Discovery of Fluoromethylketone-based Peptidomimetics as Covalent ATG4B (autophagin-1) Inhibitors

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Supporting Information

Contents

General Chemistry Experimental Conditions

Synthesis of 1-amino-3-fluoro-propan-2-ol hydrochloride (**5**)

Synthesis and analytical data of **3a** (or Ac-ETFG-FMK)

Synthesis and analytical data of analogues **4a–n**

Synthesis and analytical data of analogues **8a–k**

Synthesis and analytical data of analogues **9a–j**

Predicted binding models of Z-FG-FMK (**2**) and **9a**

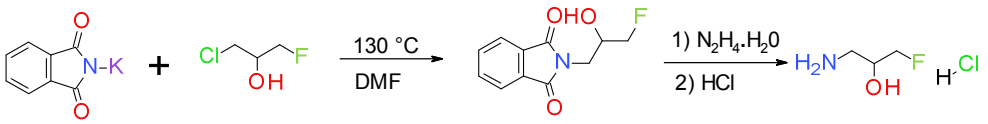
General assay protocols for evaluation of protease selectivity

General Chemistry Experimental Conditions

All of the starting materials were obtained commercially and were used without further purification. All of the reported yields are for isolated products and are not optimized. All of the reactions involving air-sensitive reagents were performed under argon atmosphere. The intermediates were purified by flash chromatography using one of the following instruments: i) Biotage SP1 system and the Quad 12/25 cartridge module; ii) ISCO CombiFlash chromatography instrument. Silica gel brand and pore size: i) KP-SIL 60 Å, particle size: 40−60 μM; ii) CAS registry NO: silica gel: 63231-67-4, particle size: 47−60 μM; iii) ZCX from Qingdao Haiyang Chemical Co., Ltd, pore size: 200−300 or 300−400 mesh. LC-MS spectra were obtained using a MicroMass Platform LC (Waters Alliance 2795-ZQ2000). ^{1}H NMR spectra were obtained using a Bruker Avance 400 MHz NMR spectrometer. All of the final compounds had purities greater than 95% based upon LC–MS and ^{1}H NMR analyses. All of the reported yields are for isolated products and are not optimized.

1-Amino-3-fluoro-propan-2-ol hydrochloride (5). Amine **5** was prepared as hydrochloride salt by the procedures shown in Scheme S1.

Scheme S1



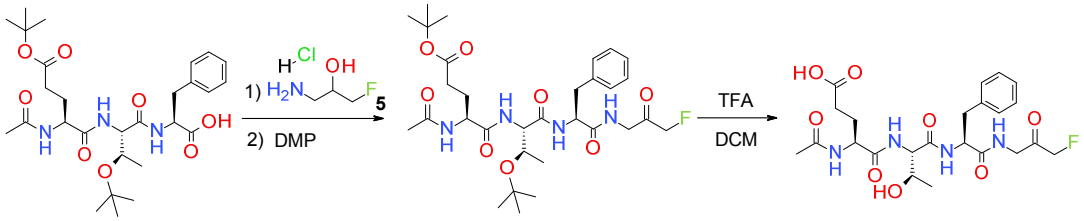
S1 5

*2-(3-Fluoro-2-hydroxy-propyl)isoindoline-1,3-dione (**S1**).* To a suspension solution of potassium phthalimide (3.7 g, 20 mmol) in DMF (15 mL) was added 1-chloro-3-fluoro-propan-2-ol (2.25 g, 20 mmol), and the mixture was stirred at 130 °C for 4 h. After cooling, the mixture was diluted with EtOAc (200 mL) and washed with water (50 mL). The organic layer was separated and concentrated under vacuum. The residue was purified by column chromatography to give **S1** (3.5 g, 78% yield). MS: calc'd (MH^+) 224.1, exp (MH^+) 224.1. ^{1}H NMR (400 MHz, Methanol-d_4) δ ppm 7.80–7.88 (m, 4H), 4.34–4.55 (m, 2H), 4.05–4.20 (m, 1H), 3.72–3.88 (m, 2H).

*1-Amino-3-fluoro-propan-2-ol hydrochloride (**5**).* To a solution of **S1** (3.5 g, 15.7 mmol) in MeOH (150 mL) was added hydrazine hydrate (0.95 eq.), and the mixture was stirred at rt for 24 h. After filtration to remove the precipitate, concentrated HCl (4 mL) was added to the filtrate and the mixture was stirred for 4 h. The precipitate was removed and the aqueous phase was concentrated to give **5** (1.5 g, 70% yield) as light yellow solid which was used for next reactions without further purification. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.00 (br. s., 2H), 4.23−4.56 (m, 2H), 3.89−4.05 (m, 1H), 2.95 (ddd, J = 12.9, 6.1, 3.6 Hz, 1H), 2.74 (ddd, J = 12.9, 8.8, 5.9 Hz, 1H).

Ac-ETFG-FMK or 3a. Tetrapeptide **3a** was synthesized by the method shown in Scheme S2.

Scheme S2



S2 S3 3a

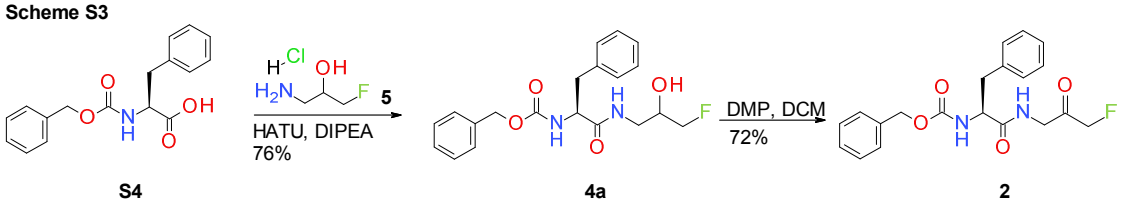
*tert-Butyl (4S)-4-acetamido-5-[[(1S,2R)-1-[[(1S)-1-benzyl-2-[(3-fluoro-2-oxo-propyl)amino]-2-oxo-ethyl]carbamoyl]-2-tert-butoxy-propyl]amino]-5-oxo-pentanoate (**S3**).* To a mixture of peptide intermediate **S2** (175 mg, 0.3 mmol) and amine **5** (50 mg, 0.4 mmol) in DMF (5 mL) were added HATU (171 mg, 0.45 mmol) and DIPEA (78 mg, 0.6 mmol). The mixture was stirred at rt for 4 h. After removal of the solvent, the residue was dissolved in EtOAc (50 mL) and the solution was washed with aqueous NaOH (0.5%, 10 mL). The organic layer was separated, dried over anhydrous Na_2SO_4, and concentrated. The obtained residue was dissolved in DCM (5 mL) and DMP (150 mg, 0.35 mmol) was added into the solution. The mixture was stirred at rt for 4 h, before it was diluted

with EtOAc (50 mL) and H_2O (20 mL). The organic layer was separated and concentrated. The residue was purified by column chromatography to give compound **S3** (150 mg, 60% yield). MS: calc'd (MH+) 623.3, exp (MH+) 623.3. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.33 (t, J = 5.4 Hz, 1H), 8.13 (d, J = 8.0 Hz, 1H), 7.82 (d, J = 8.3 Hz, 1H), 7.53 (d, J = 8.0 Hz, 1H), 7.15−7.31 (m, 5H), 5.13 (d, J = 3.5 Hz, 1H), 5.0 (d, J = 3.5 Hz, 1H), 4.6 (d, J = 4.8 Hz, 1H), 4.31 (d, J = 5.3 Hz, 1H), 4.16 (dd, J = 7.9, 3.9 Hz, 1H), 3.95−4.05 (m, 2H), 3.85 (dd, J = 6.2, 3.9 Hz, 1H), 3.07 (dd, J = 14.2, 4.9 Hz, 1H), 2.83−2.90 (m, 1H), 2.22 (t, J = 8.5 Hz, 2H), 1.86 (s, 3H), 1.64−1.76 (m, 2H), 1.40 (s, 9H), 1.05 (s, 9H), 0.95 (d, J = 6.3 Hz, 3H).

