KSRP/FUBP2 is the binding protein of GO-Y086, a cytotoxic curcumin analogue

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Experimental Procedures for Biological Analysis

Cell culture

Cells of the colorectal carcinoma line HCT116 were cultured in RPMI1640 supplemented with 10% fetal bovine serum (FBS). The HEK-293T cell line was cultured in DMEM supplemented with 10% FBS.

Cell growth suppression analysis

HCT116 was obtained from the Cell Resource Center for Biomedical Research (Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan). The growth-suppressive effects of the compounds were measured for 48 h. Cell viability was assayed by quantitation of the uptake and digestion of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt (MTT) according to the manufacturer's instructions (Dojindo Laboratories, Kumamoto, Japan) by using a 96-well plate reader, MPR-4Ai (Tosoh Corp., Tokyo, Japan). The percentage cell growth of the control, which was treated with 1% DMSO alone, was calculated and plotted, and then the mean growth inhibitory concentration (GI_{50}) value was determined.

Plasmid construction

The GFP-KSRP-FL (full-length), NT (amino-terminus), KH (KH domains), and CT (carboxy-terminus) have been described by Hall M. P. *et al*. (*Mol. Biol. Cell*, **2004**, *15*, 774). KSRP point mutations were generated by site-directed mutagenesis using primers containing a corresponding mutation (C436A: 5'-CATCCCCACTCACAAGGCTGGGCTGGTCATC-3'; K473R: 5'-GGGACCCCAACTTCAGGTTGTT CACATC-3'; C500A: 5'-GATCGAGGGTCCTCTCGCCCCAGTTGGACCAGGC-3'; K564R: 5'-GCCG CTGCAGCTGCTCTGCTTGGGTCATG-3'; K620R: 5'-TACTCTTCCCAGGCCCTAGTGTAGTCCGA C-3'; K627R: 5'-TGCTGGCCGATCTTTCTGTAATACTCTTC-3'). GFP-KSRP-FL was digested with Pst I or Not I and Kpn I. The PCR products were cloned into the digested GFP-KSRP-FL (C436A, K473R, C500A and K564R: Pst I; K620R and K627R: Not I and Kpn I), respectively. For RNAi-Ready pSIREN-RetroQ-KSRP, complementary oligonucleotides 5'-GATCCGGATCAACCGGAGAGCAAGATT CAAGAGATCTTGCTCTCCGGTTGATCCTTTTTTT-3' and 5'-AATTCTCTAGAAAAAAAGGATCAA CCGGAGAGCAAGATCTCTTGAATCTTGCTCTCCGGTT-3' were annealed. The annealed oligonucleotide was ligated into the BamHI and EcoRI sites of RNAi-Ready pSIREN-RetroQ in BD Knockout RNAi Systems (Clontech, Palo Alto, CA). For the RNAi-Ready pSIREN-RetroQ-control, Luciferase shRNA Annealed Oligonucleotide (Clontech) was ligated. All constructs were verified by DNA sequencing.

Reagents and antibodies

The antibodies used for Western blotting were anti-actin monoclonal antibody (A2066; Sigma-Aldrich, St. Louis, MO), anti-c-Myc monoclonal antibody (9E10; Santa Cruz Biotechnology, Santa Cruz, CA), anti-biotin monoclonal antibody (BN-34; Sigma-Aldrich), anti-GFP monoclonal antibody (B34; Covance, Princeton, NJ), anti-FBP2 (KSRP) polyclonal antibody (D-12; Santa Cruz Biotechnology), ECL-anti-mouse IgG (NA931V; GE Healthcare, Buckinghamshire, UK), and anti-goat IgG-HRP (sc-2020; Santa Cruz Biotechnology). The resin used for precipitation was SoftLinkTM Soft Release Avidin Resin (Promega, Madison, WI).

Transfection

Transfection with Effectene Transfection Reagent (Qiagen, Valencia, CA) was performed according to the manufacturer's instructions.

Precipitation

HCT116 cells were treated with 2 μM GO-Y086 or GO-Y088 for 9 h. After washing three times with PBS, cells were lysed with low salt lysis buffer (10 mM Hepes, 100 mM NaCl, 0.2% NP40, 10 mM EDTA, pH 7.6) and protease inhibitor mixture (Sigma, St. Louis, MO). The lysed cells were centrifuged at 12,000 rpm for 10 min and the supernatant was collected as a cell lysate. The cell lysate was incubated with Avidin Resin at 4 °C for 30 min. The reacted resins were washed with low salt lysis buffer, and the bounded proteins were eluted with SDS-polyacrylamide gel electrophoresis sample buffer (125 mM tris-HCl, 10% 2-mercaptoethanol, 4% SDS, 10% sucrose, 0.004% bromophenol blue, pH 6.8) at 95 °C for 5 min. Samples were subjected to electrophoresis in 8% sodium dodecyl sulfate (SDS)-polyacrylamide gels, probed with primary and secondary antibodies, and detected by enhanced chemiluminescence.

Cell fractionation

Cell fractionation with Proteoextract™ was performed according to the manufacturer's instructions.

PMF analysis

One half of the large scale sample (HCT116, 90Φdish x12) was subjected to electrophoresis in 7.5% SDS-polyacrylamide gel, and stained with Coomassie Brilliant Blue (CBB). The protein-containing region detected in this manner was excised and was washed successively with MilliQ (37 °C, 5 x 400 μL), acetonitrile (2 x 200 μL), and decolorant (50% acetonitrile, 100 mM NH_4HCO_3) (2 x 100 μL); the sample was then dried in vacuo. The dried gel was reduced by incubation with 50 μL of reducing agent (100 mM NH_4HCO_3, 100 mM dithiothreitol) at 56 °C for 30 min. After removing the excess reducing agent, 100 μL of an alkylating aqueous solution (100 mM NH_4HCO_3, 100 mM iodoacetoamide) was added, and the mixture was incubated at 37 °C for 30 min. The gel was washed with 100 mM aqueous NH_4HCO_3 (200 μL) for 10 min, and dried in vacuo. The dried gel was digested by incubation with 10 μL of digest solution (10 mM Tris-HCl, 25 μg/mL trypsin, pH 8.8) at 4 °C for 45 min. After excess solution was removed, 20 μL of 10 mM Tris-HCl buffer (pH 8.8) was added. The mixture was incubated at 37 °C overnight. The extract and gel were then separated. 20 μL of extract solution (5% formic acid, 50% aqueous acetonitrile) was added to the gel, and the mixture was incubated for 10 min; the extract was then separated from the gel. After this extraction cycle was repeated, the gel was further extracted with acetonitrile (2 x 20 μL). The combined extracts were concentrated until the total volume became ca. 5 μL. To the concentrate was added 20 μL of 0.1% aqueous trifluoroacetic acid. Extracted peptides in the concentrate ware absorbed on a ZipTip® pipette tip (Millipore Corporation, Bedford, MA), and the tip was washed three times with 0.1% aqueous trifluoroacetic acid. The washed peptides were eluted with 5 μL of 75% aqueous acetonitrile containing 0.1% trifluoroacetic acid. The extracted peptides were loaded onto the MALDI target plate by mixing 0.5 μL of solution with the same volume of a matrix solution, which was prepared fresh by dissolving 10 mg/mL α-cyano-4-hydroxycinnamic acid in 50% aqueous acetonitrile containing 0.1% trifluoroacetic acid. The samples were then allowed to dry. MALDI-TOF mass and tandem mass measurements were carried out with Ultraflex TOF/TOF mass

spectrometer (Bruker Daltonics GmbsH, Bremen, Germany) in reflection mode. The Mascot Search® program (Matrix Science Inc., Boston, MA) was used to search the SWISS-PLOT database for the peptide masses.

Quantitative RT-PCR

The total cellular RNA was isolated using an RNeasy Mini Kit (Qiagen). cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real time-PCR was performed on a CFX96 system (Bio-Rad, Hercules, CA) using primers of *c-myc* (Assay IDs: Hs00153408_m1 and Hs99999903_m1) of the TaqMan (r) Gene Expression Assays (Applied Biosystems). To analyze the expression level of *c-myc* mRNA, normalization across samples was performed using the expression of *β-actin.*

PMF analysis results for the GO-Y086-binding protein

Figure SI-1. MALDI-TOF mass spectrum of digested peptides of the GO-Y086-binding protein

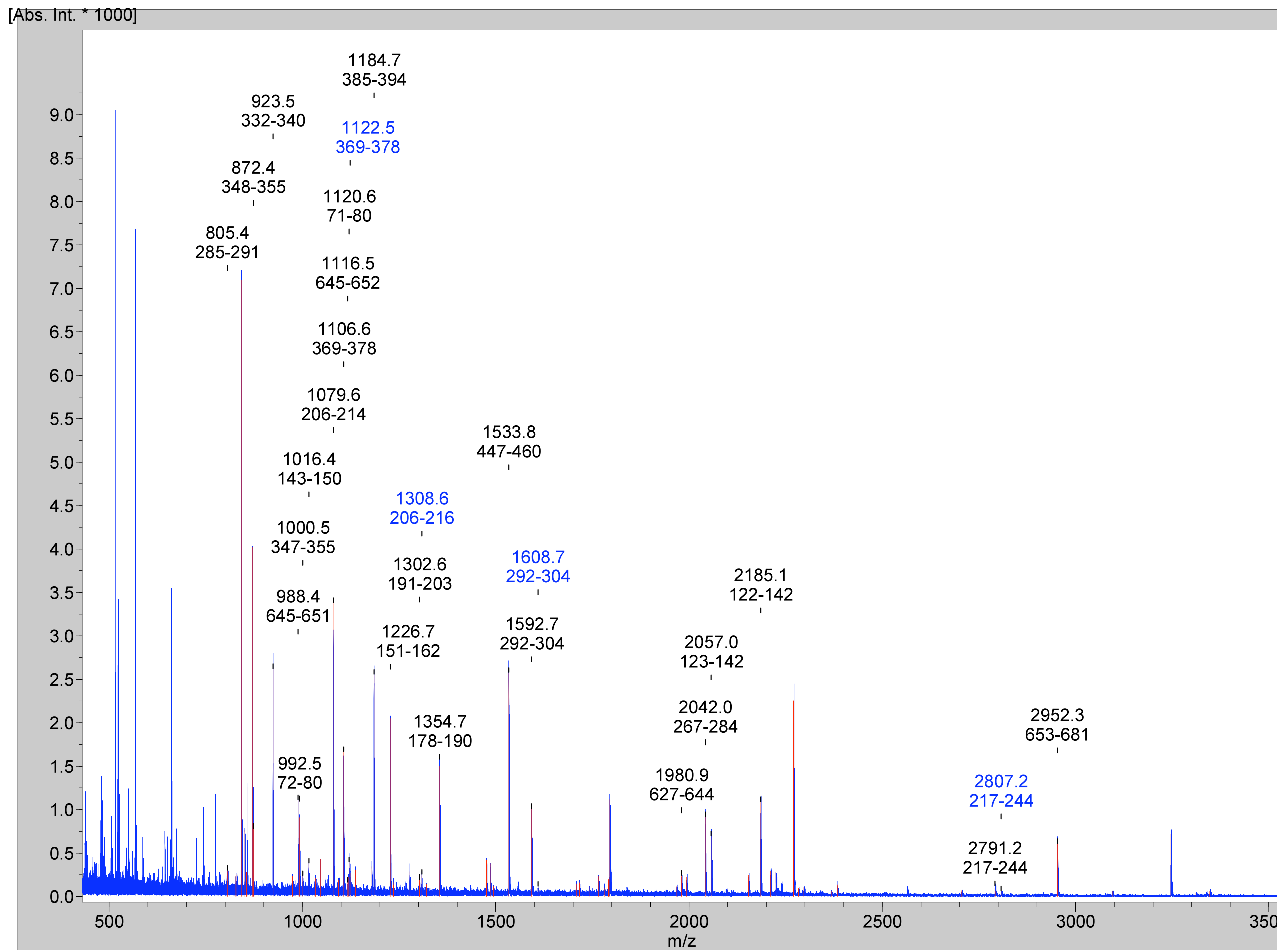

Table SI-1. PMF analysis results

Match to : **FUBP2_HUMAN**
Mass : **73063**
Score : **177**
Sequence Coverage : **36%**

