Synthesis and Pharmacology of Novel Analogues of Oxytocin and Deaminooxytocin: Directed Methods for the Construction of Disulfide and Trisulfide Bridges in Peptides[†,‡,§]

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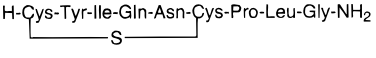
Using as models the neurohypophyseal nonapeptide hormone oxytocin and its analogue deaminooxytocin, several directed routes to formation of sulfur−sulfur bridges have been developed and evaluated. The linear sequences (through common octapeptide-resin intermediates) were assembled smoothly on tris(alkoxy)benzylamide (PAL) poly(ethylene glycol)−polystyrene (PEG−PS) graft supports, using stepwise Fmoc solid-phase chemistry. Side-chain protection of β-mercaptopropionic acid (Mpa) and/or cysteine (Cys) was provided by *S*-2,4,6-trimethoxybenzyl (Tmob), *S*-acetamidomethyl (Acm), and/or a series of sulfenyl thiocarbonate and carbamoylsulfenyl protecting/activating groups: *S*-(methoxycarbonyl)sulfenyl (Scm), *S*-(methoxycarbonyl)disulfanyl (Sscm), *S*-(*N*-methyl-*N*-phenylcarbamoyl)sulfenyl (Snm), and *S*-(*N*-methyl-*N*-phenylcarbamoyl)disulfanyl (Ssnm). Thiolytic displacement of *S*-Snm (preferred) or *S*-Scm provided intramolecular cyclized peptide disulfides, and homologation of the chemistry with *S*-Ssnm (again preferred) and *S*-Sscm provided the corresponding trisulfides along with smaller amounts of disulfides and tetrasulfides. These chemistries could be implemented both in solution and in solid-phase modes. Various parameters were studied systematically and optimized, and the novel trisulfides of oxytocin and deaminooxytocin were synthesized and purified to homogeneity. The trisulfide compounds were evaluated in three assays: uterotonic *in vitro*, uterotonic *in vivo*, and pressor tests, and they showed substantial potencies, ranging from 5% to 40% of the parent (disulfide) activities, as well as protracted actions. The affinities of the peptide trisulfides to uterine membrane receptors were only 3.3−3.6-fold lower than those of the parent disulfides. Possible explanations of the biological results are discussed.

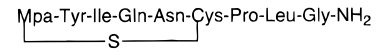
Introduction

Much is known about the importance of disulfide bridges in peptides and proteins, and many elegant ways to form these structures have been reported.[3,4] In contrast, the corresponding *trisulfide* variants reflect a rather newly appreciated area of chemical and biological interest. Several interesting natural products,[3,5,6] including allyl methyl trisulfane (garlic active principle),[5,7] the mixed trisulfide between cysteine and glutathione,[8] sporidesmin,[9] and the esperamicin/calichemicin family,[10−14] all contain trisulfide bridges which may play a role in their biological actions. Recently, it was discovered[15,16] that as much as 10% of recombinant human growth hormone produced in *Escherichia coli* contains an extra sulfur in the bridge between Cys[182] and Cys[189], and several unusual pathways to amino acid and peptide trisulfides have been reported.[17−19]

Our experience with trisulfides dates back more than a decade, starting with the development of a general method for their synthesis[5] and continuing with the successful albeit low-yield preparation by a relatively primitive synthetic route of the long-elusive trisulfide analogue of oxytocin, [β,β'-trithiobis(alanine)[1,6]]oxytocin.[20,21] This molecule (see first structure) provided the first example where a 21-membered ring was accommodated into a structure with appreciable oxytocic activity.[22−25]



Oxytocin Trisulfide



Deaminooxytocin Trisulfide

The present report indicates how we have overhauled substantially the earlier chemistry to prepare again this oxytocin derivative, as well as the related novel deaminooxytocin trisulfide analogue (see second structure). The new chemistry relies on the base-labile 9-fluorenylmethoxycarbonyl (Fmoc) group for N^{α}-amino protection and corresponding anchors and side-chain-protect-

[†] Abbreviations: Acm, acetamidomethyl; Boc, *tert*-butyloxycarbonyl; DBU, 1,8-diazobicyclo[5.4.0]undec-7-ene; DIEA, *N*,*N*-diisopropylethylamine; DIPCDI, *N*,*N*′-diisopropylcarbodiimide; DMF, *N*,*N*-dimethylformamide; EIMS, electron ionization mass spectrometry; FABMS, fast atom bombardment mass spectrometry; Fmoc, 9-fluorenylmethyloxycarbonyl; HOAc, acetic acid; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; Mpa, β-mercaptopropionic acid; NMM, *N*-methylmorpholine; PAL, 5-(4-Fmoc-aminomethyl-3,5-dimethoxyphenoxy)valeric acid handle; PEG−PS, poly(ethylene glycol)−polystyrene (graft support); Pfp, pentafluorophenyl; Scm, *S*-(methoxycarbonyl)sulfenyl; Snip, (*N*-piperidylcarbamoyl)sulfenyl; Snm, (*N*-methyl-*N*-phenylcarbamoyl)sulfenyl; Sscm, (methoxycarbonyl)disulfanyl; Ssnm, (*N*-methyl-*N*-phenylcarbamoyl)disulfanyl; TFA, trifluoroacetic acid; Tmob, 2,4,6-trimethoxybenzyl. All solvent ratios are vol/vol. All amino acids used were of the L-configuration.

[‡] Taken in part from the Ph.D. Thesis of L. Chen, University of Minnesota, Minneapolis, MN, January 1997.

[§] Portions of this work were reported in preliminary form; see refs 1 and 2.

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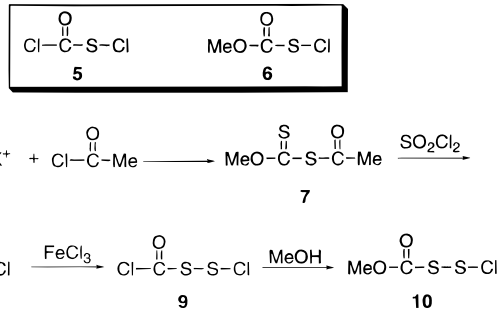
ing groups that are cleaved by a moderate strength acid, i.e., trifluoroacetic acid (TFA). This milder synthetic design was developed with the aim to lessen problems such as HF-promoted desulfurization and scrambling observed in our earlier work, which used chemistry centered around the acidolyzable *tert*-butyloxycarbonyl (Boc) N^{α}-amino-protecting group. Advances in the *orthogonal*[26,27] protection and management of sulfhydryl groups[4] are key aspects of the present work, and the relative merits of solid-phase[2,4,28,29] and solution[2,4] cyclization strategies have been assessed for both the parent disulfides and the novel trisulfide analogues. We have opted for *directed*[4,30] methods of sulfur−sulfur formation, based on the conviction that these offer greater control by comparison to the widely used symmetrical cooxidation approaches.

The novel and unusual synthetic trisulfide peptides have been evaluated in an extensive array of biological assays. Substantial potencies, ranging from 5% to 40% of the parent activities, were observed, and the affinities of the peptide trisulfides to uterine membrane receptors were only 3.3−3.6-fold lower than those of the parent disulfides. Of greatest interest were the protracted actions of the oxytocin trisulfide peptides. Several hypotheses regarding the molecular and pharmacological bases of these effects are under consideration and will likely lead to a more complete understanding of the biological properties of oxytocin and related peptides.

Results and Discussion

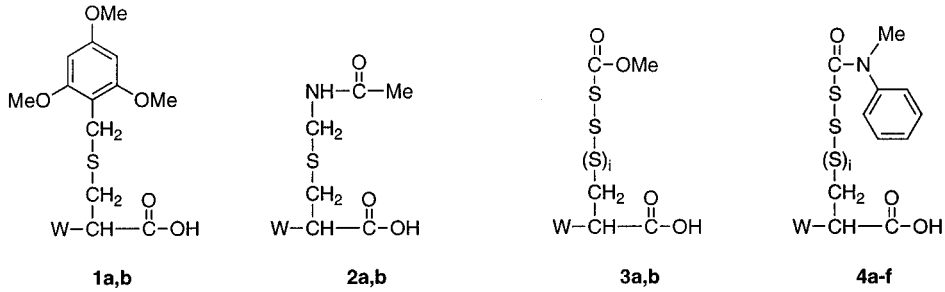
Protection (and Activation) of Cysteine (Cys) and β-Mercaptopropionic Acid (Mpa). This work applied an assortment of previously described[31−37] and new derivatives which (for Cys) had N^{α}-Fmoc or N^{α}-Boc protection and/or were extra-sulfur homologues of known derivatives (see Chart 1). Those derivatives with two sulfurs, i.e., sulfenyl thiocarbonate *S*-Scm **3a** and carbamoyl disulfide *S*-Snm **4a**−**c** (one sulfur atom derived from Cys or Mpa, the other sulfur atom part of protecting group), were prepared by general methods that were reported previously.[31] Keys to these procedures are the selective electrophilic reactions of the parent thiols or their *S*-acetamidomethyl (Acm)[37] derivatives with (chlorocarbonyl)sulfenyl chloride (**5**)[38] or (methoxycarbonyl)-sulfenyl chloride (**6**)[38] (Scheme 1, inset box). The homologues of **5** and **6**, i.e., (chlorocarbonyl)disulfanyl chloride (**9**) and (methoxycarbonyl)disulfanyl chloride (**10**), respectively, are known;[39−43] they were applied in the synthesis of the appropriate *S*-Sscm (**3b**) and *S*-Ssnm (**4d**−**f**) derivatives. Preparation of **8** as currently practiced in our laboratory (Scheme 1) involves use of sulfuryl chloride (SO_2Cl_2), a more readily managed liquid, in place of gaseous chlorine as described in the literature.

