Occurrence and Minimization of Cysteine Racemization during Stepwise Solid-Phase Peptide Synthesis[1,2]

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Contrary to the conventional wisdom of the peptide synthesis field, *N*,*S*-protected derivatives of cysteine can undergo substantial levels of racemization with widely-used reagents and protocols for stepwise incorporation. A systematic study of this problem has been carried out as a function of coupling conditions and *β*-thiol protecting groups, *i.e.*, *S*-acetamidomethyl (Acm), *S*-triphenylmethyl (trityl or Trt), *S*-2,4,6-trimethoxybenzyl (Tmob), and *S*-9*H*-xanthen-9-yl (Xan), taking advantage of a convenient and quantitative model system assay involving HPLC resolution of H-Gly-L-Cys-Phe-NH_2 from H-Gly-D-Cys-Phe-NH_2. For example, standard protocols for couplings mediated by phosphonium and aminium salts, *e.g.*, (benzotriazolyloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP), *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU), *N*-[[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]-pyridin-1-yl]methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU), and (7-azabenzotriazol-1-yloxy)tris(pyrrolidino)phosphonium hexafluorophosphate (PyAOP), typically involve 5-min preactivation times and are conducted in the presence of suitable additives such as 1-hydroxybenzotriazole (HOBt) or 7-aza-1-hydroxybenzotriazole (HOAt) plus a tertiary amine base such as *N*,*N*-diisopropylethylamine (DIEA) or *N*-methylmorpholine (NMM). Under such conditions, the levels of racemization in the model peptide, expressed as the ratio of D:L peptide formed, were in the entirely unacceptable range of 5−33%. However, these levels were in general reduced by a factor of 6- or 7-fold by avoiding the preactivation step. Additional strategies to reduce racemization involved change to a weaker base, with 2,4,6-trimethylpyridine (TMP, collidine) being substantially better than DIEA or NMM; 2-fold reduction in the amount of base; and change in solvent from neat *N*,*N*-dimethylformamide (DMF) to the less polar CH_2Cl_2−DMF (1:1). Coupling methods for the safe incorporation of cysteine with minimal racemization (<1% per step) in 9-fluorenylmethyloxycarbonyl (Fmoc) solid-phase peptide synthesis include BOP (or HBTU or HATU)/HOBt (or HOAt)/TMP (4:4:4) without preactivation in CH_2Cl_2−DMF (1:1), DIPCDI/HOBt (or HOAt) (4:4) with 5-min preactivation, and preformed pentafluorophenyl (Pfp) esters in CH_2Cl_2−DMF (1:1).

A series of careful experimental studies from the 1970's showed that during stepwise peptide synthesis both in solution[5] and solid-phase modes,[6] activation/coupling of protected amino acid building blocks proceeds with the exquisite stereochemical fidelity that is an essential requirement for the successful assembly of long chains.[7] However, the special susceptibility of *S*-benzyl (Bzl)-protected cysteine residues to racemization has been noted in solution synthesis,[8] and *C*-terminal cysteine protected with any of a variety of groups, *i.e.*, *S*-*tert*-butyl (*t*-Bu), *S*-acetamidomethyl (Acm), *S*-triphenylmethyl (trityl or Trt), *S*-*tert*-butylthio (S-*t*-Bu), *S*-(trimethylacetamido)methyl (Tacm), and *S*-4-methylbenzyl (MeBzl), has been shown to undergo racemization upon anchoring *as an ester* in solid-phase synthesis; further epimerization is reported to occur during repetitive N^{α}-deprotection steps in which treatment with the base piperidine removes the 9-fluorenylmethyloxycarbonyl (Fmoc) group.[9] While applying our recently developed *S*-9*H*-xanthen-9-

