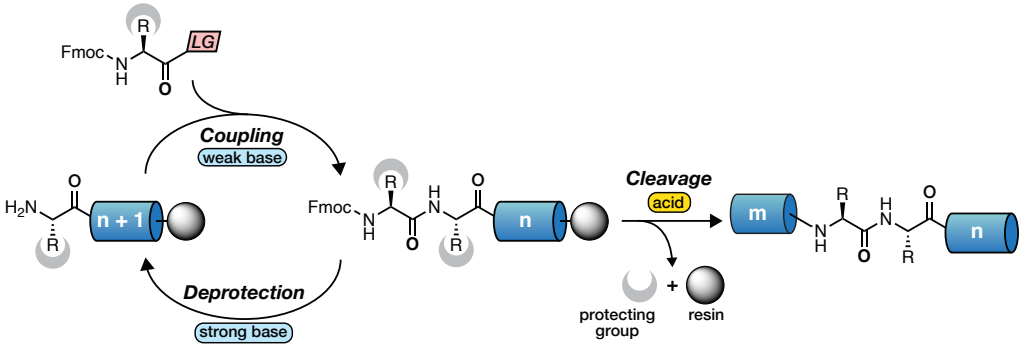
Background knowledge necessary to start on peptide projects. © VanVeller Lab 2025
Points highlighted in grey must have written answers. All other questions can be discussed.

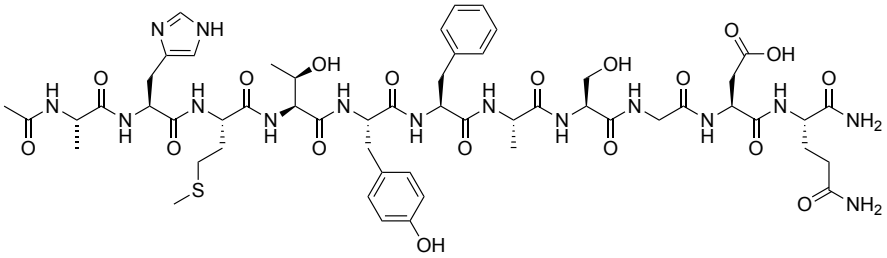
1. Solid-Phase Peptide Synthesis is a two-stage cycle under base followed by acidic cleavage.



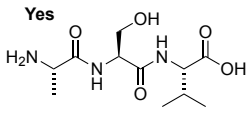
2. Each complete turn of the cycle adds one amino acid in a sequence-specific manner determined by the user.
3. Peptides are read/numbered from left to right, from the *N*-terminus to the *C*-terminus (nature adds residues to the *C*-terminus, building them from left to right)

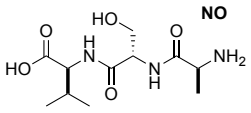
Ac-AHMTYFASGDQ-NH_2

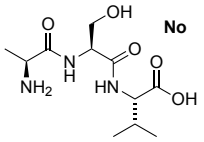
Y, Tyr, tyrosine
is the 5th residue

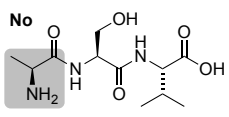


4. Peptides are chemically synthesized by SPPS in the reverse! Adding residues to the *N*-terminus so that they are <u>built</u> from right to left, but still <u>read</u> from left to right.
5. Peptides must always be drawn from *N*- to *C*-terminus with *N*-terminus on the left. The backbone should be on one axis (horizontal), side chains coming off the backbone (vertical)



