Identification and Characterization of β-Sitosterol Target Proteins

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Supplementary Material

Biological assays

Reagents.

Cholesterol, β-sitosterol, biotin, DMSO, and LPS were purchased from Sigma. Streptavidin agarose resin (cat. #20353) was purchased from Thermo Scientific. 10 mM stock solutions of cholesterol, β-sitosterol, biotinylated cholesterol, and biotinylated β-sitosterol were prepared by dissolving in ethanol while biotin was dissolved in DMSO. Antibodies to E-Syt1 (GTX88786), 17β-HSD4 N-terminal (GTX114978), and 17β-HSD4 C-terminal (GTX103864) were purchased from Genetex and the antibody to SRD5A1 was from Abnova (H00006715-D01P).

Cell culture.

Raw264.7 cells were grown in RPMI Medium 1640 with 10% FBS. PC-3 and DU-145 cells were grown in Eagle's Minimum Essential Medium (EMEM) with 10% FBS. MDA-MB-231 and A549 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS. All cell cultures were grown in a humidified 5% CO_2 incubator at 37 °C.

Affinity chromatography.

Sub-confluent cells grown in complete media in 15cm dishes were put on ice, washed twice with 2 mL cold PBS, and lysed by scraping in 1 mL cold Modified RIPA buffer (1% NP-40, 0.1% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl pH 7.5) plus protease and phosphatase inhibitors (Roche Complete Mini protease inhibitor cocktail, 1 mM β-glycerophosphate, 2.5 mM sodium pyrophosphate, 2 mM NaF, 1 mM sodium vanadate) per dish. For the experiment with Raw264.7 macrophages, the complete media was replaced with RPMI Medium 1640 containing 1% FBS and 50 ng/mL Lipopolysaccharide (LPS) and the cells grown for an additional 6 hours before lysis. Lysates from multiple plates were combined and clarified by centrifugation at 18,000 g for 10 minutes at 4°C. The protein concentration of the clarified lysate (supernatant) was determined by BCA assay, and the lysates diluted to 2 mg/mL with Modified RIPA buffer.

Affinity chromatography was performed by incubating 1 mL lysate with the indicated concentrations of biotinylated cholesterol, biotinylated β-sitosterol, or biotin for 2 hours while rotating end-over-end at 4°C, followed by the addition of streptavidin agarose resin (15 µL for 200 nM and 30 µl for 600 nM) that had been prewashed three times for 5 min each with 1 mL Modified RIPA buffer. Samples were then rotated end-over-end with the resin overnight at 4°C. The resin was pelleted at 1000 g for 1 minute at 4°C and washed three times with 600 µl Modified RIPA buffer by gently inverting each tube by hand 15 times. Proteins were then eluted with SDS-PAGE sample buffer by heating to 70°C for 10 minutes and separated on 4-12% Bis-Tris gels (Life Technologies). Gels were then either silver-stained using ProteoSilver Plus kit (Sigma) or transferred to Immobilon-P PVDF membranes (Millipore) for western blotting. Silver-stained bands for proteins bound specifically to biotinylated β-sitosterol were cut out and destained using the reagents provided in the ProteoSilver Plus kit according to the manufacturer's protocol.

MALDI mass spectrometry

Proteins were identified using Pick 'n Post Protein identification service (Alphalyse, Inc., Palo Alto, CA) essentially as previously described.[S1,2] Briefly, gel-excised protein spots were reduced, alkylated with iodoacetamide, and digested with trypsin. The resulting peptides were concentrated on a ZipTip C18 column (Millipore) and eluted onto an anchorchip target for analysis on a Bruker Autoflex Speed MALDI TOF/TOF instrument. The peptide mixture was analyzed in positive reflector mode for accurate peptide mass determination and some of the peptides analyzed by MS/MS fragmentation for partial peptide sequencing. For acquisition of peptide mass fingerprint spectra (PMF, MS), 3000 single shot spectra were averaged and the peak finding was undertaken using the SNAP algorithm. Peptide fragmentation spectra (PFF, MS/MS) were acquired of the 15 most abundant peptides. The MS and MS/MS spectra were combined and used for a Mascot (version 2.2.03) database search in the NCBI nrdb protein database with the following conditions: cysteine carbamidomethylation as a fixed modification, methionine oxidation as a variable modification, 60 ppm peptide tolerance, and 1 missed cleavage allowed.

References

S1. Shevchenko, A.; Wilm, M.; Vorm, O.; Mann, M. *Anal. Chem.* **1996**, *68*, 850.

S2. Mortz, E.; Krogh, T. N.; Vorum, H.; Görg, A. *Proteomics* **2001**, *1*, 1359.

MALDI Mass Spectrometry Data

Protein name: peroxisomal multifunctional enzyme type 2 [Mus musculus]

Mascot score: 282

Sequence coverage: 20%

Matched peptides shown in bold

1 MASPLRFDGR **VVLVTGAGGG LGRAYALAFA ER**GALVIVND LGGDFKGIGK

51 GSSAADKVVA EIRRKGGKAV ANYDSVEAGE KLVKTALDTF GR**IDVVVNNA**

101 **GILR**DRSFSR **ISDEDWDIIH R**VHLRGSFQV TRAAWDHMKK QNYGRILMTS

151 SASGIYGNFG QANYSAAK**LG ILGLCNTLAI EGRK**NNIHCN TIAPNAGSRM

201 TETVLPEDLV EALKPEYVAP LVLWLCHESC EENGGLFEVG AGWIGKLRWE

251 RTLGAIVRKR NQPMTPEAVR DNWEKICDFS NASKPQTIQE STGGIVEVLH

301 KVDSEGISPN R**TSHAAPAAT SGFVGAVGHK** LPSFSSSYTE LQSIMYALGV

351 GASVKNPKDL KFVYEGSADF SCLPTFGVIV AQKSMMNGGL AEVPGLSFNF

401 AK**ALHGEQYL ELYKPLPR**SG ELKCEAVIAD ILDKGSGVVI VMDVYSYSGK

451 ELICYNQFSV FVVGSGGFGG KRTSEKLK**AA VAVPNRPPDA VLRDATSLNQ**

501 **AALYR**LSGDW NPLHIDPDFA SVAGFEKPIL HGLCTFGFSA R**HVLQQFADN**

551 **DVSR**FKAIKV RFAKPVYPGQ TLQTEMWKEG NRIHFQTKVH ETGDVVISNA

601 YVDLVPASGV STQTPSEGGE LQSALVFGEI GRRLKSVGRE VVK**KANAVFE**

651 **WHITK**GGTVA AKWTIDLKSG SGEVYQGPAK GSADVTIIIS DEDFMEVVFG

701 KLDPQKAFFS GRLKARGNIM LSQKLQMILK DYAKL

Peptides used for identification

Peptides shown in bold have been analyzed by MS/MS sequencing

Start – End	Observed	Mr(expt)	Mr(calc)	Delta	Sequence
11 – 23	1155.67	1154.66	1154.68	-15.00	R.VVLVTGAGGGLGR.A
24 – 32	**1011.50**	**1010.50**	**1010.52**	**-23.00**	**R.AYALAFAER.G (Ions score 24)**
93 – 104	**1282.72**	**1281.72**	**1281.74**	**-18.00**	**R.IDVVVNNAGILR.D (Ions score 27)**
111 – 121	**1398.64**	**1397.64**	**1397.66**	**-16.00**	**R.ISDEDWDIIHR.V (Ions score 58)**
169 – 183	1599.87	1598.86	1598.88	-11.00	K.LGILGLCNTLAIEGR.K
169 – 184	1727.95	1726.94	1726.98	-21.00	K.LGILGLCNTLAIEGRK.N
312 – 330	1765.82	1764.82	1764.89	-41.00	R.TSHAAPAATSGFVGAVGHK.L
403 – 418	**1927.03**	**1926.02**	**1926.04**	**-8.00**	**K.ALHGEQYLELYKPLPR.S (Ions score 27)**

479 – 493	**1545.86**	**1544.86**	**1544.88**	**-14.00**	**K.AAVAVPNRPPDAVLR.D (Ions score 31)**
494 – 505	1322.66	1321.65	1321.66	-11.00	R.DATSLNQAALYR.L
542 – 554	**1528.73**	**1527.72**	**1527.74**	**-15.00**	**R.HVLQQFADNDVSR.F (Ions score 25)**
644 – 655	1443.74	1442.73	1442.77	-25.00	K.KANAVFEWHITK.G
645 – 655	1315.66	1314.65	1314.67	-18.00	K.ANAVFEWHITK.G

