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A Modular Approach to Enzymatic Ligation of Peptides and Proteins with Oligonucleotides

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Supporting Information

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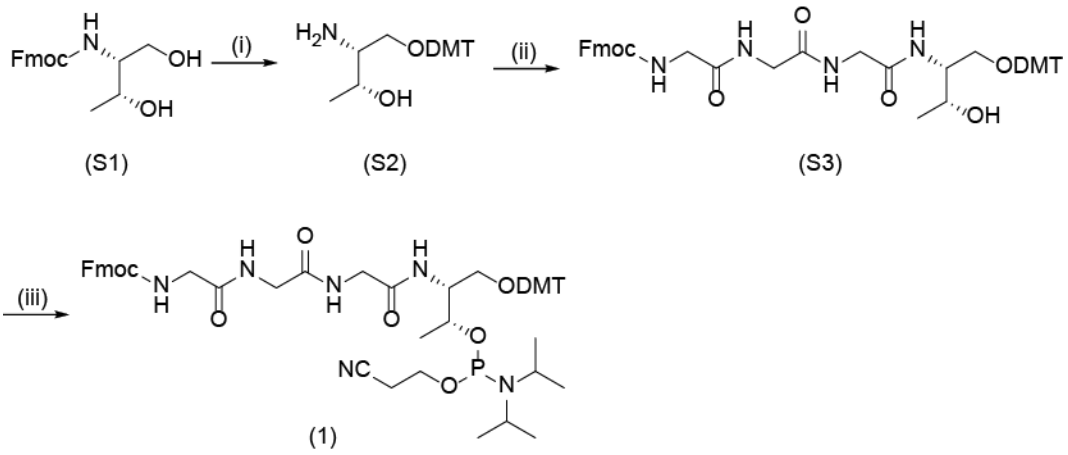
I. General methods

Reagents and solvents were purchased from commercial sources (Sigma Aldrich, Acros, Merck, Alfa Aesar). DNA phosphoramidite monomers and thio-modifier C6 S-S phosphoramidite were purchased from Glen Research. Fmoc protected amino acids were purchased from GL Biochem. All reagents were used without further purification. Flash column chromatography was performed using Grace Davisil chromatographic silica media (40-63 µm). Reaction were monitored using Merck TLC silica gel 60 F_{254} aluminium plates. TLC were visualized by UV light, *p*-anisaldehyde stain or ninhydrin stain. NMR spectra were recorded on a Bruker Avance 300 spectrometer. HRMS analysis were performed on a Waters Q-tof Premier MS.

II. Synthetic procedure for (Gly-Gly-Gly) phosphoramidite tag



Scheme S1. Synthesis of Gly-Gly-Gly phosphoramidite tag. (i) 1) DMTrCl, pyridine, 2) piperidine, DMF, 98%; (ii) Fmoc-Gly-Gly-Gly-OH, HBTU, HoBt, DIPEA, DMF, 91%; (iii) 2-cyanoethyl N,N-diisopropylchlorophosphoramidite, DIPEA, DCM, 70%.

(2*R*,3*R*)-3-amino-4-(bis(4-methoxyphenyl)(phenyl)methoxy)butan-2-ol (S2)

Fmoc-threoninol **S1** (300 mg, 916 µmol) was dissolved in anhydrous pyridine (4.00 mL) and added with DMTr-Cl (342 mg, 1.01 mmol) in 3 portions at 10 min interval. The resulting solution was stirred under nitrogen for 5 hours. Solvent was removed under reduced pressure until half volume and diluted with ethyl acetate. The mixture was extracted with saturated $NaHCO_3$ solution twice followed by brine. The organic phase was dried over Na_2SO_4, filtered and solvent removed under reduced pressure. Anhydrous DMF (1.44 mL) was then added to dissolve the oil. Piperidine (312.1 mg, 0.36 mL, 3.665 mmol) was added slowly and the resulting solution was stirred under nitrogen for 2 h at room temperature. The reaction mixture was diluted with chloroform and extracted with saturated $NaHCO_3$ solution twice followed by brine. The organic layer was dried over Na_2SO_4, filtered and solvent removed under reduced pressure. The product was purified by flash column chromatography (0-2% MeOH/DCM) to give **S2** (366 mg, 98%) as a yellow oil. ^{1}H NMR (300 MHz; $CDCl_3$): δ 1.07 (3H, d, *J* 6.27, Threoninol CH_3), 2.60-2.68 (1H, m, threoninol Hα), 3.08 (1H,

dd, *J* 5.85 9.41 threoninol CH_2), 3.24 (1H, dd, *J* 4.17 9.41 threoninol CH_2), 3.65 (1H, quintet, threoninol Hβ), 3.77 (6H, s, 2 x OCH_3), 6.82 (4H, d, *J* 8.82, H-Ar), 7.15-7.35 (7H, m, H-Ar), 7.38-7.46 (2H, m, H-Ar). ^{13}C NMR (75 MHz, $CDCl_3$): δ 19.96, 55.26, 57.16, 65.97, 68.04, 86.20, 113.21, 126.88, 127.91, 128.16, 130.08, 136.01, 136.10, 144.90, 158.57. HRMS calc. for $C_{25}H_{30}NO_4$: 408.2175; found: 408.2174.

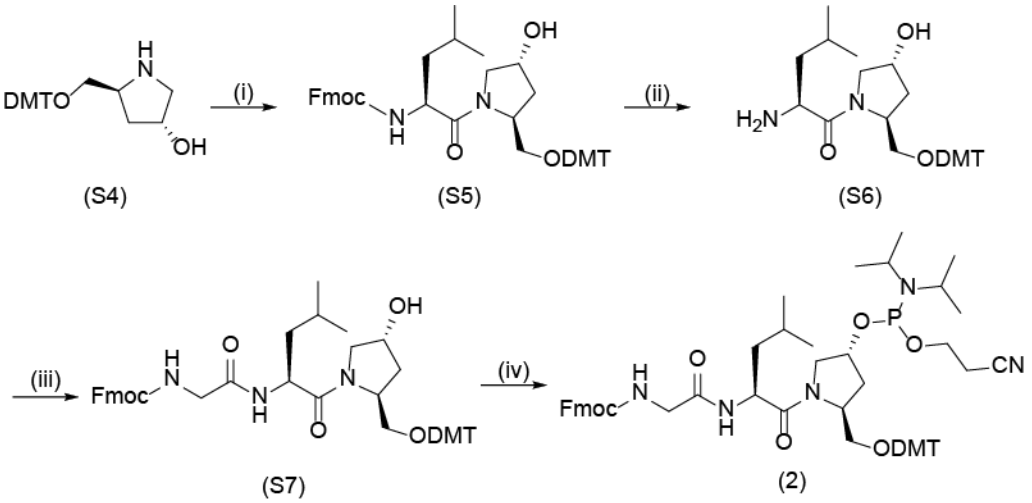
(9*H*-fluoren-9-yl)methyl ((*R*)-4-((*R*)-1-hydroxyethyl)-1,1-bis(4-methoxyphenyl)-6,9,12-trioxo-1-phenyl-2-oxa-5,8,11-triazatridecan-13-yl)carbamate (S3)

A solution of Fmoc-Gly-Gly-Gly-OH (505 mg, 1.23 mmol), DIPEA (476 mg, 0.64 mL, 3.68 mmol) , HoBt (166 mg, 1.23 mmol) and HBTU (512 mg, 1.35 mmol) dissolved in anhydrous DMF (3.5 mL) was added slowly to a solution of **S2** (500 mg, 1.23 mmol) in anhydrous DMF (3 mL). The resulting solution was stirred under nitrogen for 2 h at room temperature. The reaction mixture was poured into ice cold water and extracted twice with ethyl acetate. The combine organic layer was washed with water followed by brine. The organic layer was then dried over Na_2SO_4, filtered and solvent removed under reduced pressure. The product was purified by flash column chromatography (0-6% MeOH/DCM) to afford the product **S3** (899 mg, 92%) as a faint yellowish solid. ^1H NMR (300 MHz; MeOD): δ 1.08 (3H, d, *J* 6.38, threoninol -CH_3), 3.06-3.18 (1H, m, threoninol CH_2),3.24-3.33 (1H, m, threoninol CH_2), 3.73 (6H, s, 2 x OCH_3), 3.82 (2H, s, Gly -CH_2) 3.87-4.03 (5H, m, threoninol Hα and 2 x Gly -CH_2) 4.03-4.13 (1H, m, threoninol Hβ), 4.17 (1H, t, *J* 6.45, Fmoc -CH), 4.36 (2H, d, *J* 6.76, Fmoc -CH_2), 6.83 (4H, d, *J* 8.84, H-Ar), 7.14-7.48 (13H, m, H-Ar), 7.57-7.69 (2H, m, H-Ar), 7.79 (2H, d, *J* 7.50, H-Ar). ^{13}C NMR (75 MHz, MeOD): δ 20.33, 43.46, 43.88, 45.15, 48.29, 55.67, 56.57, 87.32, 114.06, 120.90, 126.18, 127.70, 128.15, 128.73, 128.78, 129.28, 131.24, 137.21, 137.29, 142.53, 145.16, 145.19, 146.42, 159.29, 159.99, 171.62, 172.09, 173.14. HRMS calc. for $C_{46}H_{49}N_4O_9$: 801.3500; found: 801.3503.

(9*H*-fluoren-9-yl)methyl ((4*R*)-4-((1*R*)-1-(((2-cyanoethoxy)(diisopropylamino)phosphaneyl)oxy)ethyl)-1,1-bis(4-methoxyphenyl)-6,9,12-trioxo-1-phenyl-2-oxa-5,8,11-triazatridecan-13-yl)carbamate, (1)

8 (347 mg, 433 µmol) that was suspended in anhydrous DCM (3.34 mL) was added with DIPEA (140 mg, 189 µL, 1.08 mmol). 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (123 mg, 116 µL, 520 µmol) was then added dropwise over several min and the resulting solution was then allowed to stir at room temperature under nitrogen for 30 min. The reaction mixture was diluted with DCM and extracted with saturated KCl solution. The organic layer was dried over Na_2SO_4, filtered and solvent removed under reduced pressure. The crude product was then purified by flash column chromatography (0-5% MeOH/DCM) to give **1** (307 mg, 71%) as a white foam. ^{31}P NMR (162 MHz; $CDCl_3$): δ 148.13, 148.82.

III. Synthetic procedures for (Gly-Leu) phosphoramidite tag



Scheme S2. Synthesis of Gly-Leu phosphoramidite tag. (i) Fmoc-L-Leu-OH, HBTU, HoBt, DIPEA, DMF, 85%; (ii) piperidine, DMF, 62%; (iii) Fmoc-Gly-OH, HBTU, DIPEA, DMF, 61%; (iv) 2-cyanoethyl N,N-diisopropylchlorophosphoramidite, DIPEA, DCM, 72%.

