

Supporting Information

Discovery of SHR0687, a Highly Potent and Peripheral Nervous System-restricted KOR Agonist

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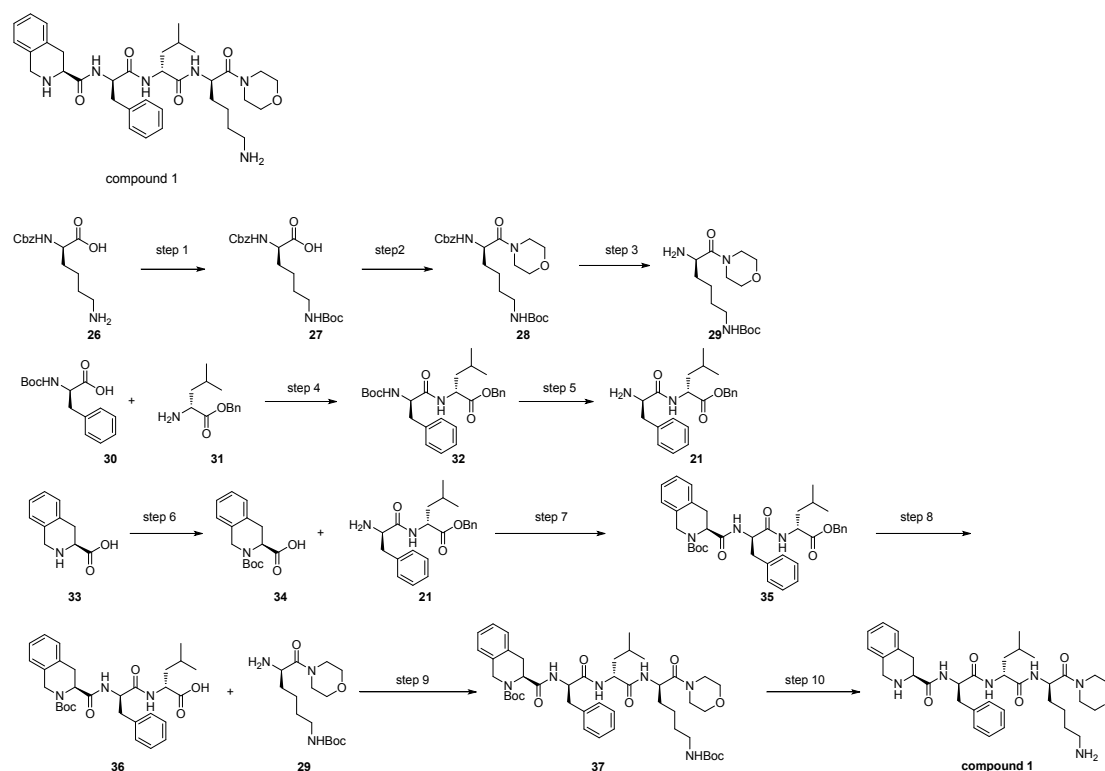
General Information

^1H spectra were recorded on a Bruker Avance-400 NMR spectrometer (^1H : 400 MHz, ^{13}C : 100 MHz) using CDCl_3 , $\text{CD}_3\text{OD}-d_4$ or $\text{DMSO}-d_6$ as a solvent and tetramethylsilane (TMS) as an internal standard. Chemical shift (δ) are reported in parts per million (ppm) relative to TMS ($\delta = 0.00$ ppm) and coupling constant (J) are reported in Hertz (Hz). Splitting patterns are indicated as follows: s, singlet; d, doublet; t, triplet; m, multiplet; brs, broad singlet. Mass spectra were determined on a Finnigan LCQAd (ESI) spectrometer (Finnigan LCQ advantage MAX, Thermo). Products were purified by column chromatography (200-300 mesh, Yantai Huanghai), or prep-TLC (0.4-0.5 mm, GF-254, Liangchen Guiyuan).

Synthetic procedure and characterization for new compounds

Compound 1:

(*S*)-*N*-((*R*)-1-(((*R*)-1-(((*R*)-6-amino-1-morpholino-1-oxohexan-2-yl)amino)-4-methyl-1-oxopentan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)-1,2,3,4-tetrahydroisoquinoline-3-carboxamide



Step 1: (*R*)-2-(((benzyloxy)carbonyl)amino)-6-((tert-butoxycarbonyl)amino)hexanoic acid (compound 27)

A mixture of compound 26 (5.00 g, 17.84 mmol), $(\text{Boc})_2\text{O}$ (8.95 g, 41.02 mmol) and NaHCO_3 (3.47 g, 41.02 mmol) in THF (50 mL) and water (50 mL) was stirred at room temperature overnight. Organic solvent was removed under reduced pressure and the resulting aqueous solution was neutralized with 2N HCl solution. The mixture was extracted with EtOAc (100 mL \times 3) and the combined organic layers were washed with brine, dried over Na_2SO_4 and concentrated to colorless oil. After removal of excess $(\text{Boc})_2\text{O}$ by Kugelrohr distillation, crude title compound (6.7 g) was obtained as colorless oil, which was used directly to the next step without further purification.

MS m/z (ESI): $\text{C}_{19}\text{H}_{27}\text{N}_2\text{O}_6$ [$\text{M}-\text{H}$] $^-$ 379.2, found 378.9.

Step 2: (*R*)-benzyl *tert*-butyl (6-morpholino-6-oxohexane-1,5-diyl)dicarbamate (compound 28)

To a solution of compound 27 (1.14 g, 3.00 mmol) in DMF (15 mL) was added morpholine (319.00 mg, 3.60 mmol), HATU (1.70 g, 4.50 mmol) and DIPEA (581.00 mg, 4.50 mmol) and the mixture was stirred at

room temperature overnight. After adding water and EtOAc (10 mL×3), the combined organic layers were washed with brine, dried over Na₂SO₄. After concentration, the residue was purified by silica gel chromatography to afford title compound (1.3g, 95% yield) as yellow oil.

MS m/z (ESI): C₂₃H₃₆N₃O₆ [M+H]⁺ 450.3, found 450.2.

Step 3: (*R*)-*tert*-butyl (5-amino-6-morpholino-6-oxohexyl)carbamate (compound 29)

A solution of compound 28 (1.30 g, 3.40 mmol) and Pd/C (350 mg) in MeOH (15 mL) was stirred under H₂ atmosphere for 1.5 h. After filtration and removal solvent under reduced pressure, the crude title compound (1.07 g) was obtained as yellow oil, which was used directly to the next step without further purification.

MS m/z (ESI): C₁₅H₃₀N₃O₄ [M+H]⁺ 316.2, found 316.2.

Step 4: (*R*)-benzyl 2-((*R*)-2-((*tert*-butoxycarbonyl)amino)-3-phenylpropanamido)-4-methylpentanoate (compound 32)

To a solution of compound 30 (500.00 mg, 1.89 mmol) and (*R*)-benzyl 2-amino-4-methylpentanoate *p*-toluenesulfonate salt (compound 31) (741 mg, 1.89 mmol) in DMF (10 mL) at room temperature was added HATU (788 mg, 2.07 mmol) and DIPEA (486 mg, 3.77 mmol). The mixture was stirred overnight. After adding water and EtOAc (10 mL×3), the combined organic layers were washed with brine dried over Na₂SO₄. After concentration, the residue was purified by silica gel chromatography to afford title compound (836 mg, 95% yield) as white solid.

MS m/z (ESI): C₂₇H₃₇N₂O₅ [M+H]⁺ 469.3, found 469.3.

Step 5: (*R*)-benzyl 2-((*R*)-2-amino-3-phenylpropanamido)-4-methylpentanoate (compound 21)

To a solution of compound 32 (22.0 g, 46.95 mmol) in DCM (200 mL) was added HCl (117.4 mL, 469.6 mmol, 4M in dioxane) and the mixture was stirred for 2 hours. After concentration under reduced pressure, the crude title compound (19.1 g) was obtained as white solid, which was used directly to the next step without further purification.

MS m/z (ESI): C₂₂H₂₉N₂O₃ [M+H]⁺ 369.2, found 369.2.

Step 6: (*S*)-2-((*tert*-butoxycarbonyl)-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (compound 34)

A solution of compound 33 (1.00 g, 5.64 mmol), (Boc)₂O (1.35 g, 6.21 mmol) and Et₃N (1.14g, 11.28 mmol) in DCM (10 mL) at room temperature was stirred overnight. After concentration under reduced pressure, the residue was purified by silica gel chromatography to afford title compound (500 mg, 32% yield) as white solid.

MS m/z (ESI): C₁₅H₁₈NO₄ [M-H]⁻ 276.1, found 276.0.

Step 7: (*S*)-*tert*-butyl

3-(((*R*)-1-(((*R*)-1-(benzyloxy)-4-methyl-1-oxopentan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)carbamoyl)-3,4-dihydroisoquinoline-2(1H)-carboxylate (compound 35)

To a solution of compound 34 (544 mg, 2.00 mmol) and (*R*)-benzyl 2-((*R*)-2-amino-3-phenylpropanamido)-4-methylpentanoate HCl salt (compound 21) (800 mg, 2.00 mmol) in DMF (15 mL) at room temperature was added HATU (1.14g, 3 mmol) and E₃N (404 mg, 4.00 mmol) and the mixture was stirred overnight. After adding water and EtOAc (20 mL×3 mL), the combined organic layers were washed with brine dried over Na₂SO₄. After concentration, the residue was purified by silica gel chromatography to afford title compound (701 mg, 57% yield) as yellow oil.

MS m/z (ESI): C₃₇H₄₆N₃O₆ [M+H]⁺ 628.3, found 628.3.

Step 8:

(*R*)-2-((*R*)-2-((*S*)-2-((*tert*-butoxycarbonyl)-1,2,3,4-tetrahydroisoquinoline-3-carboxamido)-3-phenylpropanamido)-4-methylpentanoic acid (compound 36)

A mixture of compound 35 (200 mg, 0.32 mmol) and Pd/C (20 mg) in MeOH (6 mL) was stirred at room temperature under H₂ atmosphere overnight. After filtration and concentration under reduced pressure, the

crude title compound (172 mg) was obtained as brown oil, which was used directly to the next step without further purification.

MS *m/z* (ESI): C₃₀H₃₈N₃O₆ [M-H]⁻ 536.3, found 536.3.

Step 9:

(*S*)-*tert*-butyl

3-(((10*R*,13*R*,16*R*)-13-isobutyl-2,2-dimethyl-10-(morpholine-4-carbonyl)-4,12,15-trioxo-17-phenyl-3-oxa-5,11,14-triazaheptadecan-16-yl)carbamoyl)-3,4-dihydroisoquinoline-2(1*H*)-carboxylate (compound 37)

To a solution of compound 36 (69 mg, 0.16 mmol) and compound 29 (50 mg, 0.16 mmol) in DMF (5 mL) was added HATU (90 mg, 0.24 mmol) and E₃N (32 mg, 0.32 mmol). The mixture was stirred overnight at room temperature. After adding water and EtOAc (5 mL×3), the combined organic layers were washed with brine dried over Na₂SO₄. After concentration under reduce pressure, the residue was purified by silica gel chromatography to afford title compound (118 mg, 100% yield) as yellow oil.

MS *m/z* (ESI): C₄₅H₆₇N₆O₉ [M+H]⁺ 835.5, found 835.4.

Step 10: (*S*)-*N*-((*R*)-1-(((*R*)-6-amino-1-morpholino-1-oxohexan-2-yl)amino)-4-methyl-1-oxopentan-2-yl)

amino)-1-oxo-3-phenylpropan-2-yl)-1,2,3,4-tetrahydroisoquinoline-3-carboxamide (compound 1)

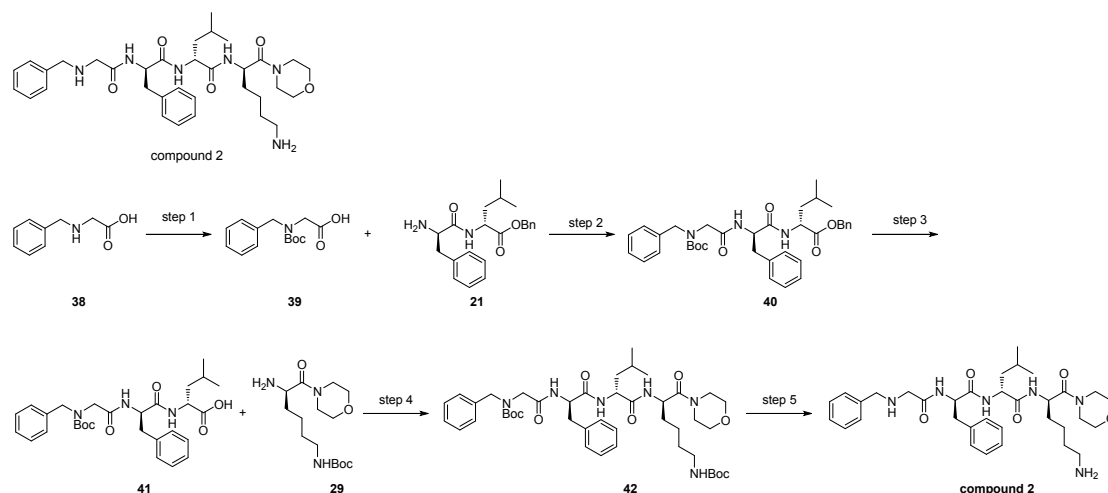
To a solution of compound 37 (300 mg, 0.36 mmol) in DCM (18 mL) at room temperature was added HCl (1 mL, 4 mmol, 4M in dioxane) and the mixture was stirred for 1 hour. After concentraion under reduced pressure, the residue was purified by high performance liquid chromatography to obtain the title compound (80 mg, 40% yield) as white solid.

HRMS *m/z* (ESI): C₃₅H₅₁N₆O₅ [M+H]⁺ 635.4, found 635.6.

