

Automation of Peptide Modifications : A Click-Chemistry Case Study



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Research Scientist, Life Science Division

CEM Corporation



We Simplify Science

Collaboration with



Outline

Introduction

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



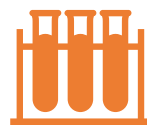
Case Study: Click Chemistry

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



Examples of Peptide Modifications

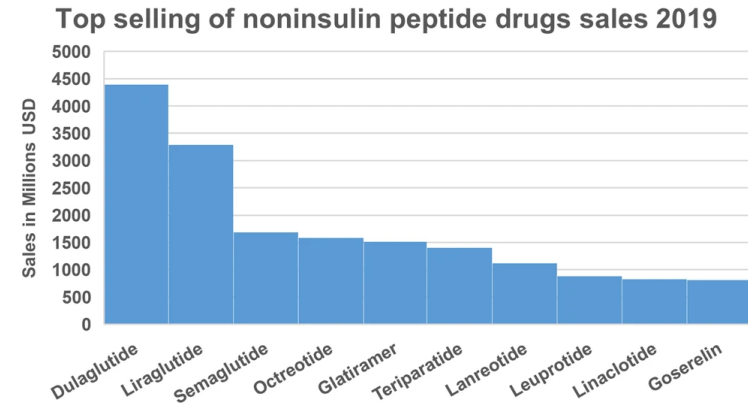
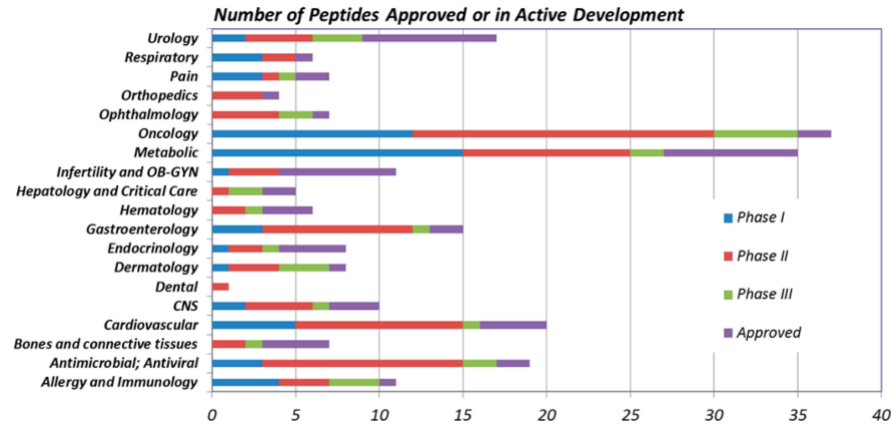
- i. Hindered Amino Acid Incorporations
- ii. Disulfide Bridging
- iii. Hydrocarbon Cyclization
- iv. Peptoid Synthesis



Conclusions and Future Directions

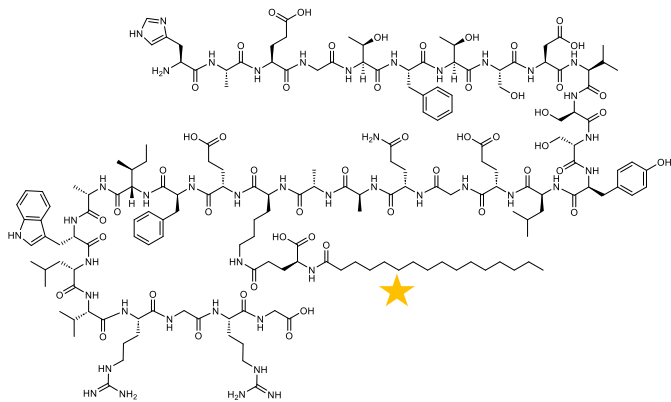


Peptides in the Drug Discovery Pipeline

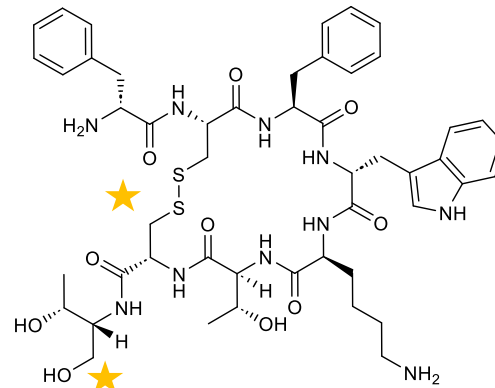


Top-selling non-insulin peptides worldwide in 2019. Data analysis according to Njardarson's group²⁷

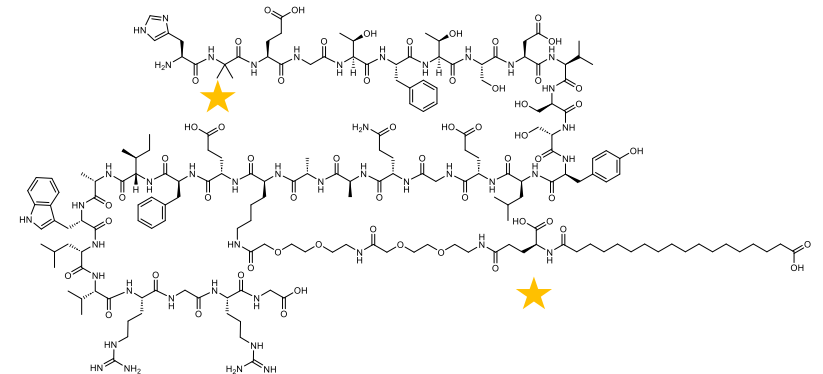
Liraglutide



Octreotide

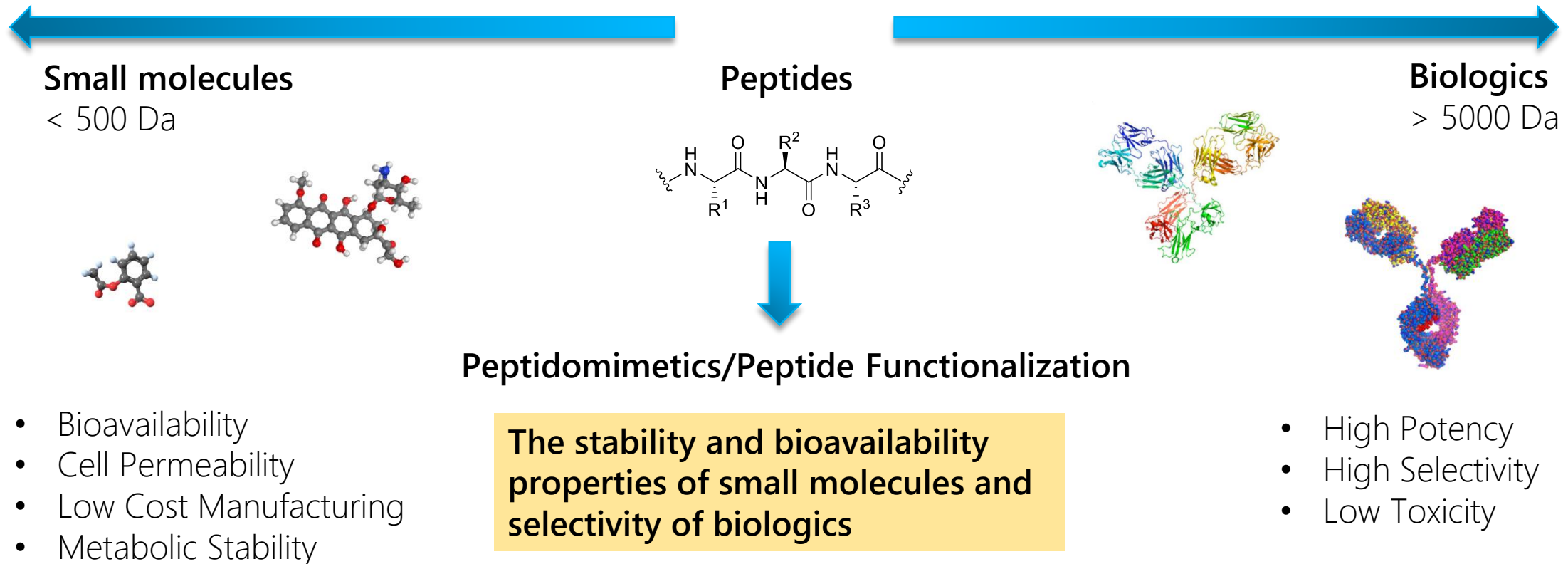


Semaglutide



Peptides as Therapeutics

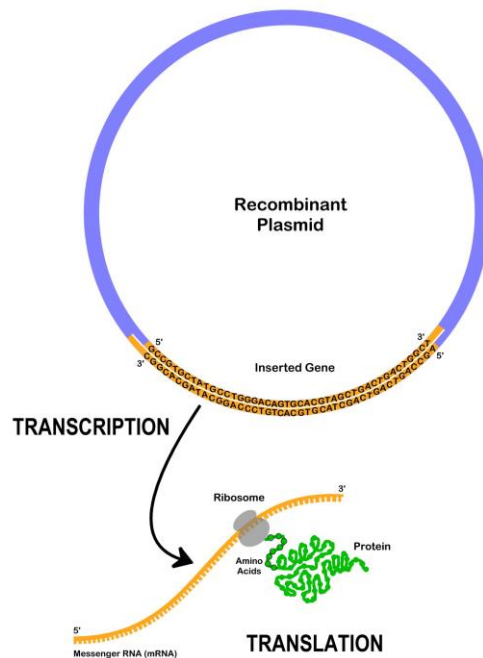
Peptide-based Drugs: Filling the Gap



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

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

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

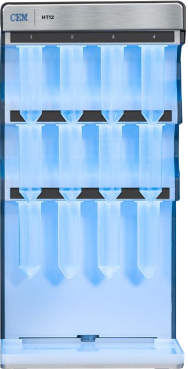


Life Science Division

- 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 – 300 μ mol
- 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



Introduction

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Case Study: Click Chemistry

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

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



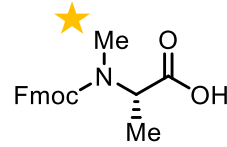
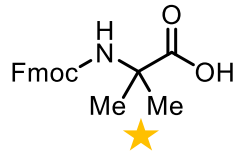
Conclusions and Future Directions



