SUPPORTING INFORMATION

Tetrazole as a Replacement of the Electrophilic Group in Characteristic Prolyl Oligopeptidase Inhibitors

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1. Synthesis

General information

All synthesized compounds were characterized by ^{1}H and ^{13}C NMR spectroscopy using a Bruker Avance III 400 MHz or Varian Mercury 300 MHz spectrometer. Chemical shifts are reported in parts per million (ppm), and spectra were calibrated using residual solvent signals ($CDCl_3$: δ_H = 7.26 ppm and δ_C = 77.16 ppm; CD_3OD: δ_H = 3.31 ppm and δ_C = 49.00 ppm). The progress of the reactions was monitored by thin-layer chromatography (TLC) on silica gel 60-F_{254} plates. Flash chromatography was performed on silica gel (SiO_2) 60 (230–400 mesh). Mass spectrometric analysis was carried out with a Waters Synapt G2 HDMS mass spectrometer using electrospray ionization (ESI). The purity was determined by UPLC-MS with diode-array. Melting points were determined for non-amorphous solids by Stuart SMP40 automatic melting point instrument.

Amide bonds on the amino-side of 2-substituted pyrrolidines (such as a prolyl group) or N-methylated aminoacyl groups have cis and trans isomers (also called rotamers) of almost the same stability, and both isomers can be normally identified from the NMR spectra of these compounds although some signals are overlapping.

The purity of all in vitro tested compounds was 95 % or higher, except for **15b** for which the purity was only 90 %.

4-Phenylbutanoyl chloride

4-Phenylbutanic acid (10 mmol, 1.64 g) and thionyl chloride (15 mmol, 1.1 mL) were placed in a small round flask under a $CaCl_2$ drying tube and stirred at 67 °C for 2 h, followed by a temperature elevation to 90 °C for 15 minutes. Excess of thionyl chloride was evaporated under reduced pressure (ca 25 mbar for 30 min). The success of evaporation of excess of thionyl chloride was checked by weighing, and the crude product was used without further purification.

L-Proline methyl ester

L-Proline (2.5 g, 22 mmol) was dissolved in methanol (50 mL) and thionyl chloride (55 mmol, 4.0 mL) was added dropwise at 0 °C, where after the reaction was heated at reflux for 1h. The solvent was evaporated under reduced pressure. The crude product was stored at -20 °C and used without further purification.

D-Proline methyl ester

Prepared the same way as L-proline methyl ester using D-proline (6 mmol, 0.98 g).

2-Aminoisobutyric acid methyl ester (2g)

Prepared the same way as L-proline methyl ester using 2-aminoisobutyric acid (5 mmol, 0.77 g).

Boc-*N*-methyl-L-alanine hydroxysuccinimide ester (5c)

N-Methyl-L-alanine (4.0 mmol, 0.41 g) was dissolved in aqueous NaOH (2.3 mL, 4 M) and di-*tert*-butyl-dicarbonate (12.0 mmol, 2.6 g) in Et_2O (1.0 mL) was added dropwise at 0 °C, and then reacted for 16 h at room temperature. Phases were separated and product was extracted from the Et_2O phase with aqueous saturated $NaHCO_3$. The aqueous phase was made acidic with 1 M HCl and extracted with EtOAc. The EtOAc phase was washed with water and saturated NaCl, dried over anhydrous $NaSO_4$, and evaporated under reduced pressure to yield Boc-*N*-methyl-L-alanine as a white powder (0.70 g, 86 %). Boc-*N*-methyl-L-alanine (3.45 mmol, 0.7 g) and HOSu (3.45 mmol, 0.39 g) were dissolved in anhydrous acetonitrile and DCC (3.45 mmol, 0.71 g) in anhydrous acetonitrile was added dropwise under argon. The reaction mixture was filtered, evaporated, the residue was triturated with heptane, and evaporated to give a white powder (0.90 g, quant). The crude product was used without further purification.

Procedure A: Synthesis of 4-phenylbutanoyl amino acids

The amino acid (11 mmol) was dissolved in an aqueous Na_2CO_3 (25 mL 10 % (w/V), 20 mmol) and Et_2O (25 mL) was added. 4-Phenylbutanoyl chloride (10 mmol, 1.83 g) was dissolved in a small amount of Et_2O and added dropwise to reaction. The reaction was stirred vigorously for 16 h. The phases were separated and the aqueous phase was washed with Et_2O. The aqueous phase was made acidic with 2 M HCl. The product was extracted with EtOAc (or alternatively with DCM). The organic phase was washed with 0.1 M HCl, dried with anhydrous Na_2SO_4 and evaporated under reduced pressure.

Procedure B1: Amide coupling with pivaloyl chloride

Pivaloyl chloride (1.0 mmol, 0.12 mL) was added dropwise to a solution of the carboxylic acid (1.0 mmol) and Et_3N (1.1 mmol, 0.15 mL) in anhydrous DCM (20 mL) at 0 °C under an argon and stirred for 60 min at 0 °C. Et_3N (1.1 mmol, 0.15 mL) and the amine (1.1 mmol) was added at 0 °C, where after the reaction mixture was allowed to react 2-16 h at room temperature. The DCM phase was washed with aqueous 30% citric acid, saturated NaCl and saturated $NaHCO_3$. The DCM phase was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure.

Procedure B1: Amide coupling with ethyl chloroformate

Ethyl chloroformate (1.0 mmol, 0.095 mL) was added dropwise to a solution of the carboxylic acid (1.0 mmol) and Et_3N (1.1 mmol, 0.15 mL) in anhydrous DCM (20 mL) at 0 °C under an argon and stirred for 60 min at 0 °C. Et_3N (1.1 mmol 0.15 mL) and the amine or the amine hydrochloride (1.1 mmol) was added at 0 °C where after the reaction mixture was allowed to react 2-16 h at room temperature. The same work up as in procedure B1.

Procedure C: Dehydration of primary amide to nitrile using TFAA

The primary amide (1.0 mmol) was dissolved in anhydrous THF (40 mL) and Et_3N (2.4 mmol, 0.33 mL) was added under argon atmosphere. TFAA (1.2 mmol, 0.17 mL) was added dropwise at 0 °C, and the reaction was stirred for 2-3 h at room temperature. The reaction was quenched with water (3.0 mL) and the solvent was evaporated under reduced pressure. The residue was dissolved in DCM and washed with aqueous 30% citric acid, saturated NaCl and saturated $NaHCO_3$. The DCM phase was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure.

Procedure D: Formation of tetrazole from nitrile

The nitrile (1.0 mmol), NH_4Cl (2.0 mmol, 0.107 g) and NaN_3 (2.0 mmol, 0.13 g) was dissolved in anhydrous DMF (4.0 mL). The reaction was heated at 100 °C for 7-16 h. The solvent was evaporated under reduced pressure.

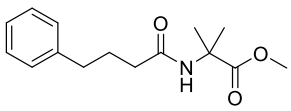
Procedure E: Removal of Boc group from amine

The Boc protected amine (1.0 mmol) was dissolved in DCM (4-8 mL) and TFA (2-4 mL) was added at 0 °C. The reaction was stirred at 0 °C for 2 h. The solvent was evaporated under reduced pressure to yield the TFA salt of the amine.

Procedure F: Hydrolysis of a carboxylic acid methyl ester

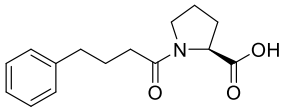
The methyl ester (1.0 mmol) and $LiOH \cdot H_2O$ (1.5 mmol, 0.063 g) was dissolved in mixture of water (2 mL) and methanol (6 mL). The mixture was allowed to react for 2-16 h at room temperature. The methanol was evaporated under reduced pressure and the aqueous remainder was diluted with water and washed with DCM. The aqueous phase was then made acidic with 2 M HCl and the product was extracted with EtOAc (or alternatively DCM). The organic phase was washed with 0.1 M HCl, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure.

4-Phenylbutanoyl-2-aminoisobutyric acid methyl ester (3g)



2-Aminoisobutyric methyl ester (**2g**) (5.0 mmol, 0.73 g) was dissolved in anhydrous DCM (50 mL) and DIPEA (20 mmol, 3.42 mL) under argon atmosphere. 4-Phenylbutanoyl chloride (5.0 mmol) was added slowly dropwise at 0 °C. The reaction was stirred for 16 h at room temperature. The DCM phase was washed with aqueous 30% citric acid, saturated NaCl and saturated $NaHCO_3$. The DCM phase was dried with anhydrous $NaSO_4$ and evaporated under reduced pressure. Purification by flash chromatography (*n*-hexane/EtOAc 2:1) gave colorless amorphous product (0.68 g, 52 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.35–7.10 (m, 5H), 6.01 (s, 1H), 3.73 (s, 3H), 2.64 (t, *J* = 7.5 Hz, 2H), 2.15 (t, *J* = 7.4 Hz, 2H), 2.01–1.88 (m, 2H), 1.52 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 175.18, 172.11, 141.64, 128.61, 128.47, 126.03, 56.38, 52.72, 35.85, 35.06, 27.06, 24.97.

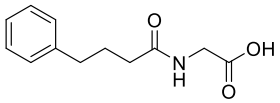
4-Phenylbutanoyl-L-proline (4a)



Prepared following procedure A using L-proline (7.2 mmol, 0.830 g), resulting in a light red amorphous compound (1.235 g, 79 %). ^{1}H NMR (400 MHz, CD_3OD) δ 7.36 – 7.06 (m, 5H), 4.40 (dd, *J* = 8.6, 3.3 Hz, 1H), 3.65–3.35 (m, 2H), 2.73–2.53 (m, 2H), 2.49–2.09 (m, 3H),

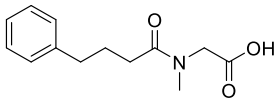
2.09–1.73 (m, 5H). ^{13}C NMR (101 MHz, CD_3OD) δ 175.73, 174.19, 143.07, 129.55, 129.37, 126.93, 60.13, 48.36, 35.99, 34.31, 30.34, 27.58, 25.61 (and an additional set of lower intensity signals (ca 30 %) from minor rotamer).

4-Phenylbutanoyl-glycine (4d)



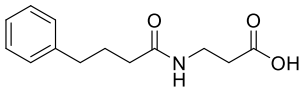
Prepared following procedure A using glycine (13.0 mmol, 1.16 g), resulting in a white crude powder (1.80 g, 82 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39–7.08 (m, 5H), 6.01 (d, *J* = 46.5 Hz, 1H), 5.63 (s, 1H), 4.06 (d, *J* = 5.2 Hz, 2H), 2.67 (td, *J* = 7.4, 5.0 Hz, 2H), 2.38 (t, *J* = 7.4 Hz, 1H), 2.26 (t, *J* = 7.5 Hz, 1H), 2.07–1.91 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.06, 173.52, 141.47, 128.74, 128.68, 126.29, 41.63, 35.61, 35.26, 27.10. The crude product contained 4-phenylbutyric acid as an impurity (ca 30%) and it was used in the next step without further purification.

4-Phenylbutanoyl-sarcosine (4e)



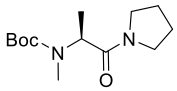
Prepared following procedure A using sarcosine (11 mmol, 0.98 g), resulting in a light orange powder (2.12 g, 82 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.71 (s, 1H), 7.36–7.12 (m, 5H), 4.13 (s, 1.7H), 3.99 (s, 0.3H), 3.01 (s, 2.5H), 2.97 (s, 0.5H) 2,73–2,62 (m, 2H), 2,38 (t, *J* = 7.4 Hz, 2H), 2.08–1.89 (m, 2H) (two rotamers 5:1). ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.55, 173.28, 141.71, 128.66, 128.52, 126.09, 49.96, 36.90, 35.24, 32.28, 26.40 (and an additional set of signals with lower intensity from minor rotamer).

4-Phenylbutanoyl-β-alanine (4h)



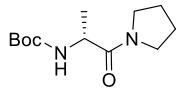
Prepared following procedure A using β-alanine (10.0 mmol, 0.98 g), resulting in a white powder (1.36 g, 58 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.38–7.09 (m, 5H), 6.09 (s, 1H), 3.51 (q, J = 5.9 Hz, 2H), 2.63 (m, 4H), 2.18 (t, J = 7.5 Hz, 2H), 2.11–1.87 (m, 2H).^{13}C NMR (75 MHz, $CDCl_3$) δ 176.98, 173.51, 141.48, 128.61, 128.55, 126.15, 35.96, 35.27, 34.95, 34.03, 27.13.

Boc-*N*-methyl-L-alanyl-pyrrolidine (6c)



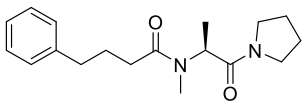
Boc-*N*-methyl-L-alanine hydroxysuccinimide ester (**5c**) (3.5 mmol, 0.7 g) was dissolved in anhydrous DCM (70 mL). Pyrrolidine (7.0 mmol, 0.6 mL) was added dropwise at 0 °C and the mixture was stirred for 16 h at room temperature. The organic phase was washed with aqueous 30% citric acid, saturated NaCl and saturated $NaHCO_3$. The DCM phase was dried over anhydrous $NaSO_3$ and evaporated Flash chromatography (*n*-heptane/EtOAc 1:2) gave a white powder (0.42 g, 52%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.97 (d, *J* = 6.5 Hz, 1H), 3.30-3.55 (m, 4H), 2.75 (s, 3H), 1.75-2.00 (m, 4H), 1.45 (s, 9H), 1.26 (d, *J* = 6.9 Hz, 3H).

Boc-D-alanyl-pyrrolidine (6f)



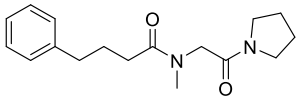
Prepared similar to **6c** using Boc-D-Ala-Osu (3.35 mmol, 0.97 g). Flash chromatography (*n*-heptane/EtOAc 2:1) gave a colorless amorphous compound (0.65 g, 80 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.45 (d, *J* = 7.5 Hz, 1H), 4.42 (q, *J* = 7.1 Hz, 1H), 3.67–3.31 (m, 4H), 2.03–1.78 (m, 4H), 1.41 (s, 9H), 1.29 (d, *J* = 6.8 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.32, 155.25, 79.52, 47.97, 46.41, 46.08, 28.50, 26.19, 24.25, 18.89.

