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Nitro-based inhibitors against thermolysin

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S1: Materials and Methods

2-Substituted 3-nitropropionic acids of (±)-, (*R*)- and (*S*)-form were synthesized as reported. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), 1-hydroxybenzotriazole hydrate (HOBt) and other reagents were purchased from Acros Chemical Co. Flash chromatography was performed with 100-200 mesh silica gel (Qingdao, China) and thin layer chromatography (TLC) was carried out on silica coated glass sheets (Qingdao silica gel 60 F-254). Melting points were taken on a Thomas-Hoover capillary melting point apparatus and uncorrected. ^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra were recorded with a Bruker AV 300 (300 MHz) instrument using tetramethylsilane as the internal standard. IR spectra were recorded on a Perkin-Elmer 1300 FT-IR spectrometer. High-resolution mass spectra were taken on a Shimadzu GC-MS-QP2010. Elemental analyses were performed at Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun, China.

All solutions for kinetic study were prepared using doubly distilled and deionized water. Stock assay solutions were filtered (GHP Acrodic syringe filter, pore size 0.2 mM) before use. TLN was purchased from Sigma Chemical Co. (Type X) and used without further purification. TLN stock solution was prepared by dissolving TLN in 0.1 M Tris-HCl, 10 mM $CaCl_2$, pH 7.2 buffers. TLN concentration was determined from the absorbance at 278 nm (ε_{278} = 66400 $M^{-1}cm^{-1}$). TLN substrate, FA-Gly-L-Leu-NH_2, was purchased from Sigma Chemical Co. The stock solution of substrate was prepared in DMF. The decrease in the absorbance at 345 nm was followed with UV spectrometer at 25 ºC. HP 8453 UV/VIS spectrometer was used in enzyme inhibition kinetic studies.

S2: Synthesis

Synthesis of (±)-2-benzyl-3-nitropropionic amide (±)-1 To a solution of 2-benzyl-3-nitropropionic acid (130 mg, 0.62 mmol) in 2 mL THF was simultaneously added *iso*-butyl chloroformate (90 μL, 0.62 mmol) and 4-methyl morphorine (70 μL, 0.62 mmol) at 0 °C. Further stirring for 30 minutes followed by addition of aqueous ammonia (300 μL, 2.5 mmol), the reaction mixture was then allowed to stand at room temperature for 1 hour. The reaction mixture was poured into 50 mL Et_2O. After washed by 10 % aqueous citric acid (10 mL × 3) and brine (15 mL × 3), the organic layer was dried over anhydrous Na_2SO_4. Removing the solvents under reduced pressure provided an oil, which was then separated by flash chromatography (MeOH : $CHCl_3$ = 1:10). Recrystallization in Et_2O provided a white solid as product. Mp: 106-107 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.34-7.16 (m, 5H), 5.61 (s, 1H), 5.48 (s, 1H), 4.81 (dd, J = 4.8, 9.7 Hz, 1H), 4.49 (dd, J = 4.8, 9.7 Hz, 1H), 3.37-3.30 (m, 1H), 3.00 (dd, J = 5.8, 7.7 Hz, 1H), 2.78 ppm (dd, J = 6.0, 7.7 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 173.2, 136.8, 129.0, 128.9, 127.4, 75.1, 45.8, 36.1 ppm; IR (KBr): v^{-1} = 3457, 1736, 1570, 1363 cm^{-1}; Anal. calcd for $C_{10}H_{12}N_2O_3$: C 57.68,; H 5.81, N 13.45, found: C 57.69, H 5.69, N 13.40.

(2*R*)-2-Benzyl-3-nitropropionic amide (2*R*)-1 $[\alpha]_D^{13}$ = + 40.5 (c = 0.49 in MeOH); Anal. calcd for $C_{10}H_{12}N_2O_3$: C 57.68, H 5.81, N 13.45, found: C 57.57, H 5.65, N 13.25.

(2*S*)-2-Benzyl-3-nitropropionic amide (2*S*)-1 $[\alpha]_D^{13}$ = – 40.0 (c = 0.48 in MeOH); Anal. Calcd for $C_{10}H_{12}N_2O_3$: C 57.68, H 5.81, N 13.45, found: C 57.64, H 5.75, N 13.28.

