and the oil was separated into three fractions (SiO_2, EtOAc). Fraction 2 was separated further by HPLC (SiO_2, EtOAc; C_{18} column, MeOH–H_2O, 14:1) to give **6b** as an oil: $[\alpha]^{25}_D$ +9° (*c* 0.2, $CHCl_3$); ^1H NMR, see Figure 2.

Anal. Found for $C_{13}H_{24}NO_5$: 274.1653 (M + H) (HRFABMS).

Formation of 6b and Lactam 15. A sample of synthetic *N*-Boc-(3*S*,4*R*,5*S*)-Ist-OEt (37 mg) was hydrolyzed with 1 N NaOH (0.1 mL) in dioxane (1 mL) at rt for 2.5 h. Solvent was removed in vacuo, and the resulting oil was treated with TFA in CH_2Cl_2 (0.1 mL) for 40 min. Excess solvent was removed under N_2, and the residual material was heated with MeOH–AcCl (40:1) at 65 °C for 25 min. MeOH and HCl were removed in vacuo, and the oil was treated with Ac_2O and C_5H_5N (0.2 mL each) at rt for 1 h. The product was passed through a small SiO_2 column (EtOAc) and then was subjected to HPLC (SiO_2, EtOAc) to give **6b**, the less polar oil (4.6 mg, 11%): $[\alpha]^{20}_D$ +11° (*c* 0.6, $CHCl_3$); ^1H NMR (200 MHz, $CDCl_3$), Figure 2. The more polar fraction gave lactam **15** (4.2 mg, 15%): $[\alpha]^{20}_D$ −5° (*c* 0.5, $CHCl_3$); IR (film) 1740, 1699 cm^{-1}; ^1H NMR, Table II; FABMS *m/z* 200 (M + H); EIMS *m/z* (rel intensity) 156 (6.4), 142 (100), 111 (64.8), 100 (36.6), 82 (100), 43 (24.0).

Anal. Found for $C_{10}H_{18}NO_3$ (M + H): 200.1287 (HRFABMS).

NOE Difference Experiment on Lactams 15 and 16. Solutions of lactams **15** (4.4 mg) and **16** (3.5 mg), each in $CDCl_3$ (0.5 mL), were degassed with dry Ar, and their qualitative NOE difference spectra were recorded with an XL-200 spectrometer: relaxation delay = 10 s; number of transients = 180 (Figure 13S).

Preparation of *N,O*-Bis(trifluoroacetyl)isostatine Methyl Esters. Synthetic samples of all eight Boc-isostatine methyl ester isomers were treated individually with TFAA and TFA at 100 °C for 5 min. Excess acid was removed under N_2, and each product was purified by HPLC (SiO_2, hexane–EtOAc, 5:1) to give **6c–13c**. Optical rotations and GC retention times are listed in Table IV.

Acid Treatment of Boc-(3*S*,4*S*,5*S*)-isostatine Ethyl Ester. Four samples (13-mg each) were treated with 6 N HCl at 110 °C for 4, 11, 24, and 38 h, respectively. Solvent was removed, and each residue was treated with MeOH–AcCl (10:1) at 110 °C for 15 min. The methanolic HCl was removed, and the resulting oil was treated with TFAA and TFA at 110 °C for 5 min. Each product was dissolved in 2-propanol (1 mL) for GC analysis.

Acid Treatment of Boc-(3*R*,4*S*,5*S*)-isostatine Ethyl Ester. Four samples (4-mg each) were treated with 6 N HCl at 110 °C for 4, 12, 36, and 42 h, respectively. The products were converted to the TFA ethyl ester derivatives using the procedure described above. Each sample was dissolved in 2-propanol (1 mL) for GC analysis.

Synthesis of Boc-(4*S*,5*S*)-2,3-anhydroisostatine Methyl Ester (20). A mixture of Boc-(3*S*,4*S*,5*S*)- and Boc-(3*R*,4*S*,5*S*)-Ist-OMe (25 mg, 0.086 mmol) was treated with 6 N HCl (1 mL) at 110 °C for 20 h. Aqueous HCl was removed under N_2, and the residue was treated with mixture of MeOH–AcCl (10:1) and concentrated and then treated with Boc-ON (30 mg) and Et_3N (20 μL) in CH_2Cl_2 at rt for 10 h. Solvent was removed, and the product was purified by HPLC using a phenyl column and hexane–2-propanol (20:1) to give pure **20** (3.6 mg, 14%): needles, mp 60 °C; $[\alpha]^{20}_D$ +2° (*c* 0.03, $CHCl_3$); ^1H NMR, Figure 21S.

Anal. Calcd for $C_{14}H_{26}NO_4$: 272.1862 (M + H). Found: 272.1859 (M + H) (HRFABMS).

Acid Treatment of 20. Four samples of **20** (0.7 mg each) were treated with 6 N HCl at 110 °C for 4, 12, 24, and 36 h. The resulting hydrolyzates were converted to TFA methyl ester derivative **20a** using the procedure described above for GC analyses: CIMS *m/z* 268 (M + H), 236, 208, 155, 141, 93, 71, 57.

Acknowledgment. This work was supported in part by grants from the National Institute of Allergy and Infectious Diseases (AI 04769, AI 01278) and the National Institute of General Medical Sciences (GM27029). We thank S. Sakemi, HBOI, for recording 2D-NMR spectra; Dr. L. H. Li, The Upjohn Company, Kalamazoo, MI, for L1210 testing; Dr. R. M. Milberg, L.-S. Rong, and P. E. Sanders for MS; Dr. S. E. Denmark for providing the polarimeter; A. G. Thompson for assistance with quadrupole GC/MS; and Dr. J. R. Carney and L. S. Shield for reading the manuscript, all at the University of Illinois.

Supplementary Material Available: Structures of **1–5**, ^{13}C NMR spectra for Boc-L-*allo*-isoleucinol and Boc-D-isoleucinol, and ^1H NMR spectra for **6a, 7a, 8a, 9a, 10a, 10b, 11a, 11b, 12a, 12b, 13a, 13b, 15** and **16** (and their NOE difference spectra), **17–20**, Boc-D-*allo*-isoleucinol, and Boc-D-isoleucinol (22 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

S-2,4,6-Trimethoxybenzyl (Tmob): A Novel Cysteine Protecting Group for the N^{α}-9-Fluorenylmethoxycarbonyl (Fmoc) Strategy of Peptide Synthesis[1-3]

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The *S*-2,4,6-trimethoxybenzyl (Tmob) group can be introduced onto sulfhydryl functions from the corresponding alcohol, with acid catalysis, and is in turn removed rapidly by treatment with 30% trifluoroacetic acid–dichloromethane in the presence of phenol, thioanisole, and water (5% each) *or* 6% trifluoroacetic acid–dichloromethane in the presence of triethylsilane or triisopropylsilane (0.5%). The appropriate cysteine derivative was prepared and applied with other N^{α}-Fmoc protected amino acids to the solid-phase syntheses of several model peptides. Acidolytic deblocking in the presence of cation scavengers and reducing agents gave the free thiol, whereas oxidative deblocking with iodine or thallium(III) trifluoroacetate provided an intramolecular disulfide. The chemistry of the *S*-Tmob group compares favorably to established chemistries with the acid-labile and oxidizable *S*-triphenylmethyl (trityl, Trt) group, as well as with the oxidizable *S*-acetamidomethyl (Acm) group.

