CHAPTER 7

Formation of Disulfide Bonds in Synthetic Peptides and Proteins

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1. Introduction

Disulfide bridges play a crucial role in the folding and structural stabilization of many important extracellular peptide and protein molecules, including hormones, enzymes, growth factors, toxins, and immunoglobulins *(1–10)*. In addition, the artificial introduction of extra disulfide bridges into peptides or proteins allows the creation of conformational constraints that can improve biological activity *(11–15)* or confer thermostability *(5,16–19)*. Given this intrinsic biological interest, disulfide-containing peptides have long been attractive targets for chemical synthesis. Starting with the pioneering work of du Vigneaud on oxytocin *(20)*, the challenge to reproduce and engineer increasingly complex arrays of disulfide bridges as are found in natural peptides and proteins *(7,10,21–23)* has stimulated the efforts and ingenuities of many peptide chemists. Table 1 provides a representative, but by no means exhaustive, listing of noteworthy syntheses of peptides or small proteins with one or more disulfides. The methods can be readily generalized to analogs in which cysteine residues are replaced by homologs, such as homocysteine, or by sterically restricted derivatives, such as penicillamine (β,β-dimethylcysteine).

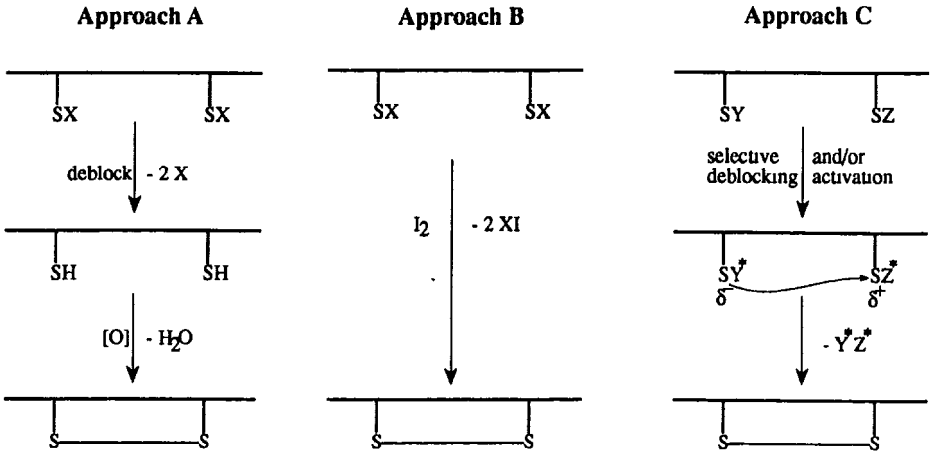
Both conformational and chemical considerations determine the ease of disulfide bond formation in synthetic peptides. The conformational aspect becomes especially important with small intramolecular disul-

From *Methods in Molecular Biology, Vol. 35: Peptide Synthesis Protocols*
Edited by: M. W. Pennington and B. M. Dunn

Table 1
Representative Examples of Synthetic Disulfide-Containing Peptides and Proteins

Name	Residues	*SS* bonds	References
Oxytocin, vasopressin, and analogs	9	1	*20,24–29*
Somatostatin and analogs	13	1	*30–35*
α-Atrial natriuretic factors/peptides (ANF/ANP)	26–33	1	*36–39*
Calcitonin	32	1	*40*
Conotoxins and analogs	13–15	2	*41–49*
Apamin and analogs	18	2	*50–57*
Endothelin and analogs	21	2	*58–63*
Posterior pituitary peptide	21	2	*64*
Sarafatoxin	21	2	*65*
β-hANP (antiparallel dimer of α-ANP)	56	2	*38,39*
E. coli enterotoxin active fragment and analogs	13	2–3	*66–68*
μ-Conotoxins (geographutoxins)	22	3	*69–72*
ω-Conotoxin	27	3	*73–77*
E. elaterium trypsin inhibitor II	28	3	*78*
C. maxima trypsin inhibitor	29	3	*79*
Defensins	29–34	3	*80–83*
Charybdotoxin and analogs	37	3	*84–86*
Bombyxins and analogs	48	3	*87–89*
Sea anemone neurotoxin and analogs	48	3	*90,91*
Human insulin, relaxin, and analogs	51–53	3	*92–97*
Transforming and epidermal growth factors	50–55	3	*98–103*
Bovine pancreatic trypsin inhibitor and analogs	58	3	*104–106*
CCK-releasing peptide	61	3	*107*
C5a anaphylatoxin	74	3	*108*
Angiogenin	123	3	*109*
ω-Agatoxin IVA	48	4	*110*
Echistatin	49	4	*111*
Na^+, K^+-ATPase inhibitor-1 (SPAI-1)	49	4	*112*
Elafin	57	4	*113*
Ribonuclease A	124	4	*114,115*

fides. For example, the minimally sized eight-membered ring formed between two adjacent half-cystinyl residues is only rarely found in nature *(116–119)*, and can be somewhat difficult to form in the laboratory (*see* refs. *120–122* for elegant applications of strained peptide disulfides). With additional intervening residues n between a pair of half-cystines, the resultant $(3n + 8)$ size rings are relatively unstrained and have considerable conformational flexibility *(3,6,8,10,12,13)*. Still, the exact sequence can make a difference; for example, small "local" loops must



Scheme 1. Synthetic approaches to intramolecular disulfide bridges. X, Y, and Z designate sulfhydryl-protecting groups that are stable to chain assembly. The activation and/or deprotection steps in approach C provide a nucleophilic SY* and an electrophilic SZ* (in the simplest case, Y* = H and Z* = Z). As discussed in the text, these approaches can be generalized to varying extents for the syntheses of molecules with multiple disulfide bridges.

include residues compatible with β- or γ-turns *(47,123–125)*, and in larger structures, the steric environment about cysteine residues affects their accessibility and pK_a, and hence, the ease of oxidation *(126–129)*.

Even for sequences in which the conformations corresponding to disulfide bridging are readily accessible, the success of synthetic efforts may still hinge on the appropriate choice of cysteine-protecting groups and corresponding deblocking conditions (*vide infra*). The present chapter covers some current alternatives for cysteine protection and then turns to procedures for efficient disulfide bond formation. Both solution and solid-phase chemistries are discussed. Three principal approaches to *intramolecular* disulfide formation in synthetic peptides can be envisaged (Scheme 1).

In approach A, both of the cysteine residues to be paired have the same protecting group, which is removed to give the dithiol form of the peptide. Oxidation by molecular oxygen or other appropriate reagents provides the intramolecular disulfide. The disulfide can also be established from the dithiol in the presence of redox mixtures, which catalyze thiol-disulfide exchange reactions. In approach B, the cysteine-protecting groups are removed oxidatively by iodine as shown, or by alternative

electrophilic reagents, to furnish the disulfide directly. Finally, the chemically more demanding directed approach C requires two cysteine-protecting groups that can be selectively cleaved and differentially activated; a subsequent displacement reaction gives the disulfide bond. Both of the symmetrical approaches A and B, as well as the unsymmetrical approach C, can suffer from intermolecular dimerization and oligomerization side reactions. These can be mitigated to various extents, depending on the sequence, by the use of *dilute* conditions in solution, or by the *pseudo-dilution* phenomenon that applies to polymer-supported reactions *(124,130–132)*.

The indicated approaches can be extended to the formation of peptides or proteins containing multiple disulfide bridges. Despite the statistical obstacles (3, 15, and 105 intramolecular disulfide isomers theoretically possible for molecules with 4, 6, and 8 half-cystine residues, respectively), approach A is often practical. Scrupulous attention to experimental details (pH, ionic strength, temperature, time, concentration) is critical, and in the cases when such random oxidations are successful, it is because the polypeptide chains in their reduced polythiol form can apparently fold into native-like conformations that favor the proper alignments of disulfides *(5,9)*. However, nonnatural isomers may form inadvertently because of misfolding (or even as obligatory intermediates in the folding pathway!), and intractable oligomers and polymers are often observed as well *(41,99,133,134)*.

Chemical control over the specificity of half-cystine pairings is clearly a desirable goal. For example, the native alignments of multiple bridges are sometimes not known with certainty from biochemical and/or instrumental methods of peptide or protein analysis, and synthetic methods for regioselective construction of the bridges in several alternative ways can become an invaluable tool for deciding the correct structure *(41,43,58, 67,68,77,94,135,136)*.

Typically, approaches A and/or B are applied in series using two or more orthogonally removable sets of pairwise cysteine-protecting groups. Although in principle such strategies generate unambiguous disulfide connectivities, the experimental procedures must be designed and executed with great care in order to prevent or minimize *scrambling* of disulfides through any of the consecutive chemical manipulations.

It is often of interest to pair half-cystine residues that are on separate linear chains. Relevant applications include conjugation of peptides to

carriers for immunological studies, preparation of standards corresponding to proteolytic fragments isolated during structural elucidation work on large proteins, development of discontinuous epitopes, and generation of active site models *(36,135–149)*. Formation of the required heterodisulfides cannot be carried out efficiently by approaches A or B, because the statistically predicted distribution that includes both symmetrical homodimers will always form upon random symmetrical oxidation. Therefore, *intermolecular* disulfides are created, whenever possible, by approach C. Because of the ready propensity of unsymmetrical disulfides to disproportionate to the symmetrical species, exposure of the desired products to strong acid or weak base should be minimized.

Although formation of disulfide bridges is usually carried out toward the end of a synthetic plan, it is sometimes advantageous to couple and/or elongate chains that include a preformed disulfide *(93,131,138,150,151)*. This can be done either in solution or on the solid phase, and requires careful choice of deprotection and cleavage conditions (*see* Chapters 4 and 5) that do not affect disulfide bonds. An interesting artifice has been devised to prepare multiply disulfide-linked chains corresponding to putative partial structures encountered in structural work *(72,73)*. A single parent chain is designed that includes a site for proteolysis; intramolecular disulfide formation (by regioselective methods, if the case warrants) followed by cleavage gives the desired product chains.

Several of the themes of the present chapter have been covered within earlier reviews *(131,152–158)*; the fundamental organosulfur chemistry literature can also be a rich source of inspiration in this field *(159,160)*. We assume herein that the required linear peptide sequences can be assembled adequately and with minimal racemization, by stepwise and/or convergent solution *(161–163)* or solid-phase methodology *(131,164–166)*; hence, our focus is on the management of cysteine residues. The subject matter of this chapter is organized parallel to the present introduction, and additionally, modern methods for determining disulfide connectivities are covered. Although the success of any synthetic effort directed at properly folded disulfide-bridged peptides and small proteins often depends critically on the purification and analytical characterization procedures that are applied, we do not address these areas because they are well described in the original literature and in more general reviews and monographs. Similarly, the elegant thiol capture method of Kemp *(167)* demonstrates some interesting disulfide chemistry, but is

outside the scope of the present chapter. In accordance with the editorial policy of this volume, emphasis is placed on practical experimental considerations, as filtered through the authors' experiences, and citations to the literature are weighted toward articles providing full details.

2. Cysteine Protection

Side-chain protection for the β-thiol of cysteine is selected foremost in the context of compatibility with the temporary N^{α}-amino protecting group used in stepwise assembly of the linear peptide sequence, and permanent groups protecting other side-chain functions. As an added complication, hydrogenolyzable protecting groups, such as N^{α}-benzyl–oxycarbonyl (Z), are generally incompatible because sulfur-containing residues poison the catalyst. For solution syntheses, strategies depend on graduated acid lability with N^{α}-4-methoxybenzyloxycarbonyl (Moz), N^{α}-*tert*-butyloxycarbonyl (Boc), N^{α}-triphenylmethyl (trityl or Trt), N^{α}-2-(4-biphenylyl)propyl(2) oxycarbonyl (Bpoc), or N^{α}-*ortho*-nitrophenylsulfenyl (Nps), together with side-chain benzyl (Bzl) or *tert*-butyl (*t*Bu) and related permanent groups, whereas current solid-phase syntheses invariably apply either "classical" Boc/Bzl strategies or orthogonal schemes with base-labile N^{α}-9-fluorenylmethyloxycarbonyl (Fmoc) and acidolysable *t*Bu and related permanent groups *(131,161–166)*.

The most widely used or promising cysteine-protecting groups are listed in Table 2, together with indications of stabilities and removal conditions. Note that this tabulation is but a subset of the many ingenious cysteine-protection chemistries that have been proposed and evaluated for peptide chemistry *(131,152,153,156,157,166,167,209–212)*. Deprotection of cysteine can be concurrent with removal of all other side-chain-protecting groups (and simultaneous cleavage from the support in the case of solid-phase peptide synthesis), or, under appropriate circumstances, protected cysteine residues can survive the final cleavage/deprotection step. In further variations, orthogonal deblocking of cysteine can be carried out selectively while retaining other side-chain protection; for solid-phase procedures, this can be done while the peptide chain remains anchored to the support. Orthogonality issues become particularly critical in designing regioselective schemes for syntheses of peptides with multiple disulfides.

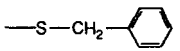
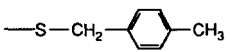
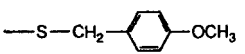
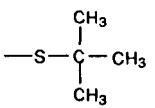
Unique to cysteine, protecting group removal can be conducted either to generate the free thiol or to provide directly a disulfide bond. In the

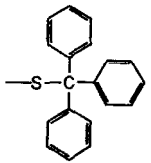
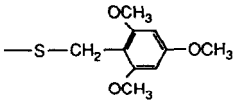
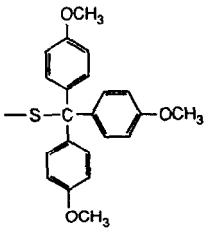
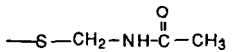
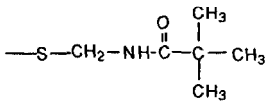
former case, the deblocking modes are acid (care must be taken to prevent realkylation of deblocked cysteine residues by carbocations generated under the cleavage conditions), base, reducing agents, or metals, such as Ag(I) or Hg(II). Metal mercaptides need to be treated in a separate step, e.g., with excess β-mercaptoethanol or hydrogen sulfide, to release the free thiol. However, sometimes the metal is very tightly bound and difficult to remove completely. Deprotection procedures should be carried out under an inert atmosphere, to the extent possible, in order to minimize inadvertent or premature oxidation. Intentional oxidative methods to give disulfides directly are covered subsequently, as are treatments with sulfenyl chlorides or other electrophilic reagents to give mixed disulfide intermediates.

The classical *S*-benzyl (Bzl) group developed by du Vigneaud and coworkers has had a distinguished history for the preparation of peptides in the oxytocin family, and was also popular in the early days of the solid-phase methodology *(20,164,168)*. However, *S*-Bzl is applied relatively infrequently in current practice because of the relative harshness of deblocking conditions, e.g., sodium in liquid ammonia or anhydrous hydrogen fluoride (HF) at 25°C (*see* ref. *213* for description of dehydroalanine residue formation upon HF-promoted elimination of benzyl mercaptan from Cys[Bzl]-containing peptide). Introduction of slightly electron-donating substituents on the aromatic ring has led to protecting groups, such as *S*-4-methylbenzyl (Meb; *169*) and *S*-4-methoxybenzyl (Mob; *171*), which have the proper balance of acid lability to allow their removal concurrent to other Bzl-type side-chain-protecting groups by use of HF–anisole (9:1) at 0°C, or acid/scavenger combinations of comparable strength *(170)*. Metal-assisted or oxidative cleavages of *S*-Meb or *S*-Mob appear to be quite difficult when the goal is preparative removal. Nevertheless, cleavage or oxidation of *S*-Acm and related groups (*see* Section 4.) is rarely carried out in the presence of intact *S*-Bzl, *S*-Meb, or *S*-Mob *(155)*.

Given the well-known lability of most *tert*-butyl (*t*Bu) derivatives to acids, it is interesting that the *S*-*t*Bu thioether survives neat HF at 0°C (although it is cleaved at 20°C; *see* ref. *175*). Relatively recently, conditions have been devised for selective acid cleavage of *S*-*t*Bu with HF, in the presence of *certain* efficient scavengers, such as anisole, *m*-cresol, or thioanisole *(39,178)*. Otherwise, adequate removal of *S*-*t*Bu can be achieved with metals or strong electrophilic reagents *(172)*. The conversion of *S*-*t*Bu to

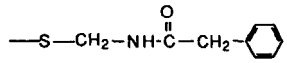
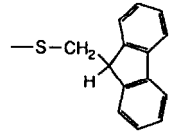
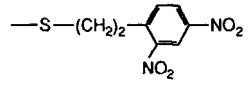
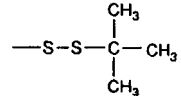
Table 2
Cysteine-Protecting Groups Currently Used in Peptide Synthesis[a,b]

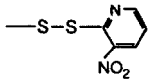
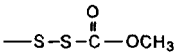
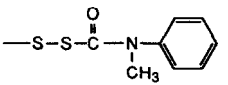
Protecting group	Abbrev.	Partial structure[a]	Stability	Removal	Compatibility[c]	References[a]
S-benzyl	Bzl		HF (0°C) Tl(III) Base RSCl	Na/liq. NH_3 HF (25°C)	Boc	*164,168*
S-4-methylbenzyl	Meb		TFA Ag(I) Base RSCl	HF (0°C) Tl(III) (partial)	Boc	*131,155,* *169,170*
S-4-methoxybenzyl	Mob		TFA Base RSCl I_2	HF (0°C) Hg(II), Ag(I) Tl(III) $(BrC_6H_4)_3N^+$	Boc	*131,155* *171–174*
S-*tert*-butyl	*t*Bu		HF (0°C) Base I_2 Ag(I)	HF (20°) HF/scavengers (0°C) Hg(II) NpsCl	Boc, Fmoc	*34,39,172,* *175–179*

S-triphenylmethyl	Trt		Base Nucleophiles	TFA/scavengers Hg(II), Ag(I) I_2,Tl(III) RSCl	Fmoc, Trt	*92,152,153, 155,179–184*
S-2,4,6-trimethoxy-benzyl	Tmob		Base Nucleophiles	Dilute TFA/ scavengers I_2, Tl(III)	Fmoc	*49,185,186*
S-4,4',4"-trimethoxy-triphenylmethyl	TMTr		Base Nucleophiles	Very dilute TFA	Fmoc	*186*
S-acetamidomethyl	Acm		HF (0°C) Base	Ag(I), Hg(II) I_2, Tl(III) RSC1	Boc, Fmoc	*48,146,153 155,174,177, 179,182,183, 187–189*
S-trimethylaceta-midomethyl	Tacm		HF Base	Hg(II), Ag(I), I_2	Boc, Fmoc	*190*

(continued)

Table 2 *(continued)*

Protecting group	Abbrev.	Partial structure[a]	Stability	Removal	Compatibility[c]	References[a]
S-phenylacetamidomethyl	Phacm		HF Base	Hg(II), I_2, Tl(III) Penicillin amidohydrolase	Boc, Fmoc	*48,191–193*
S-9-fluorenylmethyl	Fm		HF I_2	NH_3 in CH_3OH Piperidine Dilute DBU	Boc	*124,* *194–196*
S-2-(2,4,dinitrophenyl)ethyl	Dnpe		HF	Piperidine, Dilute DBU	Boc	*48,197*
S-*tert*-butylmercapto	S*t*Bu		TFA HF (partial) Base RSCl	RSH, Bu_3P, or other reducing agents	Boc, Fmoc, Nps	*35,50,138,* *161,177,* *198–200*

S-3-nitro-2-pyridine-sulfenyl	Npys		HF ("high") HOBt	RSH, Bu_3P; *see also* Scheme 3B	Boc	*137,143, 148,178, 202–204*
S-alkoxycarbonyl-sulfenyl	Scm		HF	RSH or other reducing agents; *see* Scheme 3A	Note *c*	*93,132, 205–207*
S-[(*N'*-methyl-*N'*-phenylcarbamoyl)-sulfenyl]	Snm		HF	RSH, specifically DTT and 2-mercaptopyridine	Boc	*207*

[a]The protecting group structure is drawn to include the sulfur of the cysteine that is being protected. Groups are listed in the same order as text discussion Unless indicated otherwise, conditions or reagents outlined under "Removal" are intended for quantitative deprotection and/or oxidative cleavage, the products from these reactions may be free thiols, mercaptides, or disulfides (details in text). For metal-mediated removals, the counterion and solvent are sometimes critical, for acidolytic cleavages with HF or similar strong acids, the nature and amounts of scavengers added may affect significantly the stability and/or lability of the protecting group References listed in the far right column cover discovery of the protecting group, its introduction onto cysteine, stability properties, and deprotection procedures, and may include relevant review articles in addition to or in place of the primary literature.

[b]Most of the protecting groups listed in this table have been applied to significant targets, as described throughout this chapter. Atherton and coworkers *(208)* have documented the risk of racemization for *C*-terminal-protected cysteinyl *esters* during Fmoc solid-phase synthesis, most serious for S*t*Bu, and less so for Trt, Acm, and *t*Bu The problem is *not* serious for internal protected Cys residues involved in *amide* linkages.

[c]This column provides the compatible repetitively removable N^{α}-amino-protecting group(s) for introduction of the appropriate protected cysteine derivative, and subsequent stepwise chain elongation. There is no entry for Scm, since this protecting group is always introduced indirectly once a cysteine residue has already been incorporated in the peptide chain

an aromatic mixed disulfide *(176)* by treatment with 2-nitrophenylsulfenyl chloride (NpsCl) is a prototype for a general two-step deprotection method, since further reductive transformations of mixed disulfides are easily achieved (discussed subsequently, with Scheme 3B in Section 5.).

The level of acid lability required to be compatible with Fmoc chemistry is provided by *S*-triphenylmethyl (trityl or Trt; *152,181*), *S*-2,4,6-trimethoxybenzyl (Tmob; *185*), and *S*-4,4',4"-trimethoxytriphenylmethyl (TMTr; *186*) groups (*see also* ref. *214* for description of *S*-9-phenylxanthen-9-yl [pixyl] group, which has properties similar to *S*-Trt]. The *S*-Trt, *S*-Tmob, and *S*-TMTr groups are released by treatment with varying levels of trifluoroacetic acid (TFA) in the presence of appropriate scavengers (relative lability to acid is TMTr > Tmob > Trt). The relatively stable carbocations due to these protecting groups can realkylate cysteine (an acid-mediated equilibrium process), as well as irreversibly modify sensitive tryptophan side chains. Trityl groups are removed generally with cocktails of concentrated TFA that include a mixture of aromatic and/or sulfur-containing scavengers, e.g., Reagent K *(215)*, TFA–phenol–water–thioanisole–1,2-ethanedithiol (82.5:5:5:5:2.5), or Reagent R *(216)*, TFA–thioanisole–1,2-ethanedithiol–anisole (90:5:3:2), 1–4 h, 25°C. Efficient *S*-detritylation can also be achieved by using trialkylsilane scavengers, which quench the trityl carbocation to form triphenylmethane *(217)* and have the added virtue of being less likely to interfere with subsequent oxidation/refolding steps. Reagent B *(218)*, TFA–phenol–water–triisopropylsilane (88:5:5:2), represents an effective and convenient cocktail for removal of *S*-Trt and related acid-labile side-chain-protecting groups used in Fmoc chemistry. The *S*-Tmob and *S*-TMTr groups can be cleaved in the presence of the same scavengers, but at substantially reduced TFA concentrations, e.g., 7 and 1% TFA, respectively, for ~15 min at 25°C *(185,186)*. Hence, it becomes possible to release free sulfhydryls in the presence of other side-chain-protecting groups, or for some solid-phase synthesis applications, while the peptide chain remains anchored to the polymeric support. *S*-Trt, *S*-Tmob, and *S*-TMTr can be used in orthogonal combination with *S*-Acm (discussed subsequently), and can also be removed selectively in the presence of more acid-sensitive groups, such as *S*-Bzl and *S*-Meb. Electrophilic removal conditions to generate disulfides directly are covered separately.

The *S*-acetamidomethyl (Acm; *187*) and related groups (Table 2) are particularly valuable, because they are essentially stable to both acid and

base and hence reasonably compatible with both Boc and Fmoc chemistries (*see* refs. *37,38,51,* and *219* for description of partial loss of Acm groups during repetitive chain assembly and final HF cleavage steps of Boc chemistry; the Tacm group described in ref. *190* might be more stable). The *S*-Acm group survives the conditions for acid removal of *S*-Meb, *S*-Mob, *S*-Trt, and *S*-Tmob; conversely, metal-assisted conditions or electrophilic reagents in properly chosen solvents can serve to remove Acm selectively in the presence of relatively acid-stable groups as *S*-Bzl or *S*-*t*Bu. Recent reports indicate that under a variety of N^{α}-Boc or *S*-Acm deblocking conditions, $S \rightarrow N$ or $S \rightarrow O$ transfer of Acm onto side-chain carboxamide or hydroxyl functions can occur; glycerol was suggested as a helpful scavenger to suppress such side reactions *(220,221).*

With the goal of obtaining a free sulfhydryl, *S*-Acm is removed typically by treatment with mercuric salts in *acidic* aqueous solutions, e.g., mercuric acetate ($Hg[OAc]_2$, 1 Eq; ~0.05*M*]) in pH 4.0 buffer for 1 h at 25°C *(187).* Alternatively, it is possible to treat *S*-Acm-blocked peptides with silver trifluoromethanesulfonate (10 Eq) in the presence of anisole (10 Eq), using TFA as solvent for 1 h at 0°C *(174).* In either case, the initial mercaptide is treated in the usual ways, e.g., excess hydrogen sulfide, β-mercaptoethanol, or dithiothreitol, to remove the metal. *S*-Acm removal can also be carried out in solid-phase synthesis while the peptide remains anchored to the support, by treatment with $Hg(OAc)_2$ (0.06*M*) in DMF for 3 h at 20°C (in the dark), followed by washes with DMF and DMF–β-mercaptoethanol (9:1, v/v) to remove Hg^{2+} from the deblocked peptide-resin *(48,146,189).*

The *S*-9-fluorenylmethyl (Fm; *194,195*) and *S*-2-(2,4-dinitrophenyl) ethyl (Dnpe; *197*) groups are compatible with Boc chemistry and fully orthogonal with *S*-Meb and *S*-Acm groups. *S*-Fm and *S*-Dnpe are stable to strong acids, such as HF, and are removed under basic conditions by β-elimination reactions, generally as promoted by piperidine (10–50%, v/v) or 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (2% v/v) in DMF. In solid-phase synthesis, it is possible either to remove these protecting groups while the peptide chain remains anchored to the support, prior to further transformations, or else first to cleave, isolate, and purify *S*-Fm or *S*-Dnpe-protected peptides. Although the chemical properties of *S*-Fm and *S*-Dnpe are similar, the latter group is more polar and less hindered, thereby conferring improved solubility properties to the resultant protected peptides. Also, HF cleavage yields are better with *C*-terminal

Cys(Dnpe), by comparison to Cys(Fm). Base-catalyzed removal of *S*-Fm or *S*-Dnpe in the presence of β-mercaptoethanol provides free thiol functions *(48,195)*; otherwise disulfides are obtained directly (*see* Sections 3., 6., and 8.7.).

An important class of cysteine-protecting groups is mixed alkyl, aryl, and acyl disulfides, such as *S-tert*-butylmercapto (*St*Bu; refs. in Table 2; *see* refs. *50,198,199,222,223* for information about the more acid-stable, base/nucleophile-sensitive ethyl analog), *S*-3-nitro-2-pyridinesulfenyl (Npys; refs. in Table 2), *S*-alkoxycarbonylsulfenyl (Scm shown in Table 2; *see* refs. *150* and *224* for ethyl [Sce], benzyl [Sz], and *tert*-butyl [Scb] homologs), and *S*-([*N'*-methyl-*N'*-phenylcarbamoyl]sulfenyl) (Snm, *207*) that are cleaved under orthogonal reductive conditions. The *S*-S*t*Bu group is compatible with all aspects of Boc or Fmoc chemistries, whereas *S*-Npys is compatible only with Boc, and *S*-Scm and related groups require modified strategies because of the risk of $S \rightarrow N$ acyl shifts *(207)*. Since the disulfide-containing protecting groups are reasonably stable to acid, their removal can be accomplished either selectively while a protected peptide remains anchored to a solid-phase synthesis support, or subsequent to acidolytic release from the support and cleavage of all other side-chain-protecting groups (*see* refs. *51* and *201* for discussion of problems with *S*-S*t*Bu in HF with various scavengers). Cleavage of *S*-S*t*Bu in aqueous media occurs with the usual reagents for disulfide bond scission, e.g., β-mercaptoethanol, dithiothreitol, or tri-*n*-butylphosphine (*see* Section 3. for specific examples of conditions for solution deprotection); typical conditions for solid-phase deprotection include treatment with β-mercaptoethanol–DMF (1:1) for 5 h at 25°C *(200)*. Note that these reductions are all designed to provide cysteine residues in the reduced form. In contrast, *S*-Npys, *S*-Scm, and similar groups are set up for regiospecific displacement by free sulfhydryl groups to form unsymmetrical disulfides, as covered in more detail in Section 5., Schemes 3A and B.