¹H NMR (400 MHz, Methanol-*d*₄) δ 7.39 – 7.25 (m, 7H), 7.24 – 7.17 (m, 1H), 7.13 – 7.06 (m, 1H), 4.86 – 4.78 (m, 2H), 4.46 – 4.39 (m, 1H), 4.35 (s, 2H), 4.13 (dd, *J* = 11.9, 5.0 Hz, 1H), 3.73 – 3.63 (m, 5H), 3.63 – 3.43 (m, 2H), 3.36 – 3.27 (m, 2H), 3.02 – 2.83 (m, 4H), 2.67 – 2.54 (m, 1H), 1.85 – 1.55 (m, 7H), 1.53 – 1.36 (m, 2H), 0.98 (d, *J* = 6.4 Hz, 3H), 0.94 (d, *J* = 6.4 Hz, 3H).

Compound 2:

(*R*)-*N*-((*R*)-6-amino-1-morpholino-1-oxohexan-2-yl)-2-((*R*)-2-(benzylamino)acetamido)-3-phenylpropanamido)-4-methylpentanamide



Step 1: 2-(benzyl(*tert*-butoxycarbonyl) amino) acetic acid (compound 39)

A mixture of compound 38 (1.00 g, 6.00 mmol), (Boc)₂O (3.62g, 12.00 mmol) and NaHCO₃ (1.51 g, 18.00 mmol) was stirred in THF (10 mL) and water (10 mL) at room temperature overnight. Organic solvent was removed under reduced pressure and the resulting aqueous solution was neutralized with 2*N* HCl solution. The mixture was extracted with EtOAc (10 mL×3) and the combined organic layers were washed with brine,

dried over Na₂SO₄ and concentrated to afford crude title compound (1.50 g) as white solid, which was used directly to the next step without further purification.

MS m/z (ESI): C₁₄H₁₈NO₄ [M-H]⁻ 264.1, found 264.1.

Step 2: (9*R*,12*R*)-benzyl

5,9-dibenzyl-12-isobutyl-2,2-dimethyl-4,7,10-trioxo-3-oxa-5,8,11-triazatridecan-13-oate (compound 40)

To a solution of compound 39 (265.00 mg, 0.64 mmol) and compound 21 (236 mg, 0.64 mmol) in DMF (6 mL) was added HATU (365 mg, 0.96 mmol) and DIPEA (331 mg, 2.56 mmol) at room temperature and the mixture was stirred overnight. After adding water and EtOAc (5 mL×3), the combined organic layers were separated, washed with brine dried over Na₂SO₄. After concentration, the residue was purified by silica gel chromatography to afford title compound (184 mg, 67% yield) as white solid.

MS m/z (ESI): C₃₆H₄₄N₃O₆ [M-H]⁻ 614.3, found 614.3.

Step 3: (9*R*,12*R*)-5,9-dibenzyl-12-isobutyl-2,2-dimethyl-4,7,10-trioxo-3-oxa-5,8,11-triazatridecan-13-oic acid (compound 41)

A mixture of compound 40 (184 mg, 0.30 mmol) and Pd/C (20 mg) in MeOH (10 mL) was stirred under H₂ atmosphere overnight. After filtration and concentration under reduced pressure, the crude title compound (146 mg) was obtained as white solid, which was used directly to the next step without further purification.

MS m/z (ESI): C₂₉H₃₈N₃O₆ [M-H]⁻ 524.3, found 524.3.

Step 4: tert-butyl

benzyl((10*R*,13*R*,16*R*)-16-benzyl-13-isobutyl-2,2-dimethyl-10-(morpholine-4-carbonyl)-4,12,15,18-tetraoxo-3-oxa-5,11,14,17-tetraazanonadecan-19-yl)carbamate (compound 42)

To a solution of compound 41 (110 mg, 0.21 mmol) and compound 29 (66 mg, 0.21 mmol) in DMF (4 mL) was added HATU (120 mg, 0.35 mmol) and DIPEA (810 mg, 0.63 mmol) and the mixture was stirred overnight. After adding water and EtOAc (10 mL×3), the organic phase was separated, washed with brine dried over Na₂SO₄. After concentration, the residue was purified by silica gel chromatography to afford title compound (130 mg, 75% yield) as yellow oil.

MS m/z (ESI): C₄₄H₆₇N₆O₉ [M+H]⁺ 823.5, found 823.5.

Step 5:

(*R*)-*N*-((*R*)-6-amino-1-morpholino-1-oxohexan-2-yl)-2-((*R*)-2-(2-(benzylamino)acetamido)-3-phenylpropanamido)-4-methylpentanamide (compound 2)

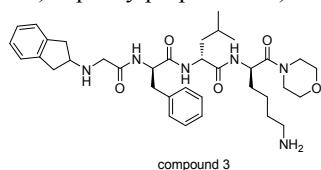
To a solution of compound 42 (130 mg, 0.16 mmol) in DCM (3 mL) was added TFA (3 mL) and the mixture was stirred for 2 hours. After concentration under reduced pressure, the residue was purified by high performance liquid chromatography to obtain the title compound (52 mg, 40% yield) as white solid.

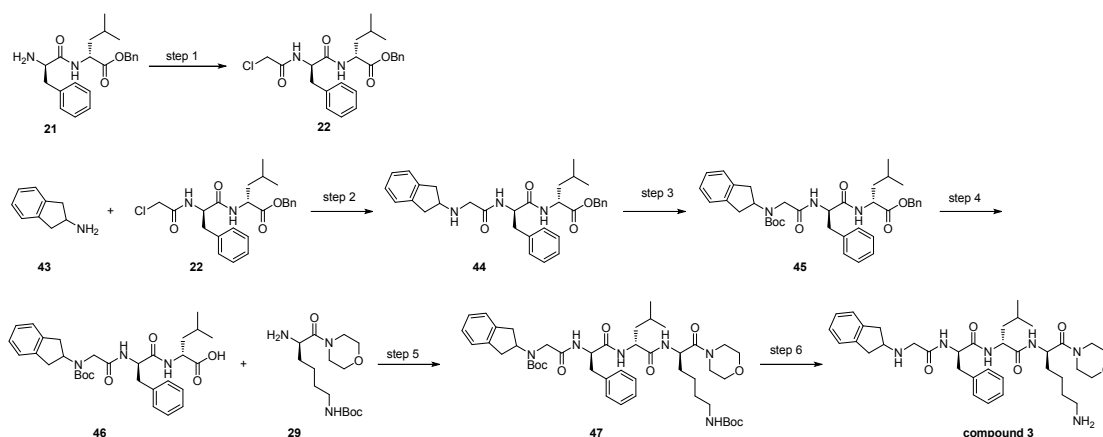
MS m/z (ESI): C₃₄H₅₁N₆O₅ [M+H]⁺ 623.4, found 623.6.

¹H NMR (400 MHz, Methanol-*d*₄) δ 7.39 – 7.17 (m, 10H), 4.80 (dd, *J* = 8.8, 5.0 Hz, 1H), 4.66 (dd, *J* = 9.4, 4.9 Hz, 1H), 4.39 (dd, *J* = 9.8, 5.0 Hz, 1H), 3.71 – 3.59 (m, 7H), 3.59 – 3.45 (m, 2H), 3.25 – 3.13 (m, 3H), 2.96 – 2.86 (m, 3H), 1.81 – 1.51 (m, 7H), 1.42 (dt, *J* = 13.8, 6.5 Hz, 2H), 1.29 (t, *J* = 7.3 Hz, 1H), 0.95 (d, *J* = 6.2 Hz, 3H), 0.92 (d, *J* = 6.3 Hz, 3H).

Compound 3:

(*R*)-*N*-((*R*)-6-amino-1-morpholino-1-oxohexan-2-yl)-2-((*R*)-2-((2,3-dihydro-1*H*-inden-2-yl)amino)acetamido)-3-phenylpropanamido)-4-methylpentanamide





Step 1: (*R*)-benzyl 2-((*R*)-2-(2-chloroacetamido)-3-phenylpropanamido)-4-methylpentanoate (compound 22)
Compound 21 (500 mg, 1.36 mmol) and Et₃N (275 mg, 2.72 mmol) was dissolved in DCM (10 mL) and then chloroacetyl chloride (230 mg, 2 mmol) was added dropwise. After stirring for 12 hours at room temperature, the reaction solution was poured into water and washed with saturated ammonium chloride solution. The organic phase was dried over anhydrous sodium sulfate and filtered. The filtrate was concentrated under reduced pressure to obtain the crude title compound (500 mg) as yellow solid, which was used directly to the next step without further purification.

MS *m/z* (ESI): C₂₄H₃₀ClN₂O₄ [M+H]⁺ 445.2, found 445.1.

Step 2: benzyl

(2*R*)-2-[[[(2*R*)-2-[[2-(indan-2-ylamino)acetyl]amino]-3-phenyl-propanoyl]amino]-4-methyl-pentanoate (compound 44)

A mixture of compound 43 (127 mg, 0.75 mmol), compound 22 (222 mg, 0.50 mmol), KI (415 mg, 2.50 mmol) and K₂CO₃ (345 mg, 2.50 mmol) in DMF (10 mL) was stirred at 60 °C overnight. After adding water and EtOAc (30 mL), the organic phase was separated, washed with brine dried over Na₂SO₄. After concentration under reduced pressure, the residue was purified by silica gel chromatography to afford title compound (300 mg, 71% yield) as yellow solid.

MS *m/z* (ESI): C₃₃H₄₀N₃O₄ [M+H]⁺ 542.3, found 542.4.

Step 3: (9*R*,12*R*)-benzyl

9-benzyl-5-(2,3-dihydro-1*H*-inden-2-yl)-12-isobutyl-2,2-dimethyl-4,7,10-trioxo-3-oxa-5,8,11-triazatridecan-13-oate (compound 45)

A mixture of compound 44 (300 mg, 0.55 mmol), (Boc)₂O (181 mg, 0.83 mmol) and Et₃N (213 mg, 1.65 mmol) was stirred in DCM (10 mL) at room temperature overnight. After concentration, the residue was purified by silica gel chromatography to afford title compound (170 mg, 48% yield) as white solid.

MS *m/z* (ESI): C₃₈H₄₈N₃O₆ [M+H]⁺ 642.4, found 642.4.

Step 4:

(2*R*)-2-[[[(2*R*)-2-[[2-[(*tert*-butoxycarbonyl(indan-2-yl)amino]acetyl]amino]-3-phenyl-propanoyl]amino]-4-methyl-pentanoic acid (compound 46)

A mixture of compound 45 (170 mg, 0.26 mmol) and Pd/C (50 mg) was stirred in MeOH (10 mL) under H₂ atmosphere overnight. After filtration and concentration under reduced pressure, the crude title compound (112 mg) was obtained as colorless oil, which was used directly to next without further purification.

MS *m/z* (ESI): C₃₁H₄₀N₃O₆ [M-H]⁻ 550.3, found 550.3.

Step 5: *tert*-butyl

N-[2-[[[(1*R*)-1-benzyl-2-[[[(1*R*)-1-[[[(1*R*)-5-(*tert*-butoxycarbonylamino)-1-(morpholine-4-carbonyl)pentyl]carb

amoyl]-3-methyl-butyl]amino]-2-oxo-ethyl]amino]-2-oxo-ethyl]-*N*-indan-2-yl-carbamate (compound 47)

To a solution of compound 46 (112 mg, 0.20 mmol) and compound 29 (63 mg, 0.20 mmol) in DMF (4 mL) was added HATU (114 mg, 0.3 mmol) and DIPEA (77.40 mg, 0.60 mmol) and the mixture was stirred overnight. After adding water and EtOAc (20 mL), the organic phase was separated, washed with brine dried over Na₂SO₄. After concentration, the residue was purified by silica gel chromatography to afford title compound (169 mg, 100% yield) as brown solid.

MS *m/z* (ESI): C₄₆H₆₉N₆O₉ [M+H]⁺ 849.5, found 849.4.

Step 6:

(*R*)-*N*-((*R*)-6-amino-1-morpholino-1-oxohexan-2-yl)-2-((*R*)-2-((2,3-dihydro-1*H*-inden-2-yl)amino)acetamido)-3-phenylpropanamido)-4-methylpentanamide (compound 3)

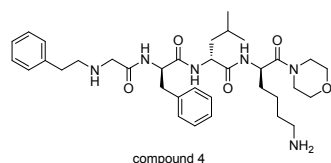
To a solution of compound 47 (85 mg, 0.1 mmol) in DCM (10 mL) was added TFA (2 mL) at room temperature and the mixture was stirred for 2 hours. After concentration under reduced pressure, the residue was purified by high performance liquid chromatography to obtain the title compound (31 mg, 45% yield) as white solid.

MS *m/z* (ESI): C₃₆H₅₃N₆O₅ [M+H]⁺ 649.4, found 649.4.

¹H NMR (400 MHz, Methanol-*d*₄) δ 7.31 – 7.20 (m, 9H), 4.61 (dd, *J* = 9.2, 5.3 Hz, 1H), 4.43 – 4.36 (m, 1H), 4.10 – 3.96 (m, 2H), 3.87 (d, *J* = 15.9 Hz, 1H), 3.69 – 3.60 (m, 3H), 3.59 – 3.45 (m, 4H), 3.38 (ddd, *J* = 16.6, 8.1, 2.9 Hz, 2H), 3.23 – 3.05 (m, 3H), 3.04 – 2.87 (m, 3H), 1.81 – 1.48 (m, 7H), 1.44 – 1.28 (m, 4H), 0.95 (d, *J* = 6.0 Hz, 3H), 0.90 (d, *J* = 5.9 Hz, 3H).

Compound 4:

(*R*)-*N*-((*R*)-6-amino-1-morpholino-1-oxohexan-2-yl)-4-methyl-2-((*R*)-2-(2-(phenethylamino)acetamido)-3-phenylpropanamido)pentanamide



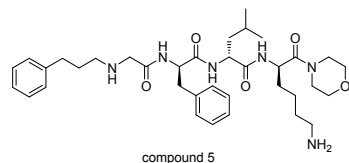
Compound 4 (white solid) was synthesized followed by the similar procedure of compound 3, while the starting material 2,3-dihydro-1*H*-inden-2-amine was replaced by 2-phenylethanamine instead.

MS *m/z* (ESI): C₃₅H₅₃N₆O₅ [M+H]⁺ 637.4, found 637.6.

