Azide–Alkyne Cycloadditions

Head-to-Tail Peptide Cyclodimerization by Copper-Catalyzed Azide–Alkyne Cycloaddition**

*Sreenivas Punna, Jane Kuzelka, Qian Wang, and M. G. Finn**

Cyclic peptides and related structures have received attention in a variety of fields pertinent to drug discovery and biochemistry.[1] Peptide cyclization[2] has been managed most commonly by the formation of amide,[3] ester,[4] disulfide,[3b,5] olefin,[6] and C–C[7] bonds. Most methods include cyclization as part of solid-phase peptide synthesis, with ring closure performed on the resin support.[8] Olefin metathesis is particularly attractive for ring closure, because terminal alkenes and the transition-metal catalysts used to manipulate them are generally unreactive with protein functional groups.[6] Thus, the "handles" for cyclization can be installed and ignored until the time comes for their connection. We report herein a conceptually similar approach with different chemistry—the copper(I)-catalyzed azide–alkyne cycloaddition reaction[9]—and describe the propensity of this process to give selective dimerization in the ring-closure step.

In the course of constructing linear and cyclic versions of the same peptide sequence for display on supramolecular protein scaffolds, we needed a cyclization method compatible with the requirements of side-chain protection/deprotection and with the installation of a reactive group to enable subsequent bioconjugation. The 11-mer and 19-mer Arg-Gly-Asp (RGD)-containing peptides **1** and **2** (Scheme 1) contain sequences taken from an adenovirus serotype that binds several α_v integrins.[10] They were synthesized by standard 9-fluorenylmethyloxycarbonyl (Fmoc) methods, starting with Fmoc-Phe-Wang resin and L-propargylglycine as the second residue installed. The end of each chain was terminated with side-chain Boc-protected Fmoc-lysine (Boc = *tert*-butoxycarbonyl), followed by Fmoc deprotection and capping of the N terminus with 5-azidopentanoic acid. The syntheses were also performed with a standard Boc-based protocol, starting

[*] Dr. S. Punna, Dr. J. Kuzelka, Dr. Q. Wang,[+] Prof. M. G. Finn
Department of Chemistry
The Scripps Research Institute
10550 N. Torrey Pines Rd., La Jolla, CA 92037 (USA)
Fax: (+1) 858-784-8850
E-mail: mgfinn@scripps.edu

[+] Present address:
Department of Chemistry & Biochemistry
University of South Carolina (USA)

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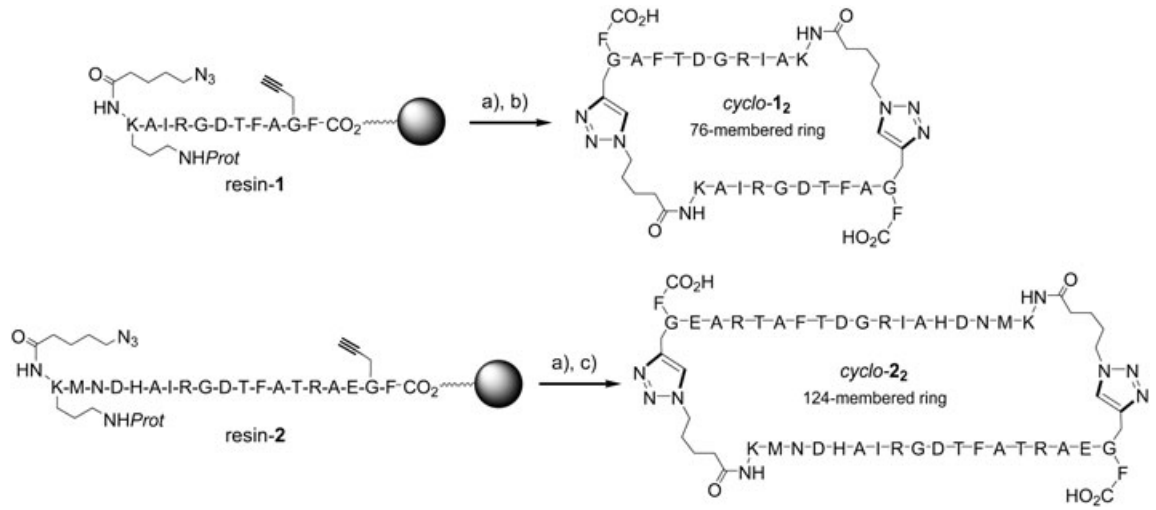


