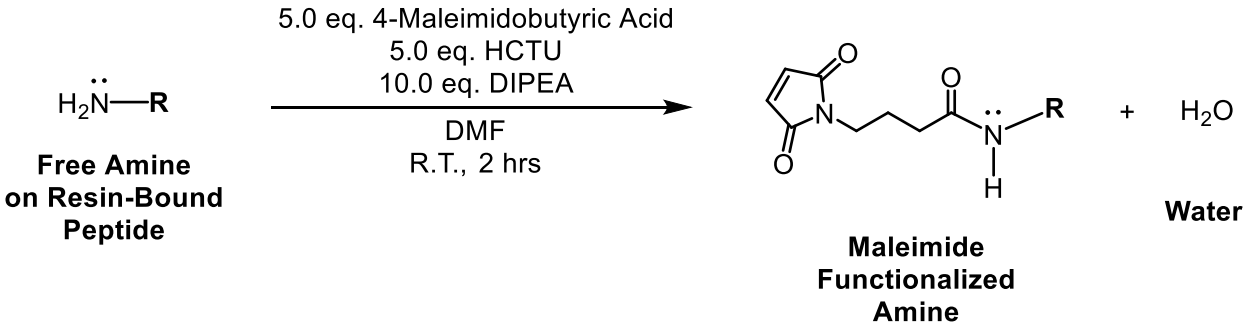
Maleimide/Carboxylic Acid Coupling Protocol

About

For certain applications it is valuable to add a reactive handle to the N-terminus of a peptide. The following protocol details the reaction of an amine, typically on the N-terminus of peptides, with a carboxylic acid molecule that contains a reactive handle. This yields a functionalized reactive handle on the N-terminus, or amine location, of a peptide on resin. If it is desired that the reactive handle be off the side of the peptide, it is often necessary to protect a side-chain amine through the allyloxycarbonyl (Alloc) group. The alloc group can then be removed to yield a free amine that can participate in the reaction detailed in this protocol. In this protocol, the coupling of a maleimide reactive group to a free amine on the peptide is detailed on resin. Another carboxylic acid can be substituted for the 4-maleimidobutyric acid, including coupling an amino acid by hand. This is because the chemistry for the reaction is similar to that for solid-phase peptide synthesis (SPPS) amide bond formation, utilizing different coupling reagents.[1] The following protocol is based on work used in multiple different bundlemer peptide applications.[2–6]

Reaction Scheme



Note: **R** represents the rest of the peptide, connected to the resin.

Glassware and Equipment

- 1 x Coarse Fritted Peptide Synthesis Reaction Vessel with Rubber Stopper
- 1 x Red Peptide Synthesis Reaction Vessel Cap
- 1 x 250 mL Erlenmeyer Flask with Side-Arm Connected to Vacuum Pump
- 1 x Vortex Mixer with Tube Foam Insert or Stir Bar, Clamp, and Stir Plate
- 1 x Fume Hood
- 1 x Analytical Balance
- 1 x Scoopula or Spatula
- 1 x Adjustable 1000-µL Micropipette
- 1 x Small Weigh Boat
- 1 x 8-mL Scintillation Vial

Materials

The materials needed for this protocol are provided below. The Fisher Scientific catalog numbers are provided in parentheses.

- Dichloromethane (AC610050040)
- Methanol (A412-4)
- N,N-Dimethylformamide (BP1160-4)
- 4-Maleimidobutyric Acid (M23371G)
- O-(1H-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium Hexafluorophosphate Novabiochem®, HCTU (C19881G)
- N,N-Diisopropylethylamine, DIPEA (AC367840250)

Reagent Tables

Table 1 – The reagent table for making the coupling solution for a 0.10 mmol peptide synthesis. The highlighted cells represent the mass or volume of the given component that should be combined.

