Supporting Information

Content

General information

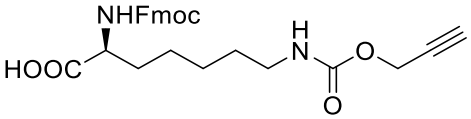
The chemicals were obtained from commercial suppliers and were used without further purification. Reactions with air- and moisture-sensitive reactants were performed in anhydrous solvents under nitrogen or argon atmosphere. Column chromatography was carried out on silica gel 60A (particle size: 40-60 µm) from *Acros Organics*. Mixtures of solvents are each stated as volume fractions. For flash column chromatography a Combi*Flash*® Rf+ from *Teledyne ISCO* was used. Thin-layer chromatography was performed on aluminum sheets from *Merck* (silica gel 60 F254, 20 × 20 cm). ^{1}H- and ^{13}C-NMR spectra were measured on Avance III™ HD 600 MHz or Bruker Avance III™ HD 400 MHz NMR system

equipped with Prodigy cryo-probe. The signals were assigned based on 2D NMR experiments. Chemical shifts δ are quoted in ppm in relation to the chemical shift of the residual non-deuterated solvent peak ($CDCl_3$: δ(^{1}H) = 7.26, δ(^{13}C) = 77.16, CD_3OD: δ(^{1}H) = 3.31, δ(^{13}C) = 49.0). High-resolution mass spectra were recorded on an *Agilent* 5975C MSD Quadrupole or LTQ Orbitrap XL from *Thermo Fisher Scientific*. HPLC-MS measurements were performed on an LCMS-2020 system from *Shimadzu* equipped with a CORTECS C18 column (2.7µm, 50 × 4.6 mm) or Luna® C18 column (3u, 100A, 100 x 4.6 mm).

Synthetic procedures

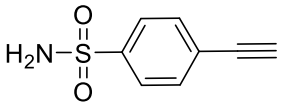
Synthesis of small molecules

Fmoc-L-Lys(Poc)



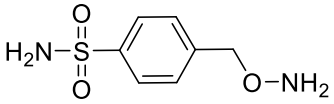
Fmoc-L-Lys(Poc) was prepared according to published procedure.[1] The compound is also commercially available (CAS: 1584133-25-4).

Inh1



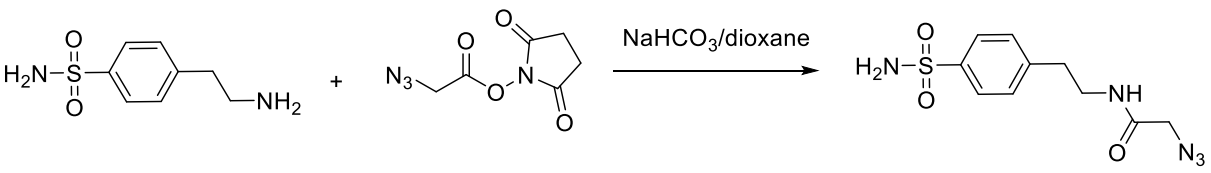
This compound is commercially available (CAS 1788-08-5). The one used in this study was from ABCR (AB443299).

Inh2



4-((Aminooxy)methyl)benzenesulfonamide was prepared according to published procedure.[2]

Inh3



The NHS active ester of azido acetic acid[3] (125 mg, 0.63 mmol) was dissolved in dry dioxane (2 mL) and the solution was dropwise added to ice cold suspension of 4-(2-Aminoethyl)benzenesulfonamide (CAS: 35303-76-5) (190 mg, 0.95 mmol, 1.5 equiv.) in saturated solution of $NaHCO_3$ (2 mL). The resulting solution was stirred at room temperature for 1.5 hours (TLC in DCM/MeOH = 9/1, anisaldehyde staining). The reaction mixture was concentrated under reduced pressure and the residue was

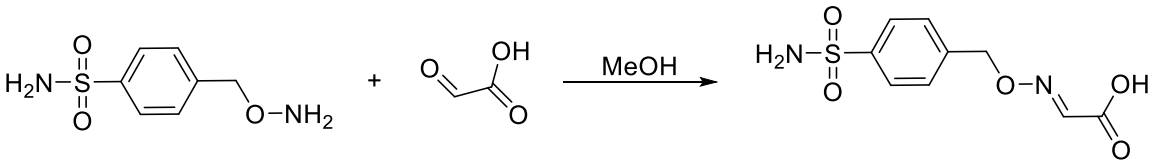
extracted with AcOEt. The organic phase was dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The residue was purified by flash column chromatography using gradient of MeOH in DCM (5 to 10%). Fractions containing the product were combined giving Inh3 as beige solid (139 mg, 78 %). mp 116-120 °C

^{1}H NMR (400 MHz, DMSO) δ: 8.20 (t, *J* = 5.6 Hz, 1H), 7.81 – 7.66 (m, 2H), 7.46 – 7.36 (m, 2H), 7.29 (s, 2H), 3.79 (s, 2H), 3.35 (q, *J* = 7.2 Hz, 2H), 2.81 (t, *J* = 7.2 Hz, 2H).

^{13}C NMR (101 MHz, DMSO) δ: 167.24, 143.42, 142.10, 129.09, 125.69, 50.77, 39.80, 34.60.

LRMS (ESI): m/z calcd. for $C_{10}H_{14}N_5O_3S$ $[M+H]^+$ 284.1, found: 284.1.

Inh2-COOH



4-((Aminooxy)methyl)benzenesulfonamide[2] (580 mg, 2.87 mmol) was dissolved in methanol (20 mL) and glyoxylic acid monohydrate (264 mg, 2.87 mmol, 1.0 equiv.) was added. The resulting solution was stirred at room temperature for 2.5 hours (TLC in DCM/MeOH = 10/1, UV detection). The reaction mixture was evaporated to dryness under reduced pressure. The residue was suspended in acetonitrile, sonicated and filtered. The resulting solution was evaporated under reduced pressure to yield Inh2-COOH as white solid (720 mg, 97 %). mp 153-157 °C

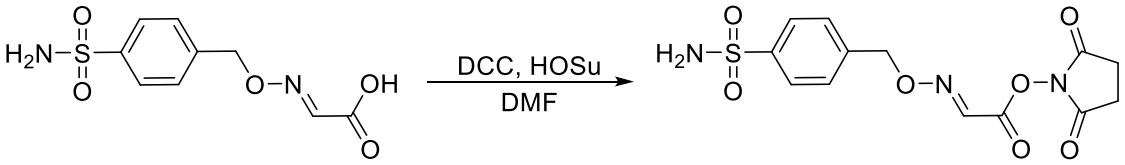
^{1}H NMR (400 MHz, DMSO) δ: 7.83 (d, *J* = 8.4 Hz, 2H), 7.66 (s, 1H), 7.54 (d, *J* = 8.4 Hz, 2H), 7.36 (s, 2H), 5.30 (s, 2H).

^{13}C NMR (101 MHz, DMSO) δ: 162.8, 143.7, 143.2, 140.8, 128.5, 125.8, 75.5.

LRMS (ESI): m/z calcd. for $C_9H_9N_2O_5S$ $[M-H]^-$ 257.0, found: 257.1.

HRMS (ESI): m/z calcd. for $C_9H_{10}N_2O_5S$ $[M-H]^-$ 257.02377, found: 257.02372.

Inh2-COOSu



Inh2-COOH (700 mg, 2.71 mmol) and *N*-Hydroxysuccinimide (405 mg, 3.52 mmol, 1.3 equiv.) were dissolved in anhydrous DMF (5 mL). *N*,*N*´-Dicyclohexylcarbodiimide (614 mg, 2.98 mmol, 1.1 equiv.) was added. The resulting solution was stirred at room temperature overnight. The suspension was filtered, filtrate evaporated to dryness under reduced pressure and suspended in methanol. This suspension was sonicated and filtered. The solid residue was washed with methanol and dichloromethane to yield Inh2-COOSu as white solid (850 mg, 89 %). mp 168-173 °C

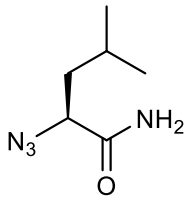
^{1}H NMR (400 MHz, DMSO) δ: 1H NMR (401 MHz, DMSO) δ 8.21 (s, 1H), 7.85 (d, *J* = 8.4 Hz, 2H), 7.58 (d, *J* = 8.4 Hz, 2H), 7.37 (s, 2H), 5.46 (s, 2H), 2.84 (s, 4H).

^{13}C NMR (101 MHz, DMSO) δ: 169.93, 157.35, 143.97, 140.01, 139.08, 128.67, 125.91, 76.78, 25.51.

LRMS (ESI): m/z calcd. for $C_{13}H_{12}N_3O_7S$ $[M-H]^-$ 354.0, found: 354.0.

HRMS (ESI): m/z calcd. for $C_{13}H_{12}N_3O_7S$ $[M-H]^-$ 354.04014, found: 354.03998.

N_3-*L*-Leu-NH_2



Azido-leucinamide was synthesized using imidazole-1-sulfonyl azide hydrogen sulfate (ISA), similar to the procedure originally reported.[4]

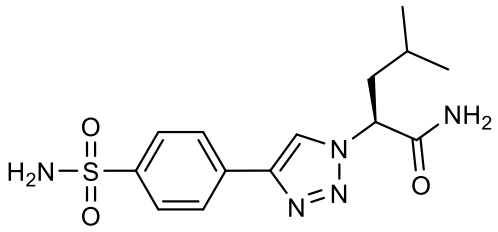
67 mg L-leucinamide hydrochloride (0.40 mmol) were dissolved in 0.90 mL MeOH/H_2O 5:1 in a 2 mL plastic vial and cooled in an ice bath. 0.20 g K_2CO_3 (1.4 mmol, 3.5 eq) were added, followed by 8 µL of a 0.50 M solution of $CuSO_4 \cdot 5\ H_2O$ in water (4 µmol, 0.01 eq and 0.13 g ISA · H_2SO_4 (0.48 mmol, 1.2 eq). After shaking for 45 min at room temperature, the solvents were evaporated using a SpeedVac vacuum concentrator. The residue was transferred to a larger flask and suspended in 20 mL water. The mixture was extracted 5x with 10-15 mL DCM. After washing the combined organic layers with brine and drying over Na_2SO_4, the solvent was evaporated using a rotary evaporator. The residue was re-dissolved in MeOH, distributed to three Eppendorf cups and evaporated again to yield solid white pellets (total: 56 mg, 0.36 mmol, 90%).

^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.26 (s, 1H), 5.85 (s, 1H), 3.96 (dd, *J* = 9.3, 4.5 Hz, 1H), 1.81 – 1.91 (m, 1H), 1.84 – 1.71 (m, 1H), 1.74 – 1.63 (m, 1H), 0.99 (d, *J* = 0.8 Hz, 3H), 0.98 (d, *J* = 0.9 Hz, 3H).^{13}C NMR (100 MHz, $CDCl_3$) δ: 172.8, 62.9, 41.4, 25.1, 23.2, 21.6.

HRMS (ESI): m/z calcd. for $C_6H_{12}N_4ONa$ $[M+Na]^+$ 179.09033, found: 179.09402.

IR (ATR-FITR, cm^{-1}): 3364 (m, NH), 3188 (m, NH), 2958 (w), 2105 (vs, N_3), 1636 (vs, C=O), 1468 (m), 1431 (m), 1410 (m), 1390 (m), 1372 (m), 1343 (m), 1313 (m), 1296 (m), 1247 (s), 1228 (m), 1171 (w), 1141 (w), 1125 (w), 1097 (w), 1047 (w), 966 (w), 787 (m), 722 (s), 665 (s), 599 (s), 563 (m), 522 (m), 439 (m), 394 (m).

Hit1



18 mg N_3-*L*-Leu-NH_2 (0.12 mmol) were dissolved in 100 µL MeOH/THF 1:1 and 20 mg 4-ethynylbenzenesulfonamide (0.11 mmol) in 30 µL DMSO. A CuAAC mixture was prepared from $CuSO_4$ in water (4 µL, 0.50 M, 2 µmol), BTTP in DMSO (10 µL, 0.20 M, 2 µmol) and sodium ascorbate in water (40 µL, 0.50 M, 20 µmol), and was added to the solution of the alkyne, together with 150 µL MeOH, 150 µL H_2O, and 100 µL THF, in an attempt to improve solubility. The azide solution was added last. LC-MS suggested completion of the reaction after 0.5 h, the total reaction time was 1 h. The solvents were evaporated using a SpeedVac vacuum concentrator and the crude product purified by reverse-phase chromatography (50 g C18 silica, A: H_2O + 0.05% FA, B: ACN + 0.05% FA, 5 → 100% B). Fractions containing the click product were combined and, after evaporation of most of the mobile phase using a rotary evaporator, distributed to three plastic vials and dried using a SpeedVac vacuum concentrator to yield Hit1 as beige powder (total yield: 27 mg, white solid, 0.08 mmol, 73%).

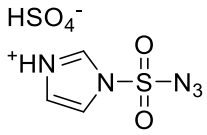
^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.86 (s, 1H), 8.10 – 8.06 (m, 2H), 7.97 (s, 1H), 7.91 – 7.86 (m, 2H), 7.46 (s, 1H), 7.38 (s, 2H), 5.41 (dd, *J* = 10.1, 5.7 Hz, 1H), 2.09 (ddd, *J* = 13.8, 10.1, 5.6 Hz, 1H), 1.92 (ddd, *J* = 14.0, 8.4, 5.7 Hz, 1H), 1.35 – 1.24 (m, 1H), 0.91 (dd, *J* = 9.8, 6.6 Hz, 6H).

^{13}C NMR (100 MHz, DMSO) δ: 169.8, 144.9, 143.1, 134.0, 126.4, 125.3, 121.9, 61.3, 40.4, 24.5, 22.5, 21.4.

HRMS (ESI): m/z calcd. for $C_{14}H_{20}N_5O_3S$ $[M+H]^+$ 338.12814, found: 338.12816.

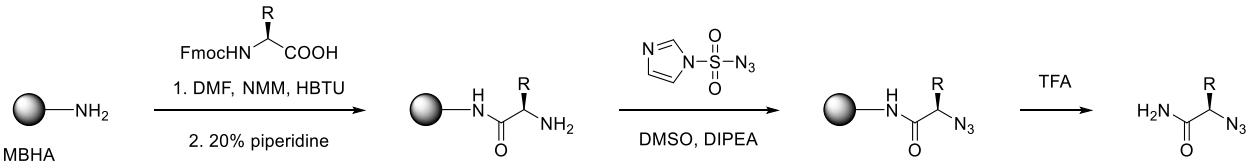
Synthesis of azido amide library

Imidazole-1-sulfonyl azide hydrogen sulfate (ISA)



Imidazole-1-sulfonyl azide hydrogen sulfate was prepared according to published procedures.[4-6]

Synthesis of the azido amide library and templated click



Overview:

Instead of synthesizing and purifying all azido amides separately, an equimolar mixture of the respective Fmoc amino acids was coupled to MBHA resin. The Fmoc group was removed and the liberated N-termini converted to azides using imidazole-1-sulfonyl azide hydrogen sulfate (ISA), similar to the procedure described previously.[7] After deprotection and cleavage from the resin using TFA, the library was semi-purified by preparative HPLC, lyophilized, and used in the templated click experiment as obtained.

Azido amide library:

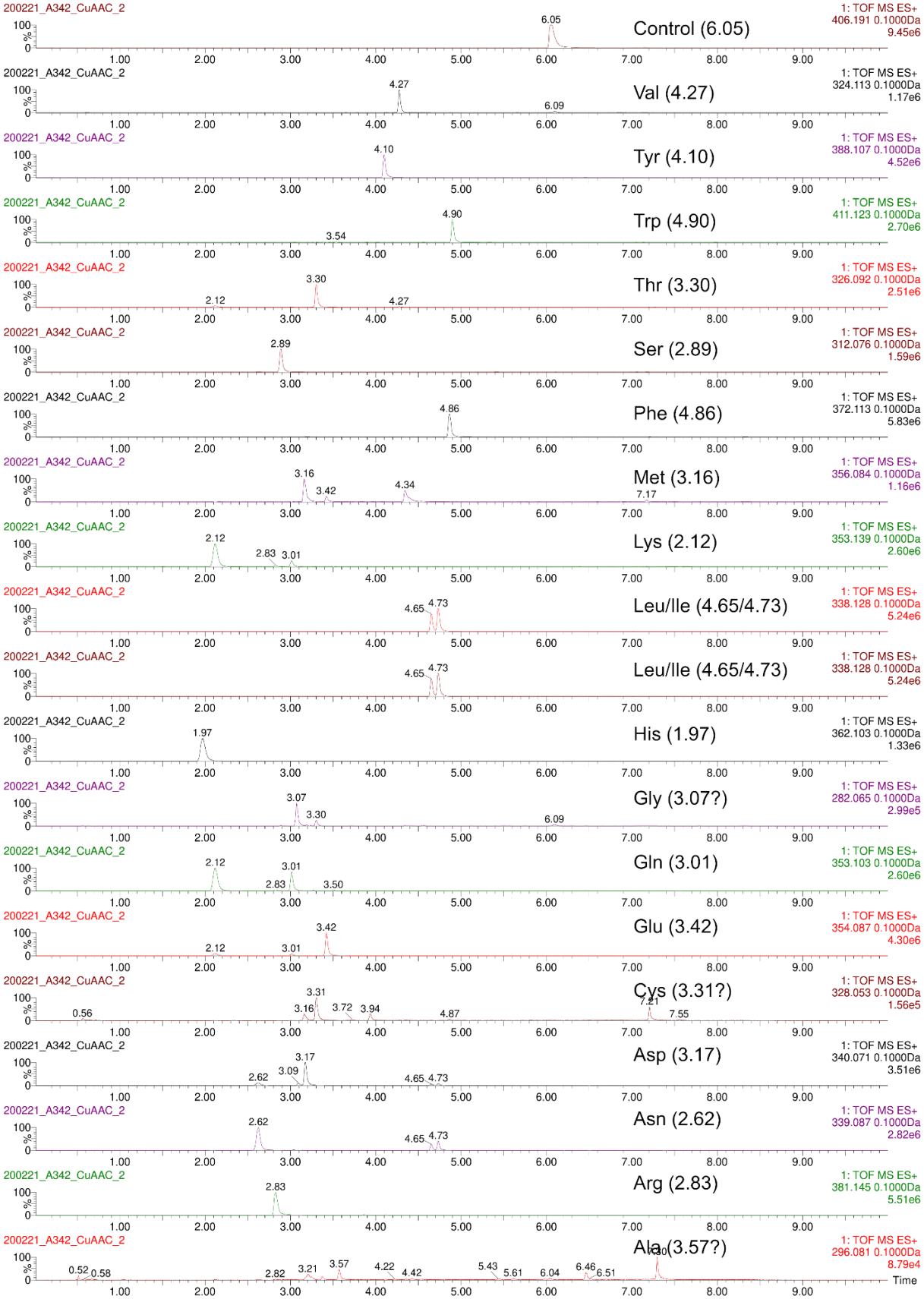
0.21 g Fmoc-MBHA resin (capacity 0.69 mmol/g; 0.14 mmol) were weighed into a 3 mL plastic syringe with a filter, washed with DCM, MeOH/DIPEA and DMF and deprotected with 20% piperidine in DMF (3x 2 mL, at least 5 min each). The resin was washed extensively with DMF, DCM, and again DMF.

0.70 mL of a solution containing each of the twenty standard Fmoc amino acids at 10 mM in DMF (0.14 mmol total Fmoc amino acids, 1 eq relative to the resin) were mixed with 50 µL NMM (0.46 mmol, ca. 3 eq) and 53 mg HBTU (0.14 mmol, 1 eq), added to the resin, and allowed to react while shaking at room temperature for 21 h. A second coupling step (6 h) was performed after a chloranil test still indicated the presence of free amine groups on the resin after that time. The following chloranil test was only slightly positive, suggesting that most of the amines had been acylated. Remaining amines were capped with Ac_2O/NMM/DMF 1:1:3 for 10 min. The Fmoc protecting group was removed with 20% piperidine in DMF (2x 2 mL, 5 min each); the following chloranil test was positive.

After washing the resin with DMSO, 0.12 g ISA · H_2SO_4 (0.44 mmol, ca. 3 eq) in 0.80 mL DMSO, 0.21 mL DIPEA (1.2 mmol, ca. 9 eq) and 0.20 mL DMF were added. Mild bubbling was observed, assumed to be nitrogen released from decomposing ISA. The mixture was shaken for 45 min at room temperature in darkness and then washed with DMF. A chloranil test was still slightly positive, so the resin was washed again with DMF/DIPEA 4:1 and the diazotransfer repeated with 59 mg ISA (0.22 mmol) in 0.80 mL DMSO and 75 µL DIPEA (0.44 mmol) for 70 min, after which a chloranil test was negative.

The library was cleaved from the resin with 3x 1 mL cleavage cocktail (TFA/TIPS/ H_2O 90:2:8) over a total of 1.5 h. After evaporation of the acid the library was "purified" by preparative HPLC by combining all fractions after the one containing the most polar azido amides (Arg, Lys, His) up the least polar ones (Phe, Trp) and discarding all fractions outside that range.

The presence of each compound was confirmed indirectly by performing a copper-catalyzed alkyne-azide click reaction with 4-ethynylbenzenesulfonamide and searching for the click products by UPLC-MS. This also provided the retention times of the compounds to be formed in the templated click experiment. 16 out of the 19 expected compounds (Pro was part of the amino acid mixture but cannot be transformed into an azide) were found, only the presence of the Gly, Ala and Cys compounds seemed questionable. The azido amide library was not expected to contain all compounds in perfectly equal ratios and the ratios were not quantified.

