Design and synthesis of triazole-based peptidomimetics of a PSD-95 PDZ domain inhibitor

Anders Bach, *a Thomas B. Pedersen a and Kristian Strømgaard a

Department of Drug Design and Pharmacology, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen, Denmark.

*Tel.: +45 3533 6242; E-mail: anders.bach@sund.ku.dk

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CHEMISTRY

1. General

All reagents were obtained from commercial suppliers and used without further purification or characterization, except for dry solvents (CH₂Cl₂, THF, DMF), which were freshly distilled, and organometallic reagents, which were titrated prior to use. Melting points were determined on an MPA100 OptiMelt melting point apparatus and are presented as averaged values based on at least two measurements. ¹H NMR spectra were recorded on a Varian Mercury Plus (300 MHz), a Bruker Avance III (400 MHz), or a Bruker Avance III HD (600 MHz) instrument; and ¹³C NMR spectra were recorded on a Varian Gemini 2000 spectrometer (75 MHz), a Bruker Avance III (101 MHz), or a Bruker Avance III HD (152 MHz) instrument. Spectra were acquired at 300 K, using deuterated dimethylsulfoxide (DMSO-d₆), water (D₂O) or chloroform (CDCl₃) as solvents. Chemical shifts for ¹H and ¹³C spectra were recorded in parts per million using the residual non-deuterated solvent as the internal standard. The following abbreviations are used for the proton spectra multiplicities: s, singlet; bs, broad singlet; d, doublet; dd, double doublet, t, triplet and m, multiplet. Coupling constants (J) are reported in Hertz (Hz). Analytical HPLC was performed on an Agilent 1100 system with a C18 reverse phase column (Zorbax 300 SB-C18 column, 4.6 mm × 150 mm), flow rate of 1 mL/min, and a linear gradient of the binary solvent system of H₂O/CH₃CN/Trifluoroacetic acid (TFA) (A: 95/5/0.1, and B: 5/95/0.1). Preparative reverse phase HPLC was performed on a Agilent 1200 system using a C18 reverse phase column (Zorbax 300 SB-C18, 21.2 mm × 250 mm; or 9.4 mm × 250 mm) with a linear gradient of the binary solvent system of H₂O/CH₃CN/TFA (A: 95/5/0.1, and B: 5/95/0.1) with a flow rate of 20 mL/min. LC-MS mass spectra were obtained with an Agilent 6410 Triple Quadrupole Mass Spectrometer instrument using electron spray ionization (ESI) coupled to an Agilent 1200 HPLC system (ESI-LC/MS) with a C18 reverse phase column (Zorbax Eclipse XBD-C18, 4.6 mm × 50 mm), autosampler and diode array detector using a linear gradient of the binary solvent system of H₂O/CH₃CN/formic acid (A: 95/5/0.1, and B: 5/95/0.086) with a flow rate of 1 mL/min. During ESI-LC/MS analysis, evaporative light scattering (ELS) traces were obtained with a Sedere Sedex 85 Light Scattering Detector. GC-MS analyses were performed on a Shimadzu QP5050A instrument. Automated flash column chromatography purifications were done using a Teledyne ISCO apparatus (CombiFlash® Rf) with pre-packed silica gel columns (from 4 g up to 40 g) and a linear gradient of heptane-ethyl acetate (EtOAc) as eluent. All final compounds (9-18 and Indole 1-2) showed ≥95% purity by NMR (¹H, ¹³C, ¹H-¹H COSY, ¹H-¹³C HSQC), LC-MS (ELSD, UV), and analytical HPLC (UV). PBS-D₂O stock solutions of final compounds (5-25 mM) used for biological tests were evaluated prior to tests (NMR, LC-MS), and concentrations were assessed by quantitative ¹H-NMR (qNMR).

2.1.1. (2R,3R)-3-(Tert-butoxy)-2-((tert-butoxycarbonyl)amino)butyl methanesulfonate (20).

Methanesulfonyl chloride (1.01 g, 0.681 mL, 8.81 mmol) was added dropwise over 10 min to an ice-cold solution of 19 (1.92 g, 7.35 mmol) and triethylamine (1.34 g, 1.84 mL, 13.2 mmol) in dry CH₂Cl₂.
(10 mL) under stirring and nitrogen. The resulting mixture was warmed to room temperature and stirred for 4 h. The reaction was diluted with CH₂Cl₂ (25 mL) and washed with water (3x20 mL) and brine (20 mL), dried over MgSO₄, and concentrated in vacuo. Title compound was obtained by flash column chromatography as a clear colorless and viscous oil (2.23 g, 89%). ¹H NMR (300 MHz, CDCl₃) δ 1.16–1.21 (m, 12H), 1.45 (s, 9H), 3.04 (s, 3H), 3.6–4.0 (m, 2H), 4.14 (d, J = 7.0 Hz, 2H), 4.93 (d, J = 8.8 Hz, 1H). MS (ESI) m/z: 184.1 [M–Boc–t-Bu+H]⁺.

