Novel *S*-Xanthenyl Protecting Groups for Cysteine and Their Applications for the N^{α}-9-Fluorenylmethyloxycarbonyl (Fmoc) Strategy of Peptide Synthesis[1−3]

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The 9*H*-xanthen-9-yl (Xan) and 2-methoxy-9*H*-xanthen-9-yl (2-Moxan) groups can be introduced onto sulfhydryl functions by *S*-alkylation reactions involving the corresponding xanthydrols, plus trifluoroacetic acid (TFA) as catalyst. Conversely, these groups are removed rapidly by acid in the presence of appropriate silane or thiol scavengers. The 3-methoxy-9*H*-xanthen-9-yl (3-Moxan) derivative was also studied, but abandoned for several reasons including challenging synthesis, excessive lability to acid, and insufficient stability in the presence of base. The N^{α}-9-fluorenylmethyloxycarbonyl (Fmoc), *S*-Xan or 2-Moxan-protected cysteine derivatives were prepared and applied to the solid-phase syntheses of several model peptides. Selective removal of *S*-Xan and *S*-2-Moxan groups, while retaining tris(alkoxybenzyl)amide (PAL) anchoring, is best accomplished with TFA−CH_2Cl_2−Et_3SiH (1:98.5:0.5), 25 °C, 2 h. Alternatively, oxidative deprotection of *S*-Xan or *S*-2-Moxan with iodine (10−20 equiv) or thallium(III) tris(trifluoroacetate) [$Tl(tfa)_3$] (1−3 equiv) to provide disulfides can be carried out on peptide substrates both in solution and while polymer-bound. Compared to established chemistries with the acid-labile and oxidizable *S*-triphenylmethyl (Trt) group, *S*-Xan and *S*-2-Moxan gave equal or superior results in terms of peptide purities (including no detectable tryptophan alkylation) and overall yields.

Disulfide bridges play critical roles in stabilizing the tertiary structures of peptides and proteins. Much effort has been devoted toward the controlled construction of disulfide bridges, which requires optimal protection strategies for chain assembly and for selective cleavage of the sulfhydryl protecting groups.[4] Of the *S*-protecting groups compatible with the base-labile N^{α}-9-fluorenylmethyloxycarbonyl (Fmoc) group,[5] the ones most often used are *S*-acetamidomethyl (Acm),[6] *S*-triphenylmethyl (Trt),[7] and *S*-*tert*-butylmercapto (S*t*Bu).[8] In addition, the *S*-2,4,6-trimethoxybenzyl (Tmob) group developed by our research laboratory has shown excellent properties for Fmoc solid-phase peptide synthesis.[9] Despite considerable progress in developing mild and/or selective conditions for removal of *S*-protection, problems have been observed on occasion with anchoring, chain assembly, deprotection, or direct oxidation.[4b,5c,9] We report here the preparation and applications of two novel Fmoc-compatible *S*-protecting groups: *S*-9*H*-xanthen-9-yl (Xan) and *S*-2-methoxy-9*H*-xanthen-9-yl (2-Moxan), which are more labile toward acidolysis and more readily oxidized than *S*-Trt and *S*-Tmob.[10,11]

Results and Discussion

Preparation of *S*-Xanthenyl Protected Cysteine Derivatives. *S*-Xanthenyl groups were introduced onto the side-chain sulfhydryl of cysteine by two different

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(1) A preliminary account of this work was reported at the 14th American Peptide Symposium, Columbus, OH, June 18−23, 1995. Solé, N. A.; Han, Y.; Vágner, J.; Gross, C. M.; Tejbrant, J.; Barany, G. In *Peptides−Chemistry, Structure and Biology*: Proceedings of the Fourteenth American Peptide Symposium; Kaumaya, P. T. P., Hodges, R. S., Eds.; ESCOM Science Publishers: Leiden, The Netherlands, 1996; pp 113−114.

(2) Taken in part from the Ph.D. thesis of Y. Han, University of Minnesota, October, 1996.

(3) Abbreviations used for amino acids and the designation of peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in *J. Biol. Chem.* **1972**, *247*, 977−983. The following additional abbreviations are used: Acm, acetamidomethyl; Ac_2O, acetic anhydride; *β*ME, *β*-mercaptoethanol; BOP, benzotriazolyl *N*-oxytris-(dimethylamino)phosphonium hexafluorophosphate; DIEA, *N*,*N*-diisopropylethylamine; DIPCDI, *N*,*N*′-diisopropylcarbodiimide; DME, 1,2-dimethoxyethane; DMF, *N*,*N*-dimethylformamide; Et_2O, diethyl ether; EtOAc, ethyl acetate; FABMS, fast atom bombardment mass spectrometry; Fmoc, 9-fluorenylmethyloxycarbonyl; HOAc, acetic acid; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; MeOH, methanol; 2-Moxan, 2-methoxy-9*H*-xanthen-9-yl; 3-Moxan, 3-methoxy-9*H*-xanthen-9-yl; Nle, norleucine; NMM, *N*-methylmorpholine; PAL, peptide amide linker: [5-(4-((9-fluorenylmethyloxycarbonyl)aminomethyl)-3,5-dimethoxyphenoxy)valeric acid; PEG-PS, polyethylene glycol−polystyrene (graft support); reagent M*, TFA−CH_2Cl_2−anisole−*β*ME (70:25:3:2); reagent R, TFA−thioanisole−1,2-ethanedithiol−anisole (90:5:3:2); TFA, trifluoroacetic acid; Tmob, 2,4,6-trimethoxybenzyl; $Tl(tfa)_3$, thallium(III) tris(trifluoroacetate); Trt (or trityl), triphenylmethyl; Xan, 9*H*-xanthen-9-yl. Amino acid symbols denote the L-configuration unless indicated otherwise.

(4) Reviews: (a) Barany, G.; Merrifield, R. B. In *The Peptides, Analysis, Synthesis, Biology;* Gross, E., Meienhofer, J., Eds.; Academic Press: New York, 1979; Vol. 2, pp 1−284. (b) Andreu, D.; Albericio, F.; Solé, N. A.; Munson, M. C.; Ferrer, M.; Barany, G. In *Methods in Molecular Biology*; Pennington, M. W., Dunn, B. M., Eds.; Humana Press: Totowa, NJ, 1994; Vol. 35; pp 91−169. (c) Moroder, L.; Besse, D.; Musiol, H.-J.; Rudolph-Böhner, S.; Siedler, F. *Biopolymers (Peptide Science)* **1996**, *50*, 207-234.

(5) (a) Carpino, L. A.; Han, G. Y. *J. Am. Chem. Soc.* **1970**, *92*, 5748−5749. Reviews: (b) Atherton, E.; Sheppard, R. *Solid Phase Peptide Synthesis: A Practical Approach*; IRL Press: Oxford, 1989. (c) Fields, G. B.; Tian, Z.; 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.

(6) Veber, D. G.; Milkowski, J. D.; Varga, S. L.; Denkewalter, R. G.; Hirschmann, R. *J. Am. Chem. Soc.* **1972**, *94*, 5456−5461.

(7) (a) Photaki, I.; Taylor-Papadimitriou, J.; Sakarellos, C.; Mazarakis, P.; Zervas, L. *J. Chem. Soc., Chem. Commun.* **1970**, 2683−2687. (b) Sieber, P.; Kamber, B.; Hartmann, A.; Jöhl, A.; Riniker, B.; Rittel, W. *Helv. Chem. Acta* **1974**, *57*, 2617−2621. (c) McCurdy, S. N. *Pept. Res.* **1989**, *2*, 147−152.

(8) (a) Weber, U.; Hartter, P. *Hoppe-Seyler's Z. Physiol. Chem.* **1970**, *351*, 1384−1388. (b) Atherton, E.; Pinori, M.; Sheppard, R. C. *J. Chem. Soc., Perkin Trans. 1* **1985**, 2057−2064. (c) Eritja, R.; Ziehler-Martin, J. P.; Walker, P. A.; Lee, T. D.; Legesse, K.; Albericio, F.; Kaplan, B. *Tetrahedron* **1987**, *43*, 2675−2680.

(9) Munson, M. C.; García-Echeverría, C.; Albericio, F.; Barany, G. *J. Org. Chem.* **1992**, *57*, 3013-3018, and references cited therein.

(10) We have also shown that Xan and 2-Moxan are valuable for protection of the N^{ω}-amide function of asparagine and glutamine. See: Han, Y.; Solé, N. A.; Tejbrant, J.; Barany, G. *Pept. Res.* **1996**, *9*, 166−173.

