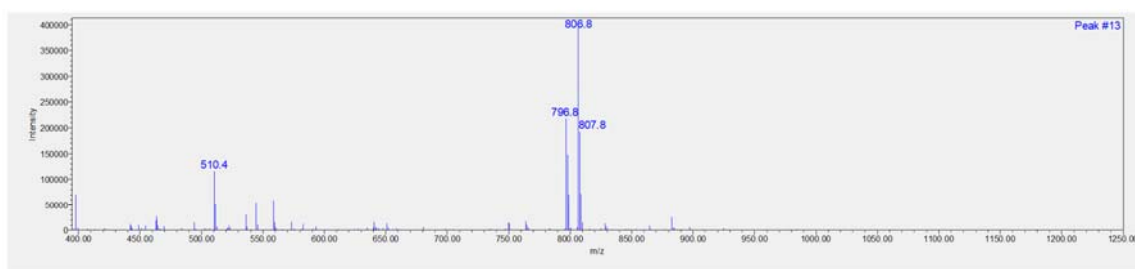


Supplementary Fig. 43. MS of MK13-MK15.

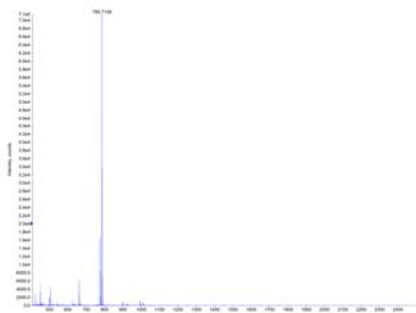


Supplementary Fig. 44. MS of TM1-TM4.

