# Chemical Syntheses and Biological Activities of Lactam Analogues of α-Conotoxin SI[¶,+,#]

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Bicyclization represents an effective method for the introduction of conformational constraints into small, biologically important peptides. Several strategies have been developed for the preparation of bicyclic lactam analogues of α-conotoxin SI, a 13-residue peptide neurotoxin found in cone snail venom. Four analogues of the natural regioisomer of α-conotoxin SI were designed and synthesized, each with one of the two paired cysteines of the parent peptide being replaced by a side-chain lactam bridged glutamic acid/lysine pair. Solid-phase lactamization was studied to determine rates of formation of the two possible loops and to document the extent of dimerization and higher oligomerization. Radioligand binding assays were carried out on all synthesized peptides, including the naturally occurring two-disulfide form, in order to determine their affinities for nicotinic acetylcholine receptors (nAChRs). Replacement of the $Cys^2$-$Cys^7$ loop of α-conotoxin SI with a lactam bridge resulted in complete loss of activity, whereas replacement of the $Cys^3$-$Cys^{13}$ disulfide loop resulted in a ∼60-fold reduction in affinity for one orientation and a ∼70-fold increase in affinity for the other. The two active lactam analogues retain the selectivity exhibited by the naturally occurring peptide for the α/δ subunit of nAChRs, as judged by competition experiments with the curariform antagonist metocurine.

## Introduction

Numerous naturally occurring peptide antibiotics and toxins have one or more rings, which are completed by peptide (lactam), ester (lactone), ether, disulfide, or thioether bridges.[4] Cyclization, be it natural or artificial, represents a significant avenue to restrict the structural, conformational, and dynamic properties that contribute to the biological potency, efficacy, and receptor (acceptor) selectivity, metabolic stability, and bioavailability of bioactive peptides and proteins.[2,5−9] Bicyclic peptide targets offer chemical challenges and may demonstrate interesting biological features.[2,3,5,7,10−13] Parent naturally occurring molecules have either one or two disulfides, meaning that the cited analogues have either an unnatural disulfide and/or side-chain lactam bridge introduced into their structures.[1,9−12,14,15] Purified yields reported for bicyclic peptides with both a disulfide and a lactam bridge range from 5 to 45%, with the major yield-diminishing side reaction apparently being incomplete cyclization and/or formation of dimers and oligomers.[5,8,10−12,16]

The present paper is directed at the chemical synthesis and biological characterization of lactam analogues of α-conotoxin SI (Scheme 1);[17] the parent compound is a member of a family of short peptides found in the venom of predatory cone snails.[18,19] These studies build on our earlier work on naturally occurring α-conotoxin SI and its two disulfide-mispaired regioisomers.[20,21] The α-conotoxins paralyze vertebrate muscle via postsynaptic inhibition and are referred to as "α" because they mimic physiologically the effects of the well-characterized snake α-neurotoxins (e.g., α-bungarotoxin).[22] Although both snake α-toxins and cone snail α-conotoxins inhibit muscle contraction by blocking nicotinic acetylcholine receptors, α-conotoxins act faster and are more quickly reversible than snake α-toxins. In this respect, the α-conotoxins more closely resemble the small curariform antagonists, with which they may share certain structural features.[23]

¶ Abbreviations used for amino acids and the designation of peptides follow the rules of the IUPAC−IUB Commission of Biochemical Nomenclature in *J. Biol. Chem.* **1972**, *247*, 977−983. The following additional abbreviations are used: Acm, acetamidomethyl; Al, allyl; Aloc, allyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; BOP, benzotriazol-1-yl-*N*-oxy-tris(dimethylamino)phosphonium hexafluorophosphate; BTX, α-bungarotoxin; DIPCDI, *N*,*N*′-diisopropylcarbodiimide; DIEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; ESMS, electrospray mass spectrometry; FABMS, fast atom bombardment mass spectrometry; Fmoc, 9-fluorenylmethyloxycarbonyl; HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HBTU, *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; HOAc, acetic acid; HOAt, 1-hydroxyazabenzotriazole; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; MTC, metocurine; nAChR, nicotinic acetylcholine receptor; NMM, *N*-methylmorpholine; $Pd(PPh_3)_4$, tetrakis(triphenylphosphine) palladium; PAL, 5-[[(4-amino)methyl]-3,5-dimethoxyphenoxy]valeric acid handle (peptide amide linker); PEG-PS, poly(ethylene glycol)-polystyrene (graft resin support); *t*Bu, *tert*-butyl; TFA, trifluoroacetic acid; Tmob, 2,4,6-trimethoxybenzyl; Trt, triphenylmethyl (trityl); Xan, 9*H*-xanthen-9-yl. Amino acids denote the L-configuration, unless stated otherwise.