Scheme 1. Sulfenyl and Disulfanyl Chlorides Used in These Studies



General Plans for Peptide Synthesis and Sulfur−Sulfur Bond Formation. Protocols used were based on earlier precedents[28,29,31−33] and on model studies first reported herein. The first eight residues common to the oxytocin and deaminooxytocin linear sequences were assembled smoothly by Fmoc chemistry with tris-(alkoxy)benzylamide (PAL) anchoring[44] on commercially available poly(ethylene glycol)−polystyrene (PEG−PS) supports.[45−47] Couplings of all N^{α}-Fmoc amino acid derivatives were mediated by *N*,*N*′-diisopropylcarbodiimide/1-hydroxybenzotriazole (DIPCDI/HOBt) in *N*,*N*-dimethylformamide (DMF), except for Fmoc-Asn-OH and Fmoc-Gln-OH (unprotected ω-carboxamide side chain) which were incorporated as their pentafluorophenyl (Pfp) active esters in DMF in the presence of HOBt. Tyr was protected as its *O*-*tert*-butyl ether. Depending on the further experimental design, the internal Cys[6] was protected as either its *S*-2,4,6-trimethoxybenzyl (Tmob)[32] or its *S*-Acm derivative. When

Chart 1



1a, W = H, Mpa(Tmob)-OH

1b, W = Fmoc-NH, Fmoc-Cys(Tmob)-OH

2a, W = H, Mpa(Acm)-OH

2b, W = Fmoc-NH, Fmoc-Cys(Acm)-OH

3a, W = H, i = 0, Mpa(Scm)-OH

3b, W = H, i = 1, Mpa(Sscm)-OH

4a, W = H, i = 0, Mpa(Snm)-OH

4b, W = Boc-NH, i = 0, Boc-Cys(Snm)-OH

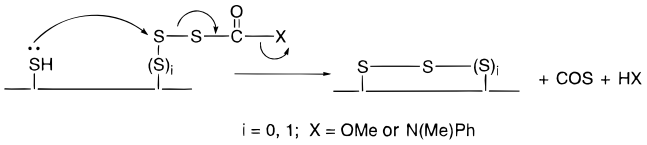
4c, W = Fmoc-NH, i = 0, Fmoc-Cys(Snm)-OH

4d, W = H, i = 1, Mpa(Ssnm)-OH

4e, W = Boc-NH, i = 1, Boc-Cys(Ssnm)-OH

4f, W = Fmoc-NH, i = 1, Fmoc-Cys(Ssnm)-OH

Scheme 2. Directed Formation of Sulfur−Sulfur Bonds



sulfenyl thiocarbonate (*S*-Scm, *S*-Sscm) or carbamoylsulfenyl (*S*-Snm, *S*-Ssnm) protection/activation was needed at the internal Cys, this was achieved by transformation[4,5,30,31,34−36] of *S*-Acm with the appropriate sulfenyl chloride (i.e., **5**, **6**, **9**, **10**) followed (as necessary) by quenching of the pendant acid chloride with methanol or *N*-methylaniline. As elaborated in the next section, such strategies were developed due to concerns about the survival of *S*-Scm or *S*-Snm during further cycles of Fmoc chemistry. On the other hand, the *N*-terminal Boc-Cys[1] (for oxytocin) or Mpa[1] (for deaminooxytocin) was readily accommodated with any one of *S*-Tmob, *S*-Acm, *S*-Scm, *S*-Snm, *S*-Sscm, or *S*-Ssnm protection of the sulfur. Here, the standard DIPCDI/HOBt couplings worked well, and the protecting group lability became irrelevant since elongation past that point was not needed.

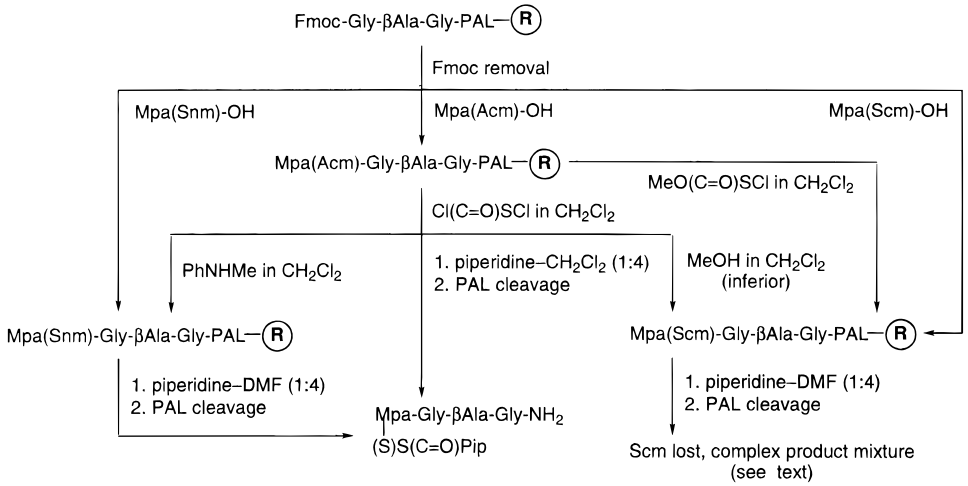
Directed reactions to form sulfur−sulfur bonds (Scheme 2) were carried out both in solution and while the peptide remained anchored to the polymeric support. In the latter cases, we sought to exploit the kinetic *pseudodilution* phenomenon[27,48] which favors intramolecular cyclization. Selective acidolytic removal[29,33] of *S*-Tmob as a prerequisite to solid-phase cyclizations was accomplished with TFA−CH_2Cl_2−Et_3SiH−phenol−H_2O (7:90:1:1:1) ("low" TFA), at 25 °C for 2 × 15 min. Acidolytic cleavage/deprotection of completed peptide-resins with TFA−CH_2Cl_2−Et_3SiH−phenol−H_2O (92:5:1:1:1) ("high" TFA), at 25 °C for 2 h, gave the corresponding linear and/or cyclized peptide amides in high recoveries (90−94% released from support, unless specifically indicated otherwise); see text and accompanying tables for distribution and purities of products.

Model Studies on Stabilities of Carbamoyl and Methoxycarbonyl Disulfides under Conditions Relevant to Peptide Synthesis. Despite the demonstrated usefulness of the *S*-Scm protecting/activating group in peptide chemistry,[31,34−36] essentially all reported chemistry involves *indirect* establishment of the group rather than straightforward incorporation of the appropriate building block in *stepwise* peptide synthesis. Even with indirect introduction, further chain elongation steps are limited generally to zero or one further cycle. In earlier work,[31] we developed conditions for one cycle of *safe* coupling/deprotection (Boc chemistry) of Boc-Cys(Scm)-OH; keys to success were to minimize the time of the neutralization step after TFA-promoted removal of Boc and to carry out coupling of the incoming protected amino acid residue in CH_2Cl_2 as solvent rather than DMF. The results were extended to Boc-Cys(Snm)-OH (**4b)**, which proved to be a somewhat superior derivative with respect to resisting the $S \rightarrow N$ migration presumed to be responsible for decreased incorporation under suboptimal conditions.[31] With this as background, a series of experiments were designed (Scheme 3) to address whether *S*-Scm and/or *S*-Snm could be used in conjunction with Fmoc chemistry, which features piperidine-promoted deprotection steps.

For these model studies, a tripeptide spacer (Gly-βAla-Gly) was built onto a PAL−PEG−PS support, and portions were extended by Mpa blocked with each of Snm, Scm, and Acm. In the first two cases, acidolytic release of the resultant tetrapeptides from the supports provided the needed *S*-Snm and *S*-Scm standards for HPLC and mass spectral analysis, while in the third case, the *S*-Acm group was converted in several ways to resin-bound *S*-Scm, *S*-Snm, or a novel *S*-(*N*-piperidylcarbamoyl)sulfenyl (Snip) derivative, all of which were evaluated by HPLC and FABMS after acidolytic cleavage. Most reliable was to treat the Acm-containing peptide with (chlorocarbonyl)sulfenyl chloride (**5**) followed by *N*-methylaniline or piperidine *in* CH_2Cl_2. The corresponding *S*-Snm or *S*-Snip derivatives were formed in good yields and purities, with negligible cleavage of the PAL linkages throughout the on-resin reaction sequence. The *S*-Scm group was best established from the *S*-Acm precursor by direct reaction with (methoxycarbonyl)sulfenyl chloride (**6**), with the two-step procedure of reaction first with **5** followed by methanol giving a less homogeneous product mixture.

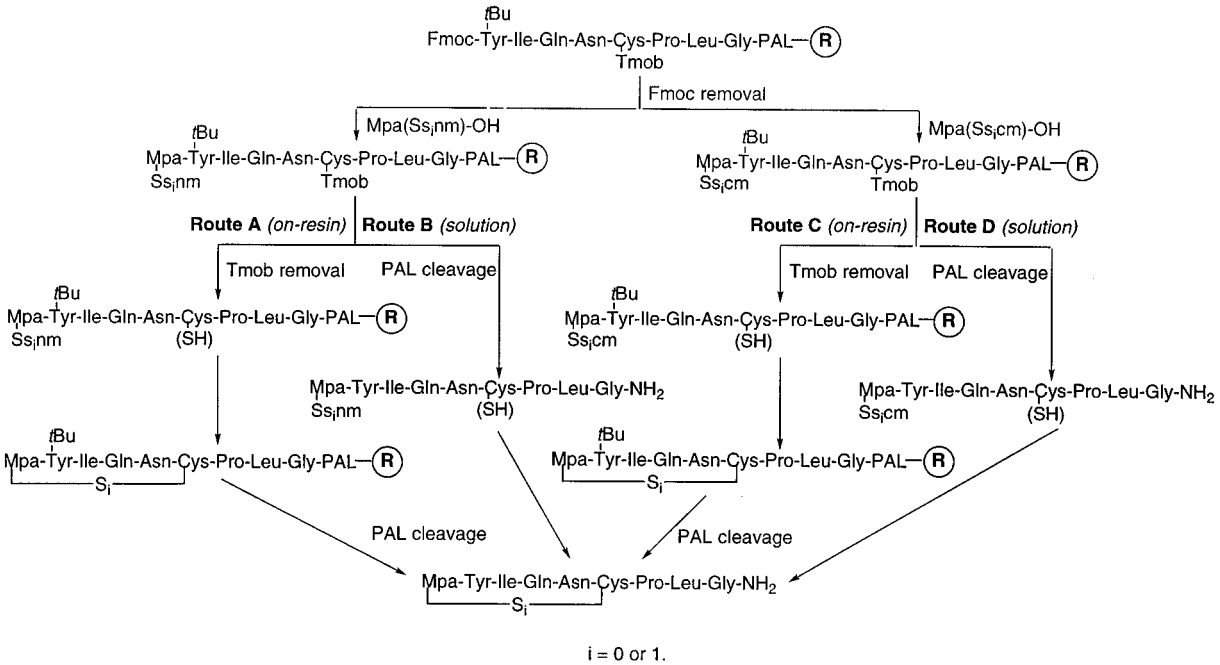
Treatment of the protected model tetrapeptides under standard Fmoc removal conditions, i.e., piperidine−DMF (1:4), 10 min, 25 °C, revealed rapid and complete

Scheme 3. Model Studies on *S*-Protecting Group Stability[a]



[a] See text and the Experimental Section for conditions of protected amino acid incorporation, Fmoc removal, transformations of the *S*-Acm group, and final PAL cleavage. The (S) in parentheses implies that it is the sulfur part of the *N*-terminal Mpa residue.