An additional level of orthogonality is provided by the use of protecting groups that can be removed enzymatically *(192,225)*. Optimal enzymes for this purpose should operate at near neutral pH values in aqueous media, and should have high selectivity for the bond-cleaving reactions that they catalyze as well as broad substrate specificity with regard to recognizable structures. Hermann and coworkers have suggested that acylamidomethyl cysteine-protecting groups could be

deblocked in two stages: (1) enzymatic cleavage of the acylamido bond; followed by (2) spontaneous hydrolysis of the aminomethylmercapto intermediate (for *N*-terminal Cys, this cyclizes further to a thiazolidine-4-carboxyl residue). These concepts were tested initially on Cys(Acm) using an ω-aminoacylase from chicken kidney. The most promising results were obtained using *S*-phenylacetamidomethyl (Phacm) *(48,191–193)* for cysteine protection, and penicillin G acylase from *E. coli*, an enzyme with a P_1 specificity for phenylacetyl moieties. Deprotection is carried out typically for ~24 h at 35°C, using enzyme that is immobilized on acrylic beads, and with the Cys(Phacm) peptide dissolved in pH 7.8 phosphate buffer. When the enzymatic hydrolysis buffer includes β-mercaptoethanol (2% v/v), the product peptide has a free sulfhydryl, whereas in the absence of a reducing agent, the symmetrical homodimer forms. The *S*-Phacm group is compatible with both Boc/Bzl and Fmoc/*t*Bu synthetic strategies, and is fully orthogonal with base-labile *S*-Fm and *S*-Dnpe, and acid-labile *S*-Meb, *S*-Trt, and *S*-Tmob groups. *S*-Phacm can also be applied with *S*-Acm, which survives the initial enzymatic removal of *S*-Phacm *(48,193)*. Thus, the enzymatic approach has the potential to extend substantially the range of options in regiospecific synthesis of disulfide-containing peptides. Possible limitations include relatively long reaction times, and solubility restrictions given the narrow pH optimum of the enzyme.

3. Formation of Disulfides from Free Thiol Precursors

The conceptually simplest approach to prepare disulfide-containing peptides involves complete deprotection, purification of the linear precursor in the free poly(thiol) form, and then careful oxidation to the properly folded product with the correct pairings (Scheme 1, Approach A). This often successful strategy offers the clear advantage of requiring only one type of *S*-protecting group for all cysteine residues, thus minimizing the number of chemical steps that must be conducted following completion of chain assembly.

The crude cleaved material generally needs to be treated with suitable reducing agents to ensure that the chain is a linear monomeric species, free of intra- or intermolecular crosslinks. Toward this end, treatments are generally carried out with: (1) 10–100 m*M* dithiothreitol or (2) 0.1–0.3*M* β-mercaptoethanol, either reducing agent in an appropriate buffer, e.g., 0.1*M* Tris-HCl, 1 m*M* EDTA, at pH 8–9, usually for 2–20 h at 25°C

(226) (*see* ref. *227* for example where heating to 37°C was necessary; *see* refs. *228–230* for alternative dithiols that serve as reducing agents); or (3) tri-*n*-butylphosphine (5- to 10-fold excess over disulfide) in 0.5*M* aqueous sodium bicarbonate–*n*-propanol (1:1), pH 8.0–8.3, at 20–25°C for 1 h (ref. *231*; alcohol needed to dissolve phosphine reagent; however, *see* ref. *232* for an alternative, water-soluble, phosphine: tris[2-carboxyethyl]phosphine [TCEP]). Sometimes, reduction is carried out in the presence of denaturants, such as 6*M* guanidium hydrochloride or 8*M* urea (the latter is less preferred because of partial decomposition to cyanate at basic pH).

Once reduction of crude synthetic material is complete, purification should be carried out at acidic pH, to minimize oxidation and/or disulfide interchange. Low-mol-wt species, e.g., denaturants, salts, and excess reducing agents, should then be removed by dialysis or gel filtration, setting the stage for the folding/renaturation/reoxidation steps. Reversed-phase HPLC (*see* Chapter 3, *PAP*) and/or ion-exchange steps (*see* Chapters 2 and 5, *PAP*) can also separate the desired reduced material efficiently from byproducts. In our experiences, disulfide formation procedures are more likely to be successful if the original reduced polypeptide has undergone initial purification step(s) (*see* ref. *233* for an example where an oxidized product reverted to unwanted polymers on lyophilization, because of concentration of trace thiol scavengers remaining from a peptide-resin cleavage step). Also, some workers prefer to carry out purification at the poly(*S*-sulfonate) level; the required intermediates are generated by oxidative sulfitolysis and subsequently reduced to the poly(thiol) by procedures similar to those already described (*see* refs. *114,131,234* for examples and leading references).

Oxidation in solution needs to be carried out at high dilution, typically at 10–100 μ*M*, in order to avoid aggregation and intermolecular side reactions. Nevertheless, solubility can often be a problem. Commonly, molecular oxygen serves to promote disulfide formation under slightly alkaline conditions, e.g., pH 7.5–8.5; this is done by simple aeration, under gentle stirring, or with slow bubbling, through the dilute peptide solutions. Most likely, a radical mechanism applies *(159,160,235,236)*. Occasionally, moderate levels of denaturants, e.g., 0.5–3*M* urea or 0.1–1.5*M* guanidine hydrochloride, are added to avoid aggregation; a possible added benefit may be to cause a partial unfolding and expedite the renaturation process (*see* ref. *237* for a recent report of intramolecular oxidation of peptide

bis(thiols) carried out in 8*M* Gu-HCl). Reactions are conducted at 5–25°C for 2 h to 4 d, and monitored by HPLC, capillary electrophoresis (*see* Chapter 6, *PAP*), and/or other analytical techniques, including Ellman or related tests *(238–240)* to determine disappearance of free thiols. Considerable experimentation is required to devise optimal conditions, and it is not uncommon for misfolded species or oligomers to accumulate in solution and/or precipitate out. Examples of the overall approach and some of the pitfalls, together with experimental documentation, are provided in the syntheses of a variety of toxins, growth factors, bovine pancreatic trypsin inhibitor, and ribonuclease, already listed with references in Table 1.

Air oxidation, under conditions already discussed, is sometimes too slow to achieve useful yields of monomeric products. The organic and biochemical literature includes numerous examples of relatively mild oxidizing agents that can serve to convert thiols to disulfides *(159,160, 236,241)*. Potassium ferricyanide *(24,26,33,242)* has been particularly useful in the preparation of small-size, intramolecular single-disulfide peptides in the oxytocin or somatostatin families. The procedure has also been used recently in the orthogonal formation of the first disulfide bond of several peptides containing two disulfide bonds *(44,46)*. Dimethyl sulfoxide (DMSO)-promoted oxidation of thiols to disulfides was described first by Wallace *(243)*, and the use of DMSO to circumvent some of the difficulties that are encountered occasionally by classical oxidation approaches was recently brought to the fore by the independent studies of Tam, Fujii, and their respective coworkers *(81,146,244)*. Advantages of DMSO oxidation include applicability over the extended pH range of 3–8, faster reaction rates, the effect of DMSO as a denaturing cosolvent, and improved solubility characteristics for the materials being oxidized (*see* Note 2). Further applications of the method have been demonstrated in the conotoxin and trypsin inhibitor families; these experiments were encouraging, although difficulties in removing DMSO from the final products were also noted *(49,106)*.

An interesting way *(245)* to achieve rapid intramolecular cyclization of dithiol peptides involves use of ethoxycarbonylsulfenyl chloride (SceCl). Crude peptides, obtained directly after lyophilization of HF cleavage products, were taken up in mildly acidic (pH 4–7) aqueous media, and combined with the reagent in a 2:1 SH:SceCl ratio (in practice, the amount of SceCl needed was greater). In this method, SceCl acts

nominally as an oxidizing agent, although the actual mechanism presumably involves formation of an *S*-Scm derivative of one thiol, which then becomes displaced by a second thiol (compare to Scheme 3A in Section 5.). Because some closely related impurities were noted in several cases tested, the SceCl methodology has been advocated for cases where other methods proved inadequate *(78,245)*. Azo-oxidizing agents, e.g., diethyl azodicarboxylate (DEAD) also react by a net two-step mechanism (compare to Scheme 3C and accompanying discussion in Section 5.).

Another significant strategy for renaturing peptides and small proteins with multiple disulfide bonds involves the use of redox buffers that mimic physiological conditions *(246,247)*. These procedures are carried out with dilute poly(thiol) peptide at pH 7.3–8.7, and proceed by thiol-disulfide exchange mechanisms *(4,9)*. Thus, even should nonnative disulfide bridges be favored kinetically, they can equilibrate ultimately to pair in the thermodynamically preferred arrangements found in the correctly folded structure. Most commonly, reduced and oxidized glutathione are added at 3 and 0.3 m*M*, respectively *(247)*, although in some cases, oxidized glutathione alone has been adequate *(106)*. Other redox systems are based on dithiothreitol *(105,248)* or on the protein thioredoxin *(249)*. Methods involving protein disulfide isomerase *(250–252)* have not been applied as yet to synthetic peptides, but may be important for future work.

The previous section included a description of orthogonal deprotection modes in solid-phase synthesis that allow retention of free cysteine-containing peptides on the polymeric support. This opens up possibilities for carrying out in the solid-phase mode some of the oxidation chemistries covered in the present section. The pseudo-dilution phenomenon will then favor intramolecular cyclization reaction to provide monomeric disulfides as the major (>60%) products, although dimers and higher oligomers also form (all peptide products identified and quantitated *after* cleavage from the support). Other advantages of solid-phase cyclizations include straightforward removal of oxidizing agents and/or solvents by filtration and washings, and the fact that reaction conditions are independent of the solubility characteristics of the sequence to be oxidized.

The principles of resin-bound oxidation of peptide dithiols have been demonstrated in several laboratories. Early examples used air or 1,2-diiodoethane as oxidizing agents *(44,223,253,254)*. An instructive example with Fmoc chemistry used TFA-labile *p*-alkoxybenzyl ester anchoring as a starting point for synthesis of a decapeptide with *S*-S*t*Bu

cysteine protection; orthogonal reduction was followed by overnight oxidation with 1*M* aqueous potassium ferricyanide–DMF (1:10 v/v), at 25°C, to form a 26-membered cyclic disulfide *(200)*. An optimal example from Boc chemistry involves oxytocin, which was prepared on an HF-labile MBHA-resin using *S*-Fm protection for the cysteine residues *(124)*. Fm removal under an argon atmosphere with piperidine–DMF–β-mercaptoethanol (10:10:0.7), at 25°C for 3 h, gave the resin-bound dithiol which was oxidized for 1 h with air or with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, 0.5 Eq) while the resin was suspended in "buffered" DMF (containing 0.1*M* each of acetic acid [HOAc] and *N*-methylmorpholine [NMM], with further NMM added to achieve a nominal pH of 7.5 at a glass electrode). The overall solid-phase yield was considerably better than when corresponding chemistry was carried out in solution, and marginally improved over a procedure where solid-phase Fm removal with piperidine–DMF (1:1), 25°C, 3 h, gave *directly* the 20-membered cyclic disulfide. Results with *S*-Dnpe in place of *S*-Fm were similar *(197)*. Lastly, resin-bound dithiols from several sequences could be generated by selective removal in dilute TFA of *S*-Tmob from peptides prepared by Fmoc synthesis on tris(alkoxy)benzylamide (PAL) supports *(185,186)*. Subsequent oxidation occurred under novel conditions adapted from Wenschuh and coworkers *(255)*: ~20 m*M* carbon tetrachloride–triethylamine (2 Eq each) in *N*-methylpyrrolidinone, at 20°C for 4 h (49, 186). These conditions were optimized carefully to maximize intramolecular cyclization, and represent a balance between negligible reaction and excessive oligomerization. Interestingly, the same oxidation procedure when applied to resin-bound peptides containing a *single* free cysteine residue represents a key first step in efficient *intermolecular* routes to parallel homodimers (*see* Scheme 5F in Section 6., and ref. *256*).

4. Symmetrical Formation of Disulfides from S-Protected Precursors

A number of oxidizing agents take *S*-protected cysteine derivatives directly to the corresponding disulfides (Scheme 1, Approach B). The prototype reaction in this regard is the conversion with iodine (1–10 Eq = 0.01–0.1*M*, for 1 min to 2 h at 25°C) of *S*-*T*rt, *S*-Tmob, or *S*-Acm groups *(122,182,185,257)*. (On completion of the deprotection, excess iodine is generally destroyed by reaction with solid or aqueous ascorbate or thiosulfate [titration]; with powdered zinc dust; or by dilution with water

Table 3
"Half-times" (t_h) for Iodine Oxidation of (**A**) Boc-Cys(Trt)-Gly-Glu(OtBu)$_2$ and (**B**) Boc-Cys(Acm)-Gly-Glu(OtBu)$_2$ in Various Solvents[a]

	Solvent	t_h for **A**, *S*-Trt	t_h for **B**, *S*-Acm
Group I	MeOH	3–5 s	1 min
t_h(*S*-Trt) < t_h(*S*-Acm)	MeOH–H_2O (4·1)	<1 s	4–6 s
	HOAc	70–80 s	40–45 min
	HOAc-H_2O (4:1)	1–3 s	50–60 s
	Dioxane	1 min	1.5–2 h
	Dioxane–H_2O (4:1)	5–10 s	5–10 min
	MeOH–$CHCl_3$ (1:1)	2–4 s	15 min
Group II	$CHCl_3$, CH_2Cl_2	1–2 s	1.5–2 h
t_h(*S*-Trt) << t_h(*S*-Acm)	HFIP–$CHCl_3$ (1:1)	1–2 s	>2 h
	HFIP–$CHCl_3$ (3:1)	<1 s	>2 h
	TFE–$CHCl_3$ (1:1)	5–6 s	>2 h
	TFE–$CHCl_3$ (3:1)	4–5 s	>2 h
Group III	DMF	25–35 s	2–3 s
t_h(*S*-Trt) > t_h(*S*-Acm)	DMF–H_2O (4.1)	30–40 s	3–5 s

[a]Adapted from Kamber et al. *(182)*. Reactions were carried out at 20–25°C, with 5 m*M* peptide and 15 m*M* iodine Group II solvents allow selective and quantitative oxidation of *S*-Trt in the presence of *S*-Acm With Group I solvents, co-oxidation of two different linear peptides, one protected with *S*-Trt and the other with *S*-Acm, showed a surprising preference (as much as 70–80%) for open-chain *asymmetrical* heterodimer formation (*see* refs. *59,144,147,151,182*). The corresponding experiments with Group III solvents (DMF or DMF–H_2O mixtures) gave the expected random statistical mixtures The recent work of Tesser and coworkers *(258)* illustrates solid-phase variations on this chemistry, taking advantage of cysteine bound as a thioether to an appropriate trityl resin

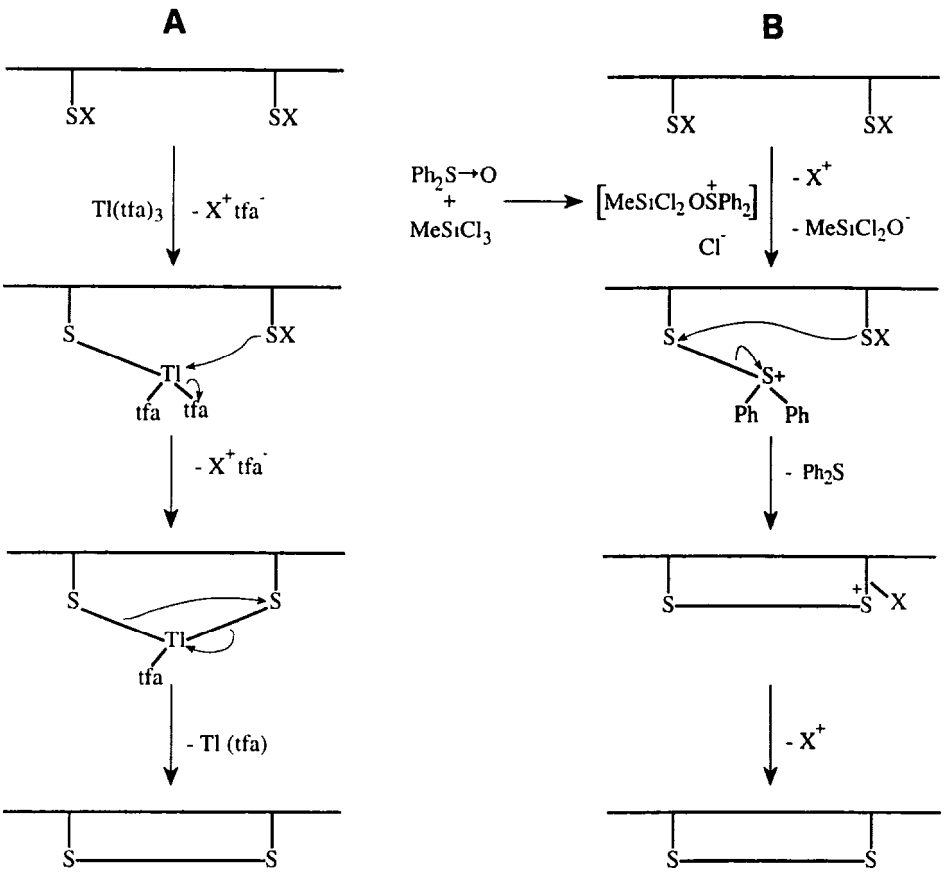
followed by extraction with carbon tetrachloride.) Suitable neutral solvents for iodine oxidation include aqueous and neat methanol or trifluoroethanol, dioxane, chloroform, and DMF; in some cases, it is advantageous to carry out reactions with anhydrous or aqueous carboxylic acid solvents or cosolvents such as acetic acid, propionic acid, and trifluoroacetic acid (water often accelerates reaction rates). In fact, the classic studies of Kamber and coworkers *(182)* show that remarkable selectivities (from 10-fold to >1000-fold) between *S*-Trt and *S*-Acm, in either direction, can be achieved by appropriate choices of solvent (Table 3).

As documented throughout this chapter, pairwise iodine oxidations have become the cornerstone of numerous successful applications in the peptide field. Occasionally, sensitive residues, such as tryptophan,

tyrosine, or methionine, suffer modification under the conditions used *(62,85,182,221,242)*; overoxidation of cystine bridges has also been noted *(257)*. Such side reactions can be mitigated by appropriate scavengers, control of pH (acid media are preferable), and minimization of reaction times (as shown in ref. *259*, disulfide dimerization can be suppressed, and intramolecular tryptophan-2-thioether formation favored, when the amount of iodine is limiting and "Group II" solvents [*see* Table 3] are used). Iodine oxidations of Cys(Acm) residues at or near the *N*-terminus of a peptide are said to proceed better when the endgroup is blocked *(136)*. Other sources of iodonium ion (I^+), such as cyanogen iodide *(260)* or *N*-iodosuccinimide *(57)*, may be advantageous alternatives to iodine.

Fujii and coworkers *(183)* introduced thallium (III) trifluoroacetate ($Tl[tfa]_3$) as a particularly mild oxidizing agent for removing a range of *S*-protecting groups (Scheme 2A). Typically, reactions are successful with thallium reagent (1–2 Eq), in the presence of anisole (2 Eq), at 0°C for 1–2 h, using TFA as the solvent. Tryptophan and methionine residues should be protected (the latter as its sulfoxide), since under the conditions cited Trp is destroyed and Met is oxidized partially to the sulfoxide (but not sulfone). In relevant side-by-side comparisons, thallium oxidations have been found to proceed more rapidly and give cleaner results than reactions with iodine; however, it should be stressed that the storage sensitivity and toxicity of thallium precludes its use on larger than laboratory scales. Also, as with other metals, thallium can be difficult to remove entirely from sulfur-containing peptides.

Another novel oxidizing milieu (Scheme 2B), discovered independently by Kiso *(261,263)* and Fujii *(262)*, is a mixture of alkyltrichlorosilanes and sulfoxides. Typical reaction conditions involve dialkyl or diaryl sulfoxide (0.4*M*; diphenyl sulfoxide [DPSO] as shown in Scheme 2B, or DMSO or tetramethylene sulfoxide [TMSO]) and silyl chloride (1.0*M*; $MeSiCl_3$ as shown in Scheme 2B, or TmsCl, Tms triflate, or $SiCl_4$) for 4 h at 4°C, in TFA as solvent. The method is compatible with most amino acid side-chains; Trp must be used as its N^{in}-formyl derivative (later removed by rapid base treatment without affecting disulfide) because unprotected Trp becomes fully chlorinated under the reaction conditions *(263)*. An interesting variation involves treatment of peptides containing acid-labile cysteine-protecting groups such as *S*-Trt and even *S*-Mob (but not *S*-Meb) with TFA in the presence of dimethyl sulfoxide



Scheme 2. Suggested mechanisms for mild oxidation methods of *S*-protected cysteine residues to form disulfide bonds. **(A)** Mediated by $Tl(tfa)_3$; adapted from ref. *183*. The initial thallium reagent is in the (III) oxidation state, and the final thallium salt coproduct is at the (I) level. Reactions are conducted typically in TFA (*183*), although DMF and acid–DMF mixtures can also be used (*124*). The chemistry has been demonstrated for X = *t*Bu, Mob, Acm, Trt, and Tmob (listed in approximative order of reactivity, from least reactive to most). **(B)** Mediated by silyl chloride/sulfoxide; adapted from ref. *261*. The chemistry has been demonstrated for X = Acm, Meb, Mob, Tacm, and *t*Bu (listed alphabetically without regard to relative reactivity). *See* refs. *261–263* and text for further discussion.

(DMSO) (10%, v/v) and anisole (1%); disulfide bonds are formed directly in good yields *(264)*.

A final example for oxidation of *S*-protected cysteine was given by Platen and Steckhan *(173)*, who showed that treatment of Cys(Mob)-

containing peptides in acetonitrile (in the presence of solid $NaHCO_3$) with the homogeneous electron-transfer agent tris(4-bromophenyl) ammoniumyl (a radical cation with antimony hexachloride counterion) (3 Eq), rapidly gives the corresponding intramolecular disulfides in high yields. Because Boc, Z, and *tert*-butyl groups are stable to these conditions, and because *S*-Mob survives manipulations of *S*-Trt (e.g., oxidation with iodine), this methodology shows considerable promise for regioselective disulfide formation.

Some of the oxidation chemistry just discussed can be transferred to the solid-phase mode (general principles already discussed in the previous section). It is preferable to use solvents that effectively swell the peptide-resin; DMF is near optimal in this regard for synthetic schemes based on either Boc or Fmoc. Typical conditions involve iodine (3–10 Eq = 0.03 – 0.08*M*) at 25°C for 1–2 h, or $Tl(tfa)_3$ (~1.2 Eq = 6 m*M*) at 0°C for 1–2 h; peptides protected with *S*-Acm, *S*-Trt, and *S*-Tmob have been oxidized this way *(48,49,124,185,265–270)*. Yields of monomeric intramolecular disulfide-bridged peptides can be as high as 60–90% by these solid-phase approaches. Everything else being equal, results are generally better using thallium rather than iodine for oxidation *(124,185,269)*, but it should be noted that thallium is not compatible with *S*-Trt *(124)*.

When acid-stable anchoring linkages, such as *p*-methylbenzhydrylamine (MBHA) or *o*-nitrobenzylamide (Nb), are used, TFA (which promotes good swelling) can be a helpful solvent for on-resin cyclization. Interestingly, the same oxidizing reagents (I_2, $Tl[tfa]_3$, DMSO) in TFA can be applied with acid-labile anchoring linkages, such as *p*-alkoxybenzyl (PAB, PAC, or "Wang" resin) or tris(alkoxy)benzylamide (PAL); in these cases, excellent yields of disulfide-cyclized peptides can be obtained *concurrently* with deprotection and release of material into solution *(185)*. In a different illustration of the general principle, iodine can be added to the dilute-acid cocktail for cleavage of 2-chlorotrityl resins; the resultant peptides include a disulfide bridge from oxidation of Cys(Trt), but other side-chain-protecting groups remain intact *(271)*.

5. Unsymmetrical Formation of Disulfides by Directed Methods

The symmetrical methods of disulfide formation discussed so far are applicable primarily to cases where the required bonds are either intramolecular cyclic or intermolecular homodimeric. When the goal is to con-

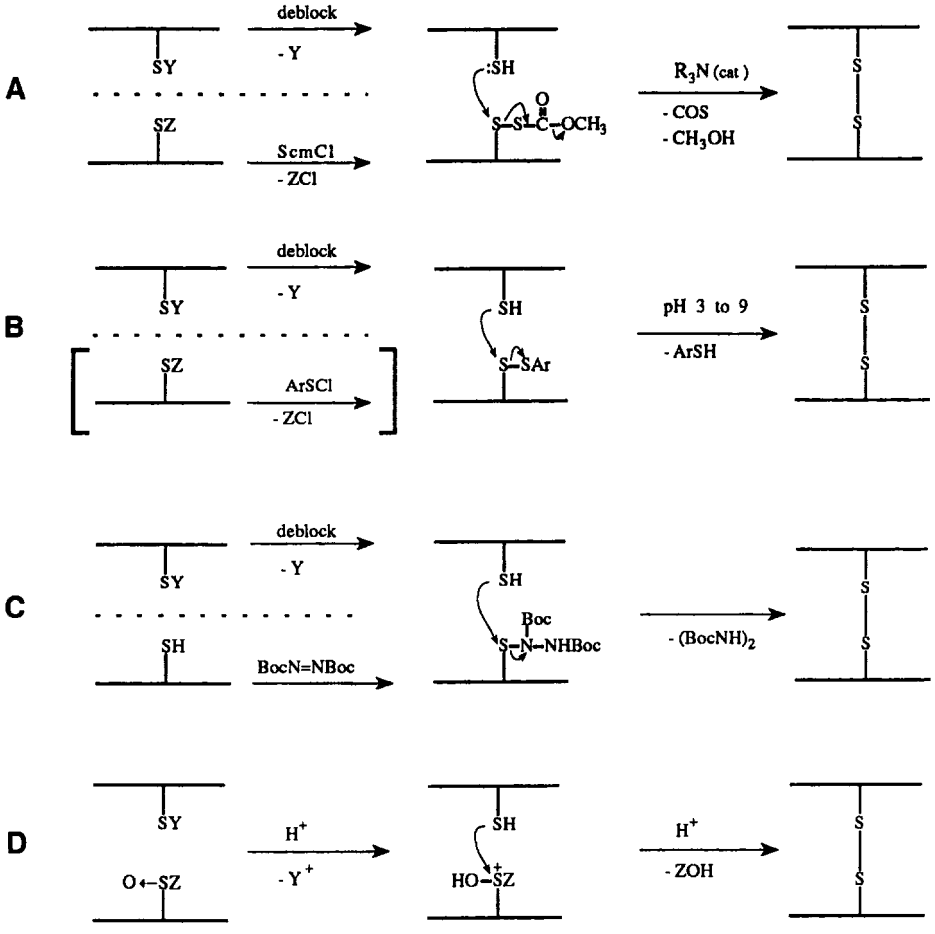
nect two different peptide chains by a disulfide bridge, co-oxidation methods become impractical, since the desired heterodimer is often a minor product in the presence of unwanted homodimers (*see*, however, the careful work of Kamber and coworkers, ref. *182*, which shows that in appropriate solvents, co-oxidation of two linear peptides protected with Cys[Trt] and Cys[Acm] respectively, can lead predominantly to open-chain asymmetrical cystine peptides, as noted in footnote to Table 3). Control of regioselectivity is possible, in principle, by directed disulfide formation methods (Scheme 1, Approach C). Directed methods are sometimes applied to internal disulfide bridges, although their main application is to heterodimeric and multiple disulfide-containing peptides.

Selective activation of a given cysteine residue (formation of SZ* in Scheme 1) can be carried out:

1. *In situ*;
2. As a discrete step leading to an isolable, characterizable intermediate; or
3. With a choice of a cysteine-protecting group that is sufficiently stable to chain elongation conditions, yet suitably activated for disulfide formation (i.e., Z = Z*).

Initial examples for category (1) are due to the pioneering work of Hiskey *(153)*, in which thiocyanogen $(SCN)_2$ was reacted with peptides containing either a free or protected thiol. The resultant activated peptides (Z = –SCN, used without isolation) were then reacted further with second peptides containing a free thiol, to form heteromeric disulfide-bridged products. Substantial levels of homodimeric byproducts form when the activation step is either too slow or incomplete, and the high electrophilic reactivity of thiocyanogen poses significant risks for a variety of side reactions. These problems provided an impetus for efforts to devise milder chemistries according to category (2) above.

