Novel Solid-Phase Reagents for Facile Formation of Intramolecular Disulfide Bridges in Peptides under Mild Conditions[1,2]

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Received April 2, 1998

Abstract: The controlled formation of intramolecular disulfide bridges in peptides, while avoiding unwanted oligomerization, is a significant challenge. Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid), was developed originally in the context of an assay for measuring free thiol concentration under physiological conditions. The present studies demonstrate that this reagent, when bound through two sites to a suitable solid support (PEG-PS, modified Sephadex, or controlled-pore glass), is an effective mild oxidizing reagent that promotes the formation of disulfide bridges. Rates and yields of the reactions were determined as a function of pH, excess of oxidizing reagent, resin loading, and parent support, for the preparation of oxytocin and deamino-oxytocin (9 residues, disulfide bridge between residues 1 and 6), somatostatin (14 residues, disulfide bridge between residues 3 and 14), α-conotoxin SI (13 residues, disulfide bridges between residues 2 and 7; 3 and 13), and apamin (18 residues, disulfide bridges between residues 1 and 11; 3 and 15). Cystine dimers of these peptide models formed, if at all, in relatively low amounts. Use of solid-phase Ellman's reagents to oxidize the linear precursors of conotoxin or apamin (tetrathiols) gave as the main products the correctly paired regioisomers. Particular advantages of the overall approach include fast reaction rates over a wide range of pH, from 2.7 to 6.6; easy purification of disulfide-containing products; and the specificity and reusability of the reagents.

Introduction

Disulfide bridges represent significant structural motifs in many biologically important peptides and proteins.[3] Intramolecular disulfides serve to covalently cross-link portions of the polypeptide chain that are apart in the linear sequence but come together in three dimensions. Introduction of disulfide bridges into natural or designed peptides and small proteins is a valuable tactic toward improving biological activities/specificities and stabilities. Despite extensive research, the controlled formation of intramolecular disulfide bridges from cysteine-containing precursors remains a significant challenge.[4] Thus, while currently available methods have facilitated progress, low yields are frequently observed due to problems of solubility, intermolecular dimerization/oligomerization, scrambling, and/or modification of sensitive amino acid side chains (Tyr, Met, or Trp).[4] Stimulated by the myriad applications of solid-phase methodologies in chemistry and biochemistry,[5] we seek polymer-bound reagents that can be used to overcome some of the aforementioned problems.[6,7]

We report here how Ellman's reagent, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB, **1**), developed originally in the

(1) Abbreviations and conventions are defined at first use in the text and are summarized in full in the Supporting Information. Abbreviations used repeatedly for this work include: CPG, controlled pore glass; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); El, 5-thio-2-nitrobenzoyl; PAC, *p*-alkoxybenzyl ester (Peptide Acid Linker); PAL, 5-[[(4-amino)methyl]-3,5-dimethoxyphenoxy]valeric acid handle (Peptide Amide Linker); PEG-PS, poly(ethylene glycol)−polystyrene (graft resin support); TNB, 5-thio-2-nitrobenzoic acid; and Xan, 9*H*-xanthen-9-yl.

(2) Portions of this work were reported in preliminary form: (a) Barany, G.; Chen, L. In *Innovation and Perspectives in Solid-Phase Synthesis & Combinatorial Libraries, 1996*, Collected Papers, Fourth International Symposium, Edinburgh, Scotland, September 12−16, 1995; Epton, R., Ed.; Mayflower Scientific: Kingswinford, U.K., 1997; pp 181−186. (b) Annis, I.; Barany, G. In *Peptides−Chemistry, Structure and Biology: Proceedings of the Fifteenth American Peptide Symposium*, Nashville, TN, June 14−19, 1997; Tam, J. P., Kaumaya, P. T. P., Eds.; Kluwer: Dordercht, The Netherlands, 1998, in press. (c) Annis, I.; Chen, L.; Barany, G. In *Innovation and Perspectives in Solid-Phase Synthesis & Combinatorial Libraries, 1998*, Collected Papers, Fifth International Symposium, London, U.K., September 2−6, 1997; Epton, R., Ed.; Mayflower Scientific Ltd.: Kingswinford, U.K., 1998, in press.

(3) For reviews, see: (a) Richardson, J. S. *Adv. Prot. Chem.* **1981**, *34*, 167−339. (b) Thornton, J. M. *J. Mol. Biol.* **1981**, *151*, 261−287. (c) Creighton, T. E. *BioEssays* **1988**, *8*, 57−63. (d) Raines, R. T. *Nat. Struct. Biol.* **1997**, *4*, 424−427.

(4) For reviews, see: (a) König, W.; Geiger, R. In *Perspectives in Peptide Chemistry*; Eberle, A., Geiger, R., Wieland, T., Eds.; S. Karger: Basel, 1981; pp 31−44. (b) Büllesbach, E. E. *Kontakte (Darmstadt)* **1992**, *1*, 21−29. (c) Andreu, D.; Albericio, F.; Solé, N. A.; Munson, M. C.; Ferrer, M.; Barany, G. In *Methods in Molecular Biology, Vol. 35, Peptide Synthesis Protocols;* Pennington, M. W., Dunn, B. M., Eds; Humana: Totowa, NJ, 1994; pp 91−169. (d) Moroder, L.; Besse, D.; Musiol, H.-J.; Rudolph-Böhner, S.; Siedler, F. *Biopolymers* **1996**, *40*, 207−234. (e) Annis, I.; Hargittai, B.; Barany, G. *Methods Enzymol.* **1997**, *289*, 198−221.

(5) For monographs and reviews, see: (a) *Biochemical Aspects of Reactions on Solid Supports;* Stark, G. R., Ed.; Academic: New York, 1971. (b) *Polymer-Supported Reactions in Organic Synthesis;* Hodge, P., Sherrington, D. C., Eds.; John Wiley & Sons: New York, 1980. (c) Merrifield, R. B. *Science* **1986**, *232*, 341−347. (d) Früchtel, J. S.; Jung, G. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 17−42. (e) Barany, G.; Kempe, M. In *A Practical Guide to Combinatorial Chemistry*; Czarnik, A. W., DeWitt, S. H., Eds.; American Chemical Society: Washington, DC, 1997; pp 51−97.

(6) Initial efforts in this regard are reported in refs 2a−c and Chen, L.; Barany, G. *Lett. Pept. Sci.* **1996**, *3*, 283−292.

(7) We are aware of two additional, possibly related, solid-phase approaches to facilitate disulfide formation. In one approach, poly(thiol) snake toxin substrates were passed over a commercially available agarose glutathione-2-pyridyl disulfide support, and the oxidized products were eluted by gradients of reduced and oxidized glutathione at pH 8.7. See: (a) Smith, D. C.; Hider, R. C. *Biophys. Chem.* **1988**, *31*, 21−28. In the second approach, a commercially available methacrylate resin, derivatized in an unspecified manner, gave a support termed Ekathiox. This was incubated with solutions of dihydrooxytocin and dihydrocalcitonin in dilute acetic acid and said to promote rapid, high-yield transformations to the corresponding intramolecular disulfides. See: (b) Clark, B. R.; Pai, M. Presented at the 14th American Peptide Symposium, Columbus, OH, June 18−23, 1995; Poster 90. (c) Clark, B. R.; Pai, M. *Chem. Abstr. (Selects Plus: Amino Acids, Peptides and Proteins*) **1996**, *14*, 23, 125: 12365k.

S0002-7863(98)01111-1 CCC: $15.00
Published on Web 07/09/1998

context of an assay for measuring free thiol concentration under physiological conditions,[8,9] can be adapted successfully to the new application of mediating intramolecular disulfide formation in the solid-phase mode. (Schemes 1 and 3, both later in this paper, present the overall approach and depict the structure of **1**.) Our approach offers advantages described for other polymeric reagents, including circumvention of potential problems due to solubility characteristics of substrates as well as reagent, the chance to carry out reactions under mild conditions conducive to product formation and to drive reactions to completion with excess reagent, the ready separation of reagent and concomitant isolation of product by simple filtration, and the recovery of reagent suitable for regeneration and reuse. In addition, the *pseudodilution* principle for polymer-supported reactions[10] is expected to favor intramolecular reactions and to decrease the extent of oligomerization, important considerations for the desired application to the creation of disulfides.[11] Finally, issues unique to solid-phase oxidations, for example, compatible solid supports and reaction milieus, loading and relative site isolation of supports, and a yield-diminishing intermolecular side reaction involving covalent adsorption to the support, have been addressed with respect to optimizing intramolecular disulfide formation.

Results and Discussion

Concept, Experimental Design, and Overview. In aqueous solution, at pH 6.8−8.2, thiol functions from low molecular weight organic compounds, as well as from peptides or proteins, react cleanly and rapidly with excess DTNB (**1**) to displace 5-thio-2-nitrobenzoic acid (TNB, **2**) and form the corresponding mixed aliphatic−aromatic disulfide intermediates.[8,9,12,13] This thiol−disulfide exchange reaction[14] is driven by the stability of the aromatic thiolate leaving group, as reflected by the low pK_a, ~4.75,[12a] of the conjugate aromatic thiol; the original titer of free aliphatic thiol groups is quantified on the basis of the TNB dianion chromophore at 412 nm. Depending on structural and conformational features of the mono- or poly(thiol) substrate that is reacted with DTNB, it is possible for the initially formed mixed disulfide to undergo disproportionations and/or further nucleophilic displacements. Such secondary transformations may provide a combination of intra- and intermolecular disulfides, and will not change the thiol titer reported by TNB. Studies with a range of organic thiols have shown that the rates of bimolecular thiolytic displacements of TNB from mixed aliphatic−aromatic disulfides are 1−2 orders of magnitude slower than the rates of the original displacements from the aromatic homodisulfide DTNB,[12] but when the attacking thiol is in the same molecule, an intramolecular cyclization step ensues which is substantially faster than the reaction with DTNB (i.e., no intermediate is observed from reaction of DTNB with organic dithiols).[12a] Ellman's chemistry can be harnessed for the creation of intramolecular peptide disulfides in solution; this approach is limited by the sparing solubility of DTNB at pH below 7, and is inevitably accompanied by the formation of *S*-TNB-containing byproducts.[15−17]

We envisaged that similar chemistry, but with important differentiating features, would occur upon treating a peptide substrate containing an even number of thiol groups with DTNB bound covalently to a solid support through two points of attachment (Scheme 1, top line). This allows the use of a much wider pH range than in the solution precedents, because now the reagent is on the solid phase and its solubility is no longer an issue. At the same time, the reaction milieu and compatible support can be optimized for proper folding and adequate solubility of the peptide substrate. Our proposed oxidation mechanism (Scheme 1) involves an initial "capture" step, that is, reaction of one of the peptidyl-thiol groups with the solid-phase reagent to provide a support-bound activated intermediate (Scheme 1, middle). Next, this intermediate undergoes intramolecular "cyclization" through attack by the other peptidyl-thiol group, resulting in formation of the desired disulfide bridge and concomitant release of the monomeric oxidized peptide product back into solution (Scheme 1, bottom). During this second step, the substrate is relatively sequestered (pseudodilution) from other potential thiol nucleophiles in solution or at other sites on the support, lessening the likelihood of competing intermolecular attacks which would lead to dimeric and oligomeric byproducts.[6,10,17] However, when intramolecular cyclization is retarded for conformational/steric reasons, an on-resin intermolecular side reaction involving attack by the second peptidyl-thiol group on a separate polymer-bound DTNB site can occur, despite pseudodilution.[11c,d] This side reaction,

(8) Ellman, G. L. *Arch. Biochem. Biophys.* **1959**, *82*, 70−77.

(9) (a) Habeeb, A. F. S. A. *Methods Enzymol.* **1972**, *25*, 457−464. (b) Riddles, P. W.; Blakeley, R. L.; Zerner, B. *Methods Enzymol.* **1983**, *91*, 49−61.

(10) This term, which implies the *kinetic* basis for minimizing intersite reactions, was introduced along with experimental documentation for a benzyne system by (a) Mazur, S.; Jayalekshmy, P. *J. Am. Chem. Soc.* **1979**, *101*, 677−683. See also: (b) Barany, G.; Merrifield, R. B. In *The Peptides−Analysis, Synthesis, Biology;* Gross, E., Meienhofer, J., Eds.; Academic: New York, 1979; Vol. 2, pp 1−284, especially pp 27−29.

(11) The approach taken in the present study is reciprocal to other work in which the peptide substrate for disulfide formation is attached to the support, as reviewed in refs 4c and 4e and also covered in (a) 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**, *37*, 402−413. (b) Munson, M. C.; Barany, G. *J. Am. Chem. Soc.* **1993**, *115*, 10203−10216, and references therein. Due to pseudodilution, intramolecular cyclization is favored, but intermolecular processes do compete. Also, when intramolecular pathways are not available to the resin-bound peptide substrates, conditions can often be found under which intermolecular disulfide formation occurs in reasonably high yield, as shown in (c) Bhargava, K. K.; Sarin, V. K.; Le Trang, N.; Cerami, A.; Merrifield, R. B. *J. Am. Chem. Soc.* **1983**, *105*, 3247−3251. (d) Munson, M. C.; Lebl, M.; Slaninovà, J.; Barany, G. *Pept. Res.* **1993**, *6*, 155−159.

(12) (a) Whitesides, G. M.; Lilburn, J. E.; Szajewski, R. P. *J. Org. Chem.* **1977**, *42*, 332−338. (b) Wilson, J. M.; Bayer, R. J.; Hupe, D. J. *J. Am. Chem. Soc.* **1977**, *99*, 7922−7926.