(3*R*,5*S*)-5-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)pyrrolidin-3-ol (S4)

Compound **(S4)** was synthesized according to procedure reported by Prakash *et al*.[1]

(9*H*-fluoren-9-yl)methyl ((*S*)-1-((2*S*,4*R*)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-4-hydroxypyrrolidin-1-yl)-4-methyl-1-oxopentan-2-yl)carbamate (S5)

A solution of **S4** (200 mg, 477 µmol) in anhydrous DMF (1 mL) was added to a stirring solution of Fmoc-L-Leu-OH (168 mg, 477 µmol), DIPEA (185 mg, 0.25 mL, 1.43 mmol), HBTU (199 mg, 524 µmol) and HoBt (64 mg, 477 µmol) in anhydrous DMF (1.8 mL). The resulting mixture was stirred under nitrogen for 1 h at room temperature. The reaction mixture was poured into ice cold water and extracted twice with ethyl acetate. The combine organic layer was washed with water followed by brine and dried over Na_2SO_4. Solvent was removed under reduced pressure and the crude product was purified by silica flash column chromatography (30-50% EA/Hex with 1% TEA) to give **S5** (305 mg, 85%) as a white foam. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.81-1.05 (6H, m, Leu 2 x CH_3), 1.49-1.60 (2H, m, Leu Hβ), 1.70-1.80 (1H, m, Leu Hγ), 1.91-2.23 (2H, m, prolinol H3'/H3"), 3.07-3.16 (1H, m, prolinol H1'), 3.45-3.60 (1H, m, prolinol H1"), 3.65-3.73 (1H, m, prolinol H5'), 3.78 (6H, s, 2 x OCH_3), 3.88-4.10 (1H, m, prolinol H5"), 4.14-4.25 (1H, m, Fmoc CH), 4.27-4.40 (2H, m, Fmoc CH_2), 4.40-4.67 (3H, m, prolinol H2, H4, Leu Hα), 5.52 (1H, d, *J* 8.52, NH), 6.64-6.88 (4H, m, H-Ar), 7.17-7.43 (13H, m, H-Ar), 7.52-7.63 (2H, m, H-Ar), 7.76 (2H, d, *J* 7.35, H-Ar). ^{13}C NMR (75 MHz; $CDCl_3$): δ 8.53, 22.11, 23.26, 24.65, 36.45, 42.06, 42.51, 45.63, 47.16, 50.87, 55.20, 55.73, 56.02, 59.97, 63.01, 67.16, 70.49, 86.01, 113.08, 119.95, 125.17, 126.79, 127.08, 127.69, 127.77, 128.09, 129.14, 130.01, 135.95, 136.13, 141.29, 143.74, 143.78, 143.91, 144.96, 158.46, 171.60 . HRMS calc. for $C_{47}H_{50}N_2O_7$: 755.3696; found: 755.3687

(*S*)-2-amino-1-((2*S*,4*R*)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-4-hydroxypyrrolidin-1-yl)-4-methylpentan-1-one (S6)

To a solution of **S5** (369 mg, 489 µmol) in anhydrous DMF (1.7 mL) was added piperidine (167 mg, 193 µL, 1.96 mmol). The resulting solution was stirred at room temperature under nitrogen for 2 h. The reaction mixture was diluted with chloroform and extracted with saturated sodium bicarbonate solution twice followed by brine. The organic layer was dried over Na_2SO_4, filtered and solvent removed under reduced pressure. The crude product was then purified by silica flash column chromatography (0-5% MeOH/DCM with 1% TEA) to give **S6** (184 mg, 70%) as a white foam. ^{1}H NMR (300 MHz; $CDCl_3$): δ 0.80-0.97 (6H, m, Leu 2 x CH_3), 1.41-1.61 (2H, m, Leu Hβ), 1.67-1.87 (1H, m, Leu Hγ), 1.89-2.04 (1H, m, prolinol H3'), 2.05-2.20 (1H, m, prolinol H3"), 2.96-3.15 (1H, m, prolinol H1'), 3.46-3.64 (2H, m, prolinol H1", Leu Hα), 3.67-3.82 (8H, m, 2 x OCH_3, H5', H5",), 3.82-3.96 (2H, m, NH_2), 4.39-4.48 (1H, m, prolinol H2), 4.51 (1H, s, prolinol H4), 6.74-6.86 (4H, m, H-Ar), 7.13-7.30 (7H, m, H-Ar), 7.30-7.38 (2H, d, *J* 7.68, H-Ar). ^{13}C NMR (75 MHz, $CDCl_3$): δ 22.08, 23.29, 24.67, 36.10, 43.79, 44.76, 50.79, 55.22, 55.28, 55.84, 56.16, 62.87, 70.42, 85.94, 113.10, 126.78, 127.78, 128.11, 130.02, 135.98, 136.19, 145.00, 158.46. HRMS calc. for $C_{32}H_{40}N_2O_5$: 533.3015; found: 533.3016.

(9*H*-fluoren-9-yl)methyl (2-(((*S*)-1-((2*S*,4*R*)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-4-hydroxypyrrolidin-1-yl)-4-methyl-1-oxopentan-2-yl)amino)-2-oxoethyl)carbamate (S7)

To a stirring solution of **S6** (294 mg, 551 µmol) in anhydrous DMF (1.6 mL) was added a solution of Fmoc-Gly-OH (164 mg, 551 µmol), DIPEA (285 mg, 0.38 mL, 2.20 mmol) and HBTU (230 mg, 606 µmol) in anhydrous DMF (1.5 mL). The resulting mixture was stirred under nitrogen for 1h at room temperature. The reaction mixture was poured into ice cold water and extracted twice with ethyl acetate. Combined organic layer was washed with water followed by brine. The organic layer was dried over Na_2SO_4, filtered and solvent removed under reduced pressure. The crude product was purified by silica flash column chromatography (50-100% EA/Hex with 1% TEA) to give **S7** (322 mg, 72%) as a yellow foam. ^{1}H NMR (300 MHz; $CDCl_3$): δ 0.78-1.02 (6H, m, Leu 2 x CH_3), 1.46-1.73 (3H, m, Leu Hβ, Leu Hγ), 1.84-2.24 (2H, m, prolinol H3'/H3"), 2.93-3.11 (1H, m, prolinol H1'), 3.51-3.67 (2H, m, prolinol H1", prolinol H5'), 3.60-3.69 (1H, m, prolinol H5"), 3.71 (6H, s, 2 x OCH_3), 3.79-3.91 (2H, m, Gly Hα), 3.96-4.08 (1H, m, prolinol H5"), 4.09-4.24 (1H, m, Fmoc CH), 4.33 (2H, d, *J* 6.54, Fmoc CH_2), 4.52-4.40 (2H, m, prolinol H2', prolinol H4'), 4.72-4.92 (1H, m, Leu Hα), 6.18 (1H, s, Gly NH), 6.75 (4H, d, *J* 8.49, H-Ar) 7.09-7.28 (10H, m, H-Ar and Leu NH), 7.29-7.43 (4H, m, H-Ar), 7.56 (2H, d, *J* 7.17, H-Ar), 7.72 (2H, d, *J* 7.53, H-Ar). ^{13}C NMR (75 MHz, $CDCl_3$): δ 22.19, 23.12, 24.66, 35.98, 42.05, 44.15, 47.06, 49.49, 55.13, 56.04, 56.22, 62.60, 67.20, 70.36, 85.85, 113.02, 119.89, 125.15, 126.73, 127.09, 127.67, 127.72, 128.03, 129.95, 129.99, 135.85, 136.13, 141.22, 143.81, 144.93, 158.40, 169.50, 171.43. HRMS calc. for $C_{49}H_{53}N_3O_8$: 812.3909; found: 812.3911.

(2*S*,4*R*)-1-((((9*H*-fluoren-9-yl)methoxy)carbonyl)glycyl-L-leucyl)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-4-(((2-cyanoethoxy)(diisopropylamino)phosphaneyl)oxy)pyrrolidin-3-ylium (2)

DIPEA (119.1 mg, 0.16 mL, 921.2 µmol) was added to a stirring solution of **S7** (299 mg, 369 µmol) in anhydrous DCM (2.85 mL) under nitrogen. N,N-diisopropylchlorophosphoramidite (105 mg, 99 µL, 442 µmol) was then added dropwise over several min to the reaction mixture and the resulting solution was stirred at room temperature under nitrogen for 30 min. The reaction mixture was diluted with anhydrous DCM and extracted with saturated KCl solution. The organic layer was dried over Na_2SO_4, filtered and solvent removed under reduced pressure. The crude product was purified by silica flash column chromatography (50-65% EA/Hex with 1% TEA) to give **2** (333 mg, 89%) as a white foam. ^{31}P NMR (162 MHz; $CDCl_3$): δ 147.45, 147.96, 148.16, 148.57.

IV. Oligonucleotide synthesis

All DNA oligonucleotides were chemically synthesized on the Applied Biosystems Inc. (ABI) 394 DNA/RNA synthesizer using standard phosphoramidite chemistry. A solution of phosphoramidite **1** in anhydrous DCM (0.12 M) or **2** in anhydrous ACN (0.12M) was used for the coupling, with an extended coupling time of 10 min. Prepacked 1 µmol functionalized controlled pore glass column (*Glen Research*) was used for 5' or internal tagged oligonucleotide and 1 µmol UnyLinker (*Chemgenes*) was used for 3' tagged oligonucleotide. The coupling efficiency of phosphoramidite **1** and **2** was similar to that of standard DNA phosphoramidites. Oligonucleotides were cleaved from the support and deprotected with concentrated aq. NH_3 at 55 °C for 16 h. The oligonucleotide was then purified by reverse phase HPLC (250 x 10 mm, Waters, XBridge BEH C18) with a gradient 5% to 35% ACN over 8 min followed by 35% to 100% ACN in 12 min using ACN/TEAA mobile phase mixture. DMT was removed using 2% or 3% TFA solution, for PO or PS oligonucleotide respectively, on a PolyPak cartridge (*Glen Research*). The purified oligonucleotide was then desalted using Glen Pak 2.5 desalting column (*Glen Research*). The desalted oligonucleotide was then lyophilized, dissolved in deionized water and quantified by UV absorbance at 260 nm. Isolated yield of the modified oligonucleotides was comparable to that of unmodified oligonucleotides having similar length.

V. Peptides preparation

All peptides were purchased from *Chempeptide Limited*. The peptide was dissolved in DI water to make up a stock solution of 5 mM for *Pep1* and *Pep3*, 9.95 mM for *Pep2* and 0.72 mM for *Pep4*.

VI. Sortase A expression

The plasmid containing gene fragment encoding the Sortase A mutant, Sortase-7M, was purchased from *Addgene* and transformed into Rosetta T1R cells. The cells were grown in LB containing antibiotics to OD_{600} 0.6 – 0.8, and induced with addition of 0.5 mM IPTG at 18 °C overnight. Cells were harvested by centrifugation and the cell pellet resuspended in lysis buffer containing 50mM Tris-HCl, pH7.5, 150mM NaCl, 10% (v/v) glycerol. Cells were lysed by sonication, and the crude lysate clarified by centrifugation at 21,000 rpm at 4 °C for 1 hour. The soluble protein was purified by Ni-affinity chromatography (HisTrap, GE Healthcare) followed by size exclusion chromatography (GE Healthcare) pre-equilibrated with 50mM Tris-HCl, pH7.5, 150mM NaCl, 10% (v/v) glycerol. The sortase-7M protein was concentrated using 10 kDa MWCO centricon (Millipore), concentration determined using bicinchoninic acid protein assay (Pierce) and stored at -80 °C.

VII. OaAEP1b expression

A gene fragment encoding for an N-terminal histidine-tag with human ubiquitin (1 – 76 aa), and OaAEP1b (24 – 474 aa) with C247A mutation was synthesized (IDT), cloned into pET-28b vector (Novagen) and transformed into Rosetta T1R cells. The cells were expressed and purified as described by Yang and coworkers[2] with slight modification to the procedures. Briefly, cells were grown in LB containing antibiotics to OD_{600} 0.6 – 0.8, and induced with addition of 0.5 mM IPTG, shaking at 220 rpm at 16 °C overnight. Cells were harvested by centrifugation at 10,000 rpm at 4 °C for 10 mins and the cell pellet resuspended in lysis buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 % (v/v) glycerol and 0.05 % (v/v) Nonidet P-40. Cells were lysed by sonication, and the crude lysate clarified by centrifugation at 21,000 rpm at 4 °C for 20 min. The soluble protein was purified by Ni-affinity chromatography (HisTrap, GE Healthcare) and anion exchange chromatography (HiTrapQ, GE Healthcare). The purified protein fractions were combined in buffer containing 20 mM HEPES pH 7.0, 2 mM DTT, 10 % (v/v) glycerol. The OaAEP1b protein was activated by the removal of the cap domain of OaAEP1b with the addition of glacial acetic acid to achieve pH 4.0 in the sample. The protein was left at room temperature overnight, and the completeness of the reaction monitored by SDS-PAGE. The activated OaAEP1b protein was concentrated using 10 kDa MWCO centricon (Millipore) and the protein concentration determined using bicinchoninic acid protein assay (Pierce), and stored at -80 °C.

