Application of a Proteolysis/Mass Spectrometry Method For Investigating the Effect of Inhibitors on Hydroxylase Structure

Supplementary Information

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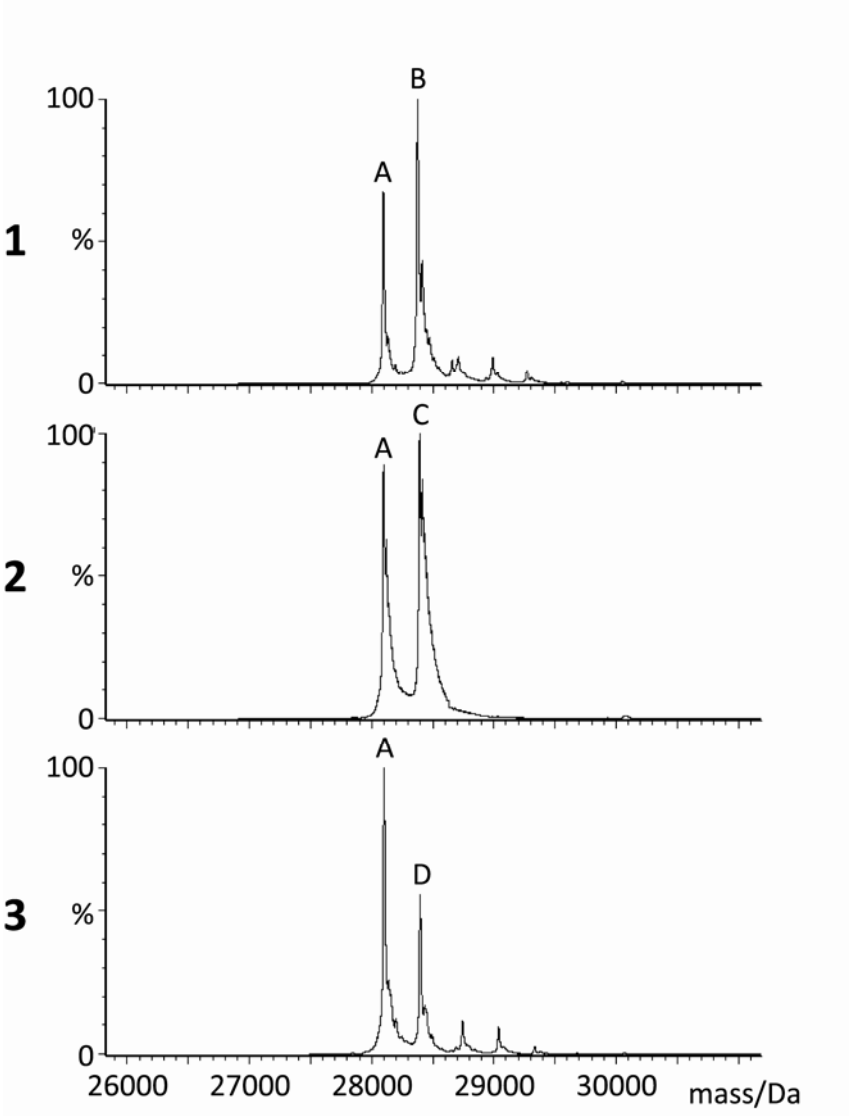
Supplementary Figure 1. Non-denaturing electrospray-ionization mass spectrometry comparing binding of D- and L-Alanine derivatives of BIC to PHD2.



