TOWARD THE DESIGN, SYNTHESIS, AND CHARACTERIZATION OF ABIOTIC COILED-COIL PEPTIDES VIA SOLID-PHASE COPPER-CATALYZED AZIDE-ALKYNE CYCLOADDITION (SP-CUAAC) CLICK REACTION FOR THE PREPARATION OF CONTROLLED SELF-ASSEMBLY MOLECULAR BUILDING BLOCK

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ABIOTIC COILED-COIL PEPTIDES VIA SOLID-PHASE COPPER-
CATALYZED AZIDE-ALKYNE CYCLOADDITION (SP-CUAAC) CLICK
REACTION FOR THE PREPARATION OF CONTROLLED SELF-ASSEMBLY
MOLECULAR BUILDING BLOCK

by

LIAQUAT ALI

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Chemistry
Department of Chemistry and Biochemistry

Sean C. Butler, Ph.D., Committee Chair

College of Arts and Science

The University of Texas at Tyler
April 2023
The University of Texas at Tyler
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April 6, 2023
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Dean, College of Arts and Science
Acknowledgements

In the name of GOD, the Most Gracious and the Most Merciful.

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
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<tbody>
<tr>
<td>Ala (A)</td>
<td>alanine</td>
</tr>
<tr>
<td>Arg (R)</td>
<td>arginine</td>
</tr>
<tr>
<td>Asp (D)</td>
<td>aspartic acid</td>
</tr>
<tr>
<td>Asn (N)</td>
<td>asparagine</td>
</tr>
<tr>
<td>Cys (C)</td>
<td>cysteine</td>
</tr>
<tr>
<td>Gln (Q)</td>
<td>glutamine</td>
</tr>
<tr>
<td>Glu (E)</td>
<td>glutamic acid</td>
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<td>Gly (G)</td>
<td>glycine</td>
</tr>
<tr>
<td>His (H)</td>
<td>histidine</td>
</tr>
<tr>
<td>Ile (I)</td>
<td>isoleucine</td>
</tr>
<tr>
<td>Leu (L)</td>
<td>leucine</td>
</tr>
<tr>
<td>Lys (K)</td>
<td>lysine</td>
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<td>Met (M)</td>
<td>methionine</td>
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<td>Phe (F)</td>
<td>phenylalanine</td>
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<td>Pro (P)</td>
<td>proline</td>
</tr>
<tr>
<td>Ser (S)</td>
<td>serine</td>
</tr>
<tr>
<td>Thr (T)</td>
<td>threonine</td>
</tr>
<tr>
<td>Trp (W)</td>
<td>tryptophan</td>
</tr>
</tbody>
</table>
Tyr (Y) tyrosine
Val (V) valine

Abbreviations

\( \alpha \) alpha
AcOH acetic acid
AcOEt ethyl acetate
Al ethylbenzaldehyde
AIDS acquired immunodeficiency syndrome
\( \beta \) beta
Boc tert-butoxycarbonyl
Boc\(_2\)O di-tert-butyl decarbonate (Boc-anhydride)
n-BuOH 1-butanol
CuAAC copper catalyzed azide-alkyne reaction
D\(_{\text{Ax}}\)–D\(_{\text{Ay}}\) covalently crosslinked peptides D\(_{\text{Ax}}\) and D\(_{\text{Ay}}\)
\( ^\circ \text{C} \) degree(s) Celsius
\( \text{cm}^{-1} \) wavenumber (IR)
DIC diisopropylcarbodiimide
DCM dichlorormethane
DMF \( N,N' \)-dimethylforamide
DNA deoxyribonucleic acid
DIPEA \( N,N' \)-diisopropylethylamine
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>DC</td>
<td>dynamic covalent</td>
</tr>
<tr>
<td>d</td>
<td>doublet (NMR)</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DAX</td>
<td>DAY</td>
</tr>
<tr>
<td>δ</td>
<td>delta; chemical shift in ppm downfield from tetramethylsilane</td>
</tr>
<tr>
<td>equiv</td>
<td>equivalents</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionization</td>
</tr>
<tr>
<td>FPLC</td>
<td>fast protein liquid chromatography</td>
</tr>
<tr>
<td>Fmoc</td>
<td>fluorenylmethoxycarbonyl protecting group</td>
</tr>
<tr>
<td>Fmoc-OSu</td>
<td>N-(9-Fluorenylmethoxycarbonyloxy)succinimide</td>
</tr>
<tr>
<td>g</td>
<td>gram(s)</td>
</tr>
<tr>
<td>GEP</td>
<td>green, fluorescent t protein</td>
</tr>
<tr>
<td>GCN4</td>
<td>transcription factor for gene expression</td>
</tr>
<tr>
<td>Hy</td>
<td>N-Boc-4-ethynylbenzhydrazides</td>
</tr>
<tr>
<td>Hz</td>
<td>hertz (NMR)</td>
</tr>
<tr>
<td>1H</td>
<td>proton</td>
</tr>
<tr>
<td>IR</td>
<td>infrared</td>
</tr>
<tr>
<td>J</td>
<td>coupling constant in hertz (NMR)</td>
</tr>
<tr>
<td>K</td>
<td>kelvin (temperature)</td>
</tr>
<tr>
<td>Kg</td>
<td>Kilo gram</td>
</tr>
<tr>
<td>Symbol</td>
<td>Abbreviation</td>
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<td>--------</td>
<td>--------------</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LHRH</td>
<td>synthetic luteinizing hormone releasing hormone</td>
</tr>
<tr>
<td>LCMS</td>
<td>liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>min</td>
<td>minute(s)</td>
</tr>
<tr>
<td>MBB</td>
<td>molecular building block</td>
</tr>
<tr>
<td>μL</td>
<td>microliter(s)</td>
</tr>
<tr>
<td>μm</td>
<td>micrometer(s)</td>
</tr>
<tr>
<td>MW-SPPS</td>
<td>microwave-assisted solid phase peptide synthesis</td>
</tr>
<tr>
<td>mg</td>
<td>milligram(s)</td>
</tr>
<tr>
<td>mL</td>
<td>milliliter(s)</td>
</tr>
<tr>
<td>mmol</td>
<td>millimole(s)</td>
</tr>
<tr>
<td>mol</td>
<td>mole(s)</td>
</tr>
<tr>
<td>[M+H]^+</td>
<td>molecular ion plus hydrogen (mass spectrometry)</td>
</tr>
<tr>
<td>m/z</td>
<td>mass to charge ratio.</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer(s)</td>
</tr>
<tr>
<td>MAP</td>
<td>multiple antigen presenting</td>
</tr>
<tr>
<td>m</td>
<td>multiplet (NMR)</td>
</tr>
<tr>
<td>NMP</td>
<td>N-methyl-2-pyrrolidone</td>
</tr>
<tr>
<td>NCL</td>
<td>native chemical ligation</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
</tbody>
</table>
PBS phosphate-buffered saline
PDB Protein Data Bank
Π Pi
pH potential of hydrogen
ppm parts per million
rcf relative centrifugal force
RNA ribonucleic acid
Rf retention factor
siRNA small interfering RNA
SPPS solid-phase peptide synthesis
SPAAC strain-promoted azide-alkyne cycloaddition
SNARE soluble N-ethylmaleimide-sensitive factor attachment receptor
SEC size-exclusion chromatography.
PET positron emission tomography
POCs peptide-oligonucleotide conjugates
TOF time-of-flight
TFA trifluoroacetic acid
TIS triisopropylsilane
TBTA tris((1-benzyl-4-triazolyl)methyl)amine
tRNA transfer Ribonucleic acid
TLC thin layer chromatography
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Tm</td>
<td>thermal denaturing temperature</td>
</tr>
<tr>
<td>TMS-acetylene</td>
<td>trimethylsilylacetylene</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet radiation</td>
</tr>
<tr>
<td>Z</td>
<td>amino azide</td>
</tr>
</tbody>
</table>
Abstract

TOWARD THE DESIGN, SYNTHESIS, AND CHARACTERIZATION OF ABIOTIC COILED-COIL PEPTIDES VIA SOLID-PHASE COPPER-CATALYZED AZIDE-ALKYNE CYCLOADDITION (SP-CUAAC) CLICK REACTION FOR THE PREPARATION OF CONTROLLED SELF-ASSEMBLY MOLECULAR BUILDING BLOCK

Liaquat Ali
Thesis Chair: Sean C. Butler, Ph.D.
The University of Texas at Tyler
April 2022

The production of cutting-edge materials, the development of novel medications, drug delivery systems, technological advancements, and biosynthesizing all depend on molecular building blocks. Proteins are required for the creation of intricate, well-organized structures, and coiled-coil protein domains are vital subunits for the oligomerization of protein complexes, gene expression, and the structural components of biological materials. The numerous interactions between a wide variety of amino acids make it difficult to assemble protein complexes with a particular shape.

In the current study, we successfully designed and synthesized four different 32-residue peptides, each of which had two modified amino azide residues. These peptides were then used in solid-phase copper-catalyzed azide-alkyne cycloaddition (SP-CuAAC) click reactions to incorporate aryl aldehyde and acyl
hydrazide functionalities into peptide oligomers. Although, results from LCMS indicate that individual peptides and click reaction product were successfully synthesized. Furthermore, we analyzed the orthogonality of peptide pairs by size exclusion chromatography (SEC) for self-assembly of paired coiled coil peptide and show successful association of dimeric coiled coils.
CHAPTER ONE

AN OVERVIEW OF COILED-COIL PEPTIDE AND ITS IMPLICATIONS

1.1 The Fundamentals of Coiled-Coil Sequence and Structure

Coiled coils are protein structural motifs that wrap around each other to form supercoiled helices (Fig. 1.1 a, b). Helix-helix interactions are guided and solidified via so-called knobs-into-holes (KIH) interactions, as outlined by Crick,\(^1,2\) (Fig. 1.1 c, d). A sidechain (the knob) from one helix fits into a diamond-shaped arrangement (the hole) extending from another helix in these tight connections. To qualify as \(\alpha\)-helical coiled-coil structures, helical assemblies must include contiguous sections of KIH contacts; otherwise, they are merely globular \(\alpha\)-helical domains or bundles, with alternative and less-intimate packing patterns operating. Proteins with \(\alpha\)-helical structural elements are important components that oligomerize protein complexes engaged in biological processes or serve as structural elements of biological materials. Basic leucine-zipper proteins, for example, have coiled-coil motifs that enable dimerization,\(^3\) which alters biochemical processes such as gene regulation in a variety of species.\(^4\) Tropomyosin, an actin cytoskeleton regulator, polymerizes along actin filaments\(^5\) and the stiffness and stability of the coiled coil are linked to muscle activity.\(^6\) Coiled coils have been studied molecularly over the past three decades, in addition to their important biological functions.
The basic sequence structure relationship of coiled coils will be described in order to understand how coiled coils with specific attributes may be built. Two (up to seven) \( \alpha \)-helices wrap around each other to form coiled coils. These \( \alpha \)-helices feature a distinctive heptad \( abcd\text{efg} \) sequence that aids their assembly into supramolecular structures.\(^7\)\(^9\) The hydrophobic core of the structure is defined by KIH interactions.