(13) (a) Butterworth, P. H. W.; Baum, H.; Porter, J. W. *Arch. Biochem. Biophys.* **1967**, *118*, 716−723. (b) Riddles, P. W.; Blakeley, R. L.; Zerner, B. *Anal. Biochem.* **1979**, *94*, 75−81. (c) Wilson, J. M.; Wu, D.; Motiu-DeGrood, R.; Hupe, D. J. *J. Am. Chem. Soc.* **1980**, *102*, 359−363. (d) Li, T.-Y.; Minkel, D. T.; Shaw, C. F., III; Petering, D. H. *Biochem. J.* **1981**, *193*, 441−446. (e) Kuwata, K.; Uebori, M.; Yamada, K.; Yamazaki, Y. *Anal. Chem.* **1982**, *54*, 1082−1087. (f) Robey, F. A. *Protides Biol. Fluids* **1986**, *34*, 47−50. (g) Savas, M. M.; Shaw, C. F., III; Petering, D. H. *J. Inorg. Biochem.* **1993**, *53*, 235−249.

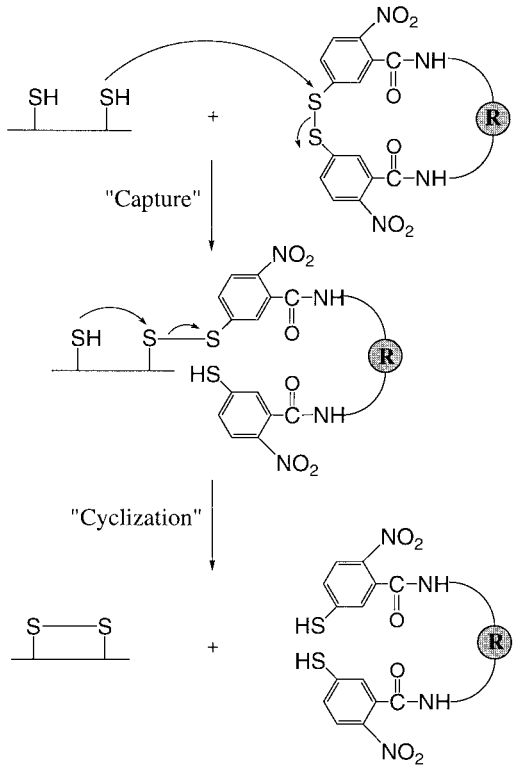
(14) For a review, see: Gilbert, H. F. *Adv. Enzymol.* **1990**, *63*, 69−172.

(15) The first explicit application of DTNB to intramolecular disulfide formation was reported in ref 11a, although in hindsight, several aspects of the experiment were suboptimal and not all of the observed oxidation can be attributed to DTNB. Thus, a linear oxytocin sequence assembled by solid-phase peptide synthesis was selectively deblocked, and the resin-bound peptide dithiol was treated with DTNB (0.5 equiv) in "buffered" DMF, pH 7.5, for 1 h. Cleavage from the support revealed monomeric oxytocin in 65% absolute yield, along with a major byproduct, detected by HPLC, which was believed to contain TNB on the basis of UV absorbance at 220, 280, and 340 nm.

(16) A preparative solution experiment used DTNB (10 equiv) to carry out disulfide cyclization at pH 6.8 on a peptide (2 mM) that was labile and sluggishly reactive under standard alkaline pH air-oxidation conditions. The desired product was isolated in 36% yield, and an extra HPLC peak was observed and postulated to be the peptide with both Cys thiol groups linked to TNB groups through disulfide bonds. See: Engebretsen, M.; Agner, E.; Sandosham, J.; Fischer, P. M. *J. Peptide Res.* **1997**, *49*, 341−346.

(17) Our own preliminary studies (see Experimental Section) showed that solution oxidations with DTNB in the oxytocin, somatostatin, and conotoxin families provide, at best, 50−85% monomeric intramolecular disulfide, and identified significant experimental difficulties that limit the practical usefulness of the method.

Scheme 1. Proposed Mechanism for Intramolecular Disulfide Formation Mediated by Solid-Phase Ellman's Reagents[a]



[a] Solid supports Ⓡ demonstrated in this work include poly(ethylene glycol)−polystyrene (PEG-PS) graft, Sephadex G-15, and controlled pore glass (CPG). For preparative experiments, DTNB was attached directly to the amino-functionalized supports, whereas for some mechanistic experiments, appropriate spacers and/or cleavable linkers were used between DTNB and the support backbone. Details in text.

documented later in this paper (Scheme 6 and accompanying discussion), is the solid-phase equivalent of bis(TNB) byproduct formation presumed to occur in solution;[16,17] it leads to covalent retention of the peptide on the support, and lowers the overall yield of product obtained in solution without affecting its purity.

Implementation of our concept required first the development of efficient methods for preparation of solid-phase Ellman's reagents. Note the significance of attaching DTNB through two sites; if this were not the case, approximately half of the capture events (Scheme 1, first step) would result in a mixed disulfide intermediate released back into solution, and hence the suggested pseudodilution benefit would be forfeited. As shown later, creation of an aromatic disulfide cross-link as part of the structure of the polymer-bound reagent was achieved in both inter- and intramolecular fashion; the intermolecular cross-linking is more difficult because of the relative site isolation implicit in pseudodilution.[10,11] In the next phase of this research, solid-phase Ellman's reagents made by several methods and on different types of parent supports were evaluated for their capability to mediate disulfide formation in a representative menu of peptide substrates (e.g., Table 1). Transformations were monitored by chromatographic analysis of species remaining in solution; polymer-bound intermediates and byproducts were also quantified (Figure 1). The data do not permit a complete kinetic description of heterogeneous multistep reactions with competing pathways, but a good idea of relative rates is provided by $t_{1/2}^{app}$, the time at which the material remaining in solution comprises equal amounts of starting reduced substrate and oxidized product(s). Our overall concept is validated by the fact that conversion can reach completion and provides the expected intramolecular cyclic disulfides as the predominant products found in *solution*. Moreover, the observed absolute yields are a gauge of how readily the peptide structure can assume a structure leading to cyclization, because the principal alternative pathways result in byproducts that are retained on the support.

Solid Supports. Our approach should be viable using, as parent supports, materials with a variety of chemical compositions and physical characteristics and architectures; experimental results as a function of choice of support are expected to reflect the extent of site isolation in the solid phase. Initial studies were focused on three types of commercially available supports that have been well established for synthesis and/or chromatography applications involving peptides, small proteins, and/or oligonucleotides: (1) microporous poly(ethylene glycol)−polystyrene (PEG-PS) grafts,[18] (2) cross-linked dextran with a molecular weight exclusion of 1500 (Sephadex G-15),[19] and (3) controlled pore glass (CPG).[20] PEG-PS has advantageous physical and mechanical properties for batchwise and continuous flow reactions, and its grafted PEG chains impart good swelling in a range of organic solvents as well as in water. Sephadex is more fragile, but has excellent compatibility with aqueous systems and exhibits significant swelling in water. CPG provides a rigid (nonswelling) support, and its functional sites are located on the walls of uniformly distributed pores.

DTNB derivatives contain pendant carboxyl groups which are suited for attachment, via amide bond forming reactions, to complementary amino sites on appropriate functionalized supports. Amino-PEG-PS (0.15−0.38 mmol/g loading) and aminopropyl-CPG (1000 Å pore size, 35 m^2/g, 0.02−0.09 mmol/g loading) are commercially available. However, introduction onto Sephadex G-15 of amino groups at a sufficiently high loading level required development of a new procedure (Scheme 2).[21] Bromoethylamine hydrobromide (**3**) was neutralized and treated with di-*tert*-butyl dicarbonate to provide the Boc-protected derivative *tert*-butyl *N*-(2-bromoethyl)carbamate (**4**).[22] Subsequently, Sephadex G-15 was activated with base

(18) (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: Leiden, 1993; pp 267−268. (b) Zalipsky, S.; Chang, J. L.; Albericio, F.; Barany, G. *React. Polym.* **1994**, *22*, 243−258. (c) Barany, G.; Albericio, F.; Kates, S. A.; Kempe, M. In *Chemistry and Biological Application of Polyethylene Glycol*, ACS Symposium Series 680; Harris, J. M., Zalipsky, S., Eds.; American Chemical Society Books: Washington, DC, 1997; pp 239−264.

(19) (a) Köster, H.; Heyns, K. *Tetrahedron Lett.* **1972**, *16*, 1531−1534. (b) Vlasov, G. P.; Bilibin, A. Y.; Skvortsova, N. N.; Kalejs, U.; Kozhevnikova, N. Y.; Aukone, G. In *Peptides 1994. Proceedings of the Twenty-Third European Peptide Symposium*; Maya, H. L. S., Ed.; ESCOM Science: Leiden, 1995; pp 273−274.

(20) (a) Caruthers, M. H.; Barone, A. D.; Beaucage, S. L.; Dodds, D. R.; Fisher, E. F.; McBride, L. J.; Matteuci, M.; Stabinski, Z.; Tang, J.-Y. *Methods Enzymol.* **1987**, *20*, 2571−2580. (b) Beaucage, S. L.; Caruthers, M. H. In *Bioorganic Chemistry: Nucleic Acids*; Hecht, S. M., Ed.; Oxford University: New York, 1996; pp 36−74. (c) Blackburn, G. M.; Gait, M. J. In *Nucleic Acids in Chemistry and Biology*, 2nd ed.; Blackburn, G. M., Gait, M. J., Eds.; Oxford University: Oxford, 1996; pp 83−145.

(21) Several methods for activating Sephadex have been reported previously, but they give maximal loadings of 0.1−60 μmol/g. See: (a) Axén, R.; Porath, J.; Ernback, S. *Nature* **1967**, *214*, 1302−1304. (b) Parikh, I.; March, S.; Cuatrecasas. *Methods Enzymol.* **1975**, *34*, 77−102. (c) Brocklehurst, K.; Carlsson, J.; Kierstan, M. P. J.; Crook, E. M. *Methods Enzymol.* **1975**, *34*, 531−547. As this work was being readied for publication, a method for converting the commercially available HiTrap Sepharose derivative to a form with 0.2 mmol/g of amino functionalization was reported; see: (d) Tegge, W.; Frank, R. *J. Peptide Res.* **1997**, *49*, 355−362.

(22) This compound has been prepared previously by a similar method: Beylin, V. G.; Goel, O. P. *Org. Prep. Proced. Int.* **1987**, *19*, 78−80.

Table 1. Overview of Rates and Absolute Yields of Oxidation of Representative Peptide Substrates by Solid-Phase Ellman's Reagent[a]

	pH 2.7		pH 6.6	
substrate	$t_{1/2}^{app}$ (min)	yield (%)	$t_{1/2}^{app}$ (min)	yield (%)
H-CYIQNCPLG-NH_2 (oxytocin)	5	72	<1	77
Mpa-YIQNCPLG-NH_2 (deamino-oxytocin)	10	62	<1	73
H-AGCKNFFWKTFTSC-OH (somatostatin)	330	70	5	75
H-ICC(Acm)NPACGPKYSC(Acm)-NH_2 (conotoxin SH 2&7)	150	80	3	90
H-IC(Acm)CNPACGPKYSC(Acm)-NH_2 (conotoxin SH 3&7)	330	70	4	90
H-IC(Acm)CNPAC(Acm)GPKYSC-NH_2 (conotoxin SH 3&13)	255	86	3	89
H-ICCNPACGPKYSC-NH_2 (α-conotoxin SI)	54	79	3	90
H-CNCKAPETALCARRCQQH-NH_2 (apamin)	120	96	5	92

[a] Reaction conditions: 15-fold excess of oxidizing reagent El-Lys(El)-PEG-PS disulfide (prepared by route of Scheme 5, right side), 0.21 mmol/g loading, and reduced peptide substrate 0.5 μmol, at a concentration of ~1 mg/mL in buffer−CH_3CN−CH_3OH (2:1:1). The apparent half-time, $t_{1/2}^{app}$, represents the time when the soluble peptide comprises equal amounts of reduced and oxidized species. Reactions were carried out until negligible amounts of reduced substrate were observed by HPLC (a time referred to in later tables as t_{end}; this proved empirically to be 2- to 5-fold longer than the reported $t_{1/2}^{app}$ for most of the peptides studied). The absolute yields reported represent monomeric oxidized product(s); in the cases of apamin and α-conotoxin SI they are the sum of yields of all disulfide regioisomers formed (refer to Table 7 for regioselectivity studies). With oxytocin and deamino-oxytocin, formation of dimers accounts for an additional 3−19% conversion of the initial reduced substrate (details in Table 8). With other peptides, dimers were not observed, and the major yield-diminishing side reaction was covalent adsorption to the support (details later).

