Cyclic Peptides Hot Paper

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Vicinal Disulfide Constrained Cyclic Peptidomimetics: a Turn Mimetic Scaffold Targeting the Norepinephrine Transporter**

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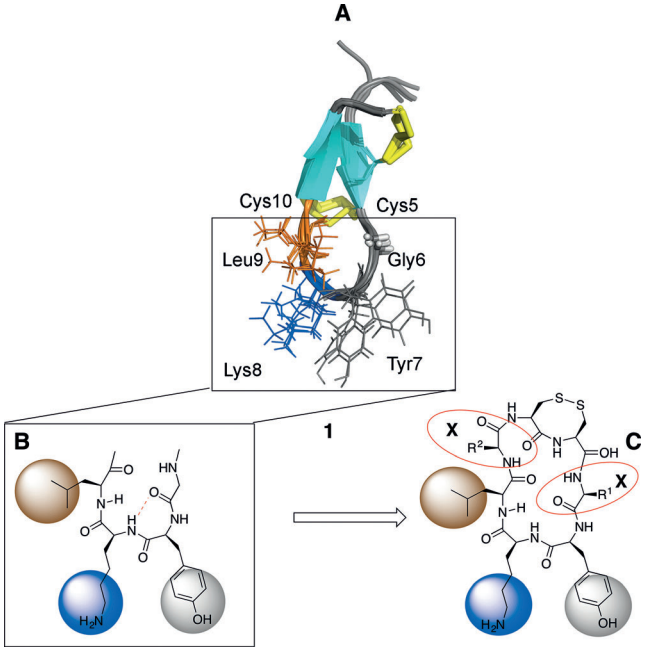
The design of peptidomimetics[1] of peptide–protein or protein–protein interactions needs to take into consideration the specific amino acid interactions between the ligand and its protein receptor.[2] Peptide turns play an essential role in many recognition events,[2] including receptor recognition in the most abundant receptor class, G protein coupled receptors (GPCRs),[3] with many bioactive peptides adopting γ or β turn conformations.[3] Strategies for making peptide turn mimetics range from local peptide amino acid modification to the development of small-molecule turn mimetics.[1–2,4] However, these approaches often generate overly flexible scaffolds that reduce receptor selectivity and potency[5], or require complex synthetic strategies.[6] In the absence of precise three-dimensional structures for ligands in complex with membrane proteins[7], the introduction of conformational constraints (cyclization and turn mimetics) is a promising approach for the development of bioactive peptidomimetics.[8] Such strategies have produced clinically relevant[9] and potent peptidomimetics of somatostatin, angiotensin II receptor antagonists, integrin inhibitors, and oxytocin and vasopressin analogues.[3]

To date, there is no design strategy that can generate readily accessible diverse sets of turn mimetics with properties appropriate for further drug development. Here, we describe a broadly applicable combinatorial chemistry approach that addresses some of these shortcomings, and use it to design a norepinephrine reuptake inhibitory peptide mimetic of exceptional metabolic stability.

The χ-conotoxin mrIa (**1**, sequence NGVCCGYKLCHOC; Scheme 1 A) noncompetitively inhibits the norephinephrine transporter (NET), and a closely related analogue Xen2174 (**1a**, sequence UGVCCGYKLCHOC where U = pyroglutamyl) is currently under development for the treatment of severe pain.[10a,11] These χ-conotoxins contain the sequence GYKL, which is essential for high-affinity NET inhibition.[10a] Structural studies reveal that the rigid framework formed by two disulfide bonds between the cysteine residues (1–4, 2–3) induces a β hairpin with two antiparallel strands (Scheme 1 A) connected by an inverse γ turn comprising GYK,[10b] thereby further constraining the pharmacophore (Scheme 1 B).



Scheme 1. A) NMR structure of χ-conopeptide **1**[10] highlighting the pharmacophore of this norepinephrine reuptake inhibitor. B) Pharmacophore (GYKL) of **1** represented by an inverse turn. C) Design of N-to-C cyclic vicinal disulfide constrained peptide turn mimetics with X indicating the modification sites.

