

Disulfide-rich peptides

Optimizing and automating syntheses and regioselective formation of disulfide bonds

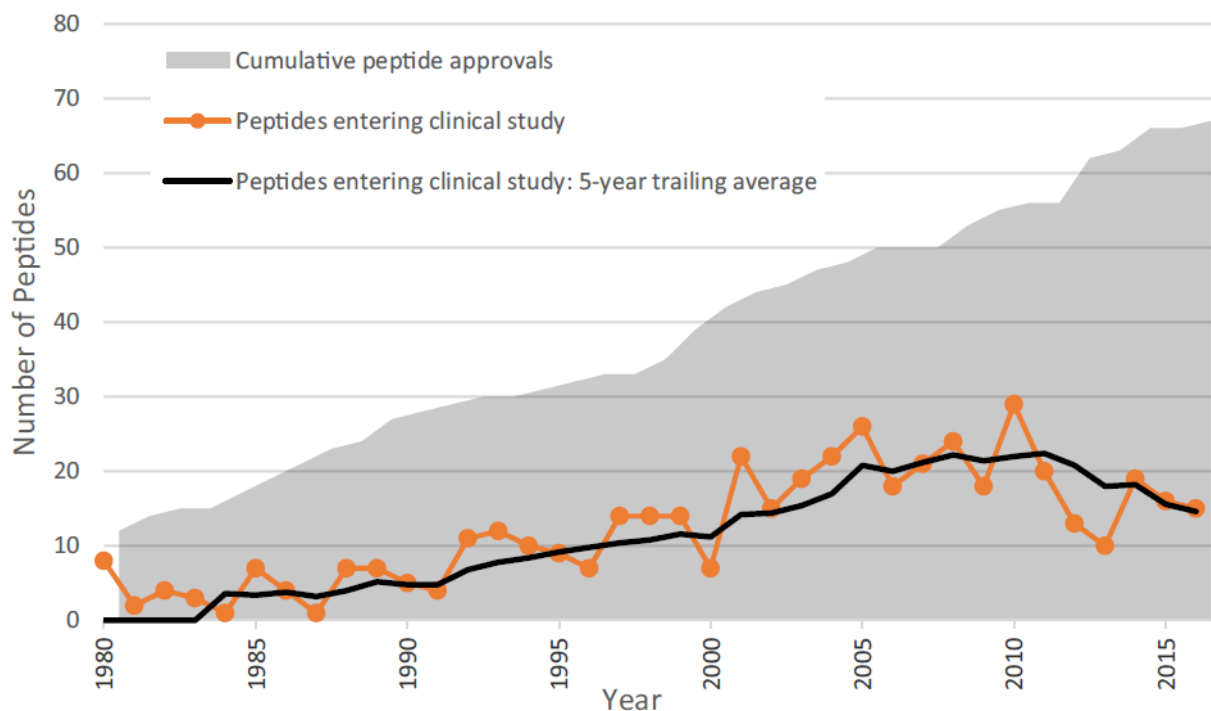
Elizabeth Denton, PhD

31 October 2018

Peptide therapeutics continues to grow

Delivery and bioavailability still largest hurdles

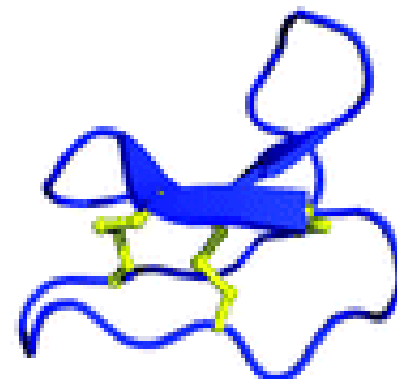
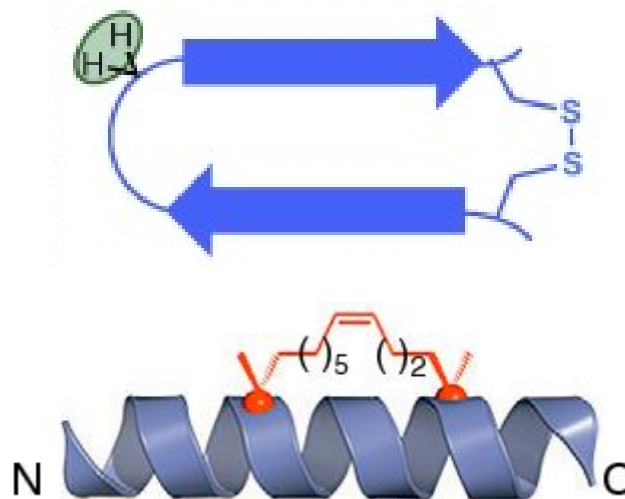
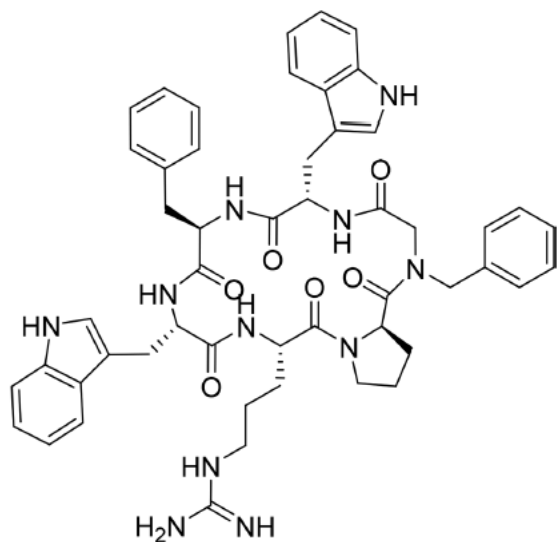
- » Expecting approximately \$50 bil market for peptide therapeutics by 2025
 - » More than 60 approved therapeutics
 - » >150 in active clinical trials



Lau, J. L. and Dunn, M. K. *BioOrg. and Med. Chem.* **2018**, *10*, 2700-2707.

Structural stabilization improves biological activity

- » Head-to-tail cyclization reduces proteolytic degradation
- » Secondary structure stabilization improves binding affinity
- » Small macrocycles seem to be passively cell permeable
- » Disulfide rich peptides present loop regions for binding



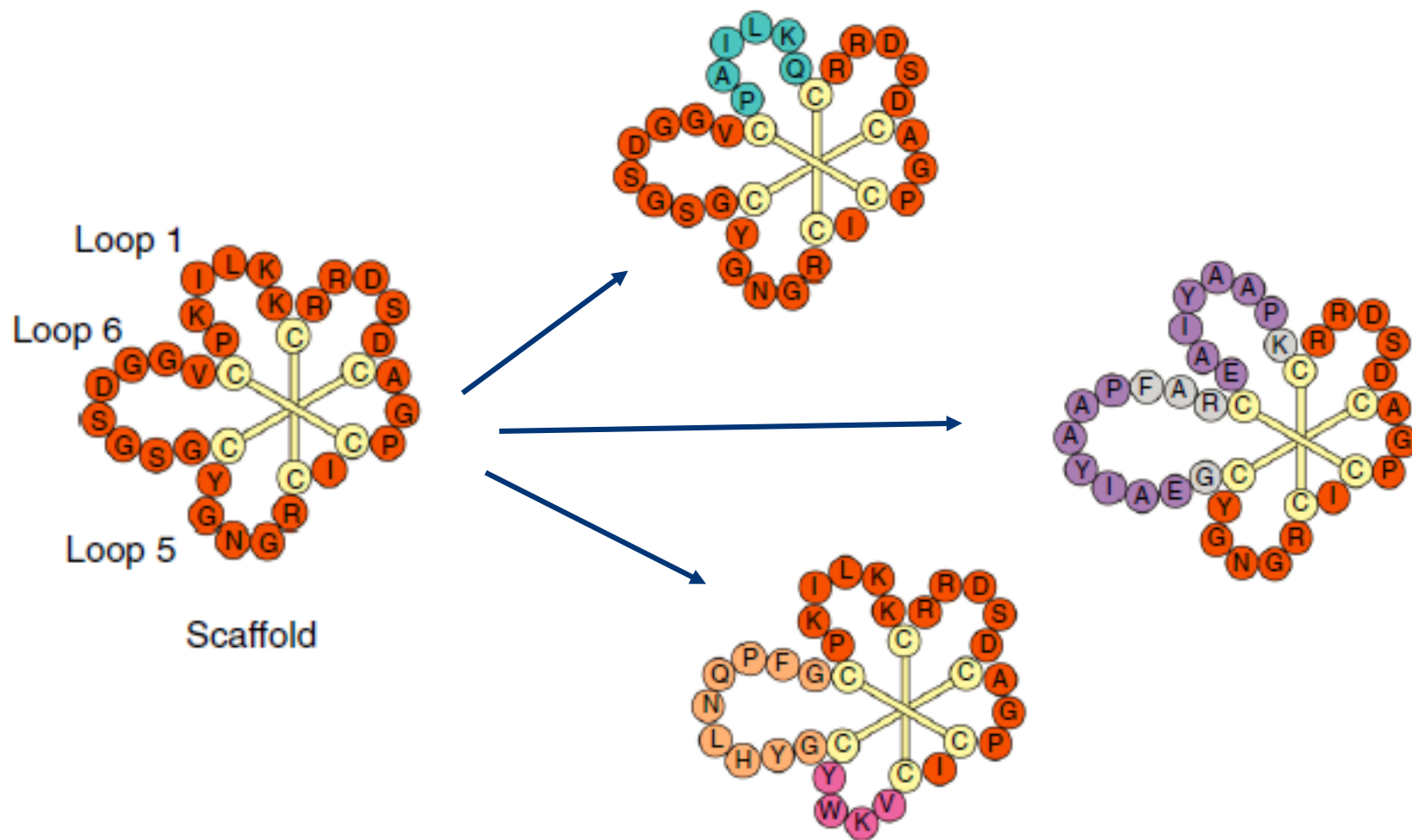
Boehm, M. et al. *J. Med. Chem* **2017**, 60, 9653-9663.

Sarnowski, M. P. et al, *Bio. and Med. Chem.* **2018**, 26, 1162-1166.