Matched peptides are shown in **Bold Red**

1 MSDYSTGGPP PGPPPPAGGG GGAGGAGGGP PPGPPGAGDR GGGGPCGGGP
51 GGGSAGGPSQ PPGGGGPGIR **KDAFADAVQR** ARQIAAKIGG DAATTGNNST
101 PDFGFGGQKR QLEDGDQPES K**KLASQGDSI** **SSQLGPIHPP** **PRTSMTEEYR**
151 **VPDGMVGLII** **GR**GGEQINKI QQDSGCK**VQI** **SPDSGGLPER** **SVSLTGAPES**
201 **VQK**AK**MMLDD** **IVSRGRGGPP** **GQFHDNANGG** **QNGTVQEIMI** **PAGK**AGLVIG
251 KGGETIKQLQ ERAGVK**MILI** **QDGSQNTNVD** **KPLRIIGDPY** **KVQQACEMVM**
301 **DILR**NVTKAG FGDRNEYGSR IGGGIDVPVP R**HSVGVVIGR** SGEMIK**KIQN**
351 **DAGVR**IQFKQ DDGTGPEK**IA** **HIMGPPDR**CE HAAR**IINDLL** **QSLR**SGPPGP
401 PGGPGIPGGR GRGRGQGNWG PGGEMTFSIP THKCGLVIGR GGENVK**AINQ**
451 **QTGAFVEISR** QLPPTGTPTS KLFIIRGSPQ QIDHCRQLIE EKIEGPLCPV
501 GPGPGGPGPA GPMGPFNPGP FNQGPPGAPP HAGGPPPHQY PPQGWGNTYP
551 QWQPPAPHDP SKAAAAAADP NAAWAAYYSH YYQQPPGPVP GPAPAPAAPP
601 AQGEPPQPPP TGQSDYTKAW EEYYKK**IGQQ** **PQQPGAPPQQ** **DYTKAWEEYY**
651 **KKQAQVATGG** **GPGAPPGSQP** **DYSAAWAEYY** **R**QQAAYYGQT PVPGPQPPPT
701 QQGQQQQ

Matched peptide list (27 peptides in total)

(Start-End)	Obsd.	Mr (expt.)	Mr (calcd)	ppm	Miss*	Sequence
(71-80)	1120.5583	1119.5510	1119.5673	-15	1	R.KDAFADAVQR.A
(72–80)	992.4568	991.4495	991.4723	-23	0	K.DAFADAVQR.A
(122–142)	2185.1253	2184.1181	2184.1651	-22	1	K.KLASQGDSISSQLGPIHPPPR.T
(123–142)	2057.0389	2056.0316	2056.0701	-19	0	K.LASQGDSISSQLGPIHPPPR.T
(143–150)	1016.4279	1015.4206	1015.4280	-7	0	R.TSMTEEYR.V
(151–162)	1226.6572	1225.6500	1225.6853	-29	0	R.VPDGMVGLIIGR.G
(178–190)	1354.6821	1353.6748	1353.6888	-10	0	K.VQISPDSGGLPER.S
(191–203)	1302.6283	1301.6210	1301.6827	-47	0	R.SVSLTGAPESVQK.A
(206–214)	1079.5664	1078.5591	1078.5151	41	0	K.MMLDDIVSR.G
(206–216)	1308.6401	1307.6328	1307.6326	0	1	K.MMLDDIVSRGR.G (Ox.)**
(217–244)	2791.2276	2790.2203	2790.3144	-34	0	R.GGPPGQFHDNANGGQNGTVQEIMIPAGK.A
(217–244)	2807.2376	2806.2303	2806.3093	-28	0	R.GGPPGQFHDNANGGQNGTVQEIMIPAGK.A (Ox.)**
(267–284)	2042.0251	2041.0178	2041.0626	-22	0	K.MILIQDGSQNTNVDKPLR.I
(285–291)	805.3559	804.3486	804.4381	-111	0	R.IIGDPYK.V

(292–304)	1592.7383	1591.7311	1591.7520	-13	0	K.VQQACEMVMDILR.N
(292–304)	1608.6999	1607.6927	1607.7470	-34	0	K.VQQACEMVMDILR.N (Ox.)**
(332–340)	923.5075	922.5002	922.5349	-38	0	R.HSVGVVIGR.S
(347–355)	1000.5039	999.4967	999.5461	-49	1	K.KIQNDAGVR.I
(348–355)	872.4142	871.4069	871.4512	-51	0	K.IQNDAGVR.I
(369–378)	1106.5573	1105.5500	1105.5702	-18	0	K.IAHIMGPPDR.C
(369–378)	1122.5148	1121.5075	1121.5652	-51	0	K.IAHIMGPPDR.C (Ox.)**
(385–394)	1184.6847	1183.6774	1183.6924	-13	0	R.IINDLLQSLR.S
(447–460)	1533.7790	1532.7717	1532.7947	-15	0	K.AINQQTGAFVEISR.Q
(627–644)	1980.8871	1979.8798	1979.9701	-46	0	K.IGQQPQQPGAPPQQDYTK.A
(645–651)	988.4015	987.3942	987.4338	-40	0	K.AWEEYYK.K
(645–652)	1116.5340	1115.5267	1115.5287	-2	1	K.AWEEYYKK.Q
(653–681)	2952.3230	2951.3157	2951.3474	-11	0	K.QAQVATGGGPGAPPGSQPDYSAAWAEYYR.Q

*Miss : number of missed cleavage; **(Ox.) : oxidation at M residue

Figure SI-2. Alignment of FUBP2/KSRP protein sequences found in the SWISS-PLOT database used in the PMF analysis (PMF) and that used this study (Vector). Highlights in gray are the sites of amino acid mutation. The highlight in yellow indicates the C-terminal region. The underlined sequence corresponds to peptide fragments observed in the PMF analysis. The lysine residues designated as K were found intact in the peptide fragments. The lysine residue designated as K must be intact in order for the peptide fragments to be observed in the PMF analysis. Thus, the possible GO-Y086-binding sites are indicated by the blue highlights.

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PMF    MSDYSTGGPP PGPPPPAGGG GGAGGAGGGP PPGPPGAGDR GGGGPCGGGP GGGSAGGPSQ 60
Vector MSDYSTGGPP PGPPPPAGGG GGAGGAGGGP PPGPPGAGDR GGGGPCGGGP GGGSAGGPSQ 60

PMF    PPGGGGPGIR KDAFADAVQR ARQIAAKIGG DAATTGNNST PDFGFGGQKR QLEDGDQPES 120
Vector PPGGGGPGIR KDAFADAVQR ARQIAAKIGG DAATTVNNST PDFGFGGQKR QLEDGDQPES 120

PMF    KKLASQGDSI SSQLGPIHPP PRTSMTEEYR VPDGMVGLII GRGGEQINKI QQDSGCKVQI 180
Vector KKLASQGDSI SSQLGPIHPP PRTSMTEEYR VPDGMVGLII GRGGEQINKI QQDSGCKVQI 180

PMF    SPDSGGLPER SVSLTGAPES VQKAKMMLDD IVSRGRGGPP GQFHDNANGG QNGTVQEIMI 240
Vector SPDSGGLPER SVSLTGAPES VQKAKMMLDD IVSRGRGGPP GQFHDNANGG QNGTVQEIMI 240

PMF    PAGKAGLVIG KGGETIKQLQ ERAGVKMILI QDGSQNTNVD KPLRIIGDPY KVQQACEMVM 300
Vector PAGKAGLVIG KGGETIKQLQ ERAGVKMILI QDGSQNTNVD KPLRIIGDPY KVQQACEMVM 300

PMF    DILRNVTKAG FGDRNEYGSR IGGGIDVPVP RHSVGVVIGR SGEMIKKIQN DAGVRIQFKQ 360
Vector DILRERDQGG FGDRNEYGSR IGGGIDVPVP RHSVGVVIGR SGEMIKKIQN DAGVRIQFKQ 360

PMF    DDGTGPEKIA HIMGPPDRCE HAARIINDLL QSLRSGPPGP PGGPGIP-GG RGRGRGQGNW 419
Vector DDGTGPEKIA HIMGPPDRCE HAARIINDLL QSLRSGPPGP PGGPGMPPGG RGRGRGQGNW 420

PMF    GP-GGEMTFS IPTHKCGLVI GRGGENVKAI NQQTGAFVEI SRQLPPTGTP TSKLFIIRGS 478
Vector GPPGGEMTFS IPTHKCGLVI GRGGENVKAI NQQTGAFVEI SRQLPPNGDP NFKLFIIRGS 480

PMF    PQQIDHCRQL IEEKIEGPLC PVGPGPGGPG PAGPMGPFNP GPFNQGPPGA PPHAGGPPPH 538
Vector PQQIDHAKQL IEEKIEGPLC PVGPGPGGPG PAGPMGPFNP GPFNQGPPGA PPHAGGPPPH 540
                         ↑497
PMF    QYPPQGWGNT YPQWQPPAPH DPSKAAAAAA DPNAAWAAYY SHYYQQPPGP VPGPAPAPAA 598
Vector QYPPQGWGNT YPQWQPPAPH DPSKAAAAAA DPNAAWAAYY SHYYQQPPGP VPGPAPAPAA 600

PMF    PPAQGEPPQP PPTGQSDYTK AWEEYYKKIG QQPQQPGAPP QQDYTKAWEE YYKKQAQVAT 658
Vector PPAQGEPPQP PPTGQSDYTK AWEEYYKKIG QQPQQPGAPP QQDYTKAWEE YYKKQAQVAT 660

PMF    GGGPGAPPGS QPDYSAAWAE YYRQQAAYYG QTPVPG-PQP PPTQQGQQQQ - 707
Vector GGGPGAPPGS QPDYSAAWAE YYRQQAAYYG QTPGPGGPQP PPTQQGQQQA Q 711
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Effects of KSRP knockdown

Figure SI-3. siRNA knockdown of KSRP reduced the expression of c-Myc. HEK293T cells were transfected with RNAi-Ready pSIREN-RetroQ-control (lane 1) or RNAi-Ready pSIREN-RetroQ-KSRP (lane 2) to produce control siRNA or KSRP siRNA in cells, respectively. Cell lysates were prepared 48 h after transfection, and then analyzed by Western blotting using anti-KSRP, anti-c-Myc, and β-actin antibodies.

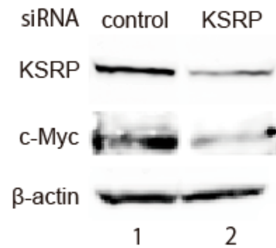
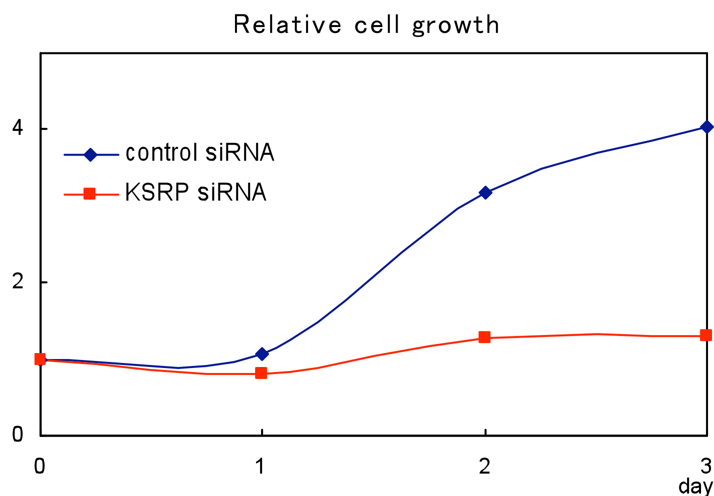


Figure SI-4. Relative cell growth of the HEK-293T cells that were transfected with RNAi-Ready pSIREN-RetroQ-control or RNAi-Ready pSIREN-RetroQ-KSRP. Cell growth was evaluated by MTT assay.



General Experimental Procedures for Chemical Synthesis

All reactions were carried out under an atmosphere of argon unless otherwise specified. Anhydrous solvents were transferred via syringe to flame-dried glassware, which had been cooled under a stream of dry nitrogen. Ethereal solvents and dichloromethane (anhydrous; Kanto Chemical Co. Inc., Osaka, Japan) were used as received. All other solvents were dried and distilled by standard procedures. Yields refer to chromatographically and spectroscopically (^{1}H NMR) homogeneous materials unless otherwise stated. Reagents were purchased at the highest commercial quality and used without further purification unless otherwise stated.

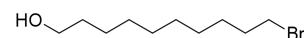
The reactions were monitored by thin-layer chromatography (TLC) carried out on 0.25 mm Merck silica gel plates (60F-254) using UV light as the visualizing agent and *p*-anisaldehyde in ethanol/aqueous H_2SO_4/CH_3CO_2H for staining. Column chromatography was performed using silica gel 60 particle size 0.063-0.210 mm. The eluents employed are reported as volume : volume percentages.

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded using a JEOL JMN-AL400 (400 MHz), and a JEOL JNM-ECA500 (500 MHz) spectrometer. Chemical shift (δ) is reported in parts per million (ppm) downfield relative to tetramethylsilane (TMS). Coupling constants (J) are reported in Hz. Multiplicities are reported using the following abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad; app, Apparent. Carbon-13 nuclear magnetic resonance (^{13}C NMR) spectra were recorded using a JEOL JMN-AL400 (100 MHz) and a JEOL JNM-ECA500 (125 MHz) spectrometer. Chemical shifts are reported in ppm relative to the center of $CDCl_3$.

