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Peptide Cyclization and Cyclodimerization by Cu^{I}-Mediated Azide-Alkyne Cycloaddition

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Abstract

Head-to-tail cyclodimerization of resin-bound oligopeptides bearing azide and alkyne groups occurs readily by 1,3-dipolar cycloaddition upon treatment with Cu(I). The process was found to be independent of peptide sequence, sensitive to the proximity of the alkyne to the resin, sensitive to solvent composition, facile for α- and β-peptides but not for γ-peptides, and inhibited by the inclusion of tertiary amide linkages. Peptides shorter than hexamers were predominantly converted to cyclic monomers. Oligoglycine and oligo(β-alanine) chains underwent oligomerization by 1,3-dipolar cycloaddition in the absence of copper catalyst. These results suggest that cyclodimerization depends on the ability of the azido-alkyne peptide to form in-frame hydrogen bonds between chains in order to place the reacting groups in close proximity and lower the entropic penalty for dimerization. The properties of the resin and solvent are crucial, giving rise to a productive balance between swelling and inter-strand H-bonding. These findings allow for the design of optimal substrates for triazole-forming ring closure, and for the course of the reaction to be controlled by the choice of conditions.

Keywords

Azide-alkyne cycloaddition; cyclic peptides; peptide synthesis; cyclodimerization; click chemistry

Introduction

Cyclic peptides and related structures are of longstanding interest as biologically active compounds because of their ability to display protein-like epitopes with restricted conformational flexibility.[1-3] Relative to their linear analogues, cyclic peptides are often more bioavailable, more stable toward metabolic degradation, and more selective in receptor binding.[4-11] It is therefore not surprising that peptide sequences are frequently evaluated for function in both linear and cyclic forms, creating a need for effective cyclization strategies.

Peptide cyclization is most often accomplished by the formation of amide, ester, thioester, disulfide, olefin, or C-C bonds.[12-14] Recently the copper(I)-mediated azide-alkyne cycloaddition (CuAAC) reaction has been employed as a means of orthogonal modification[15] and cyclization of peptides, [16-18] glycopeptides,[19] polymers,[20] and dendrimers.[21,22] In the context of peptides, this "click reaction"[23-25] methodology offers

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Supporting Information **Available**. Experimental details and representative HPLC and MALDI mass spectrometry data. This material is available free of charge via the Internet at http://pubs.acs.org.

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two important advantages. (1) Azide and alkyne units can be incorporated into peptide sequences during solid-phase peptide synthesis (SPPS) without protection, as both of these groups are stable to typical conditions of peptide synthesis and cleavage. (2) Azides and alkynes react selectively with each other, but do so very slowly unless catalyzed, allowing their ligation to be triggered at will.

We previously described attempts to cyclize two peptides on resin via the CuAAC reaction (Figure 1).[26] The sequences used in this preliminary study were derived from an adenovirus serotype that binds several α_v integrins and contains the canonical Arg-Gly-Asp (RGD) triad. The alkyne, introduced as a propargylglycine unit, was located near the resin-bound C-terminus, while the azide group was incorporated as a 5-azidopentanoyl unit at the N-terminus. On-resin cyclization mediated by cuprous iodide gave high yields of head-to-tail cyclic dimer rather than the expected cyclic monomer, even for sequences of substantial length and no obvious conformational, steric, or torsional constraints.

Several examples of solution-phase Cu-mediated cyclodimerization of peptides and carbohydrates have been reported, all with azido-alkyne substrates for which monomeric ring closure is disfavored because of torsional or geometric factors (described in more detail in Supporting Information).[27-30] Other investigators have described mixtures of peptide cyclic monomers and dimers as a function of ring strain and concentration,[31,32] as well as clean macrocyclization under the influence of conformational constraints.[17,18,33] These observations show that the CuAAC process is not inherently predisposed toward ring closure by head-to-tail dimerization. The unexpected and clean cyclodimerization of the two resin-bound peptides shown in Figure 1 therefore signaled to us the presence of an unexpected mechanistic feature. We suggested a sequence-dependent conformational bias and/or amide-Cu binding as two possible contributing factors in our original report.[26] We describe here experimental tests that disprove these hypotheses and suggest a crucial role for inter-chain hydrogen bonding. In addition, we wanted to explore the scope and limitations of the cyclodimerization method, since its products are both unique and potentially useful. When compared with monocyclic analogues, cyclic dimers test the contributions of both conformational rigidity and valency in the binding of peptide motifs to their targets.

Results and Discussion

Sequence Tolerance

The dependence of cyclodimerization on sequence identity was probed with a set of 32 peptides synthesized by batch splitting after coupling every second residue (Table 1). All proteinogenic amino acids were included except proline; the sequences tested the importance of several residues in the original sequence that were suspected of playing a role in the cyclodimerization outcome. Peptides were synthesized on Rink amide MBHA (4-methylbenzhydrylamine) resin with an initial loading of 0.70 mmol/g, similar to that in our previous report. As before, alkynes were installed near the C-terminus, although by *N*-propargylation rather than in propargylglycine for synthetic convenience, and azides were introduced by acylation of the *N*-terminus. Each linear sequence was individually cleaved and lyophilized after a standard work-up procedure. All 32 peptides were obtained with good to excellent purity (>75%, estimated by MALDI mass spectrometry analysis of the lyophilized crude powders).

Each resin-bound protected peptide was subjected to cyclization in 20 mM solutions of cuprous iodide under the convenient reaction conditions employed in our previous report (20% DMSO in MeCN, room temperature, 40 h, in screw-cap reaction vials closed immediately after bubbling with a stream of dry N_2). Successful cyclodimerization was indicated by a clear transition from a dominant peak of monomer mass to a dominant peak of dimeric mass in the MALDI mass spectrum of the lyophilized powders. This result was observed for all of the 32

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sequences shown in Table 1, although some were slower to react than others due to inconsistencies in the exclusion of oxygen, thereby giving rise to different amounts of active Cu(I) catalyst. To confirm the expected cyclic nature of the products, most of the samples were subjected to careful analysis by analytical reversed-phase (RP) HPLC after cleavage from the resin. In each case, a significantly shorter retention time was observed for the major product macrocycle in comparison with corresponding linear monomers. In contrast, linear dimers synthesized independently showed uniformly longer reversed-phase HPLC retention times than their monomeric counterparts. Moreover, the chromatogram and mass spectrum of each product was unchanged after treatment with excess triphenylphosphine, which would reduce the azide in a linear dimer. To the extent that our survey of sequence space is representative, it appears that the CuAAC-mediated peptide cyclodimerization process is largely insensitive to the nature of the amino acids present in the sequence, keeping in mind that side chain functional groups are protected.

Solution Phase Cyclization

To illuminate the role of the resin in the peptide cyclodimerization process, solution phase reactions were performed with a protected peptide sequence similar to that in our original studies (Figure 2). Following solid-phase synthesis, cleavage (without deprotection), and purification, structure **33** was subjected to Cu^{I}-mediated cycloaddition in a deoxygenated $MeOH/CH_2Cl_2$ mixture at concentrations of 0.8 mM and 3 mM, to promote intra- and intermolecular reaction, respectively.[32] After side-chain deprotection, resin cleavage, and ether precipitation, the samples were analyzed by analytical HPLC and MALDI mass spectrometry. The major product peak in both reactions had monomer molecular weight, significantly shorter retention time on RP-HPLC than the linear deprotected starting material, and was insensitive to reduction with phosphine, showing that monocyclization to structure **34** was the main pathway in the solution phase reaction. A small amount of dimeric product **35** was also observed (approximately 10% and 20% when conducted at 0.8 and 3 mM, respectively, as determined by HPLC integration) which co-eluted with an authentic sample of cyclic dimer. Therefore, selective cyclodimerization of peptides of this length is facilitated by attachment to a resin. However, a particular resin is not required. The use of three different polystyrene supports with different loading levels (chlorotrityl resin at 1.4 mmol/g starting functional group density, Rink amide MBHA resin at 0.70 mmol/g, and the PEGylated Rink-modified TentaGel (TGR) resin at 0.26 mmol/g) induced no substantial difference in the course of the reaction (Supporting Information).