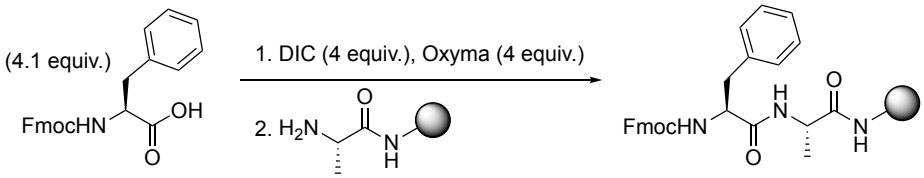




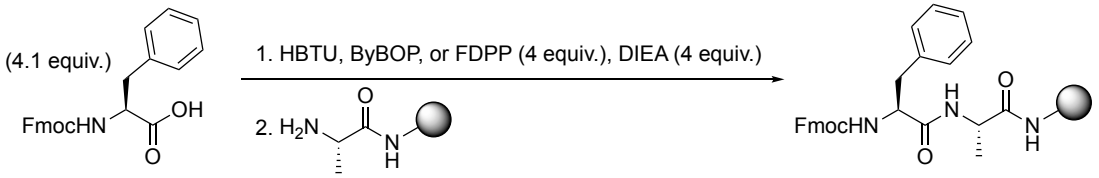


The text-to-structure tool in Chemdraw (ala-ser-val) always draws the *N*-term residue incorrectly. "Chemdraw drew it that way" is never an acceptable explanation for anything!

6. Fmoc-Amino acids are coupled through activation of the *C*-term carboxylic acid through a variety of coupling agents.
    a. Draw the structures of DIC/Oxyma, HBTU, PyBOP, FDPP
    b. Draw the curly arrow mechanism for activation of Fmoc-Phe-OH with DIC/Oxyma followed by reaction with H-Ala-Rink.



c. Draw the curly arrow mechanism for activation of Fmoc-phenylalanine with HBTU, PyBOP, FDPP, followed by reaction with H-Ala-Rink. DIEA or NMM is added to mixtures with HBTU, PyBOP, FDPP but not DIC. Explain. (hint: the simplest mistake a peptide chemist can make is not counting their proton equivalents).



d. Uronium (HBTU) and Phosphonium (PyBOP) reagents can vary based on the LG they install. (i) Explain the difference between HBTU/PyBOP, HCTU/PyClOP, HATU/PyAOP, PyBrOP. (ii) Order them in terms of their reactivity with the amine nucleophile that is on the solid-phase support bead (resin). (iii) Explain difference in their reactivity/electrophilicity.
e. What is the purpose of Oxyma, HOBt, HOCt, HOAt?
f. Why do you need to be careful about excess equivalents of HBTU/HCTU/HATU relative to the Fmoc-Phe-OH prior to adding H-Ala-Rink but not excess equivalents of PyBOP?

7. After coupling, there should no longer be any reactive amine on the resin. The sequence has been elongated by one amino acid and the new reactive amine is Fmoc-protected. This protected stage is generally considered to be safe for storage.

8. Difficult couplings: For problematic couplings (sterically hindered or anything significantly outside of conventional 20 Fmoc-amino acids) it is prudent to do the following.
    a. Double couple: It is generally more effective to drain the SPPS funnel and apply fresh activated amino acid rather than allowing a stale coupling to extend for a longer time.
    b. Acetate capping: Whenever in doubt, application of Ac_2O and NR_3 will cap unreacted amines, preventing any further elongation. Explain what happens if acetate capping is not applied following an incomplete coupling and SPPS elongation is continued. Explain why Ac capped chains are effectively waste (lowering yield) but simplify chromatographic purification.

You should now have a beginner-level understanding of the coupling phase of SPPS. Now we turn to the deprotection phase.

9. Fmoc deprotection is relatively simple compared the variety of conditions for coupling. There are only two common bases used in Fmoc deprotection: 10-20% piperidine and 2-5% DBU with DMF v/v. In general, 3° amines such as DIEA, NMM are used for coupling because they do not remove the Fmoc. 2° Amines reliably remove Fmoc. 1° Amines remove Fmoc at a slower rate than 2° amines. (see table on next page)

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Fmoc Deprotection Conditions