Activation to form an isolable intermediate is exemplified by *S*-sulfenylphthalimide derivatives of cysteine peptides *(272)*. This interesting advance has had limited application, perhaps because of difficulties in the preparation of the activated compounds. In contrast, *S*-alkoxycarbonylsulfenyl derivatives (e.g., Scm in Table 2 and Scheme 3A) can be prepared readily by reaction of either free or protected (e.g., *S*-Trt, *S*-Acm) thiols with the appropriate alkoxycarbonylsulfenyl chlorides *(150,205–207,224,245,273)*. These base-labile *S*-sulfenyl derivatives must be handled under neutral or slightly acidic conditions where there is, however, a risk of undesired modification of unprotected tryptophan. Forma-



Scheme 3. Approaches for directed formation of disulfide bonds. *See* accompanying text for discussion and leading references. Reactions can be conducted when the two cysteines to be paired are on separate chains (heterodimer formation) or on the same chain (intramolecular cyclization). Transformations above and below the horizontal dotted lines are carried out separately. The square brackets for entry B indicate that the *S*-SAr intermediate is optionally prepared as shown, but can also be obtained by using Cys(SAr) as a building block in chain assembly. Numerous options exist for selective removal of protecting group Y; check Table 2 for reagents, conditions, and compatibility restrictions.

tion of the desired unsymmetrical disulfide occurs in a mild separate step catalyzed by mild base: A second free thiol displaces the *S*-alkoxycarbonylsulfenyl moieties, which are lost irreversibly as inert carbonyl sulfide

(COS) and alcohol (hence driving the reaction to completion) (Scheme 3A; ref. *273*). The references given already in this paragraph demonstrate and document the use of this chemistry for modification and disulfide-linking of cysteine-containing peptides in solution. Kemp's thiol capture method also makes use of Scm groups to facilitate connections to a 4-mercaptodibenzofuran template *(167)*. Conversions of *S*-Acm to *S*-Scm, and/or reactions of cysteine thiols with Cys(Scm) in the same or different peptides, can also be carried out in the solid-phase mode *(132,274)*. The products are intramolecular cyclic disulfides and disulfide heterodimers, respectively. Solid-phase variations have the usual advantages of simplicity, convenience of product isolation, and the possibility to drive reactions by use of excesses, but suffer from the difficulty of monitoring adequately completeness of the desired reactions and avoidance of side reactions.

A further way to activate free or protected cysteine residues involves reaction with aromatic sulfenyl halides, such as 2-nitrophenylsulfenyl chloride (Nps; Z = –S-[2-nitrophenyl])*(176,275)* or 2-pyridinesulfenyl chloride (SPyr; Z = –S-2-pyridyl) (276) (Scheme 3B). Directed disulfide formation is now driven by the low pK_a of the aromatic thiol. Unfortunately, the required sulfenyl halides can be difficult to obtain and/or suffer from extreme sensitivity to hydrolysis from atmospheric moisture. On the other hand, peptides containing cysteine in the free thiol form can be activated directly, both in solution and on the solid phase, by application of 2,2'-dithiodipyridine ($[PyrS]_2$) (*239*; *see* refs. *39,87,141,149* for examples in the peptide field). The highly specific transformation of *S*-Snm to *S*-SPyr on treatment with 2-mercaptopyridine in chloroform represents another mild avenue to the required stable *S*-arenesulfenyl intermediates *(207)*. Finally, cysteine-containing peptides can be reacted in aqueous pH 7.4 buffer with Ellman's reagent (DTNB; *238*) to provide the appropriate mixed disulfide intermediates; these react further *in situ* with a second cysteine peptide to provide significant levels of heterodimer *(277)*.

Although most of the *S*-activation procedures described in the preceding paragraphs can be performed relatively efficiently, the additional synthetic step complicates the synthetic strategy and practice both in solution and solid-phase modes. An advantageous alternative, category (3), takes advantage of the dual nature of certain groups, e.g., the 3-nitro-2-pyridinesulfenyl (Npys; Z = –S-[3-nitro-2-pyridyl; *see* Table 2) group for both protection and activation. The commercially available Boc-

Cys(Npys)-OH *(137)* derivative is incorporated readily into synthetic peptides by Boc chemistry, survives successive deprotection/coupling cycles, and is stable to final cleavage/deprotection with strong acids (e.g., HF, TFMSA) *(204)*. Asymmetric disulfide bond formation with a second peptide that contains a free thiol takes place over a wide pH range in aqueous buffers and can be monitored by spectrophotometric titration of the released 3-nitro-2-pyridinethiol *(278)*. Times for complete reaction have been reported *(137,140)* to vary from several hours at pH as low as 4, to 30 min or less at pH 8–9 (caution required at the high end of pH range, because of disproportionation, which provides symmetrical homodimers); another study gave substantially faster rates *(141,149)*. Reactions can also be carried out in the solid-phase mode *(178)*. Recent applications of the Npys group have included the synthesis of intramolecularly cyclized disulfide peptides *(178,203)*, disulfide heterodimers *(137,279)*, parallel and antiparallel bis-cystine peptides *(141,149,280)*, and conjugates of immunopeptides to carrier proteins *(140,143,145,281; see also* Chapter 10, *PAP*). Although the lability of *S*-Npys to piperidine prevents its use in Fmoc solid-phase protocols, peptides otherwise synthesized from N^{α}-Fmoc-amino acids can incorporate Boc-Cys(Npys)-OH as an *N*-terminal residue to be used subsequently in conjugation to a carrier. Additionally, internal Cys(Npys) residues can be formed after chain assembly is complete, by solution or solid-phase conversion of internal free or protected (with *S*-Acm, *S*-Trt, or *S*-*t*Bu) cysteine residues to *S*-Npys by methods covered in the previous paragraph *(148)*.

Additional approaches to directed disulfide formation exist. One attractive option, so far applied only in solution, represents in effect a controlled stepwise oxidation (Scheme 3C; refs. *138,139,282,283*). Peptides containing a free cysteine residue are dissolved in argon-saturated DMF and reacted with bis(*tert*-butyl)azodicarboxylate (2–3 Eq), for 4–12 h at 25°C, to provides isolable sulfenylhydrazides (Z = –N[Boc]NHBoc); following concentration and trituration to remove excess oxidizing agent, these are redissolved and combined under similar conditions with a *second* peptide containing deblocked cysteine to give disulfide heterodimers. Another elegant approach allows directed pairing of protected cysteine residues under strong acidic conditions (Scheme 3D; refs. *155,284,285*). One of the pair is not only protected (e.g., with Z = Mob or Acm), but oxidized further as the sulfoxide (in fact, the sulfoxide is present in the amino acid building block used during assembly of the

entire peptide chain). Once the acid-labile group (e.g., Y = Mob) protecting the other Cys is removed, the liberated thiol attacks the electropositive sulfur atom of the protonated sulfoxide, and a disulfide bond results. The method has been applied for both inter- and intramolecular reactions.

6. Regioselective Formation of Disulfides

This chapter has so far presented various alternatives for selective protection and deprotection of cysteine residues, together with methods for disulfide formation. The most demanding tests of these chemistries arise when the goal is to prepare peptides containing multiple disulfide bridges. The general approach is for graduated deprotection and/or co-oxidation of pairwise half-cystine residues, as specified by the original protection scheme. The present section describes a few representative examples of viable strategies, as applied to some of the targets already presented in Table 1. The best work in the field provides unambiguous syntheses of the correct structures, although it should be noted that absolute yields are often quite low (or not reported). As with random oxidation approaches discussed earlier, the regioselective routes require considerable care in the selection of experimental conditions, particularly with regard to appropriate solvents (*see* ref. *63* for an ingenious regioselective random oxidation, based on the preference of cysteine and penicillamine residues to form mixed disulfides).

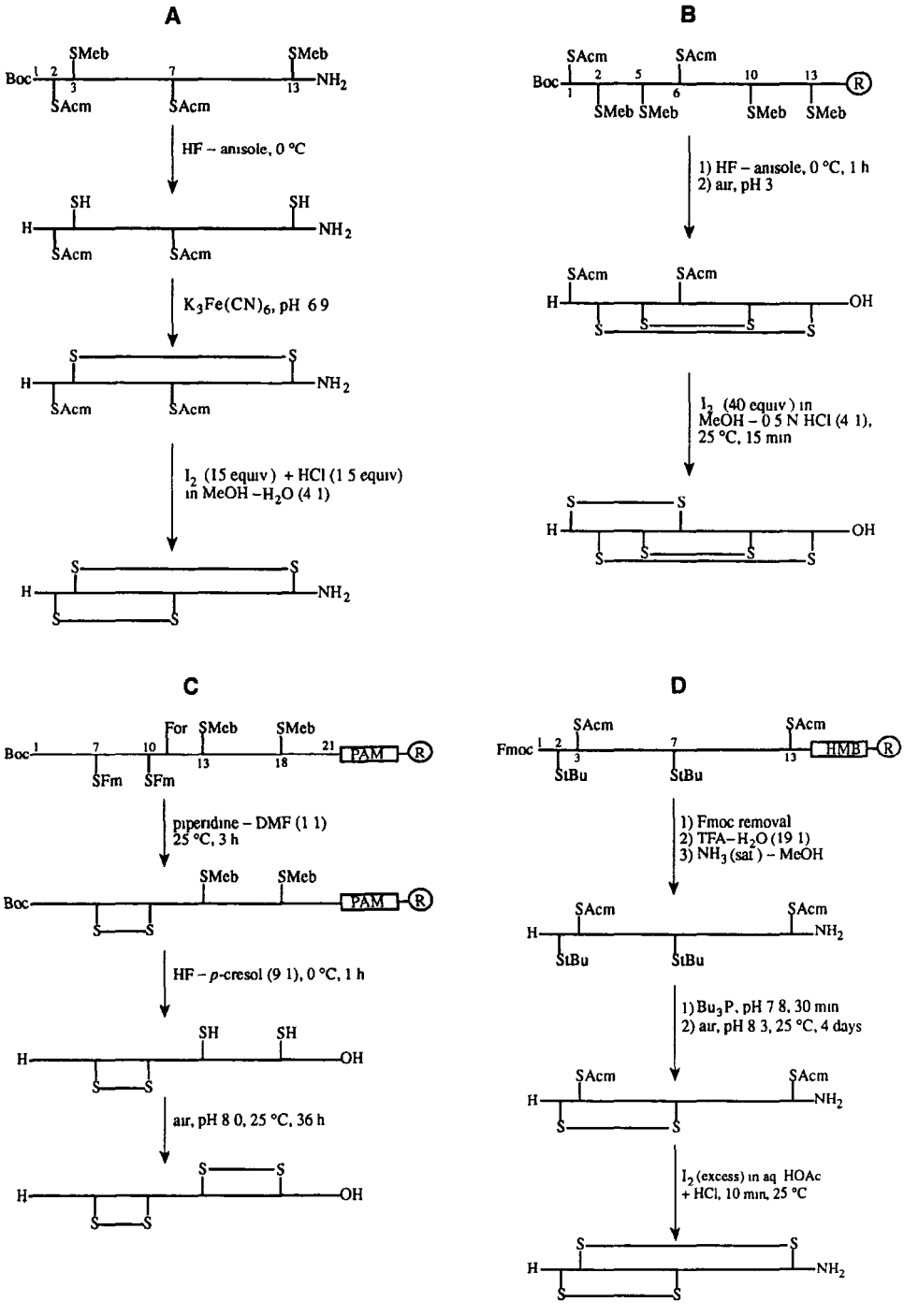
To achieve regioselective disulfide formation in single-chain peptides (Scheme 4), the desired linear protected peptide sequences are assembled by the usual solution or solid-phase methods, generally with two classes of cysteine-protecting groups. Nishiuchi and Sakakibara (Scheme 4A) used a combination of *S*-Meb and *S*-Acm groups in a synthesis of conotoxin GI that confirmed the disulfide alignment of the native peptide. The fully protected sequence prepared by solution methods was deprotected in HF and oxidized with $K_3Fe(CN)_6$ to give an intermediate retaining Acm protection at Cys^2 and Cys^7. After purification by gel filtration, the second disulfide was formed by treatment with iodine in acidic aqueous methanol. Nearly identical protection strategies have been used by other workers to prepare peptides in the conotoxin family *(42,44,46,47)*, the major differences being that initial chain assembly was by Boc solid-phase chemistry, and in some cases, *S*-Mob was used in place of *S*-Meb and oxidation at the bis(Acm), bis(thiol) stage was carried out in dilute solution with air instead of $K_3Fe(CN)_6$. The Boc/Meb/

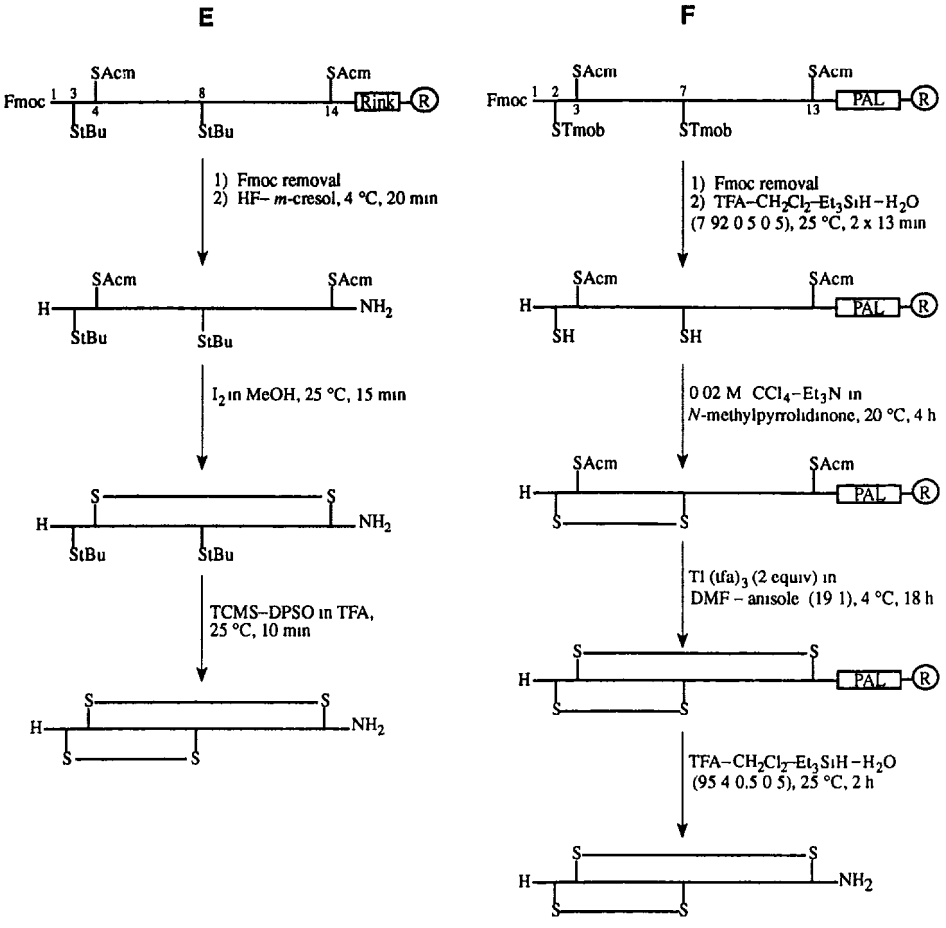
Acm solid-phase strategy can be extended to peptides containing three disulfide bridges, as exemplified by a 13-residue *E. coli* enterotoxin fragment (Scheme 4B). In this partially regioselective approach of Shimonishi and coworkers, two of the disulfide bridges were found to pair correctly by air oxidation of a bis(Acm) precursor, and the third bridge was formed by subsequent iodine treatment. Permutations to the initial protection scheme failed to give the native folded enterotoxin, hence ruling out some alternative possible half-cystine pairings.

Other cysteine-protecting group combinations can also be used for regioselective disulfide formation together with Boc solid-phase synthesis. Ponsati and coworkers demonstrated an orthogonal protection scheme with *S*-Fm and *S*-Meb for the synthesis of a bovine pituitary peptide (Scheme 4C). The novel feature of this synthesis was formation of the first disulfide while the protected peptide remained anchored to the polymeric support (earlier sections of this chapter have discussed advantages of this approach for peptides with single disulfides). The piperidine used to promote simultaneous Fm removal and disulfide formation concomitantly deblocked a Trp(For) in the sequence. Next, the peptide-resin, as well as all remaining protecting groups, were fully cleaved by HF treatment; the strong acid did not affect the already-formed disulfide bridge. Finally, the second disulfide was formed by air oxidation at high dilution. The indicated route was more convenient and provided better results than a synthesis of the same sequence using a Boc/Acm/Meb strategy. Importantly, the unambiguous product formed by regiospecific methods coincided with the *major* thermodynamic product from random oxidation of the tetrathiol peptide, a process that also gave minor regioisomers.

The first regiospecific synthesis compatible with Fmoc chemistry was demonstrated by Atherton and coworkers (Scheme 4D). The conotoxin GI sequence was assembled on a 4-hydroxymethylbenzoyl polyamide support, using *S*-Acm and *S*-S*t*Bu protection. Ammonolysis released the protected peptide amide, and then Cys^2 and Cys^7 were selectively deblocked by phosphine-mediated reduction. The first disulfide formed by slow air oxidation, and the second was formed in the usual way with iodine in acidic aqueous acetic acid.

Akaji and coworkers used Fmoc chemistry to prepare conotoxin MI on Rink resin with *S*-Acm and *S*-*t*Bu (Scheme 4E). It should be noted that all aspects of their strategy appear to be compatible with Boc chem-


A
SMeb
SMeb
Boc
1 2 7 13
NH2
3
SAcm
SAcm
HF – anisole, 0 °C
SH
SH
H
NH2
SAcm
SAcm
K3Fe(CN)6, pH 6 9
S
S
H
NH2
SAcm
SAcm
I2 (15 equiv) + HCl (1 5 equiv)
in MeOH –H2O (4 1)
S
S
H
NH2
S
S
B
SAcm
SAcm
Boc
1 2 5 10 13
R
6
SMeb SMeb
SMeb
SMeb
1) HF – anisole, 0 °C, 1 h
2) air, pH 3
SAcm
SAcm
H
OH
S
S
S
S
I2 (40 equiv) in
MeOH – 0 5 N HCl (4 1),
25 °C, 15 min
S
S
H
OH
S
S
S
S
C
For
SMeb
SMeb
Boc
1 7 10 13 18 21
PAM
R
SFm
SFm
piperidine – DMF (1 1)
25 °C, 3 h
SMeb
SMeb
Boc
PAM
R
S
S
HF – p-cresol (9 1), 0 °C, 1 h
SH
SH
H
OH
S
S
air, pH 8 0, 25 °C, 36 h
S
S
H
OH
S
S
D
SAcm
SAcm
Fmoc
1 2 7 13
HMB
R
3
StBu
StBu
1) Fmoc removal
2) TFA–H2O (19 1)
3) NH3 (sat) – MeOH
SAcm
SAcm
H
NH2
StBu
StBu
1) Bu3P, pH 7 8, 30 min
2) air, pH 8 3, 25 °C, 4 days
SAcm
SAcm
H
NH2
S
S
I2 (excess) in aq HOAc
+ HCl, 10 min, 25 °C
S
S
H
NH2
S
S



Scheme 4. Regioselective schemes for the preparation of single-chain peptides with multiple disulfide bridges. Only end-group positions and locations of half-cystine residues are shown, and lines in the schematic are not drawn to scale. *See text* for further details. (**A**) Conotoxin GI *(41)*. (**B**) Enterotoxin fragment ST_h (6-18) (*67*; note that for purposes of this scheme, residues have been renumbered). (**C**) Posterior pituitary peptide *(64)*. (**D**) Conotoxin GI *(45)*. (**E**) Conotoxin MI *(39)*. (**F**) α-Conotoxin SI *(48,49)*.

istry with appropriate HF-labile supports. In one variation (not shown in Scheme 4), cleavage with HF–anisole at 4°C for 1 h removed selectively *S*-*t*Bu and all side-chain protecting groups except for *S*-Acm, thus providing the bis(Acm), bis(thiol) linear chain. This intermediate is analogous to

ones already described from Boc/Acm/Meb strategies, so sequential air oxidation and Acm deprotection/co-oxidation (the latter using chemistry of Scheme 2B) gave the desired bicyclic peptide. Alternatively (Scheme 4E), *both* classes of cysteine-protecting groups were stable to HF–*m*-cresol for 20 min at 4°C, under which conditions the anchoring linkage and all other side-chain-protecting groups were cleaved. In this strategy, the first disulfide bridge was formed in highly dilute solution by iodine oxidation of *S*-Acm; following purification, the silyl chloride-sulfoxide method (*see* Scheme 2B) was applied to cleave simultaneously the *t*Bu groups and form the second disulfide. Thus, a common linear precursor can be used to form regioselective disulfide bridges in either of two orders, as controlled by the deprotection conditions.

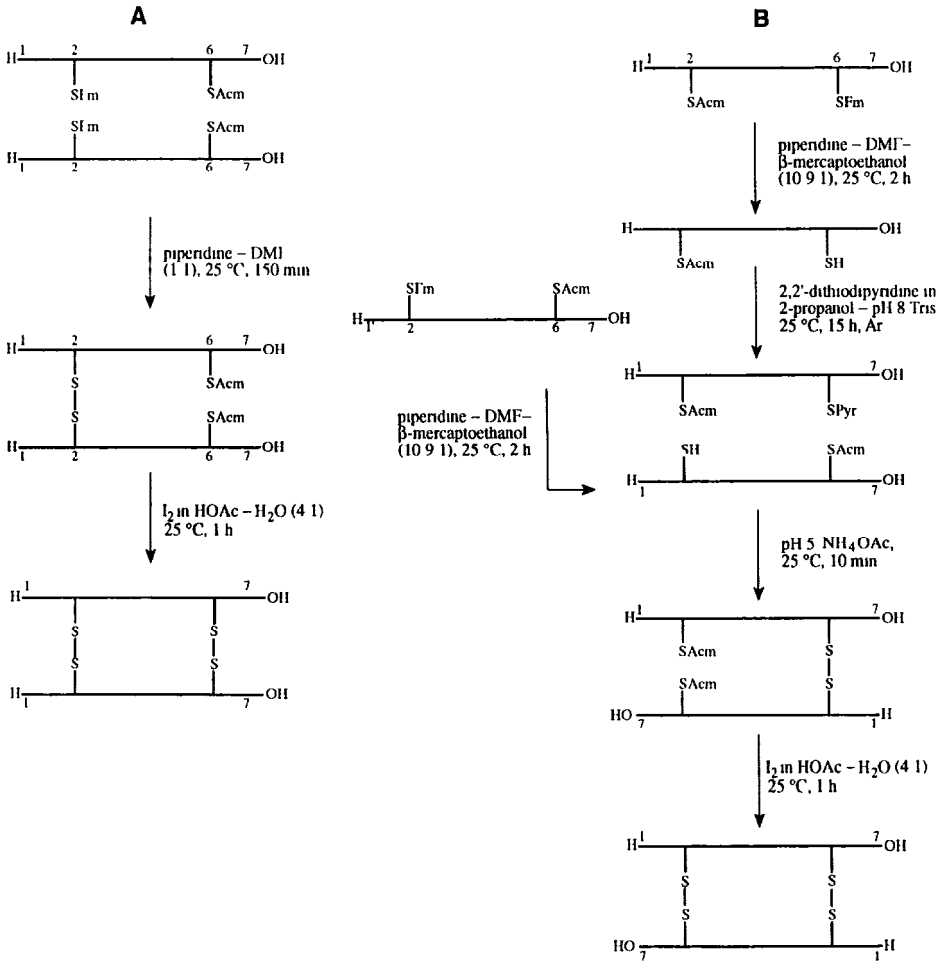
Further concepts are illustrated in several recently devised alternative routes to α-conotoxin SI (Scheme 4F). The linear sequence was assembled by Fmoc chemistry, and cysteine residues were protected by an orthogonal Tmob/Acm combination. Treatment with dilute acid removed *S*-Tmob selectively, to provide the resin-bound bis(Acm), bis(thiol) intermediate suitable for oxidation under mild conditions to close the first disulfide bridge. The resin-bound monocyclic bis(Acm) intermediate was oxidized further with $Tl(tfa)_3$ in DMF–anisole (19:1) (Scheme 2A), and finally the desired bicyclic peptide was released from the acid-labile tris(alkoxy)benzylamide (PAL) support with a TFA/scavengers cocktail. The general approach was varied to explore the order of cyclization (dictated by location of *S*-Tmob and *S*-Acm groups in initial protected sequence), and to assess the relative merits of fully solid-phase vs solution disulfide-forming strategies.

Methods for controlled formation of disulfides also come into play for the synthesis of parallel and antiparallel bis(cystine) dimers, which are worthwhile targets for a variety of applications *(149)*. For instance, some biologically active molecules, such as the hormone β-atrial natriuretic factor (β-ANP) *(36,39,286)* and the progesterone-binding protein uteroglobin *(141,287)*, consist of two identical polypeptide chains linked in antiparallel fashion by two disulfide bridges; the hinge region of immunoglobulins contains a parallel dimer *(139)*; some artificially designed dimers have interesting binding properties due to their symmetry *(280,288)*; and a parallel dimer of deamino-oxytocin has been shown recently to act as a long-lasting prohormogen, presumably by slow disproportionation under physiological conditions *(256)*. Given the regular-

ity with which dimers arise as unwanted byproducts of syntheses directed at monomeric cyclic disulfide peptides, it is interesting how challenging the intentional synthesis of dimers can be (for example, disulfide exchange during formation of the second bridge can give the corresponding cyclic monomer; *see* refs. *256* and *289*).

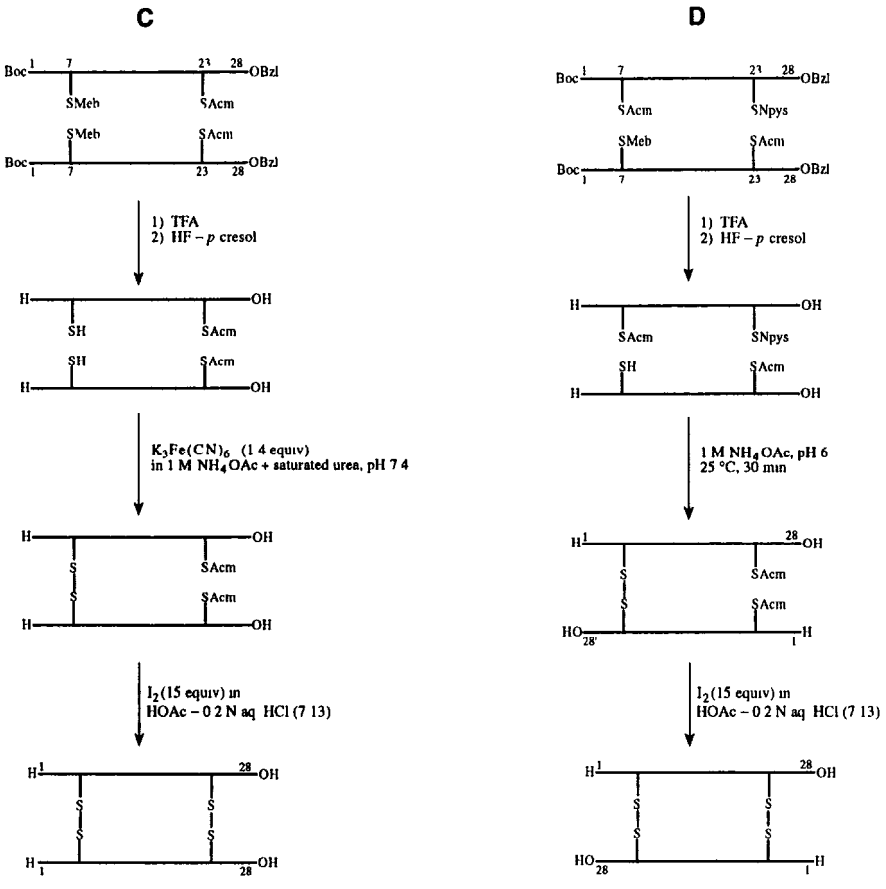
In a prototype example of the kinds of approaches that are possible for the preparation of dimers, Ruiz-Gayo and coworkers carried out Boc solid-phase syntheses of a linear heptapeptide with acid-stable *S*-Fm and *S*-Acm protection (Scheme 5A,B). This was straightforward when the goal was the parallel dimer. For the antiparallel synthesis, the two chains with inverted protection were required; these were obtained after simultaneous elongation carried out on two polymeric supports with different flotation properties. To obtain the parallel dimer, the single linear chain was released from the support by HF, then *S*-Fm removal and simultaneous oxidation was carried in solution by treatment with piperidine–DMF (1:1), and lastly, codeprotection/oxidation of *S*-Acm with iodine in aqueous acetic acid gave the desired product. The orthogonal alternative of oxidizing *S*-Acm first was considered less desirable because of the poor solubility of the resultant bis(Fm) dimer intermediate. To prepare the antiparallel dimer, both of the monomeric chains obtained after HF cleavage were treated with piperidine in the presence of β-mercaptoethanol to furnish the corresponding mono(Acm) peptides with free thiols. One of these chains was activated to furnish its *S*-pyridyl derivative, which was purified and reacted with the second chain in its free thiol form to give the first disulfide (chemistry of Scheme 3B). The second disulfide was closed as before with iodine.