Scheme 4. Routes for Directed Formation of Sulfur−Sulfur Bridges in Deaminooxytocin[a]



[a] See text and the Experimental Section for conditions of protected amino acid incorporation, Fmoc removal, *S*-Tmob removal, and PAL cleavage. All of the indicated routes were implemented in the deaminooxytocin (disulfide) system ($i = 0$; *S*-Scm or *S*-Snm used to protect *N*-terminal Mpa residue). The (S) in parentheses implies that it is the sulfur part of the Cys incorporated within the peptide chain. For deaminooxytocin trisulfide ($i = 1$; *S*-Sscm or *S*-Ssnm used to protect *N*-terminal Mpa residue), route B was the most successful; results with other routes are discussed in the text. Extensions of this chemistry to the oxytocin family are also covered in the text.

loss of protection with dichotomous outcomes depending on what the initial *S*-protecting group was. The *S*-Scm group was converted to a complex mixture of products, which were difficult to identify at the peptide level. An organic model system with $CDCl_3$ as solvent showed that the Scm carbonyl was susceptible to nucleophilic cleavage, by piperidine displacing either methanol or the dithio moiety, followed by sequential further reactions of the presumed intermediates and initial products.[49−52] On the other hand, both the peptide and organic[53] model systems revealed that the *S*-Snm group was transformed rather cleanly to the *S*-Snip group (authentic standard available by alternative route; Scheme 3). This result implies that nucleophilic attack by piperidine at the Snm carbonyl results in unidirectional displacement of *N*-methylaniline. In the peptide model, switching the solvent for Fmoc removal from DMF to CH_2Cl_2[53−55] showed a considerably slower rate for *S*-Snm to *S*-Snip conversion ($t_{1/2} \sim 8.5$ min). It follows that we have yet to identify conditions whereby *S*-Snm can survive fully even a single cycle of Fmoc chemistry. Nevertheless, this realization does not present an insurmountable obstacle, in light of our subsequent discovery that *S*-Snip derivatives can also participate (as does *S*-Snm) in intramolecular directed disulfide formation reactions (see Scheme 5, later, for an application of this insight).

Formation of Deaminooxytocin (Disulfide). We have developed an effective mild orthogonal solid-phase synthesis of deaminooxytocin, using *N*-terminal Mpa(Snm) and internal Cys(Tmob) (Scheme 4, route A; Table 1, entry 4). The *S*-Tmob group was removed selectively under the "low" TFA conditions, and cyclization mediated by dilute (1%) *N*-methylmorpholine (NMM) in DMF led to formation of principally the desired intramolecular disulfide (80% of the crude product directly after cleavage with "high" TFA). Interestingly,

Table 1. Cyclization of Mpa(Snm)-Tyr(*t*-Bu)-Ile-Gln-Asn-Cys-Pro-Leu-Gly-PAL-PEG-PS in Various Solvent Systems[a]

entry no.	cyclization milieu (25 °C, 2 h)	product distribution (% by HPLC)		
		disulfide	trisulfide	linear
1	untreated control	2		92
2	HOAc−CH_2Cl_2 (1:9)	3		85
3	NMM−CH_2Cl_2 (1:99)	34	5	47
4	NMM−DMF (1:99)[b]	80	10	
5	solution (pH 8)[b,c]	85	5	

[a] Protocols are in the Experimental Section. Product distribution is based on total integration of peaks detected by analytical HPLC (e.g., Supporting Information Figure 1). Retention times are disulfide, t_R 14.7 min; trisulfide, t_R 17.0 min; linear disulfide precursor, t_R 22.1 min. [b] This experiment was repeated on a larger scale. [c] Linear peptide concentration was 2 mM in CH_3CN−10 mM phosphate buffer (1:1).

the major byproduct from the cyclization was the corresponding trisulfide (5−10%, *vide infra*; further 2% tetrasulfide noted). As controls, negligible disulfide was observed when the linear precursor was cleaved directly under "high" TFA conditions (Table 1, entry 1), nor was disulfide noted when solid-phase treatment with HOAc−CH_2Cl_2 (1:9) preceded the cleavage (entry 2). The role of the solvent was illustrated by the sluggish cyclization promoted by NMM in CH_2Cl_2 (34% disulfide and 47% unreacted linear; see Table 1, entry 3, and compare to entry 4 observed in DMF). The experiment using NMM in DMF to promote solid-phase cyclization was carried out preparatively (5 μmol scale), and semipreparative high-performance liquid chromatography (HPLC) purification gave analytically pure (>99% by HPLC) deaminooxytocin (disulfide) in an absolute isolated yield of ~33% based on the initial loading of PAL−PEG−PS.

The desired cyclization was also carried out readily in solution (Scheme 4, route B; Table 1, entry 5). The linear precursor with *N*-terminal Mpa(Snm) and internal Cys (free thiol), directly after acidolytic release from

Table 2. Cyclization of Mpa(Scm)-Tyr(*t*-Bu)-Ile-Gln-Asn-Cys-Pro-Leu-Gly-PAL-PEG-PS in Various Solvent Systems[a]

entry no.	cyclization milieu (25 °C, 2 h)	product distribution (% by HPLC)		
		disulfide	trisulfide	linear
1	untreated control	36	1	56
2	HOAc−CH_2Cl_2 (1:9)	36	2	50
3	NMM−CH_2Cl_2 (1:99)	58	2	28
4	CH_3I (10 equiv)−CH_2Cl_2	54	3	25
5	DMF	79	5	
6	NMM−DMF (1:99)	70	1	
7	CH_3I (10 equiv)−DMF	80	2	

[a] Details match those in the corresponding footnote of Table 1.

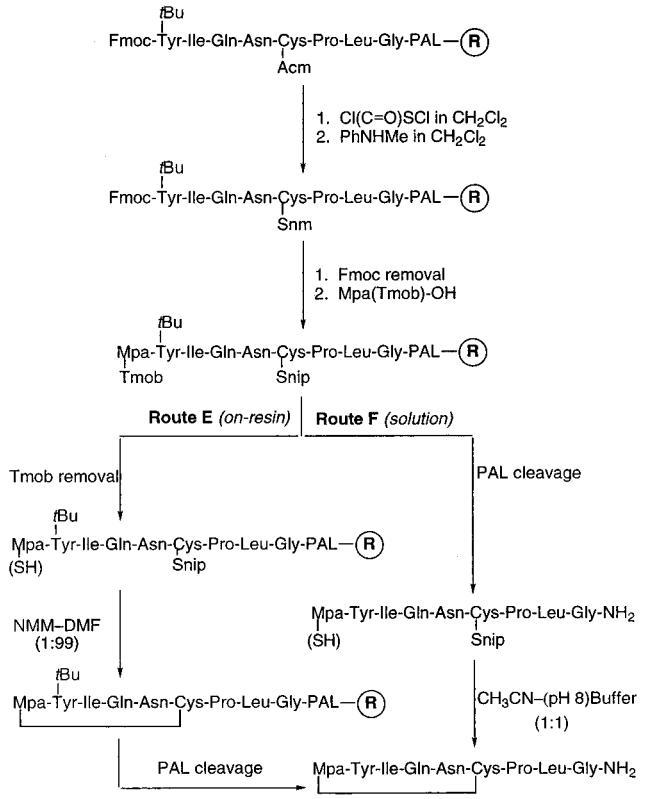
the support, was dissolved in CH_3CN−10 mM phosphate buffer (1:1) at pH 8 (final concentration ~ 2 mM). Disulfide was the predominant product (87%), with small amounts of tri- and tetrasulfide. The absolute isolated yield of the solution experiment was 56%, somewhat better than the solid-phase results (same scale) already cited.

In contrast to the clear-cut nature of the results reported in the preceding paragraphs, corresponding experiments using *N*-terminal Mpa(Scm) gave more ambiguous data (Scheme 4, route C; Table 2). Thus, mere "high" TFA cleavage already revealed that about one-third of the chains had cyclized (Table 2, entry 1; unchanged when the peptide-resin was incubated first in the presence of HOAc−CH_2Cl_2 (1:9)). In the presence of NMM (1%), these values increased to almost 60% with CH_2Cl_2 as solvent (entry 3) and 70% with DMF as solvent (entry 6; note that in DMF, the linear material was transformed completely). Indeed, the amount of cyclic disulfide was almost 80% when the cyclization milieu was DMF, without added base (entry 5). We drew the indirect conclusion that some on-resin cyclization occurs and sought to provide further mechanistic evidence by adding iodomethane in an attempt to trap free sulfhydryl groups. However, no *S*-alkylated intermediates were formed, and the results in CH_2Cl_2 (Table 2, entry 4) and DMF (entry 7), respectively, were nearly identical with those in the same solvent without CH_3I. In total, these data indicate that *S*-Scm is more labile than *S*-Snm and that application of the latter derivative allows more control over the chemistry of multistep schemes.

We also explored the reciprocal strategy[56] for deaminooxytocin using *N*-terminal Mpa(Tmob) and internal Cys(Snm) (Scheme 5). As indicated earlier, Cys[6] in the linear octapeptide-resin precursor was blocked initially by the *S*-Acm group, which was converted to *S*-Snm. Preparatory to coupling the Mpa residue, a piperidine step to deblock Tyr[2] resulted in the simultaneous conversion of *S*-Snm to *S*-Snip. After selective acidolytic removal of *S*-Tmob, on-resin cyclization was promoted with dilute NMM in DMF. The desired disulfide was the major product (~67%), and the absolute isolated yield was ~19%.[56] Results were comparable when cyclization was carried out in solution at pH 8 (reaction complete in 30 min: initial purity 57%, isolated yield 27%).[56]

Formation of Oxytocin (Disulfide). Since the most unambiguous methods for making deaminooxytocin had *N*-terminal Mpa(Snm) and internal Cys(Tmob) (Scheme 4, route A), we repeated selected experiments for the oxytocin structure. For on-resin cyclizations, the

Scheme 5. Reciprocal Strategies for Directed Synthesis of Sulfur−Sulfur Bridges in Deaminooxytocin[a]



[a] See text and the Experimental Section for conditions of conversion of *S*-Acm to *S*-Snm, Fmoc removal, protected amino acid incorporation, *S*-Tmob removal, and PAL cleavage. The (S) in parentheses implies that it is the sulfur part of the *N*-terminal Mpa residue.