4-Phenylbutanoyl-N-methyl-L-alanyl-pyrrolidine (7c)



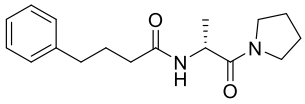
Boc-*N*-methyl-L-alanyl-pyrrolidine (**6c**) (1.67 mmol, 0.38 g) was deprotected according to procedure E. The resulting TFA salt was dissolved in anhydrous DCM (16 ml) and Et_3N (14.5 mmol, 2.1 mL) and 4-phenylbutanoylchloride (2.5 mmol) was added dropwise at 0 °C under argon, where after it was stirred at room temperature for 16 h. The DCM solution was washed with aqueous 30% citric acid, saturated NaCl and saturated $NaHCO_3$, dried over anhydrous Na_2SO_4, and evaporated. Flash chromatography (EtOAc/MeOH 9:1) gave colorless amorphous compound (0.40 g, 79 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.37–7.10 (m, 5H), 5.44 (q, *J* = 7.0 Hz, 1H), 3.50–3.38 (m, 4H), 2.89 (s, 3H), 2.68 (t, *J* = 7.5 Hz, 2H), 2.32 (t, *J* = 7.5 Hz, 2H), 2.10–1.68 (m, 6H), 1.27 (d, *J* = 7.0 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.72, 170.13, 141.78, 128.60, 128.50, 126.06, 50.06, 46.44, 46.12, 35.42, 32.87, 30.50, 26.49, 26.34, 24.22, 14.51. HRMS (ESI-QTOF) *m/z* $[M+H]^+$ calcd for $C_{18}H_{26}N_2O_2$: 303.2073, found: 303.2068.

4-Phenylbutanoyl-sarcosyl-pyrrolidine (7e)



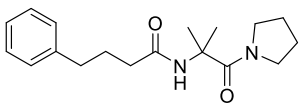
Prepared according to procedure B1 using 4-phenylbutanoyl-sarcosine (**4e**) (5.0 mmol, 1.18 g). Flash chromatography (EtOAc/MeOH 9:1) gave a colorless amorphous compound (1.25 g, 87 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.35–7.12 (m, 5H), 4.10 (s, 1.8H), 3.88 (s, 0.2H) 3.50-3.38 (m, 4H), 3.06 (s, 2.6H), 2.97 (s, 0.4H) 2.71–2.61 (m, 2H), 2.39 (t, *J* = 7.5 Hz, 1.8H), 2.21 (t, *J* = 7.5 Hz, 0.2H) 2.09–1.77 (m, 6H) (two rotamers 9:1). ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.52, 166.72, 141.93, 128.62, 128.39, 125.88, 50.01, 45.95, 45.76, 36.96, 35.27, 32.33, 26.47, 26.25, 24.16 (and an additional set of signals with lower intensity from minor rotamer). HRMS (ESI-QTOF) *m/z* $[M+H]^+$ calcd for $C_{17}H_{24}N_2O_2$: 289.1916, found: 289.1920.

4-Phenylbutanoyl-D-alanyl-pyrrolidine (7f)



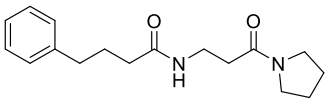
Boc-D-alanyl-pyrrolidine (**6f**) (2.6 mmol, 0.65 g) was deprotected according to procedure E. The resulting TFA salt was dissolved in Et_2O (30 mL) and 1 M NaOH-solution (30 mL) and 4-phenylbutanoylchloride (5.2 mmol) was added dropwise at 0 °C. The reaction mixture was left stirring vigorously for 2 h at room temperature. Phases were separated and the Et_2O phase was washed with aqueous 30% citric acid, saturated NaCl and saturated $NaHCO_3$. The Et_2O was dried over $NaSO_3$ and evaporated. Flash chromatography (EtOAc/MeOH 99:1) gave a colorless amorphous compound (0.50 g, 66 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.33–7.09 (m, 5H), 6.49 (d, *J* = 7.0 Hz, 1H), 4.80–4.62 (m, 1H), 3.68–3.34 (m, 4H), 2.70–2.53 (m, 2H), 2.25–2.14 (m, 2H), 2.03–1.80 (m, 6H), 1.31 (d, *J* = 6.6 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.97, 171.06, 141.65, 128.63, 128.49, 126.04, 46.85, 46.50, 46.16, 36.02, 35.38, 27.25, 26.17, 24.25, 18.61. HRMS (ESI-QTOF) *m/z* $[M+H]^+$ calcd for $C_{17}H_{24}N_2O_2$: 289.1916, found: 289.1916.

4-Phenylbutanoyl-2-aminoisobutyl-pyrrolidine (7g)



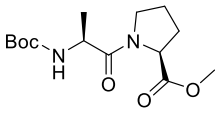
4-Phenylbutanoyl-2-aminoisobutyric acid methyl ester (**3g**) (2.5 mmol 0.66 g) was hydrolyzed according to procedure F. The reaction was continued following procedure B2 using the resulting 4-phenylbutanoyl-2-aminoisobutyric acid (2.0 mmol, 0.50 g) and pyrrolidine (4.2 mmol) without any Et_3N in the second step. Flash chromatography (EtOAc/MeOH 9:1) gave a white hygroscopic powder (0.33 g, 44 %). ^{1}H NMR (300 MHz, CD_3OD) δ 7.35–7.07 (m, 5H), 3.44 (t, *J* = 6.7 Hz, 4H), 2.62 (t, *J* = 7.6 Hz, 2H), 2.21 (t, *J* = 7.6 Hz, 2H), 1.99–1.65 (m, 6H), 1.43 (s, 6H). ^{13}C NMR (75 MHz, CD_3OD) δ 174.52, 173.89, 142.87, 129.39, 129.37, 126.96, 57.57, 48.93, 48.63, 36.33, 35.91, 28.45, 28.07, 25.53, 23.95. HRMS (ESI-QTOF) *m/z* $[M+H]^+$ calcd for $C_{18}H_{26}N_2O_2$: 303.2073, found: 303.2071.

4-Phenylbutanoyl-β-alanyl-pyrrolidine (7h)



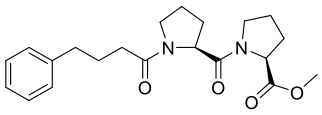
Prepared according to procedure B1 using 4-phenylbutanoyl-β-alanine (**4h**) (10.0 mmol, 2.35g). Flash chromatography (EtOAc/MeOH 19:1) gave a colorless amorphous compound (0.61 g, 41.0 %)^{1}H NMR (300 MHz, $CDCl_3$) δ 7.33–7.11 (m, 5H), 6.48 (s, 1H), 3.54 (dd, *J* = 11.2, 5.8 Hz, 2H), 3.44 (t, *J* = 6.8 Hz, 2H), 3.35 (t, *J* = 6.7 Hz, 2H), 2.63 (t, *J* = 7.5 Hz, 2H), 2.45 (t, *J* = 5.6 Hz, 2H), 2.18–2.11 (m, 2H), 2.02 –1.76 (m, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.73, 170.45, 141.72, 128.59, 128.44, 126.00, 46.62, 45.69, 36.21, 35.41, 34.91, 34.30, 27.34, 26.11, 24.47. HRMS (ESI-QTOF) *m/z* $[M+H]^+$ calcd for $C_{17}H_{24}N_2O_2$: 289.1916, found: 289.1920.

Boc-L-alanyl-proline methyl ester (8b)



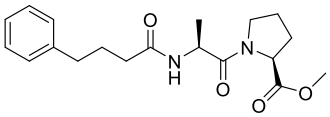
L-Proline methyl ester hydrochloride (13 mmol, 2.08 g) and DIPEA (26 mmol, 4.5 mL) were dissolved in anhydrous DCM (30 mL) at 0 °C and the resulting solution was added dropwise to a solution of Boc-L-Ala-Osu (8,6 mmol, 2,40 g) in anhydrous DCM (30 ml) at 0 °C under argon, where after it was stirred at room temperature for 3 h. The DCM phase was washed with aqueous 30% citric acid, saturated NaCl and saturated $NaHCO_3$, dried over $NaSO_3$ and evaporated. Flash chromatography (*n*-heptane/EtOAc 3:2) gave a colorless amorphous compound (2.25 g, 87 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.31 (d, *J* = 7.6 Hz, 1H), 4.55-4.37 (m, 2H), 3.72 (s, 3H), 3.69–3.54 (m, 2H), 2.25–1.91 (m, 4H), 1.41 (s, 9H), 1.34 (d, *J* = 6.9 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.35, 171.62, 155.16, 79.48, 58.65, 52.13, 47.70, 46.68, 28.87, 28.32, 24.87, 18.28.

4-phenylbutanoyl-L-prolyl-L-proline methyl ester (9a)



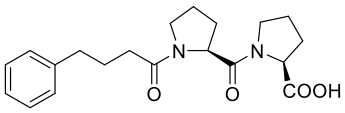
Prepared according to procedure B2 using 4-phenylbutanoyl-L-proline (**4a**) (0.9 mmol, 0.235 g) and L-proline-methyl ester (1.1 mmol, 0.182 g). Flash chromatography (EtOAc/MeOH 19:1) gave colorless amorphous compound (0.262g, 78 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42–7.02 (m, 5H), 4.68 (dd, *J* = 8.1, 3.8 Hz, 1H), 4.57 (dd, *J* = 8.6, 4.3 Hz, 1H), 3.94-2.83 (m, 1 H), 3.77–3.66 (m, 3H), 3.66–3.56 (m, 2H), 3.50-3.35 (m, 1H), 2.67 (t, *J* = 7.5 Hz, 2H), 2.44–1.75 (m, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.02, 171.58, 171.01, 141.93, 128.64, 128.38, 125.89, 58.75, 57.62, 52.22, 47.32, 46.75, 35.31, 33.62, 28.94, 28.53, 26.04, 25.09, 24.77.

4-Phenylbutanoyl-L-alanyl-proline methyl ester (9b)



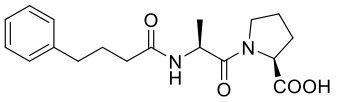
Boc-L-alanyl-proline methyl ester (**8b**) (7.5 mmol, 2.25 g) was deprotected according to procedure E. The resulting residue and DIPEA (32 mmol, 5.6 ml) was dissolved in anhydrous DCM (30 ml). 4-Phenylbutanoyl chloride (8.25 mmol) was added dropwise at 0 °C, where after it was stirred for 16 h at room temperature. The DCM phase was washed with aqueous 30% citric acid, saturated NaCl and saturated $NaHCO_3$. The DCM phase was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. Flash chromatography (EtOAc/MeOH 99:1) gave a colorless amorphous compound (0.46 g, 56 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.32–7.10 (m, 5H), 6.40 (d, *J* = 7.5 Hz, 1H), 4.74 (p, *J* = 7.0 Hz, 1H), 4.55–4.46 (m, 1H), 3.72 (t, *J* = 1.3 Hz, 3H), 3.70–3.56 (m, 2H), 2.63 (t, *J* = 7.5 Hz, 2H), 2.25–1.90 (m, 8H), 1.36 (d, *J* =6.8 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.39, 172.07, 171.53, 141.60, 128.59, 128.48, 126.03, 58.90, 52.33, 46.96, 46.66, 35.94, 35.34, 29.05, 27.20, 25.06, 18.24.

4-Phenylbutanoyl-L-prolyl-L-proline (10a)



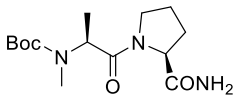
4-Phenylbutanoyl-L-prolyl-L-proline methyl ester (**9a**) (0.7 mmol 0.262 g) was hydrolyzed according to procedure F, resulting in a colorless amorphous compound (0.192 g, 76 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40–7.11 (m, 5H), 6.28 (s, 1H), 4.69–4.51 (m, 2H), 3.85 (q, *J* = 8 Hz, 1H), 3.71 – 3.49 (m, 2H), 3.43 (dt, *J* = 9.7, 6.9 Hz, 1H), 2.77–2.55 (m, 2H), 2.45–1.70 (m, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.52, 172.93, 172.07, 141.79, 128.65, 128.45, 126.00, 59.94, 57.68, 47.55, 47.45, 35.21, 33.56, 28.70, 27.61, 26.05, 25.16, 24.99. HRMS (ESI-QTOF) m/z $[M+H]^+$ calcd for $C_{20}H_{26}N_2O_4$: 359.1971, found: 359.1971.

4-Phenylbutanoyl-L-alanyl-L-proline (10b)



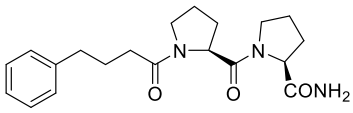
4-Phenylbutanoyl-L-alanyl-proline methyl ester (**9b**) (4.2 mmol, 1.45 g) was hydrolyzed according to procedure F, resulting in a white powder (1.33 g 95 %). mp: 128-130 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.33–7.10 (m, 5H), 6.45 (d, *J* = 7.5 Hz, 1H), 4.78 (p, *J* = 7.1 Hz, 1H), 4.57 (dd, *J* = 7.6, 4.6 Hz, 1H), 3.83–3.41 (m. 2H), 2.73–2.54 (m, 2H), 2.39–1.82 (m, 8H), 1.35 (d, *J* = 6.9 Hz, 2.4H), 1.31 (d, *J* = 6.9 Hz, 0.6H) (two rotamers 4:1). ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.17, 173.16 172.56, 141.58, 128.63, 128.54, 126.11, 59.48, 47.46, 46.69, 35.82, 35.34, 28.32, 27.14, 25.03, 18.07 (and an additional set of lower intensity signals from minor rotamer). HRMS (ESI-QTOF) *m/z* $[M+H]^+$ calcd for $C_{18}H_{24}N_2O_4$: 333.1814, found: 333.1811.

Boc-*N*-methyl-L-alanyl-L-prolineamide (12c)



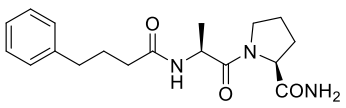
Prepared according to procedure B2 using Boc-*N*-methyl-L-alanine (10.0 mmol, 2.04 g) and L-prolineamide (12 mmol, 1.37 g), resulting in a white powder (1.99 g, 67 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.82 (s, 1H), 5.51 (s, 1H), 5.06–4.38 (m, 2H), 3.55 (m, 2H), 2.82 (d, *J* = 9.9 Hz, 3H), 2.39–1.83 (m, 4H), 1.46 (s, 9H), 1.30 (d, *J* = 7.2 Hz, 3H).

