Electronic Supplementary Material (ESI) for Analyst.

Supplementary Information

Identification of Lipid-Specific Proteins with High-Density Lipid-Immobilized Beads

Masayuki Morito, Hiroki Yasuda, Takaaki Matsufuji, Masanao Kinoshita, Nobuaki Matsumori*

Department of Chemistry, Graduate School of Science, Kyushu University, Fukuoka, 819-0395, Japan.

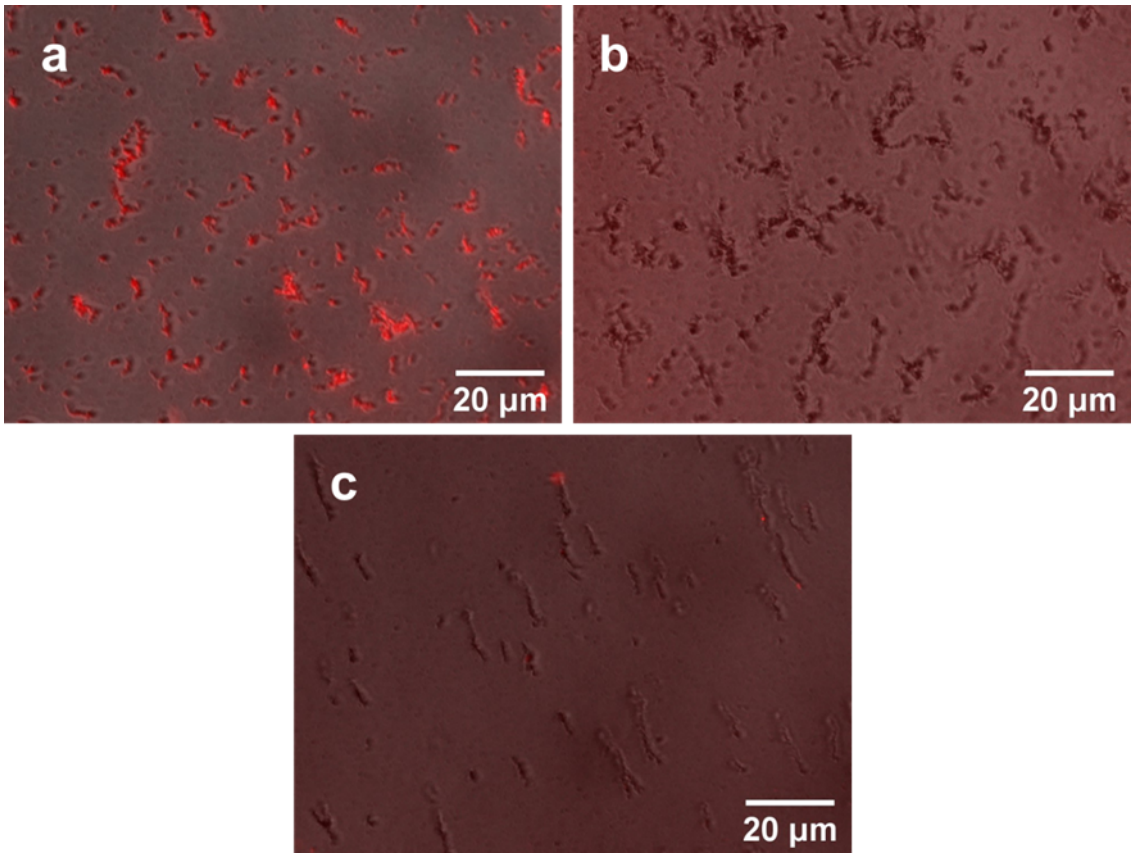

Figure S1. Fluorescence images of RFP-lysenin bound to each type of beads (a: SM beads, b: Cer beads, c: control beads). After incubation with RFP-lysenin and washing with PBS, the beads were resuspended in PBS and observed using a fluorescence microscope (BZ-X700, Keyence, Osaka, Japan). RFP-lysenin specifically bound to SM beads and not to the other beads. The brightness and contrast were adjusted for clarity.

Table S1. Amount of immobilized SM molecules per milligram of beads (***x***) and an occupation area per SM molecule on the bead surface (***s***).

	x (nmol mg^{-1})	s (Å^2)
1	76.6	49.1
2	72.5	51.8
3	70.6	53.2
Average	73.2	51.4
S.D.	3.0	2.1

Table S2. Identified lipid binding proteins from Neuro2a cells in each band.

#1 Cer

Protein name	*Score*	*Mass*	*Number of peptides*
Keratin, type I cytoskeletal 42	123	50444	4
Bone marrow stromal antigen 2	91	19311	3
Nesprin-2	87	787997	5

#2 SM

Protein name	*Score*	*Mass*	*Number of peptides*
Proline-, glutamic acid- and leucine-rich protein 1	99	119306	4
YLP motif-containing protein 1	80	155146	4
GRB10-interacting GYF protein 2	79	149387	3

#3 SM

Protein name	*Score*	*Mass*	*Number of peptides*
Tumor protein D54	805	24085	32
MICOS complex subunit Mic19	229	26546	6
Vimentin	222	53712	11
Peptidase inhibitor 16	179	54243	5