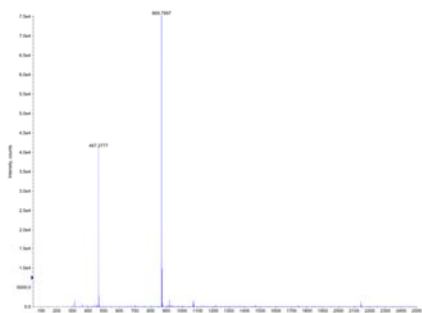
TM5



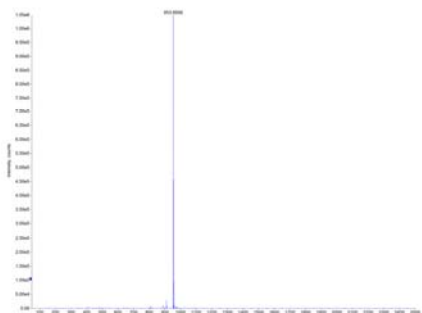
TM6



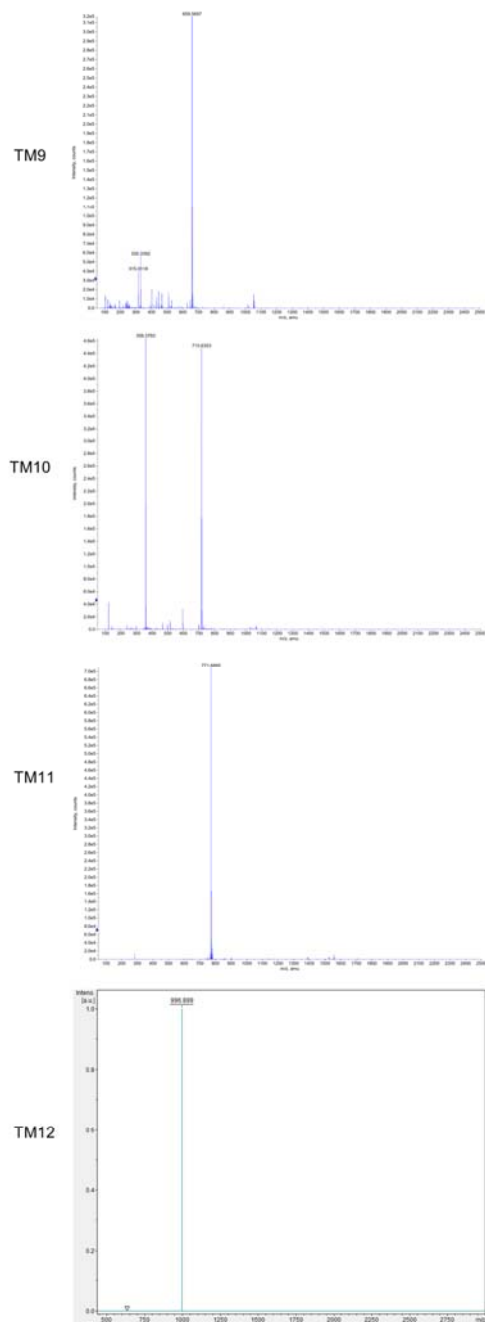
