An efficient cell-free protein synthesis platform for producing proteins with pyrrolysine-based non-canonical amino acids

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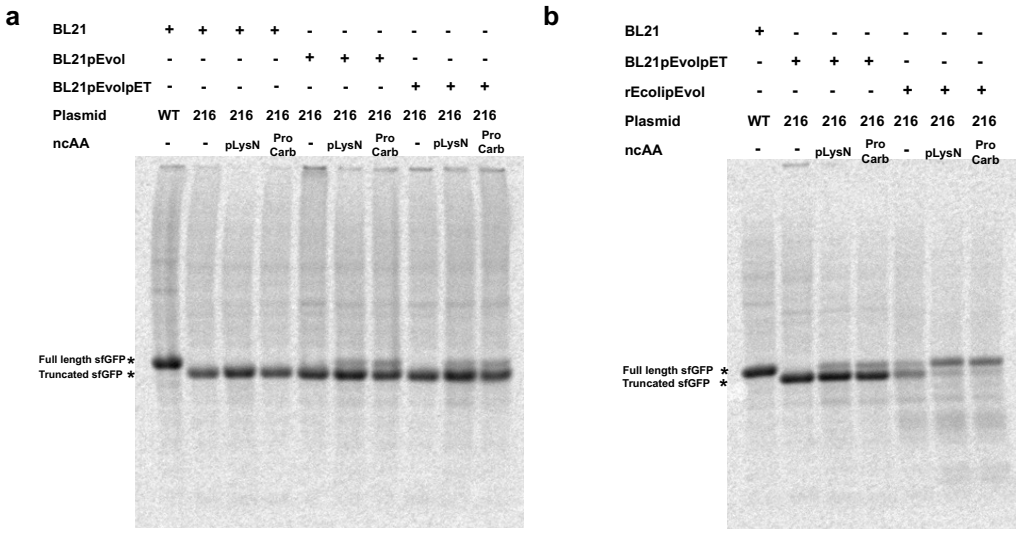
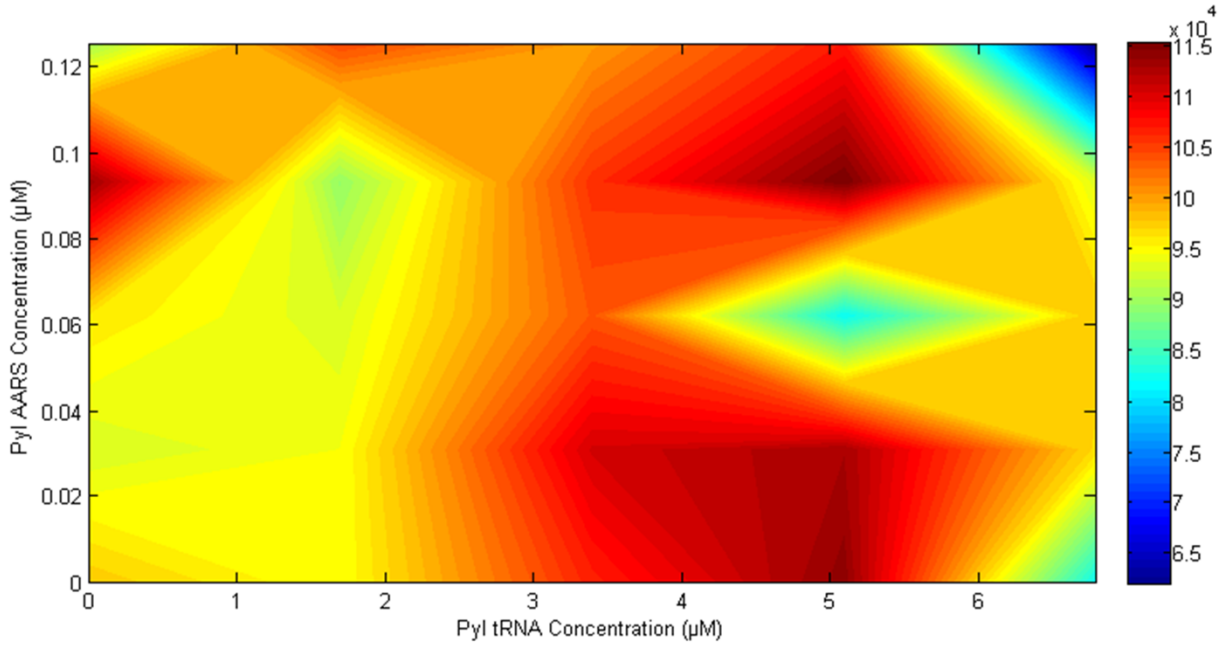