4-Phenylbutanoyl-L-prolyl-L-prolineamide (13a)



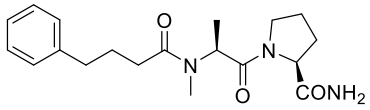
Prepared according to procedure B1 using 4-phenylbutanoyl-L-proline (**4a**) (3.83 mmol, 1.0 g) and L-prolineamide (4.21 mmol, 0.481 g). Flash chromatography (EtOAc/MeOH 9:1) gave a colorless amorphous compound (0.910 g, 66 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.19 (s, 0.5H), 7.32–7.13 (m, 5H), 6.90 (s, 0.5H), 5.71 (s, 0.5H), 5.41 (s, 0.5H), 4.67–4.59 (m, 1H), 4.45–4.33 (m, 0.5H), 4.28 (d, *J* = 7.9 Hz, 0,5H), 3.89–3.78 (m, 0,5H), 3.66–3.49 (m, 2.5H), 2.74–2.51 (m, 2H), 2.62–1.71 (m, 13H) (two rotamers 1:1). ^{13}C NMR (400 MHz, $CDCl_3$) δ 173.97, 173.80, 172.53, 172.19, 171.68, 170.80, 141.80, 141.63, 128.64, 128.63, 128.45, 128.43, 126.03, 125.97, 60.92, 59.57, 58.77, 57.81, 47.81, 47.41, 47.37, 46.84, 35.27, 35.20, 33.75, 33.62, 31.63, 28.88, 28.67, 27.02, 26.16, 26.05, 25.41, 25.15, 24.98, 22.27 (two rotamers). HRMS (ESI-QTOF) *m/z* $[M+H]^+$ calcd for $C_{20}H_{27}N_3O_3$: 358.2131, found: $[M+H]^+$ 358.2129.

4-Phenylbutanoyl-L-alanyl-L-prolineamide (13b)



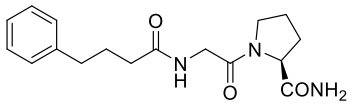
4-Phenylbutanoyl-L-alanyl-L-proline (**10b**) (4.0 mmol, 1.33 g) and Et_3N (4.0 mmol, 0.53 ml) were dissolved in anhydrous THF (40 mL) under argon. Ethylchloroformate (4.0 mmol, 0.38 mL) was added dropwise at -10 °C and allowed to react for 20 min. Ammonia (20 mmol, 2.85 mL 7 M solution in MeOH) was added at -10 °C and left stirring for 48 h at room temperature. The solvent was evaporated under reduced pressure. The residue was dissolved in DCM and the solution was washed with aqueous saturated $NaHCO_3$, dried over anhydrous $NaSO_4$, and evaporated under reduced pressure. Flash chromatography (EtOAc/MeOH 9:1) gave a white hygroscopic powder (0.94 g, 71 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.58 (s, 0.2 H), 7.37–6.99 (m, 5H), 6.67 (s, 0.8 H), 6.53 (d, *J* = 7.7 Hz, 1H), 6.02 (s, 0.2 H), 5.88 (s, 0.8H), 4.80–4.67 (m, 1H), 4.59–4.47 (m, 1H), 3.80–3.50 (m, 2H), 2.64 (t, *J* = 7.5 Hz, 2H), 2.37–1.85 (m, 8H), 1.33 (d, *J* = 6.9 Hz, 2.4H), 1.28 (d, *J* = 6.9 Hz, 0.6 H) (two rotamers 4:1). ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.51, 172.91, 172.29, 141.57, 128.58, 128.48, 126.05, 59.71, 47.42, 46.65, 35.77, 35.33, 27.58, 27.14, 25.16, 18.22 (and an additional set of lower intensity signals from minor rotamer). HRMS (ESI-QTOF) *m/z* $[M+H]^+$ calcd for $C_{18}H_{25}N_3O_3$: 332.1974, found: 332.1969.

4-Phenylbutanoyl-*N*-methyl-L-alanyl-L-prolineamide (13c)



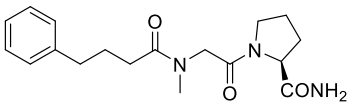
Boc-*N*-methyl-L-alanyl-L-prolineamide (**12c**) (6.64 mmol, 1.99 g) was deprotected according to procedure E and the resulting TFA salt was reacted the same way as for **7c**. Flash chromatography (EtOAc/MeOH 9:1) gave a white hygroscopic powder (1.51 g, 66 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.23 (m, 5H), 6.80 (s, 0.2 H), 6.75 (s, 0,8 H) 5.60 (s, 1H), 5.39 (q, *J* = 7.1 Hz, 0.8H), 5.39 (q, *J* = 7.1 Hz, 0.2H) 4.59–4.35 (m, 1H), 3.73–3.50 (m, 2H), 2.95 (s, 2.4H), 2.85 (s, 0.6H), 2.65 (m, 2H), 2.29 (m, 3H), 2.19–1.78 (m, 5H), 1.32 (d, *J* = 7.1 Hz, 2,4H), 1.28 (d, *J* = 7.2 Hz, 0.6H) (two rotamers 4:1). ^{13}C NMR (400 MHz, $CDCl_3$) δ 173.59, 173.22, 172.36, 141.67, 128.58, 128.49, 126.07, 59.66, 50.06, 47.32, 35.32, 32.95, 30.77, 27.47, 26.36, 25.18, 14.65 (and an additional set of lower intensity signals from minor rotamer). HRMS (ESI-QTOF) *m/z* $[M+Na]^+$ calcd for $C_{19}H_{27}N_3O_3$: 368.1945, found: 368.1936.

4-Phenylbutanoyl-glycyl-L-prolineamide (13d)



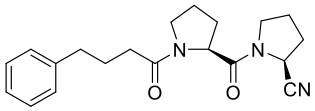
Prepared according to procedure B1 using 4-phenylbutanoyl-glycine (**4d**) (6.8 mmol, 1.50 g) and L-prolineamide (8.8 mmol, 0.93 g). Flash chromatography (EtOAc/MeOH 6:1) gave a white hygroscopic powder (0.97 g, 45 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39–7.04 (m, 5H), 6.75 (s, 1H), 6.48 (s, 1H), 5.67 (s, 1H), 4.55 (dd, J=7.6, 2.4 Hz, 1H), 4.07 – 3.99 (m, 1H), 3.69–3.49 (m, 1H), 3.49–3.36 (m, 1H), 2.66 (t, J=7.6 Hz, 2H), 2.41–2.29 (m, 1H), 2.29–2.18 (m, 2H), 2.11 (m, 1H), 2.06–1.86 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.28, 173.22, 168.57, 141.56, 128.62, 128.53, 126.10, 60.06, 46.63, 42.19, 35.69, 35.35, 27.99, 27.21, 24.88. HRMS (ESI-QTOF) *m/z* calcd for $C_{17}H_{23}N_3O_3$: 318.1818, found: 318.1820.

4-Phenylbutanoyl-sarcosyl-L-prolineamide (13e)



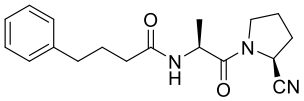
Prepared according to procedure B1 using 4-phenylbutanoyl-sarcosine (**4e**) (1.9 mmol, 0.450 g) and L-prolineamide (2.4 mmol, 0.282 g). Flash chromatography (EtOAc/MeOH 9:1) gave a white hygroscopic powder (0.330 g, 52 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.44–7.23 (m, 5H), 7.07 (s, 1H), 5.59 (s, 1H), 4.69–4.63 (m, 1H), 4.26 (d, *J* = 15.8 Hz, 1H), 4.05 (d, *J* = 15.8 Hz, 1H), 3.85–3.73 (m, 1H), 3.66–3.52 (m, 1H), 3.19 (s, 2,6H), 3.18 (s, 0,4H), 2.77 (t, *J* = 7.5 Hz, 2H), 2.51–2.40 (m, 2H), 2.15–2.00 (m, 6H) (two rotamers 5:1). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.14, 173.77, 168.60, 141.74, 128.61, 128.49, 126.04, 60.05, 50.80, 46.85, 37.64, 35.29, 32.35, 28.18, 26.45, 24.86 (and an additional set of lower intensity signals from minor rotamer). HRMS (ESI-QTOF) *m/z* $[M+H]^+$ calcd for $C_{18}H_{25}N_3O_3$: 332,1974, found: 332,1974.

4-Phenylbutanoyl-L-prolyl-2(*S*)-cyanopyrrolidine (14a)



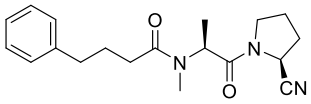
Prepared according to procedure C using 4-phenylbutanoyl-L-prolyl-L-prolineamide (**13a**) (1.82 mmol, 0.650 g) resulting in a colorless amorphous product (0.58 g, 94 %), the crude product was used without further purifications in the next step and not as the tested KYP-2047 ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37–7.07 (m, 5H), 4.89–4.76 (m, 1H), 4.56 (dd, *J* = 8.2, 4.0 Hz, 1H), 3.95–3.81 (m, 1H), 3.70–3.53 (m, 2H), 3.49–3.37 (m, 1H), 2.66 (t, *J* = 7.5 Hz, 2H), 2.38–2.07 (m, 8H), 2.03–1.87 (m, 4H) . ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.78, 171.49, 141.75, 128.63, 128.44, 125.99, 118.77, 57.48, 47.35, 46.61, 46.47, 35.21, 33.55, 29.81, 28.86, 26.04, 25.49, 25.01.

4-Phenylbutanoyl-L-alanyl-2*(S)*-cyanopyrrolidine (14b)



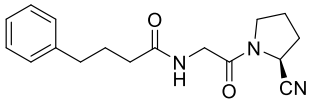
Prepared according to procedure C using 4-phenylbutanoyl-L-alanyl-L-prolineamide (**13b**) (1.0 mmol, 0.331 g). Flash chromatography (*n*-heptane/EtOAc 1:1) gave a colorless amorphous compound (0.25 g, 80 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.43–6.98 (m, 5H), 6.53-6.25 (m, 1H), 4.82–4.52 (m, 2H), 3.91–3.42 (m, 2H), 2.80–2.52 (m, 2H), 2.34–2.10 (m, 6H), 2.02–1.90 (m, 2H), 1.43 (d, *J* = 6.9 Hz, 0.3 H), 1.36 (d, *J* = 6.9 Hz, 2.3H), 1.31 (d, *J* = 6.9 Hz, 0.5H) (three rotamers 15:3:2) ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.39, 171.97, 141.51,128.60, 128.51, 126.09, 118.20, 46.63, 46.61, 46.49, 35.69, 35.25, 29.92, 27.09, 25.35, 18.15 (and two additional sets of lower intensity signals from minor rotamers). HRMS (ESI-QTOF) *m/z* $[M+H]^+$ calcd for $C_{18}H_{23}N_3O_2$: 314.1869, found: 314.1872. (NB. This compound give three rotamers although only one amide bond is N-alkylated)

4-Phenylbutanoyl-*N*-methyl-L-alanyl-*(S)*-cyanopyrrolidine (14c)



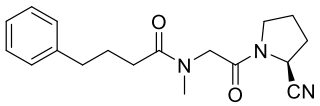
Prepared according to procedure C using 4-phenylbutanoyl-*N*-methyl-L-alanyl-L-prolineamide (**13c**) (3.51 mmol, 1.21 g). Flash chromatography (gradient *n*-heptane/EtOAc 9:1 → EtOAc) gave a colorless amorphous compound (1.04 g, 91 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.56–6.90 (m, 5H), 5.56 (q, *J* = 7.2 Hz, 0.4H), 5.35 (q, *J* = 7.2 Hz, 0.6H), 4.76 (d, *J* = 6.7 Hz, 0.4H), 4.73–4.65 (m, 0.6H), 3.69–3.46 (m, 2H), 2.94 (s, 1.8H), 2.83 (s, 1.2H), 2.80–2.53 (m, 2H), 2.40–1.90 (m, 8H), 1.35 (d, *J* = 7.1 Hz, 1.8H), 1.27 (d, *J* = 6.8 Hz, 1.2H) (two rotamers 3:2). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.27, 171.31, 141.62, 128.57, 128.50, 126.09, 118.44, 49.98, 46.65, 46.47, 35.30, 32.90, 30.72, 29.97, 26.36, 25.40, 14.48 (and an additional set of lower intensity signals from minor rotamer). HRMS (ESI-QTOF) *m/z* $[M+H]^+$ calcd for $C_{19}H_{25}N_3O_2$: 328.2025, found: 328.2028.

4-Phenylbutanoyl-glycyl-2*(S)*-cyanopyrrolidine (14d)



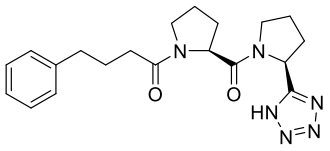
Prepared according to procedure C using 4-phenylbutanoyl-glycyl-L-prolineamide (**13d**) (2.1 mmol, 0.67 g). Flash chromatography (EtOAc) gave a colorless amorphous compound (0.364 g, 58 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32–7.15 (m, 5H), 6.39 (s, 1H), 4.77–4.69 (m, 1H), 4.16-4.06 (m, 2H), 3.75–3.58 (m, 1H), 3.53–3.41 (m, 1H), 2.66 (t, *J* = 7.6 Hz, 2H), 2.39–2.09 (m, 6H), 2.04–1.95 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.06, 167.63, 141.52, 128.63, 128.53, 126.11, 118.01, 46.70, 45.64, 42.13, 35.70, 35.34, 30.03, 27.18, 25.16. HRMS (ESI-QTOF) *m/z* $[M+H]^+$ calcd for $C_{17}H_{21}N_3O_2$: 300.1712, found: 300.1713.

4-Phenylbutanoyl-sarcocyl-2*(S)*-cyanopyrrolidine (14e)



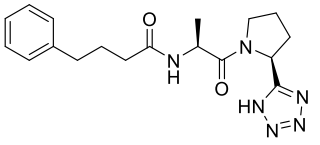
Prepared according to procedure C using 4-phenylbutanoyl-sarcosyl-L-prolineamide (**13e**) (3.6 mmol, 1.2 g). Flash chromatography (EtOAc/MeOH 19:1) gave a colorless amorphous compound (0.780 g, 69 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.31–7.16 (m, 5H), 4.96 (dd, *J* = 8.4, 1.9 Hz, 0.2H), 4.79–4.73 (m, 0.8H), 4.50 (m, 1H), 3.96–3.84 (m, 0.2H), 3.73 (d, *J* = 16.1 Hz, 0.8H), 3.67–3.40 (m, 2H), 3.10 (s, 0.6H), 3.08 (s, 2.4H), 2.68 (t, *J* = 7.5 Hz, 2H), 2.46–2.05 (m, 6H), 2.04–1.93 (m, 2H) (two rotamers 4:1). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.82, 167.87, 141.82, 128.65, 128.49, 126.02, 118.42, 49.92, 46.39, 45.90, 37.08, 35.32, 32.29, 29.93, 26.44, 25.37 (and an additional set of lower intensity signals from minor rotamer). HRMS (ESI-QTOF) *m/z* $[M+H]^+$ calcd for $C_{18}H_{23}N_3O_2$: 314.1869, found: 314.1869.