Our initial design strategy involved incorporating the YKL pharmacophore into a cyclic peptide. To minimize the size of the ring and restrict flexibility, we incorporated a vicinal disulfide bond as a further constraint (Scheme 1 C) and then varied the amino acids (indicated as X in Scheme 1 C) adjacent to the YKL pharmacophore to modu-

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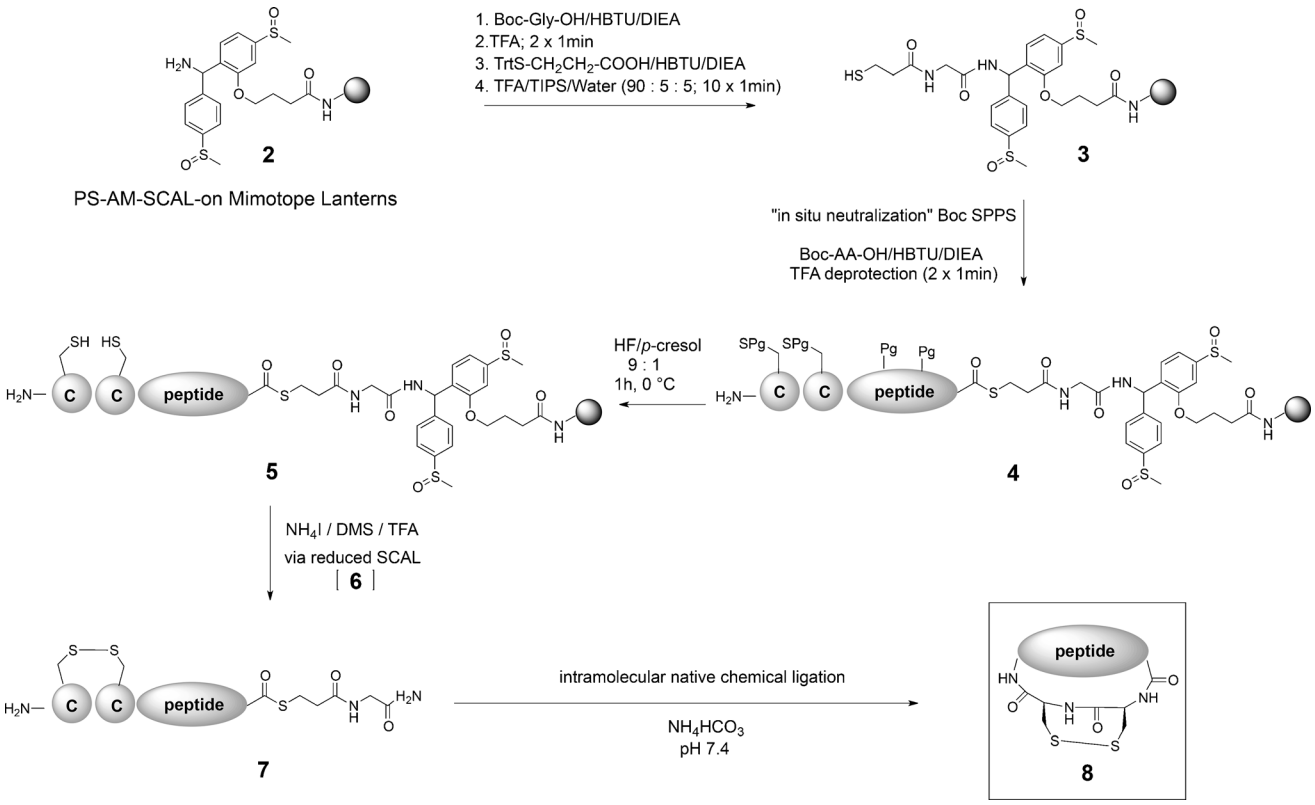
late structure and gain additional structure–activity information. Vicinal disulfide bonds are found in the active sites of enzymes[12] and near the binding sites of receptors,[12,13] as well as in toxin peptides.[14] In proteins, this unique 8-membered ring prefers a type VIII turn with a distorted *trans*-amide conformation,[12] while in small model peptides, *cis* or *trans* amide bond conformations are observed.[15] In receptors, vicinal disulfide bonds may function as regulatory switches through changes in their oxidation states.[12,16]

We used intramolecular native chemical ligation (NCL) to access these designed turn mimetics. Conveniently, the NCL approach[17] allows access to a diverse range of cyclopeptides without the need for complex side chain protection strategies.