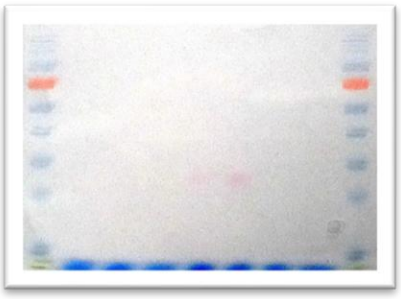

200221_A342_CuAAC_2
1: TOF MS ES+
406.191 0.1000Da
9.45e6
Control (6.05)
6.05
200221_A342_CuAAC_2
1: TOF MS ES+
324.113 0.1000Da
1.17e6
Val (4.27)
4.27
6.09
200221_A342_CuAAC_2
1: TOF MS ES+
388.107 0.1000Da
4.52e6
Tyr (4.10)
4.10
200221_A342_CuAAC_2
1: TOF MS ES+
411.123 0.1000Da
2.70e6
Trp (4.90)
4.90
3.54
200221_A342_CuAAC_2
1: TOF MS ES+
326.092 0.1000Da
2.51e6
Thr (3.30)
3.30
2.12
4.27
200221_A342_CuAAC_2
1: TOF MS ES+
312.076 0.1000Da
1.59e6
Ser (2.89)
2.89
200221_A342_CuAAC_2
1: TOF MS ES+
372.113 0.1000Da
5.83e6
Phe (4.86)
4.86
200221_A342_CuAAC_2
1: TOF MS ES+
356.084 0.1000Da
1.16e6
Met (3.16)
3.16
3.42
4.34
7.17
200221_A342_CuAAC_2
1: TOF MS ES+
353.139 0.1000Da
2.60e6
Lys (2.12)
2.12
2.83
3.01
200221_A342_CuAAC_2
1: TOF MS ES+
338.128 0.1000Da
5.24e6
Leu/Ile (4.65/4.73)
4.65
4.73
200221_A342_CuAAC_2
1: TOF MS ES+
338.128 0.1000Da
5.24e6
Leu/Ile (4.65/4.73)
4.65
4.73
200221_A342_CuAAC_2
1: TOF MS ES+
362.103 0.1000Da
1.33e6
His (1.97)
1.97
200221_A342_CuAAC_2
1: TOF MS ES+
282.065 0.1000Da
2.99e5
Gly (3.07?)
3.07
3.30
6.09
200221_A342_CuAAC_2
1: TOF MS ES+
353.103 0.1000Da
2.60e6
Gln (3.01)
2.12
3.01
2.83
3.50
200221_A342_CuAAC_2
1: TOF MS ES+
354.087 0.1000Da
4.30e6
Glu (3.42)
3.42
2.12
3.01
200221_A342_CuAAC_2
1: TOF MS ES+
328.053 0.1000Da
1.56e5
Cys (3.31?)
3.31
0.56
3.16
3.72
3.94
4.87
7.21
7.55
200221_A342_CuAAC_2
1: TOF MS ES+
340.071 0.1000Da
3.51e6
Asp (3.17)
3.17
2.62
3.09
4.65
4.73
200221_A342_CuAAC_2
1: TOF MS ES+
339.087 0.1000Da
2.82e6
Asn (2.62)
2.62
4.65
4.73
200221_A342_CuAAC_2
1: TOF MS ES+
381.145 0.1000Da
5.51e6
Arg (2.83)
2.83
200221_A342_CuAAC_2
1: TOF MS ES+
296.081 0.1000Da
8.79e4
Ala (3.57?)
3.57
0.52
0.58
2.82
3.21
4.22
4.42
5.43
5.61
6.04
6.46
6.51
7.30
100
%
0
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
9.00
Time

Labeling of hCA-II with Cy3-NHS

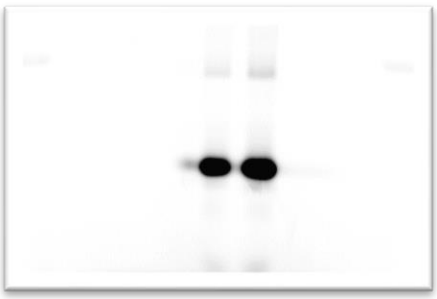
Ca. 3 mL hCA-II (0.8 mg/mL (ca. 27 µM) in 50 mM Tris-H_2SO_4, pH 7.8; M = 29246 Da) were dialyzed against 2x 1 mL HEPES buffer (10 mM, 200 mM NaCl, pH 8.3; 4 h + 16 h) at 4 °C. The protein concentration after centrifugation (21 000 g, 10 min, 4 °C) was estimated using a NanoDrop One spectrometer to 0.8—0.9 mg/mL at a total volume of approximately 3 mL (-> ca. 80—90 nmol).

To 250 µL of this solution (ca. 7 nmol) 1 µL of Cy3 NHS ester solution (50 mM in DMSO) was added and the reaction left to proceed for 2 h on ice. Excess labeling reagent was removed using Zeba spin columns conditioned with HEPES buffer (20 mM, 200 mM NaCl, pH 7.4).

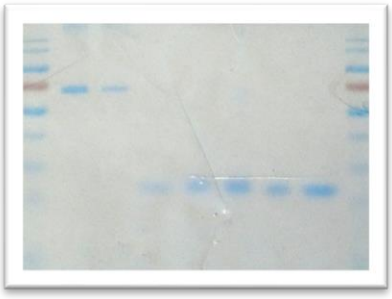
Verification of labeling: SDS-PAGE (12%; 30 mA):
Lanes 1 and 9: PageRuler
Lanes 2—3: BSA, ca. 1 µg and 0.5 µg
Lane 4: hCA-II without label, ca. 0.8 µg
Lanes 5 and 6: hCA-II/Cy3, ca. 0.5 and 1 µg
(Lanes 7 and 8: hCA-II treated with DMSO as control)



Visible light image of the gel after electrophoresis. The Cy3-labeled hCA-II is visible as pink spots in lanes 5 and 6.



Fluorescence image of the gel after electrophoresis. The Cy3-labeled hCA-II lights up in lanes 5 and 6.



Visible light image of the gel after staining with Coomassie Blue.

Preparation of the synthetic peptide library

Preparation of topologically segregated library:[8] 3g (0.78 mmol) of Tentagel resin (S-NH_2, 130 µm) was swollen overnight in ddH_2O. The resin was drained and quickly resuspended in DCM/Et_2O = 55/45

mixture (20 mL) containing Fmoc-Met-OPbf (0.1 equiv., 42 mg), Boc-Phe-OSu (0.4 equiv., 113 mg) and DIPEA (0.5 equiv., 68 µL). The resin was rotated for 30 min. The resin was then washed with DCM/Et_2O, DMF and DCM again. Quality of the segregation was verified by chloranil test. Then, the resin was swollen in DMF and reacted with Fmoc-Met-OH (1.5 equiv, 435 mg), HBTU (1.5 equiv, 444 mg), DIPEA (4 equiv.) in DMF (20 mL) for 2 hours. After washing with DMF and DCM, the Boc group from Phe was removed by TFA/DCM = 1/1 mixture (20 mL) for 1 hour. After washing the resin with DMF, DCM, DCM + DIPEA the capping of the free amino groups was performed with Ac_2O (50 equiv., 3.7 mL) and DIPEA (2 mL) in DMF (13.5 mL) for 45 min. The resin was washed with DMF, DCM, MeOH, DCM and DMF. The Fmoc group was removed by 20% piperidine in DMF (30 min + 10 min).

The library was prepared using the split-and-mix protocol:[9] The above library (ca. 7g of dry library) was equally divided into 13 polypropylene vials with a frit at the bottom. This step is best done by suspending the library in DMF and pipetting. One of the following Fmoc-protected amino acids was then added to each vial: Ala, Glu, Phe, Gly, His, Lys, Leu, Pro, Gln, Ser, Thr, Trp, Tyr. 1.5 equiv of each amino acid were used (0.78 mmol/13 = 0.06 mmol = 1 equiv.). The coupling was performed with NMM as base (3 equiv. to amino acid) and HBTU (1.05 equiv. to amino acid) in DMF (4.5 mL/vial). Coupling of each amino acid was performed 2 times for 1 hour. After each coupling step the library was combined, Fmoc deprotection was carried out with 20% piperidine in DMF and divided again into 13 vials for coupling of the next amino acid. In this manner, a 7-mer peptide library was constructed.

700 mg (ca. 0.078 mmol) of this library was treated with 20% piperidine in DMF (2x2.5 mL) to remove the *N*-terminal Fmoc group. Next, the resin was modified with Fmoc-L-Lys(Poc) (1 equiv., 35 mg), HATU (1 equiv., 30 mg) and NMM (26 uL) in DMF (2 mL) for 1 hour. This coupling step was repeated once again using the same amount of reagents. The Fmoc group was deprotected with 20% piperidine in DMF (2x2.5 mL) for 2x20 min. The resin was washed with DMF, DCM and drained. The side chain protecting groups were removed by treating the resin with a mixture of TFA/TIPS/H_2O = 95/2.5/2.5 (4 mL) for 1.5 hours. The library was then washed with DCM, DMF, DCM and dried for long term storage in the freezer.

150 mg (ca. 0.026 mmol) of the alkyne-containing peptide library was then click modified with the sulfonamide Inh3 as follows: The resin was washed with a mixture of H_2O/tBuOH = 3/2 several times. The sulfonamide Inh3 (2.5 equiv. 18.5 mg) dissolved in DMSO (0.5 M) was added to the library suspended in H_2O/tBuOH = 3/2 (1 mL). In a plastic vial, $CuSO_4x5H_2O$ (20 µL of 100 mM in water) with BTTP ligand[10] (30 µL of 100 mM in DMSO) was combined. To this deep blue solution was added solution of Na-ascorbate (30 µL of 100 mM in water). This colorless solution was added to the resin with the azide and the mixture was rotated overnight at room temperature under argon atmosphere followed by incubation at 40 °C for 2 hours. The resin was then washed with DMF, water, EDTA solution, water and DMF. For long term storage the resin was washed with DCM, vacuum dried and stored in a freezer.

Resynthesis of hit peptides

Peptide conjugates Hit2-5 were assembled in a solid-phase synthesizer Liberty Blue (CEM, USA) by stepwise coupling of the corresponding Fmoc-amino acids to the growing chain on TENTA GEL R RAM resin (200-400 mesh, 0.19 mmol/g) or Rink Amide MBHA resin (200-400 mesh, 0.68 mmol/g) from IRIS,

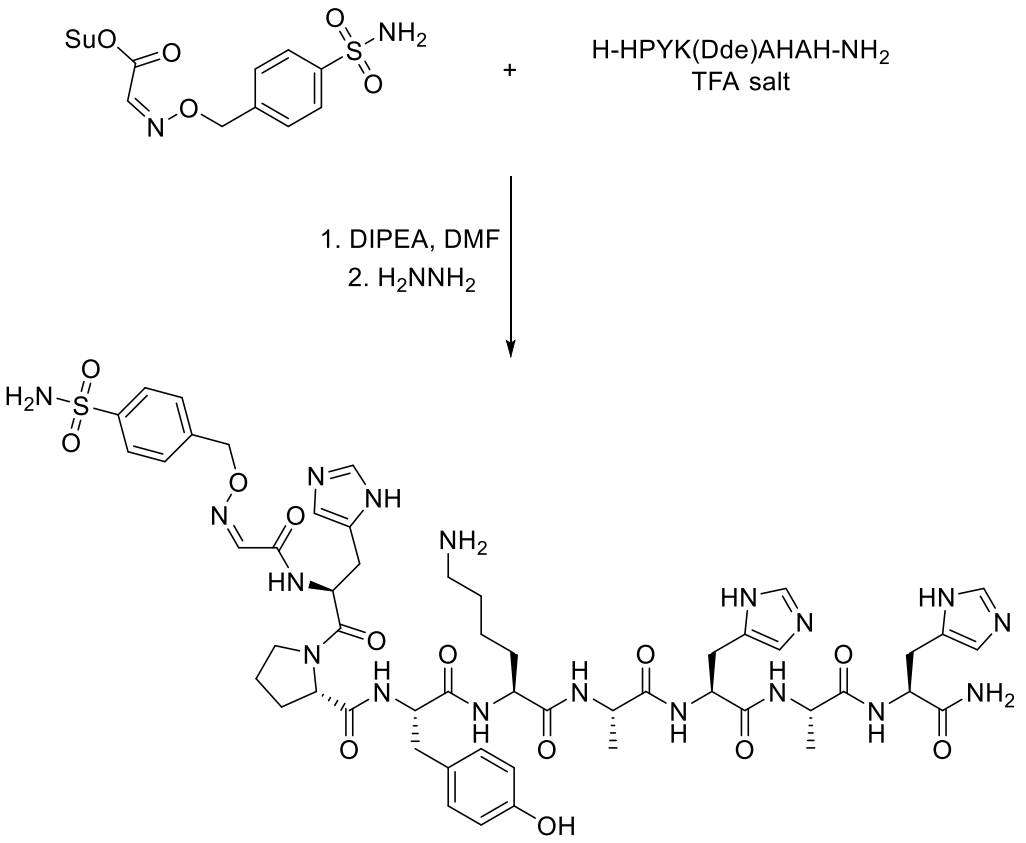
Biotech GmbH, Marktredwitz, Germany. Fully protected peptide resins were synthesized according to a standard procedure involving (i) cleavage of the Nα- Fmoc protecting group with 20% piperidine in DMF, (ii) coupling, mediated by mixtures of coupling reagents DIC/Oxyma in DMF.

On completion of syntheses, deprotection and detachment of linear peptides from the resins were carried out simultaneously, using a TFA/H2O/TIS = 95/2.5/2.5 cleaving mixture. Each of the resins was washed with DCM and the combined filtrates were evaporated at room temperature. The precipitated residues were triturated with *t*-butyl-methylether, collected by suction and dried by lyophilization. The linear peptides were purified by RP HPLC using a Waters instrument with Delta 600 pump, 2489 UV/VIS detector.

Peptides Hit3-t1-4were synthesized using liquid-phase peptide synthesis. Standard Boc-protecting group strategy was employed, while O*t*Bu group was used to protect serine hydroxyl residue. Typical procedure was: Boc-AA-OH (1 equiv.) and HATU (1 equiv.) were dissolved in DMF. This solution was cooled to 0 °C and DIPEA (3 equiv.) was added. After stirring for 10 min at 0 °C, H-$(AA)_x$-NH_2 (0.8 equiv.) was added and the reaction mixture was stirred at 0 °C for 1 hour, then warmed to room temperature. After completion, solvent was evaporated under reduced pressure and the residue was redissolved in EtOAc. The organic phase was washed with sat. $NaHCO_3$ (2x), water (2x), 10% $KHSO_4$ (2x) and brine (1x). It was then dried with $MgSO_4$ and solvent was removed under reduced pressure to obtain a crude peptide. Peptides could be purified by normal phase silica gel chromatography, if necessary. Boc-protecting group removal was performed under standard conditions. The peptide was dissolved in TFA/DCM = 1/1 and stirred for 1 hour. Then solvents were removed to obtain a crude peptide as TFA salt.

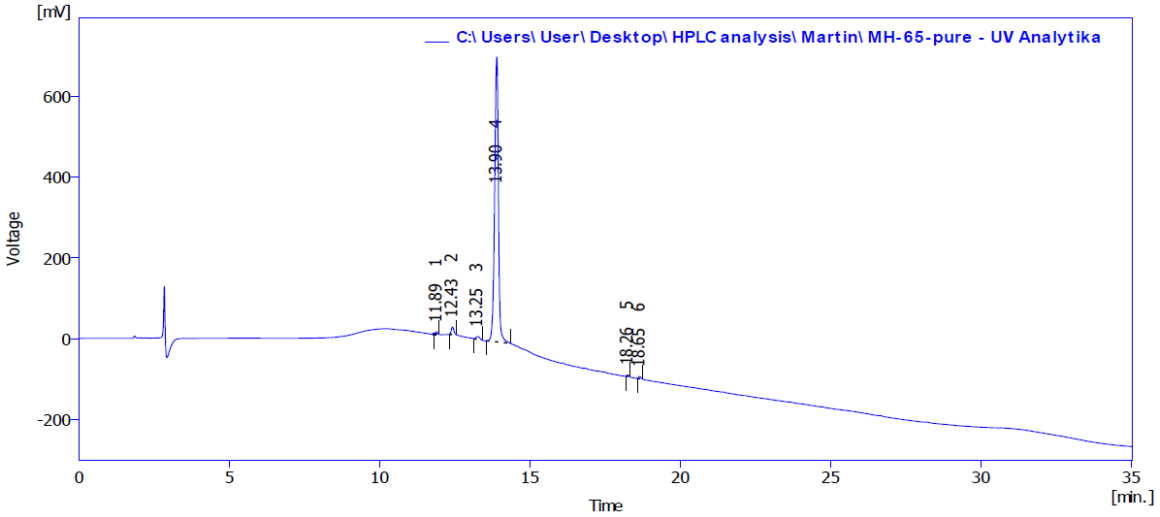
Hit2

H-HPYK(Dde)AHAH-NH_2 TFA salt (14 mg, 9 µmol) and DIPEA (17 µL, 90 µmol, 10 equiv.) were dissolved in anhydrous DMF (0.8 mL). Solution of Inh2-COOSu (2.8 mg, 8 µmol, 0.9 equiv.) in anhydrous DMF (0.2 mL) was added dropwise. The resulting mixture was stirred at room temperature for 1 hour. Dde deprotection was achieved by addition of hydrazine (1M in THF, 45 µL, 45 µmol) into the crude mixture. After 2 hours, the reaction mixture was evaporated to dryness under reduced pressure and the crude product was purified by RP HPLC to obtain product as a white lyophilizate (2.5 mg, 19 %).



LRMS (ESI): m/z calcd. for $C_{53}H_{71}N_{18}O_{13}S$ $[M+H]^+$ 1199.5, found: 1199.5.

HRMS (ESI): m/z calcd. for $C_{53}H_{71}N_{18}O_{13}S$ $[M+H]^+$ 1199.51632, found: 1199.51673.

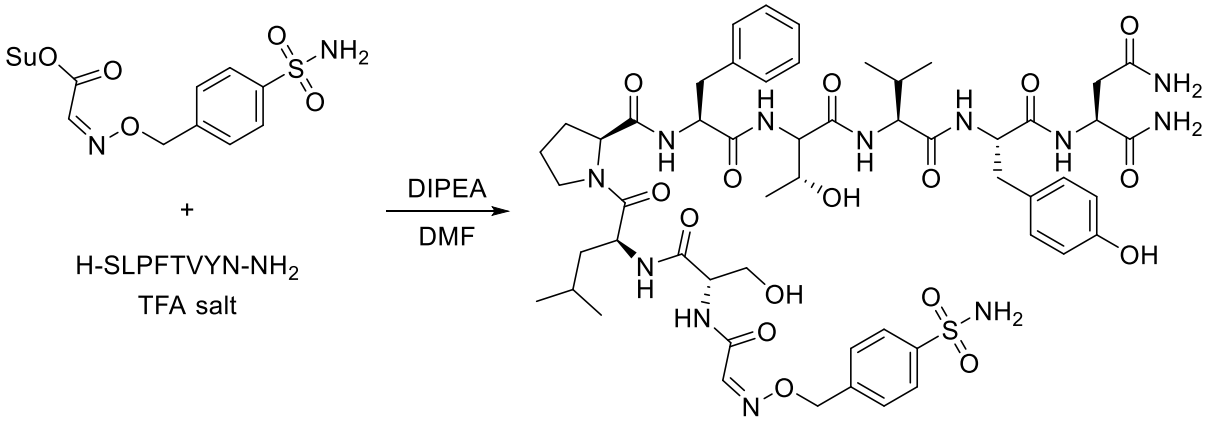


Result Table (Uncal - C:\Users\User\Desktop\HPLC analysis\Martin\MH-65-pure - UV Analytika)

	Reten. Time [min]	Area [%]
1	11.892	0.6
2	12.428	1.8
3	13.252	0.9
4	13.900	96.0
5	18.256	0.3
6	18.652	0.5
	Total	100.0

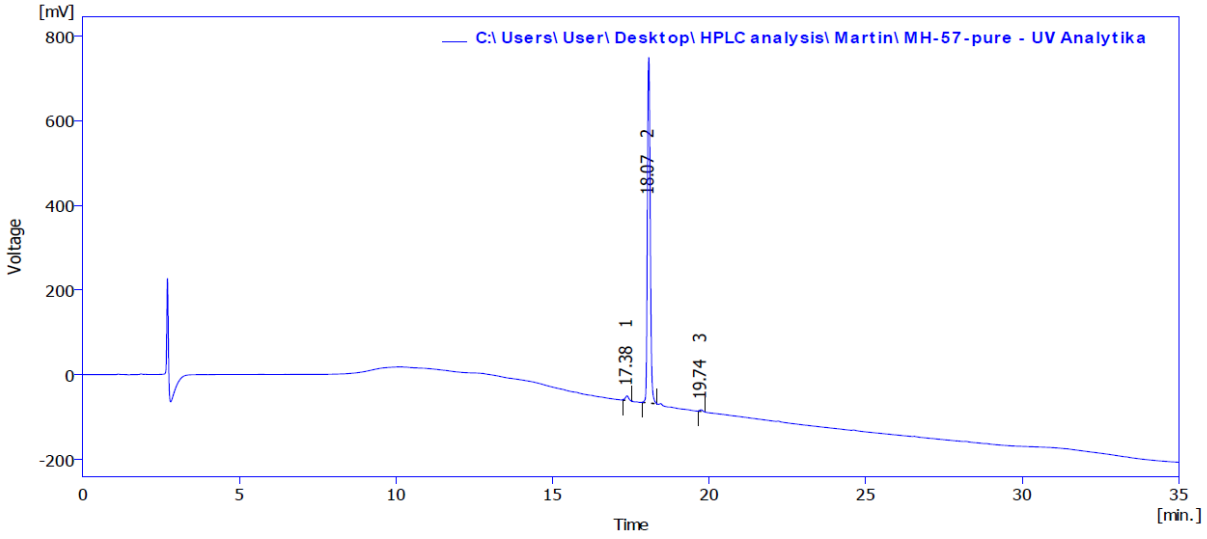
Hit3

H-SLPFTVYN-NH_2 TFA salt (15 mg, 14 µmol) and DIPEA (13 µL, 70 µmol, 5.0 equiv.) were dissolved in anhydrous DMF (0.8 mL). Solution of Inh2-COOSu (2.5 mg, 14 µmol, 0.5 equiv.) in anhydrous DMF (0.2 mL) was slowly added to the solution over 1 hour. The resulting mixture was stirred at room temperature for 2 hours. The reaction mixture was then evaporated to dryness under reduced pressure and the crude product was purified by RP HPLC to obtain product as a white lyophilisate (3 mg, 36 %).



LRMS (ESI): m/z calcd. for $C_{54}H_{75}N_{12}O_{16}S$ $[M+H]^+$ 1179.5, found: 1179.5.

HRMS (ESI): m/z calcd. for $C_{54}H_{75}N_{12}O_{16}S$ $[M+H]^+$ 1179.51392, found: 1179.51428.



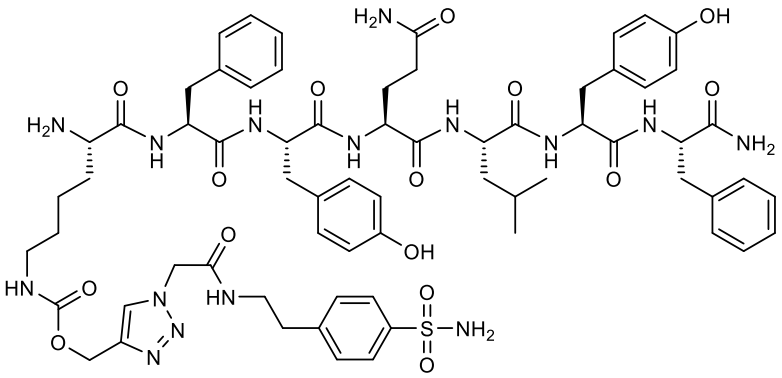
Result Table (Uncal - C:\Users\User\Desktop\HPLC analysis\Martin\MH-57-pure - UV Analytika)

	Reten. Time [min]	Area [%]
1	17.380	1.6
2	18.072	97.9
3	19.740	0.5
	Total	100.0

Hit4

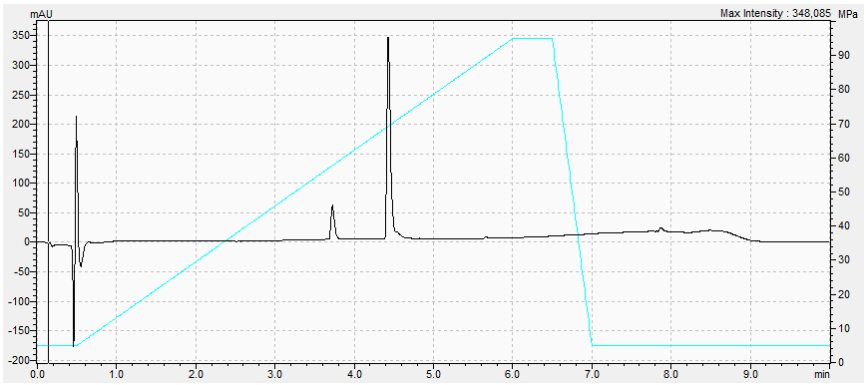
Fmoc-L-Lys(Poc) (20.5 mg, 46 µmol) and TSTU (13.7 mg, 46 µmol, 1.0 equiv.) were dissolved in anhydrous DMF (1 mL). After addition of DIPEA (30 µL, 172 µmol, 3.7 equiv.) reaction mixture was stirred at room temperature for 2 hours. The peptide H-FYQLYF-NH_2 (40 mg, 46 µmol, 1.0 equiv.) was then added and the reaction mixture was stirred overnight. Fmoc deprotection was achieved by addition of 0.1 mL of piperidine into the crude mixture. After 1.5 hours, reaction mixture was evaporated to dryness under reduced pressure and the crude product was purified by RP HPLC to obtain product as a TFA salt (14 mg, 28 %).