2.1.2. Tert-butyl ((2R,3R)-1-azido-3-(tert-butoxy)butan-2-yl)carbamate (21). NaN₃ (2.535 g, 39.0 mmol) was added portion-wise to a solution of 20 (2.23 g, 6.57 mmol) in dry DMF (10 mL). The suspension was stirred for 18 h at 50 °C, followed by cooling to room temperature, and addition of Et₂O (15 mL) and H₂O (15mL). The aqueous layer was extracted with Et₂O (3x10 mL), and the combined organic layers were dried over MgSO₄ and concentrated in vacuo. The product was purified by flash chromatography and fractions were collected based on TLC (EtOAc/heptane 1:1 containing 1% w/v PPh₃; Ninhydrin-stain) and LC-MS analysis, resulting in pure title compound as a clear, colorless oil (0.681 g, 36%). ¹H NMR (300 MHz, CDCl₃) δ 1.13 (d, J = 6.2 Hz, 3H), 1.17 (s, 9H), 1.44 (s, 9H), 3.19–3.26 (m, 1H), 3.35–3.41 (m, 1H), 3.52–3.59 (m, 1H), 3.77–3.82 (m, 1H), 4.86 (d, J = 8.5 Hz, 1H). ¹³C NMR (75 MHz, CDCl₃) δ 20.5, 28.6 (3C), 28.9 (3C), 52.1, 55.2, 65.4, 74.0, 79.7, 156.9. MS (ESI) m/z (%): 309.2 [M+Na]⁺ (17), 130.1 [M–Boc–t-Bu+H]⁺ (100). Anal. Calcd for C₁₃H₂₆N₄O₃: C, 54.52; H, 9.15; N, 19.56. Found: C, 54.45; H, 8.71; N, 19.13.

2.1.3. (2R,3R)-3-Amino-4-azidobutan-2-ol (2). Intermediate 21 (0.340 g, 1.19 mmol) was treated with 4 M HCl in dioxane (15 mL) at room temperature for 1.5 h. The product was obtained as TFA salt by concentrating the solution in vacuo, co-evaporating in CH₂Cl₂ (2×15 mL), and HPLC purification as a clear, sticky oil (151 mg, 52%). ¹H NMR (300 MHz, CD₃OD) δ 1.27 (d, J = 6.5 Hz, 1H), 3.08–3.14 (m, 1H), 3.64 (dd, J = 13.5, 7.0 Hz, 1H), 3.77–3.89 (m, 2H). ¹³C NMR (75 MHz, CD₃OD) δ 19.2, 50.0, 56.5, 64.5. MS (ESI) m/z (%): 131.2 [M+H]⁺.

2.1.4. ((S)-2-Azidopropanoic acid)-L-valine (3). A portion of Fmoc-Val-Wang resin (0.286 g, 0.2 mmol; Loading: 0.7 mmol/g) was washed (10x2 mL, 1 min each) and swelled in DMF (2 mL) for 10 min under shaking. The resin was drained and treated with 20% piperidine in DMF (2×10 min, DMF-wash in-between and after). (S)-2-Azidopropanoic acid (0.0464 g, 0.403 mmol) is activated with HATU (0.149 g, 0.392 mmol), HOAt (0.0535 g, 0.392 mmol) and collidine (0.099 g, 0.106 mL, 0.814 mmol) in dry DMF (2 mL) in a separate vial and transferred to the resin after 2 min. The resin was shaken for 3 h, washed in DMF (10×2 mL) and CH₂Cl₂ (10×2 mL) and concentrated in vacuo, followed by treatment with TFA/Tips/H₂O (90/5/5) (2.5 mL) for 2 h. The TFA solution was collected and concentrated in vacuo followed by two co-evaporations in CH₂Cl₂, addition of water (15 mL) and freeze-drying. Pure compound 3 was obtained by HPLC purification (33 mg, 77%) as a white solid. Mp 130–131 °C. ¹H NMR (300 MHz, CD₃OD) δ 0.96 (d, J = 4.7 Hz, 3H), 0.99 (d, J = 4.7 Hz, 3H), 1.44 (d, J = 7.0 Hz, 3H),
2.14–2.25 (m, 1H), 4.0 (q, J = 6.7 Hz, 1H), 4.31–4.35 (m, 1H), 8.12 (d, J = 7.3 Hz, 1H). $^{13}$C NMR (75 MHz, CD$_3$OD) δ 15.9, 17.0, 18.3, 30.4, 57.7 (2C), 172.0, 173.1. MS (ESI) m/z: 215.2 [M+H]$^+$.  

**2.1.5. (2S,3R)-2-Amino-N-(S)-1-azidopropan-2-yl)-3-hydroxybutanamide (4).** DIPEA (0.231 g, 0.311 mL, 1.79 mmol), Boc-Thr(tBu)-OH (0.493 g, 1.79 mmol) and HBTU (0.679 g, 1.79 mmol) were added to a solution of (S)-1-azidopropan-2-amine (24) (0.179 g, 1.79 mmol) in dry DMF (10 mL). The solution was stirred overnight (~12 h) at room temperature, and Et$_2$O (50 mL) and H$_2$O (50 mL) were added. The aqueous layer was extracted with Et$_2$O (3×30 mL), and the combined organic layers were dried over MgSO$_4$, concentrated in vacuo and purified by flash chromatography to obtain the Boc- and tert-butyl protected derivative of title compound (0.278 g, 43%) as a white solid (TLC: $R_f$ = 0.69 in 1:1 EtOAc/heptane). The material was treated directly with 4 M HCl in dioxane (15 mL) at room temperature for 1.5 h. The solution was concentrated in vacuo and co-evaporated in CH$_2$Cl$_2$ (2×20 mL). Title compound (4) was obtained as TFA salt by HPLC purification (127 mg, 23%, white solid). Mp 150.7–152.2 °C. $^1$H NMR (300 MHz, CD$_3$OD) δ 1.32 (d, J = 6.7 Hz, 3H), 1.42 (d, J = 6.2 Hz, 3H), 3.43 (dd, J = 12.6, 6.7 Hz, 1H), 3.57 (dd, J = 12.4, 4.8 Hz, 1H), 4.04–4.13 (m, 1H), 4.16–4.24 (m, 1H). $^{13}$C NMR (75 MHz, CD$_3$OD) δ 16.6, 19.2, 45.4, 55.3, 59.4, 66.3, 166.9. MS (ESI) m/z: 202.1 [M+H]$^+$.  

