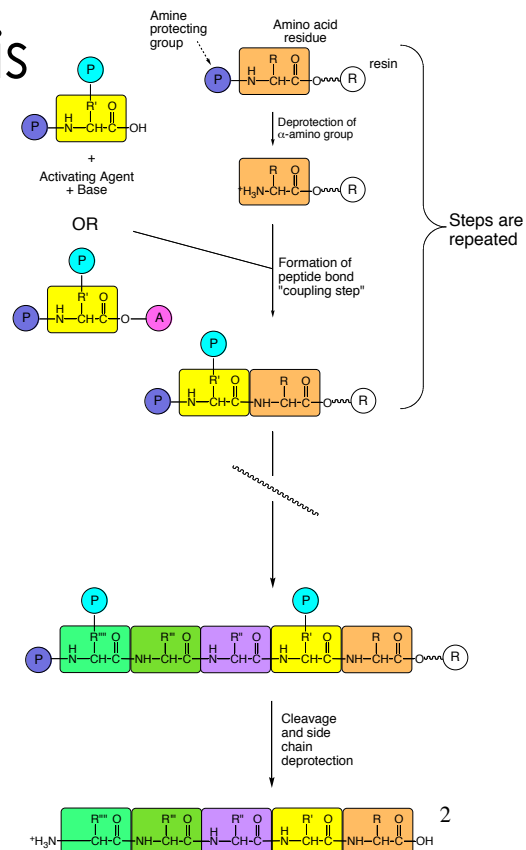


Chemical Peptide Synthesis

September 12, 2010

Chemical Synthesis of Peptides

- Chemical peptide synthesis capitalizes on old-established chemistry.
- Allows the incorporation of nonstandard and labeled amino acids and nonstandard linkages into peptides.
- Provides basis for the generation of combinatorial libraries.
- Stepwise process (can be automated)
- Routine syntheses limited to polypeptides up to ~60 amino acid residues.
- Techniques to extend this limit:
 - Native ligation of synthetic peptides.



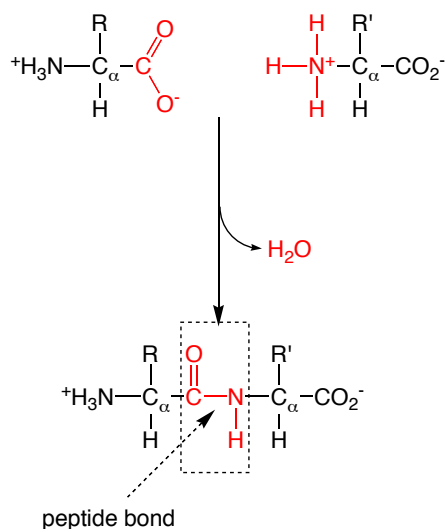
Peptide Synthesis

- Amide bond formation
- Protection schemes
- Side Reactions
- Solid phase, supports and linkers
- Cleavage and scavengers
- Libraries and other applications

3

Amide Bond Formation

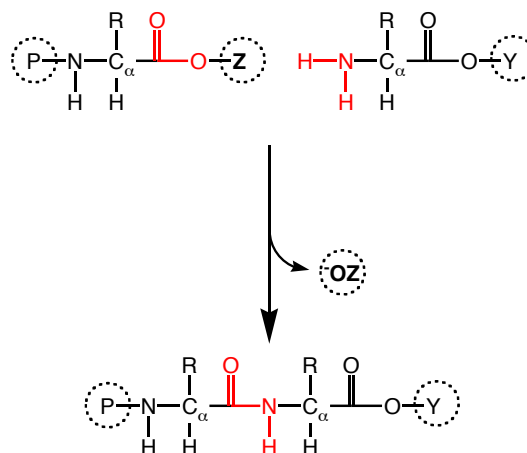
- Peptides and proteins are linear polymers of amino acids linked by amide “peptide” bonds.
- Peptide bonds are amide bonds between amino acids.
- Formation of peptide bond produces a water molecule.
- Carboxylic acid and carboxylate groups are normally not very reactive.



4

Amide Bond Formation: A Synthetic Approach

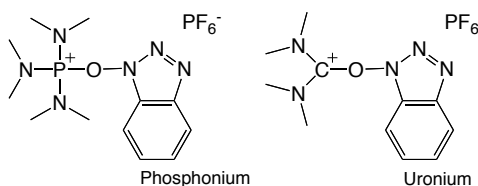
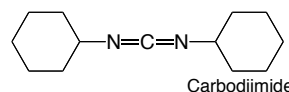
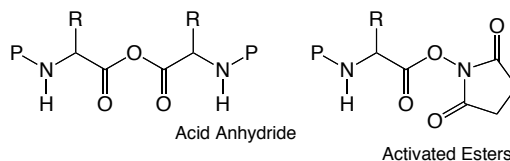
- Carboxylic acid and carboxylate groups are normally not very reactive.
- Formation of the amide ("peptide") bond requires activation of the carboxylic acid.
- Controlled synthesis requires selective protection and deprotection of the various functional groups:
 - The α -amino group.
 - The α -carboxyl group.
 - Side chain functional groups.



5

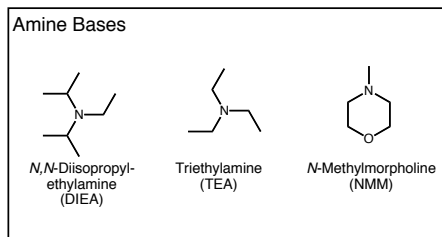
Activation of Carboxylic Acid Groups

- Activation involves converting the O-H/O⁻ of the carboxylic acid group to a suitable leaving group.
- Approaches to activating carboxyl groups.
 - Carbodiimide reagents
 - Symmetric anhydrides
 - Activated esters
 - Phosphonium reagents
 - Uronium reagents
- Each has unique considerations and capabilities.

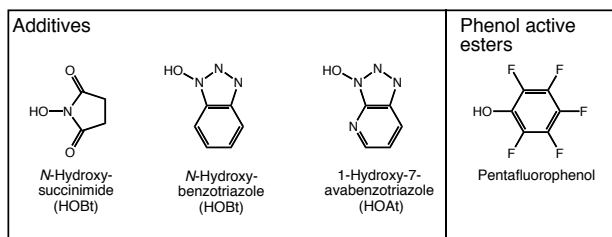


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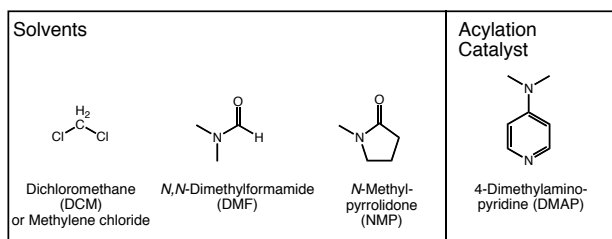
Additional Reagents



- **Bases:** tertiary amine bases. If the base is too strong it increases the risk of racemization.



- **Additives:** are often used to reduce side reactions and increase reaction efficiency.

