Supplementary Material (ESI) for Chemical Communications

ELECTRONIC SUPPLEMENTARY INFORMATION FOR:

Incorporation of chlorinated analogues of aliphatic amino acids during cell-free protein synthesis

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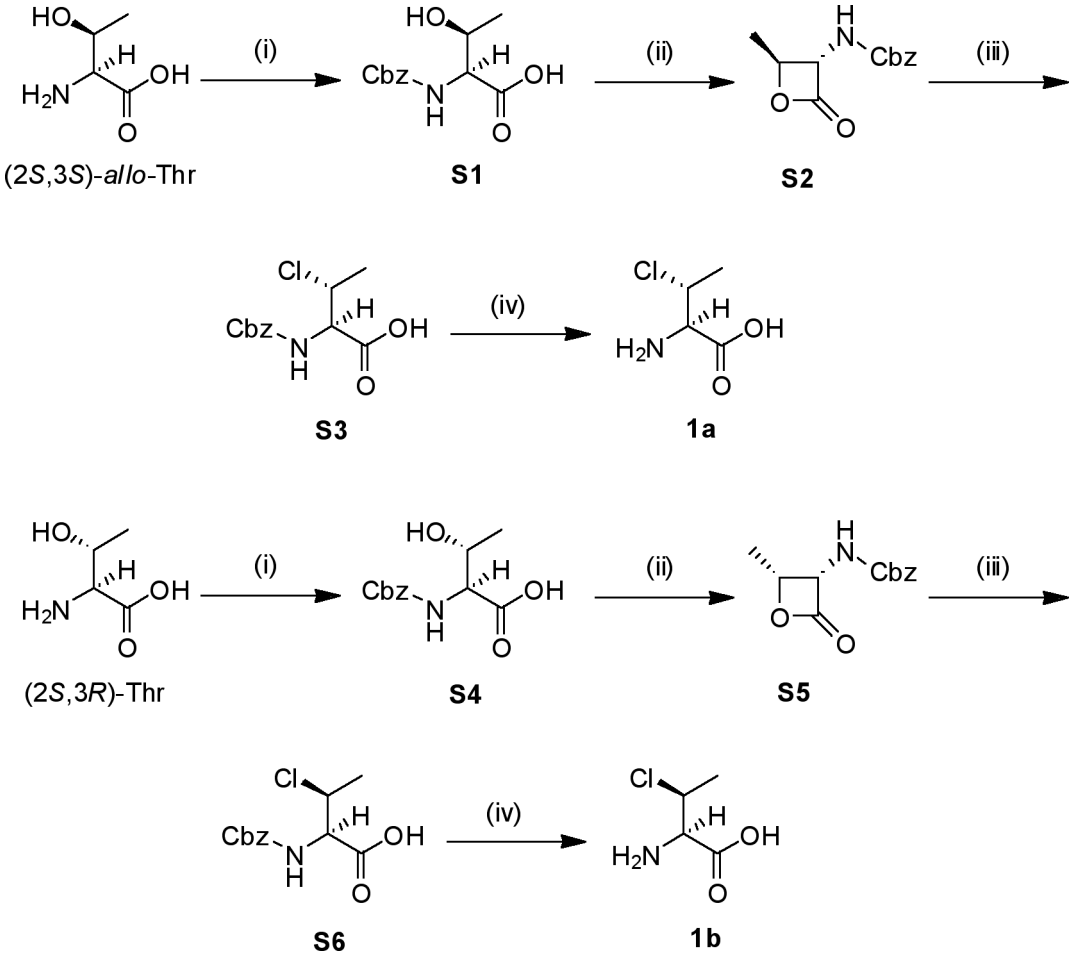
1. GENERAL EXPERIMENTAL