VIII. Cloning and expression of cyan fluorescent protein (CFP).

Cyan fluorescent protein (CFP_{PAL})

MGSSHHHHHHSQDPMVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLP
VPWPTLVTTLTWGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRI
ELKGIDFKEDGNILGHKLEYNYISHNVYITADKQKNGIKANFKIRHNIEDGSVQLADHYQQNTPIGDGPVLL
PDNHYLSTQSKLSKDPNEKRDHMVLLEFVTAAGITLGMDELYKNGL

A gene fragment encoding CFP_{PAL} was cloned into pETDuet-1 vector (Novagen) and transformed into Rosetta T1R cells. The cells were grown in LB containing antibiotics to OD_{600} 0.6 – 0.8, and induced with addition of 0.5 mM IPTG, shaking at 220 rpm at 18 °C overnight. Cells were harvested by centrifugation at 10,000 rpm at 4 °C for 7 mins and the cell pellet resuspended in lysis buffer containing 20mM Kpi pH 7.0, 500 mM KCl, 2 mM DTT, 10% (w/v) glycerol, 20mM imidazole. Cells were lysed by sonication, and the crude lysate clarified by centrifugation at 21,000 rpm at 4 °C for 1 hour. The crude lysate was filtered before loaded into the Ni-NTA column (Qiagen) for affinity purification. The purified protein fractions were combined and concentrated in buffer containing 20 mM Kpi pH 7.0, 150mM KCl. using 10 kDa MWCO centricon (Millipore).

Cyan fluorescent protein (CFP_{SORT})

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTLTWGVQ
CFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGH
KLEYNYISHNVYITADKQKNGIKANFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSKLSKDP
NEKRDHMVLLEFVTAAGITLGMDELYKLPETGLEHHHHHH

A gene fragment encoding CFP_{SORT} was cloned into pET-28b vector (Novagen) and transformed into Rosetta T1R cells. The cells were grown in LB containing antibiotics to OD_{600} 0.6 – 0.8, and induced with addition of 0.5 mM IPTG, shaking at 220 rpm at 18 °C overnight. Cells were harvested by centrifugation at 10,000 rpm at 4 °C for 7 mins and the cell pellet resuspended in lysis buffer containing 20mM Kpi pH 7.0, 500 mM KCl, 2 mM DTT, 10% (v/v) glycerol, 20mM imidazole. Cells were lysed by sonication, and the crude lysate clarified by centrifugation at 21,000 rpm at 4 °C for 1 hour. The crude lysate was filtered before loaded into the Ni-NTA column (Qiagen) for affinity purification. The purified protein fractions were combined and concentrated in buffer containing 50 mM Tris HCl, pH 7.5, 150mM NaCl, using 10 kDa MWCO centricon (Millipore).

IX. Ligation reaction with peptides

The ligation reactions using *Oa*AEP1b were performed in 20 µL reaction mixtures containing 20 mM phosphate buffer, *Oa*AEP1b ligase (0.01 to 0.3 eq.), peptide substrate (200 µM) and oligonucleotide substrate (500 uM to 1 mM) at 37°C unless otherwise stated.
The ligation reactions using Sortase A were performed in 20 µL reaction mixtures containing 50 mM Tris HCl, 150mM NaCl (pH 7.5) buffer, Sortase A ligase (1 to 3 eq), peptide substrate (200 µM) and oligonucleotide substrate (1 mM) at 4°C unless otherwise stated.

The conjugated chimeric product was purified using a reversed-phase analytical column (250 x 4.6 mm, YMC-Triart C18) with a linear gradient from 5% to 40% acetonitrile/TEAA over 35 min on a Nexera UHPLC system (*Shimadzu*). A linear fit of *ODNref* peak area at 260 nm against concentration (10, 50, 100, 150 and 200 µM) was used as the calibration plot. The peak at 260 nm corresponding to the conjugated chimeric product was integrated and the yield was then derived from the linear calibration plot. The identity of the HPLC peaks were verified by MALDI-TOF MS (JEOL JMS-S3000) analysis.

X. Ligation reaction with CFP

The ligation reactions using OaAEP1b were performed in 5 µL reaction mixtures containing 20 mM phosphate buffer pH 7.4, protein substrate (100 µM), oligonucleotide substrate (1 mM) and OaAEP1b ligase (0.02 eq) at 37°C for 1 h. The reaction was monitored by SDS-PAGE.
The ligation reactions using Sortase A were performed in 5 µL reaction mixtures containing 50 mM Tris HCl, 150mM NaCl (pH 7.5) buffer, protein substrate (50 µM), oligonucleotide substrate (500 µM) and Sortase A ligase (1 eq) at 4°C for 16 h. The reaction was monitored by SDS-PAGE.

Table S1. All oligonucleotide sequences used in this study.

Oligo[a]	Sequence (5' → 3')[b],[c],[d]	Remarks
ODNref	CTATTTGGATGTCAGC	Native
ODN1	/Tag$_{SORT}$/CTATTTGGATGTCAGC	5′-Gly-Gly-Gly tagged
ODN2	/Tag$_{SORT}$/*C*T*A*T*T*T*G*G*A*T*G*T*C*A*G*C	5′-Gly-Gly-Gly tagged PS backbone
ODN3	CTATTTGGATGTCAGC/Tag$_{SORT}$/	3′-Gly-Gly-Gly tagged
ODN4	CTATTTGG/Tag$_{SORT}$/ATGTCAGC	Internal Gly-Gly-Gly tagged
ODN S1	/Tag$_{SORT}$/TTTTTTTTTT	5′-Gly-Gly-Gly tagged poly dT
ODN S2	/Tag$_{SORT}$/GCATTCTAATAGCAGC	5′-Gly-Gly-Gly tagged
ODN S3	/Tag$_{SORT}$/TTGGGTGGGTGGGTGGGT	5′-Gly-Gly-Gly tagged G4
ODN S4	TTGGGTGGG/Tag$_{SORT}$/GGGTGGGT	Internal loop Gly-Gly-Gly tagged G4
ODN5	/Tag$_{PAL}$/CTATTTGGATGTCAGC	5′-Gly-Leu tagged
ODN6	/Tag$_{PAL}$/C*T*A*T*T*T*G*G*A*T*G*T*C*A*G*C	5′-Gly-Leu tagged PS backbone
ODN7	/Tag$_{PAL}$/C*T*A*T*T*T*G*G*A*T*G*T*C*A*G*C	5′-Gly-Leu tagged PS LNA gapmer
ODN8	CTATTTGGATGTCAGC/Tag$_{PAL}$/	3′-Gly-Leu tagged
ODN9	CTATTTGG/Tag$_{PAL}$/ATGTCAGC	Internal Gly-Leu tagged
ODN S5	/Tag$_{PAL}$/*C*T*A*T*T*T*G*G*A*T*G*T*C*A*G*C	5′-Gly-Leu tagged PS backbone
ODN S6	/Tag$_{PAL}$/TTTTTTTTTT	5′-Gly-Leu tagged poly dT
ODN S7	GCATTCTAATAGCAGC/Tag$_{PAL}$/	3′-Gly-Leu tagged
ODN S8	/Tag$_{PAL}$/SS/CTATTTGGATGTCAGC	5′-Gly-Leu tagged disulfide containing oligo
ODN S9	TTGGGTGGG/Tag$_{PAL}$/GGGTGGGT	Internal loop Gly-Leu tagged G4

[a] *ODN1–4* and ODN S1-S4 are labeled with (Gly-Gly-Gly) tag, while *ODN5–8 and ODN S5-S9* are labeled with (Gly-Leu) tag. [b] Ligation tags for sortase A (Gly-Gly-Gly) and *Oa*AEP1b (Gly-Leu) are represented as /Tag$_{SORT}$/ and /Tag$_{PAL}$/, respectively. [c] Phosphorothioate-modified internucleotide linkages are marked with asterisks (*). [d] /SS/ represents C_6-disulfide-C_6 modifier.[3]

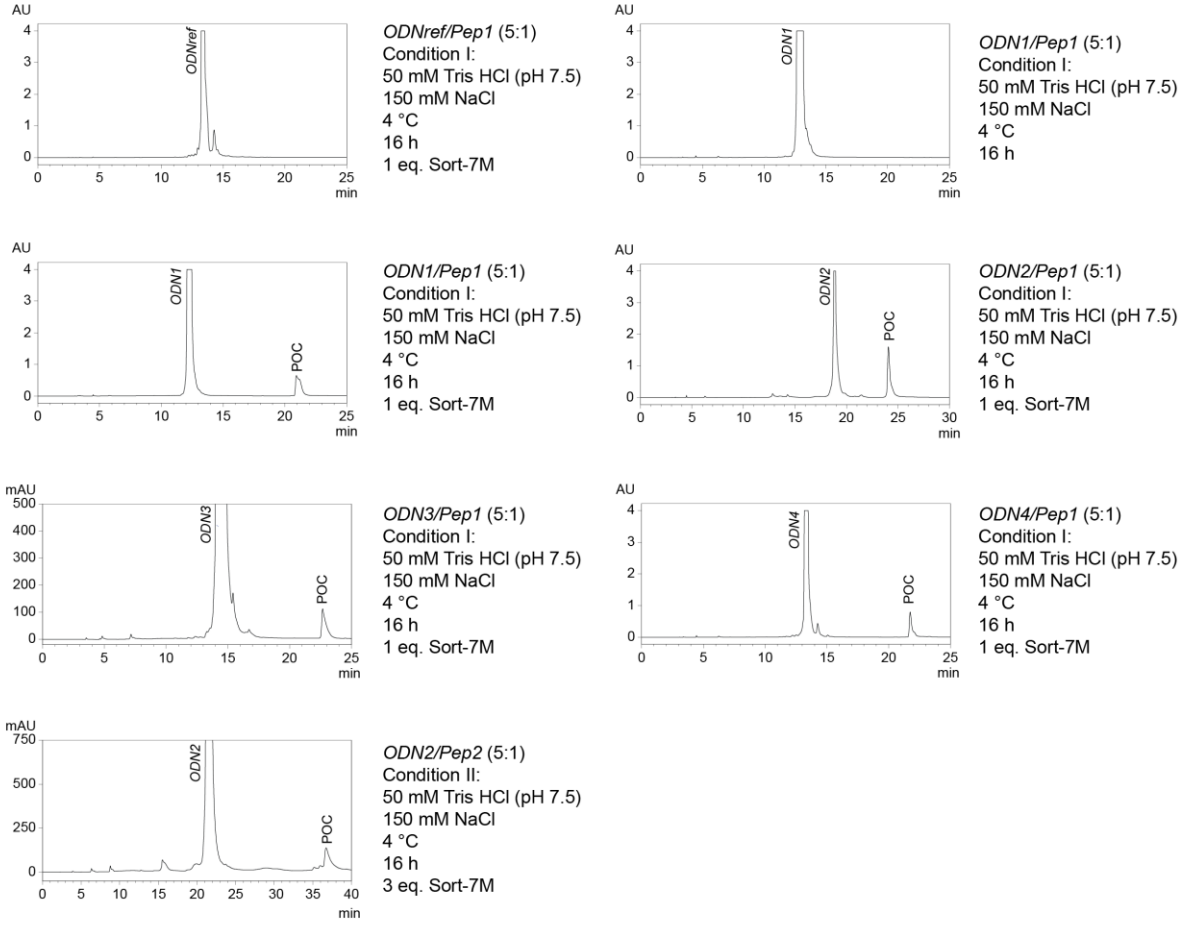


Figure S1. HPLC traces for POC ligation reaction of peptides using sortase-7M.

