Automation of Peptide Modifications : A Click-Chemistry Case Study

CEM We Simplify Science

Collaboration with Iris Biotech

Quibria Guthrie, PhD

Research Scientist, Life Science Division

CEM Corporation

Outline

Introduction

i. Peptide Therapeutics
ii. Peptide Synthesis Methods
iii. CEM Corporation Instrumentation

Examples of Peptide Modifications

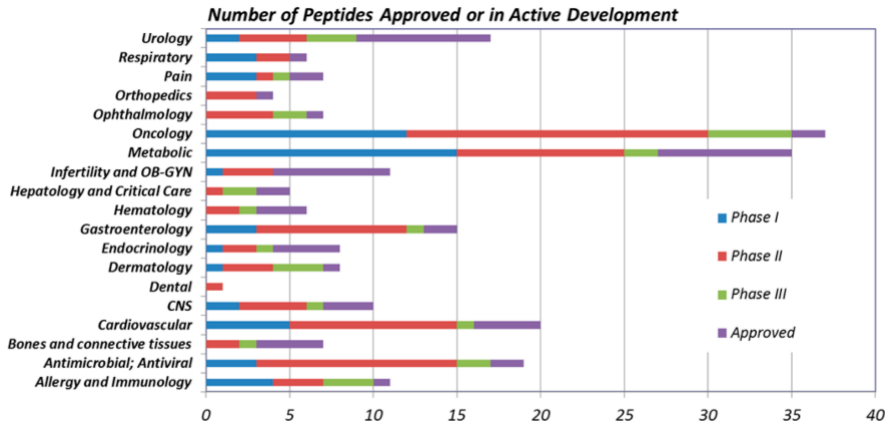
i. Hindered Amino Acid Incorporations
ii. Disulfide Bridging
iii. Hydrocarbon Cyclization
iv. Peptoid Synthesis

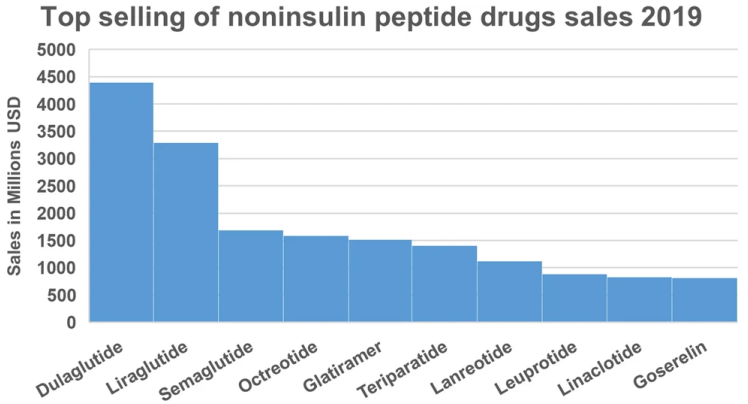
Case Study: Click Chemistry

i. Click Chemistry Introduction
ii. Automation Considerations
iii. Incorporation of Azide- and Alkyne-Containing Monomers
iv. Automation of Click Chemistry

Conclusions and Future Directions

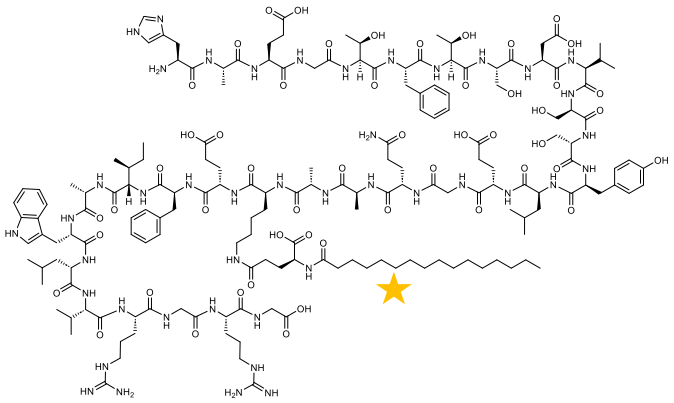
Peptides in the Drug Discovery Pipeline



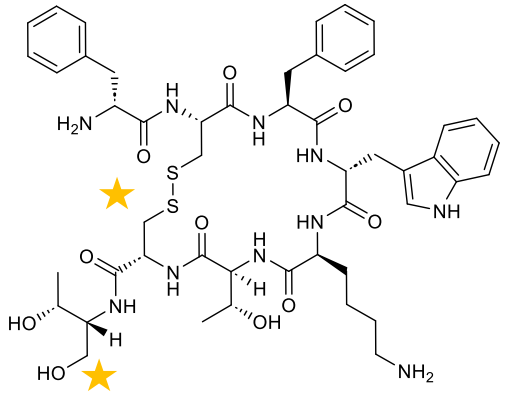


Top-selling non-insulin peptides worldwide in 2019. Data analysis according to Njardarson's group[27]

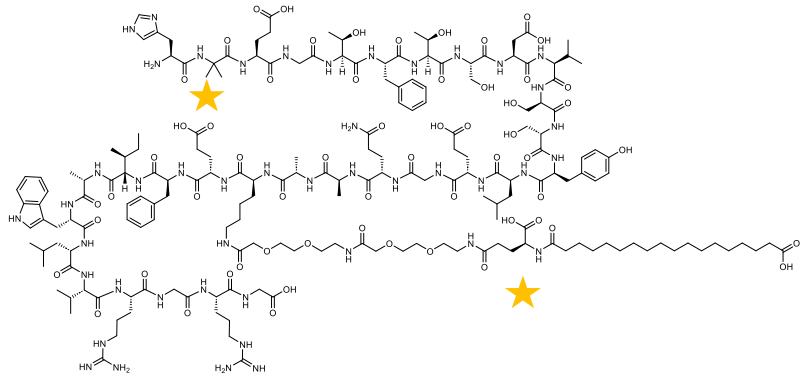
Liraglutide



Octreotide



Semaglutide

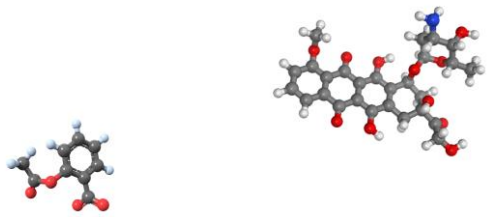


a) Henninot, A.; Collins,J. C.; Nuss, J. M. *J. Med. Chem.* **2018,** *61*, 1382-1414. b) Wang, L., Wang, N., Zhang, W. *et al. ig Transduct Target Ther* .**2022**,*7*, 48.

Peptides as Therapeutics

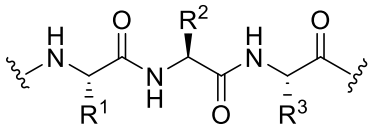
Peptide-based Drugs: Filling the Gap

Small molecules
< 500 Da



- Bioavailability
- Cell Permeability
- Low Cost Manufacturing
- Metabolic Stability

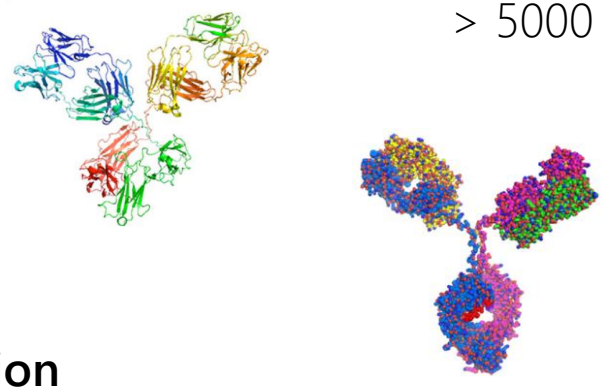
Peptides



Peptidomimetics/Peptide Functionalization

The stability and bioavailability properties of small molecules and selectivity of biologics

Biologics
> 5000 Da

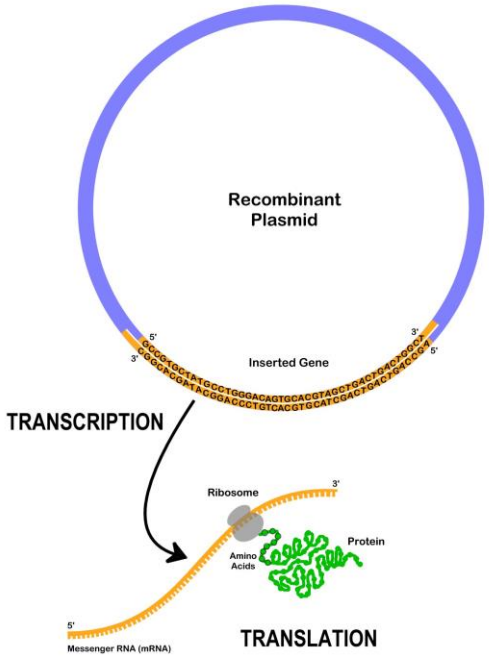


- High Potency
- High Selectivity
- Low Toxicity

Peptide Synthesis Methods

Approaches to Peptide Synthesis:

- **Recombinant Synthesis**



Strengths

- Synthesis of Longer Peptides
- No Insertion and Deletion Impurities

Limitations

- High Cost and Time Requirements
- No Non-Standard AAs or Peptidomimetics

Peptide Synthesis Methods

Approaches to Peptide Synthesis:

- **Recombinant Synthesis**
- **Solution-Phase Synthesis**

Strengths

- Non-Standard AAs or Peptidomimetics
- High Reaction Efficiency

Limitations

- Labor and Time Intensive
- Synthetic Optimization Required

Peptide Synthesis Methods

Approaches to Peptide Synthesis:

- **Recombinant Synthesis**
- **Solution-Phase Synthesis**
- **Solid-Phase Synthesis**

Strengths

- Non-Standard AAs or Peptidomimetics
- High Reaction Efficiency
- Single Purification Required
- Suitable for Automation

Limitations

- Synthetic Optimization Required

CEM Corporation

Leading Provider of Laboratory Microwave Systems

- 45 years designing and manufacturing microwave instrumentation

- Headquarters in Charlotte, North Carolina, USA
  - R&D, Manufacturing, Applications, Sales, and Service
  - Subsidiaries in Germany, UK, France, Italy, Japan, Singapore
  - Global dealer network of more than 70 countries

CEM Corporation

Life Science Division

- Team of more than 15 PhD, MS, and BS chemists
- Instrumentation spanning many applications
  - Proteomics
  - In-Situ Hybridization
  - General Synthesis
  - Peptide Synthesis
  - Purification
- Goal: Develop increasingly effective and efficient SPPS workflow solutions

CEM Corporation's Peptide Synthesizers

CEM Corporation's Peptide Synthesizers

MultiPep 2 – Parallel Peptide Synthesizer

- Exchangeable workspaces
- Cellulose and Resin-based synthesis
- Synthesis scale range of ~4nmol – 300µmol
- Can make up to 2400 peptides on cellulose at a time
- Can make up to 384 peptides on resin at a time
- Fmoc SPPS
- Uronium Chemistry: HBTU/DIEA coupling reagents