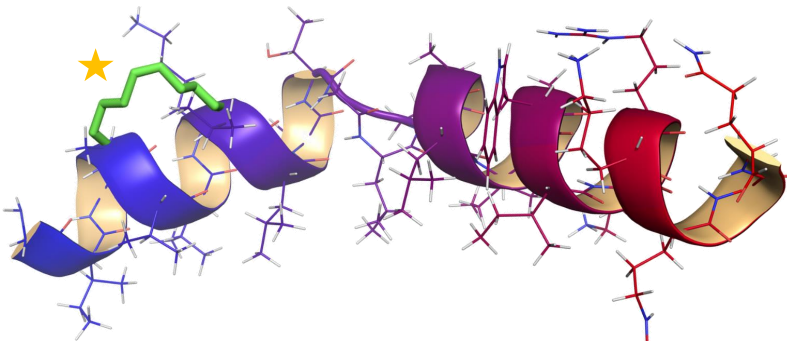
Peptide Modification Examples



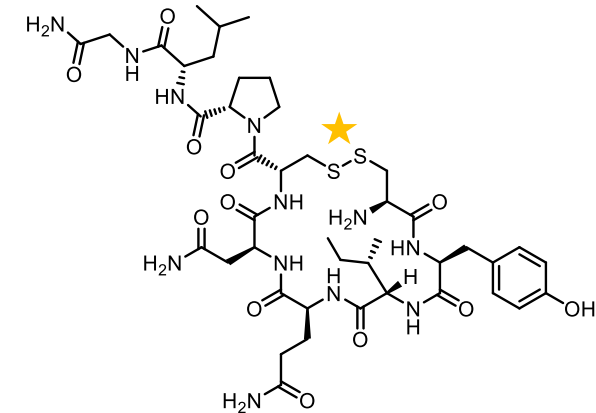
Hindered Amino Acid Incorporation



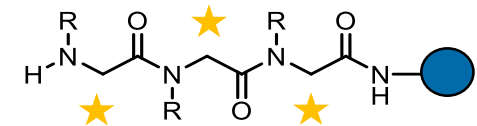
Hydrocarbon Stapling



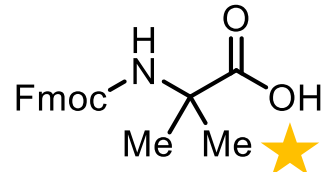
Disulfide Bridging



Peptoid Synthesis



Hindered Amino Acid Incorporation



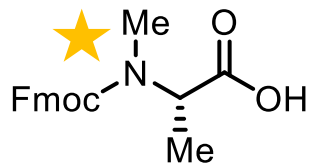
Fmoc-Aib-OH

CAS No. 94744-50-0

Product Code: FAA1613



- Induces α -Helix Formation



Fmoc-N-Me-Ala-OH

CAS No. 84000-07-7

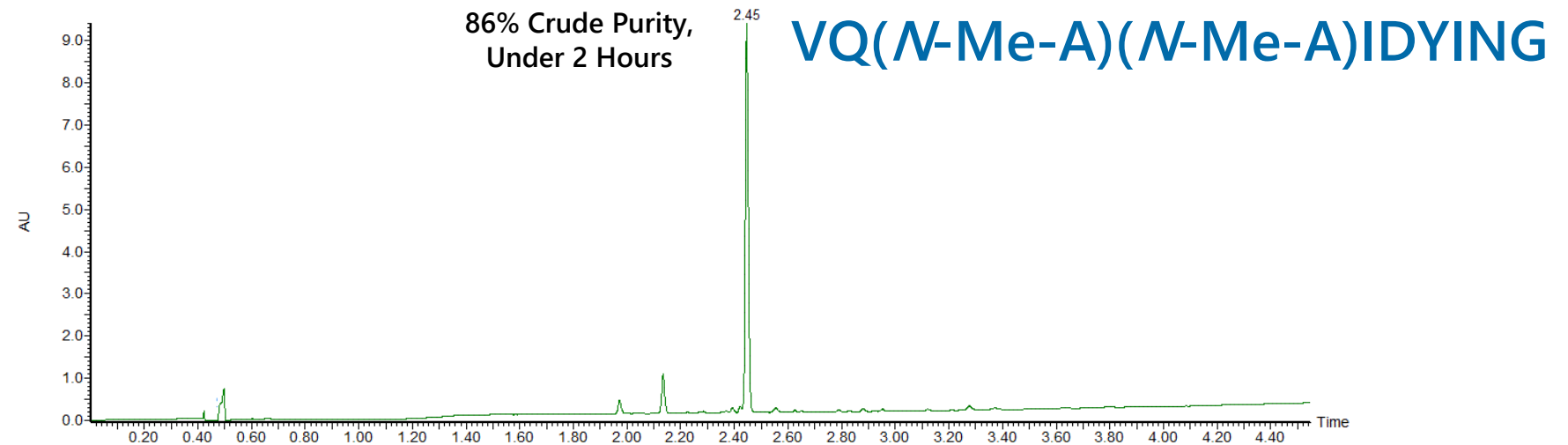
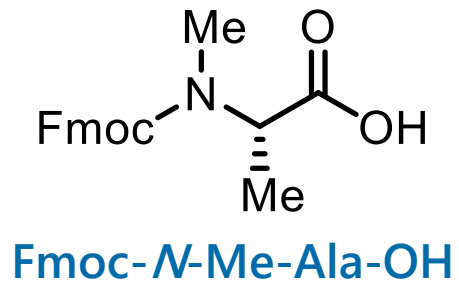
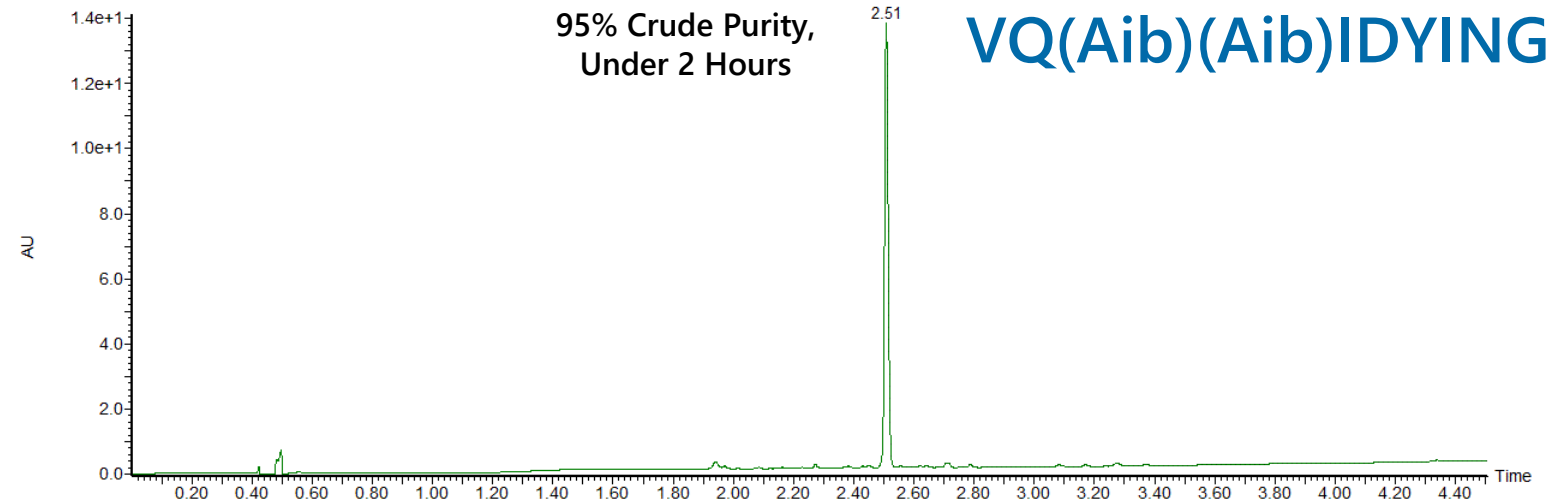
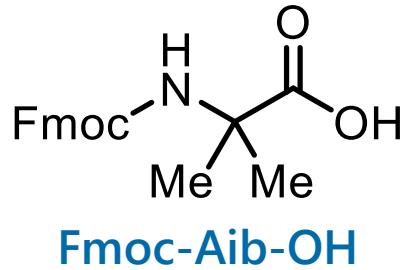
Product Code: FAA1395



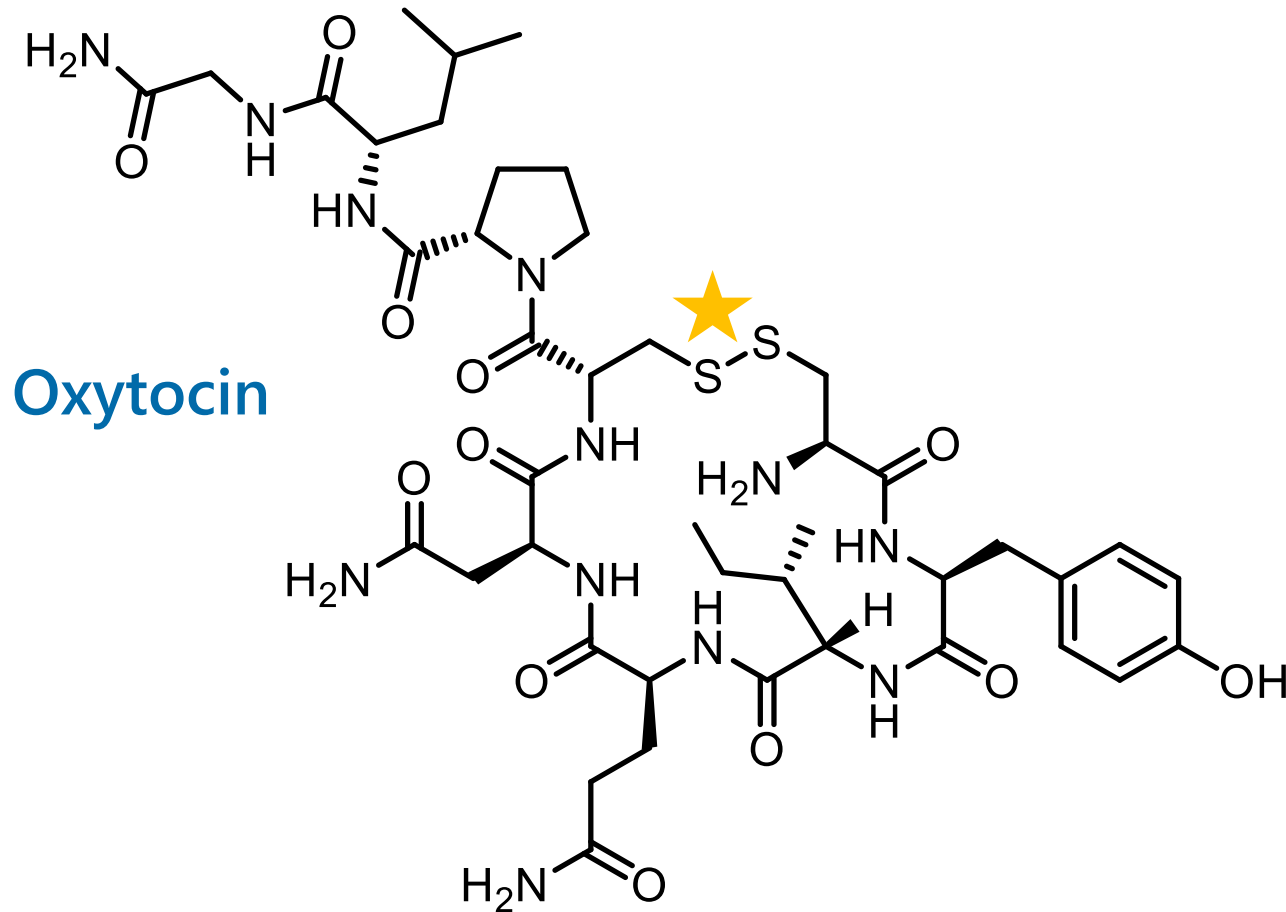
- Increases Protease Resistance
- Alters Secondary Structure