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(1) Abbreviations used for amino acids and the designation of peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in *J. Biol. Chem.* **1972**, *247*, 977-983. The following additional abbreviations are used: AA, amino acid; Acm, acetamidomethyl; BOP, (benzotriazolyloxy)tris(dimethylamino)phosphonium hexafluorophosphate; DIEA, *N*,*N*-diisopropylethylamine; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DIPCDI, *N*,*N*′-diisopropylcarbodiimide; DMF, *N*,*N*-dimethylformamide; Et_2O, diethyl ether; Fmoc, 9-fluorenylmethyloxycarbonyl; HATU, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-yl]methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HBTU, *N*-[1*H*-(benzotriazol-1-yl)-(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HOAt, 7-aza-1-hydroxybenzotriazole; HOBt, 1-hydroxybenzotriazole; 2-Moxan, 2-methoxy-9*H*-xanthen-9-yl; NMM, *N*-methylmorpholine; PAL, peptide amide linker, 5-[4-[(9-fluorenylmethyloxycarbonyl)aminomethyl]-3,5-dimethoxyphenoxy]valeric acid; PEG-PS, poly(ethylene glycol)−polystyrene graft resin support; Pfp, pentafluorophenyl; PyAOP, (7-azabenzotriazol-1-yloxy)tris(pyrrolidino)phosphonium hexafluorophosphate; Tmob, 2,4,6-trimethoxybenzyl; TFA, trifluoroacetic acid; TFE, trifluoroethanol; Tmob, 2,4,6-trimethoxybenzyl; TMP, 2,4,6-trimethylpyridine ≡ collidine; Trt, triphenylmethyl (trityl); Xan, 9*H*-xanthen-9-yl. Amino acid symbols denote the L-configuration unless indicated otherwise.

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

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yl (Xan) protecting group[10] to the synthesis by Fmoc chemistry of dihydrooxytocin with a (benzotriazolyloxy)-tris(dimethylamino)phosphonium hexafluorophosphate (BOP)/1-hydroxybenzotriazole (HOBt)/*N*-methylmorpholine (NMM) coupling protocol,[11] we observed[10b] a satellite to the major peak with the same mass as the desired product. This surprising result led us to hypothesize that, *contrary to the conventional wisdom of the field*, racemization of *internal* cysteine residues incorporated in *amide* linkages could be a serious concern in Fmoc solid-phase synthesis.[12] Given the increasing popularity of a newer generation of *in situ* activating agents that are phosphonium and aminium salts (details and literature *vide infra*), and considering advances in the protection and management of cysteine residues,[13] we decided to carry out a systematic study of this putative cysteine racemization problem as a function of coupling conditions and β-thiol protecting groups. Coupling strategies for the safe incorporation of cysteine with minimal racemization (<1% per step) in Fmoc solid-phase peptide synthesis have been identified in this work.

Results and Discussion

Pilot Observations of Racemization during Solid-Phase Synthesis of Dihydrooxytocin. The linear oxytocin sequence was assembled on a PAL-PEG-PS support[11b,15] by Fmoc chemistry with a BOP/HOBt/NMM (4:4:8 equiv with respect to peptide−resin; 5-min preactivation) protocol.[14] Both Cys[1] and Cys[6] were protected by *S*-Xan.[10] Upon completion of chain assembly, the Fmoc group was removed, cleavage/deprotection was carried out by reagent R[11b] (TFA−thioanisole−1,2-dithioethane−anisole = 90:5:3:2), and the *crude* peptide was analyzed by HPLC (Figure 1A). The major peak was identical to the expected dihydrooxytocin by several criteria, including comparison to an authentic standard.

