Gyros Protein Technologies

SPPS Tips for Success: A Guide to Better Peptide Synthesis

GYROS PROTEIN Technologies

Overview

- Introduction to Gyros Protein Technologies (GPT)
  - Instrument Portfolio
  - Introducing PurePep® Chorus
  - Symphony® X
- SPPS Tips for Success:
  - Designing a Synthesis
  - Strategies for Minimizing Side Reactions
  - Optimizing Synthesis of SARS-CoV-2 Epitopes for GMP Manufacture

Automating Peptide Synthesis throughout Drug Discovery & Development Since 1985

1985 Protein Technologies, Inc. founded by researchers affiliated with the University of Arizona, Tucson

1990 PS3® peptide synthesizer

1993 Symphony® with 12 independent RVs

1996 Sonata®, up to 200 mmol SPPS

2006 Prelude® with 6 parallel RVs, Single-Shot™ and e-mail notifications

2007 Tribute® with 2 parallel RVs, IntelliSynth™ Real-time UV monitoring and IR heating

2012 Symphony® X with 12 independent or 24 parallel RVs

2015 Prelude® X with 6 parallel RVs with individual heat control (induction heating) and oscillation mixing

2016 Gyros, AB and Protein Technologies, Inc. merge – expanding global footprint

2019 PurePep® Chorus; All-in-one, scalable peptide synthesizer that can grow with your chemistry needs

Automated Peptide Synthesis Solutions

Discovery & Lead Selection

Lead Optimization & Preclinical in vivo/in vitro

Preclinical IND enabling

PurePep® Chorus

Symphony® X

Sonata® XT

Increasing scale and throughput

Increasing flexibility to meet multiple demands

CRO/CMO on-demand

Automated peptide synthesizers designed for chemistries, from combinatorial to small organic molecules, peptide (Fmoc or t-Boc), peptoid and nucleic acids

Introducing: PurePep Chorus

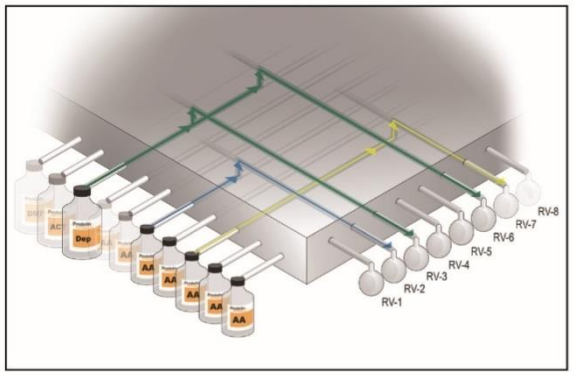
- Up to 6 parallel independent heated reaction vessels; 3 with pre-activation chemistry
- Programmable, independent induction heating (25°C – 90°C)
- IntelliSynth™ real-time UV deprotection monitoring on all channels
- Single-Shot™ Deliveries

- In-lab upgradeable from 2 to 4 to 6 reaction vessels
- On-board programmable cleavage
- Intuitive software with icon driven workflow
- Detachable tablet controller

SYMPHONY® X
HIGH THROUGHPUT MULTI-PEPTIDE SYNTHESIZER

KEY INSTRUMENT FEATURES

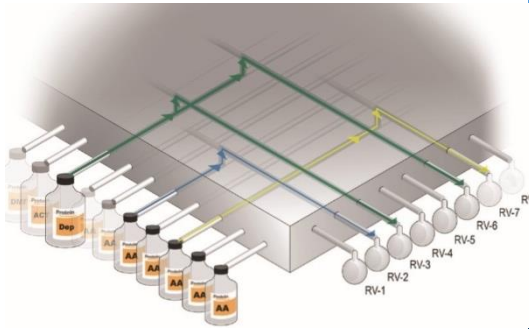
- 24 parallel RVs (12 w/ pre-activation)
- Single-Shot™ additions for special monomers – no priming required
- Ultra PurePep® pathway for simultaneous, independent reagent additions and washes in different RVs
- On-board automated cleavage
- IntelliSynth™ real-time UV monitoring
- Rapid infrared heating
- Intuitive, easy-to-use software
- Email notifications and Safe Response keep your reactions safe if interruptions occur
- 21 CFR Part 11 Compliance

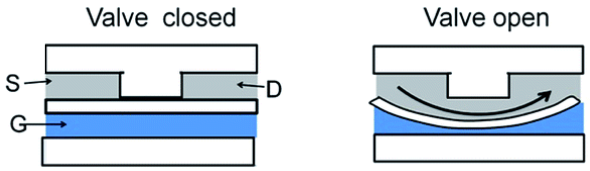


Reagent Delivery - Ultra PurePep® Pathway

Ultra PurePep pathway – proprietary

- High crude purity peptides and yields with ultra PurePep® pathway
  - Dedicated reagent lines, no dead volumes and no carryover
  - No ability for cross contamination because each fluid pathway is physically separate
- Dedicated microfluidic matrix valve block path for each reaction vessel
- Minimize side reactions with accurate fixed loop microfluidics
  - no volume or reagent ratio worries
- More robust than off-the-shelf valves
  - No leaking or corrosion
  - N2 and vacuum move all fluids
  - membranes resistant to harsh chemicals
  - 100% full strength TFA proof valves
- Delivery verification via optical sensors





S: source G: gate D: drain

Gyros Protein Technologies

Tips for Success: Designing Synthesis

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Global Product Manager
(Peptides)

Cyf Ramos Colón, Ph.D.
Senior Scientist

GYROS PROTEIN Technologies

Overview

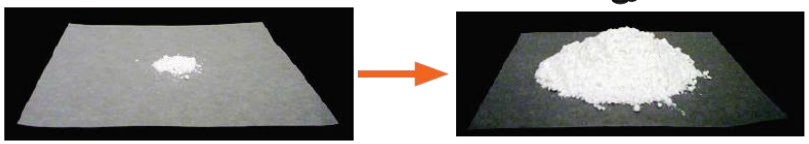
- Designing a Synthesis
  - Instrument Selection
  - Sequence Analysis
  - Resin Selection
  - Synthesis Scale
  - Chemistry Selection
  - Synthetic Protocol
  - Cleavage & Isolation

Designing A Synthesis

Instrument Selection

Instrument Selection

- Points to consider:
  - How many syntheses are going to be performed?
    - Condition screening
    - Heat Scans
    - Resin Screening
    - How many RVs will be used?
  - What scale is your synthesis?
    - How much peptide do you need?
    - Size of RV
    - Volume deliveries and reagent concentrations
  - Do you need to monitor the synthesis?
    - UV monitoring capability

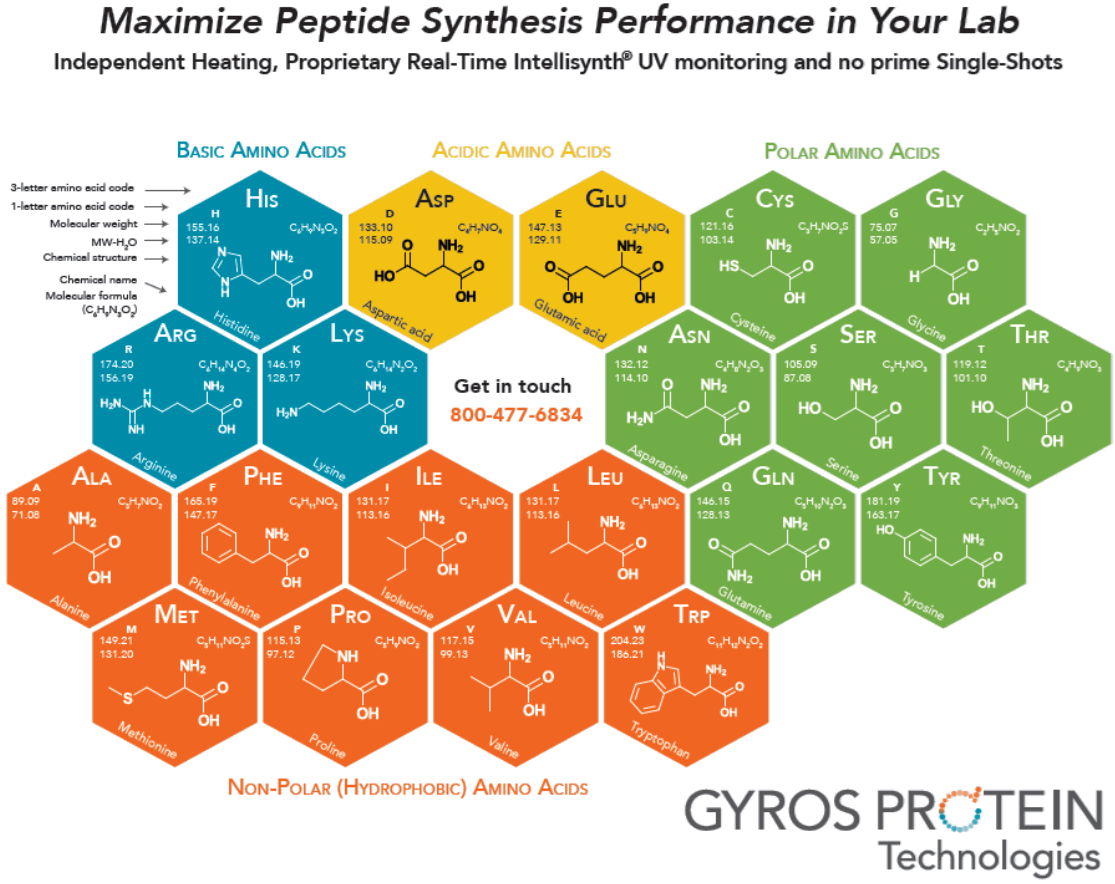


Designing A Synthesis

Sequence Analysis

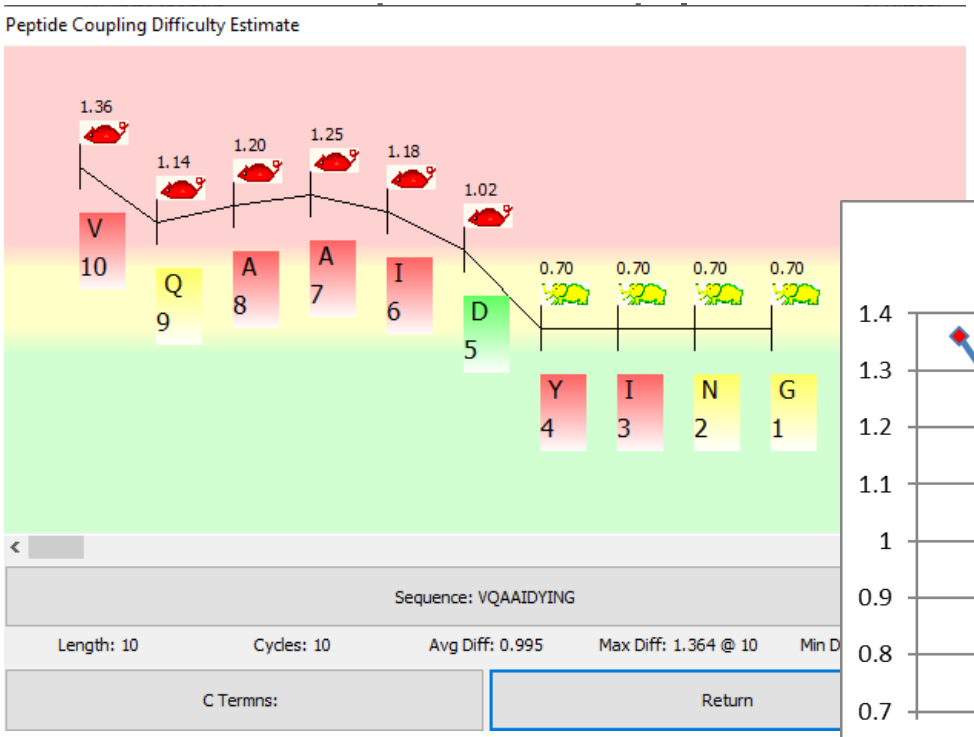
Sequence Analysis - Prediction

- Different Amino Acids have different physical attributes based on the side chain present:
  - Acidic / basic
  - Hydrophobic
  - Hydrophilic
- Some of these side chains may be protected during synthesis.
- As the peptide chain grows, aggregation then becomes a factor…

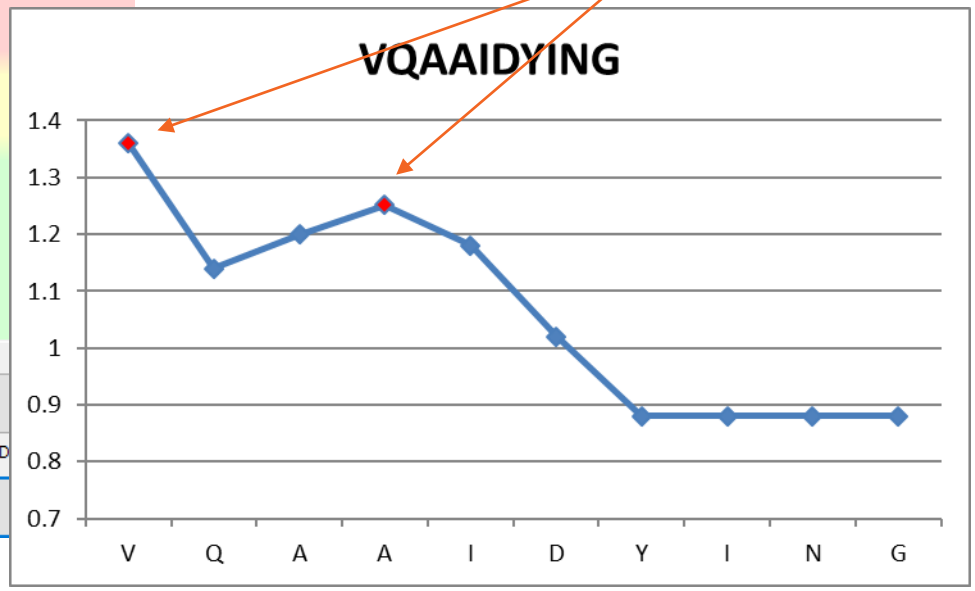


Sequence Analysis - Prediction

- To check aggregation, use a peptide predictor tool (GPT, Peptide Companion, SpyderInstitute.com)
- Gives an estimation of coupling difficulty based on



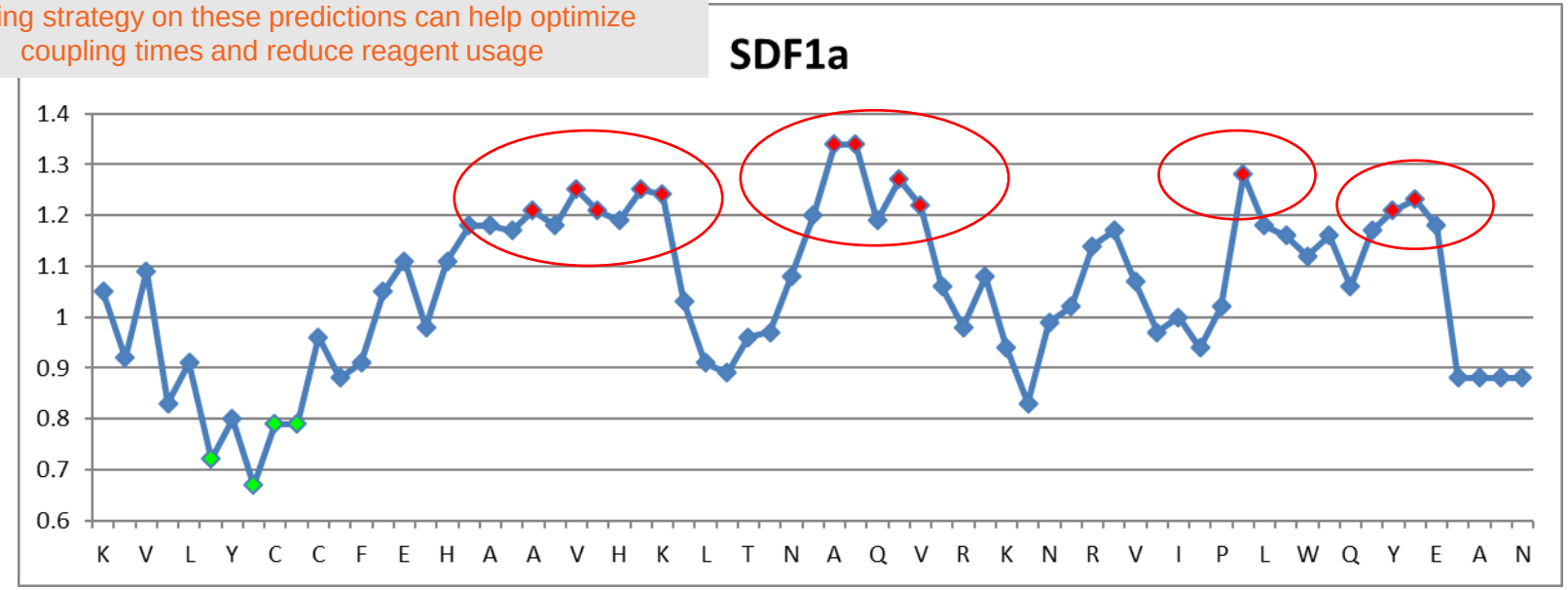
Even for a small peptide like ACP, using a predictor reveals some difficult couplings



SEQUENCE ANALYSIS - PREDICTION

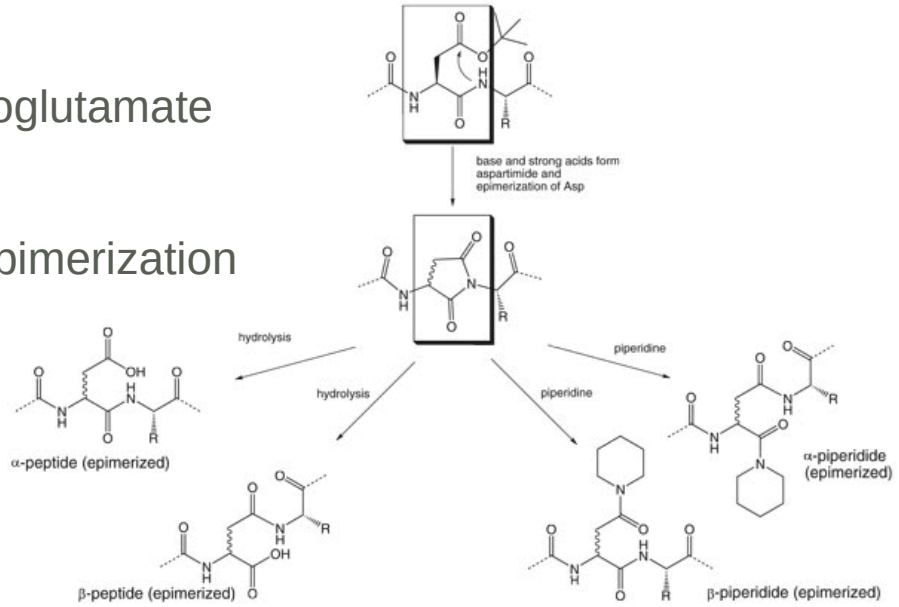
- This then gives the user an idea of what coupling strategy to use
  - Red regions = Difficult coupling
- For difficult couplings, consider extending the coupling time, adding more equivalents or changing the chemistry!
- Useful for longer sequences without double coupling all residues.

For long peptides, such as chemokines or histones, basing a coupling strategy on these predictions can help optimize coupling times and reduce reagent usage



Sequence Analysis – Potential Side Reactions

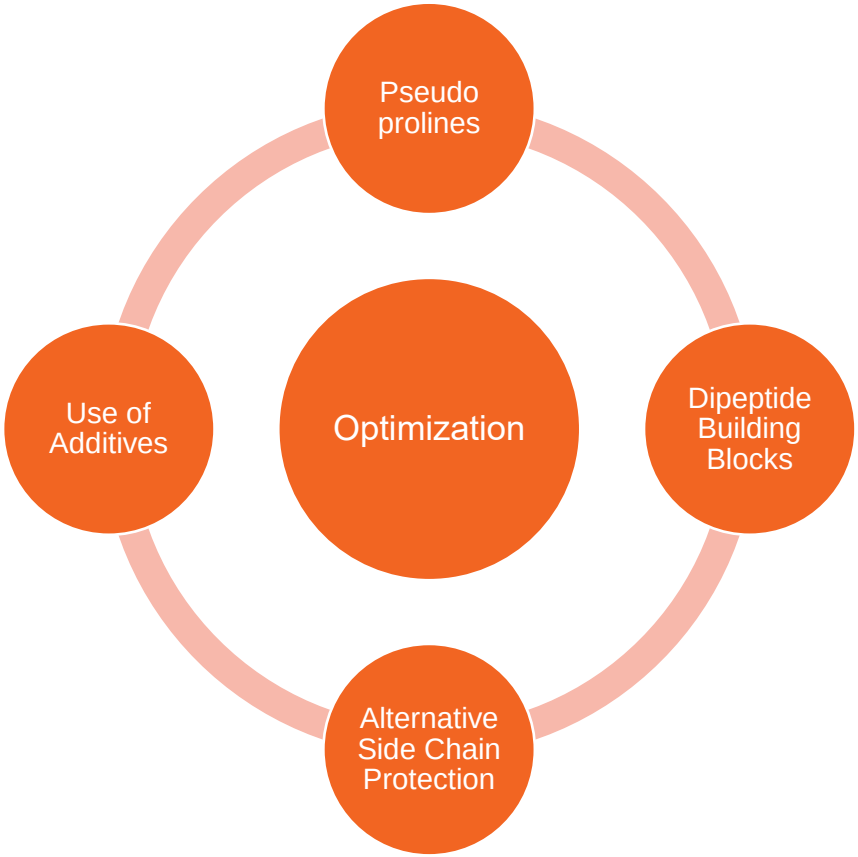
- 2,5-Diketopiperazine Formation
  - N-terminal nucleophilic attack on C-terminal residues leading to 6-membered ring formation and intramolecular cleavage, (loss of peptide from resin!)
- Aspartimide Formation
  - Treating peptides containing Asp residues with base repeatedly (e.g. piperidine) can lead to a side reaction with up to 9 different by-products!
- Pyroglutamate Formation
  - N-terminal Gln residue forms Pyroglutamate
- Racemization
  - Certain residues more prone to epimerization

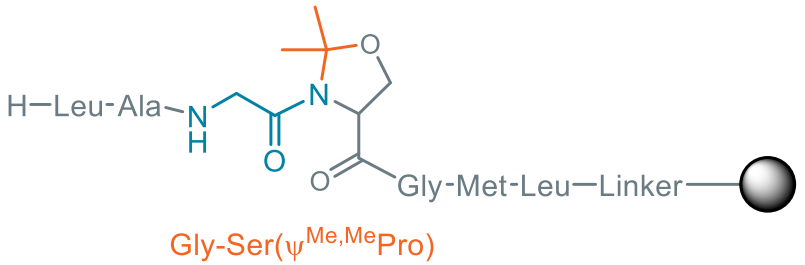


Novabiochem® Innovations 1.16

Sequence Analysis – Optimization

- There are a number of different strategies that can be employed to help reduce the risk of side reactions, or to help increase the success of individual couplings:





Gly-Ser($\psi^{Me,Me}$Pro)

www.merckmillipore.com

SEQUENCE ANALYSIS – OPTIMIZATION

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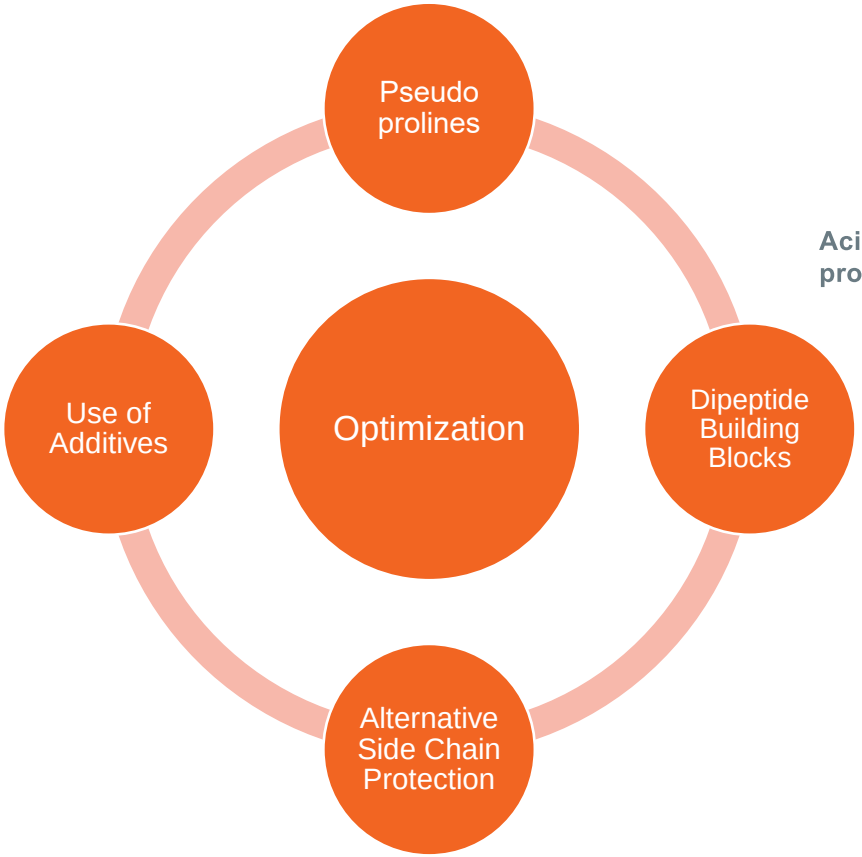
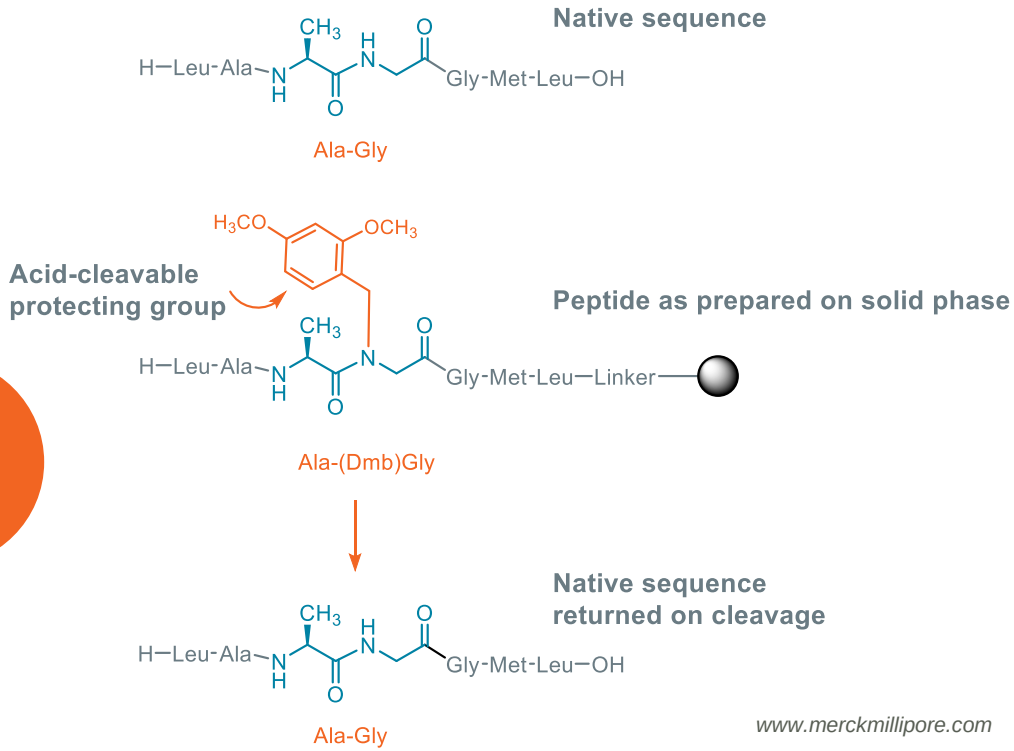
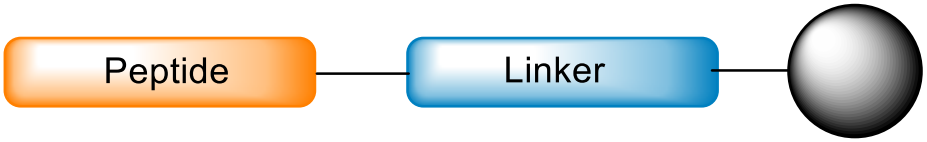




Designing A Synthesis

Resin Selection & Synthesis Scale

Resin Basics



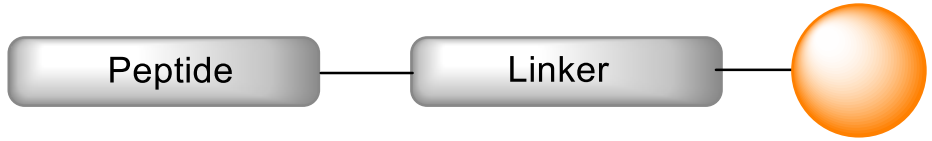
How the resin is built

- **Resin Core** (what is main resin material)
- **Resin Linkers** (influences C-terminus of final peptide)

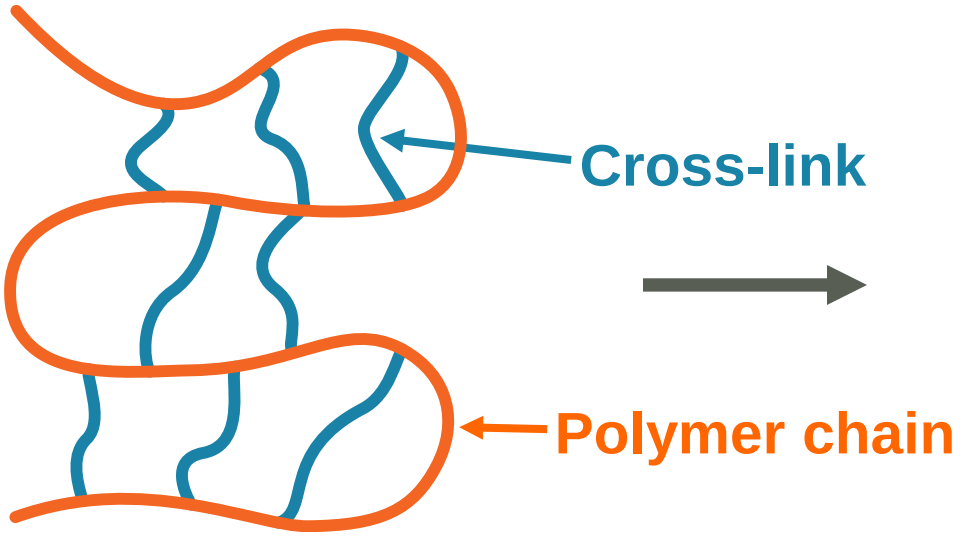
Physical Properties of Resin

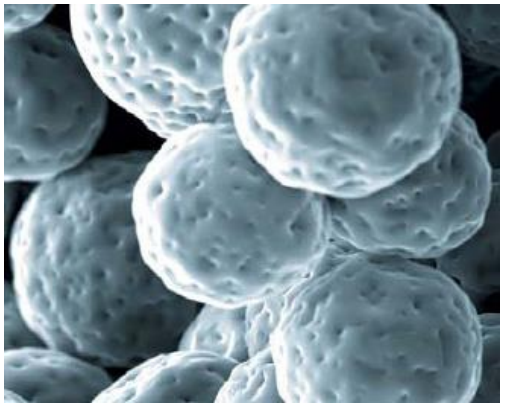
- **Resin Mesh Size** (what is the size of resin beads)
- **Resin Loading** (how much peptide can be synthesized / g of resin)
- **Resin Swelling** (how much space resin will take in Reaction Vessel and its influence on solvent consumption)

What is a Resin?