14-3-3 protein gamma	178	28456	11
Iron-sulfur protein NUBPL	169	34402	5
Mitochondrial import inner membrane translocase subunit TIM44	165	51401	6
MICOS complex subunit Mic27	157	29356	5
Beta-actin-like protein 2	155	42319	7
HAUS augmin-like complex subunit 1	154	31416	8
MICOS complex subunit Mic25	153	30175	6
Bone marrow stromal antigen 2	153	19311	5
60S ribosomal protein L24	139	17882	3
Calcium-binding mitochondrial carrier protein SCaMC-1	134	53096	4
Transcription initiation factor TFIID subunit 9B	133	27269	5
Single-strand selective monofunctional uracil DNA glycosylase	127	31091	4
Dynamin-like 120 kDa protein, mitochondrial	126	111783	5
14-3-3 protein sigma	124	27803	7
Glutamate--cysteine ligase regulatory subunit	124	30858	4
Coiled-coil domain-containing protein 127	120	30661	7
Peripherin OS=Mus musculus	117	54349	5
Cytosolic Fe-S cluster assembly factor NUBP2	114	29898	3
Heat shock protein HSP 90-beta	113	83571	3
ADP/ATP translocase 1	112	33111	6
Proteasome subunit alpha type-3	107	28615	4
Ras suppressor protein 1	95	31531	8
B-cell receptor-associated protein 31	94	27996	7
Neurosecretory protein VGF	94	68248	4
Myeloid leukemia factor 2	93	28094	3
Epimerase family protein SDR39U1	91	31496	3

Keratin, type II cytoskeletal 79	90	57802	3
Isocitrate dehydrogenase [NADP], mitochondrial	87	51330	3
D-beta-hydroxybutyrate dehydrogenase, mitochondrial	85	38617	4
Isocitrate dehydrogenase [NADP] cytoplasmic	83	47044	3
40S ribosomal protein S17	80	15571	3
X-linked lymphocyte-regulated protein PM1	77	24711	3
Peroxisomal membrane protein PEX16	75	38710	5
Tubulin alpha-1A chain	75	50788	4
tRNA selenocysteine 1-associated protein 1	74	32744	5
60S acidic ribosomal protein P0	73	34366	3
IgE-binding protein	72	63221	5
Cytochrome c1, heme protein, mitochondrial	67	35533	3
Myosin light polypeptide 6	65	17090	3
Proteasome subunit alpha type-1	54	29813	3

#4 Cer

Protein name	***Score***	***Mass***	***Number of peptides***
Protein FAM3C	200	25022	8
Protein SCO2 homolog, mitochondrial	183	29097	7
Bone marrow stromal antigen 2	167	19311	4
Membrane-associated progesterone receptor component 2	139	23434	3
Major prion protein	133	28131	4
Keratin, type II cytoskeletal 2 epidermal	126	71336	6
Bcl-2 homologous antagonist/killer	123	23394	7
Peroxisomal membrane protein 11C	122	27533	3
Retinol dehydrogenase 11	119	35525	4

Keratin, type I cytoskeletal 15	112	49278	7
4F2 cell-surface antigen heavy chain	105	58414	6
RNA transcription, translation and transport factor protein	99	28249	7
MICOS complex subunit Mic60	98	84247	5
Keratin, type II cytoskeletal 5	95	61957	7
Peptidase inhibitor 16	92	54243	3
Tubulin beta-5 chain	90	50095	3
Melanoregulin	90	25450	3
Alpha-internexin	85	55520	3
m-AAA protease-interacting protein 1, mitochondrial	83	33249	6
ZW10 interactor	81	28866	3
Endoplasmic reticulum chaperone BiP	76	72492	4
Adenine nucleotide translocase lysine N-methyltransferase	75	25009	3
Alpha-enolase	74	47453	3
Keratin, type II cytoskeletal 2 oral	70	63319	4
Tubulin beta-4B chain	63	50255	3
Acyl-protein thioesterase 2	58	25120	3
Peroxisomal membrane protein PEX16	53	38710	3

#5 SM

Protein name	*Score*	*Mass*	*Number of peptides*
ATP synthase subunit g, mitochondrial	273	11417	13
Dynein light chain 1, cytoplasmic	160	10530	4
ATP synthase subunit e, mitochondrial	144	8230	6
Tubulin beta-5 chain	137	50095	3
ADP/ATP translocase 2	133	33138	8
Enhancer of rudimentary homolog	127	12422	4
ADP/ATP translocase 1	124	33111	10
Keratin, type II cytoskeletal 1	124	66079	4

Keratin, type I cytoskeletal 16	114	51973	5
Ubiquitin-like protein 5	107	8655	4
Small integral membrane protein 4	106	9740	5
Keratin, type I cytoskeletal 15	104	49278	5
Histone H4	90	11360	4
40S ribosomal protein S18	85	17708	4
Cytochrome c oxidase subunit NDUFA4	61	9321	4

Supplementary Methods

General Method for Synthesis

EggSM was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Sphingosine was purchased from Toronto Research Chemicals (Toronto, Canada). Any other reagents were all purchased from FUJIFILM Wako Pure Chemical Corp. (Osaka, Japan), Tokyo Chemical Inc. (Tokyo, Japan), Nacalai Tesque Inc. (Kyoto, Japan) or Sigma-Aldrich (St. Louis, MO, USA). Thin-layer chromatography was performed on Merck precoated silica gel 60 F-254 plates, which were visualized by UV irradiation (254 nm) or staining with anisaldehyde/sulfuric acid. ^{1}H NMR spectra were obtained on a JEOL ECA 600 (600 MHz) spectrometer. High-resolution mass spectra (HRMS) were acquired on a Bruker micrOTOF II ESI-TOF mass spectrometer.