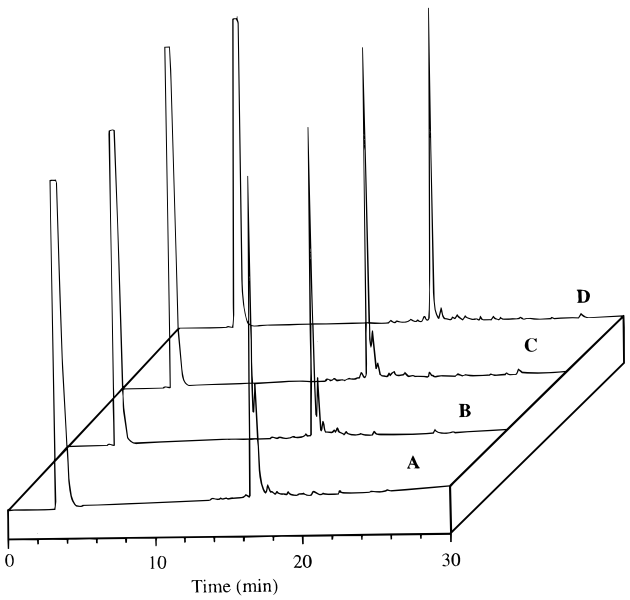

Figure 1. Detection of racemization during synthesis of dihydrooxytocin. The *crude* peptide was analyzed by HPLC on a Vydac C-18 column using a linear gradient of 0.1% TFA in CH_3CN and 0.1% aqueous TFA from 1:19 to 2:3 over a period of 30 min, flow rate 1.0 mL/min, detection at 220 nm. Authentic dihydrooxytocin elutes at 16.7 min; and the presumed racemized peptides all elute at 17.0 min. (A) Both Cys[1] and Cys[6] were coupled by BOP/HOBt/NMM in DMF, D-peptide:L-peptide = 0.35. (B) Cys[6] was coupled with DIPCDI/HOBt in DMF (4:4 equiv with respect to peptide−resin) (5-min preactivation), D-peptide:L-peptide = 0.17. (C) Cys[1] was coupled with DIPCDI/HOBt, D-peptide:L-peptide = 0.19. (D) Both Cys[1] and Cys[6] were coupled with DIPCDI/HOBt, racemization negligible.

A second peak eluting immediately thereafter was observed, at about one-third the intensity of the major peak. Both peaks were isolated by preparative HPLC and shown to have the same mass, matching the theoretical value for dihydrooxytocin. Retaining the protection scheme but substituting a coupling protocol using *N*,*N*′-diisopropylcarbodiimide (DIPCDI)/HOBt (4:4 equiv with respect to peptide−resin; 5-min preactivation) *specifically* for the two Cys(Xan) residues, the satellite peak became negligible (Figure 1D). Finally, when the Cys(Xan) residue at position 1 was incorporated with BOP/HOBt/NMM while that at position 6 was incorporated with DIPCDI/HOBt, or vice versa, the amounts of the satellite peak were about half of what they were in the all-BOP assemblies (Figure 1B,C).

The data in the previous paragraph are consistent with the occurrence of racemization at Cys, tied to the use of BOP/HOBt/NMM coupling. The *all*-L-dihydrooxytocin elutes slightly before any of the three Cys-racemized dihydrooxytocins, which do not appear to be resolved further. When the dihydro species are oxidized to oxytocin, the D-Cys-containing forms are not resolved from the desired *all*-L species. Perhaps for this reason, racemization has been often overlooked in the past.[14a,b]

Development of Model Assay for Cysteine Racemization. The literature lists several methods for the quantification of racemization of amino acids in general, and of cysteine in particular.[16] In some variations, a test or model peptide is hydrolyzed to its constituent amino acids, followed by derivatization with a suitable chiral reagent and chromatographic resolution of the resultant mixture of diastereomers by HPLC or on an amino acid

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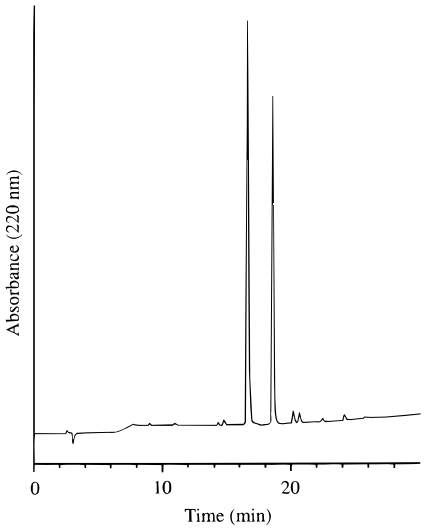

Figure 2. HPLC of a mixture of H-Gly-L-Cys-Phe-NH_2 (t_R = 16.5 min) and H-Gly-D-Cys-Phe-NH_2 (t_R = 18.8 min). Base-line separation was achieved on a Vydac C-18 column using a linear gradient of 0.1% TFA in CH_3CN and 0.1% aqueous TFA from 0:10 to 3:2 over 30 min, flow rate 1.0 mL/min, detection at 220 nm.

analyzer. The applicability of these methods to cysteine is compromised by the instability and redox chemistry of cysteine. Furthermore, procedures involving derivatization of cysteine, as a residue incorporated in the peptide (before hydrolysis) or as the free amino acid (after hydrolysis), can be quite laborious and difficult. In contrast, we have developed a convenient, quantitative, and unambiguous model system assay involving solid-phase assembly of a model tripeptide Gly-Cys-Phe on a PAL support.[11b] After cleavage/deprotection, H-Gly-L-Cys-Phe-NH_2 was base-line separated from H-Gly-D-Cys-Phe-NH_2 by HPLC [Figure 2; the tripeptide model was chosen after the corresponding des(Gly)-dipeptides were shown to elute within 1 min of each other, which severely limited the dynamic range of such a method]. This system was used to study cysteine racemization as a function of synthesis design and reaction conditions (Tables 1−3).

