Electronic Supplementary Information

A naphthalimide-based [12]aneN$_3$ compound as an effective and real-time fluorescence tracking non-virus gene vector

Yong-Guang Gao,[a] You-Di Shi,[a] Ying Zhang,[a] Jing Hu,[a] Zhong-Lin Lu,[a*] Lan He[b*]

1. Materials and instruments

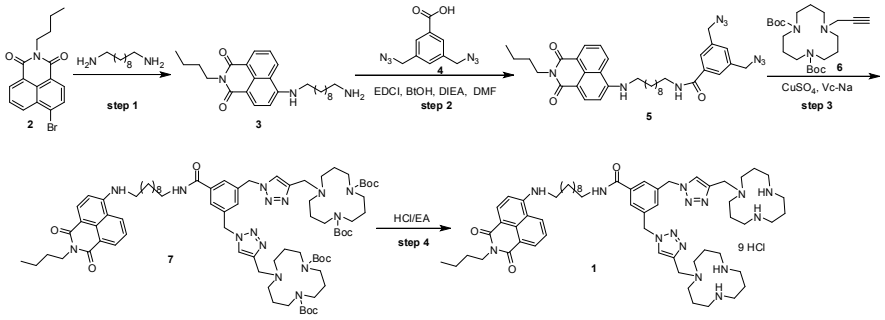
1.1. Materials

Anhydrous ethanol, methanol, tetrahydrofuran (THF), dichloromethane (DCM) and dimethyl formamide (DMF) were dried and purified under nitrogen by using standard methods and were distilled immediately before use. 3,5-bis(azidomethyl)benzoic acid and di-tert-butyl 9-(prop-2-ynyl)-1,5,9-triazacyclododecane-1,5-dicarboxylate (compound **6**) were prepared according to the literature.[1-2] Electrophoresis grade agarose, 6 × loading buffer (30 mM EDTA, 40% glycerol, 0.03% xylene cyanol FF, and 0.05% bromophenol blue), Goldview II, 4,6-diamidino-2-phenylindole (DAPI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bormide (MTT), plasmid DNA (pUC 18) were purchased from Solarbio Company. Dioleoyl phosphatidyl ethanolamine (DOPE) was from Santa Cruz Biotechnology.Lipofectamine 2000^{TM} and Cy5-labeled double-stranded DNA oligomer 5′-GGTCGGAGTCAACGGATTTGGTCG-3′-(Cy5-DNA) were from Invitrogen. Ultrapure milli-Q water (18.25 MΩ) was used in all DNA condensation assays.

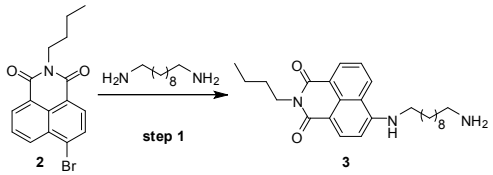
1.2. Instruments

^{1}H and ^{13}C NMR spectra were obtained on a Bruker Avance III 400 MHz spectrometer at 25 ºC. The infrared spectra were taken on a Nicolet 380 spectrometer. Mass spectra were acquired on a Waters Quattro Mocro spectrometer and high resolution mass spectra were acquired on a Waters LCT Premier XE spectrometer. Electrophoresis apparatus was a BG-subMIDI sub marine system (BayGene Biotech Company Limited, Beijing, China). Hydrodynamic diameters were determined using a Brookhaven ZetaPlus Partical Size and Zeta Potential Analyzer. The morphologies of the lipoplexes were observed by SEM(aHitachi, X650). Fluorescence spectra were measured on a Varian Cary Eclipse spectrometer.

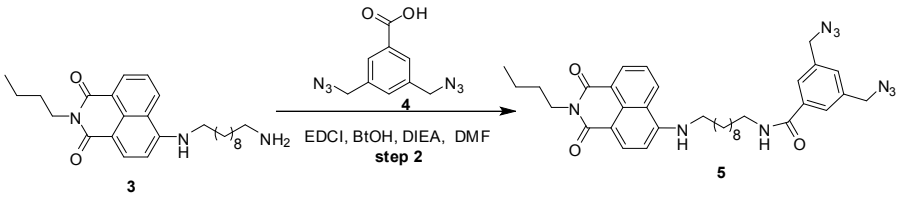
2 Synthesis



Scheme 1 Synthesis of cationic lipid **1**

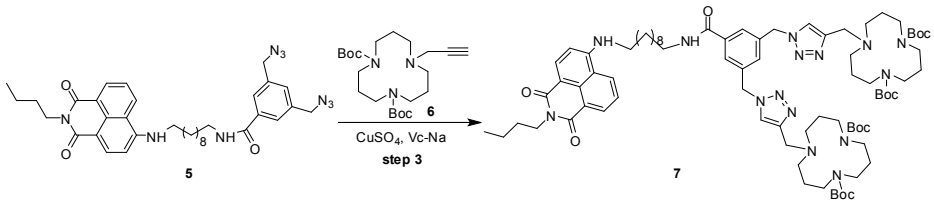


To a stirred solution of the *N*-butyl-4-bromine-1,8-naphthalimide (1.0 g, 3.0 mmol) in 2-methoxyethanol (15 mL) was added 1,10-diaminodecane (2.6 g, 15.00 mmol). The mixture was refluxed for 24 h and monitored by TLC. After completion, the reaction mixture was cooled to room temperature and concentrated under vacuum until most of the solvent was removed. The residue was washed with water and then purified by column chromatography on silica gel (CH_2Cl_2/MeOH = 5/1) to provide product 0.52 g (41%).1H NMR (400 MHz, DMSO) δ 8.71 (d, J = 8.4 Hz, 1H), 8.43 (d, J = 7.2 Hz, 1H), 8.26 (d, J = 8.6 Hz, 1H), 7.78 (s, 1H), 7.67 (t, J = 7.8 Hz, 1H), 6.76 (d, J = 8.6 Hz, 1H), 4.01 (t, J = 7.3 Hz, 2H), 3.36-3.33 (m, 2H), 1.75-1.66 (m, 2H), 1.59-1.54 (m, 2H), 1.44-1.19 (m, 18H), 0.92 (t, J = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 164.16, 163.31, 151.11, 134.65, 131.00, 129.88, 129.05, 124.54, 122.28, 120.57, 107.90, 104.10, 43.30, 31.43, 29.38, 29.34, 29.27, 29.16, 28.28, 28.04, 27.09, 26.66, 26.55, 22.42, 14.31. HR-MS: m/z = 424.2957 ([M+H]$^+$).