CEM Corporation's Peptide Synthesizers

Liberty Blue 2.0 – Microwave Peptide Synthesizer

- Resin-based synthesis
- Synthesis scale range of 0.005 – 5mmol
- HT12 and HT24 upgrades allow for up to 24 sequential peptides
- Fmoc SPPS
- 4 minute standard coupling times
- Carbodiimide Approach: Oxyma/DIC coupling reagents

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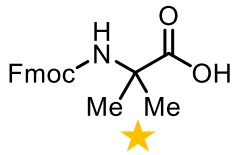
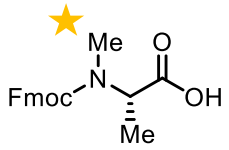
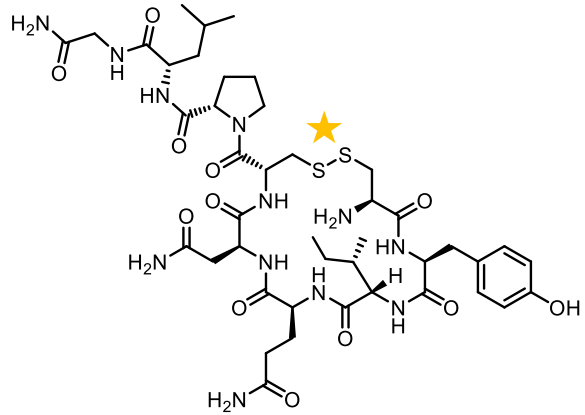
Peptide Modification Examples

Hindered Amino Acid Incorporation

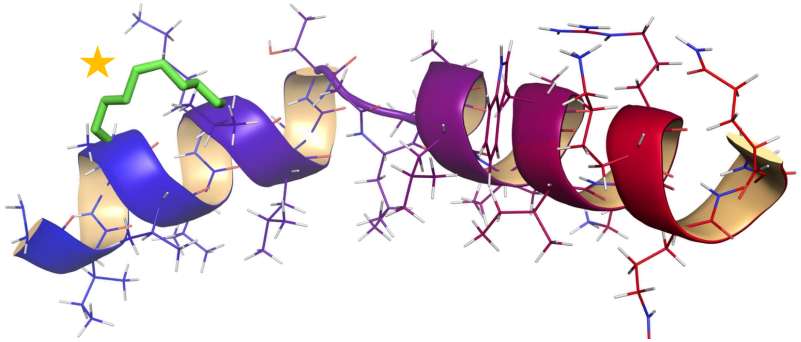




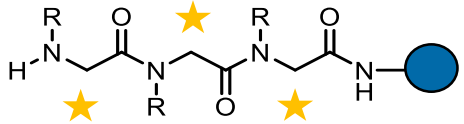
Disulfide Bridging



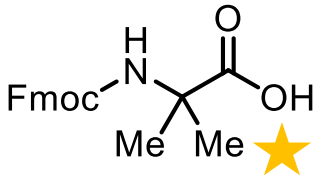
Hydrocarbon Stapling



Peptoid Synthesis



Hindered Amino Acid Incorporation



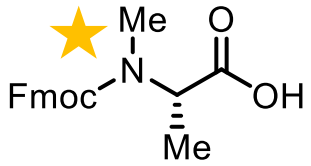
Fmoc-Aib-OH

CAS No. 94744-50-0

Product Code: FAA1613

Iris Biotech

- Induces α-Helix Formation



Fmoc-*N*-Me-Ala-OH

CAS No. 84000-07-7

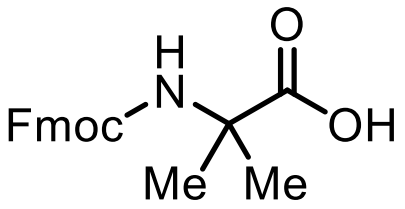
Product Code: FAA1395

Iris Biotech

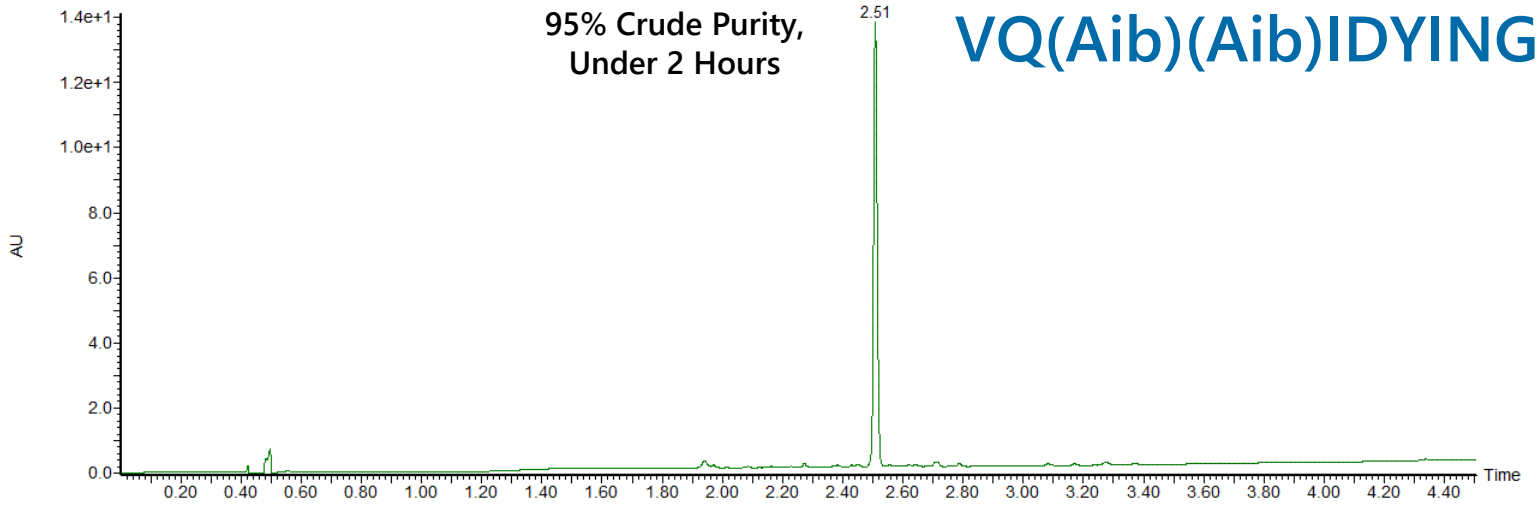
- Increases Protease Resistance
- Alters Secondary Structure

- **Hindered AA to Natural AA N-Terminus → Single Coupling**
  90°C, 2 Min
- **Hindered AA to Hindered AA N-Terminus → Double Coupling**
  90°C, 5 Min
- **Natural AA to Hindered AA N-Terminus → Double Coupling**
  90°C, 2 Min