¹H NMR (400 MHz, Methanol-*d*₄) δ 7.43 – 7.21 (m, 9H), 7.21 – 7.10 (m, 1H), 4.87 – 4.78 (m, 1H), 4.78 – 4.69 (m, 1H), 4.45 – 4.34 (m, 1H), 3.88 (d, *J* = 16 Hz, 1H), 3.79 – 3.43 (m, 9H), 3.27 – 3.04 (m, 3H), 3.01 – 2.81 (m, 5H), 1.88 – 1.33 (m, 9H), 0.97 (d, *J* = 6.4 Hz, 3H), 0.93 (d, *J* = 6.4 Hz, 3H).

Compound 5:

(*R*)-*N*-((*R*)-6-amino-1-morpholino-1-oxohexan-2-yl)-4-methyl-2-((*R*)-3-phenyl-2-((3-phenylpropyl)amino)acetamido)propanamido)pentanamide



Compound 5 (white solid) was synthesized followed by the similar procedure of compound 3, while the starting material 2,3-dihydro-1*H*-inden-2-amine was replaced by 3-phenylpropan-1-amine instead.

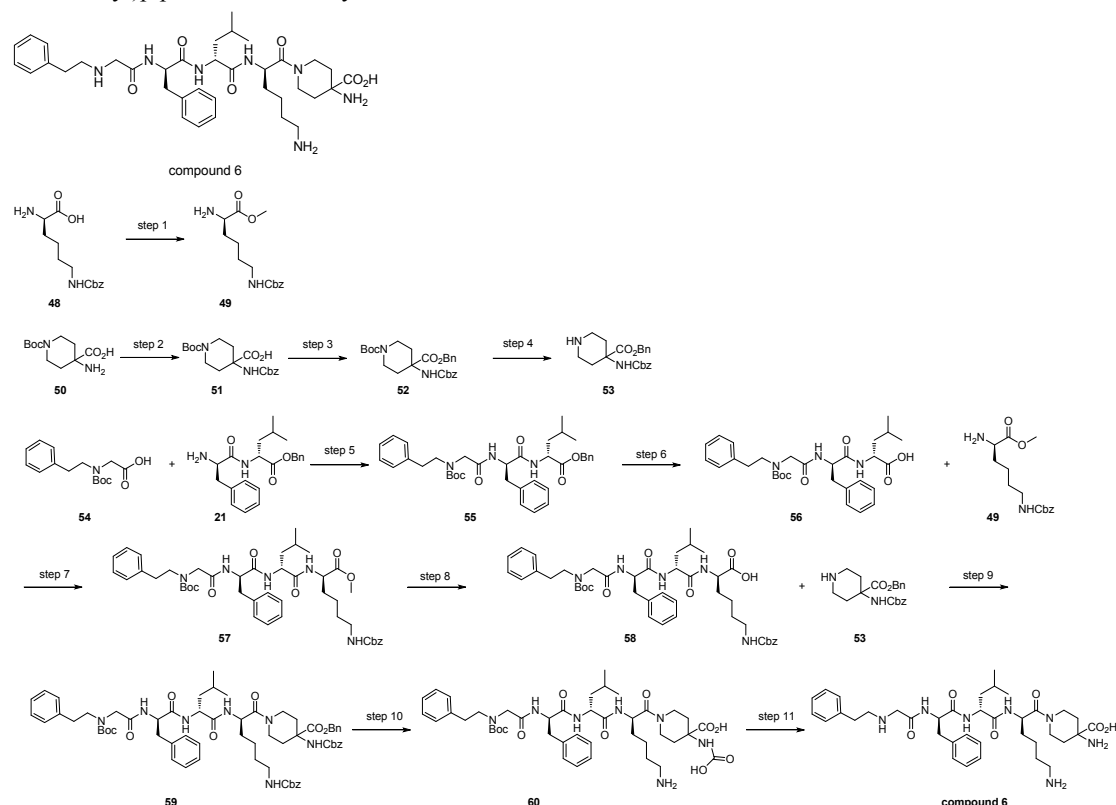
MS *m/z* (ESI): C₃₆H₅₅N₆O₅ [M+H]⁺ 651.4, found 651.5.

¹H NMR (400 MHz, Methanol-*d*₄) δ 7.39–7.19 (m, 6H), 7.18 – 7.07 (m, 2H), 7.04 – 6.92 (m, 2H), 4.83 – 4.75 (m, 1H), 4.43 – 4.23 (m, 3H), 3.84 – 3.44 (m, 9H), 3.31 – 3.20 (m, 1H), 3.00 – 2.79 (m, 3H), 2.74 – 2.50 (m,

1H), 2.43-2.25 (m, 1H), 2.16-2.02 (m, 1H), 2.02-1.33 (m, 12 H), 0.96 (d, $J = 6.4$ Hz, 3H), 0.92 (d, $J = 6.4$ Hz, 3H).

Compound 6:

4-amino-1-((2*R*,5*R*,8*R*)-2-(4-aminobutyl)-8-benzyl-5-isobutyl-4,7,10-trioxo-14-phenyl-3,6,9,12-tetraazatetra decan-1-oyl)piperidine-4-carboxylic acid



Step 1: (*R*)-methyl 2-amino-6-(((benzyloxy)carbonyl)amino)hexanoate hydrochloride (compound 49)

1.3 mL of dichlorosulfoxide was dissolved in 20 mL of methanol, and cooled to 0°C in a ice bath. Then compound 48 (2.00 g, 7.10 mmol) was added. After stirring for 12 hours at room temperature, the reaction solution was concentrated under reduced pressure to obtain the crude title compound (2.09 g) as white solid, which was used directly in the next step without purification.

MS m/z (ESI): $C_{15}H_{23}N_2O_4$ $[M+H]^+$ 295.2, found 295.2.

Step 2: 4-Benzyloxycarbonylamino-piperidine-1,4-dicarboxylic acid mono-*tert*-butyl ester (compound 51)

To a solution of compound 50 (2.00 g, 8.19 mmol) in dioxane (30 mL) and water (30 mL) at room temperature was added CbzCl (2.10 g, 12.3 mmol) and K_2CO_3 (3.40 mmol, 24.60 mmol). After stirring overnight, the organic solvent was removed under reduced pressure. The aqueous was extracted with EtOAc (30 mL×3). The combined organic layers were washed with brine and dried over Na_2SO_4 . After concentration under reduced pressure, the residue was purified by silica gel chromatography to afford title compound (2.2 g, 71% yield) as white solid.

MS m/z (ESI): $C_{19}H_{27}N_2O_6$ $[M+H]^+$ 379.2, found 379.2.

Step 3: 4-benzyloxycarbonylamino-piperidine-1,4-dicarboxylic acid 4-benzyl ester 1-*tert*-butyl ester (compound 52)

To a solution of compound 51 (1.20 g, 3.17 mmol) in DMF (20 mL) was added BnBr (650 mg, 3.80 mmol) and CS_2CO_3 (2.10 g, 6.40 mmol) at room temperature. The mixture was stirred overnight. After adding water and EtOAc (60 mL), the organic phase was separated, washed with brine dried over Na_2SO_4 . After concentration, the residue was purified by silica gel chromatography to afford title compound (800 mg, 53% yield) as colorless oil.

MS m/z (ESI): $C_{26}H_{31}N_2O_6$ $[M-H]^-$ 467.2, found 467.1.

Step 4: 4-benzyloxycarbonylamino-piperidine-4-carboxylic acid benzyl ester (compound 53)

To a solution of compound 52 (800 mg, 1.71 mmol) in DCM (10 mL) was added HCl (2 mL, 8 mmol, 4M in dioxane) at room temperature. The mixture was stirred for 2 hours. After concentration, the crude title compound (800mg) was obtained as yellow solid, which was used directly to next step without further purification.

MS m/z (ESI): $C_{21}H_{25}N_2O_4$ $[M+H]^+$ 369.2, found 369.2.

Step 5: (9*R*,12*R*)-benzyl 9-benzyl-12-isobutyl-2,2-dimethyl-4,7,10-trioxo-5-phenylethyl-3-oxa-5,8,11-triazatridecan-13-oate (compound 55)

To a solution of compound 54 (332 mg, 1.19 mmol) and compound 21 (439 mg, 1.19 mmol) in DMF (7 mL) was added HATU (679 mg, 1.785 mmol) and DIPEA (615 mg, 4.76 mmol). The solution was stirred at 0°C for 2 hours, then concentrated under reduced pressure. The resulting residue was added with water and extracted with EtOAc (30 mL×3). The combined organic layers were washed with brine, dried over anhydrous sodium sulfate and filtered. The filtrate was concentrated under reduced pressure. The resulting residue was purified by thin layer chromatography to obtain the title compound (410 mg, 55% yield) as yellow solid.

MS m/z (ESI): $C_{37}H_{48}N_3O_6$ $[M+H]^+$ 630.4, found 630.1.

Step 6:

(9*R*,12*R*)-9-benzyl-12-isobutyl-2,2-dimethyl-4,7,10-trioxo-5-phenylethyl-3-oxa-5,8,11-triazatridecan-13-oic acid (compound 56)

A solution of compound 55 (410 mg, 0.65 mmol) and Pd/C (60 mg) in MeOH (20 mL) was stirred for 12 hours at room temperature under H_2 atmosphere. The reaction solution was filtered through celite and the filtrate was concentrated under reduced pressure to obtain the crude title compound (313 mg) as yellow solid, which was used directly to next step without further purification.

MS m/z (ESI): $C_{30}H_{42}N_3O_6$ $[M+H]^+$ 540.3, found 540.3.

Step 7: (9*R*,12*R*,15*R*)-methyl 9-benzyl-15-(4-(((benzyloxy)carbonyl)amino)butyl)-12-isobutyl-2,2-dimethyl-4,7,10,13-tetraoxo-5-phenylethyl-3-oxa-5,8,11,14-tetraazahexadecan-16-oate (compound 57)

To a solution of compound 56 (100 mg, 0.125 mmol) and compound 49 (85 mg, 0.231 mmol) in DMF (5 mL) was added HATU (105 mg, 0.277 mmol) and DIPEA (72 mg, 0.555 mmol). After stirring for 2 hours at room temperature, the reaction solution was concentrated under reduced pressure. Water was added and the aqueous was extracted with EtOAc (30 mL×3). The combined organic layers were washed with saturated NH_4Cl solution, saturated $NaHCO_3$ solution and brine. Then the organic phase was dried over Na_2SO_4 and filtered. The filtrate was concentrated under reduced pressure to obtain the crude title compound (140 mg) as yellow solid, which was used directly to next step without further purification.

MS m/z (ESI): $C_{45}H_{62}N_5O_9$ $[M+H]^+$ 816.5, found 816.4.

Step 8:

(9*R*,12*R*,15*R*)-9-benzyl-15-(4-(((benzyloxy)carbonyl)amino)butyl)-12-isobutyl-2,2-dimethyl-4,7,10,13-tetraoxo-5-phenylethyl-3-oxa-5,8,11,14-tetraazahexadecan-16-oic acid (compound 58)

A mixture of compound 57 (140 mg, 0.17 mmol) and lithium hydroxide monohydrate (40 mg, 0.85 mmol) in THF (3 mL), MeOH (3 mL) and water (1 mL) was stirred for 0.5 hour at room temperature. The reaction solution was concentrated under reduced pressure. Water was added, then 1M hydrochloric acid was added dropwise to adjust the pH to 6. The reaction solution was extracted with dichloromethane (30 mL×3). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4 and filtered. The filtrate was concentrated under reduced pressure to obtain the crude title compound (220 mg) as white solid, which was used directly to next step without further purification.

MS m/z (ESI): $C_{44}H_{60}N_5O_9$ $[M+H]^+$ 802.4, found 802.2.

Step 9: benzyl 1-((9*R*,12*R*,15*R*)-9-benzyl-15-(4-(((benzyloxy)carbonyl)amino)butyl)-12-isobutyl-2,2-dimethyl-4,7,10,13-tetraoxo-5-phenylethyl-3-oxa-5,8,11,14-tetraazahexadecan-16-oyl)-4-(((benzyloxy)carbonyl)amino)piperidine-4-carboxylate (compound 59)

To a solution of compound 58 (220 mg, 0.17 mmol) and compound 53 (130 mg, 0.26 mmol) in DMF(5mL) was added HATU (100 mg, 0.26 mmol) and DIPEA(66 mg, 0.51 mmol). After stirring for 3 hours at room temperature, the reaction solution was concentrated under reduced pressure. The residue was added with water and extracted with EtOAc (30 mL×3). The combined organic layers were washed with saturated NH₄Cl, saturated NaHCO₃ solution and brine successively, dried over Na₂SO₄ and filtered. The filtrate was concentrated under reduced pressure. The resulting residue was purified by thin layer chromatography to obtain the title compound (170 mg, 87% yield) as yellow solid.

MS m/z (ESI): C₆₅H₈₂N₇O₁₂ [M+H]⁺ 1152.6, found 1152.4.

Step 10:

1-((9*R*,12*R*,15*R*)-15-(4-aminobutyl)-9-benzyl-12-isobutyl-2,2-dimethyl-4,7,10,13-tetraoxo-5-phenylethyl-3-oxa-5,8,11,14-tetraazahexadecan-16-oyl)-4-(carboxyamino)piperidine-4-carboxylic acid (compound 60)
A mixture of compound 59 (170 mg, 0.147 mmol) and Pd/C (50 mg) in MeOH (5 mL) was stirred for 12 hours at room temperature under H₂ atmosphere. The reaction solution was filtered through celite, and the filtrate was concentrated under reduced pressure to obtain the crude title compound (140 mg) as yellow solid, which was used directly to the next step without further purification

MS m/z (ESI): C₄₃H₆₄N₇O₁₀ [M+H]⁺ 838.5, found 838.4.

Step 11:

4-amino-1-((2*R*,5*R*,8*R*)-2-(4-aminobutyl)-8-benzyl-5-isobutyl-4,7,10-trioxo-14-phenyl-3,6,9,12-tetraazatetradecan-1-oyl)piperidine-4-carboxylic acid (compound 6)

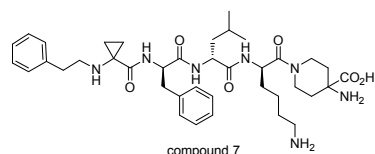
Compound 60 (140 mg, 0.167 mmol) was dissolved in DCM (3 mL). Then HCl (0.5 mL, 2.00 mmol, 4M in dioxane) was added. After stirring for 1 hour at room temperature, the reaction solution was concentrated under reduced pressure. The residue was purified by high performance liquid chromatography to obtain the title compound (57 mg, 52.2% yield) as white solid.

