PEPTIDE SYNTHESIS

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- Solution phase chemistry

-Time consuming: isolation and purification at each step

-Low yield: can't drive reaction to complete

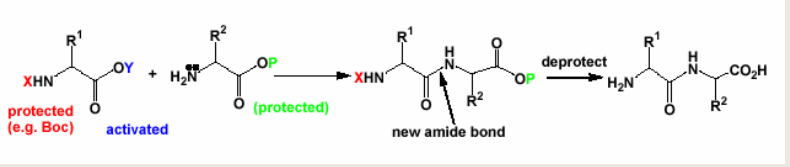
-Use excess reagent to improve yield

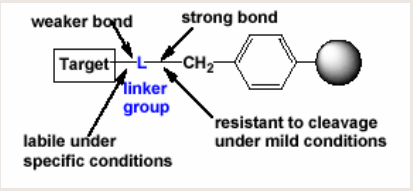
Solid phase peptide synthesis (SPPS)

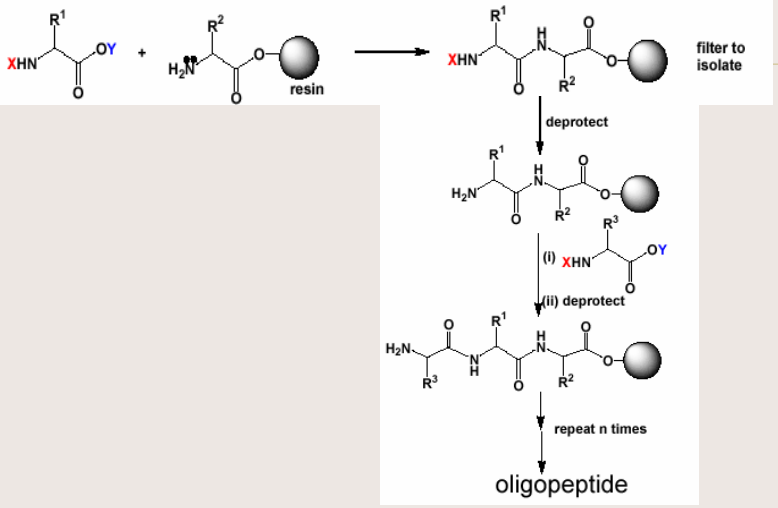
The Nobel Prize in Chemistry 1984

--for his development of methodology for chemical synthesis on a solid matrix

Robert Bruce Merrifield
Rockefeller University





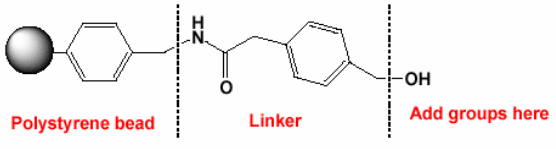


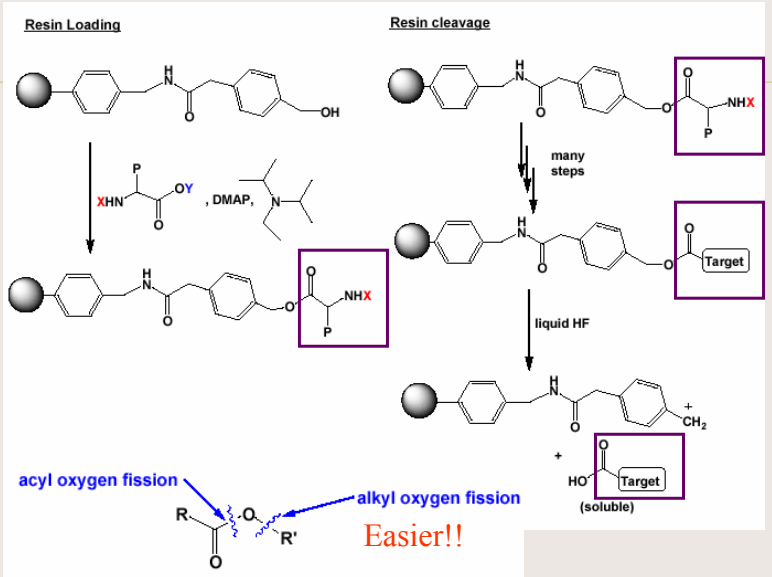
1. **Synthesis occurs on the surface of the bead and inside the bead**
2. **Bead swells when solvent is absorbed. Synthesis occurs on multiple surfaces inside the bead**

