

Unit 2: Chemistry of Peptides: Coupling Reactions, Solid Phase and Solution Phase Synthesis

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2.1 Peptides as drugs and the problem of making them

A peptide is a chain of amino acids joined by amide (peptide) bonds. The bond has partial double bond character, is planar, and almost always trans. Peptide drugs range from oxytocin (9 residues) and insulin (51 residues in two chains) to modern blockbusters, and peptide synthesis is therefore a manufacturing discipline, not an academic curiosity. The synthetic problem has three layers: twenty or so different side chains must survive the synthesis untouched, so they are protected; each new peptide bond must be formed between a carboxyl group of one amino acid and the amino group of the next without scrambling either stereocentre; and a chain of n residues needs $n - 1$ successful couplings, so even 99 percent per step gives only about 60 percent of correct full-length molecules at 50 steps. Every method in this unit is an answer to those three layers.

2.2 Protecting the building blocks

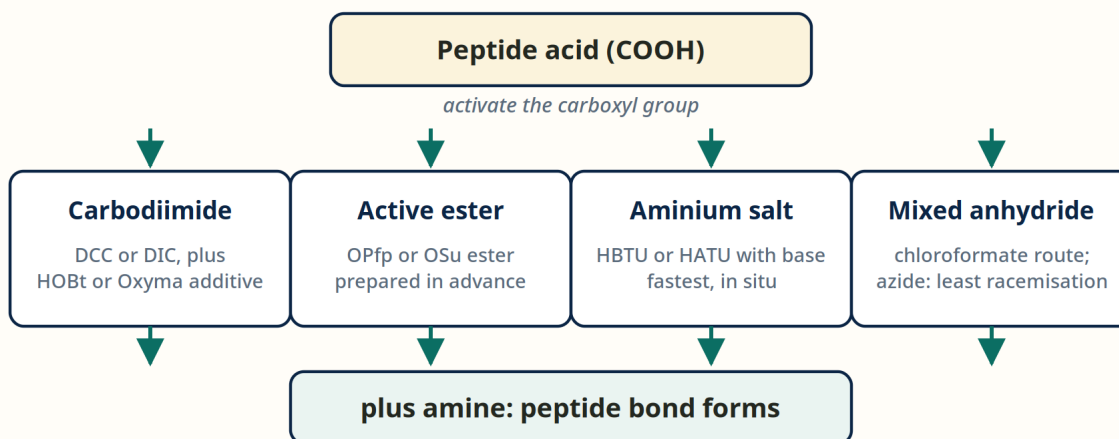
The alpha-amino group is protected with a group that can be removed under conditions that leave everything else intact. The three classical choices define the strategies of this unit:

- **Boc (tert-butyloxycarbonyl):** removed by moderate acid (trifluoroacetic acid). Acid-labile, the heart of the Boc strategy.
- **Fmoc (9-fluorenylmethoxycarbonyl):** removed by base (piperidine), through beta-elimination. Base-labile, the heart of the Fmoc strategy.
- **Cbz or Z (benzyloxycarbonyl):** removed by catalytic hydrogenolysis; the older solution phase standard.

Side chains carry protection matched to the strategy: in Boc chemistry, benzyl-based groups (benzyl ethers and esters, tosyl on arginine) that withstand repeated TFA treatment and are removed only by strong acid (HF) at the end; in Fmoc chemistry, tert-butyl-based groups (tert-butyl ethers and esters, Boc on lysine, trityl on cysteine and histidine, the sulfonyl group Pbf on arginine) that fall off in the final trifluoroacetic acid cleavage. A protection scheme in which each class of group is removed by a different mechanism is called **orthogonal**, and orthogonality is what makes controlled, stepwise synthesis possible at all.

2.3 Coupling reactions: forming the peptide bond

Activating the carboxyl group for coupling



Same product, four activation chemistries: the choice is racemisation risk, speed and cost.

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Figure: Every coupling reagent solves the same problem, activating the carboxyl group, but they differ in racemisation risk, speed, by-products and cost.