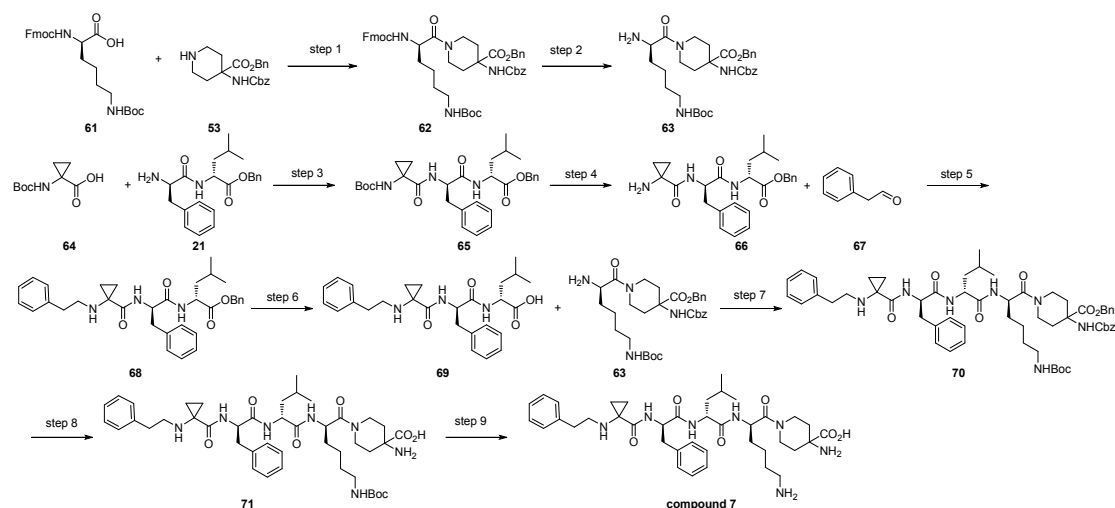
MS m/z (ESI): C₃₇H₅₆N₇O₆ [M+H]⁺ 694.4, found 694.4.

¹H NMR (400 MHz, Methanol-*d*₄) δ 7.39 – 7.21 (m, 9H), 7.15 (q, *J* = 7.2 Hz, 1H), 4.88 – 4.81 (m, 1H), 4.68 (ddd, *J* = 23.6, 10.1, 4.7 Hz, 1H), 4.43 – 4.31 (m, 1H), 4.01 (d, *J* = 15.5 Hz, 1H), 3.95 – 3.80 (m, 2H), 3.79 – 3.69 (m, 2H), 3.44 – 3.36 (m, 1H), 3.26 – 3.09 (m, 3H), 3.04 – 2.90 (m, 4H), 2.85 (s, 2H), 2.39 – 2.17 (m, 2H), 2.08 – 1.84 (m, 2H), 1.82 – 1.63 (m, 5H), 1.63 – 1.54 (m, 1H), 1.53 – 1.39 (m, 2H), 0.97 (t, *J* = 6.3 Hz, 3H), 0.91 (d, *J* = 6.3 Hz, 3H).

Compound 7:

4-amino-1-((*R*)-6-amino-2-((*R*)-4-methyl-2-((*R*)-2-(1-(phenethylamino)cyclopropanecarboxamido)-3-phenylpropanamido)pentanamido)hexanoyl)piperidine-4-carboxylic acid





Step 1: benzyl

4-(benzyloxycarbonylamino)-1-[(2*R*)-6-(*tert*-butoxycarbonylamino)-2-(9*H*-fluoren-9-ylmethoxycarbonylamino)hexanoyl]piperidine-4-carboxylate (compound 62)

To a solution of compound 61 (381 mg, 0.81 mmol) and compound 53 (300, 0.81 mmol) in DMF (16 mL) was added HATU (462 mg, 1.2 mmol) and DIPEA (209.00 mg, 1.62 mmol) and the mixture was stirred overnight. After adding water and EtOAc (20 mL×3), the combined organic layers were washed with brine, dried over Na₂SO₄. After concentration, the residue was purified by silica gel chromatography to afford title compound (500 mg, 75% yield) as yellow solid.

MS *m/z* (ESI): C₄₇H₅₅N₄O₉ [M+H]⁺ 819.4, found 819.4.

Step 2: benzyl

1-[(2*R*)-2-amino-6-(*tert*-butoxycarbonylamino)hexanoyl]-4-(benzyloxycarbonylamino)piperidine-4-carboxylate (compound 63)

A solution of compound 62 (480 mg, 0.59 mmol) and piperidine (501 mg, 5.9 mmol) was stirred in DMF (5 mL) at room temperature overnight. After adding water and EtOAc (10 mL×3), the combined organic layers were washed with brine, dried over Na₂SO₄. After concentration, the residue was purified by silica gel chromatography to afford title compound (350 mg, 100% yield) as yellow oil.

MS *m/z* (ESI): C₃₂H₄₅N₄O₇ [M+H]⁺ 597.3, found 597.4.

Step 3: benzyl

(2*R*)-2-[[[(2*R*)-2-[[1-(*tert*-butoxycarbonylamino)cyclopropanecarbonyl]amino]-3-phenyl-propanoyl]amino]-4-methyl-pentanoate (compound 65)

To a solution of compound 64 (1.10 g, 5.50 mmol) and compound 21 (2.20 g, 5.50 mmol) in DMF (25 mL) was added HATU (4.10 g, 11.00 mmol) and DIPEA (1.42 g, 11.00 mmol) and the mixture was stirred overnight at room temperature. After adding water and EtOAc (75 mL), the organic phase was separated, washed with brine dried over Na₂SO₄. After concentration, the residue was purified by silica gel chromatography to afford title compound (2 g, 67% yield) as yellow oil.

MS *m/z* (ESI): C₃₁H₄₂N₃O₆ [M+H]⁺ 552.3, found 552.3.

Step 4: benzyl

(2*R*)-2-[[[(2*R*)-2-[(1-aminocyclopropanecarbonyl)amino]-3-phenyl-propanoyl]amino]-4-methyl-pentanoate (compound 66)

To a solution of compound 65 (850 mg, 1.50 mmol) in DCM (20 mL) was added HCl (10 mL, 40 mmol, 4M in dioxane) and the reaction was stirred for 2 hours at room temperature. After concentration under reduced

pressure, the crude title compound (696 mg) was obtained as yellow oil, which was used directly to next step without further purification.

MS m/z (ESI): $C_{26}H_{34}N_3O_4$ $[M+H]^+$ 452.3, found 452.3.

Step 5: benzyl

(2*R*)-4-methyl-2-[[[(2*R*)-3-phenyl-2-[[1-(2-phenylethylamino)cyclopropanecarbonyl]amino]propanoyl]amino]pentanoate (compound 68)

To a solution of compound 66 (696 mg, 1.54 mmol) in MeOH (10 mL) was added 2-phenylacetaldehyde (compound 67) (185 mg, 1.54 mmol) and sodium cyanoborohydride (290 mg, 4.60 mmol) at 0 °C and the mixture was stirred at room temperature overnight. After concentration under reduced pressure, the residue was purified by silica gel chromatography to afford title compound (400 mg, 47% yield) as yellow solid.

MS m/z (ESI): $C_{34}H_{42}N_3O_4$ $[M+H]^+$ 556.3, found 556.3.

Step 6:

(2*R*)-4-methyl-2-[[[(2*R*)-3-phenyl-2-[[1-(2-phenylethylamino)cyclopropanecarbonyl]amino]propanoyl]amino]pentanoic acid (compound 69)

A mixture of compound 68 (400 mg, 0.72 mmol) and Pd/C (100 mg) in EtOH (20 mL) was stirred at room temperature under H_2 atmosphere overnight. After filtration and concentration under reduced pressure, the crude title compound (300 mg) was obtained as white solid, which was directly to next step without further purification.

MS m/z (ESI): $C_{27}H_{36}N_3O_4$ $[M+H]^+$ 466.3, found 466.2.

Step 7: benzyl

4-(benzyloxycarbonylamino)-1-[(2*R*)-6-(*tert*-butoxycarbonylamino)-2-[[[(2*R*)-4-methyl-2-[[[(2*R*)-3-phenyl-2-[[1-(2-phenylethylamino)cyclopropanecarbonyl]amino]propanoyl]amino]pentanoyl]amino]hexanoyl]piperidine-4-carboxylate (compound 70)

To a solution of compound 69 (200 mg, 0.43 mmol) and compound 63 (255 mg, 0.43 mmol) in DMF (8 mL) was added HATU (245 mg, 0.65 mmol) and DIPEA (111 mg, 0.86 mmol) and the mixture was stirred at room temperature overnight. After adding water and EtOAc (75 mL), the organic phase was separated, washed with brine dried over Na_2SO_4 . After concentration, the residue was purified by silica gel chromatography to afford title compound (150 mg, 33% yield) as yellow solid.

MS m/z (ESI): $C_{59}H_{78}N_7O_{10}$ $[M+H]^+$ 1044.6, found 1044.6.

Step 8:

4-amino-1-[(2*R*)-6-(*tert*-butoxycarbonylamino)-2-[[[(2*R*)-4-methyl-2-[[[(2*R*)-3-phenyl-2-[[1-(2-phenylethylamino)cyclopropanecarbonyl]amino]propanoyl]amino]pentanoyl]amino]hexanoyl]piperidine-4-carboxylic acid (compound 71)

A mixture of compound 70 (150 mg, 0.14 mmol) and Pd/C (10 mg) in EtOH was stirred at room temperature under H_2 atmosphere overnight. After filtration and concentration, the crude title compound (118 mg) was obtained as yellow solid, which was directly to next step without further purification.

MS m/z (ESI): $C_{44}H_{64}N_7O_8$ $[M-H]^-$ 818.5, found 818.6.

Step 9:

4-amino-1-((*R*)-6-amino-2-((*R*)-4-methyl-2-((*R*)-2-(1-(phenethylamino)cyclopropanecarboxamido)-3-phenylpropanamido)pentanamido)hexanoyl)piperidine-4-carboxylic acid (compound 7)

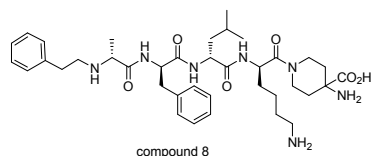
To a solution of compound 71 (118 mg, 0.14 mmol) was added TFA (1 mL) in DCM (10 mL) and the reaction was stirred at room temperature for 1.5 hour. After concentration under reduced pressure, the residue was purified by high performance liquid chromatography to obtain the title compound (60 mg, 57% yield) as white solid.

MS m/z (ESI): $C_{39}H_{58}N_7O_6$ $[M+H]^+$ 720.4, found 720.5.

^1H NMR (400 MHz, Methanol- d_4) δ 7.40 – 7.32 (m, 2H), 7.32 – 7.19 (m, 7H), 7.16 – 7.06 (m, 1H), 4.78 – 4.68 (m, 1H), 4.43 – 4.32 (m, 1H), 4.14 – 4.05 (m, 1H), 3.97 – 3.67 (m, 3H), 3.60 – 3.48 (m, 1H), 3.27 – 2.99 (m, 3H), 2.99 – 2.81 (m, 5H), 2.41 – 2.16 (m, 2H), 2.00 – 1.52 (m, 10H), 1.52 – 1.32 (m, 5H), 0.96 (d, J = 6.2 Hz, 3H), 0.91 (d, J = 6.2 Hz, 3H).

Compound 8:

4-amino-1-((2*R*,5*R*,8*R*,11*R*)-2-(4-aminobutyl)-8-benzyl-5-isobutyl-11-methyl-4,7,10-trioxo-14-phenyl-3,6,9,12-tetraazatetradecan-1-oyl)piperidine-4-carboxylic acid



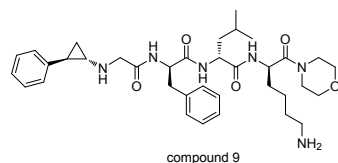
Compound 8 (white solid) was synthesized followed by the similar procedure of compound 7, while the starting material 1-((*tert*-butoxycarbonyl)amino)cyclopropanecarboxylic acid was replaced by (*R*)-2-((*tert*-butoxycarbonyl)amino)propanoic acid instead.

MS m/z (ESI): $\text{C}_{38}\text{H}_{58}\text{N}_7\text{O}_6$ $[\text{M}+\text{H}]^+$ 708.4, found 708.6.

^1H NMR (400 MHz, Methanol- d_4) δ 7.42 – 7.35 (m, 2H), 7.35 – 7.28 (m, 1H), 7.26 – 7.20 (m, 2H), 7.20 – 7.13 (m, 2H), 7.08 (t, J = 7.7 Hz, 2H), 6.89 – 6.81 (m, 1H), 4.85 – 4.79 (m, 1H), 4.45 – 4.35 (m, 1H), 4.22 – 4.10 (m, 1H), 3.97 – 3.85 (m, 1H), 3.84 – 3.71 (m, 3H), 3.27 – 3.17 (m, 1H), 2.93 (t, J = 7.6 Hz, 2H), 2.89 – 2.72 (m, 4H), 2.66 (s, 1H), 2.60 – 2.46 (m, 1H), 2.41 – 2.17 (m, 2H), 2.00 – 1.84 (m, 1H), 1.83 – 1.55 (m, 8H), 1.52 – 1.39 (m, 5H), 0.97 (dd, J = 6.6, 2.8 Hz, 3H), 0.93 (d, J = 6.4 Hz, 3H).

Compound 9:

(*R*)-*N*-((*R*)-6-amino-1-morpholino-1-oxohexan-2-yl)-4-methyl-2-((*R*)-3-phenyl-2-(2-(((1*S*,2*R*)-2-phenylcyclopropyl)amino)acetamido)propanamido)pentanamide



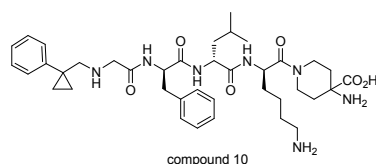
Compound 9 (white solid) was synthesized followed by the similar procedure of compound 3, while the starting material 2,3-dihydro-1*H*-inden-2-amine was replaced by (1*S*,2*R*)-2-phenylcyclopropanamine instead.

