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Solving the α-Conotoxin Folding Problem: Efficient Selenium-Directed On-Resin Generation of More Potent and Stable Nicotinic Acetylcholine Receptor Antagonists

Markus Muttenthaler,[†] Simon T. Nevin,[‡] Anton A. Grishin,[II,‡] Shyuan. T. Ngo,[§] Peng T. Choy,[§] Norelle L. Daly,[†] Shu-Hong Hu,[†] Christopher J. Armishaw,[⊥,†] Ching-I. A. Wang,[†] Richard J. Lewis,[†] Jennifer L. Martin,[†] Peter G. Noakes,[‡,§] David J. Craik,[†] David J. Adams,[II,‡] and Paul F. Alewood*[,†]

Institute for Molecular Bioscience, Division of Chemistry and Structural Biology, The University of Queensland, Brisbane, Queensland 4072, Australia; Queensland Brain Institute, The University of Queensland, Brisbane, Queensland 4072, Australia; and School of Biomedical Sciences, The University of Queensland, Brisbane, Queensland 4072, Australia

Received December 16, 2009; E-mail: p.alewood@imb.uq.edu.au

Abstract: α-Conotoxins are tightly folded miniproteins that antagonize nicotinic acetylcholine receptors (nAChR) with high specificity for diverse subtypes. Here we report the use of selenocysteine in a supported phase method to direct native folding and produce α-conotoxins efficiently with improved biophysical properties. By replacing complementary cysteine pairs with selenocysteine pairs on an amphiphilic resin, we were able to chemically direct all five structural subclasses of α-conotoxins exclusively into their native folds. X-ray analysis at 1.4 Å resolution of α-selenoconotoxin PnIA confirmed the isosteric character of the diselenide bond and the integrity of the α-conotoxin fold. The α-selenoconotoxins exhibited similar or improved potency at rat diaphragm muscle and α3β4, α7, and α1β1$\delta\gamma$ nAChRs expressed in *Xenopus* oocytes plus improved disulfide bond scrambling stability in plasma. Together, these results underpin the development of more stable and potent nicotinic antagonists suitable for new drug therapies, and highlight the application of selenocysteine technology more broadly to disulfide-bonded peptides and proteins.

1. Introduction

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated cationic channels that mediate fast synaptic transmission within the peripheral and central nervous system.[1,2] The nAChR is a functional pentamer in which the five transmembrane spanning subunits are symmetrically arranged around the central pore.[1] Channel opening is induced by the endogenous neurotransmitter acetylcholine (ACh) or exogenous agonists such as nicotine.[2] The neuromuscular nAChR is made up of combinations of four different subunits α1, β1, γ (or ϵ in the adult), and δ, with a predicted $(\alpha1)_2\beta1\gamma\delta$ stoichiometry. By contrast, neuronal nAChRs comprise homomeric or heteromeric combinations of ligand binding α subunits (α2−α10) and structural β subunits (β2−β4).[2,3] These combinatorial possibilities generate a wide diversity of subtypes in different locations in the nervous system having distinct pharmacological and biophysical properties.[3,4] The therapeutic potential of nAChRs is broad, with individual subtypes being implicated in Alzheimer's and Parkinson's disease, Tourette's syndrome, pain, myocardial infarction, nicotinic addiction, schizophrenia, attention-deficit hyperactivity disorder, autism, epilepsy, depression, and anxiety.[2,3] Subtype selectivity of ligands is essential to sharply delineate physiological functions and to minimize side effects in therapeutic treatment of such diseases.[5]

Although much effort has focused on small molecule antagonist development, little success has been achieved so far in producing viable clinical candidates.[2,6] By contrast, recent research into marine cone snail venoms has revealed a rich source of competitive, highly potent and selective antagonists for a broad range of ion channels, including the nAChRs.[7,8] The cone snails have evolved a highly sophisticated cell machinery over 50 million years that allows the production of bioactive peptides called conotoxins in a combinatorial fashion for prey capture and survival strategies.[8] Evolutionarily selected, the α-conotoxins have nanomolar activity at target receptors

[†] Institute for Molecular Bioscience, Division of Chemistry and Structural Biology, The University of Queensland.
[‡] Queensland Brain Institute, The University of Queensland.
[§] School of Biomedical Sciences, The University of Queensland.
[II] Present Address: Health Innovations Research Institute, RMIT University, PO Box 71, Bundoora, Victoria 3083, Australia
[⊥] Present Address: Torrey Pines Institute for Molecular Studies, 11350 SW Village Pkwy, Port St Lucie, FL 34987, USA

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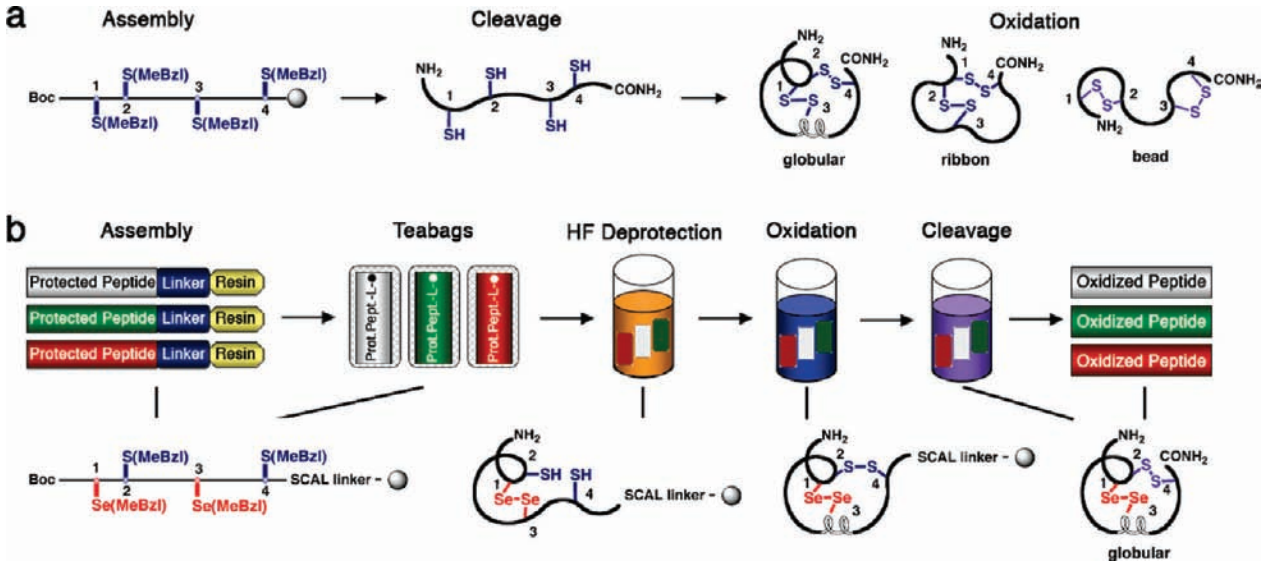