Scheme 1. Peptide cyclization by azide–alkyne cycloaddition: a) CuI (0.5 equiv), Na ascorbate (1 equiv), 2,6-lutidine (2 equiv), $CH_3CN/DMSO/H_2O$ (8:2:1), room temperature, 16 h; b) $CF_3CO_2H/H_2O/SiH(iPr)_3$ (95:2.5:2.5), room temperature, 3 h; c) CF_3CO_2H/H_2O/1,2-ethanedithiol/$SiH(iPr)_3$ (94:2.5:2.5:1), room temperature, 3 h. Initial substitution for both resins **1** and **2** was 0.77 mmol g^{-1}. *Prot* = protecting group.

with a Boc-Phe-PAM (4-hydroxymethylphenylacetamidomethyl) resin, terminated with Z- (benzyloxycarbonyl) or Fmoc-protected lysine, and finished with the appropriate capping step to install the azide group.[11]

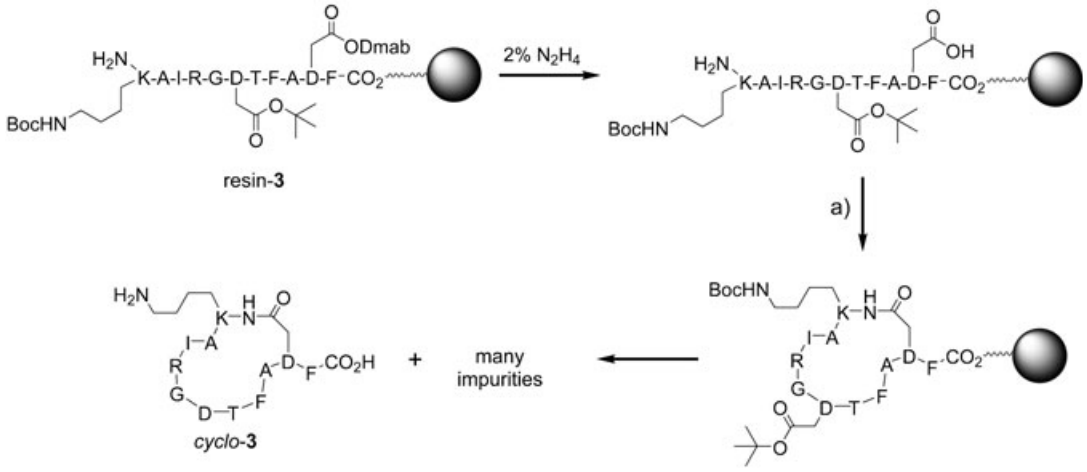
Cyclization was carried out by exposure of each resin to 0.5 equivalents of Cu^I (with respect to the maximum possible amount of alkyne) for 16 h at room temperature. The complete consumption of azide groups was verified by a modified ninhydrin test developed for the detection of resin-bound azides,[12] which is more sensitive than IR spectroscopy. In the absence of Cu^I, no reaction occurred even upon heating to 100 °C. Cleavage from the solid support and HPLC analysis showed the complete disappearance of the linear oligopeptides and the production of a single dominant species in each case, which proved to be cyclic dimers rather than monomers (Scheme 1). The yields of isolated purified linear and cyclic peptides (linear-**1**, 25–30 %; linear-**2**, 15–20 %; *cyclo*-$\mathbf{1_2}$, 15–20 %; *cyclo*-$\mathbf{2_2}$, 10–15 %, all relative to the starting resin) reflect 90–95 % yields for peptide coupling and cleavage steps, and approximately 60 % yield for cyclodimerization, including loss during purification by HPLC.