A free carboxylic acid and a free amine do not form an amide at a useful rate; the acid must be **activated** so that its carbonyl carbon becomes electrophilic enough for the amine to attack. The activation must also be gentle, because the activated species is exactly the species that racemises (section 2.4). The standard methods:

- **Carbodiimide method (Sheehan, 1955):** dicyclohexylcarbodiimide (DCC) reacts with the carboxylic acid to form an O-acylisourea, a powerful activated ester equivalent; the amine attacks and the urea (dicyclohexylurea, DCU) falls out. DCU is insoluble and contaminates products, which is why diisopropylcarbodiimide (DIC), whose urea stays soluble, is preferred on solid phase, and why the water-soluble EDC is used in solution work.
- **Additives:** 1-hydroxybenzotriazole (HOBT), its aza analogue HOAt, and the modern replacement Oxyma intercept the O-acylisourea to give an active ester that couples efficiently but racemises far less. Carbodiimide plus additive is the workhorse combination.
- **Preformed active esters:** pentafluorophenyl (OPfp) and N-hydroxysuccinimidyl (OSu) esters are stable, isolable activated forms, coupled with only mild base present.
- **Mixed anhydride method:** the acid is converted with isobutyl chloroformate and a tertiary base into a mixed carbonic anhydride, which the amine attacks at the correct carbonyl.
- **Azide method:** the peptide ester is converted to a hydrazide and diazotised to an acyl azide, which couples with exceptionally low racemisation; older, slower, but still the referee method for fragment condensations.
- **Aminium and phosphonium salts:** HBTU, HATU and PyBOP generate the active ester in situ from the acid and a tertiary base; HATU (built on HOAt) is the fastest and most racemisation-resistant of the family and is the default for difficult couplings on automated synthesizers. (The BOP reagent of MPC102T belongs to this family; its by-product issues led to PyBOP.)

2.4 Racemisation: the enemy inside the activation step

When an N-acyl amino acid or peptide acid is activated, the carbonyl oxygen of the preceding amide can attack the activated carbonyl and close a five-membered **oxazolone (azlactone)** ring. The oxazolone's

alpha proton is acidic; a base removes it to give an achiral, planar intermediate, and reprotonation returns both configurations. Ring opening by the amine then installs a mixture of the correct residue and its epimer. Urethane protection (Boc, Fmoc, Cbz) suppresses oxazolone formation because the carbamate carbonyl is less nucleophilic, which is why single protected amino acids couple almost cleanly, while **segment condensations**, where the activated component is itself a peptide with an ordinary amide behind the alpha centre, are the dangerous operations, and are planned at glycine or proline junctions where racemisation is impossible or slow. Low temperature, minimal base, and HOBt, HOAt or Oxyma additives complete the defence.

2.5 Principles of solid phase peptide synthesis

In 1963 Robert Bruce Merrifield inverted the logic of synthesis: instead of purifying the product after every step, anchor the growing peptide to an insoluble polymer bead. The C-terminal amino acid is attached to the resin through a linker; each cycle then adds one residue using a large excess of reagents to force the coupling to completion, and every excess reagent, by-product and failure is removed by simply washing the beads, operations that a machine can repeat unattended. Purification happens once, at the end. The price of the method is that chemistry must be nearly quantitative at every step (section 2.1) and that the final product mixture contains truncated and deletion sequences rather than the intermediates of classical synthesis. **Capping**, acetylating any amino groups that failed to couple, keeps those failures short and easy to separate later.

2.6 Solid supports and linkers

The original support is polystyrene cross-linked with 1 to 2 percent divinylbenzene, which swells in dichloromethane and DMF so reagents can reach the chains inside the bead. Polyethylene glycol containing resins (such as PEG-polystyrene grafts) swell in a wider range of solvents and behave more like solution chemistry. The **linker** is the molecule between bead and peptide: it decides what the C-terminus becomes on cleavage and under what conditions it is released.

- **Merrifield resin:** chloromethylated polystyrene; the first amino acid is attached as a benzyl ester; cleavage needs HF; gives peptide acids.
- **PAM resin:** a more acid stable linker for Boc chemistry, reducing losses during repeated TFA cycles.
- **Wang resin:** a p-alkoxybenzyl alcohol linker; standard for Fmoc synthesis of peptide acids, cleaved by TFA.
- **Rink amide resin:** cleaved by TFA to give a C-terminal peptide **amide**, essential for the many peptide hormones (including oxytocin) whose natural form is amidated.
- **MBHA resin:** the corresponding amide-producing support for Boc chemistry, needing HF cleavage.