+ Taken in part from the Ph.D. thesis of Balazs Hargittai, University of Minnesota, Minneapolis, MN, January, 2000.

# Preliminary syntheses of lactam analogues of α-conotoxin SI were reported in refs 1, 2, and 3.

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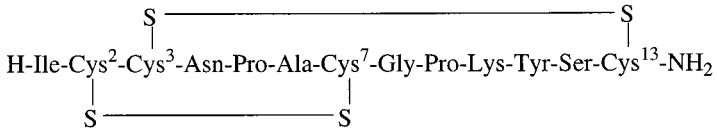
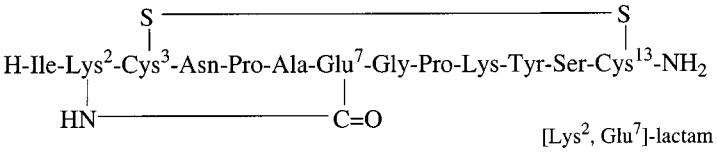
**Scheme 1.** Structures and Designations of Native α-Conotoxin SI and the Four Lactam Analogues

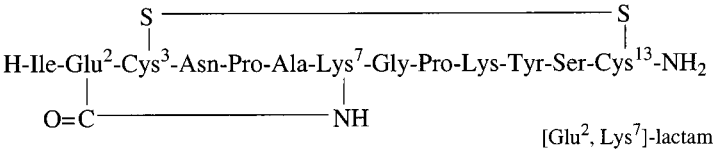
Natural isomer of α-conotoxin SI




[2,7]-analogues (replacement of small disulfide loop with lactam)

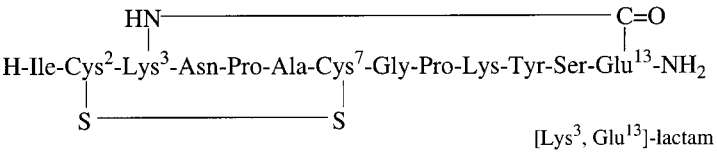



[Lys$^2$, Glu$^7$]-lactam analogue

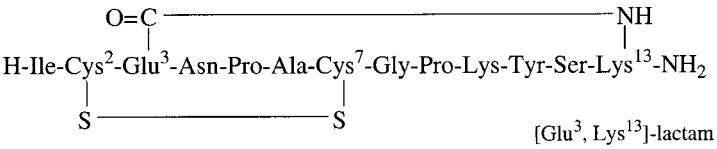



[Glu$^2$, Lys$^7$]-lactam analogue

[3,13]-analogues (replacement of large disulfide loop with lactam)




[Lys$^3$, Glu$^{13}$]-lactam analogue




[Glu$^3$, Lys$^{13}$]-lactam analogue

## Results and Discussion

**Synthesis of Lactam Analogues** (Scheme 2). The protected linear sequences were assembled by stepwise solid-phase synthesis, using (i) base-labile 9-fluorenylmethyloxycarbonyl (Fmoc) $N^{\alpha}$-amino protection, (ii) acidolyzable trityl and *tert*-butyl type side-chain protecting groups [*t*Bu ethers for Ser and Tyr, Boc urethane for Lys in position 10 (part of natural sequence), Trt amide for Asn, Trt or Xan[24] thioether for Cys, allyl ester (Al) for Glu, and allyl urethane (Aloc) for Lys (as needed for bicyclic analogues shown in Scheme 1)—In preliminary work (not described in the Experimental Section), both Cys and Asn were also protected by Tmob. Yields were comparable to what is reported herein.], and (iii) the acidolyzable tris(alkoxy)benzylamide (PAL) anchoring linkage.[25] Selective on-resin removal of the allyl-based protecting groups (Al and Aloc) was achieved, with the $N^{\alpha}$-Fmoc group still present, by treatment with $Pd(PPh_3)_4$ in NMM−HOAc−DMF (1:2:10).