**2.1.6. tert-Butyl ((3R,4R)-4-(tert-butoxy)pent-1-yn-3-yl)carbamate (26).** Dimethyl-2-oxopropylphosphonate (0.632 g, 0.526 mL, 3.8 mmol,) was added to a suspension of K$_2$CO$_3$ (1.32 g, 9.55 mmol) and p-toluenesulfonylazide (11-15% in toluene; 0.753 g, 7.63 mL, 3.8 mmol) in CH$_3$CN (45 mL). The mixture was stirred for 2 h followed by addition of the aldehyde Boc-Thr(tBu)-CHO (31, 0.809 g, 3.12 mmol) dissolved in CH$_3$OH (9 mL). The reaction was stirred overnight, dried in vacuo, and re-dissolved in Et$_2$O (40 mL). The organic solution was washed with water (2×40 mL), brine (20 mL), dried over MgSO$_4$ and concentrated in vacuo. Title compound was obtained as a clear oil (440 mg, 55%) by flash chromatography, followed by combining pure fractions, evaporation and freeze-drying for 2.5 h. $^1$H NMR (400 MHz, CDCl$_3$) δ 1.14 (d, J = 6.2 Hz, 3H), 1.19 (s, 9H), 1.42 (s, 9H), 3.76 (dt, J = 6.6, 3.3 Hz, 1H), 4.13–4.36 (m, 1H), 4.70–5.01 (m, 1H). $^{13}$C NMR (101 MHz, CDCl$_3$) δ 19.3, 28.5 (3C), 28.6 (3C), 48.6, 68.5, 71.6, 74.1, 79.9, 82.6, 155.2. MS (EI) m/z (%): 57 (100), 99 [M–Boc–t-Bu]$^+$ (20).  

**2.1.7. (2R,3R)-3-Aminopent-4-yn-2-ol (5).** Intermediate 26 (0.174 g, 0.681 mmol) was dissolved in CH$_2$Cl$_2$ (5 mL) under nitrogen. Iodotrimethylsilane (0.190 g, 0.135 mL, 0.95 mmol) was added and the reaction was stirred for 10 min, followed by quenching with CH$_3$OH (10 mL). The solution was concentrated in vacuo, co-evaporated in CH$_3$CN (20 mL), and title compound obtained as a waxy solid (80 mg, 55%; 1:1 with TFA) by purification on the automated chromatography system using a pre-packed C18 column and buffer system H$_3$O/CH$_3$CN/TFA (A: 95/5/0.1, and B: 5/95/0.1). $^1$H NMR (400 MHz, D$_2$O) δ 1.43 (d, J = 5.8 Hz, 3H), 3.10 (d, J = 2.1 Hz, 1H), 4.01–4.12 (m, 2H). $^{13}$C NMR (101 MHz, D$_2$O) δ 18.7, 48.6, 67.4, 75.6, 78.1. MS (ESI) m/z: 100.1 [M+H]$^+$.  


2.1.8. Tert-butyl (S)-but-3-yn-2-ylcarbamate (28).\(^{2,3}\) Dimethyl-2-oxopropylphosphonate (1.66 g, 1.39 mL, 10 mmol) was added to a suspension of K$_2$CO$_3$ (3.46 g, 25 mmol) and \(p\)-toluenesulfonylazide (11-15% in toluene; 1.96 g, 20.1 mL, 10 mmol) in CH$_3$CN (50 mL). The mixture was stirred for 2 h followed by addition of the aldehyde Boc-Ala-CHO (27, 1.42 g, 8.21 mmol) dissolved in CH$_3$OH (20 mL). The reaction was stirred overnight, dried in vacuo, and re-dissolved in Et$_2$O (100 mL). The organic solution was washed with water (2×80 mL), brine (40 mL), dried over MgSO$_4$ and concentrated in vacuo. Title compound was obtained as a white fluffy solid (746 mg, 54%) by flash chromatography, followed by combining pure fractions (TLC: \(R_f = 0.75\) in 1:1 EtOAc/heptane), evaporation and freeze-drying for 10 min. \(^1\)H NMR (400 MHz, CDCl$_3$) \(\delta\) 3.19 (\(d, J = 7.0\) Hz, 3H), 1.40 (s, 9H), 2.36 (d, \(J = 2.3\) Hz, 1H), 4.85 (s, 1H). \(^13\)C NMR (101 MHz, CDCl$_3$) \(\delta\) 22.7, 28.5 (3C), 38.4, 70.3, 80.1, 84.7, 154.8. MS (EI) \(m/z\): 113 [M–t-Bu]$^+$.  

