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Modulating Oxytocin Activity and Plasma Stability by Disulfide Bond Engineering

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Disulfide bond engineering is an important approach to improve the metabolic half-life of cysteine-containing peptides. Eleven analogues of oxytocin were synthesized including disulfide bond replacements by thioether, selenylsulfide, diselenide, and ditelluride bridges, and their stabilities in human plasma and activity at the human oxytocin receptor were assessed. The cystathionine (K_i = 1.5 nM, and EC_{50} = 32 nM), selenylsulfide (K_i = 0.29/0.72 nM, and EC_{50} = 2.6/154 nM), diselenide (K_i = 11.8 nM, and EC_{50} = 18 nM), and ditelluride analogues (K_i = 7.6 nM, and EC_{50} = 27.3 nM) retained considerable affinity and functional potency as compared to oxytocin (K_i = 0.79 nM, and EC_{50} = 15 nM), while shortening the disulfide bridge abolished binding and functional activity. The mimetics showed a 1.5−3-fold enhancement of plasma stability as compared to oxytocin ($t_{1/2}$ = 12 h). By contrast, the all-D-oxytocin and head to tail cyclic oxytocin analogues, while significantly more stable with half-lives greater than 48 h, had little or no detectable binding or functional activity.

Introduction

Peptides have emerged as a commercially relevant class of drugs that offer the advantage of greater specificity and potency and lower toxicity profiles over traditional small molecule pharmaceuticals.[1] They offer promising treatment options for numerous diseases, such as diabetes, HIV, hepatitis, cancer, and others, with physicians and patients becoming more accepting of peptide-based medicines.[2] On the other hand, peptides also have several well-known drawbacks that have prevented them from becoming a mainstream source of drug candidates, including short circulation half-life, poor proteolytic stability, and low oral bioavailability.[3,4] A series of formulation and conjugation strategies have been developed to address delivery disadvantages,[3,4] while the use of unnatural and D-amino acids, cyclization, terminal capping, truncation, and disulfide bond engineering can improve stability to proteolytic degradation.[5] Peptides are metabolized by three major mechanisms: Endopeptidases break peptide bonds of non-terminal amino acids, exopeptidases cleave N- or C-terminal amino acids, and thiol−protein−disulfide oxido-reductases inactivate the peptides due to loss of secondary structure.[6] Modification of the proteolytic recognition sites within the peptide is the underlying principle of most strategies aimed at improving proteolytic stability. However, steric distortions employed to increase stability often affect the biological activity and selectivity.[7−10] Presently, no general approach has been identified that works for all target peptides, and understanding their structure−activity relationships (SAR)[a] is fundamental for identifying the appropriate strategy for each class of peptide. While cyclization, truncation, and terminal capping are widely applied techniques in the pharmaceutical industry for stabilizing short linear peptides as well as disulfide-rich peptides, this approach is only applicable to target peptides where the N and C termini are not part of the pharmacophore. Replacement of selected residues by D-amino acids has been another successful approach to enhance the half-lives of peptide drugs,[11] exemplified with octreotride, a somatostatin analogue used in the treatment of gastrointestinal tumors,[12] and with desmopressin (1-desamino-8-D-Arg-vasopressin) for the treatment of diabetes insipidus and bedwetting.[13−16] Disulfide bond engineering represents another complementary approach that protects peptides from reduction or scrambling by thiol-containing molecules such as glutathione, serum albumin, or oxido-reductases. Various disulfide bond replacements have been investigated, including lactam,[17,18] dicarba,[19−21] thioether,[22−26] and diselenide bridges,[27−35] but there exists no direct comparison of the efficacy of these modifications within the same peptide, making it difficult to draw conclusions on their relative merits. In this work, we evaluate various conservative disulfide bond replacements on the single-disulfide bond peptide oxytocin (OT) and assess the effects of these disulfide bond mimetics on peptide stability and biological activity.

OT and vasopressin (AVP) are closely related, highly conserved, multifunctional nonapeptides with N-terminal cyclic six-residue ring structures stabilized by an intramolecular disulfide bond and a flexible 3-residue C-terminal tail.[36]

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[a] Abbreviations: *, C-terminal amide; cAMP, cyclic adenosine monophosphate; AVP, vasopressin, arginine-vasopressin, antidiuretic hormone; Boc, *tert*-butyloxycarbonyl; CNS, central nervous system; DIEA, diisopropylethylamine; DTT, dithiothreitol; ESI-MS, electrospray ionization−mass spectrometry; Fmoc, fluorenylmethyloxycarbonyl; GPCR, G protein-coupled receptor; GSH, reduced glutathionine; GTP, guanosine triphosphate; HATU, 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate; HOAt, 1-hydroxy-7-azabenzotriazole; HTRF, homogeneous time-resolved fluorescence; IP, inositol phosphate; LC-MS, liquid chromatography−mass spectrometry; MBHA, 4-methylbenzhydrylamine; OT, oxytocin; OTR, oxytocin receptor; PyAOP, 7-azabenzotriazol-1-yloxy-tri(pyrrolidino)phosphonium hexafluorophosphate; SAR, structure−activity relationship; Sec, U, selenocysteine; SPPS, solid-phase peptide synthesis; Tec, tellurocysteine.

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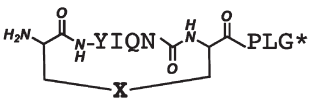
Table 1. Summary of Synthesized OT Analogues[a]

#	Synthesized Peptide	Sequence	
	Native Peptides		
1	OT	CYIQNCPLG*	
2	AVP	CY**F**QNCP**R**G*	
	Disulfide Bond Mimetics		
3	[--S]-OT iso 1, Lanthionine		**X** = S
4	[--S] OT iso 2, Lanthionine		**X** = S
5	[CH_2-S]-OT, Cystathionine		**X** = CH_2-S
6	[Se-S]-OT	**U**YIQNCPLG*	
7	[S-Se]-OT	CYIQN**U**PLG*	
8	[Se-Se]-OT	**U**YIQN**U**PLG*	
9	[Te-Te]-OT	**X**YIQN**X**PLG*	**X** = Tellurocysteine, Tec
	Cyclic Analogues		
10	N-C cyclic OT	**cyclo[**CYIQNCPLG**]**	
11	N-C cyclic OT-AGAGAG	**cyclo[**CYIQNCPLG**AGAGAG]**	
	all-D-Analogues		
12	all-D-retroinverse-OT	**glpcnqiyc***	
13	all-D-OT	**cyiqncplg***	

[a] U, selenocysteine.

OT and AVP mediate their biological function by acting on four known receptors: the OT (uterine), the vasopressin V_{1a} (vasopressor), the V_{1b} (pituitary), and the V_2 (renal) receptors, which are all members of the G protein-coupled receptor (GPCR) family.[36] Activation of the OT, V_{1a}, and V_{1b} receptors leads to a measurable intracellular increase of inositol phosphate one (IP-one), while V_2R activation results in a measurable intracellular increase of cyclic adenosine monophosphate (cAMP).[36] OT in the periphery is involved in uterine smooth muscle contraction during parturition, ejaculation, and milk ejection during lactation.[36,37] In the central nervous system (CNS), OT functions as a neurotransmitter involved in complex social behaviors, maternal care, partnership bonding, stress, and anxiety.[36−38] Recently, the OT receptor has been shown to play a role in breast cancer tumor growth[39,40] and autism,[36,41] where metabolically more stable OT analogues would be of advantage. Thus, OT was chosen as a starting point in this study with the additional advantage to be the most studied peptide in the literature with extensive structural, biological, and therapeutic data available for comparison. Importantly, the biological characterization of activity (cell-based assays, animal models, and clinical studies) is well established, and synthetic access is readily achieved via solid-phase peptide synthesis (SPPS). Its single disulfide bond with its conformational flexibility is critical for secondary structure and agonistic activity.[21,42] NMR and crystallography studies of unbound OT and desamino-OT indicate a flexible structure, which crystallizes into two well-defined conformers.[42] Recognition motifs such as the type II β-turn between the backbone −NH of Tyr2′ and the carbonyl Asn5′ and the type III β-turn between the Cys6′ carbonyl and the Gly9′ imino group are present when free but are not observed when OT is bound to its carrier protein neurophysin,[43] suggesting that binding occurs stepwise via a receptor-induced fit. Additionally, NMR studies show high conformational mobility around Tyr2′ and of the carboxylic tripeptide tail, with the disulfide bridge conformation tied to their orientation and consequently their agonist activity.[44,45] Even minor distortions such as an increase of the 20-membered ring by one methylene group,[46] reducing the disulfide bond flexibility by substitution of the cysteine at position 1 by penicillamine[45] or replacement of the disulfide bond by an amide[18] or dicarba bond,[21,47,48] lead to a significant decrease in biological activity together with either a switch from an agonist to antagonist or loss of receptor selectivity. Hence, OT can be considered as a highly sensitive model for structural modification studies, where even small changes in bond length or dihedral angles may result in biological activity changes, and thus provides an ideal platform for the SAR study of disulfide bond mimetics.

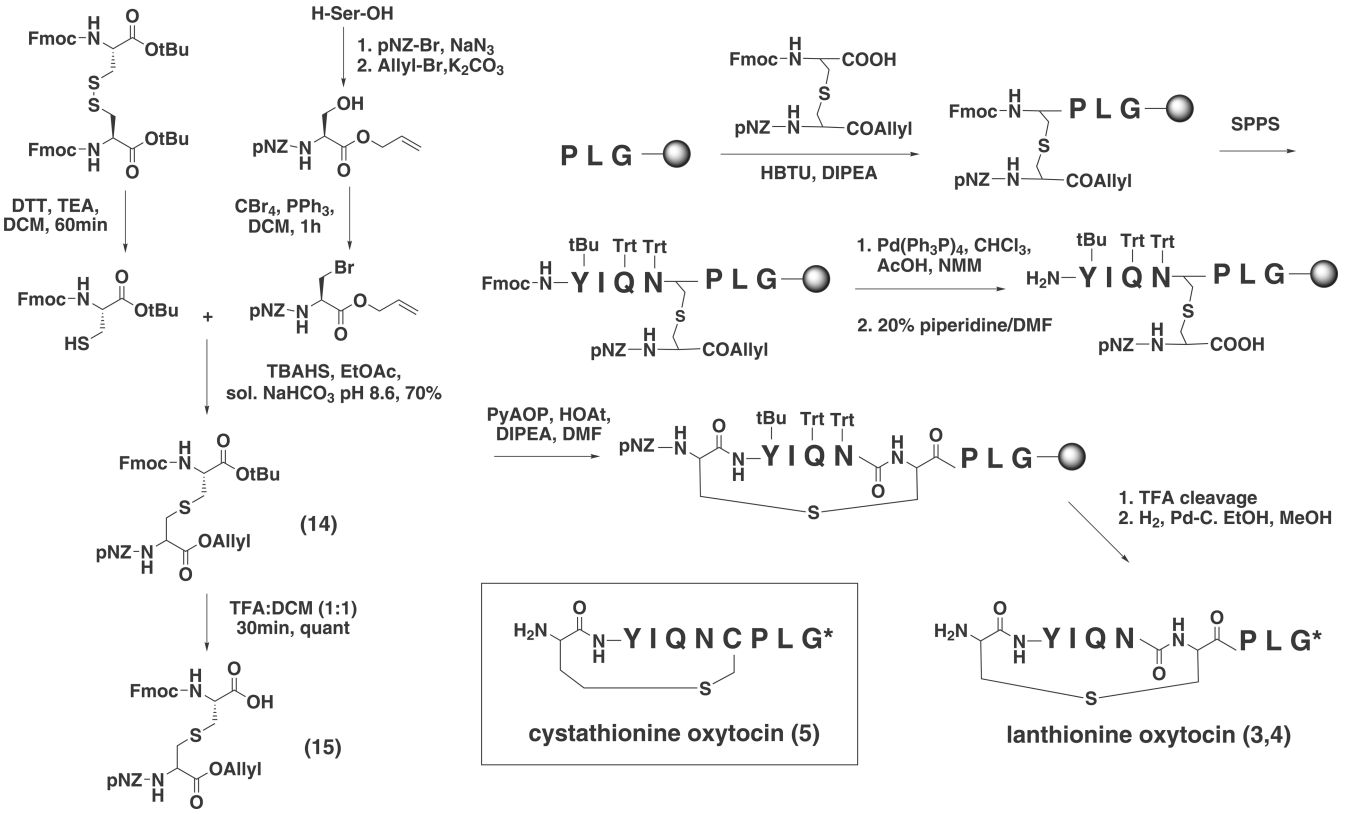
This study investigated 11 OT analogues (Table 1) including nine that contain thioether, selenylsulfide, diselenide, and ditelluride bonds to evaluate the impact of disulfide bond mimetics on biological activity at the human OT receptor and on their metabolic stability in human plasma. In addition, backbone cyclized and all-D-amino acid OT analogues were synthesized to place the disulfide engineering strategy in context with other commonly used strategies to enhance the half-life of peptides.