TM7



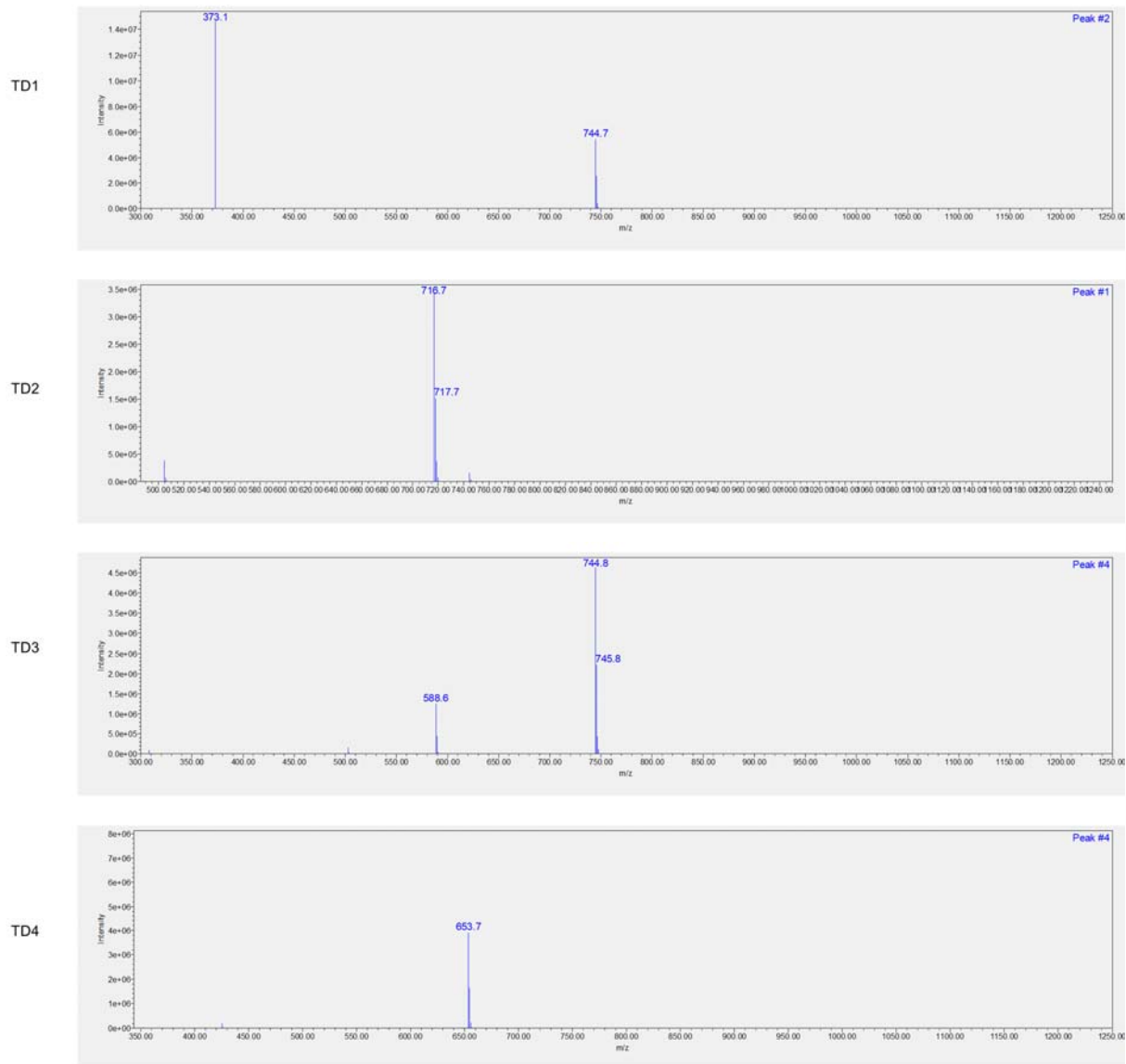
TM8



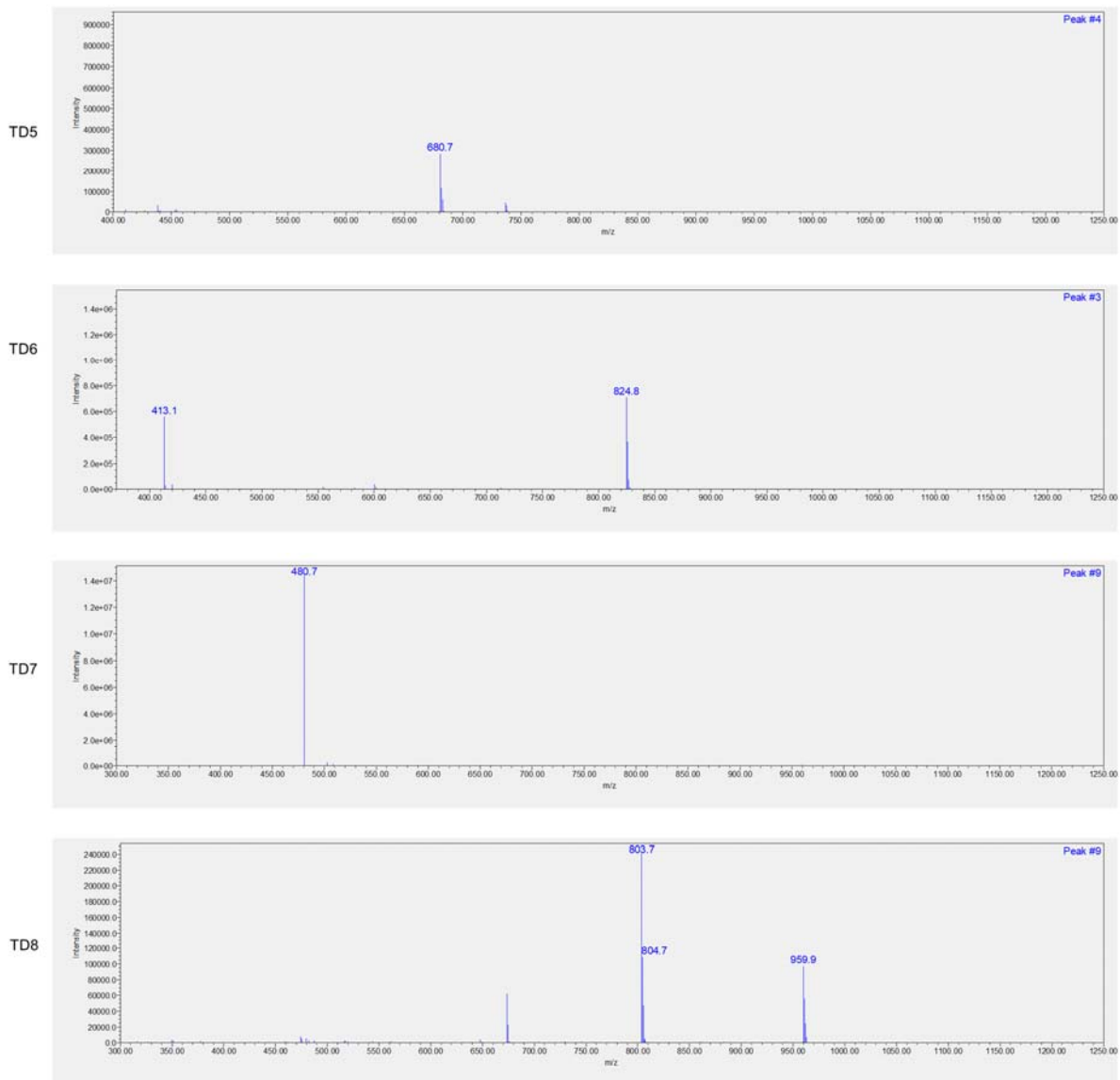
Supplementary Fig. 45. MS of TM5-TM8.



