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Supporting Information

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

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General. Fmoc-Phe-Wang (0.77 mmol/g, 100-200 mesh and 0.12 mmol/g, 100-200 mesh substitution), Boc-Phe-PAM (0.7 mmol/g substitution, 100-200 mesh), and Fmoc- and Boc-protected amino acids were purchased from Chem-Impex International and Midwest Bio-Tech, Inc. Fmoc- and Boc-L-propargylglycine were purchased from CSPS, San Diego, CA. HBTU (Senn Chemicals), $(i\text{Pr})_2\text{NEt}$ (Sigma-Aldrich), piperidine (Alfa Aesar), TFA (Halocarbon), Et_2O (EMD), DMF (OmniSolv), and trypsin (Promega) were used as received. 5-Azidopentanoic acid was prepared using reported procedure[1]. Prepative HPLC was performed with a Dynamax/Rainin Preppy SD-1 instrument and a Zorbax column (SB-C18, 21.2 mm i.d. x 25 cm), eluting with the gradient solvent mixture described on page S3. MALDI and high-resolution electrospray analyses were performed by the Mass Spectrometry Facility at The Scripps Research Institute.

Peptide Synthesis and Cyclization. Standard techniques of solid-phase Fmoc and Boc peptide synthesis were employed (Scheme S1) with the following protected amino acids in addition to those requiring no side chain protection. **Fmoc-based strategy**: Fmoc-L-Glu(O*t*Bu)-OH, Fmoc-L-Arg(Pbf)-OH, Fmoc-L-Thr (*t*Bu)-OH, Fmoc-L-Asp(O*t*Bu)-OH, Fmoc-L-His(Mtt)-OH, Fmoc-L-Asn(Trt)-OH, Fmoc-L-Lys(Boc)-OH; **Boc-based strategy**: Boc-L-Glu(Ochx)-OH, Boc-L-Arg(Tos)-OH, Boc-L-Thr(Bzl)-OH, Boc-L-Asp(Ochx)-OH, Boc-L-His(Bom)-OH, Boc-L-Asn(Xan)-OH, Boc-L-Lys(2ClZ/Z/Fmoc)-OH.

Abbreviations: Boc = *tert*-butyloxycarbonyl, Bom = benzyloxymethyl, Bzl = benzyl, chx = cyclohexyl, 2ClZ = 2-chloro benzyloxycarbonyl, Dmab = 4-{*N*-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]amino}benzyl ester, Fmoc = 9-fluorenylmethoxycarbonyl, Hmb = 2-hydroxy-4-methoxybenzyl, Mtt = 4-methyltrityl, Pbf = 2,2,4,6,7-pentamethyl-dihydrobenzofurane-5-sulfonyl , Tos = tosyl (*p*-toluenesulfonyl), *t*Bu = *tert*-butyl, Trt = trityl, Xan = xanthyl, Z = benzyloxycarbonyl.

General Peptide Synthesis Procedure: (Boc strategy) (a) The resin was swelled in DMF for 10 min. (b) The resin was drained and treated with neat TFA (2 x 1 min) to deprotect the Boc group. (c) The resin was drained and washed with DMF (6 x, each time adding DMF to 4 times the height of the resin), and was then checked for the presence of free amine by the standard Kaiser (ninhydrin) test. (Note: some residues such as propargyl-Gly, Thr, and Asp did not show positive Kaiser tests after successful Boc-deprotection, which has been reported in the literature and depends on the sequence.) (d) 0.5 M HBTU in DMF (2 mL per 0.2 mmol of resin, 5.0 equiv) was added to Boc-L-AA (amino acid, 5.5 equiv), followed be $(i\text{Pr})_2\text{NEt}$ (0.5 mL, excess), and the mixture was allowed to stand for 2-3 minutes prior to adding to the drained resin. The coupling reaction was then allowed to stand for 15 min with occasional mixing with a glass rod, after which time the resin was tested for a negative Kaiser test indicating capping of all of the resin

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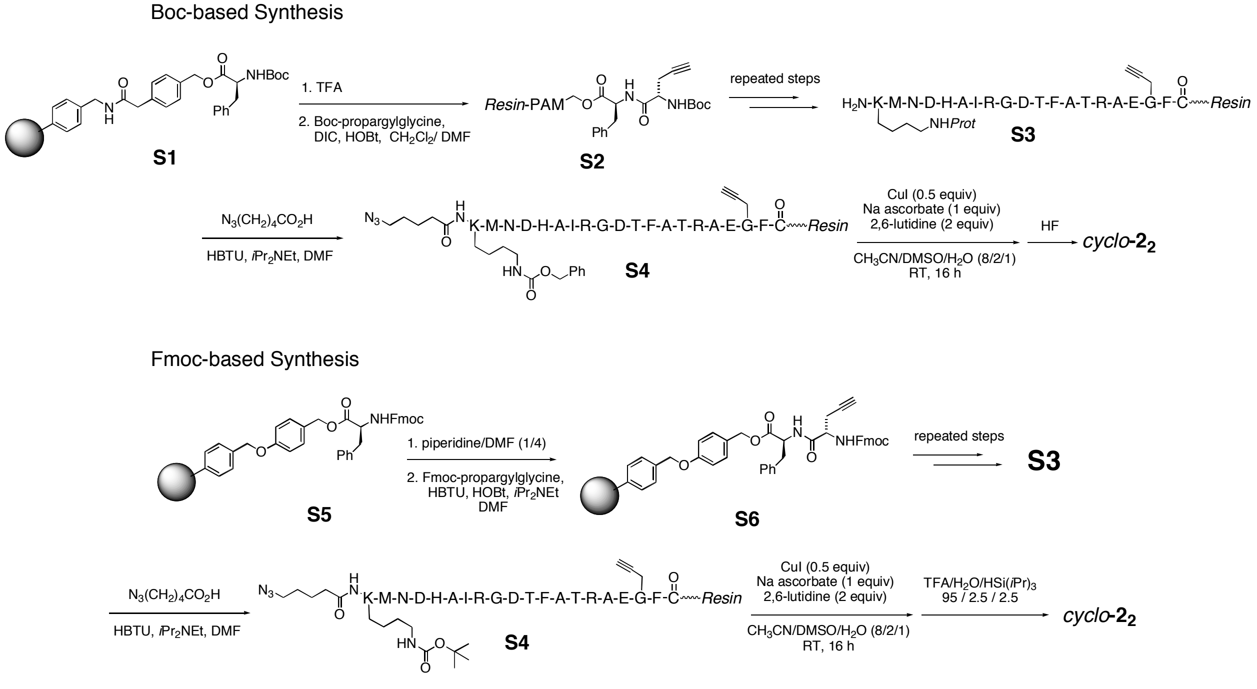
[1] R. P. McGeary, *Tetrahedron Lett.*, **1998**, *39*, 3319-3322