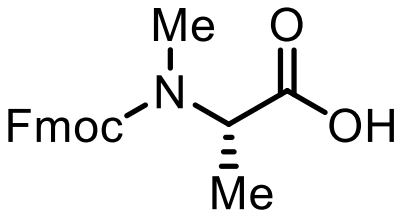
Hindered Amino Acid Incorporation



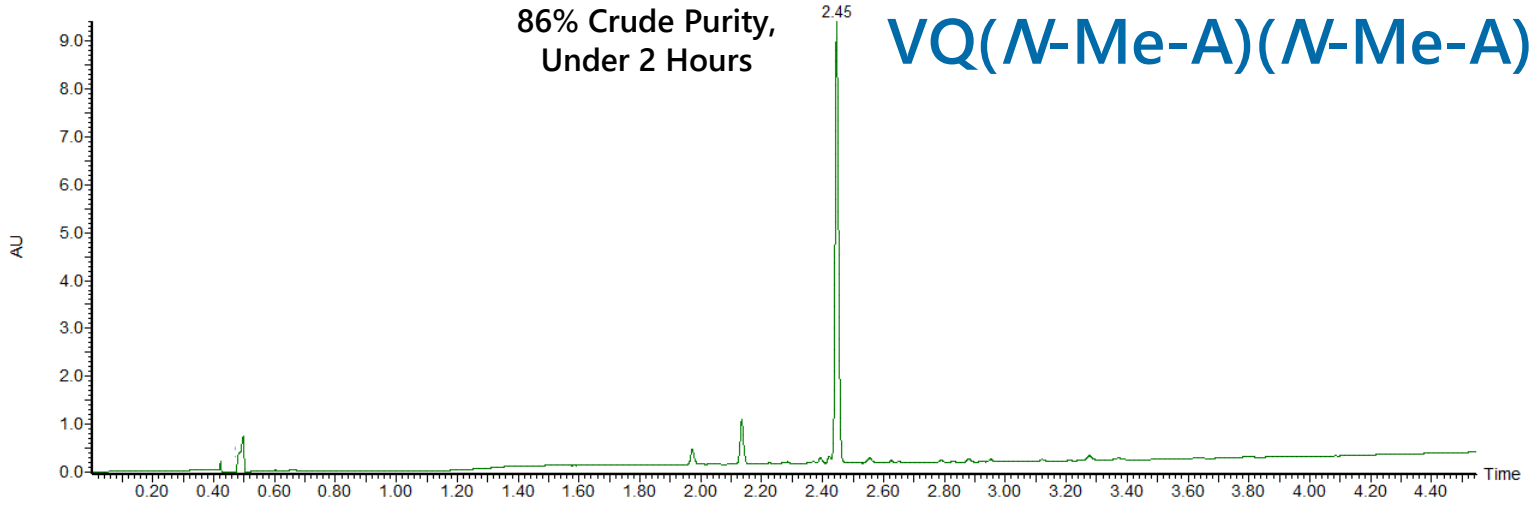
Fmoc-Aib-OH



VQ(Aib)(Aib)IDYING

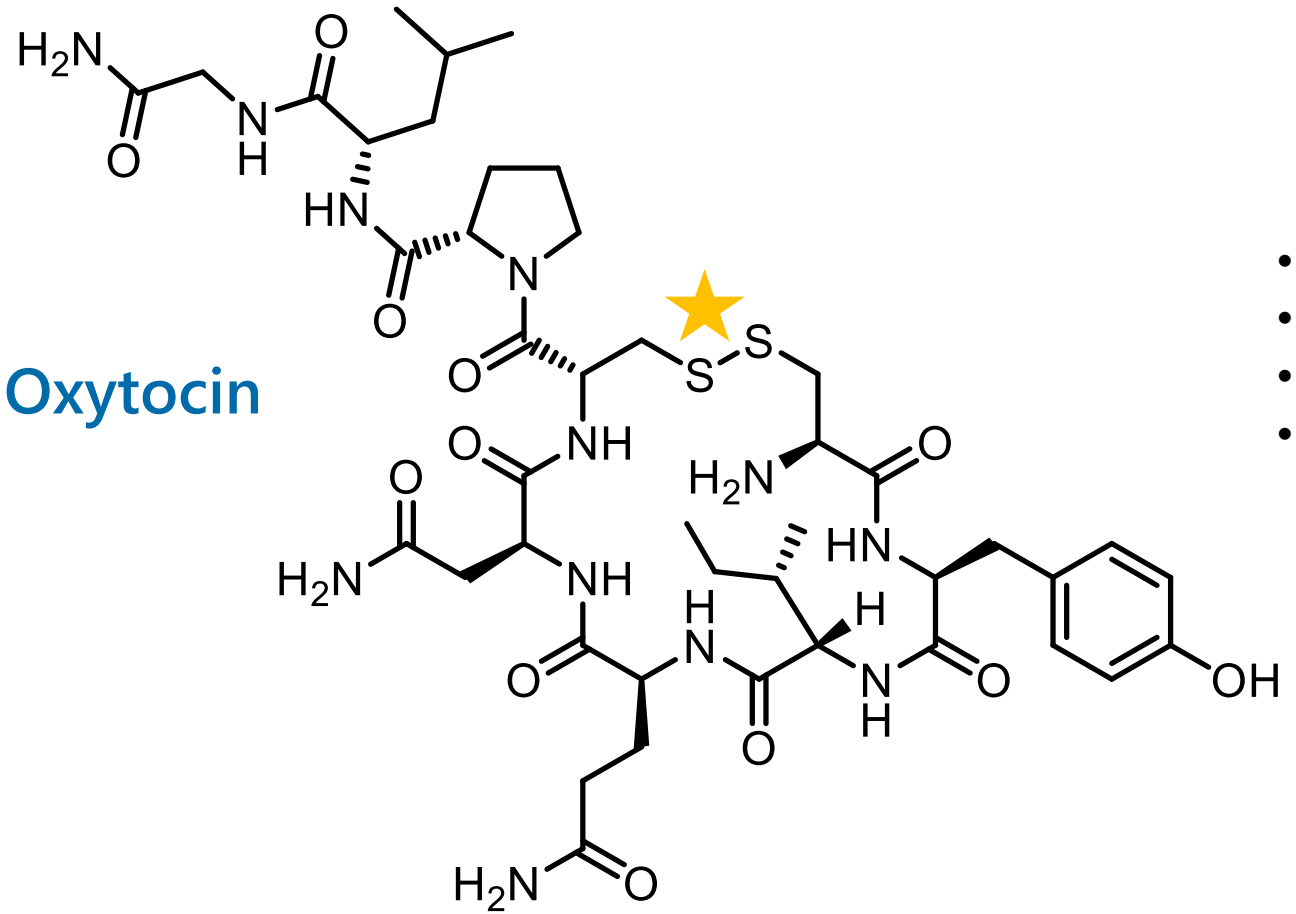


Fmoc-*N*-Me-Ala-OH



VQ(*N*-Me-A)(*N*-Me-A)IDYING

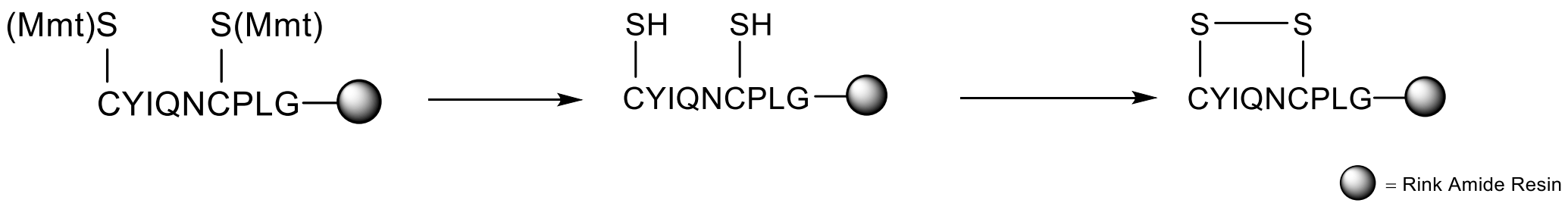
Disulfide Bridging

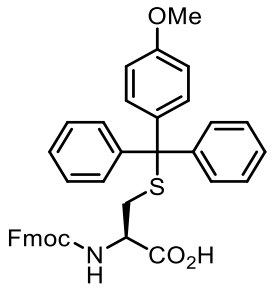


Oxytocin

- Prevalent in Biologically Active Compounds
- Stabilizes Secondary Structure
- Increases Protease Resistance
- Increases Target Affinity

Disulfide Bridging



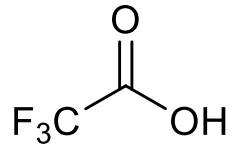


Fmoc-Cys(Mmt)-OH

Orthogonally Protected Cys Derivative

CAS No. 177582-21-7

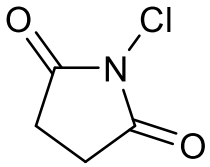
Product Code: FAA1030



Trifluoroacetic acid (TFA)

Mmt Deprotection Reagent

CAS No. 177582-21-7



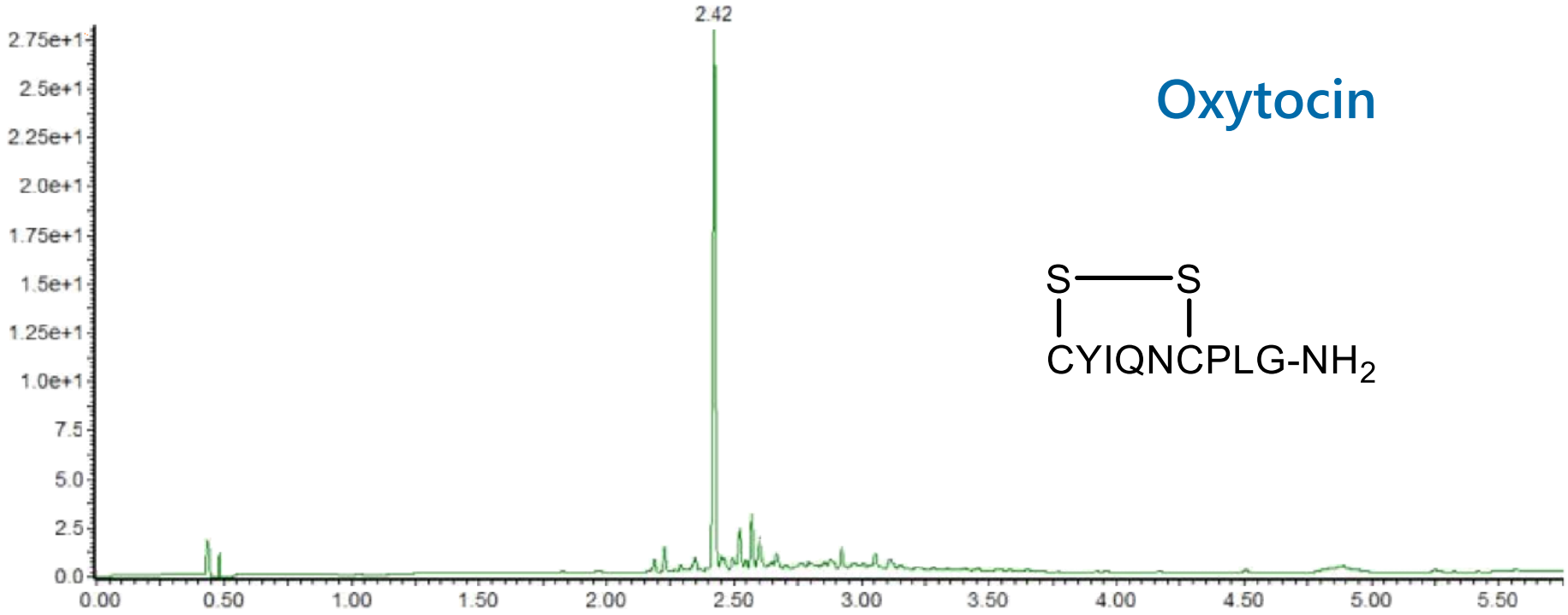
N-Chlorosuccinimide (NCS)

