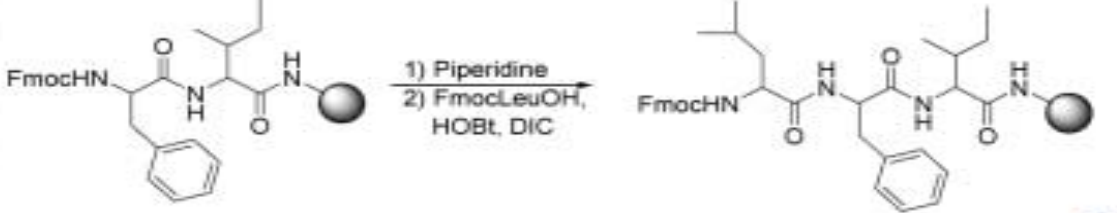
Bruce Merrifield

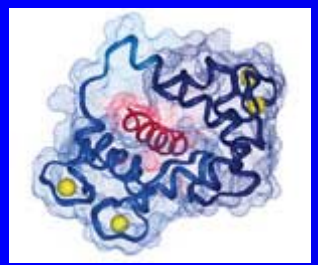


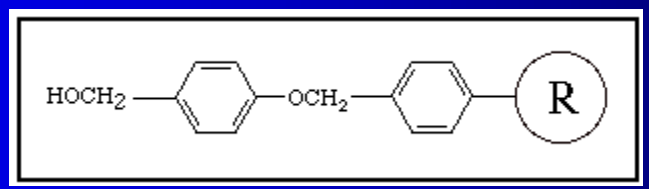
Protein Engineering
----Production of mateirals

ABIOTIC PEPTIDE SYNTHESIS

Solid Phase Peptide Synthesis (SPPS)

Bingyun Li





SPPS

For proteins:

* Consider conformation
* Obtain quantities for bio/medical research
* Obtain non-natural analogues to investigate the influence of the primary structure on the biological function

For peptides:

- Provide access to substrates and inhibitors of proteins (e.g., enzymes)
- Consider conformation
- Obtain quantities for biological investigations.

Peptide synthesis: general consideration

Equal amount of G, V
What will happen?

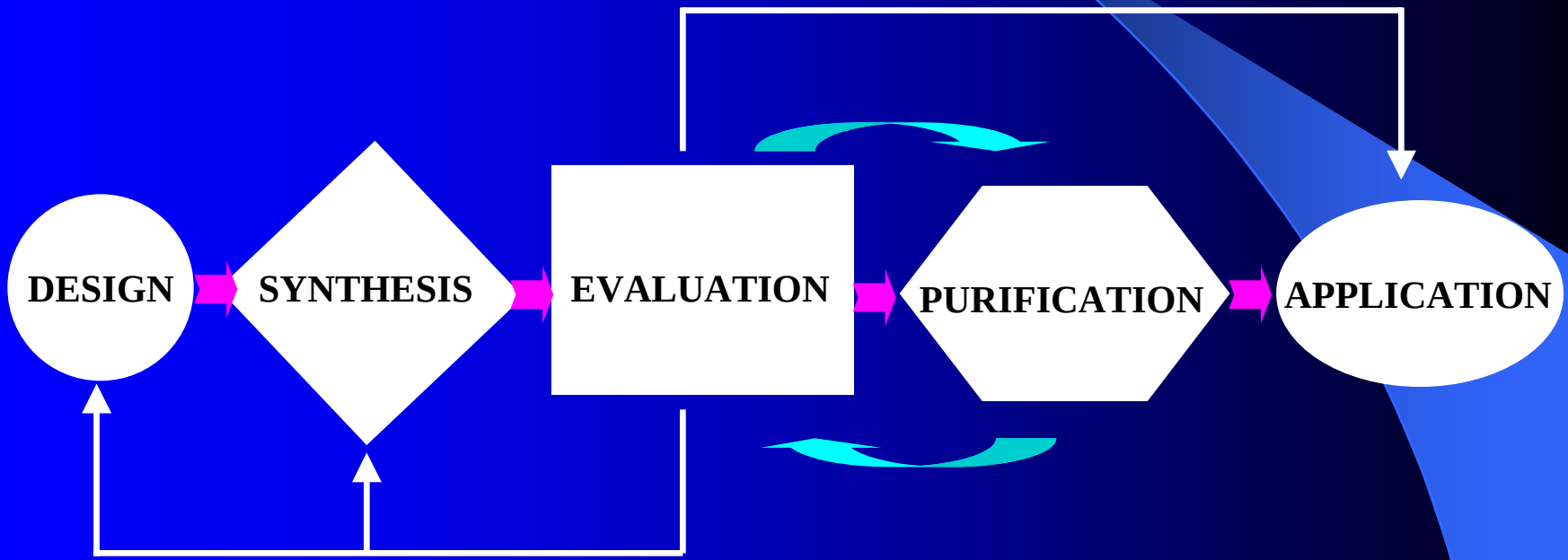
G-V, V-G, G-G, V-V,

G-G-G, G-G-V, G-G-G-G-V-V, …

G, V

Nothing happens

Diagram of the steps involved in obtaining a synthetic peptide



Synthetic Peptide Design & Tips

1. **Minimize difficult amino acids (AAs) such as multiple Cys, Met, Arg, or Trp in the sequence.**
2. **Minimize hydrophobic AAs or break up a string of them with a polar AA. If greater than 50% of the residues in a sequence are hydrophobic there can be a solubility problem.**
3. **An N-terminal glutamic acid can cyclize to pyroglutamate.**

Some useful information for peptide design:

1. ***Hydrophilic amino acids:*** **Asp, Glu, His, Lys, Asn, Gln, Arg, Ser, Thr**
2. ***Hydrophobic amino acids:*** **Ala, Phe, Ile, Leu, Met, Pro, Val, Trp, Tyr**
3. ***Amino acids that can oxidize in mild conditions:*** **Cys, Met**
4. ***Amino acids that can deamidate or dehydrate:*** **Asn, Gln**
5. ***Amino acids that can degrade during synthesis:*** **Met, Trp**
6. ***Amino acids with a (+) charge:*** **Lys, Arg, His, N-terminal end of the peptide**
   Amino acids with a (-) charge: **Asp, Glu, Tyr, C-terminal end of the peptide**