^{1}H NMR spectra were recorded using a Varian 300 spectrometer. Deuterium oxide with an isotopic purity of 99.75% was purchased from Cambridge Isotope Laboratories Inc., MA, USA. HPLC was carried out using a Waters Alliance Separation Module 2695 with a Waters 2996 photodiode array detector. Ultrapure water (resistivity >15 MΩ cm^{-1}) was prepared using a Milli-Q® reagent system. Plasmid DNA encoding for His_6-PpiB with expression under control of the phage T7 promoter (pND1098) was as described.[S1] Plasmid DNA was prepared from *E. Coli* DH5α/pND1098 with the Qiagen® Plasmid Maxi kit. T7 RNA polymerase (50,000 U/mL) was obtained from New England BioLabs Inc., MA, USA. Spectra/Por® dialysis membrane (#2, MWCO: 12-14,000) was purchased from Spectrum Laboratories, Inc. USA. SDS-PAGE gels were run on a Mini-PROTEIN® Tetra system and stained with Bio-Safe® Coomassie Blue stain from Bio-Rad, CA, USA. Acrylamide, bis-acrylamide and SDS-PAGE molecular weight standards (low range) solutions were purchased from Bio-Rad, CA, USA. Proteins were purified with a His GraviTrap® Kit from GE Healthcare and concentrated with Amicon® Ultra-4 (YM-10) centrifugal devices from Millipore, MA, USA. Protein mass spectral data were acquired by direct injection onto an Agilent 1100 series LC/MSD TOF instrument operating in the positive ESI ionisation mode. PpiB substrate (*N*-succinyl-(*S*)-Ala-(*S*)-Ala-(*S*)-Pro-(*S*)-Phe-4-nitroanilide) was purchased from Sigma Chemical Co. Ultraviolet (UV) spectra were recorded using a Shimadzu UV-2450 UV-Vis scanning spectrophotometer.

2. SYNTHETIC PROCEDURES

Scheme S1. Preparation of the amino acids **1a** and **1b**.



i) Cbz-Cl, $NaHCO_3$, H_2O; ii) HBTU, CH_2Cl_2; iii) LiCl, THF; iv) H_2, Pd/C, EtOH.

The individual diastereomers **1a** and **1b** were prepared using the previously reported[S2] sequence shown in Scheme S1. Their purification was carried out through HPLC (Phenomenex Luna C18 column, 250 mm × 4.6 mm, 5μm, 1 mL/min, 12 mM HCl mobile phase). Their ^{1}H NMR spectra are consistent with published data.[S2]

3. CELL-FREE PROTEIN SYNTHESIS

Cell-free protein synthesis was carried out following our published procedure[S3] with the following few modifications. (*S*)-Ala and RNasin were not added to the inner mixture. Commercially obtained T7 RNA polymerase (2 μL) was added to the inner mixture of each reaction instead of the plasmid encoding for this enzyme. A 5 μL quantity of total tRNA solution was added instead of 10 μL. The His_6-PpiB sequence (with an additional *C*-terminal Asn residue,[S1] mass = 19221 Da, *N*-formyl-His_6-PpiB = 19250 Da) is

MHHHHHHMVT FHTNHGDIVI KTFDDKAPET VKNFLDYCRE GFYNNTIFHR VINGFMIQGG GFEPGMKQKA TKEPIKNEAN NGLKNTRGTL AMARTQAPHS ATAQFFINVV DNDFLNFSGE SLQGWGYCVF AEVVDGMDVV DKIKGVATGR SGMHQDVPKE DVIIESVTVS EN.

The protein yields from the experiments represented by the lanes on the SDS-PAGE illustrated in Fig. 1 of the manuscript were as follows: b) 100 μg; c) 13 μg; d) 110 μg; e) 39 μg; f) 150 μg; g) 48 μg; and h) 40 μg.