Component	Ratio	Moles (mmol)	Molecular Weight (g/mol)	Mass (g)	Density (g/mL)	Volume (mL)
Peptide Resin	1.0	0.10				
4-Maleimidobutyric Acid	5.0	0.50	183.16	0.092		
HCTU	5.0	0.50	413.69	0.207		
N,N-Diispropylethylamine (DIPEA)	10.0	1.00	129.25	0.1293	0.742	0.174
N,N-Dimethylformamide (DMF)			73.09		0.944	3.826

Table 2 – The reagent table for making the coupling solution for a 0.25 mmol peptide synthesis. The highlighted cells represent the mass or volume of the given component that should be combined.

Component	Ratio	Moles (mmol)	Molecular Weight (g/mol)	Mass (g)	Density (g/mL)	Volume (mL)
Peptide Resin	1.0	0.25				
4-Maleimidobutyric Acid	5.0	1.25	183.16	0.229		
HCTU	5.0	1.25	413.69	0.517		
N,N-Diispropylethylamine (DIPEA)	10.0	2.50	129.25	0.3231	0.742	0.435
N,N-Dimethylformamide (DMF)			73.09		0.944	7.565

Safety Measures

When performing this protocol, users must wear safety glasses, laboratory gloves, pants, closed-toe shoes, and a fire-retardant laboratory coat. Everything should be performed in an efficient fume hood. These chemicals have the following hazard identifications:

Dichloromethane (DCM):

Methanol:

N,N-Dimethylformamide (DMF):

4-Maleimidobutyric Acid:

O-(1H-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium Hexafluorophosphate Novabiochem® (HCTU):

N,N-Diisopropylethylamine (DIPEA):

Procedures

1. Begin by ensuring the resin has been added to a coarse fritted peptide synthesis reaction vessel.
2. Perform a dichloromethane (DCM) wash of the resin for one minute. This is done by adding DCM to the reaction vessel, placing a red cap on the reaction vessel, and placing the reaction vessel either in the tube foam insert of a vortex mixer or adding a stir bar to the reaction vessel and holding the reaction vessel with a clamp above a stir plate.
    a. Add ~2 mL of DCM for a 0.10 mmol scale synthesis.
    b. Add ~4 mL of DCM for a 0.25 mmol scale synthesis.
3. Remove the reaction vessel from the stirring, and place the black rubber stopper on the bottom of the reaction vessel in the top of the Erlenmeyer flask connected to the vacuum. Drain the reaction vessel by taking the red cap off, opening the stopcock on the reaction vessel, and turning on the vacuum.
4. Close the reaction vessel and remove from the vacuum.
5. Repeat steps 2-4 (washing and draining the reaction vessel) for one- to two-minute washes with the following solvents:
    a. 2 x DCM
    b. 2 x Methanol
    c. 3 x DCM
6. Next, begin the preparation of the coupling solution by weighing and adding 5 equivalents of 4-maleimidobutyric acid for every equivalent of peptide to an 8-mL scintillation vial.
    a. 0.10 mmol: 0.092 g 4-maleimidobutyric acid
    b. 0.25 mmol: 0.229 g 4-maleimidobutyric acid
7. Using a small weigh boat ***in the fume hood***, measure 5 equivalents of HCTU and add to the 8-mL scintillation vial.
    a. 0.10 mmol: 0.207 g HCTU
    b. 0.25 mmol: 0.517 g HCTU
8. In the same 8-mL scintillation vial, pipette 10 equivalents of N,N-Diisopropylethylamine (DIPEA) for every equivalent of peptide.
    a. 0.10 mmol: 174 µL DIPEA
    b. 0.25 mmol: 435 µL DIPEA
9. Finally, add N,N-Dimethylformamide (DMF) to the 8-mL scintillation vial. For a 0.10 mmol scale, the total volume should be 4-mL. For a 0.25 mmol scale, the total volume should be 8-mL.
    a. 0.10 mmol: 3.826 mL DMF
    b. 0.25 mmol: 7.565 mL DMF
10. Shake the 8-mL scintillation vial till everything is mixed and dissolved. Wait five minutes.