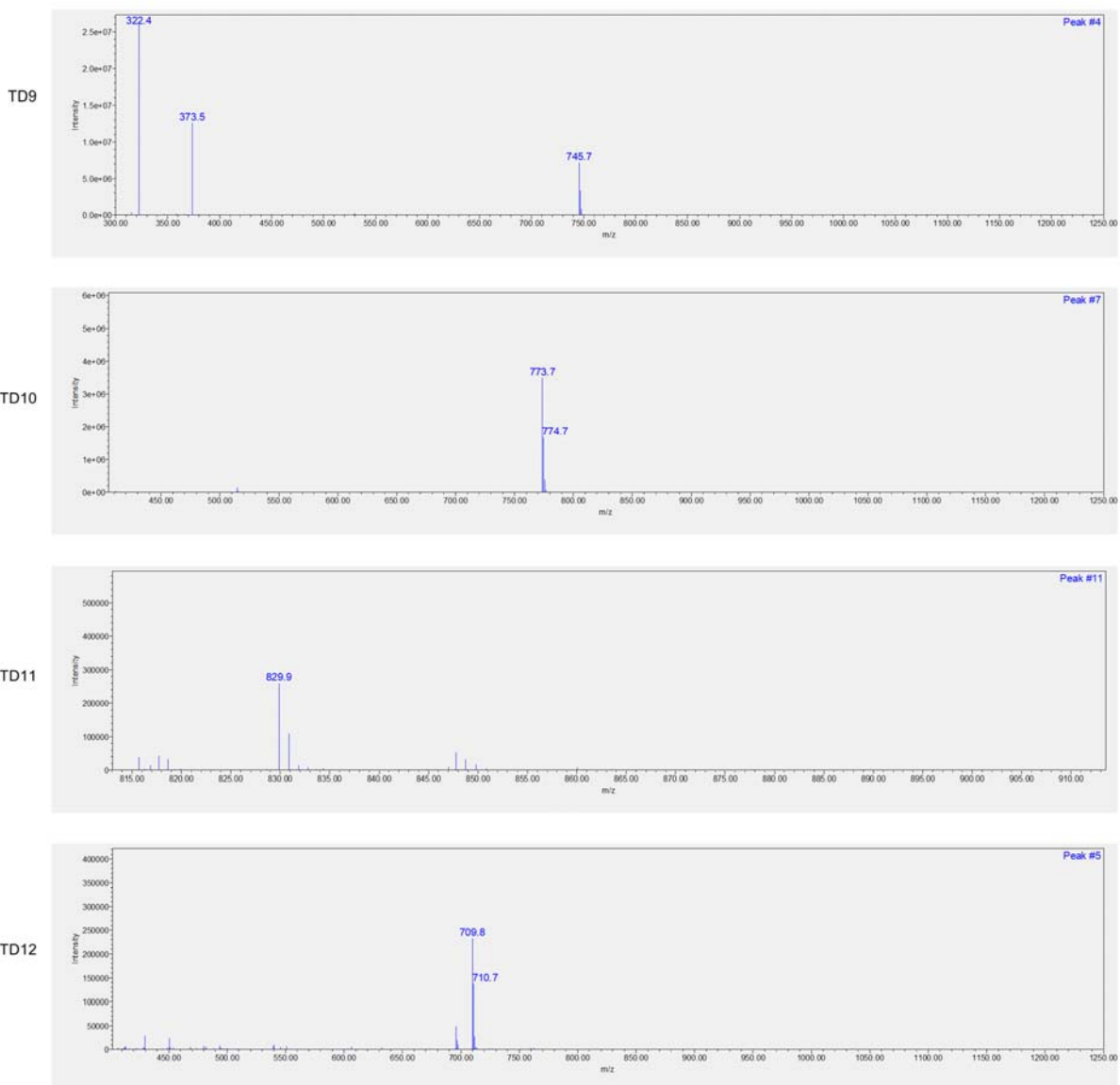
Supplementary Fig. 46. MS of TM9-TM12.