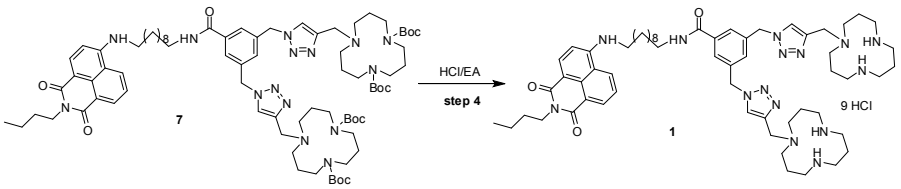
3,5-bis(azidomethyl)benzoic acid (0.16 g, 0.71 mmol), EDCI (0.15 g, 0.78 mmol), BtOH (0.11 g, 0.78 mmol) and DIEA (0.18 g, 1.42 mmol) in DMF (10 mL) stirred for 0.5 h, then compound **3**

(0.3 g, 0.71 mmol) was added, and stirred for 4h. Water (5 mL) was added and the mixture was extracted with DCM (2 x 15 mL). The organic phase was dried (Na_2SO_4), filtered, and the solvent was evaporated under reduced pressure. The crude material was purified by column chromatography on silica gel (PE/EA = 2/1) to give yellow solid 0.3 g (66%).^{1}H NMR (400 MHz, $CDCl_3$) δ 8.58 (d, *J* = 6.7 Hz, 1H), 8.46 (d, *J* = 8.4 Hz, 1H), 8.09 (d, *J* = 8.0 Hz, 1H), 7.68 (s, 2H), 7.61 (t, *J* = 7.9 Hz, 1H), 7.41 (s, 1H), 6.72 (d, *J* = 8.5 Hz, 1H), 6.19 (brs, 1H), 5.30 (t, *J* = 4.8 Hz, 1H), 4.43 (s, 4H), 4.16 (t, *J* = 7.5 Hz,, 2H), 3.47 (q, *J* = 6.9 Hz, 2H), 3.40 (q, *J* = 7.0 Hz, 2H), 1.84-1.78 (m, 2H), 1.72-1.63 (m, 4H), 1.49-1.33 (m, 14H), 0.97 (t, J = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, CDCl3) δ 166.66, 164.75, 164.21, 149.60, 136.86, 136.11, 134.52, 131.09, 130.27, 129.81, 126.36, 126.12, 124.60, 123.06, 120.19, 109.98, 104.28, 77.36, 77.10, 76.85, 54.14, 43.74, 40.29, 40.26, 31.64, 29.63, 29.39, 29.38, 29.29, 29.21, 28.86, 28.19, 27.12, 26.93, 26.89, 22.62, 14.15. HR-MS: m/z = 660.3386 ([M+Na]$^+$).



To a solution of the **5** (0.3 g, 0.47 mmol) and the **6** (0.38 g, 0.96 mmol) in THF-H_2O (10 mL/5 mL) copper sulfate (8 mg, 0.047 mmol) and Vc-Na (19 mg,0.1 mmol) were added and the mixture was stirred for overnight at room temperature. The solvent removed under reduced pressure. Water 10 mL was added and the mixturewas extracted with DCM (2 x 20 mL). The combined organic layerwas washed with saturated brine, dried over Na_2SO_4, filtered, and the solvent wasevaporated under reduced pressure. The crude material was purified by column chromatography on silica gel (CH_2Cl_2/MeOH =20/1) to give yellow solid 0.4 g (58%).^{1}H NMR (400 MHz, $CDCl_3$) δ 8.58 (d, *J* = 6.9 Hz, 1H), 8.46 (d, *J* = 8.2 Hz, 1H), 8.13 (d, *J* = 8.2 Hz, 1H), 7.70-7.57 (m, 3H), 7.37 (s, 2H), 7.31 (s, 1H), 6.72 (d, *J* = 7.2 Hz, 1H), 6.57 (s, 1H), 5.52 (s, 4H), 5.45 (s, 1H), 4.16 (s, 2H), 3.75 (s, 4H), 3.40 (s, 4H), 3.33 - 3.28 (m, 16H), 2.42 (s, 8H), 1.82 - 1.75 (m, 20H), 1.62-1.28 (m, 48H), 0.97 (d, *J* = 7.0 Hz, 3H)；^{13}C NMR (101 MHz, $CDCl_3$) δ 166.17, 164.54, 163.95, 156.14, 150.05, 144.16, 136.80, 136.33, 134.38, 130.79, 129.74, 126.83, 124.16, 122.64, 120.24, 109.22, 103.93, 79.15, 53.15, 49.76, 46.69, 45.30, 43.78, 43.56, 40.11, 39.72, 30.20, 29.40, 29.23, 29.15, 29.05,

28.56, 28.56, 28.37, 27.02, 20.27, 13.78; IR (KBr, cm^{-1}): 3406.33, 3121.69, 2969.88, 2931.93, 2812.65, 1684.94, 1646.99, 1581.93, 1552.11, 1476.20, 1413.86, 1384.04, 1365.06, 1305.42, 1243.07, 1169.88, 1050.60, 776.81; ESI-MS: m/z = 1456.5 ($[M+H]^+$).



Compound **7** (0.4 g, 0.27 mmol) was added to a saturated hydrogen chloride solution of ethyl acetate (10 mL) and the mixture was stirred for 30 minutes at room temperature. The resulting suspension was filtrated and the solid was washed with ethyl acetate, then dried in vacuum at 60 °C for 24h. 0.3 g (83%) yellow solid was obtained. ^{1}H NMR (400 MHz, D_2O) δ 8.07 - 7.85 (m, 3H), 7.77 (s, 1H), 7.55 (s, 1H), 7.45 (s, 2H), 7.12 (s, 1H), 7.03 (s, 1H), 5.84 (s, 1H), 5.31 (s, 4H), 3.96 - 3.68 (m, 4H), 3.50 (s, 2H), 3.27 - 2.28 (m, 28H), 2.18 - 1.92 (m, 4H), 1.89 – 1.65 (m, 8H), 1.26-0.34 (m, 23H); ^{13}C NMR (101 MHz, D_2O) δ 167.48, 164.58, 163.66, 150.41, 136.36, 135.99, 135.29, 130.82, 129.05, 128.44, 127.23, 124.10, 121.08, 119.63, 107.38, 103.38, 53.24, 48.81, 47.46, 43.27, 42.03, 41.13, 40.73, 40.14, 29.99, 29.20, 28.92, 28.73, 28.24, 26.73, 20.14, 18.83 , 17.99, 13.51; IR (KBr, cm^{-1}): 3419.88, 2929.22, 2853.31, 1638.86, 1576.51, 1543.98, 1459.94, 1392.17, 1386.75, 1354.22, 1243.07, 1126.51, 581.63; HR-MS: m/z = 1056.7325 ($[M+H]^+$).

3. Lipoplex formation and analysis

3.1. Agarose gel retardation

Lipoplexes at different concentrations were preparedby adding appropriate volume of the lipid (liposome) solution to 0.9 μL of pUC18 DNA (200μg/mL) and 4 μL of HEPES (100 mM，pH 7.2).Theobtained complex solution was then diluted to the total volume of 20 μL. After incubation at 37°C for 5 min, the lipoplexes were electrophoresed on a 0.7 % (w/v) agarose gel containing GelRedTM in Tris-acetate (TAE) running buffer at 120 V for 40 min. Then DNA was visualized under an ultraviolet lamp using a Vilber Lourmat imaging system.