Melting points were determined using a Yazawa BY-2 melting point apparatus and are reported uncorrected. Infrared spectra were obtained on a JASCO FT-IR-410 at 4.0 cm^{-1} resolution and are reported in wavenumbers. High resolution mass spectra (HRMS) were recorded on a JMS-AX500 or JMS-700. Low resolution mass spectra (LRMS) were recorded on a JEOL JMS-DX303.

Experimental Procedures and Compounds Data

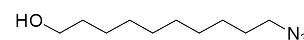
10-Bromodecanol (2)



A mixture of 1,10-decandiol (1.5 g, 8.6 mmol) and 48% HBr (aq.) (1.2 ml) in toluene (26 ml) was refluxed for 2 days. The mixture was allowed to cool to room temperature and then H_2O and AcOEt were added. The organic layer was separated, and the aqueous layer was extracted with AcOEt. The combined organic layers were washed with brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (AcOEt / hexane = 1 / 1) to afford **2** (1.9 g, 92% yield) as a colorless oil.

2: FT-IR ($CHCl_3$) ν_{max} 3336 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 3.64 (2H, t, J = 6.5 Hz), 3.41 (2H, t, J = 6.8 Hz), 1.85 (2H, quintet, J = 7.7 Hz), 1.56 (2H, quintet, J = 7.2 Hz), 1.42 (2H, m), 1.20-1.40 (12H, m); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 63.0, 34.0, 32.8, 32.8, 29.4, 29.3, 29.3, 28.7, 28.1, 25.7; LRMS (EI) m/z 235 ($[M-H]^+$); HRMS (EI) calcd. for $C_{10}H_{20}BrO$ 235.0698, found 235.0686.

10-Azidodecanol (3)

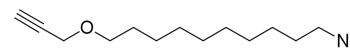


A mixture of bromide **2** (1.8 g, 7.6 mmol) and sodium azide (740 mg, 11.4 mmol) in DMF (25 ml) was heated for 6 h at 50 °C. The mixture was allowed to cool to room temperature and then H_2O and Et_2O were added. The organic layer was separated, and the aqueous layer was extracted with Et_2O. The combined

organic layers were washed with brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (AcOEt / hexane = 2 / 3) to afford **3** (1.5 g, 97% yield) as a colorless oil.

3: FT-IR ($CHCl_3$) ν_{max} 2095 cm^{-1}; ^1H-NMR (400 MHz, $CDCl_3$) δ 3.64 (2H, t, *J* = 6.7 Hz), 3.26 (2H, t, *J* = 7.0 Hz), 1.58 (4H, m), 1.20-1.40 (13H, m); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 63.0, 51.5, 32.8, 29.4, 29.4, 29.3, 29.1, 28.8, 26.7, 25.7; LRMS (FAB) m/z 200 ($[M+H]^+$); HRMS (FAB) Calcd. for $C_{10}H_{22}N_3O$ 200.1763, Found 200.1764.

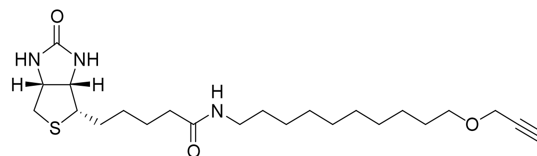
3-(10-Azidodecyloxy)propyne (4)



To a solution of alcohol **3** (500 mg, 2.5 mmol) in DMF (13 ml) was added sodium hydride (60% in oil, 130 mg, 2.8 mmol) at 0 °C. After stirring for 10 min, propargyl bromide (0.2 ml, 2.8 mmol) was added to the reaction mixture and the reaction mixture was stirred at room temperature for 6 h. The mixture was quenched with H_2O and diluted with Et_2O. The organic layer was separated, and the aqueous layer was extracted with Et_2O. The combined organic layer was washed with brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (AcOEt / hexane = 1 / 2) to afford **4** (505 mg, 85% yield) as a yellow oil.

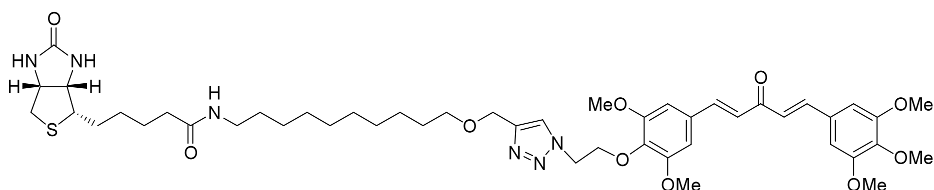
4: IR ($CHCl_3$) ν_{max} 3305, 2096 cm^{-1}; ^1H-NMR (400 MHz, $CDCl_3$) δ 4.13 (2H, d, *J* = 2.3 Hz), 3.50 (2H, t, *J* = 6.6 Hz), 3.25 (2H, t, *J* = 7.0 Hz), 2.41 (1H, t, *J* = 2.3 Hz), 1.59 (4H, m), 1.35-1.29 (12H, m); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 80.0, 74.0, 70.3, 58.0, 51.5, 29.5, 29.4, 29.4, 29.3, 29.1, 28.8, 26.7, 26.0; LRMS (FAB) m/z 238 ($[M+H]^+$); HRMS (FAB) Calcd. for $C_{13}H_{24}N_3O$ 238.1919, Found 238.1927.

5-(2-Oxohexahydrothieno[3,4-*d*]imidazol-6-yl)pentanoic acid (10-prop-2-ynyloxydecyl)amide (5)



To a solution of azide **4** (0.2 g, 0.84 mmol) in THF (5.6 ml) and H_2O was added PPh_3 (440 mg, 1.7 mmol). After stirring for 1 h at room temperature, $MgSO_4$ was added. The mixture was filtered through a filter paper and concentrated under reduced pressure. To a solution of the residue and (+)-biotin (247 mg, 1.0 mmol) in DMF (3.7 ml) were added DMAP (220 mg, 1.7 mmol) and *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (340 mg, 1.8 mmol). After stirring for 12 h at room temperature, H_2O was added and the solution was diluted with AcOEt. The organic layer was separated, and the aqueous layer was extracted with AcOEt. The combined organic layer was washed with brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (MeOH / AcOEt = 15 / 100) to afford **5** (277 mg, 75% yield) as a colorless amorphous solid.

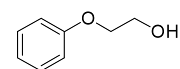
5: $[\alpha]_D^{31}$ +105.52 (*c* 0.95, MeOH); IR ($CHCl_3$) ν_{max} 3296, 1701 cm^{-1}; ^1H-NMR (400 MHz, $CDCl_3$) δ 6.26 (0.5H, s), 6.03 (0.5H, s), 5.91 (0.5H, s), 5.80 (1H, s), 5.40 (0.5H, s), 5.21 (0.5H, s), 4.52 (1H, m), 4.32 (1H, m), 4.13 (2H, d, *J* = 2.4 Hz), 3.51 (2H, t, *J* = 6.8 Hz), 3.20 (3H, m), 2.91 (1H, dd, *J* = 13.0, 5.0 Hz), 2.74 (1H, d, *J* = 12.8 Hz), 2.42 (1H, t, *J* = 2.4 Hz), 2.20 (2H, t, *J* = 7.3 Hz), 1.22-1.74 (22H, m); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 173.0, 173.0, 163.5, 163.4, 80.1, 74.1, 70.3, 61.8, 60.2, 58.0, 55.6, 55.5, 40.6, 39.6, 36.1, 29.7, 29.5, 29.5, 29.4, 29.3, 28.2, 28.1, 27.0, 26.1, 25.7, 25.7; LRMS (EI) m/z 437 (M^+); HRMS (EI) Calcd. for $C_{23}H_{39}N_3O_3S$ 437.2712, Found 437.2711.



GO-Y086

To a mixture of **GO-Y085** (0.35 g, 2.1 mmol) and alkyne **5** (156 mg, 0.36 mmol) in MeCN (10 ml) was added CuI (3 mg, 0.018 mmol). After stirring for 12 h at room temperature, H_2O was added and diluted with AcOEt. The organic layer was separated, and the aqueous layer was extracted with AcOEt. The combined organic layer was washed with brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (MeOH / $CHCl_3$ = 15 / 100) to afford **GO-Y086** (194 mg, 60% yield) as a yellow amorphous solid.

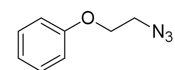
GO-Y086: $[\alpha]_D^{23}$ + 16.4 (*c* 0.25, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3296, 2929, 1701, 1648, 1618, 1583, 1503, 1462, 1419, 1278, 1244, 1629 cm^{-1}; ^{1}H-NMR (500 MHz, $CDCl_3$) δ 8.04 (1H, s), 7.67 (1H, d, *J* = 15.9 Hz), 7.64 (1H, d, *J* = 15.9 Hz), 6.98 (2H, d, *J* = 15.9 Hz), 6.85 (2H, s), 6.82 (2H, s), 5.80 (1H, brs). 5.77 (1H, brs), 5.07 (1H, brs), 4.71 (2H, t, *J* = 5.0 Hz), 4.65 (2H, s), 4.50 (1H, m), 4.39 (2H, t, *J* = 5.0 Hz), 4.31 (1H, m), 3.92 (6H, s), 3.90 (3H, s), 3.85 (6H, s), 3.53 (2H, t, *J* = 6.2 Hz), 3.21 (2H, m), 3.14 (1H, m), 2.90 (1H, dd, *J* = 12.9, 5.0 Hz), 2.72 (1H, d, *J* = 12.9 Hz), 2.18 (2H, t, *J* = 7.8 Hz), 1.68-1.58 (6H, m), 1.49-1.40 (4H, m), 1.25 (12H, m); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 188.4, 172.9, 163.6, 153.5, 153.3, 145.2, 143.5, 143.0, 140.5, 138.2, 131.0, 130.2, 125.1, 124.7, 124.1, 105.7, 105.3, 71.3, 70.8, 64.4, 61.7, 61.0, 60.1, 56.2, 56.0, 55.5, 50.5, 40.5, 39.5, 36.0, 29.6, 29.6, 29.4, 29.4, 29.4, 29.2, 28.1, 28.1, 26.9, 26.1, 25.6; LRMS (FAB) m/z 907 ($[M+H]^+$); HRMS (FAB) Calcd. for $C_{47}H_{67}N_6O_{10}S$ 907.4639, Found : 907.4626.



2-Phenoxyethanol (7)

A mixture of phenol (0.3 g, 3.2 mmol), K_2CO_3 (1.3 g, 9.6 mmol) and ethylene bromohydrin (0.34 ml, 4.8 mmol) in DMF (25 ml) was heated for 6 h at 90 °C. The mixture was cooled to room temperature and then H_2O was added and the mixture was diluted with Et_2O. The organic layer was separated, and the aqueous layer was extracted with Et_2O. The combined organic layers were washed with brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (AcOEt / hexane = 1 / 1) to afford **7** (0.21 g, 47% yield) as a colorless oil.

7: IR ($CHCl_3$) ν_{max} 3359, 1245 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$) δ7.31-7.16 (2H, m), 6.99-6.91 (3H, m), 4.08 (2H, t, *J* = 4.5 Hz), 3.96 (2H, m), 2.17 (1H, s); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 158.6, 129.5, 121.1, 114.5, 69.1, 61.5; LRMS (EI) m/z 138 ($[M-H]^+$); HRMS (EI) Calcd. for $C_8H_{10}O_2$ 138.0681, Found 138.0685.

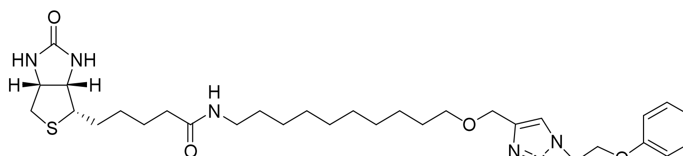


(2-Azidoethoxy)benzene (8)

To a mixture of alcohol **8** (200 mg, 1.5 mmol) and triethylamine (0.4 ml, 2.9 mmol) in CH_2Cl_2 (7 ml) was slowly added mesyl chloride (0.13 ml, 1.7 mmol). After stirring for 3 h at room temperature, H_2O was added and the solution was diluted with $CHCl_3$. The organic layer was separated, and the aqueous layer was extracted with $CHCl_3$. The combined organic layers were washed with brine, dried over $MgSO_4$ and concentrated under reduced pressure. A mixture of the residue and sodium azide (280 mg, 4.3 mmol) in DMF (3.6 ml) was heated for 3 h at 50 °C. The mixture was allowed to cool to room temperature and then H_2O and Et_2O were added. The organic layer was separated, and the aqueous layer was extracted with Et_2O.