Synthesis of 16-azidohexadecanoic acid (1)

To a solution of 16-bromohexadecanoic acid (394 mg, 1.18 mmol) in DMF (5 mL) was added NaN_3 (115 mg, 1.76 μmol). The reaction mixture was stirred at room temperature overnight and then extracted with ethyl acetate. The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated *in vacuo*. Purification by silica gel column chromatography (hexane/ethyl acetate 3:1 v/v) afforded a colorless solid (310.8 mg, 1.05 mmol, 89%). R_f = 0.22 (3/1 hexane/ethyl acetate, v/v), ^{1}H NMR (600 MHz, $CDCl_3$): δ 3.25 (t, J = 7.2 Hz, 2H), 2.34 (t, J = 7.6 Hz, 2H), 1.65-1.57 (m, 4H), 1.37-1.25 (m, 22H), HRMS (*m*/*z*): $[M + H]^+$ calcd for $C_{16}H_{31}N_3NaO_2^+$, 320.2308; found, 320.2313.

Synthesis of 16-azidohexadecanoic acid NHS easter (2)

To a solution of the azidoacylacid (**1**) (29.7 mg, 99.8 μmol) in CH_2Cl_2 (2 mL) were added *p*-nitrophenol (20.9 mg, 150 μmol) and EDC·HCl (28.8 mg, 150 μmol). The reaction mixture was stirred at room temperature for 17 h and then extracted with $CHCl_3$. Purification by silica gel

column chromatography (hexane/ethyl acetate 10:1 v/v) afforded a colorless solid (23.6 mg, 55.9 µmol, 56%). R_f = 0.39 (10/1 hexane/ethyl acetate, v/v), ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.27 (d, *J* = 12.6 Hz, 2H), 7.27 (d, *J* = 12.6 Hz, 2H), 3.25 (t, *J* = 8.6 Hz, 2H), 2.59 (t, *J* = 9.1 Hz, 2H), 1.78-1.74 (m, 2H), 1.62-1.57 (m, 2H), 1.40-1.26 (m, 22H), HRMS (*m*/*z*): $[M + Na]^+$ calcd for $C_{22}H_{34}N_4NaO_4^+$, 441.2472; found, 441.2483.

Synthesis of lysoSM (3)

A solution of egg sphingomyelin (50.4 mg, 71.7 µmol) in hydrochloric acid methanolic solution (0.5M, 2.5 mL) was stirred at 50 °C for 2 days and then the solvent was removed by evaporation. Purification by silica gel column chromatography ($CHCl_3$/MeOH/NH_4OH 4:6:1 v/v) afforded a colorless solid (13.4 mg, 28.8 µmol, 40%). R_f = 0.14 (65/35/8 $CHCl_3$/MeOH/NH_4OH, v/v), ^{1}H NMR (600 MHz, CD_3OD): δ 5.81-5.76 (m, 1H), 5.39 (q, J = 7.3 Hz, 1H), 4.51 (s, 1H), 4.18 (q, J = 6.2 Hz, 3H), 4.06-3.90 (m, 2H), 3.56 (t, J = 4.5 Hz, 2H), 3.13 (s, 9H), 2.01 (q, J = 6.9 Hz, 2H), 1.33-1.19 (m, 24H), 0.80 (t, J = 6.9 Hz, 3H), HRMS (*m*/*z*): $[M + H]^+$ calcd for $C_{23}H_{50}N_2O_5P^+$, 465.3452; found, 465.3479. $[M + Na]^+$ calcd for $C_{23}H_{49}N_2NaO_5P^+$, 487.3271; found, 487.3300.

Synthesis of azidoSM (4)

To a mixture of the lysoSM (**3**) (13.4 mg, 28.8 µmol) dissolved in CH_2Cl_2 (1 ml) and triethylamine (6.0 µL, 43.3 µmol) was added 16-hexadecanoic acid NHS ester (**2**) (18.1 mg, 43.3 µmol). The reaction mixture was stirred at room temperature for 1 day and then extracted with $CHCl_3$. Purification by silica gel column chromatography ($CHCl_3$/MeOH/NH_4OH 65:35:3 v/v) afforded a colorless solid (16.2 mg, 21.6 µmol, 75%). R_f = 0.20 (65/35/3 $CHCl_3$/MeOH/NH_4OH, v/v), ^{1}H NMR (600 MHz, CD_3OD): δ 5.64 (td, J = 14.4, 6.9 Hz, 1H), 5.38 (q, J = 7.6 Hz, 1H), 4.23 (d, J = 27.5 Hz, 2H), 4.05-3.88 (m, 3H), 3.57 (d, J = 2.7 Hz, 2H), 3.15 (s, 9H), 2.19-2.07 (m, 2H), 1.94 (d, J = 34.4 Hz, 2H), 1.51 (q, J = 7.1 Hz, 4H), 1.23-1.22 (m, 48H), 0.83 (t, J = 6.9 Hz, 3H), HRMS (*m*/*z*): $[M + Na]^+$ calcd for $C_{39}H_{78}N_5NaO_6P^+$, 766.5582; found, 766.5567.

Synthesis of aminoSM (5)

To a solution of the azidoSM (**4**) (16.2 mg, 21.8 µmol) in DMF (1 mL) were added triphenyl phosphine (17.1 mg, 65.3 µmol) and water (100 µL). The reaction mixture was stirred at room temperature for 2 days and then extracted with $CHCl_3$. Purification by silica gel column chromatography ($CHCl_3$/MeOH/NH_4OH 1:1:0.2 v/v) afforded a white solid (5.2 mg, 7.2 µmol, 33%). R_f = 0.26 (1/1/0.2 $CHCl_3$/MeOH/NH_4OH, v/v), ^{1}H NMR (600 MHz, CD_3OD): δ 5.70-5.66 (m, 1H), 5.42 (q, J = 7.6 Hz, 1H), 4.35-4.25 (m, 2H), 4.10-3.89 (m, 3H), 3.61 (t, J = 4.5 Hz, 2H), 3.20 (s, 9H), 2.74 (t, J = 7.6 Hz, 2H), 2.20-2.12 (m, 2H), 2.01 (d, J = 5.2 Hz, 2H), 1.57-1.18 (m, 48H), 0.88 (t, J = 7.2 Hz, 3H), HRMS (*m*/*z*): $[M + H]^+$ calcd for $C_{39}H_{81}N_3O_6P^+$, 718.5858; found,

718.5929.

