**Supporting Information**

# Structure-Activity Relationship Study of Leucyl-3-*epi*-Deoxynegamycin for Potent Premature Termination Codon Readthrough

Akihiro Taguchi,[†] Keisuke Hamada,[†] Masataka Shiozuka,[‡] Misaki Kobayashi,[†] Saori Murakami,[†] Kentaro Takayama,[†] Atsuhiko Taniguchi,[†] Takeo Usui,[§] Ryoichi Matsuda,[‡] and Yoshio Hayashi*[,†]

[†]Department of Medicinal Chemistry, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan
[‡]Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, 3-8-1 Komaba, Tokyo 153-8902, Japan
[§]Faculty of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba 305-8572, Japan

*Corresponding author; Yoshio Hayashi, E-mail; yhayashi@toyaku.ac.jp. Phone: +81 -42-676-3275. Fax: +81-676-4475.

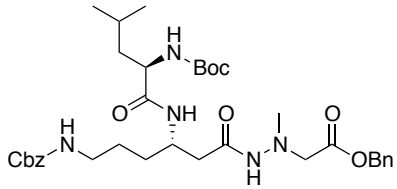
## 1. General information

All reaction mixtures were stirred magnetically. $^{1}H$ NMR spectra were measured in $CDCl_3$ and $D_2O$ solutions, and referenced to TMS (0.00 ppm), $D_2O$ (4.79 ppm) and 3-(trimethylsilyl)propionic-2, 2, 3, 3-$d_4$ acid, sodium salt (TSP-$d_4$, 0.00 ppm) using Bruker AVANCE-III (400 MHz), Bruker DPX-400 NMR Spectrometer (400 MHz). $^{13}C$ NMR spectra were measured in $CDCl_3$ and $D_2O$ solutions, and referenced to $CDCl_3$ (77.05 ppm) and TSP-$d_4$ (0.00 ppm) using Bruker AVANCE-III (400 MHz) and Bruker DPX-400 NMR (400 MHz) spectrophotometers. When peak multiplicities are reported, the following abbreviations are used: s, singlet; d, doublet; t, triplet; m, multiplet; br s, broad singlet; br d, broad doublet. Melting points were measured with Yanaco MP–500D melting point apparatuses. Mass spectra were obtained on Waters MICRO MASS LCT-premier. Optical rotations were measured with a JASCO Polarimeter P-1030 at the sodium-D line (589 nm) at the concentrations (*c*, g 100 $mL^{-1}$). The measurements were carried out between 24-25 ºC in a cell with path length (*l*) of 1 dm. Specific rotations $[\alpha]_D$ are given in $10^{-1}$ deg $cm^2$ $g^{-1}$. Column chromatography was performed on silicagel 60N (spherical, neutral) (4-50 μm or 63-210 μm), thin layer chromatography (TLC) was performed on precoated plates (0.25 mm, silica gel Merk Kieselgel $60F_{254}$), and compounds were visualized with UV light, phosphomolybdic acid stain, and ninhydrin stain. Preparative HPLC for **10a-f** and **13b** was performed using a C18 reversed-phase column (250 x 20 mm; YMC-Pack ODS-AM) with a binary solvent system. Preparative HPLC for **13a**,**c-e** was performed using a C18 reversed-phase column (19 x 150 mm; SunFire™ Prep C18 OBD™ 5 μm) with a binary solvent system. Analytical HPLC was performed using a C18 reversed-phase column (COSMOSIL Packed Column, Protein–R, 4.6ID x 150 mm) with a binary solvent system. Solvents and reagents were purchased from Kanto Chemical Co., Inc., Tokyo Chemical Industry Co., Ltd., Kokusan Chemical Co., Ltd., Wako Pure Chemical Industries, Ltd., and Watanabe Chemical Industries, Ltd..

## 2. Synthesis of derivatives 9a-f

The derivatives **9** and **10** were synthesized by previously reported synthetic procedure of Leucyl-3-*epi*-deoxynegamycin (**2**)[1].

### (7*S*,10*R*)-Benzyl 7-[3-(benzylcarbonylamino)propyl]-10-isobutyl-3,14,14-trimethyl-5,9,12-trioxo-13-oxa-3,4,8,11-tetraazapentadecan-1-oate (9a)

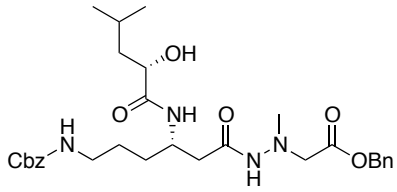



4 M HCl/dioxane (2 mL) was added to **8** (79.7 mg, 0.143 mmol) at 0 °C. After stirring for 1 h at room temperature, the mixture was removed under reduced pressure. The residue was used in the next step without further purification.

Boc-D-Leu-OH·$H_2O$ (71.3 mg, 0.286 mmol), and HOBt·$H_2O$ (43.8 mg, 0.286 mmol) were added to a solution containing above residue in DMF (2 mL) at room temperature. $Et_3N$ (39.6 μL, 0.286 mmol) and EDC·HCl (54.8 mg, 0.286 mmol) were added to the mixture at 0 °C. After stirring for overnight at room temperature, the mixture was poured into 10% citric acid aqueous solution and extracted with AcOEt. The extracts were washed with saturated aqueous $NaHCO_3$ solution, $H_2O$, brine, dried over $Na_2SO_4$, filtered and concentrated under reduced pressure. The residue was purified by silica gel flash column chromatography with $CHCl_3$ : MeOH = 60 : 1 to give **9a** (90.8 mg, 0.136 mmol, 2 steps 95%) was obtained as a white solid; $[\alpha]_D^{25}$ = –8.53 (*c* = 1.31, $CHCl_3$); m.p. 98.4–99.6 °C; $^1$H NMR (400 MHz, $CDCl_3$) δ 8.17 (s) and 7.09 (br s, total 1H), 7.45-7.22 (m, 10H), 7.18 (d, *J* = 8.7 Hz, 1H), 5.26-5.97 (m, 6H), 4.32-4.12 (m, 1H), 4.12-3.96 (m, 1H), 3.93-3.47 (m, 2H), 3.19 (br s, 2H), 2.98-2.47 (m, 4H), 2.39-2.18 (m, 1H), 1.80-1.50 (m, 7H), 1.42 (s, 9H), 1.00-0.86 (m, 6H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 174.6, 172.5, 170.7, 156.5 (2 carbons), 136.7, 135.0, 128.69 (2 carbons), 128.66 (2 carbons), 128.45 (2 carbons), 128.37 (2 carbons), 128.04, 127.98, 80.1, 66.8, 66.5, 57.8, 53.8, 46.4, 44.6, 41.2, 40.6, 39.1, 31.8, 28.3 (3 carbons), 26.4, 24.8 (2 carbons), 22.1; HRMS (ES+) calcd for $C_{35}H_{51}N_5O_8Na$ $[M+Na]^+$ 692.3635, found 692.3638.

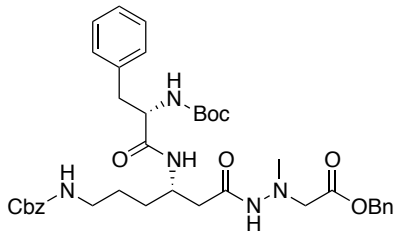
### Benzyl 2-{2-[(*S*)-6-(benzyloxycarbonylamino)-3-((*S*)-2-hydroxy-4-methylpentanamido)

**hexanoyl]-1-methylhydrazinyl}acetate (9b)**



**9b** was prepared in the same manner as described for compound **9a** using **8** (55.5 mg, 99.8 μmol) and (*S*)-2-hyrdoxy-4-pentanoic acid (26.4 mg, 0.200 mmol). **9b** (42.5 mg, 74.5 μmol, 2 steps 75%) was obtained as a white solid; $[\alpha]_D^{25}$ = –21.2 (*c* = 0.34, $CHCl_3$); m.p. 82.5–84.6 °C; $^1$H NMR (400 MHz, $CDCl_3$) δ 7.84 (s) and 7.42-7.27 (m, total 11H), 7.25-7.10 (m, 1H), 5.34-4.80 (m, 5H), 4.37-4.18 (m, 1H), 4.08 (d, *J* = 9.0 Hz, 1H), 3.84-3.46 (m, 2H), 3.30-3.14 (m, 2H), 2.85-2.59 (m, 4H), 2.42-2.18 (m, 1H), 1.98-1.70 (m, 1H), 1.69-1.40 (m, 6H), 1.25 (s, 1H), 0.95 (d, *J* = 6.5 Hz, 6H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 174.7, 170.8, 169.1, 156.57, 156.53, 136.6, 135.1, 128.8 (4 carbons), 128.55 (2 carbons), 128.53 (2 carbons), 128.1 (2 carbons), 70.7, 66.8, 66.7, 57.7, 46.3, 45.7, 44.2, 40.6, 31.7, 26.6, 24.6, 23.5, 21.4; HRMS (ES+) calcd for $C_{30}H_{42}N_4O_7Na$ $[M+Na]^+$ 593.2951, found 593.2949.

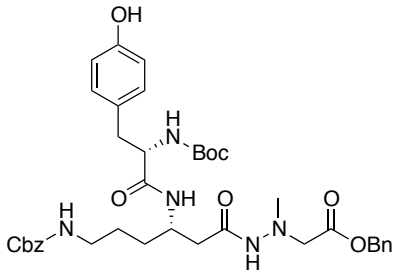
**(7*S*,10*R*)-Benzyl 10-benzyl-7-[3-(benzylcarbonylamino)propyl]-3,14,14-trimethyl-5,9,12-trioxo-13-oxa-3,4,8,11-tetraazapentadecan-1-oate (9c)**



**9c** was prepared in the same manner as described for compound **9a** using **7** (82.9 mg, 0.149 mmol) and Boc-Phe-OH (79.1mg, 0.298 mmol). **9c** (92.5 mg, 0.132 mmol, 2 steps 88%) was obtained as a white solid; $[\alpha]_D^{25}$ = –9.40 (*c* = 1.17, $CHCl_3$); m.p. 126.7–128.1 °C; $^1$H NMR (400 MHz, $CDCl_3$) δ 7.69 (s) and 7.42-7.16 (m, total 16H), 6.96 (br d, 1H), 5.25-5.00 (m, 6H), 4.40-4.24 (m, 1H), 4.23-3.99 (m, 1H), 3.78-3.46 (m, 2H), 3.26-3.10 (m, 2H), 3.02 (d, *J* = 6.1 Hz, 2H), 2.89-2.63 (m) and 2.40-2.21 (m, total 4H), 2.18-1.98 (m, 1H), 1.64-1.42 (m, 4H), 1.38 (s, 9H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 174.4, 170.6, 168.9, 156.51, 156.48, 155.4, 136.8 (2 carbons), 129.37 (2 carbons), 128.69 (2 carbons), 128.61, 128.57, 128.51 (2 carbons), 128.46 (2 carbons), 128.42 (2

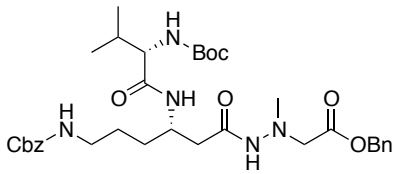
carbons), 128.04, 128.01, 126.8, 80.0, 66.7, 66.5, 57.6, 56.0, 46.4, 45.4, 44.1, 40.6, 38.5, 31.4, 28.2 (3 carbons), 26.4; HRMS (ES+) calcd for $C_{38}H_{50}N_5O_8$ $[M+H]^+$ 704.3659, found 704.3652.

**(7*S*,10*R*)-Benzyl 7-[3-(benzylcarbonylamino)propyl]-10-(4-hydroxybenzyl)-3,14,14-trimethyl-5,9,12-trioxo-13-oxa-3,4,8,11-tetraazapentadecan-1-oate (9d)**



**9d** was prepared in the same manner as described for compound **9a** using **7** (61.2 mg, 0.110 mmol) and Boc-Tyr-OH (56.1mg, 0.258 mmol). **9d** (39.3 mg, 54.6 μmol, 2 steps 50%) was obtained as a colorless solid; $[\alpha]_D^{25}$ = –11.1 (*c* = 0.49, $CHCl_3$); m.p. 130.9–132.2 °C; $^1$H NMR (400 MHz, $CDCl_3$) δ 7.74 (s) and 7.39-7.28 (m, total 11H), 6.99 (t, *J* = 7.3 Hz, 2H), 6.96-6.75 (m, 1H), 6.74-6.64 (m, 2H), 5.26-5.01 (m, 6H), 4.37-3.98 (m, 2H), 3.78-3.43 (m, 2H), 3.10-3.09 (m, 2H), 3.08-2.97 (m, 1H), 2.92-2.77 (m, 1H), 2.76-2.49 (m) and 2.36-2.28 (m, total 4H), 2.18-2.00 (m, 1H), 1.84 (br s, 1H), 1.60-1.36 (m, 13H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 174.5, 170.7, 169.2, 156.7, 155.4, 136.7, 135.2, 130.5, 128.76 (2 carbons), 128.74 (2 carbons), 128.53 (4 carbons), 128.47 (2 carbons), 128.1 (2 carbons), 115.8 (2 carbons), 80.2, 66.8, 66.7, 59.0, 57.7, 46.5, 44.1, 40.7, 38.5, 37.8, 35.6, 30.9, 28.3 (3 carbons), 26.5; HRMS (ES+) calcd for $C_{38}H_{50}N_5O_9$ $[M+H]^+$ 720.3609, found 720.3595.

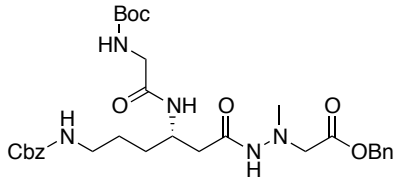
**(7*S*,10*R*)-Benzyl 7-[3-(benzylcarbonylamino)propyl]-10-isopropyl-3,14,14-trimethyl-5,9,12-trioxo-13-oxa-3,4,8,11-tetraazapentadecan-1-oate (9e)**



**9e** was prepared in the same manner as described for compound **9a** using **7** (71.7 mg, 0.129 mmol) and Boc-Val-OH (56.1 mg, 0.258 mmol). **9e** (77.8 mg, 0.119 mmol, 2

steps 92%) was obtained as a white solid; $[\alpha]_D^{25}$ = –9.25 (*c* = 0.58, $CHCl_3$); m.p. 167.1–168.3 °C; $^1$H NMR (400 MHz, $CDCl_3$) δ 7.81 (s) and 7.42-7.27 (m, total 11H), 7.04-6.93 (m, 1H), 5.28-4.88 (m, 6H), 4.39-4.10 (m, 1H), 3.87 (br s, 1H), 3.79-3.45 (m, 2H), 3.19 (br s, 2H), 2.98-2.42 (m, 4H), 2.38-2.18 (m, 1H), 2.17-2.00 (m, 1H), 1.72-1.50 (m, 4H), 1.42 (s, 9H), 1.00-0.83 (m, 6H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 174.6, 170.6, 169.1, 156.5, 155.9, 136.7, 135.1, 128.7 (4 carbons), 128.5 (2 carbons), 128.4 (2 carbons), 128.0 (2 carbons), 79.8, 66.7, 66.6, 60.2, 59.0, 46.5, 44.1, 40.7, 38.5, 31.1, 30.9, 28.3 (3 carbons), 26.5, 19.36, 19.31; HRMS (ES+) calcd for $C_{34}H_{49}N_5O_8Na$ $[M+Na]^+$ 678.3479, found 678.3481.

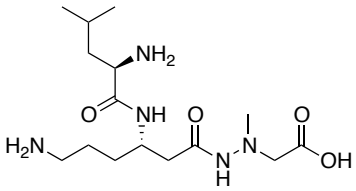
**(*S*)-Benzyl 7-[3-(benzyloxycarbonylamino)propyl]-3,14,14-trimethyl-5,9,12-trioxo-13-oxa-3,4,8,11-tetraazapentadecan-1-oate (9f)**




**9f** was prepared in the same manner as described for compound **9a** using **7** (52. 0 mg, 93.5 μmol) and Boc-Gly-OH (32.8 mg, 0.187 mmol). **9f** (51.7 mg, 84.3 μmol, 2 steps 90%) was obtained as a colorless solid; $[\alpha]_D^{25}$ = –6.21 (*c* = 0.67, $CHCl_3$); m.p. 65.3–66.6 °C; $^1$H NMR (400 MHz, $CDCl_3$) δ 7.90 (s) and 7.42-7.28 (m, total 11H), 7.17-7.04 (m, 1H), 5.38-5.22 (m, 1H), 5.21-5.00 (m, 5H), 4.35-4.12 (m, 1H), 3.82-3.36 (m, 4H), 3.27-3.12 (m, 2H), 2.98-2.47 (m, 4H), 2.40-2.17 (m, 1H), 1.60-1.49 (m, 4H), 1.43 (s, 9H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 174.7, 170.6, 169.0, 156.57, 156.53, 136.7, 135.1, 128.73 (2 carbons), 128.72 (2 carbons), 128.5 (4 carbons), 128.05 (2 carbons), 80.1, 66.8, 66.6, 57.7, 46.6, 45.8, 44.2, 40.6, 38.8, 31.7, 28.3 (3 carbons), 26.6; HRMS (ES+) calcd for $C_{31}H_{44}N_5O_8$ $[M+H]^+$ 614.3190, found 614.3175.

**3. Synthesis of derivatives 10a-f**

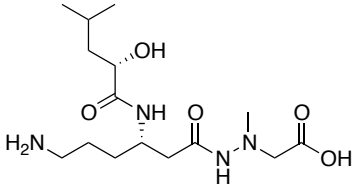
**2-{2-[(*S*)-6-Amino-3-((*R*)-2-amino-4-methylpentanamido)hexanoyl]-1-methylhydrazinyl}acetic acid·2TFA (10a)**



10 % Pd/C (4.3 mg) was added to a solution of **9a** (43.1 mg, 64.4 μmol) in MeOH (2 mL) at room temperature. The resulting reaction was subjected to three cycles of vacuum followed by flush with $H_2$ before stirring for 1 h under an atmosphere of $H_2$. The mixture was filtered through a pad of Celite® with MeOH and concentrated removed under reduced pressure.

4 M HCl/dioxane (2 mL) was added above residue at 0 ºC. After stirring for 1 h at room temperature, the solvent was removed under reduced pressure. The residue was purified by preparative HPLC (gradient: $H_2O$ (TFA 0.1%) : $CH_3CN$ (TFA 0.1%) = 90 : 10 to $H_2O$ (TFA 0.1%) : $CH_3CN$ (TFA 0.1%) = 85 : 15 over 40 min, Flow late 5 mL/min, UV: 222 nm) to give **10a** (17.2 mg, 30.0 μmol, 2 steps 47%) was obtained as a colorless solid; $[\alpha]_D^{25}$ = –23.2 (*c* = 0.73, $H_2O$); m.p. 178.6–179.6 °C; $^1$H NMR (400 MHz, $D_2O$ (N*H*$_2$, N*H*, O*H* and COO*H* (total 7H) were exchanged with $D_2O$)) δ 4.34-4.18 (m, 1H), 3.98 (t, *J* = 7.6 Hz, 1H), 3.67 (s, 2H), 3.08-2.99 (m, 2H), 2.73 (s, 3H), 2.52 (dd, *J* = 15 and 5.5 Hz, 1H), 2.41 (dd, *J* = 15 and 8.3 Hz, 1H), 1.87-1.57 (m, 7H), 1.08-0.92 (m, 6H); $^{13}$C NMR (100 MHz, $D_2O$) δ 173.1, 170.5, 170.0, 58.7, 52.0, 47.0, 44.3, 40.0, 39.0, 38.8, 30.5, 23.9, 23.6, 21.8, 20.9; HRMS (ES+) calcd for $C_{15}H_{32}N_5O_4$ $[M+H]^+$ 346.2454, found 346.2459.