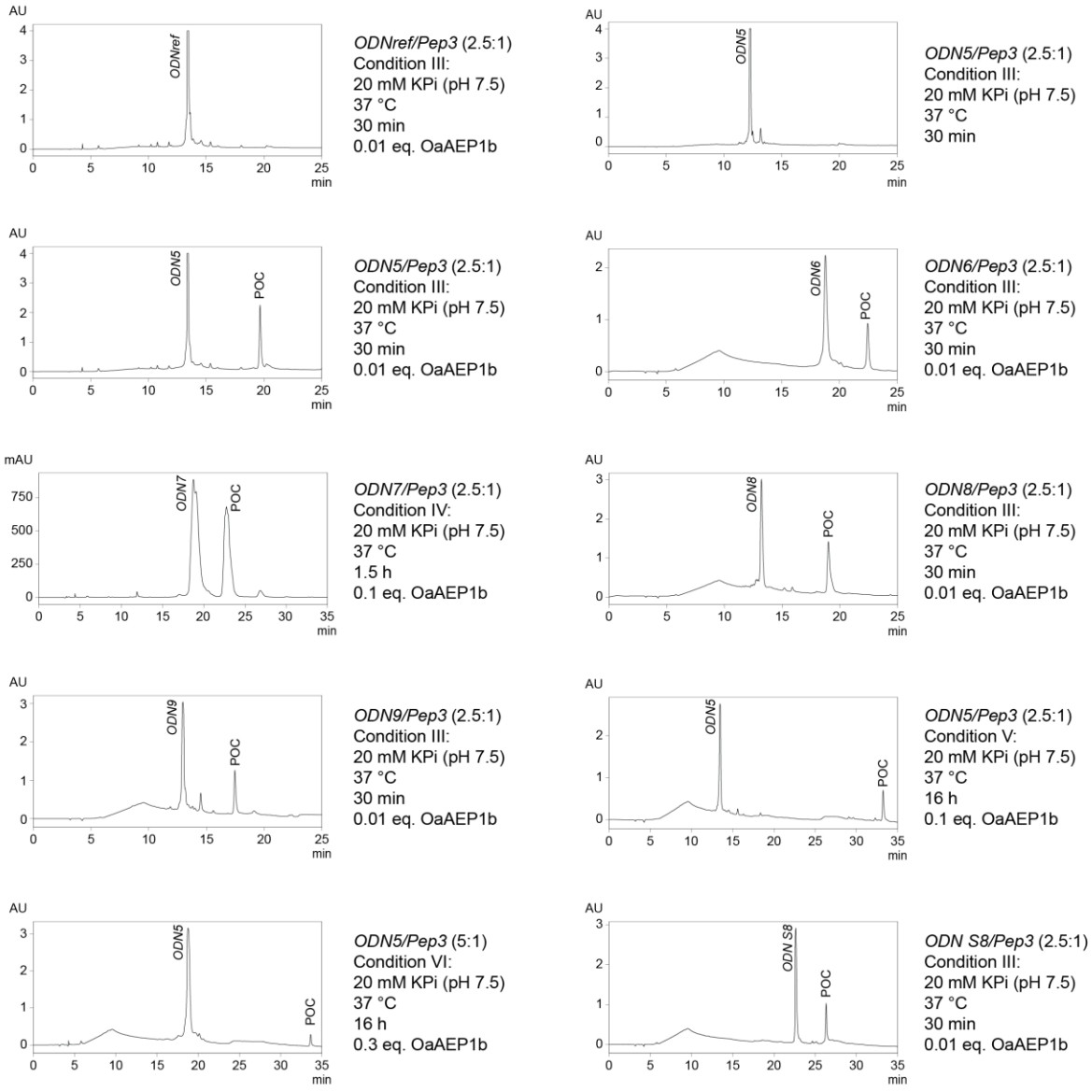


Figure S2. HPLC trace for POC reaction of peptides using *Oa*AEP1b.

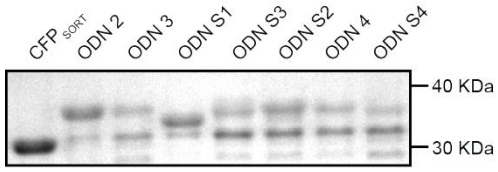


Figure S3. SDS-PAGE of ligation reaction between CFP_{SORT} protein substrate with different oligonucleotide substrate (10 eq) using sortase-7M ligase (1 eq) at 4°C for 16 h.

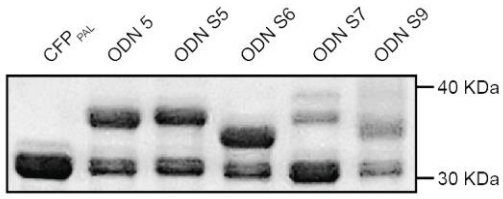


Figure S4. SDS-PAGE of ligation reaction between CFP_{PAL} protein substrate with different oligonucleotide substrate (10 eq) using *Oa*AEP1b (0.1 eq) at 37°C for 1 h.

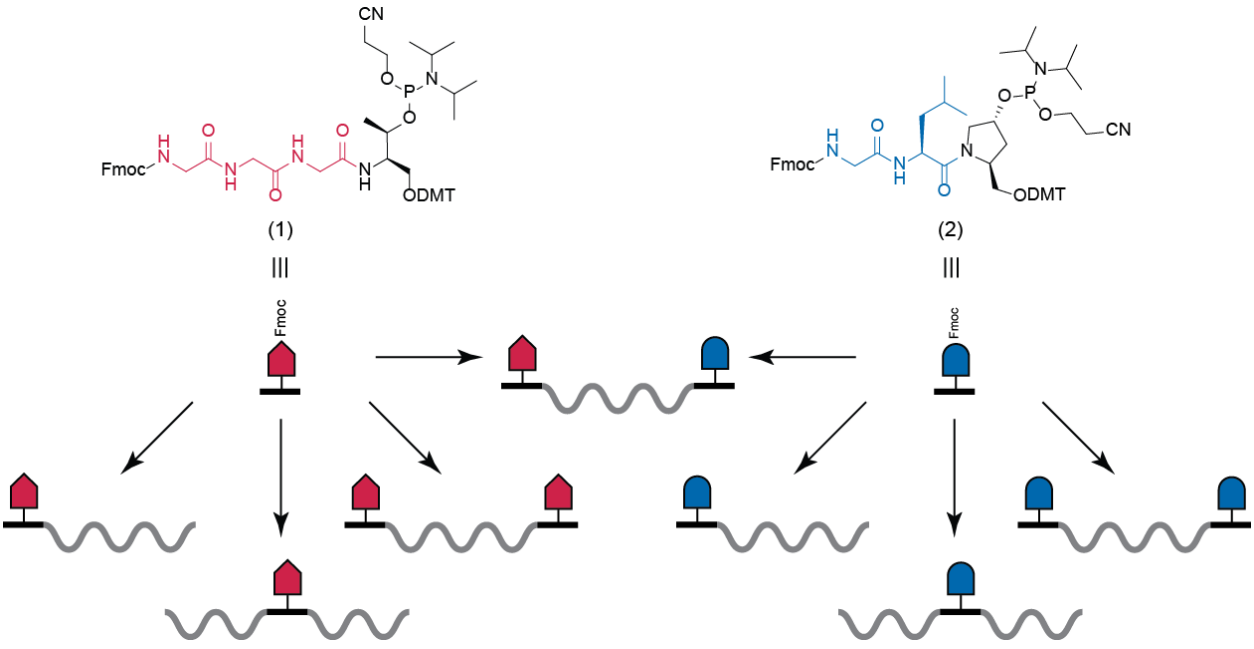


Figure S5. Both phosphoramidite tag **1** and **2** can be incorporated at either terminal or internal positions. Incorporation of multiple or a mixture of phosphoramidite tag is also possible.

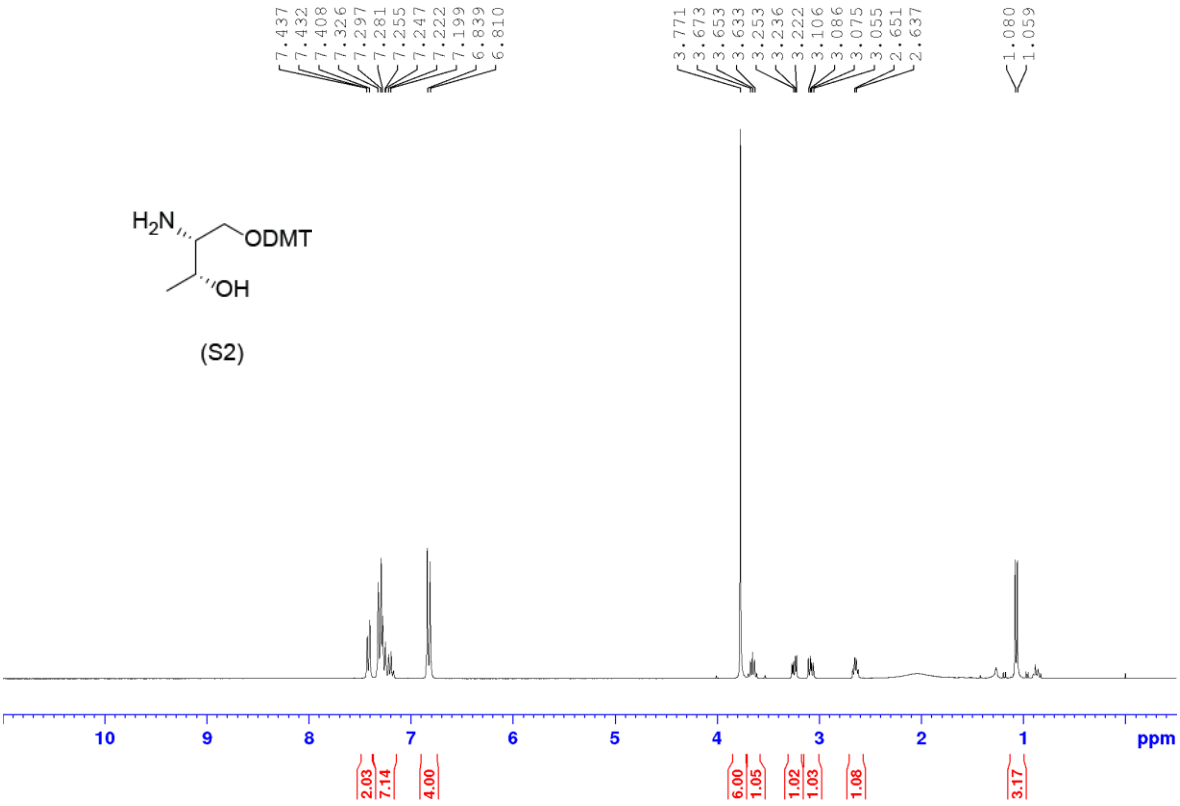


Figure S6. ^{1}H NMR spectrum of compound S2.

