Disulfide bonds are important structural motifs that play an essential role in maintaining the conformational stability of many bioactive peptides. Of particular importance are the conotoxins, which selectively target a wide range of ion channels that are implicated in numerous disease states. Despite the enormous potential of conotoxins as therapeutics, their multiple disulfide bond frameworks are inherently unstable under reducing conditions. Reduction or scrambling by thiol-containing molecules such as glutathione or serum albumin in intracellular or extracellular environments such as blood plasma can decrease their effectiveness as drugs. To address this issue, we describe a new class of selenoconotoxins where cysteine residues are replaced by selenocysteine to form isosteric and non-reducible diselenide bonds. Three isoforms of α-conotoxin ImI were synthesized by 6-butoxycarbonyl chemistry with systematic replacement of one [(Sec²⁴⁴⁴²²]ImI or [Sec⁶¹]ImI], or both [(Sec²⁴⁴⁴²²]ImI] disulfide bonds with a diselenide bond. Each analogue demonstrated remarkable stability to reduction or scrambling under a range of chemical and biological reducing conditions. Three-dimensional structural characterization by NMR and CD spectroscopy indicates conformational preferences that are very similar to those of native ImI, suggesting fully isomorphic structures. Additionally, full bioactivity was retained at the α2 nicotinic acetylcholine receptor, with each selenoanalogue exhibiting a dose-response curve that overlaps with wild-type ImI, thus further supporting an isomorphic structure. These results demonstrate that selenoconotoxins can be used as highly stable scaffolds for the design of new drugs.

A major goal in peptide-based drug design is to increase conformational stability to gain greater selectivity for a receptor target and enhanced resistance to degradation by proteases. Traditionally, cyclization is employed as a means of restricting a peptide to the bioactive conformation, which consequently increases receptor selectivity and in vivo stability (1). Nature has successfully implemented this strategy through disulfide bonds, which form single or multiple intrachain cross-links that impart considerable structural constraints and contribute to stabilizing the bioactive conformation. Some biologically important peptides containing disulfide bonds have also achieved drug status or are currently in clinical trials (Table 1).

Whereas disulfide bonds impart conformational stability to peptides and proteins, they are inherently unstable in reducing environments and are susceptible to disulfide bond exchange reactions with biological thiols such as glutathione (2). Glutathione primarily functions as an intracellular redox buffer with the intracellular concentration of reduced glutathione being in the millimolar range (2-5 mM) inside mammalian cells. Surprisingly, glutathione also occurs in the reduced form in the micro-molar range in blood plasma and may play a role in disulfide bond deactivation through scrambling or trapping processes (3). Similarly, redox-active enzymes, including thioredoxin and disulfide isomerase, which control a variety of processes through thiol redox control, can lead to reduction of disulfide bonds (4, 5). Scrambling of the disulfide bond connectivity has been shown to induce structural changes that can lead to reduced biological activity (6). From a drug design perspective, this can result in reduced potency or potentially harmful side effects.

Selenocysteine (Sec) is a naturally occurring amino acid, which forms an essential catalytic group in several redox enzymes (7). Its unique redox properties make it a useful mechanistic probe for creating artificial catalysts with novel hydrolytic and redox activities (8-10). Furthermore, selenocysteine exhibits the propensity to oxidatively form a diselenide bond analogous to the disulfide bond (11). Replacement of a disulfide bond with a diselenide bond may be viewed as one of the most highly conservative substitutions available. Early studies showed that replacement of a single disulfide bond by the diseleno isostere in oxytocin (12), somatostatin (13), and rat atrial natriuretic peptide (14) led to analogues having biological activities similar to the native peptide. Further, conformational studies by Moroder and co-workers (15) of a diselenide bond analogue of endothelin-1 indicated that considerable native secondary structure may be maintained. It would appear that the use of selenocysteine substitution of disulfide bonds has significant advantages over substitution with other chemical moieties, including carbamates (16, 17), lactam (18), and thioether bridges (19), which can impart structural distortions that may compromise bioactivity and selectivity.

Despite the similarities between cysteine and selenocysteine, their chemical properties differ quite considerably. Reactions with selenocysteine occur much faster than cysteine at pH 5.0, suggesting that selenocysteine can be selectively oxidized over cysteine at lower pH (20). The redox potential relative to glutaredoxin for the diselenide bond (–381 mV) is significantly lower than that of the disulfide bond (–180 mV), requiring stronger reducing conditions, such as a large excess of diithiothreitol or sodium borohydride, to induce its cleavage (21). Furthermore, the redox potential for a mixed sulfide/selenide bond is higher than that of a diselenide bond (–321 mV), suggesting that its formation is unfavorable. Therefore, we would anticipate that diselenide bonds,

---

* This work was supported by the Australian Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The atomic coordinates and structure factors (code 2BC7 and 2BCB) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

1 To whom correspondence should be addressed. Tel.: 61-7-3346-2222; Fax: 61-7-3346-2101; E-mail: P.Alewood@imb.uq.edu.au.