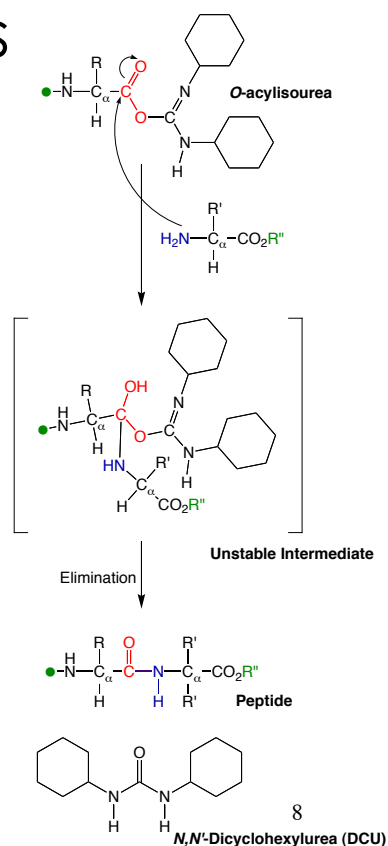


- **Solvents:** generally use anhydrous polar solvents.

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Carbodiimide Reagents and Amide Formation

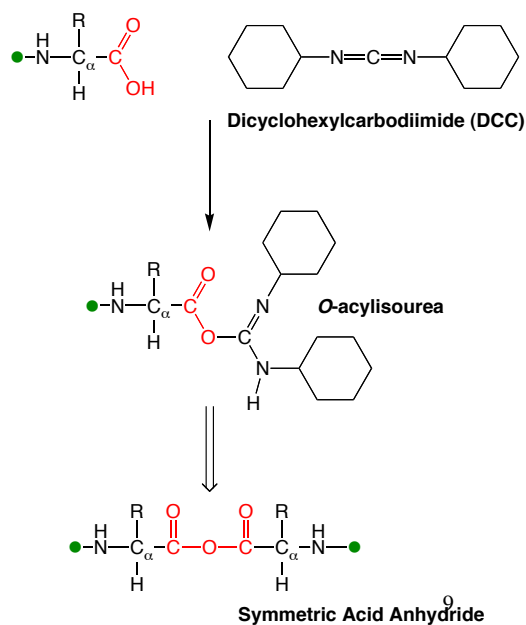
- Carbodiimides react with protected amino acids to form *O*-acylisourea, an activated intermediate.
- An amide bond is formed when the *O*-acylisourea undergoes aminolysis.
- Common carbodiimides include:
 - Dicyclohexylcarbodiimide (DCC)
 - 1-ethyl-3-(3'-dimethylamino propyl) carbodiimide (EDC)
 - Diisopropylcarbodiimide (DIPCDI)
- Side reaction results in the formation of *N*-acylurea.
- DCM is a good solvent for activation, less suitable for coupling reactions.



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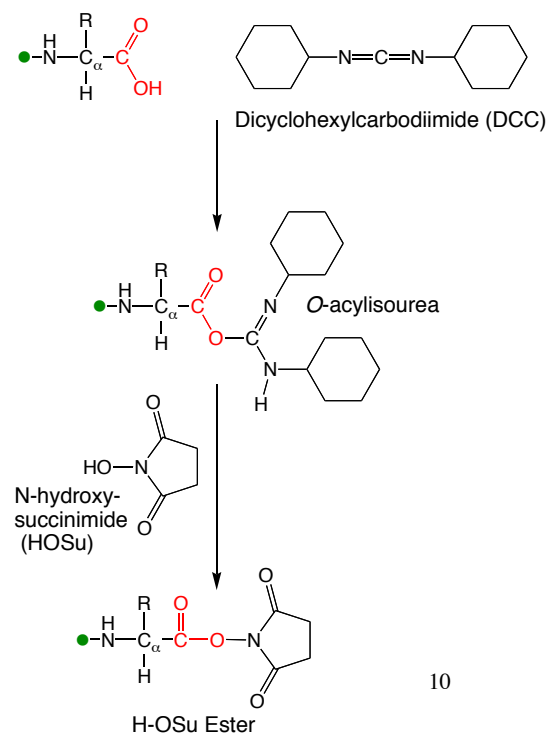
Carbodiimides and Acid Anhydrides

- Carbodiimide reagents can be used to generate symmetric anhydride.
- Formed when an excess of *N*-protected amino acid is present.
- Highly reactive species.
- Usually requires lowered reaction temperatures
- Can be isolated and used as activated amino acids for peptide synthesis.



Carbodiimides and Active Esters

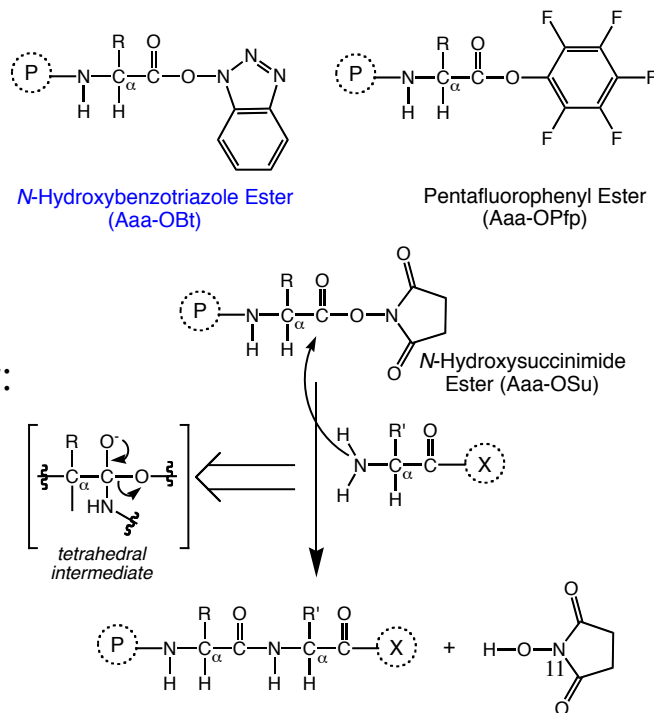
- Trapping agents can be used to reduce side reactions*.
- Trapping agents form "reactive" ester intermediates.
- Most common trapping agents:
 - *N*-Hydroxybenzotriazole
 - *N*-Hydrosuccinimide
- HOSu was found to suppress racemization and of *N*-acylisourea side reactions.
- DCC used in conjunction with HOBt has been found to be widely effective for amide bond formation.



*side reactions associated with carbodiimide and O-acylisourea.

- Active esters can be isolated and used as preactivated amino acids in peptide synthesis.
- Potential advantages:
 - Can be isolated and stored.
 - Fewer side reactions/cross reactivity.
 - Isolation of product.
- Commercial availability:
 - Pentafluorophenyl esters (-OPfp)
 - Hydroxysuccinimide esters (-OSu)
 - HOSu esters are relatively stable aqueous conditions.