Cysteine Racemization during Phosphonium or Aminium Salt-Mediated Coupling. The BOP reagent, as well as *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU), *N*-[[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-yl]methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU), and (7-azabenzotriazol-1-yloxy)tris(pyrrolidino)phosphonium hexafluorophosphate (PyAOP)], all represent useful and deservedly popular additions to the arsenal of coupling reagents employed in solid-phase peptide synthesis.[17,18] Standard coupling protocols typically involve 5-min preactivation times (*e.g.*, Table 1, entries 1 and 2) and are conducted in the presence of suitable additives such as HOBt or 7-aza-1-hydroxybenzotriazole (HOAt) plus a tertiary amine base as *N*,*N*-diisopropylethylamine (DIEA) or NMM. The levels of racemization in the model peptide, expressed as the ratio of D:L peptide formed, were in the entirely unacceptable range of 5−33%. However, these levels were in general reduced by a factor of 6- or 7-fold [*e.g.*, Table 1, entries 6, 9, and 11; curiously no significant effect with *S*-trimethoxybenzyl (*S*-Tmob)[14b] protection] by *avoiding the preactivation step*. This expedient seems to be helpful because the presumed *N*,*S*-protected *C*-activated intermediates are exposed to a base in the absence of an amine nucleophile for a minimal time; the activated species once formed becomes rapidly acylated by the amine.

Additional strategies to reduce racemization involved change to a *weaker base*, with *2,4,6-trimethylpyridine (TMP, collidine)* being substantially better[19] than DIEA or NMM (compare Table 1, entries 8 and 9 *vs* 1−3 and 5−7); *2-fold reduction* in the amount of base (compare Table 1, entry 2 *vs* 7), and *change in solvent* from neat *N*,*N*-dimethylformamide (DMF) to the less polar CH_2Cl_2−DMF (1:1) (*e.g.*, Table 1, entry 3 *vs* 9), or Sakakibara's mixture[5b,20] of 2,2,2-trifluoroethanol (TFE)−$CHCl_3$ (3:7) (Table 1, entry 4; unfortunately, this solvent cannot be recommended because couplings were found to be too slow). These findings lead to the consensus optimal conditions of BOP/HOBt/TMP (4:4:4) without preactivation in CH_2Cl_2−DMF (1:1) (Table 1, entry 9). Results with aminium salts (Table 1, entries 10−15) are very comparable to those with BOP in all regards: conditions using HBTU or HATU that are currently considered standard give substantial racemization, whereas modified conditions along lines already described are accompanied by little or no racemization.

Evaluation of Coupling Reagents That Do Not Require Added Base. Classical coupling methods that do not require added base[6] were also evaluated, in view of the known propensity of cysteine derivatives to racemize under basic conditions.[7,8] Use of *N*,*N*′-diisopropylcarbodiimide (DIPCDI), either alone or in the presence of HOBt or HOAt as additives, and in either neat DMF or CH_2Cl_2−DMF (1:1), gave racemization levels of <4%, and usually <2% (Table 2). Interestingly, a 5-min preactivation usually provided a modest *reduction* in the level of racemization. Results here did not change much from varying the solvent. Several experiments showed that couplings of symmetrical anhydrides formed *in*