Another set of (chronologically earlier) examples came from Sakakibara's laboratory, where the linear protected sequences of α-human atrial natriuretic peptide (α-hANP) were prepared by solution Boc chemistry (Scheme 5C,D). With the goal of preparing the parallel dimer, *S*-Acm- and *S*-Meb-protecting groups were used, whereas for the antiparallel dimer, the *S*-Npys group (stable to conditions of chain assembly) was needed as well. As in other syntheses described earlier, HF deprotections of these peptides gave either the mono(Acm), mono(thiol) chain, or the mono(Acm), mono(Npys) chain. For the parallel molecule, the first of these chains was oxidized sequentially with $K_3Fe(CN)_6$ and treated with iodine. For the antiparallel molecule, the first of these chains (with free thiol) attacked the second one (with Npys activation) to form a disulfide bridge, and again iodine closed the second bridge. Unfortunately,



Scheme 5. Regioselective schemes for the preparation of representative dimers (single peptide sequence, connected at two points by disulfide bridges). Only end-group positions and locations of half-cystine residues are shown, and lines in the schematic are not drawn to scale. Chains are numbered from *N*- to *C*-terminal, and the second chain has a prime (') numbering. *See text* for further details. (**A**) Parallel uteroglobin-like cavities *(141,149)*. (**B**) Antiparallel uteroglobin-like cavities *(141,149)*.

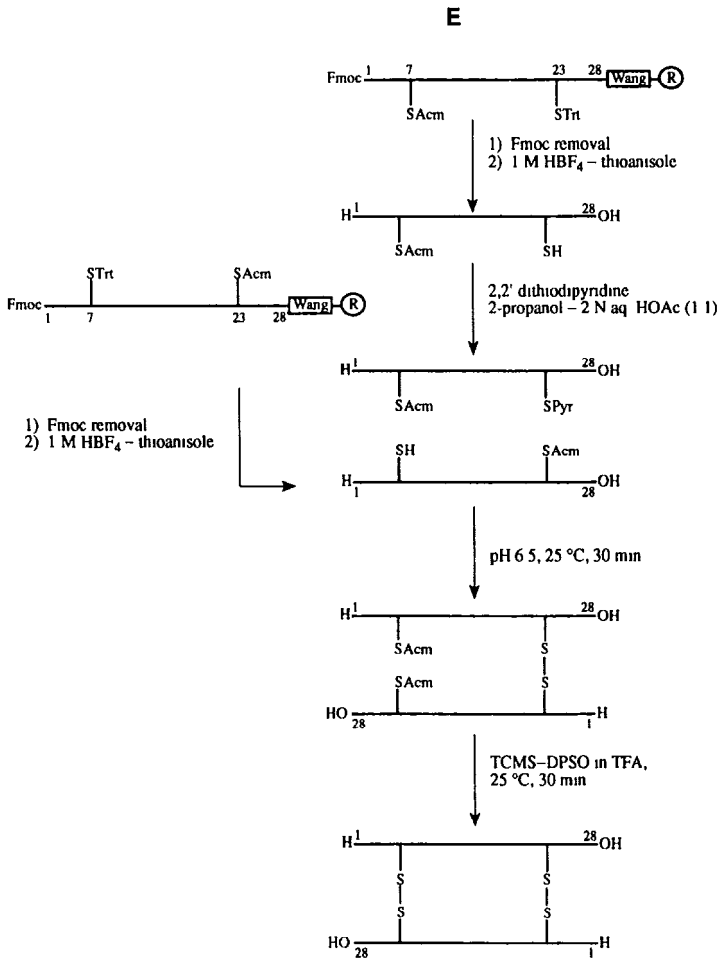
the oxidation was accompanied by several side reactions, including Met(O) formation. A potentially milder route to disulfide cyclic dimers was developed by Wünsch and coworkers *(139)* (chemistry not drawn as part of Scheme 5, but protection scheme chosen similar to Scheme 4D).



Scheme 5. *(continued)* (**C**) Parallel α-human atrial natriuretic peptide dimer *(36)*. (**D**) Antiparallel dimer of α-human natriuretic peptide dimer (synthesis in ref. *36*), which is in fact β-human atrial natriuretic peptide *(286)(continued)*.

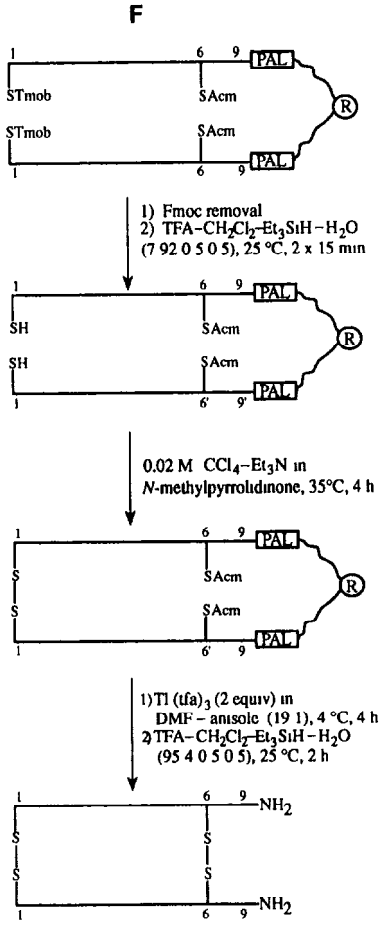
The required linear chains prepared by solution methods with *S*-Acm and *S*-S*t*Bu protection were deblocked selectively with tri-*n*-butyl-phosphine, and the azodicarboxylate oxidation procedure (Scheme 3C) was used to form the first disulfide bridge. The second bridge was closed in the usual way with iodine, and the proper design of precursors allowed preparation of the targets in both the parallel and antiparallel alignments (note, the latter compounds rearranged readily to the former under conditions conducive to thiol–disulfide interchange).

Dimers can also be prepared in conjunction with Fmoc chemistry for linear chain synthesis on *p*-alkoxybenzyl ester and related supports. The



Scheme 5. *(continued)* **(E)** Antiparallel dimer: β-human atrial natriuretic peptide dimer (synthesis in ref. *39*); sequence identical to molecule prepared in (D).

β-human atrial natriuretic peptide already mentioned (Scheme 5D) was prepared in an alternative way by Kiso and coworkers (Scheme 5E). The chains were synthesized twice, with inverted *S*-Acm/*S*-Trt protection on cysteine. Cleavage and partial deprotection with 1*M* HBF_4–thioanisole gave monomeric precursors each containing one Cys(Acm) and one Cys as the free thiol. One of the chains was activated by conversion to the *S*-pyridyl derivative, which was attacked by the free thiol of the other chain. Next, the silyl chloride/sulfoxide method (Scheme 2B) served to remove *S*-Acm and form the second bridge simultaneously.

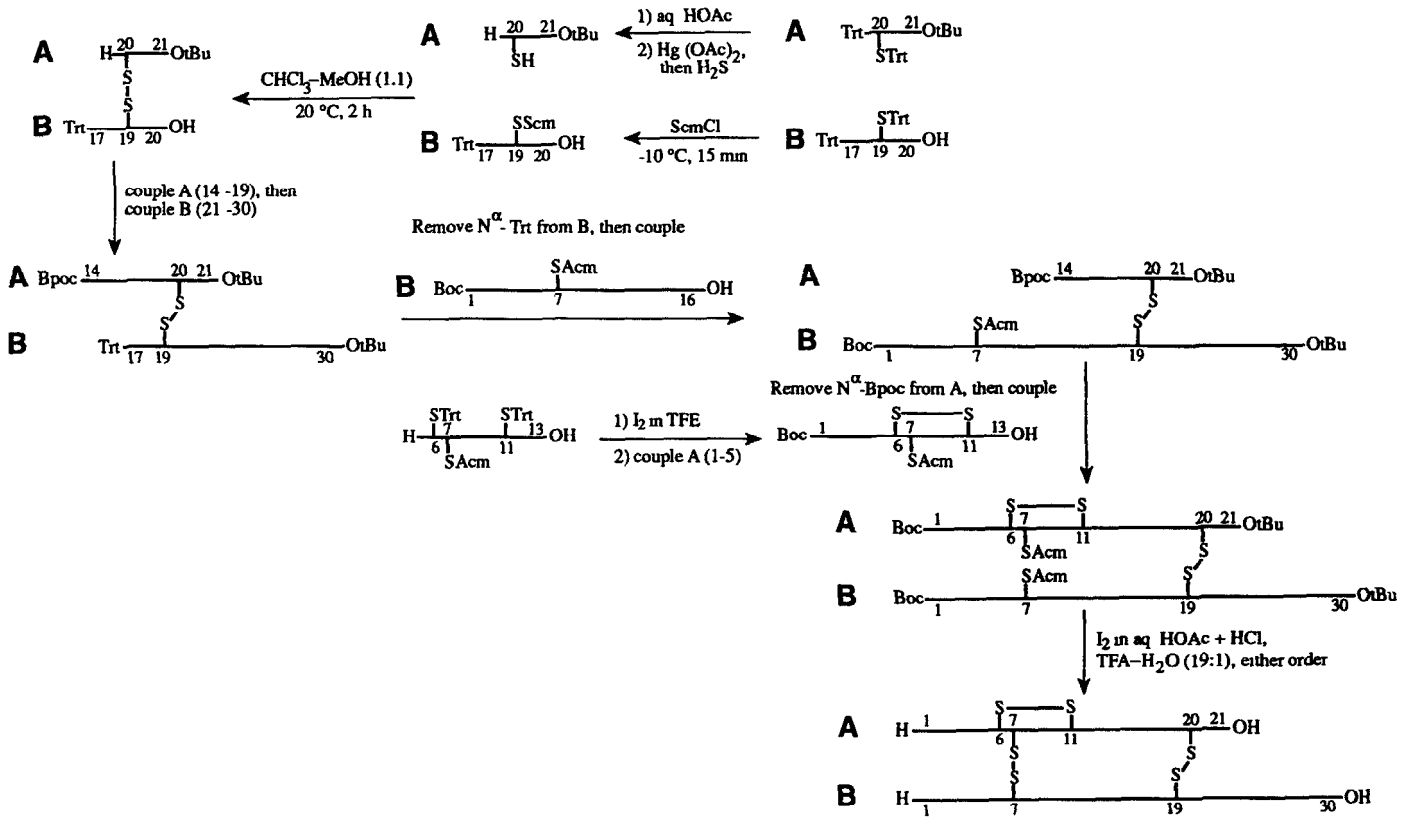


Scheme 5. *(continued)* **(F)** Parallel deamino-oxytocin dimer *(256)*.

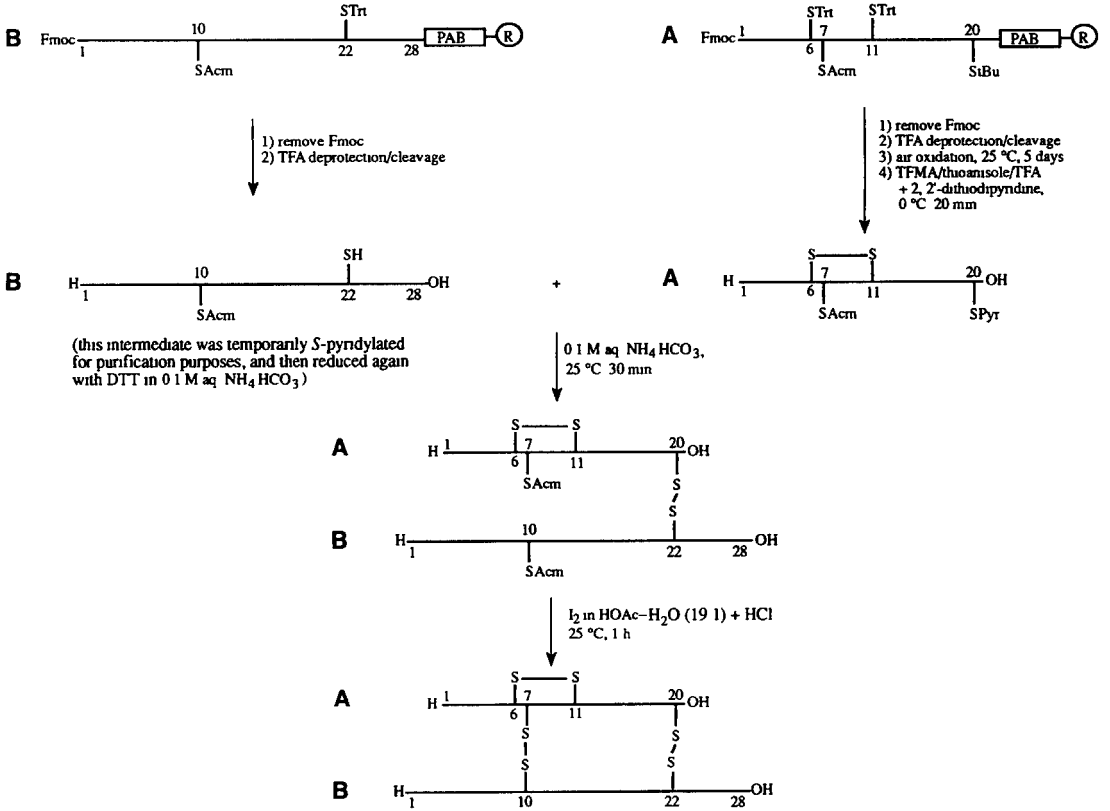
A recent orthogonal synthesis of a parallel dimer of deamino-oxytocin relied on two intermolecular polymer-supported reactions to form disulfide bridges (Scheme 5F). The protection scheme involved acid-labile *S*-Tmob for the *N*-terminal Mpa^1 residue, and *S*-Acm for the internal Cys^6 residue. Selective removal of *S*-Tmob and mild oxidation of the free thiols using CCl_4–Et_3N precluded formation of any intramolecular byproducts, and thus gave the corresponding pseudo-cyclic intermediate in high yield and purity. This resin-bound intermediate was treated with thallium to remove vicinal *S*-Acm groups oxidatively and form the desired target.

The peptide targets described so far have consisted of either a single or else two identical polypeptide chain(s), with regioselective chemistry controlling two disulfide bridges. By contrast, the impressive solution synthesis of crystalline human insulin by Sieber and coworkers from Ciba-Geigy provides a remarkable juxtaposition of regioselective disulfide-forming reactions to connect two chains with two intermolecular and a third intramolecular bridge (Scheme 6). The initial A(20-21)-B(17-20) heterodimer was formed *(205)* by the sulfenyl thiocarbonate fragmentation method (Scheme 3A), i.e., activating the *S*-Trt (or *S*-Acm)-protected B tetrapeptide with ScmCl, and carrying out reaction with the A chain dipeptide counterpart in its free thiol form. Establishment of the two remaining disulfide bonds of insulin took advantage of earlier discoveries by the same research team on the selective iodine oxidation of the *S*-Trt and *S*-Acm groups (Table 3). Thus, the key A(6-13) fragment was formed by intramolecular cyclization of two Cys(Trt) residues in the presence of Cys(Acm) (ref. *182* describes direct synthesis of corresponding A[1-13] fragment). A further noteworthy feature of the synthesis relates to the N^{α}-amino protection scheme and novel deprotection conditions, which facilitated couplings of protected insulin segments containing pre-established disulfide bridges. Similar methods provided two unnatural disulfide bond isomers, that had chromatographic, physico-chemical, and biological properties distinct from the natural material *(94)*.

The bombyxins, peptides of the insulin superfamily isolated from the silkworm, have been targets of recent synthetic efforts using regioselective disulfide formation strategies by Suzuki and coworkers. In one case (bombyxin IV, *see* Scheme 7), the A and B chains were assembled separately by Fmoc solid-phase synthesis on Wang resins and released into solution on treatment with TFA while cleaving simultaneously most of the side-chain-protecting groups. For the A chain, the internal disulfide bridge between positions 6 and 11 (both originally protected as *S*-Trt) formed spontaneously on lengthy stirring of the peptide at high dilution; meanwhile, the Cys(Acm) at position 7 and Cys(*t*Bu) at position 20 were unaffected. Acidolytic cleavage of a $Cys(Acm)^{B10}$, $Cys(Trt)^{B22}$-protected peptide-resin gave a B chain intermediate retaining *S*-Acm protection, for which the free thiol was temporarily *S*-pyridylated to prevent unwanted dimerization. Selective deprotection of *S*-*t*Bu on the aforementioned A chain intermediate already containing a single disulfide proved challenging, even in the presence of a variety of



Scheme 6. Regioselective scheme for the synthesis of human insulin. Adapted from refs. *92* and *93*. Only end-group positions and locations of half-cystine residues are shown, and lines in the schematic are not drawn to scale. A and B chains are numbered from *N*- to *C*-terminal. *See text* for further details.



Scheme 7. Regioselective scheme for the synthesis of bombyxin IV. Adapted from ref. *87*; *see* refs. *88* and *89* for related work by the same authors. Only end-group positions and locations of half-cystine residues are shown, and lines in the schematic are not drawn to scale. A and B chains are numbered from *N*- to *C*-terminal. *See text* for further details.

scavengers. Consequently, a one-pot acid deprotection/simultaneous *S*-pyridylation procedure was devised for the A chain. The *S*-pyridyl-A chain intermediate was then mixed with the free thiol form of the B chain intermediate, thus forming the first interchain disulfide connecting positions A20 and B22 (chemistry of Scheme 3B). The resultant [Cys(Acm)^{A7,B10}] bombyxin IV was purified, and the second interchain disulfide was formed by iodine oxidation under acidic conditions similar to the Ciba-Geigy insulin synthesis.

Human relaxin, another insulin-like peptide, has been prepared by regioselective methods as well, in an elegant study by Büllesbach and Schwabe *(96)*. The A and B chains were assembled by Fmoc and Boc solid-phase chemistry, respectively, and in all, four thiol-protecting groups were used: *S*-Trt, *S*-Acm, *S*-Meb, and *S*-Npys. The A chain was cleaved first with TFA–thiophenol, exposing free thiols at positions 10 and 15 (originally blocked by *S*-Trt), whereas the Cys(Acm) at position 11 and Cys(Meb) at position 24 remained intact. Intramolecular oxidation with iodine formed a cyclic disulfide in the A chain intermediate, which was treated further with HF. The stronger acid step now cleaved selectively *S*-Meb, exposing a free thiol to form an intermolecular bridge by reaction with the *S*-Npys group at position 23 of the HF-cleaved B chain intermediate. Finally, as with the two strategies already described (Schemes 6 and 7), the relaxin synthesis involved iodine oxidation to link Cys(Acm) residues (positions A11 and B11) for the third disulfide bridge. There remained deprotection steps for the Trp(HCO) and Met(O) residues present in the B chain; these were accomplished by applications of aqueous NaOH and ammonium iodide in TFA–water (9:1) respectively, without destroying the assembled disulfide array.

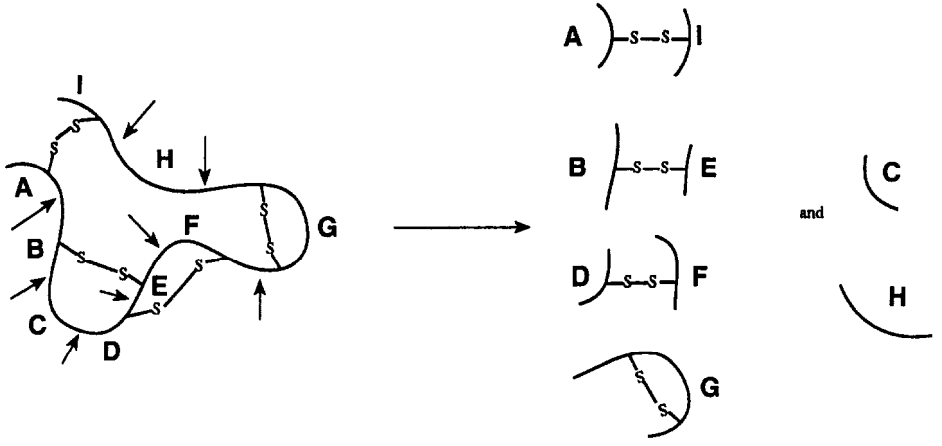
7. Analytical Methods to Establish Disulfide Pairing

The accurate and efficient assignment of cystine connectivities in natural and synthetic peptides is an essential final step for determination and/or proof of structure, yet poses a variety of analytical challenges (for analyses of synthetic peptides, availability of material is generally not a limiting factor, and the order of amino acids does not usually require extensive verification). In the classic insulin work of Sanger and coworkers *(290)*, and in numerous studies carried out since then *(291)*, the plan has been as follows: (1) work out the linear sequence(s) by using materials in which the disulfide bridges have been cleaved and irreversibly

modified, typically by reduction/*S*-alkylation (e.g., dithiothreitol [DTT] or tri-*n*-butylphosphine, followed by iodoacetic acid or 4-vinylpyridine; *see* ref. *292* for protocols and leading references), or by performic acid oxidation; and (2) separately, perform one or more rounds of proteolysis (or less commonly, chemical cleavage) on the natural unmodified material with the goal of obtaining relatively small fragments that retain intact disulfide bridges (Table 4). Once such fragments are isolated in pure form, a process often expedited by diagonal electrophoretic or chromatographic methods *(277,304,319–323)* and by disulfide-specific spectrophotometric, fluorescence, or electrochemical assays *(324–327)*, determinations of their compositions (via amino acid analysis), masses (via mass spectrometry, as described in greater detail below), and/or partial sequences (via Edman degradation) invariably provide more than adequate information to relate each fragment unambiguously to its source(s) in the primary sequence. The positions of the disulfide bridges are then readily deduced (Scheme 8).

Interpretations of fragmentation/peptide mapping data become more intricate when the linear sequence includes consecutive (or nearly so) half-cystines that are paired to different partners; it may be difficult or impossible to cut between such residues, and specialized approaches are necessary (for examples, *see* refs. *67,77,147,300,328*). Another potentially challenging task is to distinguish intermolecularly cyclized parallel and antiparallel dimers: The problem is solved by cutting specifically *between* the relevant half-cystines on *each* chain. The parallel arrangement gives two *different* homodimers, whereas antiparallel structures give a *single* heterodimer (for examples, *see* refs. *141,149,286,329*).

Edman sequential degradation is usually carried out on peptides or proteins in which the disulfide bridges have been modified. However, degradations on *unmodified* materials, either complete or after fragmentation, can be instructive with regard to the locations of multiple disulfides *(47,72,294,314,315)*. When there is a single free *N*-terminus, the cycle corresponding to the first of two paired half-cystines should give a null result, i.e., no phenylthiohydantoin (PTH) derivative is observed. Further in the sequencing process, bis(PTH)-cystine should be noted at the cycle corresponding to the *second* of the paired half-cystines. When there are two chains connected by a disulfide bridge and two *N*-termini, each Edman cycle gives primarily two PTH-derivatives in approximately equimolar amounts. Formation of a significant level of bis(PTH)-cystine



Scheme 8. Classical approach for determination of disulfide alignments in peptides and proteins. A hypothetical single-chain polypeptide with four intramolecular bridges is subjected to fragmentation under conditions that do not promote disulfide scrambling. For clear results, there should be at least one cleavage site between successive half-cystines in the linear sequence. A number of disulfide-bridged peptides (including one with an intact loop) are isolated, as are peptides that lack cysteine. Diagonal methods are based on the premise that after reductive or oxidative cleavage, disulfide-containing peptides give two new peptides (unless the disulfide is in an intramolecular loop), whereas the electrophoretic or chromatographic mobilities of peptides that lack disulfide bridging will not change following such treatments.

at one such cycle allows the conclusion that the appropriate half-cystines located the same number of residues into the sequence on both chains are paired (a situation that occurs quite frequently in natural structures, or that can be arranged by judicious choices of enzymes or reagents for fragmentation). Otherwise, bis(PTH)-cystine will appear at the cycle corresponding to the *later* of the paired half-cystines. While it should be stressed that the experimental design and interpretation requires considerable caution (particularly with regard to stepwise yields; preview and carry-over effects; avoidance of the reducing agents typically added to sequencing reactions; and identification of alternative cystine-derived PTHs, particularly adducts to PTH-dehydroalanine), the approaches outlined have been used successfully to differentiate various isomeric disulfide arrangements connecting as many as three linear chains (references at beginning of this paragraph).

Table 4
Methods for Fragmentation of Peptides and Proteins While Retaining Intact Disulfide Bridges[a]

Name	Specificity[b]	Conditions[c,d]	References[a,e]
Proteolytic enzymes			
Chymotrypsin[f]	Tyr-Y, Phe-Y, Trp-Y (Leu-Y, Met-Y, Ala-Y)	pH 6.5–8 5, 37°C, 2–24 h	*73,77,112, 293–297*
Elastase	Ala-Y, Gly-Y broad specificity	pH 8.0–8 5, 37°C, 1 m*M* $CaCl_2$, 24 h	*295,298,299*
Endoproteinase Glu-C (V8 protease)	Glu-Y (Asp-Y)[b]	pH 4–4.5 (*or* 7 8), 37°C, 2–24 h	*135,293,297, 300*
Endoproteinase Lys-C[f]	Lys-Y	pH 6.5–7.0, 37°C, 2–24 h	*65,72,112, 301*
Endoproteinase Asp-N	X-Asp	pH 7 0–8 0, 37°C, 6–24 h	*299,302*
Pepsin	Phe-Y, Leu-Y pairs of nonpolar residues	pH 1.0–2.5, 37°C, 2–16 h	*301,303–306*
Thermolysin	X-Leu, X-Phe other nonpolar residues	pH 6 5–7.5, 37–65°C, 1 m*M* $CaCl_2$, 2–12 h	*88,107, 113,136, 305–309*
Trypsin[f]	Arg-Y, Lys-Y	pH 6.5–8 5, 37°C, 2–24 h	*73,77,293,294, 297,298,301, 303,310*
Chemical cleavage			
Cyanogen bromide (CNBr)	Met-Y	70% aq. HCOOH *or* 0.1*N* HC1, 25°C, 24 h	*293,296,298, 311*
Edman degradation	N-terminal AA (sequential)	(1) PhNCS in semi-aq media, "pH" 9–10, 25–50°C, 5 min to 1 h; (2) anhydrous TFA, 40°C, 15 min	*118,296,300, 312–315*
Mild acid cleavage	Asp-Pro; other sites depending on peptide structure and acid conditions	70% aq HCOOH; 37–40°C, 24 h *or* 2% aq HCOOH, 110°C, 4 h	*118,290,291, 293,316*

[a]Modified and expanded from tables compiled by Carrey *(317)* The references provided are examples from the protein analytical literature or from analyses of synthetic peptides with multiple disulfide bridges, they are intended to be representative rather than exhaustive. Conditions are listed that minimize scrambling or disproportionation of disulfides. Thiol proteases, such as papain, are not recommended under any circumstances. *See* ref *318* for warning about transpeptidation during proteolysis as a possible source of disulfide misassignments

Another general kind of approach to working out disulfide alignments involves partial reduction of the peptide or protein, followed by peralkylation of the freed sulfhydryls with reagent RX, followed by complete reduction, followed by peralkylation with a different reagent R'X (Scheme 9). The groups R and R' serve to "tag" paired half-cystines, and are located after one or more specific steps to fragment the linearized reduced/alkylated chain. Such approaches are closely allied, in a reciprocal sense, to methodologies being used to identify disulfide-bridged folding intermediates *(4,9,44,330–333)*, and hence subject to the same potential ambiguities with regard to partial scrambling under conditions of alkylation and/or fragmentation.

