Incorporation of Fmoc-Dab(Mtt)-OH during Solid-phase peptide synthesis: A word of caution

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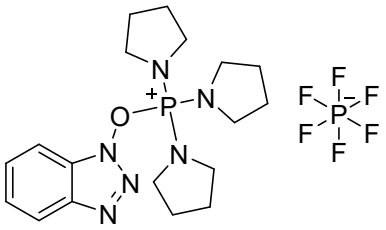
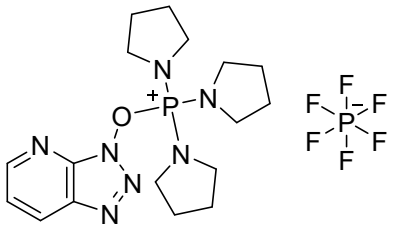
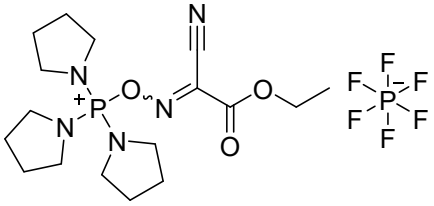
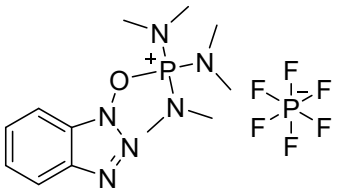
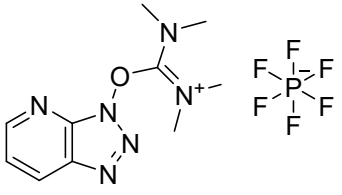
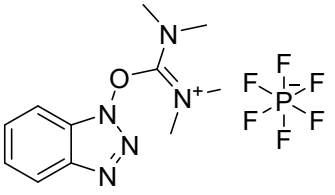
Chemistry

Reagents. All amino acid building blocks and coupling reagents for chemical reactions and solid phase peptide synthesis were purchased from Bidepharm, except Fmoc-Orn(Mtt)-OH and Fmoc-Dap(Mtt)-OH that were purchased from ChemPep. The peptide YFMVF on Rink amide resin was prepared in previous studies.[1] All solvents and other reagents were purchased from local suppliers and used without further purification. The details of coupling reagents are listed on Table S1.

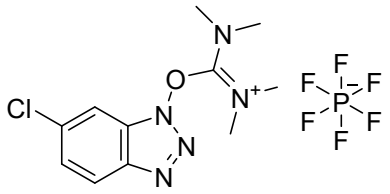
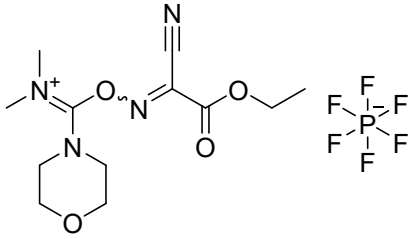
Table S1. The structure, full name, abbreviation, and CAS no. of coupling reagents are listed on Table S1.

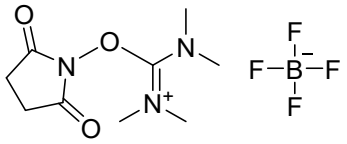
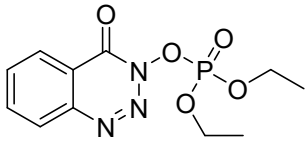
Structure		
Full name (Abbreviation)	Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP)	(7-Azabenzotriazol-1-yloxy)trispyrrolidinophosphonium hexafluorophosphate (PyAOP)
CAS Number	128625-52-5	156311-83-0

Structure		
Full name (Abbreviation)	[Ethyl cyano(hydroxyimino)acetato-O2]tri-1-pyrrolidinylphosphonium hexafluorophosphate (PyOxim)	(Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP)
CAS Number	153433-21-7	56602-33-6

Structure		

Full name (Abbreviation)	1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate (HATU)	*N,N,N′,N′*-Tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU)
CAS Number	148893-10-1	94790-37-1

Structure		
Full name (Abbreviation)	2-(6-Chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU)	(1-Cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate (COMU)
CAS Number	330645-87-9	1075198-30-9

Structure		
Full name (Abbreviation)	*N,N,N′,N′*-Tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate (TSTU)	3-(Diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one (DEPBT)
CAS Number	105832-38-0	165534-43-0

Analytical HPLC. Analytical HPLC was performed on an Agilent 1100 series HPLC system (Agilent Technologies, Stockport, UK) equipped with a diode-array detection (DAD) detector and Agilent C18 column (250 mm x 4.6 mm) at the gradients in **Table S2**.

Table S2. The timetable of gradient.

Time (min)	**A % (H_2O + 0.1 % TFA)**	**B % (MeCN + 0.1 % TFA)**	**Flow (mL/min)**
0	80	20	0.5
40	50	50	0.5
41	0	100	0.5
55	0	100	0.5

Mass spectrometry. The mass spectra, reported as m/z, were conducted by SCIEX 3200Q ESI mass spectrometer.

Nuclear magnetic resonance spectroscopy. NMR spectra were recorded on a Bruker Ultrashield 400 Plus NMR spectrometer (^{1}H NMR on 400 MHz, ^{13}C NMR on 101 MHz. The ^{1}H NMR chemical shifts were referenced to corresponding solvent peak (2.50 for DMSO-d_6). The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, m = multiplet, br = broad.

General procedure for synthesizing lactams from corresponding Fmoc-X(Mtt)-OH. To the solution of Fmoc-X(Mtt)-OH (0.1 mmol) and PyBOP (0.1 mmol) in DMF (1 mL), DIPEA (0.2 mmol) were added. The reaction mixture was stirred for 3 hours before it was diluted with ethyl acetate. The diluted reaction mixture was fully washed with water and brine. The extracted organic layer was then concentrated and purified by column chromatography on silica gel (Hex:EA = 4:1).

The lactamized Fmoc-Dab(Mtt)-OH (γ-lactam). White solid. ESI-MS calc. for $[M+H]^+$ 579.3, found 579.8; calc. for [M-Mtt+H] 322.1, found 322.7; calc. for $[Mtt]^+$ 257.1, found 257.7. MALDI-TOF HRMS calc. for $C_{39}H_{34}N_2O_3Na^+$ $[M+Na]^+$ 601.2462, found 601.2416; calc. for $C_{39}H_{34}N_2O_3K^+$ $[M+K]^+$ 617.2201, found 617.2141; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.90 (d, J = 7.9 Hz, 2H), 7.71 (dd, J = 7.6, 3.7 Hz, 2H), 7.63 (d, J = 8.2 Hz, 1H), 7.44 – 7.37 (m, 6H), 7.30 (t, J = 6.8 Hz, 2H), 7.26 – 7.22 (m, 6H), 7.14 (t, J = 7.3 Hz, 2H), 7.04 (d, J = 8.0 Hz, 2H), 4.36 – 4.26 (m, 3H), 4.20 (t, J = 7.0 Hz, 1H), 2.20 (s, 3H), 2.15 – 2.07 (m, 1H), 2.03 – 1.92 (m, 2H), 1.79 – 1.70 (m, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 174.30, 156.16, 146.14 (d, J = 3.6 Hz), 143.72 (d, J = 10.2 Hz), 142.95, 140.65 (d, J = 2.2 Hz), 134.91, 128.26, 128.23, 128.15, 127.57, 127.52, 127.01, 125.88, 125.19 (d, J = 3.0 Hz), 120.04, 70.00, 65.62, 51.90, 46.59, 39.77, 31.38, 20.39.

