

Cyclic β -sheet peptides

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Abstract

Our laboratory has developed a class of cyclic β -sheet peptides to study the folding and supramolecular assembly of β -sheets. The peptides consist of a peptide sequence of interest incorporated into a macrocycle with the β -strand mimic Hao and δ -linked ornithine units. The δ -linked-ornithine turn units help align the peptide sequence of interest with the Hao-containing template strand. The Hao β -strand mimic helps induce β -sheet folding and also blocks uncontrolled β -sheet interactions. The resulting macrocyclic peptides fold to adopt a β -sheet structure and may also undergo further supramolecular assembly to form tetramers or other oligomers through edge-to-edge and face-to-face interactions. This paper provides procedures for the synthesis of the Fmoc-protected Hao amino acid and additional procedures for the synthesis of macrocyclic β -sheet peptides containing the Hao amino acid and the δ -linked ornithine units. Fmoc-Hao is prepared on a multigram scale from hydrazine, 2-methoxybenzoic acid, and ethyl oxalyl chloride. The macrocyclic β -sheet peptides are synthesized in tens-of-milligram quantities through Fmoc-based solid-phase peptide synthesis followed by solution-phase macrocyclization, deprotection, and reverse-phase chromatographic purification.

Abbreviations

ACN	acetonitrile.
Ar	argon.
Boc-Orn(Fmoc)-OH	(2S)-2-(((tert-butoxy)carbonyl)amino)-5-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)pentanoic acid.
DCM	dichloromethane.
DI	deionized.
DIPEA	diisopropylethylamine.
DMF	dimethyl formamide.
Equiv	equivalents.
Fmoc-OSu	<i>N</i> -(9-fluorenylmethoxycarbonyloxy)succinimide.
HATU	O-(7-azabenzotriazol-1-yl)- <i>N,N,N',N'</i> -tetramethyluronium hexafluorophosphate.
HCl	hydrochloric acid.
HCTU	O-(1H-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate.
HFIP	1,1,1,3,3,3-hexafluoro-2-propanol.
HOAt	1-hydroxy-7-azabenzotriazole.
MeOH	methanol.
NaOH	sodium hydroxide.
NaCl	sodium chloride.
NaHCO₃	sodium bicarbonate.
Pd-C	palladium on carbon.
RBF	round bottom flask.
TFA	trifluoroacetic acid.
THF	tetrahydrofuran.
TIPS	triisopropylsilane.



1. Introduction

β -Sheets are secondary protein structures that contribute to protein folding, function, and protein-protein interactions. Our laboratory has used cyclic β -sheet peptides to investigate the folding and supramolecular chemistry of β -sheets, and to understand how these structures form and interact with one another (Cheng, Pham, & Nowick, 2013; Li, Rios, & Nowick, 2022; Nowick & Chung, 2003; Nowick, 2008). Although β -sheet proteins are crucial to many biological functions, their supramolecular assembly is also associated with amyloid diseases, such as the aggregation of amyloid- β ($A\beta$) in Alzheimer's disease. Our laboratory has used $A\beta$ -derived β -sheet peptides to investigate the self-assembly of the $A\beta$ peptide to form $A\beta$ oligomers, which cause neurodegeneration in Alzheimer's disease (Liu et al., 2011; Pham, Chim, Goulding, & Nowick, 2013; Pham, Demeler, & Nowick, 2014; Pham, Spencer, Chen, & Nowick, 2014; Truex & Nowick, 2016; Truex, Wang, & Nowick, 2016). We have also demonstrated that $A\beta$ -derived cyclic β -sheet peptides can serve as $A\beta$ aggregation inhibitors (Cheng, Liu, Zhao, Eisenberg, & Nowick, 2012; Lu et al., 2019).

One class of cyclic β -sheet peptides that our laboratory has developed contains the β -strand mimic Hao and δ -linked ornithine turn units, δ Orn (Cheng & Nowick, 2011; Woods et al., 2007). Hao is a tripeptide mimic composed of hydrazine, 5-amino-2-methoxybenzoic acid, and oxalic acid (Nowick et al., 2000). It is a rigid and planar molecule that presents a pattern of hydrogen-bond donor and acceptor groups to complement the hydrogen-bonding pattern of a β -strand (Nowick et al., 2002; Nowick, Cary, & Tsai, 2001). Furthermore, the δ -linked ornithine turn unit promotes hydrogen bonding between the two linked peptide chains (Nowick & Brower, 2003). A variety of different peptide sequences can be constrained to a β -sheet conformation when combined with Hao and δ Orn to form 42–66-membered ring macrocycles. In this paper, we describe procedures for the synthesis of Fmoc-protected Hao and its application to the synthesis of this class of cyclic β -sheet peptides (Figs. 1 and 2).



2. Fmoc-Hao synthesis

2.1 Materials and equipment for Hao synthesis

2.1.1 Major equipment

- Rotary vacuum evaporator (rotovap) with a heating water bath

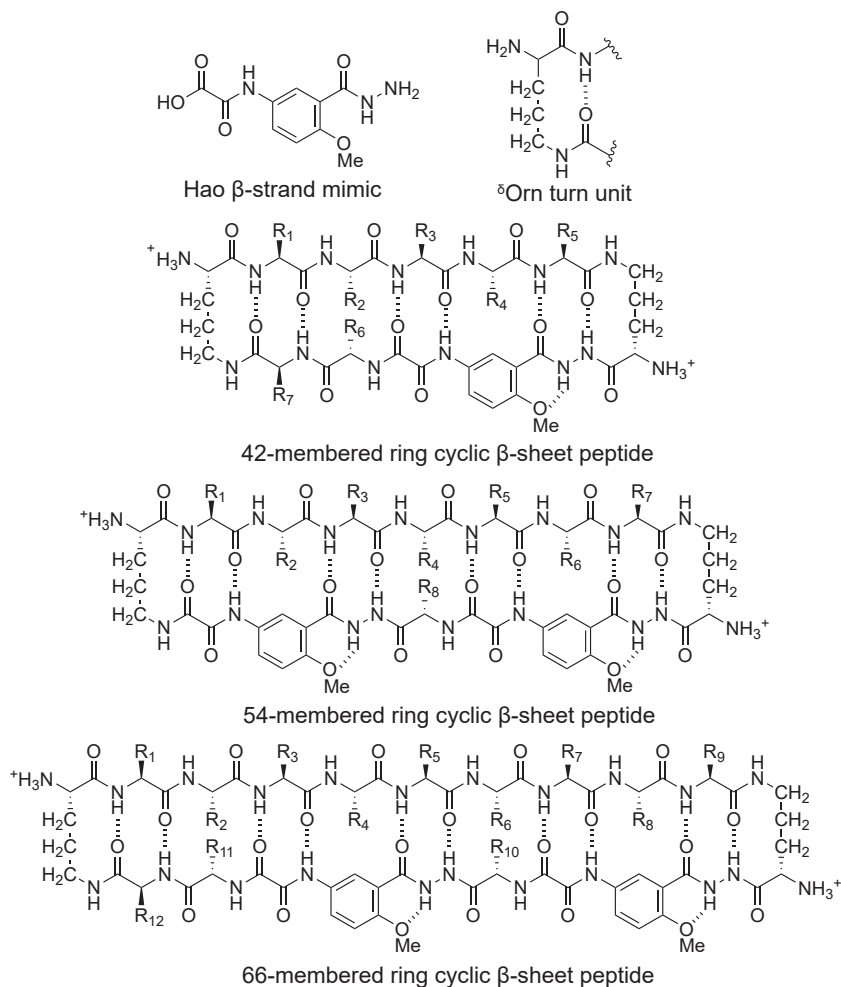


Fig. 1 Macrocyclic β -sheet peptides containing Hao and δ Orn.

