

# Peptide Library Synthesis

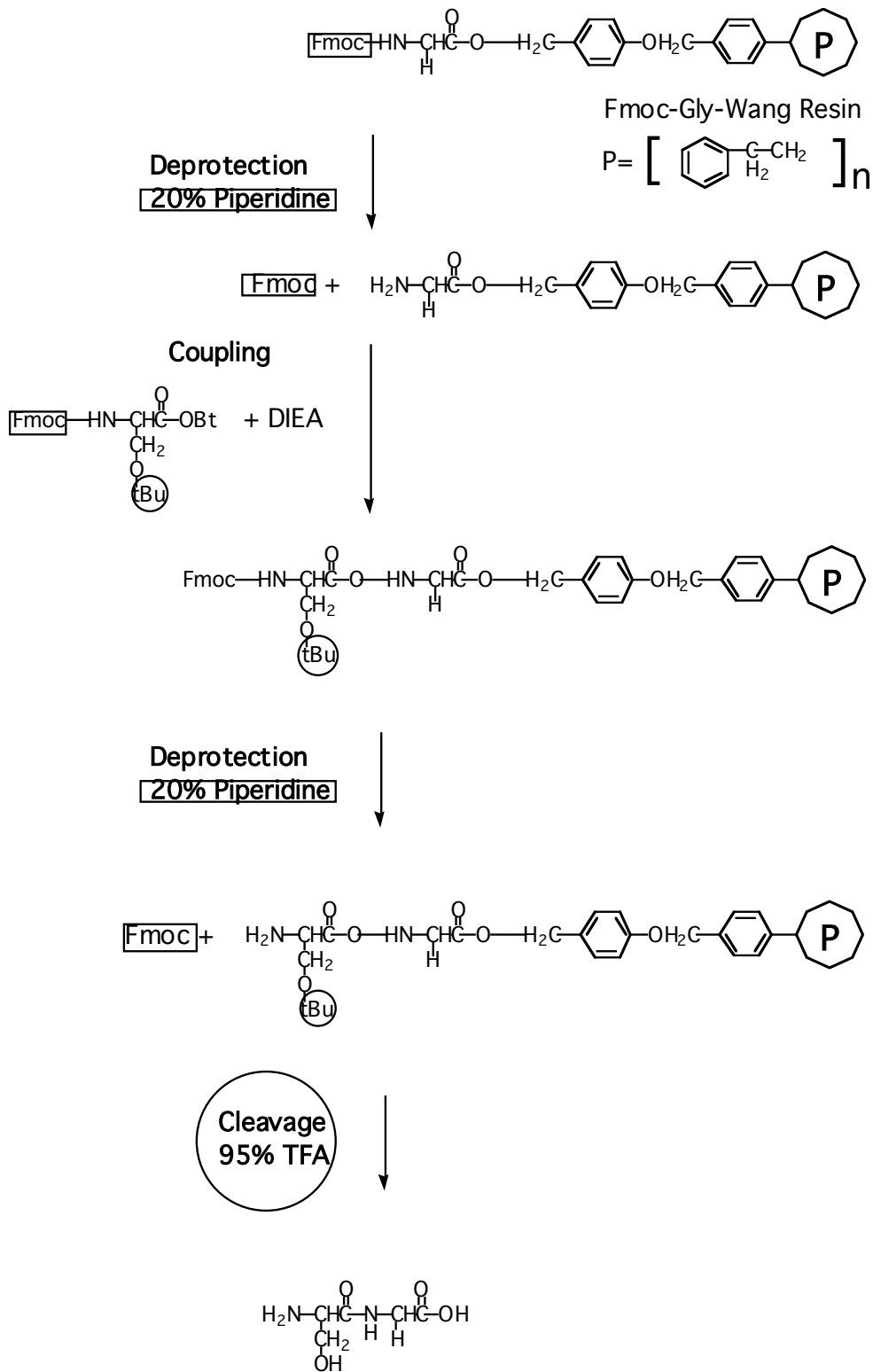
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Solid phase peptide synthesis consists of assembling amino acids from the C-terminal to the N-terminal. The  $\alpha$ -carboxyl group is attached via an acid-labile linker to a solid support, "resin" (Figure 1). Resins commonly used are composed of polystyrene (**P**). The amino terminal end of the amino acid is protected by a base-labile Fmoc (9-fluorenylmethoxycarbonyl) protecting group while the side chains are protected by acid-labile groups such as tertiary-butyl (tBu). After the first amino acid is loaded onto the resin, the Fmoc group is removed using piperidine (Deprotection). A Kaiser test is then performed to confirm that all of the Fmoc protecting groups are removed. The next Fmoc-amino acid is then attached to the growing peptide by activation of its carboxyl group (Coupling). A Kaiser test is then performed to confirm that complete coupling has occurred on all the free amines on the resin. Synthesis then proceeds through a cycle of 1) deprotection of Fmoc amino terminus groups and 2) coupling of the next amino acid until the peptide is completely synthesized. The completely synthesized peptide is then cleaved from the resin and side chain protection groups are removed using trifluoroacetic acid (Cleavage).