amine groups.

After completion of the peptide sequence by repetition of the above steps, the resin was washed with 1:1 CH_2Cl_2-MeOH (3 x) and dried under vacuum for an hour prior to HF-mediated cleavage. The procedure for Fmoc-based synthesis was the same, except that a 20% solution of piperidine in DMF was used to deprotect the Fmoc groups.

In both Fmoc and Boc methods, protected L-propargyl glycine was installed using DIC (1,3-diisoproplylcarbodiimide) and HOBt (1-hydroxybenzotriazole) in CH_2Cl_2 to prevent racemization during coupling. *Procedure:* At 0 °C, DIC (4.0 equiv) followed by HOBt (4.0 equiv) were added to Fmoc-/Boc-L-propargyl glycine (4.0 equiv with respect to amines present on the resin) in CH_2Cl_2 and the resulting suspension was stirred for 15 min at the same temperature. The mixture was then added to drained Resin-amine, and the coupling was performed for 30 min at room temperature.

Scheme S1.



In Boc-based synthesis of linear peptides, cleavage from the resin with HF proved to be incompatible with the azide group, giving peptides showing loss of 26 from the expected molecular mass. Since these peptides showed the same cyclization products as those derived from Fmoc synthesis, we presume that HF cleavage somehow compromises the azide group. For this reason, and because of the inconvenience of HF cleavage, Fmoc-based synthesis was used predominantly.

Standard peptide cyclization procedure. A solution of CuI (4.8 mg, 0.5 equiv) dissolved in 2.5 mL of 4:1 CH_3CN:DMSO was added to **Resin-1** (125 mg, maximum 0.05 mmol peptide) followed by 2,6-lutidine (11.6 μL, 0.1 mmol, 2 equiv) and sodium ascorbate (12.5 mg, 0.05 mmol, dissolved in 250 μL of H_2O).The resulting suspension was degassed with a gentle flow of

N_2 for approximately 1 min, and was then agitated by gentle tumbling for 16 h at room temperature.

The progress of the reaction was monitored by withdrawing a small portion of the resin and subjecting it to the published colorimetric test procedure [S. Punna, M.G. Finn, *Synlett* **2004**, 99-100]. An absence of color change indicated the completion of the reaction. Upon completion, the resin was transferred to a fritted reaction vessel and washed sequentially with CH_3CN, DMF, water, 0.1 M aqueous EDTA (to remove any copper that may remain bound to the resin), water, DMF, and finally with 1:1 MeOH:CH_2Cl_2. The resin was then dried under vacuum for approximately one hour.

Cleavage of the peptide from **Resin-1** was accomplished by treating the dried resin with a mixture of TFA, water, and triisopropylsilane (95:2.5:2.5) at room temperature for 3 hours, with gentle tumbling agitation. **Resin-2** required the use of a slightly different mixture because of the presence of the methionine residue: TFA, water, 1,2-ethanedithiol, and triisopropylsilane (94:2:2:1). The material was then filtered, washed with a minimum amount of TFA, and anhydrous Et_2O was added to precipitate the peptide. Dissolution of the cleaved peptide in TFA also removes any residual copper bound to the peptide. The supernatant was poured off and the resulting residue (solid or semi-solid) was washed with anhydrous Et_2O and then dried under vacuum to provide the crude peptide. In every case, a small amount of linear peptide was obtained from resin set aside before the cyclization procedure.

Analytical HPLC was performed with Hewlett Packard series 1100 instrument, using a Supelco or Vydac C18 HPLC column (25 cm x 4.6 mm, 5 μm), eluting with a gradient composed of two solvents: 0.1% aqueous TFA (solvent A) and a 60:40 mixture of acetonitrile and 0.1% aqueous TFA (solvent B). The gradient run was: time 0, A/B = 80/20; 0–30 min, linear change to A/B = 20/80; 30-31 min, linear change to A/B = 5/95; 31-40 min, A/B = 5/95; 40-41 min, linear change to A/B = 80/20; 41-50 min, re-equilibrate at A/B = 80/20. For greater resolution, 100-minute runs were performed in which the rate of change from 80/20 to 20/80 (A/B) was performed over 60 min instead of 30 min, column flushing with A/B = 5/95 was done for 19 instead of 9 min, and re-equilibration at A/B = 80/20 was performed for 19 instead of 9 min.