2.1.9. Tert-butyl ((2S,3R)-1-(((S)-but-3-yn-2-yl)amino)-3-(tert-butoxy)-1-oxobutan-2-yl)carbamate (29). Intermediate 28 (0.730 g, 4.31 mmol) was solubilized in CH$_2$Cl$_2$ (20 mL) and stirred at 0 °C under nitrogen. TFA (20 mL) was added and the reaction was stirred for 30 min, concentrated in vacuo, co-evaporated in Et$_2$O (2×40 mL), and stored at high-vacuum for 5 min resulting in a yellowish clear oil (1.5 g), which was used without further purification. Boc-Thr(tBu)-OH (1.308 g, 4.75 mmol) and HATU (1.006 g, 4.75 mmol) was solubilized in DMF (25 mL) under nitrogen at room temperature followed by addition of DIPEA (1.85 g, 2.49 mL, 14.3 mmol) and stirring for 5 min. The solution was combined with the yellowish clear oil (1.5 g) in DMF (10 mL) and DIPEA (0.614 g, 0.827 mL, 4.75 mmol). The reaction was stirred for 3 h, concentrated under vacuo (40 °C), re-dissolved in EtOAc (200 mL), and washed with saturated aqueous NaHCO$_3$ (2×75 mL) and 20% saturated NH$_3$Cl (2×75 mL). The organic layer was dried over MgSO$_4$, concentrated, and purified by flash chromatography to get title compound (1.32 g, 94%) as a white solid. TLC: \(R_f = 0.72\) in 1:1 EtOAc/heptane. Mp 78.7–82.0 °C. \(^1\)H NMR (400 MHz, CDCl$_3$) \(\delta\) 1.05 (\(d, J = 6.3\) Hz, 3H), 1.26 (s, 9H), 1.41 (\(d, J = 6.9\) Hz, 3H), 1.44 (s, 9H), 2.24 (d, \(J = 2.2\) Hz, 1H), 4.04–4.15 (m, 2H), 4.66–4.78 (m, 1H), 5.61 (s, 1H), 7.32 (s, 1H). \(^13\)C NMR (101 MHz, CDCl$_3$) \(\delta\) 17.3, 22.2, 28.4 (3C), 28.5 (3C), 37.0, 58.3, 67.2, 70.3, 75.4, 79.7, 84.0, 155.7, 169.4. MS (ESI) \(m/z\) (%): 171.2 [M–Boc–t-Bu+H]$^+$ (100), 215.1 [M–t-Bu–t-Bu+H]$^+$ (64), 271.2 [M–t-Bu+H]$^+$ (8).  

2.1.10. (2S,3R)-2-Amino-N-((S)-but-3-yn-2-yl)-3-hydroxybutanamide (6). Intermediate 29 (1.30 g, 3.98 mmol) was solubilized in CH$_2$Cl$_2$ (20 mL) and stirred at 0 °C under nitrogen. TFA (20 mL) was added and the reaction was stirred for 45 min, concentrated in vacuo, co-evaporated in Et$_2$O (2×30 mL), and stored at high-vacuum for 20 min resulting in a clear oil (2.3 g), which was solubilized in CH$_3$CN (6 mL) and water (24 mL) and lyophilized. The compound was purified on the automated chromatography system using a pre-packed C18 column and buffer system H$_2$O/CH$_3$CN/TFA (A: 95/5/0.1, and B: 5/95/0.1) resulting in pure title compound as a clear, colourless oil (1.05 g, 93%; 1:1 with TFA). \(^1\)H NMR (400 MHz, D$_2$O) \(\delta\) 1.31 (d, \(J = 6.5\) Hz, 3H), 1.44 (d, \(J = 7.0\) Hz, 3H), 2.71 (d, \(J = 2.3\) Hz, 1H), 3.74–3.79 (m, 1H), 4.08–4.16 (m, 1H), 4.65 (qd, \(J = 7.0, 2.3\) Hz, 1H), 8.26 (s, 1H), 8.93 (d, \(J = ...
2.1.11. Propioloyl-L-valine (7). A portion of 2-chlorotrityl chloride resin (0.190 g, 0.3 mmol; Loading: 1.58 mmol/g) was washed (10×2 mL, 1 min each) and swelled in DMF (2 mL) for 10 min under shaking. The resin was drained and Fmoc-Val-OH (0.068 g, 0.2 mmol) in DMF (1.6 mL) was added to the resin together with DIPEA (0.129 g, 0.174 mL, 1 mmol). The resin was shaken 1 h followed by addition of CH₃OH (0.100 mL) and shaking for another 5 min. The resin was washed in DMF (20×2 mL), drained and treated with 20% piperidine in DMF (2×10 min, DMF-wash in-between and after). Propiolic acid (0.042 g, 0.6 mmol) and EEDQ (0.148 g, 0.6 mmol) were solubilized in DMF (2 mL) in a separate vial and transferred to the drained resin after 2 min followed by shaking for 2 h. The resin washed in DMF (10×2 mL) and CH₂Cl₂ (10×2 mL) and dried in vacuo, followed by treatment with TFA/CH₂Cl₂/H₂O (47.5/47.5/5, 2.5 mL) for 2 h. The TFA solution was collected and concentrated in vacuo followed by two co-evaporations in CH₂Cl₂ (2×20 mL) to obtain crude 7. The compound was evaporated on celite and purified by flash chromatography (4 g silica gel) using a linear gradient of heptane-EtOAc/AcOH (9:1). Fractions were collected based on TLC (Rf = 0.45 in 10/10/2 EtOAc/heptane/AcOH), evaporated and purified by HPLC to obtain pure title compound as a yellowish, sticky solid (18 mg, 53%). ¹H NMR (300 MHz, D₂O) δ 0.80 (t, J = 5.9 Hz, 6H), 2.00–2.12 (m, 1H), 3.36 (s, 1H), 4.16 (d, J = 5.0 Hz, 1H). ¹³C NMR (75 MHz, D₂O) δ 17.0, 18.2, 29.8, 58.6, 75.4, 77.4, 154.5, 174.4. MS (ESI) m/z (%): 192.1 (17), 170.2 [M+H]+ (85), 152.1 (44), 124.2 (100).