MALDI MS analyses under a variety of conditions showed *cyclo*-$\mathbf{1_2}$ and *cyclo*-$\mathbf{2_2}$ to be of dimeric molecular mass, m/z $[M+H]^+ = 2690$ and 4602, respectively. Further support for the assignment of cyclic dimers was supplied by the following four observations, details of which are provided in the Supporting Information:

1) Peptides cleaved from the resin before cyclization, but not the cyclized products, were reactive with phosphine, which confirms the consumption of the azide groups in the cyclization step.
2) High-resolution ESI MS showed strong peaks at m/z values that correspond to the addition of odd numbers of protons to the dimers and that could not arise from monomers.
3) The use of a mixture of two amino acids in one of the chain-building steps toward resin-**1** provided a mixture of two sequences on the resin, which gave the three cyclized products expected from cyclodimerization.
4) Trypsin digestion of *cyclo*-$\mathbf{1_2}$ was monitored as a function of time, showing one and two cleavage events to give products of greater than monomeric molecular weight, ruling out the existence of a catenated structure.

Whereas cyclic peptide dimers have been previously prepared and shown to have interesting functional properties,[13] the selective production of such large rings as *cyclo*-$\mathbf{1_2}$ and *cyclo*-$\mathbf{2_2}$ is unprecedented. The following experiments were performed to probe the mechanism of the process:

a) Cyclization reactions of analogous resins that have a lower density of peptide chains were found to be much slower. Thus, resin-**1** and resin-**2** were prepared from Fmoc-Phe-Wang resin with an initial loading of 0.12 mmol g^{-1} (1.2 derivatized styrene units per 100) instead of 0.77 mmol g^{-1} (9 functionalized monomers per 100). With the same ratio of copper to alkyne (and therefore much more dilute in both), a substantial amount of unconverted peptide remained after 24 h, but cyclic dimers were still the only products detected. This suggests that a threshold density of azides or alkynes is required on the solid support, and that the dimeric pathway is favored even when two chains are difficult to bring together.
b) Cyclodimerization was neither favored by the peptide sequence itself nor by the presence of copper ions, as shown by the attempted ring closure with amide bond formation shown in Scheme 2. HBTU-mediated cyclization of resin-**3**, incorporating orthogonally-protected aspartic acid in place of propargyl glycine, gave poor results, and no compounds of dimeric molecular weight were observed.[14] The expected monocyclic peptide *cyclo*-**3** was detected by mass spectrometry, but the material was impossible to isolate by HPLC owing to the presence of many impurities. Added copper(I) iodide did not induce dimer formation in this process.