Peptide Length

To evaluate the influence of peptide length on cyclodimerization, truncated versions of peptide **33** were prepared. When treated with Cu^{I}, resin-bound **36** was converted cleanly to cyclic dimer, but the shorter sequence **37** gave an intractable mixture with no major peak on HPLC and a MALDI mass spectrum showing both monomer and dimer masses (Figure 3 and Supporting Information). The shortest sequence **38** produced a single major peak in the HPLC chromatogram with monomeric mass and significantly shorter retention time than the linear starting material; this material was unaffected by treatment with triphenylphosphine. Accordingly, this product was assigned as the cyclic monomer of sequence **38**. Monomeric cylization of peptides of this length was also shown to be independent of sequence (Supporting Information). These results indicate that a minimum peptide length of six residues is required in this case for selective cyclodimerization.

Proximity of Alkyne to Resin

We previously demonstrated that cyclodimerization is favored by placement of the alkyne moiety at the C-terminal end of the peptide, which is attached to the resin polymer.[26] This

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phenomenon was further explored with sequences **39−43**, which provide an increasing number of glycine spacers between the resin and the alkyne (Figure 4). Under standard reaction conditions (5 or 20 mM CuI, 4:1 MeCN/DMSO with 2,6-lutidine) each of the sequences provided cyclic dimers as the sole detectable products by MALDI mass spectrometry. However, as the distance of the alkyne to the resin was increased, a progressive increase in the intensity of unresolved broad peaks was observed in the analytical RP-HPLC chromatograms. These side products are presumed to emerge from peptide degradation, which apparently becomes significant when cyclodimerization is not optimal (see Supporting Information for additional experiments). Thus, cyclodimerization becomes less efficient as the alkyne is moved to peptide positions farther away from the resin.

Availability of Alkyne

Figure 5 summarizes the outcome of experiments designed to test whether monocyclization could be made to occur if either azide or alkyne is made the limiting functional group. Resin-bound peptides were prepared in which only 25% of the chains displayed on the resin contained both groups necessary for triazole formation, and 75% lacked either the azide (**44**) or the alkyne (**45**). Upon exposure to standard CuAAC conditions, no cyclic monomers were observed – in other words, chains containing both azide and alkyne were somehow prevented from closing upon themselves, even when the rate of cyclodimerization was necessarily reduced. Resin **44** provided only the linear heterodimer and remaining non-azide peptide, whereas resin **45** returned the linear mixed dimer, cyclic dimer, and both starting peptide sequences. A significant difference was also noted in the extent of completion for the two reactions. Resin **44**, which bears alkynes on every peptide chain, gave complete consumption of the limiting (azide) group, whereas even extended treatment of **45** with Cu^{I} failed to consume all of the limiting reagent (alkyne). This is consistent with the expectation that alkynes are converted rapidly to Cu-acetylides, and that two Cu atoms are required for triazole formation.[34-36]

Non-Peptide Oligoamides

In an attempt to establish the structural requirements for cyclodimerization, we prepared resin-bound oligoamides lacking side chains and with differing numbers of main-chain methylene spacers and different lengths: glycine oligomers **46** and **47**, β-alanine oligomers **48−50**, and γ-aminobutyrate oligomers **51** and **52** (Figure 6). Mixed β-Ala/γ-Abu sequences **53−54** were also synthesized. Upon exposure to CuI under standard conditions, the penta(α-peptide) **46** and penta(β-peptide) **48** afforded complex product mixtures (linear and cyclic monomers, dimers, and oligomers), similar to pentapeptide **37** (Figure 3) used to establish the length dependence of cyclodimerization. Unfortunately, the octapeptide **47** yielded only highly insoluble material (likely due to β-sheet aggregation characteristic of long oligoglycines), the composition of which could not be analyzed. More revealingly, lengthening the β-peptide chain by one (**49**) or three (**50**) units gave rise to a change in the product distribution, providing substantial amounts of cyclic dimers. It is therefore evident that cyclodimerization does not require the presence of oligopeptide side chains.

Sequences **49** and **50** also returned significant amounts (approximately 15% total) of linear oligomers with mass values ranging from dimer to pentamer (M_2-M_5). This phenomenon was investigated with **49**, and was found to arise from triazole formation in the absence of copper with both the resin-bound protected peptide and the linear deprotected peptide after cleavage from the resin, when each was stored in the absence of solvent at room temperature. We have previously observed such solid-state (or neat oil) oligomerization of other small-molecule α,ω-azido-alkynes during the course of polymerization studies.[37] This phenomenon has interesting implications for metal-independent triazole formation[38-41] on surfaces or in the context of materials synthesis.[37]

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In contrast to the β-Ala oligomers, oligo(γ-Abu) sequences **51** and **52** were completely converted to cyclic monomers, the latter particularly notable because it contains eight backbone amide bonds in the chain, the same number that gave reliable cyclodimerization with oligopeptides. Sequences **49** and **51** are nearly the same length, but have different numbers and relative spacings of their amide bonds. To determine which of these two factors is responsible for selective cyclodimerization, mixed β-Ala and γ-Abu sequences **53–55** were prepared, where the number of amide bonds was conserved (equivalent to **49**, which readily cyclodimerizes) but their spacing along the oligoamide strand was varied. Upon exposure to Cu^{I}, sequences **53–55** all afforded cyclic monomers, suggesting that the spacing of amide bonds influences the cyclization process, with closer amide linkages (α- or β-peptides) favoring cyclodimer formation.

Tertiary Amides

Peptoids, oligomeric peptides in which the side chains are placed on the amide nitrogen atom, have attracted a great deal of interest for their resistance to proteolytic cleavage,[42] fascinating structural and conformational properties,[43-45] and their ability to display a wide array of chemical functionalities that enhance structural diversity and biomedical relevance.[46,47] We reasoned that the treatment of azido-alkynyl derivatives of such molecules with Cu(I) would provide novel cyclic structures, and would begin to test our prior hypothesis (now discredited, see below and Supporting Information) that interactions of copper with the backbone amide groups are of importance to cyclodimerization.[26] Experiments were performed using the series of *N*-benzylated octamers shown in Figure 7, designed to exhibit various properties of conformational structure and rigidity.

Homochiral α-substitution on nitrogen confers well-characterized helicity to the peptoid architecture with a helical pitch of three residues.[43-45] Sequence **56** thereby places its azide and alkyne groups in close proximity to each other on the same side of the helix, while **57** places them a greater distance apart and on different faces of the helix.[33,47] Structures **58** and **59** mimic the alkyne and azide positions of the peptides tested above. The lack of α-substituents in the latter sequence removes the steric and torsional interactions between side chains and hence any predisposition toward helicity, creating an unstructured, more flexible peptoid strand.[48] In all of these cases (**56–59**), cyclic monomers were produced exclusively from on-resin CuAAC reaction. The cyclic monomers were identified by mass spectrometry and their substantially different retention times on analytical RP-HPLC compared to the linear starting material.

Limited *N*-alkylation of a peptide was also able to abolish cyclodimerization, as shown in Figure 8. Thus, while the resin-bound decamer **62** underwent facile cyclodimerization under standard conditions, *N*-methylation of a central glycine (**63**) induced the formation of significant amounts of both cyclic monomer and cyclic dimer, along with some trimeric material. *N*-Methylation of two flanking positions (Leu and Phe, structure **64**) diverted the CuAAC reaction products to mostly cyclic monomer, along with small amounts of cyclic dimer and oligomeric materials with masses ranging from M_3 to M_5. The complete absence of cyclic dimers from the reactions of peptoids, and the decrease in cyclodimerization upon *N*-methylation of peptides, demonstrate that secondary, not tertiary, amide bonds are required for cyclodimerization.