2.1.12. (2R,3S)-3-Aminohex-5-yne-2,4-diol (8). Boc-Thr(tBu)-CHO (25, 0.722 g, 2.78 mmol) was solubilized in THF (20 mL) under argon and cooled to 0 °C. Ethynylmagnesium chloride (19.6 mL, 9.8 mmol, 0.5 M solution in THF) was added dropwise, and the solution was stirred for 1 h on ice. The reaction was quenched with aqueous ammonium chloride (15 mL) and the THF was evaporated in vacuo. The residue was extracted with EtOAc (3×25 mL), and the combined organic extracts were washed in water (50 mL) and concentrated in vacuo. The intermediate alcohol was obtained by flash chromatography as a clear yellowish oil (451 mg, 57%; TLC: Rf = 0.56 in 1:1 EtOAc/heptane), which was directly solubilized in CH₂Cl₂ (15 mL) and treated with TFA (15 mL) at 0 °C for 1 h. The solution was concentrated in vacuo, co-evaporated in CH₂Cl₂ (2×30 mL), and purified by HPLC to obtain title compound as a clear, colourless oil (318 mg, 47%; 1:1 with TFA). ¹H NMR (400 MHz, D₂O) δ 1.17 (d, J = 6.6 Hz, 3H), 2.90–2.93 (m, 1H), 3.09–3.14 (m, 1H), 4.04 (tt, J = 7.4, 6.0 Hz, 1H), 4.52 (dd, J = 6.2, 2.3 Hz, 1H). ¹³C NMR (101 MHz, D₂O) δ 19.0, 59.2, 60.9, 63.9, 76.94, 79.8. MS (ESI) m/z (%): 130.1 [M+H]+ (100), 112.1 (13).

3.1. General CuAAC reaction conditions (9-18). Azide (21 μmol, 1 eq.) and alkyne (21 μmol, 1 eq.) were dissolved in H₂O (2 mL) and t-BuOH (2 mL). CuSO₄ (8.4 μmol, 0.4 eq.) and sodium L-ascorbate (42 μmol, 2 eq.) were added and the solution was stirred for 30 min at room temperature. When
reaction was completed (LC-MS) solvents were removed by freeze-drying. The crude was purified by HPLC.

3.1.1. (S)-2-(4-((1R,2R)-1-amino-2-hydroxypropyl)-1H-1,2,3-triazol-1-yl)-3-methylbutanoic acid (9). The reaction was carried out following the general CuAAC reaction conditions, using azide 1 (3.54 mg, 24.8 μmol) and alkyne 5 (5.28 mg, 24.8 μmol), and with the modification that MeOH (1 mL) and DMSO (2 mL) were added prior to heating the reaction (55 °C) for 18 hours. Yellowish oil (3.9 mg, 66%). \(^{1}\)H NMR (600 MHz, D\(_2\)O) \(\delta\) 0.88 (d, \(J = 6.7\) Hz, 3H), 1.02 (d, \(J = 6.7\) Hz, 3H), 1.22 (d, \(J = 6.2\) Hz, 3H), 2.45 – 2.66 (m, 1H), 4.26 – 4.39 (m, 1H), 4.52 (d, \(J = 8.6\) Hz, 1H), 4.97 (d, \(J = 8.3\) Hz, 1H), 8.30 (s, 1H). \(^{13}\)C NMR (151 MHz, D\(_2\)O) \(\delta\) 17.84, 18.71, 18.96, 30.94, 53.24, 67.25, 72.97, 124.62, 141.04, 174.40. MS (ESI) \(m/z\): 243.1 [M+H]^+.

3.1.2. ((S)-2-(4-((1R,2R)-1-amino-2-hydroxypropyl)-1H-1,2,3-triazol-1-yl)propanoyl)-L-valine (10). The reaction was carried out following the general CuAAC reaction conditions, but with modifications: Azide 3 (5.30 mg, 24.8 μmol) and alkyne 5 (5.28 mg, 24.8 μmol) were dissolved in H\(_2\)O (2 mL) and CH\(_3\)CN (0.4 mL), and CuSO\(_4\) (0.798 g, 5 μmol, 0.2 eq.), sodium L-ascorbate (30 mg, 150 μmol, 6 eq.), and DIPEA (12 μL, 8.9 mg, 69 μmol, 2.8 eq.) were added. The solution was stirred for 65 hours at room temperature. Yellowish oil (5.4 mg, 67%). \(^{1}\)H NMR (600 MHz, D\(_2\)O) \(\delta\) 0.93 (dd, \(J = 6.8, 4.7\) Hz, 6H), 1.21 (d, \(J = 6.3\) Hz, 3H), 1.90 (d, \(J = 7.2\) Hz, 3H), 2.16 – 2.25 (m, 1H), 4.21 (d, \(J = 5.7\) Hz, 1H), 4.32 (dq, \(J = 8.8, 6.4\) Hz, 1H), 4.53 (d, \(J = 8.6\) Hz, 1H), 5.69 (q, \(J = 7.1\) Hz, 1H), 8.34 (s, 1H). \(^{13}\)C NMR (151 MHz, D\(_2\)O) \(\delta\) 16.57, 17.14, 18.59, 18.90, 30.18, 53.10, 59.45 (2C), 67.21, 124.22, 141.22, 170.66, 176.54. MS (ESI) \(m/z\): 314.2 [M+H]^+.