The lactamized Fmoc-Orn(Mtt)-OH (δ-lactam). White solid. ESI-MS calc. for $[M+Na]^+$ 615.3, found 615.8; calc. for $[Mtt]^+$ 257.1, found 257.7. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.88 (d, J = 7.5 Hz, 2H), 7.70 (dd, J = 7.5, 3.9 Hz, 2H), 7.40 (t, J = 7.4 Hz, 2H), 7.35 – 7.19 (m, 13H), 7.14 (t, J = 7.2 Hz, 2H), 7.06 (d, J = 8.0 Hz, 2H), 4.29 – 4.13 (m, 4H), 2.24 (s, 3H), 2.00 (d, J = 7.7 Hz, 2H), 1.56 (s, 4H).

General procedure for analyzing the coupling reaction between the Fmoc-Dab(Mtt)-OH and resin-bound YFMVF. The dry resin-bound YFMVF (0.02 mmol) was loaded into a syringe with frits. The resin was swollen in DMF for at least 15 min. The coupling mixture with Fmoc-Dab(Mtt)-OH (0.08 mmol), coupling reagents (0.08 mmol) and DIPEA (0.16 mmol) in DMF (1 mL) were preincubated for a period (5 min for the experiment on **Fig. 2B**, and different incubation times were tried for the experiment on **Fig. 2D**). The coupling mixture was then shaken with resin-bound YFMVF for 2 h. The resin was fully washed by DMF, and the Fmoc protecting group (if any) was removed by shaking with alternative Fmoc deprotection cocktail (5% w/v piperazine, 1 % v/v DBU, 1 % v/v formic acid in DMF, 1 mL/0.1 mmol) for 25 min.[2] The resin was washed by DMF and DCM, and fully dried before global cleavage & deprotection. Global cleavage and deprotection was carried out with freshly prepared cleavage cocktail (TFA/TIPS/H_2O, v/v/v, 95/2.5/2.5, 1 mL) for 2.5 h. Upon completion, the excess

diethyl ether was added into collected post-cleavage solution, and the precipitate was collected by centrifugation (10000 rpm, 8 min). The precipitate was then dissolved in minimal MeOH, and the product was obtained after addition of diethyl ether and centrifugation (10000 rpm, 8 min). The final precipitate was dissolved in MeOH for analytical HPLC as the gradients on **Table S2**.

General procedure of solid phase peptide synthesis without pre-incubation. The dry resin-bound YFMVF (0.02 mmol), Fmoc-Dab(Mtt)-OH (0.08 mmol), and coupling reagents (0.08 mmol) was loaded into a syringe with frits. DIPEA (0.16 mmol) in DMF (1 mL) was then loaded into syringe and shaken for a period (10 min for most cases except the case when coupling reagent DEPBT used). The Fmoc deprotection, global cleavage & deprotection, precipitation, and centrifugation were performed as above procedure. The final precipitate was dissolved in MeOH for analytical HPLC as the gradients on **Table S2**.

Reference

1 Y. Wu, W. S. Tam, H. F. Chau, S. Kaur, W. Thor, W. S. Aik, W. L. Chan, M. Zweckstetter and K. L. Wong, *Chem. Sci.*, 2020, **11**, 11266–11273.

2 K. Ralhan, V. G. KrishnaKumar and S. Gupta, *RSC Adv.*, 2015, **5**, 104417–104425.

NMR Spectra of products

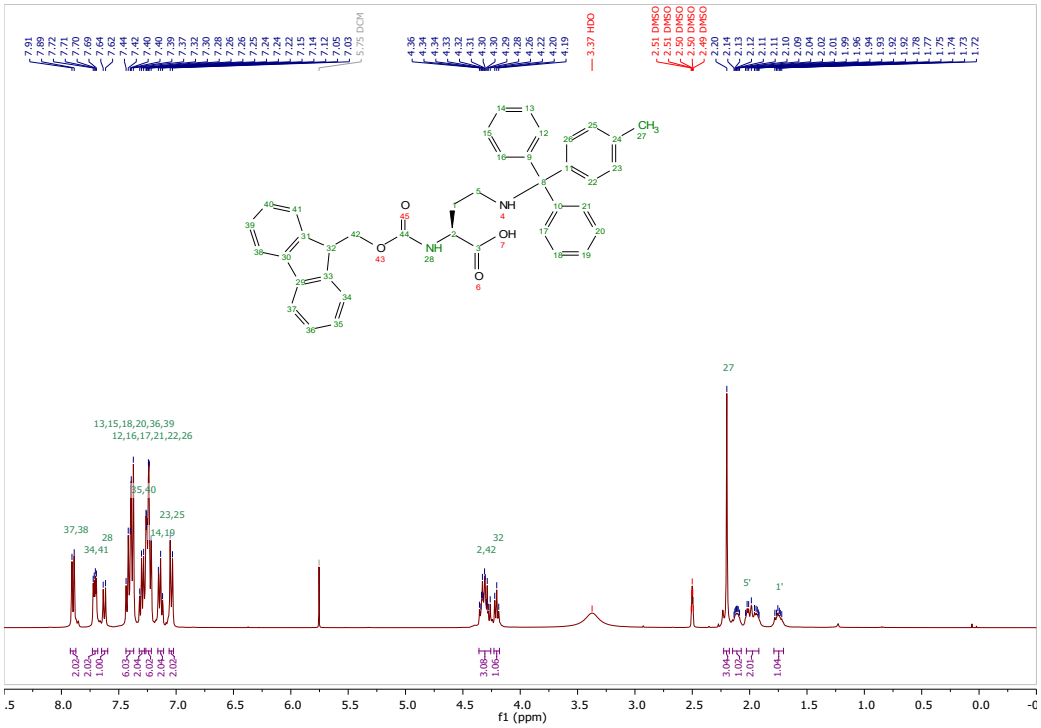


Figure S1. ^{1}H-NMR spectrum of Fmoc-Dab(Mtt)-OH.

