β-amino acids reduce ternary complex stability and alter the translation elongation mechanism

F. Aaron Cruz-Navarrete[1,2,#], Wezley C. Griffin[1,2,#], Yuk-Cheung Chan[3,#], Maxwell I. Martin[1,2], Jose L. Alejo[1,2], S. Kundhavai Natchiar[1,2], Isaac J. Knudson[4], Roger B. Altman[1,2], Alanna Schepartz[4,5,6,7,8], Scott J. Miller[3,*], and Scott C. Blanchard[1,2,*]

[1]Department of Structural Biology, St Jude Children's Research Hospital, Memphis, Tennessee, USA

[2]Department of Chemical Biology & Therapeutics, St Jude Children's Research Hospital, Memphis, Tennessee, USA

[3]Department of Chemistry, Yale University, New Haven, Connecticut, USA

[4]College of Chemistry, University of California, Berkeley, California, USA

[5]Molecular and Cell Biology, University of California, Berkeley, CA 94720, USA

[6]California Institute for Quantitative Biosciences, University of California, Berkeley, CA 94720, USA

[7]Chan Zuckerberg Biohub, San Francisco, CA 94158, USA

[8]Innovation Investigator, ARC Institute, Palo Alto, CA 94304, USA

[#]Authors contributed equally.

*To whom correspondence should be addressed: scott.blanchard@stjude.org; scott.miller@yale.edu

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure S1. Aminoacylation analysis of various aa-tRNA^{Phe}-Cy3B. A) Cartoon schematic of tRNA^{Phe}-Cy3B charging and purification workflow (see methods for details). De-acylated tRNA^{Phe}-Cy3B (variegated) is aminoacylated **(1)** mixed with an excess of flexizyme or tRNA synthetase. Addition of the specified parent (blue) or non-natural (red) amino acid initiates the acylation reaction and is allowed to proceed for the indicated time detailed in the Methods section. Aminoacylated species are then prepped and purified by hydrophobic interaction (HIC) **(2)** by monitoring the absorbance at 260 nm as outlined in the Methods. **B)** Representative HIC purification chromatograms of various parent and non-natural -tRNA^{Phe}-Cy3B (various colors and indicated monomers) compared to deacyl-tRNA^{Phe}-Cy3B (grey).

Supplemental Figure S2. Ternary complex formation assay comparing Flexizyme and PheRS-charged L-α-Phe-tRNA^{Phe}-Cy3B. Ternary complex formation assay as described in Figure 2 comparing tRNA^{Phe}-Cy3B charged by PheRS (blue) or flexizyme (green), and de-acylated tRNA^{Phe}-Cy3B (grey). Error bars represent S.D. from two separate replicates.

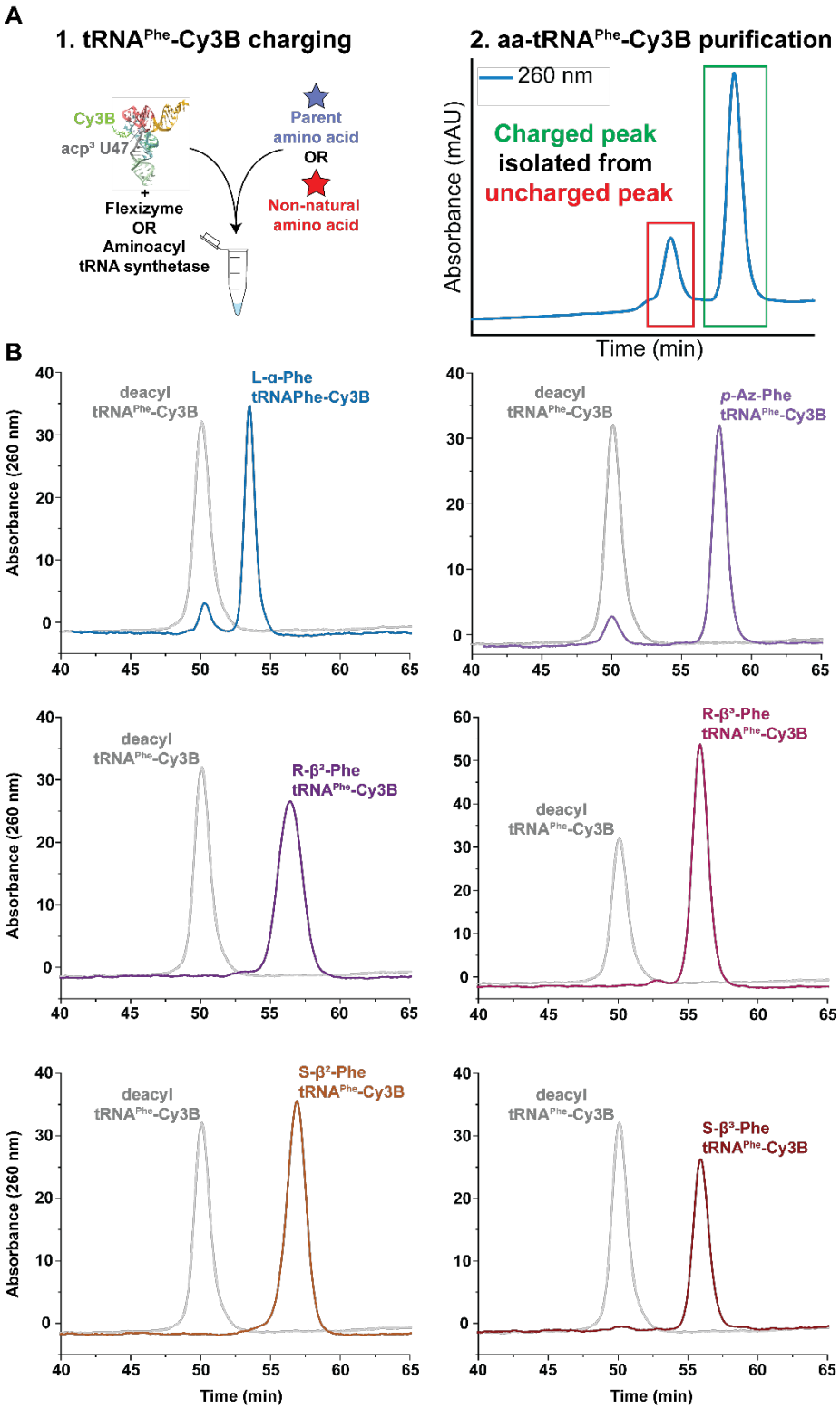
Supplemental Figure S3. Mutant tRNA^{Phe} (C49A G65U) and/or EF-Tu (N273A) examined by stopped-flow ternary complex formation. A) Sequences of WT (left) and mutant (right) *E coli* tRNA^{Phe} used for stopped-flow kinetic analysis. **B)** Structure of EF-Tu (PDB: 1OB2) with the N273 amino acid residue in the amino acid binding pocket shown next to the Phe aminoacylated to the A76 on the 3'-end of tRNA^{Phe} (yellow). **C-E)** Stopped-flow ternary complex formation assays using Cy3B-labeled mutant aa-tRNA^{Phe} (MUT) with the indicated monomers inset. **F-H)** Stopped-flow ternary complex assays with both tRNA^{Phe} MUT and mutant EF-Tu N273A with the indicated monomers inset. Error bars represent S.D. of 3-5 replicates.

Supplemental Figure S4. Kinetic simulations of ternary complex formation with S-β^3-Phe. A) Minimal reaction scheme for kinetic simulations. Contour plots of **B)** L-α-Phe and **C)** S-β^3-Phe ternary complex abundance simulated at different physiologically relevant aa-tRNA and ET-Tu/Ts concentrations. Simulations were done using measured rate constants from experiments reported here and from the literature. Ternary complex fraction was calculated as [aa-tRNA]$_{bound}$/[aa-tRNA]$_{total}$

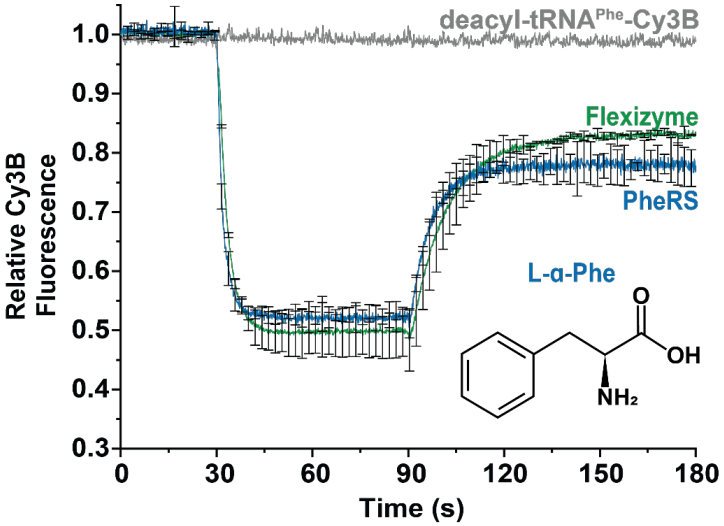
Figure S5. Manual modelling of aa-tRNA^{Phe} into the EF-Tu binding pocket. Manual modelling of **A)** L-α-Phe, **B)** *p*-Az-Phe, **C)** S-β^2-Phe, **D)** S-β^3-Phe, **E)** R-β^2-Phe, **F)** R-β^3-Phe into the amino acid binding pocket of EF-Tu formed by the DI-DII interface. Steric clashes are represented in red. The 2D model of non-natural amino acids was created using the Chemical Sketch Tool provided by the RCSB Protein Data Bank. Subsequently, the 3D atomic models and RNA-peptide links were constructed using JLigand [80]. The figures were generated using PYMOL, the PyMOL Molecular Graphics System, Version 2.5.7 Schrödinger, LLC.

