Design & Synthesis of Peptidomimetics Adopting Secondary Structures for Inhibition of p53/MDM2 Protein-protein Interaction and Multiple Myeloma Cell Adhesion

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Design & Synthesis of Peptidomimetics Adopting Secondary Structures for Inhibition of
P53/MDM2 Protein-protein Interaction and Multiple Myeloma Cell Adhesion

by

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LIST OF ABBREVIATIONS

$\beta$ = Amyloid beta

Boc = tert-Butyloxycarbonyl

Cbz = Carbobenzyloxy

CD = Circular dichoism

DBU = 1,8-Diazabicyclo[5.4.0]unec-7-ene

DCC = $N, N´$-Dicyclohexylcarbodiimide

DCM = Dichloromethane

DIC = $N, N´$-Diisopropylcarbodiimide

DIEA = $N, N´$-Diisopropylethylamine

DNA = Deoxyribonucleic acid

Fmoc = 9-Fluorenylmethoxycarbonyl

gB = Glycoprotein B

HCTU = $N, N, N´, N´$-Tetramethyl-$O$-(6-chloro-$1H$-benzotriazol-1-yl)uronium hexafluorophosphate

HDM2 = Human homologue of murine double minute 2

HF = Hydrofluoric acid

HOBt = 1-Hydroxybenzotriazole

HOSu = 1-Hydroxypyrrolidine 2,5-dione
HPLC = High-performance liquid chromatography
MDM2 = Murine double minute 2
MM = Multiple myeloma
NMR = Nuclear magnetic resonance
Nsch = N-(S)-(1-cyclohexylethyl)glycine
Nsna = N-(S)-(1-Naphthalenethyl)glycine
Nspe = N-(S)-(1-phenylethyl)glycine
Nrch = N-(R)-(1-cyclohexylethyl)glycine
PPIs = Protein-protein interactions
RNA = Ribonucleic acid
ROS = Reactive oxygen species
ROSEY = Rotating-frame overhauser effect spectroscopy
S_N2 = Nucleophilic substitution bimolecular
SPPS = Solid-phase peptide synthesis
TEA = Triethylamine (Et_3N)
TLC = Thin layer chromatography
TFA = Trifluoroacetic acid
THF = Tetrahydrofuran
T_3P = 2, 4, 6-Tripropyl-1, 3, 5, 2, 4, 6-trioxatriphosphorinane-2, 4, 6-trioxide
ABSTRACT

The protein-protein interactions (PPIs) occur when two or more proteins are bound together. Also, this protein-protein interactions (PPIs) cause the various biological processes in the body. Due to this reason, abilities of controlling or inhibiting PPIs can give us promising advantages like (1) better understanding of biological systems, (2) development of new diagnostic approaches for health or disease, and (3) establishment of novel molecular therapeutics. Many proteins adopt the secondary structures, where most of protein-protein interactions take place. α-Helices and β-sheets are the prevalent secondary conformations, but there are extended secondary structures such as β-hairpins, β-turns, 3^{10} helix, and so on. As a result, construction of molecules mimicking these protein secondary structures is tractable target for drug design.

Moreover, in drug discovery, designing peptidomimetics or non-peptidic mimetics is a popular strategy instead using peptides or truncated peptides because peptides or truncated peptides are prone to proteolysis and degraded in the body. Also, peptidomimetics and non-peptidic mimetics have not only the similar topology as peptides but also resistance to proteolysis. Due to these advantages, in this study, peptidomimetics or non-peptidic mimetics were synthesized and tested for different targets: (1) synthesis of non-peptidic α-helical mimetics for p53-MDM2 inhibition, (2) solution-phase synthesis of β-hairpin
peptide for the inhibition of multiple myeloma cells (MM) adhesion, and (3) synthesis of β-hairpin peptoid-peptide hybrids.

The synthesis in all three different studies was succeeded, but they still need some improvements. For instance, non-peptidic α-helical mimetics, terpyrimidyl derivatives, were synthesized successfully, but they did not show any bioactivity against p53-MDM2. Also, they have a solubility problem. Based on these results, it is necessary to improve the pharmacokinetic properties and bioactivity by changing the substituents on the rings or structures.

The β-hairpin peptide for the second case already showed good bioactivity against multiple myeloma (MM). For the next level of bio-study, the considerable amount of a β-hairpin peptide was demanded. In order to make the substantial β-hairpin peptide, the solution phase peptide synthesis was chosen instead of the solid phase peptide synthesis because of the cost-effect. Two methodology were tried for the solution-phase peptide synthesis: (1) segment ligation and (2) continuous synthesis. In the former case, the β-hairpin peptide synthesis was successful, but, in the latter case, it is necessary to investigate the appropriate coupling reagents for each step.

Peptoid-peptide hybrids has been one of the popular peptidomimetics in the last two decades. Also, mimicking the peptide secondary structure in peptoids has been studied extensively these days. The combination of these two factors was the goal for the third case. Because peptoid-peptide hybrids with a secondary structure can be recognizable by native proteins and resistant to proteolysis. So far, three sets of peptoid-peptide hybrids were synthesize and checked the secondary structure formation by using NMR. However, there was no indication of the secondary structure formation in the three sets of peptoid-
peptide hybrids. This result suggests that it is necessary to introduce the more constrained components in peptoid-peptide hybrids.

In the above three chapters, it has been tried to find the new drug candidates by synthesizing peptidomimetics or non-peptidic mimetics. Even though the synthesis was successful, some intended results such as the bioactivity or the secondary structure formation were not obtained. However, these results can give us the inspirations to improve properties of peptidomimetics or non-peptidic mimetics for a certain purpose, which leads to earn the intended results and eventually find new drug candidates.
CHAPTER ONE:
INTRODUCTION

1.1. Amino acids, peptides, and proteins

Proteins are the most abundant biological molecules in every cell. Proteins also have diversity, not only thousands of different kinds, but also huge differences in size. Moreover, their biological functions in the cells are the most important since their outcomes have an effect on the biological pathways and genetic expressions. Despite their key functions, combinations of relatively simple monomer subunits is the fundamental element of protein structure. Proteins are built from a set of 20 amino acids are covalently linked in linear sequences. Every amino acid is a $\alpha$-amino acid which has carboxylic acid, amino group, and its side chain. The presence of carboxylic acid and amino group makes amino acids act as acids and bases. Also, side chain of amino acids gives different identity to each amino acid and side chains occur in a variety of structure, size, and electric charge. Two amino acids can be covalently linked through a peptide bond to obtain dipeptide. The peptide bond is formed by removal of water from carboxylic acid of one amino acid and amino group of another amino acid. In a similar way, tripeptide, tetrapeptide, and so on are formed from amino acids. When a few amino acids are involved in this fashion, the structure is termed as an oligopeptide. When many peptides are involved, the product is termed as a polypeptides which has
molecular weight below 10,000. Proteins contain thousands of amino acid residues and have larger molecular weights (1).

1.2. Protein structures

The primary structure of a protein is the linkage of individual amino acids which are joined covalently through peptide bonds. The peptide bonds in protein is planar as a result of resonance structure (Figure 1). This prevents the peptide bond rotation and gives significant double bond character. Even though there are two possible configurations for the peptide bond, trans configuration is frequently found out in proteins due to benefit from steric hindrance.

The secondary structure of protein consists of the ordered regions adopted by polypeptide chains. The secondary structure is normally about folding patterns of polypeptide backbone. Moreover, most of proteins contain secondary structure regions. There are three common types of secondary structures: the α-helix, the β-pleated sheet, and the β-turn.

The tertiary structure of proteins is the three-dimensional layout of all atoms. As the length of a polypeptide gets longer, it will fold to be more ordered and stable. Because amino acids which are apart can interact within folded protein structure through weak bonding interactions or covalent bonds.

Some proteins such as hemoglobin consist of two or more subunits which may be same or different types. Only proteins which contain more than two subunits are able to have quaternary structures. The quaternary structure of proteins refers to the arrangement of these subunits in three-dimensional complexes (1,2).
1.3. α-helix mimetics in drug discovery

The α-helix was first characterized by Linus Pauling in 1951 (3). A typical α-helix has 3.6 amino acids per turn, a rise 5.4 Å/turn, and backbone dihedral angles of $\Psi = -50^\circ$ and $\Phi = -60^\circ$. The helix can be considered to have three distinct faces because the side chains from certain residues are overlapped vertically every 3-4 residues. In other words, side chains from helical residues at the position $i$, $i+3$, $i+4$, and $i+7$ are positioned along the same face of α-helix. In α-helix formation, there is a large entropy cost, but this can be cancelled out due to intramolecular hydrogen bonds between the carbonyl oxygen at the $i$ position and the carboxamide hydrogen at the $i+4$ position (4). The principal character of α-helix is the conformational hindrance of the side chains and steric restriction. In numerous proteins, α-helices are the bioactive regions which mediate protein-protein interactions along with protein-DNA and protein-RNA interactions (5).

The protein-protein interactions (PPIs) plays a key role in aspects of biological processes. Due to this reason, abilities of controlling or inhibiting PPIs can give us promising advantages like (1) better understanding of biological systems, (2) development of new diagnostic approaches for health or disease, and (3) establishment of novel molecular therapeutics. Over 30% protein secondary conformation adopts α-helices, allowing it the most abundant secondary structure in nature. As a result, a number of PPIs are involved in α-helices and mimicking α-helical templates is tractable target for drug design (6, 7).
Azzarito and co-workers classified three primary design strategies on the review paper based on helix-mediated interactions and structural features (8): (1) type I mimetics which are short oligomers and copy the local contour of \( \alpha \)-helical structural motif (9), (2) type II mimetics which are small non-peptidic molecules but not always imitation of the \( \alpha \)-helix structure of protein receptors, and (3) type III mimetics which are non-peptide scaffolds and only interact with key residues of peptide receptors not the whole \( \alpha \)-helical conformation (10, 11).

Type I mimetics are the derivatives of peptide backbone to replicate the local region of \( \alpha \)-helical conformation when protein-protein interactions occur. Peptides are not therapeutically appropriate forms because of their poor transport properties and easy proteolytic degradation (12). However, due to their complex structures, small molecules are not able to mimic completely their functions during the biological process (13). Moreover, there is a study to show that synthetic peptides are not organized in solution and adopt random conformation which delay the binding to the partner proteins (14). Despite of these limitations, there have been several approaches for oligopeptides to maximize their \( \alpha \)-helical properties and disrupt protein-protein interactions.

The first strategy is stabilization \( \alpha \)-helix conformation of short peptides by using salt bridges, metal chelates, and covalent cyclization (Figure 2). Aldert and Hamilton studied the restriction of helical structure of short peptides through hydrophobic interactions. This can be accomplished by incorporating two \( \varepsilon \)-(3,5-dinitrobenzoyl)Lys residues at various positions. They found that the helical stability is the highest for the peptide when a pair of modified residues are positioned at the \( i \) and \( i + 4 \) (15). Scholtz and co-workers showed Glu-Lys interactions at the position \( (i, i + 3) \) and \( (i, i + 4) \) stabilize \( \alpha \)-helical
structures regardless of orientation of the side chains (16). Tsou and co-workers found -0.4 kcal/mole energy decrease for $\alpha$-helical peptide through Phe-Lys cation-$\pi$ interaction (17).

The Mierke and Spatola groups have demonstrated the inhibition of disulfide-bridged nonapeptide to estrogen receptor $\alpha$/co-activator interaction. Even though circular dichroism spectra of the peptide indicated the minimal propensity of $\alpha$-helix in aqueous condition, X-ray crystal structure confirmed disulfide-bridged peptide experienced the receptor-induced conformational change and bound to receptor with $\alpha$-helix conformation (18, 19, 20). Rosenblatt and co-workers reported a parathyroid-hormone-related protein (PTHrP) analogue (21) and Fairlie and co-workers demonstrated short $\alpha$-helical peptides (22). Both of approaches utilized lactam link between lysine and aspartic acid in the $i$ and $i+4$ positions to stabilize $\alpha$-helical structure.

Despite of the stabilization of $\alpha$-helices through disulfide or lactam links, these natively occurring components may be prone to cellular degradation. To overcome this disadvantage, Grubbs group showed carbon-carbon bond restraint initially via a ring-closing metathesis reaction from unnatural O-ally serine residues (hydrocarbon stapling) (23). Arora and co-workers also exploited ring-closing metathesis to olefin bearing residues at the $i$ and $i+4$ positions (hydrogen-bonding surrogates) (24). However, the second approach is more attractive because the constraining element after ring-closing metathesis does not block any faces of $\alpha$-helix conformation unlike the result of first approach (Figure 3).

Even though all the above strategies have developed the promising peptide-based inhibitors with $\alpha$-helix conformation, it is impossible to control the activity of these
restrained peptides. Alleman group established that external stimuli can govern the protein-binding activity in a reversible way. This can be achieved by cis/trans isomerization of azobenzene under radiation exposure. An azobenzene bridge was places through cystein residues at the $i, i+4, i+7$ or $i+11$ positions and this bridge makes the peptides switch between random coil-like and $\alpha$-helical conformations under photon emission (25).

Another approach for generation of type I mimetics is the use of foldamers. Gellman and co-workers design $\beta$-peptides by adding methylene unit to $\alpha$-amino acids to cure human cytomegalovirus (HCMV, alternatively know as herpesvirus-5) infection. They identified a 12-helical inhibitor derived from the HCMV protein gB with an IC$_{50}$ of 30 $\mu$M (26). The $\beta$-peptides have not only better resistance to proteolysis but also more favorable pharmacodynamic properties. This is because an additional degree of freedom form extra methylene group makes $\beta$-peptides more flexible and suitable for folding into more defined secondary structures than $\alpha$-peptides (27). There are two well-known secondary structures in $\beta$-peptides, the 14-haelix and the 12-helix (named after the number of backbone atoms per hydrogen-bond ring) (28). The group of Schepartz showed $\beta^3$-homodecamer 14-helical peptide originated from p53 with slightly lower affinity than that of the original peptide to hDM2 (2.5-fold lower) (29). Other than $\beta$-peptide design, mixed $\alpha/\beta$-peptides were used to mimic the $\alpha$-helical conformation. Gellman and co-workers designed $\alpha/\beta$-foldamers for inhibition of anti-apoptotic and pro-apoptotic members of the Bcl-2 family and gp41 (30, 31, 32, 33, 34, 35, 36). One of examples is the development of gp41 inhibitors (35). The form of repetition was prepared through the insertion of $\beta^3$-residues into the $\alpha$-sequence of the C-terminal
heptads repeat domain (CHR) of gp41. Later, recurrent $\beta^3$-residues were replaced by constrained $\beta$-residues to compensate the entropic penalty originated from the pre-organization of more flexible analogues. This approach produced the structural mimetics of 10 turns of an $\alpha$-helical CHR domain and the most potent peptide with a $K_i$ of 9 nM improved the ability of inhibition more (~380-fold) than that of the acyclic $\beta$-peptide analogue (35). Afterwards, the Gellman group transformed cyclic constraints to the ion pairings on $i$, $i + 3$, and/or $i$, $i + 4$ positions to stabilize helical structures (36).

Type II mimetics are the small non-peptidic molecules which bind to the protein receptors. However, molecules of this class are more like functional mimetics, not necessarily mimicry of the original helix structure. Even though it is difficult to identify inhibitors in this class due to different occupation or layout from traditional drug molecules, there have been several discoveries of compelling inhibitors of PPIs (37-45) (Figure 4).

One of examples is the finding of Nutlins, a family of tetra-substituted imidazoles after a high-throughput screen. These compounds were optimized to inhibit p53-murine double minute 2 (p53-MDM2) interactions and the most effective compound, Nutlin-3 showed IC$_{50}$ value of 90 nM (37). Shaomeng Wang and co-workers demonstrated potent inhibitors based on a spirooxindole template after virtual screening. They found compounds from this family selectively bound to MDM2 protein (10,000-fold) over mDMX (murind double minute X) as well as MI-219 is the most potent inhibitor ($K_i = 5$ nM) among this pool of compounds (39).
Figure 2. Examples of helical stabilization: π-π interaction (1), salt-bridge (2), cation-π interaction (3), disulfide (4), lactam (5), azobenzene (6), olefin (7).

Abbott laboratories discovered the inhibitors of the Bcl-xL/Bak via fragment-based SAR (structure-activity relationship), a modern drug discovery tool. Despite of the highest affinity of ABT-737 ($K_i = 0.6 \text{ nM}$) for Bcl-xL among all the derivatives (42), ABT-737 was not suitable for clinical use due to poor pharmacokinetic properties. Improvement of oral availability resulted in the discovery of ABT-283 with the comparable affinity ($K_i < 1 \text{ nM}$) as that of ABT-737 (43). In a recent reports, optimized analogues of this inhibitor
showed suppression of cell growth in cancer cell lines with IC$_{50}$ values of 60 - 90 nM (44, 45)

Figure 4. Examples of type II mimetics.

Type III mimetics are the non-peptidic scaffolds which mimic the spatial orientation of the critical recognition residues from the original helix instead of recapping the helical structure like type I mimetics (46). Compounds of this class have not only a simple rod shaped-like pharmacophore with side chains oriented in a similar way to that of a native $\alpha$-helix but also the comparable protein-protein interaction areas unlike that of type II mimetics.

The groups of Rees and Willems pioneered this concept by building trisubstituted indanes as inhibitors of tachykinin receptors (47, 48). However, the first true $\alpha$-helix mimetic was built by the Hamilton group (49). They showed the synthesis and conformational analysis of trisubstituted 3,2',2''-terphenyl derivatives via alternating alkyl or aryl groups on ortho-positions of the aryl cores. The side chains on ortho-position were projected in a similar way of $i$, $i+4$, and $i+7$ positions of the $\alpha$-helix. Analogues
of this family were shown inhibition to calmodulin / phosphodiesterase, Bcl-xL / Bak, and gp41 interactions with IC₅₀ values of nanomolar ranges (49 - 51).

Despite the potent inhibition results, derivatives of this series have a poor polarity. In order to overcome this disadvantage, Hamilton and co-workers reported the terephthalamide and 4,4-dicarboxamide templates (Figure 5a, b) which have easier synthesis procedure and more drug-like property (52, 53). The inhibition results of these derivatives against Bcl-xL / Bak interaction were close or lower than the terphenyl family (Kᵢ = 0.78 µM 5a₁, 1.8 µM 5b₂, and 0.11 µM terphenyl).

![Figure 5. Examples of type III mimetics.](image)

The group of Rebek demonstrated a series of oxazole-pyridazine-piperazines with hydrophobic side chains to mimic the i, i + 4, and i + 7 positions of the helix (Figure 5c). The incorporation of heterocyclic rings increased the hydrophilicity and this surface would project to the solvent exposed area. This simultaneously allowed surface of hydrophobic side chains to turn toward recognition area of protein receptor (54). In a similar concept of pre-rigidity conformation, Hamilton and co-workers reported a trispyridylamide scaffold by using aromatic oligoamides which are easy to synthesize via
amide bond formation (Figure 5d) (55). X-ray crystallography study confirmed this template showed pre-organized conformation because of hydrogen-bonding among the NH group of the amides, the ortho-alkoxy of pyridyl rings, and nitrogen on pyridyl rings. This made side chains of the scaffold project to the same face as $i$, $i + 4$, and $i + 7$ positions of the helix (56).

Although structural rigidity is helpful to stabilize $\alpha$-helix conformation, a degree of flexibility can improve interactions with the target protein via 'induced fit'. Based on Hamilton’s original pyridyl carboxamide derivatives, several groups demonstrated an oligobenzamide scaffold by replacing pyridine rings to benzene rings, which potentially decrease the number of hydrogen-bonds within this structure of mimetics (57 - 60). Fletcher and co-workers reported distribution of pyridine and benzene rings can control intramolecular hydrogen bonding and inhibition activity against Bak (Figure 5e) (61).

The Wilson group described the first solid-phase synthesis for $\alpha$-helix mimetics. They built N-alkylated oligobenzamides via this method (Figure 5f) and tested against to p53 / hDM2 interaction (62, 63). The development of this strategy can bring the diversity libraries for $\alpha$-helix mimetics with easy and effective way.

Although there have been significant progress for design and development of $\alpha$-helix mediated protein-protein inhibitors, it is still essential to understand $\alpha$-helix mediated interactions and generate therapeutically relevant molecules with higher affinity and selectivity to protein receptors.
1.4. β-sheets, β-turns, and β-hairpins in drug discovery

Although tertiary structures of folded proteins are diverse, only limited secondary structures are observed with long-range order: helices (α and 310) and sheets (parallel and anti-parallel) (64). As a result, it is critical to study the characteristic propensity of helix and sheet conformational stability in order to predict protein folding preferences. The α-helix has been examined extensively to determine variables for helix stability such as the effects of sequence, side chain - side chain interactions, solvent, length, and other factors. This was possible because of the development of model system which adopts helical conformation in solution (65). However, the factors that contributed to β-sheet structure and stability are less well-documented due to the difficulty of model system development in aqueous solution (66, 67). There was a first discovery of modest β-hairpin model system in 1993. This resulted in the development of recent well-defined β-hairpins and the increased research pace of β-secondary structures (68).

A polypeptide with lack of side chains (poly-glycine) adopts a fully extended conformation or "zig-zag" conformation that every peptide bond is located in a common plane and a repeat distance between C_i and C_{i+2} is 7.4 Å (69). However, polypeptides with non-glycyl residues relieve the steric tension by rotating slightly in both the N – C and C – C’ bonds. A repetition of this fold in a regular fashion results in β-structure, one of the most common structures in proteins. In β-structure, N – H and C = O of the backbone are aligned approximately perpendicular to the direction of the chain and involved in inter-strand hydrogen bonding. There are two layouts depending on hydrogen bonding arrangement between strands of polypeptide. When hydrogen bonding interaction between N – H and C = O occurs collinear, β-strands are arrayed in an anti-parallel
fashion. However, deviation from co-linearity of hydrogen bonding leads to parallel arrangement of strands (Figure 6). The determinant of parallel or anti-parallel arrangement is the side chain interactions between strands (70). The side chains of \( \beta \)-conformation are toward to right angles alternatively above and below the plane and this characteristic alignment results in a \( \beta \)-sheet. Also, manipulating polar or non-polar residues provides an amphiphilic property.

![Hydrogen bonding in parallel and anti-parallel of \( \beta \)-sheet.](image)

When \( \beta \)-strand changes its overall direction, a few residues are involved in a chain reversal. The chain reversal, known as \( \beta \)-turn is formed with two corner residues \((i + 2\) and \(i + 3\)) and hydrogen bonding between \(i\) and \(i + 3\) (Figure 7). This hydrogen bond constrains the incoming and outgoing \( \beta \)-strands to maintain the \( \beta \)-sheet. The \( \beta \)-turn was
found initially in silk proteins by Geddes and co-workers (71) and defined stereochemically by Venkatachalam group (72).

Based on the stereochemistry and conformation of $\beta$-turns, they can be classified into seven different types: type I, type II, type I’, type II’, type III, type VI, and IG1 (G1 $\beta$-bulge type I) (73 - 81). Type I, most frequently occurring in native proteins has L-residues on both $i + 1$ and $i + 2$ positions (76, 77). However, proline favors to be located on $i + 2$ position. In type II turns, proline as well as L-residue is inclined to be found on position $i + 1$. For the $i + 2$ position, glycine, small polar L-residue, or D-residue is found because of steric hindrance caused by a side chain in the L-configuration. As a result, the differences between type I and type II is the location of proline due to the tension on the $\phi$ angle from the cyclic side chain.

Type I’ and type II’ are the mirror images of type I and type II and they have the opposite stereochemistry on the equivalent positions. For example, D-proline instead of L-proline would appear frequently on $i + 1$ for type I’ or on position $i + 2$ for type II’. Also, if residues with different chirality take up the corresponding sequence position, their energy level is comparable.
There is a unique turn feature of $3_{10}$ helix (82, 83). It still maintains hydrogen bonding between $i$ and $i+3$, but the incoming and outgoing strands are twisted seriously as regards to each other. The turn in this feature is called a type III turn.