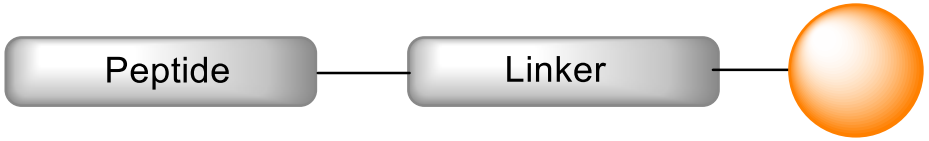


- Collection of gel beads made out of polymer chains, cross-linked by a spacer molecule = a gel-like solid
- Standard resins:
  - polystyrene (PS) cross-linked with 1% divinylbenzene (DVB)





Resin Core

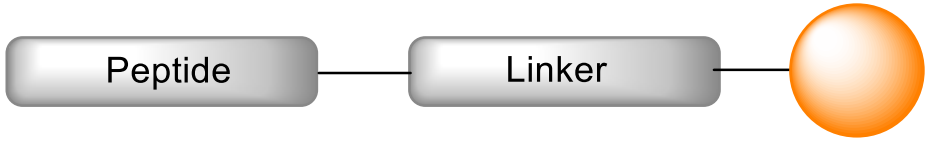


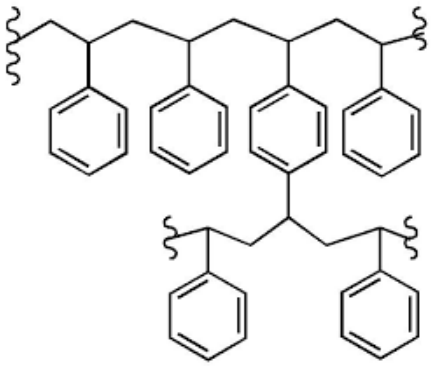
- Hydrophobic (water-incompatible),
- Hydrophilic (water-compatible) materials ,
- Combination of the two
- Common materials
  - polystyrene (PS, hydrophobic)
  - polyethylene glycol (PEG, hydrophilic)

Some common resins are listed below:

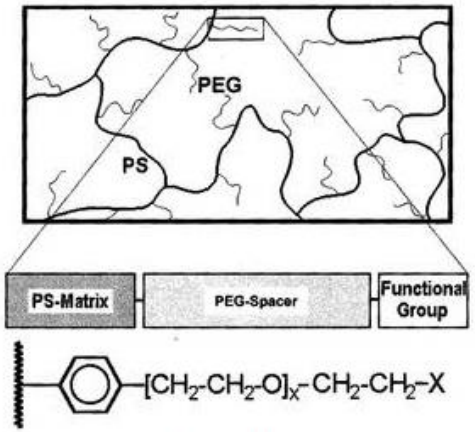
Polymer	Commercial examples	Water Compatibility
PS (1%DVB)	Merrifield, Wang, Rink	Incompatible
PS-PEG (50-70%)	TentaGel	Partially Compatible
PEG based	ChemMatrix, PEGA, CLEAR	Fully Compatible
Poly-ε-Lysine	Spheritide	Fully Compatible

Resin Core

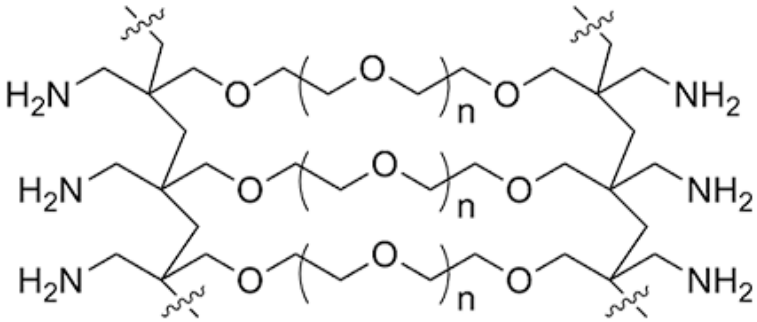




PS w/DVB Crosslinks Resin



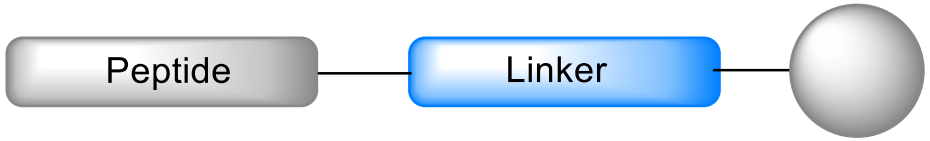
TentaGel Resin



ChemMatrix Resin

Core Resins by themselves offer a good foundation for peptide building but linker selection adds other properties in peptide synthesis

Resin Linkers



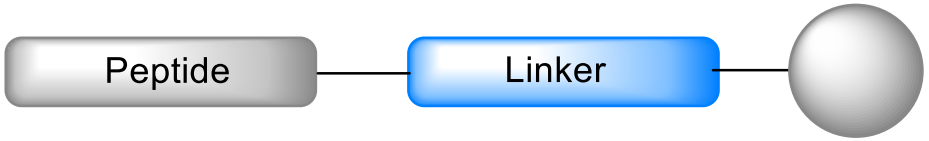
What does the linker do?

- Provide a reversible linkage between the synthetic peptide chain and the solid support
- Protect the C-terminal α-carboxyl group during the process of chain extension

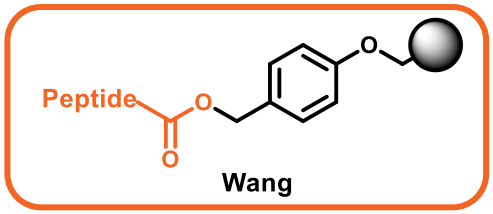
Importance

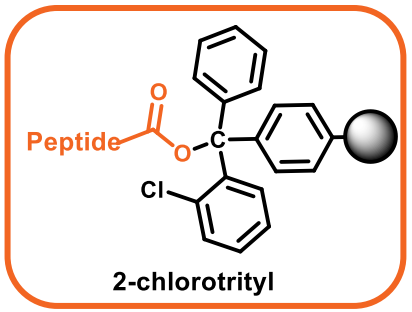
- The choice of linker determines the C-terminal functional group in the final product
  - Peptide Acids/Protected Peptide Acids
  - Peptide Amides/Protected Peptide Amides
  - Special C-terminal modifications: Peptide Aldehydes, Peptide Thioesters, Peptide Esters, Peptide Hydrazides, Cyclic Peptides
- There are a variety of linkers available that can be bound to the different core resins that affect the Cleavage properties of the peptide and peptide C-terminus

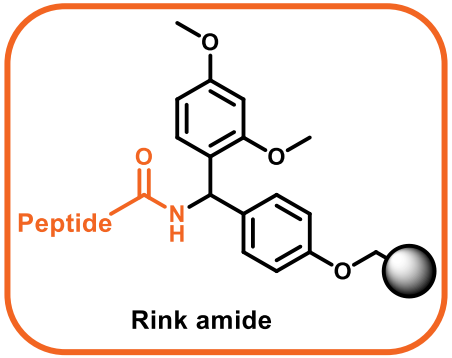
Resin Linkers

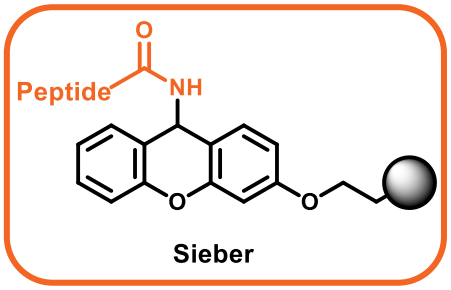


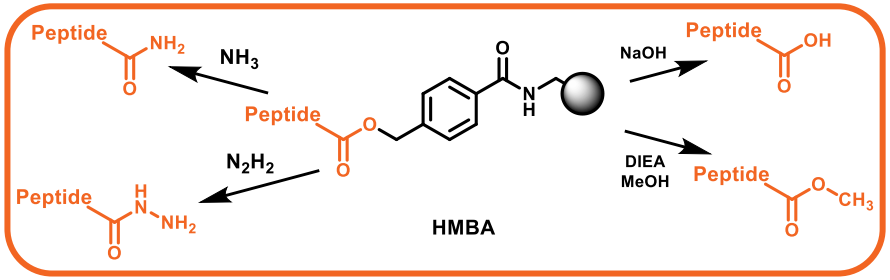
- Wang & TentaGel PHB Resin-Results in **Peptide Acids** when cleaved
- 2-Chlorotrityl, TentaGel Trt, and HMPB Resins- Results in **Protected Peptide Acids** under mild acidic conditions
- Rink Amides and PAL Resins- Results in a **Peptide Amides** when cleaved
- Sieber Amide Resin- Results in a **Protected Peptide Amides** under mild acidic conditions
- HMBA Resins (TFA stable) – Results in **Peptide amides**, **hydrazides, esters, alcohols or acids** depending on used nucleophilic reagents for cleavage
- BAL Resins – Results in **Peptide acids, aldehydes, thioesters**
- Safety catch 4-sulfamylbutyryl/Kenner safety-catch Resins – Results in **Peptide Thioesters**

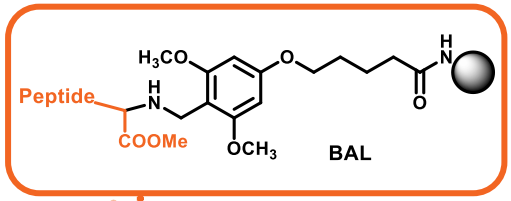


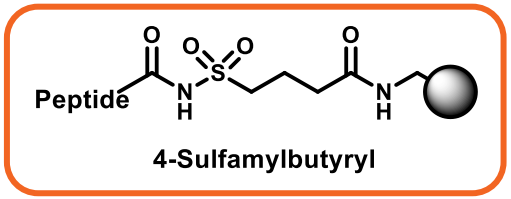












Preloaded resins or unloaded resins?

Benefits of Preloaded Resins

- Racemization is minimized
- Saves time, solvents, and reagents by eliminating a complete cycle
- Amino Acids attached to 2-Chlorotrityl resins and ChemMatrix resins are N-terminal free eliminating a further deprotection step

Anchoring of First Amino Acid

- Different procedures/chemistries depending on linker
  - C-terminal amide – standard amide coupling reactions
  - Chlorotrityl resins (C-terminal acid): absence of epimerization during loading of the first amino acids. Straight forward protocol. Recommended in particular for C-terminal Cys, Pro, Met, and Trp
  - Hydroxymethyl-based resin (C-terminal acid, e.g.Wang): special protocol, prone to epimerization
  - BAL resin: Reductive amination, anchoring through Nα

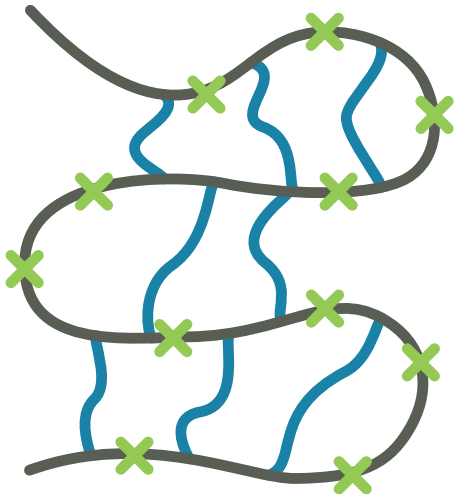
Resin Mesh Size

- Smaller beads provide better reaction kinetics because of the higher surface area to volume ratios.
  - Consider longer filtration times and possibility to clog the filter
- 100-200 mesh resin are standard
  - Recommended with standard frit sizes
  - Good on synthesizer!

Mesh	Micron	Inches
4	4760	0.185
6	3360	0.131
8	2380	0.093
12	1680	0.065
16	1190	0.046
20	840	0.0328
30	590	0.0232
40	420	0.0164
50	297	0.0116
60	250	0.0097
70	210	0.0082
80	177	0.0069
100	149	0.0058
140	105	0.0041
200	74	0.0029
230	62	0.0023
270	53	0.0021
325	44	0.0017
400	37	0.0015
625	20	0.0008
1250	10	0.0004
2500	5	0.0002

Resin Loading

- The density of reactive sites is called its **loading** and is given in mmol/g or meq/g of resin
- Ratio between scale of the synthesis and the amount of resin needed for it



Rink Amide (0.20 mmol/g)

$$\frac{\text{Scale (0.05mmol)}}{\text{Subs. (0.20mmol/g)}} = 0.25 \text{ g of Resin}$$

Rink Amide (0.64 mmol/g)

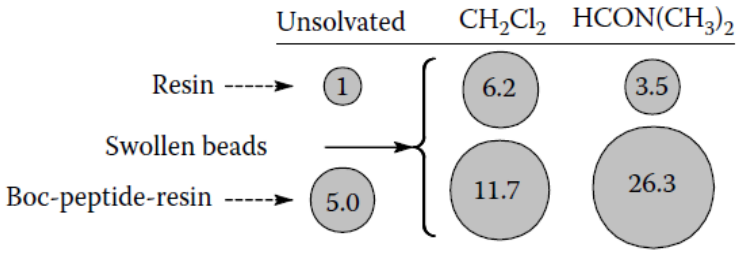
$$\frac{\text{Scale (0.05mmol)}}{\text{Subs. (0.64mmol/g)}} = 0.078 \text{ g of Resin}$$

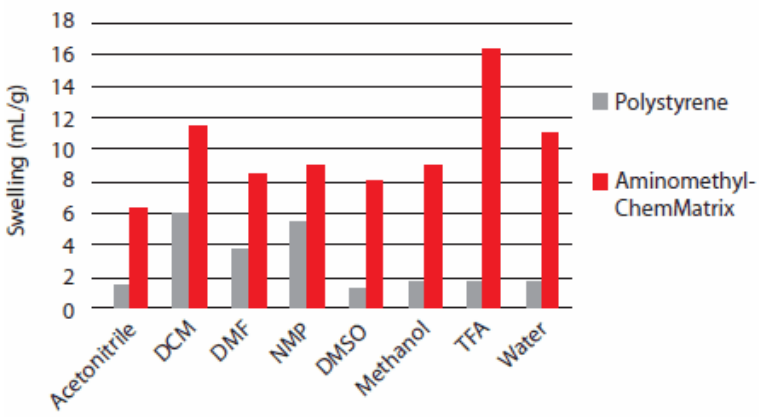
x = functional group

- Higher loading allow for less resin and solvent use with the active sites more “packed” = decreased reactivity with longer sequences
- Lower Loaded (substituted) resins (LL) – for longer (>30 AAs) and difficult peptides
- Higher Loaded resins (HL) – for shorter, easier peptides

Resin Swelling

Kinetics of the reactions involved in elongation of peptide chain are diffusion controlled therefore resins are often described by its swelling properties. The higher swelling of the resin the more accessible reaction sites but at the same time more volume of solvents needed.





- Typically the hydrophobicity of the resin determines the swelling seen
- For a basic polystyrene (PS) resin we use a ratio between resin and solvent to be…

1 ml of solvent/100mg of resin

- This ratio varies by application and for PEG based resins can go up to 1.5-2x

Resin Calculations - How much peptide?

- Example:

$^{65\text{-}74}$**ACP** H-VQAAIDYING-NH_2; **MW** = 1062.19 g/mol (mg/mmol)

Expected crude weight: 100 mg

Calculations:

Expected crude weight (mg) / MW of peptide (mg/mmol) = Scale of synthesis (mmol)

100 mg / 1062.19 mg/mmol = 0.094 mmol = 94 µmol

0.1 mmol = 100 µmol

Resin chosen: Rink amide; loading: 0.27 mmol/g

Calculations:

Scale of synthesis (mmol) / Resin loading (mmol/g) = Resin weight (g)

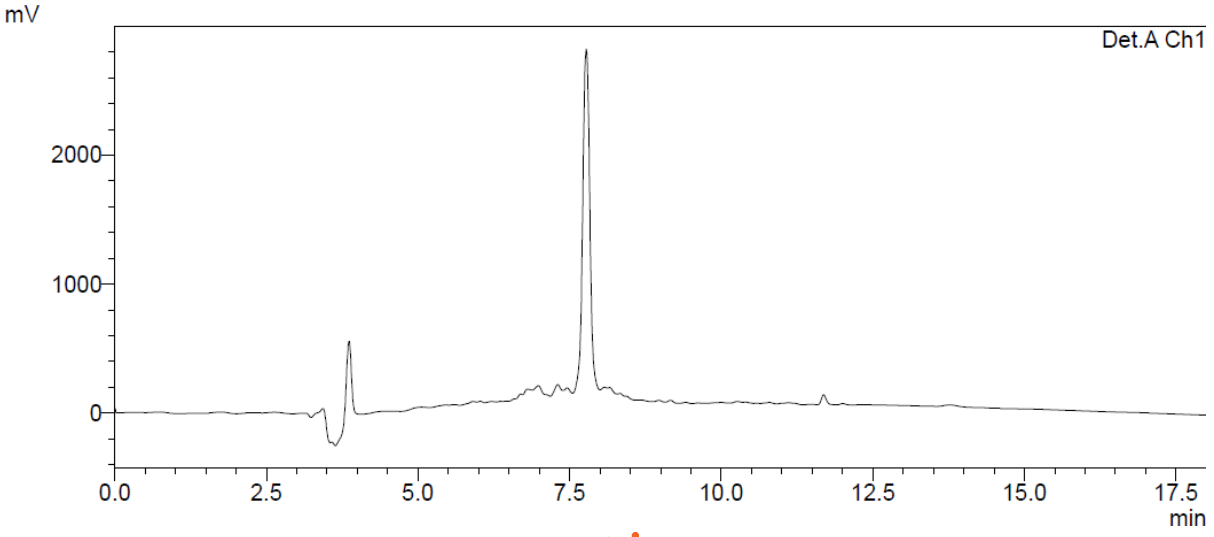
0.1 mmol / 0.27 mmol/g = 0.370 g

Importance of Resin selection: Synthesis of MK2 inhibitor analog

MK2i analog sequence:
H-AAVGLQRALAKARAQRAAARAY-OH

- Resin selection is an important factor when determining synthesis success

Resin	% Crude Purity	
	25°C	90°C
Wang Tentagel (PS-PEG)	18.1	91.4
HMPB ChemMatrix (PEG)	48.7	66.0
Wang PS	50.8	70.9
Rink PS	11.2	72.0



Designing A Synthesis

Chemistry Selection & Synthetic Protocol

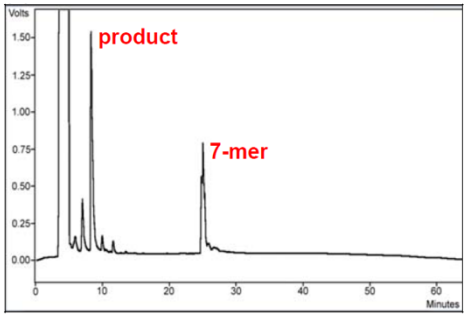
FMOC- DEPROTECTIONS

- Options include:
  - 20% Piperidine
  - 20% Piperidine + 2% DBU
  - 4-Me-piperidine
  - 10% piperazine in 1:9 EtOH:NMP – useful in one-pot synthesis

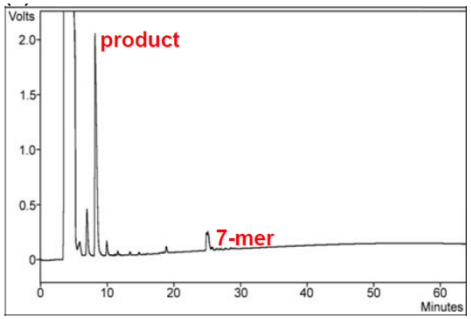
(Ala)10Lys-OH

With 20% piperidine or 2% DBU/20% piperidine using Xtendw/Reps function for the deprotection and 10 minutes using 1:1:2 amino acid/HCTU/NMM coupling

20% PIPERIDINE



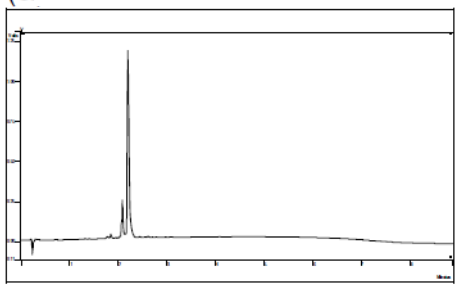
2% DBU / 20% PIPERIDINE



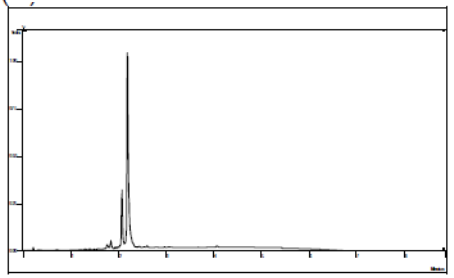
$^{65\text{-}74}$ACP

synthesized with 20% piperidine or 20% 4-methylpiperidine for 2 x 2.5 min

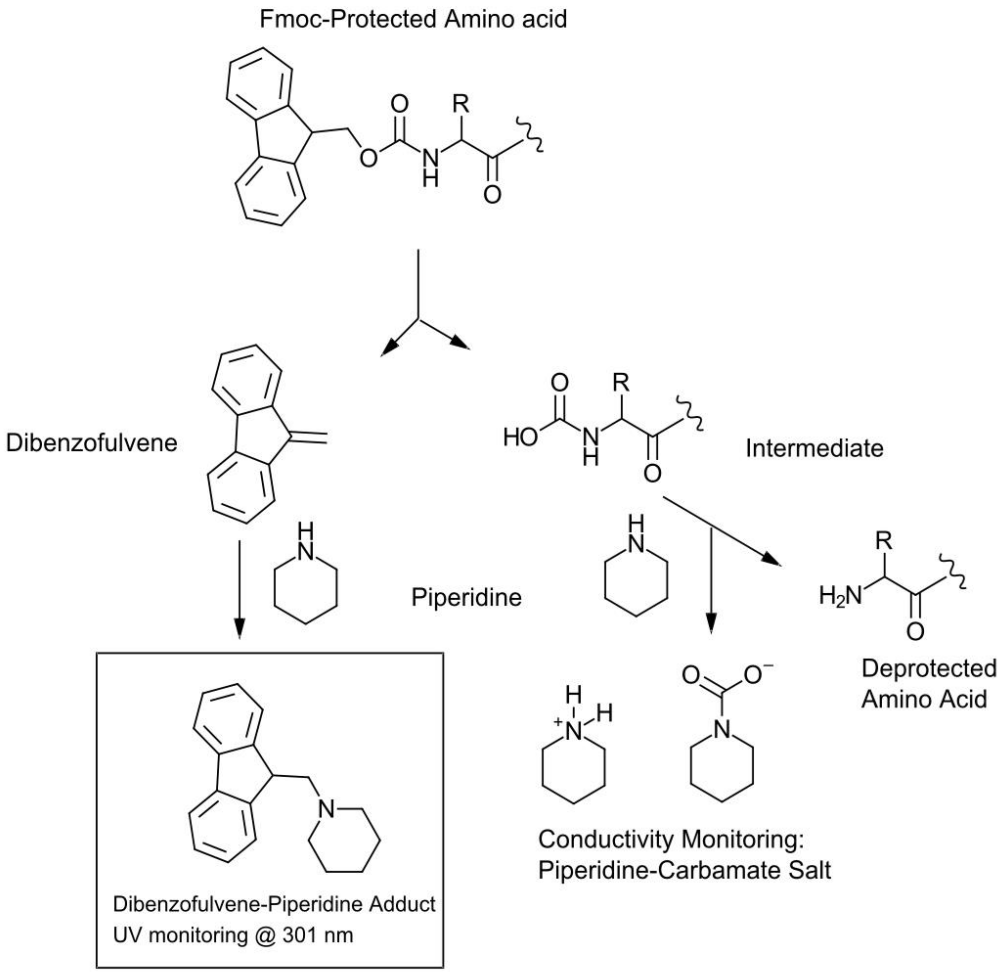
(a **20% Piperidine**



(**!0% 4-Me-Piperidine**

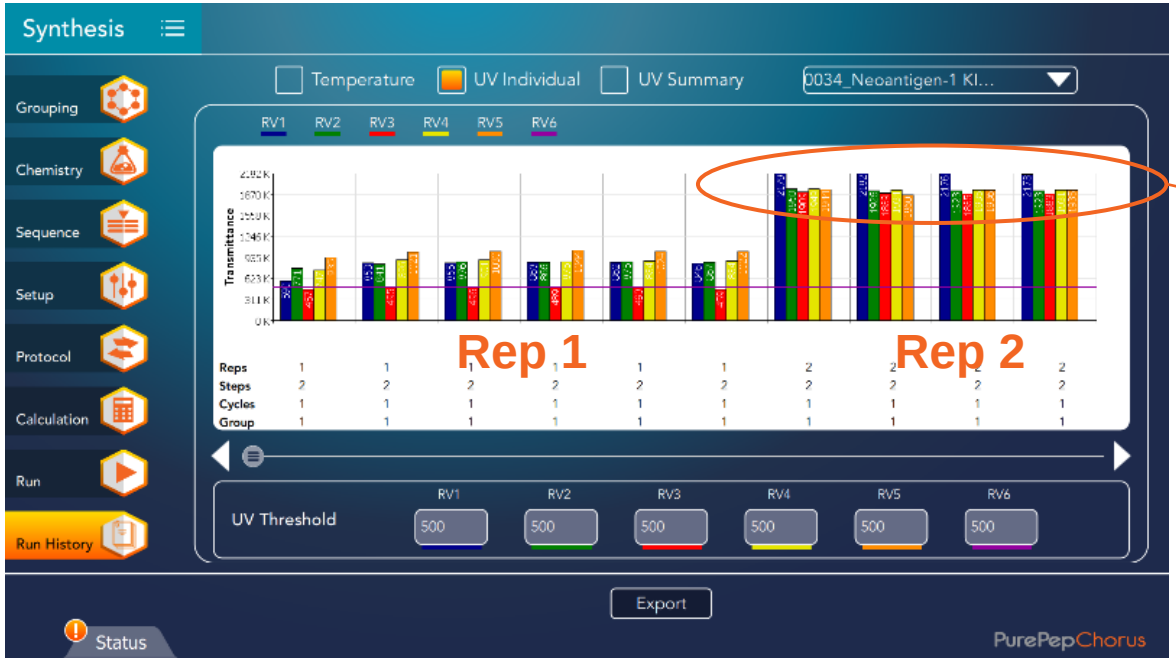


Monitoring Deprotection



- Different monitoring options
  - UV at 301 nm
  - Conductivity
  - Kaiser Test
    - Manual Intervention
- Real-time monitoring
  - Measurements done during the reaction – not waste stream
- Considerations
  - Using additives interferes with UV signal
    - HOBt
    - OxymaPure

Using UV Deprotection Monitoring in the synthesis of Neoantigen peptides



Reaction done!