2 The abbreviations used are: Sec, selenocysteine; Boc, tert-butoxycarbonyl; DBU, 1,8-diazabicyclo(5.4.0)undec-7-ene; Fmoc, N-(9-fluorenylmethoxycarbonyl) (Fmoc); HBTU, 2-(1H)-benzotriazol-1-yl-1,1,3,3-tetramethyluronium hexafluorophosphate; ImI, α-conotoxin ImI(s); nACHR, nicotinic acetylcholine receptor; RP-HPLC, reversed-phase high performance liquid chromatography; SSPPS, solid phase peptide synthesis; MeBnZ, methylbenzyl; WT, wild type; TOCSY, total correlation spectroscopy; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy.
Improving the Biological Stability of Conotoxins

TABLE 1
Representative peptide-based drugs that contain disulfide bonds

| Name               | Status     | Disulfide bonds | Mode of action                  | Disease state          |
|--------------------|------------|-----------------|---------------------------------|------------------------|
| Somatostatin       | Approved   | 1               | Inhibits hormone release         | GIantism and acromegaly|
| Oxytocin           | Approved   | 1               | Oxytocin receptor agonist        | Uterotonic             |
| Carperidic         | Approved   | 1               | Natural peptide receptor agonist | Cardiovascular (heart failure) |
| Integrin           | Approved   | 1               | Platelet glycoprotein IIb/IIIa inhibitor | Cardiovascular (antiangiial) |
| Vasopressin        | Approved   | 1               | Vasopressin receptor agonist     | Cardiovascular (antihypertensive) |
| Xen-2174 (x-Ctx Mr1A) | Phase II Clinical Trial | 2 | Noradrenaline transporter inhibitor | Analgesic |
| ACV1 (x-Ctx Vcl1.1) | Preclinical | 2                 | nAChR antagonist                  | Analgesic              |
| Insulin            | Approved   | 3               | Insulin receptor agonist         | Diabetes               |
| Aprotinin          | Approved   | 3               | Plasmin inhibitor                 | Cardiovascular (antihemorrhagic) |
| Alfimprasc         | Phase III Clinical Trial | 3 | Degradase fibroblast             | Analgesic              |
| Prialt (x-Ctx MVIIA) | Approved   | 3               | Ca²⁺ channel antagonist (N-type) | Analgesic              |
| AM-336 (x-Ctx CVID) | Phase II Clinical Trial | 3 | Ca²⁺ channel antagonist (N-type) | Analgesic              |

![FIGURE 1](image)

Once formed, would eliminate the possibility of scrambling under biological reducing conditions.

Conotoxins are a class of disulfide-rich peptide neurotoxins isolated from the venom of carnivorous marine snails. They target a wide range of ion channels with a high degree of selectivity and potency and have profound therapeutic potential, with at least one example recently approved as a drug for the treatment of chronic pain and several others currently undergoing clinical trials (see Table 1) (22). In the present study, we have investigated the impact of substituting disulfide bonds with diselenide bonds on the structure, activity, and conformational stability of a model cysteine-rich peptide in relevant biological reducing environments. Conotoxin ImI is a potent antagonist of the neuronal α7-nicotinic acetylcholine receptor (nAChR) (23), and its structure and function are well characterized (24–26). It comprises 12 amino acid residues with two internal disulfide bonds (Fig. 1) that are substantially “buried” within the three-dimensional structure. In its native form, disulfide bonds occur between residues 2 and 8 and residues 3 and 12; however, the presence of two disulfide bonds gives rise to the formation of two additional monomeric isomers, namely the 2–12 and 3–8 (ribbon) or 2–3 and 8–12 (beads) isomers through thiol-induced scrambling. Here we describe (i) the development of a general chemical procedure for the synthesis of selenocysteine- and diselenide-containing peptides that overcomes previous chemical limitations; (ii) the synthesis of novel analogues of α-conotoxin ImI, where one ([Sec2,8]ImI and [Sec3,12]ImI) and both ([Sec2,3,8,12]ImI) disulfide bonds are systematically replaced with diselenide bonds; (iii) evaluation of the three-dimensional structures of the above analogues; (iv) characterization of their potency on rat α7-nAChRs expressed in Xenopus oocytes; and (v) evaluation of their stability to reduction and scrambling in several biological environments. On the basis of the experimental data, we suggest that this new class of bioactive peptides, the selenoconotoxins, has significantly improved properties that enhance their potential for drug development.

MATERIALS AND METHODS

Synthesis of Boc-Sec(MeBzl)-OH

Disodium Diselenide (Na₂Se₂)—A 1 M solution of Na₂Se₂ (50 ml) was prepared according to the method of Klayman and Griffin (27) and was immediately used for subsequent reactions.