4-Phenylbutanoyl-prolyl-2*(S)*-tetrazolyl-pyrrolidine (15a)



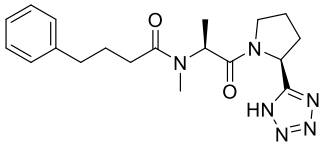
Prepared according to procedure D using 4-phenylbutanoyl-L-prolyl-2*(S)*-cyanopyrrolidine (**14a**) (1.65 mmol, 0.560 g). Flash chromatography (EtOAc/MeOH 4:1) gave a brown amorphous compound (0.564 g, 89 %). 1H NMR (400 MHz, CD_3OD) δ 7.43–7.03 (m, 5H), 5.40 (dd, *J* = 8.2, 3.5 Hz, 1H), 4.70–4.60 (m, 1H), 3.97–3.88 (m, 1H), 3.87–3.37 (m, 3H), 2.73–2.57 (m, 2H), 2.47–2.09 (m, 6H), 2.09–1.66 (m, 6H). ^{13}C NMR (101 MHz, CD_3OD) δ 174.01, 173.38, 159.79, 143.07, 129.53, 129.37, 126.93, 59.38, 52.94, 48.67, 48.23, 36.08, 34.38, 32.00, 29.58, 27.57, 25.82, 25.67 (and an additional set of lower intensity signals (ca 10 %) from minor rotamer) . HRMS (ESI-QTOF) m/z $[M+H]^+$ calcd for $C_{20}H_{26}N_6O_2$: 383,2195 found: 383,2196.

4-Phenylbutanoyl-L-alanyl-2*(S)*-tetrazolyl-pyrrolidine (15b)



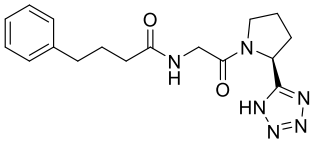
Prepared according to procedure D using 4-phenylbutanoyl-L-alanyl-2*(S)*-cyanopyrrolidine (**14b**) (0.8 mmol, 0.25 g). Flash chrmoatography (EtOAc/MeOH/AcOH 749:250:1) gave a yellowish amorphous compound (64 %, 0.182 g). ^{1}H NMR (300 MHz, CD_3OD) δ 7.30–7.06 (m, 5H), 5.49–5.33 (m, 1H), 4.71–4.33 (m, 1H), 4.01–3.46 (m, 2H), 2.66–2.54 (m, 2H), 2.48–2.00 (m, 6H), 1.95–1.79 (m, 2H), 1.33 (m, 0.7H), 1.28 (d, *J* = 7.0, 2.3H) (two rotamers 3:1). ^{13}C NMR (75 MHz, CD_3OD) δ 175.58, 173.82, 160.95, 142.98, 129.47, 129.34, 126.89, 53.43, 48.43, 48.23, 36.24, 35.96, 32.17, 28.57, 25.75, 16.72 (and an additional set of lower intensity signals from minor rotamer). HRMS (ESI-QTOF) *m/z* $[M+H]^+$ calcd for $C_{18}H_{24}N_6O_2$: 357.2039, found: $[M+H]^+$ 357.2036. Purity 90 % according UPLC-MS.

4-Phenylbutanoyl-*N*-methyl-L-alanyl-2*(S)*-tetrazolyl-pyrrolidine (15c)



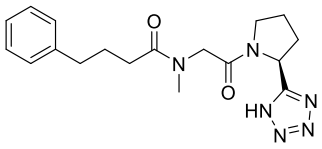
Prepared according to procedure D using 4-phenylbutanoyl-*N*-methyl-L-alanyl-2*(S)*-cyanopyrrolidine (**14c**) (2.06 mmol, 0.673 g). Flash chromatography (gradient EtOAc/MeOH 9:1 → 3:1) gave a brown amorphous compound (0.706 g, 93 %). ^{1}H NMR (400 MHz, CD_3OD) δ 7.32–7.12 (m, 5H), 5.50–5.40 (m, 0.4 H), 5.39–5.34 (m, 0.8 H), 5.28 (q, *J* = 7.0 Hz, 0.8H) 3.85–3.50 (m, 2H), 2.90 (s, 2.3 H), 2.81 (s, 0.2H), 2.60 (s, 0.5H) 2.71–2.63 (m, 2H), 2.46–2.30 (m, 3H), 2.20–1.85 (m, 5H), 1.30 (d, *J* = 6.8 Hz, 0.2H),1.25 (d, *J* = 6.8 Hz, 2.3H) 1.20 (d, *J* = 6.8 Hz, 0.5H) (three rotamers 77:17:6). ^{13}C NMR (101 MHz, CD_3OD) δ 175.47, 172.73, 159.71, 143.01,129.60, 129.49, 126.99, 53.32, 52.79, 48.13, 36.18, 33.63, 32.15, 31.30, 27.82, 25.67, 14.14 (and two additional sets of lower intensity signals from other rotamers). HRMS (ESI-QTOF) m/z $[M+H]^+$ calcd for $C_{19}H_{26}N_6O_2$: 371.2195, found: $[M+H]^+$ 371.2198.

4-Phenylbutanoyl-glycyl-2*(S)*-tetrazolyl-pyrrolidine (15d)



Prepared according to procedure D using 4-phenylbutanoyl-glycyl-2*(S)*-cyanopyrrolidine (**14d**) (0.76 mmol, 0.23 g). Flash chromatography (EtOAc/MeOH 20:7) gave a white powder (0.154 g, 60 %). ^{1}H NMR (400 MHz, CD_3OD) δ 7.33–7.06 (m, 5H), 5.50-3.59 (m, 1 H), 4.18-4.08 (m, 1H), 3.98 (d, *J* = 16.8 Hz, 0.6H), 3.83-3.70 (m, 1H), 3.69–3.53 (m, 1H), 3.35 (d, *J* = 16,8, 0.4 H), 2.72–2.53 (m, 2H), 2.46–1,85 (m, 8H) (two rotamers 3:2). ^{13}C NMR (101 MHz, CD_3OD) δ 176.38, 169.79, 162.65, 142.98, 129.49, 129.36, 126.90, 54.40, 47.90, 42.91, 36.22, 34.95, 32.51, 28.61, 23.16 (and an additional set of lower intensity signals from minor rotamer). HRMS (ESI-QTOF) *m/z* $[M+H]^+$ calcd for $C_{17}H_{22}N_6O_2$: 343.1882, found: $[M+H]^+$ 343.1883.

4-Phenylbutanoyl-sarcosyl-2*(S)*-tetrazolyl-pyrrolidine (15e)



Prepared according to procedure D using 4-phenylbutanoyl-sarcocyl-2*(S)*-cyanopyrrolidine (**14e**) (1.1 mmol, 0.35 g). Flash chromatography (EtOAc/MeOH 3:1) gave a brown amorphous compound (0.345 g, 88 %). ^{1}H NMR (400 MHz, CD_3OD) δ 7.34–7.08 (m, 5H), 5.49–5.30 (m, 1H), 4.40–4.09 (m, 2H), 3.90–3.47 (m, 2H), 3.01 (s, 1.6H), 2.93 (s, 0.5H), 2.89 (s, 0.6H), 2.81 (s, 0.3H), 2.70–2.49 (m, 2H), 2.49–2.20 (m, 3H), 2.20–1.71 (m, 5H) (four rotamers 53:20:17:10).^{13}C NMR (101 MHz, CD_3OD) δ 176.33, 169.66, 160.33, 143.11, 129.51, 129.37, 126.92, 53.67, 51.33, 47.45, 37.51, 36.14, 33.17, 32.27, 27.87, 25.52 (and three additional sets of lower intensity signals from minor rotamers). HRMS (ESI-QTOF) *m/z* $[M+Na]^+$ calcd for $C_{18}H_{24}N_6O_2$: 379.1853, found: 379.1850.

2. In vitro assay for inhibitory activity

Purfication of porcine PREP. Purification of recombinant porcine PREP is previously described in Venäläinen et al. 2002.[1]

Preparation of mice homogenates. Mice brain homogenates were prepared from cortexes of C57BL/6 mice. Brains were dissected and frozen in -80 °C. The brain cortex samples were homogenized in ten volumes of assay buffer (0.1 M Na-K-phosphate buffer, pH 7.0). The homogenate was centrifuged at 10,000 × g, +4 °C, for 20 min. Aliquots of supernatant were frozen and stored at -80 °C until assayed. The protein concentration of the supernatants were determined with Bio-Rad BCA-protein assay kit.

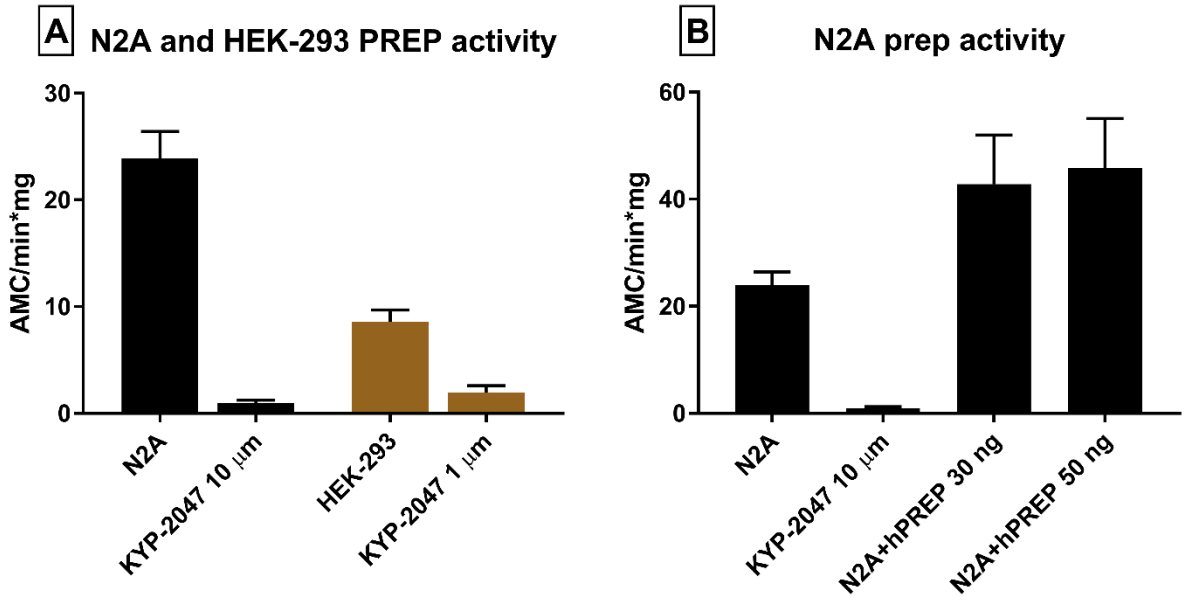
Determination of IC_{50} values. In the microplate assay procedure, 10 μL of the enzyme dilution or brain homogenate was preincubated with 65 μL of 0.1 M sodium–potassium phosphate buffer (pH 7.0) containing the compound at different concentrations 30 °C for 30 min. The enzyme reaction was initiated by adding 25 μL of 4 mM Suc-Gly-Pro-amido-4-methylcoumarin dissolved in 0.1 M sodium– potassium phosphate buffer (pH 7.0), and the mixture was incubated at 30° C for 60 min. The reaction was terminated by adding 100 μL of 1 M sodium acetate buffer (pH 4.2). Formation of 7-amido-4-methylcoumarin was determined fluorometrically with microplate fluorescence reader (excitation at 360 nm and emission at 460 nm). All activity measurements were made in triplicate. The final concentration of the compounds in the assay mixture varied from 1 mM-1 nM and the final concentration of the enzyme was approximately 2 nM and were measured using Bradford's method. The inhibitory activities (percent of control) were plotted against the log concentration of the compound, and the IC_{50} value was determined by non-linear regression utilizing GraphPad Prism 3.0 software.

3. Dimerization assay for αSyn

DNA constructs The split *Gaussia princeps* luciferase (GLuc) expression plasmids used in this study were previously described in Savolainen et al 2015[2]. Henri Huttunen (University of Helsinki) lab provided us αSyn-GLuc1 and αsyn-GLuc2

constructs. GLuc constructs used in this study have the GLuc reporter fragment placed at the N terminus separated by a (GGGGS)2SG linker.

Cell culture and transfection Mouse Neuro-2A (N2A) neuroblastoma cells were used throughout the whole study. N2A cells were cultured in Dulbecco's modified Eagle's medium (DMEM) Glutamax^{TM} with pyruvate supplement (gibco), with an additional 10% (v/v) FBS (Invitrogen), 1 % (v/v) non-essential aminoacids solution (gibco), 1% (v/v penicillin-streptomycin solution (Thermo Fischer) at 37 °C and 5% CO2, water-saturated air. Transfection of N2A was done using Lipofectamine 3000 (Thermo Fischer) according to the manufacturer's instruction. Used mouse N2A cells have high endogenous PREP activity so there were no need for over expressing PREP in this study (**Suppl. Fig. 1.**).



Suppl. Fig 1. (A) PREP activity of mouse N2A cells used in PCA-assay. N2A cells has high PREP activity which can be inhibited fully with 10 μM KYP-2047. HEK-293 cells are used as a comparison. (B) hPREP transfection increased PREP activity in N2A cells.