- Mutated kinesin family member 2C (KIF2C) antigen peptides

Chemistry

- Deprotection - 2 x 2 min
- Coupling - 3 min @ 75°C
- Reagents – 6-fold excess HCTU with DIPEA

Sequence	Purity (%)
RLFPGLTIKI	86.6
RLFPGLTI	98.6
LTIKIQRSNGL	98.1
LQARLFPGLTI	96.9
LQARLFPGLT	98.3

Lu, Y et al. Clin Cancer Res. 2014 July 1; 20(13)

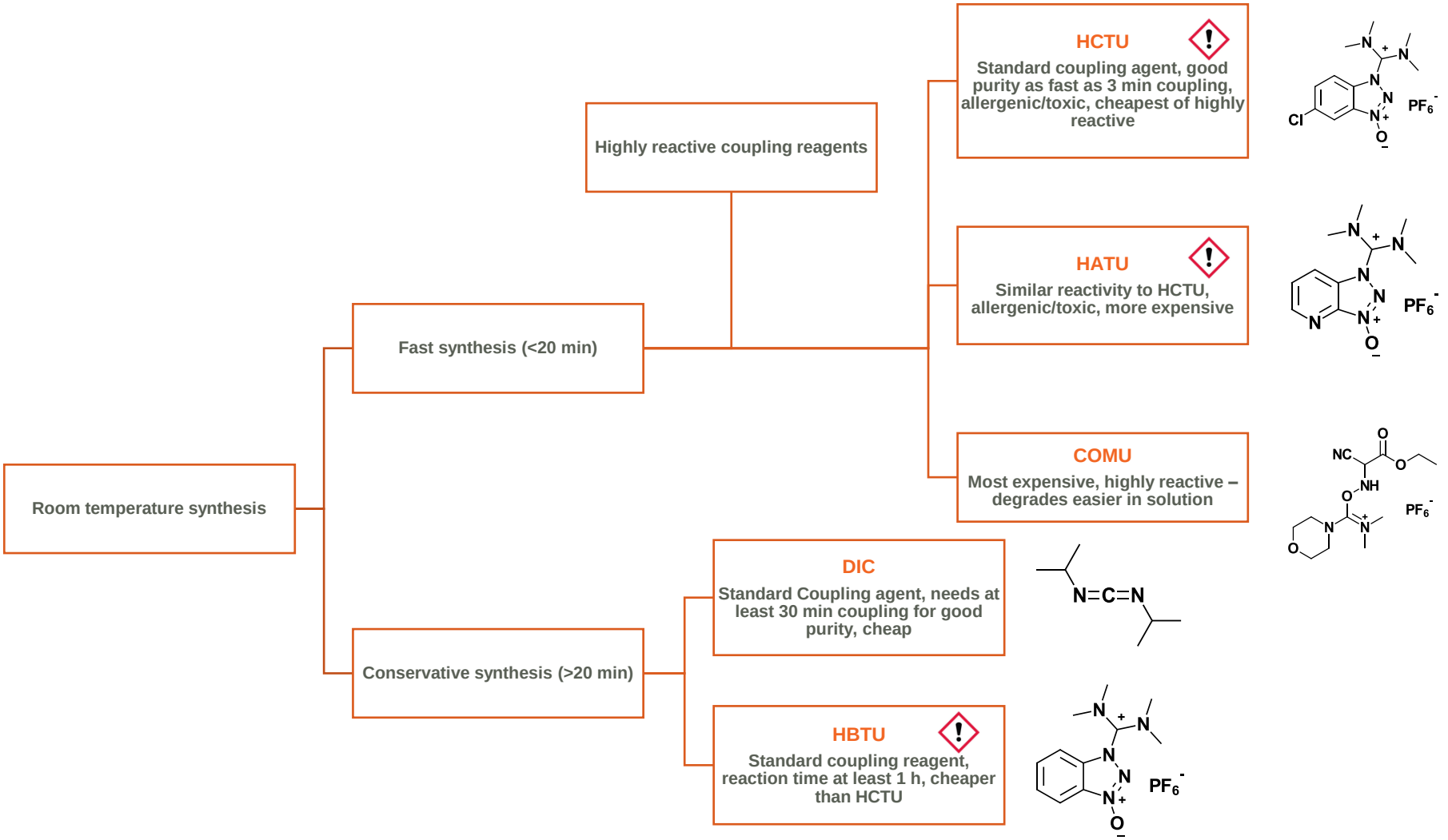
Coupling Chemistry

- Considerations:
  - Time of reaction? – reagent type, their reactivity and stability in solution
  - Double or single couplings? – activator reactivity
  - Side reactions? – guanidinium side reaction during cyclizations = step away from uronium salts

Common activators, bases, and additives.

Category	Uses	Activators	Bases	Additives
Carbodiimide	Standard	DIC (DIPCDI), EDC DCC	none	HOBt, Cl-HOBt, HOAt, OxymaPure, K-Oxyma
Phosphonium Salts	Ideal for in situ activation = no guanidinylation	AOP, BOP, PyAOP, PyBOP, PyBrOP	DIEA (DIPEA), NMM	
Uronium Salts	Standard, highly reactive	HATU, HBTU, HCTU, TATU, TBTU, TCTU		
Uronium-type	Standard, highly reactive – less stable in DMF	COMU		

WHICH COUPLING REAGENT TO CHOOSE?



Sterically hindered peptide synthesis
Aib-ACP synthesis @ room temp

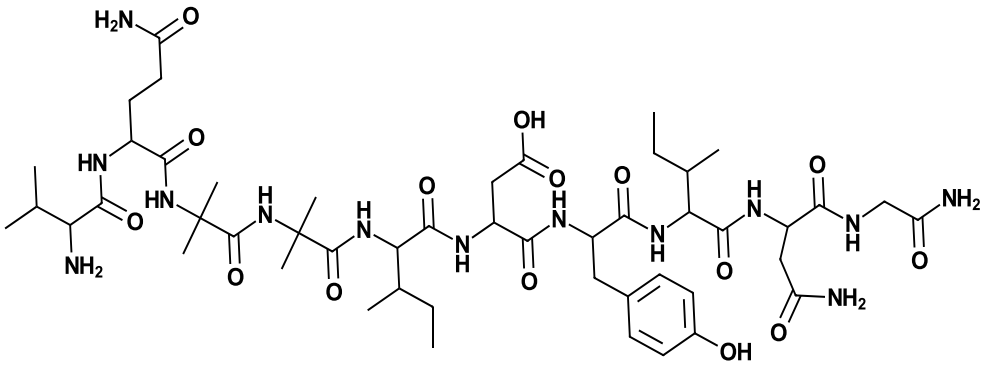
- Fast coupling reactions of sterically hindered residues

Chemistry

- Deprotections - 2 x 1 min
- Coupling
  - 2 x 3 min or 2 x 30 min @ 25°C
- Coupling reagents – 6-fold excess HCTU or COMU® with DIPEA

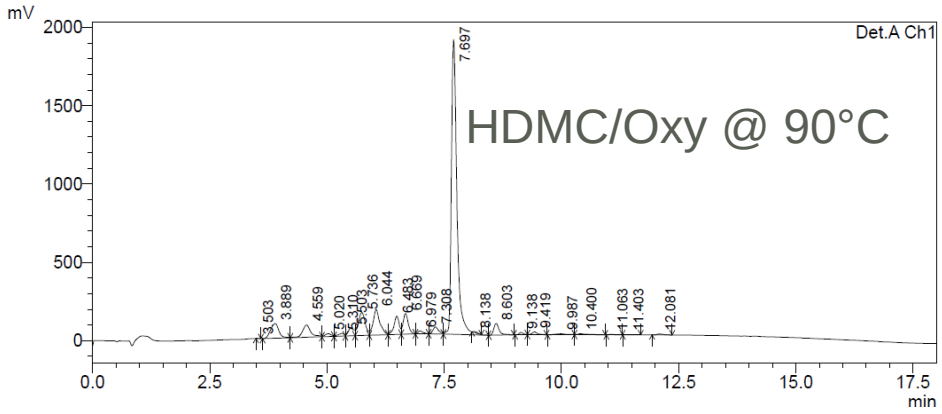
Aib-ACP

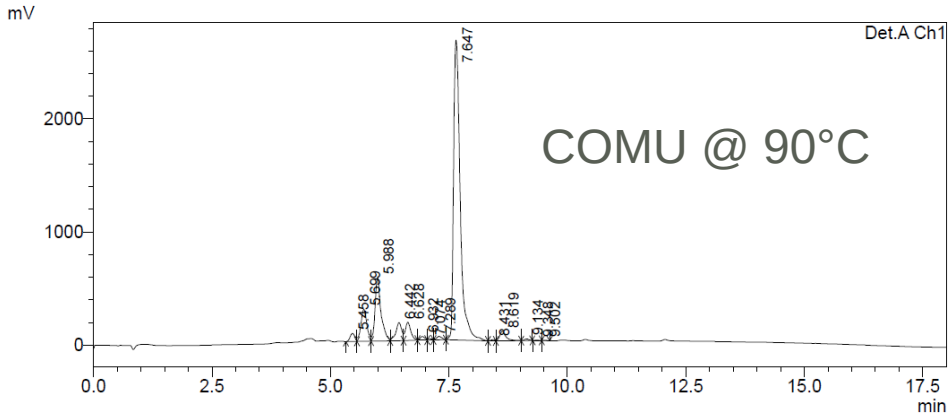
H-VQ-**Aib-Aib**-IDYING-NH_2



Coupling time	Crude Purity (%)	
	COMU	HCTU
2 x 3 min	45.2	7.8
2 x 30 min	74.3	35.5

USING DIFFERENT REAGENTS: WHAT HAPPENS AT ELEVATED TEMPERATURE?





JR 10-mer

WFTTLISTIM-NH_2

Reagent	25°C	60°C	90°C
HDMC + Oxy	21%	53%	60%
HDMC	22%	45%	51%
COMU	24%	56%	67%
DIC + Oxy	0%	49%	65%

- Heat improves purity using fast reaction times of even the "slower" reagents

CHEMISTRY

- TEMPERATURES – 25°C, 60°C or 90°C
- DEPROTECTION – 1 MINUTE
- COUPLING – 2 MINUTES

Collaboration with Dr. Fernando Albericio & Dr. Beatriz De La Torre (University of Barcelona/University Kwa-Zulu)

What about Microwave?

- Using microwave allows for faster syntheses with equal or higher purity, why?
  - Is it the magic of electromagnetic waves or just temperature?
  - Bacsa et al. investigated this in 2008

TABLE 4. Synthesis of GILTVSVAV Using Microwave and Conventional Heating at the Same Temperature on Tentagel and ChemMatrix Resins[a]

		Fmoc-amino acid		coupling			deprotection			
entry		equiv	conc (M)	temp[b] (°C)	power[c] (W)	time (min)	temp[b] (°C)	power[c] (W)	Time (min)	purity[d] (%)
					Tentagel					
1	CONV	5	0.18	67		21	67		1.5 + 3.5	77
2	MW	5	0.18	67	5	20	67	20	0.5 + 2.5	82/83
3	CONV	5	0.18	86		11	86		1.5 + 3.5	89
4	MW	5	0.18	86	10	10	86	20	0.5 + 2.5	92/93
					ChemMatrix					
5	CONV	5	0.38	67		21	67		1.5 + 3.5	90
6	MW	5	0.38	67	5	20	67	20	0.5 + 2.5	91/89
7	CONV	3	0.23	86		11	86		1.5 + 3.5	91
8	MW	3	0.23	86	10	10	86	20	0.5 + 2.5	95/91

[a] Peptide synthesis was performed on a 0.036 mmol scale using RAM-Tentagel resin (loading 0.24 mmol/g) or 0.075 mmol of RAM-ChemMatrix resin (loading 0.50 mmol/g) in a 10 mL solid-phase reaction vessel (ca. 1.1 mL of solvent for the coupling step; 2 mL of 30% piperidine in DMF for the deprotection step), CEM Discover SPS (MW) or Advanced ChemTech PLS 4 × 6 (CONV). [b] Average temperature monitored by internal fiber-optic probe (OpSens FO, see Figure 3 and S9, Supporting Information). [c] Maximum magnetron microwave output power for pulsing. [d] Purity of crude peptides (analytical RP-HPLC peak area %, UV absorbance at 215 nm). Peaks between 5 and 8 min retention time were used for integration. The chromatograms are reproduced in Figure 4 and the Supporting Information (Figure S13). The identity of the target peptide was established by MALDI-TOF MS.

J. Org. Chem. **2008**, *73*, 7532–754

What about Microwave?

- Using microwave allows for faster syntheses with equal or higher purity, why?
  - Is it the magic of electromagnetic waves or just temperature?
  - Bacsa et al. investigated this in 2008

As expected from recently published racemization studies concerning microwave-assisted SPPS at elevated temperatures,[54] a significant amount of racemization was found for both His (ca. 7% D-His) and Cys (ca. 2% D-Cys) in the synthesis of the 24-mer using the DIC/HOBt coupling conditions at 86 °C. Importantly, the racemization levels were very similar comparing peptide samples obtained from microwave and conventionally heating experiments at 86 °C (Table S3, Supporting Information). This again indicates that the mode of heating in SPPS at elevated temperature does not have an effect on peptide purity and racemization and therefore supports the notion that nonthermal microwave effects are not involved. As demonstrated previously,[54] the racemization of sensitive amino acids in microwave-assisted SPPS can be minimized by carrying out problematic coupling steps (mainly for His and Cys) at a lower temperature regime (<50 °C).

Concluding Remarks

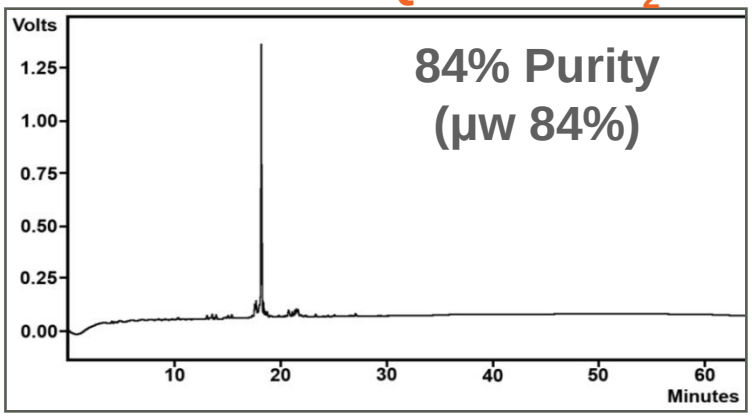
In summary, a critical investigation of microwave-assisted Fmoc/*t*-Bu solid-phase peptide synthesis (SPPS) under carefully controlled conditions was performed. A number of reports in the literature have advocated the use of this technology to obtain peptides not only faster but also in higher purity as compared to conventional room temperature SPPS. However, adequate control experiments performing comparison studies involving

actually a few degrees higher than measured by the fiber-optic probe. This hypothesis was supported by a control experiment performing a conventionally heated SPPS at 70 °C (instead of 67 °C), delivering the desired peptide in exactly the same purity as under microwave conditions (see above). The close match between microwave- and conventional heating in SPPS was not only evident by comparing peptide purities but did also extend to racemization studies. For peptides containing racemization-prone amino acids such as His and Cys the determined racemization levels at 86 °C (microwave or conventional heating) were nearly identical.

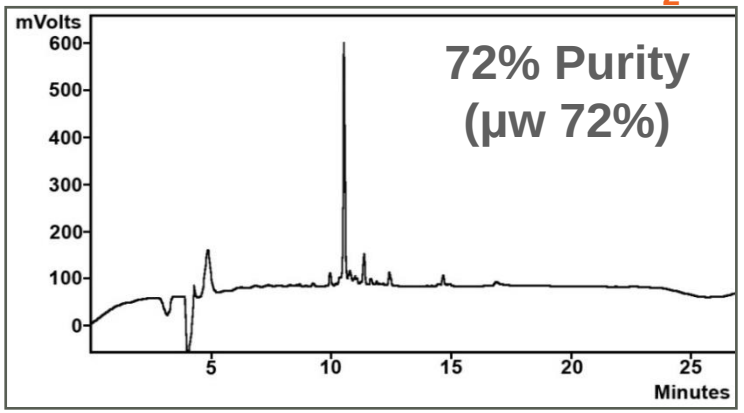
It can therefore be concluded that the observed enhancement effects in the microwave-assisted SPPS of the specific peptides investigated in this study are of purely thermal nature and not related to the microwave field. No evidence for a proposed deaggregation of the peptide backbone via direct interaction of the peptide chain with the microwave field could therefore be obtained for the comparatively short peptides studied herein.[14] Finally, it should be emphasized that increasing the reaction temperature from ambient conditions by 60 °C for both coupling and deprotection steps represents an estimated 50-fold increase in the reaction rate for both processes based on the Arrhenius equation. This kinetic effect is probably responsible for the highly efficient coupling and deprotection in microwave-assisted solid-phase peptide synthesis, providing peptides in high speed and purity.

Comparison of Rapid Heating Methods

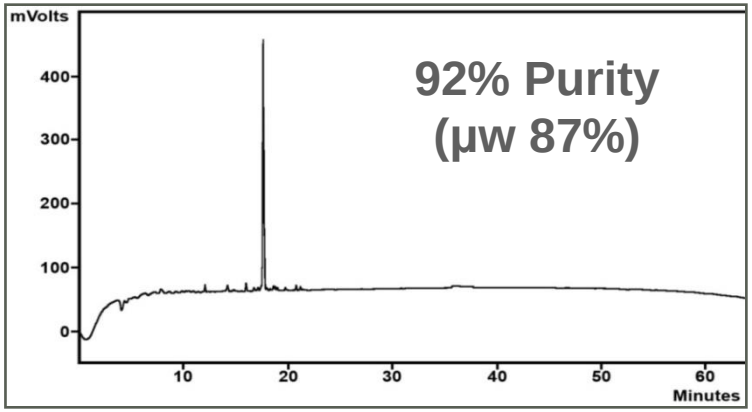
CD44 v6: KEKWFENEWQGKNP-NH_2



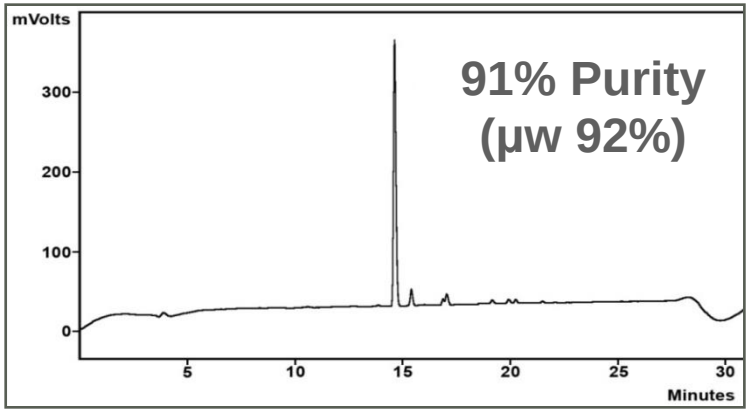
Cerebellin: SGSAKVAFSAIRSTNH-NH_2



$^{65-74}$ACP: VQAAIDYYING-OH



Aib-Enkephalin: Y-Aib-Aib-F-L-



Using Heat? – Keep in mind…

- Racemization
  - DIC/Oxy has been shown in the literature to have reduced racemization potential than base mediated reagents
  - In our experiments HCTU/DIPEA for 3 min couplings was comparable to DIC/Oxy
- Side reactions
  - Increased kinetics can also increase side reactions thus keep reaction times short
- Save time and solvents
  - Do single couplings with similar purities reducing time and waste generated

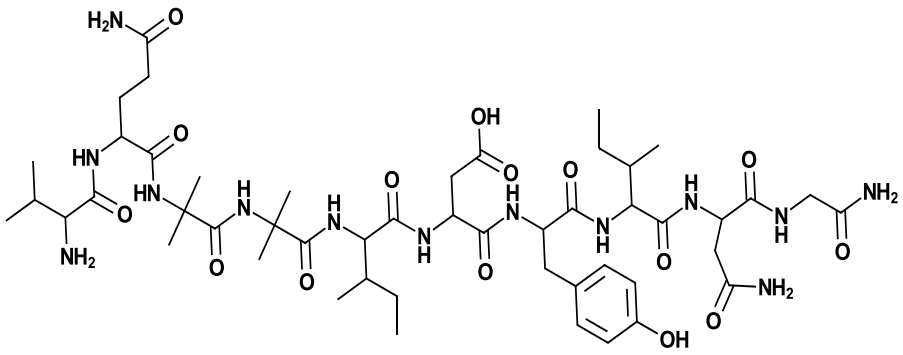
Sequence Dependent!

Aib-ACP synthesis with Heat

- Save time and solvents when using heat

Aib-ACP

VQ-**Aib**-**Aib**-IDYING-NH_2

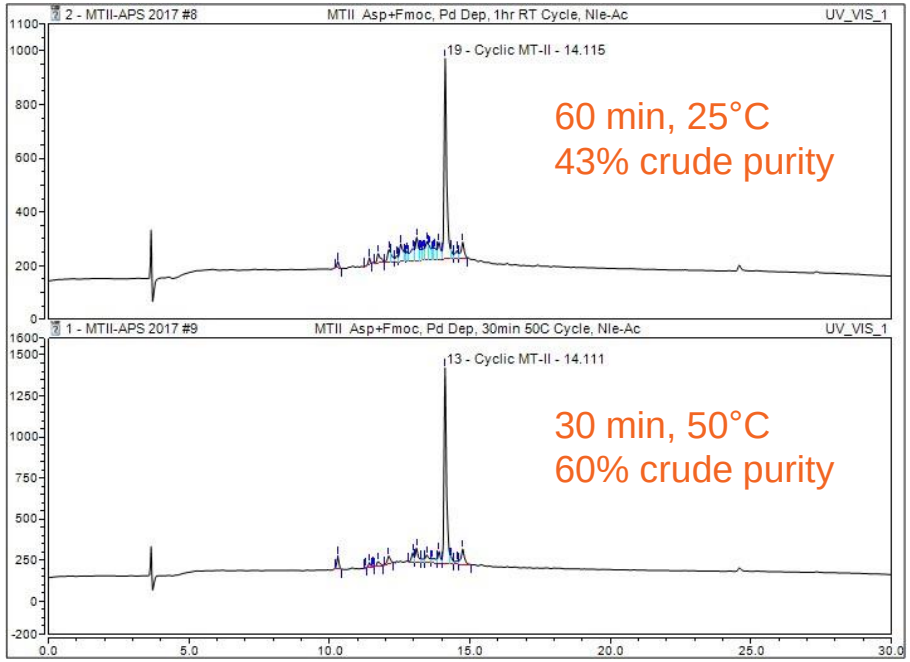


Chemistry

- Resin – Rink Amide MBHA resin at 25 umol scale
- Deprotections – 2 x 1 min
- Coupling
  - 3 min for standard AAs with 10 min for the coupling of the 2nd Aib with single or double couplings
- Coupling reagents – 6-fold excess COMU® with DIPEA
- Capping – 5 min

Temperatures	Crude Purity (%) of the synthesis using COMU	
	Double couplings	Single couplings
25 °C	74.5	36.7
75 °C	91.1	84.1
90 °C	78.3	78.3

Automated Lactam Bridge Cyclization MT-II using Heat



Melanotan II (MT-II)

Ac-Nle-cyclo[Asp-His-D-Phe-Arg-Trp-Lys]-NH_2

- Flexibility to use different reagents for special cycle without manual intervention
- Test different temperature conditions simultaneously

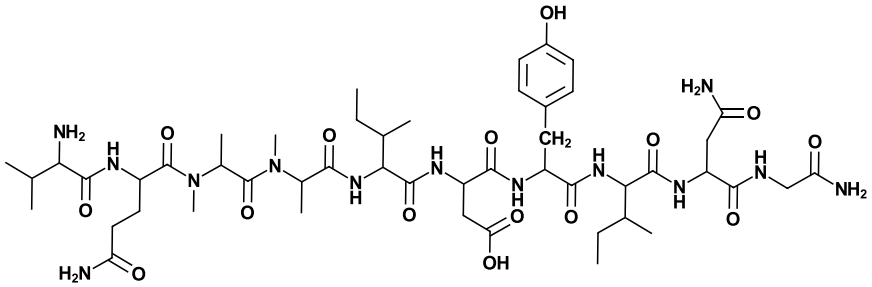
Chemistry

- Fully automated synthesis and Alloc/OAll Pd deprotection
- Cyclization (lactam) using 30 or 60 min, at 50°C or 25°C, using PyOxim/DIPEA

Sterically Hindered N-Me-Ala^{3,4}-ACP Synthesis

[N-Me-Ala^{3,4}]-ACP

VQ-NMe-Ala-NMe-Ala-IDYING-NH_2



	Bottle Set Up	
	Bottle 1	DMF
	Bottle 2	DCM
	Bottle 3	20% Piperidine
	Bottle 4	Capping
	Bottle 5	DIPEA
All Cycles	**Bottle 6**	**COMU**
Hindered cycles	**Bottle 7**	**PyOxim**
	Bottle 8	TFA

Temperature	Crude purity (%)	
	COMU	COMU/PyOxim
25 °C	85.5	83.4
75 °C	78.9	69.2
90 °C	70.0	59.9

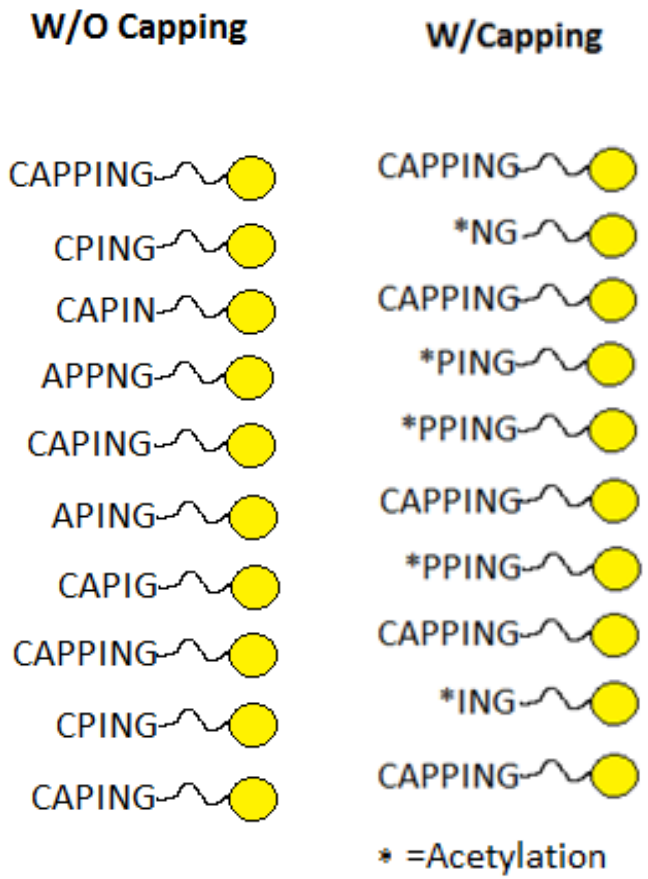
Heating for crude purity improvements is sequence dependent!