(4S)-4-acetamido-5-[[(1S,2R)-1-[[(1S)-1-benzyl-2-[(3-fluoro-2-oxo-propyl)amino]-2-oxo-ethyl]carbamoyl]-2-hydroxy-propyl]amino]-5-oxo-pentanoic acid ***(3a)***. To a solution of **S3** (62 mg. 0.1 mmol) in DCM (2 mL) was added TFA (0.5 mL), and the mixture was stirred at rt overnight. After removal of the solvent and excess TFA, a light yellow solid was obtained and it was washed with H_2O and dried to give **3a** (45 mg, 88% yield). MS: calc'd (MH^+) 511.2, exp (MH^+) 511.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 12.10 (br. s., 1H), 8.34 (t, J = 5.4 Hz, 1H), 8.08 (d, J = 7.8 Hz, 1H), 8.01 (d, J = 8.0 Hz, 1H), 7.66 (d, J = 8.3 Hz, 1H), 7.13−7.30 (m, 5H), 5.13 (d, J = 2.8 Hz, 1H), 5.01 (d, J = 2.8 Hz, 1H), 4.93 (br. s., 1H), 4.56 (td, J = 8.3, 5.3 Hz, 1H), 4.26−4.34 (m, 1H), 4.18 (dd, J = 8.3, 4.5 Hz, 1H), 3.89−4.04 (m, 3H), 3.06 (dd, J = 13.6, 4.8 Hz, 1H), 2.85 (dd, J = 13.9, 8.9 Hz, 1H), 2.23 (t, J = 8.0 Hz, 2H), 1.85 (s, 3H), 1.70 (dd, J = 13.7, 7.6 Hz, 2H), 0.97 (d, J = 6.3 Hz, 3H).

Benzyl *N*-[(1S)-1-benzyl-2-[(3-fluoro-2-hydroxy-propyl)amino]-2-oxo-ethyl]carbamate (4a) and benzyl *N*-[(1S)-1-benzyl-2-[(3-fluoro-2-oxo-propyl)amino]-2-oxo-ethyl]carbamate (2). Compounds **4a** and **2** were synthesized by the method shown in Scheme S3.

Scheme S3



S4 4a 2

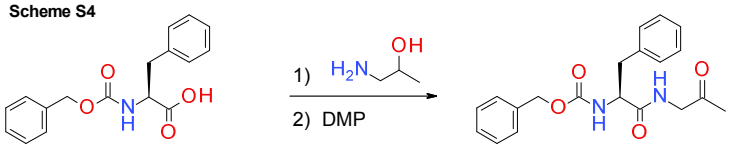
Benzyl N-[(1S)-1-benzyl-2-[(3-fluoro-2-hydroxy-propyl)amino]-2-oxo-ethyl]carbamate ***(4a)***. To a mixture of (2S)-2-(benzyloxycarbonylamino)-3-phenyl-propanoic acid **S4** (90 mg, 0.3 mmol) and **5** (50 mg, 0.4 mmol) in DMF (5 mL) were added HATU (171 mg. 0.45 mmol) and DIPEA (78 mg, 0.6 mmol). The mixture was stirred at rt for 4h. After removal of the solvent, the residue was dissolved in EtOAc (50 mL) and washed with aqueous NaOH (0.5%, 10 mL). The organic layer was separated, dried over anhydrous Na_2SO_4, and concentrated. The obtained residue was purified by column chromatography to give compound **4a** (85 mg, 76% yield). MS: calc'd (MH^+) 375.2, exp (MH^+) 375.2.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.08 (m, 1H), 7.51 (dd, J = 8.8, 4.0 Hz, 1H), 7.02−7.43 (m, 10H), 5.24 (dd, J = 5.1, 2.6 Hz, 1H), 4.93 (s, 2H), 4.16−4.34 (m, 2H), 3.68 (m, 1H), 3.05−3.20 (m, 2H), 2.92−3.01 (m, 1H), 2.70−2.81 (m, 1H).

Benzyl N-[(1S)-1-benzyl-2-[(3-fluoro-2-oxo-propyl)amino]-2-oxo-ethyl]carbamate ***(2)***. FMK-analogue **2** was obtained by treating compound **4a** with DMP as described before in 72% yield. MS: calc'd (MH^+) 375.2, exp (MH^+) 375.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.41 (t, J = 5.3 Hz, 1 H), 7.60 (d, J = 8.5 Hz, 1H), 7.10−7.40 (m, 10H), 5.17 (s, 1H), 5.05 (s, 1H), 4.90−5.00 (m, 2H), 4.26−4.36 (m, 1H), 3.95−4.10 (m, 2H), 3.04 (dd, J = 13.8, 4.3 Hz, 1H), 2.77 (dd, J = 13.7, 10.9 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 202.2 (d, J = 15.4 Hz), 172.7, 156.4, 138.5, 137.4, 129.7, 128.8, 128.5, 128.2, 127.9, 126.8, 84.7 (d, J = 178.3 Hz), 65.7, 56.5, 45.9, 37.8.

Benzyl *N*-[(1S)-2-(acetonylamino)-1-benzyl-2-oxo-ethyl]carbamate (4b). Compound **4b** was synthesized by the coupling of **S4** and **5** and then oxidation by DMP (Scheme S4).

Scheme S4

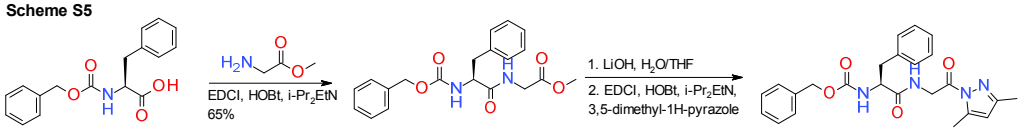


S4 **4b**

MS: calc'd (MH^+) 355.2, exp (MH^+) 355.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.08 (br s, 1H), 7.51 (br dd, J = 8.8, 4.0 Hz, 1H), 7.02−7.43 (m, 10H), 5.24 (dd, J = 5.1, 2.6 Hz, 1H), 4.85−5.01 (m, 2H), 4.16−4.34 (m, 2H), 3.68 (br s, 1H), 3.05−3.20 (m, 2H), 2.92−3.01 (m, 1H), 2.70−2.81 (m, 1H).

Benzyl *N*-[(1S)-1-benzyl-2-[[2-(3,5-dimethylpyrazol-1-yl)-2-oxo-ethyl]amino]-2-oxo-ethyl]carbamate (4c). Compound **4c** was synthesized from **S4** and methyl 2-aminoacetate by the method shown in Scheme S5.

Scheme S5



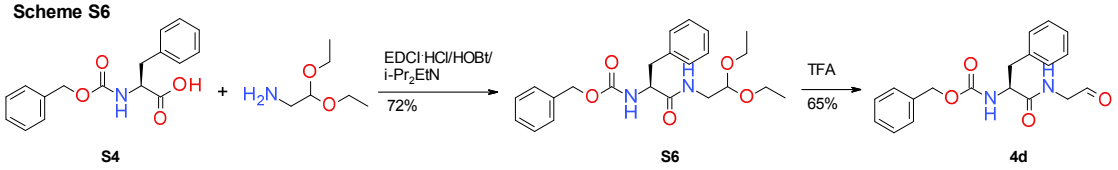
S4 **S5** **4c**

Methyl 2-[(2S)-2-{[(benzyloxy)carbonyl]amino}-3-phenylpropanamido]acetate ***(S5)***. To a mixture of **S4** (500 mg, 1.7 mmol) and TFA salt of methyl 2-aminoacetate (373 mg, 1.8 mmol) in THF (10 mL) were added DIPEA (1.0 mL, 5.8 mmol), EDCI (388 mg, 2.5 mmol) and HOBt (338 mg, 2.5 mmol) and the mixture was stirred at rt for 16 h. The reaction mixture was diluted with water (20 mL) and extracted with EtOAc (40 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by silica gel column chromatography to afford **S5** (400 mg, 65%) as off-white solid. MS: (MH^+) 371.2.

*Benzyl N-[(1S)-1-benzyl-2-[[2-(3,5-dimethylpyrazol-1-yl)-2-oxo-ethyl]amino]-2-oxo-ethyl]carbamate (**4c**).* To a solution of **S5** (500 mg) in THF-H_2O mixture (35 mL, 2.5:1) was added LiOH hydrate (68 mg) and the mixture was stirred at rt for 45 min. The reaction mixture was acidified with 2N HCl to pH 2 and extracted with EtOAc. The organic layer was dried over anhydrous Na_2SO_4 and concentrated in vacuum to afford crude carboxylic acid intermediate. To a mixture of the obtained acid product (250 mg, 0.7 mmol) and 3,5-dimethyl-1H-pyrazole (74 mg, 0.8 mmol) in THF (10 mL) were added DIPEA (0.44 mL, 2.4 mmol), EDCI (202 mg, 1.0 mmol) and HOBt (142 mg, 1.0 mmol) and the mixture was stirred at rt for 16 h. The reaction mixture was partitioned between water and EtOAc, and the aqueous phase was extracted with EtOAc. The combined organic phase was dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by column chromatography to afford **4c** (30 mg, 10%) as off-white solid. MS: calc'd (MH^+) 435.2, exp (MH^+) 435.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.49 (br t, J = 5.8 Hz, 1H), 7.57 (d, J = 8.8 Hz, 1H), 7.16−7.36 (m, 10H), 6.23 (s, 1H), 4.94 (AB, J = 13.0 Hz, 2H), 4.62 (m, 2H), 4.36 (m, 1H), 3.09 (m, 1H), 2.78 (t, J = 11.2 Hz, 1H), 2.45 (s, 3H), 2.20 (s, 3H).