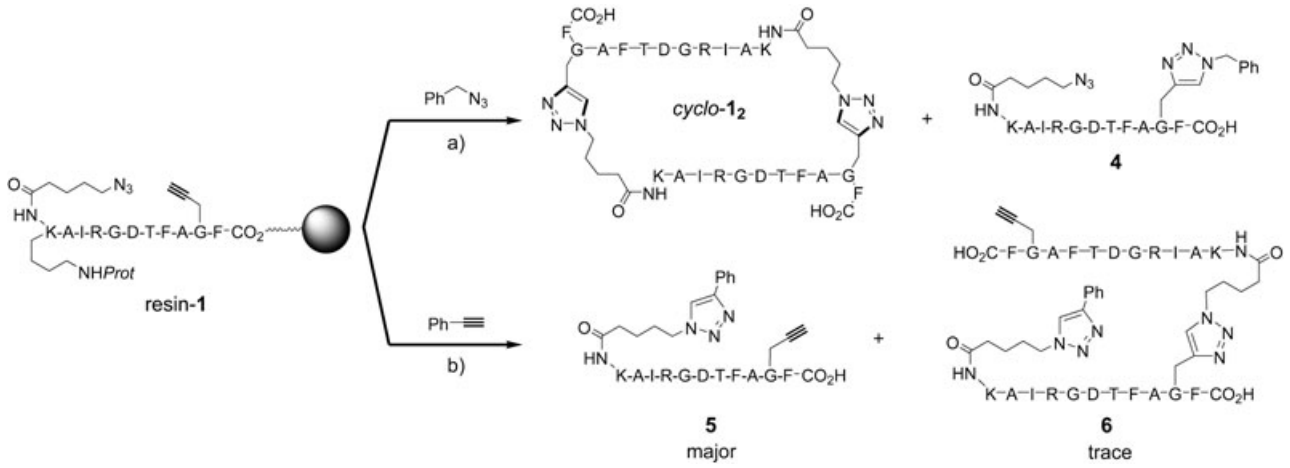
Scheme 2. Peptide cyclization by amide bond formation: a) Performed with and without Cu^{I} (0.5 equiv); HBTU (5 equiv), iPr_2NEt, DMF, room temperature. DMF = dimethylformamide; HBTU = *O*-(1*H*-benzotriazoyl-1-yl)-*N*,*N*,*N*′,*N*,′-tetramethyluronium hexafluorophosphate.

c) The use of more than 0.5 equivalents of Cu with respect to the maximum number of alkyne groups was deleterious. For example, when 10 equivalents of Cu were employed with resin-**1**, many peaks were observed by HPLC, none of them representing *cyclo*-$\mathbf{1_2}$ or linear-**1**. With 1.5 equivalents of Cu, cyclic dimer was recovered in lower yield than before, along with some of the linear unconverted peptide. Small peaks in the chromatogram suggested peptide decomposition in the presence of excess copper.[15] The addition of the copper-binding ligand 4,4′-dimethylbipyridine to the latter process (1.5 equiv Cu to alkyne; 2 equiv ligand to Cu)[16] suppressed much of the reaction, giving unaltered linear peptide as the major recovered material.

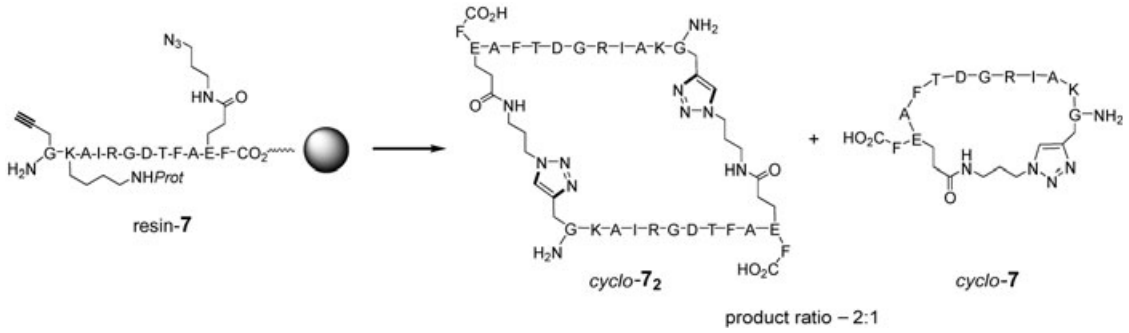
d) The cyclization of resin-**1** performed in the presence of added benzyl azide provided *cyclo*-$\mathbf{1_2}$ and the azide-trapped monomer **4** as major products (Scheme 3). In contrast, the addition of phenylacetylene completely suppressed cyclization, giving rise to the alkyne-trapped monomer **5** as the major product, with a trace of the linear dimer **6**, detected by MS. The different outcomes correlate with the much greater affinity of Cu^{I} for alkyne over azide, as discussed below.

e) Switching the positions of the azide and alkyne fragments allowed some monocyclization to occur. Resin-**7** was constructed with a side-chain-derivatized glutamic azide in the second amino acid position and propargyl glycine at the end of the chain (Scheme 4). Subjection of this material to the standard cyclization conditions gave an approximate 2:1 mixture of cyclic dimer *cyclo*-$\mathbf{7_2}$ and cyclic monomer *cyclo*-**7**, along with a greater amount of impurities than were produced from the analogous resin-**1**.