2.7 The Boc protocol

Each cycle runs: remove the Boc group with about 50 percent trifluoroacetic acid in dichloromethane; wash; neutralise the resulting ammonium salt with a tertiary base such as diisopropylethylamine; couple the next Boc amino acid (with DCC and HOBt, or HBTU). Side chains wear benzyl-type protection throughout. At the end, the peptide is cut from the resin and all side chain protection is removed in one strong acid step with liquid **HF**, using scavengers such as anisole and p-cresol to trap the benzyl cations released. Because HF is exceptionally hazardous, the **low-high HF protocol** (Tam) was developed: a first treatment with HF diluted in dimethyl sulfide and p-cresol removes most protecting groups by a gentler, more SN₂-like pathway with fewer side reactions, and a second, concentrated HF step completes the cleavage. Boc chemistry survives today where Fmoc struggles, notably for long, aggregation-prone sequences, because

the repeated acid treatments keep the growing chain protonated and better solvated.

2.8 The Fmoc protocol

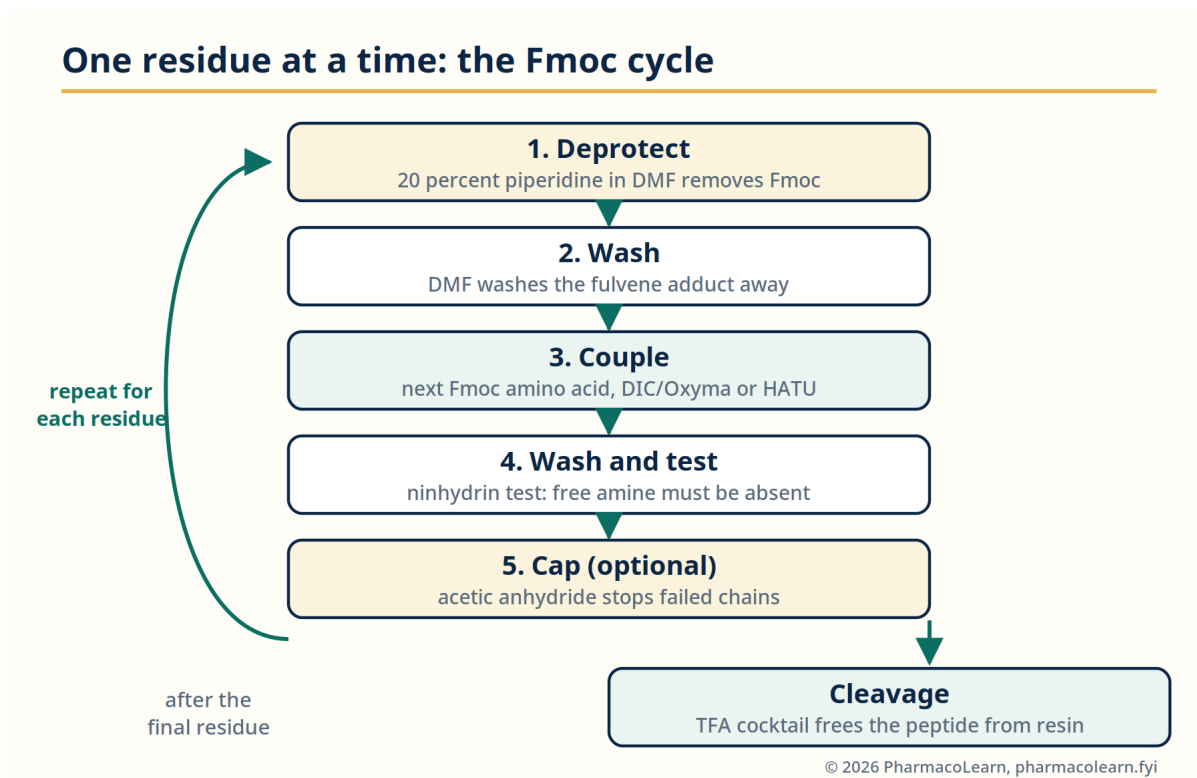


Figure: The Fmoc cycle: deprotect, wash, couple, wash and test, cap if needed, and repeat until the chain is finished, then cleave with TFA.