Benzyl *N*-[(1S)-2-[[2-(1,3-benzoxazol-2-yl)-2-oxo-ethyl]amino]-1-benzyl-2-oxo-ethyl]carbamate (4d). Compound **4d** was synthesized from **S4** and 2,2-diethoxyethan-1-amine by the method shown in Scheme S6.

Scheme S6



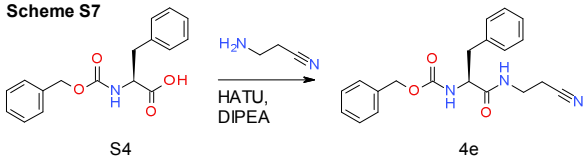
To a stirred mixture of **S4** (250 mg, 0.84 mmol) and 2,2-diethoxyethan-1-amine (123 mg, 0.92 mmol) in THF (25 mL) were added DIPEA (0.53 mL, 2.9 mmol), EDCI·HCl (240.4 mg, 1.2 mmol) and HOBt (169.4 mg, 1.2 mmol). The reaction mixture was stirred at rt for 48 h, and then it was diluted with water (20 mL) and extracted with EtOAc (30 mL). The organic layer was separated, washed with water (20 mL), brine (20 mL), dried over anhydrous Na_2SO_4 and concentrated to give **S6** (250 mg, 72% yield) as off-white solid. MS: calc'd (MH^+) 415.3, exp (MH^+) 415.3. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.03 (t, J = 5.3 Hz, 1H), 7.46 (d, J = 8.5 Hz, 1H), 7.19−7.34 (m, 10H), 4.93 (s, 2H), 4.44 (t, J = 5.4 Hz, 1H), 4.25 (dt, J = 10.0, 4.2 Hz, 1H), 3.59 (m, 2H), 3.41−3.50 (m, 2H), 3.19 (m, 1H), 3.14 (m, 1H), 2.95 (dd, J = 13.4, 3.9 Hz, 1H), 2.74 (m, 1H), 1.11 (m, 6H).

A solution of **S6** (1.0 g, 2.4 mmol) in 20 mL of TFA was stirred at 0 °C for 5 min. The mixture was diluted with EtOAc and water, and the aqueous phase was extracted with EtOAc. The organic phase was washed with saturated aqueous $NaHCO_3$ (100 mL) and brine (50 mL), dried over

anhydrous Na_2SO_4, and concentrated under reduced pressure. The crude compound was purified by triturating with CH_2Cl_2 and n-pentane to afford **4d** (540 mg, 65% yield) as off-white solid. MS: calc'd (MH^+) 341.2, exp (MH^+) 341.2. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 9.54 (s, 1H), 7.17−7.51 (m, 11H), 6.41 (br. s., 1H), 5.26 (m, 1H), 5.08 (s, 2H), 4.47 (m, 1H), 4.02−4.16 (m, 1H), 3.0−3.09 (m, 2H).

Benzyl *N*-[(1S)-1-benzyl-2-(2-cyanoethylamino)-2-oxo-ethyl]carbamate (4e). Compound **4e** was synthesized from **S4** and 3-aminopropanenitrile in analogy to **4a** (Scheme S7).

Scheme S7

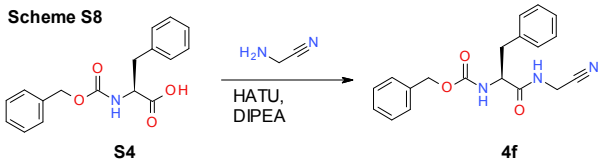


S4 4e

MS: calc'd (MH^+) 352.2, exp (MH^+) 352.2. ^{1}H NMR (400 MHz, Methanol-d_4) δ ppm 7.18−7.38 (m, 10H), 5.04 (q, J = 12.6 Hz, 2H), 4.36 (dd, J = 8.8, 6.0 Hz, 1H), 3.40 (td, J = 12.9, 6.8 Hz, 2H), 3.13 (dd, J = 13.7, 5.9 Hz, 1H), 2.90 (dd, J = 13.6, 9.0 Hz, 1H), 2.50−2.61 (m, 2H).

Benzyl *N*-[(1S)-1-benzyl-2-(cyanomethylamino)-2-oxo-ethyl]carbamate (4f). Compound **4f** was synthesized from **S4** and 2-aminoacetonitrile in analogy to **4a** (Scheme S8).

Scheme S8

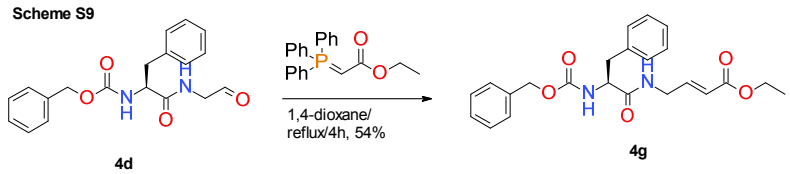


S4 4f

MS: calc'd (MH^+) 338.2, exp (MH^+) 338.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.79 (t, J = 5.4 Hz, 1H), 7.67 (d, J = 8.5 Hz, 1H), 7.05−7.44 (m, 10H), 4.85−5.04 (m, 2H), 4.19−4.29 (m, 1H), 4.16 (d, J = 5.5 Hz, 2H), 3.00 (dd, J = 13.6, 4.5 Hz, 1H), 2.77 (dd, J = 13.7, 10.4 Hz, 1H).

Benzyl *N*-[(1S)-1-benzyl-2-[[(E)-4-(methylamino)-4-oxo-but-2-enyl]amino]-2-oxo-ethyl]carbamate (4g). Compound **4g** was synthesized from **4d** and ethyl 2-(triphenyl-□^{5}-phosphanylidene)acetate by the method shown in Scheme S9.

Scheme S9



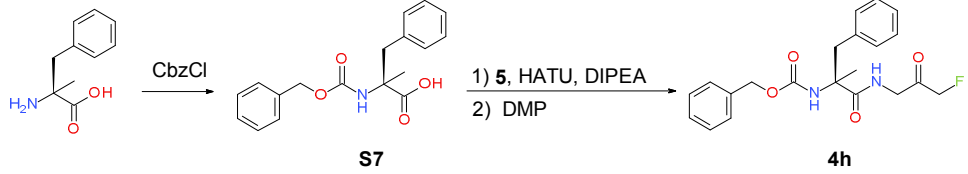
4d 4g

To a solution of **4d** (200 mg, 0.588 mmol) in 1,4-dioxane (3 mL) was added ethyl 2-(triphenyl-□^{5}-phosphanylidene)acetate (409.4 mg, 1.176 mmol) and the mixture was stirred at refluxing for 4 h.

The solvent was removed and the residue was purified by flash chromatography to afford **4g** (130 mg, 54% yield) as off-white solid. LCMS: calc'd (MH^+) 411.1, exp (MH^+) 411.1. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.31 (m, 1H), 7.56 (d, J = 8.0 Hz, 1H), 7.19−7.33 (m, 10H), 6.79 (dt, J = 15.8, 4.2 Hz, 1H), 5.83 (d, J = 15.6 Hz, 1H), 4.96 (s, 2H), 4.24 (m, 1H), 4.12 (dd, J = 13.4, 6.5 Hz, 2H), 3.88 (s, 2H), 2.99 (dd, J = 13.2, 4.6 Hz, 1H), 2.78 (dd, J = 12.4, 10.9 Hz, 1H), 1.21 (t, J = 7.0 Hz, 3.H).

Benzyl *N*-[(1S)-1-benzyl-2-[(3-fluoro-2-oxo-propyl)amino]-1-methyl-2-oxo-ethyl]carbamate (4h). Compound **4h** was synthesized by the method shown in Scheme S10.