Verdine, G. L. and Hilinski, G. J. *Drug Discov Today Tech.* **2012**, 9, e1-e70.

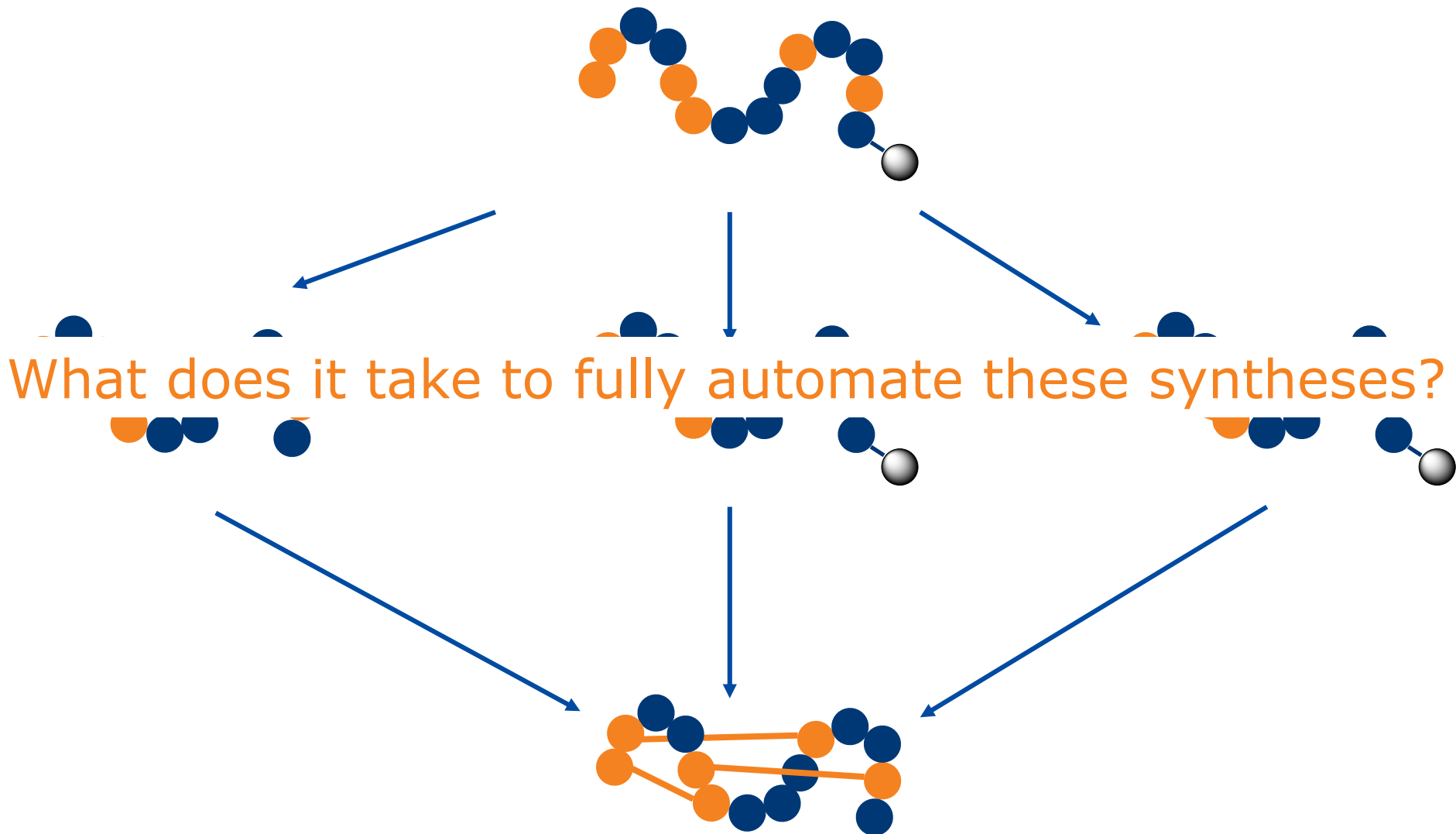
Cascales, L. and Craik, D. J. *Org. Biomol. Chem.* **2010**, 8, 5035-5047.

Disulfide Rich peptides as structural scaffolds

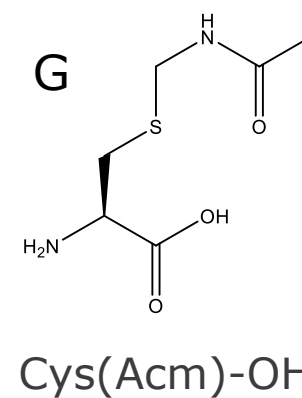
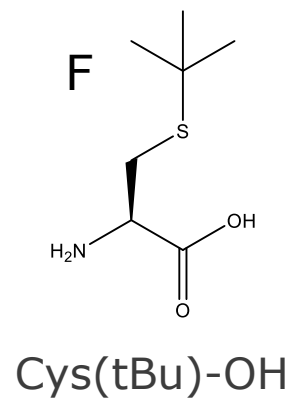
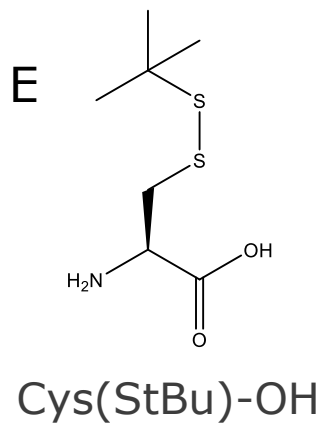
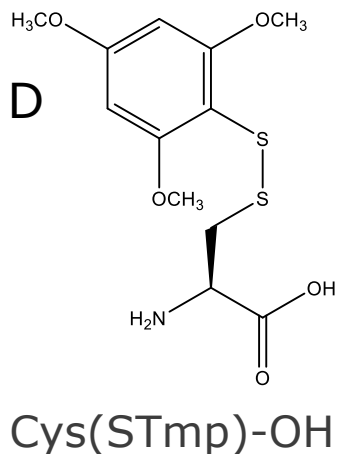
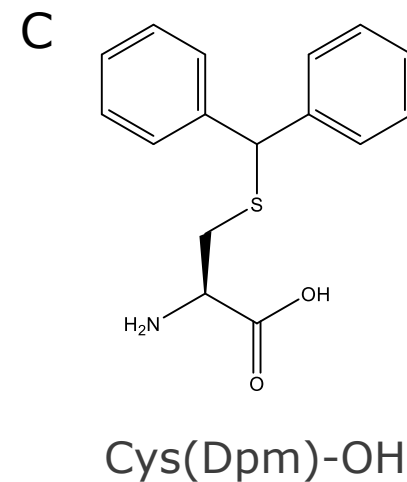
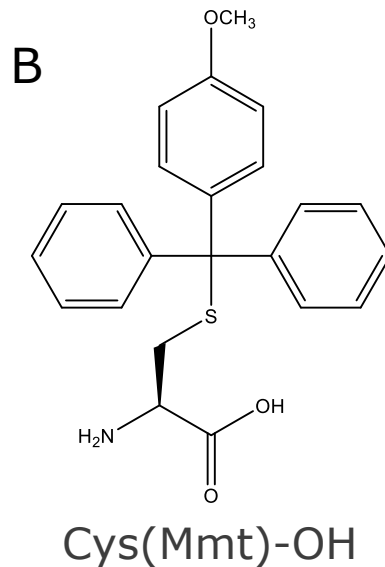
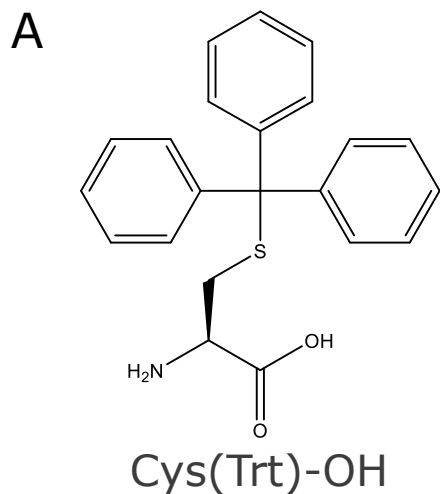


Wang, C. and Craik, D. J. *Nat. Chem. Bio.* **2018**, *14* 417-427.