Common resins for solid-phase peptide synthesis (SPPS)

Polystyrene – swells in non-polar solvents (widely used, cheap)

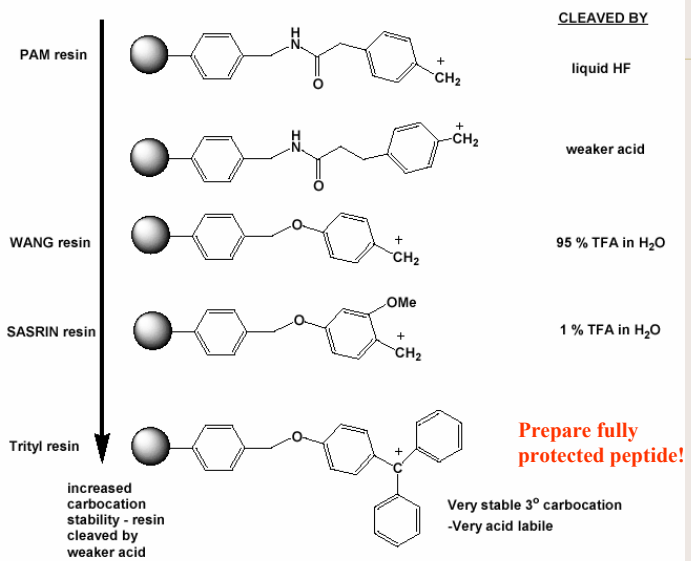
Tentagel (PS-PEG) – swells under a range of solvents (H_2O, MeOH, MeCN, DMF, DCM), expensive, suitable for bioassays on resin (can test on resin)



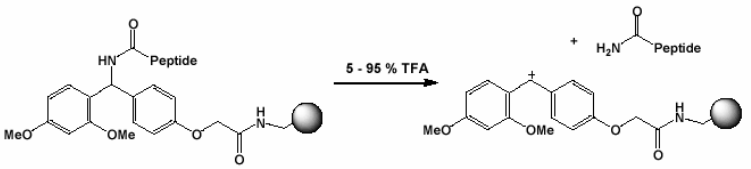


1. Choose resin!

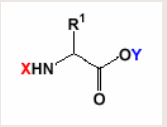
The ease of cleavage from the resin depends on the linker used and the stability of the final carbocation:



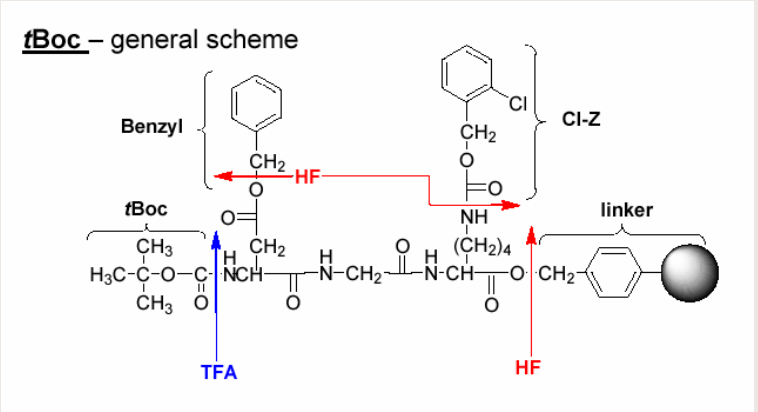
C-terminal amides are commonly prepared using the **Rink amide linker**:



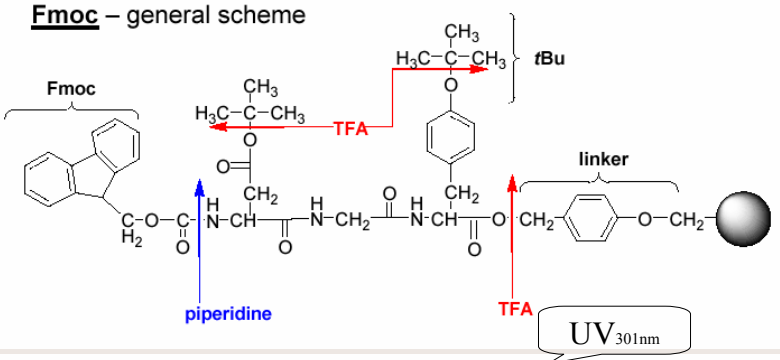
N-terminal protecting group : X

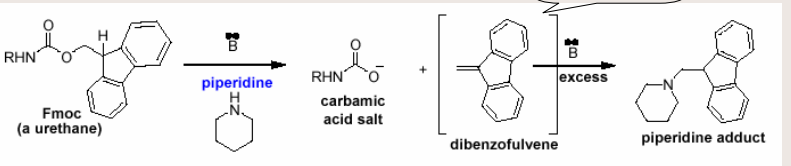


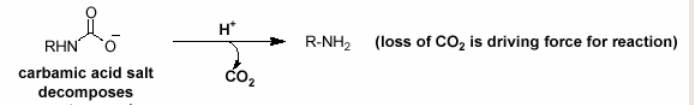
- t-Boc (t-butoxycarbonyl-)
- Fmoc (fluorenylmethoxycarbonyl)



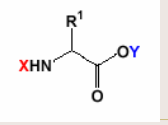
Fmoc – general scheme







Amino acid activation….. Y



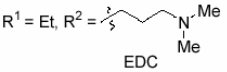
Generally use carbodiimides, phosphonium and uronium salts

General Structures

carbodiimides

R^1–N=C=N–R^2

R^1=R^2 = isopropyl
R^1=R^2 = cyclohexyl (DCC)

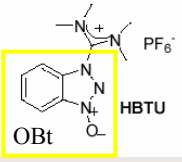


EDC
water soluble

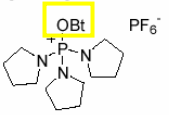
uronium

PF_6^- or BF_4^-

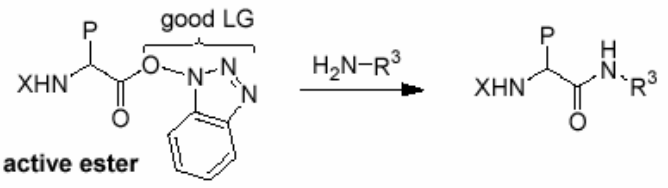
HBTU (also HOAT)



phosphonium

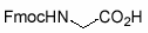


pyBOP

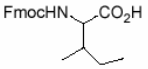


2. Choose amino acid!

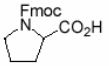
1. side-chain = H, alkyl, aryl –no side-chain protecting groups required



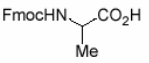
(Glycine, Gly, G)



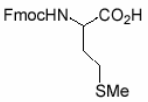
(isoleucine, ile, I)



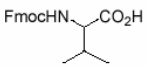
(proline, pro, P)



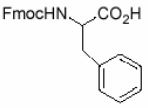
(alanine, ala, A)



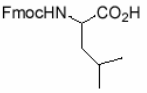
(methionine, met, M)



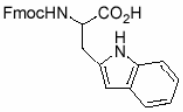
(valine, val, V)



(phenylalaniine, phe, F)

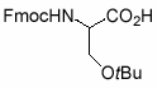


(leucine, leu, L)

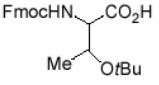


(tryptophan, trp, W)

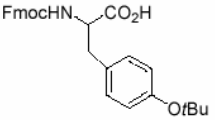
2. side-chain contains OH, protect as *t*Bu ether



(serine, ser, S)

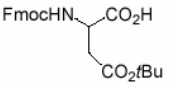


(threonine, thr, T)

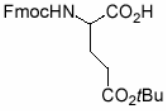


(tyrosine, tyr, Y)

3. side-chain contains CO_2H protected as *t*Bu-ester.