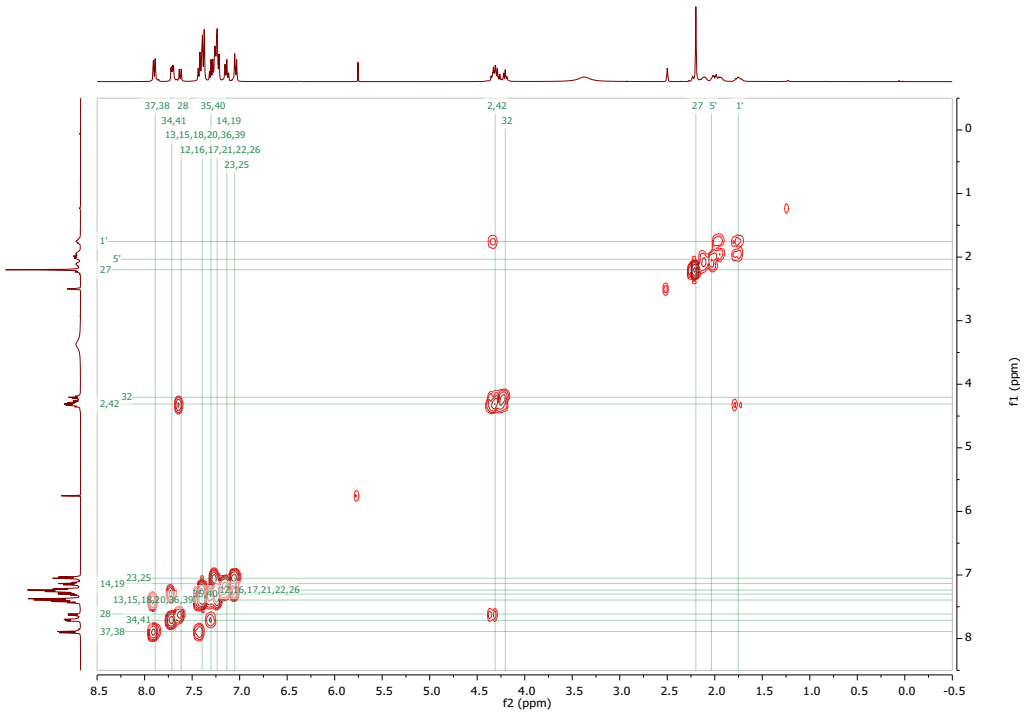


Figure S2. COSY spectrum of Fmoc-Dab(Mtt)-OH.

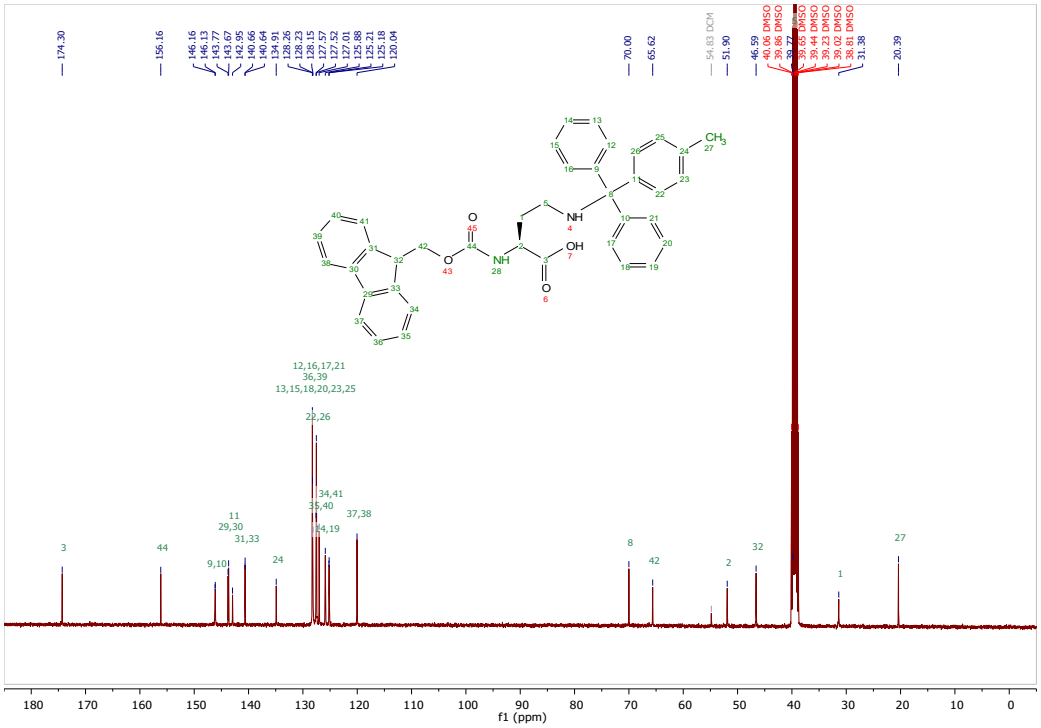


Figure S3. ^{13}C-NMR spectrum of Fmoc-Dab(Mtt)-OH.

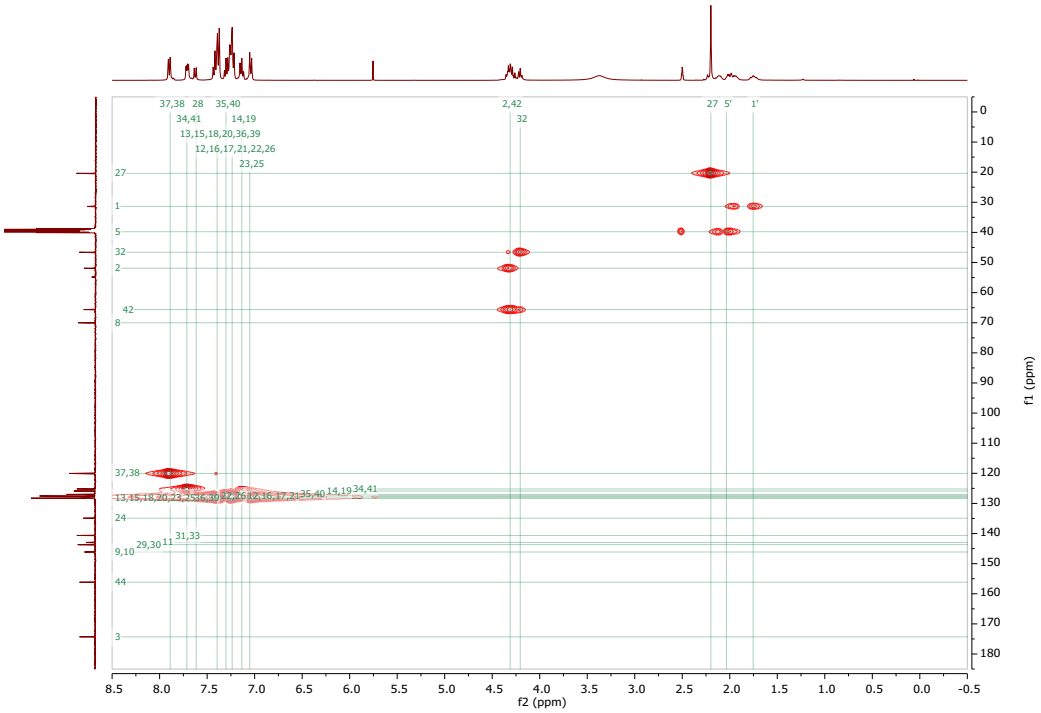


Figure S4. HSQC spectrum of Fmoc-Dab(Mtt)-OH.