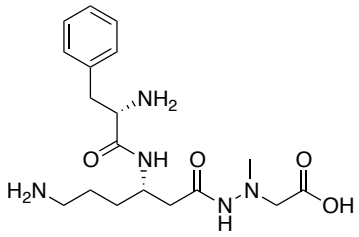
**2-{2-[(*S*)-6-Amino-3-((*S*)-2-hydroxy-4-methylpentanamido)hexanoyl]-1-methylhydrazinyl}acetic acid·TFA (10b)**



10 % Pd/C (3.4 mg) was added to a solution of **9b** (33.5 mg, 64.4 μmol) in MeOH (2 mL) at room temperature. The resulting reaction was subjected to three cycles of

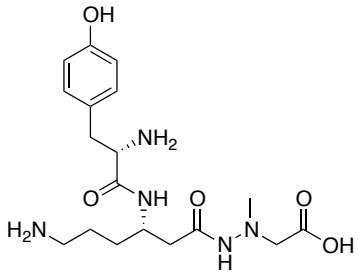
vacuum followed by flush with $H_2$ before stirring for 3 h under an atmosphere of $H_2$. The mixture was filtered through a pad of Celite® with MeOH and concentrated removed under reduced pressure. The residue was purified by preparative HPLC (gradient: $H_2O$ (TFA 0.1%) : $CH_3CN$ (TFA 0.1%) = 90 : 10 to $H_2O$ (TFA 0.1%) : $CH_3CN$ (TFA 0.1%) = 85 : 15 over 40 min, Flow late 5 mL/min, UV: 222 nm) to give **12b** (17.5 mg, 38.0 μmol, 65%) as a colorless solid; $[\alpha]_D^{25}$ = –25.8 (*c* = 0.74, $H_2O$); m.p. 112.9–113.7 °C; $^1$H NMR (400 MHz, $D_2O$ (N*H*$_2$, N*H*, O*H* and COO*H* (total 6H) were exchanged with $D_2O$)) δ 4.20 (br s) and 4.14 (t, *J* = 6.8 Hz, total 2H), 3.50 (s, 2H), 307-2.92 (m, 2H), 2.63 (s, 3H), 2.44 (dd, *J* = 14 and 5.2 Hz, 1H), 2.35 (dd, *J* = 14 and 8.6 Hz, 1H), 1.82-1.56 (m, 5H), 1.51 (t, *J* = 6.8 Hz, 2H), 0.97-0.87 (m, 6H); $^{13}$C NMR (100 MHz, $D_2O$) δ 180.1, 177.8, 173.6, 73.0, 62.3, 49.3, 46.9, 46.0, 42.2, 41.9, 33.7, 26.9, 26.5, 25.6, 25.4; HRMS (ES+) calcd for $C_{15}H_{31}N_4O_5$ $[M+H]^+$ 347.2294, found 347.2292.

**2-{2-[(*S*)-6-Amino-3-((*S*)-2-amino-3-phenylpropanamido)hexanoyl]-1-methylhydrazinyl}acetic acid·2TFA (10c)**



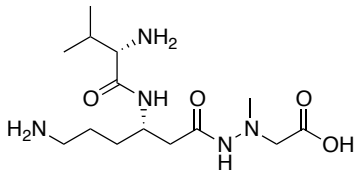

**10c** was prepared in the same manner as described for compound **10a** using **9c** (43.7 mg, 62.1 μmol). **10c** (11.2 mg, 18.4 μmol, 2 steps 30%) was obtained as a colorless solid; $[\alpha]_D^{25}$ = 17.5 (*c* = 0.43, $H_2O$); m.p. 149.3–150.8 °C; $^1$H NMR (400 MHz, $D_2O$ (N*H*$_2$, N*H* and COO*H* (total 7H) were exchanged with $D_2O$)) δ 7.47-7.37 (m, 3H), 7.32-7.25 (m, 2H), 4.24-4.09 (m, 2H), 3.57 (s, 2H), 3.23-3.09 (m, 2H), 2.98 (t, *J* = 7.0 Hz, 2H), 2.66 (s, 3H), 2.24-2.11 (m, 2H), 1.76-1.38 (m, 4H); $^{13}$C NMR (100 MHz, $D_2O$) δ 173.7, 170.2, 168.5, 133.7, 129.4 (2 carbons), 129.2 (2 carbons), 128.1, 58.8, 54.4, 46.6, 44.1, 38.9, 38.5, 37.1, 30.2, 23.3; HRMS (ES+) calcd for $C_{18}H_{30}N_5O_4$ $[M+H]^+$ 380.2298, found 380.2290.

**2-{2-[(*S*)-6-Amino-3-((*S*)-2-amino-3-(4-hydroxyphenyl)propanamido)hexanoyl]-1-methylhydrazinyl}acetic acid·2TFA (10d)**



**10d** was prepared in the same manner as described for compound **10a** using **9d** (27.8 mg, 38.6 μmol). The residue was purified by preparative HPLC (gradient: $H_2O$ (TFA 0.1%) : $CH_3CN$ (TFA 0.1%) = 95 : 5 to $H_2O$ (TFA 0.1%) : $CH_3CN$ (TFA 0.1%) = 90 : 10 over 40 min, Flow late 5 mL/min, UV: 222 nm) to give **10d** (8.25 mg, 13.2 μmol, 2 steps 34%) as a colorless solid; $[\alpha]_D^{25}$ = 6.34 (*c* = 0.29, $H_2O$); m.p. 167.7–168.5 °C; $^1$H NMR (400 MHz, $D_2O$ (N*H*$_2$, N*H*, O*H* and COO*H* (total 8H) were exchanged with $D_2O$)) δ 7.17 (d, *J* = 8.3 Hz, 2H), 6.90 (d, *J* = 8.4 Hz, 2H), 4.18-4.08 (m, 2H), 3.55 (s, 2H), 3.09 (d, *J* = 7.5 Hz, 2H), 2.98 (t, *J* = 7.5 Hz, 2H), 2.65 (s, 3H), 2.25-2.10 (m, 2H), 1.73-1.38 (m, 4H); $^{13}$C NMR (100 MHz, $D_2O$) δ 174.0, 170.2, 168.6, 155.2, 130.8 (2 carbons), 125.5, 115.9 (2 carbons), 59.0, 54.5, 46.6, 44.0, 38.9, 38.4, 36.3, 30.1, 23.3; HRMS (ES+) calcd for $C_{18}H_{30}N_5O_5$ $[M+H]^+$ 396.2247, found 396.2258.

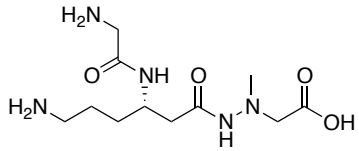
**2-{2-[(*S*)-6-Amino-3-((*S*)-2-amino-3-methylbutanamido)hexanoyl]-1-methylhydrazinyl}acetic acid·2TFA (10e)**



**10e** was prepared in the same manner as described for compound **10a** using **9e** (40.4 mg, 61.6 μmol). **10e** (11.2 mg, 20.0 μmol, 2 steps 33%) was obtained as a colorless solid; $[\alpha]_D^{25}$ = 11.7 (*c* = 0.42, $H_2O$); m.p. 197.7–198.6 °C; $^1$H NMR (400 MHz, $D_2O$ (N*H*$_2$, N*H* and COO*H* (total 7H) were exchanged with $D_2O$)) δ 4.36-4.23 (m, 1H), 3.78 (d, *J* = 5.3 Hz, 1H), 3.69-3.57 (m, 2H), 3.02 (t, *J* = 6.9 Hz, 2H), 2.68 (s, 3H), 2.50 (dd, *J* = 15 and 5.1 Hz, 1H), 2.38 (dd, *J* = 15 and 8.8 Hz, 1H), 2.23-2.12 (m, 1H), 1.80-1.54 (m, 4H), 1.01 (t, *J* = 6.7 Hz, 6H); $^{13}$C NMR (100 MHz, $D_2O$) δ 173.1, 170.3, 168.6, 58.6, 58.4, 46.6, 44.0, 39.0, 38.6, 30.7, 29.9, 23.4, 17.6, 16.8; HRMS (ES+) calcd for

$C_{14}H_{30}N_5O_4$ $[M+H]^+$ 332.2298, found 332.2286.

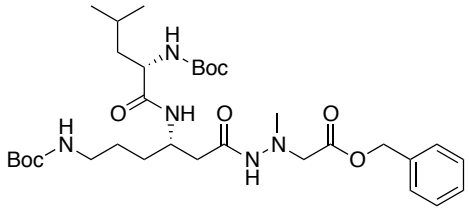
**(*S*)-2-(2-(6-Amino-3-(2-aminoacetamido)hexanoyl)-1-methylhydrazinyl)acetic acid·2TFA (10f)**



**10f** was prepared in the same manner as described for compound **10a** using **9f** (30.8 mg, 50.2 μmol). **10f** (12.3 mg, 23.8 μmol, 2 steps 47%) was obtained as a colorless solid; $[\alpha]_D^{25}$ = –9.30 (*c* = 0.42, $H_2O$); m.p. 148.0–149.9 °C; $^1$H NMR (400 MHz, $D_2O$ (N*H*$_2$, N*H* and COO*H* (total 7H) were exchanged with $D_2O$)) δ 4.28-4.19 (m, 1H), 3.89-3.75 (m, 2H), 3.68 (s, 2H), 3.01 (t, *J* = 6.0 Hz, 2H), 2.71 (s, 3H), 2.47 (dd, *J* = 14 and 5.3 Hz, 1H), 2.34 (dd, *J* = 14 and 9.0 Hz, 1H), 1.80-1.49 (m, 4H); $^{13}$C NMR (100 MHz, $D_2O$) δ 172.5, 170.5, 166.4, 58.4, 46.9, 44.3, 40.4, 38.98, 38.96, 30.6, 23.4; HRMS (ES+) calcd for $C_{11}H_{24}N_5O_4$ $[M+H]^+$ 290.1828, found 290.1829.

**4. Synthesis of derivatives 12a-e**

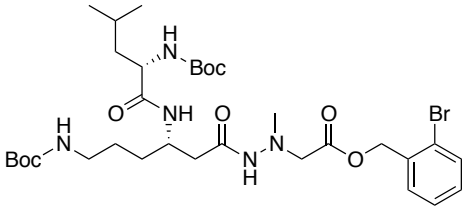
**(7*S*,10*S*)-Benzyl 7-[3-(*tert*-butoxycarbonylamino)propyl]-10-isobutyl-3,14,14-trimethyl-5,9,12-trioxo-13-oxa-3,4,8,11-tetraazapentadecan-1-oate (12a)**



**12a** was prepared in the same manner as described for compound **12b** using **11** (155 mg, 0.231 mmol) and benzylalcohol (28.8 μL, 0.277 mmol). **12a** (99.4 mg, 0.156 mmol, 3 steps 68%) was obtained as a white solid; $[\alpha]_D^{25}$ = –22.3 (*c* = 1.27, $CHCl_3$); m.p. 134.7–135.3 °C; $^1$H NMR (400 MHz, $CDCl_3$) δ 7.90 (s, 1H), 7.43-7.29 (m, 5H), 7.09 (br s, 1H), 5.27-5.13 (m, 2H), 5.13-4.94 (m) and 4.77 (br s, total 2H), 4.31-4.10 (m, 1H), 4.10-3.94 (m, 1H), 3.77-3.46 (m, 2H), 3.24-3.06 (m, 2H), 2.97-2.44 (m, 4H), 2.37-2.22 (m, 1H), 1.88-1.24 (m, 25H), 1.00-0.86 (m, 6H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 172.4, 170.5, 169.0, 156.1, 155.7, 135.1, 128.7 (2 carbons), 128.5 (2 carbons), 128.0, 79.9, 79.1, 66.7, 58.9, 57.8, 53.5, 45.8, 44.1, 41.5, 40.2, 31.0, 28.4 (3 carbons), 28.3 (3

carbons), 26.7, 24.8, 23.0, 21.9; HRMS (ES+) calcd for $C_{32}H_{53}N_5O_8Na$ $[M+Na]^+$ 658.3792, found 658.3795.

**(7*S*,10*S*)-2-Bromobenzyl 7-[3-(*tert*-butoxycarbonylamino)propyl]-10-isobutyl-3,14,14-trimethyl-5,9,12-trioxo-13-oxa-3,4,8,11-tetraazapentadecan-1-oate (12b)**



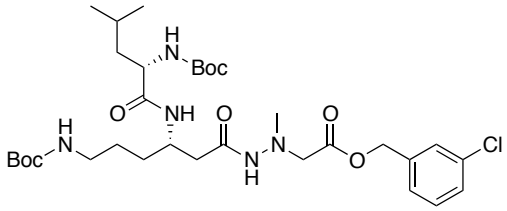
10 % Pd/C (10.6 mg) was added to a solution of **11** (106 mg, 0.158 mmol) in MeOH (2 mL) at room temperature. The resulting reaction was subjected to three cycles of vacuum followed by flush with $H_2$ before stirring for 35 min under an atmosphere of $H_2$. The mixture was filtered through a pad of Celite® with MeOH and concentrated removed under reduced pressure. The residue was used in the next step without further purification.

$Et_3N$ (43.8 μL, 0.316 mmol) and $Boc_2O$ (69.0 mg, 0.316 mmol) were added to a solution containing above residue in DMF (2 mL) at 0 °C. After stirring for 2.5 h at room temperature, 1 M HCl was added at 0 °C and extracted with AcOEt. The extracts were washed with brine, dried over $Na_2SO_4$, filtered and concentrated under reduced pressure. The residue was used in the next step without further purification.

*N*,*N*-dimethyl-4-aminopyridine (DMAP, 1.93 mg, 15.8 μmol) and *N*,*N*'-dicyclohexylcarbodiimide (DCC, 35.9 mg, 0.174 mmol) were added to a solution containing above residue and *o*-bromobenzyl alcohol (35.5 mg, 0.190 mmol) in DMF (2 mL) at 0 °C. After stirring for 3.5 h at room temperature, the mixture was concentrated under reduced pressure. The residue was filtered with $CHCl_3$. The extracts were washed with $H_2O$, brine, dried over $Na_2SO_4$, filtered and concentrated under reduced pressure. The residue was purified by silica gel flash column chromatography with $CHCl_3$ : MeOH = 100 : 1 to give **12b** (60.8 mg, 85.2 μmol, 3 steps 54%) as a white solid; $[\alpha]_D^{25}$ = –18.8 (*c* = 1.51, $CHCl_3$); m.p. 124.0–124.9 °C; $^1$H NMR (400 MHz, $CDCl_3$) δ 7.82 (s, 1H), 7.59 (d, *J* = 7.9 Hz, 1H), 7.45-7.29 (m, 3H), 7.23 (t, *J* = 6.3 Hz, 1H), 7.05 (br s, 1H), 5.35-5.20 (m, 2H), 5.03-4.90 (m, 1H), 4.80-4.63 (m, 1H), 4.35-4.12 (m, 1H), 4.12-3.96 (m, 1H), 3.81-3.51 (m, 2H), 3.26-3.03 (m, 2H), 2.97-2.43 (m, 4H), 2.39-2.20

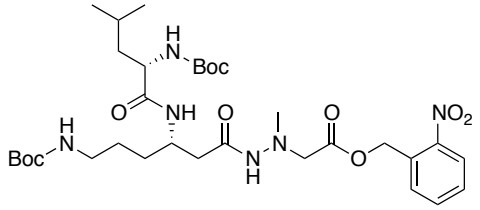
(m, 1H), 1.78-1.50 (m, 6H), 1.43 (s, 18H), 0.93 (s, 6H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 172.4, 170.3, 169.0, 156.1, 155.7, 134.5, 133.0, 130.4, 128.5, 127.7, 123.6, 79.9, 79.1, 66.2, 58.8, 57.7, 53.4, 45.7, 44.1, 41.4, 40.2, 31.5, 28.4 (6 carbons), 26.7, 24.8, 23.0, 21.9; HRMS (ES+) calcd for $C_{32}H_{52}N_5O_8NaBr$ $[M+Na]^+$ 736.2897, found 736.2919.

**(7*S*,10*S*)-3-Chlorobenzyl 7-[3-(*tert*-butoxycarbonylamino)propyl]-10-isobutyl-3,14,14-trimethyl-5,9,12-trioxo-13-oxa-3,4,8,11-tetraazapentadecan-1-oate (12c)**



**12c** was prepared in the same manner as described for compound **12b** using **11** (168 mg, 0.251 mmol) and *m*-chlorobenzylalcohol (35.5 μL, 0.301 mmol). **12c** (72.5 mg, 0.108 mmol, 3 steps 43%) was obtained as a white solid; $[\alpha]_D^{25}$ = –20.0 (*c* = 0.96, $CHCl_3$); m.p. 97.5–97.9 °C; $^1$H NMR (400 MHz, $CDCl_3$) δ 7.89 (s, 1H), 7.40-7.20 (m, 3H), 7.08 (br s, 1H), 5.24-4.97 (m, 2H), 4.77 (br s, 1H), 4.34-4.09 (m, 1H), 4.09-3.91 (m, 1H), 3.80-3.38 (m, 3H), 3.25-3.04 (m, 2H), 2.98-2.42 (m, 4H), 2.42-2.16 (m, 1H), 2.14-1.32 (m, 26H), 1.02-0.86 (m, 6H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 172.5, 171.1, 169.1, 156.1, 155.7, 137.1, 134.5, 130.0, 128.7, 128.5, 126.4, 79.8, 79.0, 65.7, 58.8, 57.7, 53.5, 45.8, 44.2, 41.4, 40.0, 31.0, 28.4 (3 carbons), 28.3 (3 carbons), 26.7, 24.8, 23.0, 21.9; HRMS (ES+) calcd for $C_{32}H_{53}N_5O_8Cl$ $[M+H]^+$ 670.3583, found 670.3585.

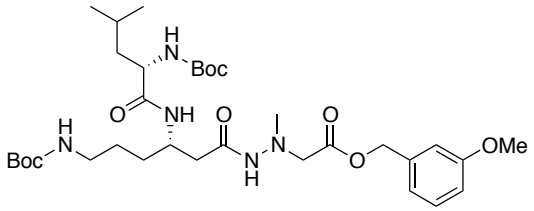
**(7*S*,10*S*)-2-Nitrobenzyl 7-[3-(*tert*-butoxycarbonylamino)propyl]-10-isobutyl-3,14,14-trimethyl-5,9,12-trioxo-13-oxa-3,4,8,11-tetraazapentadecan-1-oate (12d)**



**12d** was prepared in the same manner as described for compound **12b** using **11** (171 mg, 0.255 mmol) and *o*-nitrobenzylalcohol (46.9 mg, 0.306 mmol). **12d** (143 mg, 0.209 mmol, 3 steps 82%) was obtained as a white solid; $[\alpha]_D^{25}$ = –16.8 (*c* = 3.14, $CHCl_3$);

m.p. 114.8–116.2 °C; $^1$H NMR (400 MHz, $CDCl_3$) δ 8.16-8.08 (m, 1H), 7.99 (s, 1H), 7.74-7.49 (m, 2H), 7.37-7.31 (m, 1H), 7.14 (br s, 1H), 5.62-5.52 (m, 2H), 5.16-5.03 (m) and 4.89-4.79 (m, total 2H), 4.32-4.12 (m, 1H), 4.12-3.92 (m, 1H), 3.88-3.51 (m, 2H), 3.24-3.03 (m, 2H), 2.97-2.38 (m, 4H), 2.38-2.26 (m, 1H), 1.87-1.32 (m, 25H), 1.02-0.88 (m, 6H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 172.5, 170.0, 169.2, 156.1, 155.7, 147.6, 133.9, 131.2, 129.2, 128.4, 125.1, 79.8, 79.0, 63.3, 58.8, 57.7, 53.4, 45.5, 44.1, 41.4, 39.9, 31.1, 28.4 (3 carbons), 28.3 (3 carbons), 26.7, 24.7, 22.9, 21.9; HRMS (ES+) calcd for $C_{32}H_{52}N_6O_{10}Na$ $[M+Na]^+$ 703.3643, found 703.3642.