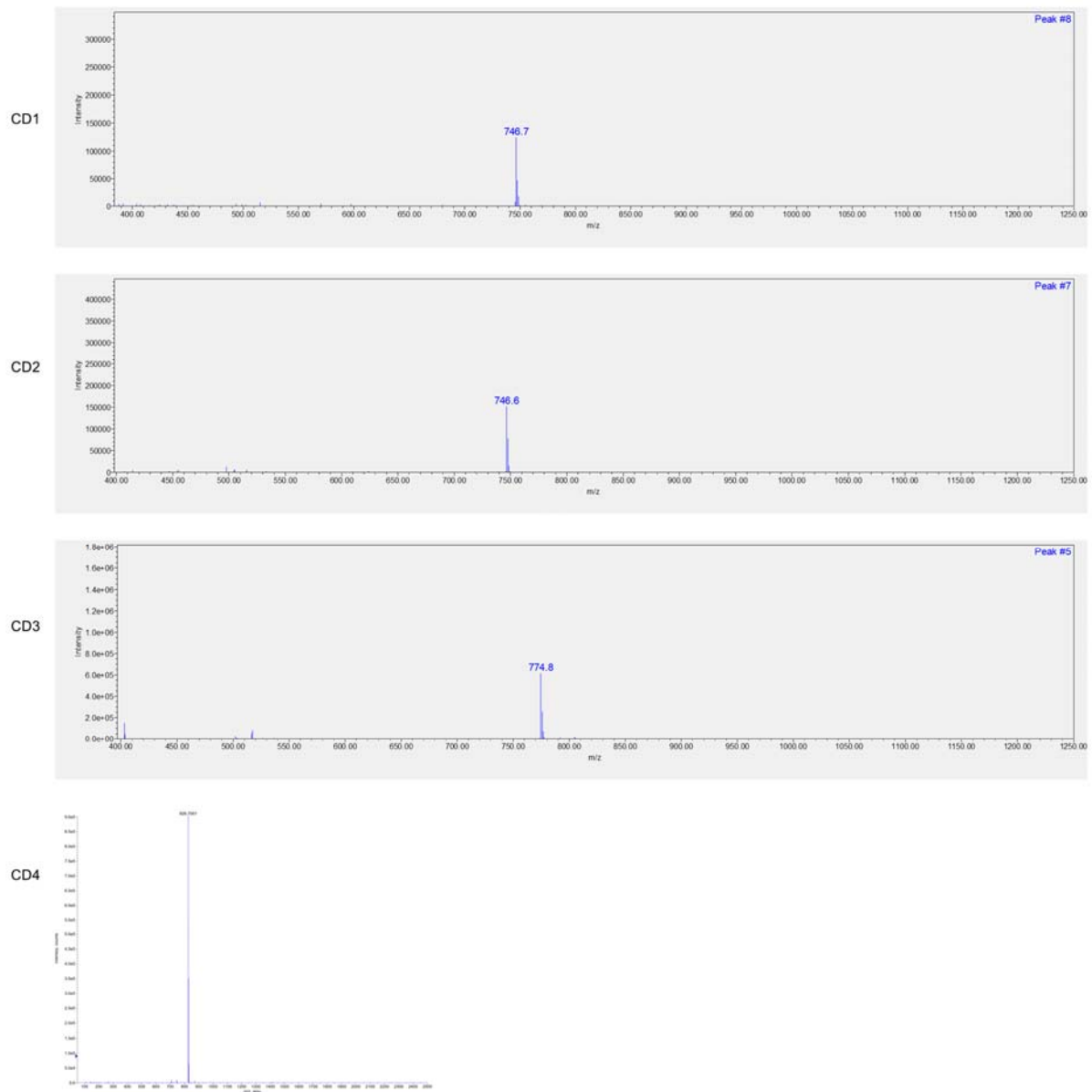
Supplementary Fig. 47. MS of TD1-TD4.