SUPPLEMENTAL FIGURES

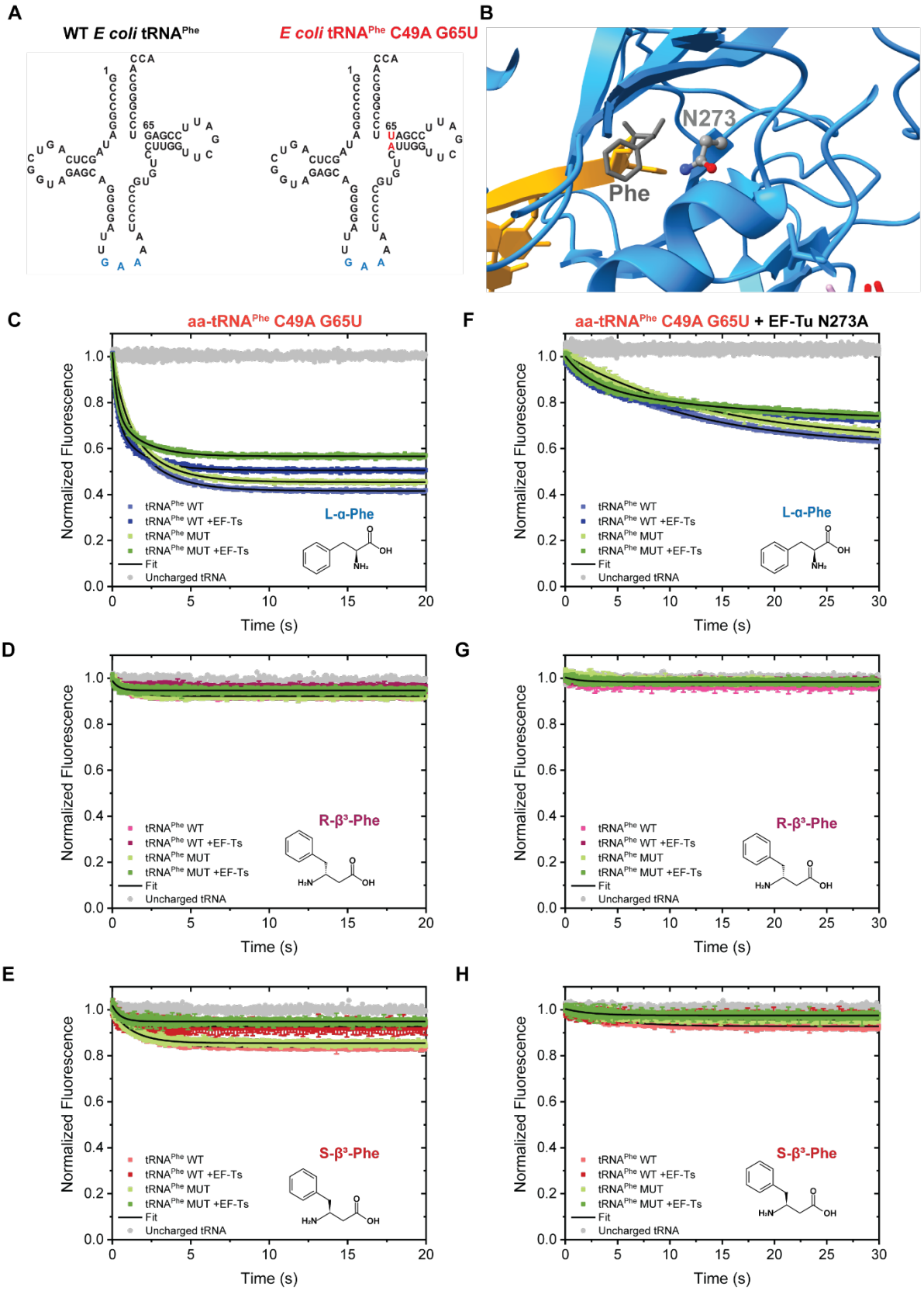
Supplemental Figure 1



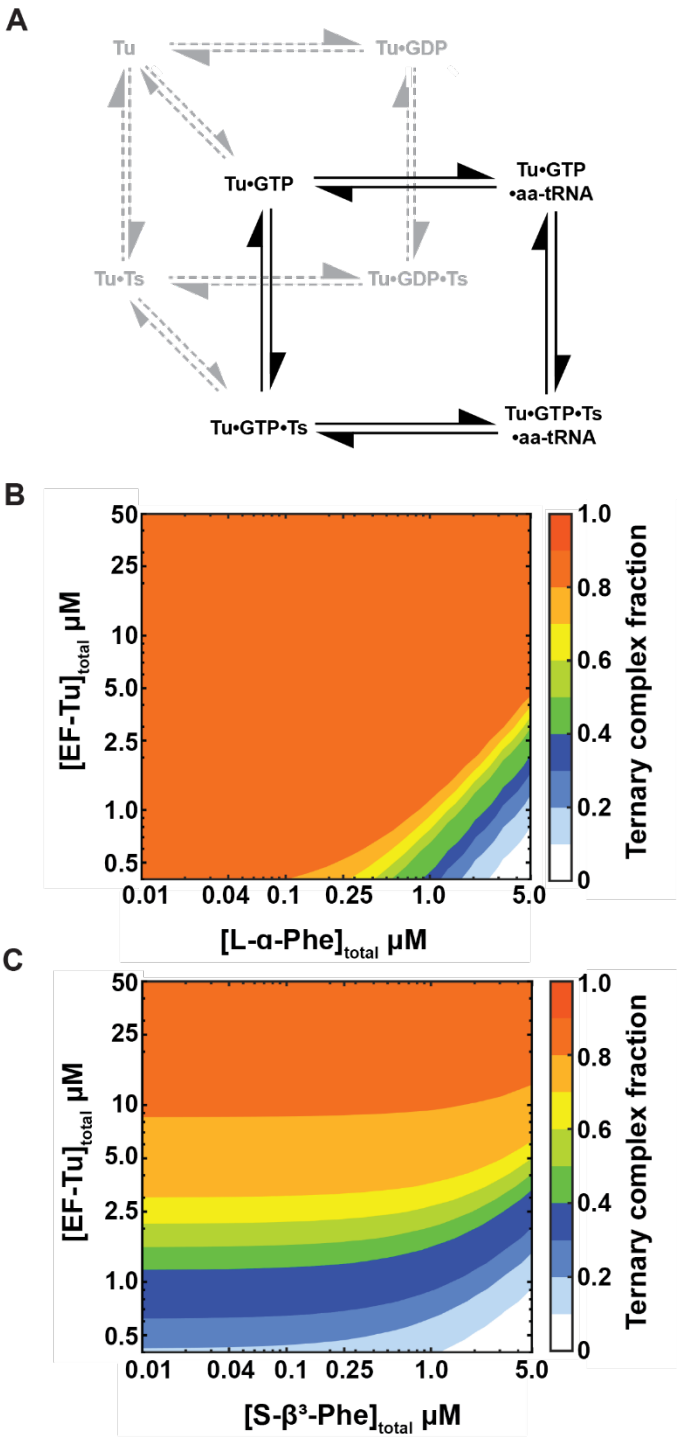
Supplemental Figure 2



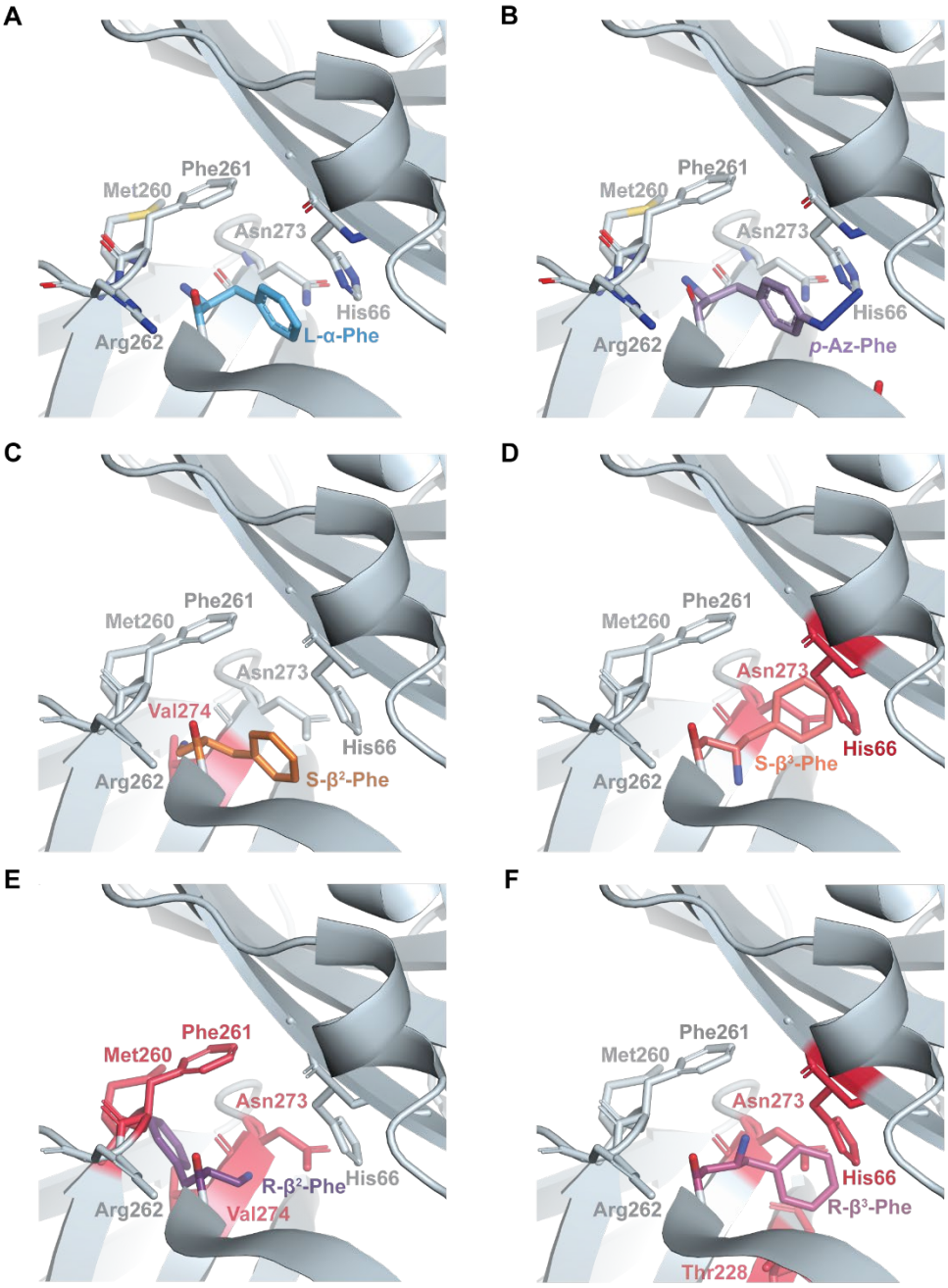
Supplemental Figure 3



Supplemental Figure 4

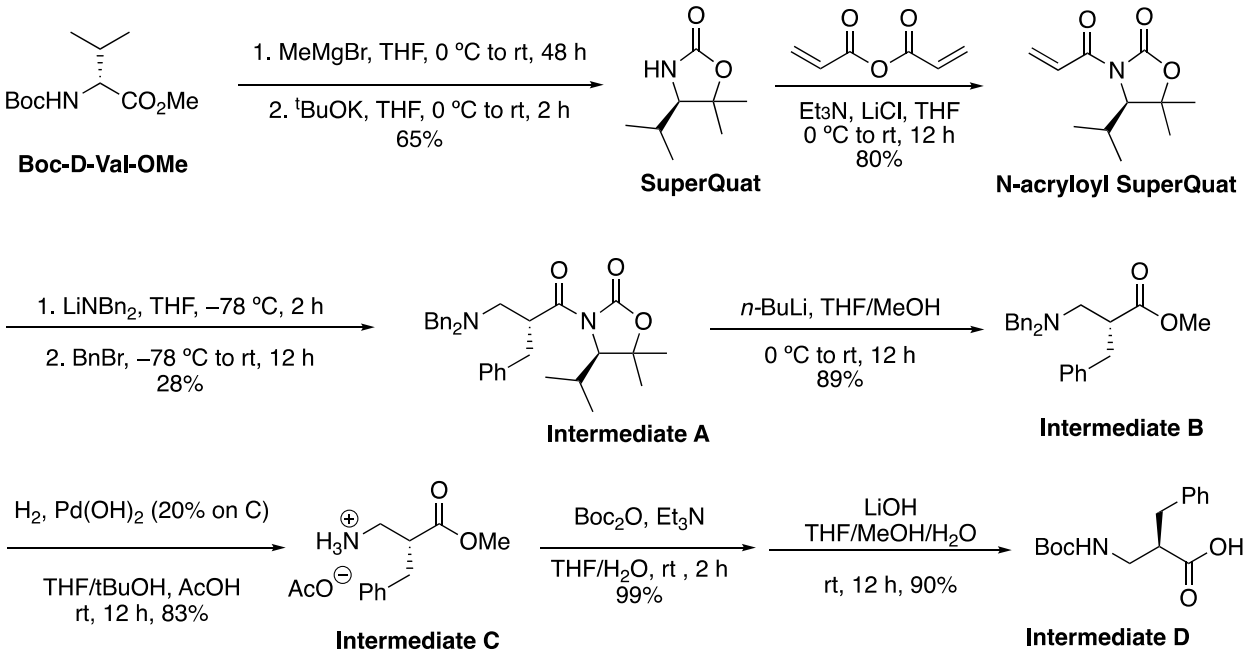


Supplemental Figure 5



SUPPLEMENTAL METHODS

Synthesis of Boc-Protected β²-amino acid (Both enantiomeric forms)



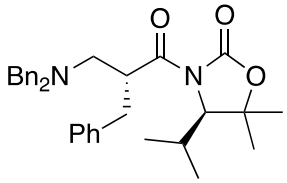
Note: The enantiomer of **Intermediate D** was prepared from Boc-L-Val-OMe.

SuperQuat and **N-acryloyl SuperQuat** were prepared according to literature procedure (Asymmetric synthesis of b2-amino acids: 2-substituted-3-aminopropanoic acids from N-acryloyl SuperQuat derivatives. *Org. Biomol. Chem*., **2007**, *5*, 2812–2825.) **Intermediate A** and **B** were also prepared according to the same literature. For **Intermediate A**, we used a different eluent system (hexanes:EtOAc 10 :1 to 7:1, instead of hexanes:Et_2O 20:1 in the literature condition) for the SiO_2 column chromatographic purification to afford **Intermediate A** as pale yellow oil (980 mg, 1.96 mmol) in 28% yield (from 7 mmol **N-acryloyl SuperQuat**). For **Intermediate B**, same literature procedure was followed to afford **Intermediate B** as colorless oil (650 mg,1.74 mmol) in 89% yield (from 1.96 mmol of **A**).

Note: The enantiomer of **Intermediate A** and **B** were prepared from Boc-L-Val-OMe as the starting material by the same procedure.