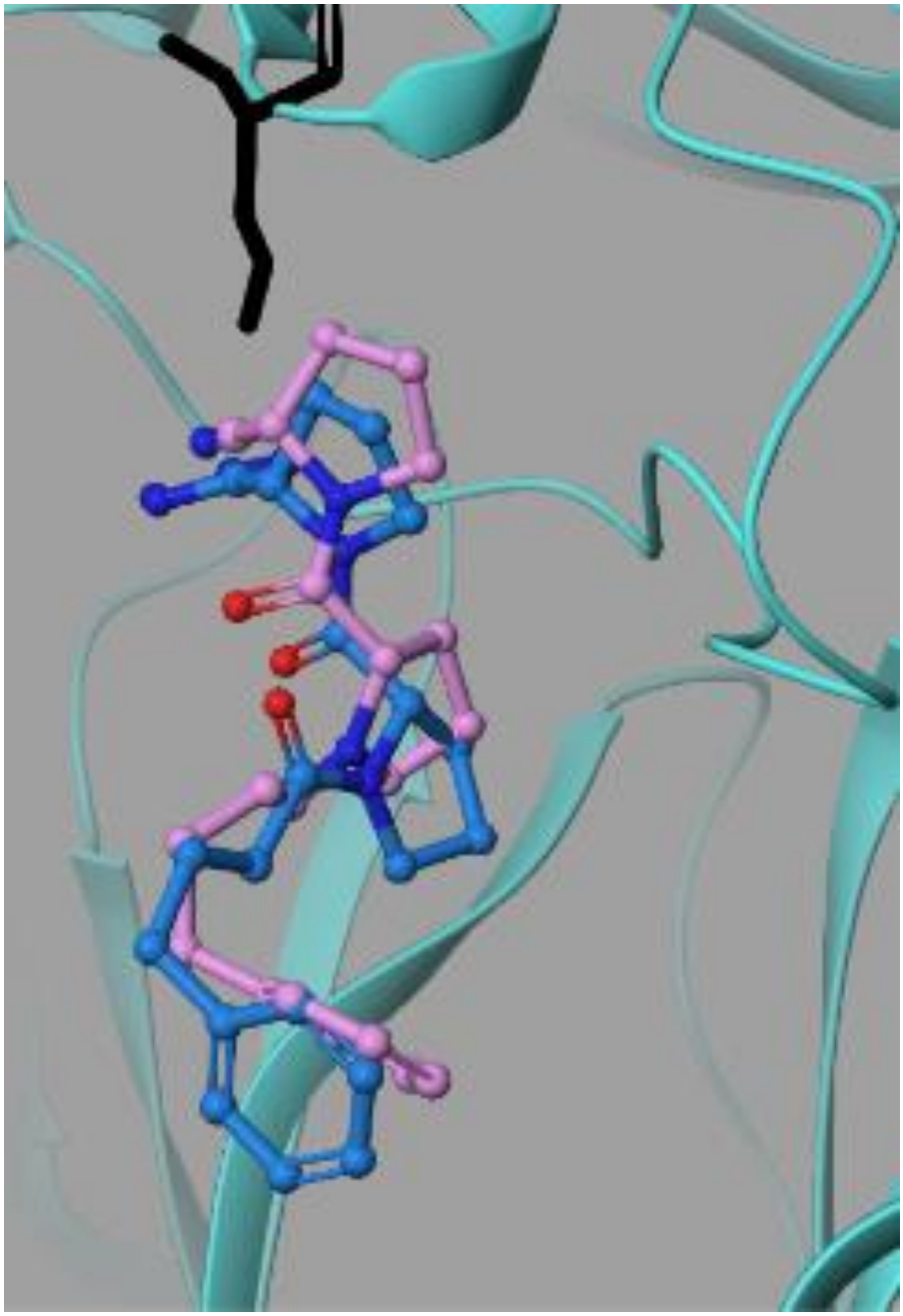
PCA (Protein-fragment complementation assay) N2A cells were plated on poly-L-lysine-coated 96-well plates (PerkinElmer Life Sciences, white wall, clear bottom) at a density of 13,000 cells per well. 24 h post-plating, reporter plasmids were transfected (100 ng of total plasmid DNA per well, 25 ng of both αSyn-Gluc1 and αSyn-Gluc2 and 50 ng mock-plasmid). 20 hours post-transfection medium was changed to phenol red free DMEM without serum containing the tested compounds at 10 μM concentration or proteasome inhibitor lactacystin (AG scientific) as a positive control. PCA signal was read 4 h post-transfection. A GLuc PCA signal was detected by injecting 25 μl of native coelenterazine (Nanolight Technology) per well (final concentration of 20 μM), and the emitted luminescence was read by Varioskan LUX multimode microplate reader (Thermo Scientific). For each experimental condition, 4 replicate wells were used, and 3– 5 replicates of independent experiments were performed. A 10 mM stock solution of compounds was prepared in DMSO and further diluted to phenol red free DMEM at 10 μM concentration. The corresponding amount of DMSO was used as vehicle control.

4. Molecular docking studies

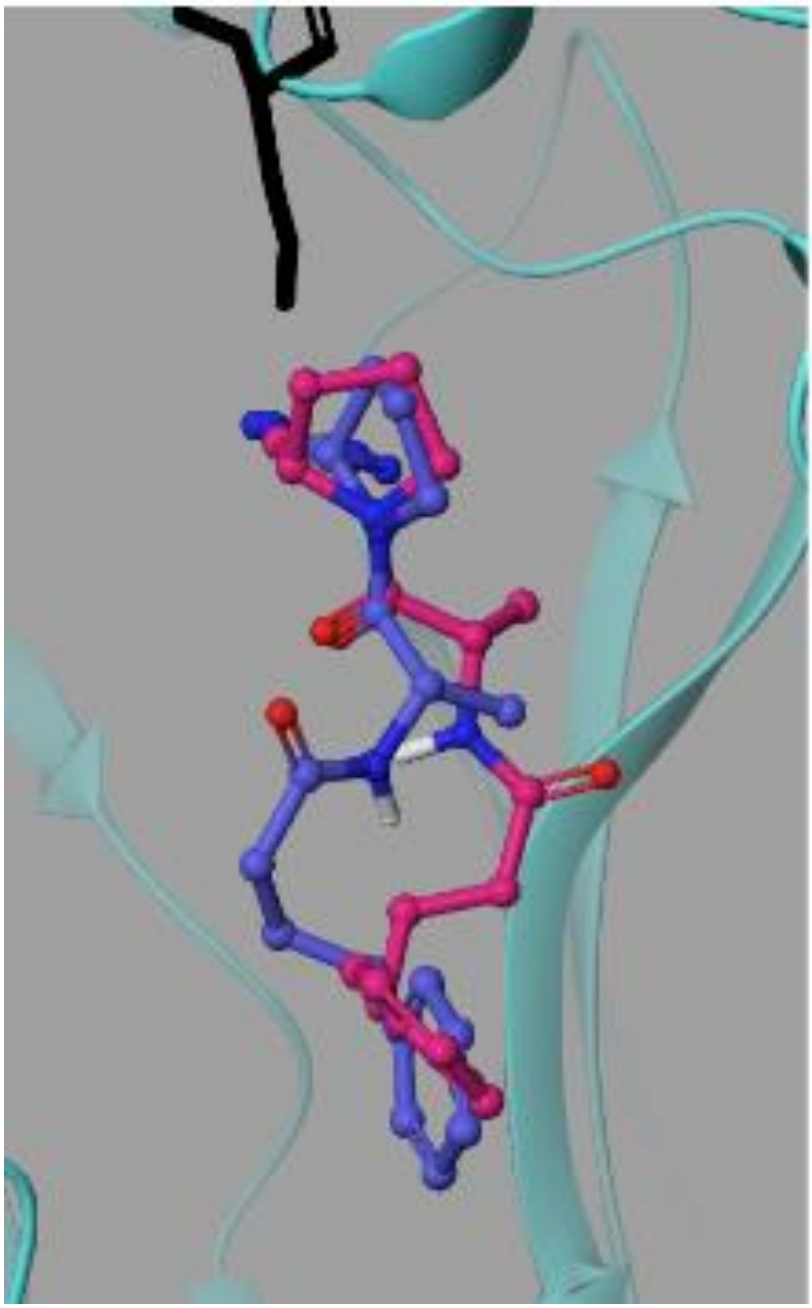
Schrödinger Maestro molecular modeling software was used in the molecular docking studies.[3] The crystal structure of PREP (PDB:3DDU)[4] was selected for these studies as it had good resolution of 1.56 Å and it included a cocrystallized ligand, which helped in determining the binding site. The protein structure was prepared with Maestro Protein Preparation Wizard. Preprocessing was done with default settings with few exceptions: missing loops and sidechains were constructed with Prime, and heteroatom states were generated with Epik using pH of 7.4. Acetate ions and glycerol were removed from the structure. H-bonds were assigned with default settings using PROPKA pH of 7.4. Waters with less than 3 H-bonds to non-waters were removed and the structure was minimized with default settings using OPLS3e force field.

Nitriles **14a-e** and tetrazoles **15a-e** were prepared with LigPrep using OPLS3e force field. Possible ionization states were generated at target pH 7.4, which resulted in non-ionized nitriles and negatively charged tetrazoles, where the tetrazole moiety had a negative charge. No tautomers or stereoisomers were generated. For docking, receptor grids with different settings were generated with Glide. The grid producing the best pose for the co-crystallized ligand was chosen to be used in the dockings.. This grid was generated to be suitable for peptide docking and the center of the grid was determined to be in the center of cocrystallized ligand. The length of the ligand diameter midpoint box was set to be 15 Å in X and Y directions and 10 Å in Z direction. The ligands were docked with Glide using SP-peptide precision and default settings. . In this method, the receptor is rigid while the ligand is flexible. OPLS3e force field was applied.

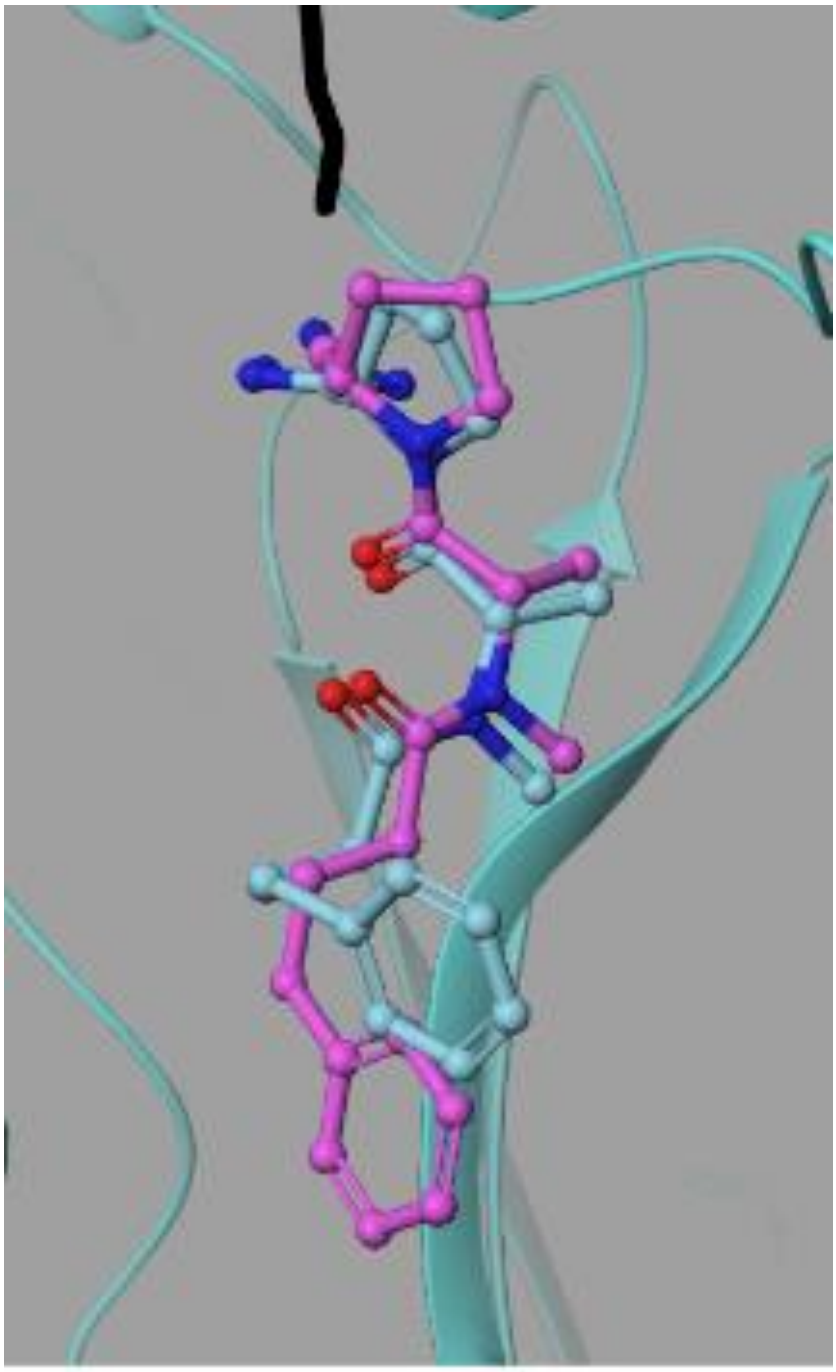
5. Binding poses of nitriles and tetrazoles



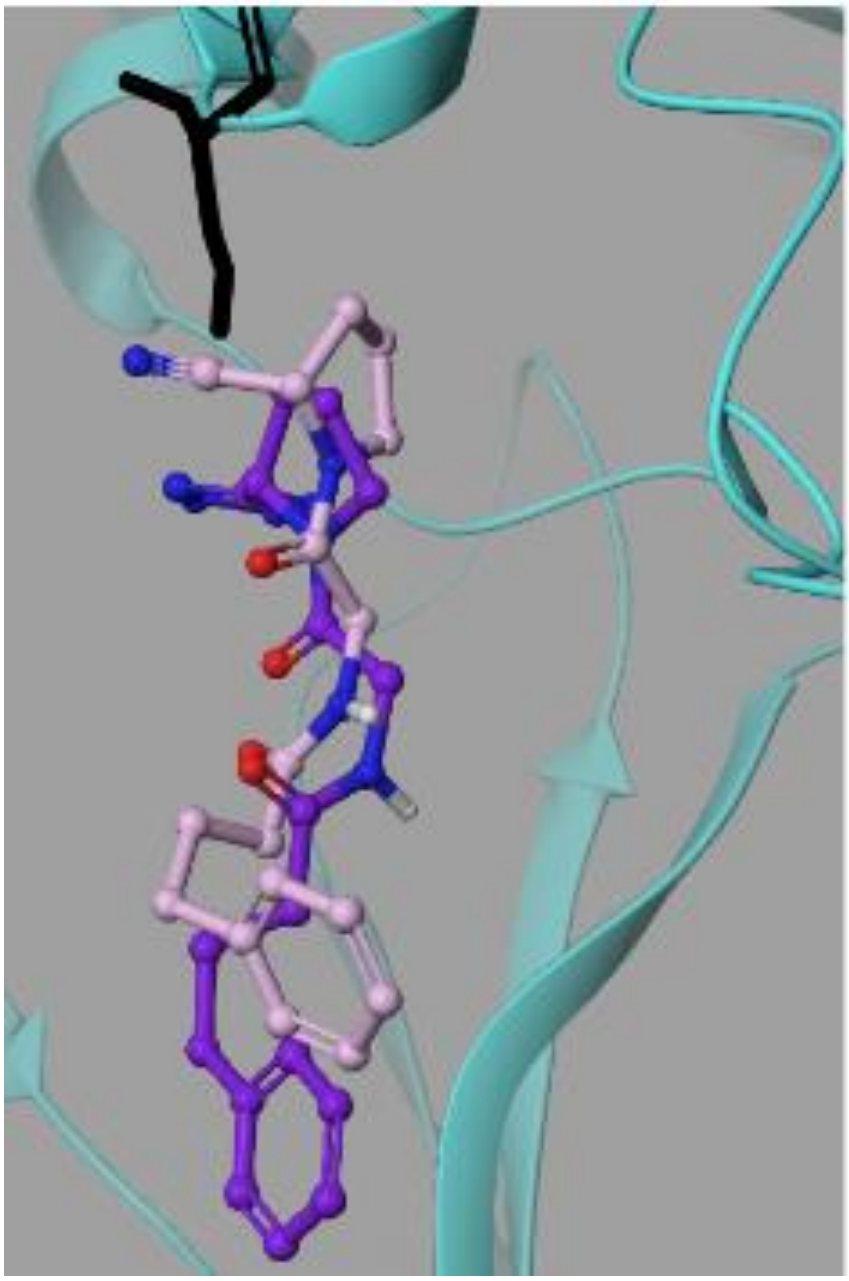
Suppl. Fig. 2. Representative binding poses of nitrile **14a** (light red ligand) and the corresponding tetrazole **15a** (blue ligand). Catalytically active serine (Ser554) is marked with black.