Supplementary Figure 1: Proteins produced in rEcoli extract have less truncated product than proteins produced in BL21 derived lysates. Autoradiogram of ^{14}C-leucine labeled proteins in (**a**) BL21 based extracts and (**b**) BL21 versus rEcoli extracts. Full length and truncated sfGFP proteins are indicated. WT indicates wt-sfGFP plasmid was used; 216 indicates sfGFP-T216 plasmid was used. Gels representative of three independent experiments.



Supplementary Figure 2: Addition of extra PylRS or Pyl $tRNA_{CUA}^{Pyl}$ does not significantly improve the incorporation of ncAAs in rEcoli based extracts. sfGFP yields (RFUs) of samples in rEcolipEvol extract are plotted using MatLab software with increasing PylRS concentration on the y-axis and increasing $tRNA_{CUA}$Pyl**.** Two independent reactions were performed for each data point.

Supplementary Tables

Supplementary Table 1. Monoisotopic masses of the different proteins in this work.

Figure	GFP species	mass (Da, Exper)	mass (Da, Theor)	error (ppm)	Shift from WT GFP (Da, Exper)	Shift from WT GFP (Da, Theor)
5a	WT GFP*	26847.52	26847.45	2.6	--	--
5a	Bl21pEvolpET extract: 1TAG + pLysN *	27010.63	27010.55	3.0	163.18	163.10
5a	Bl21pEvolpET extract: 1TAG + ProCarb*	26956.57	26956.51	2.2	109.12	109.05
5a	rEcolipEvol extract: 1TAG + pLysN	27141.7	27141.59	4.1	163.21	163.10
5a	rEcolipEvol extract: 1TAG + ProCarb *	26956.55	26956.51	1.5	109.10	109.05
5b	WT GFP*	26847.52	26847.45	2.6	--	--
5b	rEcolipEvol extract: 2TAG + pLysN	27318.78	27318.71	2.6	340.29	340.22
5b	rEcolipEvol extract: 3TAG + ProCarb	27306.71	27306.67	1.5	328.22	328.18

Supplementary Table 2. Oligonucleotide sequences used in this work.

Oligo name	Sequence
AR109	gatatccatatggataaaaaaccactaaacactctg
AR108	ggtagcgcggccgccaggttggtagaaatcccg
AR045	tggcggaaaccccgggaatc
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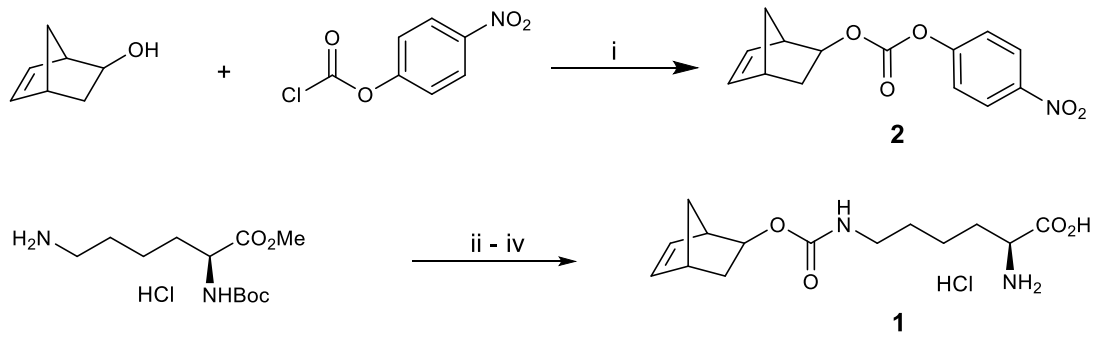
T7500up	ccgaaggtaactggcttcagcagag
S2-f	tagaaaggtgaagaactgtttac
S2-r	catatgtatatctccttcttaaagttaaac