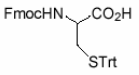


(aspartic acid, asp, D)

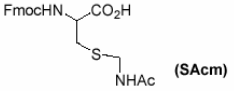


(glutamic acid, glu, E)

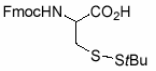
4. side-chain contains thiol (SH) group – depends on application.



(cysteine, cys, C)



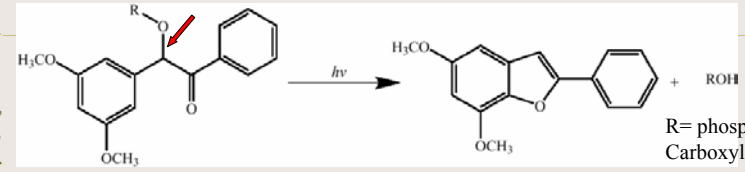
(acid stable, cleaved with Hg^{2+})



(acid stable, cleaved with thiols)

Fmoc-cys(mmt)-OH, mmt: methoxytrityl
Cleaved by 1 % TFA in DCM containing 5 % TIS

Development of the photolabile linker

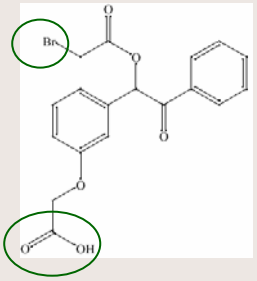


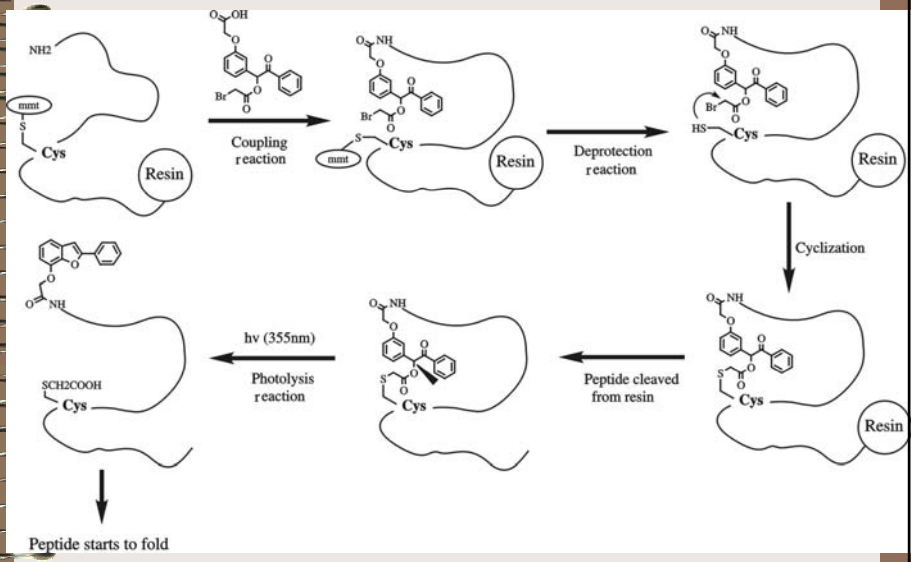
R= phosphate, amine
Carboxylic acid

3',5'-dimethoxybenzoin (DMB) 2-phenyl-5,7-dimethoxybenzofuran

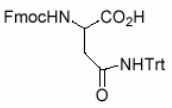
Sheehan JC, Wilson RM, and Oxford AW (1971) JACS 93, 7222-7228.

BrAc-CMB

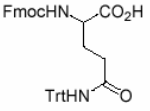




5. side-chain contains $CONH_2$ group – protect with trityl group.

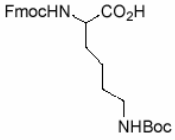


(asparagine, asn, N)

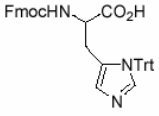


(glutamine, gln, Q)

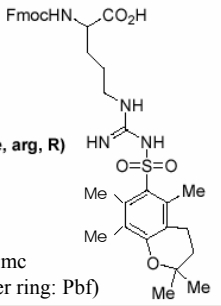
6. side-chains contain basic or imidazole groups.