Results

The native neuropeptides OT and AVP (**1** and **2**) as well as the all-D-analogues (**12** and **13**) were synthesized via *tert*-butyloxycarbonyl (Boc) chemistry SPPS using the 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate (HBTU)-mediated in situ neutralization protocol for chain assembly.[49] The seleno analogues (**6**−**8**) were synthesized as described by Muttenthaler et al.,[27,50] and the N to C cyclic analogues (**10** and **11**) followed the procedure described by Clark et al.[9] Synthesis of the thioether analogues proved to be more challenging, and a novel on-resin strategy was developed to produce the thioether analogues (**3**−**5**). The synthesis of the ditelluride analogue (**9**) involved novel chemistry and will be reported in detail elsewhere. Metabolic stability was determined by human plasma degradation studies, and the biological activity was investigated via radioligand binding and IP-one functional studies on the human OT receptor.

Synthesis of OT Thioether Analogues (3−5). Two thioether OT analogues, cystathionine (**5**) and lanthionine (**3** and **4**) OT, have been prepared as outlined in Scheme 1. The cystathionine analogue (**5**) was synthesized following the procedure described by Mayer et al.,[51] where thioether cross-linking was achieved by on-resin cyclization/alkylation of an internal cysteine thiol with a N-terminal bromo-homoalanine residue.

Scheme 1. Lanthionine Building Block Synthesis Followed by Assembly and Formation of Lanthionine OT (**3** and **4**)[a]



[a] The structure of cystathionine OT (**5**) is also shown.

In a different approach, the thioether linkage of lanthionine OT was constructed prior to resin assembly. For this purpose, the orthogonally protected lanthionine building block **15** was synthesized and later incorporated into the solid-phase synthesis. The lanthionine building block **15** was generated following the methodology reported by Zhu and Schmidt,[52] where the thioether bond was formed by reaction of protected β-bromo-L-alanine with cysteine derivatives under phase-transfer conditions at pH 8.5 (Scheme 1). Their proposed route was somewhat modified here to suit the installation of the more appropriate orthogonal protecting groups: fluorenylmethyloxycarbonyl (Fmoc), *t*Bu, *p*NZ, and allyl. Although this method has been reported to give enantiopure lanthionine compounds,[52] the thioalkylation step in our case afforded a mixture of two diastereoisomers in a ratio of 2:3 as indicated by NMR spectroscopy. Racemization at the α-carbon position of the *p*NZ-β-Br-O*t*Bu precursor is believed to have occurred as a result of an elimination−Michael addition process. The isomers could not be separated by flash chromatography; therefore, the lanthionine building block was further employed as an isomeric mixture.

The synthesis of lanthionine OT was carried out by standard Fmoc/*t*Bu-based SPPS, using HBTU/DIEA activation protocols (Scheme 1). The linear peptide was assembled manually on a Rink amide 4-methylbenzhydrylamine (MBHA) resin. The building block **15** was incorporated at the fourth residue followed by linear extension of the peptide chain. Removal of the allyl protecting group with Pd(0) and Fmoc group with piperidine was followed by intramolecular amide bond cyclization assisted by the 7-azabenzotriazol-1-yloxy-tri-(pyrrolidino)phosphonium hexafluorophosphate (PyAOP)/1-hydroxy-7-azabenzotriazole (HOAt)/DIEA coupling cocktail.[53] Attempts to remove the *N*-terminal *p*NZ protecting group while on resin using the procedure reported by Albericio[54] were unsuccessful. Deprotection was achieved as follows: The cyclic peptide was first cleaved from the resin in the *p*NZ-protected form where two products having the same expected molecular weight for the lanthionine OT were obtained. The crude material was then submitted to hydrogenolysis to afford two isomers (isomers **3** and **4**) of the fully unprotected lanthionine OT that could be separated by RP-HPLC. As the two lanthionine OT isomers showed considerable reduction in activity (Table 2), we were discouraged to further investigate their stereochemical properties in detail.

Biological Activity. The peptides were tested for activity at the human OT receptor (Table 2 and Figure 1). Binding data were obtained via radioligand binding assays, which measured the displacement of ^{3}H-OT from the oxytocin receptor (OTR) expressed in COS-1 cells. The ability of the analogues to signal through G_q and generate IP-one in COS1-cells transfected with the OTR was investigated using an HTRF (homogeneous time-resolved fluorescence) assay.[55] Hill slope analysis was done using the sigmoidal curves (variable slope) fitted to individual data points by nonlinear regression using the software package Prism (GraphPad Software) showing that the Hill slopes were not statistically significantly different from −1.

Thioether Analogues (3−5). Both lanthionine [--S]-OT isomers showed a 1000-fold drop in binding affinity (K_i = 856 nM, 1547 nM) at the OTR as compared to OT (K_i = 0.79 nM). The lanthionine isomers also failed to stimulate IP-one signaling at concentrations up to 10 μM. Cystathionine OT,

Table 2. Potencies of OT Analogues at the OTR Tested in Radioligand Binding Assay and Functional IP-One Assay and Metabolic Half-Lives in Human Serum[a]

		human OT receptor		human serum
no.	analogues	binding K_i (nM)	functional EC_{50} (nM)	half-life (h)
1	OT	0.79 ± 0.16	15 ± 2.5	11.6 ± 1.9
2	vasopressin	12 ± 2.8	41 ± 3.6	1.7 ± 0.6
3	[--S]-OT iso 1	1547 ± 457	$>10^5$	ND
4	[--S]-OT iso 2	856 ± 201	$>10^5$	ND
5	[CH_2-S]-OT	1.5 ± 0.29	32.0 ± 9.4	36.9 ± 2.8
6	[Se-S]-OT	0.29 ± 0.02	2.6 ± 1.0	18.6 ± 3.0
7	[S-Se]-OT	0.72 ± 0.31	154 ± 86	ND
8	[Se-Se]-OT	11.8 ± 4.1	18.0 ± 12	25.3 ± 5.9
9	[Te-Te]-OT	7.6 ± 2.7	27.3 ± 7.4	24.2 ± 2.2
10	N-C cyclic-OT	1404 ± 243	$>10^5$	>48
11	N-C cyclic-OT-AGAGAG	$>10^5$	$>10^5$	>48
12	all-D-retroinverse-OT	$>10^5$	$>10^5$	5.5 ± 1.3
13	all-D-OT	$>10^5$	$>10^5$	>48

[a] ND, not determined; $n = 3-6$.

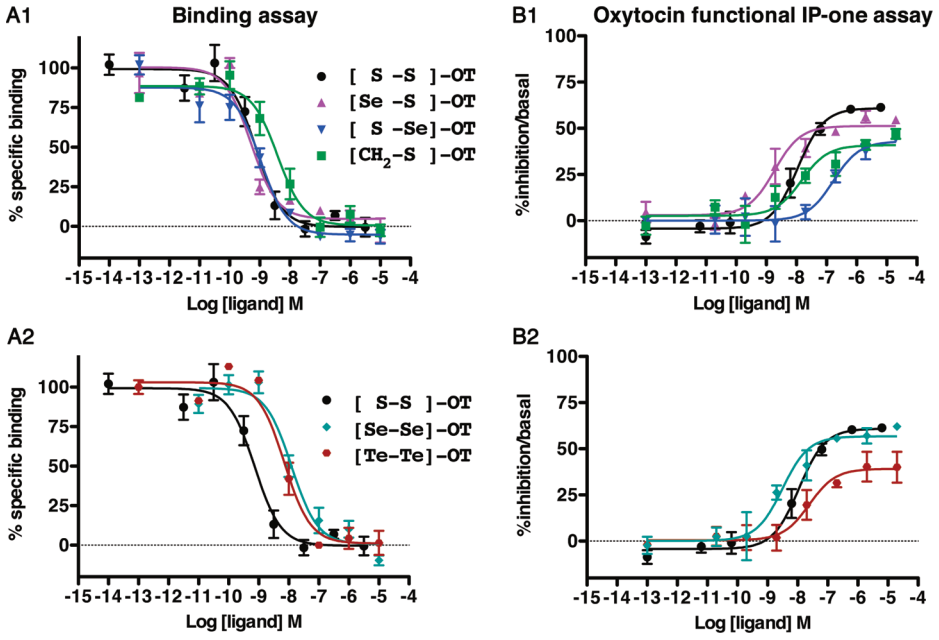


Figure 1. Binding and functional data of the disulfide bond mimetics of OT on the human OT receptor. Affinity data (**A1** and **A2**) were obtained via radioligand binding assay, which measured the displacement of ^{3}H-OT by the analogues from the human OT receptor expressed in COS-1 cells. The ability of the analogues to signal through G_q and generate IP-one in COS-1 cells transfected with the human OT receptor was investigated using a homogeneous time-resolved fluorescence assay (**B1** and **B2**). The exact K_i and EC_{50} values of each analogue are listed in Table 2. Results are the means ± SEs of three separate experiments, each performed in triplicate.

[CH_2-S]-OT, retained binding affinity (K_i = 1.5 nM) and the ability to signal (EC_{50} = 32 nM) at the OTR.

Selenium Analogues (6−8). [Se-S]-OT and [S-Se]-OT retained both their binding affinity and [Se-S]-OT also its functional efficacy, while a 10-fold decrease was observed with [S-Se]-OT (EC_{50} = 154 nM) as compared to OT (EC_{50} = 15 nM). [Se-Se]-OT showed a 10-fold decrease in binding affinity (K_i = 11.8 nM) as compared to OT (K_i = 0.79 nM); however, its functional efficacy was retained (EC_{50} = 18 nM).