Figure 1



### **Reagents**

- Acetic Anhydride
- Acetonitrile
- Alpha cyano 4-hydroxy cinnamic acid (alpha CHC acid), Agilent Technologies
- Fmoc-Amino Acids (Table 1), Novabiochem or Advanced Chemtech
- Ammonium iodide
- Dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>)
- Diethylether
- Anhydrous *N,N*-Dimethylformamide (DMF), J.T. Baker
- Diisopropylethylamine (DIEA), Acros
- Dimethylsulphide
- 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), Quantum Biotech
- Ethanedithiol (EDT)
- Ethanol
- 5-iodoacetamido fluorescein (5-IAF), Molecular Probes
- β-mercaptoethanol
- 1-(Mesitylene-2-sulfonyl)-3-nitro-1H-1,2,4-triazole (MSNT), Novabiochem
- 1-Methylimidazole
- Ninhydrin
- Phenol
- Piperidine
- Potassium cyanide
- Pyridine
- Thioanisole
- Trifluoroacetic acid (TFA)
- Wang Resin (100-200 mesh), Novabiochem
- Wang Preloaded Resin (Table 2), Novabiochem

**Note:** Unless otherwise stated, reagents are purchased from Fisher.

### **Stock Solutions**

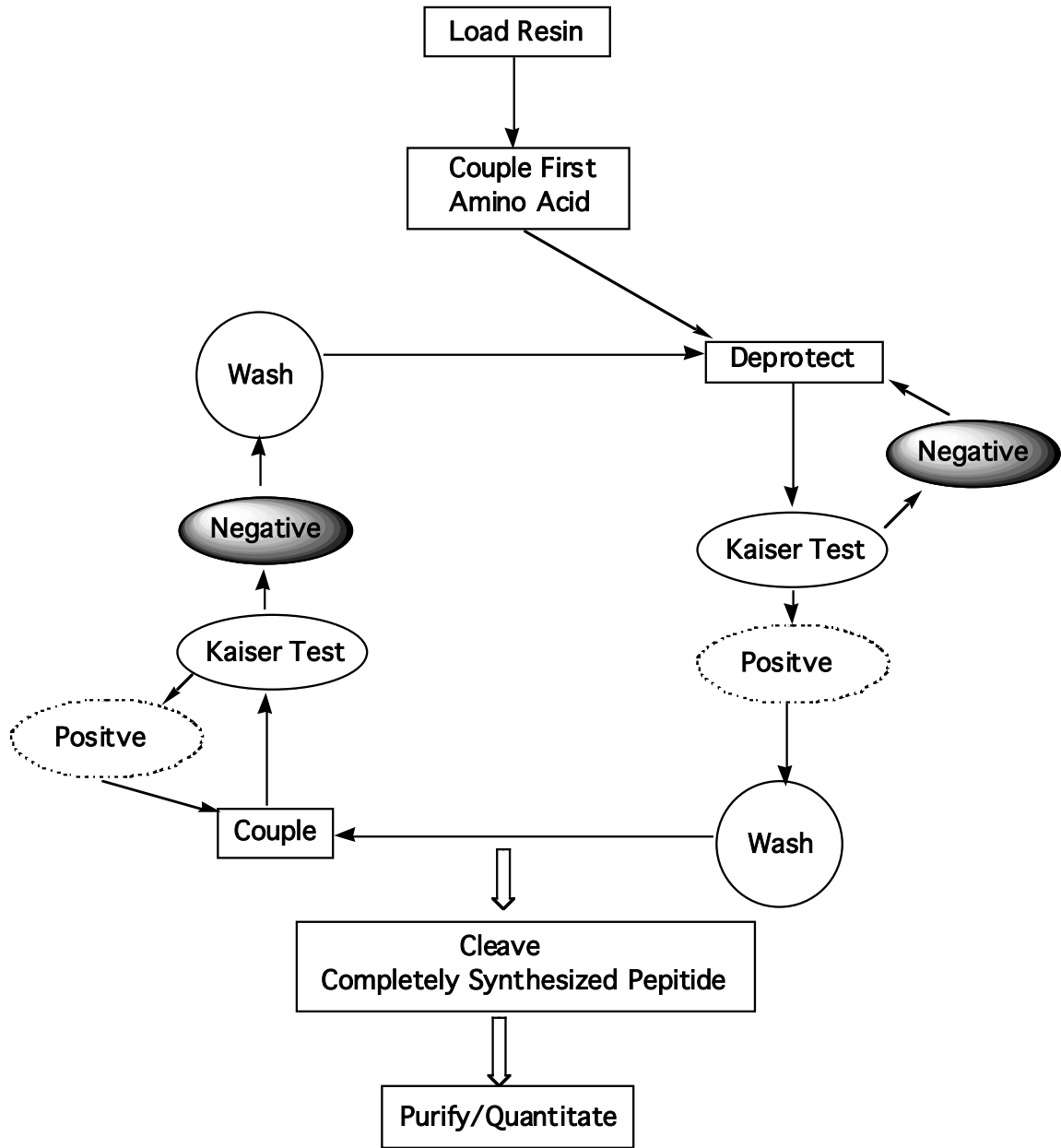
- **20% Piperidine**  
20% piperidine in DMF (v/v)
- **Kaiser Test Solutions**
  - A. Ninhydrin (5%, w/v) in ethanol
  - B. Phenol (4:1, w/v) in ethanol
  - C. Potassium cyanide (2%, v/v, of a 1 mmole/liter aqueous solution) in pyridine