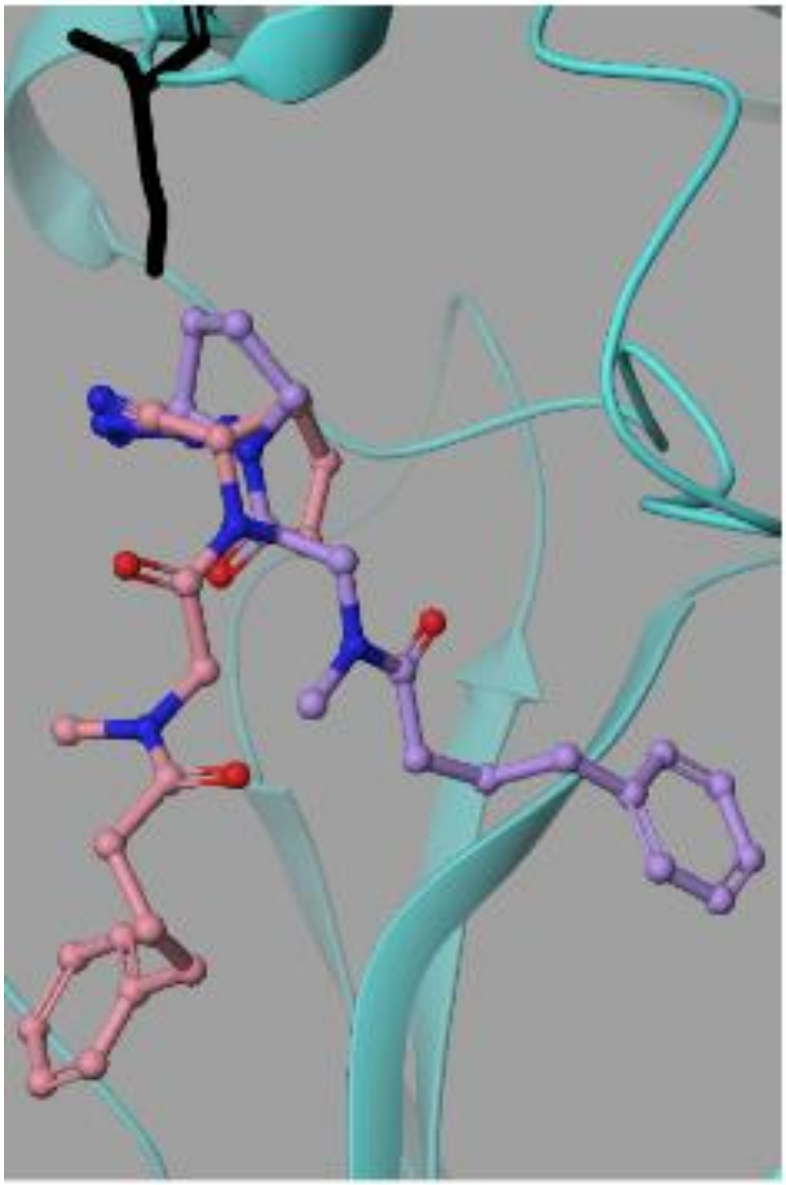
Suppl. Fig. 3. Representative binding poses of nitrile **14b** (pink ligand) and the corresponding tetrazole **15b** (violet ligand). Catalytically active serine (Ser554) is marked with black.



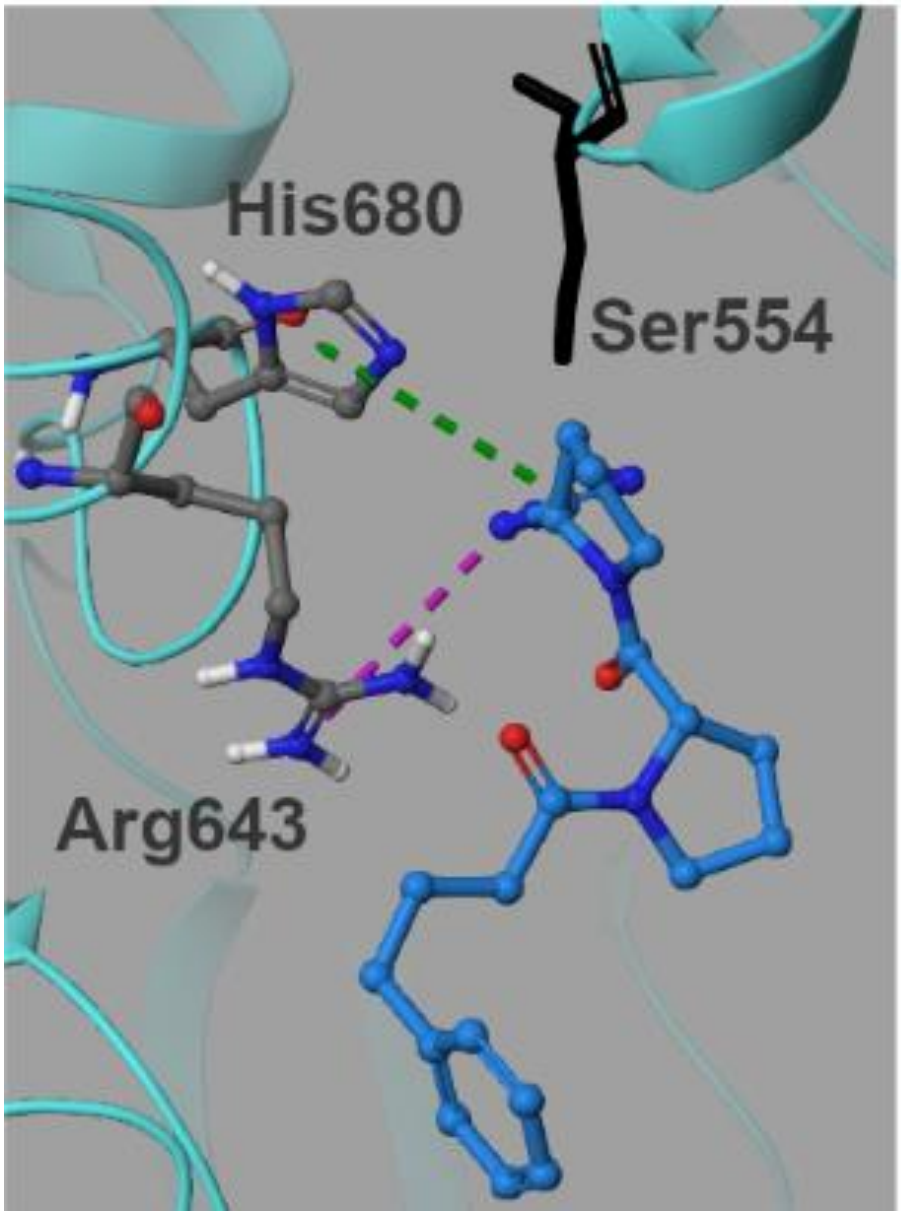
Suppl. Fig. 4. Representative binding poses of nitrile **14c** (magenta ligand) and the corresponding tetrazole **15c** (light turquoise ligand). Catalytically active serine (Ser554) is marked with black.



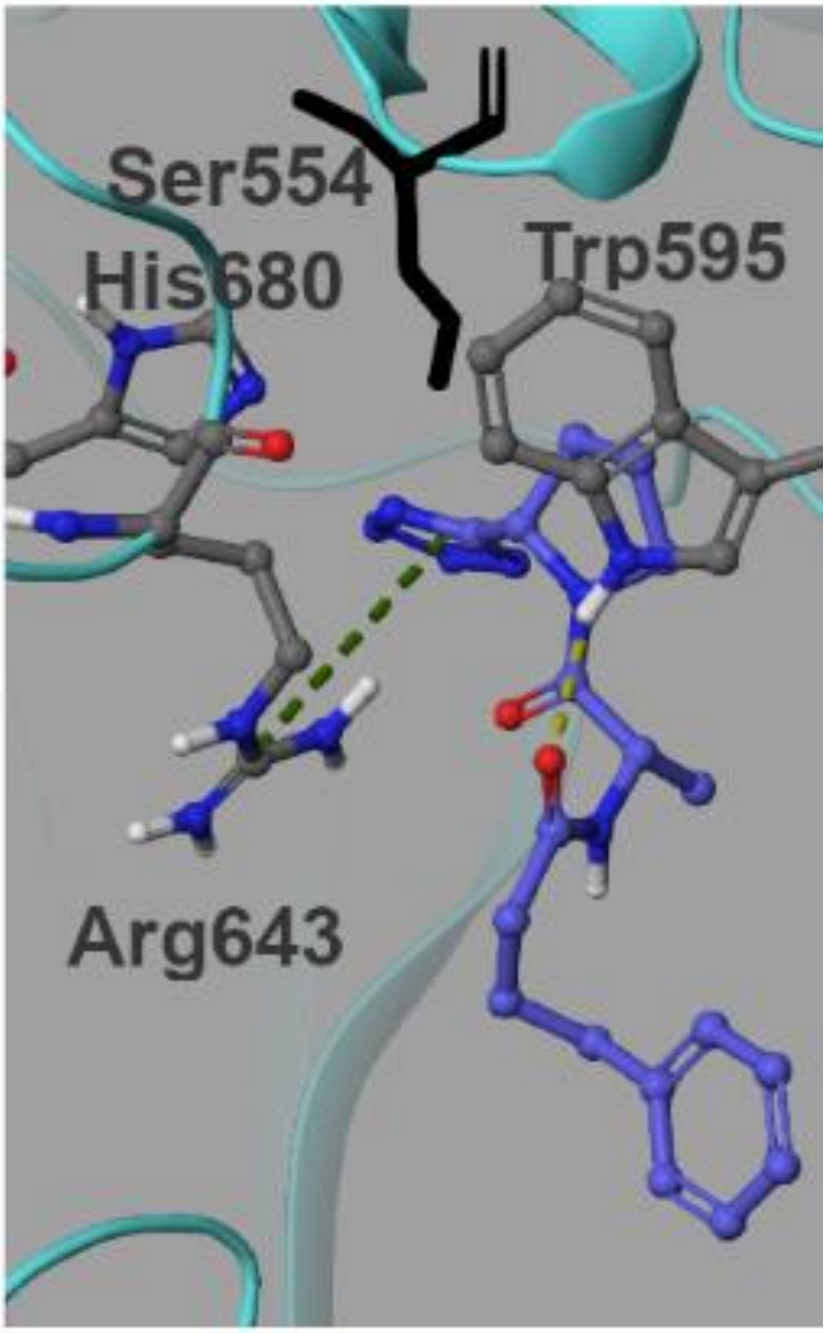
Suppl. Fig. 5. Representative binding poses of nitrile **14d** (light red ligand) and the corresponding tetrazole **15d** (purple ligand). Catalytically active serine (Ser554) is marked with black.



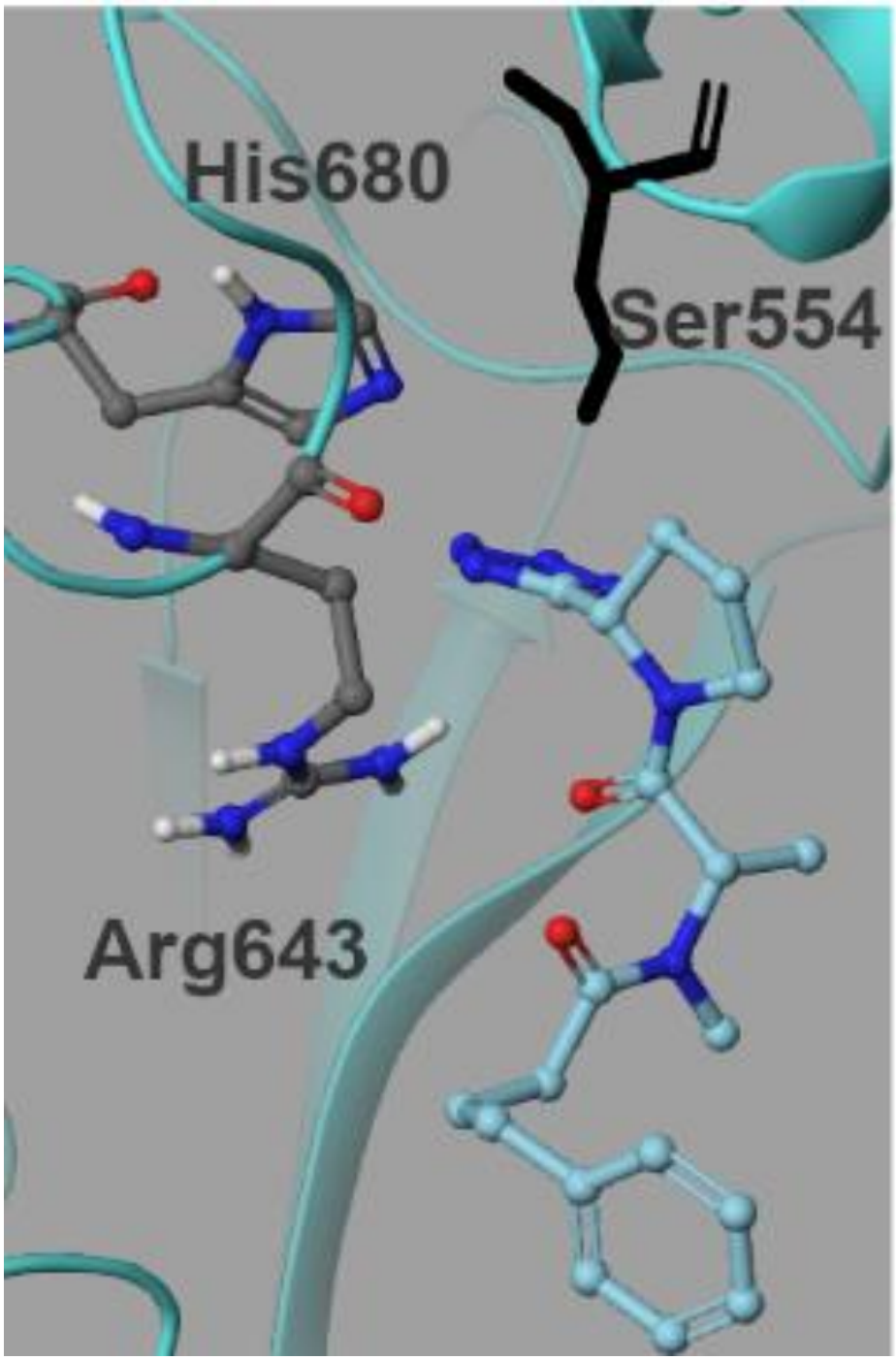
Suppl. Fig. 6. Representative binding poses of nitrile **14e** (coral red ligand) and the corresponding tetrazole **15e** (violet). Catalytically active serine (Ser554) is marked with black.