Proposed Mechanism of Cyclodimerization

An examination of the resin-bound protected peptides by IR spectroscopy revealed an important solvent-dependent change in the state of hydrogen bonding by the backbone amide groups. Both CH_2Cl_2 and DMSO swell polystyrene resins, but the latter solvent is far better at disrupting inter- and intrachain peptide H-bonding. Figure 9 shows the representative case

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of resin-bound protected peptide **39**, which in CH_2Cl_2 exhibited an amide carbonyl stretching band at 1625 cm^{-1} and an amide N-H absorbance at 3277 cm^{-1}, both characteristic of strong hydrogen bonding. In DMSO, new bands appeared at 1654 cm^{-1} and 3461 cm^{-1}, consistent with the expected loss of H-bonding due to interactions of the peptide chains with the solvent.[49-52] In the standard reaction solvent system of 4:1 MeCN/DMSO, the IR spectrum matched that in CH_2Cl_2, showing that this mixture contains an insufficient amount of DMSO to disrupt peptide H-bonding. We therefore propose that head-to-tail inter-chain H-bonding is the organizing phenomenon responsible for cyclodimerization by CuAAC.

Two important corollaries to this hypothesis concern the role of resin and solvent: (a) the resin provides a relatively hydrophobic environment that promotes hydrogen bonding,[53,54] and (b) the 4:1 MeCN:DMSO solvent mixture solvates (swells) the resin, allowing Cu^I access to all the reactants, without disrupting H-bonding between pairs of chains. This solvent system was originally chosen for a very different reason – to provide the Cu^I center with what was thought to be advantageous coordination by the acetonitrile ligand – a notion that later kinetic studies on solution-phase CuAAC reactions proved to be erroneous. The crucially balanced role of the solvent was verified by the CuAAC reaction of **Resin-62** in pure DMSO (Figure 8), which is commonly used for solid-phase peptide synthesis due to its H-bond disrupting ability. Correlating with the observed change in H-bonding (Figure 9), this change in solvent changed the outcome of the reaction from cyclodimerization to monomeric cyclization, with small amounts of cyclic dimer observed along with some degraded material. We also note that monocyclization of two azido-alkyne oligopeptides has recently been reported using the highly polar and protic combination of 20% H_2O in *N*-methylpyrrolidinone (NMP) as solvent.[55]

A summary of the experimental factors favoring cyclodimerization *vs.* monomeric cylization is given in Table 2. The hypothesis of head-to-tail preorganization by inter-strand H-bonding, along with the above two corollaries, are consistent with these findings, as follows.

1. Backbone secondary amide groups in sufficient numbers are required. When H-bonding cannot occur, as with tertiary amides (Figures 7 and 8), or is too weak, as with shorter peptides (Figure 3), monomeric cyclization is favored as each alkyne finds itself in closest proximity to the azide of its own chain.
2. Being a function of main-chain amide linkages, cyclodimerization is independent of the identity of protected peptide side chains, at least in the series of 9-mers tested in Table 1.
3. Solution-phase cyclization of a protected peptide afforded predominantly cyclic monomer, whereas on-resin reaction of the same species gave cyclic dimer (Figure 2). When performed in solution (in this case, in MeOH-CH_2Cl_2), hydrogen bonding between chains is likely to be disrupted. Contrariwise, variation in resins loadings of 0.26−1.4 mmol/g did not affect the cyclodimerization efficiency, suggesting that H-bonding in the resin environment is a powerful force.
4. Cu-mediated cyclodimerization occurs for resin-bound α- and β-oligopeptides, but not γ-peptides (Figure 6). A potential antiparallel arrangement for each of these amide oligomers is shown in Figure 10; head-to-head alignments are somewhat less favorable and would not be productive in terms of azide-alkyne ligation. Sufficiently stable inter-chain interactions could arise from a combination of H-bonding density and dipolar stabilization. Oligo(α-peptides) have the greatest number of H-bonds per unit length, enough to overcome a less favorable dipolar arrangement derived from the alternating pattern of H-bond donors and acceptors.[56] Oligo(β-peptides)[57] compensate for the lesser H-bond density with a favorable H-bond dipolar arrangement.[56] Oligo(γ-peptides) have their H-bonds too far apart to stabilize inter-chain interactions, and do not undergo cyclodimerization.

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5. Cyclic dimers are less efficiently produced when peptides are inserted between the alkyne and the polystyrene backbone (Figure 4). We suggest that when H-bonding is maximized in these extended sequences, alkyne and azide are not placed optimally for cyclodimerization, as shown at the left of Figure 11. The productive alignment (Figure 11, right) is less favorable with respect to H-bonding, and so a poor yield of cyclic dimer is obtained.

6. Uncatalyzed azide-alkyne cycloaddition reactions are extremely slow when the alkyne is not electron-deficient, and so require the reactants to be held in close proximity in order to occur, even to a small degree, at room temperature.[38,39,58] The observation of Cu-free solid-phase oligomerization for oligo(β-Ala) species **49** and **50** (and probably oligo(Gly) **47**), but not for sequences containing γ-peptides, therefore supports the proposed existence of a preorganizing interaction between peptide chains. We suggest that the slow reaction in the absence of copper begins to link the head-to-tail oriented chains, and that cyclic dimers are formed when triazole formation is accelerated under conditions where H-bonding is maintained.

Peptide Cyclization and Cyclodimerization on Preparative Scale

To demonstrate the capability of the CuAAC process to produce substantial quantities of a large cyclic peptide, 240 mg of resin-**65** was prepared (Figure 12), and cleaved to obtain 57 mg of crude linear peptide (approximately 60% of the maximum yield based on the initial functional group loading of the resin), and 30 mg of purified peptide after reversed-phase HPLC. Cyclization of a parallel batch under standard dimerization conditions (20 mL of 4:1 MeCN:DMSO, 495 mg resin-**62**, 5 mM CuI, 30 μL 2,6-lutidine, sparged with N_2) followed by cleavage and deprotection returned 52 mg of crude cyclized material (compound **66**, 91% of the amount of the linear peptide obtained without CuAAC treatment), and 32 mg after purification. Thus, the cyclodimerization process was conducted with little or no loss of material. A third parallel batch of resin-**65** was cyclized under conditions to promote monomeric ring closure (20 mL DMSO + 1 drop H_2O, 5 mM CuI, 30 μL 2,6-lutidine, sparged with N_2) and cleaved from the resin to give only 25 mg of crude product, reflecting a difficulty in precipitating the more soluble cyclic monomer, and 8.5 mg of monocyclic triazole **67** after HPLC purification. In each case, HPLC and MALDI analysis showed the expected species (linear azido-alkyne peptide, cyclic monomer, or cyclic dimer) as the dominant species in the unpurified product mixture, and the purified products were characterized by HPLC, NMR, and high-resolution mass spectrometry (Supporting Information).

Conclusions

The CuAAC cyclodimerization process is quite robust for peptides six residues or longer, allowing the convenient synthesis of large bivalent[59] cyclic peptides. Furthermore, the analogous cyclic monomers can be obtained from the same resin-bound precursor with a simple change in solvent.

The success of peptide cyclodimerization derives from a fortuitous combination of resin and solvent properties. The discovery of the beneficial effects of the 4:1 MeCN:DMSO solvent mixture provides a useful lesson in the properties of resin-bound peptides, or rather reinforces an old one. Merrifield's focus on the role of resin in maintaining oligopeptide solubility as the key enabling feature of solid-phase peptide synthesis[53] is perhaps underappreciated today even by some who use Merrifield's methods. Our reaction conditions maintain a state intermediate between the full resin swelling desired for SPPS and the collapsed aggregates that bedevil peptide-bearing resins when insufficiently solvated. Furthermore, we failed to initially recognize inter-chain hydrogen bonding as the key organizing event for cyclodimerization because we assumed that 20% DMSO was enough to disrupt all H-bonding interactions.