L-Selenocystine was prepared as described previously by Chocat et al. (28) from H-β-chloro-l-alanine and sodium diselenide (4.62 g, 70.2%).

| Name               | Status     | Disulfide bonds | Mode of action                  | Disease state          |
|-------------------|------------|-----------------|---------------------------------|------------------------|
| Somatostatin      | Approved   | 1               | Inhibits hormone release         | GIantism and acromegaly|
| Oxytocin          | Approved   | 1               | Oxytocin receptor agonist        | Uterotonic             |
| Carperidic        | Approved   | 1               | Natural peptide receptor agonist | Cardiovascular (heart failure) |
| Integrin          | Approved   | 1               | Platelet glycoprotein IIb/IIIa inhibitor | Cardiovascular (antiangiial) |
| Vasopressin       | Approved   | 1               | Vasopressin receptor agonist     | Cardiovascular (antihypertensive) |
| Noradrenaline transporter inhibitor | Analgesic |
| nAChR antagonist  | Analgesic  |                 | Insulin receptor agonist         | Diabetes               |
| Plasmin inhibitor | Analgesic  |                 | Degradase fibroblast             | Analgesic              |
| Ca²⁺ channel antagonist | Analgesic |
| Ca²⁺ channel antagonist | Analgesic |

H NMR (300 MHz, D₂O + DCl + DSS) δ 4.5 (m, 1H), 3.5 (m, 2H); 13C NMR (75.4 MHz, D₂O + DCl + DSS) δ 168.8, 51.6, 25.3.

Se-(4-methylbenzyl)-l-selenocysteine—Selenocystine (3.80 g, 9.3 mmol) was suspended from petroleum spirits/diethyl ether. (2.8 g, 75.3%)1H NMR (300 MHz, CD₃OD) δ 7.1 (d, 2H), 4.1 (q, 1H) 3.8, (s, 2H), 3.0 (m, 1H), 2.3 (s, 3H);13C NMR (75.4 MHz, CD₃OD + DCl) δ 170.7, 138.0, 136.8, 130.3, 130.1, 53.9, 28.7, 23.2, 21.4.

N*-tert-Butyloxycarbonyl-Se-(4-methylbenzyl)-l-selenocysteine—Se-(4-methylbenzyl)-l-selenocysteine (3.24 g, 10 mmol) and K₂CO₃ (3.4 g, 15 mmol) were dissolved in water (25 ml) with gentle heating. Boc-dicarbonate (2.30 g, 0.11 mol) in 1,4-dioxane (25 ml) was added, and the mixture was stirred vigorously at room temperature for 1 h before water was added (100 ml). The mixture was washed with diethyl ether (2 × 100 ml), and the aqueous layer was acidified to pH 4 with solid citric acid and extracted with ethyl acetate (3 × 100 ml). The combined extracts were washed with 10% citric acid (3 × 100 ml), brine (100 ml) and then dried over MgSO₄ and filtered, and solvent was removed in vacuo to give Boc-Sec(MeBzl)-OH as a white crystalline solid, which was recrystallized from petroleum spirits/diethyl ether. (2.8 g, 75.3%)

Peptide Assembly

Assembly of WT ImI, [Sec2,8]ImI, [Sec3,12]ImI, and [Sec2,3,8,12]ImI was performed manually using HBTU-mediated Boc in situ neutralization solid phase peptide synthesis (SPPS) (29) on 4-methylbenzhydylamine resin. Deformylation of tryptophan was carried out prior to cleavage from the resin by treating with 20% ethanolamine, 10% H₂O, and dimethylformamide. Cleavages were performed by treating the peptide-resin (300 mg) with 10 ml of HF/p-cresol/p-thiocresol (18:1.1, v/v/v) for 2 h at 0 °C. Following evaporation of the HF, in vacuo the crude peptides were precipitated and washed with cold ethyl acetate degassed with nitrogen (30 ml). The peptides were filtered under a blanket of nitrogen and immediately oxidized by dissolving in 300 ml
of 0.1 M ammonium formate, 50% isopropyl alcohol (pH 4.2). For
[Sec<sup>2,8</sup>ImI and [Sec<sup>3,12</sup>ImI], the solution was adjusted to pH 8.2,
which allowed further oxidation to form the disulfide bond. Prior to
purification, the solution was acidified to pH 2 and analyzed by RP-
HPLC and electrospray ionization mass spectrometry to ensure com-
plete oxidation. The isopropyl alcohol was removed in vacuo, and the
peptide was isolated using preparative RP-HPLC. Yields from the pep-
tide-resin were as follows: 22% for [Sec<sup>2,8</sup>ImI], 27% for [Sec<sup>3,12</sup>ImI], 30%
for [Sec<sup>2,3,8,12</sup>ImI].