Intermediate A

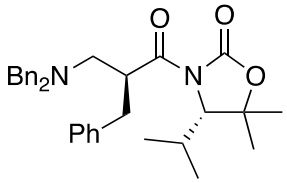
(*R*)-3-((*R*)-2-benzyl-3-(dibenzylamino)propanoyl)-4-isopropyl-5,5-dimethyloxazolidin-2-one



^{1}H NMR (400 MHz, CD_2Cl_2) δ 7.42 – 7.26 (m, 15H), 4.94 (tt, *J* = 8.6, 6.0 Hz, 1H), 4.23 (d, *J* = 2.8 Hz, 1H), 3.73 (d, *J* = 13.6 Hz, 2H), 3.65 (d, *J* = 13.7 Hz, 2H), 3.11 – 2.89 (m, 3H), 2.59 (dd, *J* = 12.6, 5.6 Hz, 1H), 2.05 (pd, *J* = 6.9, 2.8 Hz, 1H), 1.53 (d, *J* = 3.0 Hz, 6H), 0.83 (d, *J* = 7.0 Hz, 3H), 0.69 (d, *J* = 6.8 Hz, 3H).
^{13}C NMR (101 MHz, CD_2Cl_2) δ 175.6, 153.8, 139.6, 139.4, 129.7, 129.6, 128.8, 128.5, 127.3, 126.7, 82.7, 66.7, 58.9, 56.6, 43.1, 37.6, 29.9, 29.1, 21.5, 21.4, 16.6.

***enantiomer*-Intermediate A**

(*S*)-3-((*S*)-2-benzyl-3-(dibenzylamino)propanoyl)-4-isopropyl-5,5-dimethyloxazolidin-2-one



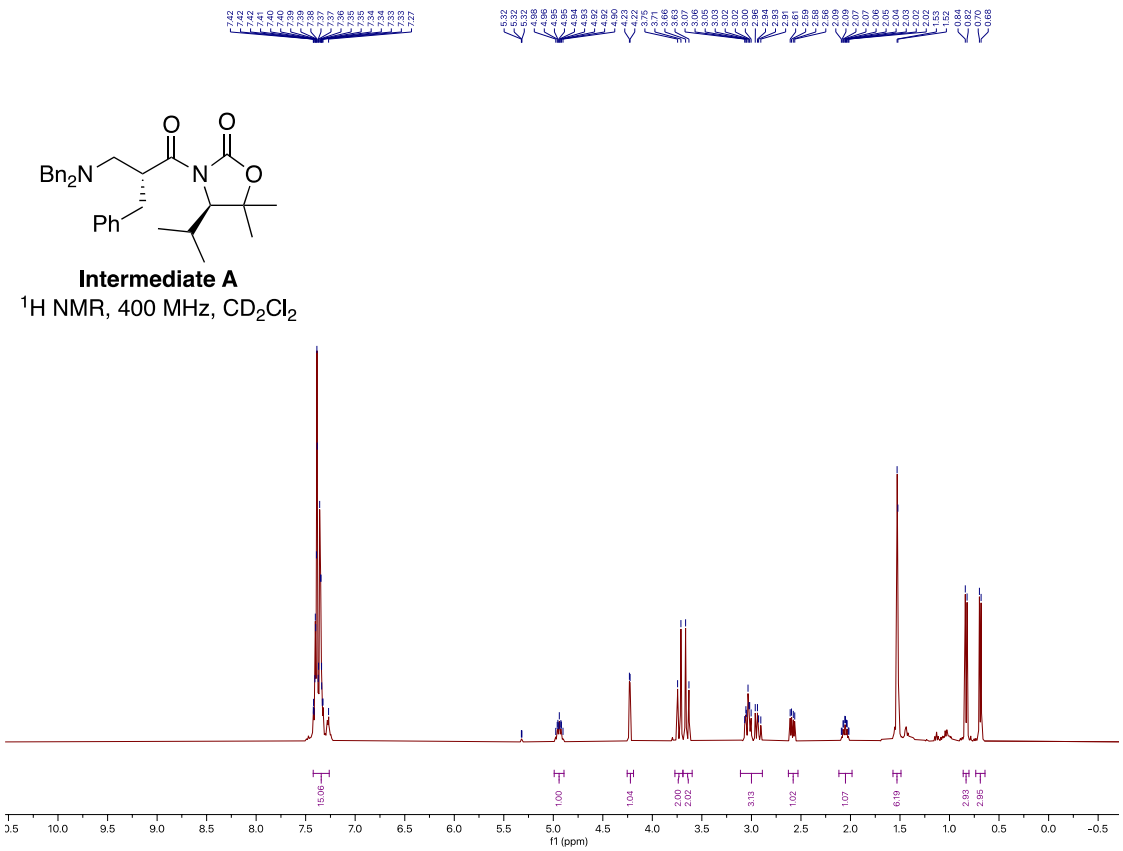
^{1}H NMR (500 MHz, CD_2Cl_2) δ 7.39 – 7.18 (m, 15H), 4.84 (tt, *J* = 8.5, 6.0 Hz, 1H), 4.16 (d, *J* = 2.8 Hz, 1H), 3.65 (d, *J* = 13.6 Hz, 2H), 3.58 (d, *J* = 13.6 Hz, 2H), 3.02 – 2.90 (m, 2H), 2.85 (dd, *J* = 13.6, 8.8 Hz, 1H), 2.51 (dd, *J* = 12.6, 5.7 Hz, 1H), 2.06 – 1.95 (m, 1H), 1.49 (d, *J* = 13.8 Hz, 6H), 0.77 (d, *J* = 7.0 Hz, 3H), 0.63 (d, *J* = 6.8 Hz, 3H).
^{13}C NMR (126 MHz, CD_2Cl_2) δ 175.6, 153.8, 139.6, 139.5, 129.6, 129.5, 128.7, 128.5, 127.3, 126.6, 82.8, 66.8, 58.9, 56.5, 43.1, 37.6, 29.9, 29.1, 21.5, 21.3, 16.6.