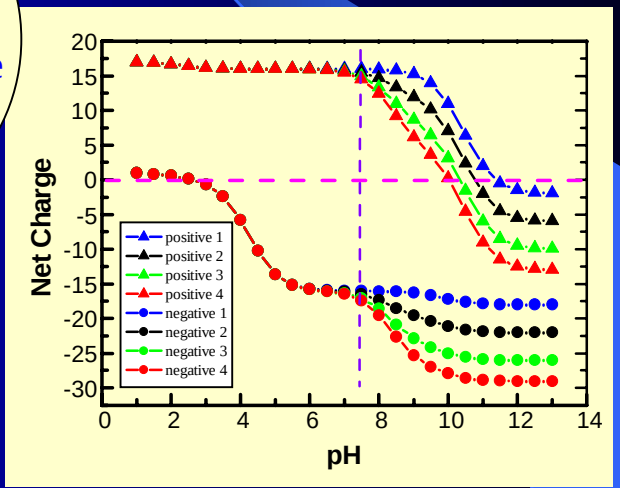
Peptide Design: an example

Cysteine-containing Peptide Design

1. Compositions
2. Sequence
   e.g., KVKG, KCKV, KVKG, KCKV
   KVKG, KCKV, KVKG, KCKY
3. Fmoc side-chain protecting groups
   C (Trt), K (Boc), E (OBut), Y (But)
4. Cleavage: Reagent K
   (TFA, phenol, thioanisole, ethanedithiol, H_2O)

V	K/E	G	C
3/8	1/2	1/8	0
1/4	1/2	1/8	1/8
1/8	1/2	1/8	1/4
0	1/2	1/8	3/8

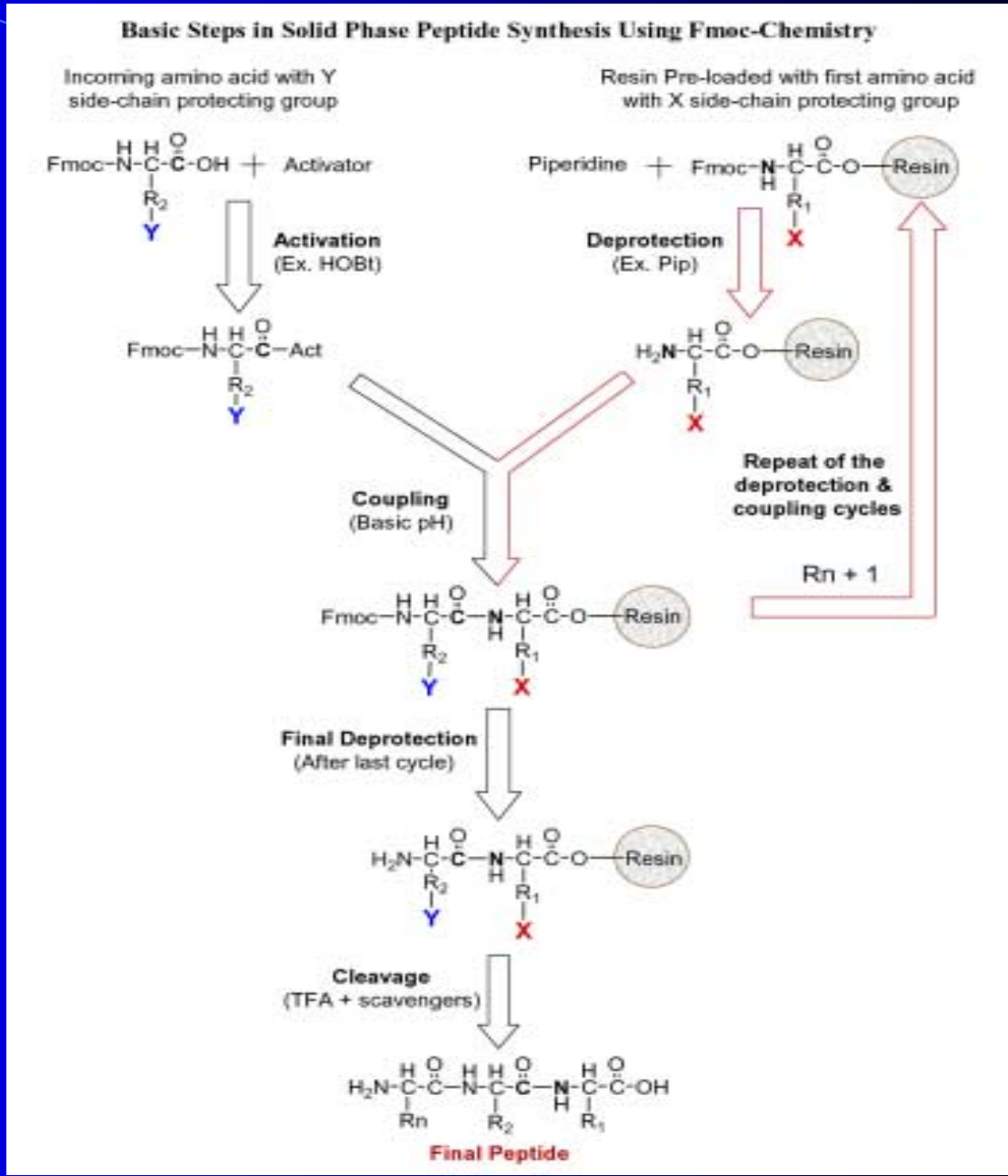
Charge, pKa
Amino acids, length
Charge distribution
Secondary structure
Hydrophobicity
……



SPPS is done from the C-terminus to the N-terminus
QUESTION?

