Supporting information for:

Diastereomeric Resolution Yields Highly Potent Inhibitor of SARS-CoV-2 Main Protease

Mark S. Cooper,[1,‡] Linlin Zhang,[2,‡] Mohamed Ibrahim,[2] Kaixuan Zhang,[2] Xinyuanyuan Sun,[2] Judith Röske,[2] Matthias Göhl,[3] Mark Brönstrup,[3,4] Justin K. Cowell,[1] Lucie Sauerhering,[5] Stephan Becker,[5,6] Laura Vangeel,[7] Dirk Jochmans,[7] Johan Neyts,[7] Katharina Rox,[3,4] Graham P. Marsh,[1,*] Hannah J. Maple,[1,*] Rolf Hilgenfeld [2,8,*]

Author Affiliations:

[1]Bio-Techne (Tocris), The Watkins Building, Atlantic Road, Bristol, BS11 9QD, UK.

[2]Institute of Molecular Medicine, University of Lübeck, 23562 Lübeck, Germany.

[3]Department of Chemical Biology, Helmholtz Centre for Infection Research (HZI), 38124 Braunschweig, Germany

[4]German Center for Infection Research (DZIF), Partner site Braunschweig-Hannover, 38124 Braunschweig, Germany

[5]Institute of Virology, University of Marburg, 35043 Marburg, Germany

[6]German Center for Infection Research (DZIF), Marburg-Gießen-Langen Site, 35043 Marburg, Germany

[7]Rega Institute, Department of Microbiology, Immunology and Transplantation, KU Leuven, B-3000 Leuven, Belgium

[8]German Center for Infection Research (DZIF), Hamburg-Lübeck-Borstel-Riems Site, University of Lübeck, 23562 Lübeck, Germany.

[‡]co-first authors

*co-corresponding authors: graham.marsh@bio-techne.com, hannah.maple@bio-techne.com and rolf.hilgenfeld@uni-luebeck.de

List of Contents:

Figure S1. ^{1}H NMR spectrum of compound **11** (**13b**). Inset, chemical structure of compound **11** (**13b**). Highlighted regions indicate where two sets of peaks are observed that correspond to a single proton. Assignment of these peaks is indicated by the numbering.

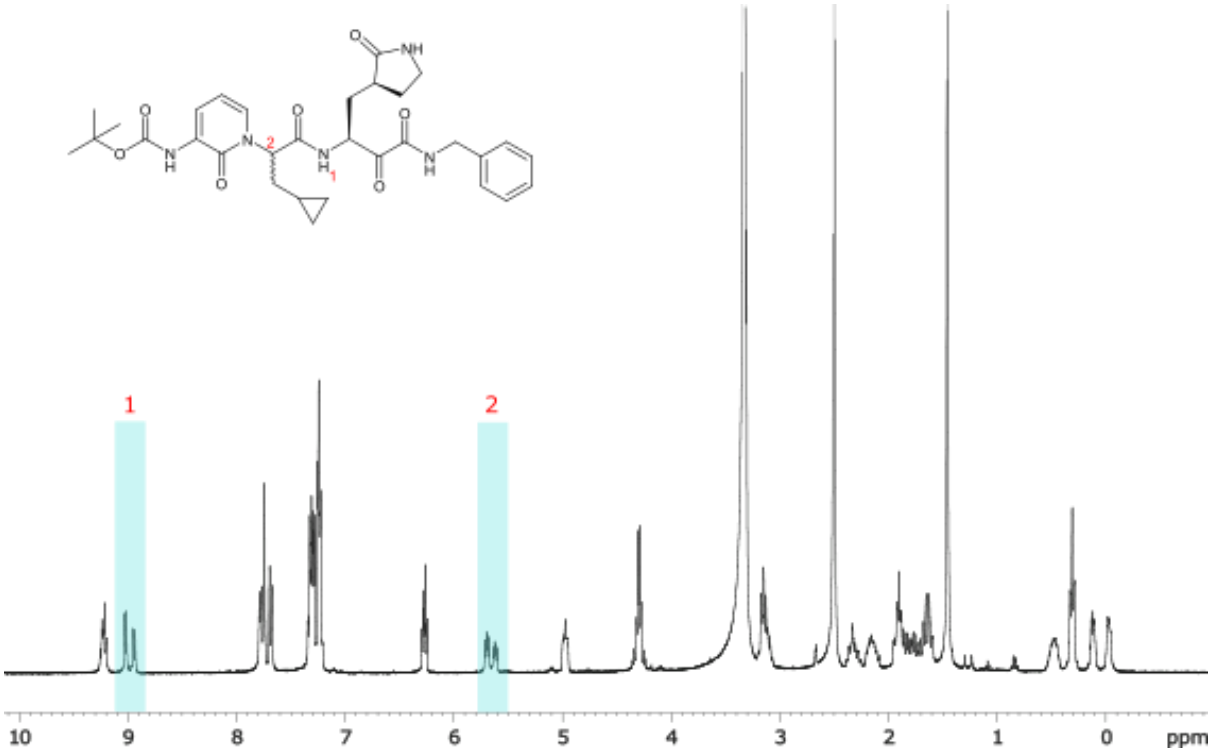


Figure S2. Single-crystal X-ray diffraction data for compound **12** (**13b-H**).