Solid-phase cyclization of the lactam bridge in the [Glu$^3$, Lys$^{13}$]-analogue of α-conotoxin SI was tested under a variety of conditions in DMF (see Supporting Information Table 1). Reactions were assessed as a function of time by qualitative ninhydrin tests,[26] and by high performance liquid chromatography (HPLC) analyses after cleavage. Cyclization yields of 90% or better within 1 h were achieved upon activation with either HBTU−NMM (1:2)[27] or HATU−NMM (1:2),[28] using DMF as solvent in both cases. The rate of lactam bridge formation was also the fastest with these reagents, while cyclizations using only DIPCDI gave very low yields and much slower reactions. Activation with HOBt or HOAt gave better results, but the yields were still lower than with either HBTU or HATU in the presence of NMM. Experiments to compare cyclization rates of the small loop ([2,7]-loop) vs the large loop ([3,13]-loop) revealed that the small loop formed relatively quickly (often complete within 30 min), whereas closing of the large lactam loop was slower, with possible side reactions. The orientation of the carboxyl component of the lactam could be interchanged without affecting rates of lactam bridge formation.

Syntheses continued by removal of the *N*-terminal Fmoc group using piperidine−DMF (1:4), and then Reagent K[29] released peptide chains from the support, concomitant with deblocking of all remaining side-chain protecting groups. The crude peptide products were analyzed by gel permeation chromatography at pH 2. These studies revealed that irrespective of the reagent used for lactam cyclization, the products were predominantly the monocyclic and monomeric bis(thiol) intermediates. However, cyclodimers were also formed (as

**Scheme 2.** Orthogonal Syntheses of the Four Lactam Analogues of α-Conotoxin SI, Demonstrated on the Synthesis of the [Glu$^3$, Lys$^{13}$]-Analogue