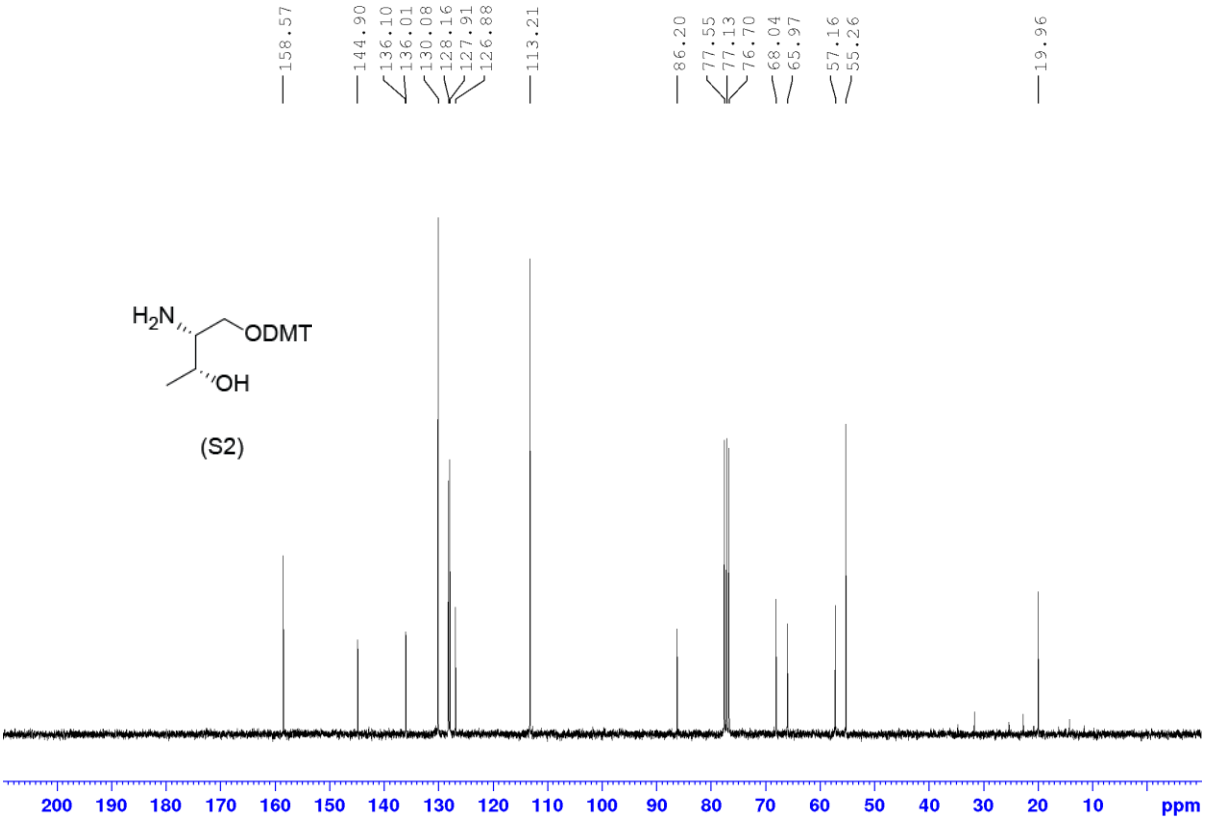


Figure S7. ^{13}C NMR spectrum of compound S2.

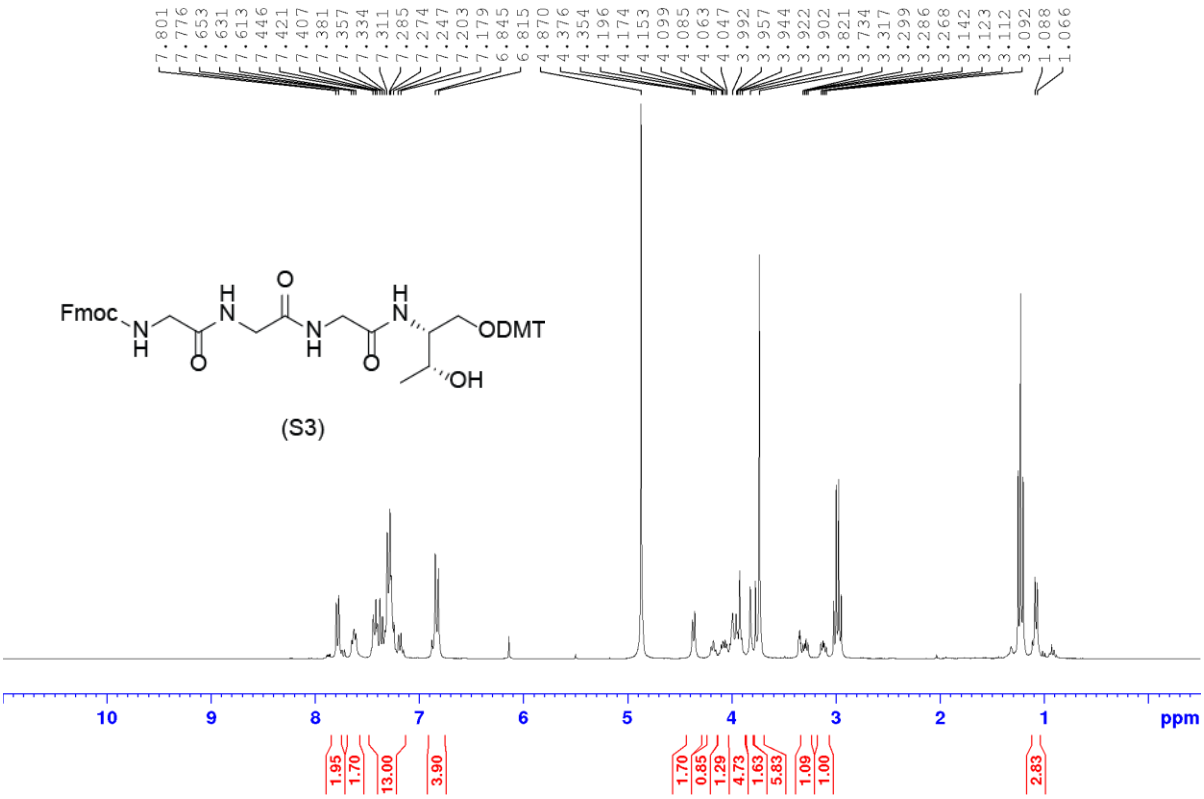


Figure S8. ^{1}H NMR spectrum of compound S3.

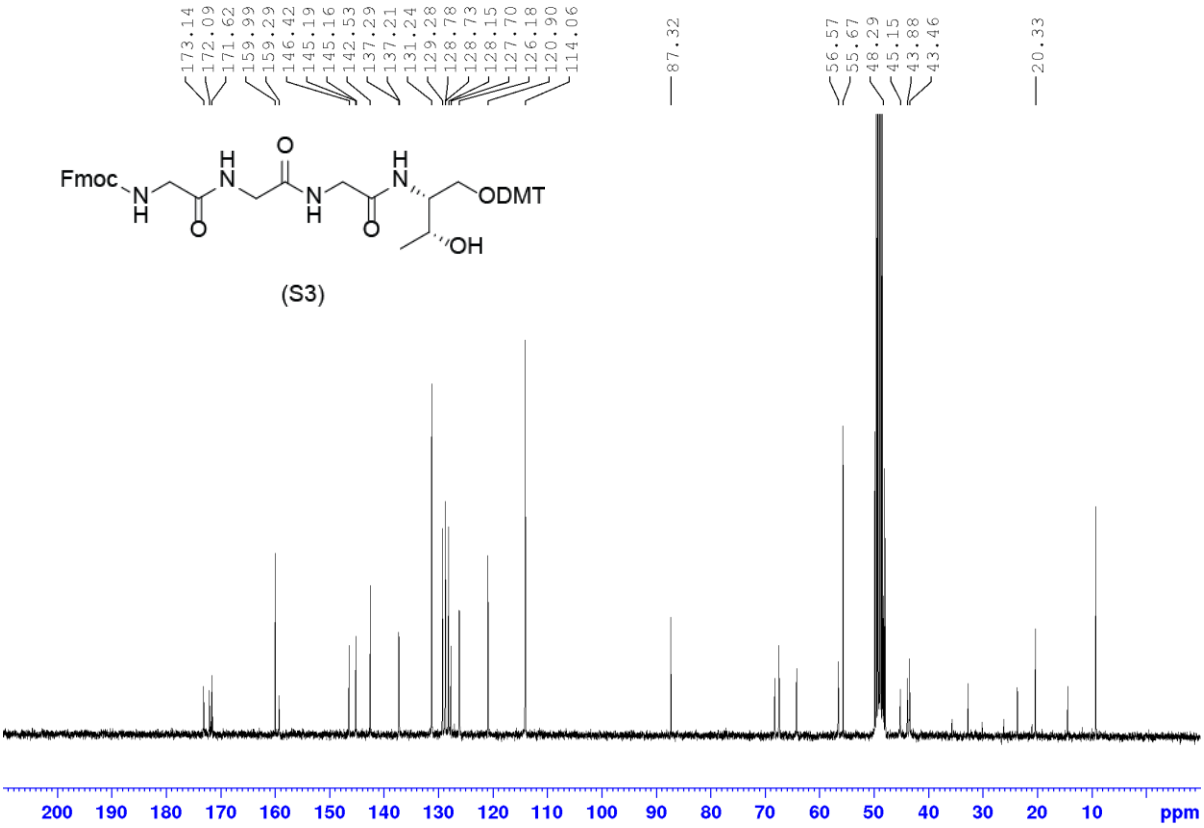


Figure S9. ^{13}C NMR spectrum of compound S3.

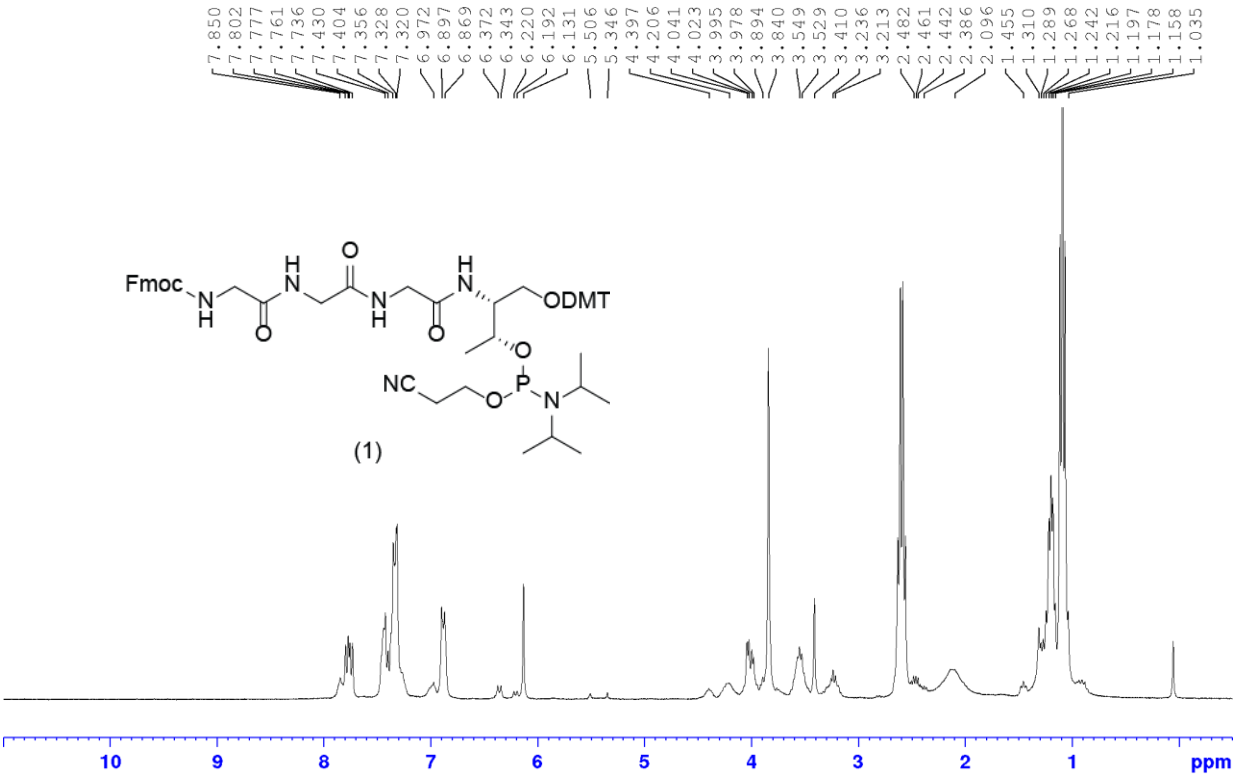


Figure S10. ^{1}H NMR spectrum of compound 1.