The combined organic layers were washed with brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (AcOEt / hexane = 1 / 4) to afford **8** (0.23 g, 97% yield) as a colorless oil.

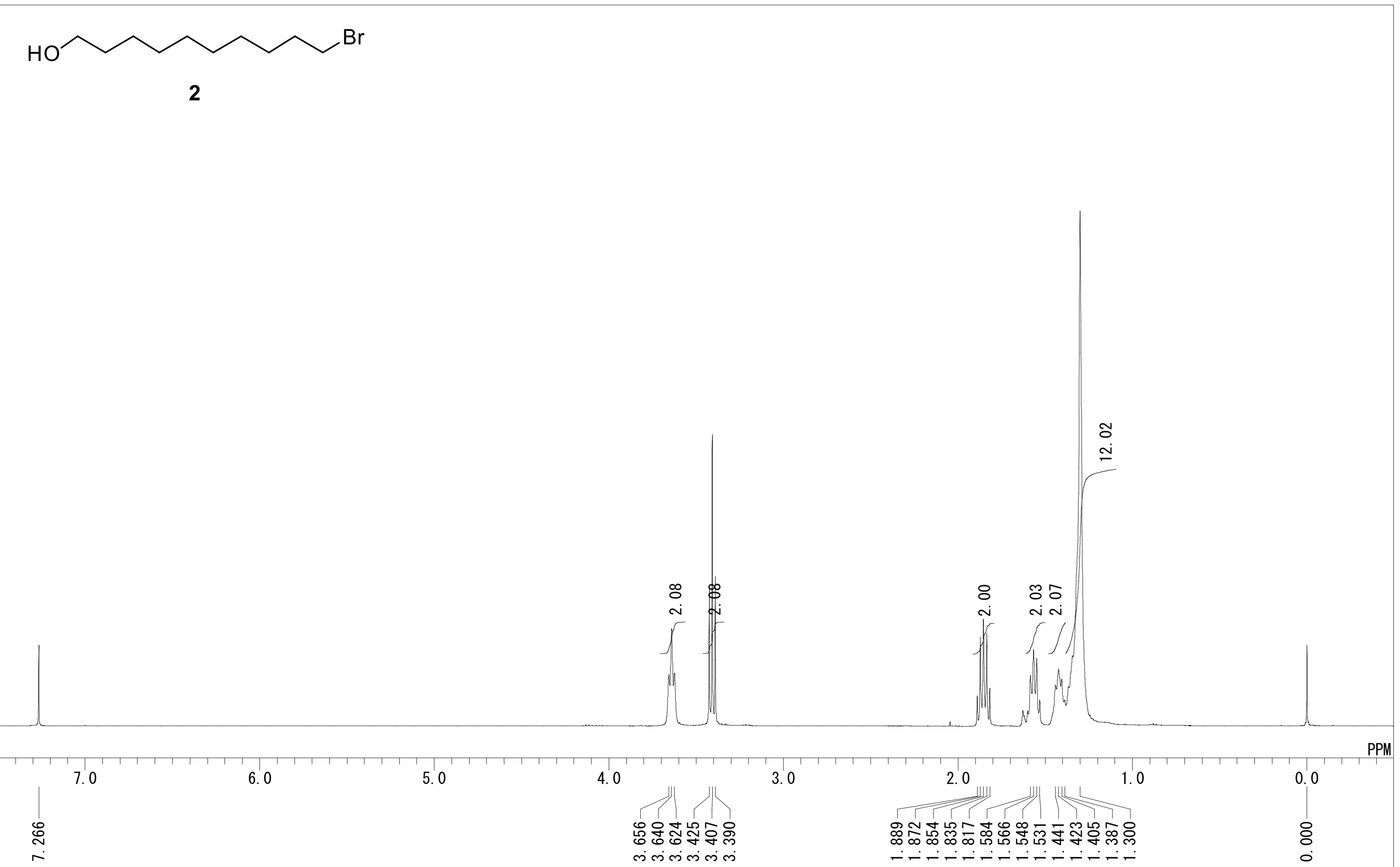
8: IR ($CHCl_3$) ν_{max} 2109, 1242 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.29 (2H, m), 7.00 (1H, t, *J* = 7.5 Hz), 6.93 (2H, m), 4.15 (2H, t, *J* = 5.0 Hz), 3.59 (2H, t, *J* = 5.0 Hz); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 158.2, 129.5, 121.3, 114.6, 66.8, 50.2; LRMS (EI) m/z 163 ($[M-H]^+$); HRMS (EI) Calcd. for $C_8H_9N_3O$ 163.0746, Found 163.0740.



GO-Y088

To a mixture of azide **8** (20 mg, 0.012 mmol) and **5** (18 mg, 0.041 mmol) in MeCN (0.4 ml) was added CuI (0.4 mg, 0.002 mmol). After stirring for 5 days at room temperature, the mixture was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (MeOH / $CHCl_3$ = 1 / 10) to afford **GO-Y088** (12 mg, 50% yield) as a colorless amorphous oil.

GO-Y088: $[\alpha]_D^{23}$ + 34.8 (*c* 0.20, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3303, 1702, 1639, 1462, 1215 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.75 (1H, s), 7.27 (2H, dd, *J* = 8.0, 7.5 Hz), 6.98 (1H, t, *J* = 7.5 Hz), 6.87 (2H, d, *J* = 8.0 Hz), 6.30 (1H, brs), 5.97 (1H, brs), 5.52 (1H, brs). 4.77 (2H, t, *J* = 4.9 Hz), 4.61 (2H, s), 4.50 (1H, m), 4.36 (2H, t, *J* = 4.9 Hz), 4.31 (1H, m), 3.50 (2H, t, *J* = 6.8 Hz), 3.16 (2H, m), 3.14 (1H, m), 2.90 (1H, dd, *J* = 12.8, 4.9 Hz), 2.73 (1H, d, *J* = 12.8 Hz), 2.20 (2H, t, *J* = 7.5 Hz), 1.77-1.42 (10H, m), 1.26 (12H, m); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 173.0, 163.8, 157.8, 145.6, 129.7, 123.6, 121.7, 114.5, 70.8, 66.3, 64.2, 61.8, 60.2, 55.5, 49.8, 40.6, 39.6, 36.1, 29.6, 29.4, 29.4, 29.4, 29.3, 28.2, 28.1, 26.9, 26.1, 25.7; LRMS (EI) m/z 600 (M^+); HRMS (FAB) Calcd. for $C_{31}H_{48}N_6O_4S$ 600.3458, Found 600.3466.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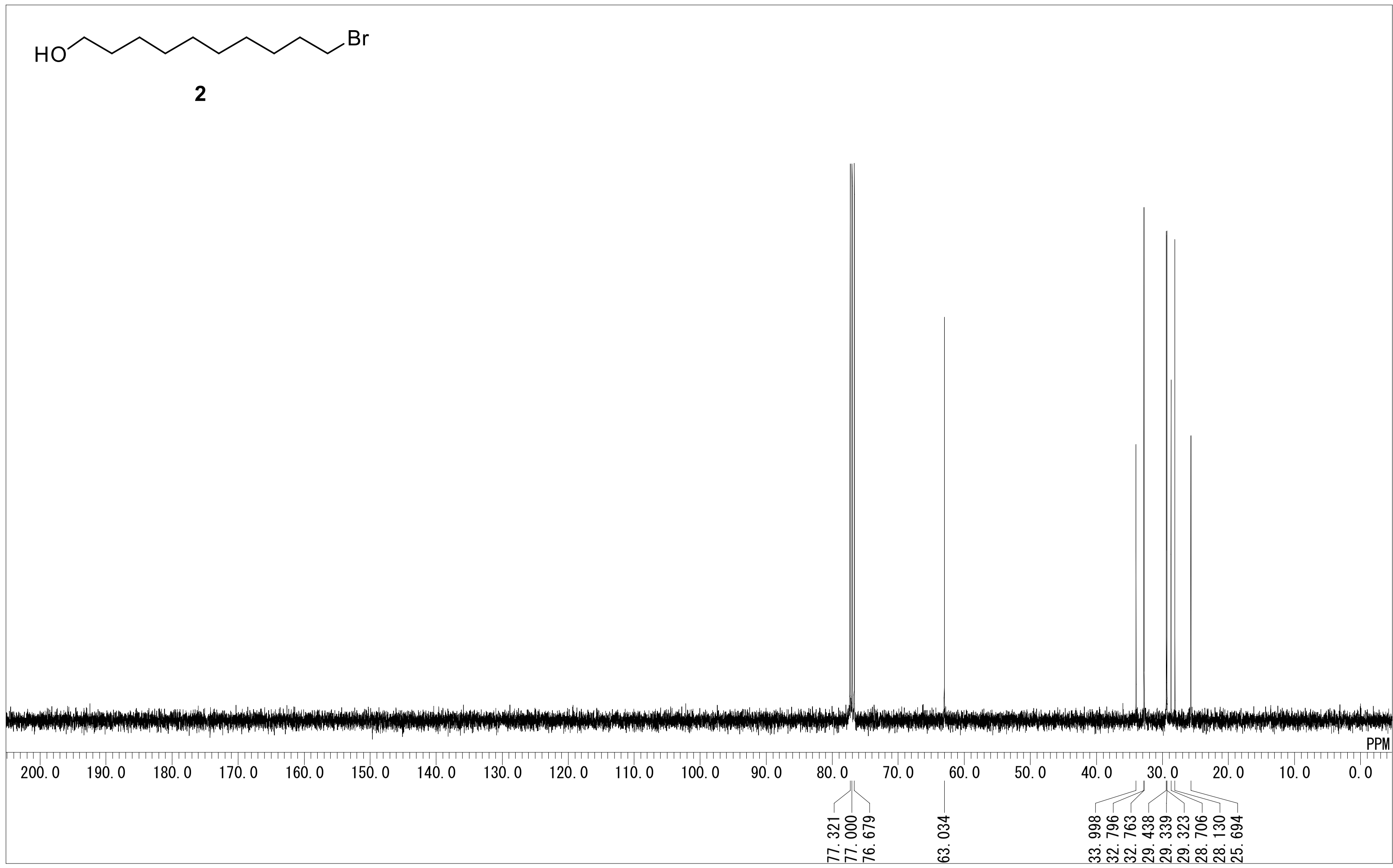

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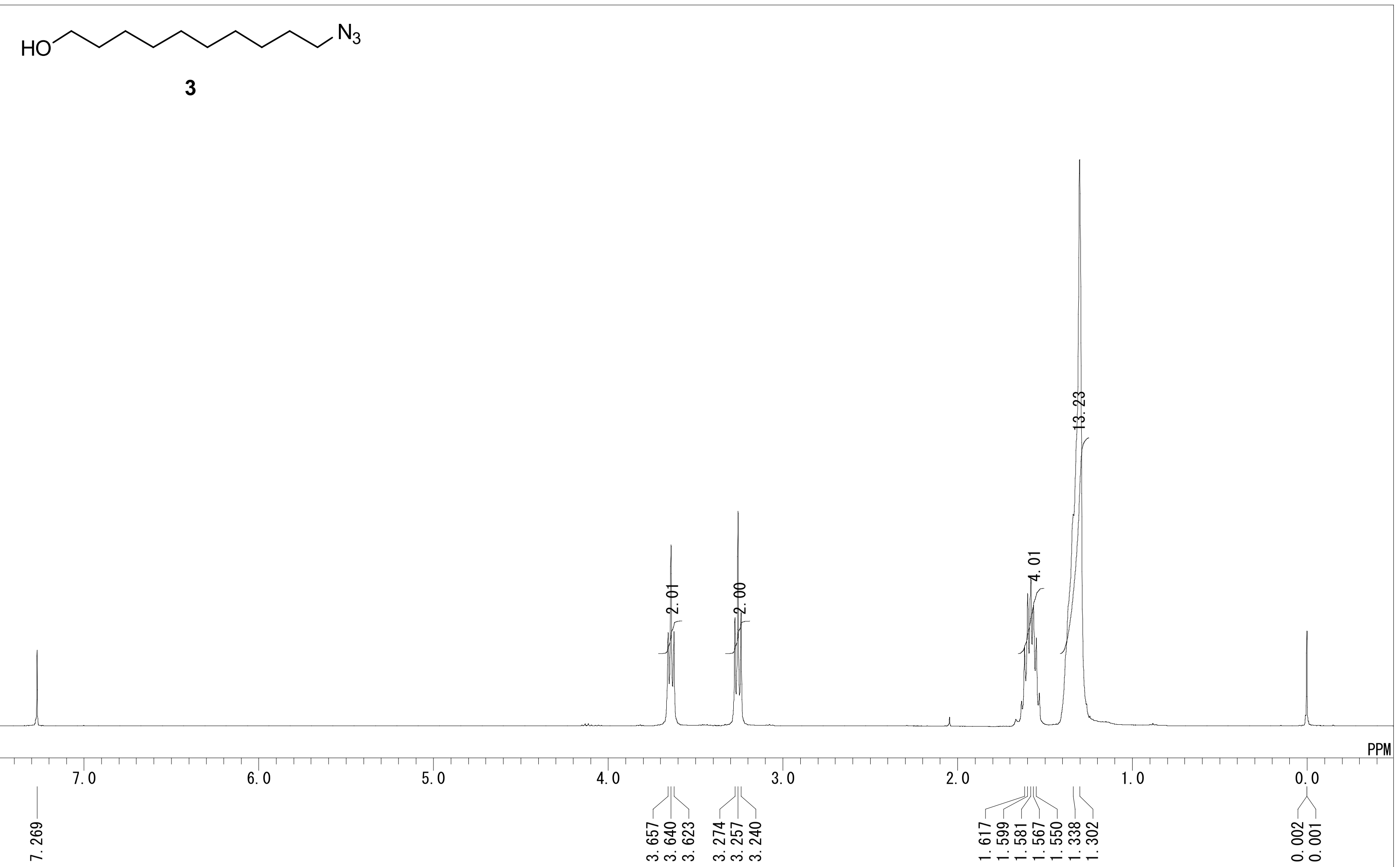