3.1.3. (S)-2-(4-((1S,2R)-2-amino-3-hydroxybutanamido)ethyl)-1H-1,2,3-triazol-1-yl)-3-methylbutanoic acid (11). The reaction was carried out following the general CuAAC reaction conditions, using azide 1 (3.01 mg, 21 μmol) and alkyne 6 (4.48 mg, 21 μmol). White solid (2.5 mg, 38%). \(^{1}\)H NMR (600 MHz, D\(_2\)O) \(\delta\) 0.87 (d, 3H), 1.00 (d, 3H), 1.20 (m, 3H), 1.62 (m, 3H), 2.61 (m, 1H), 3.81 (dd, 1H), 4.08 (m, 1H), 5.14 (d, 1H), 5.26 (q, 1H), 8.13 (s, 1H). \(^{13}\)C NMR (151 MHz, D\(_2\)O) \(\delta\) 17.56, 18.44, 18.68, 18.73, 30.99, 41.98, 58.87, 66.18, 70.35, 123.08, 148.59, 166.79, 172.55. MS (ESI) \(m/z\): 314.2 [M+H]^+.

3.1.4. ((S)-2-(4-((1S,3R)-2-amino-3-hydroxybutanamido)ethyl)-1H-1,2,3-triazol-1-yl)propanoyl)-L-valine (12). The reaction was carried out following the general CuAAC reaction conditions, using azide 3 (6.1 mg, 28.5 μmol) and alkyne 6 (8.1 mg, 28.5 μmol). White sticky solid (3.3 mg, 30%). \(^{1}\)H NMR (400 MHz, DMSO-d6) \(\delta\) 0.85 (dd, \(J = 12.3, 6.8\) Hz, 6H), 1.10 (d, \(J = 4.4\) Hz, 3H), 1.46 (d, \(J = 6.9\) Hz, 3H), 1.64 (d, \(J = 7.1\) Hz, 3H), 2.08 (h, \(J = 6.8\) Hz, 1H), 3.47 (d, \(J = 6.8\) Hz, 1H), 3.84 (p, \(J = 6.4\) Hz, 1H), 4.12 (dd, \(J = 8.5, 5.5\) Hz, 1H), 5.11 (p, \(J = 7.1\) Hz, 1H), 5.56 (q, \(J = 7.1\) Hz, 1H), 8.04 (s, 1H), 8.53 (d, \(J = 8.5\) Hz, 1H), 8.83 (d, \(J = 8.1\) Hz, 1H). \(^{13}\)C NMR (101 MHz, DMSO-d6) \(\delta\) 17.80, 17.96, 19.04,
19.94, 20.41, 29.83, 41.17, 57.39, 57.68, 58.50, 65.97, 120.91, 148.24, 166.41, 168.86, 172.56. MS (ESI) m/z: 385.3 [M+H]+.

3.1.5. **(1-((S)-1-carboxy-2-methylpropyl)-1H-1,2,3-triazole-4-carbonyl)-L-valine (13).** The reaction was carried out following the general CuAAC reaction conditions, using azide 1 (1.43 mg, 10 μmol) and alkyne 7 (1.69 mg, 10 μmol). White solid (1.58 mg, 51%). $^1$H NMR (600 MHz, D$_2$O) δ 0.91 (d, J = 6.7 Hz, 3H), 1.02 (d, J = 6.7 Hz, 3H), 1.06 (dd, J = 6.8, 4.8 Hz, 6H), 2.33 (h, J = 6.7 Hz, 1H), 2.62 (h, J = 6.9 Hz, 1H), 4.45 (d, J = 5.7 Hz, 1H), 5.07 (d, J = 8.0 Hz, 1H), 8.62 (s, 1H). $^{13}$C NMR (151 MHz, D$_2$O) δ 17.32, 17.76, 18.66, 18.68, 30.38, 31.00, 59.70, 72.66, 126.91, 141.51, 161.93 (COOH carbons not observed). MS (ESI) m/z: 313.2 [M+H]+.

3.1.6. **(1-((2R,3R)-2-amino-3-hydroxybutyl)-1H-1,2,3-triazole-4-carbonyl)-L-valine (14).** The reaction was carried out following the general CuAAC reaction conditions, using azide 2 (8.8 mg, 36 μmol) and alkyne 4 (6.1 mg, 36 μmol). White sticky solid (10.7 mg, 99%). $^1$H NMR (400 MHz, DMSO-d$_6$) δ 0.94 (d, J = 6.7 Hz, 6H), 1.22 (d, J = 6.3 Hz, 3H), 2.23 (h, J = 6.7 Hz, 1H), 3.51 – 3.58 (m, 1H), 3.74 (p, J = 6.2 Hz, 1H), 4.35 (dd, J = 8.4, 6.0 Hz, 1H), 4.62 (dd, J = 14.6, 7.4 Hz, 1H), 4.71 (dd, J = 14.7, 4.9 Hz, 1H), 5.62 (s, 1H), 8.05 (d, J = 8.5 Hz, 1H), 8.65 (s, 1H). $^{13}$C NMR (101 MHz, DMSO-d$_6$) δ 18.19, 19.16, 19.77, 29.87, 49.36, 55.43, 57.06, 63.94, 127.94, 142.23, 159.47, 172.63. MS (ESI) m/z: 300.1 [M+H]+.