The kinetic parameters of the reaction provide the basis for understanding the factors that cause cyclization to involve two peptide chains rather than one. In the previous paper in this issue,[17] we showed that the solution-phase reaction between benzyl azide and phenylacetylene exhibits a second-order dependence on Cu^{I}. Peptide cyclodimerization occurs cleanly only when less than 1 equivalent of copper per alkyne unit is present—when most or all of the metal is tied up in the



Scheme 3. Cu^{I}-mediated peptide cyclization in the presence of added azide and alkyne: a) Cu^{I} (0.5 equiv), benzyl azide (0.5 equiv), then cleavage from resin; b) Cu^{I} (0.5 equiv), phenylacetylene (0.5 equiv), then cleavage from resin.



Scheme 4. Cyclization of peptide bearing azide and alkyne in "switched" positions.

form of Cu–acetylide on the resin. If the reaction of these resin-bound species also requires the participation of two copper centers, then only adjacent chains that happen to have copper bound to their alkyne residues will engage in the cycloaddition reaction. (The 1% cross-linked polystyrene used herein is a highly flexible material,[18] so the term "adjacent" refers simply to those chains for which the alkyne groups can reach each other without too great an energetic cost.) In other words, the mechanism of the copper-mediated reaction demands the interaction of two peptide chains.

As the peptides used in this case are long and flexible, there must be some additional factor(s) that induce the azide of one chain to engage the alkyne of its neighbor rather than its own. We suggest at least three potential contributing interactions (Figure 1):

1) As the solution-phase reaction showed a rate order in PhC≡CH of between 1 and 2, a second-order pathway in alkyne is possible, with one alkyne playing a structural role. If, for example, one Cu–acetylide of associated chains undergoes π complexation to the other, as shown in structure **8**, the reactivities of the two acetylides toward azide will be different.
2) An interaction of a Cu^I center with the peptide chain may fold the oligopeptide in such a way as to make it more difficult for the azide at the terminus to interact with the Cu–acetylide at its base, also represented in structure **8**. Since no clear Cu^I-binding side chains (His, Trp, Cys, Met) were present in structure **1** (one Met residue exists in **2**), we believe that Cu binding to the peptide may occur at the backbone amide groups.[19] Other possibilities that derive from conformational bias imposed by the peptide sequence are being explored, but we do not see how such factors could generally favor dimeric cyclization.
3) Once the directed cycloaddition occurs to make one triazole connection, the organometallic Cu–triazole intermediate **9** may bind and direct the second azide to engage the remaining acetylide and complete the macrocyclization. A similar phenomenon is proposed in the accompanying article to explain the anomalously fast second cycloaddition process in the conversion of tethered diazides into ditriazoles. Although this does not bear on the question of monomeric versus dimeric cyclization, it may help explain why linear dimers are not observed.