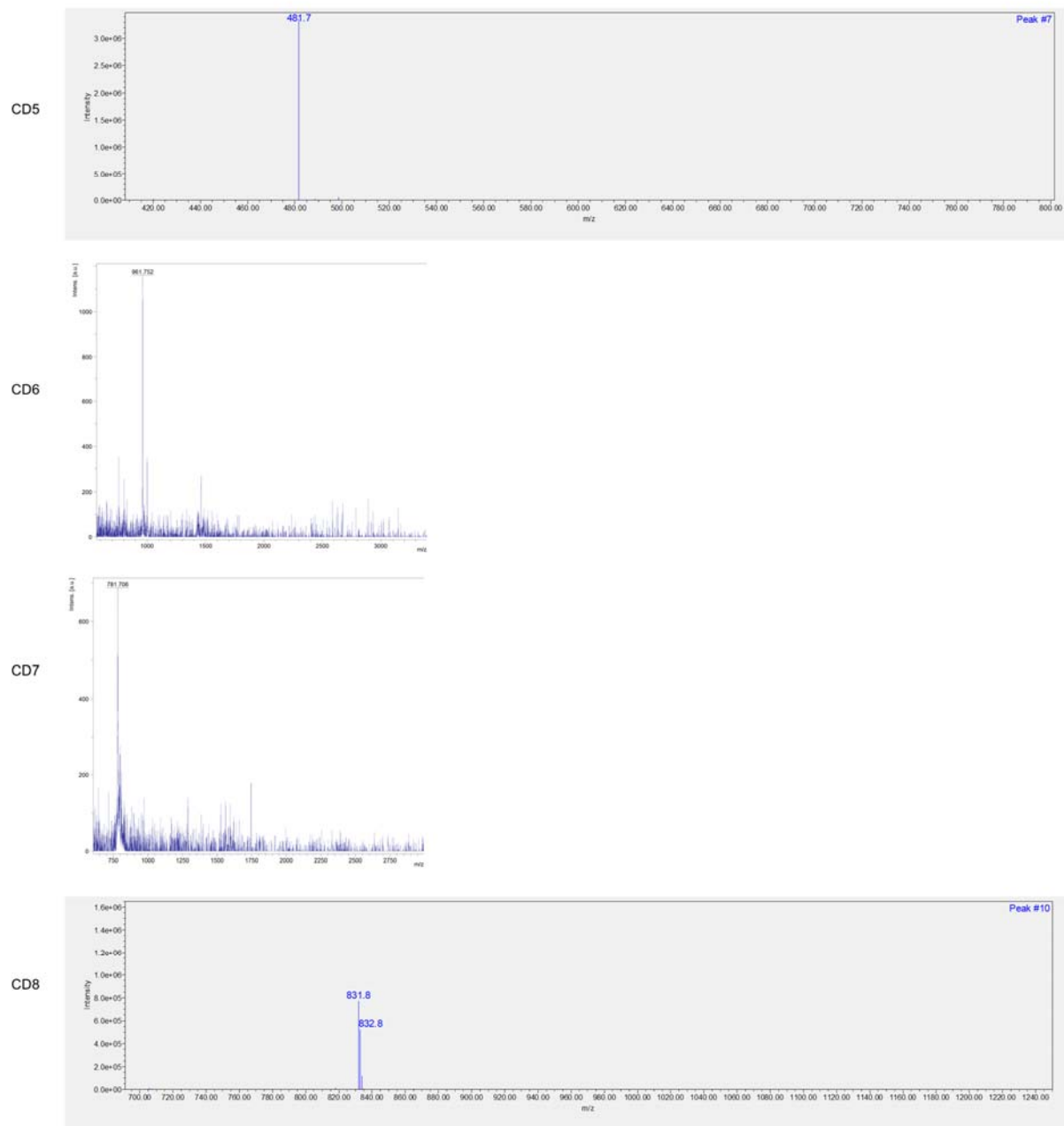
Supplementary Fig. 48. MS of TD5-TD8.