Table 3. Cyclization of (Boc/H)-Cys(Snm)-Tyr(*t*-Bu)-Ile-Gln-Asn-Cys-Pro-Leu-Gly-PAL-PEG-PS in Various Solvent Systems[a]

entry no.	cyclization milieu (25 °C, 2 h)	product distribution (% by HPLC)	
		disulfide	linear
1	untreated control	31	66
2	HOAc−CH_2Cl_2 (1:9)	43	53
3	NMM−CH_2Cl_2 (1:99)	80	
4	solution (pH 8)[b]	87	

[a] Details match those in the corresponding footnote of Table 1. It is uncertain to what extent the *N*-terminal Boc protecting group is removed under the conditions for selective on-resin *S*-Tmob removal. [b] Linear peptide concentration was 2 mM in CH_3CN−10 mM phosphate buffer (1:1).

N-terminal Cys(Snm) may have partially retained N^{α}-Boc protection (see Table 3, footnote a). A further complication was that "high"-TFA cleavage by itself resulted in ~30% cyclized oxytocin (Table 3, entry 1); the level of cyclization increased somewhat to ~40% after preincubation in the presence of HOAc−CH_2Cl_2 (1:9). Nevertheless, the result from cyclization promoted by dilute NMM in CH_2Cl_2 clearly suggests that the solid-phase mode is effective (Table 3, entry 3). In addition, solution cyclization (CH_3CN−10 mM phosphate buffer (1:1) at pH 8) gave an excellent yield and purity of the desired disulfide.

Formation of Deaminooxytocin Trisulfide. Experiments to homologate the successful deaminooxytocin syntheses reported earlier in this paper used *N*-terminal Mpa(Ssnm) but were otherwise comparable (Scheme 2). Solid-phase cyclization mediated by NMM in DMF (Scheme 4, route A; Table 4, entry 10) revealed, after

Table 4. Cyclization of Mpa(Ssnm)-Tyr(*t*-Bu)-Ile-Gln-Asn-Cys-Pro-Leu-Gly-PAL-PEG-PS in Various Solvent Systems[a]

entry no.	cyclization milieu (25 °C, 2 h)[b]	product distribution (% by HPLC) di-sulfide	tri-sulfide	tetra-sulfide	linear
1	untreated control	3	7		84
2	HOAc−CH_2Cl_2 (1:4)	1	8		77
3	HOAc−CH_2Cl_2 (1:9)[b]	3	9		67
4	HOAc−CH_2Cl_2 (1:9)[b]	8	24	3	45
5	HOAc−CH_2Cl_2 (1:9)[b]	11	28	4	56
6	HOAc−CH_3CN (1:9)	3	9		61
7	collidine−CH_3CN (1:99)	8	15		70
8	DMF	9	14	1	61
9	HOAc−DMF (1:9)	5	14	2	63
10	NMM−DMF (1:99)	27	24	18	5

[a] Details match those in the corresponding footnote of Table 1. [b] For lines 3−5, reaction times were respectively 2, 24, and 72 h.

Table 5. Solution Cyclization of Mpa(Ssnm)-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2 as a Function of pH[a]

entry no.	pH	product distribution (% by HPLC) disulfide	trisulfide	tetrasulfide	linear
1	1	3	8	0.1	48
2	1[b]	24	57	9	
3	2[c]	27	51	9	0.2
4	4	30	39	15	1.6
5	6	43	29	12	0.2
6	7	42	27	11	0.7
7	8	39	25	7	

[a] Linear peptide concentrations were 2 mM in CH_3CN−10 mM phosphate buffer (1:1), and reactions were carried out for 2 h unless indicated otherwise (see note b). Other details match those in the corresponding footnote of Table 1. [b] Reaction for 60 h. [c] This experiment was repeated on a larger scale.

cleavage in "high" TFA, that the linear precursor had reacted to form an approximately equimolar mixture of three products. The major of these was coeluted with deaminooxytocin (disulfide), the second one was identified as the novel desired trisulfide, and the third product was assigned to be the corresponding cyclic tetrasulfide. Although acidic conditions were found to favor the formation of trisulfide over disulfide (Table 4, entries 2 and 3, 6 vs 7, 9 vs 8), such solid-phase reactions were slow and could not be driven to completion (Table 4, entries 3 vs 4 vs 5).

The problems with the solid-phase route to the deaminooxytocin trisulfide target led us to consider solution cyclizations (Scheme 4, route B). The linear precursor directly after acidolytic release from the support was dissolved (final concentration ∼2 mM) in CH_3CN−10 mM phosphate buffer (1:1) at a variety of pH values (Table 5), and further transformations were monitored by HPLC (cf. Supporting Information Figure 1). The relative amount of desired trisulfide increased with decreasing pH. At pH 1, the ratio of di-:tri-:tetrasulfide was 3:8:1, but the cyclization reactions required 60 h to reach completion. Consequently, we chose to conduct solution cyclization at pH 2, where reaction was complete in 2 h to provide a mixture of di-, tri-, and tetrasulfides in an acceptable ratio of 1:2:0.3 (e.g., Supporting Information Figure 1C). The experiment was carried out preparatively (12 μmol scale), and semipreparative HPLC purification gave analytically pure (>99% by HPLC; see Supporting Information Figure 1D) deaminooxytocin trisulfide in an absolute isolated yield of ∼40% based on the initial loading of PAL−PEG−PS. At the same time, analytically pure (>99% by HPLC) deaminooxytocin (disulfide, 24%) and deaminooxytocin tetrasulfide (7%) were also isolated and characterized.

Table 6. Cyclization of Mpa(Sscm)-Tyr(*t*-Bu)-Ile-Gln-Asn-Cys-Pro-Leu-Gly-PAL-PEG-PS in Various Solvent Systems[a]

entry no.	cyclization milieu (25 °C, 2 h)	product distribution (% by HPLC) di-sulfide	tri-sulfide	tetra-sulfide	linear
1	untreated control	13	29	11	6
2	HOAc−CH_2Cl_2 (1:9)	17	33	11	6
3	NMM−CH_2Cl_2 (1:99)	20	29	10	8
4	collidine−CH_2Cl_2 (1:99)	23	30	10	

[a] Details match those in the corresponding footnote of Table 1.

In view of the earlier results in the disulfide system showing the greater reliability of *S*-Snm over *S*-Scm, it was not too surprising to learn that homologations with *N*-terminal Mpa(Sscm) showed no benefit to carrying out solid-phase incubations prior to cleavage (Scheme 4, routes C and D; Table 6). The most important practical result of those experiments was that the desired trisulfide was the major product, along with lesser amounts of di- and tetrasulfides, and little linear material remained.

Formation of Oxytocin Trisulfide. As before, various strategies already discussed for the deaminooxytocin structure were tested. Cyclizations were more facile than in the deamino system, but there was no clear advantage to on-resin cyclization (problem alluded to earlier with "untreated controls"). Using Fmoc-Cys(Ssnm)-OH (**4f**) to introduce the *N*-terminal residue, carrying out on-resin cyclization in the presence of HOAc−CH_2Cl_2 (1:9), and cleaving from the support, the *N*-Fmoc derivative of oxytocin trisulfide was obtained.[57] However, when from the same experiment the Fmoc group was removed with either piperidine−DMF (1:4), 1,8-diazobicyclo[5.4.0]undec-7-ene (DBU)−piperidine−DMF (1:1:48),[58] or 0.1 M tetrabutylammonium fluoride in DMF,[59] followed by cleavage, the major product was oxytocin (disulfide). Thus, it is unlikely that the trisulfide survives[60] the base treatment that is normally used to remove Fmoc. The strategy was therefore modified to use Boc-Cys(Ssnm)-OH (**4e**) for introduction of the *N*-terminal residue, and the experiment was carried out preparatively (12 μmol scale). Without any on-resin pretreatment to effect cyclization, the crude peptide obtained after cleavage was dissolved in HOAc−H_2O (1:4; final concentration ∼ 2 mM). Analytical HPLC revealed a 1:1 mixture of di- and trisulfides. Semipreparative HPLC purification gave analytically pure (>99% by HPLC) oxytocin trisulfide in an absolute isolated yield of ∼25% based on the initial loading of PAL PEG−PS. At the same time, oxytocin (disulfide) and oxytocin dimers[61] were also isolated and characterized.

Stabilities of Oxytocin and Deaminooxytocin Trisulfides. Samples for biological testing (see below) journeyed safely from Minneapolis to Prague by express mail and were dissolved first in 1 mM aqueous HCl to create 1 mg/mL stock solutions. These stocks were diluted further 10- and 100-fold in 0.154 M NaCl (physiological saline); stock solutions and dilutions were stored at −20 °C, and samples were periodically thawed and evaluated by HPLC. There was no change for either oxytocin or deaminooxytocin trisulfide throughout 4 weeks, the time frame by which biological assays were

Table 7. Biological Activities of Some Oxytocin Analogues

analogue	activities (IU/mg) uterus *in vitro*	uterus *in vivo*	pressor	K_i (nM)
oxytocin	510	450	5.0	1.7
oxytocin trisulfide	27.8 ± 8.0	180[a] ± 12	1.8[a] ± 0.6	6.0
deaminooxytocin	576	541[a]	5.0	0.9
deaminooxytocin trisulfide	32.7 ± 10	135[a] ± 21	0.6[a] ± 0.2	3.0

[a] Protracted action (discussed in text).

carried out on these samples. Even after one-half year of cold storage with occasional thawing and refreezing, the deaminooxytocin trisulfide stock was >90% pure while the oxytocin trisulfide was ~50%. The more dilute (0.1 mg/mL) solution of oxytocin trisulfide was >90% pure after the very extended storage. The deaminooxytocin trisulfide proved to be surprisingly stable as a lyophilized powder at 25 °C (>97% remaining after 2 weeks, ~70% after 5 weeks with the remainder 22% disulfide and 8% tetrasulfide).

Pharmacological Properties of Novel Oxytocin Trisulfides. Activities of purified peptides were measured in three assays: uterotonic *in vitro*,[62,63] uterotonic *in vivo*,[64] and pressor tests[65] (Table 7). Synthetic oxytocin and deaminooxytocin (disulfides) made by the directed methods described in this paper showed activities quite close to the literature-expected values.[66] The corresponding trisulfide analogues also had substantial potencies, ranging from 5% to 40% of the parent activities, depending on the assay. Activities of trisulfides were prolonged (with respect to oxytocin) *in vivo* (Supporting Information Figure 2). In the case of the uterotonic *in vivo* test, the magnitude of the contractions induced by either trisulfide is somewhat lower by comparison to the oxytocin control, but the overall activity *integrated over time* is quite high. The dose–response curves (as typified in Supporting Information Figure 3) for the trisulfides hence have much steeper slopes than those for oxytocin and are quite comparable to those observed for deaminooxytocin. In the case of the *in vivo* pressor test which monitors interactions of peptides with a different receptor, enhancement of blood pressure due to administration of oxytocin or deaminooxytocin trisulfides is less pronounced than upon administration of the corresponding disulfides (see Supporting Information Figure 4 for representative data). However, the response peaks from trisulfides are quite broad in contrast to the sharper peaks with the disulfides: for a given maximal response (dosages adjusted accordingly) the time after application of hormone to reach the maximum is 4–5-fold longer with trisulfides, and the rate of return to baseline levels is 3–4-fold decreased (with a medium dose of peptide, the time interval for the drop to half-maximal response is 3 min for disulfide and 10 min for trisulfide; see Supporting Information Figure 4). The prolongation effects were also observed from the *in vitro* uterotonic test, which involves cumulative dosages (peptide added at 1-min intervals until maximal contractile response reached). Whereas, in the case of oxytocin, removal of the peptide from the bath medium leads to *immediate* cessation of uterine muscle contractions, the trisulfide-induced contractions were observed to continue for 5–10 min.