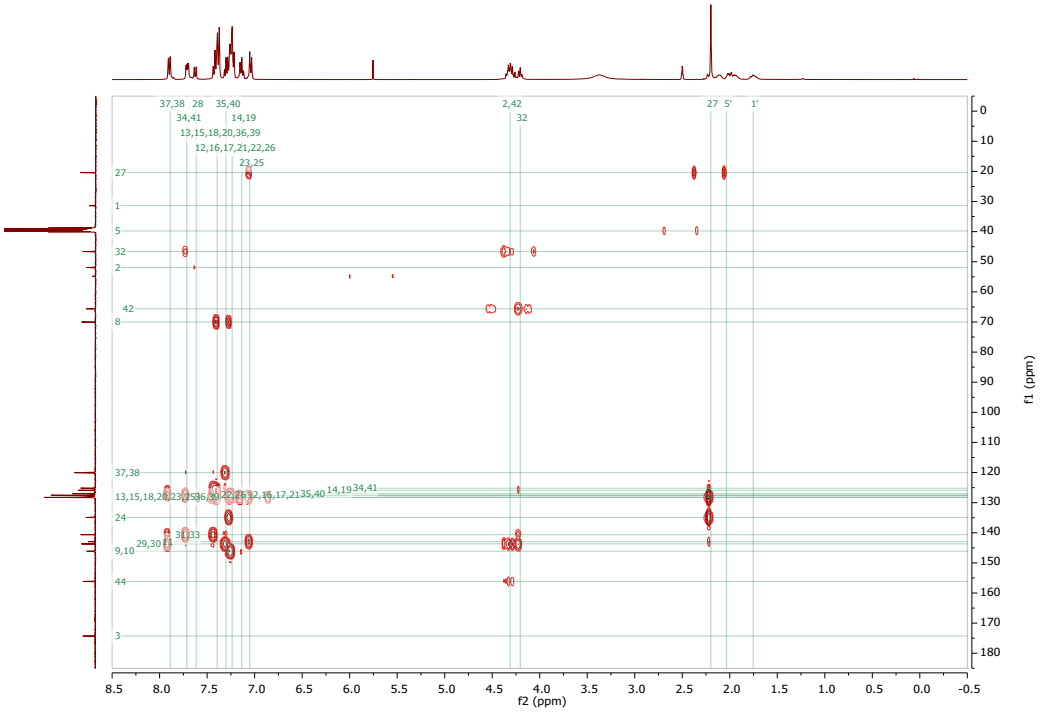


Figure S5. HMBC spectrum of Fmoc-Dab(Mtt)-OH.

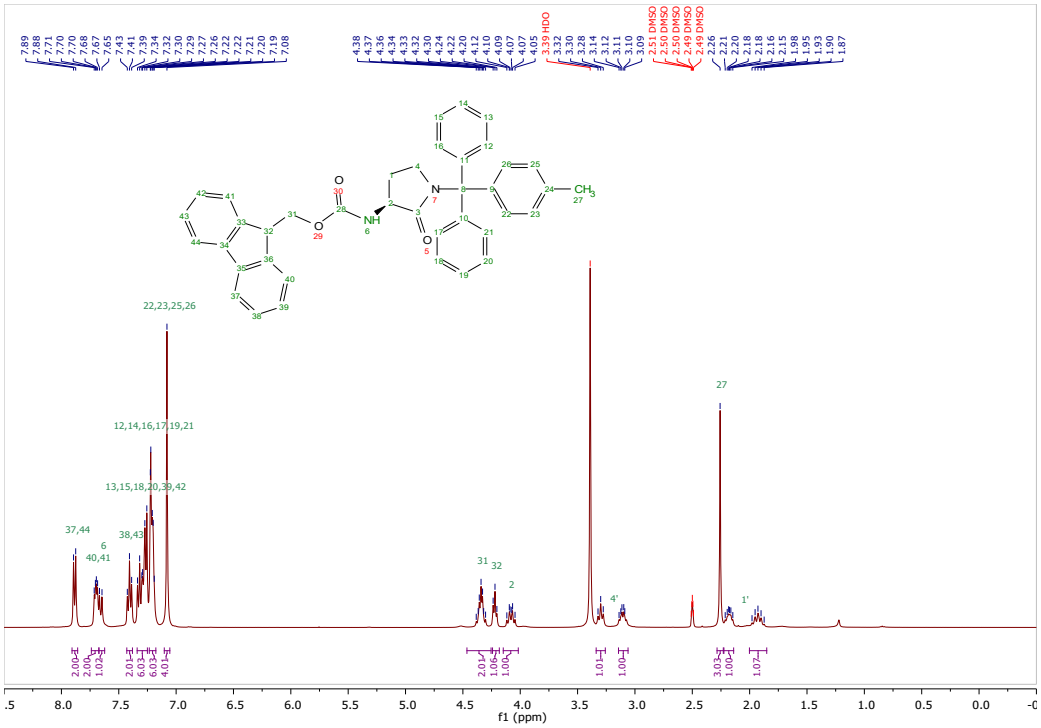


Figure S6. ^{1}H-NMR spectrum of the lactamized Fmoc-Dab(Mtt)-OH.

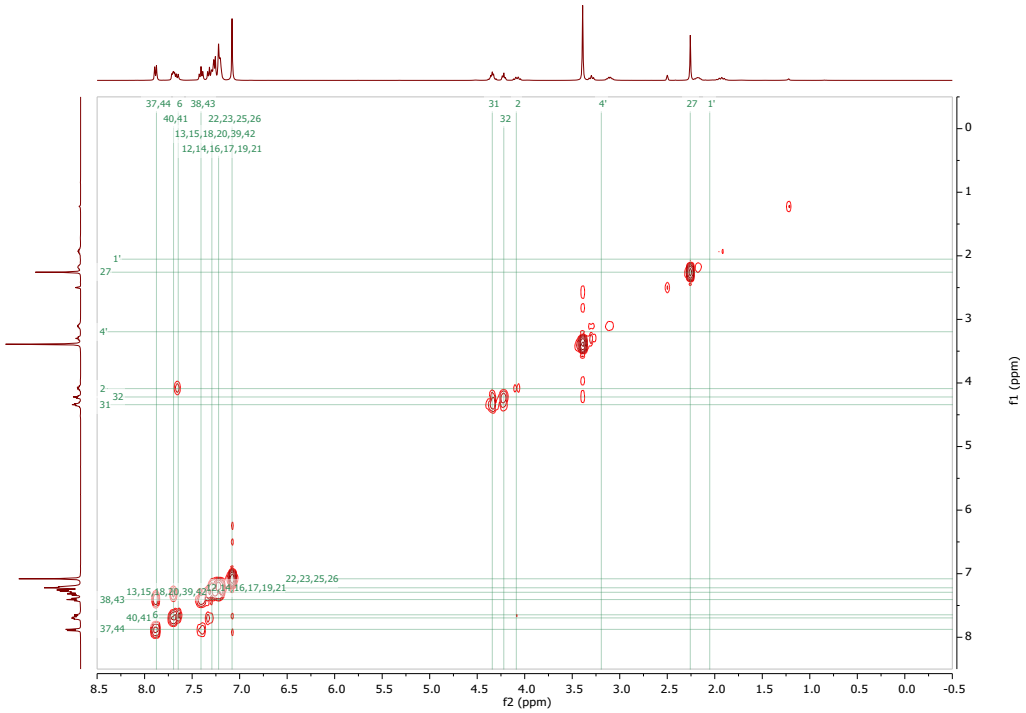


Figure S7. COSY spectrum of the lactamized Fmoc-Dab(Mtt)-OH.