Protein name: extended synaptotagmin-1 [Mus musculus]

Mascot score: 271

Sequence coverage: 27%

Matched peptides shown in bold

1 MEHSPEEGAS PEPSGQPPAT DSTRDGGSGV PPAGPGAASE ALAVLTSFGR
51 RLLVLVPVYL AGAAGLSVGF VLFGLALYLG WRRVRDGKER SLRAARQLLD
101 DEERITAETL YMSHRELPAW VSFPDVEKAE WLNKIVAQVW PFLGQYMEK**L**
151 **LAETVAPAVR GANPHLQTFT FTRVELGEKP LR**IIGVKVHP SQRKDQILLD
201 LNVSYVGDVQ IDVEVKKYFC KAGVK**GMQLH GVLR**VILEPL TGDLPIVGAV
251 SMFFIKRPTL DINWTGMTNL LDIPGLSSLS DTMIMDSIAA FLVLPNR**LLV**
301 **PLVPDLQDVA QLR**SPLPRGI IR**IHLLAAR**G LSSKDKYVKG LIEGK**SDPYA**
351 **LVRVGTQTFC SR**VIDEELNP HWGETYEVIV HEVPGQEIEV EVFDKDPDKD
401 DFLGRMKLDV GKVLQAGVLD NWYPLQGGQG QVHLRLEWLS LLPDAEKLDQ
451 VLQWNRGITS RPEPPSAAIL VVYLDRAQDL PLKK**GNKEPN PMVQLSVQDV**
501 **TR**ESK**ATYST NSPVWEEAFR FFLQDPRSQE LDVQVKDDSR ALTLGALTLP**
551 **LAR**LLTASEL TLDQWFQLSS SGPNSRLYMK LVMRILYLDY SEIRFPTVPG
601 AQDWDRESLE TGSSVDAPPR PYHTTPNSHF GTENVLR**IHV LEAQDLIAKD**
651 **RFLGGLVK**GK SDPYVKLKVA GKSFR**THVVR EDLNPR**WNEV FEVIVTSIPG
701 QELEIEVFDK DLDKDDFLGR YK**VSLTTVLN SGFLDEWLTL EDVPSGRLHL**
751 **RLER**LTPRPT AAELEEVLQV NSLIQTQKSS ELAAALLSVF LERAEDLPLR
801 KGTKPPSPYA TITVGETSHK TK**TVSQSSAP VWEESASFLI RKPHAESLEL**
851 **QVR**GEGTGTL GSVSLPLSEL LQEDQLCLDH WFALSGQGQV LMRAQLGILV
901 SQHSGVEAHS HSYSHSHSSS SLNDEPEALG GPTHPASPVL EVR**HRLTHGD**
951 **SPSEAPVGPL GQVK**LTVWYH SDEQK**LISII HSCR**ALRQNG R**DLPDPYVSV**
1001 **LLLPDKNR**ST KRKTPQKKR**T LNPEFNER**FE WDLPLDGTLR RKLDVSVKSN
1051 SSFMSREREL LGKVQLDLAE IDLSQGAAQW YDLMDDRDKG GS

Peptides used for identification

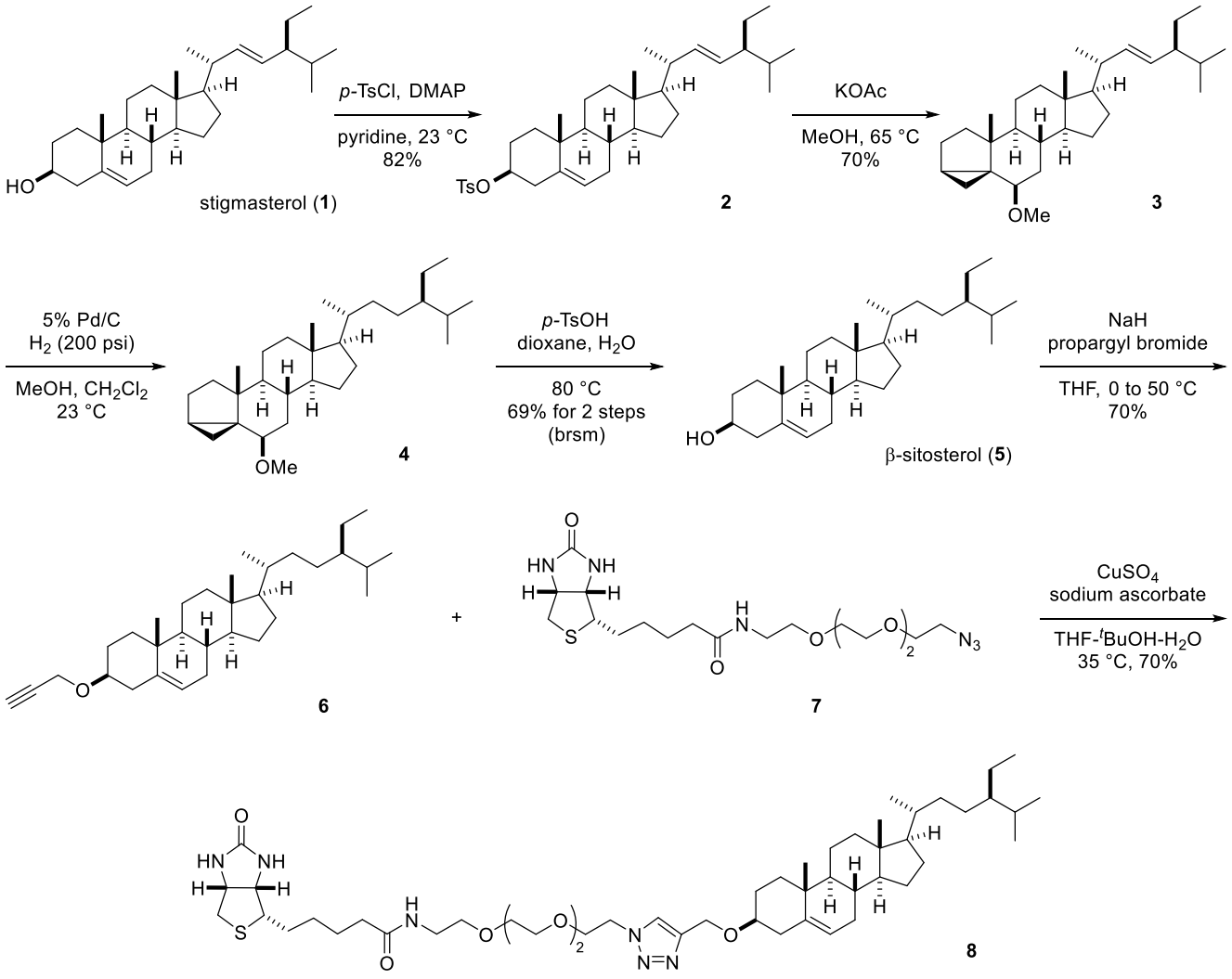
Peptides shown in bold have been analyzed by MS/MS sequencing

Start – End	Observed	Mr(expt)	Mr(calc)	Delta	Sequence
150 – 160	**1139.68**	**1138.67**	**1138.67**	**2.00**	**K.LLAETVAPAVR.G (Ions score 20)**
161 – 173	**1489.77**	**1488.76**	**1488.75**	**7.00**	**R.GANPHLQTFTFTR.V (Ions score 30)**
174 – 182	1040.61	1039.60	1039.60	1.00	R.VELGEKPLR.I