Base/cocktail	Reported rate metric	Notes	Source
20% Piperidine	$t_{1/2}$ = 7 s; $t_{99\%}$ = 1.5 min	DMF, rt, std method	*RSC Adv*, **2015**,*5*, 104417
10% Piperidine	$t_{1/2}$ = 20 s; $t_{99\%}$ = 4.4 min	DMF, rt	*RSC Adv*, **2015**,*5*, 104417
5% Piperidine	$t_{1/2}$ = 39 s; $t_{99\%}$ = 8.6 min	DMF, rt	*RSC Adv*, **2015**,*5*, 104417
2% Piperazine	$t_{1/2}$ = 139 s; $t_{99\%}$ = 31 min	DMF, rt	*RSC Adv*, **2015**,*5*, 104417
5% Piperazine	$t_{1/2}$ = 50 s; $t_{99\%}$ = 11 min	DMF, rt	*RSC Adv*, **2015**,*5*, 104417
10% Piperazine	$t_{1/2}$ = 21 s; $t_{99\%}$ = 4.5 min	EtOH:NMP 1:9	*RSC Adv*, **2015**,*5*, 104417
5% Piperazine + 0.5% DBU	$t_{1/2}$ = 12 s; $t_{99\%}$ = 2.4 min	DMF, rt	*RSC Adv*, **2015**,*5*, 104417
5% Piperazine + 1% DBU	$t_{1/2}$ = 7 s; $t_{99\%}$ = 1.5 min	DMF, rt	*RSC Adv*, **2015**,*5*, 104417
5% Piperazine + 2% DBU	$t_{1/2}$ = 4 s; $t_{99\%}$ = <1 min	DMF, rt	*RSC Adv*, **2015**,*5*, 104417
5% Piperazine + 1% DBU + 1% FA	$t_{1/2}$ = 29 s	DMF, rt	*RSC Adv*, **2015**,*5*, 104417
20% Piperidine + 1% FA	$t_{1/2}$ = 27 s	Suppress Asp-imide	*RSC Adv*, **2015**,*5*, 104417
50% Morpholine	10 + 20 min to 100%	Suppress Asp-imide	*J Pep Sci* **2024**, e3538
2% DBU	Instantaneous in practice	DMF, rt	
50% Morpholine	$t_{1/2}$ = 1 min	DMF, rt	*Perk Trans 1* **1981**, 538
50% Dicyclohexylamine	$t_{1/2}$ = 35 min	DMF, rt	*Perk Trans 1* **1981**, 538
50% DIEA	$t_{1/2}$ = 10 h	DMF, rt	Perk Trans 1 1981, 538
10% DMAP	$t_{1/2}$ = 85 min	DMF, rt	Perk Trans 1 1981, 538
50% NMM	Trace	DMF, rt	Patent EP0623626B1
50% Triethylamine	>18 h to 100%	DCM, rt	*Int J Pep Res* **1980**,*15*,59
50% Pyrrolidine	<5 min to 100%	DCM, rt	*Int J Pep Res* **1980**,*15*,59
2 M NaOH	<5 min to 100%	Dioxane:MeOH 3:1	*Int J Pep Res* **1980**,*15*,59
50% Ammonia	>18 h to 100%	DCM immiscible	*Int J Pep Res* **1980**,*15*,59
50% Diethylamine	$t_{99\%}$ = 3 h in DCM	faster in DMF	*Int J Pep Res* **1980**,*15*,59
50% Ethanolamine	<5 min to 100%	DCM, rt	*Int J Pep Res* **1980**,*15*,59
174 mM LiOH	0% Fmoc deprot in 5 h 95% hydrolysis of –OMe ester	IPA:water:THF 25:7:6 658 mM $CaCl_2$	*Bioconj Chem* **2011**, *22*, 605 *Biopolym* **2015**, *104*, 674

Other resource. Greg Fields, *Methods for Removing the Fmoc Group*, *Chapter 2*, from *Methods in Molecular Biology*, *Vol. 35*, *Peptide Synthesis Protocols*. Edited by M. W. Pennington and B. M. Dunn. Copyright ©1994 Humana Press Inc., Totowa, NJ.

Fmoc-removal in solution and on solid support using 20% piperidine

	Solution phase (100% removal)[b]	Solid phase[d] (yield, %)			
Solvents	t^c (min)	Rink Amide PS (45 s)	Rink Amide ChemMatrix® (45 s)	Rink Amide PS (7 min)	Rink Amide ChemMatrix® (7 min)
DMF	2	97.7	65.6	100	100
NFM	4	29.3	66.9	47.9	96.3
2-MeTHF	10	13.2	15.9	27.5	29.5
CPME	10	4.9	26.3	N/A	N/A
DMIE	15	2.3	47.3	N/A	N/A
EtOAc	15	33.9	9.2	N/A	N/A
DMC	15	30.4	4.6	N/A	N/A
GVL	4	43.3	89.1	95.5	100
IPA	>30 min[e]	1.0	3.2	N/A	N/A
α,α,α,-Trifluorotoluene	6	12.1	31.1	47.7	58.6

Martin and Pedersen, *RSC Adv* **2020**, *10*, 42457.