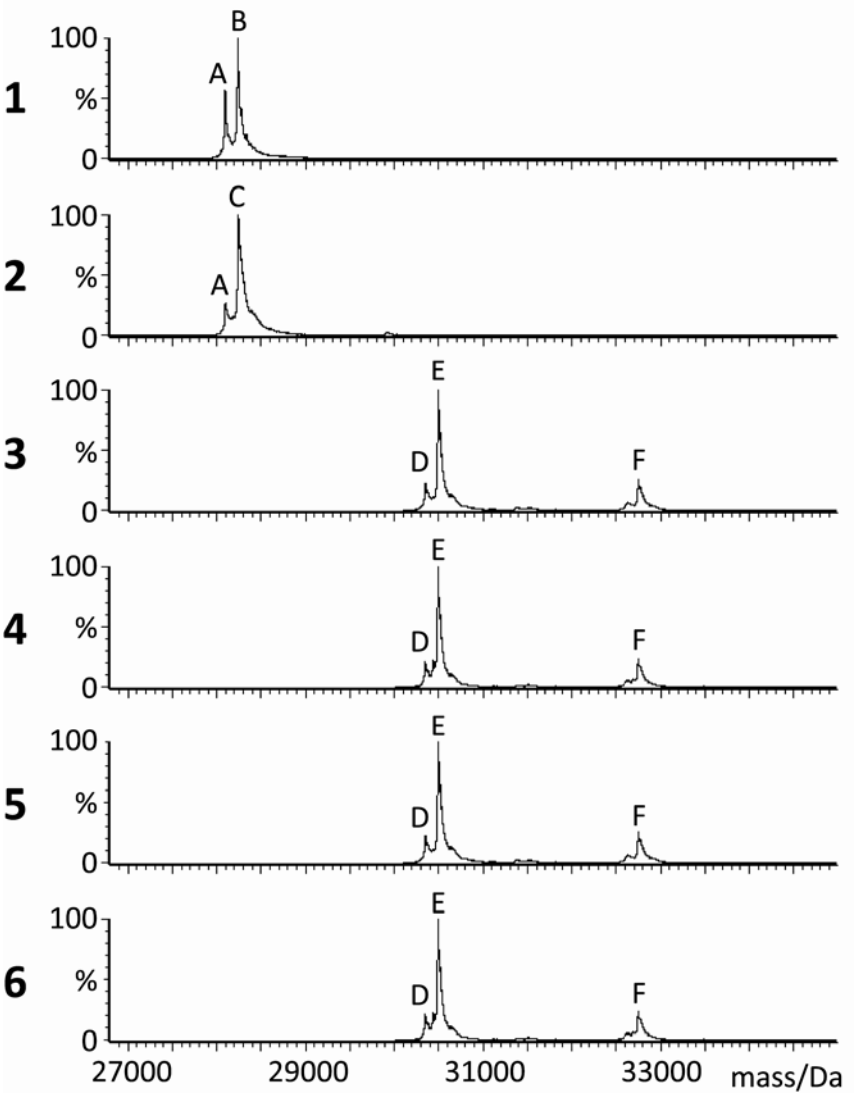
The limited proteolysis/MALDI-MS method indicated that a PHD2·Fe(II)·D-Ala-BIC complex was unstable (with respect to proteolysis) when compared to a PHD2·Fe(II)·BIC or a PHD2·Fe(II)·L-Ala-BIC complex. IC_{50} values also revealed that BIC and its L-Alanine derivative were good inhibitors of PHD2, whereas D-Ala-BIC was a poor inhibitor. To determine whether the reduced stabilization / poor inhibition were related to

an inability of D-Ala-BIC to bind tightly to PHD2, we carried out non-denaturing mass spectrometry to analyze the complexes formed under the experimental conditions used. PHD2 and Fe(II) (15 μM) were incubated for 30 min at 4 °C prior to addition of inhibitor (150 μM) before mass spectrometry was carried out (see below). Panel **1** = complexes formed with BIC; Panel **2** = complexes formed with L-Ala-BIC; Panel **3** = D-Ala-BIC. Species seen are **A**, PHD2·Fe(II), 28110 Da; **B**, PHD2·Fe(II)·BIC, 28390 Da; **C**, PHD2·Fe(II)·L-Ala-BIC, 28404 Da; **D**, PHD2·Fe(II)·D-Ala-BIC, 28404 Da.

It is apparent that D-Ala-BIC binds less tightly to PHD2·Fe(II) than BIC or L-Ala-BIC, although binding is not completely abolished by the position of the methyl group in the D-configuration. Nevertheless the trypsinolysis data suggest that the PHD2·Fe(II)·D-Ala-BIC complex is significantly destabilized, possibly due to steric clashes with the side chain of Val376[1].

Non-denaturing mass spectrometry was performed on a Q-ToF mass spectrometer (Q-ToF micro, Waters, Altrincham, UK) interfaced with a Nanomate (Advion Biosciences, Ithaca, NY, USA) with a chip voltage of 1.70 kV and a delivery pressure 0.25 psi (1 psi = 6.81 kPa). The sample cone voltage was 80 V with a source temperature of 40 °C and an acquisition/scan time of 10s/1s. Calibration and sample acquisition were performed in the positive ion mode in the range of 500–5,000 *m/z*. The pressure at the interface between the atmospheric source and the high vacuum region was fixed at 6.60 mbar. External instrument calibration was achieved by using sodium iodide. Data were processed with MassLynx v4.0 (Waters).