4. HYDROLYSIS OF THE CHLORIDES 1a,b, 2a,b AND 3a,b

A mixture of the chlorides **2a,b** (54 mg, 0.2 mmol) dissolved in D_2O (2 mL) adjusted to pH 7 through the addition of a solution of NaOD in D_2O (2.5 M), was placed in an NMR tube at a constant temperature of 37 ºC for 6 h. ^1H NMR spectra were recorded periodically. Integration of the spectra, relative to an internal standard, was used to determine the half-lives. Under the same conditions, no reaction of the chlorides **1a,b** or **3a,b** was evident.

5. ESI MASS SPECTROMETRY OF PURIFIED PROTEINS

Protein solution (50 μL) was diluted with a 50:50 (v/v) solution of 0.1% formic acid in MeCN:0.1% aqueous formic acid (150 μL) followed by direct injection into the spectrometer. Mass spectra of PpiB synthesised in the presence of the chlorides **2a,b** (2 mM) instead of (*S*)-Leu (**5**) (1 mM), and native protein are shown in Figure S1. Mass spectra of protein produced with the chloride **1a** (0.2 or 0.02 mM) instead of (*S*)-Val (**4**) (1 mM), and native protein are shown in Figure S2. Mass spectra of protein produced with the chloride **1b** (0.2 or 0.02 mM) instead of (*S*)-Val (**4**) (1 mM) and native protein are shown in Figure S3.

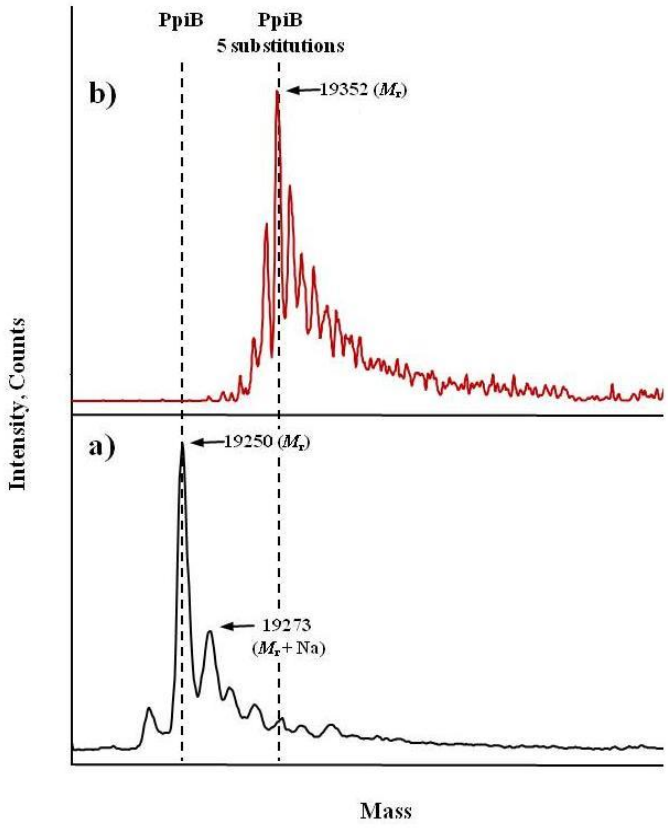


Figure S1. Mass spectra of a) native PpiB and b) PpiB produced using the chlorides **2a,b** (2 mM) instead of (*S*)-Leu (**5**) (1 mM).