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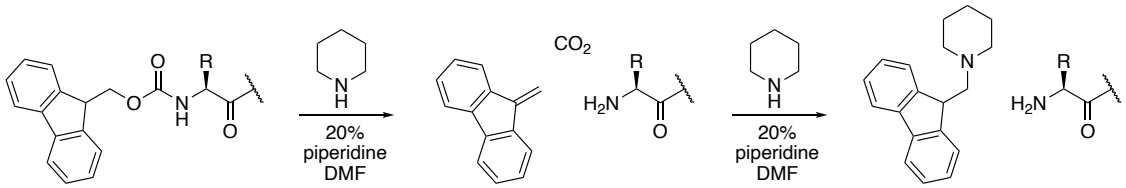
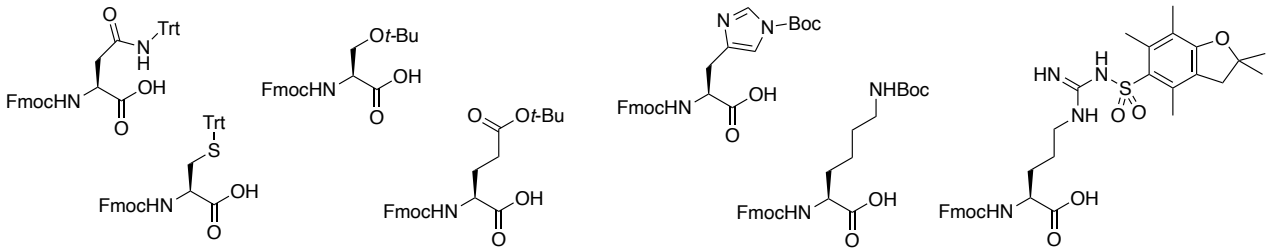
10. Draw the mechanism for Fmoc deprotection with piperidine below.



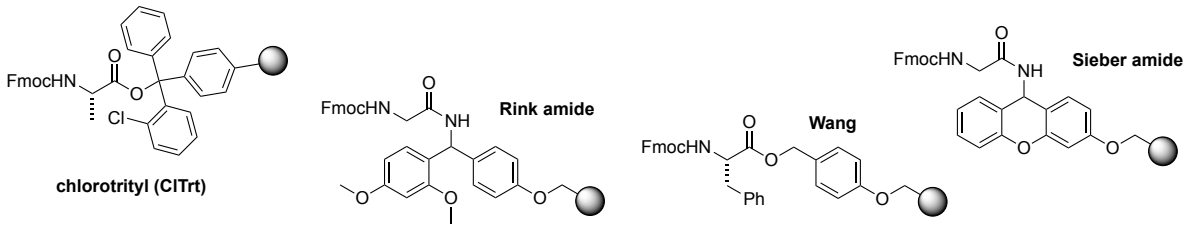
Repeated coupling and deprotection <u>under basic conditions</u> get you to your final desired sequence.

Now you need to consider getting your peptide off of the resin and removing side-chain protecting groups. This process is achieved with <u>acid</u>.

11. Any amino acid with a potentially reactive functional group needs to be capped with an acid-cleavable protecting group to survive the basic conditions of elongation. Side-chain deprotection is achieved by treating the resin with the strong acid trifluoroacetic acid (TFA). Draw the mechanism for the removal of the protecting groups below with TFA.



12. The linkage between the *C*-terminus of the peptide and the resin is also acid-labile. Draw the mechanism for cleavage of the peptide from the following resins linkers. Notice how different linkers return different *C*-term chemistries (1° amide or acid).