Although the peptide bond of polypeptide prefers trans-configuration because of benefits from steric effect, it is also able to accommodate cis-configuration. The type VI turn is one of the instances. The type VI turn has hydrogen bond ($i$ and $i+3$) with ability to link two $\beta$-strands, but mediating peptide bond between $i+2$ and $i+3$ adopts cis conformations.

The last turn type, as known G1 bulge type I, has three corner residues instead of typical two corner residues (Figure 8). This results in the hydrogen bond between $i$ and $i+4$ and $\alpha\alpha_L$ conformation in the $i+3$ position (84). Sibanda and Thorton reported that homologous proteins are structurally mutated frequently by replacing a $\beta$-bulge over a tighter two-residue turn (75). Normally, $\beta$-bulge turn is less related to cross-strand interactions (85) which are crucial for peptides and proteins folding pattern (86). This propensity considers $\beta$-bulge turn type as an element of negative design (87).

![Figure 8. G1 $\beta$-bulge type I turn.](image)
The stereochemical type of side chains in β-turns can be defined in a similar way as R-group arrangements on cyclohexyl rings (73). When side chain exists in the same plane as polypeptide backbone, it has equatorial orientation. However, side chains exists out of plane as polypeptide backbone, it can be defined as axial. Depending on turn type and amino acids configuration in the turn position, orientation of side chains can be predicted (Table 1).

<table>
<thead>
<tr>
<th>Turn Type</th>
<th>Position $i + 1^a$</th>
<th>Position $i + 2^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L-Residue</td>
<td>D-Residue</td>
</tr>
<tr>
<td>I</td>
<td>eq</td>
<td>ax (down)</td>
</tr>
<tr>
<td>I´</td>
<td>ax (up)</td>
<td>eq</td>
</tr>
<tr>
<td>II</td>
<td>eq</td>
<td>ax (down)</td>
</tr>
<tr>
<td>II´</td>
<td>ax (up)</td>
<td>eq</td>
</tr>
<tr>
<td>III</td>
<td>intermed</td>
<td>intermed</td>
</tr>
<tr>
<td>VI ($cis$ peptide bond)</td>
<td>eq</td>
<td>eq</td>
</tr>
</tbody>
</table>

$^a$ Eq: equatorial; ax: axial; intermed: intermediate.

β-hairpin motifs are consisted of β-strands and β-turns and structurally diverse because of variations in the hairpin constraints, variations in sequences, variations in the loop size, and variations in the appearance of β-bulges in the β-strands (Figure 9). Also, β-hairpins are occurring widely not only in native polypeptides and proteins but also bioactive cyclic acids such as gramicidin S and peptide hormones oxytocin and vasopressin.
There have been several studies about stability factors of isolated β-hairpins (88 - 95) and they proposed two key elements: cross-strand interactions and interactions within turn residues. Cross-strand interactions include aromatic-aromatic (96), electrostatic (97), or hydrophobic packing interactions (88, 98). The one of important interactions within turn residues is combination of type I β-turn and a G1 bulge which appears to stabilize in many peptide hairpins (99 - 103). Recently, it is found that strands length of five or more resides are required for β-hairpin stability (104). Despite of these discoveries, many studies are still undergoing to determine the relative contribution of these factors to β-hairpin stability.

Santiveri et al compared the contributions of turn, strand, and cross-strand side chain - side chain interactions via mutations on both loop and strand and residue swapping within a strand (105). They found that side chain - side chain interactions across the strand may be more important for the hairpin stability. This result is crucial because statistical analyses of protein structure have not considered these effects.

Perez-Paya group reported favor for an aromatic versus aliphatic residue at the i + 3 position in a type I´ turn (106). They used the sequence Tyr - Asn - Gly - X as a type I´ turn and found isoleucine and valine make β-hairpin most stable despite of the
appearance of tyrosine on the cross-strand from X. This result does not follow a typical β-sheet character, but the author explained this with context dependence. In another study, Balaram and co-workers studied aromatic side chain - side chain interactions in organic solvent in order to examine their importance without hydrophobic driving force (107). They found that aromatic interactions contribute to hairpin stability through weak electrostatic effects instead of the hydrophobic effects.

Hughes and Waters investigated the role of lysine methylation in mediating protein – protein interactions within a β-hairpin system (108). Methylation of lysine in histone tails causes the binding of chromodomain proteins and eventually regulates chromatin condensation. This can be achieved by packing the methylated lysine within an aromatic pocket. Based on this interaction, the interaction of a trimethylated lysine and tryptophan within β-hairpin was investigated to resolve the nature of this type of cation - π interaction (Figure 10a). They found that trimethylation of lysine improves the interaction with tryptophan by approximately 0.7 kcal/mol and hairpin structure shows the compelling thermal stability. This interaction can contribute to structural stability for hairpin loops within designed proteins.

The groups of Cochran, Wu, and Eidenschink suggested tryptophan zipper motif to stabilize β-hairpin structure (109 - 111). This motif can resolve the disadvantage of a disulfide bridge constraint. Even though using a disulfide bridge has been valuable to select specific peptide loop structures for certain protein targets in phage-display technology (112), this bridge is susceptible to be cleaved in vivo in the presence of a free thiol group. Also, the disulfide link is not able to stabilize β-hairpin structure due to many degrees of rational freedom which cost the loss of other stabilizing interactions
(113). However, tryptophan zipper motifs induce $\pi - \pi$ stacking interactions between cross-strand tryptophans at non-hydrogen bonding positions and make $\beta$-hairpins stable (Figure 10c).

Many groups have investigated two-residue turn sequences to stabilize $\beta$-hairpin structures. For example, Asn – Gly, D-Pro – Gly, Aib – Gly, D-Pro – L-Pro, and Aib – D-Pro turns promote the formation of $\beta$-hairpins because of their character to form type I’ or type II’ turn (114, 115). Even though three-residue turn is considered as an element of negative design (87), there have been a few successful three-residue turn design recently in a $\beta$-hairpin structure. One of examples is G1 bulge in a ubiquitin-based hairpin sequence that resulted in a non-natural strand registry (116). However, Balaram and co-workers reported a three-residue turn which maintains native strand registry in methanol (Figure 10b). This can be achieved by insertion of D-Ala after L-Pro in a two-residue type II $\beta$-turn, D-Pro - L-Pro that leads to a left-handed helical conformation (117).

So far, the factors which are naturally occurring and stabilizing $\beta$-hairpin structure have been discussed. Recently, many groups have investigated the possibility of incorporation of peptidomimetics into $\beta$-sheet or $\beta$-hairpin structures because these artificial elements may improve the proteolytic resistance. Many groups have examined all aspects of $\beta$-hairpin structure including the turn, strands, backbone, hydrogen bonding, side chain - side chain interactions, and chirality and found the tolerance of $\beta$-hairpins from the a wide range of alterations.
Bartlett and co-workers have suggested an amide isostere consisting of a 1,6-dehydro-3(2H)pyridinone ring for templation of β-strands, known as @-tide (118 - 120). This @-tide provides not only rigid template but also a characteristic CD signal at 280 nm (Figure 11a). This signal produced by vinylogous amide is sensitive to conformational changes that acts as an indicator of sequence and side chain effects on β-hairpin folding. Based on the CD signal at 280 nm, the authors determined intrinsic stability of each residue and contribution of the side chain - side chain interaction (120).

Another example of a template b-sheet is the introduction of a guanidine-pyrrole moiety at the N terminus of a tripeptide (Figure 11b). Schmuk and colleagues found that
this peptidomimetic model was bound to a tetra peptide in water through electrostatic and hydrogen bonding between the guanidinopyrrole and the terminal carboxylate of tetra peptide (121). This study showed sequence selectivity regarding charge pairing along the strands.

![Figure 11. Structures of peptidomimetics.](image)

Kelly and co-workers have showed the replacement of strand residues in Aβ peptide with alkene functionality. They switched Cα and the NH to alkene moiety and investigated the effect of backbone hydrogen bonding in protein aggregation (Figure 11c). Even though formation of spherical aggregates was observed, they found that alkene isostres on the Phe 19 and Phe 20 in Aβ peptide prevent amyloid formation (122).

Last example of incorporation of peptidomimetic to β-hairpin structure is the utilization of a photoswitchable azobenzene as the turn sequence (Figure 11d). Two groups studied and found β-hairpin structure was maintained in the presence of cis-azobenzene configuration (123-125). However, depending on the difference in the strand sequences, two groups showed different result: the system designed by Hilvert and co-workers was
shown aggregation in the trans-configuration, but the model made by Renner and colleagues was not shown any sign of aggregation in either configuration.

As discussed in the previous topic, α-helix, the design of protein epitope mimetics is considered as an innovative approach for the development of protein - protein and protein - nucleic acid interaction inhibitors. This is because molecular recognition of proteins is normally occurring on the surface exposed secondary structure motifs such as β-hairpins and α-helices. There have been many studies about the β-hairpin structure, that contributed to the development of β-hairpin motifs which can mimic the partial contour of the restrained macro cyclic molecules (126, 127). Also, using parallel synthetic chemistry in solid-phase promotes the diversity of β-hairpin mimetics as well as optimization of their propensities (128).

The group of Fasan found that the distance between the Cα atoms of two residues i and i + 2 along one strand of a β-hairpin is comparable to the α-helical side chains at the i and i + 4 residues (129). In order to test this design against HDM2, a series of β-hairpins with different peptide sequences was prepared. After a surface Plasmon resonance (SPR) assay, β-hairpin with a sequence of Phe1, Trp3, and Leu4 showed inhibition of p53 binding to HDM2 (IC$_{50}$ = 125 ± 8 μM) (Figure 12). For further investigation, Phe or Trp residue was replaced by Ala and it was found that β-hairpin lost the inhibition ability of p53 binding. This result indicated the significance of the side-chain complementarily.

In many organisms, it is found that the large family of cationic host-defense peptide is involved in anti-infective defense mechanism (130). These short cationic peptides typically have 10-50 residues and a net positive charge of +2 to +10. They have
amphipathic properties, but different sequences and secondary structures. Due to their participation in immune response, this family of peptides has been considered as a potential pool for development of novel antibiotics.

Figure 12. β-Hairpin inhibitor disrupting the HDM2-P53 interaction.

Among many cationic peptides, PG-1 was taken as a initial model for β-hairpin mimetic design (131). Because PG-1 not only adopts β-hairpin structure via two cross-strand disulfide bridges but also displays wide spectrum of antimicrobial activity in micromolar range. However, due to its weak potency, it is necessary to modify sequences or propensity of the peptide. Recently, a family of protein epitope mimetic (PEM) based on PG-1 was demonstrated that show the improvement of their potency including the lead compound L27-11 (Figure 13) which shows nanomolar range activity (132). These PEM compounds have the stabilized β-hairpin structure through a D-Pro-L-Pro turn template and attract to the negatively charged bacterial surface because of their positive charges in the strands. After discovery of L27-11, another optimization was followed to improve drug-like properties such as the plasma stability, target stability, and
toxicology. This resulted in the discovery of compound POL7001 (Figure 14) and the a clinical lead candidate POL7080 (132). The safety of POL7080 is under testing in healthy humans in a phase I clinical study, but it is already considered as the first in a new class of antibiotics against Gram-negative bacteria (133).

Figure 13. The template of β-hairpin antibiotic L27-11.

Figure 14. The template of β-hairpin antibiotic POL7080.
CXCR4 is characterized as the class-A family of G-protein coupled receptors (GPCRs) and plays a role as a co-receptor for CD4-dependent HIV infection of human T-cells as well as a helper in hematopoietic stem cells (HSCs) mobilization from the bone marrow (BM) to peripheral blood. Since CXCR4 appears on the surface of the majority of HSCs and interacts with its native ligand SDF-1 in BM, CXCR4 antagonist are considered as promising novel therapeutic for the efficient mobilization and harvest peripheral blood HSCs by interruption of the SDF-1/CXCR4 interaction (134) both in cancer therapy as antimetastatic agents (135) and in inflammation and tissue repair (136).

Polyphemusin II isolated from the American horseshoe crab (*Limulus polyphemus*) has β-hairpin structure with 18-amino acid residues and shows inhibition to CXCR4. This peptide was taken as a starting point and a few of PEM molecules were designed and optimized in biological assays. For example, POL3026 (Figure 15), POL5551, and POL6326 are highly potent and selective CXCR4 antagonists (127, 137). POL6326 showed very limited or no mobilization of tumor cells in the leukapheresis and has already moved into a phase II clinical trial for the transplantation of autologous stem cells in newly diagnosed multiple myeloma patients.

Also, based on the recent X-ray structure, the β-hairpin is bound to CXCR4 through residues in the inward-facing projecting walls of the seven transmembrane helical bundle, a few extracellular loops, and the N-terminal segment (138). For the selective high affinity, a network of polar, hydrogen bonding, and hydrophobic interactions is important between the ligand and the receptor. According to the structural similarity between this bound ligand and POL3026, the macro cyclic b-hairpin mimetic may interact with CXCR4 in a fundamentally identical way.
The fundamental understanding of β-hairpin structure and folding has been improved with the discovery of well-defined β-hairpin model systems. Based on this improvement, protein epitope mimetics (PEM) with β-hairpin structures have come to the light in drug discovery. With some early success, this field is now well on its way to discovering well-folded β-hairpin structure with high therapeutic potency against diverse disease.

1.5. Solid-phase peptide synthesis

The first synthesis of peptides was tried by Theodor Curtius in 1882 (139). He made a dipeptide, Bz-Gly-Gly, by mixing benzoyl chloride and the silver salt of glycine. However, Emil Fisher was generally considered as the first scientist who demonstrated the total chemical synthesis of proteins (140). In 1907, Fisher reported an octadecapeptide comprising of fifteen glycine and three leucine residues (141). Another improvement of peptide synthesis after Fischer’s era was built by Bergmann and Zervas.
They suggested a reversible protecting groups for the α-amino groups in early 1930s. This discovery allowed to control the sequences in peptide synthesis as well as to avoid the racemization of amino acid in the amide coupling. With the carbodiimide coupling methods (143) along with protecting group strategies, small peptides were able to be synthesized in solution-phase such as glutathione (144) and carnosine (145). The group of du Vigneaud showed the synthesis of oxytocin in 1954 (Figure 16) (146).

Although the above approaches of peptides and proteins have been performed in solution-phase, the majority of peptide synthesis have been accomplished in solid-phase by using the solid support such as resin bead. This is because solid-phase synthesis has several advantages in comparison with the solution-phase methods (147). First, since the synthetic intermediate is always bound to the resin, the excess reagents or unbound by-products can be washed away easily. This leads to minimize the handling losses and avoid further purification. Second, the use of large excess of reagents can force the reaction to the completion. As a result, the couplings are fast and close to quantitative. Last, depending on cleavage conditions and appropriate anchor or linker groups, the polymeric resins are occasionally able to be regenerated and recycled.

Solid phase peptide synthesis (SPPS) was introduced by Merrifield first in 1963 (148). In SPPS, the C-terminal amino acid of the intended peptide is bound covalently to a solid support. The elimination of N(α)-protecting group is followed in the attached peptide and the next amino acid in N(α)-and side-chain protected, carboxyl-activated form is added after washing the resin-bound amino acid (Figure 17). Once the expected peptide bond is formed successfully, the excess reagents and by-products are removed via filtration and washing. These steps are repeated until the desirable peptide is formed on
the resin. In the last step, every protecting group is eliminated in the presence of hydrofluoric acid (HF) or trifluoroacetic acid (TFA). During this process, the linker bound to the solid support covalently is also cleaved to give the crude peptide which needs to be purified through diverse methods.

![Chemical structures of Glutathione, Carnosine, and Oxytocin](image)

Figure 16. Synthesis of small peptide.

Although the synthesis of peptides containing about 40-50 amino acids is still limited through the most optimized SPPS, the invention of SPPS has changed the history of peptides and protein chemistry. Even the SPPS is extended its application for the synthesis of small non-peptide compounds. Despite of its useful usage, a few of essential conditions are required for the successful solid phase peptide synthesis (149).

The first requirement is cross-linked insoluble polymeric supports such as resin beads are stable to the synthetic conditions. The initial resin form used by Merrifield was
polystyrene beads where the styrene is fractionally cross linked with 1% divinylbenzene and the chloromethyl group acts as an anchor or a linker (Figure 18). The next amino acid can be coupled to the linker through an ester group. This ester group is stable to the reaction conditions during the peptide synthesis and cleaved by using harsh acidic condition at the last step of the synthesis. One disadvantage of polystyrene beads is its hydrophobicity. Because the growing peptide is hydrophilic, the growing peptide is not solvated well and folds itself to form internal hydrogen bonds. This eventually prevents the next amino acid from accessing to the end of growing peptide chains. To resolve this problem, it is necessary to develop more polar solid supports such as Sheppard’s polyamide resin. In addition, the conditions for the non-peptide synthesis are different from those of the peptide synthesis. This demands for the development of new class of resins like Tentagel resin consisting of 80% polyethylene glycol implanted to cross-linked polystyrene. Regardless which polymer resin is chosen for the synthesis, they have to be swelling well and robust to the synthetic conditions for the better synthesis efficiency.

The second requirement is about a linker or an anchor which is bound to the polymer chain of the solid support. It should contain the reactive functional group to the starting material in the intended synthesis and the resulting linker should be inert throughout the whole synthetic process. However, it can be cleaved readily to give the final molecule. Depending on the functional groups on the starting material or the final product, different linkers are used. The Wang resin has the suitable linker for the addition and release of carboxylic acids. An N-protected amino acid is bound to the
linker via an ester link and this link is robust to the coupling and deprotection steps during the peptide synthesis.

Once the synthesis is completed, the final peptide is cleaved from the bead by using trifluoroacetic acid (Figure 19a). However, starting materials with carboxylic acid are bound to the Rink resin through an amide link. After all the aimed reactions are finished, the final product is released with a primary amide group instead of the original carboxylic acid under the TFA treatment (Figure 19b). If a starting material has the primary or secondary alcohol group, a linker on a dihydropyran derivatized resin is suitable. The alcohol group can be linked to the resin in the presence of pyridinium 4-toluenesulfonate in dichloromethane and the cleavage of final product will be done with the treatment of TFA (Figure 19c).

The last requirement is protecting functional groups, which are not involved in any synthetic reactions during SPPS. The selection of appropriate protecting groups is very crucial. Not only are protecting groups stable to the reaction conditions during the whole synthetic process but also they can be removed efficiently under the mild conditions. Regarding the peptide synthesis, two primary protecting group strategies are used. The first strategy is the use of Boc/benzyl protecting pair useful for the Merrifield resin (Figure 18). The Boc-N(α)-protected amino acid is attached to the growing peptide chain in the synthesis and the Boc (tert-butyloxycarbonyl) group is deprotected with TFA to make amino group available for the next coupling with another Boc-N(α)-protected amino acid. Other functional groups on the amino acid residues also need to be protected during the synthesis. However, this protecting group should be robust to TFA. Normally, benzyl-type protecting groups are used because they are stable to TFA but
susceptible to hydrofluoric acid. In addition, the bond between the growing peptide chain and the linker is affected by hydrofluoric acid. As a result, treatment with hydrofluoric acid cleaves the final product as well as deprotects functional groups on the residues at the last step. However, this strategy has one major disadvantage which is the use of hydrofluoric acid. Because hydrofluoric acid is a corrosive chemical which might jeopardize human health. To avoid the serious health risk, Fmoc/t-Bu strategy is used as an alternative. In this case, N(α)-terminus of every amino acid involved in couplings to the growing peptide chain is protected by 9-fluorenylmethoxycarbonyl (Fmoc) group instead of Boc-group. Because Fmoc-group can be deprotected with a mild base such as piperidine. Other functional groups on amino acid residues are protected by t-butyl group which can be removed easily with TFA rather than hydrofluoric acid. Moreover, the suitable resin (e.g. Wang resin) is chosen where the bond between linker and the growing peptide is cleaved with TFA.

Protein synthesis has been challenging subject in organic chemistry, but it is a very critical topic in synthetic biology. There have been numerous modifications and changes, such as invention of SPPS for the generation of proteins. Despite of huge efforts, the efficiency of protein synthesis is still relatively low. To resolve this problem, it is necessary to develop more efficient and flexible peptide chemical synthesis which contributes to the further study in biology and drug discovery.
1.6. Solution-phase peptide synthesis

As mentioned in the previous chapter, Curtius and Fisher are the first pioneers in simple peptide chemical synthesis in early twentieth century. Since then, the chemistry
has been developed based on selection of protecting groups for amino acids, their deprotection, and peptide bond formation. These days, two main peptide synthesis approaches are applied. One is solution-phase peptide synthesis and the other is solid-phase peptide synthesis that have the fundamentally same principles. In this chapter, principles for the peptide synthesis in solution will be covered.

Figures 18, 19. Merrifield resin (a) and types of resins (b, c, d).

It is important to prepare optically pure products, but racemization can occur during amino acid deprotection as well as amino acid activation steps. Two racemization mechanisms were considered: (1) direct proton abstraction (150) and (2) racemization through oxazol-5(4H)-ones (151, 152). In the former case, it is a very rare situation such as the rapid racemization of phenylglycine derivatives. The strong base triggers the racemization by abstracting the \( \alpha \)-proton on amino acid (Figure 20). This can be solved with the application of suitable conditions like the use of a tertiary amine (153, 154). In the latter case, the formation of oxazol-5(4H)-ones is involved in racemization mechanism.
When the carbonyl moiety of N-acyl amino acids (acyl = acetyl, benzoyl, piperidyl, and so on) is activated, oxazol-5(4H)-ones is formed which gives chirally unstable intermediates through tautomerization of the oxazol-5(4H)-ones (Figure 20). In order to avoid the formation of oxazol-5(4H)-ones, urethane-protected amino acids (e.g. benzyloxy carbonyl, tert-butyloxycarbonyl etc) has been used for the peptide chemical synthesis because of their resistance to racemization during activation and coupling.

As mentioned earlier, suitable protecting groups on amino acids is critical to avoid racemization. Not only that, they are also important to control the peptide formation. N(α)-protected amino acid and C(α)-protected amino acid are used to control the peptide bond formation. After peptide formation, protected dipeptide can be isolated, purified, and characterized. For the next coupling, N(α)-protecting group is removed and another N(α)-protected amino acid is coupled to this dipeptide. As a result, in order to obtain desired peptide, appropriate protecting groups should be selected for the N(α)-amino, carboxyl moiety, or side-chain functional groups. In the following chapters, different kinds of protecting groups and their deprotection remedy are described in detail.

Bergmann and Zervas introduced Cbz-group (Figure 21) for the protection of amino groups (155). Z-amino acid can be prepared via the reaction between benzyloxy carbonyl chloride and amino acid under the Schotten-Bauman condition. This protecting group can be removed by hydrogenolysis, reduction with sodium in liquid ammonia, liquid HF and so on. However, hydrogenolysis (155) and acidolysis with HBr (156) are the most conventionally used procedures. However, if a peptide chain accommodates sulfur-containing amino acids such as Cys and Met, the hydrogenolysis of Cbz-group fails due to catalyst poisoning. To overcome this problem many attempts were made, for example use an
additive (e.g. BaSO₄ (157), boron trifluoride etherate (158)) or a proton donor (e.g. 1,4-cyclohexadiene (159), ammonium formate (160), cyclohexene (161)). This was reported to improve hydrogenolysis only for the Met-containing peptides. Kuromizu and Meienhofer demonstrated catalytic hydrogenolysis of Cbz-group from Cys-containing peptide in liquid ammonia (162). This method was applied for the somatostatin synthesis (163). There are a few protecting groups which structurally similar to Cbz-group, but more stable in acidic condition (Figure 21). The incorporation of an electron-donating group on the aromatic ring improves the acid liability of the Cbz-group. (164 - 170). These groups can be removed by hydrogenolysis or mild acid.