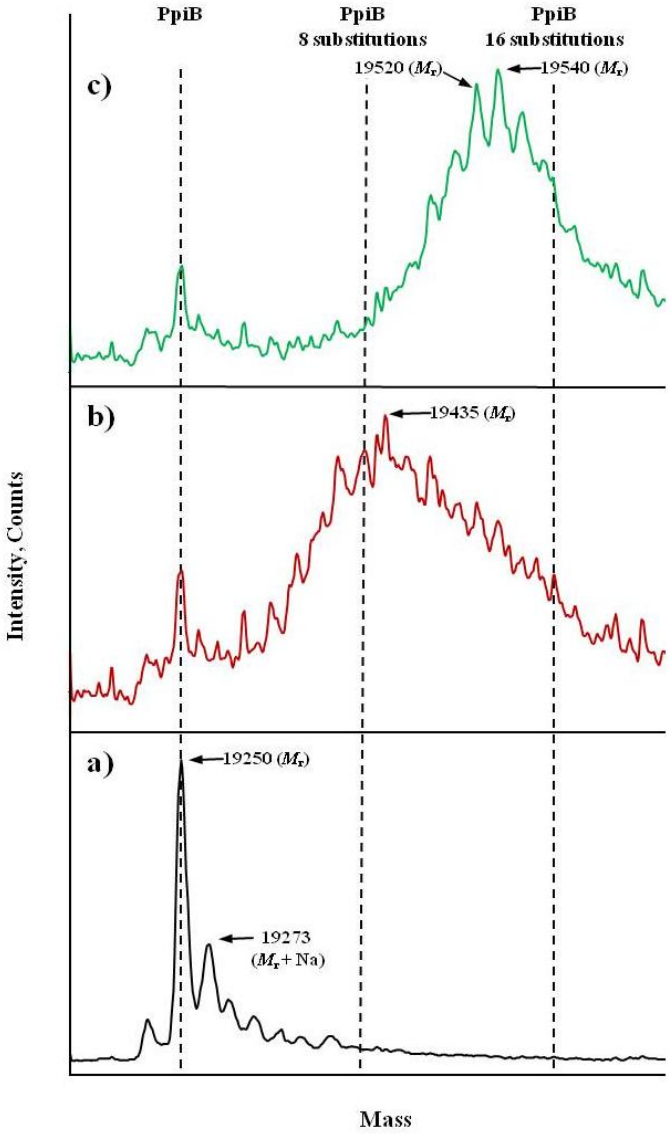


Figure S2. Mass spectra of: a) native PpiB; b) PpiB produced using the chloride **1a** (0.02 mM) instead of (*S*)-Val (**4**) (1 mM); and c) PpiB produced using the chloride **1a** (0.2 mM) instead of (*S*)-Val (**4**) (1 mM).