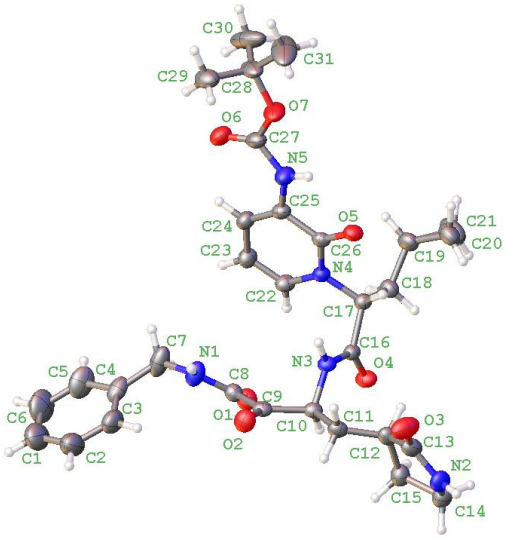


Figure S3. Dose-response curves from the FRET-based cleavage assay, for **13b-K** (A), **13b-H** (B) and an equimolar mixture of **13b-K** and **13b-H** (C).

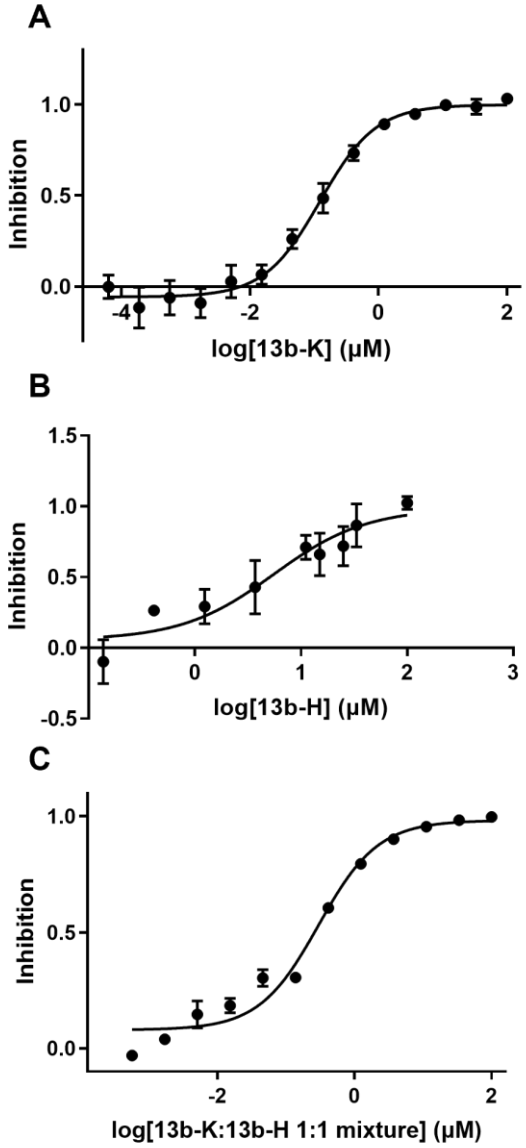


Figure S4. The crystal structures of **13b-K** (A) and **13b-H** (B) show the substrate-binding cleft of the M^{pro}. The same orientation of the view was chosen in both cases (A and B). Panel (C) shows the surface of pocket S2 in gold, and the cyclopropyl (P2) perfectly fits inside, in the case of **13b-K**. Additional representation of the carbon atoms of the cyclopropyl (P2) as spheres (depicted in red) representing the Van der Waal radii of the carbon atoms of the cyclopropyl. The carbon atoms of the compounds are depicted in yellow and those of the protein in cyan, with the exception of the Ser1 of the other monomer (green). Blue symbols (S1, S2) indicate the canonical binding pockets for moieties P1-P4 (red symbols) of the inhibitors. The 2Fo-Fc map carved around the inhibitor is depicted as a blue mesh (contoured at 1.0). Polar contacts are represented as red dashed lines.