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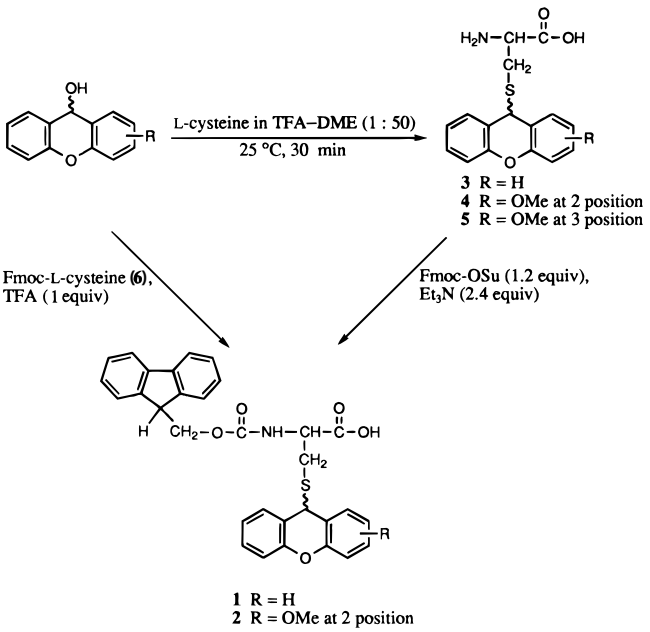
Scheme 1[a]



[a] As described in text, results were poor with R = OMe at 3 position.

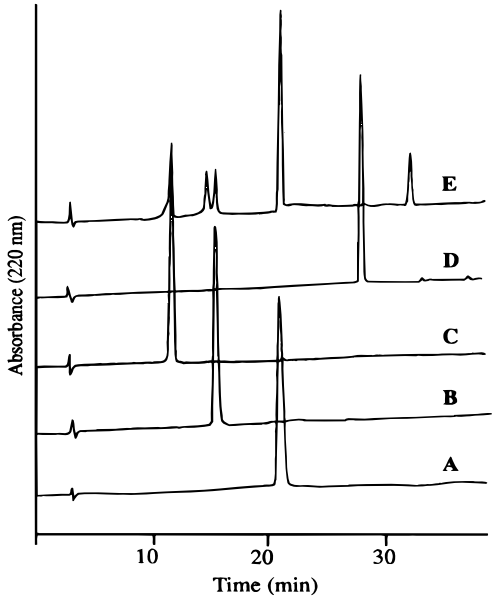


Figure 1. HPLC analysis of the transformation of H-Cys(3-Moxan)-OH upon treatment with piperidine−DMF (1:4), 25 °C, 24 h. (A) Standard of 3-methoxy-9*H*-xanthen-9-one, t_R = 21.1 min. (B) Standard of 3-methoxy-9*H*-xanthen-9-ol, t_R = 15.6 min. (C) Standard of H-Cys(3-Moxan)-OH, t_R = 11.7 min. (D) Standard of 3-methoxy-9*H*-xanthene, t_R = 27.3 min. (E) Reaction mixture after 24 h treatment of H-Cys(3-Moxan)-OH with piperidine−DMF (1:4). See Experimental Section for HPLC conditions.

approaches (Scheme 1). The method preferred on a laboratory scale started with the quantitative reduction of bis(Fmoc)cystine to Fmoc-cysteine by use of Zn (10 equiv) in trifluoroacetic acid (TFA)−MeOH (1:19) at 25 °C, followed by acid-catalyzed reactions with the corresponding xanthydrols[10,12] in appropriate solvents. Alternatively, acid-catalyzed *S*-xanthenylation of cysteine was carried out first, followed by introduction of the Fmoc group onto the free N^{α}-amino group by use of Fmoc-succinimide (Fmoc-OSu). Yields and purities by both routes were high for the Xan and 2-Moxan derivatives. The H-Cys(3-Moxan)-OH derivative (TFA salt) was made readily, but attachment of the Fmoc group was problematic due to the borderline stability of the desired final product during the purification steps; similar difficulties marred the route *via* Fmoc-cysteine.

Stabilities/Labilities of *S*-Xan, *S*-2-Moxan, and *S*-3-Moxan Groups. Given our goal to apply these *S*-protecting groups to Fmoc chemistry, the appropriate derivatives were exposed to an Fmoc deblocking milieu, piperidine−DMF (1:4, v/v) at 25 °C. Whereas H-Cys-(Xan)-OH and H-Cys(2-Moxan)-OH were found to be stable to the secondary amine base for more than 24 h, H-Cys(3-Moxan)-OH decomposed extensively under these conditions (Figure 1). Decomposition products derived from the 3-Moxan group include the corresponding xanthone and xanthydrol; the fate of the cysteine residue was not established. Due to the observed instability of H-Cys(3-Moxan)-OH to base, this derivative was not evaluated further. The Fmoc-Cys(Xan or 2-Moxan)-OH derivatives were stable to 0.3 M 1-hydroxybenzotriazole (HOBt) in *N*,*N*-dimethylformamide (DMF), 25 °C, 24 h.

Acidolytic Removal of *S*-Xan and *S*-2-Moxan Groups. The *S*-Xan and *S*-2-Moxan groups are removed rapidly from cysteine derivatives by exposure to dilute TFA in the presence of silane or thiol scavengers (Table 1; Figure 2). Under otherwise identical acidolytic conditions, the *S*-2-Moxan group is removed somewhat more readily than *S*-Xan. Commonly used aromatic scavengers such as phenol and thioanisole are not at all effective (Table 1, entries 1 and 2), but β-mercaptoethanol used in great excess allows cleavages to go to completion (Table 1, entry 3). Silanes, *e.g.*, Et_3SiH and $i Pr_3SiH$, are highly efficient scavengers since they can convert 9*H*-xanthen-9-yl cations irreversibly to the corresponding xanthene derivatives.[13] The relative usefulness of scavengers for solution cleavages is reflected by the concentration of acid needed for *S*-Xan/*S*-2-Moxan removal: 0.1% TFA for 1 h or 0.2% TFA for 10 min is sufficient when either silane is used [Et_3SiH slightly better than $i Pr_3SiH$; *e.g.*, Table 1, entry 4 *vs* 8, 5 *vs* 9; Figure 2]; 10% TFA is needed in conjunction with β-mercaptoethanol (βME) (Table 1, entry 3); and even 50% TFA is not enough when phenol and thioanisole are used (Table 1, entry 2). However, the much weaker acid HOAc does not remove *S*-Xan even

(11) Independent of our studies, Voelter has reported that the 9-phenylxanthen-9-yl (pixyl) group, used widely in *O*-protection in nucleoside chemistry, can be used for thiol protection. See: Echner, H; Voelter, W. In *Innovation and Perspectives in Solid Phase Synthesis: Peptides, Polypeptides and Oligonucleotides;* Epton, R., Ed.; Intercept Ltd.: Andover, England, 1992; pp 371−375.

(12) Han, Y.; Bontems. S. L.; Hegyes, P.; Munson, M. C.; Minor, C. A.; Kates, S. A.; Albericio, F.; Barany, G. *J. Org. Chem.* **1996**, *61*, 6326-6339, and references cited therein.

(13) 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. Cleavage of *S*-Xan in the presence of Et_3SiH or $i Pr_3SiH$ gave 9*H*-xanthene as a coproduct, as verified by TLC and coinjection with a commercial standard on HPLC. Cleavage of *S*-2-Moxan gave 2-methoxy-9*H*-xanthene, an authentic sample of which was prepared as follows (see ref 10 for precedent): 2-methoxy-9*H*-xanthen-9-ol (0.28 g, 1.2 mmol) was added in 5 portions to TFA−CH_2Cl_2−Et_3SiH (2:93:5; 15 mL). The reaction mixture was stirred at 25 °C for 30 min, concentrated under reduced pressure, and purified further by silica gel chromatography. Yield: 0.24 g (92%); white solid, mp 68−69 °C; R_f [hexane−EtOAc (10:1)] 0.47; ^{1}H NMR (CD_3SOCD_3) δ 7.23 (d, J = 7.0 Hz, 1H), 7.19 (d, J = 7.5 Hz. 1H), 6.98−7.05 (m, 3H), 6.81 (d, J = 2.5 Hz, 1H), 6.78 (dd, J = 2.5 and 8.5 Hz, 1H), 4.63 (s, 2H), 3.78 (s, 3H).