Scheme S10



To a solution of (2S)-2-amino-2-methyl-3-phenyl-propanoic acid (180 mg, 1 mmol) in H_2O/CH_3CN (1;1, 20 mL) was added NaOH (80 mg, 2 mmol) and CbzCl (205 mg, 1.2 mmol) at rt and the mixture was stirred at rt for 4 h. The reaction was quenched with citric acid (5 mL) and then extracted with DCM (20 mL) three times. The organic phase was concentrated to give compound **S7** (128 mg, 41% yield). MS: calc'd (MH^+) 314.1, exp (MH^+) 314.1.

To a mixture of **S7** (94 mg, 0.3 mmol) and **5** (50 mg, 0.4 mmol) in DMF (5 mL) was added HATU (171 mg. 0.45 mmol) and DIPEA(78 mg, 0.6 mmol). The mixture was stirred at rt for 4 h. After removal of the solvent, the residue was dissolved in EtOAc (50 mL) and washed with aqueous NaOH (0.5%, 10 mL). The organic layer was separated, dried over anhydrous Na_2SO_4, and concentrated. The residue was dissolved in DCM (5 mL), to the solution was added DMP (150 mg, 0.35 mmol) and the mixture was stirred at rt for 4 h. The mixture was diluted with EtOAc (50 mL) and washed with H_2O (20 mL). The organic layer was separated and concentrated. The residue was purified by flash chromatography to give compound **4h** (75 mg, 65% yield). MS: calc'd (MH^+) 387.2, exp (MH^+) 387.2. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.43−7.33 (m, 5H), 7.28−7.19 (m, 3H), 7.10−7.00 (m, 2H), 6.99−6.89 (m, 1H), 5.19 (d, J = 11.8 Hz, 2H), 5.14−5.06 (m, 1H), 5.03−4.98 (m, 1H), 4.89 (s, 1H), 4.32 (m, 2H), 3.45−3.32 (m, 1H), 3.23−3.11 (m, 1H), 1.64 (s, 3H).

Benzyl *N*-[(1S)-2-[(3-fluoro-2-oxo-propyl)amino]-2-oxo-1-phenyl-ethyl]carbamate (4i). Compound **4i** was synthesized from (2S)-2-[(2-benzyloxyacetyl)amino]-2-phenyl-acetic acid and **5** in analogy to **4h**. MS: calc'd (MH^+) 359.2, exp (MH^+) 359.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.52−8.58 (m, 1H), 7.99 (d, J = 8.0 Hz, 1H), 7.46 (d, J = 7.0 Hz, 2H), 7.25−7.42 (m, 1H), 7.25−7.42 (m, 8H), 5.34 (d, J = 8.0 Hz, 1H), 5.13 (s, 1H), 5.05−5.07 (m, 1H), 5.01−5.05 (m, 1H), 4.01 (d, J = 5.5 Hz, 2H), 3.18 (d, J = 5.3 Hz, 1H).

Benzyl *N*-[(1S)-1-[(3-fluoro-2-oxo-propyl)carbamoyl]-2-methyl-propyl]carbamate (4j). Compound **4j** was synthesized from (2S)-2-[(2-benzyloxyacetyl)amino]-3-methyl-butanoic acid and **5** in analogy to **4h**. MS: calc'd (MH^+) 325.2, exp (MH^+) 325.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.26 (t, J = 5.52 Hz, 1H), 7.29−7.43 (m, 6H), 5.19 (s, 1H), 5.00−5.12 (m, 3H), 3.97−4.08 (m, 2H), 3.86−3.93 (m, 1H), 1.93−2.01 (m, 1H), 0.88 (dd, J = 9.5, 6.8 Hz, 6H).

Benzyl *N*-[(1R)-1-benzyl-2-[(3-fluoro-2-oxo-propyl)amino]-2-oxo-ethyl]carbamate (4k). Compound **4k** was synthesized from (2R)-2-(benzyloxycarbonylamino)-3-phenyl-propanoic acid and **5** in analogy to **4h**. MS: calc'd (MH^+) 373.2, exp (MH^+) 373.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.41 (t, J = 5.5 Hz, 1H), 7.60 (d, J = 8.5 Hz, 1H), 7.14−7.39 (m, 10H), 5.17 (s, 1H), 5.05 (s, 1H), 4.91−5.01 (m, 2H), 4.26−4.36 (m, 1H), 3.96−4.09 (m, 2H), 3.04 (dd, J = 13.9, 3.9 Hz, 1H), 2.77 (dd, J = 13.7, 10.9 Hz, 1H).

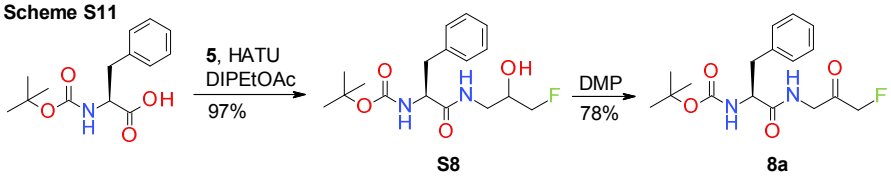
Benzyl *N*-[(1S)-1-[(2-chlorophenyl)methyl]-2-[(3-fluoro-2-oxo-propyl)amino]-2-oxo-ethyl]carbamate (4l). Compound **4l** was synthesized from (2S)-2-amino-3-(2-chlorophenyl)propanoic acid and **5** in analogy to **4h**. MS: calc'd (MH^+) 407.1, exp (MH^+) 407.1. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.35 (s, 6H), 7.26−7.17 (m, 3H), 6.59−6.49 (m, 1H), 5.46−5.37 (m, 1H), 5.08 (s, 2H), 4.98 (s, 1H), 4.86 (s, 1H), 4.59 (d, J = 6.4 Hz, 1H), 4.44−4.22 (m, 2H), 3.36−3.28 (m, 1H), 3.23−3.11 (m, 1H).

Benzyl *N*-[(1S)-1-[(3-chlorophenyl)methyl]-2-[(3-fluoro-2-oxo-propyl)amino]-2-oxo-ethyl]carbamate (4m). Compound **4m** was synthesized from (2S)-2-amino-3-(3-chlorophenyl)propanoic acid and **5** in analogy to **4h**. MS: calc'd (MH^+) 407.1, exp (MH^+) 407.1. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.44−7.29 (m, 5H), 7.24 (m, 3H), 7.15−7.05 (m, 1H), 6.84−6.49 (m, 1H), 5.54−5.42 (m, 1H), 5.17−5.04 (m, 2H), 4.95 (s, 1H), 4.83 (s, 1H), 4.59−4.46 (m, 1H), 4.40−4.17 (m, 2H), 3.26−2.94 (m, 2H).

Benzyl *N*-[(1S)-2-[(3-fluoro-2-oxo-propyl)amino]-1-[(3-fluorophenyl)methyl]-2-oxo-ethyl]carbamate (4n). Compound **4n** was synthesized from ((2S)-2-amino-3-(3-fluorophenyl)propanoic acid and **5** in analogy to **4h**. MS: calc'd (MH^+) 391.1, exp (MH^+) 391.1. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.42−7.30 (m, 5H), 7.28−7.24 (m, 1H), 7.04−6.90 (m, 3H), 6.63−6.49 (m, 1H), 5.39−5.28 (m, 1H), 5.11 (s, 2H), 4.98 (s, 1H), 4.90−4.83 (m, 1H), 4.52 (d, J = 6.0 Hz, 1H), 4.42−4.22 (m, 2H), 3.26−3.01 (m, 2H).

***tert*-Butyl *N*-[(1S)-1-benzyl-2-[(3-fluoro-2-oxo-propyl)amino]-2-oxo-ethyl]carbamate (8a).** Compound **8a** was synthesized from (2S)-2-(*tert*-butoxycarbonylamino)-3-phenyl-propanoic acid and **5** (Scheme S11).