**(7*S*,10*S*)-3-Methoxybenzyl 7-[3-(*tert*-butoxycarbonylamino)propyl]-10-isobutyl-3,14,14-trimethyl-5,9,12-trioxo-13-oxa-3,4,8,11-tetraazapentadecan-1-oate (12e)**

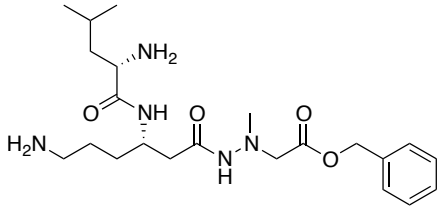



**12e** was prepared in the same manner as described for compound **12b** using **11** (170 mg, 0.254 mmol) and *m*-methoxybenzylalcohol (42.1 mg, 0.305 mmol). **12e** (84.3 mg, 0.127 mmol, 3 steps 50%) was obtained as a white solid; $[\alpha]_D^{25}$ = –18.3 (*c* = 1.40, $CHCl_3$); m.p. 96.4–97.1 °C; $^1$H NMR (400 MHz, $CDCl_3$) δ 7.88 (s, 1H), 7.40-7.28 (m, 2H), 7.08 (br s, 1H), 6.96-6.84 (m, 1H), 5.26-4.92 (m, 3H), 4.85-4.65 (m, 1H), 4.34-4.10 (m, 1H), 4.10-3.97 (m, 1H), 3.86-3.40 (m, 5H), 3.26-3.00 (m, 2H), 3.00-2.38 (m, 4H), 2.38-2.12 (m, 1H), 1.84-1.38 (m, 26H), 1.02-0.85 (m, 6H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 172.4, 170.5, 169.0, 159.8, 156.1, 155.7, 136.6, 129.8, 128.5, 120.5, 114.0, 79.8, 79.1, 66.6, 58.8, 57.8, 55.3, 53.5, 46.7, 44.1, 41.4, 40.2, 31.1, 28.4 (3 carbons), 28.3 (3 carbons), 26.7, 24.8, 23.0, 21.9; HRMS (ES+) calcd for $C_{33}H_{55}N_5O_9Na$ $[M+Na]^+$ 688.3897, found 688.3898.

**5. Synthesis of derivatives 13a-e**

**Benzyl**

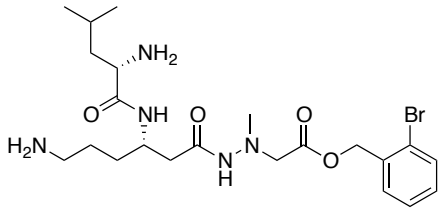
**2-(2-((*S*)-6-amino-3-((*S*)-2-amino-4-methylpentanamido)hexanoyl)-1-methylhydrazinyl)acetate·2TFA (13a)**



**13a** was prepared in the same manner as described for compound **12b** using **12a** (55.7 mg, 87.6 μmol). The residue was purified by preparative HPLC (gradient: $H_2O$ (TFA 0.1%) : $CH_3CN$ (TFA 0.1%) = 80 : 20 to $H_2O$ (TFA 0.1%) : $CH_3CN$ (TFA 0.1%) = 56 : 44 over 12 min, Flow late 5 mL/min, UV: 222 nm) to give **13a** (13.5 mg, 20.3 μmol, 2 steps 23%) as a colorless solid; $[\alpha]_D^{25}$ = 2.48 (*c* = 0.39, $H_2O$); m.p. 90.3–91.3 °C; $^1$H NMR (400 MHz, $D_2O$ (N*H*$_2$ and N*H* (total 6H) were exchanged with $D_2O$)) δ 7.53-7.38 (m, 5H), 5.23 (s, 2H), 4.26-4.15 (m, 1H), 3.94 (t, *J* = 6.0 Hz, 1H), 3.73-3.59 (m, 2H), 3.03-2.91 (m, 2H), 2.64 (s, 3H), 2.31 (dd, *J* = 5.2 and 15 Hz, 1H), 2.21 (dd, *J* = 8.7 and 15 Hz, 1H), 1.76-1.46 (m, 7H), 1.00-0.88 (m, 6H); $^{13}$C NMR (100 MHz, $D_2O$) δ 173.7, 173.1, 172.8, 138.2, 131.8 (2 carbons), 131.6, 131.4 (2 carbons), 70.1, 61.5, 54.8, 49.6, 47.2, 43.1, 41.8, 41.6, 33.5, 26.7, 26.3, 24.7, 23.6; HRMS (ES+) calcd for $C_{22}H_{38}N_5O_4$ $[M+H]^+$ 436.2924, found 436.2916.

**2-Bromobenzyl**

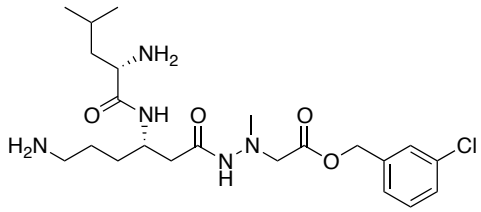
**2-(2-((*S*)-6-amino-3-((*S*)-2-amino-4-methylpentanamido)hexanoyl)-1-methylhydrazinyl)acetate·2TFA (13b, TCP-199)**



4 M HCl/dioxane (2 mL) was added to **12b** (40.6 mg, 56.9 μmol) at 0 °C. After stirring for 1 h at room temperature, the solvent was removed under reduced pressure. The residue was purified by preparative HPLC (gradient: $H_2O$ (TFA 0.1%) : $CH_3CN$ (TFA 0.1%) = 75 : 25 to $H_2O$ (TFA 0.1%) : $CH_3CN$ (TFA 0.1%) = 55 : 45 over 40 min, Flow late 5 mL/min, UV: 222 nm) to give **13b** (21.7 mg, 29.3 μmol, 51%) as a colorless

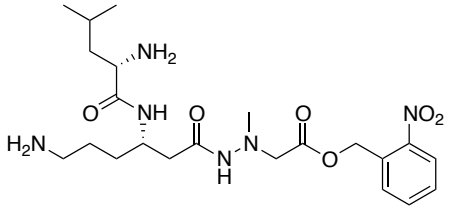
solid; $[\alpha]_D^{25}$ = 2.70 (*c* = 0.22, $H_2O$); m.p. 101.7–102.2 °C; $^1$H NMR (400 MHz, $D_2O$ ($NH_2$ and N*H* (total 5H) were exchanged with $D_2O$)) δ 8.35 (d, *J* = 9.0 Hz, 1H), 7.69 (d, *J* = 7.9 Hz, 1H), 7.55-7.47 (m, 1H), 7.43 (t, *J* = 7.4 Hz, 1H), 7.33 (t, *J* = 7.5 Hz, 1H), 5.30 (s, 2H), 4.30-4.24 (m, 1H), 4.02-3.85 (m, 1H), 3.80-3.60 (m, 2H), 3.07-2.90 (m, 2H), 2.64 (s, 3H), 2.34 (dd, *J* = 15 and 5.2 Hz, 1H), 2.23 (dd, *J* = 15 and 8.7 Hz, 1H), 1.80-1.44 (m, 7H), 1.03-0.84 (m, 6H); $^{13}$C NMR (100 MHz, $D_2O$) δ 173.6, 173.0, 172.8, 137.2, 135.9, 133.9, 133.6, 131.0, 126.3, 69.9, 61.4, 54.8, 49.6, 47.3, 43.2, 41.9, 41.7, 33.6, 26.8, 26.4, 24.8, 23.7; HRMS (ES+) calcd for $C_{22}H_{37}N_5O_4Br$ $[M+H]^+$ 514.2029, found 514.2032.

**3-Chlorobenzyl 2-(2-((*S*)-6-amino-3-((*S*)-2-amino-4-methylpentanamido)hexanoyl)-1-methylhydrazinyl)acetate (13c)**



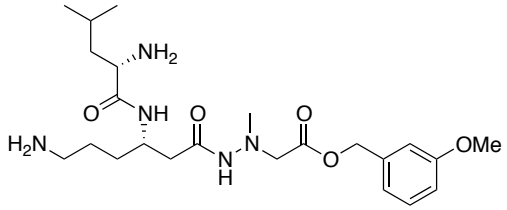

**13c** was prepared in the same manner as described for compound **13a** using **12c** (38.1 mg, 56.8 μmol). **13c** (12.5 mg, 17.9 μmol, 2 steps 31%) was obtained as a hygroscopic solid; $[\alpha]_D^{25}$ = 3.07 (*c* = 0.37, $H_2O$); $^1$H NMR (400 MHz, $D_2O$ ($NH_2$ and N*H* (total 6H) were exchanged with $D_2O$)) δ 7.48-7.31 (m, 4H), 5.20 (s, 2H), 4.23-4.16 (m, 1H), 3.98-3.85 (m, 1H), 3.73-3.60 (m, 2H), 3.03-2.90 (m, 2H), 2.63 (s, 3H), 2.30 (dd, *J* = 5.2 and 15 Hz, 1H), 2.21 (dd, *J* = 8.7 and 15 Hz, 1H), 1.75-1.42 (m, 7H), 1.01-0.85 (m, 6H); $^{13}$C NMR (100 MHz, $D_2O$) δ 173.6, 173.1, 172.8, 140.4, 136.8, 133.2, 131.5, 131.0, 129.6, 69.2, 61.6, 54.8, 49.6, 47.3, 43.2, 41.9, 41.7, 33.6, 26.8, 26.4, 24.8, 23.7; HRMS (ES+) calcd for $C_{22}H_{37}N_5O_4Cl$ $[M+H]^+$ 470.2534, found 470.2529.

**2-Nitrobenzyl 2-(2-((*S*)-6-amino-3-((*S*)-2-amino-4-methylpentanamido)hexanoyl)-1-methylhydrazinyl)acetate (13d)**



**13d** was prepared in the same manner as described for compound **13a** using **12d** (41.9 mg, 61.5 μmol). **13d** (24.1 mg, 34.0 μmol, 2 steps 55%) was obtained as a colorless solid; $[\alpha]_D^{25}$ = 3.11 (*c* = 0.74, $H_2O$); m.p. 85.6–86.5 °C; $^1$H NMR (400 MHz, $D_2O$ (N*H*$_2$ and N*H* (total 6H) were exchanged with $D_2O$)) δ 8.17 (d, *J* = 8.2 Hz, 1H), 7.78 (t, *J* = 7.7 Hz, 1H), 7.69-7.57 (m, 2H), 5.55 (s, 2H), 4.32-4.15 (m, 1H), 3.99-3.86 (m, 1H), 3.79-3.63 (m, 2H), 3.05-2.91 (m, 2H), 2.65 (s, 3H), 2.40 (dd, *J* = 5.1 and 15 Hz, 1H), 2.67 (dd, *J* = 8.7 and 15 Hz, 1H), 1.77-1.45 (m, 7H), 0.99-0.81 (m, 6H); $^{13}$C NMR (100 MHz, $D_2O$) δ 173.5, 173.2, 172.9, 150.3, 137.5, 133.5, 133.2, 132.7, 128.2, 117.9, 67.0, 61.3, 54.9, 49.7, 47.3, 43.2, 41.9, 33.6, 26.8, 26.4, 24.8, 23.7; HRMS (ES+) calcd for $C_{22}H_{37}N_6O_6$ $[M+H]^+$ 481.2775, found 481.2771.

**3-Methoxybenzyl**
**2-(2-((*S*)-6-amino-3-((*S*)-2-amino-4-methylpentanamido)hexanoyl)-1-methylhydrazinyl)acetate·2TFA (13e)**



**13e** was prepared in the same manner as described for compound **13a** using **12e** (41.0 mg, 61.6 μmol). **13e** (10.7 mg, 15.5 μmol, 2 steps 25%) was obtained as a colorless solid; $[\alpha]_D^{25}$ = 2.98 (*c* = 0.31, $H_2O$); m.p. 81.8–82.4 °C; $^1$H NMR (400 MHz, $D_2O$) δ 7.39 (t, *J* = 7.8 Hz, 1H), 7.10-6.99 (m, 3H), 5.20 (s, 2H), 4.26-4.16 (m, 1H), 3.97-3.87 (m, 1H), 3.85 (s, 3H), 3.74-3.60 (m, 2H), 3.04-2.90 (m, 2H), 2.64 (s, 3H), 2.31 (dd, *J* = 5.1 and 15 Hz, 1H), 2.21 (dd, *J* = 8.8 and 15 Hz, 1H), 1.80-1.42 (m, 7H), 1.03-0.82 (m, 6H); $^{13}$C NMR (100 MHz, $D_2O$) δ 173.7, 173.1, 172.8, 162.0, 140.0, 133.1, 124.0, 117.2, 116.8, 69.8, 61.5, 58.3, 54.8, 49.6, 47.2, 43.2, 41.9, 41.6, 33.6, 26.8, 26.4, 24.7, 23.7; HRMS (ES+) calcd for $C_{23}H_{40}N_5O_5$ $[M+H]^+$ 466.3029, found 466.3024.

**5. Biological evaluation**

5-1. Chemicals

Geneticin (G418) solution was purchased from Roche Diagnostics K.K., Switzerland.

5-2. Plasmid

The previously reported plasmids[1] were used in this study. The dual-reporter plasmid for mammalian cells expression encodes the β-galactosidase and luciferase genes connected with the premature termination codon (PTC), a 27-mer stretch of DNA that contains the sequence surrounding the PTC in exon 23 of the *mdx* gene for mouse dystrophin. The PTC was originally TAA. We used TGA, TAG and TAA in this study. Furthermore, for the readthrough frequency of **13b**, the plasmid, which is containing TGG (tryptophan coding) sequence instead of PTC as a wild type sequence was constructed according to previous study[1].

5-3. The cell-based readthrough activity evaluation

The cell-based readthrough activity was evaluated by previously reported procedure[1].

COS-7 cells were maintained in D-MEM (high glucose, Wako Pure Chemical Industries, Ltd., Japan) containing 10% fetal bovine serum (FBS, Nichirei Biosciences Inc., Japan) at 37 ºC in a humidified 5% $CO_2$ atmosphere. Cells were plated in 96-well plates at 8000 cells/well. After incubation at 37 °C for 12 h, cells were transfected with above-mentioned plasmid with PTC using the FuGene® HD Transfect reagent (Promega K.K., USA). The medium was removed from the well, and the medium containing the compounds was added to the well (final concentration, 10-200 μM). As a control, the medium without compounds was also added. The cells were incubated at 37 °C for 48 h, cells were collected, and β-galactosidase activity in the cell lysates was measured according to the manufacturer's protocol for the β-Galactosidase Enzyme Assay with Reporter Lysis Buffer (Promega K.K., USA). The β-galactosidase activity was measured by TECAN SAFIRE (TECAN Japan Co., Ltd, Japan) at 420 nm (reference 0 nm). The luciferase activity in the cell lysates was measured according to the manufacturer's protocol for using the PicaGene® BrillianStar-LT (TOYO INK CO., LTD., Japan). The luciferase activity was measured using a Berthold Luminometer MicroLumat Plus LB96V (Berthold Japan K.K., Japan). The readthrough activity was determined as the ratio of luciferase activity to β-galactosidase activity. The activities of

compounds were expressed as a ratio relative to control (=1). Moreover, readthrough frequency was calculated the ratio of readthrough activity of TGA sequence (Luciferase activity/β-galactosidase activity) divided by the readthrough activity of TGG sequence (Luciferase activity/β-galactosidase activity).

5-4. *In vitro* cytotoxic assay of derivatives **13a-e**

HDF (Human dermal fibroblast) or COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Nichirei Biosciences Inc., Japan) in a humidified atmosphere containing 5% $CO_2$. For viability assay, HDF cells were seeded at $3 \times 10^3$ cells per 100 μL per well in a 96-well micro plate, and treated with 200 μM concentration of derivatives **3**, **13a-e** and G418 for 48 h at 37 ºC. Cytotoxicity values were determined using the cell counting reagent WST-1 (Roche Diagnostics K.K., Switzerland).

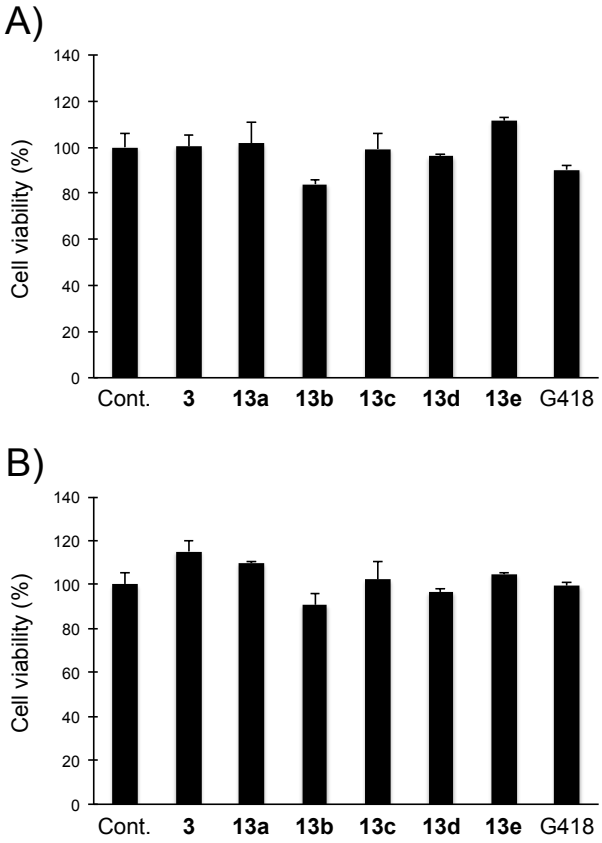



**Figure S1.** Cytotoxicity of derivatives **3**, **13a-e** and G418 against A) human dermal fibroblast, and B) COS-7 cells. Error bars indicate ± SD (n = 3).

5-5. Hydrolysis of **13b** by porcine liver esterase

The hydrolysis of **13b** was performed by previously reported procedure[2].

The stock solution of **13b** (20 mM in 100 mM phosphate buffer, pH 7.4) was diluted to 2 mM with 100 mM phosphate buffer (pH 7.4). The suspension of the porcine liver esterase (9 μL; containing 110 unit/mL; PLE: carboxylic-ester hydrolase, Aldrich, USA) was added to the solution of **13b** (2 mM, 450 μL). The residual solution of **13b** (2 mM, 50 μL) was used as a sample of time zero without addition of esterase. Each solution (2 mM, 50 μL) containing PLE was incubated at 37 ºC for appropriate times. After the incubation, the mixture was filtered through the centrifugal filter (0.2 μm filter unit, NANOSEP® MF centrifugal devices, PALL) and immediately frozen at –78 ºC to neutralize a PLE. After melting, the filtrate containing **13b** and its metabolites were analyzed by RP-HPLC and high-resolution mass spectrometry for identification of some metabolites appeared as a new HPLC peak.

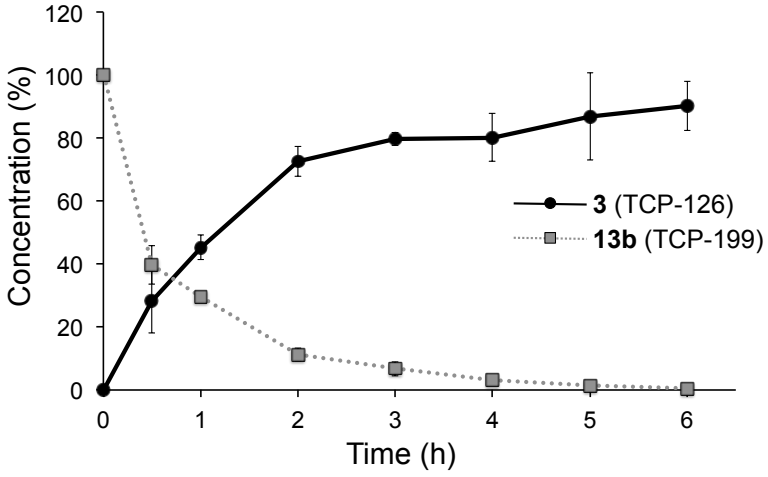



**Figure S2.** Hydrolysis of **13b** by porcine liver esterase. Compound concentration (%) was determined by RP-HPLC. Error bars indicate ± SD (n = 3).

5-6. Human plasma stability test of **13b**

The human plasma stability test of **13b** was performed according to the reference 3.

Briefly, the stock solution of **13b** (20 mM in double diluted water, 0.5 μL) was diluted to human plasma (49.5 μL, Plasma from human, containing 4% trisodium citrate as anticoagulan, Aldrich, USA) which was preincubated at 37 °C. The residual solution of **13b** (200 μM, 50 μL) was incubated at 37 °C for appropriate times. After the incubation, 200 μL acetonitrile was added and vortex vigorously. After centrifuge at

14,000 round/min at 4 °C, the supernatant was filtered through the centrifugal filter (0.2 μm filter unit, NANOSEP® MF centrifugal devices, PALL) and immediately frozen at –78 ºC. After melting, the filtrate containing **13b** and its metabolites were analyzed by RP-HPLC and high-resolution mass spectrometry.