Yields of unpurified peptides cleaved from the resin were in the range of 60-70% by mass, and isolated yields of purified products from preparative HPLC were 10-30% by mass, based on the maximum possible yield from the starting resin. As is common in solid-phase peptide synthesis, the great majority of the impurities were derived from shorter-length peptides caused by imperfect peptide coupling. It also appeared that the presence of copper led to some peptide bond hydrolysis, depending on the copper concentration and reaction time. Such hydrolysis occurred to a very small degree, if at all, under standard conditions.

For reactions in the presence of added azide and alkyne (Scheme 3), the following reagents were employed using the above protocol: **Resin-1** (62.5 mg, representing a maximum of 0.025 mmol of alkyne, designated as 1 equiv, cuprous iodide (0.5 equiv.), sodium ascorbate (1.0 equiv.), 2,6-lutidine (2.0 equiv.), benzyl azide or phenylacetylene (0.5 equiv.), CH_3CN/DMSO (4/1, total volume 1.25 mL). The same results were observed when 1.5 equiv CuI or 1.0 equiv. BnN_3 were used.

No reaction was observed (a positive colorimetric azide test and unchanged HPLC retention time) when either copper or sodium ascorbate was omitted from the reaction mixture. Attempted cyclization of **1** in solution was attempted with 75 μM linear **1**, 1.0 equiv $CuSO_4$, 2.0 equiv sodium ascorbate, in H_2O containing Et_3N, pH $\approx$ 8, at room temperature for 14 h.

Control Experiments

The same cyclic dimeric products (***cyclo*-$\mathbf{1_2}$** and ***cyclo*-$\mathbf{2_2}$**) were formed using resins obtained from both Fmoc and Boc synthesis protocols, showing that the cyclization process is not somehow dependent on the solid phase synthesis method. Attempted thermal cyclization of **Resin-1** and **Resin-2** in the absence of Cu^I (microwave reactor, 100 °C, DMF, 1 h) gave no reaction, as evidenced by a positive azide colorimetric test and the isolation of clean **1** and **2** upon peptide cleavage from the resin. Attempted copper-mediated cyclization of free **1** and **2** at approximately 75 µM also gave little or no reaction; reactions between azides and alkynes in solution at these concentrations requires the use of accelerating ligands.[2]

Proof of the Cyclic Dimeric Nature of the Products

(1) HPLC of a mixture of purified **1** and *cyclo*-$\mathbf{1_2}$ showed baseline resolution (Figure S1), with the cyclic material eluting first. This was true for both peptides, perhaps due in part to the relatively hydrophilic nature of the triazole moiety, which has a strong dipole moment and excellent hydrogen bond accepting properties.

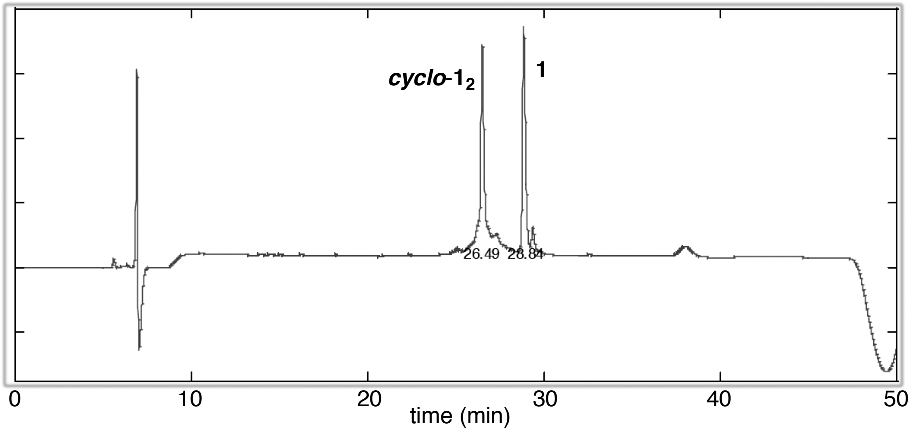


Figure S1. HPLC analysis of a mixture of the purified major products from cleavage of **Resin-1** before and after cyclization. **Resin-2** gave very similar results.

(2) MALDI mass spectrometry of the purified major products derived from **Resin-1** and **Resin-2**, under a variety of conditions (varying the amount of deposited material, nature of the matrix material, and laser desorption energy) gave peaks of dimeric mass, suggesting that these mass values do not arise from noncovalent association on the MALDI plate or in the gas phase.

(3) High-resolution electrospray ionization mass spectrometry of *cyclo*-$\mathbf{1_2}$ and *cyclo*-$\mathbf{2_2}$ revealed strong peaks at *m*/*z* values corresponding to odd numbers of protons added to both species, which can arise only from dimeric peptides [(*cyclo*-$\mathbf{1_2}$+$3H^+$), (*cyclo*-$\mathbf{2_2}$+$3H^+$), and (*cyclo*-$\mathbf{2_2}$+$5H^+$)] (Figure S2). Mass values and peak intensities corresponded closely to calculated values, supporting the compositional assignments.

(4) Treatment of purified **1** and **2** with tris(carboxyethyl)phosphine (TCEP) in Tris buffer gave a single product in each case showing a shift in HPLC retention time and a mass loss of 25 daltons (reduction of azide to amine). In contrast, *cyclo*-$\mathbf{1_2}$ and *cyclo*-$\mathbf{2_2}$ were unchanged by TCEP. This confirms that an azide group is not present in the cyclized compounds, ruling out

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[2] W. G. Lewis, F. G. Magallon, V. V. Fokin, M. G. Finn, *J. Am. Chem. Soc.* **2004**, *126*, 9152-9153.

linear dimer structures having only one triazole unit connecting the two chains. Furthermore, *cyclo*-$\mathbf{1}_2$ and *cyclo*-$\mathbf{2}_2$ prepared by the Boc strategy showed exactly the same mass spectra as the compounds isolated from Fmoc synthesis. This is noteworthy because, as mentioned above, all the linear peptide samples having an azide group showed a loss of 26 mass units after the HF cleavage required by Boc-based synthesis (as mentioned above). Since the Cu^{I}-treated peptides showed no such mass change as the result of HF cleavage, they are unlikely to possess an unreacted azide unit, and are therefore cyclized.