Each cycle runs: remove the Fmoc group with 20 percent piperidine in DMF, which also traps the dibenzofulvene released as a stable adduct whose ultraviolet absorbance lets the machine monitor deprotection; wash; couple the next Fmoc amino acid (with DIC and Oxyma, or HATU and base). Side chains wear tert-butyl-type protection. At the end, one treatment with a trifluoroacetic acid cocktail (classically TFA with water and triisopropylsilane as scavengers) cleaves the peptide from a Wang or Rink linker and strips every side chain group in the same operation. The product is precipitated in cold ether, collected and freeze dried. Fmoc chemistry is the modern default: no HF, milder conditions throughout, and continuous spectrophotometric monitoring of each deprotection.

One Fmoc solid phase cycle

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resin bound peptide, N-terminal Fmoc group on
-> 20 percent piperidine in DMF: Fmoc group off, fulvene adduct washed away
-> wash: DMF several times, bead ready
-> Fmoc amino acid + DIC/Oxyma (or HATU/base): coupling, 30 to 60 minutes
-> wash: DMF several times
-> optional capping with acetic anhydride: failed chains stopped here
-> repeat for the next residue
after the last residue: TFA cocktail cleaves peptide from resin
-> precipitate in cold ether, purify by preparative HPLC
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2.9 Cleavage, free peptides and peptide amides

Cleavage is a chemical step in its own right, not just unhooking. The cocktail must supply acid strong enough to break the linker and the side chain groups, plus scavengers (water, triisopropylsilane, anisole, p-cresol, ethanedithiol in HF work) to absorb the reactive carbocations before they alkylate tryptophan,

tyrosine or methionine in the product. The choice of linker made at the start fixes the output: acid linkers give **free peptides** ending in a carboxylic acid; amide linkers (Rink, MBHA) give **peptide amides** ending in CONH₂, a difference that decides biological activity for many hormones. After cleavage the crude peptide is precipitated, dissolved and taken to purification.

2.10 Purification and case studies

Crude solid phase products are purified by preparative reversed phase HPLC, exploiting the large polarity differences between the target and its deletion sequences, and characterised by mass spectrometry and analytical HPLC. Two case studies from the history of the method belong in every account:

- **Bradykinin:** Merrifield's solid phase synthesis of this nonapeptide in 1964 demonstrated the speed of the approach, days instead of the months a solution synthesis of the same molecule had required, and converted a sceptical field.
- **Enfuvirtide (T-20):** the 36 residue HIV fusion inhibitor was manufactured commercially by a hybrid strategy, solid phase preparation of protected fragments followed by solution phase fragment condensation, showing that SPPS scales to tonne quantities of a complex peptide drug when the economics demand it. (The total synthesis of the enzyme ribonuclease A, 124 residues, by Gutte and Merrifield in 1969 remains the landmark proof that even a protein-sized chain can be assembled this way.)

2.11 Site-specific chemical modifications of peptides

Peptides are frequently modified at defined positions to improve stability or add function:

- **Terminal modifications:** N-terminal acetylation and C-terminal amidation, which block exopeptidases and often mimic the natural hormone.
- **Cyclisation:** disulfide bridges between cysteines, head-to-tail lactams, and side chain to side chain lactam or hydrocarbon staples that lock a helix in place and resist proteolysis.
- **Lipidation:** a fatty acid chain attached through a spacer to a lysine side chain makes the peptide bind albumin and last for hours or days instead of minutes; the GLP-1 drugs liraglutide and semaglutide are built this way.
- **PEGylation:** attachment of polyethylene glycol chains, increasing hydrodynamic size, solubility and circulation time.
- **Labels and payloads:** biotin, fluorescent dyes, chelators for radioisotopes, and cytotoxic payloads in peptide-drug conjugates, all installed at one chosen residue using an orthogonally protected building block (for example a lysine whose side chain carries an acid-labile group removable while the chain is still on resin).