### **Apparatus**

- Robbins Flexchem Reactor Block (48 or 96 well)

- Sealing Covers (top-red, bottom-blue)
- Rubber gasket (2)
- Viton gasket (2)
- Flexchem rotator
- Robbins Resin Loader
- Speed Vac (GeneVac HT-4 or Mega)
- Deep well plates (48 or 96)
- Preparatory HPLC (Biotage Parallel Flex)
- MALDI-TOF (Perseptives Voyager)
- Analytical HPLC (Waters Alliance 2695)
- LCMS (Waters Micromass ZQ)
- UV Spectrophotometer (Spectronic Genesys 5)

### Peptide Synthesis Flow Chart



## **1. Loading of C-terminal Amino Acid to Wang Resin**

(Skip to Step 3 if using preloaded Wang Resin)

- 1.1. Place appropriate amount of Wang resin in wells of reactor block (96 well: 50mg, 48 well: 100mg) either using the Robbins Resin loader or manually with weighing paper.
- 1.2. Mix 3.3 equivalents (eq.) of Fmoc-amino acid to be coupled with 0.1M MSNT in  $\text{CH}_2\text{Cl}_2$ , and 2.25 eq. 1-Methylimidazole. Add to appropriate wells in the reactor block, place block on rotator, and allow to couple for at least 1 1/2 hours and maximally for 8 hours. (volume per well: 48 well, 2mL; 96 well, 1mL,).
- 1.3. Remove block from rotator. Manually invert block quickly then wait 1-2 minutes to allow resin to fall into solution. Remove bottom cover followed by top cover. This step is necessary to remove excess resin from the cover.
- 1.4. Allow the solution to drain and wash resin with DMF two times (2x 1mL).
- 1.5. Repeat steps 1.2-1.4 two times.
- 1.6. Wash thoroughly with DMF and  $\text{CH}_2\text{Cl}_2$ . (See Step 2)
- 1.7. Add acetic anhydride to acetylate any remaining functional groups of the resin.
- 1.8. Wash thoroughly with DMF and  $\text{CH}_2\text{Cl}_2$ . (See Step 2)
- 1.9. Proceed to Step 4.

## **2. Wash Step**

- 2.1. First rinse with DMF along all four walls of the well.
- 2.2. Rinse again with DMF forcefully on the center of the well to agitate resin using approximately 1 mL. Rotate block. Pull off DMF completely.
- 2.3. Repeat 2.2 2-3 times.
- 2.4. Rinse with  $\text{CH}_2\text{Cl}_2$  following the same procedure outlined in 2.1.
- 2.5. Rinse again with  $\text{CH}_2\text{Cl}_2$  forcefully on the center of the well to agitate resin.
- 2.6. Repeat step 2.5 2-3 times.)
- 2.7. Rinse both top and bottom seal with  $\text{CH}_2\text{Cl}_2$  making sure to remove any resin.

**Note:** When performing washes, the force of the reagent and the number of washes is more critical than the volume of reagent. In between washes, allow solutions to drain completely.

## **3. Preloaded Wang Resin**

- 3.1. Place appropriate amount of Wang resin in wells of reactor block (96 well: 50mg, 48 well: 100mg) either using the Robbins Resin loader or manually with weighing paper.
- 3.2. Proceed to Step 4.

## **4. Deprotection - Removal of Fmoc Amino-Terminal Protecting Group**

- 4.1. Add 20% piperidine in DMF (v/v) to each well. (volume per well: 48 well, 2mL; 96 well, 1mL,).
- 4.2. Rotate block on rotator and allow deprotection to proceed for 30 minutes.

- 4.3. Remove block from rotator. Manually invert block quickly then wait 1-2 minutes to allow resin to fall into solution. Remove bottom cover followed by top cover. This step is necessary to remove excess resin from the cover.
- 4.4. Allow the solution to drain completely.
- 4.5. Wash (Step 2).
- 4.6. Perform Kaiser Test (Step 5).
  - 4.6.1. Negative Kaiser Test: repeat steps 4.1-4.3.
  - 4.6.2. Positive Kaiser Test:
    - 4.6.2.1. If peptide is not completely synthesized proceed to coupling Step 6.
    - 4.6.2.2. If peptide is completely synthesized proceed to cleavage Step 7.