Recent advances in mass spectrometry have been a major boon to structural work on peptides and small proteins (*291,334–337*, *see also* Chapter 7, *PAP*). When the appropriate instrumentation is on hand and operating well, results are obtained quickly and with relatively little material. An obvious benefit of mass spectrometric techniques is the accurate elucidation of molecular masses of charged biomolecules in the range of up to 10,000 amu for fast atom bombardment mass spectrometry (FABMS), and in excess of 100,000 amu for electrospray mass spectrometry (ESMS) and matrix-assisted laser desorption mass spectrometry (MALD). Furthermore, modern instruments make it possible to analyze *mixtures* (direct probe insertion or on HPLC/MS), and to deduce sequences based on the fragmentation of molecular ions (directly or by tandem MS/MS techniques), hence shortcutting tedious fractionation and selective cleavage or degradation steps that characterize wet protein analytical chemistry (compare to Schemes 8 and 9; *see* refs. *338–343* for specific examples).

The methods for ionization and determination of molecular mass, as listed in the previous paragraph, are readily applicable for the study of

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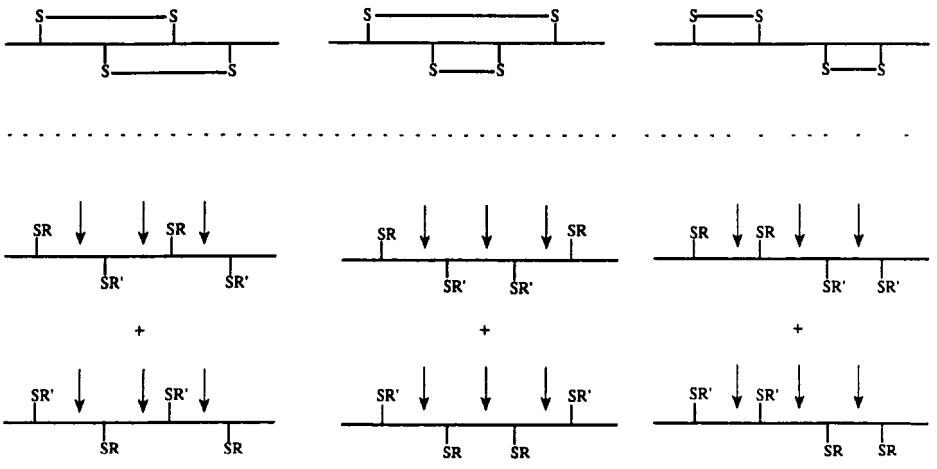
[b]Primary cleavage sites are indicated, followed where appropriate by secondary cleavage sites in parentheses. Digestion with V8 protease at acidic pH is specific adjacent to Glu, whereas at alkaline pH, the specificity is extended to both Glu and Asp peptide bonds

[c]For proteolysis, the enzyme substrate ratio is typically 1 5 to 1 100 (w/w); this table specifies reaction pH, salt if critical, temperature, and time. Cyanogen bromide cleavage is carried out with a 30- to 100-fold molar excess of reagent over Met cleavage sites.

[d]For safe digestion on the higher pH end, an alkylating agent, such as iodoacetate or iodoacetamide, is often added to inhibit disulfide scrambling (e.g., refs. *301,305*).

[e]In many of the references cited, more than one enzyme or chemical reagent is used to achieve secondary cleavage(s) of isolated peptide fragments

[f]Often, trypsin and chymotrypsin are used in combination



Scheme 9. A general approach for distinguishing alternative possible disulfide arrays. In the example schematized above, a hypothetical single-chain polypeptide with two intramolecular bridges is first subjected to *partial* reduction and then alkylated to introduce group R'. There follows complete reduction and further alkylation to introduce group R. To distinguish R and R', they should have different charge and/or mass and/or radiolabels, e.g., R = $[^{14}C]$-CH_2CONH_2 and R' = $[^{3}H]$-CH_2CO_2H, as was chosen by Gray et al. *(44)*. Below the horizontal dotted line are given two possible linear products (not shown are the linear sequence with four R, corresponding to complete reduction, or the linear sequence with four R', corresponding to no reduction, since these will provide no information concerning the original disulfide array). The arrows show desirable sites of cleavage after single or serial cleavages by enzymes or chemical means. Application of one or more of these cleavage procedures will clearly distinguish the alternative disulfide pairings.

disulfide-containing synthetic polypeptides. Clearly, the material to be analyzed needs to be dissolved in a *nonreducing* matrix, e.g., glycerol, *acidified* thioglycerol, *m*-nitrobenzyl alcohol, and mixtures thereof; beyond that, the standard technology can be used *(296,344,345)*. Oxidized and reduced forms of *intramolecularly* linked peptides and proteins are easily distinguished, since the mass differences (2 amu × number of disulfides) are covered by the resolution of routine spectrometers set up for FABMS (provides MH^+) and for ESMS (provides families of ions with $m/z = [M + nH]^+/n$). For asymmetrical intermolecular disulfides, it is difficult to avoid reduction under FABMS conditions, which leads to

weaker quasi-molecular ions (and sometimes pairing information *en passant*; *see* ref. *291* and discussion below), but reduction is *not* a problem with ESMS (e.g., refs. *106,346*). In fact, ESMS can be used to "count" the numbers of free cysteine residues and disulfide-bridged half-cystines, based on mass measurements on unmodified, alkylated, and reduced/alkylated materials *(347)*.

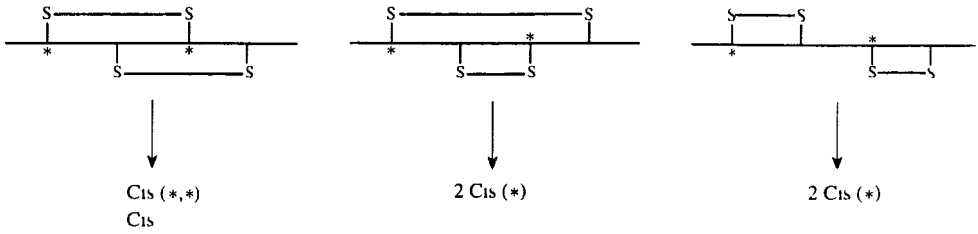
Mass spectrometry can be applied to work out disulfide pairing. In the mass spectrometric analog of diagonal methodology (*see* legend to Scheme 8), disulfide-containing peptides are recognized easily in FABMS or ESMS by the weight change after performic acid oxidation *(348)* or reduction *(349,350)*. Reduction can be carried out as a discrete chemical procedure, or it can occur *in situ* with matrices such as alkaline dithiothreitol *(349,351,352)*. Scission of an intramolecular bridge leaves a single polypeptide, whereas cleavage of intermolecular disulfides gives two daughter peptides. Moreover, in the tandem mass spectrometry mode, intact intermolecular disulfides give rise to strong disulfide cleavage peaks (generally a triplet, for RS^+, RSH^+, and RSH_2^+; *see* ref. *352*). In all of these cases, it then becomes possible to obtain at least partial sequence information on the linearized forms of the previously bridged peptide fragment(s), and this is often enough to make unambiguous assignments.

An elegant approach to disulfide alignment developed by Hidaka and Shimonishi combines chemical synthesis and mass spectrometry (Scheme 10). Deuterio-cysteine is incorporated at specific positions in the linear chain, followed by acid hydrolysis and determination of the isotope distribution in cystine.

Finally, it should be noted that when experimentally feasible, it is desirable to apply NMR or X-ray structural analysis to confirm disulfide connectivities deduced by the chemical and mass spectrometric methods covered in this section.

8. Representative Experimental Procedures

This chapter has been organized in a way that the range of deprotection and oxidation conditions used for the preparation of disulfide-containing peptides and proteins are indicated and discussed extensively within the appropriate sections of the text. For the convenience of the reader who would like a starting point for her or his own research applications, we offer in this section a *few* selected procedures that have been used successfully in our laboratories and/or are reported in the literature (refer-



Scheme 10. A synthetic/mass spectrometric strategy for determining disulfide pairings. Adapted from Hidaka and Shimonishi *(353,354)*. Regular (unlabeled) and heavy (*) cysteine is incorporated into the linear polypeptide by chemical synthesis, and the chain is then allowed to oxidize and fold spontaneously. There follows total acid hydrolysis, under conditions shown in control experiments to minimize (<20%) scrambling of disulfides. Amino acids are then converted to their Fmoc derivatives, and bis(Fmoc)-cystine is isolated by HPLC and analyzed by FABMS. Bis(Fmoc)-cystine gives a monoisotopic MH^+ at 685. Observation of an ion at 689 corresponding to bis(Fmoc)-cystine (*,*) is taken as evidence for the disulfide array at the left of the scheme. Observation of ions at 687 is consistent with the middle and right disulfide arrays. The experiment can be set up in other ways to differentiate further among various possibilities and to solve the problem for molecules with more than four half-cystines.

ences given in the following are not necessarily the primary ones; also for transformations not included, the reader is referred to the publications cited throughout this chapter). We must reiterate and stress that structural and conformational factors are often highly critical, as are solubility considerations and solvent effects. Consequently, experimental conditions for disulfide bond formation usually need to be optimized on a case-by-case basis. Also, workup and purification procedures will vary widely, depending on the properties of the target peptide or protein. Finally, the reader is reminded of the need for careful analytical work to characterize intermediates and completed products *(131,166,292)*.

8.1. Removal of S-Acm *with Hg(II), Followed by Air Oxidation*

This text was adapted from refs. *55* and *187*.

1. Prepare a pH 4.0 solvent by adding a few drops of HOAc to water, and then carry out degassing.
2. Use 2 mL of this degassed solvent to dissolve 20 μmol of an *S*-Acm-protected peptide, in a screw-cap test tube.

3. Add solid $Hg(OAc)_2$ (mol wt 318.7) corresponding to 2.5 Eq/*S*-Acm function. A gray precipitate is noted.
4. The reaction mixture is purged by bubbling through N_2, the tube is sealed and covered with aluminum foil, and stirring is carried out for 70 min at 25°C.
5. 0.45 mL of β-mercaptoethanol is added (at this point the precipitate becomes solubilized), and the mixture is stirred overnight at 25°C.
6. Gel filtration chromatography on a Bio-Gel P2 column (1.2 × 100 cm) equilibrated with 0.1*M* aqueous HOAc removes excess mercury salt and thiol.
7. The column fractions corresponding to the unprotected, reduced peptide are poured directly into 2 L of 0.1*M* Tris-HCl buffer, pH 8.0, and air oxidation is allowed to proceed with slow stirring. Aliquots are removed at different times to monitor the oxidation by HPLC (*see* Chapter 3, *PAP*), Ellman's assay *(238)* and/or biological assay.
8. When oxidation is judged to be complete (e.g., after 2 d), the peptide is purified further by ion-exchange chromatography (*see* Chapters 2 and 5, *PAP*).

8.2. Simultaneous Deprotection/Oxidation of S-Acm *with Iodine*

This text was adapted from refs. *37* and *149*; *see* also ref. *182* for alternative procedures.

1. Prepare a mixture of HOAc and water in a 4:1 ratio (optionally add 1.5 Eq of HCl/*S*-Acm function).
2. Use 100 mL of the aforementioned mixture to dissolve 100 μmol of an *S*-Acm-protected peptide.
3. Add solid iodine (mol wt 253.8) corresponding to 5 Eq/*S*-Acm function. The reaction proceeds with vigorous mixing at 25°C
4. Quench the reaction after 10 min to 1 h (based on HPLC monitoring) by dilution with 100 mL of water.
5. Extract with CCl_4 (4 × 50 mL) to remove the iodine.
6. Lyophilize the aqueous phase, which contains the oxidized (disulfide cyclized) peptide.

8.3. Simultaneous Deprotection/Oxidation of S-Acm *with Tl(III)*

This text was adapted from refs. *49* and *288*.

1. Prepare a mixture of anisole and TFA in a 1:19 ratio.
2. Use 5 mL of this mixture to dissolve 5 μmol of an *S*-Acm-protected peptide, in a screw-cap test tube. Chill to 4°C in an ice bath.
3. Add solid $Tl(tfa)_3$ (mol wt 543.4) **(Caution: handle this reagent with care)** corresponding to 0.6 Eq/S-Acm function.

4. Stir the reaction for 1 h at 4°C.
5. Add 15 mL of ethyl ether to precipitate the peptide, and triturate for 2 min.
6. The peptide is collected by centrifugation, the ether is decanted, and the trituration/centrifugation cycle is repeated two more times to ensure complete removal of the toxic thallium salt.

8.4. Intramolecular Disulfide Cyclization by Potassium Ferricyanide Oxidation

This text was adapted from refs. *24*, *26*, and *33*.

1. The peptide dithiol obtained after a cleavage/deprotection procedure that leaves Cys residues deblocked is dissolved to a concentration of 0.1–1 mg/mL in a suitable buffer, pH 7–8.
2. Over a 30-min period, the peptide solution is titrated at 25°C with 0.01*M* aqueous $K_3Fe(CN)_6$ solution, until a slight yellow color persists (the oxidation can be followed by Ellman analysis, ref. *238*). The amount of oxidant used is typically in 20% or so excess over theory. (An inverse addition procedure at pH 7 is advocated to prevent intermolecular disulfide bond formation by keeping the concentration of sulfhydryl as low as possible during the oxidation.)
3. The pH is adjusted to 5 with 50% aqueous HOAc.
4. The oxidant is removed with AG-3 anion-exchange resin.

8.5. Disulfide Formation Mediated by Glutathione Redox Buffers

This text was adapted from refs. *75*, *79*, *90*, *91*, *101*, and *134*.

1. A buffer of 0.1*M* Tris-HCl, pH 8.0–8.5, is prepared, and used to dissolve both reduced (1–10 m*M*) and oxidized (0.1–1.0 m*M*) glutathione (the molar ratio of reduced to oxidized glutathione is *typically* 10:1, but other ratios can be tried as well).
2. The aforementioned redox buffer is used to dissolve a poly(thiol) peptide that has been previously reduced (and preferably purified, including removal of excess salts and reducing agents). The final concentration should be approx 50 µg/mL.
3. The oxidation reaction is allowed to proceed at 25°C (higher temperature reaction, at 30–35°C, has also been described), and monitored by HPLC.
4. Once an end point is determined, typically in 16 h to 2 d, the oxidized peptide is concentrated by lyophilization, and purified by gel filtration chromatography on Sephadex G-10 or G-25, developed with aqueous buffers at acidic pH. (It is sometimes noted that during this procedure, perma-

nently intractable precipitates form. In such cases, an alternative folding procedure is recommended, in which the peptide is oxidized against a series of redox buffers with a slow pH gradient from 2.2 to 7.0 or 8.0.)

8.6. DMSO-Mediated Disulfide Formation

This text was adapted from ref. *81*.

1. Use HOAc and water (as required) to dissolve 50–100 μmol of a crude peptide that is obtained directly from a cleavage/deprotection procedure that leaves Cys residues deblocked (e.g., HF reaction, followed by extractions to remove organic scavengers).
2. Bring the overall volume to 250 mL, so that the final ratio of HOAc to water is 1:19.
3. Use $(NH_4)_2CO_3$ to bring the pH to 6, and add 50 mL of DMSO.
4. The oxidation reaction is run for 1–4 h at 25°C, and its progress is monitored by HPLC.
5. The reaction mixture is diluted with 2 vol of CH_3CN–0.05% aqueous TFA (1:19) (Buffer A), and loaded directly onto a preparative reversed-phase HPLC column (Vydac, 10 × 25 cm, 5μ particle size).
6. The HPLC purification is developed with a linear gradient of Buffer A and CH_3CN-0.04% aqueous TFA (3:2) (Buffer B).

8.7. Solid-Phase Simultaneous Deprotection/ Oxidation of S*-Fm*

This text was adapted from refs. *64* and *124*.

1. Following completion of solid-phase chain assembly, 0.5 g of a peptide-resin with a substitution level of 0.4–0.6 mmol/g is placed into a reaction vessel for manual synthesis and swollen by repeated washes with CH_2Cl_2 and DMF (10 mL/wash).
2. Five milliliters of a freshly prepared 1:1 mixture of piperidine and DMF are added.
3. Reaction is allowed to proceed for 3 h at 25°C (monitored by a convenient qualitative solid-phase adaptation of Ellman analysis: A 3–5 mg resin aliquot is treated with 1 mL of a 1:1 mixture of DTNB in pH 8.0 phosphate buffer with DMF; a soluble yellow color indicates that free thiol groups remain on the resin, i.e., oxidation is still incomplete; both positive and negative [i.e., with protected Cys] peptide-resin controls should be run concurrently).
4. The anchoring linkage connecting the peptide to the support is cleaved by appropriate methods, and the disulfide-containing peptide is released into solution and isolated further.

9. Conclusions and Future Challenges

The goal of this chapter has been to illustrate different chemical approaches to form one or more disulfide bonds in synthetic peptides and small proteins. Current success in this area (Table 1) can be attributed to a repertoire of cysteine-protecting groups (Table 2) that are applied to selective synthetic strategies. Peptides with a single disulfide bond, e.g., cyclic monomers, homodimers, and heterodimers, can be obtained via a range of the approaches discussed in these pages. For some cyclic structures, the ring size is such (either too small or too large) that the desired intramolecular process is slow with respect to unwanted intermolecular dimerization and oligomerization. Nevertheless, modifications in synthetic strategies and reaction conditions usually suffice to provide at least modest yields of the target molecules. The synthesis of peptides with two disulfides, in any of the three possible intramolecular arrangements as well as parallel or antiparallel dimers, requires considerable experimental skill for optimal results. Regioselective methods for disulfide bond formation have been especially useful in this field. Although it would be premature to conclude that successful results within the endothelin, apamin, conotoxin, and other families can be readily generalized to any given two-disulfide structure, the substantial collective experience acquired to date justifies a certain measure of optimism. By far the greatest challenges to peptide chemists are posed by molecules with three or more disulfide bridges. Increasingly the literature provides accounts of syntheses of this complexity, mostly relying on simultaneous air oxidation for the crucial folding steps. Purifications are often tedious and result in relatively low overall yields, and some published work has been difficult to reproduce in other systems and/or laboratories. This state of affairs punctuates the lack of generality of the modern art, and supports a compelling urgency for the development of improved chemistries, for example multiorthogonal cysteine-protection schemes. With the continued discovery and characterization of biologically active peptides containing multiple disulfide bridges *(135,136,294,295,306,355–358)*, there can be little doubt that the synthetic challenge for peptide chemists will remain undiminished in the coming years.

Acknowledgments

We thank our coworkers and colleagues for sharing their experiences on peptide disulfide chemistry, and single out Miriam Royo and Robert

P. Hammer for especially valuable discussions. Preparation of this chapter and the underlying experimental work from our Barcelona and Minneapolis laboratories were supported by CICYT (PB89-257, PB91-266, PB91-283, and SAL90-828), NIH (GM 28934 and 43552), Commission of the European Communities (SC1-CT91-0748), NATO (Collaborative Research Grants 0841/88 and 92095), and CIRIT (travel grant EE92/1-142).

Abbreviations

Abbreviations used for amino acids and the designations of peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in *J. Biol. Chem.* (1972) **247,** 977–983. The following additional abbreviations are used: Acm, acetamidomethyl; ANF or ANP, atrial natriuretic factor (peptide); Ar, aryl; Boc, *tert*-butyloxycarbonyl; Bpoc, 2-(4-biphenylyl)propyl(2)oxycarbonyl; Bzl, benzyl; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DEAD, diethyl azodicarboxylate; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; Dnpe, 2-(2,4-dinitrophenyl)ethyl; DPSO, diphenyl sulfoxide; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetracetic acid; ESMS, electrospray mass spectrometry; FABMS, fast atom bombardment mass spectrometry; Fm, 9-fluorenylmethyl; Fmoc, 9-fluorenylmethyloxycarbonyl; For, formyl; Gu, guanidine; HF, hydrogen flouride; HFIP, hexafluoroisopropanol; HMB, hydroxymethylbenzoyl linker; HOAc, acetic acid; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; MALD, matrix-assisted laser desorption mass spectrometry; MBHA, 4-methylbenzhydrylamine (resin); Meb, 4-methylbenzyl; MeOH, methanol; Mob, 4-methoxybenzyl; Moz, 4-methoxybenzyloxycarbonyl; Mpa, β-mercaptopropionic acid; Nb, *o*-nitrobenzylamide; NMM, *N*-methylmorpholine; NMP, *N*-methylpyrrolidinone; Nps, 2-nitrophenylsulfenyl; Npys, 3-nitro-2-pyridinesulfenyl; PAB or PAC, *p*-alkoxybenzyl (ester) linker (Wang resin); PAL, tris(alkoxy)benzylamide linker [5-(4-FMOC-)aminomethyl-3,5-dimethoxyphenoxy)valeric acid]; PAM, phenylacetamidomethyl (resin); Ph, phenyl; Phacm, phenylacetamidomethyl; Pixyl, 9-phenylxanthen-9-yl; PTH, phenylthiohydantoin; Pyr, 2-pyridyl; Rink, 4-(2',4'-dimethoxyphenylaminomethyl)phenoxy acid linker; Scb, *tert*-butyloxycarbonylsulfenyl; Sce, ethyloxycarbonylsulfenyl; Scm, *S*-methyloxycarbonylsulfenyl; Snm, (*N'*-methyl-*N'*-phenylcarbamoyl)sulfenyl; S*t*Bu, *tert*-butylmercapto; Sz, benzyloxycarbonylsulfenyl; Tacm, trimethylacetamidomethyl; *t*Bu,

tert-butyl; TCEP, tris(2-carboxyethyl)phosphine; TCMS, trichloromethylsilane; TFA, trifluoroacetic acid; TFE, trifluoroethanol; TFMSA, trifluoromethanesulfonic acid; $Tl(tfa)_3$, thallium (III) trifluoroacetate; Tmob, 2,4,6-trimethoxybenzyl; TMTr, 4,4',4''-trimethoxytriphenylmethyl; Trt, triphenylmethyl; Z, benzyloxycarbonyl. Unless stated otherwise, amino acid symbols denote the L-configuration, and all solvent ratios and percentages are vol/vol.

References

1 Schulz, G. E. and Schirmer, R H (1979) *Principles of Protein Structure*. Springer-Verlag, New York, pp 53–55.

2 Richardson, J. S (1981) The anatomy and taxonomy of protein structure *Adv Prot Chem.* **34,** 167–339.

3. Thornton, J. M. (1981) Disulfide bridges in globular proteins *J Mol. Biol* **151,** 261–287.

4. Creighton, T. E (1986) Disulfide bonds as probes of protein folding pathways. *Methods Enzymol.* **131,** 83–106.

5 Creighton, T. E. (1988) Disulphide bonds and protein stability *BioEssays* **8,** 57–63

6 Srinivasan, N., Sowdhamini, R , Ramakrishnan, C., and Balaram, P (1990) Conformations of disulfide bridges in proteins. *Int. J. Peptide Protein Res* **36,** 147–155, and references cited therein.

7. Branden, C. and Tooze, J. (1991) *Introduction to Protein Structure*. Garland, New York.

8. Rizo, J. and Gierasch, L. M. (1992) Constrained peptides: models of bioactive peptides and protein substructures. *Annu. Rev. Biochem.* **61,** 387–418.

9. Creighton, T. E. (ed.) (1992) *Protein Folding*. W. H. Freeman, New York, especially Chapter 7, Folding pathways determined using disulfide bonds, by Creighton, T. E., pp. 301–351.

10. Creighton, T. E. (1993) *Proteins–Structure and Molecular Properties*, 2nd ed , W. H. Freeman, New York.

11. Schiller, P. W., Eggimann, B., DiMaio, J., Lemieux, C., and Nguyen, T M -D. (1981) Cyclic enkephalin analogs containing a cystine bridge. *Biochem Biophys. Res. Commun.* **101,** 337–343.

12. Hruby, V. J. (1982) Conformational restrictions of biologically active peptides via amino acid side chain groups. *Life Sciences* **31,** 189–199.

13. Hruby, V J , Al-Obeidi, F., and Kazmierski, W. (1990) Emerging approaches in the molecular design of receptor-selective peptide ligands. conformational, topographical and dynamic considerations. *Biochem J.* **268,** 249–262.

14. Bolin, D R., Cottrell, J., Garippa, R., O'Neill, N., Simko, B., and O'Donnell, M. (1993) Structure-activity studies of vasoactive intestinal peptide (VIP). cyclic disulfide analogs. *Int. J. Peptide Protein Res.* **41,** 124–132, and references cited therein

15. Pohl, M., Ambrosius, D., Grötzinger, J., Kretzschmar, T , Saunders, D., Wollmer, A., Brandenburg, D , Bitter-Suermann, D., and Höcker, H. (1993) Cyclic disul-

fide analogues of the complement component C3a. Synthesis and conformational investigations. *Int. J. Peptide Protein Res.* **41,** 362–375.
16. Wetzel, R. (1987) Harnessing disulfide bonds using protein engineering. *Trends Biochem. Sci.* **12,** 478–482, and references cited therein.
17. Hodges, R. S., Zhou, N. E., Kay, C. M , and Semchuk, P. D. (1990) Synthetic model proteins: contribution of hydrophobic residues and disulfide bonds to protein stability. *Peptide Res* **3,** 123–137, and other recent contributions from this research team.
18. Kanaya, S., Katsuda, C., Kimura, S., Nakai, T., Kitakuni, E., Nakamura, H., Katayanagi, K., Morikawa, K., and Ikehara, M. (1991) Stabilization of *Escherichia coli* ribonuclease H by introduction of an artificial disulfide bond. *J. Biol. Chem.* **266,** 6038–6044, and references cited therein.
19. Shortle, D. (1992) Mutational studies of protein structures and their stabilities. *Quart. Rev. Biophys.* **25,** 205–250, and references cited therein.
20. du Vigneaud, V., Ressler, C., Swan, J. M., Roberts, C. W., Katsoyannis, P G., and Gordon, S. (1953) The synthesis of a peptide with the hormonal activity of oxytocin *J. Am. Chem. Soc.* **75,** 4879–4880.
21. Kikuchi, T , Némethy, G., and Scheraga, H. A. (1986) Spatial geometric arrangements of disulfide-crosslinked loops in proteins *J Comp. Chem.* **7,** 67–88
22. Mao, B. (1989) Molecular topology of multiple-disulfide polypeptide chains *J. Am. Chem. Soc.* **111,** 6132–6136.
23. Warne, N. W. and Laskowski, Jr., M (1990) All fifteen possible arrangements of three disulfide bridges in proteins are known. *Biochem. Biophys. Res. Commun* **172,** 1364–1370.
24. Hope, D. B., Murti, V V S , and du Vigneaud, V. (1962) A highly potent analogue of oxytocin, desamino-oxytocin *J Biol. Chem.* **237,** 1563–1566
25 Hruby, V. J., Upson, D. A , and Agarwal, N. S. (1977) Comparative use of benzhydrylamine and chloromethylated resins in solid-phase synthesis of carboxamide terminal peptides. Synthesis of oxytocin derivatives. *J. Org. Chem.* **42,** 3552–3556.
26. Live, D. H., Agosta, W. C., and Cowburn, D. (1977) A rapid, efficient synthesis of oxytocin and 8-arginine-vasopressin. Comparison of benzyl, *p*-methoxybenzyl, and *p*-methylbenzyl as protecting groups for cysteine *J Org. Chem.* **42,** 3556–3561.
27. Hruby, V. J. and Smith, C. W. (1987) Structure-activity relationships of neurohypophyseal peptides, in *The Peptides–Analysis, Synthesis, Biology,* vol. 8 (Udenfriend, S. and Meienhofer, J., eds.; Smith, C. W., vol. ed.), Academic, New York, pp. 77–207.
28. Jost, K., Lebl, M , and Brtník, F. (eds.) (1987) *Handbook of Neurohypophyseal Hormone Analogs,* vols. I and II, CRC, Boca Raton, FL.
29. Manning, M. and Sawyer, W. H. (1993) Design, synthesis and some uses of receptor-specific agonists and antagonists of vasopressin and oxytocin. *J Receptor Res.* **13,** 195–214
30. Yamashiro, D. and Li, C. H. (1973) Synthesis of a peptide with full somatostatin activity. *Biochem. Biophys. Res. Commun.* **54,** 882–888.