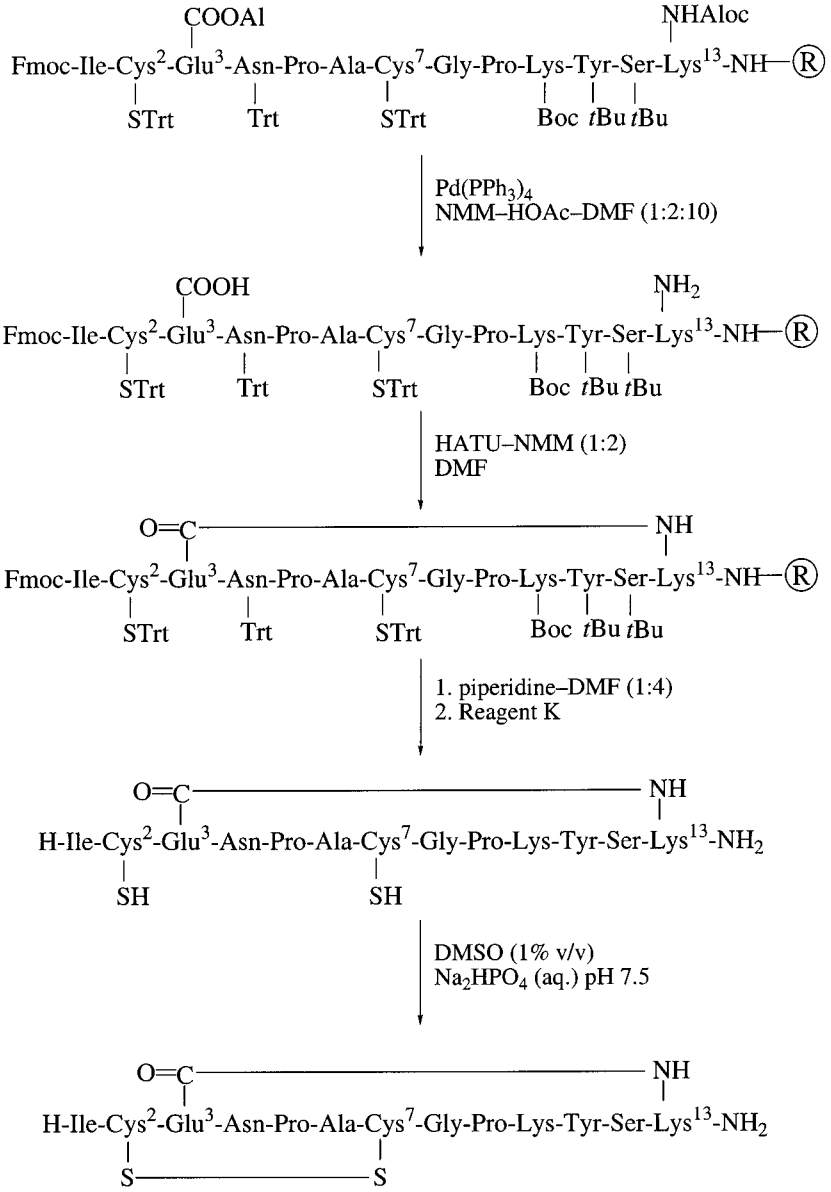



**Table 1.** Affinities of Lactam Analogues of α-Conotoxin SI for the Muscle Subtypes of Nicotinic Acetylcholine Receptors on $BC_3H$-1 Cells[a]

| toxin | $IC_{50}^{\alpha/\delta}$ (M) | $IC_{50}^{\alpha/\gamma}$ (M) |
|---|---|---|
| α-conotoxin SI | $2.4 \pm 0.64 \times 10^{-6}$ | nd |
| [Glu$^3$,Lys$^{13}$]SI analogue | $3.4 \pm 1.6 \times 10^{-8}$ | $2.3 \pm 0.64 \times 10^{-5}$ |
| [Lys$^3$,Glu$^{13}$]SI analogue | $1.4 \pm 0.34 \times 10^{-4}$ | nd |
| metocurine (MTC) | $1.5 \pm 0.27 \times 10^{-5}$ | $2.6 \pm 0.20 \times 10^{-7}$ |
| MTC (in the presence of SI) | na | $6.8 \times 10^{-7}$ |
| MTC (in the presence of [Glu$^3$,Lys$^{13}$]SI analogue) | na | $7.4 \times 10^{-7}$ |

[a] nd = not determined; na = not applicable.

supported by FABMS and ESMS mass spectral data); these unwanted byproducts were removed preparatively by size exclusion gel permeation chromatography.

Finally, the single disulfide bridge in each of the desired bicyclic analogues (Scheme 1) was closed either by air oxidation in 0.01 M NaOAc, pH 8, or by using 1% (v/v) DMSO in 0.01 M $Na_2HPO_4$ buffer, pH ~7.5.[30] During air oxidation, the monocyclic lactam precursor was present in a concentration of 10 $\mu$M, and oxidation was carried out with vigorous stirring at 25 °C for 5 h to 3 days (monitored by Ellman test and HPLC). For DMSO oxidation, the peptide was present in 0.6 mM concentration, and the oxidation was carried out with stirring at 25 °C for 15 h. As with lactam formation, the rate of disulfide formation from the bis(thiol) lactam intermediate was found to be sequence dependent: oxidation to form the small disulfide loop (i.e., [3,13]-lactam analogues) was 3-fold faster than formation of the large disulfide loop (i.e., [2,7]-lactam analogues) (see Supporting Information Figure 1).

All desired intermediates and products from these syntheses were obtained with excellent initial purities, as judged by reversed-phase HPLC (see Supporting Information Figure 2) and by analytical characteristics including amino acid analysis and molecular ions upon FABMS and ESMS. Isolated yields of the final pure bicyclic products (Scheme 1), after preparative chromatography, were up to 40%.