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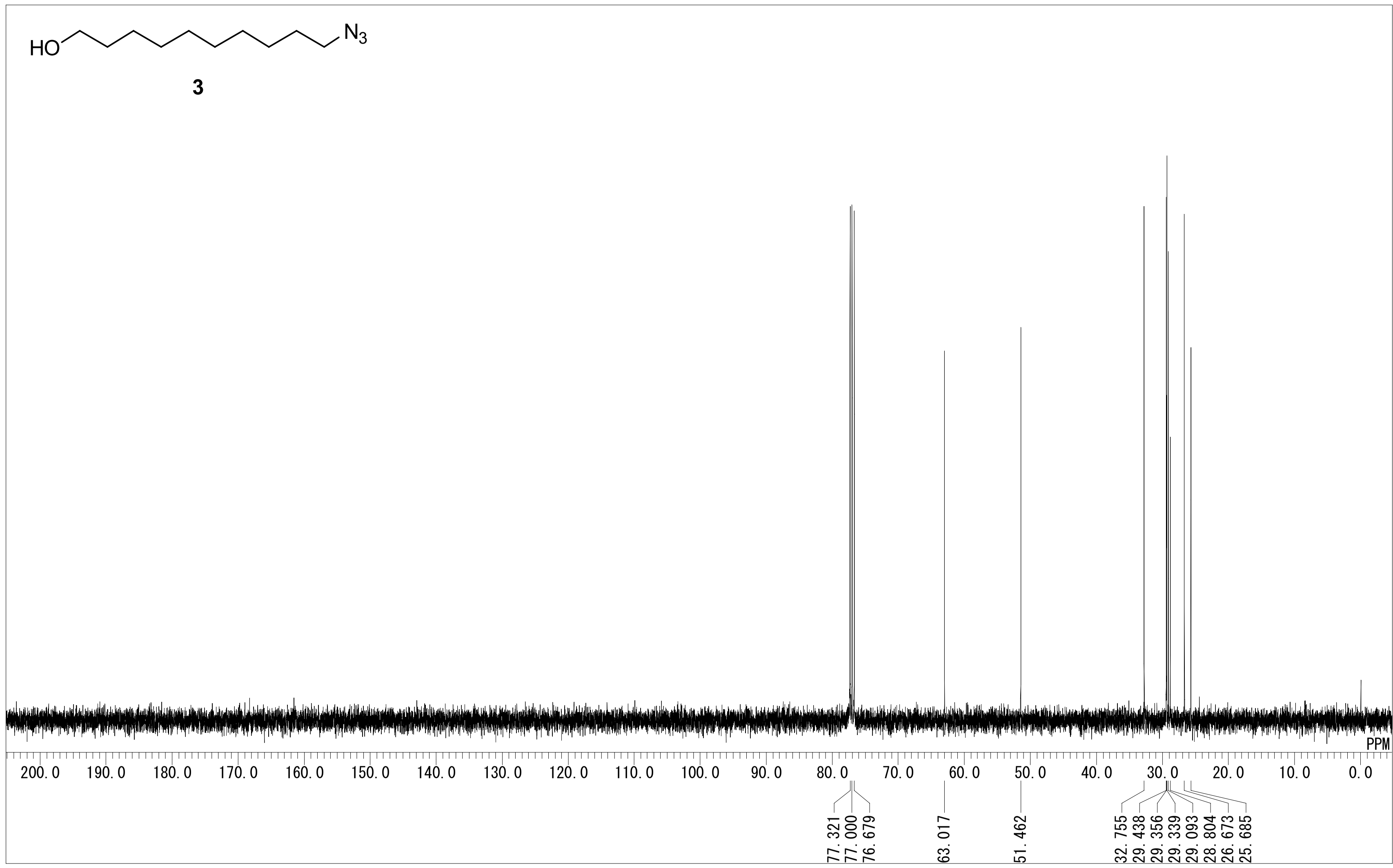
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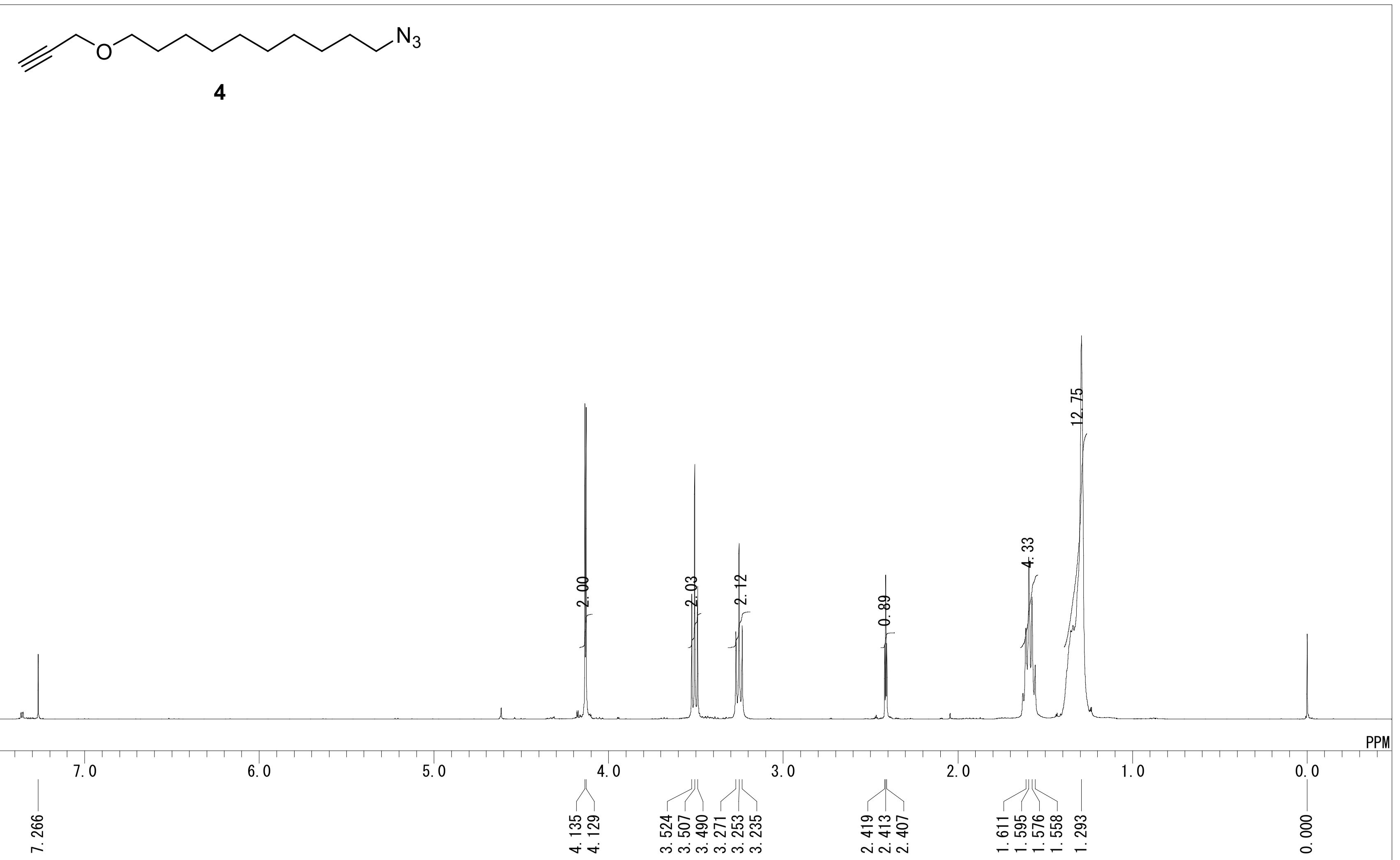

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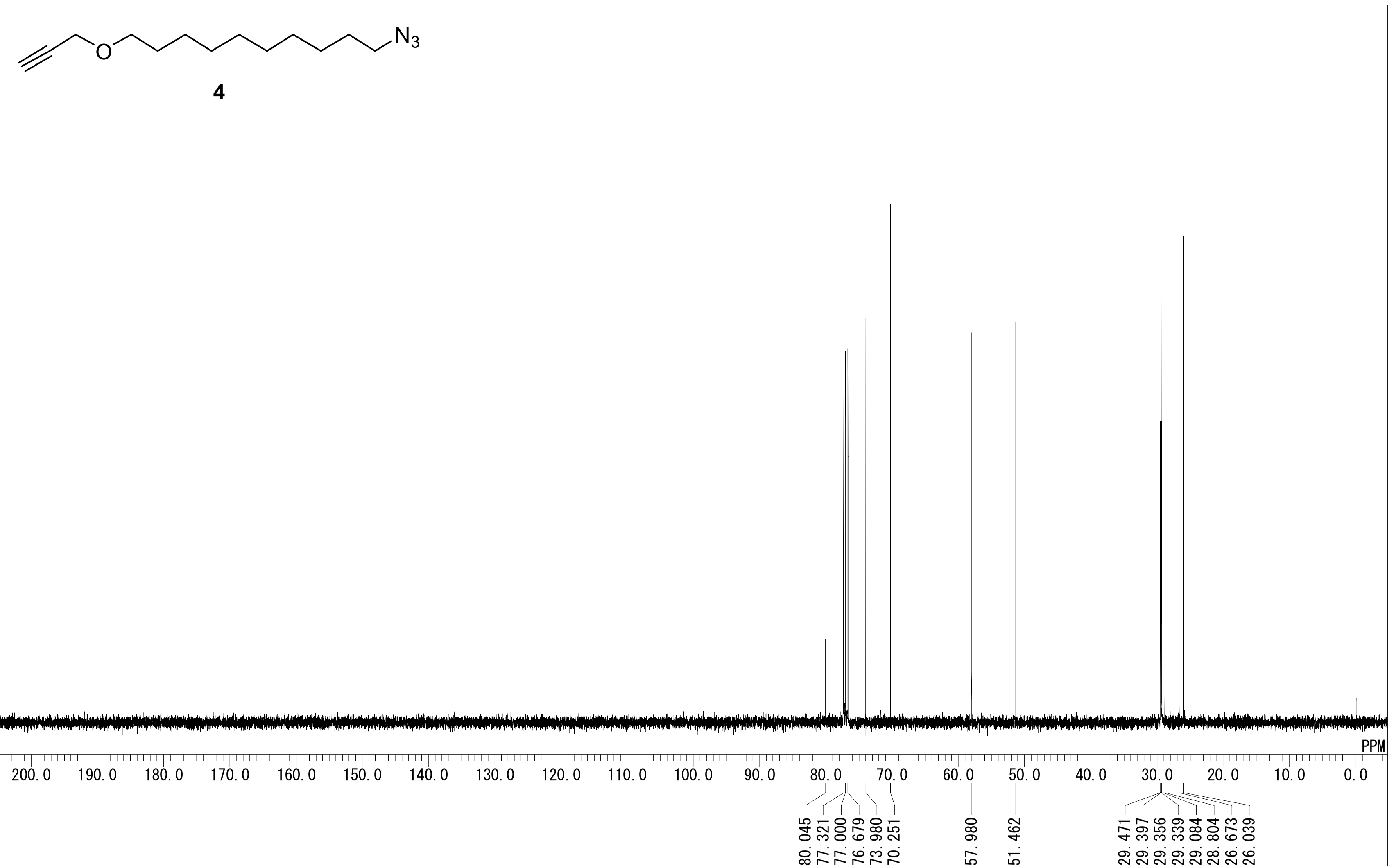