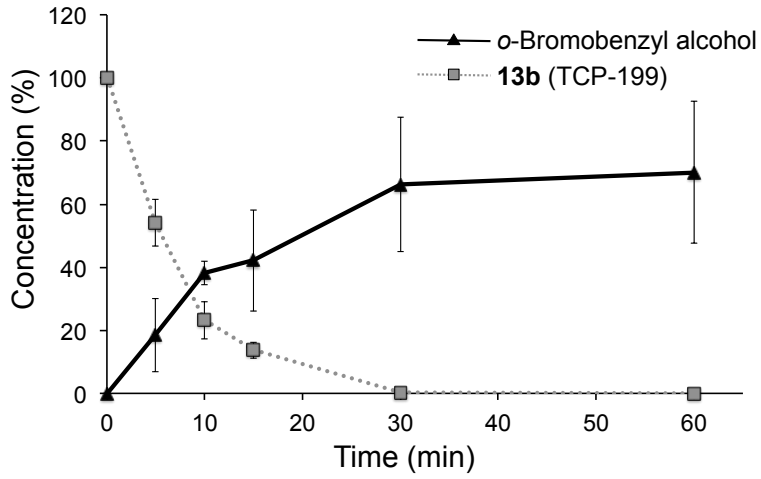



**Figure S3.** Human plasma stability of **13b**. Compound concentration (%) was determined by RP-HPLC. Error bars indicate ± SD (n = 3).

5-7. Animals

READ mouse strain on a C57/BL6 background expressed a dual-reporter gene, which was composed of the *lacZ* and *luc* genes connected with a premature termination codon region derived from exon 23 of the *mdx* mouse, a model of Duchenne muscular dystrophy, dystrophin gene. Although the premature termination codon was originally TAA, TGA-type PTC was used in this study. For further details of this mouse, see Shiozuka *et al*.,[3]. The mice were housed individually under controlled conditions of temperature and humidity and had free access to water and food. All experiments using mice were conducted under the approval of the University of Tokyo Animal Ethics Committee (Permit Number: 24-6).

5-8. *In vivo* readthrough activity of **13b** by READ mice

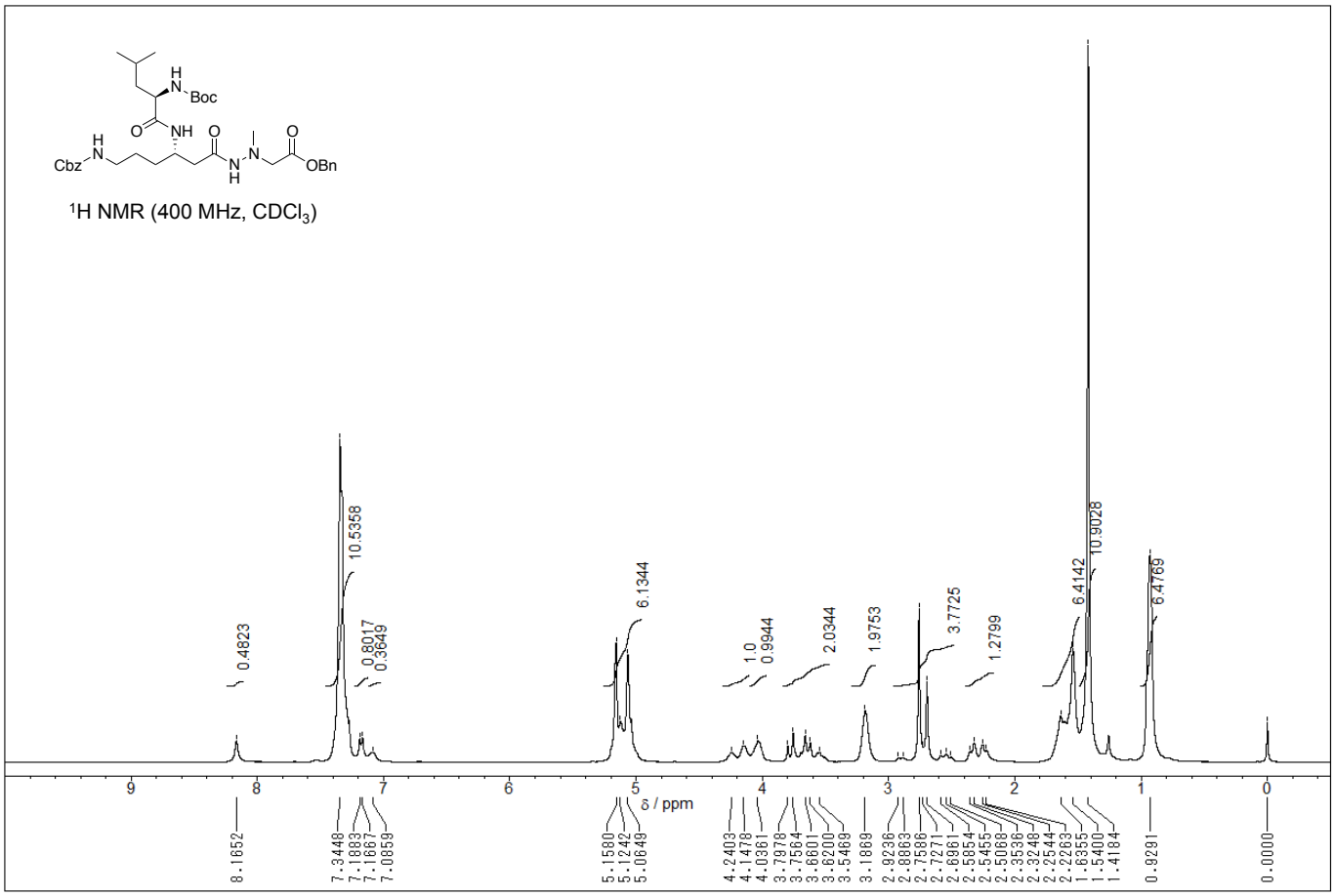
*In vivo* readthrough activity was evaluate with dual-reporter gene expression system using above-mentioned transgenic READ mice[3]. A solution of compound **13b** (1 mg) in saline (0.2 mL, the pH of the solution was adjusted to 8.0 by $NaHCO_3$ was administered) or a solution of arbekacin (1 mg, Meiji seika; trade name Habekacin) was

subcutaneously injected in the abdominal region of READ mice (each n = 4, 5 weeks old; approximately 20 g body weight) for 7 days. As a control, the saline-treated READ mice (n = 3, 5 weeks old; approximately 20 g body weight) were also tested. At the completion of the administration, the mice were euthanized with an overdose of ether. Tissue samples were collected from the rectus femoris, gastrocnemius and soleus. Dissected tissues were minced with scissors and homogenized in three volumes of the reporter lysis buffer (Promega, Madison, WI, USA) using a tissue grinder (Physcotron; MICROTEC CO., LTD, Japan). Tissue homogenates were subjected to one round of freeze-thawing. The lysate supernatants were collected after centrifugation at 17,710 x g for 10 min, and then analyzed using the Beta-Glo and Bright-Glo luciferase assay systems (Promega). The β-galactosidase and luciferase activities were measured according to the manufacturers' instructions using a luminometer (Luminescencer-JNRII AB-2300; Atto, Japan). The readthrough activity was determined as the ratio of luciferase activity to β-galactosidase activity.

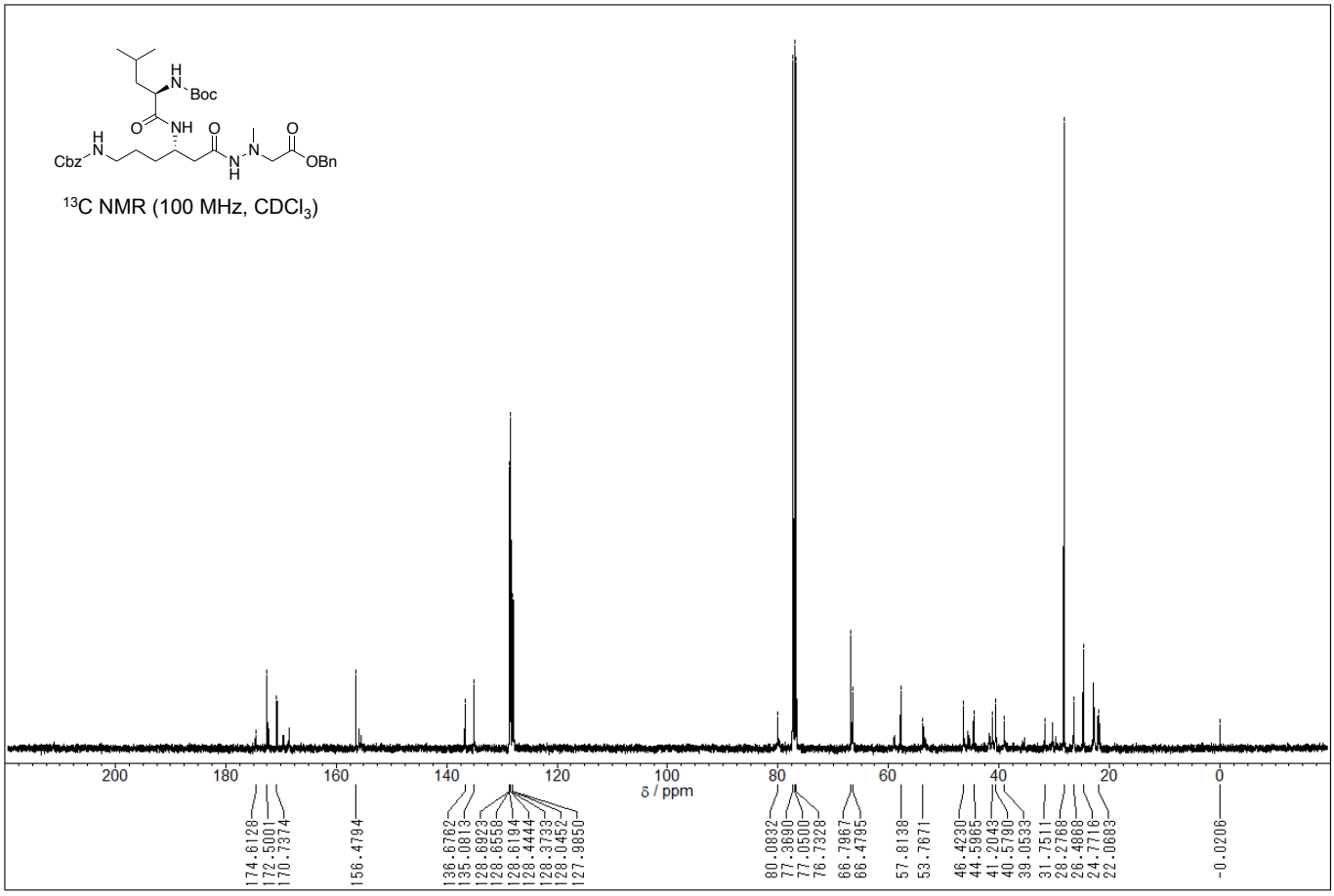
5-9. The acute toxicity test of **13b**-treated mice

After single subcutaneously injection of **13b** (10 mg/day/20 g body-weight) at the abdominal region of B10 mice (n = 4), the body weight of **13b**-treated B10 mice was measured on the 1-7 and 14 days. As a control, the body weight of saline-treated B10 mice (n = 3) was also measured.

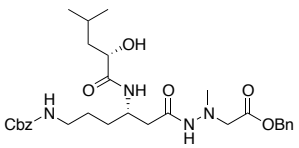
Compound 9a proton




Compound 9a carbon



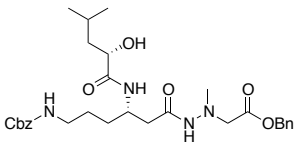
Compound 9b proton



$^{1}$H NMR (400 MHz, $CDCl_3$)

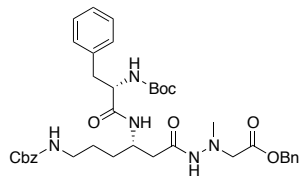



Compound 9b carbon



$^{13}$C NMR (100 MHz, $CDCl_3$)

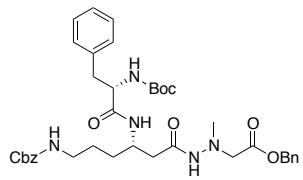
Compound 9c proton




$^{1}$H NMR (400 MHz, $CDCl_3$)

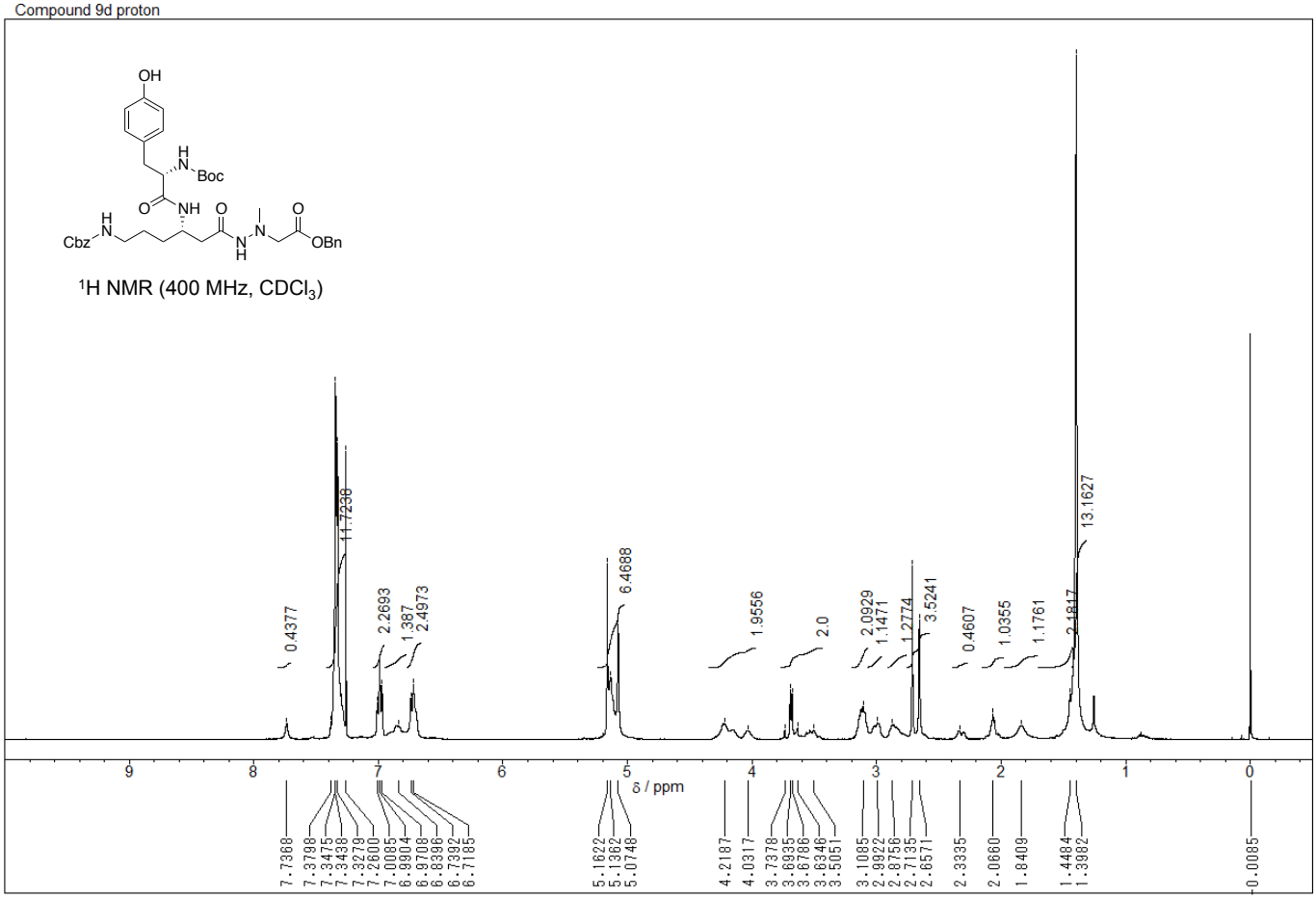



Compound 9c carbon



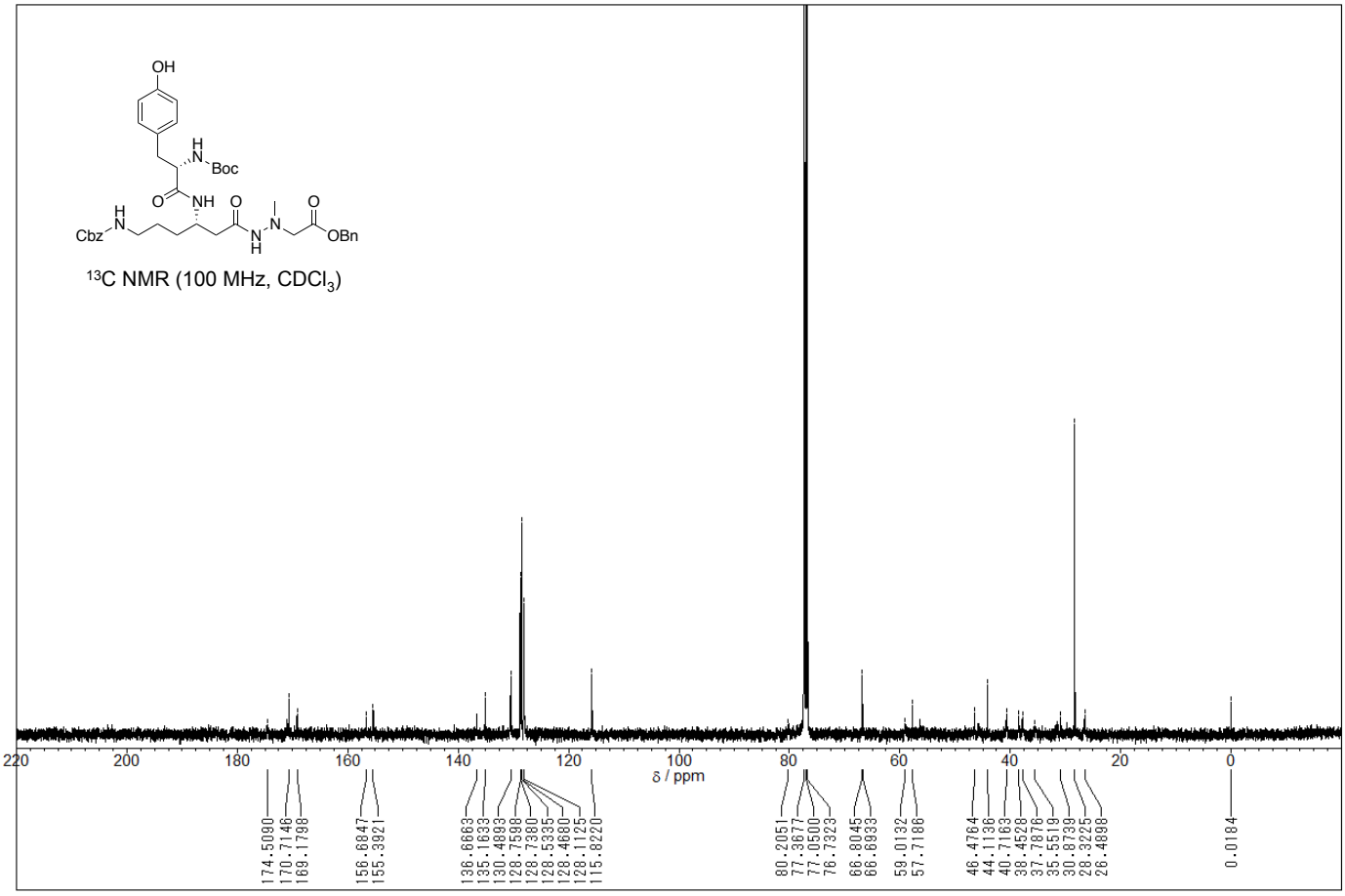

$^{13}$C NMR (100 MHz, $CDCl_3$)