Thio Oxidation Reagent

CAS No. 177582-21-7

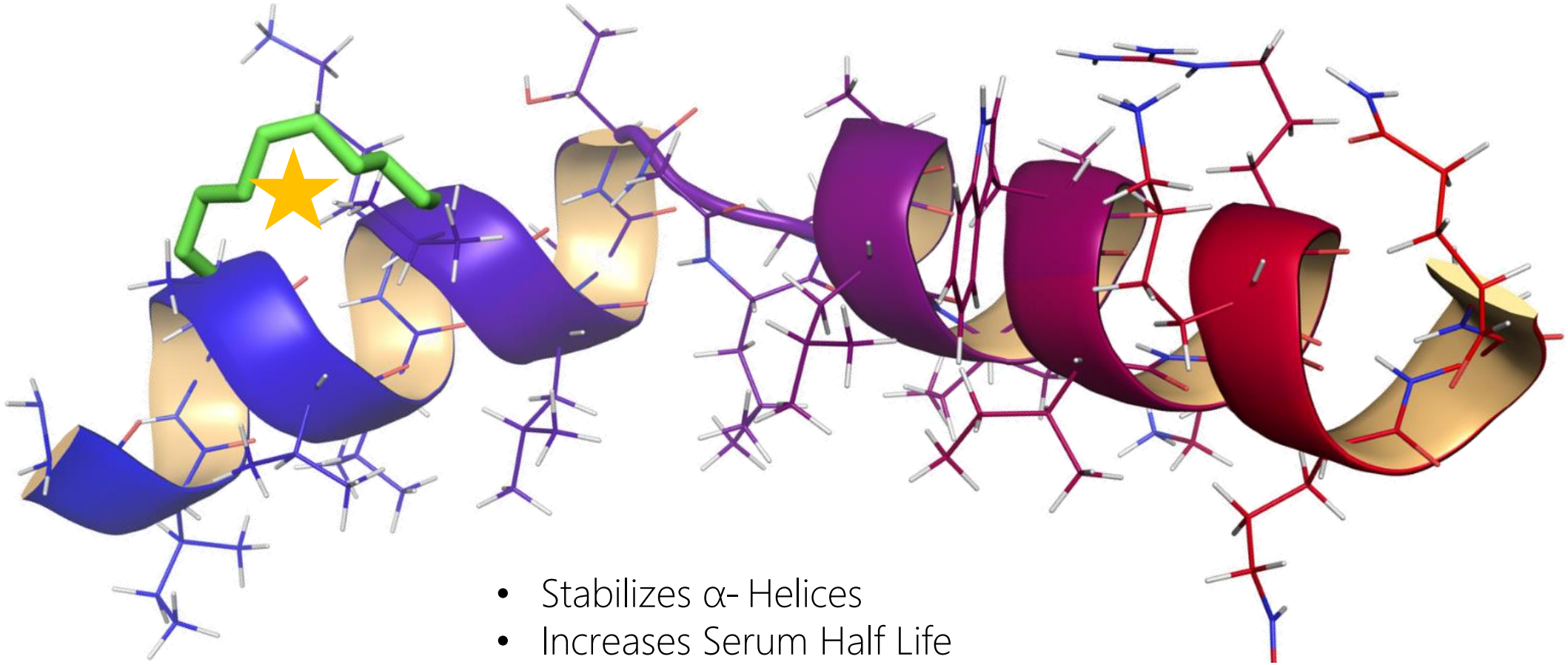
Disulfide Bridging

69% Crude Purity,
Under 3 Hours



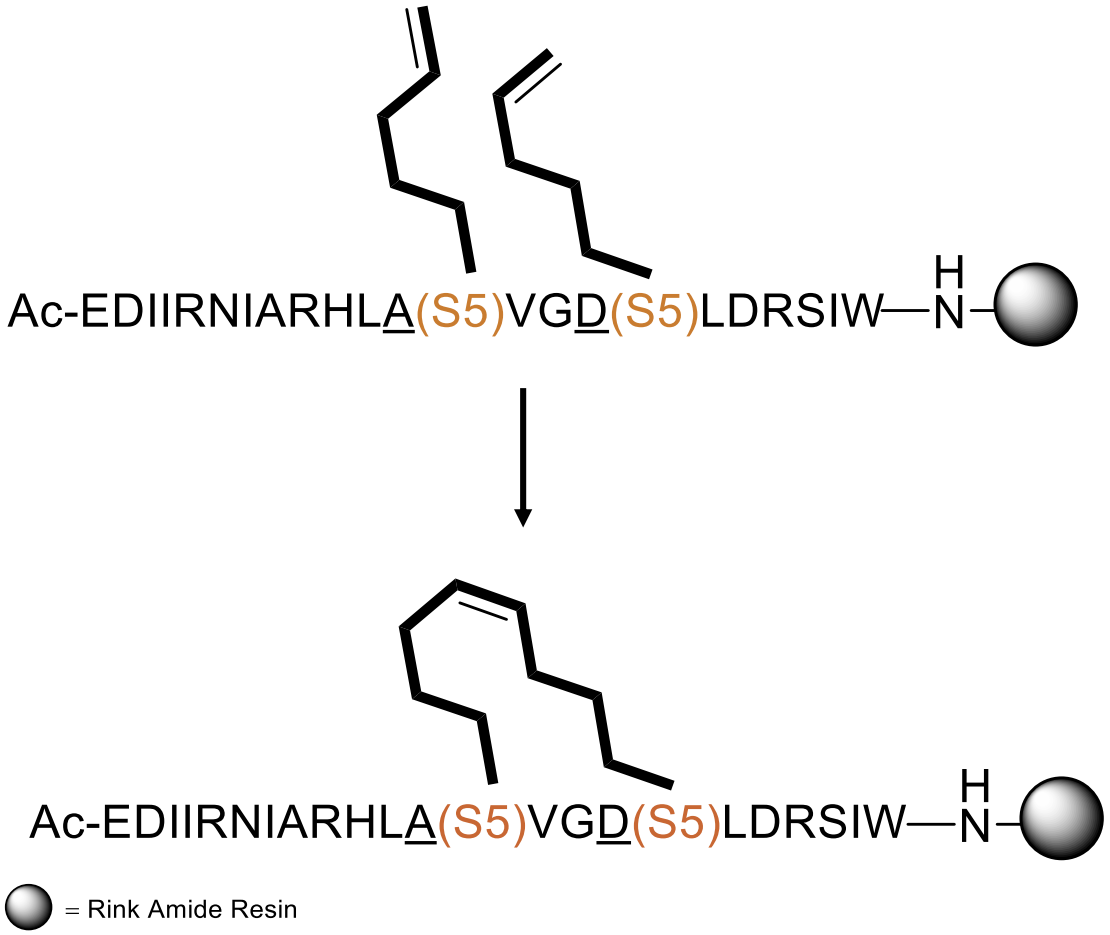
Oxytocin

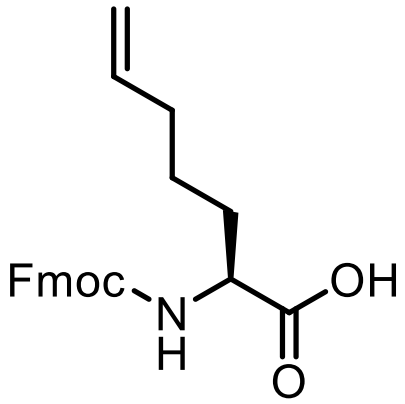
Hydrocarbon Stapling



- Stabilizes α- Helices
- Increases Serum Half Life
- Increases Cell Permeability

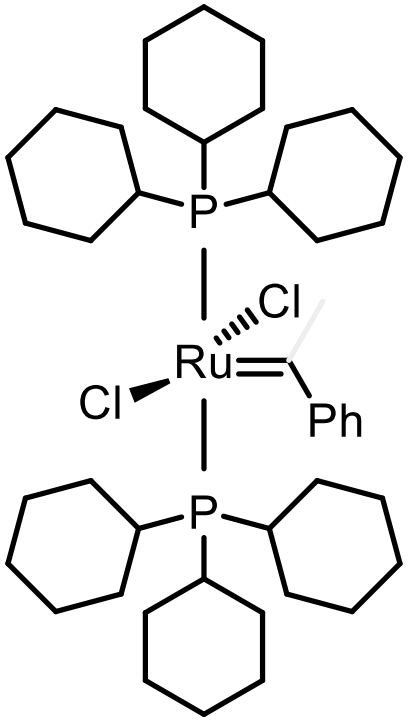
Hydrocarbon Stapling





Fmoc-(*S*)-2-(4-pentenyl)Ala-OH (S5)

Alkenyl Building Block

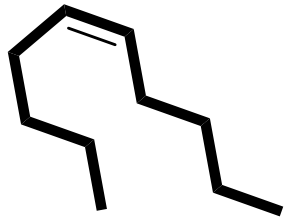


Grubb's Catalyst

Ring Closing Metathesis Catalyst

Hydrocarbon Stapling

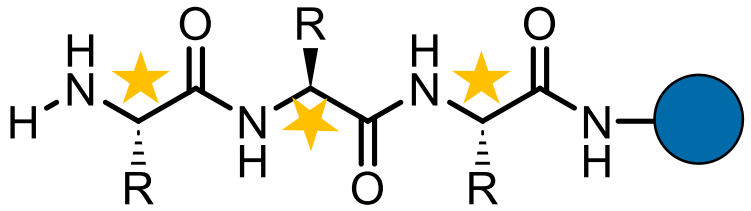
Hydrocarbon-Stapled BID SAHB



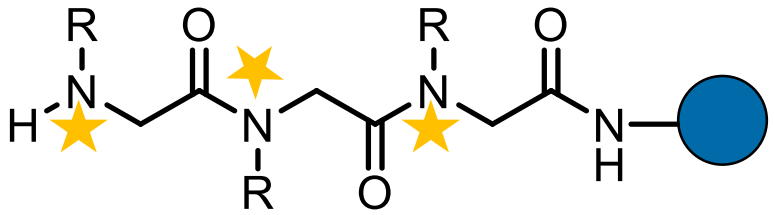
Ac-EDIIRNIARHLA(S5)VGD(S5)LDRSIW-NH_2

80% Crude Purity,
Under 4 Hours

Peptoid Synthesis



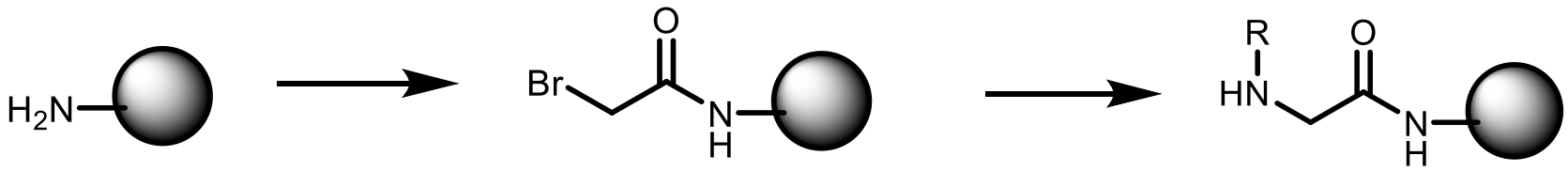
Peptide

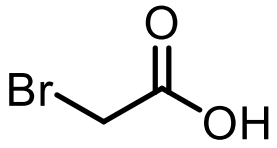


Peptoid

- Access to Unique Secondary Structure
- Increases Protease Resistance
- Increases Target Affinity and Potency