3.1.7. **(1-((S)-1-((S)-1-carboxy-2-methylpropyl)amino)-1-oxopropan-2-yl)-1H-1,2,3-triazole-4-carbonyl)-L-valine (15).** The reaction was carried out following the general CuAAC reaction conditions, using azide 3 (4.9 mg, 23 μmol) and alkyne 7 (3.9 mg, 23 μmol). White solid (5.6 mg, 64%). $^1$H NMR (400 MHz, DMSO-d$_6$) δ 0.86 (dd, J = 12.8, 6.8 Hz, 6H), 0.93 (d, J = 6.8 Hz, 6H), 1.72 (d, J = 7.1 Hz, 3H), 2.03 – 2.13 (m, 1H), 2.16 – 2.26 (m, 1H), 4.14 (dd, J = 8.5, 5.5 Hz, 1H), 4.32 (dd, J = 8.5, 5.8 Hz, 1H), 5.64 (q, J = 7.1 Hz, 1H), 8.02 (d, J = 8.5 Hz, 1H), 8.60 (d, J = 8.5 Hz, 1H), 8.73 (s, 1H). $^{13}$C NMR (101 MHz, DMSO-d$_6$) δ 17.72, 17.79, 18.20, 19.02, 19.19, 29.89, 29.94, 57.19, 57.42, 58.13, 125.93, 141.80, 159.52, 168.56, 172.52, 172.80. MS (ESI) m/z: 384.2 [M+H]+.

3.1.8. **(1-((S)-2-((2S,3R)-2-amino-3-hydroxybutanamido)propyl)-1H-1,2,3-triazole-4-carbonyl)-L-valine (16).** The reaction was carried out following the general CuAAC reaction conditions, using azide 4 (3.31 mg, 10.5 μmol) and alkyne 7 (1.78 mg, 10.5 μmol). Reddish solid (1.30 mg, 33%). $^1$H NMR (600 MHz, D$_2$O) δ 1.02 (dd, J = 11.6, 6.9 Hz, 6H), 1.08 (d, J = 6.4 Hz, 3H), 1.34 (d, J = 6.1 Hz, 3H), 2.28 (dq, J = 13.3, 6.7 Hz, 1H), 3.67 (d, J = 6.0 Hz, 1H), 3.97 (p, J = 6.4 Hz, 1H), 4.34 (d, J = 5.6 Hz, 1H), 4.51 – 4.60 (m, 2H), 4.74 (t, J = 9.4 Hz, 1H), 8.51 (s, 1H). $^{13}$C NMR (151 MHz, D$_2$O) δ 16.88, 17.24, 18.63, 18.93, 30.75, 46.02, 54.22, 58.95, 60.60, 66.29, 127.65, 141.98, 161.31, 168.53, 178.21. MS (ESI) m/z: 371.3 [M+H]+.

3.1.9. **(2S)-2-(4-((2S,3R)-2-amino-1,3-dihydroxybutyl)-1H-1,2,3-triazol-1-yl)-3-methylbutanoic acid (17).** The reaction was carried out following the general CuAAC reaction conditions, using azide 1
(3.01 mg, 21 μmol) and alkyne 8 (5.1 mg, 21 μmol). White solid (3.1 mg, 54%). \( ^1H \) NMR (600 MHz, DMSO-\(d_6\)) \( \delta \) 0.91 (d, 3H), 1.02 (d, 3H), 1.32 (d, 3H), 2.65 (m, 1H), 3.60 (q, 1H), 3.96 (m, 1H), 5.21 (d, 1H), 5.22 (s, 1H), 8.27 (s, 1H). \( ^13C \) NMR (151 MHz, DMSO-\(d_6\)) \( \delta \) 17.63, 18.45, 19.26, 29.59, 30.93, 60.58, 63.18, 63.94, 70.41, 124.48, 146.45, 172.61. MS (ESI) \( m/z \): 273.2 [M+H]+.

3.1.10.  ((2S)-2-(4-((2S,3R)-2-amino-1,3-dihydroxybutyl)-1H-1,2,3-triazol-1-yl)propanoyl)-L-valine (18). The reaction was carried out following the general CuAAC reaction conditions, using azide 3 (4.6 mg, 21 μmol) and alkyne 8 (5.2 mg, 21 μmol). White solid (3.5 mg, 49%). \( ^1H \) NMR (400 MHz, DMSO-\(d_6\)) \( \delta \) 0.85 (dd, \( J = 10.8, 6.8 \) Hz, 6H), 1.12 (d, \( J = 6.5 \) Hz, 3H), 1.67 (d, \( J = 7.1 \) Hz, 3H), 2.08 (h, \( J = 6.7 \) Hz, 1H), 3.20 – 3.25 (m, 1H), 3.63 (dt, \( J = 10.7, 5.3 \) Hz, 1H), 4.11 (dd, \( J = 8.5, 5.4 \) Hz, 1H), 4.88 (d, \( J = 7.3 \) Hz, 1H), 5.58 (q, \( J = 7.1 \) Hz, 1H), 8.17 (s, 1H), 8.47 (d, \( J = 8.5 \) Hz, 1H). \( ^13C \) NMR (101 MHz, DMSO-\(d_6\)) \( \delta \) 17.84, 18.01, 19.09, 20.57, 29.90, 57.54, 57.81, 59.97, 63.06, 63.41, 122.61, 147.14, 168.73. MS (ESI) \( m/z \): 344.2 [M+H]+.