General method to synthesize the nitro-based dipeptides Typically, a solution of (2*R*)-2-benzyl-3-nitropropanoic acid (68 mg, 0.306 mmol) in CH_2Cl_2 (4 mL) was added EDC (57.6 mg, 0.340 mmol) and HOBt (43.2 mg, 0.306 mmol) at room temperature. After stirring for 30 minutes, a mixture of (*S*)-phenylanaline methyl ester hydrochloride (104 mg, 0.482 mmol) and triethylamine (48 mg, 0.482 mmol) in THF (2 mL) was added. Stirring overnight followed by removing the solvents under reduced pressure, the residue was dissolved in 30 mL CH_2Cl_2. The organic layer was washed by brine (20 mL × 2) and dried over anhydrous Na_2SO_4 and then condensed. The obtained oil was purified by flash chromatography (*n*-hexane : EtOAc = 3:1) to provide a white solid as the product (15 mg, 25.3 %).

(2′*R*, 2*S*)-*N*-(2-Benzyl-3-nitropropionyl)phenylanaline methyl ester (2′*R*, 2*S*)-2 $[\alpha]_D^{20}$ = – 3.00 (c = 0.08 in $CHCl_3$); mp: 79-80 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.20-7.05 (m, 10H), 5.92(d, J = 7.5 Hz, 1H), 4.82-4.70 (m, 1H), 4.76(dd, J = 4.2, 14.7 Hz, 1H), 4.22 (dd, J = 4.2, 14.7 Hz, 1H), 3.67 (s, 3H), 3.21-3.19 (m, 1H), 3.08 (d, J = 5.7 Hz, 2H), 2.93 (dd, J = 7.8, 13.8 Hz, 1H), 2.86 ppm (dd, J = 5.4, 13.8 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.3, 170.3, 136.8, 135.4, 129.2, 129.0, 128.6, 127.4, 127.2, 75.2, 53.0, 52.4, 46.6, 37.8, 36.0 ppm; IR (film,): v^{-1} = 3307, 1739, 1654, 1553 cm^{-1}; Anal. calcd for $C_{20}H_{22}N_2O_5$: C 64.85, H 5.99, N 7.56, found: C 64.78, H 5.71, N 7.54.

(2′*S*, 2*R*)-*N*-(2-Benzyl-3-nitropropionyl)phenylanaline methyl ester (2′*S*, 2*R*)-2 $[\alpha]_D^{20}$ = + 2.70 (c = 0.07 in $CHCl_3$); Anal. calcd for $C_{20}H_{22}N_2O_5$: C 64.85, H 5.99, N 7.56, found: C 64.67, H 5.79, N 7.71.

(2′*S*, 2*S*)-*N*-(2-Benzyl-3-nitropropionyl)phenylanaline methyl ester (2′*S*, 2*S*)-2 Mp: 77-78 °C; $[\alpha]_D^{20}$ = – 4.40 (c = 0.16 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.30-7.05 (m, 10H), 5.92 (d, *J* = 7.5 Hz, 1H), 4.81 (dd, *J* = 3.9, 14.7 Hz, 1H), 4.86-4.78 (m, 1H), 4.28 (dd, *J* = 3.9, 14.7 Hz, 1H), 3.68 (s, 3H), 3.21-3.15 (m, 1H), 3.08 (d, *J* = 5.4 Hz, 2H), 2.95 (dd, *J* = 7.8, 13.8 Hz, 1H), 2.74 ppm (dd, *J* = 7.8, 13.8 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.2, 170.4, 136.6, 135.4, 129.3, 128.9, 128.8, 128.6, 127.3, 127.2, 74.8, 53.3, 52.3, 46.3, 38.0, 36.0 ppm; IR (film): ν^{-1} = 3307, 1740, 1653, 1553 cm^{-1}; Anal calcd for $C_{20}H_{22}N_2O_5$: C 64.85, H 5.99, N 7.56, found: C 65.23, H 5.80, N 7.50.

(2′*R*, 2*R*)-*N*-(2-Benzyl-3-nitropropionyl)phenylanaline methyl ester (2′*R*, 2*R*)-2 $[\alpha]_D^{20}$ = + 3.80 (c = 0.15 in $CHCl_3$). Anal. calcd for $C_{20}H_{22}N_2O_5$: C 64.85, H 5.99, N 7.56, found: C 64.57, H 5.75, N 7.62.

