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Article

Diversity-Oriented A^3-Macrocyclization for Studying Influences of Ring-Size and Shape of Cyclic Peptides: CD36 Receptor Modulators

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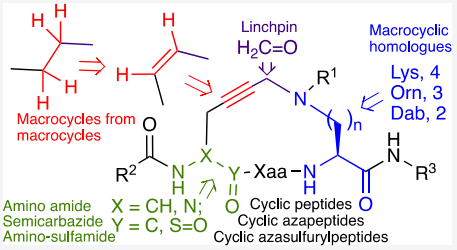
ABSTRACT: Cyclic peptide diversity has been broadened by elaborating the A^3-macrocyclization to include various di-amino carboxylate components with different N^{ϵ}-amine substituents. Triple-bond reduction provided new cyclic peptide macrocycles with *Z*-olefin and completely saturated structures. Moreover, cyclic azasulfurylpeptides were prepared by exchanging the propargylglycine (Pra) component for an amino sulfamide surrogate. Examination of such diversity-oriented methods on potent cyclic azapeptide modulators of the cluster of differentiation 36 receptor (CD36) identified the importance of the triple bond as well as the N^{ϵ}-allyl lysine and azaPra residues for high CD36 binding affinity. Cyclic azapeptides which engaged CD36 effectively reduced pro-inflammatory nitric oxide and downstream cytokine and chemokine production in macrophages stimulated with a Toll-like receptor-2 agonist. Studying the triple bond and amine components in the multiple-component A^3-macrocyclization has given a diverse array of macrocycles and pertinent information to guide the development of ideal CD36 modulators with biomedical potential for curbing macrophage-driven inflammation.



INTRODUCTION

Cyclic peptides are a proven class of clinically used drugs[1] due in part to their success in stabilizing active secondary structures, improving metabolic stability, and enhancing cellular penetration.[2,3] Efforts to make side-chain-to-side-chain cross-linked (so-called bridged or stapled) peptides have intensified as their promise to act as inhibitors of intracellular protein−protein interactions becomes realized with stapled-α-helix peptides advancing in clinical trials.[4] Cyclic peptides can achieve bioavailability in spite of violating rules typically associated with small-molecules, but few have exhibited significant oral absorption.[5] The uniformity of contemporary cross-linking chemistry, however, limits the product variety and may undermine the efforts to develop drug-like cyclic peptides.

Diversity-oriented methods for making cyclic peptides have garnered significant interest because of their potential to provide a variety of macrocycles from related chemical reactions and common intermediates.[6−10] In the so-called "A^3-macrocyclization", a multiple-component reaction takes place between alkyne, aldehyde, and amine components (Figure 1).[11] The amine and aldehyde condense to form an imine, which is attacked by an acetylide nucleophile from activation of the alkyne by the metal catalyst.[12] Preorganization from metal chelation of the acetylene and amine components may reduce entropic costs of folding to favor A^3-macrocyclization.[13] Diversity can in principle be introduced in the A^3-macrocyclization by modification of the peptide sequence, amine substituent, di-amino carboxylate chain length, and carbonyl linchpin.

The cluster of differentiation 36 receptor (CD36) is a membrane-bound glycoprotein expressed in many cell types: skeletal, cardiac muscle, microvascular endothelial, and retinal cells, as well as mononuclear phagocytes such as macrophages and microglia cells.[14−16] The single 472-amino acid peptide chain of CD36 is cross-linked by three disulfide bonds and post-translationally modified by significant glycosylation and phosphorylation.[17] Implicated in the regulation of physiological processes vital for cardiovascular biology and innate immunity, CD36 functions as a class B scavenger receptor and binds a broad variety of endogenous ligands: long-chain fatty acids, thrombospondin 1, oxidative low-density lipoprotein (oxLDL), apoptotic cells, and photoreceptor outer segments.[14−16,18−20] In macrophage-driven inflammation, CD36 acts as a co-receptor and sustains the signaling of the Toll-like receptor (TLR)-2/6 heterodimer assembly on the surface of membranes of mononuclear phagocytes.[21−23] Ligands that bind and modulate the activity of CD36 offer potential for treating cardiovascular, metabolic, and immunological disorders.[24]

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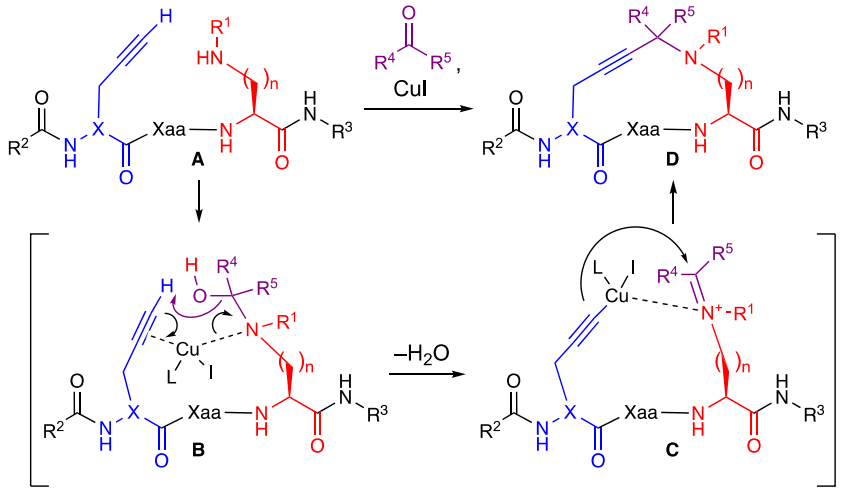
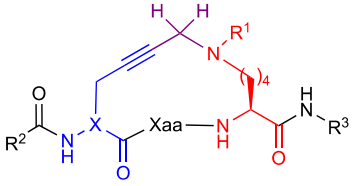


Figure 1. Diversity-oriented A^3-macrocyclization may be favored by metal chelation of the acetylene and amine components: R^1, R^4, and R^5 = diverse amine and carbonyl substituents, R^2CO-, $-NHR^3$, and Xaa = peptide chains, *n* = different di-amino acid side-chain lengths.

In efforts to develop CD36 ligands based on the linear lead growth hormone-releasing peptide-6 (GHRP-6, H-His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2), A^3-macrocyclization was conceived and employed to synthesize both cyclic peptide and azapeptide analogues (Figure 2).[24] Potent CD36 ligands, cyclic azapeptides (e.g., **3a** and **7a**, Figure 2), exhibited significant binding affinity and efficacy in reducing TLR-2 agonist-induced nitric oxide (NO) overproduction and in diminishing pro-inflammatory cytokine and chemokine production in macrophages at concentrations that were 10-fold lower (10^{-7}) than those at which linear counterparts were active.[11,24,25] Conformational analysis using NMR spectroscopy and computational analysis revealed that the semicarbazide sp^2-configuration provided the best representation of the aza-residue geometry in the active macrocycles.[25] Moreover, the active conformer of azacyclopeptide **7a** was deduced to have a compact topology featuring a type II′ β-turn geometry centered about the D-Trp-Ala dipeptide possessing an additional bridging hydrogen bond between the C-terminal amide NH and D-Trp carbonyl oxygen.[25]



R^1 = **a**, $CH_2CH{=}CH_2$; **b**, CH_3; **c**, *i*-Pr

	R^2-CO-	X	Xaa	$NH\text{-}R^3$
1	H-His-D-Trp-Ala	N	D-Phe	NH_2
2	H-His-D-Trp	N	Trp-D-Phe	NH_2
3	H-Ala	N	Ala-Trp-D-Phe	NH_2
(*R*)-**4**	H-Ala	(*R*)-CH	Ala-Trp-D-Phe	NH_2
(*S*)-**4**	H-Ala	(*S*)-CH	Ala-Trp-D-Phe	NH_2
5	H	N	D-Trp-Ala-Trp-D-Phe	NH_2
6	Ac	N	D-Trp-Ala-Trp-D-Phe	NH_2
7	H-Ala	N	D-Trp-Ala-Trp-D-Phe	NH_2
(*R*)-**8**	H-Ala	(*R*)-CH	D-Trp-Ala-Trp-D-Phe	NH_2
(*S*)-**8**	H-Ala	(*S*)-CH	D-Trp-Ala-Trp-D-Phe	NH_2
9	Ac-Ala	N	D-Trp-Ala-Trp-D-Phe	NH_2
10	H	N	D-Trp-Ala	D-Phe-Lys-NH_2
11	Ac	N	D-Trp-Ala	D-Phe-Lys-NH_2
12	H-Ala	N	D-Trp-Ala	D-Phe-Lys-NH_2
13	Ac-Ala	N	D-Trp-Ala	D-Phe-Lys-NH_2

Figure 2. Representative examples of previously synthesized cyclic (aza)peptide CD36 ligands from A^3-macrocyclization.

Previously, cyclic (aza)peptides having 16-, 19-, 22-, and 25-atom ring sizes were, respectively, synthesized using A^3-macrocyclization and formaldehyde to cross-link different N^{ϵ}-substituted lysine side chains with (*R*)- and (*S*)-propargylglycine (Pra) and their azaPra counterpart (Figure 2).[11,24−26] The semicarbazide served as an amino amide surrogate in the azapeptides.[27] Examining the scope of the A^3-macrocyclization more deeply, the diversity of the di-amino carboxylate component has now been expanded. The significance of the allyl substituent on the N^{ϵ}-amine of lysine (Lys) has been investigated using cyclopropylmethyl and *n*-propyl groups. Shorter side-chain lengths have been studied using ornithine (Orn), diaminobutyrate (Dab), and diaminopropionate (Dap). Moreover, macrocycles have been produced by reduction of the triple bond to *Z*-olefin and completely saturated counterparts. In addition, the azaPra residue was substituted by an amino-sulfamide (azasulfurylpropargylglycine, AsPra) to synthesize a cyclic azasulfurylpeptide isostere.

The analogues were initially examined for their modulatory efficiency in reducing NO production induced by the TLR-2 agonist fibroblast-stimulating lipopeptide (R-FSL-1) in macrophage cells. Promising macrocycles, which reduced NO production, were subsequently evaluated in a competition binding assay using the photo-activated agonist [^{125}I]-Tyr-Bpa-Ala-hexarelin as a radiotracer to characterize the binding affinity. Moreover, the capacity to modulate R-FSL-1-induced pro-inflammatory cytokine and chemokine release was examined by measuring the reduction of secreted levels of tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β), and monocyte chemoattractant protein-1 (MCP1, CCL-2) in cell culture media of one of two macrophage cell types: RAW 264.7 cells for TNFα and CCL-2; bone marrow-derived macrophages (BMA 3.1 A7) primed to M1 phenotype for IL-1β.

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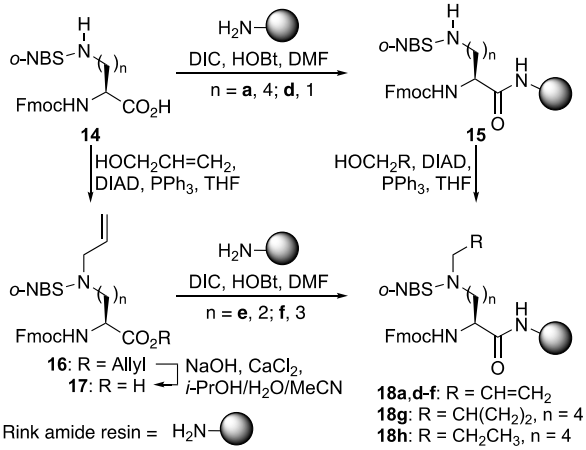
The utility of A^3-macrocyclization for the diversity-oriented synthesis of cyclic peptide analogues has been further elaborated by broadening the scope of di-amino acid and Pra components. Different azacyclopeptide CD36 modulators have been identified exhibiting interesting promise for mitigating TLR-2 agonist-induced pro-inflammatory responses. Investigating their structure−activity relationships (SARs) has provided information for designing more potent CD36 modulators with therapeutic potential and illustrated the effectiveness of this diversity-oriented method for peptide-based drug discovery.

RESULTS AND DISCUSSION

A series of macrocyclic GHRP-6 analogues was previously synthesized by A^3-macrocyclization of linear GHRP-6 analogues possessing N^{ε}-alkyl-Lys[6] and an azaPra residue, which was systematically moved from the 1- to the 4-position in the peptide (e.g., **1**−**3**, **5**−**7**, and **9**).[11] The Mitsunobu reaction on the N^{ε}-*o*-nitrobenzenesulfonamido (*o*-NBS)-Lys residue proved effective for installing a set of N^{ε}-substituents (Me, allyl, and *i*-Pr).[11] Moreover, macrocycles **10**−**13** were prepared by inserting a second Lys residue into the sequence.[11]

The length of the di-amino carboxylate in A^3 macrocyclization has now been examined by replacement of Lys[6] with lower homologues Orn, Dab, and Dap (Scheme 1). As

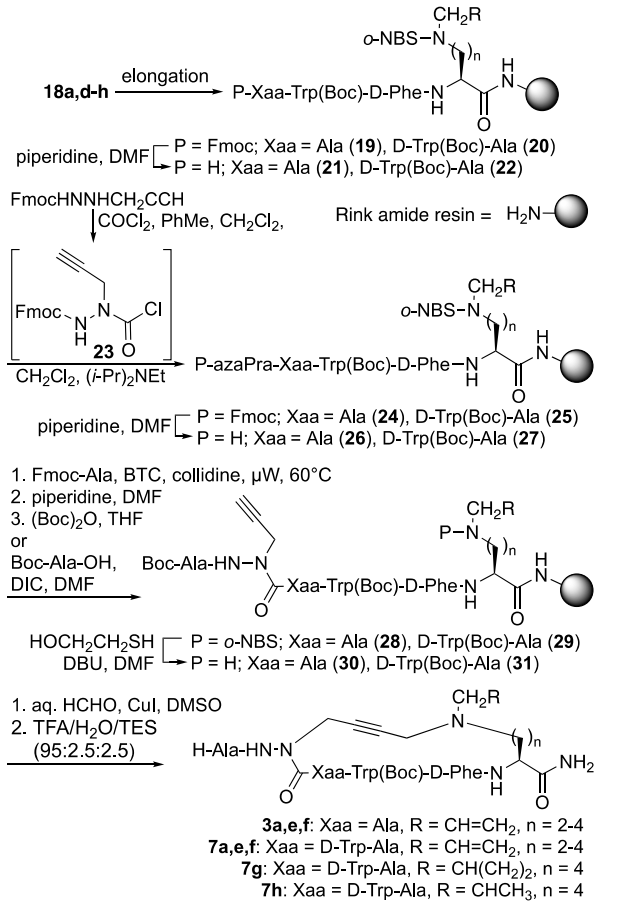
Scheme 1. Anchoring of Different Di-amino Carboxylates on Rink Amide Resin



previously described for the synthesis of Fmoc-Lys(*o*-NBS, allyl) resin **18a**, Fmoc-Dap(*o*-NBS)-OH (**14d**) was coupled to Rink amide resin using *N*,*N*′-diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole (HOBt) and converted to N^{β}-allyl derivative **18d** under Mitsunobu reaction conditions.[11] Attempts to couple Fmoc-Dab(*o*-NBS)-OH (**14e**) and Fmoc-Orn(*o*-NBS)-OH (**14f**) to the Rink amide resin gave however significantly lower loadings of **18e** and **18f** (0.10 and 0.19 mmol/g) likely due to intramolecular cyclization on N^{ω}-sulfonamide to afford the corresponding pyrrolidinone and piperidinone side products. Improved resin loadings (**18e** = 0.34 and **18f** = 0.33 mmol/g) were obtained from couplings of Fmoc-Dab(*o*-NBS, allyl)-OH and Fmoc-Orn(*o*-NBS, allyl)-OH (**17e** and **17f**), which were prepared from acids **14e** and **14f** by treatment with allyl alcohol, DIAD, and triphenylphosphine in THF, followed by ester hydrolysis without Fmoc removal using NaOH and $CaCl_2$ in a solution of *i*-PrOH/H_2O/MeCN.[28] In addition, the importance of the electron density of the N^{ε}-allyl group for the potency of cyclic azapeptides (e.g., **7a**) was explored by the synthesis of N^{ε}-cyclopropylmethyl and *n*-propyl Lys derivatives **18g** and **18h** using Mitsunobu reactions with the respective alcohols on resin **15a**.