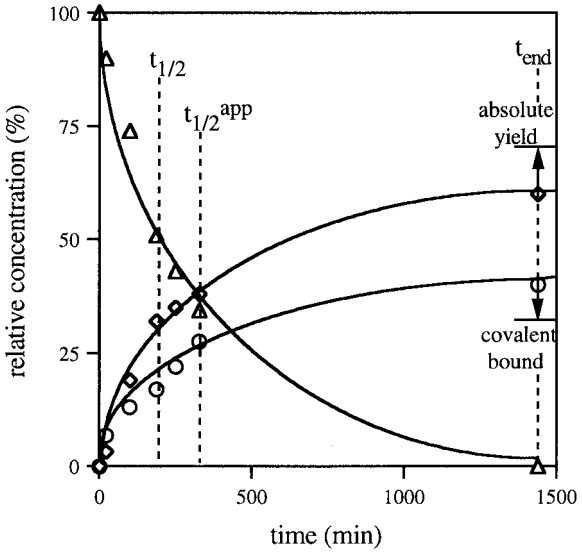
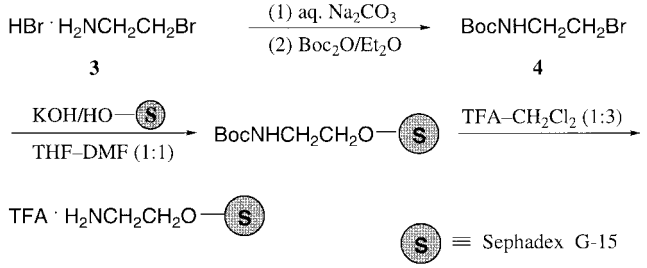


Figure 1. Conversion of reduced somatostatin (△) to its disulfide form (◇), as mediated by El-Lys(El)-PEG-PS disulfide at pH 2.7. Conditions and data correspond to entry 3 in Table 1. The zero timepoint for starting reduced substrate (prior to addition of the solid-phase reagent), as well as the endpoints for all species, were calibrated by HPLC and amino acid analysis. The amounts of soluble species during the time course of the reaction were monitored by HPLC analysis, and the amount adsorbed to the support (○), either covalently or physically, was calculated to add to 100%. Dotted lines mark and define $t_{1/2}^{app}$ (330 min for this example), the time at which the curves for loss of dithiol peptide and formation of disulfide peptide overlapped; $t_{1/2}$ (230 min for this example), the time at which half of the dithiol peptide has been lost from solution [this is calculated by ln 2/*k*, where the apparent pseudo-first-order rate constant *k* is the negative of the slope of a graph of ln(% reduced somatostatin) vs time]; and t_{end}, the time for complete conversion. The final yield of disulfide peptide is typically 10−15% higher, because upon completion of the oxidation, draining of the Ellman's reagent-resin under positive air pressure results in release of additional product that was contained within the swollen reaction volume and hence not sampled by the HPLC assay of soluble components. The residual drained resin still contains covalently bound peptide (30% for this example), as elaborated later (Scheme 6 and accompanying discussion).

(wash with 2 N aqueous NaOH), washed, and subjected to an alkylation reaction with **4** in the presence of a slight excess of KOH. Finally, acidolytic removal of the Boc group with trifluoroacetic acid (TFA)−CH_2Cl_2 (1:3) generated aminated Sephadex, the loading of which (0.2−1.0 mmol/g) was controlled by the reaction time and reagent excess in the earlier alkylation step (~50−70% of **4** becomes incorporated).

Scheme 2. Amination of Sephadex G-15

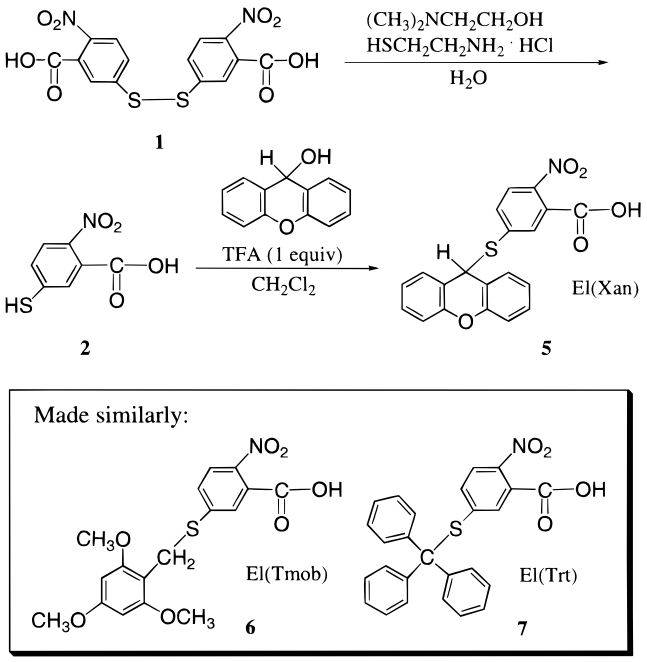


Each of the three families of functionalized supports selected for study was transformed further to create dually attached solid-phase DTNB, as described later (Schemes 4 and 5, and accompanying discussion). Some of the routes that were worked out required an on-resin oxidation step, and a number of reagents were tested for this purpose. Iodine oxidation was the most convenient, and the method of choice when the support was Sephadex or CPG. However, when iodine treatment was applied to PEG-PS, either loaded with oxidizable functions or (as a control) unsubstituted, it proved to be difficult to wash out all of the excess reagent. The resultant (presumably noncovalent) I_2···PEG-PS complex itself promoted disulfide formation from peptide dithiol substrates, hence complicating interpretations of experiments involving covalently bound Ellman's reagent. Consequently, oxidations to form DTNB attached to PEG-PS were carried out with aqueous ferricyanide; controls showed that ferricyanide-treated PEG-PS did not have residual oxidizing properties.

DTNB Derivatization and Formation of Solid-Phase Ellman's Reagents. The plan to use amide bond formation involving the DTNB carboxyl and amino-functionalized supports has been described in the preceding section. However, DTNB (**1**) is bifunctional, and we expected that coupling of a monofunctional DTNB derivative might provide more control. Toward this end, **1** was conveniently reduced by treatment with aqueous mercaptoethylamine in the presence of dimethylaminoethanol to provide aromatic thiol **2** in quantitative yield (Scheme 3, top line; reagents chosen to facilitate workup). Subsequently, acid-catalyzed reaction of **2** with 9*H*-xanthen-9-ol[23] gave the *S*-9*H*-xanthen-9-yl (Xan)-protected derivative **5**

(23) Han, Y.; Barany, G. *J. Org. Chem.* **1997**, *62*, 3841−3848.

Scheme 3. Derivatization of DTNB[a]

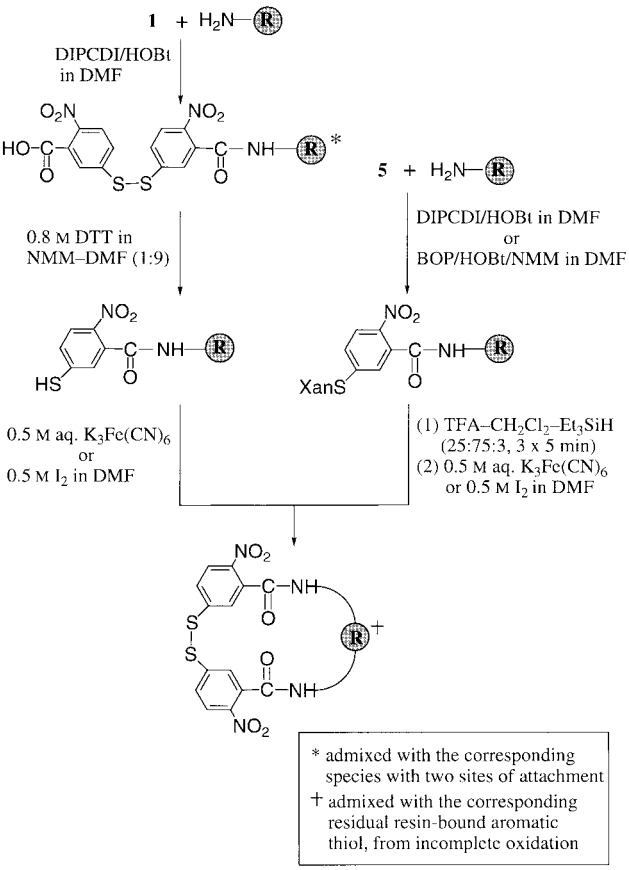


[a] Compounds **6** and **7** were somewhat more difficult to prepare with respect to **5**, and they require a higher acid concentration to remove the *S*-protecting group. Nevertheless, all three derivatives were taken further to provide solid-phase Ellman's reagents (Schemes 4 and 5), which gave equivalent performance in mediating intramolecular disulfide formation.

(Scheme 3, second line); similarly **2** could be protected as its *S*-2,4,6-trimethoxybenzyl (Tmob)[24] (**6**) and *S*-triphenylmethyl (Trt)[25] (**7**) derivatives.

The most straightforward way to immobilize Ellman's reagent functionalities is by coupling of DTNB (**1**) to amino-functionalized supports (Scheme 4, left side).[26] Initial experiments started with amino-PEG-PS, and coupling of **1** was mediated by *N*,*N*′-diisopropylcarbodiimide (DIPCDI) in *N*,*N*-dimethylformamide (DMF), in the presence of 1-hydroxybenzotriazole (HOBt). This provided a mixture of DTNB with one site and with two sites of attachment to the support. The ratio of these species ranged from ∼1:3 to ∼1:1, depending on the original degree of excess of DTNB carboxyls over resin amines (as expected, more cross-linking was achieved with a smaller excess). Because our intended application requires that DTNB have two attachment sites, it was necessary to carry out a reduction step—providing the 5-thio-2-nitrobenzoyl-resin intermediate—followed by reoxidation. Alternatively, the monofunctional *S*-Xan protected derivative **5** was coupled, followed by acidolytic deblocking to give again a resin-bound aromatic thiol, and finally oxidation (Scheme 4, right side).[27] However, in both of these initial routes, the intermolecular oxidation to provide the final reagent bound through two sites went, at best,

Scheme 4. Initial Approaches for Preparation of Solid-Phase Ellman's Reagents[a]



[a] $K_3Fe(CN)_6$ oxidation used for Ⓡ = PEG-PS, and I_2 oxidation used for Ⓡ = Sephadex G-15 or CPG. Acidolytic removal of *S*-Xan also possible with substantially less TFA, i.e., TFA−CH_2Cl_2−Et_3SiH (3:94:3, 2 × 1 h); this was done when the support included an acid-labile PAL linker. Oxidative removal of *S*-Xan with concurrent disulfide formation is discussed in ref 27. Experiments were also carried out replacing **5** by **6** [*S*-Tmob removal conditions the same] or **7** [*S*-Trt removal by TFA−CH_2Cl_2−Et_3SiH (50:50:3, 3 × 10 min)].

to ∼80−90%. Restated, ∼10−20% or more of the aromatic thiol sites in PEG-PS could not pair up and form disulfides due to site isolation effects (the thiols were still reactive, since alkylation with iodoacetamide gave the corresponding *S*-carboxamidomethyl derivatives).

The difficulty in carrying out on-resin intermolecular disulfide formation to completion led us to consider an intramolecular alternative (Scheme 5, right side). Thus, incorporation of a lysine spacer was followed by coupling of monofunctional *S*-Xan-protected derivative **5** to both the α- and ϵ-amino groups. Subsequent acidolytic deprotection generated two aromatic thiols in close proximity to each other, and these were easily bridged in quantitative yield to form the final solid-phase reagent (Scheme 5, bottom structure). Another way to access the same lysine-containing solid-phase reagent started with some solution reactions (Scheme 5, left side): DTNB (**1**) was converted to its

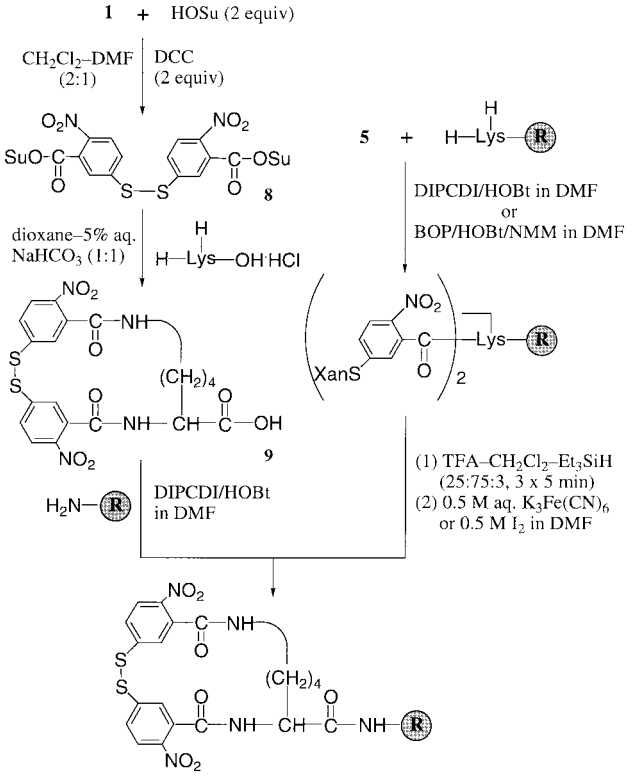
(24) Munson, M. C.; García-Echeverría, C.; Albericio, F.; Barany, G. *J. Org. Chem.* **1992**, *57*, 3013−3018.

(25) (a) Hiskey, R. G.; Mizoguchi, T.; Igeta, H. *J. Org. Chem.* **1966**, *31*, 1188−1192. (b) Photaki, I.; Taylor-Papadimitriou, J.; Sakarellos, C.; Mazarakis, P.; Zervas, L. *J. Chem. Soc., Chem. Commun.* **1970**, 2683−2687.

(26) It is instructive to compare our approach to the work of Fridkin and collaborators, whose goal was to prepare solid-phase aromatic thiols. Both symmetrical and unsymmetrical carboxyl-containing disulfides were coupled, following which borohydride reduction gave the desired products. Information about the ratio of single and dual attachments at the disulfide stage was not reported. See: (a) Stern, M.; Warshawsky, A.; Fridkin, M. *Int. J. Pept. Protein Res.* **1981**, *17*, 531−538. (b) Stern, M.; Fridkin, M.; Warshawsky, A. *J. Polym. Sci., Polym. Chem. Ed.* **1982**, *20*, 1469−1487.

(27) It is known (ref 23) that pairwise oxidation of *S*-Xan-protected thiols with iodine gives disulfides directly, raising the possibility to save a step in the chemistry of Scheme 4, right side. However, this conversion was ambiguous for Ⓡ = PEG-PS due to problems already discussed with iodine, and for Ⓡ = Sephadex G-15, the reaction was slow and difficult to carry out to completion. The residual (unoxidized) *S*-Xan-protected sites on the support led to a new problem at the next stage, when such supports were used in experiments to oxidize peptide substrates with two (or for that matter, one) thiol function(s). Thus, under the semiaqueous reaction conditions, the *S*-Xan group apparently transferred from the resin-bound aromatic thiol to the aliphatic peptide thiol.