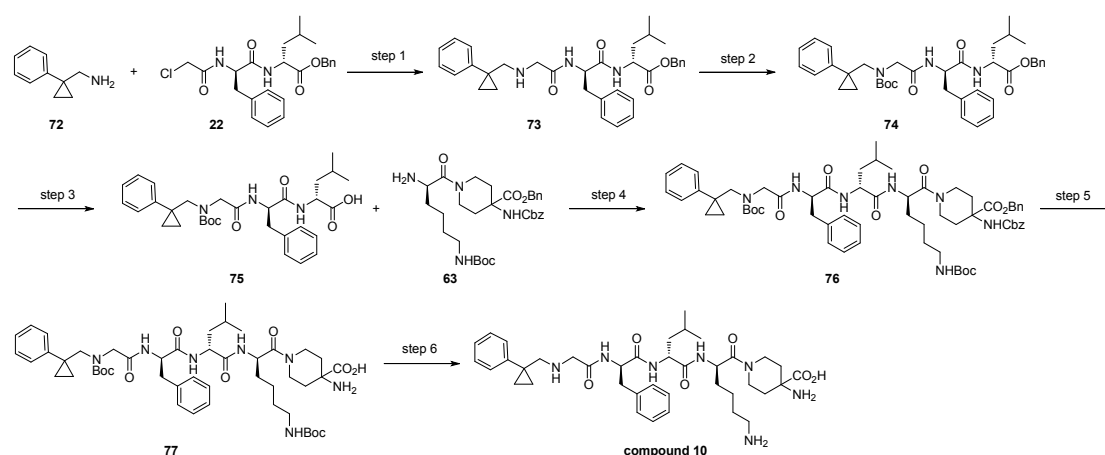
MS m/z (ESI): $\text{C}_{36}\text{H}_{53}\text{N}_6\text{O}_5$ $[\text{M}+\text{H}]^+$ 649.4, found 649.4.

^1H NMR (400 MHz, Methanol- d_4) δ 7.36 – 7.11 (m, 10H), 4.85 – 4.78 (m, 1H), 4.70 (d, J = 10.6, 4.9 Hz, 1H), 4.45 – 4.34 (m, 1H), 4.14 – 4.03 (m, 1H), 3.94 – 3.85 (m, 1H), 3.70 – 3.61 (m, 7H), 3.60 – 3.42 (m, 2H), 3.26 – 3.17 (m, 1H), 2.98 – 2.83 (m, 3H), 2.56 – 2.46 (m, 1H), 1.83 – 1.60 (m, 6H), 1.53 – 1.39 (m, 3H), 1.35 – 1.24 (m, 2H), 0.96 (d, J = 6.1 Hz, 3H), 0.91 (d, J = 6.1 Hz, 3H).

Compound 10:

4-amino-1-((6*R*,9*R*,12*R*)-12-(4-aminobutyl)-6-benzyl-9-isobutyl-4,7,10-trioxo-1-(1-phenylcyclopropyl)-2,5,8,11-tetraazatridecan-13-oyl)piperidine-4-carboxylic acid





Step 1: (*R*)-benzyl 4-methyl-2-((*R*)-3-phenyl-2-(2-(((1-phenylcyclopropyl)methyl)amino)acetamido)propanamido)pentanoate (compound 73)

To a solution of compound 72 (74 mg, 0.40 mmol) and compound 22 (150 mg, 0.33 mmol) in DMF (5 mL) was added KI (110.00 mg, 0.67 mmol) and K₂CO₃ (139 mg, 1.00 mmol) and the mixture was stirred at 60 °C overnight. After adding water and EtOAc (20 mL), the organic phase was separated, washed with brine and dried over Na₂SO₄. After concentration under reduced pressure, the residue was purified by silica gel chromatography to afford title compound (187 mg, 100% yield) as yellow oil.

MS *m/z* (ESI): C₃₄H₄₂N₃O₄ [M+H]⁺ 556.3, found 556.3.

Step 2: (9*R*,12*R*)-benzyl 9-benzyl-12-isobutyl-2,2-dimethyl-4,7,10-trioxo-5-((1-phenylcyclopropyl)methyl)-3-oxa-5,8,11-triazatridecan-13-oate (compound 74)

A solution of compound 73 (187 mg, 0.34 mmol), (Boc)₂O (147 mg, 0.67 mmol) and DIPEA (130 mg, 1.01 mmol) was stirred at room temperature overnight. After concentration under reduced pressure, the residue was purified by silica gel chromatography to afford title compound (100 mg, 46% yield) as yellow solid.

MS *m/z* (ESI): C₃₉H₄₈N₃O₆ [M-H]⁻ 654.4, found 654.4.

Step 3:

(9*R*,12*R*)-9-benzyl-12-isobutyl-2,2-dimethyl-4,7,10-trioxo-5-((1-phenylcyclopropyl)methyl)-3-oxa-5,8,11-triazatridecan-13-oic acid (compound 75)

A solution of compound 74 (100 mg, 0.15 mmol) and Pd/C (10 mg) in EtOH (10 mL) was stirred at room temperature under H₂ atmosphere. After filtration and concentration under reduced pressure, the crude title compound (86 mg, 100% yield) was afforded as colorless oil, which was directly to next step without further purification.

MS *m/z* (ESI): C₃₂H₄₄N₃O₆ [M+H]⁺ 566.3, found 566.3.

Step 4: benzyl 1-((9*R*,12*R*,15*R*)-9-benzyl-15-(4-((*tert*-butoxycarbonyl)amino)butyl)-12-isobutyl-2,2-dimethyl-4,7,10,13-tetraoxo-5-((1-phenylcyclopropyl)methyl)-3-oxa-5,8,11,14-tetraazahexadecan-16-oyl)-4-(((benzyloxy)carbonyl)amino)piperidine-4-carboxylate (compound 76)

To a solution of compound 75 (86 mg, 0.15 mmol) and compound 63 (91 mg, 0.15 mmol) in DMF (5 mL) was added HATU (115 mg, 0.3 mmol) and DIPEA (59 mg, 0.46 mmol) and the mixture was stirred overnight at room temperature. After adding water and EtOAc (20 mL), the organic phase was separated, washed with brine and dried over Na₂SO₄. After concentration under reduced pressure, the residue was purified by silica gel chromatography to afford title compound (100 mg, 58% yield) as yellow solid.

MS *m/z* (ESI): C₆₄H₈₆N₇O₁₂ [M+H]⁺ 1144.6, found 1144.6.

Step 5:

4-amino-1-((9*R*,12*R*,15*R*)-9-benzyl-15-(4-((*tert*-butoxycarbonyl)amino)butyl)-12-isobutyl-2,2-dimethyl-4,7,

10,13-tetraoxo-5-((1-phenylcyclopropyl)methyl)-3-oxa-5,8,11,14-tetraazahexadecan-16-oyl)piperidine-4-carboxylic acid (compound 77)

A mixture of compound 76 (100 mg, 0.09 mmol) and Pd/C (10 mg) in EtOH (10 mL) was stirred at room temperature under H₂ atmosphere overnight. After filtration and concentration under reduced pressure, the crude title compound (80 mg, 100% yield) was obtained as colorless oil, which was directly to next step without further purification.

MS m/z (ESI): C₄₉H₇₄N₇O₁₀ [M+H]⁺ 920.6, found 920.6.

Step 6:

4-amino-1-((6*R*,9*R*,12*R*)-12-(4-aminobutyl)-6-benzyl-9-isobutyl-4,7,10-trioxo-1-(1-phenylcyclopropyl)-2,5,8,11-tetraazatridecan-13-oyl)piperidine-4-carboxylic acid (compound 10)

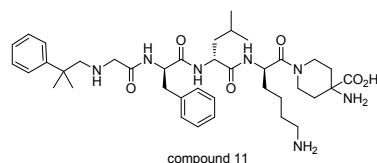
To a solution of compound 77 (80 mg, 0.09 mmol) was added TFA (2 mL) in DCM (10 mL) and the mixture was stirred for 1 hour at room temperature. After concentration, the residue was purified by high performance liquid chromatography to obtain the title compound (30 mg, yield 48%) as white solid.

MS m/z (ESI): C₃₉H₅₈N₇O₆ [M+H]⁺ 720.4, found 720.4.

¹H NMR (400 MHz, Methanol-*d*₄) δ 7.44 – 7.33 (m, 4H), 7.33 – 7.20 (m, 6H), 4.75 – 4.66 (m, 1H), 4.44 – 4.34 (m, 1H), 4.13 – 4.02 (m, 1H), 3.97 – 3.85 (m, 1H), 3.83 – 3.66 (m, 3H), 3.57 (dd, *J* = 16.1, 4.6 Hz, 1H), 3.53 – 3.43 (m, 1H), 3.24 – 3.11 (m, 2H), 3.09 – 2.99 (m, 1H), 2.92 (t, *J* = 7.6 Hz, 2H), 2.88 – 2.77 (m, 1H), 2.38 – 2.16 (m, 2H), 1.97 – 1.83 (m, 1H), 1.83 – 1.52 (m, 8H), 1.53 – 1.36 (m, 2H), 1.04 – 0.95 (m, 7H), 0.92 (d, *J* = 6.4 Hz, 3H)

Compound 11:

4-amino-1-((2*R*,5*R*,8*R*)-2-(4-aminobutyl)-8-benzyl-5-isobutyl-14-methyl-4,7,10-trioxo-14-phenyl-3,6,9,12-tetraazapentadecan-1-oyl)piperidine-4-carboxylic acid



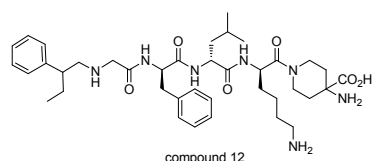
Compound 11 (white solid) was synthesized followed by the similar procedure of compound 10, while the starting material (1-phenylcyclopropyl)methanamine was replaced by 2-methyl-2-phenylpropan-1-amine instead.

MS m/z (ESI): C₃₉H₆₀N₇O₆ [M+H]⁺ 722.5, found 722.6.

¹H NMR (400 MHz, Methanol-*d*₄) δ 7.43 – 7.35 (m, 4H), 7.33 – 7.24 (m, 5H), 7.23 – 7.14 (m, 1H), 4.87 – 4.79 (m, 1H), 4.77 – 4.68 (m, 1H), 4.43 – 4.34 (m, 1H), 4.18 – 4.08 (m, 1H), 4.01 – 3.84 (m, 1H), 3.81 – 3.71 (m, 2H), 3.59 (dd, *J* = 16.1, 5.5 Hz, 1H), 3.50 – 3.37 (m, 1H), 3.26 – 3.11 (m, 2H), 3.06 – 2.97 (m, 1H), 2.93 (t, *J* = 7.6 Hz, 2H), 2.83 (q, *J* = 12.0, 11.5 Hz, 1H), 2.41 – 2.14 (m, 2H), 1.99 – 1.89 (m, 2H), 1.85 – 1.62 (m, 7H), 1.61 – 1.52 (m, 1H), 1.48 – 1.37 (m, 7H), 1.00 – 0.95 (m, 3H), 0.92 (d, *J* = 6.4 Hz, 3H).

Compound 12:

4-amino-1-((2*R*,5*R*,8*R*)-2-(4-aminobutyl)-8-benzyl-5-isobutyl-4,7,10-trioxo-14-phenyl-3,6,9,12-tetraazahexadecan-1-oyl)piperidine-4-carboxylic acid



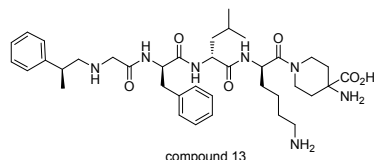
Compound 12 (white solid) was synthesized followed by the similar procedure of compound 10, while the starting material (1-phenylcyclopropyl)methanamine was replaced by 2-phenylbutan-1-amine instead.

MS m/z (ESI): C₃₉H₆₀N₇O₆ [M+H]⁺ 722.5, found 722.6.

¹H NMR (400 MHz, Methanol-*d*₄) δ 7.42 – 7.35 (m, 2H), 7.34 – 7.14 (m, 8H), 4.85–4.78 (m, 1H), 4.75 – 4.64 (m, 1H), 4.42 – 4.33 (m, 1H), 4.11 – 3.98 (m, 1H), 3.95 – 3.66 (m, 3H), 3.65 – 3.45 (m, 1H), 3.24 – 3.12 (m, 3H), 3.12 – 3.01 (m, 1H), 2.92 (t, *J* = 7.6 Hz, 2H), 2.89 – 2.76 (m, 2H), 2.39 – 2.12 (m, 2H), 1.98 – 1.52 (m, 11H), 1.50 – 1.35 (m, 2H), 1.00 – 0.93 (m, 3H), 0.93 – 0.88 (m, 3H), 0.77 (td, *J* = 7.4, 1.5 Hz, 3H).

Compound 13:

4-amino-1-((2*R*,5*R*,8*R*,14*S*)-2-(4-aminobutyl)-8-benzyl-5-isobutyl-4,7,10-trioxo-14-phenyl-3,6,9,12-tetraaza pentadecan-1-oyl)piperidine-4-carboxylic acid

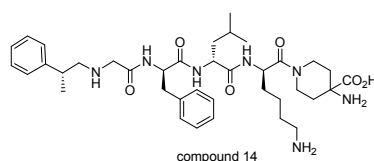


Compound 13 (white solid) was synthesized followed by the similar procedure of compound 10, while the starting material (1-phenylcyclopropyl)methanamine was replaced by (*S*)-2-phenylpropan-1-amine instead. MS *m/z* (ESI): C₃₈H₅₈N₇O₆ [M+H]⁺ 708.4, found 708.6.