**Human Blood Plasma Stability**

Whole human blood containing 1% EDTA was centrifuged at 14,000
rpm for 30 min. The supernatant was then transferred to an Eppendorf
tube and centrifuged for an additional 30 min at 14,000 rpm. Peptide
samples were dissolved in plasma (200 μl) to an initial peptide concen-
tration of ~0.25 mM. Aliquots (30 μl) were removed at various time
intervals and quenched with extraction buffer (30 μl). The aliquot was
then vortexed, diluted with additional water (60 μl), and chilled in an ice
bath for 5 min prior to centrifuging at 14,000 rpm for 15 min. The
supernatant was then analyzed by RP-HPLC.

**RESULTS**

**General Strategy**—The synthesis of selenocysteine-containing pep-
tides has been limited due to the considerable chemical difficulties expe-
rienced by several laboratories (35). Most of the chemistry developed to
date has employed the Fmoc-SPPS strategy and the Se-4-methoxyben-
yzyl derivative. The (normal) use of auxiliary bases in the coupling and
deprotection steps is reported to induce β-elimination and racemiza-
tion of the protected selenocysteine derivative (35). Despite the develop-
ment of optimized procedures that utilize minimal treatment with
piperidine for the stepwise removal of N<sup>ω</sup>-Fmoc protecting group (35),
these methods still lack general applicability. The N<sup>ω</sup>-Fmoc protecting
group can suffer from incomplete removal, thus reducing the quality
and yield of the final product, particularly in difficult peptide sequences
(36, 37). Moreover, it has been suggested that protected selenocysteine
derivatives can racemize, unless pentafluorophenyl esters are used in
subsequent chain elongation steps (35). The use of such derivatives is
also limited by their lack of commercial availability.

Recently, it has been suggested that Boc chemistry can overcome the
problems associated with the synthesis of selenocysteine-containing pep-
tides (38). In contrast to Fmoc chemistry, Boc-SPPS has the advan-
tage of clean and reliable N<sup>ω</sup>-Boc deprotection under acidic conditions,
thus ensuring high yields during chain assembly (39) and avoiding
excessive exposure to base, which compromises selenocysteine integrity
(35). Additionally, the use of Boc in situ neutralization coupling chem-
istry (29) would be expected to suppress racemization, since couplings
are performed at neutral pH, allowing the use of commonly available
protected amino acid derivatives. The S-4-methylbenzyl (S-Mebzl)
protecting group is generally used in Boc-SPPS for the protection of cyste-
ine (40). In early studies, S-Mebzl was successfully employed by Soda
and co-workers (41) for protection of selenocysteine, although no fur-
ther examples of its use have been reported, and no experimental data
were reported for the synthesis of the selenocysteine derivative. In our
opinion, Boc-Sec-Mebzl-OH is the derivative of choice for Boc-SPPS,
since the protecting group is stable to the repetitive trifluoroacetic acid
treatments required for the stepwise removal of the N<sup>ω</sup>-Boc protecting
group, and its deprotection by HF acidolysis yields the free selenol func-
tionality under nonoxidizing conditions.

Boc-1-Sec(Mebzl)-OH was prepared in three steps from β-chloro-1-
alanine as previously described (41) and fully characterized. Following
synthesis, the dipeptide with 1-leucine was formed on resin, and analysis
of the cleaved product by RP-HPLC confirmed that no racemization
had occurred, as judged by the presence of a single peak. The propensity
for the Se-Mebzl protected derivative to undergo β-elimination and
racemization under a variety of conditions was further investigated.

**Thiol Stability**

Peptide samples (0.25 mM) were dissolved in a solution containing
both 0.25 mM reduced glutathione, 12.3 μM reduced thioredoxin (Pro-
mega, Madison, WI) or 0.5 mM human serum albumin (Sigma) in 100
mM phosphate buffer plus 1 mM EDTA, pH 7.2 (300 μl), and incubated at
37 °C. Thioredoxin was reduced by treating the oxidized form with
0.9 eq of diethiothreitol for 15 min immediately prior to use. Aliquots (30
μl) were taken at various time intervals; quenched with extraction buffer consisting of 50% aqueous acetonitrile, 100 mM NaCl, and 1%
trifluoroacetic acid (30 μl), and analyzed by RP-HPLC. For conotoxin
ImI, the ratio of the nonnative isomer to the native isomer was deter-
mined by measuring the peak area.

**Electrophysiology Assay**

RNA preparation, oocyte preparation, and expression of α<sub>c</sub> nAChRs
in Xenopus oocytes were performed as described previously (33). Briefly,
plasmids with cDNA encoding the rat

**Improving the Biological Stability of Conotoxins**

Two-dimensional NMR Structural Studies

Samples used for 1H NMR measurements contained ~3 mM peptide
in 90% H<sub>2</sub>O, 10% D<sub>2</sub>O at pH 3.5. TOCSY and NOESY spectra were
acquired at 290 K using a Bruker 500- or 750-MHz NMR spectrometer.
NOESY spectra were acquired using 150-, 250-, and 300-ms mixing
times, and TOCSY spectra were acquired with an 80-ms mixing time.
1H chemical shifts were referenced to an internal sodium 2,2-dimethyl-
2-silapentane-5-sulfonate standard. Structures were calculated using
previously described or standard methods (30). Briefly, initial structures
were calculated using DYANA (31), and these were refined using CNS
(32) to obtain an ensemble of 50 structures.
cant amount of racemization was detected under any of these conditions, ~1% racemization was observed following a 24-h treatment with 50% DBU. No evidence of β-elimination products was observed under any of these conditions. The absence of problematic side reactions demonstrates that the Se-MeBzl protecting group is highly suitable for use in solid phase peptide synthesis.