Scheme 5. Optimized Approaches for Preparation of Solid-Phase Ellman's Reagents[a]



[a] $K_3Fe(CN)_6$ oxidation used for Ⓡ = PEG-PS, and I_2 oxidation used for Ⓡ = Sephadex G-15 or CPG. Acidolytic removal of *S*-Xan also possible with substantially less TFA, i.e., TFA−CH_2Cl_2−Et_3SiH (3:94:3); this was done when the support included an acid-labile PAL linker.

bis(succinimidyl) ester **8**, which was added under high dilution conditions to lysine in aqueous bicarbonate to provide analytically pure DTNB−lysine macrocyclic adduct **9**. The preparation concluded by the quantitative coupling of **9** to amino-functionalized supports. Both of these improved routes (Scheme 5) were repeated on supports that included a tris(alkoxy)benzylamide (PAL) handle[28] as well as two protected aspartyl residues (to help with solubility during the subsequent analysis). After all indicated transformations had been conducted, the final derivatized supports were cleaved with Reagent B[29] [TFA−CH_2Cl_2−H_2O−Et_3SiH−PhOH (92:5:1:1:1)] and were shown in each case to be the expected model peptide amide [lysine derivative substructure corresponding to **9** residue, followed by Asp-Asp, and concluding with C-terminal (C═O)NH_2], homogeneous by analytical HPLC and providing the proper mass upon FABMS. Such experiments prove the efficacy of these preparative methods. Moreover, we document later in this paper that when applied to the overall goal of this work, that is, to mediate intramolecular disulfide bridge formation, the lysine-containing solid-phase Ellman's reagents (Scheme 5) provided *significantly* better performance by comparison to that of any of the earlier formulations (Scheme 4).

The solid-phase Ellman's reagents made in any of the ways described in this section can be readily regenerated and recycled. Thus, after the polymeric reagent has been used to transform a suitable peptide substrate, there follows reduction with dithiothreitol to strip any covalently bound byproducts and simultaneously restore the aromatic thiol (TNB) functions on the support. Oxidation by the same methods already described then provides again resin-bound DTNB. We were able to complete three cycles of use/regeneration; after each stage, the overall rates and yields of peptide disulfide formation with regenerated reagent were unchanged.

Peptide Substrates. Several peptide substrates, the linear reduced precursors of which were obtained readily by 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase synthesis,[30,31] were chosen to study the efficiency of intramolecular disulfide bridge formation mediated by solid-phase Ellman's reagents (Table 1 and subsequent tables): (a) oxytocin (9 residues, disulfide bridge between residues 1 and 6),[32] (b) deamino-oxytocin (same as oxytocin except for N-terminal β-mercaptopropionyl residue),[32c] (c) somatostatin (14 residues, disulfide bridge between residues 3 and 14),[33] (d) α-conotoxin SI (13 residues, disulfide bridges between residues 2 and 7; 3 and 13),[11b] and (e) apamin (18 residues, disulfide bridges between residues 1 and 11; 3 and 15).[34] Oxytocin is considered to be a particularly easy sequence to oxidize, while oxidation to deamino-oxytocin is somewhat more difficult, and oxidation to somatostatin is particularly sluggish under acidic and neutral conditions. Dimers of oxytocin and deamino-oxytocin are known from previous work,[11d,35] allowing us to evaluate the relative occurrence of inter- and intramolecular oxidations in the present system. Conotoxin and apamin were chosen to study the selectivity of our proposed method in forming the correctly paired disulfide regioisomers. In the case of conotoxin, we also prepared several differentially protected derivatives [e.g., "conotoxin SH 2&7" has free thiols at residues 2 and 7, while 3 and 13 are protected by acetamidomethyl, Acm] in order to investigate selective formation of a predetermined (single) bridge.

Intramolecular Disulfide Formation Mediated by Solid-Phase Ellman's Reagents. Oxidations were carried out with peptide substrates dissolved in aqueous buffer−CH_3CN−CH_3OH (2:1:1) at a concentration of 0.7−2.0 mM, on scales of 0.5−5 μmol. Excess solid-phase Ellman's reagent resins, 2- to 50-fold over the amount of peptide, with loadings of 0.03−1.0 mmol/g, were added, and reactions were carried out at 25 °C until HPLC analysis of the soluble portion indicated an endpoint in the conversion of starting to product peptide(s)

(28) (a) Albericio, F.; Barany, G. *Int. J. Pept. Protein Res.* **1987**, *30*, 206−216. (b) Albericio, F.; Kneib-Cordonier, N.; Biancalana, S.; Gera, L.; Masada, R. I.; Hudson, D.; Barany, G. *J. Org. Chem.* **1990**, *55*, 3730−3743.

(29) Solé, N. A.; Barany, G. *J. Org. Chem.* **1992**, *57*, 5399−5403.

(30) For reviews, see: (a) Barany, G.; Kneib-Cordonier, N.; Mullen, D. G. *Int. J. Pept. Protein Res.* **1987**, *30*, 705−739. (b) Fields, G. B.; Noble, R. L. *Int. J. Pept. Protein Res.* **1990,** *35*, 161−214. (c) Fields, G. B.; Tian, Z.; Barany, G. In *Synthetic Peptides: A User's Guide;* Grant, G. A., Ed.; W. H. Freeman: New York, 1992; pp 77−183.

(31) The reduced linear precursors were 64−92% pure (<5% disulfide) upon cleavage from the support by appropriate TFA−scavanger mixtures and workup. For much of our work, these materials were used in oxidation reactions *directly*, that is, without any preliminary purification. Portions of both reduced and oxidized substrates were purified to homogeneity, quantified by amino acid analysis, and used to determine accurate HPLC response factors.

(32) (a) du Vigneaud, V.; Ressler, C.; Swan, J. M.; Roberts, C. W.; Katsoyannis, P. G.; Gordon, S. *J. Am. Chem. Soc.* **1953**, *75*, 4879−4880. (b) Live, D. H.; Agosta, W. C.; Cowburn, D. *J. Org. Chem.* **1977**, *42*, 3556−3561. (c) Chen, L.; Zoulíková, I.; Slaninová, J.; Barany, G. *J. Med. Chem.* **1997**, *40*, 864−876, and references therein.

(33) Rivier, J.; Kaiser, R.; Galyean, R. *Biopolymers* **1978**, *17*, 1927−1938, and references therein.

(34) To the best of our knowledge, this is the first successful synthesis of apamin by an Fmoc strategy. For stepwise synthesis or segment condensation with Boc chemistry, see: (a) Van Rietschoten, J.; Granier, C.; Rochat, H.; Lissitzky, S.; Miranda, F. *Eur. J. Biochem.* **1975**, *56*, 35−40. (c) Sandberg, B. E. B.; Ragnarsson, U. *Int. J. Pept. Protein Res.* **1978**, *11*, 238−245. (d) Albericio, F.; Granier, C.; Labbé-Juillié, C.; Seagar, M.; Couraud, F.; Van Rietschoten, J. *Tetrahedron* **1984**, *40*, 4313−4326.

(35) Chen, L.; Bauerová, H.; Slaninová, J.; Barany, G. *Pept. Res.* **1996**, *9*, 114−121.

Table 2. Oxidation of Conotoxin SH 3&7 by Solid-Phase Ellman's Reagent as a Function of pH[a]

pH	$t_{1/2}^{app}$ (min)	t_{end} (min)	resin-bound (%)	yield (%)
2.7	330	1320	25	70
3.4	55	150	17	80
4.0	22	53	18	78
5.0	4	20	16	81
6.6	3.5	15	6	90

[a] Reaction conditions and definitions for these studies are the same as in Table 1; in addition, the column labeled "resin-bound" reports the amount of peptide substrate that becomes attached covalently to the solid-phase Ellman's reagent.

Table 3. Comparison of Solid-Phase Ellman's Reagents Prepared by Different Routes for Efficacy in Promoting Oxidation of Several Peptide Substrates at pH 2.7[a]

	Scheme 4 (right) route (0.4 mmol/g, on PEG-PS)			Scheme 5 (right) route (0.2 mmol/g, on Lys-PEG-PS)		
substrate	$t_{1/2}^{app}$ (h)	yield (%)	resin-bound (%)	$t_{1/2}^{app}$ (h)	yield (%)	resin-bound (%)
somatostatin	15	50	50	5.5	70	23
conotoxin SH 2&7	7	78	14	2.5	80	12
conotoxin SH 3&7	21	54	40	5.5	70	25
conotoxin SH 3&13	9	80	15	4.3	86	14

[a] Reaction conditions and definitions for these studies are the same as in Tables 1 and 2. Loadings of the starting supports were chosen so that solid-phase Ellman's reagents prepared by the two routes have comparable loadings of DTNB sites.

(e.g., Figure 1). Rates and yields of oxidation were found to depend on the linear sequence of the peptide substrate and on a number of factors which were investigated systematically as reported in the following paragraphs.

Incubation of reduced peptides with solid-phase Ellman's reagents resulted in the desired formation of disulfides over a wide pH range (Table 1). The capability of achieving complete reactions under acidic conditions is one of the major advantages of our approach, since more basic conditions can promote scrambling and disproportionation. However, to maximize rates as well as yields by this method, a higher pH for reactions was preferred (Table 2, determined with conotoxin SH 3&7 as substrate). In the pH range 2.7−5.0, rates varied linearly with hydrogen ion concentration (data from Table 2 was graphed), demonstrating the involvement of thiolate ions in the rate-determining step. At pH closer to neutral, rates were very fast and had leveled off, reflecting complete ionization of the TNB leaving group. As expected, the peptides which oxidize more rapidly at a given pH with solid-phase Ellman's reagents are those known to have conformations more compatible with disulfide formation (as described in the previous section). Moreover, these same peptides give higher yields of disulfide product in solution (more so at higher pH), and correspondingly, lower amounts of resin-bound byproducts.

The low pH conditions allowed a critical test of how the method by which the solid-phase Ellman's reagent is prepared influences its eventual usefulness in the disulfide-forming application (Table 3). PEG-PS at 0.4 mmol/g, transformed by coupling of protected precursor **5**, followed by deblocking and on-resin oxidation (Scheme 4, right side) contains ∼85% oxidized DTNB functions. This level of functionality is comparable to El-Lys(El)-PEG-PS disulfide derived from PEG-PS at 0.2 mmol/g (Scheme 5, right side). Nevertheless, the applications of the Lys-containing formulation gave reaction rates that were 2- to 4-fold faster; in addition, absolute yields were somewhat better and the amounts of resin-bound byproducts were similarly lower. Solid-phase Ellman's reagent made in the original way starting with PEG-PS at 0.2 mmol/g gave even slower reactions and lower yields (data not shown), and the reproducibility of results was tempered by the incomplete oxidation to create DTNB functions. Our conclusion from these studies was to use the Lys-containing variants for all further mechanistic and preparative work.

Table 4. Oxidation of Several Peptide Substrates by Solid-Phase Ellman's Reagent as a Function of Reagent Excess[a]

substrate	pH	excess (fold)	$t_{1/2}^{app}$ (min)	t_{end} (min)	soluble reduced (%)	resin-bound (%)	yield (%)
somatostatin	6.6	5	13	60	13	15	72
	6.6	15	5	25	0	25	75
conotoxin SH 2&7	6.6	3	8	35	9	2	89
	6.6	15	3	20	4	6	90
	2.7	15	150	420	8	12	80
	2.7	40	60	210	8	17	75
conotoxin SH 3&7	6.6	3	10	45	6	4	90
	6.6	15	4	15	4	6	90
	2.7	15	330	1320	5	25	70
	2.7	30	150	480	4	26	70
conotoxin SH 3&13	6.6	3	8	40	10	2	88
	6.6	15	3	20	1	10	89

[a] Reaction conditions and definitions for these studies are the same as in Tables 1 and 2, except the number of equivalents of El-Lys(El)-PEG-PS disulfide has been varied. In addition, the column labeled "soluble reduced" reports the amount of peptide substrate remaining at t_{end}.

Our standard conditions use a 15-fold molar excess of Ellman's functions over the linear peptide to be oxidized. As might be expected, the reaction rate varied linearly with the reagent excess, while the overall yield was more or less the same (Table 4). At the low end of the pH scale, i.e., pH 2.7, rates were faster in proportion to the use of a greater excess of solid-phase Ellman's reagent. Conversely, at pH 6.6, it was possible to use lower excesses and still achieve reasonable rates and excellent absolute yields. However, these latter reactions apparently reached an equilibrium short of completion, as reflected by the relatively low amounts of starting reduced peptide substrate that did not oxidize even upon substantially longer incubation. Control experiments at pH 6.6 involved treatment of the various peptide substrates under study with acetylated PEG-PS; at most 15% background disulfide formation was observed during the time frame required to achieve full conversion with the El-Lys(El)-PEG-PS disulfide reagent.