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

Hindered Amino Acid Incorporation



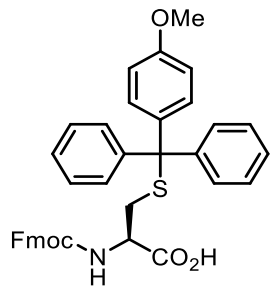
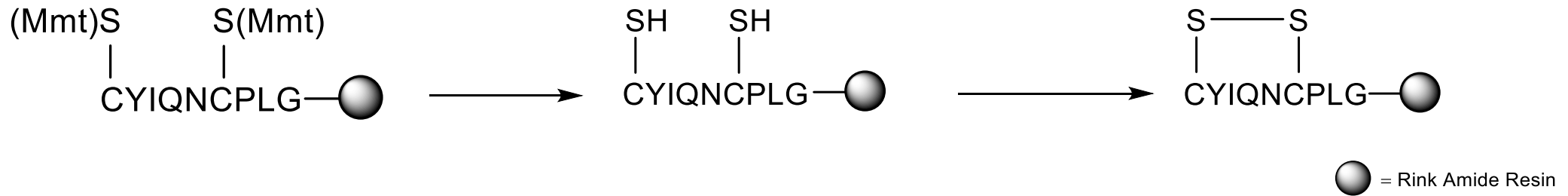
Disulfide Bridging



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

Disulfide Bridging

CEM

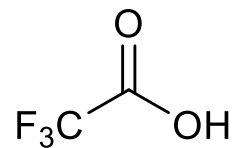


Fmoc-Cys(Mmt)-OH

Orthogonally Protected Cys Derivative

CAS No. 177582-21-7

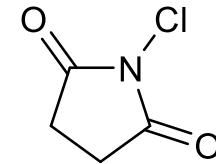
Product Code: FAA1030



Trifluoroacetic acid (TFA)

Mmt Deprotection Reagent

CAS No. 177582-21-7



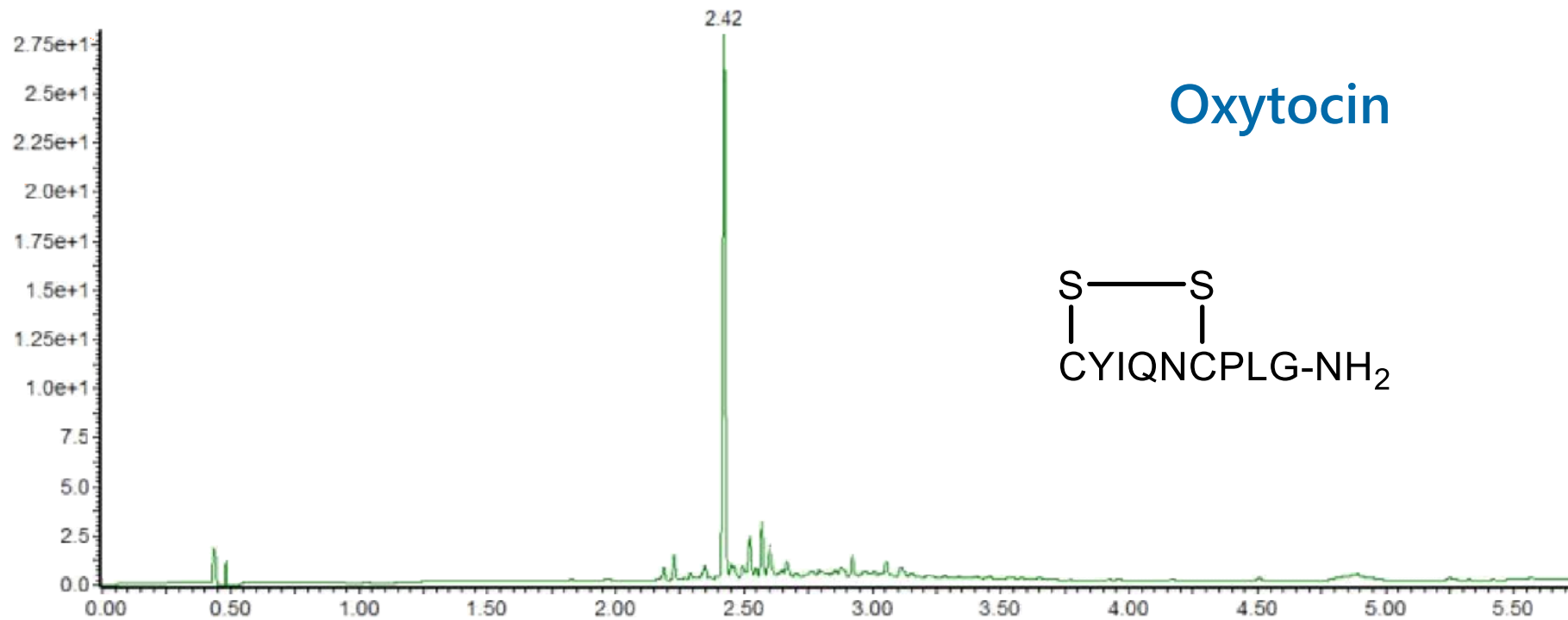
N-Chlorosuccinimide (NCS)