13. ClTrt and Sieber cleave with 1-3% TFA in DCM (v/v), while Rink and Wang require 75–100% TFA to cleave the peptide from support. Explain.
14. Resins or solid supports are crosslinked polymeric beads. The structure of the polymers used to construct the bead determine how well the resin swells with a particular solvent applied during reaction. The more that solvent can interact with the polymers of the bead, swelling or solvating the polymeric chains, the closer we 'think' that we can approach solution-like or more efficient coupling/deprotection behavior.

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Resin	Backbone / Structure	Crosslinker / Support	Key Features	Typical Uses
Polystyrene (Merrifield)	Polystyrene chain, **–CH_2–Cl** benzylic functional group	Crosslinked with 1–2% divinylbenzene (DVB)	Rigid, swells best in non-polar solvents (DCM, toluene), less in DMF/NMP; poor in aqueous solvents	Standard SPPS in early methods; simple hydrophobic peptides
Wang Resin	Polystyrene backbone with **p-alkoxybenzyl alcohol** linker	1% DVB crosslinker	Acid-labile linker (TFA cleavage)	General-purpose peptide SPPS with carboxy-terminal acids
Rink Amide Resin	Polystyrene with **p-alkoxybenzylamine** linker	1% DVB crosslinker	Yields **C-terminal amides**; TFA-labile	Peptide amidation, especially for bioactive peptides
TentaGel	Polystyrene core grafted with **PEG chains**	1% DVB PS + PEG (5–10 kDa grafts)	Amphiphilic, swells in both polar (DMF, MeOH, water) and non-polar solvents; robust beads	Long/charged peptides, peptide libraries, hydrophilic sequences
ChemMatrix®	Crosslinked **PEG-only** support	PEG (poly(oxyethylene)) crosslinked	Fully hydrophilic, excellent swelling in aqueous and organic solvents, chemically inert	Difficult peptides, hydrophilic or aggregation-prone sequences, peptide-drug conjugates
SASRIN Resin	Polystyrene with **safety-catch sulfonamide linker**	1% DVB crosslinker	Acid-labile but more stable than Wang; allows selective linker cleavage	Synthesis of protected fragments for fragment condensation
HMBA Resin	Polystyrene with **4-hydroxymethylbenzoic acid linker**	1% DVB crosslinker	Cleaves under mild acidic conditions (HFIP)	Fragment synthesis, peptide thioesters
Polyamide Resin	Crosslinked **polyamide matrix** (nylon-like)	No PS; polyamide backbone	More polar, moderate swelling in polar solvents, higher stability under harsh conditions	Used in Boc-SPPS and when stronger acid stability is needed
Novasyn TG	TentaGel variant with optimized PEG grafting	PS-PEG hybrid	Similar to TentaGel but optimized for higher loading and swelling	High-throughput SPPS, peptide libraries
CLEAR Resin	Cross-linked polyacrylamide (cross-linked ethoxylate acrylate resin)	PEG-like acrylamide	Very good swelling in polar/aqueous solvents, less hydrophobic than PS	Peptides with solubility/aggregation issues