McKay and Albertson was introduced Boc group (Figure 22) as another amino protecting group (171). Di-tert-butyl bicarbonate is preferably used to prepare for Boc-amino acids (172, 173) and the Boc group can be removed under mild acidic conditions like TFA. During the Boc cleavage by acidic treatment, tert-butyl cation is formed which causes many side reactions such as alkylation. To prevent the side reaction, anisole is used as a cation scavenger.

When Lys(Boc), Glu(OtBu), and Asp(OtBu) as well as sulfur-containing amino acids (Met and Cys) are involved in the peptide chain, both Cbz- and Boc-protecting groups cannot be applied for α-amino groups. In order to avoid the dilemma, triphenylmethyl (trityl : Tri) group (174 - 176) can be employed as a α-amino protecting group because this group is able to be removed with milder acids such as dilute acetic acid or 1 equivalent hydrochloric acid (Figure 22).
Figure 20. Two racemization mechanisms.

Chirally unstable oxazol-5(4H)-one

Figure 21. Structures of the Cbz and substituted Cbz groups.
Protecting groups removed by strong bases are not suitable for the peptide synthesis due to possibility of recamization. However, protecting groups removable under mild basic conditions is very valuable because selective cleavage of Bpoc or Tri group is especially difficult in the presence of Lys (Boc). Among diverse base-labile protecting groups, the 9-fluorenylmethyloxycarbonyl (Fmoc) group (Figure 22) is the most well-known protecting group in solid phase synthesis (177). Fmoc-amino acid can be prepared by reaction between free amino acid and Fmoc-Cl, but a side product, Fmoc-dipeptides, can be also formed in the course of this reaction (178). This resulted in the development of several additional Fmoc reagents such as Fmoc-OSu (179, 180), Fmoc-OPCP (179), and Fmoc-OBt (179). Deprotection of Fmoc group occurs easily by various amines like piperidine (181, 182) via β-elimination mechanism. The cleavage of Fmoc group by piperidine leads formation of a fulvene-piperidine adduct difficult to remove in reaction mixture in solution-phase synthesis. In order to resolve the disadvantage, polymer-bonded amines (183 - 186) have been use to remove Fmoc group in solution.

The allyloxycarbonyl (Alloc) group can also provide an effective N(α)-protecting group (187, 188). The Alloc group (Figure 22) can be removed in the presence of palladium catalyst in neutral conditions and the tBu group and fluorine-9-ylmethyl (Fm) groups are intact during the deprotection process. The 2,2,2-trichloroethoxycarbonyl (Troc) group (Figure 22) is also one of very useful N(α)-protecting groups (189 - 192) because it is stable in both acid and base presence. It can be removed by mild conditions such as zinc dust in acetic acid.

As described earlier, protecting the C-terminus on amino acids is also important as much as protecting the N-terminus due to controlling peptide bond formation. Regarding C-terminal protection, there are major difference between solid-phase...
synthesis and solution phase synthesis. In the former, the insoluble polymeric support may act as a C-terminal protecting group, whereas in the latter, more typical protecting groups are used. The most commonly used C-terminus protecting groups are alkyl ester, aryl ester, hydrazides, or protected hydrazides.

Methyl ester (-OMe) and ethyl ester (-OEt) (Figure 23) can be prepared by two steps: (1) activation alcohol (e.g. methanol, ethanol) by thionyl chloride and (2) reaction between a free amino acid and a previously activated species. Methyl and ethyl ester can be removed by saponification and modified to different functional groups for further couplings. Preparation of amino acid tBu esters (Figure 23) can be proceeded by a couple of different methods (193 - 198). For example, reaction between amino acids and isobutylene can provide amino acid tBu ester in the presence of sulfuric acid as a catalyst (193). tBu esters are inert to base-catalyzed hydrolysis, hydrogenolysis, and nucleophiles. However, they can be removed by acidolysis with moderately strong acid such as TFA or solutions of HCl in organic solvents. The 1-adamantyl esters of amino acids has the similar characters as tBu and it can be also deblocked by TFA.
The benzyl ester (-OBzl) of amino acids is prepared by a couple of mild conditioned methods (199 - 201). Cleavage of the benzyl ester (Figure 23) can be proceeded through acidolysis with strong acid or hydrogenolysis (202). Amino acid phenacyl ester (Figure 23) are obtained by the treatment of N-protected amino acid carboxylates with bromoacetophenone (203). The phenacyl group is very stable to acidic condition even to liquid HF. Due to this propensity, the phenacyl group is used along with the Boc group as N-protection. The phenacyl group can be removed with zinc in acetic acid (203) or sodium thiophenoxide in an inert solvent (204).

Peptidic hydrazides are prepared by reacting peptide methyl, ethyl, or benzyl esters to hydrazine hydrates (205, 206). The hydrazide can act as a C-terminal protecting group as well as maybe be transferred into the acyl azide with suitable treatments (207, 208). However, if the protected peptide alkyl ester contains Arg(NO₂), Asp(OBzl), Asp(OrBu), or Glu(OBzl), treatment of hydrazine hydrates causes side reactions like hydrazinolysis of side chain functional groups. In order to suppress this side reactions, it is more suitable to synthesize protected peptide hydrazides from the comparable substituted hydrazide.

![Figure 23. C-terminus protecting groups.](image-url)

- Methyl ester (-OME)
- Ethyl ester (-OEt)
- tert-butyl ester (-O-t-Bu)
- 1-adamantyl ester (-O-Ada)
- Benzyl ester (-O-Bzl)
- Phenacyl ester (-OPac)
Some native amino acids have a third functional groups. As a result, in the synthesis of peptides, it is necessary to protect the ε-amino group of Lys (or δ-amino group of Orn) in either carbonyl or amino component and the β-mercapto group of Cys in either component. However, this rule is not applied to some functional groups on amino acids such as the carbonyl groups of Asp and Glu, the guanidine group of Arg, the phenolic hydroxyl group of Tyr, the aliphatic hydroxyl group of Ser and Thr, the imidazole of His, the primary carboxamide of Asn and Gln, the aliphatic thioether of Met, and the indole ring of Trp. The group of Okada demonstrated the synthesis of eglin c containing 70 amino acid residues by following the minimum protection method (209, 210). In contrast, Sakakibara recommended “solution synthesis of peptides by the maximum protection procedure" via the DCC coupling method and a HF final deprotection method (211). "Maximum protection" strategy represents protecting maximum numbers of functional groups to avoid the side reactions. Despite less occurrence of side reaction, it is challenging to build highly soluble segments with full protection. Nakao and co-workers showed the synthesis of osteocalcin containing 40 amino acid residues by the maximum protection approach (212).

There are two main strategies to construct peptide molecules in solution: (1) stepwise elongation and (2) segment condensation. Even though their approaches are different, the chemical methods used on both are fundamentally similar.

It is critical to prevent epimerization during the peptide synthesis. Stepwise elongation from the C-terminal by one amino acid at a time using urethane-protected amino acids such as Z-amino acids and Boc-amino acids is beneficial to avoid epimerization while the peptide bond is dormed (212 - 214). The stepwise elongation
method is suitable for small peptide synthesis and preparation peptide segments to build larger peptides and proteins.

In 1952, Khorana used DCC for nucleotide synthesis (215) and Sheehan and Hess employed DCC for peptide synthesis in 1955 (216). Since then, DCC (Figure 24) has been the most effective and attractive coupling reagent both in solid phase peptide synthesis and solution phase peptide synthesis because of its high reactivity and high efficiency within short time. The defect of the use of DCC is the production of the insoluble dicyclohexylurea during activation/coupling. To overcome this disadvantage, diisopropylcarbodiimide (DIPCDI) has been utilized and is comparably effective and forms a more soluble urea by-product (216).

![Figure 24. Structures of carbodiimides.](image)

Another principle applied to stepwise elongation is mixed anhydride method which was reported by Curtius over 100 years ago, but Wieland was the first to use this approach for peptide synthesis (217). This method has an regioselective problem due to the use a mixed anhydride, containing two carbonyl groups, formed from protected amino acids or peptides and carboxylic acids. In order to obtain the desired product, the amino component has to attack to the correct carbonyl group (Figure 25). As a result, the success of this approach depends on the reduced the possibility of undesired attack on the carbonyl group. This can be achieved by using sterically hindered carboxylic acids or their analogues. The most effective coupling reagents for
this case is containing isovaleryl (218) or pivaloyl residues (219). Also, another popular type of mixed anhydrides is formation of carbonic acids rather than carboxylic acids as intermediates. Generally, ethyl chloroformate (220, 221) and isobutyl chloroformate (222) are used. The advantage of this approach is easy removal of by-products made, carbon dioxide and the corresponding alcohol, from the reaction mixture.

Based on the principle of the mixed anhydride method, Wieland and colleagues showed an amino acid thiophenyl ester (223), activated the ester moiety that has a tendency to form the peptide bond under mild conditions (224). The fundamental concept of this principle is making the carbonyl carbon more vulnerable to the amino component attack by increasing the electron-withdrawing character of the alcohol moiety of the ester. This can be achieved by using phenols with electron withdrawing group at ortho or para positions such as p-nitrophenyl ester (223), 2,4,5-trichlorophenyl ester (225), pentachlorophenyl ester (226), or penafluorophenyl ester (227). Anderson group demonstrated N-hydroxysuccinimide (HOSu) ester as one example of N-hydroxylamine type active ester (Figure 26) (228). The advantages of the N-hydroxylamine derivative are the suppression of epimerization and the easy removal with water. Another N-hydroxylamine analogue is N-hydroxybenzotriazole (HOBr) which is widely used in peptide synthesis (Figure 26) (229, 230).

![Two possible products via a mixed anhydride.](image-url)
The group of Kenner first postulated the use of acylphosphonium salts as reactive intermediates for the amide bond formation (231). However, this method was not used widely until Castro developed (benzotriazol-1-yl)oxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) reagent (232). Since then, phosphonium salts has been used widely in peptide chemistry. BOP (Figure 27) is easy to handle and promotes fast coupling, but produces the toxic byproduct, hexamethylphosphorotriamide (HMPA). In order to avoid the toxic byproduct formation, Castro replaced dimethylamine by pyrrolidine and developed (benzotriazol-1-yl)tripyrrolidinophosphonium hexafluorophosphate (PyBOP) (Figure 27) (233, 234).

The related uronium salts have also been employed in solid phase peptide synthesis. The use of uronium salts reduces the occurrence of side reaction such as epimerization (235). The group of Dourtoglou introduced 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) reagent (Figure 27) (234, 236). Subsequently, a few other uronium salts were developed. Knorr and coworkers showed the 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (HBTU) (Figure 27) (235). Later, the aza derivative of HBTU, 2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) (Figure 27) was reported (237). For years, HBTU and HATU were considered as uronium salts, but X-ray crystallography analysis confirmed their true structure as guanidium salts (238).

The condensation of peptide segments is an attractive approach for the larger peptides or proteins synthesis in solution. The separation of the peptide from truncated or deleted sequences is relatively easy. The main problem of this method is the epimerization of C-terminal residue of carboxylic segment except glycine or proline. As a result, the most critical requirement for coupling is reducing
epimerization of the C-terminal amino acid of the carboxyl moiety. The appropriate coupling methods to diminish epimerization in segment condensation are: (1) the azide procedure, (2) DCC methodology using an acidic additive, and (3) native chemical ligation (239).

\[ \text{Y} - \text{H} - \text{O} - \text{R} \quad \text{X}: \quad \begin{array}{c}
\text{p-nitrophenyl ester} \\
\text{2,4,5-trichlorophenyl ester} \\
\text{pentfluorophenyl ester} \\
\text{N-hydroxysuccinimide ester (HOSu)} \\
\text{1-hydroxybenzotriazole ester (HOBT)}
\end{array} \]

Figure 26. Structures of the active esters.

The azide procedure is favorably applied when the C-terminus does not contain a Gly or Pro residue. With certain precautions, the amount of epimerization can be maintained at very low levels (240). Another advantage of this approach is minimum protection principle can be employed because the azides do not acylate hydroxyl groups. However, its major defect is the low efficiency of coupling which leads to the rearrangement reaction to give carbamide peptides difficult to remove (208). The group of Shioiri introduced diphenylphosphoryl azide (DPPA) as an acyl azide forming reagent that constructs a peptide bond without any side chain functional groups protection as well as produces a low level of the epimeric peptide (241 - 243).

It was found that the coupling of protected peptide segments experienced a significant epimerization of the C-terminal amino acid when carbodiimides were used.
alone (244). The group of Weygand reported the segment coupling with the combination of DCC and HOSu dramatically reduced epimerization (less than 1%) (245, 246) and Wuensch and colleagues employed this approach to the synthesis of glucagon (247 - 249). Later, Koenig and Geiger demonstrated a more powerful reagent than HOSu, as known as HOBt (230) and 3,4-dihydro-3-hydroxy-4-oxo-1,2,4-benzotriazine (HOOBt) (250, 251). The DCC/HOBt procedure was successfully applied to the synthesis of human adrenocorticotropic hormone (ACTH) (252).

Native chemical ligation is the most useful method to produce long peptide chains. Native chemical ligation is remarkably chemoselective reaction between unprotected peptides containing a C-terminal thioester and N-terminal Cys residue under mild conditions in aqueous solution (253) (Figure 28). After ligation, treatment of selective desulfurization with H\textsubscript{2}/metal reagents converts Cys to Ala (254). Since Ala residue is one of the common amino acids in proteins, ligation at Ala residues can be utilized broadly.

The final step of peptide synthesis is removing all protecting groups. As mentioned earlier, all protecting groups on \(\alpha\)-amino, carboxyl component or side chain of amino acids have characteristic propensities. As a result, it is important to choose suitable deprotecting reagents to remove every protecting group. The final deprotection procedures are mainly: (1) catalytic hydrogenolysis with palladium (152, 255), (2) sodium treatment in liquid ammonia (256 - 258), (3) TFA treatment (259, 260), (4) HF treatment (261, 262), or (5) the "hard-soft acid-base" remedy (HSAB)" (263 - 270).

Solution phase peptide synthesis has been a classical approach in peptide synthesis. Even though solid phase peptide synthesis has replaced in most labs, it is still useful to produce peptide in large-scale for industrial purposes.
1. 7. Peptoids

There have been many cases that short peptide and nucleic acid oligomers with great affinity to various molecular targets suffered from poor pharmacokinetic properties. As a result, developing peptidomimetics close to poly peptide structure with the chemical diversity, spacing of side chains, a polar backbone, and the resistance to proteolysis is one of crucial tasks in drug discovery (271). In late 1980s, a new class of peptidomimetics was introduced as known as pepoids where connectivity of side chain point was moved from the alpha carbon to the amide nitrogen (272). In other words, the resulting structures are repetition of N-substituted glycine units, essentially regioisomer of peptides (Figure 29). This slight change of structures leads to the loss of a hydrogen bond donor (the NH group) and chiral center at the α-carbon and the gain of flexibility. Also, it gave peptoids a defiance to the proteolysis.

Figure 27. Structures of Phosphonium, uronium, and guanidium salts.
The initial approach of peptoid synthesis was Merrifield method of solid-phase peptide synthesis (273) by using N-substituted monomer units. The monomer units of peptoids have secondary amines, which is more hindered than the primary amines of peptides, so their coupling reaction time is longer in SPPS. Also, preparation of the monomer units is challenging because SPPS demands the considerable amount of N-substituted glycine. In order to resolve these problems, submonomer method was employed for peptoid synthesis. The submonomer concept was originally utilized by Emil Fischer who conceived peptide synthesis by using ammonia to displace haloalkylamides (274), but the group of Kent actually inspired the application of submonomer method for the peptoid synthesis. They demonstrated the ligation and cyclization of peptide fragments based on coupling nucleophiles to peptidic bromoacetamides (275). Based on the suggestion, the idea of peptoid synthesis was to grow the main chain by an acylation reaction with a haloacetic acid and then attach the side chain with a primary amine (Figure 30). This approach avoids the preparation of fully protected monomer units and uses smaller submonomer units instead. The first step is acylation of a secondary amine, which can be slower as compared to a primary amine in peptide synthesis. However, using an activating agent such as DIC can promote the reaction speed. The second step is a simple S_N2 reaction to incorporate the peptoid side chain functionality. The advantages of this step are that primary amines have not only good reactivity but also commercially-available diversity.

α-helices and β-sheets are the most prevalent secondary structures for proteins, but various other secondary structures are also well-defined for proteins, including alternative types of helices (β-helix, π-helix, 3_10 helix, polyproline-helix, and collagen helix), extended structures (α-sheets, β-hairpins, and β-bulges), and different kinds of
turns (α-turn, β-turn, γ-turn, and π-turn) (276). These secondary structures are normally characterized according to one of two ordinary methodologies: (1) hydrogen bonding pattern (277) and (2) the values of the three bond angles of the peptide backbone (φ, ψ, and ω) (278). In peptoids, it is impossible to define the secondary structure based on the former criteria due to the lack of the amide hydrogen, but assessing constrained backbone φ, ψ, and ω angles and the side chain conformations can contribute to the classification of peptoid secondary structures. The secondary structure of peptoid is generally evaluated by circular dichroism (CD) spectroscopy which enables rapid analysis relative to characterization of NMR. Moreover, due to the flexible structure of peptoids, their crystallization has been very difficult. Excessive CD studies has been established in order to correlate relationship between CD spectra and bioactivity changes and evaluate the peptoid structures. However, it is careful when comparing CD data for peptoids because different side chain compositions, especially aromatic side chains, affect on the CD spectra shape.

In peptoids, the polyproline-type-I-like helix is the most commonly reproductive secondary structure component by using the chiral and steric side chains, as already predicted by computational considerations (279). There are two available structures of this type of peptoid helix (280, 281) and their pitch differs by 10%. With the absence of the regular backbone hydrogen bonding effect in peptoids, the variation of the helical pitch may be attributed by the different side chain used (Nspc and Nrch) (Figure 31). Since the length of the peptoid backbone is fixed, a difference in helical pitch directly affect on varying numbers of residues/turn.

Despite of the original effect of sterically bulky side chains such as Nspe- and Nsch- (Figure 31) on the structure generating properties, further studies reported depending on side chains, n-π* interactions can affect on the cis/trans amide ratios
For example, one side chain (Nsna, Figure 31) preferred the cis-amide conformation, that likely results in more highly structured peptoid helices (282). In contrast, incorporation of aniline in peptoid side chains forces cis/trans amide equilibrium to over 90% trans according to the measurement of NMR spectroscopy (284). By using these side chains and a monomer capable of backbone-side chain hydrogen bonding, the characteristic acyclic peptoid reverse turn have been generated recently (285).

Besides backbone constraints, Nspe derivatives with para-substitutents can create the contacts (e.g. disulfide bonds, salt bridges, and hydrogen bonds) which are able to stabilize specific tertiary and quaternary structures (286). Alternatively, Nspe analogues with ortho- and para-substitutents can stabilize the peptoid helix via intramolecular $i \rightarrow i+3$ interactions. For instance, Wetzler and Barron proposed that the a pyridine Nspe analogue on position $i$ of a peptoid helix would be protonated at close to pH 7 because hydrogen bond acceptor on the para-position of a substituted Nspe at $i+3$ position could form hydrogen bonding with the protonated pyridine ring (276, Figure 32).

More recently, turn components have been revealed that can be achieved either by macrocyclization (287) or by utilization of heterocyclic turn-inducing unit (288). Kirshenbaum and colleagues showed head-to-tail macrocyclization of achiral peptoid oligomers and found macrocyclic hexamer or octamer peptoids are alike as peptide $\beta$-turns (287). The configurations of the turn region in each macrocycle were cis and the rest of amides were trans. The superposition of hexamer and octamer peptoids are highly similar to each other in the turn regions as well as the original peptide $\beta$-turns. Appella and co-workers demonstrated a triazole monomer acting as a turn mimic in 2007 (288). The triazole moiety is able to constrain the peptoid backbone
similar to a cis double bond that leads to a rigid turn in the peptoid structures. This outcome can facilitate the design of biomimetic peptoids using turn motif stabilization.

Figure 28. Native chemical ligation for peptide synthesis.

Figure 29. structures of peptide and peptoid.
Even though peptides and proteins are involved in important biological processes, they have not been developed for clinical use due to their poor oral bioavailability, short half-life in the body, and an immune response induction (289). These disadvantages of peptides gave the great opportunity for the peptoid therapeutics because peptoids are resistant to proteolysis and prepared in large scale with low cost relative to $\alpha$-peptides. Also, as mentioned earlier, peptoids can adopt the folded conformations such as helices (290), loops (291, 292), and turns (287, 288) in peptoids. Many researchers have taken these advantages of peptoids and developed diverse peptoid mimetics.

The group of Liskamp showed peptoid mimetics of peptide amylin which is involved in the beginning of type II diabetes (293). Amylin generates amyloid fibrils and easily aggregates in the insulin-forming islet $\beta$-cells (294) through cross $\beta$-sheet topology (295). Peptoid 1 and retropeptoid 2 (residues in reverse order) as analogs of
the amylin core region (20-29) were synthesized and studied their structures and inhibition of amylin aggregation (Table 2). In order to evaluate the structures of amylin (20-29), peptoid 1, and retropeptoid 2, CD spectroscopy was used: amylin (20-29) showed characteristic CD spectrum of β-sheet, but peptoid 1 and retropeptoid 2 displayed weak or random CD spectra indicating no defined secondary structure. Based on these CD spectra difference, the ability of the peptoids was studied whether peptoids can inhibit β-sheet and amyloid fibril formation in amylin (20-29). A 1:1 (w/w) mixture of amylin (20-29) and peptoid 1 did not show any unique CD spectrum of β-sheet, that support peptoid 1 was able to disrupt the β-sheet formation of amylin (20-29). Also, this mixture was ~20 % opaque in a comparison of that of amylin (20-29) alone, that represents inhibition of amyloid aggregation. However, the retropeptoid 12 showed moderate inhibition of aggregation by displaying ~50 % turbidity in a mixture (w/w) of amylin (20-29) and retropeptoid. This may be attributed from the supramolecular assemblies of retropeptoid 2 which interfered the effective inhibition of aggregation.

![Proposed model of intrahelix stabilizing hydrogen bond between ortho- and para-substituted Nspe analogues (X=Cl, OCH3, etc.).](image)

Kodadeck and colleagues found out three peptoids (3-5) bound to the coactivator CREB-binding protein (CBP) in vitro with a low micromolar range among a library
of ~100,000 peptoid hexamers (296, 297). This coactivator protein plays a key role in mammalian genes transcription. After further studies, among three peptoids, only peptoid 3 was selective for CBP while peptoids 4 and 5 were selective for bovine serum albumin (Figure 33). In other words, peptoid 4 and 5 failed to induce transcription, but peptoid 3 managed to activate transcription by serving as an activation domain ($EC_{50} = 8\mu M$). Moreover, the cell permeability of all three peptoids was evaluated. The peptoid 3 showed good cell permeability, which correlated well to its relative hydrophilicity. Surprisingly, peptoid 4 is most hydrophobic among three peptoids, but its cell permeability was moderate. Although peptoid 5 has intermediate hydrophobicity, it showed the poor cell permeability. Based on this permeability result, the researchers explained the reason of the transcription induction failure by peptoid 5 due to no accumulation inside cells (Figure 33).

Peptoid mimetics play an important role in drug discovery because of the fast synthesis and structural similarity to polypeptides. Many researchers already have worked on the design and application of peptoid mimics of bioactive molecules and will continue to pursue the same goal.