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Table 1. Racemization during Phosphonium- or Aminium Salt-Mediated Synthesis of Model Peptide H-Gly-Cys-Phe-NH_2 as a Function of Coupling Conditions and Cysteine Protecting Group[a]

	coupling conditions[a]					D-Peptide:L-Peptide × 100			
no.	reagent	additive	base	solvent	preact	Xan[b]	Tmob	Trt	Acm
1	BOP (4)	HOBt (4)	DIEA (8)	DMF	5	18.9	6.0	17.1	11.0
2	BOP (4)	HOBt (4)	NMM (8)	DMF	5	17.2	7.3	22.2	11.0
3	BOP (4)	HOBt (4)	NMM (8)	CH_2Cl_2−DMF[c]	5	11.4	5.9	5.9	3.3
4	BOP (4)	HOBt (4)	NMM (8)	TFE−$CHCl_3$[c,d]	5	<0.2	0.2	<0.2	<0.2
5	BOP (4)	HOBt (4)	NMM (8)	DMF	2	9.2	5.6	15.2	4.0
6	BOP (4)	HOBt (4)	NMM (8)	DMF	0	2.1	4.8	3.1	1.0
7	BOP (4)	HOBt (4)	NMM (4)	DMF	5	6.6	4.6	10.4	3.3
8	BOP (4)	HOBt (4)	TMP (8)	DMF	5	**0.8**	3.5	2.2	**1.0**
9	**BOP (4)**	**HOBt (4)**	**TMP (4)**	**CH_2Cl_2−DMF[c]**	**0**	**0.8**	**0.9**	**0.8**	**0.4**
10	HBTU (4)	HOBt (4)	NMM (8)	DMF	5	14.3	3.7	24.7	8.8
11	**HBTU (4)[e]**	**HOBt (4)**	**TMP (4)**	**CH_2Cl_2−DMF[c]**	**0**	**0.6**	**0.6**	**0.8**	**0.4**
12	HATU (4)	HOBt (4)	NMM (8)	DMF	5	19.6	5.2	36.6	16.1
13	**HATU (4)[e]**	**HOBt (4)**	**TMP (4)**	**CH_2Cl_2−DMF[c]**	**0**	**0.9**	**0.8**	**0.7**	**0.6**
14	HATU (4)	HOAt (4)	NMM (8)	DMF	5	32.8	7.9	51.3	29.0
15	**HATU (4)[e]**	**HOAt (4)**	**TMP (4)**	**CH_2Cl_2−DMF[c]**	**0**	**0.8**	**0.6**	**0.6**	**0.3**

[a] See Experimental Section for general procedures; special details are presented in subsequent footnotes. Conditions that are considered "safe" for introduction of cysteine, *i.e.*, acceptably low levels of racemization, are highlighted in **bold**. [b] A few experiments used *S*-2-methoxy-9*H*-xanthen-9-yl (2-Moxan) in place of Xan. With BOP/HOBt/NMM (4:4:8)-mediated-coupling in DMF (compared to entries 2, 5, and 6), racemization levels (ratio of D:L) were 2.1% for no preactivation, 11.2% for 2-min preactivation, and 21.2% for 5-min preactivation. [c] CH_2Cl_2−DMF mixtures were 1:1 (v/v); TFE−$CHCl_3$ mixture was 3:7 (v/v) (see refs 5b and 20). [d] Extent of coupling during the indicated time was ~50%. [e] Final coupling concentration was 0.05 M due to the poor solubility of HBTU and HATU in CH_2Cl_2−DMF (1:1).

Table 2. Racemization During *N,N′*-Diisopropylcarbodiimide (DIPCDI)-Mediated Synthesis of Model Peptide H-Gly-Cys-Phe-NH_2 as a Function of Coupling Conditions and Cysteine Protecting Group[a]

	coupling conditions[a]				D-Peptide:L-Peptide × 100			
no.	reagent	additive	solvent	preact	Xan	Tmob	Trt	Acm
1	DIPCDI (4)		DMF	0	1.3	3.5	0.8	0.4
2	DIPCDI (4)	HOBt (4)	DMF	0	1.3	2.9	0.9	0.4
3	DIPCDI (4)	HOAt (4)	DMF	0	1.6	3.2	0.8	0.4
4	**DIPCDI (4)**		**DMF**	5	**0.6**	3.0	**0.6**	**0.6**
5	**DIPCDI (4)**	**HOBt (4)**	**DMF**	5	**0.4**	3.4	**0.6**	**0.5**
6	**DIPCDI (4)**	**HOAt (4)**	**DMF**	5	**0.3**	2.4	**0.6**	**0.5**
7	DIPCDI (4)		CH_2Cl_2−DMF	0	1.2	1.8	0.9	0.7
8	DIPCDI (4)	HOBt (4)	CH_2Cl_2−DMF	0	1.0	1.2	0.9	0.7
9	DIPCDI (4)	HOAt (4)	CH_2Cl_2−DMF	0	1.4	1.4	0.8	0.6
10	**DIPCDI (4)**		**CH_2Cl_2−DMF**	**5**	**0.3**	**0.5**	**0.5**	**0.4**
11	**DIPCDI (4)**	**HOBt (4)**	**CH_2Cl_2−DMF**	**5**	**0.3**	**0.2**	**0.5**	**0.2**
12	**DIPCDI (4)**	**HOAt (4)**	**CH_2Cl_2−DMF**	**5**	**0.3**	**0.4**	**0.5**	**0.3**