**Figure 1.1.** The foundation of coiled coils is a knob-into-holes packing of \( \alpha \)-helices. An \( \alpha \)-helical coiled-coil dimer is shown in (a & b) orthogonal projections of its X-ray crystal structure.\(^9\) Orthogonal projections of an \( \alpha \)-knob from (c & d) the same structure as the panels into a d’g’a’d ‘hole is shown in (a & b). Key: a red, d green, and g violet, www.pymol.org was used to create the protein-structure pictures.)

**1.2 Simple Guidelines for Coiled-Coil Design and Prediction**

The basic sequence structure relationship of coiled coils will be described in order to understand how coiled coils with specific attributes may be built. Two (up to seven) \( \alpha \)-helices wrap around each other to form coiled coils. These \( \alpha \)-helices feature a distinctive heptad \( abcd\text{efg} \) sequence that aids their assembly into supramolecular structures.\(^7\)\(^9\) The hydrophobic core of the structure is defined by KIH interactions.
between $a$ and $d$, which are hydrophobic amino acids (Ile, Val, or Leu). $e$ and $g$ are often charged with amino acids (Glu, Arg, or Lys) that create interhelical salt bridges, defining the coiled coil topology (oligomerization state and helix orientation). Finally, $b$, $c$, and $f$ are polar amino acids that are exposed to solvents (Ser, Asn, Gln, etc.). Their proclivity for helix adds significantly to the coiled coil stability.\(^{10}\) Figure 1.2 is a common helical wheel diagram that represents this heptad design. For forecasting distinct coiled coil properties, a set of design principles has been devised.\(^{8,11–13}\) These include position-specific information on the influence of various amino acids on coiled coil stability, techniques for creating specificity and orthogonality in hetero-oligomers, and the length of a thermodynamically stable coiled coil.

**Figure 1.2.** The Coiled coil surface's hydrophobic interface, ionic interactions (or salt bridges), and amino acids exposed to solvent are all depicted in the diagram. The image shows a single heptad with the repeat pattern abcdefg.
The most common residues to occupy a coiled coil motif in conjunction with hydrophobic amino acids are amino acids with long and charged side chains (Table 2). Leucine is the most common hydrophobic amino acid. The most common polar amino acid is glutamine, which may be found on the solvent-exposed surface of coiled coils. A polar or charged residue may be put in a conventional hydrophobic location, and vice versa, without disrupting the overall fold of the coiled coil.\textsuperscript{14}

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>% Frequency in Coiled Coils</th>
</tr>
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<tbody>
<tr>
<td>Ile (I)</td>
<td>4</td>
</tr>
<tr>
<td>Leu (L)</td>
<td>12.5</td>
</tr>
<tr>
<td>Met (M)</td>
<td>2</td>
</tr>
<tr>
<td>Phe (F)</td>
<td>1</td>
</tr>
<tr>
<td>Trp (W)</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Tyr (Y)</td>
<td>1</td>
</tr>
<tr>
<td>Val (V)</td>
<td>4</td>
</tr>
<tr>
<td>Pro (P)</td>
<td>0.5</td>
</tr>
<tr>
<td>Ala (A)</td>
<td>7.5</td>
</tr>
</tbody>
</table>

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<thead>
<tr>
<th>Amino acid</th>
<th>% Frequency in Coiled Coils</th>
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<tbody>
<tr>
<td>Arg (R)</td>
<td>7.5</td>
</tr>
<tr>
<td>Asp (D)</td>
<td>4.5</td>
</tr>
<tr>
<td>Glu (E)</td>
<td>16</td>
</tr>
<tr>
<td>Lys (K)</td>
<td>10</td>
</tr>
<tr>
<td>His (H)</td>
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<th>Amino acid</th>
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<tr>
<td>Asn (N)</td>
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<tr>
<td>Cys (C)</td>
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</tr>
<tr>
<td>Gln (Q)</td>
<td>10</td>
</tr>
<tr>
<td>Gly (G)</td>
<td>2.5</td>
</tr>
<tr>
<td>Ser (S)</td>
<td>6</td>
</tr>
<tr>
<td>Thr (T)</td>
<td>4</td>
</tr>
</tbody>
</table>

\textbf{Table 1.} Amino acid composition of coiled coils (Oxford University Press: Evolutionary patterns in coiled coils, Surkont Jaroslaw: Pereira-Leal Jose B. (Copyright 2015, Oxford University Press))\textsuperscript{15}
Coiled coil stability is affected not only by sequence, but also by length: increasing coiled coil length from 3 to 4 and 5 heptads improves coiled coil stability.\textsuperscript{16} Helix propagation from the N-terminus becomes more beneficial as length rises, while helix propagation from the C-terminus becomes less favorable or stays unaltered.\textsuperscript{17} Furthermore, because the terminal heptads are prone to fraying, adding central heptads results in a disproportionately high level of coiled coil stability.\textsuperscript{16}

1.2.1 Additional Design Features

The design criteria listed above have typically been examined for dimeric parallel coiled coils, but they may now be used to anticipate the characteristics of these structures based on their main amino acid sequence. They have been used in programs like SOCKET,\textsuperscript{18} as well as structural design applications like coiled coil builder.\textsuperscript{19} Higher order structures and antiparallel coiled coils have recently been developed.

Antiparallel coiled coils have been studied in more depth as a result of the emergence of prediction tools and databases such as SYNZIP,\textsuperscript{20} and various de novo designs exist. Negron, devised a computer approach for predicting coiled coil orientation,\textsuperscript{21} which they used to create orthogonal antiparallel homodimers. These antiparallel coiled coils were subsequently employed as designed building pieces to construct protein-origami nanocages.\textsuperscript{22}

Despite significant advances in de novo protein and peptide creation, it has been recognized that using solely natural amino acids has apparent limitations. This is especially important for creating coiled coils, as the residue kinds must be limited to specified locations in the canonical heptad pattern. Introducing non-natural components into the amino acid sequence has emerged as a novel method for
overcoming the restrictions imposed by the number of available amino acid combinations. This technique broadens the sequence space and expands the range of options for modifying hydrophobicity and helix stability.

Synthetic bridges and staples are two non-natural alterations that are frequently utilized. These limit and maintain the helical structure, boosting the coiled coil's thermodynamic stability. Noncanonical amino acids, such as fluorine-modified amino acids or cyclohexyl alanine, can be inserted into hydrophobic core modifications. Azido amino acids are flexible building blocks in peptide synthesis. The latter is particularly intriguing since it has been utilized to improve heterotrimeric assembly selectivity.

In general, both natural and non-natural amino acid changes have been effectively used in the field of protein design to improve protein characteristics, such as in biomedicine and pharmacology. These alterations, on the other hand, are typically introduced to improve protein stability or increase the affinity or specificity of protein interactions. Attempts to regulate the mechanical characteristics of proteins have just lately been made. Understanding how to apply these methodologies to create proteins with controlled mechanical characteristics is especially important for coiled coils, which have been shown to have mechanical function in nature.

1.3 Self-Assembly Nanomaterials Based on Peptides

Peptides made up of amino acids may self-assemble into a variety of nanostructures that are typically biocompatible and biologically active. Functional peptide nanoparticles are regarded as a type of adaptable materials with vast
application prospects in the field of materials science due to their inherent benefits. Many scientists have studied peptide self-assembly extensively in recent decades and have achieved a basic knowledge of it. Furthermore, well-ordered nanostructured supramolecules are highly appealing "bottom-up" biomaterials with applications in nanotechnology and nanomedicine.\textsuperscript{26-27} Biomimetics and biologically-inspired nanomaterials research is an important topic that is quickly growing.\textsuperscript{27}

The peptide-based nanomaterial is appealing for a variety of reasons: The first is that peptides manufactured using solid-phase techniques might be molecularly changed, resulting in peptide-based nanomaterials with tailored features.\textsuperscript{28} Second, peptide-based nanomaterials can be further functionalized by inserting other molecules, such as enzymes, into the peptide nanostructure.\textsuperscript{29} Finally, by adjusting the secondary structures of peptide building blocks such as α-helices and β-sheets, the self-assembly process might be well-designed.\textsuperscript{30}

The creation of peptide-based nanomaterials has accelerated in recent decades. Recent achievements in peptide-based nanomaterials have been presented in a few reviews from various angles. Levin et al., for example, reviewed recent conceptual and experimental progress in self-assembling artificial peptide materials.\textsuperscript{31} The present state-of-the-art short peptide-based therapeutical advancements were described by Apostolopoulos et al.\textsuperscript{32}

1.3.1 The Non-Covalent Interactions that Cause Peptide Self-Assembly

Non-covalent interactions are common in nature and aid in the formation of complex, sophisticated biomolecular structures such as the DNA double helix,
secondary or higher-order protein structures, and cell membrane phospholipid bilayer structures.\textsuperscript{33} Non-covalent interactions are weaker and reversible than covalent bonding. They may help peptide monomers build stable structures, despite the fact that each non-covalent link has a low bond energy.\textsuperscript{34} Thermodynamics and kinetics drive the spontaneous self-assembly of peptides. Stacking, hydrophobic, electrostatic, van der Waals, and hydrogen-bonding interactions among intermolecular non-covalent interactions might preserve the structural integrity and stability of self-assembly systems (Fig. 1.3).\textsuperscript{35,36} The synergistic influence of non-covalent contacts determines the thermodynamic stability and lowest energy state of final nanostructures.\textsuperscript{37–39}

Hydrogen-bonding interactions are the main mechanisms controlling the self-assembly processes and the structure of peptide assemblies.\textsuperscript{40} At 298 K, hydrogen bonds have a strength of 10-40 kJ mol\textsuperscript{-1},\textsuperscript{41} and can exist in gaseous, liquid, solid, or supercritical phases. Peptides have a lot of hydrogen bonding sites, and hydrogen bonds are important for building and maintaining the secondary structure of peptides. They have excellent selectivity and directionality, which can lead to different nanostructures.\textsuperscript{42} In peptide self-assembly, π-π stacking, another type of weak noncovalent contact, is also frequent. There are two types of interactions that the aromatic residues of peptide building blocks can engage in: hydrophobic interactions and interactions. It has been discovered that the organization pattern of aromatic residues in hydrophobic interactions is typically disordered, whereas the organization pattern of aromatic residues in π-π interactions is ordered.\textsuperscript{43}
Figure 1.3. The forces that self-assembly uses to form its structures, and their patterns.