Thio Oxidation Reagent

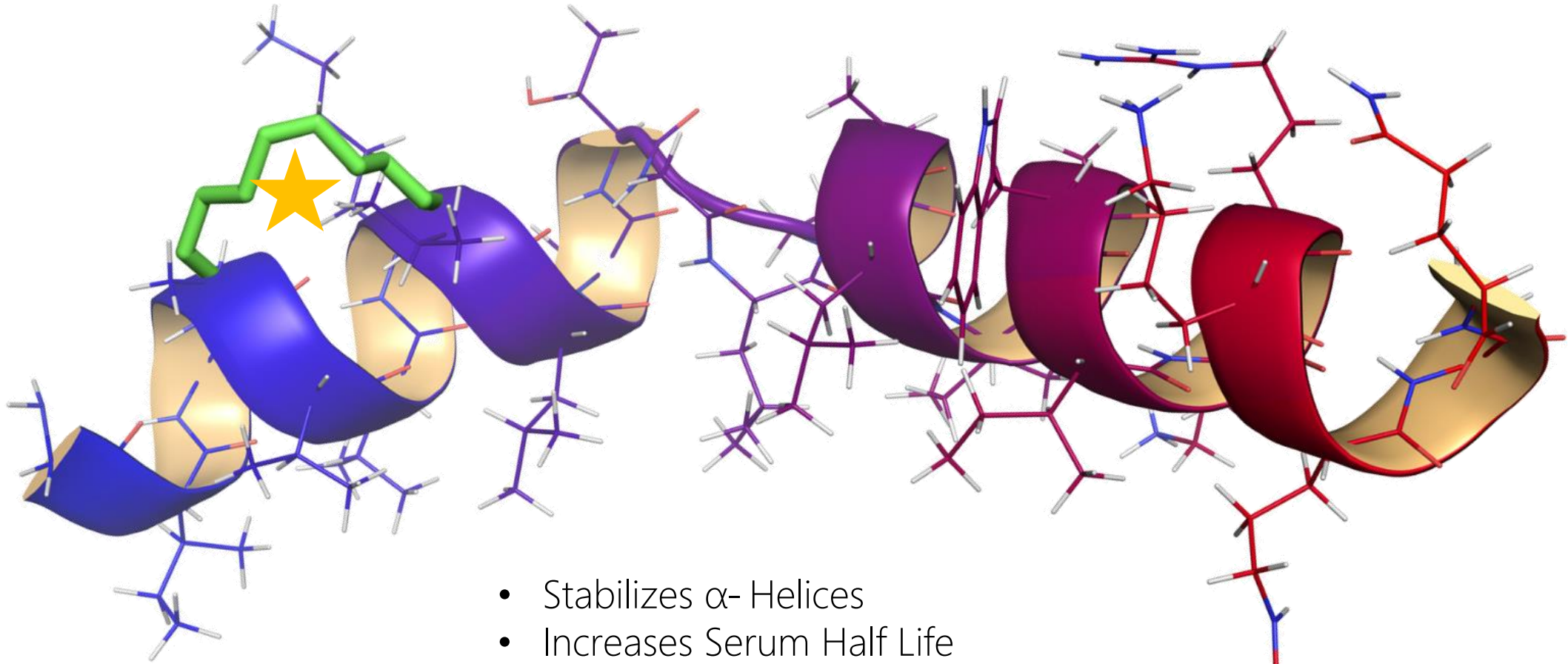
CAS No. 177582-21-7

Disulfide Bridging

69% Crude Purity,
Under 3 Hours

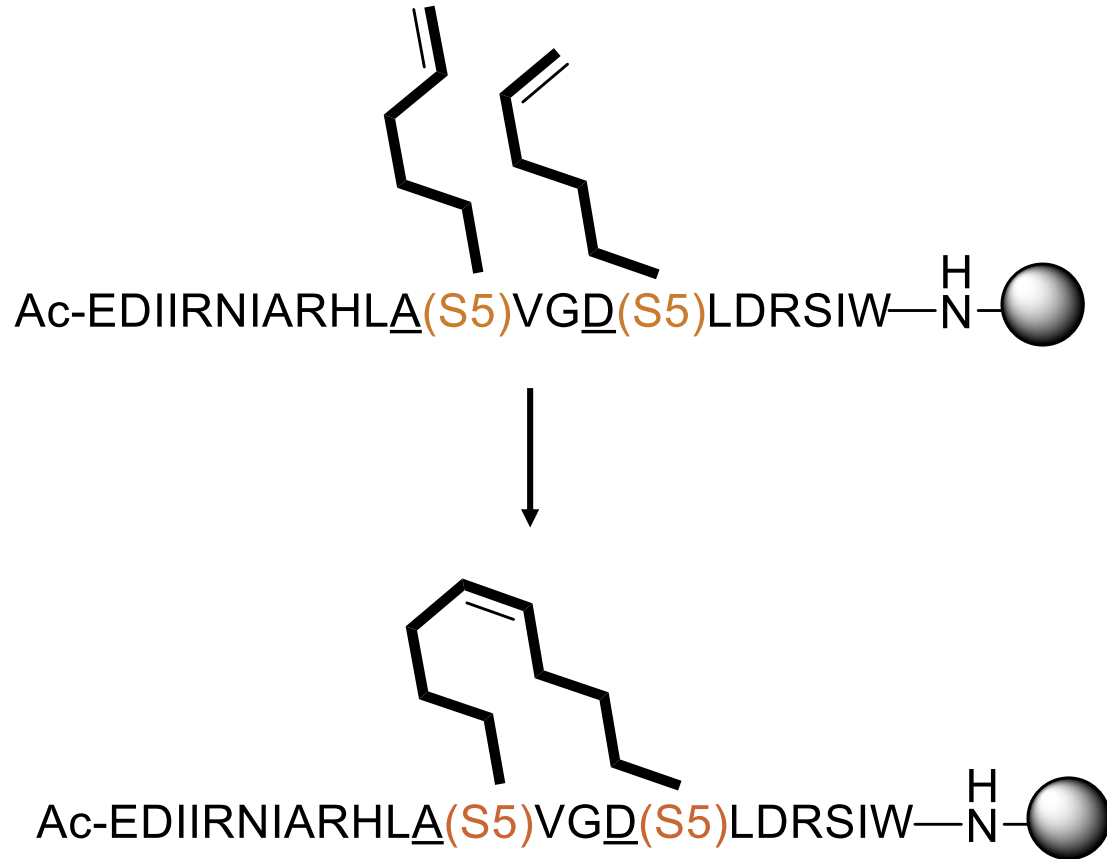


Hydrocarbon Stapling

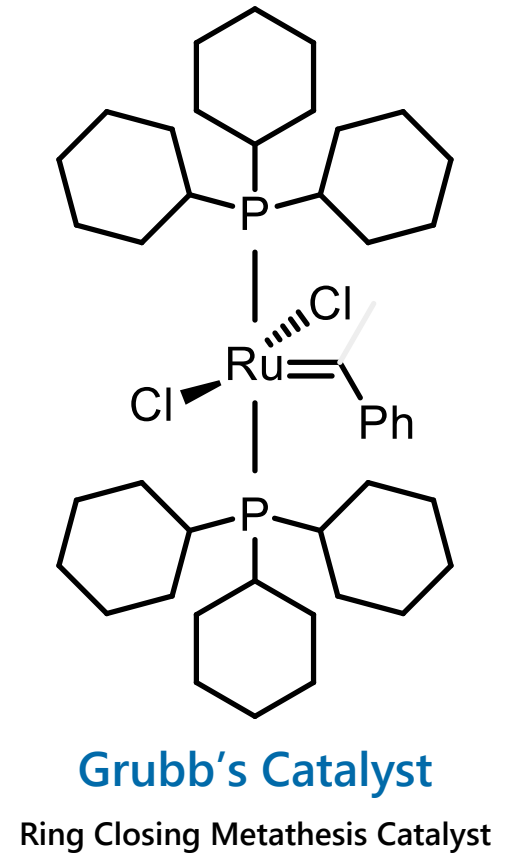
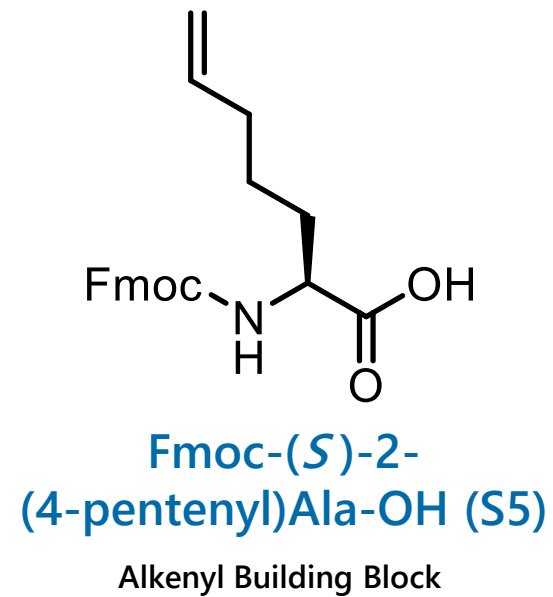


- Stabilizes α - Helices
- Increases Serum Half Life
- Increases Cell Permeability

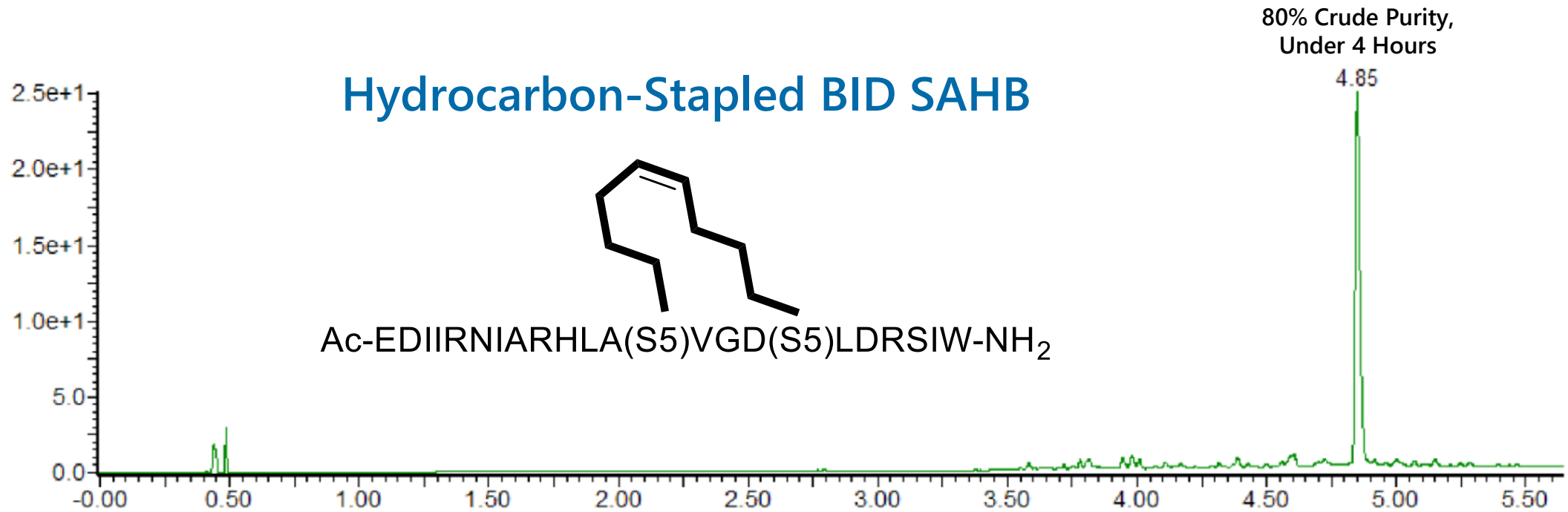
Hydrocarbon Stapling



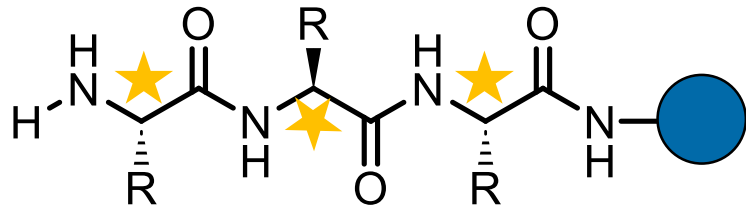
 = Rink Amide Resin



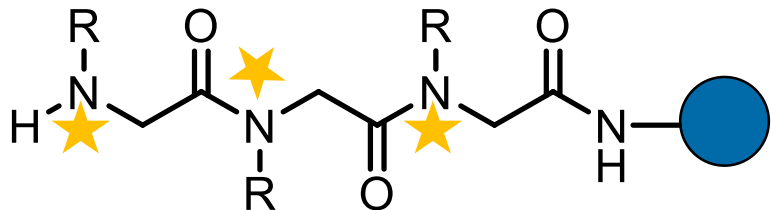
Hydrocarbon Stapling



Peptoid Synthesis



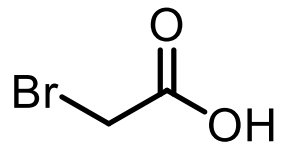
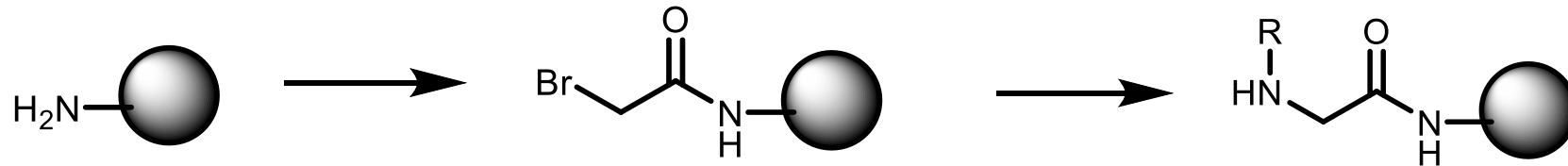
Peptide



Peptoid

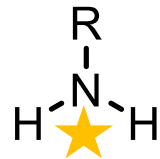
- Access to Unique Secondary Structure
- Increases Protease Resistance
- Increases Target Affinity and Potency