226 – 234	1010.56	1009.56	1009.55	6.00	K.GMQLHGVLR.V
226 – 234	1026.56	1025.55	1025.54	10.00	K.GMQLHGVLR.V Oxidation (M)
298 – 313	1789.07	1788.06	1788.05	5.00	R.LLVPLVPDLQDVAQLR.S
323 – 329	793.52	792.51	792.50	17.00	R.IHLLAAR.G
346 – 353	920.49	919.48	919.48	6.00	K.SDPYALVR.V
354 – 362	1055.53	1054.52	1054.49	31.00	R.VGTQTFCSR.V
485 – 502	2012.06	2011.05	2011.02	19.00	K.GNKEPNPMVQLSVQDVTR.E
506 – 520	1757.84	1756.83	1756.81	15.00	K.ATYSTNSPVWEEAFR.F
521 – 527	**922.48**	**921.48**	**921.47**	**6.00**	**R.FFLQDPR.S (Ions score 18)**
528 – 540	1518.76	1517.75	1517.73	15.00	R.SQELDVQVKDDSR.A
541 – 553	1309.82	1308.82	1308.81	2.00	R.ALTLGALTLPLAR.L
638 – 651	1620.91	1619.90	1619.90	3.00	R.IHVLEAQDLIAKDR.F
650 – 658	1004.60	1003.60	1003.58	14.00	K.DRFLGGLVK.G
676 – 686	**1335.72**	**1334.71**	**1334.71**	**5.00**	**R.THVVREDLNPR.W (Ions score 14)**
723 – 747	2748.34	2747.34	2747.40	-24.00	
	K.VSLTTVLNSGFLDEWLTLEDVPSGR.L				
748 – 754	936.58	935.57	935.57	6.00	R.LHLRLER.L
823 – 841	2094.06	2093.06	2093.04	7.00	K.TVSQSSAPVWEESASFLIR.K
842 – 853	**1406.78**	**1405.77**	**1405.77**	**1.00**	**R.KPHAESLELQVR.G (Ions score 17)**
944 – 964	2182.16	2181.15	2181.13	9.00	R.HRLTHGDSPSEAPVGPLGQVK.L
946 – 964	1888.97	1887.96	1887.97	-3.00	R.LTHGDSPSEAPVGPLGQVK.L
976 – 984	1098.61	1097.61	1097.60	5.00	K.LISIIHSCR.A
992 – 1008	1954.07	1953.06	1953.06	1.00	R.DLPDPYVSVLLLPDKNR.S
1020 – 1028	1119.56	1118.56	1118.54	19.00	R.TLNPEFNER.F

Experimental Procedures

All reactions were performed in glassware under a positive pressure of argon. The normal-phase flash column chromatography was performed as described by Still (Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923–2925), employing EMD silica gel 60 (230–400 mesh ASTM). TLC analyses were performed on EMD 250 μm Silica Gel 60 F254 plates and visualized by quenching of UV fluorescence (λ_{max} = 254 nm), or by staining ceric ammonium molybdate. ^{1}H and ^{13}C NMR spectra were recorded on Varian Inova-400. Chemical shifts for ^{1}H and ^{13}C NMR spectra are reported in ppm (δ) relative to the ^{1}H and ^{13}C signals in the solvent ($CDCl_3$: δ 7.26, 77.16 ppm; methanol-d4: δ 3.31, 49.00 ppm) and the multiplicities are presented as follows: s = singlet, d = doublet, t = triplet, m = multiplet. Mass spectra were acquired on Agilent 6120 Single Quadrupole LC/MS.



Scheme S1. Synthesis of biotin labeled β-sitosterol (**8**)

β-sitosterol (**5**) was synthesized using the methods developed by McCarthy (McCarthy, F. O.; Chopra, J.; Ford, A.; Hogan, S. A.; Kerry, J. P.; O'Brien, N. M.; Ryan, E.; Maguire A. R. *Org. Biomol. Chem.* **2005**, *3*, 3059–3065).

To a solution of stigmasterol **1** (200 mg, 0.46 mmol, 95% pure), 4-(dimethylamino)pyridine (6 mg, 10 mol %) in pyridine (2.0 mL) was added tosyl chloride (175 mg, 0.92 mmol) at room temperature. After 6 h, the reaction was quenched by saturated aq. sodium bicarbonate (8 mL) and the precipitate was collected, washed with water, dried and recrystallized from acetone to give the **2** as white needles (213 mg, 82%).
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (d, 2H, *J* = 8.4 Hz), 7.33 (2H, d, *J* = 8.0 Hz), 5.32-5.27 (m, 1H), 5.14 (dd, 1H, *J* = 14.8, 8.4 Hz), 5.01 (dd, 1H, *J* = 15.2, 8.8 Hz), 4.38-4.27 (m, 1H), 2.44 (s, 1H), 2.30-2.23 (m, 1H), 1.01 (d, 3H, *J* = 6.8 Hz), 0.97 (s, 3H), 0.84 (d, 3H, *J* = 6.4 Hz), 0.82-0.77 (m, 6H), 0.67 (s, 3H).

The tosylate **2** (192 mg, 0.34 mmol) and potassium acetate (183 mg, 1.86 mmol) were dissolved in anhydrous methanol (8.5 mL) and refluxed for 3 h. The solution was then evaporated. The resulting white solid was dissolved in ethyl acetate (15 mL), which was then washed by water (2×10 mL) and saturated aq. sodium chloride (2 × 10 mL). The organic layer was dried over anhydrous sodium sulfate. The residue was purified by chromatography on silica gel (10% ethyl acetate in hexanes) to give *i*-stigmasterol methyl ether **3** (100 mg, 70%) as white solid.
^{1}H NMR (400 MHz, $CDCl_3$) δ 5.15 (dd, 1H, *J* = 15.2, 8.4 Hz), 5.01 (dd, 1H, *J* = 15.2, 8.8 Hz), 3.24 (s, 3H), 2.82-2.75 (m, 1H), 0.73 (s, 3H), 0.65 (t, 1H, *J* = 4.4 Hz), 0.43 (dd, 1H, *J* = 8.0, 5.2 Hz).

To a solution of *i*-stigmasterol methyl ether **3** (44 mg, 0.10 mmol) in methanol (0.8 mL) and methylene chloride (1.2 mL) was added 5% palladium on carbon (2.2 mg, 5% by wt.). The resulting suspension was stirred under hydrogen (200 psi) at room temperature for 24 h. ^{1}H NMR of the crude showed the completion of hydrogenation. The reaction mixture was then filtered through celite and concentrated to give the crude methyl ether **4.** Crude **4** was used directly without purification.

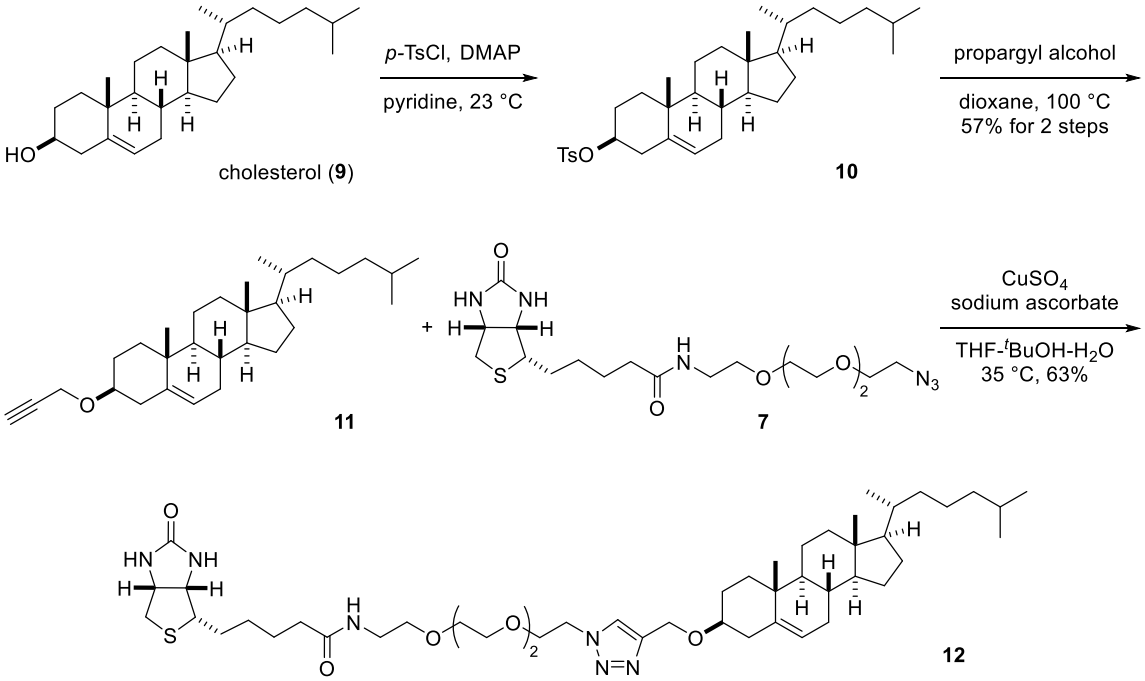
To a solution of crude **4** (42 mg) in water (0.3 mL) and 1,4-dioxane (1.0 mL) was added *p*-toluenesulfonic acid (2 mg, 0.012mmol) . The solution was heated at 80 °C for 3 h before evaporation of the solvent. The residue was then dissolved in ethyl acetate (15 mL). The organic layer was washed with water (2 × 10 mL) and saturated aqueous sodium chloride (2 × 10 mL), and then dried over anhydrous sodium sulfate. The residue was purified by chromatography on silica gel (10% ethyl acetate in hexanes) to give β-sitosterol **5** (36 mg, 84%, 2 steps) as white solid.
^{1}H NMR (400 MHz, $CDCl_3$) δ 5.37-5.33 (m, 1H), 3.57-3.47 (m, 1H), 2.34-2.18 (m, 2H), 2.06-1.93 (m, 2H), 1.90-1.78 (m, 3H), 1.01 (s, 3H), 0.92 (d, 3H, *J* = 6.4 Hz), 0.87-0.79 (m, 9H), 0.68 (s, 3H).