Figure 1. (a) Standard α-conotoxin synthesis. Assembly on resin using Boc-SPPS, followed by HF cleavage and random oxidation in aqueous buffer solutions, yields all three possible disulfide isomers. (b) Parallel directed-folding strategy using selenocysteine. Parallel chain assembly was performed using the HF stable SCAL linker on an amphiphilic ChemMatrix resin. Upon assembly, the conotoxins on solid support are transferred into labeled resin-bags, followed by HF deprotection and oxidation in aqueous 0.1M NH_4HCO_3 buffers. Regioselective folding is induced upon HF deprotection due to selective formation of the diselenide over the disulfide bond. Depending on the synthetic strategy, the peptides can then be separated, cleaved and purified individually, or be cleaved and purified simultaneously via a single RP-HPLC run.

and an enormously refined selectivity that can differentiate between individual subtypes of nAChRs.[8,9] These peptides contain 12−19 amino acids and have well-defined three-dimensional structures, predominantly due to the bracing of the backbone by two disulfide bonds in a Cys 1−3 and Cys 2−4 connectivity (referred to as the native globular connectivity). Two loops are formed with a short turn-helix-turn backbone feature that comprises most of the functional groups responsible for activity and potency.[9] The numbers of residues encompassed within these two loops (*m*/*n*) represent the basis for a further division into several structural subfamilies as the loop size pairings correlate with the nAChR subtype target selectivity.[8] Hence the α-conotoxins are ideal probes for receptor studies, and are considered promising therapeutic agents for the treatment of pain and other neurological disorders.[2,7] However, the challenge of obtaining correctly folded peptides has ensured that in-depth investigations of α-conotoxins remain a low-throughput process, thus hindering a plethora of potential structure−activity studies.[10]

The major challenge is outlined in Figure 1a, where three distinct folds with different disulfide bond connectivities and functions may be obtained from the same linear sequence under oxidative conditions. Although the oxidative folding process may be partly influenced to give a dominant isomer by adjusting oxidation buffers via variation in organic cosolvents, shuffling agents, solute concentration, temperature and time, this empirical approach often fails once non-native structural modifications are embedded in the synthetic design.[10,11] Furthermore, validation of the correct isomer is time intensive, as it involves structure and disulfide bond connectivity determination or biological characterization, if the biological target is known. The purification process also adds to the complexity due to the similar nature of the isomers, significantly lowering the yields. Given that the primary reason to select chemical approaches over peptide expression is to introduce non-native modifications for improved structure−function, fluorescent tagging, ligation chemistry, cyclization or PEGylation, it is essential to have efficient regioselective control over disulfide bond formation. Currently, this is achieved in a laborious fashion via orthogonal thiol-protecting groups,[12] which have long workup times and low yields. We describe here a methodology that takes advantage of preferential diselenide bond formation over disulfide bond formation to selectively generate α-conotoxins in their native fold either in solution or in a high throughput parallel methodology. This approach gave easy access to all five classes of nicotinic α-conotoxin antagonists with enhanced stability and potency.

Selenocysteine (Sec, U) is considered to be the most conservative substitution for cysteine and its isosteric character has been exploited in a range of bioactive peptides to either elucidate structure activity relationships (SAR) or to improve their stability in reducing environments.[13] Despite the resemblance between the elements selenium and sulfur, the amino acids Sec and Cys exhibit significantly distinct chemical properties. While Sec and Cys have similar electronegativity (EN(S) = 2.58, EN(Se) = 2.55), Sec is a stronger nucleophile and a better leaving group than its sulfur counterpart.[14] Furthermore, Sec exhibits a much higher acidity than Cys (pK_a (Sec) = 5.24−5.63, pK_a (Cys) = 8.25).[15–17] Comparative studies on diselenide, selenylsulfide, and disulfide bonds in linear unconstrained glutaredoxin fragments as well as in folded glutaredoxin 3 showed a difference of 111−166 mV in redox

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Table 1. Summary of Sequence and Bioactivity Data for α-Conotoxins[a]

Selenium Analogue	Sequence	Selectivity	IC_{50} (nM)	nAChR subtype
MI	GRCC-HPACGKNYS--C*	80%	26.0 ± 10.7	$(\alpha1)_2\beta1\delta\gamma$
Sec[1,3]-MI	GRUC-HPAUGKNYS--C*	100%	n.d.	$(\alpha1)_2\beta1\delta\gamma$
Sec[2,4]-MI	GRCU-HPACGKNYS--U*	100%	11.5 ± 2.3	$(\alpha1)_2\beta1\delta\gamma$
BuIA	GCCSTPPCAVLY---C*	75%	0.258[a]	α6α3β2
Sec[1,3]-BuIA	GUCSTPPUAVLY---C*	100%	n.d.	α6α3β2
AuIB	GCCSYPPCFATNPD-C*	44%	3100 ± 1002	α3β4
Sec[1,3]-AuIB	GUCSYPPUFATNPD-C*	90%	260 ± 20	α3β4
Sec[2,4]-AuIB	GCUSYPPCFATNPD-U*	86%	1100 ± 359	α3β4
Vc1.1	GCCSDPRCNYDHPEIC*	46%	4200 ± 1600[b]	α3β4
Sec[1,3]-Vc1.1	GUCSDPRUNYDHPEIC*	100%	n.d.	α3β4
[A10L]PnIA	GCCSLPPCALNNPDYC*	100%	23.3 ± 0.2	α7
Sec[1,3]-[A10L]PnIA	GUCSLPPUALNNPDYC*	100%	15.5 ± 2.7	α7
Sec[2,4]-[A10L]PnIA	GCUSLPPCALNNPDYU*	100%	n.d.	α7
ImI	GCCSDPRCAWR----C*	54%	69.3 ± 15.5[c]	α7
Sec[1,3] - ImI	GUCSDPRUAWR----C*	100%	49.5 ± 16.2[c]	α7
Sec[2,4] - ImI	GCUSDPRCAWR----U*	100%	50.3 ± 8.4[c]	α7

[a] Taken from ref 43.[b] Taken from ref 40.[c] Taken from ref 21.[a] Shown are the amino acid sequence, the replacement of pairs of cysteine (red C) by isosteric selenocysteine (blue U), the selectivity towards correctly folded material (globular isomer), and the IC_{50} values (mean ± SEM, $n = 6$) for inhibition of ACh-evoked currents mediated by $(\alpha1)_2\beta1\delta\gamma$, α6α3β2, α3β4, α9α10, and α7 nAChR subtypes expressed in *Xenopus* oocytes. n.d., not determined; * C-terminal amide.