<table>
<thead>
<tr>
<th>Oligomer</th>
<th>Sequence (N- to C-terminus)</th>
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<tr>
<td>Amylin (20-29)</td>
<td>SNNFGAILSS</td>
</tr>
<tr>
<td>Peptoid 1</td>
<td>NSer-NAsn-NAsn-NPhe-Gly-NAla-NIle-NLeu-Nser-NSer</td>
</tr>
<tr>
<td>Retropeptoid 2</td>
<td>NSer-NSer-NLeu-NIle-NAla-Gly-NPhe-NAsn-NAsn-NSer</td>
</tr>
</tbody>
</table>

Table 2. Structures of Amylin (20-29), Peptoid 1, and Retropeptoid 2
Figure 33. Peptoid hexamers 3, 4, and 5.

1.8. References


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CHAPTER TWO:

DESIGN OF NON-PEPTIDIC OLIGO-PYRIMIDINES

MIMICKING i, i+4(3), i+7 POSITIONS OF α-HELICES

2. 1. Introduction

P53 is a tumor suppressor which is inactive or mutated in tumor cells (1). In unstressed normal cells, p53 is expressed in low levels by MDM2 (murine double minute protein 2) through two ways (2). P53 is a transcription factor inhibited by MDM2. This can be achieved by binding to N-terminal domain of p53 and preventing transcriptional activity. P53 can be also decreased via ubiquitin-dependent proteasome pathway. MDM2 promotes ubiquitination of lysine residues of p53 C-terminal region by acting as an ubiquitin E3 ligase. However, stresses such as DNA damage, ultra violet light, and oncogenes induce the activation of wild type of p53 protein (3, 4). This results in the presence of target genes in different regulatory regions. These target genes lead to various effects like inhibition of angiogenesis, cell cycle arrest, or apoptosis (Fig 1). Hence, over expression of MDM2 makes wild-type p53 disabled and tumor cells elongate their lives (5).

Due to the negative feedback between MDM2 and p53, MDM2 is an attractive therapeutic target for anti-cancer treatment. Two initial approaches were validated as cancer targets, MDM2,. MDM2 expression was inhibited by antisense oligonucleotides. This results in strong activation of p53 and cell cycle arrest or apoptosis (6, 7). Also,
injecting a monoclonal antibody to MDM2 leads to activation of p53 and cell growth arrest by disrupting interaction between p53 and MDM2 (8). These studies promote development of antagonists of MDM2, leading p53 active.

Prior to antagonists synthesis, it is important to check key interactions between p53 and MDM2. According to the crystallography, the region of MDM2 that binds to NH$_2$-terminal peptide of p53 showed characteristic interactions between these two proteins. Two pairs of $\alpha$-helices from p53 and MDM2 form a hydrophobic cleft where p53 binds like a pocket-ligand (9). Stabilization of p53-MDM2 complex is achieved through interactions between hydrophobic cleft and hydrophobic residues (Phe19, Trp23, Leu26) along with one-faced p53 helical peptide. Further experimental (10, 11) and computational (12, 13) studies have confirmed the important binding
residues of p53-MDM2 complex and this leads to study the possibility that small molecules can be an anti-cancer alternative by competing for MDM2 binding (14).

Several strategies have been studied to develop MDM2 antagonists. Peptides which can inhibit MDM2 were discovered after phage display at the 100 nmol/L of IC$_{50}$ level in vitro (15). A recent study showed an improved peptide which has better binding affinity and p53 activation (15). A high-throughput screen of chemical libraries has been used to identify Nutlins, a group of cis-imidazoline derivatives which were able to inhibit p53-MDM2 binding with high affinity (16). This study has promoted development of small molecules that act as p53-MDM2 inhibitors.

Recently, the group of Dr. Hamilton has showed terphenyl scaffold which is small non-peptidic molecules and mimics of the NH$_2$-terminal $\alpha$-helix of p53 (17). In order to make $\alpha$-helix structure of terphenyl derivatives, side chains on the phenyl rings are projected like $i$, $i+4$, and $i+7$ fashion. This mimic of structure can be achieved by substituting appropriate alkyl or aryl groups on ortho-position of each phenyl ring. These terphenyl analogues also showed inhibition of p53-MDM2 interaction in vitro and p53 activation in cell culture. This study showed the possibility to develop a novel $\alpha$-helical mimetics as MDM2 antagonists.

Here, terpyrimidine derivatives which have a $\alpha$-helical mimetic structure and better polarity based on terphenyl scaffold.

2.2. Results and discussion

From the previous study, terphenyl scaffold with mimic of $\alpha$-helical structure showed inhibition p53-MDM2 interaction and activation p53. Despite of the potential, these terphenyl derivatives have poor solubility or polarity. Not only improving this disadvantage but also finding better potency, a new group of $\alpha$-helical mimetics of
the NH$_2$-terminal p53 $\alpha$-helix was developed by using pyrimidyl rings instead of phenyl rings. The terpyrimidyl derivatives project their side chains in a similar geometry of those of a-helices. Also, containing nitrogens on pyrimidyl rings results in the increase of polarity (Scheme 1).

Scheme 1. General synthesis procedure of terpyrimidyl derivatives.
Initially, various R groups (e.g. -Ph, -NH₂, -S-CH₃, -S-CH₂-C₆H₅) were used to make terpyrimidyl derivatives. Every terpyrimidyl product was obtained as a precipitate except when R is phenyl group. Due to easy purification, more terpyrimidyl libraries were built for the case of NH₂, S-CH₃, S-CH₂-C₆H₅ as R groups.

Monomeric pyrimidyl analogues (1a-1j) were prepared by Michael addition (Scheme 2; Table 1). Pyrimidyl monomers were obtained as a precipitate.

![Scheme 2. Synthesis of 1a-1j.](image)

<table>
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<th>Compound</th>
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<th>R₁</th>
<th>Yield</th>
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<td>S-CH₃</td>
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</tr>
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<td>Ph</td>
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<tr>
<td>3</td>
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<td>4</td>
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</table>
Dimeric pyrimidyl derivatives were synthesized from compounds, 1a-1j (Scheme 3). Depending on which atom of hydroxylamine is involved in $S_2$ reaction, monomeric pyrimidines (1) were transformed into mixture of 2 and 2’. Under controlled condition, derivatives of 2 were only reduced to compounds 3. The Michael addition of 3 with different Michael acceptors followed and afforded a novel group of terpyrimidyl dimers, 4a-4j (Table 2). Dimeric terpyrimidyl analogues were formed as a precipitate.

### Table 4. Percent Yield of Terpyrimidyl Dimers Synthesis

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>1h</td>
<td>NH$_2$</td>
<td>iBu</td>
<td>21%</td>
</tr>
<tr>
<td>9</td>
<td>1i</td>
<td>NH$_2$</td>
<td>Ph</td>
<td>88%</td>
</tr>
<tr>
<td>10</td>
<td>1j</td>
<td>NH$_2$</td>
<td>Benzyl</td>
<td>50%</td>
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</table>

![Scheme 3. General synthesis of terpyrimidyl dimers.](image-url)
<table>
<thead>
<tr>
<th>Entry</th>
<th>Compound</th>
<th>R</th>
<th>R₁</th>
<th>R₂</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4a</td>
<td>S-CH₃</td>
<td>iPr</td>
<td>iBu</td>
<td>13%</td>
</tr>
<tr>
<td>2</td>
<td>4b</td>
<td>S-C₆H₅</td>
<td>1-Naphthalene</td>
<td>iBu</td>
<td>86%</td>
</tr>
<tr>
<td>3</td>
<td>4c</td>
<td>NH₂</td>
<td>iBu</td>
<td>Benzyl</td>
<td>25%</td>
</tr>
<tr>
<td>4</td>
<td>4d</td>
<td>NH₂</td>
<td>Benzyl</td>
<td>1-Naphthalene</td>
<td>8%</td>
</tr>
<tr>
<td>5</td>
<td>4e</td>
<td>NH₂</td>
<td>iBu</td>
<td>1-Naphthalene</td>
<td>58%</td>
</tr>
</tbody>
</table>

Nucleophilic substitution reaction (SN₂) of dimeric terpyrimidyl derivatives with hydroxylamine hydrochloride afforded the mixture of 5 and 5′. Analogues of 5 were transformed into compounds 6 when reduction reaction was carried out in methanol using potassium formate, acetic anhydride, and 10% palladium/carbon as reducing reagents. Addition reaction with Michael donors 6 in the presence of Michael acceptors and triethylamine occurred and afforded trimeric oligo-pyrimidyl derivatives 7a-7d (Scheme 4). Oligo-pyrimidyl trimers 7a-7d were precipitated after reaction was done (Table 3).

Terminal groups (-CN, and -NH₂) of oligo-pyrimidyl analogues were transformed into carboxylic acid derivatives in order to elevate their polarity and dissolve better in aqueous solution (Scheme 5). Terminal cyano group was hydrolyzed in methanol (reflux) using NaOH. Another terminal group -NH₂ was involved in nucleophilic substitution reaction (SN₂) when succinic anhydride was employed in THF. Products after hydrolysis or SN₂ reaction were obtained as precipitation (Table 4).

In conclusion, terpyrimidyl compounds were synthesized successfully after SN₂ reaction, reduction, and Michael addition. Despite of nitrogen atoms on pyrimidyl
rings, oligo-pyrimidyl derivatives have solubility problems in aqueous solution. Based on this result, development of novel MDM2 antagonists based on different scaffolds need modifications which are in compliance with therapeutic properties.


Table 5. Percent Yield of Terpyrimidine Trimers Analogues

<table>
<thead>
<tr>
<th>Entry</th>
<th>Compound</th>
<th>R</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7a</td>
<td>NH₂</td>
<td>iBu</td>
<td>1-</td>
<td>iBu</td>
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</tr>
<tr>
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<td>Benzyl</td>
<td>1-</td>
<td>iBu</td>
<td>28%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Naphthalene</td>
</tr>
<tr>
<td>3</td>
<td>7c</td>
<td>NH₂</td>
<td>iBu</td>
<td>1-</td>
<td>iPr</td>
<td>51%</td>
</tr>
</tbody>
</table>
Naphthalene

\[
\begin{array}{ccccccc}
4 & 7d & \text{NH}_2 & \text{iBu} & \text{Benzyl} & \text{iBu} & 37%
\end{array}
\]

Scheme 5. Synthesis of 8-10.

Table 6. Percent Yield of synthesis 8-10

<table>
<thead>
<tr>
<th>Entry</th>
<th>compound</th>
<th>R</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
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<td>NH₂</td>
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<td></td>
<td>Benzyl</td>
<td>5%</td>
</tr>
<tr>
<td>2</td>
<td>9</td>
<td></td>
<td>iBu</td>
<td></td>
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<td>10</td>
<td>NH₂</td>
<td>iBu</td>
<td></td>
<td>Benzyl</td>
<td>iBu</td>
</tr>
</tbody>
</table>
2. 3. Experimental section

2. 3. 1. General comments

The chemicals were purchased from Aldrich-Sigma and Acros. $^1$H (400 MHz) and $^{13}$C (100 MHz) NMR spectra were obtained in deuterated solvents using Inova-400. For high resolution mass spectrometry (HRMS) spectra, Agilent 1100 series was used in the ESI-TOF mode.

2. 3. 2. Synthesis of monomeric pyrimidyl derivatives, 1a-1j

4-isopropyl-2-(methylthio)pyrimidine-5-carbonitrile (1a)

A mixture of Michael acceptor (6.02 mmol, 1 g), NaOEt (6.02 mmole, 0.45g) and 2-methyl-2-thiopseudourea sulfate (2.04 mmol, 1.67 g) in methanol (absolute, 200 proof, 10mL) was located under microwave conditions until the TLC indicated completion of the reaction. The mixture was brought to room temperature and the formed precipitate was filtered by vacuum and rinsed with ice cold methanol (5 mL x 3). Yield: 51%. $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 1.32-1.34 (d, 6H, $J = 0.02$), 2.60 (s, 3H), 3.33-3.38 (m, 1H), 8.60 (s, 1H). $^{13}$C NMR (CDCl$_3$, 400 MHz): $\delta$ 14.61, 21.15, 29.93, 159.83. HR-MS: $m/z = 194$ [M+H]$^+$. 

2-(methylthio)-4-phenylpyrimidine-5-carbonitrile (1b)

A mixture of Michael acceptor (4.99 mmol, 1 g), triethylamine (6.48 mmole, 0.9 ml) and 2-methyl-2-thiopseudourea sulfate (9.98 mmol, 1.39 g) in methanol (absolute, 200 proof, 10mL) was stirred under refluxing conditions until the TLC indicated completion of the reaction. The mixture was brought to room temperature and the formed precipitate was filtered by vacuum and rinsed with ice cold methanol (5 mL x 3). Yield: 92%. $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 2.66 (s, 3H), 7.54-7.59 (m, 3H), 8.09-8.11
(m, 2H), 8.78 (s, 1H). $^{13}$C NMR (CDCl$_3$, 400 MHz): δ 14.75, 129.19, 132.41, 161.56. HR-MS: $m/z = 228.05$ [M+H]$^+$. 

4-isobutyl-2-(methylthio)pyrimidine-5-carbonitrile (1c)

A mixture of Michael acceptor (11.1 mmol, 2 g), triethylamine (14.4 mmole, 2.0 ml) and 2-methyl-2-thiopseudourea sulfate (11.1 mmol, 1.54 g) in methanol (absolute, 200 proof, 10mL) was stirred under refluxing conditions until the TLC indicated completion of the reaction. The mixture was brought to room temperature and the formed precipitate was filtered by vacuum and rinsed with ice cold methanol (5 mL x 3). Yield: 13%. $^1$H NMR (CDCl$_3$, 400 MHz): δ 0.91-0.93 (d, 6H), 2.17 (m, 1H), 2.62-2.64 (d, 2H), 3.49 (s, 3H), 8.25 (s, 1H). $^{13}$C NMR (CDCl$_3$, 400 MHz): δ 8.87, 29.95, 44.59, 45.77, 121.60. HR-MS: $m/z = 208$ [M+H]$^+$. 

2-(benzylthio)-4-(naphthalene-1-ylmethyl)pyrimidine-5-carbonitrile (1d)

A mixture of Michael acceptor (6.43 mmol, 1.70 g), triethylamine (9.64 mmole, 1.3 ml) and 2-benzyl-2-thiopseudourea sulfate (9.64 mmol, 1.96 g) in methanol (absolute, 200 proof, 10mL) was stirred under refluxing conditions until the TLC indicated completion of the reaction. The mixture was brought to room temperature and the formed precipitate was filtered by vacuum and rinsed with ice cold methanol (5 mL x 3). Yield: 13%. $^1$H NMR (CDCl$_3$, 400 MHz): δ 4.17 (s, 2H), 2.21 (s, 2H), 7.10-8.17 (m, 12H), 8.64 (s, 1H). $^{13}$C NMR (CDCl$_3$, 400 MHz): δ 44.59, 45.58, 123.98, 125.53, 125.91, 126.28, 126.48, 127.40, 129.03, 134.94, 159.91, 160.16, 161.62, 162.53, 168.76, 172.27, 176.63. HR-MS: $m/z = 383$ [M+NH$_4$]$^+$. 

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2-amino-4-isopropylpyrimidine-5-carbonitrile (1e)

A mixture of guanidine hydrochloride (7.23 mmol, 0.69 g) and potassium carbonate (7.23 mmol, 1 g) in ethanol (5 mL) was added Michael acceptor (4.82 mmol, 0.8 g) suspended in ethanol (1 mL). The reaction mixture was placed on a microwave reactor for 25 min at 125 °C. A colorless crystal-like precipitate formed in the solution upon cooling to rt. The precipitate was filtered and rinsed with ice cold ethanol (3 mL x 2) to isolate compound. Yield: 29%. HR-MS: \( m/z = 163.09 \) [M+H]⁺.

2-amino-4-(naphthalene-1-ylmethyl)pyrimidine-5-carbonitrile (1f)

To a mixture of Michael acceptor (3.03 mmol, 0.8 g) and guanidine hydrochloride (4.55 mmol, 0.43 g) in ethanol (absolute, 200 proof, 12 mL) was added triethylamine (4.55 mmol, 0.6 ml). The mixture was stirred under refluxing conditions until the TLC indicated the complete consumption of starting materials. The mixture was brought to room temperature and the formed precipitate was filtered by vacuum and rinsed with ice cold methanol (5 mL x 3). Yield: 90%. HR-MS: \( m/z = 261.11 \) [M+H]⁺.

2-amino-4-(tert-butyl)pyrimidine-5-carbonitrile (1g)

A mixture of Michael acceptor (5.56 mmol, 1 g), triethylamine (8.34 mmole, 1.2 ml) and guanidine hydrochloride (8.34 mmol, 0.8 g) in methanol (absolute, 200 proof, 10 mL) was stirred under refluxing conditions until the TLC indicated completion of the reaction. The mixture was brought to room temperature and the formed precipitate was filtered by vacuum and rinsed with ice cold methanol (5 mL x 3). Yield: 55%. \(^{13}\text{C}\) NMR (CDCl₃, 400 MHz): \( \delta 28.28, 39.31, 95.45, 117.79, 161.75, 163.41, 181.20 \). HR-MS: \( m/z = 177.11 \) [M+H]⁺.
2-amino-4-isobutylpyrimidine-5-carbonitrile (1h)

A mixture of guanidine hydrochloride (8.91 mmol, 0.85 g) and triethylamine (8.91 mmol, 1.2 ml) in ethanol (5 mL) was added Michael acceptor (5.94 mmol, 1.07 g) suspended in ethanol (1 mL). The reaction mixture was placed on a microwave reactor for 25 min at 125 ºC. A colorless crystal-like precipitate formed in the solution upon cooling to rt. The precipitate was filtered and rinsed with ice cold ethanol (3 mL x 2) to isolate compound. Yield: 20%. HR-MS: \( m/z = 177.11 \) [M+H]\(^+\).

2-amino-4-phenylpyrimidine-5-carbonitrile (1i)

Microwave-assisted reactions were also done in the presence of potassium carbonate (6.00 mmole, 0.83g) to obtain the desired pyrimidines. Michael acceptor (4.00 mmol, 0.8 g) and guanidine hydrochloride (6.00 mmol, 0.57 g) was mixed in ethanol (5 mL). The reaction mixture was placed on a microwave reactor for 40 min at 120 ºC. A colorless crystal-like precipitate formed in the solution upon cooling to rt. The precipitate was filtered and rinsed with ice cold ethanol (3 mL x 2) to isolate compound 1i as needle-like crystals. Yield: 88%. \(^1\)H NMR (CDCl\(_3\), 400 MHz): \( \delta \) 6.89 (s, 2H), 7.42-7.60 (m, 5H), 7.87 (s, 1H). \(^{13}\)C NMR (CDCl\(_3\), 400 MHz): \( \delta \) 128.30, 128.45, 131.76, 159.60. HR-MS: \( m/z = 197.08 \) [M+H]\(^+\).

2-amino-4-benzylpyrimidine-5-carbonitrile (1j)

To a mixture of Michael acceptor (3.92 mmol, 0.8 g) and guanidine hydrochloride (5.88 mmol, 0.56 g) in ethanol (absolute, 200 proof, 12 mL) was added triethylamine (5.88 mmol, 0.8 ml). The mixture was stirred under refluxing conditions until the TLC indicated the complete consumption of starting materials. The mixture was
brought to room temperature and the formed precipitate was filtered by vacuum and rinsed with ice cold methanol (5 mL x 3). Yield: 50%. HR-MS: \( m/z = 211.09 \ [\text{M}+\text{H}]^+ \).

2. 3. 3. Synthesis of dimeric pyrimidyl derivatives, 4a-4e

4-(tert-butyl)-4′isobutyl-2′(methylthio)-[2,5′bipyrimidine]-5-carbonitrile (4a)

A mixture of compound 1a (2.07 mmol, 0.4 g), hydroxylamine (5.18 mmol, 0.36 g) and triethylamine (5.18 mmol, 0.7 ml) in ethanol (absolute, 200 proof, 10mL) was stirred under refluxing conditions until the TLC indicated completion of the reaction. The mixture was brought to room temperature and evaporate every ethanol under the reduced pressure. White solid are formed. With this solid 2 (0.36 mmol, 0.11 g), start reduction reaction. Dissolve solid 2 with methanol and add acetic anhydride (0.47 mmol, 0.04 ml). After five minutes, add potassium formate (1.44 mmol, 0.12g) and palladium/carbon (0.11 g). Heat the reaction solution and keep stirring overnight. By using diatomaceous earth, filter the reaction solution and evaporate every solvent. White compound 3 can be observed. With compound 3, start another coupling reaction. Dissolve solid 3 with methanol and add acetic anhydride (0.47 mmol, 0.04 ml). After five minutes, add potassium formate (1.44 mmol, 0.12g) and palladium/carbon (0.11 g). Heat the reaction solution and keep stirring overnight. By using diatomaceous earth, filter the reaction solution and evaporate every solvent. White compound 3 can be observed. With compound 3, start another coupling reaction. A mixture of compound 3 and 2′ (1.90 mmol, 0.4 g) and Michael acceptor (2.85 mmol, 0.29 g) in ethanol (absolute, 200 proof, 10mL) was stirred under refluxing conditions until the TLC indicated
completion of the reaction. The mixture was brought to room temperature and the formed precipitate was filtered by vacuum and rinsed with ice cold methanol (5 mL x 3). Yield: 13%. $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 1.30 (d, 6H), 1.57 (s, 9H), 2.64 (s, 3H), 4.05 (m, 1H), 8.98 (s, 1H), 9.14 (s, 1H). $^{13}$C NMR (CDCl$_3$, 400 MHz): $\delta$ 14.53, 22.05, 29.93, 32.24, 40.28, 104.28, 116.77, 121.65, 159.96, 162.34, 164.54, 174.27, 175.47, 179.55. HR-MS: $m/z = 328$ [M+H]$^+$. 

2′-(benzylthio)-4-isobutyl-4′-(naphthalen-1-ylmethyl)-[2,5′-bipyrimidine]-5-carbonitrile (4b)

A mixture of compound 1d (0.76 mmol, 0.28 g), hydroxylamine (1.90 mmol, 0.13 g) and triethylamine (1.90 mmol, 0.3 ml) in ethanol (absolute, 200 proof, 10mL) was stirred under refluxing conditions until the TLC indicated completion of the reaction. The mixture was brought to room temperature and remove every ethanol under the reduced pressure. White solid are formed. With this solid 2 (0.75 mmol, 0.13 g), start reduction reaction. Dissolve solid 2 with methanol and add acetic anhydride (0.98 mmol, 0.1 ml). After five minutes, add potassium formate (3.00 mmol, 0.25g) and palladium/carbon (0.30 g). Heat the reaction solution and keep stirring overnight. By using diatomaceous earth, filter the reaction solution and evaporate every solvent. White compound 3 can be observed. With compound 3, start another coupling reaction. Dissolve solid 3 with methanol and add acetic anhydride (0.98 mmol, 0.1 ml). After five minutes, add potassium formate (3.00 mmol, 0.25g) and palladium/carbon (0.30 g). Heat the reaction solution and keep stirring overnight. By using diatomaceous earth, filter the reaction solution and evaporate every solvent. White compound 3 and 2′ can be observed. With mixture of 3 and 2′, start another coupling reaction. A mixture of compound 3 and 2′ (0.75 mmol, 0.288 g), triethylamine (1.13 mmole, 0.2 ml) and
another Michael acceptor (1.13 mmol, 0.23 g) in ethanol (absolute, 200 proof, 10mL) was stirred under refluxing conditions until the TLC indicated completion of the reaction. The mixture was brought to room temperature and the formed precipitate was filtered by vacuum and rinsed with ice cold methanol (5 mL x 3). Yield: 86%. $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 0.79-0.81 (d, 6H), 1.98 (m, 1H), 2.67-2.69 (d, 2H), 4.15 (s, 2H), 5.12 (s, 2H), 6.98-7.50 (m, 12H), 8.86 (s, 1H), 9.32 (s, 1H). $^{13}$C NMR (CDCl$_3$, 400 MHz): $\delta$ 22.36, 28.89, 35.34, 40.17, 45.55, 115.19, 121.61, 124.16, 125.18, 125.54, 125.87, 126.25, 126.64, 127.31, 127.40, 128.60, 128.97, 129.12, 132.44, 133.96, 134.73, 160.09. HR-MS: $m/z$ = 502 [M+H]$^+$. 