Synthesis of α-Selenoconotoxin Analogues—Solid phase assembly of each peptide was performed using HBTU-mediated couplings with Boc in situ neutralization chemistry on 4-methylbenzhydramine cross-linked polystyrene resin (29). Deformylation of tryptophan prior to HF cleavage was achieved using 20% ethanolamine, 10% H₂O in dimethylformamide. Acidolytic cleavage of the peptides was performed using HF/p-cresol/p-thiocresol (90:5:5) for 2 h at 0 °C. Precipitation of the crude peptide with ethyl acetate afforded the reduced peptide, which was shown to be free of β-elimination and racemization products.
Whereas the fully reduced peptides were successfully isolated following cleavage with HF, common laboratory handling and storage resulted in oxidative degradation of the reduced material. In considering the difficulties associated with long term storage, the reduced selenocysteine-containing peptides were immediately oxidized in aqueous buffer following HF cleavage (Fig. 2, top). Crude reduced peptide was dissolved in aqueous 0.1M ammonium formate buffer at pH 4.2, containing 50% isopropyl alcohol and oxidized in an open vessel. The oxidation progress was efficiently monitored by electrospray ionization mass spectrometry, where the loss of 2 atomic mass units in the M+H/2 ion corresponds to the formation of the diselenide bond (Fig. 2, bottom). Oxidation of [Sec2,8]ImI proceeded rapidly to completion at pH 4.2, to yield one major product. A second product with identical mass corresponding to a second isomer of [Sec2,8]ImI was designated as the 1–4, 2–3 isomer (ribbon), since its retention time was found to be similar to the WT ImI ribbon isomer (Fig. 2, top). The formation of the strained 1–2, 3–4 (beads) is not observed in the oxidation of WT ImI and was not observed in the oxidation of the selenoconotoxins.

At pH 4.2, the diselenide bond in [Sec2,8]ImI and [Sec2,3,8,12]ImI is selectively formed over the disulfide bond, to give the partially oxidized peptide. The solution was adjusted to pH 8.2 in situ, allowing formation of the disulfide bond to give fully oxidized native [Sec2,8]ImI and [Sec2,3,12]ImI almost exclusively. However, a small amount of a second isomer (<1%) was also attributed to the ribbon isomer due to its retention time nearly identical to that of the WT ribbon isomer and mass identical to that of the native selenoconotoxins. Similarly, the presence of the beads isomer was not detected. The native isomer of each analogue was isolated in high yield and purity by preparative RP-HPLC (Fig. 2, center).

A comparison of RP-HPLC retention times showed that although [Sec2,8]ImI co-eluted with WT ImI, [Sec2,3,12]ImI and [Sec2,3,8,12]ImI demonstrated a slightly longer retention time. This is presumably a result of the exposure of the relatively hydrophobic diselenide bond to residues 3 and 12 to the hydrophobic stationary phase of the RP-column.

**Structural Studies**—CD spectroscopy was used to confirm that successful folding of the analogues was achieved. The CD spectra of each analogue indicated a conserved overall fold, displaying characteristic helical elements that indicate successful folding to achieve the correct connectivity between respective cysteine and selenocysteine residues (Fig. 3A).

$^1$H NMR was used to determine the three-dimensional structures of [Sec2,8]ImI and [Sec2,3,8,12]ImI for comparison with WT ImI. Each ana-
Improving the Biological Stability of Conotoxins

Electrophysiology—The biological activity of each analogue compared with WT ImI was examined in an electrophysiology assay by measuring the inhibition of each analogue at the rat α7 nAChR expressed in Xenopus oocytes. Membrane currents were activated with 200 μM acetylcholine, a maximally effective concentration (Fig. 4A). A comparison of concentration-response relationships for each analogue further demonstrated a negligible difference in activity compared with WT ImI at the α7 nAChR (Fig. 4B). Calculation of the IC50 values and Hill coefficients using the empirical Hill equation showed that bioactivity is conserved for each of the analogues within the limits of error (Table 2). The retention of bioactivity in all three diselenide-ImI analogues further supports structures isomorph to WT ImI.