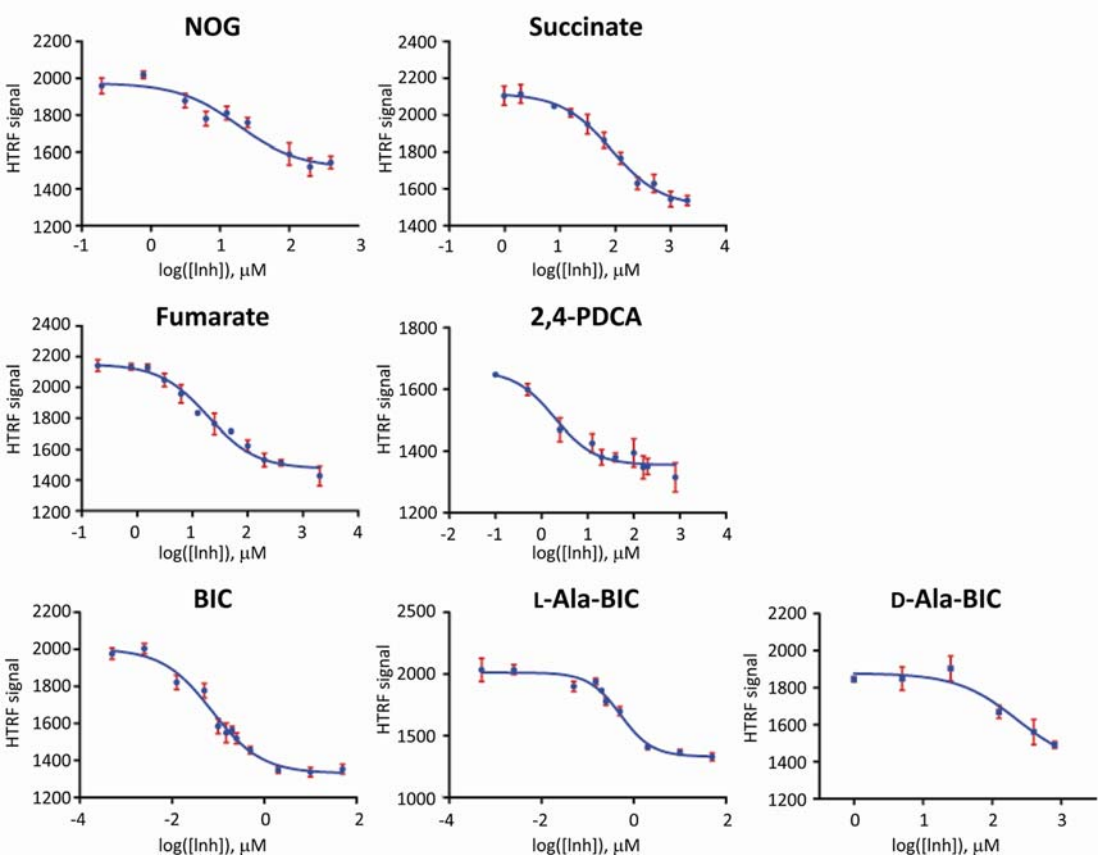
Supplementary Figure 2. Non-denaturing electrospray-ionization mass spectrometry verifying conditions for formation of enzyme·(co)substrate/inhibitor complexes.



Under the protocol used in the limited proteolysis experiments, PHD2 and Fe(II) were incubated for 30 min at 4 °C prior to addition of inhibitor/(co)substrate. 4 °C was selected to minimize the probability of protein degradation, and the inhibitor and/or (co)substrate