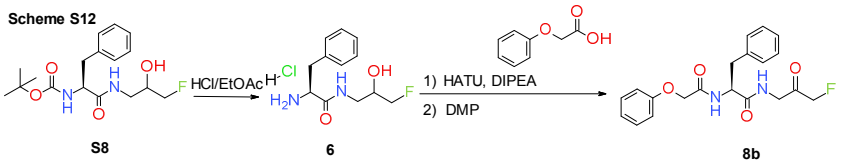
Scheme S11



To a mixture of (2*S*)-2-(tert-butoxycarbonylamino)-3-phenyl-propanoic acid (2.65 g, 10 mmol) and 1-amino-3-fluoro-propan-2-ol hydrochloride **5** (1.3 g, 10 mmol) in DMF (50 mL) was added HATU (4.56 g, 12 mmol) and DIPEA (2.6 g, 20 mmol). The mixture was stirred at rt for 4 h. After removal of the solvent, the residue was dissolved in EtOAc (200 mL) and the solution was washed with water (50 mL) and aqueous NaOH (0.5%, 50 mL). The organic phase was separated, dried over anhydrous Na_2SO_4, and concentrated. The obtained residue was purified by flash chromatography to give amide **S8** (3.1 g, 90% yield). Next, **S8** was treated with DMP as described before to give **8a**. MS: calc'd (MH^+) 339.2, exp (MH^+) 339.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.30 (t, J = 5.4 Hz, 1H), 7.28 (d, J = 4.3 Hz, 4H), 7.16−7.23 (m, 1H), 7.03 (d, J = 8.5 Hz, 1H), 5.17 (s, 1H), 5.05 (s, 1H), 4.16−4.25 (m, 1H), 4.00 (t, J = 4.9 Hz, 2H), 3.00 (dd, J = 13.4, 4.4 Hz, 1H), 2.76 (dd, J = 13.4, 10.9 Hz, 1H), 1.31 (s, 9H).

(2S)-*N*-(3-Fluoro-2-hydroxy-propyl)-2-[(2-phenoxyacetyl)amino]-3-phenyl-propanamide (8b). Compound **8b** was synthesized from **S8** by the method shown in Scheme S12.

Scheme S12



*(2S)-2-Amino-N-(3-fluoro-2-hydroxy-propyl)-3-phenyl-propanamide (**6**).* A solution of **S8** (3.1 g, 9.1 mmol) and 1N HCl in EtOAc (50 mL, 50 mmol) was stirred at rt overnight. After removal of the solvent, amine **6** was obtained as HCl salt (2.5 g, quant. yield) and was used in subsequent step directly. MS: exp (MH^+) 241.1.

*(2S)-N-(3-Fluoro-2-hydroxy-propyl)-2-[(2-phenoxyacetyl)amino]-3-phenyl-propanamide (**8b**).* To a mixture of **6** (55 mg, 0.2 mmol) and 2-phenoxyacetic acid (31 mg, 0.2 mmol) was added HATU (114 mg. 0.3 mmol) and DIPEA (52 mg, 0.4 mmol).The mixture was stirred at rt for 4hr. After removal of the solvent, the residue was dissolved in EtOAc (50 mL) and the organic phase was washed with 0.5% aqueous NaOH (10 mL). The organic phase was dried over anhydrous Na_2SO_4 and concentrated. The residue was dissolved in DCM (5 mL), to which DMP (100 mg, 0.24 mmol) was added. After stirred at rt for 4 h, the mixture was diluted with EtOAc (50 mL) and washed with water and brine. The organic phase was dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by column chromatography to give compound **8b** (45 mg, 60% yield). MS: calc'd (MH^+) 373.2, exp (MH^+) 373.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.47 (t, J = 5.5 Hz, 1H), 8.23 (d, J =

8.3 Hz, 1H), 7.13−7.33 (m, 7H), 6.95 (t, *J* = 7.4 Hz, 1H), 6.77−6.86 (m, 2H), 5.17 (s, 1H), 5.06 (s, 1H), 4.58−4.71 (m, 1H), 4.44 (s, 2H), 4.03 (d, *J* = 5.5 Hz, 2H), 3.09 (dd, *J* = 13.8, 4.5 Hz, 1H), 2.90 (dd, *J* = 13.8, 9.8 Hz, 1H).

(2S)-*N*-(3-Fluoro-2-oxo-propyl)-3-phenyl-2-(3-phenylpropanoylamino)propanamide (8c). Compound **8c** was synthesized from **6** and 3-phenylpropanoic acid in analogy to **8b**. MS: calc'd (MH^+) 371.2, exp (MH^+) 371.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.34 (t, *J* = 5.6 Hz, 1H), 8.19 (d, *J* = 8.5 Hz, 1H), 7.11−7.28 (m, 10H), 5.14 (d, *J* = 1.8 Hz, 1H), 5.03 (d, *J* = 1.8 Hz, 1H), 4.51−4.62 (m, 1H), 3.99 (d, *J* = 5.3 Hz, 2H), 3.02 (dd, *J* = 13.8, 4.8 Hz, 1H), 2.75−2.80 (m, 1H), 2.66−2.72 (m, 2H), 2.36 (dd, *J* = 8.8, 7.0 Hz, 2H).

(2S)-*N*-(3-Fluoro-2-oxo-propyl)-3-phenyl-2-[(2-phenylacetyl)amino]propanamide (8d). Compound **8d** was synthesized from **6** and 2-phenylacetic acid in analogy to **8b**. MS: calc'd (MH^+) 357.2, exp (MH^+) 357.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.35−8.46 (m, 2H), 7.14−7.29 (m, 8H), 7.03−7.11 (m, 2H), 5.15 (d, *J* = 1.0 Hz, 1H), 5.03 (s, 1H), 4.53−4.62 (m, 1H), 4.01 (d, *J* = 5.3 Hz, 2H), 3.34−3.46 (m, 2H), 3.05 (dd, *J* = 13.7, 4.4 Hz, 1H), 2.79 (dd, *J* = 13.68, 10.16 Hz, 1H).

***N*-[(1S)-1-Benzyl-2-[(3-fluoro-2-oxo-propyl)amino]-2-oxo-ethyl]benzamide (8e).** Compound **8e** was synthesized from **6** and benzoic acid in analogy to **8b**. MS: calc'd (MH^+) 343.1, exp (MH^+) 343.1. ^{1}H NMR (400 MHz, Methanol-d_4) δ ppm 8.21−8.25 (m, 2H), 7.99−8.04 (m, 1H), 7.89−7.94 (m, 2H), 7.75−7.84 (m, 4H), 7.67−7.73 (m, 1H), 5.56 (s, 1H), 5.45 (s, 1H), 5.39−5.44 (m, 1H), 4.67 (d, *J* = 1.0 Hz, 2H), 3.78−3.84 (m, 1H), 3.59 (dd, *J* = 13.8, 9.5 Hz, 1H).

***N*-[(1S)-1-Benzyl-2-[(3-fluoro-2-oxo-propyl)amino]-2-oxo-ethyl]cyclopentanecarboxamide (8f).** Compound **8f** was synthesized from **6** and cyclopentanecarboxylic acid in analogy to **8b**. MS: calc'd (MH^+) 335.2, exp (MH^+) 335.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.29 (t, *J* = 5.4 Hz, 1H), 8.00 (d, *J* = 8.5 Hz, 1H), 7.26 (d, *J* = 4.5 Hz, 4H), 7.15−7.21 (m, 1H), 5.15 (s, 1H), 5.03 (s, 1H), 4.51−4.60 (m, 1H), 4.00 (d, *J* = 5.5 Hz, 2H), 3.04 (dd, *J* = 13.7, 4.6 Hz, 1H), 2.74−2.82 (m, 1H), 2.53−2.60 (m, 1H), 1.38−1.69 (m, 7H), 1.30 (dd, *J* = 12.2, 7.4 Hz, 1H).

***N*-[(1S)-1-Benzyl-2-[(3-fluoro-2-oxo-propyl)amino]-2-oxo-ethyl]-3-chloro-benzamide (8g).** Compound **8g** was synthesized from **6** and 3-chlorobenzoic acid in analogy to **8b**. MS: calc'd (MH^+) 377.1, exp (MH^+) 377.1. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.83 (d, *J* = 8.3 Hz, 1H), 8.48 (br. s., 1H), 7.86 (s, 1H), 7.75 (d, *J* = 7.5 Hz, 1H), 7.60 (d, *J* = 7.8 Hz, 1H), 7.45−7.52 (m, 1H), 7.35 (d, *J* = 7.3 Hz, 2H), 7.27 (t, *J* = 7.3 Hz, 2H), 7.13−7.21 (m, 1H), 5.19 (s, 1H), 5.07 (s, 1H), 4.76 (d, *J* = 6.0 Hz, 1H), 4.04 (d, *J* = 4.0 Hz, 2H), 3.17 (dd, *J* = 13.6, 3.3 Hz, 1H), 2.93−3.06 (m, 1H).