To a solution of β-sitosterol **5** (36 mg, 0.086 mmol, 1.0 equiv) in anhydrous tetrahydrofuran (4 mL) was added sodium hydride (4.3 mg, 0.18 mmol, 2.1 equiv) at 0 °C. After 0.5 h at room temperature, the suspension was cooled to 0 °C followed by the addition of propargyl bromide (0.019 mL, 0.17 mmol, 2.0 equiv). The mixture was then allowed to warm at 50 °C and stirred for 24 h. The reaction was quenched by icy water at 0 °C and extracted by ethyl acetate (2 × 15 mL). The combined organic layers were washed by saturated aqueous sodium chloride (2 × 10 mL) and dried over anhydrous sodium sulfate. The residue was then purified by chromatography on silica gel (5% ethyl acetate in hexanes) to give **6** (12 mg, 69% base on recovered starting material) as a white solid in addition to **5** (20 mg).

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.39-5.34 (m, 1H), 4.19 (d, 1H, *J* = 2.4 Hz), 3.43-3.34 (m, 1H), 2.40 (t, 1H, *J* = 2.4 Hz), 2.38-2.35 (m, 1H), 2.27-2.17 (m, 1H), 1.00 (s, 3H), 0.92 (d, 3H, *J* = 6.8 Hz), 0.87-0.80 (m, 10H), 0.68 (s, 3H).^{13}C NMR (100 MHz, $CDCl_3$) δ 140.6, 121.9, 80.4, 78.2, 73.7, 56.8, 56.0, 55.1, 50.1, 45.8, 42.3, 39.8, 38.7, 37.1, 36.8, 36.1, 33.9,31.93, 31.86, 29.1, 28.2, 28.1, 26.0, 24.3, 23.0, 21.1, 19.8, 19.3, 19.0, 18.8, 12.0, 11.9.

To a solution of **6** (12 mg, 0.0265 mmol, 1.1 equiv) and **7** (11 mg, 0.024 mmol, 1.0 equiv) in tetrahydrofuran (0.1 mL), *t*-butanol (0.05 mL) and water (0.03 mL) was added a solution of copper(II) sulfate pentahydrate (1.3 mg, 0.052 mmol, 0.22 equiv) in water (0.01 mL) and a solution of sodium ascorbate (2.1 mg, 0.0106 mmol, 0.44 equiv) in water (0.01 mL), respectively. After stirring at 35 °C for 24 h, the solvent was removed and the residue was extracted by ethyl acetate (2 × 5 mL). The combined organic layers were washed by water (3 × 5 mL), saturated aqueous sodium chloride (2 × 10 mL) and dried over anhydrous sodium sulfate. The residue was then purified by chromatography on silica gel (5% methanol in chloroform) to give biotin labeled sitosterol **8** (15 mg, 70%) as a white solid.

^{1}H NMR (400 MHz, CD_3OD) δ 8.01 (s, 1H), 5.39-5.34 (m, 1H), 4.64 (s, 2H), 4.59 (t, 2H, *J* = 5.2 Hz), 4.49 (dd, 1H, *J* = 7.6, 4.8 Hz), 4.30 (dd, 1H, *J* = 7.6, 4.4 Hz), 3.90 (t, 2H, *J* = 5.2 Hz), 3.62-3.58 (m, 8H), 3.53 (t, 2H, *J* = 5.2 Hz), 3.39-3.33 (m, 2H), 3.23-3.17 (m, 1H), 2.92 (dd, 1H, *J* = 12.8, 4.8 Hz), 2.73-2.67 (m, 1H), 2.44-2.36 (m, 1H), 2.26-2.16 (m, 3H), 1.03 (s, 3H), 0.96 (d, 3H, *J* = 6.8 Hz), 0.90-0.82 (m, 10H), 0.72 (s, 3H). ^{13}C NMR (100 MHz, CD_3OD) δ 176.1, 166.1, 146.4, 141.8, 125.7, 122.9, 80.2, 71.6, 71.5, 71.4, 71.3, 70.6, 70.4, 63.4, 61.9, 61.6, 58.2, 57.5, 57.0, 51.7, 51.4, 47.3, 43.5, 41.1, 41.1, 40.4, 40.1, 38.4, 38.0, 37.4, 36.7, 35.1, 33.2, 33.1, 30.4, 29.8, 29.5, 29.4, 27.2, 26.9, 25.3, 24.2, 22.2, 20.2, 19.9, 19.4, 19.4, 12.36, 12.34. MS(ES)$^+$calcd for $C_{50}H_{85}N_6O_6S$ $(M+H)^+$ 897.6, found 897.5; $C_{50}H_{84}N_6O_6SNa$ $(M+Na)^+$ 919.6, found 919.5.

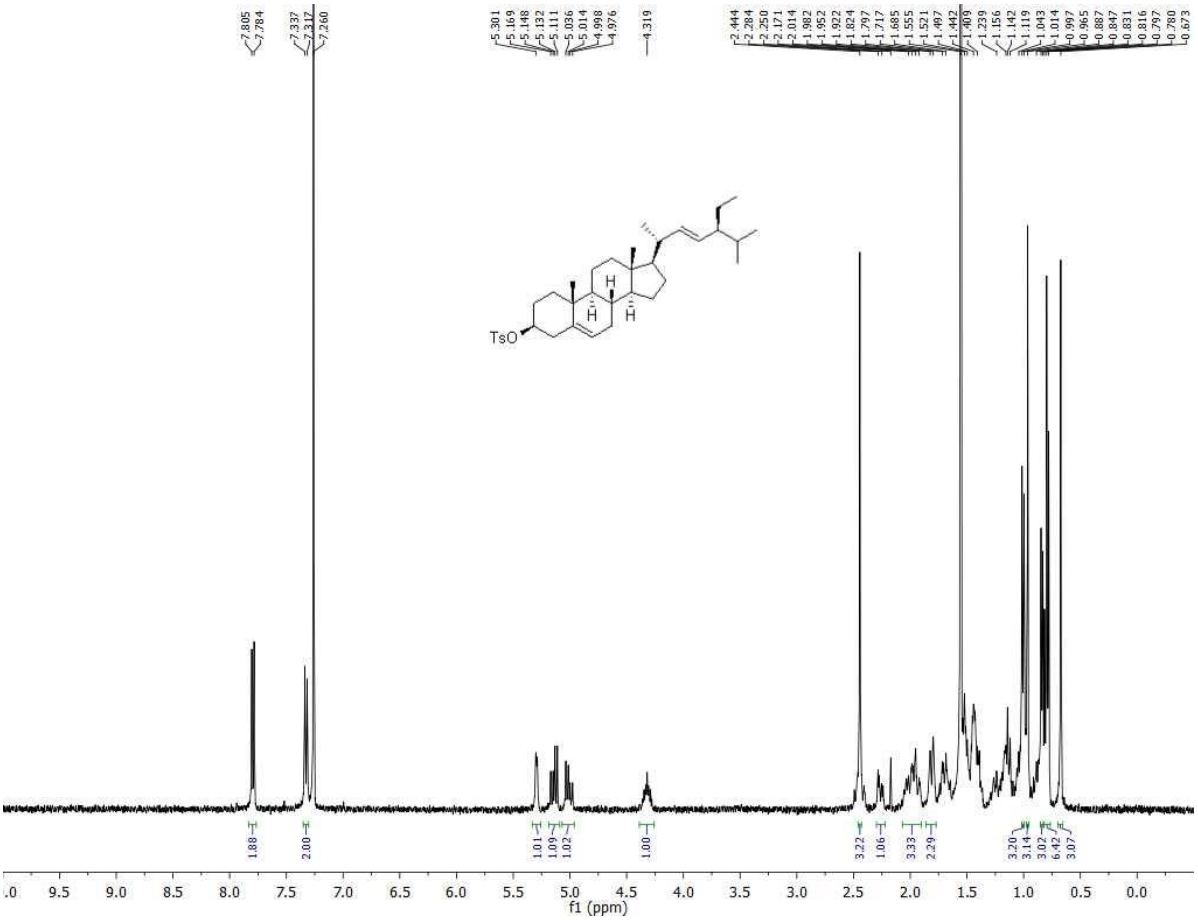


Scheme S2. Synthesis of biotin labeled cholesterol **12**.