Peptoid Synthesis



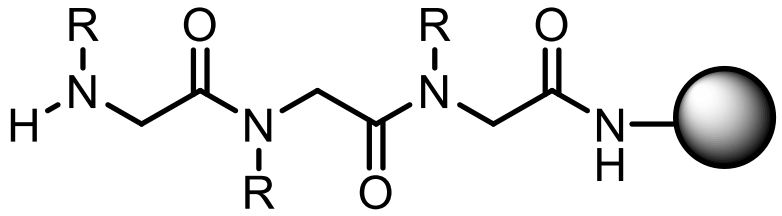


Bromoacetic Acid

Acylation of N-Terminus

Primary Amine

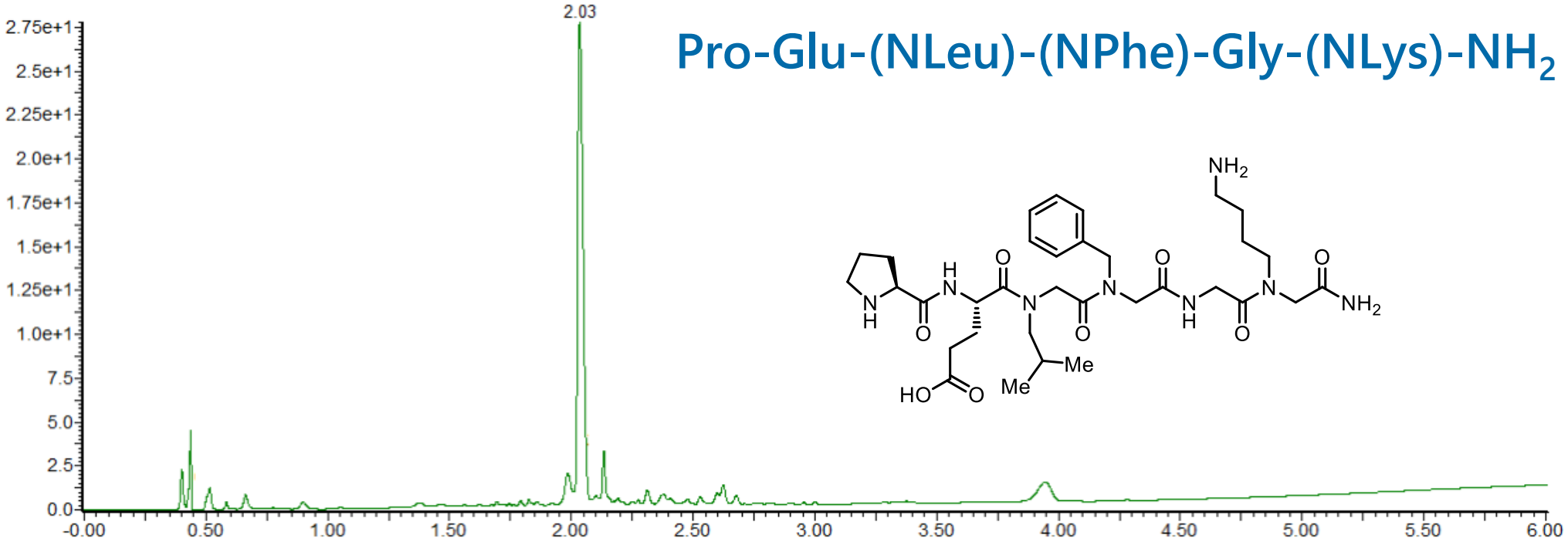
Displacement of Bromide



= Rink Amide Resin

Peptoid Synthesis

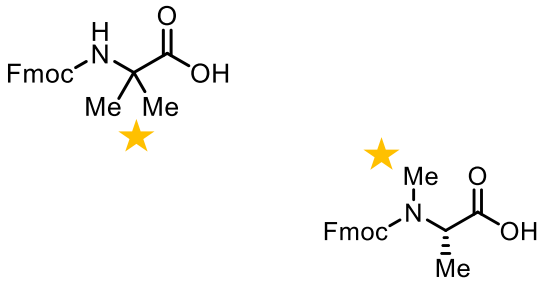
81% Crude Purity,
Under 2 Hours



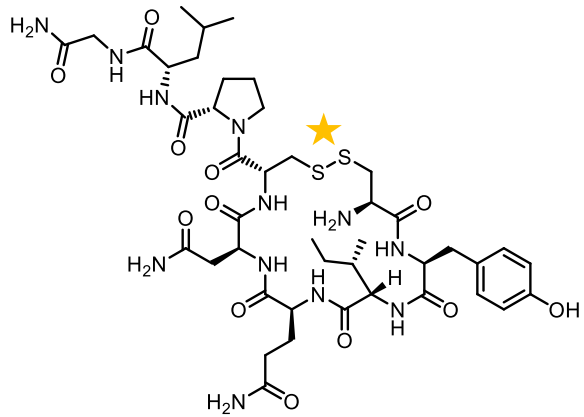
Pro-Glu-(NLeu)-(NPhe)-Gly-(NLys)-NH_2

Peptide Modification Examples

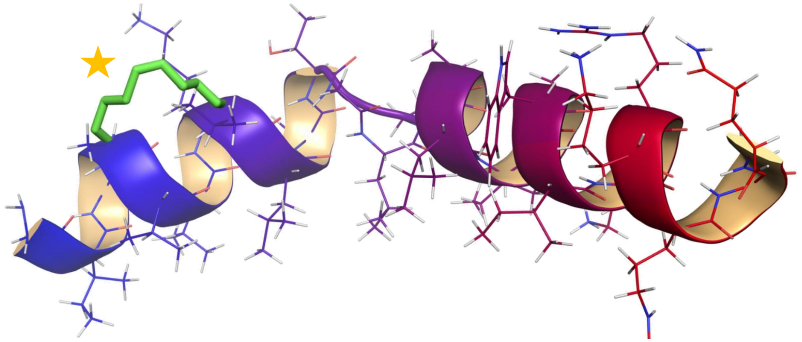
Hindered Amino Acid Incorporation



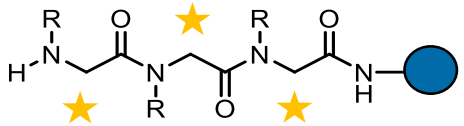
Disulfide Bridging



Hydrocarbon Stapling



Peptoid Synthesis



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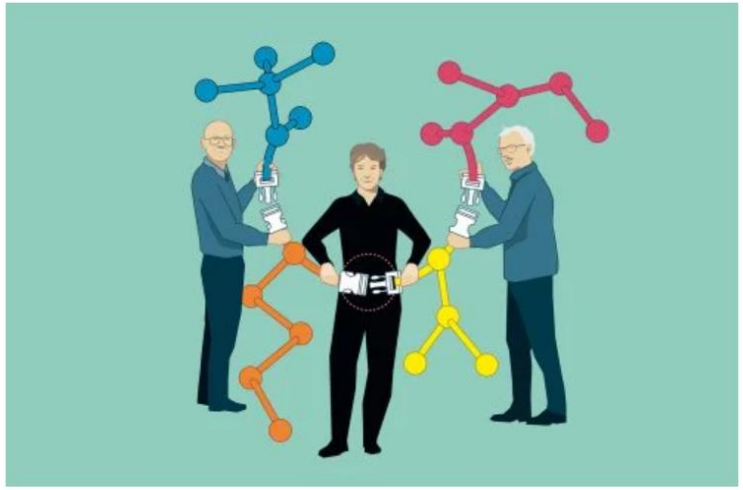
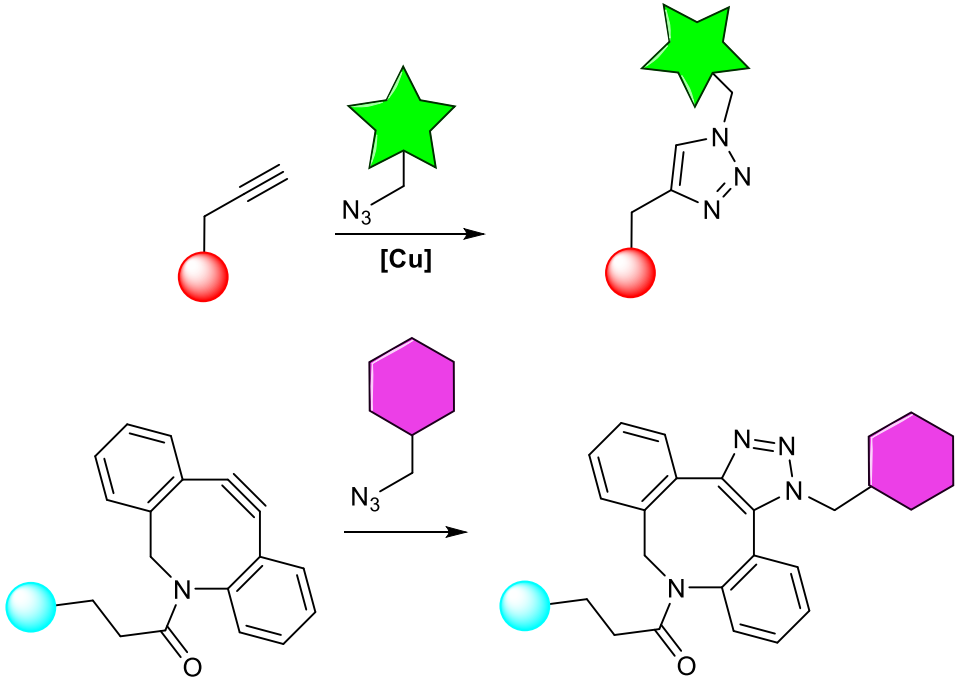


© Johan Jarnestad/The Royal Swedish Academy of Sciences

Barry Sharpless, Morten Meldal and Carolyn Bertozzi, were awarded the Nobel Prize in Chemistry 2022 for their click chemistry work!



- Orthogonal and biostable functional groups
- Strain-promoted systems allow for copper free click chemistry conditions

https://www.nobelprize.org/prizes/chemistry/2022/popular-information/

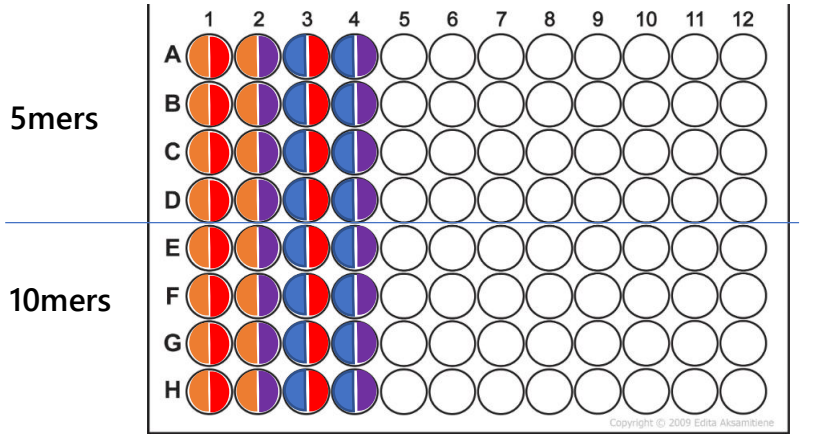
Parallel Library Synthesis

Variables

Sequence Length
- 5mer (4 each)
- 10mer (4 each)

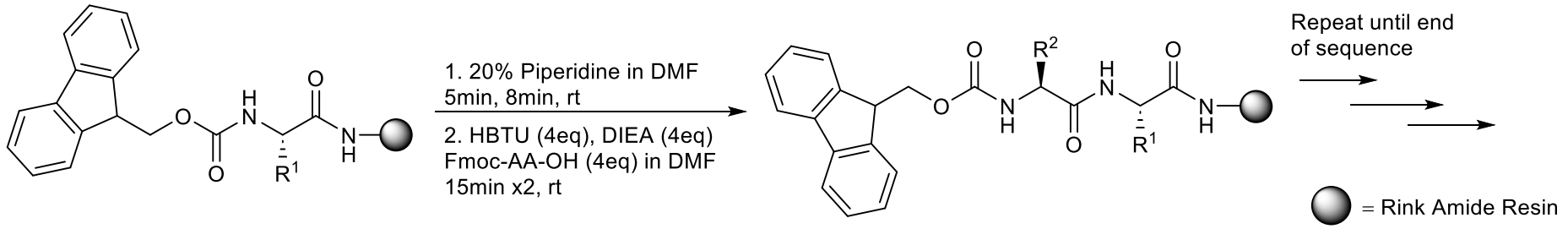
Location of Functional Group in Sequence
- N-Term (N)
- Middle (M)

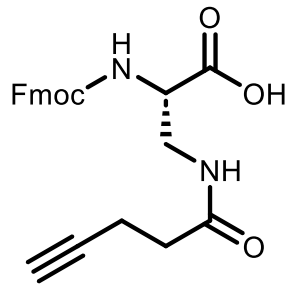
Which Functional Group is on Peptide
- Alkyne (X)
- Azide (Z)



Random Peptide Sequence Generator: https://www.bioinformatics.org/sms2/random_protein.html

Parallel Library Synthesis





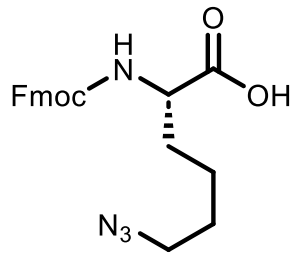
Fmoc- L Dap(Pentynoyl)-OH

Alkyne Monomer

CAS No. 2250436-47-4

Product Code: FAA4170

Iris Biotech



Fmoc- L-Lys(N_3)-OH

Azide Monomer

CAS No. 159610-89-6

Product Code: FAA4170

Iris Biotech

Incorporation of click monomers at room temperature

Alkyne-containing peptides

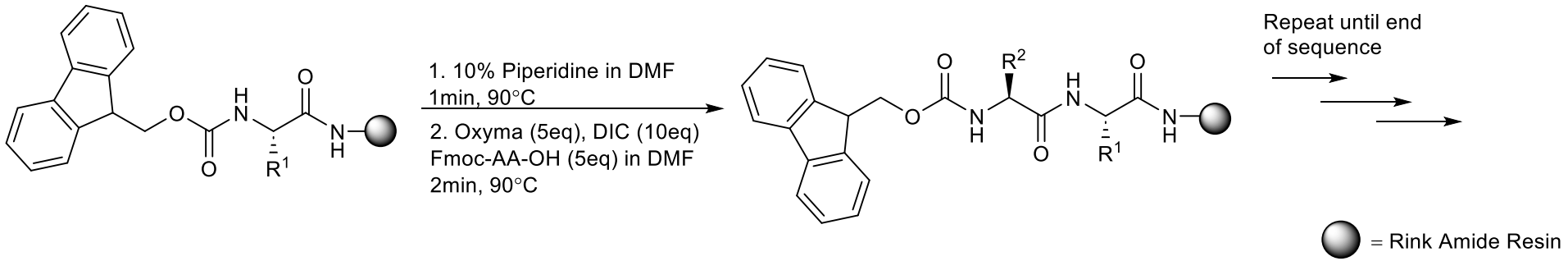
Peptide Sequences	Crude Purity (%)
XTFYN	78
XVLTI	70
XGYAQ	90
XDEIY	65
TFXYN	92
VLXTI	95
GYXAQ	76
DEXIY	77

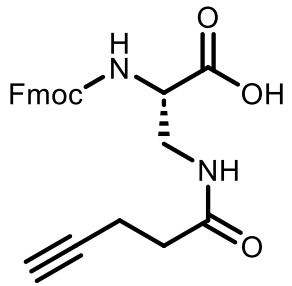
Azide-containing peptides

Peptide Sequences	Crude Purity (%)
ZTFYN	87
ZVLTI	80
ZGYAQ	85
ZDEIY	58
TFZYN	64
VLZTI	84
GYZAQ	74
DEZIY	47

- Notice that 10mer peptide crude purities were relatively low.
  - Could be a sequence dependent issue/ synthesis optimization may be required.