***N*-[(1S)-1-Benzyl-2-[(3-fluoro-2-oxo-propyl)amino]-2-oxo-ethyl]-3-methoxy-benzamide (8h).** Compound **8h** was synthesized from **6** and 3-methoxybenzoic acid in analogy to **8b**. MS: calc'd (MH^+) 373.2, exp (MH^+) 373.2. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.33−7.25 (m, 8H), 7.06 (m, 1H), 6.88

(m, 1H), 6.75 (m, 1H), 4.99−4.94 (m, 2H), 4.83 (s, 1H), 4.32−4.27 (m, 2H), 3.11 (s, 3H), 3.27−3.18 (m, 2H).

***N*-[(1S)-1-Benzyl-2-[(3-fluoro-2-oxo-propyl)amino]-2-oxo-ethyl]-3-(trifluoromethyl) benzamide (8i).** Compound **8i** was synthesized from **6** and 3-(trifluoromethyl)benzoic acid in analogy to **8b**. MS: calc'd (MH^+) 411.1, exp (MH^+) 411.1. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.99 (m, 1H), 7.89−7.87 (m, 1H), 7.76−7.74 (m, 1H), 7.53 (m, 1H), 7.32−7.21 (m, 6H), 6.85 (s, 1H), 5.03 (m, 1H), 4.95 (s, 1H), 4.83 (s, 1H), 4.33−4.28 (m, 2H), 3.25−3.23 (m, 2H).

***N*-[(1S)-1-Benzyl-2-[(3-fluoro-2-oxo-propyl)amino]-2-oxo-ethyl]pyrazine-2-carboxamide (8j).** Compound **8j** was synthesized from **6** and pyrazine-2-carboxylic acid in analogy to **8b**. MS: calc'd (MH^+) 345.1, exp (MH^+) 345.1. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 9.36 (d, J = 1.2 Hz, 1H), 8.77 (d, J = 2.5 Hz, 1 H) 8.55 (dd, J = 2.3, 1.5 Hz, 1 H) 8.33 (d, J = 8.0 Hz, 1 H) 7.21−7.34 (m, 6 H) 6.54 (br. s., 1H), 4.87−4.98 (m, 2H), 4.83 (s, 1H), 4.21−4.41 (m, 2H), 3.24 (d, J = 7.5 Hz, 2H).

***N*-[(1S)-1-Benzyl-2-[(3-fluoro-2-oxo-propyl)amino]-2-oxo-ethyl]pyridazine-3-carboxamide (8k).** Compound **8k** was synthesized from **6** and pyridazine-3-carboxylic acid in analogy to **8b**. MS: calc'd (MH^+) 345.1, exp (MH^+) 345.1. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 9.42 (dd, J = 5.0, 1.5 Hz, 1H), 9.19 (d, J = 8.5 Hz, 1H), 8.56 (t, J = 5.3 Hz, 1H), 8.14 (dd, J = 8.4, 1.63 Hz, 1H), 7.90 (dd, J = 8.5, 5.0 Hz, 1H), 7.20−7.33 (m, 4H), 7.13−7.19 (m, 1H), 5.20 (s, 1H), 5.08 (s, 1H), 4.88 (dt, J = 8.7, 4.9 Hz, 1H), 4.06 (d, J = 5.5 Hz, 2H), 3.20 (dd, J = 16.3, 9.3 Hz, 2H).

***N*-[(1S)-1-Benzyl-2-[(3-fluoro-2-oxo-propyl)amino]-2-oxo-ethyl]naphthalene-1-carboxamide (9a)**. Compound **9a** was synthesized from **6** and naphthalene-1-carboxylic acid in analogy to **8b** with 78% yield over 2 steps. MS: calc'd (MH^+) 393.2, exp (MH^+) 393.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.82 (d, J = 8.5 Hz, 1H), 8.50 (t, J = 5.3 Hz, 1H), 7.96 (dd, J = 18.6, 8.0 Hz, 2H), 7.78 (d, J = 8.5 Hz, 1H), 7.23−7.56 (m, 9H), 5.24 (s, 1H), 5.13 (s, 1H), 4.88−4.97 (m, 1H), 4.12 (d, J = 3.3 Hz, 2H), 3.21 (dd, J = 13.7, 3.6 Hz, 1H), 2.88−2.99 (m, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 202.3 (d, J = 15.4 Hz), 172.5, 169.0, 138.7, 135.0, 133.4, 130.2, 130.1, 129.8, 128.6, 128.4, 126.9, 126.8, 126.6, 126.0, 125.6, 125.3, 84.8 (d, J = 178.3 Hz), 54.9, 46.1, 37.6.

***N*-[(1S)-1-Benzyl-2-[(3-fluoro-2-oxo-propyl)amino]-2-oxo-ethyl]naphthalene-2-carboxamide (9b).** Compound **9b** was synthesized from **6** and naphthalene-2-carboxylic acid in analogy to **8b**. MS: calc'd (MH^+) 393.2, exp (MH^+) 393.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.84 (d, J = 8.3 Hz, 1H), 8.51 (t, J = 5.4 Hz, 1H), 8.43 (s, 1H), 7.94−8.04 (m, 3H), 7.89 (dd, J = 8.5, 1.5 Hz, 1H), 7.56−7.65 (m, 2H), 7.36−7.43 (m, 2H), 7.27 (t, J = 7.5 Hz, 2H), 7.13−7.20 (m, 1H), 5.20 (s, 1H), 5.08 (s, 1H), 4.78−4.87 (m, 1H), 4.06 (d, J = 5.5 Hz, 2H), 3.16−3.24 (m, 1H), 3.07 (dd, J = 13.6, 11.0 Hz, 1H).

***N*-[(1S)-1-Benzyl-2-[(3-fluoro-2-oxo-propyl)amino]-2-oxo-ethyl]-4-fluoro-naphthalene-1-carboxamide (9c).** Compound **9c** was synthesized from **6** and 4-fluoronaphthalene-1-carboxylic acid in analogy to **8b**. MS: calc'd (MH^+) 411.1, exp (MH^+) 411.1. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.86 (d, J = 8.8 Hz, 1H), 8.52 (t, J = 5.3 Hz, 1H), 8.07 (d, J = 8.5 Hz, 1H), 7.84 (d, J = 8.3 Hz, 1H), 7.64 (t, J = 7.2 Hz, 1H), 7.51−7.57 (m, 1H), 7.49 (dd, J = 7.9, 5.6 Hz, 1H), 7.29−7.42 (m, 5H), 7.23−7.29 (m, 1H), 5.24 (s, 1H), 5.12 (s, 1H), 4.87−4.95 (m, 1H), 4.11 (d, J = 5.5 Hz, 2H), 3.21 (dd, J = 13.9, 4.1 Hz, 1H), 2.92 (dd, J = 13.6, 11.3 Hz, 1H).

***N*-[(1S)-1-Benzyl-2-[(3-fluoro-2-oxo-propyl)amino]-2-oxo-ethyl]-5-fluoro-naphthalene-1-carboxamide (9d).** Compound **9d** was synthesized from **6** and 5-fluoronaphthalene-1-carboxylic acid in analogy to **8b**. MS: calc'd (MH^+) 411.1, exp (MH^+) 411.1. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.90 (d, J = 8.5 Hz, 1H), 8.52 (t, J = 5.4 Hz, 1H), 8.12 (d, J = 8.5 Hz, 1H), 7.61−7.67 (m, 1H), 7.52−7.60 (m, 2H), 7.23−7.47 (m, 7H), 5.24 (s, 1H), 5.12 (s, 1H), 4.88−4.97 (m, 1H), 4.07−4.18 (m, 2H), 3.21 (dd, J = 13.8, 4.0 Hz, 1H), 2.92 (dd, J = 13.6, 11.3 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 202.3 (d, J = 15.4 Hz), 172.4, 168.6, 138.6, 135.1, 131.5 (d, J = 4.4 Hz), 129.7, 128.6, 127.0 (d, J = 8.8 Hz), 126.8, 126.7, 126.2, 123.3 (d, J = 16.1 Hz), 122.4, 122.0 (d, J = 5.9 Hz), 110.5, 110.3, 84.8 (d, J = 178.3 Hz), 54.9, 46.0, 37.7.