Next, the effect of support loading was examined, using again the Lys-PEG-PS variant of the solid-phase Ellman's reagent (Table 5). The amount of polymeric reagent introduced to the reaction was reduced concordantly, so as to maintain the same overall excess of DTNB functions. While for a number of solid-phase applications higher loading is a benefit, for our goal of mediating intramolecular disulfide formation the higher loading led to slower reaction rates and dramatically lower absolute yields of desired solubilized monomeric disulfide. Over a 2-fold loading range, substantially more peptide was adsorbed covalently to the higher-loaded solid-phase reagent. These results, and similar findings with Sephadex and CPG (footnote to Table 5 and the next paragraph of this paper), are entirely consistent with our understanding of the principal side reaction—an intermolecular process occurring within the swollen volume of the solid-phase reagent—which competes with the desired intramolecular cyclization. The side reaction is favored in the higher-loaded supports, which have a greater effective concentration of DTNB sites. In addition, the slower overall rate observed with the higher-loaded support may be plausibly explained by noting that less resin was used (rationale earlier in this paragraph) and that the rate-determining "capture" step

Table 5. Oxidation of Several Peptide Substrates by Solid-Phase Ellman's Reagent as a Function of Support Loading[a]

		0.21 mmol/g		0.38 mmol/g	
peptide	pH	$t_{1/2}^{app}$ (min)	resin-bound (%)	$t_{1/2}^{app}$ (min)	resin-bound (%)
somatostatin	2.7	145	30	290	80
conotoxin SH 3&7	2.7	150	26	690	50
conotoxin SH 3&7	3.4	28	19	105	30

[a] Reaction conditions and definitions for these studies are the same as in Tables 1 and 2, except a 30-fold excess of the El-Lys(El)-PEG-PS disulfide reagent was used for this set of data. Additional experiments to oxidize some of these substrates were also carried out with El-Lys(El)-PEG-PS disulfide (0.08 mmol/g), $(El)_2$-PEG-PS disulfide (loading range of DTNB sites: 0.1−0.2 mmol/g), El-Lys(El)-CPG disulfide (loading range: 0.02−0.05 mmol/g), and El-Lys(El)-Sephadex G-15 disulfide (loading range: 0.2−1.0 mmol/g). The results were qualitatively in accord with the ones shown, i.e., slower rates and greater levels of resin adsorption with higher loading (see Tables 3 and 6 for baseline values).

Table 6. Oxidation of Several Peptide Substrates by Solid-Phase Ellman's Reagent as a Function of Parent Support at pH 2.7[a]

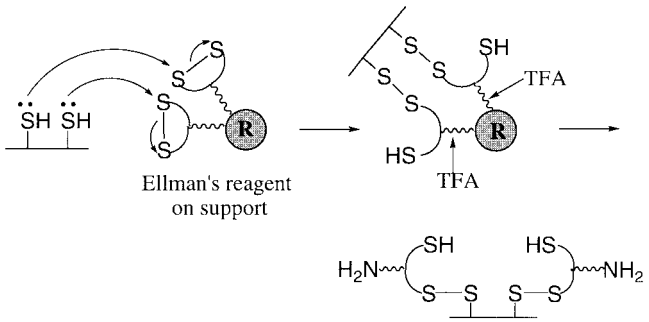
	Lys-PEG-PS		Lys-Sephadex G-15		Lys-CPG	
substrate	$t_{1/2}^{app}$ (h)	yield (%)	$t_{1/2}^{app}$ (h)	yield (%)	$t_{1/2}^{app}$ (h)	yield (%)
somatostatin	5.5	70	18	50	7	11
conotoxin SH 2&7	2.5	80	3.5	76	3.5	57
conotoxin SH 3&7	5.5	70	13	45	15	15
conotoxin SH 3&13	4.3	86	11	63	7.5	30

[a] Reaction conditions and definitions for these studies are the same as in Table 1. The loadings for Lys-PEG-PS and Lys-Sephadex G-15 were 0.2 mmol/g, and the excess of oxidizing reagent was 15-fold. In the case of Lys-CPG, the loading was 0.03 mmol/g, and a 2-fold excess of oxidizing reagent was used (rates were faster with greater excesses, consistent with observations on PEG-PS reported in Table 4). For each yield reported in this table, the amount of resin-bound byproduct was determined separately and the two values, that is, yield + resin-bound, were found to add to nearly 100%. For the CPG experiments, because the amount of material bound to the resin was so high, $t_{1/2}^{app}$ is 4- to 6-fold longer than $t_{1/2}$ (both of these terms are defined in Figure 1); the values for $t_{1/2}$ were 1.6, 0.8, 2.5, and 1.6 h for somatostatin, conotoxin SH 2&7, conotoxin SH 3&7, and conotoxin SH 3&13, respectively.

(defined in Scheme 1) is the *heterogeneous* reaction of a soluble peptide with a solid phase that contains DTNB functions. This point of view assumes that the most accessible, and hence most reactive, sites on the polymeric support are occupied first, in the course of preparing solid-phase reagents of lower loading, and that the further sites introduced to achieve higher loading contribute less to the overall kinetics.

Our findings with PEG-PS extended qualitatively to Sephadex and CPG, although there were meaningful quantitative differences (Table 6). With Sephadex, under otherwise comparable conditions (pH, loading, and substrate), rates were 1.5- to 4-fold slower, and overall yields were lower compared with those of PEG-PS. Thus, there is no advantage to using Sephadex when working with the kinds of peptide substrates that were the topic of the present studies, but it is still conceivable that our approach may be of value for Sephadex in conjunction with protein substrates and/or more aqueous media (the current work uses mixed aqueous−polar organic milieus). With CPG, we noted that reactions were 1−2 orders of magnitude faster than those with the other supports examined, and hence the excesses of El-Lys(El)-CPG disulfide needed to achieve complete conversion were as low as 2-fold. However, even with such a low excess of DTNB functions, we found that surprisingly little of the peptide substrate was oxidized to the disulfide; instead, much of the material was adsorbed covalently onto the CPG support. This observation was exploited in the mechanistic experiment described next; it is disappointing with regard to the prospects for CPG being a useful support with our method. The results with CPG indicate that despite the very low loadings on this material, the density of functional groups on the active surfaces is exceedingly high, ~12 Å apart on a square lattice. By way of comparison, functional groups in swollen PEG-PS or Sephadex are distributed in three dimensions and the separation between sites can be estimated as ~30−40 Å. The comparison among supports also suggests that reaction rates are affected by diffusion into microporous materials, for example, PEG-PS and Sephadex, whereas diffusion to functional sites on CPG is rapid and does not limit the rates.

Scheme 6. Proposed Mechanism of Formation and Analytical Proof of Covalently Bound Byproduct Leading to Lower Overall Yield during Oxidation with Solid-Phase Ellman's Reagents[a]



[a] The shorthand for the Ellman's reagent is self-explanatory in context, and the wavy line represents Lys-PAL [after TFA treatments, the PAL handle is cleaved to provide the derivatized lysine *C*-terminal amides]. This Scheme does not intend to imply that the two thiol−disulfide exchange steps depicted with the first set of structures are concerted. Further explanation in text.

Intermolecular Side Reaction to Application of Solid-Phase Ellman's Reagents: Formation of Covalently Bound Byproducts. A yield-diminishing side reaction, involving covalent retention of peptide on the solid-phase Ellman's supports, has been mentioned and documented throughout this paper. Adsorption to the support clearly involved one or more disulfide bonds, since (a) the bound peptide was released upon reduction with dithiothreitol, and (b) negligible physical adsorption was observed with control peptides of the same sequence but with the cysteine residues blocked or replaced. To elucidate further the chemical structures involved and develop a corresponding mechanistic understanding, we designed an experiment where conditions were chosen to maximize the level of the side reaction (Scheme 6). When reduced somatostatin was treated with El-Lys(El)-PAL-CPG disulfide (0.05 mmol/g; 5 equiv) at pH 2.7, ~95% of the peptide became bound to the support. Cleavage of the PAL handle with Reagent B[29] released into solution a peptide−reagent conjugate which was analyzed by MALDI mass spectrometry and shown to comprise two molecules of Ellman's spacer bound through two disulfide bonds to one molecule of peptide. Formation of this species is consistent with a mechanism for the side reaction in which the second peptidyl-thiol group attacks a second Ellman's reagent site on the solid phase (Scheme 6), rather than the desired intramolecular attack to displace Ellman thiolate from the solid-phase activated intermediate (Scheme 1). The competition between intra- and intermolecular processes correlates with those cases where yields are lower due to peptide conformation, and also explains earlier reported loading and support effects. We also rule out the possibility that this same activated intermediate does not undergo complete reaction, because in such a case we would expect to detect a conjugate comprised of one molecule

Table 7. Yields and Regiochemistry in Oxidation of Tetrathiol Substrates by Solid-Phase Ellman's Reagent[a]

substrate	pH	$t_{1/2}^{app}$ (min)	t_{end} (min)	resin-bound (%)	natural isomer (%)	isomer 1 (%)	isomer 2 (%)
conotoxin SI	6.6	3	20	10	81	5	4
	2.7	54	180	21	63	5	11
apamin	6.6	5	30	8	76	4	12
	2.7	120	600	4	70	1	25
	2.7(Gd·HCl)	160	900	20	28	19	33

[a] Reaction conditions and definitions for these studies are the same as in Tables 1 and 2. In the case of conotoxin SI, mispaired isomer 1 has disulfide bridges connecting Cys 2&3 and 7&13; mispaired isomer 2 has disulfide bridges connecting Cys 2&13 and 3&7. In the case of apamin, the two mispaired regioisomers have not been assigned; they are listed in order of retention upon HPLC. The oxidation reported on the bottom line of the table was carried out in the presence of 8 M guanidinium hydrochloride.

of Ellman's spacer bound through a single disulfide bond to one molecule of peptide.

As a practical matter, the side reaction just discussed does not affect the purity of the product from oxidation with solid-phase Ellman's reagents. Moreover, the covalently bound peptide is readily released from the support in reduced form by treatment with dithiothreitol, and can be recycled through the oxidation procedure. Regeneration and recycling of the polymeric reagent can also be done, as described in a previous section.

Regioselectivity in the Use of Solid-Phase Ellman's Reagents to Oxidize Tetrathiol Substrates. When our method was applied to the linear precursors of conotoxin SI or apamin, the naturally occurring regioisomers were the major products, formed in 63−81% absolute yields (Table 7). A further 16−26% (for apamin) and 9−16% (for conotoxin SI) of mispaired isomers[36] were observed, with relatively little material covalently adsorbed. Improved selectivities were observed at higher pH values. For both of these peptides, control oxidations carried out in solution at the same pH, in the presence of 1−20% (v/v) DMSO (dimethyl sulfoxide),[37] required considerably more time to reach completion and gave substantially higher levels of mispaired regioisomers.

A hint that dynamic disulfide exchange equilibria are involved during solid-phase oxidation may be found by comparing the overall rates, at pH 2.7, in the conotoxin SI system. Thus, formation of *both* disulfide bridges in the natural regioisomer actually occurs 3- to 6-fold faster than oxidation of the single disulfide bridge in models of the same sequence (Table 1). Another instructive result, in the apamin system, compares oxidation at pH 2.7 in the absence and in the presence of the denaturant guanidinium hydrochloride (Table 7). When the peptide is unfolded during disulfide formation, the distribution of regioisomers is relatively statistical, rather than conformationally driven to favor the naturally occurring disulfide array.

Dimerization. A complete accounting of the course of reaction of reduced oxytocin or deamino-oxytocin with the solid-phase Ellman's reagent (Table 8) revealed that 3−19% of the peptide was converted to dimers (mixture of both parallel and antiparallel). The amounts of dimers formed were less at lower pH values and under more dilute conditions [compare results at 0.5 mg/mL of peptide substrate with those at 1−2 mg/mL concentration; n.b., these concentrations are about an order of magnitude higher than what is typically used for air oxidation]. Dimerization in the oxytocin family might be correlated to their flexible conformation, which readily forms disulfides. Even after the desired monomeric product is obtained, it may undergo further equilibration in solution. The present results show that the pseudodilution advantage of our solid-phase approach is not absolute. However, it should be pointed out that other than oxytocin and deamino-oxytocin, none of the peptides examined in our studies gave evidence for dimer formation upon oxidation with solid-phase Ellman's reagents.

Table 8. Distribution of Soluble Products Formed during Oxidation of Oxytocin and Deamino-oxytocin with Solid-Phase Ellman's Reagent[a]

substrate	pH	concn (mg/mL)	time (min)	soluble reduced (%)	yield (%)	parallel dimer (%)	antiparallel dimer (%)
oxytocin	2.7	0.5	15	14	69	1	1
			60	1	81	1	2
	2.7	1.0	15	17	58	3	7
			90	2	72	4	7
			330	0	72	5	9
	2.7	2.0	15	11	63	4	7
			60	2	65	6	12
	3.4	1.0	5	7	71	5	5
			45	2	67	10	9
	6.6	1.0	1	0	77	8	7
deamino-oxytocin	2.7	1.0	15	22	42	4	2
			45	4	62	2	2
	3.4	1.0	5	13	62	2	2
			45	1	69	6	3
	6.6	1.0	3	1	73	4	10

[a] Reaction conditions and definitions for these studies are the same as in Tables 1 and 4. The excess of El-Lys(El)-PEG-PS disulfide reagent was 15-fold in all cases. The heterogeneous reaction mixture was sampled at various points in time (not all data shown) for HPLC analysis. Resin-bound material, determined separately, ranged from 8 to 30% and accounts for the remaining starting reduced substrate (values in table, plus resin-bound, add up to 100%).

Summary and Conclusions

The solid-phase reagents presented here offer an attractive route to the formation of disulfide bridges in a variety of peptide substrates. The methodology may be particularly beneficial for substrates that are challenging to oxidize for reasons of conformational restrictions, limited solubility at pH values above neutral, and/or the presence of labile amino acid residues. The procedure is easy to carry out—particularly with regard to the facile isolation of the desired disulfide products by simple filtration; it is selective over a wide pH range; and it allows recycling and reuse of both the solid-phase reagent and of any linear substrate that becomes covalently bound to the support. In addition, studies related to the generation and the application of the reagents on a variety of supports with differing morphologies have provided qualitative insights into the pseudodilution phenomenon that governs the relative levels of intra- and intermolecular processes in solid-phase systems.