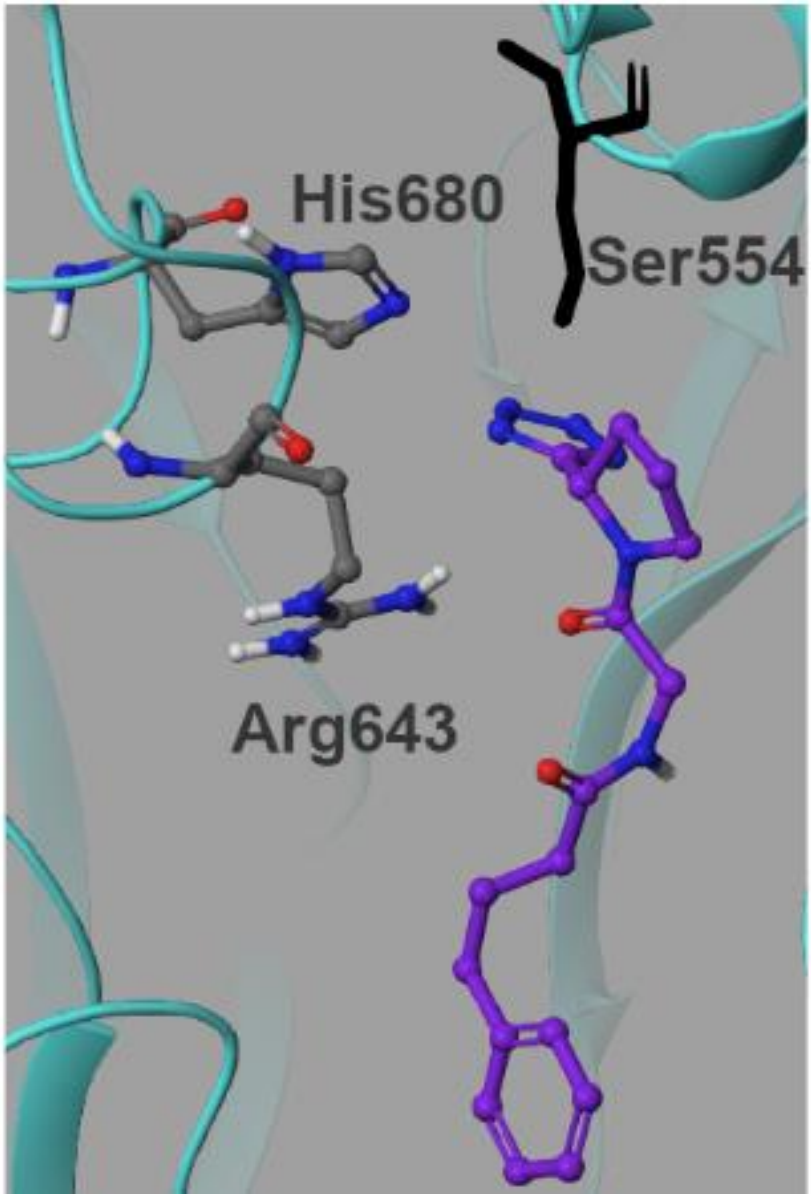
Suppl. Fig. 7. The pose and interactions of tetrazole **15a** with tetrazole ring towards S1. Interactions are marked with dashes: green for π- π stacking and magenta for salt bridge (ionic interaction).



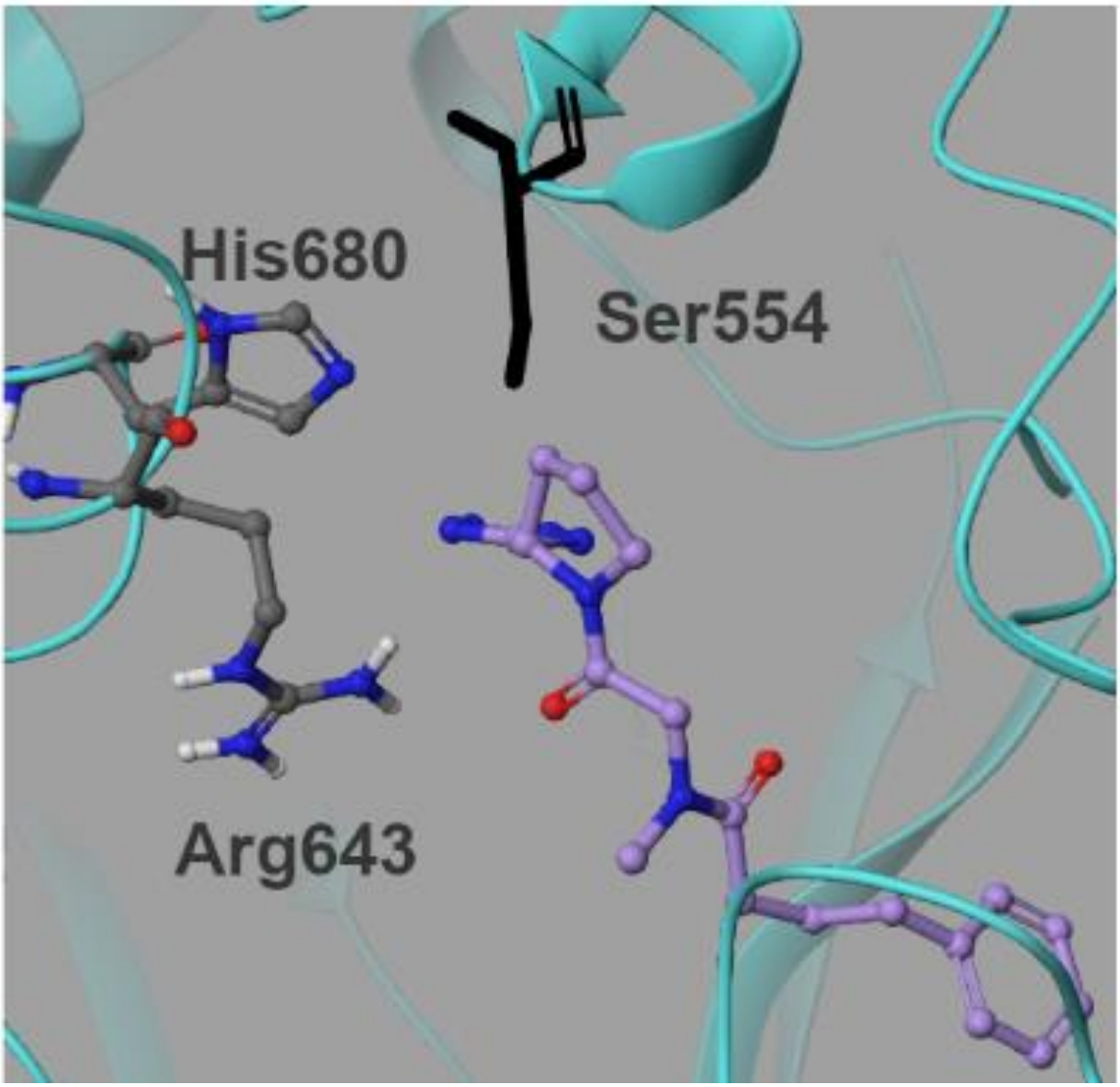
Suppl. Fig. 8. The pose and interactions of tetrazole **15b** with tetrazole ring towards S1. Interactions are marked with dashes: green for π-cation interaction and yellow for hydrogen bonding.



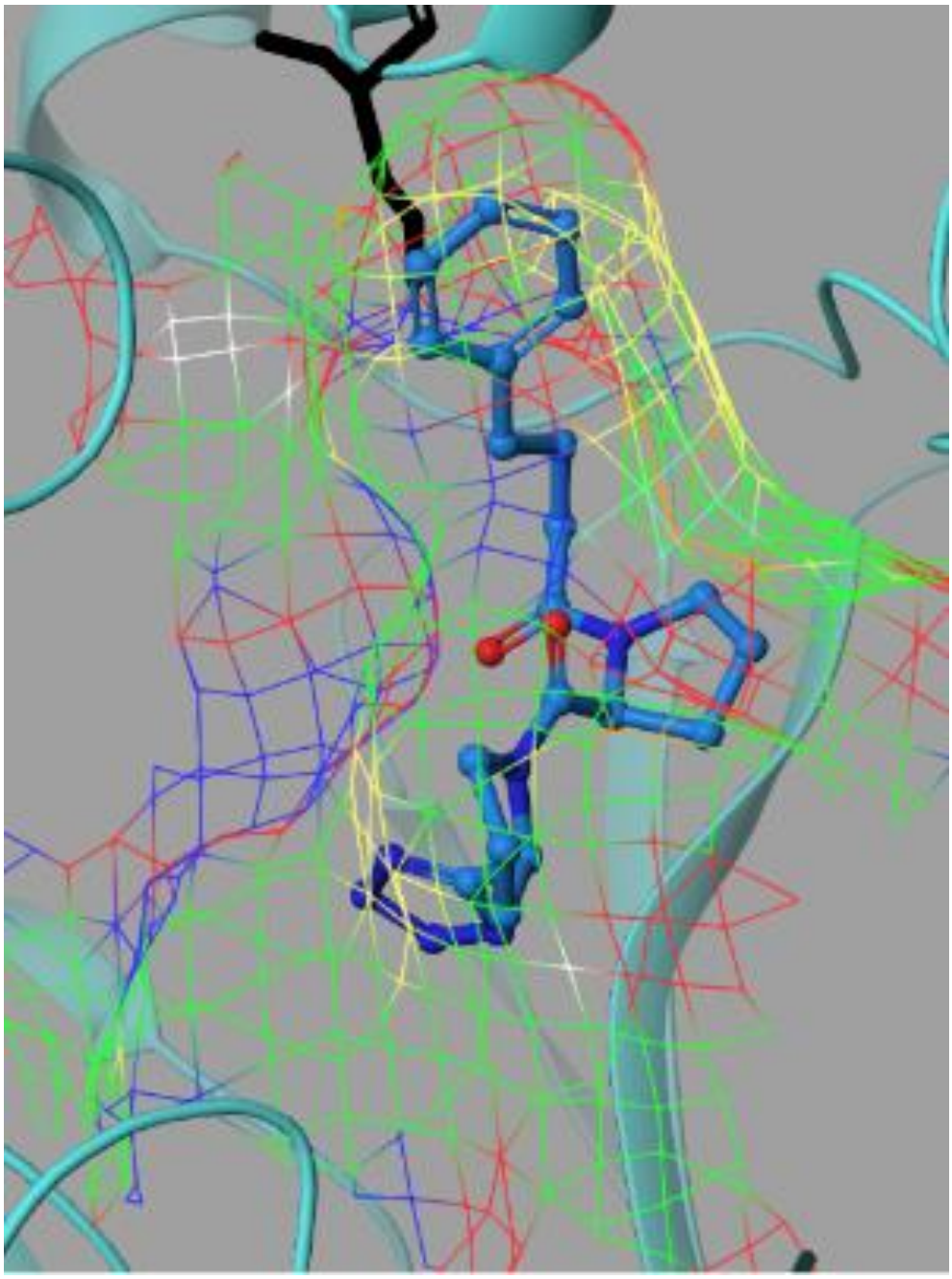
Suppl. Fig. 9 The pose of tetrazole **15c** with tetrazole ring towards S1.



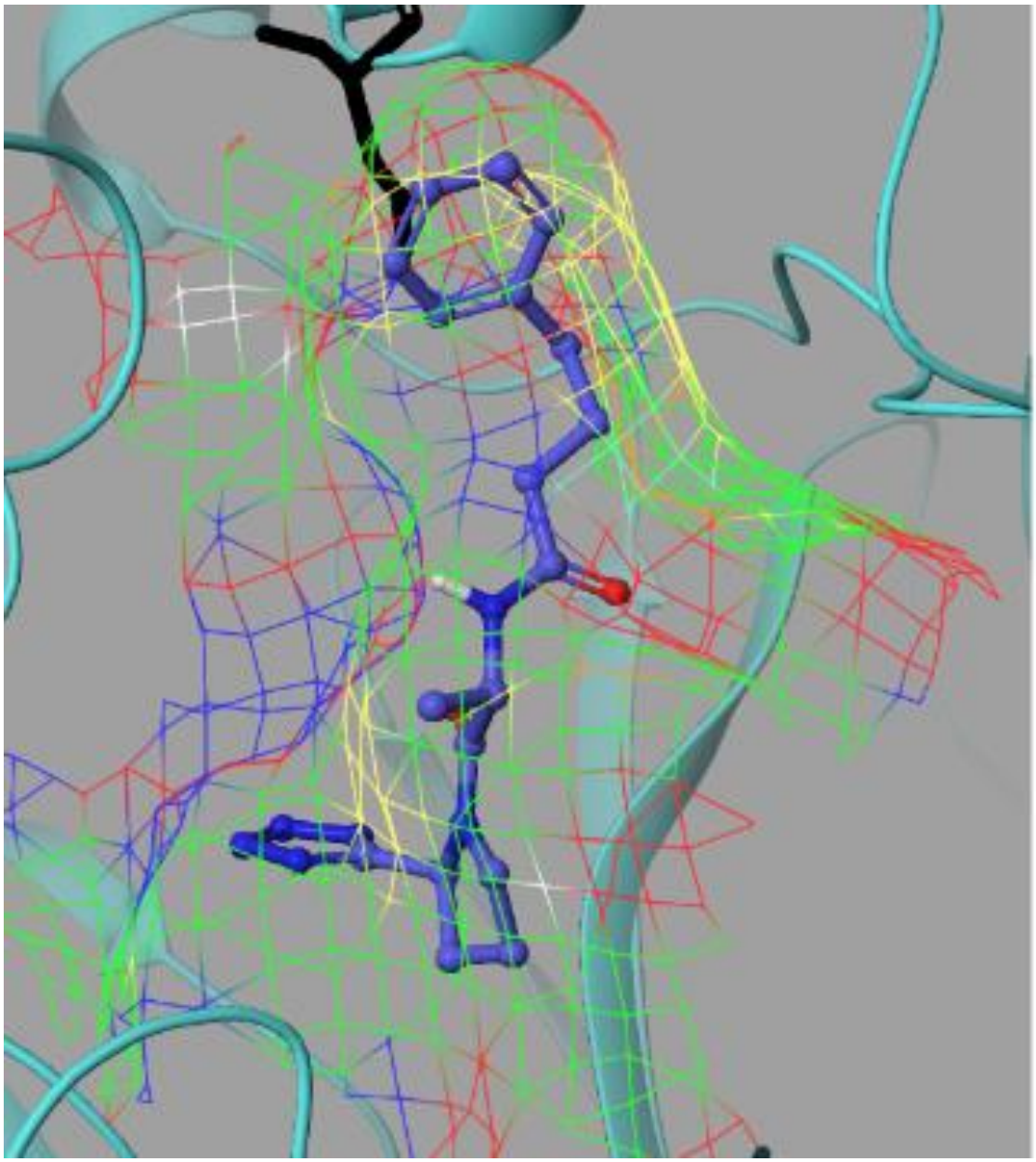
Suppl. Fig. 10. The pose of tetrazole **15d** with tetrazole ring towards S1.