Supplementary methods

Synthesis of N^6-(5-Norbornen-2-yloxycarbonyl)-L-Lysine Hydrochloride

The synthesis of N^6-((5-norbornen-2-yl)oxy)carbonyl-L-lysine (**1**) has previously been described[i], however the modified route shown in Scheme 1 was employed to avoid the use of phosgene. Condensation of p-nitrophenyl chloroformate with 5-norbornen-2-ol (mixture of isomers) in the presence of pyridine gave 5-norborn-5-en-2-yl (4-nitrophenyl) carbonate (**2**) in 28-35% isolated yield. Commercial N^2-Boc-L-lysine methyl ester hydrochloride was treated with **2** and triethylamine in DMF followed by sequential removal of the protecting groups to give compound **1** as the hydrochloride salt. Compound **1** is a ~ 3:1 mixture of the endo to exo isomers determined by ^{1}H NMR.



Scheme 1 - Reagents: i. pyridine/DCM, r.t.; ii. **2**, Et_3N, DMF; iii. LiOH, MeOH/H_2O; iv. 4M HCl/dioxane.

N^2-Boc-L-lysine methyl ester hydrochloride was purchased from ChemImpex Chemical Company and was used as received. All other reagents were purchased from Aldrich Chemical Company and were used as received. NMR spectra were obtained on Bruker Avance III Ag500 spectrometer at 500 MHz (proton) and 125 MHz (carbon). LCMS spectra were obtained using a Waters Acquity-H Single Quad UPLC-MS system, eluting on a Waters BEH C-18 column (50 x 2.1 mm, 1.7 micron) using 5 – 95% acetonitrile in water modified with 0.1% formic acid @ 0.8 mL/min over 2 minutes with a 1-minute hold at 95% acetonitrile in water.

5-norbornen-2-yl (4-nitrophenyl) carbonate (2):

A dry 100 mL flask was charged with 5-norbornylen-2-ol (0.441 g, 4 mmol), 4-nitrophenyl chloroformate (1.048 g, 5.20 mmol) and DCM (Volume: 24 ml). The flask was placed under nitrogen, cooled to 0 °C and pyridine (0.404 ml, 5.00 mmol) was added. The reaction was stirred at 0°C for 2 hours then allowed to warm to room temperature and stirred overnight. The reaction was diluted with DCM (30 mL) and washed with brine (3 x 50 mL). The organic phase was separated, dried over sodium sulfate, filtered and concentrated under vacuum. The residue was purified by flash column chromatorgraphy (Biotage Isolera, 50 g silica column, eluting with 0-100% DCM in hexanes over 10 CV @ 40 mL/min) to give a white solid determined by 1H NMR to be the product in a ~ 3:1 mixture of endo to exo isomers. ^{1}H NMR (500 MHz, CHLOROFORM-d) δ 8.25 – 8.32 (m, 2H), 7.35 – 7.43 (m, 2H), 6.44 (dd, *J* = 2.99, 5.52 Hz, 0.7H), 6.32* (dd, *J* = 2.84, 5.67 Hz, 0.2H), 6.07 (dd, *J* = 2.84, 5.67 Hz, 0.7H), 6.01* (dd, *J* = 3.31, 5.52 Hz, 0.2H), 5.38 (td, *J* = 3.27, 7.96 Hz, 0.7H), 4.75* (d, *J* = 6.94 Hz, 0.2H), 3.29 (br. S., 0.7H), 3.09* (br. S., 0.2H), 2.94 (br. S., 1H), 2.25 (ddd, *J* = 3.63, 8.43, 12.53 Hz, 0.7H), 1.83* (ddd, *J* = 2.52, 6.94, 12.93 Hz, 0.2H), 1.73 – 1.78* (m, 0.2H), 1.67 – 1.72* (m, 0.2H), 1.53 – 1.65 (m, 1.1H), 1.39 (d, *J* = 8.83 Hz, 0.7H), 1.14 (td, *J* = 3.15, 12.93 Hz, 0.7H) * indicates exo isomer.