31 Coy, D. H., Coy, E J , Arimura, A., and Schally, A V. (1973) Solid phase synthesis of a growth hormone-release inhibiting factor. *Biochem. Biophys. Res. Commun* **54,** 1267–1273.
32 Rivier, J. E. F. (1974) Somatostatin. Total solid phase synthesis. *J Am Chem Soc.* **96,** 2986–2992.
33. Rivier, J., Kaiser, R., and Galyean, R. (1978) Solid-phase synthesis of somatostatin and glucagon-selective analogs in gram quantities. *Biopolymers* **17,** 1927–1938, and references cited therein.
34. Chang, C.-D., Felix, A. M., Jimenez, M. H., and Meienhofer, J. (1980) Solid-phase synthesis of somatostatin using mild base cleavage of N^{α}-9-fluorenylmethyloxycarbonylamino acids. *Int. J. Peptide Protein Res.* **15,** 485–494.
35. Moroder, L , Gemeiner, M , Goehring, W., Jaeger, E., Thamm. P., and Wunsch, E. (1981) New synthesis of somatostatin according to the *S-tert*-butylthiocysteine procedure. *Biopolymers* **20,** 17–37.
36. Chino, N., Yoshizawa-Kumagaye, K , Noda, Y., Watanabe, T. X., Kimura, T., and Sakakibara, S. (1986) Synthesis and biological properties of antiparallel and parallel dimers of α-human atrial natriuretic peptide. *Biochem Biophys Res Commun.* **141,** 665–672.
37. Wade, J D , Fitzgerald, S. P., McDonald, M. R , McDougall, J. G., and Tregear, G. W (1986) Solid-phase synthesis of α-human atrial natriuretic factor: comparison of the Boc-polystyrene and Fmoc-polyamide methods. *Biopolymers* **25,** S21–S37.
38. Lyle, T. A., Brady, S. F., Ciccarone, T. M., Colton, C. D., Paleveda, W. J., Veber, D. F., and Nutt, R. F. (1987) Chemical synthesis of rat atrial natriuretic factor by fragment assembly on a solid support *J Org Chem.* **52,** 3752–3759, and references cited therein.
39. Akaji, K., Fujino, K., Tatsumi, T , and Kiso, Y. (1992) Regioselective double disulfide formation using silylchloride-sulfoxide system. *Tetrahedron Lett* **33,** 1073–1076.
40 Sieber, P., Brugger, M., Kamber, B., Riniker, B., and Rittel, W. (1968) Menschliches calcitonin. IV. Die synthese von calcitonin M. *Helv Chim. Acta* **51,** 2057–2061.
41. Nishiuchi, Y. and Sakakibara, S. (1982) Primary and secondary structures of conotoxin GI, a neurotoxic tridecapeptide from a marine snail. *FEBS Lett.* **148,** 260–262.
42. Gray, W. R., Rivier, J. E , Galyean, R., Cruz, L J , and Olivera, B. M. (1983) Conotoxin MI: disulfide bonding and conformational states. *J. Biol. Chem.* **258,** 12,247–12,251.
43. Nishiuchi, Y. and Sakakibara, S (1984) Synthesis of conotoxin MI and GII: structure-activity relationship of conotoxins, in *Peptide Chemistry 1983* (Munekata, E., ed.), Protein Research Foundation, Osaka, Japan, pp. 191–196.
44. Gray, W. R., Luque, F. A., Galyean, R., Atherton, E., Sheppard, R. C., Stone, B. L., Reyes, A., Alford, J., McIntosh, M., Olivera, B. M., Cruz, L. J , and Rivier, J. (1984) Conotoxin GI: disulfide bridges, synthesis, and preparation of iodinated derivatives. *Biochemistry* **23,** 2796–2802.
45. Atherton, E., Sheppard, R. C., and Ward, P. (1985) Peptide synthesis. Part 7. Solid-phase synthesis of conotoxin G1. *J. Chem. Soc , Perkin Trans* **I,** 2065–2073.

46. Myers, R. A., Zafaralla, G. C., Gray, W. R., Abbott, J., Cruz, L. J., and Olivera, B M. (1991) α-Conotoxins, small peptide probes of nicotinic acetylcholine receptors. *Biochemistry* **30,** 9370–9377.
47. Zhang, R and Snyder, G H. (1991) Factors governing selective formation of specific disulfides in synthetic variants of α-conotoxin. *Biochemistry* **30,** 11,343–11,348, and references cited therein.
48 Albericio, F , Royo, M., Munson, M. C., Solé, N. A., Van Abel, R J , Alsina, J , García-Echeverría, C., Slomczyńska, U., Eritja, R., Pons, M., Giralt, E., and Barany, G. (1993) Strategies and tactics for the solid-phase synthesis of cystine-containing peptides, in *Peptide Chemistry 1992* (Yanaihara, N , ed.), Escom, Leiden, The Netherlands, pp. 19–23.
49 Munson, M. C. and Barany, G. (1993) Synthesis of α-conotoxin SI, a bicyclic tridecapeptide amide with two disulfide bridges: illustration of novel protection schemes and oxidation strategies. *J. Am. Chem. Soc.* **115,** 10,203–10,216.
50. Van Rietschoten, J., Granier, C., Rochat, H., Lissitzky, S., and Miranda, F. (1975) Synthesis of apamin, a neurotoxic peptide from bee venom. *Eur. J. Biochem.* **56,** 35–40.
51 Van Rietschoten, J., Pedroso Muller, E., and Granier, C. (1977) Cysteine protection in solid phase synthesis of apamin, in *Peptides Proceedings of the Fifth American Peptide Symposium* (Goodman, M. and Meienhofer, J , eds.), Wiley, New York, pp. 522–524.
52 Cosand, W. L. and Merrifield, R B. (1977) Concept of internal structural controls for evaluation of inactive synthetic peptide analogs: synthesis of [Orn^{13,14}]apamin and its guanidination to an apamin derivative with full neurotoxic activity *Proc. Natl. Acad. Sci. USA* **74,** 2771–2775.
53. Granier, C., Pedroso Muller, E., and Van Rietschoten, J (1978) Use of synthetic analogs for a study on the structure-activity relationship of apamin. *Eur J. Biochem.* **82,** 293–299, and references cited therein
54. Sandberg, B. E. B. and Ragnarsson, U. (1978) Solid phase synthesis of apamin, the principal neurotoxin in bee venom. Isolation and characterization of acetamidomethyl apamin. *Int. J. Peptide Protein Res.* **11,** 238–245.
55. Albericio, F., Granier, C., Labbé-Juillié, C., Seagar, M., Couraud, F., and Van Rietschoten, J. (1984). Solid phase synthesis and HPLC purification of the protected 1-12 sequence of apamin for rapid synthesis of apamin analogues differing in the C-terminal region. *Tetrahedron* **40,** 4313–4326.
56. Koide, T., Otaka, A., Arai, H., Funakoshi, S., Fujii, N., and Yajima, H. (1990) Synthetic studies on cystine-containing peptides using regioselective disulfide bond forming reactions, in *Peptide Chemistry 1989* (Yanaihara, N , ed.), Protein Research Foundation, Osaka, Japan, pp. 171–174.
57. Shih, H. (1993) New approaches to the synthesis of cystine peptides using *N*-iodosuccinimide in the construction of disulfide bridges. *J. Org. Chem.* **58,** 3003–3008.
58. Kumagaye, S.-I , Kuroda, H., Nakajima, K., Watanabe, T. X., Kimura, T , Masaki, T., and Sakakibara, S. (1988) Synthesis and disulfide structure determination of porcine endothelin: an endothelium-derived vasoconstricting peptide. *Int. J. Peptide Protein Res.* **32,** 519–526.

59. Immer, H., Eberle, I., Fischer, W , and Moser, E (1989) Solution-synthesis of endothelin, in *Peptides 1988· Proceedings of the Twentieth European Peptide Symposium* (Jung, G. and Bayer, E., eds.), de Gruyter, Berlin, pp 94–96.
60 Morimoto, H., Fujii, N., Otaka, A., Kuramochi, K., and Yajıma, H. (1989) Silver trifluoromethanesulphonate, as an Acm-deprotecting reagent and its application to the synthesis of endothelin and endothelın-like peptide, in *Peptıde Chemistry 1988* (Ueki, M., ed.), Protein Research Foundation, Osaka, Japan, pp. 211–214.
61. Tam, J. P., Liu, W., Zhang, J.-W , Galantino, M., and de Castıglione, R. (1991) D-Amino acid and alanine scans of endothelin: an approach to study refoldıng intermediates, in *Peptides 1990: Proceedings of the Twenty-First European Peptide Symposium* (Giralt, E. and Andreu, D., eds.), Escom, Leiden, The Netherlands, pp. 160–163.
62. Casaretto, R and Nyfeler, R. (1991) Isolation, structure and activity of a side product from the synthesis of human endothelin, in *Peptides 1990: Proceedings of the Twenty-First European Peptide Symposium* (Gıralt, E and Andreu, D., eds), Escom, Leiden, The Netherlands, pp. 181–182.
63. Hunt, J. T., Lee, V. G., Liu, E. C. K., Moreland, S., McMullen, D., Webb, M. L., and Bolgar, M (1994) Control of peptide disulfide regioisomer formation by mixed cysteine-penicıllamine bridges: applicatıon to endothelin-1. *Int J Pept. Prot Res.* **42,** 249–258.
64. Ponsati, B , Giralt, E., and Andreu, D. (1990) Solid-phase approaches to regiospecific double disulfide formation. Application to a fragment of bovine pituitary peptide *Tetrahedron* **46,** 8255–8266, and references cited therein.
65. Aimoto, S., Hojoh, H., and Takasaki, C. (1990) Studies on the disulfide bridges of sarafotoxins. Chemical synthesis of sarafotoxin S6B and its homologue with dıfferent disulfide bridges. *Bıochemistry Int.* **21,** 1051–1057.
66. Gariépy, J., Judd, A K., and Schoolnik, G. K. (1987) Importance of disulfide bridges in the structure and activity of *Escherichia coli* enterotoxin ST1b. *Proc. Natl. Acad. Sci. USA* **84,** 8907–8911.
67. Shimonıshi, Y., Hidaka, Y., Koizumi, M., Hane, M., Aimoto, S., Takeda, T , Miwatani, T., and Takeda, Y. (1987) Mode of disulfide bond formatıon of a heat-stable enterotoxin (ST_h) produced by a human strain of enterotoxigenic *Escherichia coli. FEBS Lett.* **215,** 165–170.
68. Hidaka, Y., Kubota, H., Yoshımura, S., Ito, H , Takeda, Y., and Shimonishı, Y (1988) Disulfide linkages in a heat-stable enterotoxin (ST_p) produced by a porcıne strain of enterotoxigenic *Escherichia coli. Bull. Chem Soc. Jpn.* **61,** 1265–1271, and other contributions from this research team.
69. Cruz, L. J., Kupryszewski, G., LeCheminant, G. W., Gray, W. R., Olıvera, B. M., and Rivier, J. (1989) μ-Conotoxin GIIIA, a peptide lıgand for muscle sodıum channels: chemical synthesis, radiolabeling, and receptor characterization. *Biochemistry* **28,** 3437–3442
70. Becker, S., Atherton, E., and Gordon, R. D. (1989) Synthesıs and characterizatıon of μ-conotoxın IIIa *Eur J. Biochem* **185,** 79–84

71. Hatanaka, Y., Yoshida, E., Nakayama, H., and Kanaoka, Y. (1990) Synthesis of μ-conotoxin GIIIA: a chemical probe for sodium channels *Chem. Pharm. Bull.* **38,** 236–238.
72. Kubo, S., Chino, N., Watanabe, T. X , Kimura, T., and Sakakibara, S. (1993) Solution synthesis of μ-conotoxin GIIIB: optimization of the oxidative folding reaction. *Peptide Res.* **6,** 66–72.
73. Nishiuchi, Y., Kumagaye, K., Noda, Y., Watanabe, T. X., and Sakakibara, S. (1986) Synthesis and secondary-structure determination of ω-conotoxin GVIA: a 27-peptide with three intramolecular disulfide bonds. *Biopolymers* **25,** S61–S68, and references cited therein.
74. Rivier, J., Galyean, R., Gray, W. R., Azimi-Zonooz, A., McIntosh, J. M., Cruz, L. J., and Olivera, B. M. (1987) Neuronal calcium channel inhibitors Synthesis of ω-conotoxin GVIA and effects on ^{45}Ca uptake by synaptosomes *J Biol. Chem.* **262,** 1194–1198.
75. Pennington, M. W., Festin, S. M., and Maccecchini, M. L. (1991) Comparison of folding procedures on synthetic ω-conotoxin, in *Peptides 1990: Proceedings of the Twenty-First European Peptide Symposium* (Giralt, E. and Andreu, D., eds.), Escom, Leiden, The Netherlands, pp. 164–166.
76. Sabo, T., Gilon, C., Shafferman, A., and Elhanaty, E. (1992) Structure-activity studies of ω-conotoxin: the importance of disulfide bridges for biological activity, in *Peptides–Chemistry and Biology: Proceedings of the Twelfth American Peptide Symposium* (Smith, J. A. and Rivier, J. E., eds.), Escom, Leiden, The Netherlands, pp. 159–160
77 Pennington, M. W., Festin, S. M., Maccecchini, M. L., and Kem, W. R (1992) Synthesis and characterization of a disulfide bond isomer of omega-conotoxin GVIA. *Toxicon* **30,** 755–764
78. Le-Nguyen, D., Nalis, D , and Castro, B. (1989) Solid phase synthesis of a trypsin inhibitor isolated from the Cucurbitaceae *Ecballium elaterium. Int. J. Peptide Protein Res.* **34,** 492–497.
79 Kupryszewski, G., Ragnarsson, U., Rolka, K., and Wilusz, T. (1986) Solid-phase synthesis of trypsin inhibitor CMTI III from squash seeds *(Cucurbita maxima). Int. J. Peptide Protein Res.* **27,** 245–250.
80. Fujii, N., Okamachi, A., Funakoshi, S , Kuroda, M , Hayashi, Y., Yajima, H., Fukada, J., Imura, H., Bessalle, R., and Fridkin, M. (1990) Synthesis and biological activities of rabbit corticostatin (rCS), in *Peptides–Chemistry, Structure and Biology: Proceedings of the Eleventh American Peptide Symposium* (Rivier, J. E. and Marshall, G. R., eds), Escom, Leiden, The Netherlands, pp. 241–243.
81. Tam, J. P., Wu, C.-R , Liu, W , and Zhang, J.-W. (1991) Disulfide bond formation in peptides by dimethyl sulfoxide. Scope and applications. *J. Am. Chem. Soc* **113,** 6657–6662, and references cited therein.
82. Chow, M.-S., Tran, D., Dobbs, C. H., Rao, V. S. V., Hsieh, M. M., Harwig, S. S. L., and Selsted, M. E. (1992) Fmoc solid phase peptide synthesis of defensins, in *Abstracts of the Sixth Symposium of the Protein Society*, p. 135.
83. Rao, A. G , Rood, T., Maddox, J., and Duvick, J (1992) Synthesis and characterization of defensin NP-1. *Int. J. Peptide Protein Res.* **40,** 507–514.

84. Lambert, P., Kuroda, H., Chino, N., Watanabe, T. X., Kimura, T., and Sakakibara, S (1990) Solution synthesis of charybdotoxin (ChTX), a K^+ channel blocker. *Biochem. Biophys Res. Commun.* **170,** 684–690.
85 Cotton, R., Dutta, A. S., Giles, M. B., and Hayward, C F. (1992) Solid phase synthesis of a number of venom toxins containing two to six cysteine residues, in *Peptides–Chemistry and Biology. Proceedings of the Twelfth American Peptide Symposium* (Smith, J. A. and Rivier, J E , eds.), Escom, Leiden, The Netherlands, pp. 639–640.
86. Vita, C., Bontems, F., Roumestand, C., Ménez, A., and Toma, F. (1993) Solid-phase synthesis. structural and functional characterisation of charybdotoxin and of two truncated analogues, in *Peptides 1992: Proceedings of the Twenty-Second European Peptide Symposium* (Schneider, C. H and Eberle, A. N., eds.), Escom, Leiden, The Netherlands, pp. 641–642
87 Maruyama, K , Nagata, K., Tanaka, M , Nagasawa, H., Isogai, A., Ishizaki, H , and Suzuki, A (1992) Synthesis of bombyxin-IV, an insulin superfamily peptide from the silkworm, *Bombyx mori*, by stepwise and selective formation of three disulfide bridges. *J Protein Chem.* **11,** 1–12.
88. Maruyama, K., Nagasawa, H., Isogai, A., Ishizaki, H., and Suzuki, A. (1992) Determination of disulfide bond arrangement in bombyxin-IV, an insulin superfamily peptide from the silkworm, *Bombyx mori*, by combination of thermolysin digestion of natural peptide and selective synthesis of disulfide bond isomers *J Protein Chem* **11,** 13–20.
89. Nagata, K , Marayuma, K., Nagasawa, H , Urushibata, I , Isogai, A., Ishizaki, H , and Suzuki, A. (1992) Bombyxin-II and its disulfide bond isomers: synthesis and activity. *Peptides* **13,** 653–662.
90. Pennington, M. W , Kem, W. R , Norton, R S , and Dunn, B. M. (1990) Chemical synthesis of a neurotoxic polypeptide from the sea anemone *Stichodactyla helianthus. Int. J. Peptide Protein Res.* **36,** 335–343.
91. Pennington, M. W., Kem, W. R., and Dunn, B. M. (1990). Synthesis and biological activity of six monosubstituted analogs of a sea anemone polypeptide neurotoxin. *Peptide Res.* **3,** 228–232.
92. Sieber, P., Kamber, B., Hartmann, A , Johl, A., Riniker, B., and Rittel, W. (1974) Totalsynthese von humaninsulin unter gezielter bildung der disulfidbindungen *Helv. Chem. Acta* **57,** 2617–2621
93 Sieber, P., Kamber, B., Hartmann, A., Jöhl, A , Riniker, B , and Rittel, W. (1977) Totalsynthese von humaninsulin IV. Beschreibung der endstufen. *Helv. Chem Acta* **60,** 27–37, and preceding papers in this series.
94 Sieber, P., Eisler, K , Kamber, B., Riniker, B., Rittel, W , Märki, F., and De Gasparo, M (1978) Synthesis and biological activity of two disulfide bond isomers of human insulin [A7-A11, A6-B7-cystine]- and [A6-A7, A11-B7-cystine]insulin (human). *Hoppe-Seyler's Z Physiol. Chem.* **359,** 113–123.
95. Birr, C and Pipkorn, R. (1979) Fully active insulin by selective formation of the disulfide bridges between a synthetic A-chain and natural B-chain. *Angew. Chem Int. Ed. Engl.* **18,** 536–538.

96. Büllesbach, E. E. and Schwabe, C. (1991) Total synthesis of human relaxin and human relaxin derivatives by solid-phase peptide synthesis and site-directed chain combination. *J. Biol. Chem.* **266,** 10,754–10,761.
97. Wade, J. D., Vandlen, R. L., and Tregear, G. W. (1992) Continuous flow Fmoc-polyamide solid phase synthesis of human gene 1 relaxin, in *Innovation and Perspectives in Solid Phase Synthesis: Peptides, Polypeptides and Oligonucleotides. 1992* (Epton, R., ed.), Intercept Ltd., Andover, England, pp. 485–487.
98. Tam, J. P., Marquardt, H., Rosberger, D. F., Wong, T. W., and Todaro, G. J. (1984) Synthesis of biologically active rat transforming growth factor I. *Nature* **309,** 376–378.
99. Heath, W. F. and Merrifield, R. B (1986) A synthetic approach to structure-function relationships in the murine epidermal growth-factor molecule. *Proc. Natl. Acad. Sci. USA* **83,** 6367–6371.
100. Tam, J. P., Sheikh, M. A., Solomon, D. S , and Ossowski, L. (1986) Efficient synthesis of human type α transforming growth factor: its physical and biological characterization. *Proc. Natl. Acad. Sci. USA* **83,** 8082–8086.
101. Tam, J. P. (1987) Synthesis of biologically active transforming growth factor alpha. *Int. J. Peptide Protein Res.* **29,** 421–431.
102. Lin, Y.-Z , Caporaso, G , Chang, P.-Y., Ke, X.-H , and Tam, J P (1988) Synthesis of biological active tumor growth factor from the predicted DNA sequence of Shope fibroma virus *Biochemistry* **27,** 5640–5645.
103. Tam, J. P., Lin, Y.-Z , Liu, W., Wang, D.-X., Ke, X.-H , and Zhang, J.-W (1991) Mapping the receptor-recognition site of human transforming growth factor-α. *Int. J. Peptide Protein Res.* **38,** 204–211
104. Tan, N. H. and Kaiser, E. T. (1976) Studies on the solid-phase synthesis of bovine pancreatic trypsin inhibitor (Kunitz) and the characterization of the synthetic material. *J. Org. Chem* **41,** 2787–2793.
105. Aimoto, S., Mizoguchi, N., Hojo, H., and Yoshimura, S. (1989) Development of a facile method for polypeptide synthesis. Synthesis of bovine pancreatic trypsin inhibitor (BPTI). *Bull. Chem. Soc. Jpn.* **62,** 524–531.
106. Ferrer, M., Woodward, C., and Barany, G. (1992) Solid-phase synthesis of bovine pancreatic trypsin inhibitor (BPTI) and two analogues. *Int. J. Peptide Protein Res.* **40,** 194–207.
107. Lin, Y -Z., Isaac, D. D., and Tam, J. P. (1990) Synthesis and properties of cholecystokinin-releasing peptide (monitor peptide), a 61-residue trypsin inhibitor. *Int. J. Peptide Protein Res.* **36,** 433–439.
108. Chino, N., Kubo, S., Nishiuchi, Y., Kumagaye, S.-I., Yoshizawa-Kumagaye, K., Takai, M., Kimura, T., and Sakakibara, S. (1988) Synthesis of porcine C5a anaphylatoxin by the solution procedure and confirmation of the reported structure. *Biochem. Biophys. Res. Commun.* **161,** 1285–1292
109. Kimura, T., Chino, N., Kumagaye, S.-I., Kuroda, H., Emura, J , and Sakakibara, S. (1990) Total synthesis of human angiogenin by the solution procedure, in *Peptide Chemistry 1989* (Yanaihara, N., ed), Protein Research Foundation, Osaka, Japan, pp. 303–308

110. Nishio, H., Kumagaye, K. Y., Chen, Y -N., Kimura, T., and Sakakıbara, S. (1994) Synthesis and disulfıde structure determination of ω-agatoxin IV, ın *Peptides–Chemistry, Structure and Biology: Proceedings of the Thirteenth Amerıcan Peptide Symposium* (Hodges, R. S. and Smıth, J. A., eds), Escom, Leıden, The Netherlands, pp. 31–33.
111. Garsky, V. M., Lumma, P. K., Freidinger, R. M., Pitzenberger, S. M., Randall, W C , Veber, D. F., Gould, R J., and Friedman, P. A. (1989) Chemical synthesis of echistatin, a potent inhibitor of platelet aggregatıon from *Echıs carinatus*· synthesis and biological activity of selected analogs. *Proc. Natl. Acad. Sci. USA*, **86,** 4022–4026
112 Nishio, H., Kumagaye, S., Kuroda, H., Chino, N., Emura, J., Kimura, T., and Sakakibara, S. (1992) Solution synthesis of Na^+, K^+-ATPase inhıbitor-1 (SPAI-1). *Peptide Res.* **5,** 227–244.
113. Tsunemi, M., Kato, H., Nishiuchi, Y , Kumagaye, S -I., and Sakakibara, S. (1992) Synthesis and structure-actıvity relationships of elafin, an elastase-specific inhıbitor. *Bıochem. Biophys. Res. Commun.* **185,** 967–973.
114. Gutte, B. and Merrifield, R. B. (1971) The synthesıs of rıbonuclease A. *J. Bıol. Chem.* **246,** 1922–1941.
115. Yajıma, H. and Fujii, N. (1981) Studıes on peptides. 103. Chemıcal synthesıs of a crystalline protein with the full enzymatıc actıvity of ribonuclease A *J. Am. Chem. Soc.* **103,** 5867–5871.
116. Bodanszky, M. and Stahl, G. L. (1974) The structure and synthesis of malformin A *Proc. Natl. Acad. Sci. USA* **71,** 2791–2794.
117. Kao, P. N. and Karlin, A. (1986) Acetylcholine receptor binding site contains a disulfide cross-link between adjacent half-cystınyl residues. *J. Bıol. Chem.* **261,** 8085–8088.
118. Marti, T., Rösselet, S. J., Titanı, K , and Walsh, K. A. (1987) Identıfication of disulfide-bridged substructures within human von Willebrand factor. *Biochemıstry* **26,** 8099–8109, and references cited therein
119 Mosckovıtz, R. and Gershon, J. M. (1988) Three possıble disulfides ın the acetylcholine receptor α-subunit. *J. Bıol. Chem.* **263,** 1017–1022
120 Prorok, M. and Lawrence, D S. (1990) An affinity label of absolute peptidıc orıgin. *J. Am. Chem. Soc.* **112,** 8626–8627.
121 Sukumaran, D. K , Prorok, M., and Lawrence, D S (1991) A molecular constraint that generates a cıs peptide bond. *J. Am. Chem Soc.* **113,** 706–707, and references cited therein
122. Brady, S. F , Paleveda, Jr., W J., Arison, B. H , Saperstein, R , Brady, E. J , Raynor, K., Reısıne, T., Veber, D F., and Freıdinger, R M (1993) Aproaches to peptidomimetıcs which serve as surrogates for the *cis* amıde bond novel disulfide-constrained bicyclic hexapeptıde analogs of somatostatin. *Tetrahedron* **49,** 3449–3466.
123. Zhang, R. and Snyder, G H (1989) Dependence of formation of small disulfıde loops in two-cysteine peptides on the number and types of ıntervening amino acids. *J. Biol. Chem.* **264,** 18,472–18,479, and references cited therein
124 Albericio, F., Hammer, R. P., García-Echevarría, C., Molins, M. A., Chang, J. L., Munson, M. C., Pons, M., Giralt, E , and Barany, G. (1991) Cyclızation of

disulfide-containing peptides in solid-phase synthesis. *Int. J. Peptide Protein Res.* **37,** 402–413, and references cited therein.

125. Falcomer, C. M , Meinwald, Y. C., Choudhary, I., Talluri, S , Milburn, P. J., Clardy, J , and Scheraga, H. A. (1992) Chain reversals in model peptides: studies of cystine-containing cyclic peptides. 3 Conformational free energies of cyclization of tetrapeptides of sequence Ac-Cys-Pro-X-Cys-NHMe. *J. Am. Chem. Soc* **114,** 4036–4062, and references cited therein.
126. Creighton, T. E. and Goldenberg, D. P. (1984) Kinetic role of a meta-stable native-like two-disulphide species in the folding transition of bovine pancreatic trypsin inhibitor. *J. Mol. Biol.* **179,** 497–526.
127. Altmann, K.-H. and Scheraga, H. A. (1990) Local structure in ribonuclease A. Effect of amino acid substitutions on the preferential formation of the native disulfide loop in synthetic peptides corresponding to residues Cys^{58}-Cys^{72} of bovine pancreatic ribonuclease A. *J. Am. Chem. Soc.* **112,** 4926–4931, and references cited therein.
128. Noszál, B., Guo, W , and Rabenstein, D. L. (1992) Characterization of the macroscopic and microscopic acid-base chemistry of the native disulfide and reduced dithiol forms of oxytocin, arginine-vasopressin, and related peptides. *J. Org. Chem.* **57,** 2327–2334, and references cited therein.
129. Fiser, A., Cserzo, M , Tudos, E., and Simon, I. (1992) Different sequence environments of cysteines and half cystines in proteins. Application to predict disulfide forming residues *FEBS Lett.* **302,** 117–120, and references cited therein.
130. Mazur, S. and Jayalekshmy, P (1979) Chemistry of polymer-bound *o*-benzyne. Frequency of encounter between substituents on cross-linked polystyrenes. *J Am Chem. Soc.* **101,** 677–683
131. Barany, G and Merrifield, R B. (1979) Solid-phase peptide synthesis, in *The Peptides–Analysis, Synthesis, Biology,* vol 2 (Gross, E. and Meienhofer, J., eds.), Academic, New York, pp. 1–284.
132. Mott, A. W., Slomczyńska, U., and Barany, G. (1986) Formation of sulfur-sulfur bonds during solid-phase peptide synthesis: application to the synthesis of oxytocin, in *Forum Peptides Le Cap d'Agde 1984* (Castro, B and Martinez, J., eds.), Les Impressions Dohr, Nancy, France, pp. 321–324.
133. Tsuji, T., Nakagawa, R., Sugimoto, N., and Fukuhara, K.-I (1987) Characterization of disulfide bonds in recombinant proteins: reduced human interleukin 2 in inclusion bodies and its oxidative refolding. *Biochemistry* **26,** 3129–3134, and references cited therein
134 Sabatier, J.-M., Darbon, H., Fourquet, P., Rochat, H., and Van Rietschoten, J. (1987) Reduction and reoxidation of the neurotoxin II from the scorpion *Androctonus australis Hector. Int. J. Peptide Protein Res.* **30,** 125–134.
135. Iwai, M., Kobayashi, M., Tamura, K., Ishii, Y., Yamada, H., and Niwa, M. (1989) Direct identification of disulfide bond linkages in human insulin-like growth factor I (IGF) by chemical synthesis. *J. Biochem.* **106,** 949–951.
136 Kataoka, H , Li, J. P., Lui, A S. T., Kramer, S. J., and Schooley, D. A. (1992) Complete structure of eclosion hormone of *manduca sexta.* Assignment of disulfide bond location. *Int. J. Peptide Protein Res.* **39,** 29–35.