**Biological Studies on Lactam Analogues.** Nicotinic acetylcholine receptors from mouse muscle-derived

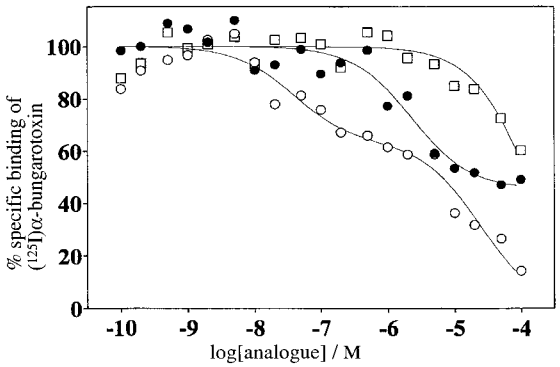



**Figure 1.** Inhibition by native α-conotoxin SI and two lactam analogues of the binding of ($^{125}$I)α-bungarotoxin to nicotinic acetylcholine receptors on $BC_3H$-1 cells: ●, competition curve of α-conotoxin SI; ○, competition curve of [Glu$^3$, Lys$^{13}$]-analogue; □, competition curve of [Lys$^3$, Glu$^{13}$]-analogue of α-conotoxin SI.

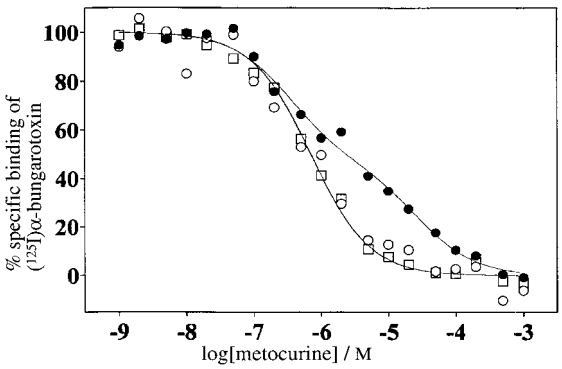



**Figure 2.** Inhibition by metocurine of the binding of ($^{125}$I)α-bungarotoxin to nicotinic acetylcholine receptors on $BC_3H$-1 cells, in the absence and presence of α-conotoxin SI and its lactam analogues: ●, affinity of metocurine in the absence of α-conotoxin analogues; ○, affinity of metocurine in the presence of 20 $\mu$M α-conotoxin SI; □, affinity of metocurine in the presence of 2 $\mu$M [Glu$^3$, Lys$^{13}$]-analogue of α-conotoxin SI.

$BC_3H$-1 cells have been used as model systems for understanding nicotinic receptor structure and function.[31,32] The embryonic muscle subtype of nicotinic acetylcholine receptor is a ligand-gated ion channel composed of four homologous subunits ($\alpha_2\beta\gamma\delta$) in a pentameric arrangement that spans the lipid bilayer. The two acetylcholine-binding sites located near the $\alpha/\gamma$ and $\alpha/\delta$ subunit interfaces ($\alpha/\gamma$ and $\alpha/\delta$ sites) are pharmacologically distinct due to sequence differences between the $\gamma$- and $\delta$-subunits. Some α-conotoxins are highly site-selective competitive antagonists at one or the other of the two acetylcholine binding sites. For example, α-conotoxins GI, MI, and SI have a greater affinity for the $\alpha/\delta$ site on mouse muscle-derived $BC_3H$-1 receptors. In contrast, when assayed against nicotinic receptors derived from the *Torpedo* electric organ, α-conotoxin GI and MI have higher affinity for the $\alpha/\gamma$ site, and α-conotoxin SI does not differentiate between the two sites.[19,33]

Both orientations of the [2,7]-lactam analogues showed negligible capability to inhibit the binding of ($^{125}$I)α-bungarotoxin to the muscle subtype of nicotinic acetylcholine receptors found on intact mouse muscle-derived $BC_3H$-1 cells (data not shown). In marked contrast, however, the [Glu$^3$, Lys$^{13}$]-analogue was ~70-fold more potent than the native α-conotoxin SI (Figure 1 and Table 1 of main text). Furthermore, although one might predict that a simple directional change of the [3,13]-lactam would result in relatively minor changes in affinity, the [Lys$^3$, Glu$^{13}$]-analogue was ~60-fold less potent than the native peptide. Restated, there was a dramatic 4000-fold difference in the potencies between the two possible orientations of that lactam bridge, despite the fact that the overall dimensions of the [3,13]-lactam ring remain constant.