Fmoc Chemistry
Boc Chemistry

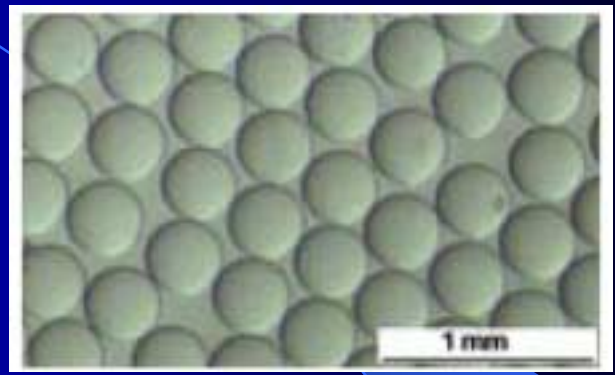
An example of Fmoc SPPS



Solid supports, linkers, and swelling

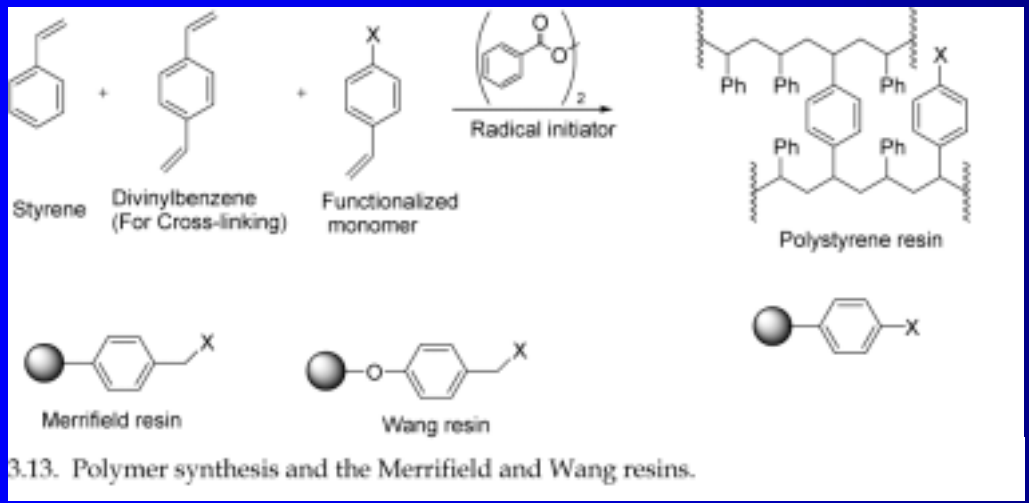
Solid support: resin

Stability, diffusion, and swelling properties

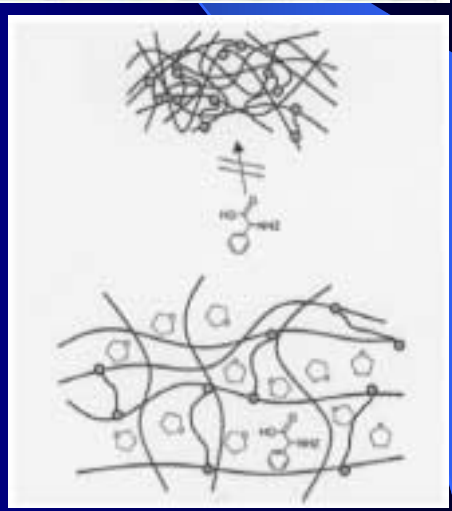


Swelling:

To provide access to the inner core of the resin



3.13. Polymer synthesis and the Merrifield and Wang resins.



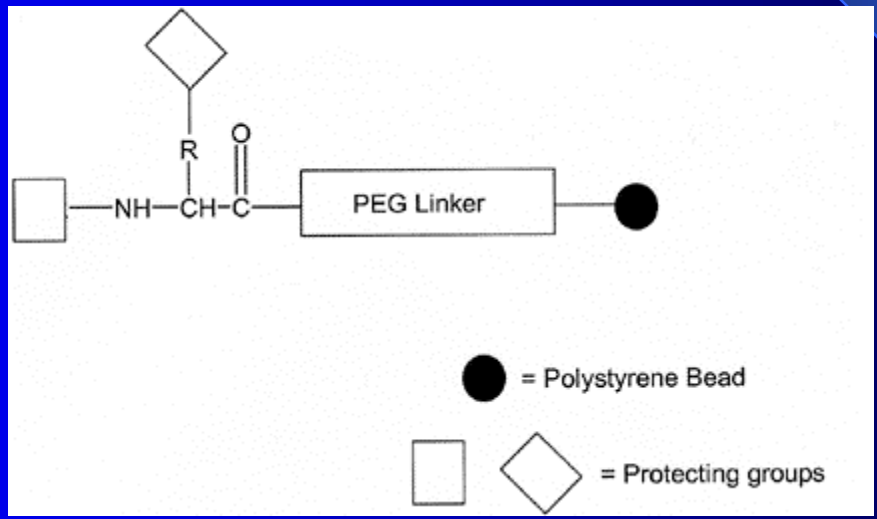
Types of resin:

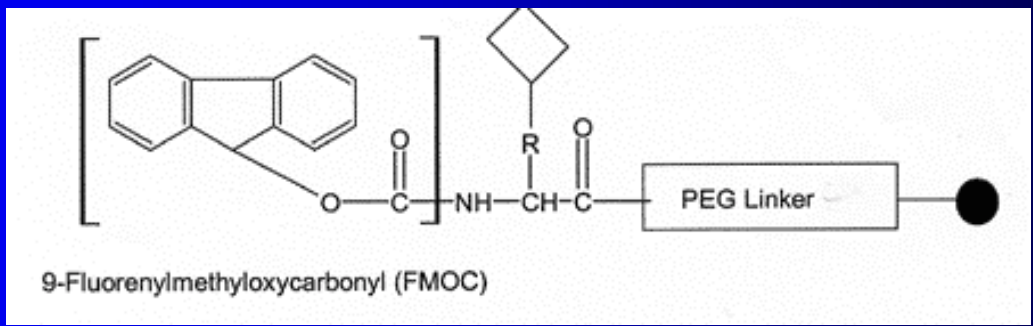
Wang resin, Rink resin, MBHA resin …

Linkers

Stable to the reaction conditions
Cleavable without destroying the synthesized peptide

Resins are available with amino acids preloaded





Protection

Protection of the N-α group is required

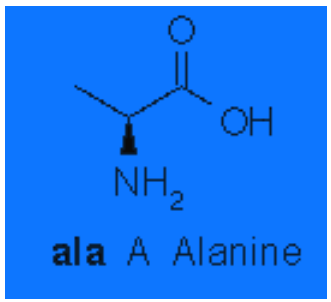
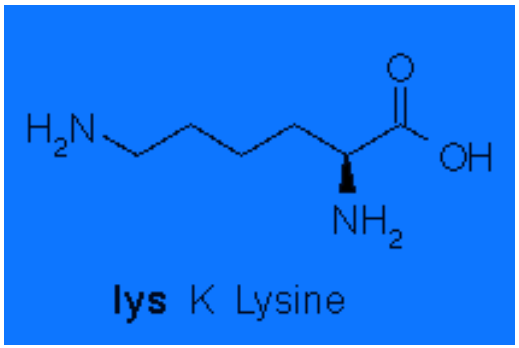
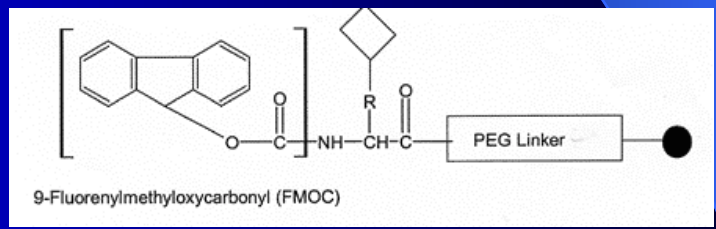