Elongation of Dap, Dab, Orn, and Lys resins **18a**,**d**−**h** using standard Fmoc-based solid-phase peptide synthesis gave tetra- and pentapeptide resins **21** and **22** (Scheme 2). The azaPra

Scheme 2. Synthesis of Azacyclopeptides 3a,e,f and 7a,e−h



residue was introduced by employing *N*-Fmoc-azaPra-Cl (**23**), which was prepared from the corresponding carbazate with bis(trichloromethyl)carbonate (BTC).[26] After Fmoc group removal, semicarbazides **26a** and **27a**,**f**−**h** were coupled to the symmetric anhydride from Boc-Ala-OH to furnish azapeptide **28a** and **29a**,**f**−**h**.[26] Semicarbazides **26e**,**f** and **27e** were reacted under microwave irradiation at 60 °C with *N*-Fmoc-Ala-Cl, which was generated using BTC and collidine in THF, to give Fmoc-protected linear peptides, which were converted to Boc counterparts **28e**,**f** and **29e**.

After removal of the *o*-NBS protection with 2-mercaptoethanol and DBU,[26] cyclic azapeptides **3a**,**e**,**f** and **7a**,**e**−**h** were prepared from linear counterparts **30a**,**e**,**f** and **31a**,**e**−**h** by A^3-macrocyclization using aqueous formaldehyde and CuI in DMSO,[11] cleaved from the resin using a TFA/TES/H_2O cocktail, and purified by HPLC (Table 1). On the other hand, attempts to perform A^3-macrocyclization on Dap azapeptides **30d** and **31d** failed to give the corresponding azacyclopeptides. A cursory attempt to increase the azaPra length using *N*′-

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Table 1. Purity, Retention Times, and Mass of Cyclic Azapeptides[a]

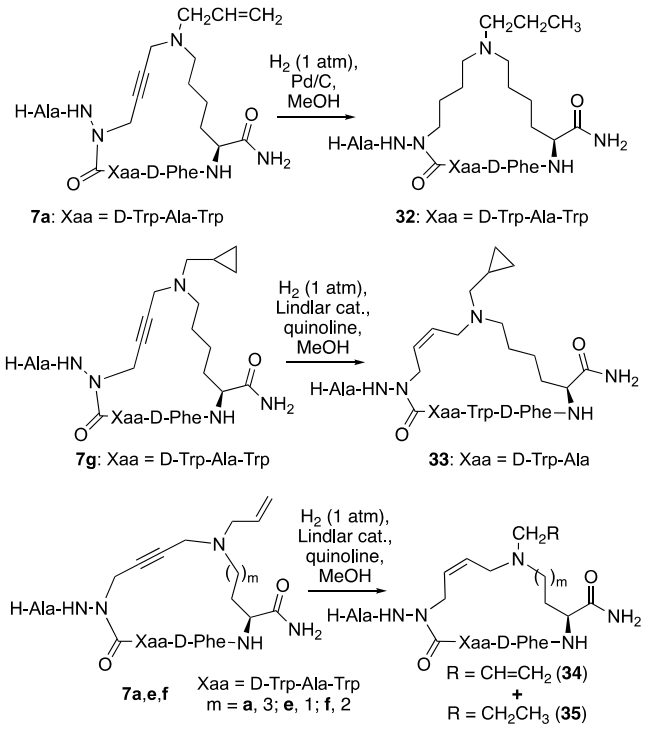
	cyclic azapeptide	R_t (min) MeOH	R_t (min) MeCN	purity at 214 nm	MS [M + 1] m/z (calc.)	MS [M + 1] m/z (obs.)
3e	*c*-{[azaPra(δC)−CH_2-Dab(γN)]H-Ala-azaPra-Ala-Trp-D-Phe-Dab(allyl)-NH_2}	5.51[b]	4.16[b]	>99	741.3831	741.3842
3f	*c*-{[azaPra(δC)−CH_2-Orn(δN)]H-Ala-azaPra-Ala-Trp-D-Phe-Orn(allyl)-NH_2}	5.72[b]	4.26[b]	>99	755.3988	755.3990
7e	*c*-{[azaPra(δC)−CH_2-Dab(γN)]H-Ala-azaPra-D-Trp-Ala-Trp-D-Phe-Dab(allyl)-NH_2}	6.91[b]	4.87[b]	>97	927.4624	927.4643
7f	*c*-{[azaPra(δC)−CH_2-Orn(δN)]H-Ala-azaPra-D-Trp-Ala-Trp-D-Phe-Orn(allyl)-NH_2}	6.85[b]	4.79[b]	>97	963.4600	963.4573
7g	*c*-{[azaPra(δC)−CH_2-Lys(εN)]H-Ala-azaPra-D-Trp-Ala-Trp-D-Phe-Lys[$CH_2CH(CH_2)_2$]-NH_2}	8.12[c]	8.09[c]	99	969.5094	969.5057
7h	*c*-{[azaPra(δC)−CH_2-Lys(εN)]H-Ala-azaPra-D-Trp-Ala-Trp-D-Phe-Lys(*n*-Pr)-NH_2}	8.01[c]	5.86[c]	99	955.5094	955.5100
32	*c*-{[azaGly(αN)−$(CH_2)_4$−Lys(εN)]H-Ala-azaGly-D-Trp-Ala-Trp-D-Phe-Lys(*n*-Pr)-NH_2}	6.04[b]	5.71[b]	>99	961.5407	961.5391
33	*c*-{[aza-hexenylglycine(εC)-Lys(εN)]H-Ala-*Z*-aza-hexenylglycinyl-D-Trp-Ala-Trp-D-Phe-Lys(cyclopropylmethyl)-NH_2}	6.64[b]	4.93[b]	>99	971.5250	971.5254
36	*c*-{[AsPra(δC)−CH_2-Lys(εN)]H-Ala-AsPra-D-Trp-Ala-Trp-D-Phe-Lys(allyl)-NH_2}	8.12[c]	5.99[c]	>99	991.4607	991.4657

[a]Isolated purity ascertained by LC−MS using gradients of X−Y% [MeOH (0.1% FA)/H_2O (0.1% FA)] or [MeCN (0.1% FA)/H_2O (0.1% FA)] over *Z* min. [b]10−90%/10. [c]5−50%/9.

homopropargyl-flourenylmethyl-carbazate proved more challenging than anticipated and was abandoned.

The chemistry of propargyl amines offers rich potential for supplementary modifications after A^3-macrocyclization.[29] Partial saturation and full saturation of the triple bond were both explored to alter the macrocycle ring-shape. Azapeptide **7a** was completely saturated by hydrogenation using Pd/C as a catalyst in MeOH to give macrocycle **32** in 74% yield (Scheme 3). Partial reduction of the triple bond of the cyclopropyl methyl analogue **7g** using the Lindlar catalyst and quinoline in MeOH gave Z-olefin **33** in 33% yield after HPLC purification. Partial hydrogenation of cyclic alkynes **7a**, **7e**, and **7f** without terminal olefin saturation was attempted using the Lindlar catalytic conditions but produced inseparable mixtures of allyl and *n*-propyl analogues **34** and **35**.

Scheme 3. Full and Partial Acetylene Saturation by Macrocycle Hydrogenation



The scope of the propargylglycine component was further explored in A^3-macrocyclization by employing azasulfurylpropargylglycine (AsPra; As = azasulfuryl). Previously, replacement of the semicarbazide in the linear analogue [aza-(4-F)F^4]-GHRP-6 by an amino-sulfamide gave [As-(4-F)F^4]-GHRP-6, which had comparable CD36 binding affinity and exhibited similar activity in suppressing TLR-agonist-induced pro-inflammatory NO, cytokine, and chemokine production in macrophages and reducing retinal inflammation by activated mononuclear phagocytes upon photo-oxidative stress in a mouse model.[30] In contrast to [aza-(4-F)F^4]-GHRP-6, which inhibited microvascular sprouting mediated through CD36 in the mouse choroidal explant model, the azasulfurylpeptide counterpart preserved microvascular survival.[30] Subtle chemical modification from a carbonyl to a sulfuryl residue effected significantly specific aspects of CD36-mediated chemical biology.[30] In comparison to the planar urea in the semicarbazide moiety, the sulfamide adopts tetrahedral geometry, increases NH Brønsted acidity, and adds a second Lewis basic oxygen.[31] In crystal structures of model analogues, such structural changes altered the β-turn conformation adopted by azapeptides to a γ-turn geometry in azasulfurypeptides.[31] The impact of such a change has now been investigated by the synthesis of cyclic azasulfurylpeptide **36** (Scheme 4).

Azasulfuryl tripeptide **42** was first prepared in solution by coupling Boc-Ala-OH to H-azaPra-OFm (**37**) using isobutyl chloroformate and *N*-methylmorpholine in THF to prepare Boc-Ala-azaPra-OFm (**38**) in 72% yield.[32] After Fmoc group removal, hydrazide **39** was reacted with sulfamidate **40** and triethylamine in dichloroethane under microwave irradiation to provide Boc-Ala-AsPra-D-Trp(Boc)-ODmb (**41**).[33] Selective Dmb ester hydrolysis was achieved without removal of acid labile Boc groups using 1% TFA in DCM to provide acid **42**. Coupling of acid **42** to tetrapeptide resin **43** using DIC and HOBt furnished linear azasulfurylpeptide resin **44**. After *o*-NBS removal, cyclic azasulfurylpeptide **36** was obtained uneventfully by A^3-macrocyclization of azasulfurylheptapeptide **45**, followed by resin and protecting group cleavage and HPLC purification (Table 1).

Biology. In earlier studies of linear and cyclic azapeptides,[24] CD36 modulator activity was initially evaluated by the ability to reduce overproduction of NO in macrophage cells induced with the TLR-2 agonist R-FSL-1.[24,34] Produced by macrophages to battle against pathogenic microbes but potentially destructive to host tissue, NO is a useful marker of the

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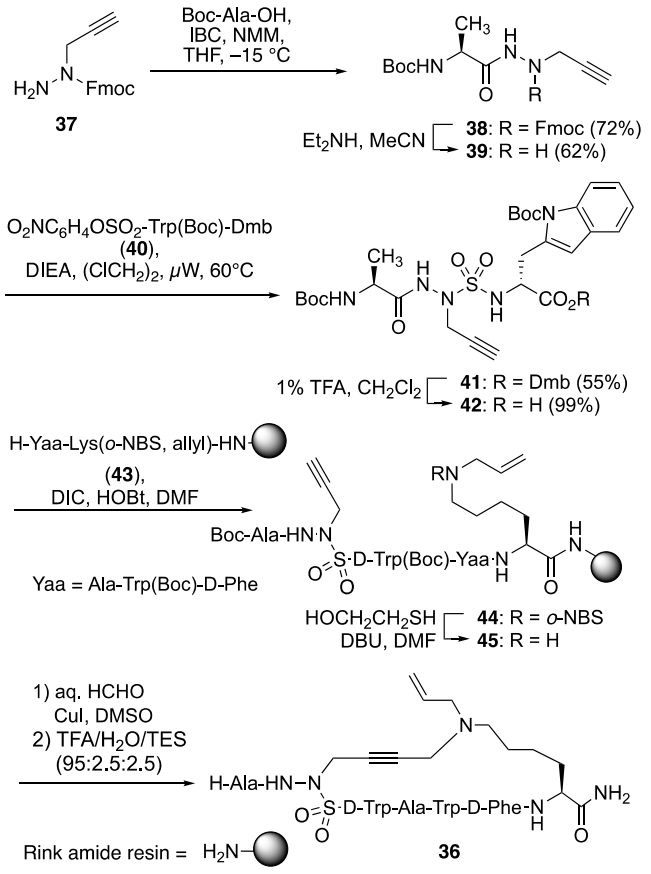
Scheme 4. Synthesis of Cyclic Azasulfurylpeptide 36



modulation of the inflammatory pathway and can be effectively measured by formation of a fluorescence naphthotriazole adduct using 2,3-diaminonaphthalene.[35] Reduction of NO overproduction by CD36 ligands was correlated with binding affinity and with decreased production of pro-inflammatory cytokines and chemokines induced by R-FSL-1 in macrophage cells.[24]

Among the five different macrocycle ring sizes previously studied (e.g., **1**−**13**, Figure 2), the larger 22- and 25-membered cyclic azapeptides (e.g. **3**, **4**, **6**−**9**) gave consistently higher percentages of NO reduction compared to the 16- and 19-membered ring systems (e.g., **1**, **2**, and **10**−**13**).[24] A basic protonated amine at the N-terminal appeared key for reducing NO overproduction by the 25-membered cyclic azapeptides (10^{-7} M, Table 2): N-terminal alanine analogue **7a** (35%) > *N*-acetyl [for example, **9a** (23%) > **6a** (18%)] ≈ N-terminal semicarbazide **5a** (5%),[11] which is likely not protonated under physiologic conditions.[36] The N^{ε}-substituent of the Lys component of the 25-membered cyclic azapeptides has now been shown to influence the ability to reduce NO overproduction. A comparison of N^{ε}-allyl (**7a**, 35%), cyclopropylmethyl (**7g**, 14%), and *n*-propyl (**7h**, 4%) derivatives illustrates that the saturated analogues gave significantly lower inhibitory activity and binding affinity compared to the unsaturated derivative. The intermediate abilities to reduce NO overproduction shown by cyclic azapeptide **7g** may be due to the greater electron density of the cyclopropyl "bent" bonds, which are situated between the σ- and π-bonds in **7h** and **7a** (Figure 3).