137. Bernatowicz, M. S., Matsueda, R., and Matsueda, G. R (1986) Preparation of Boc-[*S*-(3-nitro-2-pyridinesulfenyl)]-cysteine and its use for unsymmetrical disulfide bond formation. *Int. J Peptide Protein Res.* **28,** 107–112
138. Romani, S., Moroder, L , Gohring, W., Scharf, R., Wunsch, E., Barde, Y A., and Thoenen, H. (1987). Synthesis of the trypsin fragment 10-25/75-88 of mouse nerve growth factor. *Int. J. Peptide Protein Res.* **29,** 107–117
139. Wünsch, E., Moroder, L., Göhring-Romani, S , Musiol, H.-J., Göhring, W., and Bovermann, G. (1988) Synthesis of the bis-cystinyl-fragment 225-232/225'-232' of the human IgG1 hinge region. *Int. J. Peptide Protein Res.* **32,** 368–383
140. Drijfhout, J. W., Perdijk, E. W., Weijer, W J , and Bloemhoff, W. (1988) Controlled peptide-protein conjugation by means of 3-nitro-2-pyridinesulfenyl protection-activation. *Int. J Peptide Protein Res.* **32,** 161–166
141. Ruiz-Gayo, M., Albericio, F., Pons, M , Royo, M , Pedroso, E., and Giralt, E (1988) Uteroglobin-like peptide cavities I. Synthesis of antiparallel and parallel dimers of bis-cysteine peptides. *Tetrahedron Lett* **29,** 3845–3848.
142. Ponsati, B., Giralt, E., and Andreu, D (1989) A synthetic strategy for simultaneous purification-conjugation of antigenic peptides. *Anal. Biochem.* **181,** 389–395.
143. Albericio, F , Andreu, D , Giralt, E , Navalpotro, C , Pedroso, E , Ponsati, B., and Ruiz-Gayo, M. (1989) Use of the Npys thiol protection in solid phase peptide synthesis. Application to direct peptide-protein conjugation through cysteine residues. *Int. J. Peptide Protein Res.* **34,** 124–128.
144 Chaturvedi, S and Bahl, O. P. (1990) Synthesis of cystine peptides 21-25/70-73 and 35-39/56-59 of the β-subunit of human choriogonadotropin. *Int. J. Peptide Protein Res.* **35,** 133–140.
145. Drijfhout, J. W. and Bloemhoff, W (1991) A new synthetic functionalized antigen carrier. *Int. J. Peptide Protein Res.* **37,** 27–32
146 Tam, J. P. and Shen, Z.-Y (1992) Efficient approach to synthesis of two-chain asymmetric cysteine analogs of receptor-binding region of transforming growth factor-α. *Int. J. Peptide Protein Res* **39,** 464–471.
147 Fischer, P. M. and Howden, M. E. H. (1993) Application of anti-peptide antibodies to the assignment of the inter-chain disulphide bond in tetanus toxin. *Int J Peptide Protein Res.* **41,** 415–419, and references cited therein
148. Simmonds, R. G., Tupper, D. E., and Harris, J. R. (1994) Synthesis of disulfide-bridged fragments of ω-conotoxins GVIA and MVIIA. Use of Npys as a protecting/activating group for cysteine in Fmoc syntheses. *Int J Peptide Protein Res.* **43,** 363–366.
149. Ruiz-Gayo, M., Royo, M., Fernández, I., Albericio, F , Giralt, E , and Pons, M. (1993) Unequivocal synthesis and characterization of a parallel and an antiparallel bis-cystine peptide. *J. Org. Chem* **58,** 6319–6328
150. Kullmann, W. and Gutte, B. (1978) Synthesis of an open-chain asymmetrical cystine peptide corresponding to the sequence A^{18-21}–B^{19-26} of bovine insulin by solid phase fragment condensation. *Int. J Peptide Protein Res.* **12,** 17–26, and references cited therein.

151. Büllesbach, E. E and Schwabe, C. (1992) Sequential synthesis of an unsymmetrical two-chain disulfide peptide on solid-phase *Tetrahedron Lett* **33,** 5881–5884, and references cited therein.
152 Photaki, I. (1976) The role of sulfur in amino acid protective group chemistry, in *Topics in Sulfur Chemistry*, vol. 1 (Senning, A , ed.), Georg Thieme, Stuttgart, pp. 111–183.
153. Hiskey, R. G. (1981) Sulfhydryl group protection in peptide synthesis, in *The Peptides–Analysis, Synthesis, Biology*, vol. 3 (Gross, E. and Meienhofer, J., eds.), Academic, New York, pp. 137–167.
154. König, W and Geiger, R. (1981) Selective formation of the disulfide bond in peptide synthesis, in *Perspectives in Peptide Chemistry* (Eberle, A , Geiger, R., and Wieland, T., eds.), S. Karger, Basel, pp. 31–44.
155. Yajima, H., Fujii, N., Funakoshi, S., Watanabe, T , Murayama, E , and Otaka, A. (1988) New strategy for the chemical synthesis of proteins. *Tetrahedron* **44,** 805–819.
156. Cavelier, F., Daunis, J., and Jacquier, R. (1989) Les réactions régiosélectives de formation de ponts disulfure en synthèse de peptides contenant des cystines. *Bull. Soc. Chim. Fr.*, 788–798.
157. Cavelier, F., Daunis, J., and Jacquier, R. (1990) Etude bibliographique et critique de la protection de la fonction thiol en synthèse peptidique (mise au point). *Bull. Soc. Chim. Fr.*, 210–225.
158 Büllesbach, E. E. (1992) Site-directed disulfide formation in peptide synthesis *Kontakte (Darmstadt)* **1,** 21–29.
159. Oae, S (ed.) (1977) *Organic Chemistry of Sulfur*, Plenum, New York, especially Chapter 4, Thiols, by Ohno, A. and Oae, S , pp. 119–187, and Chapter 7, Disulfides and polysulfides, by Field, L , pp. 303–382.
160 Oae, S. (1991) *Organic Sulfur Chemistry: Structure and Mechanism.* CRC, Boca Raton, FL.
161. Wünsch, E. (ed.) (1974) Synthese von Peptiden, in *Houben-Weyl's Methoden der Organischen Chemie*, vol. 15, 4th ed., parts 1 and 2 (Müller, E., ed.), Georg Thieme, Stuttgart.
162. Bodanszky, M. (1984) *Principles of Peptide Synthesis.* Springer-Verlag, Berlin.
163. Bodanszky, M. and Bodanszky, A. (1984) *The Practice of Peptide Synthesis.* Springer-Verlag, Berlin.
164. Stewart, J. M. and Young, J. D. (1984) *Solid Phase Peptide Synthesis*, 2nd ed., Pierce Chemical Co , Rockford, IL.
165. Atherton, E. and Sheppard, R. C (1989) *Solid Phase Peptide Synthesis· A Practical Approach.* IRL, Oxford.
166. Fields, G. B., Tian, Z., and Barany, G. (1992) Principles and practice of solid-phase peptide synthesis, in *Synthetic Peptides: A User's Guide* (Grant, G A , ed.), W. H. Freeman, New York, pp. 77–183.
167. Fotouhi, N., Galakatos, N G., and Kemp, D. S. (1989) Peptide synthesis by prior thiol capture. 6. Rates of the disulfide bond forming capture reaction and demonstration of the overall strategy by synthesis of the C-terminal 29-peptide sequence

of BPTI. *J. Org. Chem* **54,** 2803–2817, and previous contributions from this research team.

168. du Vigneaud, V , Audrieth, L. F , and Loring, H S. (1930) The reduction of cystine in liquid ammonia by metallic sodium. *J. Am. Chem. Soc.* **52,** 4500–4504.
169 Erickson, B. W. and Merrifield, R. B. (1973) Acid stability of several benzylic protecting groups used in solid-phase peptide synthesis. Rearrangement of *O*-benzyltyrosine to 3-benzyltyrosine. *J Am. Chem. Soc.* **95,** 3750–3756.
170. Heath, W. F , Tam, J. P., and Merrifield, R. B. (1986) Improved deprotection of cysteine-containing peptides in HF. *Int. J. Peptide Protein Res* **28,** 498–507, and references cited therein.
171. Akabori, S., Sakakibara, S., Shimonishi, Y., and Nobuhara, Y. (1964) A new method for the protection of the sulfhydryl group during peptide synthesis. *Bull Chem Soc. Jpn.* **37,** 433–434
172 Nishimura, O., Kitada, C , and Fujino, M (1978) A new method for removing the *S*-*p*-methoxybenzyl and *S*-*t*-butyl groups of cysteine residues with mercuric trifluoroacetate. *Chem. Pharm. Bull.* **26,** 1576–1585.
173 Platen, M. and Steckhan, E. (1984) Oxidative deblocking of the 4-methoxybenzyl thioether protecting group: application to the directed synthesis of poly-cystinyl peptides. *Liebigs Ann. Chem.,* 1563–1576.
174. Fujii, N., Otaka, A , Watanabe, T , Okamachi, A., Tamamura, H , Yajima, H , Inagaki, Y., Nomizu, M., and Asano, K (1989) Silver trifluoromethanesulphonate as an S-deprotecting reagent for the synthesis of cystine peptides. *J. Chem. Soc., Chem. Comm.,* 283–284.
175. Sakakibara, S , Shimonishi, Y , Kishida, Y , Okada, M , and Sugihara, H (1967) Use of anhydrous hydrogen fluoride in peptide synthesis. I. Behavior of various protective groups in anhydrous hydrogen fluoride. *Bull. Chem Soc Jpn* **40,** 2164–2167
176. Pastuszak, J J and Chimiak, A. (1981) *tert*-Butyl group as thiol protection in peptide synthesis. *J. Org. Chem.* **46,** 1868–1873, and references cited therein.
177 Atherton, E., Pinori, M., and Sheppard, R. C. (1985) Peptide synthesis. Part 6 Protection of the sulphydryl group of cysteine in solid-phase synthesis using N_{α}-fluorenylmethoxycarbonylamino acids. Linear oxytocin derivatives. *J Chem Soc., Perkin Trans I,* 2057–2064
178 Ploux, O., Chassaing, G., and Marquet, A (1987) Cyclization of peptides on a solid support Application to cyclic analogs of substance P *Int J. Peptide Protein Res.* **29,** 162–169.
179 McCurdy, S N (1989) The investigation of Fmoc-cysteine derivatives in solid phase peptide synthesis *Peptide Res.* **2,** 147–152
180. Kamber, B and Rittel, W (1968) Eine neue, einfache methode zur synthese von cystinpeptiden. *Helv. Chim. Acta* **51,** 2061–2064
181. Photaki, I., Taylor-Papadimitriou, J., Sakarellos, C., Mazarakis, P., and Zervas, L (1970) On cysteine and cystine peptides. Part V. *S*-trityl- and *S*-diphenylmethylcysteine and -cysteine peptides. *J Chem. Soc. (C),* 2683–2689, and other papers from this research team.
182 Kamber, B., Hartmann, A., Eisler, K , Riniker, B., Rink, H., Sieber, P , and Rittel, W (1980) The synthesis of cystine peptides by iodine oxidation of *S*-trityl-cysteine

and *S*-acetamidomethyl-cysteine peptides. *Helv. Chim. Acta* **63,** 899–915, and references cited therein.

183. Fujii, N., Otaka, A., Funakoshi, S., Bessho, K , Watanabe, T., Akaji, K., and Yajima, H. (1987) Studies on peptides. CLI. Syntheses of cystine-peptides by oxidation of *S*-protected cysteine-peptides with thallium (III) trifluoroacetate *Chem Pharm. Bull.* **35,** 2339–2347, and references cited therein
184. Frankel, A. D., Biancalana, S., and Hudson, D (1989) Activity of synthetic peptides from the Tat protein of human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. USA* **86,** 7397–7401.
185. Munson, M. C., García-Echeverría, C., Albericio, F., and Barany, G. (1992) *S*-2,4,6-Trimethoxybenzyl (Tmob). a novel cysteine protecting group for the N^{α}-9-fluorenylmethyloxycarbonyl (Fmoc) strategy of peptide synthesis. *J. Org. Chem.* **57,** 3013–3018, and references cited therein.
186. Munson, M. C., García-Echeverría, C., Albericio, F., and Barany, G. (1992) Novel cysteine protecting groups for the N^{α}-9-fluorenylmethyloxycarbonyl (Fmoc) strategy of peptide synthesis, in *Peptides–Chemistry and Biology. Proceedings of the Twelfth American Peptide Symposium* (Smith, J. A and Rivier, J. E , eds.), Escom, Leiden, The Netherlands, pp. 605–606.
187 Veber, D., Milkowski, J D , Varga, S L , Denkewalter, R G , and Hirschmann, R. (1972) Acetamidomethyl A novel thiol protecting group for cysteine *J Am Chem. Soc.* **94,** 5456–5461.
188. Kamber, B. (1971) Cystinpeptide aus (*S*-acetamidomethyl-cystein)-peptiden durch oxydation mit jod· die synthese von *cyclo*-L-cystin. *Helv. Chim. Acta* **54,** 927–930.
189 Liu, W., Shiue, G H , and Tam, J P. (1989) A novel strategy for the deprotection of *S*-acetamidomethyl containing peptides: an approach to the efficient synthesis of endothelin, in *Peptides–Chemistry, Structure and Biology: Proceedings of the Eleventh American Peptide Symposium* (Rivier, J. E. and Marshall, G R , eds.), Escom, Leiden, The Netherlands, pp. 271–272.
190 Kiso, Y., Yoshida, M., Kimura, T., Fujiwara, Y, and Shikomura, M. (1989) A new thiol protecting trimethylacetamidomethyl group. Synthesis of a new porcine brain natriuretic peptide using the *S*-trimethylacetamidomethyl-cysteine. *Tetrahedron Lett* **30,** 1979–1982.
191 Greiner, G. and Hermann, P. (1991) Application of penicillin amidohydrolase (PAH) for sulphur and amino deprotection in peptide synthesis, in *Peptides 1990· Proceedings of the Twenty-First European Peptide Symposium* (Giralt, E. and Andreu, D., eds.), Escom, Leiden, The Netherlands, pp. 277–278.
192. Hermann, P. and Schillings, T. (1993) Enzymatic manipulation of protecting groups, in *Peptides 1992: Proceedings of the Twenty-Second European Peptide Symposium* (Schneider, C. H. and Eberle, A. N., eds.), Escom, Leiden, The Netherlands, pp. 411–412, and references cited therein.
193. Royo, M., Alsina, J., Giralt, E., Slomczyńska, U., and Albericio, F (1994). *S*-Phenylacetamidomethyl (Phacm). A versatile cysteine protecting group for Boc and Fmoc solid-phase synthesis strategies, in *Peptides–Chemistry, Structure and Biology: Proceedings of the Thirteenth American Peptide Symposium* (Hodges, R. S. and Smith, J. A., eds.), Escom, Leiden, The Netherlands, pp. 116–118.

194. Bodanszky, M and Bednarek, M. A (1982) Derivatives of S-9-fluorenylmethyl-L-cysteine *Int. J. Peptide Protein Res.* **20,** 434–437.
195 Ruiz-Gayo, M., Albericio, F., Pedroso, E., and Giralt, E. (1986) (*S*)-9-Fluorenylmethyl-L-cysteine, a useful HF-stable derivative for peptide synthesis. *J. Chem. Soc., Chem. Commun.,* 1501–1502.
196. Albericio, F., Nicolás, E., Rizo, J , Ruiz-Gayo, M., Pedroso, E., and Giralt, E. (1990) Convenient syntheses of fluorenylmethyl-based side chain derivatives of glutamic and aspartic acids, lysine, and cysteine. *Synthesis,* 119–122.
197. Royo, M., García-Echeverría, C., Giralt, E., Eritja, R., and Albericio, F. (1992) *S*-2-(2,4-dinitrophenyl)ethyl-L-cysteine: a new derivative for solid-phase peptide synthesis *Tetrahedron Lett* **33,** 2391–2394.
198. Weber, U. and Hartter, P. (1970) *S*-Alkylmercapto-gruppen zum schutz der SH-funktion des cysteins, I: synthese und stabilität einiger *S*-(alkylmerkapto)cysteine *Hoppe-Seyler's Z Physiol. Chem* **351,** 1384–1388
199 Wunsch, E. and Spangenberg, R (1971) Eine neue *S*-schutzgruppe fur cystein, in *Peptides 1969* (Scoffone, E., ed.), North-Holland Publ., Amsterdam, pp. 30–34
200 Eritja, R., Ziehler-Martin, J P , Walker, P. A., Lee, T D , Legesse, K , Albericio, F , and Kaplan, B E. (1987) On the use of *S-t*-butylsulphenyl group for protection of cysteine in solid-phase peptide synthesis using Fmoc-amino acids *Tetrahedron* **43,** 2675–2680
201. Hemmasi, B., Zeng, W , Waidelich, D , and Bayer E (1993) Reaction of HF with peptides containing Cys(StBu) in the presence of different scavengers, in *Peptides 1992: Proceedings of the Twenty-Second European Peptide Symposium* (Schneider, C. H. and Eberle, A. N., eds), Escom, Leiden, The Netherlands, pp. 202–204, and references cited therein.
202. Matsueda, R., Kimura, T , Kaiser, E. T , and Matsueda, G. R (1981) 3-Nitro-2-pyridinesulfenyl group for protection and activation of the thiol function of cysteine. *Chem. Lett.* **6,** 737–740.
203. Ridge, R. J , Matsueda, G. R., Haber, E , and Matsueda, R. (1982) Sulfur protection with the 3-nitro-2-pyridinesulfenyl group in solid-phase peptide synthesis Synthesis of lysine^{8}-vasopressin *Int. J. Peptide Protein Res.* **19,** 490–498.
204. Matsueda, R., Higashida, S., Albericio, F , and Andreu, D (1992) Compatibility of the *S*-(3-nitro-2-pyridinesulfenyl) protecting group with DCC/HOBt coupling chemistry. *Peptide Res.* **5,** 262–263, and references cited therein.
205 Kamber, B. (1973) Die gezielte synthese offenkettiger asymmetrischer cystinpeptide mittels thiol-induzierter fragmentierung von sulfenylthiocarbonaten. Insulinfragmente mit intakter disulfidbrucke A^{20}-B^{19} *Helv. Chim. Acta* **56,** 1370–1381.
206. Hiskey, R. G , Muthukumaraswamy, N., and Vunnam, R. R. (1975) Sulfur-containing polypeptides XVII. The *S*-carbomethoxysulfenyl derivative as a protective group for cysteine. *J. Org. Chem.* **40,** 950–953
207. Schroll, A. L. and Barany, G. (1989) A new protecting group for the sulfhydryl function of cysteine *J Org. Chem.* **54,** 244–247, and references cited therein
208. Atherton, E., Hardy, P. M., Harris, D. E , and Matthews, B H. (1991) Racemization of C-terminal cysteine during peptide assembly, in *Peptides 1990. Proceed-*

ings of the Twenty-First European Peptide Symposium (Giralt, E. and Andreu, D., eds.), Escom, Leiden, The Netherlands, pp. 243–244.

209. Ueki, M. and Shinozaki, K. (1983). Phosphinyl and phosphinothioyl amino acids and peptides. VII. The use of the dimethylphosphinothioyl group as a thio protecting group of cysteine. *Bull Chem. Soc. Jpn.* **56,** 1187–1191.
210 Galakatos, N G. and Kemp, D. S. (1985) New S-protection from known N-protection: thio esters of *N*-urethanyl-*N*-methyl-γ-aminobutyric acid as a class of protective groups for thiols in peptide synthesis. *J. Org. Chem.* **50,** 1302–1304, and references cited therein.
211. Otaka, A., Morimoto, H., Fujii, N., Koide, T., Funakoshi, S., and Yajima, H. (1989) S-Benzyloxymethylcysteine, its properties and application in the synthesis of porcine brain natriuretic peptide (pBNP). *Chem. Pharm. Bull.* **37,** 526–528, and references cited therein.
212. Blake, J., Woodworth, B. A., Litzi-Davis, L., and Cosand, W. L. (1992) Ethylcarbamoyl protection for cysteine in the preparation of peptide-conjugate immunogens. *Int. J. Peptide Protein Res.* **40,** 62–65, and references cited therein.
213. Hallinan, E. A. (1991) Formation of a dehydroalanyl residue from S-benzylcysteine upon HF cleavage of a [Sar^1, Cys^8]-angiotensin II peptide resin *Int J Peptide Protein Res.* **38,** 601–602.
214. Echner, H and Voelter, W. (1992) 9-Phenylxanthen-9-yl- (Pixyl): a new thiol protecting group and its use in solid phase peptide chemistry, in *Innovation and Perspectives in Solid Phase Synthesis: Peptides, Polypeptides and Oligonucleotides. 1992* (Epton, R., ed.), Intercept Ltd., Andover, England, pp. 371–375.
215. King, D S., Fields, C. G., and Fields, G. B. (1990) A cleavage method which minimizes side reactions following Fmoc solid phase peptide synthesis. *Int. J. Peptide Protein Res.* **36,** 255–266.
216. Albericio, F., Kneib-Cordonier, N., Biancalana, S., Gera, L., Masada, R. I., Hudson, D , and Barany, G. (1990) Preparation and application of the 5-(4-(9-fluorenylmethyloxycarbonyl)aminomethyl-3,5-dimethoxyphenoxy)valeric acid (PAL) handle for the solid-phase synthesis of *C*-terminal peptide amides under mild conditions. *J. Org. Chem.* **55,** 3730–3743.
217. Pearson, D. A., Blanchette, M., Baker, M. L., and Guindon, C. A. (1989) Trialkylsilanes as scavengers for the trifluoroacetic acid deblocking of protecting groups in peptide synthesis. *Tetrahedron Lett.* **30,** 2739–2742.
218. Solé, N. A. and Barany, G. (1992) Optimization of solid-phase synthesis of [Ala^8]-dynorphin A. *J. Org. Chem.* **57,** 5399–5403.
219. Brady, S., Paleveda, W. J., and Nutt, R. (1989) Studies of acetamidomethyl as cysteine protection: application in synthesis of ANF analogs, in *Peptides–Chemistry and Biology· Proceedings of the Tenth American Peptide Symposium* (Marshall, G. R., ed.), Escom, Leiden, The Netherlands, pp. 192–194.
220. Mendelson, W. L., Tickner, A. M., Holmes, M. M., and Lantos, I. (1990) Efficient solution phase synthesis of [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid)-2-(*O*-ethyl-D-tyrosine)-4-valine-9-desglycine]arginine vasopressin. *Int J Peptide Protein Res.* **35,** 249–257.

221 Lamthanh, H., Roumestand, C , Deprun, C , and Ménez, A (1993) Side reaction during the deprotection of (*S*-acetamidomethyl)cysteine in a peptide with a high serine and threonine content. *Int. J Peptide Protein Res.* **41,** 85–95, and references cited therein.

222 Inukai, N., Nakano, K , and Murakami, M. (1967) The peptide synthesis. I. Use of the *S*-ethylmercapto group for the protection of the thiol function of cysteine *Bull Chem. Soc. Jpn.* **40,** 2913–2918.

223. Inukai, N., Nakano, K., and Murakami, M. (1968) The peptide synthesis. II. Use of the phenol resin for the peptide synthesis. *Bull. Chem. Soc. Jpn.* **41,** 182–186

224 Nokihara, K. and Berndt, H. (1978) Studies on sulfur-containing peptides: *tert*-butyloxycarbonylsulfenyl and benzyloxycarbonylsulfenyl derivatives as protecting groups for cysteine. *J. Org. Chem.* **43,** 4893–4895

225. Glass, J. D. (1987) Enzymatic manipulation of protecting groups in peptide synthesis, in *The Peptides–Analysis, Synthesis, Biology*, vol 9 (Udenfriend, S and Meienhofer, J., eds.), Academic, San Diego, pp. 167–184

226. Jaenicke, R. and Rudolph, R. (1989) Folding proteins, in *Protein Structure: A Practical Approach* (Creighton, T. E., ed.), IRL, Oxford, pp. 191–223, and references cited therein

227. Narhi, L O., Arakawa, T., McGinley, M. D , Rohde, M F , and Westcott, K. R (1992) Circular dichroism of reduced and oxidized recombinant human epidermal growth factor. *Int. J. Peptide Protein Res.* **39,** 182–187

228. Singh, R and Whitesides, G. M. (1991) A reagent for reduction of disulfide bonds in proteins that reduces disulfide bonds faster than does dithiothreitol. *J. Org. Chem.* **56,** 2332–2337

229. Ranganathan, S. and Jayaraman, N. (1991) Highly efficient propane-1,3-dithiol mediated thiol-disulphide interchange. a facile and clean methodology for S–S reduction in peptides. *J. Chem. Soc., Chem. Commun* , 934–936.

230. Lamoureux, G. V. and Whitesides, G M. (1993) Synthesis of dithiols as reducing agents for disulfides in neutral aqueous solution and comparison of reduction potentials *J. Org. Chem* **58,** 633–641

231. Rüegg, U. T. and Rudinger, J. (1977) Reductive cleavage of cystine disulfides with tributylphosphine *Methods Enzymol.* **47,** 111–116

232. Burns, J. A., Butler, J. C., Moran, J., and Whitesides, G. M. (1991) Selective reduction of disulfides by tris(2-carboxyethyl)phosphine *J Org. Chem* **56,** 2648–2650.

233. Weldon, M. A. and Packman, L C. (1992) Disulphide bridge formation in synthetic peptides—rapid monitoring of the process by capillary electrophoresis, in *Innovation and Perspectives in Solid Phase Synthesis. Peptides, Polypeptides and Oligonucleotides 1992* (Epton, R , ed.), Intercept Ltd., Andover, England, pp 489–493.

234 Chance, R. E., Hoffmann, J. A., Kroeff, E P., Johnson, M. G., Schirmer, E. W , and Bromer, W. W. (1981) The production of human insulin using recombinant DNA technology and a new chain combination procedure, in *Peptides–Synthesis, Structure, Function. Proceedings of the Seventh American Peptide Symposium* (Rich, D. H. and Gross, E., eds.), Pierce Chemical Co , Rockford, IL, pp 721–728.

235. Tarbell, D. S. (1961) The mechanism of oxidation of thiols to disulfides, in *Organic Sulfur Compounds,* vol. 1 (Kharasch, N., ed.), Pergamon, Oxford, pp. 97–102, and references cited therein.
236. Jocelyn, P. C. (1972) *Biochemistry of the SH Group.* Academic, London, especially Chapter 4, Oxidation of thiols, pp. 94–115, and references cited therein.
237. Adamson, J. G. and Lajoie, G. A. (1994) Guanidine hydrochloride assists intramolecular disulfide bond formation in cysteine peptides, in *Peptides–Chemistry, Structure and Biology: Proceedings of the Thirteenth American Peptide Symposium* (Hodges, R. S. and Smith, J. A., eds.), Escom, Leiden, The Netherlands, pp. 44,45.
238. Ellman, G. L. (1959) Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **82,** 70–77
239 Grassetti, D. R. and Murray, J. F., Jr. (1967) Determination of sulfhydryl groups with 2,2'- or 4,4'-dithiodipyridine. *Arch. Biochem. Biophys.* **119,** 41–49.
240. Riddles, P. W., Blakeley, R. L., and Zerner, B. (1979) Ellman's reagent: 5,5'-Dithiobis(2-nitrobenzoic acid)—a reexamination. *Anal. Biochem.* **94,** 75–81.
241. Ashworth, M. R. F. (1976) *The Determination of Sulphur-Containing Groups, vol. 2, Analytical Methods for Thiol Groups.* Academic, London.
242. Zonta, A. and Adermann, K. (1993) Disulfide bridge formation in synthetic urodilatin (CDD/ANP-95-126), in *Peptides 1992 Proceedings of the Twenty-Second European Peptide Symposium* (Schneider, C H. and Eberle, A. N , eds), Escom, Leiden, The Netherlands, pp. 397–398.
243. Wallace, T. J. and Mahon, J. J. (1965) Reactions of thiols with sulfoxides III. Catalysis by acids and bases. *J. Org. Chem.* **30,** 1502–1506, and references cited therein
244. Fujii, N., Otaka, A., Okamachi, A., Watanabe, T., Arai, H., Tamamura, H., Funakoshi, S., and Yajima, H. (1989) Synthetic studies on cystine-containing peptides, in *Peptides 1988: Proceedings of the Twentieth European Peptide Symposium* (Jung, G. and Bayer, E., eds.), Walter de Gruyter & Co., Berlin, pp. 58–60.
245. Le-Nguyen, D. and Rivier, J. (1986) Use of carboethoxysulfenyl chloride for disulfide bond formation *Int. J. Peptide Protein Res.* **27,** 285–292.
246 Hantgan, R R , Hammes, G. G., and Scheraga, H. A. (1974) Pathways of folding of reduced bovine pancreatic ribonuclease. *Biochemistry* **13,** 3421–3431.
247. Karim Ahmed, A., Schaffer, S. W., and Wetlaufer, D. B. (1975) Nonenzymic reactivation of reduced bovine pancreatic ribonuclease by air oxidation and by glutathione oxidoreduction buffers. *J. Biol. Chem.* **250,** 8477–8482
248. Rothwarf, D. M. and Scheraga, H. A. (1993) Regeneration of bovine pancreatic ribonuclease A 1. Steady-state distribution. *Biochemistry* **32,** 2671–2679, and three successive articles in same journal.
249. Pigiet, V. P. and Schuster, B. J. (1986) Thioredoxin-catalyzed refolding of disulfide-containing proteins. *Proc. Natl. Acad. Sci. USA* **83,** 7643–7647.
250. Hillson, D. A., Lambert, N., and Freedman, R. B. (1984) Formation and isomerization of disulfide bonds in proteins: protein disulfide-isomerase. *Methods Enzymol.* **107,** 281–294.
251 Edman, J. C., Ellis, L., Blacher, R. W., Roth, R A., and Rutter, W J. (1985) Sequence of protein disulphide isomerase and implications of its relationship to thioredoxin. *Nature* **317,** 267–270, and references cited therein.