Despite considerable recent progress for stepwise solid-phase synthesis (SPPS) of peptides under mild conditions using the base-labile N^{α}-9-fluorenylmethyloxycarbonyl (Fmoc) protecting group,[6] there is to date no entirely

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satisfactory general strategy for protection of the sulfhydryl function of cysteine.[6-10] For each of the principal available groups, *S*-acetamidomethyl (Acm), *S*-triphenylmethyl (trityl, Trt), and *S*-*tert*-butylsulfenyl (S-*t*-Bu), problems

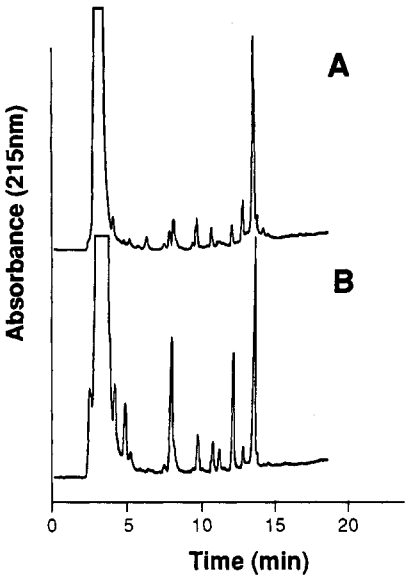

Figure 1. Analytical HPLC of samples from solid-phase syntheses of the model pentapeptide H-Trp-Met-Asp-Phe-Cys-NH_2, cleaved with TFA–DCM–anisole–βME (70:25:3:2), 1 h, 25 °C. Panel A, synthesis with Cys(Tmob); panel B, synthesis with Cys(Trt). HPLC was performed on a Vydac C-18 reversed-phase column (5 μm, 4.6 × 250 mm); linear gradient over 20 min using CH_3CN and 0.01 N aqueous HCl from 1:9 to 6:4, flow rate 1.2 mL/min. The large peaks at the front of the chromatogram are solvent, and the desired peptide elutes at t_R 13.0 min.

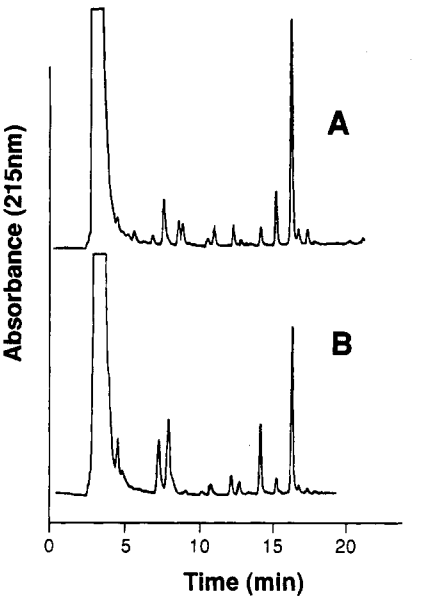

Figure 2. Analytical HPLC of samples from solid-phase syntheses of the model pentapeptide H-Trp-Met-Asp-Phe-Cys-NH_2, cleaved with TFA–DCM–phenol–thioanisole–water (70:15:5:5:5), 1 h, 25 °C. Panel A, synthesis with Cys(Tmob); panel B, synthesis with Cys(Trt). HPLC conditions were nominally the same as for Figure 1, but in these runs, the desired peptide was found to elute at t_R 15.8 min.

(1) A preliminary account of portions of this work was presented: (a) 12th American Peptide Symposium, Cambridge, MA, June 16–22, 1991; Munson, M. C.; Garcia-Echeverria, C.; Albericio, F.; Barany, G. In *Peptides: Chemistry and Biology*; Smith J. A., Rivier, J. E., Eds.; Escom: Leiden, The Netherlands, 1991; pp 605–606. (b) 2nd International Symposium on Solid Phase Synthesis, Canterbury, England, Aug 27–31, 1991; Royo, M.; Garcia-Echeverria, C.; Munson, M. C.; Słomczyńska, U.; Eritja, R.; Giralt, E.; Barany, G.; Albericio, F. In *Innovation and Perspectives in Solid Phase Synthesis and Related Technologies: Peptides, Polypeptides and Oligonucleotides 1992*; Epton, R., Ed.; Intercept: Andover, England, 1992; in press.

(2) Taken in part from the Ph.D. thesis of C.G.-E., University of Barcelona, Sept, 1990.

(3) Abbreviations used are as follows: Acm, acetamidomethyl; Ac_2O, acetic anhydride; βME, β-mercaptoethanol; BOP, benzotriazolyl *N*-oxytris(dimethylamino)phosphonium hexafluorophosphate; DCM, dichloromethane; DIEA, *N*,*N*-diisopropylethylamine; DIPCDI, *N*,*N*'-diisopropylcarbodiimide; DMF, *N*,*N*-dimethylformamide; DMS, dimethyl sulfide, Et_2O, diethyl ether; EtOAc, ethyl acetate; Fmoc, 9-fluorenylmethyloxycarbonyl; Fmoc-N_3, 9-fluorenylmethyloxycarbonyl azide; Fmoc-OSu, 9-fluorenylmethoxycarbonyl succinimide; HOAc, acetic acid; HOBt, 1-hydroxybenzotriazole; MBHA, 4-methylbenzhydrylamine (resin); MeOH, methanol; Nle, norleucine; NMM, *N*-methylmorpholine; PAL, tris(alkoxy)benzylamide linker of ref 6e [5-(4-((9-fluorenylmethyloxycarbonyl)aminomethyl)-3,5-dimethoxyphenoxy)valeric acid]; PEG, polyethylene glycol spacer; PS, copoly(styrene–1%-divinylbenzene) polymeric support; SPPS, solid-phase peptide synthesis; Tmob, 2,4,6-trimethoxybenzyl; TFA, trifluoroacetic acid; $Tl(Tfa)_3$, thallium(III) trifluoroacetate; Trt, triphenylmethyl (trityl); t_R, retention time. Amino acid symbols denote the L-configuration unless indicated otherwise. All solvent ratios and percentages are volume/volume unless stated otherwise.