The peptide was dissolved in 400 µL tBuOH/H_2O 2:3, 400 µL DMF and 300 µL H_2O. To 38 µL of a 100 mM $CuSO_4$ solution in H_2O (3.8 µmol), 150 µL of a 50 mM solution of BTTP in DMSO (7.5 µmol) were added to form a dark blue copper(II) complex. Addition of 150 µL of an aqueous solution of sodium ascorbate (100 mM, 15 µmol) under argon reduced the copper and rendered the solution colorless. This CuAAC mixture was added to the peptide solution, followed by 280 µL of a 100 mM solution of Inh3 in DMSO (28 µmol, ca. 2.5 eq). The mixture was left to react under argon for 100 min at room temperature and purified by semi-preparative HPLC to yield 10 mg of a white, fluffy powder after lyophilization (7 µmol).



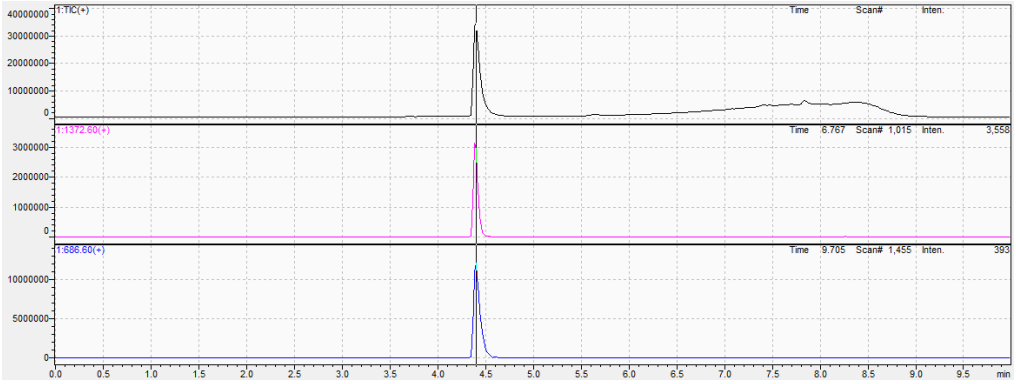
Chemical Formula: $C_{67}H_{85}N_{15}O_{15}S$
Exact Mass: 1371.6070
Molecular Weight: 1372.5670

PDA at 200 nm



The impurity at 3.7 min in the UV trace was not identified. At 200 nm the ratio of the peaks is approximately 14:86.

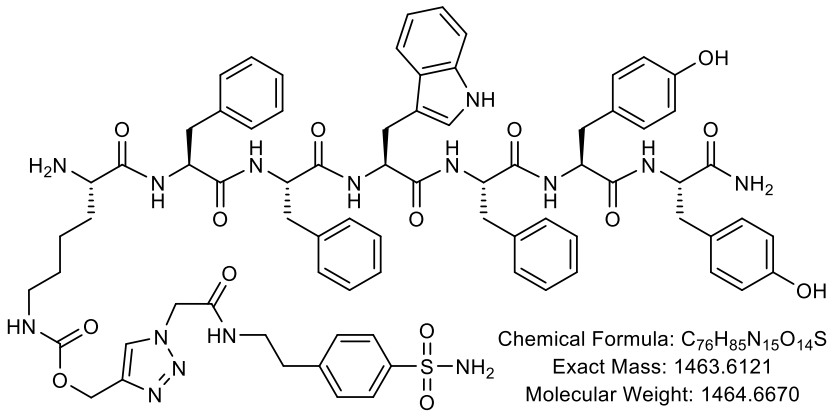
MS (TIC, 1372.6 and 686.6 m/z)



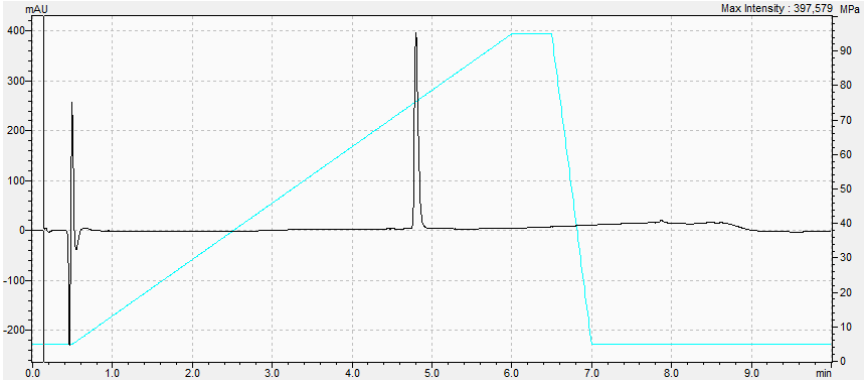
Hit5

Fmoc-L-Lys(Poc) (18.5 mg, 41 µmol) and TSTU (12.4 mg, 41 µmol, 1.0 equiv.) were dissolved in anhydrous DMF (2 mL). After addition of DIPEA (40 µL, 232 µmol, 5.5 equiv.) reaction mixture was stirred at room temperature for 2 hours. The peptide H-FFWFYY-NH_2 (40 mg, 41 µmol, 1.0 equiv.) was then added and the reaction mixture was stirred overnight. Fmoc deprotection was achieved by addition of 0.15 mL of piperidine into the crude mixture. After 1 hour, reaction mixture was evaporated to dryness under reduced pressure and the crude product was purified by RP HPLC to obtain product as a TFA salt (9 mg, 17 %).

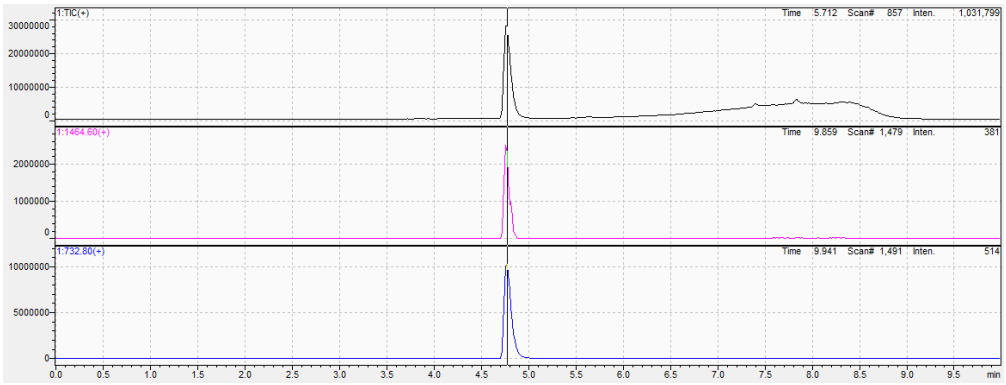
The peptide was dissolved in 1.2 mL DMF/H_2O 1:1 mixture. To 25 µL of a 100 mM $CuSO_4$ solution in H_2O (2.5 µmol), 100 µL of a 50 mM solution of BTTP in DMSO (5 µmol) were added to form a dark blue copper(II) complex. Addition of 100 µL of an aqueous solution of sodium ascorbate (100 mM, 10 µmol) under argon reduced the copper and rendered the solution colorless. This CuAAC mixture was added to the peptide solution, followed by 185 µL of a 100 mM solution of Inh3 in DMSO (18.5 µmol, ca. 2.5 eq). The mixture was left to react under argon for 100 min at room temperature and purified by semi-preparative HPLC to yield 6 mg of a white, fluffy powder after lyophilization (4 µmol).



PDA at 200 nm

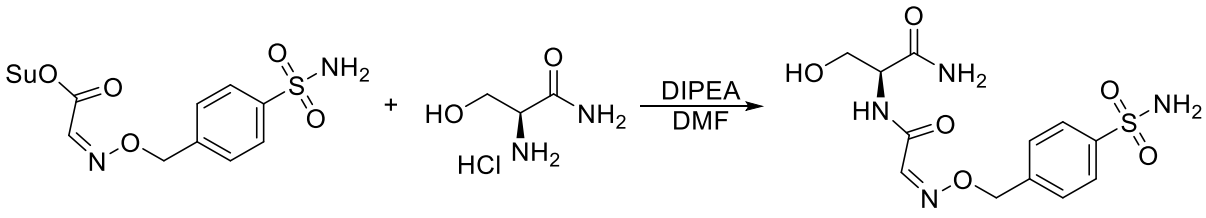


MS (TIC, 1464 and 732 m/z)



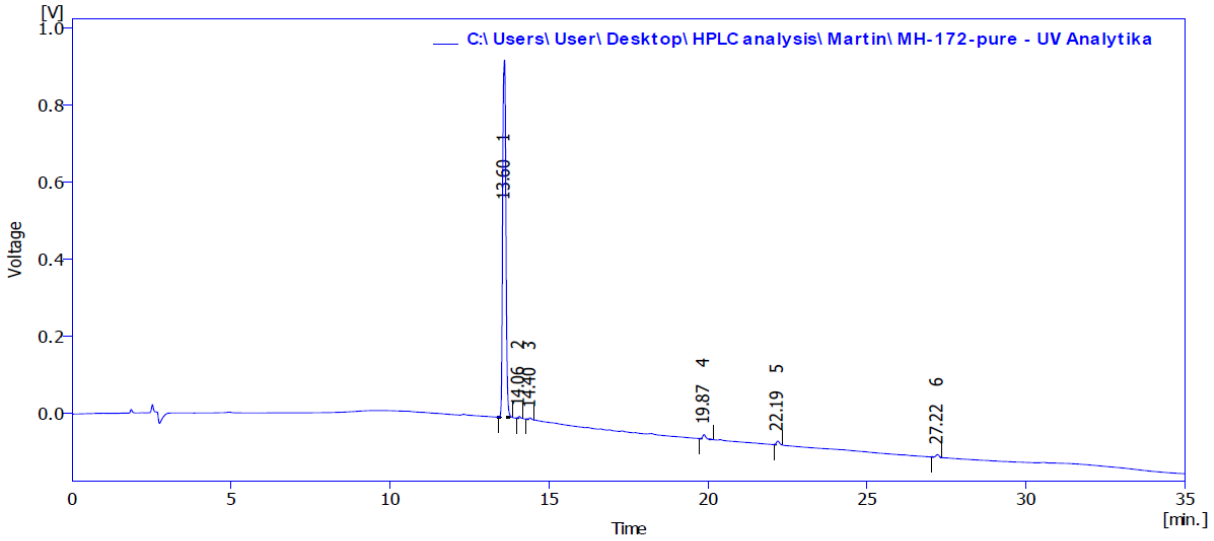
Hit3-t1

H-Ser-NH_2 HCl salt (14 mg, 0.10 mmol) and TEA (36 µL, 0.25 mmol, 2.5 equiv.) were dissolved in anhydrous DMF (0.8 mL). Solution of Inh2-COOSu (30 mg, 0.08 mmol, 0.8 equiv.) in anhydrous DMF (0.2 mL) was added to the solution dropwise. The resulting mixture was stirred at room temperature for 2 hours. The reaction mixture was then evaporated to dryness under reduced pressure and the crude product was purified by RP HPLC to obtain the product as a white powder after lyophilization (13 mg, 45 %).



LRMS (ESI): m/z calcd. for $C_{12}H_{16}N_4O_6SNa$ $[M+Na]^+$ 367.1, found: 367.1.

HRMS (ESI): m/z calcd. for $C_{12}H_{16}N_4O_6SNa$ $[M+Na]^+$ 367.06828, found: 367.06833.

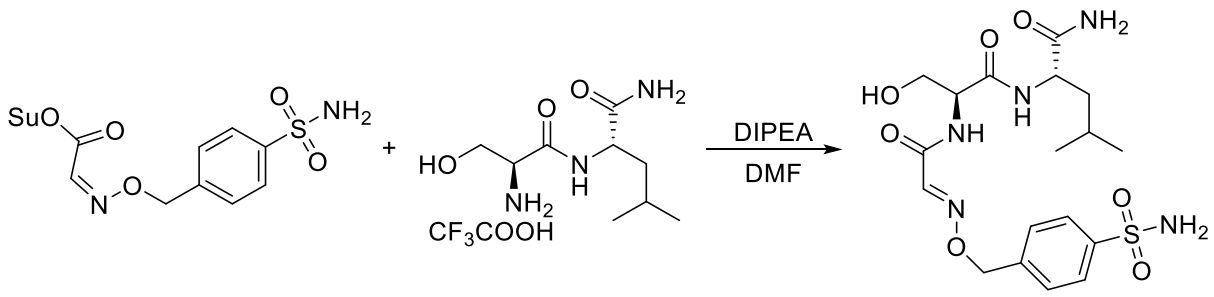


Result Table (Uncal - C:\Users\User\Desktop\HPLC analysis\Martin\MH-172-pure - UV Analytika)

	Reten. Time [min]	Area [%]
1	13.596	96.5
2	14.060	0.3
3	14.404	0.3
4	19.872	1.2
5	22.192	1.0
6	27.216	0.7
	Total	100.0

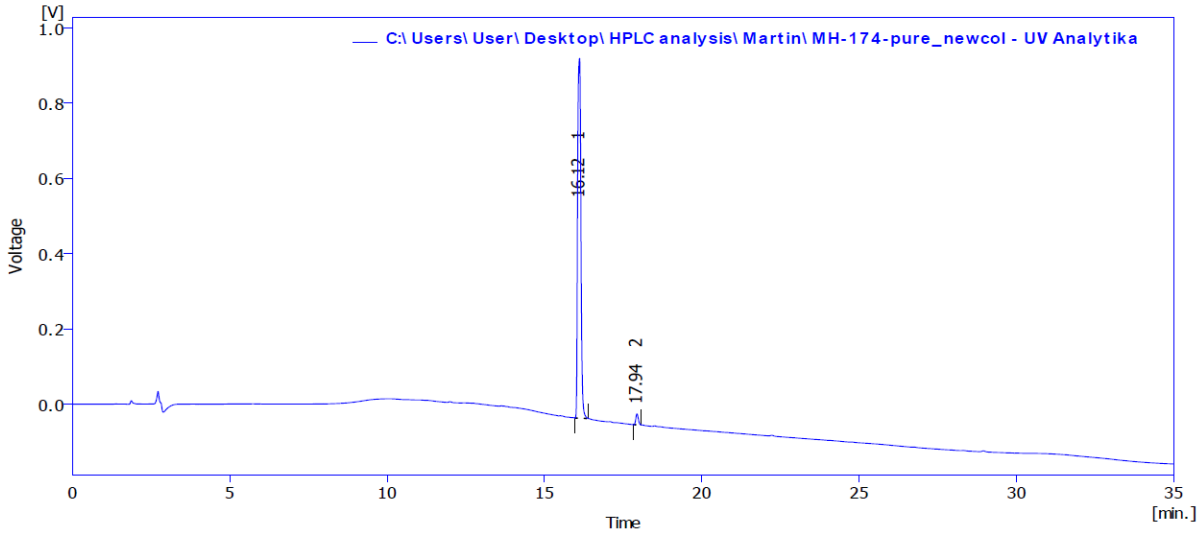
Hit3-t2

H-Ser-Leu-NH_2 TFA salt (33 mg, 0.10 mmol) and TEA (36 µL, 0.25 mmol, 2.5 equiv.) were dissolved in anhydrous DMF (0.8 mL). Solution of Inh2-COOSu (30 mg, 0.08 mmol, 0.8 equiv.) in anhydrous DMF (0.2 mL) was added to the solution dropwise. The resulting mixture was stirred at room temperature for 2 hours. The reaction mixture was then evaporated to dryness under reduced pressure and the crude product was purified by RP HPLC to obtain the product as a white powder after lyophilization (12 mg, 32 %).



LRMS (ESI): m/z calcd. for $C_{18}H_{27}N_5O_7SNa$ $[M+Na]^+$ 480.2, found: 480.2.

HRMS (ESI): m/z calcd. for $C_{18}H_{27}N_5O_7SNa$ $[M+Na]^+$ 480.15234, found: 480.15199.

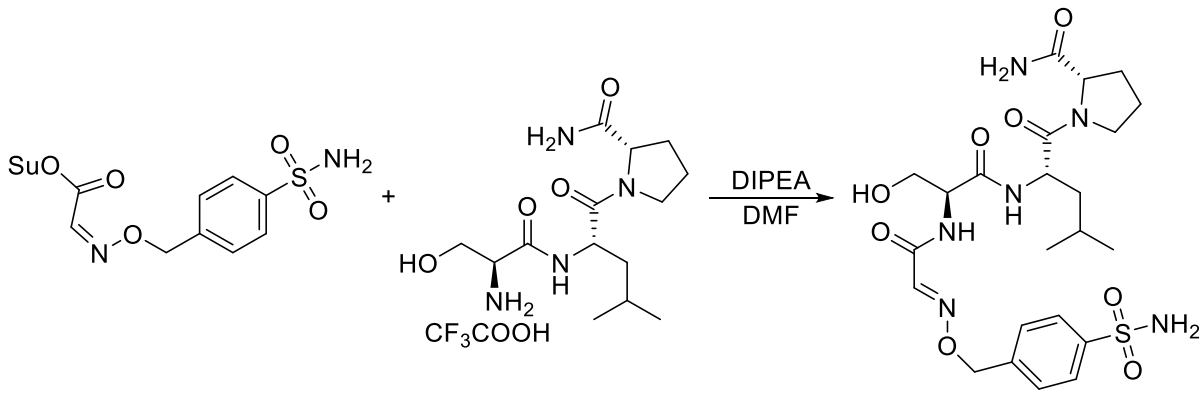


Result Table (Uncal - C:\Users\User\Desktop\HPLC analysis\Martin\MH-174-pure_newcol - UV Analytika)

	Reten. Time [min]	Area [%]
1	16.116	97.8
2	17.940	2.2
	Total	100.0

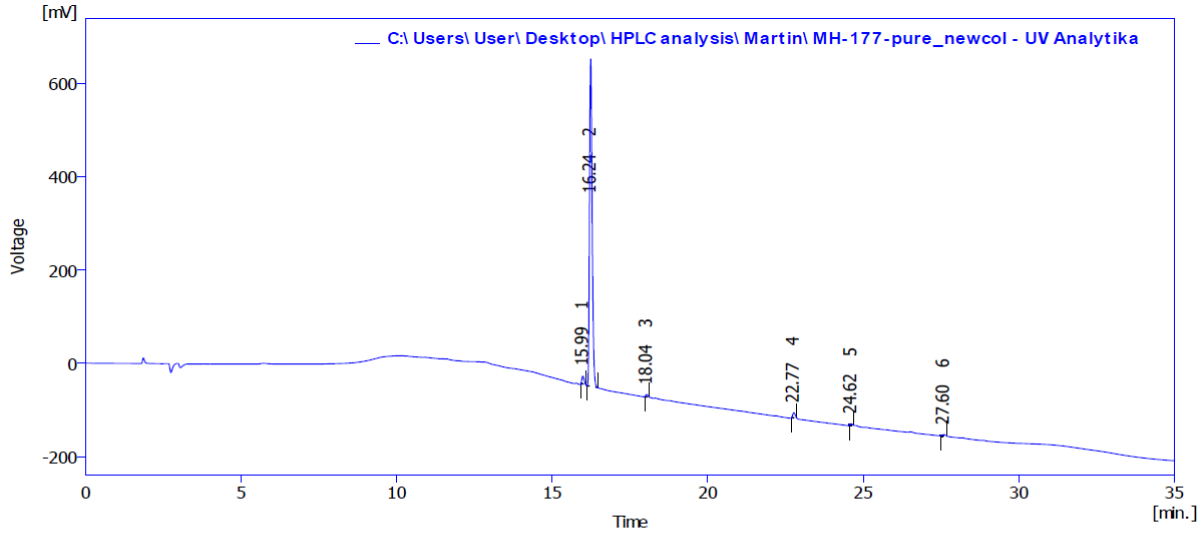
Hit3-t3

H-Ser-Leu-Pro-NH_2 TFA salt (29 mg, 68 µmol) and TEA (24 µL, 170 µmol, 2.5 equiv.) were dissolved in anhydrous DMF (0.8 mL). Solution of Inh2-COOSu (19 mg, 54 µmol, 0.8 equiv.) in anhydrous DMF (0.2 mL) was added to the solution dropwise. The resulting mixture was stirred at room temperature for 2 hours. The reaction mixture was then evaporated to dryness under reduced pressure and the crude product was purified by RP HPLC to obtain the product as a white powder after lyophilization (18 mg, 60 %).



LRMS (ESI): m/z calcd. for $C_{23}H_{34}N_6O_8SNa$ $[M+Na]^+$ 577.2, found: 577.2.

HRMS (ESI): m/z calcd. for $C_{23}H_{34}N_6O_8SNa$ $[M+Na]^+$ 577.20510, found: 577.20485.