11. After waiting five minutes, add the vial's contents to the peptide synthesis reaction vessel. Place the red cap on the reaction vessel, and let the vessel shake on the vortex mixer or on the stir plate for two hours.
12. After two hours, drain the reaction vessel by placing the reaction vessel rubber stopper into the top of the Erlenmeyer flask vacuum setup, taking the red cap off, opening the stopcock on the reaction vessel, and turning on the vacuum.
13. Once the coupling solution has been removed from the reaction vessel, perform the following washes, shaking for one to two minutes per wash:
    a. 1 x DCM
    b. 1 x Methanol
    c. 1 x DCM
14. After completing those washes, repeat steps 6-11, once more, to perform a second coupling.
15. After the second coupling, drain the reaction vessel as before. Perform the following washes, shaking for two minutes per wash:
    a. 3 x DCM
    b. 1 x Methanol
    c. 3 x DCM
16. The maleimide or other carboxylic acid handle coupling is now completed. Further modifications can be performed, or the peptide can be cleaved from resin.

Reaction Mechanism

The reaction mechanism for the reaction between a free amine and a carboxylic acid utilizing HCTU and DIPEA as coupling reagents is given in **Figure 1** on the following page. The carboxylic acid is first activated by HCTU. In this process, a urea byproduct is formed. The free amine then continues the reaction through nucleophilic attack of the activated carbonyl carbon. This leads to the formation of a tetrahedral intermediate that forces the HCTU derivative to act as the leaving group. DIPEA steals a hydrogel atom from the amine to yield a positively charged DIPEA derivative, a negatively charged HCTU derivative, and the functionalized amine, which in this case is a maleimide click reactive handle.