(2′*R*, 2*S*)-*N*-(2-*iso*-Butyl-3-nitropropionyl)phenylanaline methyl ester (2′*R*, 2*S*)-3 mp: 128-129 °C; $[\alpha]_D^{20}$ = – 0.95 (c = 0.21 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.32-7.10 (m, 5H), 6.10 (d, *J* = 7.8 Hz, 1H), 4.96-4.89 (m, 1H), 4.70 (dd, *J* = 9.3, 14.1 Hz, 1H), 4.28 (dd, *J* = 4.8, 14.4 Hz, 1H), 3.76 (s, 3H), 3.21 (dd, *J* = 5.4, 13.8 Hz, 1H), 3.06 (dd, *J* = 7.5, 14.4 Hz, 1H), 2.99-2.92 (m, 1H), 1.53-1.49 (m, 1H), 1.20-1.10 (m, 2H), 0.85 ppm (d, *J* = 4.9 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.4, 135.7, 129.1, 128.6, 127.2, 76.0, 53.1, 52.5, 42.7, 38.7, 37.9, 25.4, 22.8, 21.9 ppm. IR (film): ν^{-1} = 3325, 1710, 1675, 1550 cm^{-1}; Anal. calcd for $C_{17}H_{24}N_2O_5$: C 60.70, H 7.19, N 8.33, found: C 60.53, H 7.55, N 8.64.

(2′*S*, 2*S*)-*N*-(2-*iso*-Butyl-3-nitropropionyl)phenylanaline methyl ester (2′*S*, 2*S*)-3 mp: 80.5-81.5 °C. $[\alpha]_D^{20}$ = – 1.30 (c = 0.23 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.35-7.11 (m, 5H), 6.08 (d, *J* = 7.8 Hz, 1H), 4.96-4.90 (m, 1H), 4.74 (dd, *J* = 9.9, 14.4 Hz, 1H), 4.29 (dd, *J* = 3.9, 14.1 Hz, 1H), 3.75 (s, 3H), 3.15-3.13 (d, *J* = 5.4 Hz, 2H), 3.04-2.94 (m, 1H), 1.62-1.51 (m, 2H), 1.25-1.20 (m, 1H), 0.90 ppm (d, *J* = 4.9 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 172.1, 171.2, 135.4, 129.3, 128.7, 127.3, 75.9, 53.1, 52.4, 42.7, 38.8, 37.9, 31.9, 29.7, 25.4, 22.7, 22.0 ppm; IR (film): ν^{-1} = 3338, 1715, 1650, 1550 cm^{-1}; Anal. calcd for $C_{17}H_{24}N_2O_5$: C 60.70, H 7.19, N 8.33, found: C 61.08, H 7.33, N 8.67.

(2′*R*, 2*S*)-*N*-(2-Benzyl-3-nitropropionyl)leucine methyl ester (2′*R*, 2*S*)-4 Mp.: 50-51 °C; $[\alpha]_D^{20}$ = – 2.60° (c = 0.16 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.32-7.18 (m, 5H), 5.76 (d, *J* = 7.8 Hz, 1H), 4.81 (dd, *J* = 5.4, 14.4 Hz, 1H), 4.54-4.46 (m, 1H), 4.40 (dd, *J* = 5.4, 14.4 Hz, 1H), 3.70 (s, 3H), 3.30-3.25 (m, 1H), 2.95 (dd, *J* = 6.3, 13.8 Hz, 1H), 2.82 (dd, *J* = 6.3, 13.5 Hz, 1H), 1.59-1.51 (m, 1H), 1.45-1.40 (m, 2H), 0.81 ppm (d, *J* = 4.8 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 172.9, 170.4, 134.8, 129.1, 128.9, 127.3, 126.7, 75.4, 52.4, 50.1, 46.7, 41.3, 37.1, 30.0, 24.4, 22.7 ppm; IR (film): ν^{-1} = 3308, 1744, 1651, 1555 cm^{-1}; Anal. calcd for $C_{17}H_{24}N_2O_5$: C 60.70, H 7.19, N 8.33, found: C 60.79, H 6.96, N 8.04.