Synthesis of azidoCer (6)

To a solution of *D-erythro*-sphingosine (10 mg, 33.4 µmol) in CH_2Cl_2/DMF (2:1, v/v, 2 mL) were added 16-hexadecanoic acid NHS ester (**2**) (21.0 mg, 50.0 µmol) and triethylamine (7.0 µL, 50.0 µmol). The mixture was stirred at room temperature for 1 day and then extracted with $CHCl_3$. Purification by silica gel column chromatography ($CHCl_3$/MeOH 30:1 v/v) afforded a colorless solid (12.6 mg, 13.0 µmol, 39%). R_f = 0.28 (30/1 $CHCl_3$/MeOH, v/v), ^{1}H NMR (600 MHz, $CDCl_3$): δ 6.24 (d, J = 7.6 Hz, 1H), 5.79-5.75 (m, 1H), 5.52 (dd, J = 15.8, 6.2 Hz, 1H), 4.31 (d, J = 4.1 Hz, 1H), 3.96-3.88 (m, 2H), 3.70-3.63 (m, 1H), 3.24 (t, J = 7.2 Hz, 2H), 2.21 (q, J = 7.1 Hz, 2H), 2.06-2.00 (m, 2H), 1.65-1.56 (m, 6H), 1.39-1.24 (m, 44H), 0.86 (t, J = 6.9 Hz, 3H), HRMS (*m*/*z*): $[M + Na]^+$ calcd for $C_{34}H_{66}N_4NaO_3^+$, 601.5027; found, 601.5020.

Synthesis of aminoCer (7)

To a solution of the azidoCer (**6**) (12.6 mg, 21.8 µmol) in THF (1 mL) were added triphenyl phosphine (17.1 mg, 65.3 µmol) and water (20 µL). The reaction mixture was stirred at room temperature for 2 days and then extracted with $CHCl_3$. Purification by silica gel column chromatography ($CHCl_3$/MeOH/NH_4OH 40:20:4 v/v) afforded a white solid (5.8 mg, 10.5 µmol, 48%). R_f = 0.39 (40/20/4 $CHCl_3$/MeOH/NH_4OH, v/v), ^{1}H NMR (600 MHz, $CDCl_3$): δ 6.27 (d, J = 6.9 Hz, 1H), 5.79-5.74 (m, 1H), 5.52 (dd, J = 15.5, 6.5 Hz, 1H), 4.29 (s, 1H), 3.95-3.88 (m, 2H), 3.68 (dd, J = 11.7, 3.4 Hz, 1H), 2.66 (t, J = 7.2 Hz, 2H), 2.23-2.16 (m, 2H), 2.06-1.99 (m, 2H), 1.63 (q, J = 7.3 Hz, 4H), 1.42-1.24 (m, 44H), 0.86 (t, J = 6.9 Hz, 3H), HRMS (*m*/*z*): $[M + H]^+$ calcd for $C_{34}H_{69}N_2O_3^+$, 553.5303; found, 553.5353.