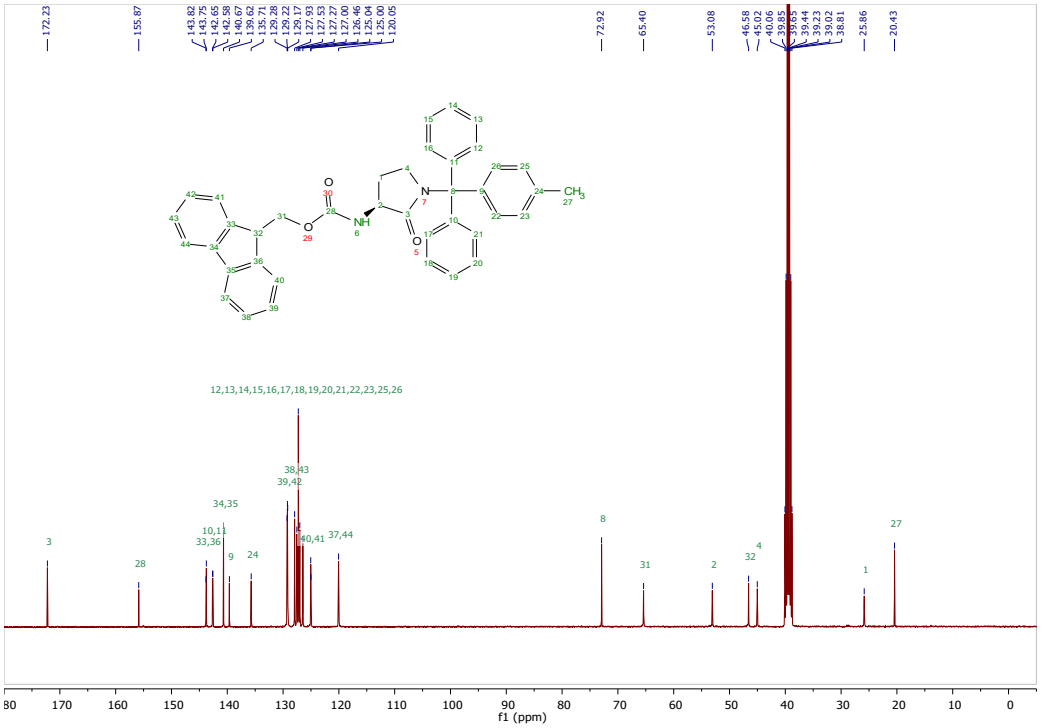


Figure S8. ^{13}C-NMR spectrum of the lactamized Fmoc-Dab(Mtt)-OH.

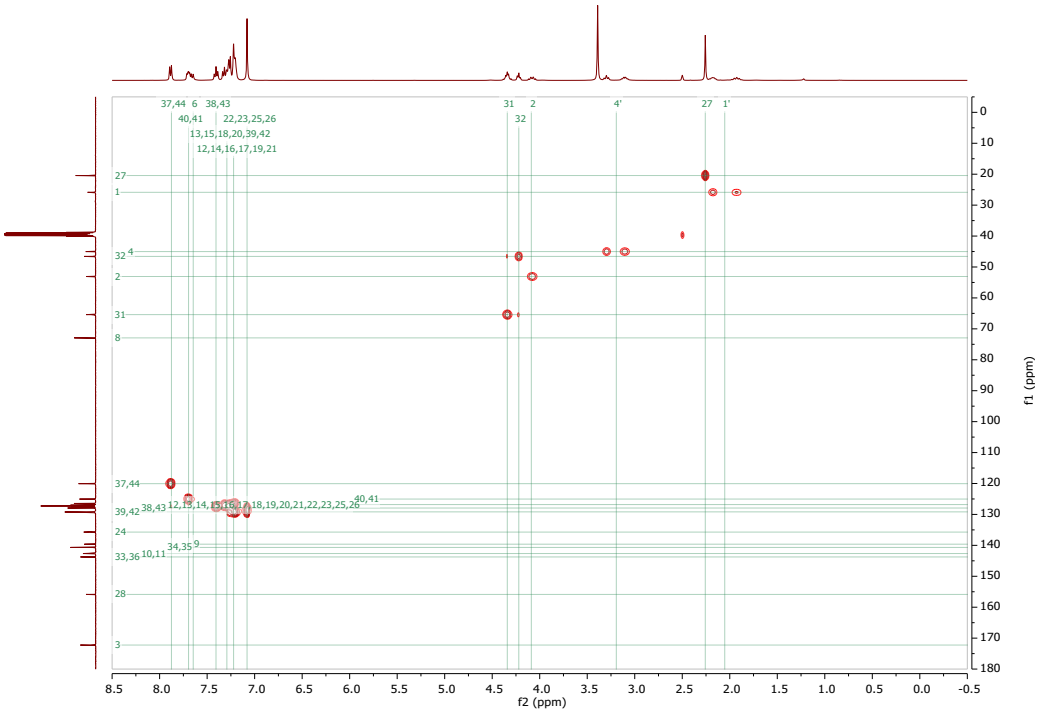


Figure S9. HSQC spectrum of the lactamized Fmoc-Dab(Mtt)-OH.