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The CuAAC reaction is well suited to joining together of such noncovalently organized fragments into larger structures. Azides and alkynes are incorporated with ease into peptide building blocks and remain undisturbed under conditions of standard protection, deprotection, and coupling steps. The rate of the reaction is fast, which is crucial to the overall outcome. However, these features are not unique to CuAAC, and it should be possible to find other ligation reactions such as olefin metathesis,[60-62] nitrile imine-olefin cycloaddition,[63,64] or thiol-ene condensation,[65] to accomplish the same task. In addition to such studies, we will continue the development of cyclic triazole-linked peptides for biological and materials science.

Experimental Section

Peptide synthesis—All peptides were prepared by Fmoc SPPS methods using Rink amide MBHA resin with an initial loading of 0.70 mmol/g, unless otherwise noted. Resins were swollen in DMF for 45 min prior to synthesis. For sequence extension, the Fmoc-protected amino acid (4 equiv) was activated by treatment with HBTU or HCTU (3.9 equiv) and *N,N*-diisopropylethylamine (500 μL per mmol of amino acid) in DMF (4 mL per mmol of amino acid) for 2 min. This solution was added to the free amine on resin, and the coupling reaction was allowed to proceed for 15 min (longer for difficult couplings) with intermittent stirring. After washing with DMF, Fmoc deprotection was achieved with 20% 4-methylpiperidine in DMF (2×7 min). The resin was washed once again and the process was repeated for the next amino acid.

The alkyne group was usually incorporated as an N-propargylglycine unit by the method shown in Figure 13. The symmetric anhydride of 2-bromoacetic acid (5 equiv of anhydride relative to free amine) was prepared by mixing the acid (10 equiv) with *N,N′*-diisopropylcarbodiimide (DIC, 5 equiv) in DMF at room temperature for 15 min. This mixture was then added to the resin and allowed to react for 1 h with intermittent stirring. The resin was washed and then treated with propargylamine (20 equiv) in DMF for 12−16 h. After washing, coupling of the next residue to the resultant secondary amine was achieved by preparing the symmetrical anhydride of that protected residue (5 equiv of anhydride) with DIC in DMF at 0°C for 15 min, and coupling this mixture to the amine for 1 h with occasional stirring. This same coupling method was used for all instances when acylation of a secondary amine was required. Where the alkyne was incorporated by coupling Fmoc-L-propargylglycine, preactivation was carried out by mixing the protected unnatural amino acid with HOBt and DIC (4 equiv each) in DMF at 0°C for 20 min. Azide was incorporated by the attachment of either 5-azidopentanoic acid or 6-azidohexanoic acid by the standard peptide coupling procedure.

The following Fmoc-protected amino acids with side chain protecting groups were obtained from NovaBiochem and used as received: Fmoc-γAbu-OH, Fmoc-Ala-OH, Fmoc-βAla-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Gly-OH, Fmoc-His(Mtt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Phe-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Val-OH.

Peptide Cyclization—On-resin cyclization was carried out at the bench with solvents freshly deoxygenated by bubbling with nitrogen for at least five minutes. A CuI solution (usually 5 mM, but 20 mM was used in early experiments) was prepared using a solvent mixture of 20% DMSO in MeCN. Acetonitrile was required for solublizing the copper salt, while DMSO was crucial for resin swelling and peptide accessibility. A 4 mL vial was charged with 50 mg of peptide-loaded resin, to which was added 2 mL CuI solution and 5 μL of 2,6-lutidine. Cyclizations on larger scale were carried out in 20 or 40 mL scintillation vials as required. Nitrogen was gently bubbled through the mixture for 3 min, and the vial was capped, sealed

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with parafilm or electrical tape, and gently agitated on a rotisserie at room temperature for 16 or 40 h. After the reaction, the resin was washed successively with DMF, MeCN, and water. The resin was then gently stirred in a saturated aqueous solution of disodium EDTA (2 × 10 min) to remove residual bound copper, followed by sequential washing with water, MeCN, CH_2Cl_2, and diethyl ether, and drying in a vacuum desiccator.

Peptide Cleavage—Linear and cyclized peptides were cleaved from the resin with 2% triisopropylsilane (TIS) in trifluoroacetic acid (TFA, approx. 1 mL TFA/TIS per g resin) for 2.5 h. The cleavage mixture (including resin) was mixed with cold ether to precipitate the peptide and then filtered. The filtrate was washed with cold ether, and the peptide was extracted from the residue with H_2O/MeCN mixtures (typically 5−20% MeCN in water, 50% for peptoids) containing 0.1% TFA. The resulting solution was frozen and lyophilized to afford a white, solid product. A similar procedure was used for release of fully protected peptides from the resin, using 4% TFA in CH_2Cl_2 for 4 h. The peptide was precipitated from cold ether, filtered, extracted with 20% MeOH in CH_2Cl_2, evaporated to dryness, and purified by preparatory HPLC to give a white powder.

Analytical Methods—Peptides were analyzed by reversed phase analytical HPLC (RP-HPLC) at 1.0 mL/min flow rate using a Hewlett Packard Series 1100 instrument and a 4.7 × 150 mm Zorbax 300SB-C18 column (5 μm particle size), with detection at 220 nm. Gradients of 0−35% or 15−50% MeCN in H_2O over 20 min (with constant 0.1% TFA) were used. Preparatory HPLC was performed at 10 mL/min on a Dynamax SD-1 instrument using a Vydac 218TP series C18 column (22 × 250 mm, 10 μm particle size) and MeCN/H_2O with 0.1% TFA gradients as needed. MALDI mass spectra were obtained on a PerSeptive Biosystems Voyager-DE instrument.

Peptoid Synthesis—*N*-substituted glycine oligomers (peptoids) were synthesized on solid-phase support using modified submonomer synthesis protocols reported previously by Zuckermann *et al.*[66] Peptoid oligomers were synthesized by manual techniques as well as automated procedures on a robotic peptide synthesizer (Charybdis Instruments) with software program files written in-house. All reactions were completed at room temperature.

Rink amide resin (100 mg, 0.69 mmol/g) was swollen in 3 mL CH_2Cl_2 for 45 min before Fmoc deprotection. Typically, the resin was washed with DMF (4 × 2 mL) and CH_2Cl_2 (3 × 2 mL) between each synthetic step described below. Stoichiometries are based on the loading of the starting resin functional groups; that is, assuming quantitative yield at each coupling step. Fmoc deprotection was performed by treatment of the resin with 20% piperidine in DMF (20 min, 15 mL per g resin). The resin was washed and 20 equiv of bromoacetic acid (1.2 M in DMF, 8.5 mL per g resin) and 24 equiv DIC (2 mL per g resin) were added. The reaction mixture was agitated at room temperature for 20 min. After another resin wash, 20 equiv of the monomer amine (1.0 M in DMF, 10 mL per g resin) was added and the reaction mixture was shaken for 20 min at room temperature. Subsequent bromoacylation and amine displacement steps were performed to prepare peptoids of desired length.

Linear and cyclized peptoid products were cleaved from the solid support by treatment with 50% TFA in CH_2Cl_2 (40 mL per g resin) for 10 min at room temperature. The cleavage cocktail was evaporated under nitrogen gas flow and the products were resuspended at an approximate concentration of 2 mM in HPLC solvent (50% acetonitrile in water, with 0.1% TFA) for analysis by RP-HPLC and electrospray ionization mass spectrometry.