were added before trypsin to prevent uncoupled 2OG turnover. To ensure that a PHD2·Fe(II)·inhibitor/(co)substrate complex was formed prior to trypsinolysis, non-denaturing mass spectrometry was used to analyze the complexes produced under the pre-trypsinolysis incubation conditions. Panels 1–3 show complexes formed under these conditions (without trypsin addition) for (**1**) PHD2·Fe(II)·2OG, (**2**) PHD2·Fe(II)·NOG and (**3**) PHD2·Fe(II)·NOG·$HIF_{556-574}$. Panels 4–6 show PHD2·Fe(II)·NOG·$HIF_{556-574}$ complexes formed when (**4**) PHD2, Fe(II), NOG and $HIF_{556-574}$ were all pre-incubated for 30 min at 4 °C, (**5**) PHD2·Fe(II) was pre-incubated at 37 °C prior to addition of NOG and $HIF_{556-574}$, and (**6**) PHD2, Fe(II), NOG and $HIF_{556-574}$ were all pre-incubated for 30 min at 37 °C. The species seen are **A**, PHD2·Fe(II), 28110 Da; **B**, PHD2·Fe(II)·2OG, 28255 Da; **C**, PHD2·Fe(II)·NOG, 28256 Da; **D**, PHD2·Fe(II)·$HIF_{556-574}$, 30364 Da; **E**, PHD2·Fe(II)·NOG·$HIF_{556-574}$, 30510 Da; **F**, PHD2·Fe(II)·NOG·2eq$HIF_{556-574}$, 32764 Da. For experiments 1 and 2, the predominant species has 2OG/NOG bound. For experiments 3–6, the predominant species is PHD2·Fe·NOG·$HIF_{556-574}$ and in each case, the observed ratio of complexes is very similar. We conclude from these results that the incubation conditions used prior to proteolysis were sufficient to form the enzyme-inhibitor complexes.

Supplementary Figure 3. Dose-response curves showing inhibition of PHD2 by NOG, succinate, fumarate, 2,4-PDCA, BIC and its L-Ala and D-Ala derivatives.



IC_{50} values for the inhibition of PHD2 by NOG, succinate, fumarate, 2,4-PDCA, BIC and its L- and D-Alanine derivatives were determined using a time-resolved fluorescence resonance energy transfer (FRET)-based assay[2]. NOG IC_{50} = 18.47 ± 1.79 μM (95% confidence interval 10.31 to 33.06 μM); succinate IC_{50} = 85.31 ± 2.95 μM (95% confidence interval 62.29 to 116.8 μM); fumarate IC_{50} = 19.09 ± 0.93 μM (95% confidence interval 14.25 to 25.57 μM); 2,4-PDCA IC_{50} = 1.912 ± 0.933 μM (95% confidence interval 0.9994 to 3.657 μM);BIC IC_{50} = 0.0734 ± 0.004 μM (95%

confidence interval 0.05301 to 0.1016 μM); L-Ala-BIC IC_{50} = 0.5116 ± 0.104 μM (95% confidence interval 0.3871 to 0.6761 μM); D-Ala-BIC IC_{50} = 230.3 ± 22.1 μM (95% confidence interval 75.73 to 700.3 μM). D-Ala-BIC was not soluble above 4 mM, thus the IC_{50} value for this inhibitor was an approximation based on curve fitting of the available data.

The assay was carried out essentially as described [2]. Briefly, inhibition of PHD2 activity was analyzed by determining the binding of biotinylated HIF-1$\alpha_{556-574}$ peptides[3] to ternary VCB (pVHL, Elongins C and B) complex. The ternary VCB complex was produced as described[4], purified by successive GST and gel filtration chromatography, thrombin cleavage, and further GST and gel filtration chromatography. VCB was labeled with a Eu^{3+}-cryptate linker (LANCE Eu-W1024-ITC, PerkinElmer; ~6 Eu per VCB). Inhibition assays were performed at 50 μl total volume with 20 nM PHD2, 300 nM HIF-1$\alpha_{556-574}$, 750 nM 2OG, 100 μM Fe^{2+} and 2 mM ascorbate in HTRF buffer (50 mM Tris pH 7.5, 100 mM NaCl, 0.05 % purified BSA, 0.05 % Tween-20), for one hour shaking at 25 °C, then quenched using 150 mM succinate in HTRF buffer. The quenched peptide (100 nM) was allowed to reach binding equilibrium with streptavidin-allophycocyanin (75 nM) and Eu^{3+}-VCB (0.9 nM) at 20 μl total volume in 384 well plates by shaking for one hour before detection using three repeats per well on an EnVision Multilabel plate reader (PerkinElmer) at 25 ºC. The data output ("HTRF signal") is the ratio of the 665 nm and 615 nm emission signals resulting from the FRET excitation of streptavidin-allophycocyanin at 615 nm and the laser excitation of Eu^{3+} at 320 nm, respectively,