Stability Studies—Blood plasma stability was used to assess the stability of the analogues in vivo. Reducing thiols are well known to be present in human blood plasma with their concentrations ranging from low to medium micromolar levels (43), which can potentially reduce or scramble the disulfide bond framework of disulfide-rich peptides (Fig. 5A) (44). Incubation of 0.25 mM WT ImI in human blood plasma showed that the low levels of thiols present are indeed sufficient to rearrange the disulfide framework, as characterized by the presence of an earlier eluting peak over time that was identified as the ribbon isomer (Fig. 5B). The ratio of the peak areas can be represented as percentage scrambling, where a 1:1 ratio of ribbon to native isomer represents 100% scrambling (Fig. 5C). The presence of an additional peak on RP-HPLC, corresponding to the ribbon isomer was not observed for each of the α-selenoconotoxin analogues, upon incubation in blood plasma. Since the ribbon isomer of each α-selenoconotoxin analogue is well resolved by RP-HPLC, this suggests total stability to scrambling or reduction for these analogues in blood plasma.

To examine the extent by which external thiols influence the scrambling of the WT ImI disulfide framework, a 0.25 mM solution of WT ImI was treated with 1 eq of glutathione. Under these conditions, it was shown that complete scrambling of the disulfide bond framework occurs rapidly for WT ImI (approximately 6 h) (Fig. 5D). Importantly, no evidence of scrambling or reduction was detected for any of the diselenide analogues.

Table 2

| Analogue | IC50 (nM) | H | n |
|----------|-----------|---|---|
| WT ImI   | 69.3 ± 15.5 | 0.8 ± 0.2 | 10 |
| [Sec2,8]ImI | 49.5 ± 16.2 | 0.7 ± 0.2 | 9 |
| [Sec3,12]ImI | 50.3 ± 8.4 | 0.7 ± 0.1 | 12 |
| [Sec2,3,8,12]ImI | 49.7 ± 6.0 | 0.8 ± 0.1 | 8 |

Albumin constitutes the most significant blood protein, occurring at a concentration of 0.6 mM (45). Approximately 70% of albumin is present as mercaptoalbumin, which consists of 17 disulfide bonds and one free and highly reactive cysteine residue, Cys34 (45). It is known that Cys34 can interact with other disulfide-containing compounds, notably cystine or oxidized glutathione, through an intramolecular disulfide exchange reaction at alkaline pH (46). Preliminary experiments in our laboratory have indicated that mercaptoalbumin induces significant disulfide bond scrambling in disulfide-containing peptides. The drug candidates carperitide (human atrial natriuretic peptide) and AM-336 (ω-conotoxin CVID) (See Table 1) were incubated with 0.6 mM albumin. Improving the Biological Stability of Conotoxins
Improving the Biological Stability of Conotoxins

human mercaptalbumin to determine the effect on their disulfide bond frameworks. In those experiments, several disulfide bond isomers were present in the AM-336 reaction mixture, and a time-dependent decrease (trapping) was observed for human atrial natriuretic peptide (data not shown). Similarly, WT ImI was shown to undergo disulfide scrambling. By contrast, none of the selenocysteine-containing mutants showed any evidence of scrambling under identical conditions (Fig. 5E).

Thioredoxin is a redox-active enzyme that is secreted by cells and is important in a variety of biological processes, including DNA replication and redox control (47). This redox enzyme contains two cysteine residues in its active site. In its reduced form, thioredoxin is a powerful protein disulfide reductase and therefore was used in these stability studies. The selenocysteine-containing α-conotoxin ImI analogues and WT ImI were treated with thioredoxin at a concentration of 12.3 μM. It was shown that whereas scrambling is induced in the two disulfide bond-containing WT ImI (Fig. 5F) none of the diselenide analogues showed any evidence of reduction or scrambling.

**DISCUSSION**

Although we are witnessing the increased use of cystine-stabilized bioactive peptides as therapeutic candidates, the susceptibility of those candidates to reduction in vivo has, until now, failed to be addressed. This study describes the synthesis and application of a new class of bioactive peptides, the α-selenoconotoxins, with properties suitable for their use as peptide drugs.

The synthesis of diselenide-containing α-conotoxin ImI analogues was undertaken by highly optimized Boc chemistry, where introduction of the selenocysteine residues was achieved using activated Boc−S−(MeBzl)−OH. The products obtained using these methods were free of racemic and β-elimination side products that have plagued researchers employing Fmoc chemical strategies. These procedures may also be extended to other classes of selenocysteine-containing peptides, including use in the synthesis of larger selenoproteins through selenocysteine-mediated native chemical ligation and expressed protein ligation (20, 48, 49). A major advantage of our synthetic procedure is that formation of the diselenide bonds is readily achieved by mild aerobic oxidation of the reduced selenocysteine-containing peptide following cleavage with HF. This is in contrast to previous methods that utilize oxidative protection of the Se-methoxybenzyl protected selenocysteine using iodine or dimethyl sulfoxide, increasing the risk of potential side reactions that are known to occur in sensitive side chains, such as Trp, His, Met, and Tyr (50, 51).