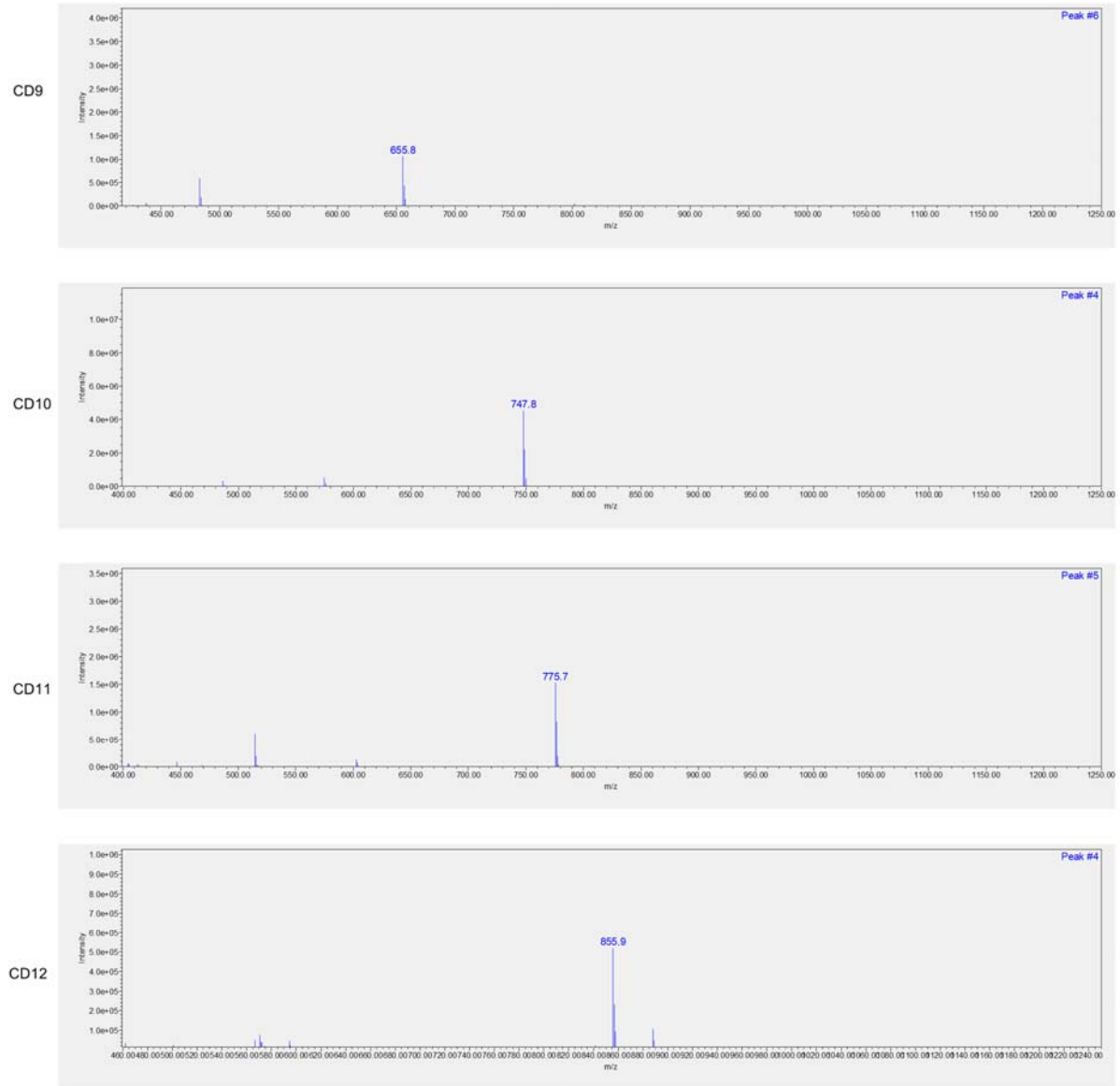


Supplementary Fig. 49. MS of TD9-TD12.



Supplementary Fig. 50. MS of CD1-CD4.





Supplementary Fig. 52. MS of CD9-CD12.

