Supporting Information

Interactions between Human Glutamate Carboxypeptidase II and Urea-based Inhibitors: Structural Characterization

Cyril Barinka,[†] Youngjoo Byun,[‡] Crystal Dusich,[‡,#] Sangeeta Ray,[‡,#] Ying Chen,[‡,#] Mark Castanares,[§] Ronnie Mease,[‡], Martin G. Pomper,[‡,§,] and Jacek Lubkowski[†,*]*

† Center for Cancer Research, National Cancer Institute at Frederick, Frederick, Maryland 21702, USA,‡ Russell H. Morgan Department of Radiology and Radiological Sciences, [§]Department of Pharmacology & Molecular Sciences, Johns Hopkins Medical Institutions, Baltimore, Maryland 21231

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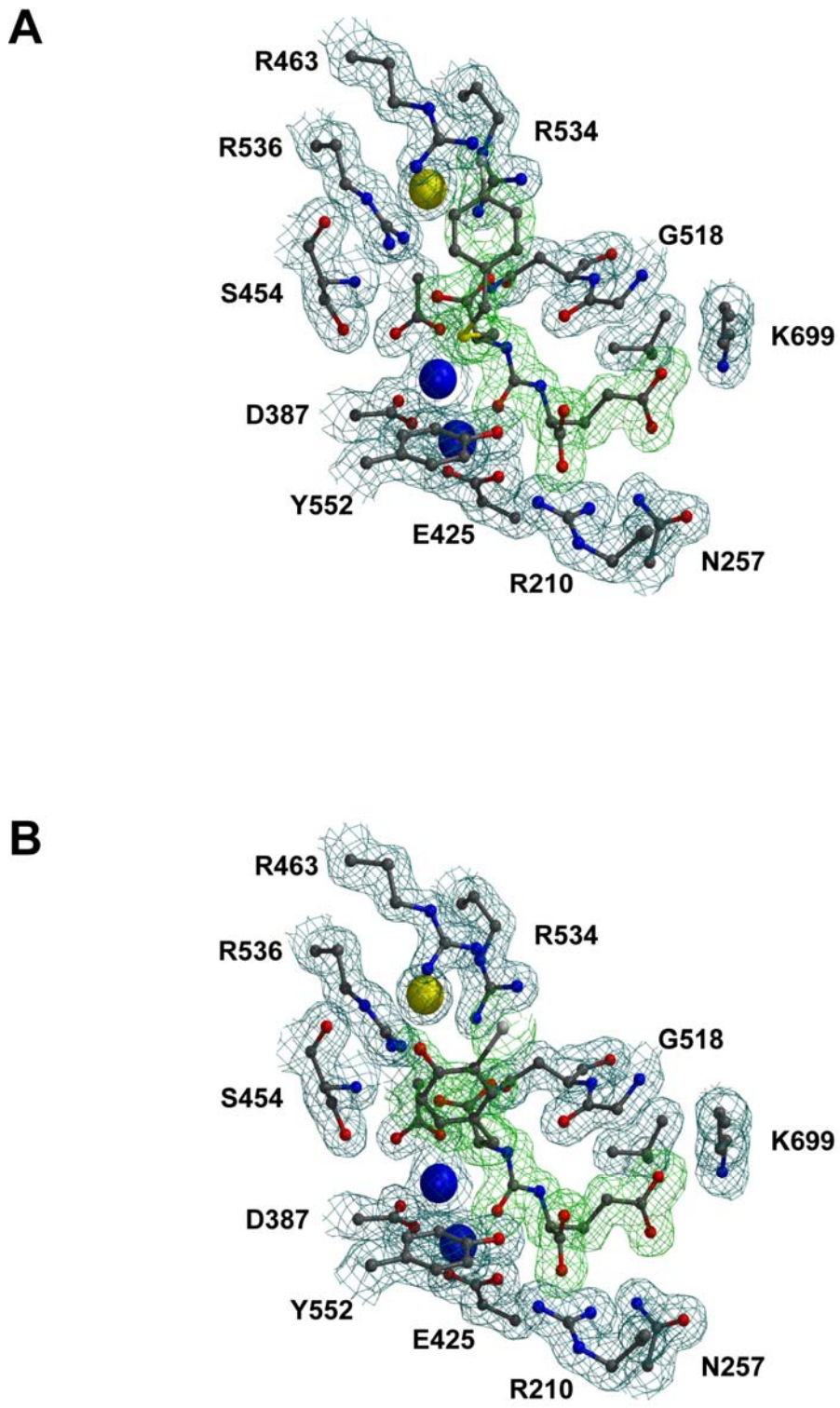
Figure S1: The electron density maps of the substrate binding cavity of human GCPII in complex with 3 (DCFBC, Panel A), 2 (DCIT, Panel B), 1 (DCMC, Panel C), and 4 (DCIBzL, Panel D). The protein and inhibitor residues are shown in ball-and-stick representation, while Zn^{2+} and Cl^- ions are depicted as blue and yellow spheres, respectively. The F_o-F_c electron density omit maps around inhibitor molecules are contoured at the 3σ level (green) and the $2F_o$-F_c electron density maps at the 1σ level (blue). The picture was generated using Molscript, Bobscript and rendered with PovRay.