Hydrophobic interactions are essential for the rational design of water-soluble amphiphilic polypeptide molecules. The hydrophobic tails of amphipathic molecules prefer to collect in the center of aggregates, while the hydrophilic heads are exposed on the aggregates' edge and in contact with water. These interactions are sustained due to favorable entropy rather than favorable enthalpy and are critical for peptide self-assembly. The aromatic residues of peptide building blocks may function via hydrophobic interactions or π-π interactions. Electrostatic interaction is a non-covalent interaction used to create structural specificity in charged peptides. It involves electrostatic attraction and repulsion, as well as intramolecular and intermolecular electrostatic contact of points. Electrostatic contact is influenced by pH and ionic strength and can be altered by altering the pH or ionic strength of the solution.
1.3.2 Dynamic Covalent Bonding

Non-covalent and dynamic covalent interactions may be involved in peptide self-assembly. Reversible covalent chemistries, such as the disulfide bond created by cysteine residues and the imine bond formed by the reaction of aldehyde and amine groups, have been studied extensively as a mixture of non-covalent contacts that help peptide self-assembly. Zhang et al. recently published a paper describing a helical fibril structure made up of C₃-peptides with glycine–cysteine (Gly–Cys) dipeptide pendants. The oxidation of thiol groups produces disulfide connections between neighboring building blocks, which can help to maintain the left-handedness of long helical threads and improve overall nanostructure stability. Synthetic chemists have been very interested in mechanically interlocked molecules as prototypes of molecular machines. Link et al. recently reported the synthesis of mechanically interlocked peptides, which were made possible by the self-assembly of completely peptidic, cysteine-decorated building blocks in water, resulting in a variety of disulfide-bonded dynamic interlocked molecular libraries. Peptides decorated with aryl aldehyde and acyl hydrazide functions were employed to produce peptide quaternary assemblies in addition to disulfide chemistry. In moderate circumstances, a combination of complementary peptides might react to generate peptide-peptide intermolecular macrostructures with customizable ring diameters. Controlling the quantities of reactive peptides might be used to create more complex ladder structures. The research establishes a standard strategy for constructing complicated abiotic quaternary structures.
1.4 Application of Peptide Building Block

Having understood the sequence structure mechanics relationship and being able to design coiled coils with defined mechanical properties de novo, what can we "build" with them? Many applications of coils and coil coils have already been developed, such as protein-based materials, biosensors, and protein-protein interactions.\(^4\)

As an affinity tag for protein purification, the EK heterodimer was developed by Hodges et al.\(^5\) and can immobilize proteins in biosensors and be used for protein immobilization.\(^5\) A more interesting application of it is to develop membrane fusion methods inspired by SNAREs for synthetic vesicles.\(^45\) It recognizes transport vesicles and initiates membrane fusion by binding to SNARE (Soluble NSF Attachment Protein REceptor) proteins. A four-helix bundle of SNAREs is formed as a result of the zipper-like assembly of SNARE molecules. Coil-coiled membranes have been used to mimic SNARE-mediated membrane fusion. They have ability to trigger the development of synthetic lipid vesicles, making it easier to employ them as drug carriers.

Peptide-based nanostructures offer some benefits over alternative delivery systems, such as their chemical variety, biocompatibility, and precise binding to target proteins, which makes them a perfect candidate for biological applications such as drug administration. It has been suggested that doxorubicin-conjugated peptide nanoparticles are an effective cancer therapeutic option. To create microparticles, Rubert et al. suggested a technique of hierarchically constructing peptide-based nanofibers with alginate,\(^5\) where the alginate shell is functionalized to bind the folate
receptor and the doxorubicin is conjugated to the alginate core.\textsuperscript{55,56} Drugs containing nucleic acids have been used to treat AIDS, cancer, and viral infections. Theoretically, small interfering (siRNA) may target any protein and is simple to assemble. In recent years, siRNA medicines have drawn increasing amounts of interest.\textsuperscript{57} Admittedly, RNA medications are readily degraded and cannot cross cell membranes; thus, effective and biocompatible nucleic acid delivery mechanisms are essential.\textsuperscript{58} Nowadays, the polypeptide carrier system is often lengthy (more than 20 amino acids), which makes it expensive and complicated to create. A new siRNA-induced peptide co-assembly nanocarrier with high RNA silencing effectiveness, minimal cytotoxicity, and good biocompatibility both in vitro and in vivo was described by Li et al.\textsuperscript{59} The peptide-nucleotide nanoparticle in this system has just nine amino acids and is based on peptides that have been methionine-modified. Polypeptides are a class of self-existing proteins found in living things. They are extremely biocompatible and efficient nucleic acid transporters, and their use in biomedicine has garnered a lot of interest.\textsuperscript{60}
CHAPTER TWO
DESIGN OF COILED-COIL PEPTIDE FOR PROTEIN BUILDING BLOCK

2.1 Peptide Base Material

Proteins are naturally complex entities made up of basic building elements. Despite their essential function as the building blocks of life, protein-based bulk materials were widely used to create textiles made from animals (wool) or insects (silk). The desire for low-cost synthetic substitutes arises from the fact that collecting resources from living species is occasionally seen as inefficient and/or unethical, even though these natural materials are strong but biodegradable and find use in various sectors. Synthetic protein-based materials continue to be in the limelight despite the fact that equivalent synthetic materials can also be made from organic polymers because they are flexible, biodegradable, and in some cases, highly accurate replicas of natural materials.

Although proteins have been used to create several fascinating and practical materials, there is a specific size threshold at which they become difficult to research. Prokaryotic systems can produce huge amounts of proteins that can be produced and collected, but these methods are constrained by the canonical amino acids. As the number of unnatural residues rises, incorporating non-canonical amino acids into expressed proteins quickly becomes a challenging task. Nevertheless, many labs continue to use post-purification modifications, amber and ochre codons, and non-
canonical side chains to incorporate unnatural side chains into large proteins for study.\textsuperscript{62}

Synthetic proteins, in contrast, permit the maximum inclusion of non-canonical amino acids but suffer from time-consuming synthesis, challenging purification, and low yield because of several tandem processes. Targeting synthetic peptides, which are shorter and can be produced in higher quantities with less difficulties, can help to alleviate these issues. Synthetic peptides are capable of folding into a wide variety of secondary and tertiary structures, however the range of folds that may be achieved is constrained by short sequence length. Simplified design principles for their construction, however, are a benefit of simpler folding motifs. Additionally, because single mutations can have more noticeable impacts on the structure and because foreign residues can be inserted anywhere in the sequence, peptides are more flexible than proteins in terms of fine-tuning.

Similar to proteins, self-assembled peptides also rely on folding-directed assembly, but because of their shorter length, the local folding motifs that may be used by the monomer are fewer. Synthetic peptides are nevertheless widely used in supramolecular materials despite this problem. The amino acid sequence should ideally contribute to secondary structure and self-assembly. Several self-assembling $\beta$ sheet peptides have been investigated\textsuperscript{63} for use in antibacterial agents,\textsuperscript{64} responsive hydrogel,\textsuperscript{65} and nanofibers.\textsuperscript{66} Moreover, as strong peptide-based nanomaterials, collagen and other polyproline structures have been thoroughly investigated.\textsuperscript{67–69} In materials chemistry, $\alpha$-helices have also had some success. Peptides as little as seven amino acids can form helices,\textsuperscript{70} which can span hundreds of residues without
In terms of materials, the tendency of α-helices to form coiled coils is the most prominent characteristic.

Following the publication of Woolfson's self-assembling fiber peptides, a lot of research groups have focused on coiled coils. According to a review by Klok from 2010, coiled coils have been designed with a variety of properties that aid in supramolecular assembly, including disulfide bond induced stability, small molecule induced self-assembly, pH, salt, and temperature sensitivity (some of which cause to shift in secondary structure), and others.

2.2 Design of Individual Peptide

All the peptides utilized in this investigation were adapted from earlier research by Gradišar and Jerala. The four heptads that make up a peptide have an additional Ser-Pro-Glu-Asp (SPED) extension at the N-terminus, and this portion of the coiled-coil has unique properties because the capping interactions at the ends of α-helices play a significant role in determining the stability of the protein's secondary and tertiary structure. The most common residues are found at the N-terminal locations, where they either exhibit unique dihedral angles, stabilize helical dipoles, or create hydrogen bonds between their side chains and the polypeptide backbone. Table 2 outlines the process of creating tailored peptides.
Table 2. Inventive sequences created by Gradišar and Jerala to produce the coiled-coil peptide pairs DA3|DA4 and DA5|DA6 that are orthogonal and parallel. The one-letter amino acid code is used to represent the sequences. The bolded leucine residues at position d make up the leucine zipper, residues in position a created a hydrophobic pattern. Electrostatic pattern formed from residues at positions e and g.

<table>
<thead>
<tr>
<th>Sequence</th>
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<tbody>
<tr>
<td></td>
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<tr>
<td><strong>DA3</strong></td>
</tr>
<tr>
<td>SPEDE</td>
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<tr>
<td>EIQLKE</td>
</tr>
<tr>
<td>EIQLKEQ</td>
</tr>
<tr>
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</tr>
<tr>
<td>KIAQLKQ</td>
</tr>
<tr>
<td>ENQLEEE</td>
</tr>
<tr>
<td>ENNALEY</td>
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<td>IINN</td>
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<tr>
<td>KKEE</td>
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<tr>
<td><strong>DA5</strong></td>
</tr>
<tr>
<td>SPEDE</td>
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<tr>
<td>ENAALEE</td>
</tr>
<tr>
<td>KIAQLKQ</td>
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<tr>
<td>KNAALKE</td>
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<tr>
<td>EIALEY</td>
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<tr>
<td>NINI</td>
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<tr>
<td>KKEE</td>
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<td><strong>DA6</strong></td>
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<tr>
<td>NINI</td>
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<td>KEKE</td>
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According to the rule that hydrophobic residues at positions a or d and oppositely charged residues at positions e and g between the two helices stabilize, while polar residues (such as Asn) buried at positions a or d and the same charge at positions e and g destabilize the coiled-coil dimer, we also designed sequences for four heptads that make up a peptide. In this simplified model, we only took into account a small number of variable residues at the a, d, e, and g locations, ignoring their impact on other places (positions b, c and f).