¹H NMR (400 MHz, Methanol-*d*₄) δ 7.41 – 7.32 (m, 2H), 7.33 – 7.23 (m, 7H), 7.22 – 7.16 (m, 1H), 4.86 – 4.78 (m, 1H), 4.74 – 4.64 (m, 1H), 4.43 – 4.33 (m, 1H), 3.98 (s, 1H), 3.83 – 3.71 (m, 3H), 3.65 (dd, *J* = 15.8, 8.3 Hz, 1H), 3.49 – 3.36 (m, 1H), 3.23 – 3.14 (m, 2H), 3.14 – 3.02 (m, 2H), 2.92 (t, *J* = 7.8 Hz, 2H), 2.88 – 2.80 (m, 1H), 2.40 – 2.15 (m, 2H), 1.98 – 1.50 (m, 9H), 1.50 – 1.37 (m, 2H), 1.36 – 1.27 (m, 3H), 0.99 – 0.94 (m, 3H), 0.91 (d, *J* = 6.4 Hz, 3H).

Compound 14:

4-amino-1-((2*R*,5*R*,8*R*,14*S*)-2-(4-aminobutyl)-8-benzyl-5-isobutyl-4,7,10-trioxo-14-phenyl-3,6,9,12-tetraaza pentadecan-1-oyl)piperidine-4-carboxylic acid

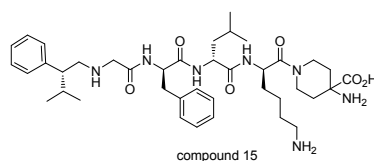


Compound 14 (white solid) was synthesized followed by the similar procedure of compound 10, while the starting material (1-phenylcyclopropyl)methanamine was replaced by (*R*)-2-phenylpropan-1-amine instead. MS *m/z* (ESI): C₃₈H₅₈N₇O₆ [M+H]⁺ 708.4, found 708.6.

¹H NMR (400 MHz, Methanol-*d*₄) δ 7.41 – 7.34 (m, 2H), 7.33 – 7.21 (m, 7H), 7.19 – 7.12 (m, 1H), 4.76 – 4.64 (m, 1H), 4.44 – 4.32 (m, 1H), 4.20 – 4.08 (m, 1H), 4.02 – 3.92 (m, 1H), 3.91 – 3.68 (m, 3H), 3.69 – 3.58 (m, 1H), 3.51 – 3.38 (m, 1H), 3.25 – 3.15 (m, 1H), 3.15 – 3.03 (m, 3H), 2.92 (t, *J* = 7.7 Hz, 2H), 2.89 – 2.75 (m, 1H), 2.38 – 2.15 (m, 2H), 1.98 – 1.52 (m, 9H), 1.52 – 1.38 (m, 2H), 1.38 – 1.25 (m, 3H), 1.00 – 0.94 (m, 3H), 0.92 (d, *J* = 6.4 Hz, 3H).

Compound 15:

4-amino-1-((2*R*,5*R*,8*R*,14*R*)-2-(4-aminobutyl)-8-benzyl-5-isobutyl-15-methyl-4,7,10-trioxo-14-phenyl-3,6,9,12-tetraazahexadecan-1-oyl)piperidine-4-carboxylic acid



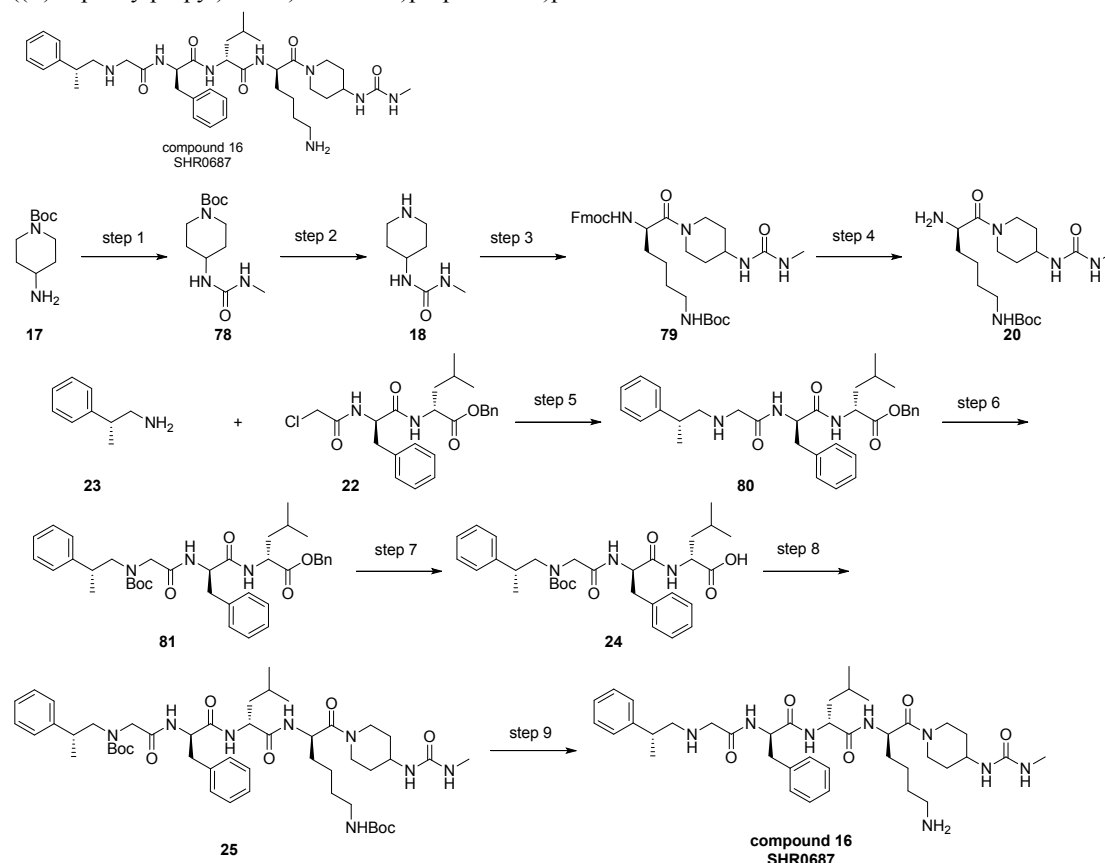
Compound 15 (white solid) was synthesized followed by the similar procedure of compound 10, while the starting material (1-phenylcyclopropyl)methanamine was replaced by (*R*)-3-methyl-2-phenylbutan-1-amine instead.

MS *m/z* (ESI): C₄₀H₆₂N₇O₆ [M+H]⁺ 736.5.

¹H NMR (400 MHz, Methanol-*d*₄) δ 7.42 – 7.35 (m, 2H), 7.35 – 7.29 (m, 1H), 7.30 – 7.17 (m, 7H), 4.87 – 4.78 (m, 1H), 4.68 (td, *J* = 9.4, 4.6 Hz, 1H), 4.43 – 4.33 (m, 1H), 4.08 – 3.99 (m, 1H), 3.96 – 3.83 (m, 1H), 3.84 – 3.67 (m, 2H), 3.62 (dd, *J* = 15.9, 6.3 Hz, 1H), 3.54 – 3.45 (m, 1H), 3.37 – 3.33 (m, 1H), 3.19 (ddd, *J* = 13.7, 8.6, 4.7 Hz, 1H), 2.92 (t, *J* = 7.6 Hz, 2H), 2.84 (dt, *J* = 14.1, 10.5 Hz, 1H), 2.74 – 2.64 (m, 1H), 2.38 – 2.15 (m, 2H), 1.95 – 1.61 (m, 9H), 1.61 – 1.35 (m, 4H), 0.99 – 0.94 (m, 6H), 0.92 (d, *J* = 6.5 Hz, 3H), 0.71 (d, *J* = 6.7 Hz, 3H).

Compound 16:

(*R*)-*N*-((*R*)-6-amino-1-(4-(3-methylureido)piperidin-1-yl)-1-oxohexan-2-yl)-4-methyl-2-((*R*)-3-phenyl-2-(2-((*R*)-2-phenylpropyl)amino)acetamido)propanamido)pentanamide



Step 1: *tert*-butyl 4-(3-methylureido)piperidine-1-carboxylate (compound 78)

Compound 17 (870.0 mg, 4.30 mmol) was dissolved in DCM (15 mL), then DIPEA (1.67 g, 12.9 mmol) was added. Methylcarbamic chloride (400.0 mg, 4.3 mmol) was added to the reaction at 0°C and the reaction was stirred for 2 hours at room temperature. To the reaction solution was added 200 mL of saturated sodium bicarbonate solution. The aqueous was extracted with dichloromethane (20 mL×3). The combined organic layers were washed with brine, dried over Na₂SO₄ and filtered. The filtrate was concentrated under reduced pressure to obtain the crude title compound (1 g) as yellow oil, which was used directly to next step without further purification.

MS *m/z* (ESI): C₁₂H₂₄N₃O₃ [M+H]⁺ 258.2, found 258.2.

Step 2: 1-methyl-3-(piperidin-4-yl)urea (compound 18)

Compound 78 (1 g, 4 mmol) was dissolved in DCM (10 mL), then HCl (2 mL, 8 mmol, 4M in dioxane) was added. After stirring for 2 hours at room temperature, the reaction solution was concentrated under reduced pressure to obtain the crude title compound (1 g) as white solid, which was used directly to next step without further purification.

MS m/z (ESI): C₇H₁₆N₃O [M+H]⁺ 158.2.

Step 3: (*R*)-(9*H*-fluoren-9-yl)methyl *tert*-butyl

(6-(4-(3-methylureido)piperidin-1-yl)-6-oxohexane-1,5-diyl)dicarbamate (compound 79)

Compound 18 (1 g, 5.16 mmol) and commercially available

(*R*)-2-(((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-6-((*tert*-butoxycarbonyl)amino)hexanoic acid

(compound 19) (2.42 g, 5.16 mmol) were dissolved in DMF (20 mL), then HATU (2.94 g, 7.74 mmol) and Et₃N (1.03 g, 10.32 mmol) were added. After stirring for 4 hours at room temperature, After adding water and EtOAc (60 mL), the organic phase was separated, washed with brine and dried over Na₂SO₄. After concentration under reduced pressure, crude title compound (1.00 g) was obtained as yellow solid, which was used directly to next step without further purification.

MS m/z (ESI): C₂₈H₃₈N₅O₄ [M+2H-Boc]⁺ 508.3, found 508.2.

Step 4: (*R*)-*tert*-butyl (5-amino-6-(4-(3-methylureido)piperidin-1-yl) -6-oxohexyl) carbamate (compound 20)

Compound 79 (1.00 g, 1.97 mmol) was dissolved in DMF (6 mL), then 6 mL of Et₃N was added. The reaction solution was stirred for 12 hours at room temperature. After removal of DMF under reduced pressure, the residue was purified by silica gel chromatography to afford title compound (530 mg, 32% yield in 4 steps) as yellow oil.

MS m/z (ESI): C₁₈H₃₆N₅O₄ [M+H]⁺ 386.3, found 386.2.

Step 5: (*R*)-benzyl 4-methyl-2-((*R*)-3-phenyl-

2-(2-(((*R*)-2-phenylpropyl)amino)acetamido)propanamido)pentanoate (compound 80)

To a solution of compound 22 (500 mg, 1.12 mmol) and commercially available (*R*)-2-phenylpropan-1-amine (compound 23) (228 mg, 1.68 mmol) in DMF (10 mL) was added KI (372 mg, 2.24 mmol) and K₂CO₃ (309 mg, 2.24 mmol). The reaction solution was warmed to 60°C and stirred for 12 hours. After the reaction solution was cooled to room temperature, water was added. The organic phase was extracted with DCM (90 mL). The organic phases were combined, dried over Na₂SO₄ and filtered. The filtrate was concentrated under reduced pressure to obtain the crude title compound (600 mg) as yellow solid, which was used directly to next step without further purification.

MS m/z (ESI): C₃₃H₄₂N₃O₄ [M+H]⁺ 544.3, found 544.2.

Step 6: (9*R*,12*R*)-benzyl 9-benzyl-12-isobutyl-2,2-dimethyl-4,7,10-trioxo-5-((*R*)-2-phenylpropyl)-3-oxa-5,8,11-triazatridecan-13-oate (compound 81)

Compound 80 (600 mg, 1.1 mmol) was dissolved in DCM (20 mL), then (Boc)₂O (361 mg, 1.66 mmol) and Et₃N (222 mg, 2.2 mmol) were added. After stirring for 12 hours at room temperature, water and DCM (60 mL) was added. The organic phase was separated, washed with brine and dried over Na₂SO₄. After concentration, the crude title compound was obtained (580 mg) as yellow solid, which was used directly to next step without further purification.

MS m/z (ESI): C₃₈H₅₀N₃O₆ [M+H]⁺ 644.4, found 644.4.

Step 7:

(9*R*,12*R*)-9-benzyl-12-isobutyl-2,2-dimethyl-4,7,10-trioxo-5-((*R*)-2-phenylpropyl)-3-oxa-5,8,11-triazatridecan-13-oic acid (compound 24)

A mixture of compound 81 (580 mg, 0.9 mmol) and Pd/C (60 mg) in MeOH (10 mL) was stirred for 12 hours at room temperature under H₂ atmosphere. The reaction solution was filtered through celite and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel chromatography to obtain the title compound (510 mg, 82% yield in four steps) as yellow solid.

MS m/z (ESI): C₃₁H₄₂N₃O₆ [M-H]⁻ 552.3, found 552.3.

Step 8: *tert*-butyl ((10*R*,13*R*,16*R*)-16-benzyl-13-isobutyl-2,2-dimethyl-10-(4-(3-methylureido)piperidine-1-carbonyl)-4,12,15,18-tetraoxo-3-oxa-5,11,14,17-tetraazanonadecan-19-yl)((*R*)-2-phenylpropyl)carbamate (compound 25)

To a solution of compound 20 (193 mg, 0.50 mmol) and compound 24 (109.60 mg, 0.20 mmol) in DMF (15 mL) was added HATU (152 mg, 0.4 mmol) and DIPEA (52.00 mg, 0.4 mmol) and the reaction was stirred for 12 hours at room temperature. The reaction solution was added with saturated citric acid solution. The organic phase was extracted with ethyl acetate (30 mL×3). The organic phases were combined, washed with saturated sodium bicarbonate solution and brine successively, dried over Na₂SO₄ and filtered. The filtrate was concentrated under reduced pressure to obtain the crude title compound (184.0 mg) as yellow solid, which was used directly to next step without further purification.