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(5) Current addresses: (a) University of Wisconsin—Madison, School of Pharmacy, 425 North Charter Street, Madison, WI 53706. (b) Millipore Corporation, 75A Wiggins Avenue, Bedford, MA 01730.

(6) Reviews: (a) Barany, G.; Kneib-Cordonier, N.; Mullen, D. G. *Int. J. Peptide Protein Res.* **1987**, *30*, 705–739. (b) Atherton, E.; Sheppard, R. *Solid Phase Peptide Synthesis: A Practical Approach*, IRL Press, Oxford, 1989. (c) Fields, G. B.; Noble, R. L. *Int. J. Peptide Protein Res.* **1990**, *35*, 161–214. (d) Fields, G. B.; Tian, Z. T.; Barany, G. In *Synthetic Peptides: A User's Guide*; Grant, G., Ed.; W. H. Freeman and Co.: Salt Lake City, UT, 1992; pp 77–183. For a representative recent experimental paper, see: (e) Albericio, F.; Kneib-Cordonier, N.; Biancalana, S.; Gera, L.; Masada, R. I.; Hudson, D.; Barany, G. *J. Org. Chem.* **1990**, *55*, 3730–3743 and references cited therein.

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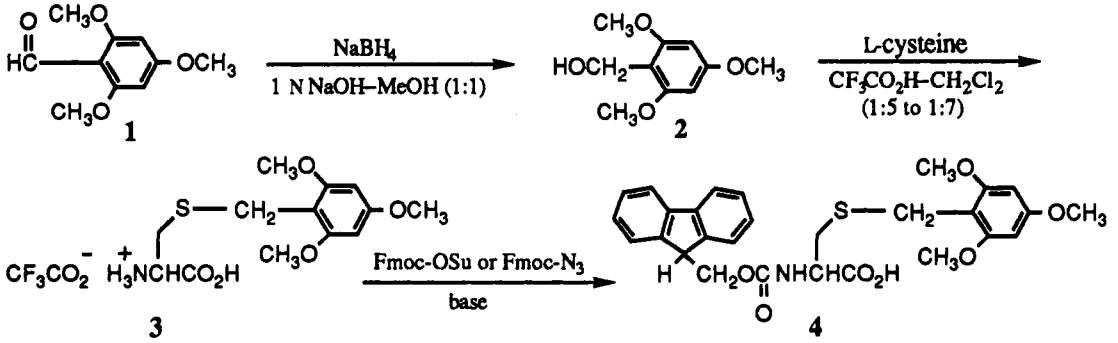
(8) Previous papers in this area from our Minnesota and Barcelona laboratories are: (a) Albericio, F.; Grandas, A.; Porta, A.; Pedroso, E.; Giralt, E. *Synthesis* **1987**, 271–272. (b) Eritja, R.; Ziehler-Martin, J. P.; Walker, P. A.; Lee, T. D.; Legesse, K.; Albericio, F.; Kaplan, B. E. *Tetrahedron* **1987**, *43*, 2675–2680. (c) Schroll, A.; Barany, G. *J. Org. Chem.* **1989**, *54*, 244–247. (d) Ponsati, B.; Ruiz-Gayo, M.; Giralt, E.; Albericio, F.; Andreu, D. *J. Am. Chem. Soc.* **1990**, *112*, 5345–5347. (e) Albericio, F.; Hammer, R. P.; García-Echeverría, C.; Molins, M. A.; Chang, J. L.; Munson, M. C.; Pons, M.; Giralt, E.; Barany, G. *Int. J. Peptide Protein Res.* **1991**, *33*, 402–413. These publications also provide detailed overviews of the earlier literature.

(9) Representative papers using Fmoc chemistry include refs 8a,b,e and: (a) Chang, C. D.; Felix, A. M.; Jimenez, M. H.; Meienhofer, J. *Int. J. Peptide Protein Res.* **1980**, *15*, 485–494. (b) Atherton, E.; Pinori, M.; Sheppard, R. C. *J. Chem. Soc., Perkin Trans. 1* **1985**, 2057–2064. (c) Atherton, E.; Sheppard, R. C.; Ward, P. *J. Chem. Soc., Perkin Trans 1* **1985**, 2065–2073. (d) Wade, J. D.; Fitzgerald, S. P.; McDonald, M. R.; McDougall, J. G.; Tregear, G. W. *Biopolymers* **1986**, S21–S37. (e) McCurdy, S. N. *Peptide Res.* **1989**, *2*, 147–152. (f) Atherton, E.; Hardy, P. M.; Harris, D. E.; Matthews, B. H. In *Peptides 1990: Proceedings of the 20th European Peptide Symposium*; Giralt, E., Andreu, D., Eds.; Escom: Leiden, The Netherlands, 1991; pp 243–244. Some of these publications also provide details about difficulties associated with incorporation of cysteine and/or cystine residues into peptides prepared by chemical synthesis.

(10) Recent relevant contributions, *not* using Fmoc chemistry, include refs 8c,d and (a) van Rietschoten, J.; Pedroso, E.; Granier, C. In *Peptides: Proceedings of the Fifth American Peptide Symposium*; Goodman, M.; Meienhofer, J., Eds.; John Wiley and Sons: New York, 1977; pp 522–524. (b) Brady, S.; Paleveda, W.; Nutt, R. In *Peptides: Chemistry and Biology*; Marshall, G. R., Ed.; Escom: Leiden, The Netherlands, 1988; pp 192–194. (c) Kemp, D. S.; Carey, R. J. *J. Org. Chem.* **1989**, *54*, 3640–3646. Some of these publications also provide details about difficulties associated with incorporation of cysteine and/or cystine residues into peptides prepared by chemical synthesis.

Scheme I



have been noted on occasion at the steps for anchoring, chain elongation, deblocking to the free thiol, or direct oxidation to form disulfides. The question of proper cysteine residue management is of special importance for experiments directed at controlled syntheses of peptides containing two or more disulfide bonds.[6d,7b-d,8e,9c,11] The present paper reports on the preparation and applications of the new acid-labile S-2,4,6-trimethoxybenzyl (Tmob)[12] protecting group, which may offer some advantages for Fmoc solid-phase synthesis of cysteine-containing peptides.