The binding affinity (IC_{50}) of analogues exhibiting high activity in reducing NO overproduction was evaluated in a competition binding assay using the photo-activated agonist [^{125}I]-Tyr-Bpa-Ala-hexarelin as the radiotracer and rat heart membrane preparation as the source of CD36 binding sites (Table 2 and Figure 4).[24] Some correlation of the binding affinity of the 25-membered macrocycles with their NO inhibitory activity was observed (r = −0.55; P = 0.0159, Supporting Information): **7a** (35%, 0.1 μM) > **9a** (23%, 0.67 μM) > **6a** (18%, 0.89 μM) > **7g** (14%, 3.06 μM) > **7h** (4%, 1.86 μM) (Figure 4A,B). Moreover, the related 22-membered macrocycle **3a** (25%, 0.24 μM) with an N-terminal alanine featured relatively high NO inhibitory potency and binding

Table 2. Cyclic Peptide Binding Affinity and Modulatory Effect on R-FSL-1-Induced Release of NO, TNFα, and CCL-2 in RAW 264.7 Macrophage Cells and of IL-1β in Bone Marrow Macrophages BMA 3.1 A7[a]

	NO			TNF-α		CCL-2		IL-1β	
#	mean ± SD (μM)	%[f]	IC_{50} (μM)	mean ± SD (pmol)	%[f]	mean ± SD (pmol)	%[f]	mean ± SD (pmol)	%[f]
basal	0.774 ± 0.235			851 ± 22.08		238 ± 127[c]		71 ± 9	
R-FSL-1 stimulation as control	2.821 ± 0.1			4966 ± 35		1097 ± 26		410 ± 40	
3a	2.302 ± 0.137[c]	25	0.24	3837 ± 221[e]	27	758 ± 114	39	291 ± 17[e]	35
3e	2.506 ± 0.356	15	10.2	4848 ± 79	3	954 ± 177	17	389 ± 31	6
3f	2.812 ± 0.241	0	22.8	4756 ± 165	5	1028 ± 179	8	381 ± 33	9
5a	2.723 ± 0.241	5	>50	4901 ± 163	2	1042 ± 92	6	388 ± 34	7
6a	2.451 ± 0.177	18	0.89	4558 ± 146[b]	10	785 ± 178[c]	36	351 ± 7	18
7a	2.113 ± 0.284[e]	35	0.1	3943 ± 109[e]	25	672 ± 34[d]	50	253 ± 28[e]	46
7e	2.26 ± 0.216[d]	27	4.75	4178 ± 108[e]	19	857 ± 54	28	271 ± 24[e]	41
7f	2.52 ± 0.428	15	13.1	4654 ± 135	8	971 ± 167	15	411 ± 33	0
7g	2.533 ± 0.181	14	3.06	4623 ± 150	8	984 ± 69	21	366 ± 38	13
7h	2.748 ± 0.21	4	1.86	4462 ± 405[c]	12	918 ± 78	13	313 ± 33[d]	29
9a	2.346 ± 0.197[c]	23	0.67	4042 ± 167[e]	22	707 ± 98[d]	45	304 ± 18[d]	32
32	2.078 ± 0.236[e]	36	2.26	4113 ± 141[e]	21	802 ± 49[b]	34	312 ± 17[d]	29
33	2.566 ± 0.085	12	9.05	4823 ± 296	3	903 ± 73	23	312 ± 17	9
36	2.739 ± 0.273	4	10.2	5120 ± 133	−4	969 ± 89	15	378 ± 37	6

[a]SD: standard deviation. Statistical evaluation for cyclic azapeptide comparison was performed using one-way ANOVA with post hoc Dunnett's test. [b]P < 0.05. [c]P < 0.01. [d]P < 0.001. [e]P < 0.0001 *vs* R-FSL-1 stimulatory condition as control. N = 4 replicates per experiment. [f]% of inhibition relative to R-FSL-1 as control.

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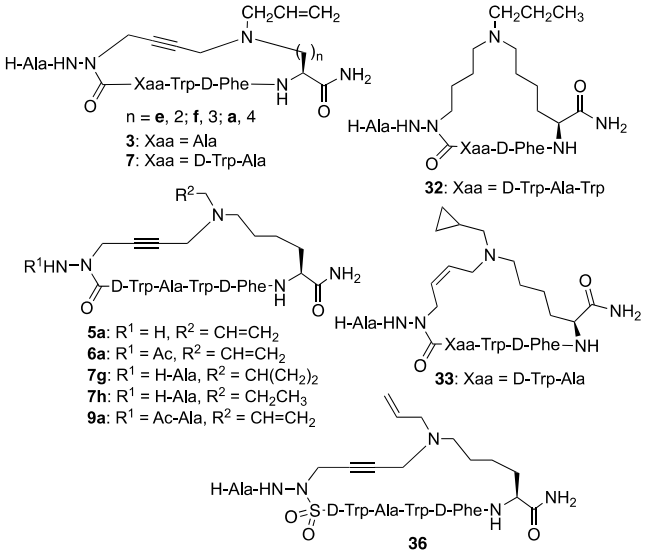

Figure 3. Cyclic azapeptides evaluated for CD36 binding affinity and biological activity.

affinity. On the other hand, cyclic azapeptide **5a** with a N-terminal semicarbazide did not show any competitive binding affinity or effect on cytokine and chemokine expression (*vide infra*) and has been suggested to likely reduce NO production by an off-target mechanism.[11]

Considering the high NO inhibitory potency and binding affinity of the 22- and 25-membered macrocycles, efforts were focused on modifying cyclic azapeptides **3a** and **7a** by shortening the di-amino carboxylate component, reducing the triple bond, and swapping of the urea for a sulfamide moiety. These structural modifications had significant consequences on pharmacological activity and binding affinity. For example, replacement of the Lys residue by the shorter di-amino acids, Dab (**3e** and **7e**) and Orn (**3f** and **7f**), caused profound drops in NO inhibitory potency. Substitution of Lys in macrocycles **7a** and **3a** by Orn resulted, respectively, in 131-fold (**7f**) and 95-fold (**3f**) losses of binding affinity, illustrating the significant importance of the di-amino acid chain length (Figure 4B,D).

Complete hydrogenation of the acetylene and N^{ϵ}-allyl groups of cyclic azapeptide **7a** gave saturated N^{ϵ}-propyl analogue **32**, which exhibited a similar NO inhibitory potency but a 22.6-fold loss in binding affinity. Compared to N^{ϵ}-propyl cyclic alkyne **7h**, saturated N^{ϵ}-propyl analogue **32** had a ninefold better potency on NO inhibition but 1.2-fold less binding affinity (Table 2 and Figure 4B,C). Partial hydrogenation of the acetylene of the N^{ϵ}-cyclopropylmethyl macrocycle **7g** to *Z*-olefin **33** caused only a subtle drop in NO inhibitory potency but a threefold loss in binding affinity. In the cyclic alkyne series, the N^{ϵ}-allyl analogue **7a** exhibited, respectively, 2.5- and 8.75-fold higher NO inhibitory potencies as well as 30.6- and 18.6-fold better binding affinities relative to the N^{ϵ}-*n*-propyl and N^{ϵ}-cyclopropylmethyl analogues **7h** and **7g** (Figure 4B). The losses in binding affinity caused by hydrogenation to saturated and *Z*-olefin analogues **32** and **33** relative to their corresponding N^{ϵ}-*n*-propyl and N^{ϵ}-cyclopropylmethyl alkyne analogues **7h** and **7g** highlight the significance of the triple bond for binding affinity. On the

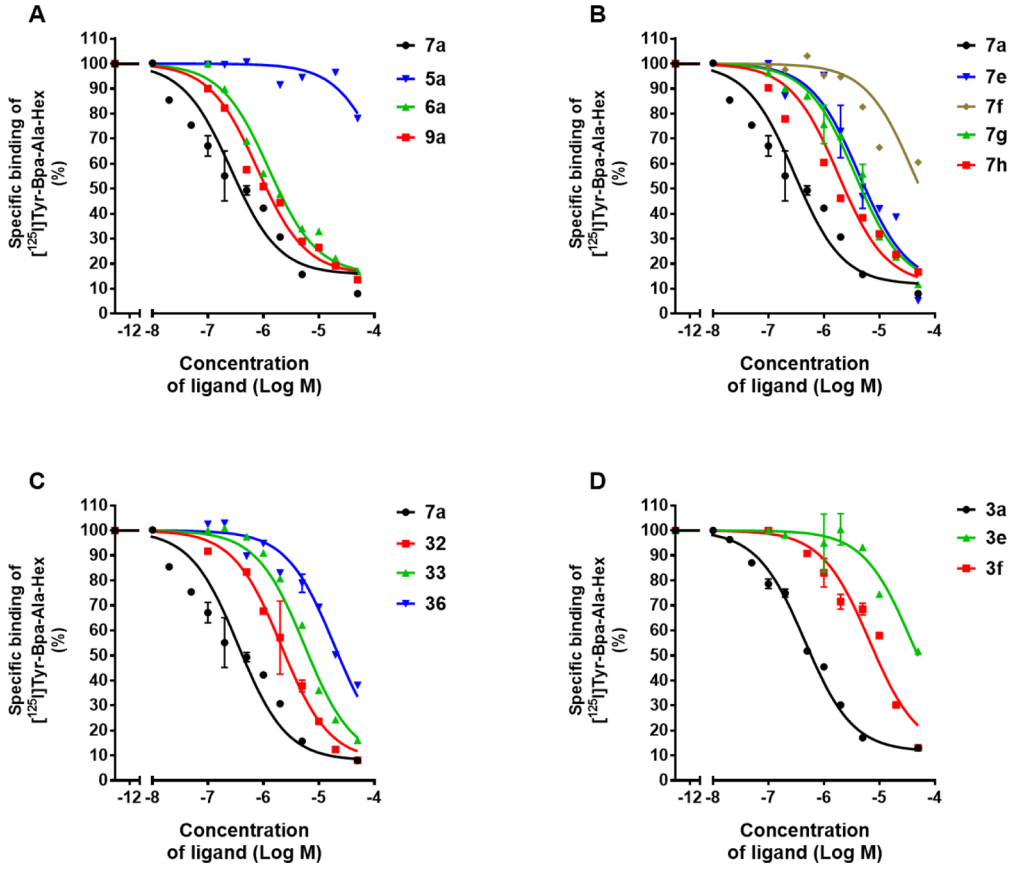

Figure 4. A–D) Assessment of cyclic azapeptides' binding affinity for CD36 in the presence of [^{125}I]Tyr-Bpa-Ala-hexarelin.

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other hand, improved and retained NO inhibitory potency of **32** and **33** relative to **7h** and **7g** indicates that increased flexibility may give rise to alternative active conformers.

In linear aza- and azasulfurylpeptide analogues, such as [aza-(4-F)F[4]]- and [As-(4-F)F[4]]-GHRP-6, the exchange of the urea moiety by a sulfamide had a limited effect on binding affinity.[30] In contrast, replacement of semicarbazide **7a** by *N*-amino sulfamide **36** caused a 102-fold drop in binding affinity. Conformational analyses of cyclic azapeptide **7a** and cyclic peptide counterparts (*R*)- and (*S*)-**8a** using NMR spectroscopy and computation have previously indicated that the semicarbazide sp^2-configuration provided a better representation of the aza-residue geometry in the active macrocycle than the pseudo-sp^3 (*R*)- and (*S*)-hybridizations.[25] The preferred sp^3 geometry of the *N*-amino sulfamide may account in part for the diminished binding affinity of cyclic azasulfurylpeptide **36** (Figure 4C).

Cyclic azapeptide with macrocycles of 22- (**3a**) and 25-members (**6a**, **7a**, **7g**, **7h**, **9a**, and **33**) were further tested for capacity to modulate R-FSL-1-induced production of pro-inflammatory cytokines (TNFα, IL-1β) and chemokine (CCL-2) in macrophage-type cell models. Cultured cells were treated with cyclic azapeptide at 10^{-7} M, and levels of TNF-α and CCL-2 were measured by ELISA kit assays on the collected cell culture supernatants.[37] Secreted levels of IL-1β were ascertained in BMA macrophages by measuring the activity of the NLRP3-caspase-1 inflammasome cascade of the innate immune system.[37−39]

Modifications on macrocycles that led to loss of binding affinity resulted typically in the loss of inhibitory effect on NO as well as reduced ability to inhibit the release of pro-inflammatory cytokines (TNF-α and IL-1β) and the chemokine (CCL2) induced by the TLR-2 agonist in macrophages. A negative correlation was, respectively, observed between the binding affinity and the inhibitory effects of the cyclic azapeptides on TLR2-mediated secretion of pro-inflammatory cytokines IL-1β and TNF-α and chemokine CCL-2 ($r = -0.61$, $r = -0.57$ and $r = -0.69$ Supporting Information). Inhibitory potency on NO production strongly correlated with the inhibition of pro-inflammatory cytokines (IL-1β, $r = 0.71$ and TNF-α, $r = 0.82$) and chemokine (CCL-2, $r = 0.83$, Supporting Information). For example, the drop of inhibitory potency on NO release upon replacement of Lys (**3a**) by Dab (**3e**) and Orn (**3f**) caused between 2.3-fold and 9-fold diminishment of inhibitory effects on TNF-α, IL-1β, and CCL-2. Substitution of Lys (**7a**) by Orn (**7f**) precipitated, respectively, the inhibitory effects on the release of CCL2, IL-1β, and TNF-α by 3.3-, 9.3-, and 3.1-fold. In contrast, the corresponding Dab modification (**7e**) attenuated moderately inhibitory potency on the release of proinflammatory cytokines and chemokine. Similarly, the critical role of the allyl N^{ϵ}-substituent of **7a** was evidenced by replacement with N^{ϵ}-cyclopropylmethyl (**7g**) and *n*-propyl (**7h**) groups, which decreased by 1.6-fold to 6.25-fold the inhibitory effects on TNF-α, CCL-2, and IL-1β (Table 2). Finally, saturated N^{ϵ}-propyl analogue **32** exhibited activity in reducing TNF-α, CCL-2, and IL-1β by 21, 34, and 29%, respectively. These inhibitory levels were similar to that of N^{ϵ}-allyl cyclic alkyne **7a** and higher than that of N^{ϵ}-propyl cyclic alkyne **7h**. The subtle effects of the cyclic peptides in reducing pro-inflammatory cytokine and chemokine production highlighted the importance of N-terminal and N^{ϵ}-substituents, ring size, and flexibility on downstream signaling once effective CD36 binding affinity is achieved.

CONCLUSIONS

Diversity-oriented synthesis of cyclic peptides by way of A^3-macrocyclization offers power to provide an array of cyclic peptides with subtle differences in macrocycle substitution, size, and shape for studying SAR. A^3-macrocyclization could be applicable for developing high-affinity binders for various biological targets. Moreover, the methods described for selective alkyne reduction may be of value for further exploration of cyclic peptide analogues possessing unsaturated cross-linkers.[40,41]

The scavenger receptor CD36 plays roles in the regulation of the TLR2/6 heterodimer assembly, the activation of transcription of proinflammatory cytokines, and the production of NO and reactive oxygen species.[37,42] Cyclic azapeptides offer notable potency for reducing TLR-2 agonist-induced pro-inflammatory cytokines and chemokines by binding CD36 and inducing dissociation from the TLR complex.[24] The employment of A^3-macrocyclization has provided a set of cyclic azapeptide CD36 modulators. Employing different N^{ϵ}-substituents on Lys as well as shorter Dab and Orn homologues, substituent electron density and macrocycle size were, respectively, shown to be significant for CD36 binding affinity and NO inhibitory potency. By reducing the cyclic alkyne to *Z*-olefin and saturated counterparts, the macrocycle shape and conformational flexibility were similarly demonstrated to influence CD36 binding affinity and anti-inflammatory activity. Moreover, the relevance of the semicarbazide geometry for binding and activity was illustrated by replacement with an amino-sulfamide counterpart through the synthesis of azasulfurylpeptide **36** using A^3-macrocyclization.

Notably, cyclic azapeptides exhibited a correlation between CD36 binding affinity and modulation of anti-inflammatory responses. Further study is planned to examine the influences of such subtle structural changes on other CD36-mediated actions, such as cholesterol efflux from macrophages and lipid metabolism. The diversity-oriented utility of A^3-macrocyclization for generating cyclic peptide ligands has been further validated by the production of a valuable set of CD36 ligands exhibiting biomedical potential for modulating both immune and metabolic responses.

EXPERIMENTAL SECTION

General Protocols. Chromatography was performed on 230−400 mesh silica gel. Analytical thin-layer chromatography (TLC) was performed on glass-backed silica gel plates (Merck 60 F254). Visualization of TLC plates was performed by UV absorbance or staining with potassium permanganate stain. ^{1}H and ^{13}C NMR spectra were, respectively, measured in $CDCl_3$ or DMSO-d_6 at 500 (125) MHz and referenced to $CDCl_3$ [7.26 (77.16) ppm] and DMSO-d_6 [2.50 (39.52) ppm]. Coupling constant *J* values were measured in Hertz (Hz) and chemical shift values in parts per million (ppm). Specific rotations, $[\alpha]_D$ values, were measured at 25 °C at the specified concentrations (*c* in g/100 mL) using a 1 dm cell length on a PerkinElmer Polarimeter 589 and expressed using the general formula: $[\alpha]^{25}_{589} = (100 \times \alpha)/(d \times c)$. High-resolution mass spectrometric analyses were performed at the Centre Regional de Spectrométrie de Masse de l'Universite de Montreal. Protonated molecular ions $[M + H]^+$ and sodium adducts $[M + Na]^+$ were used for empirical formula confirmation.