**Note:** Washing all of the piperidine out is crucial before proceeding to next step.

## 5. Kaiser Test

- 5.1. Make up three test solutions:
  - A. Ninhydrin (5% w/v) in ethanol
  - B. Phenol (4:1, w/v) in ethanol
  - C. Potassium cyanide (2%, v/v, of a 1 mmole/liter aqueous solution) in pyridine
- 5.2. The test is carried out by adding 2 drops of A, 1 drop of B and 1 drop of C to the test sample (usually 1-2 mg of resin, 10-20 resin particles) contained in a small glass test tube and then heating the tube for 2-5 minutes on a hot plate.
- 5.3. A blue coloration of the resin is a positive result indicating that there are free amines on the growing peptide i.e.: coupling is incomplete or deprotection is complete. Occasionally, some amino acid residues can give unusual coloration ranging from red to blue (Asn, Cys, Ser, Thr).
- 5.4. If the resin does not change color, this is a negative test i.e.: coupling is complete or deprotection is incomplete.

**Note:** The result of the Kaiser test is based on the color of the resin and not the color of the solution. If solution turns blue, it could be due the presence of DIEA, piperidine, or too much DMF.

## 6. Coupling - Peptide Bond Formation

- 6.1. Add in order 1) 3 eq. of Fmoc-amino acid, 2) 2.5 eq. of HBTU, and 3) DMF (volume per well: 48 well, 1.5 mL; 96 well, 1 mL) allow mixture to react for 2-3 minutes (agitation may be required to get Fmoc-amino acid into solution) Alternatively, a pre-made solution of HBTU in DMF can be made fresh each day and stored at 5°C, then added to Fmoc-amino acid.
- 4) Add 5 eq. of DIEA after Fmoc-amino acid is in solution, mix.
- 6.2. Add mixture to appropriate wells.
- 6.3. Place reactor block on rotator and allow coupling to occur for a minimum of 1 1/2 hours and a maximum of 8 hours.

- 6.4. Remove block from rotator. Manually invert block quickly then wait 1-2 minutes to allow resin to fall into solution. Remove bottom cover followed by top cover. This step is necessary to remove excess resin from the cover.
- 6.5. Allow the solution to drain.
- 6.6. Wash (Step 2).
- 6.7. Perform Kaiser Test (Step 5).
  - 6.7.1. Negative Kaiser Test: proceed to deprotection Step 4.
  - 6.7.2. Positive Kaiser Test:
    - 6.7.2.1. Repeat Steps 6.1-6.2.
    - 6.7.2.2. Allow coupling to proceed for 30 minutes.
    - 6.7.2.3. Remove top cover, do not draw off solution, and perform Kaiser testing resin from at least 3 different wells.
    - 6.7.2.4. For negative Kaiser Test proceed to deprotection Step 4.
    - 6.7.2.5. For positive Kaiser Test, replace top cover and allow coupling to continue until a negative Kaiser test is obtained. If Kaiser Test remains positive after 8 hours, Repeat Step 6.7.2.

## 7. **Cleavage of Peptides from Resin**

- 7.1. After performing the deprotection Step 4, further wash the resin with  $\text{CH}_2\text{Cl}_2$  (3 times).
- 7.2. Wash resin with methanol to shrink the resin.
- 7.3. Allow the resin to dry under vacuum for at least four hours or overnight.
- 7.4. Cleavage Cocktail
  - 7.4.1. For peptides containing methionine and cysteine: 81% TFA, 5% phenol, 5% thioanisole, 2.5% EDT, 3% water, 2% dimethylsulphide, 1.5% ammonium iodide (w/w).

**Note: This is a very stinky solution!!** Perform everything in the hood and wash everything this solution touches with bleach prior to removing from hood including gloves.
  - 7.4.2. For all other peptides: 95% TFA, 1% thioanisole, 2% phenol, 2% water.
- 7.5. Add appropriate cleavage cocktail to wells (volume per well: 96 well, 1mL; 48 well, 1.5mL).
- 7.6. Allow peptides to cleave for at least 2 hours up to 24 hours. Note: Peptides containing > 2 arginines require longer cleaving times to remove protecting groups.
- 7.7. After cleavage time is complete, collect filtrate in deep well plates (46 or 96 well).
- 7.8. Wash resin twice using TFA, collecting the filtrate.
- 7.9. Remove TFA using Genvac.
- 7.10. Dissolve the precipitate in < 50% ACN/water.
- 7.11. Purify peptide using HPLC-MS.