Microwave synthesis





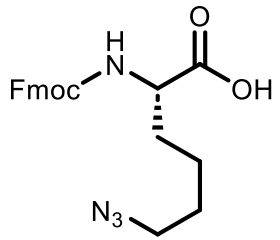
Fmoc- L Dap(Pentynoyl)-OH

Alkyne Monomer

CAS No. 2250436-47-4

Product Code: FAA4170

Iris Biotech



Fmoc- L-Lys(N_3)-OH

Azide Monomer

CAS No. 159610-89-6

Product Code: FAA4170

Iris Biotech

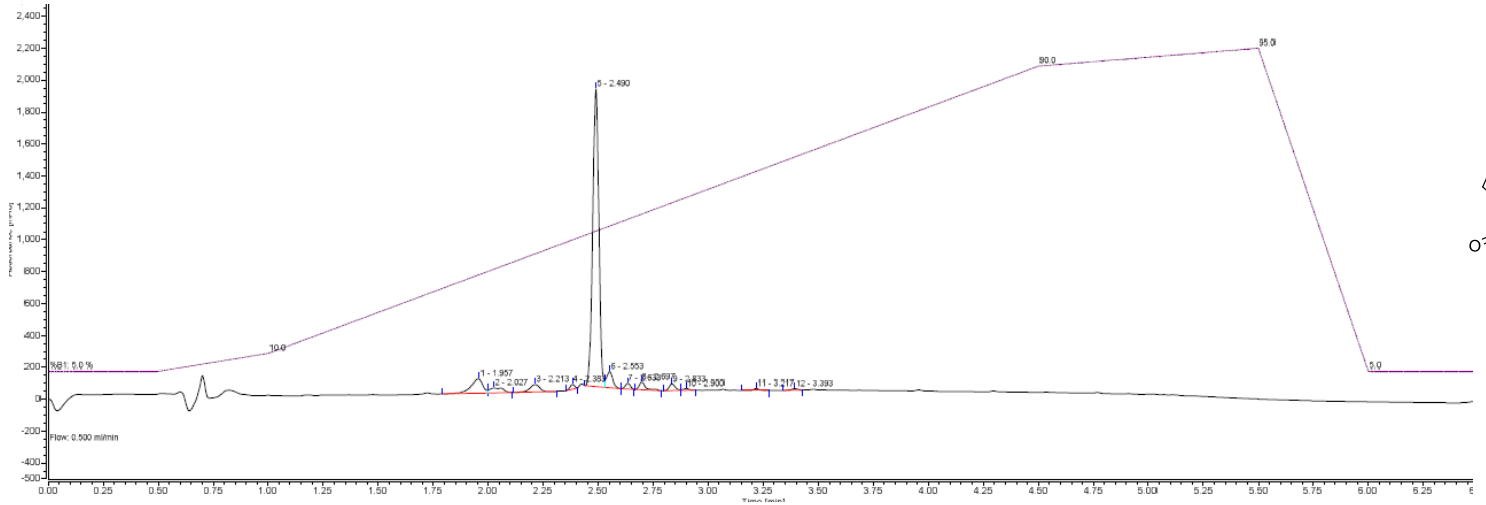
Incorporation of click monomers at elevated temperatures

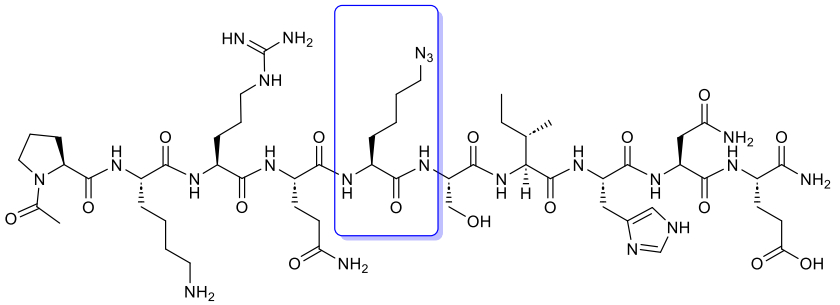
Alkyne-containing peptides

Peptide Sequences	Crude Purity (%)
XTFYN	90
TFXYN	92
XPKRQSIHNE	84
PKRQXSIHNE	85

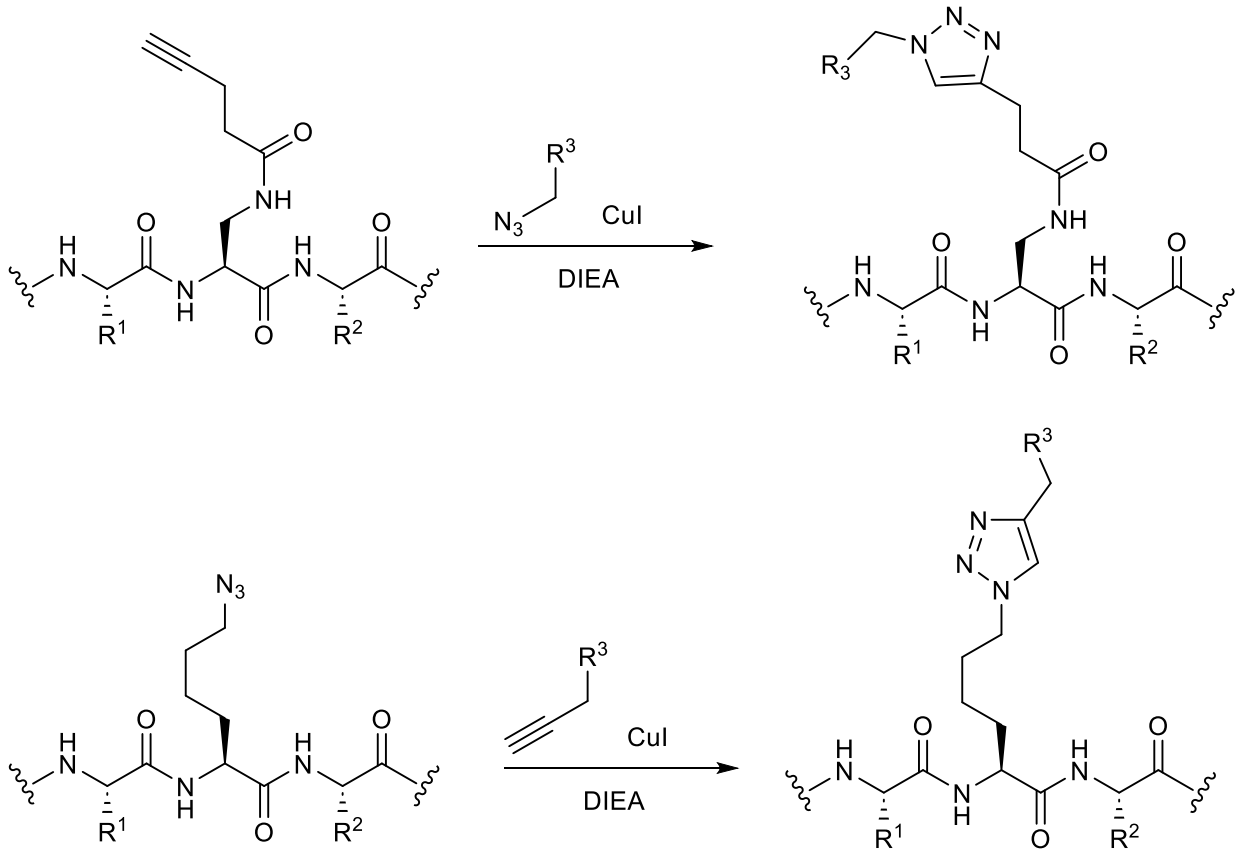
Azide-containing peptides

Peptide Sequences	Crude Purity (%)
ZTFYN	79
TFZYN	79
ZPKRQSIHNE	-
PKRQZSIHNE	76





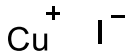
Click chemistry Considerations for Automation



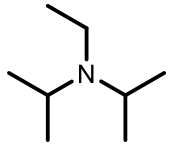
Things to consider when trying new chemistry on an automated synthesizer:

- Could the reagents harm the instrument?
- Solubility of all reagents
- Stability of all reagents
- Scalability of the chemistry

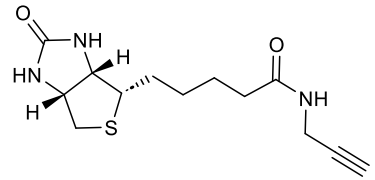
Reagent Solubility in Various Solvents



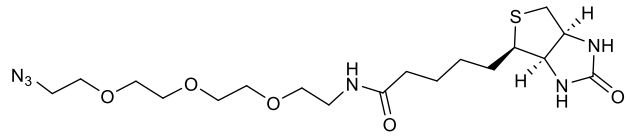
Copper Iodide
Copper Catalyst
CAS No. 773888-45-2



N,N-Diisopropylethyl amine (DIEA)
Base
CAS No. 773888-45-2



Biotin-Propargylamide
Azide click partner
CAS No. 773888-45-2
Product Code: RL-3490