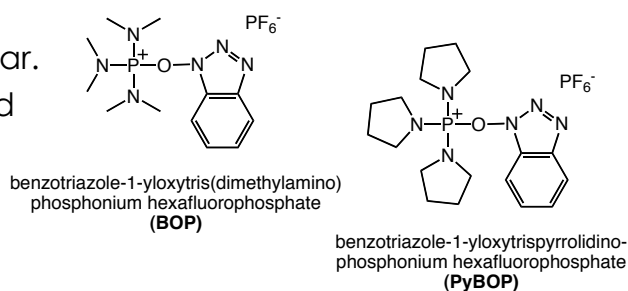
Active Esters and Amide Bonds



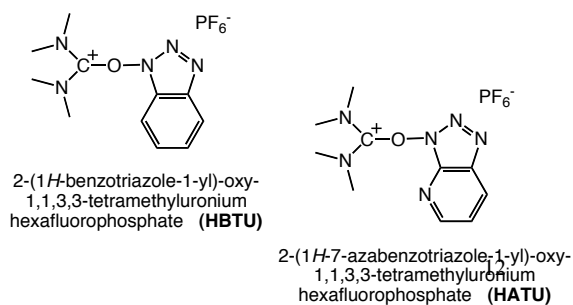
Phosphonium and Uronium Reagents

- Phosphonium and uronium reagents are chemically similar.
- Organic soluble salts (PF_6^- and BF_4^-)
- High reactivity and easy to handle.
- Frequently used in "one pot" reactions.
- No significant side reactions associated with phosphonium or uronium coupling reactions.
- HMPA is a byproduct of reactions involving Bop.
- In HATU, the N at 7 positions, accelerates reaction

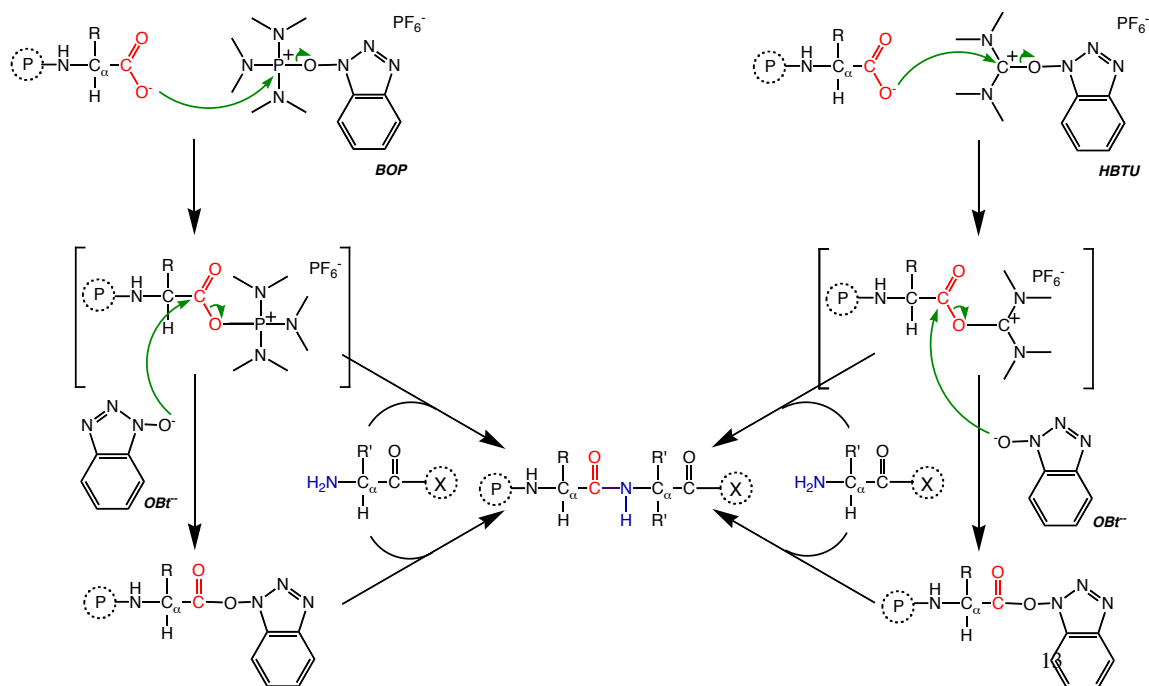
Phosphonium



Uronium

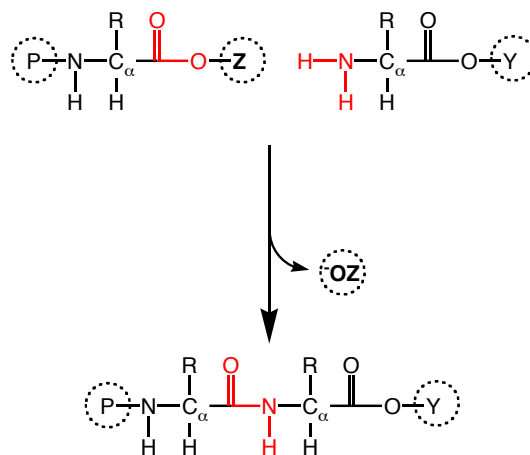


Phosphonium/Uronium Activation



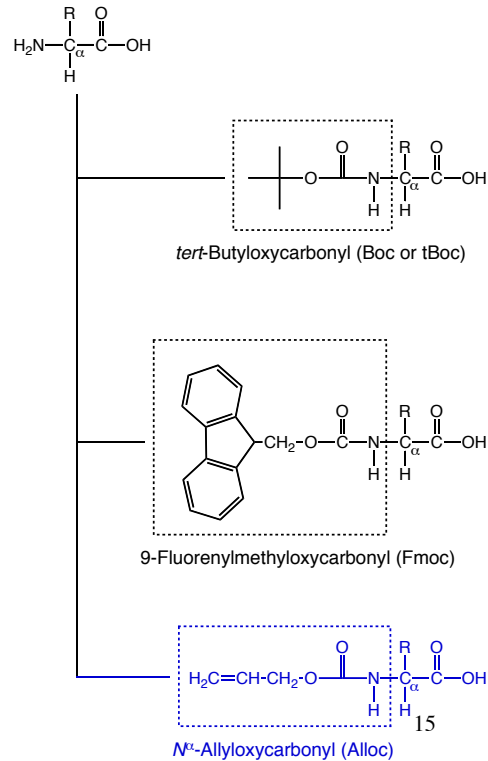
Amide Bond Formation: A Synthetic Approach

- Carboxylic acid and carboxylate groups are normally not very reactive.
- Formation of the amide (“peptide”) bond requires activation of the carboxylic acid.
- Controlled synthesis requires selective protection and deprotection of the various functional groups:
 - **The α -amino group.**
 - The α -carboxyl group.
 - Side chain functional groups.



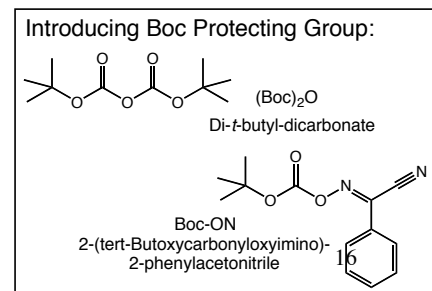
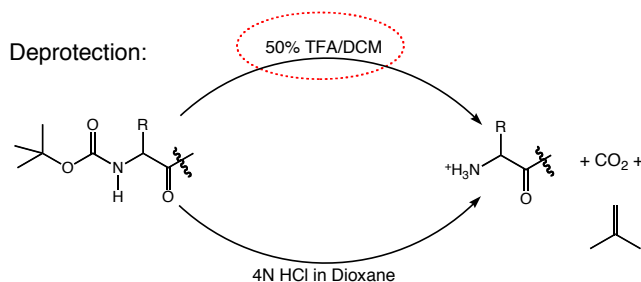
Amino Acid Protection

- The controlled synthesis of peptides and formation of amide bonds requires the use of reversible ion of the α -amino group.
- Three common protection chemistries are:
 - *tert*-Butoxycarbonyl (tBoc)
 - 9-Fluorenylmethyloxycarbonyl (Fmoc)
 - *N*-Allyloxycarbonyl (Alloc)
- These represent different protection and deprotection chemistries.
- It is also necessary to reversibly mask reactive side chain functional groups.