3.2 Dynamic light scattering (DLS)

The liposome/DNA complexes with various concentrations were prepared by adding 0.9 μL of

pUC18 DNA (200 μg/mL) to the appropriate volume of the liposome solution.Then the complex solution was vortexed for 30 s before being incubated at 37 °C for 5 min andthen diluted up to 1 mL by ultrapurity water solution prior to be measured. Data were shown asmean ± standard deviation (SD) based on three independent measurements.

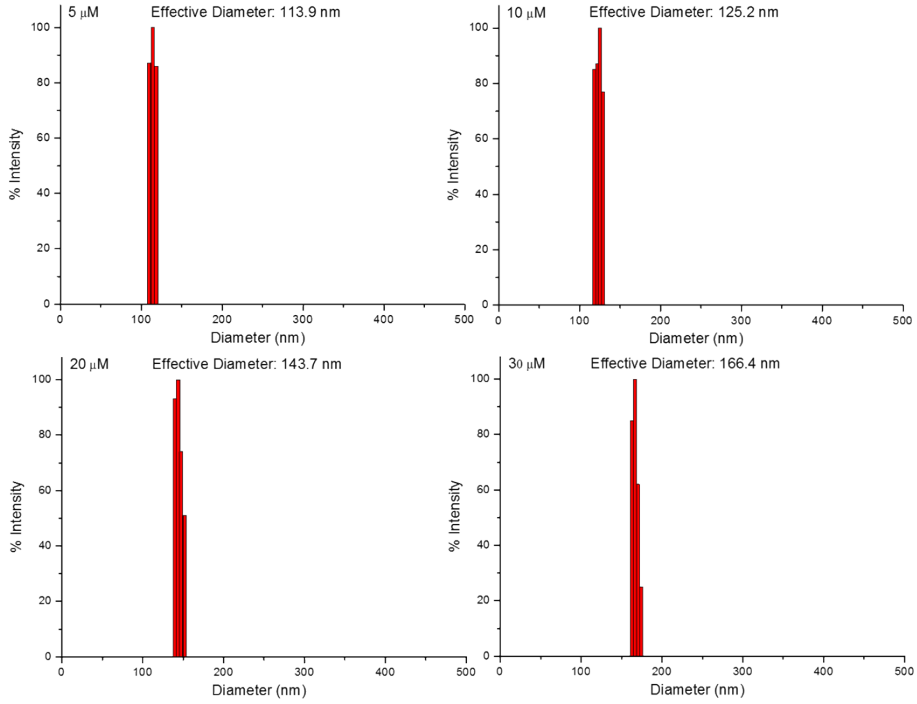


Fig. S1 Hydrodynamic diameter distributions of pUC18 DNA particles condensed by complexes of **1** withDOPEat different concentrations by DLS. The molar ratio of lipid**1**/DOPE was 1:2. The DNA concentration is 9 μg/mL

3.3 Scanning electron microscope (SEM) images

0.9 μL of pUC18 DNA (200 μg/mL)was mixed with the appropriate volume of liposome **1** solution to form complexes, diluted by water to a total volume of 20 μL, and incubated at 37 °C for 5 min. The lipoplexes was added dropwise to the silicon slice. The slice was dried at room temperature at atmosphericpressure for several hours before observation.

4. Biological studies

4.1. In vitro transfection experiments

Gene transfection of liposome **1** was investigated in A549 cells. Cells were seeded in 24-well plates (8 x 10^4 cells/well) and grown to reach 70-80% cell confluence at 37 °C for 24 h in 5% CO_2.Before transfection, the medium was replaced with a serum-free DMEM culture medium

containing liposome/DNA complexes at various concentrations. After 4 hunder standard incubator conditions, the medium was replaced with fresh medium containingserum and incubated for another 20 h.

4.2. Confocal laser scanning microscopy (CLSM)

The cellular uptake of Cy5-labeled dsDNA condensates was observed by fluorescence microscope. A549 cells were cultured in DMEM medium supplemented with 10% FBS in a humid atmosphere containing 5% CO_2 at 37 ºC. The cells were seeded in Glass Bottom Cell Culture Dishes at 1000 cells per dish and cultured for 24 h. After washed three times with DMEM, the cells were treated with freshly prepared Cy5-DNA condensates and the controls (500 μL). The blue fluorescence dye DAPI (5 μg/mL) was also added to each dish for nuclear staining, after that the cells were cultured for 4 h (or different hours). Finally, the cells were washed for 6 times with PBS buffer, observed using a Zeiss Inverted Fluorescence Microscope with a 40× objective and DAPI filter for DAPI (blue), GFP filter for lipid **1** (green), and Rhodamine filter for Cy5 (red), respectively.

4.3.Cytotoxicity assay

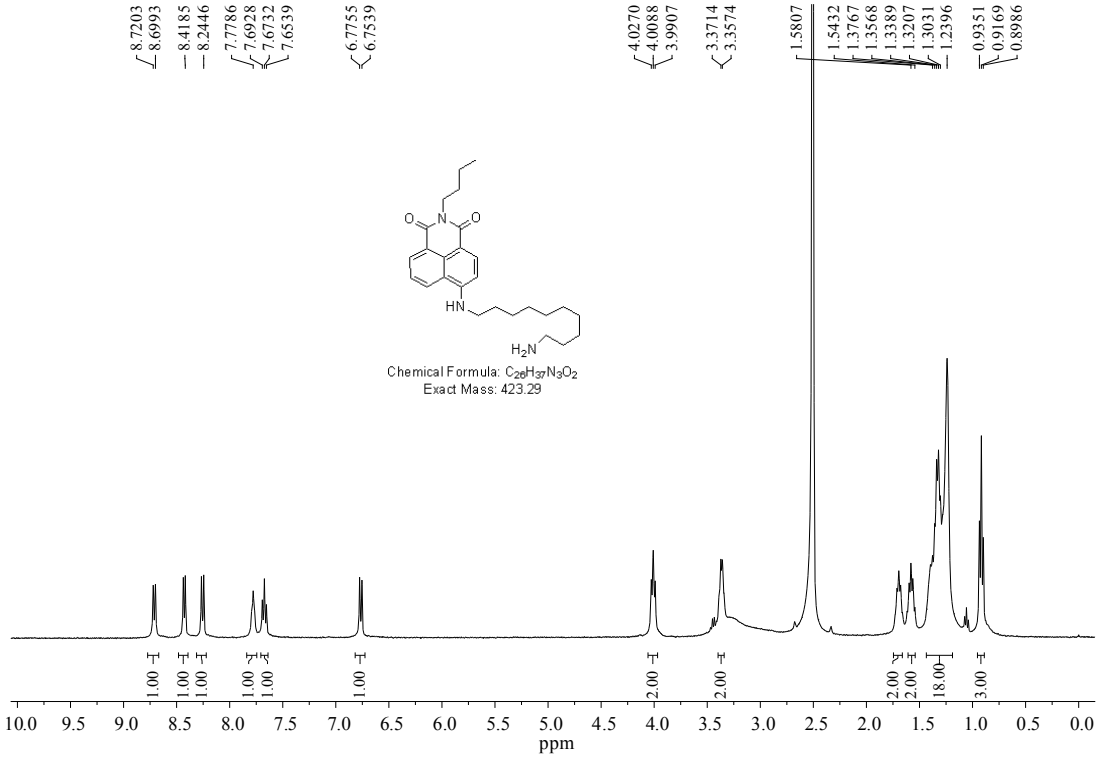
The cytotoxicity of lipoplex **1**toward A549 cell lines were tested by MTT assays (MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with fetal bovine serum (FBS, 10%, v/v) in a humid atmosphere containing 5% CO_2 at 37 ºC. After 48 h of incubation in the medium, the cells were seeded in 96-well plates at 5000 cells and 100 μL medium per well and cultured for another 24 h. Then the cells were treated with different concentrations of **1**in 100 μL DMEM, 100 μL DMEM with 10% FBS was added to each well 4 h later, and cells were further cultured for 20 h. After that the medium was removed and 20 μL of MTT (5 mg/mL) was added to wells, the cells were incubated for another 4 h. Finally MTT was replaced with 200 μL of DMSO, the plates were oscillated for 10 min to fully dissolve the formazan crystal formed by living cells in the wells. The absorbance of the purple formazan was recorded at 490 nm using a Thermo Scientific Multiskan GO. The relative viability of the cells was calculated based on the data of five parallel tests by comparing to the controls.