Conformational studies by CD and NMR spectroscopy indicate that correct cross-linking was achieved for [Sec^{2,8}]ImI and [Sec^{3,12}]ImI. This was anticipated given that correct pairing was driven by the differences in redox potential between cysteine and selenocysteine and that the formation of a mixed sulfide/selenide bond is generally unfavorable. Moreover, the correct pairing of selenocysteine residues in the two diselenocysteine bond-containing [Sec^{2,3,8,12}]ImI was achieved as the major product without the use of regioselective pairing of selenocysteine residues (24). This suggests that formation of the diselenide bonds is a cooperative process with the correct fold driven by similar factors that influence the cysteine-rich homolog. Three-dimensional structure calculations of the diselenide-containing α-conotoxin ImI analogues indicate structures isomorphic with WT ImI. Structural integrity was further supported by electrophysiology assays at the rat α2 nAChR, in which each of the diselenide-containing analogues exhibited similar activity to WT ImI. Furthermore, complete replacement of the disulfide-bonding framework with diselenide bonds yielded a fully isomorphic molecule. The results show that the use of selenocysteine is a highly conservative substitution relative to other modifications, and, in buried cysteine-rich frameworks such as the α-conotoxins, selenocysteine can be accommodated even given the marginally longer carbon-selenium (~1.80 Å) and selenium-selenium (~2.02 Å) bond lengths.3

Through a careful examination of the stability of the wild type and diselenide-containing α-conotoxin ImI isoforms in human blood plasma, we observed significant scrambling in WT ImI, with a 1:1 ratio of native/ribbon scrambling. By contrast, none of the selenocysteine-containing mutants showed any evidence of scrambling under identical conditions (Fig. 5E).

![Figure 5](https://example.com/figure5.jpg)  
**FIGURE 5.** Stability studies of α-selenoconotoxin ImI analogues. A, proposed mechanism for scrambling of WT ImI to the ribbon isomer by external thiols. B, RP-HPLC analysis of scrambling of 0.25 mM WT ImI in human blood plasma (containing 1% EDTA) over time. Shown is the disulfide framework stability of 0.25 mM ImI analogue in human blood plasma (C). 0.25 mM glutathione (D); 0.6 mM human serum albumin (E), and 12.3 mM thioredoxin (F). 50 mM phosphate buffer plus 1 mM EDTA, pH 7.2, incubated at 37 °C. WT ImI: , [Sec^{2,8}]ImI; , [Sec^{3,12}]ImI; C.  

3 Estimated by measurement of various diselenide-containing organic compounds deposited in the Cambridge crystallographic data base (53).
Improving the Biological Stability of Conotoxins

isomer occurring within 24 h. Importantly, under identical conditions, each of the diselenide analogues maintained complete structural integrity with no evidence of scrambling occurring. Whereas we anticipated that [Sec\textsuperscript{2,3}Im\textsubscript{I}]Im\textsubscript{I} would demonstrate complete stability in the presence of reducing thiols, the substitution of just one disulfide bond was found to be sufficient to achieve complete structural integrity. Thus, [Sec\textsuperscript{3}Im\textsubscript{I}]Im\textsubscript{I} and [Sec\textsuperscript{3,12}Im\textsubscript{I}]Im\textsubscript{I} exhibited complete resistance to reducing thiols, with no evidence of scrambling present. This can be attributed to the difference in redox potential between cysteine and selenocysteine, which disallows the formation of mismatched isomers (52).

In summary, we anticipate that the isomorphic nature and high oxidation potential of diselenide bonds may find increasing use for probing disulfide bonds in proteins and peptides. Furthermore, the superior oxidation potential of diselenide bonds may find increasing use for probing selenocysteine, which disallows the formation of mismatched isomers (52).