Chemistry

- Deprotections - 2 x 1 min
- Coupling – 2 x 3 min and 2 x 10 min for coupling of residues after the N-Me Ala
- Coupling reagents –
  - Run 1 – COMU®/DIPEA
  - Run 2 – **COMU®/DIPEA** for standard AAs and **PyOxim/DIPEA** for NMe-Ala

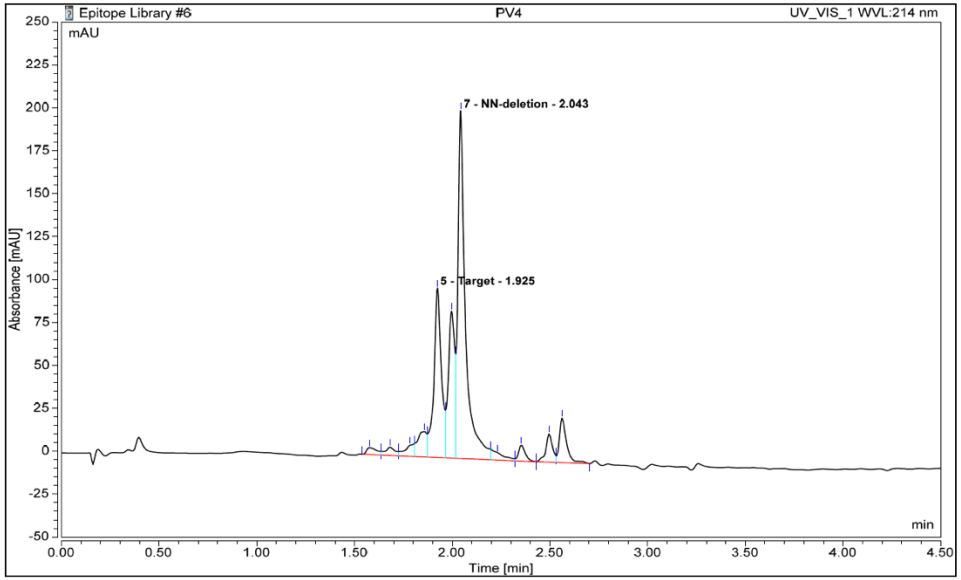
CAPPING

- Useful for synthesis optimization
  - Helps identify problematic couplings that lead to deletions/truncations
- Capping solutions
  - Ac_2O:Pyridine:DMF
  - Ac_2O:DIPEA:DMF
- Ratios or equivalents
  - 1/1/3 for 5 min
  - 50 equivalents in 1:1 Ac_2O:Pyridine for 1 h

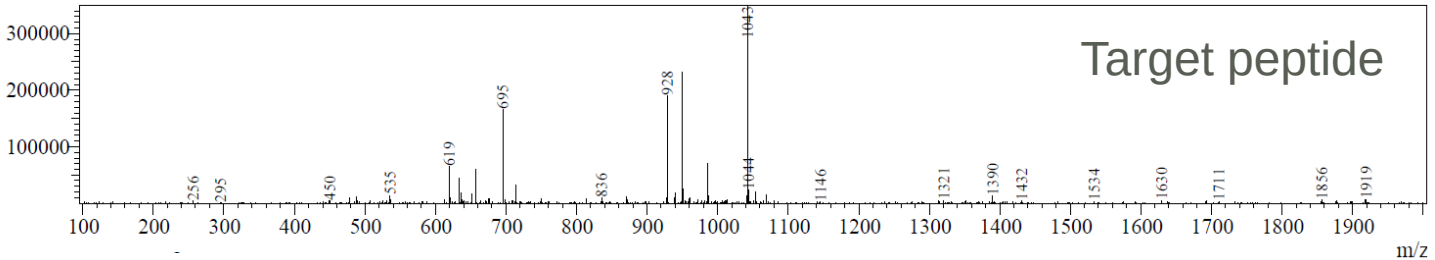


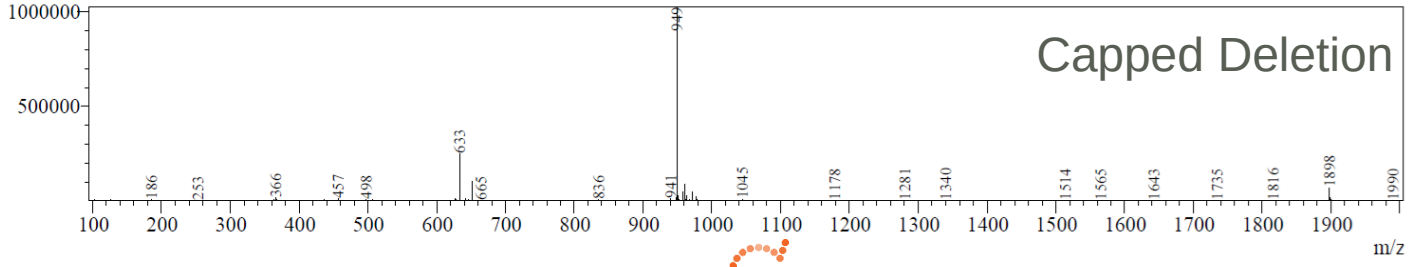
CAPPING EXAMPLE – SARS-COV EPITOPE

Thanks to N-Acetylation on truncated peptide we can see the deletion as a separate peak



Target Peptide
H-NNNAATVLQLPQGTTLPKGF-NH_2





Before starting up your synthesis make sure…

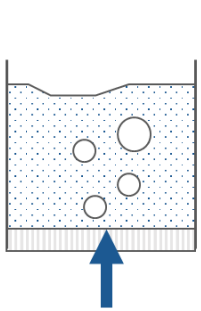
- SPPS is normally carried out at ≥ 3 equivalents
  - Automated peptide synthesis is typically done with 4-6 equivalents
  - It is important that the final concentration in the RV is high for high efficiency couplings
- Total volume should cover resin and allow for proper resin mixing
  - Good standard: 1 mL/ 100 mg of resin – works very well for PS resins; use about 1.5x the volume for CM resin (it swells a LOT!)
- Proper RV size is selected
  - Consider resin growth when doing long peptides at a higher scale

Protocol Example

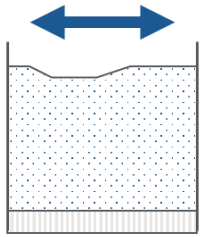
Name: Stand_3ml_HT | Cleave | Ops | Mix | Comments | Ins | Add | Del

Step	Operation	Solvent	Volume (µL)	Time	Drain	PV	UVDM	Reps
1	Bottom Delivery	20% pip [Btl3]	3000	00:01:00	■	☐	Basic	1
2	Top Delivery	DMF [Btl1]	3000	00:00:30	■	☐	None	3
3	AA Building Block	None	1000	00:00:00	☐	☐	None	1
4	Bottom Delivery	HCTU [Btl6]	1000	00:00:00	☐	☐	None	1
5	Bottom Delivery	NMM [Btl5]	1000	00:03:00	■	☐	None	1
6	Top Delivery	DMF [Btl1]	3000	00:00:30	■	☐	None	3

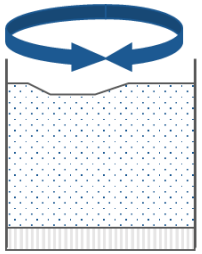
Save | Save As | Report | Cancel | Done



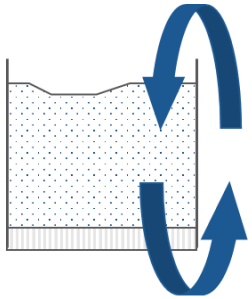
Nitrogen Bubbling



Shaker



Vortex Mixing



Recirculation

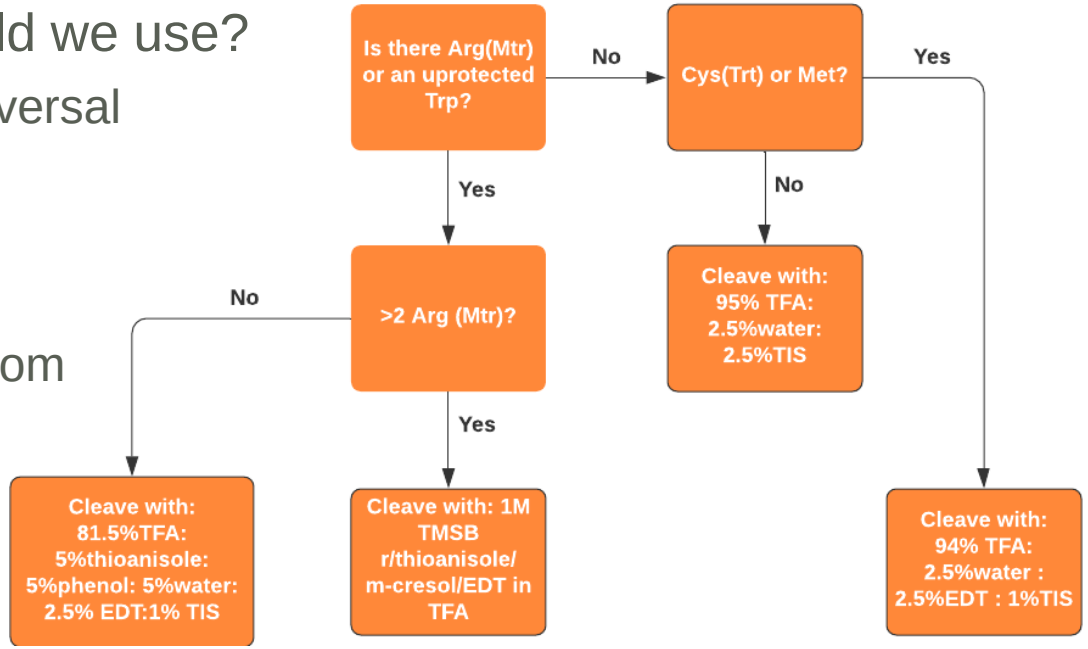
- Mixing can have a direct effect on the purity of the peptide being synthesized

Designing A Synthesis

Cleavage and Isolation

Cleavage & Isolation

- Which scavengers should we use?
  - Reagent K is quite universal
- For how long?
  - Standard is 2-3 h at room temperature
  - Faster with heat



@ 38°C for 30-40 min → Precipitate in ether

Cleavage & Isolation

- Automated cleavage
  - Cleavage is ready for precipitation at a set time
- Precipitating peptides
  - Cold diethyl ether
  - Petroleum ether
  - Mixture of solvents
  - Rotovap and lyophilize

GYROS PROTEIN TECHNOLOGIES

SPPS TIPS FOR SUCCESS: STRATEGIES FOR MINIMIZING SIDE-REACTIONS

Łukasz Frankiewicz
Senior Product Specialist
(Peptides)

GYROS PROTEIN Technologies

Overview

- SPPS Side Reactions
  - Racemization
  - DKP formation
  - Pyroglutamate formation
  - Aspartimide formation
  - Deamidation of Asn/Gln
  - Peptide Guanidination
  - Arg δ-Lactam formation
  - Oxidation reactions
  - Amino Acids Additions/Insertions
  - Amino Acids Deletions
  - Amino Acids Impurities
  - Cleavage Side reactions

SPPS Side Reactions

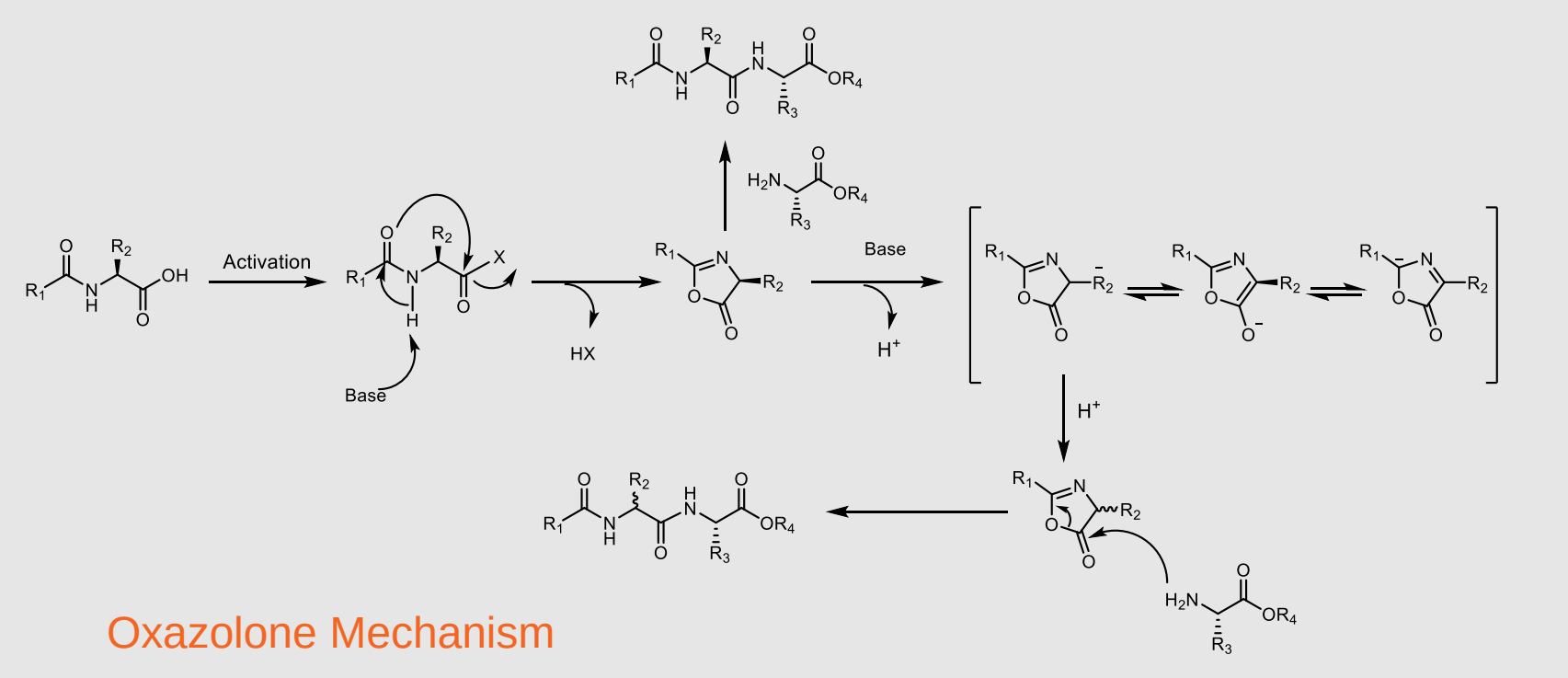
Racemization

RACEMIZATION

Significantly important in the synthesis of peptide therapeutics

AAs prone to racemization:

- His,
- Cys,
- N-alkylated AAs



Oxazolone Mechanism

Yi Yang. Side Reactions in Peptide Synthesis. 2016

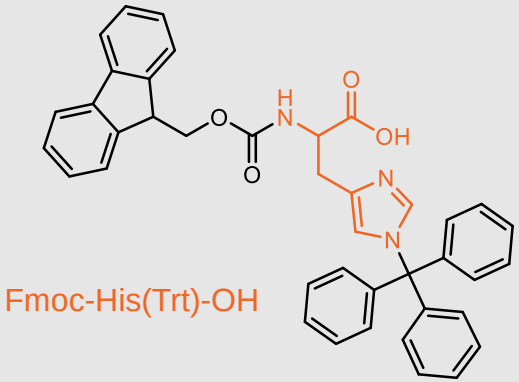
What can we do about racemization?

Racemization usually occurs during base-mediated activation methods (especially high with pre-activation and heating)

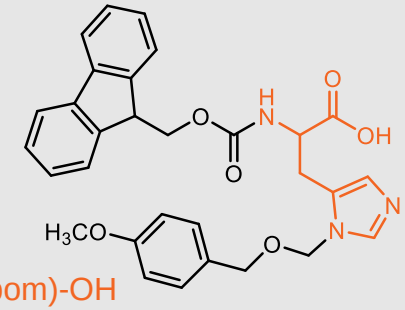
- Try carbodiimide activation instead to lower the pH
- HCTU – good coupling efficiency and racemization suppression
- COMU – superior racemization suppression relative to HOBt-based coupling reagents
- 2,4,6-collidine – reduces substantially racemization compared to DIPEA and NMM

Using an alternative side chain protecting groups to the usual trityl protecting group,

- Histidine: Mbom, may help reduce racemization
- Cysteine: S-StBu> S-Trt >> S-Tacm >S-Acm >S-MeBzl > S-tBu



Fmoc-His(Trt)-OH

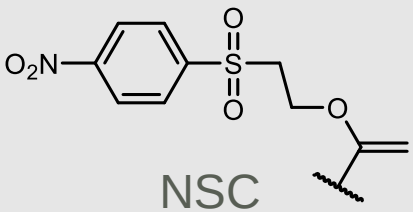


Fmoc-His(Mbom)-OH

Behrendt, R., White, P., & Offer, J. *J. Pept. Sci. 2016*

What can we do about racemization?

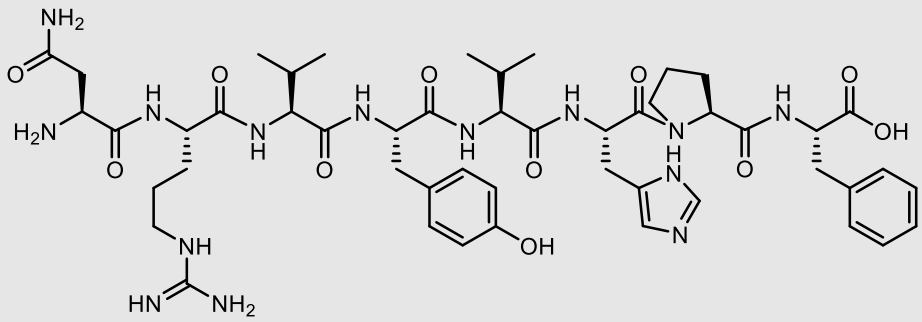
- Lowering the temperature if using heat and incorporating additives if not already in use
- Solvent polarity can have an effect on racemization:
  - Adding DCM to DMF/NMP as cosolvent can help reduce racemization
- Cu(II) Salt additive
  - Adding $CuCl_2$ as additive in carbodiimide, phosphonium or uronium coupling reagents
- N^{α} protecting group – NSC for Cys, His, and Ser
  - Shown to reduce racemization extent compared to Fmoc-protected when using HBTU/DIPEA chemistry
  - NSC removed same as Fmoc – slower deprotection



NSC

Racemization Results: Angiotensin

Angiotensin H-NRVYVHPF-OH



Chemistry

Deprotections

1:30 min @ 90°C

Coupling

2:30 min @ 90°C

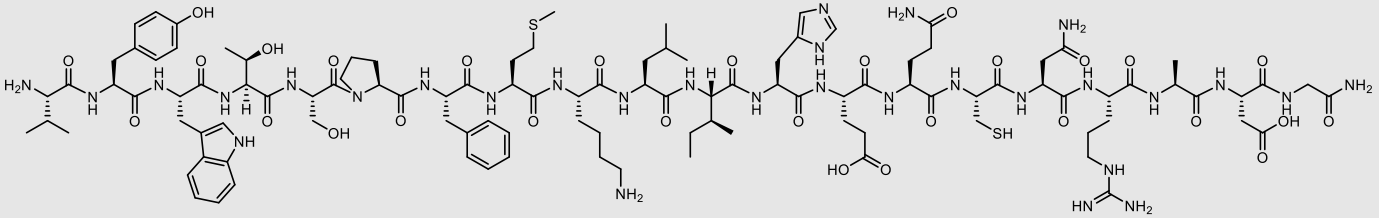
Coupling reagents

6-fold DIC/OxymaPure

Amino Acid	% D-enantiomer
Val	< 0.10
Pro	0.16
Asp/Asn	< 0.10
Phe	1.67
Tyr	n.d. – only traces
Arg	0.25
His	4.27

Racemization Results: ABC-20mer

H-VYWTSPFMKLIHEQCNRADG-NH_2



Sample sequence with all 20 natural occurring amino acids

Chemistry

Deprotections

1:30 min @ 90°C

Coupling

2:30 min @ 90°C

Coupling reagents

6-fold DIC/OxymaPure or HCTU/DIPEA

Amino Acid	% D or Impurity with DIC	% D or Impurity with HCTU
Ala	0.24	0.29
Val	< 0.10	< 0.10
Thr	< 0.10 D-Thr	< 0.10 D-Thr
Ile	< 0.10 D-Ile	< 0.10 D-Ile
Pro	0.21	0.23
Leu	0.22	0.24
Ser	< 0.10	0.10
Cys	0.36	0.52
Asp	7.25	7.69
Met	0.17	0.18
Phe	0.18	0.16
Glu	1.89	1.64
Tyr	0.24	0.32
Lys	0.14	0.13
Arg	0.31	0.24
Trp	0.15	0.28
His	5.30	5.76

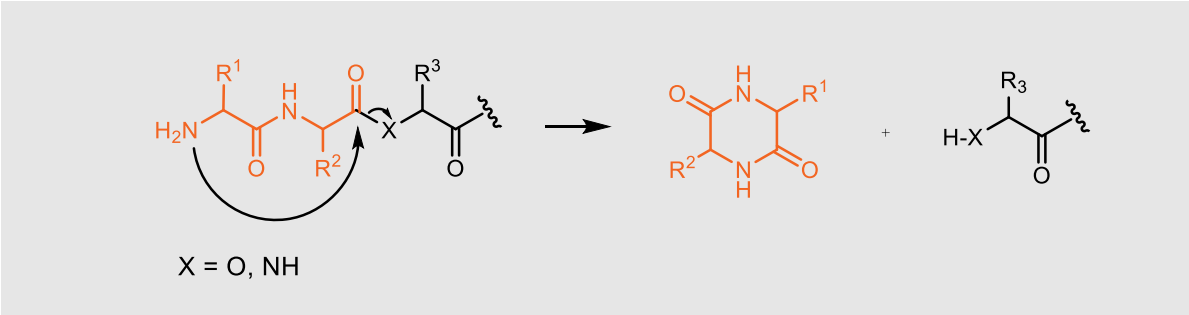
SPPS Side Reactions

DKP Formation

2,5-Diketopiperazine Formation

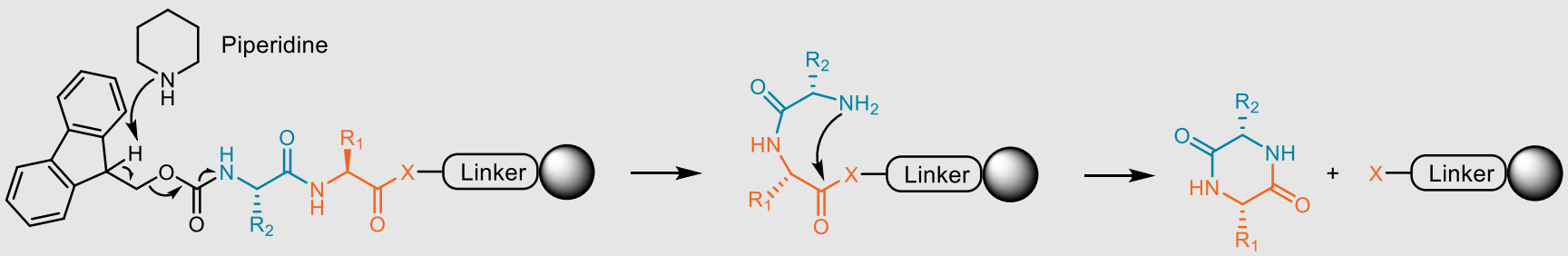
One of the most prevalent side reactions in SPPS

- N-terminal nucleophilic attack on C-terminal residues leading to 6-membered ring formation and intramolecular cleavage, (loss of peptide from resin!)



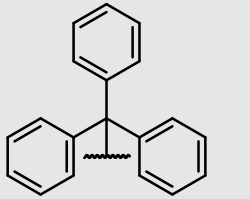
- DKP formation is accelerated if the two Aas involved in the reaction are in the cis-configuration – this alignment promotes nucleophilic attack.
- DKP is exacerbated by the following motifs: N-Me-Xaa, alternating D- and L- Aas, Pro at C-terminus.

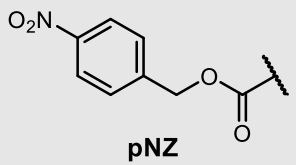
2,5-Diketopiperazine Formation

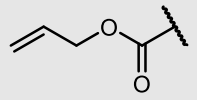


DKP Prevention

- DKP is more likely to happen during Fmoc deprotection, so reducing the deprotection time is critical
- Addition of 0.1M HOBt (or OxymaPure) to the 20% piperidine solution can also help
- Use CTC resin when Pro is at C-terminus (steric hindrance)
- Use of carbodiimide coupling agents can also reduce the risk of DKP formation (absence of tertiary bases)
- Use of alternative to Fmoc protecting groups (Trt, pNZ, Alloc)
- Employment of the dipeptide building blocks







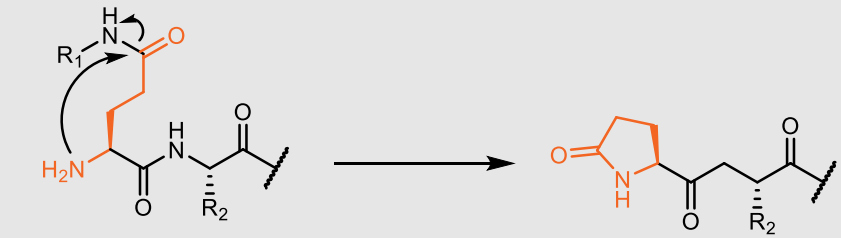
SPPS Side Reactions

Pyroglutamate Formation

Pyroglutamate Formation

Pyroglutamate is formed when N-terminal Gln or Glu residues undergo intramolecular lactam formation.

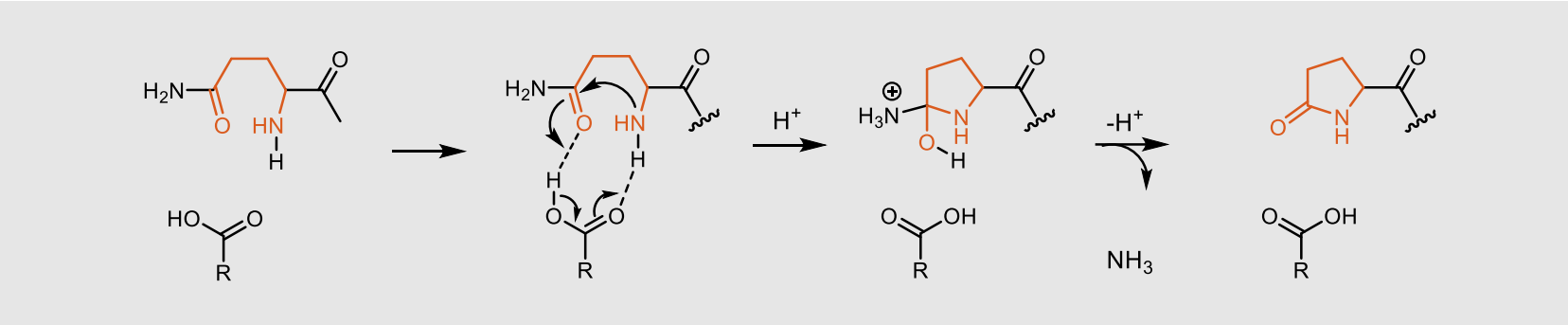
- Gln is more susceptible than Glu
- This N-terminal cyclization can cause termination of the peptide chain, or formation of two species if Gln/Glu is the final residue.



R^1 = H or protecting group

- Pyroglutamate formation takes place predominantly during the process of acylation of the N-terminal Gln peptide, therefore accelerating this step could help minimize Pyr formation…

Pyroglutamate Formation



Measures to reduce Pyr formation

- Using high AA excess
- Using highly reactive coupling agents (e.g. COMU)
- Use of symmetric anhydride coupling
- Preactivation of the incoming AA

If Gln is at the N-terminus

- Use Pyr building block to form only the cyclic derivative (not a mixture of two species).