(5) **Resin-S1**, analogous to **Resin-1**, was prepared with a mixture of glycine and aspartic acid used in the fifth peptide coupling step, creating a mixture of RGD- and RGG-containing sequences (Scheme S2). Cyclization under the standard copper conditions gave rise to the expected two homodimers (*cyclo*-$\mathbf{1}_2$ and *cyclo*-**S1b**) and the single crossed dimer (*cyclo*-**S1a**), as determined by MALDI-MS (Scheme S2). Interestingly, *cyclo*-$\mathbf{1}_2$ was apparently produced in only minor amounts relative to the other two, even though it is obtained without difficulty from **Resin-1**. While not definitive, these results cast additional doubt on the notion that monomers are produced but are detected as dimers by mass spectrometry.

Scheme S2. Cyclization of a mixture of two peptides on a single resin.

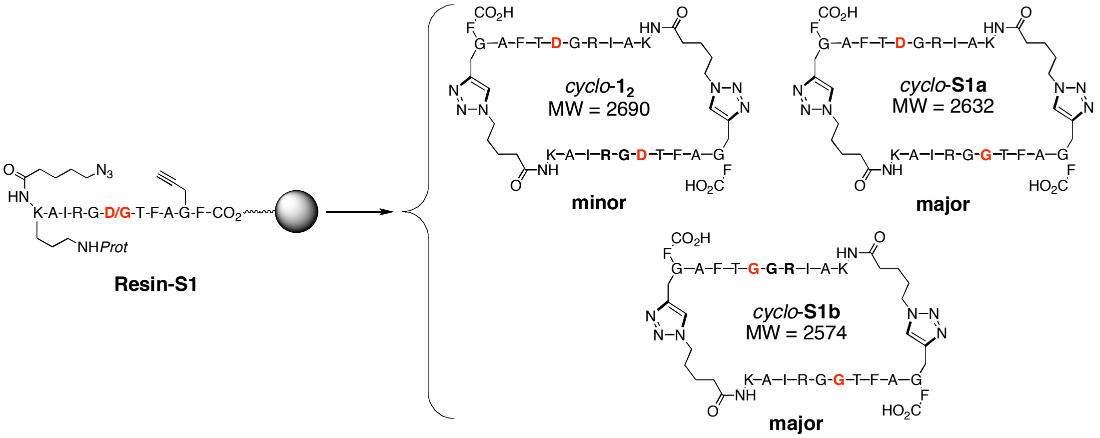
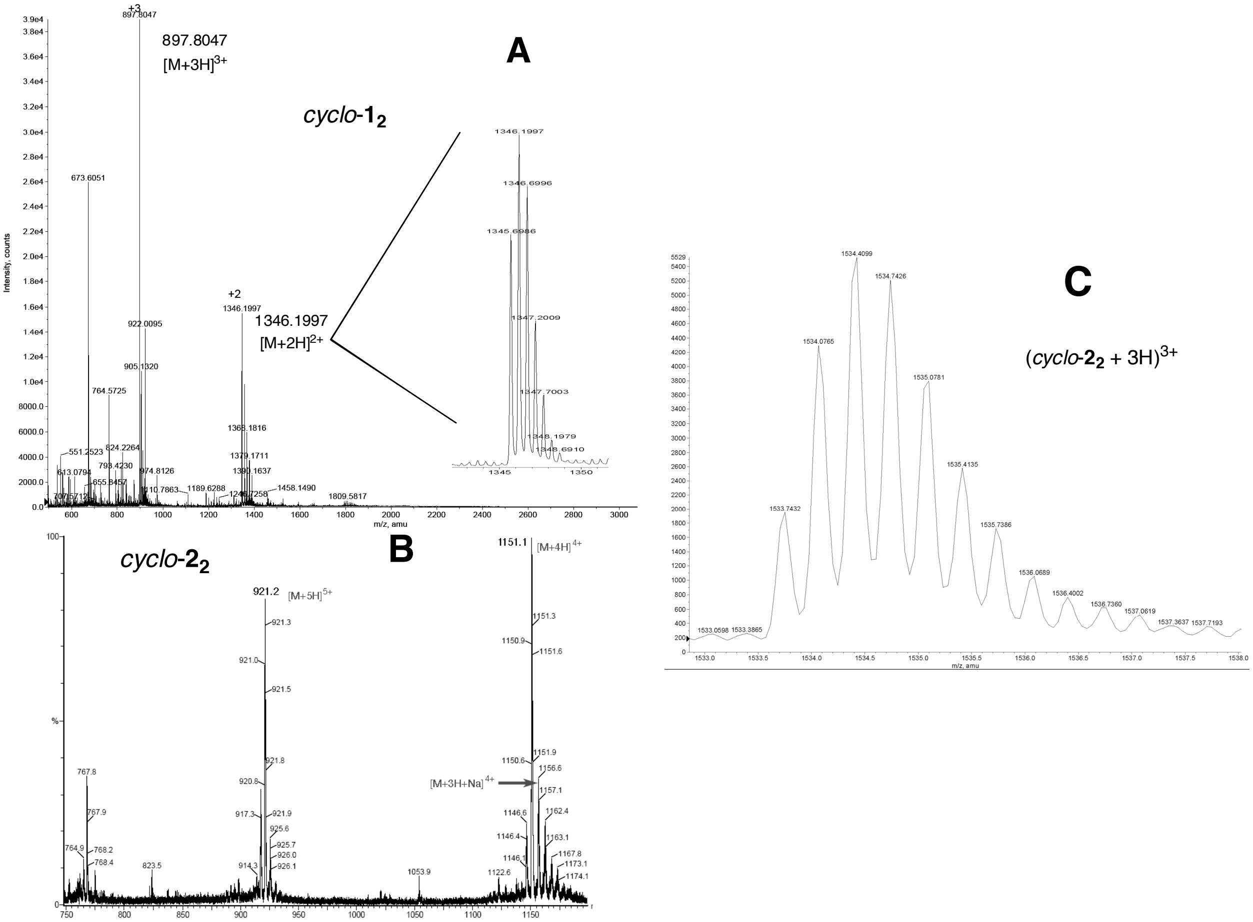