Compound 9d proton



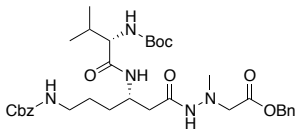

$^{1}$H NMR (400 MHz, $CDCl_3$)

Compound 9d carbon




$^{13}$C NMR (100 MHz, $CDCl_3$)

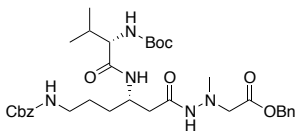
Compound 9e proton



$^{1}$H NMR (400 MHz, $CDCl_3$)

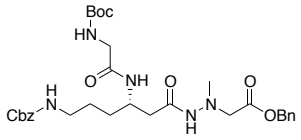



Compound 9e carbon



$^{13}$C NMR (100 MHz, $CDCl_3$)

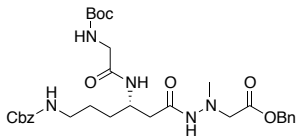
Compound 9f proton




$^{1}$H NMR (400 MHz, $CDCl_3$)

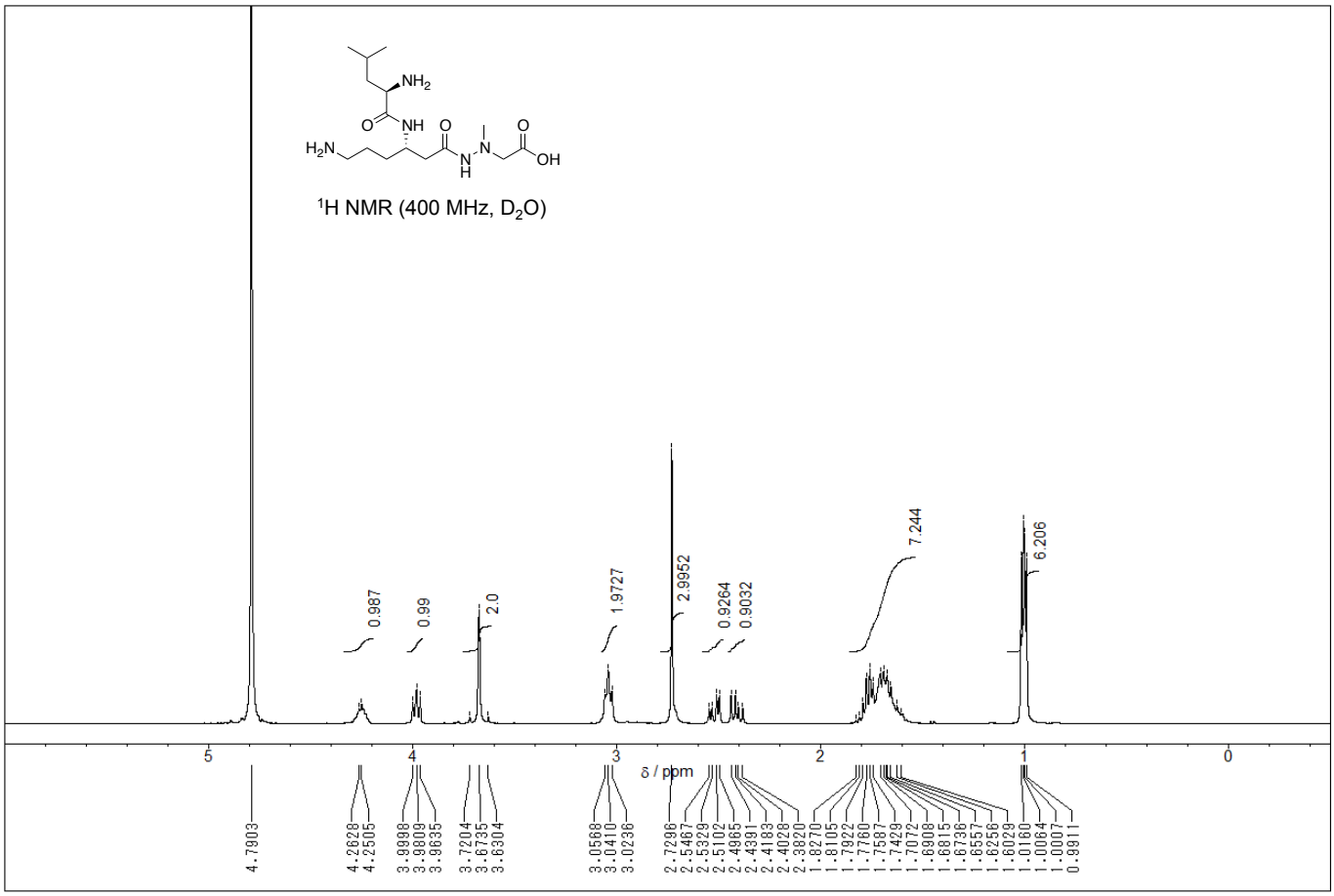



Compound 9f carbon

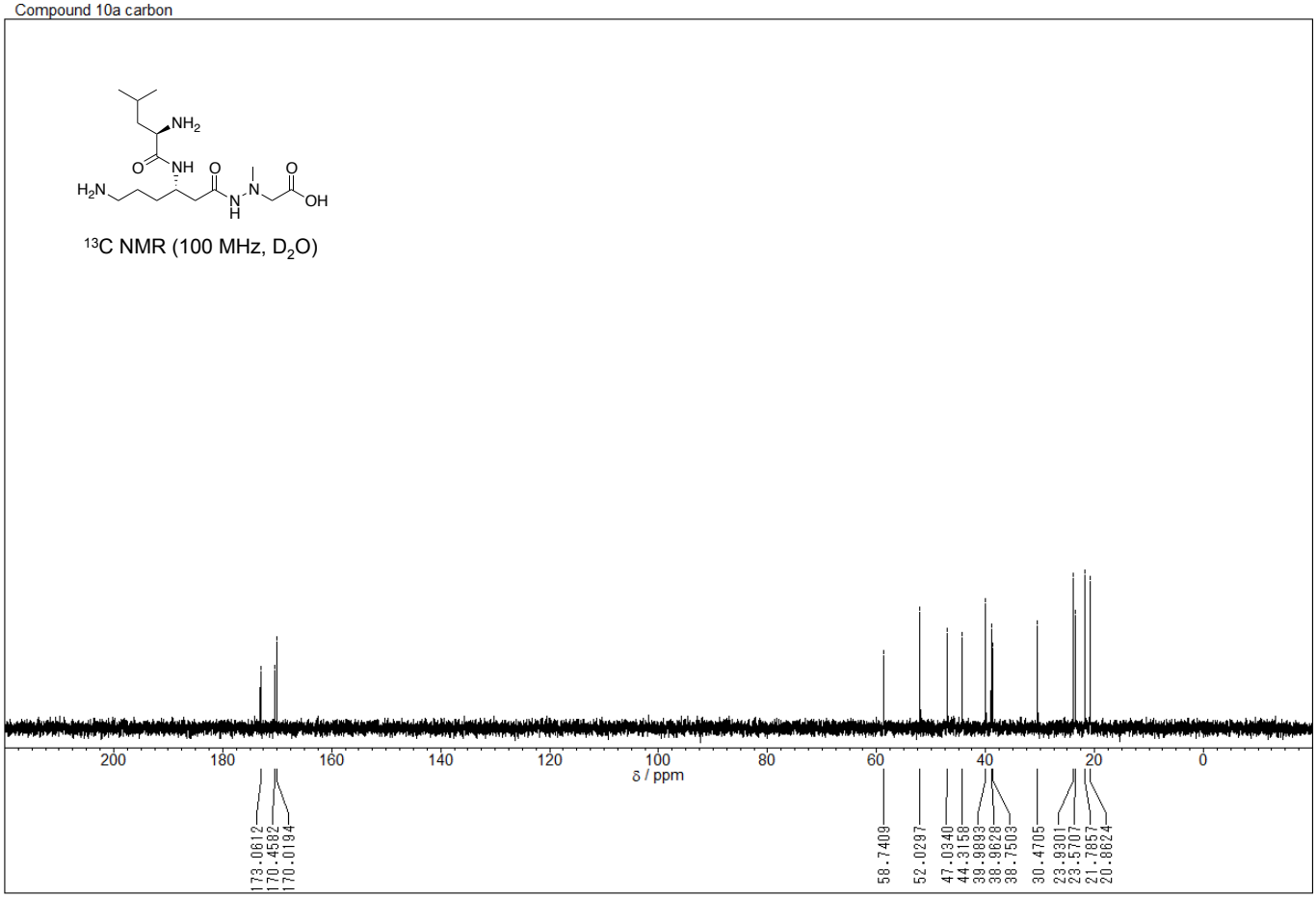



$^{13}$C NMR (100 MHz, $CDCl_3$)

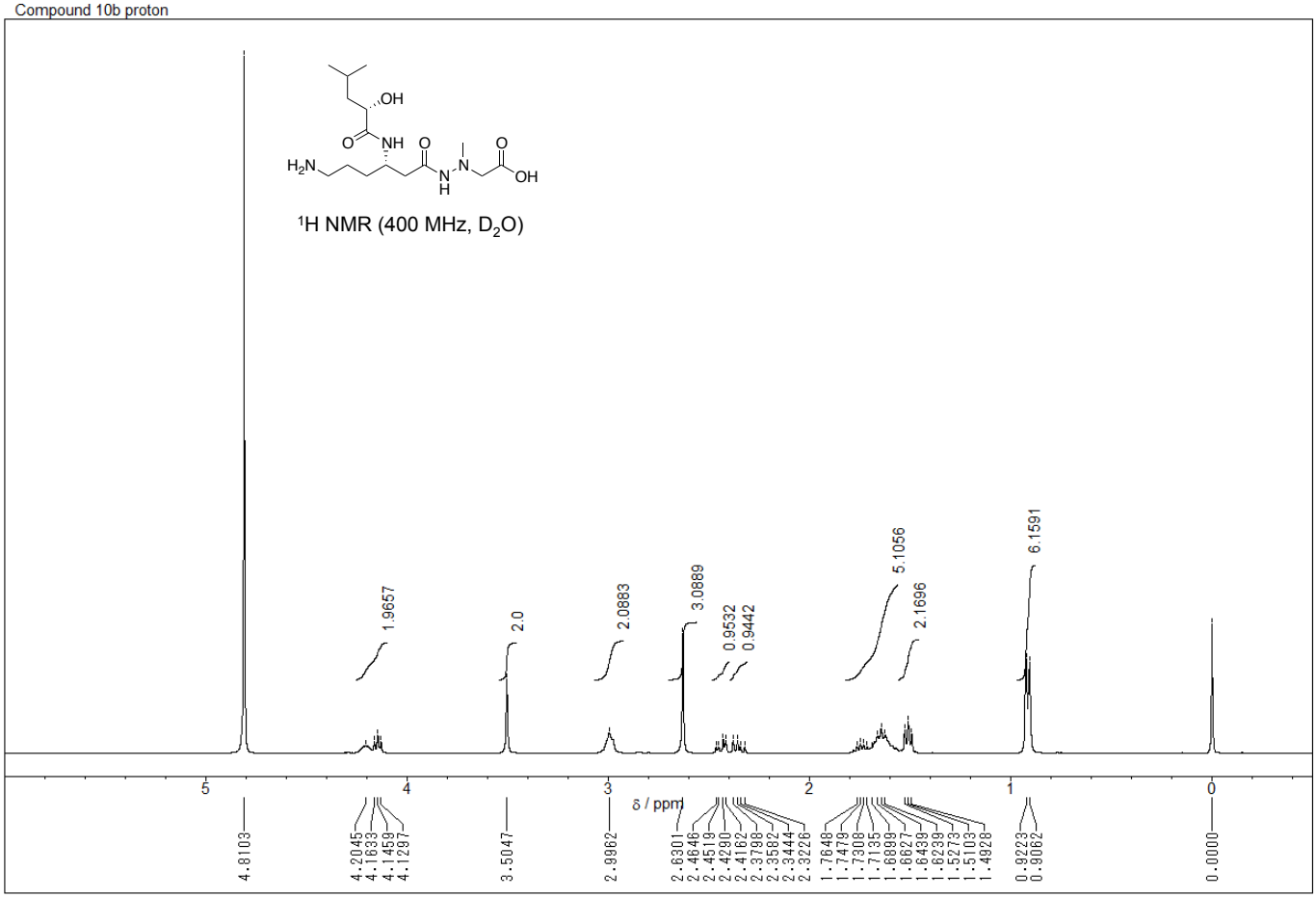
Compound 10a proton



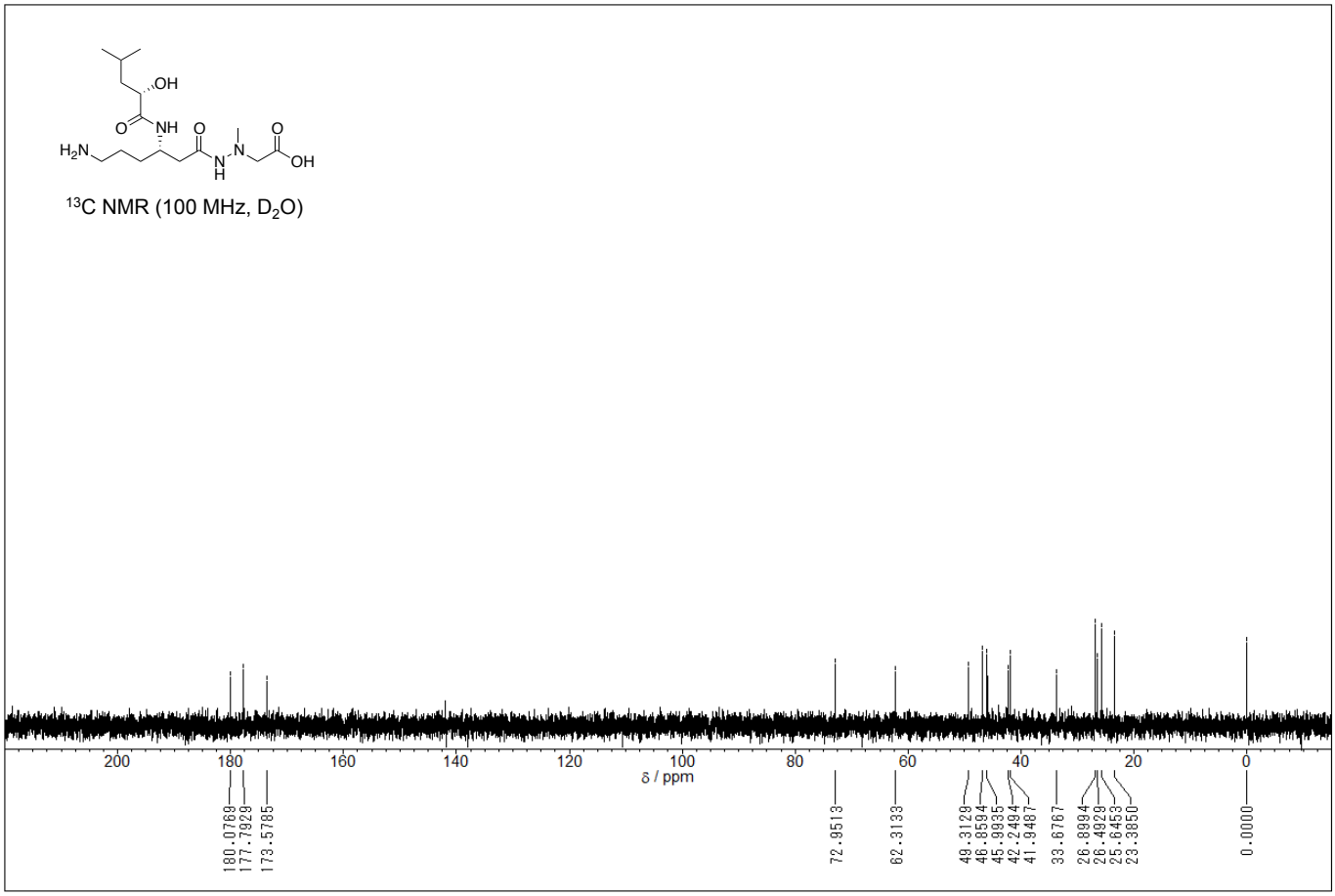

Compound 10a carbon



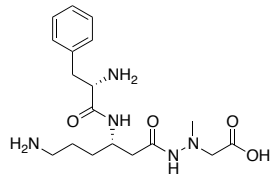
Compound 10b proton




Compound 10b carbon



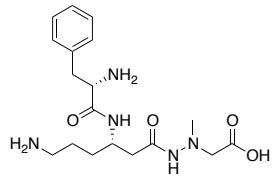
Compound 10c proton



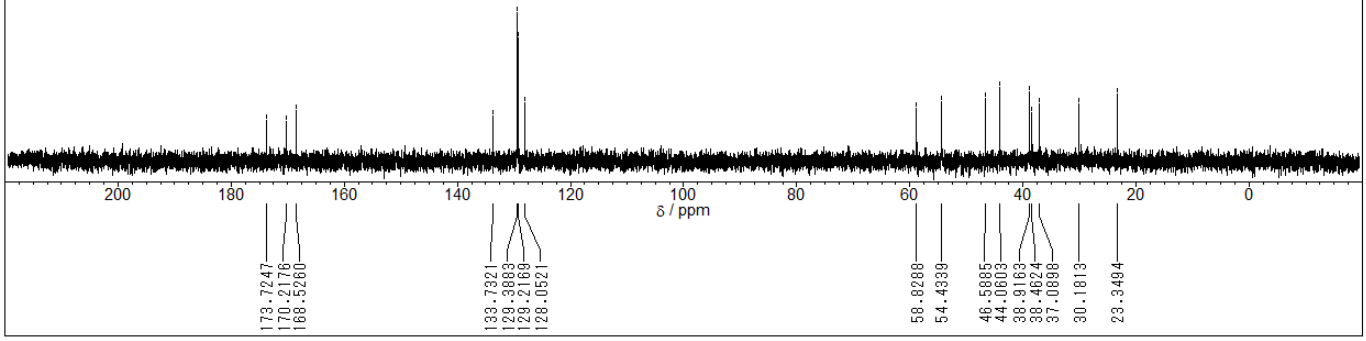
$^{1}$H NMR (400 MHz, $D_2O$)



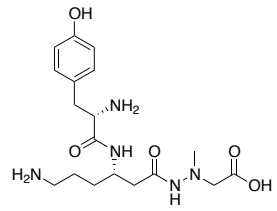

Compound 10c carbon



$^{13}$C NMR (100 MHz, $D_2O$)



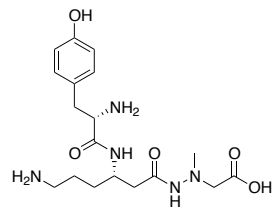
Compound 10d proton



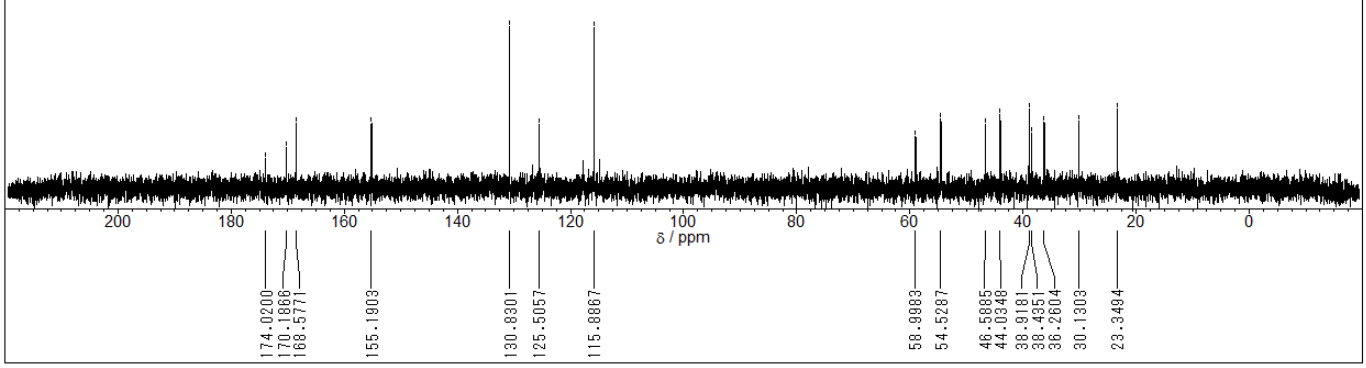
$^1$H NMR (400 MHz, $D_2O$)