potential between the disulfide and the diselenide bond.[18,19] This difference, in combination with the higher nucleophilicity and lower pK_a of Sec, suggests that diselenide or selenylsulfide bond formation is highly favored over disulfide bond formation as was observed in earlier studies.[20] The lower redox potential of the diselenide bond should at the same time provide higher stability in a reductive environment.[21]

2. Results

2.1. Directed Folding using Selenocysteine—In Solution. To investigate the potential of selenocysteine to direct oxidative folding in a straightforward and efficient manner, well studied α-conotoxins from all five existing functional classes with differing nAChR selectivity (α3/5-MI, α4/3-ImI, α4/4-BuIA, α4/6-AuIB, α4/7-Vc1.1, α4/7-[A10L]PnIA) were chosen and complementary pairs of cysteine residues in each sequence were replaced by selenocysteine (see Figure 1b and Table 1). The α-conotoxins AuIB, Sec[1,3]-AuIB, Sec[2,4]-AuIB, MI, Sec[1,3]-MI, Vc1.1, Sec[1,3]-Vc1.1, [A10L]PnIA, Sec[1,3]-[A10L]PnIA, Sec[2,4]-[A10L]PnIA, ImI, Sec[1,3]-ImI, Sec[2,4]-ImI, were assembled using Boc-SPPS incorporating selenocysteine via Boc-L-Sec(MeBzl)−OH. This chemical approach was chosen as reports in the literature suggest that Sec racemization may occur using Fmoc chemistry strategies. Furthermore, Sec was shown to have a high tendency to deselenate via β-elimination during iterative piperidine Fmoc-deprotection steps that result in dehydroalanine and consequently piperidyl adducts.[22] Boc-L-Sec(MeBzl)−OH was synthesized following the protocol of Armishaw et al..[21] Chain assembly of all α-conotoxin sequences proceeded smoothly using HBTU-mediated Boc in situ neutralization SPPS[23] on a 4-methylbenzhydrylamine resin. The linear peptides were deprotected and cleaved by hydrofluoric acid (HF) in a single step. Intriguingly, the Sec residues were found to be fully oxidized directly upon deprotection via HF (pH < 1) and it was not possible to isolate the fully reduced species. Attempts to do so in the presence of an excess of reducing agents such as TCEP, TBP, or $NaBH_4$ led to significant deselenation. Oxidation of the diselenide, dithiol-containing α-conotoxin intermediates was performed in 0.1M NH_4HCO_3 at pH 8.4 and the solution was analyzed by RP-HPLC and LCMS (see Supporting Information Figure S2). Additional oxidation trials on the selenium analogs of AuIB, MI, and [A10L]PnIA revealed that once the diselenide is formed, disulfide bond formation occurred readily at pH 5 in contrast to the native all Cys peptides, which remain as free thiols (see Supporting Information Figure S3). In general, oxidations (pH 8.4, 25 °C) were complete within 10 min without any folding enhancers and only a single purification step was necessary. This makes Sec-directed oxidative folding far more efficient than standard oxidation protocols or orthogonal protecting group strategies.

2.2. Directed Folding using Selenocysteine—On Resin. With regioselective folding established in solution we set out to develop resin-supported chemistry to underpin high throughput parallel processing of peptides and unify time-intensive tasks such as side-chain deprotection, oxidation, cleavage, and purification to give fast and efficient access to correctly folded α-conotoxins (see Figure 1b). α-Conotoxins MI, Sec[2,4]-MI, Vc1.1, Sec[1,3]-Vc1.1, BuIA, Sec[2,8]-BuIA, and [A10L]PnIA were assembled using Boc-chemistry on an amphiphilic resin (ChemMatrix) with a C-terminal HF stable safety-catch acid

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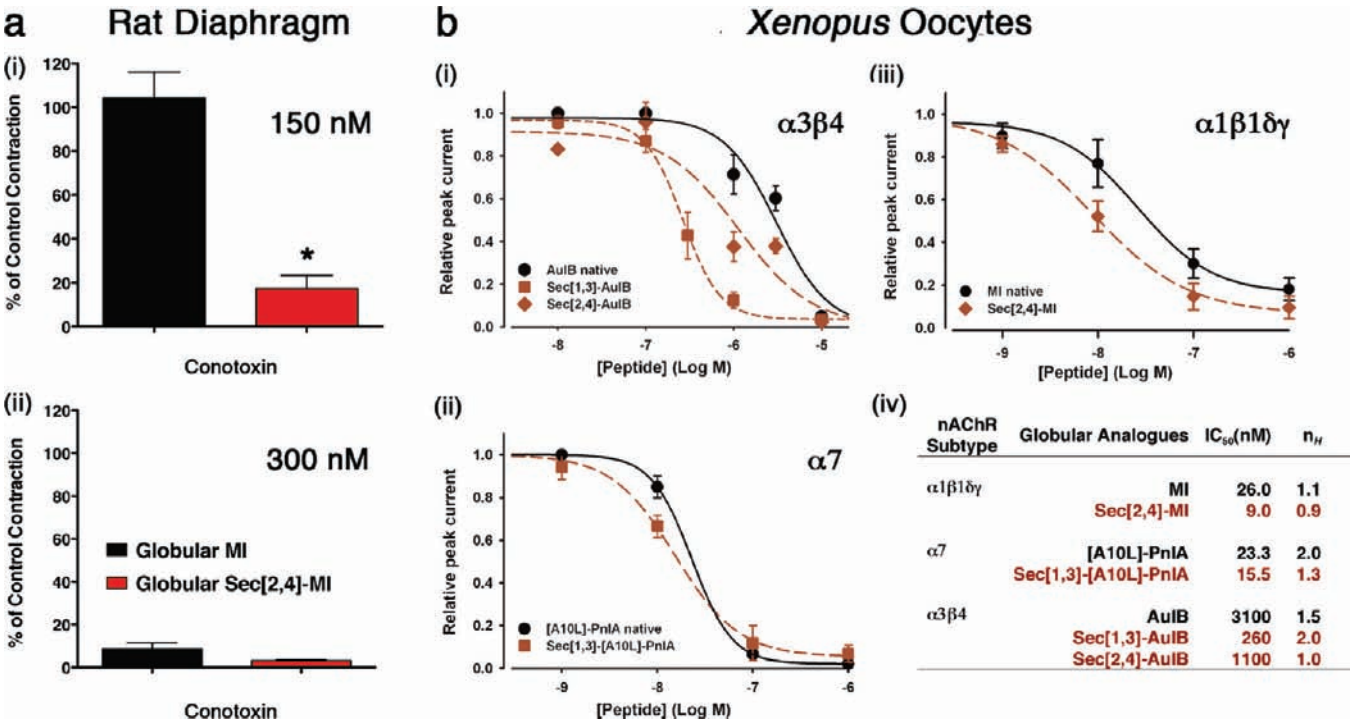