Unless otherwise specified, reagents were used as received. Polystyrene Rink amide resin (0.5 mmol/g) and HOBt were purchased from Advanced Chemtech, Boc-Ala-OH, Fmoc-Ala-OH, Fmoc-Dab(Boc)-OH, Fmoc-Dap(Boc)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Lys-OH, Fmoc-Orn(Boc)-OH, Fmoc-D-Phe-OH, Fmoc-D-Trp-(Boc)-OH, Fmoc-Trp(Boc)-OH, Fmoc-chloride 1,8-

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diazabicyclo[5.4.0]undec-7-ene (DBU), di-isopropyl azodicarboxylate (DIAD), and triphenylphosphine were all purchased from Chem-Impex, acetic anhydride, allyl alcohol, copper iodide, di-isopropyl carbodiimide (DIC), diisopropylethylamine (DIEA), 37% aqueous formic acid (FA), 65% aqueous hydrazine hydrate solution, 80% propargyl bromide solution in toluene and 2-mercaptoethanol were all purchased from Sigma-Aldrich, and 2-nitrobenzenesulfonyl chloride (*o*-NBSCl) was purchased from Acros Organics. All solvents were obtained from VWR International. Anhydrous solvents (CH_2Cl_2 and THF) were obtained by passage through solvent filtration systems (GlassContour, Irvine, CA). Fmoc-Lys(*o*-NBS)-OH and Fmoc-Dap(*o*-NBS)-OH were synthesized according to literature procedures.[43] Cyclic azapeptides **3a**, **5a**, **6a**, **7a**, and **9a** were synthesized as previously described.[11,26]

Cyclic Peptide Nomenclature. Side-chain-to-side-chain cross-linked cyclic peptides are described as follows: *c*-{[N-terminal residue (atom linked)—linker—C-terminal residue (atom linked)]linear sequence without linker}. No linker is given in cases in which the N- and C-terminal residues are directly linked.

Peptide Purification and Analysis. Azapeptides were purified on a semipreparative column (C18 Gemini column) using the appropriate gradient from 10% to a higher % of MeOH (0.1% FA) in water (0.1% FA) at a flow rate of 10 mL/min. Purity (>95%) was assessed using analytical HPLC on a 5 μM, 50 mm × 4.6 mm C18 Phenomenex Gemini column with a flow rate of 0.5 mL/min using the appropriate gradient in two different solvent systems: MeCN (0.1% FA) in water (0.1% FA) and MeOH (0.1% FA) in water (0.1% FA).

Solid-Phase Chemistry, Removal of Fmoc Protection, and Peptide Analysis. Fmoc-based peptide synthesis was performed on an automated shaker using the polystyrene Rink amide resin (0.5 mmol/g, 75−100 mesh) and standard conditions.[44] The loading was calculated from the UV absorbance for Fmoc-deprotection after the coupling of the first amino acid. Couplings of amino acids (3 equiv) were performed in DMF using DIC (3 equiv) and HOBt (3 equiv) for 3−6 h. Fmoc-deprotections were performed by treating the resin with 20% piperidine in DMF for 30 min. The resin was washed after each coupling and the deprotection step performed sequentially with DMF (×3), MeOH (×3), THF (×3), and CH_2Cl_2 (×3). The reaction quality was assessed with a Kaiser test for the coupling of the amino acids. Coupling reaction conversion was assessed by LC−MS analysis on a cleaved 2−5 mg dried resin sample after treatment with TFA/TES/H_2O (95:2.5:2.5, v/v/v, 0.5 mL) for 15 min and filtration. The filtrate was evaporated under reduced pressure, dissolved in MeOH, evaporated to dryness, and then examined by LC−MS.

***c*-{[azaPra(δC)−CH_2−Dab(γN)]H-Ala-azaPra-Ala-Trp-D-Phe-Dab(allyl)-NH_2} (3e).** Cyclic azapeptide **3e** was prepared from linear azahexapeptide resin **30e** (∼500 mg, 0.18 mmol) according to the protocol described for the synthesis of cyclic azapeptide **3f** using CuI (6.0 mg, 0.032 mmol) and aqueous formaldehyde (80 μL, 1.07 mmol, 37% in H_2O). Examination by LCMS of a cleaved resin sample (5 mg) showed complete conversion and a peak with molecular ion consistent with cyclic azapeptide **3e**: LCMS R_t = 4.76 min [10−90% MeOH (0.1% FA) in water (0.1% FA) over 10 min]; MS *m/z*: calcd for $C_{38}H_{49}N_{10}O_6^+$ [M-2Boc + H]$^+$, 741.4; found, 741.5. Resin cleavage using a freshly made solution of TFA/H_2O/TES (95:2.5:2.5, v/v/v, 5 mL) and purification by preparative HPLC gave cyclic azahexapeptide **3e** (2.5 mg, 2%) as a white foam: HPLC R_t = 5.51 min [10−90% of MeOH (0.1% FA) in H_2O (0.1% FA) over 10 min and then 10% MeOH (0.1% FA) for 5 min,]; R_t = 4.16 min [10−90% MeCN (0.1% FA) in H_2O (0.1% FA) over 10 min and then 10% MeCN (0.1% FA) for 5 min]; HRMS *m/z*: calcd for $C_{38}H_{49}N_{10}O_6^+$ [M + H]$^+$, 741.3831; found, 741.3842.

***c*-{[azaPra(δC)−CH_2−Orn(δN)]H-Ala-azaPra-Ala-Trp-D-Phe-Orn(allyl)-NH_2} (3f).** In a plastic syringe tube equipped with a Teflon filter, stopcock, and stopper, Boc-Ala-azaPra-Ala-Trp(Boc)-D-Phe-Orn(allyl) Rink amide resin **30f** (∼500 mg, 0.15 mmol) was swollen in DMSO (6 mL) for 30 min, treated with CuI (6.0 mg, 0.032 mmol) and aq. formaldehyde (70 μL, 0.94 mmol, 37% in H_2O), shaken on an automated shaker for 42 h, and filtered. After filtration, the resin was washed sequentially with AcOH/H_2O/DMF (5:15:80, v/v/v, ×3), DMF (×3), THF (×3), MeOH (×3), and DCM (×3). Examination by LCMS of a cleaved resin sample (5 mg) showed complete conversion and a peak with a molecular ion consistent with cyclic azapeptide **3f**: LCMS R_t = 4.50 min [10−90% MeOH (0.1% FA) in water (0.1% FA) over 10 min], MS *m/z*: calcd for $C_{39}H_{51}N_{10}O_6^+$ [M-2Boc + H]$^+$, 755.4; found, 755.5. Resin-bound Boc-protected cyclic azahexapeptide was deprotected and cleaved from the support using a freshly made solution of TFA/H_2O/TES (95:2.5:2.5, v/v/v, 5 mL) at room temperature for 2 h. The resin was filtered and rinsed with TFA (5 mL). The filtrate and rinses were concentrated to an oil, from which a precipitate was obtained by addition of cold ether (10 mL). After centrifugation (1200 rpm for 10 min), the supernatant was removed, and the precipitate was taken up in aqueous MeOH (10% v/v) and freeze-dried prior to purification. The resulting light brown foam was purified by preparative HPLC to give cyclic azahexapeptide **3f** (1.6 mg, 1%) as a white foam: HPLC, R_t = 5.72 min [10−90% of MeOH (0.1% FA) in H_2O (0.1% FA) over 10 min and then 10% MeOH (0.1% FA) for 5 min]; R_t = 4.26 min [10−90% MeCN (0.1% FA) in H_2O (0.1% FA) over 10 min and then 10% MeCN (0.1% FA) for 5 min]; HRMS *m/z*: calcd for $C_{39}H_{51}N_{10}O_6^+$ [M + H]$^+$, 755.3988; found, 755.3990.

***c*-{[azaPra(δC)−CH_2−Dab(γN)]H-Ala-azaPra-D-Trp-Ala-Trp-D-Phe-Dab(allyl)-NH_2} (7e).** Azapeptide **7e** was prepared according to the protocol described for cyclic azapeptide **7f** from linear azaheptapeptide resin **31e** (∼500 mg, 0.164 mmol) using CuI (6.0 mg, 0.032 mmol) and aq. formaldehyde (70 μL, 0.94 mmol, 37% in H_2O). Examination by LCMS of a cleaved resin sample (5 mg) showed complete conversion and a peak with a molecular ion consistent with cyclic azapeptide **7f**: LCMS R_t = 5.36 min [10−90% MeOH (0.1% FA) in water (0.1% FA) over 10 min]; MS *m/z*: calcd for $C_{49}H_{59}N_{12}O_7^+$ [M-3Boc + H]$^+$, 927.5; found, 927.5. Resin cleavage using a freshly made solution of TFA/H_2O/TES (95:2.5:2.5, v/v/v, 5 mL) and purification by preparative HPLC gave cyclic azaheptapeptide **7e** (3.4 mg, 2%) as a white foam: HPLC R_t = 6.91 min [10−90% of MeOH (0.1% FA) in H_2O (0.1% FA) over 10 min and then 90% MeOH (0.1% FA) for 5 min]; R_t = 4.87 min [10−90% MeCN (0.1% FA) in H_2O (0.1% FA) over 10 min and then 90% MeCN (0.1% FA) for 5 min]; HRMS *m/z*: calcd for $C_{49}H_{59}N_{12}O_7^+$ [M + H]$^+$, 927.4624; found, 927.4643.

***c*-{[azaPra(δC)−CH_2−Orn(δN)]H-Ala-azaPra-D-Trp-Ala-Trp-D-Phe-Orn(allyl)-NH_2} (7f).** In a plastic syringe tube equipped with a Teflon filter, stopcock, and stopper, resin **31f** (∼1 g, 0.20 mmol) was swollen in DMSO (8 mL) for 30 min, treated with CuI (7.0 mg, 0.04 mmol) and aq. formaldehyde (90 μL, 1.2 mmol, 37% in H_2O), shaken on an automated shaker for 31 h, filtered, and washed sequentially with AcOH/H_2O/DMF (5:15:80, v/v/v, ×3), DMF (×3), THF (×3), MeOH (×3), and DCM (×3). Examination by MS of a cleaved resin sample (5 mg) showed a peak with a molecular ion consistent with cyclic azaheptapeptide **7f**: MS *m/z*: calcd for $C_{50}H_{61}N_{12}O_7^+$ [M + H]$^+$, 941.5, found 941.4. The resin was treated with a freshly made solution of TFA/H_2O/TES (95:2.5:2.5, v/v/v, 5 mL) at room temperature for 2 h, filtered, and washed with TFA (5 mL). The filtrate and washes were combined and concentrated to an oil, from which a precipitate was obtained by addition of cold ether (10 mL). After centrifugation (1200 rpm for 10 min), the supernatant was decanted and the precipitate was taken up in aqueous MeOH (10% v/v) and freeze-dried to a light brown foam, which was purified by preparative HPLC to give cyclic azaheptapeptide **7f** (1.3 mg, 1%) as a white foam: HPLC R_t = 6.85 min [10−90% of MeOH (0.1% FA) in H_2O (0.1% FA) over 10 min and then 10% MeOH (0.1% FA) for 5 min]; R_t = 4.79 min [10−90% MeCN (0.1% FA) in H_2O (0.1% FA) over 10 min and then 10% MeCN (0.1% FA) for 5 min]; HRMS *m/z*: calcd for $C_{50}H_{60}N_{12}O_7Na^+$ [M + Na]$^+$, 963.4600; found, 963.4573.

***c*-{[azaPra(δC)−CH_2−Lys(εN)]H-Ala-azaPra-D-Trp-Ala-Trp-D-Phe-Lys[$CH_2CH(CH_2)_2$]-NH_2} (7g).** Cyclic azapeptide **7g** was prepared from resin **18g** according to protocols described for the synthesis of **7f**. Purification provided a 2.1% yield of material of 99% purity: LCMS analysis R_t = 8.12 min [5−50% of MeOH (0.1% FA) in H_2O (0.1% FA) over 9 min and then 5% MeOH for 5 min]; R_t = 8.09

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min [5−50% of MeCN (0.1% FA) in H_2O (0.1% FA) over 9 min and then 5% MeCN for 5 min]; HRMS *m/z* calcd for $C_{52}H_{62}N_{12}O_7$ $[M + H]^+$, 969.5094; found, 969.5057

***c*-{[azaPra(δC)−CH_2−Lys(εN)]H-Ala-azaPra-D-Trp-Ala-Trp-D-Phe-Lys(*n*-Pr)-NH_2} (7h).** Cyclic azapeptide **7h** was prepared from resin **18h** according to protocols described for the synthesis of **7f**. Purification provided a 1.9% yield of material of 99% purity: LCMS analysis R_t = 8.01 min [5−50% of MeOH containing 0.1% FA in H_2O (0.1% FA) over 9 min and then at 5% MeOH for 5 min]; R_t = 5.86 min [5−50% of MeCN (0.1% FA) in H_2O (0.1% FA) over 9 min and then 5% MeCN for 5 min]; HRMS *m/z* calcd for $C_{51}H_{65}N_{12}O_7$ $[M + H]^+$, 957.5094; found, 955.5100.

Fmoc-Dab(*o*-NBS)-OH (14e). A solution of Fmoc-Dab(Boc)-OH (1.01 g, 2.29 mmol) in CH_2Cl_2 (30 mL) was treated with TFA (20 mL), stirred at room temperature for 3 h, and evaporated on a rotary evaporator. The resulting yellow oil was dissolved in MeOH and water and lyophilized. The resulting white foam was dissolved in THF (25 mL) and water (25 mL), treated with DIEA (4.00 mL, 23.0 mmol) and *o*-NBS-Cl (577 g, 2.60 mmol), stirred at room temperature for 4 h, and diluted with EtOAc (50 mL). The organic phase was washed with aqueous HCl (1 M, 50 mL × 3), water (50 mL), and brine (50 mL). The organic layer was dried over $MgSO_4$, filtered, and evaporated to give **14e** as a light-yellow gum (1.15 g, 96%): $[\alpha]_D$ −6.9 (*c* 0.026, MeOH); ^{1}H NMR (300 MHz, DMSO): δ 8.18 (t, *J* = 5.4 Hz, 1H), 7.97 (dq, *J* = 5.7, 3.1 Hz, 2H), 7.92−7.81 (m, 4H), 7.68 (dd, *J* = 13.4, 7.8 Hz, 3H), 7.41 (t, *J* = 7.1 Hz, 2H), 7.31 (t, *J* = 7.4 Hz, 2H), 4.32−4.17 (m, 3H), 4.02 (tt, *J* = 8.9, 4.4 Hz, 1H), 2.97 (dd, *J* = 14.2, 7.6 Hz, 2H), 2.02−1.91 (m, 1H), 1.86−1.70 (m, 1H). ^{13}C NMR (75 MHz, DMSO): δ 173.4, 172.0, 156.1, 147.8, 143.8, 140.7, 134.0, 132.6, 129.4, 127.7, 127.1, 125.3, 124.5, 120.1, 65.7, 51.3, 46.6, 30.8. LCMS, R_t = 10.01 min [10−90% MeOH (0.1% FA) in water (0.1% FA) over 10 min]; ESI-HRMS *m/z*: calcd for $C_{25}H_{23}N_3O_8SNa^+$ $[M + Na]^+$, 548.1098; found, 548.1105.