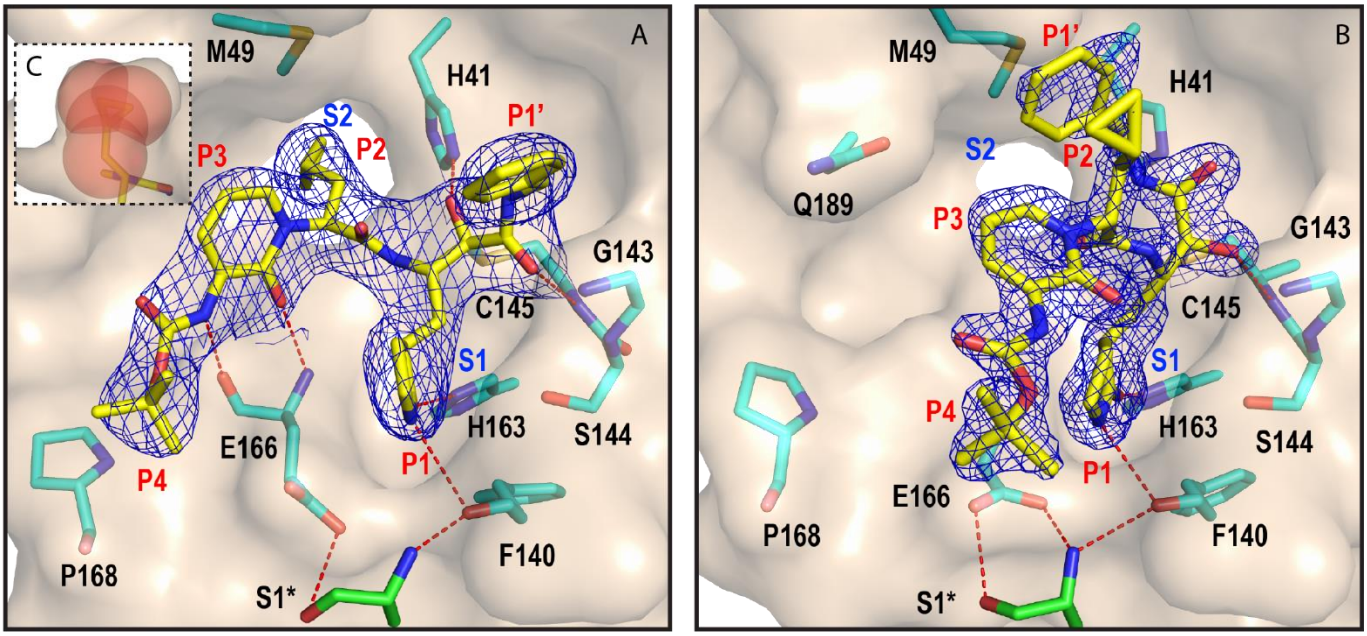
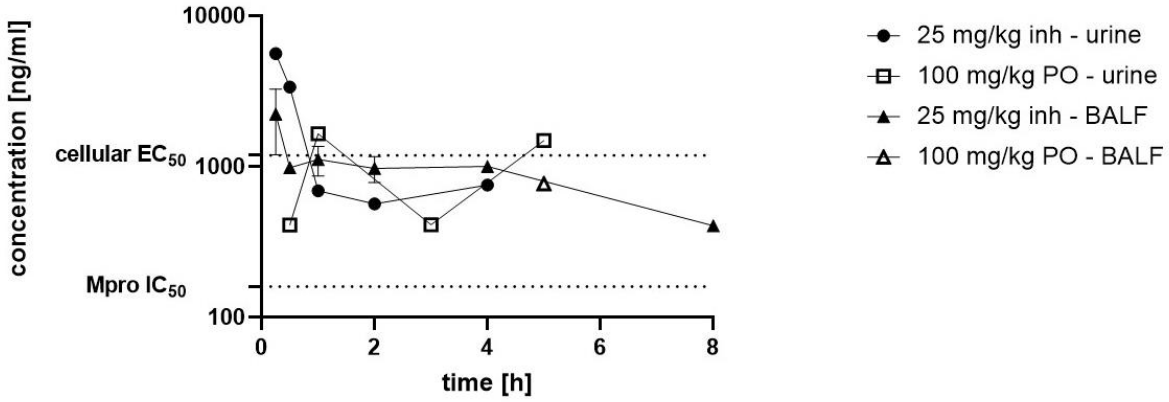
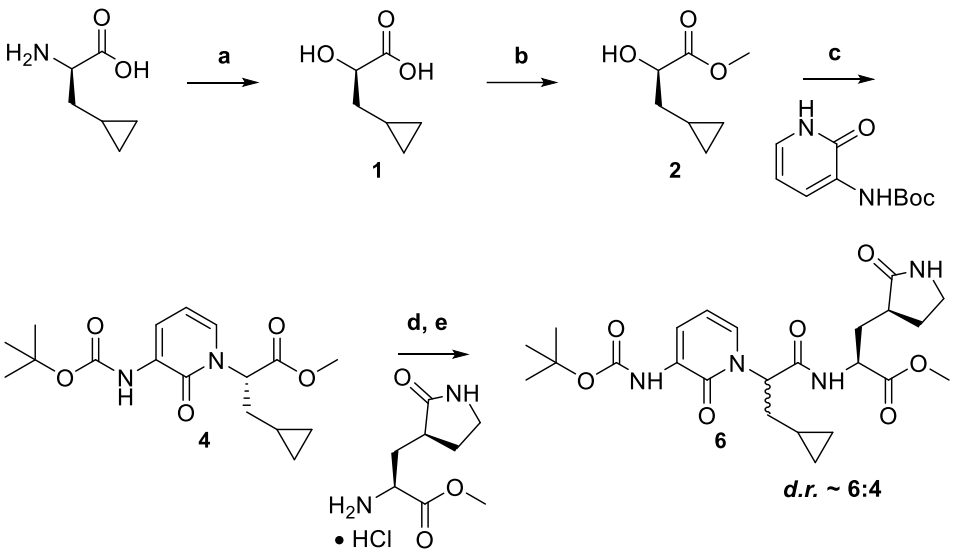


Figure S5. Levels of **13b-K** in urine and BALF after 25 mg/kg inhalative and 100 mg/kg peroral administration.