Some α-conotoxins display a marked capability to differentiate between the two acetylcholine-binding sites found on each receptor.[33] Those α-conotoxin analogues that retained significant activity (i.e., the native toxin and the two orientations of the [3,13]-lactam analogues) were evaluated in an assay specifically designed to determine their acetylcholine-binding site preference. This assay involves blocking one of the two acetylcholine-binding sites with the site-selective α-conotoxin under investigation using a conotoxin concentration that does not block the other site [20 $\mu$M for α-conotoxin SI and 2.0 $\mu$M for the [Glu$^3$, Lys$^{13}$]-lactam analogue], and then evaluating the apparent affinity of metocurine for the remaining unoccupied acetylcholine-binding site. Curariform antagonists, like metocurine, possess two cationic centers about 10 Å apart, which are separated from each other by a rigid hydrophobic region.[34] Metocurine is known to have a higher affinity for the $\alpha/\gamma$ acetylcholine-binding site on $BC_3H$-1 cells,[32] displaying two apparent affinities for $BC_3H$-1 receptors that differ 46-fold (Figure 2 and Table 1 of main text).

In the presence of α-conotoxin SI or the [Glu$^3$, Lys$^{13}$]-analogue at concentrations sufficient to occupy their higher-affinity sites, metocurine displayed only a single high affinity for the remaining unoccupied acetylcholine-binding site on $BC_3H$-1 receptors (Figure 2 and Table 1 of main text). In the presence of the [Lys$^3$, Glu$^{13}$]-analogue, the fraction of low affinity metocurine binding sites was reduced substantially (data not shown). Thus, all three of these α-conotoxins preferentially occupy the lower affinity metocurine binding site (the $\alpha/\delta$ site) of $BC_3H$-1 receptors, leaving the higher affinity metocurine binding site (the $\alpha/\gamma$ site) unoccupied.

It has been reported that replacement of the large disulfide loop in (des-Glu$^1$)-conotoxin GI with an amide loop (Asp-Orn) does not affect the diaphragm paralytic activity in mice.[5] However, replacement of the small disulfide loop of the peptide with this amide bridge lowers the paralytic activity by approximately 150-fold. One possible explanation for this result is that the amide side-chain group forms new hydrogen bonds within the structure, and these greatly lower the capability to form the (des-Glu$^1$)-conotoxin GI nAChR-binding conformation. This preferred binding conformation of the small ring could also be adversely affected by the difference in geometry between an amide and a disulfide linkage. Alternatively, if the disulfide bridge is directly involved in the binding to the nAChR, then its replacement by a lactam bridge could greatly affect the binding of the analogues. Our results in the α-conotoxin SI system suggest that the large [3,13]-loop plays a global structural role, while the smaller [2,7]-loop is involved in more subtle changes in structure that affect activity.

## Summary and Conclusions

Four lactam analogues of the natural isomer of α-conotoxin SI were synthesized. Rates of on-resin cyclization to form side-chain lactams were sequence dependent. For the α-conotoxin SI model, formation of the small lactam loop occurred more readily than the corresponding method for the large lactam loop. The carboxyl component of the lactam can be interchanged without affecting the rates of these reactions. Among the various activating reagents tested for lactam formation on solid-phase, the best results in terms of speed and yield were obtained with HBTU or HATU, each in the presence of NMM in DMF. The second step of bicyclization, disulfide formation (with the lactams in place), proved to be also sequence-dependent. The oxidation was 3-fold more rapid for the small disulfide loop, in comparison to the large disulfide loop. In radioligand binding assays, performed to determine the affinity of these peptides for nicotinic acetylcholine receptors, no binding was observed for the [2,7]-lactam analogues, while both orientations of the [3,13]-lactam analogues showed significant binding. All active peptides that show affinity prefer the α/δ binding site on the receptors.