## 8. **Purification by HPLC on Biotage**

- 8.1. Before using the Biotage, new peptides may require method optimization on an analytical HPLC system.
- 8.2. Before using the Biotage proper training is required.
- 8.3. Once a method has been established, place crude peptides in either 96 well or 48 deep well plates.
- 8.4. Run Biotage Method and follow current Biotage Protocols
  - 8.4.1. For NCOA peptides use the following method:
    - UV detection: 214 and 280nm
    - Injection volume: 1.8 mL
    - Mobile Phase A: Water 0.05% TFA
    - Mobile Phase B: Acetonitrile 0.05% TFA
    - Flow Rate: 20mL/min
    - Gradient:

	Mobile Phase A	Mobile Phase B	Time (minutes)
Equilibrate	90%	10%	3.0
Injection	90%	10%	0.6
Gradient	10%	90%	12.0
	10%	90%	1.0
Equilibrate	90%	10%	3.0

- 8.4.2. Collect fractions based on peak absorption at 214 and 280nm.
  - 8.4.3. Identify fractions using MALDI-TOF.
    - 8.4.3.1. Add 0.5 $\mu$ L of sample to plate
    - 8.4.3.2. Add 0.5 $\mu$ L of matrix (alpha CHC Acid) on top of samples and allow to dry
    - 8.4.3.3. Detect samples on MALDI-TOF (training may be required).
  - 8.4.4. Dry down selected samples on Genevac (Mega or HT-4) using the two step acetonitrile preprogrammed method.
- 9. Attaching a thiol reactive fluorophore (5-iodoacetamido fluorescein)**
- 9.1. Dissolve 2-5mg of peptide (minimum 2 mg) in 2 Ml of phosphate buffer, (50 Mm sodium phosphate, 154 Mm NaCl, Ph 7.2). If peptide is very hydrophobic dissolve in DMF. Final concentration should be approximately 250 $\mu$ M.
  - 9.2. Dissolve 100mg of 5-IAF in 1 Ml of DMF (194Mm)
  - 9.3. Add 50 $\mu$ L to 2 Ml peptide solution (approximately 20-fold excess)
  - 1.4. Allow reaction to proceed for at least 2 hours.
  - 9.5. Quench with a an excess of  $\beta$ -mercaptoethanol, approximately 20  $\mu$ L.
  - 9.6. HPLC purify following step 8.0.
- 10. Quality Control Testing**
- 10.1. Test the purity of the peptides samples using LCMS.
  - 10.2. Dissolve peptides in 50:50 Acetonitrile:Water
  - 1.3. LCMS methods may need to be optimized for individual peptides and libraries
  - 10.4. For labeled NCOA peptides use the following method:

UV detection: Diode Array detection  
 Injection volume: 10  $\mu$ L  
 Mobile Phase A: Water 0.05% TFA  
 Mobile Phase B: Acetonitrile 0.05% TFA  
 Flow Rate: 0.2 MI/min  
 Gradient:

	Mobile Phase A	Mobile Phase B	Time (minutes)
Equilibrate	90%	10%	1.0
Gradient	10%	90%	15.0
	10%	90%	1.0
	90%	10%	4.0
Equilibrate	90%	10%	5.0

MS Tune Conditions:

Capillary (kV)	3.0
Cone (V)	51
Extractor (V)	5
RF Lens	0
Source Temperature ( $^{\circ}$ C)	100
Desolvation Temperature ( $^{\circ}$ C)	250

### **11. Determination of Peptide Concentration for Labeled Peptides (5-IAF)**

- 11.1. Dissolve peptides in DMSO.
- 11.2. Add 10  $\mu$ L of peptides from 11.1 into 1 mL Tris Buffer (20 mM Tris, pH 9.0).
- 11.3. Detect UV absorption at 492 nm.
- 11.4. Calculate concentration using the extinction coefficient of 78,000  $\text{cm}^{-1} \text{M}^{-1}$ .

### **12. Storage of Peptides**

- 12.1. The best storage conditions are in the dried state at  $-80^{\circ}$ C.  
 Alternatively, store stock solutions in DMSO at  $-80^{\circ}$ C.
- 12.2. Working stock solutions can be made in buffer and stored at  $-80^{\circ}$ C.