MS m/z (ESI): C₄₄H₆₉N₈O₇ [M+2H-Boc]⁺ 821.5, found 821.5.

Step 9:

(*R*)-*N*-((*R*)-6-amino-1-(4-(3-methylureido)piperidin-1-yl)-1-oxohexan-2-yl)-4-methyl-2-((*R*)-3-phenyl-2-(2-((*R*)-2-phenylpropyl)amino)acetamido)propanamido)pentanamide (compound 16)

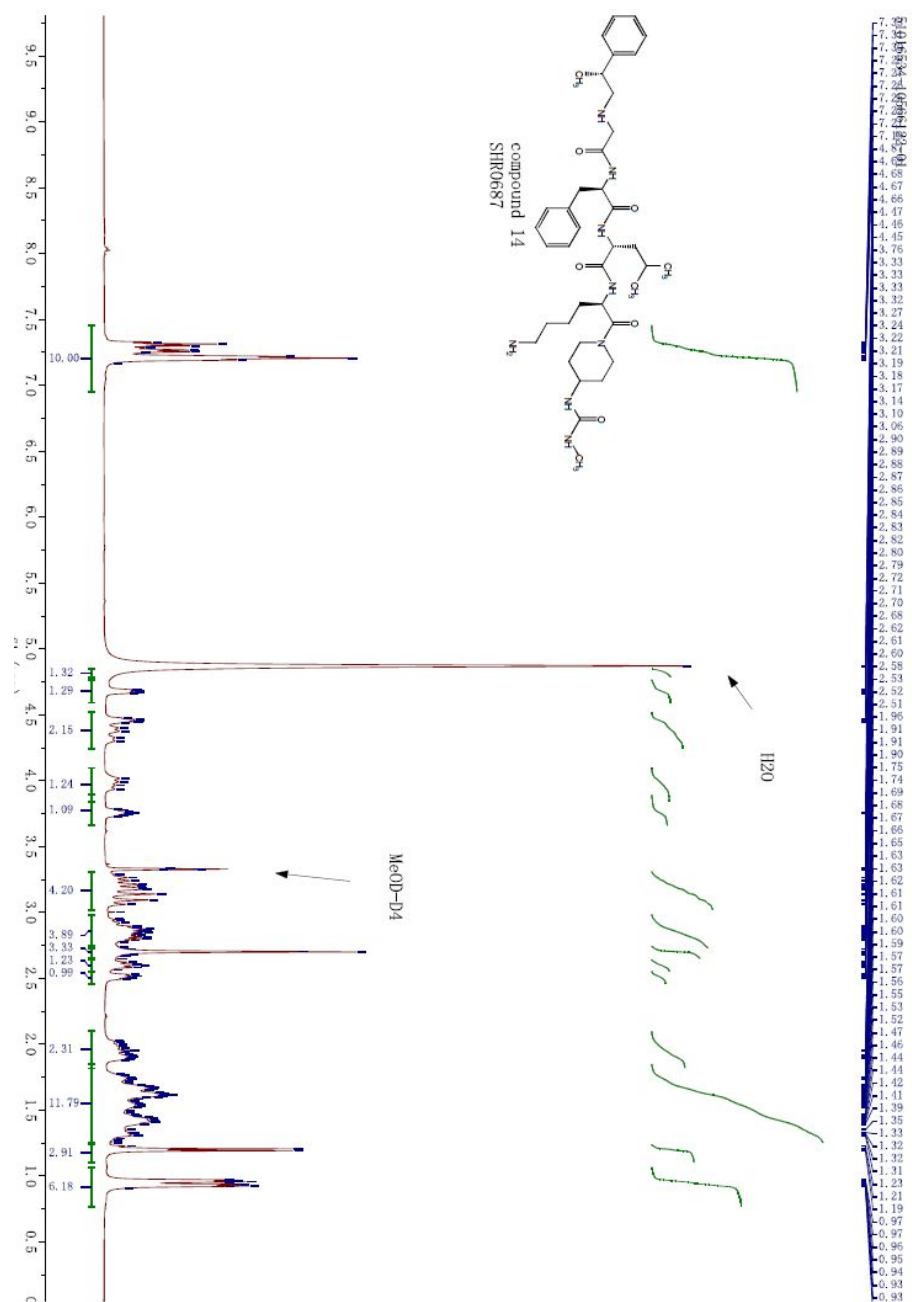
Compound 25 (184 mg, 0.2 mmol) was dissolved in DCM (10 mL), then HCl (2 mL, 8 mmol, 4M in dioxane) was added. After stirring for 12 hours at room temperature, the reaction solution was concentrated under reduced pressure. The resulting residue was purified by high performance liquid chromatography to obtain the title compound (60 mg, 42% yield in two steps) as white solid.

MS m/z (ESI): C₃₉H₆₁N₈O₅ [M+H]⁺ 721.5, found 721.5.

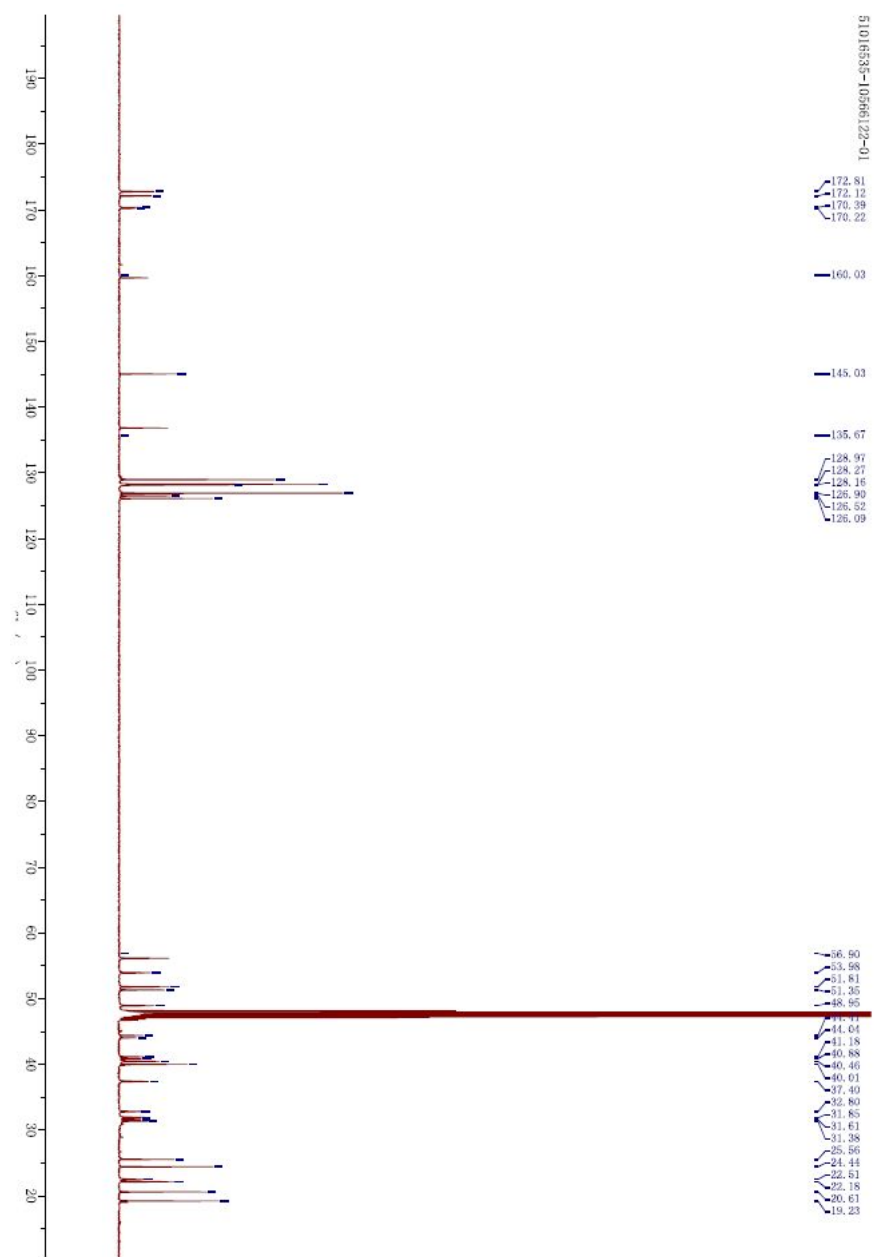
¹H NMR (500 MHz, Methanol-*d*₄) δ 7.37-7.11 (m, 10H), 4.88-4.75 (m, 1H), 4.73-4.63 (m, 1H), 4.51-4.25 (m, 2H), 4.06-3.89 (m, 1H), 3.82-3.70 (m, 1H), 3.30-3.01 (m, 4H), 2.99-2.76 (m, 4H), 2.70 (s, 3H), 2.65-2.56 (m, 1H), 2.56-2.46 (m, 1H), 2.10-1.83 (m, 2H), 1.81-1.26 (m, 12H), 1.20 (d, *J*=8 Hz, 3H), 0.97 (d, *J*=8 Hz, 3H), 0.93 (d, *J*=8 Hz, 3H).

¹³C NMR (100 MHz, Methanol-*d*₄): 172.81, 172.12, 170.39, 170.22, 159.67, 145.01, 136.85, 128.97, 128.27, 127.17, 126.91, 126.52, 126.09, 59.90, 53.98, 51.81, 51.35, 48.95, 44.41, 44.04, 41.18, 40.88, 40.46, 40.01, 37.4, 32.80, 31.85, 31.61, 31.38, 25.56, 24.44, 22.51, 22.18, 20.61, 19.23.

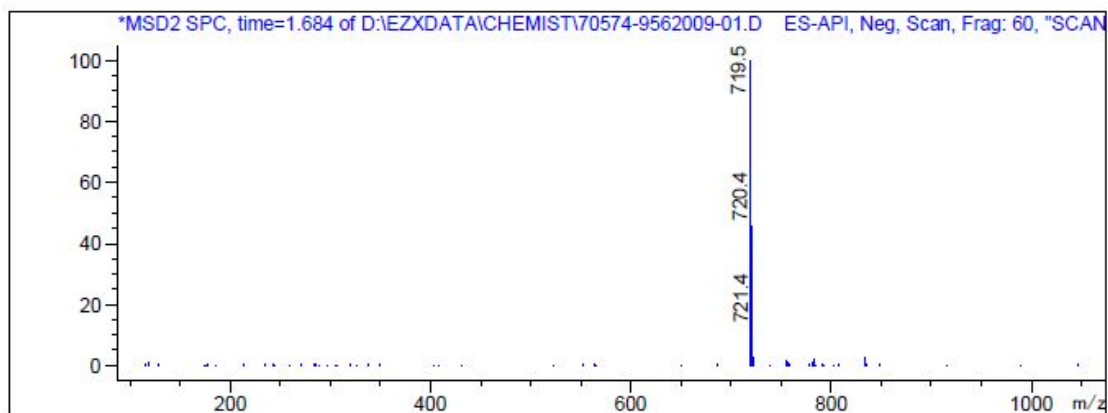
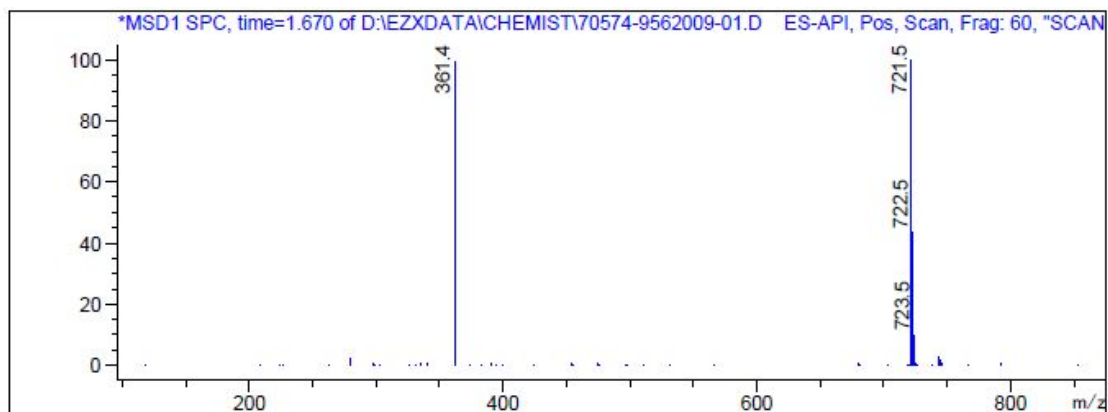
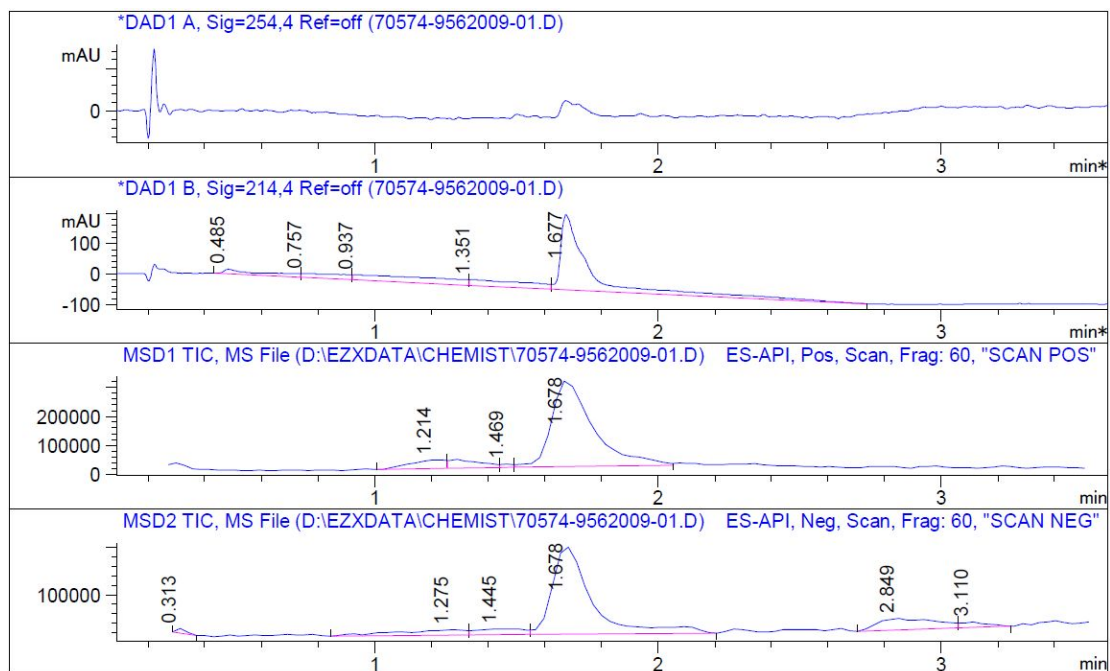
¹H NMR of SHR0687