## Experimental Section

**General.** Most of the materials, solvents, instrumentation, and general synthesis methods have been described and summarized in our previous publications.[20,21,24,25] Most protected Fmoc-L-amino acid derivatives, along with Fmoc-PAL-PEG-PS resin (0.16−0.18 mmol/g), were purchased from PerSeptive Biosystems GmbH (Hamburg, Germany). Fmoc-Cys(Xan)-OH was prepared in our laboratory.[24] DMF was purchased from Fisher Scientific (Fair Lawn, NJ) and dried over 4 Å molecular sieves while nitrogen was bubbled through it for at least 24 h prior to use. $CH_2Cl_2$ (Aldrich Chemical, Milwaukee, WI) was distilled from anhydrous calcium hydride. Piperidine, trifluoroacetic acid (TFA), and 1-hydroxybenzotriazole (HOBt) were also from Fisher Scientific. All other starting materials and reagents were from Aldrich (Milwaukee, WI) and used without purification.

Analytical HPLC was performed using a Vydac analytical C-18 reversed phase column (10 μm particle size; 0.46 × 25.0 cm) on a Beckman system configured with two Model 112 pumps and a Model 165 variable wavelength detector controlled from an IBM computer with Beckman System Gold software. Peptide samples were chromatographed at 1.2 mL/min using 0.1% aqueous TFA−0.1% TFA in $CH_3CN$ (1:0 to 11:9 over 20 min), detection at 220 nm. Preparative HPLC was performed using a Vydac semipreparative C-18 reversed phase column (10 μm particle size; 2.2 × 25.0 cm) on a Waters DeltaPrep system using manual injections (5 mL loop sizes). Low resolution fast atom bombardment mass spectrometry (FABMS) was carried out on a VG Analytical 707E-HF low resolution double focusing mass spectrometer equipped with a VG 11/250 data system.

Synthesis of the natural form of α-conotoxin SI proceeded by a previously published orthogonal strategy.[21]

**Synthesis of Lactam Analogues of α-Conotoxin SI.** Manual solid-phase peptide synthesis was carried out in a sterile plastic syringe (12 mL volume) containing a porous polypropylene frit, starting with Fmoc-PAL-PEG-PS (500 mg, 0.16−0.18 mmol/g loading). Fmoc removal was achieved by treatment with piperidine−DMF (1:4, 8 + 3 min), followed by washes with DMF (5 × 2 min). Fmoc-amino acids (4−5 equiv) were incorporated by 1 h couplings (completion verified by ninhydrin tests),[26] mediated by DIPCDI/HOBt (4−5 equiv each) in DMF (∼3 mL). A portion of the protected peptide-resin (100 mg, 0.10 mmol/g loading based on amino acid analysis) was swollen in DMF (5 mL) for 5 min and treated with $Pd(PPh_3)_4$ (1.11 g, 4 equiv, relative to protecting groups to be removed) in NMM−HOAc−DMF (1:2:10, 20 mL), at 25 °C for 3 h under nitrogen, to remove allyl protecting groups (Al and Aloc). This was followed by washings with a solution of DIEA [0.5% (v/v), 0.25 mL] and sodium diethyldithiocarbamate [0.5% (w/v), 0.25 mg] in DMF (50 mL).[2] Next, side-chain cyclization was mediated by a number of activating reagents in DMF. The best cyclization yields (up to 94%) were achieved upon activation with HATU (228 mg, 5 equiv)/NMM (0.13 mL, 10 equiv) in DMF (7.5 mL; 1.3 mM effective concentration of peptide on resin) at 25 °C for 1 h. Syntheses continued by removal of the N-terminal Fmoc group from the cyclic intermediate by piperidine−DMF (1:4, 7 mL) at 25 °C for 8 + 3 min. Treatment with Reagent K, TFA−phenol−water−thioanisole−1,2-ethanedithiol (82.5:5:5:5:2.5, 7 mL), at 25 °C for 2 h, released peptide chains from the support concomitant with deblocking of all side-chain protecting groups. The peptides were precipitated by diethyl ether, and subsequently redissolved in 0.01 M $Na_2HPO_4$ buffer, pH 7.5 (16 mL; 0.6 mM final peptide concentration), and DMSO [1% (v/v), 0.16 mL] was added. After the mixture was stirred at 25 °C for 15 h, the oxidation was quenched by lyophilization, to provide the bicyclic compound. The identities of the intermediates and the final products were verified by FABMS: *m/z* calcd for $C_{61}H_{97}N_{17}O_{18}S_2$ (unprotected intermediate, no side-chain bridges) 1407.7, found 1408.7 [$MH^+$]; *m/z* calcd for $C_{61}H_{95}N_{17}O_{17}S_2$ (bis(thiol), lactam intermediate) 1389.7, found 1390.6 [$MH^+$]; *m/z* calcd for $C_{61}H_{93}N_{17}O_{17}S_2$ (bicyclic product) 1387.7, found 1388.7 [$MH^+$] for [2,7]-lactam analogues, 1388.6 [$MH^+$] for [3,13]-lactam analogues.