Scheme S1. Alternative synthetic sequence towards **6** employing a Mitsunsobu protocol.[a]



[a] Reagents and conditions: (a) H_2SO_4, $NaNO_2$, H_2O, -10°C-RT; (b) 2,2-dimethoxypropane, TMSCl, MeOH, RT; (c) *tert*-butyl (2-oxo-1,2-dihydropyridin-3-yl)carbamate, DEAD, PPh_3, PhMe/THF (3:1), 0°C-RT; (d) LiOH, MeOH, H_2O, RT; (e) methyl (*S*)-2-amino-3-((*S*)-2-oxopyrrolidin-3-yl)propanoate hydrochloride, HATU, DIPEA, DMF, 0°C.

Associated synthetic procedures

(R)-3-Cyclopropyl-2-hydroxypropanoic acid (1) D-Cyclopropylalanine (20.50 g, 158.7 mmol, 1.00 eq) was dissolved in 1 M sulfuric acid (158.7 mL, 158.7 mmol, 1.00 eq) and chilled in a NaCl/ice cooling bath. A solution of sodium nitrite (87.61 g, 1.270 mol, 8.00 eq) in water (200 mL) was added in a manner that the internal temperature stays around −10°C. The reaction mixture was allowed to warm to room temperature and stirred for further 14 hours. The mixture was extracted with MTBE (3 x 300 mL) and the combined organic extracts were dried over Na_2SO_4. Evaporation of the solvent afforded the title compound (19.68 g, assumed quantitative), which was used without any further purification. All spectroscopic data matched those previously reported.

Methyl (R)-3-cyclopropyl-2-hydroxypropanoate (2) (*R*)-3-Cyclopropyl-2-hydroxypropanoic acid **(1)** (19.68 g, 151.2 mmol, 1.00 eq) was dissolved in a mixture of MeOH (150 mL) and 2,2-dimethoxypropane (50.0 mL, 408 mmol, 2.70 eq), and TMSCl (2.88 mL, 22.7 mmol, 15 mol%) was added. The mixture was stirred for 16 hours. After careful removal of the volatiles the crude product was purified by flash chromatography, eluting with 25% diethyl ether/pentane increased to 40% diethyl ether/pentane. Careful removal of the solvent furnished the title compound as colorless oil (17.82 g, 123.6 mmol, 78% over 2 steps). All spectroscopic data matched those previously reported.

Methyl (S)-2-(3-((tert-butoxycarbonyl)amino)-2-oxopyridin-1(2H)-yl)-3-cyclopropylpropanoate (4) Methyl (*R*)-3-cyclopropyl-2-hydroxypropanoate **(2)** (3.400 g, 21.70 mmol, 1.00 eq), *tert*-butyl 2-oxo-1,2-dihydropyridin-3-ylcarbamate (5.107 g, 24.29 mmol, 1.03 eq), and triphenylphosphine (8.820 g, 33.63 mmol, 1.55 eq) were dissolved in a mixture of toluene (75 mL) and THF (25 mL) under an inert gas atmosphere. The reaction mixture was chilled in an ice bath and DEAD (15.31 mL, 40 wt.% in PhMe, 33.63 mmol, 1.55 eq) was added over 60 minutes. After 5 minutes the mixture was allowed to warm to room temperature, stirred for 1 hour and the volatiles were removed under reduced pressure. Purification by flash chromatography, eluting with 20% EtOAc/petroleum ether increased to 40% EtOAc/petroleum ether, afforded the title compound (5.823 g, 17.31 mmol, 80%) as a colorless oil. All spectroscopic data matched those previously reported.

(S)-2-(3-((tert-Butoxycarbonyl)amino)-2-oxopyridin-1(2H)-yl)-3-cyclopropylpropanoic acid (5) Methyl (*S*)-2-(3-((*tert*-butoxycarbonyl)amino)-2-oxopyridin-1(2H)-yl)-3-cyclopropylpropanoate **(4)** (11.38 g, 33.83 mmol, 1.00 eq) was dissolved in methanol (80 mL), diluted with water (40 mL), and lithium hydroxide (1.499 g, 62.59 mmol, 1.85 eq) was added. After stirring the emulsion vigorously for 6 hours until complete consumption of the starting material by TLC, most of the methanol was removed at 40°C under reduced pressure. The resulting mixture was partitioned between MTBE (100 mL) and H_2O (200 mL). After separation of the phases the aqueous layer was stirred, adjusted to pH = 2 with 4 M HCl, and a fluffy colorless solid began to precipitate. The mixture was stirred for further 30 min and the crystallization was continued at 4 °C for 3 hours. The solid was collected by vacuum filtration and washed with ice cold water (2 x 50 mL) followed by rinsing with pentane (2 x 50 mL). Drying the solid at 40°C under vacuum afforded title compound (9.394 g, 29.14 mmol, 86%) as a colorless solid. All spectroscopic data matched those previously reported.