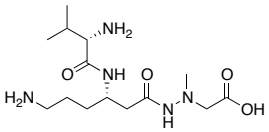

Compound 10d carbon



$^{13}$C NMR (100 MHz, $D_2O$)



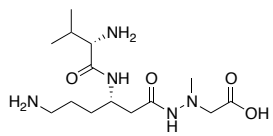
Compound 10e proton



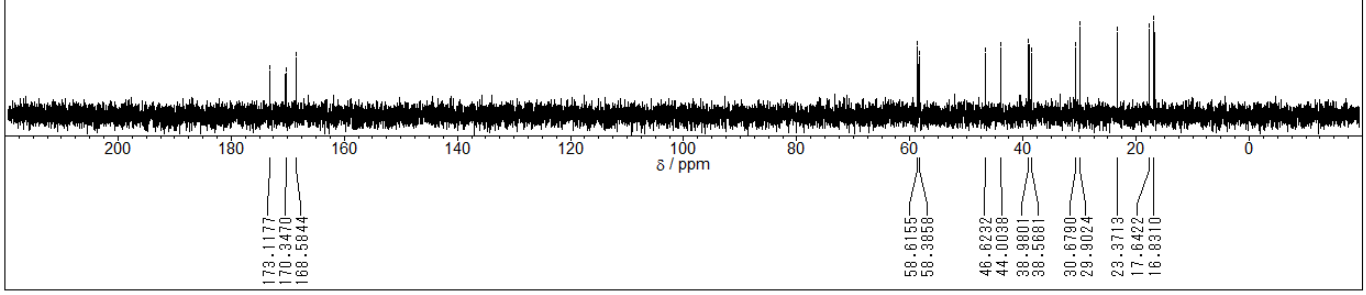
$^{1}$H NMR (400 MHz, $D_2O$)



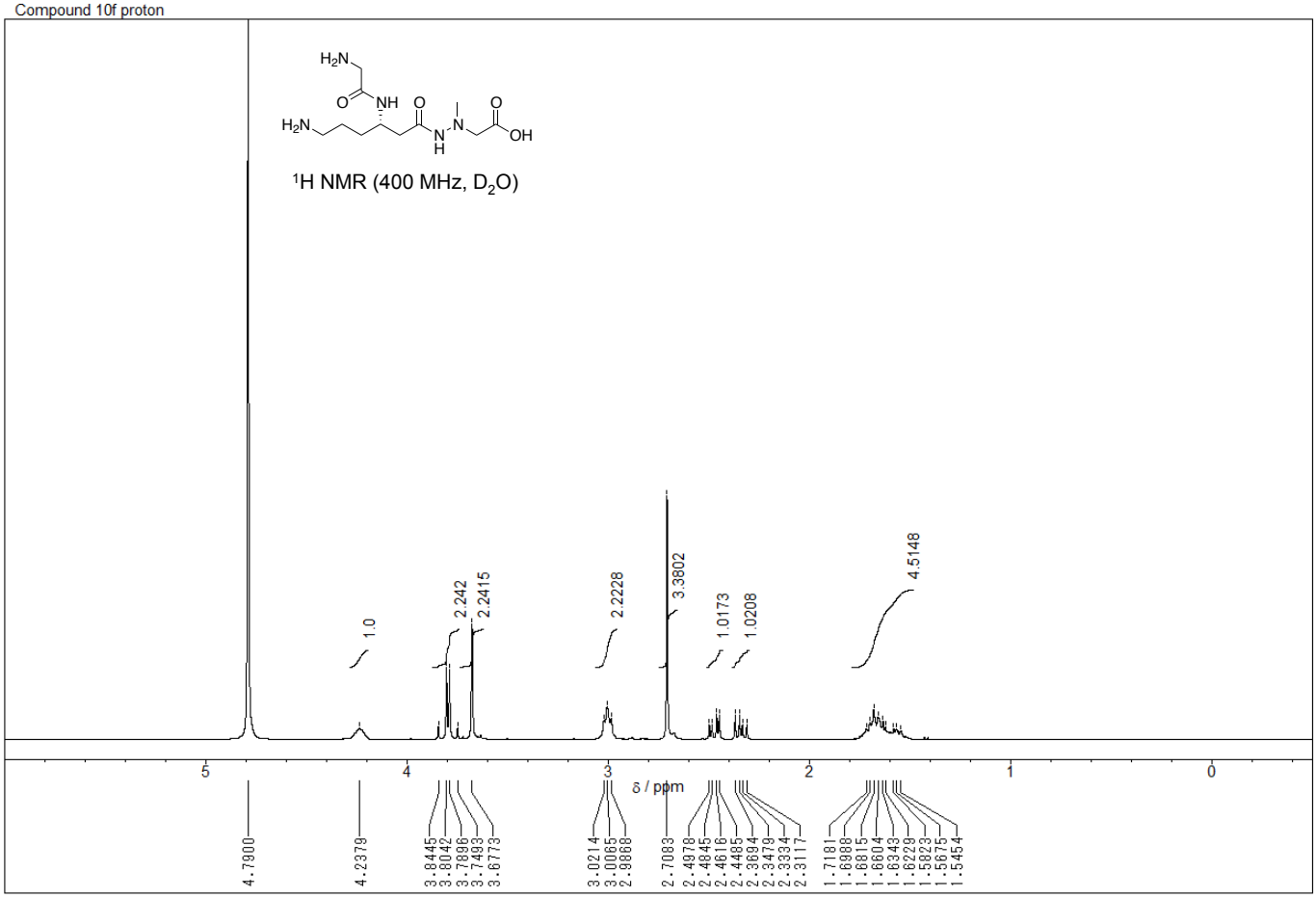

Compound 10e carbon



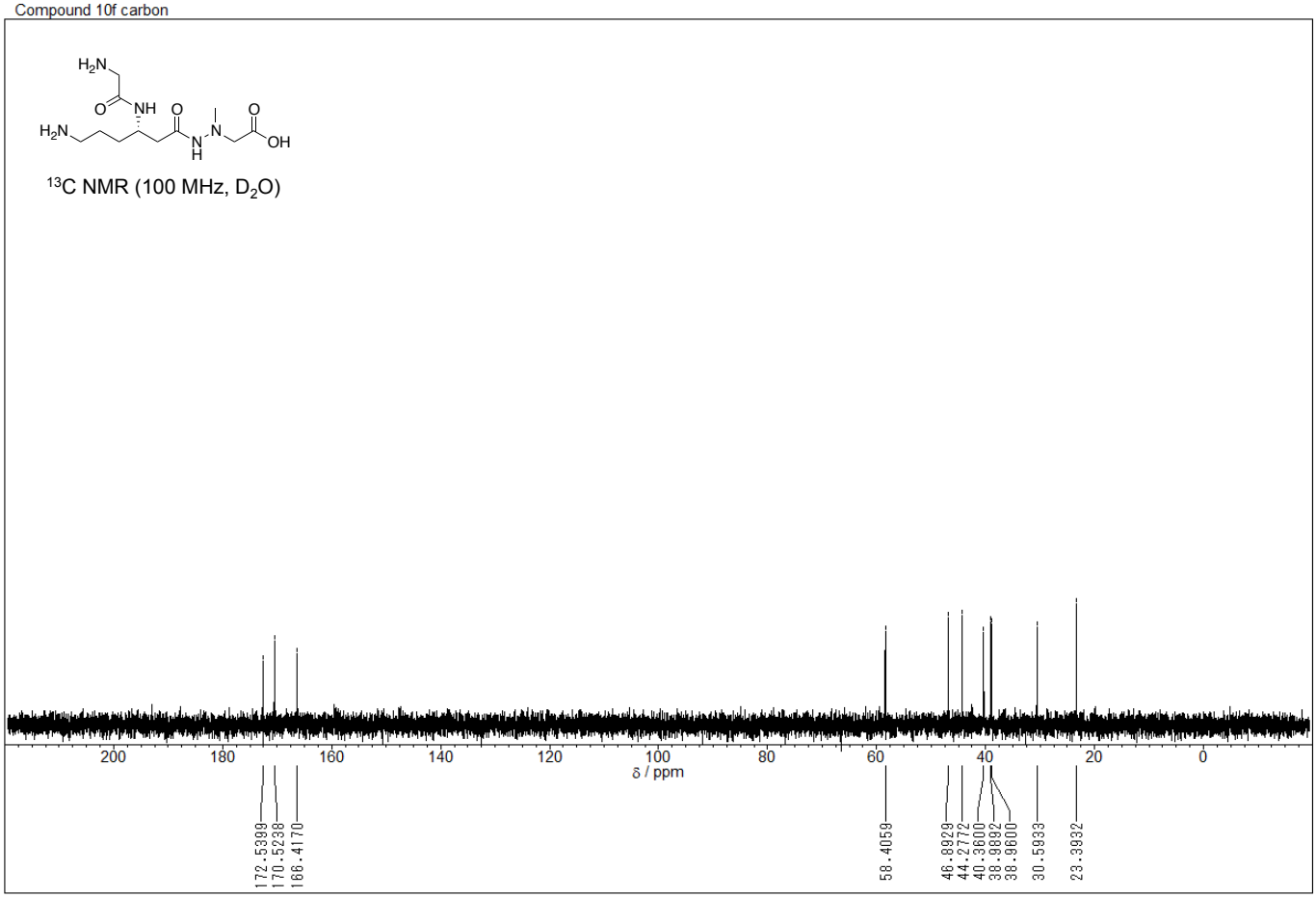
$^{13}$C NMR (100 MHz, $D_2O$)



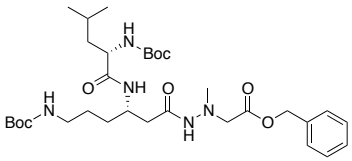
Compound 10f proton




Compound 10f carbon



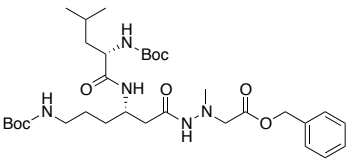
Compound 12a proton



$^{1}$H NMR (400 MHz, $CDCl_3$)

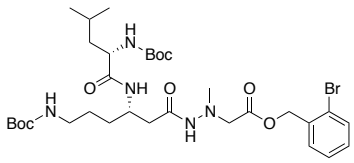



Compound 12a carbon



$^{13}$C NMR (100 MHz, $CDCl_3$)

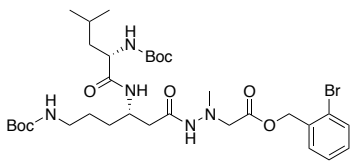
Compound 12b proton



$^{1}$H NMR (400 MHz, $CDCl_3$)

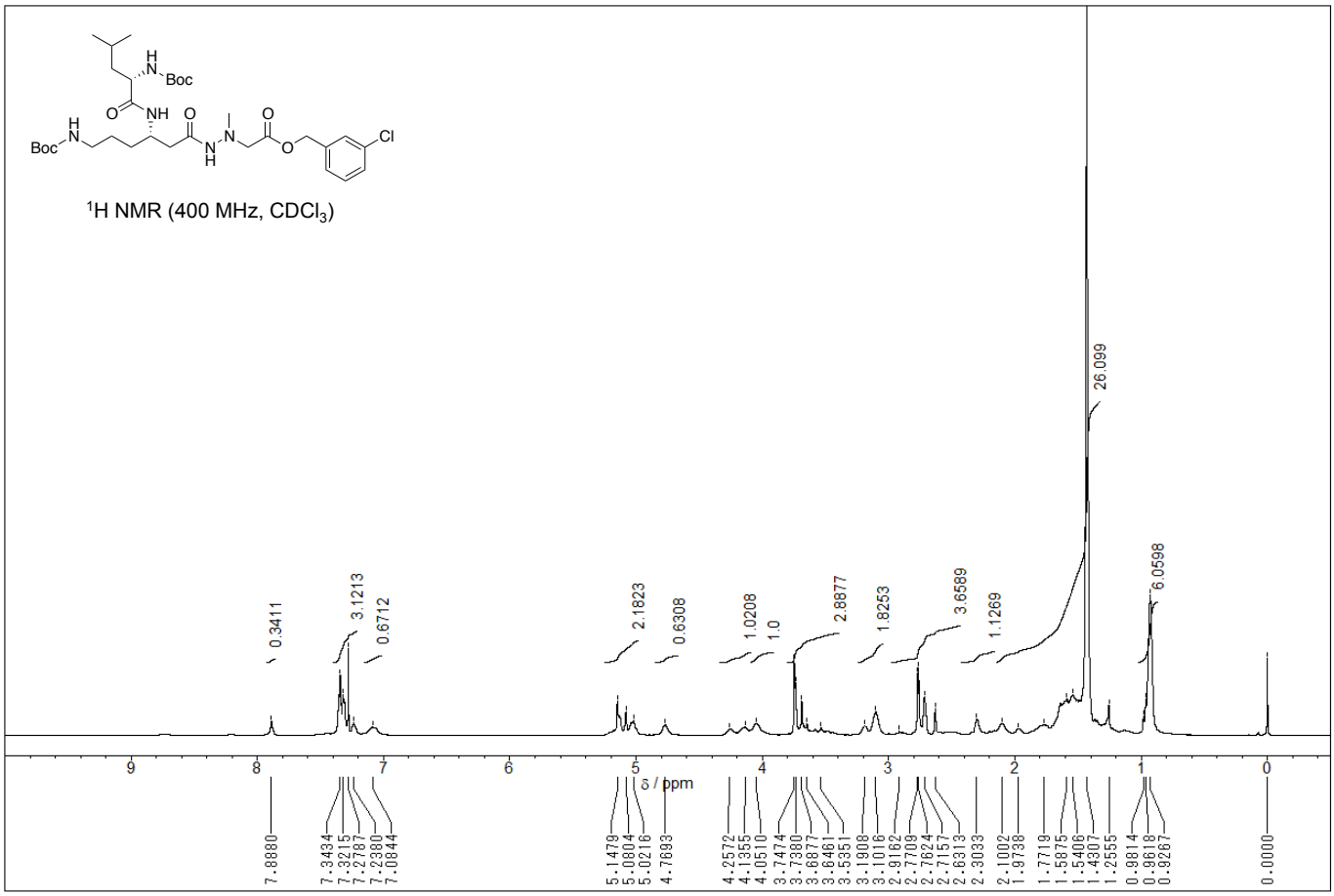



Compound 12b carbon

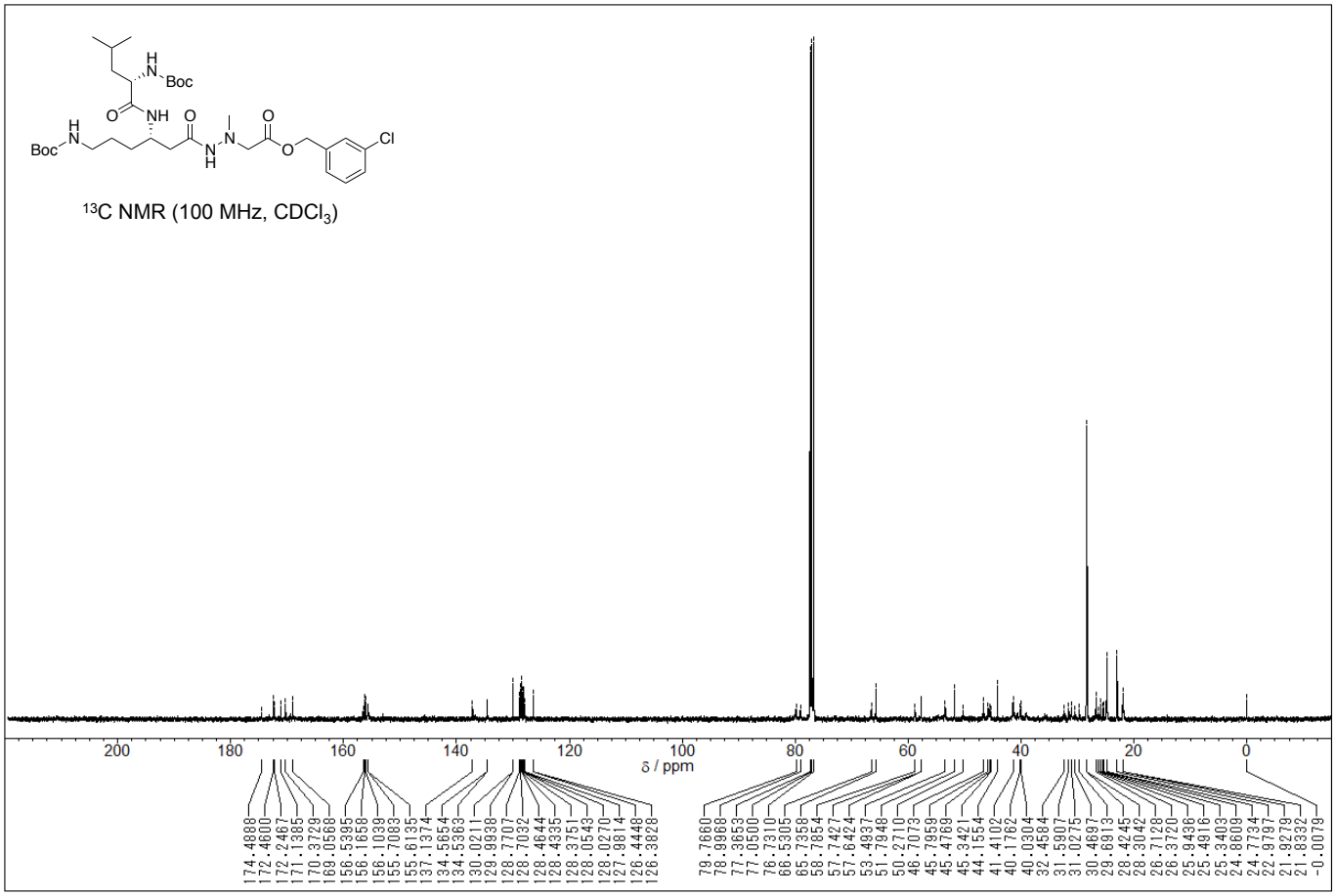


$^{13}$C NMR (100 MHz, $CDCl_3$)