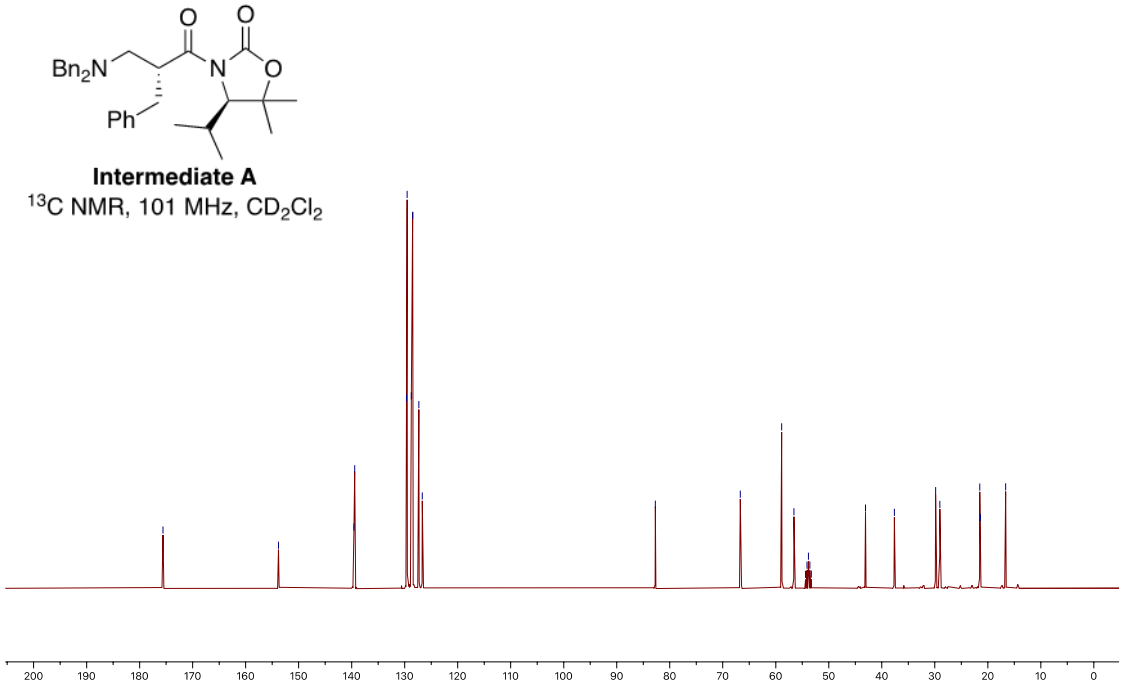
For **Intermediate B** to **Intermediate C**, we modified the condition (Literature: *Org. Biomol. Chem*., **2007**, *5*, 2812–2825.) from Pd/C in MeOH/AcOH into $Pd(OH)_2$/C (10% by weight w.r.t. substrate) in THF/*t*-BuOH (v:v, 3:1, 0.1 M) with AcOH (10% by volume) as additive.

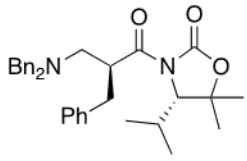
Intermediate B (650 mg, 1.74 mmol) was dissolved in THF/*t*-BuOH (v:v, 3:1, total 16 mL, *ca*. 0.1 M) and AcOH (1.6 mL). $Pd(OH)_2$ (20% on carbon) (65 mg) was added to the solution. The reaction was degassed, connected to a H_2 balloon and stirred at rt for overnight before filtration over Celite. The filtrate was concentrated under reduced pressure to afford **Intermediate C** as oil (364 mg, 1.44 mmol, 83%) which was subjected to next step without further purification. Same experimental sequence was carried out to prepare the enantiomer of **Intermediate C**.

For **Intermediate C** to **Intermediate D**, **C** (364 mg, *ca*. 1.44 mmol, 1 equiv.) was dissolved in THF/H_2O (6 mL:2 mL). Et_3N (0.6 mL, 4.3 mmol, 3 equiv.) and Boc_2O (377 mg, 1.7 mmol, 1.2 equiv.) were added. The solution was stirred at rt for 2 h before dilution with EtOAc (10 mL). The organic phase was washed successively with 10% citric acid solution, saturated $NaHCO_3$ solution

and brine. The organic phase was dried over anhydrous Na_2SO_4, filtered and concentrated to afford (*R*)-Boc-β2-Phe-OMe (423 mg, 1.44 mmol), which was subjected to next step without further purification. LiOH•H_2O (242 mg, 7.2 mmol, 4 equiv.) and THF/MeOH/H_2O (4 mL/1 mL/1 mL) were added to the crude product. The mixture was stirred at rt for overnight before dilution with 4 mL H_2O. The aqueous phase was washed against Et_2O (5 mL × 2), followed by acidifying with 1N HCl to adjust the pH into ~2. The aqueous phase was extracted with EtOAc (15 mL × 2), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to afford **Intermediate D** (363 mg, 1.3 mmol, 90%) as a colorless oil. The same experimental sequence was carried out to prepare the enantiomer of **Intermediate D**.