Figure S2. High-resolution (ESI-TOF) MS. (A) *cyclo*-**1**$_2$ (inset is expanded view of $(M+2H)^{2+}$ ion); (B) *cyclo*-**2**$_2$, focusing on tetra- and pentaprotonated ions; (C) *cyclo*-**2**$_2$, focusing on triprotonated ion; (D) table of calculated and observed *m/z* and intensity values for *cyclo*-**1**$_2$ ions taken from spectrum A; (E) table of calculated and observed *m/z* and intensity values for triply protonated *cyclo*-**2**$_2$ ion taken from spectrum C.



D

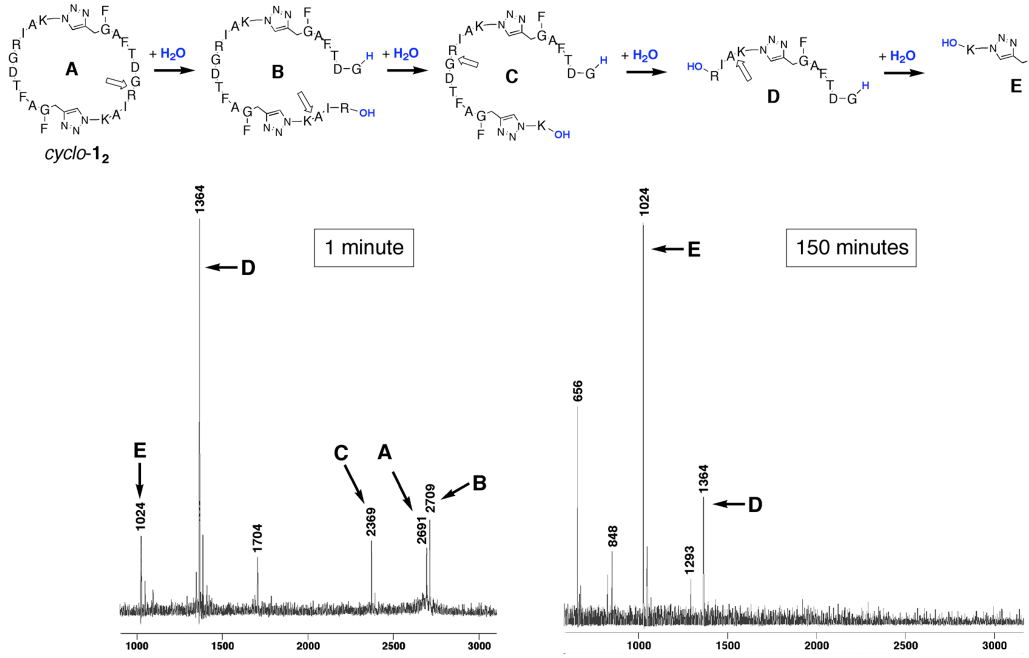
m/z (calc)	*m/z* (obs)	intens. (calc)	intens. (obs)
897.8047	897.8047	$[M+3H]^{3+}$	
1345.7017	1345.6986	64.1	68.8
1346.2031	1346.1997	100	100
1346.7045	1346.6996	81.7	81.3
1347.2059	1347.2009	46.3	43.8
1347.7073	1347.7003	20.4	21.9

E

m/z (calc)	*m/z* (obs)	intens. (calc)	intens. (obs)
1533.7356	1533.7432	29.3	33.2
1534.0699	1534.0765	74.3	77.8
1534.4040	1534.4099	100	100
1534.7381	1534.7426	94.3	95.8
1535.0722	1535.7081	69.6	70.5
1535.4062	1535.4135	42.6	47.9
1535.7402	1535.7386	22.5	28.0

(6) The reaction of *cyclo*-$\mathbf{1}_2$ with trypsin was found to give a distribution of species proving the existence of a monocyclic dimer and not a catenane (Figure S3). Thus, MS analysis after short reaction time showed a small amount of uncleaved *cyclo*-$\mathbf{1}_2$ (peak A), a peak corresponding to cleaved cyclic dimer (B), and then smaller fragments derived from sequential hydrolysis at Arg and Lys residues (C, D, and E). Longer exposure to trypsin funneled the distribution of species to the lower molecular weight fragments. The peaks of greater than monomeric molecular weights (B and C) could not arise from a catenated dimer. *Cyclo*-$\mathbf{2}_2$ proved to be much more reactive toward trypsin than *cyclo*-$\mathbf{1}_2$, such that reaction even under much more dilute conditions gave extensive peptide cleavage.

Figure S3. Trypsin-catalyzed hydrolysis of *cyclo*-$\mathbf{1}_2$, and MALDI-MS analysis.