Table 1. Acidolytic Removal of *S*-Xan and *S*-2-Moxan Groups[a]

no.	cleavage cocktail	time (min)	cleavage yield (%) *S*-Xan	cleavage yield (%) *S*-2-Moxan
1	TFA−CH_2Cl_2−phenol−thioanisole−H_2O (10:75:5:5:5)	60	< 1	< 1
2	TFA−CH_2Cl_2−phenol−thioanisole−H_2O (50:35:5:5:5)	60	19	53
3	TFA−CH_2Cl_2−βME (10:85:5)	30	98	100
4	TFA−CH_2Cl_2−Et_3SiH (0.1:99.4:0.5)	5	52	87
5	TFA−CH_2Cl_2−Et_3SiH (0.1:99.4:0.5)	15	87	93
6	TFA−CH_2Cl_2−Et_3SiH (0.1:99.4:0.5)	30	93	100
7	TFA−CH_2Cl_2−Et_3SiH (0.1:99.4:0.5)	60	100	100
8	TFA−CH_2Cl_2−iPr$_3$SiH (0.1:99.4:0.5)	5	38	42
9	TFA−CH_2Cl_2−iPr$_3$SiH (0.1:99.4:0.5)	15	70	79
10	TFA−CH_2Cl_2−iPr$_3$SiH (0.1:99.4:0.5)	30	95	100
11	TFA−CH_2Cl_2−iPr$_3$SiH (0.1:99.4:0.5)	60	100	100
12	TFA−CH_2Cl_2−Et_3SiH (0.2:99.3:0.5)	10	100	100
13	TFA−CH_2Cl_2−iPr$_3$SiH (0.2:99.3:0.5)	10	100	100
14	HOAc−CH_2Cl_2−Et_3SiH (50:48:2)	60	< 1	< 1

[a] Experimental procedures and HPLC conditions in text. For the Xan series, mixtures of authentic 9*H*-xanthene and Fmoc-Cys(Xan)-OH were used to establish calibration curves, and cleavage yield was determined by:

$$\%\ \text{cleavage yield} = \frac{(100)\ 9H\text{-xanthene (mol)}}{[\text{Fmoc-Cys(Xan)-OH} + 9H\text{-xanthene}]\ \text{(mol)}} = 100\left[\left(\frac{\text{Fmoc-Cys(Xan)-OH}}{9H\text{-xanthene}}\right) + 1\right]^{-1}$$

The corresponding standards were run and calculations carried out in the 2-Moxan series.

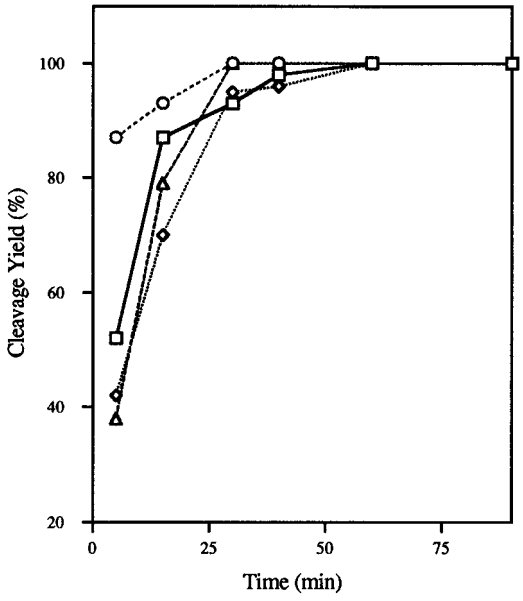


Figure 2. Cleavage kinetics in 0.1% TFA in CH_2Cl_2. Squares: *S*-Xan, Et_3SiH scavenger; diamonds: *S*-Xan, iPr$_3$SiH scavenger; circles: *S*-2-Moxan, Et_3SiH scavenger; triangles: *S*-2-Moxan, iPr$_3$SiH scavenger. Experimental details in text.

in the presence of silane scavengers (Table 1, entry 14). Interestingly, the amount of acid required to effect complete cleavage is considerably higher in the solid-phase mode: thus 0.1% TFA in the presence of Et_3SiH or iPr$_3$SiH, which led to complete removal of *S*-Xan/*S*-2-Moxan in solution model studies, gave only 18% and 25% removal, respectively, of *S*-Xan and *S*-2-Moxan from a protected Cys-PEG-PS model. However, removal is complete with 1% TFA for 2 h (Figure 3). It is possible that some TFA binds to the polyether moieties of the support backbone, hence lowering the effective concentration of acid.

Selective Acidolysis of *S*-Xan and *S*-2-Moxan Groups in the Presence of the Tris(alkoxy)benzylamide (PAL) Anchoring Linkage. For some applications, it is important to achieve selective release of free sulfhydryl groups while a peptide chain remains attached to a polymer support.[4b,14] Since the PAL anchor is used widely for the synthesis of peptide amides,[15] we studied the stability of PAL to the acid/scavenger combinations that cleave *S*-Xan and *S*-2-Moxan. No detectable PAL cleavage was observed in 2 h at 25 °C from treatment with 1% TFA in CH_2Cl_2 in the presence of 0.5% Et_3SiH as scavenger (Table 2, entry 2); such conditions were established previously to lead to quantitative removal of *S*-Xan and *S*-2-Moxan (Figure 3). However, higher levels of TFA did lead to some PAL cleavage, *i.e.*, 6% and 45%, respectively, with 2% and 5% of TFA (Table 2, entries 3 and 4).

Oxidative Cleavage of *S*-Xan and *S*-2-Moxan Groups. The *S*-Xan and *S*-2-Moxan groups are removed from cysteine by iodine (I_2) or thallium tris(trifluoroacetate) [Tl(tfa)$_3$], concomitant with disulfide formation (Tables 3 and 4). Suitable solvents for the I_2 reaction include MeOH, DMF, and HOAc [listed in order of increasing effectiveness], and 10 equiv of I_2 are preferred (Table 3). Similar to the conclusion from the acidolysis studies, the *S*-2-Moxan group is somewhat more labile than *S*-Xan. In the case of Tl(tfa)$_3$ oxidation, reactions are rapid and proceed in high yield, with little differentiation among results upon varying *S*-protecting group, solvent, or temperature (Table 4).

Use of Fmoc-Cys(Xan)-OH and Fmoc-Cys(2-Moxan)-OH in Solid-Phase Peptide Synthesis. Ideally, protecting groups for cysteine should be compatible with schemes that finally provide the free sulfhydryl functions, as well as with schemes that give rise directly to desired disulfide bridges. Toward these ends, three previously studied model peptides were prepared.

The pentapeptide H-Trp-Met-Asp-Phe-Cys-NH_2 incorporates the challenging tetragastrin sequence and tests the compatibility of cysteine protecting groups with alkylation-sensitive tryptophan residues.[9] Starting from an Fmoc-PAL-PEG-PS resin,[15,16] peptide chain assembly was carried out by DIPCDI/HOBt (4:4)-mediated cou-

(14) Munson, M. C.; Barany, G. *J. Am. Chem Soc.* **1993**, *115*, 10203−10216, and references cited therein.

(15) Albericio, F.; Kneib-Cordonier, N.; Biancalana, S.; Gera, L.; Masada L. I.; Hudson, D.; Barany, G. *J. Org. Chem.* **1990**, *55*, 3730−3743.

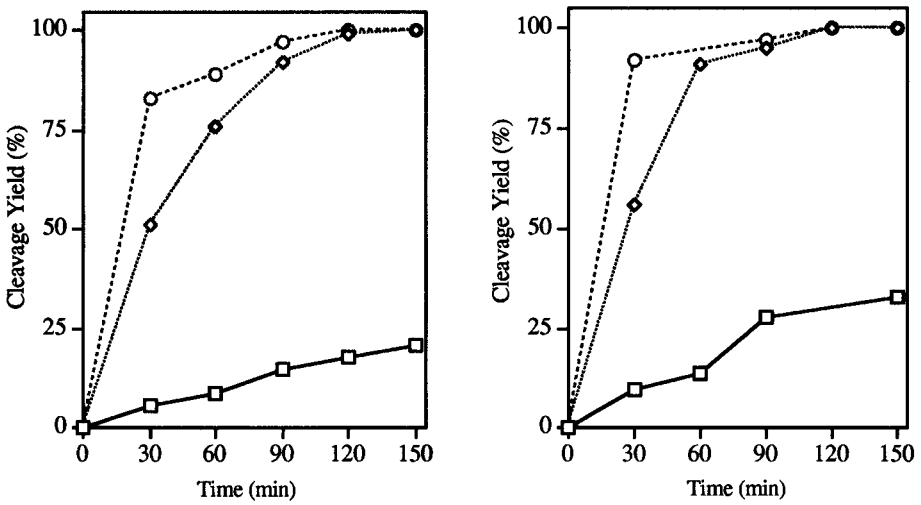


Figure 3. Cleavage kinetics of *S*-Xan (left side) and *S*-2-Moxan (right side) from Fmoc-Cys(Xan or 2-Moxan)-PEG-PS. All experiments in CH_2Cl_2 plus 0.5% Et_3SiH as scavenger. Squares: 0.1% TFA; diamonds: 0.5% TFA; circles: 1% TFA.