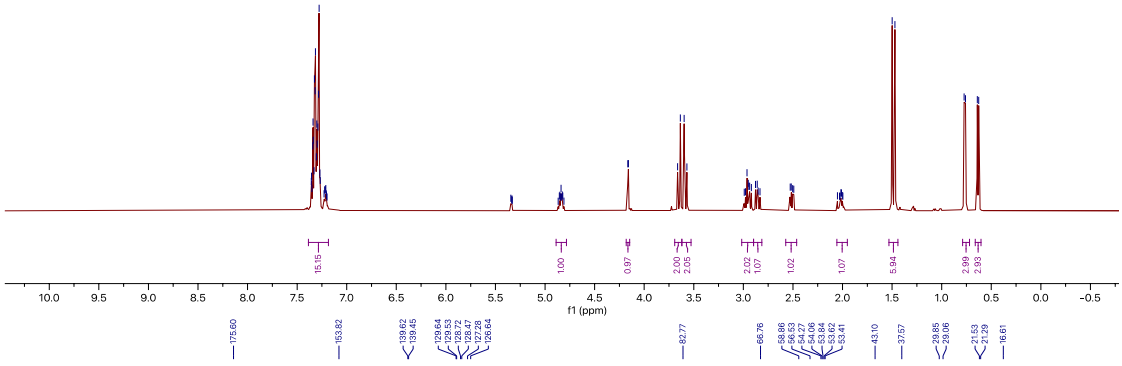


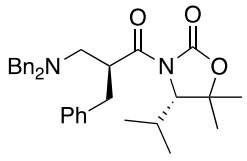




***ent*-Intermediate A**

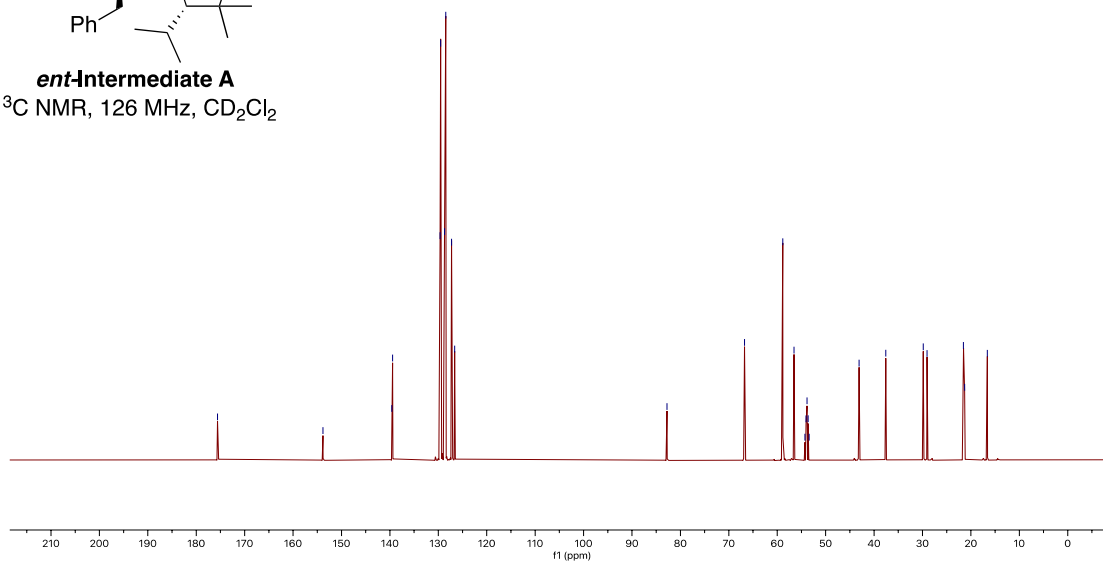
^{1}H NMR, 500 MHz, CD_2Cl_2



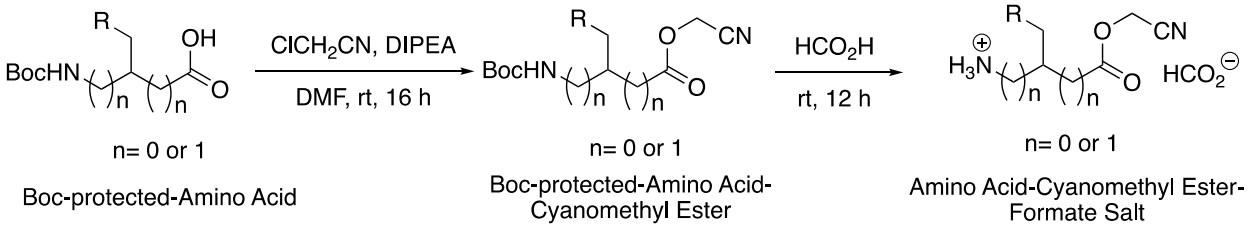


***ent*-Intermediate A**

^{13}C NMR, 126 MHz, CD_2Cl_2



General procedure for synthesis of β-amino acid-cyanomethyl ester-formate salt



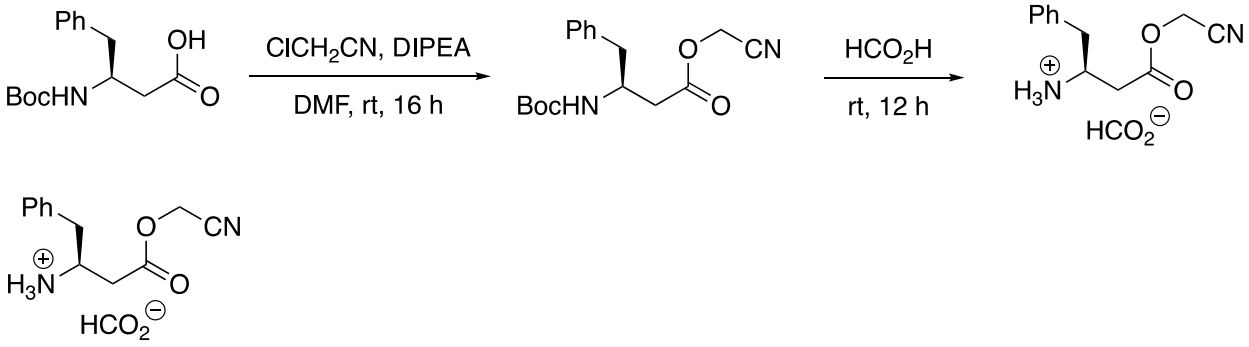
Boc-protected-Amino Acid

Boc-protected-Amino Acid-Cyanomethyl Ester

Amino Acid-Cyanomethyl Ester-Formate Salt

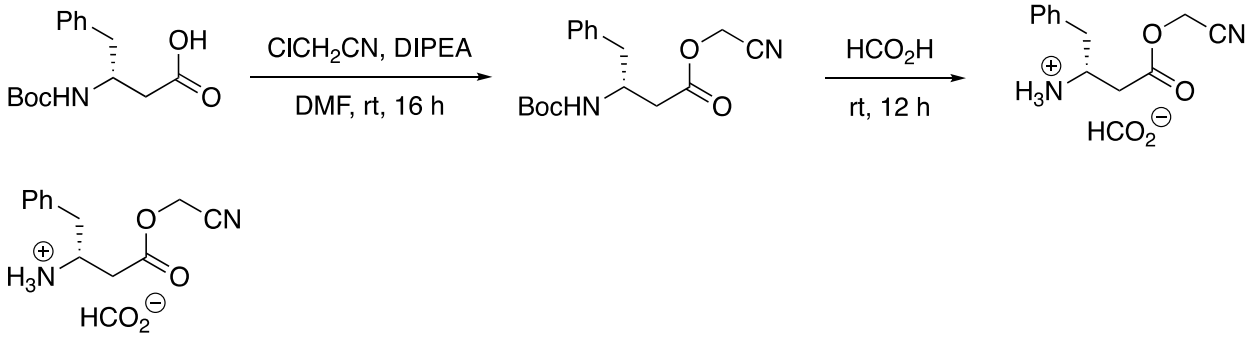
To a stirred solution of Boc-protected-amino acid (0.5 mmol, 1 equiv.) was added dry DMF (1 mL), $ClCH_2CN$ (0.75 mmol, 1.5 equiv.) and dry DIPEA (1 mmol, 2 equiv.), the solution was stirred at rt for 16 h before dilution with EtOAc (10 mL). The organic phase was washed successively with 10% citric acid aq. solution, 5 % LiCl aq. solution and brine. The organic phase was dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to afford the corresponding Boc-protected-Amino Acid-Cyanomethyl ester, which was used without further purification. Note: The cyanomethyl ester is very sensitive to aqueous basic medium.

The Boc-protected-Amino Acid-Cyanomethyl ester (*ca* 0.5 mmol) was treated with neat formic acid (2 mL). The solution was stirred at rt for 12 h before removing all the formic acid under reduced pressure by azeotropic distillation with $CHCl_3$ to afford a pale-yellow oil. The oil was dissolved in minimum amount of THF (*ca.* 2 mL), triturated with excess MTBE or Et_2O until white solid was formed persistently. All the residual solvent was removed under reduced pressure. The white solid was crushed into fine powder, rinsed thoroughly with Et_2O (10 mL) and dried over vacuum for overnight. The typical yield over two steps was 50%. Note: The final product is very sensitive to water and alcohol which cause saponification or transesterification.



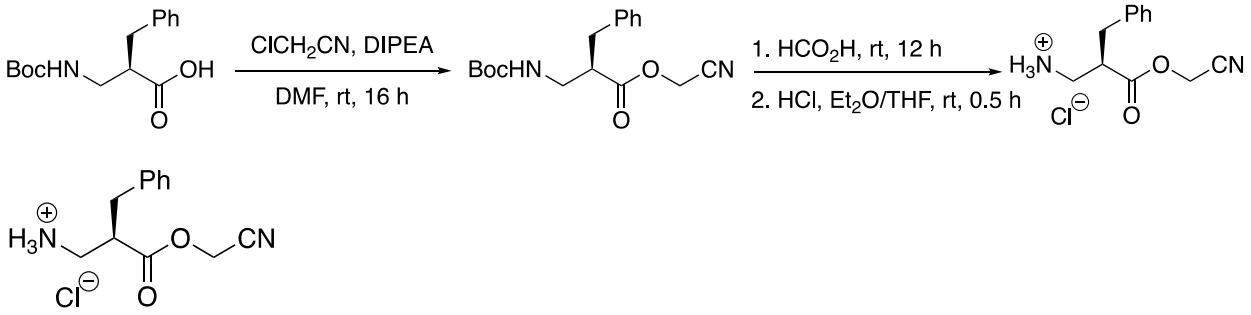
(*S*)-4-(cyanomethoxy)-4-oxo-1-phenylbutan-2-aminium formate

^{1}H NMR (500 MHz, $CDCl_3$) δ 9.22 (s, 3H), 8.37 (s, 1H), 7.41 – 7.26 (m, 5H), 4.72 (s, 2H), 3.89 (s, 1H), 3.23 (dd, *J* = 14.0, 5.6 Hz, 1H), 3.04 – 2.74 (m, 3H).
^{13}C NMR (126 MHz, $CDCl_3$) δ 169.9, 168.2, 135.4, 129.5, 129.2, 127.7, 114.4, 49.4, 49.1, 39.3, 35.8.
HRMS-ESI: calculated for $[C_{12}H_{15}N_2O_2]^+$ 219.1128, found 219.1150.



(*R*)-4-(cyanomethoxy)-4-oxo-1-phenylbutan-2-aminium formate

^{1}H NMR (500 MHz, $CDCl_3$) δ 9.15 (s, 3H), 8.36 (s, 1H), 7.43 – 7.20 (m, 5H), 4.70 (s, 2H), 3.87 (s, 1H), 3.22 (dd, *J* = 13.6, 5.6 Hz, 1H), 3.01 – 2.70 (m, 3H).
^{13}C NMR (126 MHz, $CDCl_3$) δ 169.8, 168.3, 135.5, 129.5, 129.2, 127.7, 114.4, 49.3, 49.0, 39.3, 35.9.
HRMS-ESI: calculated for $[C_{12}H_{15}N_2O_2]^+$ 219.1128, found 219.1144.