Tellurium Analogue (9). The binding affinity of [Te-Te]-OT decreased 10-fold (K_i = 7.6 nM) when compared to OT, although functional activity was maintained (EC_{50} = 27.3 nM).

N- to C-Terminal Cyclic and D-OT Analogues (10−13). The bicyclic and the all-D OT analogues all showed drastic decreases in binding affinities (>2000-fold) and also failed to stimulate signaling at concentrations up to 10 μM on the human OTR.

Stability in Human Plasma. The stability of peptides **1**, **2**, **5**, **6**, and **8**−**13** in human plasma was assessed by LC-MS and RP-HPLC analysis (Figure 2 and Table 2). AVP showed a short half-life of 2 h as compared to OT with 12 h and was not detectable by analytical RP-HPLC or LC-MS after 4 h. As the positive controls, OT and AVP were incubated in phosphate buffer at pH 7.4 and 37 °C, and no degradation was observed over a period of 48 h. The selenium (**6** and **8**), tellurium (**9**), and thioether (**5**) analogues exhibited an 1.5−3-fold increase in stability in relation to OT, although not as distinct as the two bicyclic analogues (**10** and **11**) with half-lives greater than 48 h. While all-D-OT was the most stable analogue (only 20% degradation after 48 h), the all-D-retroinverse OT surprisingly exhibited a half-life of only 4 h and was less stable than L-OT. No traces of linear reduced analogues were observed throughout the study.

Reduction and Alkylation Study. Peptides **1**, **5**, and **8** were exposed to a variety of reduction and alkylation conditions

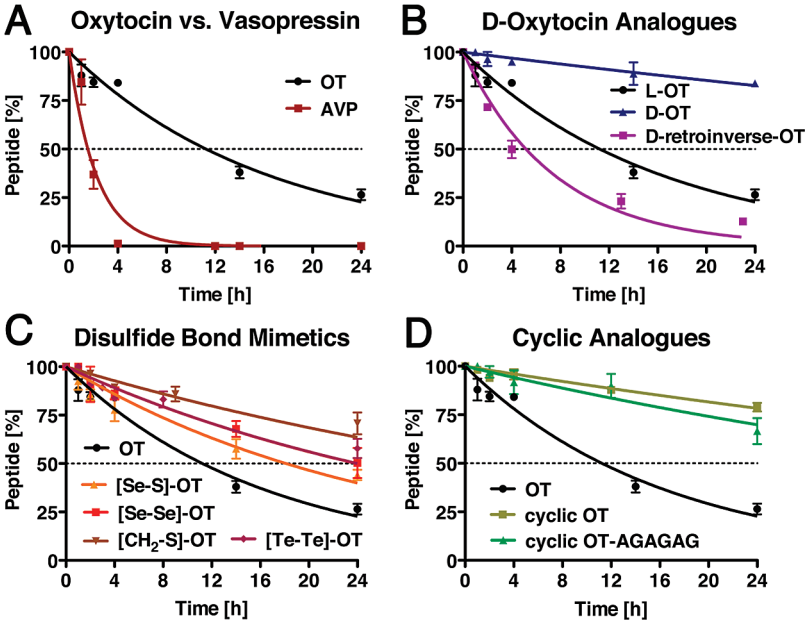


Figure 2. Metabolic stability of OT analogues in human serum. OT vs AVP (**A**), OT vs D-analogues all-D-OT and all-D-retroinverse-OT (**B**), OT vs selenium analogues [Se-S]-OT and [Se-Se]-OT (**C**), and OT vs cyclic analogues cyclic-OT and cyclic-OT-AGAGAG (**D**).

to compare the stability of the disulfide, diselenide, and thioether bond in reducing environments (Figure 3). The thioether bond (Figure 3, **A3**−**D3**) was stable to all tested reduction and alkylation conditions, including 100-fold excess dithiothreitol (DTT) and 1000-fold excess glutathione. The diselenide bond (Figure 3, **A2**−**D2**) was completely reduced and alkylated by a 10-fold excess of DTT, followed by the addition of excess of iodoacetamide at pH 7.4 and 37 °C. The reduced species (selenol) could not be detected by analytical means of MS, LC-MS, nor HPLC, indicating that reduction/reoxidation occurs rapidly. The disulfide bond (Figure 3, **A1**−**D1**) was readily reduced and alkylated by a 10-fold excess of DTT or a 1000-fold excess of reduced glutathione (GSH) in the presence of iodoacetamide. Reduction with 10-fold DTT or 1000-fold GSH of [Te-Te]-OT resulted in a complex mixture of GSH adducts and products with tellurium deletions (data not shown).

Discussion and Conclusion

Eleven OT analogues were synthesized and characterized on the human OT receptor and in human serum to determine the impact of disulfide bond engineering on biological activity and stability (Table 1). Modifications to OT included replacement of the disulfide bond by thioether, selenylsulfide, diselenide, and ditelluride bridges, as well as bicyclic and all-D-amino acid analogues selected as controls for metabolic stability assessment. Given that OT activity appears highly sensitive to structural changes (Table 2), it permitted a close evaluation of isosteric replacements of either one or both sulfur atoms in the bridge with closely related elements such as carbon, selenium, and tellurium (Table 3). Changes in the bond lengths of the disulfide bridge replacements ranged from approximately 0.77 (lanthionine analogues) to 2.68 Å (ditelluride analogues), and changes in torsion angles [from 180° (thioether bridge) to 89−98° (disulfide, diselenide, and ditelluride bridges)] gave insight into the influence of bond length and torsion angles on conformational flexibility and activity.

The biological analysis of the disulfide bond mimetics on the human OT receptor confirmed the hypothesis that thioether, selenylsulfide, diselenide, and ditelluride substitutions can be considered conservative disulfide bond replacements as full binding and functional activity (±10-fold) was retained in these analogues (Table 2 and Figures 1, 4, and 5). The affinity and functional activity of OT at the human OT receptor correlate well with literature values, displaying binding affinity ∼10-fold higher than agonist potency.[55−58] Surprisingly, [Se-S]-OT enhanced both binding affinity and potency 3−6-fold, while [S-Se]-OT had no affect on binding affinity but produced a 10-fold reduction in agonist activity as compared to OT (Table 2). Even the ditelluride analogue with the largest changes in bond length (+0.65 Å) retained full binding affinity and functional efficiency. Reduction in ring size by deletion of one sulfur in the bridge as in case of the lanthionine analogues (**3** and **4**) resulted in a drastic loss of binding and functional activity. The cystathionine on the other hand maintained full binding and functional activity. N- to C-terminal cyclization of OT (**10**) led to a complete loss of activity, which has also been observed in earlier work.[59] Incorporation of a six-residue linker (AGAGAG, **11**) to induce more flexibility also resulted in an inactive analogue.

The observed 10−100-fold shift between binding affinity and agonist potency of OT and analogues has been observed previously for other agonists of GPCRs including vasopressin analogues acting at the V_{1b} receptor, where small changes in ligand structure resulted in a 30-fold shift between binding affinity and potency to stimulate IP.[60] The affinity/potency difference observed in these studies could arise from cross-talk between G-proteins, receptors, and ligand that influences the conformation of the receptor in a well-described guanosine triphosphate (GTP)-dependent manner.[61−64] Hill slope analysis showed no significant evidence for two binding sites or cooperative binding effects.

Overall, it is interesting to observe that changes in bond length of the disulfide bridge by −0.21 Å up to +0.65 Å can be achieved without any major losses in activity, even in such sensitive systems as OT, where β-turns need to be preserved for receptor recognition.[44,65] The observed changes due to torsion angles or individual positions (Se in position 1

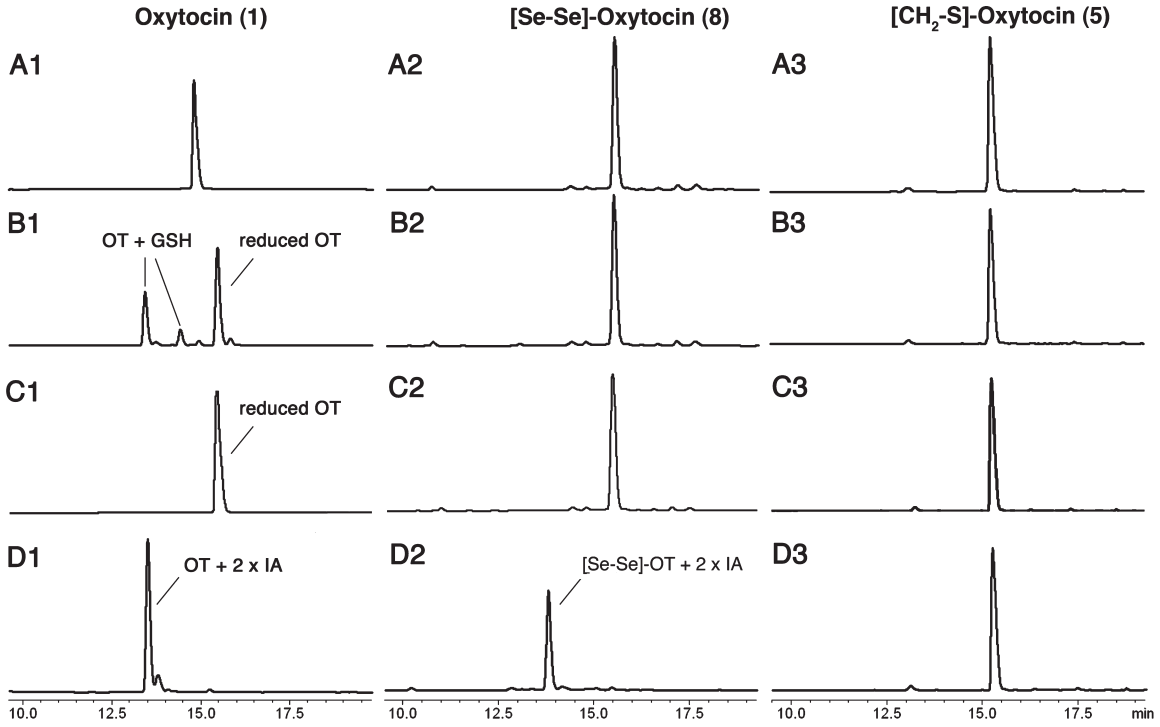


Figure 3. Reduction and alkylation study of OT (**1**), diselenide OT (**8**), and cystathionine OT (**5**). This study illustrates that the thioether bond (**A3**−**D3**) is stable to all tested reduction and alkylation conditions. The diselenide bond (**A2**−**D2**) seems to be stable to reduction up to 1000-fold GSH and 100-fold DTT according to MS/LCMS/HPLC analysis, but by addition of alkylating agent, the diselenide bond is reduced and alkylated, indicating that reduction/reoxidation occurs rapidly and cannot be detected by MS/LCMS/HPLC. The disulfide bond is readily reduced by 10-fold DTT or 100-fold GSH. Conditions A: Compounds **1**, **5**, and **8** in PBS buffer, pH 7.4, room temperature at time = 0 min. Conditions B: 1000-fold glutathione at 37 °C, pH 7.4, after 12 h. Conditions C: 100-fold DTT at 37 °C, pH 7.4, 1 h. Conditions D: 10-fold DTT at 37 °C, pH 7.4, 1 h, followed by addition of 100-fold iodoacetamide (IA) and incubated for 1 h at 37 °C, pH 7.4.