(lysine, lys, K)



(histidine, his, H)



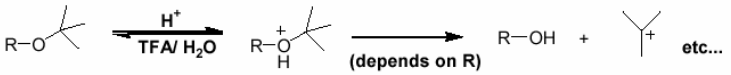
(arginine, arg, R)

Pmc
(5-member ring: Pbf)

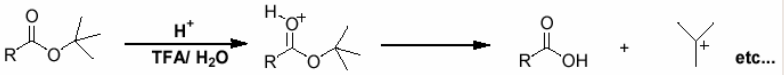
Fmoc-Lys(mtt)-OH mtt: methyltrityl
Cleaved by 1 % TFA in DCM containing 5 % TIS

3. Choose cleavage reagents!

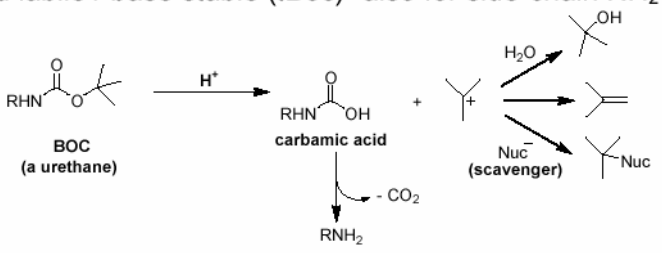
Protection of alcohols (Fmoc chemistry) as *t*Butyl ethers.



Protection of acids (Fmoc chemistry) as *t*Butyl esters.



acid labile / base stable (*t*Boc)- also for side-chain NH_2



Scavenger!!!!

- EDT (Ethanedithiol) – scavenger for t-butyl cation, help to remove Trt from Cys
- EDT, Thioanisole – avoid Met oxidation
- Phenol – protect Tyr, Trp
- TIS (Triisopropylsilane) – quench highly stable Trt cation

Side reaction during cleavage….

- Alkylation for Met, Cys, Trp (by t-Butyl cation)
- Sulfonation for Trp (by Mtr, Pmc): Use Trp(Boc)

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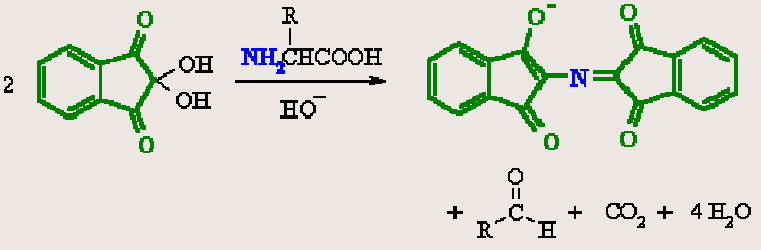
ABI 433A Peptide Synthesizer

Coupling efficiency and final yield

	Yield (%)					
efficiency (%)	10-mer	20-mer	30-mer	40-mer	50-mer	60-mer
99	90	82	74	67	61	55
98	82	67	55	45	36	30
95	60	36	21	13	8	5
80	11	1	0	0	0	0

Ninhydrin test



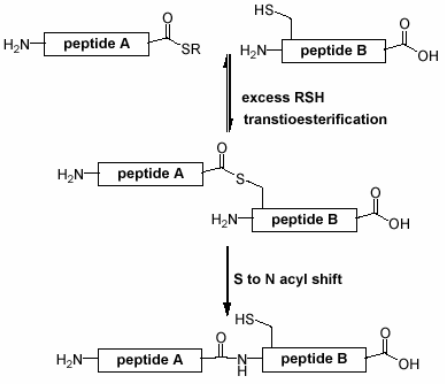
110 C, 4-6 min

A blue to blue-violet color is given by α-amino acids and constitutes a positive test. Other colors (yellow, orange, red) are negative.