Fmoc-Orn(*o*-NBS)-OH (14f). Sulfonamide **14f** was prepared according to the protocol for the synthesis of **14e** above from Fmoc-Orn(Boc)-OH (2.02 g, 4.44 mmol) using *o*-NBSCl (1.13 g, 5.08 mmol) and obtained as a light-yellow solid (2.4 g, quantitative): mp 108−110 °C; $[\alpha]_D$ −1.6 (*c* 0.036, MeOH); ^{1}H NMR (300 MHz, DMSO): δ 8.10 (t, *J* = 5.6 Hz, 1H), 8.03−7.92 (m, 2H), 7.92−7.81 (m, 4H), 7.72 (d, *J* = 7.4 Hz, 2H), 7.61 (d, *J* = 8.0 Hz, 1H), 7.41 (t, *J* = 7.2 Hz, 2H), 7.32 (t, *J* = 7.1 Hz, 2H), 4.34−4.16 (m, 3H), 3.89 (td, *J* = 8.7, 4.6 Hz, 1H), 2.90 (q, *J* = 6.3 Hz, 2H), 1.73 (s, 1H), 1.65−1.43 (m, 3H). ^{13}C NMR (75 MHz, DMSO): δ 173.7, 156.1, 147.8, 143.8, 140.7, 134.0, 132.7, 132.6, 129.4, 127.7, 127.1, 125.3, 124.4, 120.1, 65.6, 53.5, 46.7, 42.3, 27.9, 26.0. LCMS, R_t = 11.04 min {10−90% MeOH [0.1% formic acid (FA)] in water (0.1% FA) over 10 min}. ESI-HRMS *m/z*: calcd for $C_{26}H_{26}N_3O_8S^+$ $[M + H]^+$, 540.1435; found, 540.1449.

Fmoc-Lys(*o*-NBS)-Rink Amide Resin 15a. In a syringe fitted with a Teflon filter, stopcock, and stopper, the Fmoc group was removed from the Rink amide resin (3.00 g) using 20% piperidine in DMF solution for 30 min. The resin was filtered and washed sequentially with DMF (×3), MeOH (×3) and CH_2Cl_2 (×3). A separate solution of Fmoc-Lys(*o*-NBS)-OH (1.62 g, 2.93 mmol) in DMF (20 mL) was treated with DIC (0.7 mL, 4.52 mmol) and HOBt (611 mg, 4.52 mmol), stirred for 3 min, and added to the syringe containing the resin. The resin mixture was shaken for 14 h, filtered, and sequentially washed with DMF (×3), MeOH (×3), and CH_2Cl_2 (×3) and dried. The loading of resin **15a** was measured at 0.345 mmol/g resin.

Fmoc-Dab(allyl, *o*-NBS)-$OCH_2CH{=}CH_2$ (16e). Under an argon atmosphere, Fmoc-Dab(*o*-NBS)-OH (**14e**, 2.39 g, 4.54 mmol) was dissolved in THF (dry, 50 mL), treated sequentially with PPh_3 (3.58 g, 13.7 mmol), allyl alcohol (1.9 mL, 27.9 mmol), and DIAD (2.7 mL, 13.7 mmol), stirred at room temperature for 2 h, and diluted with EtOAc (100 mL). The organic phase was washed with water (100 mL × 3) and brine (100 mL), dried over $MgSO_4$, filtered, and evaporated to a residue, which was purified by chromatography using a CombiFlash instrument and 0−100% EtOAc in hexane as the eluent. Evaporation of the collected fractions gave amino ester **16e** as a colorless oil (1.81 g, 66%): $[\alpha]_D$ 5.9 (*c* 0.011, MeOH); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.02−7.96 (m, 1H), 7.78 (d, *J* = 7.4 Hz, 2H), 7.71−7.55 (m, 5H), 7.41 (t, *J* = 7.4 Hz, 2H), 7.32 (dd, *J* = 8.1, 6.9 Hz, 2H), 5.92 (qd, *J* = 11.0, 5.8 Hz, 1H), 5.68 (ddt, *J* = 16.5, 9.9, 6.5 Hz, 1H), 5.52 (d, *J* = 8.1 Hz, 1H), 5.41−5.15 (m, 4H), 4.67 (d, *J* = 5.6 Hz, 2H), 4.39 (d, *J* = 7.6 Hz, 3H), 4.24 (t, *J* = 7.1 Hz, 1H), 4.03−3.86 (m, 2H), 3.53−3.31 (m, 2H), 2.29−2.13 (m, 1H), 2.10−1.95 (m, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.3, 156.0, 148.2, 144.0, 143.9, 141.5, 133.8, 133.4, 132.5, 131.8, 131.5, 131.0, 127.9, 127.3, 125.3, 124.4, 120.1, 119.4, 67.4, 66.6, 52.0, 50.5, 47.2, 43.5, 30.8. LCMS R_t = 11.01 min [10−90% MeOH (0.1% FA) in water (0.1% FA) over 10 min]; ESI-HRMS *m/z*: calcd for $C_{31}H_{32}N_3O_8S^+$ $[M + H]^+$, 606.1905; found, 606.1923.

Fmoc-Orn(allyl, *o*-NBS)-$OCH_2CH{=}CH_2$ (16f). Ester **16f** was synthesized using the protocol described above for **16e** from Fmoc-Orn(*o*-NBS)-OH (**14f**, 2.4 g, 4.44 mmol), PPh_3 (3.46 g, 13.2 mmol), allyl alcohol (1.8 mL, 26.5 mmol), and DIAD (2.6 mL, 13.2 mmol). After column chromatography using 20−50% EtOAc in petroleum ether as the eluent, evaporation of the collected fractions provided amino ester **16f** (1.70 g, 62%) as a pale-yellow oil: $[\alpha]_D$ −2.6 (*c* 0.0115, MeOH). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.05−7.99 (m, 1H), 7.78 (d, *J* = 7.4 Hz, 2H), 7.69−7.55 (m, 5H), 7.41 (t, *J* = 7.4 Hz, 2H), 7.32 (td, *J* = 7.4, 1.1 Hz, 2H), 5.99−5.84 (m, 1H), 5.76−5.59 (m, 1H), 5.39−5.31 (m, 3H), 5.28−5.20 (m, 1H), 5.19−5.13 (m, 1H), 4.65 (d, *J* = 5.7 Hz, 2H), 4.46−4.32 (m, 3H), 4.23 (t, *J* = 7.0 Hz, 1H), 3.91 (d, *J* = 6.1 Hz, 2H), 3.32 (t, *J* = 6.3 Hz, 2H), 1.85 (d, *J* = 10.1 Hz, 1H), 1.70−1.55 (m, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.9, 156.0, 148.1, 144.0, 143.9, 141.5, 133.7, 133.6, 132.7, 131.8, 131.5, 131.1, 127.9, 127.2, 125.2, 124.3, 120.1, 119.5, 119.3, 67.2, 66.3, 53.6, 50.1, 47.3, 46.7, 29.8, 23.9. LCMS [10−90% MeOH (0.1% FA) in water (0.1% FA) over 10 min]; R_t = 10.01 min. ESI-HRMS *m/z*: calcd for $C_{32}H_{33}N_3O_8SNa^+$ $[M + Na]^+$, 642.1881; found, 642.1898.

Fmoc-Dab(allyl, *o*-NBS)-OH (17e). A solution of Fmoc-Dab(allyl, *o*-NBS)-$OCH_2CH{=}CH_2$ (**16e**, 1.44 g, 2.38 mmol) in MeCN (10 mL) was treated with a 0.8 M $CaCl_2$ solution in 7:3 *i*-PrOH:H_2O (60 mL), followed by aqueous NaOH (1 M, 2.4 mL, 2.4 mmol), stirred at room temperature for 6 h, and diluted with EtOAc (100 mL). The organic phase was washed with aqueous HCl (1 M, 100 mL). The aqueous layer was extracted with EtOAc (100 mL). The combined organic layers were washed with brine (100 mL), dried over $MgSO_4$, filtered, and evaporated to a residue that was purified by chromatography using a CombiFlash instrument and 0−100% EtOAc in hexane. Evaporation of the collected fractions gave acid **17e** (850 mg, 63%) as a light-yellow oil: $[\alpha]_D$ −15.2 (*c* 0.01, MeOH); ^{1}H NMR (300 MHz, DMSO): δ 8.01 (ddd, *J* = 16.9, 7.7, 1.4 Hz, 2H), 7.92−7.77 (m, 4H), 7.74−7.60 (m, 3H), 7.41 (t, *J* = 7.4 Hz, 2H), 7.31 (t, *J* = 7.4 Hz, 2H), 5.66 (dq, *J* = 10.4, 6.2 Hz, 1H), 5.19 (dd, *J* = 25.1, 13.7 Hz, 2H), 4.33−4.17 (m, 3H), 4.00−3.87 (m, 3H), 3.46−3.19 (m, 2H), 2.05−1.91 (m, 1H), 1.91−1.77 (m, 1H). ^{13}C NMR (75 MHz, DMSO): δ 173.7, 156.5, 147.9, 144.2, 141.2, 135.1, 133.2, 133.0, 132.2, 130.4, 128.1, 127.5, 125.7, 124.8, 120.6, 119.7, 66.1, 51.9, 50.3, 47.1, 44.8, 30.0. LCMS R_t = 10.54 min [10−90% MeOH (0.1% FA) in water (0.1% FA) over 10 min]. ESI-HRMS *m/z*: calcd for $C_{28}H_{28}N_3O_8S^+$ $[M + H]^+$, 566.1592; found, 566.1608.

Fmoc-Orn(allyl, *o*-NBS)-OH (17f). Acid **17f** was synthesized from ester **16f** (1.70 g, 2.75 mmol) using the protocol described for **17e** with a 0.8 M $CaCl_2$ solution in 7:3 *i*-PrOH:H_2O (70 mL) and NaOH (1 M, 2.7 mL, 2.7 mmol). The residue was purified twice by column chromatography using EtOAc/*n*-hexane (4:1) and then MeOH/CH_2Cl_2 (1:9) to give amino acid **17f** (623 mg, 39%) as a light-yellow oil: $[\alpha]_D$ 12.0 (*c* 0.0075, MeOH); ^{1}H NMR (300 MHz, DMSO): δ 8.04 (dd, *J* = 7.5, 1.6 Hz, 1H), 7.96 (dd, *J* = 7.6, 1.5 Hz, 1H), 7.92−7.78 (m, 4H), 7.72 (d, *J* = 7.1 Hz, 2H), 7.60 (d, *J* = 7.9 Hz, 1H), 7.42 (t, *J* = 7.3 Hz, 2H), 7.32 (t, *J* = 7.3 Hz, 2H), 5.71−5.56 (m, 1H), 5.27−5.10 (m, 2H), 4.33−4.17 (m, 3H), 3.90 (d, *J* = 6.0 Hz, 2H), 3.50−3.15 (m, 3H), 1.74−1.45 (m, 4H). ^{13}C NMR (75 MHz, DMSO): δ 173.6, 156.1, 147.5, 143.9, 143.8, 140.7, 134.5, 132.8, 132.5, 131.9, 129.9, 127.6, 127.1, 125.3, 124.2, 120.1, 119.1, 65.6, 53.6, 49.3, 46.7, 27.9, 24.1. LC−MS [30−95% MeOH (0.1% FA) in

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water (0.1% FA) over 10 min]; R_t = 8.85 min. ESI-HRMS *m/z*: calcd for $C_{29}H_{30}N_3O_8S^+$ $[M + H]^+$, 580.1748; found, 580.1763.

Fmoc-Lys(*o*-NBS, allyl)-Rink Amide Resin 18a. In a syringe fitted with a Teflon filter, vacuum dried Fmoc-Lys(*o*-NBS)-resin **15a** (0.441 mmol) was swollen in dry THF (5 mL), treated sequentially with solutions of allyl alcohol (206 μL, 3.03 mmol) in THF (dry, 1 mL), PPh_3 (397 mg, 1.51 mmol) in THF (dry, 1 mL), and DIAD (298 μL, 1.51 mmol) in THF (dry, 1 mL), shaken for 90 min, filtered, and sequentially washed with DMF (×3), MeOH (×3), THF (×3), and CH_2Cl_2 (×3). Examination by LCMS of a cleaved resin sample (5 mg) showed complete allylation: LCMS R_t = 8.65 min [30−95% MeOH (0.1% FA) in water (0.1% FA) over 10 min]; ESI-MS *m/z*: calcd for $C_{30}H_{33}N_4O_7S^+$ $[M + H]^+$, 593.2; found, 593.2.

Fmoc-Dab(allyl, *o*-NBS) Rink Amide Resin 18e. In a plastic syringe equipped with a stopcock and Teflon filter, the Fmoc group was removed from the Rink amide resin (850 mg) using 20% piperidine in DMF solution for 30 min. The resin was filtered and washed sequentially with DMF (×3), MeOH (×3), and CH_2Cl_2 (×3). A solution of Fmoc-Dab(allyl,*o*-NBS)-OH (**17e**, 450 mg, 0.796 mmol) in DMF (10 mL) was treated in a separate flask with DIC (0.19 mL, 1.23 mmol) and HOBt (166 mg, 1.30 mmol), stirred for 3 min, and transferred to the syringe containing the swollen resin. The resin mixture was shaken for 23 h, filtered, washed sequentially with DMF (×3), MeOH (×3) and CH_2Cl_2 (×3), and was dried. The loading was measured to be 0.327 mmol/g resin.

Fmoc-Orn(allyl, *o*-NBS) Rink Amide Resin 18f. As described for resin **18e**, the Fmoc group was removed from the Rink amide resin (902 mg), which was then reacted with a premixed mixture of Fmoc-Orn(allyl, *o*-NBS)-OH (**17f**, 510 mg, 0.879 mmol), DIC (0.2 mL, 1.29 mmol), and HOBt (178 mg, 1.32 mmol) in DMF (8 mL), shaken for 18 h, filtered, washed, and dried to give a resin with a loading of 0.338 mmol/g.

Fmoc-Lys(*o*-NBS, cyclopropylmethyl) Rink Amide Resin 18g. Resin **18g** was prepared using cyclopropylmethanol in the protocol described for the synthesis of **18a**: LCMS R_t = 10.46 min [10−95% MeOH (0.1% FA) in water (0.1% FA) over 10 min]; ESI-MS *m/z* calcd for $C_{31}H_{34}N_4O_7S$ $[M + H]^+$, 607.2; found, 607.2.

Fmoc-Lys(*o*-NBS, propyl)-Rink Amide Resin 18h. Resin **18g** was prepared using *n*-propanol in the protocol described to make resin **18a**: LCMS R_t = 8.74 min [10−95% MeOH (0.1% FA) in water (0.1% FA) over 10 min]; ESI-MS *m/z* calcd for $C_{30}H_{34}N_4O_7S$ $[M + H]^+$, 595.2; found, 595.2.

Peptide Elongation. Peptides were elongated by standard Fmoc peptide synthesis methods.[44]

Fmoc-Ala-Trp-D-Phe-Dab(*o*-NBS, allyl) Rink Amide Resin 19e. LCMS R_t = 6.63 min [30−95% MeOH (0.1% FA) in water (0.1% FA) over 10 min]; ESI-MS *m/z*: calcd for $C_{36}H_{43}N_8O_8S^+$ [M-Fmoc-2Boc + H]$^+$, 747.3; found, 747.2.