To a solution of cholesterol **9** (39 mg, 0.1 mmol, 1.0 equiv), 4-(dimethylamino)pyridine (1.2 mg, 0.01 mmol, 0.1 equiv) in pyridine (1.0 mL) was added *p*-toluenesulfonyl chloride (21 mg, 0.11 mmol, 1.1 equiv) at room temperature. After stirring for 6 h, the reaction was quenched by saturated aqueous sodium bicarbonate (5 mL) and extracted with ethyl acetate (2 × 5 mL). The combined organic layers were washed by water (2 × 3 mL), saturated aqueous sodium chloride (2 × 3 mL) and dried over anhydrous sodium sulfate. The crude was used for the next step without purification.

To a solution of crude tosylate **10** obtained above in anhydrous 1,4-dioxane (1 mL) was added propargyl alcohol (0.046 mL, 0.8 mmol, 8.0 equiv). The solution was then heated to reflux. After stirring for 12 h, the reaction was cooled to room temperature, quenched by water and extracted by ethyl acetate (2 × 5 mL). The combined organic layers were washed by saturated aqueous sodium chloride (2 × 5 mL) and dried over anhydrous sodium sulfate. The residue was then purified by chromatography on silica gel (5% ethyl acetate in hexanes) to give **11** (24 mg, 57%, two steps) as a colorless oil.
^{1}H NMR (400 MHz, $CDCl_3$) δ 5.40-5.34 (m, 1H), 4.19 (d, 2H, *J* = 2.0 Hz), 3.43-3.34 (m, 1H), 2.43-2.34 (m, 2H), 2.27-2.18 (m, 1H), 1.01 (s, 3H), 0.91 (d, 1H, *J* = 5.2 Hz), 0.87 (d, 3H, *J* = 2.0 Hz), 0.85 (d, 2H, *J* = 1.6 Hz), 0.67 (s, 3H).