(2′*S*, 2*S*)-*N*-(2-Benzyl-3-nitropropionyl)leucine methyl ester (2′*S*, 2*S*)-4 Mp: 49-50 °C; $[\alpha]_D^{20}$ = – 0.65 (c = 0.23 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.35-7.17 (m, 5H), 5.86 (d, *J* = 8.0 Hz, 1H), 4.85 (dd, *J* = 4.5, 14.4 Hz, 1H), 4.60-4.57 (m, 1H), 4.31 (dd, *J* = 4.5, 14.4 Hz, 1H), 3.70 (s, 3H), 3.25-3.23 (m, 1H), 3.01 (dd, *J* = 7.5, 13.8 Hz, 1H), 2.75 (dd, *J* = 7.5, 13.8 Hz, 1H), 1.59-1.55 (m, 2H), 1.52-1.49 (m, 1H), 0.84 ppm (d, *J* = 4.9 Hz, 6H); ^{13}C NMR

(75 MHz, $CDCl_3$): δ = 172.9, 170.6, 136.7, 127.3, 126.8, 74.9, 52.3, 50.9, 41.3, 35.9, 31.9, 29.7, 24.6, 22.1 ppm; IR (film): v^{-1} = 3307, 1742, 1651, 1555 cm^{-1}; Anal. calcd for $C_{17}H_{24}N_2O_5$: C 60.70, H 7.19, N 8.33, found: C 60.81, H 7.08, N 8.11.

(2′*R*, 2*S*)-*N*-(2-*iso*-Butyl-3-nitropropionyl)leucine methyl ester (2′*R*, 2*S*)-5 Mp: 100.5-101.5 °C; $[\alpha]_D^{20}$ = – 1.25 (c = 0.35 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.14 (d, *J* = 8.2 Hz, 1H), 4.34 (dd, *J* = 4.7, 14.1 Hz, 1H), 3.74 (s, 3H), 3.07 (m, 1H), 1.68-1.63 (m, 5H), 1.26-1.24 (m, 1H), 0.94 ppm (d, *J* = 4.9 Hz, 12H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 172.9, 171.6, 52.4, 51.0, 42.9, 41.5, 38.7, 30.0, 25.7, 24.9, 22.8, 22.7, 22.0, 21.9 ppm; IR (film): v^{-1} = 3425, 3275, 1750, 1650, 1500, 1275 cm^{-1};Anal. calcd for $C_{14}H_{26}N_2O_5$: C 55.61, H 8.67, N 9.26, found: C 55.85, H 8.73, N 8.98.

(2′*S*, 2*S*)-*N*-(2-*iso*-Butyl-3-nitropropionyl)leucine methyl ester (2′*S*, 2*S*)-5 Mp: 78-79 °C; $[\alpha]_D^{20}$ = – 0.30 (c = 0.20 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.98 (d, *J* = 8.2 Hz, 1H), 4.77 (dd, *J* = 10.1, 14.2 Hz, 1H), 4.68-4.66 (m, 1H), 4.32 (dd, *J* = 4.0, 14.2 Hz, 1H), 3.74 (s, 3H), 3.05-3.02 (m, 1H), 1.68-1.62 (m, 5H), 1.25-1.22 (m, 1H), 0.93 ppm (d, *J* = 4.9 Hz, 12H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 173.2, 171.5, 62.8, 52.3, 50.8, 43.0, 41.6, 38.8, 25.4, 24.7, 22.8, 22.1, 21.7 ppm; IR (film): v^{-1} = 3420, 3275, 1750, 1650, 1500, 1250 cm^{-1}; Anal. calcd for $C_{14}H_{26}N_2O_5$: C 55.61, H 8.67, N 9.26, found: C 56.23, H 8.60, N 9.08.

General Method to synthesize *N*-(2-*iso*-butyl-3-nitropropionyl)-α-amino acid methyl esters

Condensation of 2-*iso*-butyl-3-hydroxypropionic acid with α-amino acid methyl ester hydrochloride Typically, a solution of (±)-2-*iso*-butyl-3-hydroxypropionic acid (102 mg, 0.7 mmol) in 5 mL CH_2Cl_2 was added EDC (134 mg, 0.7 mmol), HOBt (94.2 mg, 0.7 mmol) and triethylamine (177 mg, 1.75 mmol) at 0 °C. Further stirring for 30 minutes followed by the addition of (2*S*)-phenylanaline methyl ester hydrochloride (165.7 mg, 0.77 mmol), the reaction mixture was allowed overnight at room temperature. The organic layer was washed by 10 % citric acid (2 mL × 3), saturated aqueous $NaHCO_3$ (2 mL × 3), and brine (2 mL × 3). Dried over anhydrous Na_2SO_4, the solvents were removed under reduced pressure. The residue was separated by flash chromatography (*n*-hexane : EtOAc = 3:1). The two diastereomers were then obtained as white solids.