To produce cyclic peptide libraries where X is variable (Scheme 1 C), we employed *tert*-butoxycarbonyl (Boc) chemistry to synthesize the peptide thioesters necessary for the NCL cyclization. Although Boc chemistry is well established and robust, throughput is limited by the final HF cleavage step.[17] To overcome this bottleneck, we employed the combination of a safety-catch amide linker and a resin support that is compartmentalized to allow easy manual handling and sorting (Scheme 2).

Starting with Mimotopes aminomethylated lanterns, a safety-catch linker (SCAL)[18] was attached (**2**), followed by a Gly spacer and a mercaptopropionic acid linker. The thiol resin **3** was then used to produce the cyclic-peptide libraries (Scheme 1 C) of type **8** (Scheme 2) in a split-and-mix fashion using standard peptide coupling conditions. After chain assembly was complete, the thioester-loaded lanterns **4** were transferred together into a single HF cleavage vessel, which allowed removal of the side-chain protecting groups, while the HF stable SCAL maintained the deprotected thioester on the resin (**4**→**5**). The free thioester peptides **7** were obtained after sorting the lanterns into individual vials and adding NH_4I/DMS/TFA, which both reduces the SCAL sulfoxide (**5**→**6**) and renders the substituted benzhydrylamine linkage TFA labile, while oxidizing the vicinal disulfide bond to yield **7**.[18–19] The crude peptide thioesters **7** were readily N-C cyclized in NH_4HCO_3 buffer (0.1 M) to yield **8**, the formation of which is autocatalyzed by the thiol released from the linker moiety during cyclization (see the Supporting Information).

Cyclic peptides **8a**–**8j** were tested using a functional human norepinephrine transporter assay. Table 1 shows that our design strategy successfully generated active mimetics of peptide **1**, with the vicinally constrained cyclic heptapeptide **8a** (CCGYKLG) being equipotent to **1**. Other analogues showed that we could manipulate potency by varying either the pharmacophoric residues or residues at positions X. To confirm that the vicinal disulfide bond is a structurally



Scheme 2. High-throughput synthesis of peptide thioester **7** and intramolecular native chemical ligation, yielding cyclic peptide turn mimetics (**8**): A combination of a safety-catch linker/thiol linker (**2**→**3**) together with compartmentalized resin (lanterns) allows for split-and-mix Boc synthesis (**3**→**4**) and parallel hydrogen fluoride deprotection (→**5**). After sorting and safety-catch linker activation (**5**→**6**), peptide thioester **7** is obtained. Native chemical ligation yields cyclic peptides of type **8**. HBTU = *O*-(benzotriazol-l-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, DIEA = diisopropylethylamine, Trt = trityl, TFA = trifluoroacetic acid, TIPS = triisopropylsilane, DMS = dimethyl sulfide, Pg = protecting group, PS-AM-SCAL = safety-catch amide linker on aminomethylated polystyrene.

Table 1: Norepinephrine reuptake inhibition ($IC_{50} \pm$ SEM) by vicinal disulfide constrained cyclic peptidomimetics **8a–8j**, and χ-conopeptide **1** (1–4, 2–3 disulfide bond). Reduced and alkylated products **9** and **10** show decreased activity owing to loss of the vicinal disulfide constraint.