Supplementary Material

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Acknowledgments

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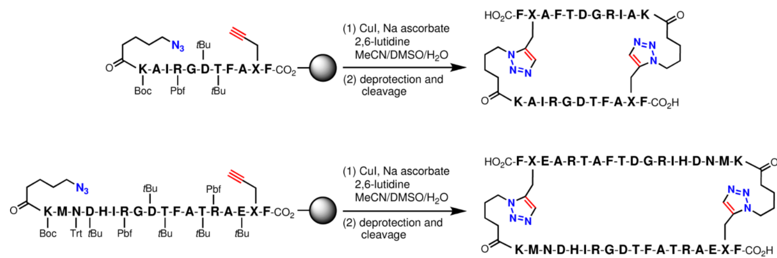

Figure 1.

On-resin peptide cyclodimerization of two sequences originally reported by Finn et al.[26] Amino acids are represented by their single-letter codes in bold print (X = propargylglycine).

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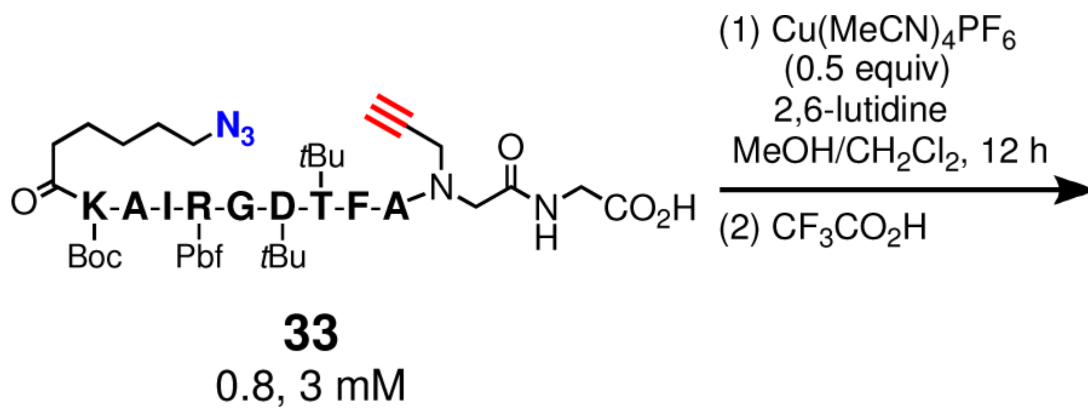

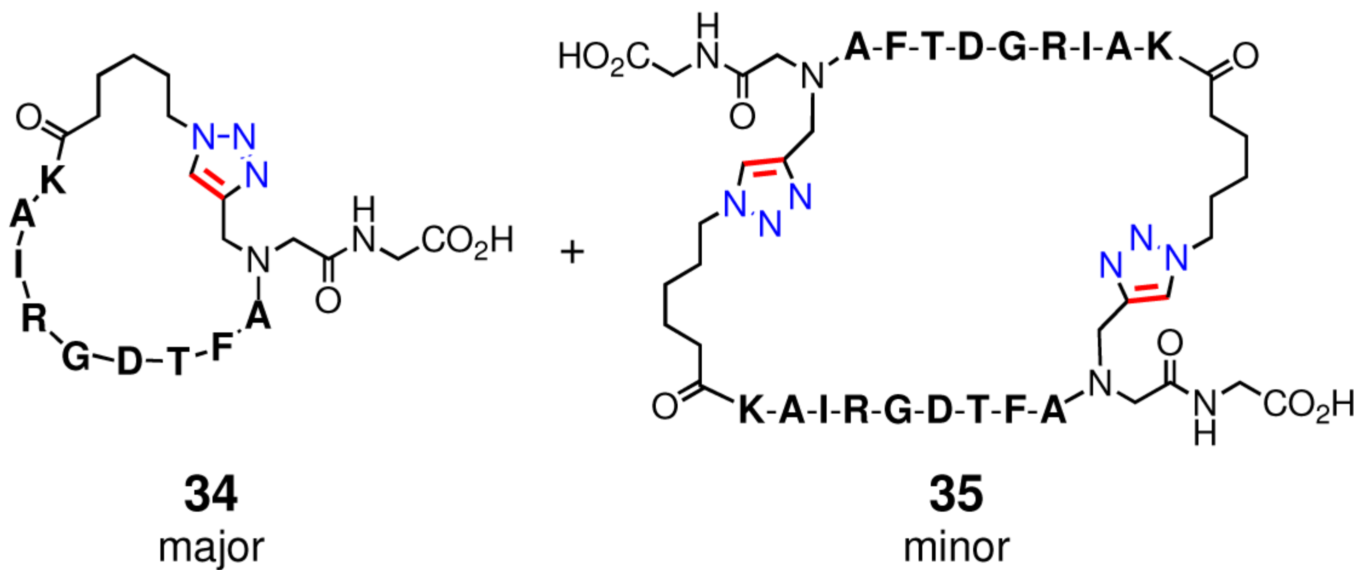

Figure 2.
Solution-phase cyclization of an alkyne- and azide-derivatized peptide.

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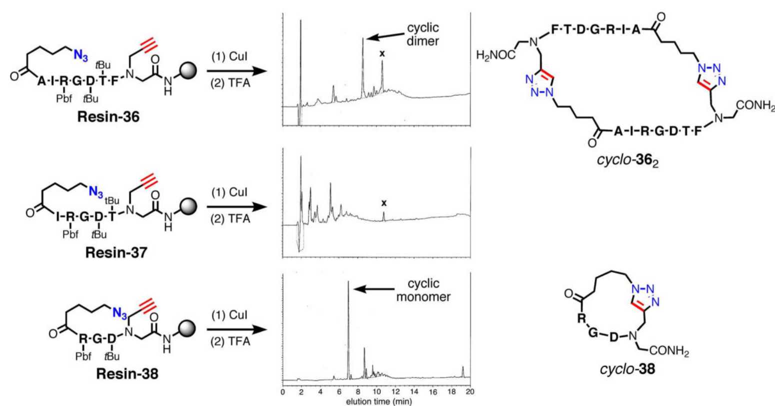

Figure 3.

Test of the dependence of sequence length on cyclization. RP-HPLC chromatograms of the crude products are shown. No linear monomers were observed, verified by co-injection of peptides before cyclization (not shown); “x” indicates peak due to a small amount of a chromophore with high extinction coefficient.

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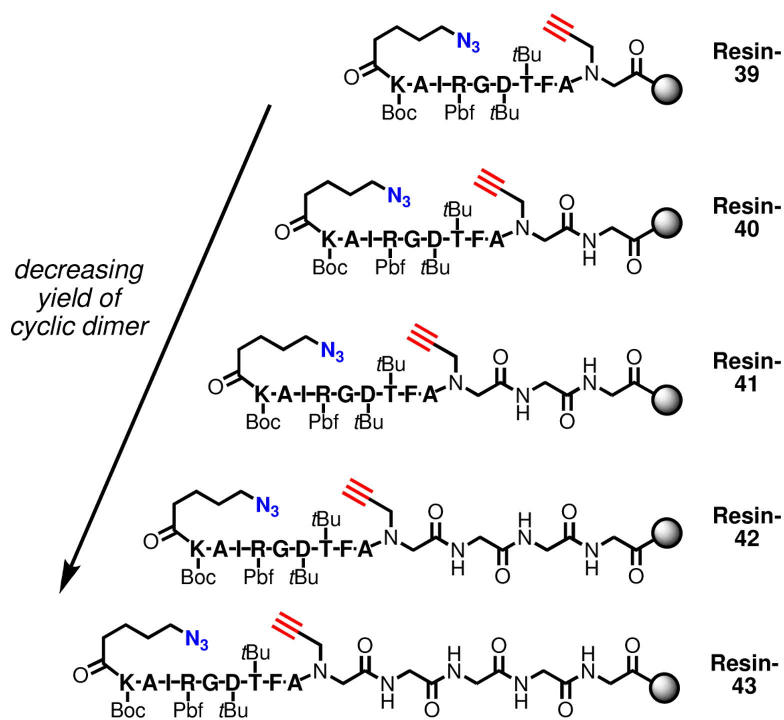

Figure 4.

