Electronic Supplementary Material (ESI) for RSC Medicinal Chemistry.

ELECTRONIC SUPPLEMENTARY INFORMATION

Discovery, affinity maturation and multimerization of small molecule ligands against human Tyrosinase and Tyrosinase Related Protein 1

Marco Catalano,† Gabriele Bassi,† Giulia Rotondi, Lyna Khettabi, Maria Dichiara, Patrizia Murer, Jörg Scheuermann, Montserrat Soler-Lopez, and Dario Neri*

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General remarks

High-Resolution Mass Spectrometry (HRMS) spectra and analytical Reversed-Phase Ultra Performance Liquid Chromatography (UPLC) were recorded on a Waters Xevo G2-XS QTOF coupled to a Waters Acquity UPLC H-Class System with PDA UV detector, using an ACQUITY UPLC BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm × 50 mm at a flow rate of 0.6 ml/min with linear gradients of solvents A and B (A = Millipore water with 0.1% FA, B = MeCN with 0.1% FA). Preparative reversed-phase medium-pressure liquid chromatography (RP-MPLC) were performed on a Büchi Sepacore system supported by two pump modules C-601 and with a PDA UV detector, flash cartridge (FlashPure ID C18, 12g) at a flow rate of 30 mL/min with different gradients of solvents A and B (A = Millipore water with 0.1% TFA, B = MeCN with 0.1% TFA). Preparative reversed-phase high-pressure liquid chromatography (RP-HPLC) were performed on a Waters Alliance HT RP-HPLC with PDA UV detector, using a Synergi 4µm, Polar-RP 80Å 10 × 150 mm C18 column at a flow rate of 4 ml/min with linear gradients of solvents A and B (A = Millipore water with 0.1% TFA, B = MeCN with 0.1% TFA). Preparative reversed-phase high-pressure liquid chromatography (HPLC) for the LNA conjugates was performed on a Waters Alliance HT RP-HPLC with PDA UV detector, using a Synergi 4 µm, Polar-RP 150 × 10 mm C18 column using a gradient of eluent A (TEAA 100 mM) and eluent B (TEAA 100 mM in 80% ACN). Proton (^{1}H) nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AV400 (400 MHz). Carbon (^{13}C) NMR spectra were recorded on a Bruker AV400 (100 MHz) spectrometer. Shifts are given in ppm using residual solvent as the internal standard. Coupling constants (*J*) are reported in Hz with the following abbreviations used to indicate splitting: s = singlet, d = doublet, t = triplet, dd = doublet of doublets.

Reagents

All compounds and chemical reagents were obtained from Sigma-Aldrich, TCI Europe, Enamine or ABCR, and used without further purification. Peptide grade N,N-dimethylformamide (DMF) for solid phase synthesis was bought from ABCR. Complementary Locked Nucleic Acid (LNA) and DNA starting materials were purchased from LGC Biosearch Technologies. H-Rink amide ChemMatrix® resin was purchased from Sigma Aldrich (Cat. Nr. 727768-5G). Fmoc-Lys(Boc)-Wang resin, 200-400mesh was purchased from Bachem (Cat. Nr. 4003241.0005). Fmoc-Cys(Trt)-Wang resin (100-200 mesh, 0.40-1.00 mmol/g) was purchased from Bachem (Cat. Nr. 4028211.0001). 4-(2-aminothiazol-4-yl)benzene-1,3-diol was purchased from Fluorochem (Cat. Nr. 495142-1g).

Protein expression

Overexpression and purification of the human intramelanosomal domain of hTYR (residues 19 to 456) and hTYRP1 (residues 25 to 471) respectively were done according to already published procedures.[1,2] A final purification step by size exclusion chromatography was carried out using a Superdex 200 10/300 GL column (GE Healthcare) equilibrated with buffer 10 mM Tris-HCl, pH 7.8, 100 mM NaCl. The 0.5 mL elution fractions containing the monomeric pure proteins as shown in **Figure 1b** were subsequently pooled and concentrated using a 30 kDa cut-off Amicon membrane (Merck Millipore, Burlington, Massachusetts, U.S.A.) to a concentration of ≈7.5 mg/mL for hTYRP1 and ≈3 mg/ml for hTYR, and stored at 193 K.

Selections

Affinity selections were performed with both single (ss) and double stranded (ds) libraries with 10^7 copies of individual library members for each selection.[3] Selections against hTYR/hTYRP1 was performed in duplicate. The ss-library and ds-library were diluted to 110 nM in protein-specific buffer, containing also 0.05% tween-20 and 20 *μ*g/mL herring sperm DNA (100 μL). The selections against immobilized protein targets were performed with the automated system King Fisher (Thermo Fisher) as reported by Decurtins et al.[4]

Enzymatic assay

The enzymatic activity of Tyrosinase was measured at 25 °C following already described procedures using L-DOPA as substrate.[5–7] In 384-well microtiter clear plates (Greiner non-binding), a mixture of 1 mM L-DOPA, 4 mM MBTH, 200 nM human Tyrosinase and the inhibitor was prepared (final volume 30 μL in PBS 1% DMSO, pH=6.8). The increase of the absorption at 490-510 nm, due to the formation of the L-dopaquinone-MBTH complex, was recorded using a Spectra Max Paradigm multimode plate reader (Molecular Devices) over 10 minutes. Experiments were performed in duplicates and the values were fitted using Graphpad Prism software. The obtained OD values were plotted as a function of time. Linear regression of the plotted points was performed. IC50 values were calculated fitting the obtained slope as function of the inhibitor concentration.

Fluorescence polarization

The dilution series were done in a non-binding black 384-well microplate (Greiner Bio On.). In a final volume of 30 μL, 50 nM of the fluorescein-conjugated ligand was

incubated with a 1:1 dilution series of hTYRP1 in PBS pH 7.4 The fluorescence anisotropy was measured on a Spectra Max Paradigm multimode plate reader (Molecular Devices). Experiments were performed in triplicate and the anisotropy values fitted using Graphpad Prism software.

Fluorescence polarization on-DNA

From 1 μM solutions, the A12-LNA and the B12-DNA conjugated complementary strands were annealed in a 1:1.5 ratio in presence of PBS (pH 7.4). The annealing was performed at 65 °C for 4 minutes and then left cooling down at RT for ~0.5 hour. The dilution series were done in a non-binding black 384-well microplate (Greiner Bio On.). In a final volume of 30 μL, 50 nM of the fluorophore-labelled double stranded LNA were incubated with a 1:1 dilution series of hTYRP1 in PBS pH 7.4 The fluorescence anisotropy was measured on a Spectra Max Paradigm multimode plate reader (Molecular Devices). Experiments were performed in triplicate and the anisotropy values fitted using Graphpad Prism software.

ELISA

The Enzyme-Linked ImmunoSorbent Assay (ELISA) was performed following already described procedures.[8] Nunc MaxiSorp™ ELISA stripes (ThermoFisher) were incubated over night at 4 °C with 100 μL of 100 nM solution in PBS of protein (i.e., hTYR and hTYRP1). The supernatant was removed and 200 μL of a 4% solution of Milk powder in PBS (M-PBS) was used as blocking agent for 30-45 minutes. Wells were then washed three times with 200 μL PBS and dried. 50 μL of a pre-made serial dilution of the fluorescein-ligand conjugate was added and incubated for 45 minutes in the dark. Carefully, the supernatant was removed and the wells were quickly

washed twice with 200 µL PBS. 100 µL of anti-fluorescein IgG (200 nM) in 2% M-PBS were added and incubated for 45 minutes. The supernatant was removed and wells washed three times with 200 µL PBS. 100 µL of a solution of 1:1000 diluted enzyme-linked protein (i.e., proteinA-HRP) in 2% M-PBS was added and incubated for 45 minutes in the dark. The supernatant was removed and wells washed three times with 200 µL of a 0.1% solution of Tween 20 in PBS and three times with PBS. Finally, 60 µL of 3,3',5,5'-tetramethylbenzi- dine (TMB) was added to each well and left for 10 to 50 seconds at room temperature developing in the dark until formation of a visible intense blue. The reaction was then quenched upon addition of 30 µL of 1 M $H_2SO_{4(aq)}$. Absorbance at 450 nm and 620 nm were measured with Spectra Max Paradigm multimode plate reader (Molecular Devices) and values were fitted using Graphpad Prism software.

Cell culture

B16F10 melanoma cell line was kindly provided by the group of Michael Detmar (Swiss Federal Institute of Technology, Zurich, Switzerland). Cells were grown according to the manufacturer's protocol.

Flow Cytometry

Adherent B16F10 cells cultured in T-150 flasks were detached with 2 mM EDTA. Resuspended cells were counted, centrifuged, and resuspended in FACS buffer (2 mM EDTA + 0.5% BSA in PBS, filtered solution) to reach a concentration of 2.5 mio/mL. In 96-well plate (Greiner Bio On.), 500.000 cells were incubated with 10 µM solutions of fluorescein linked ligands in FACS buffer. After 60 minutes, 1x wash was performed and then cells were incubated with Cy5-conjugated anti-Fluorescein IgG

(100 nM) for 60 minutes. Cells were 2x washed and sorted by FACS (CytoFLEX, BeckmanCoulter, Brea, CA) and analyzed using FlowJo software (FlowJo,Ashland, OR).

Fluorescence polarization assay A26-B389

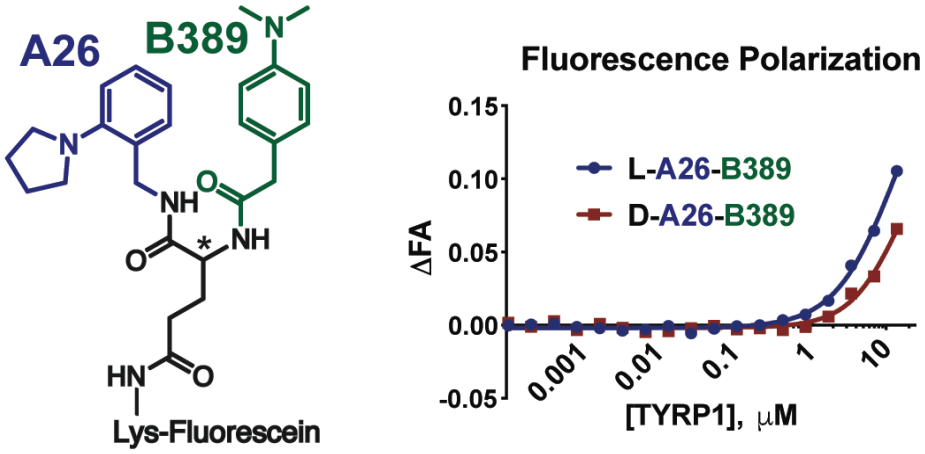


Figure S1. Fluorescence polarization binding assay of Fl-Lys-L-Gln-A26-B389 and Fl-Lys-D-Gln-A26-B389 in presence of hTYRP1. The complete structure and synthetic procedures are described below.

hTYR-based enzymatic assays

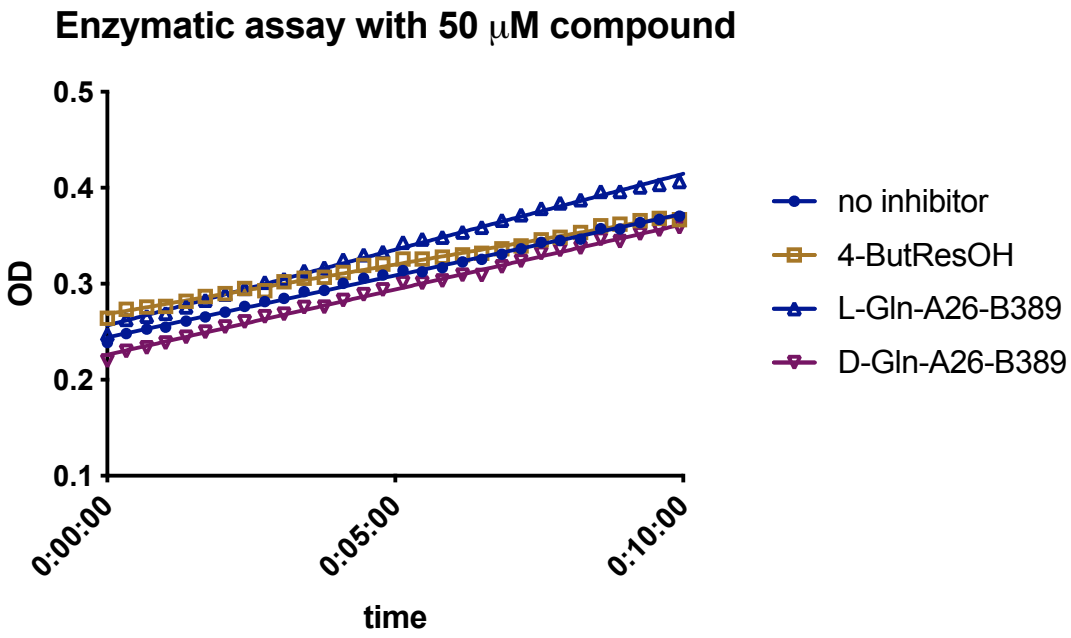


Figure S2. The enzymatic activity of human-TYR was measured with or without inhibitors. 4-butylresorcinol was used as positive control. Inhibitors were incubated at a fixed concentration (50 μM) at pH 6.8.