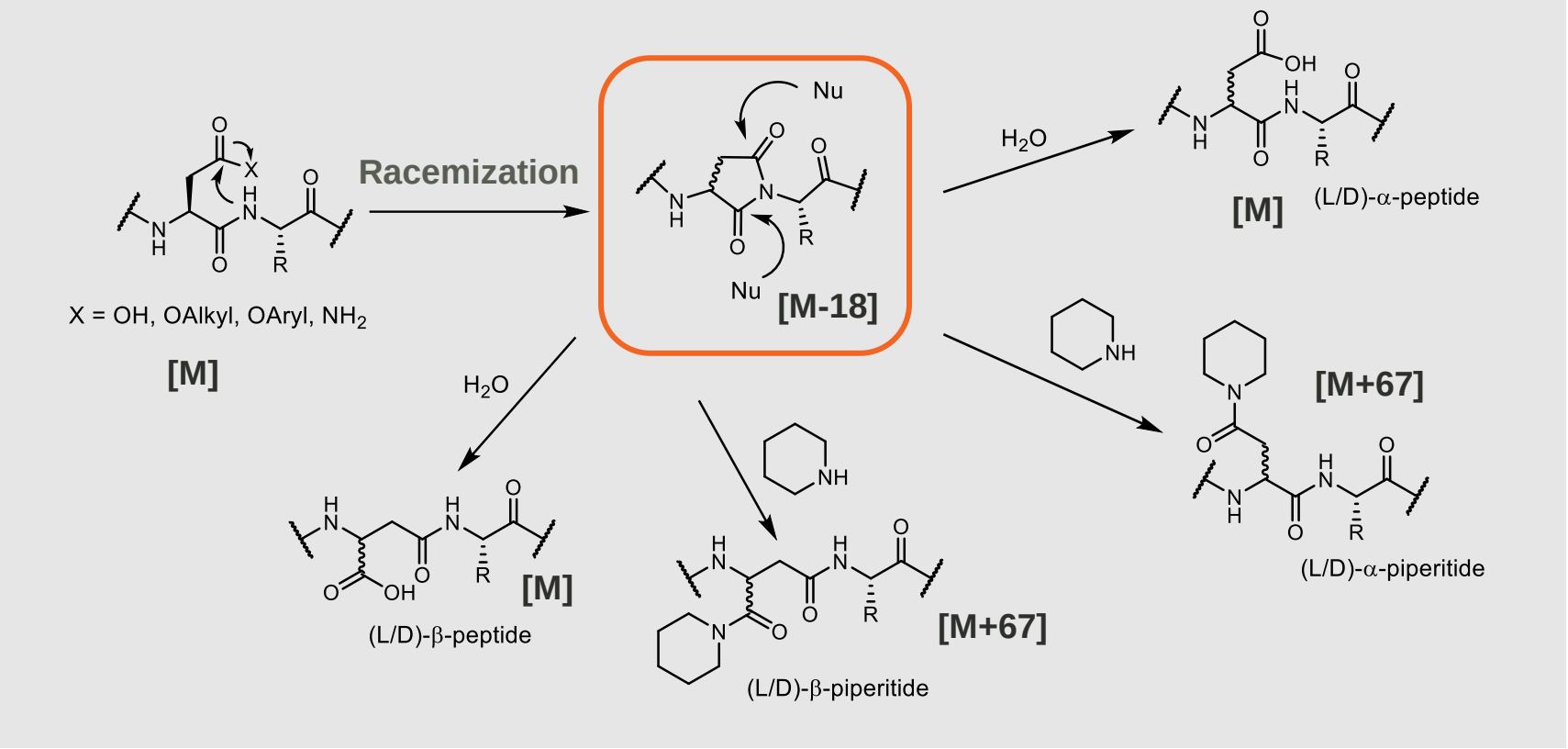
SPPS Side Reactions

Aspartimide Formation

Aspartimide Formation

Aspartimide formation

Treating peptides containing Asp residues with base repeatedly (e.g. piperidine) can lead to a side reaction with up to **9 different by-products!**



Aspartimide Formation

What is influencing aspartimide formation ?

- Bases (piperidine, DIEA, TEA)
- Acids (HF, TfMSA, 6N HCl, TFA)
- Asp side chain protecting groups – Asp(OtBu), Asp(OAll)
- Temperature – higher temperature, higher aspartimide content
- Solvent – purity, content of secondary amines, DMSO > **DMF** > >THF > DCM
- Peptide sequence: **-Asp(X)-Gly-**, -Asp(X)-Asn(Trt/Mtt), -Asp(OtBu)-Ala/Gln(Trt)-, -Asp(X)-His(Y)- (pH dependant)
- Microwave

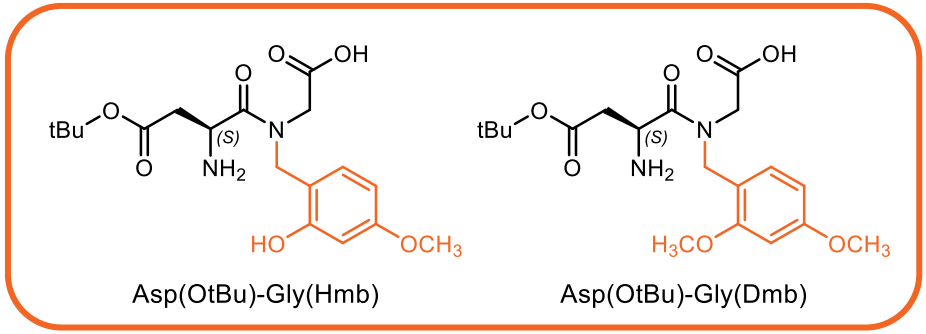
Asp-Axx	Sensitivity to Aspartimide Formation
Gly	+++++
Asn(Trt)	+++
Asp(OtBu)	++
Ser/Thr	++
Cys(Acm)	++
Cys(Trt)	+
Thr(tBu)	+
Ala	+

Aspartimide Formation - Solutions

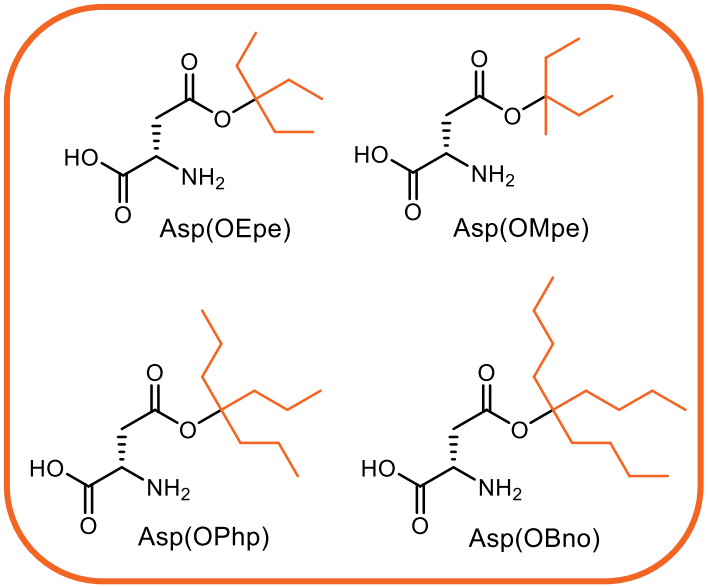
How to deal with aspartimide?

- Utilization of alternative to piperidine bases – piperazine (with addition of 0.1M HOBt)
- Alternative side chain protecting groups – Asp(OMpe), Asp(OEpe), Asp(OPhp), Asp(OBno)
- Backbone amide protection -Asp(OtBu)-*N*-Dmb-Xaa-, -Asp(OtBu)-*N*-Hmb-Xaa-
- Employment of pseudoproline building blocks

Conditions	% Target product[a]	
	Peptide **I**	Peptide **II**
Piperidine	15.8	30.8
Piperidine + HOBt	n.d[b]	63.4
Piperidine + Hmb	99.5	n.d[b]
Piperazine	58.3	70.5
Piperazine + HOBt	74.9	89.7
Piperazine + Hmb	99.1	n.d[b]
1-Hydroxypiperidine	58.8	70.3
1-Hydroxypiperidine + Hmb	99.2	n.d[b]
TBAF	n.d[c]	20
TBAF + Hmb	99.2	n.d[b]
DBU	n.d[c]	11
DBU + Hmb	89.7	n.d[b]



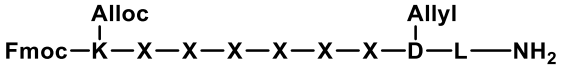
Asp(OtBu)-Gly(Hmb) Asp(OtBu)-Gly(Dmb)



Asp(OEpe) Asp(OMpe) Asp(OPhp) Asp(OBno)

Behrendt, R.; Huber, S.; White, P. *Journal of peptide science* ***2016, 22, 92.***
Wade, J. D.; Mathieu, M. N.; Macris, M.; Tregear, G. W. *Letters in Peptide Science* ***2000, 7, 107.***

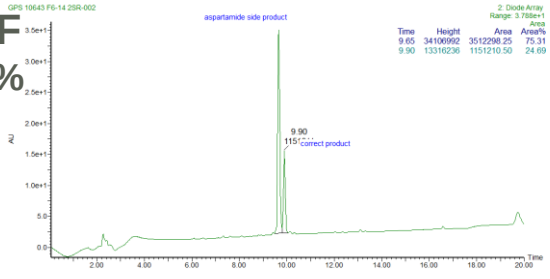
ASPARTIMIDE FORMATION - EXAMPLES



TECHNICAL DMF

Correct product: **25%**

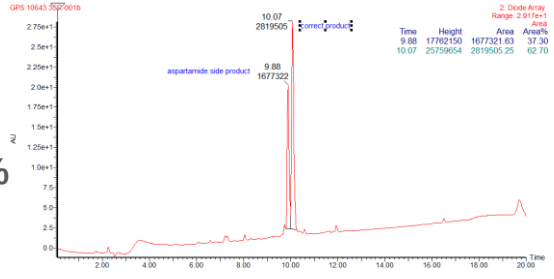
Aspartimide: **75%**



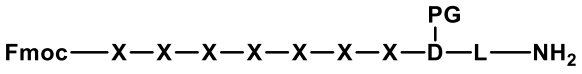
PEPTIDE SYNTHESIS GRADE DMF

Correct product: **63%**

Aspartimide: **37%**



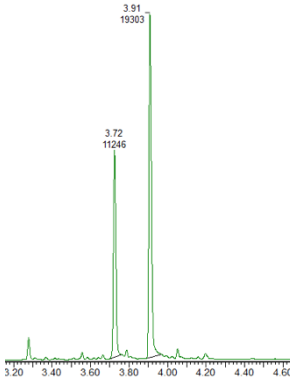
PEPTIDE SYNTHESIS GRADE DMF



Allyl protection on Asp

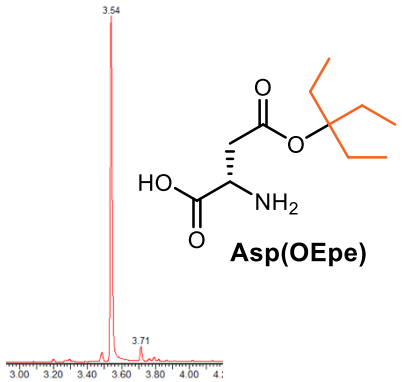
Correct product: **63%**

Aspartimide: **37%**



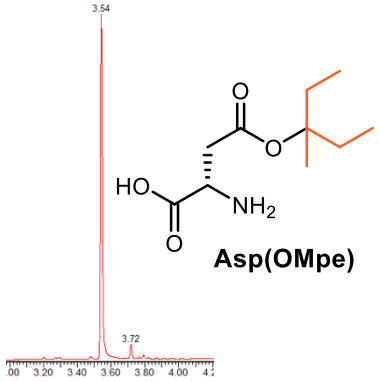
Epe protection on Asp

Correct product: **>95%**



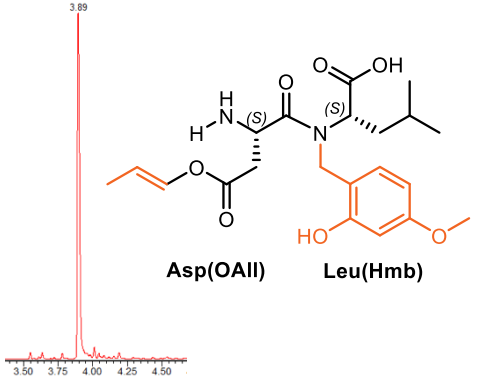
Mpe protection on Asp

Correct product: **>95%**



Allyl protection on Asp
FmocHmb protection on Leu

Correct product: **>95%**



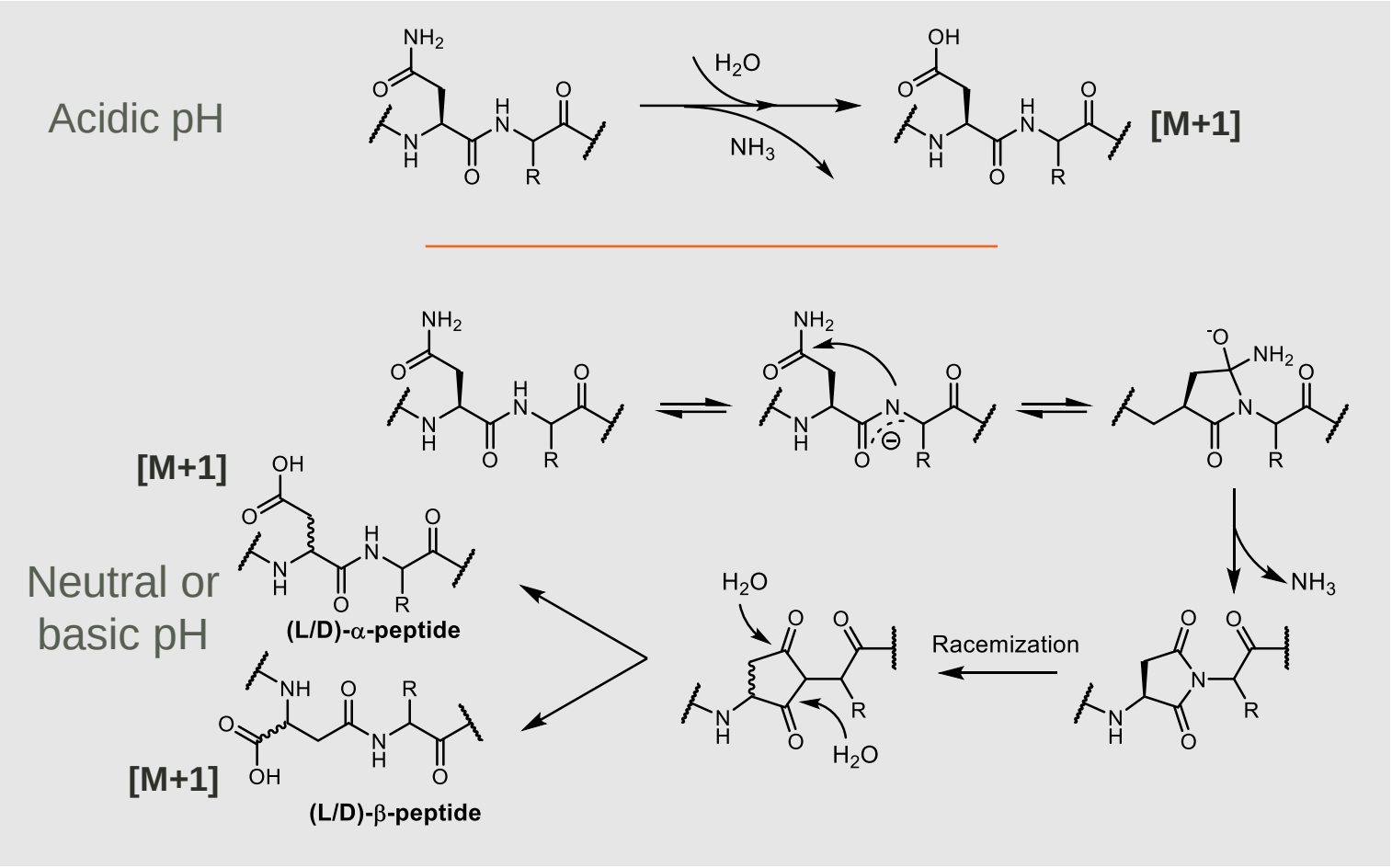
GYROS PROTEIN Technologies

SPPS Side Reactions

Deamidation of Asn/Gln

Deamidation of Asn/Gln

Mechanism: Direct hydrolysis or through succinimide intermediate



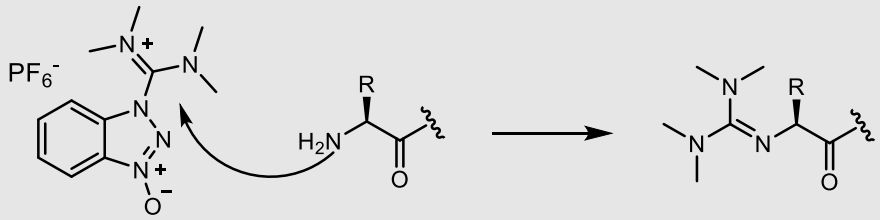
Catak, S.; Monard, G.; Aviyente, V.; Ruiz-López, M. F. *The Journal of Physical Chemistry A* ***2009, 113, 1111.***

SPPS Side Reactions

Peptide Guanidination

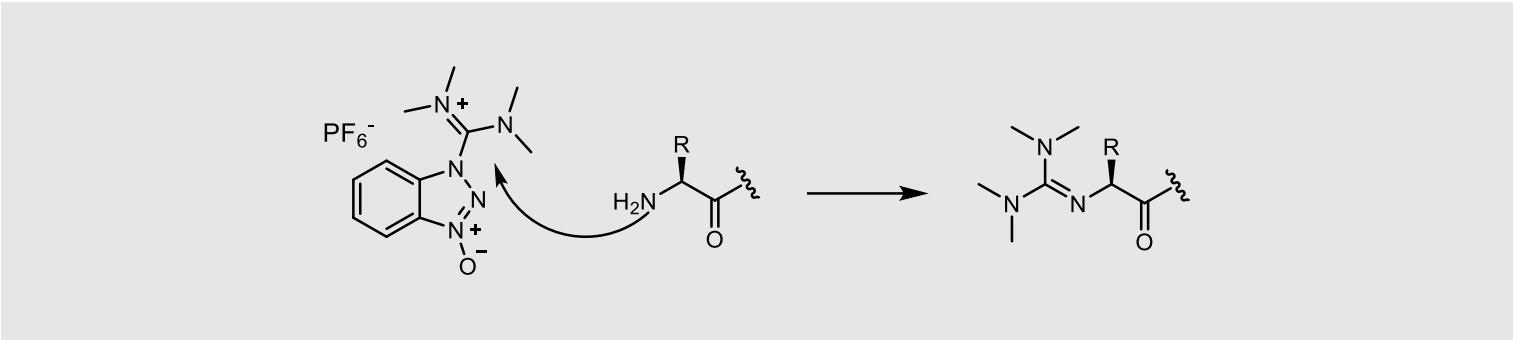
GUANIDINATION

- Uronium/Guanidinium salt coupling reagents such as TBTU, HBTU, and HATU can contribute towards unintended guanidination of the amino group of the amino acids



- This guanidino derivative is chemically stable to subsequent synthesis steps, even to TFA during cleavage, and so this truncation of the peptide chain effectively terminates the synthesis.

GUANIDINATION

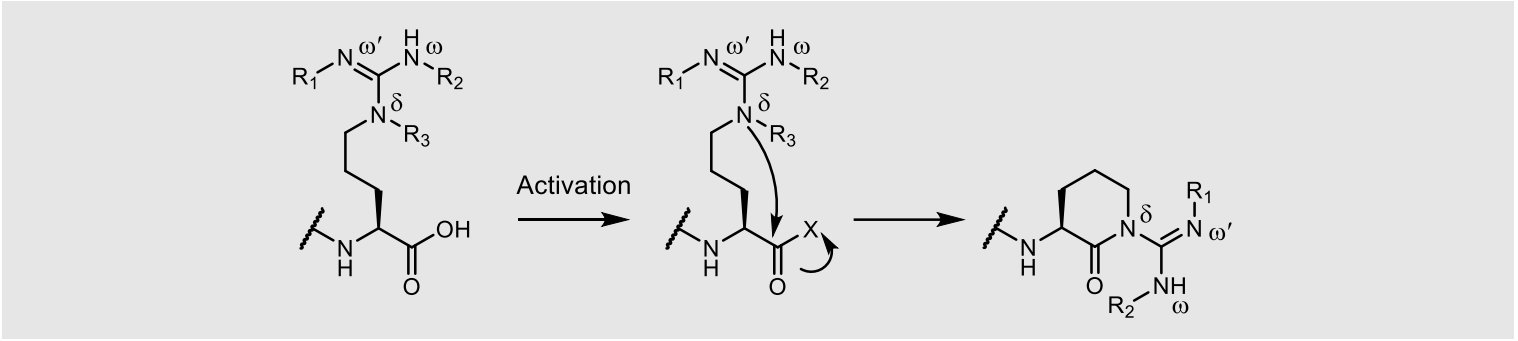


- Improper stoichiometry can lead to guanidination (too much HATU vs AA), so lowering the excess can help reduce its formation (e.g. 0.95eq HATU vs 1eq AA).
- Preactivation of the incoming AA can also help eliminate guanidination as the coupling agents are consumed before reaching the free peptide chain… however be careful of increased risk of racemization!

SPPS Side Reactions

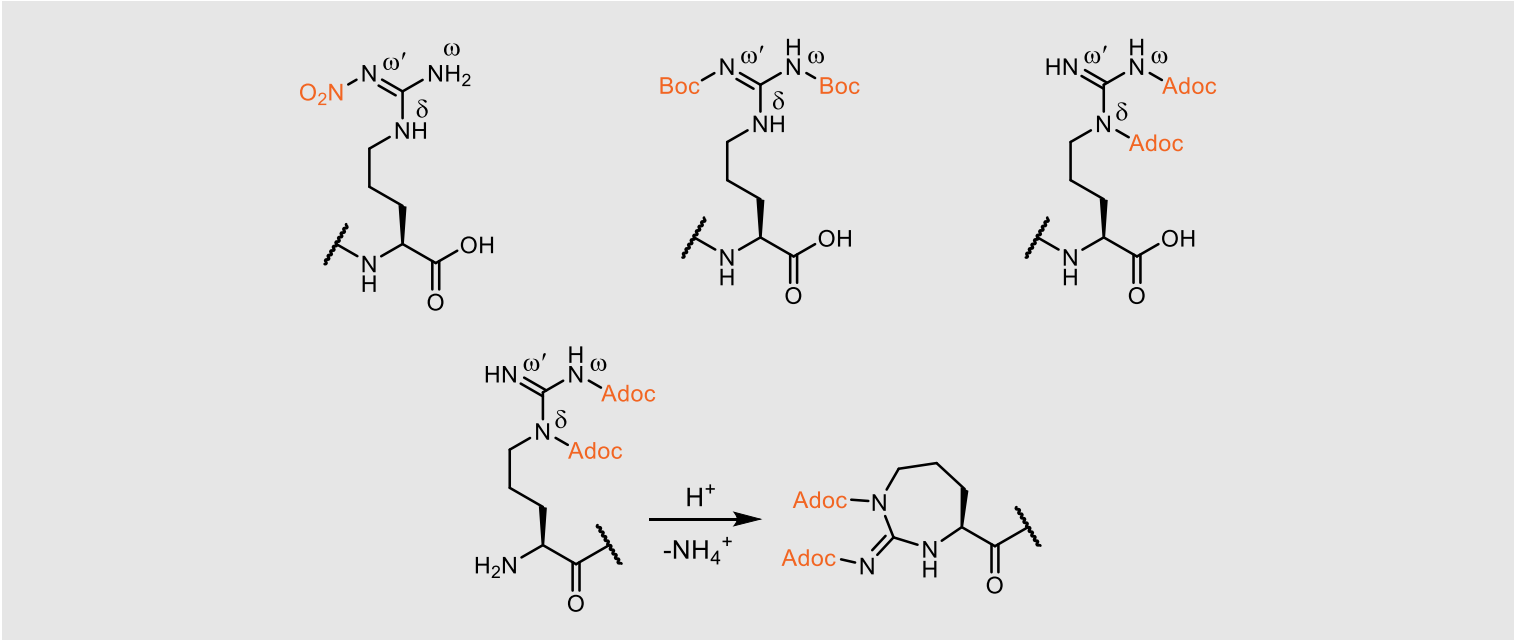
Arg ACTIVATION - δ-LACTAM FORMATION

Arg Activation - δ-Lactam Formation



- Happens during Arg activation
- Can create substoichiometric quantities and cause Arg deletions – des Arg derivatives
- Creates the necessity for Arg recoupling
- Can be drastically increased during microwave assisted synthesis

Arg Activation - δ-Lactam Formation



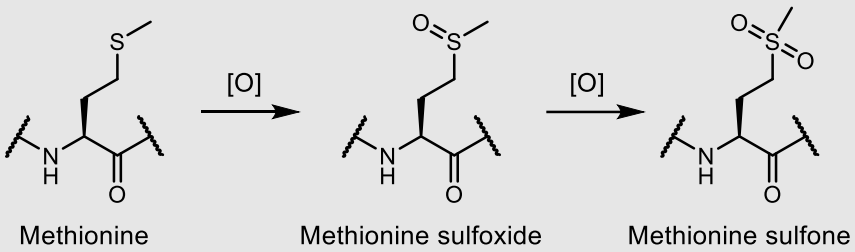
Possible Solutions

- Incorporation of proper Arg side chain protection: *N*ω' NO_2, *N*ω, ω' bis-(Boc), *N*δ,ω-bis(Adoc)
- Short preactivation time
- Low coupling temperature

SPPS Side Reactions

Oxidation Side Reactions

Oxidation Side Reactions - Met



Known methionine oxidants:

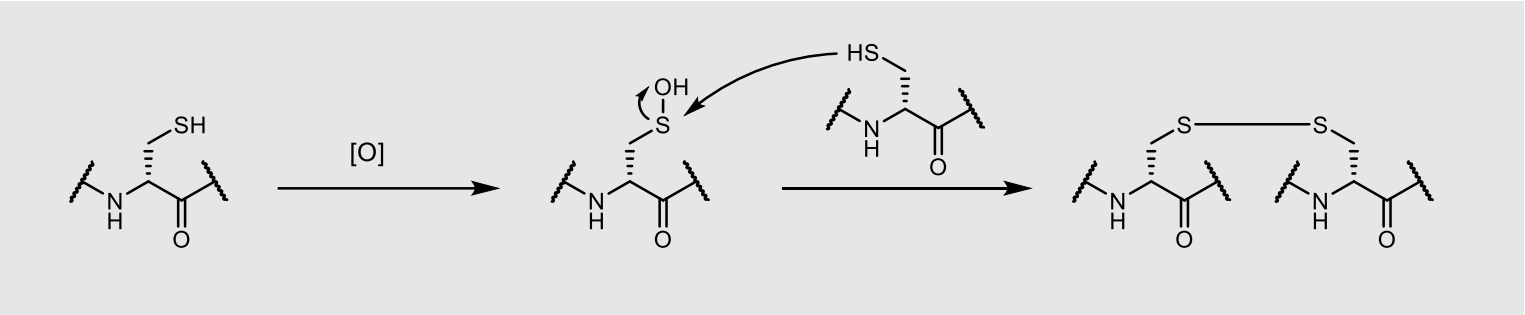
- Air (purification, isolation process)
- NBS, NCS, H_2O_2
- DMSO/HCl

Methods for the reduction of Met[O] to Met

- NH_4I/Me_2S – mild reaction, compatible with disulfide derivatives, Cys(Acm)
- TMSBr/EDT/thioanisole
- Bu4NBr/TFA/thioanisole/anisole/EDT

Taboada, L.; Nicolás, E.; Giralt, E. *Tetrahedron Letters* ***2001, 42, 1891.***

Oxidation Side Reactions - Cys



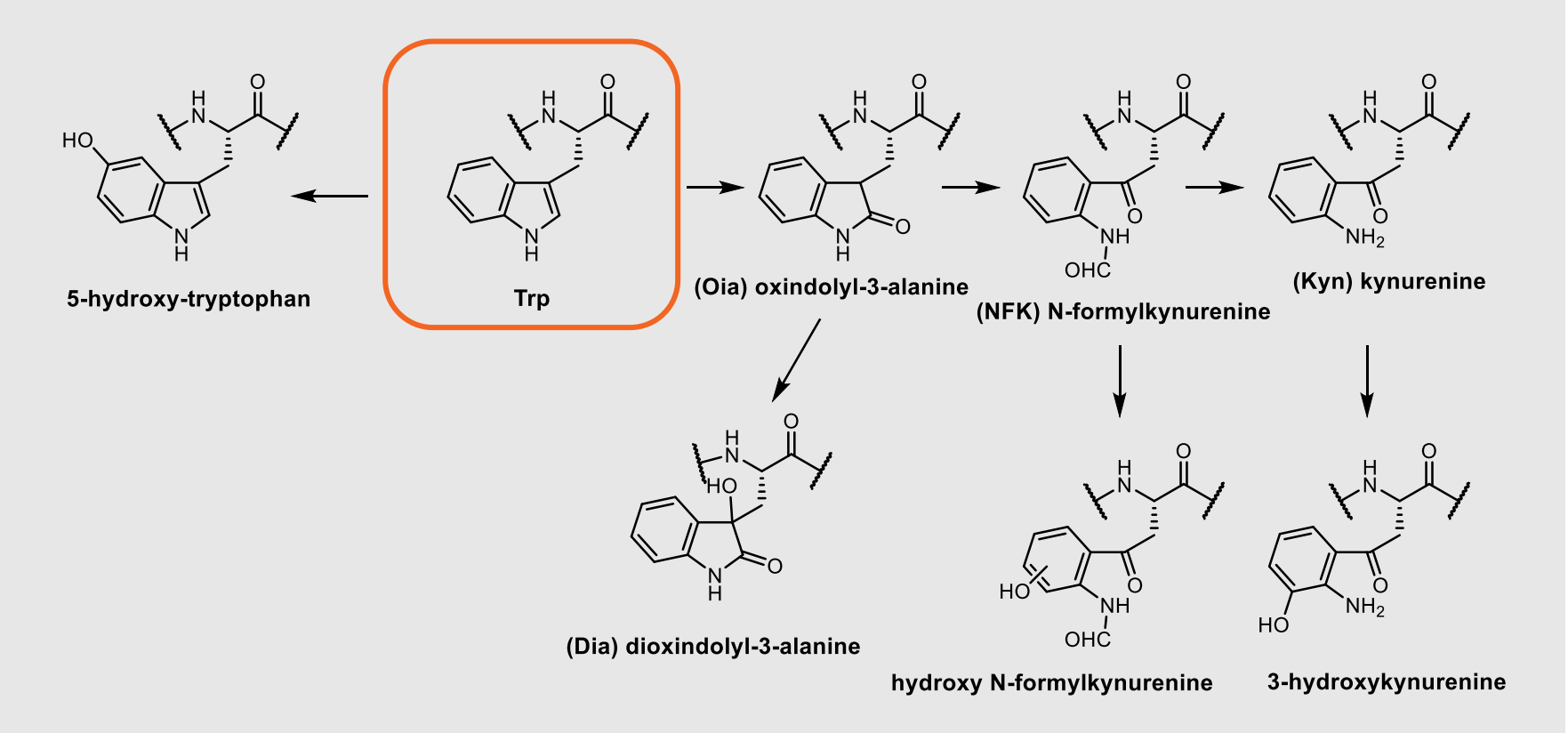
Cysteine to disulfide bonds:

- Air (neutral or higher pH)
- I_2, H_2O_2, $Tl(CF_3COO)_3$
- DMSO

Reducing disulfide bridges

- Dissolve in acidic pH
- Add DTT

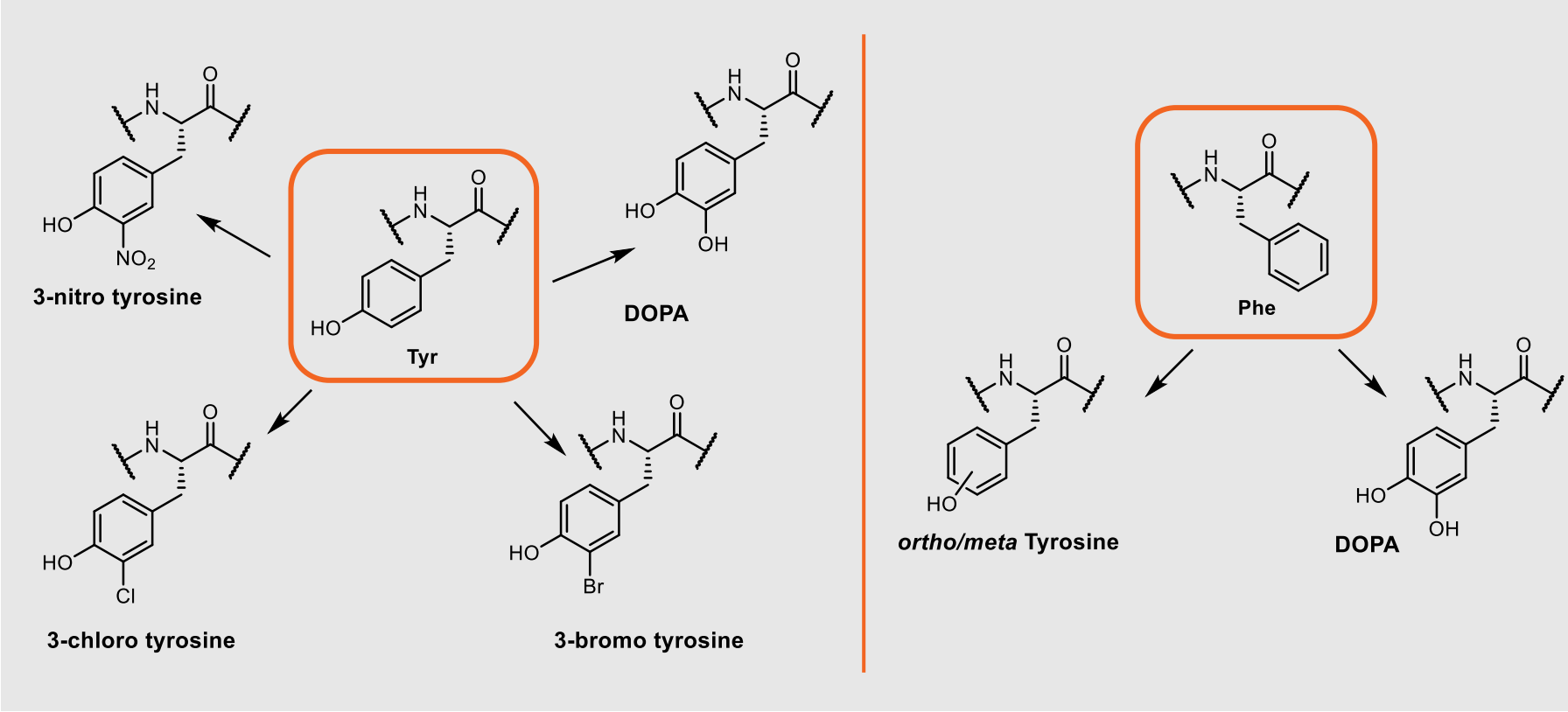
Oxidation Side Reactions - Trp



Common oxidants: H_2O_2, O_2, DMSO/HCl, cleavage process

To suppress: addition of DTT or methylindole during TFA cleavage

Oxidation Side Reactions – Tyr, Phe



- Oxidation by air or radicals

SPPS Side Reactions

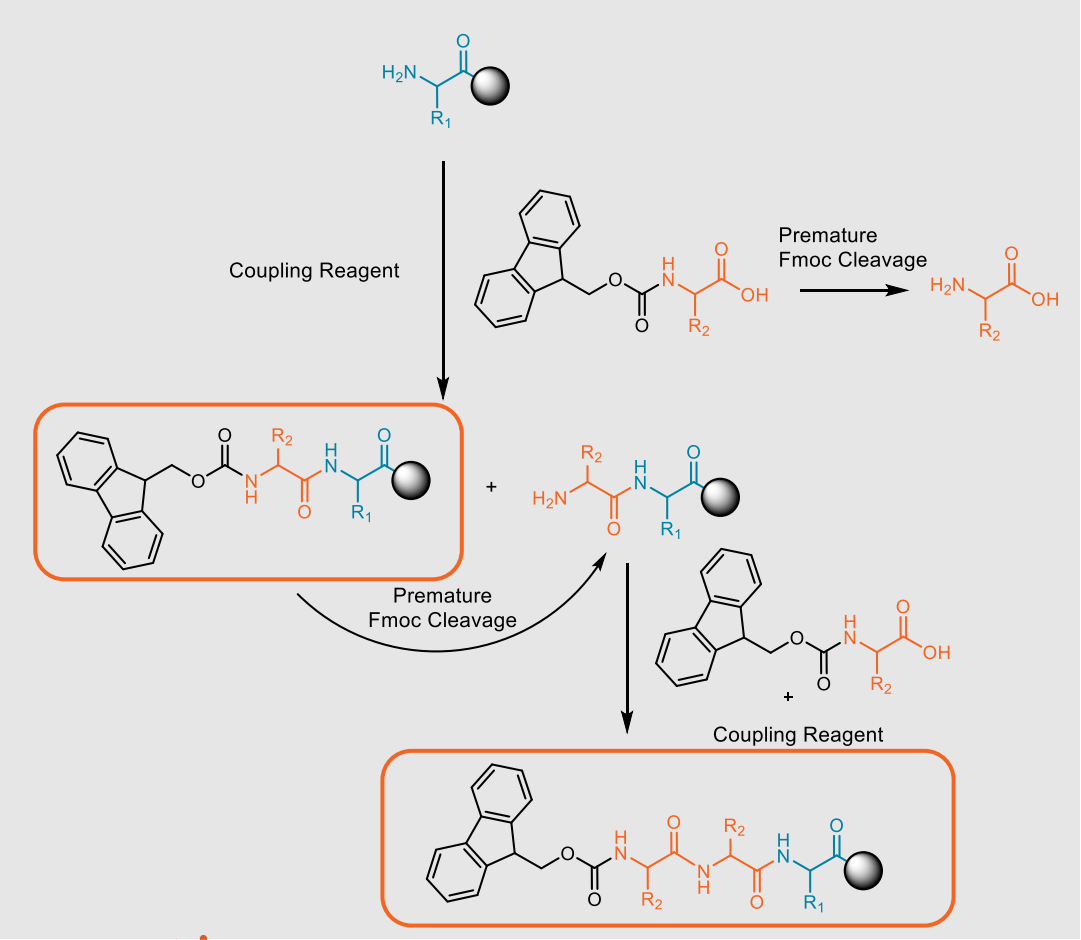
Amino Acids Additions/Insertions

Amino Acid Insertion/Addition

Amino acid additions can occur due to premature Fmoc-deprotection of the Fmoc-AA-OH

What can induce this?

- Lys-N$^{\varepsilon}$
- N$^{\alpha}$-Proline
- DMF/NMP degradation
- DMAP/DIEA
- Residual Pip/
  Insufficient washes



Lys-Nε-induced Fmoc premature deprotection

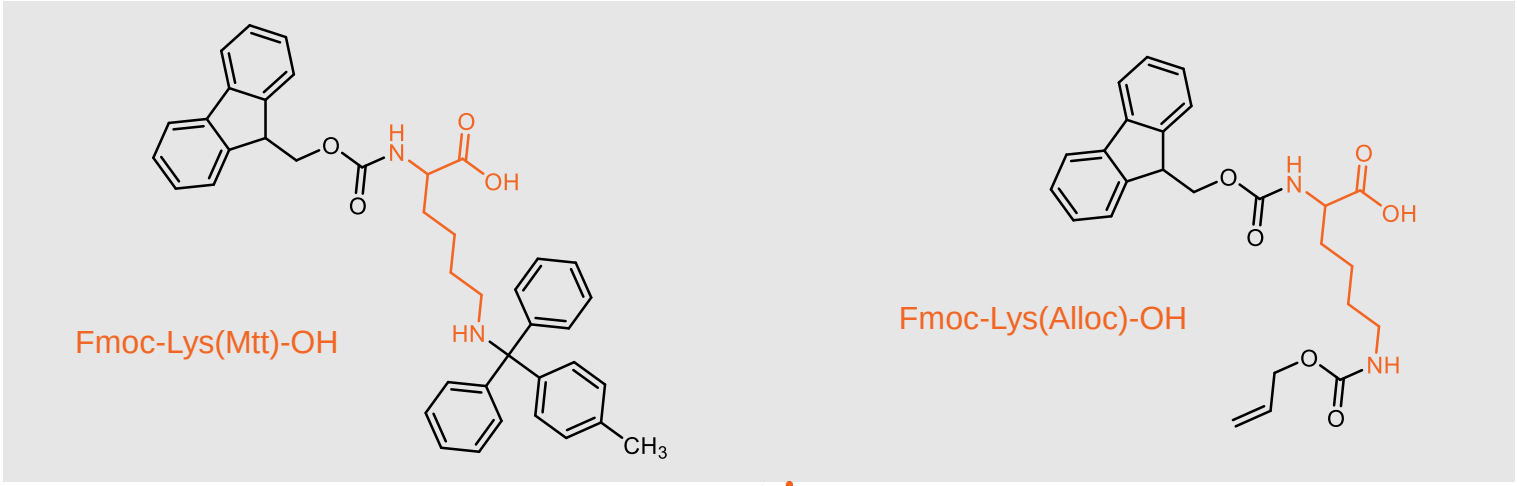
Nα-Fmoc can be removed during the selective deprotection of orthogonal Lys side chain:

Lys(Mtt)

- Skip DIPEA/DCM neutralization step
- Neutralization done with base in next coupling step in presence of reagent like PyAOP

Lys(Alloc)

- Limit the reaction time to reduce exposure of liberated amino group, reducing potential Fmoc deprotection



Lys-Nε-induced Fmoc premature deprotection Synthesis of Cyclic MTT

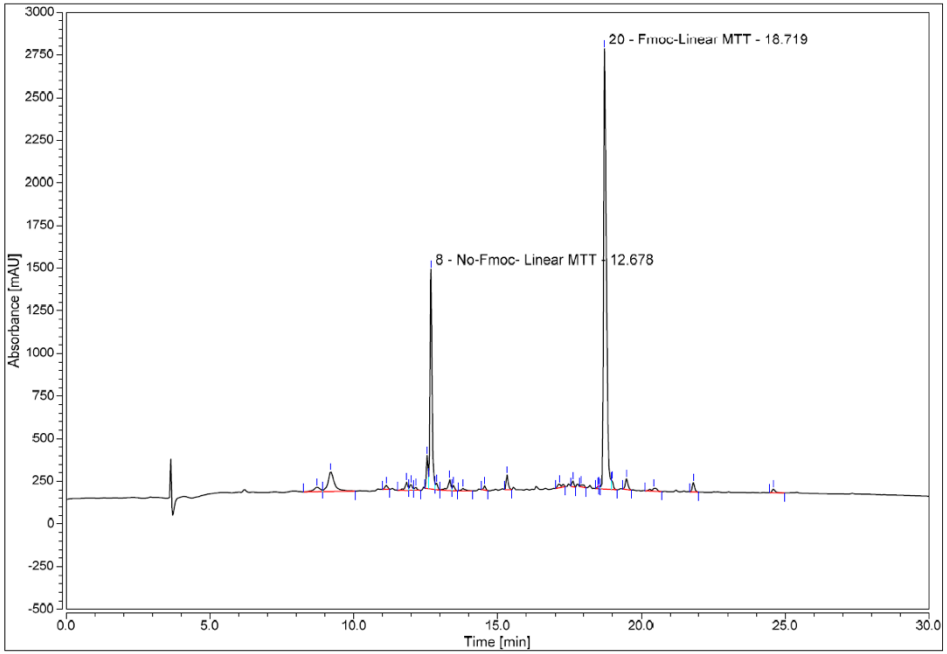
MTT

Ac-Nle-cyclo[Asp-His-D-Phe-Arg-Trp-Lys]-NH_2

Linear MTT up to Asp

Side chain deprotections/ cyclization

Chain elongation



Chemistry

Resin

Rink Amide MBHA LL

Deprotections

2 x 2 min

Coupling

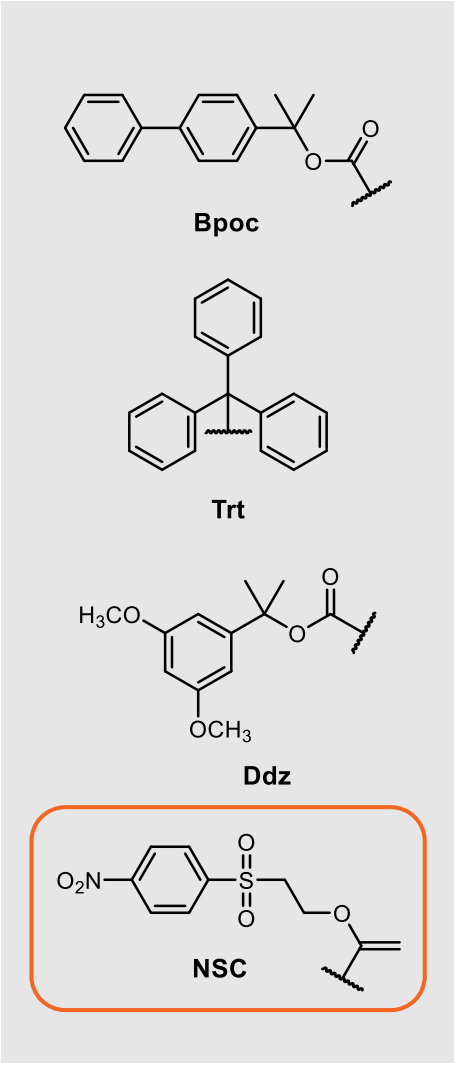
30 min COMU/DIPEA

Intermediate

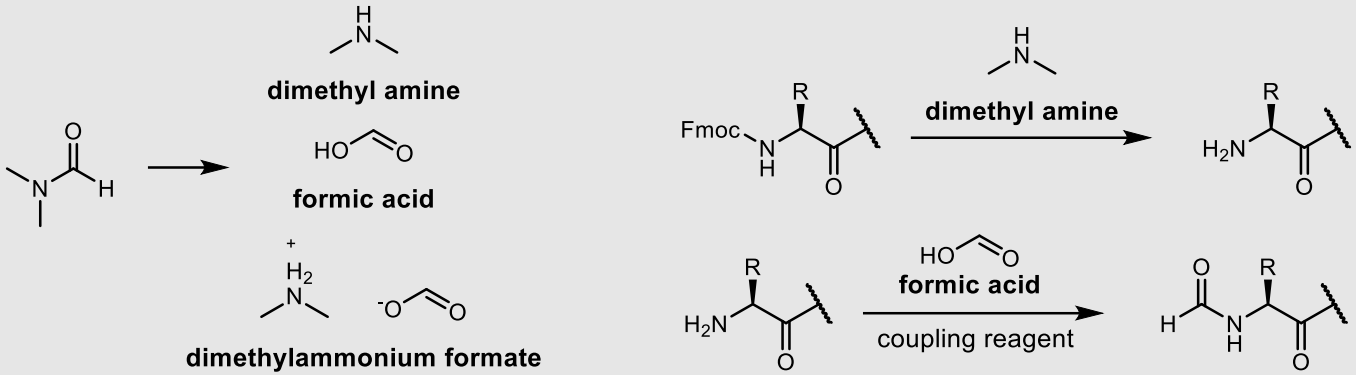
Fmoc-Asp(OAll)-His-D-Phe-Arg-Trp-Lys(Alloc)-NH_2

Nα-Proline induced Fmoc deprotection

- Proline and its derivatives are significant contributors to premature Fmoc deprotection
- Proline **pKa 10.6**, piperidine **pKa 11.2**
- Fmoc deprotection via β-elimination mechanism – similar to piperidine
- Alternative goups for Nα protection have been developed: **Bpoc, Trt, or Ddz**
- **NSC** most promising: stable to base 3-10x higher than Fmoc, less susceptible to premature deprotection
- Adjusting the reaction's acidity is another option
  - Adding OxymaPure



DMF INDUCED PREMATURE FMOC DEPROTECTION /PEPTIDE FORMYLATION



Degradation of DMF/NMP release diverse byproducts that can interfere with SPPS

- Heat, increased H_2O content, UV – increase degradation
- Residual starting material of solvent production

Dimethylamine/methylamine from degradation or from starting material lead to deprotection

Control quality of solvents

- Vacuum degassing
- N2 gas flow bubbling
- Aluminum oxide treatment

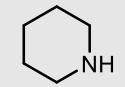
Solvent induced premature Fmoc deprotection

Residual piperidine from insufficient washings

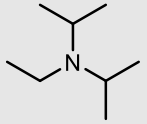
- Include extensive washes
- Chloranil test – to look for piperidine presence in washes

Tertiary amines like DMAP and DIPEA, particularly in fragment condensation reactions or hindered couplings

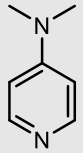
- Evaluate if these are necessary for reaction



piperidine



DIPEA



DMAP

SPPS Side Reactions

Amino Acids Deletions

Peptide Fragmentation/Deletions

Decreased solubility of growing peptide chain

- Double couplings
- Resin selection

Secondary Structure - β-sheet formation during synthesis

- Use pseudoproline dipeptides
- Special N-protecting groups that can be removed during cleavage
- Addition of DBU to the piperidine deprotection solution

DKP - formation

TFA mediated fragmentation

Acidolysis

- -Asp-Pro- bond
- H-His-Pro-Xaa
- N-terminal FITC
- Thioamide peptide
- N-Ac-N-alkyl-Xaa motif
- C-terminal N-Me-Xaa

Autodegradation

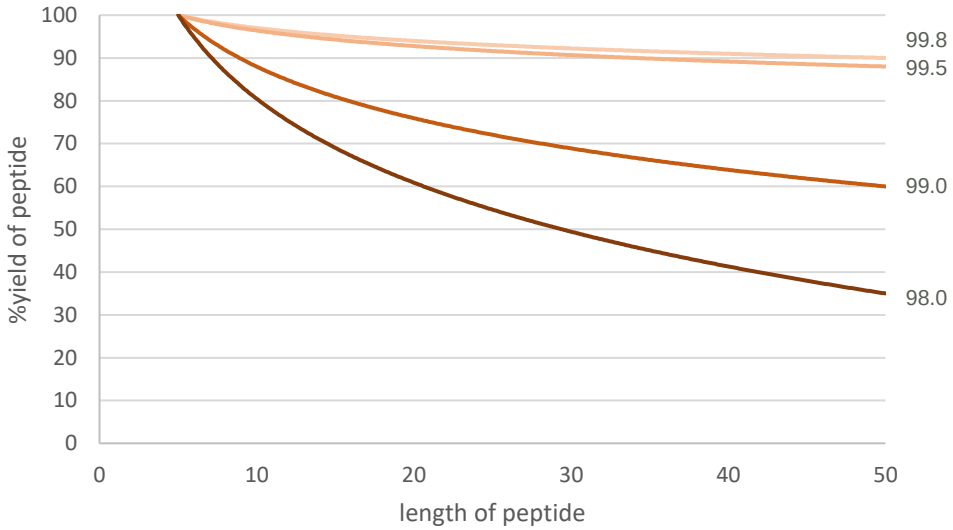
- -N-acyl-N-alkyl-Aib-Xaa bond

SPPS Side Reactions

Amino Acids Impurities

Amino Acid Impurities

Impurities present in SPPS building blocks have a significant effect on peptide step-wise yield.



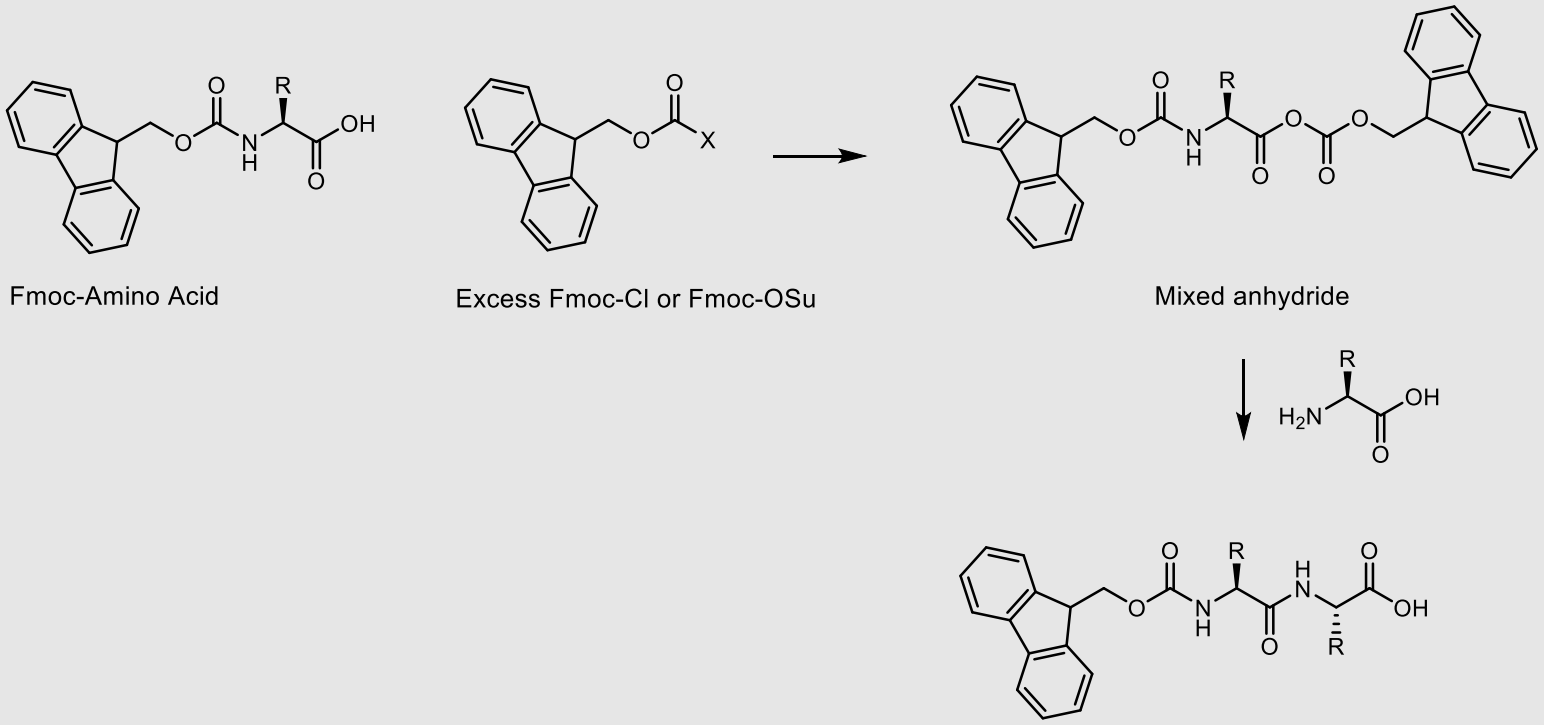
Synthesis of Fmoc-amino acids produces predictable amino-acid related impurities which can negatively affect the outcome of peptide synthesis.

Novabiochem® Enhanced specification Fmoc-amino acids 2014

Amino Acid Impurities

Dipeptides

- These are formed when excess Fmoc-OSu or Fmoc-Cl react with the already formed Fmoc-amino acid.
- Leads to double insertion of the target amino acid

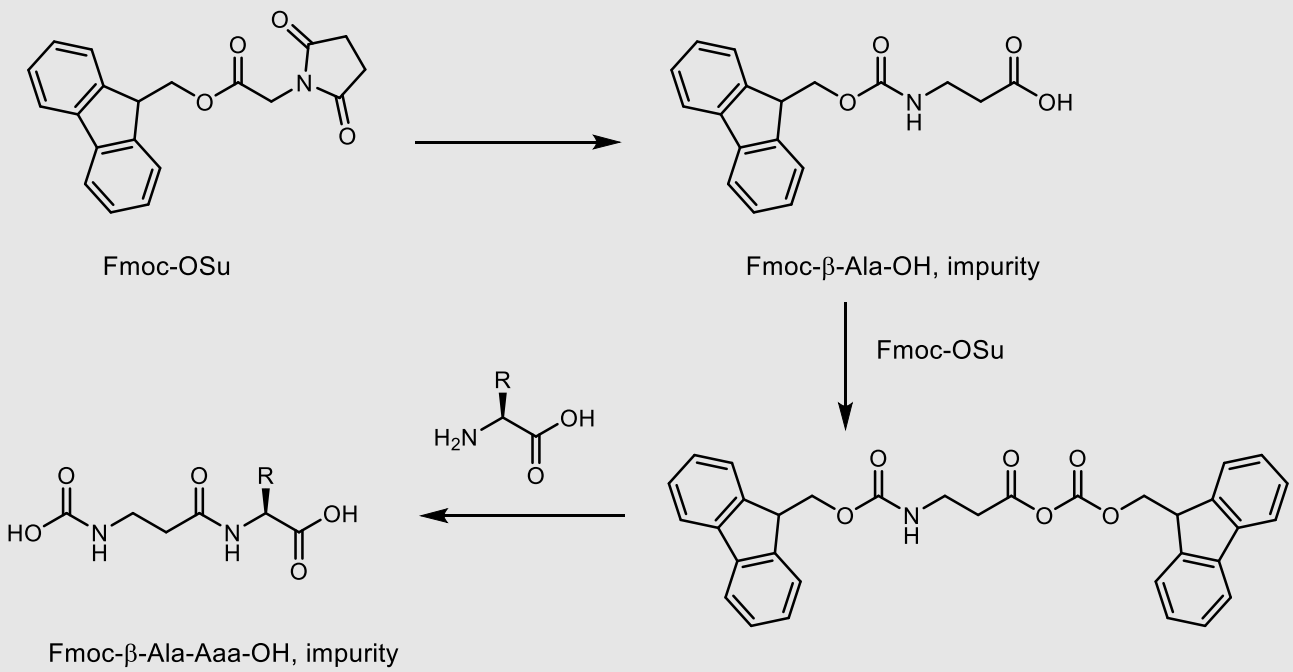


Novabiochem® Enhanced specification Fmoc-amino acids 2014

Amino Acid Impurities

β-Alanyl Impurities

- Formed via ring opening of Fmoc-OSu
- Leads to insertion of β-alanine instead of target amino acid into the peptide.