Results and Discussion

Preparation and Properties of Cys(Tmob). The Tmob group was introduced onto cysteine by acid-catalyzed S-alkylation[13] of the carbonium ion generated from 2,4,6-trimethoxybenzyl alcohol[14] (2) (Scheme I). Conversely, Tmob is removed rapidly from cysteine derivative 3 by trifluoroacetic acid (TFA)-promoted acidolysis. Cleavage of Tmob, and its transfer from sulfur onto certain scavengers, is a reversible process; thus, a variety of cocktails[15] containing different combinations of phenol, thioanisole, anisole, and/or β-mercaptoethanol (βME) were unsuccessful in preventing reattachment of the reactive carbocation back onto cysteine. However, the addition of water (5%) to a mixture of phenol (5%) and thioanisole (5%) in trifluoroacetic acid–dichloromethane (TFA–DCM; TFA at least 30% v/v) *or* the use of triethylsilane or triisopropylsilane[16] (0.5% v/v) in TFA–DCM (at least 6% v/v) allowed complete deblocking within 5 min at 25 °C. When water was the only scavenger employed, Tmob removal was incomplete, even at very high TFA concentration. Furthermore, when DCM was replaced by cosolvents such as *N*,*N*-dimethylformamide (DMF) or methanol, Tmob removal was partial at a variety of acid concentrations. Only silanes[16] were effective scavengers at lower TFA concentrations *in dichloromethane* because they react irreversibly with the Tmob cation to form 2,4,6-trimethoxytoluene. In summary, successful removal of Tmob from cysteine requires careful attention to both the choice of cation scavenger(s) and the concentration of acid.

Use of Cys(Tmob) in Fmoc SPPS (Free Sulfhydryl). Additional solution model studies on 3 showed the Tmob group to be stable to standard conditions[17] for Fmoc removal in solid-phase peptide synthesis. Consequently, 3 was readily converted[18] to the corresponding Fmoc derivative 4 (Scheme I), which was in turn evaluated in stepwise Fmoc solid-phase synthesis.[6] The model pentapeptide H-Trp-Met-Asp-Phe-Cys-NH_2 was selected as a target, since it incorporates the challenging tetragastrin sequence[19] added onto a protected cysteine which is required to survive four cycles of amino acid incorporation. Cysteine was blocked with Tmob, and in a parallel experiment, with Trt. In each case, aspartic acid was protected as its *tert*-butyl ester, whereas methionine and tryptophan were unprotected. Chain assembly was carried out on an automated continuous-flow synthesizer using a novel polyethylene glycol–polystyrene (PEG-PS) graft support[20] and a "PAL"[6e] handle for establishing the C-terminal peptide amide. Amino acid analyses on both

(11) (a) Kamber, B.; Hartmann, A.; Eisler, K.; Riniker, B.; Rink, H.; Sieber, P.; Rittel, W. *Helv. Chim. Acta* **1980**, *63*, 899–915. (b) Chino, N.; Yoshizawa, K.; Noda, Y.; Watanabe, T. X.; Kimura, T.; Sakakibara, S. *Biochem. Biophys. Res. Comm.* **1986**, *141*, 665–672. (c) Ruiz-Gayo, M.; Albericio, F.; Pons, M.; Royo, M.; Pedroso, E.; Giralt, E. *Tetrahedron Lett.* **1988**, *29*, 3845–3868. (d) Wünsch, E.; Moroder, L.; Gohring-Romani, S.; Musiol, H. J.; Gohring, W.; Bovermann, G. *Int. J. Peptide Protein Res.* **1988**, *32*, 368–383. (e) Ponsati, B.; Giralt, E.; Andreu, D. *Tetrahedron* **1990**, *46*, 8255–8266 and references to earlier literature cited in all of these articles.

(12) The 2,4,6-trimethoxybenzyl (Tmob) protecting group for the N^{ω}-carboxamide side chains of asparagine and glutamine was reported by D. Hudson at the 10th American Peptide Symposium, St. Louis, MO, May 23–28, 1987. See Biosearch Technical Bulletin 9000-01, Milli-Gen/Biosearch Division of Millipore, Bedford, MA.

(13) The conditions used are precedented in publications by Photaki and co-workers, reviewed in ref 7a; see particularly: Photaki, I.; Taylor-Papadimitriou, J.; Sakarellos, C.; Mazaraki, P.; Zervas, L. *J. Chem. Soc., Chem. Commun.* **1970**, 2683–2687. See also ref 23, in Experimental Section.

(14) Alcohol 2 is quite labile (hydroxymethyl further reduced to methyl, or polymerization with slight acid catalysis) but can be prepared readily by borohydride reduction of commercially available aldehyde 1 (see text). Alcohol 2 was originally reported by a more awkward reduction procedure applied to 1, see: Freudenburg, K.; Harder, M. *Liebigs Ann. Chem.* **1926**, *451*, 213–222.

(15) For an excellent discussion of possible scavengers, see: King, D.; Fields, C.; Fields, G. *Int. J. Peptide Protein Res.* **1990**, *36*, 255–266.

(16) Silanes were introduced as scavengers for peptide chemistry by: Pearson, D. A.; Blanchette, M.; Baker, M. L.; Guindon, C. A. *Tetrahedron Lett.* **1989**, *30*, 2739–2742. Since triethylsilane can reduce the indole side chain of tryptophan, these authors suggest use of triisopropylsilane for sequences containing this residue. We find that Tmob removal by a cocktail of TFA–DCM–$(i\text{-Pr})_3$SiH (5:94.5:0.5) is incomplete after 5 min at 25 °C but complete in 30 min. The coproduct is 2,4,6-trimethoxytoluene, verified by comigration with an authentic standard (Aldrich) on HPLC.

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(18) (a) Tessier, M.; Albericio, F.; Pedroso, E.; Grandas, A.; Eritja, R.; Giralt, E.; Granier, C.; van Rietschoten, J. *Int. J. Peptide Protein Res.* **1983**, *22*, 125–128. (b) Ten Kortenaar, P. B. W.; van Dijk, B. G.; Peters, J. M.; Raaben, B. J.; Adams, P. J. H. M.; Tessier, G. I. *Int. J. Peptide Protein Res.* **1986**, *27*, 398–400.