- Schlenk line connected to an argon tank.
- Vacuum line (<1 mmHg)
- Water purification system to generate high purity (18M Ω) deionized water (Barnstead Nanopure, cat. #50131922)

2.1.2 Labware

- 1-L pressure-equalizing addition funnel,
- 60-mL pressure-equalizing addition funnel,

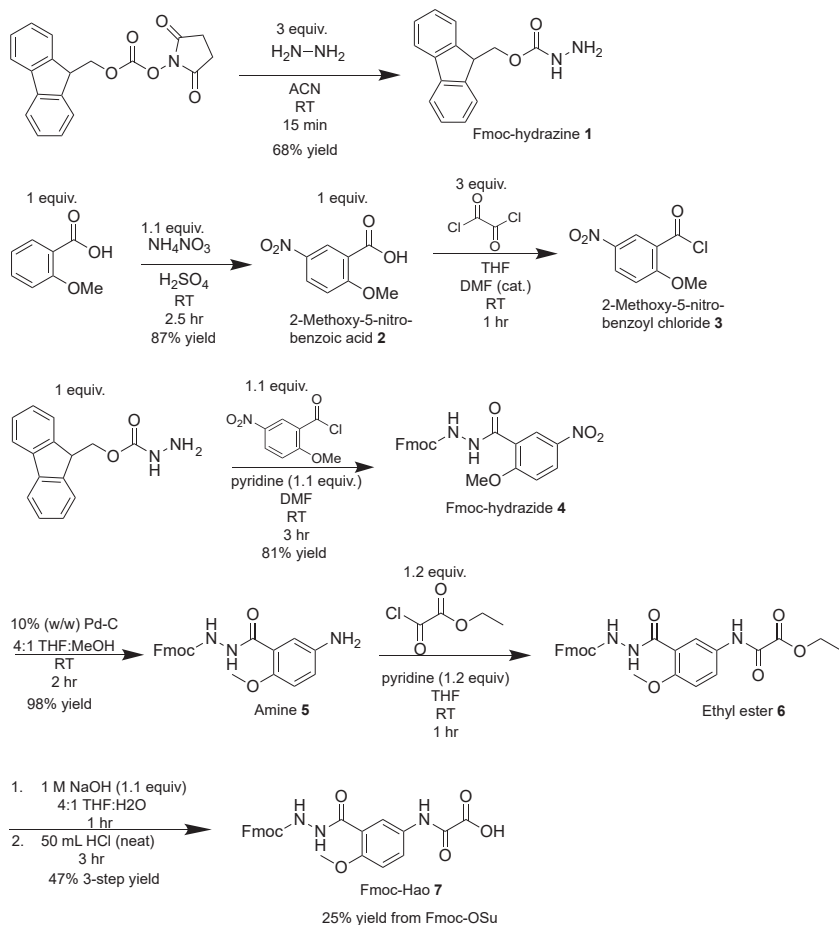


Fig. 2 Synthesis of Fmoc-Hao.

- 1-L 3-neck RBF (Morton style RBF is preferred if available) equipped with a Schlenk line gas inlet, stopcock gas valve inlet, and a rubber septum
- 2-L RBF
- 500-mL RBF
- TLC plates and jar
- Mortar and pestle
- Glass frit vacuum filter
- Stir plate and stirring bars
- Ice bath

2.1.3 Reagents

- Fmoc-OSu
- Anhydrous hydrazine
- 2-methoxybenzoic acid
- Ammonium nitrate
- Sulfuric acid
- Oxalyl chloride
- Ethyl oxoacetate
- Pd-C
- NaHCO₃
- Pyridine
- NaOH
- Concentrated aqueous HCl
- Celite

2.1.4 Solvents

ACN, THF, MeOH, diethyl ether, ethyl acetate, hexanes, DCM, DMF (Alfa Aesar, cat.#A13547).

2.2 Fmoc-hydrazine 1

1. Add ACN (300 mL) and anhydrous hydrazine (3.11 mL, 100 mmol) to a 1-L RBF.
2. Dissolve Fmoc-OSu (16.9 g, 50.1 mmol) in 300 mL of ACN and add this solution to the 1-L RBF dropwise over 10 min through a 1-L pressure-equalizing addition funnel while magnetically stirring. Stir for an additional hour.
3. Both reagents are fully soluble in ACN, but the product is not. A precipitate will form. Collect the product through vacuum filtration.
4. Wash the filter cake with 200 mL of ACN, 400 mL of DI water, 200 mL of ACN, and 200 mL of diethyl ether.
5. Dry the product under vacuum to yield a fluffy, white solid. Yield: 8.60 g (33.8 mmol, 68 %).

Caution: Hydrazine is extremely flammable and care should be taken to remove nearby ignition sources.

2.3 2-Methoxy-5-nitrobenzoic acid 2

Note: This reagent is prepared at a 250 mmol scale and stored for subsequent usage. This procedure can be scaled down as needed.

1. Dissolve 2-methoxybenzoic acid (38.0 g, 250 mmol) in 600 mL of concentrated H_2SO_4 in a 1-L RBF. Once fully dissolved, chill the solution to 0 °C in an ice bath.
2. Add ammonium nitrate (22.0 g, 275 mmol) to the RBF in two half-portions. Add the first portion and stir for 30 min. Add the second portion and stir for 2 h.
3. Fill a 2-L RBF with ice. Slowly pour the reaction mixture over the ice. Pour in increments and swirl in between to avoid heating the 2-L RBF. The product will precipitate.
4. Collect the product through filtration. Wash the filter cake twice with 500 mL of DI water and dry under vacuum overnight to yield a white powder. Grind the solid with a mortar and pestle to facilitate drying and transfer it to a vacuum line (<1 mmHg) for complete drying. Yield: 42.8 g (217 mmol, 87 %).

2.4 2-Methoxy-5-nitrobenzoyl chloride 3

1. Dissolve 2-methoxy-5-nitrobenzoic acid **2** (7.36 g, 37.4 mmol, or 1.1 equiv relative to Fmoc-hydrazine **1**) in 150 mL of THF and 30 μL of DMF (catalytic amount) in a 500-mL RBF.
2. Add oxalyl chloride (9.61 mL, 112 mmol, or 3 equiv relative to acid **2**) in a single portion while stirring.
3. Stir for 1 h or until bubbling ceases (this reaction generates CO_2 and CO).
4. Remove the THF and excess oxalyl chloride using a rotary vacuum evaporator at 40 °C to yield a white solid. Move on to the next step immediately.