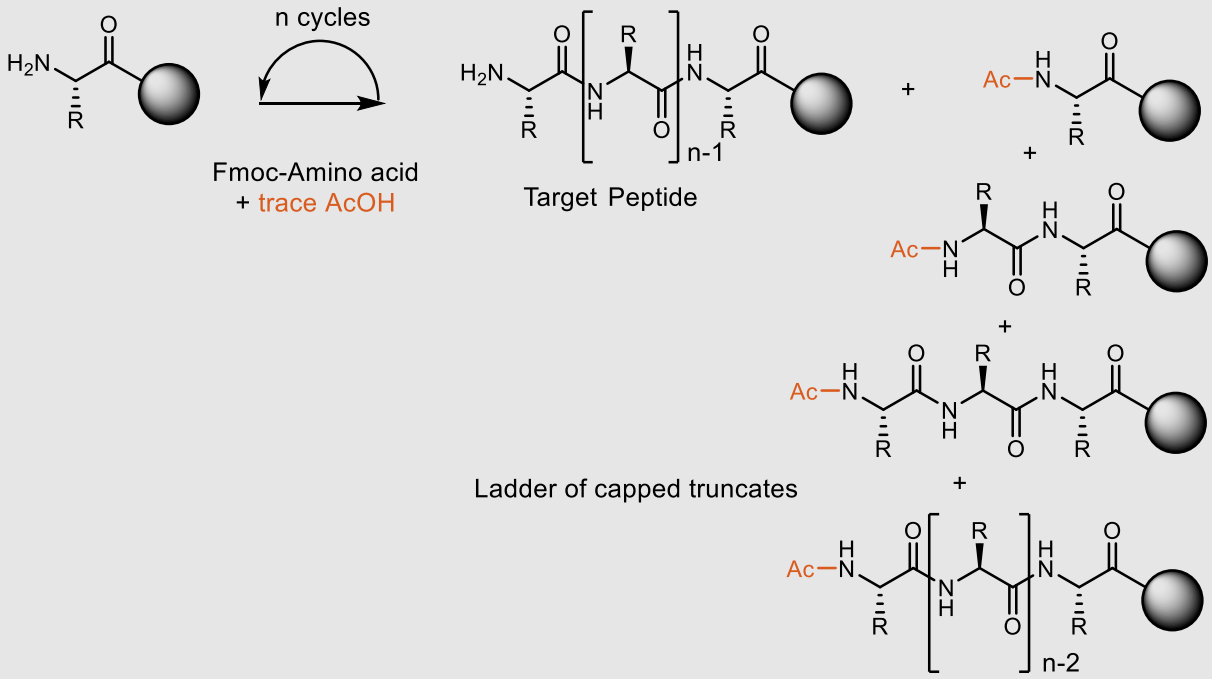


Novabiochem® Enhanced specification Fmoc-amino acids 2014

Amino Acid Impurities

Acetic Acid

– AcOH contamination of Fmoc-amino acids results in truncation of the peptide chain during SPPS.

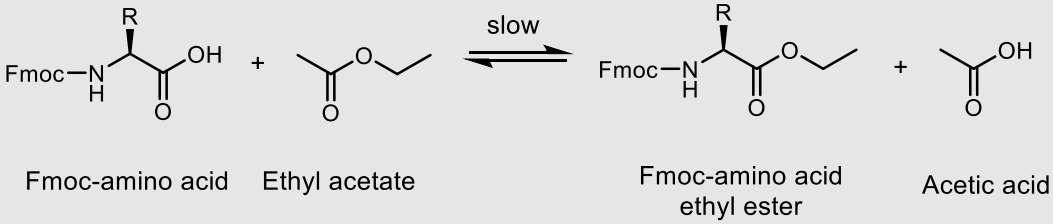


Novabiochem® Enhanced specification Fmoc-amino acids 2014

Amino Acid Impurities

Acetic Acid

- Originates from ethyl acetate used in preparation and crystallization of Fmoc-amino acids. Hydrolysis of this solvent forms acetic acid.
- Residual ethyl acetate can also trans-esterify with the solid Fmoc-amino acid resulting in acetic acid formation on storage.



- Specification of Fmoc-amino acids is important!
- Look for both acetate and ethyl acetate spec of Certificates of Analysis

Garbage in = Garbage out!

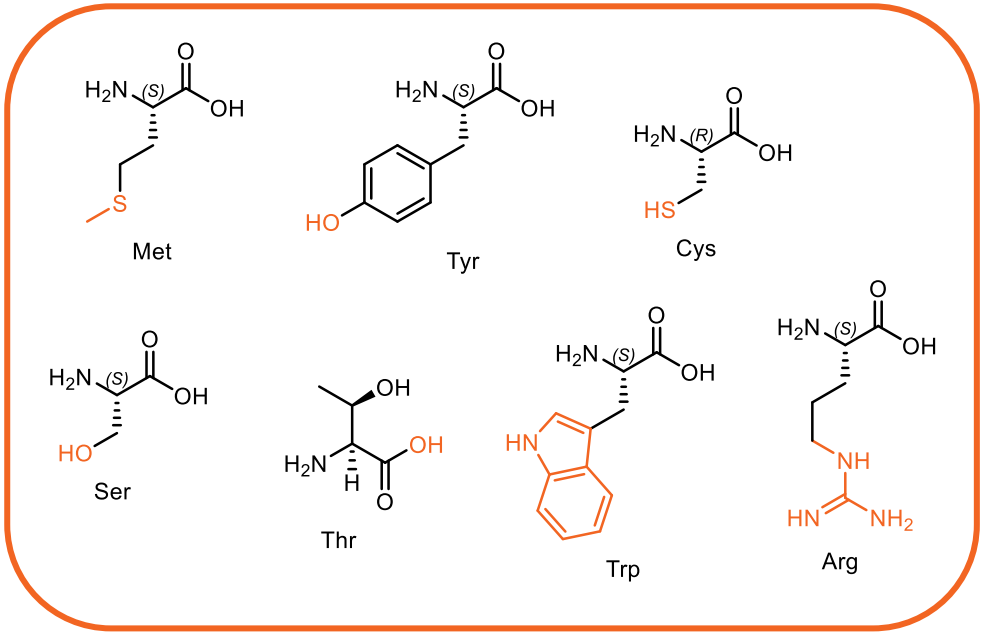
Novabiochem® Enhanced specification Fmoc-amino acids 2014

SPPS Side Reactions

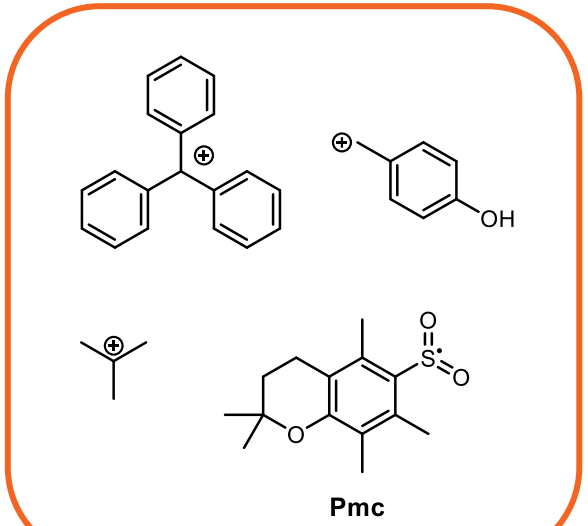
Cleavage Side Reactions

CLEAVAGE SIDE REACTIONS

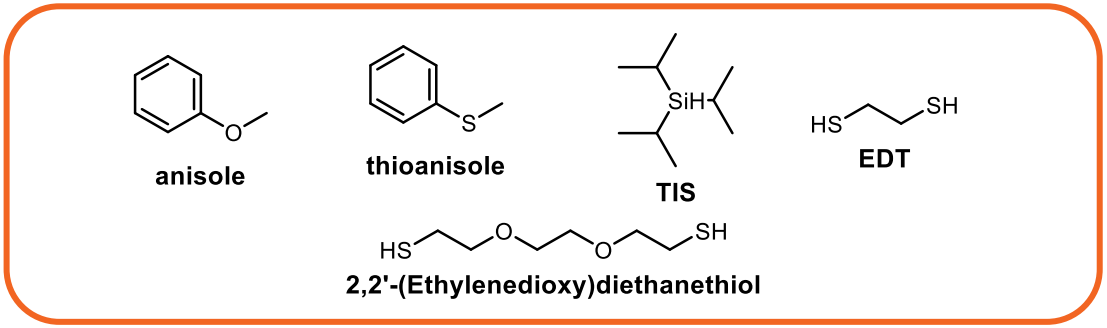
Sensitive Amino Acids



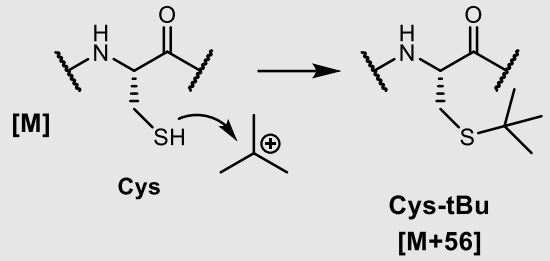
Carbocations

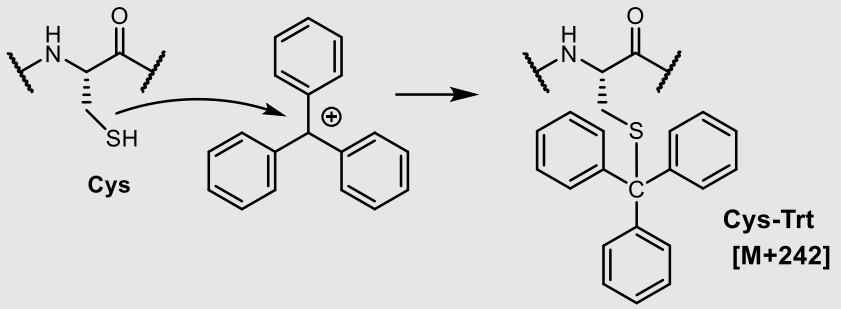


Scavengers

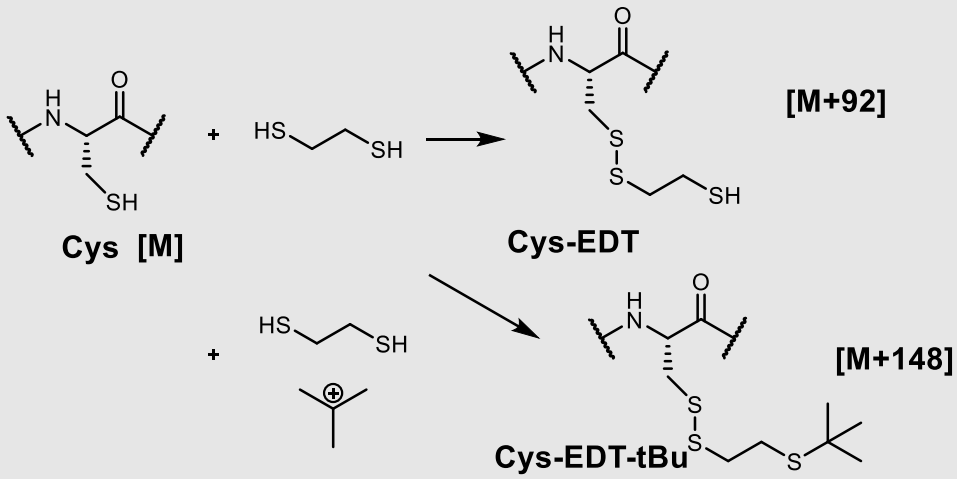


Cys Alkylation



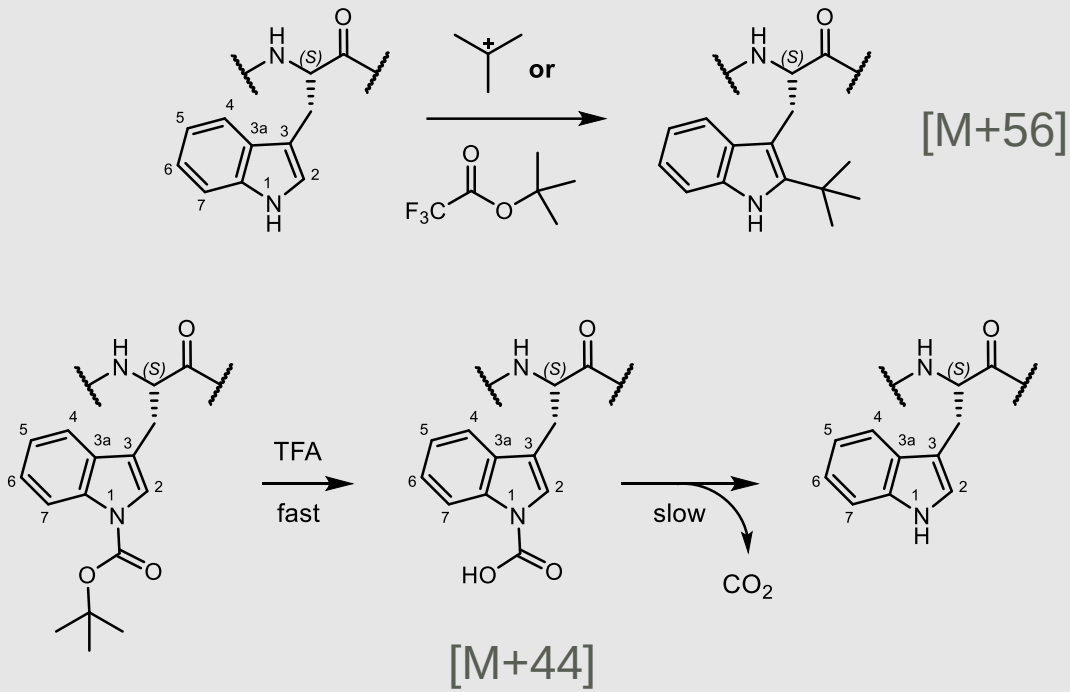


- Not properly scavenged *tert*-butyl or trityl cations come from Boc,tBu and Trt protections and will alkylate SH of Cys
- Use EDT for tBu and TES or TIS for Trt
- In case of EDT adduct, alternative scavengers like DODT, DTT could be used. Treatment with TCEP will regenerate peptide to target product



Singh, P. R.; Rajopadhye, M.; Clark, S. L.; Williams, N. E. *Tetrahedron Letters* ***1996, 37, 4117.***

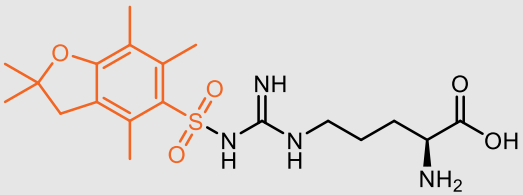
TERT-BUTYLATION OF TRP

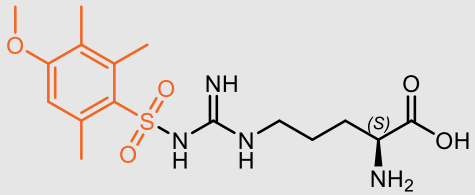


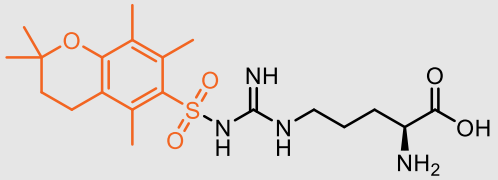
- tBu cations come from different amino acids protecting groups (Ser, Thr, Tyr, Asp or Glu) or from decomposition of Boc group
- **Solution:** protect indole wiht Boc group, in this case stepwise deprotection

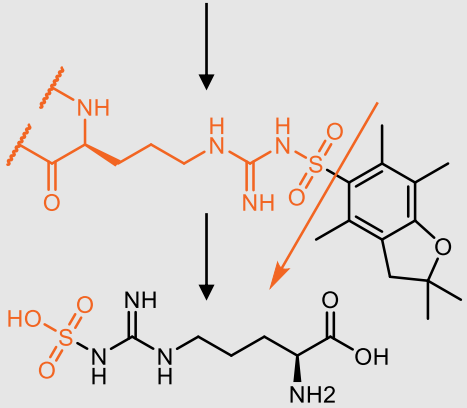
Arginine Deprotection/Peptide Sulfonation

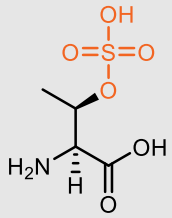
- Residual Pbf group on peptide [M+252u]
- Undesired sulfonation of Trp, Tyr, Ser/Thr, Arg coming from the arginine guanidine protecting groups during cleavage

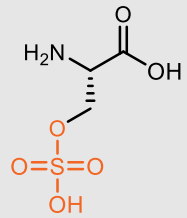


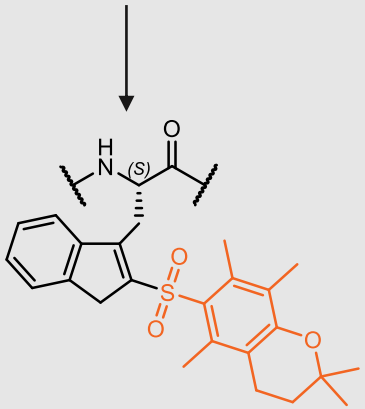












GYROS PROTEIN TECHNOLOGIES

SPPS TIPS FOR SUCCESS: OPTIMIZING SYNTHESIS OF SARS-COV-2 EPITOPES FOR GMP MANUFACTURE

Cyf Ramos Colón, Ph.D.
Senior Scientist

GYROS PROTEIN Technologies

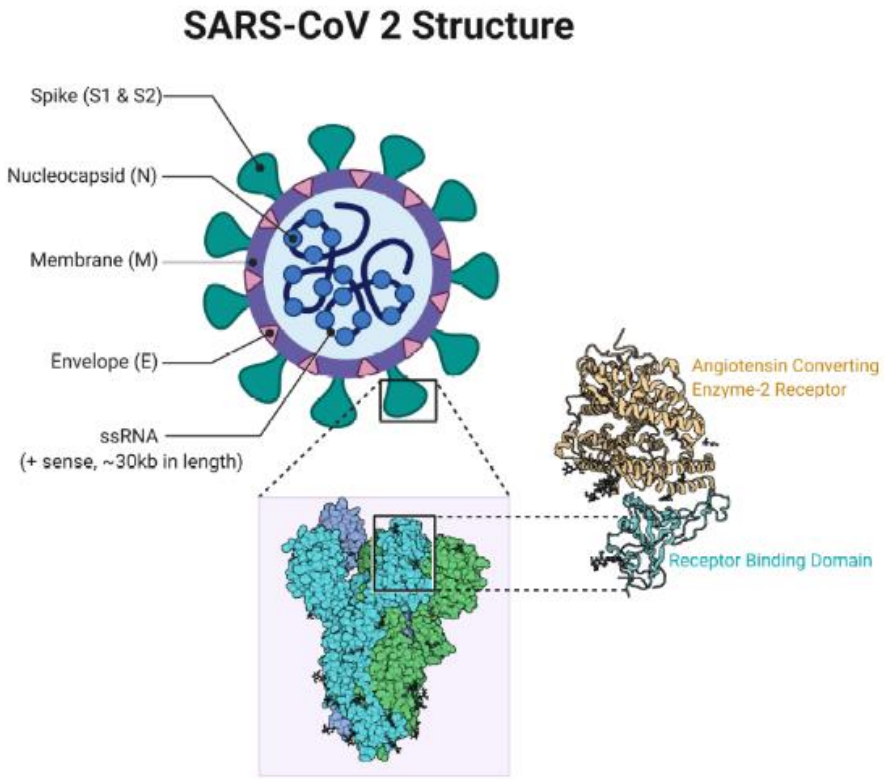
Overview

- Optimizing Syntheses for GMP Manufacture
  - SARS-COV-2 Epitope Synthesis
  - GLP-1 Receptor Agonists
  - Neoantigen Synthesis
  - Instrument features for GMP
  - 21CFR Part 11 Compliance
  - IQ/OQ/PQ Documentation
  - Reference Materials

SARS-COV-2 EPITOPE SYNTHESIS

COVID-19 AND PEPTIDES

- SARS-CoV-2 infection binds to the human angiotensin-converting enzyme 2 (ACE2) receptor via its spike protein
  - Can we target this PPI with peptides?
- Recent studies have shown peptides can reduce infectivity of SARS-CoV in cells
- ACE2 peptide fragment has also been shown to bind with high affinity to the S receptor binding domain of SARS-CoV-2



Zheng B-J et al. Antiviral Therapy 10 393-403. 2005
Zhang G. et al. bioRxiv preprint
An experimental peptide could block Covid-19. MIT News

EPITOPES SYNTHESIZED

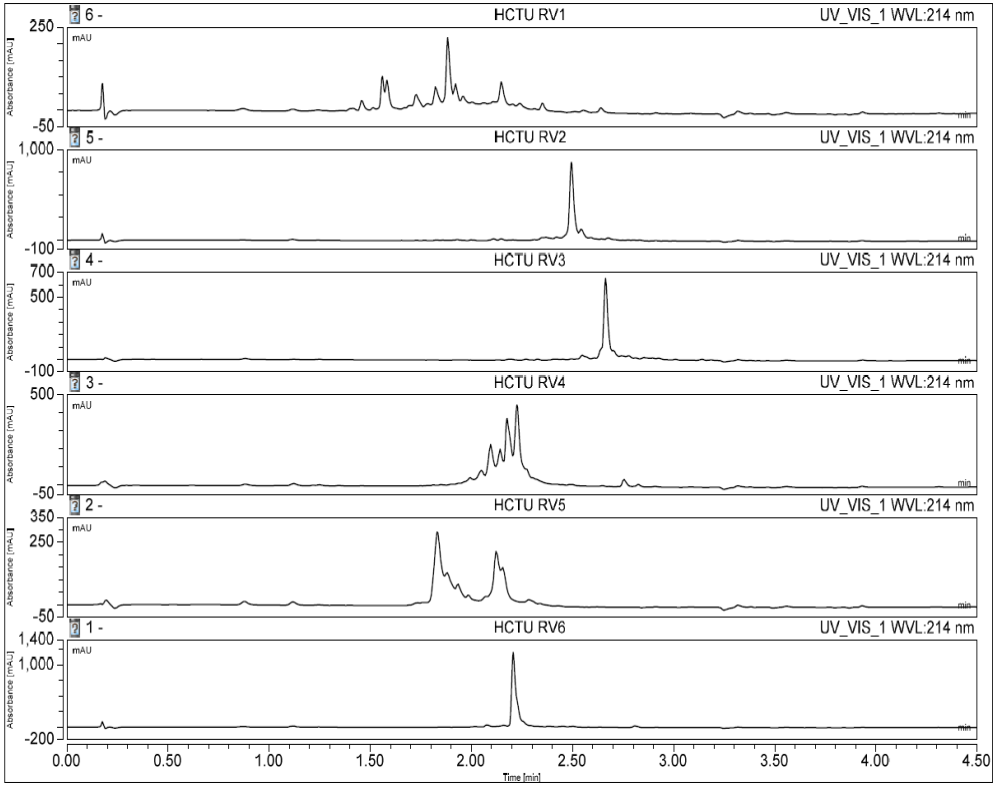
PV	Sequences	RV	Sequences
PV1	EVPVAIHADQLTPTWRVYSTGS	**RV1**	DAVDCALDPLSETKCTLKSFTVEKGIYQTSN
PV2	FSQILPDPSKPSKRSFIE	**RV2**	FGAGAALQIPFAMQMAYRFNGI
PV3	PLLESELVIGAVILRGHLRI	**RV3**	MADSNGTITVEELKKLLEQWNLVI
PV4	NNNAATVLQLPQGTTLPKGF	**RV4**	RPQGLPNNTASWFTALTQHGK
PV5	IRGWIFGTTLDSKTQSLL	**RV5**	NKHIDAYKTFPPTEPKKDKKKKTDEAQPLPQRQKKQPTVTLLPADM
PV6	QPFLMDLEGKQGN	**RV6**	CTFEYVSQPFLMD
PV7	KSFTVEKGIYQTSNFRVQ	**RV7**	TRFQTLLALHRSYLTPGDSSSGW
PV8	KLPDDFTGCV	**RV8**	SASFSTFKCYGVSPTKL
PV9	YLYRLFRKSNLKPFERDI	**RV9**	NLDSKVGGNYNYLYRLFR
PV10	KPFERDISTEIYQ	**RV10**	QSIIAYTMSLGAENSVAY
PV11	SIIAYTMSL	**RV11**	TECSNLLLQYGSFCTQL
PV12	DSLSSTASALGKLQDVV	**RV12**	VKQIYKTPPIKDFGGFNF

Epitope Synthesis – Run 1

- First pass at synthesizing a library of peptides using the Symphony X

Chemistry

- Deprotections - 2 x 1 min
- Coupling
  - 1 x 5 min @ 25°C
- Coupling reagents – 6-fold excess HCTU with DIPEA
- Capping – 5 min with 1:1:3 of Ac2O/Pyridine/DMF

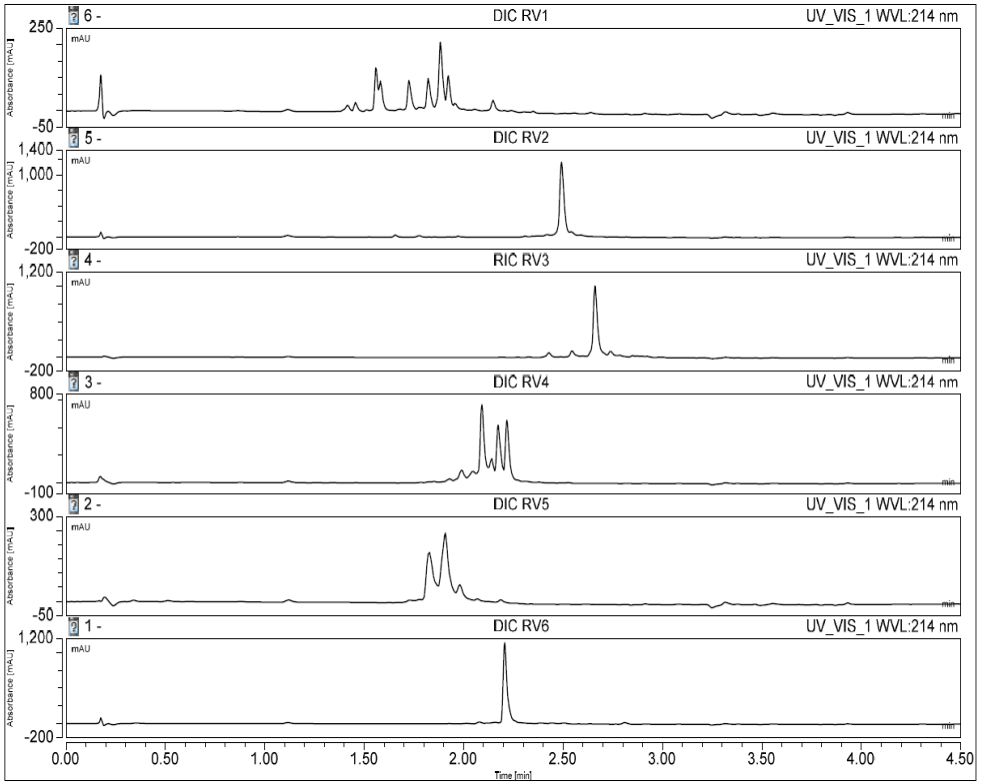


Epitope Synthesis – Run 2

- Synthesis optimization of epitope library using the Symphony X

Chemistry

- Deprotections - 2 x 1 min
- Coupling
  - 1 x 30 min @ 25°C
- Coupling reagents – 6-fold excess DIC and OxymaPure
- Capping – 5 min with 1:1:3 of Ac2O/Pyridine/DMF