A Range of Strategies for Folding

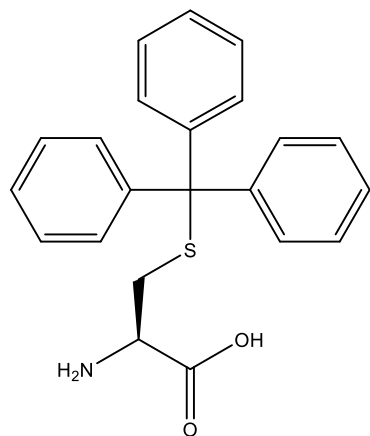


Suite of orthogonally protected Cys



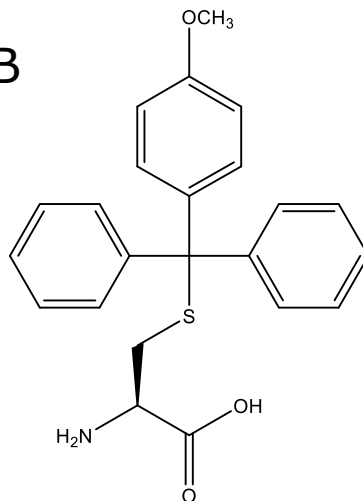
Suite of orthogonally protected Cys

A



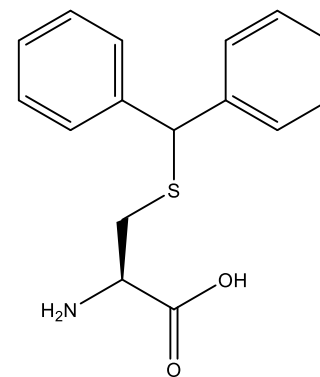
Cys(Trt)-OH

B



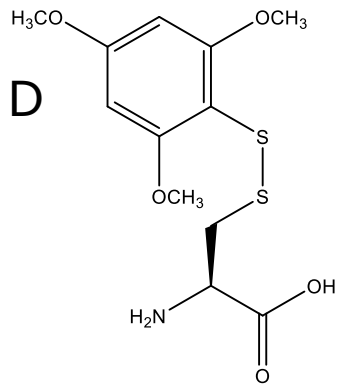
Cys(Mmt)-OH

C



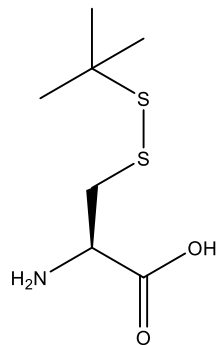
Cys(Dpm)-OH

D



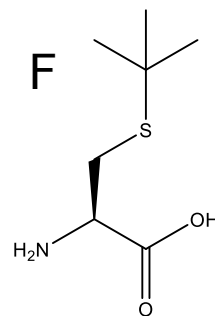
Cys(STmp)-OH

E



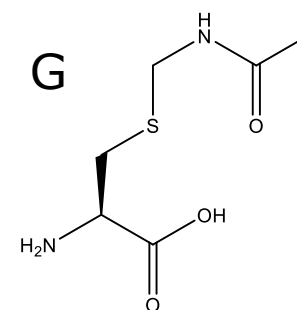
Cys(StBu)-OH

F



Cys(tBu)-OH

G



Cys(Acm)-OH

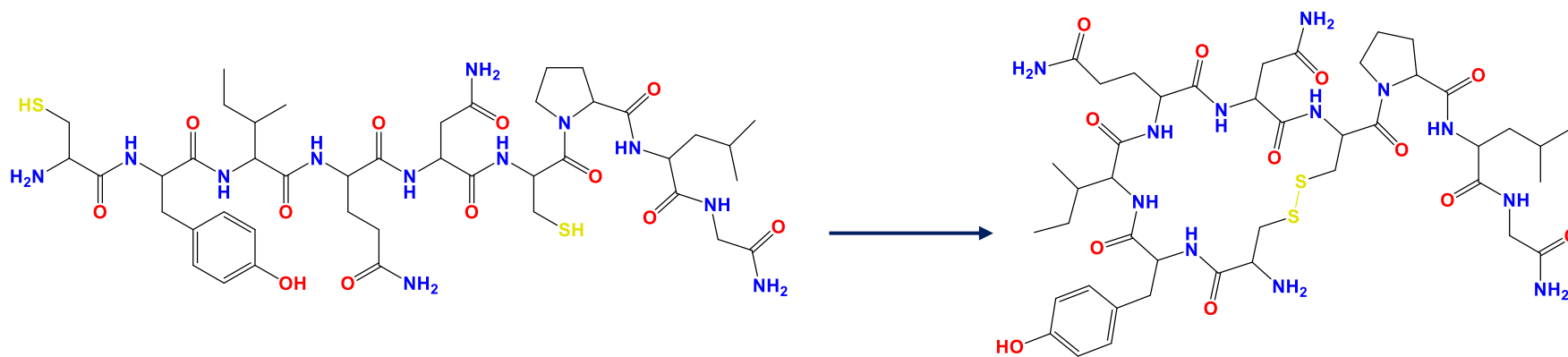
Where to start?

Managing instrumentation specifications

- » Instruments perform tasks differently than you do manually
 - » Volume limitations
 - » Mixing mechanisms
 - » Scaling?
- » What we need:
 - » Cysteine oxidation conditions
 - » Mmt removal conditions
 - » STmp removal conditions
 - » Acn removal conditions
 - » Model systems to evaluate efficacy
- » Concerns:
 - » Gentle oxidation to prevent disulfide shuffling
 - » Efficient protecting group removal

Optimizing disulfide bond formation

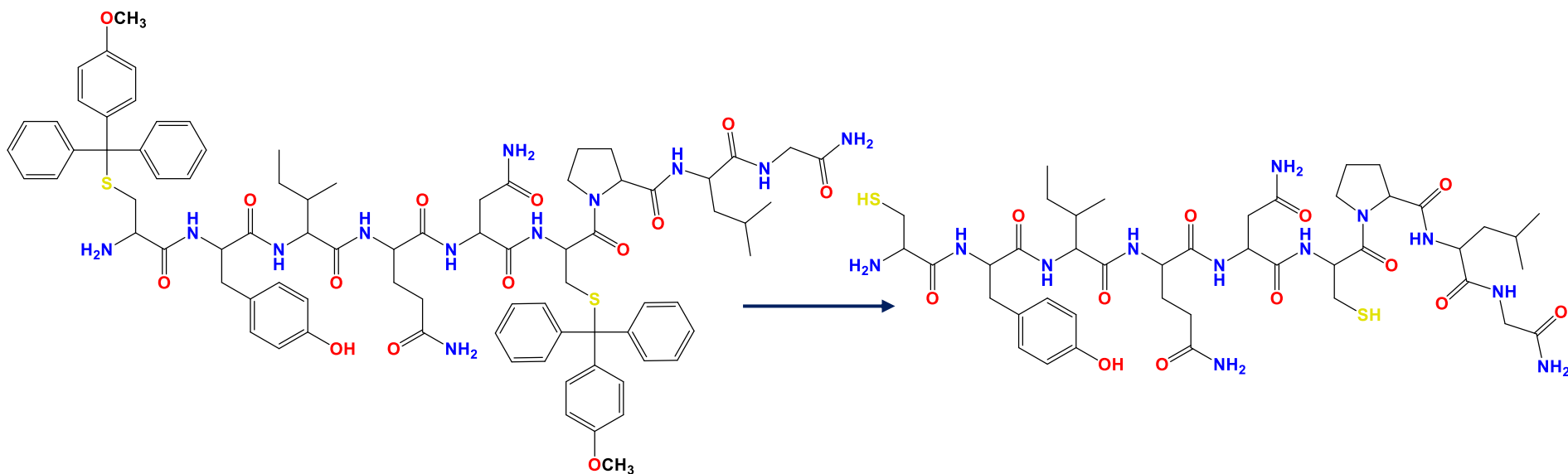
| Reagents | Equivalents | Temperature | Time (min) | Percent completion |
|--|-------------|-------------|------------|--------------------|
| NH ₃ /H ₂ O ₂ | 2/1.2 | r.t. | 30 | 50 |
| NH ₃ /H ₂ O ₂ | 4/2.4 | r.t. | 30 | 50 |
| NCS | 2 | r.t. | 15 | 100 |
| NCS | 4 | r.t. | 5 | 100 |
| NCS | 2 | 50 °C | 5 | 100 |
| NCS | 1 | 50 °C | 5 | 100 |



Postma, T. M. and Albericio, F. *Org. Lett.* **2012**, 15, 616-619.

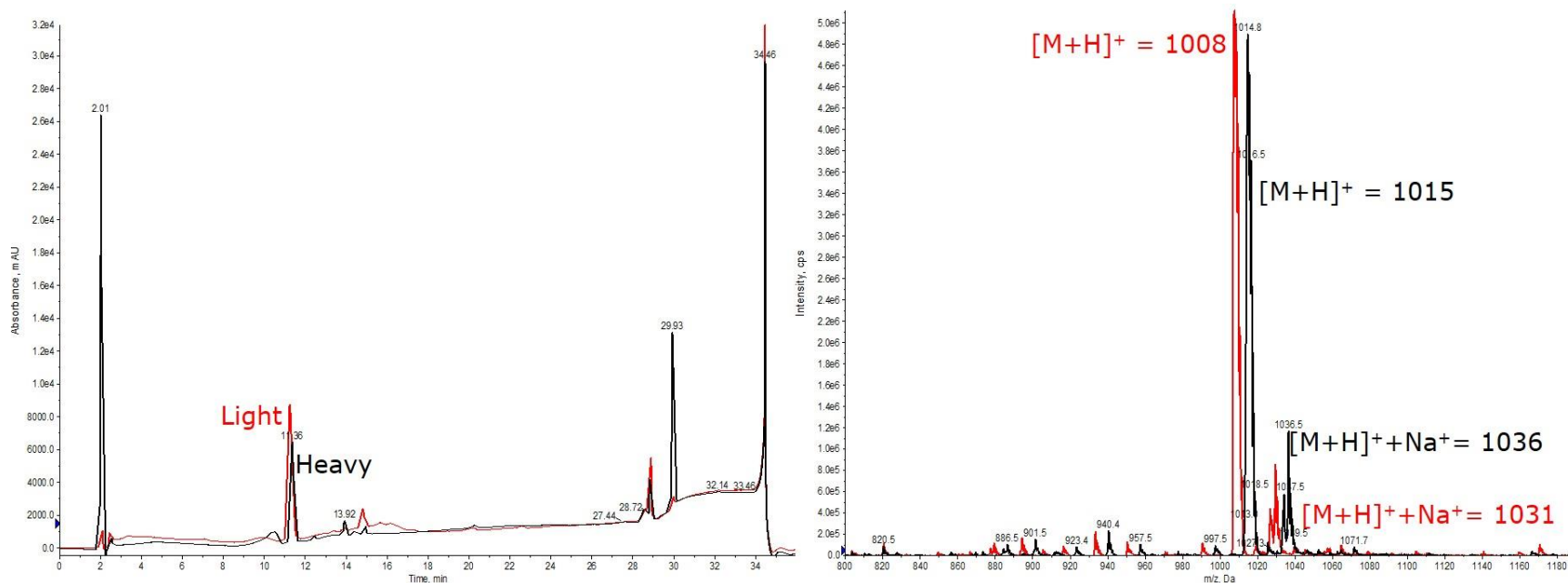
Mmt removal efficiency varies with scale

| Scale (mmol) | TFA in DCM + 5% TIPS | Volume (mL) | Time (min) | Attempts |
|--------------|----------------------|-------------|------------|----------|
| 0.025 | 2% TFA | 1.5 | 20 | 2 |
| 0.235 | 2% TFA | 4.5 | 20 | 4 |
| 0.4 | 2% TFA | 9 | 30 | 6 |