Methyl (S)-2-(2-(3-((tert-butoxycarbonyl)amino)-2-oxopyridin-1(2H)-yl)-3-cyclopropylpropanamido)-3-((S)-2-oxopyrrolidin-3-yl)propanoate (6) A flask was charged

with (*S*)-2-(3-((*tert*-butoxycarbonyl)amino)-2-oxopyridin-1(2*H*)-yl)-3-cyclopropylpropanoic acid **(5)** (4.125 g, 12.80 mmol, 1.00 eq), methyl (*S*)-2-amino-3-((*S*)-2-oxopyrrolidin-3-yl)propanoate hydrochloride (3.191 g, 14.33 mmol, 1.12 eq) and HATU (5.595 g, 14.72 mmol, 1.15 eq), and DMF (50 mL) was added under an inert atmosphere. The mixture was stirred 5 min at ambient temperature and then chilled in an ice bath. DIPEA (7.021 mL, 40.31 mmol, 3.15 eq) was added and the mixture was stirred at 0°C for 6 hours. All volatiles were removed at 40°C, and the residue was partitioned between EtOAc (250 mL) and saturated K_2CO_3 solution (aq) (150 mL). The organic phase was separated, and the aqueous component was further extracted with EtOAc (3 x 200 mL). The combined organic extracts were dried over anhydrous sodium sulfate and concentrated under reduced pressure. Purification by flash chromatography, eluting with 30% EtOAc/Petroleum ether increased to 50% EtOAc/Petroleum ether, afforded the title compound (5.091 g, 10.38 mmol, 81%) as a mixture of diastereomers (*d.r.* = 6:4) in form of an almost colorless oil. All spectroscopic data matched those previously reported.

Methyl (R)-3-cyclopropyl-2-((triisopropylsilyl)oxy)propanoate TIPSCl (3.018 g, 15.65 mmol, 1.12 eq) was added under an inert atmosphere to NMI (3.00 mL, 37.6 mmol, 2.69 eq), followed by addition of methyl (*R*)-3-cyclopropyl-2-hydroxypropanoate **(2)** (2.015 g, 13.98 mmol, 1.00 eq). The biphasic reaction mixture and was stirred vigorously with a SmCo stirring bar to ensure a microemulsion. After a reaction time of 14 hours and complete consumption of the starting material by TLC, methanol (1 mL) was added and the reaction was stirred vigorously for further 15 minutes. The crude mixture was partitioned between pentane (150 mL) and H_2O (75 mL), and the organic phase was separated. The aqueous component was extracted pentane (75 mL), and the combined organic extracts were dried over anhydrous sodium sulfate and concentrated under reduced pressure. Finally, MeOTIPS was removed on the rotary evaporator at 50°C and a final pressure of 2 mbar to provide the title compound as a colorless oil (4.199 g, quantitative), which was used in the next step without further purification. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 4.40 (t, *J* = 6.0 Hz, 1H), 3.70 (s, 3H), 1.64 (m_c, 2H), 1.12 − 1.03 (m, 21H), 0.83 (m_c, 1H), 0.43 (m_c, 2H), 0.04 (m_c, 2H). ^{13}C NMR (126 MHz, $CDCl_3$): δ = 174.4, 72.8, 51.7, 41.0, 17.99, 17.97, 12.3, 6.8, 4.7, 4.2.

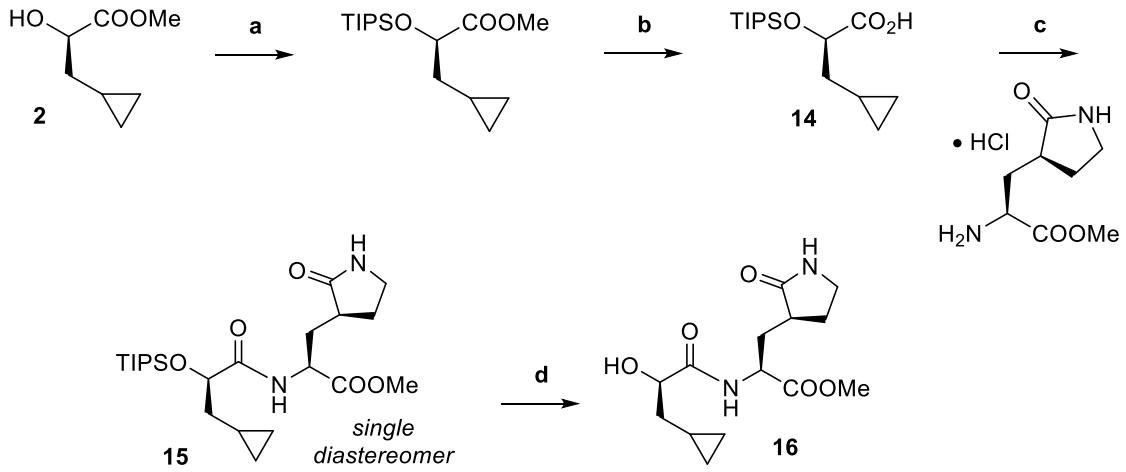
(R)-3-Cyclopropyl-2-((triisopropylsilyl)oxy)propanoic acid (14) Methyl (*R*)-3-cyclopropyl-2-((triisopropylsilyl)oxy)propanoate (4.190 g, 13.94 mmol, 1.00 eq) was dissolved in THF (20 mL), diluted with methanol/water(1:1, 40 mL), and lithium hydroxide (617.7 mg, 25.79 mmol, 1.85 eq) was added. After stirring the emulsion vigorously for 8 hours until complete consumption of the starting material by TLC, most of the THF and methanol was removed at ambient temperature under reduced pressure, and the resulting mixture was partitioned between MTBE (100 mL) and H_2O (250 mL). After separation of the phases, the aqueous layer was adjusted to pH = 2 with 2 M HCl, extracted with petrol ether (2 x 250 mL) and the combined organic phases were dried over anhydrous sodium sulfate. Removal of all volatiles afforded the title compound as a colorless oil (3.657 g, 12.77 mmol, 91% over two steps), which was used directly in the next step without further purification. ^{1}H NMR (700 MHz, $CDCl_3$): δ = 4.48 (dd, *J* = 5.6, 3.9 Hz, 1H), 1.95 (td, *J* = 14.6, 5.6 Hz, 1H), 1.55 (ddd, *J* = 14.6, 8.7, 3.9 Hz, 1H), 1.17 (m_c, 3H), 1.11−1.08 (m, 19H), 0.88 (m_c, 1H), 0.48 (m_c, 2H), 0.13 (m_c, 2H).