252 Lyles, M. M. and Gilbert, H F. (1991) Catalysis of the oxidative folding of ribonuclease A by protein disulfide isomerase: dependence of the rate on the composition of the redox buffer. *Biochemistry* **30,** 613–619, and references cited therein.

253 Bondi, E., Fridkin, M., and Patchornik, A (1968) The use of polymeric carriers in the synthesis of cyclic peptides containing S–S bonds *Isr. J. Chem.* **6,** 22p.

254 Buchta, R., Bondi, E , and Fridkin, M. (1986) Peptides related to the calcium binding domains II and III of calmodulin. Synthesis and calmodulin-like features *Int. J. Peptide Protein Res.* **28,** 289–297.

255. Wenschuh, E., Heydenreich, M., Runge, R , and Fischer, S. (1989) CCl_4 als mildes oxidans in der schwefelchemie: oxidation von thiolen zu disulfiden *Sulfur Lett.* **8,** 251–260.

256. Munson, M C., Lebl, M., Slaninová, J., and Barany, G. (1993) Solid-phase synthesis and biological activity of the parallel dimer of deamino-oxytocin. *Peptide Res.* **6,** 155–159

257 Ruiz-Gayo, M , Albericio, F , Royo, M , García-Echevarría, C., Pedroso, E , Pons, M., and Giralt, E. (1989) A convenient procedure for synthesis of cystine peptides via iodine oxidation of S-acetamidomethyl-cysteine peptides *An Quim.* **85C,** 116–118.

258. Van Vliet, A., Smulders, R. H. P. H , Rietman, B. H , and Tesser, G I (1992) Protected peptide intermediates using a trityl linker on a solid support, in *Innovation and Perspectives in Solid Phase Synthesis: Peptides, Polypeptides and Oligonucleotides. 1992* (Epton, R., ed.), Intercept, Andover, England, pp 475–477.

259. Sieber, P., Kamber, B., Riniker, B., and Rittel, W (1980) Iodine oxidation of *S*-trityl- and *S*-acetamidomethyl-cysteine-peptides containing tryptophan· conditions leading to the formation of tryptophan-2-thioethers *Helv Chem Acta* **63,** 2358–2363

260 Bishop, P. and Chmielewski, J. (1992) Cyanogen iodide· a new reagent for disulfide bond formation in peptides. *Tetrahedron Lett.* **42,** 6263–6266.

261 Akaji, K., Tatsumi, T., Yoshida, M , Kimura, T., Fujiwara, Y., and Kiso, Y. (1991) Synthesis of cystine-peptide by a new disulfide bond-forming reaction using the silyl chloride–sulphoxide system. *J. Chem. Soc., Chem Commun.,* 167–169.

262. Koide, T , Otaka, A., Suzuki, H , and Fujii, N. (1991) Selective conversion of *S*-protected cysteine derivatives to cystine by various sulphoxide–silyl compound / trifluoroacetic acid systems *Syn. Lett ,* 345–346

263. Akaji, K., Tatsumi, T , Yoshida, M., Kimura, T , Fujiwara, Y , and Kiso, Y. (1992) Disulfide bond formation using the silyl chloride–sulfoxide system for the synthesis of a cystine peptide. *J Am. Chem. Soc* **114,** 4137–4143, and references cited therein.

264. Otaka, A., Koide, T , Shide, A., and Fujii, N. (1991) Application of dimethylsulphoxide (DMSO) / trifluoroacetic acid (TFA) oxidation to the synthesis of cystine-containing peptide. *Tetrahedron Lett.* **32,** 1223–1226

265. Funakoshi, S., Murayama, E , Guo, L., Fujii, N , and Yajima, H. (1988) A modified benzhydrylamine as a handle reagent for the solid phase synthesis of peptide amides based on the fluorenylmethoxycarbonyl method *J. Chem. Soc., Chem Commun.,* 382–384.

266. Seidel, C., Klein, C., Empl, B., Bayer, H., Lin, M., and Batz, H.-G. (1990) Synthesis of cyclic peptide for diagnostic use by oxidation of its resin-bound intermediate, in *Peptides 1990: Proceedings of the Twenty-First European Peptide Symposium* (Giralt, E and Andreu, D., eds.), Escom, Leiden, The Netherlands, pp. 236–237.
267. Albrecht, E., Harada, Y., Cooper, G. J. S., Jones, H., and Lehman de Gaeta, L. S. (1992) Synthesis and activity of human amylin and analogues, in *Peptides–Chemistry and Biology. Proceedings of the Twelfth American Peptide Symposium* (Smith, J. A. and Rivier, J. E., eds.), Escom, Leiden, The Netherlands, pp 441–442.
268. Canas, M., Jodas, G., Albericio, F., Andreu, D., García-Antón, J. M., Parente, A., and Ponsati, B. (1993) Optimization of iodine oxidation methods for S-Acm, S-Trt dicysteinyl-peptides on the resin, in *Peptides 1992. Proceedings of the Twenty-Second European Peptide Symposium* (Schneider, C H. and Eberle, A. N., eds.), Escom, Leiden, The Netherlands, pp. 401–402.
269 Edwards, W. B., Anderson, C. J , Welch, M. J., Fields, C. G., and Fields, G. B. (1994) The synthesis of DTPA-D-Phe^1-octreotide by solid-phase synthesis. *J. Labelled Compds. Radiopharm.* **35,** 359–361.
270. Deadman, U., Lu, X , Moreno, A , Rahman, S., Chino, N , Claeson, G , Kakkar, V. V., and Williams, J A (1993) Novel procedure for on-resin cyclisation via disulphide bond formation in large rings Abstract P314, Thirteenth American Peptide Symposium, Edmonton, Canada
271 Barlos, K., Gatos, D., Kutsogianni, S , Papaphotiou, G., Poulos, C., and Tsegenidis, T. (1991) Solid phase synthesis of partially protected and free peptides containing disulphide bonds by simultaneous cysteine oxidation-release from 2-chlorotrityl resin *Int. J Peptide Protein Res.* **38,** 562–568.
272. Harpp, D. N. and Back, T. G. (1971) The synthesis of some new cysteine-containing unsymmetrical disulfides. *J. Org. Chem.* **36,** 3828–3829.
273. Brois, S. J., Pilot, J F., and Barnum, H. W. (1970) A new pathway to unsymmetrical disulfides. The thiol-induced fragmentation of sulfenyl thiocarbonates. *J. Am. Chem Soc* **92,** 7629–7631
274. Ten Kortenaar, P B W and van Nispen, J. W. (1988) Formation of open-chain asymmetrical cystine peptides on a solid support. Synthesis of pGlu-Asn-Cyt-Pro-Arg-Gly-OH. *Coll Czech. Chem. Commun.* **53,** 2537–2541.
275. Moroder, L., Marchiori, F., Borin, G., and Scoffone, E. (1973) Studies on cytochrome c. Part II. Synthesis of the protected heptapeptide (sequence 17–23) of baker's yeast iso-1-cytochrome c. *Biopolymers* **12,** 493–505.
276. Castell, J. V. and Tun-Kyi, A. (1979) The removal of *S*-cysteine protection by means of 2-pyridine sulfenyl chloride and the subsequent formation of disulfide bonds *Helv. Chim. Acta* **62,** 2507–2510
277. Crimmins, D. L. (1989) Analysis of disulfide-linked homo- and hetero-peptide dimers with strong cation-exchange sulfoethyl aspartamide column. *Peptide Res* **2,** 395–401, and references cited therein.
278. Kimura, T., Matsueda, R , Nakagawa, Y., and Kaiser, E. T. (1982) New reagents for the introduction of the thiomethyl group at sulfhydryl residues of proteins with

concomitant spectrophotometric titration of the sulfhydryls: methyl 3-nitro-2-pyridyl disulfide and methyl 2-pyridyl disulfide. *Anal Biochem.* **122,** 274–282.

279. Ponsati, B., Ruiz-Gayo, M , Giralt, E., Albericio, F., and Andreu, D. (1990) Solid-phase-mediated peptide heterodisulfide formation. *J. Am. Chem. Soc.* **112,** 5345–5347.
280. Royo, M., Albericio, F., Giralt, E., and Pons, M. (1993) Use of cyclic dimers of palindromic peptides for the study of coiled-coils, in *Peptides 1992: Proceedings of the Twenty-Second European Peptide Symposium* (Schneider, C H. and Eberle, A. N., eds.), Escom, Leiden, The Netherlands, pp. 487–488.
281. Baleux, F. and Dubois, P (1992) Novel version of multiple antigenic peptide allowing incorporation on a cysteine functionalized lysine tree. *Int. J. Peptide Protein Res.* **40,** 7–12
282. Mukaiyama, T. and Takahashi, K. (1968) A convenient method for the preparation of unsymmetrical disulfides by the use of diethyl azodicarboxylate *Tetrahedron Lett.* **56,** 5907–5908.
283. Wünsch, E. and Romani, S. (1982) A new method for the selective synthesis of unsymmetrical cystine peptides. *Hoppe Seyler's Z Physiol Chem.* **363,** 449–453.
284. Fujii, N , Otaka, A., Watanabe, T., Arai, H., Funakoshi, S., Bessho, K., and Yajima, H. (1987) Sulphoxide-directed disulphide bond-forming reaction for the synthesis of cystine peptides. *J. Chem. Soc., Chem. Commun.,* 1676–1678.
285. Fujii, N , Watanabe, T., Aotake, T , Otaka, A., Yamamoto, I., Konishi, J., and Yajima, H. (1988) Studies on peptides. CLXII. Synthesis of chicken calcitonin-gene-related peptide (cCGRP) by application of sulphoxide-directed disulfide-bond-forming reaction *Chem. Pharm. Bull.* **36,** 3304–3311, and other contributions from this research team.
286 Kangawa, K., Fukuda, A., and Matsuo, H. (1985) Structural identification of β- and γ-human atrial natriuretic polypeptides. *Nature* **313,** 397–400
287. Nieto, A., Postingl, H., and Beato, M. (1977) Purification and quaternary structure of the hormonally induced protein uteroglobin. *Arch. Biochem. Biophys.* **180,** 82–92.
288 García-Echeverría, C., Albericio, F., Giralt, E., and Pons, M. (1993) Design, synthesis, and complexing properties of (^{1}Cys-$^{1'}$Cys, ^{4}Cys-$^{4'}$Cys)-dithiobis(Ac-L-^{1}Cys-L-Pro-D-Val-L-^{4}Cys-NH_2). The first example of a new family of ion-binding peptides. *J. Am Chem. Soc.* **115,** 11,663–11,670.
289 Hiskey, R. G., Davis, G. W., Safdy, M. E., Inui, T , Upham, R. A., and Jones, W. C., Jr. (1970) Sulfur-containing polypeptides. XIII. Bis cystine peptide derivatives. *J. Org. Chem.* **35,** 4148–4156.
290. Ryle, A. P., Sanger, F., Smith, L. F., and Kitai, R. (1955) The disulphide bonds of insulin. *Biochem. J.* **60,** 541–556.
291. Smith, D. L. and Zhou, Z. (1990) Strategies for locating disulfide bridges in proteins. *Methods Enzymol.* **193,** 374–389, and references cited therein.
292. Inglis, A. S. (1983) Single hydrolysis method for all amino acids, including cysteine and tryptophan. *Methods Enzymol.* **91,** 26–36.
293. McMullen, B A., Fujikawa, K., and Davie, E. W (1991) Location of the disulfide bonds in human plasma prekallikrein. the presence of four novel apple domains in the amino-terminal portion of the molecule. *Biochemistry* **30,** 2050–2056.

294. Sardana, M., Sardana, V., Rodkey, J., Wood, T., Ng, A., Vlasuk, G. P., and Waxman, L. (1991) Determination of disulfide bond pairs and stability in recombinant tick anticoagulant peptide. *J. Biol. Chem.* **266,** 13,560–13,563.
295. Calvete, J. J., Schäfer, W., Soszka, T., Lu, W., Cook, J. J., Jameson, B. A., and Niewiarowski, S (1991) Identification of the disulfide bond pattern in albolabrin, an RGD-containing peptide from the venom of *trimeresurus albolabris*: significance for the expression of platelet aggregation inhibitory activity. *Biochemistry* **30,** 5225–5229.
296. Ishibashi, Y., Kikuchi, T., Wakimasu, M., Mizuta, E., and Fujino, M (1991) Assignment of disulfide bonds in synthetic endothelin-1 isomers by fast atom bombardment mass spectrometry. *Biol. Mass Spectrometry* **20,** 703–708.
297. Bendixen, E., Halkier, T., Magnusson, S., Sottrup-Jensen, L., and Kristensen, T. (1992) Complete primary structure of bovine β_2-glycoprotein I: localization of the disulfide bridges. *Biochemistry* **31,** 3611–3617.
298. Töpfer-Petersen, E., Calvete, J., Schäfer, W., and Henschen, A. (1990) Complete localization of the disulfide bridges and glycosylation sites in boar sperm acrosin. *FEBS Lett.* **275,** 139–142.
299. Calvete, J. J., Wang, Y., Mann, K., Schafer, W., Niewiarowski, S., and Stewart, G. J (1992) The disulfide bridge pattern of snake venom disintegrins, flavoridin and echistatin. *FEBS Lett.* **309,** 316–320, and references cited therein
300. Axelsson, K., Johansson, S., Eketorp, G., Zazzi, H., Hemmendorf, B., and Gellefors, P. (1992) Disulfide arrangement of human insulin-like growth factor I derived from yeast and plasma. *Eur. J. Biochem* **206,** 987–994, and references cited therein.
301. Taniyama, Y., Yamamoto, Y., Kuroki, R., and Kikuchi, M. (1990) Evidence for difference in the roles of two cysteine residues involved in disulfide bond formation in the folding of human lysozyme. *J. Biol. Chem.* **265,** 7570–7575
302. Fabri, L., Nice, E. C., Ward, L. D., Maruta, H., Burgess, A. W., and Simpson, R. J. (1992) Characterization of bovine heparin-binding neurotrophic factor (HBNF): assignment of disulfide bonds. *Biochem. Internat.* **228,** 1–9.
303. Smith, M. C., Cook, J. A., Furman, T. C., and Occolowitz, J. L. (1989) Structure and activity dependence of recombinant human insulin-like growth factor II on disulfide bond pairing. *J. Biol. Chem.* **264,** 9314–9321.
304. Carr, C., Aykent, S , Kimack, N. M., and Levine, A. D. (1991) Disulfide assignments in recombinant mouse and human interleukin 4. *Biochemistry* **30,** 1515–1523
305. Hess, D., Schaller, J., and Rickli, E. E. (1991) Identification of the disulfide bonds of human complement C1s. *Biochemistry* **30,** 2827–2833
306. Ng, N. F. L. and Hew, C. L. (1992) Structure of an antifreeze polypeptide from the sea raven. Disulfide bonds and similarity to lectin-binding proteins. *J. Biol Chem.* **267,** 16,069–16,075.
307. Kumazaki, T. and Ishii, S.-I. (1990) Disulfide bridge structure of ascidian trypsin inhibitor I: similarity to Kazal-type inhibitors. *J Biochem.* **107,** 414–419.
308. Lepage, P., Bitsch, F., Roecklin, D., Keppi, E., Dimarcq, J.-L , Reichhart, J.-M., Hoffman, J. A., Roitsch, C., and Van Dorsselaer, A. (1991) Determination of

disulfide bridges in natural and recombinant insect defensin A. *Eur. J. Biochem.* **196,** 735–742.

309. Violand, B. N., Tou, J. S., Vineyard, B. D., Siegel, N. R., Smith, C E., Pyla, P D , Zobel, J F., Toren, P. C., and Kolodziej, E W. (1991) Determination of the disulfide bond pairings in bovine transforming growth factor-α *Int. J Peptide Protein Res* **37,** 463–467.
310. Huth, J. R., Mountjoy, K., Perini, F., and Ruddon, R. W. (1992) Intracellular folding pathway of human chorionic gonadotropin β subunit. *J. Biol. Chem* **267,** 8870–8879.
311. Gross, E. and Witkop, B. (1962) Nonenzymatic cleavage of peptide bonds. the methionine residues in bovine pancreatic ribonuclease *J. Biol. Chem* **237,** 1856–1860.
312. Edman, P. and Henschen, A. (1975) Sequence determination, in *Protein Sequence Determination· A Sourcebook of Methods and Techniques* (Needleman, S B , ed), Springer-Verlag, New York, pp 232–279.
313. Hunkapiller, M. W. (1988) Gas phase sequence analysis of proteins/peptides, in *Protein/Peptide Sequence Analysis: Current Methodologies* (Brown, A S , ed), CRC, Boca Raton, FL, pp 87–117
314. Burman, S., Wellner, D., Chait, B., Chaudhary, T , and Breslow, E. (1989) Complete assignment of neurophysin disulfides indicates pairing in two separate domains. *Proc. Natl. Acad Sci. USA* **86,** 429–433
315. Haniu, M., Acklin, C., Stoney, K , Kenney, W. C., and Rohde, M F (1994) Direct assignment of disulfide bonds by Edman degradation of selected peptide fragments. *Int. J. Peptide Protein Res.* **43,** 81–86 and references cited therein.
316 Landon, M. (1977) Cleavage at aspartyl-prolyl bonds *Methods Enzymol.* **47,** 145–149
317. Carrey, E A. (1989) Peptide mapping, in *Protein Structure: A Practical Approach* (Creighton, T. E., ed.), IRL, Oxford, pp. 117–144, and references cited therein.
318. Canova-Davis, E , Kessler, T. J., and Ling, V T (1991) Transpeptidation during the analytical proteolysis of proteins. *Anal. Biochem.* **196,** 39–45.
319 Brown, J R. and Hartley, B. S (1966) Location of disulphide bridges by diagonal paper electrophoresis The disulphide bridges of bovine chymotrypsinogen A *Biochem. J* **101,** 214–228
320. Walsh, K A , McDonald, R. M., and Bradshaw, R A (1970) Automatic systems for detecting cystine and cystinyl peptides during column chromatography *Anal Biochem.* **35,** 193–202.
321 Thannhauser, T. W., McWherter, C A., and Scheraga, H. A. (1985) Peptide mapping of bovine pancreatic ribonuclease A by reverse-phase high-performance liquid chromatography. II. A two-dimensional technique for determination of disulfide pairings using a continuous-flow disulfide-detection system. *Anal. Biochem.* **149,** 322–330.
322 Creighton, T. E (1989) Disulphide bonds between cysteine residues, in *Protein Structure: A Practical Approach* (Creighton, T. E., ed.), IRL, Oxford, pp 155–167, and references cited therein.
323 Stone, K. L., Elliott, J. I., Peterson, G., McMurray, W , and Williams, K. R. (1990) Reversed-phase high-performance liquid chromatography for fractionation of

enzymatic digests and chemical cleavage products of proteins. *Methods Enzymol* **193,** 389–412.

324 Thannhauser, T. W., Konishi, Y., and Scheraga, H. A. (1984) Sensitive quantitative analysis of disulfide bonds in polypeptides and proteins. *Anal. Biochem.* **138,** 181–188.

325. Sueyoshi, T., Miyata, T , Iwanaga, S., Toyo'oka, T., and Imai, K (1985) Application of a fluorogenic reagent, ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate for detection of cystine-containing peptides. *J. Biochem.* **97,** 1811–1813.

326. Lazure, C., Rochemont, J., Seidah, N. G., and Chrétien, M. (1985) Novel approach to rapid and sensitive localization of protein disulfide bridges by high-performance liquid chromatography and electrochemical detection *J. Chromatography* **326,** 339–348.

327 Sun, Y , Smith, D. L., and Shoup, R. E (1991) Simultaneous detection of thiol- and disulfide-containing peptides by electrochemical high-performance liquid chromatography with identification by mass spectrometry. *Anal. Biochem.* **197,** 69–76, and references cited therein.

328. Selsted, M. E. and Harwig, S. S. L. (1989) Determination of the disulfide array in the human defensin HNP-2. A covalently cyclized peptide. *J. Biol. Chem.* **264,** 4003–4007, and references cited therein.

329 Hiskey, R. G., Li, C -D., and Vunnam, R R. (1975) Sulfur-containing polypeptides. XVIII Unambiguous synthesis of the parallel and antiparallel isomers of some bis-cystine peptides. *J. Org. Chem.* **40,** 3697–3703.

330. Weissman, J. S. and Kim, P. S (1991) Reexamination of the folding of BPTI: predominance of native intermediates. *Science* **253,** 1386–1393, and references cited therein.

331. Chatrenet, B. and Chang, J.-Y. (1992) The folding of hirudin adopts a mechanism of trial and error. *J. Biol. Chem.* **267,** 3038–3043.

332. Andersson, M., Ostman, A., Bäckström, G., Hellman, U., George-Nascimento, C., Westermark, B., and Heldin, C.-H. (1992) Assignment of interchain disulfide bonds in platelet-derived growth factor (PDGF) and evidence for agonist activity of monomeric PDGF. *J. Biol. Chem.* **267,** 11,260–11,266

333. Huth, J. R , Mountjoy, K , Perini, F., Bedows, E., and Ruddon, R. W. (1992) Domain-dependent protein folding is indicated by the intracellular kinetics of disulfide bond formation of human chorionic gonadotropin β subunit. *J. Biol. Chem.* **267,** 21,396–21,403.

334. Fenn, J. B., Mann, M., Meng, C. K , Wong, S. F., and Whitehouse, C. M. (1989) Electrospray ionization for mass spectrometry of large biomolecules. *Science* **246,** 64–71, and references cited therein.

335 Desiderio, D. M. (ed) (1991) *Mass Spectrometry of Peptides* CRC, Boca Raton, FL.

336 Burlingame, A L , Baillie, T. A., and Russell, D. H. (1992) Mass spectrometry. *Anal. Chem.* **64,** 467R–502R, and references cited therein

337. Chait, B. T. and Kent, S. B. H. (1992) Weighing naked proteins· practical, high-accuracy mass measurement of peptides and proteins. *Science* **257,** 1885–1894, and references cited therein.

338 Yazdanparast, R., Andrews, P C., Smith, D. L , and Dixon, J. E (1987) Assignment of disulfide bonds in proteins by fast atom bombardment mass spectrometry *J. Biol. Chem.* **262,** 2507–2513.

339. Raschdorf, F., Dahinden, R., Maerki, W., Richter, W. J., and Merryweather, J. P. (1988) Location of disulphide bonds in human insulin-like growth factor (IGFs) synthesized by recombinant DNA technology. *Biomed. Environ. Mass Spectrom.* **16,** 3–8.

340. Griffin, P. R., Shabanowitz, J., Yates, III, J. R., Zhu, N. Z., and Hunt, D. F. (1989) Laser photodissociation Fourier transform mass spectrometry: new methodology for sequence analysis of oligopeptides and location of disulfide bonds, in *Techniques in Protein Chemistry* (Hugli, T. E., ed.), Academic, San Diego, pp 160–167.

341. Sørenson, H. H., Thomsen, J., Bayne, S., Højrup, P., and Roepstorff, P. (1990) Strategies for determination of disulphide bridges in proteins using plasma desorption mass spectrometry *Biomed. Env Mass Spectrom* **19,** 713–720.

342 Despeyroux, D., Bordas-Nagy, J., and Jennings, K. R. (1991) Determination of the amino acid sequence of cystine-containing peptides by tandem mass spectrometry *Rapid Commun. Mass Spectrom.* **5,** 156–159

343. Stewart, A. E., Raffioni, S., Chaudhary, T., Chait, B T., Luporini, P , and Bradshaw, R. A. (1992) The disulfide bond pairing of the pheromones Er-1 and Er-2 of the ciliated protozoan *Euplotes raikovi. Protein Sci.* **1,** 777–785.

344 Mancini, M. L. (1989) Enhancing the stability of disulfide-bond containing peptides under fast-atom bombardment conditions. *Biomed Environ. Mass Spectrom.* **18,** 1102–1104.

345. Visentini, J., Gauthier, J., and Bertrand, M. J. (1989) Effect of trifluoroacetic acid on the reduction of disulfide bridges in peptides analyzed by fast-atom bombardment mass spectrometry *Rapid Commun. Mass Spectrom.* **3,** 390–395.

346 Bolgar, M. S. and DiDonato, G. C. (1992) Disulfide bond assignment of endothelin-1 and analogues by ionspray mass spectrometry, in *Proceedings of the 40th ASMS Conference on Mass Spectrometry and Allied Topics,* pp. 1799–1800.

347 Feng, R., Bell, A., Dumas, F., and Konishi, Y. (1992) A fast and simple method for accurate counting of cysteines, disulfide bridges and free SH groups in proteins using ionspray™ mass spectrometry, in *Biotechnology International* (North, K., ed.), Century, London, pp. 155–158.

348. Sun, Y. and Smith, D. L (1988) Identification of disulfide-containing peptides by performic acid oxidation and mass spectrometry. *Anal. Biochem.* **172,** 130–138 and references cited therein

349. Morris, H. R. and Pucci, P. (1985) A new method for rapid assignment of S–S bridges in proteins. *Biochem. Biophys. Res. Commun.* **126,** 1122–1128.

350. Savoy, L.-A., Greer, F M., and Morris, H. R. (1993) Peptide and protein analysis by electrospray mass spectrometry, in *Peptides 1992: Proceedings of the Twenty-Second European Peptide Symposium* (Schneider, C. H. and Eberle, A. N., eds.), Escom, Leiden, The Netherlands, pp. 441–442

351 Rodriguez, H., Nevins, B., and Chakel, J. (1989) Evaluation of methods for the analysis of disulfide containing peptides by fast atom bombardment mass spec-

trometry, in *Techniques in Protein Chemistry* (Hugli, T. E., ed.), Academic, San Diego, pp. 186–194

352. Bean, M. F. and Carr, S. A. (1992) Characterization of disulfide bond position in proteins and sequence analysis of cystine-bridged peptides by tandem mass spectrometry. *Anal Biochem* **201,** 216–226, and references cited therein.
353. Hidaka, Y. and Schimonishi, Y. (1989) A new method for determination of disulfide pairing in peptides. *Bull. Chem. Soc. Jpn.* **62,** 1986–1994.
354 Hidaka, Y., Sato, K , Nakamura, H., Kobayashi, J., Ohizumi, Y , and Shimonishi, Y. (1990) Disulfide pairings in geographutoxin I, a peptide neurotoxin from *Conus geographus. FEBS Lett.* **264,** 29–32.
355. Grant, G. A. and Chiappinelli, V. A. (1985) κ-Bungarotoxin: complete amino acid sequence of a neuronal nicotinic receptor probe. *Biochemistry* **24,** 1532–1537.
356. Kopeyan, C., Martinez, G , and Rochat, H. (1985) Primary structure of toxin IV of *Leiurus quinquestriatus quinquestriatus.* Characterization of a new group of scorpion toxins. *FEBS Lett.* **181,** 211–217
357. Ryan, R J., Charlesworth, M. C., McCormick, D. J , Milius, R. P., and Keutmann, H T. (1988) The glycoprotein hormones: recent studies of structure-function relationships. *FASEB J.* **2,** 2661–2669.
358 Nakaya, K., Omata, K , Okahashi, I., Nakamura, Y., Kolkenbrock, H., and Ulbrich, N (1990) Amino acid sequence and disulfide bridges of an antifungal protein isolated from *Aspergillus giganteus. Eur. J. Biochem* **193,** 31–38.