Sequences prepared to determine the efficacy of cyclodimerization when the distance between alkyne and solid support (Rink amide MBHA resin) is increased.

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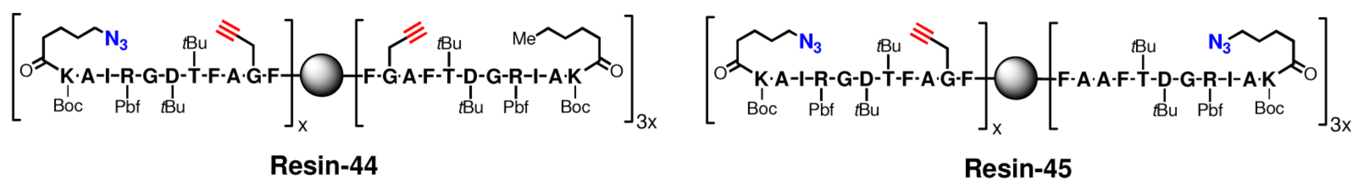

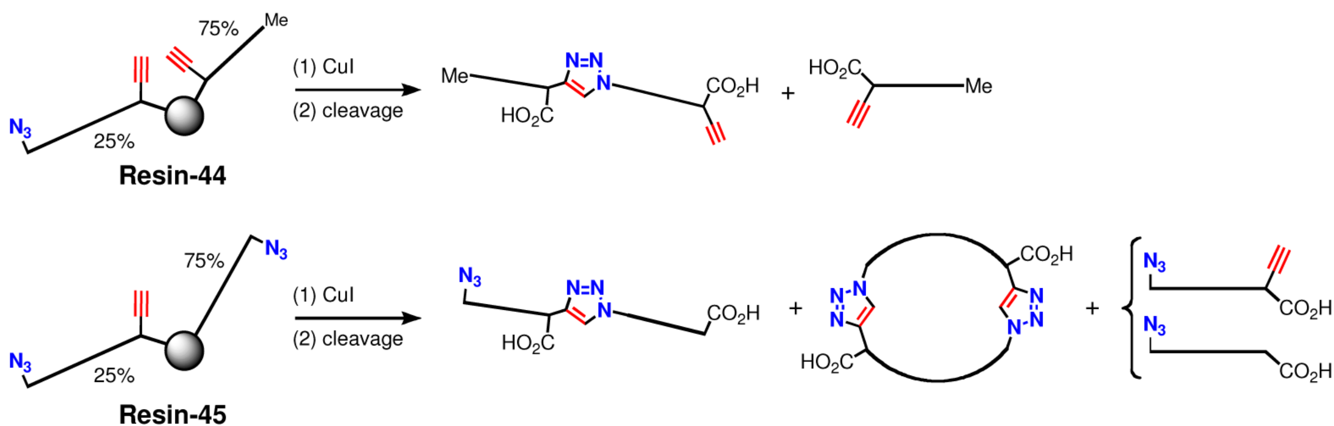

Figure 5.
(Top) Mixed peptide sequences with sub-stoichiometric quantities of azide or alkyne groups. (Bottom) Summary of the results from exposure to cyclization reaction conditions.

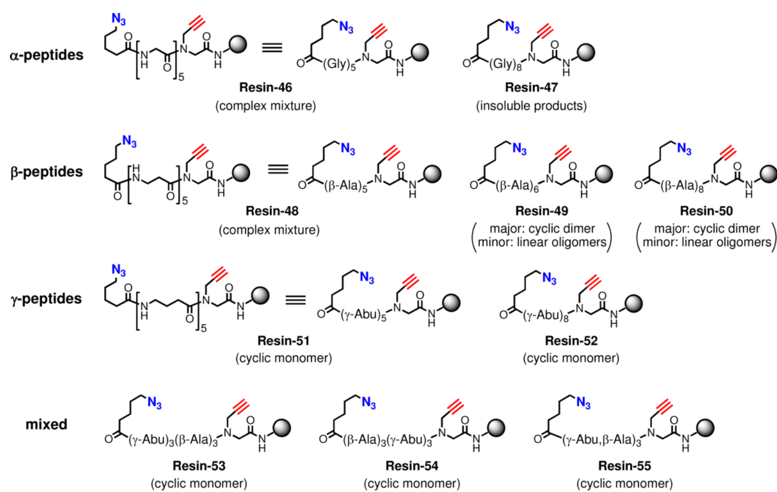

Figure 6.
Oligomers of β-Ala, γ-Abu, and Gly tested for on-resin CuAAC cyclization. The observed products of standard Cu^I treatment are indicated in parentheses.

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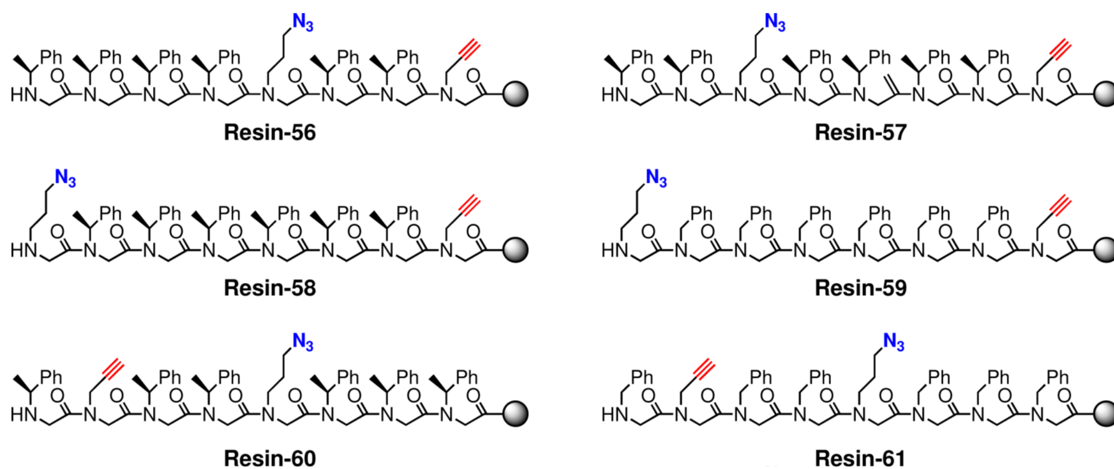

Figure 7.
Resin-bound peptoid sequences subjected to standard CuAAC conditions and subsequent cleavage with TFA. All returned cyclic monomers in clean fashion, with the exception of **61**, which gave cyclic monomer + oligomers.