The contact between the residues at locations g and e of the matching heptads directs the electrostatic interactions between heptads in the parallel orientation. Whereas residues with the same charge at such locations would be detrimental for the pair formation, oppositely charged residues stabilize the proper pair. The electrostatic instability of the homodimers caused by this design method also encourages the development of heterodimers. The residues Gln, Glu, or Ala are
occupy positions b, c, and f, respectively and they have the highest tendency to form helices, give enough solubility for the peptide, and inhibit the development of aggregates above dimers, which may also be controlled by residues at those locations.\textsuperscript{10,77}

The best conditions for the production of parallel coiled-coil dimers seem to be Ile or Asn at position and Leu at position d. As a result, Ile or Asn residue was utilized for position a, while Leu residue was put for position d in all heptads. In contrast, pairing Asn and Ile is severely punished, which offers a crucial mechanism for ensuring the right combination. This design choice also precludes the creation of antiparallel heptads, where the Asn at position a would be coupled with Leu at location d, in addition to destabilizing the wrong parallel pairings.

2.3 Design of Orthogonal Binding Peptide Pairs

Four distinct arrangements are conceivable for each heptad based on the design principles mentioned above: two based on various charged residues at positions g and e, (same or opposite charge), multiplied by two options depending on the residue at position a. (either Asn or Ile). With the help of these bases, we were able to generate the combinatorial space for four heptads, which may be chosen from four combinations with two Asn and two Ile residues as well as four electrostatic combinations. The set of two peptide pairings with the highest degree of orthogonality among the various combinations was chosen in order to maximize the energy difference between the proposed sequence and any other undesirable combinations. Negative design, on the other hand, tends to dramatically lessen the intensity of the
interaction between two peptides, especially for the unwanted combinations and orientations. As a result, the energy gap between the desired and undesirable combinations widens.

As a result, we created a pair of two coiled-coil-forming pairs, and Table 2 shows their sequences. Our heptads provide the basis of each peptide. Each peptide has two Asn(N) insertions at the equivalent a site of the same pair of coiled-coil-forming peptides, which is different from the places in other orthogonal pairings and adds to the pairing specificity. With the layout IINN, IIIN, NINI and NINI for pairings DA3|DA4 and DA5|DA6, respectively, each peptide at locations an of four heptads comprises two Asn (N) and two Ile (I) residues. Acidic Glu (E) and basic Lys (K) residues were arranged as follows for electrostatic coding: EEKK and EKKE for peptides DA3 and DA5, and the oppositely charged residues for peptides DA4 and DA6, which served as their complementary pairing partners. The design of the set of orthogonal pairings was confirmed by the size exclusion result, which shows that peptide pairs DA3-DA4 and DA5-DA6 are the most stable.

2.4 Design of Heterodimer Peptide

In this project, four peptides were synthesized, and each was intended to mate with a single complimentary peptide in α-helical coiled-coil pattern while keeping their specificity. Nevertheless, the focus of this work was on the controlled self-assembly of many heterodimer peptide pair molecular building blocks that linearly bond in α-helical coiled-coil pattern. To accomplish this, two peptides were first
covalently bonded together, and then these crosslinked peptidic building blocks were linked to the appropriate peptide pair.

The previously mentioned heptad sequence design oversaw controlling the parameters affecting the selectivity and stability of the peptide interaction. One further modification was made to the first and third heptads, adding an unnatural amino acid residue at position f to enable crosslinking between certain peptide partners. These heptads' position f is in opposition to the hydrophobic core that develops during peptide interaction. This makes it possible to covalently couple two peptides while still allowing either side of the newly formed pair's heptad sequence to interact with other peptides. The crosslinked peptide pair additionally maintains the specified selectivity and specificity on either side since each peptide has also been engineered for orthogonal interaction. Two of the peptides are further linked together by a 1,3-dipolar cycloaddition click reaction after azido-L-ornithine modified position f, introducing the capacity to covalently connect with complementary aldehyde and hydrazide functionalities. as shown in Table 3, the individual peptide sequences synthesized for this project were labeled as DA3 through DA6, respectively.
Table 3. created peptide sequences that are utilized as molecular building blocks. Azido-L ornithine is a modified amino acid Z that is located at position f in the second and third heptad. The relationships of the peptides DA3|DA4 and AD5|DA6 have the same hydrophobic and electrostatic patterns.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Hydrophobic Pattern</th>
<th>Electrostatic Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>DA3</td>
<td>SPD EIQQLKZ</td>
<td>EIQQLEQ</td>
</tr>
<tr>
<td></td>
<td>KNAALKZ</td>
<td>KNQALKY</td>
</tr>
<tr>
<td></td>
<td>INNN</td>
<td>EKK</td>
</tr>
<tr>
<td>DA4</td>
<td>SPD KIAQLKZ</td>
<td>KIQALKQ</td>
</tr>
<tr>
<td></td>
<td>ENIQQLEZ</td>
<td>ENAASEY</td>
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<td>IINN</td>
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<tr>
<td>DA5</td>
<td>SPD ENAASEY</td>
<td>KIAQLKQ</td>
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<td></td>
<td>KNAALKZ</td>
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<td>DA6</td>
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</table>

2.5 Enhanced Synthesis of Fmoc Azido Amino Acids

The discovery of the 1,4-disubstituted 1,2,3-triazole-forming Huisgen 1,3-dipolar cycloaddition reaction of azides and alkynes, which is CuI-catalyzed, has sparked a boom in these kinds of click reactions, which have now found a wide range of uses in peptide and protein chemistry. It is due to the motif that 1,2,3-triazoles exhibit, which has peptide bond-like structural and electrical properties. Other uses for 1,2,3-triazoles include cyclization, the induction of β-turns, β-hairpins, helical structures, or the creation of peptide disulfide bond mimics. A further application of azides is in a Staudinger ligation variation to produce peptides and proteins. However, the high expense of azido or alkyne precursors, namely Fmoc-protected azido, and alkyne amino acids, which are typically available for around $250-300 per 250 mg, frequently hinders the synthesis of peptides containing azido or alkyne moieties. As we just discovered, this can make the creation of bigger series of
azido/alkyne peptides exceedingly costly. Due to this, we chose to prepare Fmoc-protected azido amino acids internally from L-ornithine. To synthesize 5-azido-2-(9-fluorenylmethyloxycarbonylamino) pentanoic acid 5, which are L-ornithine derivatives, we used the orthogonal functional group protection method (Scheme 2.1).87–91

**Scheme 2.1.** Synthesis of Fmoc azido amino pentanoic acid (5) from L-ornithine. HCl (1).

L-Ornithine.HCl 1 was used as the starting material for the synthesis, and under basic circumstances, the reaction produced the copper (II) complex of tert-butoxycarbonyl-L-ornithine 2, which was then purified by precisely filtering off its insoluble copper complexes. Utilizing 8-quinolinol, metal was removed quantitatively to produce intermediate 5-(tertButoxycarbonylamino)-2-aminopentanoic acid 3 that were specifically Boc-protected and acquired the form of zwitterions. The 5-
(tertButoxycarbonylamino)-2-(9fluorenylmethyloxycarbonylamino) pentanoic acid 4 that were produced after the alpha amino group was acylated with 9-fluorenylsuccinimidyl carbonate (Fmoc-OSu) were then treated with TFA to release the free amine. A diazotransfer reaction, which produces the necessary 5-azido-2-(9-fluorenylmethyloxycarbonylamino) pentanoic acid 5, is the last step. The comprehensive physicochemical characterization of all intermediates and final products was supplied in the experimental section, along with detailed descriptions of all synthetic processes.
CHAPTER THREE

PEPTIDE CONJUGATION VIA CUAAC ‘CLICK’ CHEMISTRY AND SELF-ASSEMBLY

3.1 Peptide Conjugation

At the turn of the twentieth century, peptides and proteins were identified. Scientists did not figure out their natural biosynthetic process for another 50 years. The transcription of deoxyribonucleic acid (DNA) sequences to ribonucleic acid (RNA) sequences, also known as messenger RNA (mRNA), is the first step in the production of peptides and proteins in cells. The ribosome converts these mRNA transcripts to peptides or proteins. Amino acids are conjugated to one another with the aid of transfer RNA (tRNA) in this biological mechanical ligation factory inside living cells. The proteins that arise serve as enzymes, structural proteins, signaling proteins, and other important functions in living organisms. Proteins are a popular component of modern pharmaceutics because of their wide range of biological functions. Insulin, for example, was first taken from cows and pigs, refined, and utilized in humans as a medicinal treatment to manage blood sugar levels in patients with Type 1 diabetes. Scientists have attempted to duplicate the conjugation process in vitro in order to optimize this procedure. When Theodor Curtius succeeded in conjugating the first N-protected dipeptide, benzoyl glycylglycine, in 1881, chemical peptide synthesis began. Emil Fischer proposed an alternate glycylglycine dipeptide synthesis through
a hydrolysis mechanism twenty years later. Since then, interest in peptide synthesis has grown, and with the advent of temporary protective groups like carbobenzyloxy (Cbz) by Bergmann and Zerwas in 1931, Vigneaud et al. were able to synthesize the first physiologically active peptide hormone (oxytocin). Finally, Merrifield's invention of solid support (also known as solid phase peptide synthesis or SPPS) in the early 1960s simplified peptide synthesis. The SPPS method enabled the production of complex, chemically manufactured physiologically active peptides like human insulin and the enzyme ribonuclease. SPPS can effectively synthesize peptides up to 50 amino acids in length in a short amount of time. The solubility of the increasing peptide and the buildup of by-products on the solid substrate were major limitations of SPPS for the synthesis of longer peptide (>50 amino acids), resulting in low purity and yield.