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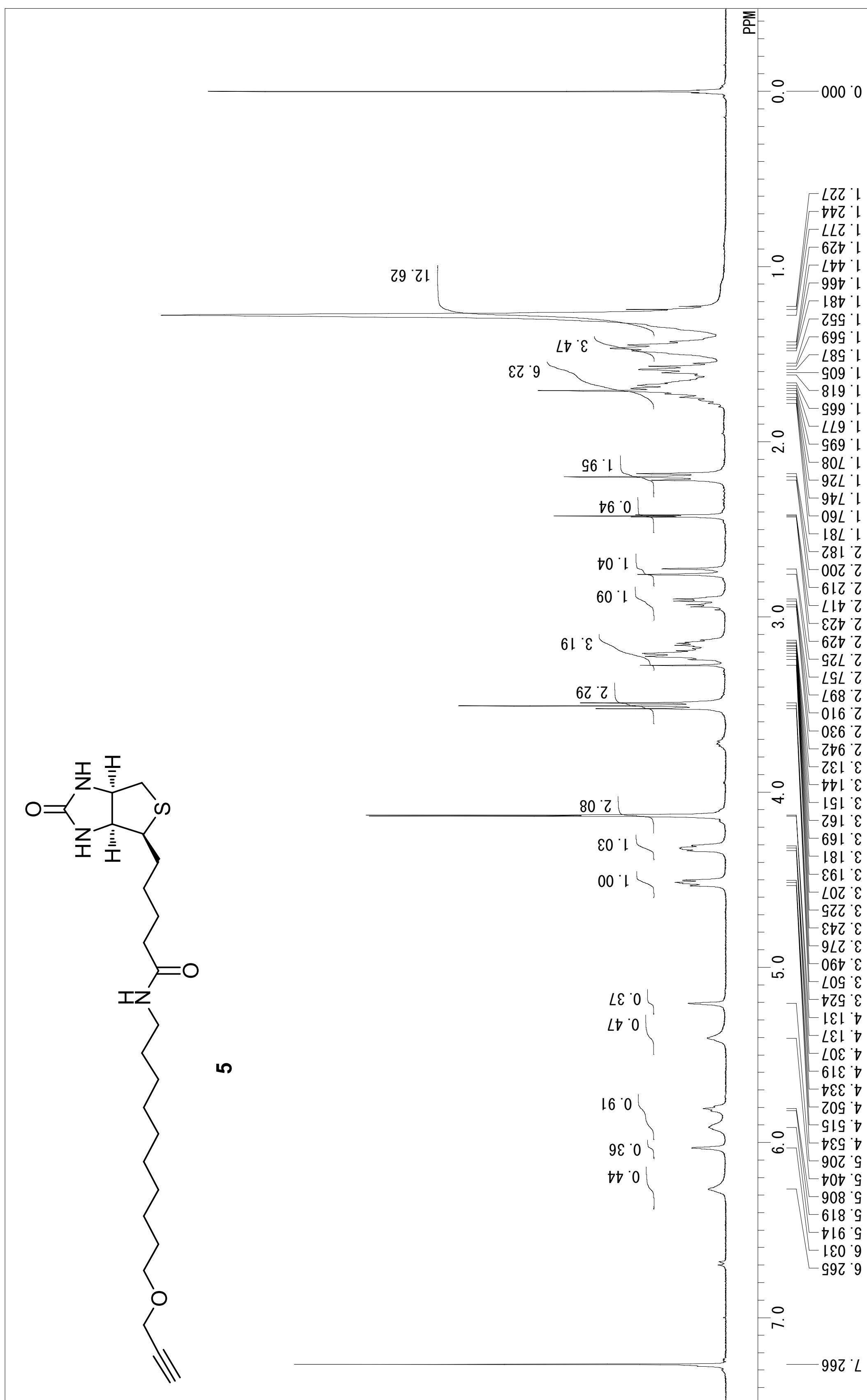

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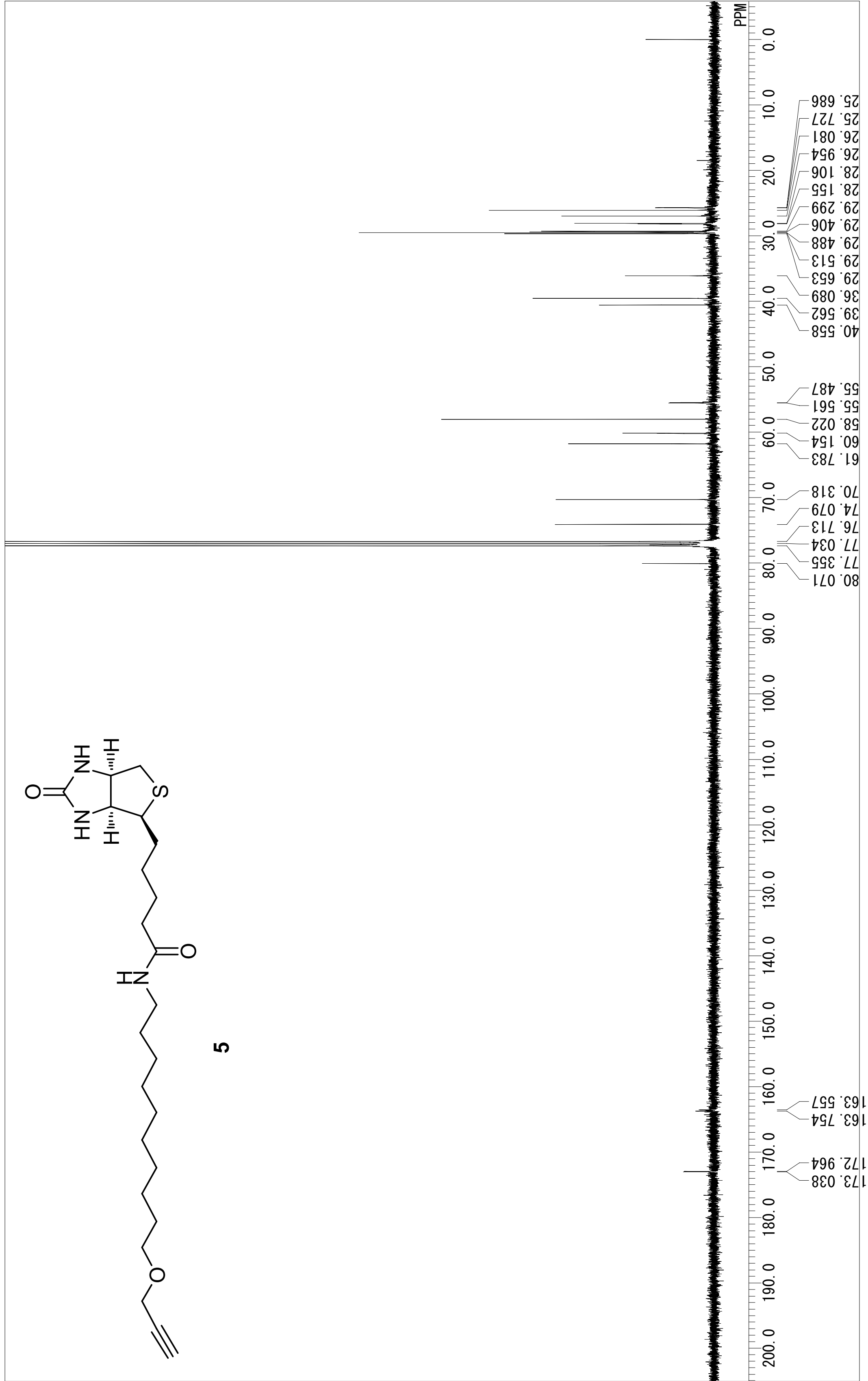
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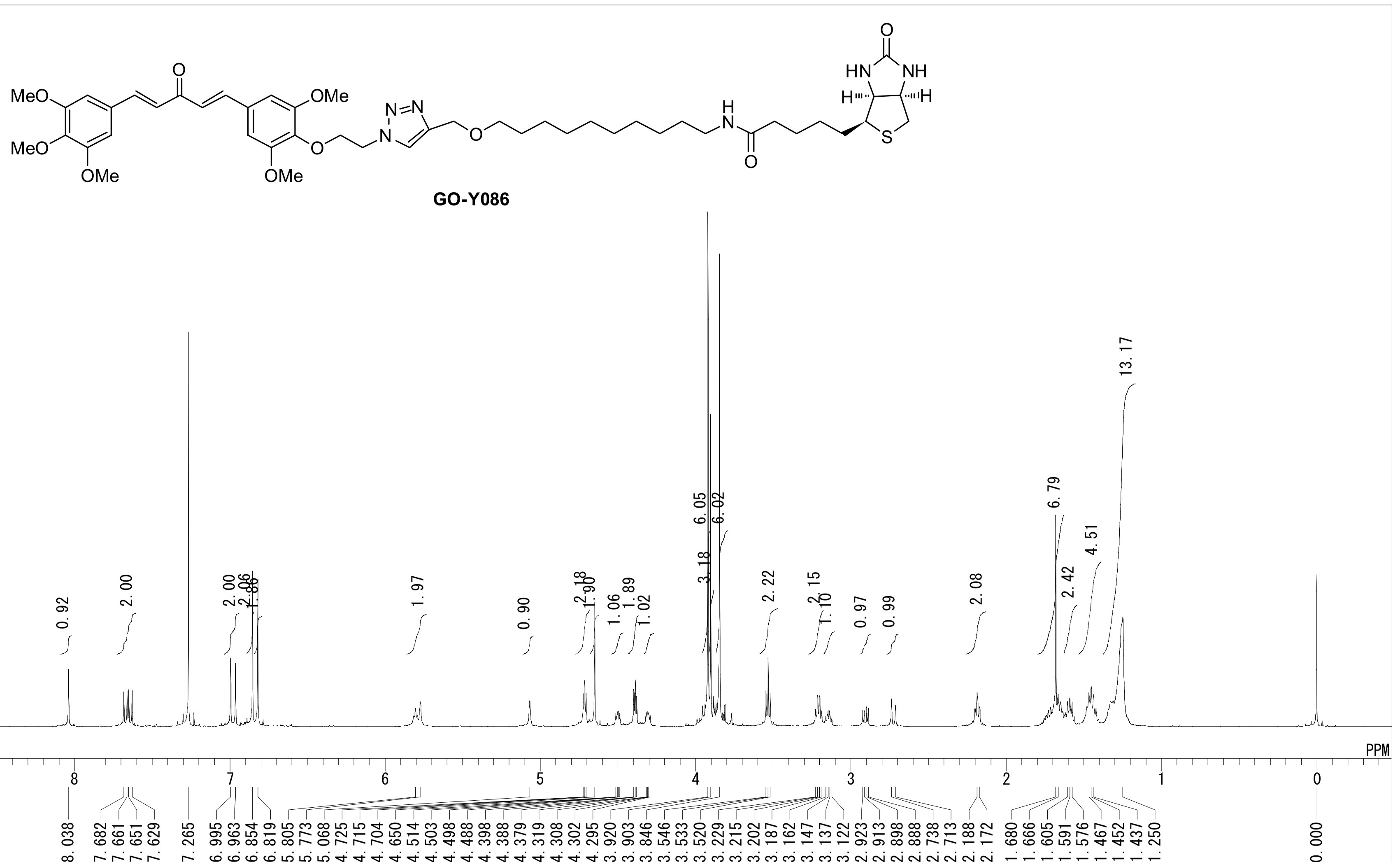
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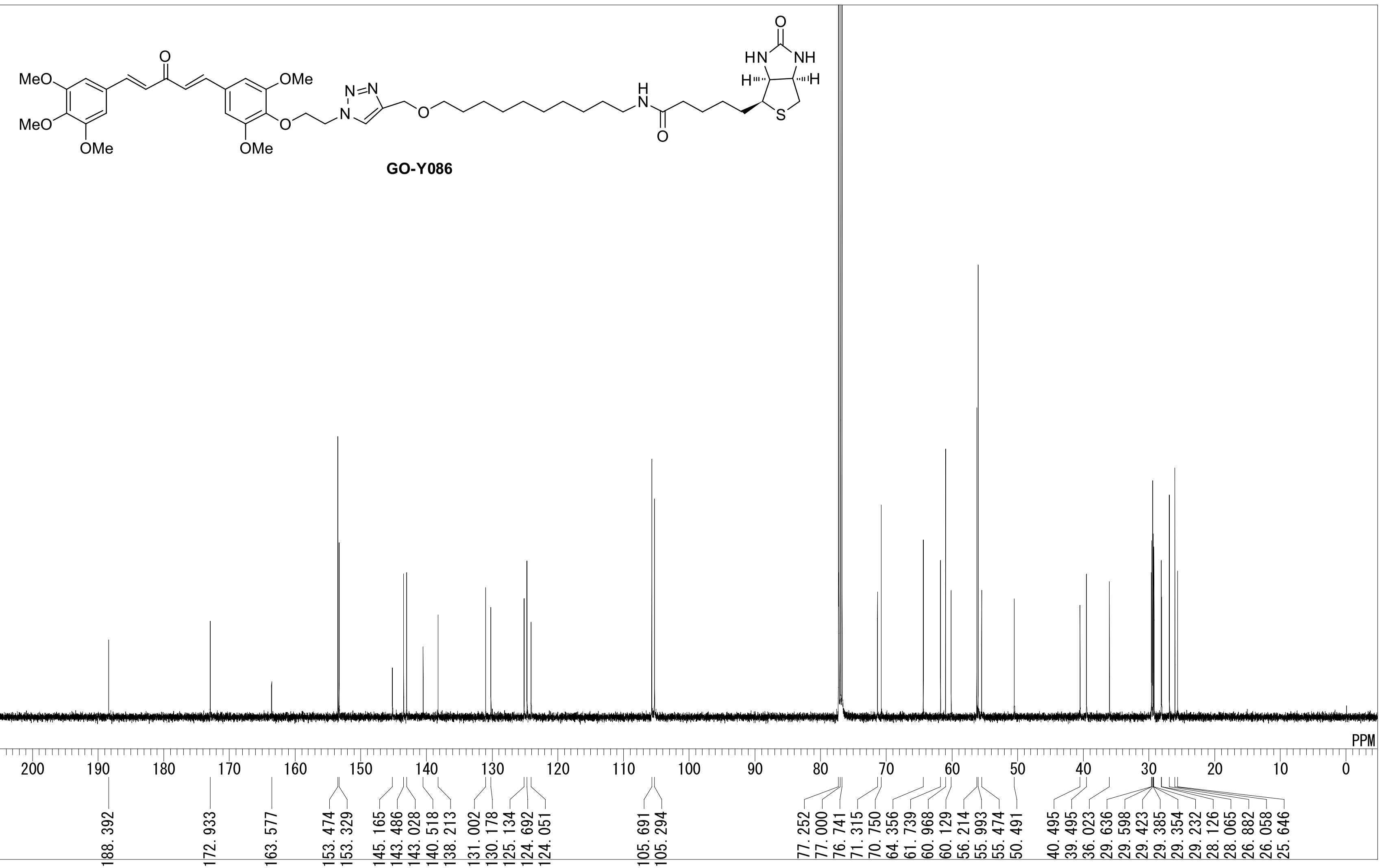
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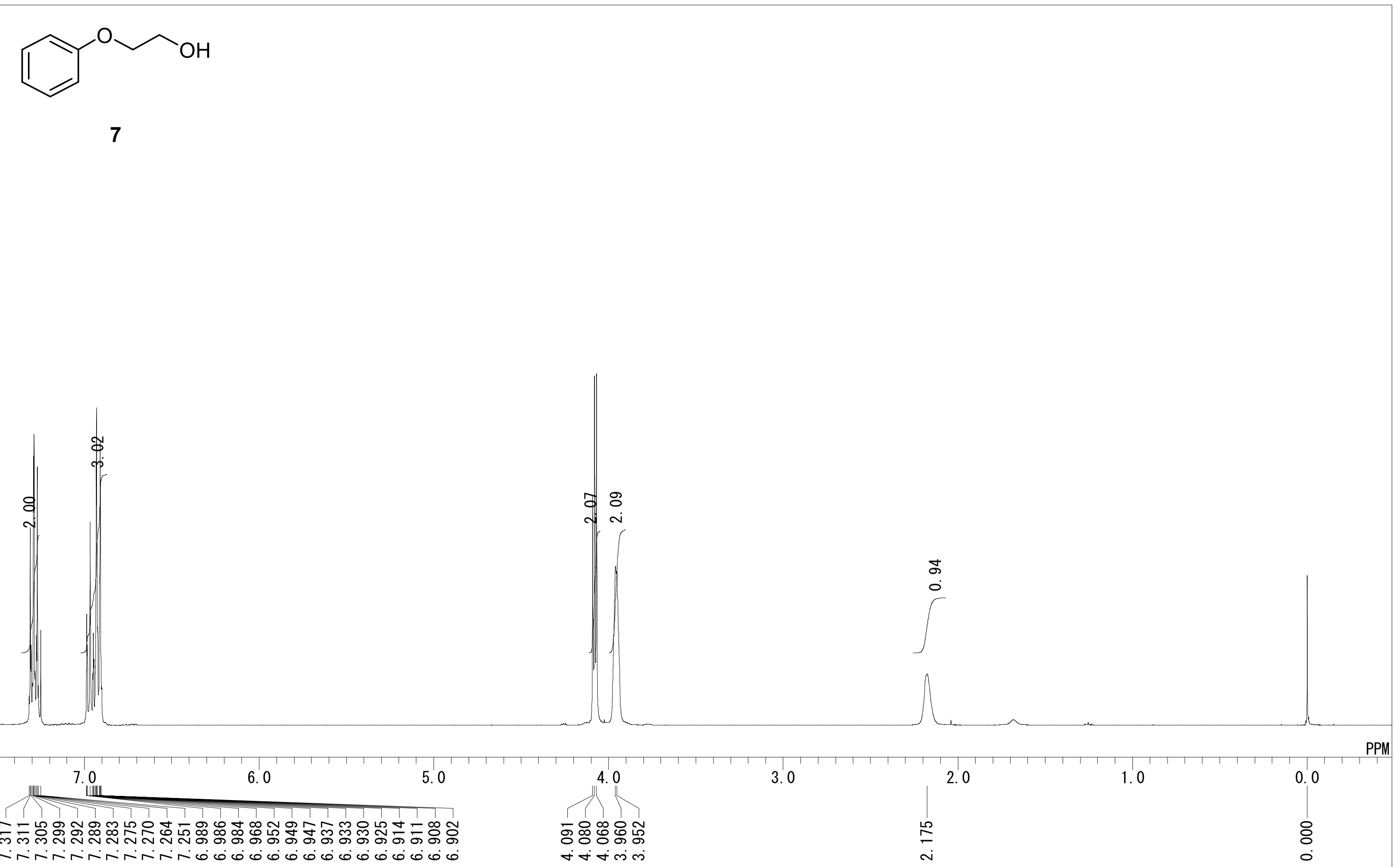