Figure S2: Structural similarity of glutamate binding in the S1' pocket. The X-ray structures of rhGCPII/EPE (PDB code 3bi0), rhGCPII/glutamate complex (PDB code 2c6g) and the rhGCPII/**2** complexes were superimposed on corresponding Cα atoms. The active-site bound ligands are in stick representation, residues shaping the S1' pocket are shown as lines, Zn^{2+} ions as cyan spheres and conserved water molecules as red spheres. H-bonds are depicted as broken lines with interatomic distances in Angstroms (from the rhGCPII/**2** complex).

Figure S3: Flexibility of S1 arginines 463 and 536. While Arg534 is kept in an invariant position, arginines 463 and 536 can adopt different, yet distinct, conformations depending on the structural characteristics of the P1 moiety of an active-site bound ligand. The Arg536 side chain can adopt either binding (R536b) or stacking (R536s) conformation and the transition between these two conformations is associated with a 4.5 Å shift (the Cζ…Cζ distance) of the guanidinium group. In crystal structures the Arg463 side chain is observed in either 'up' (R463u) or 'down' (R463d) position with the positional difference of ~ 2.5 Å for corresponding Cηatoms. The X-ray structures of ligand-free GCPII (PDB code 2oot), the rhGCPII/**1** complex and the rhGCPII/**4** complex were superimposed on corresponding Cα atoms. The S1 arginines are shown in stick representation and colored red (R463), blue (R534), and yellow (536). The S1-bound chloride anion is shown as a green sphere.

Figure S4: Thermal displacement parameters of urea-based inhibitors. Protein residues are shown as lines, urea-based inhibitors are in stick representation, the active-site Zn^{2+} and S1-bound Cl^- ions are depicted as blue spheres. The atoms are colored according to values of their thermal displacement factors (B-factors). Notice overall higher B-factor values for the P1 moieties of inhibitors. rhGCPII in complex with **3** (**A**), **1** (**B**), **2** (**C**), and **4** (**D**).

Supplementary Figure S1



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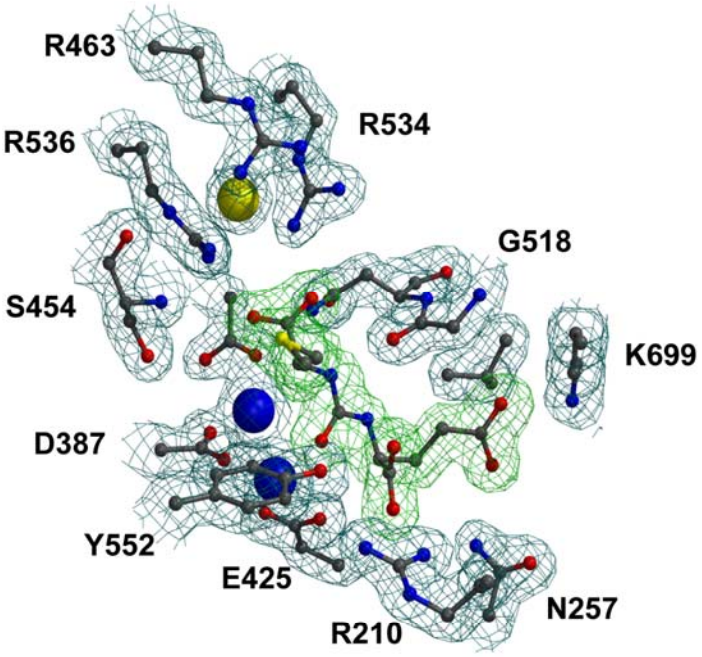
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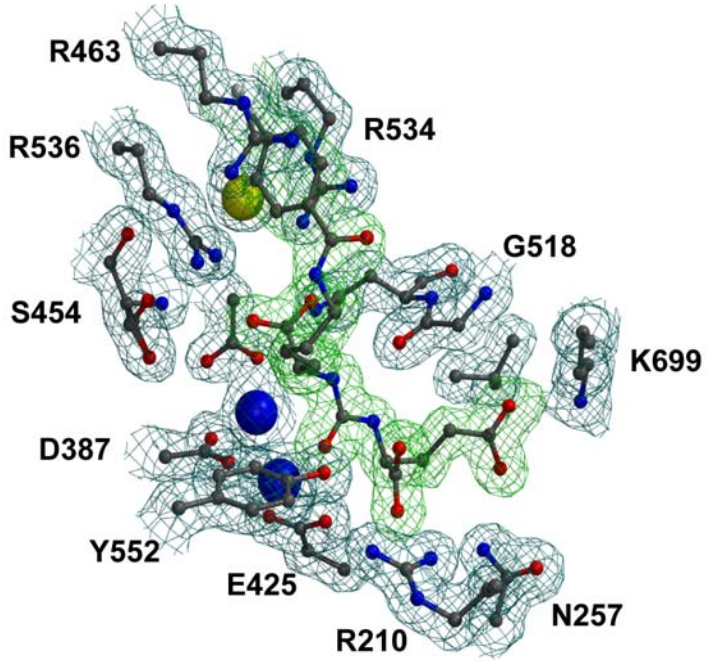
R210