Wieland et al. established native chemical ligation (NCL) in 1953, which was a more beneficial chemical approach. NCL involves the conjugation of a C-terminal thioester peptide to an N-terminal cysteinyl peptide. However, this procedure was significantly more efficient since it did not need the protection of peptide side chains, and the end product was generated with a high yield and purity. However, the peptide of interest had to have a cysteine residue (or its derivatives) in its native sequence, or else a desulfurization procedure would be necessary to remove the 'unwanted' sulfur group. As a result, other sulfur moieties within the peptide construct must endure the desulfurization process if they are present, or else the sulfur groups will be eliminated, resulting in an unwanted peptide build. Despite the fact that NCL is an extremely successful approach for producing big peptides and proteins,
challenges with ligation of hydrophobic target products have been reported. NCL has also shown to be problematic to use as a conjugation approach between peptides and non-peptidic compounds like polymer. Alternative techniques for peptide modification have been devised, including the Staudinger ligation, the Diels-Alder reaction, strain promoting alkyne-azide cycloaddition (SPAAC), and copper catalyzed alkyne-azide cycloaddition reaction (CuAAC).

3.2 Peptide Conjugation via CuAAC ‘Click’ Chemistry

3.2.1 Copper (I) Catalyzed Alkyne-azide 1,3-Dipolar cycloaddition (CuAAC)

Author Michael discovered and reported the synthesis of triazole in 1893. Rolf Huisgen conducted comprehensive investigations on the nature of this reaction in 1961, and it was dubbed the 1,3-dipolar cycloaddition (Scheme 3.1). L'Abbé originally reported the use of copper as a catalyst for the Hüisgen azide-alkyne 1,3-dipolar cycloaddition (CuAAC) in 1984 as a side reaction during the synthesis of azidoallenes complex. There was no further examination into this discovery until 2001, when two separate laboratories led by Sharpless in the United States and Meldal in Denmark introduced the response. The CuAAC reaction, often known as the ‘click' reaction, is a regioselective copper (I) catalytic process that produces 1,4-disubstituted 1,2,3-triazoles under moderate circumstances (Scheme 3.1, comprehensive probable mechanisms were described by Jones et al. and Himo et al.). The CuAAC reaction became a popular conjugation approach soon after its discovery, owing to its high robustness, selectivity, and resistance to pH and temperature variations. CuAAC is
being employed in a variety of disciplines, including biomolecular and pharmaceutical chemistry, as well as polymer sciences. 103–105

Although copper (I) is required for the cycloaddition reaction, in some cases, temperature control and the addition of ligand molecules or a reducing agent, as well as the introduction of ligand molecules or a reducing agent, can push the reaction even further towards its desired product. The reaction was catalyzed by a variety of copper (I) sources that were evaluated. Copper (I) iodide (CuI), copper (I) bromide (CuBr), copper (II) sulfide (CuSO₄), and copper (0) are some of these (such as copper wire, powder and palette). Meldal and colleagues, for example, utilized CuI and N, N-diisopropylethylamine (DIPEA), (a base that pre activates CuI by creating a copper-acetylene complex) in N, N-dimethylformamide (DMF) at 25 °C to produce 1,4-disubstituted 1,2,3-triazole structures. 106 Jang et al. proposed an alternate technique that comprised the addition of sodium ascorbate, a reducing agent that converted in situ copper (II) to copper (I), as well as the substitution of DIPEA with pyridine, which resulted in the production of the triazole structure. 107 The removal of the base from the reaction mixture was shown to have no effect on the reaction yield, hence a base-free CuAAC is often reported. 104,108,109 The addition of a copper ligand, which was optional, aided the reaction's progress while also preserving the Cu(I) ions from oxidation. Meldal has evaluated the adaptability of this cycloaddition process. 110

CuAAC's usage for biological molecules, however, is contentious due to copper toxicity and the use of the reducing agent. Active copper species, for example, have been shown to quickly produce radicals that may (partially) breakdown or destroy peptides and protein complexes during CuAAC reactions, whereas copper complexes
may be taken up by cells in an in vitro system, affecting cellular metabolisms and functions. Compatibility can be increased in CuAAC-cell by using water-soluble ligands (e.g., bis-(L-histidine) or, in certain circumstances, accelerating CuI-ligands that permitted modest CuI loading during catalytic reaction. Copper wires have also been found to catalyze the CuAAC reaction without the need of any additional ligands or reducing agents. Copper toxicity is well known; yet, because copper is an important element for human health, the amount of copper traces present in biologically relevant material must be accurately evaluated (the suggested copper health standard level is below 15 ppm).

![Scheme 3.1](image)

**Scheme 3.1.** A triazole ring produced via the general CuAAC reaction.

### 3.2.2 Amide Bond and 1,4-Disubstituted Triazole Structure Studies

General reaction for CuAAC reaction producing triazole ring amide bonds serve a crucial function in regulating a protein's bioactivity. Amino acids, which are the building blocks of proteins, are linked by amide bonds. These bonds have a limited range of flexibility, allowing for different protein conformations. Intramolecular interactions between nearby peptide chains, such as hydrogen bonding, disulfide
bridge formation, or hydrophobic interactions, increase this structural shape. As a result, even a single amino acid replacement at any location throughout the protein might cause structural changes. When an unnatural element is inserted into a peptide or protein, the synthetic construct is ability to imitate the native structure is critical in ensuring that the synthetic constructs preserve the required biological function. For example, it was revealed that changing a single amino acid in a synthetic luteinizing hormone releasing hormone (LHRH) lowers its activity by substantially changing the peptide folding. Similarly, for the immune system to identify and develop a protective antibody against the intended pathogen, an antigen in a subunit peptide vaccination must fold into its natural shape.

Because of the significance of peptide and protein conformation, it is difficult to replace artificial components for peptide bonds. Peptide bonds can be substituted by functional groups that imitate them (e.g., ester), but not all of them (e.g., alkene) can preserve the peptide's secondary structure when inserted into the sequence. These findings generated specific attention in the application of triazole moiety for this purpose. Horne et al. were the first to study the structure of amides and triazoles in 2004 using modified pLI-GCN4 sequences, which have α-helix coiled coil structure. They found that despite a 1.1-fold increase in the length of the amide bond in the peptide backbone and this changed peptide was still able to maintain its helical conformation. The researchers used triazole analogs of a peptide-based HIV protease inhibitor and discovered that the modified constructions still had nanomolar inhibitory action. Furthermore, the triazole structure has nearly identical polarizing characteristics to amides, including the locations of hydrogen bonding donor and
acceptor, as well as a similar electrophoretic dipole (5 debye vs. 4 debye in amide bonds). In addition, the triazole ring can form hydrogen bonds with other amide groups, analogous to how an amide group aligns with other amides in peptide secondary structure. Triazoles, on the other hand, are resistant to proteolytic amide breakdown, unlike native amide bonds. To summarize, structural alteration of peptides and proteins utilizing the CuAAC process may result in a structural mimic of native amide linkages.

Triazoles have been discovered to be effective peptide bond substituents, owing to their potential to boost peptide biological stability while preserving activity in vivo. Various proteases are extremely vulnerable to the naturally occurring amide bond.\textsuperscript{119} Thus, replacing the amide bond with a triazole gives another option for increasing the target compound's bioavailability in vivo. Another benefit of triazole substitution is that it improves the CuAAC reaction. CuAAC is selective for terminal azide and alkyne functional groups, unlike peptide coupling processes. As a result, unprotected peptides with azide/alkyne groups can be used in this procedure. The CuAAC reaction is very simple to carry out, with a wide variety of reaction mediums and copper sources to select from. As a result, the reaction condition may be changed to meet the conjugation reaction circumstances. CuAAC is largely employed for peptide-to-biomolecular conjugation, despite its flexibility and the triazole moiety is ability to imitate the amide bond.
3.3 CuAAC is Used in the Modification of Peptides.

Tornoe et al. reported the first use of the CuAAC reaction to generate peptide derivatives in 2002 with the synthesis of peptidotriazoles and neoglycopeptide-linked-triazoles on solid support. A wide range of azido groups were investigated, yielding compounds with crude purities ranging from 75% to 95%. Because this reaction may be used to conjugate molecules not just between them (intermolecular coupling), but also within them (intramolecular coupling), the number of publications that have employed it has increased tremendously. The enormous range of applications for this approach has lately been expanded to include both intermolecular and intramolecular conjugations.

3.3.1. Intermolecular Coupling with a Single Site

Tagging biomolecules is one of the goals of azide-alkyne single scaffold conjugation. The target of interest can be visualized using positron emission tomography (PET) or fluorescent imaging (fluorescent microscopy) by labeling peptides or proteins with radioactive molecules (such as iodine-125 or fluorine-18 and their derivatives) or fluorescent compounds (green, fluorescent t protein, GFP). The arginine-aspartic acid-glycine (RDG) peptide was employed by Sewald and colleagues as a targeting moiety for anticancer medication delivery. Cryptophycins, an apoptosis-promoting and tubulin-inhibiting depsipeptide (anticancer drug), were conjugated to cyclic RDG peptides using the CuAAC process. Conjugation of RDG to cryptophycins, however, lowers the drug's effectiveness. Due to steric hindrance,
adding a fluorescein derivative to the medication reduced its affinity for the microtubule in cancer cells.

The CuAAC process was utilized to change the biological characteristics of peptide-oligonucleotide conjugates (POCs) in addition to tagging. Astakhova et al. used the CuAAC process to bind enkephalin peptides to oligonucleotides (deoxyribonucleic acid, DNA) and studied the structure and characteristics of the oligonucleotides. The procedure was extremely repeatable, and the conjugation yields were over 95%. POCs were stable at greater temperatures (up to 10 °C) than free oligonucleotides, according to thermal denaturing temperature (Tm) study. In comparison to locked-nucleic acid DNA (locked-DNA) and unmodified DNA (control), which decomposed after 1 hour and 30 minutes, structural study of double POC conjugates revealed structural durability of the POCs for up to 8 hours in diluted human blood (90 percent).