2'-amino-4-benzyl-4'-isobutyl-[2,5'-bipyrimidine]-5-carbonitrile (4c)

A mixture of compound 1h (2.09 mmol, 0.368 g), hydroxylamine (5.23 mmol, 0.36 g) and triethylamine (5.23 mmol, 0.7 ml) in ethanol (absolute, 200 proof, 10mL) was stirred under refluxing conditions until the TLC indicated completion of the reaction. The mixture was brought to room temperature and evaporate every ethanol under the reduced pressure. White solid are formed. With this solid 2 (2.09 mmol, 0.44 g), start reduction reaction. Dissolve solid 2 with methanol and add acetic anhydride (2.72 mmol, 0.3 ml). After five minutes, add potassium formate (8.36 mmol, 0.70g) and palladium/carbon (0.44 g). Heat the reaction solution and keep stirring overnight. By using diatomaceous earth, filter the reaction solution and evaporate every solvent. White compound 3 can be observed. With compound 3, start another coupling reaction. 

A mixture of compound 3 (2.09 mmol, 0.403 g), triethylamine (3.13 mmole, 0.4 ml) and Michael acceptor (3.13 mmol, 0.64 g) in ethanol (absolute, 200 proof, 10mL) was stirred under refluxing conditions until the TLC indicated completion of the reaction. The
mixture was brought to room temperature and the formed precipitate was filtered by vacuum and rinsed with ice cold methanol (5 mL x 3). $^1$H NMR (CDCl$_3$, 400 MHz): δ 0.79 (d, 6H), 1.98 (m, 1H), 2.97 (d, 2H), 4.26 (s, 2H), 5.30 (s, 2H), 7.22-7.34 (m, 5H), 8.83 (s, 1H), 9.00 (s, 1H). $^{13}$C NMR (CDCl$_3$, 400 MHz): δ 22.72, 28.66, 43.35, 45.05, 127.68, 129.16, 129.51, 160.41, 162.29. HR-MS: m/z = 345.18 [M+H]$^+$.  

2'-amino-4'-benzyl-4-(naphthalene-1-ylmethyl)-[2,5'-bipyrimidine]-5-carbonitrile (4d)  

A mixture of compound 1j (1.90 mmol, 0.40 g), hydroxylamine (7.60 mmol, 0.52 g) and triethylamine (7.60 mmol, 1.1 ml) in ethanol (absolute, 200 proof, 10mL) was stirred under refluxing conditions until the TLC indicated completion of the reaction. The mixture was brought to room temperature and remove every ethanol under the reduced pressure. White solid are formed. With this solid 2 (1.90 mmol, 0.46 g), start reduction reaction. Dissolve solid 2 with methanol and add acetic anhydride (2.47 mmol, 0.2 ml). After five minutes, add potassium formate (7.60 mmol, 0.64g) and palladium/carbon (0.44 g). Heat the reaction solution and keep stirring overnight. By using diatomaceous earth, filter the reaction solution and evaporate every solvent. White compound 3 can be observed. With compound 3, start another coupling reaction. A mixture of compound 3 (1.90 mmol, 0.43 g), triethylamine (2.85 mmole, 0.4 ml) and Michael acceptor (2.85 mmol, 0.60 g) in ethanol (absolute, 200 proof, 10mL) was stirred under refluxing conditions until the TLC indicated completion of the reaction. The mixture was brought to room temperature and the formed precipitate was filtered by vacuum and rinsed with ice cold methanol (5 mL x 3). $^1$H NMR (CDCl$_3$, 400 MHz): δ 4.21 (s, 2H), 4.69 (s, 2H), 5.25 (s, 2H), 6.82-8.07 (m, 12H), 8.82 (s, 1H), 8.93 (s, 1H). $^{13}$C NMR (CDCl$_3$, 400 MHz): δ 40.41, 42.11, 124.19, 125.72, 126.13, 126.32, 126.69, 128.33, 128.67, 128.79, 129.12, 162.60. HR-MS: m/z = 429 [M+H]$^+$.  

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**2′-amino-4′-isobutyl-4′-(naphthalene-1-ylmethyl)-[2,5′-bipyrimidine]-5-carbonitrile (4e)**

A mixture of compound 1h (1.02 mmol, 0.18 g), hydroxylamine (2.55 mmol, 0.18 g) and triethylamine (2.55 mmol, 0.4 ml) in ethanol (absolute, 200 proof, 10mL) was stirred under refluxing conditions until the TLC indicated completion of the reaction. The mixture was brought to room temperature and evaporate every ethanol. White solid are formed. With this solid 2 (1.02 mmol, 0.21 g), start reduction reaction under the reduced pressure. Dissolve solid 2 with methanol and add acetic anhydride (1.33 mmol, 0.1 ml). After five minutes, add potassium formate (4.08 mmol, 0.34g) and palladium/carbon (0.21 g). Heat the reaction solution and keep stirring overnight. By using diatomaceous earth, filter the reaction solution and evaporate every solvent. White compound 3 can be observed. With compound 3, start another coupling reaction. 

A mixture of compound 3 (1.02 mmol, 0.19 g), triethylamine (1.33 mmole, 0.2 ml) and Michael acceptor (1.33 mmol, 0.35 g) in ethanol (absolute, 200 proof, 10mL) was stirred under refluxing conditions until the TLC indicated completion of the reaction. The mixture was brought to room temperature and the formed precipitate was filtered by vacuum and rinsed with ice cold methanol (5 mL x 3).  

$^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 0.71 (d, 6H), 1.91 (m, 1H), 2.85 (d, 2H), 4.80 (s, 2H), 5.28 (s, 2H), 7.50-8.10 (m, 7H), 8.91 (s, 1H), 8.93 (s, 1H). $^{13}$C NMR (CDCl$_3$, 400 MHz): $\delta$ 22.58, 28.58, 40.56, 44.79, 124.33, 125.76, 126.17, 126.69, 128.68, 128.75, 129.12, 132.09, 132.17, 134.22, 160.54. HR-MS: m/z = 395 [M+H]$^+$. 

HR-MS: m/z = 395 [M+H]$^+$. 

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2. 3. 4. Synthesis of trimeric terpyrimidyl derivatives, 7a-7d

2'-амино-4,4'-изобутил-4'-(напthalен-1'-илметил)-[2,5':2',5''-терпиримидин]-5'-карбонитрил (7a)

A mixture of compound 4e (0.73 mmol, 0.288 g), hydroxylamine (2.92 mmol, 0.20 g) and triethylamine (2.92 mmol, 0.4 ml) in ethanol (absolute, 200 proof, 10mL) was stirred under refluxing conditions until the TLC indicated completion of the reaction. The mixture was brought to room temperature and remove every ethanol under the reduced pressure. White solid are formed. With this solid (0.73 mmol, 0.31 g), start reduction reaction. Dissolve solid with methanol and add acetic anhydride (0.95 mmol, 0.1 ml). After five minutes, add potassium formate (2.92 mmol, 0.25g) and palladium/carbon (0.31 g). Heat the reaction solution and keep stirring overnight. By using diatomaceous earth, filter the reaction solution and evaporate every solvent. White compound 6 can be observed. With compound 6, start another coupling reaction. A mixture of compound 6 (0.73 mmol, 0.30 g), triethylamine (2.92 mmole, 0.4 ml) and Michael acceptor (1.09 mmol, 0.20 g) in ethanol (absolute, 200 proof, 10mL) was stirred under refluxing conditions until the TLC indicated completion of the reaction. The mixture was brought to room temperature and the formed precipitate was filtered by vacuum and rinsed with ice cold methanol (5 mL x 3). Yield: 57%. $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 0.71-0.77 (d, 6H), 0.77-0.81 (d, 6H), 1.85-2.00 (m, 2H), 2.68-2.70 (d, 2H), 2.89-2.91 (d, 2H), 5.17 (s, 1H), 5.24 (s, 1H), 6.97-8.03 (m, 7H), 8.90 (s, 1H), 8.91 (s, 1H), 9.53 (s, 1H). $^{13}$C NMR (CDCl$_3$, 400 MHz): $\delta$ 22.37, 22.62, 28.77, 28.96, 40.15, 44.60, 45.58, 115.11, 123.98, 125.91, 126.28, 126.48, 129.03, 132.27, 134.94, 159.91, 160.16, 161.63, 162.53, 164.63, 168.75, 172.26, 173.63. HR-MS: $m/z = 529$ [M+H]$^+$. 

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A mixture of compound 4d (0.11 mmol, 0.05 g), hydroxylamine (0.44 mmol, 0.03 g) and triethylamine (0.44 mmol, 0.1 ml) in ethanol (absolute, 200 proof, 10mL) was stirred under refluxing conditions until the TLC indicated completion of the reaction. The mixture was brought to room temperature and evaporate every ethanol under the reduced pressure. White solid are formed. With this solid 5 (0.11 mmol, 0.05 g), start reduction reaction. Dissolve solid 5 with methanol and add acetic anhydride (0.14 mmol, 0.01 ml). After five minutes, add potassium formate (0.44 mmol, 0.04g) and palladium/carbon (0.05 g). Heat the reaction solution and keep stirring overnight. By using diatomaceous earth, filter the reaction solution and evaporate every solvent. White compound 6 can be observed. With compound 6, start another coupling reaction.

A mixture of compound 6 (0.11mmol, 0.05 g), triethylamine (0.28 mmole, 0.04 ml) and Michael acceptor (0.17 mmol, 0.03 g) in ethanol (absolute, 200 proof, 10mL) was stirred under refluxing conditions until the TLC indicated completion of the reaction. The mixture was brought to room temperature and the formed precipitate was filtered by vacuum and rinsed with ice cold methanol (5 mL x 3). Yield: 28%. ¹H NMR (CDCl₃, 400 MHz): δ 0.82 (d, 6H), 2.05 (m, 1H), 2.73 (d, 2H), 4.29 (s, 2H), 5.15 (s, 2H), 5.21 (s, 2H), 6.98-7.97 (m, 12H), 8.91 (s, 1H), 9.01 (s, IH), 9.52 (s, 1H). ¹³C NMR (CDCl₃, 400 MHz): δ 22.40, 28.96, 40.16, 41.46, 45.60, 104.99, 105.36, 107.25, 115.12, 116.34, 121.60, 123.99, 125.44, 125.86, 126.12, 126.77, 128.24, 129.03, 129.27, 132.28, 133.99, 134.84, 141.20, 154.44, 159.90, 160.18, 162.11, 162.79, 164.61, 168.85, 170.71, 173.61, 173.88. HR-MS: m/z = 563 [M+H]+.
A mixture of compound 4e (0.40 mmol, 0.158 g), hydroxylamine (1.60 mmol, 0.11 g) and triethylamine (1.60 mmol, 0.2 ml) in ethanol (absolute, 200 proof, 10mL) was stirred under refluxing conditions until the TLC indicated completion of the reaction. The mixture was brought to room temperature and remove every ethanol under the reduced pressure. White solid are formed. With this solid 5 (0.40 mmol, 0.17 g), start reduction reaction. Dissolve solid 5 with methanol and add acetic anhydride (0.52 mmol, 0.05 ml). After five minutes, add potassium formate (1.60 mmol, 0.26g) and palladium/carbon (0.17 g). Heat the reaction solution and keep stirring overnight. By using diatomaceous earth, filter the reaction solution and evaporate every solvent. White compound 6 can be observed. With compound 6, start another coupling reaction. A mixture of compound 6 (0.40 mmol, 0.164 g), triethylamine (1.60 mmole, 0.2 ml) and Michael acceptor (0.52 mmol, 0.086 g) in ethanol (absolute, 200 proof, 10mL) was stirred under refluxing conditions until the TLC indicated completion of the reaction. The mixture was brought to room temperature and the formed precipitate was filtered by vacuum and rinsed with ice cold methanol (5 mL x 3). Yield: 51%. 1H NMR (CDCl3, 400 MHz): δ 0.68-0.69 (d, 6H), 1.21-1.24 (d, 6H), 1.91 (m, 1H), 2.83-2.85 (d, 2H), 3.42 (m, 1H), 5.19 (s, 4H), 7.03-7.86 (m, 7H), 8.85 (s, 1H), 8.92 (s, 1H), 9.54 (s, 1H). 13C NMR (CDCl3, 400 MHz): δ 21.16, 22.58, 28.71, 35.21, 40.06, 124.14, 125.54, 125.58, 126.27, 126.95, 127.50, 128.99, 159.95, 160.49. HR-MS: m/z = 515 [M+H]+.
A mixture of compound 4c (0.43 mmol, 0.15 g), hydroxylamine (1.72 mmol, 0.12 g) and triethylamine (1.72 mmol, 0.2 ml) in ethanol (absolute, 200 proof, 10mL) was stirred under refluxing conditions until the TLC indicated completion of the reaction. The mixture was brought to room temperature and evaporate every ethanol under the reduced pressure. White solid are formed. With this solid 5 (0.43 mmol, 0.16 g), start reduction reaction. Dissolve solid 5 with methanol and add acetic anhydride (0.56 mmol, 0.1 ml). After five minutes, add potassium formate (1.72 mmol, 0.15 g) and palladium/carbon (0.16 g). Heat the reaction solution and keep stirring overnight. By using diatomaceous earth, filter the reaction solution and evaporate every solvent. White compound 6 can be observed. With compound 6, start another coupling reaction. A mixture of compound 6 (0.43 mmol, 0.15 g), triethylamine (1.72 mmole, 0.2 ml) and Michael acceptor (0.65 mmol, 0.12 g) in ethanol (absolute, 200 proof, 10mL) was stirred under refluxing conditions until the TLC indicated completion of the reaction. The mixture was brought to room temperature and the formed precipitate was filtered by vacuum and rinsed with ice cold methanol (5 mL x 3). Yield: 37%. \(^1\)H NMR (CDCl\(_3\), 400 MHz): \(\delta\) 0.83 (d, 6H), 1.00 (d, 6H), 1.61 (s, 2H), 2.01 (m, 2H), 2.91 (d, 2H), 3.15 (d, 2H), 4.72 (s, 2H), 5.27 (s, 2H), 7.82 (m, 2H), 7.86 (m, 3H), 8.99 (s, 1H), 9.02 (s, 1H), 9.44 (s, 1H). \(^13\)C NMR (CDCl\(_3\), 400 MHz): \(\delta\) 14.10, 14.81, 22.79, 22.83, 25.80, 43.92, 48.82, 53.45, 121.61, 128.60, 129.29, 155.52, 157.58. HR-MS: \(m/z = 479\ [M+H]^+\).
2. 3. 5. Synthesis of 8-10

2-’amino-4-benzyl-4-’isobutyl-[2,5-’bipyrimidine]-5-carboxylic acid (8)

A mixture of compound 4c (0.58 mmol, 0.2 g) and sodium hydroxide (5.8 mmol, 0.23 g) in methanol (absolute, 200 proof, 10mL) was stirred under refluxing conditions. Check TLC and remove every solvent under the reduced pressure. Dissolve the white solid with water and add 1M HCl until pH reaches to 2–3. Extract the product with ethyl acetate and recrystallize the product with ether and ethyl acetate. The yield is 5 %.

1H NMR (MeOH, 400 MHz): δ 0.73-0.77 (d, 6H), 1.90-1.94 (m, 1H), 2.94-2.98 (d, 2H), 4.39 (s, 2H), 4.61 (s, 2H), 7.17-7.32 (m, 5H), 8.79-8.85 (t, 2H), 9.19 (s, 1H).

13C NMR (MeOH, 400 MHz): δ 21.57, 28.56, 40.93, 41.37, 43.99, 44.15, 104.98, 126.34, 126.51, 128.26, 128.36, 129.23, 129.32, 155.86, 156.01, 164.51. HR-MS: m/z = 364 [M+H]+.

4-benzyl-2-’(3-carboxypropanamido)-4-’isobutyl-[2,5-’bipyrimidine]-5-carboxylic acid (9)

Dissolve compound 8 (0.13 mmole, 0.05 g) with THF add succinic anhydride (0.13 mmole, 0.013 g) and triethylamine (0.26 mmole, 0.1 ml) into the same flask. Reflux the mixture and check TLC. Evaporate every solvent under the reduced pressure and perform the column to purify the compound 9. (ethyl acetate -> MeOH : ethyl acetate (1:4)). The yield is 37 %.

1H NMR (MeOH, 400 MHz): δ 0.66-0.67 (d, 6H), 0.82-0.84 (d, 2H), 1.01-1.03 (d, 2H), 1.80-1.85 (m, 1H), 2.84-2.86 (d, 2H), 4.54 (s, 2H), 6.93 (s, 2H), 7.13-7.29 (m, 5H), 8.68 (s, 1H), 8.94 (s, 1H).

13C NMR (MeOH, 400 MHz): δ 22.93, 23.16, 28.56, 29.82, 30.63, 32.82, 128.79, 129.90, 151.75, 174.72, 202.97, 203.02, 204.18, 205.50, 205.57, 205.59, 206.21. HR-MS: m/z = 464 [M+H]+.
A mixture of compound 7d (0.43 mmol, 0.2 g) and sodium hydroxide (5.8 mmol, 0.23 g) in methanol (absolute, 200 proof, 10mL) was stirred under refluxing conditions. Check TLC and remove every solvent under the reduced pressure. Dissolve the white solid with water and add 1M HCl until pH reaches to 2~3. Extract the product with ethyl acetate and recrystallize the product with ethyl acetate. The yield is 14%. $^1$H NMR (MeOH, 400 MHz): $\delta$ 0.73-0.77 (m, 12H), 0.91-0.93 (d, 2H), 1.08-1.13 (m, 1H), 1.29-1.30 (d, 2H), 1.89-1.99 (m, 1H), 4.72-4.76 (d, 2H), 4.39 (s, 1H), 4.61 (s, 1H), 7.13-7.31 (m, 5H), 9.21 (s, 1H), 9.31 (s, 1H), 9.35 (s, 1H). $^{13}$C NMR (MeOH, 400 MHz): $\delta$ 20.92, 21.57, 21.69, 28.53, 28.55, 28.60, 40.94, 41.28, 41.32, 41.39, 43.81, 43.98, 44.16, 126.22, 126.35, 126.50, 128.26, 128.35, 128.97, 129.00, 129.22, 129.32, 159.57, 160.80, 165.35. HR-MS: $m/z = 498.27$ [M+H]$^+$. 

2. 4. References


CHAPTER THREE:

SOLUTION-PHASE SYNTHESIS OF β-HAIRPIN PEPTIDE INHIBITING MULTIPLE MYELOMA CELLS ADHESION

3. 1. Introduction

Many classes of blood cells are formed from blood stem cells in bone marrow. Plasma cells, a type of white cell, can make antibodies which fight against infections. Plasma cells follow the normal cell cycle. When genes of plasma cells are changed, they turn into myeloma cells. In contrast to plasma cells, myeloma cells do not follow normal cell cycle. Overtime, myeloma cells outgrow plasma cells and form a mass of myeloma cells which is called a solitary plasma cytoma in bone marrow. This can result in invading bone tissue and spreading throughout the body.

Multiple myeloma (MM) is a cancer of plasma cells. Even though treatment for multiple myeloma (MM) has been highly improved, the disease still remains incurable because myeloma cells can develop drug-resistance by being exposed to chemotherapy over long periods of time. There have been numerous studies of both solid and hematological cancer cells from different tissues showed drug resistance (1-8). Even though dominant or multiple factors are involved in multidrug resistance, a few drug transporters (e.g., P-glycoprotein, multidrug resistance-associated protein, breast cancer resistant protein) and proteins which control or regulate cell growth or cell death (e.g., Bcl-2 family) as cellular drug-resistant factors were studied intensively (9). Despite of
the fact that current standard therapy for cancers, including MM, is targeting apoptotic pathway, these studies suggested that activating multiple cell death pathways may avoid cancer drug resistance and cure cancers better (10). Also, Jason et al found out cell-adhesion mediated drug resistance (CAM-DR) of multiple myeloma cells is not related to two well-known factors of drug resistance, active drug transport and increased expression of Bcl-2 family members which activate the apoptotic pathway (11).

There are three different types of cell death based on the morphological appearance (12). According to characteristic morphological aspects, apoptosis (type 1 cell death) shows nuclear pyknosis (chromatin condensation) and karyorthexis (nuclear fragmentation) (13). Autophagy (type 2 cell death) includes the massive autophagic vacuolization and the digestion of partial cytoplasm by lysosomal hydrolases (14). Necrosis (type 3 cell death) is characterized by rupture of the plasma membrane by deflation of cell volume and the random dismantle of swollen organelles. Currently, most conventional anticancer drug discovery strategy is inducing apoptosis because of plethora knowledge of molecular mechanism of apoptosis regulation (15). A delicate balance between proapoptotic and antiapoptotic proteins of apoptosis pathways plays an important role to control the survival of long-lived cells and termination of short-lived cells in a number of tissues, including the bone marrow. However, imbalance of proapoptotic and antiproptotic regulators causes various diseases. Especially, for cancers, cells which facilitate neoplasia and malignance can prolong their survival by thwarting action of antiapoptotic proteins. Although in a variety of accumulating evidences validate that apoptosis induction can be a potential drug discovery strategy, it is necessary to optimize selectivity of compounds which interact with more than two members of apoptosis protein families along with improving pharmacokinetic
properties. This disadvantage is another reason to induce alternative cell death for anti-cancer therapy.

Previously, it was reported that HYD-1 (kikmviswkg), a synthetic D-amino acid peptide, can act as a ligand mimetic and block tumor cell adhesion (16). This cascades preventing migration of prostate tumor cells on laminin 322 (laminin 5) and changing the cellular signals coming from a laminin 322 (laminin 5) matrix (17, 18). Later, Rajesh et al discovered HYD-1 can block binding $\alpha_4\beta_1$ integrin-dependent multiple myeloma cells to extracellular matrix, fibronectin, as well as induce cell death in H929, 8226, and U266 multiple myeloma (MM) cell lines but not in CD34+ normal hematopoietic progenitor cells or surrounding blood mono-nuclear cells (19). HYD-1 induced necrosis (type 3 cell death) in MM cells that is different from apoptosis or autophagy by observing the loss of $\Delta\psi_m$, total cellular ATP decrease, increased ROS expression. However, because of modest antitumor activity of HYD-1 ($IC_{50} = 33 \mu M$) in vivo, it was necessary to modify HYD-1 to improve bioavailability or antitumor activity (e.g. PEGylation, cyclization).

![Figure 35. Structure of HYD-1.](image-url)
Hazlehurst and co-workers discovered that MVISW were the minimal elements of HYD-1 for bioactivity after creating N- and C-terminal deletion peptides and replacement isoleucine to valine increased bioactivity. Based on these findings, first modification was designing β-hairpin structure by applying MVVSW in the recognition strand and KLKLK in the non-recognition strand (20). Advantages of β-hairpin design that contribute to better bioactivity include: (1) amphiphilic functionality and (2) rigid conformational structure. After a structure-activity relationship (SAR) study, methionine (M) was changed to norleucine and serine (S) was replaced by alanine (A). For further optimization, conformational search and energy minimization for β-turn promoters were studied by using the macromodel and the GLIDE software distributed by Schrodinger, Inc.. This result indicated that ether-peptidomimetic proline derivative at one turn and methylsulfonamide amino ethyl glycine at the other turn showed an impressive bioactivity. By applying every optimization condition, a cyclic peptide referred as MTI-101 showed 1.08 μM (=IC₅₀) bioactivity after TOPRO3 assay.