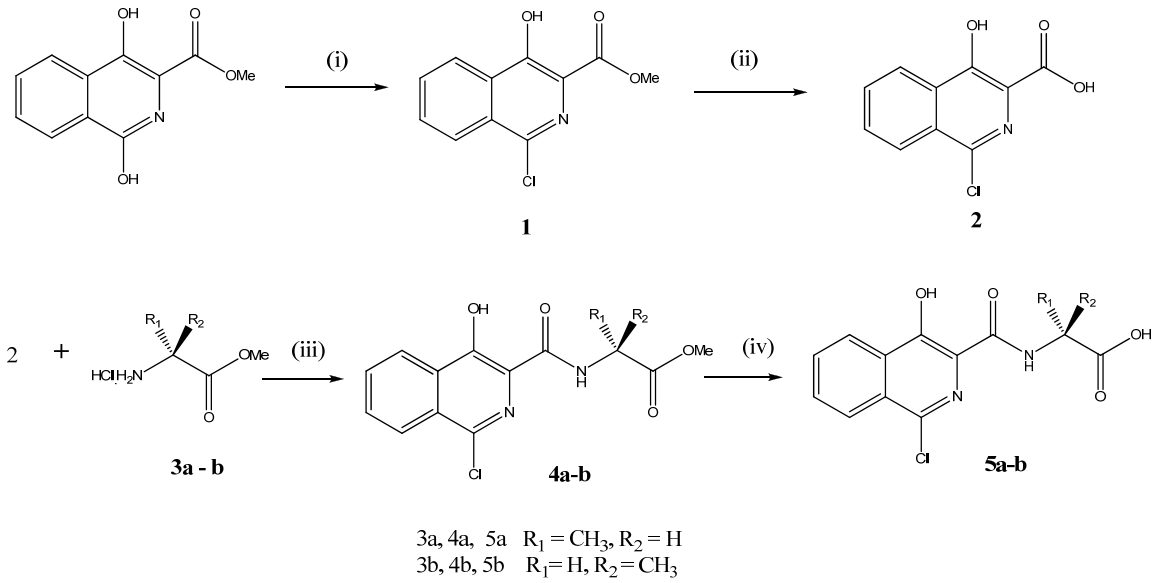
multiplied by 10,000. Analyses of the binding curves were performed using dose-response curve-fitting procedures (GraphPad Prism software).

Supplementary Experimental Details. Synthesis of L-Ala and D-Ala derivatives of BIC

General Protocols

Melting points were recorded on a Kofler hot block. Proton and carbon nuclear magnetic resonance (δ_H, δ_C) spectra were recorded on Bruker AVC 500 (500 MHz) or Bruker AMX 500 (500 MHz) spectrometers. Chemical shifts are quoted on the δ-scale in ppm, using residual solvent as an internal standard. Low resolution mass spectra were recorded on a Micromass Platform 1 spectrometer using electrospray ionization (ESI) in either positive or negative ion modes (ES^+ or ES^-), or using a VG Micromass spectrometer. High resolution mass spectra were recorded using a Waters 2790-Micromass LCT ESI mass spectrometer, using either ESI (NH_3, Cl) technique as stated. *M/z* values are reported in Daltons and are followed by their percentage abundance in parentheses. Optical rotations were measured using a Perkin-Elmer 241 polarimeter with a path length of 1 dm. Concentrations are given in g / 100 mL. Microanalyses were performed by the Science Centre Elemental Analysis Service, London Metropolitan University, London. Thin Layer Chromatography (TLC) was carried out using Merck Kieselgel $60F_{254}$ pre-coated glass-backed plates. Visualization of the plates was achieved using a UV lamp (λ_{max} = 254 or 365 nm), and/or ammonium molybdate (5% in 2 M sulfuric acid), or sulfuric acid (5% in

ethanol). Flash column chromatography was carried out using Sorbsil C60 40/60 silica. Chemicals and solvents were purchased from Aldrich Chemical Co.