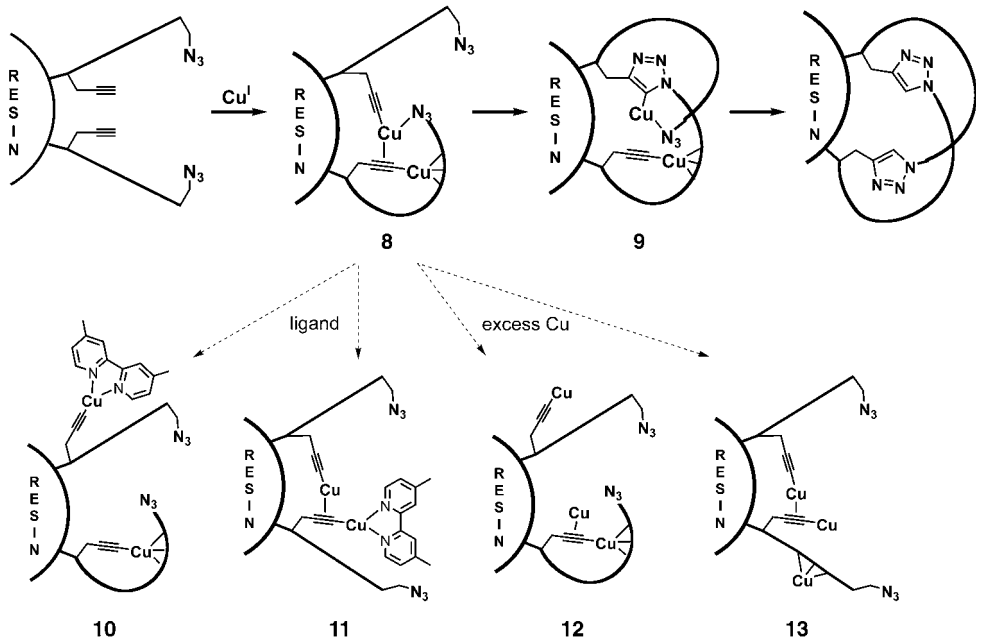


Figure 1. Schematic illustration of potential interactions leading to selective cyclodimerization.

The experimental observations are accommodated by this mechanistic scheme as follows: the slow rate of reaction on a lightly loaded resin (observation (a), above) can be ascribed to a dramatic decrease in the concentration of adjacent Cu-bearing sites. The deleterious effects of bidentate ligand or excess Cu^{I} (observation c)) are similarly consistent with the loss of the required binuclear Cu–alkyne–peptide ensemble, either by disrupting Cu–alkyne–Cu binding (**10** and **12**, Figure 1) or Cu–peptide interaction (**11** and **13**). Added alkyne can also block cyclodimerization (observation d)) by competing with the resin-bound alkyne for Cu^{I}, resulting in capture of peptide azide by [Cu−C≡CPh] to afford linear monotriazole **5** (Scheme 3). Benzyl azide does not bind copper with sufficient affinity to take the metal away from the oligopeptide alkyne, so the putative $[Cu_2(alkyne)_2]$ species is either trapped by pendant azide to give the usual cyclic dimer or intercepted by free azide to give linear monomer **4** (Scheme 3). The opening of a competitive monocyclization pathway by placing the alkyne away from the polystyrene backbone (observation e)) suggests that such adjacent Cu–acetylide centers can come together with greater conformational flexibility, allowing the reactive ensemble to find either azide residue of its component chains.

The selective production of large cyclic dimers from resin-tethered peptides containing azide and alkyne residues is a striking consequence of the mechanistic constraints of the copper-catalyzed cycloaddition reaction. The process has clear utility in the construction of large and highly functional molecules if the presence of the triazole moiety can be tolerated.[20] Indeed, a solution-phase synthesis of head-to-tail cyclic dimers of trisaccharides to make ditriazole-containing cyclodextrin analogues has been recently reported.[21] The on-resin phenomena described herein and related future studies of the reaction in solution provide a versatile forum for the continued exploration of the reaction mechanism. The potential for interplay between peptide length, flexibility, secondary structure, and copper–acetylide interactions is especially interesting. We are also exploring the preparation of peptide libraries, catenanes, and nonpeptide cyclic structures.