Fully automated oxytocin synthesis

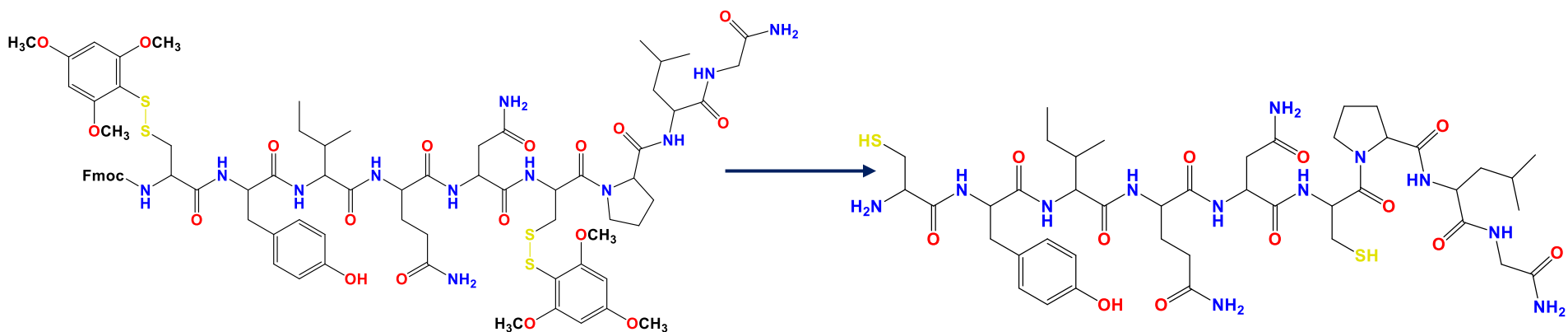
Incorporating Fmoc-Cys(Mmt)-OH and C^{13},N^{15} Leu



Isotopically-labeled oxytocin prepared with fully automated synthesis and on-resin oxidation in 90% crude purity

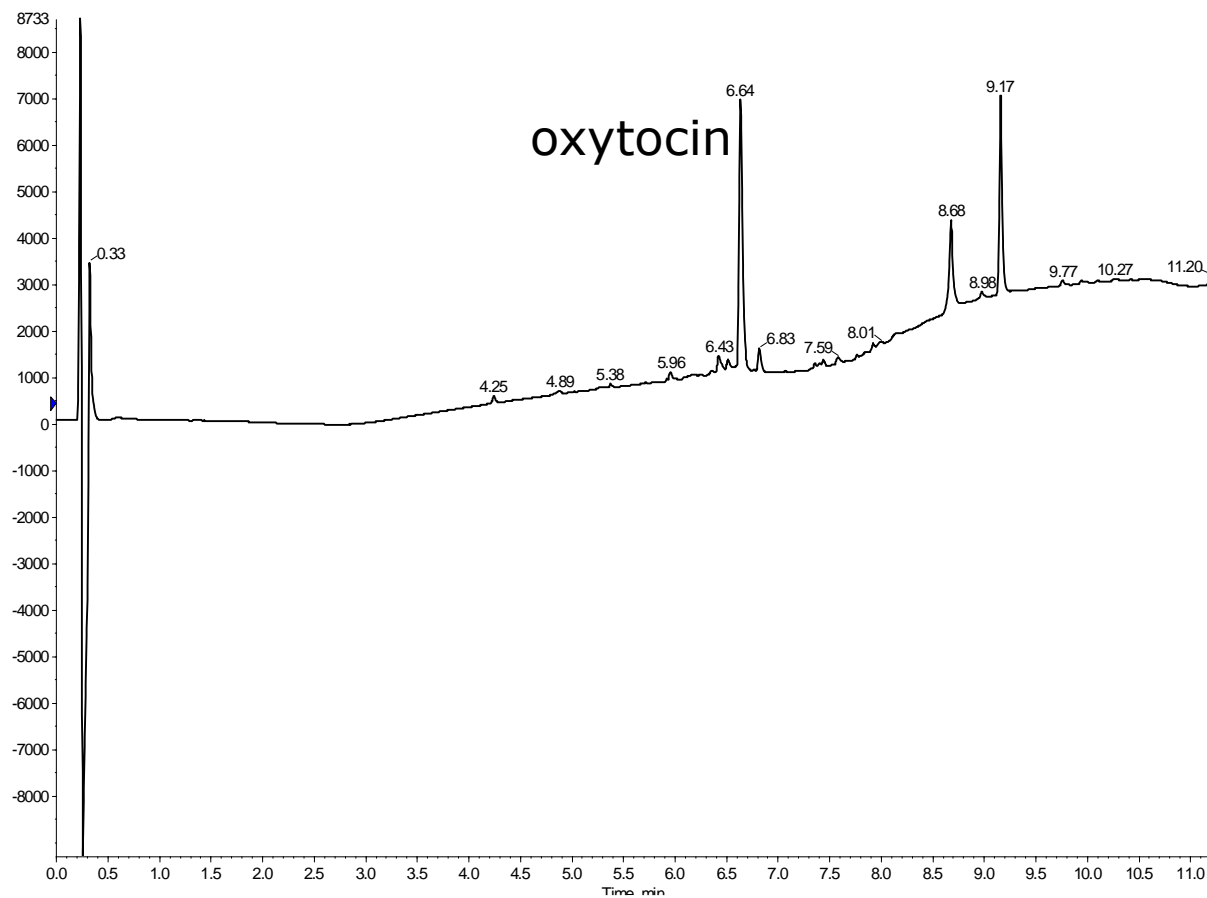
Optimizing STmp removal

| Reagent volume (mL) | Reaction time (min) | Reaction temperature | Iterations |
|---------------------|---------------------|----------------------|------------|
| 4.5 | 5 | r.t. | 3 |
| 3 | 5 | r.t. | 3 |
| 1 | 5 | r.t. | 3 |
| 0.5 | 5 | r.t. | 3 |
| 0.25 | 5 | r.t. | 3 |



Fully automated oxytocin

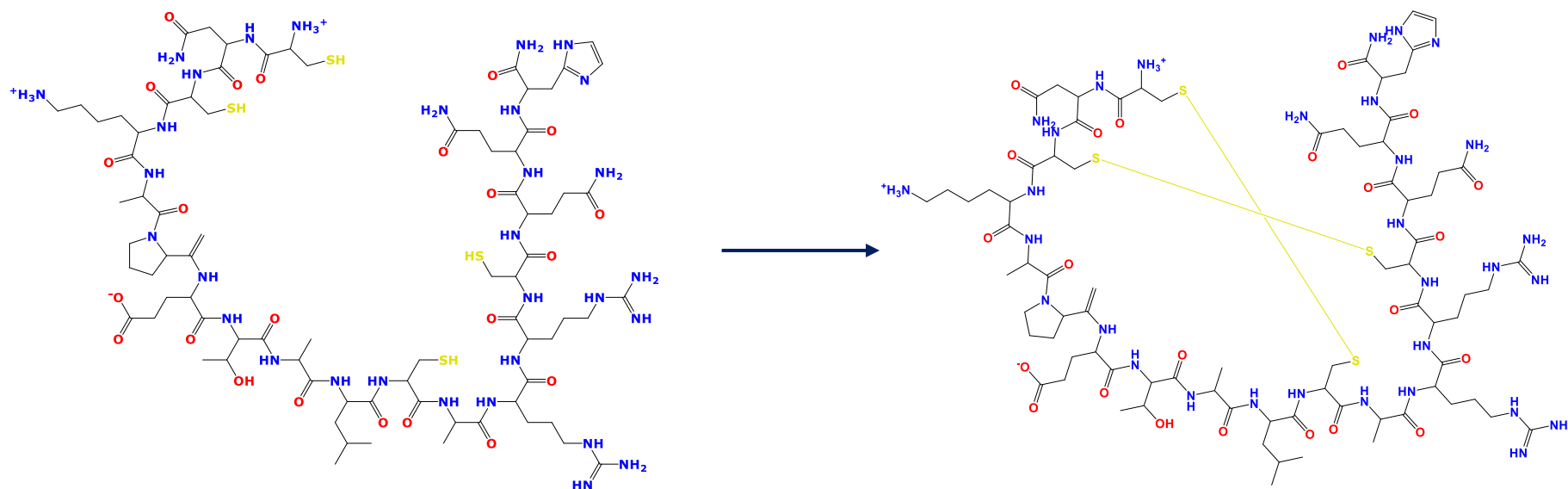
Incorporating Fmoc-Cys(STmp)-OH and optimized NCS-mediated oxidation