Figure 36. β-Hairpin structure.

Due to the promising bioactivity of MTI-101 in vitro, it is necessary to produce a massive amount of MTI-101 to investigate whether the same effect will reproduce in
vivo. The mass production of peptides can be carried out through either continuous solid-phase synthesis or conventional solution-phase synthesis. Each method has its own disadvantages, for example, classic solution-phase synthesis needs to isolate and purify intermediates and continuous solid-phase synthesis demands to handle large amount of pricy resins or large excess of reagents. In order to scale up the production of the biologically active peptides successfully, it seems better to combine the strong aspects of both methods to overcome their disadvantages (21).

In this study, solution-phase synthesis which has advantages of solution-phase and solid-phase methods was examined for the production of MTI-101. We approached in two different ways: (1) combination of recognition strand and non-recognition strand and (2) continuous solution-phase synthesis. The data suggested the possibility of larger-scale production for MTI-101 with simple purification and less use of reactants or reagents in solution phase.

![Figure 37. Structure of MTI-101.](image)

<table>
<thead>
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<th>Advantages</th>
<th>Disadvantages</th>
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Table 7. Comparison of Solid-phase Synthesis and Solution-phase Synthesis
### Results and discussion

Previously, continuous solution methods have utilized Fmoc- chemistry to build small-scaled short peptides which consist of six to eight amino acids (22). Because of inconvenience of by-product removal after Fmoc- deprotection, in this study, Cbz- or Boc- protecting groups were used. In addition, in contrast to solid-phase approach, the reaction can be monitored and analyzed at any stage. MALDI-TOF and LC-MS techniques were used for the analysis.
Synthesis of MTI-101 was approached in two different ways. One is recognition strand and non-recognition strand were synthesized separately and combined later. The other is the whole peptide structure was built in a continuous way. We investigated the former case first since short peptides of six to eight amino acid units were synthesized successfully in solution (22).

Before building the recognition strand, β-turn promoter was synthesized. Scheme 6 shows synthesis procedures of β-turn promoter, derivative of ether-peptidomimetic proline. Boc-D-proline was reduced to Boc-D-prolinol by using NaBH₄. S_N₂ reaction was followed to convert Boc-D-prolinol to Boc-D-pyrrolidine derivative. For the next coupling, Boc- group was deprotected selectively under 0 °C. The sequence of recognition strand (WAVVN) was attached to the β-turn promoter. Three different coupling reagents were used for the better yield. In order to proceed the next coupling, Cbz- group was deprotected in the presence of 10 % Pd/C and high N₂ (55 psi). After each coupling, the product was purified by column chromatography. The final deprotection step was completed via treatment with TFA (Scheme 7).

Building non-recognition strand was started with the synthesis of methylsulfonamide amino ethyl glycine linker. Excess of 1,2-ethylenediamine was reacted to di-tert-butyl dicarbonate to give one side protected product. In the next step, the unprotected amino
group attacked sulfur atom of methanesulfonyl chloride in a fashion of $S_N2$ reaction. Due to slightly more nucleophilic property of nitrogen of methylsulfonamide side, nucleophilic substitution reaction occurred only one side in the presence of methyl bromoacetate and NaH. The Boc-group was deprotected for the next coupling (Scheme 8). Scheme 9 shows all the coupling and deprotection steps for non-recognition strand. Five amino acids were coupled to the linker by using T$_3$P in an order of K(Boc)LK(Boc)LK(Boc). Boc-protected lysine was used to avoid side reactions in coupling and deprotection steps. Deblocking Cbz-group was processed under 10% Pd/C and H$_2$ (55 psi). After every coupling, the pure product was obtained through recrystallization in ether (Scheme 9).

The last step for MTI-101 synthesis is cyclization of recognition strand and non-recognition strand in solution. Linear peptide was formed between -NH$_2$ from non-recognition strand and -COOH from recognition strand under the condition of T$_3$P, DIEA, and anhydrous THF. For the final cyclization, Cbz-deprotection was proceeded earlier than that of methyl ester group. Because free carboxylic acid was transformed back into methyl ester in the presence of excess methanol which is solvent for the Cbz-deprotection step. After Cbz-deprotection, methyl ester group was deblocked by using 5% lithium hydroxide. Cyclization of linear peptide occurred via treatment with T$_3$P. The pure product was obtained as precipitation in ether. The final Boc-deprotection of MTI-101 was completed with TFA/DCM (Scheme 10).
Scheme 10. Cyclization between recognition strand and non-recognition strand.

Even though cyclization of recognition strand and non-recognition strand was successful in solution, this approach suffered from economical problem. Because recognition strand synthesis demands column chromatograph purification after every coupling. This would eventually cost more for the large-scale peptide synthesis. In order to overcome this deficiency, continuous solution-phase synthesis was examined since every product of non-recognition strand was able to be recrystallized in ether. Before launching continuous solution-phase synthesis, it is necessary to modify protecting group from previously synthesized β-turn promoter due to involvement of Boc-protected lysine. Instead of Boc-D-proline, Cbz-D-proline was reduced to Cbz-D-prolinol in the presence of N-methylmorpholine, isobutyl chloroformate, and NaBH₄. Cbz-D-prolinol was transformed into Cbz-pyrrolidine derivative by using tert-butyl
bromoacetate. tert-Butyl group was deprotected for the coupling in two different ways: (1) TFA/DCM and (2) H$_3$PO$_4$/toluene. Even though the combination of TFA and DCM is the typical method for deblocking Boc-group, coupling reagent, T$_3$P, enabled trifluoroacetic acid active and caused uncontrolled side reaction. Moreover, this side product was impossible to remove after recrystallization (Scheme 11).

\[
\begin{align*}
\text{a} : \text{isobutyl chloroformate, N-methyl morpholine, NaBH}_4, \text{THF}, & \quad \text{b} : \text{-butyl bromoacetate, TBAI, 30\% NaOH, toluene,} \\
\text{c} : \text{TFA, DCM, d} : \text{H}_3\text{PO}_4, \text{toluene} \\
\end{align*}
\]


In the beginning, continuous solution-phase synthesis followed the same procedure of non-recognition strand synthesis. A sequence of K(Boc)LK(Boc)LK(Boc) was coupled to the linker by using combination of N-methylmorpholine and isobutylchloroformate or T$_3$P as coupling reagents. Except percent yield of dipeptide 2, the most of percent yields were comparable for both coupling reagents. This is because the linker contained trifluoroacetic acids as counter ions after Boc-deprotection and they were activated by T$_3$P. This resulted in undesirable reactions and decreased percent yield. Between coupling steps, Cbz-group was deprotected under the condition of Pd/C and H$_2$ or Pd/C and triethylsilane. A combination of Pd/C and triethylsilane made Cbz-deprotection faster, however, the efficiency of deblocking completion decreased as the peptide became longer. After hexapeptide 6,
two more couplings (β-turn promoter and tryptophan) were proceeded. However, because of facing a couple of problems, for example, side reactions or low percent yield, it is implausible to proceed the rest of coupling and cyclization. (Scheme 12, Table 6, 7)

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*T₃P as coupling reagent

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*N-methylmorpholine and isobutylchloroformate as coupling reagent


In summary, it is shown the possibility of MTI-101 synthesis in solution by using two different approaches: (1) cyclization of recognition strand and non-recognition strand and (2) continuous synthesis. In the former case, synthesis of MTI-101 was successful in solution, however, column chromatography purification was necessary for
recognition strand that causes more cost for the large-scale synthesis. In the latter case, even though products after each coupling were recrystallized in ether, synthesis of MTI-101 was unable to be completed due to side reactions or low yield. Large-scale synthesis of MTI-101 would be refined by using appropriate coupling reagents or methods.

3. 3. Experimental section

3.3.1. General comments

All peptides were identified by mass spectral analysis (Bucker) and all new compounds showed consistent $^1$H, $^{13}$C NMR (INOVA-400), and high resolution mass spectral data (Agilent 1100 series).

3.3.2. β-turn promoter synthesis

3.3.2.1. Tert-butyl-2-((2-tert-butoxy-2-oxoethoxy)methyl)pyrrolidine-carboxylate

N-Boc-D-prolinol (1 g, 4.96 mmol) was dissolved in 20 ml of toluene and cooled down to 0 °C. Tetrabutylammonium iodide (0.92 g, 2.49 mmol), 30 % NaOH solution (12 ml, 90 mmol), and tert-butylbromoacetate (1.46 ml, 9.93 mmol) were added subsequently into the cooled solution. The reaction mixture was brought to room temperature after 5 hours and left overnight. 20 ml of toluene was added and the mixture was extracted. The organic layer was washed with 1 N HCl (15 ml) and brine (15 ml). The organic layer was concentrated after being dried over MgSO$_4$. Purification by flash column chromatography (Hexane-EtOAc, 3:1) gave a light yellow colored oil (1.41 g, 90 %). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ = 1.46 (s, 9 H), 1.47 (s, 9H), 1.80-1.82 (m, 1H), 1.91-1.93 (m, 2H), 2.05-2.07 (m, 1H), 3.32-3.34 (m, 2H), 3.52-3.54 (m, 1H), 3.60-3.63 (m, 1H),
3.87-3.89 (m, 1H), 3.96 (s, 2H); $^{13}$C NMR (400 MHz, CDCl$_3$): $\delta$ 27.86, 28.04, 28.34, 28.76, 56.58, 69.25, 175.3; HRMS: $m/z$ calcd for C$_{16}$H$_{29}$NO$_5$ [M + H$^+$], 316.2046; found, 316.2007.

3.3.2.2. Benzyl-2-((2-tert-butoxy-2-oxoethoxy)methyl)pyrrolidine-carboxylate

N-methylmorpholine (1.3 ml, 12.1 mmol) and isobutylchloroformate (1.6 ml, 12.1 mmol) were added to a solution of Cbz-proline (3.01 g, 12.1 mmol) in 30 ml at 0 °C. After 10 minutes, a white precipitation was removed by filtration and filtrate was transferred to three neck flask. Solution of NaBH$_4$ (0.69 g, 18.1 mmol) in 10 ml water was mixed slowly to the cool filtrate while purging Ar gas. TLC indicated the completion of reduction after 1 hour. THF was removed under reduced pressure and water was added to destroy excess of NaBH$_4$. Product was extracted with ethyl acetate (3×10 ml), washed with brine, and dried over Na$_2$SO$_4$. After evaporation of ethyl acetate, light yellow oil was obtained. N-Cbz-D-prolinol (2.84 g, 12.1 mmol) was dissolved in 20 ml of toluene and cooled down to 0 °C. Tetrabutylammonium iodide (2.23 g, 6.05 mmol), 30 % NaOH solution (29 ml, 218 mmol), and tert-butylbromoacetate (3.6 ml, 24.2 mmol) were added subsequently into the cooled solution. The reaction mixture was brought to room temperature after 5 hours and left overnight. 20 ml of toluene was added and the mixture was extracted. The organic layer was washed with 1 N HCl (15 ml) and brine (15 ml). The organic layer was concentrated after dried over MgSO$_4$. Purification by flash column chromatography (Hexane-EtOAc, 4:1) gave a light yellow colored oil (2.96 g, 70 %). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ = 1.47 (s, 9H), 1.81-1.84 (m, 1H), 1.90-1.96 (m, 2H), 2.07-2.11 (m, 1H), 3.39-3.42 (m, 2H), 3.57-3.58 (m, 1H), 3.58-3.59 (m, 1H), 3.89 (s, 1H), 3.97 (s, 1H), 4.01-4.02 (m, 1H), 5.08-5.18 (m,
2H), 7.28-7.38 (m, 5H); $^{13}$C NMR (400 MHz, CDCl$_3$): $\delta$ 23.15, 24.08, 28.17, 28.34, 28.94, 46.98, 47.26, 56.59, 57.24, 66.81, 67.02, 68.70, 69.24, 71.76, 72.38, 76.73, 76.94, 77.04, 77.26, 77.36, 77.46, 77.57, 81.76, 82.10, 128.01, 128.15, 128.68, 137.16, 155.19, 169.92; HRMS: m/z calcd for C$_{16}$H$_{29}$NO$_5$ [M + H$^+$], found.

3.3.2.3. Tert-butyl-2-((2-tert-butoxy-2-oxoethoxy)methyl)pyrrolidine-1-carboxylic acid

Tert-butyl-2-((2-tert-butoxy-2-oxoethoxy)methyl)pyrrolidine-carboxylate (1.41g, 4.47 mmol) in 10 ml of DCM was cooled down to 0 °C and 3 ml of trifluoroacetic acid was added to the cooled solution. Reaction mixture was kept at 0 °C for 5 hours. Mixture was extracted by DCM (10 ml x 3) after excess TFA was neutralized by 6 M KOH. The combined organic layers were washed with brine (10 ml) and dried over MgSO$_4$. After concentration, light yellow oil was obtained (0.91g, 95%).

3.3.2.4. 2-((1-((benzyloxy)carbonyl)pyrrolidin-2-yl)methoxy)acetic acid

i) Benzyl-2-((2-tert-butoxy-2-oxoethoxy)methyl)pyrrolidine-carboxylate (2.96g, 8.46 mmol) in 10 ml of DCM was cooled down to 0 °C and 3 ml of trifluoroacetic acid was added to the cooled solution. Reaction mixture was kept at 0 °C for 5 hours. After concentration, a light yellow oil was obtained. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ = 1.54-1.58 (m, 1H), 1.79-2.00 (m, 3H), 3.33-3.47 (m, 4H), 4.05 (s, 2H), 4.24-4.28 (m, 1H), 4.96-5.09 (dd, $J$ = 0.05, 2H), 7.27-7.35 (m, 5H); $^{13}$C NMR (400 MHz, CDCl$_3$): $\delta$ 24.02, 27.89, 29.97, 46.71, 56.79, 67.74, 68.33, 75.31, 76.93, 77.24, 77.56, 114.15, 117.02, 127.90, 128.37, 128.70, 136.14, 157.23, 161.89, 162.28, 162.27, 163.06, 175.80; HRMS: m/z calcd for C$_{15}$H$_{19}$NO$_5$ [M + H$^+$], 294.1336; found, 294.1344.
ii) Benzyl-2-((2-tert-butoxy-2-oxoethoxy)methyl)pyrrolidine-carboxylate (0.70 g, 1.99 mmol) in 1 ml of toluene was added to 1 ml of 85% phosphoric acid of another flask. Reaction mixture was kept overnight. 5 ml of water added to reaction mixture and product was extracted with ethyl acetate. The combined organic layer was washed with brine and dried over Na$_2$SO$_4$. After concentration and flush column chromatography (Hexane:ethyl acetate=1:2), a viscous light yellow oil was obtained (0.41 g, 70%).

### 3.3.3. Recognition strand synthesis

#### 3.3.3.1. β-turn promoter(O-t-Bu)-Trp-NH(Cbz)

Solution of Tert-butyl-2-((2-tert-butoxy-2-oxoethoxy)methyl)pyrrolidine-1-carboxylic acid (1.19 g, 5.53 mmol) and DIEA (2.3 ml, 13.83 mmol) was prepared in anhydrous DCM (10 ml) under argon gas. N-Cbz-Trp-OH (1.87 g, 5.53 mmol) and Propylphosphonic Anhydride (T$_3$P, 3.52 g, 11.06 mmol) were added to previously prepared solution. Leave the reaction mixture at room temperature overnight while purging argon gas. The reaction was quenched with water (10 ml) and extracted with DCM (10 ml x 3). The combined organic layers were washed with brine (10 ml), dried over MgSO$_4$, and concentrated. After flash column chromatography (Hexane-EtOAc, 1:1), a light yellow crystal was obtained (2.375 g, 80%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta = 1.44$ (s, 9 H), 1.66-1.73 (m, 2H), 1.82-1.93 (dd, $J = 0.09, 0.02, 2H$), 2.48-2.55 (dd, $J = 0.05, 0.02, 1H$), 3.06-3.12 (dd, $J = 0.05, 0.02, 1H$), 3.12-3.19 (m, 2H), 3.44-3.47 (m, 1H), 3.58-3.59 (m, $J = 0.01, 0.01, 1H$), 3.66-3.68 (m, 1H), 3.89 (s, 2H), 4.67-4.73 (q, $J = 0.02, 1H$), 5.10 (s, 2H), 7.04-7.38 (m, 10H); $^{13}$C NMR (400 MHz, CDCl$_3$): $\delta$ 23.63, 2.51, 28.33, 42.25, 47.07, 53.96, 57.18, 66.97, 69.10, 70.90, 76.68, 81.78, 110.93, 111.26,
3.3.3.2. β-turn promoter(O-t-Bu)-Trp-NH₂

Lin-Trp-NH(Cbz) (2.375 g, 4.43 mmol) and palladium carbon powder (10 %) was added to methanol (20 ml). Leave mixture under high H₂ pressure (48 psi) for 20 hours. Excess palladium carbon powder was removed over diatomaceous earth. White crystal was obtained after methanol removal under reduced pressure (1.677 g, 94 %).

3.3.3.3. β-turn promoter(O-t-Bu)-Trp-Ala-NH(Cbz)

Lin-Trp-NH₂ (2.375 g, 4.43 mmol) was dissolved in 10 ml of anhydrous DCM under argon gas. Subsequently, Cbz-Ala-OH (0.99 g, 4.43 mmol), DIEA (1.8 ml, 11.08 mmol), and Propylphosphonic Anhydride (T₃P, 5.64 g, 8.86 mmol) were added to previous solution. Reaction mixture was stirred for 22 hours while purging argon gas. The crude product was extracted with DCM (10 ml x 3) after 10 ml of water was added for quenching excess T₃p. The combined organic layers were washed with brine (10 ml), dried over MgSO₄, and concentrated. Purification by flash column chromatography (Hexane-EtOAc, 1:2) gave a light yellow crystal (1.935 g, 72 %). ¹H NMR (400 MHz, CDCl₃): δ = 1.33-1.35 (d, J = 0.02, 3H), 1.44 (s, 9 H), 1.66-1.73 (m, 2H), 1.82-1.93 (dd, J = 0.09, 0.02, 2H), 3.16-3.18 (d, J = 0.02, 2H), 3.28-3.30 (m, 2H), 3.50-3.51 (m, 1H), 3.56-3.57 (m, 1H), 3.89 (s, 2H), 3.99-4.01 (m, 1H), 6.96-7.36 (m, 10H); ¹³C NMR (400 MHz, CDCl₃): δ = 23.63, 2.51, 28.33, 42.25, 47.10, 47.12, 52.51, 57.24, 69.04, 70.82, 81.82, 110.87, 111.28, 118.95, 119.89, 122.46, 123.28, 128.42, 128.76, 136.15, 170.28 ; HRMS : m/z calcd for C₃₃H₄₂N₄O₇ [M + H⁺], 607.3053; found, 607.3120.
3.3.3.4. β-turn promoter(O-t-Bu)-Trp-Ala-NH$_2$

Lin-Trp-Ala-NH$_2$ (1.935 g, 3.19 mmol) and 10 % palladium carbon powder were added to methanol and kept under high H$_2$ pressure (53 psi) overnight. Excess palladium carbon powder was filtered over diatomaceous earth. Concentration of filtrate gave a white crystal (1.357 g, 90 %).

3.3.3.5. β-turn promoter(O-t-Bu)-Trp-Ala-Val-NH(Cbz)

Solution of Lin-Trp-Ala-NH$_2$ (2.68 g, 5.67 mmol) was prepared in anhydrous DCM (10 ml) while purging argon gas. Cbz-Val-OH (1.14 g, 4.54 mmol), DIEA (1.5 ml, 9.08 mmol), and Propylphosphonic Anhydride ($T_3P$, 5.77 g, 9.08 mmol) were added to previously prepared solution. Reaction mixture was under dry condition overnight and quenched with water (10 ml). Crude product was extracted with DCM (15 ml x 3), washed with brine (15 ml), and dried over Na$_2$SO$_4$. Purification by column flash chromatography (pure ethylacetate) gave a white crystal (3.207 g, 80 %). $^1$H NMR (400 MHz, CDCl$_3$): δ = 0.89-0.91 (d, J = 0.02, 6H), 1.44 (s, 9 H), 2.05-2.07 (d, J = 0.02, 3H), 2.62-2.68 (m, 2H), 3.01-3.03 (m, 1H), 3.16-3.17 (d, J = 0.01, 2H), 3.29-3.31 (m, 2H), 3.50-3.51 (m, 1H), 3.89 (s, 2H), 3.95-4.01 (m, 2H), 4.48-4.52 (t, J = 0.02, 1H), 4.87-4.92 (q, J = 0.02, 1H), 5.12 (s, 2H), 5.44-5.46 (d, J = 0.02, 1H), 7.06-7.36 (m, 10H); $^{13}$C NMR (400 MHz, CDCl$_3$): δ = 19.50, 23.77, 27.23, 28.33, 29.02, 31.44, 47.12, 49.15, 52.52, 57.25, 60.53, 67.34, 69.03, 70.84, 76.68, 81.84, 110.68, 111.35, 118.85, 119.84, 122.43, 123.38, 128.29, 128.41, 128.77, 136.18, 169.88, 170.17, 171.38; HRMS: m/z calcd for C$_{38}$H$_{51}$N$_5$O$_8$ [M + H$^+$], 706.3771; found, 706.3975.
3.3.3.6. $\beta$-turn promoter(O-t-Bu)-Trp-Ala-Val-NH$_2$

Lin-Trp-Ala-Val-NH(Cbz) (3.207 g, 4.54 mmol) was dissolved in methanol (20 ml) and palladium carbon powder was added. The reaction mixture was left under H$_2$ (53 psi) overnight and filtered over diatomaceous earth. A white crystal (2.464 g, 95 %) was obtained after filtrate was concentrated.

3.3.3.7. $\beta$-turn promoter(O-t-Bu)-Trp-Ala-Val-NH(Cbz)

Lin-Trp-Ala-Val-NH$_2$ (2.169 g, 3.80 mmol) and DIEA (1.3 ml, 7.60 mmol) were dissolved in 10 ml of anhydrous DCM under argon gas. Cbz-Val-OH (0.95 g, 3.80 mmol) and Propylphosphonic Anhydride (T$_3$P, 4.84 g, 7.60 mmol) were added to previously prepared mixture. The reaction was left overnight while purging argon gas. Excess Propylphosphonic Anhydride (T$_3$P) was quenched with water (15 ml) and crude product was extracted with DCM (15 ml x 3). The combined organic layers were washed with brine (15 ml), dried over Na$_2$SO$_4$, and concentrated. Purification by flash column chromatography (ethylacetate-methanol; 20:1) gave a white crystal (2.326 g, 76 %). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ = 0.90-0.96 (d, $J = 0.02$, 12H), 1.43 (s, 9 H), 1.46-1.48 (d, $J = 0.02$, 3H), 1.84-1.93 (m, 2H), 2.08-2.16 (m, 2H), 2.58-2.60 (m, 1H), 3.15-3.17 (d, $J = 0.02$, 2H), 3.22-3.29 (m, 1H), 3.41-3.45 (m, 2H), 3.54-3.61 (m, 2H), 3.55 (s, 2H), 3.99-4.01 (m, 1H), 4.11-4.18 (m, 1H), 4.34-4.38 (m, 1H), 4.74-4.76 (m, 1H), 4.91-4.95 (q, $J = 0.02$, 1H), 5.10 (s, 1H), 7.04-7.53 (m, 10H); $^{13}$C NMR (400 MHz, CDCl$_3$): $\delta$ = 18.32, 18.34, 18.47, 18.48, 19.48, 19.49, 23.69, 24.43, 27.18, 28.33, 29.28, 31.40, 47.12, 48.46, 56.53, 57.18, 58.81, 60.84, 67.13, 67.21, 69.00, 69.20, 70.88, 76.67, 81.79, 110.68, 111.31, 119.80, 122.37, 127.71, 128.17, 128.33, 128.74, 136.14, 136.62, 156.78, 169.87, 170.35, 170.84, 171.62, 171.97, 172.00; HRMS: $m/z$ calcd for C$_{43}$H$_{60}$N$_{6}$O$_{9}$ [M + H$^+$], 805.4455; found, 805.4518.
3.3.3.8. $\beta$-turn promoter(O-t-Bu)-Trp-Ala-Val-Val-NH$_2$

The solution of pentamer (2.558 g, 3.18 mmol) was prepared in methanol and palladium carbon powder was added. The reaction mixture was left under H$_2$ (53 psi) for one day and filtered over diatomaceous earth. Concentration of filtrate gave a white crystal (1.937 g, 91 %).