Trypsin Digestion Procedure. To 1μL of peptide solution (5μg/μL stock solution in 25 mM NH_4HCO_3, pH 8.0), 74 μL of 25 mM NH_4HCO_3 was added followed by 20 μL of CH_3CN (20% overall), trypsin (5 μL of 20 ng/μL stock solution in 50 mM AcOH) was then added to give a peptide/trypsin molar ratio of 50. The resulting solution was placed in a 37 °C bath for the indicated time. The reaction was stopped by the addition of one drop of neat TFA and immediate vortex mixing; the sample was analyzed directly by MALDI-MS.

Molecular Weights

cyclo-$\mathbf{1}_2$	2691.07	*cyclo*-$\mathbf{2}_2$	4601.12	*cyclo*-**3**	1222.39
4	1478.69	**5**	1447.67	**6**	2793.20
cyclo-**7**	1431.62	*cyclo*-$\mathbf{7}_2$	2863.24	*cyclo*-**S1a**	2633.04
cyclo-**S1b**	2575.00				

Observations Described in Text

Observation (a)

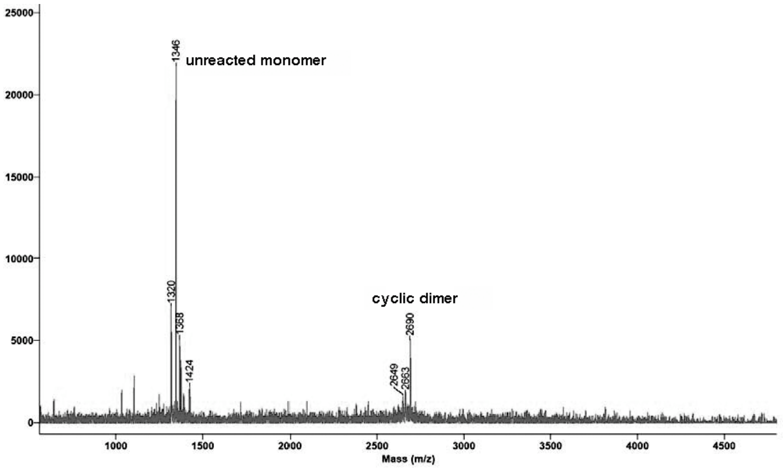
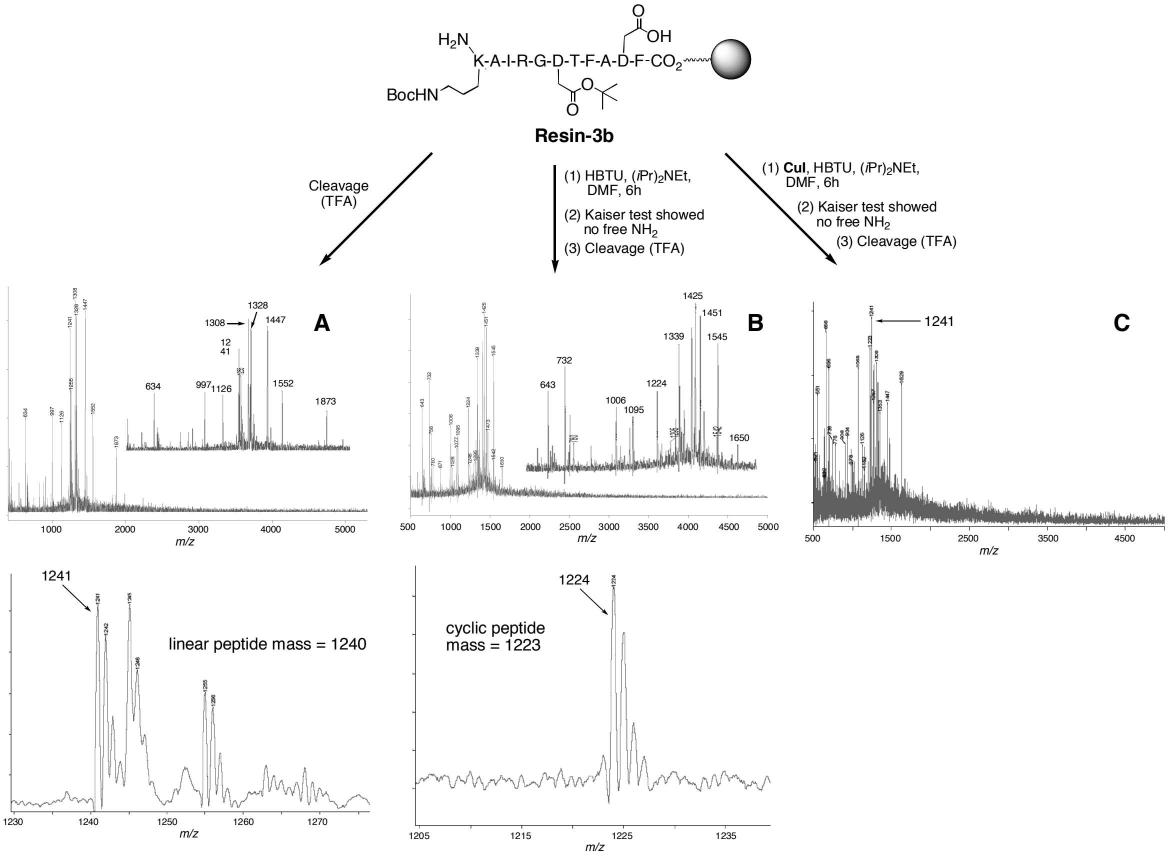


Figure S4. MALDI-MS of crude product from cyclization of **Resin-1** derived from polystyrene with a loading of 0.12 mmol/g (observation *b* in the text). **Resin-2** gives very similar results.