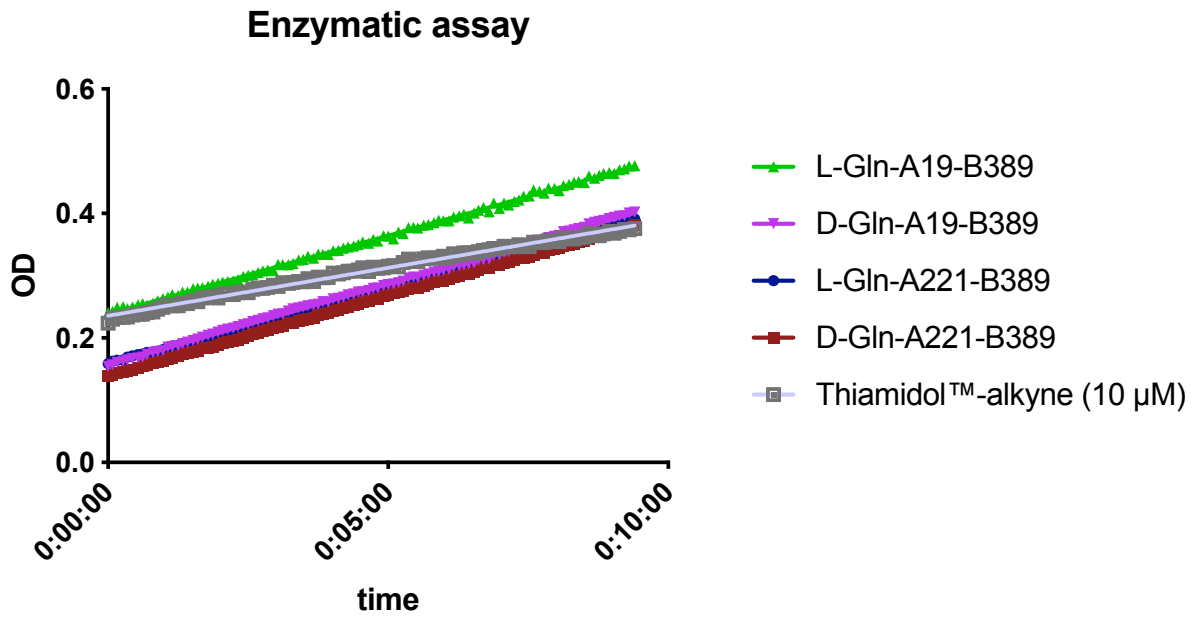


Figure S3. The enzymatic activity of human-TYR was measured in presence of different compounds. Thiamidol™-alkyne was used as positive control. Compounds were incubated at a fixed concentration (50 μM, Thiamidol™-alkyne only 10 μM) at pH 6.8.

Concentration Inhibitors

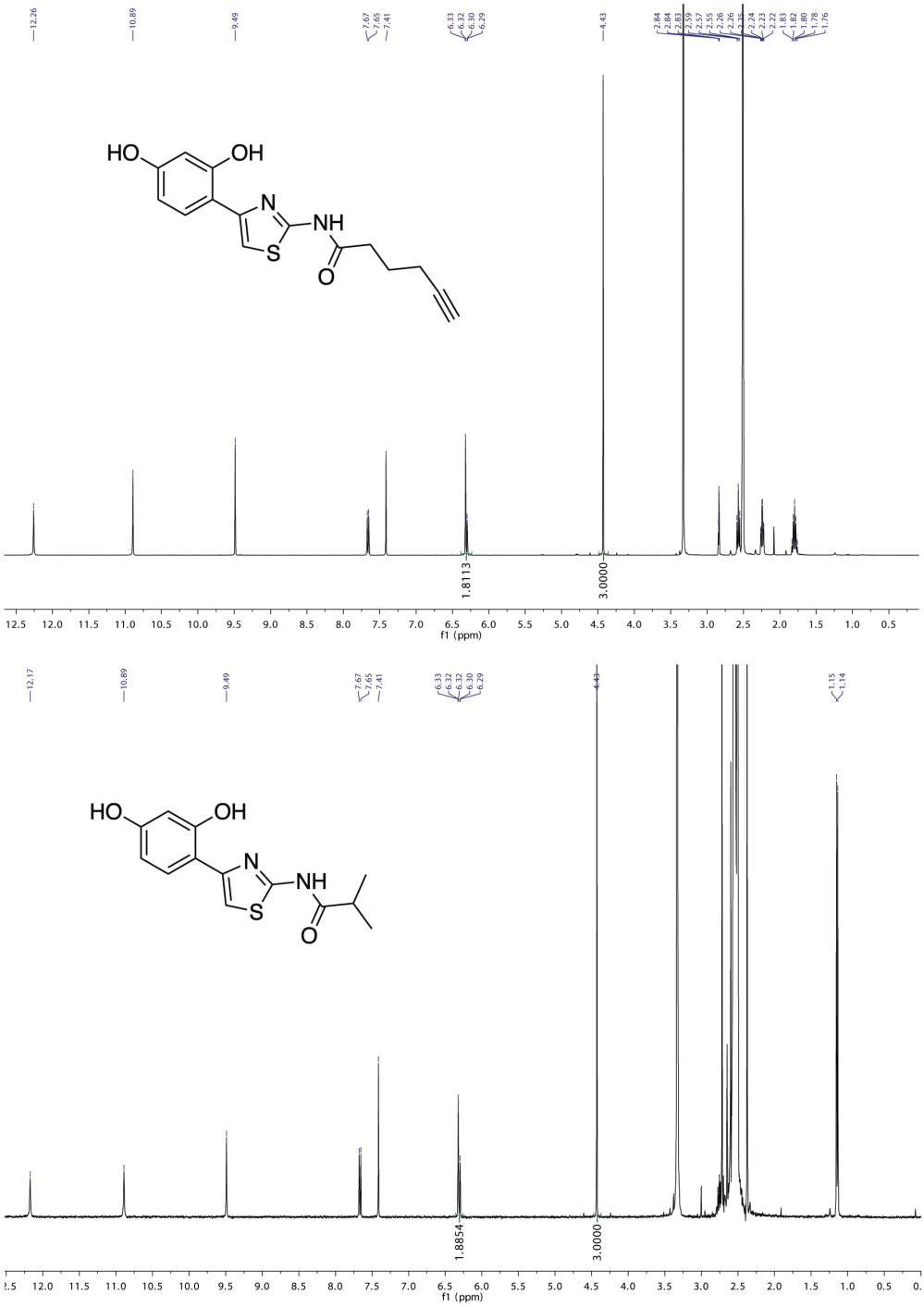


Figure S4. Concentration of Thiamidol™ and Thiamidol™-alkyne was measured *via* NMR in presence of a known concentration of nitromethane (CH_3NO_2, δ 4.43, 3H).

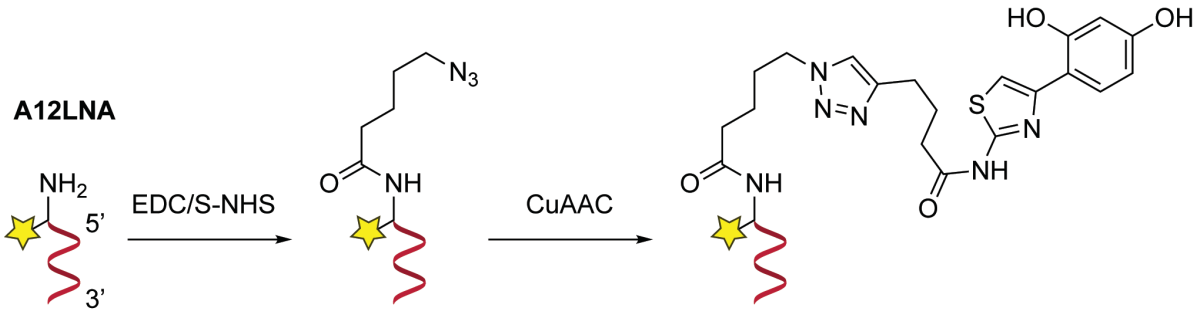
Synthesis on-DNA

Hits compounds selected from affinity maturation 2+1 selections were synthetized and validated on DNA-LNA hybrid. Two complementary oligonucleotides were used:

5' – GGA TGG CTA CTA - 3' AmMC6 (B12-DNA)

3' – CCT ACC GAT GA(dT Fluorescein) - 5' AmMC6 (A12-LNA)

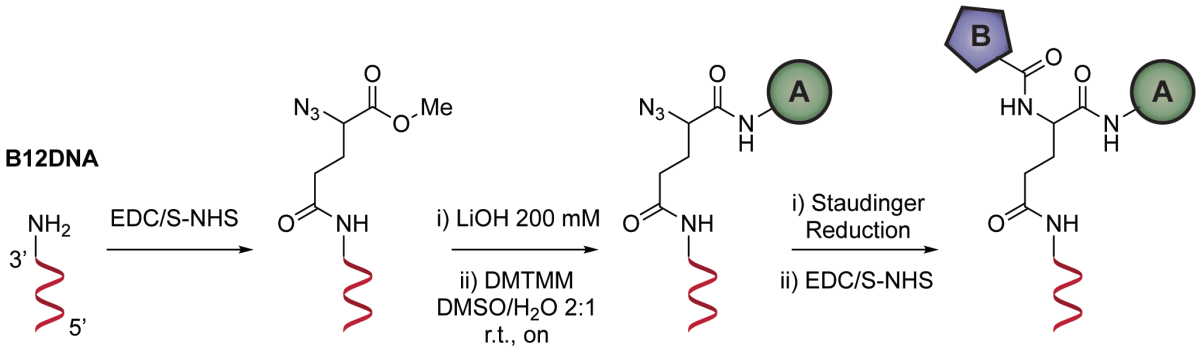
A12LNA synthetic procedure



on-DNA amide bond formation: in a 1.5 mL Eppendorf tube 42 µL (100 mM DMSO, 85eq.) of the desired carboxylic acid were mixed for 30 minutes with 90 µL DMSO, 20 µL (S-NHS 100 mM DMSO/H_2O 2:1, 40eq.), 42 µL (EDC 100 mM DMSO, 84 eq.). In a separate well 50 µL DNA (1 nmol/µL, 1eq.) in H_2O were mixed with 50 µL of TEA buffer (100 mM, pH=10). The activated carboxylic acid solution was added to the oligo solution and stirred at room temperature over-night. The reacted DNA was precipitated by adding 30 µL (NaCl 5 M) and 30 µL (AcOH/$NaAcO^-$ 3 M, pH= 4.7) followed by 900 µL EtOH absolute. The precipitated DNA was kept at -20°C for 4hours and centrifuged a 4°C for 30mins at 16.1 krpm. The supernatant was discarded and the pellet dried under vacuum.

on-DNA CuAAC click reaction: to 2.5 µL DNA (0.3 nmol), 30 µL (Borate buffer pH= 9.5) followed by 2.5 µL $CuSO_4$ (5 mM in H_2O, 42 eq) and 10 µL of the desired alkyne (10 mM in DMSO, 100 eq) were added. The reaction was then started by the addition of 2.5 µL sodium ascorbate (5 mM in H_2O, 42 eq) and stirred at room temperature overnight. The reacted DNA was precipitated by adding 12 µL 5 M NaCl and 240 µL EtOH. The precipitated DNA was kept at -20°C for 4 hours and centrifuged a 4°C for 30 mins at 16.1 krpm. The supernatant was discarded and the pellet dried under vacuum.