[a] See Experimental Section for general procedures. CH_2Cl_2−DMF mixtures were 1:1 (v/v). Conditions that are considered "safe" for introduction of cysteine, *i.e.*, acceptably low levels of racemization, are highlighted in **bold**. Special considerations with *S*-Tmob discussed in text.

Table 3. Racemization during Synthesis of Model Peptide H-Gly-Cys-Phe-NH_2 Using Preactivated Species, as a Function of Coupling Conditions and Cysteine Protecting Group[a]

	coupling conditions[a]			D-Peptide:L-Peptide × 100			
no.	reagent	additive	solvent	Xan	Tmob	Trt	Acm
1	**sym anhy (4)**		**CH_2Cl_2−DMF**	**0.4**	2.0	**0.3**	**0.4**
2	**Pfp ester (4)**		**DMF**	**0.2**	**0.2**	**0.2**	**0.4**
3	**Pfp ester (4)**	**HOBt (4)**	**DMF**	**0.3**	**0.8**	**0.6**	**0.2**
4	**Pfp ester (4)**	**HOAt (4)**	**DMF**	**0.2**	**0.2**	**0.4**	**0.2**
5	**Pfp ester (4)**	**HOBt (4)**	**CH_2Cl_2−DMF**	**0.3**	**0.8**	**0.6**	**0.2**

[a] See Experimental Section for general procedures. CH_2Cl_2−DMF mixtures were 1:1 (v/v). Conditions that are considered "safe" for introduction of cysteine, *i.e.*, acceptably low levels of racemization, are highlighted in **bold**.

situ,[21] and of pentafluorophenyl (Pfp) active esters,[22,23] resulted in negligible racemization (Table 3). These results are consistent with expectations from the literature.[6,7]

Effect of Cysteine Protecting Group. As optimized, none of the *S*-protecting groups evaluated provides much risk of racemization (always <1% in model studies), with the exception of *S*-Tmob, which for DIPCDI/HOAt, DIPCDI/HOBt, and symmetrical anhydride procedures gives slight racemization (~2−3%; *e.g.*, Table 2, entries 5 and 6; Table 3, entry 1). However, *S*-Tmob is preferred to *S*-Xan, *S*-Trt, and *S*-Acm when phosphonium and aminium salts are used (*e.g.*, Table 1, entries 1, 10, 12, and 14).

Mechanisms. It is well understood that the racemization process is a competition between activation, rearrangement of the activated species to a 5(4*H*)-oxazolone, base-catalyzed abstraction of the α-proton (enolization) from either the activated species directly or

(21) (a) Rich, D. H.; Singh, J. In *The Peptides*; Gross, E., Meienhofer, J., Eds.; Academic Press: New York, 1979; Vol. 1, pp 241−261. (b) Merrifield, R. B.; Vizioli, L.D.; Boman, H. G. *Biochemistry* **1982**, *21*, 5020−5031.

(22) Kisfaludy, L.; Schön, I. *Synthesis* **1983**, 325−427.

(23) Fmoc-Cys(Trt)-OPfp and Fmoc-Cys(Acm)-OPfp were commercially available, and Fmoc-Cys(Xan)-OPfp and Fmoc-Cys(Tmob)-OPfp were prepared as described in the Experimental Section.

from the oxazolone, and acylation of the activated species and/or the oxazolone with an incoming amine nucleophile, which is also a base. The more activated species couple more rapidly, but are also more prone to racemize. The presence of base gives dual and opposite effects, because while base catalyzes more rapid acylation, it also leads to more rapid epimerization.