Fmoc-D-Trp(Boc)-Ala-Trp(Boc)-D-Phe-Dab(*o*-NBS,allyl) Rink Amide Resin 20e. LCMS R_t = 9.58 min [30−95% MeOH (0.1% FA) in water (0.1% FA) over 10 min]; ESI-MS *m/z*: calcd for $C_{62}H_{63}N_{10}O_{11}S^+$ [M-2Boc + H]$^+$, 1155.4; found, 1155.4.

Fmoc-D-Trp(Boc)-Ala-Trp(Boc)-D-Phe-Orn(*o*-NBS, allyl) Rink Amide Resin 20f. LCMS R_t = 6.13 min [30−95% MeOH (0.1% FA) in water (0.1% FA) over 10 min]; ESI-MS *m/z*: calcd for $C_{48}H_{55}N_{10}O_9S^+$ [M-Fmoc-2Boc + H]$^+$, 947.4; found, 947.3.

Fmoc-azaPra-Ala-Trp-D-Phe-Dab(*o*-NBS, allyl) Rink Amide Resin 24e. Semicarbazide **24e** was prepared from pentapeptide **21e** (∼400 mg, 0.18 mmol) using the protocol described for the synthesis of azapeptide **24f** and Fmoc-azaPra-Cl from *N*′-propargyl-fluorenylmethylcarbazate (100 mg, 0.342 mmol) and phosgene in toluene (0.35 mL, 0.49 mmol). Examination by LCMS of a cleaved resin sample (5 mg) showed complete coupling: LCMS R_t = 7.46 min [50−90% MeOH (0.1% FA) in water (0.1% FA) over 10 min]; ESI-MS *m/z*: calcd for $C_{55}H_{57}N_{10}O_{11}S^+$ [M-Boc + H]$^+$ 1065.4; found, 1065.3.

Fmoc-azaPra-Ala-Trp(Boc)-D-Phe-Orn(*o*-NBS, allyl) Rink Amide Resin 24f. *N*′-Propargyl-fluorenylmethylcarbazate (110 mg, 0.377 mmol, prepared according to ref 26) was dissolved in dry CH_2Cl_2 (10 mL) under an argon atmosphere, cooled to 0 °C, treated with a 15% solution of phosgene in toluene (0.5 mL, 0.70 mmol), warmed to rt, stirred for 30 min, and evaporated. The residue was dissolved in CH_2Cl_2 (10 mL) and evaporated. The resulting white solid was dissolved in dry CH_2Cl_2 (6 mL), added to H-Ala-Trp(Boc)-D-Phe-Orn(*o*-NBS, allyl) Rink amide resin **21f** (∼600 mg, 0.167 mmol) swollen in DIEA (0.13 mL, 0.746 mmol) in a plastic syringe equipped with a Teflon filter, stopcock, and stopper, and shaken for 18 h. The resin was filtered and washed sequentially with DMF (×3), MeOH (×3), THF (×3), and CH_2Cl_2 (×3). Examination by LCMS of a cleaved resin sample (5 mg) showed complete coupling and formation of azapeptide: LCMS R_t = 9.25 min [30−95% MeOH (0.1% FA) in water (0.1% FA) over 10 min]; ESI-MS *m/z*: calcd for $C_{56}H_{59}N_{10}O_{11}S^+$ [M-Boc + H]$^+$, 1079.4; found, 1079.3.

Fmoc-azaPra-D-Trp(Boc)-Ala-Trp(Boc)-D-Phe-Dab(*o*-NBS, allyl) Rink Amide Resin 25e. Semicarbazide **25e** was prepared from pentapeptide **22e** (∼400 mg, 0.164 mmol) using the protocol described for the synthesis of azapeptide **25f** and Fmoc-azaPra-Cl from *N*′-propargyl-fluorenylmethylcarbazate (100 mg, 0.342 mmol) and phosgene in toluene (0.35 mL, 0.49 mmol). Examination by LCMS of a cleaved resin showed complete coupling to provide the azapeptide: LCMS R_t = 7.89 min [50−90% MeOH (0.1% FA) in water (0.1% FA) over 10 min] ESI-MS *m/z*: calcd for $C_{66}H_{67}N_{12}O_{12}S^+$ [M-2Boc +2H]$^{2+}$ 626.2; found, 626.3.

Fmoc-azaPra-D-Trp(Boc)-Ala-Trp(Boc)-D-Phe-Orn(*o*-NBS, allyl) Rink Amide Resin 25f. *N*′-Propargyl-fluorenylmethyl carbazate (248 mg, 0.849 mmol, prepared according to ref 26) was dissolved in CH_2Cl_2 (dry, 40 mL) under an argon atmosphere, cooled to 0 °C, and treated with a 20% solution of phosgene in toluene (1 mL, 1.87 mmol). The ice bath was removed. The reaction mixture was warmed to rt with stirring for 50 min and evaporated to a residue, which was dissolved in CH_2Cl_2 (10 mL) and evaporated. The resulting white solid was dissolved in dry CH_2Cl_2 (7 mL), added to a solution of H-D-Trp(Boc)-Ala-Trp(Boc)-D-Phe-Orn(*o*-NBS, allyl) Rink amide resin **22f** (∼1 g, 0.20 mmol) swollen in DIEA (0.07 mL, 0.402 mmol) in a plastic syringe equipped with a Teflon filter, stopcock, and stopper, and shaken for 28 h. The resin was filtered and washed sequentially with DMF (×3), MeOH (×3), THF (×3), and CH_2Cl_2 (×3). Examination by LCMS of a cleaved resin showed complete coupling to provide the azapeptide: LCMS R_t = 8.26 min [30−95% MeOH (0.1% FA) in water (0.1% FA) over 10 min]; ESI-MS *m/z*: calcd for $C_{52}H_{59}N_{12}O_{10}S^+$ [M-Fmoc-2Boc + H]$^+$, 1043.4; found, 1043.3.

Boc-Ala-azaPra-Ala-Trp-D-Phe-Dab(*o*-NBS, allyl) Rink Amide Resin 28e. Azapeptide **28e** was prepared from Fmoc-deprotected vacuum-dried azapeptide **26e** (∼400 mg, 0.18 mmol) according to the protocol for the synthesis of azapeptide **28f** using Fmoc-Ala-OH (279 mg, 0.897 mmol), BTC (95 mg, 0.320 mmol), and 2,4,6-collidine (0.24 mL, 1.82 mmol). Examination by LCMS of a cleaved resin showed complete coupling: LCMS R_t = 9.12 min [30−95% MeOH (0.1% FA) in water (0.1% FA) over 10 min]; ESI-MS *m/z*: calcd for $C_{58}H_{62}N_{11}O_{12}S^+$ [M-Boc + H]$^+$ 1136.4; found, 1136.3. The Fmoc group was removed and the resin (∼400 mg) was treated with Boc_2O (218 mg, 1.0 mmol).

Boc-Ala-azaPra-Ala-Trp(Boc)-D-Phe-Orn(*o*-NBS, allyl) Rink Amide Resin 28f. Dry THF (10 mL) was added to a mixture of Fmoc-Ala-OH (265 mg, 0.852 mmol) and BTC (85 mg, 0.286 mmol) in a microwave vial containing a magnetic stirring bar. The vial was sealed, cooled to 0 °C, treated with 2,4,6-collidine (0.23 mL, 1.74 mmol), and stirred for 1 min. The vial was opened to remove the magnetic stirring bar, treated with Fmoc-deprotected vacuum-dried azapeptide resin **26f** (∼600 mg, 0.167 mmol), sealed, heated with microwave irradiation at 60 °C for 1 h, cooled, and opened. The resin was transferred to a plastic syringe equipped with a Teflon filter, stopcock, and stopper, then washed sequentially with DMF (×3), MeOH (×3), THF (×3), and CH_2Cl_2 (×3). Examination by LCMS of a cleaved resin showed complete coupling and a new azapeptide: LCMS R_t = 9.22 min [30−95% MeOH (0.1% FA) in water (0.1% FA) over 10 min]; ESI-MS *m/z*: calcd for $C_{59}H_{64}N_{11}O_{12}S^+$ [M-Boc + H]$^+$, 1150.5; found, 1150.3.

After the Fmoc group removal, the resin (∼500 mg, 0.15 mmol) was treated with a solution of Boc_2O (170 mg, 0.779 mmol) in dry

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THF (5 mL) in a plastic syringe equipped with a Teflon filter, stopcock, and stopper. The resin was shaken for 1 h, filtered, and washed sequentially with DMF (×3), MeOH (×3), THF (×3), and CH_2Cl_2 (×3). The Kaiser test showed complete Boc-protection.

Boc-Ala-azaPra-D-Trp(Boc)-Ala-Trp(Boc)-D-Phe-Dab(*o*-NBS, allyl) Rink Amide Resin 29e. Azapeptide **29e** was prepared from Fmoc-deprotected azapeptide resin **27e** (~400 mg, 0.164 mmol) according to the protocol to make resin **28f** using Fmoc-Ala-OH (270 mg, 0.868 mmol), BTC (85 mg, 0.286 mmol), and 2,4,6-collidine (0.23 mL, 1.74 mmol). Examination by LCMS of a cleaved resin showed complete coupling: LCMS R_t = 9.59 min [30−95% MeOH (0.1% FA) in water (0.1% FA) over 10 min]; ESI-MS *m/z*: calcd for $C_{69}H_{72}N_{13}O_{13}S^+$ [M-2Boc +2H]$^{2+}$ 661.8; found, 661.8. After Fmoc removal, the resin was treated with Boc_2O (182 mg, 0.834 mmol).

Boc-Ala-azaPra-D-Trp(Boc)-Ala-Trp(Boc)-D-Phe-Orn(*o*-NBS, allyl) Rink Amide Resin 29f. The Fmoc group was removed from semicarbazide **25f** (~1 g, 0.20 mmol) and resin **27f** was treated twice with symmetric anhydride that was generated from Boc-Ala-OH according to the protocol described in ref 26. Examination by LCMS of a cleaved resin sample (5 mg) showed complete coupling: LCMS R_t = 6.39 min [30−95% MeOH (0.1% FA) in H_2O (0.1% FA) over 10 min]; ESI-MS *m/z*: calcd for $C_{55}H_{64}N_{13}O_{11}S^+$ [M-3Boc + H]$^+$, 1114.5; found, 1114.4.

Boc-Ala-azaPra-Ala-Trp-D-Phe-Dab(allyl) Rink Amide Resin 30e. Azapeptide **30e** was prepared from azapeptide **28e** (~400 mg, 0.18 mmol) according to the protocol to make resin **30f** using DBU (270 μL, 1.81 mmol) and 2-mercaptoethanol (60 μL, 0.86 mmol). Examination by LCMS of a cleaved resin sample (5 mg) showed complete *o*-NBS-removal: LCMS R_t = 4.52 min [10−90% MeOH (0.1% FA) in water (0.1% FA) over 10 min]; ESI-MS *m/z*: calcd for $C_{37}H_{49}N_{10}O_6^+$ [M-2Boc + H]$^+$, 729.4; found, 729.6.

Boc-Ala-azaPra-Ala-Trp(Boc)-D-Phe-Orn(allyl) Rink Amide Resin 30f. In the plastic syringe, *o*-NBS-protected azapeptide resin **28f** (~500 mg, 0.15 mmol) was swollen in DMF (6 mL), treated with DBU (220 μL, 1.47 mmol) and 2-mercaptoethanol (50 μL, 0.71 mmol), shaken for 3 h, filtered, and sequentially washed with DMF (×3), MeOH (×3), THF (×3), and CH_2Cl_2 (×3). Examination by LCMS of a cleaved resin sample (5 mg) showed complete *o*-NBS-removal and a new azapeptide: LCMS R_t = 3.54 min [10−90% MeOH (0.1% FA) in water (0.1% FA) over 10 min]; ESI-MS *m/z*: calcd for $C_{38}H_{51}N_{10}O_6^+$ [M-2Boc + H]$^+$, 743.4; found, 743.5.

Boc-Ala-azaPra-D-Trp(Boc)-Ala-Trp(Boc)-D-Phe-Dab(allyl) Rink Amide Resin 31e. Azapeptide **31e** was prepared according to the protocol to make azapeptide **31f** using resin **29e** (~400 mg, 0.164 mmol), DBU (250 μL, 1.67 mmol), and 2-mercaptoethanol (60 μL, 0.86 mmol): LCMS R_t = 5.10 min [10−90% MeOH (0.1% FA) in water (0.1% FA) over 10 min]; ESI-MS *m/z*: calcd for $C_{48}H_{59}N_{12}O_7^+$ [M-3Boc + H]$^+$, 915.5; found, 915.5.

Boc-Ala-azaPra-D-Trp(Boc)-Ala-Trp(Boc)-D-Phe-Orn(allyl) Rink Amide Resin 31f. In a plastic syringe equipped with a Teflon filter, stopcock, and stopper, *o*-NBS-protected azapeptide **29f** (~1 g, 0.20 mmol) was swollen in DMF (6 mL), treated with DBU (300 μL, 2.01 mmol) and 2-mercaptoethanol (70 μL, 1.00 mmol), shaken for 1 h, filtered, and washed sequentially with DMF (×3), MeOH (×3), THF (×3), and CH_2Cl_2 (×3). Examination by LCMS of a cleaved resin sample (5 mg) showed complete *o*-NBS-removal and a new azapeptide: LCMS R_t = 4.49 min [30−95% MeOH (0.1% FA) in water (0.1% FA) over 10 min]; ESI-MS *m/z*: calcd for $C_{49}H_{61}N_{12}O_7^+$ [M-3Boc+2Na]$^{2+}$ 487.2; found, 487.3.

***c*-{[azaGly(αN)−$(CH_2)_4$−Lys(εN)]H-Ala-azaGly-D-Trp-Ala-Trp-D-Phe-Lys(*n*-Pr)-NH_2} (32).** A solution of *c*-{[azaPra(δC)−CH_2−Lys(εN)]H-Ala-azaPra-D-Trp-Ala-Trp-D-Phe-Lys(allyl)-NH_2} (**7a**, 5 mg, 5.0 μmol) in MeOH (2 mL) was treated with Pd/C (10 wt %, 4 mg), placed under an argon atmosphere, treated with hydrogen gas bubbles, and stirred at room temperature for 1 h under an atmosphere of hydrogen. The atmosphere was exchanged for argon. The suspension was filtered through a plug of Celite. The filter cake was washed with MeOH. The filtrate and washings were combined, treated with water, and freeze-dried to a light-yellow foam, which was purified by preparative HPLC to give cyclic azapeptide **32** (3.7 mg, 74%) as a white foam: HPLC R_t = 6.04 min [10−90% of MeOH (0.1% FA) in H_2O (0.1% FA) over 10 min and then 90% MeOH (0.1% FA) for 5 min]; R_t = 5.71 min [10−90% MeCN (0.1% FA) in H_2O (0.1% FA) over 10 min and then 90% MeCN (0.1% FA) for 5 min]; HRMS *m/z*: calcd for $C_{51}H_{69}N_{12}O_7^+$ [M + H]$^+$, 961.5407; found, 961.5391.

***c*-{[Aza-hexenylglycine(εC)-Lys(εN)]H-Ala-*Z*-aza-hexenylglycinyl-D-Trp-Ala-Trp-D-Phe-Lys(cyclopropylmethyl)-NH_2} (33).** A solution of *c*-{[azaPra(δC)−CH_2−Lys(εN)]H-Ala-azaPra-D-Trp-Ala-Trp-D-Phe-Lys(cyclopropylmethyl)-NH_2} (**7g**, 6 mg, 6.2 μmol) in MeOH (2 mL) was mixed with the Lindlar catalyst (5 mg,, prepared according to ref 45). The vessel containing the suspension was flushed with argon. Quinoline (50 μL, 0.42 mmol) was added to the suspension, which was placed under an atmosphere of hydrogen and stirred at room temperature for 1 h. The atmosphere was exchanged for argon. The suspension was filtered through a plug of Celite. The filter cake was washed with MeOH. The filtrate and washings were combined, treated with water, and freeze-dried. The resulting light-yellow foam was purified by preparative HPLC to give cyclic azapeptide **33** (2 mg, 33%) as a white foam: HPLC R_t = 6.64 min [10−90% of MeOH (0.1% FA) in H_2O (0.1% FA) over 10 min and then 90% MeOH (0.1% FA) for 5 min]; R_t = 4.93 min [10−90% MeCN (0.1% FA) in H_2O (0.1% FA) over 10 min and then 90% MeCN (0.1% FA) for 5 min]; HRMS *m/z*: calcd for $C_{52}H_{67}N_{12}O_7^+$ [M + H]$^+$, 971.5250; found, 971.5254.