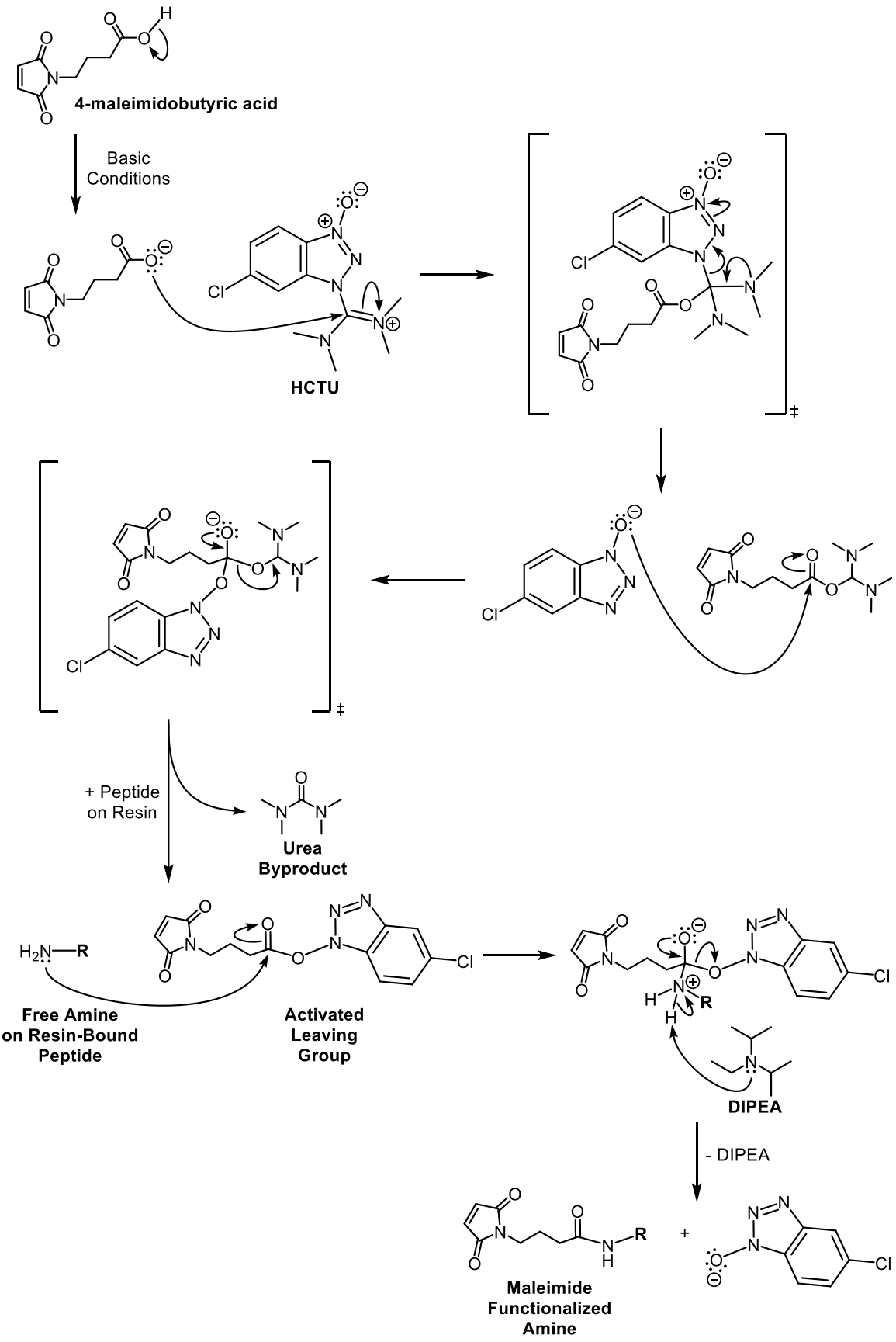

4-maleimidobutyric acid
Basic Conditions
HCTU
+ Peptide on Resin
Urea Byproduct
Free Amine on Resin-Bound Peptide
Activated Leaving Group
DIPEA
- DIPEA
Maleimide Functionalized Amine

Figure 1 – (Previous page) The reaction mechanism for amide bond formation using HCTU and DIPEA as coupling reagents. **R** represents the rest of the peptide.[1]

References

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(2) Wu, D.; Sinha, N.; Lee, J.; Sutherland, B. P.; Halaszynski, N. I.; Tian, Y.; Caplan, J.; Zhang, H. V.; Saven, J. G.; Kloxin, C. J.; Pochan, D. J. Polymers with Controlled Assembly and Rigidity Made with Click-Functional Peptide Bundles. *Nature* **2019**, *574* (7780), 658–662. https://doi.org/10.1038/s41586-019-1683-4.

(3) Sinha, N. J.; Wu, D.; Kloxin, C. J.; Saven, J. G.; Jensen, G. V.; Pochan, D. J. Polyelectrolyte Character of Rigid Rod Peptide Bundlemer Chains Constructed via Hierarchical Self-Assembly. *Soft Matter* **2019**, *15* (48), 9858–9870. https://doi.org/10.1039/C9SM01894H.

(4) Sinha, N. J.; Shi, Y.; Tang, Y.; Kloxin, C. J.; Saven, J. G.; Faraone, A.; Jensen, G. V.; Pochan, D. J. Intramolecular Structure and Dynamics in Computationally Designed Peptide-Based Polymers Displaying Tunable Chain Stiffness. *Phys. Rev. Mater.* **2021**, *5* (9), 095601. https://doi.org/10.1103/PhysRevMaterials.5.095601.

(5) Guo, R.; Sinha, N. J.; Misra, R.; Tang, Y.; Langenstein, M.; Kim, K.; Fagan, J. A.; Kloxin, C. J.; Jensen, G.; Pochan, D. J.; Saven, J. G. Computational Design of Homotetrameric Peptide Bundle Variants Spanning a Wide Range of Charge States. *Biomacromolecules* **2022**, *23* (4), 1652–1661. https://doi.org/10.1021/acs.biomac.1c01539.

(6) He, Y.; Wu, D.; Zhang, X. Bottom–up on-Surface Synthesis Based on Click-Functionalized Peptide Bundles. *Nanoscale* **2023**, *15* (20), 8996–9002. https://doi.org/10.1039/D3NR01070H.