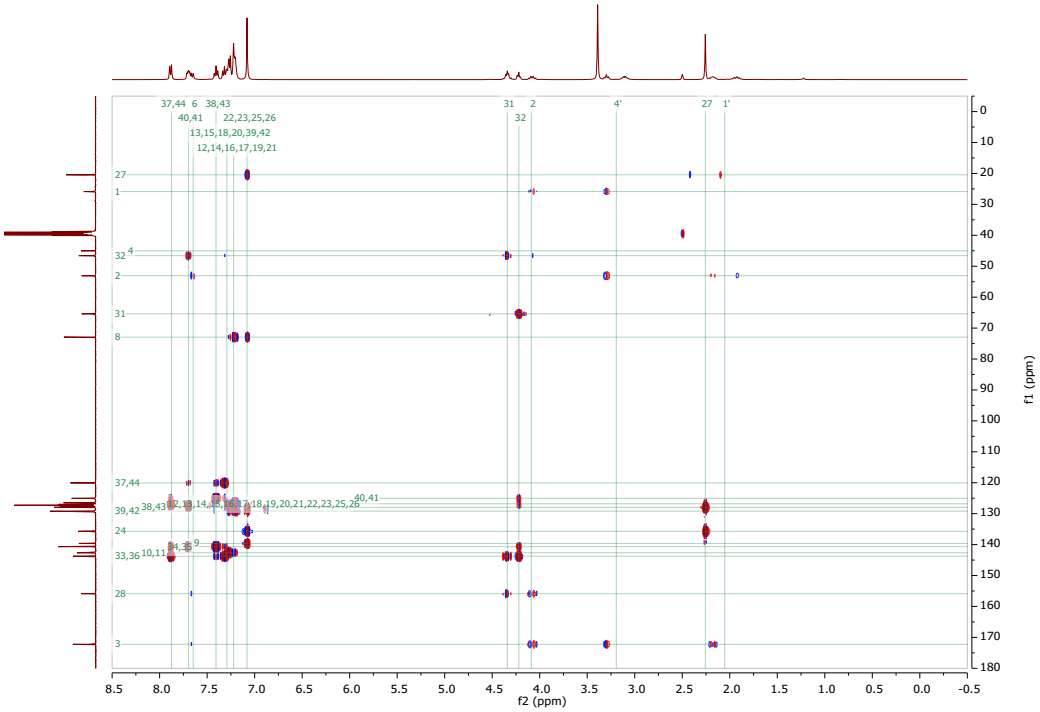


Figure S10. HMBC spectrum of the lactamized Fmoc-Dab(Mtt)-OH.

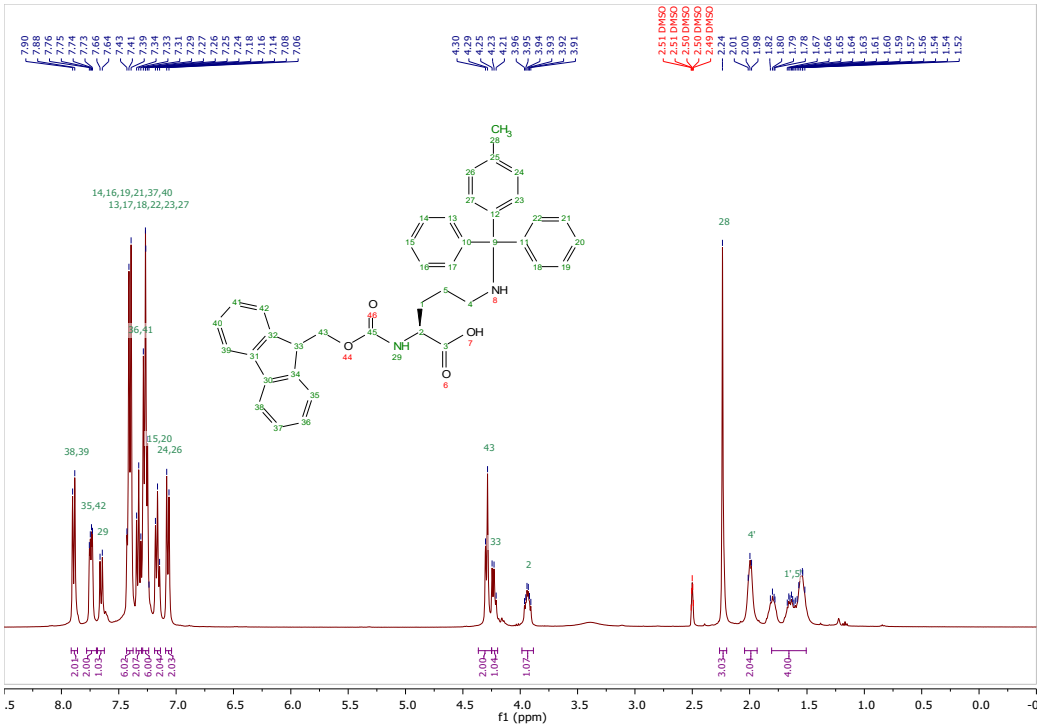


Figure S11. ^{1}H-NMR spectrum of Fmoc-Orn(Mtt)-OH.

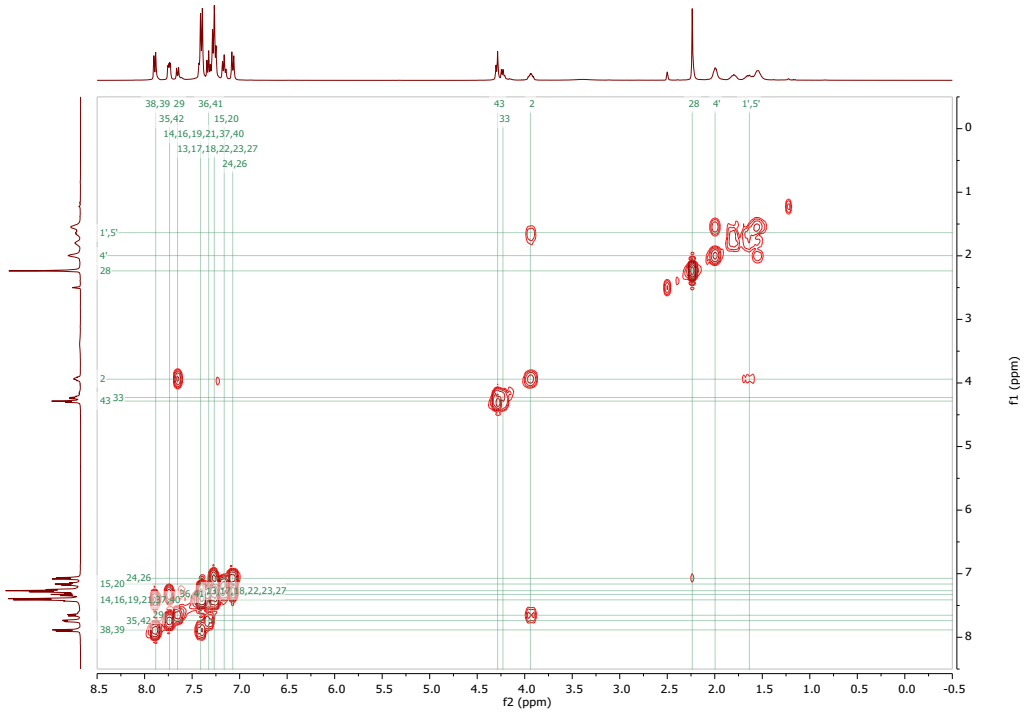


Figure S12. COSY spectrum of Fmoc-Orn(Mtt)-OH.