¹³C NMR of SHR0687



LCMS of SHR0687



Chiral HPLC of SHR0687

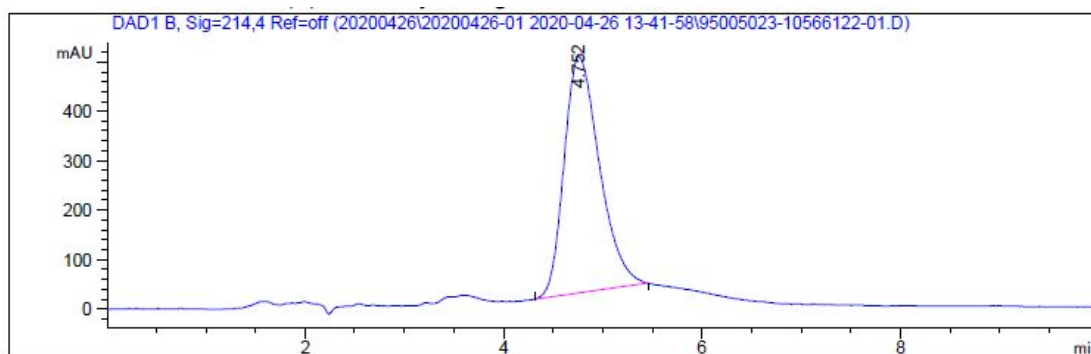
Instrument: Agilent 1260 DAD

Column: CHIRALPAK IG 150*4.6mm,5um

Column temperature: 35°C

Flow rate: 1.0 ml/min

Mobile phase: A: EtOH and 0.1% DEA:50% B:hexane: 50%



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1 B, Sig=214,4 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	4.752	MM R	0.4117	1.18441e4	479.53125	100.0000

Totals : 1.18441e4 479.53125

Methods for in vitro, in vivo, and pharmacokinetic assays

General Information

Chemicals and reagents

Recombinant human P450 isoenzymes (CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4, CYP3A5, and CYP4A11) were purchased from BD Gentest (Woburn, MA). Purified water was generated by Milli-Q gradient water purification system (Millipore, Molsheim, France). CR845 as positive control was prepared by a method disclosed in the patent.¹

The purity of compounds in biological test was more than 98%.

Other commercially available reagents were of analytical grade.

LC-MS/MS instrumentation and general conditions

LC-MS/MS for *in vitro* and *in vivo* sample bioanalysis consisted of an HPLC system (Shimadzu, Japan) equipped with a binary solvent manager, an auto sampler and an AB4000 triple quadrupole mass spectrometer (AB SCIEX) with electrospray ionization (ESI) source. Data acquisition and analysis were performed using Analyst software (AB SCIEX). A Hypersil Gold C8 column (30 mm × 2.1 mm i.d., 1.9 μm; Thermo, USA) thermostatted at 35 °C. The flow rate was set to 0.3 mL/min. The mobile phase consisted of water with 0.2% formic acid (A) and acetonitrile (B). Gradient elution started from 15% (B), followed by a linear gradient to 85% (B) over 0.6 min and held for another 0.6 min then zoomed to 15% (B) in the next 0.1 min, and finally re-equilibrated to 15% (B) in 0.9 min. MS detection was performed in a positive ESI mode with the source temperature at 550 °C and ion spray voltage at 5.5 kV.

In vitro Experiments

1. Biological assay experimental method

The objective of this experiment is to determine the agonistic effect of the compounds of the present invention on human KOR (h-KOR) receptors, and to evaluate the *in vitro* activity of the compounds according to the values of EC₅₀. The compounds of the present invention can activate h-KOR receptor, thereby reducing intracellular cAMP levels. The second messenger cAMP enters the nucleus and binds to the CRE of the DNA, thereby initiating the expression of the downstream luciferase. Luciferase reacts with its substrate to emit fluorescence, and the measured fluorescence signals reflect the agonistic activity of the compounds.

2. Experimental materials and instruments

Reagent name	Supply company	Item number
HEK293 cell line	Cell bank of the typical culture preservation Committee of Chinese Academy of Sciences	GNHu43
DMSO	Shanghai Titanchem	G75927B
DMEM high glucose medium	Thermo HyCLone	SH30243018
Fetal bovine serum (FBS)	Gibco	10099-141
CRE/pGL4.29	Promega	E8471
KOR-1/pcDNA3.1(+)	GENEWIZ Biological Technology Co., Ltd	Synthesis
ONE-Glo Luciferase Assay System	Promega	E6110

3. Experimental Procedure

1) Obtaining HEK293/KOR/CRE monoclonal cell lines

KOR/pcDNA3.1 (+) and CRE/pGL4.29 were transferred into HEK293 cells. G418 and hygromycin were added into the culture medium, and HEK293/KOR/CRE monoclonal cell lines were screened in a 96-well cell culture plate.

2) Agonistic effect of example compounds on h-KOR

HEK293/h-KOR/CRE monoclonal cells were cultured in a DMEM/high glucose medium (10% FBS, 1 mg/ml G418, 200 µg/ml hygromycin, mixed uniformly), and passaged every 3 days. On the day of the experiment, a cell suspension was prepared with a fresh cell medium, added to a 96 well plate (BD, #356692) with 20,000 cells/well, and incubated in 5% CO₂ at 37°C. On the second day, the compound was dissolved in pure DMSO at a concentration of 20 mM, then formulated with DMSO to a first concentration of 200 nM and diluted in 3 fold concentration gradient to 8 concentrations. 90 µl of DMSO was added to blank and control wells. The compound solution was diluted 20-fold with DMEM/hyperglucose (SH30243.01B, Hyclone) medium containing 10 µM Forskolin. The cell culture plates inoculated on the first day were taken out, and 10 µl of the diluted drug or the control (0.5% DMSO) was added to each well. The plate was gently shaken, and placed at 37°C for 5 hours. In a 96-well cell culture plate, 100 µl of luciferase assay solution (Promega, # E6110) was added to each well. The plate was placed for 5 minutes at room temperature. The chemiluminescence value was measured using Victor 3.0. The EC₅₀ values of the compounds were calculated using Graphpad Prism software based on each concentration of the compound and the corresponding signal value.

4. Test results

The activity of the compounds of the present invention on agonizing h-KOR and affecting downstream cAMP levels was determined by the above test.

The EC₅₀ values are mean of two or more runs at least. The EC₅₀ values of KOR agonist were shown in Table 1. CR845 was included as a positive control of each batch (Emax of CR845 was defined as 100%). The potency of CR845 was shown below.

Compound ID	CR845
hKOR EC ₅₀ (pM)	2.62±0.75, n=5
hMOR EC ₅₀ (nM)	>50000
hDOR EC ₅₀ (nM)	>50000

The positive control for hMOR and hDOR test was Morphine.

		Mean±SD
hMOR	EC ₅₀ nM	79.12±9.91, n=3
	Emax (%)	103.76±1.12, n=3

		Mean±SD
hDOR	EC ₅₀ nM	203.97±33.51, n=3
	Emax (%)	101.77±2.66, n=3

In vitro CYP inhibition assay

The inhibitory IC₅₀ values of SHR0687 for five major P450 enzymes was determined in human liver microsomes (BD Gentest) by similar conditions as described in literatures.^{2,3} The CYPs inhibitor IC₅₀ was summarized in Table 3.

***In vivo* Experiments**

***In vivo* PK Studies Procedures**

Animals utilized for preclinical studies include nude mice, rats and dogs. All animals were treated in accordance with Institutional Guide for the Care and Use of Laboratory Animals. ICR mice (around 20g, 9fe males) were purchased from Sino-British Sippr/BK Lab Animal Co. Ltd (Shanghai) (SCXK 2013-0016), Sprague Dawley (SD) rats (200-250g, 6 males and 6 females) from Sino-British Sippr/BK Lab Animal Co. Ltd (Shanghai) (SCXK 2013-0016), and beagle dogs (9-13kg, 3 males) from Beijing Marshall Biotechnology Co., Ltd (SCXK 2016-0001). Plasma samples of nude mice, SD rats and dogs were collected at pre-dose and 0.083, 0.25, 0.5, 1, 2, 4, 8, 12, 24 h after the i.v. administration.

Pharmacokinetic parameters of the compound SHR0687 were shown in Table 5.

Pharmacokinetic parameters of CR845 were shown in the table below.

Intravenous PK data of CR845 ^{a,b}

Species	Mouse	Rat	Dog
AUC _{0-t} (ng/mL*h)	891	1745	3864
T _{1/2} (h)	0.32	0.49	2.16
CL (mL/min/kg)	18.6	9.66	7.2
V _z (mL/Kg)	506	412	417

^aCL is clearance; T_{1/2} is the half-life of the compound exposure in plasma; AUC is the area under the curve. ^bThe dosage for mouse and rat was 1 mpk and for dog was 0.5mpk

***In vivo* Efficacy Study of SHR0687**

A carrageenan inflammatory pain model in rats was established to evaluate the therapeutic effect of KOR agonists on inflammatory pain in rats.

1. Experimental method and experimental materials

1)Test animals and feeding conditions

Male Wistar rats were purchased from Shanghai Slac Laboratory Animal Co., Ltd. (Shanghai, China, Certificate No. 2015000513408, License No. SCXK (Shanghai) 2012-0002). The rats were 150-180 g, and feeded at 5/cage, in a condition of 12/12 hours light/dark cycle adjustment, constant temperature of 23±1°C , humidity of 50~60%, and free access to food and water. After purchase, the animals adapted to this condition for 7 days before the experiment was started.

2)Test compound

Compound SHR0687;

λ-Carrageenan, Batch No. BCBP8978V, purchased from sigma.

0.9% Sodium chloride solution (500 mL, 4.5 g)

1% λ-Carrageenan was placed in physiological saline, and stirred overnight to form a jelly-like suspension.

The compound dose was calculated on bases.

2. Experimental design and experimental method

1)Animal grouping

After adaptive feeding, the rats were grouped as follows:

Groups of Inflammatory Pain Model	n	Molding Method	Administration Method
Blank control group	8	0.9% NS (s.c., 0.1 ml/rat, once)	0.9 % NS (i.v., once)
Model group	8	1%λ-Carrageenan (s.c., 0.1 ml/rat, once)	0.9% NS (i.v., once)
CR845 group	8	1%λ-Carrageenan (s.c., 0.1 ml/rat, once)	CR845 (0.1, 0.3 mg/kg i.v., once)
SHR0687 group (0.03, 0.1 and 0.3 mg/kg)	8	1%λ-Carrageenan (s.c., 0.1 ml/rat, once)	SH0687 (0.03, 0.1, 0.3 mg/kg i.v., once)

Note: NS: normal saline used in the preparation of carrageenan solution; i.v.: intravenous injection; s.c.: subcutaneous injection.

2)Experiment method

The experimental method was modified in accordance with the method of Document 1 (Kazunari Nakao et al.).⁴ Before inflammatory pain experiment, rats were randomly divided into the following groups according to body weight: blank control group, model group, CR845-0.1 mg/kg group and CR845-0.3 mg/kg group, SHR0687-0.03 mg/kg group, SHR0687-0.1 mg/kg group, and SHR0687-0.3 mg/kg group. There were 8 rats in each group. Inflammatory pain model was made in Wistar rat footpads that were subcutaneously injected with 1% carrageenan (100 µl). After 4 hours, the rats were subjected to a plantar tenderness test to evaluate the mechanical pain threshold. Single tail vein administration of the drug (1 ml/kg) was carried out 30 minutes before detection, and the control group and the model group were given corresponding solvents.

3)Experimental apparatus

Electronic Von Frey : UGO BASILE,type 38450.

3.Data representation and statistical processing

The experimental data were expressed as mean ± standard deviation (S.D.). Statistical comparisons were performed using the excel software t test. The data between the model group and the control group were analyzed and compared to determine whether there was a significant statistical significance. *P <0.05 indicates that there is a significant difference between the model group and the control group, ** P <0.01 indicates that there is a high significant difference between the model group and the control group, #P<0.05 indicates that there is a significant difference between the model group and the control group, ##P <0.01 indicates that there is a high significant difference between the model group and the administration group.

4.Results

Efficacy data of SHR0687 on carrageenan-induced carrageenan inflammatory pain model in rats was shown in Fig 2.

Molecular modeling

Molecular modeling studies were done using a laptop computer with an Intel i7-7700HQ, 2.8 GHz CPU and 16GB RAM running the Microsoft Windows 10 professional operating system. The software package Molecular Operating Environment (MOE), Chemical Computing Group Inc., Montreal, H3A 2R7 Canada, <http://www.chemcomp.com> was used for the modeling study. PDB structures were downloaded and prepared using Structure Preparation within MOE, including adjust hydrogens and lone pairs using Protonate3D (Labute, P. Proteins. 2009, 75, 187.). Sequence and structures alignments were performed using MOE Align/Superpose function. The agonist bound KOR protein structure (PDB: 6B73) and the DIPP-NH2 conformation in DOR co-Xtal structure (PDB code: 4RWA) were kept. SHR0687(compound 16) was modified based on the DIPP-NH2. By selecting the compound SHR0687 and the surrounding residues

within 4.5Å, iterative energy minimizations with gradually increased tether deviations were performed to relax the clash between the ligand and protein while forming multiple interactions between ligand and protein. Final pose was evaluated by using GBVI/WDA dG score and ligand strain energy with the rigid receptor and AMBER10: EHT force field with R-Field solvation.

Reference:

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