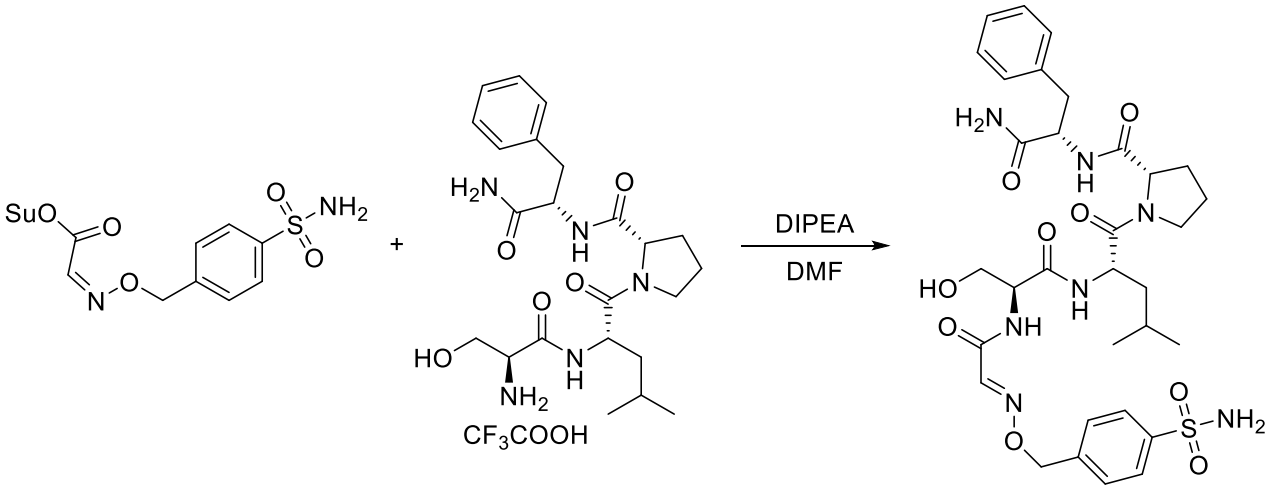


Result Table (Uncal - C:\Users\User\Desktop\HPLC analysis\Martin\MH-177-pure_newcol - UV Analytika)

	Reten. Time [min]	Area [%]
1	15.988	1.9
2	16.240	95.4
3	18.040	0.5
4	22.772	1.4
5	24.616	0.3
6	27.596	0.4
	Total	100.0

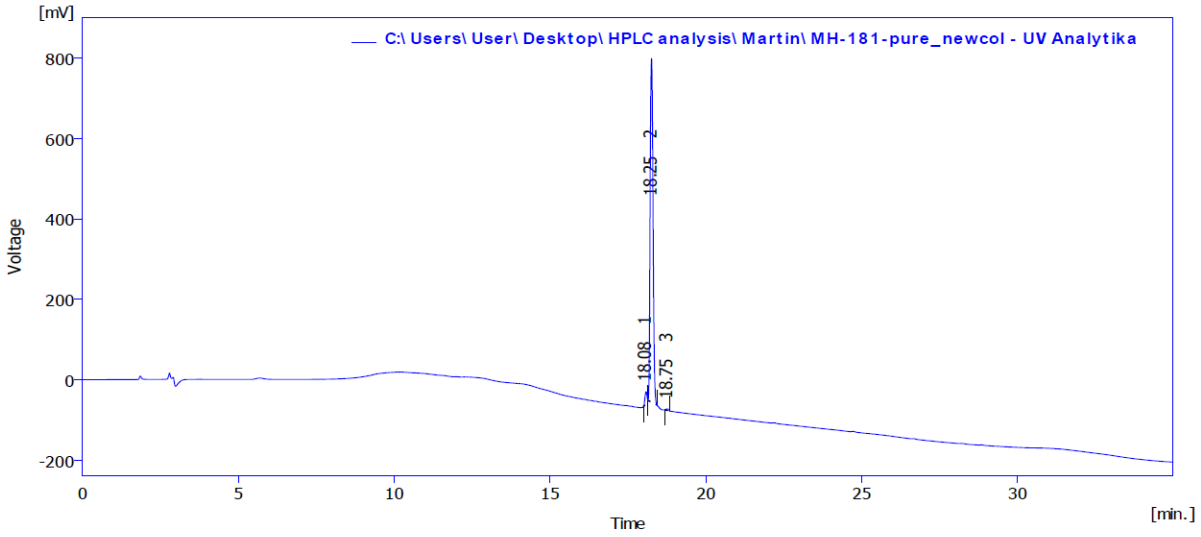
Hit3-t4

H-Ser-Leu-Pro-Phe-NH_2 TFA salt (38 mg, 66 µmol) and TEA (24 µL, 170 µmol, 2.5 equiv.) were dissolved in anhydrous DMF (0.8 mL). Solution of Inh2-COOSu (19 mg, 54 µmol, 0.8 equiv.) in anhydrous DMF (0.2 mL) was added to the solution dropwise. The resulting mixture was stirred at room temperature for 2 hours. The reaction mixture was then evaporated to dryness under reduced pressure and the crude product was purified by RP HPLC to obtain the product as a white powder after lyophilization (22 mg, 58 %).



LRMS (ESI): m/z calcd. for $C_{32}H_{43}N_7O_9SNa$ $[M+Na]^+$ 724.3, found: 724.3.

HRMS (ESI): m/z calcd. for $C_{32}H_{43}N_7O_9SNa$ $[M+Na]^+$ 724.27352, found: 724.27330.



Result Table (Uncal - C:\Users\User\Desktop\HPLC analysis\Martin\MH-181-pure_newcol - UV Analytika)

	Reten. Time [min]	Area [%]
1	18.076	2.2
2	18.252	97.5
3	18.752	0.3
	Total	100.0

In situ hCA II-templated click selection

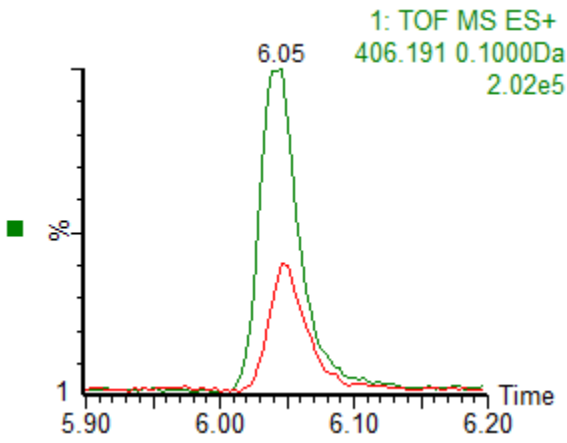
The templated click reaction of an alkyne anchor molecule (4-ethynylbenzenesulfonamide) with a selection of azido amides was performed according to previously published procedure with slight modifications.[11]

The following solutions were prepared:

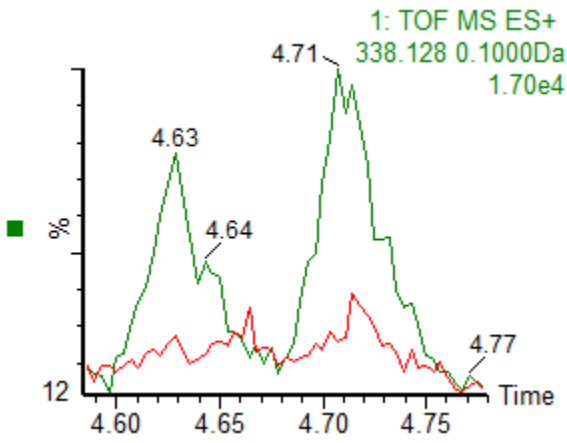
- hCA II, 1.0 mg/mL (34 µM) in 50 mM Tris · H_2SO_4 (pH 7.8)
- BSA, 1.0 mg/mL (15 µM) in 50 mM Tris · H_2SO_4 (pH 7.8)
- 4-ethynylbenzenesulfonamide, 200 mM in DMSO
- N_3-L-Val-NHCy, 20 mM in DMSO (a hit compound identified previosuly[11] was used as a control)
- azido amide library, 0.2 M in DMSO: a 2.3 mg sample of the "purified" and lyophilized library was dissolved in 70 µL DMSO; with an average molecular weight of M ~ 162 Da, the total azide concentration was estimated to be 0.2 M.

To 92.5 µL protein solution (hCA II: 3.1 nmol, BSA: 1.4 nmol) were added 3 µL of the azide library (0.6 µmol), 1.5 µL N3-L-Val-NHCy[11] (0.03 µmol), and 3 µL 4-ethynylbenzenesulfonamide (0.6 µmol), the final concentrations were therefore 6.3 mM total azides, 6 mM 4-ethynylbenzenesulfonamide, and 31 µM hCA II and 14 µM BSA respectively. The samples were then incubated at 37 °C and analyzed after 40 h.

Small molecules not bound to protein were removed by concentrating the protein on a 10 kDa MWCO spin filter membrane (45 min, 14 000 g, 8 °C) and washing 1x with 150 µL Tris buffer with 5% DMSO and 2x 200 µL water (30 min, 14 000 g, 8 °C). The concentrated protein on the membrane was denaturated with 20% TFA in water and the released small molecules collected by centrifugation. The filter was washed 1x with 50 µL aqueous TFA. The combined TFA fractions were lyophilized and re-dissolved in a total of 60 µL ACN/H_2O/0.1% FA, filtered through 0.22 µm spin filters, and then analyzed by UPLC-MS. Narrow m/z traces (± 0.1 Da) of the target compounds at the expected retention times were extracted from the UPLC-MS data of both the hCA II and BSA runs and overlaid. If the trace of the hCA II experiment showed a peak but the trace of the BSA experiment did not or the peak was considerably smaller, the respective compound was considered a hit. After 40 h, this was true only for the control (N3-L-Val-NHCy) and Leu/Ile, of which the Leu compound (Hit 1) was resynthesized for further experiments.



control (N_3-L-Val-NHCy) after 40 h



Leu/Ile after 40 h

Phage-displayed peptide library

Library construction:

S(X)8_TEV library was constructed according to NEB PhD manual. Double stranded oligonucleotide encoding for the N-terminal serine followed by random sequence and the TEV cleavage site (S-$(X)_8$-TEV) was constructed from synthetic oligonucleotide 5'-CATGTTTCGGCCGAGCCCTGAAAATACAGGTTTTCMNNMNNMNNMNNMNNMNNMNNMNNMNNNGAAGAG TGAGAATAGAAAGGTACCCGGG-3' (Sigma-Aldrich) and the 5'-CATGCCCGGGTACCTTTCTATTCTC-3' primer using the Klenow enzyme. Double stranded oligo was further digested with KpnI and EagI (Thermo), purified from 3% agarose gel using Zymoclean gel DNA recovery kit (Zymoresearch cat. #D4001) and inserted into M13KE vector at a 3:1 insert:vector ratio. Ligation mixture was cleaned using the DNA Clean and Concentrator kit (Zymoresearch cat. #D4060) and electroporated into ER2738 bacteria (Lucigen cat. #60522) after 45 minutes of incubation at 37°C the outgrowth was titrated and the yield was 10^7 clones. The initial outgrowth was used to infect 1liter of ER2738 culture for amplification. The library was amplified for 5 hours. After removing the bacteria by centrifugation 5000g/20 min/4°C the phages were precipitated from the supernatant by adding 1/6 volume of PEG/NaCl (20% polyethelene glycol/2.5M NaCl). The supernatant was incubated with PEG/NaCl at 4°C for 16 hours and centrifuged at 5000g 4°C for one hour. Pellet containing the phages was resuspended in 50 ml of TBS (50 mM Tris-HCl pH7.5, 150 mM NaCl), and precipitated again. Finally, the library was resuspended in TBS/50% glycerol and stored in -20°C.

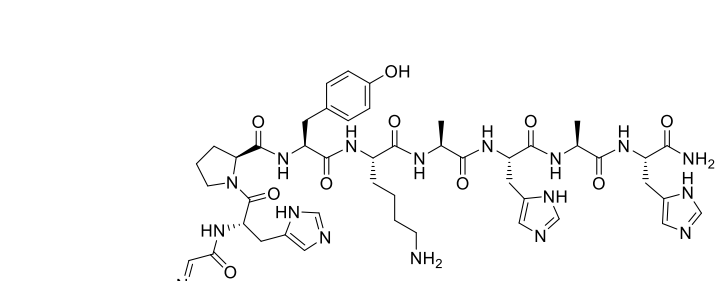
Modification of the library with sulfonamide:

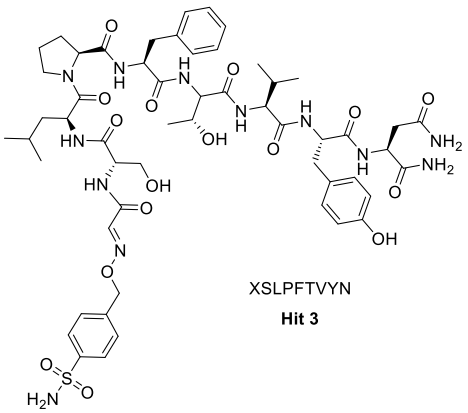
The amplified S-$(X)_8$-TEV library containing 10^{10} plaque forming units (PFU) in 100 µL of PBS was modified with Inh2 according to previously published protocol.[12] The N-terminal serine residues were first oxidized by sodium periodate. To 100 µL of library, 1 µL of 6 mM $NaIO_4$ (dissolved in H_2O) was added for 5 min and the reaction was incubated on ice in the dark. To quench the periodate 1 µL of 50 mM (dissolved in H_2O) reduced glutathione was added to the reaction and incubated for 10 minutes. Then 100 µL of 2mM Inh2 dissolved in 200 mM anilinium acetate (pH 4.7) was added to the oxidized library and the reaction was incubated for 1 hour at room temperature. After this time the reaction was diluted with 300 µL of water and precipitated by addition 1/6 of the volume (83 µL) of 20% polyethelene glycol/2.5M NaCl. After centrifugation 25 000g/10min/4°C the supernatant was removed and the pellet was resuspended in 500 µL of TBS, precipitated again and titrated. In each panning step $1x10^9$ PFU of the sulfonamide modified library was used as the starting material.

Library Panning:

Three steps of panning were performed, each using human carbonic anhydrase II (hCA II) enzyme immobilized on a bead support. To avoid selecting for nonspecific sequences binding, the bead material hCA II was immobilized on a different type of beads for each panning step. Each time NHS ester linkage was used and the beads were saturated with the enzyme according to manufacturer's protocols. First step of panning used the Sepharose (NHS Act Sepharose® 4 Fast Flow, GE17-0906-01), second the Glass beads (Sigma G8893, prepared by reacting amino functionalized glass beads (APTES)[13] with an excess of suberic acid bis(n-hydroxysuccinimide ester) and incubating the NHS-modified beads with hCA II) and the third the Tentagel beads (TentaGel® S COOSu, S30135, Rapp polymere). In each panning step 20 µL of hCA II-modified beads (packed bead volume) were pre-blocked using blocking buffer (5% BSA and 1% tryptone 0.005% Tween 20 in PBS) for one hour at room temperature. The

beads were incubated with $1x10^9$ PFU of sulfonamide modified library suspended in the blocking buffer on a rotator for 1.5 hour at room temperature. After this time, beads were washed extensively using the blocking buffer (5x1mL, each 15 minutes) followed by a short 2x in 1mL washes with H_2O. Bound phages were eluted using 0.2M glycine (pH=2.2) with subsequent neutralization with 1M Tris-HCl (pH=9). Eluted phages were amplified using ER2738 cells for 4.5 hours and precipitated from the cleared media using PEG/2.5M NaCl. The precipitation procedure was repeated again and the library was ready for the next round of panning. After 3rd round of panning 10 random plaques were picked and sequenced. 2 sequences were chosen for solid phase peptide synthesis (Hit 2 and Hit 3). The sequence of Hit 2 was found in 2 consensus sequences and that of Hit 3 in 3 consensus sequences.



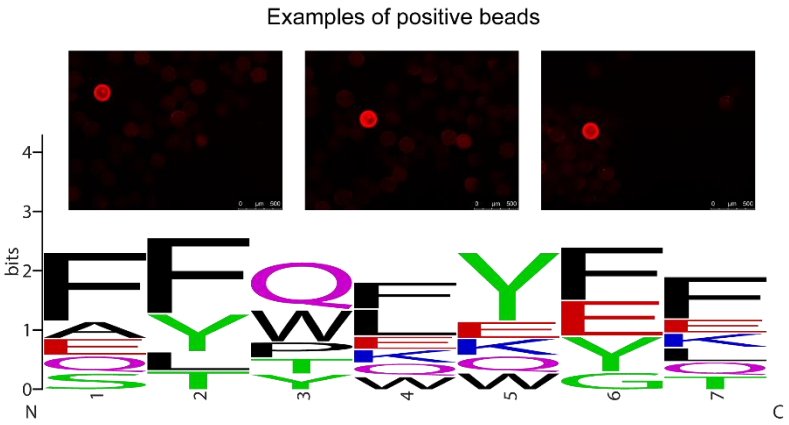


Screening of the synthetic peptide library

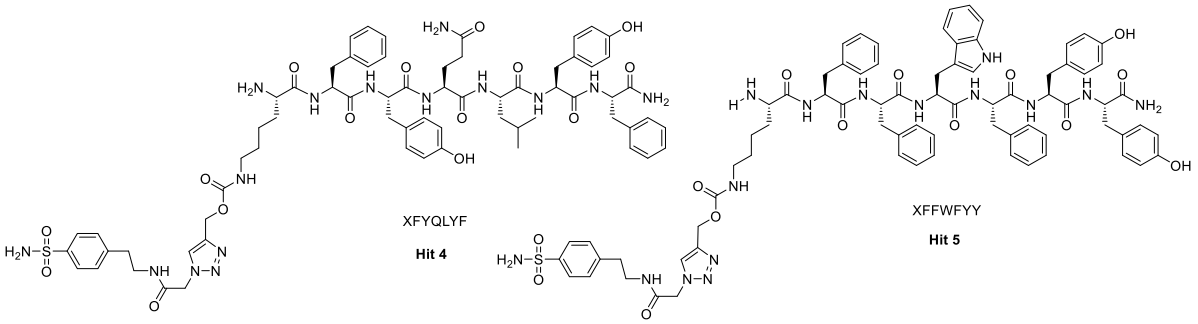
The sulfonamide inhibitor containing peptide library (75 mg) was washed with water and incubated in blocking buffer (30 mM HEPES, pH 7.4, 150 mM NaCl, 0.05% Tween 20, 0.05% gelatin) for 1 hour. After washing with water, the library was suspended in 50 mM HEPES, pH 8.3, 150 mM NaCl (1 mL) and 10 µL (ca. 0.5-1 mg/mL, ca. 30 µM solution) of Cy-3 labeled hCA II in 20 mM HEPES, 200 mM NaCl was added giving approximately 300 nM final hCA II concentration. The library was rotated in the dark at room temp. for 2 hours after which it was washed with blocking buffer for 30 min. The library was then inspected under a fluorescent stereomicroscope (Leica M205 FA) equipped with a LED fluorescence source (pE-300 white) and the most fluorescent beads were manually collected. The beads were then washed 2x 20 min with 6M quinidine HCl (pH= 1), DMF, water and DMF to remove the bound protein.

In total, 8 beads were sequenced using Edman sequencing on Procise protein sequencing system from Applied Biosystems with the following sequences found:

XQFWFQGQM
XFFQWKYFM
XFLYLYEEM
XSYQKYYFM
XFFTFYEKM
XAYPEYFLM
XETWLWFFM
XFFQQEFTM



where **X** represents the N-terminal modification containing the sulfonamide inhibitor Inh3.
Based on these sequences, the following hit peptide sequences were resynthesized.



Biochemistry and structural analysis

Protein preparation

Recombinant hCA II was prepared by heterologous expression in *E. coli* and purified as previously described.[14] For the preparation of ^{15}N-labeled protein, cells were grown in minimal medium supplemented with 1 g/l $(^{15}NH_4)_2SO_4$ (Cat. No. NLM-713-10, Cambridge Isotope Laboratories Inc., Tewksbury, MA, USA). For the preparation of $^{15}N/^{13}C$-labeled protein, cells were grown in minimal medium supplemented with 1 g/l $(^{15}NH_4)_2SO_4$ (Cat. No. NLM-713-10, Cambridge Isotope Laboratories Inc., Tewksbury, MA, USA) and 4 g/l ^{13}C-D-glucose (Cat. No. CLM-1396-5, Cambridge Isotope Laboratories Inc., Tewksbury, MA, USA).[15-16]
The extracellular part of hCA IX comprising the PG and CA domains (residues 38–391) and including the amino acid substitution C174S was expressed in HEK 293 cells and purified as previously described.[17]

Inhibition assay

A stopped-flow instrument (Applied Photophysics) was used for measuring the CA-catalyzed CO_2 hydration activity in the presence of inhibitors under conditions previously described.[18,19] The original data from the measurements are summarized in Figures S9-S12.

Protein crystallography

Complexes of hCA II with compounds were prepared by addition of a one-fold or two-fold molar excess of the compounds (dissolved in 100% DMSO) to a 20−25 mg/ml protein solution in 50 mM Tris-H_2SO_4 pH 7.8. The final concentration of DMSO in crystallization drops did not exceed 10%. Crystals were

prepared by the hanging drop vapour diffusion method at 18 °C, the drop volume were prepared by mixing 2 µl of protein solution with 1 µl of precipitating solution. The precipitation solution consisted of 1.6 M sodium citrate, 50 mM Tris-H_2SO_4, pH 7.8. Prior to data collection, the crystals were soaked for 10 seconds in the reservoir solution supplemented with 20% (v/v) sucrose and stored in liquid nitrogen. X-ray diffraction data at 100 K were collected on BL14.1 and BL14.2 operated by the Helmholtz-Zentrum Berlin (HZB) at the BESSY II electron storage ring (Berlin-Adlershof, Germany).[20] Diffraction data were processed using the XDS suite of programs.[21-22] Crystal parameters and data collection statistics are summarized in Supporting Information Table S1–S2.

The crystal structures of hCA II complexes with compounds were determined by the difference Fourier technique. Coordinates from PDB entry 3PO6[23] was used as a model for hCA II. Atomic coordinates and geometric libraries of inhibitor molecules were generated by AceDRG.[24] The Coot programme[25] was used for inhibitor fitting, model rebuilding, and the addition of water molecules. Refinement was carried out with the Refmac5 programme[26] with 5% of the reflections reserved for cross-validation. The structures were first refined with isotropic atomic displacement parameters (ADPs). Following addition of solvent atoms and zinc ions, building inhibitor molecules in the active site, and several alternate conformations for a number of residues, anisotropic ADPs were refined for nearly all atoms. The quality of the crystallographic model was validated by MolProbity.[27] The final refinement statistics are summarized in Supporting Information Table S1–S2. All the figures representing structures were created using PyMOL.[28] Analysis of contacts was conducted using the Contact programme included in the CCP4 suite.[29] Distance cut-off used to evaluate contacts between ligand and protein atoms was 4 Å.

Atomic coordinates and structure factors for the crystal structures of CA II in complex with Hit2, Hit3, Hit4, Hit3-t1, Hit3-t2, and Hit3-t4 were deposited in the PDB with accession codes 7OYM, 7OYN, 7OYO, 7OYP, 7OYQ, 7OYR, respectively.

NMR binding studies

All NMR data were acquired at 308 K on 850 MHz Bruker Avance spectrometer equipped with triple-resonance ($^{15}N/^{13}C/^{1}H$) cryoprobes (Bruker, Billerica, MA, USA). Two-dimensional $^{15}N/^{1}H$ HSQC (heteronuclear single quantum coherence) spectra were acquired for a 165 µl sample containing 30 µM ^{15}N-labeled hCA II upon addition of 500 µM compound. The sample buffer was PBS supplemented with 5% D_2O. A series of triple-resonance spectra were recorded to determine sequence-specific resonance assignments for hCA II. Essentially complete sequence-specific resonance assignment was obtained as previously described.[15-16] To determine the binding interface in hCA II, ligand-induced perturbations of ^{15}N hCA II in HNCO spectra were monitored employing the minimal backbone chemical shift method.[30] The minimum changes in the positions of HNCO peaks for the free and ligand-bound hCA II were calculated and plotted against the protein sequence. This histogram identified hCA II residues significantly affected by ligand binding. These residues were mapped onto the crystal structures of hCA II-ligand complexes.

Table S1: Diffraction data collection and refinement statistics.

CA II in complex with			
	Hit2	**Hit3**	**Hit4**
Data collection statistics			
Wavelength (Å)	0.9184	0.9184	0.9184
Space group	$P2_1$	$P2_1$	$P2_1$
Cell parameters (Å, °)	42.26 41.27 72.30 90.00 104.44 90.00	42.23 41.30 72.22 90.00 104.40 90.00	42.25 41.38 72.24 90.00 104.49 90.00
Resolution range (Å)	40.93-0.98 (1.06-0.98)	35.57-0.98 (1.06-0.98)	40.91-1.03 (1.09-1.03)
Number of unique reflection	122,587 (14,875)	116,713 (13,123)	108,711 (14,523)
Multiplicity	3.5 (2.8)	3.8 (3.2)	3.9 (3.7)
Completeness (%)	93.8 (70.8)	89.5 (62.4)	90.8 (75.4)
R_{merge}[a]	5.6 (58.8)	4.4 (58.6)	7.4 (74.4)
$CC_{(1/2)}$ (%)[b]	99.8 (79.9)	99.9 (76.0)	99.7 (72.1)
Average I/σ(I)	12.6 (1.8)	15.1 (2.0)	10.1 (1.6)
Wilson B ($Å^2$)	13.2	13.0	13.2
Refinement statistics			
Resolution range (Å)	40.93 -0.98 (1.00 -0.98)	35.56 -0.98 (1.00 -0.98)	40.90 -1.03 (1.06 -1.03)
No. of reflection in working set	122,362 (2,859)	116,661 (2,381)	107,622 (5,677)
No. of reflection in the test set	2,498 (59)	2,381 (58)	1,088 (58)
R_{work} value (%)[c]	13.6 (32.2)	12.1 (27.1)	13.6 (29.1)
R_{free} value (%)[d]	16.0 (33.1)	13.6 (28.0)	16.7 (30.8)
RMSD bond length (Å)	0.013	0.011	0.012
RMSD angle (°)	1.77	1.74	1.74
Mean ADP value ($Å^2$)	16.8	13.3	15.2
Ramachandran plot statistics[e]			
Residues in favored regions (%)	96.1	96.1	96.9
Residues in allowed regions (%)	3.9	3.9	3.1
PDB code	7OYM	7OYN	7OYO

The data in parentheses refer to the highest-resolution shell for data collection statistic.