3.3.2. Intermolecular Coupling with Multiple Site

The CuAAC process allows two molecules to be conjugated at a single location. Numerous molecules can be conjugated to a single multi-site entity in a precise way, and two molecules can be linked via multiple conjugations. Dendritic, linear, cyclic, and cross-linked assemblies are some of the several types of biomolecule conjugation (Figure 3.1). Although dendrimer and linear assemblies are normally utilized in medicinal chemistry, they have also been used in vaccine production to allow antigen inclusion in a multiple antigen presenting (MAP) system. This system
has been found to elicit greater immune responses than a single antigen presentation system.\textsuperscript{125} The SPPS approach was used to create MAP-based constructions. However, purifying the peptides to homogeneity is frequently problematic. NCL of consecutive antigens is time-consuming and attaching many epitopes at the same time might be challenging.\textsuperscript{126} To efficiently build such structures, CuAAC offers an alternative to both stepwise SPPS and NCL. CuAAC progressed faster than multivalent NCL, yielded a greater yield, and the triazole product was stable in a biological context.\textsuperscript{127}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Multiple conjugation method using the CuAAC method: (A) dendritic, (B) linear, (C) cyclic (D) cross-linked.}
\end{figure}
Reuther et al. used SP-CuAAC to effectively add complementary aldehyde and hydrazide dynamic covalent (DC) functionalities to peptide oligomers containing two, three, four, and six azide side chains, resulting in multi-DC peptides. When these complimentary peptides are combined, they create distinct, intermolecular quaternary structures via the creation of hydrazone connections in a fully abiotic way. By combining suitable complementary DC – peptides, interesting macrocyclic, zipper, triple loop, figure-of-eight, and dumbbell-shaped multi-loop macrocyclic quaternary structures were effectively created. They also succeeded in synthesizing high-MW (>14 kDa) peptide-based LPs with up to seven DC – peptides covalently assembled. To initiate polymerization, correct mismatching of reactive groups (Vernier templating) on DC - peptides was used. As a result of this study, it is now possible to not only insert dynamic covalent bonding (DCB-ing) functional groups into peptide oligomers, but also to deliberately assemble these materials into intriguing and predictable quaternary structures.

3.4 Solid-phase CuAAC Reaction of Dynamic Covalent Functionality.

Dynamic disulfide bonds are frequently used by naturally occurring peptides and proteins to impart specified tertiary/quaternary structures for the development of uniformly sized and functional binding pockets. Controlling the creation of quaternary structures remains a key problem despite the fact that peptide synthesis and modification are well understood processes. Here, we describe solid-phase copper-catalyzed azide-alkyne cycloaddition (SP-CuAAC) click reactions for
the straightforward insertion of aryl aldehyde and acyl hydrazide functionalities into peptide oligomers. A sequence of predictable quaternary configurations emerge as a result of correct matching of the number and spacing of these DCB-ing units during intermolecular hydrazone synthesis (Scheme 3.2a) between these complementary functional groups. These complimentary functional groups quickly combine to produce protein building blocks in aqueous environments with a neutral pH. In the present study \( N\)-Boc-4-ethynylbenzhydrazide synthesizes from 4-bromobenzhydrazide and used as acyl hydrazide show in scheme 2, while 4-ethynylbenzaldehyde used as aryl aldehyde.

In order to integrate the aldehyde and hydrazide functionalities onto azide-peptide side chains, we developed a solid-phase (SP) CuAAC click reaction. This straightforward procedure was utilized to combine the dynamic covalent bonding pairs into the peptide oligomers DA3 and DA4, respectively, with ethylbenzaldehyde (Al) and \( N\)-Boc-4-ethynylbenzhydrazides (Hy) (Scheme 3.2 b,c). The beginning peptides DA3Al and DA5Hy were detected in the liquid chromatography-mass spectrometry (LCMS) as show in figure D.5 and D.6.
3.5 Future Advancement

3.5.1 Formation of Hydrazone

In future progress, the synthesis of bridge between the peptide containing aldehyde functionalized DC-peptides can combine with complementary hydrazide containing DC-peptides need to be done for generating an unnatural peptide quaternary
structure. This synthesis could be done by the formation of hydrazone among two peptides as shown in scheme 3.3.

Scheme 3.3. Synthesis of hydrazone for the formation of dynamic covalent peptide.

3.5.2 Design of Molecule Building Blocks (MBBs)

Further studies of the self-assembly of multiple peptides for making molecule building block need to be done. Regulated MBB self-assembly should be designed once the hydrazone creation is finished. The regulated assembly of the MBBs will be accomplished by a stepwise addition method. As each peptide can only attach to one of the two different MBB sides, or one side of another MBB that has the appropriate peptide accessible for interaction, each side of the MBB will only interact with one of the two separate peptides. According to this approach we will associate the peptide containing
aldehyde functionality with DA4 and hydrazide functionality with DA6 respectively. The purpose MBBs for this project's are depicted in Scheme 3.4.

Scheme 3.4. Purpose self-assembly of peptide molecular building block a) DA4 associate with DA3-Al b) DA6 associate with DA5-Hy
CHAPTER FOUR
EXPERIMENT DETAIL

4.1 Synthesis of Fmoc Azido Amino Acids

4.1.1 Materials and Equipment

Solvents used in this study were of analytical grade. L-Ornithine hydrochloride, Boc-anhydride, Fmoc-OSu and sodium bicarbonate came from Chem-Impex and used as received. 8-Quinolinal, sodium azide, and triflic anhydride purchase form Oakwood chemical and used without further purification. Copper (II) acetate (Fisher chemicals) and Copper (II) sulfate pentahydrate (Lab Chem) were used as received. TLC analyses were performed on silicycle glass backed, 250 μm F-254 TLC plates using the following solvent systems (v/v): A, n-BuOH: AcOH: AcOEt: H2O (1: 1: 1: 1); B, ethyl acetate: acetone: methanol: water (6: 1: 1: 0.5), spot was visualized by exposure to UV light at 254 nm and by ninhydrin spraying. 1H NMR spectra for small molecule precursors were collected on Varian AS400 NMR spectrometer. IR spectra were recorded on Thermo Scientific Nicolet iS10 apparatus.
4.1.2 Synthesis of Copper (II) Complex with L-Ornithine (2)

The preparation of compound 2 began with a stirred solution of L-ornithine HCl 1 (1.0 equiv, 16.862 g, 100 mmol) in 2 M NaOH (100 mL). Next, a solution of Cu(CH$_3$COO)$_2$ H$_2$O (0.5 equiv, 9.982 g, 50 mmol) in water (50 mL) was added, and finally a solution of 96% Boc$_2$O (1.0 equiv, 28.73 g, 130 mmol) in 200 mL of acetone. After 24 hours, 100 mL more of the acetone was added, and stirring lasted for another 20 hours. The precipitate was removed using filtering and then rinsed with 200 mL of acetone: water (2:1) and water (2 x 500 mL). Then, compound 2 was allowed to air dry.

Yield: 19.67 g (37.3 mmol) of light blue solid, 75 %. $R_f$ (A) = 0.80, m.p. 244-245 °C. Because of the diamagnetism of copper, NMR spectra were not recorded. IR (KBr) \( \nu_{\text{max}} \) (cm$^{-1}$) 2934 m, 1385 s, 1364 (CH$_3$); 1679 vs (C=O) carbamate; 1614 vs (NH$_2$); 1520 s (amide II); 1573m, 1401s (COO$^-$); 1174 vs (C(CH$_3$)$_3$).
4.1.3 Synthesis of Protected Amino-Pentanoic Acid (3)

Compound 2 (13.152 g, 25 mmol) was well mixed in 50 mL of acetone for 15 minutes before 50 mL of water was added and stirring continued for another 10 minutes. Then 300 mL of water and 8-quinolinol (9.45g, 65 mmol) were added, and stirring was continued for a further 4 hours. The copper (II) quinolinolate precipitate was removed by filtering and rinsed with water (2x25 mL). After combining the filtrate and washings, acetone evaporated. The remaining aqueous solution was extracted with ethyl acetate (3x100 mL) and discarded. To produce 5-(tert-Butoxycarbonylamino)-2-aminopentanoic acid 3, the solvents from reaction mixtures were evaporated in vacuo on a rotating evaporator at bath temperatures below 45 °C.

Yield: 10.43 g (45 mmol) of white solid, 90%. $R_f$ (A) = 0.68, m.p. 220-222 °C.
4.1.4 Synthesis of Fmoc-OSu Protected Amino-Pentanoic Acid 

![Synthesis Diagram]

Compound 3 (1.0 equiv, 5.9 g; 25.4 mmol) was put in a round-bottom flask with a magnetic spin bar and dissolved in a solution of NaHCO₃ (4.3 g; 50.8 mmol) in 100 mL of water. The flask was submerged in an ice bath to chill it and then 9-fluorenylsuccinimidyl carbonate (Fmoc-OSu) (1.0 equiv, 8.6 g; 25.4 mmol) in 100 mL of dioxane was added dropwise under vigorous stirring during 30 minutes. After all of the Fmoc-OSu had been added, the reaction mixture was left to react for one hour at 0 °C and then for a further 24 hours at room temperature. After that, 200 mL of water was added, then concentrated citric acid was added drop by drop until the pH level was between 2-3, then with 150 mL of ethyl acetate, the reaction mixture was extracted four times. The mixed organic layers were washed twice with 150 mL of brine and twice with 150 mL of water and dried over MgSO₄. The filtrate was evaporated to get the yellow oil, which was then triturated at –20 °C in an ethyl acetate-petroleum ether solution to produce the pure product.

Yield: 8.6 g (18.9 mmol) of light blue solid, 75 %. \( R_f \) (B) = 0.81, m.p. 104-106 °C. \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): 1.37 (9H, s, (CH₃)₃), 1.42 (2H, m, CH₂–), 1.69 and 1.71 (2H, 2× m, CH₂–), 2.91 (2H, m, CH₂–N), 3.90 (1H, ddd, \( J = 9.1, 8.2 \) and 4.8, >CH–N), 4.22 (1H, dd, \( J = 7.1 \) and 6.8, >CH–), 4.26 (1H, dd, \( J = 10.0 \) and 6.3, CO–O–CH₃Hb–), 4.28 (1H, dd, \( J = 10.0 \) and 7.1, CO–O–CH₃Hb–), 6.80 (1H, t, \( J = 5.6, \) –
NH–CO–O), 7.63 (1H, br d, J = 8.2, –NH–CO–O), 7.32 (2H, m, Ar–H), 7.41 (2H, m, Ar–H), 7.71 (2H, m, Ar–H), 7.88 (2H, m, Ar–H), 12.70 (1H, br s, COOH).