**Table 1. List of common Fmoc-amino acids**

<b>Amino Acid</b>	<b>Fmoc Protected</b>	<b>g/mole</b>
<b>A</b>	Fmoc-Ala-OH	311.30
<b>R</b>	Fmoc-Arg(Mtr)-OH	608.70
<b>N</b>	Fmoc-Asn(Trt)-OH	596.70
<b>D</b>	Fmoc-Asp(OtBu)-OH	411.50
<b>C</b>	Fmoc-Cys(trt)-OH	585.70
<b>Q</b>	Fmoc-Gln(Trt)-OH	610.70
<b>E</b>	Fmoc-Glu(OtBu)-OH	425.50
<b>G</b>	Fmoc-Gly-OH	297.30
<b>H</b>	Fmoc-His(Trt)-OH	619.70
<b>I</b>	Fmoc-Ile-OH	353.40
<b>L</b>	Fmoc-Leu-OH	353.40
<b>K</b>	Fmoc-Lys(Boc)-OH	468.55
<b>M</b>	Fmoc-Met-OH	371.50
<b>F</b>	Fmoc-Phe-OH	387.00
<b>P</b>	Fmoc-Pro-OH	337.40
<b>S</b>	Fmoc-Ser(tBu)-OH	383.40
<b>T</b>	Fmoc-Thr(tBu)-OH	397.50
<b>W</b>	Fmoc-Trp(Boc)-OH	526.60
<b>Y</b>	Fmoc-Tyr(tBu)-OH	466.00
<b>V</b>	Fmoc-Val-OH	339.40

Purchased from Novabiochem or Advanced Chemtech

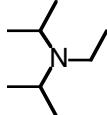
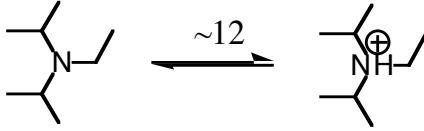
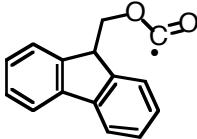
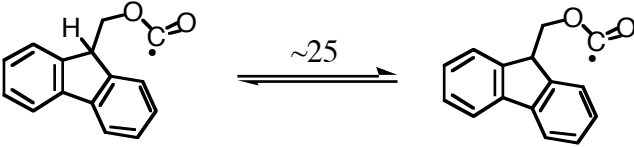
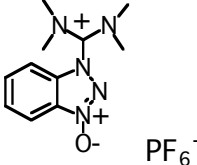

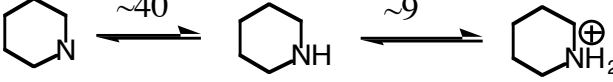
**Table 2. List of common Fmoc-amino acid wang resin**

<b>Amino Acid</b>	<b>Fmoc Amino Acid Resin</b>	<b>*Substitution (mmol/g)</b>
<b>A</b>	Fmoc-Ala-Wang	0.69
<b>R</b>	Fmoc-Arg(Pbf)-Wang	0.35
<b>N</b>	Fmoc-Asn(Trt)-Wang	0.40
<b>D</b>	Fmoc-Asp(OtBu)-Wang	0.86
<b>C</b>	Fmoc-Cys(Trt)-Wang	0.45
<b>Q</b>	Fmoc-Gln(Trt)-Wang	0.71
<b>E</b>	Fmoc-Glu(OtBu)-Wang	0.51
<b>G</b>	Fmoc-Gly-Wang	1.00
<b>H</b>	Fmoc-His(Trt)-Wang	0.39
<b>I</b>	Fmoc-Ile-Wang	0.81
<b>L</b>	Fmoc-Leu-Wang	0.88
<b>K</b>	Fmoc-Lys(Boc)-Wang	0.60
<b>M</b>	Fmoc-Met-Wang	0.43
<b>F</b>	Fmoc-Phe(4-1)-Wang	0.60
<b>P</b>	Fmoc-Pro-Wang	0.51
<b>S</b>	Fmoc-Ser(tBu)-Wang	0.94
<b>T</b>	Fmoc-Thr(tBu)-Wang	0.61
<b>W</b>	Fmoc-Trp-Wang	0.54
<b>Y</b>	Fmoc-Tyr(tBu)-Wang	0.88
<b>V</b>	Fmoc-Val-Wang	0.68