N^2-t-butoxycarbonyl-N^6-((5-norbornen-2-yl)oxy)carbonyl-L-lysine methyl ester:

A solution of N^2-Boc-L-Lysine methyl ester hydrochloride (0.757 g, 2.55 mmol) and 5-norbornen-2-yl (4-nitrophenyl) carbonate (0.772 g, 2.81 mmol) in dry DMF (Volume: 15 ml) was treated with triethylamine (1.066 ml, 7.65 mmol) and allowed to stir overnight at room temperature. The reaction was diluted with DCM (30 mL) and washed with water (5 x 25 mL). The organic phase was dried over sodium sulfate and filtered. The reaction was concentrated under vacuum and the residue taken up in DCM (3 mL). ~ 0.2 mL was loaded onto a 1g Biotage samplet and purified by

flash chromatographty (10 g KP-Sil column, 0-100 DCM in hexanes over 10 CV @ 12 mL/min followed by 0-10% methanol in DCM over 5 CV @ 12 mL/min) to give the product as a white waxy solid. With the column conditions determined, the remaining material was purified (Biotage Isolera, 25g KP-Sil column, 0-10% methanol in DCM over 10 CV @ 25 mL/min). The products from both columns were combined to give the product (0.6 g, 59%) as a waxy, white solid. ^{1}H NMR showed a ~ 3:1 mixture of isomers, endo to exo based on the starting carbonate. The product was slightly contaminated with DMF but was used as is in the next step. ^{1}H NMR (500 MHz, CHLOROFORM-d) δ 6.34 (dd, *J* = 2.84, 5.36 Hz, 0.7H), 6.24* (d, *J* = 2.52 Hz, 0.2H), 5.95 – 6.02 (m, 1H), 5.26 (d, *J* = 4.10 Hz, 0.7H), 5.11 (br. S., 0.7H), 4.64 (br. S., 1H), 4.30 (d, *J* = 4.73 Hz, 1H), 3.76 (s, 3H), 3.15 (br. S., 3H), 2.84 (br. S., 1H), 2.14 (ddd, *J* = 3.63, 8.35, 12.30 Hz, 0.7H), 1.80 (d, *J* = 5.36 Hz, 2H), 1.61 – 1.73 (m, 2H), 1.29 – 1.59 (m, 18H), 0.93 (d, *J* = 12.30 Hz, 0.7H) * indicates exo isomer. LCMS (*ESI+*) m/z calculated: 396; found: 397 (M+H), T_r = 2.16 min.

N^2-t-butoxycarbonyl-N^6-((5-norbornen-2-yl)oxy)carbonyl-L-lysine:

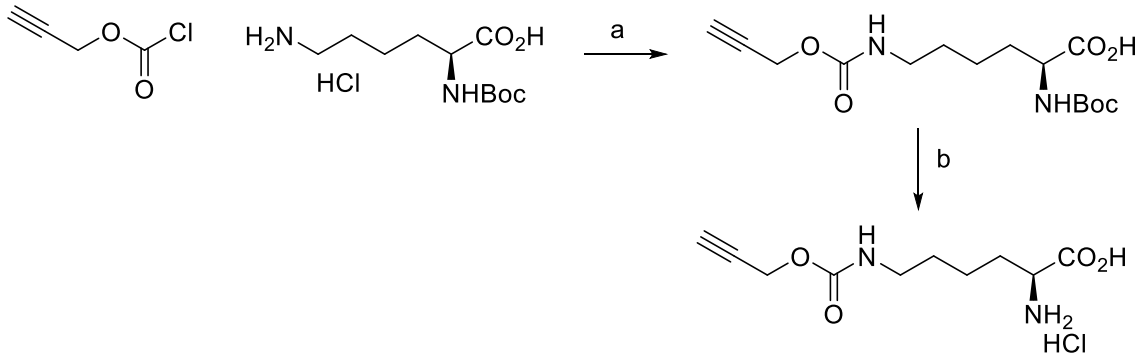
A solution of N^2-t-butoxycarbonyl-N^6-((5-norbornen-2-yl)oxy)carbonyl-L-lysine methyl ester (0.60 g, 1.513 mmol) in methanol (8.07 ml) was treated with lithium hydroxide monohydrate (0.140 g, 3.33 mmol) in water (2.018 ml). The resulting mixture was stirred at room temperature for 2 hours then concentrated under vacuum to a volume of ~ 3 mL. Water (3 mL) was added and the resulting solution washed with methylene chloride (3 x 5 mL). The aqueous phase was acidified to ~ pH 4 with 1N HCl and extracted with ethyl ether (5 x 5 mL). The combined organic phases were dried over sodium sulfate, filtered and concentrated under vacuum to give the product (0.43 g, 74%) as a white solid. ^{1}H NMR (500 MHz, CHLOROFORM-d) δ 6.35 (d, *J* = 1.89 Hz, 0.7H), 6.25* (br. s., 0.2H), 5.99 (d, *J* = 2.52 Hz, 1H), 5.27 (d, *J* = 7.88 Hz, 1.3H), 4.57 - 4.83 (m, 1H), 4.13 - 4.41 (m, 1H), 3.16 (m, 3H), 2.85 (br. s., 1H), 2.15 (ddd, *J* = 3.63, 8.35, 12.30 Hz, 0.7H), 1.67 - 1.98 (m,

3H), 1.37 - 1.61 (m, 14H), 0.94 (d, *J* = 12.61 Hz, 0.7H) * exo isomer. LCMS (*ESI*+) m/z calculated: 382; found: 393 (M+H), T_r = 2.14 min.

N^6-((5-norbornen-2-yl)oxy)carbonyl-L-lysine (1):

A solution of N^2-butoxycarbonyl-N^6-((5-norbornen-2-yl)oxy)carbonyl-L-lysine (0.425 g, 1.089 mmol) in DCM (4 mL) was treated with hydrogen chloride, 4.0M in dioxane (0.408 mL, 1.634 mmol) and stirred at room temperature for 1 hr. A white precipitate formed. LCMS showed ~ 50% conversion so additional hydrogen chloride, 4.0M in dioxane (0.408 ml, 1.634 mmol) was added. The reaction was stirred for 30 minutes at which point it was nearly complete by LCMS. The solvents were removed under vacuum and the white solid allowed to stand overnight to complete the reaction. Residual solvent and trace HCl were removed under high vacuum for 1 hour. Compound **1** was isolated as a white solid (0.35 g, 100%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.11* (t, *J* = 4.89 Hz, 0.2H), 6.92 - 7.02 (m, 0.7H), 6.29 - 6.37* (m, 0.2H), 6.27 (d, *J* = 2.84 Hz, 0.7H), 6.01* (dd, *J* = 3.15, 5.04 Hz, 0.2H), 5.94 (d, *J* = 2.21 Hz, 0.7H), 5.06 - 5.16 (m, 0.7H), 4.44 (d, *J* = 6.31 Hz, 0.2H), 3.82 (br. s., 1H), 3.05 (br. s., 0.7H), 2.92 (d, *J* = 5.36 Hz, 2H), 2.73 - 2.85 (m, 1.2H), 2.05 (ddd, *J* = 3.47, 8.28, 11.90 Hz, 0.7H), 1.76 (br. s., 2H), 1.19 - 1.64 (m, 6H), 0.80 (d, *J* = 12.30 Hz, 0.7H) ^{13}C NMR (125 MHz, DMSO-d_6) δ 171.1, 164.0*, 156.3, 156.2*, 140.9*, 138.2, 132.8*, 131.9, 74.1*, 74.0, 51.9, 47.2, 47.0*, 45.9*, 45.6, 41.7, 34.2*, 34.1, 29.7, 28.9, 21.6. * indicates exo isomer. LCMS (*ESI*+) m/z calculated: 282; found: 283 (M+H), T_r = 1.63 min.