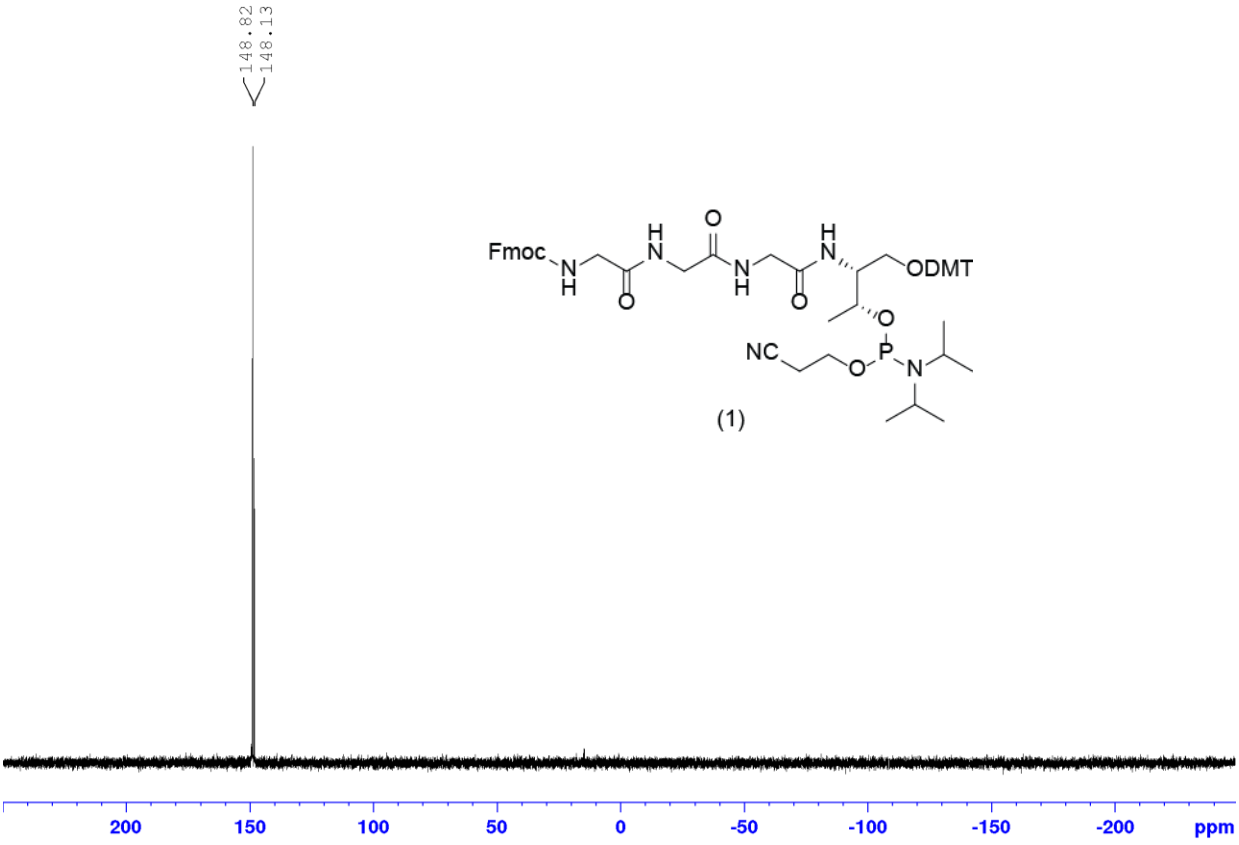


Figure S11. $^{31}P\{^{1}H\}$ NMR spectrum of compound 1.

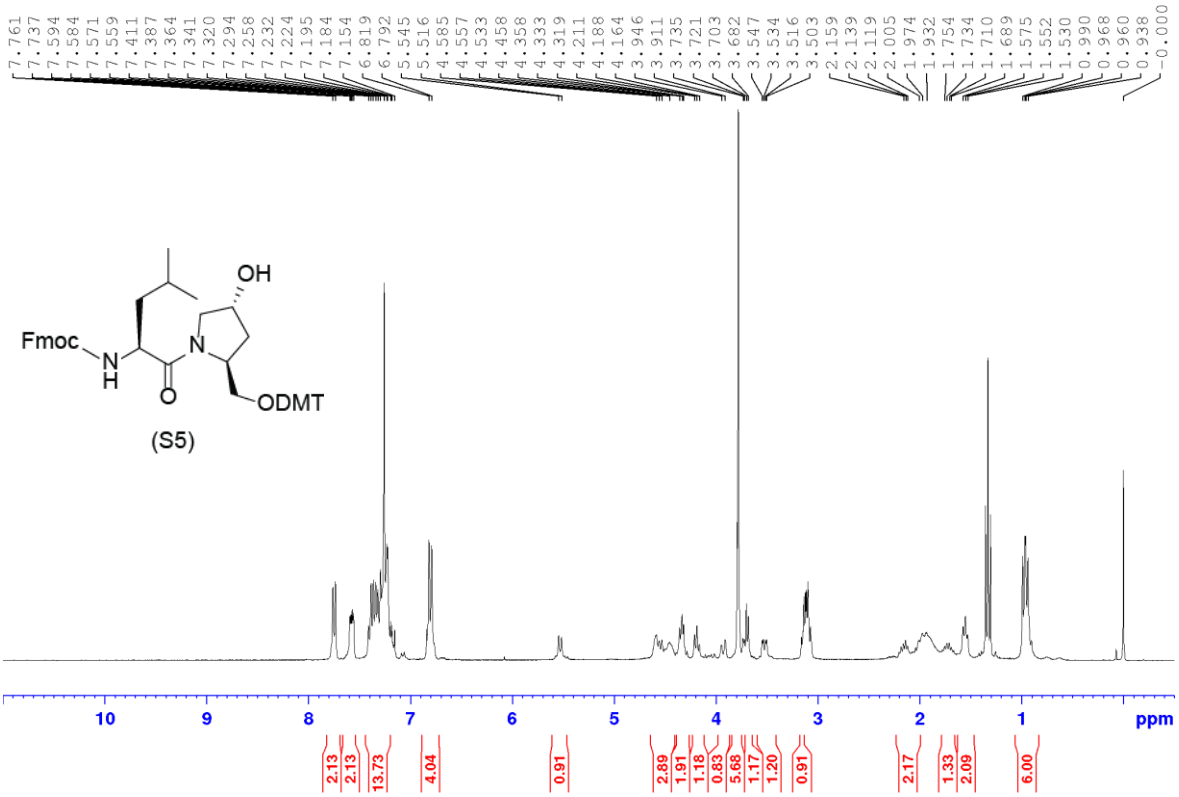


Figure S12. ^{1}H NMR spectrum of compound S5.

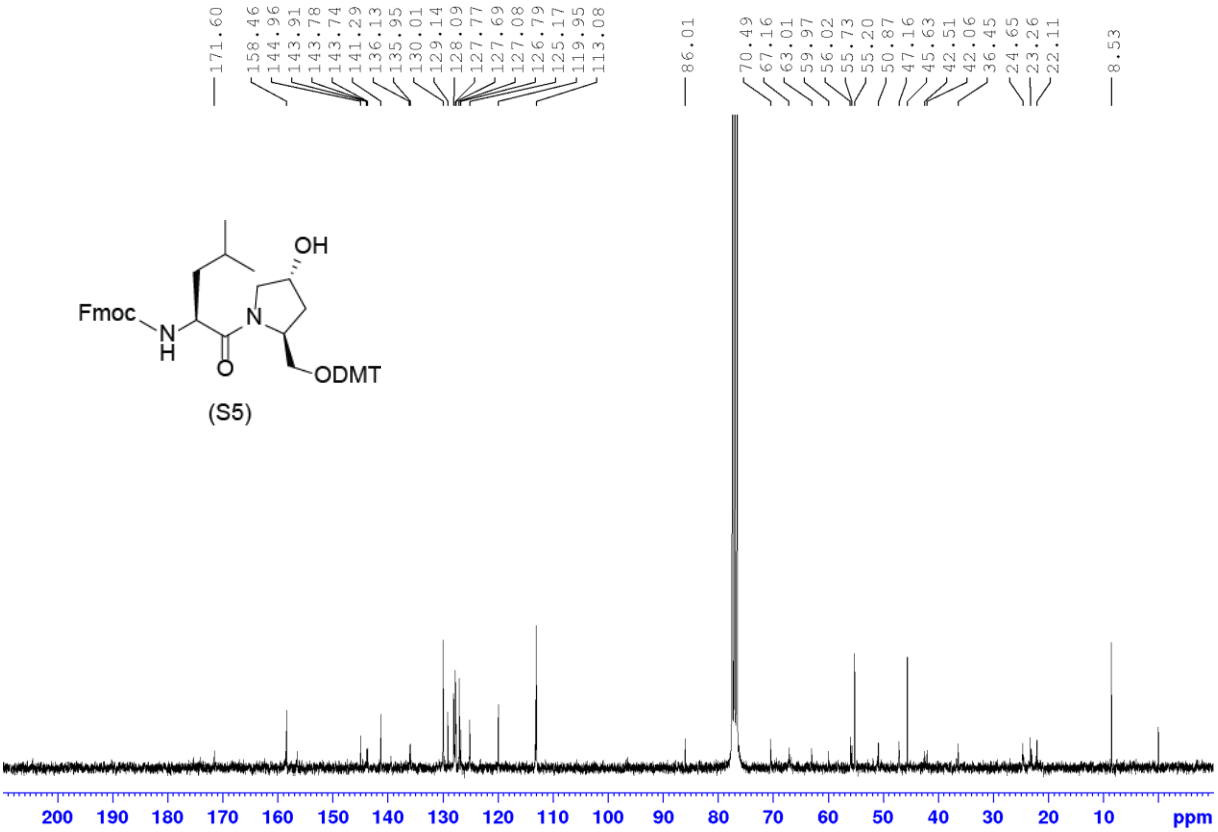


Figure S13. ^{13}C NMR spectrum of compound S5.