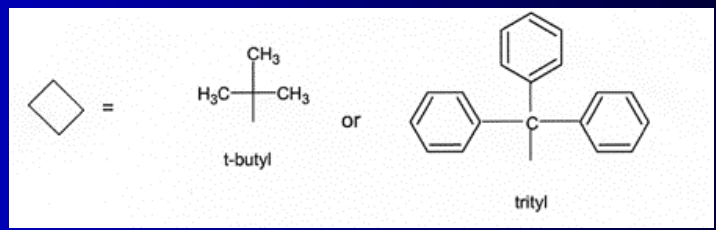
Figure 3.2. Fmoc protecting group and mechanism of cleavage.

Side chain protection: *To prevent from acylation, alkylation, …*

- the carboxyl groups in the side chains of aspartic and glutamic acids
- the ε-amino function of Iysine
- the alcohol functions of serine and threonine
- the phenol of tyrosine
- the guanidine of arginine
- the thiol of cysteine
- … …







Deprotection

Fmoc Chemistry:

N-α protection schemes: Fmoc
Base labile (25% piperidine)

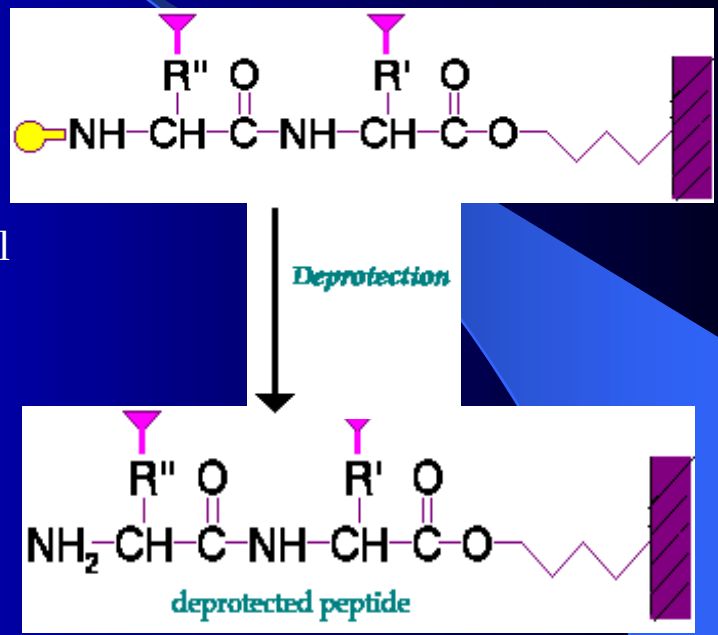
Orthogonal protection: Boc, *tert*-Butyl, trityl
Acid labile (TFA)

Scavenge Orthogonal groups
Water, EDT, …

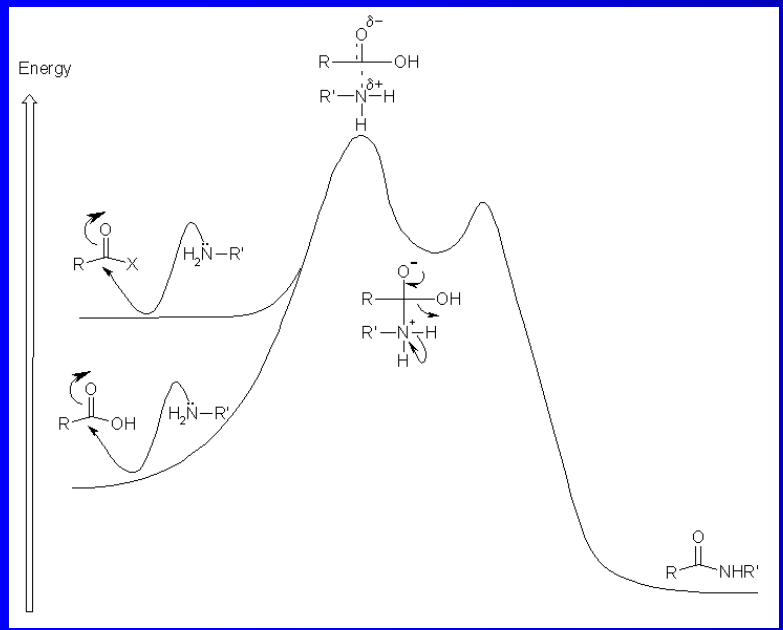
Boc Chemistry:

N-α protection schemes: Boc
Acid labile (50%TFA)

Orthogonal protection: Cbz, Tosyl, …
Acid labile (HF)