D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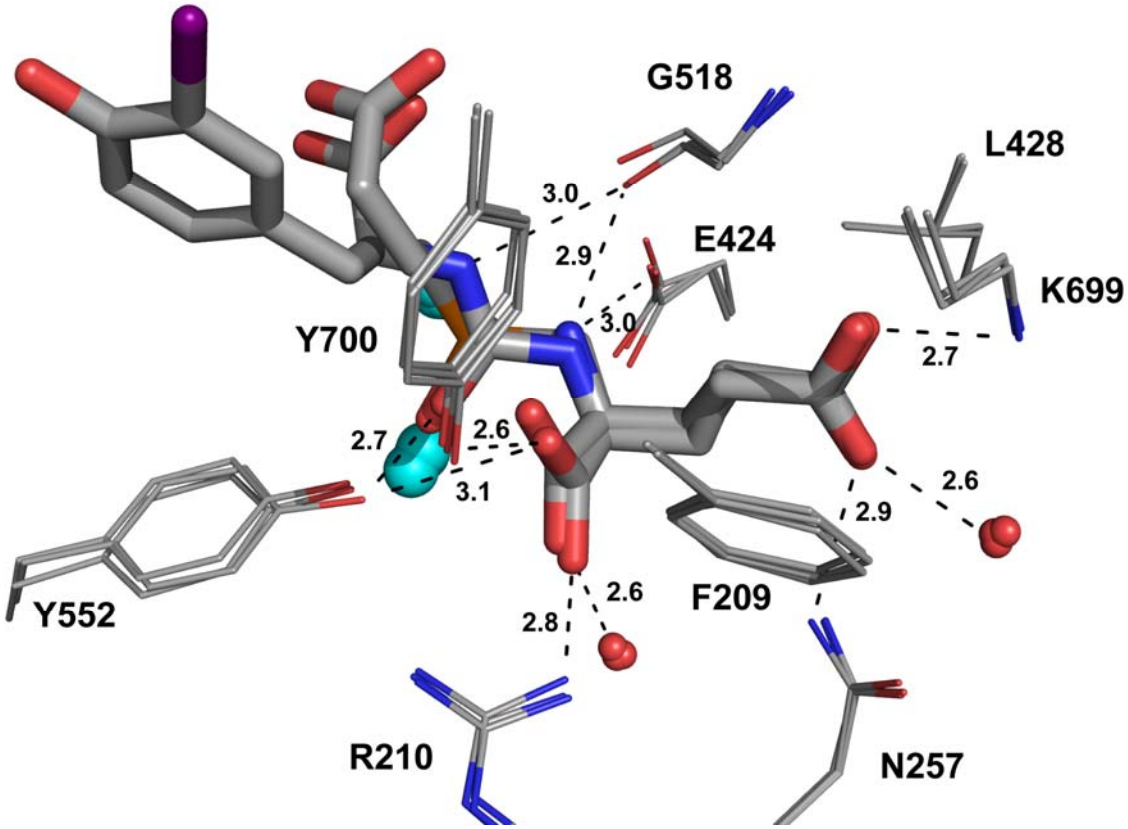