(2′*R*, 2*S*)-*N*-(2-*iso*-Butyl-3-hydroxypropionyl)phenylanaline methyl ester (2′*R*, 2*S*)-6 Mp: 97.5-99 °C; $[\alpha]_D^{20}$ = – 4.6 (c = 0.25 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.30-7.12 (m, 5H), 6.15 (d, *J* = 7.6 Hz, 1H), 4.95-4.88 (m, 1H), 3.76 (s, 3H), 3.63 (d, *J* = 5.9 Hz, 2H), 3.19 (dd, *J* = 5.5, 14.0 Hz, 1H), 3.05 (dd, *J* = 7.2, 14.0 Hz, 1H), 2.90.-2.77 (m, 1H), 2.39-2.35 (m, 1H), 1.58-1.35 (m, 2H), 1.28-1.08 (m, 1H), 0.85 ppm (d, *J* = 5.7 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 175.2, 172.6, 135.8, 129.1, 128.6, 127.2, 64.2, 53.0, 52.5, 47.0, 37.5, 37.1, 25.7, 22.8, 22.4 ppm; IR (KBr): v^{-1} = 3338, 3244, 1751, 1651, 1556, 1458 cm^{-1}; Anal. calcd for $C_{17}H_{25}NO_4$: C 66.43, H 8.20, N 4.56, found: C 66.08, H 8.37, N 4.84.

(2′*S*, 2*S*)-*N*-(2-*iso*-Butyl-3-hydroxypropionyl)phenylanaline methyl ester (2′*S*, 2*S*)-6 Mp: 52-53 °C; $[\alpha]_D^{20}$ = – 2.0 (c = 0.25 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.39-7.01 (m, 5H), 6.15 (d, *J* = 7.4 Hz, 1H), 4.95-4.86 (m, 1H), 3.75 (s, 3H), 3.62 (d, *J* = 6.3 Hz, 1H), 3.22

(dd, J = 5.5, 14.0 Hz, 1H), 3.07 (dd, J = 6.6, 14.0 Hz, 1H), 2.39-2.35 (m, 2H), 1.78-1.61 (m, 1H), 1.57-1.39 (m, 2H), 0.87 ppm (d, J = 5.9 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 174.8, 712.1, 135.9, 129.2, 128.6, 127.2, 63.4, 52.9, 52.4, 46.8, 37.8, 37.6, 29.7, 25.7, 22.7, 22.4 ppm; IR (KBr): 3365, 3298, 1747, 1649, 1545, 1438, 1371 cm^{-1}; Anal. calcd for $C_{17}H_{25}NO_4$: C 66.43, H 8.20, N 4.56, found: C 66.21, H 8.52, N 4.78.

(2′*R*, 2*S*)-*N*-(2-*iso*-Butyl-3-hydroxypropionyl)leucine methyl ester (2′*R*, 2*S*)-7 Mp: 99-100 °C; $[\alpha]_D^{20}$ = – 1.4 (c = 0.25 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.13 (d, J = 7.2 Hz, 1H), 4.64-4.54 (m, 1H), 3.75 (s, 3H), 3.67 (d, J = 6.3 Hz, 2H), 3.10-2.90 (m, 1H), 2.50-2.43 (m, 1H), 1.72-1.52 (m, 5H), 1.30-1.10 (m, 1H), 0.96-0.86 ppm (m, 12H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 175.6, 174.3, 64.6, 52.5, 51.1, 47.4, 40.7, 36.8, 26.0, 25.1, 22.8, 22.6, 22.3, 21.6 ppm; IR (KBr): v^{-1} = 3362, 3240, 1757, 1650, 1560, 1425, 1400, 1202, 1157, 1025, 750 cm^{-1}; Anal. calcd for $C_{14}H_{27}NO_4$: C 61.51, H 9.96, N 5.12, found: C 61.30, H 9.65, N 5.48.