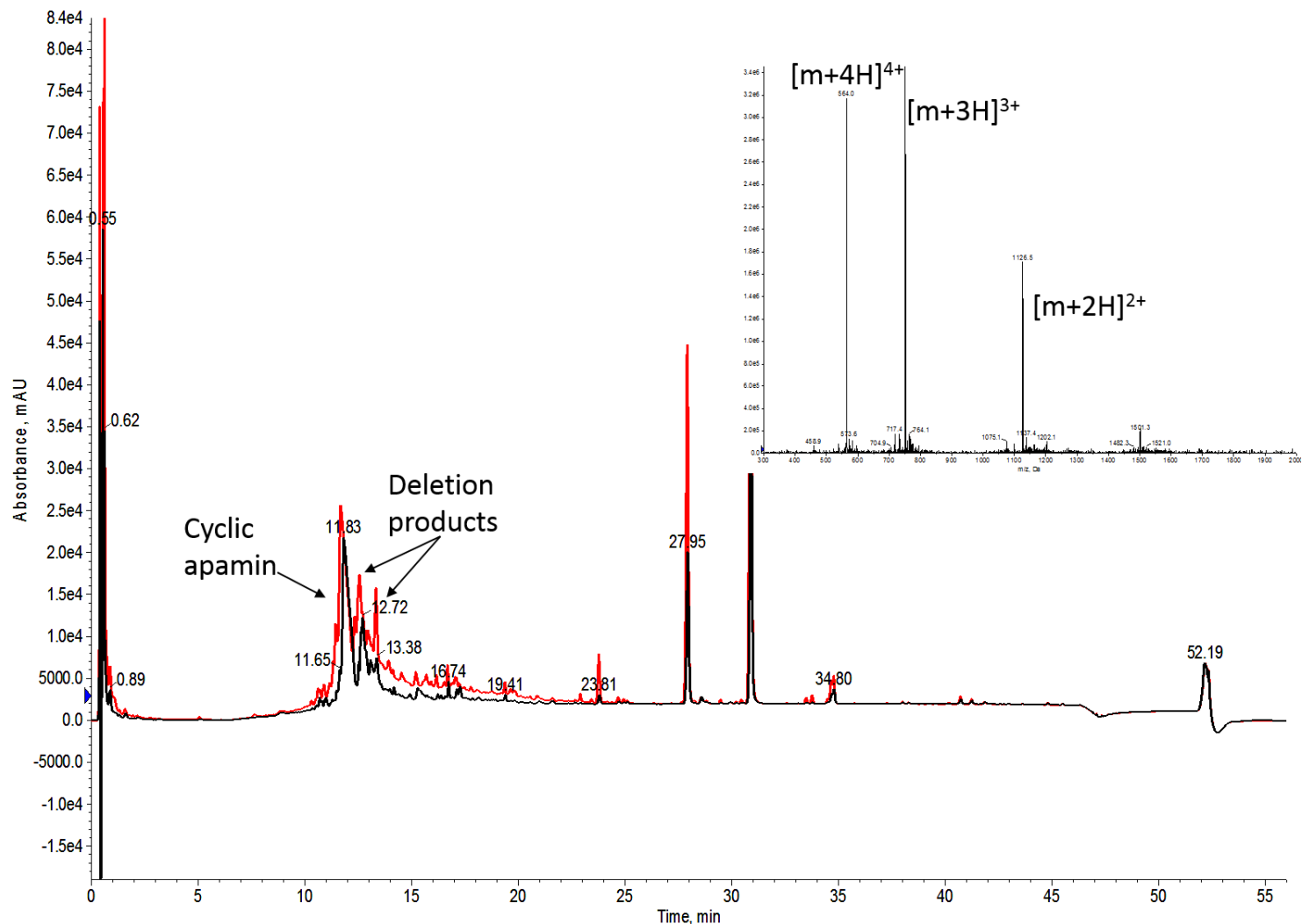
Automated synthesis and on-resin disulfide bond formation in >83% crude purity

Optimizing Acm removal with concomitant Cys oxidation

| Experiment | Time (min) | I ₂ equivalents (mmol) |
|------------|------------|-----------------------------------|
| 1 | 60 | 15 |
| 2 | 45 | 15 |
| 3 | 30 | 15 |
| 4 | 60 | 10 |
| 5 | 60 | 5 |
| 6 | 60 | 2.5 |

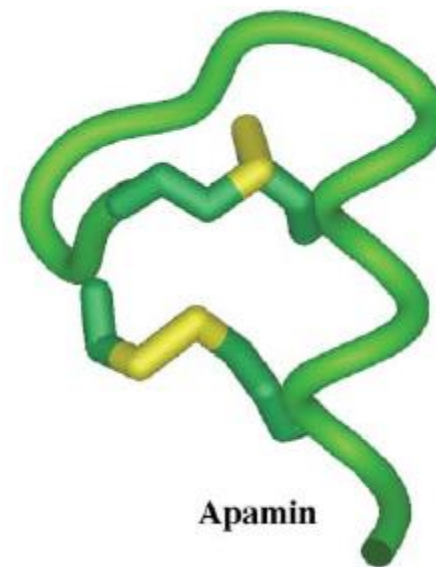
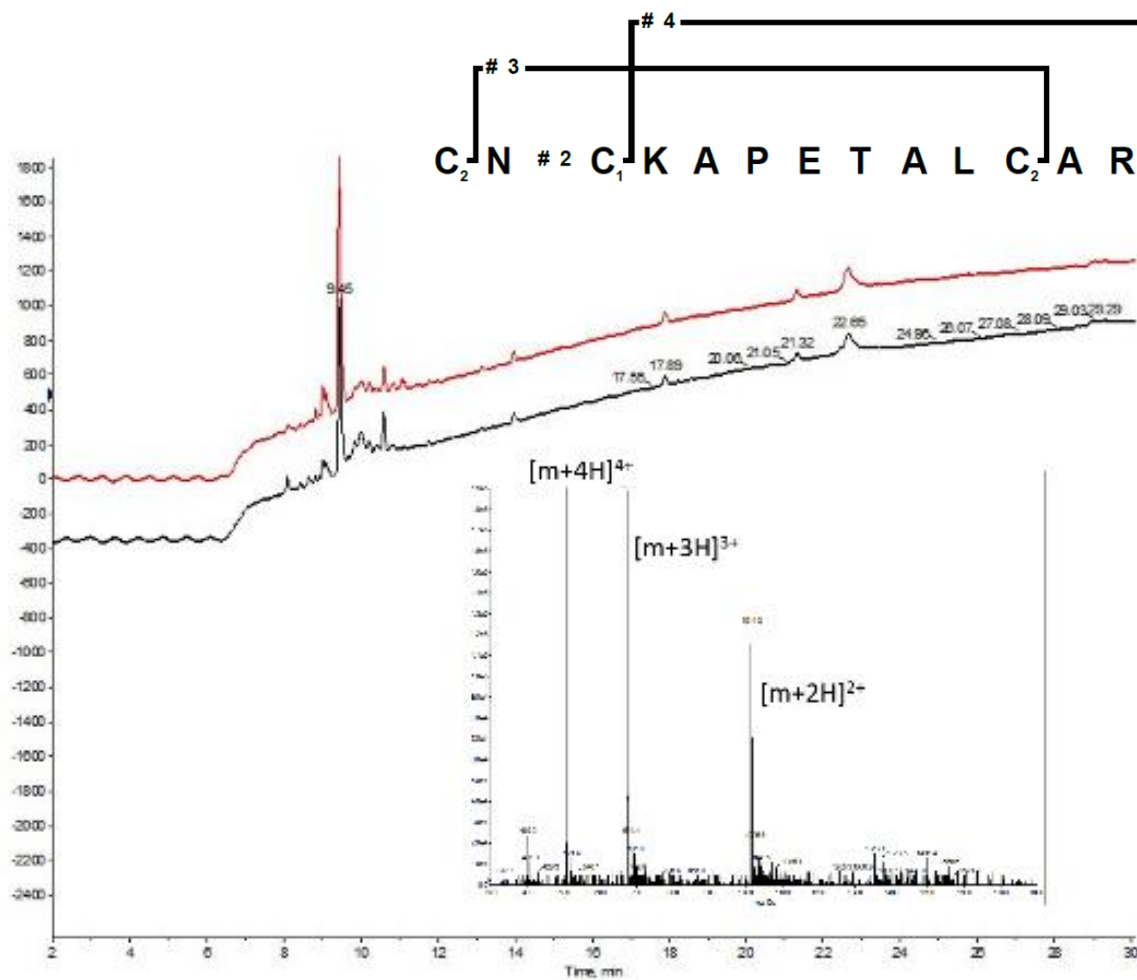


All conditions yielded desired product as majority species



Increasing complexity

Does the order of disulfide bond formation matter?



Pease, J. H. B. and Wemmer, D. E. *Biochemistry* **1988**, 27, 8491-8498.

Further increasing complexity

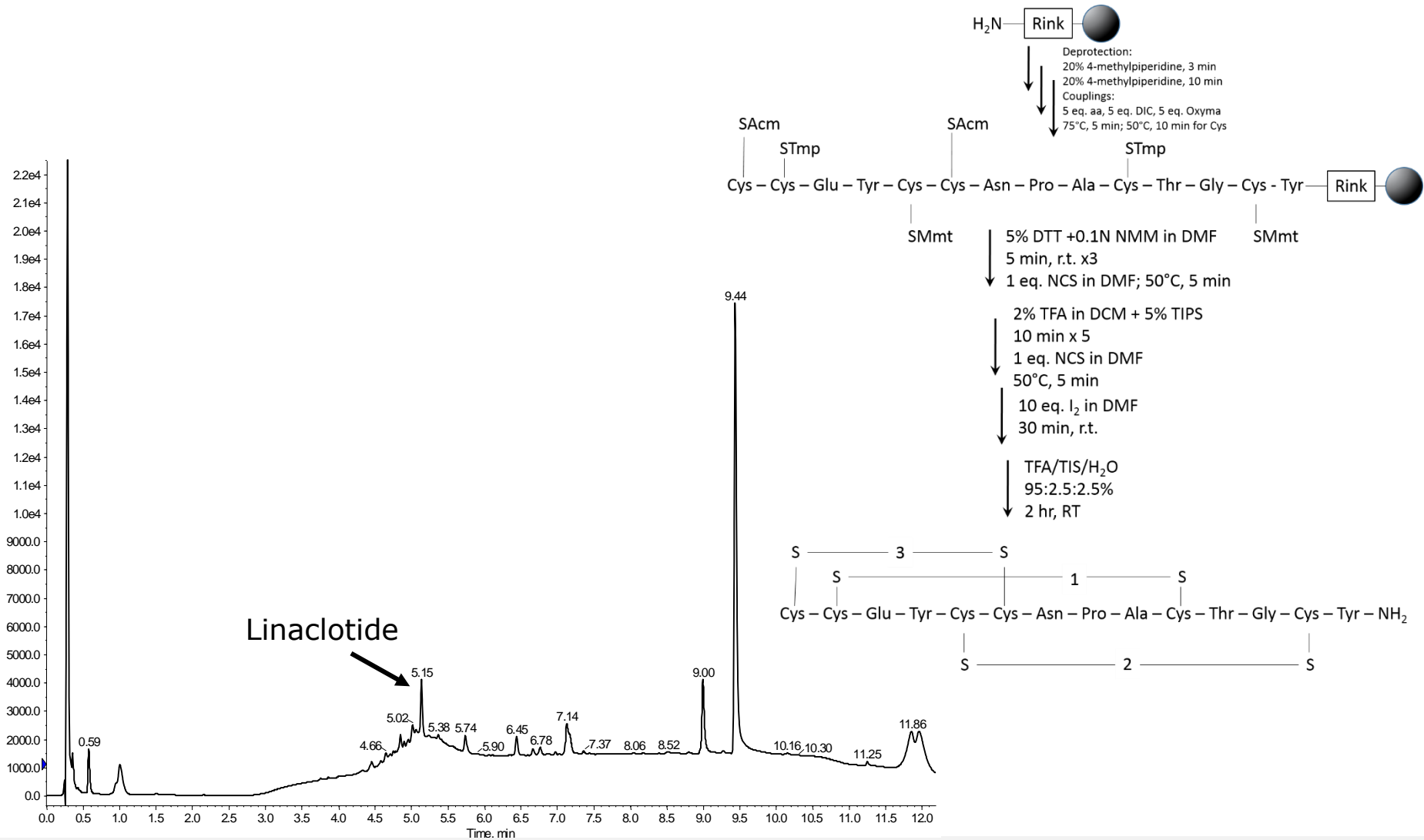
Synthesizing Linaclotide

- » Linaclotide
 - » FDA approved therapeutic
 - » 14 amino acids
 - » 3 disulfide bonds
 - » 43% Cys content
- » Goal: automate synthesis and regioselective disulfide bond formation on-resin using orthogonal protecting groups