Table 3. Physicochemical Properties of Carbon, Sulfur, Selenium, and Tellurium[27,34,80,81a]

properties	X = carbon	X = sulfur	X = selenium	X = tellurium
covalent radius (Å)	0.77	1.02	1.17	1.35
bond length $C^{\beta}-X^{\gamma}$ (Å)	1.54	1.82	1.95−1.99	2.40
bond length $X^{\gamma}-X^{\delta}$ (Å)	1.54	2.03	2.33	2.68
van der Waals radii (Å)	1.70	1.80	1.90	2.06
torsion angle $\chi^3-C^{\beta}-C^{\gamma}-X^{\delta}-C^{\varepsilon}-$ (°)	NA	180	174	180
torsion angle $\chi^3-C^{\beta}-X^{\gamma}-X^{\delta}-C^{\varepsilon}-$ (°)	NA	98	94	89

[a] NA, not available.

vs position 6) further confirm the enormous sensitivity of the OT ligand−receptor system, making it an ideal system for SAR studies.

Previous to this study, the two-disulfide bond containing α-conotoxins represented the most-studied system for disulfide bond mimetics, although the disulfide bond replacements had been conducted on different α-conotoxins, thus making interpretation and comparison of their impact difficult. Disulfide bonds stabilize and orientate the secondary loop structure (α-helix) in the α-conotoxins, which contain the residues important for receptor interaction.[66] Nevertheless, replacement of one of the disulfide bonds in α-conotoxin SI by a lactam bridge resulted in either complete or 60−70-fold loss of activity, depending on which disulfide bond was modified.[17] Substitution of a single disulfide bond in α-ImI with an unsaturated dicarba bridge resulted in both cis and trans isomers, with one retaining significantly reduced biological activity, while the other was inactive.[19] Thioether replacement of one disulfide bond in α-GI also resulted in 260−800-fold loss of activity of the two obtained isomers.[22] Similarly to our study, it appears that the most promising approach is the generation of selenotoxin mimetics. A comprehensive structural and functional study of various α-conotoxins containing selenocysteine replacements illustrated that such a modification had no significant impact on torsion angles, activity, or receptor subtype selectivity of this class of peptides.[27] X-ray analysis at 1.4 Å resolution of α-selenoconotoxin PnIA showed that the diselenide bond was 0.3 Å longer than the disulfide bond (2.03 Å) with torsion angles of 93.9 and 83.1°, respectively. Additionally, it was shown that the increased hydrophobicity and surface exposure of the diselenide bond had a beneficial effect on the activity in some of the analogues.

The second objective of this study was to compare disulfide bond mimetics with other commonly employed metabolic stability improvement strategies such as backbone cyclization and the use of D-amino acids. The driving concept behind disulfide replacement is that disulfide bond reduction and scrambling by thiol-oxido-reductases or similar free thiol-containing molecules generally leads to biological inactivation.[6] Thus, implementation of disulfide bond mimetics with redox-stable cross-links such as thioether or dicarba bridges should yield metabolically more stable analogues.[67−69] This was exemplified

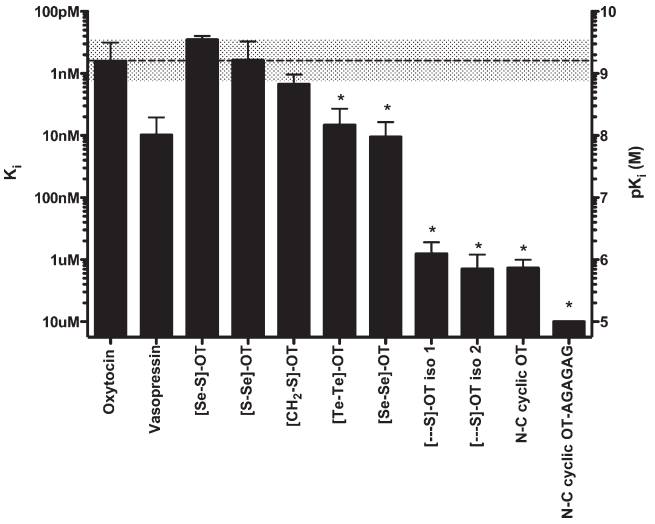


Figure 4. Binding data of OT analogues at the human OTR expressed in COS-1 cells. Binding affinities were measured as the displacement of H^3−OT radioligand by OT analogues. * indicates statistically significant changes based on OT ($p < 0.05$). Results are the means ± SEs of 3−6 experiments. Each experiment was performed in triplicate.

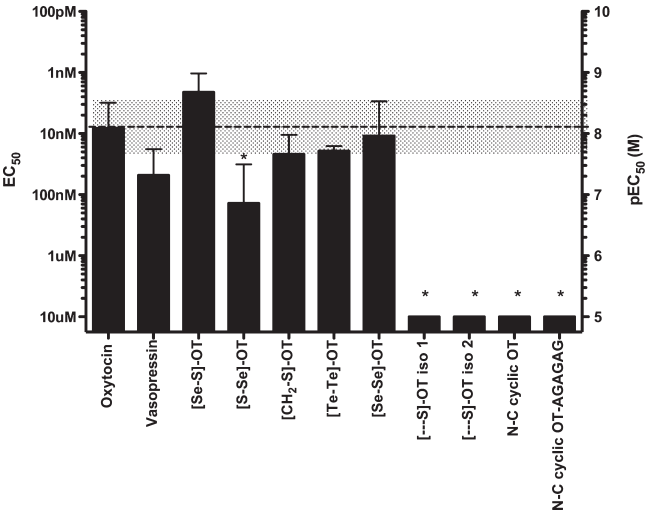


Figure 5. Functional data of OT analogues at the human OTR expressed in COS-1 cells. Functional data were measured as the accumulation of IP-one in the cell after stimulation by OT analogues. * indicates statistically significant changes based on OT ($p < 0.05$). Results are the means ± SEs of 3−6 experiments. Each experiment was performed in triplicate.

in carbetocin, clinically used to treat postpartum hemorrhage, where a thioether bridge results in a longer acting agonist than its disulfide bond counterpart.[25,26] A similar strategy replaces sulfur by selenium, as diselenides also exhibit a lower redox potential than disulfides with a difference of 111−166 mV.[30,70] There exists only limited data on ditelluride compounds regarding redox potentials, in particular within peptides; however, the ditelluride bridge is expected to have a lower redox potential than the diselenide bond. While there are currently no in vivo studies that assess a diselenide bond impact on half-life or longer lasting effects, recent studies on α-conotoxins showed that a single diselenide bond substitution in this two-disulfide bond system efficiently suppressed drug inactivation via scrambling. However, no significant half-life improvement was observed in plasma degradation studies, mainly due to the fact that plasma degradation occurs largely by endo- and exopeptidases rather than by oxido-reductases.[27,31] Moreover, a question remains as to whether the increased stability of the diselenide is due to its lower redox potential or to its rapid reoxidation kinetics. To answer this question, we conducted a comprehensive reduction and alkylation study together with stability studies in human serum.

Initially, the influence of the bridge redox potential on plasma half-life was investigated and was compared to other commonly used stabilization strategies such as backbone cyclization and the use of D-amino acids. Well-established plasma stability assays were employed, which are frequently used in peptide drug optimization processes:[71,72] Metabolic stability improvements were observed that correlate with the redox potential of the disulfide bond substitution, yielding half-lives of 12 h for OT, 19 h for [Se-S]-OT, 24 h for [Te-Te]-OT, 25 h for [Se-Se]-OT, and 37 h for [CH_2-S]-OT (Figure 2C and Table 2). This 1.5−3-fold enhancement in half-life, however, is quite moderate as compared to other strategies employed; for example, the bicyclic analogues (**10** and **11**) led to an increase in half-life (> 48 h), and all-D-OT (**13**) resulted in the greatest stability increase of all tested compounds, showing only 20% degradation over 48 h. In general terms, the cyclization approach and the use of unnatural or D-amino acids can be considered as the more effective strategies to improve the proteolytic half-lives of peptides than the often more challenging disulfide bond modifications. This is particularly the case given that plasma degradation by endo- and exopeptidases occurs more readily than by oxido-reductases or other reducing molecules.[73] However, considering the complete loss of activity of the bicyclic analogues, head to tail cyclization will only have a selective use in OT agonist design but might be practical for the development of long-acting antagonists where free N and C termini are not necessarily important for function due to distinct SARs and topographical features regarding receptor binding.[45,74−77] Selective and strategic residue exchanges with D-amino acids in combination with disulfide bond engineering can be considered as an effective approach to improve the proteolytic stability of OT analogues significantly. It has to be noted that the circulation half-life of OT in vivo is only 1−5 min due to effective renal clearance; however, proteolytic stability is important for other biomedical applications including CNS-related disorders (Autism, anxiety, and stress)[36,41] or breast cancer tumor detection,[40] where the route of administration eludes renal clearance.

The plasma stability study also revealed that although OT and AVP only differ in two amino acids (isoleucine to phenylalanine change at position three and a leucine to arginine change at position eight); OT with a half-life of 12 h was found to be six times more stable than AVP with a half-life of 2 h (Figure 2A). It seems that the L8R substitution renders AVP susceptible to Pro-Arg-cleavage by trypsinlike serine proteases such as thrombin, factor Xa, and plasmin, which are abundant in blood and play an important role in blood coagulation.[73] Replacement of arginine in position 8 by its D-amino acid analogue to increase the metabolic stability was successfully employed within the drug Desmopressin (1-desamino-8-D-Arg-vasopressin), resulting in increased stability and longer lasting effects.[14−16] All-D-retroinverse-OT showed a surprisingly short half-life of only 5.5 h as compared to the 12 h of L-OT, suggesting that the secondary structure (two β-turns) has significant contribution on the peptide's overall stability. The reduction and alkylation study on compounds **1**, **5**, and **8** was conducted to evaluate the stability of the disulfide, thioether, and diselenide bonds in a reducing environment. The thioether

bridge was as expected stable to reduction by a 1000-fold excess of GSH and DTT, while the disulfide bond was readily reduced by a 100-fold excess of GSH or a 10-fold excess of DTT. The reduction of the diselenide bond, however, could only be measured indirectly by reduction in the presence of an alkylating agent. It was not possible to observe the reduced species directly by means of MS, LC-MS, or HPLC analysis due to the high reactivity of the selenolate, which reoxidizes rapidly to the diselenide bond. However, complete reduction and alkylation were observed within 1 h in the presence of a 10-fold excess of DTT and excess of iodoacetamide, demonstrating that the diselenide bond is readily reduced by a small excess of reducing agent, but is reoxidized immediately if no other molecules are present to trap the reduced species.