Swelling properties of resins in different solvents in mL g^{-1}

Solvent/resin[b] (loading in mmol g^{-1})	Merrifield (0.6–1.0)	ParaMax (2.0)	JandaJel™ (0.8–1.2)	Tentagel™ S (0.23)	ArgoGel™ (0.48)	HypoGel200® (0.8)	NovaGel (0.74)	Chem-Matrix® (0.5–1.2)	Spheri-Tide™ (0.21)
Acetone	2.2	4.6	2.6	3.4	3.8	2.8	3.6	4.0	2.8
Anisole	5.6	5.4	8.8	3.8	6.2	4.2	3.8	5.0	2.4
[Bmim][BF_4]	1.9	1.9	1.8	2.8	3.8	1.8	1.8	5.8	1.8
Butanone	4.5	4.6	4.8	3.4	4.2	3.4	3.8	4.8	2.4
CH_2Cl_2	5.6	5.6	8.1	5.6	7.0	5.0	6.0	9.8	4.8
CPME	5.6	7.0	6.6	2.4	1.6	2.8	1.8	2.8	2.4
Cyclo-pentanone	5.6	7.0	8.8	4.6	5.8	4.8	4.6	6.6	3.6
Cyrene™	1.8	1.6	1.8	3.8	4.8	3.4	4.2	8.2	2.8
Diethyl carbonate	3.0	5.6	2.6	3.0	4.2	2.6	2.8	5.0	2.0
Dimethyl carbonate	2.8	4.2	3.8	3.8	4.8	2.8	3.2	6.4	2.8
DMIE	5.6	7.0	6.6	4.4	5.8	5.4	4.8	7.8	3.4
D-Limonene	1.9	5.4	4.9	2.6	4.2	2.4	1.8	4.8	1.8
DMF	4.6	5.5	6.1	4.0	5.2	4.4	5.2	7.6	4.8
Ethyl acetate	3.8	5.2	5.1	3.8	4.8	2.8	3.2	4.8	2.6
Ethylene carbonate	1.8	2.8	1.8	3.8	3.8	2.8	2.8	6.6	2.4
EtOH	1.9	1.9	2.1	1.6	2.2	1.8	1.8	3.8	3.8
1-Heptanol	1.9	1.9	2.1	1.6	1.6	1.8	1.8	6.4	2.8
Isobutyl acetate	3.8	5.4	4.8	2.8	3.8	2.8	1.8	2.4	2.8
Isopropanol	1.9	1.9	2.1	1.6	1.6	1.8	1.8	1.8	2.8
Isopropyl acetate	3.4	4.6	4.8	2.8	3.8	2.4	2.2	2.6	1.8
MeOH	1.9	1.9	2.1	2.8	3.8	1.8	2.4	6.4	3.8
2-MeTHF	5.4	7.0	8.8	3.6	4.2	4.0	3.6	5.6	2.7
MIBK	3.2	4.2	4.8	2.8	3.4	2.8	2.2	2.6	1.8
NMP	6.4	7.0	7.9	3.8	4.8	4.8	5.0	7.8	5.4
p-Cymene	1.9	2.1	5.2	1.6	1.8	1.8	1.8	2.0	1.8
PC	1.8	1.6	2.6	3.4	4.2	2.4	2.8	5.0	2.4
Water	1.9	1.9	1.8	2.8	2.8	1.8	1.8	7.8	2.6
γ-Valerolactone	3.1	5.0	7.8	3.8	4.8	3.4	3.8	6.4	3.0

Martin and Pedersen, *RSC Adv* **2020**, *10*, 42457 → (a lot of excellent info on SPPS)

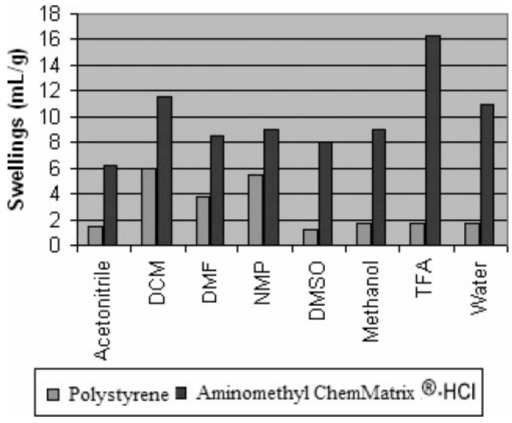

Figure 2. Swelling of polystyrene and CM resins in different solvents.

J Comb Chem **2006**, *8*, 213

15. The ‘resin loading’ is a measure of how many reactive groups are on the resin. The resin loading is usually determined by the manufacturer in mmol/g (so it can be treated like any other reagent with it’s own molar mass), but you can also determine the resin loading yourself in cases in which you have modified it and need to know it’s new loading. Lower resin loading is usually beneficial for sequences that are prone to aggregation. Explain.
16. The resin is almost always treated as the limiting reagent, where 4–5 equivalents of amino acid and coupling reagent are used for each coupling.