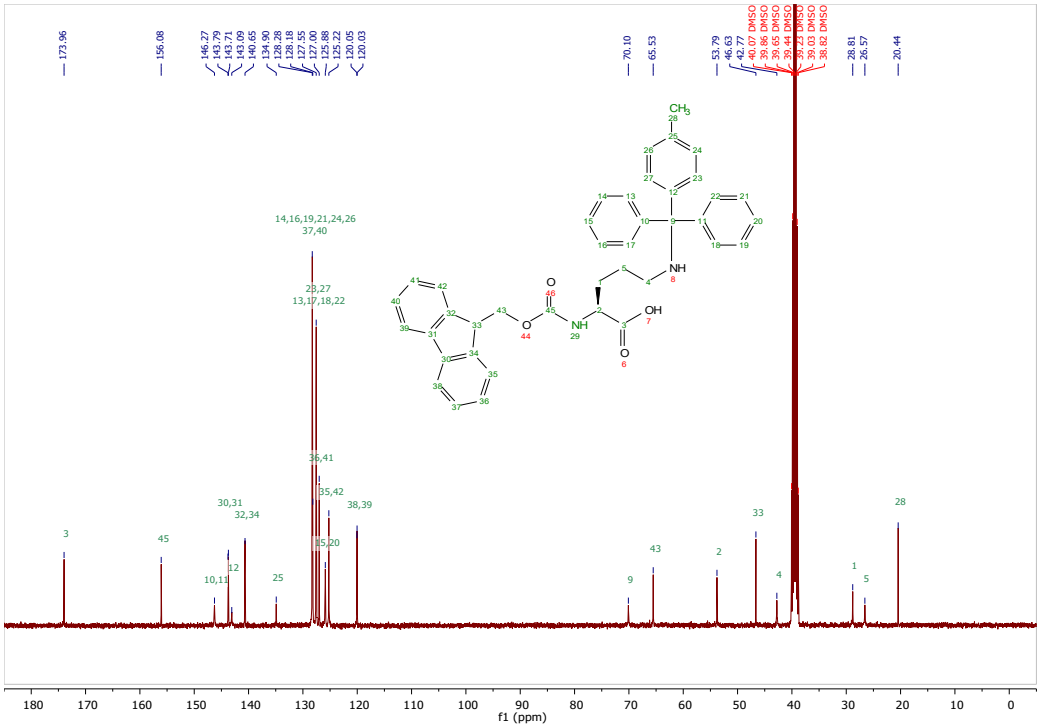


Figure S13. ^{13}C-NMR spectrum of Fmoc-Orn(Mtt)-OH.

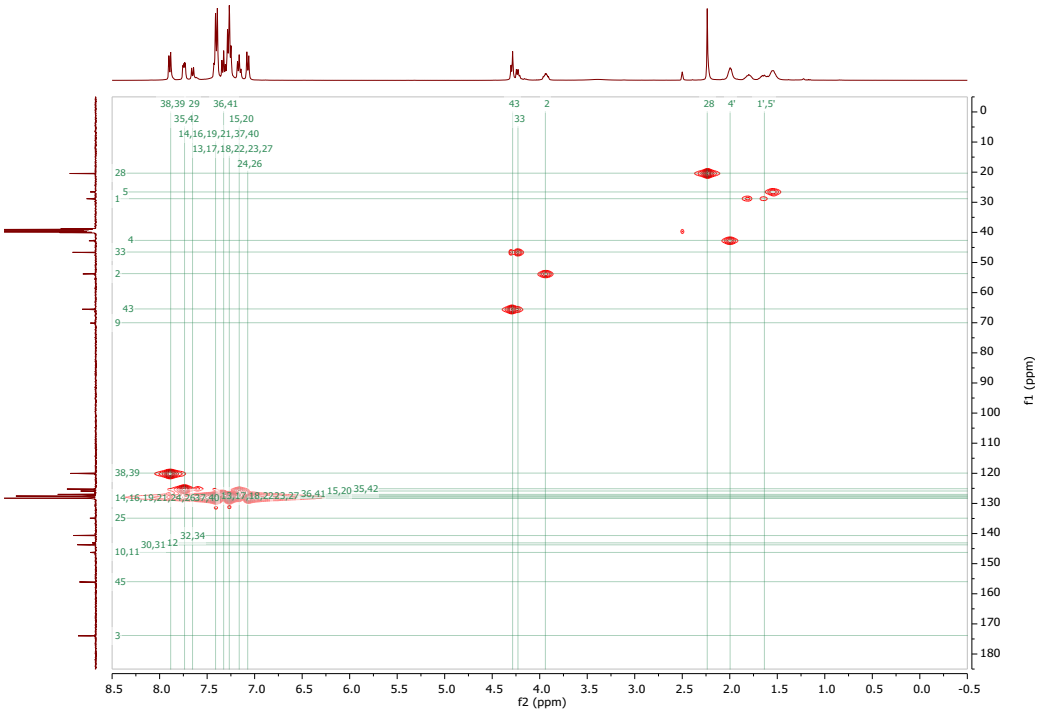


Figure S14. HSQC spectrum of Fmoc-Orn(Mtt)-OH.

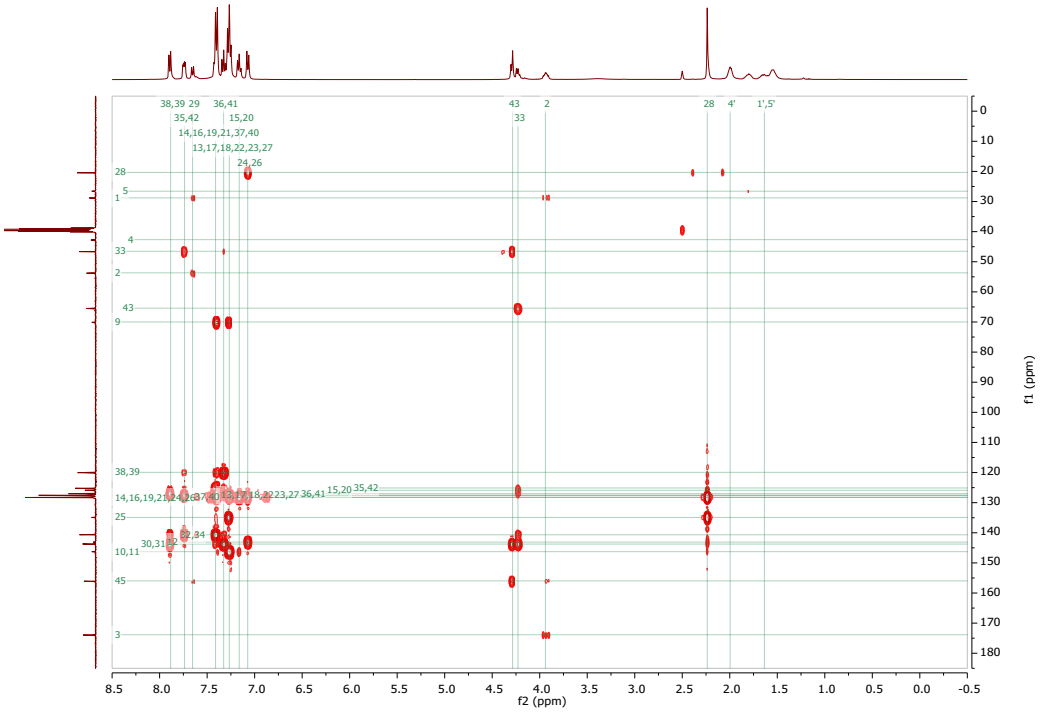


Figure S15. HMBC spectrum of Fmoc-Orn(Mtt)-OH.