Table 2. Selective Cleavage of *S*-Xan and *S*-2-Moxan Groups from PAL-PEG-PS[a]

no.	cleavage cocktail	time (min)	cleaved PAL (%)
1	TFA−CH_2Cl_2−Et_3SiH (1:98.5:0.5)	60	ND
2	TFA−CH_2Cl_2−Et_3SiH (1:98.5:0.5)	2 × 60	ND
3	TFA−CH_2Cl_2−Et_3SiH (2:97.5:0.5)	60	6
4	TFA−CH_2Cl_2−Et_3SiH (5:94.5:0.5)	60	45
5	TFA−CH_2Cl_2−Et_3SiH−H_2O−anisole (7:92:0.5:0.5:0.5)[b]	60	42

[a] Cleavage of PAL was calculated based on Fmoc level remaining on support after treatment; see Experimental Section for details. ND = not detected. [b] Condition reported by Munson and Barany (ref 14) for selective cleavage of *S*-Tmob.

Table 3. Oxidative Cleavage of *S*-Xan and *S*-2-Moxan Groups by I_2[a]

				oxidation yield (%)	
no.	I_2 (equiv)	solvent	time (min)	*S*-Xan	*S*-2-Moxan
1	2	DMF	30	67	81
2	2	DMF	60	76	82
3	2	CH_2Cl_2[b]	30	100	96
4	2	CH_2Cl_2[b]	60	100	100
5	2	CH_3OH	30	89	90
6	2	CH_3OH	60	91	94
7	2	HOAc	30	86	100
8	2	HOAc	60	100	100
9	5	DMF	30	93	99
10	5	DMF	60	100	100
11	5	CH_2Cl_2[b]	30	100	99
12	5	CH_3OH	30	94	99
13	5	HOAc	30	100	100
14	10	DMF	30	100	−
15	10	CH_3OH	30	95	97

[a] Oxidation yield was determined by HPLC peak area ratio of bis(Fmoc)cystine over the total area of bis(Fmoc)cystine and Fmoc-Cys(Xan)-OH [or Fmoc-Cys(2-Moxan)-OH]; see text for experimental details and HPLC conditions. [b] Same results were obtained in $CHCl_3$ as CH_2Cl_2.

plings. Aspartic acid was protected as its *t*Bu ester while methionine and tryptophan were left unprotected. In parallel experiments, the sulfhydryl of cysteine was blocked with Xan, 2-Moxan, or Trt. Amino acid analyses

Table 4. Oxidative Cleavage of *S*-Xan and *S*-2-Moxan Groups by $Tl(tfa)_3$[a]

			oxidation yield (%)	
no.	solvent	temp (°C)	*S*-Xan	*S*-2-Moxan
1	DMF	0	100	100
2	DMF	25	100	100
3	CH_2Cl_2[b]	0	94	90
4	CH_2Cl_2	25	94	96
5	CH_3OH	0	94	98
6	CH_3OH	25	97	98
7	HOAc	25	100	100

[a] Oxidation yield was determined as Table 3; see Experimental Section for details. [b] $Tl(tfa)_3$ is sparingly soluble in CH_2Cl_2.

of the peptide-resins agreed with theoretical values for all three cases. Final cleavage/deprotection steps used reagent M* [TFA−CH_2Cl_2−anisole−βME (70:25:3:2)][9,17] or reagent R[15] [TFA−thioanisole−1,2-ethanedithiol−anisole (90:5:3:2)], 25 °C, 1 h. Cleavage yields from PAL were 75-81%, reflecting some back-alkylation of the peptide *via* its tryptophan residue. Purities and yields were reproducibly in the order Xan > 2-Moxan > Trt, with relatively modest differences among the *S*-protecting groups [Figures 4 and 5; superiority of Xan more pronounced when Fmoc protection retained on *N*-terminus]. As judged by FABMS, none of these protecting groups migrated onto Trp, although there was evidence for some *tert*-butylation.

Ac-Cys-Pro-D-Val-Cys-NH_2[9,18] was prepared in both the dihydro and oxidized forms. For the former, difficulties were encountered in obtaining crude peptide by the normal workup that involves precipitation by diethyl ether (Et_2O). Xanthenyl-derived contaminants were removed only by addition of CH_2Cl_2, but such an expedient led to significant reductions in yield. Consequently, TFA−CH_2Cl_2−Et_3SiH (1:98.5:0.5) was applied at 25 °C for 2 h in order to remove *S*-Xan groups while the peptide remained anchored to the PAL support (<0.5% chain

(16) (a) Barany, G.; Albericio, F.; Solé, N. A.; Griffin,, G. W.; Kates, S. A.; Hudson, D. In *Peptides 1992: Proceedings of the Twenty-Second European Peptide Symposium;* Schneider, C. H.; Eberle, A. N., Eds.; ESCOM Science Publishers: Leiden, The Netherlands, 1993; pp 267−268. (b) Zalipsky, S.; Chang, J. L.; Albericio, F.; Barany, G. *React. Polym.* **1994**, *22*, 243-258, and references cited therein.

(17) The use of a combination of anisole with β-mercaptoethanol to scavenge acidolytic cleavage/deprotection reactions was first proposed by: (a) 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. Pept. Protein Res.* **1990**, *35*, 527-538.

(18) (a) García-Echeverría, C.; Albericio, F.; Pons, M.; Barany, G.; Giralt, E. *Tetrahedron Lett.* **1989**, *30*, 2441−2444. (b) 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. Pept. Protein Res.* **1991**, *33*, 402-413.

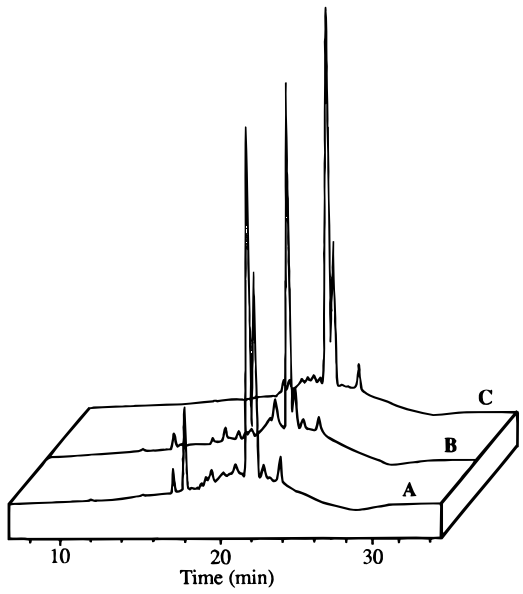


Figure 4. Analytical HPLC of crude peptide Fmoc-Trp-Met-Asp-Phe-Cys-NH_2 cleaved with reagent M* [TFA−CH_2Cl_2−anisole−βME (70:25:3:2)] (25 °C, 1 h). Cysteine side chain was protected with: (A) *S*-Trt; (B) *S*-Xan; (C) *S*-2-Moxan. HPLC analysis was carried out on a Vydac C-18 column (5 μm, 4.6 × 250 mm) using a linear gradient of 0.1% TFA in CH_3CN and 0.1% aqueous TFA from 1:9 to 3:2 over a period of 20 min, from 3:2 to 1:9 over 5 min, flow rate 1.0 mL/min, detection at 220 nm. The desired peptide elutes at t_R = 21.9 min.

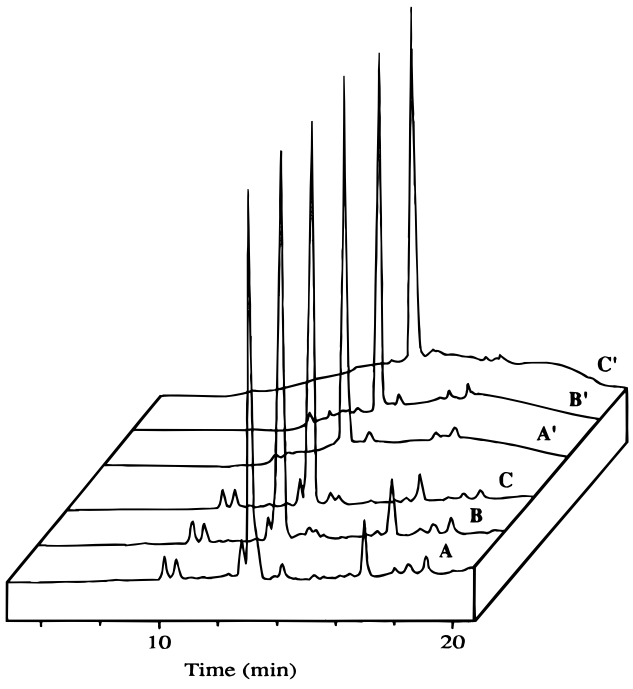


Figure 5. Analytical HPLC of crude peptide H-Trp-Met-Asp-Phe-Cys-NH_2. Final cleavage was carried out with reagent M* (25 °C, 1 h) for panels A, B, and C; with reagent R (25 °C, 1 h) for panels A′, B′, and C′. Cysteine side chain was protected with: *S*-Trt for Panels A and A′; *S*-Xan for panels B and B′; *S*-2-Moxan for Panels C and C′. HPLC conditions same as in Figure 4.

loss). The deprotected dihydro-peptide was released from the polymeric support by reagent R, 25 °C, 1 h, and precipitated by Et_2O to remove scavengers. The linear peptide was obtained in excellent yield and purity (Figure 6, absolute yield: 55% for Xan, 52% for 2-Moxan; cleavage yield: 98% for Xan, 94% for 2-Moxan; HPLC purity: 86% for Xan, 93% for 2-Moxan). On-resin oxidization by $Tl(tfa)_3$,[9,18,19] followed by cleavage and workup, gave excellent results for the desired β-turn intramolecular disulfide: cleavage yield: 80% for Xan, 78% for 2-Moxan; HPLC purity: 92% for Xan, 89% for 2-Moxan; overall yield: 45% for Xan, 34% for 2-Moxan (Figure 6).