Peptoid Synthesis



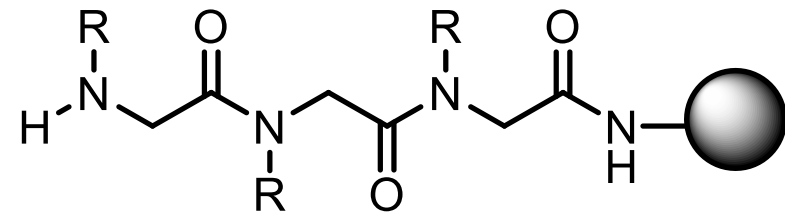
Bromoacetic Acid

Acylation of N-Terminus



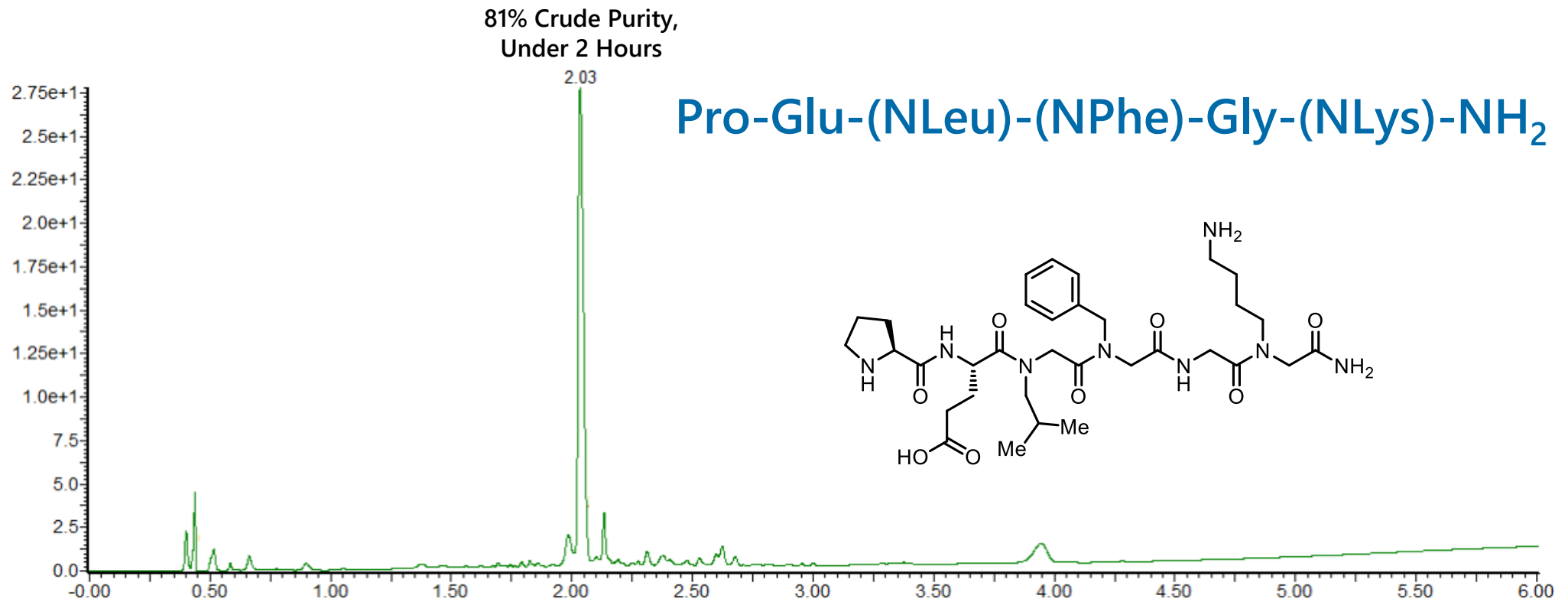
Primary Amine

Displacement of Bromide



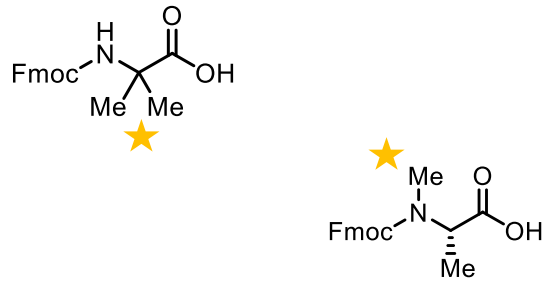
 = Rink Amide Resin

Peptoid Synthesis

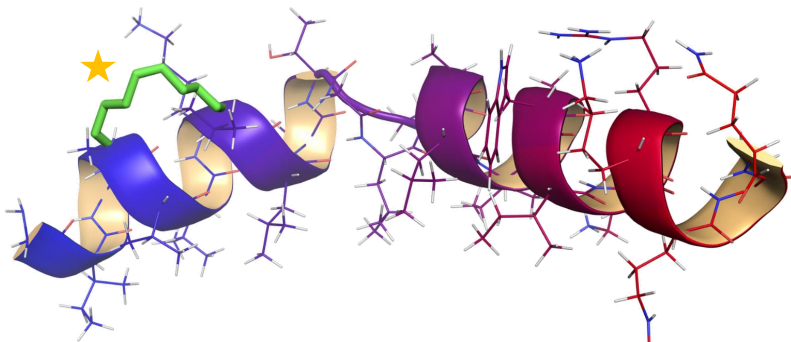


Peptide Modification Examples

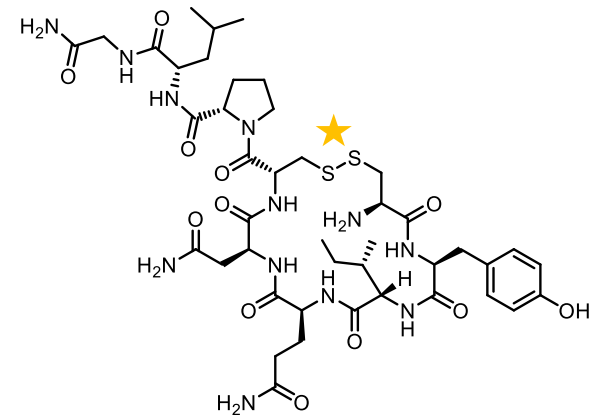
Hindered Amino Acid Incorporation



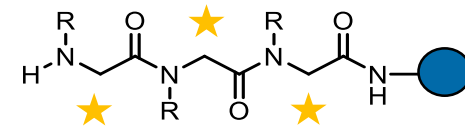
Hydrocarbon Stapling



Disulfide Bridging



Peptoid Synthesis



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Examples of Peptide Modifications

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Case Study: Click Chemistry

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Conclusions and Future Directions

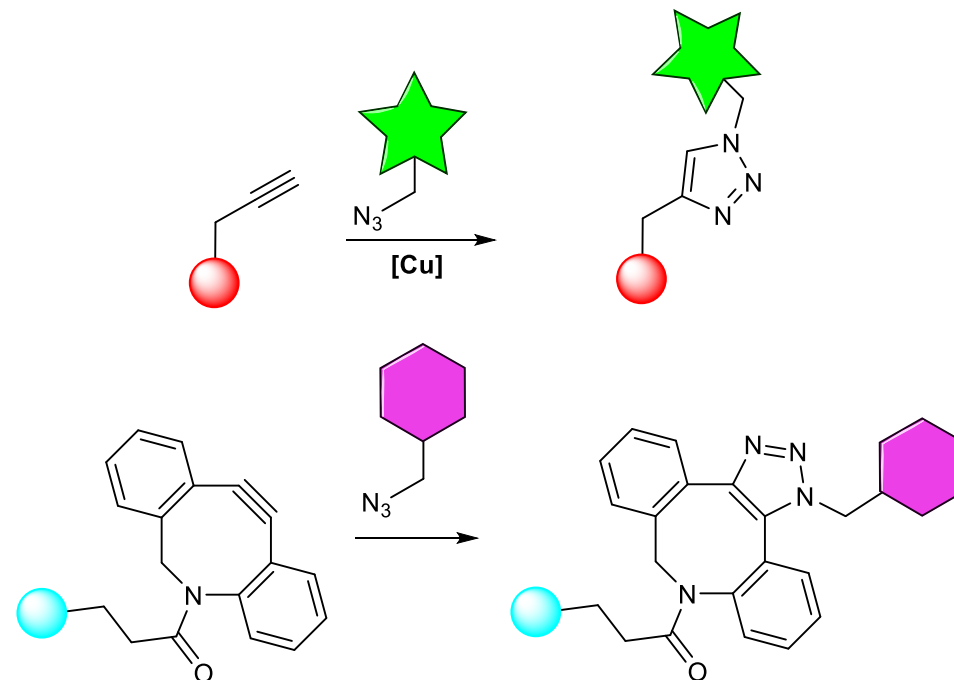


Click Chemistry



© 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

Parallel Library Synthesis



Variables

Sequence Length

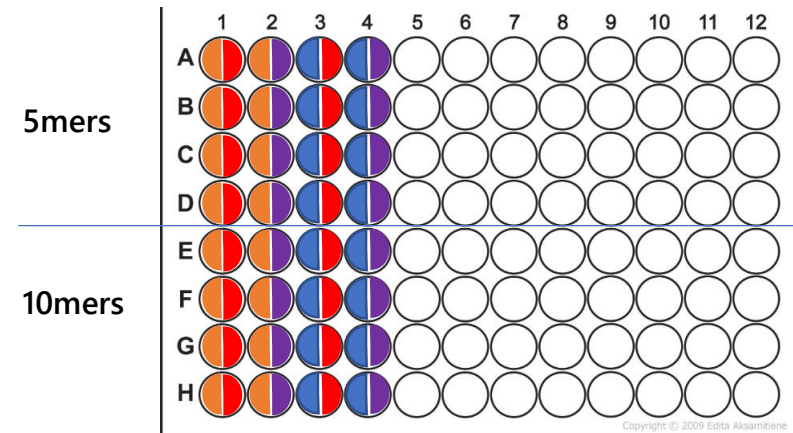
- 5mer (4 each)
- 10mer (4 each)

Location of Functional Group in Sequence

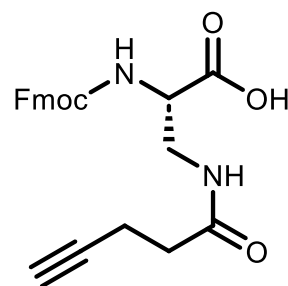
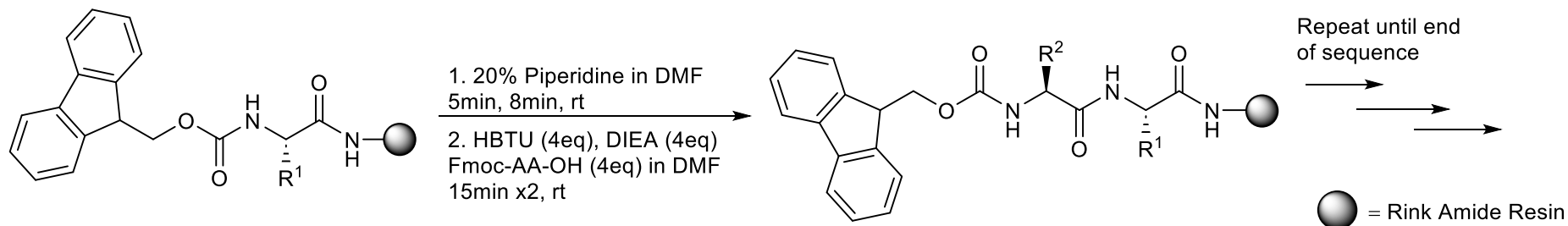
- N-Term (**N**)
- Middle (**M**)

Which Functional Group is on Peptide

- Alkyne (**X**)
- Azide (**Z**)

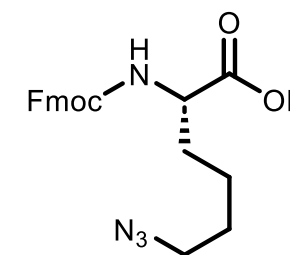


Parallel Library Synthesis



Fmoc- L Dap(Pentynoyl)-OH

Alkyne Monomer
CAS No. 2250436-47-4
Product Code: FAA4170



Fmoc- L-Lys(N₃)-OH

Azide Monomer
CAS No. 159610-89-6
Product Code: FAA4170



Incorporation of click monomers at room temperature



Alkyne-containing peptides

Peptide Sequences	Crude Purity (%)
XTFYN	78
XVLTl	70
XGYAQ	90
XDElY	65
TFXYN	92
VLXTI	95
GYX AQ	76
DEXIY	77