2.5 Fmoc-hydrazide 4

1. Dissolve Fmoc-hydrazine **1** (8.60 g, 33.8 mmol, 1 equiv) in 250 mL of DMF and pyridine (3.03 mL, 37.2 mmol, 1.1 equiv) in a 500-mL RBF. Chill to 0 °C in an ice bath.
2. Dissolve acid chloride **3** (presumably 1.1 equiv assuming 100 % conversion in reaction 2.3) in 50 mL of DMF and add this solution to the 500-mL RBF dropwise over 5 min through a 60-mL pressure-equalizing addition funnel.
3. Remove the ice bath and stir for 3 h under inert gas.
4. Add 1 L DI water to a 2-L RBF. Pour the reaction into this flask, swirl to mix, and let it sit for 3–5 min before collecting the product through filtration.

5. Wash the filter cake with 300 mL of DI water, 500 mL of 5 % aqueous NaHCO_3 , and 500 mL of DI water.
6. Pull air through the filter for 1–2 h or until you can easily transfer the product to a 1-L RBF.
7. Dissolve the product in DCM, remove DCM on a rotary vacuum evaporator and decant the residual water. Repeat 2–3 times until most water is removed. Transfer the product to a vacuum line (<1 mmHg) for further drying. Use a mortar and pestle to finely grind the material to yield an off-white solid. Yield: 12.0 g (27.7 mmol, 81 %)

2.6 Amine 5

Note: Fmoc-hydrazide **4** must be completely dry before moving on to this step.

1. Equip a 3-neck 1-L RBF as seen in [Fig. 3](#). (A Morton style 3-neck 1-L RBF is preferred if available.). All materials should be oven-dried or flame-dried prior to use.
2. Add 10 % Pd-C (1.20 g, 10 % w/w relative to Fmoc hydrazide **4**) to the flask.

Note: Pd-C is extremely flammable and can spark/ignite. Ensure that the flask is at room temperature before adding the catalyst.

3. Evacuate the flask and backfill with Ar 3x.

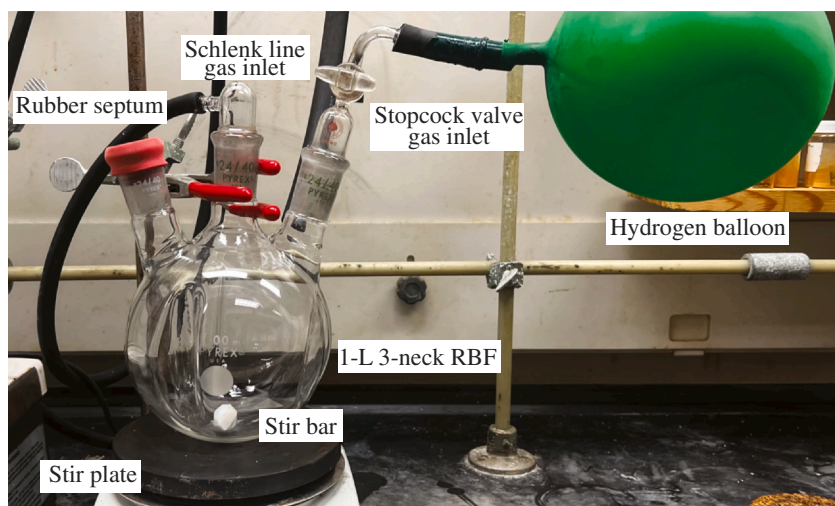


Fig. 3 Apparatus for the hydrogenation reaction in [Section 2.5](#).

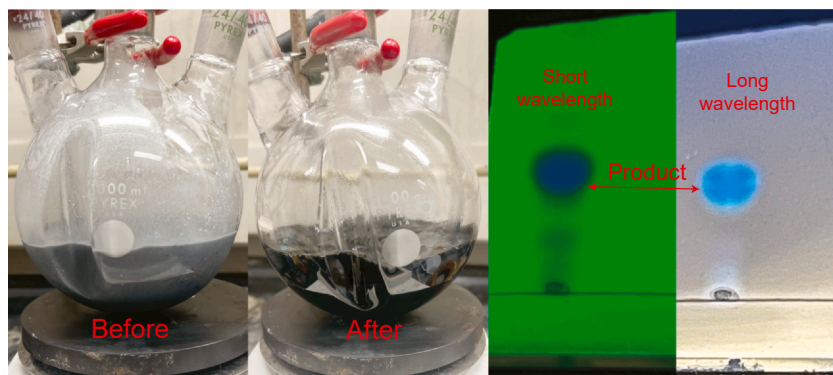


Fig. 4 Changes in appearance of the reaction mixture during hydrogenation. TLC of reaction mixture after hydrogenation illustrating the appearance of the product spot.

4. Prepare 300 mL of 4:1 THF:MeOH and add it to the flask followed by Fmoc-hydrazide **4** (12.0 g; 27.7 mmol) under a countercurrent of N_2 . Note: Your material will be sparingly soluble in the solvent mixture at this point, but the reduced product will be fully soluble (see Fig. 4). As the reduction proceeds, the suspension changes appearance from grey to black, reflecting the dissolution of the starting material.
5. Evacuate the flask and backfill with Ar 3x while magnetically stirring.
6. Evacuate the flask and backfill with H_2 . The balloon will deflate as the reaction proceeds, and also by the escape of hydrogen through the wall of the balloon. Refill the H_2 balloon as needed.
7. Stir until the reaction reaches completion. Monitor the reaction via TLC by drawing a small amount of solution through the septum with a syringe and needle. The TLC mobile phase is 50 % ethyl acetate, 50 % hexanes, and a few (~5) drops of methanol. The reaction is typically done within 1–2 h.

Note: Fmoc deprotection is a competing reaction. Be sure to monitor the reaction to maximize product conversion and minimize Fmoc-deprotection. The reduced product has a lower R_f value than the starting material. It appears as a dark spot on a fluorescent TLC plate under short-wave UV light and glows blue under long-wave UV light. The starting material does not glow under long-wave UV light.

8. Evacuate the flask and open it to air to quench the reaction.
9. Filter the reaction through a celite plug to remove Pd-C. Wash the celite plug extensively with 4:1 THF:MeOH (~300 mL) and then neat MeOH (~100 mL) until the flow-through runs clear.

10. Remove the solvent by rotary evaporation at 40 °C and place the product on a vacuum line (<1 mmHg) for further drying to yield a dark yellow to brown solid. Yield: 11.0 g (27.3 mmol, 98 %).

2.7 Ethyl ester 6

1. Dissolve amine **5** (11.0 g, 27.3 mmol, 1 equiv) in 300 mL of THF and pyridine (2.67 mL, 32.8 mmol, 1.2 equiv) in a 1-L RBF.
2. Add ethyl oxalyl chloride (3.67 mL, 32.8 mmol, 1.2 equiv) dropwise over 2 min while stirring. Stir for 1 h.