***N*-[(1S)-1-Benzyl-2-[(3-fluoro-2-oxo-propyl)amino]-2-oxo-ethyl]quinoline-4-carboxamide (9e)**. In analogy to **9a**, analogue **9e** was synthesized from **6** and quinoline-4-carboxylic acid. MS: calc'd (MH^+) 394.2, exp (MH^+) 394.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 9.10 (d, J = 8.53 Hz, 1H), 8.94 (d, J = 4.3 Hz, 1H), 8.58 (t, J = 5.5 Hz, 1H), 7.94−8.07 (m, 2H), 7.85 (d, J = 8.3 Hz, 1H), 7.68−7.80 (m, 1H), 7.63 (d, J = 8.0 Hz, 1H), 7.47−7.54 (m, 1H), 7.25−7.41 (m, 4H), 5.24 (s, 1H), 5.13 (s, 1H), 4.92−5.01 (m, 1H), 4.13 (d, J = 5.8 Hz, 2H), 3.22 (dd, J = 13.6, 3.8 Hz, 1H), 2.89 (dd, J = 13.7, 11.4 Hz, 1H).

***N*-[(1S)-1-Benzyl-2-[(3-fluoro-2-oxo-propyl)amino]-2-oxo-ethyl]quinoline-8-carboxamide (9f).** Compound **9f** was synthesized from **6** and quinoline-8-carboxylic acid in analogy to **8b**. MS: calc'd (MH^+) 394.2, exp (MH^+) 394.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.32 (d, J = 7.0 Hz, 1H), 8.99 (dd, J = 4.27, 1.8 Hz, 1H), 8.64 (t, J = 5.5 Hz, 1H), 8.58 (dd, J = 8.3, 1.8 Hz, 1H), 8.50 (dd, J = 7.3, 1.5 Hz, 1H), 8.21 (dd, J = 8.2, 1.4 Hz, 1H), 7.67−7.77 (m, 2H), 7.39 (d, J = 7.0 Hz, 2H), 7.30 (t, J = 7.4 Hz, 2H), 7.17−7.23 (m, 1H), 5.20 (s, 1H), 5.08 (s, 1H), 4.87−4.94 (m, 1H), 4.05 (d, J = 5.3 Hz, 2H), 3.24 (dd, J = 13.8, 4.8 Hz, 1H), 3.09 (dd, J = 13.8, 8.8 Hz, 1H).

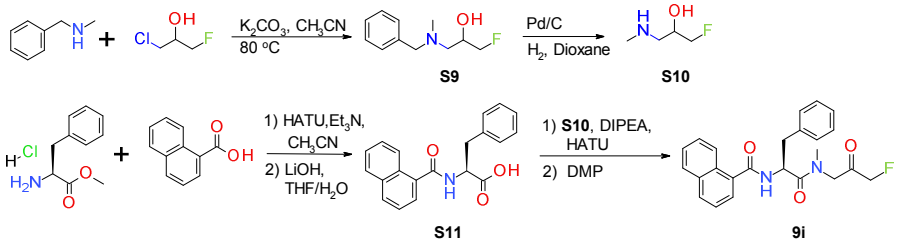
***N*-[(1S)-1-Benzyl-2-[(3-fluoro-2-oxo-propyl)amino]-2-oxo-ethyl]cinnoline-4-carboxamide (9g).** Compound **9g** was synthesized from **6** and cinnoline-4-carboxylic acid in analogy to **8b**. MS: calc'd (MH^+) 395.1, exp (MH^+) 395.1. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 9.20 (s, 1H), 8.56 -8.53 (d, J = 8.4 Hz, 1H), 8.07−8.05 (d, J = 8.4 Hz, 1H), 7.88−7.86 (d, J = 7.6 Hz, 1H), 7.79−7.75 (m, 1H),

7.38−7.12 (m, 5H), 7.12−7.11 (d, *J* = 7.2 Hz, 1H), 6.67 (s, 1H), 5.11−5.08 (m, 1H), 5.01 (s, 1H), 4.90 (s, 1H), 4.47−4.32 (m, 2H), 3.35−3.23 (m, 2H).

***N*-[(1S)-1-Benzyl-2-[(3-fluoro-2-oxo-propyl)amino]-2-oxo-ethyl]-2-methoxy-naphthalene-1-carboxamide (9h).** Compound **9h** was synthesized from **6** and 2-methoxynaphthalene-1-carboxylic acid in analogy to **8b**. MS: calc'd (MH^+) 423.2, exp (MH^+) 423.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.78 (d, *J* = 8.5 Hz, 1H), 8.24 (t, *J* = 5.3 Hz, 1H), 7.96 (d, *J* = 9.0 Hz, 1H), 7.85 (d, *J* = 7.5 Hz, 1H), 7.45 (d, *J* = 9.3 Hz, 1H), 7.24−7.39 (m, 7H), 7.15 (d, *J* = 8.3 Hz, 1H), 5.25 (s, 1H), 5.13 (s, 1H), 4.91 (ddd, *J* = 11.4, 8.0, 3.9 Hz, 1H), 4.17 (d, *J* = 5.3 Hz, 2H), 3.81 (s, 3H), 3.19 (dd, *J* = 14.1, 4.02 Hz, 1H), 2.86 (dd, *J* = 13.9, 11.2 Hz, 1H).

***N*-[(1S)-1-Benzyl-2-[(3-fluoro-2-oxo-propyl)-methyl-amino]-2-oxo-ethyl]naphthalene-1-carboxamide (9i).** Compound **9i** was synthesized by the method shown in Scheme S13.

Scheme S13



*1-[Benzyl(methyl)amino]-3-fluoro-propan-2-ol (**S9**).* A mixture of N-methyl-1-phenyl-methanamine (363 mg, 3.0 mmol), 1-chloro-3-fluoro-propan-2-ol (337 mg, 3.0 mmol) and K_2CO_3 (828 mg, 6.0 mmol) in CH_3CN (15 mL) was stirred in a sealed tube at 80 °C overnight. After removal of the solvent in vacuum, the residue was dissolved in EtOAc (30 mL) and the organic layer was washed with brine, dried and concentrated. The residue was purified by column chromatography to give **S9** as oil (475 mg, 80% yield). MS: calc'd (MH^+) 198.1, exp (MH^+) 198.1

*1-Fluoro-3-(methylamino)propan-2-ol (**S10**).* A mixture of **S9** (100 mg, 0.5 mmol) and Pd/C (10 mg) in dioxane (5 mL) was charged with a hydrogen balloon and stirred at rt for 3 h. After removal of the catalyst and the solvent, the crude product **S10** was used directly in subsequent reactions.

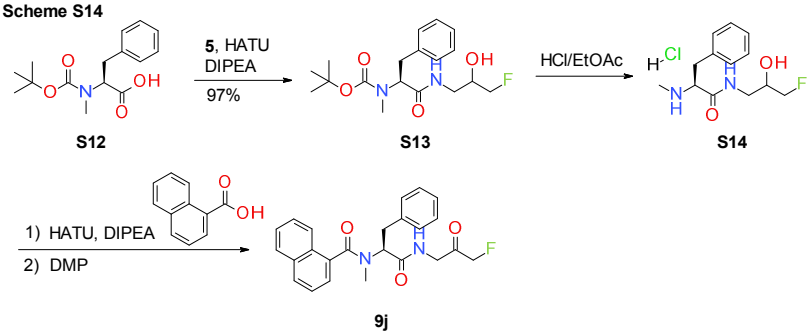
*(2S)-2-(Naphthalene-1-carbonylamino)-3-phenyl-propanoic acid (**S11**).* To a mixture of naphthalene-1-carboxylic acid (172 mg, 1 mmol) and methyl (2S)-2-amino-3-phenyl-propanoate hydrochloride salt (215 mg, 1 mmol) in CH_3CN (50 mL) were added HATU (418 mg. 1.1 mmol) and Et_3N (200 mg, 2 mmol). The reaction mixture was stirred at rt for 4h. After removal of the solvent, the residue was dissolved in EtOAc and the organic layer was washed with 0.5% aqueous NaOH, dried over anhydrous Na_2SO_4, and concentrated. The obtained residue was purified by column chromatography to give ester intermediate (2.7 g, 81% yield). To a solution of the obtained ester (2.7 g, 8.1 mmol) in THF (30 mL) and H_2O (20 mL) was added $LiOH{\cdot}H_2O$ (0.5 g, 12 mmol), and the

mixture was stirred at rt overnight. After evaporation to remove THF, the aqueous solution was acidified by HCl (1N) to pH 2, and the precipitate was collected, washed with water and dried to give acid **S11** (2.4 g, 93% yield). MS: calc'd (MH^+) 320.1, exp (MH^+) 320.1

*N-[(1S)-1-Benzyl-2-[(3-fluoro-2-oxo-propyl)-methyl-amino]-2-oxo-ethyl]naphthalene-1-carboxamide (**9i**)*. Compound **9i** was synthesized in analogy to **4h** by using **S11** and **S10** with 42% yield over 2 steps. MS: calc'd (MH^+) 407.2, exp (MH^+) 407.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.90−8.98 (m, 1H), 7.78−8.04 (m, 3H), 7.24−7.56 (m, 9H), 5.23 (s, 1H), 5.11 (s, 1H), 4.78 (d, J = 3.8 Hz, 1H), 4.21−4.36 (m, 2H), 3.20 (s, 3H), 3.09 (dd, J = 13.8, 5.0 Hz, 1H), 2.93−3.01 (m, 1H).