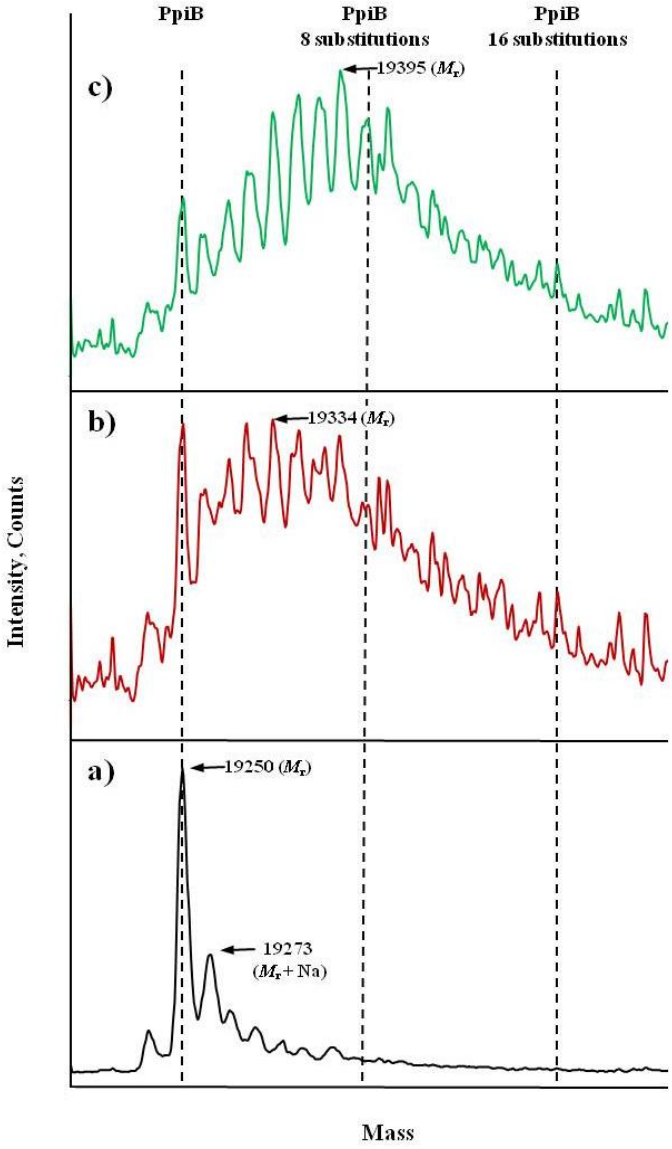


Figure S3. Mass spectra of: a) native PpiB; b) PpiB produced using the chloride **1b** (0.02 mM) instead of (*S*)-Val (**4**) (1 mM); and c) PpiB produced using the chloride **1b** (0.2 mM) instead of (*S*)-Val (**4**) (1 mM).

6. ACTIVITY ASSAY FOR PpiB

Enzyme activity was determined under similar conditions to those reported.[S4] Peptide substrate (*N*-succinyl-(*S*)-Ala-(*S*)-Ala-(*S*)-Pro-(*S*)-Phe-4-nitroanilide, 25 mg, 0.04 mmol) was dissolved in a solution of LiCl in trifluoroethanol (0.3 M, 66 mL, 0.6 mM). The assay buffer (800 μL, 50 mM HEPES, 100 mM NaCl, pH 8.0) was added to a quartz cuvette and cooled to 0 ºC, before the UV-Vis trace was recorded as a baseline. The peptide solution (25 μL) was then added and the UV-Vis absorbance was recorded followed by the addition of a chymotrypsin (CHT) solution (5 μL, 1 mg/mL in 1 mM HCl). PpiB solution (5 μL, 49 μM, 20 mM phosphate, 0.5 M NaCl, pH 7.4) was added and the absorbance at 390 nm was followed until the reaction was complete. The UV-Vis traces without the addition of PpiB are shown in Figure S4. The UV-Vis traces for native enzyme are shown in Figure S5 and those for PpiB produced with the chloride **1a** (2 mM) instead of (*S*)-Val (**4**) (1 mM) are shown in Figure S6. A comparison of the absorbances at 390 nm over time of all three experiments is shown in Figure S7.

The kinetic constants for native and chlorinated PpiB were also determined as previously reported[S4] by using twenty times the above concentration of PpiB and one thousand times the above concentration of chymotrypsin with various substrate concentrations ranging from 7.5 – 4,000 μM. Under these conditions native PpiB had K_M and k_{cat} values of 380 ± 25 μM and 1940 ± 60 s^{-1}, respectively, while the corresponding values for the chlorinated PpiB were 590 ± 50 μM and 1110 ± 50 s^{-1}.