Note: If a dark brown precipitate forms, remove it through vacuum filtration before moving on to step 3.

3. Remove THF on a rotary vacuum evaporator to yield a yellow solid.

2.8 Fmoc-Hao 7

1. Dissolve ethyl ester **6** in 300 mL of 4:1 THF:H₂O.
2. Add 1 M aqueous NaOH (30.0 mL, 30.0 mmol, presumably 1.1 equiv assuming 100 % conversion in Section 2.6) and magnetically stir for 1 h. Monitor the reaction via TLC.
3. If starting material remains after 1 h, add 1 M NaOH in 5 mL increments (while monitoring via TLC) until the reaction reaches completion.

Note: Residual pyridinium hydrochloride from Section 2.6 will consume NaOH, which is why additional NaOH may be needed. However, too much NaOH will Fmoc-deprotect your material, so it is extremely important to monitor your reaction and allow at least 20 min before each addition.

4. Add 50 mL concentrated aqueous HCl in a single portion and stir for 3 h. Ensure pH < 1.
5. Remove THF and excess HCl using a rotary vacuum evaporator. The product will precipitate out of solution and is collected through vacuum filtration.

Note: The carboxylate intermediate is brown while the carboxylic acid product is light tan. If the precipitate is dark brown, then the protonation was likely unsuccessful. Stir in 4:1 THF:H₂O pH < 1 for longer (overnight if necessary).

6. Wash the filter cake with 500 mL DI water before transferring to a RBF for complete drying on a vacuum line (<1 mmHg) to yield a light tan solid. Yield: 6.04 g (12.7 mmol, 47 % from amine **5**). NMR samples were prepared in deuterated DMSO. See Appendix [Figs. 6–13](#) for ¹H NMR spectra of the intermediated and final product.



3. Peptide synthesis

This procedure is for the synthesis of cyclic β -sheet peptides containing Hao, as the trifluoroacetate (TFA) salts. It was adapted from a procedure for synthesizing cyclic β -sheet peptides containing *N*-methylated amino acids (Guaglianone, Kreutzer, & Nowick, 2021). It is important to use high-grade (>99.9 %), amine-free DMF in the synthesis of Hao-containing peptides. Our laboratory uses Alpha Aesar DMF exclusively for these peptides.

3.1 Materials and equipment for peptide synthesis

3.1.1 Major equipment

- Automated medium-pressure reverse-phase chromatography system (Biotage Isolera One equipped with a Biotage Sfar C18 60 g D Duo 30 μ m column)
- Preparative RP-HPLC (Shimadzu equipped with an Agilent Zorbax 7 μ m 300SB-C18 column (21.2 $\times$ 250 mm) column)
- Heated water bath for heating the preparative HPLC column. We use a Sterlite plastic bin and a sous vide immersion heater (<\$100), rather than a laboratory water bath (>\$1000).
- Analytical RP-HPLC (Agilent 1260 Infinity II equipped with a Phenomonex bioZen PEPTIDE 2.6 μ m XB-C18 column (150 $\times$ 4.6 mm))
- Water purification system to generate high purity (18M Ω) deionized water (Barnstead Nanopure, cat. #50131922)
- Lyophilizer (Labconco FreeZone 4.5 L -84°C Benchtop Freeze Dryer)
- LC-MS instrument
- Rotary vacuum evaporator (rotovap) with a heating water bath
- Vacuum line (<1 mmHg)
- Table-top centrifuge
- UV-Vis spectrophotometer

3.1.2 Labware

- Solid-phase peptide synthesis vessel (Chemglass, cat. #CG-1866)
- Bio-Rad Poly-Prep chromatography column (Bio-Rad, cat. #7311550),
- Scintillation vials
- Quartz cuvette
- Pasteur pipettes
- 50-mL polypropylene conical centrifuge tubes

- 1-L RBF
- Stir plate and stir bar

3.1.3 Reagents

- 2-chlorotrityl resin
- Boc-Orn(Fmoc)-OH
- 2,4,6-collidine
- piperidine
- MeOH
- DIPEA
- HCTU
- Fmoc-protected amino acids
- HFIP
- HATU
- HOAt
- TIPS
- TFA
- Fmoc-Hao (synthesized according to Section 2)

3.1.4 Solvents

DCM, DMF (Alfa Aesar, cat. #A13547), diethyl ether (anhydrous), ACN (HPLC grade).

3.2 Loading the resin

Load resin at ~ 0.6 mmol/g for peptides containing one Hao unit. Load resin to ~ 0.4 mmol/g or less for larger peptides containing two or more Hao units.

1. Add 300 mg 2-chlorotrityl chloride resin (1.55 mmol/g loading capacity) into a 10-mL BioRad Poly-Prep column (or a similar column with a polypropylene frit).
2. Swell the resin in DCM for 15 min.
3. Dissolve 100 mg Boc-Orn(Fmoc)-OH (0.193 mmol) in 8 mL DCM and 0.3 mL 2,4,6-collidine. This generally leads to ~ 0.4 mmol/g loading, but the exact loading is calculated in Section 3.3.
4. Use an N_2 stream to expel DCM from the Poly-Prep column and add the amino acid solution.
5. Place the Poly-Prep column on a rocker for at least 8 h but no more than 24 h (usually overnight).

3.3 Capping the resin

1. Expel the amino acid solution and wash the resin with DCM (3x, ~ 7 mL each).
2. Prepare the capping solution by combining 9 mL of DCM, 1 mL of MeOH, and 0.5 mL of DIPEA.
3. Add the capping solution to the Poly-Prep column using a Pasteur pipette and let it rock for 1 h.
4. Expel the capping solution with N_2 gas and wash the resin with DCM (3x, ~ 7 mL each).
5. Wash the resin with DMF (3x, ~ 7 mL each) and transfer the resin to a coupling vessel.

3.4 Checking the resin loading

1. Weigh 1–2 mg of dry resin and transfer it to a scintillation vial. The easiest way to dry the resin is to wash it with DCM (5x, ~ 7 mL) and use an N_2 stream to evaporate the solvent.
2. Prepare 5 mL of fresh 20 % piperidine (in DMF). Blank a UV-Vis spectrophotometer at 301 nm with this solution.
3. Add 3 mL of this solution to the scintillation vial. Stir the solution for at least 5 min and transfer it to a clean quartz cuvette. Measure the absorbance at 301 nm.
4. Calculate the resin loading using the following equation:

$$\text{Resin loading} \left(\frac{\text{mmol}}{\text{g}} \right) = \left[\frac{3 \text{ mL} \times \text{Abs}_{301 \text{ nm}}}{7800 \text{ M}^{-1} \text{ cm}^{-1}} \right] \times \left[\frac{1000 \text{ mg/g}}{\text{mg of resin}} \right]$$

3.5 Coupling amino acids

Our laboratory uses coupling vessels from ChemGlass (cat #GC-1866) to perform solid-phase peptide synthesis. The coupling vessels are connected to a Schlenk line to easily alternate between vacuum (to drain the vessel) and N_2 gas (to mix solutions).