\*Substitution may vary from lot to lot

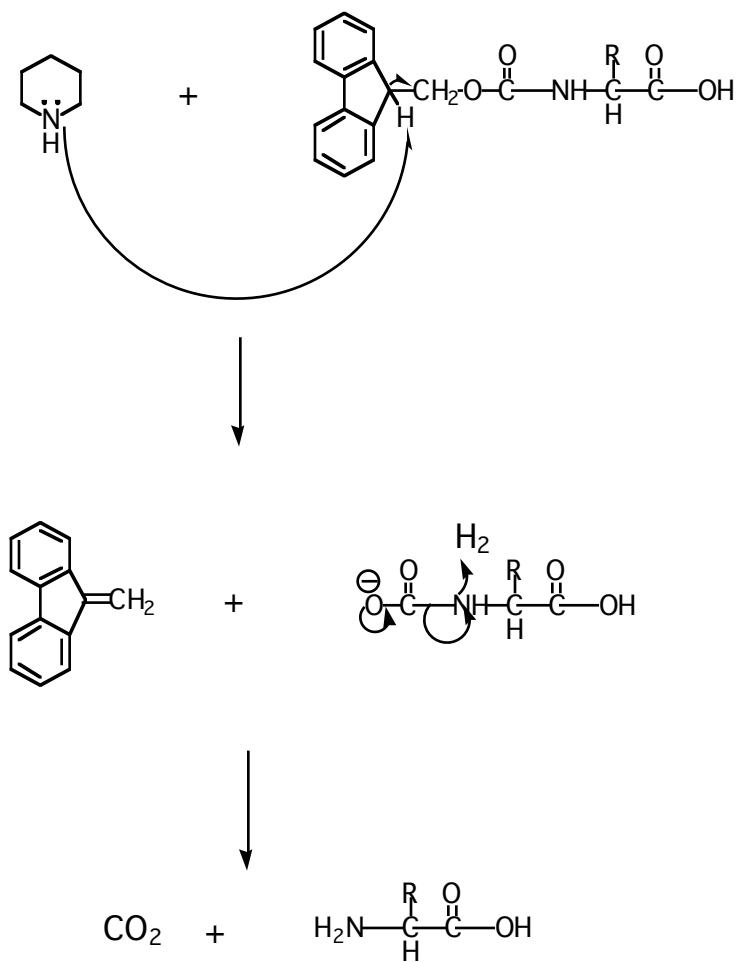
Purchased from Novabiochem or Advanced Chemtech

**Table 3. Relevant Physical Properties**

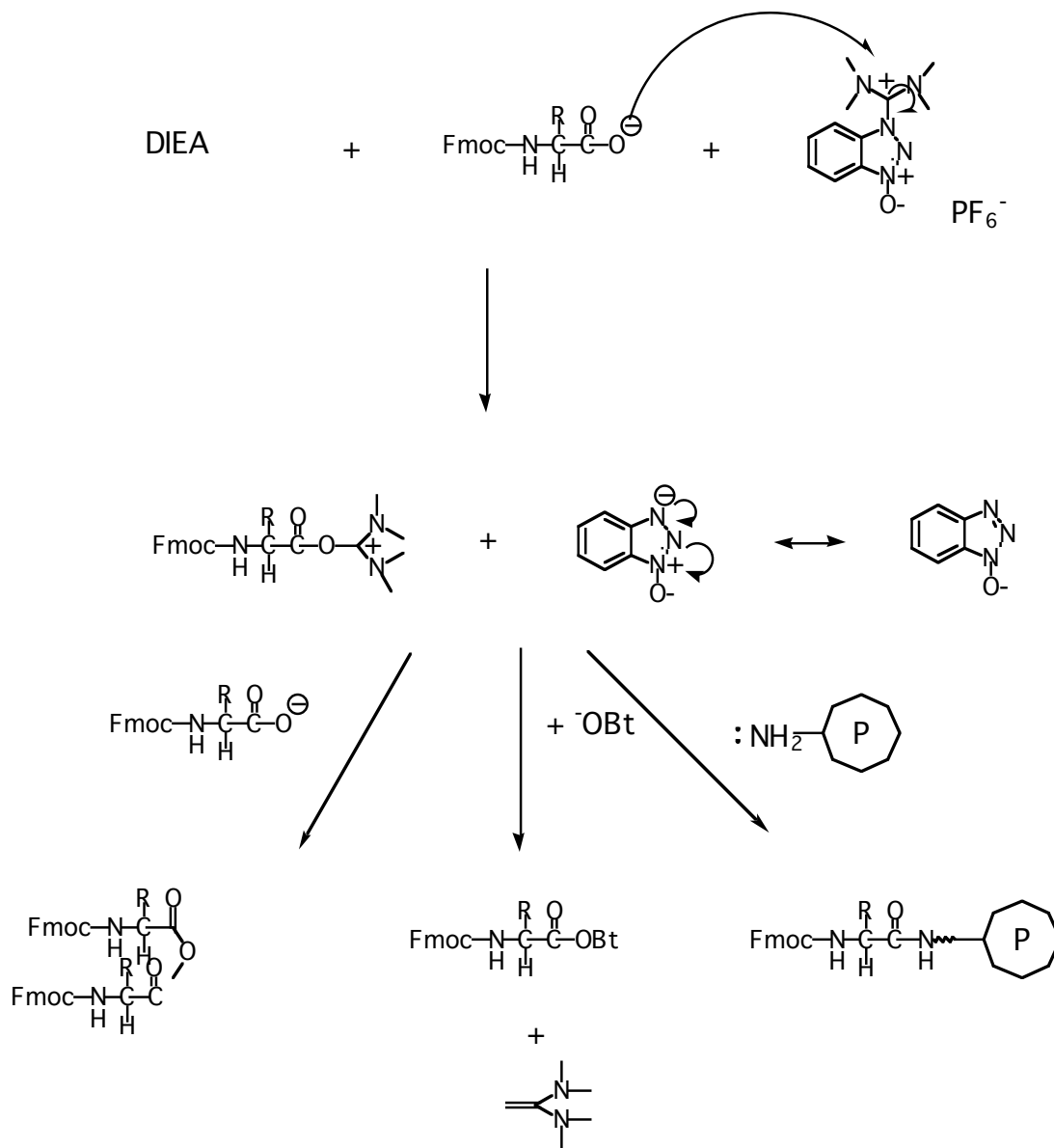
Reagent	Structure	Molecular Weight	Density	pKa
DIEA		129.24	0.74	
Fmoc				
HBTU		379.3		
Piperidine		85.15		