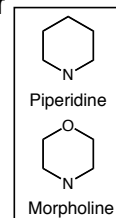
tert-Butoxycarbonyl

- Used in the standard Merrifield system, which is based on **graduated acid lability**.
- ***tert*-butoxycarbonyl (Boc) is an acid-labile α -amino protecting group.** Readily removed using TFA in DCM or 4N HCl in dioxane. The Boc group is **stable to basic conditions and nucleophiles**.
- Usually introduced onto an amino acid through $(\text{Boc})_2\text{O}$ or Boc-ON in aqueous 1,4-dioxane and NaOH or TEA.
- **Side chain protecting groups are usually ether, ester and urethane derivatives of benzyl alcohols.**
- Synthesized peptides are **cleaved from solid support and side chain protecting groups are removed using strong acids such as HF or TFMSA**. Scavengers are usually required to minimize side reactions.

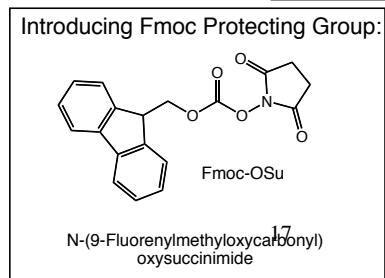
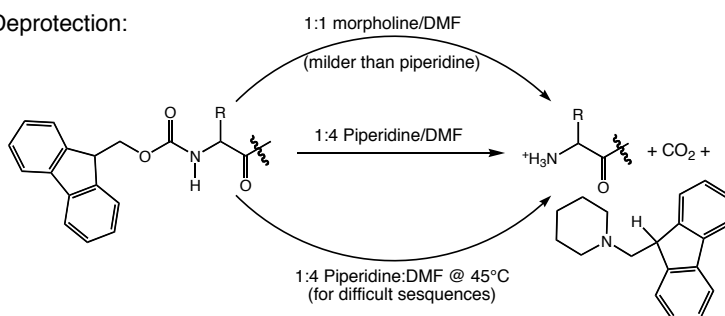


9-Fluorenylmethoxycarbonyl

- This synthetic method employs an **orthogonal protection strategy**.
- 9-Fluorenylmethoxycarbonyl (Fmoc) is a **base labile α -amino protecting group**. Usually removed using 20-50% piperidine in DMF or NMP (10-18 min).
- Usually introduced onto an amino acid through the Fmoc succinimidyl carbonate in an organic/aqueous solvent mixture and base.
- **Side chain protecting groups are usually based on ether, ester and urethane derivatives of *tert*-butanol.**
- **Peptides are cleaved from the solid support and side chain protecting groups removed using strong acid: TFA at RT.** Scavengers are usually used to minimize side reactions.



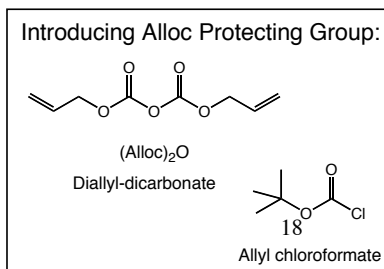
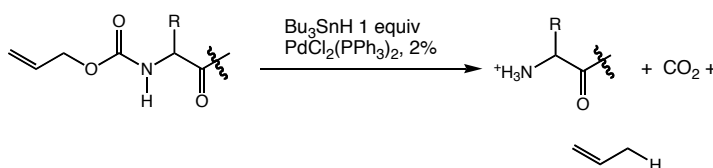
Deprotection:



Allyloxycarbonyl

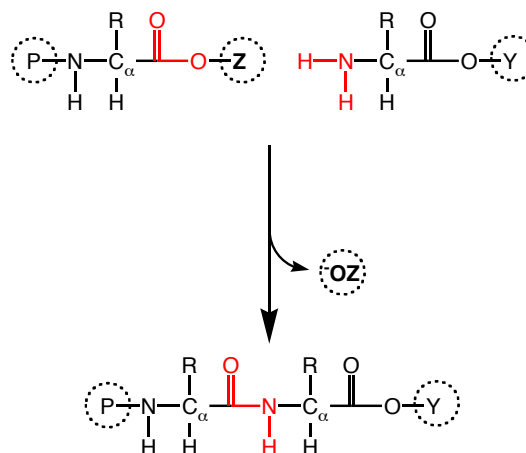
- Allyloxycarbonyl (Alloc) represents a protection and synthetic scheme that is **orthogonal to both Boc and Fmoc chemistries**.
- Alloc is an α -amino protecting group **removed by mild hydrogenation** conditions.
- Alloc is **stable to acids and bases**, and it is resistant to Boc and Fmoc reaction/deprotection conditions.
- Usually introduced onto an amino acid by means of the chloroformate.
- **Suitable for the protection of amines and alcohols. Carboxylic acids can be similarly protected as allyl esters.** Avoids the need for harsh acids such as TFA and HF.

Deprotection:



Amide Bond Formation: A Synthetic Approach

- Carboxylic acid and carboxylate groups are normally not very reactive.
- Formation of the amide ("peptide") bond requires activation of the carboxylic acid.
- Controlled synthesis requires selective protection and deprotection of the various functional groups:
 - The α -amino group.
 - The α -carboxyl group.
 - **Side chain functional groups.**



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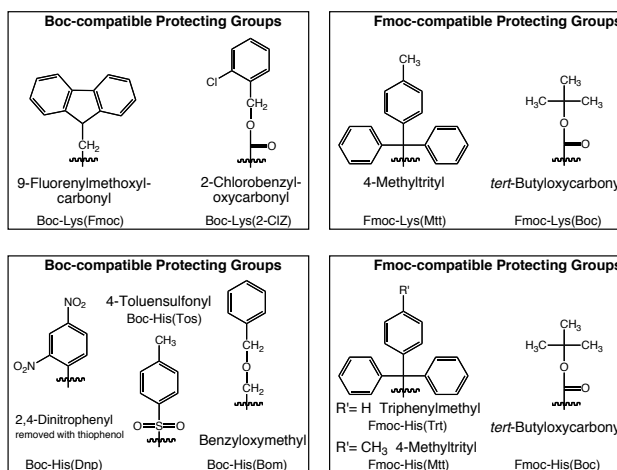
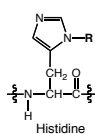
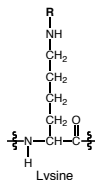
Lysine:

The ϵ -amino group of lysine is a **potent nucleophile**. Side reactions during the coupling reaction can be minimized by use of appropriate blocking groups.

Side Chain Side Reactions

Histidine:

Histidine presents several problems. **Acylation (reversible) of the histidine side chain** occurs during the coupling reaction when unprotected or temporarily protected histidine is used. Histidine is also **prone to racemization** which can be reduced by blocking the τ -nitrogen.

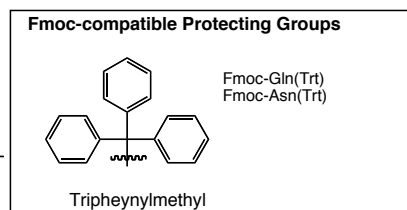
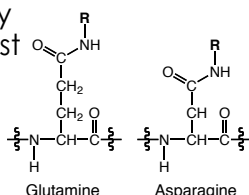


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Side Chain Side Reactions

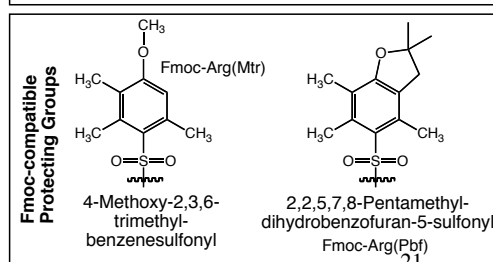
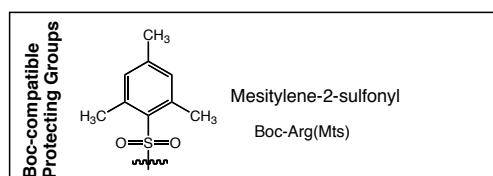
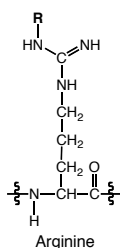
Arginine:

The **guanidine group is strongly nucleophilic** and can be easily acylated during synthesis. Most protecting groups block the ω-nitrogen.