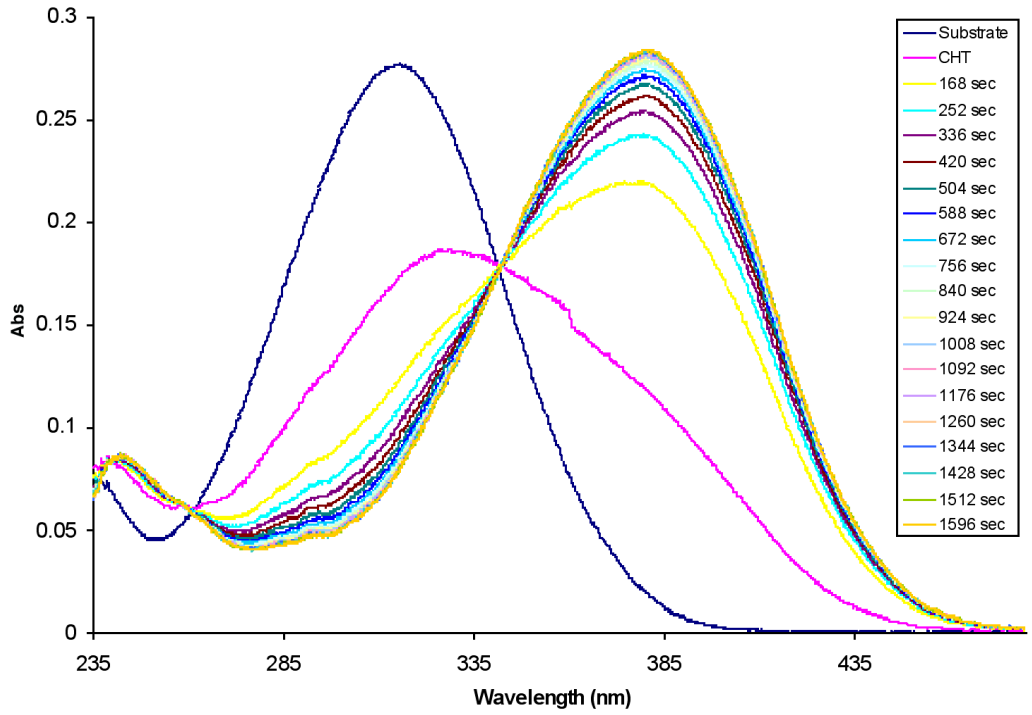


Figure S4. UV traces of the enzymatic cleavage of peptide substrate by CHT (6 μg/mL) without PpiB, at pH 8 over 26 min.

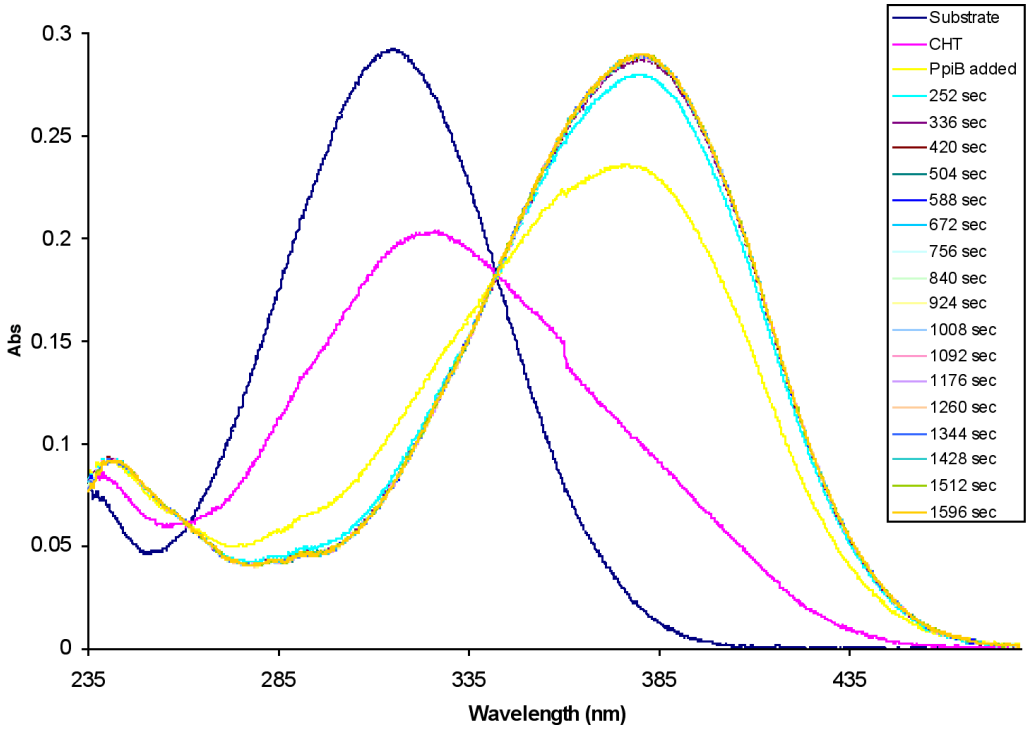


Figure S5. UV traces of the enzymatic cleavage of peptide substrate by CHT (6 μg/mL) in the presence of native PpiB (290 nM), at pH 8 over 26 min.