In conclusion, a set of conservative disulfide bond mimetics of OT, including thioether, selenylsulfide, diselenide, and ditelluride bridges, were analyzed for binding and functional activity on the human OT receptor and metabolic stability in human plasma. Single sulfur substitution by a methylene or selenium was well tolerated, along with the longer diselenide and ditelluride bond analogues, which also retained their biological activity. These analogues represent enhanced mimetics as compared to earlier studied lactam and dicarba analogues, where agonist activity was lost. In contrast, deletion of a sulfur atom from the bridge led to complete loss of activity, underscoring the importance of ring size for receptor activation. The disulfide bond mimetics showed a 1.5−3-fold improvement of metabolic plasma stability; yet, degradation in plasma occurs largely by endo- and exopeptidases,[73] and strategies such as backbone cyclization and the use of D-amino acids were shown to outperform disulfide bond engineering in plasma. This study establishes a robust platform for future OT/AVP agonist design and is fully compatible with other important classes of disulfide-rich peptides, such as neurotransmitters, growth factors, hormones, enzyme inhibitors, antimicrobial peptides, and venom toxins to create analogues with enhanced metabolic stability.

Experimental Section

Materials. N^{α}-Boc- and N^{α}-Fmoc-L-amino acids, Fmoc-Rink amide MBHA resin, and reagents used during chain assembly were peptide synthesis grade purchased from Novabiochem (Merck Pty., Kilsyth, VIC, Australia) and Bachem (Bubendorf, Switzerland). L-Selenocystine and Boc-L-Sec(MeBzl)-OH were synthesized as described by Muttenthaler et al.[27] MBHA resin was purchased from the Peptide Institute (Osaka, Japan). HBTU and PyAOP were purchased from Fluka (Buchs, Switzerland), 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate (HATU) was purchased from GenScript Corp. (Piscataway, NJ), and diisopropylethylamine (DIEA), trifluoroacetic acid (TFA), dichloromethane (DCM), and *N,N′*-dimethylformamide (DMF) were purchased from Auspep (Melbourne, VIC, Australia). Anhydrous hydrogen fluoride (HF) was purchased from BOC Gases (Sydney, NSW, Australia), and *p*-cresol and *p*-thiocresol, human plasma, as well as all other organic reagents and solvents, unless stated otherwise, were purchased in their highest purity from Sigma-Aldrich (Sydney, NSW, Australia). All solvents for SPPS were of peptide synthesis grade and used without further purification. HPLC grade acetonitrile (Lab Scan, Bangkok, Thailand) and water measuring 18.2 MΩ (ELGA, Melbourne, VIC, Australia) were used for the preparation of all solvents for liquid chromatography.

OTR cDNA was obtained from Origene technologies inc (Rockville, United States). Dulbecco's modified Eagle's medium and Lipofectamine2000 were purchased from Invitrogen (Mulgrave, VIC, Australia). Complete protease inhibitor cocktail and FuGENE 6 Transfection Reagent were from Roche Diagnostics (Castle Hill, NSW, Australia). [Tyrosyl-2,6-^{3}H]-OT; 46.3 Ci/mmol, 2200 Ci/mmol, FlashBLUE GPCR scintillating beads, TopSeal-A 96-well sealing film, and 384-well white optiplates were from PerkinElmer Life Sciences (Knoxfield, VIC, Australia). Costar 96-well white plates with clear bottoms were obtained from Corning (Lindfield, NSW, Australia). The IP-one HTRF assay kit was from CisBio International (30204 Bagnols-sur-Cèze, France).

Peptide Synthesis. Peptides **1**, **2**, **6**−**8**, and **10**−**13** were assembled manually via Boc-SPPS using the HBTU-mediated in situ neutralization protocol[49] following the procedure of Muttenthaler et al.[27] for the monocyclic (**1**, **2**, **6**−**8**, **12**, and **13**) and the procedure of Clark et al.[9] for the bicyclic OT analogues (**10** and **11**). After HF cleavage, the crude peptides were purified by preparative RP-HPLC (Vydac C_{18} column, 300 Å, 250 mm × 21.2 mm) using a linear gradient of 0−50% B (A, H_2O/0.05% TFA; B, 90% CH_3CN/10% H_2O/0.043% TFA) in 50 min at 8 mL/min while monitoring UV absorbance at 226 nm. The selenium-containing peptides were already oxidized after HF cleavage.[27] Folding and cyclization of the disulfide bond containing analogues **1**, **2**, and **10**−**13** were carried out in 0.1 M NH_4HCO_3 buffer, pH 8.4, at 25 °C and a peptide concentration of 100 μM. Peptide oxidation, deprotection, and purity were monitored by mass spectrometry (MS) on a LCT-TOF mass spectrometer equipped with an electrospray ionization source, by analytical RP-HPLC using a Vydac C_{18} column (300 Å, 5 μm, 250 mm × 4.6 mm) at 214 nm, and by ESI-LCMS on a Phenomenex Jupiter liquid chromatography mass spectrometry (LC-MS) C_{18} column (90 Å, 4 μm, 250 mm × 2 mm) on a SCIEX QSTAR Pulsar QqTOF mass spectrometer equipped with an atmospheric pressure ionization source, running a linear gradient of 0−50% solvent B over 50 min with a flow rate of 1 mL/min for RP-HPLC and 200 μL/min for LC-MS analysis. The synthesized peptides and organic compounds were of >98% purity, unless stated otherwise.

The cystathionine analogue (**5**) was synthesized following the procedure described by Mayer et al.,[51] where thioether cross-linking was achieved by on-resin cyclization/alkylation of an internal cysteine thiol with a N-terminal bromo-homoalanine residue. The synthesis of the ditelluride analogue **9** will be reported elsewhere.

Synthesis of Lanthionine OT Isomers (3 and 4). N^{α}-(9-Fluorenylmethoxycarbonyl)-L-cysteine *tert*-Butyl Ester (Fmoc-Cys-OtBu). To a solution of (Fmoc-Cys-O*t*Bu)$_2$ (0.91 g, 1.14 mmol) in DCM (20 mL), 1,4-DTT (263 mg, 1.71 mmol) and triethylamine (0.239 mL, 1.71 mmol) were added. After it was stirred at room temperature for 1 h, the reaction mixture was concentrated and purified by flash chromatography (petroleum ether:EtOAc 4:1). The product was obtained as a colorless oil in quantitative yield (0.90 g, 99%). Analytical data for this compound are in accordance with literature.[78]

N^{α}-*para*-Nitrobenzyloxycarbonyl-L-serine Allyl Ester (pNZ-Ser-OAll). *p*-Nitrobenzyl chloride (6.15 g, 28.5 mmol) was dissolved in dioxane (12 mL), and a solution of NaN_3 (2.22 g, 34.2 mmol) in 9 mL of H_2O was added. The resulting emulsion was stirred for 2.5 h. Serine (3.0 g, 28.5 mmol) was dissolved in 35 mL of dioxane/2% Na_2CO_3 (1:1) and added dropwise. The resulting emulsion was stirred for 2 days keeping the pH between 9 and 10 by addition of 10% Na_2CO_3 solution. Water was added, and the suspension was washed with diethylether (3×). The aqueous phase was acidified to pH 2 by adding 3 N HCl, and the product was extracted with 3× EtOAc. The organic layers were combined, dried over $MgSO_4$, and concentrated. The crude product (6.25 g of *p*NZ-Ser-OH, 77% yield) was used without further purification. To a solution of *p*NZ-Ser-OH (3.0 g, 10.5 mmol) in DMF (20 mL), K_2CO_3 (1.85 g, 13.4 mmol) and allyl bromide (1.7 mL, 15.8 mmol) were added. The mixture was stirred overnight at room temperature. Water was added, and the product was extracted 3× with EtOAc. The combined organic layers were washed with water (2×), brine, dried over

$MgSO_4$, and concentrated. The desired product was recrystallized from EtOAc/petroleum ether to give light yellow crystals (2.3 g, 68% yield, > 98% purity by HPLC). MS (*m*/*z*): MS (calcd): 325.1036 [M + H]; MS (found): 325.1022 [M + H]. mp 81−83 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 2.34 (1H, s, OH), 4.00 (2H, m, βCH_2), 4.46 (1H, m, αCH), 4.67 (2H, d, J = 5.5 Hz, CH_2), 5.21 (2H, m, CH_2), 5.26 (1H, d, J = 10.4 Hz, CH=CH_2), 5.34 (1H, d, J = 17.2 Hz, CH=CH_2), 5.86−5.92 (2H, m, CH=CH_2 + NH), 7.50 (2H, d, J = 8.3 Hz, Ar), 8.20 (2H, d, J = 8.5 Hz, Ar). ^{13}C NMR (150 MHz, $CDCl_3$) δ (ppm): 56.1, 63.1, 65.6, 66.4, 119.1, 123.8, 128.1, 131.2, 143.5, 147.6, 155.7, 170.0.

N^{α}-*para*-Nitrobenzyloxycarbonyl-β-bromo-L-alanine Allyl Ester (pNZ-β-Br-Ala-OAll). *p*NZ-Ser-OAll (3.23 g, 9.96 mmol) and CBr_4 (5.29 g, 15.9 mmol) were dissolved in 50 mL of DCM, and the solution was cooled down to 0 °C. PPh_3 (5.22 g, 19.9 mmol) was added portion-wise, and the reaction mixture was stirred at 0 °C for 20 min. Diethyl ether was added, and the precipitate was removed by filtration. The resulting diethyl ether mother solution was washed with saturated solution of $NaHCO_3$ (2×) and brine, dried over $MgSO_4$, and concentrated. Purification of the product was performed by flash chromatography eluting with petroleum ether:EtOAc (6:1 to 2:1) to give 1.66 g (43% yield, > 98% purity by HPLC) of a white solid. MS (*m*/*z*): MS (calcd): 387.0191/389.0171 [M + H]; MS (found): 387.0171/389.0128 [M + H]. mp 70−72 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 3.81 (2H, m, βCH_2), 4.70 (2H, m, CH_2), 4.81 (1H, m, αCH), 5.23 (2H, m, CH_2), 5.29 (1H, d, J = 10.4 Hz, CH=CH_2), 5.36 (1H, d, J = 17.2 Hz, CH=CH_2), 5.77 (1H, d, J = 7.6 Hz, NH), 5.91 (1H, m, CH=CH_2), 7.52 (2H, d, J = 8.4 Hz, Ar), 8.21 (2H, d, J = 8.0 Hz, Ar). ^{13}C NMR (150 MHz, $CDCl_3$) δ (ppm): 33.4, 54.4, 65.6, 66.9, 119.5, 123.8, 128.1, 131.0, 143.4, 147.7, 155.1, 168.4.