***c*-{[AsPra(δC)−CH_2−Lys(εN)]H-Ala-AsPra-D-Trp-Ala-Trp-D-Phe-Lys(allyl)-NH_2} (36).** Cyclic azasulfurylpeptide **36** was prepared according to the protocol described for cyclic azapeptide **7f** using linear azasulfurylpeptide resin **45** (1 equiv, 282 mg, 0.0925 mmol), CuI (0.2 equiv, 3.52 mg, 0.0185 mmol), and aq. formaldehyde (6 equiv, 41.6 μL, 0.555 mmol): LCMS R_t = 7.75 [10−90% MeOH (0.1% FA) in water (0.1% FA) over 10 min and then in 10% MeOH (0.1% FA) in water (0.1% FA) for 5 min]; ESI-MS *m/z* calcd for $C_{50}H_{63}N_{12}O_8S$ [M + H]$^+$, 991.45; found, 991.4. Resin cleavage using a freshly made solution of TFA/H_2O/TES (95:2.5:2.5, v/v/v, 5 mL) at 4 °C for 3 h and purification of the light brown foam by preparative RP-HPLC on a Gemini 5 —μm C_{18} 110A column (Phenomenex Inc., 250 × 21.2 mm, 5 μm) using a gradient of 5−60% MeOH (0.1% FA) in water (0.1% FA) with a flow rate of 10.0 mL/min and UV detection at 214 nm gave cyclic azasulfurylpeptide **36** (1.0 mg, 1%) as a white foam, which was analyzed on a Sunfire column: LCMS R_t 8.12 min [5−50% MeOH (0.1% FA) in water (0.1% FA) over 9 min and then 5% MeOH (0.1% FA) in water (0.1% FA) for 5.0 min] >99% purity; R_t 5.99 min [5−50% MeCN (0.1% FA) over 9 min and then 5% MeCN (0.1% FA) for 5.0 min] >99% purity; HRMS *m/z* calcd for [M + H]$^+$ $C_{50}H_{63}N_{12}O_8S$, 991.4607; found, 991.4657.

***N*-Propargyl Fluorenylmethyl Carbazate (H-azaPra-Ofm, 37).** A solution of *tert*-butyl carbazate (1 equiv, 2 g, 15.1 mmol) in 9:1 THF/DMF (30 mL) was treated with powdered K_2CO_3 (1.5 equiv, 3.14 g, 22.7 mmol), cooled to 0 °C, and treated dropwise with a solution of propargyl bromide (0.8 equiv, 1.3 mL, 12.1 mmol, 80% in toluene) in THF (10 mL). The ice bath was removed. After warming to room temperature with stirring overnight, the reaction mixture was washed with H_2O (100 mL) and brine (100 mL), dried over Na_2SO_4, filtered, and evaporated. The reside was purified by column chromatography using 30−40% EtOAc/hexane as the eluent to give *N′*-propargyl *tert*-butyl carbazate (1.91 g, 6.54 mmol, 54%) as a white solid: mp 54−55 °C; R_f = 0.24 (EtOAc/hexane 1:4); ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.20 (s, 1H), 4.07 (s, 1H), 3.62 (d, *J* = 2.5 Hz, 2H), 2.24 (t, *J* = 2.5 Hz, 1H), 1.46 (s, 9H); ^{13}C NMR (125 MHz, CD_3OD): δ 156.5, 81.0, 80.1, 72.5, 41.4, 28.5; HRMS *m/z* calcd for $C_8H_{15}N_2O_2$ [M + H]$^+$, 171.1128; found, 171.1122.

A solution of *N′*-propargyl *tert*-butyl carbazate (1 equiv, 0.57 g, 3.35 mmol) in CH_2Cl_2 (40 mL) was cooled to −40 °C and treated with DIEA (1.5 equiv, 0.83 mL, 5.02 mmol), followed dropwise by a solution of Fmoc-Cl (1.1 equiv, 0.95 g, 3.68 mmol) in CH_2Cl_2 (10 mL). The cooling bath was removed. After warming to room temperature with stirring overnight, the reaction mixture was washed with H_2O (100 mL) and brine (100 mL), dried over Na_2SO_4, filtered, and evaporated. The reside was purified by column chromatography

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using 10−15% EtOAc/hexane as the eluent to give *N*′-propargyl *N*′-Fmoc *tert*-butyl carbazate (1.24 g, 3.15 mmol, 94%) as a white solid: mp 93−94 °C; R_f = 0.4 (EtOAc/hexane 1:4). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.77 (d, J = 7.6 Hz, 2H), 7.62 (d, J = 7.5 Hz, 2H), 7.41 (t, J = 7.5 Hz, 2H), 7.34−7.29 (m, 2H), 6.63 (s, 1H), 4.41 (br s, 4H), 4.26 (br s, 1H), 2.30 (s, 1H), 1.50 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 156.7, 154.4, 143.7 (2C), 141.4 (2C), 128.0 (2C), 127.3 (2C), 125.4 (2C), 120.2 (2C), 82.2, 78.1, 72.9, 69.1, 47.1, 39.9, 28.3 (3C); HRMS m/z calcd for $C_{23}H_{25}N_2O_4$ $[M + Na]^+$, 415.1628; found, 415.1621.

A solution of *N*′-propargyl *N*′-Fmoc *tert*-butyl carbazate (1 equiv, 1.1 g, 2.8 mmol) in CH_2Cl_2 (50 mL) was treated with bubbles of dry HCl gas for 2 h when complete consumption of the starting material was observed by TLC. The volatiles were removed under reduced pressure to give *N*-propargyl fluorenylmethyl carbazate (**37**, 1.09 g, 2.77 mmol, 99%) as a white solid: mp 176 °C; ^{1}H NMR (500 MHz, CD_3OD): δ 7.82 (d, J = 7.6 Hz, 2H), 7.66 (dd, J = 7.5, 0.8 Hz, 2H), 7.45−7.39 (m, 2H), 7.36−7.31 (m, 2H), 4.64 (d, J = 6.3 Hz, 2H), 4.37−4.31 (m, 3H), 3.03 (t, J = 2.5 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD): δ 155.7, 144.5 (2C), 142.7 (2C), 129.1 (2C), 128.3 (2C), 126.0 (2C), 121.1 (2C), 77.1, 76.2, 70.9, 48.0, 39.7; HRMS m/z calcd for $C_{18}H_{17}N_2O_2$ $[M + H]^+$, 293.1285; found, 293.1274.

Boc-Ala-azaPra-Ofm (38). A −15 °C solution of Boc-Ala-OH (1.1 equiv, 0.513 g, 2.71 mmol) in THF (10 mL) was treated sequentially with isobutyl chloroformate (1.1 equiv, 0.353 mL, 2.71 mmol) and *N*-methylmorpholine (2 equiv, 0.542 mL, 4.93 mmol), stirred for 15 min, treated with a solution of H-azaPra-Ofm (**37**, 1 equiv, 0.72 g, 2.46 mmol) in THF (15 mL), followed by DIEA (1 equiv, 0.407 mL, 2.46 mmol), and stirred for 1 h. The volatiles were removed by rotary evaporation. The residue was purified by column chromatography eluting with 30−35% EtOAc/hexane. Evaporation of the collected fractions gave Boc-Ala-azaPra-Ofm (**38**, 0.82 g, 1.77 mmol, 72%) as a white solid: mp 78−79 °C; R_f = 0.33 (EtOAc/hexane 2:3). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.39 (br s, 1H), 7.76 (d, J = 7.5 Hz, 2H), 7.57 (s, 2H), 7.40 (t, J = 7.4 Hz, 2H), 7.34−7.27 (m, 2H), 4.81 (br s, 1H), 4.56−4.42 (m, 2H), 4.37 (s, 2H), 4.23 (br s, 2H), 2.25 (br s, 1H), 1.44 (s, 9H), 1.31 (br s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 180.1, 156.0, 143.7, 143.6 (2C), 141.4 (2C), 128.0 (2C), 127.3 (2C), 125.2 (2C), 120.2 (2C), 80.9, 77.7, 73.2, 68.5, 48.5, 47.1, 39.4, 28.5 (3C), 17.4; HRMS m/z calcd for $C_{26}H_{30}N_3O_5$ $[M + H]^+$, 464.2180; found, 464.2178.

***N*-(Boc)Alanine *N*′-Propargyl Hydrazide (39).** A solution of Boc-Ala-azaPra-Ofm (**38**, 1 equiv, 0.82 g, 1.77 mmol) in MeCN (15 mL) was treated with diethylamine (27.4 equiv, 3.55 g, 5 mL, 48.5 mmol), stirred at room temperature for 1.5 h, and evaporated to a residue that was purified by flash chromatography[46] on silica gel eluting with a gradient of 60−80% EtOAc in hexanes containing 2% triethylamine to afford hydrazide **39** (0.265 g, 1.1 mmol, 62%) as a white solid: mp 75−76 °C; R_f 0.21 (40% EtOAc in hexanes containing 2% triethylamine); ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.96 (s, 1H), 4.99 (br s, 1H), 4.71 (s, 1H), 4.16 (s, 1H), 3.61 (s, 2H), 2.23 (t, J = 2.5 Hz, 1H), 1.44 (s, 9H), 1.37 (d, J = 7.1 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 172.4, 155.6, 80.6, 79.7, 72.7, 48.9, 41.2, 28.5 (3C), 18.2; HRMS m/z calcd for $C_{11}H_{20}N_3O_3$ $[M + H]^+$, 242.1499; found, 242.1494.

2,4-Dimethoxybenzyl *N*^{in}-(Boc)-D-tryptophan 4-Nitrophenyl Sulfamidate (40). A solution of Fmoc-D-Trp(Boc)-OH (1 equiv, 4.5 g, 8.55 mmol), TBTU (1 equiv, 2.74 g, 8.55 mmol), and DIEA (2 equiv, 2.82 mL, 17.1 mmol) in DMF (20 mL) under an argon atmosphere was stirred at room temperature for 30 min, treated dropwise with a solution of 2,4-dimethoxybenzyl alcohol (1.1 equiv, 1.58 g, 9.4 mmol) in DMF (5 mL), stirred at room temperature for 4 h, and diluted with EtOAc. The organic phase was washed with water (2 × 100 mL) and brine (2 × 100 mL), dried over Na_2SO_4, filtered, and evaporated to a residue that was purified by flash chromatography[46] on silica gel eluting with 20% EtOAc in hexane. Evaporation of the collected fractions afforded Fmoc-D-Trp(Boc)-Odmb (4.97 g, 7.35 mmol, 86%) as a white solid: mp 67−68 °C; R_f 0.29 (20% EtOAc in hexane); ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.11 (br s, 1H), 7.76 (d, J = 7.6 Hz, 2H), 7.58−7.48 (m, 3H), 7.44 (s, 1H), 7.42−7.36 (m, 2H), 7.33−7.26 (m, 3H), 7.21 (t, J = 7.3 Hz, 1H), 7.11 (d, J = 8.0 Hz, 1H), 6.46−6.39 (m, 2H), 5.44 (d, J = 8.0 Hz, 1H), 5.12 (s, 2H), 4.78 (dd, J = 13.6, 5.4 Hz, 1H), 4.40−4.28 (m, 2H), 4.19 (t, J = 7.3 Hz, 1H), 3.80 (s, 3H), 3.79 (s, 3H), 3.33−3.20 (m, 2H), 1.65 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 171.7, 161.7, 159.2, 155.8, 149.7, 144.0, 143.9, 141.4 (2C), 131.7 (2C), 127.8 (2C), 127.2 (2C), 125.3 (2C), 124.6 (2C), 124.5, 124.4, 122.8, 120.1, 119.0, 115.8, 115.4, 115.1, 104.2, 98.7, 83.8, 67.3, 63.4, 55.5 (2C), 54.4, 47.3, 28.3 (3C), 28.0; HRMS m/z calcd for $C_{40}H_{41}N_2O_8$ $[M + H]^+$, 677.2857; found, 677.2859.

A solution of Fmoc-D-Trp(Boc)-Odmb (1 equiv, 4.2 g, 6.21 mmol) in MeCN (62 mL) was treated with diethylamine (18.3 mL, 334 mmol), stirred at room temperature for 2 h, and evaporated to a residue that was purified by flash chromatography[46] on silica gel eluting with 40% EtOAc in hexane containing 2% triethylamine to afford H-D-Trp(Boc)-Odmb (2.48 mg, 5.46 mmol, 88%) as an oil: ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.12 (s, 1H), 7.55 (d, J = 7.6 Hz, 1H), 7.47 (s, 1H), 7.33−7.28 (m, 1H), 7.25−7.20 (m, 1H), 7.14 (d, J = 8.1 Hz, 1H), 6.47−6.43 (m, 2.3 Hz, 2H), 5.12 (s, 2H), 3.84 (dd, J = 7.7, 5.0 Hz, 1H), 3.81 (s, 3H), 3.81 (s, 3H), 3.23−3.15 (m, 1H), 3.01−2.94 (m, 1H), 1.66 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 175.3, 161.5, 159.2, 149.7, 135.6, 131.6, 130.6, 124.5, 124.3, 122.6, 119.1, 116.4, 116.2, 115.4, 104.1, 98.7, 83.6, 62.6, 55.53, 55.50, 54.7, 30.5, 28.3 (3C); HRMS m/z calcd for $C_{25}H_{31}N_2O_6$ $[M + H]^+$, 455.2177; found, 455.2172.

A −78 °C solution of 4-nitrophenyl chlorosulfate (2 equiv, 2.3 g, 9.68 mmol, prepared according to ref 33) in DCM (25 mL) was treated dropwise with a solution of H-D-Trp(Boc)-Odmb (1 equiv, 2.2 g, 4.84 mmol), 4-nitrophenol (3 equiv, 2.02 g, 14.5 mmol), and triethylamine (TEA, 6 equiv, 4.04 mL, 29 mmol) in DCM (100 mL) and stirred for 1.5 h. The cooling bath was removed. After warming to room temperature with stirring for 1 h, the reaction mixture was evaporated to a residue that was purified by flash chromatography[46] eluting with 40% Et_2O in petroleum ether to afford fractions contaminated with 4-nitrophenol. The collected fractions were evaporated, dissolved in DCM (25 mL), washed with sat. $NaHCO_3$ (aq, 3 × 25 mL), dried over $MgSO_4$, filtered, and evaporated to afford sulfamidate **40** (1.08 g, 1.65 mmol, 34%) as a white solid: mp 48−50 °C; R_f 0.30 (40% Et_2O in petroleum ether); ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.11−8.10 (m, 1H), 8.10−8.09 (m, 1H), 7.50 (d, J = 7.7 Hz, 1H), 7.45 (s, 1H), 7.35−7.30 (m, 1H), 7.26−7.18 (m, 3H), 7.10 (d, J = 8.0 Hz, 1H), 6.92−6.88 (m, 1H), 6.46−6.41 (m, 2H), 5.63 (d, J = 8.5 Hz, 1H), 5.17−5.08 (m, 2H), 4.59−4.53 (m, 1H), 3.82 (s, 3H), 3.79 (s, 3H), 3.32−3.22 (m, 2H), 1.67 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 170.6, 162.0, 161.5, 159.4, 154.4, 149.6, 146.0, 132.2, 130.2, 126.4, 125.5, 124.91, 124.86, 122.9, 122.3, 118.9, 115.8, 115.5, 115.2, 113.7, 104.3, 98.7, 84.3, 64.2, 57.2, 55.59, 55.55, 28.8, 28.3 (3C). HRMS m/z calcd for $C_{31}H_{33}N_3O_{11}S$ $[M + Na]^+$, 678.1728; found, 678.1720.