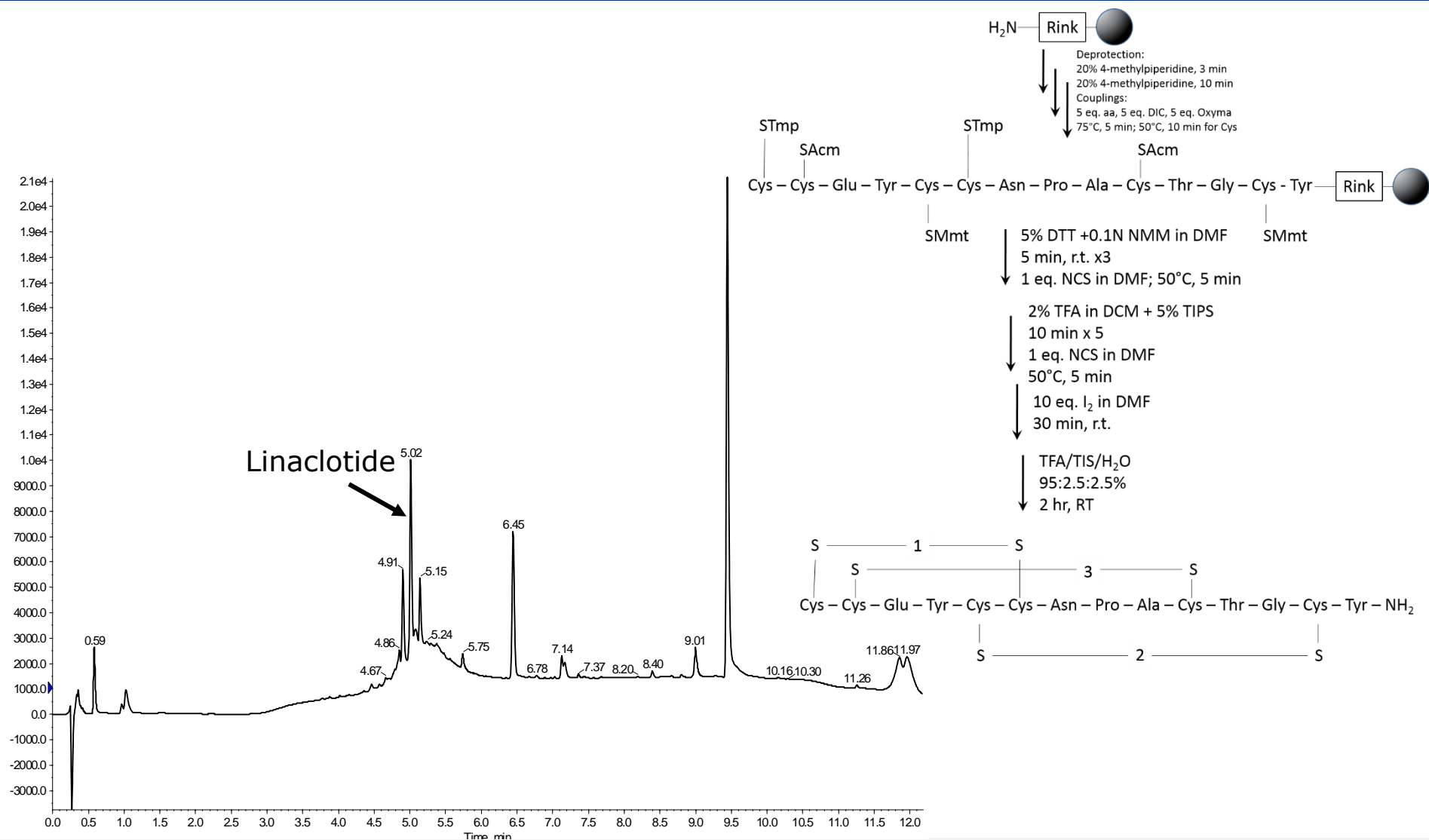
Further increasing complexity

What order should the disulfide bonds be formed?



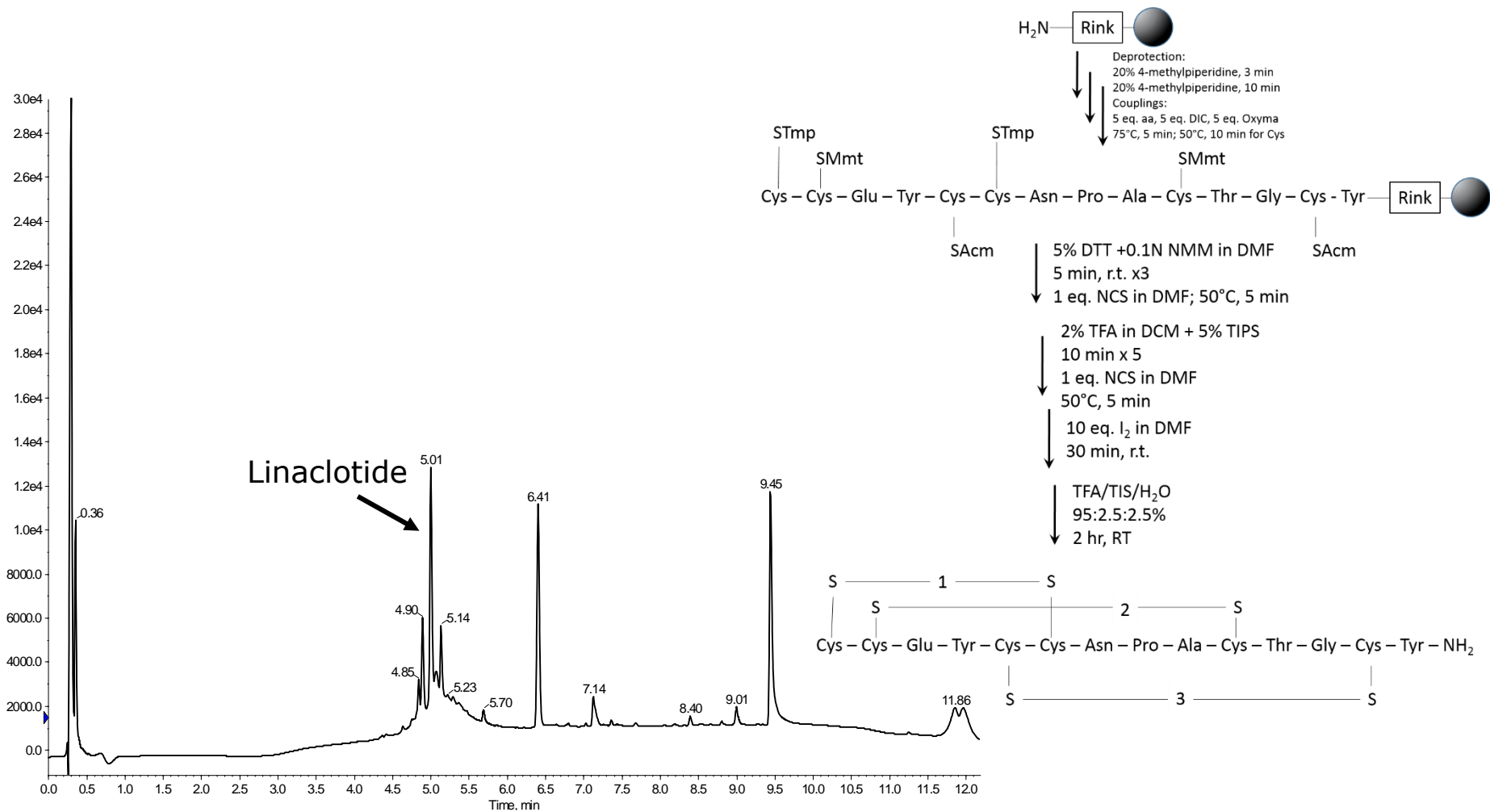
Further increasing complexity

What order should the disulfide bonds be formed?