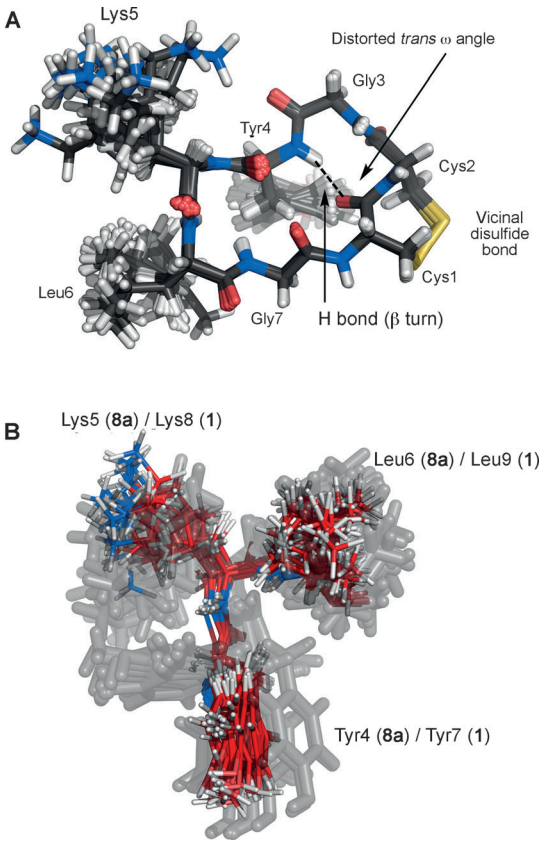
Entry	Sequence	IC_{50} [μM]
1	NGVCCGYKLCHOC[a]	2.45 ± 0.07
1a	UGVCCGYKLCHOC[a]	2.55 ± 0.18
8a	(CCGYKLG)	3.9 ± 0.2
8b	(CCGYK[CHA]G)[b]	2.3 ± 0.3
8c	(CCGYKL-)	31.6 ± 4
8d	(CCGYK[CHA]-)[b]	18 ± 1.2
8e	(CCEYKLG)	117 ± 2.8
8f	(CCFYKLG)	72 ± 6
8g	(CCH(Dnp)YKLG)[c]	24 ± 11
8h	(CCLYKLG)	63 ± 20
8i	(CCWYKLG)	28 ± 11
8j	(CCFYKL-)	87 ± 14
9	([C-Saa][C-Saa]GYKLG)[d]	20 ± 3
10	([C-SMe][C-SMe]GYKLG)[e]	49 ± 8

[a] (U = pyroglutamic acid, O = hydroxyproline), [b] CHA = cyclohexyl alanine. [c] Dnp = 2,5-dinitrophenyl, [d] Saa = S-acetamidated cysteine (= S-CH_2CONH_2), [e] SMe = S-methylated cysteine.

essential element for the cyclopeptide bioactivity, we reduced peptidomimetic **8a** using tris(2-carboxyethyl)phosphine (TCEP), and S-alkylated the cysteine residues with iodoacetamide or iodomethane. The cyclic and S-alkylated products (**9** and **10**, Table 1) showed an approximately 10-fold loss in activity, confirming the importance of the vicinal disulfide bond.

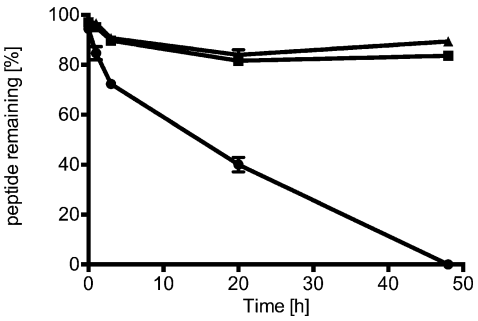
The structure of mimetic **8a** in water (with 5% D_2O) was determined by 1D ^{1}H NMR and 2D NMR spectroscopy (TOCSY, NOESY, ^{1}H-^{13}C HSQC) at a ^{1}H frequency of 900 MHz. Structure calculations were performed using the torsion angle dynamics approach of CYANA v3.0[20] with automated NOE assignments. The NOE data was supplemented with backbone angle restraints based on ^{1}H and ^{13}C chemical shift data using TALOS+.[21] Sufficient NOE interactions enabled determination of a high-resolution structure of **8a** (Scheme 3A). The vicinal disulfide bond in **8a** results in a distorted *trans*-amide bond (41° from planarity), and this observation is consistent with previous reports[22] (see also the Supporting Information). This geometry allows the formation of a hydrogen bond between Cys1-(CO) and Tyr4-(NH), an interaction that stabilizes the CCGY region in a β turn conformation (Scheme 3A). Superposition of the YKL side chains of the parent compound **1** and mimetic **8a** revealed a high degree of overlap, suggesting that the mimetic retained the pharmacophore of **1** (root-mean-square deviation (RMSD) over Cα atoms: 0.06 Å and Cβ atoms: 0.22 Å, see Scheme 3B). In contrast, NMR analysis of the reduced and methylated **10** produced results consistent with a random coil, with no long-range interactions, thus confirming that the vicinal disulfide bond provides a key structural constraint (see the Supporting Information).