B12DNA synthetic procedure



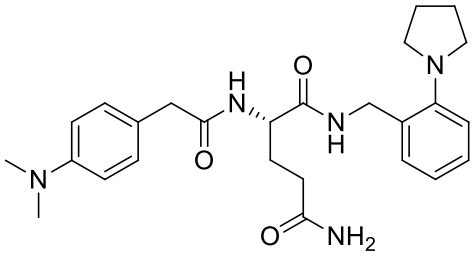
On-DNA methylester deprotection: In a 2 mL Eppendorf tube, DNA (50 nmol) was dissolved in 250 µL H_2O, 250 µL LiOH (200 mM) were added and the resulting mixture was stirred at 25°C for 4 h. The reaction was monitored by LC-MS. After the reaction was completed, LiOH was quenched by adding 16 µL AcOH (3 M). The reacted DNA was precipitated by adding 50 µL 5 M NaCl and 1375 µL EtOH. The precipitated DNA was kept at -20°C for 4 hours and centrifuged at 4°C for 30 mins at 16.1 krpm. The supernatant was discarded and the pellet dried in a speed vacuum machine.

on-DNA Reverse amide bond reactions (acid attached on-DNA): in a 2 mL Eppendorf tube DNA (50 nmol, 1 eq) was dissolved in 40 µL H_2O. 137 µL of MOPS buffer (100 mM MOPS, 1 M NaCl, pH= 7.0) was added, followed by 30 µL of DMT-MM (500 mM in MOPS buffer, 300eq). The resulting mixture was stirred at 30 °C for 30 min. 75 µL (100 mM, 150 eq) of the desired amine was added to the activation solution and stirred at 30°C for 16 hours. The reacted DNA was precipitated by adding 28 µL 5 M NaCl and 775 µL EtOH. The precipitated DNA was kept at -20°C for 4hours and centrifuged at 4°C for 30mins at 16.1 krpm. The supernatant was discarded and the pellet dried in a speed vacuum machine.

on-DNA Staudinger reduction: in a 2 mL Eppendorf tube DNA (1-100 nmol,1 eq) was dissolved in 100 µL of TCEP buffer (30 mM TCEP, in 500 mM TRIS*HCl, pH= 7.4). The resulting mixture was stirred at 30 °C for 4 hours. Reaction completion was monitored by LC-MS. The reacted DNA was precipitated by adding 25 µL 5 M NaCl and 375 µL EtOH. The precipitated DNA was kept at -20°C for 4hours and centrifuged a 4°C for 30 mins at 16.1 krpm. The supernatant was discarded and the pellet dried under vacuum.

Synthetic procedures

L-Gln-A26-B389

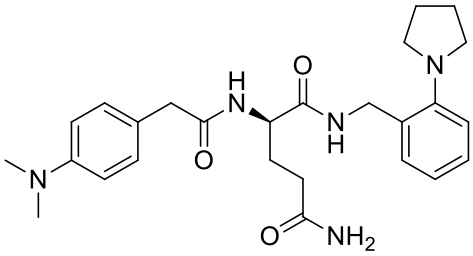


(*S*)-2-(2-(4-(dimethylamino)phenyl)acetamido)-N1-(2-(pyrrolidin-1-yl)benzyl)pentanediamide: commercially available Rink amide resin (0.2048 g, 0.1 mmol) was swollen in *N,N*-dimethylformamide (20 mL) for 2 h in a syringe provided with a filter pad. A solution of Fmoc-L-glutamic acid 5-allyl ester (4.0 equiv., 0.4 mmol), DIPEA (4.0 equiv., 0.4 mmol) and HATU (4.0 equiv., 0.4 mmol) in *N,N*-dimethylformamide (4 mL) was added to the syringe, allowed to react for 16 h and washed with N,N-dimethylformamide (5 × 4 mL × 1 min). The O-allyl deprotection was performed using palladium-tetrakis(triphenylphosphine) ($Pd(PPh_3)_4$, 0.25 equiv., 0.025 mmol) and phenylsilane ($PhSiH_3$, 24 equiv., 2.4 mmol) in dichloromethane (4 mL) for 2 h. After washing with dichloromethane (5 × 4 mL × 1 min) and N,N-dimethylformamide (5 × 4 mL × 1 min), a solution of (2-(pyrrolidin-1-yl)phenyl)methanamine (4 equiv., 0.4 mmol), DIPEA (8 equiv., 0.8 mmol) and HATU (4.0 equiv., 0.4 mmol) in N,N-dimethylformamide (4 mL) was added to the syringe and allowed to react for 16 h and washed with *N,N*-dimethylformamide (5 × 4 mL × 1 min). The Fmoc group was removed using a solution of 20% piperidine in *N,N*-dimethylformamide for 20 min and then washed with *N,N*-dimethylformamide (5 × 4 mL × 1 min). The coupling to the 2-(4-(dimethylamino)phenyl)acetic (4 equiv., 0.4 mmol) was performed using the same conditions reported before. After washing with

N,*N*-dimethylformamide (5 × 4 mL × 1 min) the resin was cleaved using a solution of TFA:TIPS:mqH_2O (95:2.5:2.5, v/v) for 1 h at room temperature. The so-obtained solution was collected in a round-bottom flask, dried under vacuum and purified over RP HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 14 min). After lyophilization the final compound was collected as a white solid; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 8.25 (t, J = 5.6 Hz, 1H), 8.12 (d, J = 8.0 Hz, 1H), 7.31 (s, 1H), 7.22 – 7.14 (m, 2H), 7.12 (d, J = 8.7 Hz, 2H), 6.98 (d, J = 8.0 Hz, 1H), 6.89 (t, J = 7.4 Hz, 1H), 6.80 (s, 1H), 6.69 (dd, J = 9.2, 2.4 Hz, 2H), 4.31 (d, J = 5.7 Hz, 2H), 4.30 – 4.27 (m, 1H), 3.39 (s, 2H), 3.11 (td, J = 8.8, 2.6 Hz, 4H), 2.91 – 2.88 (m, 6H), 2.12 (ddd, J = 8.1, 6.2, 3.4 Hz, 2H), 1.99 – 1.93 (m, 1H), 1.93 – 1.88 (m, 4H), 1.88 – 1.75 (m, 1H). ^{13}C-NMR (101, MH Z, DMSO-d_6) δ 24.9 (2 × CH2), 28.5 (CH2), 32.0 (CH2), 39.8 (2 × CH3), 40.8 (CH2), 41.7 (CH2), 51.5 (2 × CH2), 52.9 (CH), 112.9 (2 × Ar), 116.8 (Ar), 120.8 (Ar), 124.3 (Ar), 127.7 (Ar), 128.7 (Ar), 129.8 (Ar), 130.0 (2 × Ar), 148.6 (Ar), 149.6 (Ar), 171.3 (CONH2), 171.9 (CONH), 174.1 (CONH).

HRMS (ES) calculated for $[M+H]^+$ (m/z): 466.2813, found 466.2308.

D-Gln-A26-B389

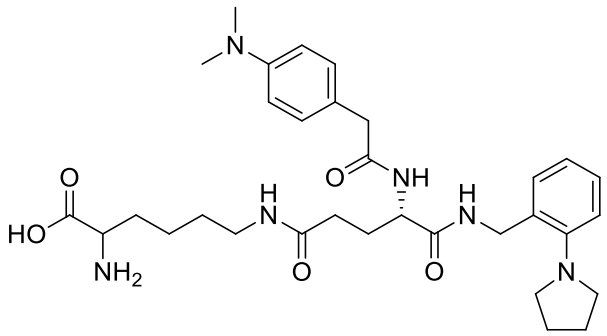


(*R*)-2-(2-(4-(dimethylamino)phenyl)acetamido)-*N*1-(2-(pyrrolidin-1-yl)benzyl)pentanediamide: commercially available Rink amide resin (0.2048 g, 0.1 mmol) was swollen in *N,N*-dimethylformamide (20 mL) for 2 h in a syringe provided with a filter pad. A solution of Fmoc-D-glutamic acid 5-allyl ester (4.0 equiv., 0.4 mmol), DIPEA (4.0 equiv., 0.4 mmol) and HATU (4.0 equiv., 0.4 mmol) in *N,N*-dimethylformamide (4 mL) was added to the syringe, allowed to react for 16 h and washed with *N,N*-dimethylformamide (5 × 4 mL × 1 min). The *O*-allyl deprotection was performed using palladium-tetrakis(triphenylphosphine) ($Pd(PPh_3)_4$, 0.25 equiv., 0.025 mmol) and phenylsilane ($PhSiH_3$, 24 equiv., 2.4 mmol) in dichloromethane (4 ml) for 2 h. After washing with dichloromethane (5 × 4 mL × 1 min) and *N,N*-dimethylformamide (5 × 4 mL × 1 min), a solution of (2-(pyrrolidin-1-yl)phenyl)methanamine (4 equiv., 0.4 mmol), DIPEA (8 equiv., 0.8 mmol) and HATU (4.0 equiv., 0.4 mmol) in *N,N*-dimethylformamide (4 mL) was added to the syringe and allowed to react for 16 h and washed with *N,N*-dimethylformamide (5 × 4 mL × 1 min). The Fmoc group was removed using a solution of 20% piperidine in *N,N*-dimethylformamide for 20 min and then washed with *N,N*-dimethylformamide (5 × 4 mL × 1 min). Thus, the coupling to the 2-(4-(dimethylamino)phenyl)acetic (4 equiv., 0.4 mmol) was performed using the same conditions reported before. After washing with *N,N*-dimethylformamide (5 × 4 mL × 1 min) the resin was cleaved using a solution

of TFA:TIPS:mqH_2O (95:2.5:2.5, *v/v*) for 1 h at room temperature. The so-obtained solution was collected in a round-bottom flask, dried under vacuum and purified over RP HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 14 min). After lyophilization the final compound was collected as a white solid; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.76-1.84 (m, 1H, CH_2), 1.89-1.92 (m, 4H, 2 × CH_2), 1.94-1.99 (m, 1H, CH_2), 2.10-2.15 (m, 2H, CH_2), 2.90 (6, 6H, 2 × CH_3), 3.08-3.14 (m, 4H, 2 × CH_2), 3.39 (s, 2H, CH_2), 4.27-4.29 (m, 1H, CH), 4.31 (d, J = 5.7 Hz, 2H, CH_2), 6.68-6.71 (m, 2H, Ar), 6.80 (s, 1H, $CONH_2$), 6.87-6.91 (m,1H, Ar), 6.97-6.99 (m, 1H, Ar), 7.12 (d, J = 8.7 Hz, 2H, Ar), 7.15-7.20 (m, 2H, Ar), 7.31(s, 1H, $CONH_2$), 8.12 (d, J = 8.0 Hz, 1H, CONH), 8.25 (t, J = 5.6 Hz, 1H, CONH); ^{13}C NMR (101, MH Z, DMSO-d_6) δ 24.91 (2 × CH_2), 28.55 (CH_2), 32.00 (CH_2), 39.77 (2 × CH_3), 40.79 (CH_2), 41.69 (CH_2), 51.55 (2 × CH_2), 52.91 (CH), 112.92 (2 × Ar), 116.78 (Ar), 120.85 (Ar), 124.34 (Ar), 127.70 (Ar), 128.74 (Ar), 129.77 (Ar), 129.96 (2 × Ar), 148.64 (Ar), 149.64 (Ar), 171.29 ($CONH_2$), 171.93 (CONH), 174.11 (CONH).

HRMS (ES) calculated for $[M+H]^+$ (*m/z*): 466.2813, found 466.2308.

Lys-L-Gln-A26-B389

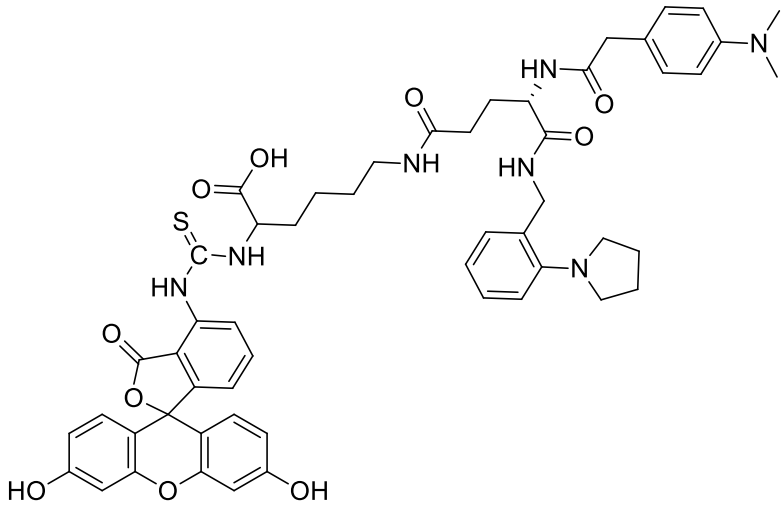


2-amino-6-((*S*)-4-(2-(4-(dimethylamino)phenyl)acetamido)-5-oxo-5-((2-(pyrrolidin-1-yl)benzyl)amino)pentanamido)hexanoic acid: commercially available Fmoc-Lys(Boc)-Wang resin (0.25 mmol) was swollen in *N,N*-dimethylformamide (10 mL) for 2 h inside a syringe provided with a filter pad. The Fmoc group was removed using a solution of 20% piperidine in *N,N*-dimethylformamide for 20 min and after washing the resin with *N,N*-dimethylformamide (5 × 10 mL × 1 min), a solution of Fmoc-L-glutamic acid 5-allyl ester (4.0 equiv., 1 mmol), DIPEA (8.0 equiv., 2 mmol) and HATU (4.0 equiv., 1 mmol) in *N,N*-dimethylformamide (10 mL) was added to the syringe, allowed to react for 16 h and washed with *N,N*-dimethylformamide (5 × 10 mL × 1 min). The *O*-allyl deprotection was performed using palladium-tetrakis(triphenylphosphine) ($Pd(PPh_3)_4$, 0.25 equiv., 0.0625 mmol) and phenylsilane ($PhSiH_3$, 24 equiv., 6 mmol) in dichloromethane (20 mL) for 2 h. After washing with dichloromethane (5 × 10 mL × 1 min) and *N,N*-dimethylformamide (5 × 10 mL × 1 min) a solution of the (2-(pyrrolidin-1-yl)phenyl)methanamine (4 equiv., 1 mmol), DIPEA (8 equiv., 2 mmol) and HATU (4.0 equiv., 2 mmol) in *N,N*-dimethylformamide (10 mL) was added to the syringe and allowed to react for 16 h. After washing with *N,N*-dimethylformamide (5 × 10 mL × 1 min) the Fmoc group was removed and the coupling to the 2-(4-

(dimethylamino)phenyl)acetic acid (4 equiv., 1.0 mmol) was performed using the same conditions reported before. After washing with *N,N*-dimethylformamide (5 × 10 mL × 1 min) the resin was cleaved using a solution of TFA:TIPS:mqH_2O (95:2.5:2.5, *v/v*) for 1 h at room temperature. The so-obtained solution was collected in a round-bottom flask, dried under vacuum and purified over RP-HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 100% over 14 min). After lyophilization the final compound was collected as a white solid.