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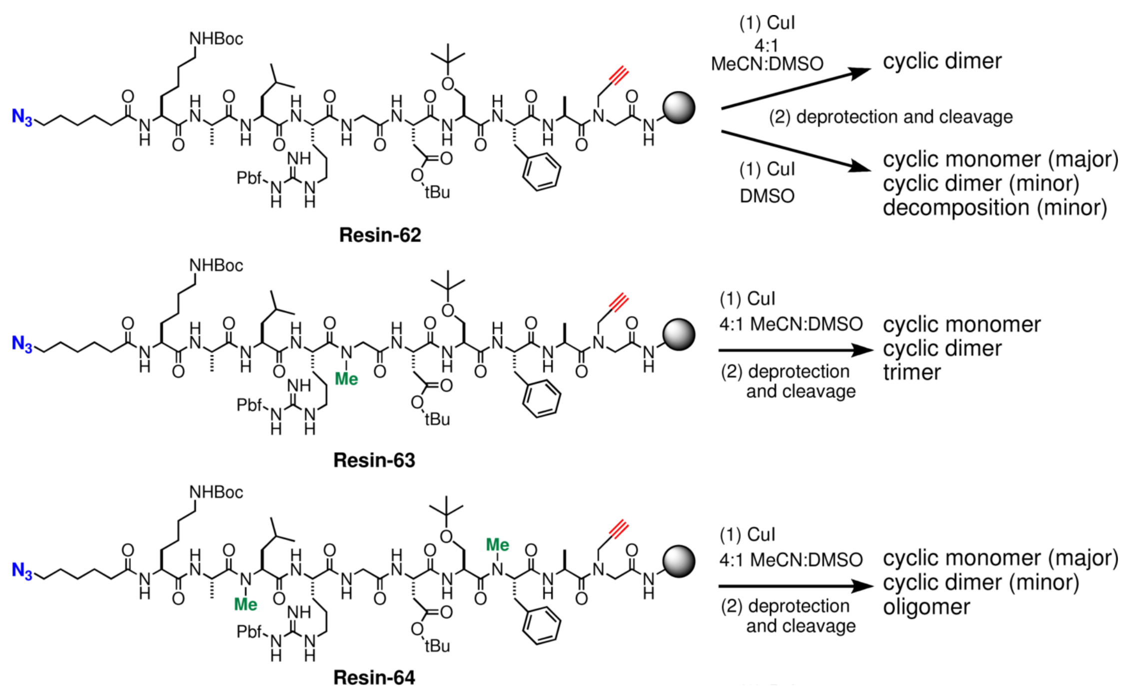

Figure 8.

Peptides used to test the dependence of backbone amide methylation on cyclodimerization, and their results after applying standard reaction conditions.

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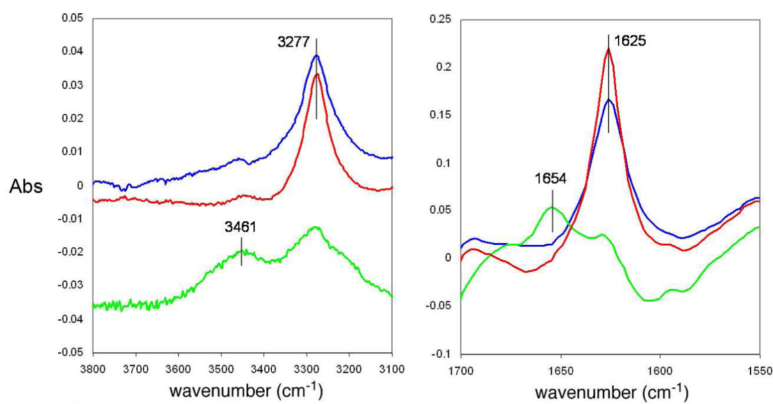

Figure 9.

IR spectra of protected resin-bound nonapeptide **39** swollen overnight in either CH_2Cl_2 (blue), DMSO (green), or the standard reaction solvent system of 4:1 MeCN/DMSO (red).

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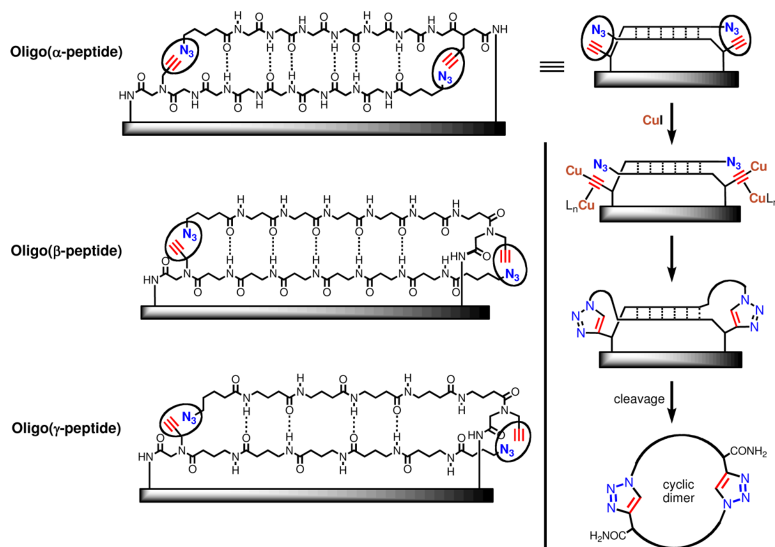

Figure 10.
(Left) Potential hydrogen patterns formed by head-to-tail alignments of oligo(Gly), oligo(β-Ala), and oligo(γ-Abu), placing inter-strand alkyne and azide moieties in close proximity to each other. The shaded bar represents the polystyrene chains to which the peptides are attached. (Right) Proposed CuAAC cyclodimerization pathway directed by head-to-tail H-bonding.

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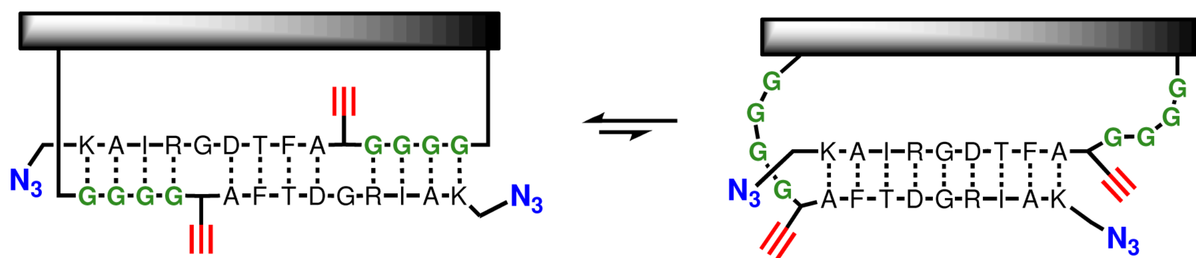

Figure 11.
Cartoon representations of proposed hydrogen-bonded alignments formed by resin-**43** (Figure 4).

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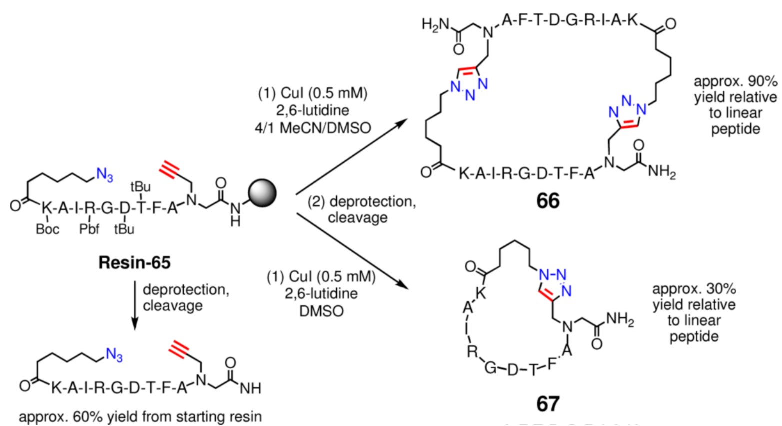

Figure 12.

Preparative-scale syntheses of a representative cyclic dimer and monomer.