Methyl (S)-2-((R)-3-cyclopropyl-2-((triisopropylsilyl)oxy)propanamido)-3-((S)-2-oxopyrrolidin-3-yl)propanoate (15) A flask was charged with methyl (*S*)-2-amino-3-((*S*)-2-

oxopyrrolidin-3-yl)propanoate hydrochloride (472.2 mg, 2.121 mmol, 1.25 eq), HATU (722.4 mg, 1.900 mmol, 1.12 eq) and DMF (5 mL), followed by (*R*)-3-cyclopropyl-2-((triisopropylsilyl)oxy)propanoic acid **(14)** (486.0 mg, 1.696 mmol, 1.00 eq), and the mixture was placed under an inert atmosphere. After cooling in an ice bath, DIPEA (990 µL, 5.683 mmol, 3.35 eq) was added and the reaction mixture was stirred at 0°C for 4 hours. The reaction mixture was concentrated at 40°C and the residue was partitioned between EtOAc (50 mL) and saturated aqueous K_2CO_3 solution (30 mL). After separation of the phases, the aqueous phase was further extracted with EtOAc (2 x 30 mL). The combined organic extracts were dried over anhydrous sodium sulfate and concentrated under reduced pressure. Purification by flash chromatography, eluting with 20% acetone/petroleum ether increased to 40% acetone/petroleum ether, afforded the title compound (569.4 mg, 1.252 mmol, 74%), a single diastereomer, as a colorless oil. ^{1}H NMR (700 MHz, $CDCl_3$): δ = 7.39 (d, *J* = 8.7 Hz, 1H), 5.87 (brs, 1H), 4.68 (m_c, 1H), 4.39 (dd, *J* = 5.2, 3.9 Hz, 1H), 3.75 (s, 3H), 3.35 (m_c, 1H), 3.31 (m_c, 1H), 2.54 (m_c, 1H), 2.40 (m_c, 1H), 2.22 (dd, *J* = 10.6, 3.7 Hz, 1H), 1.92 − 1.85 (m, 3H), 1.52 (dd, *J* = 8.7, 3.7 Hz, 1H), 1.17 − 1.12 (m, 3H), 1.09 (d, *J* = 6.8 Hz, 1H), 0,87 (m_c, 1H), 0.42 (m_c, 1H), 0.31 (m_c, 1H), 0.06 (m_c, 1H), 0.03 (m_c, 1H). ^{13}C NMR (176 MHz, $CDCl_3$): δ = 197.1, 174.5, 172.3, 73.4, 52.6, 50.1, 40.4, 40.3, 38.0, 35.1, 28.2, 18.2, 18.1, 5.7, 4.5, 3.9.