We further evaluated the suitability of analogues **8a** and **8b** as clinical leads. Unlike many peptides, we found that both peptidomimetic **8a**, which is stable in trypsin (see the Supporting Information), and peptidomimetic **8b** had exceptional stability in plasma that was not derived from serum



Scheme 3. A) NMR structure of vicinal disulfide constrained cyclic peptidomimetic **8a**. B) Superposition of the pharmacophore region of χ-conopeptide (**1**, gray) with mimetic **8a** (red).

albumin adsorption (see the Supporting Information), and remained predominantly intact for at least 50 h (Scheme 4). This stability can be attributed to the N-to-C cyclization[23] but the stability of the disulfide bond (redox potential of **8a** −313 mV, see the Supporting Information), which forms



Scheme 4. Stability of cyclic peptidomimetics **8a** (—■—) and **8b** (—▲—) in rat plasma compared with clinical candidate **1a** (—●—).

readily even under highly acidic conditions, may also contribute. The redox potential of **8a** compared well with values reported for similar *transoid* vicinal disulfides,[15] and it is surprisingly more stable than many naturally occurring disulfides.[24] We also established that mimetic **8a** inhibits NET in isolated rat brain slices (IC_{50} = 4.2 μM; see the

Supporting Information), thereby indicating stability not only in plasma but also in brain tissue.

In conclusion, we herein describe a synthesis of peptide turn mimetics that combines a novel high-throughput combinatorial strategy for peptide thioesters, and their subsequent conversion into backbone cyclic peptides containing a vicinal disulfide constraint. Our results demonstrate that it is possible to graft an active peptide turn onto a vicinal disulfide constrained cyclic scaffold to improve both chemical and biological stability while maintaining full potency. Although this work is based on a well characterized peptide turn,[10a] we believe that this approach could be broadly applied to the design of turn mimetics for other peptide turns that are less well studied. This is due to the fact that it is the topological orientation of the pharmacophore side chains that largely determines bioactivity, rather than the specific turn type.[25]

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Communications

Cyclic Peptides

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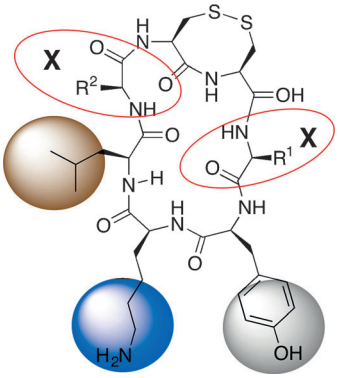
Vicinal Disulfide Constrained Cyclic Peptidomimetics: a Turn Mimetic Scaffold Targeting the Norepinephrine Transporter



Loopy peptides: Peptide turn mimetics of a clinically relevant norepinephrine reuptake inhibitor were developed employing a high-throughput synthesis approach to generate peptide thioesters, with subsequent cyclization through native chemical ligation. The vicinal disulfide constrained cyclic peptidomimetics (see scheme) show high structural and functional similarity to the parent peptide, though with superior metabolic stability.