[a] $R_{merge} = (|I_{hkl} - \langle I \rangle|)/I_{hkl}$, where the average intensity $\langle I \rangle$ is taken over all symmetry equivalent measurements and I_{hkl} is the measured intensity for any given reflection

[b] $CC_{(1/2)}$ is the correlation coefficient between random half data sets and from its value the Pearson correlation coefficient of the true level of signal can be calculated: $CC^* = \sqrt{2CC_{1/2}/1 + CC_{1/2}}$.

[c] R-value = $||F_o| - |F_c||/|F_o|$, where F_o and F_c are the observed and calculated structure factors, respectively.

[d] R_{free} is equivalent to R-value but is calculated for 5% of the reflections chosen at random and omitted from the refinement process.[31]

[e] As determined by Molprobity [32]

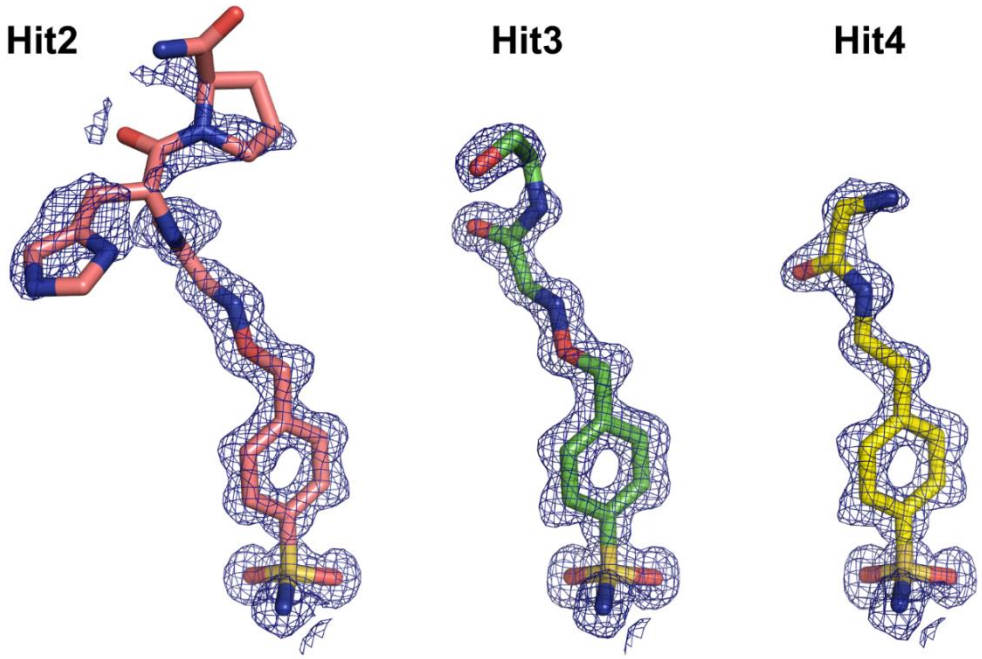


Figure S1: Structure of inhibitor-peptide conjugates bound to the active site of hCA II.

Carbon atoms are colored by different colors: Hit2 (salmon), Hit3 (green), Hit4 (yellow). Oxygen, nitrogen, and sulfur atoms are shown in red, blue, and orange, respectively. $2F_o$-F_c map contoured at 1 σ as a mesh. All compounds were modelled in one conformation with full occupancy.

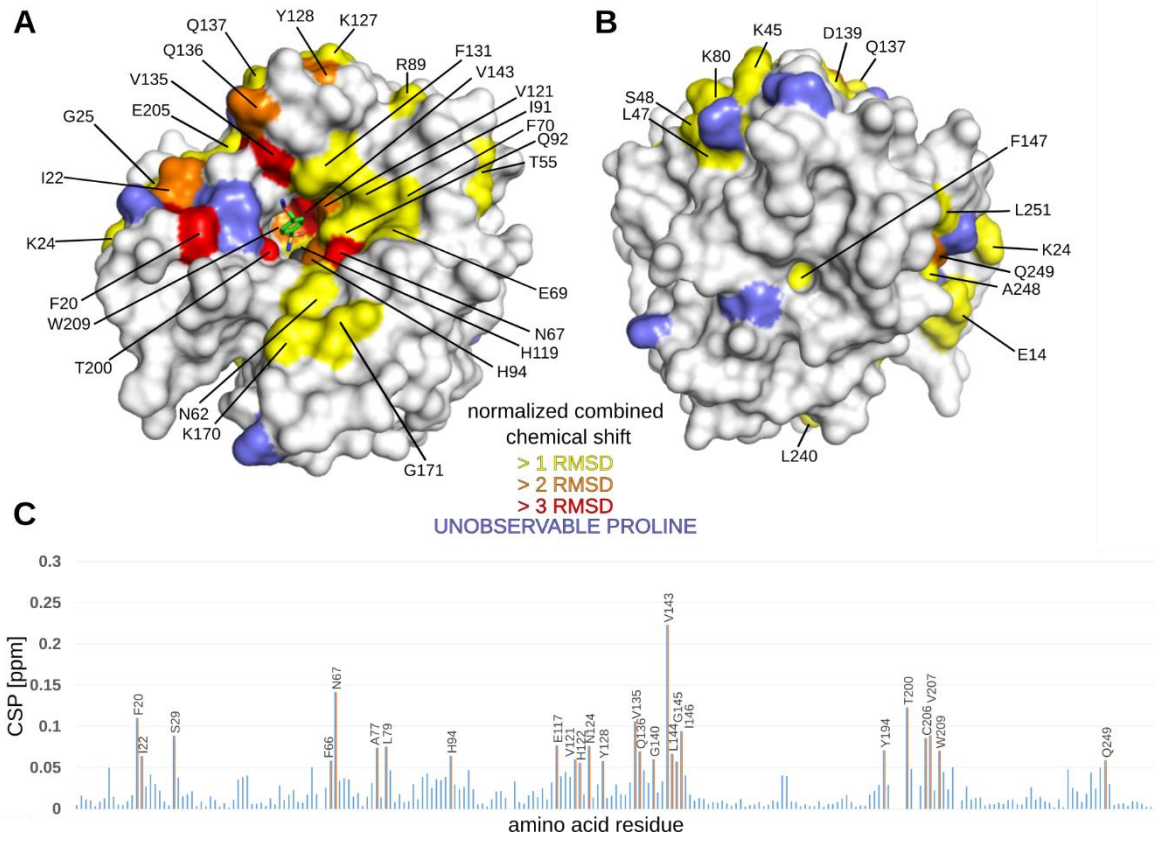


Figure S2: NMR chemical shift perturbations of ^{15}N hCA II upon binding of **Inh2**.

Residues perturbed upon binding of Inh 2 are mapped on the surface of ^{15}N hCA II from one side (A) and from the opposite side (B). Normalized combined chemical shifts with values over 1, 2 or 3 are colored yellow, orange, and red, respectively. (C) Histogram of normalized combined chemical shift upon Inh 2 binding versus the protein sequence of ^{15}N hCA II. Numbers of residues with normalized combined chemical shift values higher than 2 are indicated above the corresponding bars.Q

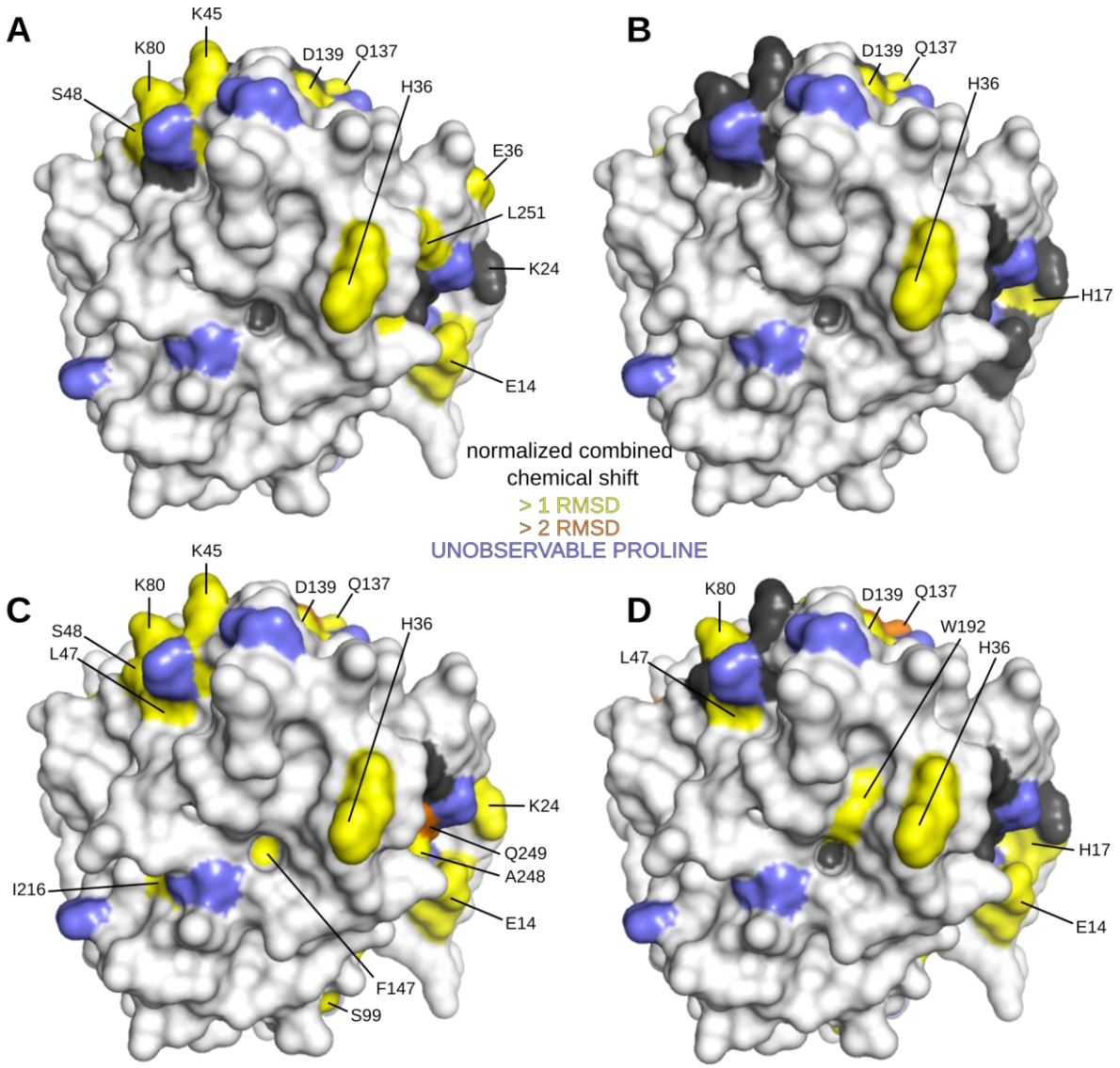


Figure S3: NMR chemical shift perturbations of ^{15}N hCA II upon binding of peptide-inhibitor conjugates **Hit2** (A), **Hit3** (B), **Hit4** (C), and **Hit 5** (D). Enzyme surface on the side opposite to the enzyme active site is shown (view is rotated by approx. 180° along vertical axis with respect to Figure 3).

Residues perturbed upon peptide-inhibitor conjugate binding are mapped on the surface of ^{15}N hCA II. Normalized combined chemical shifts with values over 1, and 2 are colored yellow, and orange, respectively. For visualization of binding for each hit conjugate, determined crystal structure was used, beside for Hit5 where crystal structure of Hit4 was used instead due to missing crystal structure.

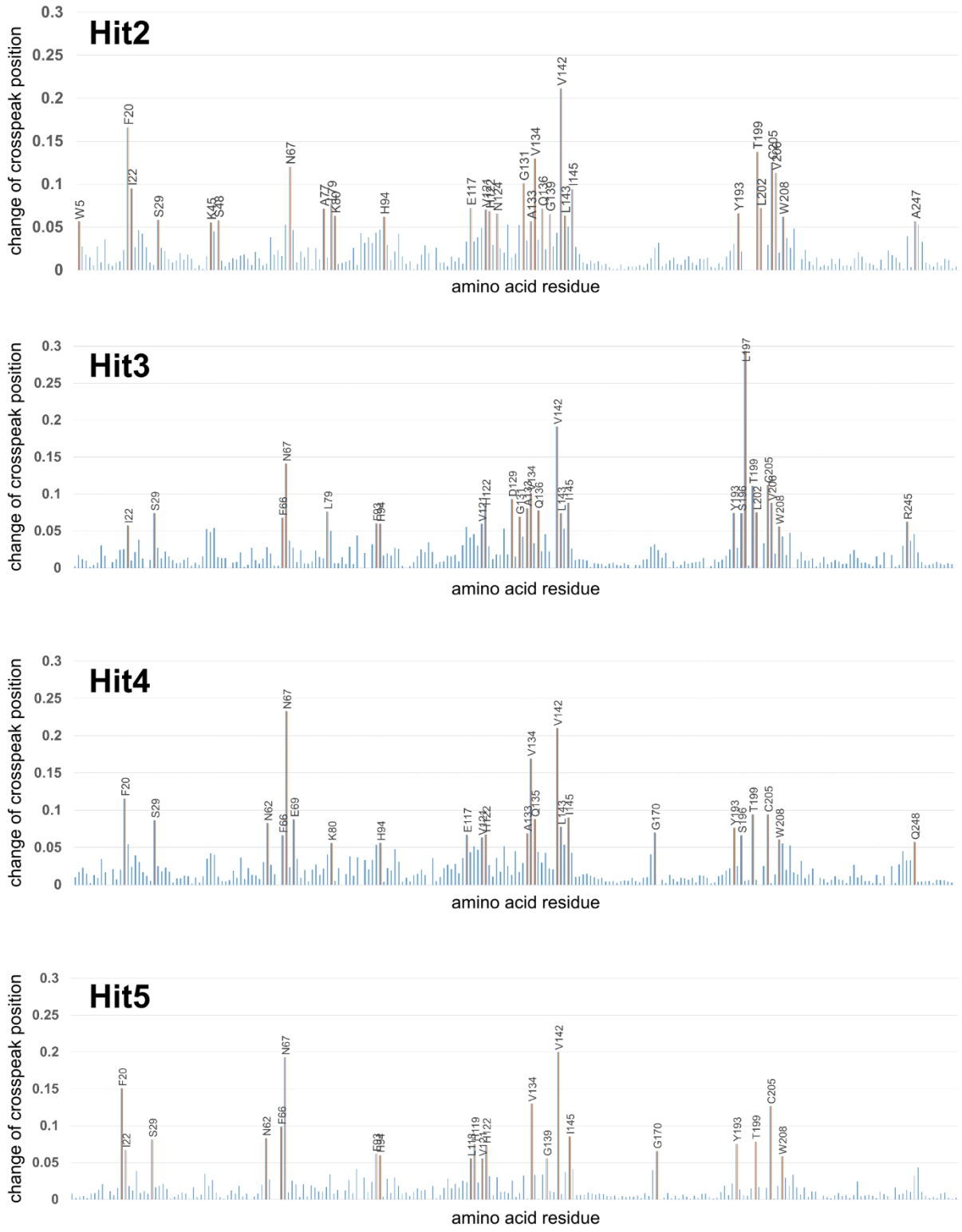


Figure S4: Histogram of normalized combined chemical shift upon binding of peptide-inhibitor conjugates Hit2, Hit3, Hit4, and Hit 5 versus the protein sequence of ^{15}N hCA II.

Numbers of residues with normalized combined chemical shift values higher than 2 are indicated above the corresponding bars.

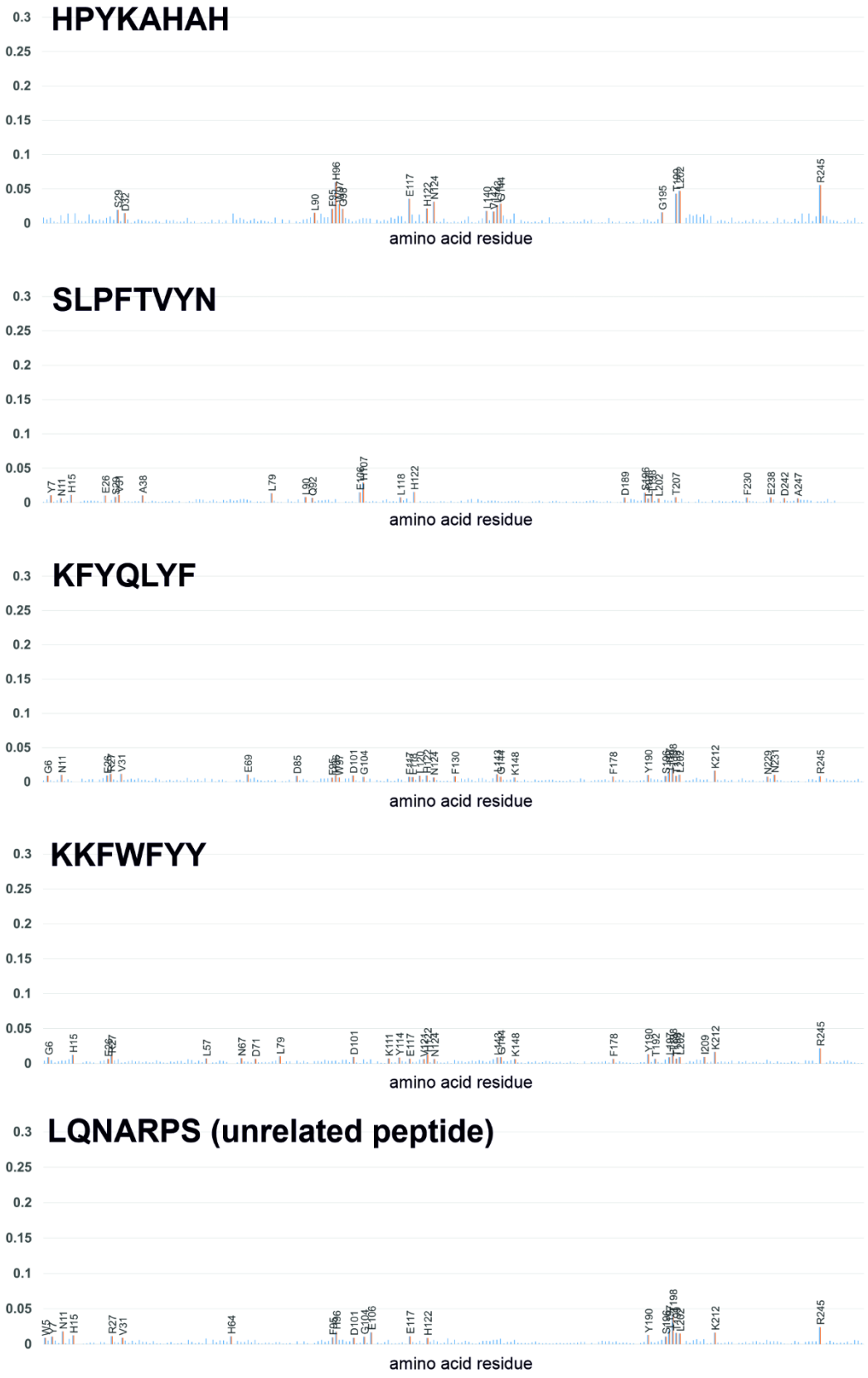


Figure S5: Histogram of normalized combined chemical shift upon binding of peptides without the inhibitory warhead HPYKAHAH (peptide from **Hit 2**), SLPFTVYN (peptide from **Hit 3**), KFYQLYF (peptide from **Hit 4**), KFFWFYY (peptide from **Hit 5**), and LQNARPS versus the protein sequence of ^{15}N hCA II.

Numbers of residues with normalized combined chemical shift values higher than 2 are indicated above the corresponding bars.

Table S2: Diffraction data collection and refinement statistics.

CA II in complex with			
	Hit3-t1	**Hit3-t2**	**Hit3-t4**
Data collection statistics			
Wavelength (Å)	0.9184	0.9184	0.9184
Space group	$P2_1$	$P2_1$	$P2_1$
Cell parameters (Å, °)	42.33 41.35 72.40 90.00 104.57 90.00	42.23 41.27 72.52 90.00 104.45 90.00	42.27 41.22 72.48 90.00 104.51 90.00
Resolution range (Å)	40.97-1.05 (1.11-1.05)	35.58-1.15 (1.22-1.15)	40.93-1.15 (1.22-1.15)
Number of unique reflection	101,502 (9,263)	86,270 (12,950)	76,454 (13,894)
Multiplicity	3.4 (2.3)	3.6 (3.4)	3.6 (2.8)
Completeness (%)	89.5 (50.6)	97.4 (93.1)	88.5 (52.9)
R_{merge}[a]	4.4 (47.0)	6.8 (109.7)	7.9 (57.8)
$CC_{(1/2)}$ (%)[b]	99.9 (77.6)	99.9 (63.5)	99.6 (78.3)
Average I/σ(I)	14.4 (1.9)	11.2 (1.3)	9.9 (2.1)
Wilson B ($Å^2$)	12.9	15.8	16.1
Refinement statistics			
Resolution range (Å)	40.96 -1.05 (1.08 -1.05)	35.58 -1.15 (1.18 -1.15)	40.92 -1.15 (1.18 -1.15)
No. of reflection in working set	99,471 (3,317)	81,776 (5,327)	74,160 (2,812)
No. of reflection in the test set	2,031 (68)	2,529 (164)	2,294 (87)
R_{work} value (%)[c]	11.8 (24.6)	14.3 (40.7)	12.3 (24.5)
R_{free} value (%)[d]	13.4 (22.7)	18.0 (41.8)	15.1 (28.7)
RMSD bond length (Å)	0.011	0.012	0.011
RMSD angle (°)	1.70	1.81	1.72
Mean ADP value ($Å^2$)	13.7	16.2	16.2
Ramachandran plot statistics[e]			
Residues in favored regions (%)	96.9	96.1	96.9
Residues in allowed regions (%)	3.1	3.9	3.1
PDB code	7OYP	7OYQ	7OYR

The data in parentheses refer to the highest-resolution shell for data collection statistic.

[a] $R_{merge} = (|I_{hkl} - \langle I \rangle|)/I_{hkl}$, where the average intensity $\langle I \rangle$ is taken over all symmetry equivalent measurements and I_{hkl} is the measured intensity for any given reflection

[b] $CC_{(1/2)}$ is the correlation coefficient between random half data sets and from its value the Pearson correlation coefficient of the true level of signal can be calculated: $CC^* = \sqrt{2CC_{1/2}/1 + CC_{1/2}}$.

[c] R-value = $||F_o| - |F_c||/|F_o|$, where F_o and F_c are the observed and calculated structure factors, respectively.