***N*-[(1S)-1-Benzyl-2-[(3-fluoro-2-oxo-propyl)amino]-2-oxo-ethyl]-N-methyl-naphthalene-1-carboxamide (9j).** Compound **9j** was synthesized by the method shown in Scheme S14.

Scheme S14



*tert-Butyl N-[(1S)-1-benzyl-2-[(3-fluoro-2-hydroxy-propyl)amino]-2-oxo-ethyl]-N-methyl-carbamate (**S13**)*. To a mixture of (2S)-2-[tert-butoxycarbonyl(methyl)amino]-3-phenyl-propanoic acid **S12** (84 mg, 0.3 mmol) and **5** (50 mg, 0.4 mmol) in DMF (5 mL) were added HATU (171 mg. 0.45 mmol) and DIPEA (78 mg, 0.6 mmol) and the reaction mixture was stirred at rt for 4h. After removal of the solvent, the residue was dissolved in EtOAc (50 mL). The organic phase was washed with 0.5% aqueous NaOH (10 mL), dried over anhydrous Na_2SO_4, and concentrated. The obtained residue was purified by column chromatography to give **S13** (98 mg, 92% yield). MS: calc'd (MH^+) 355.2, exp (MH^+) 355.2.

*(2S)-N-(3-Fluoro-2-hydroxy-propyl)-2-(methylamino)-3-phenyl-propanamide (**S14**)*. A solution of **S13** (98 mg, 0.28 mmol) and 1N HCl in EtOAc (5 mL, 5 mmol) was stirred at rt overnight. After removal of the solvent, **S14** was obtained as hydrochloride salt (81 mg, 96% yield) that was used in the next coupling reaction directly.

*N-[(1S)-1-Benzyl-2-[(3-fluoro-2-oxo-propyl)amino]-2-oxo-ethyl]-N-methyl-naphthalene-1-carboxamide (**9j**)*. Analogue **9j** was synthesized from **S14** and naphthalene-1-carboxylic acid in analogy to **8b**. MS: calc'd (MH^+) 407.2, exp (MH^+) 407.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.41 (br. s., 1H), 7.92 (d, J = 8.3 Hz, 2H), 7.43 (br. s., 8H), 7.05−7.19 (m, 1H), 6.78−6.97 (m, 1H), 5.27 (s, 1H), 5.15 (s, 1H), 4.06−4.25 (m, 2H), 3.36−3.42 (m, 1H), 3.04−3.16 (m, 2H), 2.62 (s, 3H).

Predicted binding models of Z-FG-FMK (2) and 9a

The surface representation of the predicted binding model of Z-FG-FMK (**2**) in the catalytic site of ATG4B is shown in Figure S1. The three subpockets (S1−S3) of ATG4B are indicated, with Phe residue of **2** occupying the S2 subpocket and the Cbz phenyl group forming π-stacking with the backbone amide of Tyr143−Gly144.

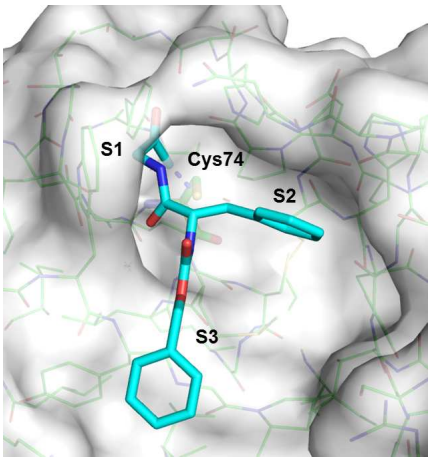


Figure S1. Surface representation of the model of Z-FG-FMK **2** (cyan) in the catalytic site of human ATG4B (green). The X-ray co-crystal structure of human ATG4B with the LC3 peptide (PDB code: 2Z0D) is used as template. The catalytic Cys74 is shown in stick mode and the three subpockets S1−S3 which are occupied by the inhibitor are annotated. See also Figure 2 in the main text.

In the predicted binding model, the naphthyl group of **9a** forms efficient π-stacking with the backbone amide group of Tyr143−Gly144. The benzyl substituent of **9a** is engaged in dispersion interactions with lipophilic side chains in the S2 pocket. A hydrogen bond is formed between the carbonyl oxygen atom of the amide linker connecting P1 and P2 fragments and a buried water molecule. And the NH functionality of the amide linker between P2 and P3 makes a hydrogen bond with the carbonyl oxygen atom of Tyr143 and the carbonyl group is solvent-exposed.

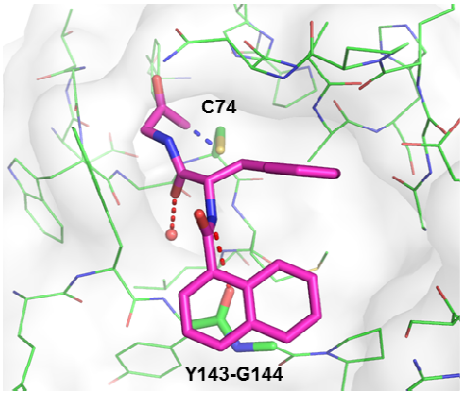


Figure S2. Model of compound **9a** (magenta) in the catalytic site of human ATG4B (green). The X-ray co-crystal structure of human ATG4B with the LC3 peptide (PDB code: 2Z0D) is used as template. The sidechain

of the catalytic Cys74 and the amide backbone of Tyr143-Gly144 are shown in stick. Intermolecular hydrogen bonds for **9a** are shown as red lines and the covalent interaction with Cys74 is shown in blue. See Reference 13 for a description of the model building.

General assay protocols for evaluation of protease selectivity

The protease panel assays were performed by Genscript USA according to their standard protocols (www.genscript.com). The stock solutions for the test compounds and the positive control articles were aliquoted and stored at -20°C. The stock solutions were further diluted with assay buffer to make final test solutions. All the final test solutions contained no more than 2.0% DMSO. The stock solution of test compounds or positive controls was diluted with assay buffer and added into plates. To the plate were added diluted proteases and/or other components, and the solution was incubated for 20 min at rt. The specified protease substrate was added into the plate to initiate reactions (Table S1). The fluorescence signal or colorimetric change of enzymatic products was recorded with PHERAstarPLUS (BMG) or FlexStation 3 (MD) using kinetics model, and the data acquisition and analyses were performed with GraphPad Prism 6. Maximum activity had enzyme, substrate and buffer in reaction system; Minimum had substrate and buffer in reaction system, no enzyme existed. Sample activity had enzyme, substrate and tested article in reaction system. The percentage Inhibition of the test article was calculated from the following equation: % inhibition= [1-(sample activity - Min)/ (Max-Min)]*100. The data from the positive control was consistent with GenScript historical data, thereby validating the performance of this study.

Table S1. The substrates and positive controls used for each protease inhibition assay

Protease	Substrate	Positive control
Cathepsin D	5-FAM/QXL 520 FRET	Pepstatin A
Cathepsin G	Suc-AAPF-pNA	Chymostatin
Tryptase, βII	Z-GPR-AMC	Gabexate
Neutrophil Elastase	MeOSu-AAPV-AMC	3,4-Dichloroisocoumarin
20S proteasome	suc-LLVY-AMC	Epoxomicin
Caspase 2	Capsae 2 (ICH-1) substrate	Ac-DEVD-CHO
Caspase-3	SensoLyte® AMC Caspase Substrate Sampler Kit	Ac-DEVD-CHO
Caspase 7, 8, 9	Ac-LEHD-AMC	Ac-DEVD-CHO
Cathepsin B	Z-FR-AMC	E-64
Calpain	Calpain substrate	B27-WT
ACE, ECE–1	Mca-RPPGFSAFK (Dnp)	Captopril
MMP1, MMP3, MMP9	Mca-PLGL-Dpa-AR	NNGH