Asparagine/Glutamine:

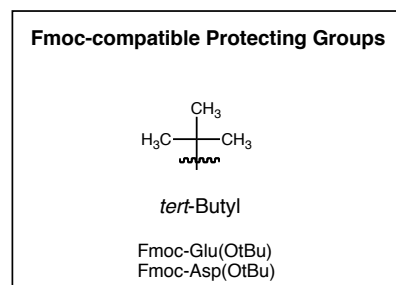
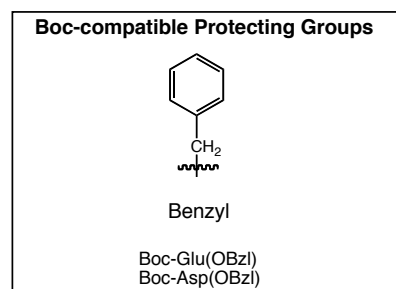
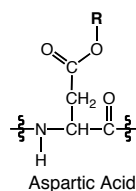
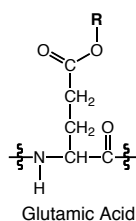
Can be incorporated without side chain protection. However, they can undergo several side reactions. The most common being dehydration of the carboxamide forming a nitrile. Can be minimized by using HOBT in the coupling reaction. Blocking the side chain carboxamide group also prevents dehydration. Gln is prone to pyroglutamate formation, which is prevented by altering deprotection and coupling conditions in Boc chemistry.



Side Chain Side Reactions

Aspartic Acid/Glutamic Acid:

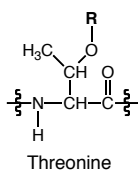
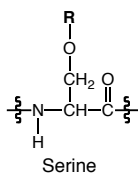
Asp is prone to cyclization forming succinimide. Opening of this ring results in β-aspartyl peptides. Blocking the side chain carboxylate reduces the probability of this. However, it still occurs, and this is sequence dependent. **Glu as well can undergo cyclization** and subsequent formation of γ-Glu peptides. Normally prevented by using same blocking groups as used with Asp.



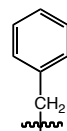
Side Chain Side Reactions

Serine/Threonine:

Side chain **hydroxyl groups may complicate syntheses** by functioning as nucleophiles during coupling reactions. Use of appropriate protecting groups can prevent these side reactions.



Boc-compatible Protecting Groups

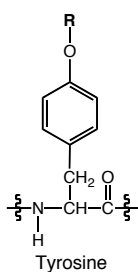


Benzyl

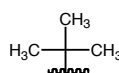
Boc-Ser(Bzl)
Boc-Thr(Bzl)
Boc-Tyr(Bzl)

Tyrosine:

As with Ser and Thr, use of appropriate blocking groups can prevent side reactions. However, care must be taken in selecting side chain protecting groups so as to prevent possible side reactions that may occur during removal of the side chain protecting group.

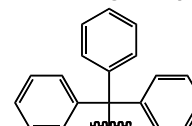


Fmoc-compatible Protecting Groups



tert-Butyl

Fmoc-Ser(*t*Bu)
Fmoc-Thr(*t*Bu)
Fmoc-Tyr(*t*Bu)



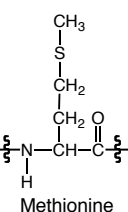
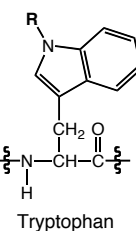
Triphenylmethyl

Fmoc-Ser(Trt)
Fmoc-Thr(Trt)

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Tryptophan:

There are **two main side reactions that Trp may undergo: Oxidation of the indole ring or alkylation of the indole ring by carbonium ions formed during cleavage.** In Boc syntheses, protecting the Trp with a formyl group generally prevents these reactions. It is resistant to these conditions. In the case of Fmoc syntheses, these side reactions have generally been minimized by using appropriate scavenger mixtures during the cleavage reaction.



Methionine:

Main side reaction is the acid **catalyzed oxidation of the thioether** to the sulfoxide. This can be reversed during cleavage by using a reducing scavenger such as thioanisole.

Side Chain Side Reactions

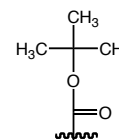
Boc-compatible Protecting Groups



Boc-Trp(For)

Formyl protection for indole ring of Trp

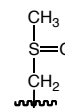
Fmoc-compatible Protecting Groups



Fmoc-Trp(Boc)

tert-Butyloxycarbonyl

Boc-compatible Protecting Groups



Methionine can be protected as the sulfoxide which is reduced by thioanisole during cleavage.

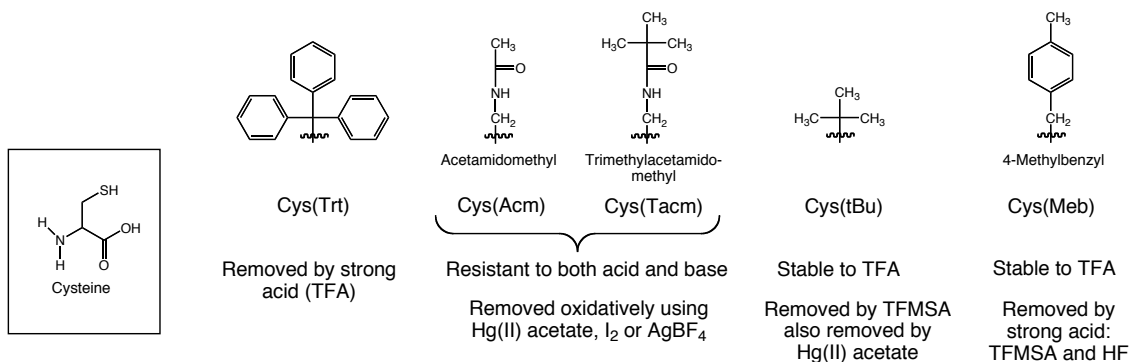
Side Chain Side Reactions

Cysteine:

The thiol side chain of cysteine is strongly nucleophilic and is prone to oxidation. Blocking this thiol usually prevents these side reactions. There are some concerns associated with side reactions that may occur during deprotection and cleavage, but these can be controlled by using appropriate protecting groups and deprotection conditions.

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Cysteine a Special Case

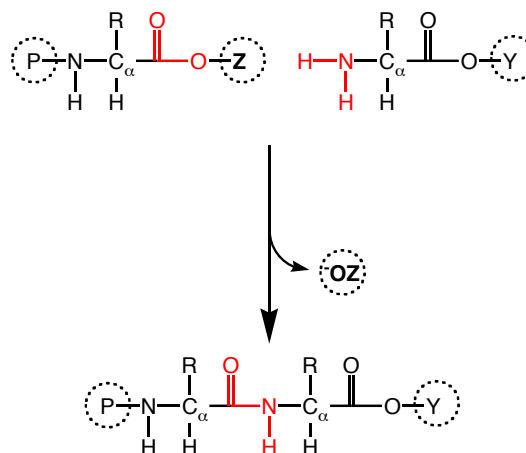


- **Fmoc syntheses: Trt, Acm, tBu and Tacm**
- **Boc syntheses: Acm, Tacm, tBu and Meb**
- Some Cys protecting groups are removed during cleavage: Cys(Trt), Cys(tbu) and Cys(Meb).
- Other Cys protecting groups are stable to these conditions (i.e. Acm and Tacm) allowing these Cys side chains to be selectively deprotected after cleavage.
- Selective deprotection of cysteine residues allows controlled formation of disulfide bonds.