4.1.5 Synthesis of Azido Amino-Pentanoic Acid (5)

Compound 4 (10.4 g; 22.9 mmol) was processed to produce amine using 20 mL of DCM, 20 mL of TFA, and 2 mL of water. The volatile components evaporated after 2 hours of shaking, producing yellow oil. Following the addition of 100 mL of toluene, 14.9 g of sodium azide (229 mmol) was dissolved in 100 mL of water. Under vigorous stirring, the mixture was cooled to 0 °C. The mixture was vigorously stirred as it cooled to 0 °C. Triflic anhydride (12.9 g, 45.8 mmol) was added dropwise and vigorously agitated for a further 30 minutes at 0 °C, the temperature was increased to 10 °C and mixture was swirled for two hours. Drop by drop, a saturated aqueous solution of NaHCO₃ was added until gas evolution stopped. The aqueous layer was extracted using toluene (2 x 40 mL) after the two phases were separated. In the next diazo transfer process, the mixed organic layers were used. Copper (II) sulfate pentahydrate (57 mg, 0.229 mmol), sodium hydrogen carbonate (19.2 g, 229 mmol), and amine were all dissolved in 100 mL of water. To create a homogenous system, the trifluoroazide stock solution was added, and then 150 mL of methanol was added. The
blue mixture was then vigorously agitated at room temperature. The reaction mixture's color changed from blue to green, which was tracked by TLC, to indicate that all the amines had been consumed. A rotating evaporator was used to extract solvents in vacuo while carefully keeping the temperature below 25 °C. Reaction mixture was once again chilled in an ice bath for the work-up, and concentrated HCl was added dropwise until pH 0-1 was achieved. Following the addition of 150 mL of water, the reaction mixture was extracted three times with 100 mL of ethyl acetate. After being rinsed with brine (2 x 100mL) and once with 100 mL of water, the mixed organic layers were dried over MgSO₄. After the filtrate evaporated, a high vacuum pump was used to dry the remaining colorless oil.

Yield: 8.2 g of white solid, 97 %. \( R_f(B) = 0.81, \) m.p. 95 to 98 °C. \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): 1.60 (2H, m, –CH\(_2\)–), 1.67 and 1.76 (2H, 2x m, –CH\(_2\)–),3.33 (2H, m, –CH\(_2\)–N\(_3\)), 3.98 (1H, ddd, \( J = 9.2, 8.2 \) and 5.0, >CH–N), 4.24 (1H, br t, \( J = 7.0, >\text{CH}–\) ), 4.30 (2H, m, CO–O–CH\(_2\)–),7.32 (2H, m, Ar–H), 7.41 (2H, m, Ar–H), 7.71 (1H, br d, \( J = 8.2, –\text{NH}–\text{CO}–\)O), 7.73 (2H, m, Ar–H), 7.88 (2H, m, Ar–H), 12.64 (1H, br s, COOH).

### 4.2 Solid Phase Synthesis and Characterization of Individual Peptide

#### 4.2.1 Materials and Equipment

All amino acids and other chemicals for peptide synthesis were purchased from Chem-Impex. In this study, analytical-grade reagents and solvents were used. Individual peptides were analyzed via liquid chromatography-mass spectrometry (LC-MS) using an Agilent 6230 LC/TOF. In Mass spectrometry (MS)
samples are charged by electrospray ionization (ESI) methods. Finally, the time-of-flight (TOF) method was used to analyze ionic species depending on their mass-to-charge ratio \((m/z)\) in the analyzer. Peptide solutions (0.5 mg in 1 ml of methanol) were auto injected (2 μL) and separated with an Agilent EXTEND C18 column (2.1 x 50 mm, 1.8 μm). Eluted components were monitored by UV absorbance at 250 nm.

### 4.2.2 Synthesis of Peptide

Standard microwave-assisted solid phase peptide synthesis (MW-SPPS) was used to create specific peptide sequences using a Biotage® Initiator+ AlstraTM peptide synthesizer. On a 0.2 mmol scale, each peptide was produced using amino acid solutions (0.1 M in DMF, 4 equiv.) Each amino acid was linked in turn, beginning with preloaded C-terminal tyrosine bound Wang resin (0.56 mmol/g), using the conventional Fmoc-piperidine deprotection chemistry, diisopropylcarbodiimide (DIC), and Oxyma amide coupling conditions. Dimethylformamide (DMF) was used to swell the resin for 20 min at 70 °C and 130 W in an oscillating mixer. The piperidine (20 vol% in DMF, 90 equiv.) was used to deprotect amines from the Fmoc, after that Using DIC (2 M in DMF, 4 equiv.) and Oxyma (4.5 M in NMP, 4 equiv.) for the coupling of all amino acids. All peptides' N-termini underwent the identical Fmoc-deprotection process. After peptide synthesis, dichloromethane was used to wash all resins (DCM, 3 x 10 mL). The resins were kept at −20 °C after being vacuum-dried.
4.2.3 LC-MS Characterization of Individual Peptide

Liquid chromatography-mass spectrometry (LC-MS) techniques are used to identify individual peptides. For LC-MS analysis each peptide was cleaved with trifluoroacetic acid (TFA), triisopropylsilane (TIS), and water (95:2.5:2.5 vol%) during two hours of agitation. Filtration was used to separate the resin from the peptide solution after that resin was washed and filtered with DCM (3 x 5 mL). All washings were combined with peptide solution then, the cleavage cocktail and DCM were lowered to around 25% of their original volume via air dry. The peptide product was precipitated with the addition of diethyl ether. A Beckman Coulter 25R Allegro Centrifuge was used to spin the peptides at 8000 rpm for 30 minutes at 20 °C. The peptides were washed with new diethyl ether after the supernatant was decanted. Four rounds of centrifugation, decantation, and washing were performed on the peptides. The peptides were kept at −20 °C after being vacuum-dried for about two hours. In order to identify the peaks of these peptides, the samples were run on LC-MS with a 50 µL concentration (0.5 mg in 1 ml of methanol). For solvent system Millipore H₂O/Formic acid (99.9:0.1 vol%) and acetonitrile/ Formic acid (99.9:0.1 vol%) were used as mobile phase A and B, respectively. The mobile phase A; 5 to 95% of B gradient over 12 min. Figures D.1 to D.4 show LC-MS spectra of distinct peptides.

4.2.4 Characterization of Orthogonal Peptide Pairs via Experiment

Regarding the creation of the secondary structure, binding selectivity, and stability of synthetic peptides, the features of proposed coiled-coil orthogonal pairs
were investigated. To examine the stability of the coiled-coils the orthogonal peptides generated, we employed size exclusion chromatography. Bio-Rad NGC™ Chromatography FPLC equipment was used to perform size-exclusion chromatography (SEC). Peptide solutions (5 mg/mL in 0.1% ammonium bicarbonate) were filtered via a 0.2 m filter using an HR10/30 Superdex 70 column, manually injected with 300 L, and eluted with PBS elution buffer (pH 7.4). The UV absorbance at 280 nm was used to track the peptides' elution for investigate the oligomerization state of equimolar mixtures of designed orthogonal pairs. The findings showed that whereas orthogonal pairings of peptides can form heterodimers, individual peptides exist as monomers. Figure C.2 to C.9 show the chromatogram for all combination forms DA3 through DA6.

4.4 Synthesis of Hydrazide

4.4.1 Materials and Equipment:

All solvents used in synthesis were purchased from Fischer Scientific and used as received. 4-bromobenzhydrazide (Oakwood Chemical) and Copper (I) iodide (Alfa Aesar, 98%) were used without further purification. Boc-anhydride, triethylamine, TMS-acetylene, and tetrakis(triphenylphosphine) palladium (0) (Pd(PPh3)₄ received form Chem-Impex. TLC analyses were performed on silica-gel-coated glass plates (Silicycle) using the following solvent systems (v/v): (1:1 Hexanes : Ethyl Acetate) spot was visualized by exposure to UV light at 254 nm and by
ninhydrin spraying. NMR solvents (DMSO-d$_6$) were purchased from Sigma-Aldrich and spectra for small molecule precursors were collected on Varian MR-400 MHz NMR spectrometer.

4.4.2 Boc-protection of 4-Bromobenzhydrazide (7)

\[ \text{In a 250 mL round bottom flask, the 4-bromobenzhydrazide (1.0 equiv, 8.41 g, 39.1 mmol) and 75 mL of DCM were added. The reaction flask was then chilled to 0 °C in an ice bath. 100 mL of DCM was used to dissolve the Boc-anhydride (Boc$_2$O; 5.0 eq. 43.1 g, 197 mmol) before being added dropwise over the course of two hours using an addition funnel. The ice bath was then eliminated, and the solution was left to stir at room temperature for 24 hours while TLC was used to track the reaction's development. The reaction mixture was finished by being rinsed with D.I. H$_2$O (3 x 50 mL) and brine (1 x 100 mL). The reaction solution was dried over anhydrous magnesium sulfate, then decanted into an empty flask further, concentrated under reduced pressure, and finally dried under high vacuum.}

Yield: 10.2 g (32.5 mmol) of white crystals, 83 %. \( R_f \) (1:1 Hexanes:Ethyl Acetate) = 0.55.
$^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ (ppm) 10.28 (N-H, s, 1H), 8.95 (N-H, s, 1H), 7.80 (Ar-H, d, $J = 8.28$ Hz), 7.72 (Ar-H, d, $J = 8.52$ Hz), 1.42 (Boc-H, s, 9H).

4.4.3 Sonogashira Reaction with TMS Acetylene (8)

![Sonogashira Reaction with TMS Acetylene](image)

$N$-Boc-4-bromobenzhydrazide (3.70 g, 11.7 mmol, 1.0 equiv.) triethylamine (5.00 mL, 36.1 mmol, 3.0 equiv.), TMS-acetylene (1.95 mL, 14.1 mmol, 1.2 equiv.), and around 30 mL of dry THF were introduced to a three-neck R.B. flask that had been N$_2$ purge. After that, the mixture received additions of copper iodide (CuI; 0.227 g, 1.19 mmol, 0.10 equiv.) and tetrakis(triphenylphosphine) palladium (0) (Pd (PPh$_3$)$_4$; 0.679 g, 0.587 mmol, 0.05 equiv.), and the reaction was let to continue stirring for 24 hours. After that reaction mixture was filtered through Celite$^{TM}$ and repeatedly rinsed with 150 mL of THF. The crude mixture was redissolved in DCM after the solvent was withdrawn under reduced pressure. The solution was then washed with aqueous sat. NH$_4$Cl (3 x 50 mL), 1 M HCl (3 x 50 mL), sat. NaHCO$_3$ (3 x 50 mL), and brine solution (1 x 100 mL). The reaction solution was then dried over anhydrous sodium sulfate, decanted into a clean flask, and then concentrated under reduced pressure. By using SiO$_2$ column chromatography, the product was further
purified before being collected and vacuum-dried and further recrystallized with hexane and get off-white solid.