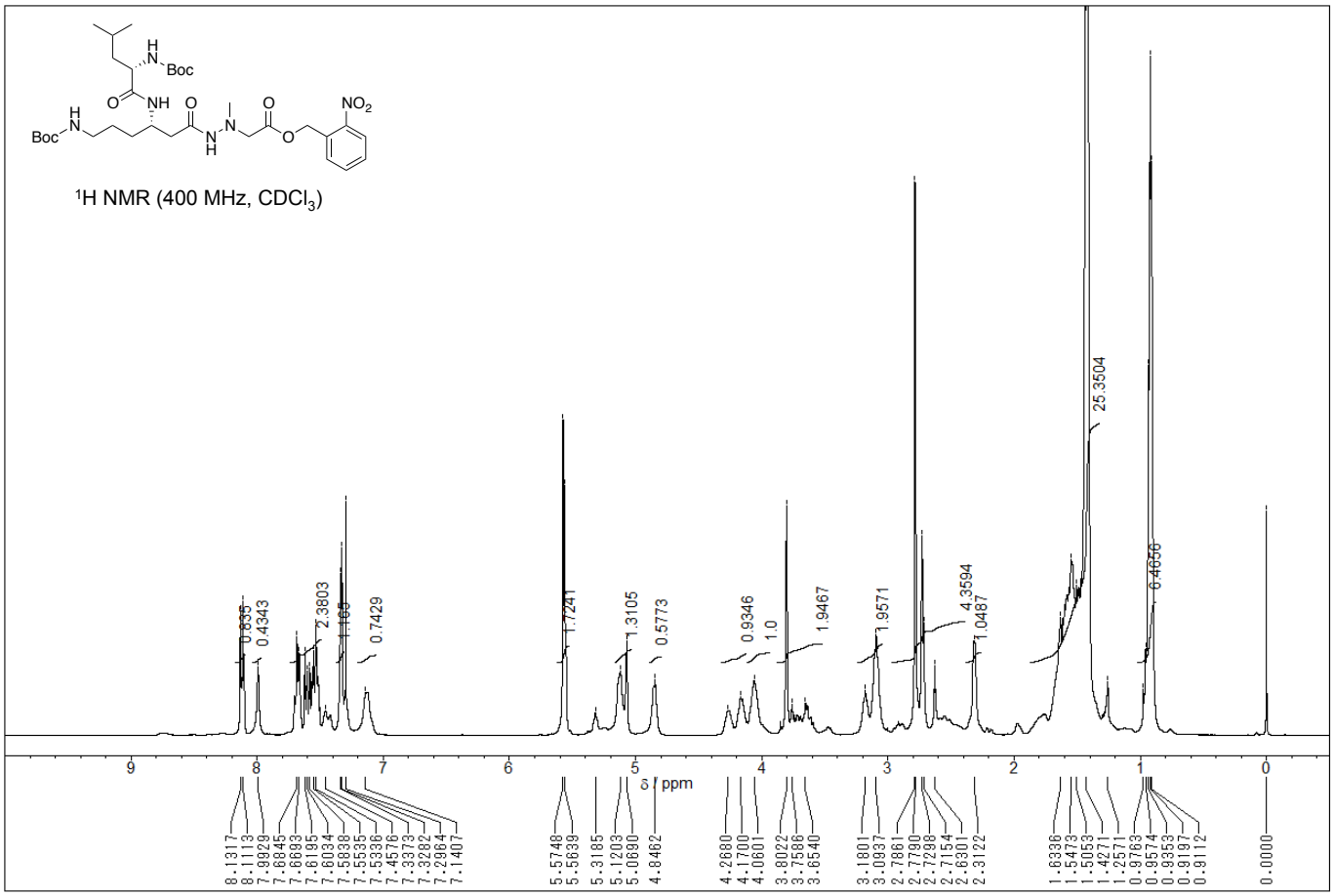
Compound 12c proton



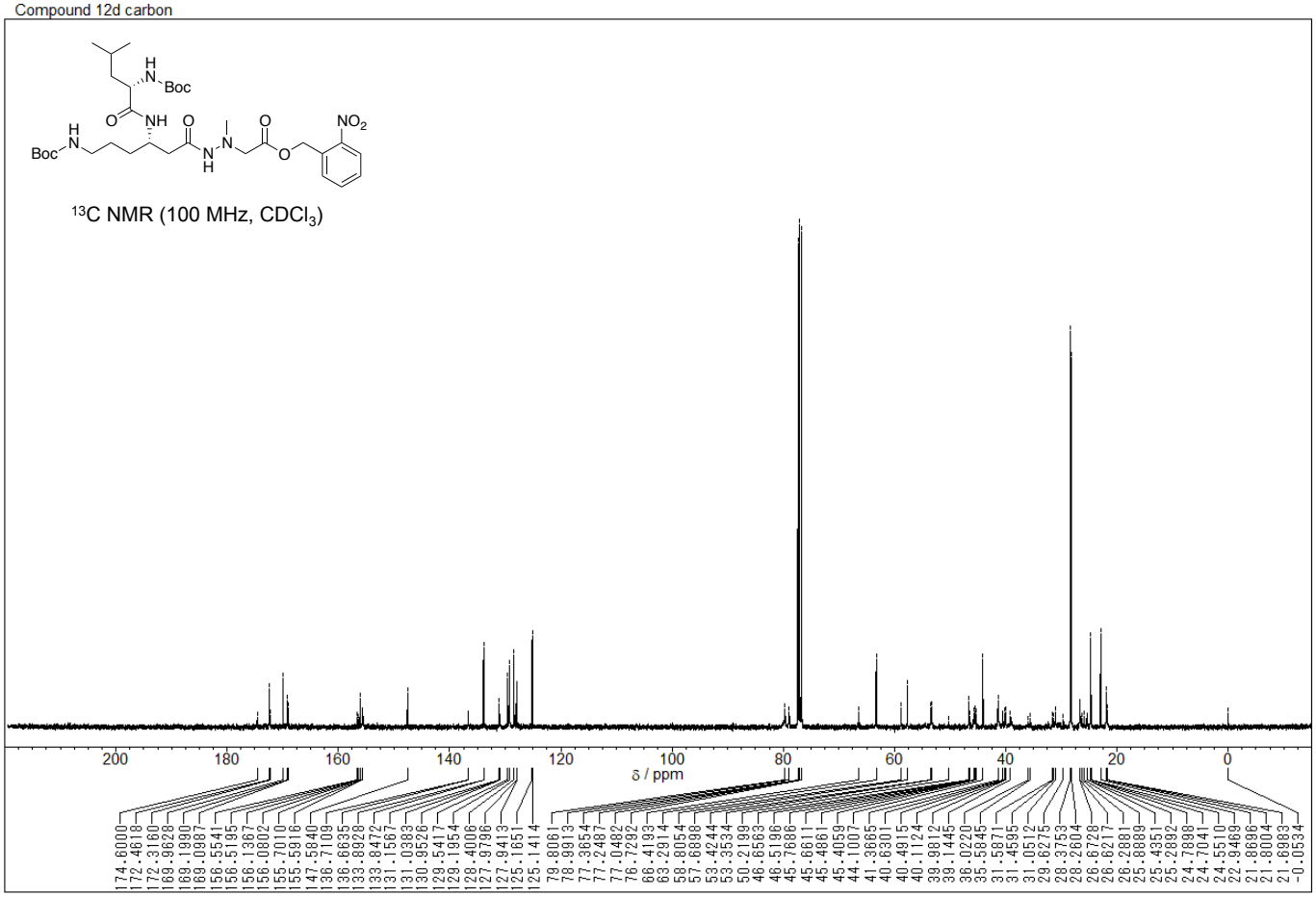

Compound 12c carbon



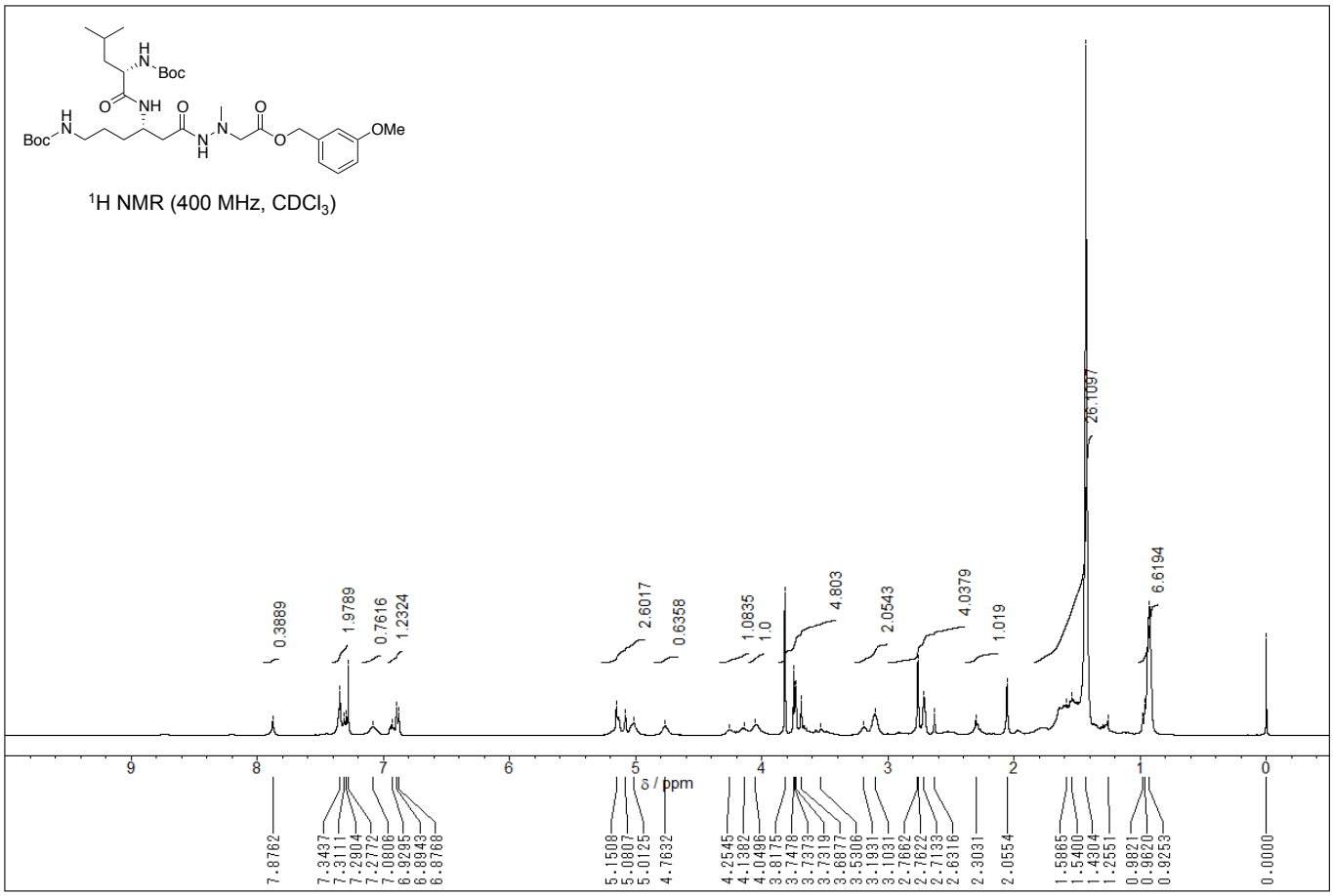
Compound 12d proton



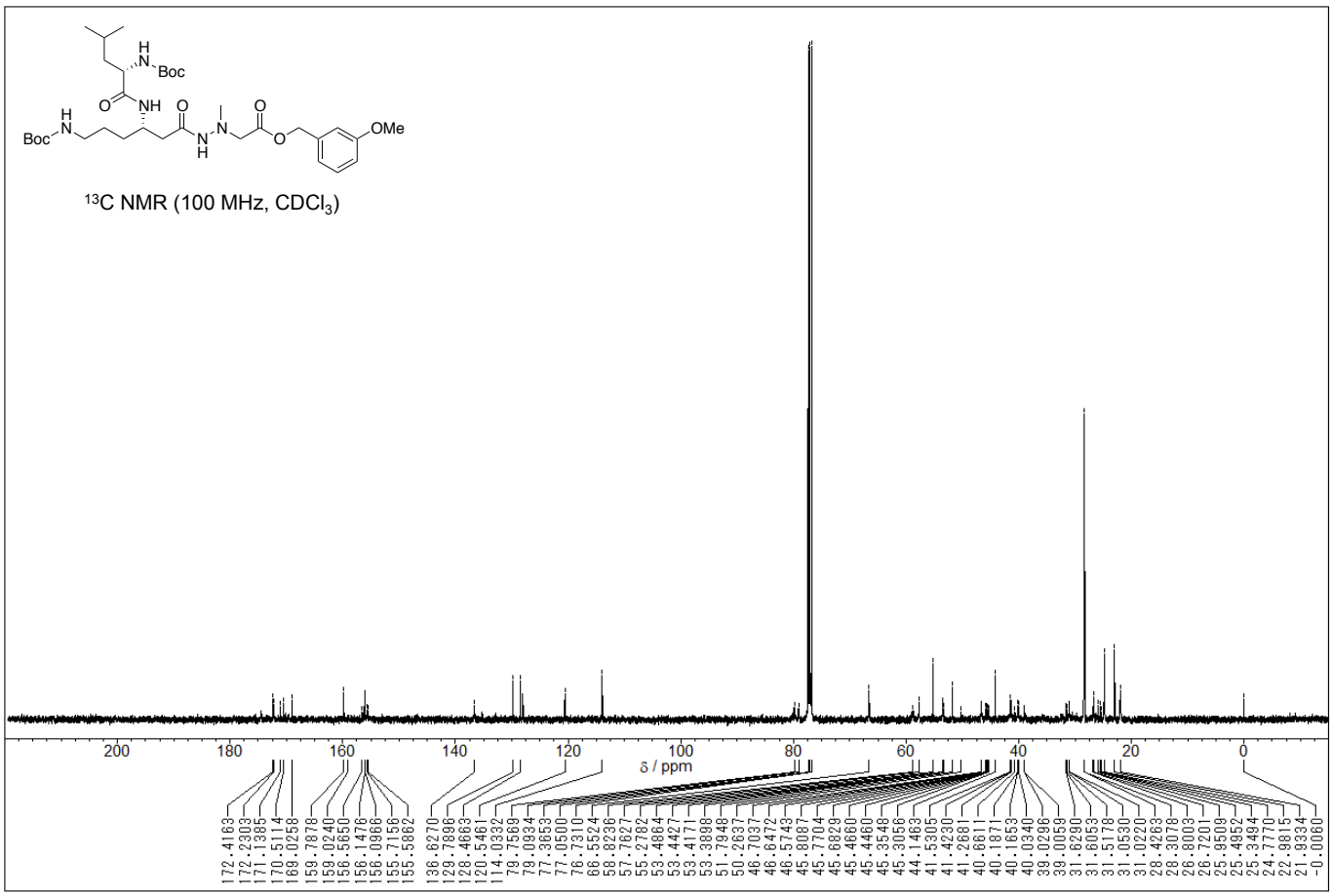

Compound 12d carbon



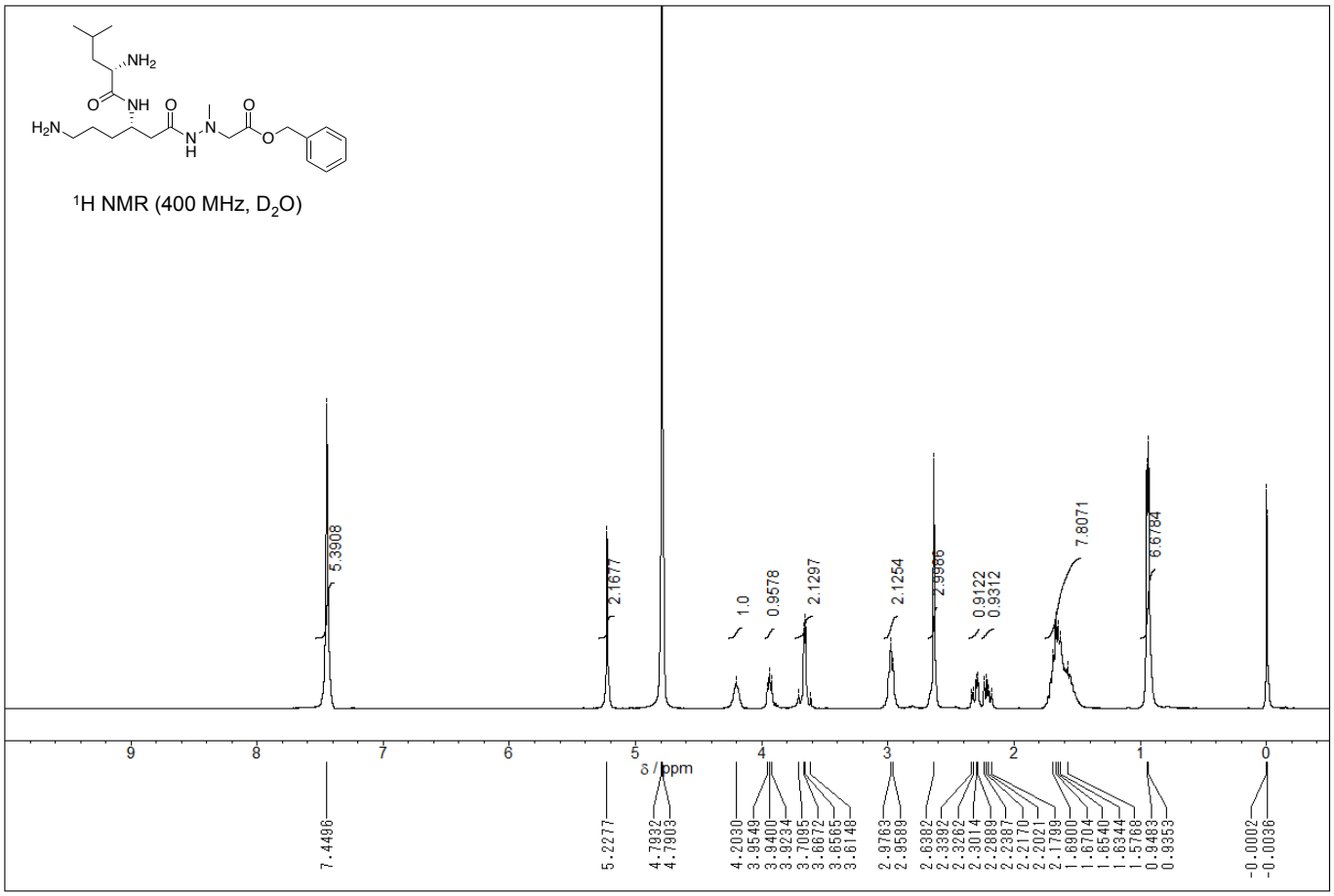
Compound 12e proton



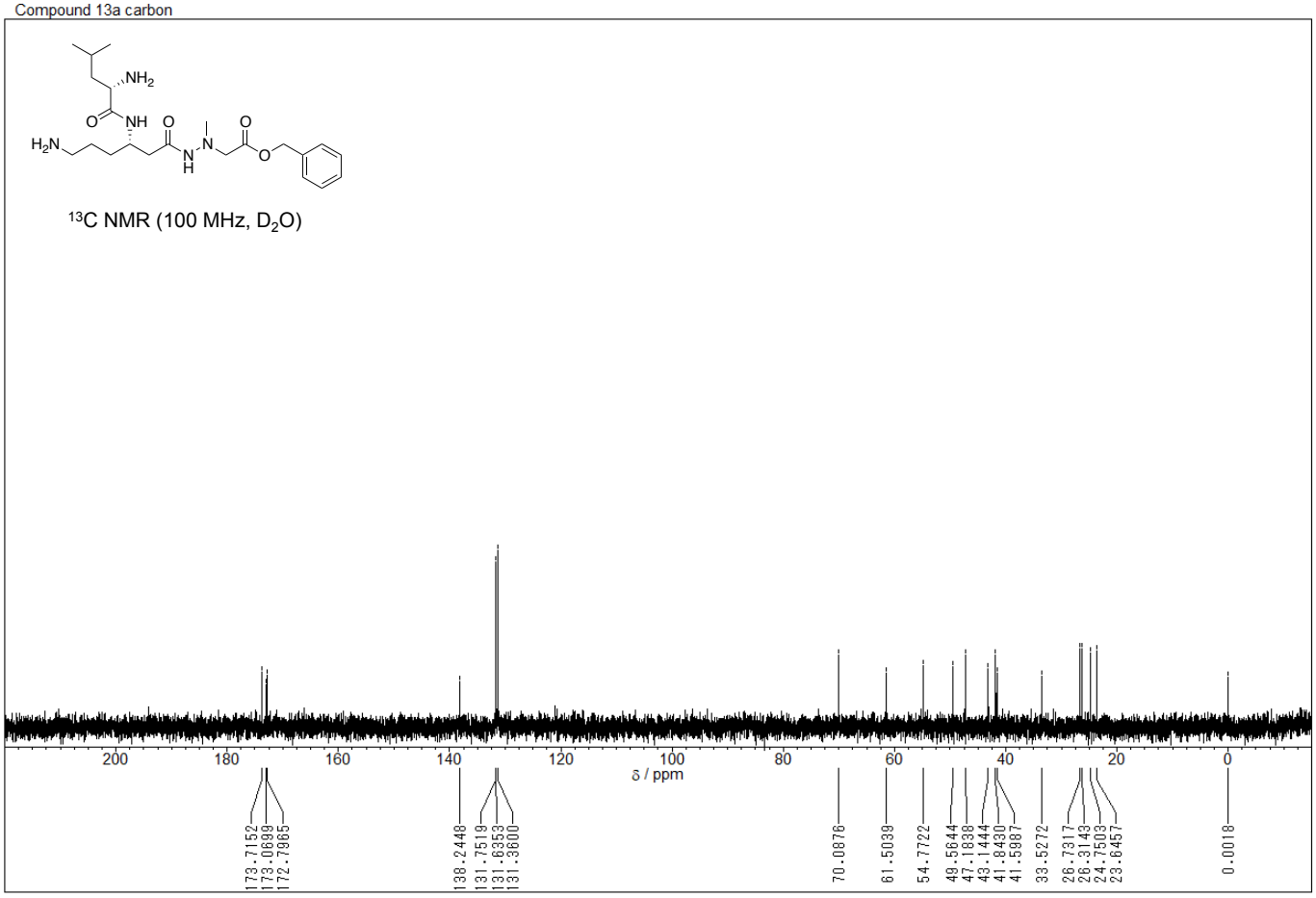

Compound 12e carbon



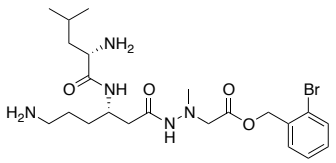
Compound 13a proton




Compound 13a carbon



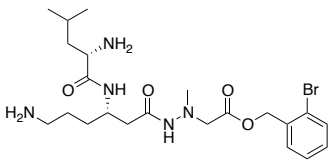
Compound 13b proton



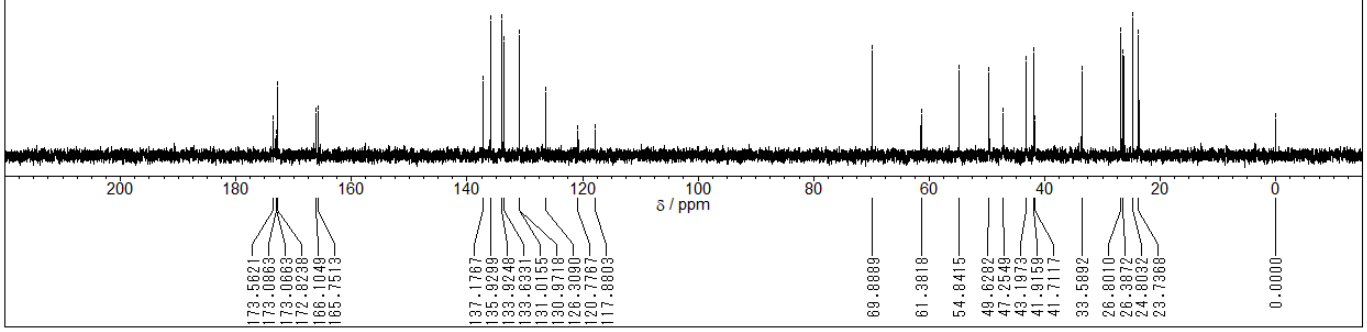
$^{1}$H NMR (400 MHz, $D_2O$)