(2′*S*, 2*S*)-*N*-(2-*iso*-Butyl-3-hydroxypropionyl)leucine methyl ester (2′*S*, 2*S*)-7 Mp: 60-61.5 °C; $[\alpha]_D^{20}$ = + 0.4 (c = 0.24 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.13 (d, J = 7.2 Hz, 1H), 4.68-4.61 (m, 1H), 3.80-3.69 (m, 2H), 3.74 (s, 3H), 2.52-2.35 (m, 2H), 1.69-1.56 (m, 5H), 1.37-1.33 (m, 1H), 0.96-0.90 ppm (m, 12H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 175.2, 173.6, 63.8, 52.3, 50.7, 46.8, 41.4, 37.7, 25.7, 25.2, 22.8, 22.7, 22.5, 21.9 ppm; IR (KBr): v^{-1} = 3375, 3275, 1750, 1650, 1550, 1425, 1250, 1220, 1075, 1050, 800 cm^{-1}; Anal. calcd for $C_{14}H_{27}NO_4$: C 61.51, H 9.96, N 5.12, found: C 61.82, H 10.13, N 4.97.

Transformation of hydroxy to bromo group via mesylate Typically, (2′*S*, 2*S*)-**7** (48.8 mg, 0.179 mmol) was dissolved in 10 mL CH_2Cl_2 and cooled down below -5 °C, methanesulfonyl chloride (61.5 mg, 0.537 mmol) and triethylamine (54.3 mg, 0.537 mmol) were added dropwise simultaneously. The reaction mixture was kept stirring below -5 °C for 30 minutes and then allowed to room temperature for further 2.5 hours. After washed by 1 N HCl (3 mL × 5) and water (3 mL × 2), the organic layer was dried over anhydrous $MgSO_4$. Removing the solvents under reduced pressure gave oil (121 mg), which was used for subsequent reaction without further purification. The above mesylate was dissolved in 10 mL anhydrous THF, and added LiBr (43.5 mg, 0.501 mmol). After refluxing overnight, the solvents were removed under reduced pressure. The resulting residue was dissolved in 15 mL EtOAc. The organic layer was washed by brine (10 mL × 3), dried over anhydrous Na_2SO_4, and then condensed under reduced pressure. The residue was separated by flash chromatography (*n*-hexane : EtOAc = 10 :1) provided (2′*R*, 2*S*)-*N*-(2-*iso*-butyl-3-bromopropionyl)leucine methyl ester as white solids (44 mg, yield: 73.2 %).

(2′*R*, 2*S*)-*N*-(2-*iso*-Butyl-3-bromopropionyl)phenylanaline methyl ester (2′*R*, 2*S*)-8 Mp: 94-95 °C; $[\alpha]_D^{20}$ = – 2.6 (c = 0.23 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.30-7.18 (m, 5H), 6.05 (d, J = 7.5 Hz, 1H), 5.00-4.94 (m, 1H), 3.73 (s, 3H), 3.55 (t, J = 9.6 Hz, 2H), 3.33 (dd, J = 4.5, 10.0 Hz, 1H), 3.16 (d, J = 5.7 Hz, 1H), 2.59-2.52 (m, 1H), 1.67-1.50 (m, 2H), 1.32-1.25 (m, 1H), 0.88 ppm (d, J = 6.0 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 172.4, 171.8, 135.6, 129.4, 128.6, 127.2, 53.0, 52.3, 48.4, 41.2, 38.1, 33.5, 25.8, 23.0, 22.0 ppm; IR

(KBr): v^{-1} = 3277, 1746, 1647, 1562, 1440, 1284, 1213, 1125, 1050, 700 cm^{-1}; Anal. calcd for $C_{17}H_{24}BrNO_3$: C 55.14, H 6.53, N 3.78, found: C 54.72, H 6.71, N, 4.05.