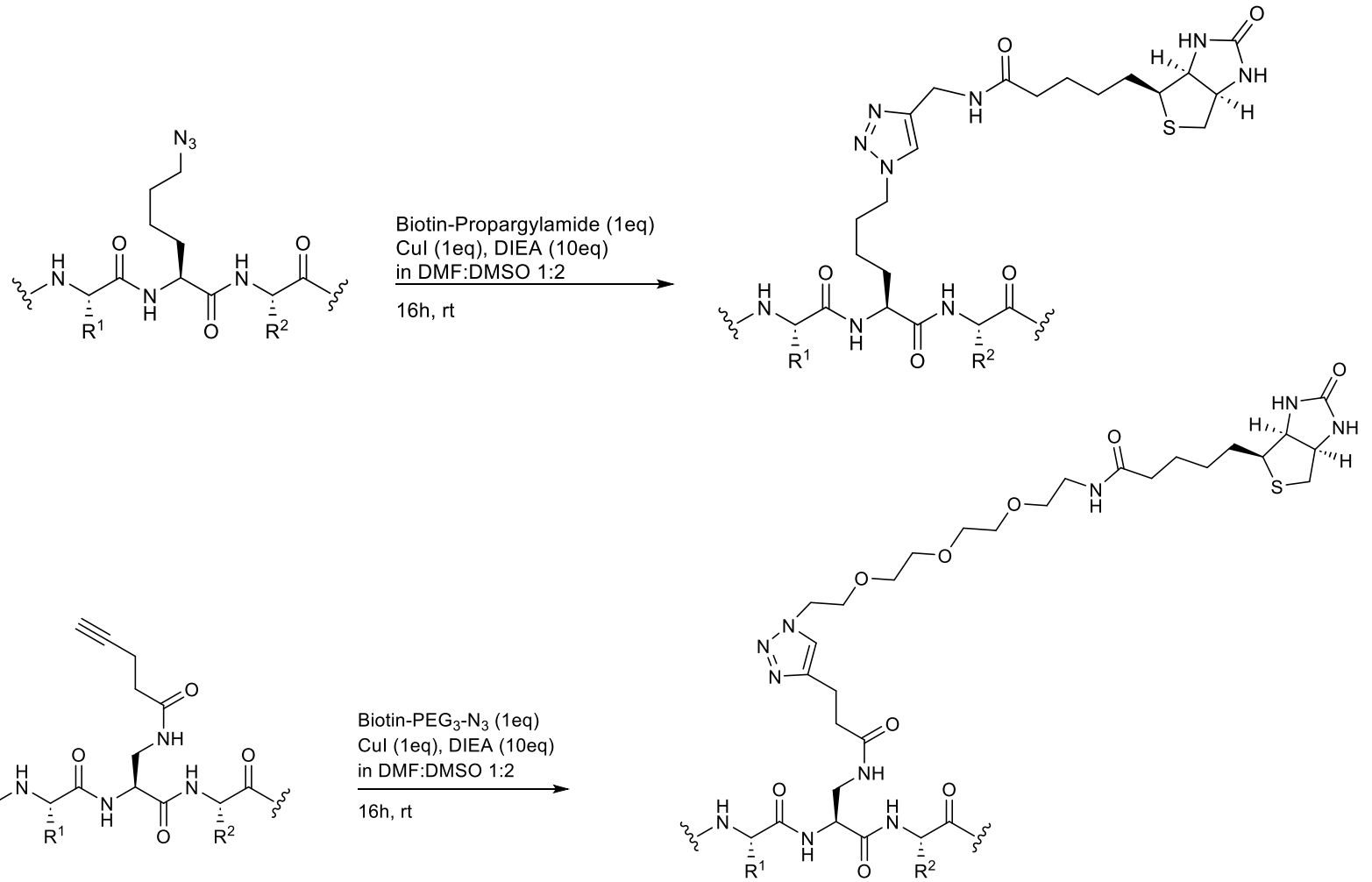
Biotin-PEG(3)-N3
Alkyne click partner
CAS No. 875770-34-6
Product Code: PEG4940

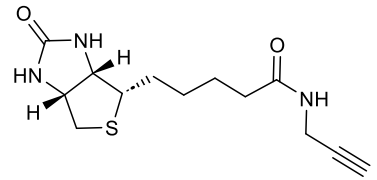
Reagents	DMF	NMP	THF	DMSO	DMF:DMSO 1:2
CuI	✗	✗	✗	✓	✓
DIEA	✓	✓	-	✗	✓
Biotin-PEG_3-N_3	✗	✗	✗	✓	✓
Biotin-Propargylamide	✗	✗	✗	✓	⚠

⚠

- Solubility became an issue when scaling up from MultiPep to Liberty Blue 2.0.
- Due to the sensitivity of Biotin Propargylamide with DMF, for 0.1mmol scale, DIEA was added neat.

Click Chemistry on MultiPep2 at Room Temperature





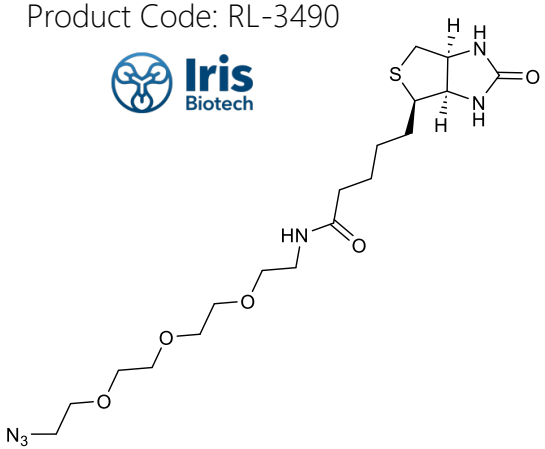
Biotin-Propargylamide

Azide click partner

CAS No. 773888-45-2

Product Code: RL-3490

Iris Biotech



Biotin-PEG(3)-N3

Alkyne click partner

CAS No. 875770-34-6

Product Code: PEG4940

Iris Biotech

Click Chemistry at Room Temperature

Alkyne-containing peptides

Peptide Sequences	Crude Purity (%)
XTFYN	70
XVLTI	50
XGYAQ	80
XDEIY	72
TFXYN	72
VLXTI	52
GYXAQ	77
DEXIY	74

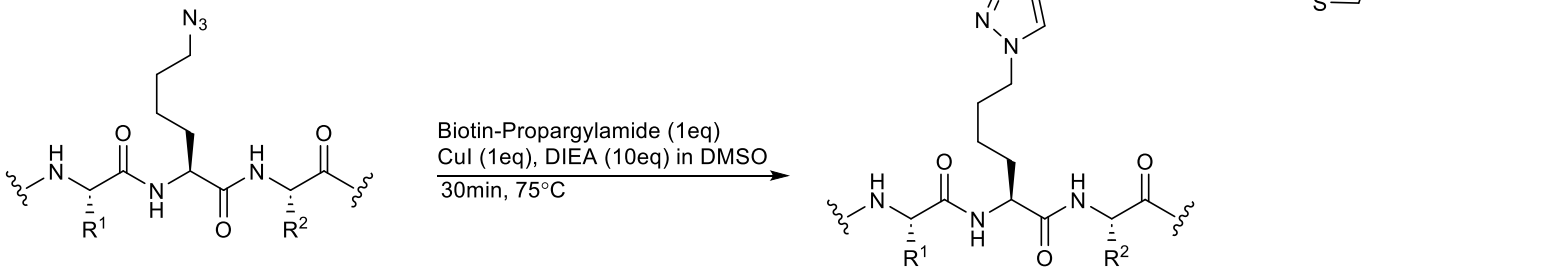
Azide-containing peptides

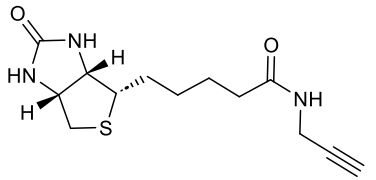
Peptide Sequences	Crude Purity (%)
ZTFYN	66
ZVLTI	55
ZGYAQ	73
ZDEIY	54
TFZYN	84
VLZTI	84
GYZAQ	77
DEZIY	78

Click Chemistry on Liberty Blue 2.0 at Elevated Temperature

Temperature	Time													
T = Conventional	1 h	2 h	3 h	4 h	6 h	8 h	10 h	12 h	16 h	18 h	24 h	48 h	96 h	172 h
T + 10 °C	30 min	1 h	1.5 h	2 h	3 h	4 h	5 h	6 h		9 h	12 h	24 h	48 h	96 h
T + 20 °C	15 min	30 min	45 min	1 h	1.5 h	2 h	2.5 h	3 h		5 h	6 h	12 h	24 h	48 h
T + 30 °C	8 min	15 min	23 min	30 min	45 min	1 h	75 min	1.5 h		2.5 h	3 h	6 h	12 h	24 h
T + 40 °C	4 min	8 min	12 min	15 min	23 min	30 min	38 min	45 min		75 min	1.5 h	3 h	6 h	12 h
T + 50 °C									30 min	38 min	45 min	1.5 h	3 h	6 h
T + 60 °C	1 min	2 min	3 min	4 min	6 min	8 min	10 min	12 min	15 min	20 min	23 min	45 min	1.5 h	3 h
T + 70 °C		1 min	2 min	2 min	3 min	4 min	5 min	6 min	8 min	10 min	12 min	23 min	45 min	1.5 h
T + 80 °C			1 min	1 min	2 min	2 min	3 min	3 min	4 min	5 min	6 min	12 min	23 min	45 min