3.3.3.9. $\beta$-turn promoter(O-t-Bu)-Trp-Ala-Val-Nlle-NH(Cbz)

Lin-Trp-Ala-Val-Val-NH$_2$ (1.937 g, 2.89 mmol) and DIEA (7.23 mmol, 1.2 ml) were added to 10 ml of anhydrous DCM under argon gas. Cbz-Nlle-OH (0.77 g, 2.89 mmol) and Propylphosphonic Anhydride (T$_3$P, 1.83.7 g, 5.78 mmol) were added to previously prepared solution. The reaction mixture was kept overnight while purging argon gas. Excess T$_3$P was quenched with water (15 ml) and crude product was extracted with DCM (15 ml x 3). The combined organic layers were washed with brine (15 ml), dried over Na$_2$SO$_4$, and concentrated. Recrystallization with Et$_2$O gave a white crystal (2.406 g, 91 %). HRMS : m/z calcd for C$_{49}$H$_{71}$N$_7$O$_{10}$ [M + H$^+$], 918.5296; found, 918.6535.

3.3.3.10. $\beta$-turn promoter(OH)-Trp-Ala-Val-Nlle-NH(Cbz)

Lin(O-t-Bu)-Trp-Ala-Val-Nlle-NH(Cbz) ( 2.406 g, 2.62 mmol) and trifluroacetic acid (TFA, 3 ml) were added to 10 ml of DCM. The mixture was kept overnight, neutralized with 6 M KOH, and extracted with DCM (15 ml x 3). The combined organic layers were washed with brine (15 ml) and dried over Na$_2$SO$_4$. Evaporation of DCM gave a white crystal (2.123 g, 94 %). C$_{45}$H$_{63}$N$_7$O$_{10}$ [M - H$^+$], 860.4564; found, 860.4544.
3.3.4. Synthesis of linker

3.3.4.1. Tert-butyl-2-aminoethylcarbamate

Ethylenediamine (55.6 mL, 831 mmol) was dissolved in THF (200 mL). A solution of Boc₂O (20 g, 91.6 mmol) in 200 mL of THF was added dropwise at 0°C overnight. The resulting solution was concentrated, dissolved in DCM (100 mL), and extracted with water (3x20 mL). The combined water layer was back-extracted with DCM (100 mL), and the organic layers were combined and dried over anhydrous Na₂SO₄. After concentrated, a yellow oil was left (14.52 g, 99%).

3.3.4.2. Tert-butyl (2-(methylsulfonamido)ethyl)carbamate

Tert-butyl-2-aminoethylcarbamate (11.19 g, 69.8 mmol) was dissolved in 100 mL of THF. Triethylamine (11.68 mL, 83.8 mmol), and methanesulfonyl chloride (5.41 mL, 69.8 mmol) were added and kept at room temperature for one hour. The reaction mixture was concentrated, extracted in ethyl acetate (EtOAc, 3x25 mL), and washed with brine (10 mL). The organic layer was concentrated, resulting in an off-white solid. Recrystallization in diethyl ether gave white solids (15.11 g, 90.8%). ¹H NMR (400 MHz, CDCl₃): δ = 1.40 (s, 9H), 2.93 (s, 3H), 3.18-3.27 (m, 4H); ¹³C NMR (400 MHz, CDCl₃): δ = 28.58, 40.35, 40.80, 40.95, 43.67, 79.62, 79.98, 156.74; HRMS: m/z calcd for C₄₃H₆₀N₆O₉ [M + H⁺], 239.106; found, 239.105.

3.3.4.3 Methyl 2-(N-(2-(tert-butoxycarbonylamino)ethyl)methylsulfonamido)acetate

Tert-butyl-(2-(methylsulfonamido)ethyl)carbamate (15.11 g, 63.4 mmol) was dissolved in 100 mL of anhydrous THF. 60% sodium hydride (2.54 g, 63.4 mmol) was added to the previous prepared solution while purging Ar gas. After 15 minutes, addition of methyl bromoacetate (5.83 mL, 63.4 mmol) was followed and the solution was stirred
for one hour. The solution was quenched with water (10 mL). The resulting solution was concentrated, dissolved in EtOAc (75 mL), and extracted five times with water (10 mL). The organic layer was concentrated, and the crude product was recrystallized in EtOAc:Hexane (1:1), producing a white solid (14.96 g, 76.0%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta = 1.38$ (s, 9H), 3.21 (s, 3H), 3.22-3.25 (m, 2H), 3.32-3.35 (m, 2H), 3.71 (s, 3H), 4.10 (s, 2H); $^{13}$C NMR (400 MHz, CDCl$_3$): $\delta = 28.55$, 38.89, 39.79, 47.81, 48.57, 52.63, 79.72, 156.27, 170.54; HRMS: m/z calcd for C$_{43}$H$_{60}$N$_6$O$_9$ [M + H$^+$], 311.127; found, 311.128.

3.3.4.4. Methyl-N-(2-aminoethyl)-N-(methylsulfonyl)glycinate

Methyl-N-(2-aminoethyl)-N-(methylsulfonyl)glycinate (2.00 g, 6.44 mmol) was dissolved in 10 ml of DCM. Trifluoroacetic acid (3 mL) was added and allowed to stir overnight. The reaction mixture was concentrated and resulting in a yellow oil (2.02 g, 96.5%).

3.3.5. Synthesis of non-recognition strand (Contributed by Dr. Philip Murray)

3.3.5.1. Linker(OMe)-Lys(Boc)-NH(Cbz)

The deprotected linker (2.02 g, 6.22 mmol) was dissolved in anhydrous DCM (40 mL) under argon. Cbz-lysine(Boc)-OH (2.37 g, 6.22 mmol), 50% propanephosphonic anhydride (7.92 mL, 12.4 mmol) and DIEA (2.57 mL, 16.0 mmol) were added subsequently. The reaction mixture was allowed to stir overnight. The resulting solution was quenched with water (10 mL), concentrated and dissolved in ethyl acetate (50 mL). The filtrate was extracted three times with water (10 mL). The organic layer was concentrated, and the crude product was recrystallized in ether (10 mL), producing a white solid (1.93 g, 54.6%). m/z [M+H]$^+$ 573.1.
3.3.5.2. Linker(OMe)-Lys(Boc)-NH$_2$

Linker(OMe)-Lys(Boc)-NHCbz (1.93 g, 3.38 mmol) was dissolved in methanol. 10 % Palladium carbon powder was added, and the solution was placed under high H$_2$ pressure (55 psi) overnight. The palladium carbon powder was filtered over diatomaceous earth, and the resulting solution was concentrated, forming a white solid.

3.3.5.3. Linker(OMe)-Lys(Boc)-Leu-NH(Cbz)

The deprotected dimer (1.48 g, 3.38 mmol) was dissolved in anhydrous DCM under argon. DIEA (1.40 mL, 8.44 mmol), Z-Leu-OH (0.896 g, 3.38 mmol), and 50% propanephosphonic anhydride (T$_3$P, 4.30 mL, 6.75 mmol) were added and allowed to stir overnight. The reaction mixture was quenched with water, concentrated and dissolved in EtOAc (50 mL). The organic layer was washed three times with water (10 mL), the organic layer was concentrated, and the resulting crude product was recrystallized in ether (10 mL) overnight, producing a white solid (1.99 g, 86.3%). m/z [M+H]$^+$ 686.34.

3.3.5.4. Linker(OMe)-Lys(Boc)-Leu-NH$_2$

Linker(OMe)-Lys(Boc)-Leu-NH(Cbz) (1.99 g, 2.91 mmol) was dissolved in methanol. 10 % Palladium carbon powder was added, and the solution was placed under H$_2$ pressure (55 psi) overnight. The palladium carbon powder was filtered over diatomaceous earth, and the resulting solution was concentrated, forming a white solid.
3.3.5.5. Linker(OMe)-Lys(Boc)-Leu-Lys(Boc)-NH(Cbz)

The deprotected product (1.60 g, 2.91 mmol) was dissolved in anhydrous DCM (10 mL) under argon. Z-Lys(Boc)-OH (1.11 g, 2.91 mmol), 50% propanephosphonic anhydride (T₃P, 3.70 mL, 5.81 mmol), and DIEA (1.20 mL, 7.27 mmol) were added at 0°C and allowed to stir overnight. The reaction mixture was quenched with water (5 mL) for 1 hr, then concentrated and dissolved in EtOAc (50 mL). The organic solution was extracted three times with water (10 mL). The organic layer was dried over Na₂SO₄ and concentrated. The crude product was recrystallized in ether (10 mL) overnight, producing a white solid (2.19 g, 82.7%). m/z [M+H]⁺ 914.49.

3.3.5.6. Linker(OMe)-Lys(Boc)-Leu-Lys(Boc)-NH₂

Linker(OMe)-Lys(Boc)-Leu-Lys(Boc)-NH(Cbz) (2.19 g, 2.40 mmol) was dissolved in methanol (100 mL). Palladium (10%) carbon powder (0.4 g) was added, and the solution was placed under H₂ pressure (55 psi) overnight. The palladium carbon powder was filtered over diatomaceous earth, and the resulting solution was concentrated, forming a white solid.

3.3.5.7. Linker(OMe)-Lys(Boc)-Leu-Lys(Boc)-Leu-NH(Cbz)

The deprotected product (1.87 g, 2.40 mmol) was dissolved in anhydrous DCM (15 mL) under argon. Z-Leu- OH (0.638 g, 2.40 mmol), propanephosphonic anhydride (T₃P, 3.06 mL, 4.79 mmol), and DIEA (1.04 mL, 6.00 mmol) were added at 0°C and allowed to stir overnight. Water (5 mL) was added to quench the excess of T₃P for 1 hour. The resulting solution was concentrated and dissolved in EtOAc (50 mL). The solution was extracted three times with water (10 mL). The organic layer was dried over
Na$_2$SO$_4$ and concentrated. The crude product was recrystallized in ether (10 mL) overnight, producing a white solid (1.83 g, 74.5%). m/z [M+H]$^+$ 1027.57.

3.3.5.8. Linker(OMe)-Lys(Boc)-Leu-Lys(Boc)-Leu-NH$_2$

Linker(OMe)-Lys(Boc)-Leu-Lys(Boc)-Leu-NH(Cbz) (1.83 g, 1.78 mmol) was dissolved in methanol (100 mL). Palladium (10%) carbon powder (0.4 g) was added, and the solution was placed under H$_2$ pressure (55 psi) overnight. The palladium carbon powder was filtered over diatomaceous earth, and the resulting solution was concentrated, forming a white solid.

3.3.5.9. Linker(OMe)-Lys(Boc)-Leu-Lys(Boc)-Leu-Lys(Boc)-NH(Cbz)

The deprotected product (1.59 g, 1.78 mmol) was dissolved in anhydrous DCM (15 mL) under argon. Z-Lys(Boc)-OH (0.678 g, 1.78 mmol), propanephosphonic anhydride (T$_3$P, 2.27 mL, 3.56 mmol), and DIEA (0.736 mL, 4.45 mmol) were added at 0°C and allowed to stir overnight. The resulting solution was quenched with water (5 mL) for 1 hour, then concentrated and dissolved in EtOAc (50 mL). The solution was extracted three times with water (10 mL). The organic layer was dried over Na$_2$SO$_4$ and concentrated. The crude product was then recrystallized in ether (10 mL) overnight, producing a white solid (2.00 g, 89.5%). m/z [M+H]$^+$ 1255.72.

3.3.5.10. Linker(OMe)-Lys(Boc)-Leu-Lys(Boc)-Leu-Lys(Boc)-NH$_2$

Linker(OMe)-Lys(Boc)-Leu-Lys(Boc)-Leu-Lys(Boc)-NH(Cbz) (2.00 g, 1.59 mmol) was dissolved in methanol (100 mL). Palladium (10%) carbon powder (0.4 g) was added, and the solution was placed under H$_2$ pressure (55 psi) overnight. The palladium
carbon powder was filtered over diatomaceous earth, and the resulting solution was concentrated, forming a white solid.

3.3.6. Cyclization of recognition strand and non-recognition strand

(Contributed by Dr. Priyesh Jain)

3.3.6.1. Synthesis of linear peptide

Hexameric recognition strand (0.14 mg, 0.125 mmol) and non-recognition strand (0.11 g, 0.125 mmol) were dissolved in anhydrous THF (10 ml). Solution of 50% propanephosphonic anhydride (T₃P, 0.16 mL, 0.25 mmol) was added to the previous solution at 0°C. The reaction was allowed to stir overnight at room temperature. Excess T₃P was quenched with water (2 mL), concentrated, and dissolved in EtOAC. The solution was extracted three times with water. The organic layer was dried over Na₂SO₄ and concentrated. The crude product was recrystallized in ether (10 mL) overnight, producing a white solid (fully protected linear peptide, 0.17 g, 68%). The coupled peptide was validated by MALDI-TOF after deprotecting all Boc groups. m/z [M+H]⁺ 1664.71

3.3.6.2. Deprotection of linear peptide

Protected linear peptide (0.17 g, 0.086 mmol) was dissolved in methanol (30 mL). Palladium (10%) carbon powder (0.4 g) was added and the solution was placed under H₂ pressure (55 psi) overnight. The palladium carbon powder was filtered over diatomaceous earth. The resulting solution was concentrated and white solid was formed (0.14 g, 88%). Cbz-deprotected linear peptide (0.14 g, 0.076 mmol) was dissolved in THF:H₂O (1:1, 5 mL). 5% LiOH solution (2 mL) was added to the previous solution at 0°C. The reaction was carried out for 30 minutes until the TLC showed complete consumption of the starting material. The reaction contents were concentrated and
freeze dried to give peptide as a white solid. Deprotected linear peptide was validated by MALDI-TOF after deprotecting all Boc groups. m/z [M+H]^+ 1516.47.

3.3.6.3. Cyclization of linear peptide

Deprotected linear peptide (0.11 g, 0.076 mmol) was dissolved in anhydrous THF (10 mL). Solution of 50% propanephosphonic anhydride (0.10 mL, 0.15 mmol) was added at 0°C. The reaction was allowed to stir overnight at room temperature. Excess T3P was quenched with water (2 mL), concentrated, and dissolved in EtOAC. The solution was then extracted three times with water. The organic layer was dried over Na2SO4 and concentrated. The crude product was recrystallized in ether (10 mL) overnight, a white solid was formed. The cyclized peptide was validated by MALDI-TOF after deprotecting all Boc groups. m/z [M+H]^+ 1499.43.

3.3.7. Linear synthesis of MTI-101

3.3.7.1. Linker(OMe)-Lys(Boc)-NH(Cbz)

Cbz-N-Lys(Boc)-OH (0.77 g, 2.03 mmol) was dissolved in anhydrous DCM (10 ml) and cool down at 0 °C. N-methylmorpholine (0.22 ml, 2.03 mmol) and isobutylchloroformate (0.27 ml, 2.03 mmol) were added to the cooled solution. Reaction mixture was allowed to stir for 15 minutes. Deprotected linker (0.43 g, 2.03 mmol) and triethylamine (0.57 ml, 4.06 mmol) were mixed to the previous solution. The reaction mixture was brought to room temperature and stirred overnight. The product was extracted with DCM (3 × 10 ml). The combined organic layers were washed with brine, dried over Na2SO4 and concentrated. The white solid was obtained after recrystallization in ether (1.09 g, 98 %). 1H NMR (400 MHz, CDCl3): δ = 1.31-1.32 (d, 2H), 1.37 (s, 9H), 1.61-1.62 (m, 1H), 1.79-1.82 (m, 1H), 2.96 (s, 3H), 3.03-3.05 (m,
\[ \text{1H NMR (400 MHz, CDCl}_3\text{): } \delta = 0.82-0.84 \text{ (t, 6H, } J = 0.02), \ 1.18-1.21 \text{ (m, 2H), } 1.34 \text{ (s, 9H), } 1.40-1.42 \text{ (m, 2H), } 1.46-1.49 \text{ (m, 1H),} \\
\ 1.56-1.59 \text{ (m, 2H), } 2.84-2.85 \text{ (m, 2H), } 2.97 \text{ (s, 3H), } 3.22-3.24 \text{ (m, 4H), } 3.39-3.41 \text{ (m, 2H),}\]

3.3.7.2. \textit{Linker(OMe)-Lys(Boc)-NH}_2

Protected dimeric peptide (1.09 g, 1.90 mmol) was dissolved in 100 ml of methanol and palladium carbon powder (10 %) was mixed. Triethylsilane (1.52 ml, 9.51 mmol) was added slowly under the hydrogen gas. The completion of reaction was monitored by TLC. Palladium carbon powder was removed over diatomaceous earth and the resulting solution was concentrated under reduced pressure.

3.3.7.3. \textit{Linker(OMe)-Lys(Boc)-Leu-NH(Cbz)}

Cbz-N-Leu-OH (0.50 g, 1.90 mmol) was dissolved in anhydrous DCM (10 ml) and cool down at 0 °C. N-methylmorpholine (0.21 ml, 1.90 mmol) and isobutylchloroformate (0.25 ml, 1.90 mmol) were added to the cooled solution. Reaction mixture was allowed to stir for 15 minutes. Deprotected dimeric peptide (0.83 g, 1.90 mmol) and triethylamine (0.53 ml, 3.80 mmol) were mixed to the previous solution. The reaction mixture was brought to room temperature and stirred overnight. The product was extracted with DCM (3 × 10 ml). The combined organic layers were washed with brine, dried over Na\textsubscript{2}SO\textsubscript{4}, and concentrated. The white solid was obtained after recrystallization in ether (1.24 g, 95 %). \textit{\textsuperscript{1}H NMR (400 MHz, CDCl}_3\text{): } \delta = 0.82-0.84 \text{ (t, 6H, } J = 0.02), \ 1.18-1.21 \text{ (m, 2H), } 1.34 \text{ (s, 9H), } 1.40-1.42 \text{ (m, 2H), } 1.46-1.49 \text{ (m, 1H),} \\
\ 1.56-1.59 \text{ (m, 2H), } 2.84-2.85 \text{ (m, 2H), } 2.97 \text{ (s, 3H), } 3.22-3.24 \text{ (m, 4H), } 3.39-3.41 \text{ (m,
8H), 3.64 (s, 3H), 4.08 (s, 2H), 4.01-4.13 (m, 2H), 5.01 (s, 2H), 7.29-7.34 (m, 5H); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta = 22.04$, 23.33, 23.75, 24.85, 28.94, 29.87, 32.40, 38.04, 39.50, 39.66, 39.71, 39.92, 40.12, 40.33, 40.44, 40.54, 40.75, 41.21, 47.50, 48.98, 52.66, 53.17, 53.83, 66.06, 78.01, 127.67, 128.32, 128.45, 129/01, 137.68, 156.20, 156.64, 170.76, 172.45, 172.87; HRMS: $m/z$ calcd for C$_{31}$H$_{51}$N$_5$O$_{10}$S [M + H$^+$], 686.342; found, 686.345.

3.3.7.4. Linker(OMe)-Lys(Boc)-Leu-NH$_2$

Protected trimeric peptide (1.24 g, 1.81 mmol) was dissolved in 100 ml of methanol and palladium carbon powder (10 %) was mixed. Triethylsilane (1.44 ml, 9.03 mmol) was added slowly under the hydrogen gas. The completion of reaction was monitored by TLC. Palladium carbon powder was removed over diatomaceous earth and the resulting solution was concentrated under reduced pressure.

3.3.7.5. Linker(OMe)-Lys(Boc)-Leu-Lys(Boc)-NH(Cbz)

Cbz-N-Lys(Boc)-OH (0.69 g, 1.81 mmol) was dissolved in anhydrous DCM (10 ml) and cool down at 0 °C. N-methylmorpholine (0.20 ml, 1.81 mmol) and isobutylchloroformate (0.24 ml, 1.81 mmol) were added to the cooled solution. Reaction mixture was allowed to stir for 15 minutes. Deprotected trimeric peptide (1.00 g, 1.81 mmol) and triethylamine (0.50 ml, 3.62 mmol) were mixed to the previous solution. The reaction mixture was brought to room temperature and stirred overnight. The product was extracted with DCM (3 $\times$ 10 ml). The combined organic layers were washed with brine, dried over Na$_2$SO$_4$, and concentrated. The white solid was obtained after recrystallization in ether (1.47 g, 89 %). $^1$H NMR (400 MHz, CDCl$_3$): $\delta = 0.88$-0.93 (dd, 6H), 1.32-1.45 (m, 4H), 1.40 (s, 9H), 1.55-1.61 (m, 5H), 1.79-1.85 (m, 3H), 151
2.98 (s, 3H), 3.05-3.10 (m, 4H), 3.38-3.48 (m, 4H), 3.72 (s, 3H), 4.14 (s, 2H), 4.28-4.36 (m, 1H), 4.80-4.83 (m, 1H), 5.07 (s, 2H), 7.30-7.35 (m, 5H); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta =$ 21.95, 23.11, 23.26, 23.55, 24.51, 28.69, 29.66, 31.94, 32.08, 37.79, 39.24, 39.44, 39.65, 39.86, 40.07, 40.18, 40.28, 40.49, 40.91, 47.24, 48.71, 51.40, 52.42, 53.02, 55.17, 65.81, 77.80, 127.33, 128.10, 128.20, 128.76, 137.40, 155.99, 166.43, 170.52, 172.14, 172.25, 172.43; HRMS: $m/z$ calcd for C$_{42}$H$_{71}$N$_7$O$_{13}$S [M + H$^+$], 914.490; found, 914.494.

3.3.7.6. Linker(OMe)-Lys(Boc)-Leu-Lys(Boc)-NH$_2$

Protected tetrameric peptide (1.47 g, 1.61 mmol) was dissolved in 100 ml of methanol and palladium carbon powder (10 %) was mixed. Triethylsilane (1.28 ml, 8.04 mmol) was added slowly under the hydrogen gas. The completion of reaction was monitored by TLC. Palladium carbon powder was removed over diatomaceous earth and the resulting solution was concentrated under reduced pressure.