(2′*S*, 2*S*)-*N*-(2-*iso*-Butyl-3-bromopropionyl)phenylanaline methyl ester (2′*S*, 2*S*)-8 Mp: 110-111 °C; $[\alpha]_D^{20}$ = – 1.6 (c = 0.49 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.39-7.12 (m, 5H), 6.15 (d, J = 7.6 Hz, 1H), 4.93-4.82 (m, 1H), 3.72 (s, 3H), 3.62 (d, J = 5.9 Hz, 2H), 3.25 (dd, J = 5.5, 14.0 Hz, 1H), 3.00 (dd, J = 2.9, 7.0 Hz, 1H), 2.84-2.72 (m, 1H), 1.83-1.61 (m, 1H), 1.62-1.50 (m, 2H), 0.87 ppm (d, J = 5.8 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 172.4, 171.9, 135.8, 129.2, 128.6, 127.1, 52.9, 52.3, 48.2, 40.9, 38.1, 33.4, 25.7, 23.0, 21.9 ppm; IR (KBr): v^{-1} = 3259, 3084, 1738, 1650, 1558, 1437, 1287, 1175, 1038, 750 cm^{-1}; Anal. calcd for $C_{17}H_{24}BrNO_3$: C 55.14, H 6.53, N 3.78, found: C 55.31, H 6.25, N 3.97.

(2′*R*, 2*S*)-*N*-(2-*iso*-Butyl-3-bromopropionyl)leucine methyl ester (2′*R*, 2*S*)-9 Mp: 123-124 °C; $[\alpha]_D^{20}$ = + 0.2 (c = 0.26 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.98 (d, J = 8.0 Hz, 1H), 4.75-4.65 (m, 1H), 3.74 (s, 3H), 3.57 (t, J = 9.8 Hz, 2H), 3.36 (dd, J = 4.3, 9.8 Hz, 1H), 2.62-2.59 (m, 1H), 1.71-1.59 (m, 5H), 1.25-1.30 (m, 1H), 0.93 ppm (d, J = 4.8 Hz, 12H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 173.2, 172.4, 52.1, 50.8, 48.6, 41.8, 41.2, 33.4, 32.8, 24.8, 22.9, 22.7, 22.0, 21.8 ppm; IR (KBr): v^{-1} = 3300, 3100, 1750, 1650, 1550, 1225, 1025, 800 cm^{-1}; Anal. calcd for $C_{14}H_{26}BrNO_3$: C 50.01, H 7.79, N 4.17, found: C 50.33, H 8.04, N 4.48.

(2′*S*, 2*S*)-*N*-(2-*iso*-Butyl-3-bromoxypropionyl)leucine methyl ester (2′*S*, 2*S*)-9 Mp: 115-116 °C; $[\alpha]_D^{20}$ = – 1.4 (c = 0.22 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.03 (d, J = 8.0 Hz, 1H), 4.71-4.66 (m, 1H), 3.75 (s, 3H), 3.56 (t, J = 9.6 Hz, 1H), 3.39 (dd, J = 4.9, 10.0 Hz, 1H), 2.65-2.59 (m, 1H), 1.64-1.57 (m, 5H), 1.37-1.34 (m, 1H), 0.95 ppm (d, J = 5.7 Hz, 12H); ^{13}C NMR (75 MHz, $CDCl_3$): δ =173.2, 172.6, 52.3, 50.7, 48.3, 41.7, 40.8, 33.6, 25.9, 24.9, 23.0, 22.0, 21.9 ppm; IR (KBr): v^{-1} = 3300, 3100, 1750, 1650, 1550, 1225, 1025, 800 cm^{-1}; Anal. calcd for $C_{14}H_{26}BrNO_3$: C 50.01, H 7.79, N 4.17, found: C 49.83, H 8.08, N 4.35.

Nitro substitution Typically, $NaNO_2$ (10.3 mg, 0.15 mmol) and phloroglucinol (11.6 mg, 0.092 mmol) were stirring in 3 mL DMF for 5 minutes and then added a solution of (2′*R*, 2*S*)-**10** (29 mg, 0.086 mmol) in 2 mL DMF. The reaction mixture was then kept at 70 °C overnight. After cooling and partition by 40 mL Et_2O and 10 mL water, the water layer was further extracted by Et_2O (10 mL × 3). The combined organic layer was then washed by brine (15 mL × 3) and dried over anhydrous Na_2SO_4. Removing the solvents under reduced pressure gave an oil, which was purified by flash chromatography (*n*-hexane : EtOAc = 10:1). Recrystallization from Et_2O provided a white solid as (2′*S*, 2*S*)-**5** (16.2 mg, yield: 61.2 %).