Fully Protected Lanthionine Building Block (14). Fmoc-Cys-O*t*Bu (730 mg, 1.83 mmol) and *p*NZ-β-Br-Ala-OAll (710 mg, 1.83 mmol) were dissolved in EtOAc (20 mL), and 20 mL of a saturated solution of $NaHCO_3$ and 2.49 g of tetrabutylammonium hydrogensulfate (7.32 mmol) were added. The reaction mixture was allowed to stir overnight at room temperature. EtOAc was added, and the organic phase was washed with saturated solution of $NaHCO_3$ (3×) and brine, dried over $MgSO_4$, and concentrated. The product was purified by flash chromatography (petroleum ether:EtOAc, gradient of 5:1 to 1:1) to afford 900 mg of a colorless oil (70% yield, > 99% purity by HPLC). MS (calcd): 706.2434 [M + H]; MS (found): 706.2452 [M + 1], 650.2005 [M-*t*Bu]. ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 1.37 (9H, s, *t*Bu), 2.75−2.86 (2H, m, βCH_2), 2.90−3.00 (2H, m, βCH_2), 4.06−4.10 (1H, m, αCH), 4.21 (1H, t, J = 7.0 Hz, CH *Fmoc*), 4.26−4.32 (3H, m, αCH + CH_2 *Fmoc*), 4.57 (2H, d, J = 1.0 Hz, CH_2), 5.17 (1H, d, J = 12 Hz, CH=CH_2), 5.19 (2H, s, CH_2), 5.29 (1H, d, J = 17 Hz, CH=CH_2), 5.83−5.90 (1H, m, CH=CH_2), 7.30 (2H, t, J = 7.4 Hz, Ar *Fmoc*), 7.39 (2H, t, J = 7.4 Hz, Ar *Fmoc*), 7.59 (2H, d, J = 8.4 Hz, Ar *pNZ*), 7.69 (2H, d, J = 7.3 Hz, Ar *Fmoc*), 7.77/7.79 (1H, d, J = 8.4 Hz, NH), 7.87 (2H, d, J = 7.5 Hz, Ar *Fmoc*), 8.01/8.02 (1H, d, J = 8.2 Hz, NH), 8.21 (2H, d, J = 8.6 Hz, Ar *pNZ*). ^{13}C NMR (150 MHz, DMSO-d_6) δ (ppm): 28.0, 33.1/33.3, 33.4/33.5, 47.1, 54.4/54.6, 54.9/55.1, 64.9, 65.6, 66.2, 81.6, 118.2, 120.6, 123.9, 125.7, 127.5, 128.1, 128.5, 132.6, 141.2, 144.2, 145.3, 147.4, 156.2, 156.4, 170.3, 170.7.

Lanthionine Building Block (15). Fully protected lanthionine **14** (730 mg, 1.06 mmol) was dissolved in 10 mL of DCM and cooled to 0 °C. TFA (10 mL) was added, and the reaction mixture was stirred at 0 °C for 30 min and then allowed to react at room temperature for 1.5 h. TFA was removed by coevaporation with toluene, and the product was dissolved in 50% ACN and lyophilized. The product was obtained as a white amorphous solid (quantitative, > 98% purity by HPLC). MS (*m*/*z*): MS (calcd): 650.1808 [M + H]; MS (found): 650.1822 [M + H]. ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 2.77−2.87 (2H, m, βCH_2), 2.96−3.00 (2H, m, βCH_2), 4.12−4.17 (1H, m, αCH), 4.21 (1H, t, J = 7.0 Hz, CH *Fmoc*), 4.25−4.31 (3H, m, αCH + CH_2 *Fmoc*), 4.57 (2H, d, J = 4.7 Hz, CH_2), 5.17 (1H, d, J = 14 Hz, CH=CH_2), 5.18 (2H, s, CH_2), 5.28 (1H, d, J = 17 Hz, CH=CH_2), 5.83−5.89 (1H, m, CH=CH_2), 7.30 (2H, t, J = 7.4 Hz, Ar *Fmoc*), 7.39 (2H, t, J = 7.4 Hz, Ar *Fmoc*), 7.58 (2H, d, J = 8.4 Hz, Ar *pNZ*), 7.71 (2H, d, J = 7.3 Hz, Ar *Fmoc*), 7.74/7.77 (1H, d, J = 8.5 Hz, NH), 7.87 (2H, d, J = 7.5 Hz, Ar *Fmoc*), 8.00/8.02 (1H, d, J = 7.5 Hz, NH), 8.21 (2H, d, J = 8.5 Hz, Ar *pNZ*). ^{13}C NMR (150 MHz, DMSO-d_6) δ (ppm): 33.1/33.3, 33.4/33.6, 47.0, 54.3/54.4, 54.5/54.7, 64.9, 65.6, 66.2,118.2, 120.6, 124.0, 125.7, 127.5, 128.1, 128.5, 132.6, 141.1, 144.2, 145.3, 147.4, 156.2, 156.5, 170.7, 172.6.

Assembly of OT Isomers (3 and 4). The OT thioether was assembled by Fmoc/*t*Bu-based manual SPPS on a Rink amide MBHA resin (Novabiochem, loading 0.64 mmol/g) using HBTU/DIEA to activate the standard residues as well as the building block **15**, and treatment with 50% piperidine/DMF (2 × 1 min) for Fmoc deprotection. Couplings were carried out with 4 equiv of Fmoc-amino acid, 4 equiv of HBTU, and 8 equiv of DIEA in DMF for a minimum of 10 min and monitored by the Kaiser test.[79] After assembly of the Gly, Leu, and Pro residues, the lanthionine building block was coupled to the peptide chain by using 2 equiv of **15**, 2 equiv of HBTU, and 4 equiv of DIEA. Following Fmoc deprotection, residues Asn, Gln, Ile, and Tyr were coupled as usual. Prior to peptide cyclization, the allyl group of the lanthionine moiety was removed by treatment with 3 equiv of $Pd[PPh_3]_4$ in 6 mL of $CHCl_3$:AcOH:*N*-methyl morpholine (37:2:1) for 2.5 h under argon and subsequent washing of the resin with DMF (5×), 0.5% DIEA in DMF (2 × 1 min), 0.5% dithiocarbamate in DMF (3 × 1 min), and finally DMF (5×). Removal of the *N*-terminal Fmoc protecting group was followed by overnight cyclization with 5 equiv of PyAOP, 5 equiv of HOAt, and 10 equiv of DIEA in DMF. Cleavage of the thioether from resin was performed under standard conditions (TFA:triisopropylsilane:water, 95:2.5:2.5, 2 h). Final deprotection of *p*NZ group was accomplished by hydrogenolysis in a Parr hydrogenation apparatus where the crude peptide was dissolved in EtOH:MeOH (1:1) and submitted to hydrogenation at 30 psi pressure in the presence of Pd−C (10% g/g) for 2 h. After removal of the Pd catalyst by filtration through Celite, the OT thioether was purified by RP-HPLC (Phenomenex C18 column, 300 Å, 10 mm, 250 mm × 21.2 mm, 0−30% solvent B in 60 min at 8 mL/min flow). Two isomers of the desired lanthionine OT (**8** and **9**, ratio 2:3) having the same molecular weight {MS (calcd): 974.5, MS (found): 975.5 [M + 1]} were obtained in pure form. Their stereochemical properties were not investigated. All steps of lanthionine assembly were monitored by MS and analytical RP-HPLC.

Stability Assay. Three hundred microliters of human plasma (Sigma Aldrich) was incubated at 37 °C for 30 min. Fifty microliters of peptide sample (0.3 mM) in 0.1 M phosphate buffer, pH 7.2, was added to the human plasma. The vortexed mixture was incubated at 37 °C. Aliquots (30 μL) were taken at 1, 2, 3, 4, 12, 24, and 48 h, quenched with extraction buffer (70 μL) consisting of 50% ACN/H_2O, 0.1 M NaCl, and 1% TFA, chilled on ice for 5 min, centrifuged (14000 rpm, 10 min), and analyzed (2 × 20 μL injection) by RP-HPLC and LC-MS. Data analysis (n = 3−6) was performed using Prism Version 5 a nonlinear fit one-phase decay model.

Reduction/Alkylation Study. Condition A: PBS buffer, pH 7.4, and room temperature. Condition B: 1000-fold GSH: 10 μL of a 1.2 mM OT, 720 μM [Se-Se]-OT, and 800 μM [CH_2-S]-OT solution was added to 90 μL of a 136, 80, and 90 mM solution of glutathione in PBS buffer, respectively, and incubated at 37 °C and at pH 7.4 over a period of 24 h. Condition C: 100-fold DTT: 5 μL of a 1.2 mM OT, 720 μM [Se-Se]-OT, and 800 μM [CH_2-S]-OT solution was added to 75 μL of a 8, 4.8, and 5.3 mM solution of DTT in PBS buffer, respectively, and incubated at 37 °C and at pH 7.4 over a period of 12 h. Condition D: 10-fold DTT−100-fold iodoacetamide: 5 μL of a 1.2 mM OT, 720 μM [Se-Se]-OT, and 800 μM [CH_2-S]-OT solution was added to 75 μL of a

800, 480, and 530 μM solution of DTT in PBS buffer, respectively, and incubated at 37 °C and at pH 7.4. After 1 h, 15 μL of a 40 mM iodoacetamide in methanol solution was added and incubated at 37 °C, pH 7.4, for another 2 h. Samples were taken every hour and analyzed by RP-HPLC and LC-MS, running a linear gradient of 2%/min of solvent B from 0 to 40% solvent B.

Transfection and Membrane Preparation. COS-1 cells grown in Dulbecco's modified Eagle's medium and 5% fetal bovine serum in 150 mm plates were transiently transfected with plasmid DNA (25 μg) encoding OTR using Lipofectamine2000 reagent (50 μL). The cells were harvested 48 h post-transfection and homogenized using an Ultra turrax homogenizer (22000/min) in OT buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4) with complete protease inhibitor cocktail. The homogenate was centrifuged at 484*g* (2000 rpm) for 10 min, and the resulting supernatant was centrifuged at 23665*g* (14000 rpm) for 30 min. The pellet was resuspended in OT buffer without protease inhibitor containing 10% glycerol and stored at −80 °C until assayed.

Radioligand Binding Assay. Receptor binding assays were performed using FlashBLUE GPCR scintillation beads. Reactions containing increasing concentrations of competing OT analogue (10 pM to 10 μM), FlashBLUE GPCR beads (100 μg), OT membrane preparation (5 μg protein), and radioligand ^{3}H-OT (2 nM) in assay buffer with 0.1% BSA were established in 96-well white polystyrene plates with clear flat bottoms. The assays were performed in triplicate, in a total reaction volume of 80 μL. The plates were sealed with TopSeal-A film and incubated with shaking for 1 h at room temperature. Radioligand binding was detected using a Wallac 1450 MicroBeta scintillation counter (PerkinElmer Life Sciences).

IP-one HTRF Assay. COS-1 cells were transiently transfected with plasmid DNA encoding the OT receptor using Lipofectamine2000/DNA ratio of 2 in Dulbecco's modified Eagle's medium. Assays measuring IP-one accumulation were performed 48 h after transfection according to the manufacturer's protocol. Briefly, cells were incubated with an increasing concentration of test compound in stimulation buffer (10 mM Hepes, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 4.2 mM KCl, 146 mM NaCl, 5.5 mM glucose, and 50 mM LiCl, pH 7.4) for 1 h in 37 °C, 5% CO_2 in white 384-well optiplates. Cells were lysed by the addition of the HTRF reagents (the europium cryptate-labeled anti-IP-one antibody and the d2-labeled IP-one analogue) diluted in lysis buffer. The assays were incubated for 1 h at room temperature. The emission signals at 590 and 665 nm were measured after excitation at 340 nm using the Envision multilabel plate reader (PerkinElmer Life Sciences).