Figure 2. Functional assays of α-conotoxins. (a) Inhibition of nerve-evoked muscle contraction of rat diaphragm by MI and its selenium analog at concentrations of (i) 150 nM and (ii) 300 nM. Error bars are mean ± SEM (* $P = 0.0123$, $n = 3$, 2 way ANOVA) **(**b) Inhibition of ACh-evoked currents mediated by nAChR subtypes expressed in *Xenopus* oocytes by α-conotoxin (i) MI, (ii) [A10L]PnIA, (iii) AuIB and their Sec-analogs. Error bars are mean ± SEM ($n = 6$). The concentration−response data are summarized in the Table (iv), where IC_{50} is the half-maximal inhibitory concentration and n_H is the Hill coefficient.

labile (SCAL) linker (see Supporting Information Figure S1).[24] Simultaneous side-chain deprotection by HF of the conotoxins separately packaged in labeled solvent-permeable 74 μm mesh polypropylene bags[25] delivered the fully deprotected peptides on-resin and was followed by oxidation in 0.1M NH_4HCO_3 buffer at pH 8.4. Similar to solution folding outcomes, different ratios of the three possible disulfide bond isomers of the all-Cys analogs could be accessed by changing the folding conditions (see Supporting Information Table S2 online).[26] Full regioselective control over multiple isomer formation was only achieved by incorporation of isosteric selenocysteine into the synthetic scheme (see Table 1 and Supporting Information Figure S4). Once the peptides folded on-resin, a chemoselective reaction (reductive acidolysis by NH_4I/TFA) activates the linker and releases the correctly folded α-conotoxins from the solid support (see Supporting Information Figure S1). Depending on the peptide library strategy, cleavage and purification can either be achieved individually (separated by the labeled teabags) or simultaneously in an efficient "one-pot" approach. Both alternatives were investigated and in both cases the correctly folded peptides were isolated in high purity (see Supporting Information Figure S5).

2.3. Biological Characterization. 2.3.1. Contraction Assay Rat Diaphragm. The biological activities of globular MI and Sec[2,4]-MI were characterized by their ability to inhibit acetylcholine (ACh)-induced muscular contractions in response to phrenic nerve stimulation in rat diaphragm preparations. The peptides were initially tested at a concentration of 150 nM, followed by 300 and 500 nM. Over a 2 h period, the globular selenium-analog Sec[2,4]-MI was the only peptide capable of 90% inhibition of the ACh-induced muscular contraction at 150 nM (see Table 1 and Figure 2a). Both peptides were able to completely block the muscle contraction at concentrations of 300 and 500 nM.

2.3.2. Electrophysiology Assays using *Xenopus* Oocytes. α-Conotoxins MI, [A10L]PnIA and AuIB are selective for the muscle $\alpha1\beta1\gamma\delta$ and the neuronal α7 and $\alpha3\beta4$ nAChR subtypes, respectively, and the activity of the native peptides a well as their selenium-analogs was examined on ACh-evoked currents mediated by recombinant nAChRs expressed in *Xenopus* oocytes (see Table 1 and Figure 2b). Globular α-conotoxin MI was tested on ACh (1 μM)-evoked currents mediated by muscle $(\alpha1)_2\beta1\gamma\delta$ nAChR. Concentration−response curves obtained for the inhibition of ACh-evoked currents by globular MI exhibited an IC_{50} of 26 nM and a Hill coefficient of 1.1 ($n = 6$). Globular Sec[2,4]-MI retained the potency with an IC_{50} of 9 nM and Hill coefficient of 0.9 ($n = 6$). Globular [A10L]PnIA was tested on ACh (100 μM) -evoked currents mediated by neuronal α7 nAChRs and exhibited an IC_{50} of 23.3 nM and a Hill coefficient of 2.0 ($n = 6$). Globular Sec[1,3]-[A10L]PnIA had a similar IC_{50} value of 15.5 nM and a Hill coefficient of 1.3 ($n = 5$). Neuronal $\alpha3\beta4$ nAChR-mediated currents evoked by 50 μM ACh were inhibited by globular AuIB giving an IC_{50} value of 3.1 μM and a Hill coefficient of 1.5 ($n = 6$). The selenium substituted globular Sec[1,3]-AuIB and globular Sec[2,4]-AuIB exhibited IC_{50}'s of 0.3 μM and 1.1 μM and Hill coefficients of 2.0 and 1.0 respectively ($n = 6$).

2.4. Structural Characterization. The enhanced potencies of the selenium analogs prompted a structural analysis of the isosteric character of selenocysteine. Circular dichroism (CD)

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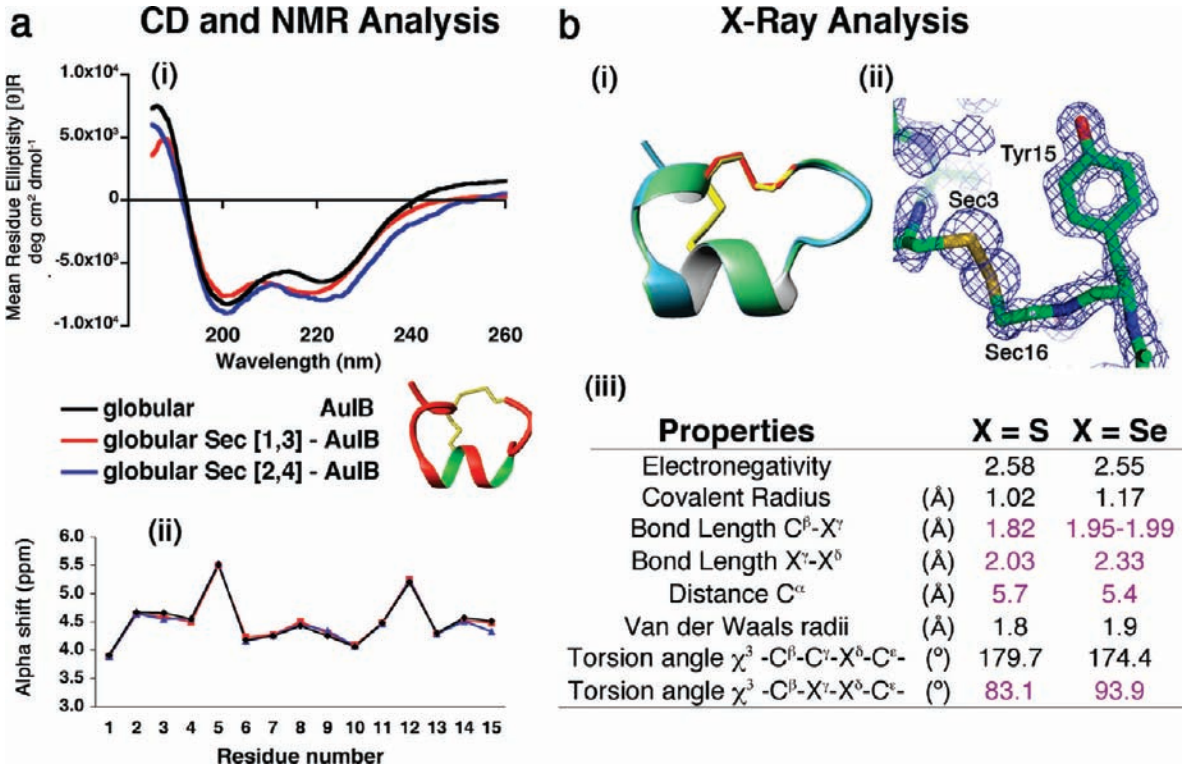