**FLUORESCENCE POLARIZATION ASSAY**

PDZ1 (61-151), PDZ2 (155-249), and PDZ3 (309-401) from PSD-95 (numbers in parenthesis refer to the residue numbers in the human full-length PSD-95α without exon 4b), containing an N-terminal His-tag sequence (MHHHHHPGRGS), were expressed in *E. coli* (BL21-DE3, pLysS) and purified using a nickel(II)-charged HisTrap column (GE Healthcare Life Sciences, Uppsala, Sweden) followed by anion-exchange chromatography or gel-filtration as described previously.\(^4\) Control peptides (TAV and SAV) and fluorescent labelled peptide probes (Cy5-GluN2B and Cy5-CRIPT) were synthesized by coupling Cy5-maleimide to the cysteine side chain of the peptide sequences CSG-YEKLSSIESDV and CSG-LDTKNYKQTSV, respectively, in solution, as previously described.\(^4\) The fluorescence polarization assay was also done as previously:\(^4\) First, saturation binding experiments were performed for measuring binding affinity (\( K_d \)) between Cy5-labelled probes and PDZ domain proteins by applying an increasing amount of PDZ domain (0-60 μM) to a fixed and low concentration of probe (50 nM). Cy5-GluN2B was used for PDZ1 and PDZ2, and Cy5-CRIPT for PDZ3. The assay was performed in 1×PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM phosphate, pH 7.4) using black flat-bottom 384-well plates (Corning Life Sciences, NY) and a Safire2 plate-reader (Tecan, Mannedorf, Switzerland). Cy5-probes were measured at excitation/emission values of 635/670 nm (bandwidth = 15 nm). The FP values were fitted to the equation \( Y = B_{max} \times X/(K_d + X) \), with \( B_{max} \) being the maximal FP value, X is the PDZ concentration, and Y is the experimental FP values. Secondly, to measure inhibitory activity of test substances and control compounds (9-18, TAV, SAV, Indole 1-2), heterologous competition binding assays were performed by adding increasing concentrations of test substance to a fixed concentration of probe and protein under the same conditions as in the saturation binding experiments. FP values were fitted to the general equation: \( Y = \text{Bottom} + (\text{Top}–\text{Bottom})/[1 + (10(X-\log IC_{50})\text{HillSlope})] \), where X is the logarithmic value of compound concentration and Y is the experimental FP values. Competitive inhibition constants, \( K_i \) values, were calculated from the IC_{50}
values to quantify affinities between compound and PDZ domain. All stock solutions were prepared using 1×PBS in deuterated water (9-18, TAV, SAV) or 10×PBS in deuterated water (Indole 1-2), and 1:1 dilutions series were performed in 1xPBS.

**ISOTHERMAL TITRATION CALORIMETRY (ITC).**

PSD-95 PDZ2 was buffer exchanged in deuterated PBS (0.01 M phosphate, 0.0027 M potassium chloride and 0.137 M sodium chloride) using Amicon Ultra Centrifugal Filters (Sigma-Aldrich) with a molecular weight cut-off of 10 kDa. The filtered buffer was used for solubilizing TAV and for sample dilution during TAV measurements. Protein and ligand stocks for ITC experiments of 10 were dialyzed against the same PBS-D₂O buffer for 3 hours at room temperature (Pur-A-Lyzer dialysis kit, 3.5 kDa cut-off, Sigma-Aldrich). Ligand and protein stocks were pH adjusted to 7.5 within 0.02 pH units. Ligand concentrations were determined by qNMR. Protein concentration was determined by UV absorption at 280 nm based on extinction coefficient of 7771 M⁻¹cm⁻¹ previously determined by amino acid analysis. ITC experiments were performed on a microcalorimeter (ITC200, Microcal, MA, USA) at 25 °C by titrating the ligand (TAV at 2305 μM and 10 at 5140 μM) into the PSD-95 PDZ2 solution (35-50 μM) at 180 s injection intervals and stirring speed of 1000 rpm. TAV was titrated into PDZ2 as 20 × 2 μL injections, and 10 as 20×2 μL, 12×3.2 μL or 15×2.5 μL injections. Heats of dilution were determined by titration of ligand into buffer and buffer into protein, but these were not subtracted from measured ligand-protein heats to avoid introducing errors by subtracting a relative large background to a small signal as especially seen for 10. ORIGIN 7.0 (Microcal, MA, USA) was used to determine the thermodynamic properties of ligand binding using the one-site model. Stoichiometry (N) was fixed to 1. The first injection was not considered during curve fitting as this is affected by titrant diffusion from syringe tip during initial equilibration.

**MOLECULAR MODELLING AND DOCKING**

Docking of compounds into PDZ2 was performed as previously described, using the Maestro platform (version 9.9, Schrödinger, LLC, New York, NY, USA). PDZ2 was aligned with PDZ3 using Prime (Version 1.6), and a homology model was created using the PDZ3 X-ray crystal structure (PDB ID: 1BE9) as template in Prime with standard parameters. The peptide ligand from 1BE9, KQTSV, was rebuilt to IESDV in the homology model. The side chains of PDZ2 and peptide ligand were minimized in Macromodel (Version 9.5, Schrödinger, LLC) using force field OPLS2005 and backbone constraining. A 20 Å grid centred at His225 was generated in Glide (Version 4.5, Schrödinger, LLC) and used for docking. Compounds were prepared and minimized using LigPrep and standard parameters followed by flexible docking in Glide using default parameters and post docking minimization. Poses were ranked according to their G-score, and top 10 poses were evaluated by manual inspection. The conserved water molecule seen in the binding pocket for PDZ3 (1BE9) was maintained in the PDZ2
model during grid preparation, minimization, and docking. The PyMOL Molecular Graphics System (Version 1.3 Schrödinger, LLC) was used for creating figures.
REFERENCES