Activation and coupling



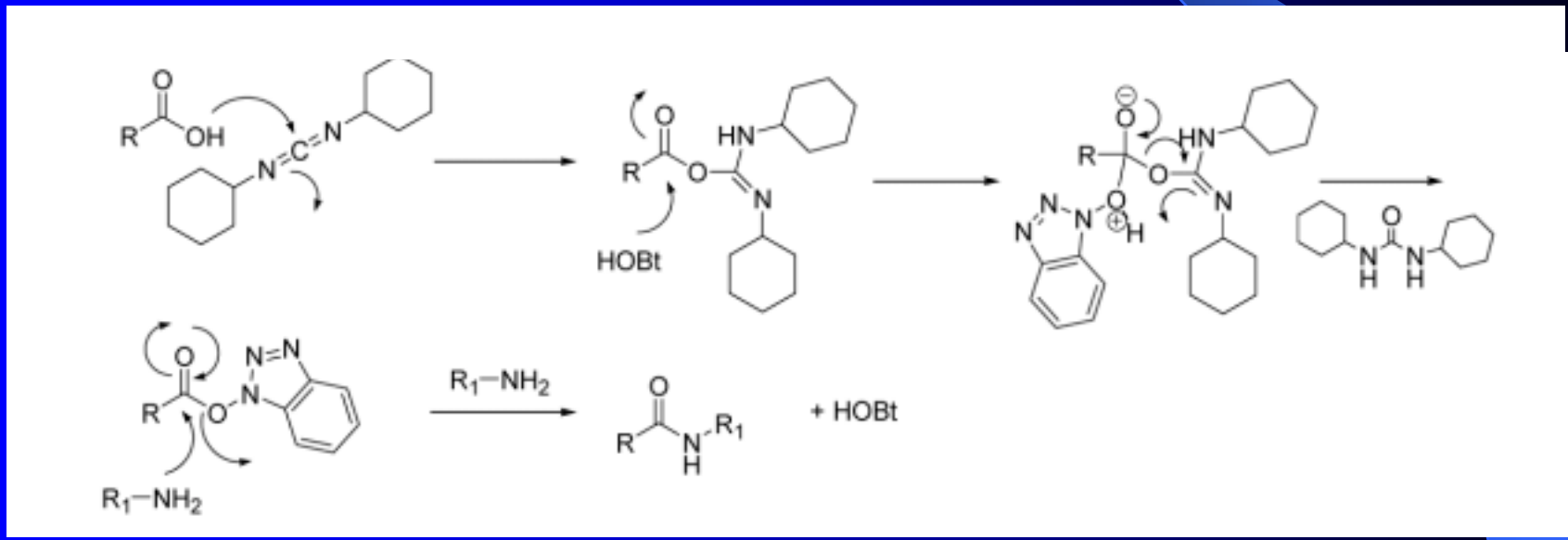
Activating agent

Function: to deprotonate the –COOH, and thus activate the AA need to be coupled.
Examples: HOBt, HATU

Coupling agent

Function: to be a catalyst for peptide bond formation.
Examples: DIC, DCC

Mechanism for carbodiimide activation

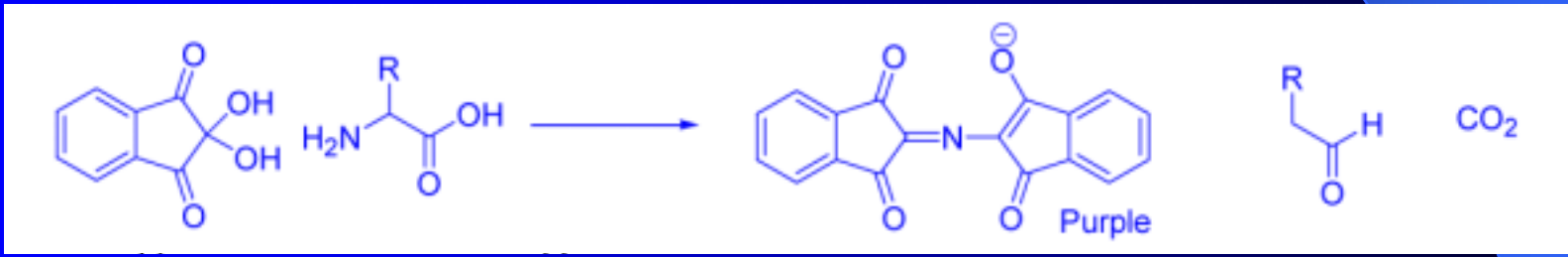


Process monitoring:

(1) HPLC and Mass Spec

(2) *In-situ* monitoring (e.g. Ninhydrin test)

Ninhydrin test: to detect *N-deprotection* and *complete peptide coupling*.



Modifications

C-terminal amidation

N-terminal acetylation

Biotin, stearate and other modifications could be added to the N-terminus.

Other modifications are also possible.

Cleavage

Cleavage cocktail solution and scavengers

Cocktail solution: TFA(>85%)+scavengers
Function: to cleave the peptides from the resin, in the same time, to remove the orthogonal protecting groups.

Scavengers:
Function: to break the equilibrium between the peptide and the orthogonal protecting groups.
Examples: Water, EDT, Anisole …

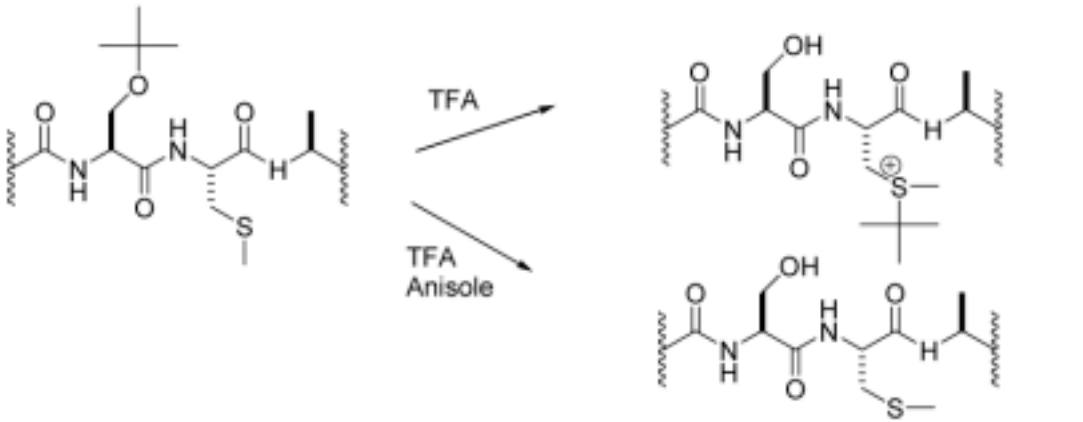


Figure 3.4. Use of scavenger.

Precipitation and isolation

Precipitation and isolation:

Function: to remove TFA, deprotection byproducts and excess scavengers.
Chemicals: pre-cold diethyl ether
Centrifuge
Repeat at least 3-4 times

Side products:

(1) Deletion products: missing residues from incomplete coupling

(2) Derivatized peptides: having residual side chains either from incomplete deprotection or from side reactions during cleavage and isolation (e.g., oxidation of Methionine, or alkylation of tryptophan)

(3) Racemization

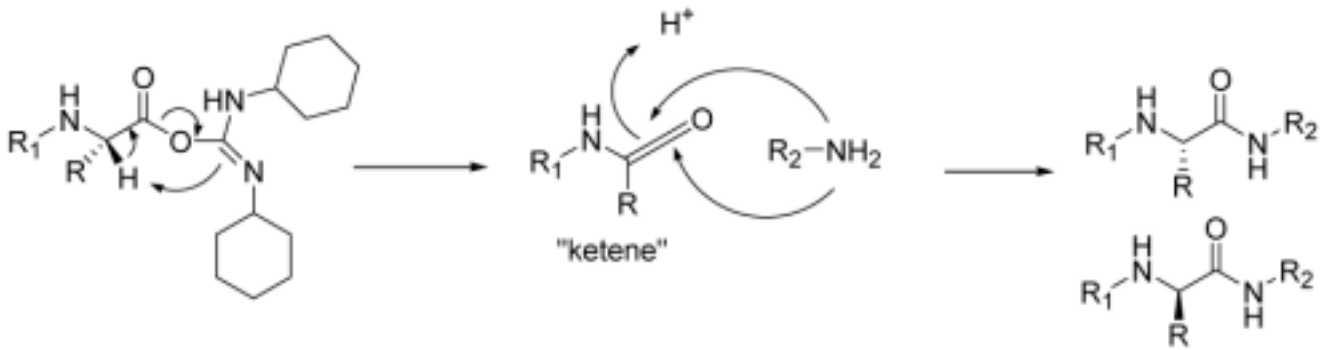


Figure 3.7. Racemization with carbodiimides.