1. Add 7 mL of 20 % piperidine (in DMF) to the coupling vessel and mix by bubbling N_2 gas for 8 min.
2. Wash the resin with DMF (5x, ~ 7 mL each).
3. Dissolve 5 equiv amino acid and 5 equiv HCTU in 5 mL of 20 % 2,4,6-collidine (in DMF).
4. Add the amino acid/HCTU solution to the coupling vessel and mix by bubbling nitrogen for 15–20 min.

5. Repeat steps 1–4 for each amino acid in your sequence. Coupling times can be extended beyond 20 min for amino acids that are sluggish to couple. Couple Hao according to [Section 3.6](#). Remove the last Fmoc group in the sequence before moving on to [Section 3.6](#).

3.6 Coupling Hao

1. Dissolve 2 equiv Fmoc-Hao and 2 equiv HCTU in 5 mL of 20 % 2,4,6-collidine (in DMF).
2. Add the solution to the coupling vessel and mix by bubbling nitrogen for 1 h.
3. Drain the vessel and repeat steps 1–2. Do not Fmoc-deprotect in between the two couplings.

3.7 Checking the linear peptide

This step is done to check the progress of the synthesis after every few coupling steps, or as desired. The Fmoc group should be removed beforehand.

1. Use the tip of a Pasteur pipette to pick up a few resin beads (20–30 beads are sufficient).
2. Place the pipette with the resin beads into a clean scintillation vial.
3. Use a clean Pasteur pipette to transfer ~1 mL of TFA to the scintillation vial and suspend all resin beads in solution.
4. Mix the vial (or sonicate) for 15–30 min.
5. Filter out the resin beads using a Pasteur pipette with a cotton plug. Transfer the TFA cleavage solution to a clean scintillation vial. Use a gentle air (or N₂) stream to evaporate the TFA.
6. Dissolve the peptide in 0.5–1 mL of 20 % ACN (in H₂O). The solution is now ready for mass spectrometry analysis (a dilution may be necessary).
7. Analyze the sample by mass spectrometry to identify the desired product and any deletion products to determine if the synthesis is progressing in a satisfactory fashion.

3.8 Cleaving from the resin

Complete this step once your linear sequence is complete, the final Fmoc group is removed and the linear peptide should be checked as described in [Section 3.7](#).

1. Transfer the resin to a 10-mL Poly-Prep column. Wash the resin with DCM (5x, ~7 mL each).

2. Prepare 10 mL of 20 % HFIP (in DCM) by combining 8 mL of DCM and 2 mL of HFIP. Add the solution to the Poly-Prep column. The resin should turn red upon addition. Place the Poly-Prep column on a rocker for 1 h.
3. Drain the 20 % HFIP solution into a clean 500-mL RBF.
4. Repeat steps 2–3. Rock the resin for 30 min this time.
5. Remove the solvent using a rotary vacuum evaporator. Your linear peptide with protected side chains is now in the flask.

3.9 Cyclizing the peptide

It is important to design the synthesis of the macrocyclic β -sheet peptide so that the cyclization occurs efficiently and without epimerization of the C-terminal residue. We design our syntheses to cyclize a δ -linked ornithine turn unit onto an α -amino acid (as shown in [Fig. 5](#)), and we avoid cyclizing a δ -linked ornithine turn unit onto the amino group of Hao. You may also design your synthesis to cyclize a glycine residue onto an α -amino acid.

1. Dissolve the peptide in 125 mL of DMF and 0.3 mL of DIPEA. Add 6 equiv HATU and 6 equiv HOAt.
2. Evacuate the flask and backfill with inert gas (3x). Cap the flask and magnetically stir for at least 24 h, but longer reaction times are okay (48 h is standard).

3.10 Global deprotection

The purpose of this step is to remove acid-labile sidechain protecting groups from the cyclized peptide.

1. Remove DMF using a rotary vacuum evaporator at 60–70 °C.
2. Place the dried peptide on a vacuum line (<1 mmHg) for at least 1 h to remove residual solvent.
3. Prepare the cleavage solution by combining 18 mL of TFA, 1 mL of TIPS, and 1 mL of H₂O.
4. Add the cleavage solution to the dried peptide and magnetically stir for 90 min under inert gas.

3.11 Ether precipitation

The peptide is collected by precipitating in ether using 45 mL of ether for 5 mL of cleavage solution.

1. Add 45 mL of ether to four 50-mL conical tubes. Chill the ether to 0 °C in an ice bath.

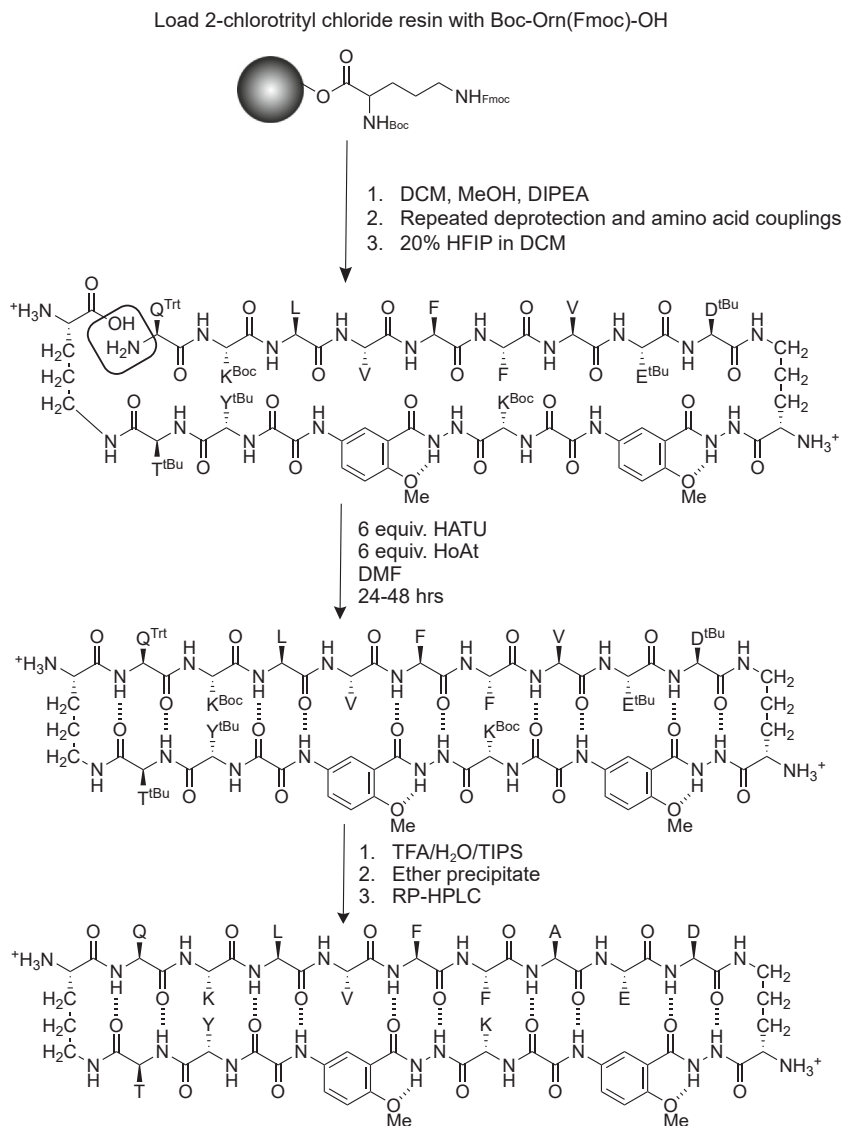


Fig. 5 Synthesis of macrocyclic β -sheet peptides.