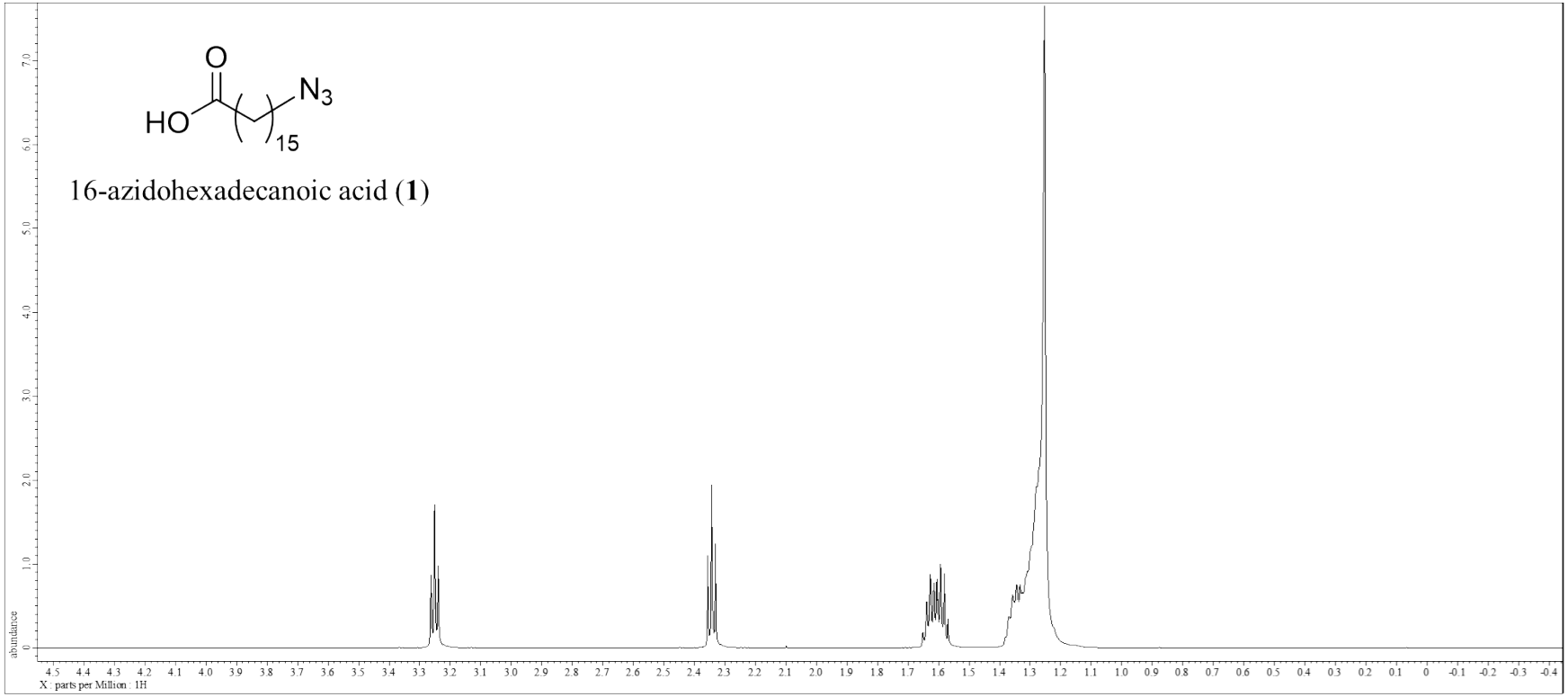

Fig. S2 600 MHz ^{1}H NMR spectrum of 16-azidohexadecanoic acid (**1**)

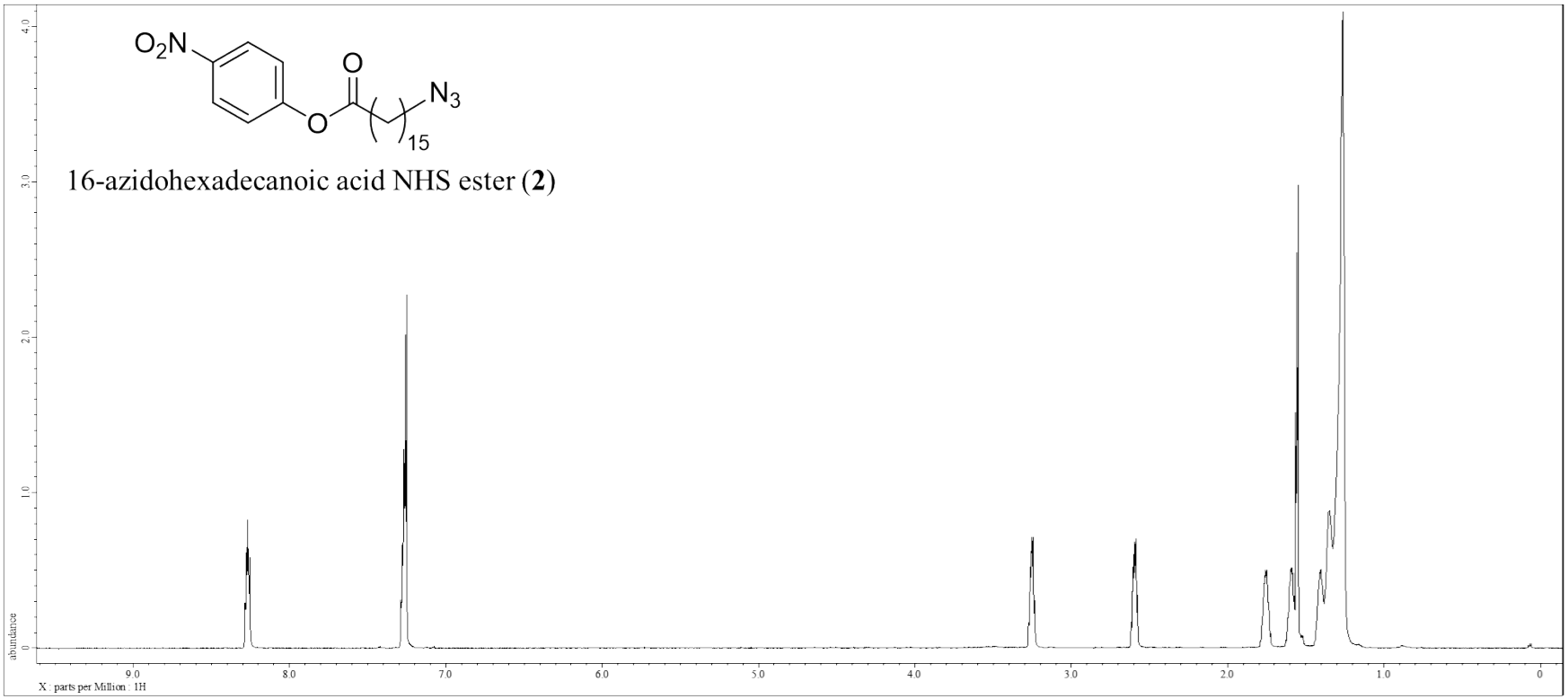

Fig. S3 600 MHz ^{1}H NMR spectrum of 16-azidohexadecanoic acid NHS ester (**2**)