Experimental Section

General. Most of the materials, solvents, instrumentation, and general methods have been described and summarized in our previous publications,[6,11a,b,d,28,29,32c,35] and are described further in the Supporting Information. Fmoc-PAL-PEG-PS and HCl·H_2N-PEG-PS supports (initial loading 0.14−0.4 mmol/g) were from the Biosearch Division

(36) In the case of conotoxin, authentic standards of unambiguously synthesized mispaired regioisomers were available; see: Hargittai, B.; Barany, G. In *Peptides−Chemistry, Structure and Biology: Proceedings of the Fifteenth American Peptide Symposium*; Tam, J. P., Kaumaya, P. T. P., Eds.; Kluwer: Dordercht, The Netherlands, 1998, in press.

(37) The DMSO oxidation procedure was developed by Tam, J. P.; Wu, C.-R.; Liu, W.; Zhang, J.-W. *J. Am. Chem. Soc.* **1991**, *113*, 6657−6662. For applications to the conotoxin system, see ref 11b and Experimental Section of this paper.

of PerSeptive Biosystems (Framingham, MA), Sephadex G-15 was from Aldrich (Milwaukee, WI), and controlled pore glass (50−100 μmol/g, 1000 Å pore size, 35 m^2/g surface area) was from Solid Phase Sciences (San Rafael, CA). Peptide chain assembly was carried out either manually or on a PerSeptive (formerly MilliGen/Biosearch) model 9050 continuous-flow synthesizer, as detailed in the Supporting Information. Completed peptide−resins were treated with piperidine−DMF (1:4) to remove the terminal Fmoc group, washed with CH_2Cl_2, and then cleaved with appropriate acid/scavenger cocktails (1 mL/100 mg of resin) for 1.5−4.5 h at 25 °C. The cleavage mixtures were then filtered and washed with the same cocktails, and the combined filtrates were concentrated under a stream of N_2. Next, cold Et_2O was added, and the resultant precipitated peptides were collected by low-speed centrifugation and washed with cold Et_2O. The peptides were dissolved in 1% aqueous HOAc for analytical HPLC and related evaluations, and then lyophilized for storage as powders at 4 °C.

Loadings of PEG-PS, Sephadex, and CPG supports were calculated, after BOP/NMM/HOBt-mediated coupling of Fmoc-Gly-OH, by quantitative spectrophotometric monitoring[30c] following Fmoc deblocking with piperidine−DMF (1:4, 2 + 2 + 8 min), and by amino acid analysis, and both methods agreed closely. (In the case of Sephadex, the process started with a resin suspension, and the weight was determined after completion of Fmoc deprotection by appropriate washing and drying in vacuo.) UV measurements were at 301 nm (ϵ = 7800 M^{-1} cm^{-1} for the piperidine−fulvene adduct). Analytical HPLC was performed using a Vydac analytical C-18 reversed-phase column (218TP54; 5 μm, 300 Å; 0.46 × 25 cm), and linear gradients of 0.1% aqueous TFA and 0.1% TFA−CH_3CN were run at 1.2 mL/min. When appropriate, crude peptide products, both reduced and oxidized, were purified by preparative HPLC using a Vydac semi-preparative C-18 reversed-phase column (218TP1010; 10 μm, 300 Å; 1.0 × 25 cm) and elution of aqueous TFA−CH_3CN gradients at 5 mL/min. 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.

For oxidation studies (Tables 1−8), the crude lyophilized powders were redissolved in one of the following buffer milieus: (a) 1% aqueous HOAc at pH 2.7; (b) pH 3.4 buffer, obtained by adding aqueous HCl (0.1 N, 55.2 mL) to disodium citrate solution (44.8 mL, in turn prepared from 0.1 M aqueous citric acid (100 mL) and 1 N aqueous NaOH (20 mL)); (c) pH 4.0 buffer, obtained by adding aqueous HCl (0.1 N, 44.9 mL) to aqueous disodium citrate (55.1 mL, prepared as described above); (d) pH 5.0 buffer, prepared by mixing aqueous potassium hydrogen phthalate (0.1 N, 50 mL), NaOH (0.1 N, 22.6 mL), and H_2O (27.4 mL); (e) pH 6.6, 7.3, 7.5, or 8.0 buffers, prepared by titrating an aqueous solution of Na_2HPO_4 (0.1 M) with concentrated H_3PO_4 (drops) while monitoring with a pH meter.

2-Nitro-5-thiobenzoic Acid [TNB or El(H)] (2). The Ellman's reagent 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, or El_2) (**1**) (1.0 g, 2.5 mmol) was suspended in distilled, degassed H_2O (25 mL), and *N*,*N*-dimethylethanolamine (1.0 mL, 7.7 mmol) was added to form a homogeneous solution. A solution of β-mercaptoethylamine hydrochloride (1.2 g, 10.4 mmol) in H_2O (25 mL) was added next, and the solution immediately became red. After 1 h of stirring at 25 °C under N_2, completion of the reaction was verified by TLC. The reaction mixture was acidified with 1 N aqueous HCl (47 mL), and the product was extracted rapidly with EtOAc (3 × 60 mL). The organic phase was dried (Na_2SO_4) and concentrated in vacuo to provide orange crystals, which were stored in a desiccator at <5 mmHg and used within 3 days of preparation. Yield 1.0 g (quantitative); mp 136−137 °C (lit.[13b] mp 137−137.5 °C). TLC, ^1H and ^{13}C NMR results are in the Supporting Information.

***tert*-Butyl *N*-(2-Bromoethyl)carbamate (4).** A solution of 2-bromoethylamine hydrobromide (**3**) (4.7 g, 22.9 mmol) in saturated aqueous Na_2CO_3 (30 mL) was extracted with a solution of di-*tert*-butyl dicarbonate (Boc_2O, 3.2 g, 14.7 mmol) in Et_2O (40 mL), followed by further extractions with Et_2O (3 × 30 mL). The combined organic phases were dried (Na_2SO_4), partially concentrated in vacuo to ∼40 mL, and stirred for 24 h at 25 °C. The product **4** was then separated from excess unreacted free amine by silica gel chromatography, eluting with EtOAc; upon in vacuo removal of solvent, **4** was a clear, viscous liquid suitable for further use (white crystals formed after several days' refrigeration). Yield 2.62 g (80%); mp 33−35 °C (lit.[22] mp 33−35 °C). TLC, ^1H NMR, and FABMS results are in the Supporting Information.

2-Nitro-5-*S*-(9*H*-xanthene-9-yl)thiobenzoic Acid [El(Xan)] (5). A solution of 2-nitro-5-thiobenzoic acid (**2**) (1.5 g, 7.5 mmol) in anhydrous CH_2Cl_2 (75 mL) was treated with TFA (0.58 mL, 7.5 mmol) and stirred for 5 min. Next, 9*H*-xanthen-9-ol (1.5 g, 7.5 mmol) was added in small portions over a 10 min period, and the homogeneous yellow reaction mixture was stirred for 2 h at 25 °C under N_2 (to prevent competing reoxidation of **2** to **1**). The reaction was quenched by addition of hexane (300 mL), whereupon off-white needles formed; these were collected after standing overnight at −20 °C. Yield 2.55 g (90%). Recrystallization was carried out by dissolving the product in a minimum amount of hot CH_2Cl_2, cooling the solution to 25 °C, adding an excess of hexane, and cooling the mixture to −20 °C. Fluffy light yellow crystals; mp 172 °C (dec). TLC, ^1H and ^{13}C NMR, FABMS, and EA results are in the Supporting Information.

2-Nitro-5-*S*-(2,4,6-trimethoxybenzyl)thiobenzoic Acid [El(Tmob)] (6). A solution of 2-nitro-5-thiobenzoic acid (**2**) (0.9 g, 4.5 mmol) in anhydrous CH_2Cl_2 (42 mL) was treated with TFA (0.35 mL, 4.5 mmol), following which a solution of 2,4,6-trimethoxybenzyl alcohol[24] (0.9 g, 4.5 mmol) in anhydrous CH_2Cl_2 (45 mL) was added dropwise over a 5 min period. The reaction was stirred under N_2; precipitation of the yellow product started within 1 h. After 5 h total reaction, TLC monitoring indicated complete reaction, and the precipitate was collected and washed with hot CH_2Cl_2 (30 mL). Yield 1.03 g (60%); dark yellow amorphous crystals; mp 182−184 °C. TLC, ^1H and ^{13}C NMR, FABMS, and EA results are in the Supporting Information.

2-Nitro-5-*S*-(triphenylmethyl)thiobenzoic Acid [El(Trt)] (7). On a 4.5 mmol scale, the same procedure as for **5** was followed, but using triphenylmethanol (1.2 g, 4.5 mmol). Yield 1.77 g (89%); flaky light yellow plates; mp 165 °C. TLC, ^1H and ^{13}C NMR, FABMS, and EA results are in the Supporting Information.

5,5′-Dithiobis(2-nitrobenzoic Acid *N*-Hydroxysuccinimide Ester) [DTNB($OSu)_2$] (8). *N*-Hydroxysuccinimide (0.58 g, 5.1 mmol) was added to a solution of DTNB (**1**) (1.0 g, 2.5 mmol) in CH_2Cl_2−DMF (10:1, 55 mL). The solution was chilled to 4 °C, and *N*,*N*′-dicyclohexylcarbodiimide (DCC) (1.05 g, 5.1 mmol) was added. The reaction mixture became cloudy immediately, and was stirred overnight at 4 °C. TLC [EtOAc−CH_3OH (7:3)] showed the disappearance of **1**, R_f 0.28, and formation of the product **8**, R_f 0.9. The reaction mixture was filtered twice, once with suction and once with gravity, to remove the white precipitate, *N*,*N*′-dicyclohexylurea (DCU). The filtrate was concentrated in vacuo at <40 °C, hence removing the CH_2Cl_2 but not all of the DMF. The resulting thick, yellow liquid was diluted with dry EtOAc (40 mL), filtered to remove the remaining urea, and concentrated again to remove EtOAc. The concentrate was diluted to the needed volume with dioxane, and used for the subsequent reaction later on the same day, without purification.

***N*$^{\alpha}$,*N*$^{\epsilon}$-Bis(5-thio-2-nitrobenzoyl)-L-lysine Disulfide [Ellman's Reagent−Lysine Adduct] (9).** The bis(*N*-hydroxysuccinimide ester) **8** derived from **1** (2.5 mmol), prepared as described above, was dissolved in dioxane (150 mL), and added dropwise, over a period of 4−5 h (1 drop/4−5 s) with *vigorous* stirring to a solution of L-lysine hydrochloride (0.46 g, 2.5 mmol) in 5% aqueous $NaHCO_3$ (160 mL). Almost at once, the reaction mixture became cloudy, and took on an increasingly dark yellow coloration. At a later stage of the addition, the reaction mixture was orange, and an orange precipitate had started to form. The reaction mixture was stirred overnight at 25 °C, and the precipitated product (first crop) was collected the next day. A second crop was collected after partial concentration of the filtrate to remove dioxane. Yield 0.65 g (51%, combined two crops); mp 270 °C, with significant darkening and possible decomposition occurring at ∼120 °C; HPLC, gradient, started after 3 min and conducted over 40 min, of 0.1% aqueous TFA and 0.1% TFA−CH_3CN from 1:0 to 1:1, t_R = 31.1 min, purity ∼87%. TLC, ^1H NMR, FABMS, and EA results are in the Supporting Information.

Amination of Sephadex G-15 (Scheme 2). The procedure which follows is typical; reaction conditions may need to be adjusted

somewhat to achieve target loadings with different batches of support. Sephadex G-15 (0.5 g, powder) was weighed into a 6-mL plastic syringe fitted with a polypropylene frit. The Sephadex was activated by washing with 2 N aqueous NaOH (3 mL, applied 4 × 2 min) and rinsed with deionized H_2O (∼10 mL) until the pH of the filtrate was neutral. The activated Sephadex was next washed with DMF (4 × 1 min) and drained, and a mixture of THF−DMF (1:1, 0.5 mL) was added. Freshly ground solid KOH (13 mg, 0.23 mmol) was then added, with aid of further THF−DMF (1:1, 0.5 mL), and the suspension was stirred gently. Amination was commenced by the addition of a solution of *tert*-butyl *N*-(2-bromoethyl)carbamate (**4**) (45 mg, 0.2 mmol) in THF−DMF (1:1, 1 mL) and was carried out with gentle stirring for 2 h at 25 °C. Subsequently, the liquid was drained, and the Sephadex was washed thoroughly with DMF (∼10 mL) and H_2O (∼15 mL) until the pH of the filtrate was neutral. The Boc-protected Sephadex G-15 was stored at 4 °C as a suspension in DMF or H_2O. Just before use, the Boc group was removed by treatment with TFA−CH_2Cl_2 (1:3, 2 mL, 3 × 5 min), at 25 °C, followed by washing with CH_2Cl_2 (3 mL, 2 × 1 min) and DMF (2 mL, 2 × 1 min). Loading, determined as described in "General", was 0.20 mmol/g of Sephadex, with 50 mg of Sephadex corresponding to a settled bed volume of 0.3 mL. The same experiment, but using a larger amount of **4** (90 mg, 0.4 mmol) and a reaction time of 3 h gave a loading of 0.44 mmol/g; use of **4** (225 mg, 1.0 mmol) and a 20 h reaction time gave a loading of 1.0 mmol/g.

Preparation of Solid-Phase Ellman's Reagents: Model Studies (Schemes 4 and 5). The results of these exploratory experiments suggested optimized procedures, which are presented subsequently, to prepare the desired solid-phase Ellman's reagents on larger scales. Hence, specific details that overlap the preparative procedures are described in the present section only in outline form.