To a solution of **11** (14 mg, 0.033 mmol, 1.14 equiv) and **7** (13 mg, 0.029 mmol, 1.0 equiv) in tetrahydrofuran (0.1 mL), *t*-butanol (0.05 mL) and water (0.03 mL) was added a solution of copper(II) sulfate pentahydrate (1.7 mg, 0.0068 mmol, 0.23 equiv) in water (0.01 mL) and a solution of sodium ascorbate (2.7 mg, 0.0136 mmol, 0.47 equiv) in water (0.01 mL), respectively. After stirring at 35 °C for 24 h, the solvent was removed by evaporation and the residue was extracted by ethyl acetate (2 × 5 mL). The combined organic layers were washed by water (3 × 5 mL), saturated aqueous sodium chloride (2 × 10 mL) and dried over anhydrous sodium sulfate. The residue was then purified by chromatography on silica gel (5% methanol in chloroform) to give biotin labeled cholesterol **12** (16 mg, 63%) as a white solid.
^{1}H NMR (400 MHz, CD_3OD) δ 8.01 (s, 1H), 5.39-5.35 (m, 1H), 4.64 (s, 2H), 4.59 (t, 2H, *J* = 4.8 Hz), 4.49 (dd, 1H, *J* = 7.6, 4.8 Hz), 4.30 (dd, 1H, *J* = 7.6, 4.8 Hz), 3.90 (t, 2H, *J* = 5.2 Hz), 3.63-3.58 (m, 8H), 3.53 (t, 2H, *J* = 5.6 Hz), 3.35 (t, 2H, *J* = 5.6 Hz), 3.23-3.16 (m, 1H), 2.92 (dd, 1H, *J* = 12.8, 5.2 Hz), 2.73-2.67 (m, 1H), 2.44-2.36 (m, 1H), 2.26-2.16 (m, 3H), 1.03 (s, 3H), 0.95 (d, 3H, *J* = 6.4 Hz), 0.88 (d, 6H, *J* = 6.8 Hz), 0.72 (s, 3H). ^{13}C NMR (100 MHz, CD_3OD) δ 176.1, 166.1, 146.4, 141.8, 125.7, 122.9, 80.1, 71.6, 71.5, 71.4, 71.3, 70.6, 70.4, 63.4, 61.9, 61.6, 58.2, 57.6, 57.0, 51.7, 51.4, 43.5, 41.2, 41.1, 40.7, 40.4, 40.1, 38.4, 38.0, 37.4, 37.1, 36.7, 33.2, 33.1, 29.8, 29.5, 29.4, 29.3, 29.2, 26.8, 25.3, 25.0, 23.2, 23.0, 22.2, 19.9, 19.3, 12.3. MS(ES)$^+$calcd for $C_{48}H_{81}N_6O_6S$ $(M+H)^+$ 869.6, found 869.5; $C_{48}H_{80}N_6O_6SNa$ $(M+Na)^+$ 891.6, found 891.5.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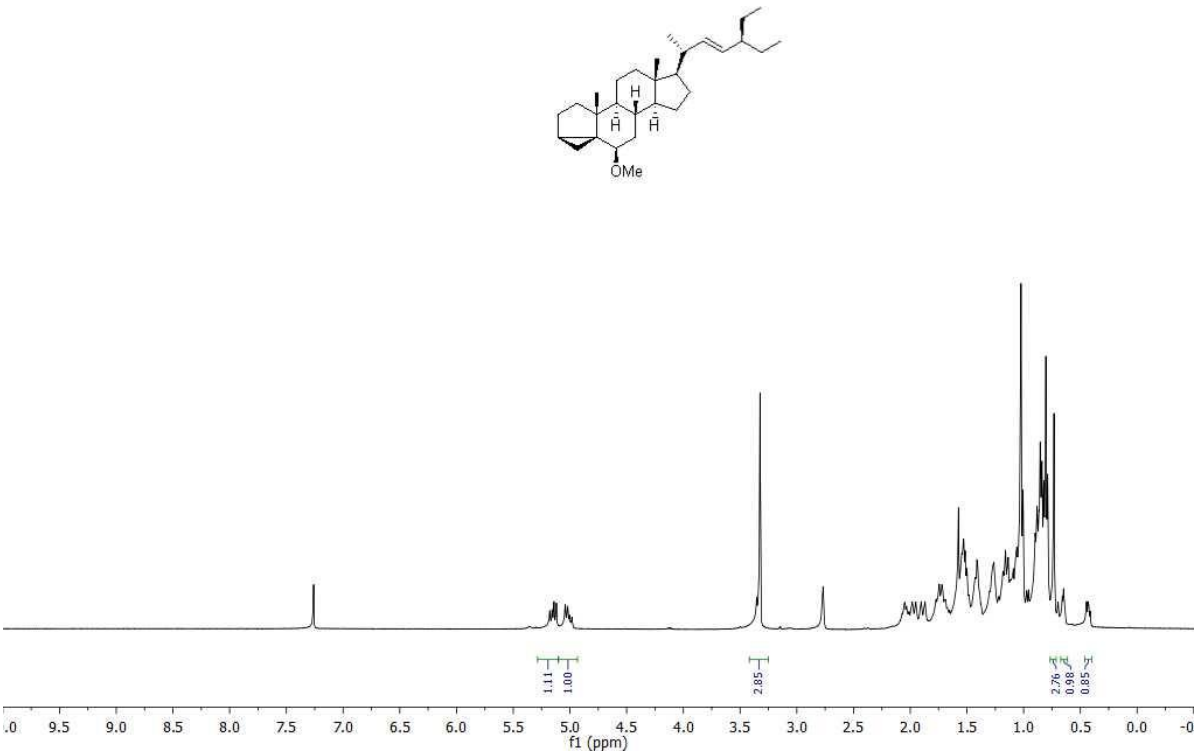