Difficult coupling

- Prolonged coupling time
- Dry solvent
- Aggregation – shrinking of resin matrix: use dipolar aprotic solvent (DMF, DMSO, NMP), resin crosslinking < 1 %
- Add chaotropic salt (0.8 M NaClO4, LiCl, 4M KSCN)
- Use different activation method (PyBOP, HOBt/HBTU, TBTU)
- Magic mixture: DCM/DMF/NMP (1:1:1) with 1 % Triton X100, and 2 M ethylenecarbonate at 55 C for solvent in acylation

Most common fragment coupling strategy - **native chemical ligation** (NCL) (Kent, *science*, 1994) involves the coupling of 2 unprotected peptide fragments in aqueous solution and in the absence of protecting groups.



- A chemoselective reaction.
- Requires 1 peptide thioester (sythesized using *t*Boc chemistry on thioester resin or by Fmoc SPPS on sulfonamide "safety-catch" resin)
- Requires 1 peptide containing an N-terminal cysteine residue (easily prepared)

Batchwise and continuous flow SPPS

- In batch instruments, reactions and washings are carried out in a shaken, stirred, vortexed, or bubbled reaction vessel. Reagents and solvents are added and removed through a filter via application of gas pressure or vacuum.
- In continuous flow mode, a glass column with filters at the top and the bottom contains the resin and acts as a reaction vessel. The system includes a positive displacement pump to enable continuous fluid flow. Continuous flow instrumentation was designed for Fmoc/*t*Bu based methods because *N* protecting group removal proceeds under milder conditions (piperidine)
- Polystyrene (PS) resins, the most traditional support used in solid phase, in conjunction with fluid delivery via a pump, create high pressures that may halt the synthetic process.
- To overcome this problem, polyethylene glycol (PEG)-PS supports, which combine a hydrophobic core of PS with hydrophilic PEG chains, have been developed

Antibody against small peptides

- Antibodies to small peptides have become an essential tool in life science research, with applications including gene product detection and identification, protein processing studies, diagnostic tests, protein localization, active site determination, protein homology studies and protein purification.
- Anti-peptide antibodies will always recognize the peptide.

Sequence epitopes in proteins generally consist of 6-12 amino acids and can be classified as continuous and discontinuous.

- Continuous epitopes are composed of a contiguous sequence of amino acids in a protein. Anti-peptide antibodies will bind to these types of epitopes in the native protein provided the sequence is not buried in the interior of the protein.
- Discontinuous epitopes consist of a group of amino acids that are not contiguous but are brought together by folding of the peptide chain or by the juxtaposition of two separate polypeptide chains. Anti-peptide antibodies may or may not recognize this class of epitope depending on whether the peptide used for antisera generation has secondary structure similar to the epitope and/or if the protein epitope has enough continuous sequence for the antibody to bind with a lower affinity.

- When examining a protein sequence for potential antigenic epitopes, it is important to choose sequences which are hydrophilic, surface-oriented, and flexible. Antibodies bind to epitopes on the surface of proteins.
- Algorithms for predicting protein characteristics such as hydrophilicity/hydrophobicity and secondary structure regions such as alpha-helix, beta-sheet and beta-turn aid selection of a potentially exposed, immunogenic internal sequence for antibody generation. Many commercial software packages such as MacVectorTM, DNAStarTM, and PC-GeneTM incorporate these algorithms. 
- length of the peptide: long peptides (20-40 amino acids in length) increases the number of possible epitopes. Peptides longer than 20 residues in length are often more difficult to synthesize with high purity because there is greater potential for side reactions, and they are likely to contain deletion sequences. On the other hand, short peptides (<10 amino acids) may generate antibodies that are so specific in their recognition that they cannot recognize the native protein or do so with low affinity. The typical length for generating anti-peptide antibodies is in the range of 10-20 residues.

Coupling the synthetic peptide to carrier protein

- Conjugation to a carrier protein is important because peptides are small molecules, that alone do not tend to be immunogenic, thus possibly eliciting a weak immune response.
- The carrier protein contains many epitopes that stimulate T-helper cells, which help induce the B-cell response. It is important to ensure the peptide is presented to the immune system in a manner similar to the way it would be presented by the native protein.
- Internal sequences can be coupled at either end. Another consideration for internal sequences is to acetylate or amidate the unconjugated end as the sequence in the native protein molecule would not contain a charged terminus.