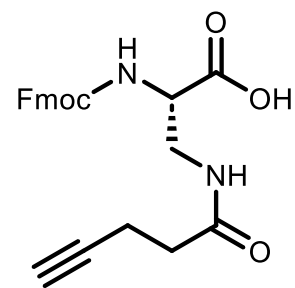
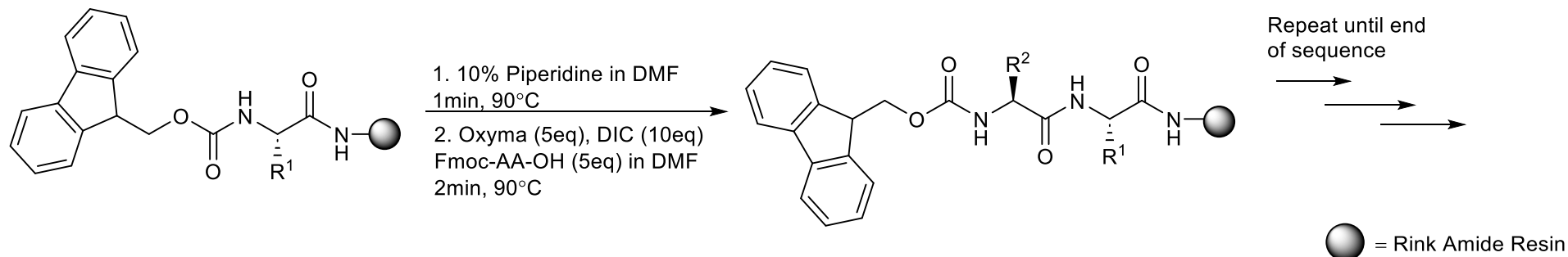
Azide-containing peptides

Peptide Sequences	Crude Purity (%)
ZTFYN	87
ZVLTl	80
ZGYAQ	85
ZDElY	58
TFZYN	64
VLZTI	84
GYZ AQ	74
DEZlY	47

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

Microwave synthesis

CEM

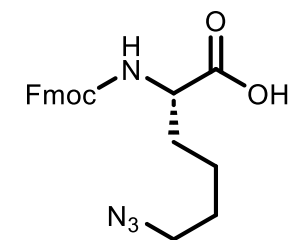


Fmoc- L Dap(Pentynoyl)-OH

Alkyne Monomer

CAS No. 2250436-47-4

Product Code: FAA4170



Fmoc- L-Lys(N₃)-OH

Azide Monomer

CAS No. 159610-89-6

Product Code: FAA4170



Incorporation of click monomers at elevated temperatures

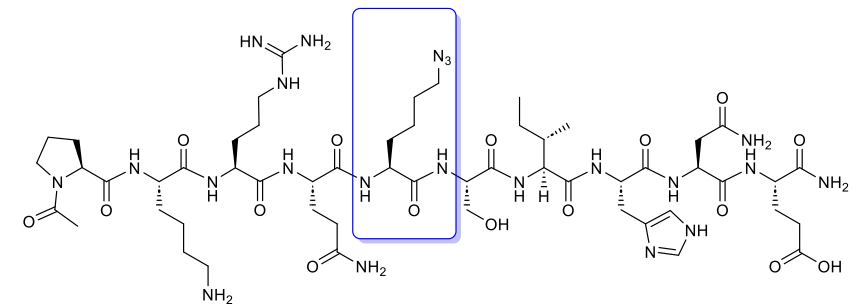
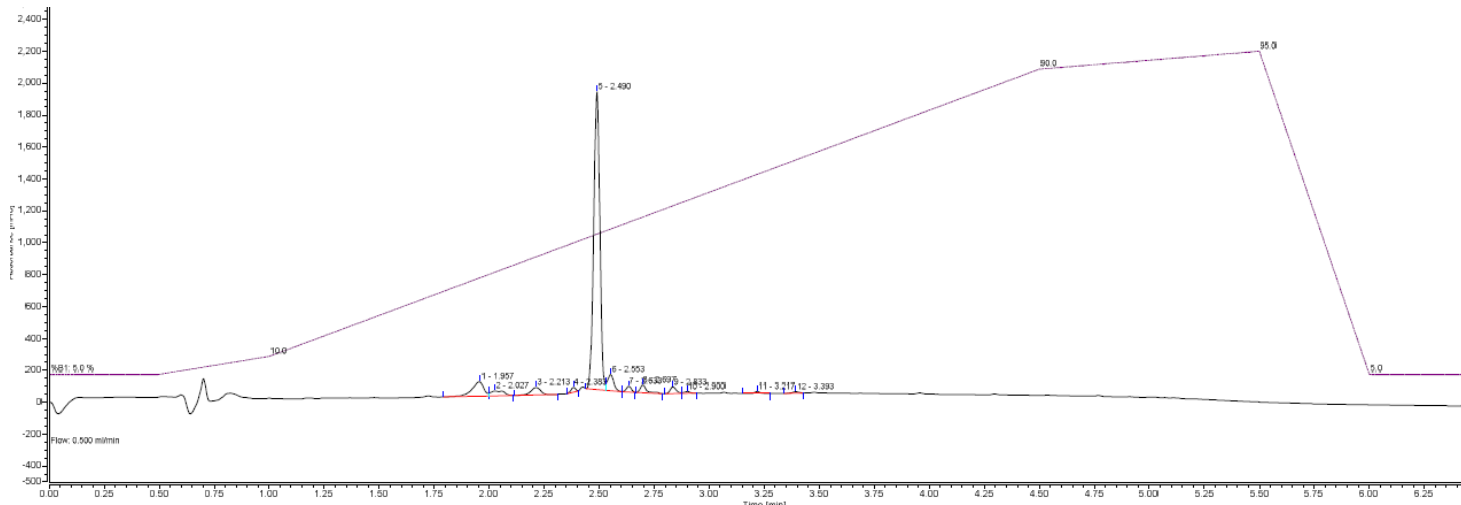


Alkyne-containing peptides

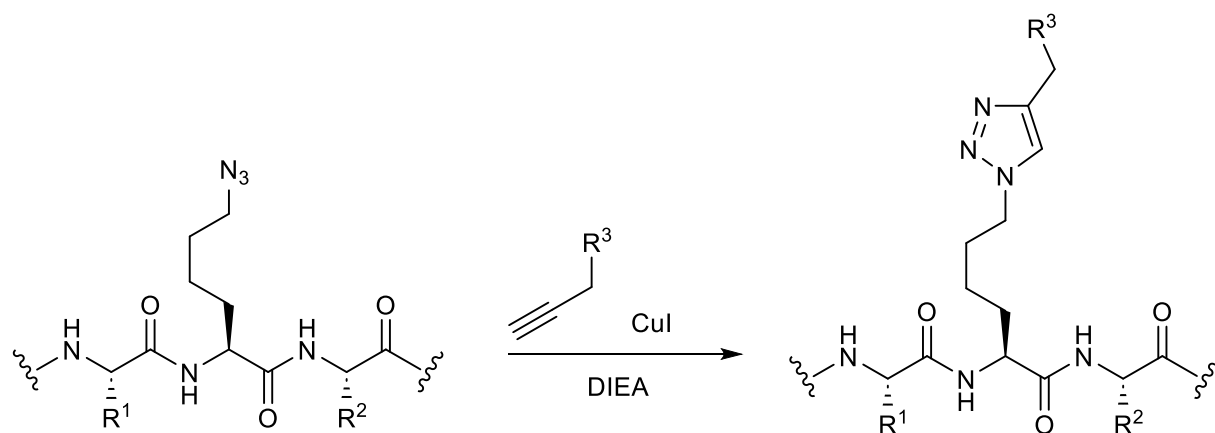
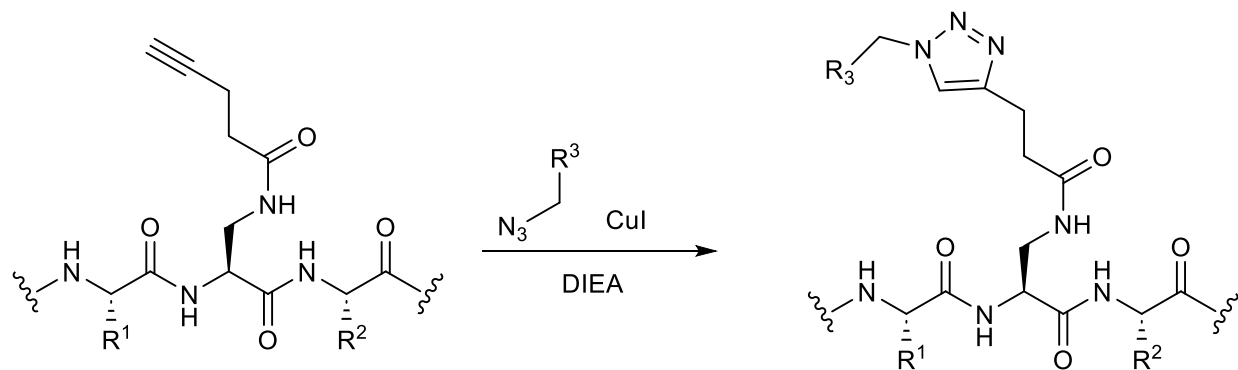
Peptide Sequences	Crude Purity (%)
XTFYN	90
TFXYN	92
XPKRQSIHNE	84
PKRQXSIHNE	85

Azide-containing peptides

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



Click chemistry Considerations for Automation

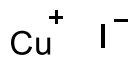


Things to consider when trying new chemistry on an automated synthesizer:

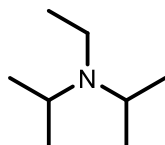
- Could the reagents harm the instrument?
- Solubility of all reagents
- Stability of all reagents
- Scalability of the chemistry

Reagent Solubility in Various Solvents

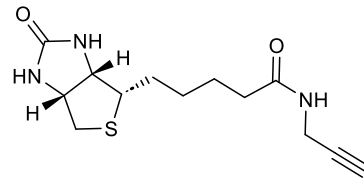
CEM



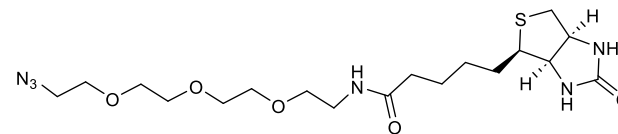
Copper Iodide
Copper Catalyst
CAS No. 773888-45-2