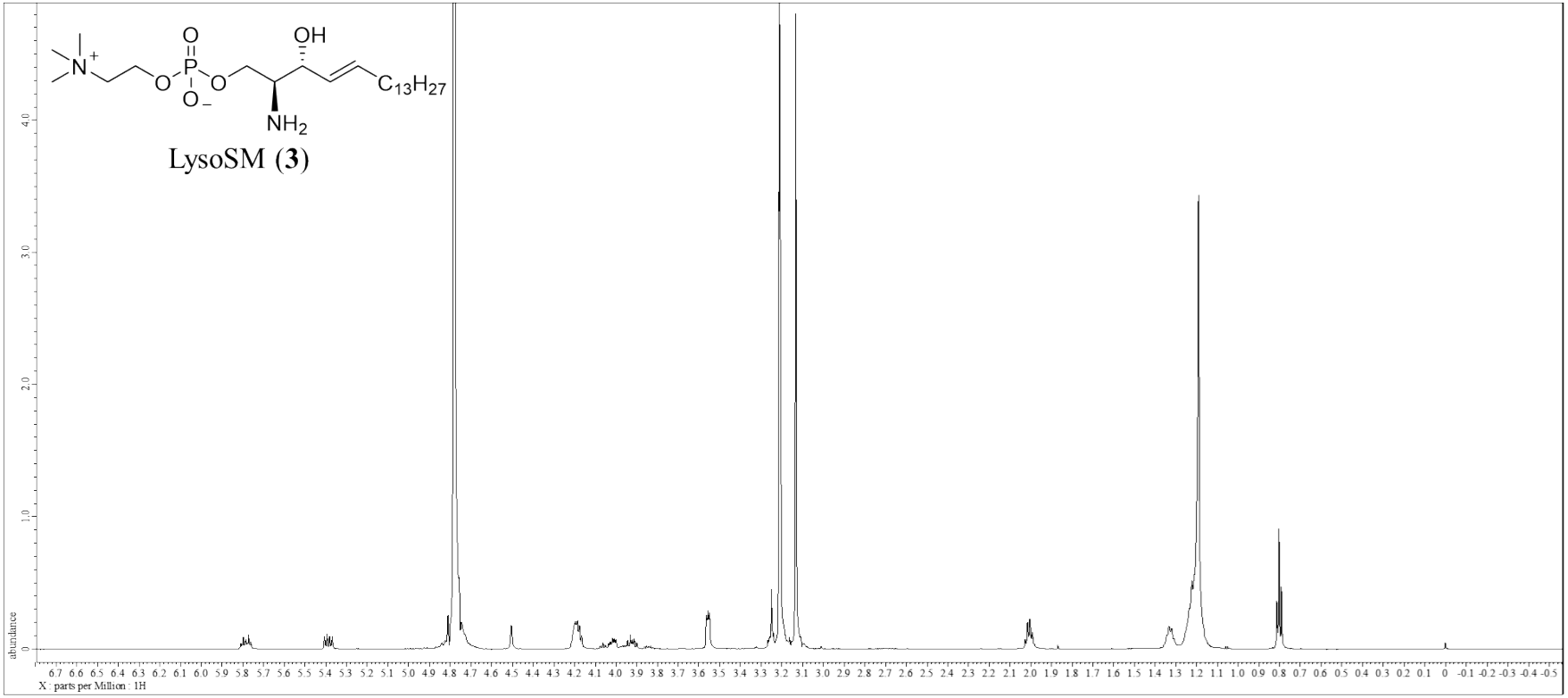

Fig. S4 600 MHz ^{1}H NMR spectrum of lysoSM (**3**)

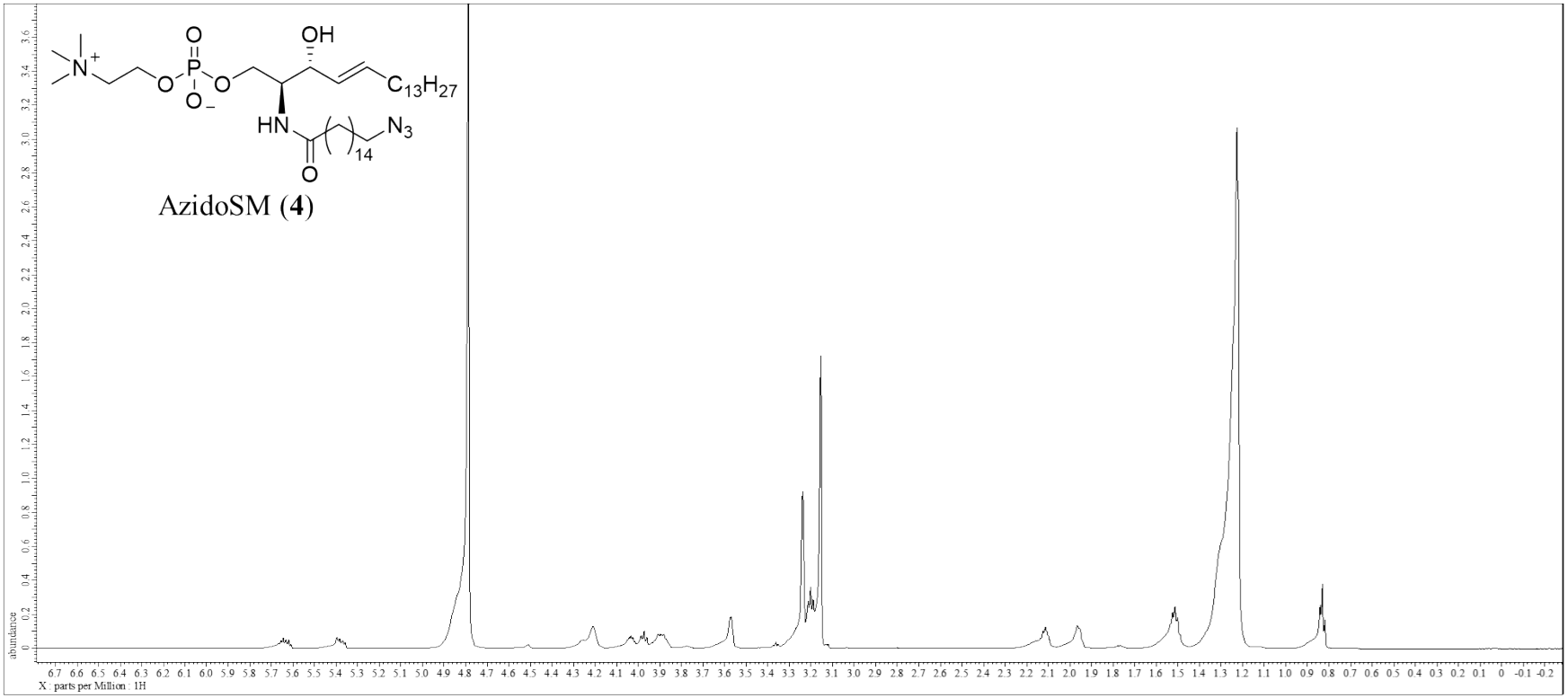

Fig. S5 600 MHz ^{1}H NMR spectrum of azidoSM (**4**)