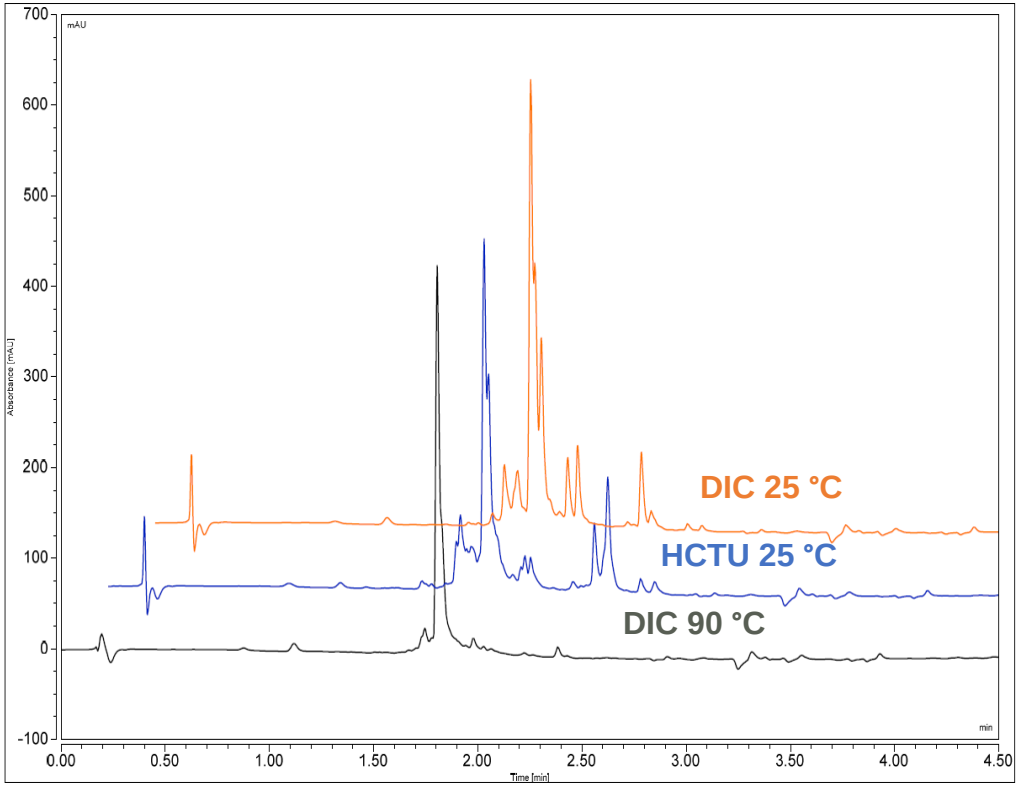


Epitope Synthesis – Synthesis Optimization with Heat

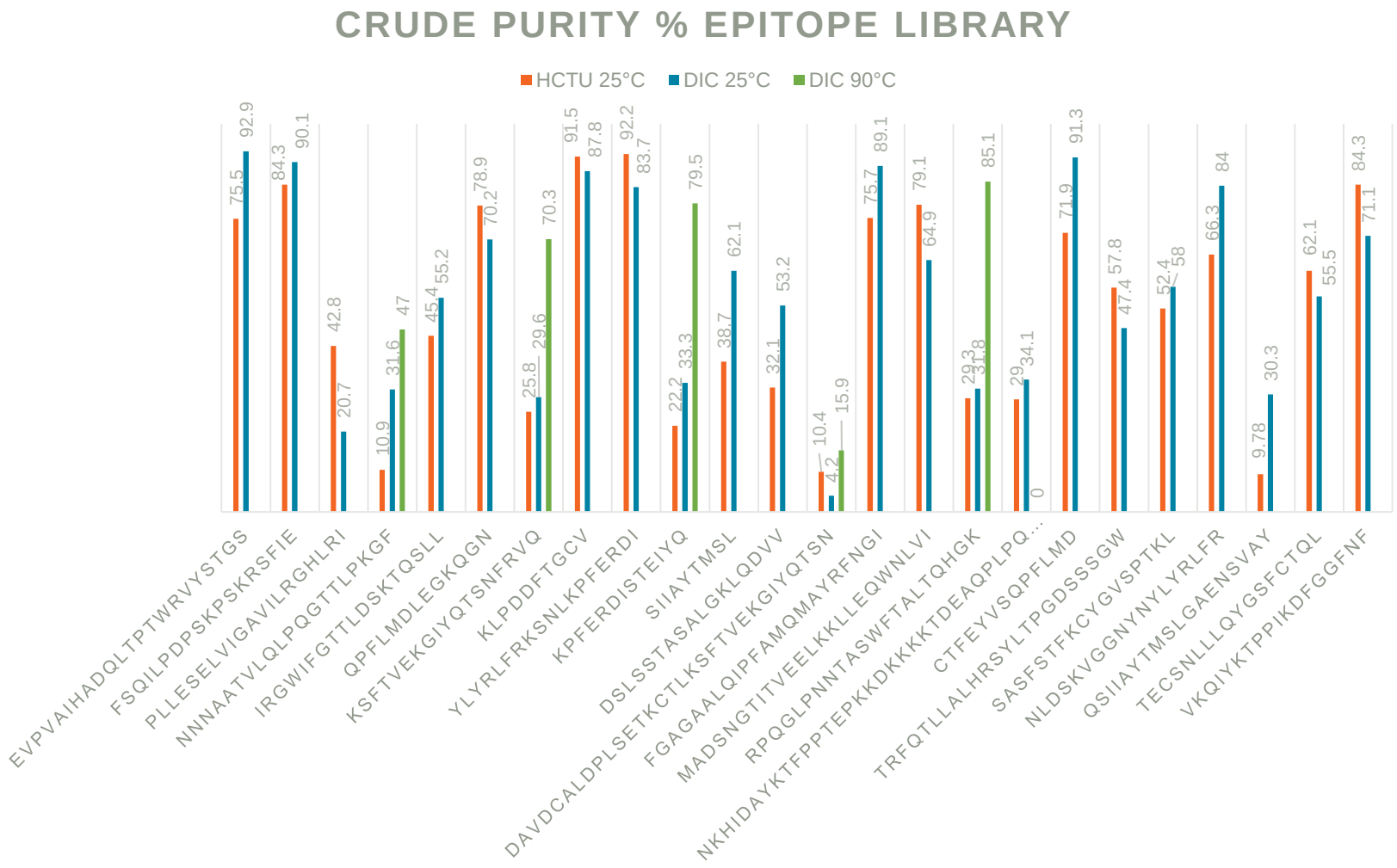
- Synthesis optimization of epitope library using heat on PurePep® Chorus

Chemistry

- Deprotections - 2 x 1 min
- Coupling
  - 1 x 3 min @ 90°C
- Coupling reagents – 6-fold excess DIC and OxymaPure
- Capping – 5 min with 1:1:3 of Ac2O/Pyridine/DMF



SUMMARY OF RESULTS



GLP-1 AGONIST SYNTHESIS

Synthesis of GLP-1 Agonists: Parallel condition screening – Resin, Coupling Reagent

- GLP-1 receptor agonists are an important treatment for the management of type 2 diabetes
- Some published patents describe optimization by fragment synthesis and solution phase condensation to reduce impurities and maximize yields

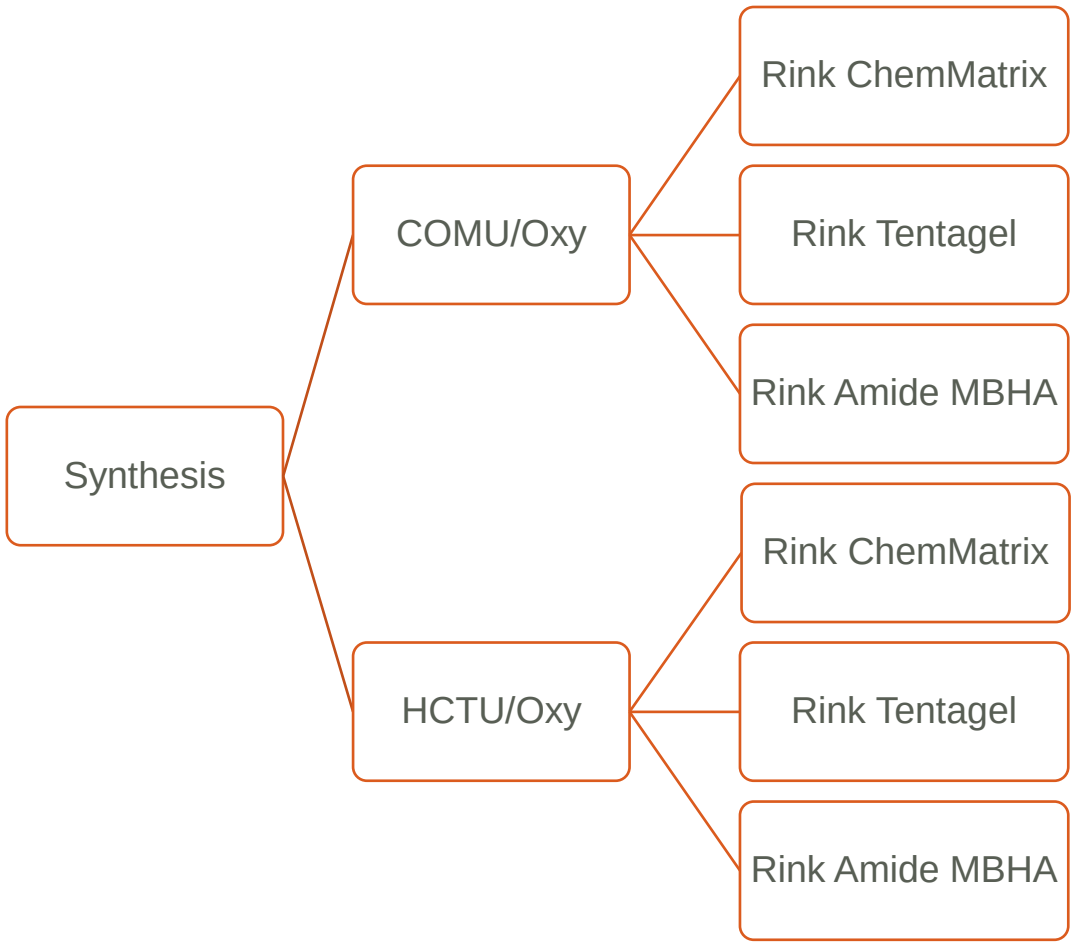
Pramlintide

H-KCNTATCATQRLANFLVHSSNNFGPILPPTNVGSNTY-NH_2

Lixisenatide

H-HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPSKKKKKK-NH_2

Synthesis of GLP-1 Agonists: Parallel condition screening – Resin, Coupling Reagent



Chemistry

- Deprotections – 2 x 30 s
- Coupling reaction – 2 x 1 min

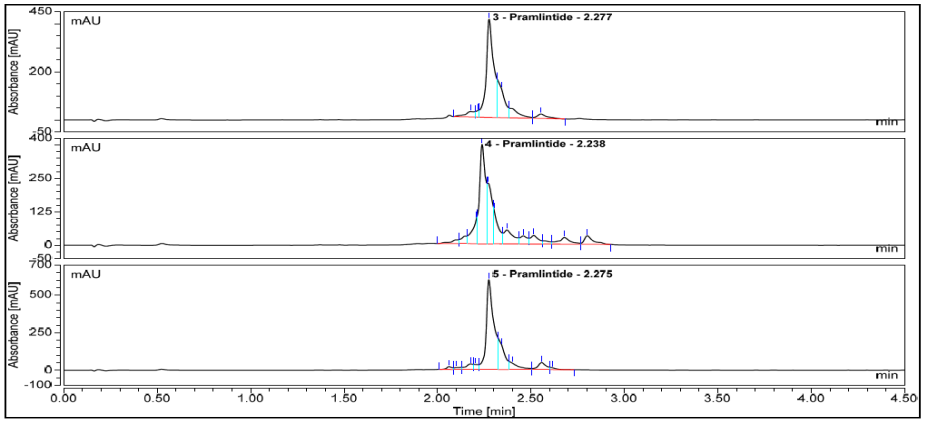
Synthesis of Pramlintide (GLP-1 Agonist): Parallel condition screening – Resin, Coupling Reagent

Pramlintide

H-KCNTATCATQRLANFLVHSSNNFGPILPPTNVGSNTY-NH_2

- Multi-variable conditions were successfully tested in parallel for the high-throughput optimization of GLP-1 receptor agonists
- Highest purity with HCTU and ChemMatrix or PS resin

Resins	% Purity	
	HCTU	COMU
R Ram Tentagel	35.1	43.0
Rink ChemMatrix	62.7	47.0
Rink MBHA Low Loaded	62.3	42.2



Crude purity profiles of Pramlintide synthesized with HCTU on Rink ChemMatrix, Rink Tentagel resins and Rink Amide MHBA resin.

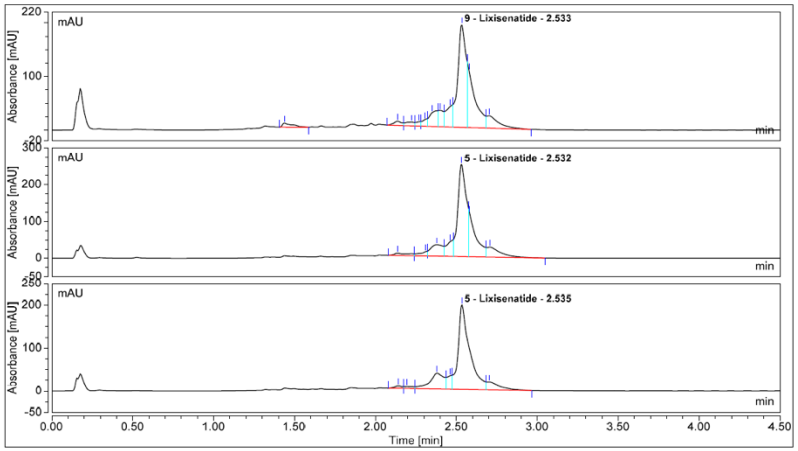
Synthesis of Lixisenatide (GLP-1 Agonist): Parallel condition screening – Resin, Coupling Reagent

Lixisenatide

H-HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPSKKKKKK-NH_2

- Multi-variable conditions were successfully tested in parallel for the high-throughput optimization of GLP-1 receptor agonists
- Highest purity with COMU and ChemMatrix or PS resin

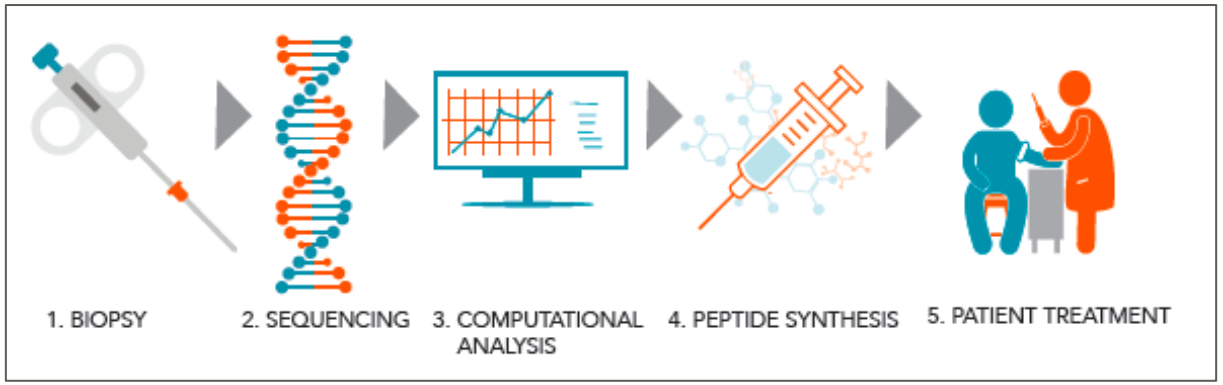
Resins	% Purity	
	HCTU	COMU
R Ram Tentagel	54.7	49.5
Rink ChemMatrix	56.4	68.2
Rink MBHA Low Loaded	45.1	41.0



Crude purity profiles of Lixisenatide synthesized with COMU on Rink ChemMatrix, Rink Tentagel resins and Rink Amide MHBA resin.

NEOANTIGEN SYNTHESIS

Neoantigen Peptides



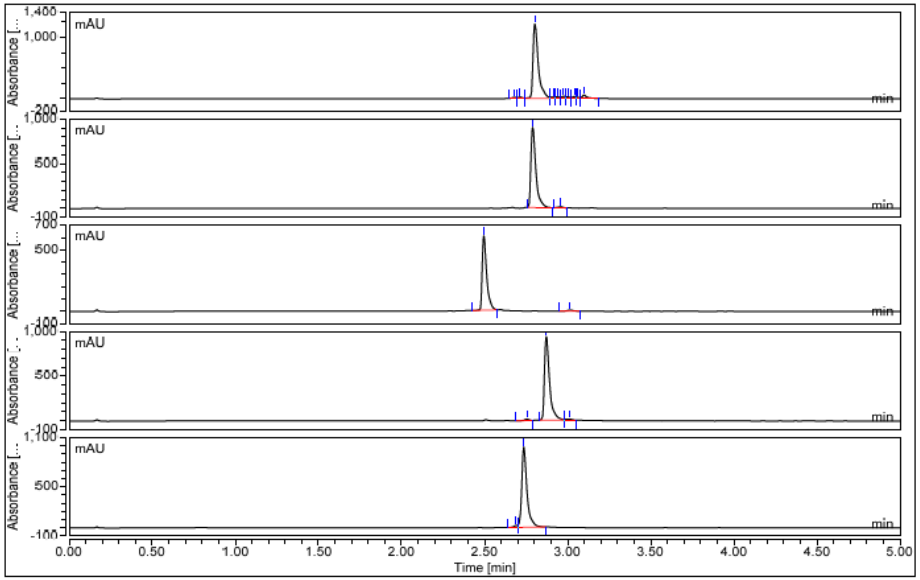
- Neoantigens can be present in a type of tumor but can also be specific to an individual patient's tumor
- Some cancers, such as melanoma, result in more mutations than others, making the production of neoantigens more likely
- Speed is crucial!
  - Trying to hit a moving target – Fast parallel synthesis can be the solution = high purity peptides in reduced amount of time

NEOANTIGEN PEPTIDES

- Fast synthesis in high purity of mutated kinesin family member 2C (KIF2C) antigen peptides for metastatic melanoma cell therapy development

CHEMISTRY

- DEPROTECTION - 2 x 2 min
- COUPLING - 3 min @ 75°C
- COUPLING REAGENTS – 6-fold excess HCTU with DIPEA



Sequence	Purity (%)
RLFPGLTIKI	86.6
RLFPGLTI	98.6
LTIKIQRSNGL	98.1
LQARLFPGLTI	96.9
LQARLFPGLT	98.3

Lu, Y et al. Clin Cancer Res. 2014 July 1; 20(13)

GMP Manufacture

cGMP Considerations

Gyros Protein Technologies provides:

- Training by qualified personnel
- IQOQ documentation
- Software designed for 21 CFR Part 11 compliance.

In combination with a comprehensive quality management system, GPT Instrument users can meet and exceed regulatory standards

Instrument Features - Hardware

- Enabling GMP manufacture:
  - Resin integrity – The resin for each synthesis is contained in a single RV and is not moved or transferred to any part of the system. This feature eliminates any risk of product cross contamination.
  - Valve block – The Ultra PurePep® Pathway on GPT's instruments ensures a dedicated reagent delivery line for each RV, with no reagent cross-over and no dead volumes.
  - Line clearances – Lines are automatically flushed with primary solvent and nitrogen after each delivery to ensure fluid channels are rinsed and dried before the next addition.

21CFR PART 11 COMPLIANCE - SOFTWARE

- GPT instruments in cGMP facilities are used for production of peptides required in clinical studies, neoantigen trials, and cosmetic formulations
- For instrumentation in these environments, the United States Food and Drug Administration (FDA) and European Medicines Agency (EMA) require certain controls and documentation for software involved in the processing of electronic data. Title 21 CFR Part 11 is the part of the Code of Federal Regulations that establishes FDA regulations on electronic records and electronic signatures, and Annex 11 is the European equivalent
- To help address these requirements, GPT software includes features related to:
  - User Management
  - Audit Trail
  - Data Integrity
  - Electronic Signatures

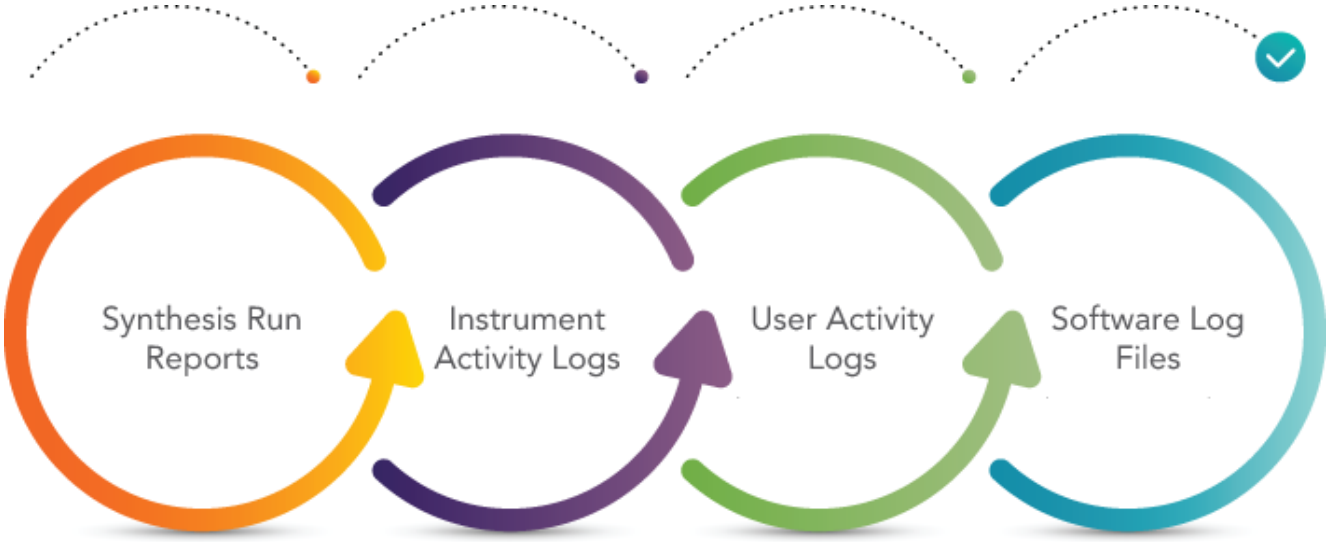
21CFR PART 11 COMPLIANCE - SOFTWARE

- Predefined roles of Administrator, Designer, Runner, Auditor, Service, and Factory with specified role rights, and individual users can be added and assigned to one of these
- Administrators can configure passwords by defining password length, complexity, frequency of change, and exclusion of used passwords.
- Administrators may define the maximum number of failed login attempts.

User Group	User Editor Privileges	Edit Settings	General Settings edits	Manual Operations	Automated synthesis runs	Synthesis editor, Program editor, Sequence Editor	Exit to OS /Shutdown system	View Audit Log Files
Administrator	✓	✓	✓				✓	✓
Designer				✓	✓	✓	✓	✓
Runner				✓	✓			✓
Auditor								✓

21CFR PART 11 COMPLIANCE - SOFTWARE

- An audit trail is a time-stamped, modification-protected electronic data file detailing all system events and record modifications. It is possible to export and print the full contents of all records including the audit trail.
- Detailed records of user and software activity as well as data from internal sensors monitoring fluid deliveries, heating, etc also make these files useful diagnostics for instrument performance and troubleshooting.



21CFR PART 11 COMPLIANCE - SOFTWARE

- Password protection and encryption of synthesis files.
- Backup and Restore points.
- Electronic signatures.

IQ/OQ/PQ – GMP Documentation

IQ
INSTALLATION QUALIFICATION

- ✓ *TESTS Correct instrument installation*
- ✓ *ENSURES Correct installation of system in-line with specifications*
- ✓ *ESTABLISHES Baseline for instrument*

OQ
OPERATIONAL QUALIFICATION

- ✓ *TESTS Correct instrument operation*
- ✓ *ENSURES Correct operation of system in-line with specifications*
- ✓ *VERIFIES System meets claims from parameters*

PQ
PERFORMANCE QUALIFICATION

- ✓ *TESTS Correct instrument performance*
- ✓ *ENSURES: Correct performance of system in-line with specifications*
- ✓ *VERIFIES: System meets user's intended purpose*

Reference Materials

- Dr. Alastair Hay, Almac Group
  - Background to use of automated parallel synthesisers
  - Use of synthesisers in the manufacture of long and complex peptides
  - Process development and route scouting
  - High Throughput cGMP manufacture

https://www.gyrosproteintechnologies.com/pti-webinars

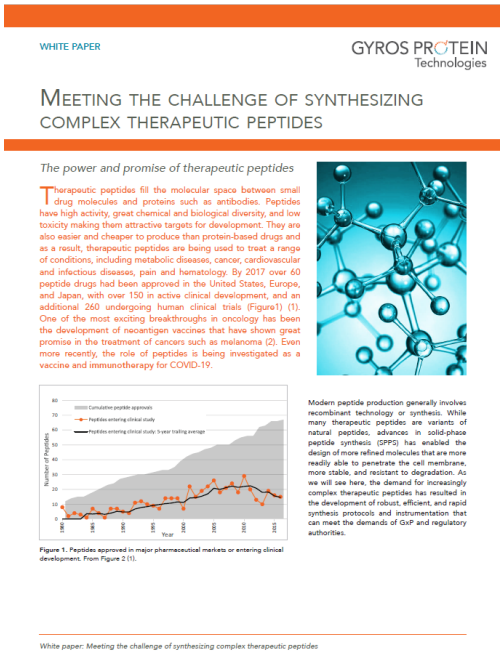
Reference Materials

- SARS-COV-2 Epitope Synthesis – TIDES eBook

https://informaconnect.com/innovating-cmc-oligonucleotide-peptide-therapeutics/

Reference Materials

- Synthesizing Complex Therapeutic Peptides – White Paper



WHITE PAPER

GYROS PROTEIN Technologies

MEETING THE CHALLENGE OF SYNTHESIZING COMPLEX THERAPEUTIC PEPTIDES

The power and promise of therapeutic peptides

Therapeutic peptides fill the molecular space between small drug molecules and proteins such as antibodies. Peptides have high activity, great chemical and biological diversity, and low toxicity making them attractive targets for development. They are also easier and cheaper to produce than protein-based drugs and as a result, therapeutic peptides are being used to treat a range of conditions, including metabolic diseases, cancer, cardiovascular and infectious diseases, pain and hematology. By 2017 over 60 peptide drugs had been approved in the United States, Europe, and Japan, with over 150 in active clinical development, and an additional 260 undergoing human clinical trials (Figure1) (1). One of the most exciting breakthroughs in oncology has been the development of neoantigen vaccines that have shown great promise in the treatment of cancers such as melanoma (2). Even more recently, the role of peptides is being investigated as a vaccine and immunotherapy for COVID-19.

Figure 1. Peptides approved in major pharmaceutical markets or entering clinical development. From Figure 2 (1).

Modern peptide production generally involves recombinant technology or synthesis. While many therapeutic peptides are variants of natural peptides, advances in solid-phase peptide synthesis (SPPS) has enabled the design of more refined molecules that are more readily able to penetrate the cell membrane, more stable, and resistant to degradation. As we will see here, the demand for increasingly complex therapeutic peptides has resulted in the development of robust, efficient, and rapid synthesis protocols and instrumentation that can meet the demands of GxP and regulatory authorities.

White paper: Meeting the challenge of synthesizing complex therapeutic peptides

With this in mind, the most important factor in a peptide synthesizer is having the flexibility, be that to choose room temperature or heated protocols, and also the ability to screen both in the same run in parallel.

Monitoring synthesis in real time

Synthesis setup is not always a case of "press the button and walk away". Being able to monitor the efficiency of, for example, the deprotection step during the run can be important in optimizing protocols.

A peptide synthesizer that handles complexity

PurePrep™ Chorus meets many of the demands made in the synthesis of complex peptides. The proprietary PurePep™ Pathway comprises proprietary fluidics that minimize cross-contamination, dead volumes, and reagent carryover. This is especially crucial for the synthesis of long sequences, in which even small amounts of impurities, side products, and incomplete reactions over many cycles can drastically reduce the final purity and yield of desired peptides.

Aggregation, secondary structure, steric hindrance, and conformational effects can still pose challenges in synthesis, and PurePep Chorus features enabling technologies that aid the synthesis of complex peptides and peptidomimetic sequences. Intellisynth™ real-time UV monitoring optimizes reaction times to ensure complete deprotection. By monitoring at 301 nm, the instrument measures the progress of the reaction – avoiding guesswork that can lead to incomplete deprotections, deletions, and side reactions. This feature is available on all reaction vessels, providing UV monitoring on up to six peptides in a single synthesis.

Other features include:

- A modular peptide synthesizer that is in-lab upgradeable, from 2 to 4 to 6 reaction vessels to meet productivity needs
- Independent induction heating, simultaneous and configurable to multiple vessels, and the ability to run multiple conditions in one run speeds up method development
- Icon-driven intuitive software platform with pre-programmed methods, ability to import sequences and reagent preparation calculators
- Designed for 21 CFR part 11 compliance, together with IQ/OQ support and PQ guidance are available to support work in regulated environments

Neoantigen vaccines promise to bring personalized cancer therapy to a new level

Cancer is characterized by a high frequency of genetic mutations that result in mutated proteins that can be processed into peptides and presented as immune signals on the surface of cancer cells. It is these peptides that are called neoantigens, which can be targeted by T cells as foreign, leading to cancer cell death.

While neoantigens can be shared amongst certain tumor types or patients, they can also be specific to the tumor in an individual patient, which makes them a highly promising target for individualized immunotherapy using cancer vaccines. Certain cancers, such as melanoma, result in more mutations than others, making the production of neoantigens more likely.

The preparation of neoantigen peptide vaccines involves the following critical steps:

1. Isolating a patient's tumor tissue
2. Genome sequencing and mutation analysis
3. Computational analysis of the mutation spectrum
4. Synthesis and pooling of key peptide mutants
5. Final preparation of vaccine ready for patient treatment

PurePep Chorus

All-in-one, scalable peptide synthesizer that can grow with your chemistry needs.

DOWNLOAD PRODUCT INFO. SHEET

White paper: Meeting the challenge of synthesizing complex therapeutic peptides

https://page.gyrosproteintechnologies.com/wp-meeting-the-challenge-of-synthesizing-complex-therapeutic-peptides

Resources

- Long peptides
- Hydrophobic peptides
- PNAs
- Peptoids
- Stapled peptides
- Cyclic peptides

- **PTH-84mer**
- **Poly-Ala^{9}**
- **Condition optimization of PNA analogs synthesis**
- **Synthesis of [des-Arg^{7}]-Dynorphin A peptoid analogue: faster methods using parallel automated synthesis and induction heat**
- **NYAD-1 peptide**
- **MT-II cyclization using heat**

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