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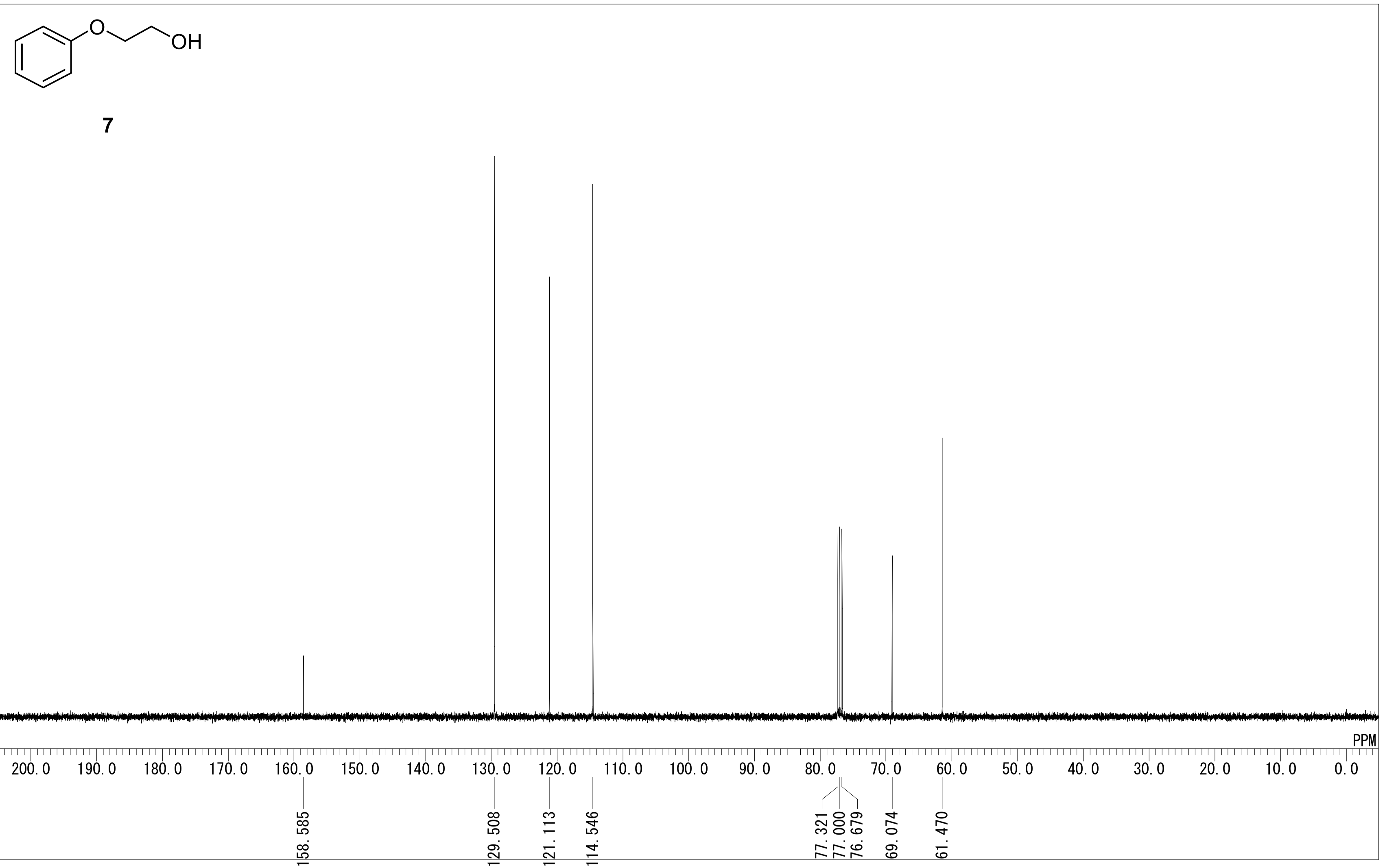
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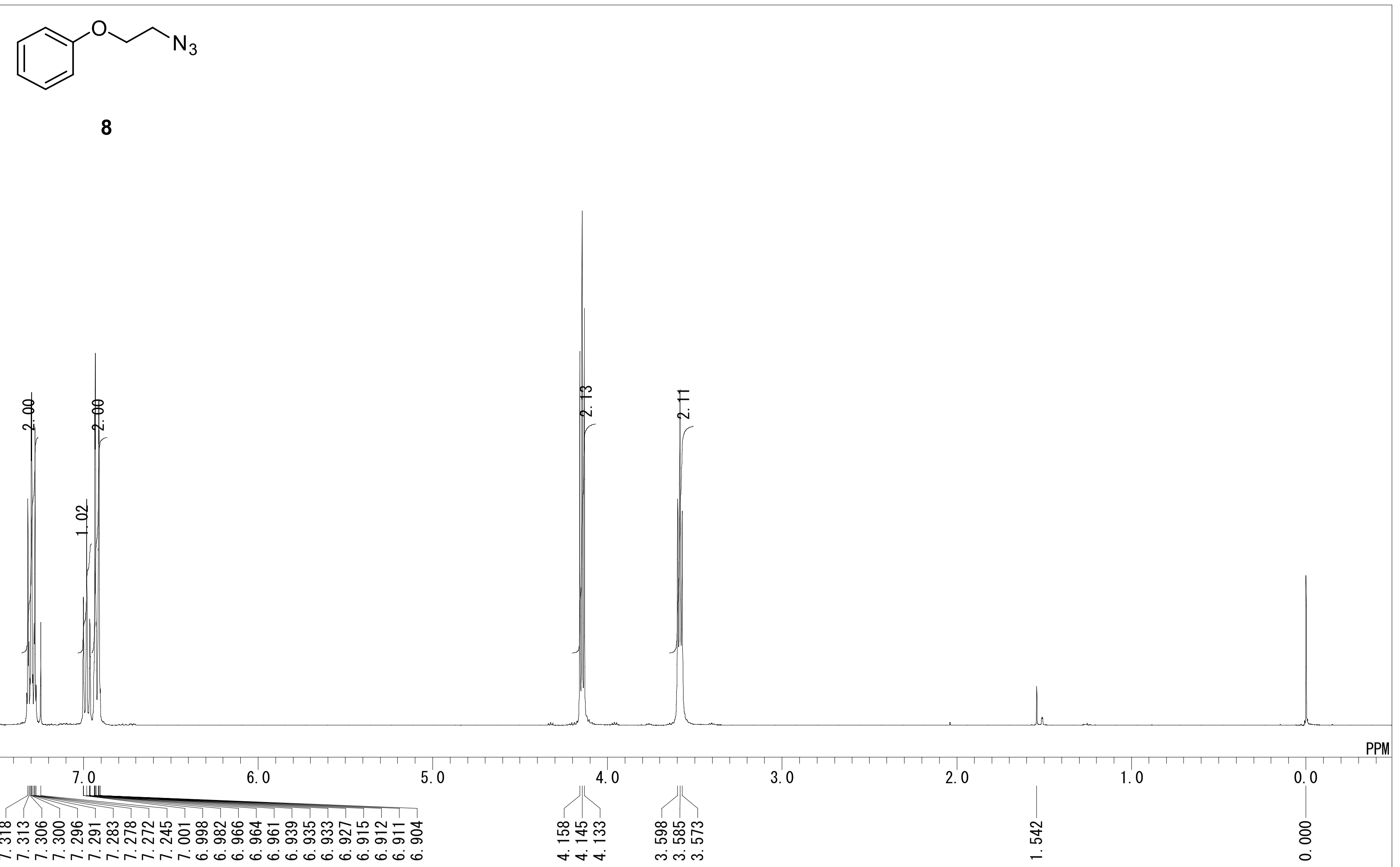
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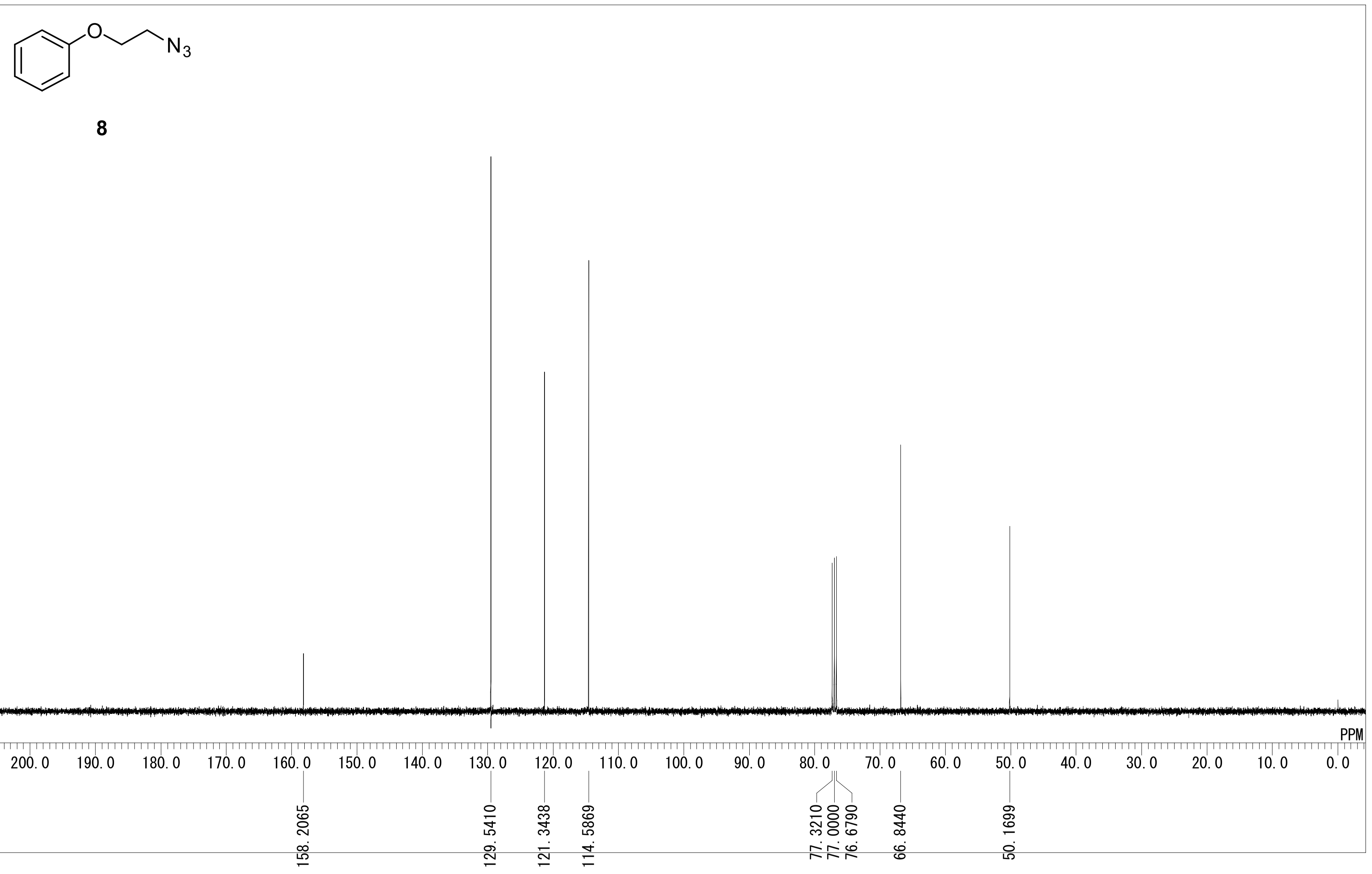