In order to understand better the reasons for the differences in activities, competition experiments with tritiated oxytocin were carried out to determine binding affinities of test peptides to uterine membranes, according to the procedure described by Fahrenholz et al.[67,68] All affinities were high (Table 7, far right column); the 3.3–3.6-fold lower relative affinity of trisulfides with respect to disulfides corresponded better to the ratio of biological activities in the *in vivo* uterotonic (2.5–4-fold difference) than in the *in vitro* uterotonic (18-fold difference) tests. We expected the agreement to be closer between *in vitro* binding and *in vitro* assay; hence the actual result is surprising and difficult to explain.

The protracted actions of the trisulfide peptides are of interest. Such phenomena have been described previously for deamino[69–71] and carba[71] analogues of oxytocin, but the reasons are not completely understood. One possible explanation is sequestration of peptide in the receptor compartment of the membrane. In principle, biologically active peptide may accumulate; it may undergo metabolic transformation; the formation as well as the dissociation of the receptor–ligand complex may have different kinetics,[72] or processing of and/or signal transduction from the complex may be different.[73–76] The present data showing differing response profiles and high affinities (same order of magnitude as native interaction) suggest that the biological activities observed are due to the trisulfide structure rather than to metabolic desulfurization to the parent disulfide under physiological conditions.

Conclusions

Novel directed methods for construction of sulfur–sulfur bridges have been developed and applied to the preparation of oxytocin and deaminooxytocin (disulfides), as well as the corresponding trisulfides (linear sequences assembled by Fmoc solid-phase chemistry). Strategies were worked out for the introduction and use of sulfenyl thiocarbonate (*S*-Scm) and carbamoyl disulfide (*S*-Snm) protecting/activating groups, as well as their homologues (*S*-Sscm, *S*-Ssnm). For deaminooxytocin, intramolecular cyclizations to form disulfide bridges proceed in the solid-phase mode or in dilute mixed aqueous solution at pH 8. In contrast, trisulfide formation was found to be best in solution at pH 2, conditions which allowed for cyclization with relatively controllable levels of di- and tetrasulfide disproportionation byproducts. For oxytocin, cyclization is much more facile, perhaps due to the increased nucleophilicity of the attacking thiol which is proximal to an N^{α}-amino group. As a consequence, linear precursors set up for intramolecular directed synthesis formed products, in whole or in part, already upon direct workup of material released from the solid support. Carbamoyl-type groups are preferable to (methoxycarbonyl)sulfenyl-type groups by several criteria. Synthetic routes which involve attack by the thiol of an internal cysteine residue on a terminal *S*-Snm or *S*-Scm moiety appear to give higher yields and purities of products than experiments carried out with the reciprocal orientations;[56] the latter studies included an unexpected twist insofar as the true electrofugal[30] species after Fmoc removal with piperidine was the new *S*-Snip carbamoyl disulfide. It remains to be seen how widely applicable the methods developed here will be to other peptide families that normally contain intramolecular disulfide bridges.

Turning to the biology, the relatively high activities and prolonged actions of the oxytocin and deaminooxytocin trisulfides in a number of assays are of considerable theoretical and practical interest. The conventional wisdom in the oxytocin field has been that a 20-membered ring is crucial for activity, much more so than the atoms comprising it. However, we have shown here that the 21-membered ring-containing trisulfide analogues of oxytocin are highly potent. We believe that the data presented herein are consistent with the hypothesis that the observed biological activities truly reflect interaction of the intact trisulfide molecules, rather than their putative metabolic products, with the cognate receptors. Further studies are required to elucidate the molecular bases of these effects and to draw conclusions about the active conformation of oxytocin. We hope that generalizations to other peptide and non-peptide structural families will prove insights on the possible significance of bridging, by three versus two sulfurs, as important motifs for natural products.

Experimental Section

General. Most of the materials, solvents, instrumentation, and general methods have been described and summarized in our previous publications.[28,29,31−33,38,41−44] In particular, (chlorocarbonyl)sulfenyl chloride (**5**) and (methoxycarbonyl)-sulfenyl chloride (**6**) were prepared as described by us previously.[38] ^{1}H NMR spectra were observed in the indicated deuterated solvents with IBM NR 200 AF, IBM NR 300 AF, or Varian VXR 300 instruments. Exchangeable protons are not reported. Electron ionization mass spectra were obtained on a Finnigan MAT 95 instrument at the indicated eV and a source temperature of 200 °C. Low-resolution fast atom bombardment mass spectroscopy (FABMS) was carried out on a VG Analytical 707E-HF low-resolution double-focusing mass spectrometer equipped with a VG 11/250 data system, operated at a resolution of 2000 and 4000. Elemental analyses were performed by M-H-W Laboratories, Phoenix, AZ.

Protected Fmoc amino acid derivatives, as well as Fmoc PAL−PEG−PS supports (initial loading 0.14−0.22 mmol/g) for peptide synthesis, were mainly from the Biosearch Division of PerSeptive Biosystems (Framingham, MA). Additional suppliers of protected derivatives, including the *S*-Acm derivatives **2a**,**b**, were Advanced Chemtech (Louisville, KY), Bachem Bioscience (Philadelphia, PA), and Peptide International (Louisville, KY). Mpa(Tmob)-OH (**1a**),[33] Fmoc-Cys(Tmob)-OH (**1b**),[32] and Boc-Cys(Snm)-OH (**4b**)[31] were prepared as described in our earlier reports. Piperidine, TFA, *N*,*N*-diisopropylethylamine (DIEA), and HOBt were from Fisher (Pittsburgh, PA). Peptide chain assembly was carried out either manually or on a PerSeptive (formerly MilliGen/Biosearch) Model 9050 continuous-flow synthesizer, according to the general plans indicated in the text. Fmoc removal was achieved with piperidine−DMF (1:4, 10 min). DIPCDI/HOBt couplings (4 equiv over resin-bound amine) involved minimal (<2 min) preactivation time and 1−2 h of reaction. Washings between chemical steps were carried out with DMF. The common peptide-resin intermediates Fmoc-Tyr(*t*-Bu)-Ile-Gln-Asn-Cys(Tmob)-Pro-Leu-Gly-PAL-PEG-PS and Fmoc-Tyr(*t*-Bu)-Ile-Gln-Asn-Cys(Acm)-Pro-Leu-Gly-PAL-PEG-PS were prepared on 1 g scales, and portions were extended with the suitable Cys or Mpa derivative and manipulated further as outlined in the text and Supporting Information figures. Upon completion of on-resin steps, the peptide-resins (∼20 mg) were washed with CH_2Cl_2, and then cleavages were carried out with TFA−CH_2Cl_2−Et_3SiH−phenol−H_2O (92:5:1:1:1) (2 mL) for 2 h. The cleavage mixtures were then filtered, and washed with the same cocktail (2 × 2 mL). The combined filtrates were concentrated by bubbling through a stream of N_2, and then Et_2O−CH_2Cl_2 (10 mL of 20:1 mixture) was added at 0 °C. The resultant precipitated peptides were collected by low-speed centrifugation, washed with cold ether (10 mL), and dissolved in a suitable medium for analytical HPLC. The 10 mM phosphate buffers used as cosolvent for solution cyclization studies were prepared starting with monobasic sodium phosphate and adjusting to the desired pH with either phosphoric acid or 10 N aqueous NaOH.

Amino acid analyses were performed on a Beckman 6300 analyzer with a sulfated polystyrene cation-exchange column (0.4 cm × 21 cm). Peptide-resins were hydrolyzed in 12 N HCl−propionic acid (1:1, v/v) + 2 drops of liquefied phenol for 1 h at 160 °C, and free peptides were hydrolyzed with 6 N aqueous HCl at 160 °C for 1 h + 2 drops liquefied phenol to prevent degradation of Tyr. Analytical HPLC was performed using a Vydac analytical C-18 reversed-phase column (218TP54; 5 μm, 300 Å, 0.46 × 25 cm) on a Beckman system configured with two Model 112 pumps and a Model 165 variable wavelength detector controlled from an IBM computer with Beckman System Gold software. Peptide samples (oxytocin and deaminooxytocin families) were chromatographed at 1.2 mL/min using a linear gradient over 20 min of 0.1% aqueous TFA and 0.1% TFA in acetonitrile from 9:1 to 3:2, detection at 220 nm. Model peptides (Scheme 3) were chromatographed in essentially the same way, except the linear gradient was from 0:10 to 7:13. Semipreparative HPLC to purify crude peptide products obtained after cleavage was performed on a Vydac semipreparative C-18 reversed-phase column (218TP1010; 10 μm, 300 Å, 1.0 × 25 cm) on a Waters Deltaprep system using manual injection and elution at 5 mL/min with a linear gradient of 0.1% aqueous TFA−CH_3CN from 83:17 to 73:27 over 45 min (deaminooxytocin system) or 17:3 to 7:3 over 40 min (oxytocin system), detection at 220 nm. Fractions with the correct peptide were pooled and lyophilized to provide white powders. The final isolated yields were calculated by comparison of amino acid analyses of the purified peptide to those on the initial loaded resin.

***S*-[(Methoxycarbonyl)sulfenyl]-β-thiopropionic Acid [Mpa(Scm)-OH, 3a].** A solution of β-mercaptopropionic acid (1.74 mL, 20 mmol) in CH_2Cl_2 (30 mL) was reacted with *N*,*O*-bis(trimethylsilyl)acetamide (5.44 mL, 22 mmol) for 1.5 h, following which the mixture was added dropwise over 20 min into an ice−salt bath-cooled solution of (methoxycarbonyl)-sulfenyl chloride (**6**) (1.80 mL, 20 mmol) in CH_2Cl_2 (10 mL). The yellow color faded, and the cloudy reaction mixture was brought to 25 °C. Stirring proceeded for 2 h, and then H_2O (50 mL) was added for a further 30 min of stirring. The organic phase was dried (Na_2SO_4) and concentrated to provide a yellow liquid (3.97 g), purified further by distillation, bp 150−152 °C (1 mmHg). Yield: 2.5 g (64%), a liquid which solidifies at −20 °C. ^{1}H NMR ($CDCl_3$): δ 3.90 (s, 3H), 3.02 (t, 2H), 2.79 (t, 2H). EIMS: *m*/*z* calcd 196.0, found 195.9 [$M^{\bullet+}$]. Anal. Calcd for $C_5H_8O_4S_2$ (mol wt 196.24): C, 30.60; H, 4.41; S, 32.67. Found: C, 30.82; H, 4.16; S, 32.90.