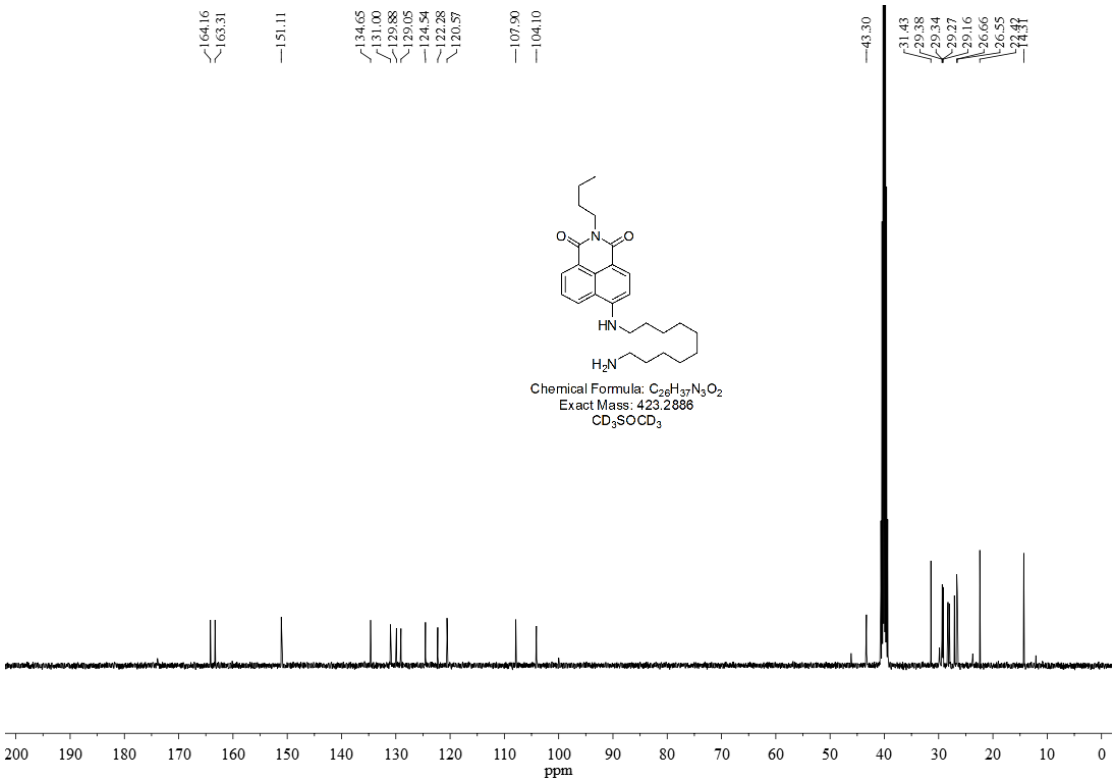
References

1.H.Y. Kuchelmeister and C. Schmuck. *Eur. J. Org. Chem.*, 2009, **2009**, 4480.

2.Z.-F. Guo, H. Yan, Z.-F. Li and Z.-L. Lu. *Org. Biomol. Chem*., 2011, **9**, 6788.

Spectra





Elemental Composition Report Page 1

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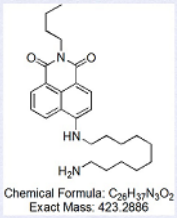

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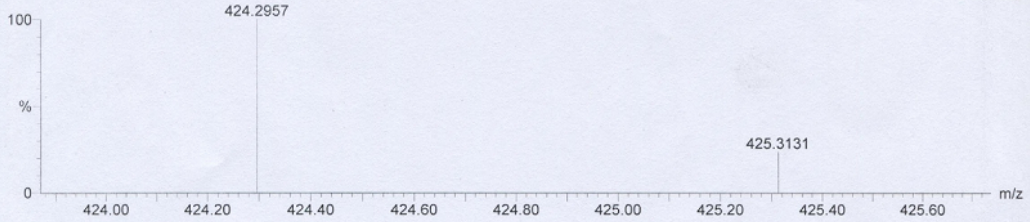

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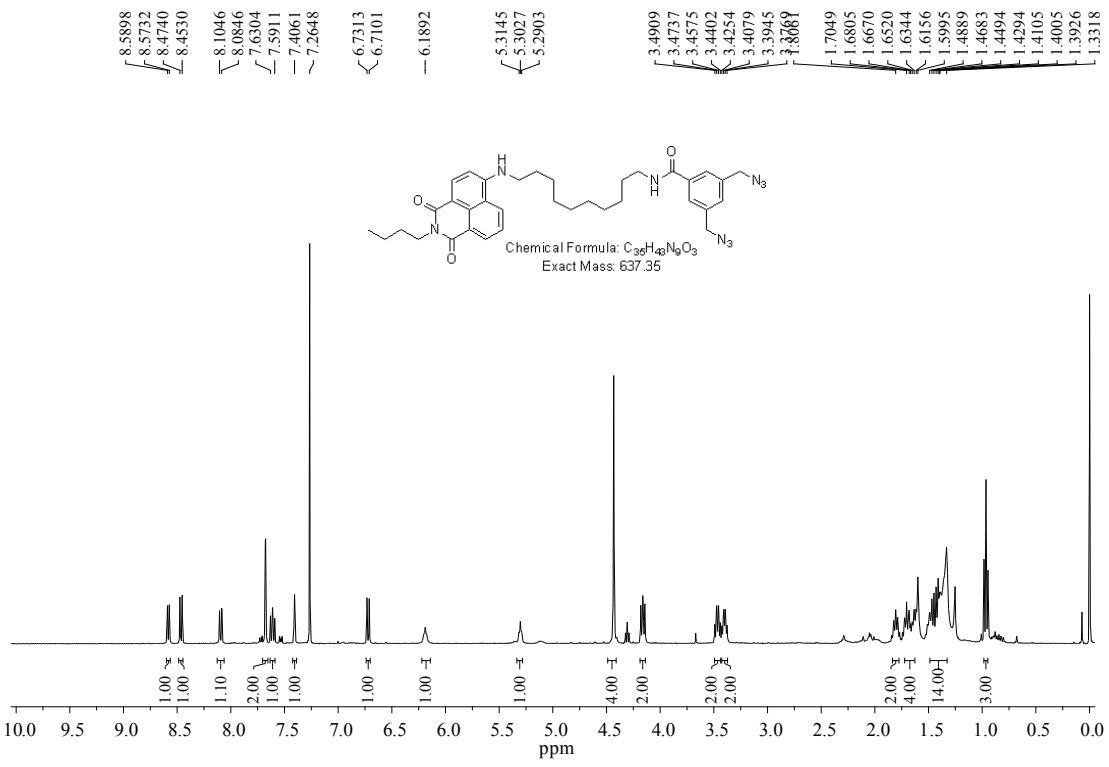