REFERENCES

1. Kessler, H. (1982) Angew. Chem. Int. Ed. 21, 512–523
2. Rabenstein, D. L., and Weaver, K. H. (1996) J. Org. Chem. 61, 7391–7397
3. Meister, A., and Anderson, M. E. (1983) Annu. Rev. Biochem. 52, 711–716
4. Holmgren, A., and Bjornstedt, M. (1995) Methods Enzymol. 252, 199–208
5. Matthias, J. L., Yarn, P. T. W., Jiang, X.-M., Vandegraaff, N., Li, P., Poumbourios, P., Donoghue, N., and Hogg, P. J. (2002) Nat. Immunol. 3, 727–732
6. Gehrmann, J., Alewood, P. F., and Craik, D. J. (1998) J. Mol. Biol. 278, 401–415
7. Stadtmann, T. C. (2002) Annu. Rev. Biochem. 71, 1–16
8. House, K. L., Dunlap, R. B., Odom, J. D., Wu, Z.-P., and Hilvert, D. (1992) J. Am. Chem. Soc. 114, 8573–8579
9. Gorlatov, S. N., and Stadtmann, T. C. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 8520–8525
10. Lee, S.-R., Bar-Noy, S., Kwon, J., Levine, R. L., Stadtmann, T. C., and Rhee, S. G. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 2521–2526
11. Müller, S., Senn, H., Gsell, B., Vetter, W., Baron, C., and Böck, A. (1994) Biochemistry 33, 3404–3412
12. Walter, R., and du Vigneaud, V. (1965) J. Am. Chem. Soc. 87, 4192–4193
13. Hartrodt, B., Neubert, K., Bierwoll, B., Blech, W., and Jakubke, H. D. (1980) Tetrahedron Lett. 21, 2393–2396
14. Koide, T., Itoh, H., Otaka, A., Furuuya, M., Kitajima, Y., and Fujii, N. (1993) Chem. Pharm. Bull. 41, 1596–1600
15. Pegoraro, S., Fiori, S., Rudolph-Böhner, S., Watanabe, T. X., and Moroder, L. (1998) J. Mol. Biol. 284, 779–792
16. Nutt, R. F., Veber, D. F., and Saperstein, R. (1980) J. Am. Chem. Soc. 102, 6539–6545
17. Stymiest, J. L., Mitchell, B. F., Wong, S., and Verderas, J. C. (2003) Org. Lett. 5, 47–49
18. Hargi, T., Solé, N. A., Groebe, D. R., Abramson, S. N., and Barany, G. (2000) J. Med. Chem. 43, 4787–4792
19. Bondieberg, J., Grunnet, M., Jespersen, T., and Meldal, M. (2003) ChemBioChem 4, 186–194
20. Honda, R. J., Nilsson, B. L., and Raines, R. T. (2001) J. Am. Chem. Soc. 123, 5140–5141
21. Besse, D., Siedler, F., Dierks, T., Kessler, H., and Moroder, L. (1997) Angew. Chem. Int. Ed. 36, 883–885
22. Armshaw, C. J., and Alewood, P. F. (2005) Curr. Protein Pept. Sci. 6, 221–240
23. Gehrmann, J., and Griffen, T. S. (1973) J. Am. Chem. Soc. 95, 197–199
24. Chocat, P., Esaki, N., Tanaka, H., and Sode, K. (1985) Anal. Biochem. 148, 485–489
25. Schönhöfer, M., Alewood, P., Jones, A., Alewood, D., and Kent, S. B. H. (1992) Int. J. Protein Pept. Res. 40, 180–193
26. Rosenkranz, K. I., Daly, N. L., Plan, M. R., Waine, C., and Craik, D. J. (2003) J. Biol. Chem. 278, 8606–8616
27. Güntert, P., Mumenthaler, C., and Wüthrich, K. (1997) J. Mol. Biol. 273, 283–298
28. Brünger, A. T., Adams, P. D., Clore, G. M., Delano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M. J., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr. Sect. D 54, 905–921
29. Hogg, R. C., Hopping, G., Alewood, P. F., Adams, D. J., and Bertrand, D. (2003) J. Biol. Chem. 278, 26908–26914
30. Glorius, S., Pongs, O., and Schmalzing, G. A. (1995) Gene (Amst.) 160, 213–217
31. Besse, D., and Moroder, L. (1997) J. Pept. Chem. 3, 442–453
32. Drysdale, A., and Sheppard, R. C. (1977) Tetrahedron 33, 7391–7397
33. Kent, S. B. H. (1988) Annu. Rev. Biochem. 57, 957–988
34. Live, D. H., Agosta, W. C., and Cowburn, D. (1977) J. Org. Chem. 42, 3556–3561
35. Sikawa, T., Esaki, N., Tanaka, H., and Soda, K. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3057–3059
36. Wüthrich, K. (1986) NMR of Proteins and Nucleic Acids, pp. 130–161, John Wiley & Sons, Inc., New York
37. Dröge, W. (2002) Exp. Gerontol. 37, 1331–1343
38. Singh, R. R., and Chang, J.-Y. (2003) Biochem. Biophys. Acta 1651, 85–92
39. Quaderer, R., Sewing, A., and Hilvert, D. (2001) Helv. Chim. Acta 84, 1197–1206
40. Lipinski, B., and Egyud, L. G. (1992) Bioorg. Med. Chem. Lett. 2, 919–924
41. Holmgren, A. (1989) J. Biol. Chem. 264, 13963–13966
42. Sieber, P., Kamber, R., Rüker, R., and Rittel, W. (1980) Helv. Chim. Acta 63, 2358–2362
43. Otaka, A., Koido, T., Shide, A., and Fujiu, N. (1991) Tetrahedron Lett. 32, 1223–1226
44. Pegoraro, S., Fiori, S., Cramer, J., Rudolph-Böhner, S., and Moroder, L. (1999) Protein Sci. 8, 1605–1613
45. Allen, F. H. (2002) Acta Crystallogr. Sect. B 58, 380–388
46. Wishart, D. S., Biggam, C. G., Holm, A., Hodges, R. S., and Sykes, B. D. (1995) J. Biomol. NMR 5, 67–81