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(20) Barany, G.; Albericio, F.; Biancalana, S.; Bontems, S.; Chang, J.; Eritja, R.; Ferrer, M.; Fields, C.; Fields, G.; Lyttle, M.; Solé, N.; Tian, Z.; Van Abel, R.; Wright, P.; Zalipsky, S.; Hudson, D. In *Peptides: Chemistry and Biology*; Smith, J. A., Rivier, J. E., Eds.; Escom: Leiden, The Netherlands, 1991; pp 603–604. The support used for the present studies was prepared by BOP/HOBt/DIEA coupling of dimaleyl-Jeffamine (MW 2200) to an MBHA-PS resin (initial loading 0.6 mmol/g); following selective acid hydrolysis, PEG-PS was obtained comprising PEG:PS = 1:1 by weight and with 0.18 mmol/g final loading of amino sites. This material is particularly suited for use in automated continuous-flow synthesizers.

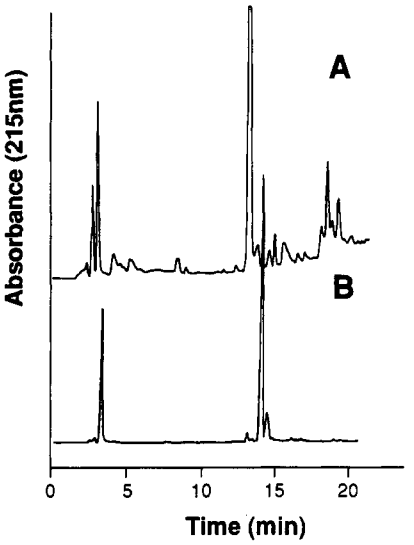

Figure 3. Analytical HPLC of samples from solid-phase syntheses of oxytocin with Cys(Tmob) protection. Panel A, the peptide-resin was oxidized with $Tl(tfa)_3$ (1.2 equiv) in DMF–anisole (18:1), 90 min, 0 °C, followed by cleavage from the support with TFA–DCM–anisole (8:1:1), 2 h, 25 °C. Panel B, direct cleavage of the protected peptide-resin (no oxidation). HPLC was performed as indicated in the legend to Figure 1. Monomeric oxidized (disulfide) and reduced (dithiol) oxytocins elute at t_R 12.9 and 14.0 min, respectively.

peptide-resins agreed with theory, and the final cleavage/deprotection steps were carried out under two sets of conditions: TFA–DCM–anisole–βME (70:25:3:2),[21] 1 h, 25 °C (Figure 1) and TFA–DCM–phenol–thioanisole–water (70:15:5:5:5), 1 h, 25 °C (Figure 2). The material synthesized with Tmob (panel A) showed substantially improved purity with respect to the material made with Trt (panel B). The correct structure of the desired pentapeptide (free sulfhydryl form) was supported by a satisfactory fast atom bombardment mass spectrum (FABMS).

Use of Cys(Tmob) in Fmoc SPPS (Disulfides). The Tmob protecting group was also evaluated with regard to chemistry for deblocking accompanied by simultaneous direct oxidation to provide a disulfide bond. These newer studies were pursued in the context of an extensive recent investigation from our laboratories[8e] that defined optimal conditions for the use of Trt and Acm in polymer-supported intramolecular cyclizations of peptides with two protected cysteine residues. Pilot experiments carried out on Fmoc-Cys(Tmob)-OH (4) in solution indicated that bis(Fmoc)-cystine with reasonable absolute yields (65–90%) upon treatment with iodine (1–5 equiv), 15–30 min, 25 °C, in chloroform, or in DMF, methanol, or acetic acid (latter three solvents neat or with 20% v/v water). Some Fmoc-cysteic acid (5–10%) was also formed during these oxidations. Extending these observations to the solid-phase case, the model peptide Ac-Cys(Tmob)-Pro-D-Val-Cys(Tmob)-NH_2, known to readily assume a type-II β-turn conformation,[8e,22] was assembled starting with an Fmoc-PAL-Nle-MBHA-resin, and the peptide-resin was

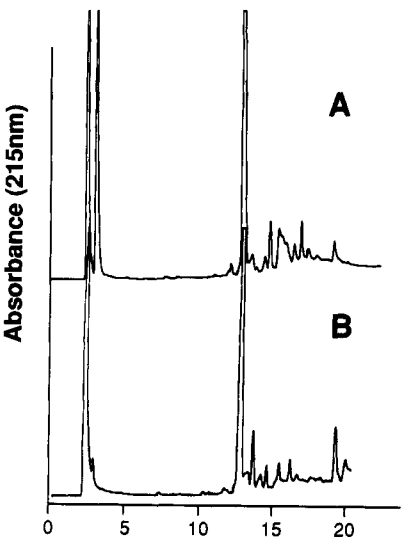

Figure 4. Analytical HPLC of samples from solid-phase syntheses of oxytocin with oxidizing agent present in cleavage cocktail. Panel A, iodine (3 equiv) in TFA–DCM–anisole (82:15:3), 1 h, 0 °C, followed by 1 h, 25 °C. Panel B, $Tl(tfa)_3$ (1.3 equiv), other conditions the same. HPLC conditions same as Figures 1 and 3.

oxidized for 90 min with iodine (10 equiv) in DMF at 0 °C. Of the material released into solution after cleavage with TFA–DCM–dimethyl sulfide (DMS) (14:7:1), 64% was the desired monomeric cyclic tetrapeptide.

Further experiments were directed toward the more difficult problem of synthesizing the nonapeptide oxytocin. The linear bis(Tmob) sequence, assembled on Fmoc-PAL-Nle-MBHA-resin, was best oxidized by thallium tris(trifluoroacetate)[7e,8e] [$Tl(tfa)_3$] (1.2–2.4 equiv), 90 min in DMF–anisole (18:1) at 0 °C. The material cleaved with TFA–DCM–DMS (8:1:1) included about 65% monomeric oxytocin (absolute amount, by comparison to standard of known concentration), with the remaining peptide material presumably dimers and oligomers (Figure 3, panel A). Omission of anisole from the oxidation cocktail was accompanied by a 2- to 3-fold reduction in absolute yield, although HPLC traces appeared comparable. Moreover, resin-bound oxidation with iodine (3.0 equiv) in DMF revealed substantially worse yields and purities. This pattern of results is comparable to observations reported earlier[8e] on $Tl(tfa)_3$ oxidation of the corresponding bis(Acm) sequence. The optimal absolute yields starting with bis(Acm) sequences depend on use of $Tl(tfa)_3$ in only slight molar excess; yields become appreciably lower with more $Tl(tfa)_3$ (i.e., 2–3 equiv).[8e] In contrast, bis(Tmob) sequences can be oxidized safely with a wider range of $Tl(tfa)_3$ excesses (this work). By way of comparing acid-labile cysteine protecting groups, it should be noted that bis(Trt) sequences *cannot* be oxidized successfully with the $Tl(tfa)_3$ reagent.