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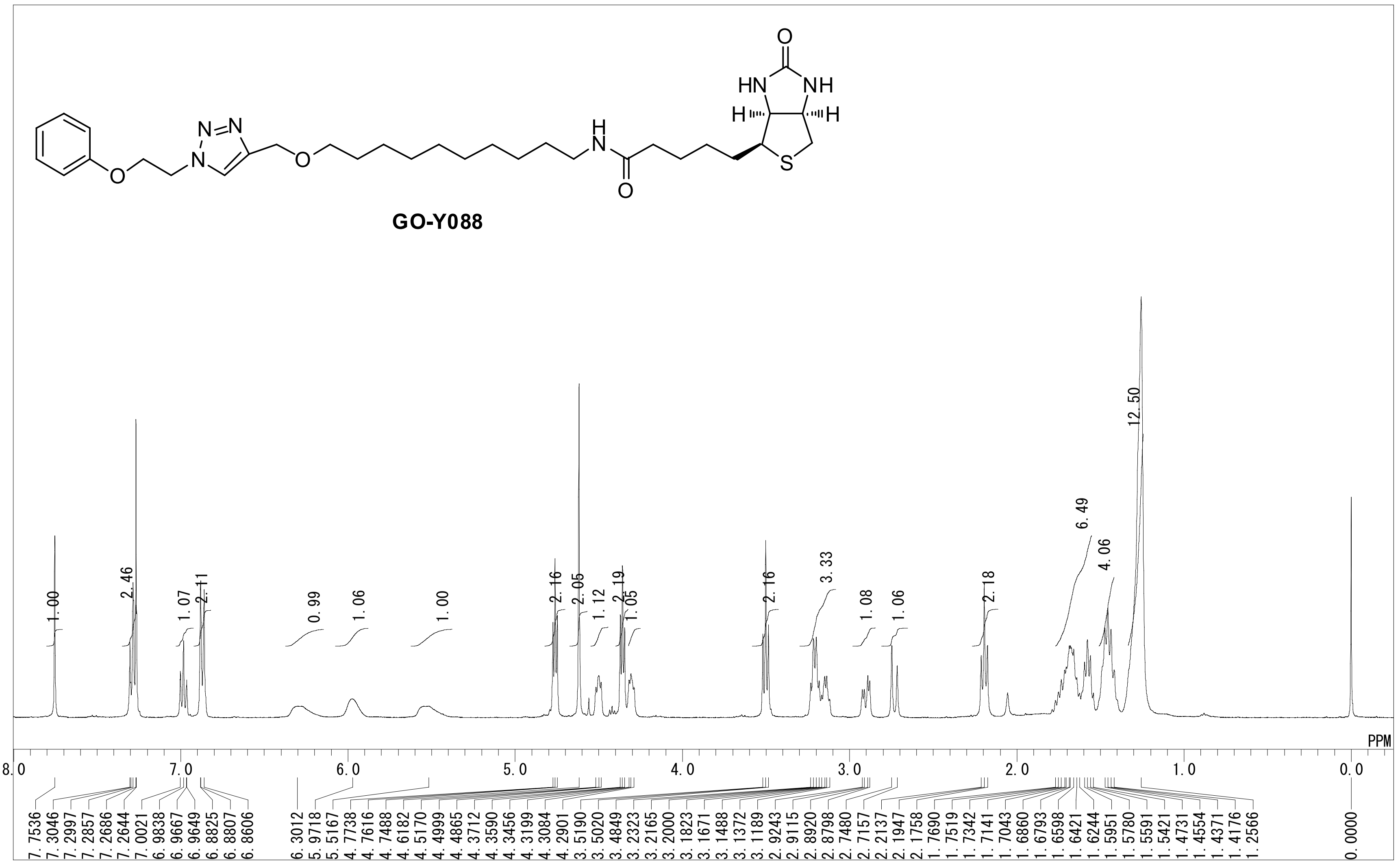
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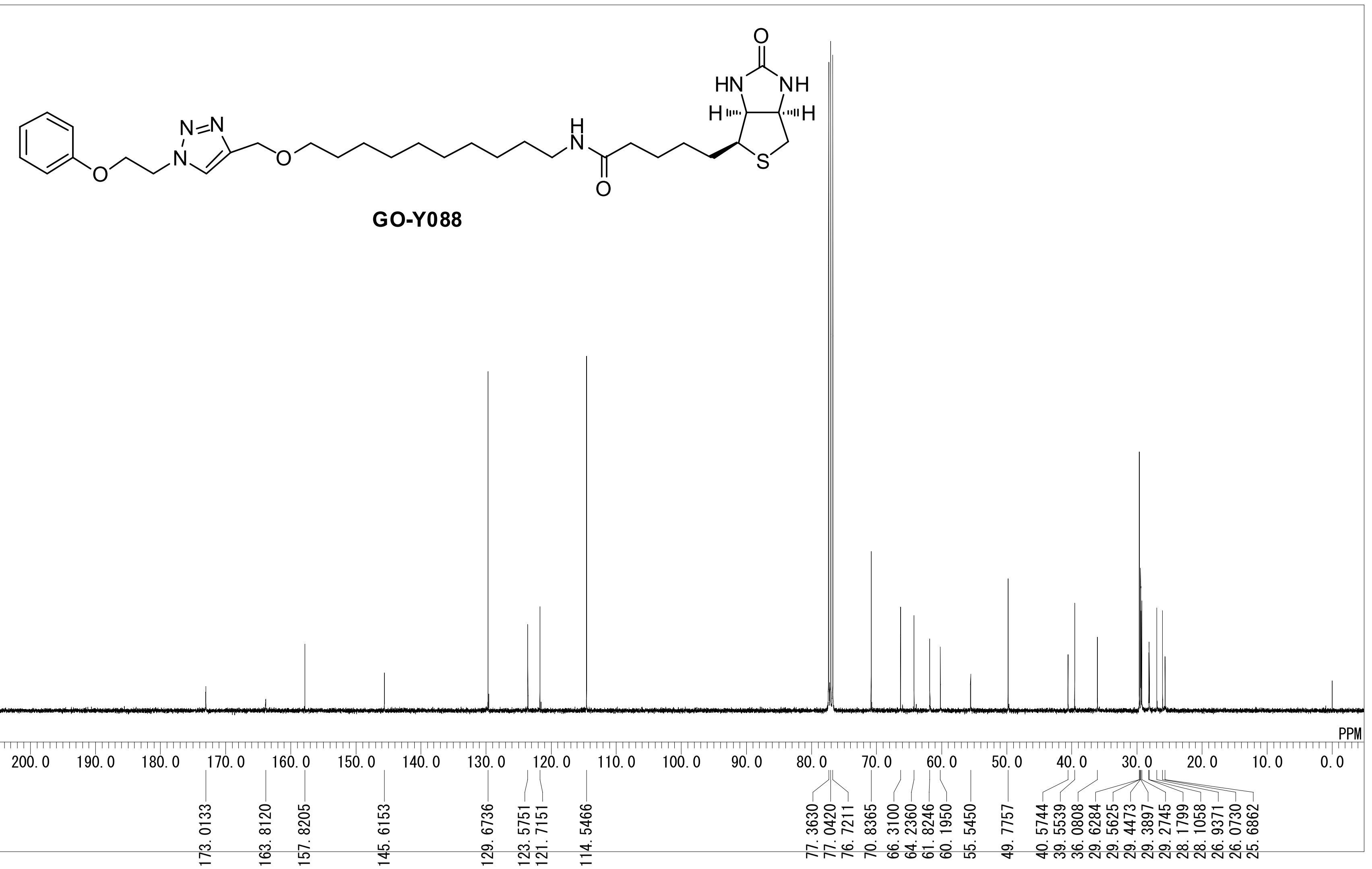
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