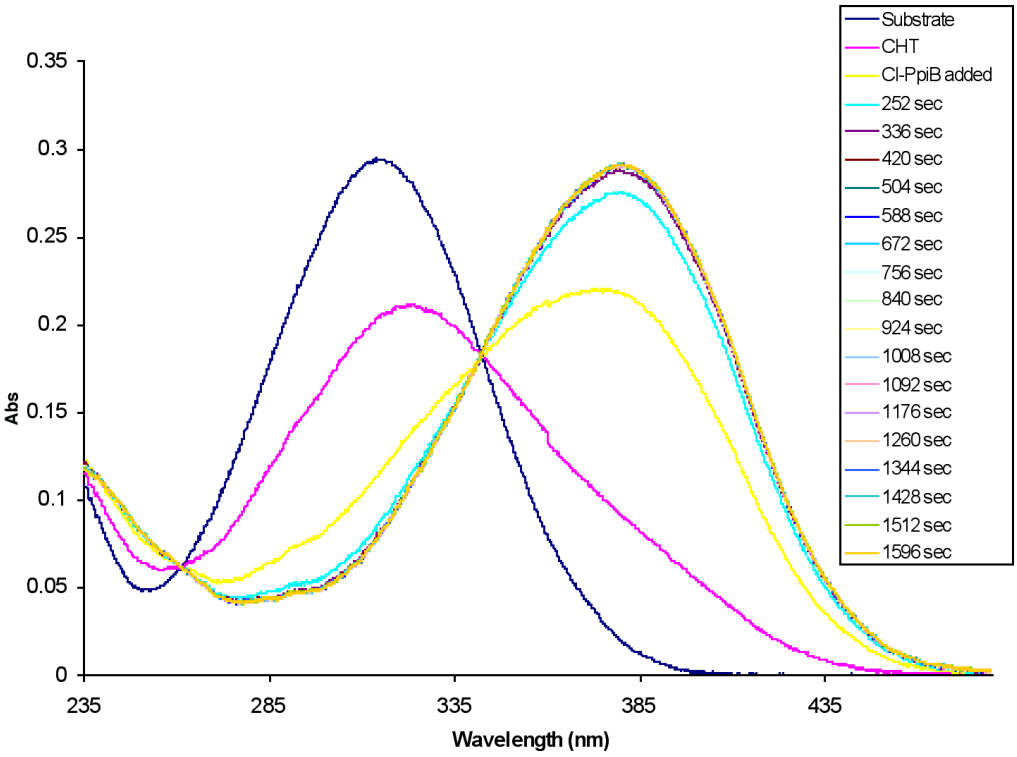


Figure S6. UV traces of the enzymatic cleavage of peptide substrate by CHT (6 μg/mL) in the presence of PpiB (290 nM) prepared from the chloride **1a** (2 mM) instead of (*S*)-Val (**4**) (1 mM), at pH 8 over 26 min.