2. Add the cleavage solution to the conical tubes dropwise (5 mL per conical).
3. Let the solution chill on ice for 10 min.
4. Centrifuge the conical tubes at $2500 \times g$ for 10 min.

5. Decant the ether into a clean flask taking care not to disturb the pellets.
Note: Save your ether solution until you are confident you have your peptide. Sometimes, the peptide will not precipitate and will stay in solution. In this case, evaporate the ether/TFA solution to recover your peptide.
6. Freeze the thoroughly dried pellets at -20°C for storage overnight, or proceed to the purification, below.

3.12 Medium-pressure reverse-phase chromatography pre-purification

The goal of this step is to remove any residual solvent, protecting groups, or coupling agents that remain in the pellets after ether precipitation. This step may be omitted if the needed chromatography system is not available.

1. Dissolve the ether precipitate pellets in 5–10 mL 10 % ACN (or 20 % ACN, depending on the solubility of the peptide). Choose the lowest percentage of ACN that is needed to dissolve the peptide.
2. Equilibrate the column and instrument lines with the same percentage of ACN.
3. Load the peptide onto the column and begin the gradient as described below.
4. Use LC-MS or HPLC analysis to determine which fractions contain the desired product.
5. Combine the fractions that contain product and lyophilize them. Remove ACN by rotatory evaporation before placing your sample on the lyophilizer. This step is necessary if your lyophilizer has a -40°C collector but is optional if it has a -84°C collector.

3.12.1 Parameters

1. Cartridge: Biotage Sfar C18 60 g D Duo 30 μm
2. Temperature: Room temperature ($\sim 22^{\circ}\text{C}$)
3. Mobile phase A: 18M Ω deionized H_2O (0.1 % TFA)
4. Mobile phase B: ACN (0.1 % TFA)
5. Flowrate: 50 mL/min
6. Max fraction volume: 15 mL
7. UV monitor: 214 and 280 nm

3.12.2 Gradient

This method is a good starting point, but the gradient can be adjusted as needed ([Table 1](#)).

Table 1 MPLC method for the pre-purification of the crude cyclic β -sheet peptide.

Step	%B start	%B end	Column volumes
1	10 %	10 %	3
2	10 %	60 %	10
3	60 %	95 %	1
4	95 %	95 %	2
5	95 %	50 %	1
6	50 %	50 %	2

Hao-containing peptides usually elute at 30–45 % ACN, but retention times can vary depending on your peptide sequence and the number of Hao units in the peptide.

3.13 RP-HPLC purification

The purpose of this step is to obtain the desired peptide in > 95 % purity. We typically heat the HPLC column in a water bath at 60 °C to minimize self-association of the peptide and thus facilitate HPLC purification.

1. Dissolve the lyophilized pre-purified peptide from the previous step in 5–10 mL 10 % ACN. Generally, the solubility of the lyophilized peptide is better than the crude ether precipitate pellets. Start at 10 % ACN and increase towards 20 % ACN if needed.
2. Equilibrate the column for 10 min at the appropriate percentage of ACN (the same percentage as the peptide sample) and temperature before injecting your peptide onto the column.
3. Run the gradient. β -Sheet peptides that self-associate typically elute as a broad peak over many fractions, rather than a sharp peak.
4. Use LC-MS to determine which fractions contain the desired product. In Section 3.13, these fractions will be further analyzed by HPLC and the pure fractions will be collected and lyophilized to give the desired peptide.
5. Store the fractions at 4 °C while further analyzing the fractions (until you are ready to combine the fractions).

3.13.1 Parameters

1. Column: Agilent Zorbax 7 μ m 300SB-C18 column (21.2 $\times$ 250 mm)
2. Temperature: 60 $^{\circ}$ C
3. Mobile phase A: 18M Ω deionized H₂O (0.1 % TFA)
4. Mobile phase B: ACN (0.1 % TFA)
5. Flowrate: 15 mL/min
6. Maximum fraction volume: 15 mL
7. UV monitor: 214 and 280 nm

3.13.2 Gradient

Table 2.

Table 2 HPLC method for the preparative purification of cyclic β -sheet peptides.

Step	% B start	% B end	Duration (minutes)
1	10 %	10 %	8
2	10 %	60 %	90
3	60 %	95 %	1
4	95 %	95 %	10
5	95 %	50 %	1

3.14 HPLC analysis and peptide lyophilization

The purpose of this section is to select pure fractions and isolate the pure cyclic β -sheet peptide as a fluffy white solid.

1. Dilute fractions 5-fold in 18M Ω deionized water before injecting onto the analytical HPLC.
2. Calculate purity using the area under the curve. Combine the fractions that are > 95 % pure.
3. Remove ACN by rotatory evaporation before placing your sample on the lyophilizer. This step is necessary if your lyophilizer has a -40° C collector but is optional if it has a -84° C collector.
4. Lyophilize the pure combined fractions to give the desired peptide.
5. Fractions containing a significant amount of product but not of > 95 % purity may be collected and lyophilized separately for a second round of preparative HPLC purification.

3.14.1 Parameters

1. Column: Phenomenex bioZen PEPTIDE 2.6 μm XB-C18 column (150 $\times$ 4.6 mm)
2. Temperature: 60 $^{\circ}\text{C}$
3. Mobile phase A: 18M Ω deionized H₂O (0.1 % TFA)
4. Mobile phase B: ACN (0.1 % TFA)
5. Flowrate: 1 mL/min
6. Injection volume: 20 μL
7. UV monitor: 214 and 280 nm

3.14.2 Gradient

Table 3.

Table 3 Analytical HPLC method for the analysis of preparative fractions.

Step	%B start	%B end	Duration (minutes)
1	5	67	15
2	100	100	3
3	5	5	5



4. Summary

Cyclic β -sheet peptides provide a useful model system with which to study the folding, supramolecular assembly, and interactions of β -sheets. Our laboratory has developed a class of cyclic β -sheet peptides that contains the β -strand mimic Hao and the β -turn mimic δ -linked ornithine (δOrn). This paper details methods for synthesizing Fmoc-Hao and this class of macrocyclic β -sheet peptides.