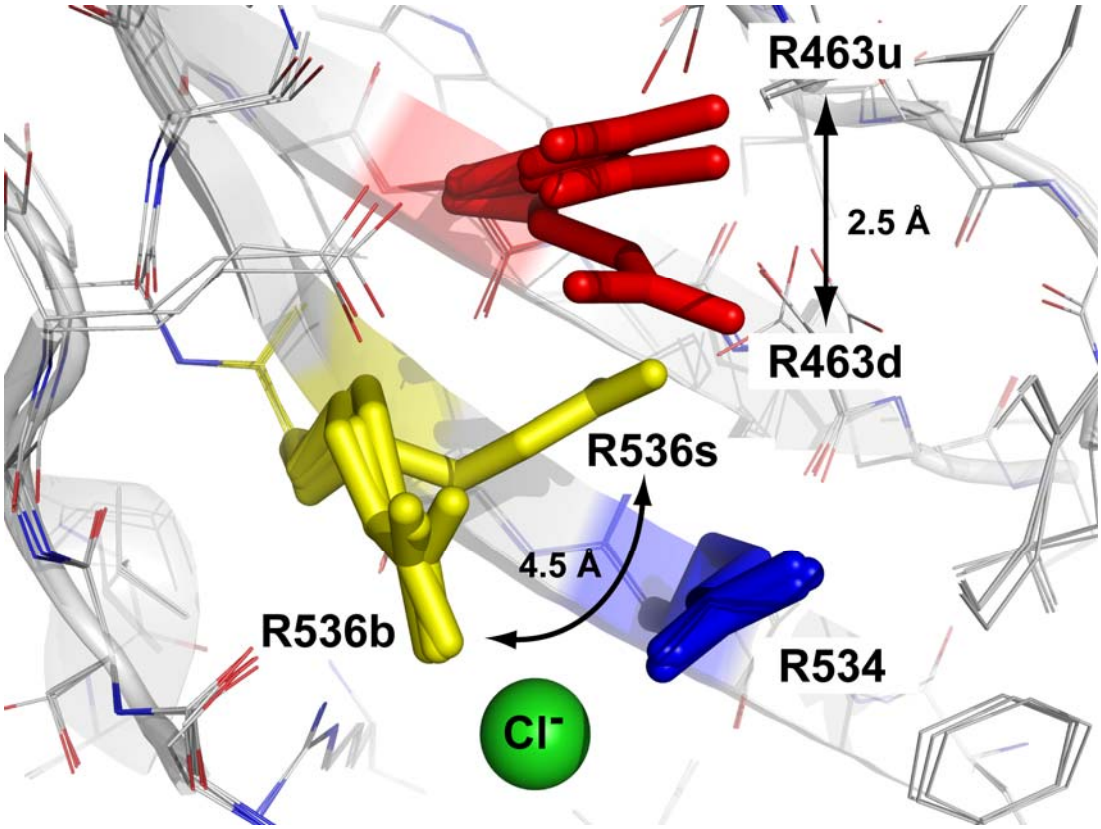
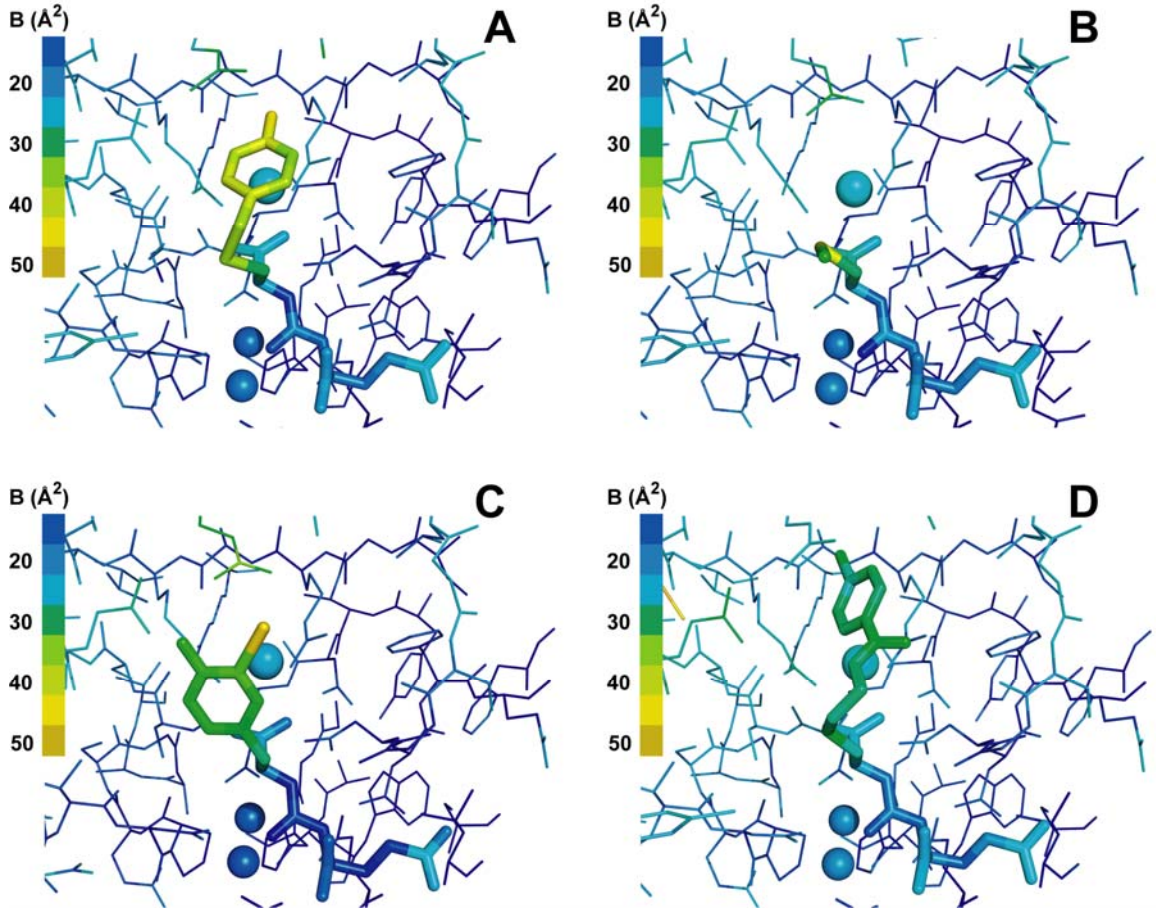
Supplementary Figure S2