***S*-[(Methoxycarbonyl)disulfanyl]-β-thiopropionic Acid [Mpa(Sscm)-OH, 3b].** In the same way as **3a** but on a 3.5 mmol scale of Mpa-OH and using (methoxycarbonyl)disulfanyl chloride (**10**) (0.5 g, 3.2 mmol) in CH_2Cl_2 (10 mL), the crude product after reaction and workup was a yellowish liquid (0.83 g) purified further by flash chromatography (CH_2Cl_2−MeOH, 30:1) to provide an off-white liquid (0.42 g, 58%) which solidified at −20 °C. ^{1}H NMR ($CDCl_3$): δ 3.92 (s, 3H), 3.17 (t, 2H), 2.96 (t, 2H). EIMS: *m*/*z* calcd 228.0, found 227.9 [$M^{\bullet+}$]. Anal. Calcd for $C_5H_8O_4S_3$ (mol wt 228.30): C, 26.31; H, 3.53; S, 42.13. Found: C, 26.12; H, 3.66; S, 42.00.

***S*-[(*N*-Methyl-*N*-phenylcarbamoyl)sulfenyl]-β-thiopropionic Acid [Mpa(Snm)-OH, 4a].** A solution of (chlorocarbonyl)sulfenyl chloride (**5**) (0.42 mL, 5 mmol) in $CHCl_3$ (5 mL) was added dropwise over 5 min at 0−5 °C into an ice bath-chilled suspension of Mpa(Acm)-OH (**2a**) (885 mg, 5 mmol) in $CHCl_3$ (20 mL). After an additional 10 min of stirring, the reaction mixture was filtered and the filtrate was added dropwise at 0 °C to a solution of *N*-methylaniline (5.42 mL, 50 mmol) in $CHCl_3$ (15 mL). After a further 20 min, the reaction mixture was washed with 1 N aqueous HCl (2 × 50 mL) and H_2O (50 mL). The organic phase was dried ($MgSO_4$), concentrated *in vacuo*, and purified by flash chromatography ($CHCl_3$−HOAc, 100:1) to provide a colorless sticky liquid which became crystalline after several weeks at −20 °C. Yield: 1.08

g (80%), mp 59−61 °C. ^{1}H NMR ($CDCl_3$): δ 7.25−7.49 (m, 5H), 3.36 (s, 3H), 2.93 (t, 2H), 2.72 (t, 2H). FABMS: *m/z* calcd 271.1, found 272.1 [$(MH)^+$]. Anal. Calcd for $C_{11}H_{13}NO_3S_2$ (mol wt 271.35): C, 48.69; H, 4.83; N, 5.16; S, 23.63. Found: C, 48.79; H, 4.83; N, 5.03; S, 23.49.

N^{α}-(9-Fluorenylmethyloxycarbonyl)-*S*-[(*N*-methyl-*N*-phenylcarbamoyl)sulfenyl]-L-cysteine [Fmoc-Cys(Snm)-OH, 4c]. Modeled on the published procedure for the corresponding Boc derivative **4b**,[31] (chlorocarbonyl)sulfenyl chloride (**5**) (81 μL, 1 mmol) in $CHCl_3$ (10 mL) was added dropwise over 5 min into an ice bath-cooled suspension of Fmoc-Cys(Acm)-OH (**2b**) (415 mg, 1 mmol) in $CHCl_3$ (10 mL). After an additional 10 min of stirring, the reaction mixture was added dropwise into an ice bath-cooled solution of *N*-methylaniline (1.1 mL, 10 mmol) in $CHCl_3$ (10 mL). After 20 min, the reaction mixture was washed with 1 N aqueous HCl (2 × 25 mL) and H_2O (25 mL). The organic phase was dried ($MgSO_4$) and purified by flash chromatography ($CHCl_3$−MeOH−HOAc, 80:1:1) to provide a yellow expanded mass. Yield: 0.26 g (51%), mp 50−53 °C. ^{1}H NMR ($CDCl_3$): δ 7.19−7.81 (m, 14H), 6.66 (d, NH), 4.43 (m, 2H), 4.28 (m, 1H), 3.44 (s, 3H), 3.35 (dd, 1H), 2.92 (dd, 1H). FABMS: *m/z* calcd 508.2, found 509.3 [$(MH)^+$]. Anal. Calcd for $C_{26}H_{24}N_2O_5S_2$ (mol wt 508.61): C, 61.4; H, 4.76; N, 5.51; S, 12.61. Found: C, 61.6; H, 4.82; N, 5.28; S, 12.83.

S-[(*N*-Methyl-*N*-phenylcarbamoyl)disulfanyl]-β-thiopropionic Acid [Mpa(Ssnm)-OH, 4d]. The title compound was made in the same way and on the same scale as **4a** but using (chlorocarbonyl)disulfanyl chloride (**9**) (0.82 g, 5 mmol); the crude product was purified by flash chromatography ($CHCl_3$−HOAc, 100:1 followed by 19:1) to give light-yellow crystals (1.0 g) which were dissolved in minimal $CHCl_3$ and recrystallized by addition of hexane. Yield: 0.86 g (57%), white needles, mp 120−122 °C. ^{1}H NMR ($CDCl_3$): δ 7.25−7.43 (m, 5H), 3.37 (s, 3H), 3.16 (t, 2H), 2.94 (t, 2H). FABMS: *m/z* calcd 303.1, found 304.0 [$(MH)^+$]. Anal. Calcd for $C_{11}H_{13}NO_3S_3$ (mol wt 303.41): C, 43.55; H, 4.32; N, 4.62; S, 31.70. Found: C, 43.36; H, 4.13; N, 4.62; S, 31.49.

N^{α}-(*tert*-Butyloxycarbonyl)-*S*-[(*N*-methyl-*N*-phenylcarbamoyl)disulfanyl]-L-cysteine [Boc-Cys(Ssnm)-OH, 4e]. (Chlorocarbonyl)disulfanyl chloride (**9**) (489 mg, 3 mmol) in $CHCl_3$ (5 mL) was added dropwise over 5 min into an ice bath-cooled suspension of Boc-Cys(Acm)-OH (**2b**) (869 mg, 3 mmol) in $CHCl_3$ (20 mL). After an additional 10 min of stirring, the reaction mixture was filtered and the filtrate was added dropwise into an ice bath-cooled solution of *N*-methylaniline (3.25 mL, 30 mmol) in $CHCl_3$ (15 mL). Further workup analogous to **4c**, featuring flash chromatography ($CHCl_3$−HOAc, 50:1 and 19:1), provided a white expanded mass. Yield: 0.62 g (49%), mp 104−107 °C. ^{1}H NMR ($CDCl_3$): δ 7.27−7.43 (m, 5H), 5.60 (d, NH), 4.42 (m, 1H), 3.50 (dd, 1H), 3.38 (s, 3H), 3.27 (dd, 1H), 1.44 (s, 9H). FABMS: *m/z* calcd 418.1, found 419.1 [$(MH)^+$]. Anal. Calcd for $C_{16}H_{22}N_2O_5S_3$ (mol wt 418.54): C, 45.92; H, 5.30; N, 6.69; S, 22.98. Found: C, 45.82; H, 5.44; N, 6.46; S, 23.17.

N^{α}-(9-Fluorenylmethyloxycarbonyl)-*S*-[(*N*-methyl-*N*-phenylcarbamoyl)disulfanyl]-L-cysteine [Fmoc-Cys(Ssnm)-OH, 4f]. Starting with Fmoc-Cys(Acm)-OH (**2b**) (415 mg, 1 mmol) but otherwise (proportionally scaled) just as for **4e**, a crude product was obtained which was purified by flash chromatography ($CHCl_3$−MeOH−HOAc, 80:1:1) to provide an off-white expanded mass. Yield: 0.47 g (87%), mp 52−55 °C. ^{1}H NMR ($CDCl_3$): δ 7.17−7.77 (m, 14H), 6.39 (d, NH), 4.39 (d, 2H), 4.26 (m, 1H), 3.45 (dd, 2H), 3.38 (s, 3H). FABMS: *m/z* calcd 540.1, found 541.2 [$(MH)^+$]. Anal. Calcd for $C_{26}H_{24}N_2O_5S_3$ (mol wt 540.67): C, 57.76; H, 4.41; N, 5.18; S, 17.79. Found: C, 57.91; H, 4.60; N, 4.95; S, 17.64.

Acetyl Methoxythiocarbonyl Sulfide (7). Acetyl chloride (34 g, 0.43 mol) was added dropwise over 25 min to an ice bath-chilled suspension of potassium methyl xanthate (60.8 g, 0.42 mol) in CCl_4 (300 mL). Due to a spontaneous exotherm, the reaction temperature reached 25 °C without removing the ice bath. After overnight stirring, the reaction mixture was filtered to remove KCl, followed by washing with CCl_4 (150 mL). The combined filtrates were concentrated at reduced pressure to provide a red-brown liquid (78.4 g), which was purified by distillation, bp 57−62 °C (0.15−0.2 mmHg) [lit.[40] bp 51 °C (0.4 mmHg); lit.[43] bp 57−60 °C (0.15 mmHg)]. Yield: 50.1 g (80%), a yellow liquid.

(Methoxydichloromethyl)disulfanyl Chloride (8). Acetyl methoxythiocarbonyl sulfide (**7**) (14.6 g, 97.5 mmol) in $CHCl_3$ (100 mL) was added dropwise over 30 min at 0 °C to a solution of sulfuryl chloride (29.0 g, 215 mmol) in $CHCl_3$ (100 mL), and stirring was continued for 3 h. Solvent was removed at reduced pressure to provide a yellow liquid (18.3 g), purified by distillation, bp 54−57 °C (∼0.2 mmHg) [lit.[41] bp 29 °C (0.09 mmHg); lit.[42] bp 49−52 °C (0.15 mmHg); lit.[43] bp 47−53 °C (0.15 mmHg)]. Yield: 10.5 g (51%).