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References

(1) Sato, A. K.; Viswanathan, M.; Kent, R. B.; Wood, C. R. Therapeutic peptides: Technological advances driving peptides into development. *Curr. Opin. Biotechnol.* **2006**, *17*, 638–642.
(2) Reichert, J. *Therapeutic Peptides in Clinical Study in 2000−07 Nearly Doubled 1990s Rate*; Tufts Center for the Study of Drug Development, Boston, 2009; pp 1−4.
(3) Kumar, T. R. S.; Soppimath, K.; Nachaegari, S. K. Novel delivery technologies for protein and peptide therapeutics. *Curr. Pharm. Biotechnol.* **2006**, *7*, 261–276.
(4) Hamman, J. H.; Enslin, G. M.; Kotze, A. F. Oral delivery of peptide drugs: Barriers and developments. *BioDrugs* **2005**, *19*, 165–177.
(5) Craik, D. J.; Adams, D. J. Chemical modification of conotoxins to improve stability and activity. *ACS Chem. Biol.* **2007**, *2*, 457–468.
(6) Claybaugh, J. R.; Uyehara, C. F. T. Metabolism of neurohypophysial hormones. *Ann. N. Y. Acad. Sci.* **1993**, *689*, 250–268.
(7) Hong, S. Y.; Oh, J. E.; Lee, K. H. Effect of D-amino acid substitution on the stability, the secondary structure, and the activity of membrane-active peptide. *Biochem. Pharmacol.* **1999**, *58*, 1775–1780.
(8) Ember, J. A.; Sanderson, S. D.; Taylor, S. M.; Kawahara, M.; Hugli, T. E. Biological activity of synthetic analogs of C5a anaphylatoxin. *J. Immunol.* **1992**, *148*, 3165–3173.
(9) Clark, R. J.; Fischer, H.; Dempster, L.; Daly, N. L.; Rosengren, K. J.; Nevin, S. T.; Meunier, F. A.; Adams, D. J.; Craik, D. J. Engineering stable peptide toxins by means of backbone cyclization: Stabilization of the α-conotoxin MII. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 13767–13772.
(10) Hill, P. S.; Smith, D. D.; Slaninova, J.; Hruby, V. J. Bicyclization of a weak oxytocin agonist produces a highly potent oxytocin antagonist. *J. Am. Chem. Soc.* **1990**, *112*, 3110–3113.
(11) Werle, M.; Bernkop-Schnuerch, A. Strategies to improve plasma half life time of peptide and protein drugs. *Amino Acids* **2006**, *30*, 351–367.
(12) Bauer, W.; Briner, U.; Doepfner, W.; Haller, R.; Huguenin, R.; Marbach, P.; Petcher, T. J.; Pless, J. SMS 201−995: A very potent and selective octapeptide analog of somatostatin with prolonged action. *Life Sci.* **1982**, *31*, 1133–1140.
(13) Janknegt, R. A.; Zweers, M. M.; Delaere, K. P. J.; Kloet, A. G.; Khoe, S. G. S.; Arendsen, H. J. Oral desmopressin as a new treatment modality for primary nocturnal enuresis in adolescents and adults: A double-blind, randomized, multicenter study. *J. Urol.* (*Baltimore*) **1997**, *157*, 513–517.
(14) Zaoral, M.; Kolc, J.; Sorm, F. Amino acids and peptides. LXXI. Synthesis of 1-deamino-8-D-gamma-aminobutyrine vasopressin, 1-deamino-8-D-lysine vasopressin, and 1-deamino-8-D-arginine vasopressin. *Collect. Czech. Chem. Commun.* **1967**, *32*, 1250–1257.
(15) Sawyer, W. H.; Acosta, M.; Manning, M. Structural changes in the arginine vasopressin molecule that prolong its antidiuretic action. *Endocrinology* **1974**, *95*, 140–149.
(16) Barberis, C.; Morin, D.; Durroux, T.; Mouillac, B.; Guillon, G.; Seyer, R.; Hibert, M.; Tribollet, E.; Manning, M. Molecular pharmacology of AVP and OT receptors and therapeutic potential. *Drug News Perspect.* **1999**, *12*, 279–292.
(17) Hargittai, B.; Sole, N. A.; Groebe, D. R.; Abramson, S. N.; Barany, G. Chemical syntheses and biological activities of lactam analogs of α-conotoxin SI. *J. Med. Chem.* **2000**, *43*, 4787–4792.
(18) Smith, C. W.; Walter, R.; Moore, S.; Makofske, R. C.; Meienhofer, J. Replacement of the disulfide bond in oxytocin by an amide group. Synthesis and some biological properties of (cyclo-(1-L-aspartic acid,6-L-α,β-diaminopropionic acid))oxytocin. *J. Med. Chem.* **1978**, *21*, 117–120.
(19) MacRaild, C. A.; Illesinghe, J.; van Lierop, B. J.; Townsend, A. L.; Chebib, M.; Livett, B. G.; Robinson, A. J.; Norton, R. S. Structure and Activity of (2,8)-Dicarba-(3,12)-cystino α-ImI, an α-Conotoxin Containing a Nonreducible Cystine Analogue. *J. Med. Chem.* **2009**, *52*, 755–762.
(20) Yamanaka, T.; Hase, S.; Sakakibara, S.; Schwartz, I. L.; Dubois, B. M.; Walter, R. Crystalline deamino-dicarba-oxytocin. Preparation and some pharmacological properties. *Mol. Pharmacol.* **1970**, *6*, 474–480.
(21) Stymiest, J. L.; Mitchell, B. F.; Wong, S.; Vederas John, C. Synthesis of oxytocin analogues with replacement of sulfur by carbon gives potent antagonists with increased stability. *J. Org. Chem.* **2005**, *70*, 7799–7809.
(22) Bondebjerg, J.; Grunnet, M.; Jespersen, T.; Meldal, M. Solid-phase synthesis and biological activity of a thioether analogue of conotoxin G1. *ChemBioChem* **2003**, *4*, 186–194.
(23) Oesapay, G.; Prokai, L.; Kim, H.-S.; Medzihradszky, K. F.; Coy, D. H.; Liapakis, G.; Reisine, T.; Melacini, G.; Zhu, Q.; Wang, S. H. H.; Mattern, R.-H.; Goodman, M. Lanthionine-Somatostatin Analogs: Synthesis, Characterization, Biological Activity, and Enzymic Stability Studies. *J. Med. Chem.* **1997**, *40*, 2241–2251.
(24) Schramme, A. R.; Pinto, C. R. F.; Davis, J.; Whisnant, C. S.; Whitacre, M. D. Pharmacokinetics of carbetocin, a long-acting oxytocin analogue, following intravenous administration in horses. *Equine Vet. J.* **2008**, *40*, 658–661.
(25) Hunter, D. J. S.; Schulz, P.; Wassenaar, W. Effect of carbetocin, a long-acting oxytocin analog on the postpartum uterus. *Clin. Pharmacol. Ther.* (*St. Louis*) **1992**, *52*, 60–67.
(26) Sweeney, G.; Holbrook, A. M.; Levine, M.; Yip, M.; Alfredsson, K.; Cappi, S.; Ohlin, M.; Schulz, P.; Wassenaar, W. Pharmacokinetics of carbetocin, a long-acting oxytocin analog, in nonpregnant women. *Curr. Ther. Res.* **1990**, *47*, 528–540.
(27) Muttenthaler, M.; Nevin, S. T.; Grishin, A. A.; Ngo, S. T.; Choy, P. T.; Daly, N. L.; Hu, S.-H.; Armishaw, C. J.; Wang, C.-I. A.; Lewis, R. J.; Martin, J. L.; Noakes, P. G.; Craik, D. J.; Adams, D. J.; Alewood, P. F. Solving the α-Conotoxin Folding Problem: Efficient Selenium-Directed On-Resin Generation of More Potent and Stable Nicotinic Acetylcholine Receptor Antagonists. *J. Am. Chem. Soc.* **2010**, *132*, 3514–3522.