Acknowledgments

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Appendix. ^1H NMR spectra of Fmoc-Hao and synthetic intermediates

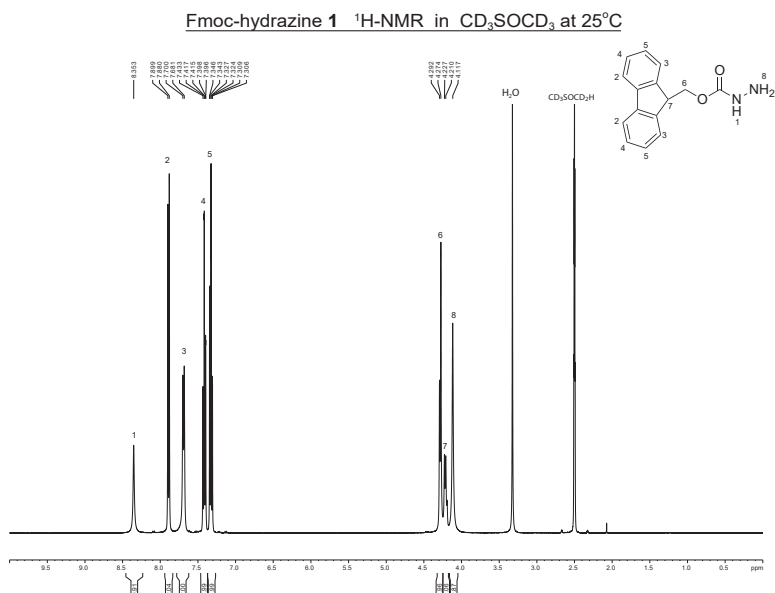


Fig. 6 ^1H NMR spectra of Fmoc-hydrazine 1.

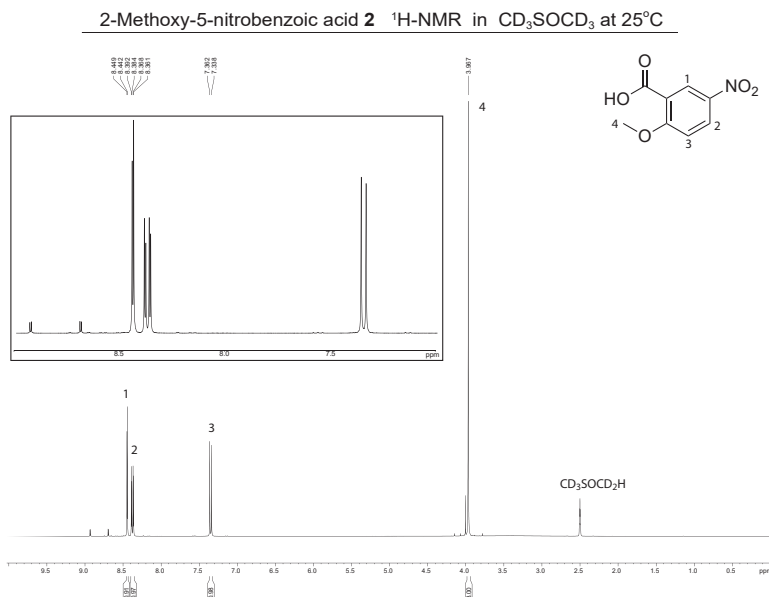


Fig. 7 ^1H NMR spectra of 2-methoxy-5-nitrobenzoic acid 2.

Fmoc-hydrazide **4** $^1\text{H-NMR}$ in CD_3SOCD_3 at 25°C

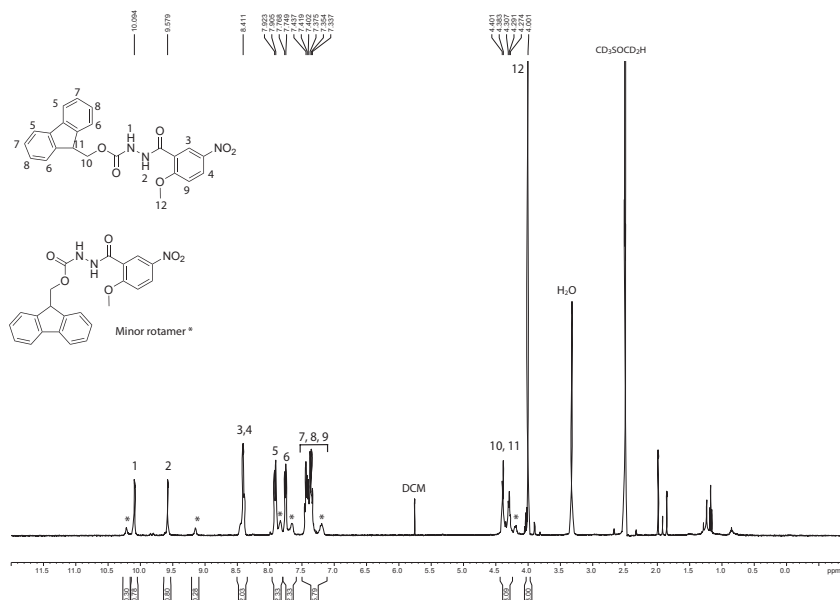


Fig. 8 $^1\text{H-NMR}$ spectra of Fmoc-hydrazide **4**.