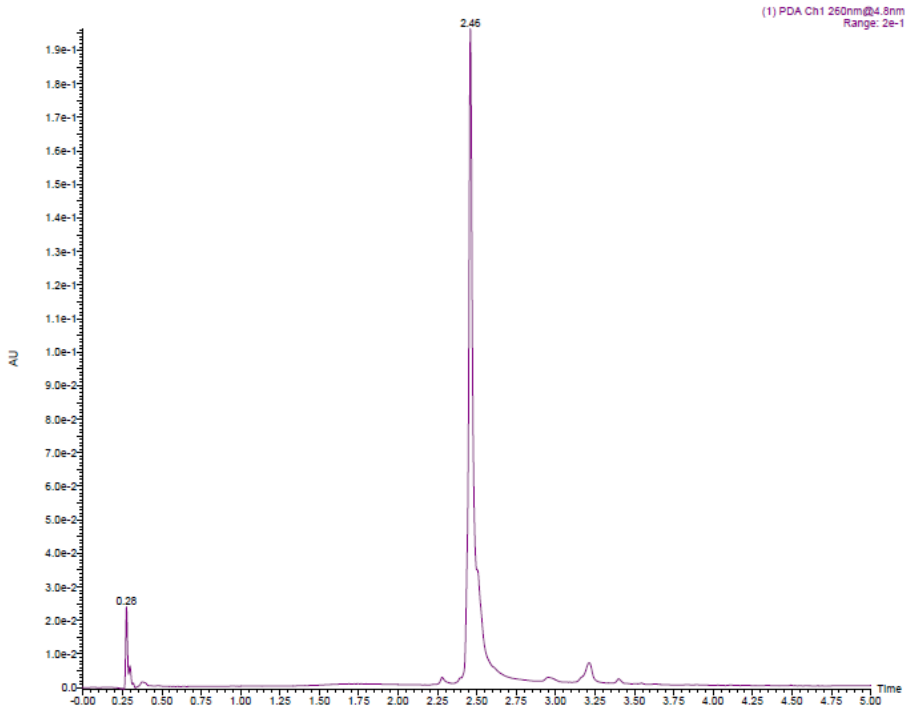
HRMS (ES) calculated for $[M+H]^+$ (*m/z*): 595.3603, found 595.3643.

Fl-Lys-L-Gln-A26-B389



To a solution of Lys-L-Gln-A26-B389 (2.8 mg, 1 equiv.) in dimethyl sulfoxide (100 µL) was added DIPEA (20 µL) and a solution of commercially available fluorescein isothiocyanate (3.7 mg, 2.0 equiv.) in dimethyl sulfoxide (100 µL). After stirring for 30 min in the dark, the reaction was diluted with a solution of DMSO:mqH_2O (1:1 *v*:*v*,) and purified over RP HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 100% over 14 min). The fractions containing the product, identified by mass spectrometry, were collected and then lyophilized to give the title compound as a yellow powder.

HRMS (ES) calculated for $[M+H]^+$ (*m/z*): 984.3961, found 984.3460.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5.0e-2
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3.0e-2
2.0e-2
1.0e-2
0.0
-0.00
0.25
0.50
0.75
1.00
1.25
1.50
1.75
2.00
2.25
2.50
2.75
3.00
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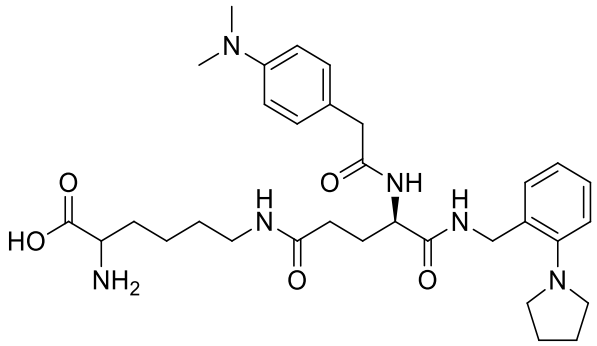
5.00

Lys-D-Gln-A26-B389



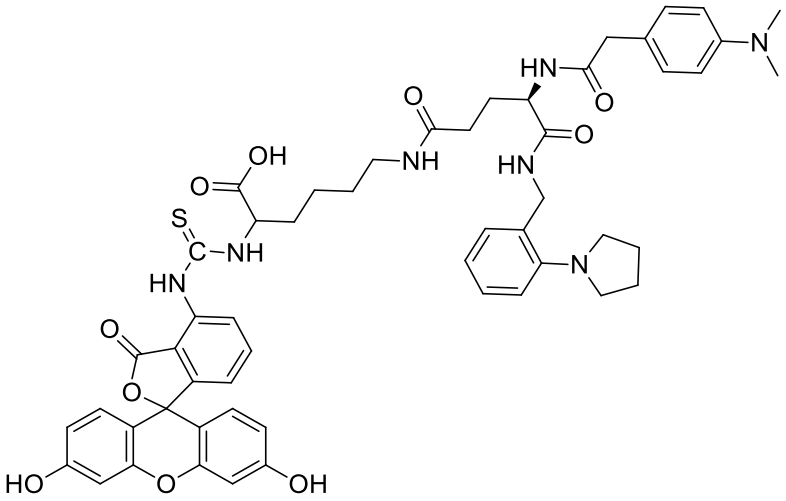
2-amino-6-((*R*)-4-(2-(4-(dimethylamino)phenyl)acetamido)-5-oxo-5-((2-(pyrrolidin-1-yl)benzyl)amino)pentanamido)hexanoic acid:

commercially available Fmoc-LysBoc-Wang resin (0.25 mmol) was swollen in *N,N*-dimethylformamide (10 mL) for 2 h inside a syringe provided with a filter pad. The Fmoc group was removed using a solution of 20% piperidine in *N,N*-dimethylformamide for 20 min and after washing the resin with *N,N*-dimethylformamide (5 × 10 mL × 1 min), a solution of Fmoc-D-glutamic acid 5-allyl ester (4.0 equiv., 1 mmol), DIPEA (8.0 equiv., 2 mmol) and HATU (4.0 equiv., 1 mmol) in *N,N*-dimethylformamide (10 mL) was prepared, added to the syringe, allowed to react for 16 h and washed with *N,N*-dimethylformamide (5 × 10 mL × 1 min). The *O*-allyl deprotection was performed using palladium-tetrakis(triphenylphosphine) ($Pd(PPh_3)_4$) (0.25 equiv., 0.0625 mmol) and phenylsilane ($PhSiH_3$) (24 equiv., 6 mmol) in dichloromethane (20 ml) for 2 h. After washing with dichloromethane (5 × 10 mL × 1 min) and *N,N*-dimethylformamide (5 × 10 mL × 1 min) a solution of the (2-(pyrrolidin-1-yl)phenyl)methanamine (4 equiv., 1 mmol), DIPEA (8 equiv., 2 mmol) and HATU (4.0 equiv., 2 mmol) in *N,N*-dimethylformamide (10 mL) was added to the syringe and allowed to react for 16 h. After washing with *N,N*-dimethylformamide (5 × 10 mL × 1 min) the Fmoc group was removed and the coupling to the 2-(4-

(dimethylamino)phenyl)acetic (4 equiv., 1.0 mmol) performed using the same conditions reported before. After washing with *N,N*-dimethylformamide (5 × 10 mL × 1 min) the resin was cleaved using a solution of TFA:TIPS:mqH_2O (95:2.5:2.5, *v/v*) for 1 h at room temperature. The so-obtained solution was collected in a round-bottom flask, dried under vacuum and purified over a reversed-phase HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 100% over 14 min). After lyophilization the final compound was collected as a white solid.

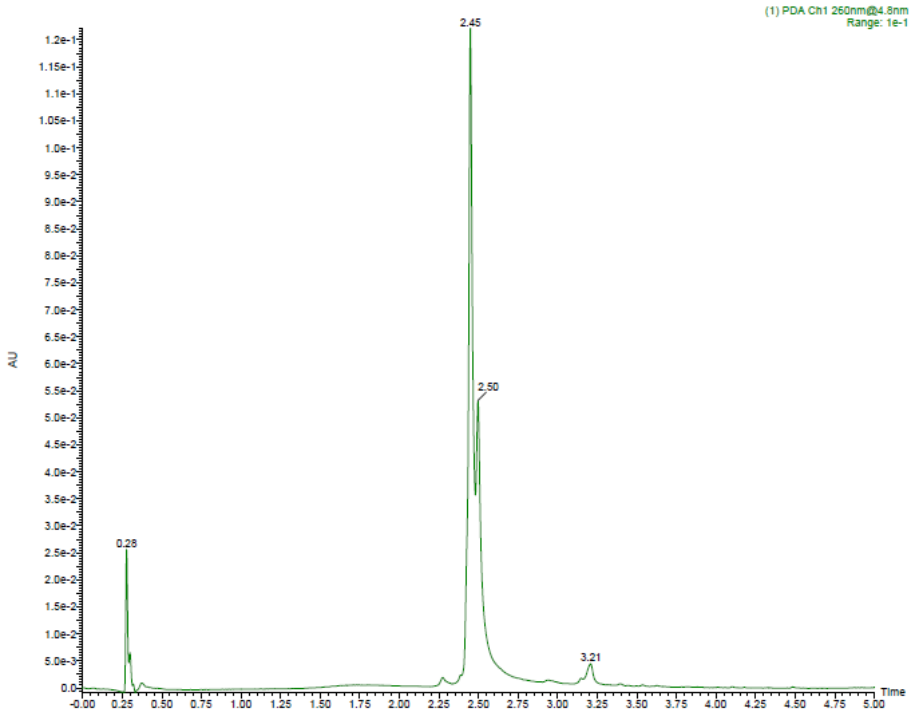
HRMS (ES) calculated for $[M+H]^+$ (*m/z*): 595.3603, found 595.3448.

Fl-Lys-D-Gln-A26-B389



To a solution of Lys-D-Gln-A26-B389 (3.3 mg, 1 equiv.) in dimethyl sulfoxide (100 µL) was added DIPEA (20 µL) and a solution of commercially available fluorescein isothiocyanate (4.3 mg, 2.0 equiv.) in dimethyl sulfoxide (100 µL). After stirring for 30 min in the dark, the reaction was diluted with a solution of DMSO:mqH_2O (1:1 *v*:*v*) and purified over a reversed-phase HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 100% over 14 min). The fractions containing the product identified by mass spectrometry, collected and then lyophilized to give the title compound as a yellow powder.