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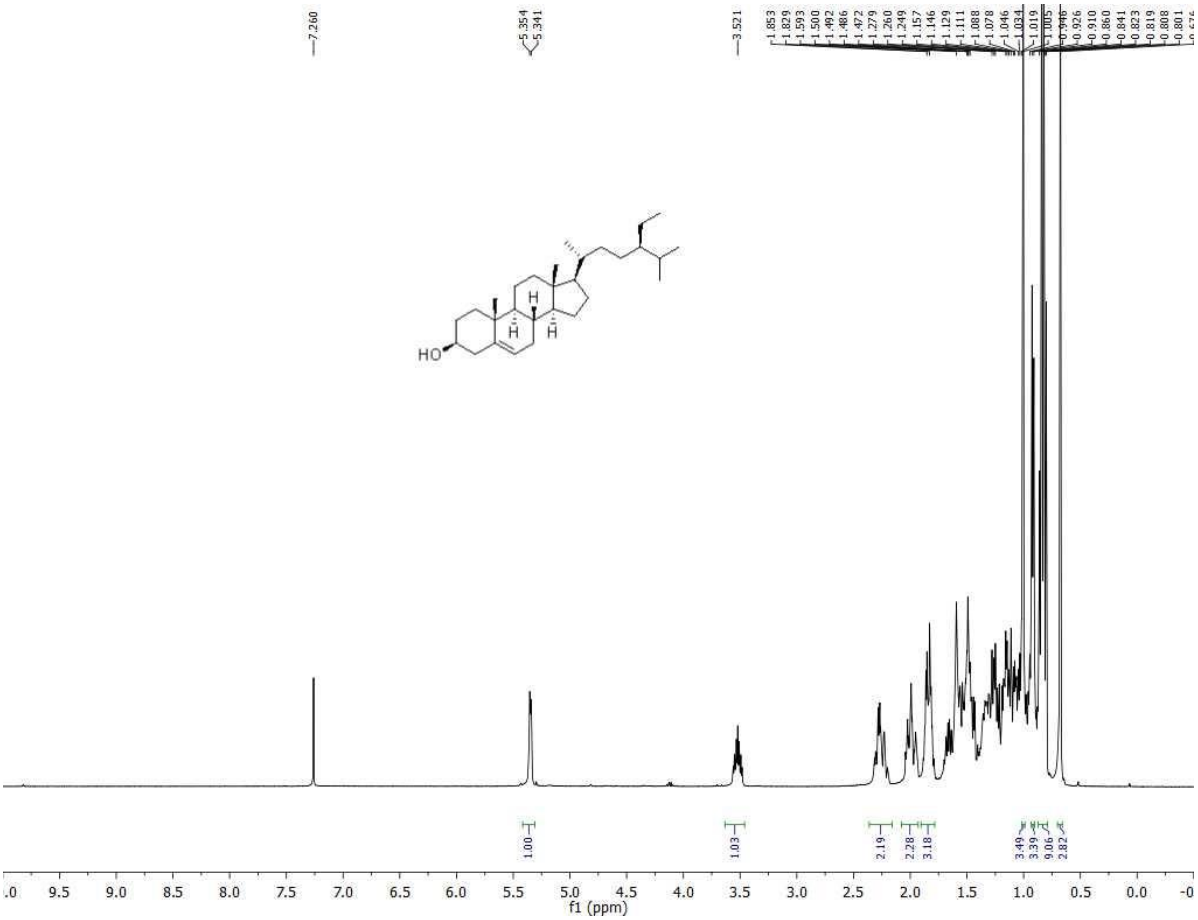
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3.39
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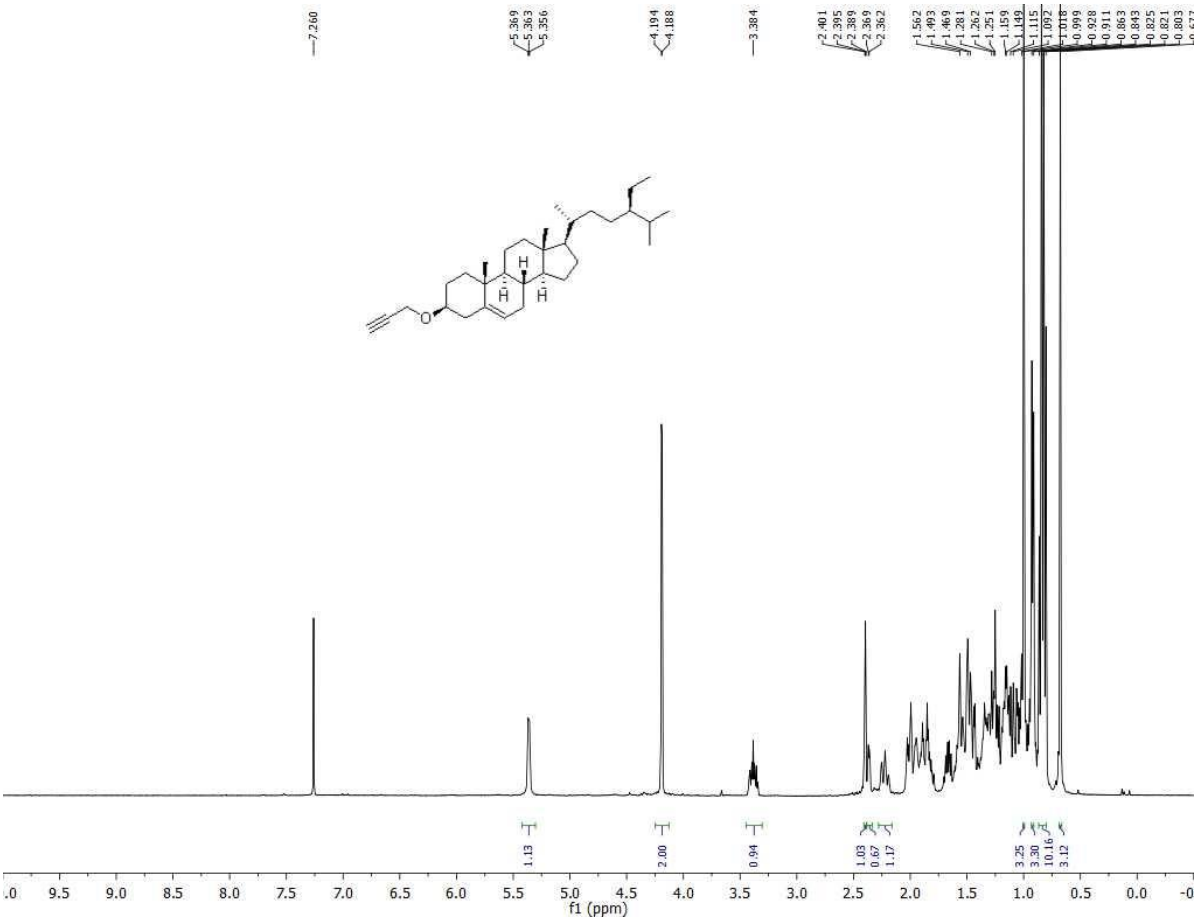
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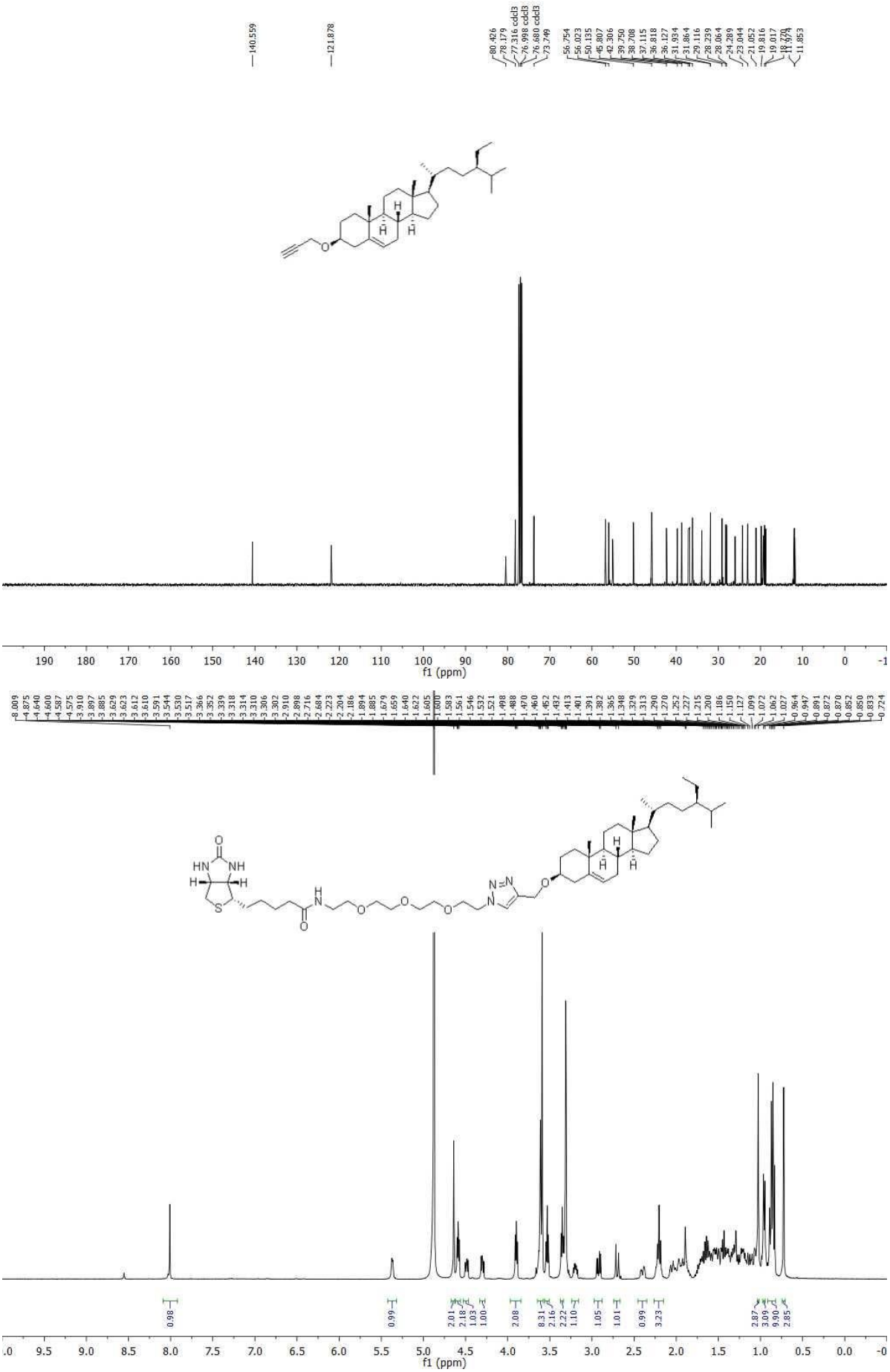
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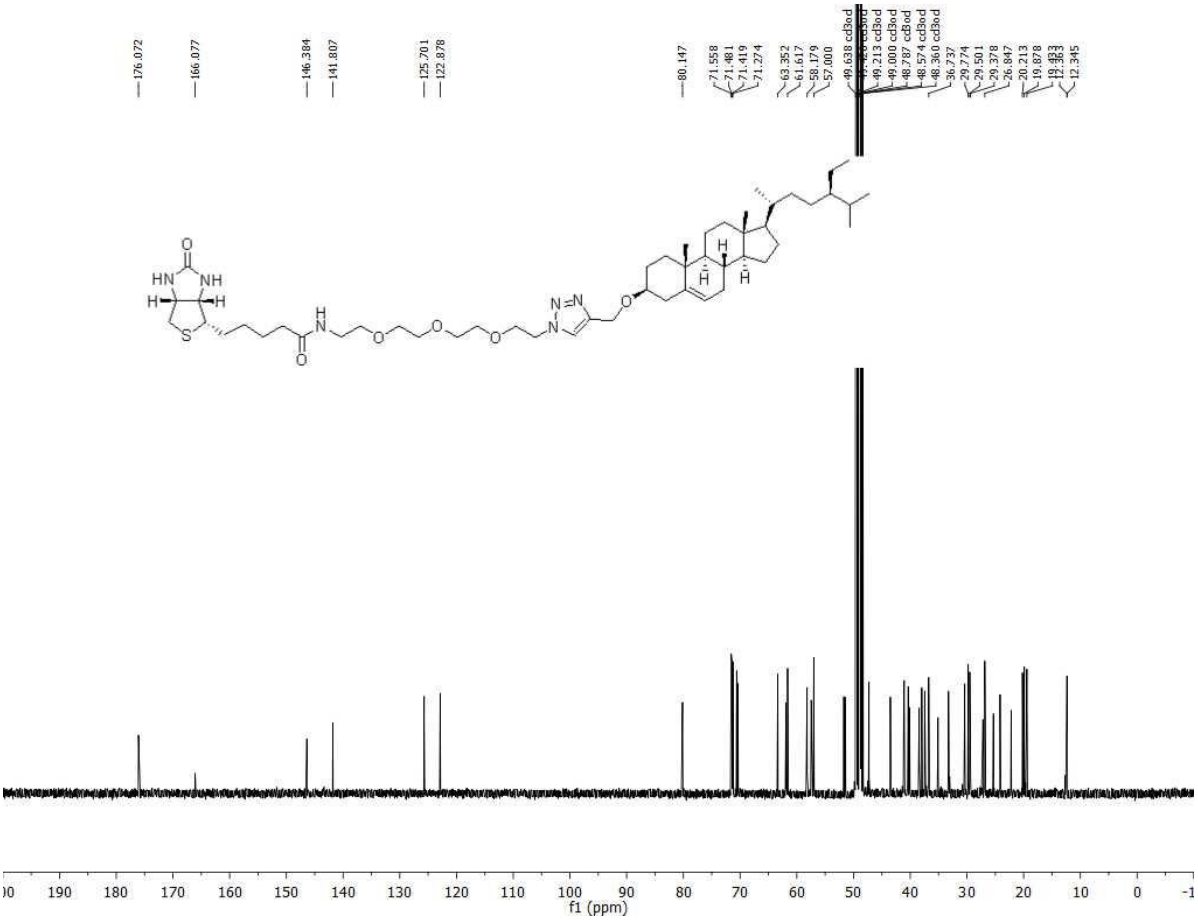
f1 (ppm)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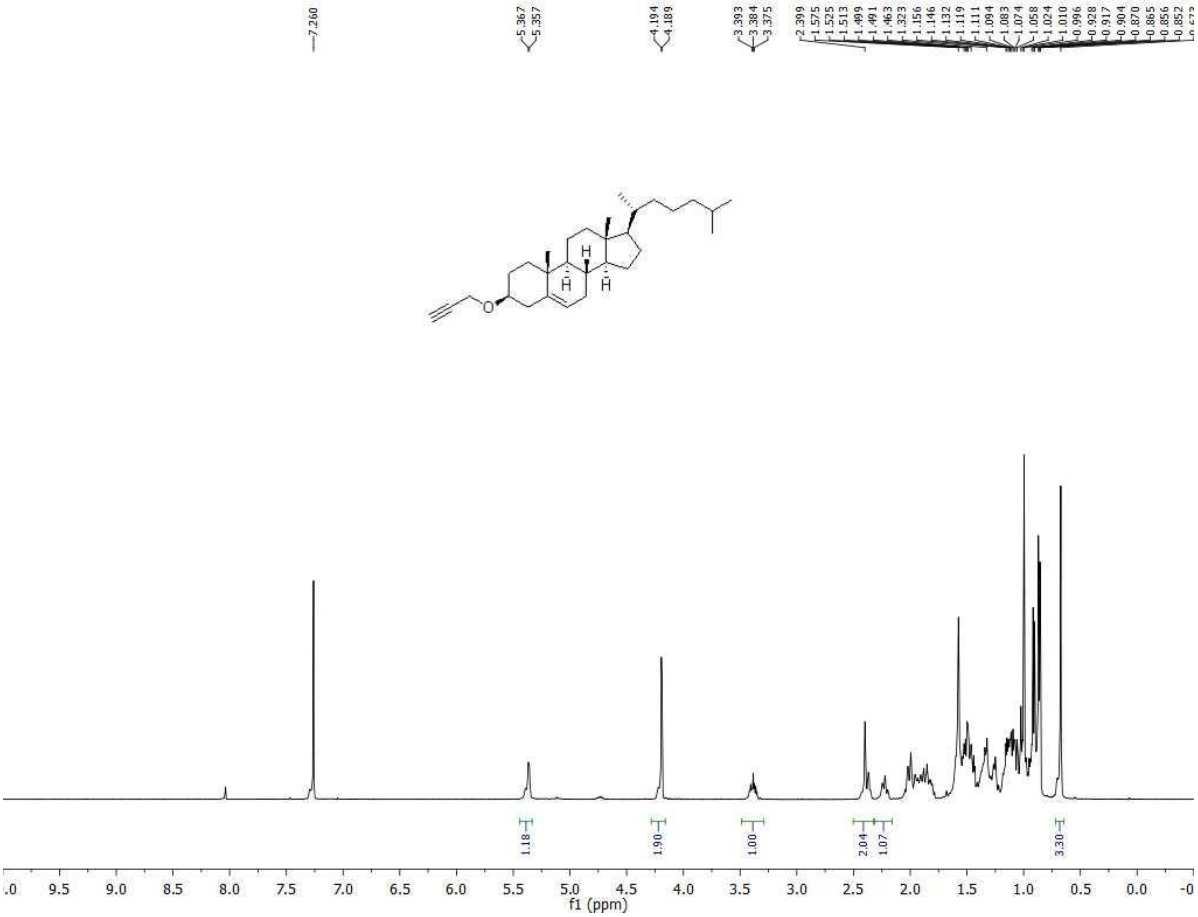
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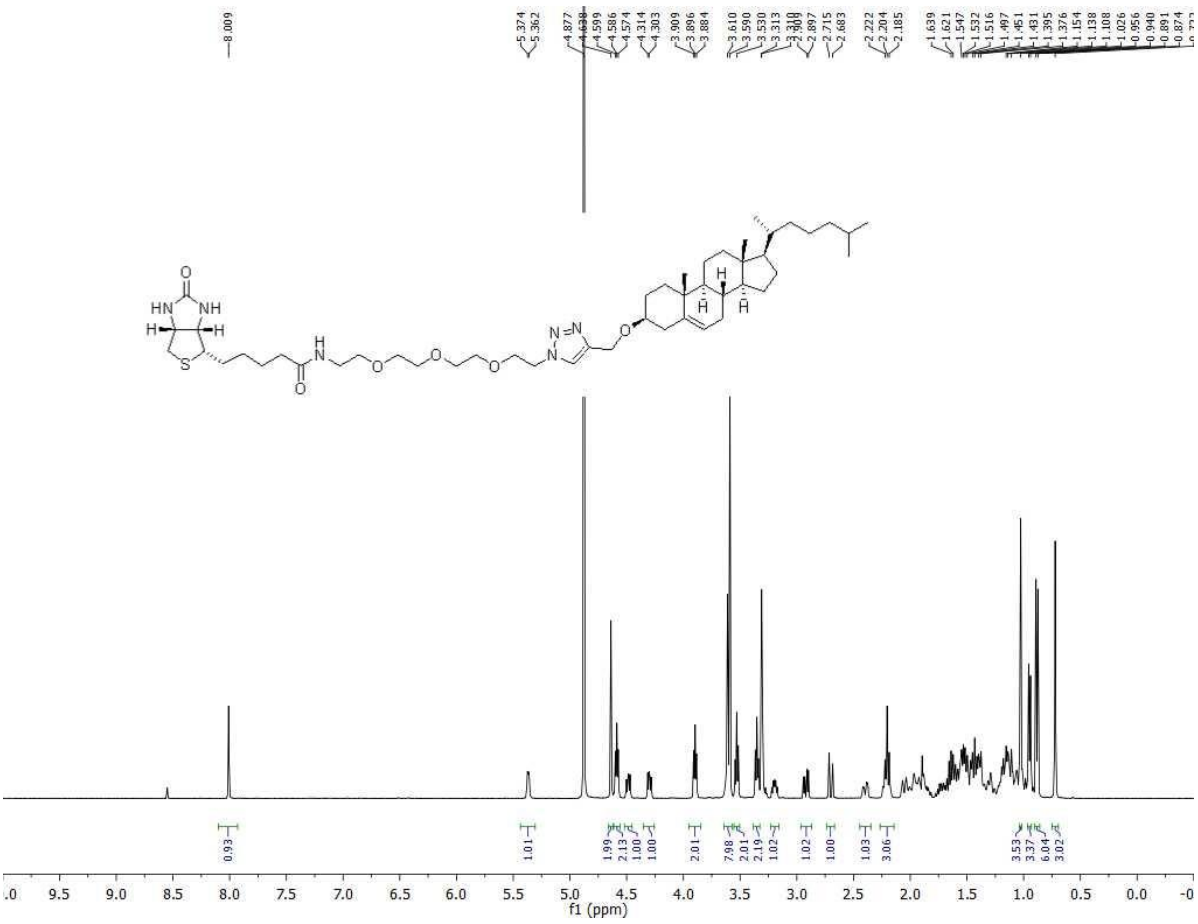
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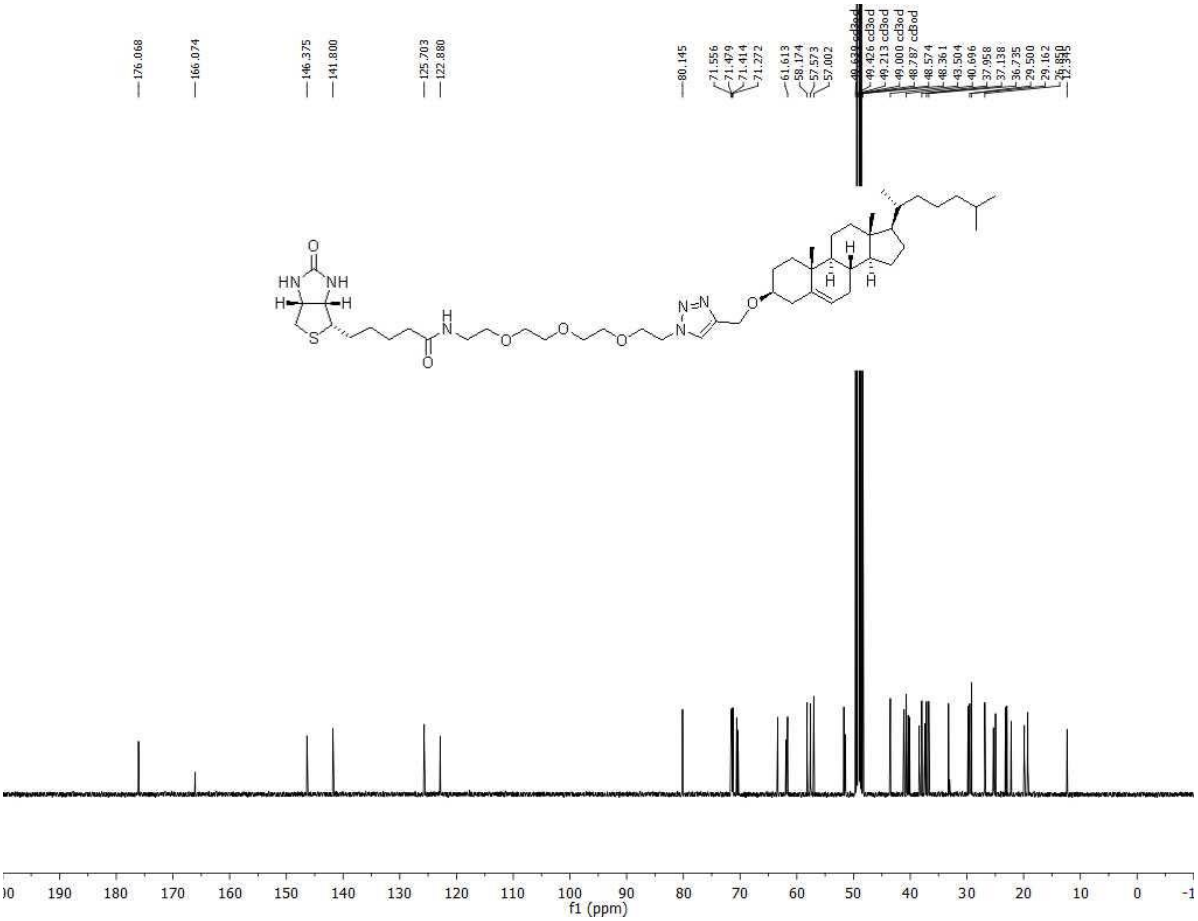
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