## Mechanisms

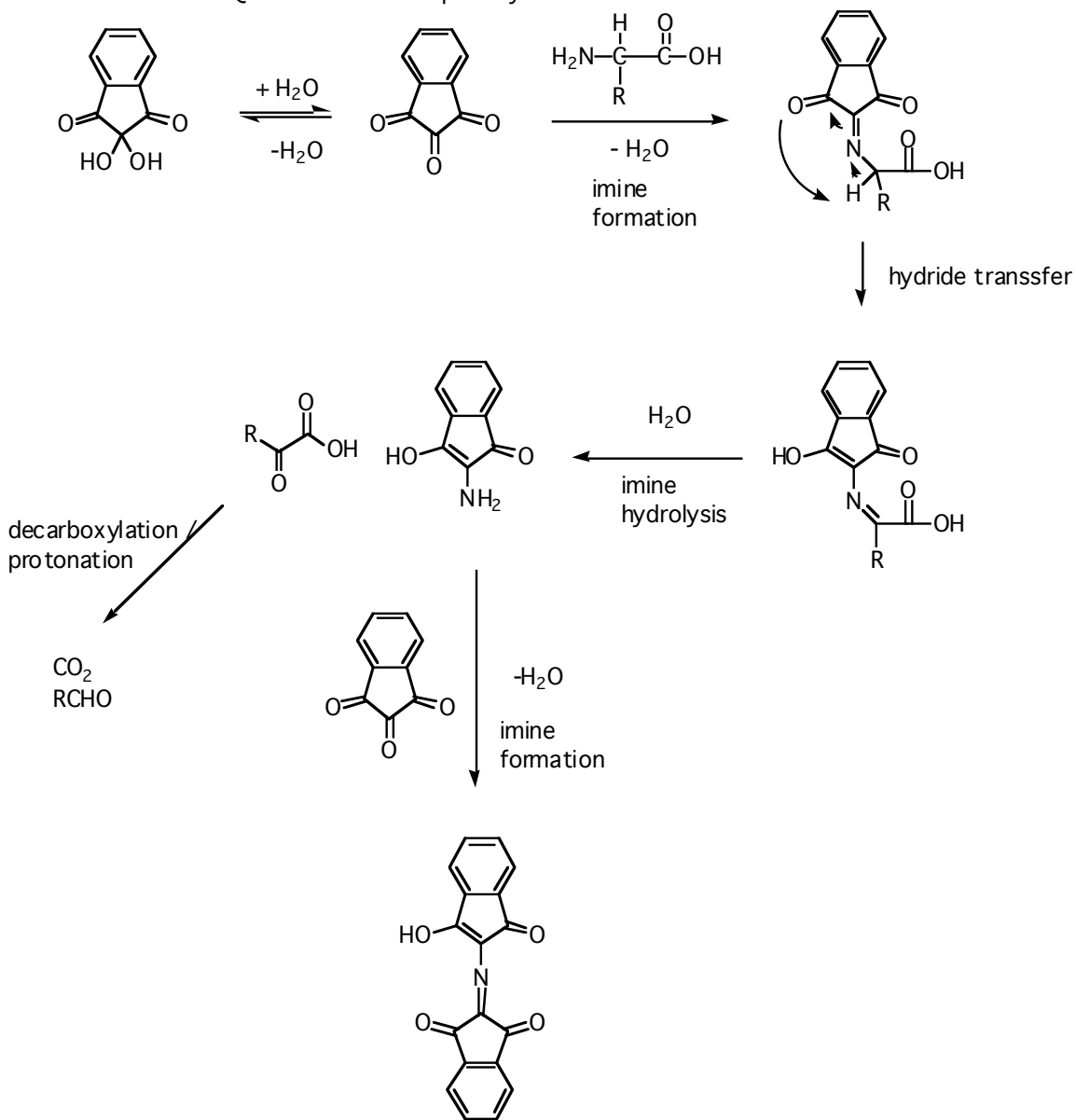
### I. Deprotection - Removal of Fmoc group using piperidine



II. Coupling Reaction - Coupling of Fmoc-amino acid to growing peptide using HBTU



III. Kaiser Test - Qualitative test for primary amines



**IV. Cleavage Reaction - Mechanism for the reduction of methionine sulphoxide to methionine with TFA, ammonium iodide, and dimethylsulphide**