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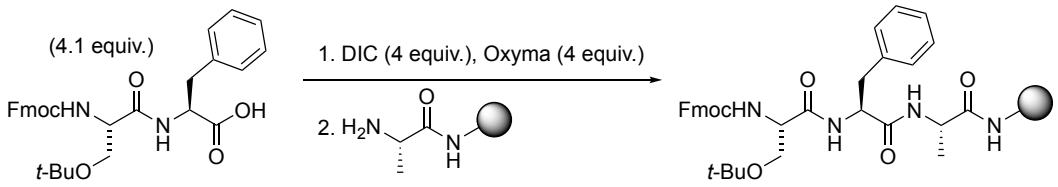
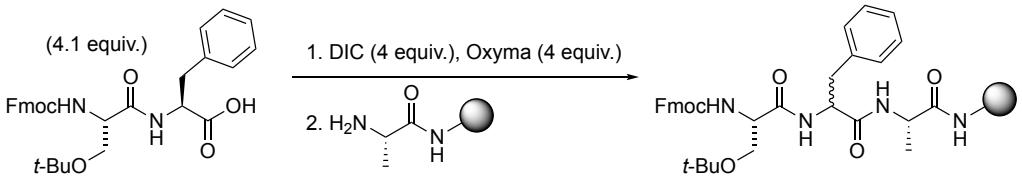
SPPS trivia and observations worth considering.

17. You could propose to halve the number of steps to reach your final peptide by simply coupling Fmoc-dipeptides…

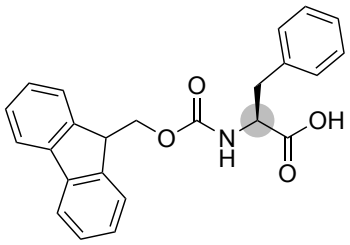


This thought exercise ignores the synthetic steps needed to make each Fmoc-dipeptide but, in theory, the synthesis of a 20-mer could be completed in only 10 SPPS couplings! There is a problem however, because the coupled dipeptide will contain significant quantities of epimer at the *C*-term residue of the dipeptide.

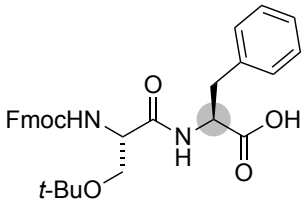


18. Propose a mechanism to explain how the Phe residue racemizes during coupling.
19. Consider the different coupling partners below. One is a standard Fmoc-protected amino acid (which obviously resists racemization during coupling); the other is a dipeptide that does racemize/epimerize. Propose a reason why carbamates (Fmoc, Boc, Cbz) behave differently than amides in this context.

Phe does not racemize during coupling



versus



Phe racemizes during coupling

Reading assignments

20. Read pages 9–37 of *Fmoc Solid Phase Peptide Synthesis – A Practical Approach* Edited by W.C. Chan and P.D. White.
21. Read *Organic Letters* **2019**, *21*, 7015-7018
    a. Explain why thioamides are more acidic than oxoamides.
    b. What problem and what stage of SPPS does the thioimidate address?
    c. How is the thioamide installed into the peptide? Draw the mechanism for all steps to convert Fmoc-Phe-OH into the thioacyl reagent for thioamide coupling.
    d. At what stage of SPPS can the thioamide be converted to a thioimidate and what is the procedure? How long does the reaction take?
    e. The approach can be used on all amino acids except those preceding a Pro. Explain this limitation.

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Points highlighted in grey must have written answers. All other questions can be discussed.

22. Read *Journal of Organic Chemistry* ***2019***, *84*, *15309-15314*
    a. What problem does this study address?
    b. Draw the mechanism of the Staudinger reaction

23. Read *Journal of Organic Chemistry* **2021**, *86*, 18287-18291
    a. Explain why thioamides impose different hydrogen-bonding geometries than amides.
24. Read *Org Lett* **2024**, *26*, 1452-1457
    a. What is the effect of thioamides on alpha helices?
25. Read *JACS* **2022**, *144*, 22397-22402
    a. Explain why thioamides are not appropriate sites for amidine installation with Ag metal in linear peptides
    b. Draw the mechanism for amidine installation showing all proton transfer steps.
    c. What are the limitations of the method in terms of approach and scope?
26. Read *Journal of Organic Chemistry* **2024**, *89*, 14755-14761
    a. What are the advantages of the presented method to access thioimidates compared to those reported in 18 above?
27. Read *Chemical Science* **2024**, *15*, 18992-18999
    a. What geometries for hydrogen bonding would you predict around an amidine?