HRMS (ES) calculated for $[M+H]^+$ (*m/z*): 984.3961, found 984.3598.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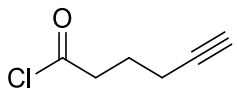
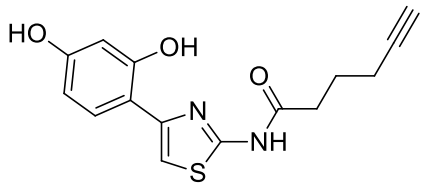
Hex-5-ynoyl chloride



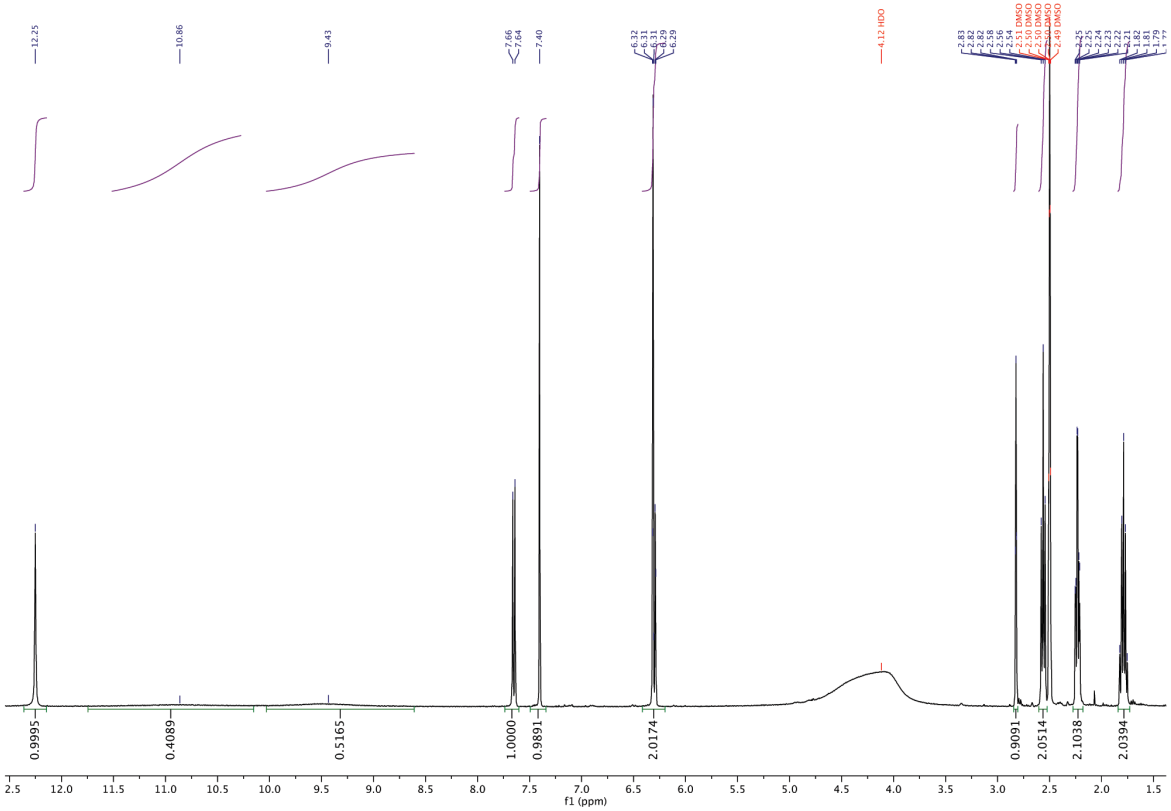
To a stirring solution of 5-hexynoic acid (1.0 equiv.) in dichloromethane (0.1 M) and in presence of a catalytic amount of *N,N*-dimethylformamide, a 2.0 M solution of oxalyl chloride in dichloromethane (1.1 equiv.) was added dropwise at 0 °C. After 1 h, the reaction was completed, evaporated under vacuum and the so obtained red precipitate resuspended in a solution of tetrahydrofuran, in presence of a catalytic amount of pyridine.

Thiamidol™-alkyne



***N*-(4-(2,4-dihydroxyphenyl)thiazol-2-yl)hex-5-ynamide**: to a stirring solution of 4-(2-aminothiazol-4-yl)benzene-1,3-diol (1.0 equiv.) in tetrahydrofuran (0.1 M) the fresh solution of hex-5-ynoyl chloride (from 3.0 to 5.0 equiv. depending on the stability of the chloride) was added drop-wise. The reaction was controlled through LC-MS analysis until the formation of a tri-functionalized derivative. After 16 h, the reaction mixture was treated with a 33 % solution of methylamine in absolute ethanol (8 M, 10 equiv.). After 2 h, the reaction was dried under vacuum, resuspended in the proper amount of CH_3CN:mqH_2O and purified over a Büchi Sepacore RP-MPLC system (2% MeCN in 0.1% aq. TFA over 10 min, 2% MeCN in 0.1% aq. TFA to 70% over 30 min). The fractions containing the product identified by mass spectrometry, were collected and then lyophilized to give the title compound as a white solid; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.25 (s, 1H), 10.86 (s, 1H), 9.43 (s, 1H), 7.65 (d, J = 8.3 Hz, 1H), 7.40 (s, 1H), 6.42 – 6.20 (m, 2H), 2.82 (t, J = 2.6 Hz, 1H), 2.56 (t, J = 7.4 Hz, 2H), 2.23 (td, J = 7.1, 2.7 Hz, 2H), 1.79 (p, J = 7.2 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ 170.6, 158.2, 156.7, 156.2, 146.3, 128.1, 110.5, 107.0, 105.1, 102.8, 83.6, 71.6, 33.6, 23.2, 17.1.

HRMS (ES) calculated for $[M+H]^+$ (*m/z*): 303.0803, found 303.0596.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6.31
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6.29
6.29
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2.82
2.82
2.58
2.56
2.54
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2.25
2.25
2.24
2.23
2.22
2.21
1.82
1.81
1.79
1.77
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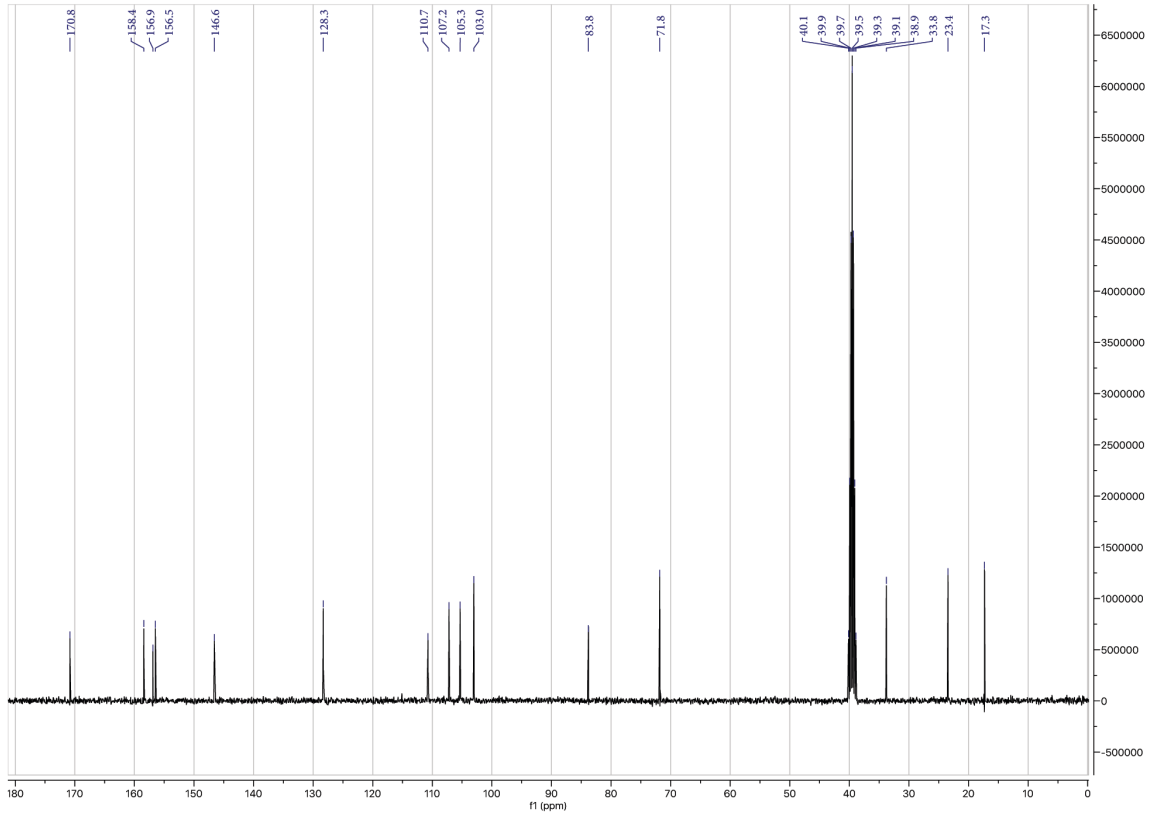
2.1038
2.0394
f1 (ppm)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128.3
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107.2
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103.0
83.8
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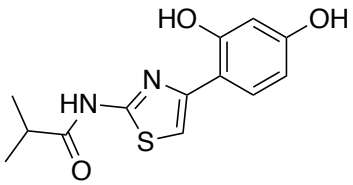
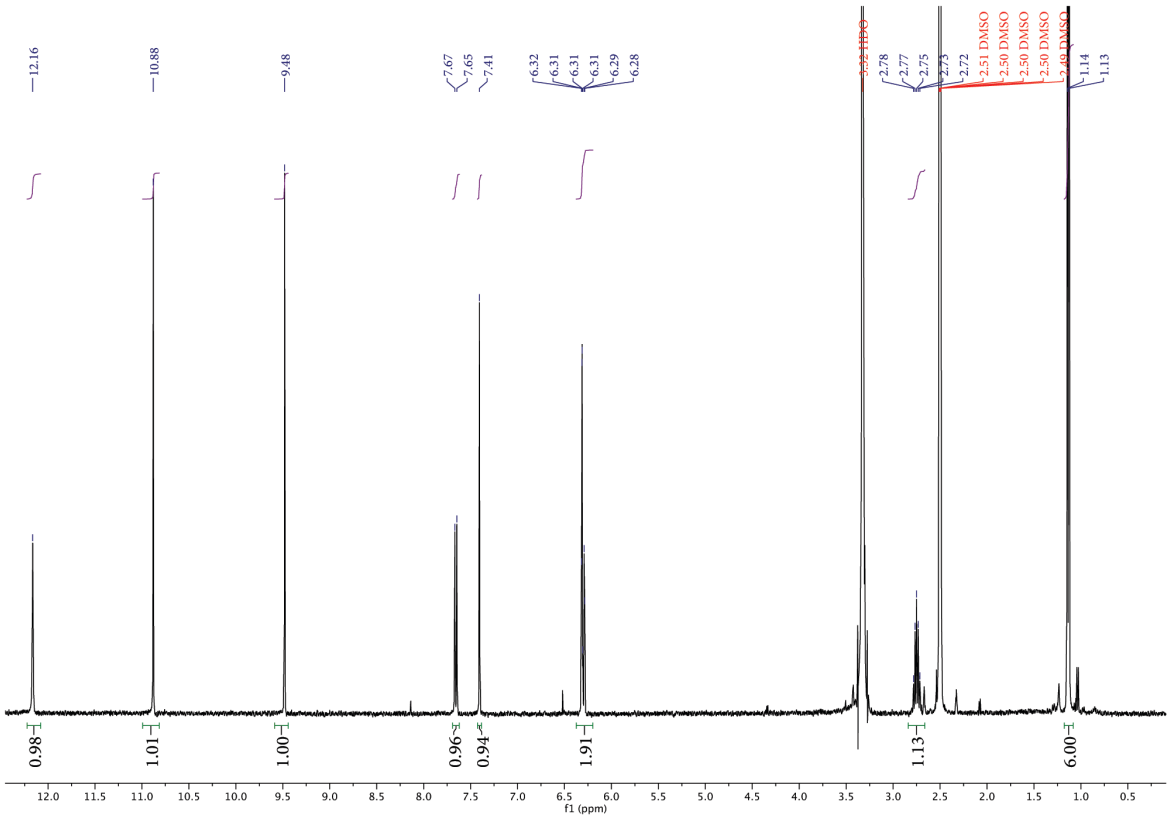
33.8
23.4
17.3
f1 (ppm)

Thiamidol™



***N*-(4-(2,4-dihydroxyphenyl)thiazol-2-yl)isobutyramide**: to a stirring solution of 4-(2-aminothiazol-4-yl)benzene-1,3-diol (1.0 equiv.) in tetrahydrofuran (0.1 M) the commercially available isobutyryl chloride (5.0 equiv.) was added drop-wise in presence of pyridine (0.1 equiv.). The reaction was controlled through LC-MS analysis until the formation of a tri-functionalized derivative. After 16 h, the reaction mixture was treated with a 33 % solution of methylamine in absolute ethanol (8 M, 10 equiv.). After 2 h, the reaction was dried under vacuum, resuspended in the proper amount of CH_3CN:mqH_2O and purified over a Büchi Sepacore RP-MPLC system (2% MeCN in 0.1% aq. TFA over 10 min, 2% MeCN in 0.1% aq. TFA to 70% over 30 min). The fractions containing the product identified by mass spectrometry, were collected and then lyophilized to give the title compound as a white solid; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.16 (s, 1H), 10.88 (s, 1H), 9.48 (s, 1H), 7.66 (d, J = 8.3 Hz, 1H), 7.41 (s, 1H), 6.37 – 6.20 (m, 2H), 2.75 (p, J = 6.8 Hz, 1H), 1.14 (d, J = 6.8 Hz, 6H). NMR data are in full agreement with previously reported values.[9]