A final variation in the oxytocin series involved *concurrent* oxidation and detachment of the peptide from the support. Treatment of the bis(Tmob) peptide-resin with solutions of either iodine (3.0 equiv) or $Tl(tfa)_3$ (1.3 equiv) in TFA–DCM–anisole (82:15:3), 1 h at 0 °C followed by 1 h at 25 °C, provided oxytocin as the monomeric disulfide in excellent yield (60–65%) and purity (Figure 4, panels A and B, respectively). As a control, direct cleavage in the absence of oxidizing agents gave reduced oxytocin (dithiol) in ~80% yield and >85% purity (Figure 3, panel B).

Conclusions

We have defined here an array of conditions for appli-

(21) The use of a combination of anisole with β-mercaptoethanol to scavenge acidolytic cleavage/deprotection reactions was first proposed by: Grandas, A.; Pedroso, E.; Giralt, E.; van Rietschoten, J. *Tetrahedron* **1986**, *42*, 6703–6711. See also: (b) Kneib-Cordonier, N.; Albericio, F.; Barany, G. *Int. J. Peptide Protein Res.* **1990**, *35*, 527–538. In the present application, cleavage for 1 h (no water; compare to results of model studies discussed in previous paragraph) was adequate to remove Tmob with negligible reattachment onto Cys or Trp.

(22) (a) García-Echeverría, C.; Albericio, F.; Pons, M.; Barany, G.; Giralt, E. *Tetrahedron Lett.* **1989**, *30*, 2441–2444. (b) García-Echeverría, C.; Siligardi, G.; Mascagni, P.; Gibbons, W.; Giralt, E.; Pons, M. *Biopolymers* **1991**, *31*, 835–843.

cation of the S-Tmob group in the preparation by Fmoc SPPS of peptides containing cysteine residues in either the free thiol or disulfide form. Our results indicate that S-Tmob may prove to be a useful alternative to the widely applied S-Acm or S-Trt groups for both manual and automated SPPS. In particular, the improved solubility characteristics of Fmoc-Cys(Tmob)-OH with respect to Fmoc-Cys(Trt)-OH should prove advantageous. Current studies in our laboratory reveal the value of a Tmob/Acm combination for orthogonal Fmoc SPPS of peptides with two disulfide bonds.

Experimental Section

General Procedures. Materials, solvents, instrumentation, and general methods were essentially as summarized in previous publications from these laboratories.[6e,8c,e] ^1H and ^{13}C NMR spectra were recorded at 300 and 75 MHz, respectively, on an IBM NT-300 instrument using $CDCl_3$, CD_3SOCD_3, or CD_3OD as solvents; peak assignments were confirmed by DEPT experiments. Optical rotations were measured on a Perkin-Elmer 141 instrument. Thin-layer chromatography was performed on Analtech silica gel GF plates (250 μm, 10 × 20 cm), developed with the following: CA, chloroform–acetic acid (19:1); BW, 2-methyl-2-propanol–water (17:3); or BPAW, n-butanol–pyridine–acetic acid–water (15:10:3:12). Compounds were visualized by (1) fluorescence quenching; (2) iodine vapor; (3) spray with 0.02% (w/v) ninhydrin in acetone or 0.3% (w/v) ninhydrin in γ-collidine–acetic acid–ethanol (1:3.3:29); (4) spray with 5,5′-dithiobis(2-nitrobenzoic acid) (0.01 M) in pH 7.0 buffer followed by spray with 0.1 N Tris·HCl buffer pH 8 (free thiol indicated by immediate formation of yellow spot). Elemental analyses were conducted by M-H-W Laboratories, Phoenix, AZ.

2,4,6-Trimethoxybenzyl Alcohol (2). Sodium borohydride (2.8 g, 75 mmol) was added portionwise to a solution of 2,4,6-trimethoxybenzaldehyde (1) (10.0 g, 51 mmol) in methanol–1 N NaOH (1:1, 100 mL) at 25 °C. After 2 h under constant stirring, the mixture was concentrated partially (~50 mL) and extracted with anhydrous Et_2O (3 × 100 mL). The combined organic layers were washed with saturated NaCl (2 × 150 mL), dried ($MgSO_4$), and concentrated in vacuo to provide an NMR- and TLC-pure white solid (9.3 g, 92%): mp 56–58 °C (lit.[14] mp 63 °C); TLC (BPAW) R_f 0.83, TLC (BW) R_f 0.82; ^1H NMR (CD_3SOCD_3) δ 6.19 (s, 2 H), 3.76 (d, J = 3.8 Hz, 2 H), 3.47 (s, 9 H); ^{13}C NMR ($CDCl_3$) δ 161.1 (aryl C4), 159.2 (aryl C2 and C6), 110.0 (aryl C1), 90.6 (aryl C3 and C5), 55.7 (ortho, 2 × OCH_3), 55.3 (para, OCH_3), 54.2 (CH_2OH). Anal. Calcd for $C_{10}H_{14}O_4$, MW 198.22: C, 60.59; H, 7.12. Found: C, 60.39; H, 7.17.

S-(2,4,6-Trimethoxybenzyl)-L-cysteine Trifluoroacetate Salt [Tfa^- $^+H_2$-Cys(Tmob)-OH] (3). *Procedure A.*[23] A suspension of L-cysteine (free base, 0.9 g, 7.4 mmol) in DCM (60 mL) was stirred at <5 °C, and TFA (12.0 mL, 156 mmol) was added dropwise to dissolve completely the amino acid. A solution of 2,4,6-trimethoxybenzyl alcohol (2) (1.5 g, 7.5 mmol) in DCM (30 mL) was added dropwise over ~10 min, following which the reaction mixture was concentrated in vacuo. The residue was chased with anhydrous diethyl ether (Et_2O, ~50 mL), triturated further with Et_2O (~200 mL), and filtered to provide a white solid (2.9 g, 84%): mp 175–185 °C; TLC (BPAW) R_f 0.72; $[\alpha]_D$ = −64.5° (c = 1.0, H_2O); ^1H NMR (CD_3OD) δ 6.22 (s, 2 H, aromatic), 3.98 (dd, J = 3.7 and 10.3 Hz, α-H), 3.88 (d, J = 13.1 Hz, 1 H, benzylic), 3.82 (s, 6 H, OCH_3, ortho), 3.80 (s, 3 H, OCH_3, para), 3.67 (d, J = 13.1 Hz, 1 H, benzylic), 3.19 (dd, J = 3.7 and 14.7 Hz, β-H), 2.72 (dd, J = 10.3 and 14.7 Hz, β-H); ^{13}C NMR (CD_3OD) δ 170.8 (COOH), 162.4 (aryl C4), 160.1 (aryl C2 and C6), 107.6 (aryl C1), 91.6 (aryl C3 and C5), 56.2 (ortho, 2 × CH_3O), 55.8 (para, CH_3O), 53.4 (α-C), 32.6 (CH_2 SAr), 24.0 (β-CH_2). Anal. Calcd for $C_{15}H_{20}F_3NO_7S$, MW 415.38: C, 43.40; H, 4.85; N, 3.37; S, 7.71. Found: C, 43.77; H, 5.19; N, 3.47; S, 7.60.