Scheme 1. Reagents and conditions: (i) $POCl_3$, reflux, 2 h; 98%; (ii) EtOH:NaOH 1:1, reflux, 2 h; 90%; (iii) benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate, Et_3N, CH_2Cl_2, r.t, 20 h; **4a**, 67%; **4b**, 59%; (iv) MeOH: NaOH 1:1, r.t, 2 h; **5a**, 98%; **5b**, 93%. Compounds **5a** (L-Ala-BIC) and **5b** (D-Ala-BIC) were prepared according to the procedure reported for BIC.[5-7]

Methyl 1-chloro-4-hydroxyisoquinoline-3-carboxylate 1[5-7]

Methyl 1,4-dihydroxyisoquinoline (1.30 g, 5.93 mmol) was added to phosphorus oxychloride (10 mL, excess). The resulting mixture was stirred under reflux at 80 ^{o}C. After 2 h, TLC analysis (hexane: EtOAc, 4:1) indicated formation of a major product (R_f 0.5), and complete consumption of starting material (R_f 0.0). The excess phosphorus oxychloride was then removed under reduced pressure and the crude residue dissolved in EtOAc (30 mL). The organic layer phase was then washed with sodium hydrogen carbonate (3 x 30 mL), and brine (30 mL), dried ($MgSO_4$), filtered, and concentrated *in vacuo* to give methyl 1-chloro-4-hydroxyisoquinoline-3-carboxylate **1** (1.39 g, 98%) as a white solid; mp 137-140 ^{o}C; ν_{max} (KBr) 3454 (OH), 1619 (C=O), 1566, 1467, 1407, 1335 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.08 (3H, s, CH_3O), 7.82-7.86 (2H, m, 2 x ArCH), 8.27-8.30 (1H, m, ArCH), 8.39-8.42 (1H, m, ArCH), 11.76 (1H, s, ArOH); ^{13}C NMR (125 MHz, $CDCl_3$) δ 53.1 (CH_3O), 119.3 (ArC), 123.7 (ArCH), 126.5 (ArCH), 129.6 (ArC), 129.7 (ArC), 131.0 (ArCH), 131.5 (ArCH), 140.9 (ArC), 156.5 (ArC), 170.2 (C=O) ; MS (ES$^-$) *m/z* 236.1 (60) [M-H]$^-$; HMRS (ESI) calculated for $C_{11}H_7ClNO_3$ [M-H]$^-$ 236.0120; found 236.0113.

1-Chloro-4-hydroxyisoquinoline-3-carboxylic acid 2[5-7]

A mixture of compound **1** (1.30 g, 5.47 mmol) and 1 N NaOH: EtOH (1:1) (50 ml, excess) was stirred under reflux at 85 ^{o}C. After 2 h, TLC analysis (hexane: EtOAc, 4:1) indicated formation of a major product (R_f 0.0), and complete consumption of starting material (R_f 0.5). The reaction mixture was then washed with EtOAc (3 x 50 mL). The

aqueous layer was acidified with 1 N HCl to pH 1 and then extracted with EtOAc (3 x 50 mL), dried ($MgSO_4$), filtered, and concentrated *in vacuo* to give 1-chloro-4-hydroxyisoquinoline-3-carboxylic acid **2** (1.10 g, 90%) as a white solid; mp 318-320 ^oC; ν_{max} (KBr) 3403 (OH), 1660 (C=O), 1570, 1475, 1315, 1235, 1173, 774 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 7.96-8.01 (2H, m, 2 x ArCH), 8.21-8.25 (1H, m, ArCH), 8.31-8.36 (1H, m, ArCH); ^{13}C NMR (125 MHz, DMSO-d_6) δ 119.5 (ArC), 123.2 (ArCH), 125.9 (ArCH), 128.8 (ArC), 129.1 (ArC), 131.8 (ArCH), 132.1 (ArCH), 138.8 (ArC), 156.0 (ArC), 171.5 (C=O) ; MS (ES^-) *m/z* 222.0 (100) $[M-H]^-$; HMRS (ESI) calculated for $C_{10}H_5ClNO_3$ $[M-H]^-$ 221.9963; found 221.9955.