Supplementary Figure S3



Supplementary Figure S4

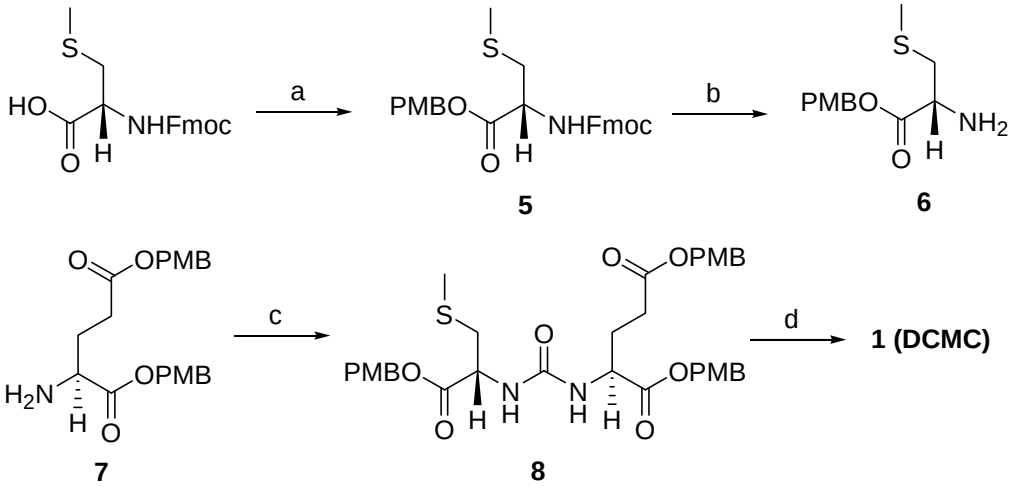


5. Supplemetary experimental section of DCMC and DCIT

5.1 General

All reactions were performed under a nitrogen atmosphere unless otherwise noted. Solvents and chemicals obtained from commercial sources were analytical grade or better and used without further purification. All experiments were performed in duplicate or triplicate to ensure reproducibility. Analytical TLC was performed using Aldrich aluminum-backed 0.2 mm silica gel Z19, 329-1 plates and visualized by ultraviolet light (254 nm), I_2 and 1% ninhydrin in ethanol. Flash column chromatography was performed on silica gel (60 Å) from MP Biomedical (Germany). HPLC purifications were performed on a Waters 625 HPLC apparatus using an Alltech Econosil C18-RP preparative column (10 x 250 mm) with H_2O/CH_3CN/TFA solvent systems. The absorbance detector was a Waters 490E set at 220 and 254 nm. The ^{1}H NMR spectra were recorded on a Varian 400 mHz NMR spectrometer or Bruker ultrashield^{TM} 400Hz Spectrometer. Chemical shifts (δ) are reported in ppm. ESI mass spectra were obtained with a Bruker Daltonics Esquire 300 plus spectrometer or with an Applied Biosystems Voyageur DE-FTR MALDI-TOF.