12.16
10.88
9.48
7.67
7.65
7.41
6.32
6.31
6.31
6.31
6.29
6.28
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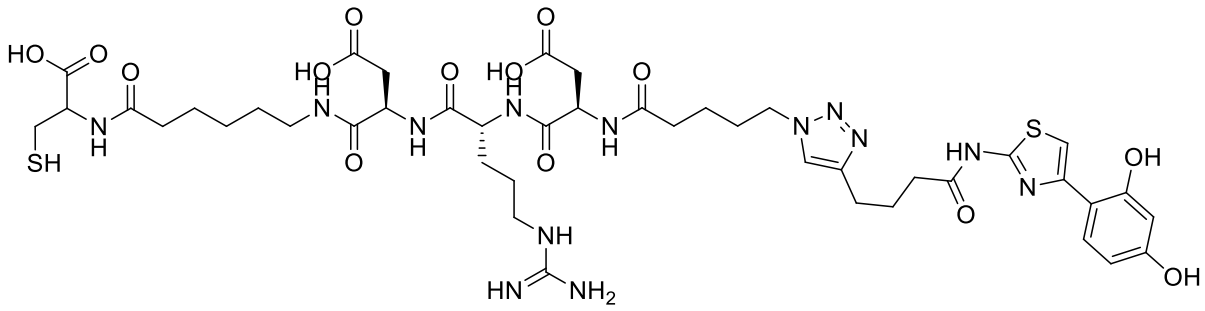
2.78
2.77
2.75
2.73
2.72
2.51 DMSO
2.50 DMSO
2.50 DMSO
2.50 DMSO
2.49 DMSO
1.14
1.13
0.98
1.01
1.00
0.96
0.94
1.91
1.13
6.00
f1 (ppm)

General Procedure on-resin

Commercial Fmoc-Cys(Trt) Wang resin (0.7 g, 0.5 mmol) was swollen in *N,N*-dimethylformamide (20 mL) for 1 h inside a syringe provided with a filter pad. The Fmoc group was removed using a solution of 20% piperidine in *N,N*-dimethylformamide for 20 min and then the resin washed with *N,N*-dimethylformamide (5 × 20 mL × 1 min). A solution of Fmoc-6-aminocaproic acid (4.0 equiv., 2 mmol), DIPEA (8.0 equiv, 4 mmol) and HATU (4.0 equiv., 2 mmol) in *N,N*-dimethylformamide (20 mL) was prepared, added to the syringe and allowed to react for 2 h. After washing with *N,N*-dimethylformamide (5 × 20 mL × 1 min) the Fmoc group was removed using a solution of 20 % piperidine in *N,N*-dimethylformamide. The peptide was extended with Fmoc-Asp(OtBu)-OH, Fmoc-Arg(Pbf)-OH and Fmoc-Asp(OtBu)-OH using the same coupling conditions (HATU/DIPEA equiv.), Fmoc deprotection (20% piperidine in DMF) and washing step as reported before.

The resin was split into different parts for the synthesis of the Thiamidol™-derivatives.

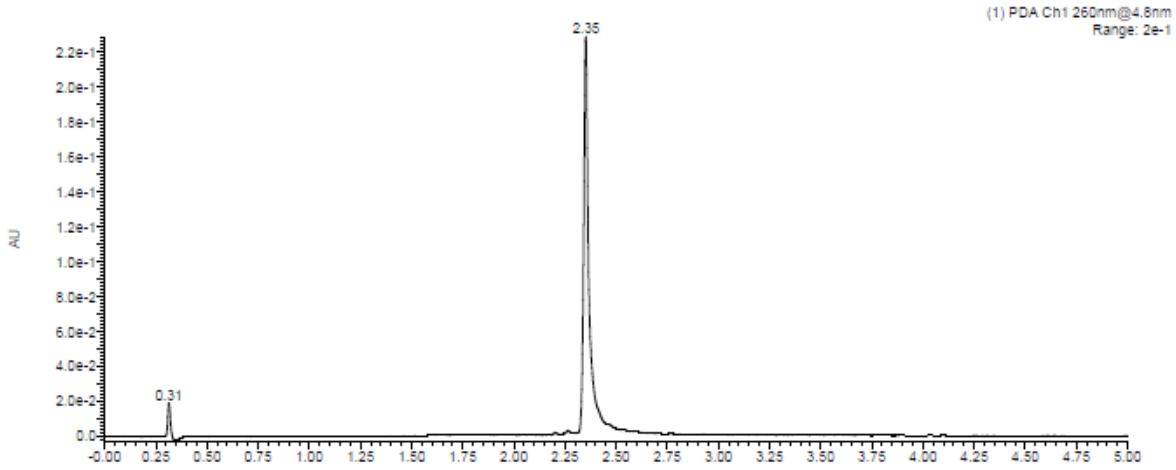
C-C6-DRD-monomer-Thiamidol™



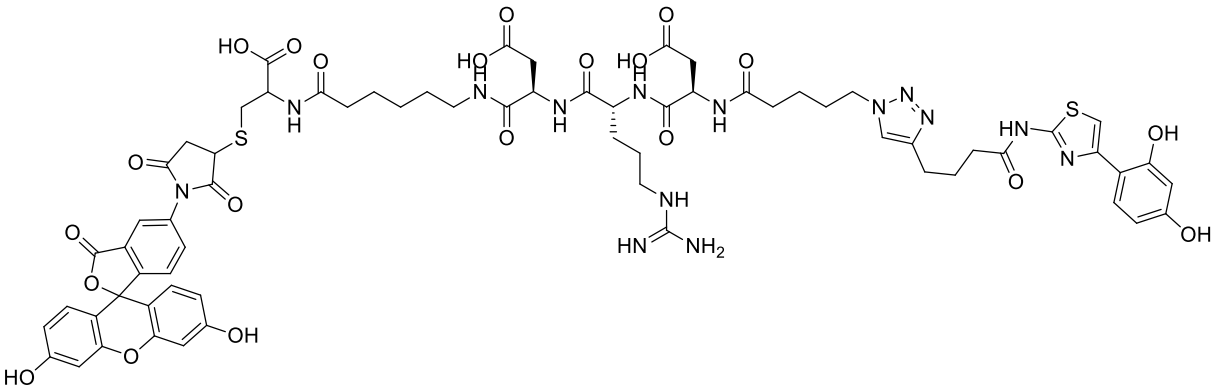
After the removal of the Fmoc-group, 0.1 mmol of the obtained resin was reacted with a solution of 5-azido pentanoic acid (4 equiv., 0.4 mmol**),** DIPEA (8.0 equiv., 0.4 mmol) and HATU (4.0 equiv., 0.4 mmol) in *N,N*-dimethylformamide (4 mL) for 2 h. After washing with *N,N*-dimethylformamide (5 × 4 mL × 1 min) the Cu-catalysed alkyne-azide cycloaddition was performed. The reaction protocol was adjusted from a previously described procedure.[10] A solution of sodium ascorbate (1 equiv.), 2,6-lutidine (10 equiv.) and DIPEA (10 equiv.) in degassed mqH_2O was added to a solution of CuI (1.0 equiv.) in *N,N*-dimethylformamide. The resulting solution and the alkyne (2.0 equiv.) were added to the resin and allowed to react for 16 h. After washing with *N,N*-dimethylformamide (5 × 4 mL × 1 min), 50 mM aq. EDTA solution pH = 8 (5 × 4 mL × 1 min), *N,N*-dimethylformamide (5 × 4 mL × 1 min) and dichloromethane (5 × 4 mL × 1 min), the resin was cleaved using a mixture of TFA (3.30 mL, 82.5 %), *m*-Cresol (200 mL, 5 %), thioanisol (200 mL, 5 %), mq-H_2O (200 mL, 5 %) and TIPS (100 mL, 2.5 %) for 2 h at room temperature and then washed with TFA (1 × 1 mL × 1 min). The combined cleavage and washing solutions were added dropwise to ice cold diethyl ether (20 mL), leading to precipitate formation that was collected by centrifugation, washed with ice cold diethyl ether (3 × 20 mL × 2 min, 2000 rpm), dried, re-dissolved in mqH_2O:DMSO and treated with Tris(2-carboxyethyl)phosphine

hydrochloride (2 equiv.). The solution was injected and purified over a RP-HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 14 min), the fractions containing the product identified by mass spectrometry collected and then lyophilized to give the title compound as a white solid.

HRMS (ES) calculated for $[M+H]^+$ (*m/z*): 1048.3980, found 1048.3822.

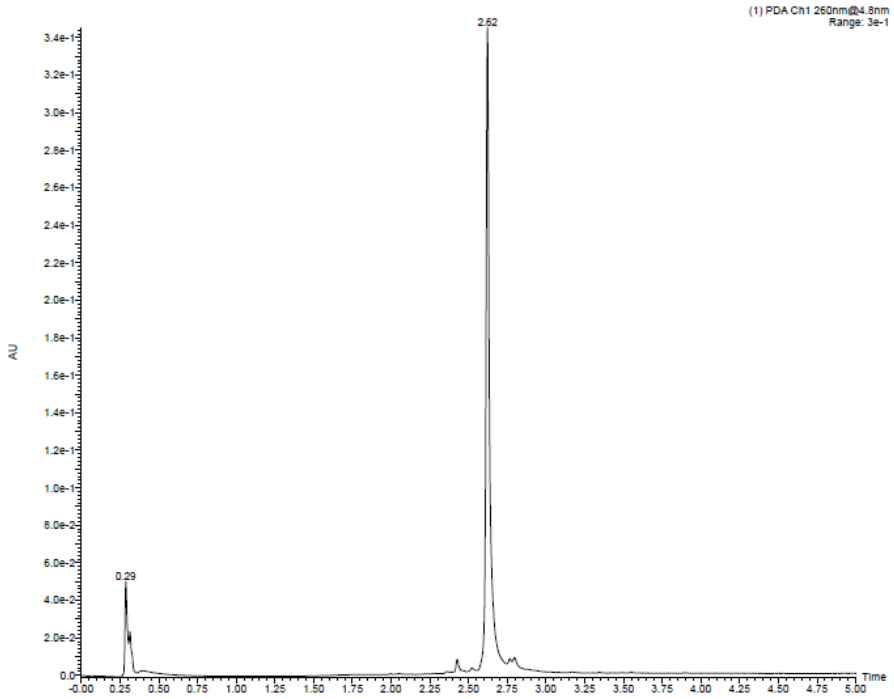


Monomer



Fl-C-C6-DRD-monoThiamidol™: to a solution of C-C6-DRD-monomer-Thiamidol™ (2.0 mg, 1 equiv.) in dimethyl sulfoxide (100 µL) was added a 0.1 M solution of commercially available fluorescein-5-maleimide (0.81557 mg, 1.0 equiv.) in dimethyl sulfoxide (19.1 µL). After stirring for 30 min in the dark, the reaction was quenched with the proper volume of DMSO:mqH_2O, injected and purified over a reversed-phase HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 70% over 14 min). The fractions containing the product were identified by mass spectrometry, collected and lyophilized to give the title compound as a yellow solid.