*(*R*)-Methyl 2-(1-chloro-4-hydroxyisoquinoline-3-carboxamido)propanoate 4a*[5-7]

To a solution of D-alanine methyl ester hydrochloride (98 mg, 0.70 mmol, 1.2 equiv) and triethylamine (0.2 mL, 1.43 mmol, 2.5 equiv.) in CH_2Cl_2 (10 mL) were added compound **2** (130 mg, 0.58 mmol, 1 equiv) followed by benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (332 mg, 0.64 mmol, 1.1 equiv).The resulting mixture was then stirred at room temperature. After 20 h, TLC (hexane: EtOAc, 4:1) indicated formation of a major product (R_f 0.5), and complete consumption of starting material (R_f 0.0). The organic solvents were removed and the residue was purified by flash chromatography (hexane: EtOAc, 4:1) to afford (R)-methyl 2-(1-chloro-4-hydroxyisoquinoline-3-carboxamido)propanoate **4a** (110 mg, 67%) as a white solid, mp 116-118 ^oC; ν_{max} (KBr) 3464 (OH), 3394 (NH), 1740 (C=O), 1638 (C=O), 1568, 1531, 1329, 955, 772 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 1.61 (3H, d, J= 6.9 Hz, CH_3), 3.82

(3H, s, CH_3O), 4.78-4.84 (1H, m, CH), 7.80-7.85 (2H, m, 2 x ArCH), 8.20 (1H, d, J= 7.6 Hz, NH), 8.27-8.31 (1H, m, ArCH), 8.39-8.42 (1H, m, ArCH), 12.96 (1H, s, ArOH); ^{13}C NMR (125 MHz, $CDCl_3$) δ 18.2 (CH_3), 47.9 (CH), 52.6 (CH_3O), 120.4 (ArC), 123.4 (ArCH), 126.5 (ArCH), 129.5 (ArC), 130.2 (ArC), 130.8 (2 x ArCH), 139.5 (ArC), 140.9 (ArC), 154.9 (ArC), 168.5 (C=O), 172.7 (C=O) ; MS (ES^-) *m/z* 307.1 (100) $[M-H]^-$; HMRS (ESI) calculated for $C_{14}H_{12}ClN_2O_4$ $[M-H]^-$ 307.0491; found 307.0493.

*(*R*)-Methyl 2-(1-chloro-4-hydroxyisoquinoline-3-carboxamido)propanoate 4b*[5-7]

To a solution of L-alanine methyl ester hydrochloride (98 mg, 0.70 mmol, 1.2 equiv) and triethylamine (0.2 mL, 1.43 mmol, 2.5 equiv) in CH_2Cl_2 (10 mL) were added compound **2** (130 mg, 0.58 mmol, 1 eq) followed by benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (332 mg, 0.64 mmol, 1.1 equiv). The resulting mixture was then stirred at room temperature. After 20 hours, TLC analysis (hexane: EtOAc, 4:1) indicated formation of a major product (R_f 0.5), and complete consumption of starting material (R_f 0.0). The organic solvents were removed and the residue was purified by flash chromatography (hexane: EtOAc, 4:1) to afford (R)-methyl 2-(1-chloro-4-hydroxyisoquinoline-3-carboxamido)propanoate **4b** (105 mg, 59%) as a white solid, mp 178-180 ^oC; ν_{max} (KBr) 3464 (OH), 3394 (NH), 1740 (C=O), 1638 (C=O), 1568, 1530, 1328, 955, 771 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 1.61 (3H, d, J= 7.3 Hz, CH_3), 3.82 (3H, s, CH_3O), 4.78-4.84 (1H, m, CH), 7.79-7.85 (2H, m, 2 x ArCH), 8.20 (1H, d, J= 7.6 Hz, NH), 8.27-8.30 (1H, m, ArCH), 8.38-8.41 (1H, m, ArCH), 12.97 (1H, s, ArOH); ^{13}C NMR (125 MHz, $CDCl_3$) δ 18.2 (CH_3), 47.9 (CH), 52.6 (CH_3O),

120.4 (ArC), 123.4 (ArCH), 126.4 (ArCH), 129.5 (ArC), 130.2 (ArC), 130.8 (2 x ArCH), 139.5 (ArC), 140.9 (ArC), 154.9 (ArC), 168.5 (C=O), 172.7 (C=O) ; MS (ES^-) *m/z* 307.1 (100) $[M-H]^-$; HMRS (ES^-) calculated for $C_{14}H_{12}ClN_2O_4$ $[M-H]^-$ 307.0491; found 307.0495.