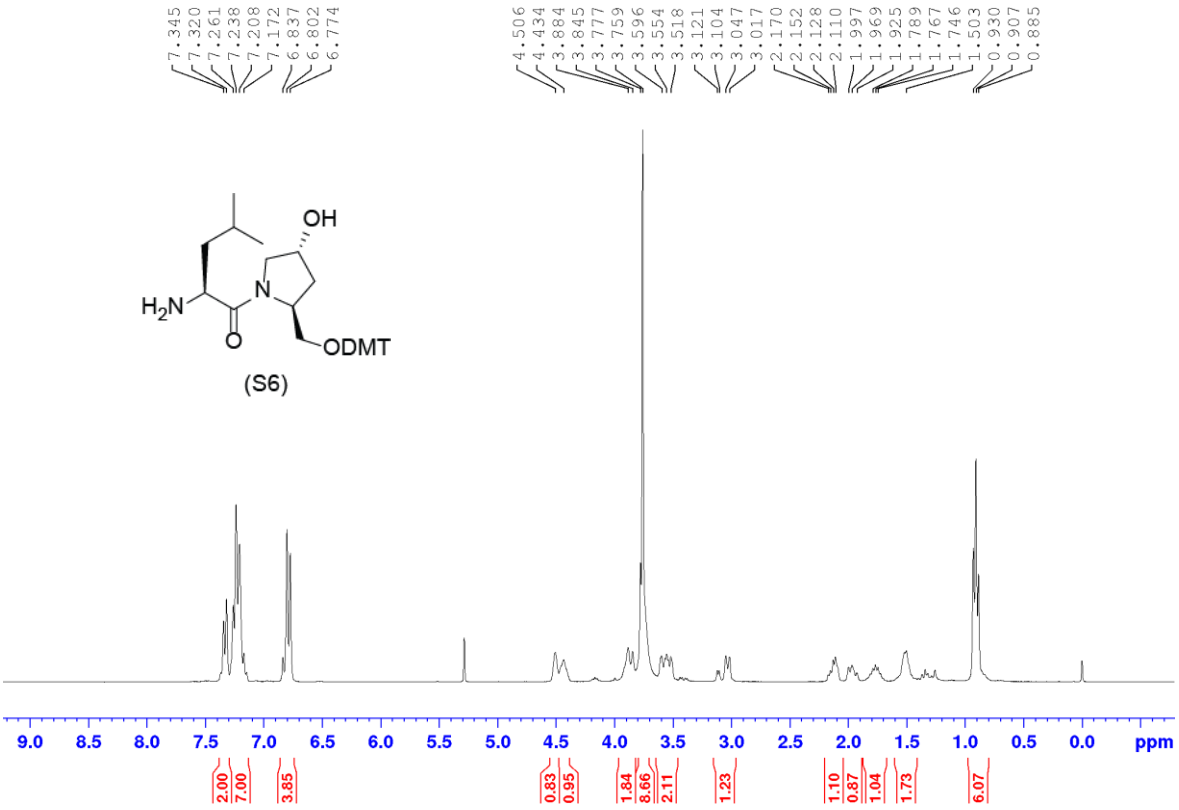


Figure S14. ^{1}H NMR spectrum of compound S6.

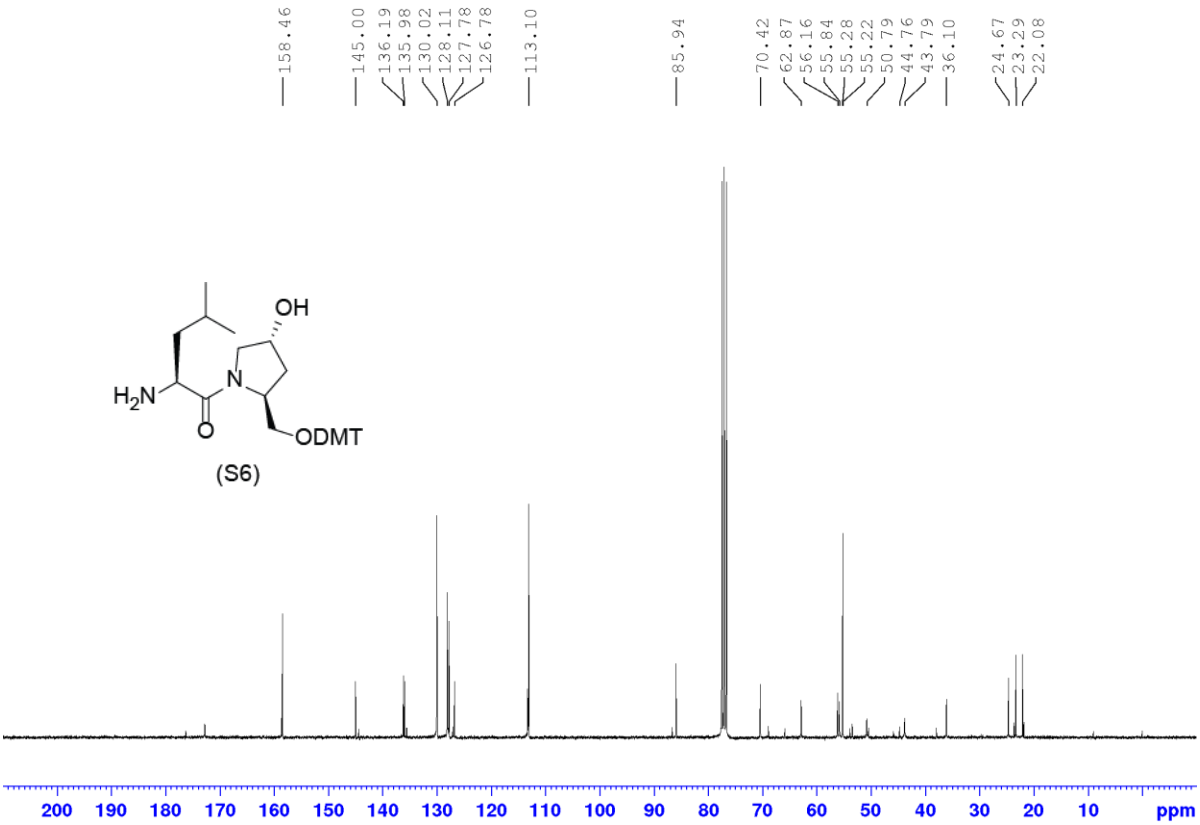


Figure S15. ^{13}C NMR spectrum of compound S6.

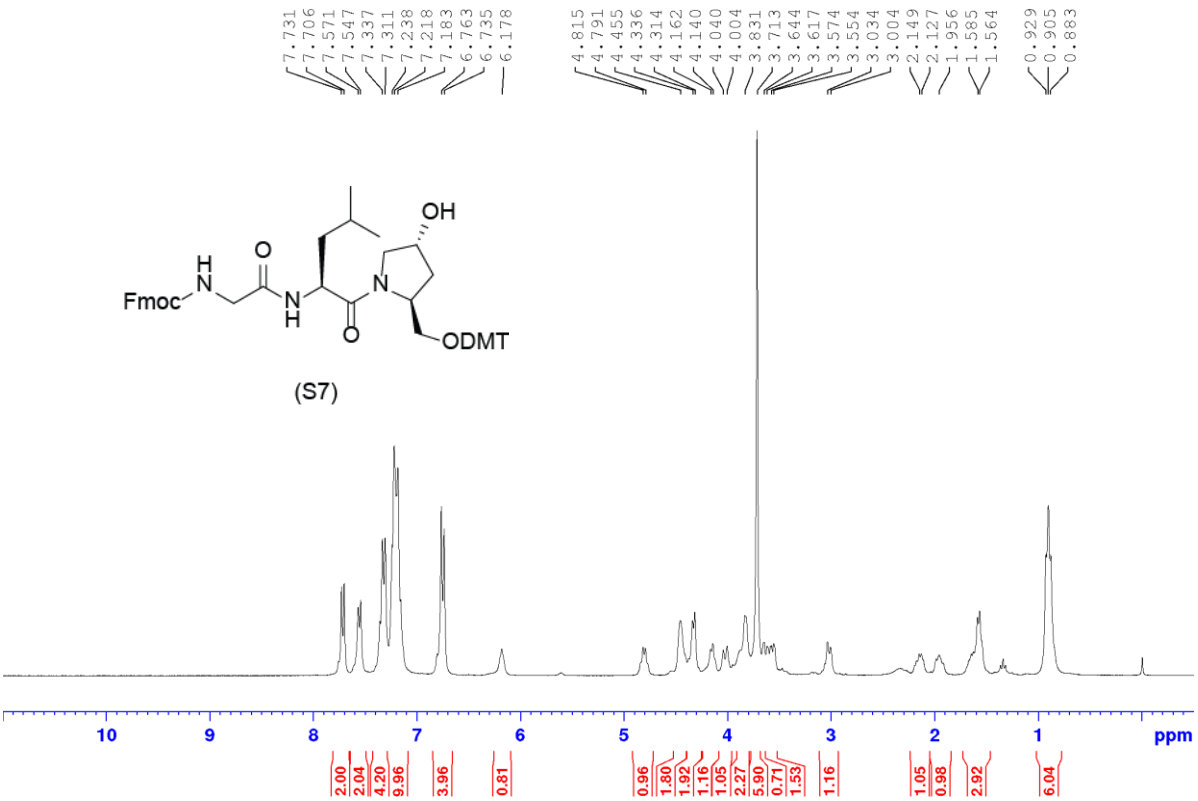


Figure S16. ^{1}H NMR spectrum of compound S7.

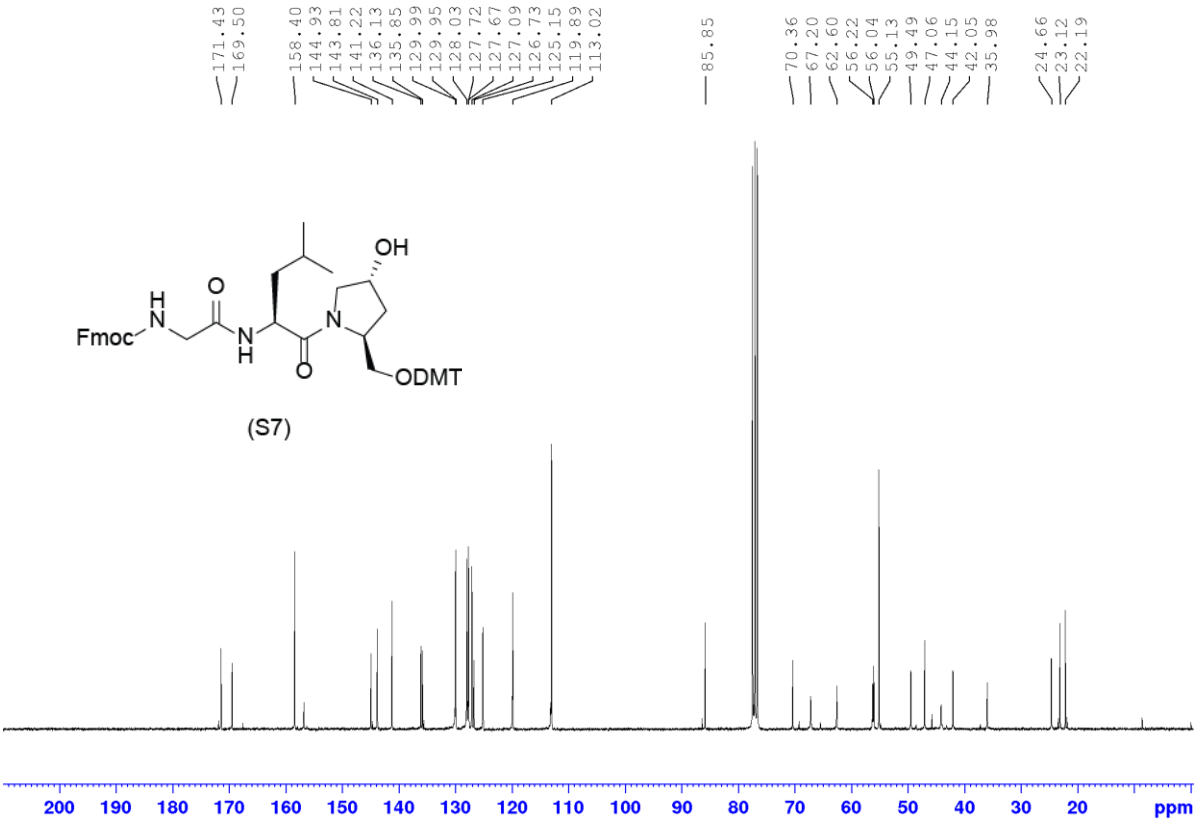


Figure S17. ^{13}C NMR spectrum of compound S7.