Procedure B. A general procedure introduced by Photaki[13] was modified. A mixture of L-cysteine hydrochloride (0.25 g, 1.6 mmol) and 2 (0.3 g, 1.6 mmol) in TFA (3.0 mL) was stirred at 25 °C for 15 min, following which the mixture was concentrated, Et_2O (5 mL) was added to the residue, and 10% (w/v) aqueous sodium acetate (~25 mL) was added. The resultant precipitate was collected, washed with water, and triturated with hot Et_2O to provide the product isolated as the zwitterion (0.3 g, 65%); mp 127–130 °C; further characteristics reported with procedure A.

$N^α$-(9-Fluorenylmethyloxycarbonyl)-S-(2,4,6-trimethoxybenzyl)-L-cysteine [Fmoc-Cys(Tmob)-OH] (4). *Procedure A.*[18b] A solution of Fmoc-OSu (2.2 g, 6.5 mmol) in acetonitrile (50 mL) was added slowly to a well-stirred solution of S-(2,4,6-trimethoxybenzyl)-L-cysteine trifluoroacetate salt (3) (2.45 g, 5.9 mmol) and triethylamine (2.5 mL, 15.0 mmol) in distilled water (80 mL). After reaction for 3 h at 25 °C, the mixture was partially concentrated in vacuo (most of the acetonitrile was removed; mixture became cloudy). Ice–water (250 mL) was added, followed by sufficient 10% (w/v) aqueous citric acid to bring the pH to 3.1. The chilled aqueous mixture, which included a white precipitate, was extracted with ethyl acetate (EtOAc; 2 × 150 mL), and the organic layers were combined, washed with pH 3 citric acid (2 × 150 mL), dried ($MgSO_4$), and concentrated in vacuo to provide an off-white solid (2.5 g, 80%): mp 98–101 °C; $[\alpha]_D$ = +6.6° (c = 1.0, $CHCl_3$); TLC (CA) R_f 0.63; ^1H NMR ($CDCl_3$) δ 7.73 (d, J = 7.6 Hz, 2 H, Fmoc), 7.49 (d, J unresolved, 2 H, Fmoc), 7.37 (t, J = 7.4 Hz, 2 H, Fmoc), 7.28 (t, J = 7.4 Hz, 2 H, Fmoc), 6.09 (s, 2 H, Tmob), 5.82 (d, J = 8.2 Hz, NH), 4.64 (m, α-H), 4.44 (d, J = 6.7 Hz, Fmoc CH_2), 4.23 (t, J = 6.7 Hz, Fmoc CH), 3.85 (d, J = 12.9 Hz, benzylic, 1 H), 3.77 (s, 9 H, OCH_3), 3.72 (d, J = 12.9 Hz, benzylic, 1 H), 2.96 (dd, J unresolved, β-H, 2 H); ^{13}C NMR ($CDCl_3$) δ 175.9 (COOH), 160.7 (aryl C4), 158.9 (aryl C2 and C6), 156.5 (Fmoc carbonyl), 143.9 and 141.4 (Fmoc-aryl C), 127.8, 127.2, 125.2, and 120.0 (Fmoc-aryl CH), 107.2 (aryl C1), 90.8 (aryl C3 and C5), 67.0 (Fmoc-CH_2), 55.8 (ortho, 2 × CH_3O), 55.4 (para, CH_3O), 54.3 (α-C), 47.2 (fluorenyl C9), 33.5 (CH_2 SAr), 24.6 (β-C). Anal. Calcd for $C_{28}H_{29}NO_7S$, MW 523.60: C, 64.23; H, 5.58; N, 2.67; S, 6.12. Found: C, 64.33; H, 5.42; N, 2.60; S, 6.00.

Procedure B.[18a] A solution of Fmoc-N_3 (0.2 g, 0.7 mmol) in dioxane (3.0 mL) was added to a solution of S-(2,4,6-trimethoxybenzyl)-L-cysteine (zwitterion, compare to structure 3) (0.2 g, 0.7 mmol) in 10% (w/v) aqueous sodium carbonate (5.0 mL) at 0 °C. The reaction was allowed to run for 1 h at 0 °C and 2 days at 25 °C, with the pH being maintained at 9.0–9.5 by addition of aqueous sodium carbonate. The mixture was quenched in ice–water (50 g) and extracted with Et_2O (3 × 25 mL), and the aqueous phase was combined with EtOAc (50 mL). Next, 1 N aqueous HCl was added until the aqueous phase reached pH ~2; the organic phase was separated and combined with further EtOAc extracts (3 × 50 mL) of the aqueous phase. The combined organic extracts were washed with 0.1 N aqueous HCl (2 × 100 mL), H_2O (2 × 100 mL), and saturated aqueous NaCl (2 × 100 mL), dried ($MgSO_4$), and concentrated. The residue was triturated with n-hexane to furnish title product (0.28 g, 76%); mp 111–113 °C; further characteristics reported with procedure A.

Studies on Tmob Removal and Stability. S-(2,4,6-trimethoxybenzyl)-L-cysteine trifluoroacetate salt (3) was dissolved in various reagent/solvent milieus (1.0 mL scale), at an overall concentration of 1–2 mM, and the fate of 3 was monitored by TLC (BW or BPAW, R_f ~ 0.7 for 3, 0.5 for free cysteine) with fluorescence quenching, ninhydrin, and Ellman detection (see General). A statement in the text that complete cleavage occurred corresponds to the observed absence of 3 and its replacement by cysteine. Cleavage cocktails containing silane scavengers (but not aromatic scavengers) were evaluated further by analytical HPLC on a Vydac C-18 column, linear gradient over 15 min of CH_3CN and 0.01 N aqueous HCl from 1:9 to 3:1, flow rate 1.2 mL/min, detection at 260 or 280 nm; t_R ~ 8.6 min for 3 and 17.2 min for 2,4,6-trimethoxytoluene.[16] Treatment of 3 with piperidine–DMF (1:1 or 1:4) or 0.1 M tetra-n-butylammonium fluoride in DMF, 48 h, 25 °C, revealed that 3 remained unchanged.