5.2. Experimental Section of 1 (DCMC)



Synthetic route for 1 (DCMC). Reagents and conditions: (a) $CsCO_3$, *p*-methoxybenzyl chloride, DMF, rt, 4 hr; (b) piperidine, DMF, rt, 15 min; (c) (i) triphosgene/TEA, CH_2Cl_2, -78 ^{o}C, 90 min, (ii) **5**, CH_2Cl_2, rt, overnight; (d) anisole, TFA, rt, 30 min.

(*R*)-4-methoxybenzyl 2-(((9*H*-fluoren-9-yl)methoxy)carbonylamino)-3-(methylthio)propanoate (5). Under a N_2 atmosphere, a suspension of Fmoc-Cys-(Me)-OH (1.00 g, 2.80 mmol), cesium carbonate (1.80 g, 5.60 mmol), and *p*-methoxybenzyl chloride (0.66 g, 4.20 mmol) in anhydrous DMF (20 mL) was stirred at room temperature for 4 hours. The suspension was filtered, the filtrate was diluted with EtOAc, and washed with H_2O (1 x 50 mL), 5% Na_2CO_3 (1 x 50 mL), and H_2O (2 x 50 mL). The organic layers were collected and dried over anhydrous $MgSO_4$, filtered, and concentrated. The sticky white solid was recrystalized with hexanes/EtOAc (7:3) to yield compound **5** (0.90 g, 69%). ^{1}H NMR ($CDCl_3$) δ 7.79 (d, 2H, *J* = 7.5 Hz), 7.63 (d, 2H, *J* = 7.4 Hz), 7.43 (t,

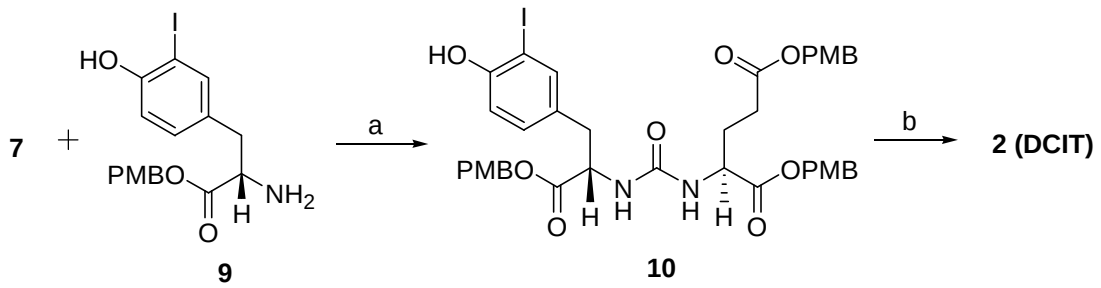
2H, *J* = 7.4 Hz, 7.5 Hz), 7.29-7.37 (m, 4H), 6.90 (d, 2H, *J* = 8.6 Hz), 5.68 (d, 1H, *J* = 8.0 Hz), 5.17 (d, 2H, *J* = 4.4 Hz), 4.66 (br, 1H), 4.42 (d, 2H, *J* = 7.1 Hz), 4.25 (t, 1H, *J* = 7.1 Hz), 3.82 (s, 3H), 2.93-3.07 (m, 2H), 2.08 (s, 3H).

(*R*)-4-methoxybenzyl 2-amino-3-(methylthio)propionate (6). Compound **5** (500 mg, 1.05 mmol) was stirred in a 20% piperidine/DMF solution (10 mL) for 15 min. at room temperature. The solution was diluted with CH_2Cl_2, then extracted H_2O (3 x 25 mL) and brine (1 x 25 mL. The organic layer was dried over anhydrous $MgSO_4$, followed by filtration and concentration under reduced pressure. Purification by column chromatography (hexanes/EtOAc gradient, 1:1 to 1:4) gave compound **6** (0.18 g, 68%). ^{1}H NMR ($CDCl_3$) δ 7.32 (d, 2H, *J* = 8.4 Hz), 6.91 (d, 2H, *J* = 8.4 Hz), 5.13 (s, 2H), 3.83 (s, 3H), 3.66-3.72 (m, 1H), 2.88-2.94 (m, 1H), 2.73-2.80 (m, 1H), 2.09 (s, 3H).