(Chlorocarbonyl)disulfanyl Chloride (9). With stirring, anhydrous $FeCl_3$ (20 mg) was added carefully to (methoxydichloromethyl)disulfanyl chloride (**8**) (10.23 g, 48 mmol) maintained at 0 °C under N_2. Vigorous gas evolution occurred, and after 30 min the weight loss was 2.28 g (95% of the theoretical amount of MeCl). The title product was obtained as a yellow liquid upon distillation, bp 54−62 °C (∼20 mmHg) [lit.[42] bp 49 °C (12 mmHg); bp[43] 53−54 °C (12 mmHg)]. Yield: 4.25 g (54%).

Model Reaction of a Carbonyl Disulfide with Piperidine.[49] A solution of *S*-ethylsulfenyl *O*-methyl thiocarbonate[38] [0.76 g, 5 mmol; ^{1}H NMR ($CDCl_3$) δ 3.89 (s, 3H), 2.81 (q, 2H), 1.33 (t, 3H)] in $CDCl_3$ (5 mL) was combined with a solution of piperidine [0.43 g, 5 mmol; ^{1}H NMR ($CDCl_3$) δ 2.6−2.8 (m, 4H), 1.3−1.6 (m, 6H)] in $CDCl_3$ (5 mL). A ^{1}H NMR spectrum recorded after 30 min showed that no starting material remained and that products were ethyl *N*-piperidinylcarbamoyl disulfane [^{1}H NMR ($CDCl_3$) δ 3.42−3.62 (m, 4H), 2.75 (q, 2H), 1.52−1.65 (m, 6H), 1.30 (t, 3H)], diethyl trisulfide [^{1}H NMR ($CDCl_3$) δ 2.89 (q, 2H), 1.39 (t, 3H); lit.[38] ^{1}H NMR δ 2.89 (q, 2H), 1.38 (t, 3H)], *O*-methyl *N*-piperidinyl carbamate [^{1}H NMR ($CDCl_3$) δ 3.68 (s, 3H), 2.39−2.43 (m, 4H), 1.44−1.53 (m, 6H)], and methanol [^{1}H NMR ($CDCl_3$) δ 3.45 (s, 3H)]. Upon standing overnight, there were also found diethyl disulfide [^{1}H NMR ($CDCl_3$) δ 2.70 (q, 2H), 1.32 (t, 3H); lit.[38] ^{1}H NMR δ 2.71 (q, 2H), 1.32 (t, 3H)] and diethyl tetrasulfide [^{1}H NMR ($CDCl_3$) δ 2.97 (q, 2H), 1.41 (t, 3H); lit.[38] ^{1}H NMR δ 2.97 (q, 2H), 1.40 (t, 3H)]. Spectra of samples that had been worked up by washing with 1 N aqueous HCl and H_2O followed by drying (Na_2SO_4), concentration, and redissolving in $CDCl_3$ were identical, except that methanol was lost. An authentic sample of $EtSS(C{=}O)NC_5H_{10}$ was prepared by reaction of (ethyldithio)carbonyl chloride, $EtSS(C{=}O)Cl$, with piperidine following the general outlined procedure of Barany et al.[38] Ratios of products, based on ^{1}H NMR integrations, are outlined in ref 49.

Model Reaction of a Carbamoyl Disulfide with Piperidine.[53] A solution of ethyl *N*′-methyl-*N*-phenylcarbamoyl disulfane[38] [1.14 g, 5 mmol; ^{1}H NMR ($CDCl_3$) δ 6.8−7.3 (m, 5H), 2.90 (s, 3H), 2.72 (q, 2H), 1.28 (t, 3H)] in $CDCl_3$ (5 mL) was combined with a solution of piperidine (0.43 g, 5 mmol) in $CDCl_3$ (5 mL). A ^{1}H NMR spectrum recorded after 30 min showed a ratio of product $EtSS(C{=}O)NC_5H_{10}$ (see preceding procedure for spectral data) to unreacted starting material of 3:7. Overnight reaction went to completion, yielding $EtSS(C{=}O)NC_5H_{10}$ plus equimolar *N*-methylaniline [^{1}H NMR ($CDCl_3$) δ 6.7−7.3 (m, 5H), 2.90 (s, 3H)]. The *N*-methylaniline product and any unreacted piperidine were removed upon workup by washing with 1 N aqueous HCl and H_2O followed by drying (Na_2SO_4), concentration, and redissolving in $CDCl_3$.

On-Resin Transformations of *S*-Acm (Scheme 3). In a typical experiment, Mpa(Acm)-Gly-βAla-Gly-PAL-PEG-PS (∼15 mg, ranging from 0.14 to 0.20 mmol/g) was treated with (chlorocarbonyl)sulfenyl chloride (**5**) (∼3 μL, ∼4 equiv, 8 mM) in CH_2Cl_2 (1 mL) for 15 min and then reacted with (i) *N*-methylaniline (∼3 μL, ∼4 equiv, 8 mM) in CH_2Cl_2, (ii) MeOH−CH_2Cl_2 (1:1), and (iii) piperidine−CH_2Cl_2 (1:4), for 15 min each; in addition, (iv) (methoxycarbonyl)sulfenyl chloride (**6**) was applied directly in place of $Cl(C{=}O)SCl$ (**5**). For each of i−iv, the peptide products were released from the support as described in General and finally taken up in CH_3CN−H_2O (1:1) for HPLC analysis. Results: (i) Major peak (∼95%), assigned to the Mpa(Snm) peptide, t_R 18.5 min; matching authentic standard from direct coupling of Mpa(Snm)-OH (**4a**)

to the tripeptide-resin; further confirmed by MS [FABMS: *m/z* calcd 455.1, found 456.1 $(MH)^+$]; (ii) major peak (∼75%), assigned to the Mpa(Scm) peptide, t_R 12.1 min; matching authentic standard from coupling Mpa(Scm)-OH (**3a**) to the tripeptide-resin; further confirmed by MS [FABMS: *m/z* calcd 380.1, found 381.1 $(MH)^+$]; (iii) major peak (∼94%), assigned to the Mpa(Spip) peptide, t_R 16.3 min; confirmed by MS [FABMS: *m/z* calcd 433.2, found 434.1 $(MH)^+$]; (iv) major peak (∼85%), assigned to the Mpa(Scm) peptide, t_R 12.1 min.

Conversion of *S*-Snm Group under Fmoc Removal Conditions (Scheme 3). Mpa(Snm)-Gly-βAla-Gly-PAL-PEG-PS (∼20 mg/experiment) was treated with an appropriate reagent (see below), after which the peptide-resin was washed with CH_2Cl_2 and processed and evaluated exactly as described in the above protocol. Treatment with either piperidine−DMF (1:4) for 10 min or piperidine−CH_2Cl_2 (1:4) for 60 min led to formation of Mpa(Snip)-Gly-βAla-Gly-NH_2, confirmed by mass spectral analysis [FABMS: *m/z* calcd 433.2, found 434.1 $(MH)^+$] and by cochromatography with the product of an alternative synthesis (Scheme 3, above procedure). Treatment with piperidine−CH_2Cl_2 (1:4) for 10 min showed a 5:4 ratio of product Snip to starting Snm. Corresponding experiments with Mpa(Scm)-Gly-βAla-Gly-PAL-PEG-PS showed that piperidine completely destroyed the *S*-Scm group (i.e., no remaining model peptide), but we could not identify any obvious corresponding product that survived the acid cleavage and workup (e.g., no Snip or free thiol derivative formed).

Pilot Studies of On-Resin Cyclizations (Tables 1−4). A suitable protected peptide-resin (∼10 mg/experiment) was swollen in CH_2Cl_2, treated with TFA−CH_2Cl_2−Et_3SiH−phenol−H_2O (7:90:1:1:1) (2 mL, 2 × 15 min), and washed with CH_2Cl_2. The resultant peptide-resin with a free thiol was then incubated in the presence of various solutions (1 mL), under conditions specified in the tables. After 2 h at 25 °C (unless indicated otherwise), the peptide-resin was washed with CH_2Cl_2, and then cleavage was carried out as described in General, with the final crude peptide mixture dissolved in 1 mL of TFA−H_2O (1:1) for analytical HPLC.

Pilot Studies of Solution Cyclizations (Table 5; selected entries in Tables 1 and 3). As controls to the experiments described above, the same peptide-resins (∼10 mg) were directly cleaved and worked up as described in General. The final peptide residues (∼0.12 μmol) were then dissolved in 1 mL of CH_3CN−10 mM phosphate buffer (1:1) at various pH values, and the further progress of reactions was monitored by analytical HPLC.

Deaminooxytocin. Scheme 4, Route A: Solid-Phase. Fmoc-Tyr(*t*-Bu)-Ile-Gln-Asn-Cys(Tmob)-Pro-Leu-Gly-PAL-PEG-PS (40 mg, initial loading 0.14 mmol/g) was treated with piperidine−DMF (1:4) (2 × 2 mL, 2 + 8 min), washed with DMF, CH_2Cl_2, and DMF, and then extended by addition of a solution of Mpa(Snm)-OH (**4a**) (6.1 mg, 22 μmol), HOBt (3.0 mg, 22 μmol), and DIPCDI (3.5 μL, 22 μmol) in DMF (0.2 mL) for 2 h. After washing with DMF and CH_2Cl_2, the peptide-resin was treated with TFA−CH_2Cl_2−Et_3SiH−phenol−H_2O (7:90:1:1:1) (2 mL, 2 × 15 min), washed with CH_2Cl_2, and then stirred for 2 h in the presence of NMM−DMF. Further cleavage/workup as described in General gave a peptide precipitate which was dissolved in 10% aqueous HOAc (4 mL) and shown by analytical HPLC to comprise deaminooxytocin (65%), deaminooxytocin trisulfide (5%), and deaminooxytocin tetrasulfide (2%). Semipreparative HPLC (see General) gave the desired peptide (1.56 mg, 33%, >99% purity).

Scheme 4, Route B: Solution. The identical procedure as immediately above, but omitting the on-resin steps with dilute TFA and with dilute NMM, gave a peptide precipitate which was dissolved in 4 mL of CH_3CN−10 mM phosphate buffer (1:1) at pH 8. Cyclization, as monitored by analytical HPLC, was complete in 10 min and showed a mixture of deaminooxytocin (87%), deaminooxytocin trisulfide (5%), and deaminooxytocin tetrasulfide (2%). Semipreparative HPLC purification gave the desired peptide (2.67 mg, 56%, >99% purity).