(**A**) Starting with Fmoc-PAL-PEG-PS (0.4 g, 0.45 mmol/g), manual DIPCDI/HOBt coupling protocols were used to add in turn Fmoc-Gly-OH, Fmoc-β-Ala-OH, Fmoc-Gly-OH, and El(Xan) (**5**) to create the resin El(Xan)-Gly-β-Ala-Gly-PAL-PEG-PS. This resin was divided into 50 mg portions [23 μmol of El(Xan) per experiment], and each portion was treated with TFA−CH_2Cl_2−Et_3SiH (3:94:3, 2 × 1 h) to remove the *S*-Xan group. Oxidation was carried out for 20 h at 25 °C with each of (a) iodine (58 mg, 0.23 mmol, 10 equiv) in DMF (0.6 mL), (b) potassium ferricyanide (76 mg, 0.23 mmol, 10 equiv) in DMF−H_2O (1:1, 0.46 mL) in the dark, and (c) Et_3N (6 μL, 43 μmol) in *N*-methylpyrrolidone (NMP) (1 mL). Subsequently, the treated polymeric reagent was washed several times each with the original reaction milieu minus reagent, followed by DMF and CH_2Cl_2, and then Reagent B, TFA−PhOH−H_2O−Et_3SiH (88:5:5:2, 1 mL), was added for 1.5 h to achieve cleavage. The cleavage mixtures were worked up as described under "General" and subjected to HPLC analysis using a gradient, started after 3 min and conducted over 40 min, of 0.1% aqueous TFA and 0.1% TFA−CH_3CN from 1:0 to 1:1. In general, two peaks were observed: t_{R1} = 12.1 min assigned to El(H)-Gly-β-Ala-Gly-NH_2 (monomeric thiol) and t_{R2} = 19.0 min assigned to (El-Gly-β-Ala-Gly-NH_2)$_2$ (homodimeric disulfide). Consistent with these assignments, the t_{R1} = 12.1 min peak disappeared while the t_{R2} = 19.0 min peak increased upon overnight stirring in pH 8 buffer; alternatively, when DTT (∼50 mM final concentration) was added to the same solution at pH 8, the t_{R2} = 19.0 min peak was reduced completely while the t_{R1} = 12.1 min peak increased. For the three oxidation conditions cited, the ratios of thiol:disulfide were (a) 1:9, (b) 1:6, and (c) 1:4. In other experiments designed to determine why oxidation had not gone to completion, the polymeric reagent (50 mg) was alkylated, after the oxidation step, with iodoacetamide (42 mg, 0.23 mmol) in the presence of NMM (25 μL, 0.23 mmol) in DMF (0.5 mL) for 5 h. Cleavage and workup gave again the t_{R2} = 19.0 min peak upon HPLC analysis, as well as a t_{R3} = 10.1 min peak assigned to El(CH_2CONH_2)-Gly-β-Ala-Gly-NH_2, in relative amounts consistent with the earlier analyses.

(**B**) Starting with Fmoc-PAL-PEG-PS (0.4 g, 0.38 mmol/g), an H-Gly-β-Ala-Gly-PAL-PEG-PS resin was assembled as described in **A**. One portion of this resin (0.15 g, 56 μmol) was extended by coupling DTNB (23 mg, 56 μmol) using DIPCDI (9 μL, 56 μmol) and HOBt (8 mg, 56 μmol) in DMF (0.3 mL) overnight; a second portion (0.25 g, 96 μmol) was extended by coupling DTNB (153 mg, 0.39 mmol) using DIPCDI (60 μL, 0.39 mmol) and HOBt (52 mg, 0.39 mmol) in DMF (0.8 mL), also overnight. Aliquots (40 mg) from both of these polymeric reagents were cleaved with Reagent B and evaluated as described in **A**. The first case (use of 1 equiv DTNB) showed a 1:2 ratio of El-El-Gly-β-Ala-Gly-NH_2 (corresponding to a single site of attachment, t_{R4} = 25.0 min) to (El-Gly-β-Ala-Gly-NH_2)$_2$ (homodimeric disulfide corresponding to two sites of attachment, t_{R2} = 19.0 min); in the second case (use of 4 equiv DTNB), the ratio was 1:1.2. A further aliquot (150 mg) of the resin from the second case was treated with DTT (180 mg, 1.16 mmol, 20 equiv) in NMM−DMF (1:9, 1.5 mL) at 25 °C for 1 h. This treatment gave El(H)-Gly-β-Ala-Gly-PAL-PEG-PS with properties (including an HPLC-homogeneous monomeric thiol derivative upon cleavage) intersecting with the resin formed after *S*-Xan removal described in **A**. Furthermore, this reduced resin was reoxidized with potassium ferricyanide by the already-described procedure, again giving results overlapping with the previous.

(**C**) Starting with Fmoc-PAL-PEG-PS (0.4 g, 0.2 mmol/g), manual DIPCDI/HOBt coupling protocols were used to add in turn Fmoc-Asp(O*t*Bu)-OH, Fmoc-Asp(O*t*Bu)-OH, Fmoc-Lys(Fmoc)-OH, and El(Xan) (**5**), to create the resin El(Xan)-Lys(El(Xan))-Asp(O*t*Bu)-Asp(O*t*Bu)-PAL-PEG-PS. Further transformations and analyses were as described in **A**; only (a) iodine and (b) potassium ferricyanide were tested for oxidation (same number of equivalents with respect to each El residue in the model). HPLC analysis, under the same conditions as in **A**, showed a single peak, t_R = 24.7 min, assigned to the disulfide of El-Lys(El)-Asp-Asp-NH_2 (confirmed by ESMS (*m*/*z*): calcd for $C_{28}H_{30}N_7O_{13}S_2$, 736.71; found, 738.0 $[MH]^+$). The corresponding reduced model peptide had t_R = 25.0 min, known from a separate DTT reduction experiment; importantly, none of the reduced peptide was observed in the described preparation of lysine-containing solid-phase Ellman's reagent, thus establishing that the oxidation had reached completion. As further confirmation of the described oxidation route, and proof of the structure of the resin-bound species, the t_R = 24.7 min peak was the only one noted (confirmed by co-injection) when Reagent B cleavage followed by HPLC analysis was performed on the derivatized peptide-resin obtained by an alternative route, the coupling of compound **9** (4 equiv) to H-Asp(O*t*Bu)-Asp(O*t*Bu)-PAL-PEG-PS by a DIPCDI/HOBt protocol.

Solid-Phase Ellman's Reagent: El-Lys(El)-PEG-PS Disulfide. The procedure which follows is representative; similar ones were used with other loadings of starting support and with other TNB precursors (i.e., **6** or **7** in place of **5**). HCl·H_2N-PEG-PS (1.0 g, 0.2 mmol/g) was weighed into a 36-mL plastic syringe fitted with a polypropylene frit. All wash volumes were 10 mL, and wash times were 1 min. The resin was swelled by washing with CH_2Cl_2 (3 min), rinsed with DMF, treated with piperidine−DMF (1:4, 10 mL, 2 × 1 min) to neutralize the hydrochloride salt, and washed again with DMF (3×). Fmoc-Lys(Fmoc)-OH (473 mg, 0.8 mmol, 4 equiv) and HOBt (108 mg, 0.8 mmol, 4 equiv) were combined and dissolved in DMF (3 mL), DIPCDI (125 μL, 0.8 mmol, 4 equiv) was added, and this mixture was added immediately to the resin. The coupling was allowed to proceed at 25 °C for 1 h, at which time the resin was negative to the Kaiser Ninhydrin test.[38] The resultant Fmoc-Lys(Fmoc)-PEG-PS was washed with DMF (3×), and treated with piperidine−DMF (1:4, 10 mL, 3 × 6 min) to remove the Fmoc groups. Next, El(Xan) (**5**) (608 mg, 1.6 mmol, 4 equiv with respect to free NH_2) and HOBt (216 mg, 1.6 mmol, 4 equiv) were combined and dissolved in DMF (4 mL), DIPCDI (250 μL, 1.6 mmol, 4 equiv) was added, and this mixture was added immediately to the H-Lys(H)-PEG-PS resin. The coupling was allowed to proceed at 25 °C for 1 h, at which time the resin was negative to the Kaiser Ninhydrin test. After washes with DMF (3×) and CH_2Cl_2 (2×), the *S*-Xan group was removed with TFA−CH_2Cl_2−Et_3SiH (25:75:3, 10 mL, 3 × 5 min), followed by further washes with CH_2Cl_2 (2×) and DMF (3×). On-resin oxidation was accomplished by treatment with potassium ferricyanide (1.3 g, 4 mmol) in DMF−H_2O (1:1, 8.0 mL) for 20 h at 25 °C in the dark. The polymeric reagent was then washed thoroughly with H_2O (3×), DMF (2×), again H_2O (2×), DMF (2×),

(38) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. *Anal. Biochem.* **1970**, *34*, 595−598.

and Et_2O (1×), following which it was dried in vacuo for 30 min and stored at 4 °C.

Solid-Phase Ellman's Reagent: El-Lys(El)-Sephadex G-15 Disulfide. Procedures were carried out in a 12-mL plastic syringe fitted with a polypropylene frit; wash volumes were 5 mL, and wash times were 1 min. Aminated Sephadex (0.5 g, 0.2 mmol/g) that had been freshly deprotected as described earlier (present as trifluoroacetate salt) was washed with DMF (3×). Fmoc-Lys(Fmoc)-OH (237 mg, 0.4 mmol, 4 equiv), BOP (177 mg, 0.4 mmol, 4 equiv), and HOBt (54 mg, 0.4 mmol, 4 equiv) were combined and dissolved in DMF (3 mL), NMM (90 μL, 0.8 mmol, 8 equiv) was added, and the mixture was added immediately to the solid support. The coupling was allowed to proceed for 1.5 h, at which time the resin was negative to the Kaiser Ninhydrin test. The resultant Fmoc-Lys(Fmoc)-Sephadex was washed with DMF (3×) and treated with piperidine−DMF (1:4, 5 mL, 3 × 6 min) to remove the Fmoc groups. Next, El(Xan) (**5**) (304 mg, 0.8 mmol, 4 equiv with respect to free NH_2), BOP (354 mg, 0.8 mmol, 4 equiv), and HOBt (108 mg, 0.8 mmol, 4 equiv) were combined and dissolved in DMF (4 mL), NMM (180 μL, 1.6 mmol, 8 equiv) was added to the solution, followed by immediate addition of the mixture to the H-Lys(H)-Sephadex. The coupling was allowed to proceed at 25 °C for 1 h, at which time the resin was negative to the Kaiser Ninhydrin test. After washes with DMF (3×) and CH_2Cl_2 (1×), the *S*-Xan group was removed with TFA−CH_2Cl_2−Et_3SiH (25:75:3, 5 mL, 3 × 5 min), followed by further washes with CH_2Cl_2 (2×) and DMF (3×). On-resin oxidation was accomplished with iodine (0.5 g, 2 mmol, 10 equiv based on each El residue) in DMF (6 mL) for 20 h at 25 °C. The polymeric reagent was washed thoroughly with DMF (2×), $CHCl_3$ (2×), and again DMF (2×) and stored in DMF at 4 °C.

Solid-Phase Ellman's Reagent: El-Lys(El)-CPG Disulfide. Wash volumes were 6 mL, and wash times were 1 min. CPG (500 mg, loading 0.05 mmol/g) was weighed into a 12-mL plastic syringe fitted with a polypropylene frit and washed with DMF. Fmoc-Lys(Fmoc)-OH (74 mg, 0.125 mmol, 5 equiv), BOP (56 mg, 0.125 mmol, 5 equiv), and HOBt (17 mg, 0.125 mmol, 5 equiv) were combined and dissolved in DMF (1.2 mL), NMM (28 μL, 0.250 mmol, 10 equiv) was added, and the mixture was added immediately to the CPG. The coupling was allowed to proceed for 18 h. The resultant Fmoc-Lys(Fmoc)-CPG was washed with DMF (3×) and treated with piperidine−DMF (1:4, 6 mL, 2 + 2 + 8 min) to remove the Fmoc group. Quantitative UV measurement of the fulvene−piperidine adduct revealed a loading of 0.03 mmol of Lys/g of CPG. Next, El(Xan) (**5**) (46 mg, 0.12 mmol, 4 equiv with respect to free NH_2), BOP (53 mg, 0.12 mmol, 4 equiv), and HOBt (17 mg, 0.12 mmol, 4 equiv) were combined and dissolved in DMF (1.2 mL), NMM (27 μL, 0.24 mmol, 8 equiv) was added to the solution, followed by immediate addition of the mixture to the H-Lys(H)-CPG. The coupling was allowed to proceed at 25 °C for 1.5 h. After washes with DMF (3×) and CH_2Cl_2 (1×), the *S*-Xan group was removed with TFA−CH_2Cl_2−Et_3SiH (25:75:3, 6 mL, 3 × 5 min), followed by further washes with CH_2Cl_2 (2×) and DMF (3×). On-resin oxidation was accomplished with iodine (77 mg, 0.3 mmol, 10 equiv based on each El residue) in DMF (3 mL) for 20 h at 25 °C. The polymeric reagent was washed thoroughly with DMF (4 x), CH_2Cl_2 (3×), and Et_2O (1×), following which it was dried in vacuo for 30 min and stored at 4 °C.