Synthesis of N^6-(propargyloxycarbonyl)-L-Lysine Hydrochloride



Conditions: a) THF, NaOH (aq), 0C, b) 4M HCl (dioxane), CHCl3, 0C

(S)-2-((tert-butoxycarbonyl)amino)-6-(((prop-2-yn-1-yloxy)carbonyl)amino)hexanoic acid. A solution of N2-Boc-L-lysine (1.232 g, 5 mmol) in THF (12.5 ml) and 1M sodium hydroxide (12.5 ml, 12.5 mmol) was cooled to 0 °C and treated with propargyl chloroformate (0.585 ml, 6.00 mmol) dropwise over 5 minutes. The resulting mixture was stirred at room temperature overnight. The reaction was cooled to 0°C and washed with ethyl ether (2 x 50 mL). The aqueous phase was acidified to ~ pH 4-5 with 1N HCl then extracted with ethyl acetate (3 x 50 mL). The combined extracts were dried over sodium sulfate, filtered and concentrated under vacuum to give the product as a colorless oil (1.42 g, 86%). 1H NMR (500 MHz, CHLOROFORM-d) δ 6.23 (br. s., 1H), 5.23 (d, J = 6.62 Hz, 1H), 4.97 (br. s., 1H), 4.71 (d, J = 1.89 Hz, 2H), 4.25 - 4.40 (m, 1H), 4.14 (bs, 1H), 3.24 (d, J = 5.67 Hz, 2H), 2.50 (br. s., 1H), 1.67 - 1.95 (m, 2H), 1.52 - 1.63 (m, 2H), 1.48 (s, 9H); 13C NMR (125 MHz, CDCl3) δ 155.99, 155.73, 80.46, 78.26, 74.68, 53.10, 52.53, 40.55, 31.64, 29.28, 28.34, 22.24, 14.23; LCMS (Method A) r.t. 1.21 min, *m/z* 329.5 (M+H).

(S)-2-amino-6-(((prop-2-yn-1-yloxy)carbonyl)amino)hexanoic acid HCl. A solution of (S)-2-((tert-butoxycarbonyl)amino)-6-(((prop-2-yn-1-yloxy)carbonyl)amino)hexanoic acid (1.420 g, 4.32 mmol) in chloroform (24 ml) was cooled to 0 °C. 4M hydrogen chloride (dioxane) (6.49 ml, 25.9

mmol) was added dropwise and the reaction allowed to warm to room temperature. After 1 hour LCMS showed no Boc cleavage. Additional HCl - dioxane (10 mL) was added and stirring continued for 3 days. The reaction was complete by LCMS. The volatiles were removed under vacuum and the resulting white powder dried for 2 hours @ 0.5 Torr. 1H NMR 500 MHz, DEUTERIUM OXIDE) δ 4.53 (s, 2H), 3.87 (t, *J* = 6.31 Hz, 1H), 3.02 (t, *J* = 6.62 Hz, 2H), 2.75 (br. s., 1H), 1.72 - 1.92 (m, 2H), 1.39 - 1.48 (m, 2H), 1.22 - 1.38 (m, 2H); 13C NMR (125 MHz, D2O) δ 172.67, 157.62, 100.00, 78.45, 75.44, 53.09, 52.29, 39.85, 29.39, 28.22, 21.32; LCMS (Method A) r.t. 0.84 min, *m/z* 229 (M+H).

Supplementary Sequences

pY71-GB1f (Transzyme Plasmid). In green is the transzyme sequence. In blue is the Kan resistance sequence.

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pEVOL-Pyl. In green is Pyl aaRS.

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pY71-sfGFP-1xTAG. In green is the amber codon.

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pY71-sfGFP-2xTAG. In green are the amber codons.

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pY71-sfGFP-3xTAG. In green are the amber codons.

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