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Amide Bond Formation: A Synthetic Approach

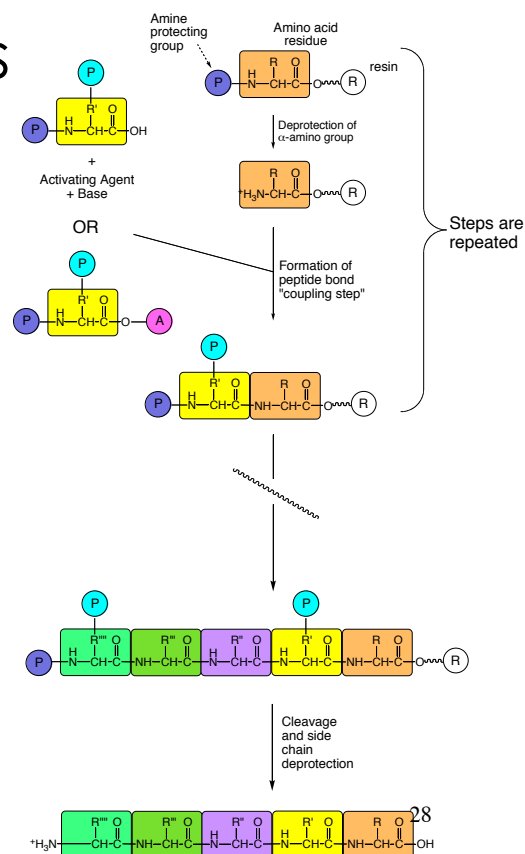
- Carboxylic acid and carboxylate groups are normally not very reactive.
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 - The α -carboxyl group.
 - **Side chain functional groups.**



27

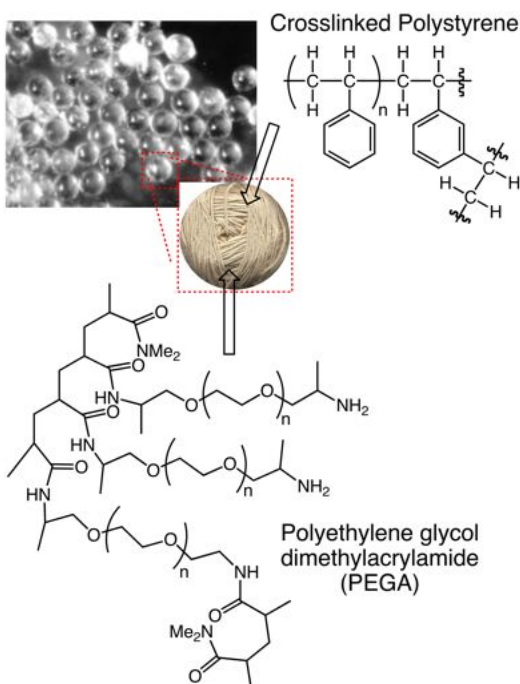
Chemical Synthesis of Peptides

- Chemical peptide synthesis capitalizes on old-established chemistry.
- In solid-phase peptides synthesis the peptide being synthesized is anchored to an insoluble solid support.
- SPPS allows efficient removal of excess reagents and soluble byproducts after each reaction cycle because the peptide remains anchored to the solid support.
 - Deprotection
 - Coupling
 - Washes
- This is not the case in solution-phase chemistry .
- Both synthetic strategies require the chemistries be optimized for efficiency.



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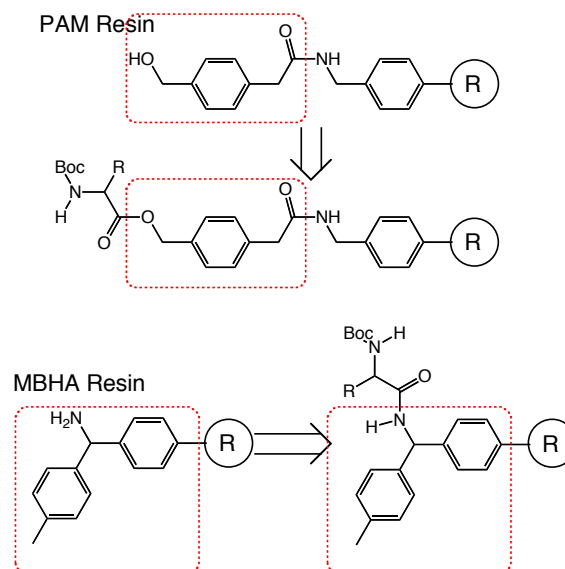
Resins and Solid Supports



- In solid-phase peptide synthesis (SPPS) the growing peptide is affixed to a “solid” support, a crosslinked polymer resin. Such as:
 - Crosslinked polystyrene
 - Polyethylene glycol dimethylacrylamide (PEGA)
- For successful syntheses, the resin cannot be rigid and static, it must be able to swell and shrink depending on environment.
- Much of the actual chemistry takes place within the resin bead as well as on the surface.
- Resin must be resistant to reaction conditions.
- The growing peptide is attached to the solid support by means of linker units, which provide for release the peptide upon completion of₂₉ assembly of the peptide.

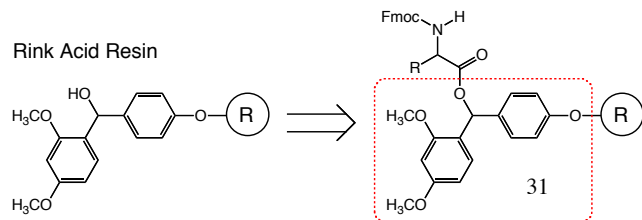
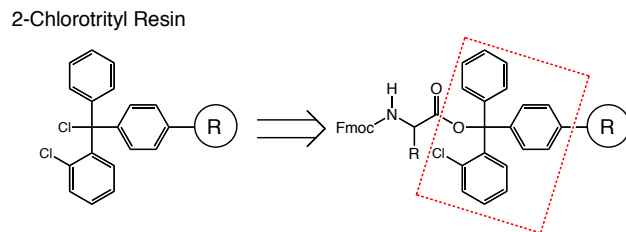
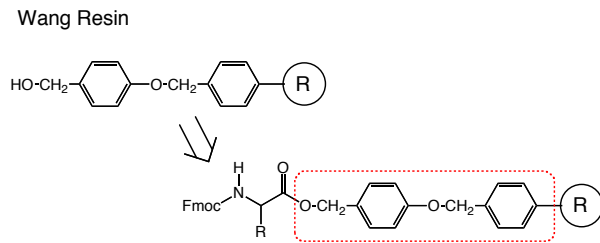
- Anchoring linkages in Boc syntheses must be resistant to TFA.
- Generally substituted benzyl ester type linkages are used when a peptide acid is desired. 4-(hydroxymethyl)phenylacetic acid (**PAM**)
- When a peptide C-terminal amide is desired a anchoring linkage based on benzhydrylamine is used. 4-methylbenzhydrylamine (**MBHA**)
- In both cases, the peptide can be **cleaved from the anchoring linker using strong acid** such as anhydrous **HF** or **TFMSA**.
- **Resins can be purchased with and without the first amino acid being attached.**