Oxidation of Peptide Substrates by Solid-Phase Ellman's Reagents. In a typical procedure, the reduced peptide (∼1 mg) was dissolved in buffer−CH_3CN−CH_3OH (2:1:1, 1 mL). An aliquot (50 μL) was taken for hydrolysis and amino acid analysis, and another aliquot (20 μL) was taken for HPLC analysis of the zero timepoint. Separately, the solid-phase reagent (e.g., ∼50 mg at 0.2 mmol/g corresponding to 15-fold excess based on DTNB functions) was weighed into a plastic syringe fitted with a polypropylene frit. When the parent support was PEG-PS, the resin was first swelled in CH_2Cl_2 (2 min), washed with DMF (2 × 1 min) and again with CH_2Cl_2 (1 min), and drained. In the cases of Sephadex and CPG, only DMF washes were used prior to draining. The syringe tip was plugged, using a small septum or a plastic lock cap, and the solution of peptide substrate was added to the syringe already containing the solid-phase Ellman's reagent. The reaction mixture was stirred magnetically or agitated on a rotary shaker, *gently*, at 25 °C. Monitoring was carried out by taking aliquots (20 μL) from the liquid phase for eventual HPLC analysis (done in real time for relatively slow reactions; for fast reactions at higher pH, aliquots were frozen in liquid N_2 and thawed just prior to HPLC injection). Upon completion of oxidation, as judged by disappearance of the reduced substrate peak in the HPLC trace, the liquid phase was drained into a vial, using positive air pressure, and aliquots were taken for hydrolysis/amino acid analysis and endpoint HPLC. The consumed solid-phase reagent was washed thoroughly with DMF, H_2O, and DMF again, rinsed with Et_2O, and dried in vacuo; a portion (∼10 mg) was then taken for hydrolysis/amino acid analysis to determine the extent of covalently bound material.

Regeneration of Solid-Phase Ellman's Reagents. The regeneration process was usually carried out by combining the recovered resins from several oxidations performed by the above procedure. The used solid-phase reagents (up to 0.5 g) were washed thoroughly with DMF, H_2O, and CH_2Cl_2, and dithiothreitol (∼20 equiv with respect to the original loading of DTNB functions; final concentration 0.8 M) dissolved in NMM−DMF (1:9) was added. Treatment for 2 h resulted in reduction of all on-resin disulfide bonds. Following washes with DMF and CH_2Cl_2, the resin-bound TNB functions were oxidized, using either iodine or $K_3Fe(CN)_6$ as appropriate for the parent solid support, in the same ways as previously described for the creation of resin-bound DTNB functions.

Controls to Rule Out Noncovalent Adsorption during Application of Solid-Phase Ellman's Reagents. The standard procedures and analyses as described were carried out using a variety of solid-phase Ellman's reagents (40 mg; Scheme 4 and Scheme 5 routes, on PEG-PS, Sephadex G-15, and/or CPG), together with the cysteine-lacking model peptide [Abu^3,Ala^{14}]-somatostatin (1 mg) dissolved in 1% aqueous HOAc−CH_3CN−CH_3OH (2:1:1, 1 mL). The amount of peptide in the liquid phase at different points in time was monitored by HPLC (gradient, conducted over 25 min, of 0.1% aqueous TFA and 0.1% TFA−CH_3CN from 4:1 to 1:1; t_R = 12.3 min) and was never more than 20% (typically 8−15%) reduced from the level before any resin had been added. Upon draining the resins under positive air pressure, typically 95−98% of the peptide was recovered. Hydrolysis/amino acid analysis on the washed and dried resin did not reveal any peptide content (<1%) in the solid phase.

Determination of Covalently Bound Byproduct Formed with Solid-Phase Ellman's Reagents (Scheme 6). Starting with CPG (70 mg, loading 0.09 mmol/g) and using procedures analogous to those reported in "Solid-Phase Ellman's Reagent: El-Lys(El)-CPG Disulfide", an El-Lys(El)-PAL-CPG disulfide support was assembled. BOP/HOBt/NMM (4:4:8) coupling protocols were used. The initial coupling of Fmoc-PAL-OH (16 mg, 0.032 mmol, 5 equiv) required 18 h; *S*-Xan removal was with TFA−CH_2Cl_2−Et_3SiH (3:94:3) for 2 × 1 h; and the final loading was determined experimentally to be 0.05 mmol/g. The solid-phase reagent was washed thoroughly with DMF (3 mL, 4 × 1 min), CH_2Cl_2 (3 mL, 3 × 1 min), drained, and a solution of reduced somatostatin (1.1 mg, 0.7 μmol) in 1% aqueous HOAc−CH_3CN−CH_3OH (2:1:1, 2 mL) was added. After 15 h of gentle shaking, HPLC (gradient, conducted over 25 min, of 0.1% aqueous TFA and 0.1% TFA−CH_3CN from 4:1 to 1:1) indicated that only ∼5% of the initial amount of peptide was present in solution (fully converted to the monomeric cyclic disulfide of somatostatin). Hydrolysis followed by amino acid analysis indicated that ∼95% of the peptide had became bound to the support. The reaction mixture was drained, and the solid support was washed thoroughly. Treatment with Reagent B, TFA−CH_2Cl_2−H_2O−PhOH−Et_3SiH (92:5:1:1:1, 3 mL), for 1.5 h cleaved the acid-labile handle PAL and provided a residue which was subjected to MALDI analysis: calcd for the structure outlined on the lower right side of Scheme 6, $C_{116}H_{148}N_{28}O_{33}S_6$, 2652.9; found, 2646.0 (within experimental error of the analytical procedure).

Somatostatin (Disulfide). The oxidations of the reduced linear somatostatin to monomeric somatostatin disulfide as mediated by solid-phase Ellman's reagents are covered in Figure 1 and Tables 1 and 3−6. In addition, linear somatostatin (8 mg, 4.9 μmol) treated with $(El)_2$-Sephadex G-15 disulfide (390 mg, 0.2 mmol/g) in pH 6.6 buffer−CH_3CN−CH_3OH (2:1:1, 8 mL) was oxidized completely in 90 min. Preparative HPLC (gradient, conducted over 40 min, of 0.1% aqueous TFA and 0.1% TFA−CH_3CN from 77:23 to 2:3) gave somatostatin

disulfide (2.4 mg, 1.5 μmol) in an overall purified yield of 30%. As a control, linear somatostatin (1 mg, 0.6 μmol) in 0.1 M phosphate buffer, pH 6.6 (1 mL), stirred open to air at 25 °C, was oxidized completely in 26 h. However, the absolute amount of disulfide, quantified by the analytical HPLC peak area (gradient, conducted over 25 min, of 0.1% aqueous TFA and 0.1% TFA−CH_3CN from 4:1 to 1:1; t_R = 12.6 min) corresponded to only 30% of the initial amount of precursor, t_R = 13.4 min. As another control, no oxidation occurred, and the starting substrate remained unchanged, after 2 days in 1% aqueous HOAc, pH 2.7.

One-Disulfide α-Conotoxin Analogues. Syntheses and characterizations of the linear precursors are described in the Supporting Information, and oxidations mediated by solid-phase Ellman's reagents are covered in Tables 1−6. In addition, a larger-scale experiment used conotoxin SH 3&7 (3.7 mg, 2.5 μmol). Treatment with El-Lys(El)-PEG-PS disulfide (190 mg, 0.2 mmol/g) in 1% aqeuous HOAc−CH_3CN−CH_3OH (2:1:1, 3.8 mL) showed complete oxidation in ∼24 h (crude yield of 78%), and preparative HPLC (gradient, conducted over 40 min, of 0.1% aqueous TFA and CH_3CN from 19:1 to 3:2) gave conotoxin SS 3&7 (1.4 mg, 0.9 μmol) in an overall purified yield of 37%. Control oxidations in solution used reduced peptide substrate (1 mg, 0.7 μmol) in buffer (1 mL), stirred at 25 °C, with the following results: (a) oxidation of conotoxin SH 2&7 was complete and quantitative in 10 h at pH 6.6, based on the analytical HPLC retention (t_R = 17.5 min for SH, t_R = 17.8 min for SS) and area (gradient, conducted over 25 min, of 0.1% aqueous TFA and 0.1% TFA−CH_3CN from 1:0 to 7:3); (b) the same finding for conotoxin SH 3&7 (t_R = 18.5 min for SH, t_R = 17.6 min for SS), except that a reaction time of 30 h was required; (c) the same finding for conotoxin SH 3&13 (t_R = 18.6 min for SH, t_R = 17.4 min for SS), except that a reaction time of 24 h was required; (d) at pH 2.7, none of these substrates oxidized to any significant extent, over a 24-h period; and (e) incubation of these peptides (1 mg), dissolved in pH 6.6 buffer−CH_3CN−CH_3OH (2:1:1, 1 mL), with acetylated PEG-PS (40 mg, 0.2 mmol/g) for 30 min gave the following relatively low amounts of oxidized products formed: 10% for conotoxin SH 3&7; 12% for conotoxin SH 3&13, and 15% for conotoxin SH 2&7.

α-Conotoxin SI. Experiments using solid-phase Ellman's reagents are summarized in Table 7, and discussed in the text. There follow descriptions of control oxidations in solution, and reference analytical data. For both the solution and solid-phase oxidations, only the starting reduced peptide (tetrathiol) and the final fully oxidized products (naturally occurring as well as mispaired regioisomers) were observed by HPLC.

(**A**) Crude linear reduced conotoxin (20 mg, 14.7 μmol) was dissolved in 0.01 M phosphate buffer, pH 7.5 (19 mL), and DMSO (0.2 mL) was added. After 20 h at 25 °C, the reaction mixture was lyophilized, and the crude peptide was analyzed by HPLC, under conditions identical to those just described for the one-disulfide α-conotoxin analogues, and was shown to comprise 84% natural regioisomer (t_R = 19.6 min), 8% mispaired isomer SS 2&3, 7&13 (t_R = 18.6 min), and 8% mispaired isomer SS 2&13, 3&7 (t_R = 18.8 min). Purification of the main product by preparative HPLC, under conditions identical to those just described for the one-disulfide α-conotoxin analogues, gave 5 mg (3.7 μmol, 25%) of material that matched a standard by co-injection and had FABMS (*m*/*z*): calcd for $C_{55}H_{84}N_{16}O_{16}S_4$: 1352.51; found, 1353.5 $[MH]^+$.

(**B**) Crude linear reduced conotoxin (1.0 mg, 0.74 μmol) was dissolved in 0.1 M phosphate buffer, pH 6.6 (0.9 mL), and DMSO (0.1 mL, 10% v/v) was added. After 19 h at 25 °C, the reaction mixture was analyzed by HPLC and was shown to comprise 86% natural regioisomer, 6% mispaired isomer SS 2&3, 7&13, and 8% mispaired isomer SS 2&13, 3&7.

(**C**) Crude linear reduced conotoxin (1.0 mg, 0.74 μmol) was dissolved in 1% aqueous HOAc, pH 2.7 (0.8 mL), and DMSO (0.2 mL, 20% v/v) was added. After 48 h at 25 °C, the reaction mixture was analyzed by HPLC and was shown to comprise 51% natural regioisomer, 28% mispaired isomer SS 2&3, 7&13, and 21% mispaired isomer SS 2&13, 3&7.

Apamin. Both solution (see following) and solid-phase oxidations (Table 7) showed complicated kinetics, including the observation of HPLC-detected intermediates at t_R = 14.6 min, t_R = 14.8 min (possibly one-disulfide intermediates), and t_R = 18.2 min (not reduced by DTT and not formed in the presence of 8 M guanidinium hydrochloride).

(**A**) Linear reduced apamin (1 mg, 0.5 μmol, t_R = 15.3 min), purified by preparative HPLC (gradient, conducted over 40 min, of 0.1% aqueous TFA and 0.1% TFA−CH_3CN from 9:1 to 11:9) was dissolved in 0.1 M phosphate buffer, pH 6.6 (1 mL). After 2 h at 25 °C, the solution was analyzed by analytical HPLC (gradient, started after 2 min and conducted over 30 min, of 0.1% aqueous TFA and 0.1% TFA−CH_3CN from 9:1 to 7:3) and was found to contain no starting substrate, 76% of a peak at t_R = 11.2 min corresponding to apamin (verified by co-injection with a standard), and several unidentified peaks.

(**B**) Linear reduced apamin (1 mg, 0.5 μmol), purified by preparative HPLC, was dissolved in 1% aqueous HOAc, pH 2.7 (0.8 mL), and DMSO (0.2 mL) was added. After 24 h at 25 °C, HPLC analysis revealed 60% of desired apamin, t_R = 11.2 min; 1% of mispaired isomer 1, t_R = 13.7 min; and 39% of mispaired isomer 2, t_R = 13.8 min.

Oxidation of Selected Peptide Substrates by Ellman's Reagent (1) in Solution. For solubility reasons, reactions were carried out in 0.1 M phosphate buffer, pH 7.3. Peptide stock solutions were 0.6 mM (200 μL used per experiment), and reactions were initiated by adding aliquots from a 4.2 mM stock solution of DTNB (**1**) to achieve peptide:DTNB ratios ranging from 10:1 to 0.5:1. Reactions were monitored in real time by UV (after 15-fold dilution: full spectra from 190−540 nm and single wavelength measurements at 412 nm) and by HPLC. The amount of TNB^- formed was deduced from the UV data; the value did not change after 5 min and was in close agreement with theoretical expectation (for peptide in excess, the amount of DTNB added; for DTNB in excess, the amount of SH groups in the reduced peptide substrate). The highest absolute amounts of desired disulfide product formed were observed within 15 min and with peptide:DTNB ratios near to 1; these values were ∼85% for deamino-oxytocin, ∼50% for somatostatin, and ∼75% for conotoxin SH 3&13. However, for all of these peptides, up to three additional peaks were observed in the HPLC, and the levels of these extra peaks increased with time while that for the desired disulfide was reduced. The additional peaks absorbed at 330 nm and were reduced by DTT; they are tentatively assigned as bis(TNB) derivatives of the parent peptide and/or its dimers. More detailed investigations of the structures of these byproducts, and the kinetics of their formation and interconversion, are beyond the scope of this work.

Acknowledgment. I.A. is grateful to the National Science Foundation for a graduate fellowship. We thank the National Institutes of Health (GM 43552, 51628) for support of this research.

Supporting Information Available: Additional experimental information, including details on general procedures and instrumentation, characterizations of all organic compounds, and methods for the assembly and characterizations of the linear peptide precursors (9 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

JA981111P