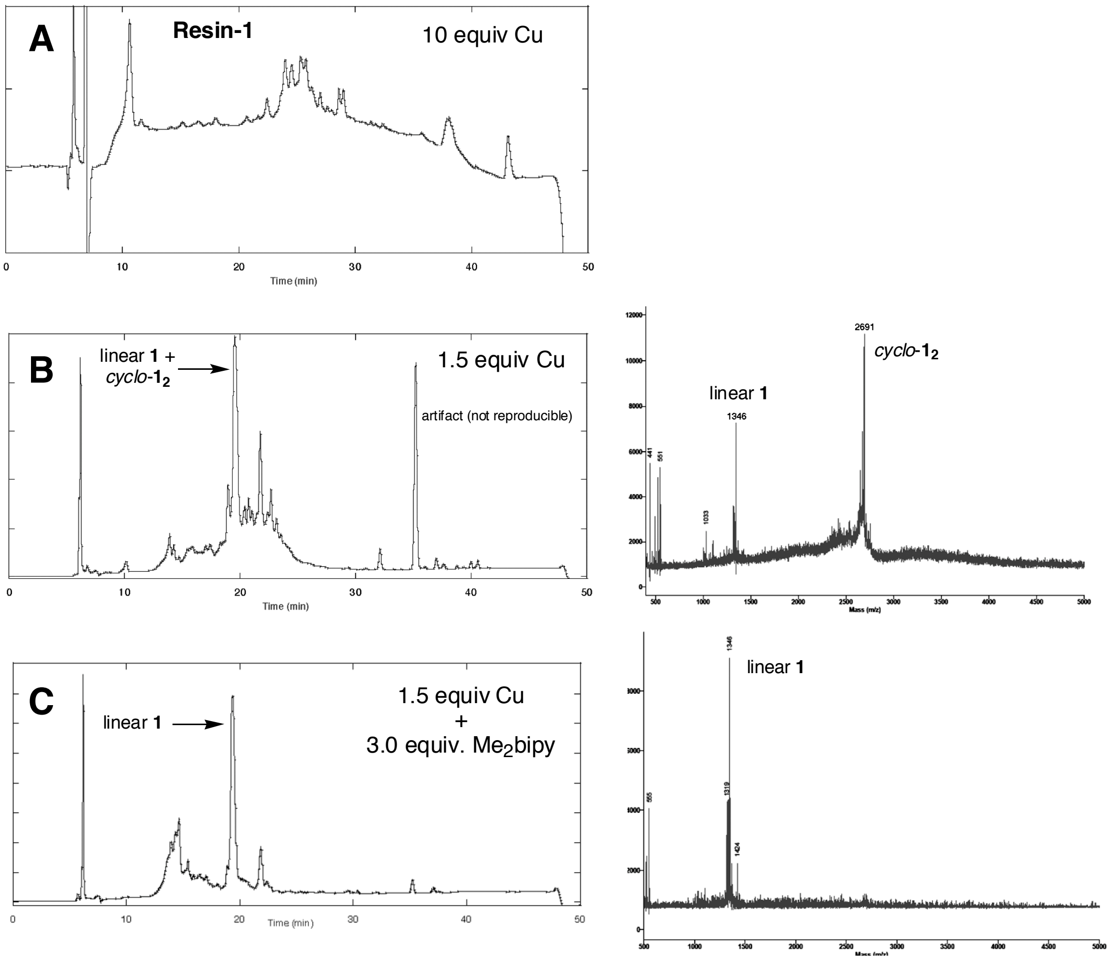
Observation (b): ring closure by amide bond formation.

Figure S5. Sample MALDI-MS data of crude products from **Resin-3**. No products of dimeric molecular weight, and many more byproducts, were detected, in contrast to the azide-alkyne ring closure route. Spectrum B shows the shift of the full peptide molecular ion from m/z = 1241 to 1224, indicative of ring closure; the rest of the spectrum shows the same level of impurity peaks as is shown in C.



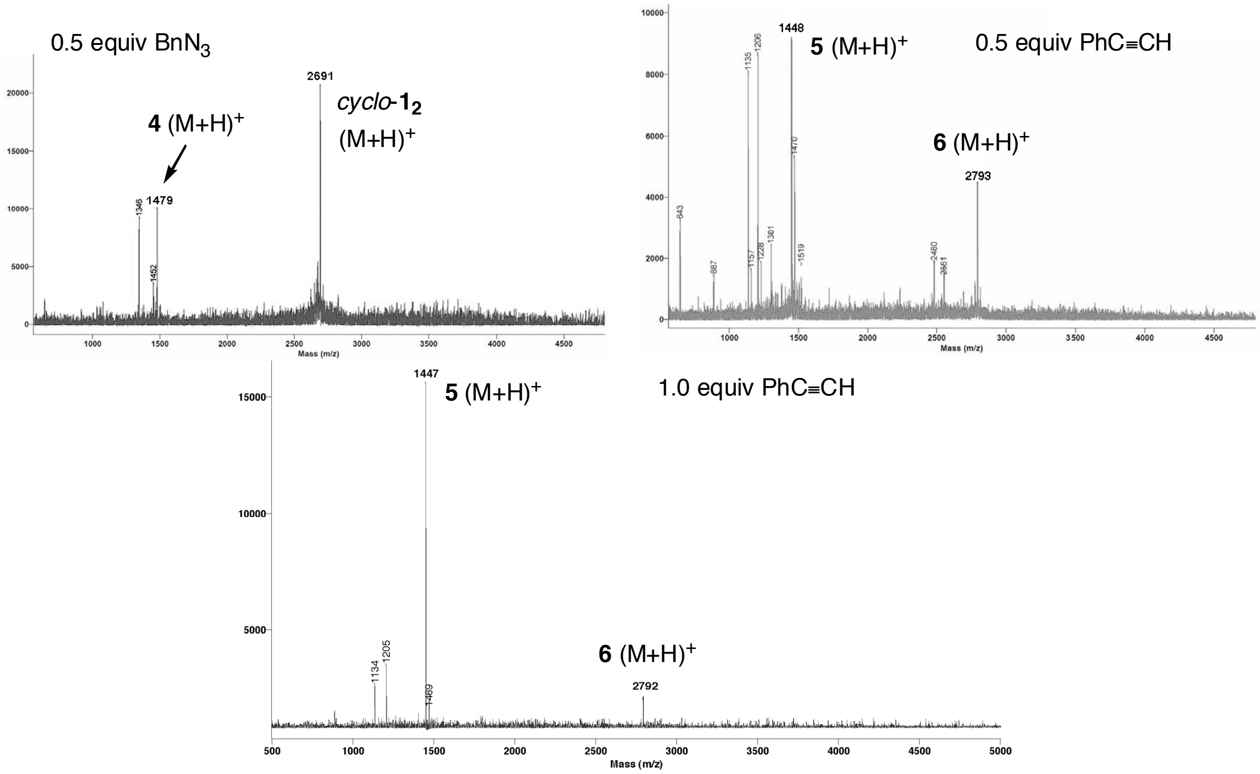
Observation (c): variable Cu.