Figure 3. Structural analysis of α-conotoxin analogs. a(i) CD analysis of the globular AuIB and its selenocysteine analogs showing the presence of an α-helix. a(ii) Overlay of the αH NMR shifts of globular AuIB and its selenium analogs showing no significant changes in overall structure. b(i) Superimposition of the crystal structures of α-PnIA and α-Sec[2,4]-[A10L]PnIA illustrating the isosteric character of the diselenide bond (red) and the disulfide bond (yellow). b(ii) 2Fo-Fc electron density around the diselenide bond of Sec[2,4]-[A10L]PnIA, contoured at 1σ. b(iii) Physicochemical properties of sulfur and selenium (black)[14] and structural data (purple) of torsion angles, Cα distances and bond lengths derived from the crystal structure analysis of Sec[2,4]-[A10L]PnIA.

analysis of globular and ribbon AuIB, and its selenium analogs showed that the α helix was preserved in the native and in the selenium analogs (see Figure 3a (i)). NMR analysis of αH chemical shifts of peptides was conducted, as it is a rapid and reliable way of detecting structural differences if present.[27,28] It is clear from comparison of the αH chemical shifts of native and selenocysteine isomers that there were no significant differences in their three-dimensional structures (see Figure 3a (ii)). X-ray analysis of Sec[2,4]-[A10L]PnIA and comparison with the crystal structure of α-PnIA allowed high resolution insight into bond lengths, Cα distances and torsion angles (see Figure 3b and Supporting Information Table S3). Sec[2,4]-[A10L]PnIA was crystallized under similar conditions to those described for PnIA,[29] but the crystals grew isomorphously with PnIB in the space group $P2_12_12_1$. The selenocysteine mutant of PnIA did not hinder the growth of well-diffraction crystals (1.42 Å resolution using in-house source). The refined structure of Sec[2,4]-[A10L]PnIA comprises all 16 residues plus 27 solvent molecules and has an overall R_{factor} of 15.6% with R_{free} of 17.1%. Overall the structure of Sec[2,4]-[A10L]PnIA is very similar to that of PnIA. The rms (Root Mean Square) deviation for superimposition of main chain atoms between Sec[2,4]-[A10L]PnIA and Pn1A is 0.4 Å. The conformation of the diselenide bridge of selenocysteine mutant of PnIA is also similar to that of the disulfide bridge of the native peptide. The dihedral angles (Cβ−S−S−Cβ or Cβ−Se−Se−Cβ) of Sec[2,4]-[A10L]PnIA are −98.3° and −93.9° for Cys1-Cys3 and Sec2-Sec4, respectively, and compares well with −98.6° and −83.1° for the disulfide counterparts in PnIA. The distance is 5.7 Å for the diselenide bridge Sec2-Sec4 Cα atoms and 5.1 Å for Cys1-Cys3 Cα atom in Sec[2,4]-[A10L]PnIA, with a bond length of 2.33 Å and 2.03 Å for the diselenide and disulfide bridge, respectively. Similar values were observed in PnIA (5.1 Å for Cys1-Cys3 Cα; 5.4 Å for Cys2-Cys4 Cα, S−S bond lengths of 2.03 Å). Structural analysis of the selenocysteine mutant of PnIA supports the notion that replacement of S for Se atoms in peptides or proteins does not induce significant distortion either in the diselenide bridge or in the overall fold.[30] Interestingly, C_{18}-RP−HPLC coelution analysis showed that the α-conotoxin with the most pronounced activity increase Sec[1,3]-AuIB was the only analog that did not coelute its native counterpart, indicating that the more pronounced hydrophobicity of the diselenide bond might contribute to the observed change in activity.

2.5. Stability Assays. RP−HPLC and LC−MS studies on AuIB and the α-selenoconotoxin Sec[1,3]-AuIB and Sec[2,4]-AuIB were performed in a solution containing equimolar glutathione and in rat plasma at physiological pH. In both systems LC−MS analysis demonstrated that a single diselenide bond was able to largely suppress scrambling (see Figure 4). The observed selenylsulfide to diselenide bond ratio during the glutathione assay correlated well with the oxidation profile observed after full reduction and reoxidation, and after oxidative

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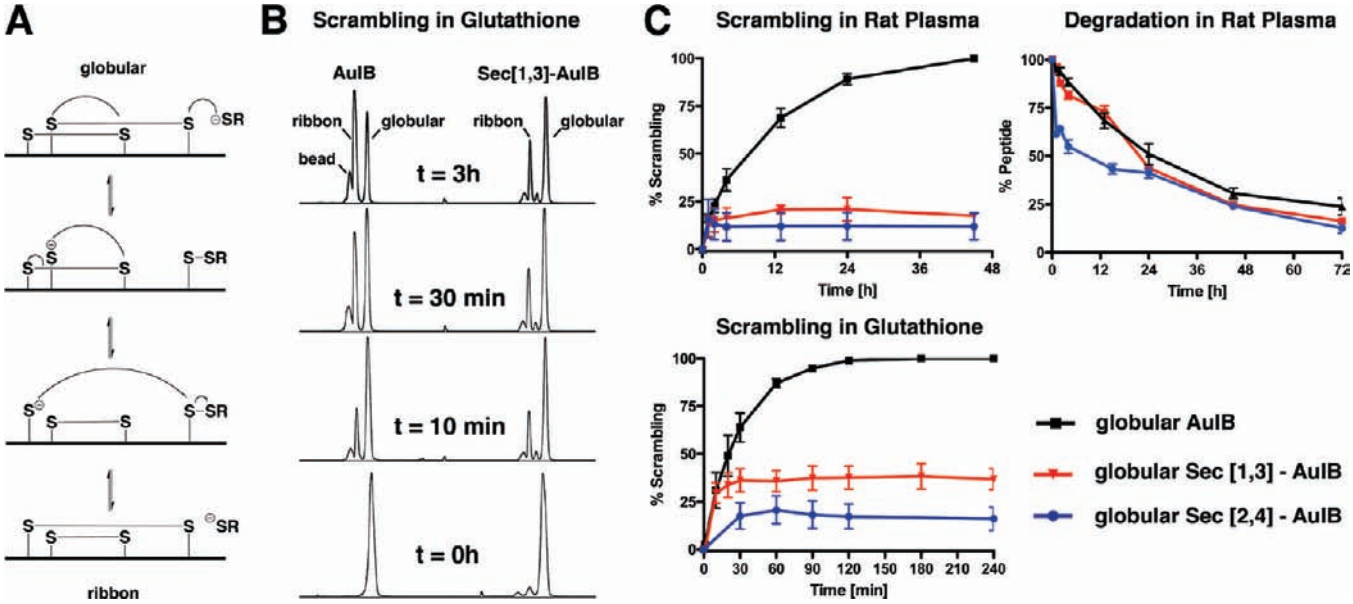