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S3: Determination of K_i value

The enzyme stock solution was added to a solution containing FaGLa (final concentration, 0.8-1.0 mM) and inhibitor in 0.1 M Tris / 0.01 M $CaCl_2$, pH 7.2 buffer (1 mL cuvette, the final content of DMF was controlled at 5 %), and the change in absorbance at 345 nm was measured immediately. The final concentration of TLN was 0.25-0.45 μM. Initial velocities were then calculated from the initial linear portion of the change in absorbance where the amount of substrate consumed was less than 10 %. The K_i values were determined from the plot of v_0/v against the concentration of inhibitors based on the equation, $v_0/v=1+[I]/K_i$, in which v_0 and v represent the initial velocity in the absence and presence of the inhibitor, respectively. In the plot, the intercept of the straight on [I] would give the K_i value.

S4: X-ray Crystallography

TLN was purchased from Sigma and further crystallized as described previously.[1] TLN was dissolved in a 0.05 M Tris buffer (pH 7.2) solution containing DMSO [45 % (v/v)] and calcium acetate (1.4 M) to have the final protein concentration of about 80 mg / mL. Crystals were grown by the hanging-drop vapour diffusion method by using a reservoir solution containing 0.01 M calcium acetate, 5 % (v/v) DMSO and 0.05 M Tris buffer (pH 7.2). Native crystals of TLN were obtained after seven days, and then equilibrated in the reservoir solution supplemented with 10 m M of (±)-**2** for another seven days at 4 °C to obtain crystals of TLN-inhibitor complex.

A crystal of TLN-inhibitor complex with approximate dimensions of 0.15 × 0.25 × 0.20 mm was quickly frozen to 100 K, and then X-ray diffraction data were collected to 1.93 Å using a Rigaku RA-Micro 7 Desktop Rotating Anode X-ray Generator with a Cu target operating at 40 kV × 20 mA and R-Axis IV^{++} imaging-plate detector at a wavelength of 1.5418 Å. A 0.5 mm collimator was used to keep the whole crystal bathed in the X-ray beam. A total of 360 images with 1.0 oscillation were collected. The collected intensities were indexed, integrated, corrected for absorption, scaled and merged using *HKL2000* with an R_{merge} of 5.0 %.[2] The crystal belongs to the hexagonal space group $P6_122$, with unit-cell parameters $a = b = 93.09$ Å, $c = 129.28$ Å, $\gamma = 120°$.

The structure of the native TLN (pdb code: 1LNF) was used as the starting model. Using reflections in the 38.48-1.93 Å resolution range, model refinement was carried out with the COOT program[3] and CNS.[4] Water and DMSO molecules were gradually added to the model with the program CCP-4.[5] The inhibitor model was included in the last stage of the refinement. Hydrogen atoms have been added in the riding positions. Final structures of TLN-inhibitor complex were analyzed using Procheck program.[6]

S5: Table Data collection and refinement statistics for the TLN-(*R*)-1 complex

space group	$P6_122$
unit cell	
a, *b*, *c* (Å)	93.09, 93.09, 129.28
α, *β*, *γ* (deg)	90.00, 90.00, 120.00
resolution range (Å)	38.48-1.93 (2.00-1.93)[d]
number of unique reflections	22886 (1856)
overall completeness (%)	94.6 (91.0)
R_{merge} (%)[a]	5.0 (15.1)
R factor (%)[b]	18.4 (working set) / 18.6 (all data)
R_{free} (%)	21.3
rms deviations[c]	
bonds (Å)	0.005
angles (deg)	1.2
dihedrals (deg)	14.1

[a] R_{merge} for data sets for replicate reflections, $R = \sum \| F_{hi} | - < | F_h | > | \ / \ \sum < | F_h | >$, $| F_{hi} |$ = scaled structure factor for reflection *h* in data set *i*, $< | F_h | >$ = average structure factor for reflection h calculated from replicate data. [b] *R* factor, $R = \sum \| F_o | - | F_c \| \ / \ \sum | F_o |$; $| F_o |$ and $| F_c |$ are the observed and calculated structure factors, respectively. [c] rms: root mean square. [d] Values in parentheses refer to the high-resolution shell.

S6: Figure Schematic representation of the occurred tetrahedron intermediate in the TLN-catalyzed hydrolysis (a) and the binding modes of the phosphate (b), the silanediol (c) and the nitro group (d) at the active site of TLN.

S7: References

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