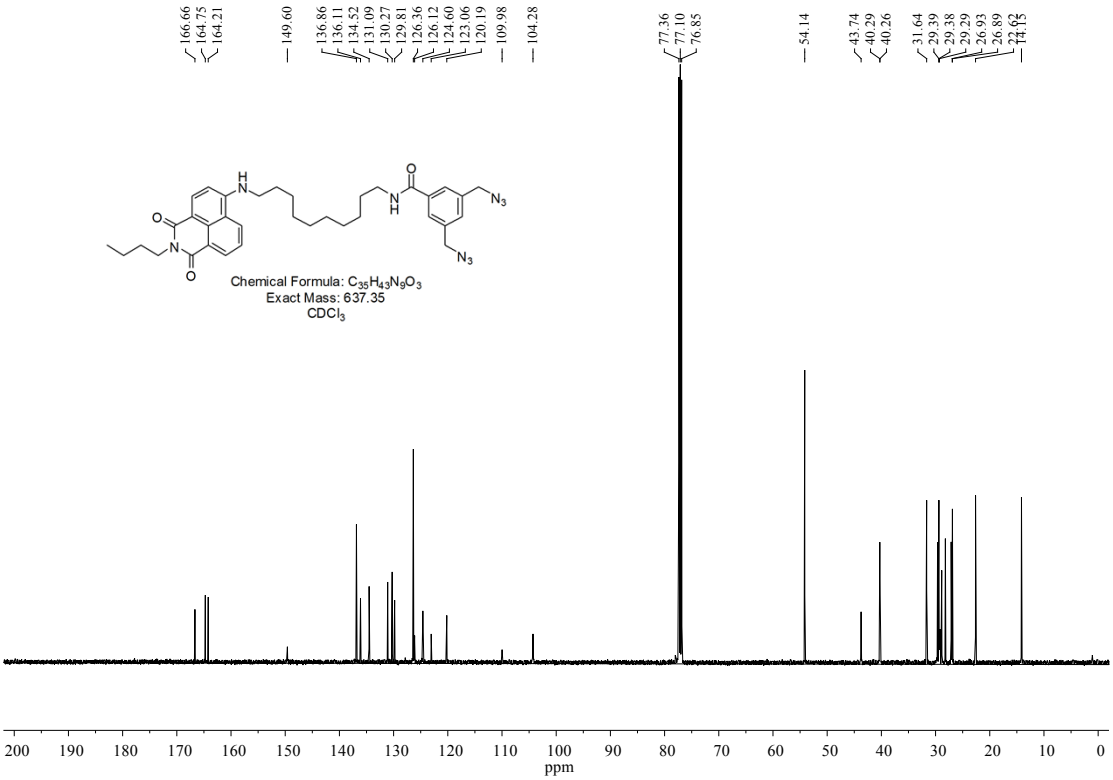

Elemental Composition Report

Page 1

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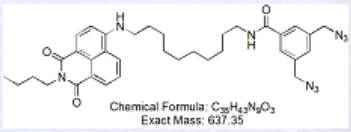

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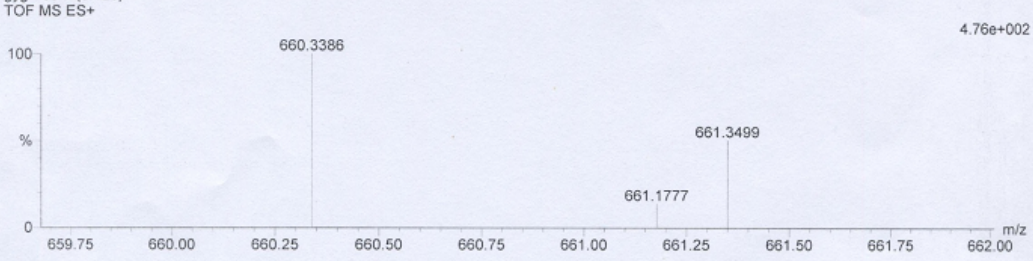