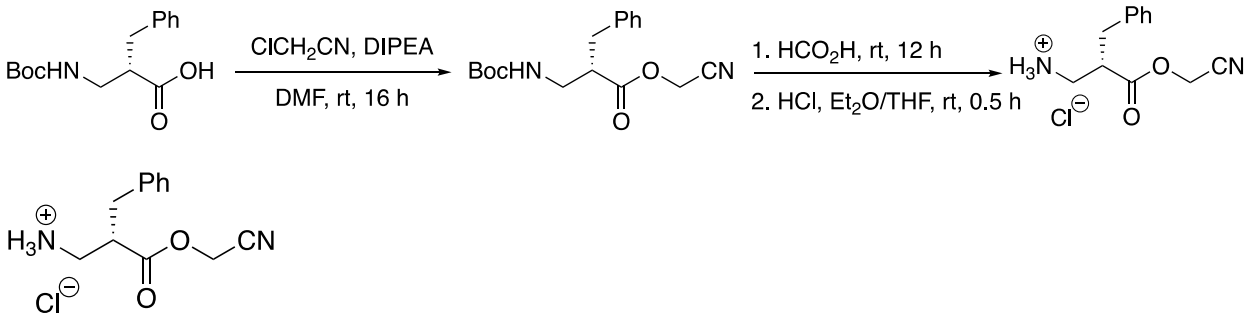


(*R*)-2-benzyl-3-(cyanomethoxy)-3-oxopropan-1-aminium chloride

Procedure for anion metathesis with chloride

After removal of residual formic acid from the Boc deprotection, the oily form product (formate salt) was dissolved in minimum amount of THF (*ca* 2 mL). HCl (1M in Et_2O) (1.5 mL, 3 equiv.) was added. The solution was stirred for 0.5 h before concentration to dryness, and repetitively triturated with excess MTBE or Et_2O until white solid was formed persistently. All the residual solvent was removed under reduced pressure. The white solid was crushed into fine powder, rinsed thoroughly with Et_2O (10 mL) and dried over vacuum for overnight.

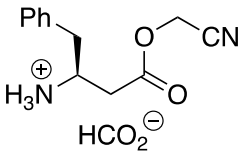
^{1}H NMR (500 MHz, DMSO-*d*6) δ 8.45 (s, 3H), 7.35 – 7.14 (m, 5H), 4.95 (s, 2H), 3.21 (ddq, *J* = 8.3, 6.0, 3.0 Hz, 1H), 3.08 – 2.96 (m, 2H), 2.90 (dd, *J* = 13.8, 8.0 Hz, 2H).
^{13}C NMR (126 MHz, DMSO-*d*6) δ 171.1, 137.3, 128.9, 128.5, 126.8, 115.6, 49.6, 44.3, 39.1.
HRMS-ESI: calculated for $[C_{12}H_{15}N_2O_2]^+$ 219.1128, found 219.1141.



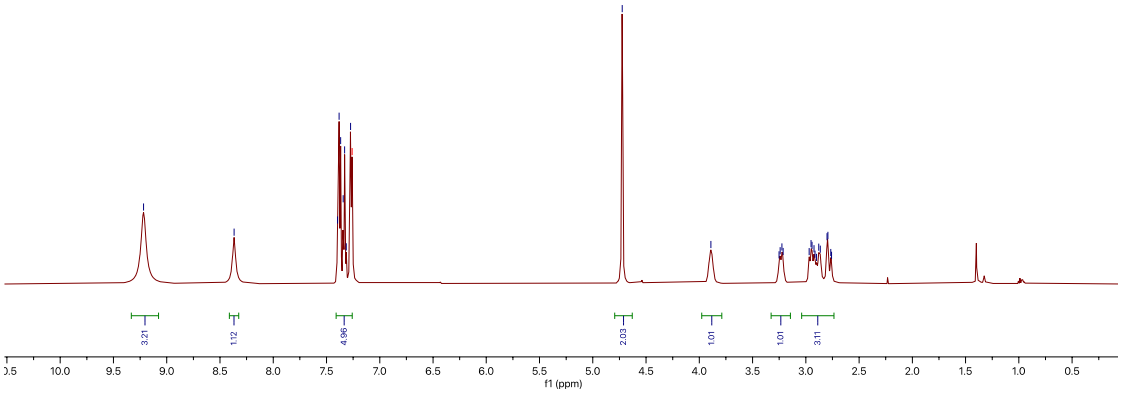
(*S*)-2-benzyl-3-(cyanomethoxy)-3-oxopropan-1-aminium chloride

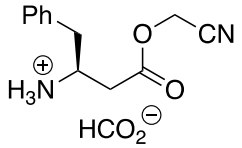
Following the procedure for anion metathesis with chloride.

^{1}H NMR (500 MHz, DMSO-*d*6) δ 8.37 (s, 3H), 7.32 – 7.20 (m, 5H), 4.95 (s, 2H), 3.19 (td, *J* = 8.2, 4.1 Hz, 1H), 3.06 – 2.87 (m, 4H).
^{13}C NMR (126 MHz, DMSO-*d*6) δ 171.1, 137.3, 128.9, 128.5, 126.8, 115.7, 49.6, 44.4, 39.1.
HRMS-ESI: calculated for $[C_{12}H_{15}N_2O_2]^+$ 219.1128, found 219.1137.