Boc Compatible Linkers



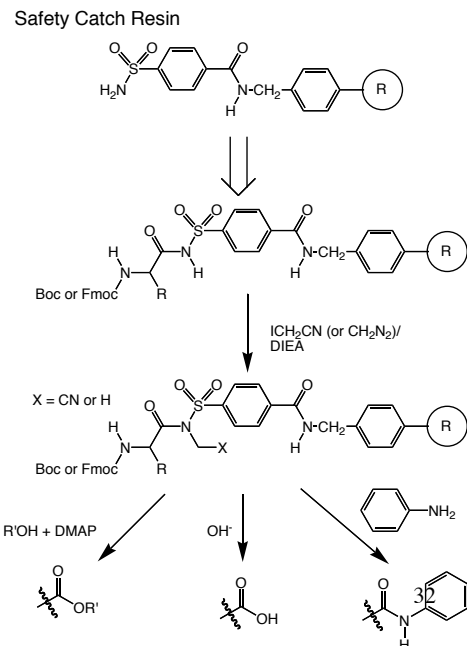
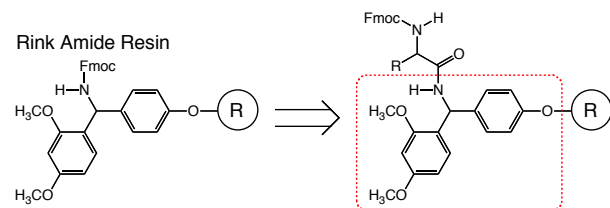
Fmoc Compatible Linkers

- An assortment of anchoring linkages are compatible with Fmoc chemistry that afford peptide acids.
 - 4-alkoxybenzyl alcohol resin (**Wang**)
 - 2-chlorotrityl-chloride resin
 - 4-(2',4'-dimethoxyphenyl-hydroxymethyl)phenoxy resin (**Rink Acid**)
- The peptide can be cleaved from these anchoring linker using TFA or in some cases acetic acid.
- Resins can be purchased with and without the first amino acid being attached.



Additional Linkers

- Using Fmoc chemistry and a C-terminal **peptide amide** is desired.
 - 4-(2',4'-dimethoxyphenyl-aminomethyl)phenoxy resin (**Rink Amide**)
- Cleavage from the resin is achieved using TFA.
- Resins for nonacidic cleavage.**
- Sulfamylbenzoyl (**Safety-Catch**) resin.
- The resin sulfonamide is stable to nucleophiles and bases.
- The anchoring linkage is activated by treating with iodoacetonitrile or diazomethane.
- Peptide can then be released by treating with a range of nucleophiles

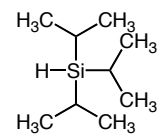
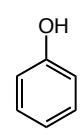
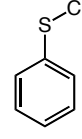
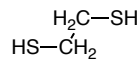
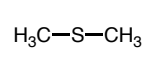
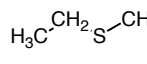
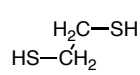
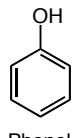
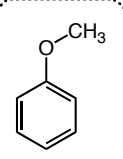
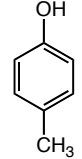
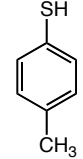
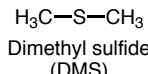
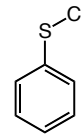
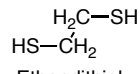
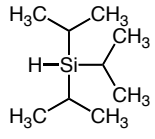
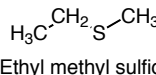
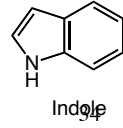


Scavengers

- During cleavage it is frequently necessary to include scavengers in the reaction mixture.
- In Fmoc chemistry--Reagent K cocktail:
TFA/water/phenol/ thioanisole/EDT
(82.5:5:5:5:2.5)
- Alternatively, protected amino acids could be selected so as to minimize potential side reactions
- Fmoc --> TFA/TIS/water (95:2.5:2.5).

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Scavengers and Additives

Alkylation of Aromatics <i>N</i> - and <i>S</i> -alkylation (Trp, and Met)				
			Phenol	Thioanisole
Sulfonation of Met, Trp, Ser and Thr in Fmoc		Additional Additives		
				
Ethanedithiol (EDT)	Phenol	Anisole	p-cresol	p-Thiocresol
Oxidation of Cys or Met				Trp oxidation and alkylation
			Thioanisole	
Ethanedithiol (EDT)	Triisopropylsilane (TIS)	Ethyl methyl sulfide (EMS)		Indole

Boc Cleavage Conditions

Standard HF cleavage:

- Resin and scavengers (anisole/DMS/p-thiocresol or DMS/anisole) combined in reaction vessel.
- Vessel cooled in dry ice methanol.
- Distil HF into flask (keep temperature between -5° and 0°C)
- At the end of the reaction, evaporated HF and DMS under a stream of N₂.

Low-high HF cleavage:

Low

- Cool peptide and reaction vessel in ice bath (5-0°C).
- Add m-cresol, TFA, TFMSA (slowly to avoid warming)
- In peptides containing Trp(For) add EDT.
- After 3 hours at 0-5°C filter reaction, wash and keep resin.
- Dry resin.

High

- Combine dried resin with EDT and thioanisole.
- Cool to 0-5°C and add TFA, then slowly add TFMSA.
- Allow reaction to warm to RT and continue for specified time.
- Filter to remove resin and wash with TFA. Combine filtrates and dilute 8-10 fold in cold ether.

Fmoc Cleavage Conditions

- **Remove the N-terminal Fmoc group prior to cleavage.**
- Place dry resin in flask and add TFA and scavengers.
- Allow to react at RT.
- Remove resin by filtration and wash with TFA.
- Combine filtrates and dilute 8-10 fold in cold diethyl ether. (anticipate precipitation of peptide)

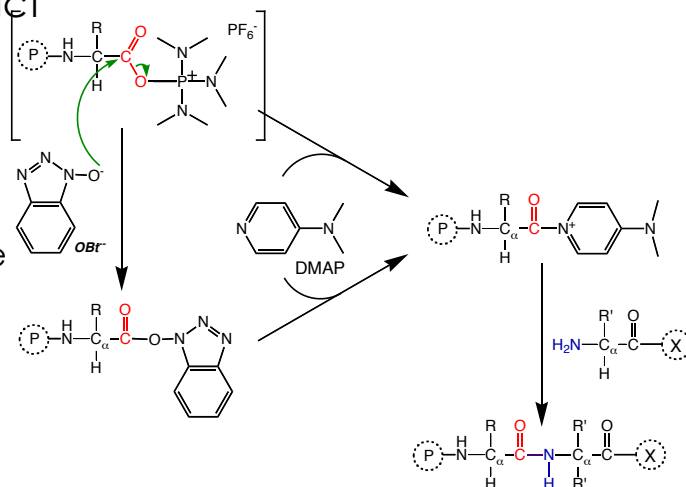
Purification of Synthetic Peptide

- The peptide is usually precipitated from the cleavage reaction mixture by the addition of diethyl ether. This removes the acid, some scavengers and byproducts.
- The peptide/resin mixture can be suspended in water or aqueous acid and filtered to remove the resin.
- Many of the salts and remaining scavengers can be removed by gel-filtration and/or ion exchange chromatography.
- The target peptide can then be isolated from other peptide products and impurities by reversed-phase HPLC. (C4, C8, C18)