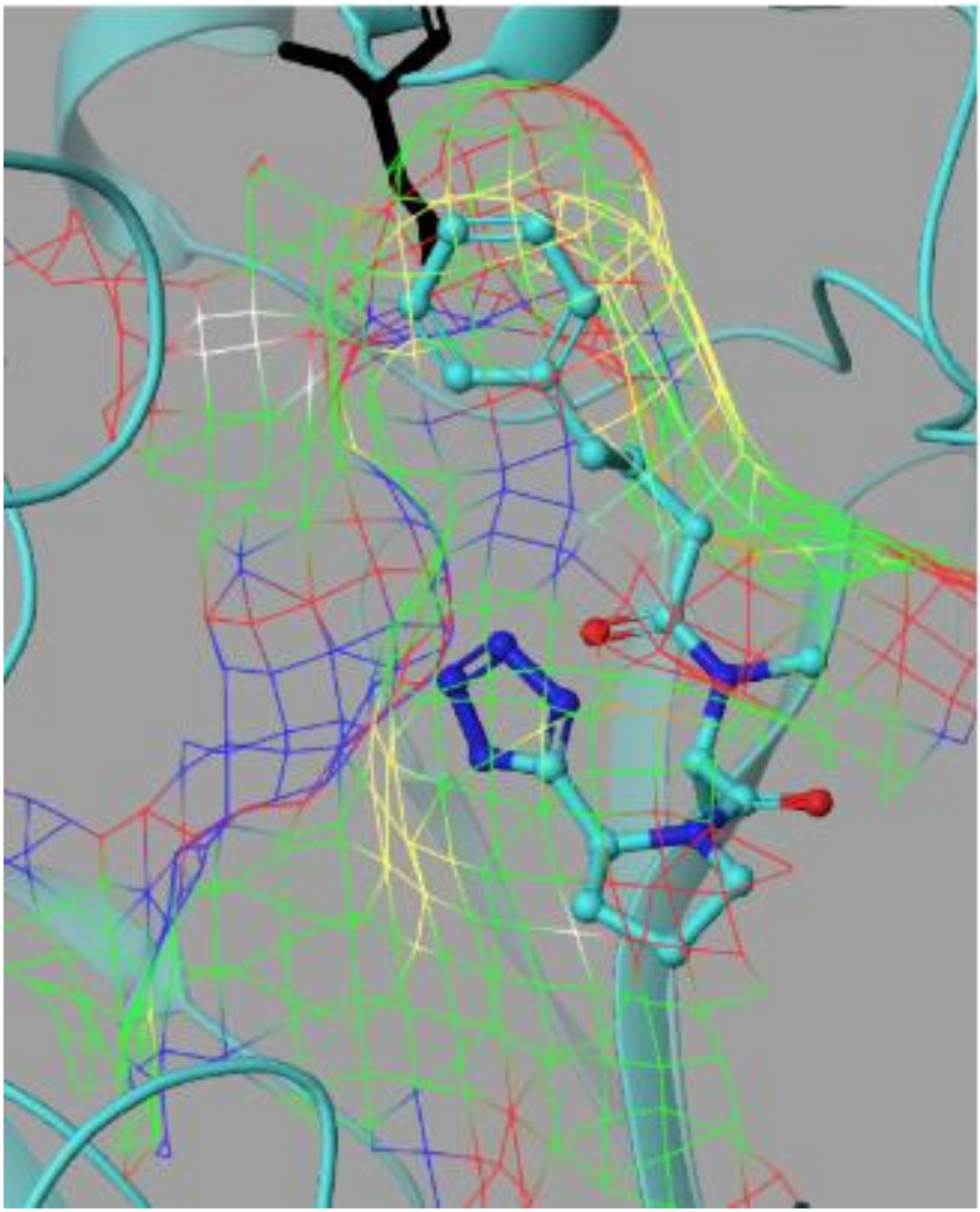
Suppl. Fig. 11. The pose of tetrazole **15e** with tetrazole ring towards S1.



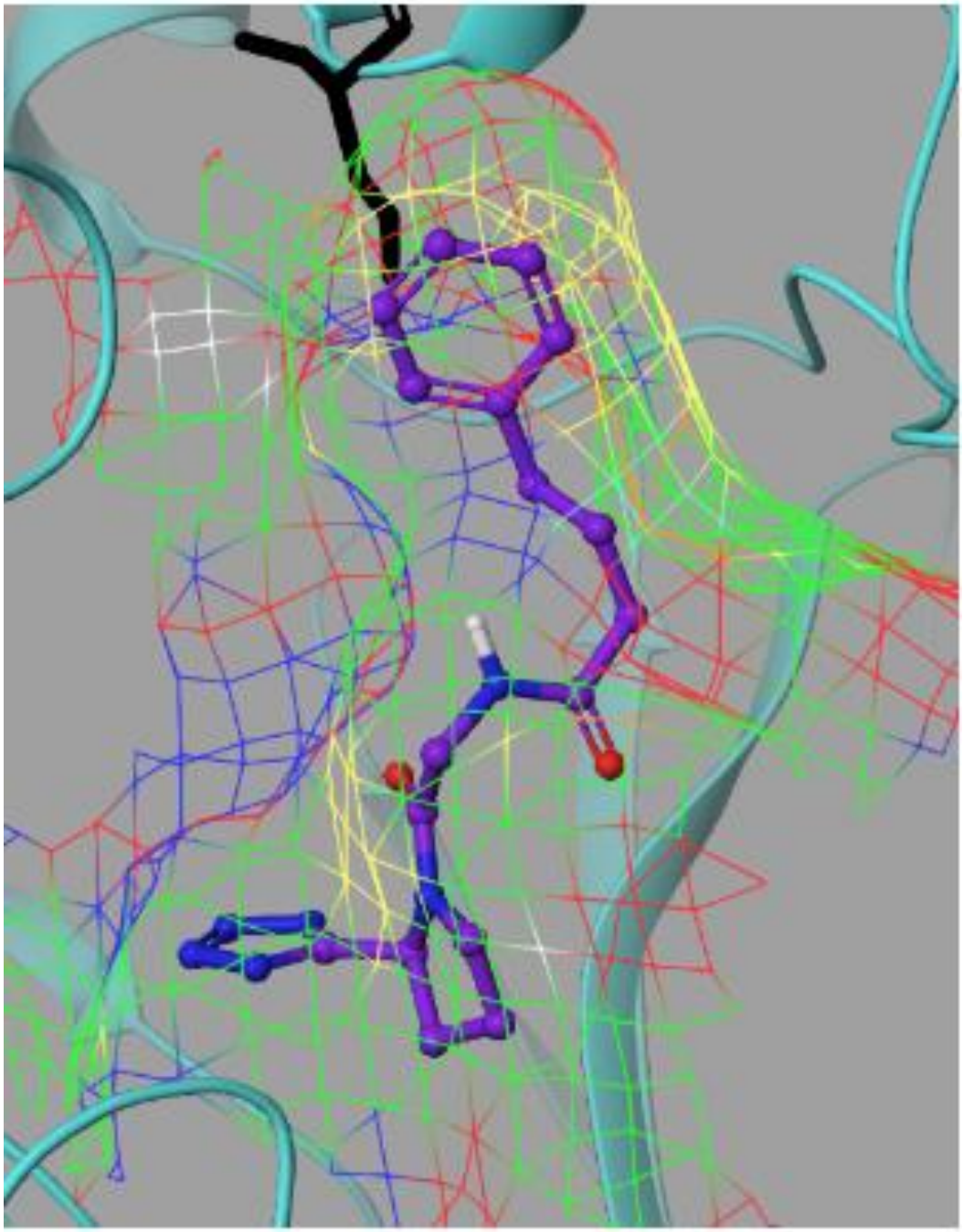
Suppl. Fig. 12. Pose of tetrazole **15a** with benzene ring at S1. The binding pocket shape is modeled with the dashed net. The colors in the net indicate different pocket are properties: green for lipophilic, yellow for aromatic, red for electronegative, and blue for electropositive. Catalytically active serine (Ser554) is marked with black.



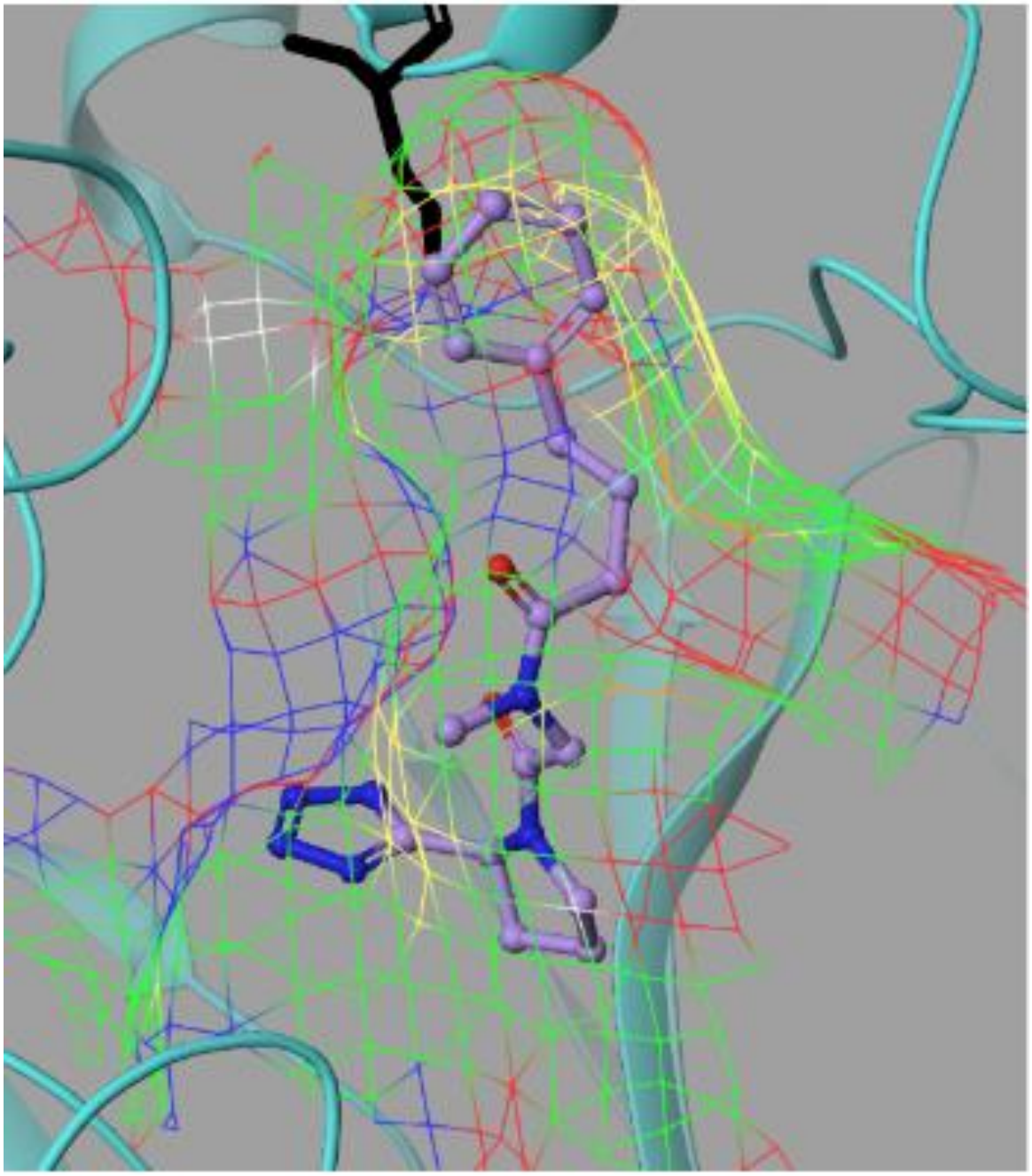
Suppl. Fig. 13 Pose of tetrazole **15b** with benzene ring at S1. The binding pocket shape is modeled with the dashed net. The colors in the net indicate different pocket are properties: green for lipophilic, yellow for aromatic, red for electronegative, and blue for electropositive. Catalytically active serine (Ser554) is marked with black.



Suppl. Fig. 14. Pose of tetrazole **15c** with benzene ring at S1. The binding pocket shape is modeled with the dashed net. The colors in the net indicate different pocket are properties: green for lipophilic, yellow for aromatic, red for electronegative, and blue for electropositive. Catalytically active serine (Ser554) is marked with black.



Suppl. Fig. 15. Pose of tetrazole **15d** with benzene ring at S1. The binding pocket shape is modeled with the dashed net. The colors in the net indicate different pocket are properties: green for lipophilic, yellow for aromatic, red for electronegative, and blue for electropositive. Catalytically active serine (Ser554) is marked with black.



Suppl. Fig. 16. Pose of tetrazole **15e** with benzene ring at S1. The binding pocket shape is modeled with the dashed net. The colors in the net indicate different pocket are properties: green for lipophilic, yellow for aromatic, red for electronegative, and blue for electropositive. Catalytically active serine (Ser554) is marked with black.

References

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