Figure S6. HPLC and MALDI-MS of products from cyclization in the presence of varying amounts of Cu(I). (A) 10 equiv, (B) 1.5 equiv, (C) 1.5 equiv Cu(I) plus 3.0 equiv bipyridyl ligand. In the latter case, the colorimetric test for azide was strongly positive, indicating the presence of a large amount of unreacted azide residues. The MALDI spectra shown at the right are of the crude samples corresponding to the HPLC traces on the left; in this case, linear and cyclized forms of **1** were found to co-elute. Note the appearance of a much cleaner HPLC trace in the presence of ligand, suggesting that Cu-mediated peptide cleavage is suppressed to some degree under these conditions.



Observation (d), Scheme 3.

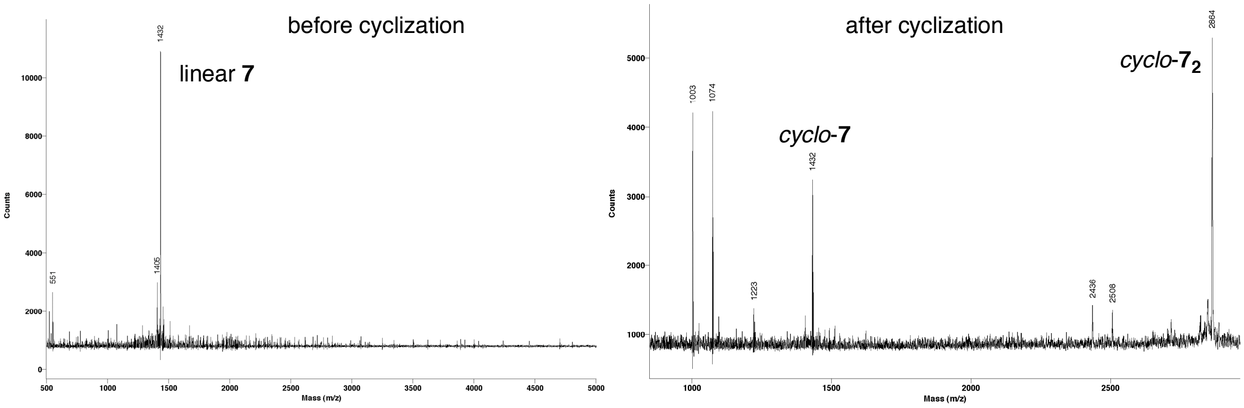
Figure S7. MALDI-MS of crude product from cyclization in the presence of 0.5 equiv benzyl azide (*top left*), 0.5 equiv. phenylacetylene (*top right*), and 1.0 equiv phenylacetylene (*bottom*); see Scheme 3.



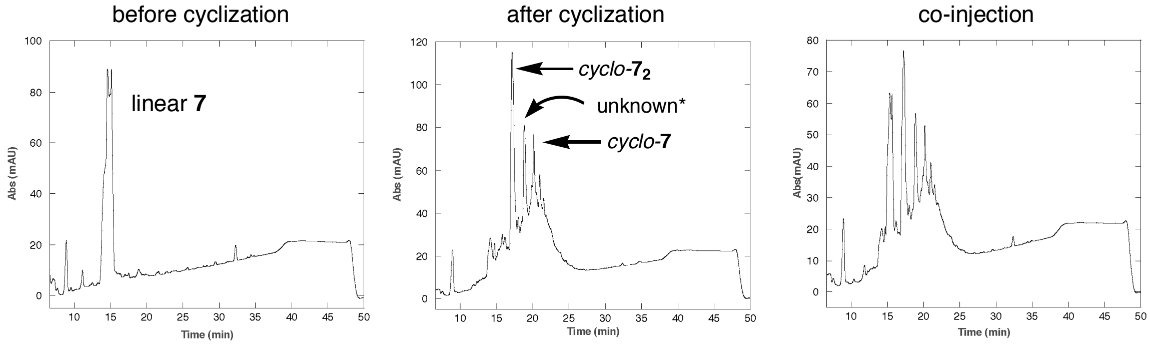
Observation (e), (Scheme 4).

Figure S8. MALDI-MS and HPLC of crude product from **Resin-7**.

MALDI-MS of crude products from **Resin-7**



HPLC of crude products from **Resin-7**



* no peaks above *m/z* 1066 in MS

A schematic representation of potential pathways leading to both monomeric and dimeric cyclization is shown in Scheme S3.

Scheme S3