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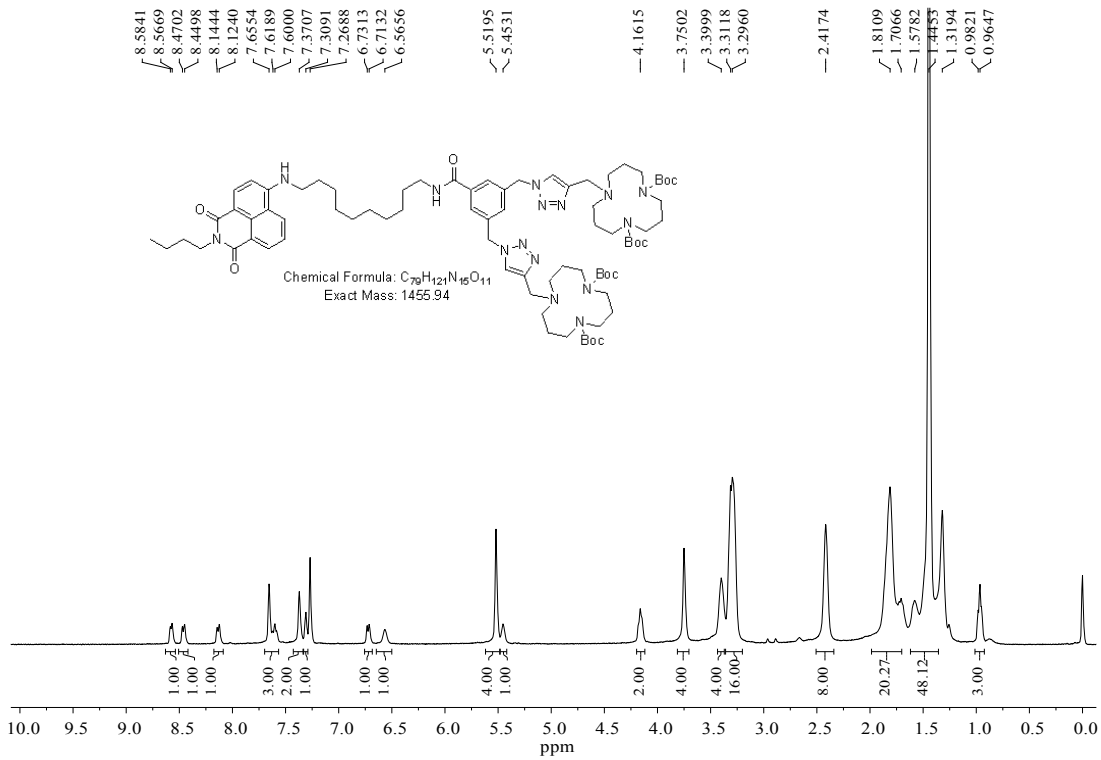
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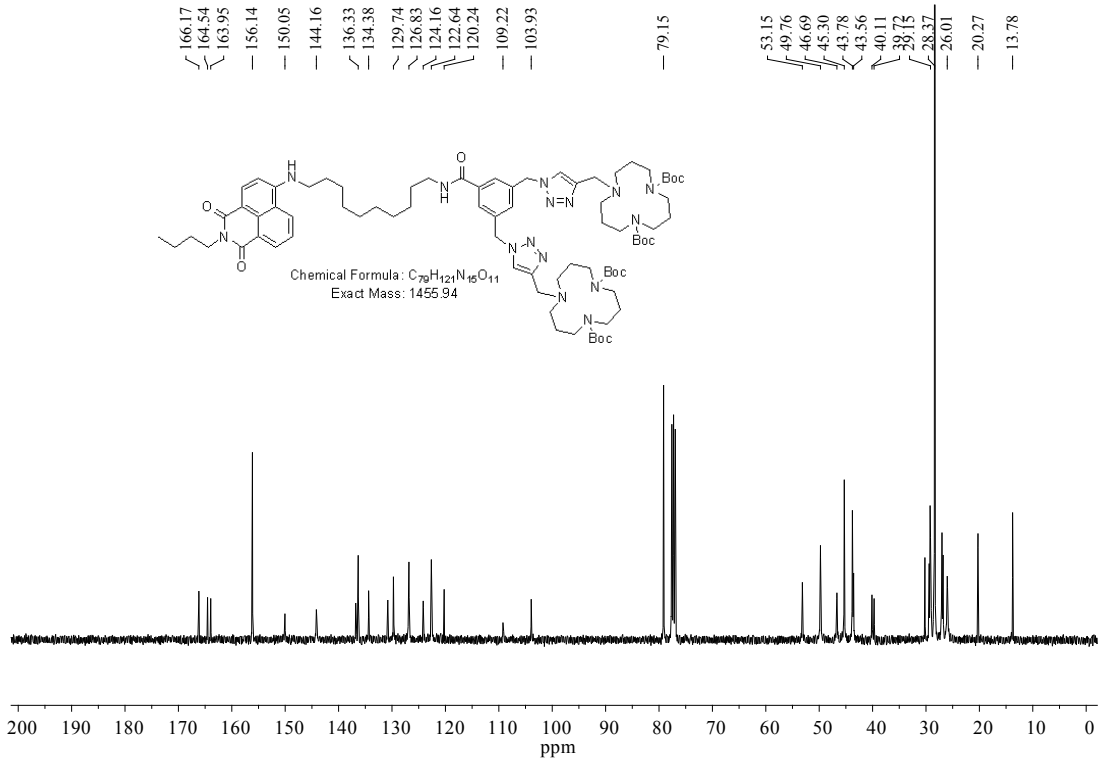
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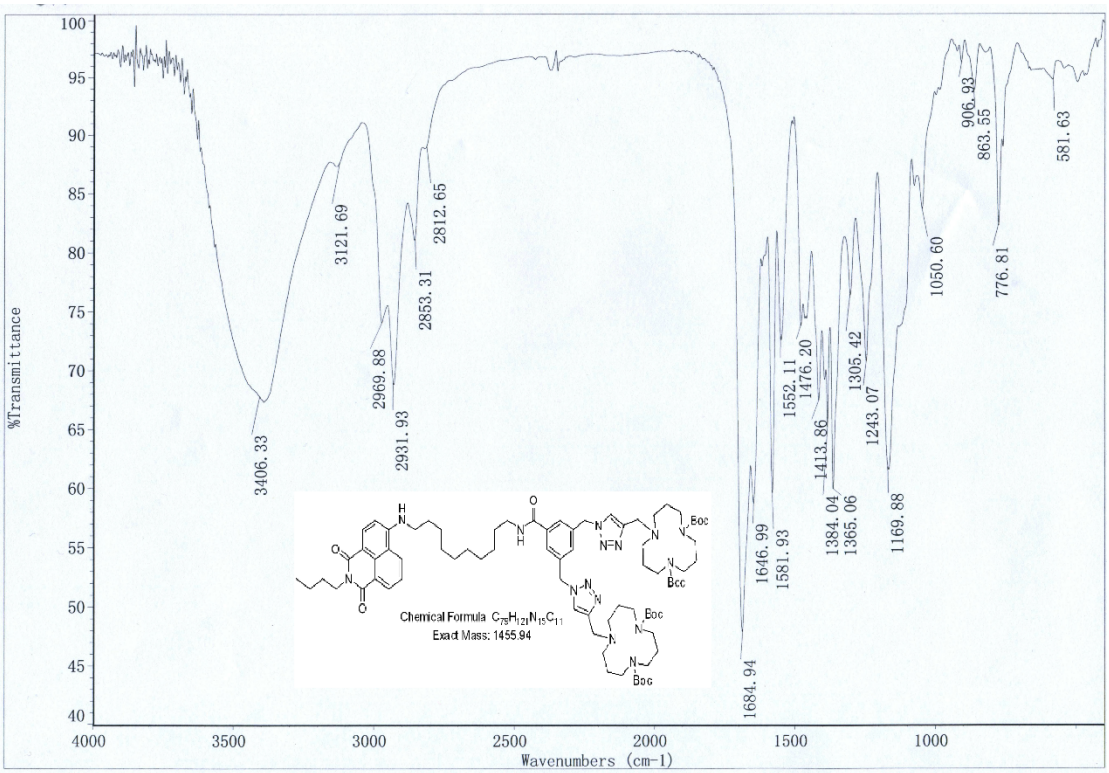