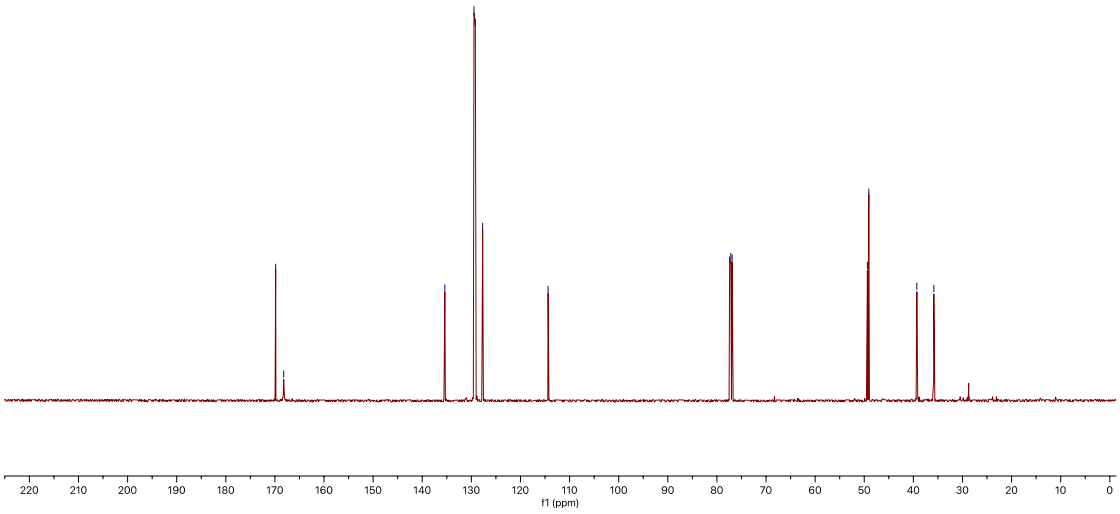


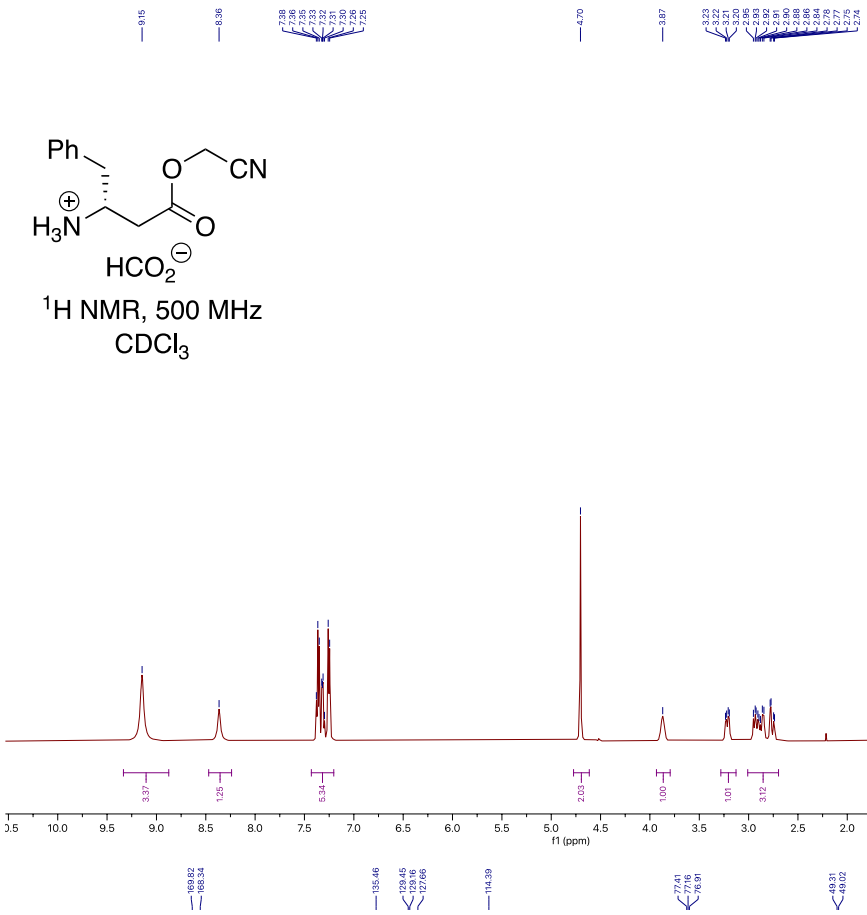
^{1}H NMR, 500 MHz
$CDCl_3$

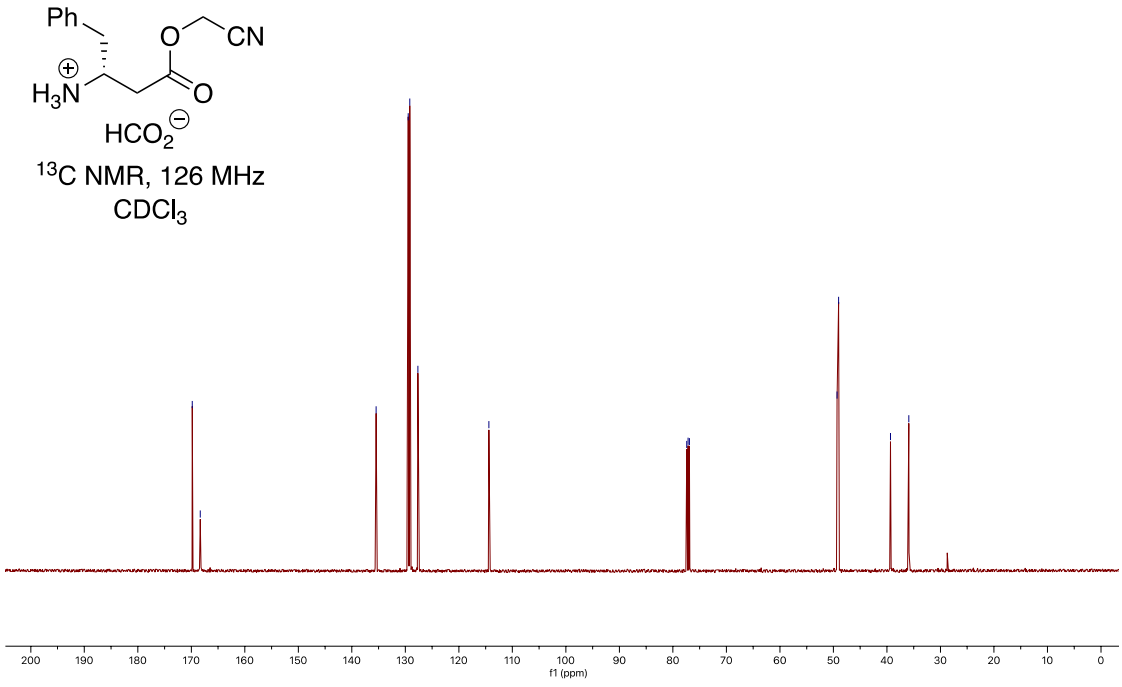


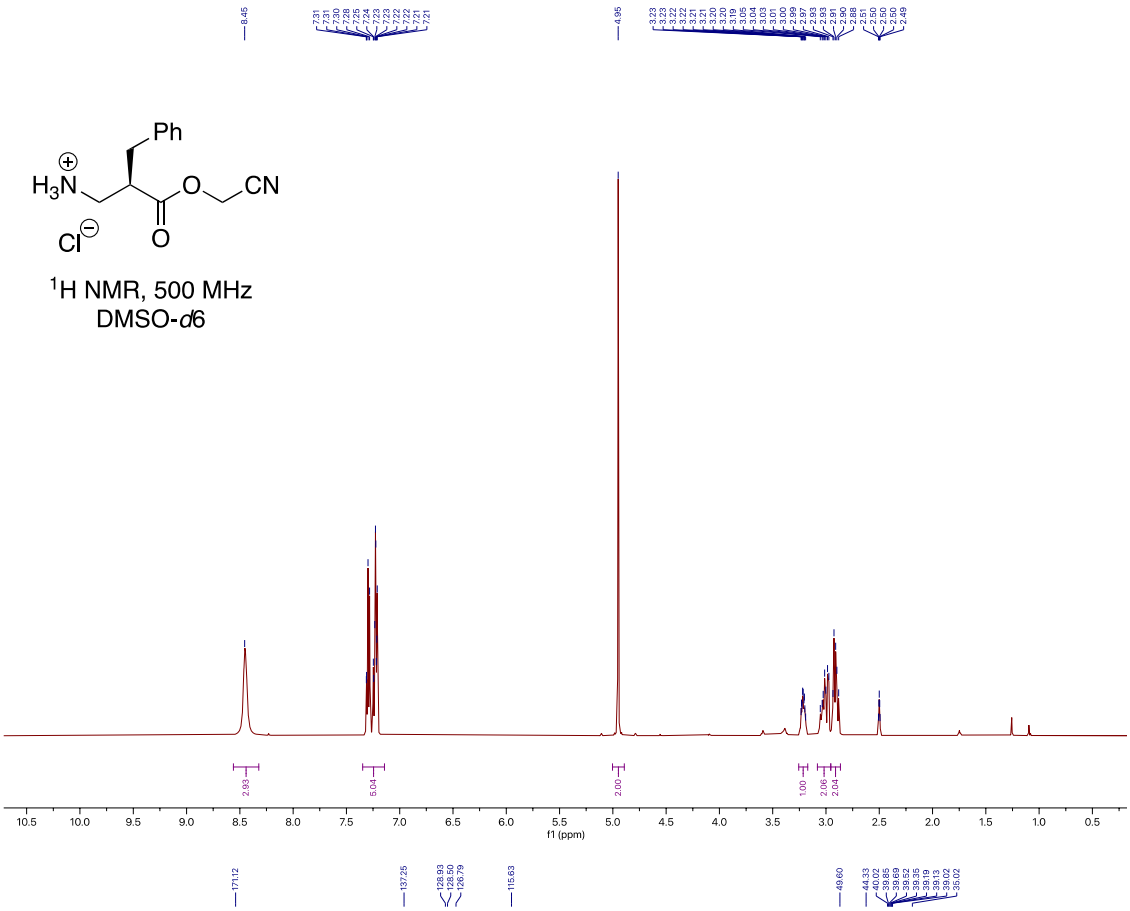


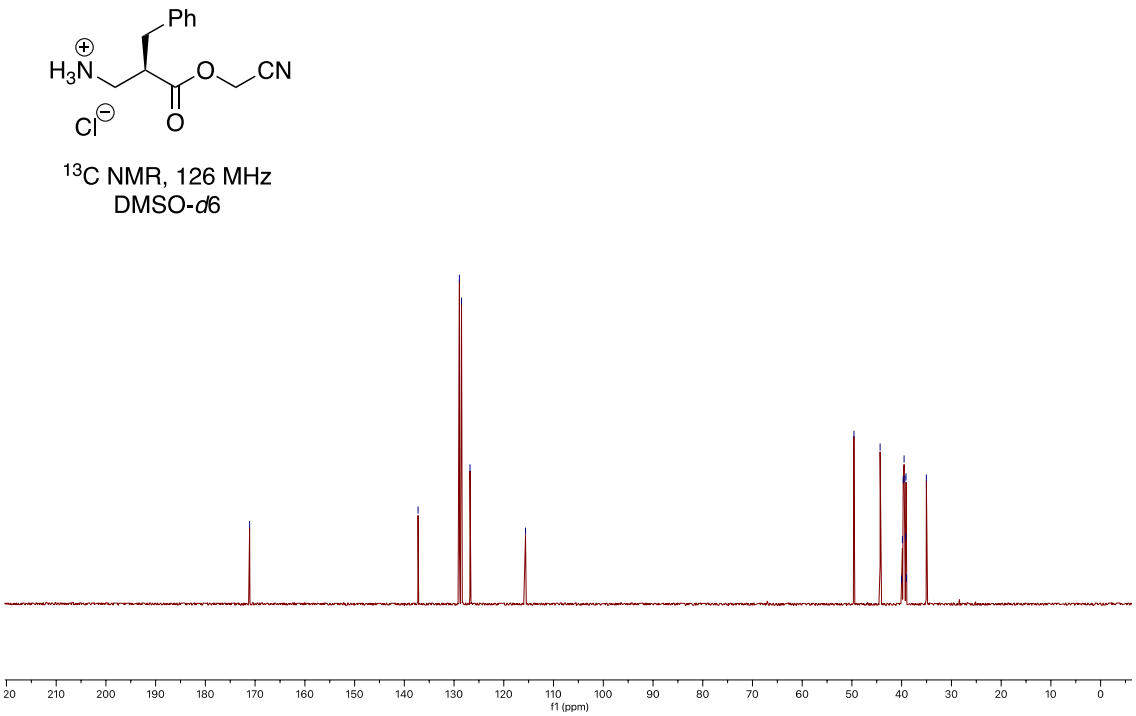
^{13}C NMR, 126 MHz
$CDCl_3$

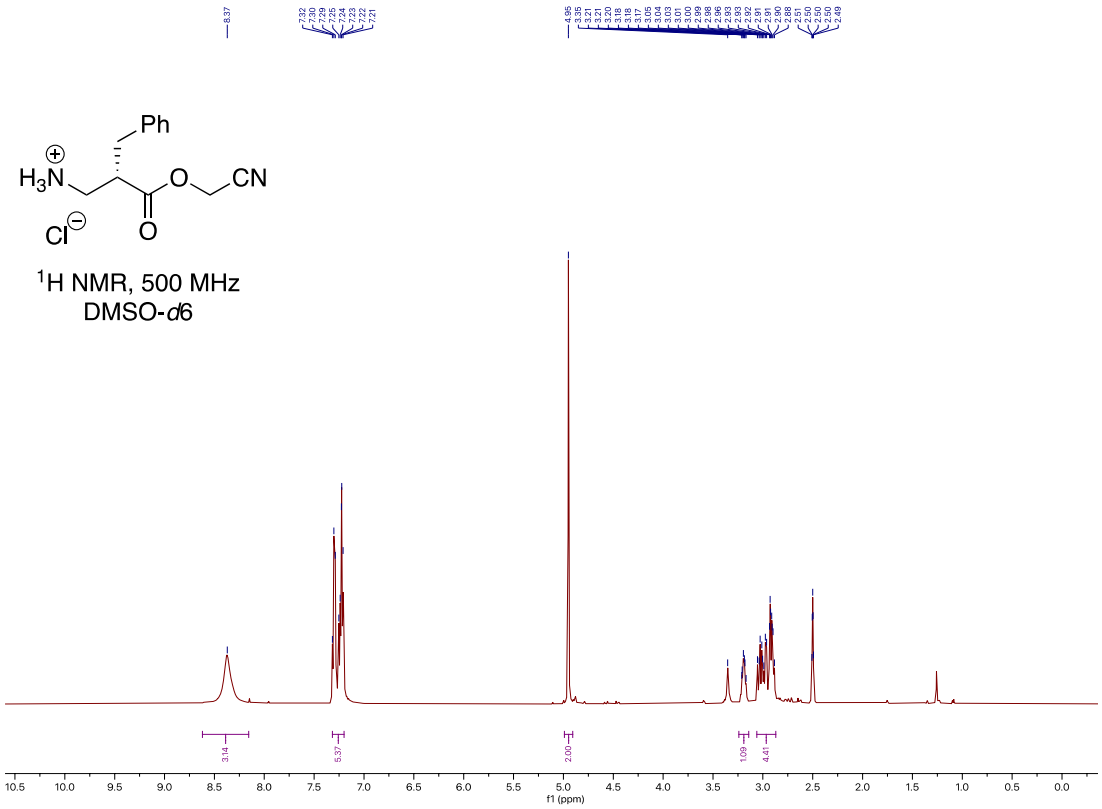


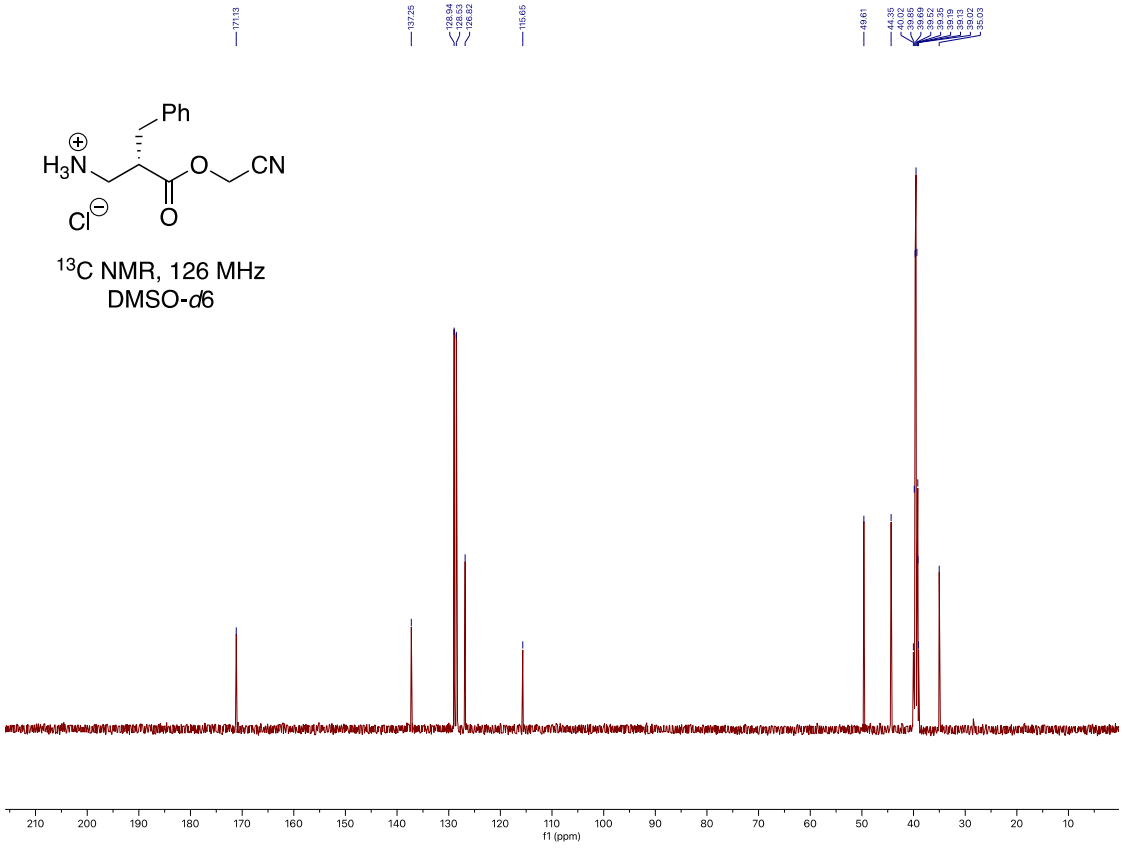




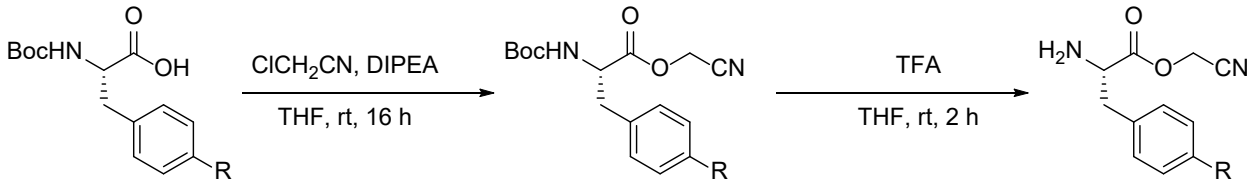






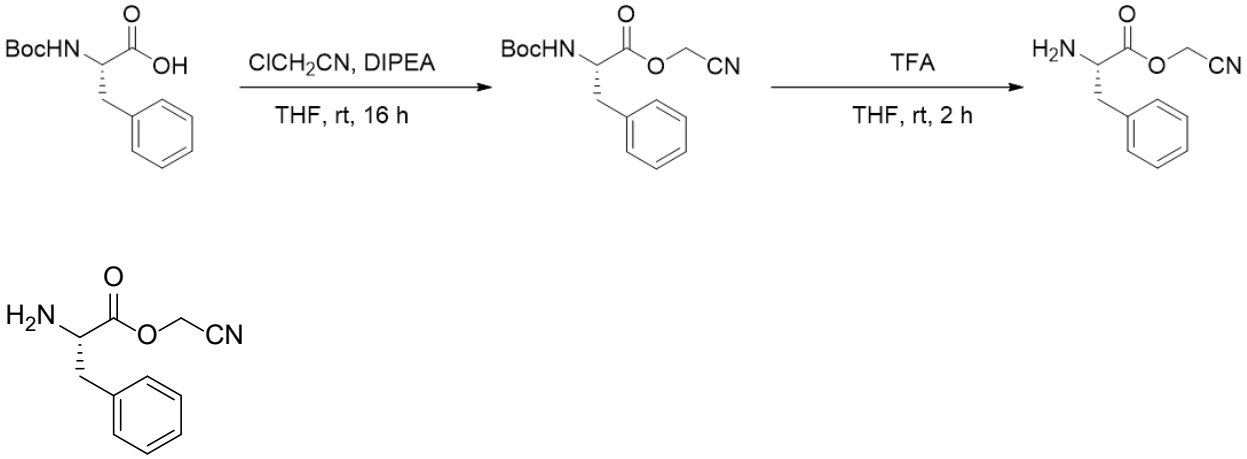


General procedure for synthesis of α-amino acid-cyanomethyl esters



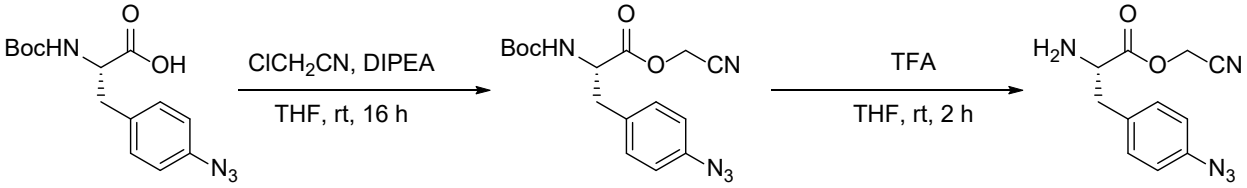
R = H or N3 R = H or N3 R = H or N3

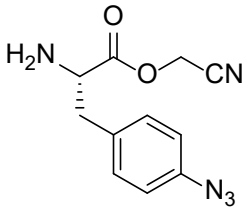
To a 5-mL round-bottom flask, N-Boc protected amino acid (0.5 mmol) was dissolved in 1 mL of tetrahydrofuran. Flask was then charged with 315 µL of chloroacetonitrile (5.0 mmol, 10 eq.), followed by addition of 100 L of N,N-diisopropylethylamine (0.6 mmol, 1.2 eq.). Flask was capped with septa and stirred at room temperature overnight, 16 hours. Solvent was removed via rotary evaporation then the crude material was purified by reverse-phase flash chromatography, 0-100% acetonitrile in water, holding at 60% acetonitrile until