Analysis and purification

Yield:

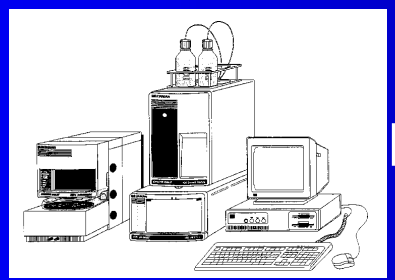
Theoretical Yield (mg) = Sresin * Mresin * MWproduct
where
Sresin — resin substitution in mmol/g
Mresin — resin dry mass in g
MWproduct — MW of the product in mg/mmol

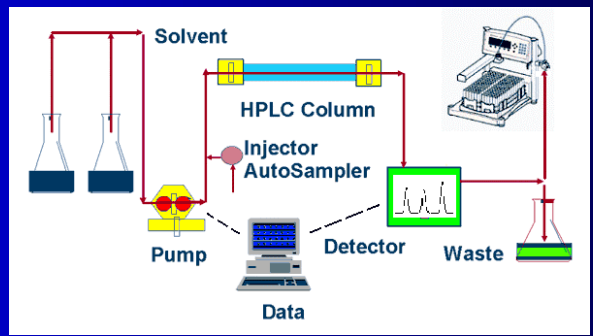
Mass Spectrometry — to determine the MW

Amino acid analysis — to determine the composition

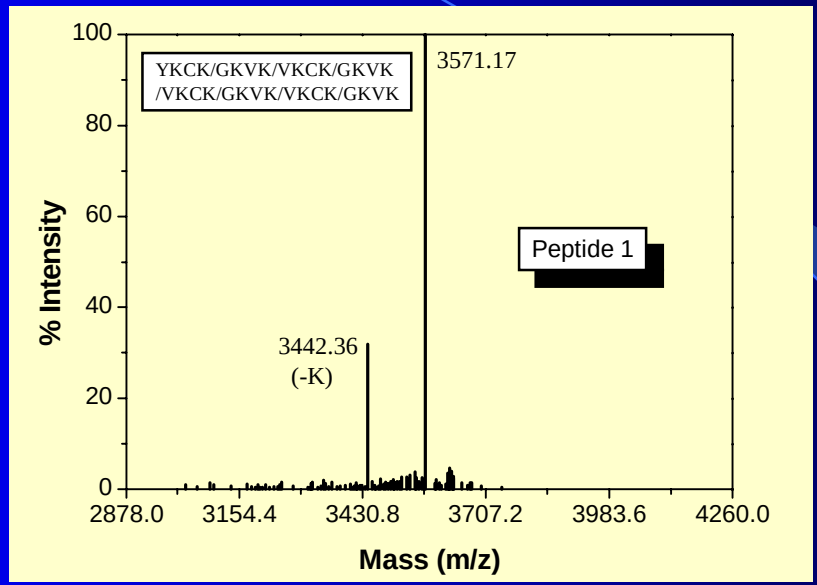
Sequencing (e.g., Edman degradation) — to determine the primary structure

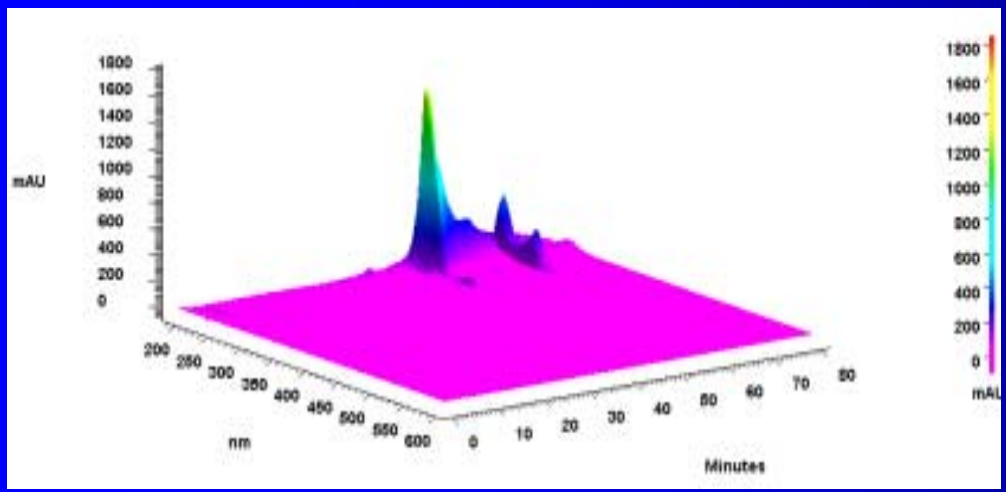
HPLC — to determine the purity and also to purify the product

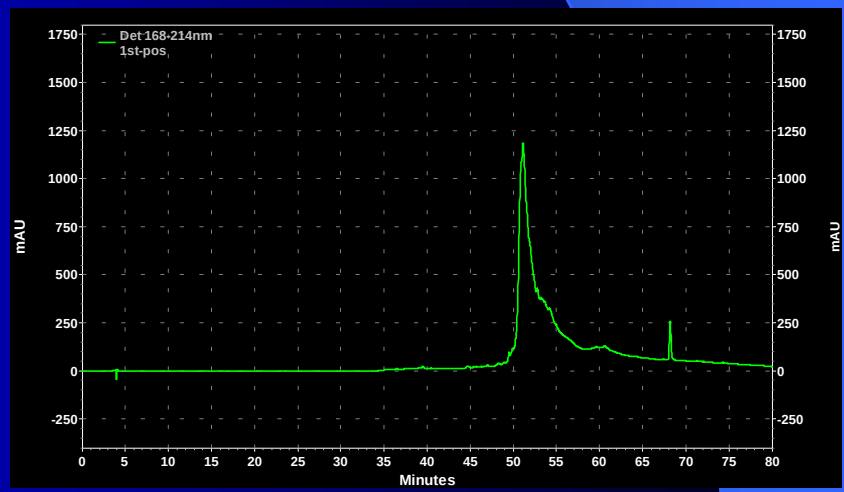




Analysis of synthesized peptide: an example







Peptide storage

Lyophilized Peptides

- Stored cool and dry, tightly sealed, either refrigerated or frozen (-70°C).
- When thawing, allow the closed container to come to room temperature before opening the vial.
- Peptides are light sensitive and wrapping them in aluminum foil is recommended. Most peptides are stable for years.