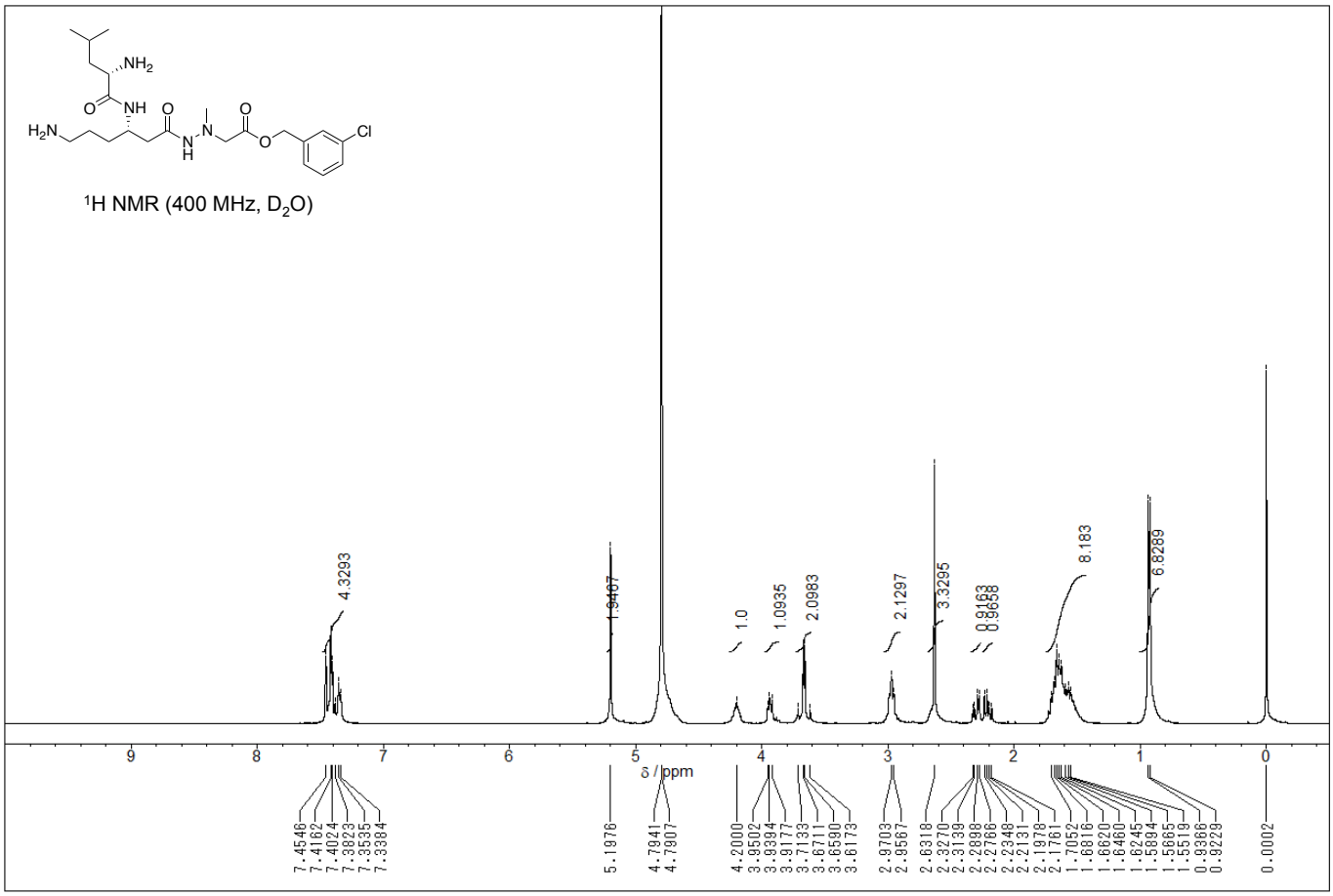

Compound 13b carbon



$^{13}$C NMR (100 MHz, $D_2O$)

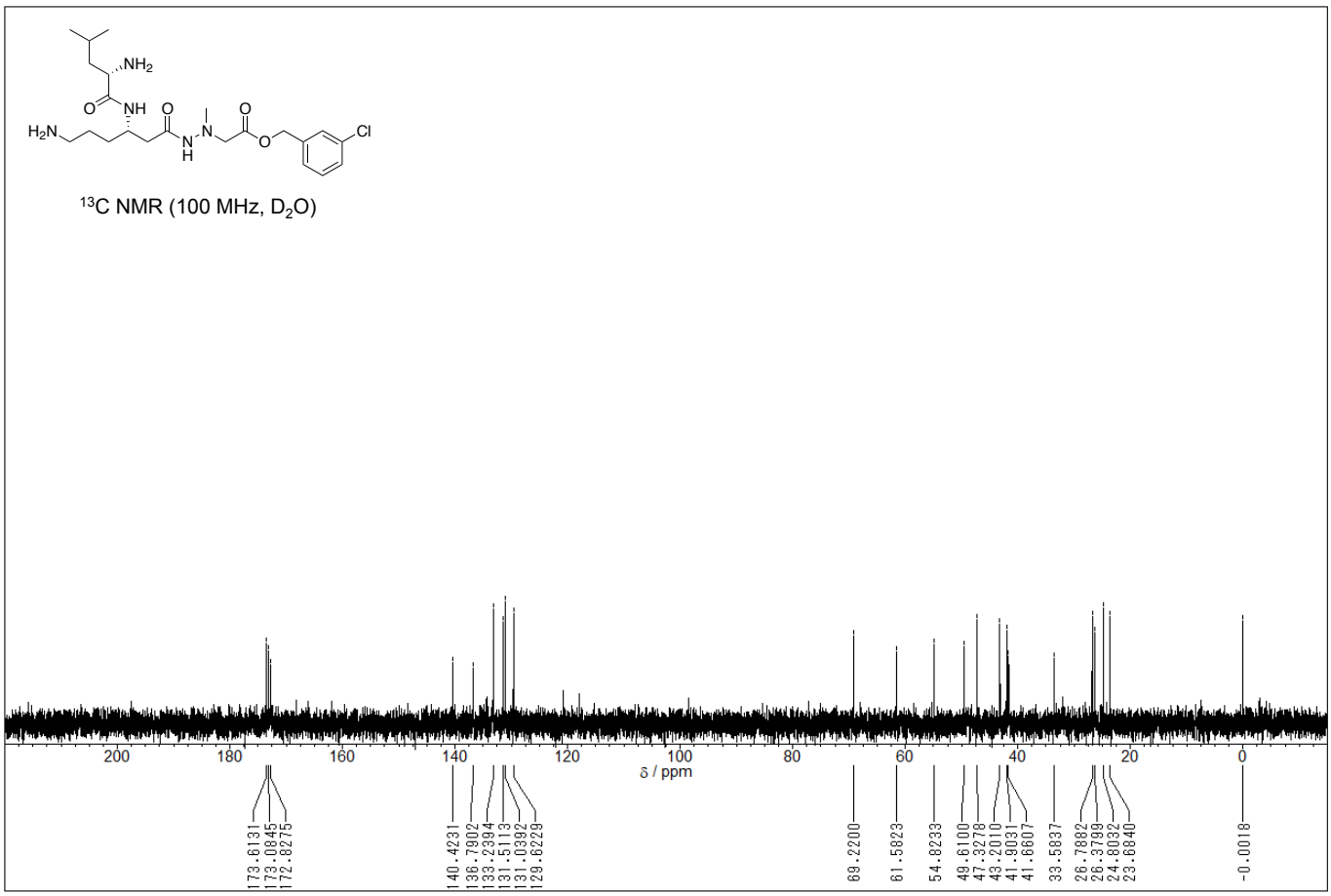


Compound 13c proton



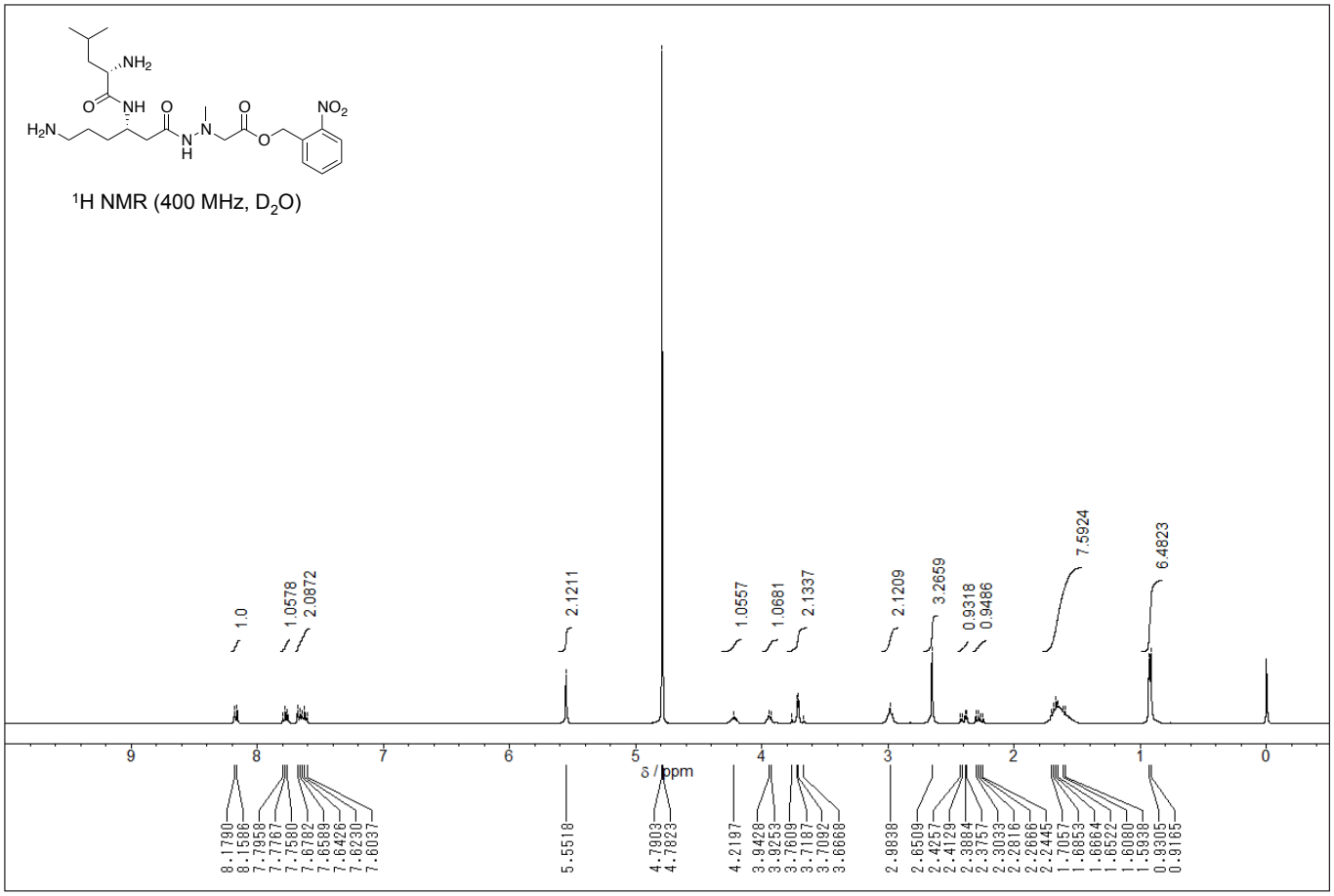

$^{1}$H NMR (400 MHz, $D_2O$)

Compound 13c carbon

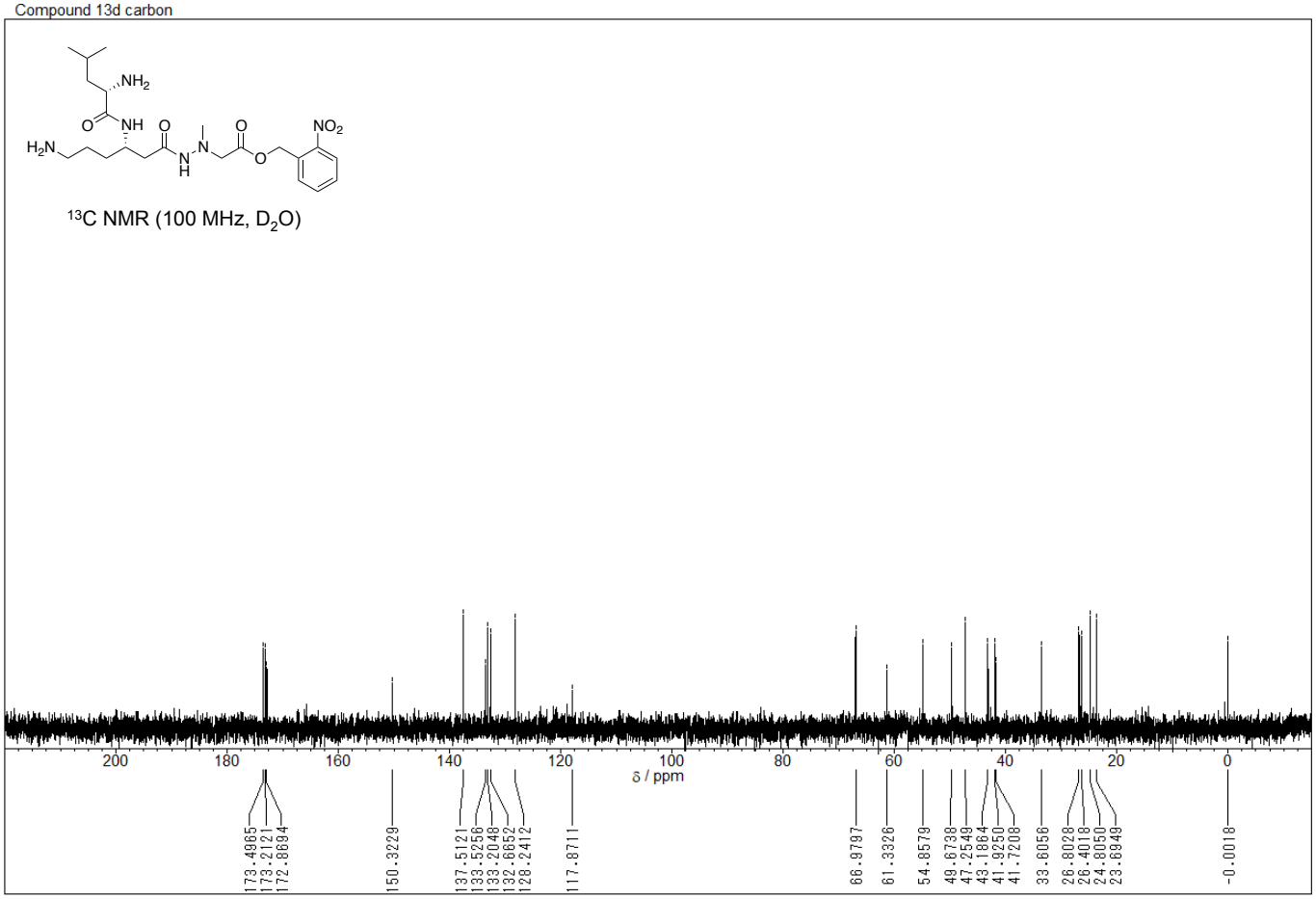



$^{13}$C NMR (100 MHz, $D_2O$)

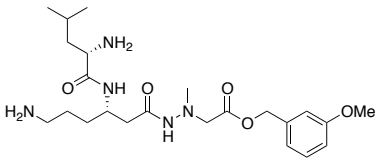
Compound 13d proton




Compound 13d carbon



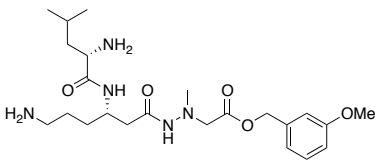
Compound 13e proton



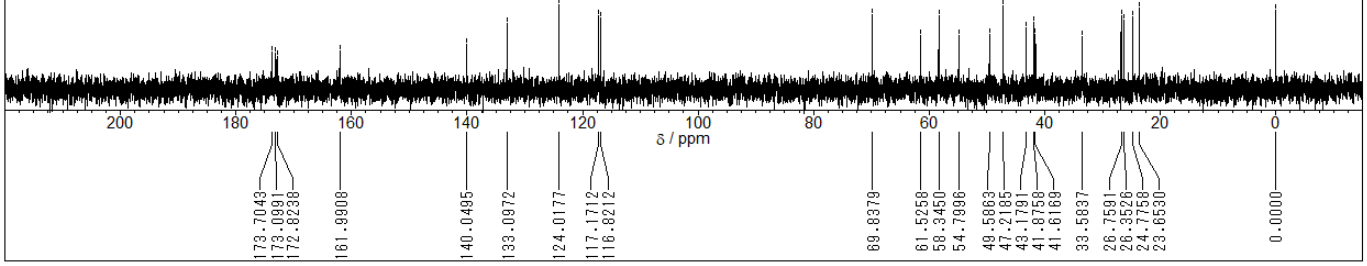
$^1$H NMR (400 MHz, $D_2O$)




Compound 13e carbon



$^{13}$C NMR (100 MHz, $D_2O$)



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2. Hamada, K.; Taguchi, A.; Kotake, M.; Aita, S.; Murakami, S.; Takayama, K.; Yakushiji, F.; Hayashi, Y. Structure-activity relationship studies of 3-*epi*-deoxynegamycin derivatives as potent readthrough drug candidates. *ACS Med. Chem. Lett.* **2015**, *6*, 689–694.
3. Konsoula, R.; Jung, M. *In nitro* plasma stability, permeability and solubility of mercaptoacetamide histone deacetylase inhibitors. *Int. J. Pharm.* **2008**, *361*, 19–25.
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