4. Supplementary references

1. Terstappen, G.C., Meyer, A.H., Bell, R.D. & Zhang, W. Strategies for delivering therapeutics across the blood–brain barrier. *Nature Reviews Drug Discovery* **20**, 362-383 (2021).
2. Arvanitis, C.D., Ferraro, G.B. & Jain, R.K. The blood–brain barrier and blood–tumour barrier in brain tumours and metastases. *Nature Reviews Cancer* **20**, 26-41 (2020).
3. Hou, X., Zaks, T., Langer, R. & Dong, Y. Lipid nanoparticles for mRNA delivery. *Nature Reviews Materials* **6**, 1078-1094 (2021).
4. Xue, Y., Che, J., Ji, X., Li, Y., Xie, J. & Chen, X. Recent advances in biomaterial-boosted adoptive cell therapy. *Chemical Society Reviews* **51**, 1766-1794 (2022).
5. Tang, W., Fan, W., Lau, J., Deng, L., Shen, Z. & Chen, X. Emerging blood-brain-barrier-crossing nanotechnology for brain cancer theranostics. *Chemical Society Reviews* **48**, 2967-3014 (2019).
6. Khare, P., Edgecomb, S.X., Hamadani, C.M., Tanner, E.E.L. & S Manickam, D. Lipid nanoparticle-mediated drug delivery to the brain. *Advanced Drug Delivery Reviews* **197**, 114861 (2023).
7. Ogawa, K. et al. Focused ultrasound/microbubbles-assisted BBB opening enhances LNP-mediated mRNA delivery to brain. *Journal of Controlled Release* **348**, 34-41 (2022).
8. Dhaliwal, H.K., Fan, Y., Kim, J. & Amiji, M.M. Intranasal delivery and transfection of mRNA therapeutics in the brain using cationic liposomes. *Molecular Pharmaceutics* **17**, 1996-2005 (2020).
9. Gao, M. et al. Targeted mRNA nanoparticles ameliorate blood–brain barrier disruption postischemic stroke by modulating microglia polarization. *ACS Nano* **18**, 3260-3275 (2024).
10. Wang, D., Tai, P.W. & Gao, G. Adeno-associated virus vector as a platform for gene therapy delivery. *Nature reviews Drug discovery* **18**, 358-378 (2019).
11. Thomas, C.E., Ehrhardt, A. & Kay, M.A. Progress and problems with the use of viral vectors for gene therapy. *Nature Reviews Genetics* **4**, 346-358 (2003).
12. Robison, A.J. et al. Behavioral and structural responses to chronic cocaine require a feedforward loop involving Δ FosB and calcium/calmodulin-dependent protein kinase II in the nucleus accumbens shell. *Journal of Neuroscience* **33**, 4295-4307 (2013).
13. Kelz, M.B. et al. Expression of the transcription factor Δ FosB in the brain controls sensitivity to cocaine. *Nature* **401**, 272-276 (1999).
14. Nabhan, J.F. et al. Intrathecal delivery of frataxin mRNA encapsulated in lipid nanoparticles to dorsal root ganglia as a potential therapeutic for Friedreich's ataxia. *Scientific Reports* **6**, 20019 (2016).
15. Delhaas, E.M. & Huygen, F. Complications associated with intrathecal drug delivery systems. *BJA Education* **20**, 51-57 (2020).
16. Jain, R. et al. MicroRNAs Enable mRNA therapeutics to selectively program cancer cells to self-destruct. *Nucleic Acid Therapeutics* **28**, 285-296 (2018).
17. Zhang, Y. et al. STING agonist-derived LNP-mRNA vaccine enhances protective immunity against SARS-CoV-2. *Nano Letters* **23**, 2593-2600 (2023).

18. Chinchilla, R., Dodsworth, D.J., Nájera, C. & Soriano, J.M. A new polymer-supported reagent for the Fmoc-protection of amino acids. *Tetrahedron Letters* **42**, 7579-7581 (2001).
19. Zhang, X. et al. Functionalized Lipid-Like Nanoparticles for in Vivo mRNA Delivery and Base Editing. *Science Advances* **6**, eabc2315 (2020).
20. Natarajan, R., Northrop, N. & Yamamoto, B. Fluorescein isothiocyanate (FITC)-dextran extravasation as a measure of blood-brain barrier permeability. *Current Protocols in Neuroscience* **79**, 9.58.51-59.58.15 (2017).
21. Chen, S., Zhou, Y., Chen, Y. & Gu, J. fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* **34**, i884-i890 (2018).
22. Kim, D., Paggi, J.M., Park, C., Bennett, C. & Salzberg, S.L. Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nature Biotechnology* **37**, 907-915 (2019).
23. Li, H. et al. The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25**, 2078-2079 (2009).
24. Liao, Y., Smyth, G.K. & Shi, W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* **30**, 923-930 (2013).
25. Love, M.I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology* **15**, 550 (2014).
26. ©[[2023]] Allen Institute for Brain Science, [Allen Mouse Brain Atlas], https://mouse.brain-map.org/experiment/thumbnails/100048576?image_type=atlas.