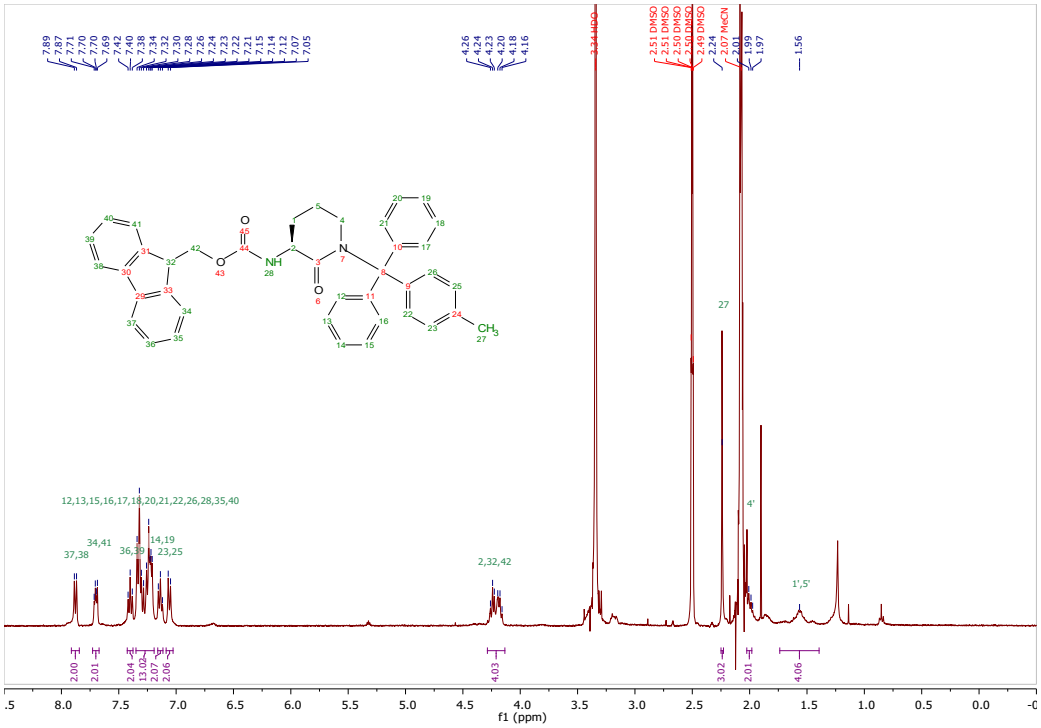


Figure S16. ^{1}H-NMR spectrum of the lactamized Fmoc-Orn(Mtt)-OH.

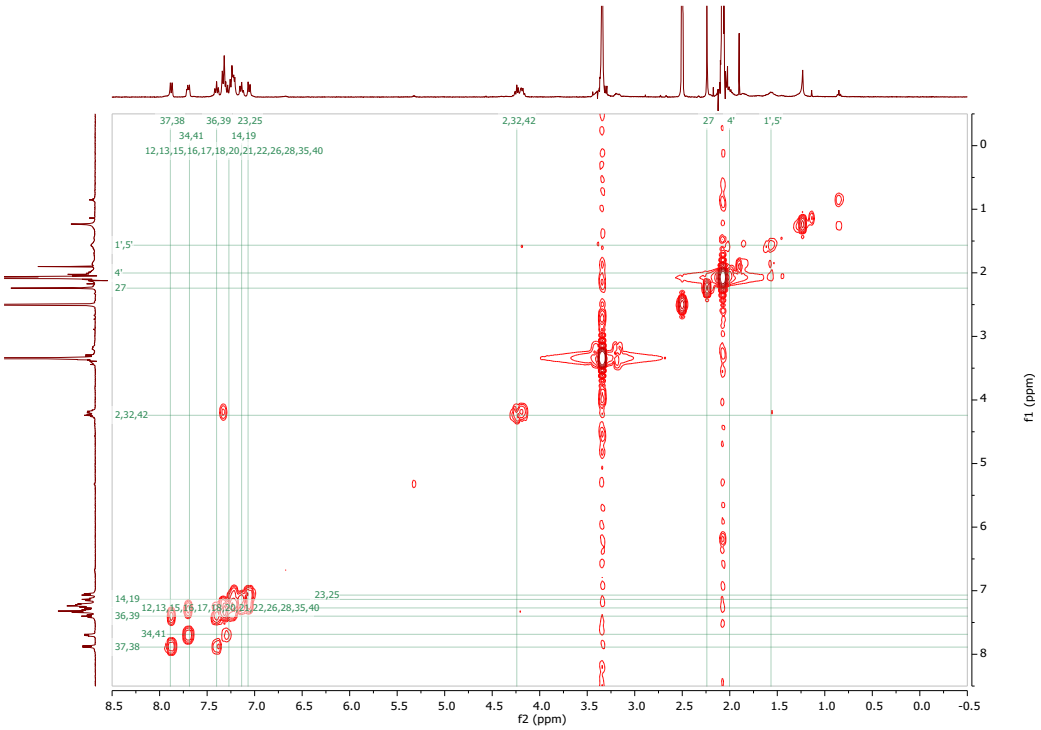


Figure S17. COSY spectrum of the lactamized Fmoc-Orn(Mtt)-OH.

HRMS of product

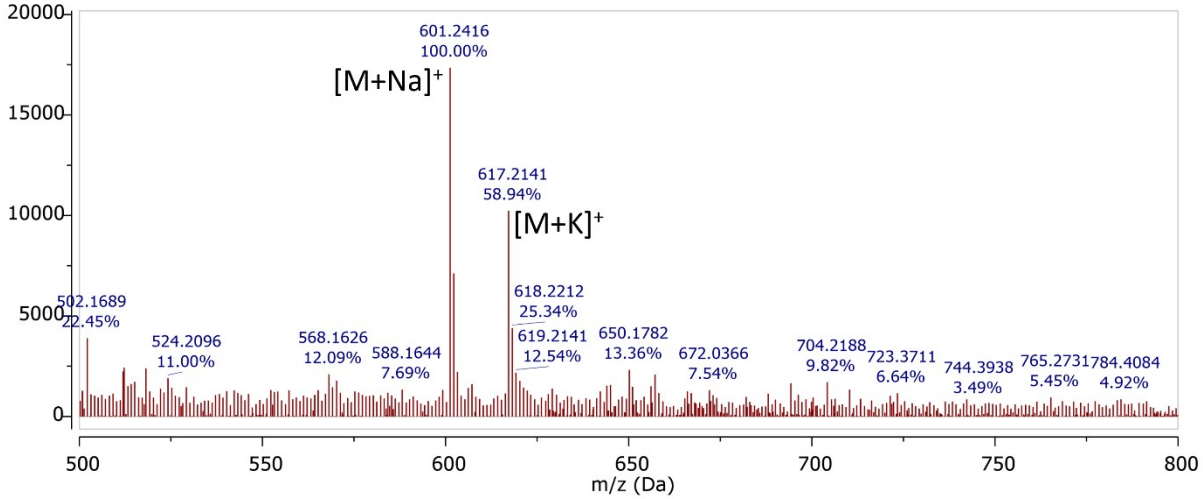


Figure S18. HRMS of the lactamized Fmoc-Dab(Mtt)-OH.