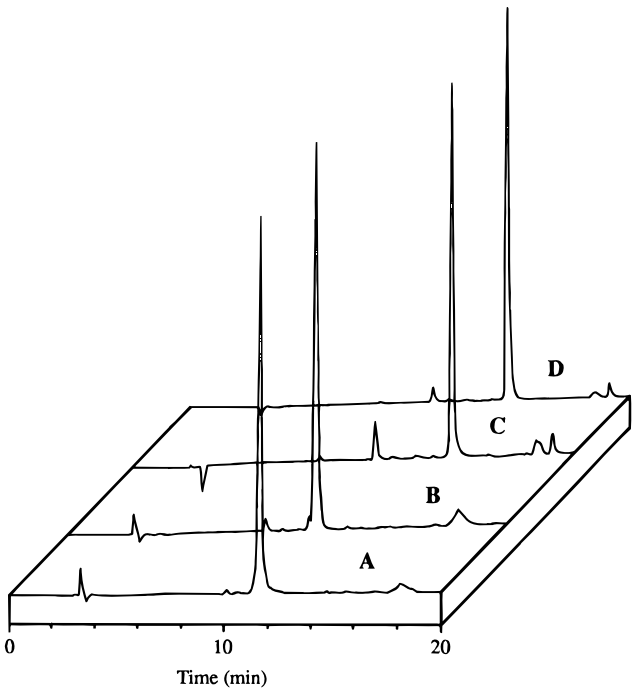


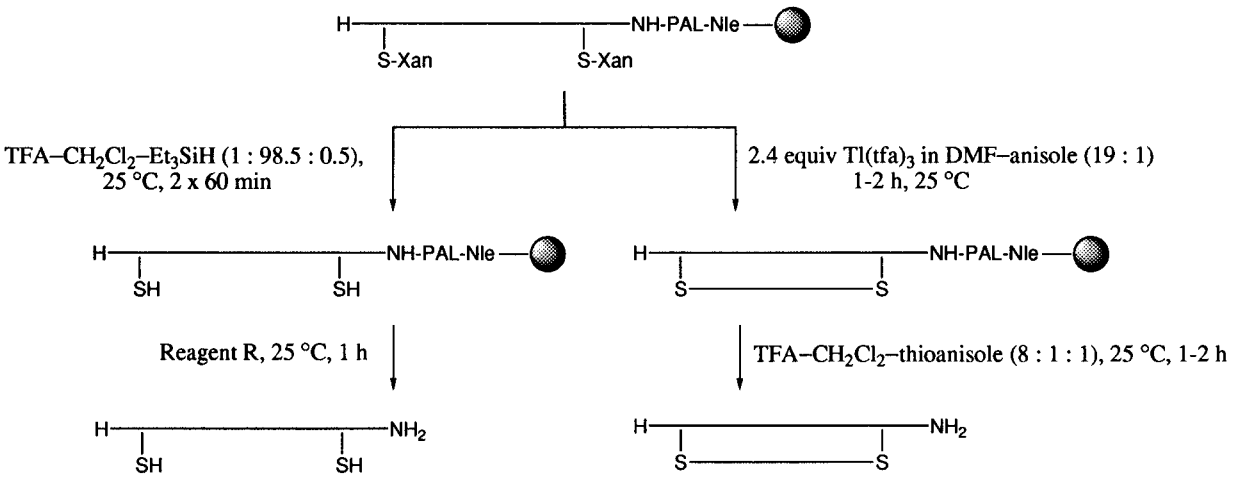
Figure 6. Analytical HPLC of crude peptide Ac-Cys-Pro-D-Val-Cys-NH_2 (both dihydro and oxidized forms). Peptide forms: Panels A and B ≡ disulfide-peptide (t_R = 11.2 min); panels C and D ≡ dihydro-peptide (t_R = 14.8 min). Cysteine side-chain was protected with: *S*-Xan for panels A and C; *S*-2-Moxan for panels B and D. HPLC analysis was carried out on a Vydac C-18 column (5 μm, 4.6 × 250 mm) using a linear gradient of 0.1% TFA in CH_3CN and 0.1% aqueous TFA from 1:9 to 1:1 over a period of 30 min, flow rate 1.0 mL/min, detection at 220 nm.

Further experiments (Scheme 2) were directed toward the more difficult problem of synthesizing the nonapeptide oxytocin (H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2).[9,18b] The linear bis(Xan/2-Moxan) sequences were assembled on Fmoc-PAL-PEG-PS-resins by BOP/HOBt/NMM (4:4:8 = 0.25 M) [method A] or DIPCDI/HOBt (6:6) [Method B] mediated couplings. Cleavage with reagent R released the linear dihydro-peptides in 75% yield; the purity was better when coupling was by method B (~90% purity for crude material), in comparison to method A (~70%) (Figure 7, panels A−C). The major byproducts from the inferior synthesis were isolated and shown to be diastereomers in which one or the other of the Cys residues had racemized.[20] To obtain oxytocin disulfide, $Tl(tfa)_3$ (2.4 equiv)-mediated on-resin oxidation[9,18b,19] was carried out in anisole−DMF (1:19), at 25 °C for 1 h. The *crude* material cleaved with TFA−CH_2Cl_2−thioanisole (8:1:1) included ~ 80-85% monomeric oxytocin (~50−60% absolute yield) (Figure 7, panel E). Moreover, resin-bound oxidation with I_2 (20 equiv) in

(19) Yajima, H.; Fujii, N.; Funakoshi, S.; Watanabe, T.; Murayama, E.; Otaka, A. *Tetrahedron* **1988**, *44*, 805-819.

(20) The occurrence of racemization, and conditions for minimization of this serious problem, are the subject of a separate report: Han, Y.; Albericio, F.; Barany, G. *J. Org. Chem.* **1997**, *62*, in press.

Scheme 2



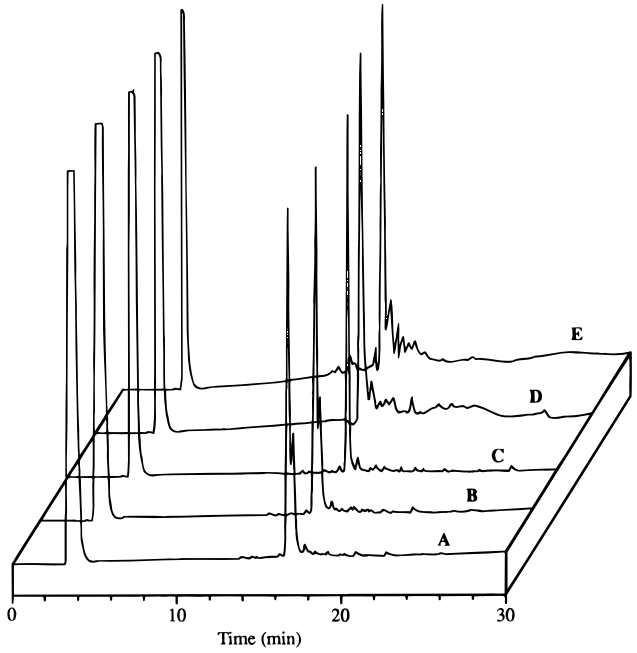


Figure 7. Analytical HPLC of oxytocin H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2 (both dihydro and oxidized forms). Peptide forms: Panels A, B, and C ≡ dihydro-oxytocin (t_R = 17.0 min); panels D and E ≡ oxytocin (t_R = 16.6 min). Identities of peaks were confirmed by coinjections with authentic standards. Coupling methods: Method A for panels A and B; method B for panels C, D, and E. Cysteine side chain was protected with: *S*-Xan for panels A, C, D, and E; *S*-2-Moxan for panel B. Panel D was oxidized with I_2; panel E with $Tl(tfa)_3$. Final cleavage was carried out with reagent R for Panels A, B, C, and D; TFA−CH_2Cl_2−thioanisole (8:1:1) for panel E. HPLC conditions same as in Figure 6.