Studies on Tmob Oxidation. Solutions of Fmoc-Cys(Tmob)-OH (4), at a concentration of 3–5 mM in the appropriate

(23) The reproducible, optimized procedure reported here reflects experiences from less successful variations (solvent, acid, temperature, order of addition, workup) in which Cys(Tmob) was contaminated with oligomers and polymers derived from the Tmob carbonium ion. The byproducts (in some cases, formed in amounts greater than desired 3) were indicated by additional ^1H NMR peaks [δ 6.09 (s), 3.7 (family of closely spaced singlets)], ions upon FABMS (thioglycerol matrix) at m/z 361.1 and 541.1, and a faster migrating spot (R_f 0.71) upon TLC (BPAW).

solvent (3.0 mL), were treated with iodine (1–5 equiv) for 15–60 min, and the reactions were quenched by addition of solid ascorbic acid or sodium thiosulfate (slight excess). Analytical HPLC on a Vydac C-18 column, linear gradient over 15 min of CH_3CN and 0.01 N aqueous HCl from 3:7 to 9:1, flow rate 1.0 mL/min, detection at 260 or 280 nm; resolved unreacted **4** from bis(Fmoc)-cystine, $t_R \sim 14.3$ and 14.7 min, respectively (base-line resolution). Alternatively, an aliquot (10 μL) from the oxidation mixture was evaporated, treated with piperidine–DMF (1:1) (0.2 mL), diluted with pH 2.2 buffer (0.5 mL), and injected onto an amino acid analyzer to quantitate cystine (36 min), as well as cysteic acid (8 min), leucine (external reference, 47 min), and H-Cys(Tmob)-OH (**3**) (60 min, generally absent since oxidations were complete).

H-Trp-Met-Asp-Phe-Cys-NH_2. The experimental design and principal conclusions are given in the text and accompanying Figures 1 and 2. Chain assembly was carried out on a MilliGen/Biosearch Model 9050 Automated Peptide Synthesizer (continuous-flow), starting with an Fmoc-PAL-PEG-PS[20] resin (100 mg, 0.18 mmol/g). Fmoc removal was with piperidine–DMF (3:7, 7 min), followed by washes with DMF (3.0 mL/min for 12 min). The required Fmoc-amino acids (5 equiv) were incorporated with the aid of *N,N'*-diisopropylcarbodiimide (DIPCDI) and HOBt (3 equiv each), 1 h, in a total circulating volume of 5.0 mL. The completed peptide-resins contained Asp 1.03; Met 0.95; Phe 1.02 [synthesis with Cys(Tmob)]; Asp 1.04; Met 0.96; Phe 1.00 [synthesis with Cys(Trt)]. The peptide-resins (20 mg per experiment) were treated with appropriate cleavage cocktails (1.0 mL), 1 h, 25 °C. The filtrate from each cleavge reaction was diluted with acetic acid–water (3:7, 2 mL) and extracted with DCM (4 mL). The aqueous layer was purged with nitrogen (to remove excess DCM) and evaluated by HPLC. Peptide material (corresponding to Figure 1, panel A) was also collected after lyophilization and submitted for FABMS (thioglycerol matrix): calculated monoisotopic mass of $C_{32}H_{41}N_7O_7S_2$ 699.25, positive spectrum *m/z* 722.1 [$(M + Na)^+$], 700.2 [MH^+], negative spectrum *m/z* 698.1 [$(M - H)^-$].

Ac-Cys-Pro-D-Val-Cys-NH_2. Chain assembly by Fmoc chemistry was performed manually (0.3 g scale, 2-mL wash volumes), starting with an Fmoc-PAL-Nle-MBHA resin (0.19 mmol/g), essentially as described previously.[8e] Fmoc removal was with piperidine–DMF (3:7, 2 × 6 min), followed by washing with DMF–DCM (1:1, 4 × 1 min). Couplings were achieved by first dissolving the Fmoc amino acid, BOP reagent, and HOBt (3.0 equiv each) in a 0.3 M solution of NMM (4.5 equiv) in DMF then waiting ~2 min for preactivation, adding the mixture to the resin, and shaking for 1.5–2.0 h (negative ninhydrin test obtained). The final acetylation was carried out with Ac_2O (0.33 M)–DIEA (0.33 M) in DMF for 1 h. Final loadings, as determined by amino acid analysis on D-valine, were ~0.15 mmol/g. For oxidation, the peptide-resin (20 mg) was suspended in DMF (1.0 mL) at 0 °C, and iodine (8 mg, 10 equiv, final concentration 3.3 mM) was added. After 90 min of reaction, the peptide-resin was washed with DMF and DCM (4 × 1 min each), and cleavage was carried out with TFA–DCM–DMS (14:7:1) (1 mL), 2 h, 25 °C. The absolute yield of the desired monomeric cyclic tetrapeptide was determined by comparing the HPLC peak area of the crude peptide to that of a standard of pure peptide of known concentration, as described previously.[8e]

H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2 (Oxytocin). Chain assembly was carried out on a MilliGen Model 9050 automated peptide synthesizer (continuous-flow), starting with an Fmoc-PAL-Nle-MBHA resin (300 mg, nominally, 0.27 mmol/g, based on Nle). Glass beads (equal mass) were added to the column in order to minimize collapse and damage of the polystyrene-based peptide-resin under continuous-flow conditions. The required Fmoc-amino acids (6 equiv) were incorporated by 1-h couplings (except Ile, 90 min) mediated by BOP/HOBt/NMM [BOP and HOBt, 6 equiv each, final concentration of 0.25 M in 5.0 mL of DMF containing NMM at 0.5 M]. Tyrosine was incorporated with *t*-Bu side-chain protection, whereas asparagine and glutamine were incorporated as the side-chain unprotected pentafluorophenyl active esters (6 equiv for 1 h in the presence of 6 equiv of HOBt). The final peptide-resins (0.15 mmol/g) comprised Asp 1.04, Glu 1.05, Pro 1.07, Gly 1.09, Ile 0.88, Leu 1.01, Tyr 0.92, Cys not determined. Oxidations were carried out at 0 °C, using peptide-resin (~20 mg) suspended in the appropriate solvent (~1.0 mL) containing iodine or $Tl(tfa)_3$ [final concentration 3–9 mM]. After 60–90 min of reaction, peptide-resins were washed with DMF and DCM (4 × 1 min each) and cleaved with TFA–DCM–anisole (8:1:1) (1 mL), 2 h, 25 °C. In other experiments, the oxidant was added directly to the cleavage cocktail. Further analysis (Figures 3 and 4) was carried out essentially as described previously.[8e]

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