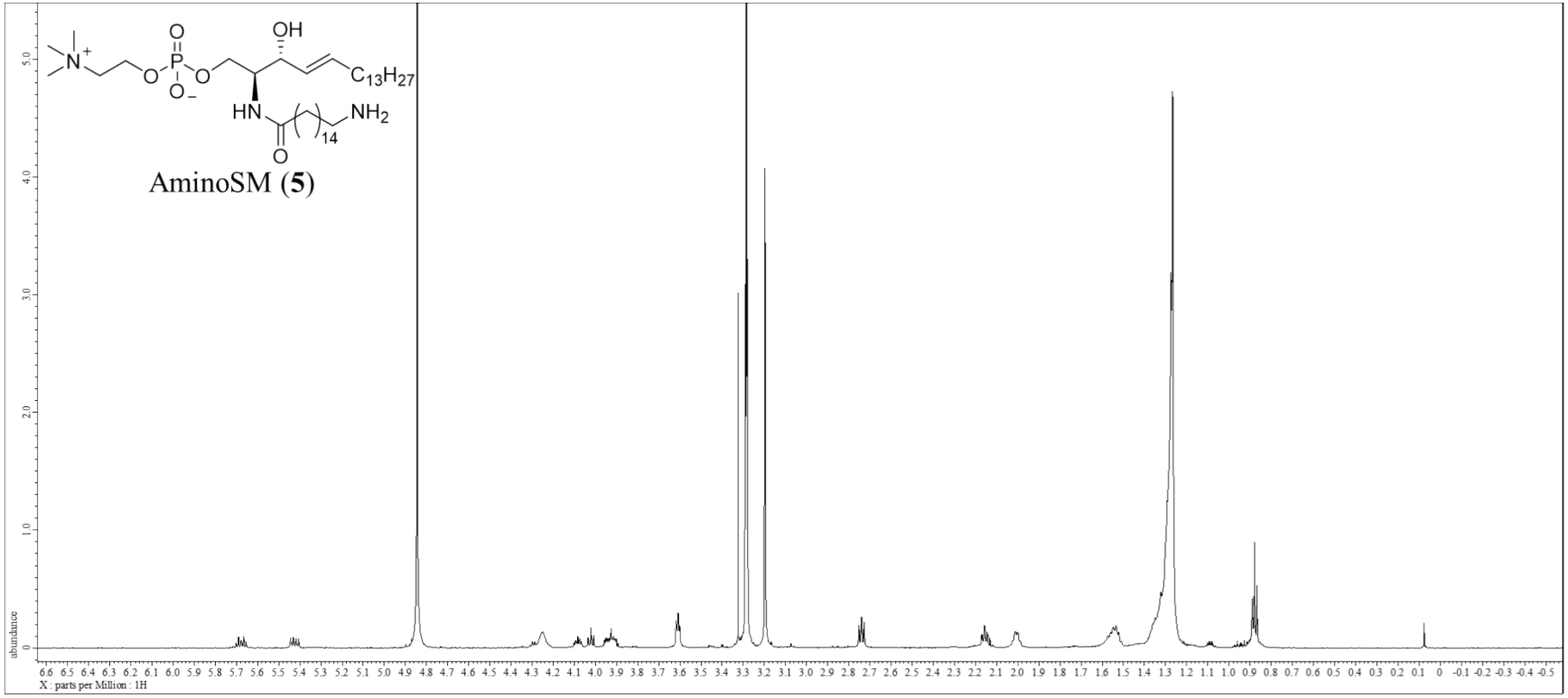

Fig. S6 600 MHz ^{1}H NMR spectrum of aminoSM (**5**)