product was collected. Solvent removed via rotary evaporation, where the resulting oil was dissolved in 1 mL of tetrahydrofuran for deprotection. To the resulting solution, 1.9 mL of trifluoroacetic acid (25 mmol, 50 eq.) was added, and allowed to stir at room temperature for 2 hours. Upon completion, the solvent was removed followed by purification by reverse-phase flash chromatography utilizing a 2% acetonitrile in water mobile phase. Solvent was removed by lyophilization to yield target materials as colorless oils at 54% and 49% yield for phenylalanine and 4-azidophenylalanine derivatives respectively. Note: The final product is very sensitive to water and alcohol which cause saponification or transesterification.



Cyanomethyl-ester-L-phenylalanine

^{1}H NMR (400 MHz, DMSO-*d*6) δ 8.58 (s, 2H), 7.33 (m, 5H), 5.10 (d, 2H), 4.47 (t, 1H), 3.12 (m, 2H). ESI-MS: calculated for $[C_{11}H_{13}N_2O_2]^+$ = 205.10, found 205.24.





4-Azido-Cyanomethyl-Ester-L-Phenylalanine

^{1}H NMR (400 MHz, DMSO-*d*6) δ 8.45 (s, 2H), 7.29 (d, 2H), 7.09 (d, 2H), 5.09 (d, 2H), 4.43 (t, 1H), 3.11 (m, 2H). ESI-MS: calculated for $[C_{11}H_{12}N_5O_2]^+$ = 246.10, found 246.15.