HOAc resulted in similar yields and purities (Figure 7, panel D). The results of the present work compare favorably with earlier experiences from our laboratory on oxidative deprotection of *S*-Acm,[18b] *S*-Trt,[9] and *S*-Tmob.[9]

Conclusions

We have described efficient protocols for preparation of N^{α}-Fmoc, *S*-xanthenyl protected cysteine derivatives and defined an array of conditions for applications of the *S*-Xan and *S*-2-Moxan groups to the preparation by Fmoc solid-phase synthesis of peptides containing cysteine residues in either the free thiol or disulfide form. Our results indicate that *S*-Xan and *S*-2-Moxan, due to their lability characteristics toward dilute acid and oxidative cleavage, may prove to be useful alternatives to the widely applied *S*-Acm or *S*-Trt groups. The selective removal of *S*-Xan and *S*-2-Moxan groups from acid-labile tris(alkoxybenzyl)amide (PAL) supports provides additional options for multidimensional protection schemes which may allow regioselectively controlled disulfide bridge formation. Finally, these *S*-protecting groups are expected to see applications for aliphatic and aromatic sulfhydryl functions beyond those encountered in the peptide arenas.[21]

Experimental Section

Most of the materials, as well as general synthetic and analytical procedures have been described in our earlier publications.[9,10,12,15,16] ^{1}H NMR spectra were recorded at 300 MHz on either an IBM NR-300 or a Varian VXR-300 instrument and at 500 MHz on a Varian VXR-500 instrument using $CDCl_3$, CD_3SOCD_3, or CD_3OD as solvents. Fast atom bombardment mass spectrometry (FABMS) to characterize synthetic peptides was carried out on a VG 7070E-HF instrument, with a glycerol matrix, to obtain both positive and negative ion spectra. Elemental analyses were performed by M-H-W Laboratories, Phoenix, AZ.

Thin-layer chromatography was performed on Analtech or Merck silica gel GF plates (250 μm, 10 × 20 cm), developed with various solvent systems. Compounds were visualized by one or more of following methods: (1) fluorescence quenching; (2) I_2 vapor; (3) spray with 0.3% (w/v) ninhydrin in γ-collidine−HOAc−EtOH (1:3.3:29). Melting points were taken on a Fisher-Johns melting point apparatus and are uncorrected. Peptides attached to PEG-PS were hydrolyzed in 12 N HCl−propionic acid (1:1 v/v) + 2 drops liquefied phenol for 1 h at 160 °C. Amino acid analyses and analytical HPLC were carried out on previously described Waters or Beckman instruments.

***N*$^{\alpha}$-(9-Fluorenylmethyloxycarbonyl)-*S*-(9*H*-xanthen-9-yl)cysteine (1). Method A.** A solution of Fmoc-OSu (0.12 g, 0.36 mmol) in CH_3CN (2.0 mL) was added over 30 min to a yellowish solution of H-Cys(Xan)-OH (**3**) (0.10 g, 0.33 mmol) plus Et_3N (0.11 mL, 0.83 mmol) in H_2O (2.0 mL). The reaction mixture was stirred at 25 °C for 3 h, concentrated by evaporation of organic solvent, diluted with H_2O (2.0 mL), and then adjusted to pH 3.5 by addition of 10% aqueous citric acid

(21) (a) Barany, G.; Chen, L.; Annis, I. ABRF '96: Biomolecular Techniques. An International Symposium Sponsored by The Association of Biomolecular Resource Facilities, San Francisco, CA, March 30−April 2, 1996. (b) Barany, G.; Chen, L. In *Innovation and Perspectives in Solid Phase Synthesis & Combinatorial Chemical Libraries*: *Biomedical & Biological Applications, 1996*; Epton, R., Ed.; Mayflower Scientific Ltd.: Kingswinford, England, 1996; pp 181−186.

solution. After extraction with EtOAc (3 × 20 mL), the combined organic layer was washed with H_2O (3 × 20 mL) and brine (1 × 20 mL) and dried (Na_2SO_4). Evaporation of organic solvent gave a white solid which was purified further by silica gel chromatography. Yield: 0.14 g (81%). **Method B (preferred on laboratory-scale)**. 9*H*-Xanthen-9-ol[10] (1.45 g, 7.3 mmol) was added in five portions to a solution of Fmoc-Cys-OH (**6**) (2.52 g, 7.3 mmol) and TFA (0.56 mL, 7.3 mmol) in CH_2Cl_2 (100 mL). The yellowish reaction solution was stirred at 25 °C for 30 min under N_2 atmosphere, and then petroleum ether (100 mL) was added. After 3 h, the resultant white precipitate was collected by filtration, washed with a mixture of CH_2Cl_2−petroleum ether (1:1; 3 × 15 mL), and dried *in vacuo* over P_2O_5. Yield: 3.54 g (90%); mp 70−72 °C; R_f [$CHCl_3$−MeOH (10:1)] 0.46; ^{1}H NMR (CD_3SOCD_3) δ 7.86 (d, J = 7.8 Hz, 2H), 7.74 (d, J = 8.4 Hz, 1H), 7.70 (d, J = 7.5 Hz, 2H), 7.10−7.47 (m, 12H), 5.48 (s, 1H), 4.19−4.30 (m, 3H), 4.02−4.09 (m, 1H), 2.82 (dd, J = 4.5 and 13.2, 1H), 2.64 (dd, J = 9.9 and 13.2 Hz, 1H); ^{1}H NMR ($CDCl_3$) δ 7.75−7.77 (m, 2H), 7.56−7.59 (m, 2H), 7.21−7.41 (m, 8H), 7.05−7.10 (m, 4H), 5.30 (s, 1H), 5.19 (d, J = 8.0 Hz, NH), 4.33−4.40 (m, 3H), 4.20 (m, 1H), 2.75−2.83 (m, 2H). Anal. Calcd for $C_{31}H_{25}NO_5S$ (523.61): C, 71.11; H, 4.81; N, 2.68; S, 6.12. Found: C, 70.89; H, 4.78; N, 2.52; S, 5.98.

***N*$^\alpha$-(9-Fluorenylmethyloxycarbonyl)-*S*-(2-methoxy-9*H*-xanthen-9-yl)-cysteine (2). Method A.** The procedure was exactly as method A for compound **1** but starting with H-Cys-(2-Moxan)-OH (1.00 g, 3.0 mmol). Yield: 1.39 g (83%). **Method B.** The procedure was exactly as method B for compound **1** but starting with 2-methoxy-9*H*-xanthen-9-ol[10,12] (1.63 g, 7.1 mmol). Yield. 3.27 g (83%); mp 63-65 °C; R_f [$CHCl_3$−MeOH (10:1)] 0.46; ^{1}H NMR ($CDCl_3$) δ 7.76 (d, J = 6.0 Hz, 2H), 7.57 (m, 2H), 7.20−7.40 (m, 6H), 7.02−7.08 (m, 3H), 6.91 (dd, J = 2.5 and 10 Hz, 1H), 6.82 (dd, J = 2.5 and 8.5 Hz, 1H), 5.29 (s, 1H), 5.23 (d, J = 8.0 Hz, NH), 4.32−4.41 (m, 3H), 4.20 (m, 1H), 3.75 (s, 3H) 2.76−2.84 (m, 2H). Anal. Calcd for $C_{32}H_{27}NO_6S$ (553.63): C, 69.42; H, 4.92; N, 2.53; S, 5.79. Found: C, 69.22; H, 4.95; N, 2.44; S, 5.54.

***S*-(9*H*-Xanthen-9-yl)cysteine (3).** A suspension of cysteine (0.61 g, 5.0 mmol) in TFA−1,2-dimethoxyethane (DME) (1:50; 100 mL) was stirred at 25 °C under N_2 until a colorless clear solution was obtained (several hours). Next 9*H*-xanthen-9-ol[10] (1.0 g, 5.1 mmol) was added in one portion. The reaction mixture was stirred at 25 °C for 30 min and then neutralized to pH 7 by addition of saturated aqueous Na_2CO_3 solution, concentrated under reduced pressure, diluted with H_2O (50 mL), treated with 10% citric acid to pH ∼ 6, and then stirred at 25 °C for 1 h. The resultant white solid was collected by filtration, washed with H_2O (3 × 10 mL) and EtOAc (3 × 10 mL), and dried *in vacuo* over P_2O_5. Yield: 1.33 g (88%); mp 165 °C (dec.); R_f [MeOH−H_2O (4:1)] 0.69; ^{1}H NMR (CD_3OD) δ 7.38−7.46 (m, 2H), 7.14−7.21 (m, 2H), 6.98−7.04 (m, 4H), 5.35 (s, 1H), 3.37−3.41 (m, 1H), 2.84 (dd, J = 4.0 and 14.2 Hz, 1H), 2.72 (dd, J = 8.1 and 14.2 Hz, 1H). Anal. Calcd for $C_{16}H_{15}NO_3S$ (301.36): C, 63.77; H, 5.02; N, 4.65; S, 10.64. Found: C, 63.80; H, 4.93; N, 4.56; S, 10.47.