Figure 4. Stability studies on AuIB and its selenium analogs. (A) Proposed disulfide bond scrambling mechanism in a 2-disulfide bond system. (B) Analytical RP−HPLC traces of AuIB and Sec[1,3]-AuIB in stoichiometric equimolar 0.25 mM glutathione solution at physiological pH 7.4 (PBS buffer) and 37 °C, showing the structural change from the globular into the ribbon isomer. (C) Selenium analogs significantly suppress scrambling in rat plasma and glutathione solution. The degradation of the selenoconotoxins is very similar to the native analogs. Error bars are mean ± SEM for $n = 3$.

workup. Further analyses showed that the stability of the Sec analogs in rat plasma was similar to their native analogs with a half-life of 24 h.

3. Discussion

Strategic replacement of pairs of cysteine residues by selenocysteine residues in α-conotoxins has a decisive impact on the oxidative folding profiles (see Table 1 and Supporting Information Figure S2). When placed into the native (globular) positions (1−3, or 2−4) nearly quantitative formation of the desired isomer was observed *independent* of the loop frameworks for all five known classes of α-conotoxins. Intriguingly, the Sec residues were found to be fully oxidized directly upon deprotection via HF (pH < 1), which was unexpected as the reported pK_a(Sec) = 5.24−5.63 would indicate that selenols should be stable in strong acidic media. This observation is, however, consistent with the paucity of literature describing that selenocysteine can be only found either in the form of a diselenide bond or as a selenolate stabilized in a catalytic triads.[31−33] The facile oxidation of the Sec residues and the rapid oxidation (<10 min) at pH 8 of the two remaining Cys residues drastically reduces workup times and makes Sec-directed oxidative folding a far more efficient method than orthogonal protecting group strategies (see Supporting Information Figure S3 online). More importantly, this approach makes experiments to determine disulfide bond connectivity superfluous and simplifies the purification process. Furthermore, this methodology is highly complementary to native chemical ligation[19,34,35] and orthogonal thiol-protecting group strategies, providing the basis for the folding of more complex disulfide rich peptides containing 3 or 4 disulfide bond bridges, a task that has not been achieved as yet.

The development of resin-supported chemistry compatible with the selenium-directed folding approach provides the basis for high-throughput production of correctly folded α-conotoxins. The parallel processing of peptide assembly, side-chain deprotection, oxidation, cleavage, and purification allows efficient production of a large number of peptides (see Figure 1b). Similar to in-solution folding, different ratios of the three possible disulfide bond isomers of the all-Cys analogs can be accessed through astute selection of folding conditions, a process that can be helpful when access to all different isomers is desired (see Supporting Information Table S2).[26] However, the use of selenocysteine induces regioselective control also on-resin, providing fast access to correctly folded α-conotoxins. This methodology avoids excessive use of solvents and reagents, and has the potential to be widely used for the synthesis of disulfide-rich peptides.

In contrast to earlier studies on the selenocystine-containing analogs of endothelin[20] or apamin[36] whose disulfide bonds do not form a hydrophobic core we were aware of the possibility that incorporation of Se−Se bond(s) could perturb the structure of the more tightly compacted α-conotoxins. Thus, the synthesized α-selenoconotoxins were examined closely to determine any impact on structure and function. Strikingly, pharmacological analysis of the α-conotoxin MI and its selenocysteine analogs to inhibit muscle contractions in response to phrenic nerve stimulation in rat diaphragm preparations revealed a significant increase in potency for the globular selenium-analog Sec[2,4]-MI (see Table 1 and Figure 2a). Quantitative electrophysiology studies on α-conotoxins MI, AuIB, [A10L]PnIA and their selenocysteine analogs using ACh-evoked currents of muscle ($(\alpha1)_2\beta1\delta\gamma$) and neuronal nAChR subtypes (α3β4, α7, α9α10)

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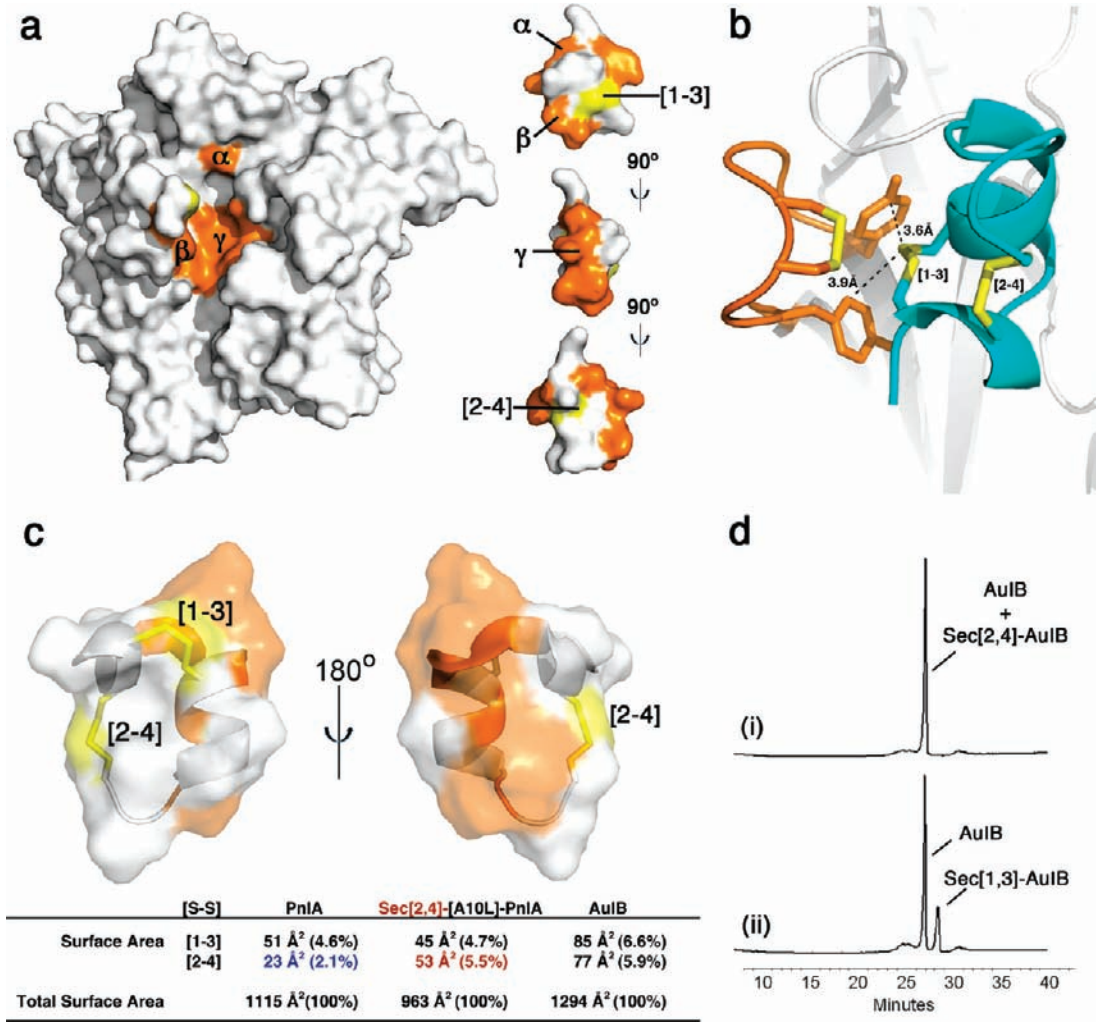