Carrier proteins

- Many different carrier proteins can be used for coupling to synthetic peptides. The most commonly selected carriers are keyhole limpet hemacyanin (KLH) and bovine serum albumin (BSA).
- The higher immunogenicity of KLH often makes it the preferred choice. Another advantage of choosing KLH over BSA is that BSA is used as a blocking agent in many experimental assays. Because antisera raised against peptides conjugated to BSA will also contain antibodies to BSA, false positives may result.
- Although KLH is large and immunogenic, it may precipitate during cross-linking, making it difficult to handle in some cases.
- Ovalbumin (OVA) is another useful carrier protein. It is a good choice as a second carrier protein when verifying whether antibodies are specific for the peptide alone and not the carrier.

Coupling methods

- The most common coupling methods rely on the presence of free amino(α-amino or Lys), sulfhydryl (Cys), or carboxylic acid groups (Asp, Glu or α-carboxyl). Coupling methods should be used that link the peptide to the carrier protein via the carboxy- or amino-terminal residue. The sequence chosen should not have multiple residues that may react with the chosen chemistry. If multiple reactive sites are present, try to shorten the peptide or choose the sequence so they are all localized at either the amino or the carboxyl terminus of the peptide. For internal sequences the end furthest from the predicted epitope is normally favored as this avoids potential masking problems.

Figure 1. The EDC Method

Activate protein or peptide

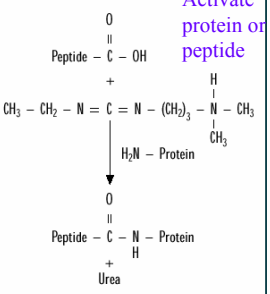


Figure 3. Glutaraldehyde Method uses the amino group of two compounds to link them together.

Glutaldehyde can react with C, Y, H too

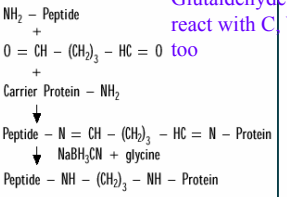
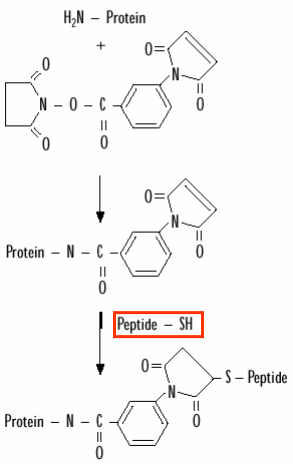
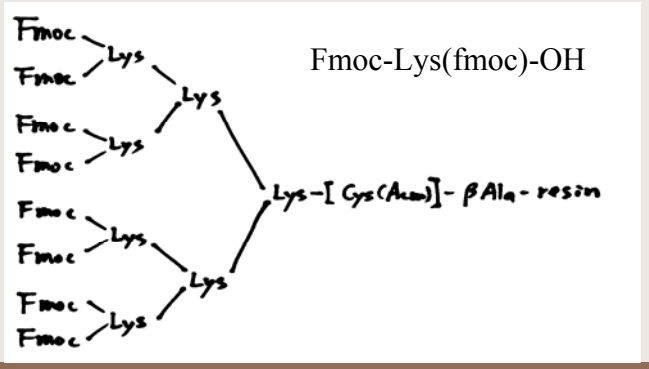


Figure 2. MBS Method
The thiol group on the cysteine residue is utilized in the MBS method.



Multiple Antigen Peptide system (MAPs)

- The MAP system represents a unique approach to anti-peptide antibody generation.
- The system is based on a small immunogenically inert branched lysine core onto which multipe peptides are synthesized in parallel.



- The result after synthesis is a three-dimensional molecule, which has a high molar ratio of peptide antigen to core molecule and therefore does not require the use of a carrier protein to induce an antibody response.
- The result is a highly immunogenic MAP, which exhibits significantly higher titers when compared to its monomeric counterpart attached to a carrier protein.
- It should be noted that there are some synthesis concerns when making a MAP complex. Steric hindrance becomes a problem during the synthesis of long peptides, resulting in some arms of the dendrimer being deletion products. The high molecular weight of the complex does not lend itself to good quality control measures (mass spec and/or analytical HPLC).