Scheme 5, Route E: Solid-Phase. Fmoc-Tyr(*t*-Bu)-Ile-Gln-Asn-Cys(Acm)-Pro-Leu-Gly-PAL-PEG-PS (50 mg, initial loading 0.14 mmol/g) was treated with (chlorocarbonyl)sulfenyl chloride (**5**) (∼9 μL, ∼4 equiv, 8 mM) in CH_2Cl_2 (1.5 mL) for 15 min and then reacted with *N*-methylaniline (∼9 μL, ∼4 equiv, 8 mM) in CH_2Cl_2 (1.5 mL) for 15 min. After washing with CH_2Cl_2 and DMF, the Fmoc group was removed and the *S*-Snm group was converted to *S*-Snip by treatment with piperidine−DMF (1:4) (2 × 2 mL, 2 + 8 min). Further washing with DMF, CH_2Cl_2, and DMF was followed by introduction of Mpa(Tmob)-OH (**1a**) (6.1 mg, 22 μmol), mediated by HOBt (3.0 mg, 22 μmol) and DIPCDI (3.5 μL, 22 μmol) in DMF (0.2 mL) for 2 h. After washing with DMF and CH_2Cl_2, the peptide-resin was treated with TFA−CH_2Cl_2−Et_3SiH−phenol−H_2O (7:90:1:1:1) (2 mL, 2 × 15 min), washed with CH_2Cl_2, and then stirred for 2 h in the presence of NMM−DMF (1:99). Further cleavage/workup as described in General gave a peptide precipitate which was dissolved in 10% aqueous HOAc (4 mL) and shown by analytical HPLC to comprise deaminooxytocin (67%) and deaminooxytocin trisulfide (13%). Semipreparative HPLC gave the desired peptide (1.1 mg, 19%, >99% purity).

Scheme 5, Route F: Solution. The identical procedure as that immediately preceding, but omitting the on-resin steps with dilute TFA and with dilute NMM, gave a peptide precipitate which was dissolved in 4 mL of CH_3CN−10 mM phosphate buffer (1:3) at pH 8. Cyclization, as monitored by analytical HPLC, was complete in 10 min to show a mixture of deaminooxytocin (57%) and deaminooxytocin trisulfide (15%). Semipreparative HPLC purification gave the desired peptide (1.6 mg, 27%, >95% purity).

Deaminooxytocin Trisulfide. The successful approach is outlined in Scheme 4, route B. Fmoc-Tyr(*t*-Bu)-Ile-Gln-Asn-Cys(Tmob)-Pro-Leu-Gly-PAL-PEG-PS (105 mg, initial loading 0.14 mmol/g) was deprotected similarly to the beginning of the corresponding procedure for deaminooxytocin and then extended with Mpa(Ssnm)-OH (**4d**) (16.9 mg), HOBt (8.6 mg), and DIPCDI (8.8 μL) in DMF (1 mL) for 2 h. Treatment with TFA−CH_2Cl_2−Et_3SiH−phenol−H_2O (92:5:1:1:1) (3 mL) for 2 h (cleavage yield 94%) and further standard workup gave a peptide precipitate which was dissolved in 8 mL of CH_3CN−10 mM phosphate buffer (3:5) at pH 2. Cyclization, as monitored by analytical HPLC (Supporting Information Figure 1), was complete in 2 h, following which the reaction mixture was lyophilized, redissolved in CH_3CN−H_2O (1:4), and purified by semipreparative HPLC. This provided the desired deaminooxytocin trisulfide (4.9 mg, 40%, t_R 17.0 min), deaminooxytocin (2.9 mg, 24%, t_R 14.7 min), and deaminooxytocin tetrasulfide (0.9 mg, 7%, t_R 17.8 min), all in >97% purity. The amino acid composition of the purified peptide trisulfide was Asn, 1.01; Gln, 1.03; Pro, 1.30; Gly, 1.06; Ile, 0.91; Leu, 0.99; Tyr, 0.93. FABMS: *m/z* calcd 1023.4, found 1024.4 $[(MH)^+]$.

Oxytocin Trisulfide. The same nominal steps were followed as for deaminooxytocin trisulfide, except that Boc-Cys(Ssnm)-OH (**4e**) (26.8 mg, 64 μmol) was used in place of **4d**. The crude cleaved peptide after ether precipitation was dissolved in 20% aqueous HOAc (6 mL) and checked by analytical HPLC within 5 min to reveal a mixture of oxytocin (disulfide), t_R 10.4 min, and oxytocin trisulfide, t_R 11.2 min, in a ratio of 13:15, as well as peaks at t_R 12.5 and 12.7 min at ∼45% and 60%, respectively, of the main peak. Semipreparative HPLC (see General) gave the desired trisulfide peptide (3.0 mg, 25%) as well as oxytocin disulfide (2.5 mg, 21%), both in >99% purity. The amino acid composition of the purified peptide trisulfide was Asn, 1.02; Gln, 1.07; Pro, 1.41; Gly, 1.08; Ile, 0.90; Leu, 1.02; Tyr, 0.91. FABMS: *m/z* calcd 1037.4, found 1038.9 $[(MH)^+]$. The later eluting peaks were separated and assigned to be dimers (details in ref 61) on the basis of FABMS: calcd (average mass) 2015.4, found 2015.6; calcd (average mass) 2047.5, found 2048.0.

Biological Activities. The following literature procedures were followed: uterotonic *in vitro* test[62,63] in magnesium-free solution, uterotonic *in vivo* test[64] using urethane-anesthetized rats, and pressor test[65] using pithed rat preparations. Wistar rats weighing 180−280 g were used in all tests. Dose−response curves were constructed (e.g., Supporting Information Figure 3), and threshold doses were compared. Synthetic oxytocin (450 IU/mg) was used as a standard for oxytocic assays; synthetic arginine vasopressin (400 IU/mg) was a standard for the pressor assay.

Oxytocin Analogue Binding to Receptors. Following the procedure of Fahrenholz et al.,[67,68] with minor modifications, a crude rat uterine membrane preparation (50−100 μg of protein) was incubated with [^{3}H]oxytocin (from NEN-DuPont; 1184 GBq/mmol, diluted from 37 MBq/mL = 31 μM stock to final concentration 2 nM) and various concentrations (0.1−1000 nM) of peptides for 30 min at 35 °C. The total volume was 0.25 mL (50 mM HEPES, pH 7.6, containing 10 mM $MnCl_2$ and 1 mg/mL bovine serum albumin). The reaction was terminated by quick filtration on a Brandel cell harvester. Binding affinities are expressed as K_i calculated according to the equation $K_i = IC_{50}/[(c/K_{dOT}) + 1]$, where K_{dOT} is taken as 1.8 nM.

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Supporting Information Available: Figures of analytical C_{18} RP-HPLC monitoring of experiments to form the trisulfide variant of deaminooxytocin (Figure 1), contractions *in vivo* of rat uterus after intravenous administration of oxytocin and deaminooxytocin trisulfide (Figure 2), dose−response curves of oxytocin and its analogues in the uterotonic *in vivo* test (typical experiment) (Figure 3), and blood pressure recordings after administration of peptides (Figure 4) (4 pages). Ordering information is given on any current masthead page.

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(49) The model compound *S*-ethylsulfenyl *O*-methyl thiocarbonate, EtSS(C═O)OMe, available from the studies of ref 38, was treated with piperidine (1 equiv, 0.5 M) in $CDCl_3$ at 25 °C for 30 min. The products observed represented a partitioning between two pathways: (a) formation of ethyl *N*-piperidinylcarbamoyl disulfane, $EtSS(C{=}O)NC_5H_{10}$, + MeOH and (b) formation of ethyl trisulfide, EtSSSEt, + *O*-methyl *N*-piperidinyl carbamate, $MeO(C{=}O)NC_5H_{10}$, + carbonyl sulfide (COS gas, assumed) + MeOH (ethyl hydrodisulfane, EtSSH, is presumed to be an intermediate in the reaction mechanism). From the ratio of Et integrations upon ^{1}H NMR, it was concluded that the ratio of pathways a:b was about 2:1. After overnight standing, the EtS-derived compounds had disproportionated and desulfurized to show a mixture of EtSSEt, EtSSSEt, and EtSSSSEt in a ratio of 1.5:1.8:1.0. In addition, the apparent ratio of pathways a:b had changed to 1:3. The model substrate for this reaction and most of the products are described in ref 38. Authentic samples of $MeO(C{=}O)NC_5H_{10}$ and $EtSS(C{=}O)NC_5H_{10}$ were made by reactions of piperidine with the corresponding chloroformate and dithiocarbonyl chloride, using methods described in ref 38.
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(54) Some of the earlier papers in Fmoc chemistry used *freshly prepared* piperidine−CH_2Cl_2 mixtures for Fmoc removal, but these deprotection cocktails are less preferred because (i) rates are slower and (ii) with time, a precipitate of piperidinium hydrochloride forms, which can lead to problems with automated synthesizers; see ref 55.
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(56) In ref 20 which applied Boc chemistry, the resin-bound intermediate for cyclization had an *N*-terminal cysteine (originally protected by *S*-trityl) and an internal Cys(Scm) obtained by conversion of internal Cys(Acm) with MeO(C═O)SCl (**6**). For the experiment of Scheme 5, which relies on Fmoc chemistry, we observed ∼20% chain loss after treatment with Cl(C═O)SCl (**5**), and in addition, the final yield for cleavage of the PAL anchoring linkage was the unusually low 70%. These factors are in part contributing to the relatively lower overall isolated yields (based on the initial loading of resin) of cyclized peptides from these experiments.
(57) This product, a single peak with t_R 19.0 min (HPLC flow 1.2 mL/min, linear gradient over 20 min of 0.1% aqueous TFA and 0.1% TFA in acetonitrile from 19:1 to 1:9, detection at 220 nm), was characterized by FABMS [m/z calcd 1259.5, found 1261.9 $(MH)^+$] (procedure matches the successful text experimental for oxytocin trisulfide, substituting Fmoc building block **4f** for Boc building block **4e**).
(58) Kates, S. A.; Solé, N. A.; Beyermann, M.; Barany, G.; Albericio, F. Optimized Preparation of Deca(L-Alanyl)-L-Valinamide by 9-Fluorenylmethyloxycarbonyl (Fmoc) Solid-Phase Synthesis on Polyethylene Glycol-Polystyrene (PEG-PS) Graft Supports, with 1,8-Diazobicyclo[5.4.0]-undec-7-ene (DBU) Deprotection. *Pept. Res.* **1996**, *9*, 106−113 and references cited therein.
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(61) We suspect, but did not prove, that these fractions comprised principally the antiparallel dimer(s) of oxytocin. FABMS showed that one of the dimer fractions had four sulfurs and a separately resolved, later eluting dimer fraction had five sulfurs. Isomeric dimers (four sulfurs) of oxytocin were resolved and characterized by: Yamashiro, D.; Hope, D. B.; du Vigneaud, V. Isomeric Dimers of Oxytocin. *J. Am. Chem. Soc.* **1968**, *90*, 3857−3860.
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