***S*-(2-Methoxy-9*H*-xanthen-9-yl)cysteine (4).** The procedure was exactly as for compound **3** but starting with 2-methoxy-9*H*-xanthen-9-ol[10,12] (3.89 g, 17.1 mmol). Yield: 5.20 g (97%); mp 185 °C dec; R_f [MeOH−H_2O (4:1)] 0.75; ^{1}H NMR (CD_3OD) δ 7.50−7.54 (two dd,[22] ratio: 1:1, J = 1.5 and 7.8 Hz, 1H), 7.24−7.31 (m, 1H), 7.02−7.14 (m, 4H), 6.86−6.87 (two dd[22], ratio: 1:1, J = 3.0 and 9.0 Hz, 1H), 5.45 (s, 1H), 3.82 (s, 3H), 3.49−3.54 (m, 1H), 2.80−3.00 (m, 2H). Anal. Calcd for $C_{17}H_{17}NO_4S$ (331.39): C, 61.62; H, 5.17; N,4.23; S, 9.67. Found: C, 61.45; H, 4.93; N, 4.23; S, 9.44.

***S*-(3-Methoxy-9*H*-xanthen-9-yl)cysteine (5).** 3-Methoxy-9*H*-xanthen-9-one[10,12] (1.50 g, 6.6 mmol) was dissolved in 95% ethanol (150 mL), following which solid NaOH (1.33 g, 33 mmol) and preactivated Zn dust[10,12] (1.72 g, 26 mmol) were added, each in one portion. The suspension was refluxed for 4 h, cooled to 25 °C, filtered to remove unreacted Zn dust, and washed with ethanol (3 × 10 mL). The pink filtrate was concentrated to give a red solid, which was dried *in vacuo* over P_2O_5 and then dissolved in a solution of TFA (5.2 mL, 68 mmol) and cysteine (0.77 g, 6.3 mmol) in DME (150 mL). The reaction solution was stirred at 25 °C for 30 min, diluted with H_2O (100 mL), neutralized to pH 7 by addition of saturated aqueous Na_2CO_3 solution, concentrated partially under reduced presure to remove DME, and then treated with 10% citric acid to ∼ pH 6. The resultant white precipitate was collected by filtration, washed with H_2O (3 × 20 mL) and Et_2O (3 × 10 mL), and dried *in vacuo* over P_2O_5. Yield: 2.03 g (97%); mp 150 °C dec; R_f [MeOH−H_2O (4:1)] 0.73; ^{1}H NMR (CD_3OD) δ 7.09−7.56 (m, 5H), 6.68−6.78 (m, 2H), 5.43 (s, 1H), 3.81 and 3.80 (adjacent s, equal height, due to diastereomers, 3H), 3.49 (dd, J = 4.2 and 8.1 Hz, 1H), 2.92−2.99 (m, 1H), 2.77−2.86 (m, 1H). Anal. Calcd for $C_{17}H_{17}O_4NS$ (331.39): C, 61.62; H, 5.17; N: 4.23; S, 9.67. Found: C, 60.99; H, 5.05; N, 3.66; S, 9.12.

***N*$^\alpha$-(9-Fluorenylmethyloxycarbonyl)cysteine (6).** TFA (25 mL, 0.32 mol) was added to a solution of bis(Fmoc)cystine (5.54 g, 8.1 mmol) in MeOH (500 mL). Zinc powder (5.32 g, 81.4 mmol) was added next in one portion, and the reaction mixture was stirred at 25 °C for 30 min [complete reduction was confirmed by TLC: $CHCl_3$−MeOH−HOAc = 30:1:0.1, R_f = 0.53 for Fmoc-cysteine; starting bis(Fmoc)cystine does not migrate under these TLC conditions]. The mixture was then diluted with H_2O (100 mL) and then partially concentrated under reduced pressure to remove MeOH. The resultant white solid was collected by filtration, washed with H_2O (until the filtrate was nonacidic to pH paper), and then dried *in vacuo* over P_2O_5. Yield: 5.01 g (90%); mp 96−98 °C; ^{1}H NMR (CD_3COCD_3) δ 7.87 (d, J = 7.5 Hz, 2H), 7.73 (d, J = 6.3 Hz, 2H), 7.33−7.42 (m, 4H), 6.82 (d, J = 6.3 Hz, NH), 4.48 (broad, 1H), 4.37 (d, J = 6.3 Hz, 2H), 4.27 (t, J = 6.3 Hz, 1H), 2.95−3.06 (m, 2H). Anal. Calcd for $C_{18}H_{17}NO_4S$ (343.40): C, 62.96; H, 4.99; N, 4.08; S, 9.34. Found: C, 63.07; H, 5.15; N, 3.89; S, 9.11.

Solution Stability/Lability Studies. Fmoc-Cys(Xan)-OH or Fmoc-Cys(2-Moxan)-OH (∼1 mg) were dissolved in various milieus, at a concentration of 1−2 mM. Acid cleavages were quenched with 5% aqueous $NaHCO_3$. The aqueous layers were extracted with CH_2Cl_2 (2 × 1 mL). Organic phases were concentrated by a stream of N_2, treated with petroleum ether (3 × 10 mL) to remove aromatic scavengers, and dissolved in MeOH (1 mL) for analysis. Possible reactions were monitored by TLC: $CHCl_3$−MeOH (10:1), R_f = 0.46 for Fmoc-Cys(Xan)-OH and Fmoc-Cys(2-Moxan)-OH; Fmoc-cysteine stays near origin; and HPLC [Vydac C-18 column (5 μm, 4.6 × 250 mm), linear gradient of 0.1% TFA in CH_3CN and 0.1% aqueous TFA from 2:3 to 4:1 over a period of 35 min, flow rate 1.2 mL/min, detection at 254 nm], t_R = 29.4 min for Fmoc-Cys(Xan)-OH; t_R = 11.7 min for Fmoc-cysteine; t_R = 26.7 min for 9*H*-xanthene; t_R = 24.3 min for Fmoc-Cys(2-Moxan)-OH; t_R = 20.4 min for 2-methoxy-9*H*-xanthene.

In other experiments, H-Cys(Xan)-OH, H-Cys(2-Moxan)-OH, or H-Cys(3-Moxan)-OH were dissolved, at a concentration of 3 mM, in piperidine−DMF (1:4). The fate of *S*-protected cysteine was monitored by TLC [MeOH−H_2O (4:1)] or analytical HPLC [Vydac C-18 column (5 μm, 4.6 × 250 mm), linear gradient of 0.1% TFA in CH_3CN and 0.1% aqueous TFA from 1:4 to 4:1 over a period of 45 min, flow rate 1.0 mL/min, detection at 220 nm]. H-Cys(3-Moxan)-OH (t_R = 11.7 min) decomposed to 3-methoxy-9*H*-xanthen-9-one (t_R = 21.1 min), and 3-methoxy-9*H*-xanthen-9-ol (t_R = 15.6 min) (Figure 1).

Solid-Phase Stability/Lability Studies. Fmoc-Cys(Xan)-OH and Fmoc-Cys(2-Moxan)-OH were incorporated onto a PEG-PS resin (loading: 0.21 mmol/g) using DIPCDI/HOBt (4:4)-mediated coupling. Portions of these resins (15−25 mg per experiment) were subjected to various *S*-Xan cleavage conditions. The filtrates from the cleavages, containing 9*H*-xanthene or 2-methoxy-9*H*-xanthene, were diluted with MeOH to a final volume of 25 mL, and absorbance was measured at 250 nm [data used for Figure 3]. To determine the stability of Fmoc-PAL-PEG-PS resin to these same conditions, the treated resins were washed with DMF (3 × 2 min) and CH_2Cl_2 (3 × 2 min), dried *in vacuo*, treated further with piperidine−DMF (1:4, 2.0 mL) at 25 °C for 20 min, and washed with

(22) Two aromatic protons were resolved because the title Cys derivative exists as a diastereomeric mixture.

DMF (3 × 2.0 mL). The filtrates were then diluted to 10.0 mL with DMF for UV analysis at 301 nm in order to quantitate the Fmoc group. In an alternative experimental design, Fmoc-Gly-Cys(Xan or 2-Moxan)-PAL-Nle-PEG-PS was treated in various ways, and the ratio of Gly to Nle "internal reference" amino acid upon hydrolysis was used to evaluate the stability of the PAL linkage [data used for Table 2].