(28) Walewska, A.; Zhang, M.-M.; Skalicky Jack, J.; Yoshikami, D.; Olivera Baldomero, M.; Bulaj, G. Integrated oxidative folding of cysteine/selenocysteine containing peptides: improving chemical synthesis of conotoxins. *Angew. Chem., Int. Ed. Engl.* **2009**, *48*, 2221–2224.
(29) Gowd, K. H.; Yarotskyy, V.; Elmslie, K. S.; Skalicky, J. J.; Olivera, B. M.; Bulaj, G. Site-Specific Effects of Diselenide Bridges on the Oxidative Folding of a Cystine Knot Peptide, ω-Selenoconotoxin GVIA. *Biochemistry* **2010**, *49*, 2741–2752.
(30) Metanis, N.; Keinan, E.; Dawson, P. E. Synthetic Seleno-Glutaredoxin 3 Analogues Are Highly Reducing Oxidoreductases with Enhanced Catalytic Efficiency. *J. Am. Chem. Soc.* **2006**, *128*, 16684–16691.
(31) Armishaw, C. J.; Daly, N. L.; Nevin, S. T.; Adams, D. J.; Craik, D. J.; Alewood, P. F. α-Selenoconotoxins, a New Class of Potent α7 Neuronal Nicotinic Receptor Antagonists. *J. Biol. Chem.* **2006**, *281*, 14136–14143.
(32) Fiori, S.; Pegoraro, S.; Rudolph-Böhner, S.; Cramer, J.; Moroder, L. Synthesis and conformational analysis of apamin analogues with natural and non-natural cystine/selenocystine connectivities. *Biopolymers* **2000**, *53*, 550–564.
(33) Pegoraro, S.; Fiori, S.; Rudolph-Böhner, S.; Watanabe, T. X.; Moroder, L. Isomorphous replacement of cystine with selenocystine in endothelin: Oxidative refolding, biological and conformational properties of [Sec3, Sec11, Nle7]-endothelin-1. *J. Mol. Biol.* **1998**, *284*, 779–792.
(34) Muttenthaler, M.; Alewood, P. F. Selenopeptide chemistry. *J. Pept. Sci.* **2008**, *14*, 1223–1239.
(35) Muttenthaler, M.; Alewood, P. F. Selenocystine Peptides—Synthesis, Folding and Applications. In *Oxidative Folding of Peptides and Proteins*; Johannes, B., Moroder, L., Eds.; The Royal Society of Chemistry: Cambridge, 2008; pp 396−418.
(36) Gimpl, G.; Fahrenholz, F. The oxytocin receptor system: structure, function, and regulation. *Physiol. Rev.* **2001**, *81*, 629–683.
(37) Zingg, H. H.; Laporte, S. A. The oxytocin receptor. *Trends Endocrinol. Metabol.* **2003**, *14*, 222–227.
(38) Donaldson, Z. R.; Young, L. J. Oxytocin, vasopressin, and the neurogenetics of sociality. *Science* **2008**, *322*, 900–904.
(39) Reversi, A.; Cassoni, P.; Chini, B. Oxytocin receptor signaling in myoepithelial and cancer cells. *J. Mammary Gland Biol. Neoplasia* **2005**, *10*, 221–229.
(40) Chini, B.; Chinol, M.; Cassoni, P.; Papi, S.; Reversi, A.; Areces, L.; Marrocco, T.; Paganelli, G.; Manning, M.; Bussolati, G. Improved radiotracing of oxytocin receptor-expressing tumours using the new [111In]-DOTA-Lys8-deamino-vasotocin analogue. *Br. J. Cancer* **2003**, *89*, 930–936.
(41) Green, J. J.; Hollander, E. Autism and oxytocin: New developments in translational approaches to therapeutics. *Neurotherapeutics* **2010**, *7*, 250–257.
(42) Wood, S. P.; Tickle, I. J.; Treharne, A. M.; Pitts, J. E.; Mascarenhas, Y.; Li, J. Y.; Husain, J.; Cooper, S.; Blundell, T. L.; et al. Crystal structure analysis of deamino-oxytocin: conformational flexibility and receptor binding. *Science* **1986**, *232*, 633–636.
(43) Rose, J. P.; Wu, C.-K.; Hsiao, C.-D.; Breslow, E.; Wang, B.-C. Crystal structure of the neurophysin-oxytocin complex. *Nat. Struct. Biol.* **1996**, *3*, 163–169.
(44) Bhaskaran, R.; Chuang, L. C.; Yu, C. Conformational properties of oxytocin in dimethyl sulfoxide solution: NMR and restrained molecular dynamics studies. *Biopolymers* **1992**, *32*, 1599–1608.
(45) Meraldi, J. P.; Hruby, V. J.; Brewster, A. I. R. Relative conformational rigidity in oxytocin and [1-penicillamine]oxytocin: A proposal for the relation of conformational flexibility to peptide hormone agonism and antagonism. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 1373–1377.
(46) Smith, C. W.; Ferger, M. F. Synthesis and some pharmacological properties of five analogs of oxytocin having L-homocysteine in position 6. *J. Med. Chem.* **1976**, *19*, 250–254.
(47) Stymiest, J. L.; Mitchell, B. F.; Wong, S.; Vederas, J. C. Synthesis of biologically active dicarba analogues of the peptide hormone oxytocin using ring-closing metathesis. *Org. Lett.* **2003**, *5*, 47–49.
(48) Keller, O.; Rudinger, J. Synthesis of (1,6-alpha,alpha′-diaminosuberic acid)oxytocin ('dicarba-oxytocin'). *Helv. Chim. Acta* **1974**, *57*, 1253–1259.
(49) Schnölzer, M.; Alewood, P.; Jones, A.; Alewood, D.; Kent, S. B. H. In situ neutralization in Boc-chemistry solid phase peptide synthesis. Rapid, high yield assembly of difficult sequences. *Int. J. Pept. Protein Res.* **1992**, *40*, 180–193.
(50) Muttenthaler, M.; Ramos, Y. G.; Feytens, D.; de Araujo, A. D.; Alewood, P. F. p-Nitrobenzyl protection for cysteine and selenocysteine: A more stable alternative to the acetamidomethyl group. *Biopolymers* **2010**, *94*, 423–432.
(51) Mayer, J. P.; Heil, J. R.; Zhang, J.; Munson, M. C. An alternative solid-phase approach to C1-oxytocin. *Tetrahedron Lett.* **1995**, *36*, 7387–7390.
(52) Zhu, X.; Schmidt, R. R. Efficient synthesis of differently protected lanthionines via beta-bromoalanine derivatives. *Eur. J. Org. Chem.* **2003**, 4069–4072.
(53) Albericio, F.; Cases, M.; Alsina, J.; Triolo, S. A.; Carpino, L. A.; Kates, S. A. On the use of PyAOP, a phosphonium salt derived from HOAt, in solid-phase peptide synthesis. *Tetrahedron Lett.* **1997**, *38*, 4853–4856.
(54) Isidro-Llobet, A.; Guasch-Camell, J.; Alvarez, M.; Albericio, F. p-Nitrobenzyloxycarbonyl (pNZ) as a temporary Nalpha-protecting group in orthogonal solid-phase peptide synthesis—Avoiding diketopiperazine and aspartimide formation. *Eur. J. Org. Chem.* **2005**, 3031–3039.
(55) Trinquet, E.; Fink, M.; Bazin, H.; Grillet, F.; Maurin, F.; Bourrier, E.; Ansanay, H.; Leroy, C.; Michaud, A.; Durroux, T.; Maurel, D.; Malhaire, F.; Goudet, C.; Pin, J.-P.; Naval, M.; Hernout, O.; Chretien, F.; Chapleur, Y.; Mathis, G. D-myo-Inositol 1-phosphate as a surrogate of D-myo-inositol 1,4,5-tris phosphate to monitor G protein-coupled receptor activation. *Anal. Biochem.* **2006**, *358*, 126–135.
(56) Kimura, T.; Tanizawa, O.; Mori, K.; Brownstein, M. J.; Okayama, H. Structure and expression of a human oxytocin receptor. *Nature* (*London*) **1992**, *356*, 526–529.
(57) Dutertre, S.; Croker, D.; Daly, N. L.; Andersson, A.; MuttenthalerM.; Lumsden, N. G.; Craik, D. J.; Alewood, P. F.; Guillon, G.; Lewis, R. J. Conopressin-T from Conus Tulipa Reveals an Antagonist Switch in Vasopressin-like Peptides. *J. Biol. Chem.* **2008**, *283*, 7100–7108.
(58) Manning, M.; Stoev, S.; Chini, B.; Durroux, T.; Mouillac, B.; Guillon, G. Peptide and non-peptide agonists and antagonists for the vasopressin and oxytocin V1a, V1b, V2 and OT receptors: Research tools and potential therapeutic agents. *Prog. Brain Res.* **2008**, *170*, 473–512.
(59) Zaoral, M.; Krchnak, V. Amino acids and peptides. Part CXLII. Cyclooxytocin, an oxytocin analog with an enhanced stability of the secondary structure. *Collect. Czech. Chem. Commun.* **1977**, *42*, 3500–3509.
(60) Orcel, H.; Albizu, L.; Perkovska, S.; Durroux, T.; Mendre, C.; Ansanay, H.; Mouillac, B.; Rabie, A. Differential coupling of the vasopressin V1b receptor through compartmentalization within the plasma membrane. *Mol. Pharmacol.* **2009**, *75*, 637–647.
(61) Kenakin, T. *Pharmacologic Analysis of Drug-Receptor Interaction*, 3rd ed.; Lippincott-Raven Publishers: Philadelphia, 1997.
(62) Kenakin, T. Ligand-selective receptor conformations revisited: the promise and the problem. *Trends Pharmacol. Sci.* **2003**, *24*, 346–354.
(63) Cottet, M.; Albizu, L.; Perkovska, S.; Jean-Alphonse, F.; Rahmeh, R.; Orcel, H.; Mejean, C.; Granier, S.; Mendre, C.; Mouillac, B.; Durroux, T. Past, present and future of vasopressin and oxytocin receptor oligomers, prototypical GPCR models to study dimerization processes. *Curr. Opin. Pharmacol.* **2010**, *10*, 59–66.
(64) Kobilka, B. K.; Deupi, X. Conformational complexity of G-protein-coupled receptors. *Trends Pharmacol. Sci.* **2007**, *28*, 397–406.
(65) Smith, C. W.; Botos, C. R.; Skala, G.; Walter, R. Conformation-activity studies of oxytocin. Effects of structural modifications at corner positions of the β-turns on the uterotonic activity. *J. Med. Chem.* **1977**, *20*, 1457–1460.
(66) Nicke, A.; Wonnacott, S.; Lewis, R. J. α-Conotoxins as tools for the elucidation of structure and function of neuronal nicotinic acetylcholine receptor subtypes. *Eur. J. Biochem.* **2004**, *271*, 2305–2319.
(67) Barth, T.; Krejci, I.; Vaneckova, J.; Jost, K.; Rychlik, I. Prolonged action of deamino-carba analogs of oxytocin on the rat uterus in vivo. *Eur. J. Pharmacol.* **1974**, *25*, 67–70.
(68) Small, C. W.; Watkins, W. B. Glutathione-oxytocin transhydrogenase of human placental origin with specificity towards neurohypophysial hormones. *Nature* (*London, U. K.*) **1974**, *251*, 237–239.
(69) Suska-Brzezinska, E.; Fruhaufova, L.; Barth, T.; Rychlik, I.; Jost, K.; Sorm, F. Inactivation of deamino-carba analogs of oxytocin in the rat uterus, liver, and kidney. *Collect. Czech. Chem. Commun.* **1972**, *37*, 2289–2294.
(70) Besse, D.; Siedler, F.; Diercks, T.; Kessler, H.; Moroder, L. The redox potential of selenocystine in unconstrained cyclic peptides. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 883–885.
(71) Powell, M. F.; Stewart, T.; Otvos, L., Jr.; Urge, L.; Gaeta, F. C. A.; Sette, A.; Arrhenius, T.; Thomson, D.; Soda, K.; Colon, S. M. Peptide stability in drug development. II. Effect of single amino acid substitution and glycosylation on peptide reactivity in human serum. *Pharm. Res.* **1993**, *10*, 1268–1273.
(72) Powell, M. F.; Grey, H.; Gaeta, F.; Sette, A.; Colon, S. Peptide stability in drug development: A comparison of peptide reactivity in different biological media. *J. Pharm. Sci.* **1992**, *81*, 731–735.

(73) Anderson, N. L.; Anderson, N. G. The human plasma proteome. History, character, and diagnostic prospects. *Mol. Cell. Proteomics* **2002**, *1*, 845–867.
(74) Hruby, V. J. Designing peptide receptor agonists and antagonists. *Nat. Rev. Drug Discovery* **2002**, *1*, 847–858.
(75) Smith, D. D.; Slaninova, J.; Hruby, V. J. Structure-activity studies of a novel bicyclic oxytocin antagonist. *J. Med. Chem.* **1992**, *35*, 1558–1563.
(76) Hruby, V. J.; Rockway, T. W.; Viswanatha, V.; Chan, W. Y. Pharmacological, conformational and dynamic properties of cycloleucine-2 analogues of oxytocin and [1-penicillamine]oxytocin. *Int. J. Pept. Protein Res.* **1983**, *21*, 24–34.
(77) Hruby, V. J. Relation of conformation to biological activity in oxytocin, vasopressin and their analogs. *Top. Mol. Pharmacol.* **1981**, *1*, 99–126.
(78) Mustapa, M. F. M.; Harris, R.; Esposito, D.; Chubb, N. A. L.; Mould, J.; Schultz, D.; Driscoll, P. C.; Tabor, A. B. Synthesis of a Cyclic Peptide Containing Norlanthionine: Effect of the Thioether Bridge on Peptide Conformation. *J. Org. Chem.* **2003**, *68*, 8193–8198.
(79) Alewood, P.; Alewood, D.; Miranda, L.; Love, S.; Meutermans, W.; Wilson, D. Rapid in situ neutralization protocols for Boc and Fmoc solid-phase chemistries. *Methods Enzymol.* **1997**, *289*, 14–29.
(80) Moroder, L. Isosteric replacement of sulfur with other chalcogens in peptides and proteins. *J. Pept. Sci.* **2005**, *11*, 187–214.
(81) Van den Bossche, G.; Spirlet, M. R.; Dideberg, O.; Dupont, L. New form of bis(p-chlorophenyl) ditelluride, $C_{12}H_8Cl_2Te_2$. *Acta Crystallogr., Sect. C: Cryst. Struct. Commun.* **1984**, *C40*, 1011–1012.