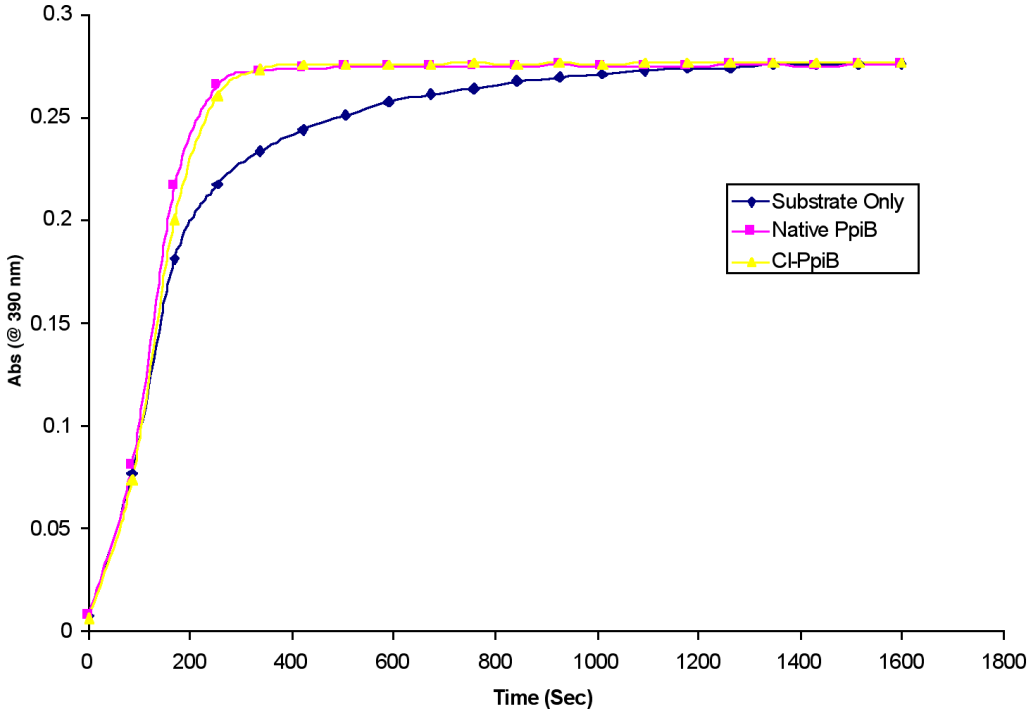


Figure S7. Enzymatic activity of native PpiB and PpiB prepared from the chloride **1a** (2 mM) instead of (*S*)-Val (**4**) (1 mM) compared with substrate cleavage without PpiB.

7. IleRS ACTIVITY ASSAY

A mixture of an aqueous solution of (2*S*,3*S*)-Ile (**6**) (6 μL, 10 mM) and one of ATP (5 μL, 6 mM) in buffer (16.2 μL, 40 mM Tris-HCl, 0.8 mM $MgCl_2$, 3.2 mM KCl, pH 7.6) was incubated at 37 ºC for 3 min before an aqueous IleRS solution (2.8 μL, 16 μM) was added. Aliquots (10 μL) were removed immediately and after 16 h, and the enzyme activity quenched through the addition of aqueous sodium dodecylsulfate (10 μL, 0.02% w/v). HPLC using an Alltech Alltima HP C18 column, eluting with a gradient of ammonium dihydrogen phosphate (60 mM) and tetrabutylammonium dihydrogen phosphate (5 mM) in water (solvent A) and tetrabutylammonium phosphate (5 mM) in methanol (solvent B), relative to standards, showed AMP production in the assay mixture to a concentration of 100 μM. The analogous experiment carried out using a 1:1 mixture of the chlorides **3a,b** (6 μL, 10 mM) instead of (2*S*,3*S*)-Ile (**6**) showed AMP production to a concentration of 210 μM.

8. REFERENCES

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S2. N. Valls, M. Borregán and J. Bonjoch, *Tetrahedron Lett*., 2006, **47**, 3701–3705.

S3. M. A. Apponyi, K. Ozawa, N. E. Dixon and G. Otting, in *Structural Proteomics: High Throughput Methods*, ed. B. Kobe, M. Guss and T. Huber, *Methods in Molecular Biology*, Humana Press, Totowa, NJ, 2008, vol. **426**, pp. 257–268.

S4. J. L. Kofron, P. Kuzmič, V. Kishore, E. Colón-Bonilla and D. H. Rich, *Biochemistry*, 1991, **30**, 6127–6134.