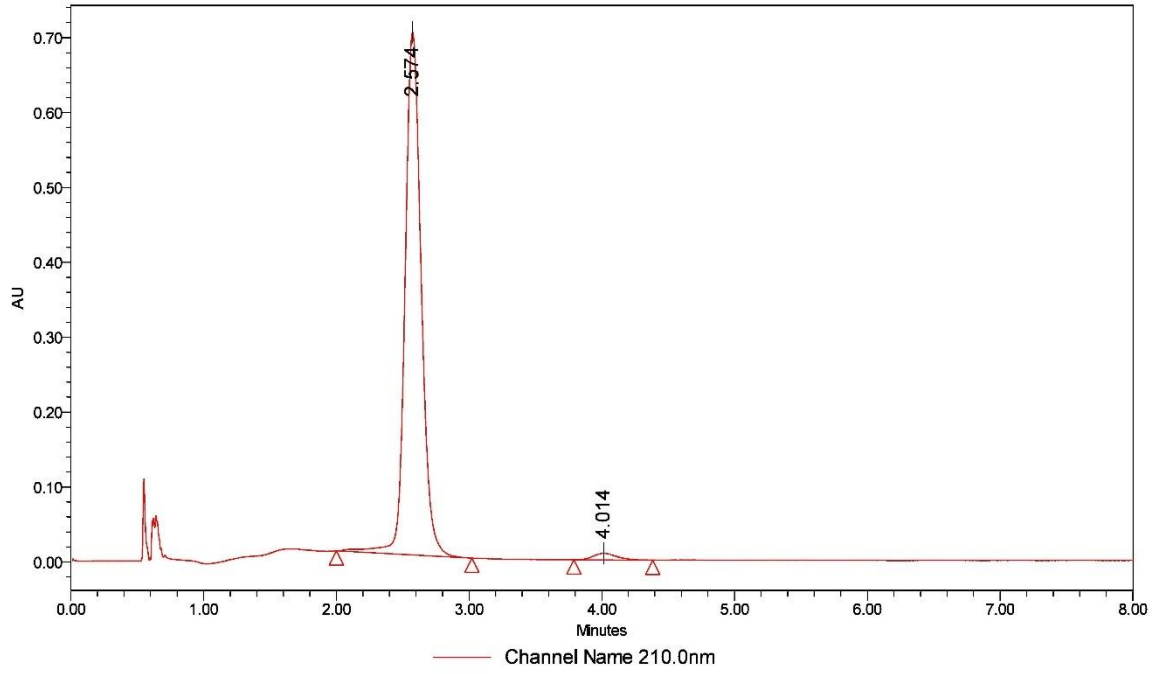
Methyl (S)-2-((R)-3-cyclopropyl-2-hydroxypropanamido)-3-((S)-2-oxopyrrolidin-3-yl)propanoate (16) Methyl (*S*)-2-((*R*)-3-cyclopropyl-2-((triisopropylsilyl)oxy)propanamido)-3-((*S*)-2-oxopyrrolidin-3-yl)propanoate **(15)** (518.1 mg, 1.139 mmol, 1.00 eq) was dissolved in MeCN (2 mL) and HF_{aq} (100 µL, 48%) was added. After stirring the reaction mixture for 4 h until complete consumption of the starting material by TLC, solid $NaHCO_3$ was added followed by the careful addition of water H_2O (1 mL).The reaction mixture was partitioned between EtOAc (50 mL) and brine (30 mL) and the aqueous phase was further extracted with EtOAc (3 x 30 mL). The combined organic extracts were dried over anhydrous sodium sulfate and concentrated under reduced pressure to provide the title compound (311.9 mg, 1.045 mmol, 92%) as a single diastereomer, which did not need further purification. ^{1}H NMR (700 MHz, $CDCl_3$): δ = 7.77 (d, *J* = 8.7 Hz, 1H), 6.25 (s, 1H), 4.62 (dd, *J* = 8.1, 3.7 Hz, 1H), 4.24 (dd, *J* = 7.6, 4.3 Hz, 1H), 3.74 (s, 3H), 3.39 − 3.34 (m, 2H), 2.19 (dd, *J* = 11.4, 4.3 Hz, 1H), 1.94 − 1.84 (m, 2H), 1.71 − 1.63 (m, 2H), 0.86 (m_c, 1H), 0.48 (m_c, 2H), 0.12 (m_c, 2H). ^{13}C NMR (176 MHz, $CDCl_3$): δ = 179.1, 173.7, 171.5, 71.7, 51.7, 49.9, 39.7, 38.3, 37.5, 32.9, 27.5, 6.1, 3.7, 2.9.

Scheme S2. A stereo-controlled approach to analogue **16** *via* alternative early stage incorporation of γ-lactam and cyclopropyl sidechains. [a]



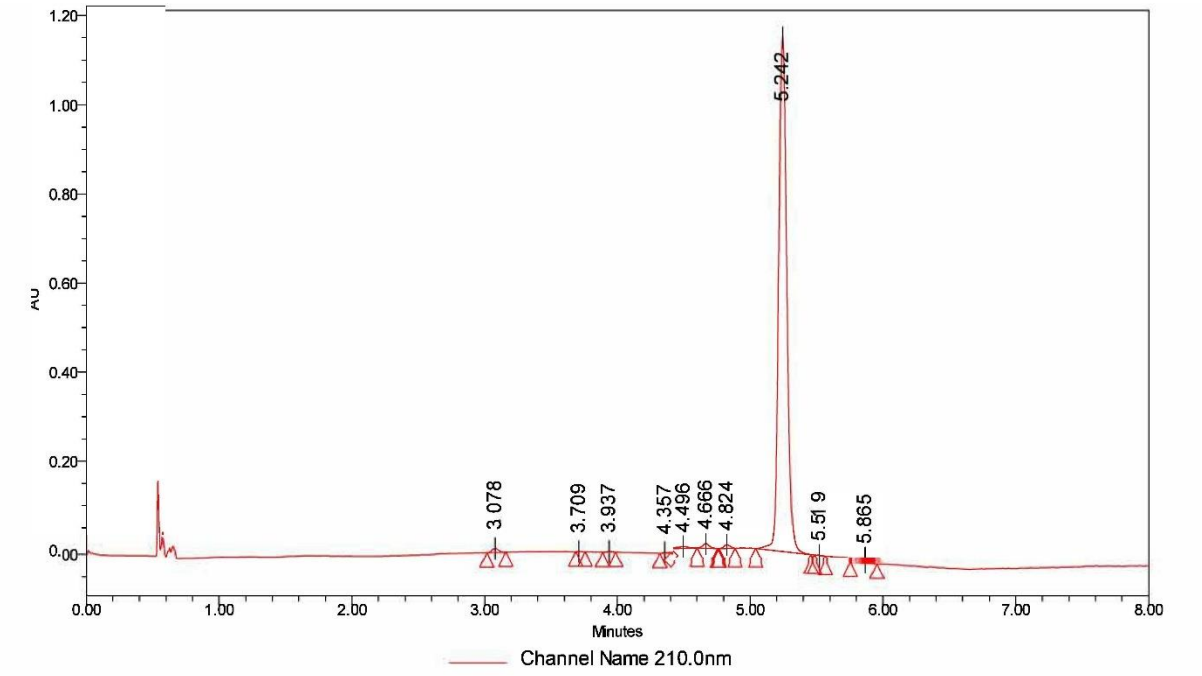
[a] Reagents and conditions: (a) TIPSCl, NMI, RT; (b) LiOH, MeOH, H_2O, RT; (c) methyl (*S*)-2-amino-3-((*S*)-2-oxopyrrolidin-3-yl)propanoate hydrochloride, HATU, DIPEA, DMF, 0°C; (d) HF (48% aq), MeCN, RT.