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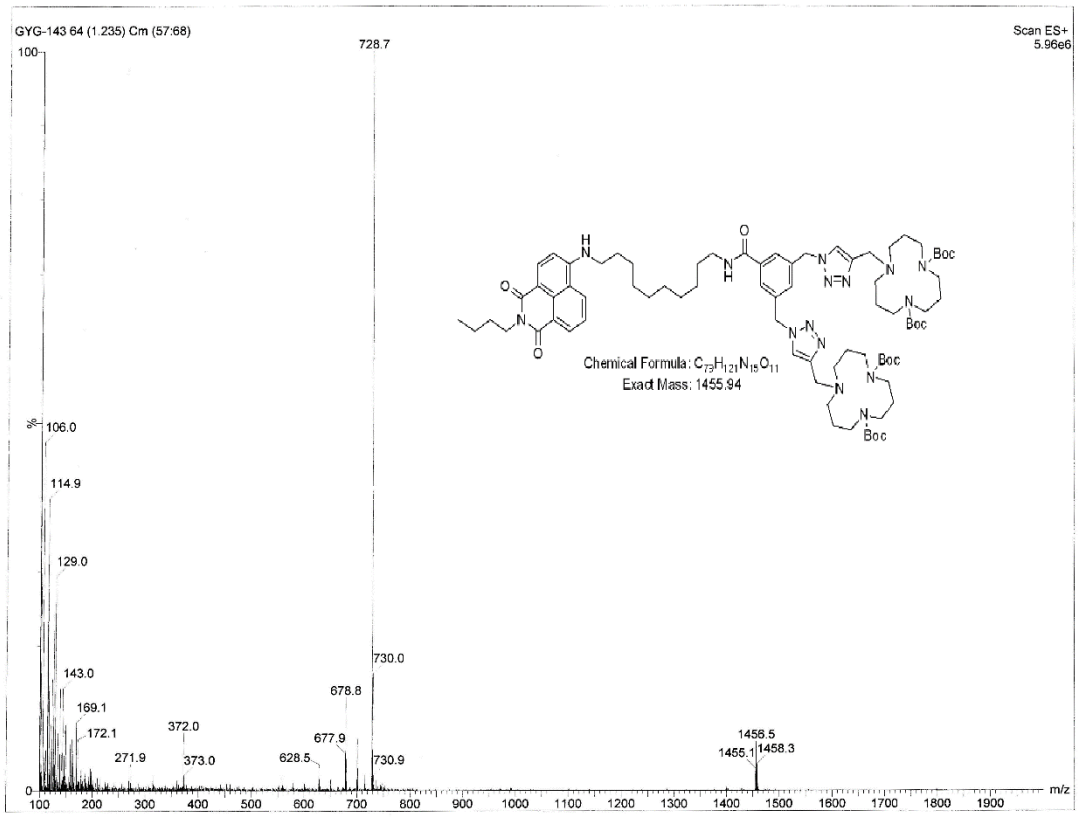
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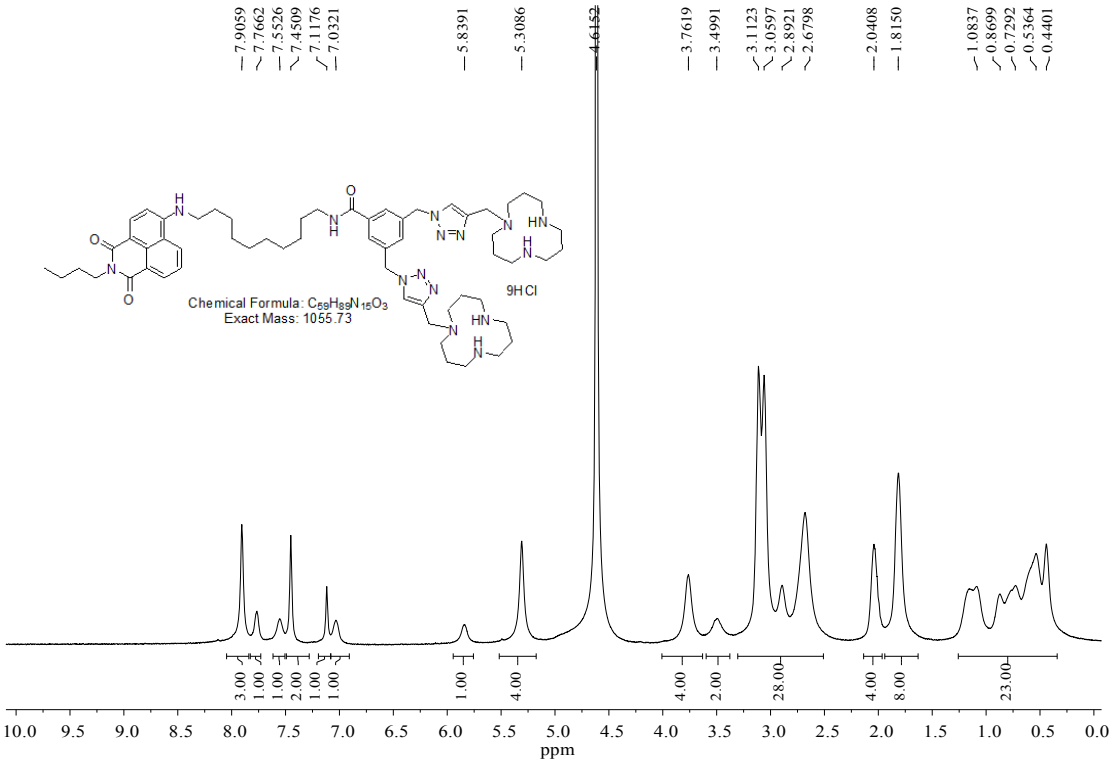
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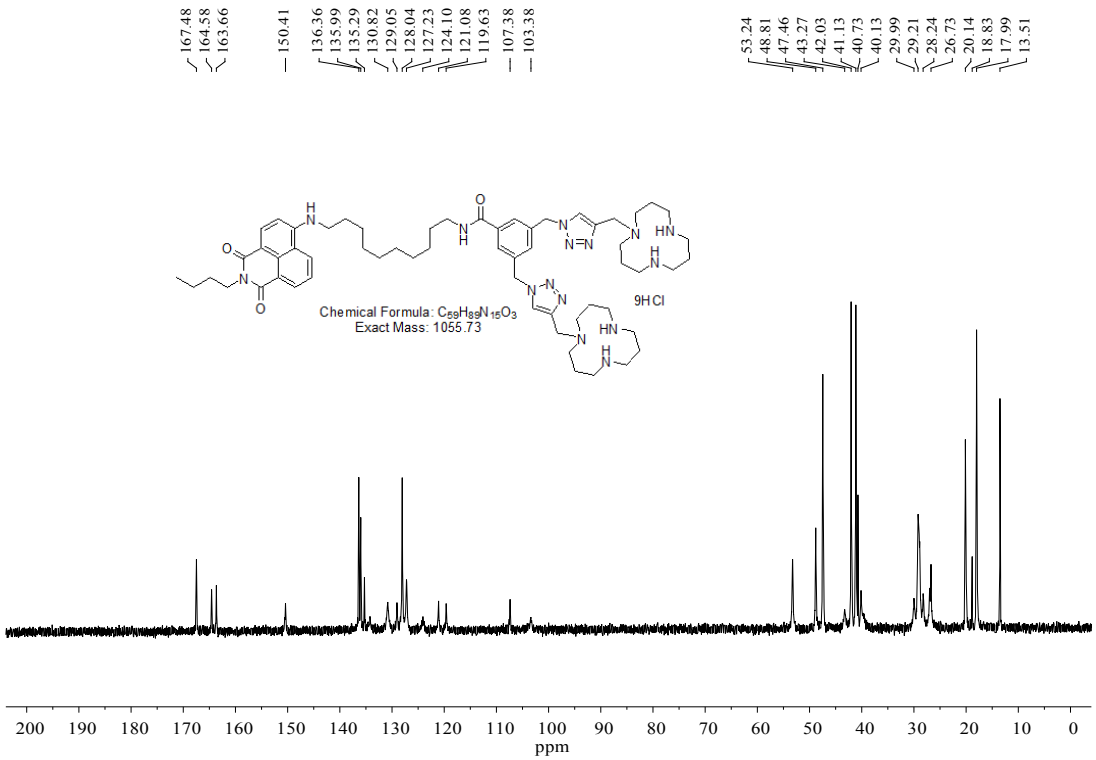
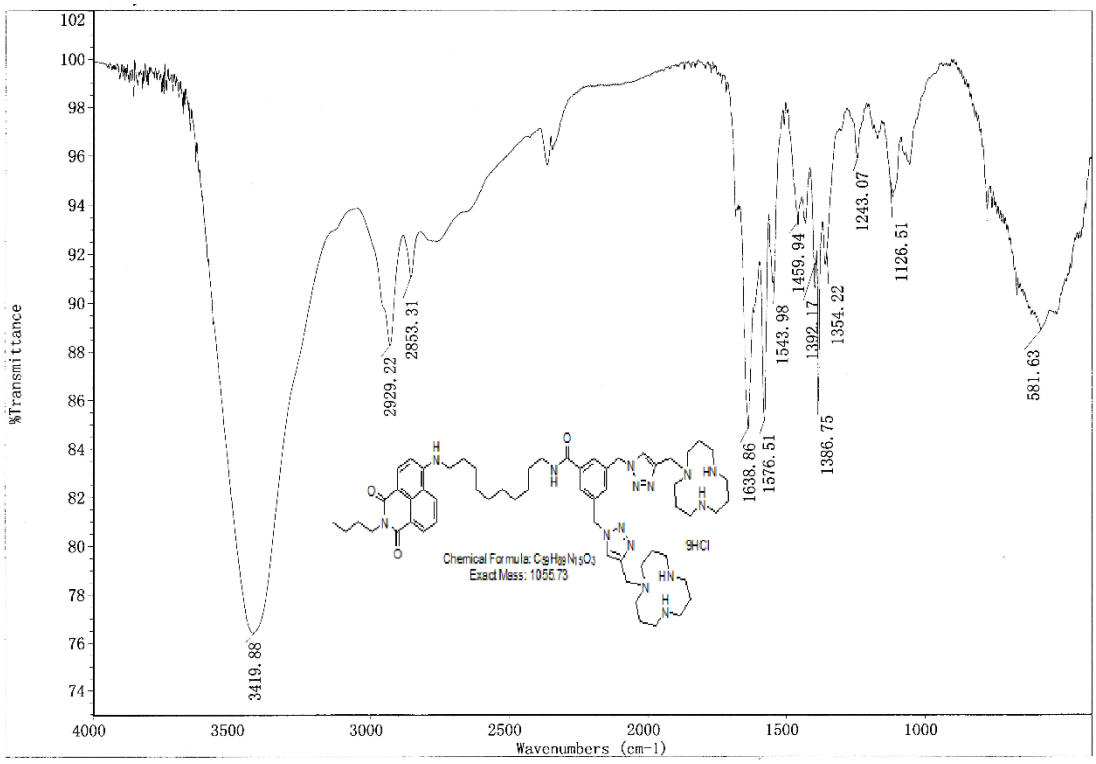
ppm