Further increasing complexity

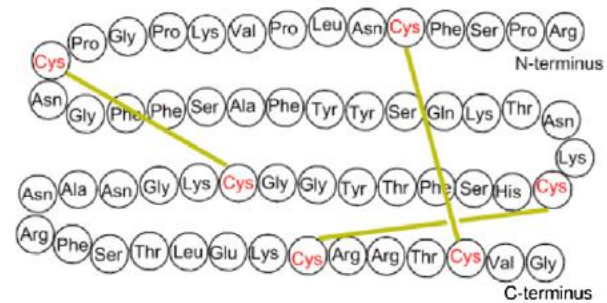
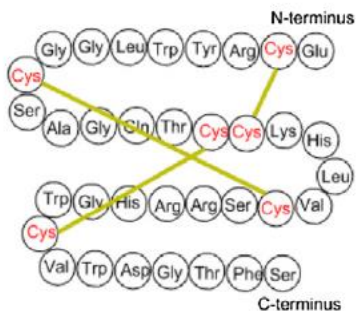
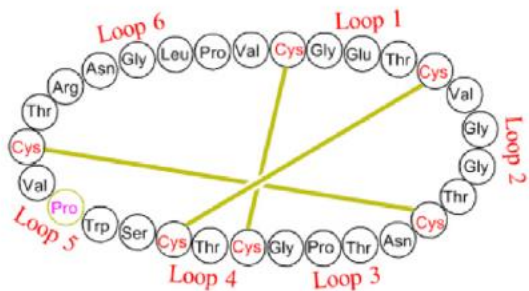
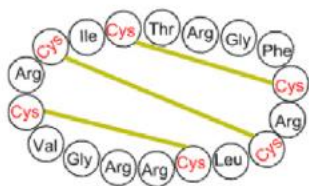
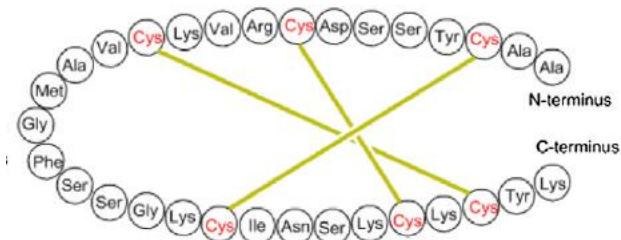
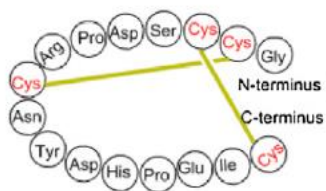
What order should the disulfide bonds be formed?



Gongora-Benitez, M et al. *Biopolymers*, **2011**, 96, 69-80.

Vast structural diversity causes challenges for synthesis, production

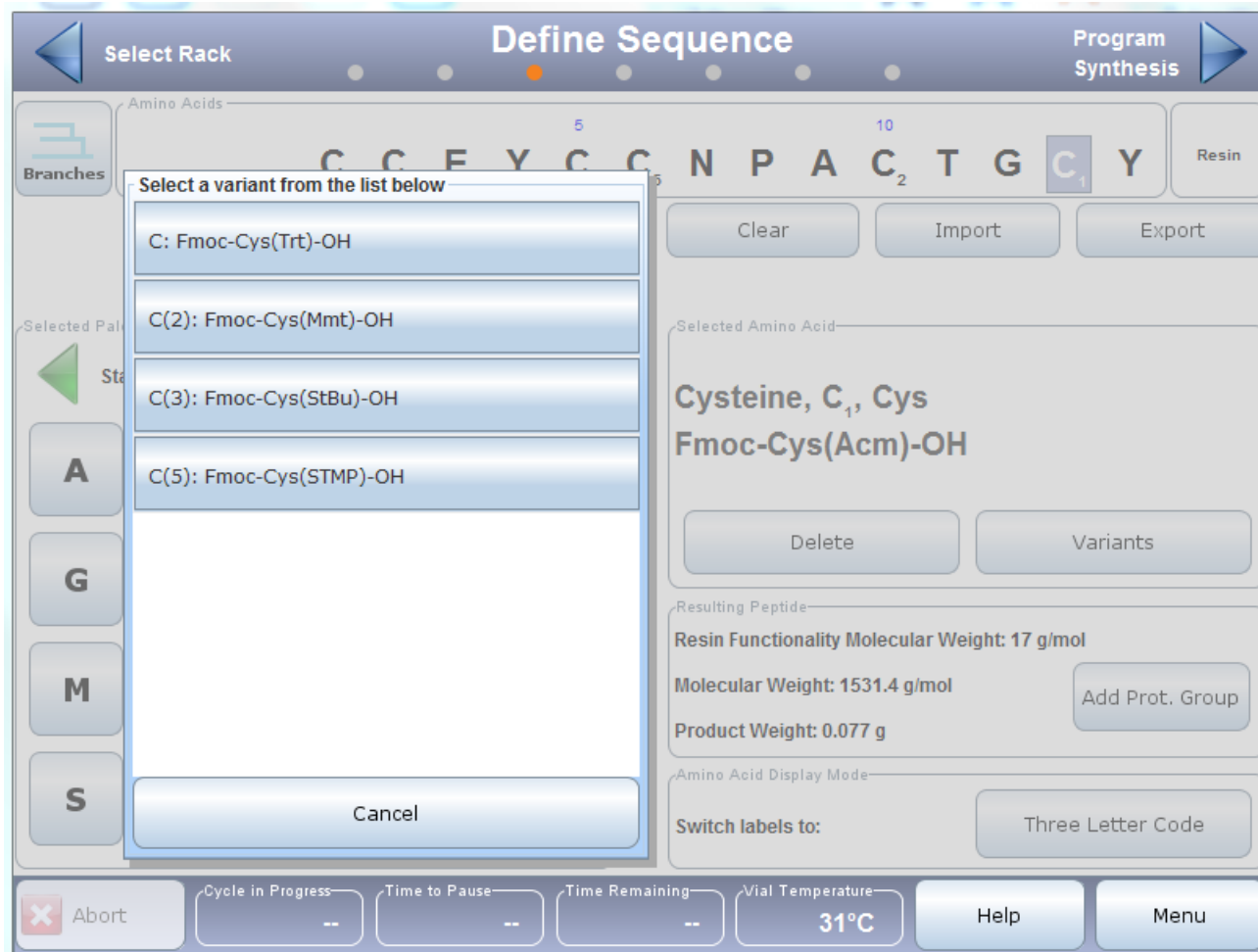
Disulfide Rich Peptides



Fang, G.-M. et al. *Chin. Chem. Lett.* **2018**, 29, 1022-1042.

Simplifying synthesis with smart software

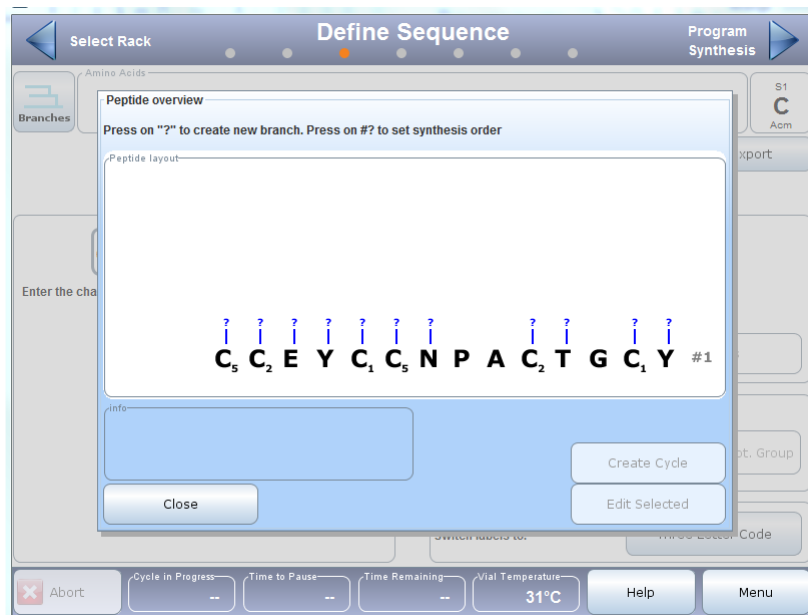
Directly visualize and specifically program everything



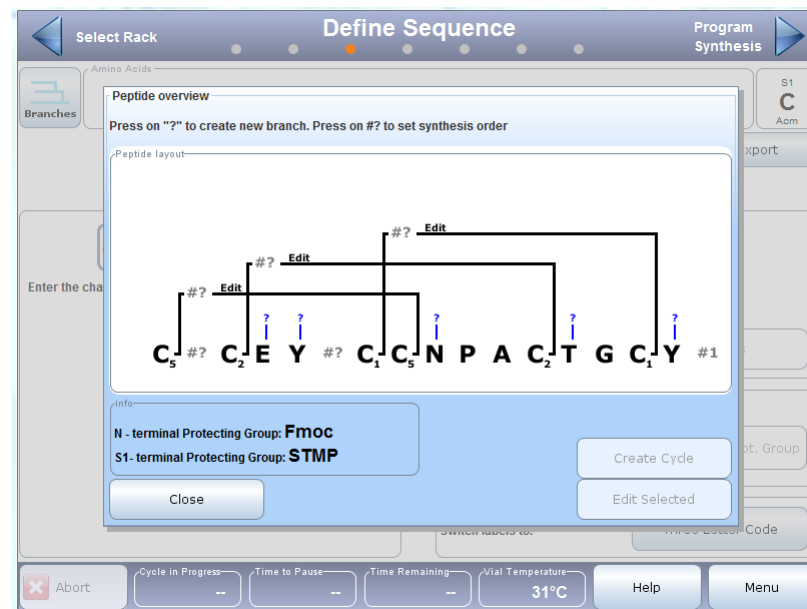
Easily match orthogonally protected Cys pairs with the Variants palette

Simplifying synthesis with smart software

Directly visualize and specifically program cyclizations



The screenshot shows the 'Define Sequence' window in the software. The 'Peptide overview' section contains the text: "Press on '?' to create new branch. Press on '#' to set synthesis order". Below this, the peptide layout is displayed as a linear sequence: C₅ C₂ E Y C₁ C₅ N P A C₂ T G C₁ Y #1. Each amino acid is preceded by a question mark icon. The interface includes a 'Close' button and a 'Create Cycle' button.