**Maintenance of $BC_3H$-1 Cells.** $BC_3H$-1 cells were maintained in growth medium (Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100 units/mL penicillin, and 0.1 mg/mL streptomycin) in a humidified atmosphere of 5% $CO_2$/95% air at 37 °C. For experiments, $BC_3H$-1 cells in growth medium were seeded into gelatin-coated 24-well culture plates at a density of 8000−12000 cells/well. The plated cells were then grown as described previously.[32,33,35] Prior to each experiment, plated cells were equilibrated to 25 °C for 30 min, washed once with 1 mL of assay buffer (140 mM KCl, 25 mM HEPES, 5.4 mM NaCl, 1.8 mM $CaCl_2$, 1.7 mM $MgSO_4$, 0.06 mg/mL bovine serum albumin, pH 7.4), and equilibrated in 1 mL of fresh assay buffer for 20 min.

**Inhibition of ($^{125}$I)α-Bungarotoxin Binding to Nicotinic Acetylcholine Receptors on $BC_3H$-1 Cells.** All experiments were performed at 25 °C. Inhibition of the association of ($^{125}$I)α-bungarotoxin [($^{125}$I)α-BTX] to nicotinic receptors on $BC_3H$-1 cells was performed as described previously.[32,33] Briefly, $BC_3H$-1 cells expressing nAChRs cultured in 24-well plates were incubated for 2 h with each α-conotoxin analogue, or 30 min with metocurine in the assay buffer. After this incubation, ($^{125}$I)α-BTX was added (final concentration = 20 nM) and incubated for 15 min. The cells were washed twice with assay buffer, and the bound ($^{125}$I)α-BTX was collected with two 0.5 mL volumes of 1% Triton X-100 in water. The two Triton X-100 washes were pooled and counted to determine the amount of bound ($^{125}$I)α-BTX, correcting for nonspecific binding. In experiments designed to identify the higher affinity α-conotoxin binding site, cells were incubated for 2 h with a concentration of α-conotoxin that selectively occupied the higher affinity α-conotoxin binding site (20 μM for α-conotoxin SI and 2.0 μM for the [Glu$^3$, Lys$^{13}$]-lactam analogue). Next, the apparent affinity of metocurine for the remaining available acetylcholine-binding sites was determined as described previously.[32,33,36] Nonspecific binding of ($^{125}$I) α-BTX was determined from cells previously incubated for 30 min with 100 nM α-BTX. The total number of ($^{125}$I)α-BTX binding sites [determined after a 60 min incubation with ($^{125}$I)α-BTX] was 130 ± 18 fmol/well ($n$ = 8). In the absence of any competing drugs, 74 ± 3.8% ($n$ = 8) of the total population of binding sites were labeled by ($^{125}$I)α-BTX in 15 min.

**Data Analysis.** Functions describing competitive binding of a ligand to either a single site or two independent sites were fit by nonlinear regression to the inhibition of ($^{125}$I)α-BTX

binding to nicotinic receptors. A two-site competition model was accepted over a single site model only when a statistical comparison between the two models gave a value of $p < 0.05$.[37] All averaged $IC_{50}$ values reported with an associated SEM included data from three to five experiments. All curves that were fit significantly better to a two-site competition model contained the two sites in approximately equal abundance. Nonlinear regression was performed on a Northgate 386 personal computer using Prism (GraphPad).

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**Supporting Information Available:** Table containing solid-phase cyclization yields for the formation of the lactam bridge in the [Glu[3], Lys[13]]-analogue, based on HPLC analysis of crude products after cleavage from support; Figure showing HPLC monitoring of oxidation reactions to form disulfide bridges using air oxidation; and Figure showing HPLC analyses of peptide intermediates. This material is available free of charge via the Internet at http://pubs.acs.org.

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