Click Chemistry on Liberty Blue 2.0 at Elevated Temperature





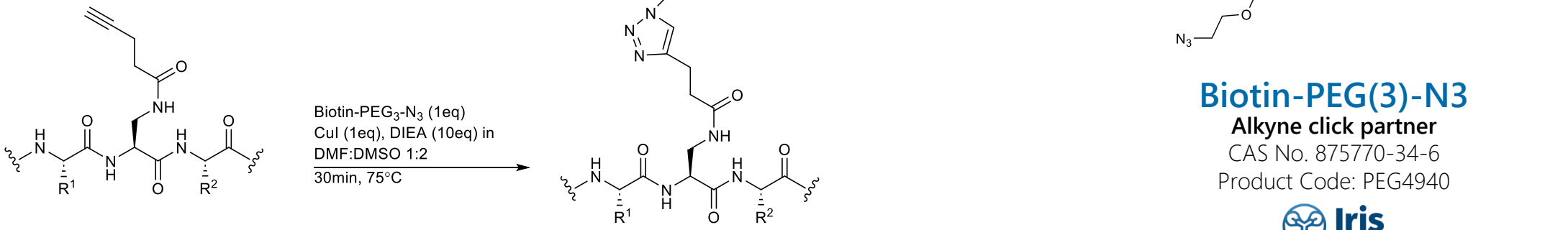
Biotin-Propargylamide

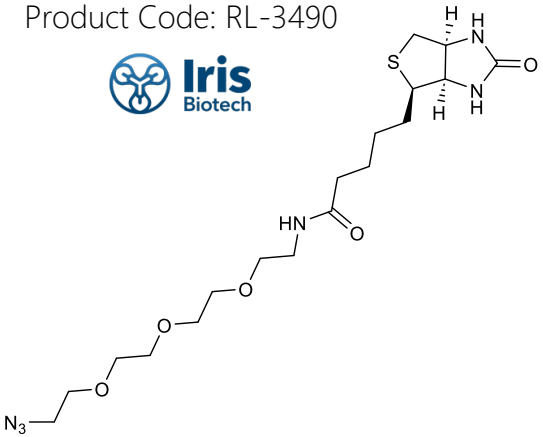
Azide click partner

CAS No. 773888-45-2

Product Code: RL-3490

Iris Biotech





Biotin-PEG(3)-N3

Alkyne click partner

CAS No. 875770-34-6

Product Code: PEG4940

Iris Biotech

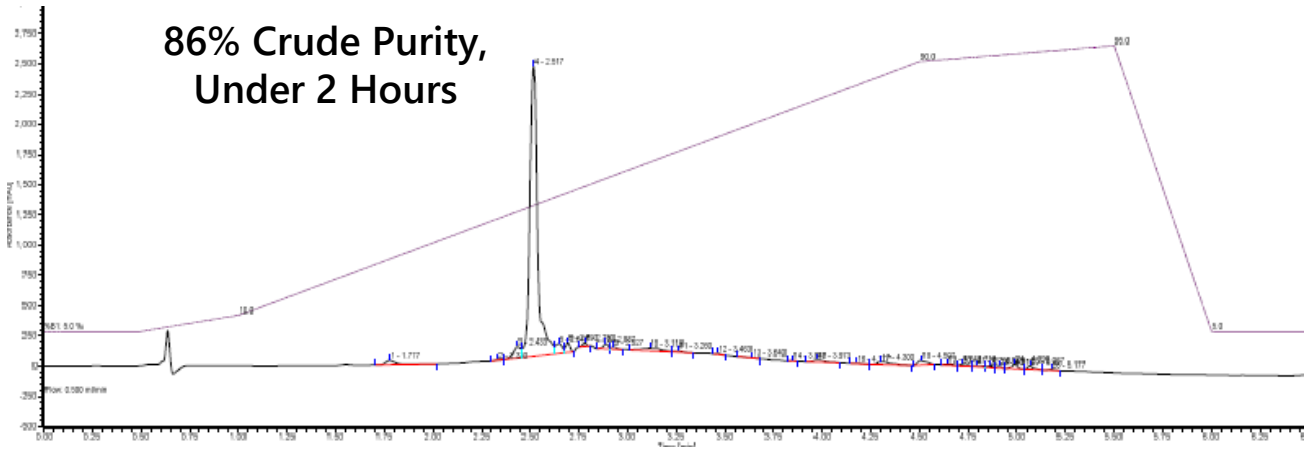
Click Chemistry at Elevated Temperature

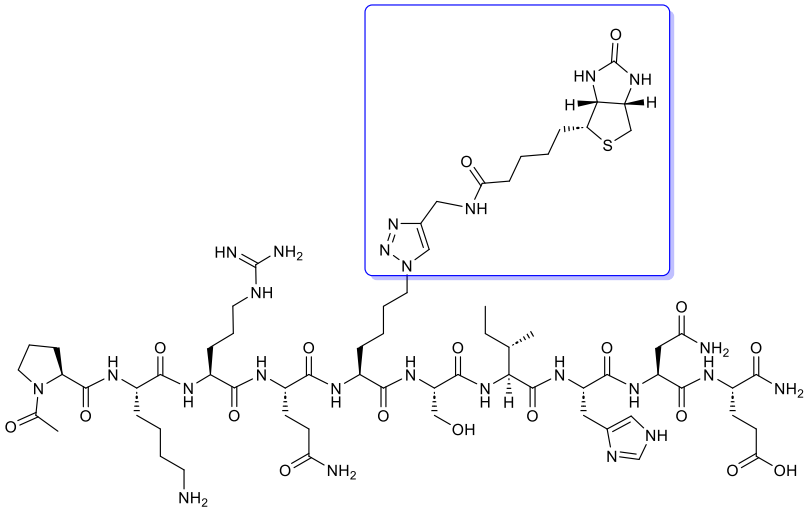
Alkyne-containing peptides

Peptide Sequences	Crude Purity (%)
TFXYN	74
PKRQXSIHNE	80

Azide-containing peptides

Peptide Sequences	Crude Purity (%)
TFZYN	61
PKRQZSIHNE	86





Up Next

Introduction

i. Peptide Therapeutics
ii. Peptide Synthesis Methods
iii. CEM Corporation Instrumentation

Examples of Peptide Modifications

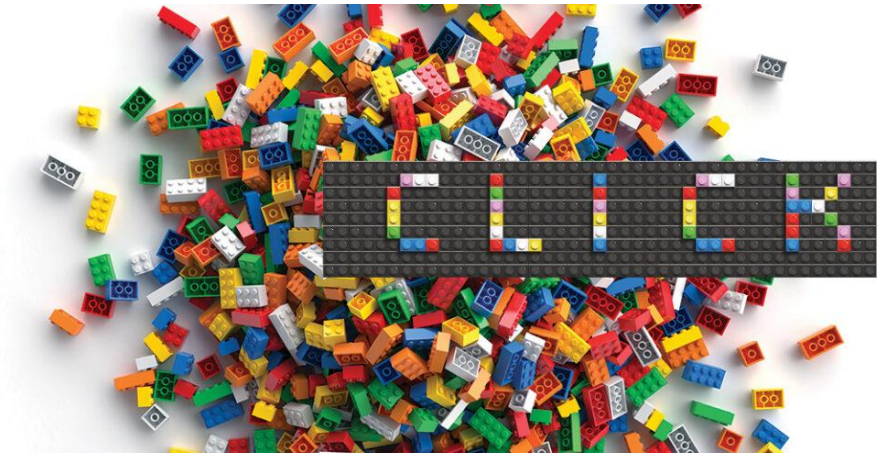
i. Hindered Amino Acid Incorporations
ii. Disulfide Bridging
iii. Hydrocarbon Cyclization
iv. Peptoid Synthesis

Case Study: Click Chemistry

i. Click Chemistry introduction
ii. Automation Considerations
iii. Incorporation of Azide- and Alkyne-containing monomers
iv. Automation of Click Chemistry

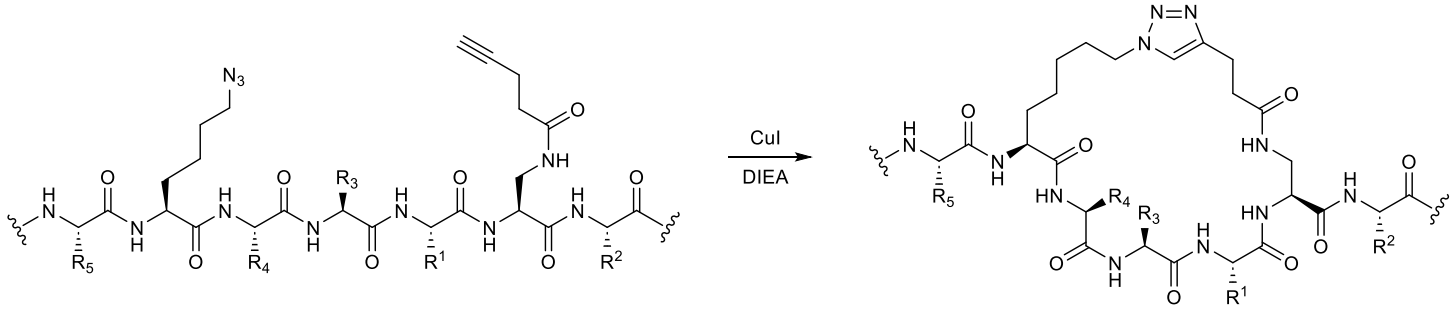
Conclusions and Future Directions

Conclusions

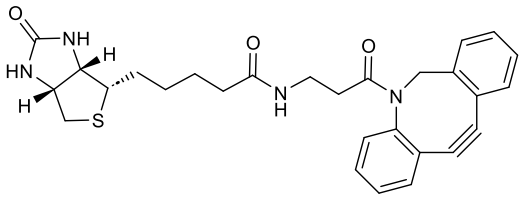


- Incorporation of azide- and alkyne-containing monomers can be achieved at both room temperature and elevated temperature
- Click chemistry was shown to be accessible at room temperature overnight
- Click chemistry can also be achieved at an elevated temperature in as little as 30 minutes.
- The placement of azide vs alkyne and location of the click chemistry seem to have little effect on crude purity.

Future Directions



- Further optimize reaction conditions
- Macrocyclization via click chemistry
- Strained-promoted click chemistry



Biotin-DBCO

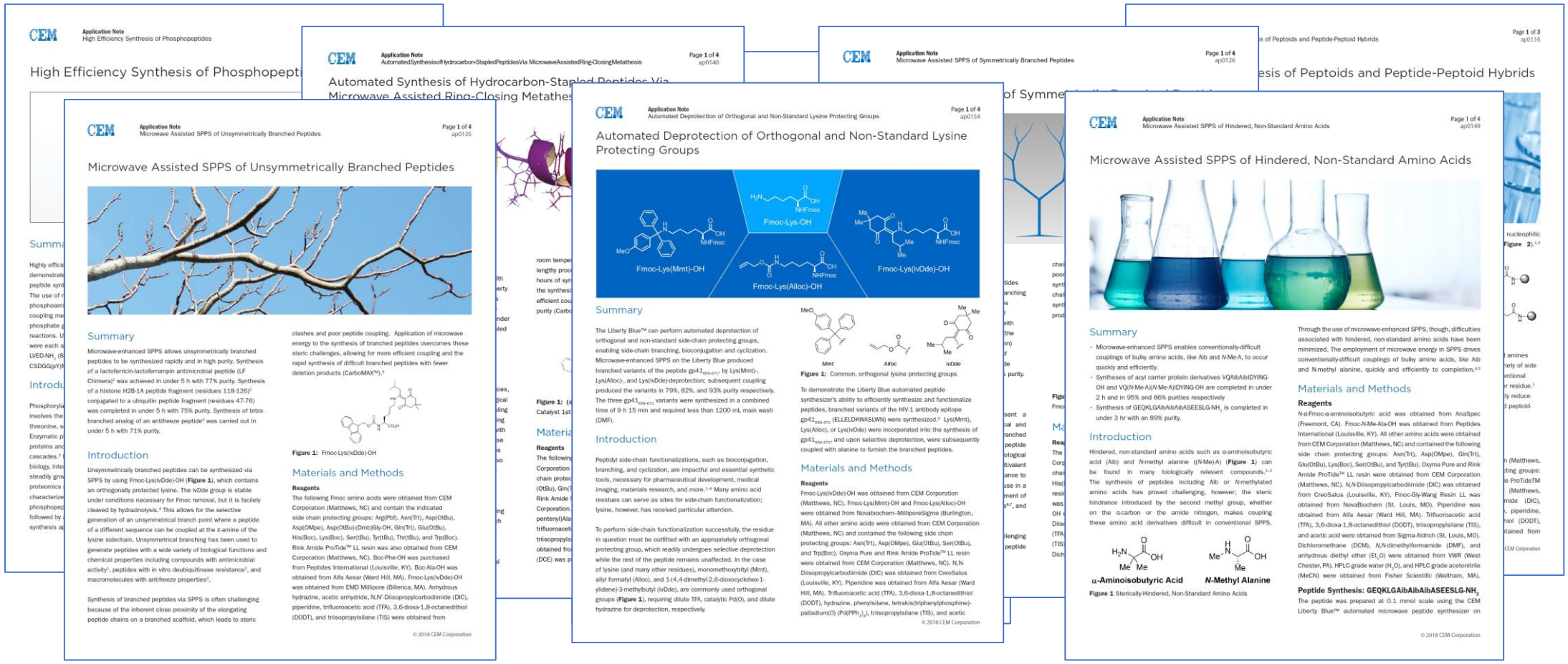
Azide Strained Promoted Click Partner

CAS No. 1418217-95-4

Product Code: LS-4270

Looking Forward

Increasing Automation of Peptide Modifications



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A Very Special Thank You to Iris Biotech !!!