[d] R_{free} is equivalent to R-value but is calculated for 5% of the reflections chosen at random and omitted from the refinement process.[31]

[e] As determined by Molprobity [32]

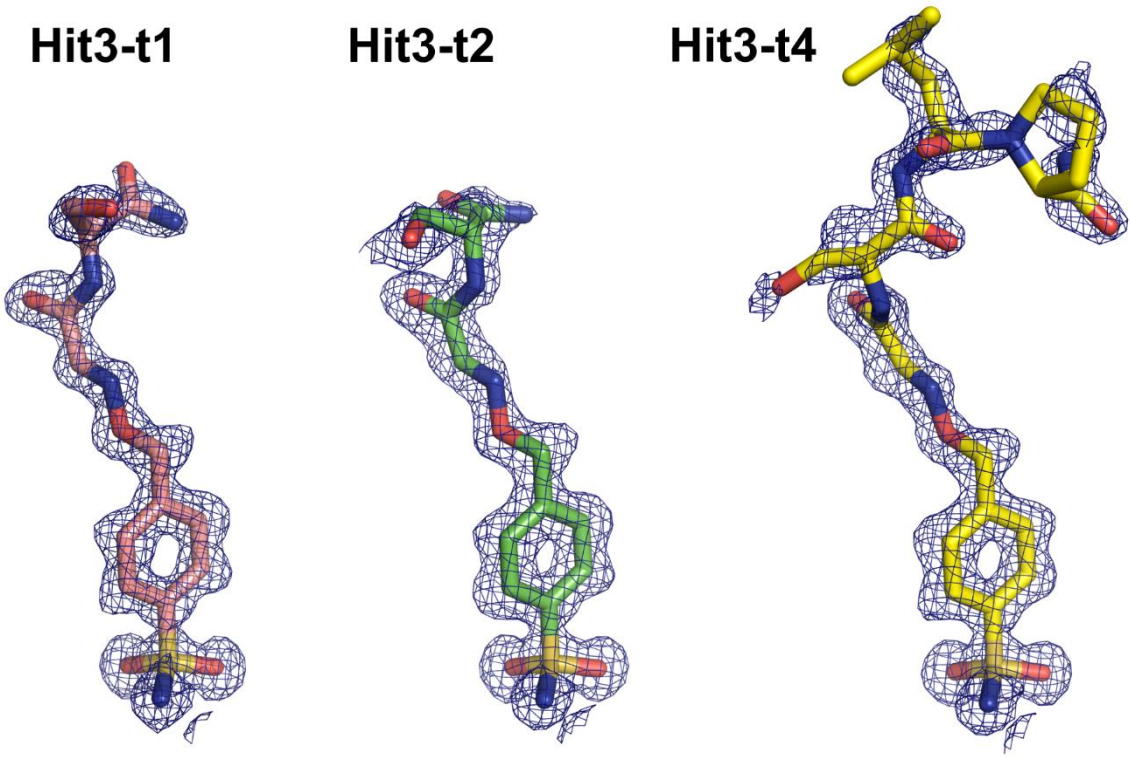


Figure S6: Structure of truncated versions of conjugates bound to the active site of hCA II.

Carbon atoms are colored by different colors: Hit3-t1 (salmon), Hit3-t2 (green), Hit3-t4 (yellow). Oxygen, nitrogen, and sulfur atoms are shown in red, blue, and orange, respectively. $2F_o$-F_c map contoured at 1 σ as a mesh. All compounds were modelled in one conformation with full occupancy.

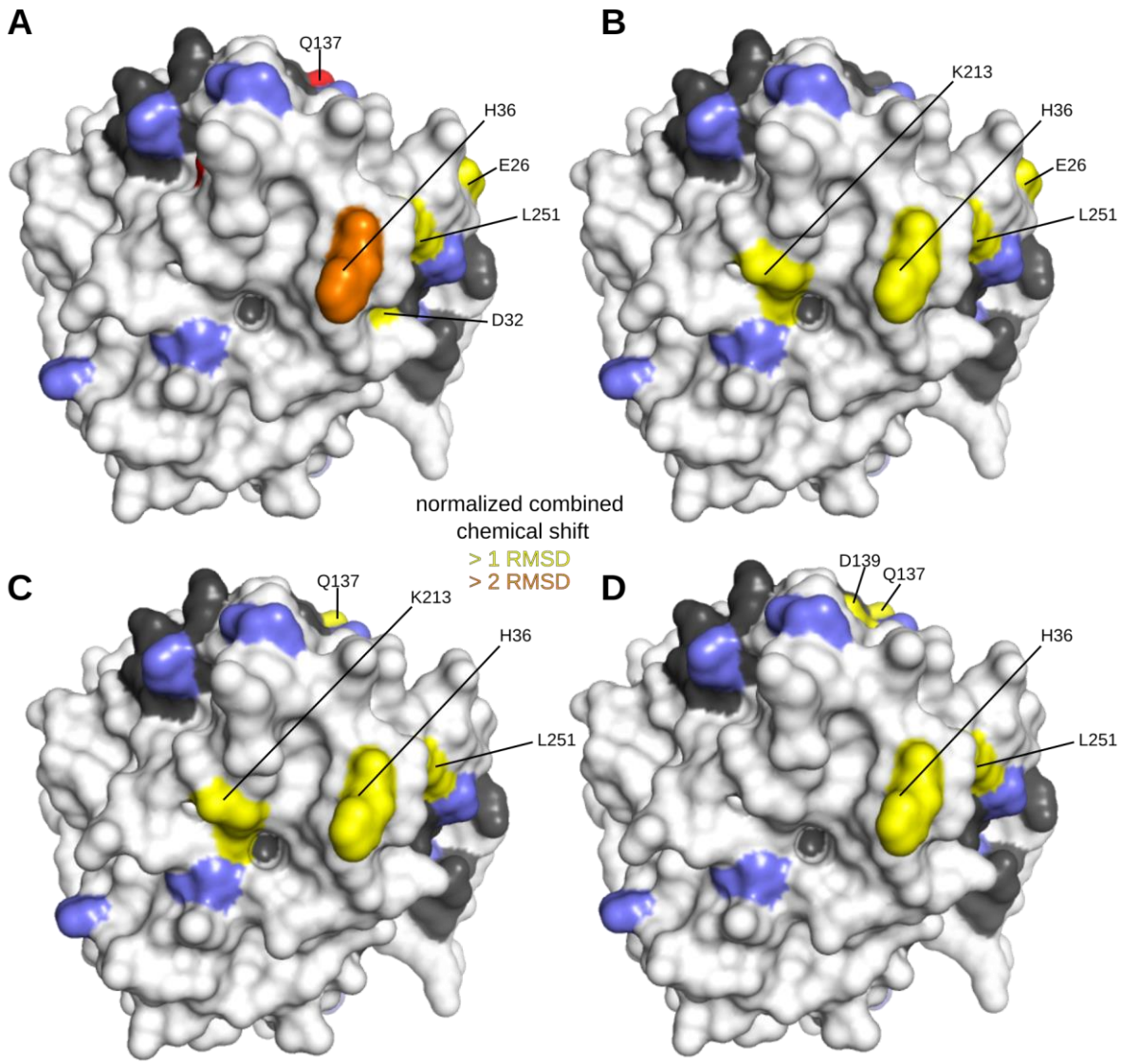


Figure S7: NMR chemical shift perturbations of ^{15}N hCA II upon binding of truncated versions of conjugates Hit3-t1 (A), Hit3-t2 (B), Hit3-t3 (C), and Hit3-t4 (D). Enzyme surface on the side opposite to the enzyme active site is shown (view is rotated by approx. 180° along vertical axis with respect to Figure 6).

Residues perturbed upon truncated versions of conjugates binding are mapped on the surface of ^{15}N hCA II. Normalized combined chemical shifts with values over 1, and 2 are colored yellow, and orange, respectively. For visualization of binding for each truncated hit conjugate, determined crystal structure was used, beside for Hit3-t3 where crystal structure of Hit3-t4 was used instead due to missing crystal structure.

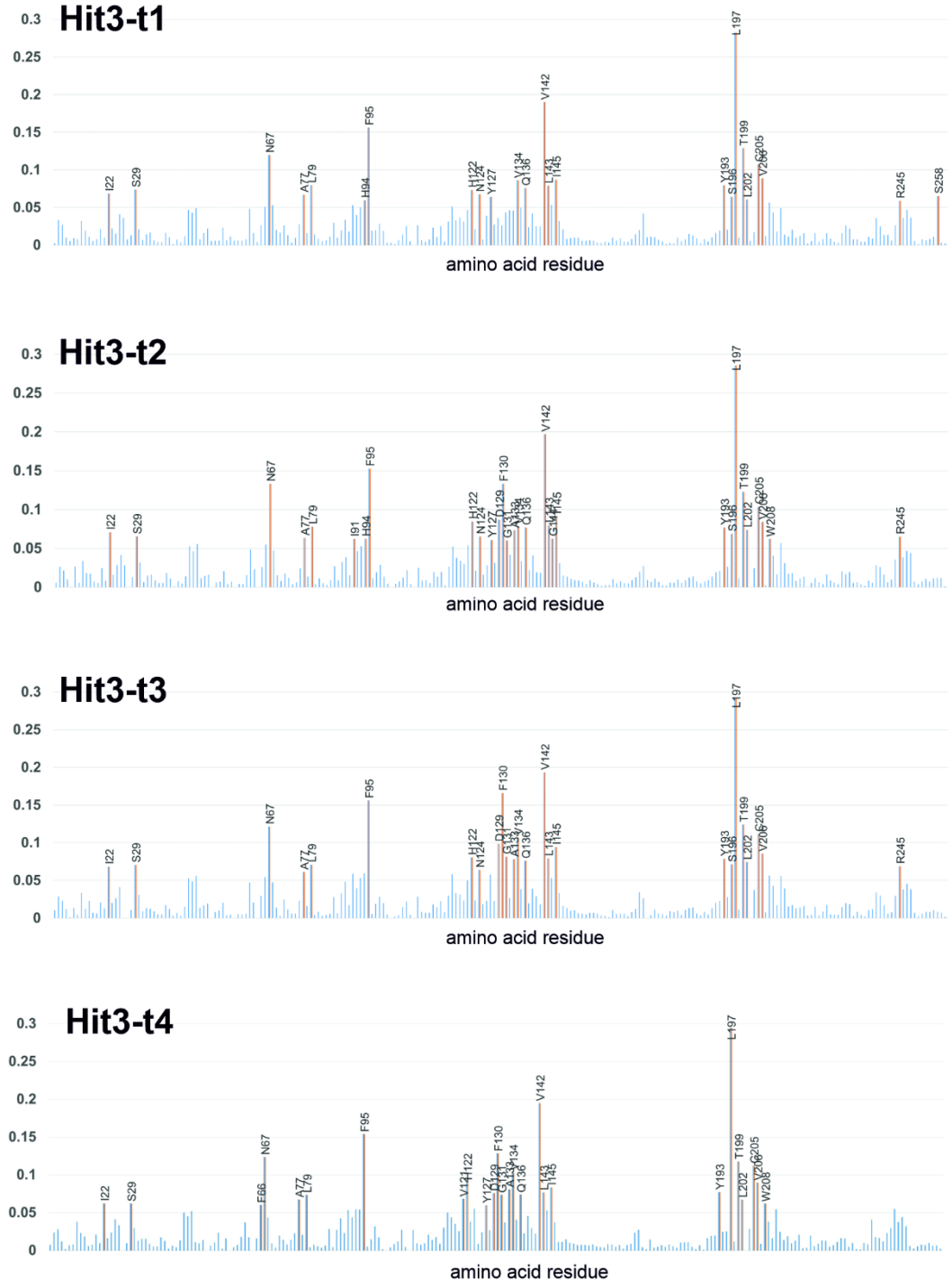


Figure S8: Histogram of normalized combined chemical shift upon binding of truncated versions of conjugates Hit3-t1, Hit3-t2, Hit3-t3, and Hit3-t4 versus the protein sequence of ^{15}N hCA II.

Numbers of residues with normalized combined chemical shift values higher than 2 are indicated above the corresponding bars.

Inhibition assay measurements

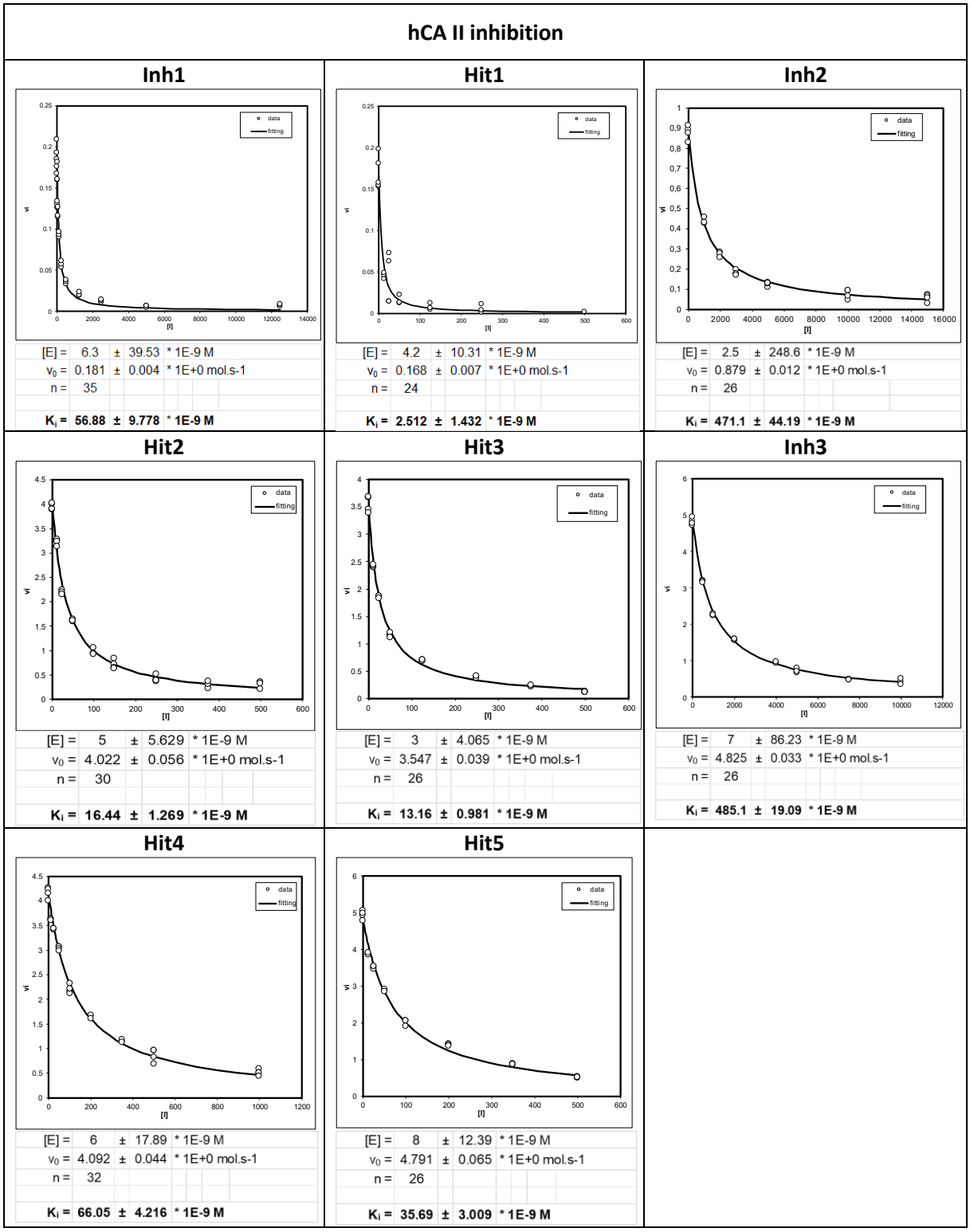


Figure S9: Inhibition of hCA II by rising concentration of compounds **Inh1-3** and **Hit1-5**.

Plots show the dose dependent decrease of catalytic activity expressed as speed of decrease in absorbance (v_i expressed in $\Delta A.s^{-1}$). The concentration of compound [I] in shown in $nmol.l^{-1}$. Each circle represents one measurement, black line shows fitted curves by the nonlinear least-squares method using Williams-Morrison equation. Enzyme concentration [E], initial velocity v_0, number of measurements (n) and K_i value are shown below each plot.

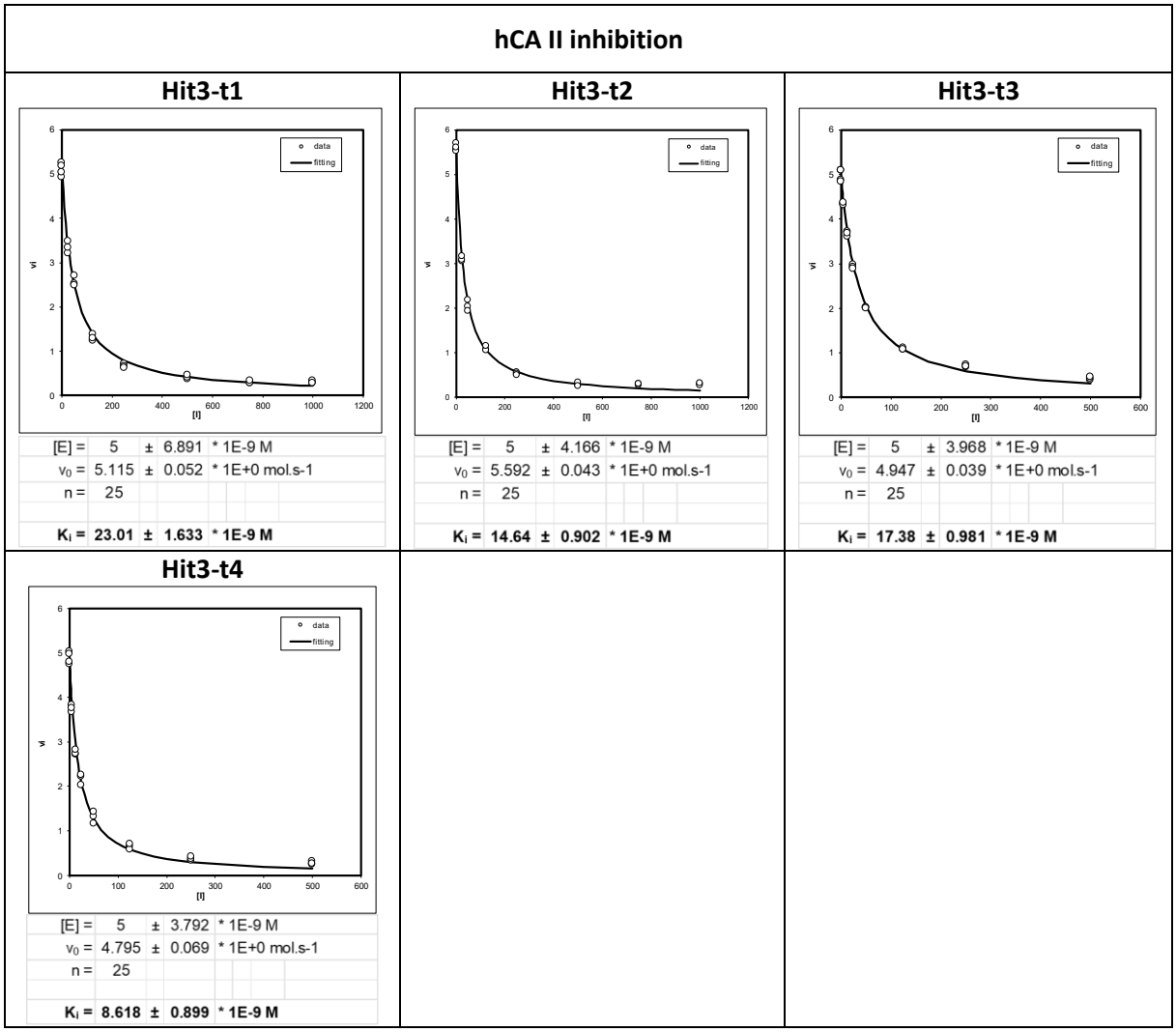


Figure S10: Inhibition of hCA II by rising concentration of compounds **Hit3-t1-4**.

Plots show the dose dependent decrease of catalytic activity expressed as speed of decrease in absorbance (v_i expressed in $\Delta A.s^{-1}$). The concentration of compound [I] in shown in $nmol.l^{-1}$. Each circle represents one measurement, black line shows fitted curves by the nonlinear least-squares method using Williams-Morrison equation. Enzyme concentration [E], initial velocity v_0, number of measurements (n) and K_i value are shown below each plot.

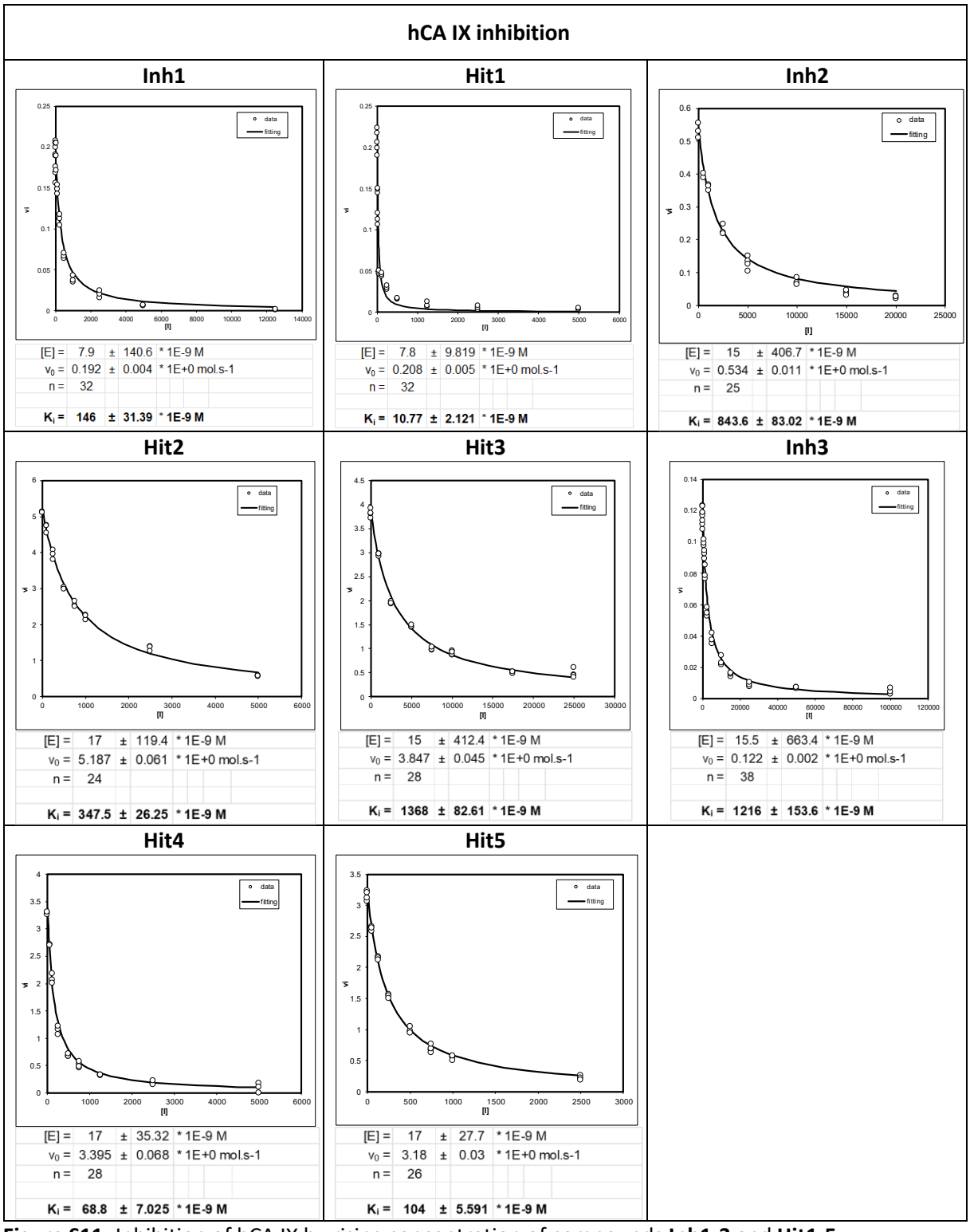


Figure S11: Inhibition of hCA IX by rising concentration of compounds **Inh1-3** and **Hit1-5**.