HRMS (ES) calculated for $[M+2H]^+$ (*m/z*): 1476.4753, found 1476.4056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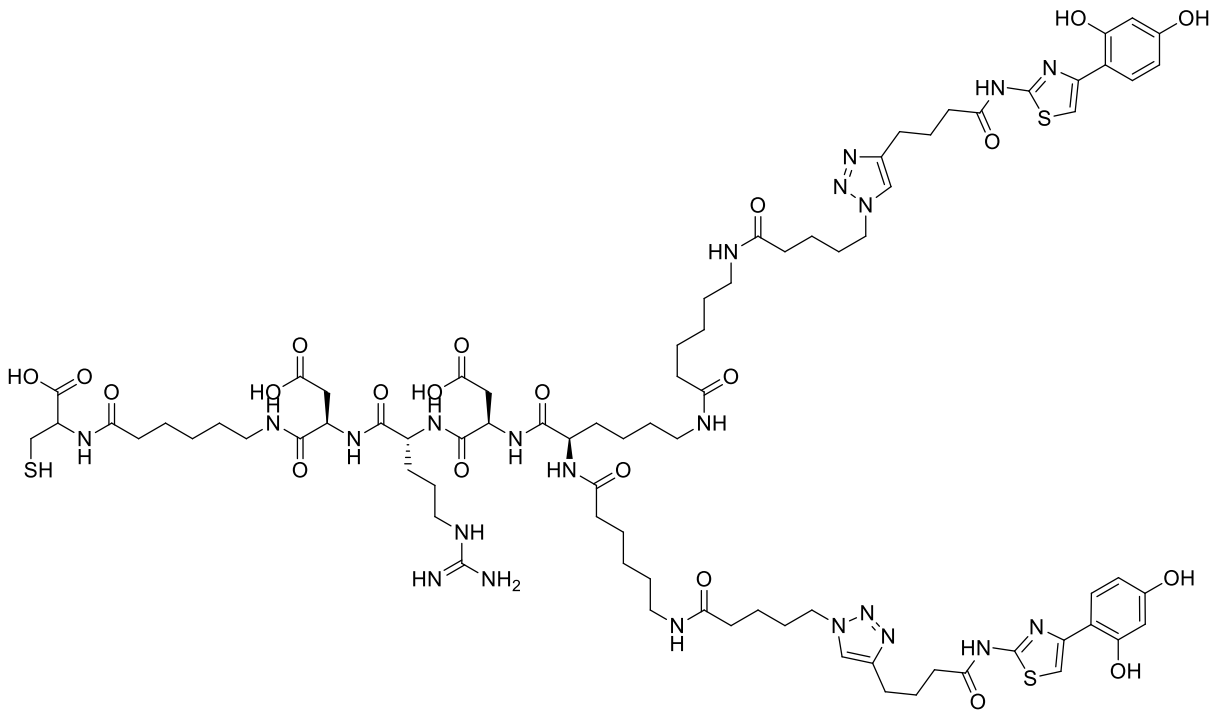
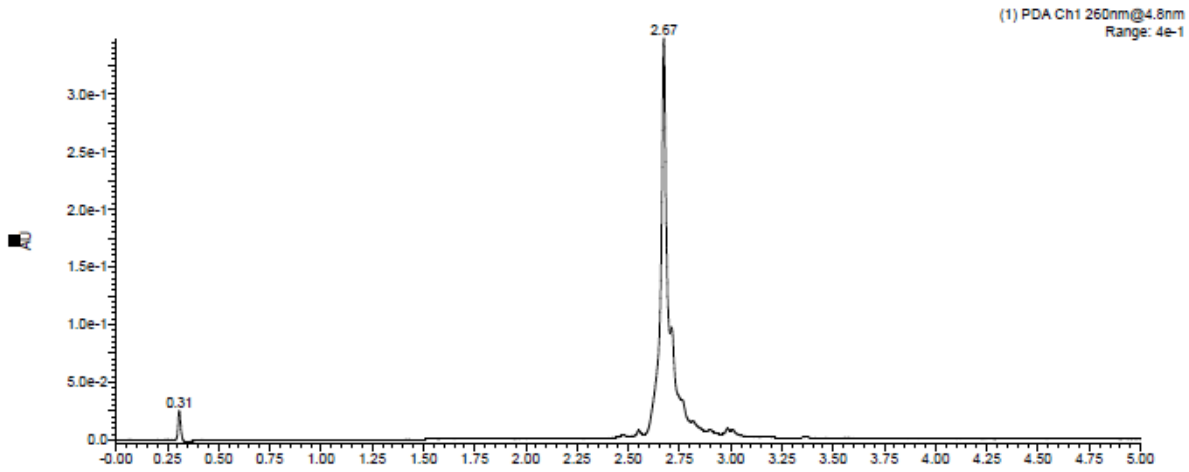
C-C6-DRD-dimer-Thiamidol™



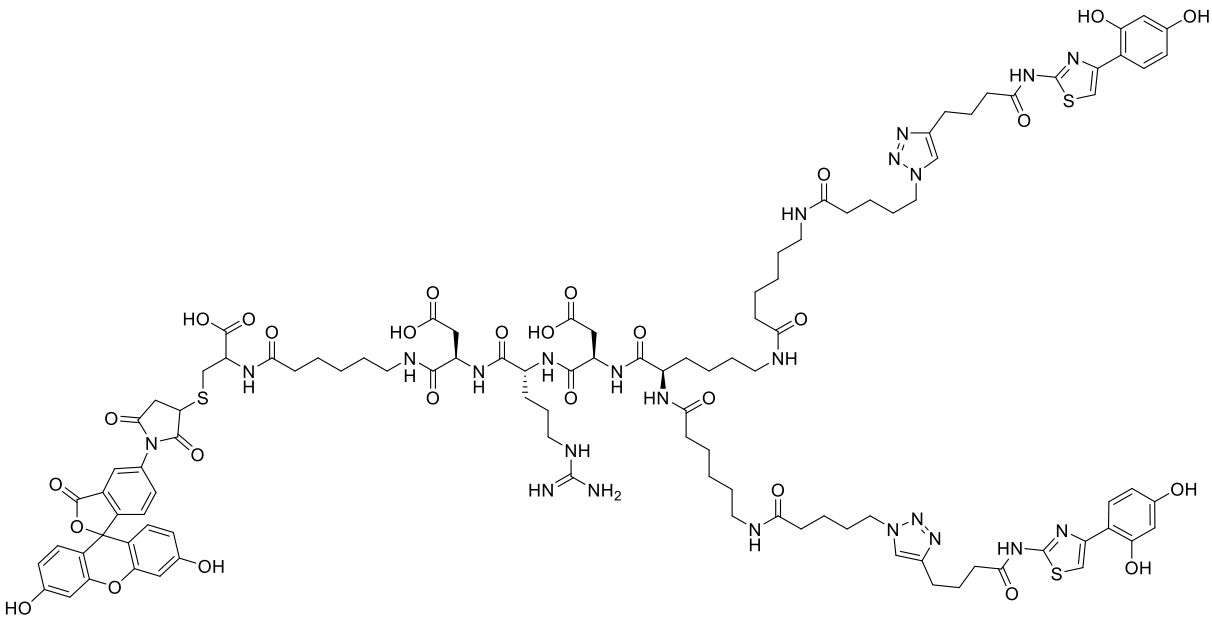
After the removal of the Fmoc-group, 0.2 mmol of the resin was reacted with a solution of Fmoc-Lys(Fmoc)-OH (4 equiv., 0.8 mmol), DIPEA (8 equiv., 1.6 mmol) and HATU (4.0 equiv, 0.8 mmol) in *N,N*-dimethylformamide (8 mL) for 2 h. After washing with *N,N*-dimethylformamide (5 × 8 mL × 1 min) the peptide was extended with Fmoc-6-aminocaproic acid and then capped with 5-azido pentanoic acid fixing the coupling conditions (Fmoc-6-aminocaproic or 5-azido pentanoic and HATU 6 equiv., DIPEA 12 equiv.), using the same Fmoc deprotection conditions (20 % piperidine in DMF) and washing step mentioned before. After the last coupling step, the Cu-catalysed alkyne-azide cycloaddition was performed. The reaction protocol was adjusted from a previously described procedure.[10] A solution of sodium ascorbate (1.5 equiv.), 2,6-lutidine (15 equiv.) and DIPEA (15 equiv.) in degassed mqH_2O was added to a solution of CuI (1.5 equiv.) in *N,N*-dimethylformamide. The resulting solution and the alkyne (3 equiv.) were added to the resin and allowed to react for 16 h. After washing with *N,N*-

dimethylformamide (5 × 8 mL × 1 min), 50 mM aq. EDTA solution pH = 8 (5 × 8 mL × 1 min), *N,N*-dimethylformamide (5 × 8 mL × 1 min) and dichloromethane (5 × 4 mL × 1 min), the resin was cleaved using a mixture of TFA (6.60 mL, 82.5 %), *m*-Cresol (400 mL, 5 %), thioanisol (400 mL, 5 %), mq-H_2O (400 mL, 5 %) and TIPS (200 mL, 2.5 %) for 2 h at room temperature and washed with TFA (1 × 4 mL × 1 min). The combined cleavage and washing solutions were added dropwise to ice cold diethyl ether (40 mL) leading to precipitate formation that was collected by centrifugation, washed again with ice cold diethyl ether (3 × 40 mL × 2 min, 2000 rpm), dried, re-dissolved in mqH_2O:DMSO and treated with Tris(2-carboxyethyl)phosphine hydrochloride (2 equiv.). The solution was injected and purified over a RP HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 14 min), the fractions containing the product identified by mass spectrometry, collected and lyophilized to give the title compound as a white solid.

HRMS (ES) calculated for $[M+2H]^+$ (*m/z*): 1830.8004, found 1830.7086.

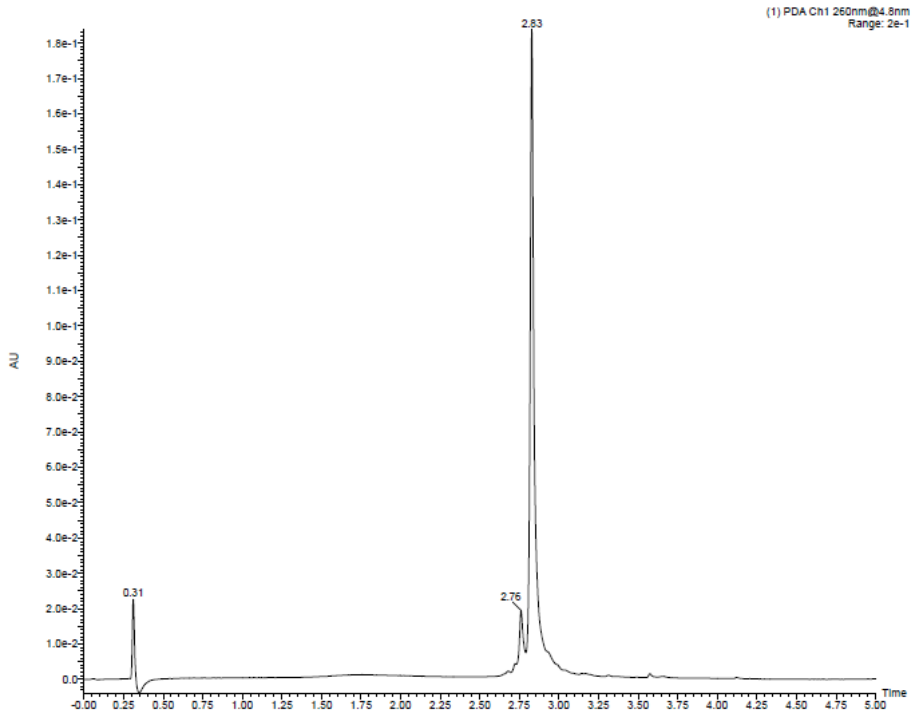


Dimer



FI-C-C6-DRD-dimerLongThiamidol™: To a solution of the so-obtained compound (3.2 mg, 1 equiv.) in dimethyl sulfoxide (100 µL) was added a 0.1 M solution of commercially available fluorescein-5-maleimide (0.8537 mg, 1.0 equiv.) in dimethyl sulfoxide (20 µL). After stirring for 30 min in the dark, the reaction was quenched with the proper volume of DMSO:mqH_2O, injected and purified over a reversed-phase HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 70% over 14 min). The fractions containing the product were identified by mass spectrometry, collected and lyophilized to give the title compound as a yellow solid.

HRMS (ES) calculated for $[M+H]^+$ (*m/z*): 2257.8651, found 2258.0203.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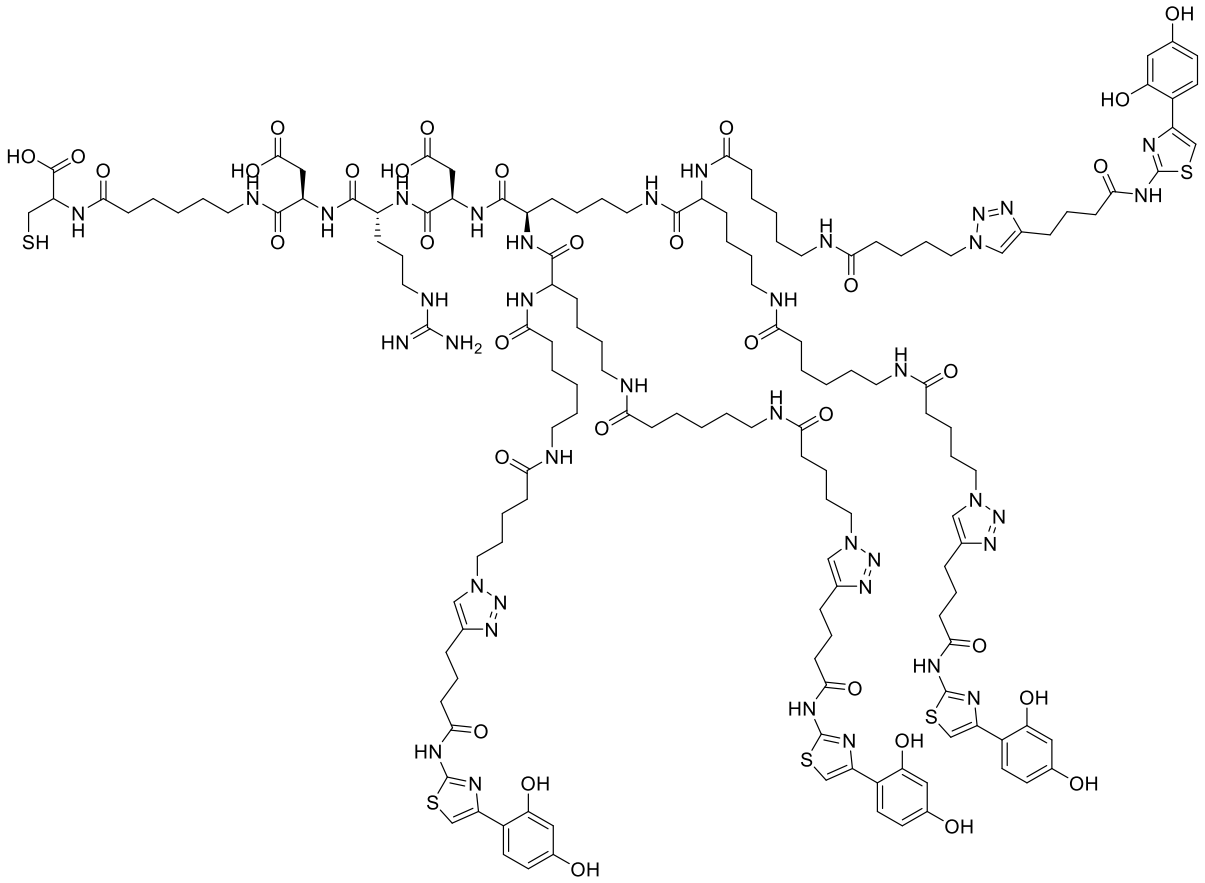
2.76
AU
Time