(*S*)-bis(4-methoxybenzyl)-2-(3-((*R*)-1-(4-methoxybenzyloxy)-3-(methylthio)-1-oxopropan-2-yl)ureido)pentanedioate (8). Bis-4-methoxybenzylglutamate hydrochloride [1,2] (**7**, 300 mg, 0.71 mmol) was dissolved in anhydrous CH_2Cl_2 (10 mL) and cooled to -78˚C. Triphosgene (74 mg, 0.25 mmol) in CH_2Cl_2 (1.5 mL) was added, followed by triethylamine (220 mg, 2.14 mmol). The reaction stirred at -78˚C for 90 minutes, then warmed to room temperature over 15 minutes. Compound **6** (180 mg, 0.71 mmol) in CH_2Cl_2 (2.5 mL) was added and stirred at room temperature overnight. The reaction was diluted with CH_2Cl_2 and washed with H_2O (2 x 30 mL) and brine (2 x 30 mL). The organic layer was dried over anhydrous Na_2SO_4, then filtered and concentrated under reduced pressure. Purification by column chromatography (CH_2Cl_2/EtOAc, 9:1) yielded compound **8** (360 mg, 77%). ^{1}H NMR ($CDCl_3$) δ 7.28 (d, 6H, *J* = 6.6 Hz), 6.89 (d, 6H, *J* = 8.6 Hz), 5.57 (d, 1H, *J* = 7.8 Hz), 5.45 (d, 1H, *J* = 8.0 Hz), 4.99-5.17 (m, 6H), 4.74, (br, 1H), 4.54 (br, 1H), 3.82 (s, 9H), 2.93 (d, 2H, *J* = 5.0 Hz), 2.32-2.49 (m, 2H), 2.11-2.24 (m, 1H), 2.02 (s, 3H), 1.90-2.04 (m, 1H).

(*S*)-2-(3-((*R*)-1-carboxy-2-methylthio)ethyl)ureido)pentanedioic acid (1, DCMC). A solution of anisole (0.2 mL) and trifluoroacetic acid (1 mL) was cooled to 0° C, then added to a flask containing compound 8 (450 mg, 0.068 mmol). The solution was stirred for 30 min before concentration under reduced pressure. RP-HPLC purification (H_2O/CH_3CN/TFA, 95:5:0.1, 4 mL/min) of the crude mixture afforded compound **1** (17 mg, 79%). ^{1}H NMR (D_2O) δ 4.37 (dd, 1H, *J* = 4.9 Hz, 2.7 Hz), 4.17 (dd, 1H, *J* = 5.1 Hz, 4.1 Hz), 2.79-2.96 (m, 2H), 2.41 (t, 2H, *J* = 7.2 Hz), 2.04-2.14 (m, 1H), 2.03 (s, 3H), 1.82-1.93 (m, 1H). MS (MALDI-TOF, positive mode, DHB matrix): *m/z* 309.04 $[M+H]^+$, 331.01 $[M+Na]^+$, calcd for $C_{10}H_{16}N_2O_7S$ 308.07.

5.3. Experimental Section of 2 (DCIT)



Synthetic route for 2 (DCIT). Reagents and conditions: (a) (i) triphosgene/TEA, CH_2Cl_2, -78 ^oC, 90 min, (ii) **9**, CH_2Cl_2, rt, overnight; (d) anisole, TFA, rt, 20 min.