Plots show the dose dependent decrease of catalytic activity expressed as speed of decrease in absorbance (v_i expressed in $\Delta A.s^{-1}$). The concentration of compound [I] in shown in $nmol.l^{-1}$. Each circle represents one measurement, black line shows fitted curves by the nonlinear least-squares method using Williams-Morrison equation. Enzyme concentration [E], initial velocity v_0, number of measurements (n) and K_i value are shown below each plot.

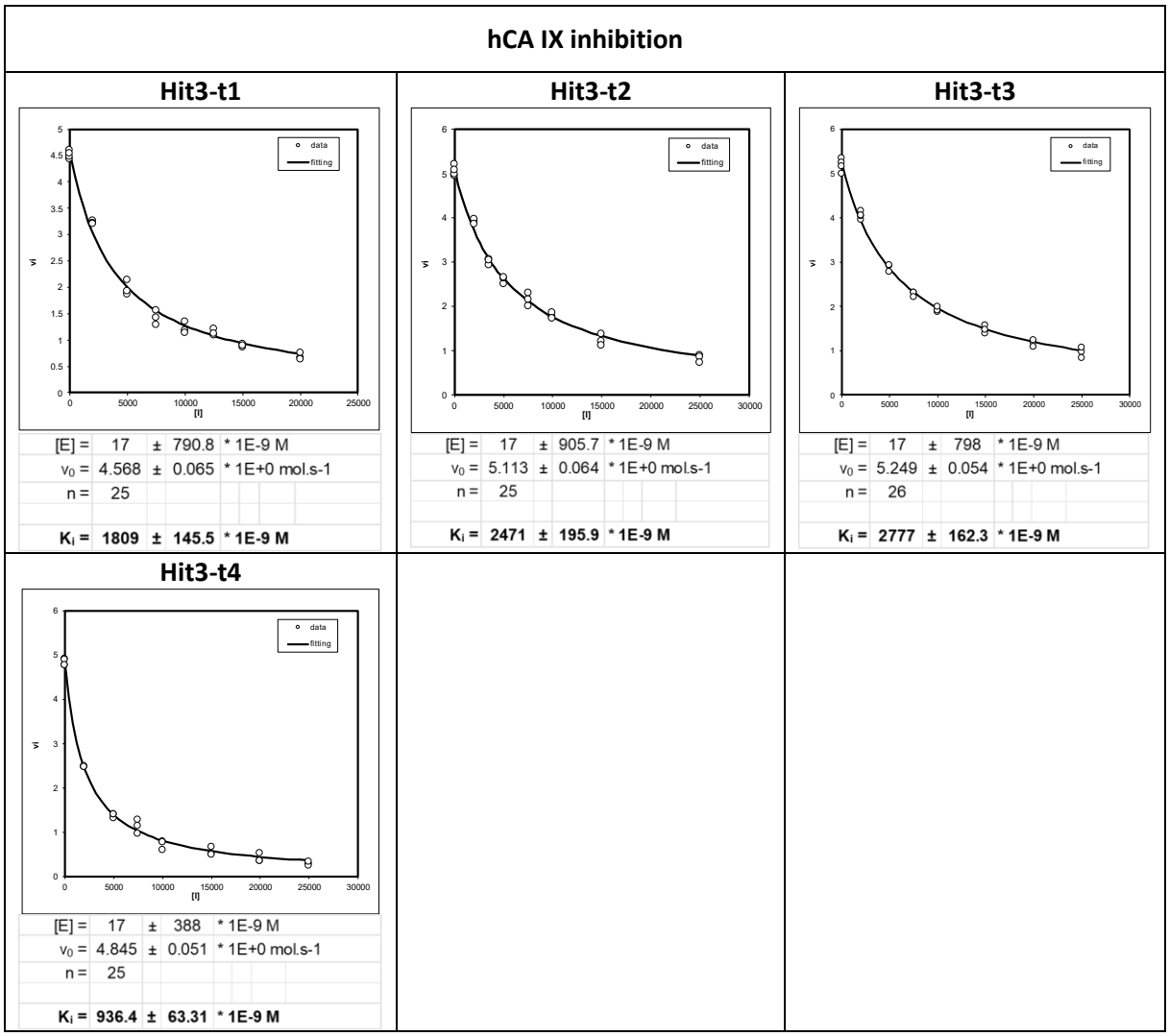


Figure S12: Inhibition of hCA IX by rising concentration of compounds **Hit3-t1-4**.

Plots show the dose dependent decrease of catalytic activity expressed as speed of decrease in absorbance (v_i expressed in $\Delta A.s^{-1}$). The concentration of compound [I] in shown in $nmol.l^{-1}$. Each circle represents one measurement, black line shows fitted curves by the nonlinear least-squares method using Williams-Morrison equation. Enzyme concentration [E], initial velocity v_0, number of measurements (n) and K_i value are shown below each plot.

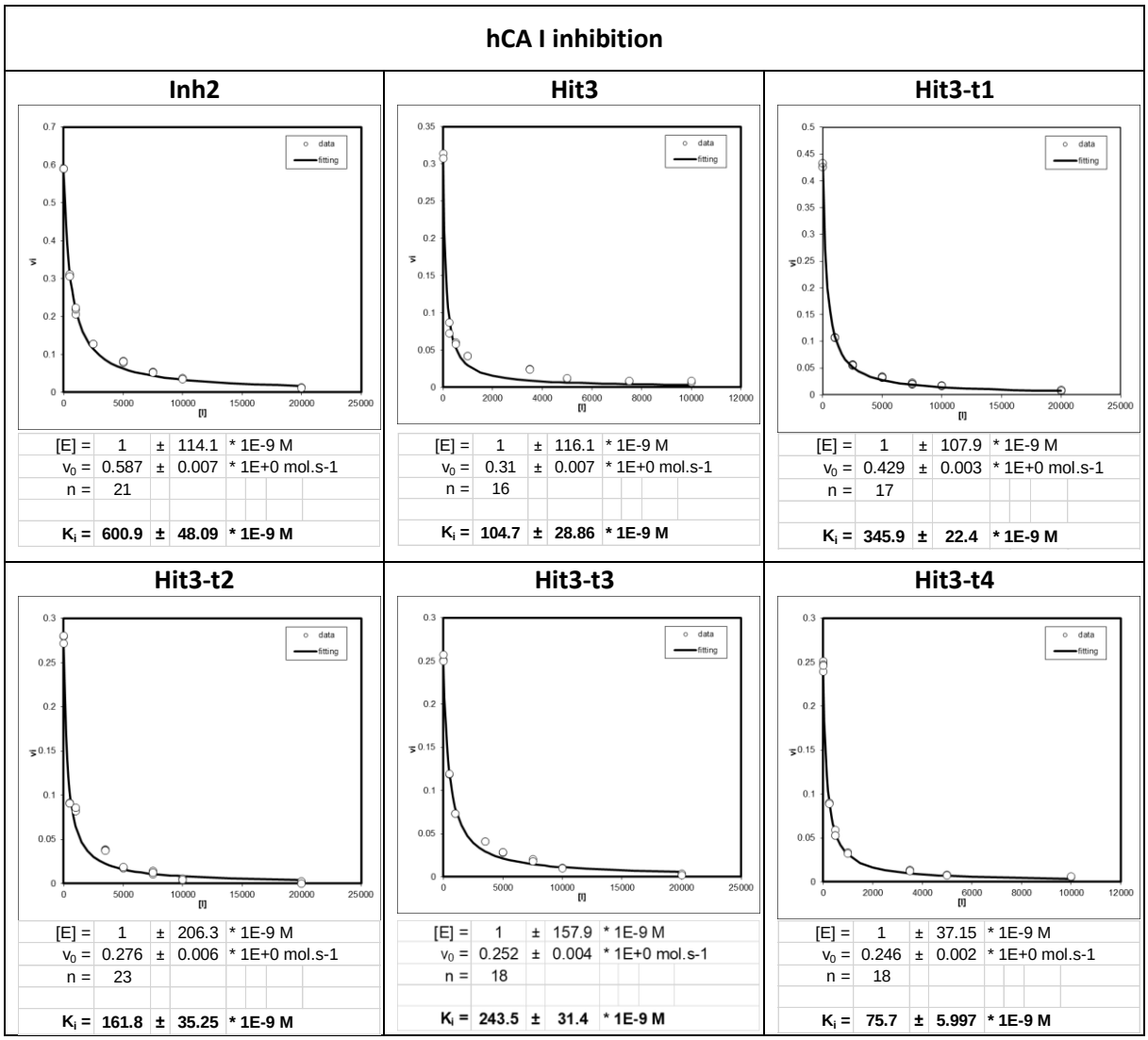


Figure S13: Inhibition of hCA I by rising concentration of compounds.

Plots show the dose dependent decrease of catalytic activity expressed as speed of decrease in absorbance (v_i expressed in $\Delta A.s^{-1}$). The concentration of compound [I] in shown in $nmol.l^{-1}$. Each circle represents one measurement, black line shows fitted curves by the nonlinear least-squares method using Williams-Morrison equation. Enzyme concentration [E], initial velocity v_0, number of measurements (n) and K_i value are shown below each plot.

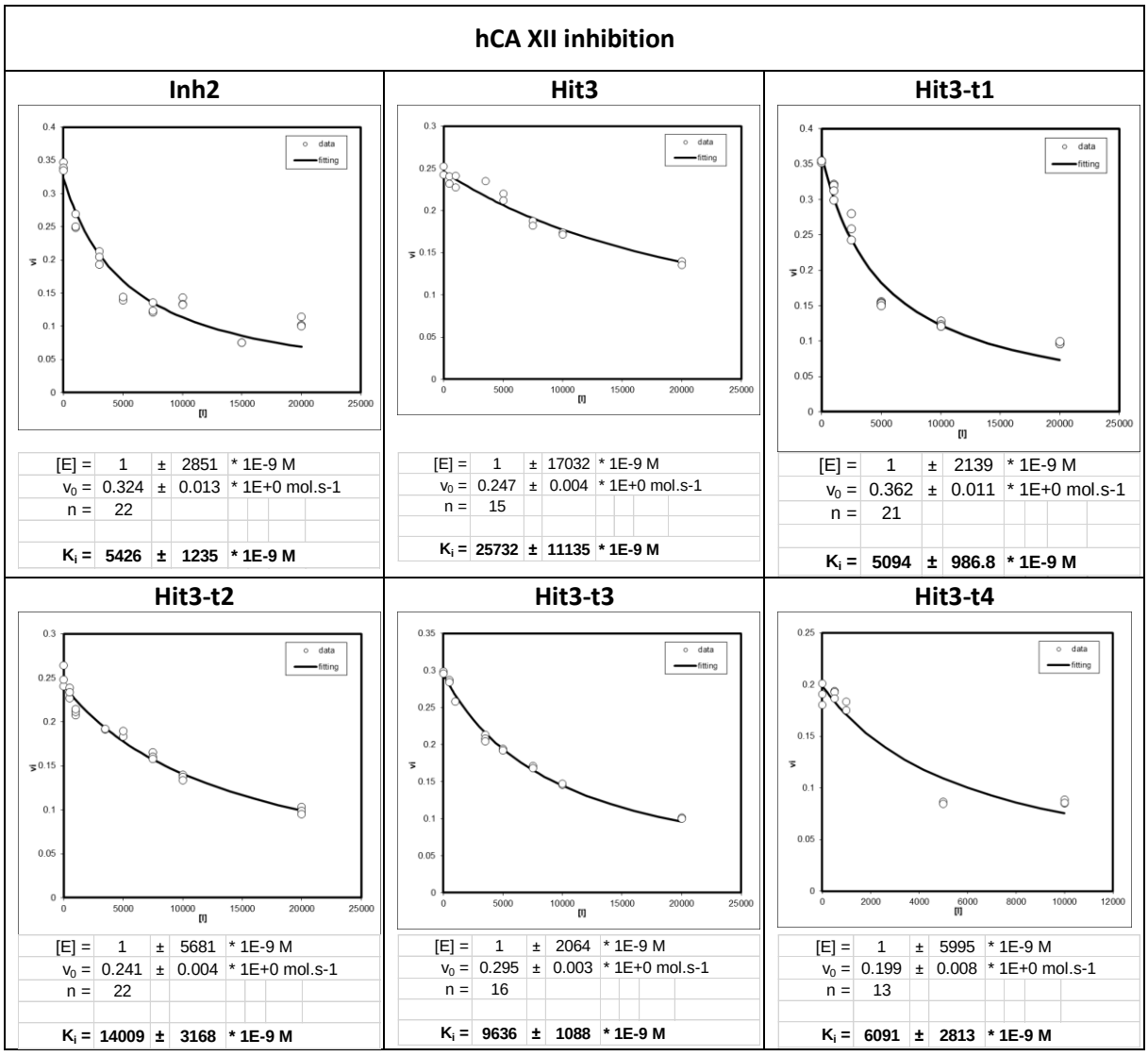


Figure S14: Inhibition of hCA XII by rising concentration of compounds.

Plots show the dose dependent decrease of catalytic activity expressed as speed of decrease in absorbance (v_i expressed in $\Delta A.s^{-1}$). The concentration of compound [I] in shown in $nmol.l^{-1}$. Each circle represents one measurement, black line shows fitted curves by the nonlinear least-squares method using Williams-Morrison equation. Enzyme concentration [E], initial velocity v_0, number of measurements (n) and K_i value are shown below each plot.

Literature

(1) Rink, W. M.; Thomas, F. *Org. Lett.* **2018,** *20* (23), 7493-7497.

(2) Tjhung, K. F.; Kitov, P. I.; Ng, S.; Kitova, E. N.; Deng, L.; Klassen, J. S.; Derda, R. *J. Am. Chem. Soc.* **2016,** *138* (1), 32-35.

(3) Ghosh, P. S.; Hamilton, A. D. *Chem. Eur. J.* **2012,** *18* (8), 2361-2365.

(4) Goddard-Borger, E. D.; Stick, R. V. *Org. Lett.* **2007,** *9* (19), 3797-3800.

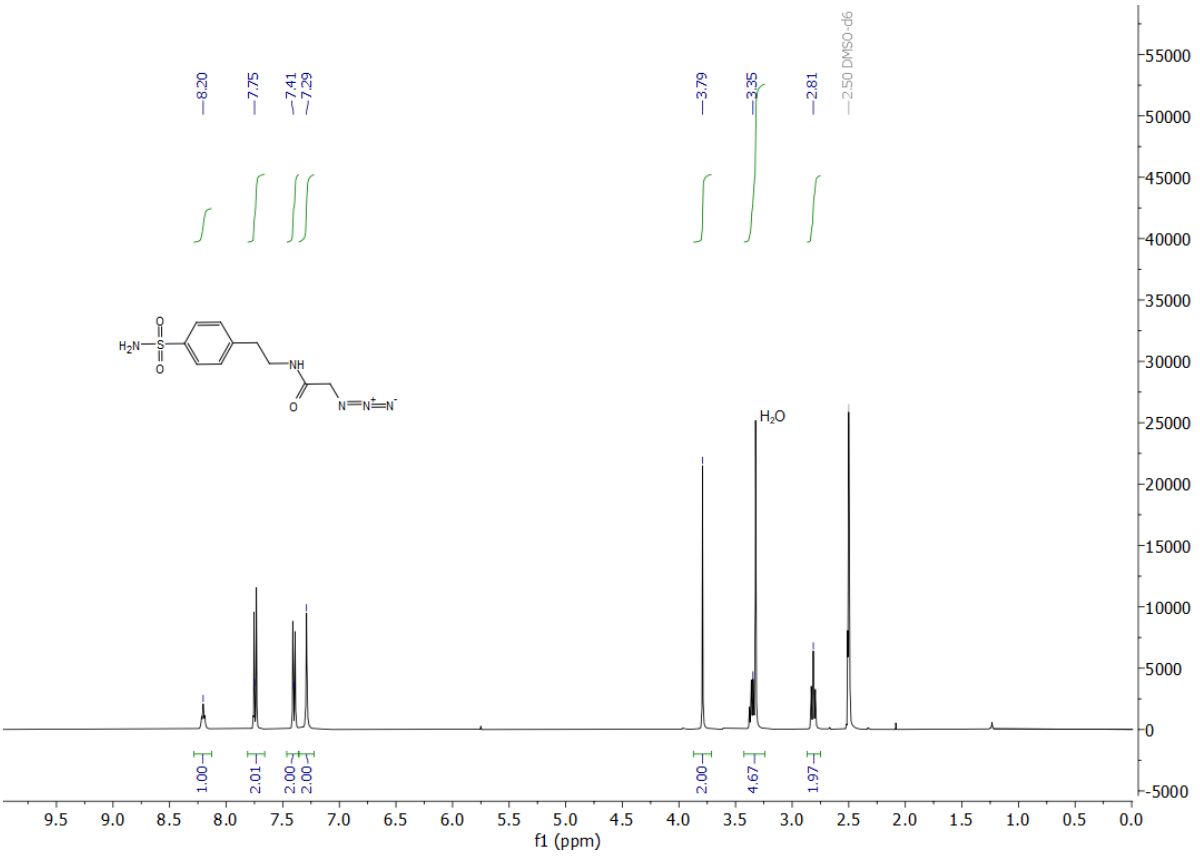
(5) Potter, G. T.; Jayson, G. C.; Miller, G. J.; Gardiner, J. M. *J. Org. Chem.* **2016,** *81* (8), 3443-3446.

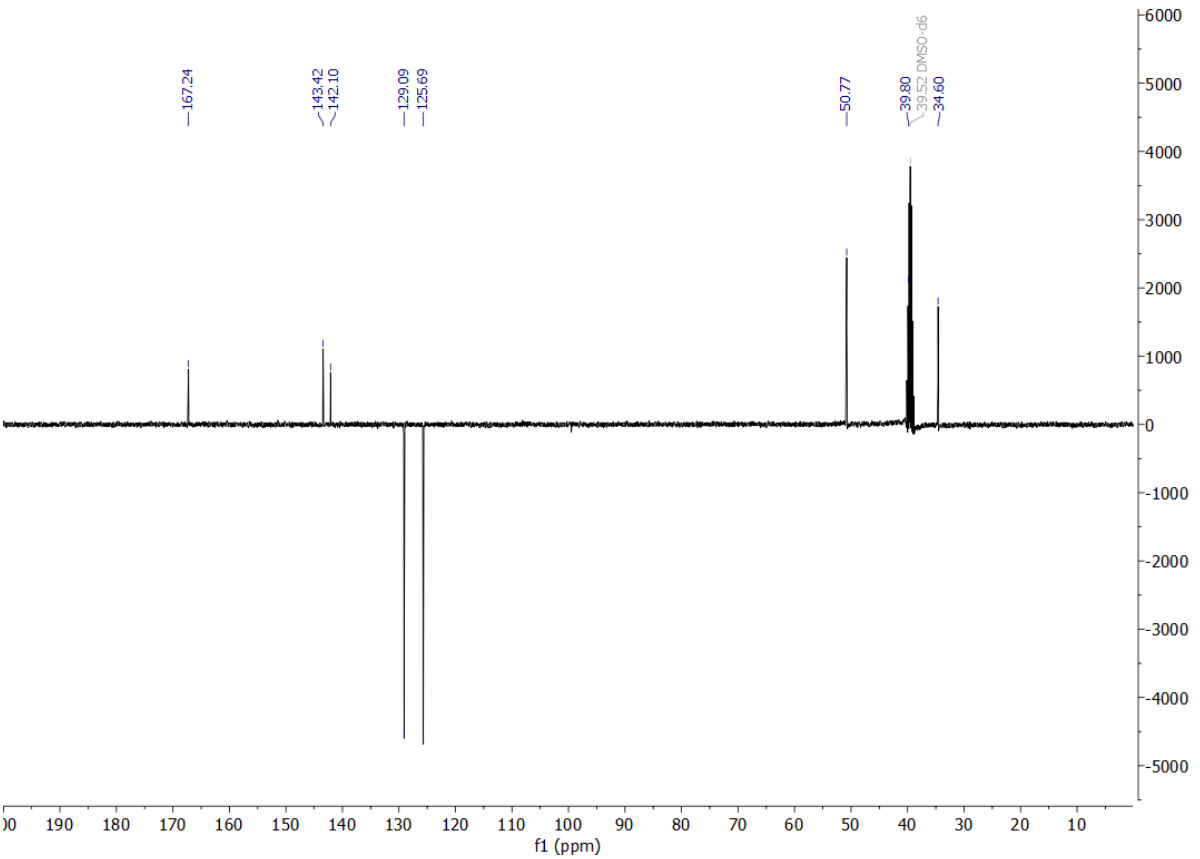
(6) Fischer, N.; Goddard-Borger, E. D.; Greiner, R.; Klapötke, T. M.; Skelton, B. W.; Stierstorfer, J. *J. Org. Chem.* **2012,** *77* (4), 1760-1764.