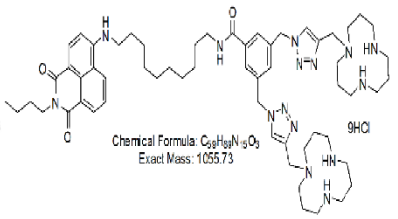

167.48
164.58
163.66
150.41
136.36
135.99
135.29
130.82
129.05
128.04
127.23
124.10
121.08
119.63
107.38
103.38
53.24
48.81
47.46
43.27
42.03
41.13
40.73
40.13
29.99
29.21
28.24
26.73
20.14
18.83
17.99
13.51
9HCl
Chemical Formula: C59H89N15O3
Exact Mass: 1055.73
ppm



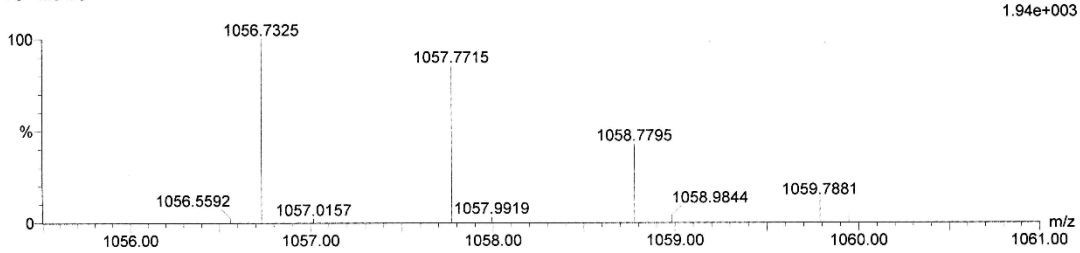
Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 2

Monoisotopic Mass, Even Electron Ions
7367 formula(e) evaluated with 34 results within limits (up to 50 closest results
Elements Used:
C: 0-100 H: 0-120 N: 0-15 O: 0-10 I: 0-4



GYG-145 8 (0.148)
TOF MS ES+
1.94e+003



Minimum:				-1.5		
Maximum:		5.0	5.0	50.0		
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
1056.7325	1056.7327	-0.2	-0.2	41.5	128.5	C41 H4 N6 O6 I3
	1056.7330	-0.5	-0.5	10.5	26.1	C57 H103 N9 O I
	1056.7319	0.6	0.6	30.5	5.9	C70 H90 N9
	1056.7319	0.6	0.6	34.5	144.3	C40 H13 N4 I4
	1056.7317	0.8	0.8	5.5	33.4	C56 H107 N5 O5 I
	1056.7313	1.2	1.1	36.5	148.0	C40 H8 N2 O10 I3
	1056.7338	-1.3	-1.2	21.5	269.3	C28 H17 N6 O7 I4
	1056.7338	-1.3	-1.2	17.5	21.0	C58 H94 N11 O7
	1056.7311	1.4	1.3	22.5	299.9	C24 H13 N12 O5 I4
	1056.7340	-1.5	-1.4	46.5	110.6	C42 N10 O2 I3
	1056.7306	1.9	1.8	25.5	7.5	C69 H94 N5 O4
	1056.7306	1.9	1.8	29.5	165.1	C39 H17 O4 I4
	1056.7304	2.1	2.0	0.5	42.0	C55 H111 N O9 I
	1056.7346	-2.1	-2.0	29.5	4.6	C74 H94 N3 O2
	1056.7300	2.5	2.4	42.5	148.6	C37 N12 O4 I3
	1056.7351	-2.6	-2.5	26.5	241.0	C29 H13 N10 O3 I4
	1056.7351	-2.6	-2.5	22.5	15.8	C59 H90 N15 O3