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Capping and Double Coupling

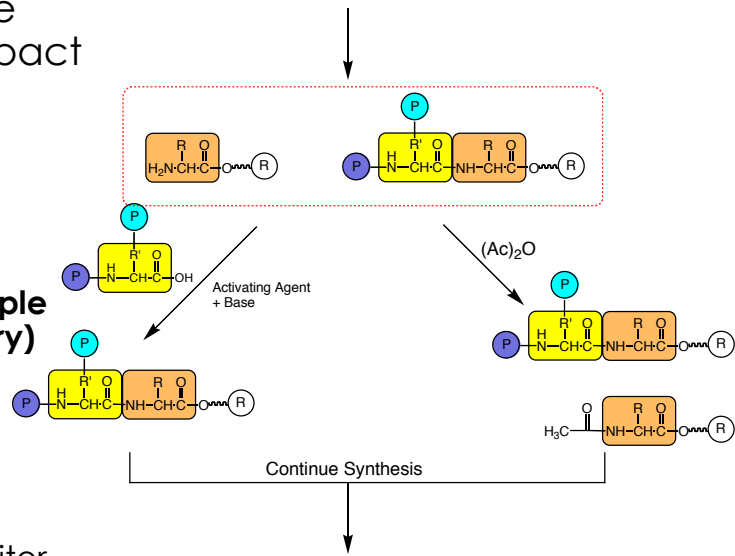
- Not all coupling steps will go to completion.
- This can complicate purification and impact yield.
- Three options:
 - Optimize reaction conditions and use **acylation catalyst**.
 - Double coupling (triple coupling if necessary)
 - Capping
- Various methods to monitor reaction efficiency:
 - Fmoc synthesis monitor 300-320 nm in flow through.



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Capping and Double Coupling

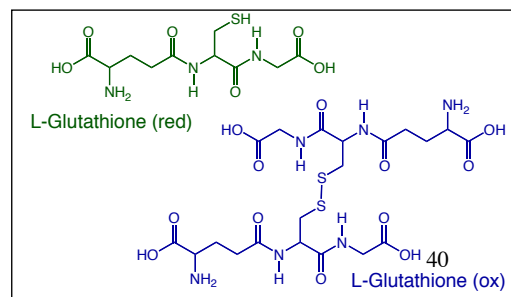
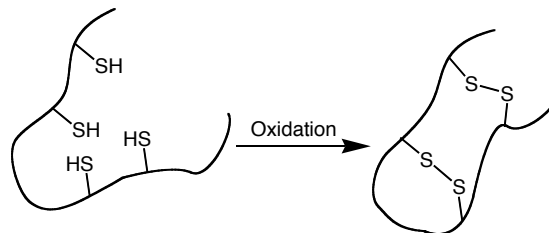
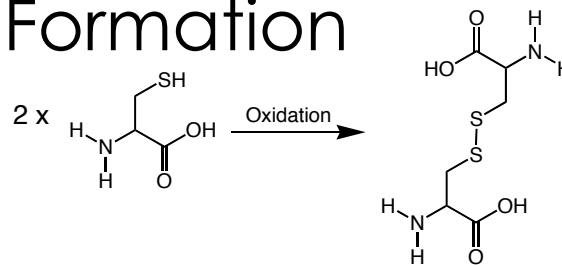
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 - Fmoc synthesis monitor 300-320 nm in flow through.



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Disulfide Formation

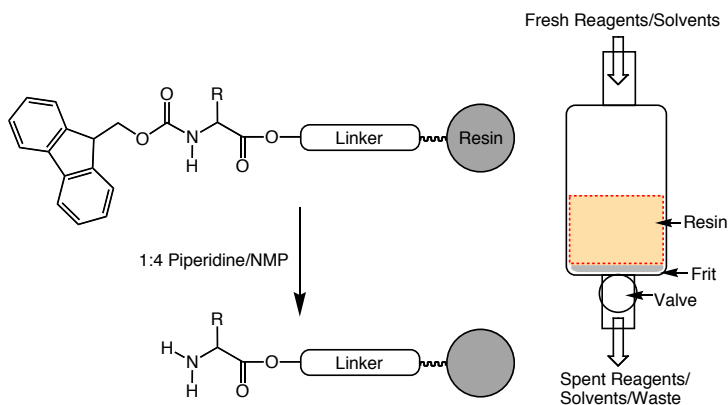
- Many approaches to forming disulfide bonds.
 - Air oxidation
 - Use of oxidizing agents such as DMSO.
- When multiple disulfide bonds are being formed:
 - Combinations of reduced and oxidized thiols promote disulfide shuffling.
 - Selective formation of disulfide bonds.



	Cleavage Conditions (Boc linkages)		Cleavage Conditions (Fmoc linkages)	
	Side Reaction	Precaution Strategies	Side Reaction	Precaution Strategies
Tyrosine	C-alkylation of aromatic ring (i.e. by O-benzyl)	substitute thioanisole for anisole as scavenger	tert-butylation Sulfonation	Use EDT as scavenger Use EDT and phenol as scavenger
Methionine	S-alkylation	Avoid thioanisole as scavenger DMS or EMS prevent Met alkylation	tert-butylation Sulfonation	Use EDT and thioanisole as Scavenger Use EDT or phenol as scavenger
	S-oxidation	DMS or EMS can minimize oxidation DMS, EMS or 2-mercaptopyridine reduce Met(O)	Oxidation to Met(O)	Use EMS, EDT and/or thioanisole as scavenger
Tryptophan	N-alkylation	Avoid thioanisole as scavenger	Acid catalyzed ozonolysis	Use EDT as scavenger
	Irrev. Oxidation	Indole as a scavenger minimizes oxidation and alkylation.	Alkylation by t-butyl	Use EDT as scavenger
			Alkylation by resin bound benzyl (Wang) or benzhydriyl (Rink)	Use EDT as scavenger Use EDT, TISP and phenol as scavengers
			Sulfonation by Pmc	Use H ₂ O and phenol as scavengers Use H ₂ O in combination with EDT
Cysteine	Oxidation	prevent exposure to air and use scavenger cocktail: HF/anisole/DMS/p-thiocresol	Deprotection or Acn and tBu by thioanisole	Avoid thioanisole
			Reattachment to cationic benzyl type linkers	Use EDT as scavenger
			Incomplete Cys(Trt) deprotection	Used EDT and TISP as scavengers
Aspartic acid	Aspartimide formation with Asp-Gly	Asp(OBzl) and Asp(OChx) cleave at -5°C or lower.		
Glutamic acid	Acylation of scavengers by Glu	Decrease cleavage reaction temp (-5-0°C) and use Glu(OChx)		
Serine/Threonine			Sulfonation by Pmc	use H ₂ O and phenol as scavengers.
Arginine			Incomplete and sluggish removal of arylsulfonyls (Pmc and Pbf)	Use thioanisole as a scavenger to accelerate the process
Asparagine			N-terminal Asn(Trt) is stable to TFA	Extend reaction time and use TISP to quench Trt.

SPPS Step by Step: Deprotection

- **Wash:** NMP or DMF [3x 1min each]
- **Deprotection:** Piperidine/NMP (or DMF) (1:4) ~20 minutes
- **Wash:** NMP (or DMF) [3x 1min each]



SPPS Step by Step: Coupling

- **Activation:**
Fmoc- amino acid (4 equiv.) in NMP (or DMF); HBTU (3.6 equiv); DIEA 97.2 equiv); HOBt (4 equiv) [5 min.]

- **Coupling:**
combine with resin [45 min]

- **Wash:** NMP (or DMF) [3x 1min each.]

- **Process is repeated....**