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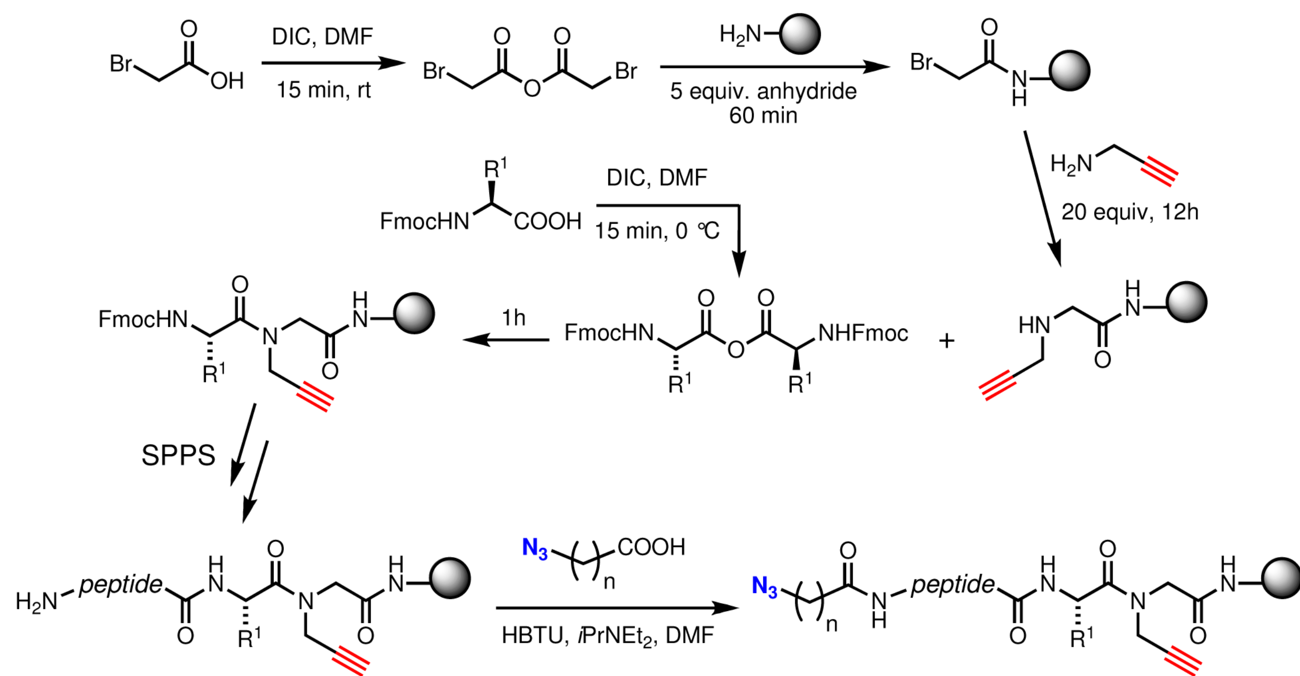

Figure 13.

Incorporation of alkyne and azide groups during solid-phase peptide synthesis.

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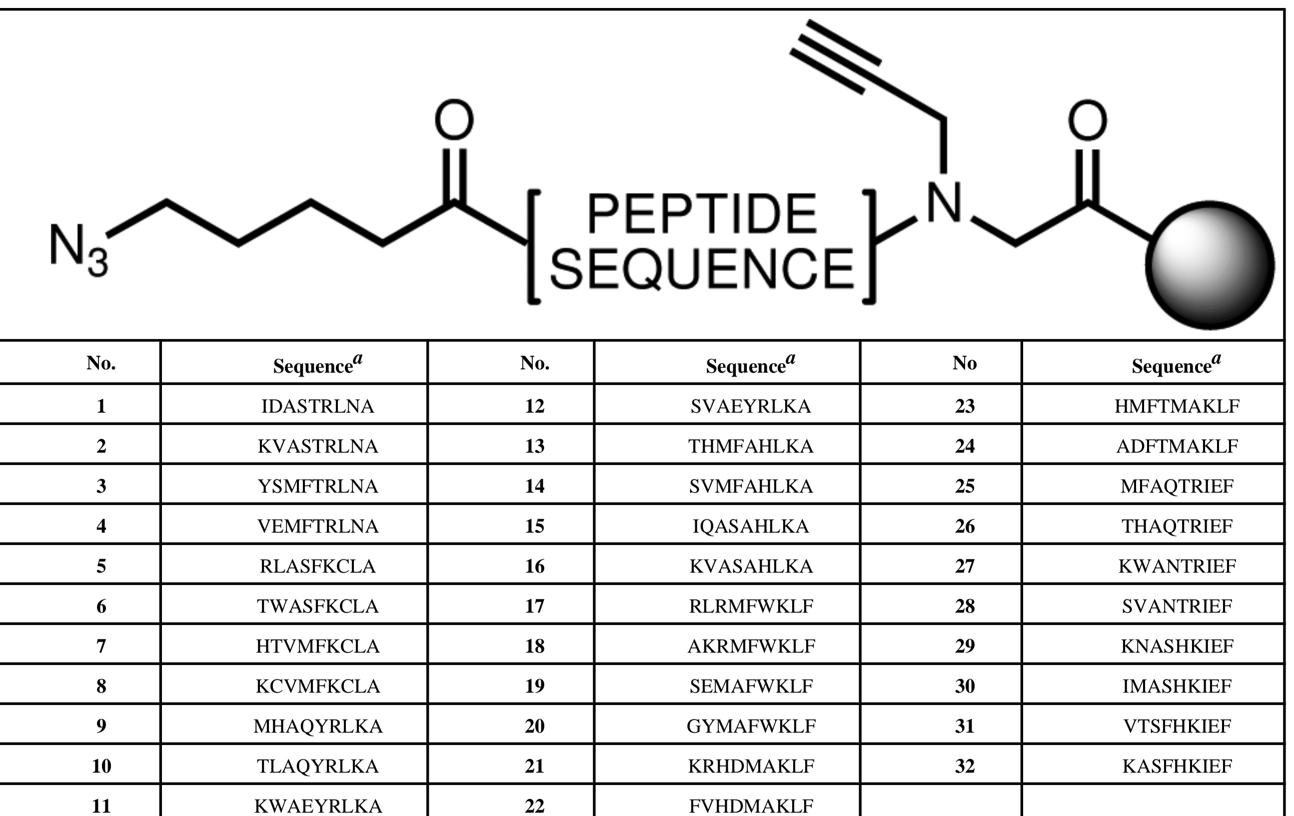
Table 1

Peptide sequences used to test the dependence of cyclodimerization on amino acid composition. All peptides bear protected side chain functional groups as listed in the Experimental section, and all were cyclodimerized successfully by treatment with CuI in 4:1 MeCN:DMSO.



No.	Sequence[a]	No.	Sequence[a]	No	Sequence[a]
1	IDASTRLNA	12	SVAEYRLKA	23	HMFTMAKLF
2	KVASTRLNA	13	THMFAHLKA	24	ADFTMAKLF
3	YSMFTRLNA	14	SVMFAHLKA	25	MFAQTRIEF
4	VEMFTRLNA	15	IQASAHLKA	26	THAQTRIEF
5	RLASFKCLA	16	KVASAHLKA	27	KWANTRIEF
6	TWASFKCLA	17	RLRMFWKLF	28	SVANTRIEF
7	HTVMFKCLA	18	AKRMFWKLF	29	KNASHKIEF
8	KCVMFKCLA	19	SEMAFWKLF	30	IMASHKIEF
9	MHAQYRLKA	20	GYMAFWKLF	31	VTSFHKIEF
10	TLAQYRLKA	21	KRHDMAKLF	32	KASFHKIEF
11	KWAEYRLKA	22	FVHDMAKLF		

[a] Single letter amino acid codes represent the resin-bound N-to-C sequence present between azide and alkyne moieties with the general structure shown above the table.

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Table 2

Summary of conditions found to promote dimeric *vs*. monomeric CuAAC cyclization of peptides.

Dimeric Cyclization	Competing Monomeric Cyclization	Reliable Monomeric Cyclization
side chain groups protected	side chain groups protected	side chain groups protected
polystyrene-based resin swelled in 4:1 MeCN:DMSO		polystyrene-based resin swelled in DMSO or protic solvent, or reaction done in solution
6 or more amino acids in length	4 or fewer amino acids in length	tertiary backbone amide bonds
α- or β-peptides	γ-peptides	
alkyne group installed close to polystyrene backbone		