Oxidative Cleavage of *S*-Xan and *S*-2-Moxan Groups. I_2 stock solutions in various solvents were ~0.1 M. Fmoc-Cys-(Xan or 2-Moxan)-OH was dissolved in these stock solutions to achieve the stated excesses. After the indicated times at 25 °C (see Table 3), reactions were quenched with a slight excess of aqueous sodium thiosulfate, and aliquots were analyzed by HPLC on a Vydac C-18 column (5 μm, 4.6 × 250 mm), using a linear gradient of 0.1% TFA in CH_3CN and 0.1% aqueous TFA over 20 min from 1:1 to 4:1, flow rate 1.2 mL/min, detection at 254 nm: $t_R = 13.0$ min for bis(Fmoc)cystine; $t_R = 16.5$ min for Fmoc-Cys(Xan)-OH; $t_R = 17.3$ min for Fmoc-Cys(2-Moxan)-OH.

For thallium(III) oxidations (Table 4), $Tl(tfa)_3$ was dissolved in the appropriate solvents to a concentration of 3.7 mM. Other aspects of the experimental design and execution were as in the preceding paragraph.

H-Trp-Met-Asp-Phe-Cys-NH_2. The detailed experimental design, HPLC documentation (Figures 4 and 5), and essential conclusions are described in the text. Starting with Fmoc-PAL-PEG-PS resin (300 mg, 0.15 mmol/g), peptide chain assembly was performed manually using DIPCDI/HOBt (4:4)-mediated couplings with 5 min preactivation in DMF, 25 °C, 1 h. Fmoc removal was carried out with piperidine−DMF (1:4, 2 + 8 min), followed by washes with DMF (5 × 2 min). The peptide-resins were treated with piperidine−DMF (1:4), and aliquots were hydrolyzed for amino acid analysis: *S*-Trt experiment: Asp 1.14, Met 0.93, Phe 0.93; *S*-Xan experiment: Asp 1.17, Met 0.87, Phe 0.96; *S*-2-Moxan experiment: Asp 1.19, Met 0.87, Phe 0.94. Final release of the peptide [either retaining *N*-terminal Fmoc protection, or with a free α-amino terminus] was carried out with reagent M* or reagent R, 25 °C, 1 h. The filtrates from the cleavage reaction were evaporated partially under a stream of N_2, diluted with CH_2Cl_2 (1 mL for 20 mg peptide-resin), and then precipitated with anhydrous Et_2O (10 mL). The procedure of adding CH_2Cl_2 followed by Et_2O precipitation was repeated for a total of three times. The resultant white peptides were dissolved in H_2O and lyophilized. Purities were evaluated by analytical HPLC and FABMS (matrix: glycerol−TFA): for free peptide calcd monoisotopic mass of $C_{32}H_{41}N_7O_7S_2$: 699.25; found positive mass spectra: $[M + H]^+$ m/z 700.2; $[M + Na]^+$ m/z 722.1; found negative mass spectrum: $[M - H]^-$ m/z 698.1. For Fmoc peptide (separate experiment), calcd monoisotopic mass of $C_{47}H_{51}N_7O_9S_2$: 921.3; found positive mass spectrum: $[M + H]^+$ m/z 922.5; found negative mass spectrum: $[M - H]^-$ m/z 920.4. Tryptophan *tert*-butylation was found in both cases: $[M + H]^+$ m/z 756.3 for free peptide; $[M + H]^+$ m/z 978.6 for Fmoc-protected peptide.

Ac-Cys-Pro-D-Val-Cys-NH_2. Starting with Fmoc-PAL-PEG-PS resin (300 mg, loading: 0.15 mmol/g), peptide chain assembly was performed manually using DIPCDI/HOBt (4:4)-mediated coupling with 5 min preactivation in DMF, 25 °C, 2 h. Fmoc group removal was carried out with piperidine−DMF (1:4, 2 + 8 min), followed by DMF washes (5 × 2 min). The final acetylation was accomplished with Ac_2O−DIEA (0.3 M each) in DMF, 25 °C, 1 h. After selective removal of *S*-Xan with TFA−CH_2Cl_2−Et_3SiH (1:98.5:0.5, 25 °C, 2 × 1 h), the linear dihydro-peptide (Cys in reduced form) was released from the support by reagent R at 25 °C for 1 h and precipitated with anhydrous Et_2O (3 × 10 mL). On-resin oxidation was carried out with $Tl(tfa)_3$ (2.4 equiv, 25 mM) in DMF−anisole (19:1) at 25 °C for 1-2 h, and the oxidized peptide was released from the support by TFA−CH_2Cl_2−Et_3SiH (8:1:1), 25 °C, 1 h. The filtrate from the cleavage reaction was evaporated partially under a stream of N_2, diluted with CH_2Cl_2 (1 mL for 20 mg peptide-resin), and then precipitated with anhydrous Et_2O (10 mL). The procedure of adding CH_2Cl_2 followed by Et_2O precipitation was repeated for a total of three times. The resultant crude peptides were analyzed by analytical HPLC and confirmed by FABMS (matrix: glycerol−TFA): calcd monoisotopic mass of $C_{18}H_{31}N_5O_5S_2$ (dihydro-peptide): 461.60; found positive mass spectrum: $[M + H]^+$ m/z 462.2; found negative mass spectrum: $[M - H]^-$ m/z 460.2; calcd monoisotopic mass of $C_{18}H_{29}N_5O_5S_2$ (disulfide peptide): 459.58; found positive mass spectrum: $[(M + H)^+]$ m/z 460.2; found negative mass spectrum: $[M - H]^-$ m/z 458.2.

H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2 (Oxytocin). Method A.[20] Peptide chain assembly was carried out manually, starting with Fmoc-PAL-PEG-PS resin (300 mg, loading: 0.15 mmol/g). Asn and Gln were incorporated as their corresponding Pfp esters (with HOBt, 1:1 ≡ 6 equiv), 25 °C, 1 h. All other protected amino acids were incorporated using BOP/HOBt/NMM (4:4:8 = 0.25:0.25:0.5 M) in DMF, 25 °C, 1 h, except for Ile with a 90-min coupling. The Tyr hydroxyl was protected as a *t*Bu ether, Asn and Gln side-chain amides were left unprotected, and the Cys side chain was protected with Xan or 2-Moxan as specified. Upon completion of chain assemblies, peptide-resins were hydrolyzed and subjected to amino acid analysis: *S*-Xan experiment: Asp 1.03, Glu 1.02, Pro 1.00, Gly 1.00, Ile 0.96, Leu 0.99, Tyr 1.00; *S*-2-Moxan experiment: Asp 1.02, Glu 1.00, Pro 1.02, Gly 1.02, Ile 0.94, Leu 1.00, Tyr 1.00 for. Final release of the dihydro-peptide was carried out using reagent R, 25 °C, 1−2 h. The filtrate from the cleavage reaction was evaporated partially under a stream of N_2, diluted with CH_2Cl_2 (1 mL for 20 mg peptide-resin), and then precipitated with anhydrous Et_2O (10 mL). The procedure of adding CH_2Cl_2 followed by Et_2O precipitation was repeated for a total of three times, in order to remove all low molecular weight organic compounds. **Method B.** As with Method A, except the incorporation of protected amino acids (other than Asn and Gln) by DIPCDI/HOBt (6:6)-mediated coupling, 25 °C, 1 h. Amino acid analysis: *S*-Xan experiment: Asp 1.03, Glu 1.00, Pro 1.06, Gly 1.04, Ile 0.95, Leu 1.01, Tyr 0.98; *S*-2-Moxan experiment: Asp 1.02, Glu 1.00, Pro 1.02, Gly 1.02, Ile 0.94, Leu 1.00, Tyr 1.00. Final cleavage of linear dihydro-oxytocin was carried out as in method A. On-resin oxidation by $Tl(tfa)_3$ (2.4 equiv) was carried out in anisole−DMF (1:19) (25 °C, 1−2 h), followed by washes with DMF (5 × 2 min) and CH_2Cl_2 (3 × 2 min). The resultant oxytocin was released from the support by TFA−CH_2Cl_2−thioanisole (8:1:1), 2 h, 25 °C. I_2 (20 equiv)-mediated on-resin oxidation was carried out in HOAc, 25 °C, 1 h, and final peptide cleavage from the support was accomplished with reagent R.

Crude linear dihydro-oxytocin (obtained *via* methods A and B) and oxytocin were evaluated by analytical HPLC and FABMS (matrix: glycerol−TFA): calcd monoisotopic mass of dihydro-oxytocin: 1008.44; found positive and negative of pure dihydro-oxytocin: $[M + H]^+$ m/z 1009.6; $[M - H]^-$ m/z 1007.7; found positive and negative of racemized dihydro-oxytocin: $[M + H]^+$ m/z 1009.6; $[M - H]^-$ m/z 1007.6. Further analysis was carried out essentially as described previously.

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