(7) Castro, V.; Blanco-Canosa, J. B.; Rodriguez, H.; Albericio, F. *ACS Comb. Sci.* **2013,** *15* (7), 331-334.
(8) Kunys, A. R.; Lian, W.; Pei, D. *Current Protocols in Chemical Biology* **2012,** *4* (4), 331-355.
(9) Lam, K. S.; Lebl, M.; Krchňák, V. *Chem. Rev.* **1997,** *97* (2), 411-448.
(10) Wang, W.; Hong, S.; Tran, A.; Jiang, H.; Triano, R.; Liu, Y.; Chen, X.; Wu, P. *Chem. Asian J.* **2011,** *6* (10), 2796-2802.
(11) Mocharla, V. P.; Colasson, B.; Lee, L. V.; Röper, S.; Sharpless, K. B.; Wong, C.-H.; Kolb, H. C. *Angew. Chem. Int. Ed.* **2005,** *44* (1), 116-120.
(12) Ng, S.; Jafari, M. R.; Matochko, W. L.; Derda, R. *ACS Chem. Biol.* **2012,** *7* (9), 1482-1487.
(13) Ozmen, M.; Can, K.; Akin, I.; Arslan, G.; Tor, A.; Cengeloglu, Y.; Ersoz, M. *J. Hazard. Mater.* **2009,** *171* (1), 594-600.
(14) Pinard, M. A.; Boone, C. D.; Rife, B. D.; Supuran, C. T.; McKenna, R. *Biorg. Med. Chem.* **2013,** *21* (22), 7210-7215.
(15) Renshaw, P. S.; Veverka, V.; Kelly, G.; Frenkiel, T. A.; Williamson, R. A.; Gordon, S. V.; Hewinson, R. G.; Carr, M. D. *J Biomol NMR* **2004,** *30* (2), 225-226.
(16) Veverka, V.; Lennie, G.; Crabbe, T.; Bird, I.; Taylor, R. J.; Carr, M. D. *J Biomol NMR* **2006,** *36* (1), 3-3.
(17) Pospíšilová, K.; Knedlík, T.; Šácha, P.; Kostka, L.; Schimer, J.; Brynda, J.; Král, V.; Cígler, P.; Navrátil, V.; Etrych, T.; Šubr, V.; Kugler, M.; Fábry, M.; Řezáčová, P.; Konvalinka, J. *ACS Omega* **2019,** *4* (4), 6746-6756.
(18) Khalifah, R. G. *J. Biol. Chem.* **1971,** *246* (8), 2561-&.
(19) Kugler, M.; Holub, J.; Brynda, J.; Pospíšilová, K.; Anwar, S. E.; Bavol, D.; Havránek, M.; Král, V.; Fábry, M.; Grüner, B.; Řezáčová, P. *J. Enzyme Inhib. Med. Chem.* **2020,** *35* (1), 1800-1810.
(20) Mueller, U.; Förster, R.; Hellmig, M.; Huschmann, F. U.; Kastner, A.; Malecki, P.; Pühringer, S.; Röwer, M.; Sparta, K.; Steffien, M.; Ühlein, M.; Wilk, P.; Weiss, M. S. *Eur. Phys. J. Plus* **2015,** *130* (7), 141.
(21) Kabsch, W. *Acta Crystallogr. Sect. D Biol. Crystallogr.* **2010,** *66*, 125-132.
(22) Kabsch, W. *Acta Crystallogr. Sect. D Biol. Crystallogr.* **2010,** *66*, 133-144.
(23) Mader, P.; Brynda, J.; Gitto, R.; Agnello, S.; Pachl, P.; Supuran, C. T.; Chimirri, A.; Řezáčová, P. *J. Med. Chem.* **2011,** *54* (7), 2522-2526.
(24) Long, F.; Nicholls, R. A.; Emsley, P.; Graæulis, S.; Merkys, A.; Vaitkus, A.; Murshudov, G. N. *Acta crystallographica. Section D, Structural biology* **2017,** *73* (Pt 2), 112-122.
(25) Emsley, P.; Cowtan, K. *Acta Crystallogr. Sect. D Biol. Crystallogr.* **2004,** *60* (12), 2126-2132.
(26) Murshudov, G. N.; Vagin, A. A.; Dodson, E. J. *Acta Crystallogr. Sect. D Biol. Crystallogr.* **1997,** *53*, 240-255.
(27) Chen, V. B.; Arendall, W. B., 3rd; Headd, J. J.; Keedy, D. A.; Immormino, R. M.; Kapral, G. J.; Murray, L. W.; Richardson, J. S.; Richardson, D. C. *Acta Crystallogr D Biol Crystallogr* **2010,** *66* (Pt 1), 12-21.
(28) Schrodinger, LLC, The PyMOL Molecular Graphics System, Version 1.8. 2015.
(29) Winn, M. D.; Ballard, C. C.; Cowtan, K. D.; Dodson, E. J.; Emsley, P.; Evans, P. R.; Keegan, R. M.; Krissinel, E. B.; Leslie, A. G. W.; McCoy, A.; McNicholas, S. J.; Murshudov, G. N.; Pannu, N. S.; Potterton, E. A.; Powell, H. R.; Read, R. J.; Vagin, A.; Wilson, K. S. *Acta Crystallogr. Sect. D Biol. Crystallogr.* **2011,** *67* (Pt 4), 235-242.
(30) Cheng, X.; Veverka, V.; Radhakrishnan, A.; Waters, L. C.; Muskett, F. W.; Morgan, S. H.; Huo, J.; Yu, C.; Evans, E. J.; Leslie, A. J.; Griffiths, M.; Stubberfield, C.; Griffin, R.; Henry, A. J.; Jansson, A.; Ladbury, J. E.; Ikemizu, S.; Carr, M. D.; Davis, S. J. *J. Biol. Chem.* **2013,** *288* (17), 11771-11785.
(31) BRÜNGER, A. T.; KURIYAN, J.; KARPLUS, M. *Science* **1987,** *235* (4787), 458-460.
(32) Chen, V. B.; Arendall, W. B., III; Headd, J. J.; Keedy, D. A.; Immormino, R. M.; Kapral, G. J.; Murray, L. W.; Richardson, J. S.; Richardson, D. C. *Acta Crystallogr. Sect. D Biol. Crystallogr.* **2010,** *66* (1), 12-21.

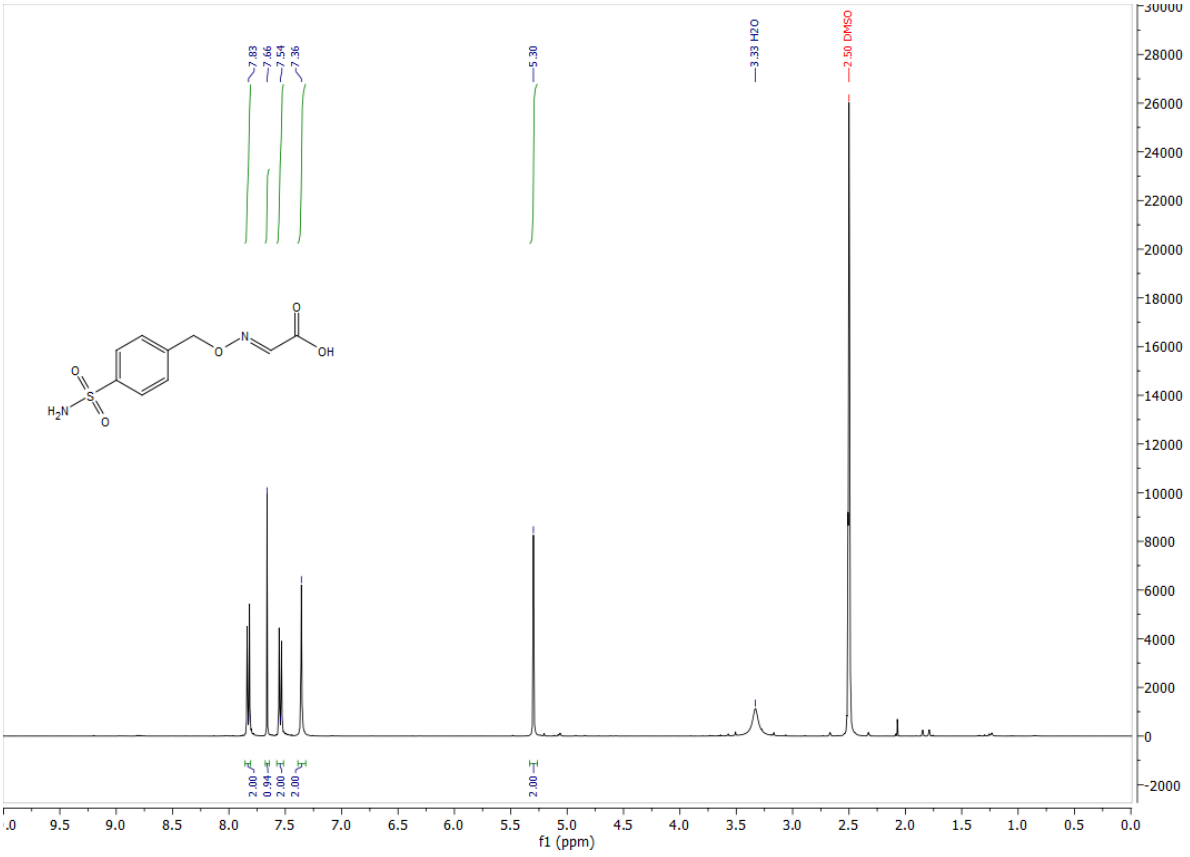
Copies of NMR spectra

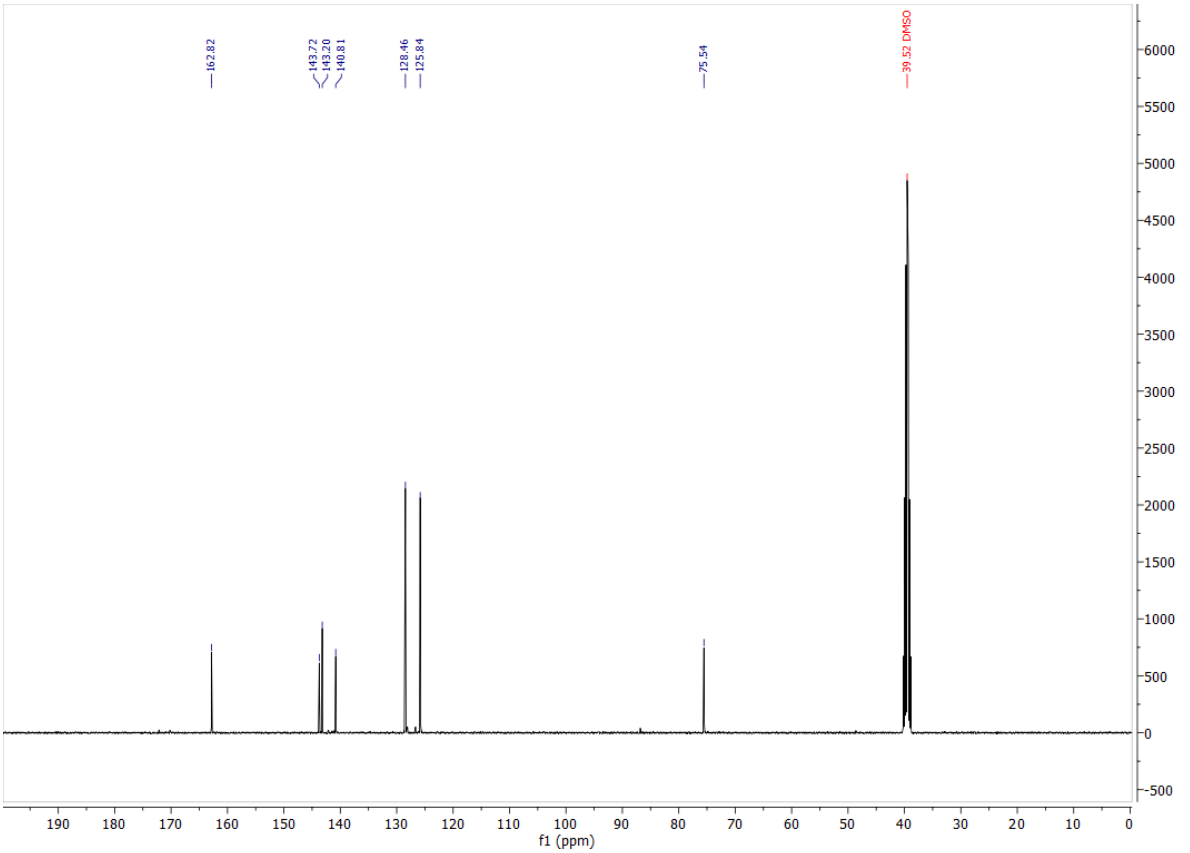
^{1}H and ^{13}C APT NMR spectra of **Inh3**



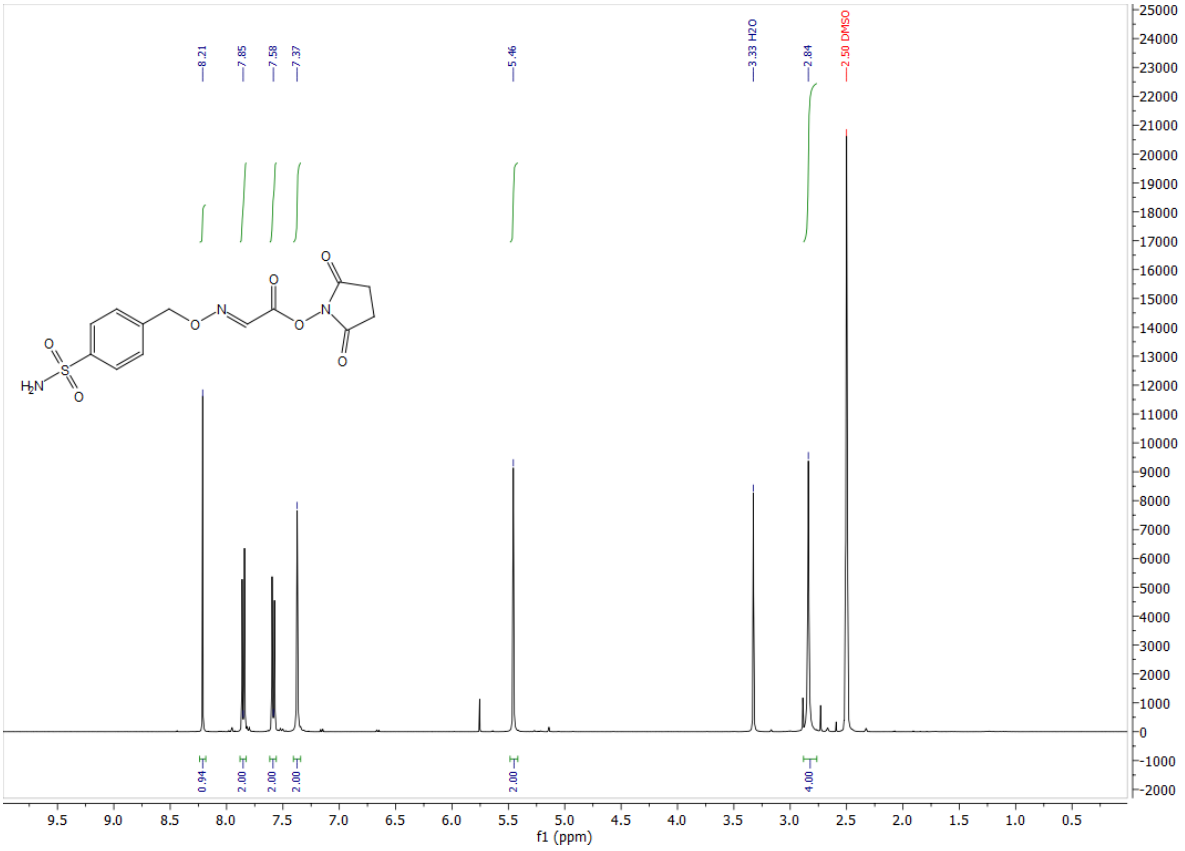


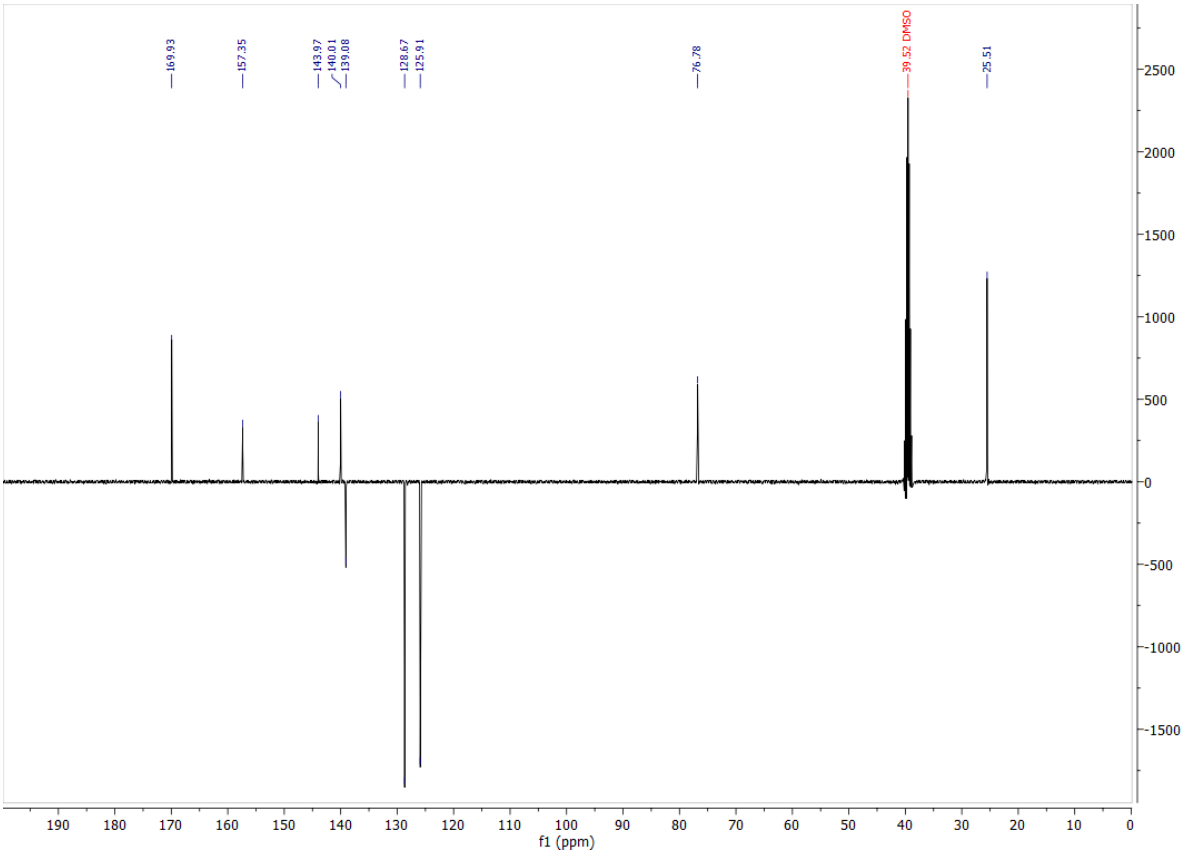
^{1}H and ^{13}C NMR spectra of **Inh2-COOH**



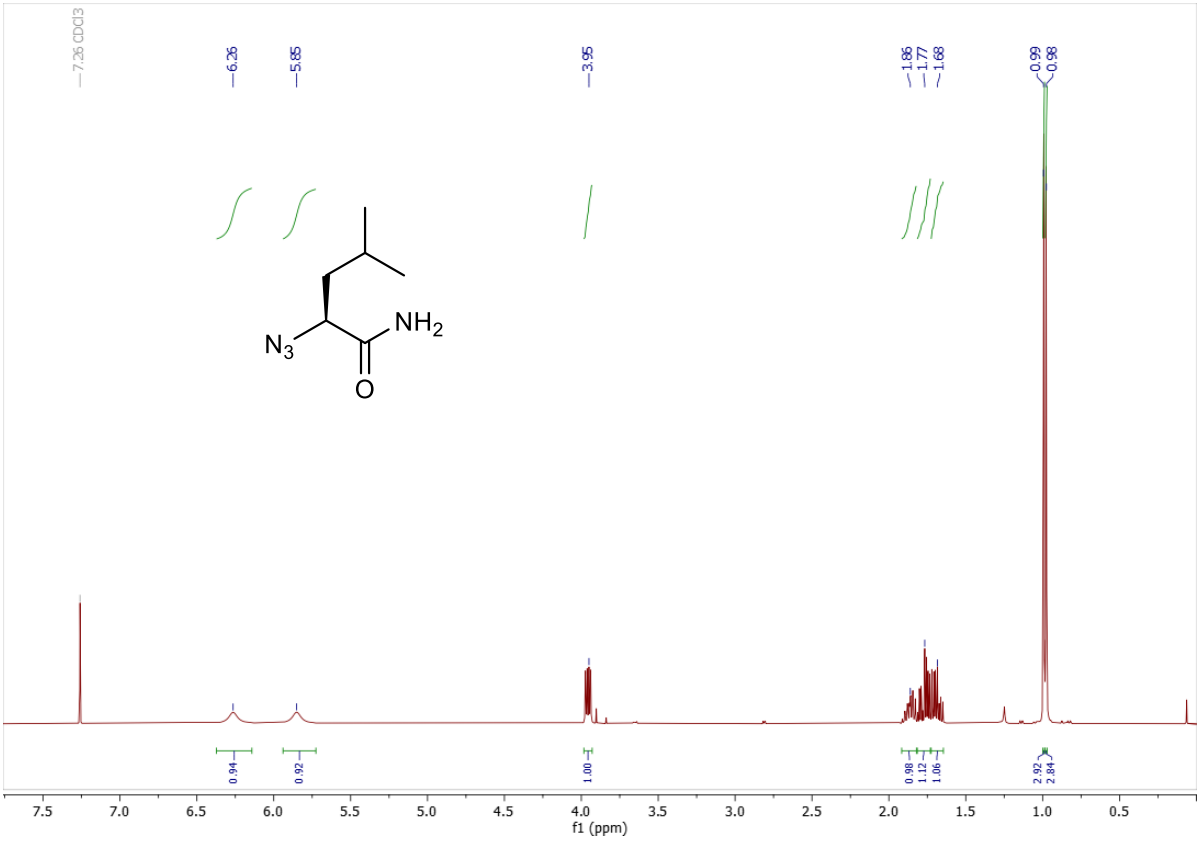


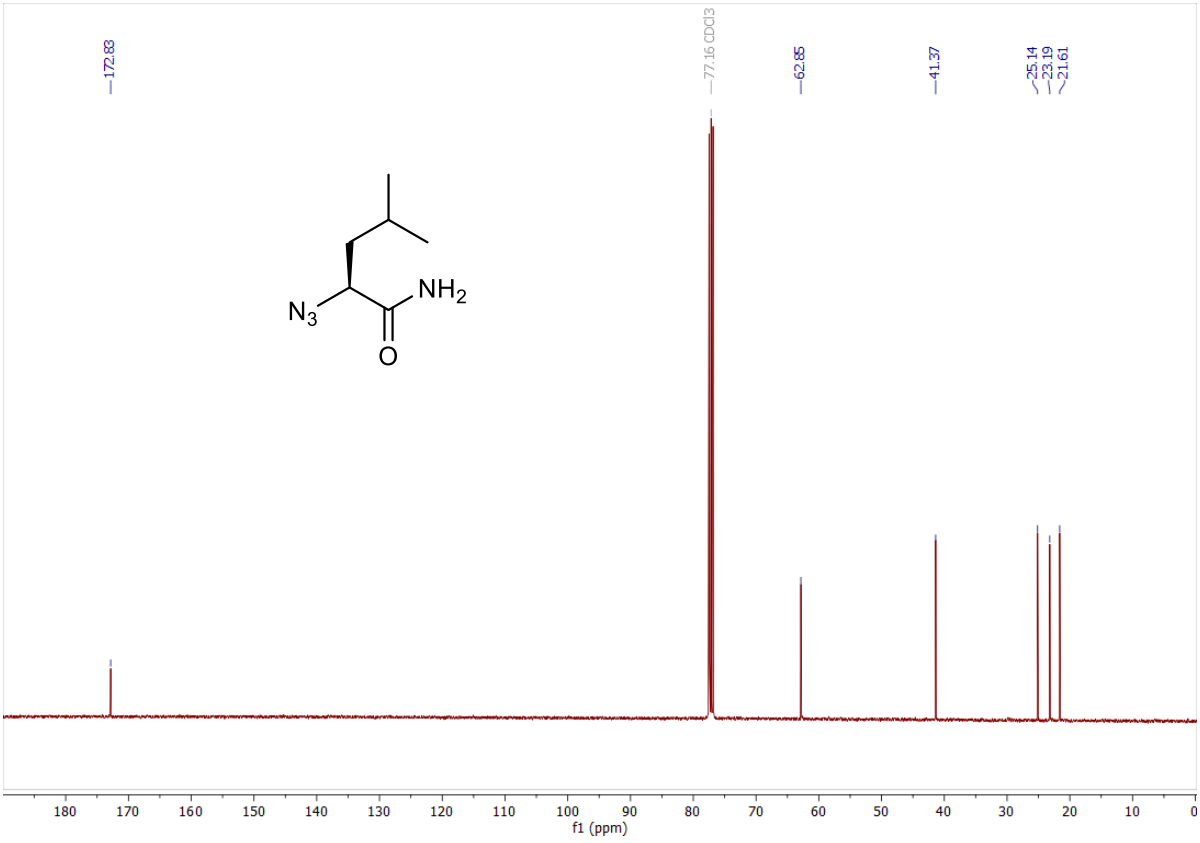
^{1}H and ^{13}C APT NMR spectra of **Inh2-COOSu**





^{1}H and ^{13}C NMR spectra of **N_3-*L*-Leu-NH_2**





^{1}H and ^{13}C NMR spectra of **Hit1**