	[S-S]	PnIA	Sec[2,4]-[A10L]-PnIA	AuIB
Surface Area	[1-3]	51 Å² (4.6%)	45 Å² (4.7%)	85 Å² (6.6%)
	[2-4]	23 Å² (2.1%)	53 Å² (5.5%)	77 Å² (5.9%)
Total Surface Area		1115 Å²(100%)	963 Å² (100%)	1294 Å² (100%)

Figure 5. Surface analysis and binding model (a) Surface hydrophobicity plot of the cocrystal structure of AChBP and α-conotoxin PnIA with the hydrophobic residues colored in orange and the disulfide bonds in yellow. α,*β*,*γ* indicate the complementary hydrophobic interaction of PnIA with the binding pocket. (b) Close up of α-conotoxin PnIA binding into the hydrophobic pocket, revealing that the first disulfide bond$^{1-3}$ is flanked by two tyrosine residues (3.6−3.9 Å). (c) NMR surface analysis of α-conotoxin AuIB, showing that the$^{1-3}$ disulfide bond is in close proximity to the conserved hydrophobic residues that are important for binding. The surface exposure of the disulfide/diselenide bonds of PnIA, Sec[2,4]-[A10L]PnIA and AuIB are analyzed in the table, showing that the surface exposure of the diselenide bond is twice as large as the disulfide bond counterpart. (d) Co-elution study of AuIB its selenium analogs. Analytical C_{18}-RP-HPLC traces of the coelution of (i) globular AuIB with globular Sec[2,4]-AuIB and (ii) globular Sec[2,4]-AuIB with globular Sec[1,3]-AuIB, revealed that Sec[1,3]-AuIB is more hydrophobic than its analogs.

expressed in *Xenopus* oocytes (see Table 1 and Figure 2b) confirmed observed results obtained using the hemidiaphragm preparation, with selenium analogs Sec[2,4]-MI, Sec[1,3]-AuIB, Sec[2,4]-AuIB, and Sec[1,3]-[A10L]PnIA exhibiting up to a 10-fold increase in inhibition compared to their respective native α-conotoxins.

With selenium having a slightly larger atomic radius (S 1.02 Å, Se 1.17 Å) and longer bond length than sulfur ($C^{\beta}-S^{\gamma}$ = 1.82 Å, $C^{\beta}-Se^{\gamma}$ = 1.95−1.99 Å)[14] we assessed the isosteric character of selenocysteine more rigorously to determine if structural differences could explain the increased biological activity. CD and αH chemical shift NMR analysis of the AuIB analogs showed that the secondary structure was highly preserved in the selenium analogs, and no significant differences in their three-dimensional structures from the native peptides were observed (see Figure 3a). This conclusion was further supported by the crystal structure of the α-selenoconotoxin, Sec[2,4]-[A10L]PnIA (resolution 1.42 Å), which was near identical to the native α-conotoxin PnIA (rmsd of 0.4 Å for the main-chain atoms).[29] Further structural analysis of Sec[2,4]-[A10L]PnIA indicated that torsion angles, bond lengths, Cα−Cα distances and electron densities of the diselenide and the homologous disulfide bridge from the native PnIA were very similar and no distortion was observed in the overall fold (see Figure 3b and Supporting Information Table S3). To determine the role of increased hydrophobicity of the Se−Se bond in binding with the nAChR, the cocrystal structure of PnIA and the acetylcholine binding protein (AChBP), a structurally and functionally homologous protein of nAChR was examined.[37] The cocrystal structure suggests that α-conotoxin PnIA binds to AChBP via complementary hydrophobic patches, with the [1,3]-disulfide bond flanked by two tyrosine residues (3.6−3.9 Å) (see Figure 5 a and b). This hydrophobic binding motif (**Y**N**CC**E**E**I**Y**) (see Figure 5b) is also highly conserved in the α3 subunit (**YSCCPEPY**), the subunit important for Sec[1,3]-AuIB recognition, which has the most pronounced enhancement

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of activity (10-fold) and hydrophobicity (see Table 1 and Figure 5 c and d). Such a change in hydrophobicity in combination with small changes in bond length and torsion angle are likely to strengthen hydrophobic interactions between the diselenide bond and the two tyrosine residues or in changing in the binding orientation, which subsequently increases the hydrophobic contacts with conserved aromatic residues in the binding site. Another anticipated advantage of selenoconotoxins is their higher stability in reducing environments due to the lower redox potential of the diselenide.[18,19,21] RP−HPLC and LC−MS studies on AuIB and the α-selenoconotoxin Sec[1,3]-AuIB and Sec[2,4]-AuIB performed in solutions containing equimolar glutathione or rat plasma at physiological pH demonstrated that a single diselenide bond was able to largely suppress scrambling (see Figure 4). However, the selenium analogs showed a similar half-life to the native peptides in plasma, confirming that the main degradation occurs mostly via enzymatic digestion. In order to improve the plasma stability of this class of peptides even more, N- to C-terminal cyclization is currently the method of choice and significant progress has been achieved in this field recently.[38,39]