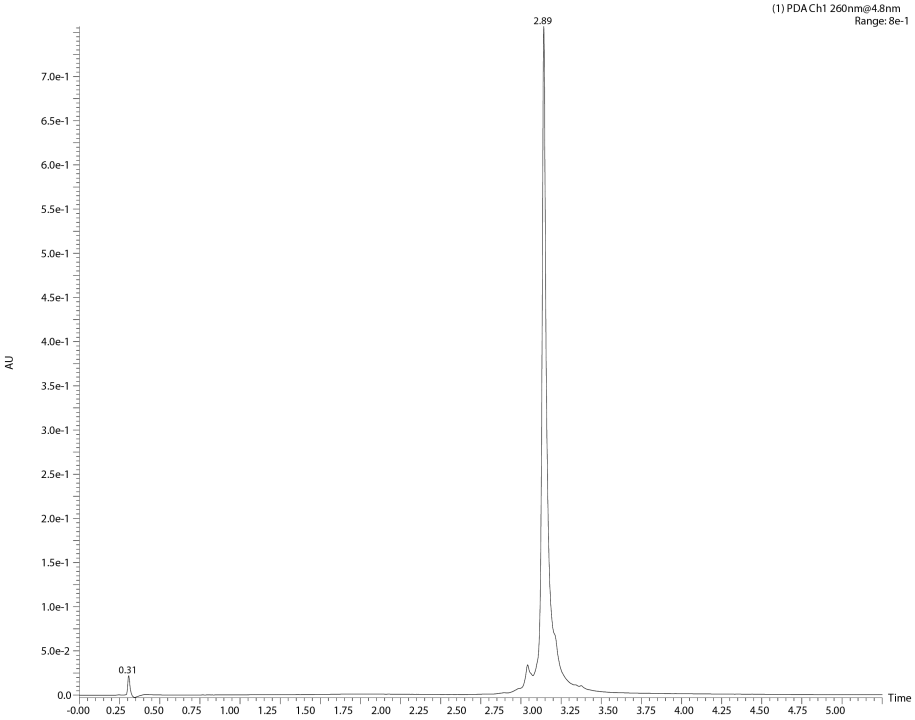
C-C6-DRD-tetramer-Thiamidol™



After the removal of the Fmoc-group, 0.2 mmol of the resin was reacted with a solution of Fmoc-Lys(Fmoc)-OH (4 equiv., 0.8 mmol), DIPEA (8 equiv., 1.6 mmol) and HATU (4.0 equiv, 0.8 mmol) in *N,N*-dimethylformamide (8 mL) for 2 h. After washing with *N,N*-dimethylformamide (5 × 8 mL × 1 min) the peptide was extended with Fmoc-Lys(Fmoc)-OH, Fmoc-6-aminocaproic acid and then capped with 5-azido pentanoic acid fixing the coupling conditions (Fmoc-Lys(Fmoc)-OH and HATU 6 equiv., DIPEA 12 equiv., Fmoc-6-aminocaproic or 5-azido pentanoic and HATU 12 equiv., DIPEA 24 equiv.), using the same Fmoc deprotection conditions (20 % piperidine in DMF) and washing step mentioned before. After the last coupling step, the Cu-catalysed alkyne-azide cycloaddition was performed. The reaction protocol was adjusted from a previously described procedure.[10] A solution of sodium ascorbate (3 equiv.), 2,6-

lutidine (30 equiv.) and DIPEA (30 equiv.) in degassed mqH_2O was added to a solution of CuI (3 equiv.) in *N,N*-dimethylformamide. The resulting solution and the alkyne (6 equiv.) were added to the resin and allowed to react for 16 h. After washing with *N,N*-dimethylformamide (5 × 8 mL × 1 min), 50 mM aq. EDTA solution pH = 8 (5 × 8 mL × 1 min), *N,N*-dimethylformamide (5 × 8 mL × 1 min) and dichloromethane (5 × 4 mL × 1 min), the resin was cleaved using a mixture of TFA (6.60 mL, 82.5 %), *m*-Cresol (400 mL, 5 %), thioanisol (400 mL, 5 %), mq-H_2O (400 mL, 5 %) and TIPS (200 mL, 2.5 %) for 2 h at room temperature and washed with TFA (1 × 4 mL × 1 min). The combined cleavage and washing solutions were added dropwise to ice cold diethyl ether (40 mL) leading to precipitate formation that was collected by centrifugation, washed again with ice cold diethyl ether (3 × 40 mL × 2 min, 2000 rpm), dried, re-dissolved in mqH_2O:DMSO and treated with Tris(2-carboxyethyl)phosphine hydrochloride (2 equiv.). The solution was injected and purified over a RP HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 14 min), the fractions containing the product identified by mass spectrometry, collected and lyophilized to give the title compound as a white solid.

HRMS (ES) calculated for $[M+2H]^+$ (*m/z*): 1584.2132, found 1584.2404.


(1) PDA Ch1 260nm@4.8nm
Range: 8e-1
2.89
0.31
AU
Time
7.0e-1
6.5e-1
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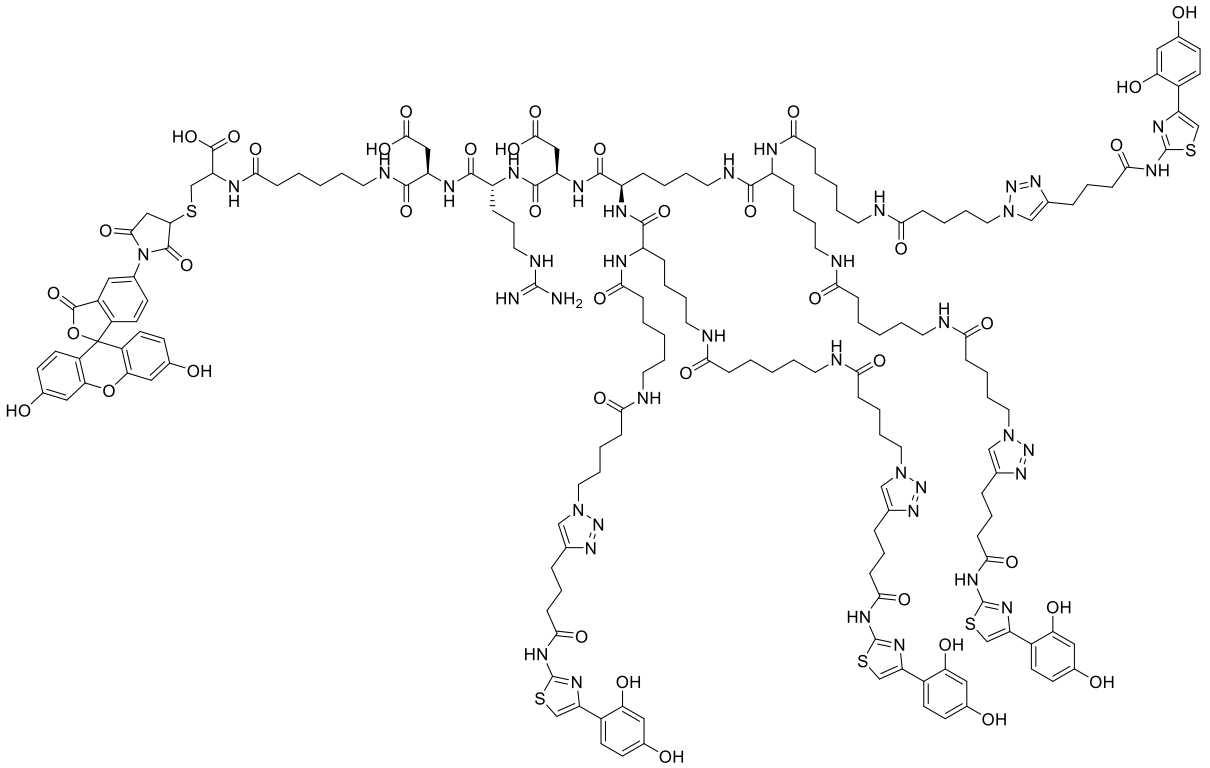
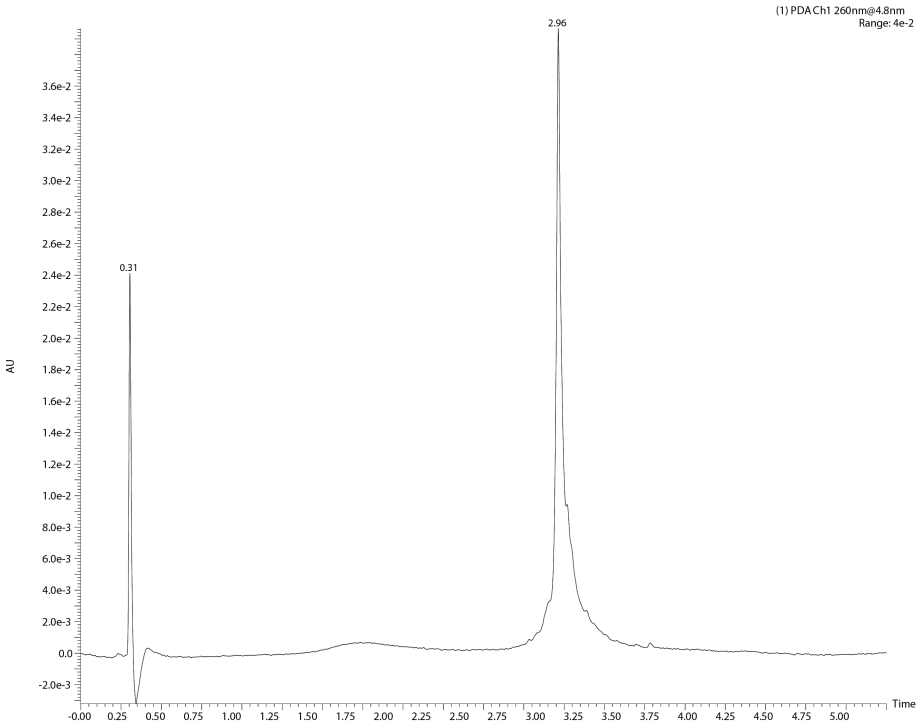
5.5e-1
5.0e-1
4.5e-1
4.0e-1
3.5e-1
3.0e-1
2.5e-1
2.0e-1
1.5e-1
1.0e-1
5.0e-2
0.0
-0.00 0.25 0.50 0.75 1.00 1.25 1.50 1.75 2.00 2.25 2.50 2.75 3.00 3.25 3.50 3.75 4.00 4.25 4.50 4.75 5.00

Tetramer



FI-C-C6-DRD-dimerLongThiamidol™: to a solution of C-C6-DRD-tetramer-Thiamidol™ (4.9 mg, 1 equiv.) in dimethyl sulfoxide (100 µL) was added a 0.1 M solution of commercially available fluorescein-5-maleimide (0.665 mg, 1.0 equiv.) in dimethyl sulfoxide (15.5 µL). After stirring for 30 min in the dark, the reaction was quenched with the proper volume of DMSO:mqH_2O, injected and purified over a reversed-phase HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 70% over 14 min). The fractions containing the product were identified by mass spectrometry, collected and lyophilized to give the title compound as a yellow solid.

HRMS (ES) calculated for $[M+2H]^+$ (*m/z*):, 1797.7523 found 1798.6615.


(1) PDA Ch1 260nm@4.8nm
Range: 4e-2
2.96
0.31
AU
3.6e-2
3.4e-2
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3.0e-2
2.8e-2
2.6e-2
2.4e-2
2.2e-2
2.0e-2
1.8e-2
1.6e-2
1.4e-2
1.2e-2
1.0e-2
8.0e-3
6.0e-3
4.0e-3
2.0e-3
0.0
-2.0e-3
-0.00 0.25 0.50 0.75 1.00 1.25 1.50 1.75 2.00 2.25 2.50 2.75 3.00 3.25 3.50 3.75 4.00 4.25 4.50 4.75 5.00
Time

References

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