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[1] a) J. S. Davies in *Cyclic Polymers*, 2nd ed. (Ed.: J. A. Semlyen), Kluwer Academic Publishers, Dordrecht, **2000**, pp. 85–124; b) H. Kessler, *Angew. Chem.* **1982**, *94*, 509–520; *Angew. Chem. Int. Ed. Engl.* **1982**, *21*, 512–523; c) J. S. Davies, *Amino Acides Pept. Proteines* **2002**, *33*, 238–294; d) F. Sarabia, S. Chammaa, A. S. Ruiz, L. M. Ortiz, F. J. L. Herrera, *Curr. Med. Chem.* **2004**, *11*, 1309–1332; e) E. Lohof, E. Planker, C. Mang, F. Burkhart, M. A. Dechantsreiter, R. Haubner, H.-J. Wester, M. Schwaiger, G. Holzemann, S. L. Goodman, H. Kessler, *Angew. Chem.* **2000**, *112*, 2868–2871; *Angew. Chem. Int. Ed.* **2000**, *39*, 2761–2764; f) Y.-Q. Tang, J. Yuan, G. Osapay, K. Osapay, D. Tran, C. J. Miller, A. J. Ouellette, M. E. Selsted, *Science* **1999**, *286*, 498–502; g) B. W. Matthews, C. S. Craik, H. Neurath, *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 4103–4105.

[2] a) P. Li, P. P. Roller, J. Xu, *Curr. Org. Chem.* **2002**, *6*, 411–440; b) J. N. Lambert, J. P. Mitchell, K. D. Roberts, *J. Chem. Soc. Perkin Trans. 1* **2001**, 471–484.

[3] a) J. T. Lundquist IV, J. C. Pelletier, *Org. Lett.* **2002**, *4*, 3219–3221; b) E. Falb, Y. Salitra, T. Yechezkel, M. Bracha, P. Litman, R. Olender, R. Rosenfeld, H. Senderowitz, S. Jiang, M. Goodman, *Bioorg. Med. Chem.* **2001**, *9*, 3255–3265; c) Y. Shao, W. Lu, S. B. H. Kent, *Tetrahedron Lett.* **1998**, *39*, 3911–3914.

[4] a) D. Ranganathan, V. Haridas, S. Kurur, R. Nagaraj, E. Bikshapathy, A. C. Kunwar, A. V. S. Sarma, M. Vairamani, *J. Org. Chem.* **2000**, *65*, 365–374; b) W. D. F. Meutermans, S. W. Golding, G. T. Bourne, L. P. Miranda, M. J. Dooley, P. F. Alewood, M. L. Smythe, *J. Am. Chem. Soc.* **1999**, *121*, 9790–9796.

[5] a) B. A. Katz, *Biochemistry* **1995**, *34*, 15421–15429; b) D. Ranganathan, V. Haridas, R. Nagaraj, I. L. Karle, *J. Org. Chem.* **2000**, *65*, 4415–4422.

[6] a) S. J. Miller, R. H. Grubbs, *J. Am. Chem. Soc.* **1995**, *117*, 5855–5856; b) S. J. Miller, H. E. Blackwell, R. H. Grubbs, *J. Am. Chem. Soc.* **1996**, *118*, 9606–9614; c) T. D. Clark, M. R. Ghadiri, *J. Am. Chem. Soc.* **1995**, *117*, 12364–12365; d) J. Pernerstorfer, M. Schuster, S. Blechert, *Chem. Commun.* **1997**, 1949–1951; e) A. S. Ripka, R. S. Bohacek, D. H. Rich, *Bioorg. Med. Chem. Lett.* **1998**, *8*, 357–360; f) H. E. Blackwell, R. H. Grubbs, *Angew. Chem.* **1998**, *110*, 3469–3472; *Angew. Chem. Int. Ed.* **1998**, *37*, 3281–3284; g) N. Schmiedeberg, H. Kessler, *Org. Lett.* **2002**, *4*, 59–62.

[7] M. Hiroshige, J. R. Hauske, P. Zhou, *J. Am. Chem. Soc.* **1995**, *117*, 11590–11591.

[8] S. A. Kates, N. A. Sole, F. Albericio, G. Barany in *Peptides* (Eds.: C. Basava, G. N. Anantharamaiah), Birkhaeuser, Boston, **1994**, pp. 39–58.