Amine **5** $^1\text{H-NMR}$ in CD_3SOCD_3 at 25°C

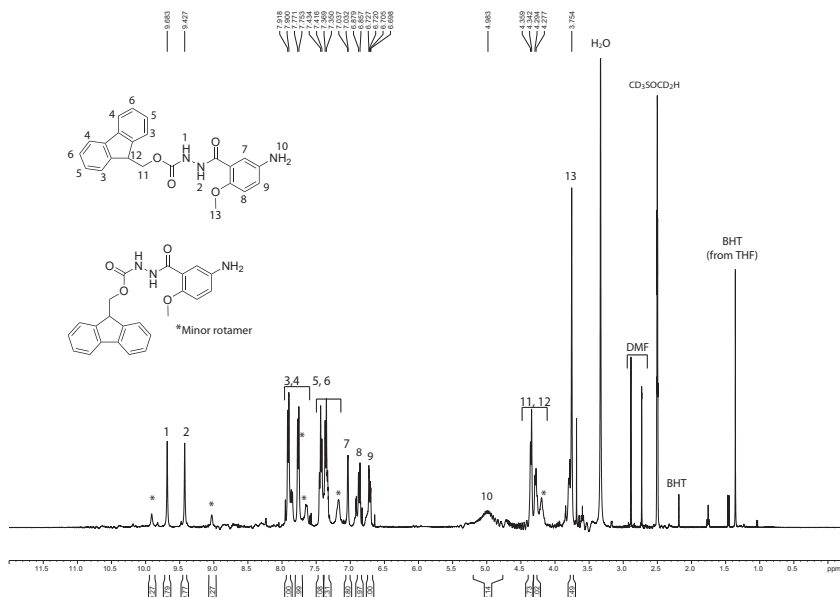
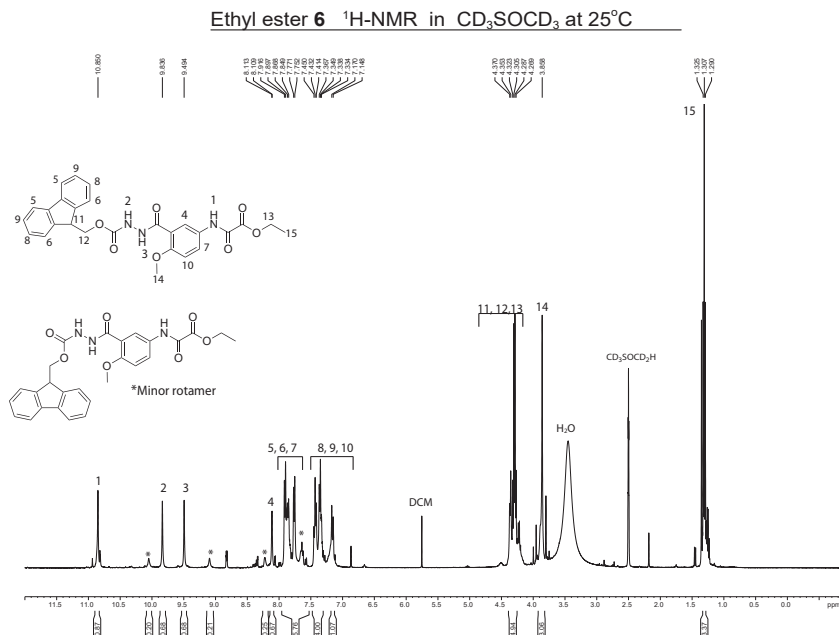
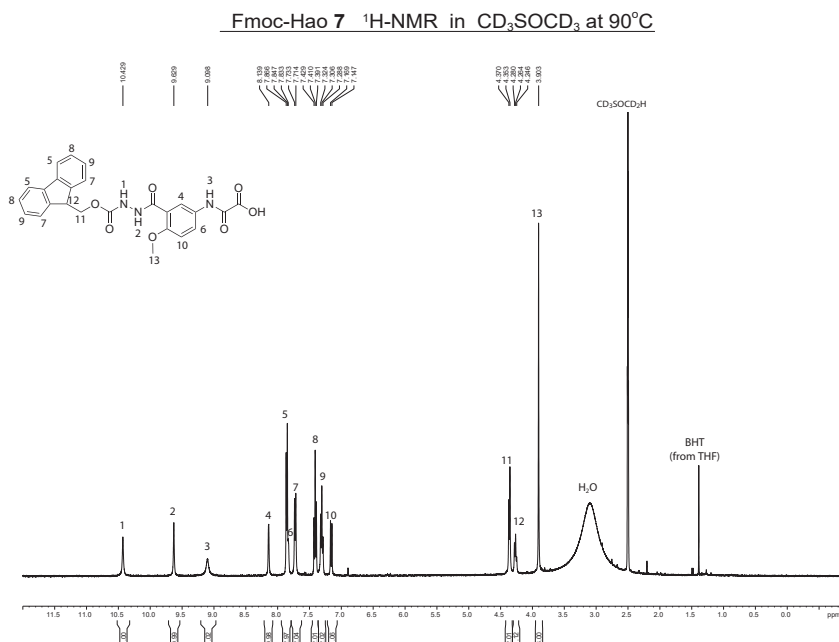


Fig. 9 $^1\text{H-NMR}$ spectra of amine **5**.

**Fig. 10** ^1H NMR spectra of ethyl ester 6.**Fig. 11** ^1H NMR spectra of Fmoc-Hao 7.

Fmoc-Hao 7 $^1\text{H-NMR}$ in CD_3SOCD_3 at 90°C (4-8 ppm)

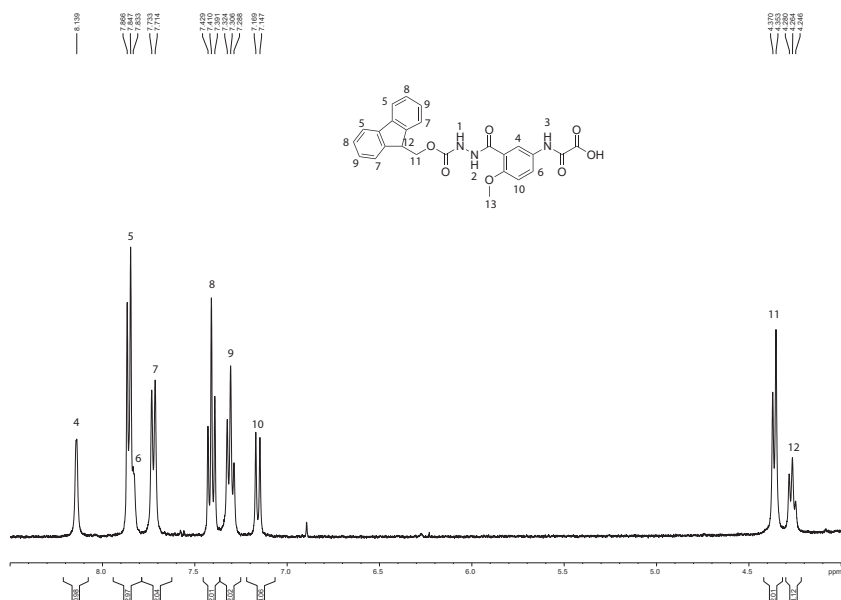


Fig. 12 ^1H NMR spectra of Fmoc-Hao 7 (4-8 ppm).

Fmoc-Hao 7 $^1\text{H-NMR}$ in CD_3SOCD_3 at 25°C

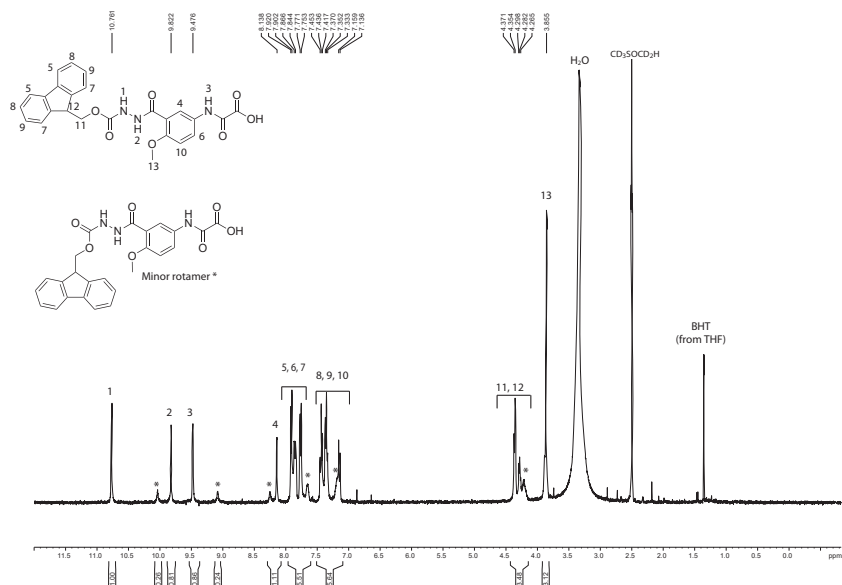


Fig. 13 ^1H NMR spectra of Fmoc-Hao at room temperature.

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