*(*R*)-2(1-Chloro-4-hydroxyisoquinoline-3-carboxamido)propanoic acid 5a*[5-7]

A mixture of compound **4a** (90 mg, 0.29 mmol) and 1 N NaOH: MeOH (1:1) (10 mL, excess) was stirred at room temperature. After 2 h, TLC analysis (hexane: EtOAc, 4:1) indicated formation of a major product (R_f 0.0), and complete consumption of starting material (R_f 0.5). The reaction mixture was then washed with EtOAc (3 x 10 mL). The aqueous layer was acidified with 1 N HCl to pH 1 and then extracted with EtOAc (3 x 10 mL), dried ($MgSO_4$), filtered, and concentrated *in vacuo* to give (R)-2(1-chloro-4-hydroxyisoquinoline-3-carboxamido)propanoic acid **5a** (84 mg, 98%) as a white solid; mp 116-118 ^oC; $[\alpha]_D^{22}$ - 26.8 (*c*, 1.0 in MeOH); ν_{max} (KBr) 3441 (OH), 3372 (NH), 1737 (C=O), 1619 (C=O), 1586, 1573, 1547, 1359, 1286, 771 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 1.50 (3H, d, J= 7.3 Hz, CH_3), 4.54-4.60 (1H, m, CH), 7.98-8.03 (2H, m, 2 x ArCH), 8.29-8.32 (1H, m, ArCH), 8.34-8.37 (1H, m, ArCH), 9.08 (1H, d, J = 7.9 Hz, NH), 13.67 (1H, s, OH); ^{13}C NMR (125 MHz, DMSO-d_6) δ 16.9 (CH_3), 47.6 (CH), 120.4 (ArC), 123.0 (ArCH), 126.1 (ArCH), 128.6 (ArC), 129.5 (ArC), 131.7 (ArCH), 131.8 (ArCH), 154.4 (ArC), 168.2 (C=O), 173.2 (C=O) ; MS (ES^-) *m/z* 293.0 (100) $[M-H]^-$; HMRS (ES^-) calculated for $C_{13}H_{10}ClN_2O_4$ $[M-H]^-$ 293.0335; found 293.0342. Anal.

($C_{13}H_{11}ClN_2O_4$) requires: C, 52.98; H, 3.76; N, 9.51%; found C, 52.93; H, 3.83; N, 9.40%.

*(*S*)-2(1-Chloro-4-hydroxyisoquinoline-3-carboxamido)propanoic acid 5b*[5-7]

A mixture of compound **4b** (85 mg, 0.28 mmol) and 1 N NaOH: MeOH (1:1) (10 mL, excess) was stirred at room temperature. After 2 h, TLC analysis (hexane: EtOAc, 4:1) indicated formation of a major product (R_f 0.0), and complete consumption of starting material (R_f 0.5). The reaction mixture was then washed with EtOAc (3 x 10 mL). The aqueous layer was acidified with 1 N HCl to pH 1 and then extracted with EtOAc (3 x 10 mL), dried ($MgSO_4$), filtered, and concentrated *in vacuo* to give (S)-2(1-chloro-4-hydroxyisoquinoline-3-carboxamido)propanoic acid **5b** (77 mg, 93%) as a white solid; m.p. 178-180 ^oC; $[\alpha]_D^{25}$ + 25.0 (*c*, 1.0 in MeOH); ν_{max} (KBr) 3441 (OH), 3372 (NH), 1737 (C=O), 1618 (C=O), 1586, 1574, 1547, 1360, 1286, 771 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 1.50 (3H, d, J= 7.3 Hz, CH_3), 4.54-4.60 (1H, m, CH), 7.98-8.02 (2H, m, 2 x ArCH), 8.28-8.31 (1H, m, ArCH), 8.33-8.36 (1H, m, ArCH), 9.07 (1H, d, J = 7.6 Hz, NH), 13.66 (1H, s, OH); ^{13}C NMR (125 MHz, DMSO-d_6) δ 16.9 (CH_3), 47.6 (CH), 120.4 (ArC), 123.0 (ArCH), 126.1 (ArCH), 128.6 (ArC), 129.5 (ArC), 131.7 (ArCH), 131.8 (ArCH), 154.4 (ArC), 168.2 (C=O), 173.2 (C=O) ; MS (ES^-) *m/z* 293.0 (100) [M-H]$^-$; HMRS (ES^-) calculated for $C_{13}H_{10}ClN_2O_4$ [M-H]$^-$ 293.0335; found 293.0335. Anal. ($C_{13}H_{11}ClN_2O_4$) requires: C, 52.98; H, 3.76; N, 9.51%; found C, 52.95; H, 3.65; N, 9.43%.

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