Boc-Ala-AsPra-D-Trp(Boc)-Odmb (41). To a microwave vessel containing a solution of sulfamidate **40** (1 equiv, 0.453 g, 0.691 mmol) in dichloroethane (4.5 mL), hydrazide **39** (1.2 equiv, 0.2 g, 0.829 mmol) was added, followed by TEA (2 equiv, 0.14 g, 0.192 mL, 1.38 mmol), at which point the solution turned yellow. The vessel was sealed, heated to 60 °C using microwave irradiation for 3 h, and cooled to room temperature. The volatiles were evaporated. The residue was purified by flash chromatography[46] on silica gel eluting with a solution of 40−50% EtOAc in hexane. The collected fractions were evaporated to a residue, which was dissolved in DCM (25 mL), washed with sat. $NaHCO_3$ (3 × 25 mL), dried over $MgSO_4$, filtered, and evaporated to afford azasulfuryl tripeptide **41** (0.29 g, 0.383 mmol, 55%) as a white solid: R_f 0.25 (40% EtOAc in hexane); mp 79 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.30 (s, 1H), 8.08 (br s, 1H), 7.58 (d, J = 7.7 Hz, 1H), 7.45 (s, 1H), 7.29 (t, J = 7.3 Hz, 1H), 7.22 (t, J = 7.2 Hz, 1H), 7.12 (d, J = 7.8 Hz, 1H), 6.46−6.41 (m, 2H), 5.47 (d, J = 7.0 Hz, 1H), 5.08 (d, J = 2.7 Hz, 2H), 4.95 (br s, 1H), 4.65−4.58 (m, 1H), 4.32−4.24 (m, 1H), 4.23−4.16 (m, 1H), 4.16−4.08 (m, 1H), 3.82 (s, 3H), 3.80 (s, 3H), 3.27 (t, J = 4.5 Hz, 2H), 2.32 (t, J = 2.4 Hz, 1H), 1.66 (s, 9H), 1.42 (s, 9H), 1.33 (d, J = 7.1 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 171.8, 171.6, 161.7, 159.2, 149.7,

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131.9, 130.7 126.3, 124.8, 124.6, 122.8, 119.4, 115.8, 115.5, 115.3, 114.4, 104.3, 98.7, 83.8, 81.0, 76.4, 75.0, 63.9, 56.4, 55.5 (2C), 49.2, 42.0, 28.49, 28.42 (3C), 28.35 (3C), 17.3; HRMS m/z calcd for $C_{36}H_{48}N_5O_{11}S$ $[M + H]^+$, 758.3066; found, 758.3061.

H-Ala-Trp(Boc)-D-Phe-Lys(*o*-NBS, allyl) Rink Amide Resin (43). In a plastic syringe equipped with a Teflon filter, stopcock, and stopper, a vacuum-dried Fmoc-Ala-Trp(Boc)-D-Phe-Lys(*o*-NBS) Rink amide resin (1 equiv, 4.26 g, 1.5 mmol, synthesized according to the protocol in ref 11) was swollen in THF (42.6 mL), placed under argon, treated sequentially with solutions of allyl alcohol (10 equiv, 1.02 mL, 15 mmol) in THF (14.2 mL), PPh_3 (5 equiv, 1.97 g, 7.5 mmol) in THF (14.2 mL), and DIAD (5 equiv, 1.49 mL, 7.5 mmol) in THF (14.2 mL), shaken for 30 min, filtered, and washed sequentially using 15 s agitations with DMF (×3), MeOH (×3), and DCM (×3). The resin was vacuum—dried. A 0.5 g resin sample was treated with 20% piperidine in DMF (10 mL) to afford resin **43**. Examination by LCMS of a cleaved resin sample (2−3 mg) showed complete allylation: LCMS R_t = 7.94 min [10−90% MeOH (0.1% FA) in water (0.1% FA) over 9 min and then 10% MeOH (0.1% FA) in water (0.1% FA) for 5 min]; ESI-MS m/z: calcd for $C_{38}H_{47}N_8O_8S$ [M-Boc + H]$^+$, 775.32; found, 775.3.

Boc-Ala-AsPra-D-Trp(Boc)-Ala-Trp(Boc)-D-Phe-Lys(*o*-NBS, allyl) Rink Amide Resin (44). 2,4-Dimethoxybenzyl ester **41** (180 mg, 0.238 mmol) was stirred in a solution of 1% TFA in DCM (5 mL) at room temperature for 1 h. The volatiles were evaporated. The residue was treated with Et_2O (5 mL) to provide a precipitate that was filtered. The filtrate was evaporated to afford Boc-Ala-AsPra-D-Trp(Boc)-OH (**42**). A solution of tripeptide **42** (1.2 equiv, 126 mg, 0.208 mmol) and HOBt (1.2 equiv, 28.1 mg, 0.208 mmol) in DMF (5 mL) was stirred for 5 min, treated with DIC (1.2 equiv, 32.4 μL, 0.208 mmol), stirred for 5 min, and transferred to a plastic syringe equipped with a Teflon filter, stopcock, and stopper containing swollen H-Ala-Trp(Boc)-D-Phe-Lys(*o*-NBS, allyl) Rink amide resin **43** (1 equiv, 460 mg, 0.173 mmol) in DMF (5 mL). The resin was shaken for 18 h at room temperature, filtered, and washed sequentially using 15 s agitations with DMF (×3), MeOH (×3), and DCM (×3). Examination by LCMS of a cleaved resin sample (2−3 mg) showed complete coupling to give azasulfurylpeptide **44**: LCMS R_t = 6.25 min [50−90% MeOH (0.1% FA) in water (0.1% FA) over 8.5 min and then 50% MeOH (0.1% FA) in water (0.1% FA) for 5.5 min]; ESI-MS m/z: calcd for $C_{55}H_{66}N_{13}O_{12}S$ [M-3Boc + H]$^+$, 1164.43; found, 1164.3.

Boc-Ala-AsPra-D-Trp(Boc)-Ala-Trp(Boc)-D-Phe-Lys(allyl) Rink Amide Resin (45). Azasulfurylpeptide resin **44** (1 equiv, 300 mg, 0.0925 mmol) was swollen in DMF (6 mL) for 30 min, treated with DBU (10 equiv, 138 μL, 0.925 mmol) and 2-mercaptoethanol (5 equiv, 32.5 μL, 0.462 mmol), shaken for 30 min, filtered, and washed sequentially using 15 s agitations with DMF (×3), MeOH (×3), and DCM (×3). Examination by LCMS of a cleaved resin sample (2−3 mg) showed complete removal of the *o*-NBS group: LCMS R_t = 7.46 min [10−90% MeOH (0.1% FA) in water (0.1% FA) over 10 min and then 10% MeOH (0.1% FA) in water (0.1% FA) for 5 min]; ESI-MS m/z: calcd for $C_{49}H_{63}N_{13}O_8S$ [M-3Boc + H]$^+$, 979.45; found, 979.4.

Effects of Cyclic Azapeptide and Cyclic Azasulfurylpeptide on the Overproduction of NO Induced by R-FSL-1 in the RAW 264.7 Macrophage Cell Line. The murine RAW 264.7 macrophage cell line (American Type Cell Collection, ATCC #TIB-71) was seeded at 1.5×10^5 cells/well in DMEM supplemented with penicillin and streptomycin on a 48-well plate and incubated at 37 °C with 5% CO_2. After 2 h, the medium of adhered cells was changed to DMEM-Pen/Strep containing 0.2% of bovine serum albumin (BSA) and supplemented with either the cyclic azapeptide or cyclic azasulfurylpeptide at a concentration of 10^{-7} M. After 1 h of pre-incubation, the cells were stimulated overnight with R-FSL-1 (Invivogen #L7022). Supernatants were collected for fluorescence determination of nitrite using 2,3-diaminonaphthalene (DAN). Briefly, 25 μL of the sample was incubated with 0.5 μg of DAN in a 100 μL final volume of phosphate buffer (50 mM, pH 7.5) at room temperature in the dark. After 15 min, the reaction was stopped with 20 μL of NaOH (2.8 N), and the plate was read using a fluorescence plate reader (TECAN Safire, λ_{exc}: 365 nm and λ_{em}: 430 nm).

Radiolabeling of the Photoactivatable Ligand as the Radiotracer for the Receptor-Binding Assay. The radioiodination of Tyr-Bpa-Ala-hexarelin was performed as previously described in ref 47. Briefly, 10 nmol of Tyr-Bpa-Ala-hexarelin was mixed with 100 ng of lactoperoxidase and 1 mCi of $Na^{125}I$ in a volume of 30 μL of 0.1 M sodium acetate buffer, pH 5.6. The reaction was started by adding 3 nmol of H_2O_2 over 5 min at room temperature. This step was repeated twice with a period of 5 min of incubation for each addition. The reaction was stopped by diluting the mixture with 1 mL of 0.1% TFA. The radio-iodinated peptide was purified by HPLC on a reverse-phase Vydac C_{18} column with a 60 min linear gradient from 20 to 50% of acetonitrile (0.1% TFA) in H_2O (0.1% TFA). The eluted radio-labeled tracer was collected, aliquoted, and stored at −80 °C.

Competition Binding Curves. The receptor binding assay of the photoactivatable ligand was performed as follows: Isolated membranes from rat hearts (50 μg) were incubated in the dark in the presence of 250,000 cpm of [^{125}I]-Tyr-Bpa-Ala-hexarelin in buffer (50 mM Tris-HCl pH 7.4 containing 2 mM EGTA and 0.05% Bacitracin) and increasing concentrations from 0.1 to 10 μM of competition ligands: hexarelin (as the reference standard), cyclic azapeptides, and cyclic azasulfurylpeptides. Nonspecific binding was defined as binding not displaced by 50 μM of the corresponding peptide. After an incubation period of 60 min at 22 °C (vortexed every 15 min), membranes were subjected to irradiation with UV lamps (365 nm) for 15 min at 4 °C. After centrifugation at 12,000*g* for 15 min, the pellets were resuspended in 40 μL of sample buffer (62 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 15% 2-mercaptoethanol, and 0.05% bromophenol blue) and proteins were separated on a 7.5% SDS-PAGE Bio-Rad Mini-Protean electrophoresis system (150 V for 1 h). The gels resulting from SDS/PAGE were fixed, colored in Coomassie Brilliant Blue R-250, dried, exposed to a storage phosphor intensifying screen (Amersham Biosciences), and analyzed using a Typhoon Phosphorimager (GE Healthcare Life Sciences). Protein bands were quantified by densitometry and the covalent binding signal of 87 kDa was analyzed by densitometry and ImageLab 6.1 software (Bio-Rad Laboratories Inc.) to set competition curves. The curves were analyzed using the Graphpad Prism version 7.05 software package.

Modulation of R-FSL-1-Induced Pro-inflammatory Cytokine (TNF-α, IL-1β) and Chemokine (CCL-2) Release by Cyclic Azapeptides in a Macrophage Model. The murine RAW264.7 macrophage cell line (American Type Cell Collection, ATCC #TIB-71) was seeded at 1.5×10^5 cells/well in a 48-well plate (Costar #3548) and weaned overnight in medium (DMEM supplemented with 1% penicillin/streptomycin) at 37 °C with 5% CO_2. The next day, the medium was changed for DMEM-1% penicillin/streptomycin supplemented with 0.2% BSA. The cells were first pretreated with either linear azapeptide [azaTyr^4]-GHRP-6 as the reference standard or cyclic azapeptide at the concentration of 10^{-7} M for 30 min and then treated with R-FSL-1 (300 ng/mL final concentration, R-FSL-1, Invivogen #L7022) for 4 h at 37 °C with 5% CO_2 for assessment of TNF-α and CCL-2 or 19 h for nitrite assay. At the end of the incubation period, the media was collected and assayed for the nitrites as previously described,[24,35] or stored at −80 °C for determination of TNF-α and CCL-2 levels using ELISA kits (EbioScience #88-7391 and #88-7324).

For the IL-1β assay, murine bone marrow-derived macrophage cells (BMA 3.1 A7, kindly provided by Dr. K. Rock, University of Massachusetts Medical School) were seeded at 5×10^5 cells/well in a 48-well plate (Costar #3548) in DMEM medium (supplemented with 1% penicillin/streptomycin and 10% of fetal bovine serum). The next day, cells were first primed with LPS (200 ng/mL) for 2 h and washed with phosphate-buffered saline. Then, cells were preincubated with cyclic azapeptide for 15 min and stimulated with R-FSL-1 (300 ng/mL) for 4 h. Thirty minutes before the end of incubation time, adenosine triphosphate (0.5 mM ATP, Sigma-Aldrich # A2383) was added to culture media to induce IL-1β release. At the end of the

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incubation period, the media was collected and stored at −80 °C for IL-1β analysis using an ELISA kit (EbioScience # 88-7013-88).

The percentages of inhibition (%) of cytokines and chemokine released by the cycloazapeptide treatment were determined by the ratio: mean levels induced by R-FSL-1 (control) minus that detected in the presence of corresponding cycloazapeptides/mean level induced by R-FSL-1 minus mean level detected in basal condition X100.

Statistical data were analyzed by analysis of variance (ANOVA) with post hoc comparisons using Dunnett's test with statistical software package and statistical evaluation of correlation between variables was assessed by a Pearson normality test (Graph Pad Prism version 7.05). $P < 0.05$ was considered statistically significant.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.1c00642.

Details of synthesis, spectroscopic data, and HPLC chromatograms for compound identification, and correlations between cyclic azapeptide inhibitory effects (PDF)

Molecular formula strings (CSV)

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R.G.O. and M.M. contributed equally to this paper. Conceptualization, investigation, and visualization were performed by W.D.L., H.O., S.C., R.G.O., R.M.C., A., M.M., and J.Z.; methodology was studied by R.G.O., R.M.C., A., M.M., and J.Z.; funding acquisition, project administration, resources, supervision, and validation were completed by W.D.L., H.O., and S.C.; writing the original draft was done by R.G.O., R.C., and W.D.L. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

azaPra, aza-propargylglycine; AsPra, azasulfurylpropargylglycine; Boc, *tert*-butyloxycarbonyl; BTC, bis(trichloromethyl)-carbonate; CCL-2, monocyte chemoattractant protein-1; CD36, cluster of differentiation 36; Dab, diaminobutyrate; Dap, diaminopropionate; DIC, *N*,*N*′-diisopropylcarbodiimide; FA, formic acid; GHRP-6, growth hormone releasing peptide 6; HOBt, 1-hydroxybenzotriazole; IL-1β, interleukin-1β; *o*-NBS, *o*-nitrobenzenesulfonamido; NMR, nuclear magnetic resonance; NO, nitric oxide; Orn, ornithine; oxLDL, oxidized low-density lipoproteins; Pra, propargylglycine; R-FSL-1, fibroblast-stimulating lipopeptide; SAR, structure−activity relationships; SI, Supporting Information; TBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; TLR, Toll-like receptor; TNFα, tumor necrosis factor-α

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