Figure S6. SFC purity trace for **12** (13b-H). Trefoil AMY1 (30mm x 150 mm, 2.5µm), IPA:CO_2 (34.5% IPA isocratic).



	RT	% Area	Area (µV*sec)
1	2.574	98.16	5916281
2	4.014	1.84	110968

Figure S7. SFC purity trace for **13** (13b-K). Trefoil AMY1 (150 x 3.0mm, 2.5µm), IPA:CO_2 (20-60% IPA gradient).



	RT	% Area	Area (µV*sec)
1	3.078	0.60	30448
2	3.709	0.05	2324
3	3.937	0.11	5555
4	4.357	0.09	4582
5	4.496	0.53	26927
6	4.666	0.72	36477
7	4.824	0.52	26721

	RT	% Area	Area (µV*sec)
8	5.242	97.09	4947748
9	5.519	0.05	2313
10	5.865	0.26	13175

Table S1. Diffraction data and model refinement statistics

Protein / Ligand	**M^{pro} with 13b-H**	**M^{pro} with 13b-K**
PDB entry	8A4Q	8A4T
Data collection statistics		
X-ray source	DESY P11	DESY P11
Wavelength [Å]	1.0332	1.0332
V_m [Å^3/Da]	2.00	2.23
Solvent content [%]	38.61	44.78
Space group	*C*2	*H*3
Unit cell dimensions [Å]	*a* = 114.91, *b* = 53.67, *c* = 44.78	*a* = 170.00, *b* = 170.00, *c* = 54.11
Unit cell dimensions [º]	α = γ = 90.0, β = 101.43	α = γ = 90.0, β = 120.0
Resolution range [a] [Å]	48.45 – 1.75 (1.84 – 1.75)	49.08 – 2.50 (2.50 – 2.64)
Number of observations	182,254 (27,056)	212,338 (29,733)
Number of unique reflections	26,554 (3,816)	20,163 (2930)
Completeness [%]	98.1 (96.8)	100.0 (100.0)
Mean I/σ(I)	11.1 (1.8)	10.7 (1.3)
Multiplicity	6.9 (7.1)	10.5 (10.1)
R_{merge} [b] [%]	0.100 (1.086)	0.145 (1.846)
R_{pim} [c] [%]	0.041 (0.435)	0.047 (0.608)
$CC_{1/2}$ [d]	0.998 (0.656)	0.997 (0.548)
Wilson *B*-factor [Å^2]	26.52	66.19
Refinement statistics		
R_{cryst} [e] /R_{free} [f] [%]	17.31/22.44	19.8/28.8
r.m.s.d. in bond lengths [Å]	0.010	0.008
r.m.s.d. in bond angles [°]	1.7	1.7
Clashscore [g]	4	5
Average B-factor for protein atoms [Å^2]	27.51	71
Average B-factor for ligand atoms [Å^2]	37.95	79
Average B-factor for water molecules [Å^2]	49	52
Number of protein atoms	2376	4700
Number of ligand atoms	43	86

Number of water molecules	228	51
Ramachandran plot		
Preferred regions [%]	93.82	88.74
Allowed regions [%]	5.62	9.94
Outlier regions [%]	0.56	1.32

[a] The highest resolution shell is shown in parantheses.

[b] $R_{merge} = \sum_{hkl} \sum_{i=1}^{n} |I_i(hkl) - \bar{I}(hkl)| / \sum_{hkl} \sum_{i=1}^{n} I_i(hkl)$

[c] $R_{pim} = \sum_{hkl} \sqrt{1/(n-1)} \sum_{i=1}^{n} |I_i(hkl) - \bar{I}(hkl)| / \sum_{hkl} \sum_{i=1}^{n} I_i(hkl)$ [1]

[d] $CC_{1/2}$ is the correlation coefficient determined by two random half data sets [2]

[e] $R_{cryst} = \sum_{hkl} |F_o(hkl) - F_c(hkl)| / \sum_{hkl} |F_o(hkl)|$

[f] R_{free} was calculated for a test set of reflections (5%) omitted from the refinement.

[g] Clashscore is defined as the number of clashes calculated for the model per 1000 atoms (including hydrogens) of the model. Hydrogens were added by MolProbity [3]

REFERENCES

(1) Weiss, M. S.; Hilgenfeld, R. On the Use of the Merging It R Factor as a Quality Indicator for X-Ray Data. *J. Appl. Crystallogr.* **1997**, *30* (2), 203–205.
(2) Karplus, P. A.; Diederichs, K. Linking Crystallographic Model and Data Quality. *Science* **2012**, *336* (6084), 1030–1033.
(3) 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. It MolProbity: All-Atom Structure Validation for Macromolecular Crystallography. *Acta Crystallogr. Sect. D* **2010**, *66* (1), 12–21.