[9] a) C. W. Tornøe, C. Christensen, M. Meldal, *J. Org. Chem.* **2002**, *67*, 3057–3062; b) V. V. Rostovtsev, L. G. Green, V. V. Fokin, K. B. Sharpless, *Angew. Chem.* **2002**, *114*, 2708–2711; *Angew. Chem. Int. Ed.* **2002**, *41*, 2596–2599.

[10] G. R. Nemerow, P. L. Stewart, *Virology* **2001**, *288*, 189–191.

[11] For details, see Supporting Information.

[12] S. Punna, M. G. Finn, *Synlett* **2004**, 99–100.

[13] a) B. P. England, P. Balasubramanian, I. Uings, S. Bethell, M.-J. Chen, P. J. Schatz, Q. Yin, Y.-F. Chen, E. A. Whitehorn, A. Tsavaler, C. L. Martens, R. W. Barrett, M. McKinnon, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 6862–6867; b) Z. Szewczuk, P. Buczek, P. Stefanowicz, K. Krajewski, Z. Wieczorek, I. Z. Siemion, *Acta Biochim. Pol.* **2001**, *48*, 121–130.

[14] The use of azide and alkyne connecting groups avoids certain protecting group problems commonly encountered in solid phase peptide synthesis. For example, orthogonal protection was required for an aspartate residue of resin-**3** to prepare for cyclization by amide bond formation. The 4-(*N*-(1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl)amino)benzyl ester (Dmab) group, most commonly used in such situations, is expensive, bulky, and chemically sensitive: T. Johnson, M. Liley, T. J. Cheeseright, F. Begum, *J. Chem. Soc. Perkin Trans. 1* **2000**, 2811–2820. The introduction and manipulation of aspartate can give rise to undesired aspartamide cyclization with neighboring residues during both Fmoc and Boc synthetic approaches and piperidide formation during Fmoc manipulations; deprotection is often relatively difficult. The standard solutions to these difficulties (such as protecting the preceding residue as *N*-Hmb; Hmb = 2-hydroxy-4-methoxybenzyl) are also somewhat cumbersome. Each of these problems were encountered in the synthesis and isolation of *cyclo*-**3**, but did not occur in the preparation and reactions of the analogous sequence **1**.

[15] J. T. Groves, R. R. Chambers, Jr, *J. Am. Chem. Soc.* **1984**, *106*, 630–638.

[16] W. G. Lewis, F. G. Magallon, V. V. Fokin, M. G. Finn, *J. Am. Chem. Soc.* **2004**, *126*, 9152 – 9153.
[17] See the preceding Communication in this issue: V. O. Rodionov, V. V. Fokin, M. G. Finn, *Angew. Chem.* **2005**, *117*, 2250; *Angew. Chem. Int. Ed.* **2005**, *44*, 2210 (DOI: 10.1002/anie.200461496).
[18] a) J. I. Crowley, T. B. Harvey, III, H. Rapoport, *J. Macromol. Sci. Part A* **1973**, *7*, 1117 – 1126; b) S. L. Regen, D. P. Lee, *J. Am. Chem. Soc.* **1974**, *96*, 294 – 296; c) J. Rebek, Jr., J. E. Trend, *J. Am. Chem. Soc.* **1979**, *101*, 737.
[19] The formation of peptide dimers with copper(II) by interaction with backbone amide nitrogen atoms has been described: H. Yokoi, A. Hanaki, *Chem. Lett.* **1983**, *8*, 1319 – 1322. This is, of course, different from copper(I), for which many protein interactions are known, but all involve anchoring to at least one "soft" side-chain ligand.
[20] Q. Su, A. B. Beeler, E. Lobkovsky, J. A. Porco, Jr., J. S. Panek, *Org. Lett.* **2003**, *5*, 2149 – 2152.
[21] K. D. Bodine, D. Y. Gin, M. S. Gin, *J. Am. Chem. Soc.* **2004**, *126*, 1638 – 1639.