Yield: 2.95 g (8.8 mmol) of off-white solid, 76%. $R_f$ (1:1 Hexanes:Ethyl Acetate) = 0.65. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ (ppm) 10.28 (N-H, s, 1H), 8.94 (N-H, s, 1H), 7.85 (Ar-H, d, $J = 8.00$ Hz, 2H), 7.57 (Ar-H, d, $J = 8.28$ Hz, 2H), 1.42 (Boc-H, s, 9H), 0.24 (TMS-H, s, 9H).

4.4.4 Deprotection of TMS-protected Alkyne (9)

The purified TIPS-protected alkyne from the preceding step (1.0 equiv, 0.918 g, 2.76 mmol) was introduced to the R.B. flask along with 30 mL MeOH. The potassium carbonate (K2CO3; 3.5 equiv, 1.31 g, 9.46 mmol) was mixed with the 10 mL MeOH and 5 mL water to dissolve it, and then it was added to the swirling mixture. The reaction was allowed to stir at room temperature for 18 hours. After removing the MeOH under reduced pressure, DCM (3 x 50 mL) was used to extract the product, and the product was subsequently washed with D.I H2O (3 x 100 mL). The DCM solution was then dried without additional purification over anhydrous magnesium sulfate, decanted into a clean flask, and dried under high vacuum.
Yield: 0.510g (1.95 mmol) of off-white solid, 70.8 %. \( R_f \) (1:1 Hexanes:Ethyl Acetate) = 0.55. \( ^1 \text{H} \) NMR (400MHz, DMSO-\( d_6 \)): \( \delta \) (ppm) 10.28 (N-H, s, 1H), 8.95 (N-H, s, 1H), 7.84 (Ar-H, d, \( J = 8.00 \) Hz, 2H), 7.57 (Ar-H, d, \( J = 8.32 \) Hz, 2H), 4.41 (C≡C-H, s, 1H), 1.42 (Boc-H, s, 9H).

4.5 CuAAC Reaction for Peptide Pair Conjugation

4.5.1 Materials and Equipment

The 4-ethynylbenzaldehyde from Oakwood chemicals and used without further purification. The \( N \)-Boc-4-ethynylbenzhydrazide was synthesized according to literature protocol.\(^{128} \) The copper (I) iodide (Alfa Aesar, 98%), sodium L-ascorbate (CHEM-IMPER) and TBTA (Tokyo chemical industry) were used as received. The Peptides Conjugation were analyzed via liquid chromatography-mass spectrometry (LC-MS) using as some series and parameter was used for individual peptides analysis.

4.5.2 CuAAC Reaction of Resin Bounded DA5 with \( N \)-Boc-4-Ethynylbenzhydrazide

The peptide DA5 on resin (typically ~100 mg; 1.0 equiv based on resin loading) and \( N \)-Boc-4- ethynylbenzhydrazide (2.0 eq. per azide) were added to a 20 mL vial along with 0.5 mL of DMF. \( \text{N}_2 \) gas was used to purge the vial after it had been septum sealed. The copper iodide (0.25 equiv), sodium ascorbate (0.5 equiv), and TBTA (0.5 equiv) were dissolved in 1.2 mL of DMF and 0.3 mL of water in a separate, \( \text{N}_2 \) purged vial. The purged resin vial was then filled with the catalyst solution, and the
vial was fitted with a N₂ balloon. The reaction was shaken at room temperature for 18 hours. After vacuum filtration, the resin was recovered and washed with 25 mL of DMF, 13 mL of DCM, and 13 mL of methanol. Acyl hydrazide peptides (DA5-Hy) were cleaved with agitation for 3 hours using 95% TFA, 2.5% triisopropylsilane (TIS), and 2.5% H₂O cleavage cocktails. Copper iodide did not dissolve, and the catalyst mixture had a cloudy, brownish-orange appearance. Once the catalyst solution was injected into the resin, the reaction mixture became very turbid. After CuAAC, it was seen that the resin's color changed from orange to dull green, however following cleavage, the dull green pigment was removed, leaving behind opaque resin beads.

4.5.3 CuAAC Reaction of DA3 with 4-Ethynylbenzaldehyde

For this reaction, N-Boc-4-ethynylbenzhydrazide (2.0 equiv per azide) and the peptide DA3 were used without resin, and a 20 mL vial also included 0.5 mL of DMF. The vial was septum sealed and then purged with N₂ gas. In a separate, N₂ purged vial, the copper iodide (0.25 equiv), sodium ascorbate (0.5 equiv), and TBTA (0.5 equiv) were dissolved in 1.2 mL of DMF and 0.3 mL of water. The catalyst solution was then added to the vial of purged resin, which was then sealed with a N₂ balloon. The reaction was shaken at room temperature for 18 hours.

4.5.4 LC-MS Characterization of Peptide Conjugation

The samples were analyzed on LC-MS with a 50 µL concentration to determine these covalently linked peptides' peaks (0.5 mg in 1 ml of methanol). Mobile phase A and B for the solvent system Millipore H₂O/Formic acid (99.9:0.1 vol%) and
acetonitrile/Formic acid (99.9:0.1 vol%) were employed, respectively. The mobile phase A; a 12-minute gradient from 5 to 95% of B. The LC-MS spectra of DA5-Hy shows the most intense peak and there is no sign of the starting material azide peptide, and the results are shown in Figure D.5. Furthermore, The LC-MS spectra of DA3-Al shows no trace of the starting material, azide peptide, and the product has the strongest peak and displays the peak of TBTA and MS peaks (Figure D.6) show mixture of single and double click reaction.
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APPENDIX (A)

METHOD FOR CALCULATING EFFECTIVE LOADING OF WANG RESIN BONDED PEPTIDE.

We created a solid-phase (SP) CuAAC click reaction to add hydrazide functionalities to azide-peptide side chains. The dynamic covalent bonding pairs were joined using this simple technique to form the peptide oligomers DA4 with N-Boc-4-ethynylbenzhydrazide. Admittedly, it was uncertain how much DA4 existed on the resin. The initial resin loading of Wang resin that was tyrosine-bound determined the quantity of DA4 on the resin. The theoretical concentration of DA5 on resin was equivalent to the reaction scale of 0.2 mmol, assuming a 100% coupling efficiency of all amino acids during synthesis. Once the resin had sufficiently dried, the mass of the DA5-containing resin was measured. By dividing the theoretical moles of DA5 by the mass of the dried resin with DA5, it was possible to calculate the new effective loading of the resin with DA5. See a calculation example below.

1. Mass of resin with DA3 (dried): 1.1235 g
2. Initial loading of Tyr-Wang resin: 0.56 mmol/g
3. Initial weight of Tyr-Wang resin: 0.357 g

\[
\frac{0.56 \text{ mmol Tyr}}{1 \text{ g Tyr - wang resin}} \times 0.357 \text{ g Tyr - wang resin} = 0.20 \text{ mmol Tyr}
\]

\[
= 0.20 \text{ mmol DA5}
\]

\[
\frac{0.20 \text{ mmol DA5}}{1.1235 \text{ g DA5 - wang resin}} = 0.1780 \frac{\text{mmol}}{g} \text{ (effective loading)}
\]
APPENDIX B

$^1$H NMR SPECTRA
Figure B.1. $^1$H NMR spectrum of Fmoc-OSu protected amino-pentanoic acid (4) (400 MHz, DMSO-d$_6$)
Figure B.2. $^1$H NMR spectrum of azido amino-pentanoic acid (5) (400 MHz, DMSO-$d_6$)
Figure B.3. $^1$H NMR spectrum of Boc-protected 4-Bromobenzhydrazide (7) (400 MHz, DMSO-$d_6$)
Figure B.4: $^1$H NMR spectrum of TMS acetylene (8) (400 MHz, DMSO-d$_6$)
Figure B.5. $^1$H NMR spectrum of Deprotection of TMS-protected Alkyne (9) (400 MHz, DMSO-$d_6$)
Figure C.1. SEC analysis of a mixture of thyroglobulin (Mr 670,000), bovine -globulin (Mr 158,000), chicken ovalbumin (Mr 44,000), horse myoglobin (Mr 17,000), and vitamin B12 (Mr 1,350) from Bio-Rad gel filtration, with a pI range of 4.5 to 6.9.
**Figure C.2.** Size Exclusion Chromatographic Analysis of Peptide DA3.

**Figure C.3.** Size Exclusion Chromatographic Analysis of Peptide DA4.
Figure C.4. Size Exclusion Chromatographic Analysis of Peptide DA5.

Figure C.5. Size Exclusion Chromatographic Analysis of Peptide DA6.
Figure C.6. Size Exclusion Chromatographic Analysis of Peptide DA3|DA4.

Figure C.7. Size Exclusion Chromatographic Analysis of Peptide DA5|DA6.
Figure C.8. Comparison of the peptides DA3 (black), DA4 (blue), and the related peptide DA3|DA4 product (Orange). The change in elution volume demonstrates how the different peptides joined together to produce the DA3|DA4 peptide pair.

Figure C.9. Comparison of the peptides DA5 (black), DA6 (blue), and the related peptide DA5|DA6 product (Orange). The change in elution volume demonstrates how the different peptides joined together to produce the DA5|DA6 peptide pair.
APPENDIX D:

LCMS CHROMATOGRAPHIC DATA
Figure D.1. LCMS of DA3 run in (Water: Acetonitrile) with 0.5% formic acid as mobile phase in gradient from 5 to 95% acetonitrile over 12 min.
Figure D.2. LCMS of DA4 run in (Water: Acetonitrile) with 0.5% formic acid as mobile phase in gradient from 5 to 95% acetonitrile over 12 min.
Figure D.3. LCMS of DA5 run in (Water: Acetonitrile) with 0.5% formic acid as mobile phase in gradient from 5 to 95% acetonitrile over 12 min.
Figure D.4. LCMS of DA6 run in (Water: Acetonitrile) with 0.5% formic acid as mobile phase in gradient from 5 to 95% acetonitrile over 12 min.
Figure D.5. LCMS of CuAAC reaction of resin bounded DA5 with N-Boc-4-ethynylbenzhydrazide (Hy) run in (Water: Acetonitrile) with 0.5% formic acid as mobile phase in gradient from 5 to 95% acetonitrile over 12 min.
Figure D.5. LCMS of CuAAC reaction of DA3 with 4-ethynylbenzaldehyde (Al) run in (Water: Acetonitrile) with 0.5% formic acid as mobile phase in gradient from 5 to 95% acetonitrile over 12 min.