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

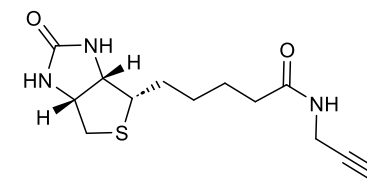
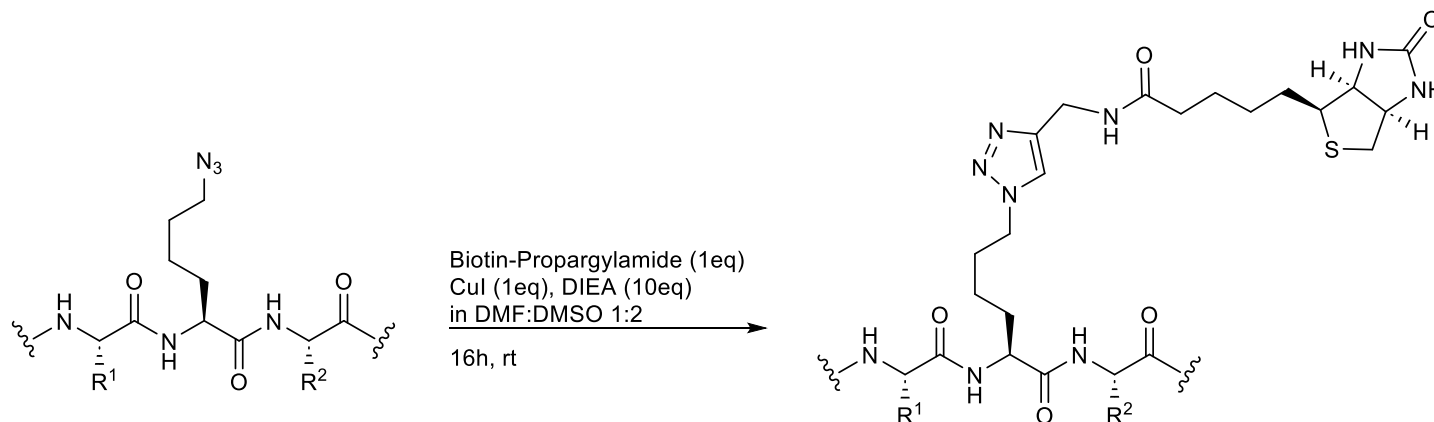
Reagents	DMF	NMP	THF	DMSO	DMF:DMSO 1:2
CuI	✗	✗	✗	✓	✓
DIEA	✓	✓	-	✗	✓
Biotin-PEG ₃ -N ₃	✗	✗	✗	✓	✓
Biotin-Propargylamide	✗	✗	✗	✓	⚠



- 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

CEM

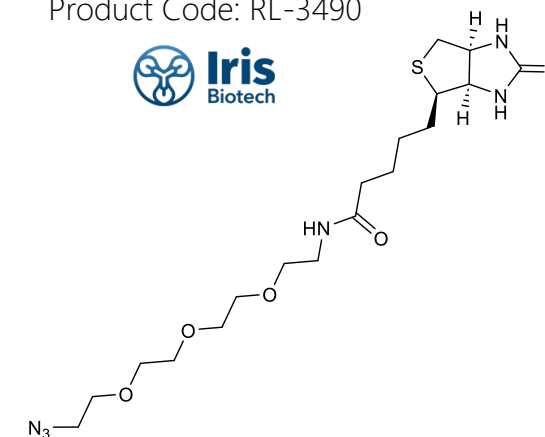


Biotin-Propargylamide

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



Click Chemistry at Room Temperature



Alkyne-containing peptides



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

Azide-containing peptides

Peptide Sequences	Crude Purity (%)
ZTFYN	66
ZVLTl	55
ZGYAQ	73
ZDEIY	54
TFZYN	84
VLZTI	84
GYZ AQ	77
DEZ IY	78

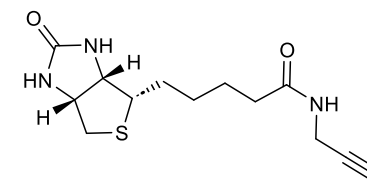
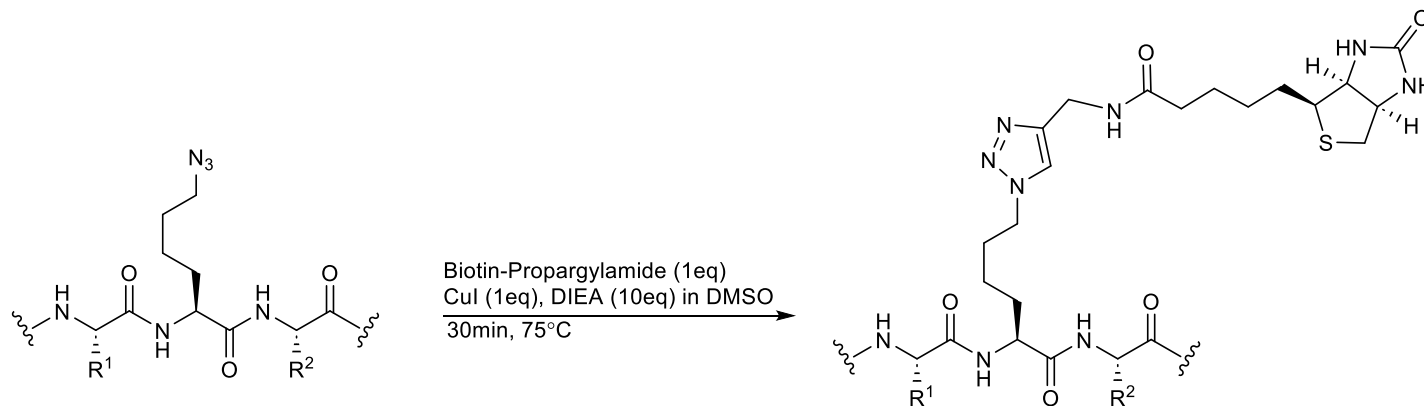
Click Chemistry on Liberty Blue 2.0 at Elevated Temperature



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

CEM

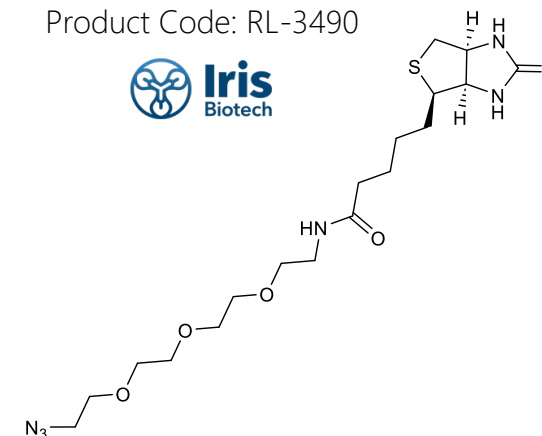


Biotin-Propargylamide

Azide click partner

CAS No. 773888-45-2

Product Code: RL-3490

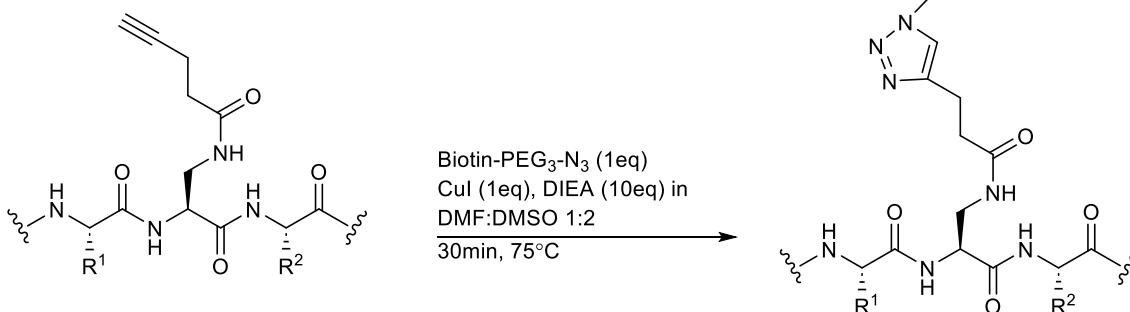


Biotin-PEG(3)-N₃

Alkyne click partner

CAS No. 875770-34-6

Product Code: PEG4940



Click Chemistry at Elevated Temperature

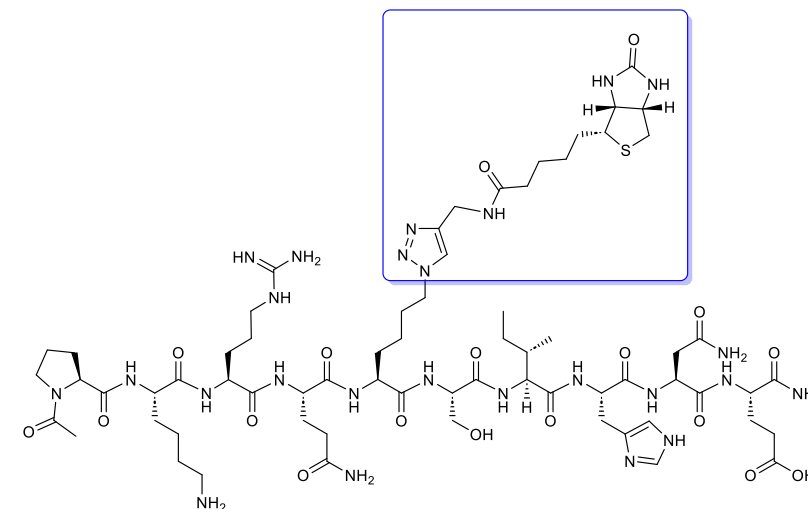
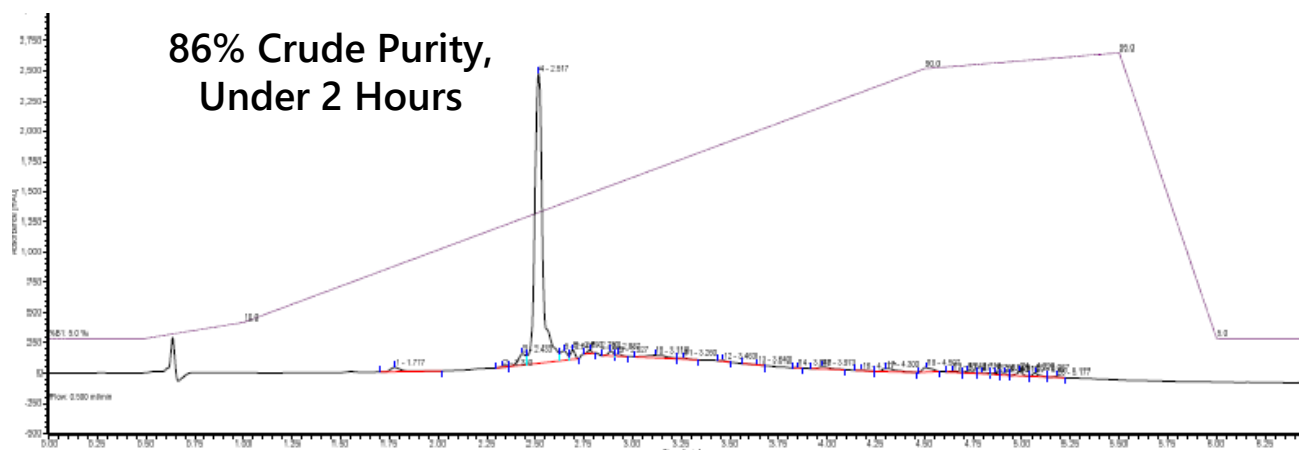