3.3.7.7. Linker(OMe)-Lys(Boc)-Leu-Lys(Boc)-Leu-Cbz

Cbz-N-Leu-OH (0.43 g, 1.61 mmol) was dissolved in anhydrous DCM (10 ml) and cool down at 0 °C. N-methylmorpholine (0.18 ml, 1.61 mmol) and isobutylchloroformate (0.21 ml, 1.61 mmol) were added to the cooled solution. Reaction mixture was allowed to stir for 15 minutes. Deprotected tetrameric peptide (1.26 g, 1.61 mmol) and triethylamine (0.45 ml, 3.22 mmol) were mixed to the previous solution. The reaction mixture was brought to room temperature and stirred overnight. The product was extracted with DCM (3 $\times$ 10 ml). The combined organic layers were washed with brine, dried over Na$_2$SO$_4$, and concentrated. The white solid was obtained after recrystallization in ether (1.42 g, 86 %). $^1$H NMR (400 MHz, CDCl$_3$): $\delta =$ 0.87-0.95
(m, 6H), 1.25-1.47 (m, 10H), 1.39 (s, 9H), 1.57-1.60 (m, 8H), 1.82-1.95 (m, 2H), 2.97 (s, 3H), 2.99-3.08 (m, 4H), 3.38-3.40 (m, 4H), 3.71 (s, 3H), 4.16 (s, 2H), 4.20-4.34 (m, 2H), 4.75-4.89 (m, 2H), 5.09 (s, 2H), 7.29-7.40 (m, 5H); $^1$H NMR (100 MHz, CDCl$_3$):

$\delta = 14.51, 21.88, 23.46, 23.56, 28.69, 39.45, 39.66, 39.87, 40.08, 40.29, 47.23, 48.70, 52.41, 52.84, 53.58, 60.20, 65.79, 77.77, 127.40, 128.05, 128.19, 128.76, 137.44, 155.96, 156.37, 170.51, 170.78, 171.92, 172.11, 172.13, 172.80; HRMS: m/z calcd for C$_{48}$H$_{82}$N$_8$O$_{14}$S [M + H$^+$], 1027.574; found, 1027.576.

3.3.7.8. **Linker(OMe)-Lys(Boc)-Leu-Lys(Boc)-Leu-NH$_2$**

Protected pentameric peptide (1.42 g, 1.38 mmol) was dissolved in 100 ml of methanol and palladium carbon powder (10 %) was mixed. Triethylsilane (1.11 ml, 6.92 mmol) was added slowly under the hydrogen gas. The completion of reaction was monitored by TLC. Palladium carbon powder was removed over diatomaceous earth and the resulting solution was concentrated under reduced pressure.

3.3.7.9. **Linker(OMe)-Lys(Boc)-Leu-Lys(Boc)-Leu-Lys(Boc)-NH(Cbz)**

Cbz-N-Lys(Boc)-OH (0.52 g, 1.38 mmol) was dissolved in anhydrous DCM (10 ml) and cool down at 0 °C. N-methylmorpholine (0.15 ml, 1.38 mmol) and isobutylchloroformate (0.18 ml, 1.38 mmol) were added to the cooled solution. Reaction mixture was allowed to stir for 15 minutes. Deprotected pentameric peptide (1.23 g, 1.38 mmol) and triethylamine (0.38 ml, 2.76 mmol) were mixed to the previous solution. The reaction mixture was brought to room temperature and stirred overnight. The product was extracted with DCM (3 $\times$ 10 ml). The combined organic layers were washed with brine, dried over Na$_2$SO$_4$ and concentrated. The white solid was obtained
after recrystallization in ether (1.58 g, 91 %). MALDI-TOF : m/z [M + H⁺] 955.387.

(MALDI-TOF was validated after deprotecting all Boc groups)

3.3.7.10. **Linker(OMe)-Lys(Boc)-Leu-Lys(Boc)-Leu-Lys(Boc)-NH₂**

Protected hexameric peptide (1.58 g, 1.26 mmol) was dissolved in 100 ml of methanol and palladium carbon powder (10 %) was mixed. The reaction mixture was located overnight under the high pressure of hydrogen gas. Palladium carbon powder was removed over diatomaceous earth and the resulting solution was concentrated under reduced pressure.

3.3.7.11. **Linker(OMe)-Lys(Boc)-Leu-Lys(Boc)-Leu-Lys(Boc)-β-turn promoter-NH(Cbz)**

Deprotected β-turn promoter (0.51 g, 1.26 mmol) was dissolved in anhydrous DCM (10 ml) and cool down at 0 °C. N-methylmorpholine (0.14 ml, 1.26 mmol) and isobutylchloroformate (0.16 ml, 1.26 mmol) were added to the cooled solution. Reaction mixture was allowed to stir for 15 minutes. Deprotected hexameric peptide (1.41 g, 1.26 mmol) and triethylamine (0.35 ml, 2.52 mmol) were mixed to the previous solution. The reaction mixture was brought to room temperature and stirred overnight. The product was extracted with DCM (3 x 10 ml). The combined organic layers were washed with brine, dried over Na₂SO₄, and concentrated. The white solid was obtained after recrystallization in ether (1.28 g, 73 %). MALDI-TOF : m/z [M + H⁺] 1096.709. (MALDI-TOF was validated after deprotecting all Boc groups)

3.3.7.12. **Linker(OMe)-Lys(Boc)-Leu-Lys(Boc)-Leu-Lys(Boc)-β-turn promoter-NH₂**

Protected heptameric peptide (1.28 g, 0.92 mmol) was dissolved in 100 ml of methanol and palladium carbon powder (10 %) was mixed. The reaction mixture was
located overnight under the high pressure of hydrogen gas. Palladium carbon powder was removed over diatomaceous earth and the resulting solution was concentrated under reduced pressure.

3.3.7.13. Linker(OMe)-Lys(Boc)-Leu-Lys(Boc)-Leu-Lys(Boc)-β-turn promoter-NH(Cbz)

Cbz-N-Trp-OH (0.31 g, 0.92 mmol) was dissolved in anhydrous DCM (10 ml) and cool down at 0 °C. N-methylmorpholine (0.10 ml, 0.92 mmol) and isobutylchloroformate (0.12 ml, 0.92 mmol) were added to the cooled solution. Reaction mixture was allowed to stir for 15 minutes. Deprotected heptameric peptide (1.16 g, 0.92 mmol) and triethylamine (0.26 ml, 1.84 mmol) were mixed to the previous solution. The reaction mixture was brought to room temperature and stirred overnight. The product was extracted with DCM (3 × 10 ml). The combined organic layers were washed with brine, dried over Na₂SO₄, and concentrated. The white solid was obtained after recrystallization in ether (0.2 g, 23 %). MALDI-TOF : m/z [M + H⁺] 1282.758. (MALDI-TOF was validated after deprotecting all Boc groups)

3. 4. References


4. 1. Introduction

Peptides play an important role in biological functions and therefore promising candidates in therapeutic development. However, their natural instability to proteases has led to numerous research in peptidomimetics recently (1). Peptidomimetics not only have similar functions and interactions to the intended target as those of natural peptides but also resist to the proteolysis. In other words, peptidomimetics would have improved stability and bioavailability.

In late 1990s, the Gellman group introduced the field of foldamers (2). Foldamers are defined as unnatural oligomers which can fold in a certain fashion and copy the behaviors known from biopolymers (3 - 7). One example of a type of foldamer is hybrid peptoid-peptide ligands. Goodman and colleagues reported the first cases of peptoid-peptide hybrids in 1994 (8). They synthesized and characterized collagen mimicry with peptide repetition H₂N-Gly-[Gly-Pro-Hyp]₁₀, which were covalently attached to the Kemp tricarboxylic acid (9 - 11). In addition to this, similar assemblies containing the N-isobutylglycine (Nleu) peptoid residue instead of hydroxyproline (Hyp) were investigated in a series of the study (12 - 16).
Hamy and co-workers showed hybrid peptoid-peptide ligands with the ability to disrupt the interactions between HIV-1 transactivation protein (Tat) and the transactivation response (TAR) RNA domain, which following HIV-1 replication in primary human lymphocytes (17 - 19). After screening in a pool of $3.2 \times 10^6$ ligands, a inhibitor of the Tat-TAR RNA complex with nanomolar activity was found (Figure 1a). The inhibitor was further studied and proved to bind TAR RNA specifically by using NMR analysis and molecular modeling (16). In addition, the group of Rocchi reported the receptor targeting case by using peptoid substituted compounds based on dermorphin, deltorphin, and endomorphin as opioid receptor agonists (Figure 1b) (20 - 22).

Most types of peptidomimetics, including peptoid-peptide hybrids, are investigated as membrane active cationic anti-microbial peptide (AMP) derivatives. There are two major strategies: (1) replacing certain residues in a known natural product or peptide and (2) constructing the de novo design of an amphiphilic template. Both of these strategies have been applied to develop AMP analogues with peptoid-peptide hybrids. Robinson and co-workers displayed a small series of peptoid-containing analogues of the antimicrobial β-hairpin structure protegrin I by using the first strategy (Figure 1c) (23). In a preliminary study, the disulfide bridges connecting native hairpin were replaced by D-Pro-L-Pro moiety through macrocyclization. Two substituted compounds were designed, synthesized, and evaluated (23). After deuterium exchange and 2D ROESY NMR spectroscopy experiment along with the constrained molecular modeling, a lead compound (Figure 1c) maintains a relatively stable β-hairpin conformation in aqueous solution. Also, this lead compound has slightly better potency than that of its nonpeptoid-containing α-peptides against the tested microorganisms, and more
importantly, it showed much more resistance to hemolysis the natural product lead protegrin I (23).

Figure 38. Peptoid-peptide hybrids with bioactivity.
Ryge, Hansen, and colleagues reported de novo designed amphiphilic peptoid-peptide hybrids as AMP mimetics and optimized their design via combinatorial libraries (24 - 26). Their most powerful molecule showed low micromolar range for the IC50 against a broad spectrum of both Gram-positive and Gram-negative bacteria (Figure 1d). In order to investigate the transmembrane segment mimetics, peptoid-peptide hybrids interacting membrane have also been studied (27, 28).

Since Goodman and co-workers introduced the first examples of peptoid-peptide hybrid, the α-amino acid combined peptoid residues have been engaged in numerous biologically active ligands. As discussed earlier, ligands for inhibition protein-protein interactions and antimicrobial activities are the examples as related research fields. The research of peptoid-peptide hybrid structures has been thrived in development of peptidomimetics and will provide more ligands with interesting bioactivities in the future.

4. 2. Results and discussion

N-substituted glycine oligomers, or peptoids, are an important class of foldamers built by tertiary amide linkages (29). The extensive chemical diversity of the peptoid side chains promotes the design strategies which can be applied to the various fields such as enenatioselctive catalysis, molecular recognition, and intracellular delivery (30 - 32). Moreover, numerous studies have been dedicated to development of folded peptoids with the distinct secondary structures by changing side chains which control the backbone conformational ordering. Recently, two well-ordered peptoid secondary structures were built using triazole linkages (33, 34). Even though this discovery opens up the possibility of the peptoid design with similar structure as small folded proteins, we seek the capability to craft functional peptoid-peptide hybrids adopting
the β-hairpin structure. β-hairpin structure is one of the extended peptide secondary structures, that two functional strands are connected through a linkage. Depending on side chains, β-hairpin can improve the target recognition ability as well as the pharmacokinetic properties.

Peptoid oligomers can be prepared efficiently via submonomer solid-phase synthesis strategy. The strategy is to elongate the main chain by an acylation reaction with a haloacetic acid and then attach the side chain with a primary amine. Because of its high efficiency, the similar approach was applied in this experiment. The whole peptoids were constructed on 2-chlorotrityl chloride resins. Before the synthesis was initiated, the resins were reactivated by using thionyl chloride in DCM. For the acylation step, bromoacetic anhydride was used instead of haloacetic acid due to its high reactivity. Also, 2,4-dimethoxybenzyl amine was selected rather than ammonia to introduce the secondary amide, hydrogen bonded to the carbonyl group on the opposite strand, because ammonia can cleave the peptoids on the resins. Also, 2,4-dimethoxytoluene was able to be removed with TFA at the last stage of synthesis. One of critical elements in β-hairpin structure is turn promoter. For this experiment, D-Pro-L-Pro template, type II′ turn, was chosen as a β-turn promoter.

In order to observe the structure changes or differences between acyclic and cyclic peptoid-peptide hybrids, the resins were divided in half before the cleavage from the resins. The divided resins were deprotected separately and processed in different ways. For the construction of acyclic peptoid-peptide hybrids, the resulting compounds after deprotection were purified by HPLC. To build the cyclic peptoid-peptide hybrids, the resulting compounds after deprotection were cyclized in the presence of HCTU in DCM and then purified by HPLC. Based on this strategy, three sets of acyclic and
cyclic peptoid-peptide hybrids were prepared (Figure 2). Three sets of peptoid-peptide hybrids were different in sizes and the number of turn promoters.

NMR study was employed to check the formation of β-hairpin structures based on the hypothesis: if the β-hairpin structure is formed, glycine α-protons of an acyclic peptoid-peptide hybrid in the 4 ppm ranges will shift to 5 ppm ranges after cyclization. Deturated acetonitrile or chloroform were used as NMR solvents. After NMR studies, there was no indication of the β-hairpin structure formation from three sets of peptoid-peptide hybrids because there was no chemical shift changes from α-protons in acyclic and cyclic peptoid-peptide hybrids.

In this study, the possibility of β-hairpin structure in peptoid-peptide hybrids was investigated. NMR studies were performed based on the hypothesis: glycine α-protons
of an acyclic peptoid-peptide hybrid in the 4 ppm ranges will shift to 5 ppm ranges after cyclization due to the \( \beta \)-hairpin structure formation. However, the NMR study results displayed that there was no indication of the \( \beta \)-hairpin structure formation in peptoid-peptide hybrids. These results suggested that the peptoid-peptide hybrid structures were still flexible despite of the introduction of intra hydrogen bonding and \( \beta \)-turn promoters. According to this finding, it is necessary to develop the components which provide the more rigid structure in peptoid-peptide hybrids such as incorporations of ring moieties in the main chain or development of staple peptoid-peptide hybrids.

4.3. Experimental section

4.3.1. General comments

All reaction intermediates were monitored by mass spectral analysis (Agilent). The complete peptoid-peptide hybrids were purified by using HPLC (DIONEX) and purified compounds were studied via \(^1\)H NMR (INOVA-400).

4.3.2. General procedure

All the cyclic peptoid-peptide hybrids were prepared using solid-phase peptide synthesis (Figure 2). On the 2-chlorotrityl chloride resin, Fmoc-D-Proline was attached via SN2 reaction and typical submonomer strategy was performed to add different peptoid residues. The treatment with TFA provided not only cleavage of linear peptoid-peptide hybrids on the resin but also deprotection of 2,4-dimethoxytoluene, transferring tertiary amine into secondary amine. The resulting peptoid-peptide hybrids were cyclized in solution and purified by HPLC for further study.
4.3.2. Synthesis of acyclic and cyclic peptoid-peptide hybrids 1

2-Chlorotrityl chloride resins (0.3 g; capacity: 0.79 mmol/g) were swelled with 3 ml of anhydrous DCM at room temperature for 20 minutes. Fmoc-D-proline (0.1 g, 0.95 mmol) and DIEA (0.28 ml, 1.58 mmol) in 2 ml of DCM were added to the synthesis reaction vessel. After 30 minutes, the solution was drained and the same washing step was repeated. The beads were washed with anhydrous DCM. Then the beads were deprotected using 2 ml of 2 % DBU in DMF for 5 minutes at room temperature. This step was repeated, and the beads were washed with DCM. The acylation step was carried out at room temperature for 20 minutes. All the beads were incubated in a mixture of bromoacetic anhydride (0.41 g, 1.58 mmol), DIEA (0.56 ml, 3.16 mmol), and 2 ml of DCM. After, that step the beads were washed with DCM. Depending on the side chain sequences, different primary amines (3.16 mmol) in 2 ml of DCM were added to the beads and the reaction mixture was stirred at room temperature for 20 minutes before the beads were washed with DMF. These acylation and S_N2 by primary amines (e.g. 2,4-dimethoxybenzyl amine, isopropyl...
amine, benzyl amine and et al) were repeated until the intended sequences are done.
In order to complete β-turn template (D-Pro-L-Pro), Fmoc-L-Proline (1.07 g, 3.16 mmol), HCTU (1.31 g, 3.16 mmol), and DIEA (1.12 ml, 6.32 mmol) in 3 ml of DCM were mixed for 1 hour, and this step was repeated. The beads were washed with DCM. Before the linear peptoids were cleaved from the resin, Fmoc group was deprotected using 2 % DBU in DMF and the beads were washed with DCM. Based on the mass, the resins were divided by in half for the preparation of acyclic and cyclic peptoids. Both of peptoids on the resins were cleaved with TFA and the resulting solution was collected in the flask. For the complete deprotection (2,4-dimethoxytoluene), the resulting solution was stirred for 2 days. After removing TFA under the reduced pressure, one of batches were purified using HPLC to collect the acyclic peptoid (Figure 2). The peptoids in the other batch were cyclized in the mixture of HCTU (0.65 g, 1.58 mmol), DIEA (0.56 ml, 3.16 mmol) in 5 ml of DCM. After proper work-up, the cyclic peptoids were obtained after HPLC purification (Figure 2). HRMS: m/z [M+H]^+ (acyclic version). HRMS: m/z [M+H]^+ (cyclic version).

![Figure 40. Structures of acyclic and cyclic peptoid-peptide hybrids 1.](image-url)
4.3.3. Synthesis of acyclic and cyclic peptoid-peptide hybrids

2-Chlorotrityl chloride resins (0.3 g; capacity: 0.79 mmol/g) were swelled with 3 ml of anhydrous DCM at room temperature for 20 minutes. Fmoc-D-proline (0.1 g, 0.95 mmol) and DIEA (0.28 ml, 1.58 mmol) in 2 ml of DCM were added to the synthesis reaction vessel. After 30 minutes, the solution was drained and the same step was repeated. The beads were washed with anhydrous DCM. The beads were deprotected using 2 ml of 2 % DBU in DMF for 5 minutes at room temperature. This step was repeated, and the beads were washed with DCM. The acylation step was carried out at room temperature for 20 minutes. All the beads were incubated in a mixture of bromoacetic anhydride (0.41 g, 1.58 mmol), DIEA (0.56 ml, 3.16 mmol), and 2 ml of DCM. The beads were washed with DCM. Depending on the side chain sequences, different primary amines (3.16 mmol) in 2 ml of DCM were added to the beads and the reaction mixture was stirred at room temperature for 20 minutes before the beads were washed with DCM. These acylation and SN2 by primary amines (e.g. 2,4-dimethoxybenzyl amine, isopropyl amine, benzyl amine and et al) were repeated until the intended sequences are done. In order to complete β-turn template (D-Pro-L-Pro), Fmoc-L-Proline (1.07 g, 3.16 mmol), HCTU (1.31 g, 3.16 mmol), and DIEA (1.12 ml, 6.32 mmol) in 3 ml of DCM were mixed for 1 hour, and this step was repeated. The beads were washed with DCM. Before the linear peptoids were cleaved from the resin, Fmoc group was deprotected using 2 % DBU in DMF and the beads were washed with DCM. Based on the mass, the resins were divided by in half for the preparation of acyclic and cyclic peptoids. Both of peptoids on the resins were cleaved with TFA and the resulting solution was collected in the flask. For the complete deprotection (2,4-dimethoxytoluene), the resulting solution was stirred for 2 days. After removing TFA under the reduced
pressure, one of batches were purified using HPLC to collect the acyclic peptoid (Figure 3). The peptoids in the other batch were cyclized in the mixture of HCTU (0.65 g, 1.58 mmol), DIEA (0.56 ml, 3.16 mmol) in 5 ml of DCM. After proper work-up, the cyclic peptoids were obtained after HPLC purification (Figure 3). MALDI-TOF: m/z [M+H]$^+$ (acyclic version). MALDI-TOF: m/z [M+H]$^+$ 929.57 (cyclic version).

![Figure 41. Structures of acyclic and cyclic peptoid-peptide hybrids 2.](image)

### 4.3.3. Synthesis of acyclic and cyclic peptoid-peptide hybrids 3

2-Chlorotrityl chloride resins (0.3 g; capacity: 0.79 mmol/g) were swelled with 3 ml of anhydrous DCM at room temperature for 20 minutes. Fmoc-D-proline (0.1 g, 0.95 mmol) and DIEA (0.28 ml, 1.58 mmol) in 2 ml of DCM were added to the synthesis reaction vessel. After 30 minutes, the solution was drained and the same step was repeated. The beads were washed with anhydrous DCM. The beads were deprotected using 2 ml of 2 % DBU in DMF for 5 minutes at room temperature. This step was repeated, and the beads were washed with DCM. The acylation step was carried out at room temperature for 20 minutes. All the beads were incubated in a mixture of bromoacetic anhydride (0.41 g, 1.58 mmol), DIEA (0.56 ml, 3.16 mmol), and 2 ml of DCM. The beads were washed with DCM. Depending on the side chain sequences, different primary amines (3.16 mmol) in 2 ml of DCM were
added to the beads and the reaction mixture was stirred at room temperature for 20 minutes before the beads were washed with DCM. These acylation and S_N2 by primary amines (e.g. 2,4-dimethoxybenzyl amine, isopropyl amine, benzyl amine and et al) were repeated until the intended sequences are done. In order to complete β-turn template (D-Pro-L-Pro), Fmoc-L-Proline (1.07 g, 3.16 mmol), HCTU (1.31 g, 3.16 mmol), and DIEA (1.12 ml, 6.32 mmol) in 3 ml of DCM were mixed for 1 hour, and this step was repeated. The beads were washed with DCM. The Fmoc group was deprotected by using 2 % DBU in DMF and the beads were washed with DCM. In order to construct the other strand, the same procedure such as SN2 reaction with Fmoc-D-Proline, acylation, SN2 reaction with primary amines, and SN2 reaction with Fmoc-L-Proline was performed. Before the linear peptoids were cleaved from the resin, Fmoc group was deprotected using 2 % DBU in DMF and the beads were washed with DCM. Based on the mass, the resins were divided by in half for the preparation of acyclic and cyclic peptoids. Both of peptoids on the resins were cleaved with TFA and the resulting solution was collected in a flask. For the complete deprotection (2,4-dimethoxytoluene), the resulting solution was stirred for 2 days. After removing TFA under the reduced pressure, one of batches were purified using HPLC to collect the acyclic peptoid (Figure 4). The peptoids in the other batch were cyclized in the mixture of HCTU (0.65 g, 1.58 mmol), DIEA (0.56 ml, 3.16 mmol) in 5 ml of DCM. After proper work-up, the cyclic peptoids were obtained after HPLC purification (Figure 4). MALDI-TOF: m/z [M+H]^+ 1677.99 (acyclic version). MALDI-TOF: m/z [M+H]^+ 1659.97 (cyclic version).
4.3.4. NMR study of peptoid-peptide hybrids

In order to investigate the chemical shift change of $\alpha$-protons in acyclic and cyclic peptoid-peptide hybrids, the proton NMR study was performed (INOVA-400 and INOVA-500). Compounds were dissolved in deturated chloroform and acetonitrile.

4.4. References


27. Tang Y. C., Deber C. M. *Biopolymers*. 2004; **76**: 110-118.


APPENDIX: 1H, 13C NMR SPECTRA, MASS SPECTRA
201
218
(The sample was checked after three Boc-groups were deprotected with TFA.)
(The sample was checked after three Boc-groups were deprotected with TFA.)
(The sample was checked after three Boc-groups were deprotected with TFA.)
(The sample was checked after three Boc-groups were deprotected.)
(The sample was checked after three Boc-groups were deprotected.)
Acyclic (top) and cyclic (bottom) peptoid-peptide hybrid 1
Acyclic (top) and cyclic (bottom) peptoid-peptide hybrid 2

4700 Reflector Spec #1 [BP = 947.5, 42414]

4700 Reflector Spec #2 [BP = 929.6, 23786]
Acyclic (top) and cyclic (bottom) peptoid-peptide hybrid 3

4700 Reflector Spec #1 [BP = 1678.0, 22654]

4700 Reflector Spec #1 [BP = 2228.6, 46481]
ABOUT THE AUTHOR

Ms. Hyun Joo Kil received dual bachelors’ degrees in the general science education and the chemistry education from Kangwon National University, South Korea in 2005. She started the masters’ program in physical organic chemistry at Kangwon National university in 2005. She received her masters degree in 2007. She pursued her doctorate degree at Kangwon National university in 2007 and transferred to University of South Florida in 2008. She has worked in Dr. Mark McLaughlin’s lab to find drug candidates to the various targets.