(*S*)-4-methoxybenzyl 2-amino-3-(4-hydroxy-3-iodophenyl)propanoate (9). To a solution of 3-iodo-L-tyrosine (3.07 g, 10 mmol) in DMF (10 mL) was added N, N, N′, N′-tetramethylguanidine (1.15 g, 10 mmol) slowly at room temperature. After stirring for 10 min, ethyl acetoacetate (1.27 mL, 10 mmol) was added and was stirred at 60º C for 45 min. To the reaction mixture was added 4-methoxybenzyl bromide (1.60 mL, 11 mmol) and the reaction mixture was stirred for another 2 hr at 60°C. The reaction mixture was then diluted with $NaHCO_3$ (1M, 50 mL) and EtOAc (70 mL). The organic phase was then washed with $NaHCO_3$ (1M, 50 mL) and water (3 x 50 mL) and dried over Na_2SO_4. After evaporation, the residue was dissolved in methanolic hydrochloric acid (1N, 20 mL) and stirred for 10 min at room temperature. The excess solvent was removed under reduced pressure and the oily residue was washed several times with diethyl ether to give compound **9** (3.70 g, 80%) as a colorless solid. ^1H NMR (400 Hz) δ : 7.53 (s, 1H), 7.24 (d, J = 8.8 Hz, 2H), 6.91 (m, 3H), 6.74 (d, J = 8.0 Hz, 1H), 5.13 (m, 2H), 4.2 (t, J =7.5 Hz, 1H), 3.80 (s, 3H), 3.04 (m, 2H). ESI-MS: $[M+1]^+$ 428.3 Calcd for $C_{17}H_{19}INO_4$, 428.0359.

(*S*)-bis(4-methoxybenzyl) 2-(3-((*S*)-3-(4-hydroxy-3-iodophenyl)-1-(4-methoxybenzyloxy)-1-oxopropan-2-yl)ureido)pentanedioate (10). To a solution of compound **7** (3.40 g, 8.0 mmol) in CH_2Cl_2 (15 mL) was added triphosgene (0.783 g, 2.64 mmol) in CH_2Cl_2 (5 mL) at -78 ^oC. After stirring for 30 min, triethylamine (6 mL, 42.5 mmol) in CH_2Cl_2 (10 mL) was added to the reaction mixture. The reaction was stirred at -78^oC for 1 hr, allowed to warm to room temperature, and stirred for 30 min. Compound **9** (3.70 g, 8.0 mmol) in CH_2Cl_2 (7 mL) was added to the reaction mixture. This mixture was stirred overnight. CH_2Cl_2 (20 mL) was added and washed with water, followed by brine. After drying over Na_2SO_4, filtration and evaporation, flash chromatography on silica gel 60 (EtOAc/CH_2Cl_2, 1:4) afforded compound **10** (4.20 g, 63%). ^1H NMR ($CDCl_3$) δ: 7.30 (s, 1H), 7.30-7.22 (m, 6H), 6.86-6.82 (m, 7H), 6.68 (d, J = 8.8 Hz, 1H), 5.80 (d, J = 8.8 Hz, 1H), 5. 61(d, J = 8.8 Hz, 1H), 5.08-4.93 (m, 4H), 4.78 (m, 1H), 4.54 (m, 1H), 4.51 (m, 1H), 3.77 (s, 9H), 2.88 (m, 2H), 2.36 (m, 2H), 2.12 (m, 1H), 1.92 (m, 1H). ESI-MS: $[M+1]^+$ 841.0, Calcd for $C_{39}H_{41}IN_2O_{11}$, 840.7

(*S*)-2-(3-((*S*)-1-carboxy-2-(4-hydroxy-3-iodophenyl)ethyl)ureido)pentanedioic acid (2, DCIT). Compound **10** (200 mg, 0.24 mmol) was dissolved in an ice-cold solution of TFA (7 mL) and anisole (0.3 mL) and was stirred at 0 ^oC for 10 min. The ice-bath was

removed and solution was warmed up to room temperature and stirred another 10 min. The solution was evaporated under reduced pressure and the light brown residue was dried under high vacuum for 2 hr. The residue was washed with diethyl ether (3 × 5 mL) and water (10 × 2 mL). The residue was purified further by RP-HPLC (H_2O/CH_3CN/TFA, 80:20:0.1, 6 mL/min) to give compound **2** (90 mg, 78 %) R_t=7.2 min. ^{1}H NMR (D_2O) δ: 7.33 (t, J = 2.0 Hz, 1H), 6.83 (d, J = 10.0 Hz, 2H), 6.60 (d, J = 10.0 Hz, 2H), 4.19 (m, 1H), 3.96 (m, 1H), 2.77 (m, 1H), 2.63 (m, 1H), 2.19 (m, 2H), 1.86 (m, 1H), 1.66 (m, 1H). ESI-MS: $[M+1]^+$ 481; HRFAB-MS: $[M+1]^+$ 481.1018, Calcd for $C_{15}H_{18}IN_2O_4$, 481.1010.

References

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2. Maclaren JA: **Some Amino-Acid Esters - Improved Preparative Method**. *Australian Journal of Chemistry* 1978, **31:**1865-1868.