In summary, we have developed a novel, highly scalable method amenable to high throughput α-conotoxin synthesis that employs resin-supported selenium-chemistry to solve a long-standing folding problem of an important class of subtype-selective nAChR antagonists. Stability studies and electrophysiological analysis revealed that the selenium analogs of α-conotoxins are more potent and stable than their native counterparts. Comprehensive structural analysis showed that the slightly larger atomic radius of selenium has no significant impact on the overall structure. Surface analysis and investigation of the binding pocket of AChBP revealed that the increase in hydrophobicity of the diselenide bond is likely to be the reason for observed increase in potency of this toxin class. This methodology is highly complementary to native chemical ligation and orthogonal thiol-protecting group strategies and is anticipated to provide access to more complex disulfide rich peptides or proteins with similar folding challenges.[19,35]

4. Methods

4.1. Peptide Synthesis. All peptides were assembled by manual Boc-SPPS using HBTU-mediated *in situ* neutralization protocol with DMF as solvent.[23] Deprotection of the 2,4-dinitrophenyl (Dnp) group of histidine was carried out prior to HF treatment with 20% 2-mercaptoethanol/ 10% DIEA/ DMF. HF deprotection or cleavage was performed by treatment of the dried peptide resin (300 mg) with 10 mL HF/*p*-cresol/*p*-thio-cresol (18:1:1, v/v/v) for 2 h at 0 °C. Following evaporation of the HF, the peptides were precipitated and washed with cold ether, filtered, and either redissolved in 30 mL of 50% ACN/1% TFA and lyophilized, or redissolved directly in 0.1 M NH_4HCO_3 (pH 8.4, $c = 0.1$ μM) for direct oxidation. Oxidation was monitored by RP−HPLC, LC−MS and MS, and the peptides were isolated using preparative C_{18} RP−HPLC.

4.2. On-Resin Folding using a Safety-Catch Acid Labile (SCAL) Linker. Peptide assembly was achieved by manual Boc-SPPS[23] using the Fmoc-SCAL linker and a three glycine spacer between the linker and the aminomethyl ChemMatrix resin. Deprotection of the Fmoc group of the SCAL linker was performed with 2× 1 min treatment of 50% piperidine/DMF. 100−500 mg of the individual peptides on-resin were transferred into labeled 74 μm mesh polypropylene bags with the dimension of 5 × 5 cm. Side-chain deprotection was achieved by 10 mL HF treatment at 0 °C for 2 h with *p*-cresol as scavengers [9:1 (v/v) HF:scavenger]. HF was evaporated and the solid support was directly transferred into TFA to maintain the swelling properties. The resins were washed with DCM, DMF, and H_2O before being placed into a 0.1 M NH_4HCO_3 solution (100 mg /10 mL) at pH 8.4. The final cleavage was performed either by reductive acidolysis with NH_4I/ TFA/DMS for 1 h at 0 °C and 1 h at room temperature.[26] TFA was then evaporated by nitrogen purging, the peptides were precipitated and the scavengers removed with cold ethyl acetate. The peptides were redissolved in H_2O, 0.1% TFA and lyophilized. After MS and analytical RP-HPLC analysis, the peptides were purified by C_{18}−RP−HPLC.

4.3. Contraction Bioassay Rat Diaphragm and Electrophysiology−*Xenopus* Oocytes. RNA preparation, oocyte preparation, and expression of nAChRs in *Xenopus* oocytes were performed as previously described.[40] The methods and experimental protocols for the rat diaphragm contraction bioassay and the electrophysiological assays using *Xenopus* oocytes can be found in the Supporting Information.

4.4. Circular Dichroism (CD) Spectroscopy. CD spectroscopy was performed on a Jasco J-810 spectropolarimeter. Spectra were recorded at room temperature under nitrogen atmosphere. Peptides were dissolved in 20 mM phosphate buffer, containing 30% trifluoroethanol at pH 7. The peptide concentration was determined by quantitative RP−HPLC. The peptides were transferred into a 0.01 cm path length demountable cell and data were recorded over 5 scans, from 260 to 185 at 10 nm/min, with a resolution of 1 nm and a response time of 0.25 s. CD data in ellipticity was converted to mean residue ellipticity ([θ]R) using the equation: $[\theta]R = \theta/(10 \times C \times Np \times l)$ where θ is the ellipticity in millidegrees, C is the peptide molar concentration (M), l is the cell path length (cm), and Np is the number of peptide residues.

4.5. NMR Spectroscopy. NMR spectra were recorded at 290 K on a Bruker Avance 600 MHz spectrometer and processed using Topspin (Bruker Corp. Billerica, MA, USA) software. The concentration for the ^{1}H NMR measurements was ∼1 mM peptide in 90% H_2O/ 10% D_2O (v/v) at pH 3. 2D NMR spectra were recorded in phase-sensitive mode using time-proportional phase incrementation for quadrature detection in the t_1 dimension.[41] The 2D experiments consisted of a TOCSY using a MLEV-17 spin lock sequence[42] with a mixing time of 80 ms, and NOESY with a mixing time of 250 ms. Solvent suppression was achieved using a modified WATERGATE sequence. Spectra were acquired over 6024 Hz with 4096 complex data points in F_2 and 512 increments in the F_1 dimension. The t_1 dimension was zero-filled to 1024 real data points, and 90° phase-shifted sine bell window functions were applied prior to Fourier transformation. Chemical shifts were referenced to internal 2, 2-dimethyl-2-silapentane- 5-sulfonate.

4.6. Glutathione Stability Assay. Peptide samples (0.3 mM) were dissolved in a solution containing 0.3 mM reduced glutathione (Sigma Aldrich) in 100 mM phosphate buffer, pH 7.2 and incubated at 37 °C. Aliquots (30 mL) were taken at different time points, quenched with extraction buffer (70 mL) consisting of 50% aqueous acetonitrile, 100 mM NaCl and 1% TFA, and analyzed by RP−HPLC and LC−MS.[21]

4.7. Rat Plasma Stability Assay. Rat plasma (Sigma Aldrich) was incubated at 37 °C for 30 min and 300 mL of plasma was added to 50 mL of 0.3 mM peptide sample in 100 mM phosphate buffer, pH 7.2. The samples were incubated at 37 °C and aliquots (30 mL) were taken at different time points, quenched with extraction buffer

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(70 mL) consisting of 50% aqueous acetonitrile, 100 mM NaCl, and 1% TFA, chilled on ice for 5 min prior to centrifugation at 14 000 rpm for 10 min and analyzed by RP−HPLC and LC−MS.[21]

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Supporting Information Available: Synthetic procedures and analytical data; general materials and methods; details on the safety-catch acid labile (SCAL) linker; additional experiments and conditions on selenium-directed folding; X-ray analysis; details on biological analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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