Peptides in Solution

- ✓ Stored at or below -20°C buffered at pH 5-7.
- ✓ Peptides with cys, met, or trp are susceptible to oxidation, peptides with gln or asn may undergo deamidation, and should not be stored in solution.
- ✓ Repeated freeze-thaw cycles should be avoided.
- ✓ Use of sterile water and buffers.
- ✓ Ideally, buffers and liquids should be degassed and sterile filtered before use.

Peptide solubility and use

A strategy if the peptide is somehow insoluble:

- ***Acidic peptides*: may be solubilized by dissolving first in a small amount of a weak base (e.g., 10% ammonium bicarbonate) followed by dilution with water to the desired concentration.**
- ***Basic peptides:* dissolve in dilute acid (e.g., 30% acetic acid) followed by dilution with water.**
- ***Hydrophobic peptides:* dissolve in DMSO followed by dilution with water.**
- ***Peptides prone to aggregation*: may require strong denaturants (e.g., 6 M urea, 6 M urea with 20% acetic acid, or 6 M guanidine -HCL)**
- **Only add in buffer salts after the peptide is dissolved!**

Peptide synthesis in solution

Solution phase peptide synthesis is quite often used for the synthesis of smaller sequences of peptides (di-, tri-), and for large-scale synthesis. However, for longer sequences a number of problems arise. 1) Solubility: Longer peptides are not very soluble in organic solvents. 2) The synthesis and purification becomes more laborious and tedious. This is especially true for sequences with protected side chains.

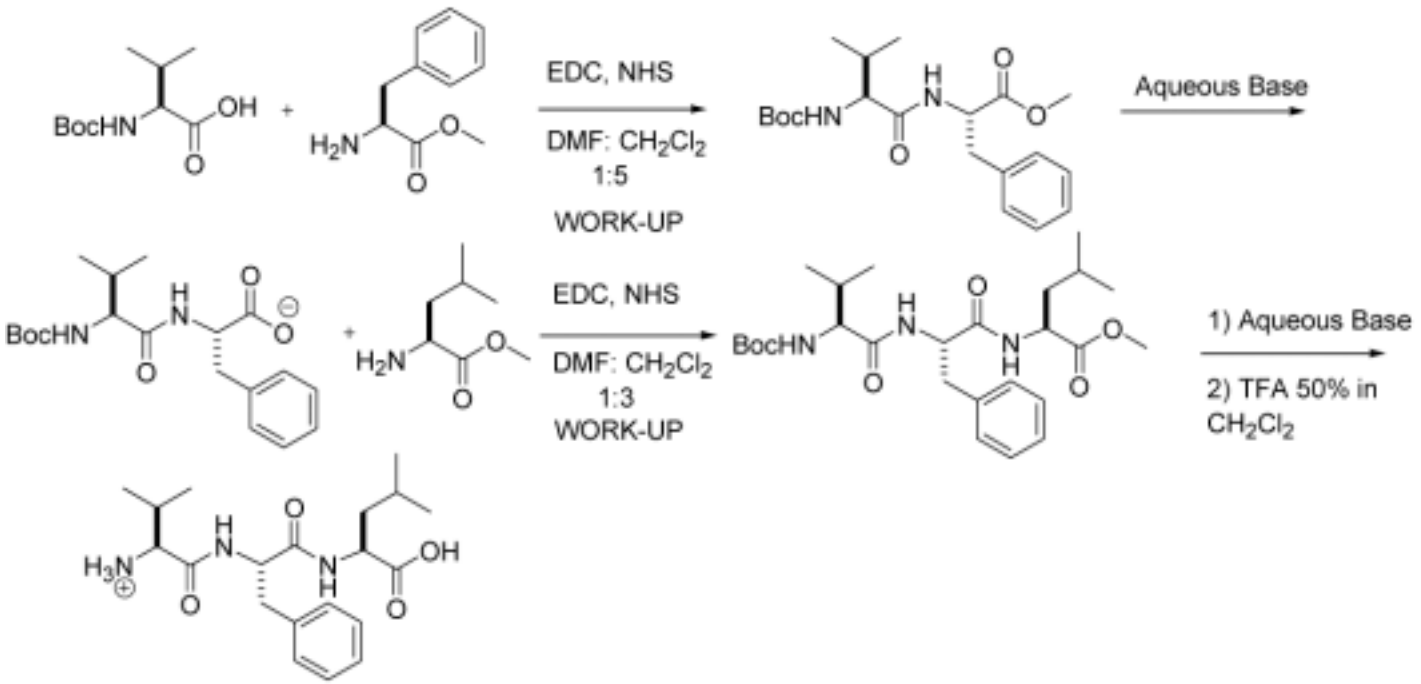


Figure 3.10. Peptide synthesis in solution (Val-Phe-Leu).

Instrument description

Detailed procedures for Fmoc peptide synthesis

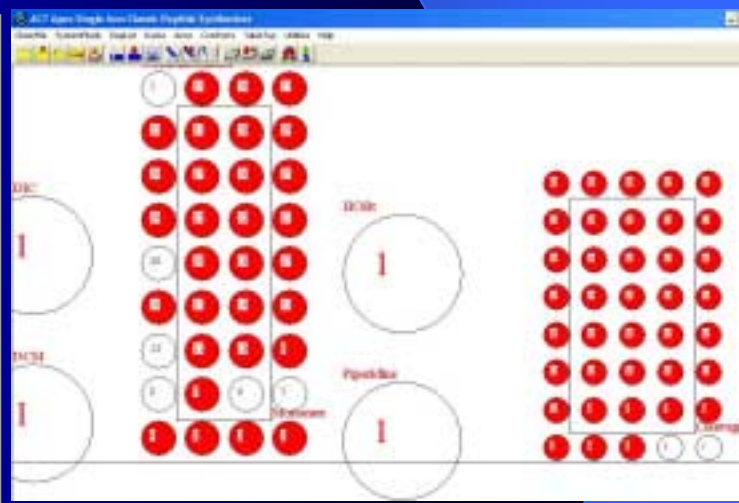
For instance, to make:

KVKG/KCKV/KVKG/KCKV/KVKG/KCKV/KVKG/KCKY

<u>Step one:</u> Calibrations

Racks needed to be calibrated: RV Block, monomer Piperidine, DIC, cleavageblock, cocktail solution

RVBlock	Diameter=20mm		X	Y
No. of Col.	**5**	1st Corner Pos	**2344**	**2036**
No. of Rows	**8**	2nd Corner Pos	**1831**	**634**
Vessel cross section area	**314.16 mm²**	Z-max Pos.	**2820**	
		Z-Start Pos	**2615**	
		Z-Dispense Pos	**2615**	
		Z-Travel Pos	**1**	
		Aspiration Speed	**18**	
		Dispense Speed	**15**	
Define Vessels: activate used vessels		**Advanced**: Can Empty.		



Calibration: System fluid definition

System fluid needed		Aspiration speed	Dispense speed
DMF	Dilutor 1	18	15
MEOH	Dilutor 2	15	15
DCM	Dilutor 1	20	18

Step two: Calculations

(0.2g Wang resin)*(resin substitution)*(5 fold excess)=A mmol
(A)*(1ml/0.5 mmol 0.5M HOBt)= B ml

Step three: Protocols

Swell.chm, deprotection.chm, wash-1.chm, couple.chm, wash-2.chm, final-deprotection.chm, cleavage.chm

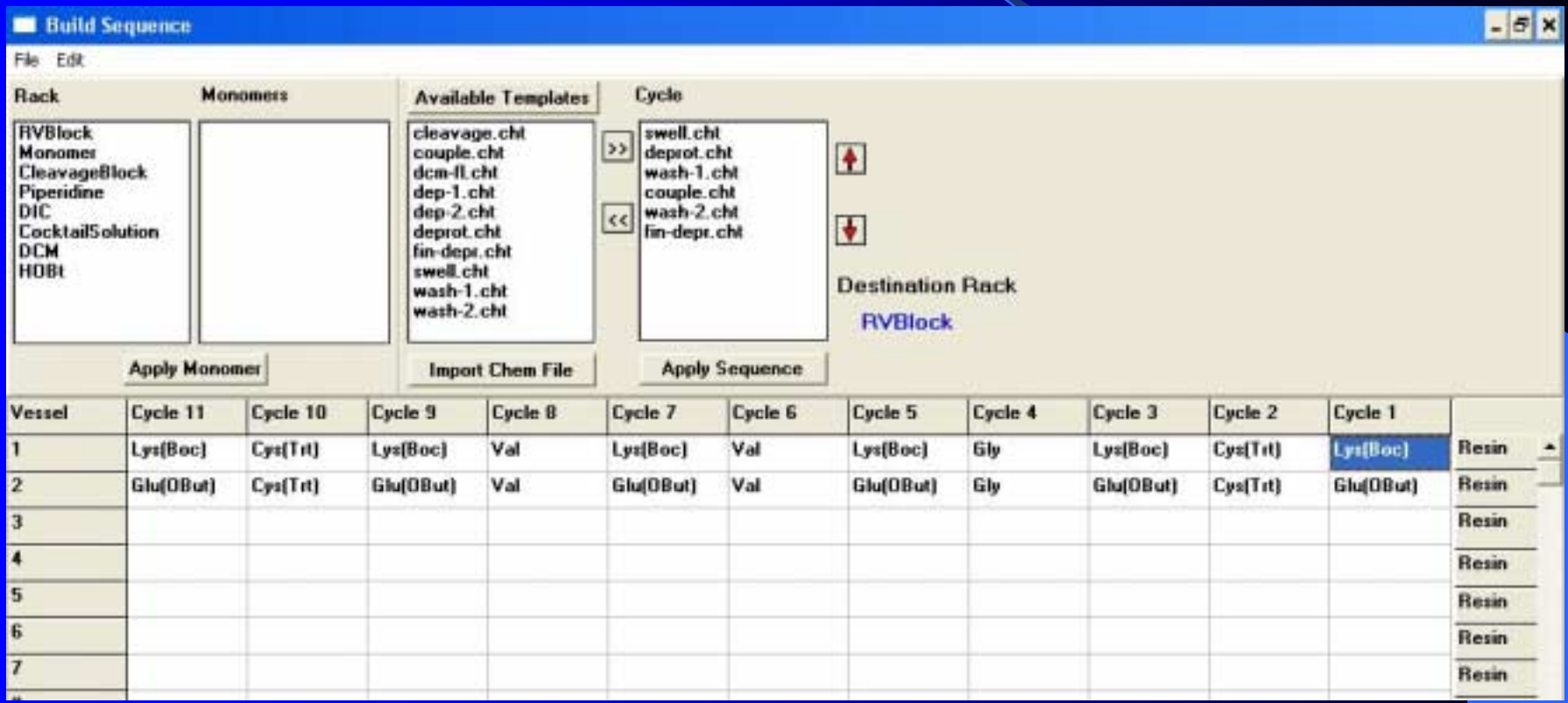
For example,

Couple.chm

1. Dispense System Fluid DMF [1] 300 µl to RVBlock[1-2]
   (the porous frit will soak up this dead volume)
2. <Dispense Matrix> (Dispense List)
   —this is a place holder in software for dispensing AAs
3. Transfer 1200 µl from DIC [1][DIC] to RVBlock[1-2] using DMF [1]
4. Mix RVBlock for 1 hour at 450 rpm
5. Empty RVBlock for 2 minutes
6.
7. Dispense System Fluid DMF [1] 300 µl to RVBlock[1-2]
8. <Dispense Matrix>
9. Transfer 1200 µl from DIC [1][DIC] to RVBlock[1-2] using DMF [1]
10. Mix RVBlock for 1 hour at 450 rpm
11. Empty RVBlock for 2 minutes

Step four: Synthesis sequence file

KVKG/KCKV/KVKG/KCKV/KVKG/KCKV/KVKG/KCKY



Step five: Chemicals preparation

0.5 M HOBt
25 vol% piperidine in DMF
0.5 M DIC in DMF
AAs dissolved in 0.5 M HOBt

Step six: Run the synthesis sequence file

Step seven: Cleave the peptides using cocktail solution

Step eight: Precipitate the peptides using cold diethyl ether and lyophilize

Step nine: Preventive maintenance