2.12 Solution phase strategies: segment and sequential

Solution synthesis never died; it remains the method of choice for short peptides at very large scale and for fragment condensations. Two organisational strategies are named in the syllabus:

- **Sequential (stepwise) strategy:** residues are added one at a time from one end, as in solid phase work but with isolation and purification at every step. The industrial synthesis of the sweetener **aspartame** (L-aspartyl-L-phenylalanine methyl ester) illustrates it at its simplest: protected aspartic acid is coupled to phenylalanine methyl ester, then deprotected.

- **Segment (fragment) strategy:** the chain is built as several protected fragments in parallel and the fragments are condensed together, convergently, so the longest linear sequence of steps is short and each fragment can be purified rigorously before the risky fragment couplings. Racemisation control dictates that fragments end in glycine or proline where possible.

Case study 1, oxytocin: du Vigneaud's 1953 synthesis of the nonapeptide hormone oxytocin, the first synthesis of a peptide hormone, used solution chemistry throughout: a protected C-terminal fragment and a protected N-terminal fragment were prepared separately and joined by fragment condensation, the disulfide bridge was closed by oxidation at the end, and C-terminal amidation was built into the design. It earned the Nobel Prize in 1955.

Case study 2, insulin: insulin's two chains (A, 21 residues; B, 30 residues) joined by two interchain disulfide bridges make a formidable target. The classical solution approach synthesised each chain separately with all cysteines protected, then combined them: deprotection and air oxidation form the bridges, with the correct pairing obtained in modest yield alongside scrambled isomers, a problem that shaped decades of work. Modern manufacture sidesteps it biologically (recombinant proinsulin in bacteria or yeast, folded as one chain, then enzymatically trimmed), but the chemical insulin syntheses remain the definitive teaching example of the segment strategy and its disulfide problem.

2.13 Side reactions in peptide synthesis

The syllabus organises side reactions by their initiating event:

- **Deletion peptides:** any coupling or deprotection that stops short of completion leaves chains missing one residue; these are the hardest impurities to remove because they differ from the product by a single internal unit. Prevention is excess reagent, double coupling of difficult residues, monitoring (ninhydrin or chloranil tests for free amine), and capping, which converts deletions into truncated chains that separate more easily.
- **Initiated by proton abstraction (base):** aspartimide formation, in which the backbone nitrogen following an aspartate attacks its side chain ester under piperidine treatment, giving a succinimide that reopens to a mixture of the normal alpha peptide and the isomeric beta (isoaspartyl) peptide, partly racemised; and diketopiperazine formation, in which a resin bound dipeptide ester cyclises onto itself and falls off the resin, a notorious chain-terminating event in sequences containing proline or glycine at the second position.
- **Initiated by protonation (acid):** cleavage cocktails generate tert-butyl and benzyl cations that alkylate electron-rich side chains (tryptophan, tyrosine, methionine, cysteine) unless scavengers intercept them; acid can also cleave sensitive bonds such as Asp-Pro.
- **Initiated by overactivation:** the O-acylisourea of the carbodiimide method rearranges to an inert N-acylurea if the amine is slow to arrive (sterically hindered couplings), losing the activated acid entirely; and aminium coupling reagents such as HBTU and HATU react directly with a free N-terminal amine when no carboxylate is available, capping it as a tetramethylguanidinium group, a chain terminating side reaction prevented by correct reagent stoichiometry.
- **Side reactions of individual amino acids:** cysteine racemises readily, oxidises and undergoes disulfide exchange; methionine oxidises to the sulfoxide and is alkylated during HF cleavage; tryptophan is oxidised and alkylated by carbocations; histidine is among the most racemisation-prone residues; asparagine and glutamine dehydrate to nitriles under carbodiimide conditions; arginine can cyclise to a lactam when activated and its side chain protection (Pbf, Pmc) is among the slowest to remove; serine and threonine can be O-acylated under forcing conditions.

Feature	Boc strategy	Fmoc strategy
Alpha-amino protection	Boc	Fmoc
Removed by	TFA (acid), every cycle	Piperidine (base), every cycle
Side chain protection	Benzyl type	tert-Butyl type
Final cleavage	HF (low-high protocol), or TFMSA	TFA cocktail with scavengers
Monitoring	After the fact tests	Continuous, fulvene adduct by UV
Characteristic strengths	Aggregation-prone and long sequences	Mild conditions, no HF, automation friendly
Characteristic hazards	HF handling	Aspartimide and diketopiperazine formation

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