Alkyne-containing peptides

Peptide Sequences	Crude Purity (%)
TFXYN	74
PKRQXSIHNE	80

Azide-containing peptides

Peptide Sequences	Crude Purity (%)
TFZYN	61
PKRQZSIHNE	86



Up Next

CEM

Introduction

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



Case Study: Click Chemistry

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



Examples of Peptide Modifications

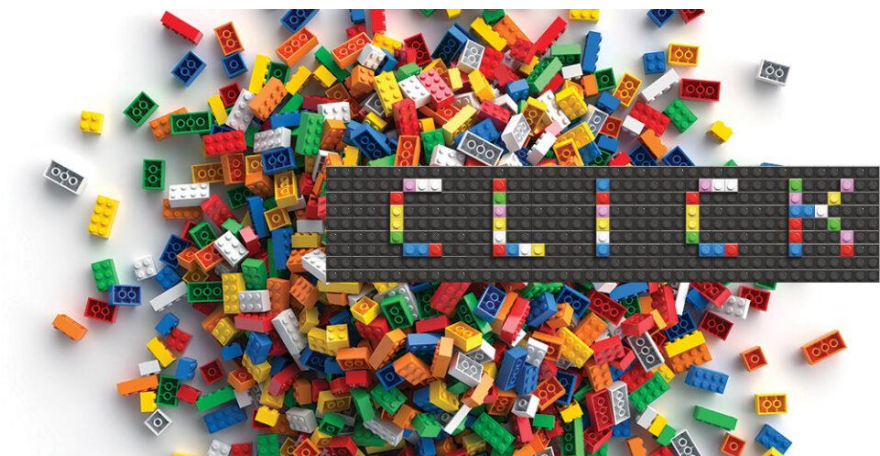
- i. Hindered Amino Acid Incorporations
- ii. Disulfide Bridging
- iii. Hydrocarbon Cyclization
- iv. Peptoid Synthesis



Conclusions and Future Directions

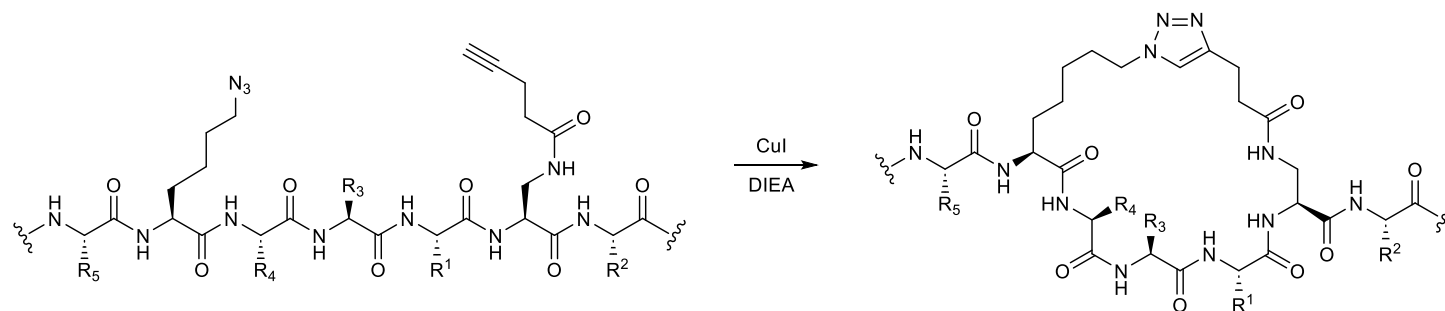


Conclusions

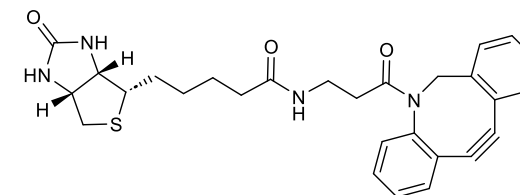


- Incorporation of azide- and alkyne-containing monomers can be achieved at both room temperature and elevated temperature
- Click chemistry was shown to be accessible at room temperature overnight
- Click chemistry can also be achieved at an elevated temperature in as little as 30 minutes.
- The placement 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

Application Note
High Efficiency Synthesis of Phosphopeptides

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ap0140

High Efficiency Synthesis of Phosphopeptides

Application Note
Automated Synthesis of Hydrocarbon-Stapled Peptides Via Microwave Assisted Ring-Closing Metathesis

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Automated Synthesis of Hydrocarbon-Stapled Peptides Via Microwave Assisted Ring-Closing Metathesis

Application Note
Microwave Assisted SPPS of Symmetrically Branched Peptides


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ap0126

Synthesis of Peptoids and Peptide-Peptoid Hybrids

Application Note
Microwave Assisted SPPS of Unsymmetrically Branched Peptides

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ap0135

Microwave Assisted SPPS of Unsymmetrically Branched Peptides



Summary

Microwave-enhanced SPPS allows unsymmetrically branched peptides to be synthesized rapidly and in high purity. Synthesis of a lactoferrin-lactoferramin antimicrobial peptide (LP Chimeric) was achieved in under 5 h with 77% purity. Synthesis of a histone H2B-5A peptide fragment (residues 118-126) conjugated to a ubiquitin peptide fragment (residues 47-76) was completed in under 5 h with 75% purity. Synthesis of tetra-branched analog of an antifreeze peptide³ was carried out in under 5 h with 73% purity.

Introduction


Unsymmetrically branched peptides can be synthesized via SPPS by using Fmoc-Lys(ivDde)-OH (Figure 1), which contains an orthogonally protected lysine. The ivDde group is stable under conditions necessary for Fmoc removal, but it is easily cleaved by hydrazinolysis.⁴ This allows for the selective generation of an unsymmetrical branch point where a peptide of a different sequence can be coupled at the same of the lysine sidechain. Unsymmetrical branching has been used to generate peptides with a wide variety of biological functions and chemical properties including compounds with antimicrobial activity,⁵ peptides with in vitro deubiquitinase resistance⁶, and macromolecules with antifreeze properties.⁷

Synthesis of branched peptides via SPPS is often challenging because of the inherent close proximity of the elongating peptide chains on a branched scaffold, which leads to steric

Application Note
Automated Deprotection of Orthogonal and Non-Standard Lysine Protecting Groups

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ap0154

Automated Deprotection of Orthogonal and Non-Standard Lysine Protecting Groups



Summary

The Liberty Blue™ can perform automated deprotection of orthogonal and non-standard side-chain protecting groups, enabling side-chain branching, bioconjugation and cyclization. Microwave-enhanced SPPS on the Liberty Blue produced branched variants of the peptide gp41₁₀₀₋₁₂₇ by Lys(Mmt), Lys(Alloc), and Lys(ivDde) deprotection; subsequent coupling produced the variants in 79%, 82%, and 93% purity respectively. The three gp41₁₀₀₋₁₂₇ variants were synthesized in a combined time of 9 h 15 min and required less than 1200 mL main wash (DMF).

Introduction


Peptidyl side-chain functionalizations, such as bioconjugation, branching, and cyclization, are impactful and essential synthetic tools, necessary for pharmaceutical development, medical imaging, materials research, and more.¹⁻⁴ Many amino acid residues can serve as sites for side-chain functionalization; lysine, however, has received particular attention.

To perform side-chain functionalization successfully, the residue in question must be equipped with an appropriately orthogonal protecting group, which readily undergoes selective deprotection while the rest of the peptide remains unaffected. In the case of lysine (and many other residues), monomethyltrityl (Mmt), allyl formate (Alloc), and 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl (ivDde), are commonly used orthogonal groups (Figure 1), requiring dilute TFA, catalytic Pd(O), and dilute hydrazine for deprotection, respectively.

Application Note
Microwave Assisted SPPS of Hindered, Non-Standard Amino Acids

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ap0149

Microwave Assisted SPPS of Hindered, Non-Standard Amino Acids



Summary

Microwave-enhanced SPPS enables conventionally-difficult couplings of bulky amino acids, like Abz and N-Me-A, to occur quickly and efficiently.

Syntheses of acyl carrier protein derivatives VQAIAbIDYING-OH and VQ(N-Me-A)N-Me-AIDYING-OH are completed in under 2 h and in 95% and 86% purities respectively.

Synthesis of GEQLGAbAibAibAEEESLGH-NH₂ is completed in under 3 h with an 89% purity.

Introduction

Hindered, non-standard amino acids such as α-aminoisobutyric acid (Aib) and N-methyl alanine ((N-Me)A) (Figure 1) can be found in many biologically relevant compounds.¹⁻⁴ The synthesis of peptides including Aib or N-methylated amino acids has proved challenging, however; the steric hindrance introduced by the second methyl group, whether on the α-carbon or the amide nitrogen, makes coupling these amino acid derivatives difficult in conventional SPPS.

Materials and Methods

N-α-Fmoc-α-aminoisobutyric acid was obtained from AnaSpec (Fremont, CA). Fmoc-N-Me-Aib-OH was obtained from Peptides International (Louisville, KY). All other amino acids were obtained from CEM Corporation (Matthews, NC) and contained the following side chain protecting groups: Asn(Trt), Asp(OMe), Gln(Trt), Glu(OtBu), Lys(Boc), Ser(OtBu), and Tyr(Trt). Oryza Pure and Rank Amide ProTide™ LL resin were obtained from CEM Corporation (Matthews, NC). N,N-Diisopropylcarbodiimide (DIC) was obtained from CreaSalus (Louisville, KY). Fmoc-9-Fluorenyl Resin LL was obtained from NovaBiochem (St. Louis, MO). Piperidine was obtained from Alfa Aesar (Ward Hill, MA). Trifluoroacetic acid (TFA), 3,6-dioxo-1,8-octanedithiol (DODT), trisopropylsilane (TIS), and acetic acid were obtained from Sigma-Aldrich (St. Louis, MO). Dichloromethane (DCM), N,N-dimethylformamide (DMF), and anhydrous diethyl ether (Et₂O) were obtained from VWR (West Chester, PA). HPLC-grade water (H₂O), and HPLC-grade acetonitrile (MeCN) were obtained from Fisher Scientific (Waltham, MA).

Peptide Synthesis: GEQLGAbAibAibAEEESLGH-NH₂

The peptide was prepared at 0.1 mmol scale using the CEM Liberty Blue™ automated microwave peptide synthesizer on

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THANK YOU!

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A Very Special Thank You to