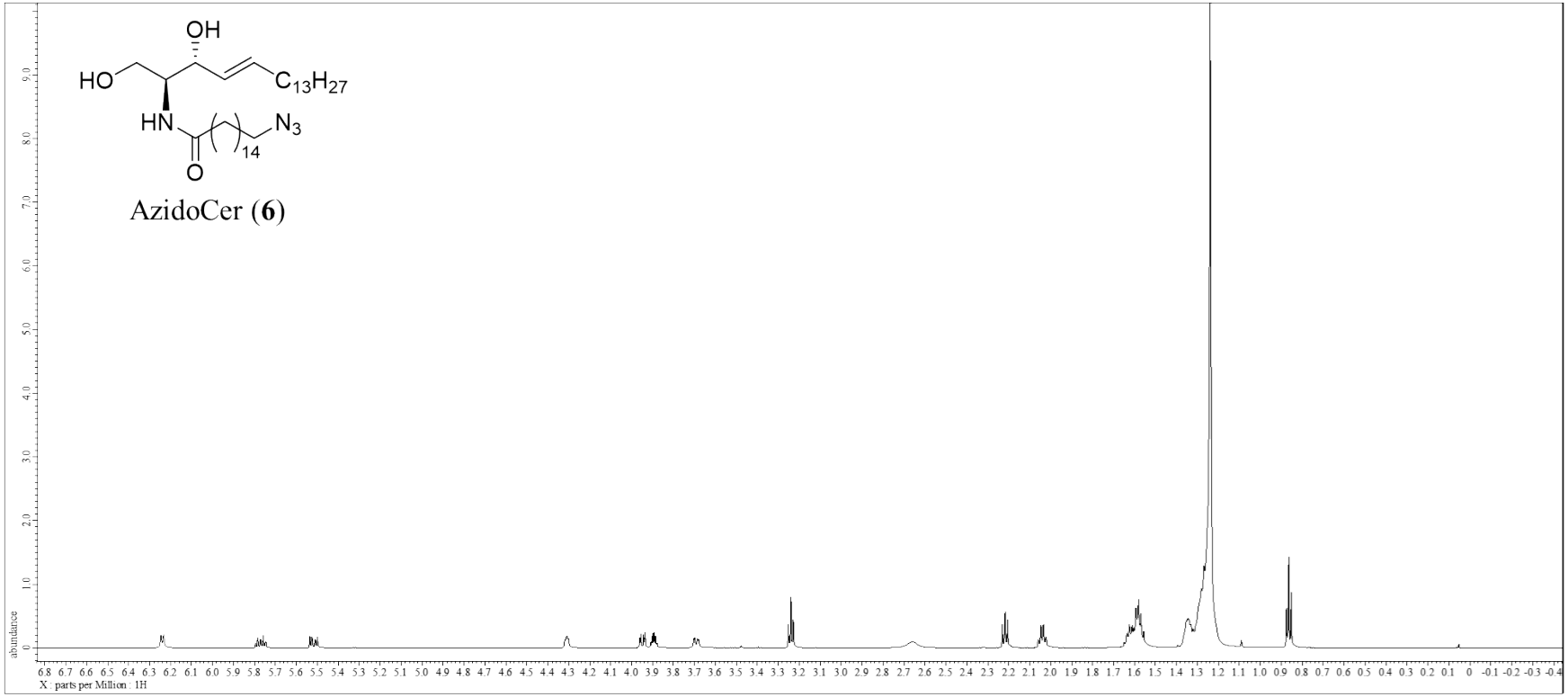

Fig. S7 600 MHz ^{1}H NMR spectrum of azidoCer (**6**)

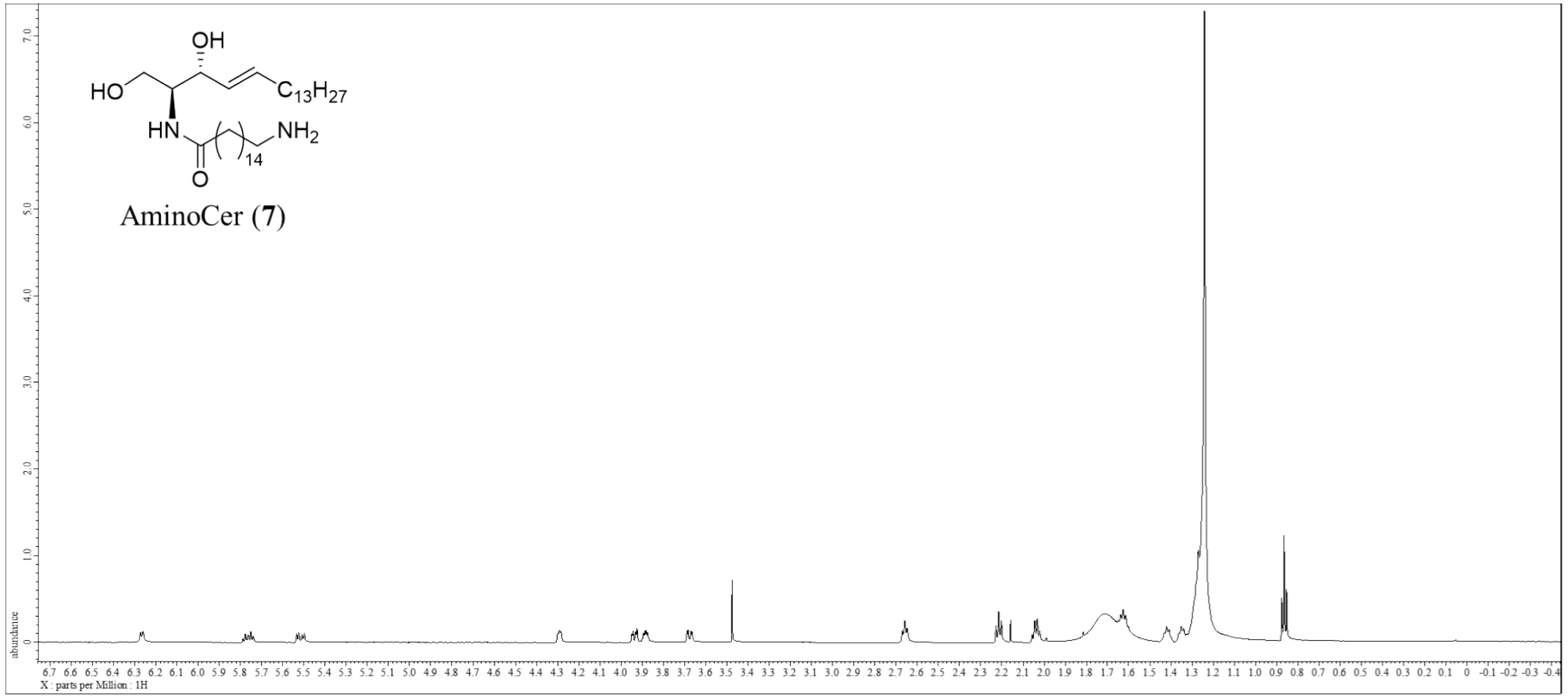

Fig. S8 600 MHz ^{1}H NMR spectrum of aminoCer (**7**)