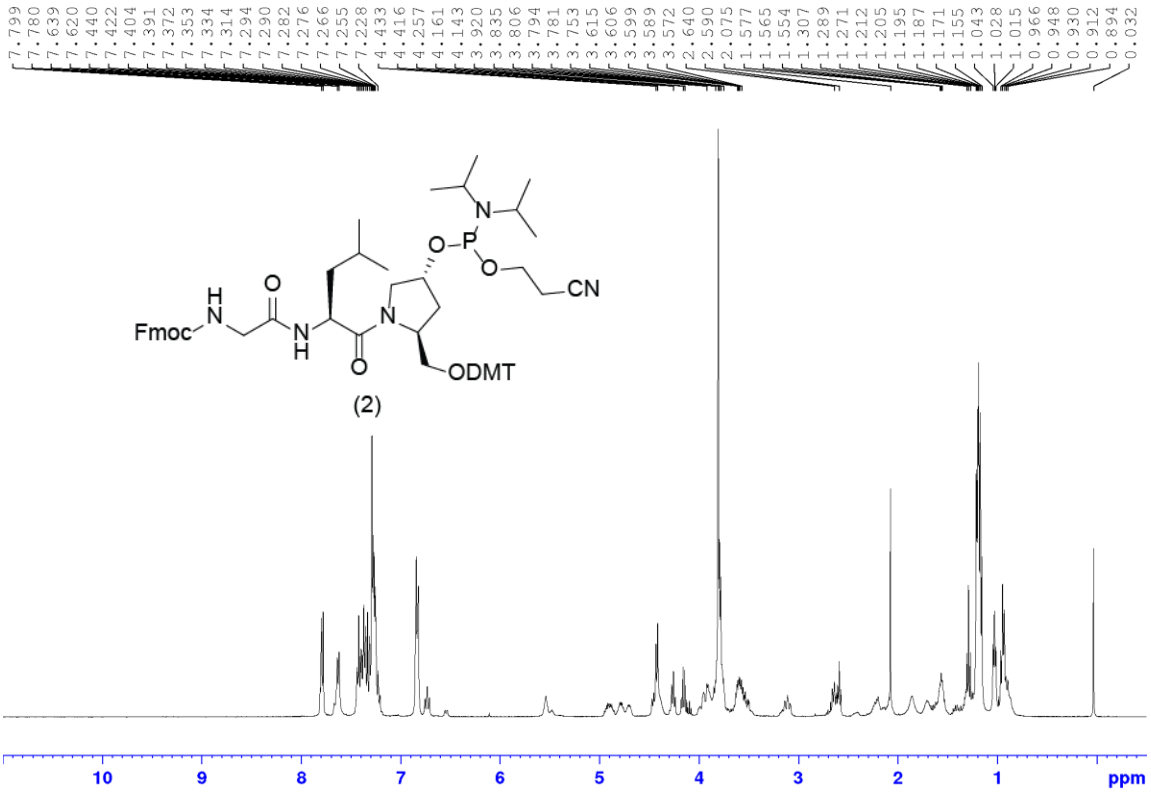


Figure S18. ^{1}H NMR spectrum of compound 2.

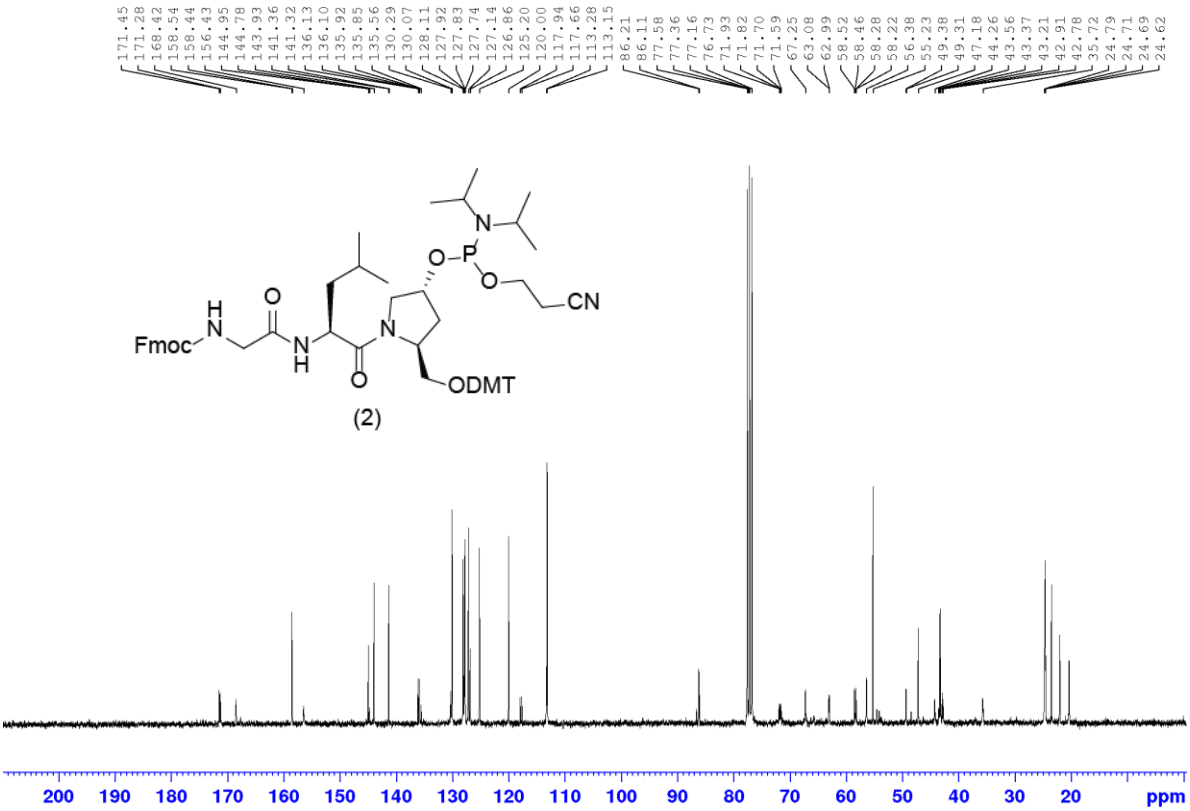


Figure S19. ^{13}C NMR spectrum of compound 2.

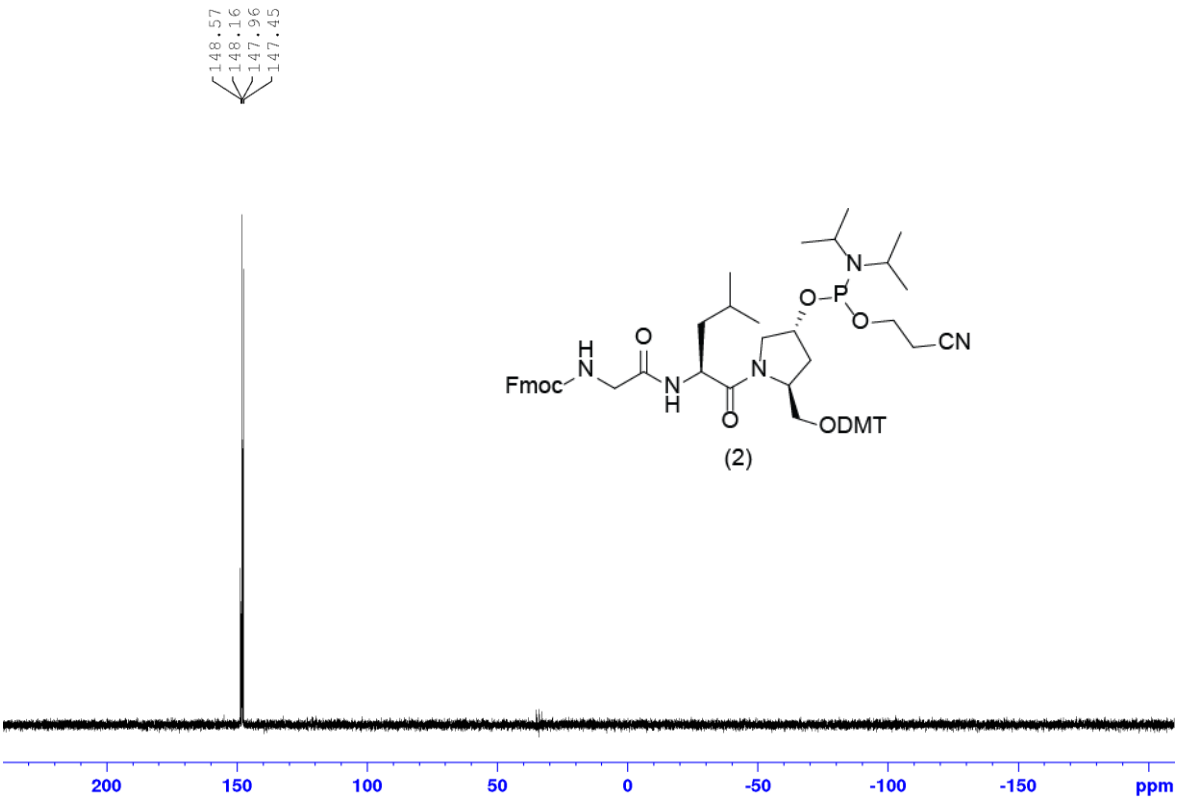


Figure S20. ^{31}P $\{^{1}H\}$ NMR spectrum of compound 2.

XI. Full SDS-PAGE gel images

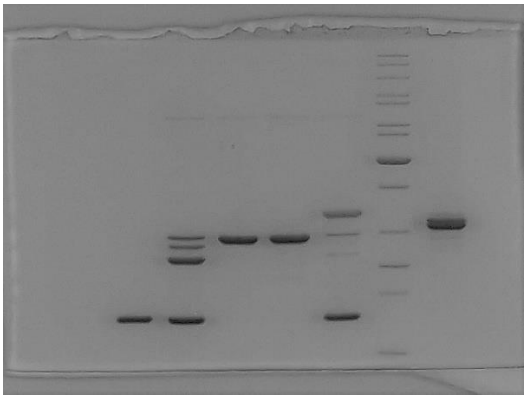


Figure S21. Full image of SDS-PAGE gel in Fig. 2b.

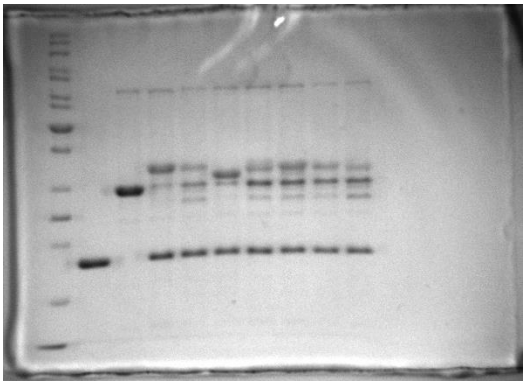


Figure S22. Full image of SDS-PAGE gel in Fig. S3.

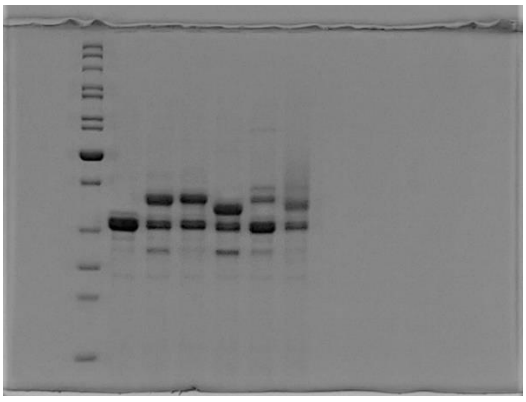


Figure S23. Full image of SDS-PAGE gel in Fig. S4.

XII. References

1. T. P. Prakash, M. J. Graham, J. Yu, R. Carty, A. Low, A. Chappell, K. Schmidt, C. Zhao, M. Aghajan, H. F. Murray, S. Riney, S. L. Booten, S. F. Murray, H. Gaus, J. Crosby, W. F. Lima, S. Guo, B. P. Monia, E. E. Swayze and P. P. Seth, *Nucleic Acids Res.*, 2014, **42**, 8796-8807.
2. R. Yang, Y. H. Wong, G. K. T. Nguyen, J. P. Tam, J. Lescar and B. Wu, *J. Am. Chem. Soc.*, 2017, **139**, 5351–5358.
3. https://www.glenresearch.com/10-1936.html.