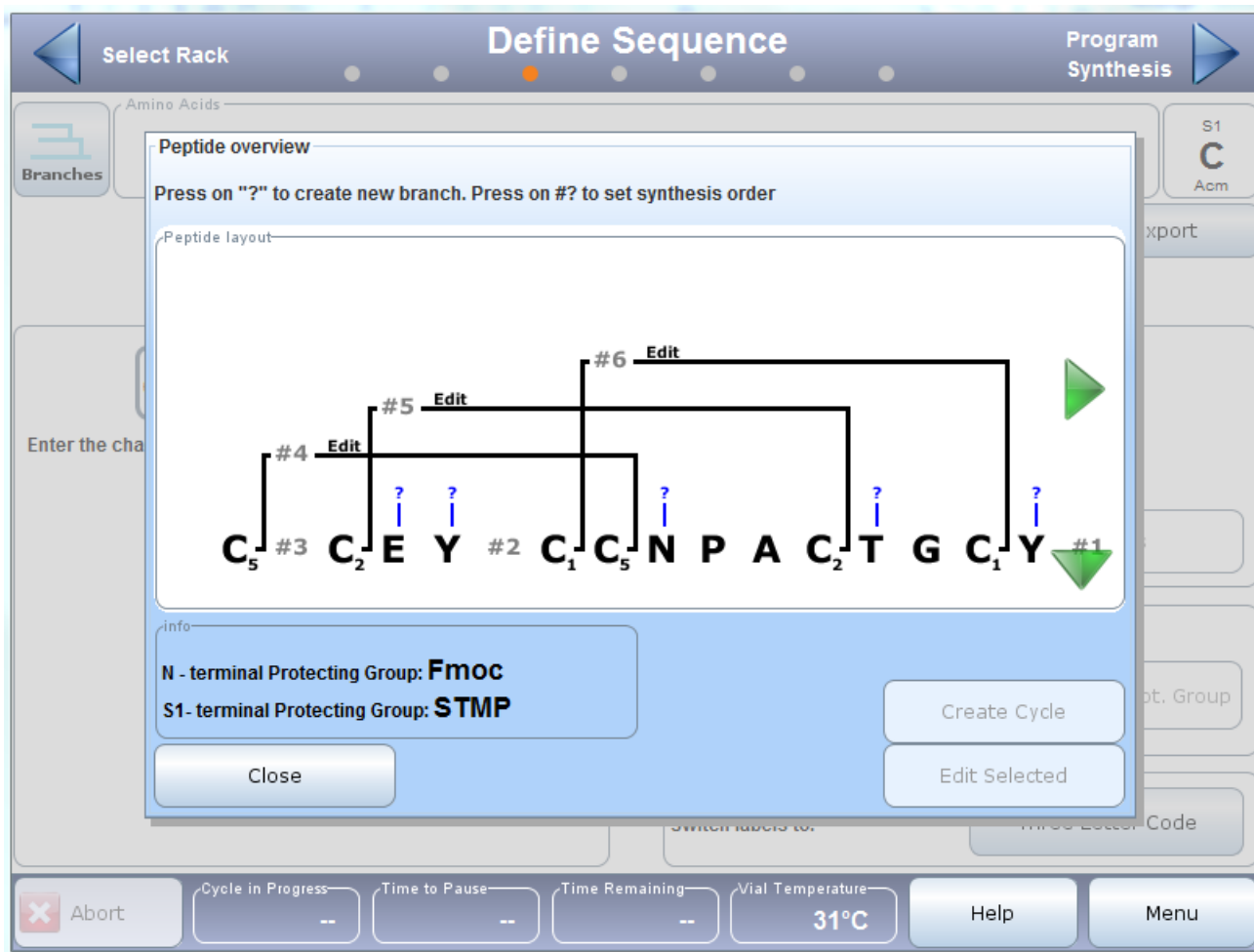


The screenshot shows the 'Define Sequence' window with a branched peptide sequence. The sequence is: C₅ #? C₂ E Y #? C₁ C₅ N P A C₂ T G C₁ Y #1. The branching is visualized with lines connecting the question marks. The 'Info' section is expanded, showing: "N-terminal Protecting Group: Fmoc" and "S1-terminal Protecting Group: STMP". The interface includes a 'Close' button and a 'Create Cycle' button.

Readily assign and *visualize* disulfide bond connectivity

Simplifying synthesis with smart software

Directly visualize and specifically program cyclizations

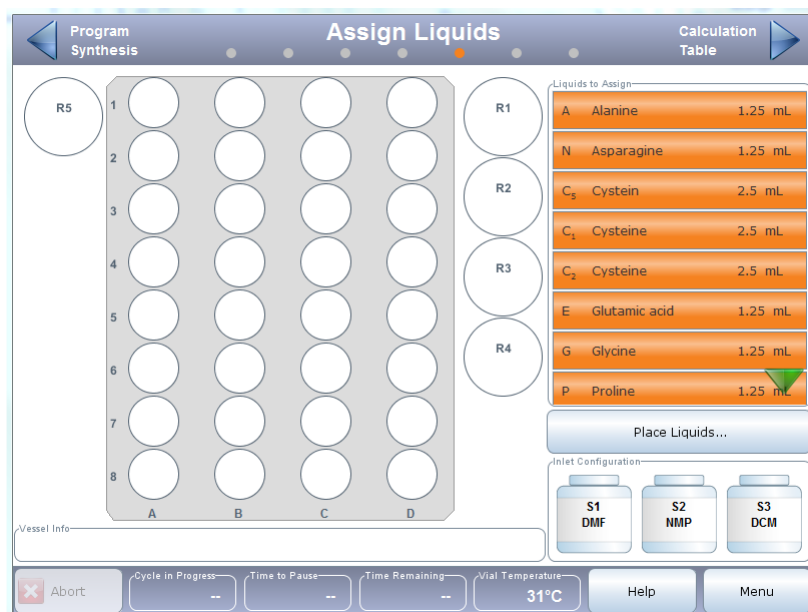


The screenshot shows the 'Define Sequence' window in a peptide synthesis software. The window has a title bar with 'Select Rack' on the left and 'Program Synthesis' on the right. Below the title bar, there are navigation arrows and a 'Peptide overview' section. The 'Peptide overview' section contains the text: 'Press on "?" to create new branch. Press on #? to set synthesis order'. Below this is a 'Peptide layout' area showing a sequence of amino acids: C₅, #3, C₂, E, Y, #2, C₁, C₅, N, P, A, C₂, T, G, C₁, Y, #1. Above the sequence, there are six 'Edit' points labeled #4, #5, and #6, which are connected by lines to the sequence, indicating cyclization points. A green arrow points to the right from the #6 Edit point. Below the sequence, there are two buttons: 'Create Cycle' and 'Edit Selected'. At the bottom of the window, there is a status bar with 'Abort', 'Cycle in Progress', 'Time to Pause', 'Time Remaining', 'Vial Temperature' (31°C), 'Help', and 'Menu' buttons.

Simply assign order in which synthesis will occur

Simplifying synthesis with smart software

Directly visualize and specifically program cyclizations



Program Synthesis Assign Liquids Calculation Table

Liquids to Assign:

| | | |
|----------------|---------------|---------|
| A | Alanine | 1.25 mL |
| N | Asparagine | 1.25 mL |
| C ₅ | Cystein | 2.5 mL |
| C ₁ | Cysteine | 2.5 mL |
| C ₂ | Cysteine | 2.5 mL |
| E | Glutamic acid | 1.25 mL |
| G | Glycine | 1.25 mL |
| P | Proline | 1.25 mL |

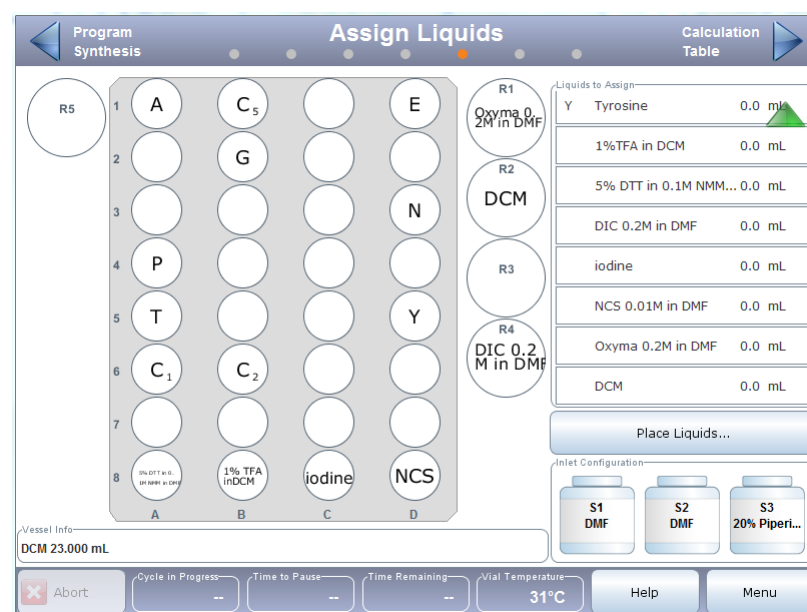
Place Liquids...

Inlet Configuration:

| | | |
|-----|-----|-----|
| S1 | S2 | S3 |
| DMF | NMP | DCM |

Vessel Info: 31°C

Buttons: Abort, Cycle in Progress, Time to Pause, Time Remaining, Vial Temperature, Help, Menu



Program Synthesis Assign Liquids Calculation Table

Liquids to Assign:

| | | |
|---|-----------------------|--------|
| Y | Tyrosine | 0.0 mL |
| | 1%TFA in DCM | 0.0 mL |
| | 5% DTT in 0.1M NMM... | 0.0 mL |
| | DIC 0.2M in DMF | 0.0 mL |
| | iodine | 0.0 mL |
| | NCS 0.01M in DMF | 0.0 mL |
| | Oxyma 0.2M in DMF | 0.0 mL |
| | DCM | 0.0 mL |

Place Liquids...

Inlet Configuration:

| | | |
|-----|-----|---------------|
| S1 | S2 | S3 |
| DMF | DMF | 20% Piperi... |

Vessel Info: DCM 23.000 mL

Buttons: Abort, Cycle in Progress, Time to Pause, Time Remaining, Vial Temperature, Help, Menu

Assign each reagent position wherever you want

- » Successfully optimized *automated* orthogonal protecting group removal
- » Successfully optimized on-resin disulfide bond chemistry with different reagents
- » Successfully applied these optimized strategies to *automate* synthesis of complex, disulfide rich apamin and linacotide peptides
- » Highlighted smart software simplicity for automating syntheses of complex peptides like these and potentially others

Questions?