All of the phenomena described in the above paragraph are exacerbated with protected *activated* cysteine; no further epimerization occurs once cysteine is incorporated within the peptide chain. Those activation protocols that involve the presence of base are likely to result in measurable cysteine racemization. As might be expected, the level of racemization decreases by using less base, weaker base, and eliminating preactivation. Jones and Kovacs, with their respective co-workers, have suggested that the β-sulfur of cysteine stabilizes, possibly via a five-membered ring, an anion at the α-carbon of an activated cysteine derivative.[8b−e] Such insights are of peripheral value in providing a semiquantitative rationalization of the experimental results reported in the present work.

Conclusions

Our work provides some cautionary information that is of both mechanistic and practical significance. We are able to define effective compromise conditions that may be adopted to manual and automated solid-phase synthesis. Racemization of cysteine by whatever coupling protocol can be minimized by the following: (i) using as solvent CH_2Cl_2−DMF, with the minimal amount of DMF consistent with solubility of derivatives; (ii) avoiding preactivation in BOP, HATU, or HBTU-mediated (and related) protocols; (iii) replacing the traditional DIEA or NMM base with TMP. Once a cysteine residue has been incorporated safely, there appears to be little risk for further racemization at steps to couple additional amino acids.

Experimental Section

Most of the materials and general synthetic and analytical procedures have been described in our earlier publications.[10b,11b,14,15] Fmoc-Cys(Tmob)-OH[14b] and Fmoc-Cys(Xan)-OH[10b] were made in our laboratory as described previously, and other protected amino acid derivatives were commercially available. ^{1}H NMR spectra were recorded at 300 MHz using either $CDCl_3$ or CD_3OD as solvents. Fast atom bombardment mass spectrometry (FABMS) to characterize synthetic peptides was carried out with glycerol or thioglycerol matrices being used to obtain both positive and negative ion spectra. Elemental analyses were performed by M-H-W Laboratories, Phoenix, AZ.

Thin-layer chromatography was performed on Analtech or Merck silica gel GF plates (250 μm, 10 × 20 cm), and compounds were observed by fluorescence quenching and by spraying with a dilute ethanolic ninhydrin solution. Analytical HPLC were carried out on C-18 columns.

***S*-(9*H*-Xanthen-9-yl)-D-cysteine [H-D-Cys(Xan)-OH].** Starting with D-cysteine·H_2O·HCl (2.0 g, 11.4 mmol), the previously described[10b] procedure for the corresponding L compound was followed exactly: yield 2.87 g (84%); mp 175 °C dec; R_f [MeOH−H_2O (4:1)] 0.78; ^{1}H NMR (CD_3OD) δ 7.51−7.58 (dd, J = 7.5 and 12.9 Hz, 2H), 7.31 (t, J = 7.5 Hz, 2H), 7.11−7.17 (m, 4H), 5.48 (s, 1H), 3.52 (dd, J = 4.2 and 7.8 Hz, 1H), 2.97 (dd, J = 4.2 and 14.4 Hz, 1H), 2.85 (dd, J = 7.8 and 14.4 Hz, 1H). Anal. Calcd. for $C_{16}H_{15}NO_3S$ (301.36): C, 63.77; H, 5.02; N, 4.65; S, 10.64. Found: C, 63.18; H, 5.93; N, 4.45; S, 9.96.

***N*$^\alpha$-(9-Fluorenylmethyloxycarbonyl)-*S*-(9*H*-xanthen-9-yl)-D-cysteine [Fmoc-D-Cys(Xan)-OH].** A solution of Fmoc-OSu (1.15 g, 3.4 mmol) in CH_3CN (20 mL) was added over 30 min to a yellowish solution of H-D-Cys(Xan)-OH (0.96 g, 3.2 mmol) in H_2O−CH_3CN (1:1, 40 mL) plus Et_3N (1.06 mL, 7.6 mmol). The reaction mixture was stirred at 25 °C for 3 h, adjusted to pH 3.5 by addition of 10% aqueous citric acid solution, and concentrated under reduced pressure. The resultant precipitate was extracted with EtOAc (3 × 100 mL), washed with H_2O (2 × 100 mL) and brine (1 × 100 mL), and dried (Na_2SO_4). Evaporation of organic solvent gave a white solid that was purified further by silica gel chromatography ($CHCl_3$−MeOH = 30:1, R_f = 0.11): yield 1.21 g (72%); mp 117−120 °C; R_f [$CHCl_3$−MeOH (10:1)] 0.48; ^{1}H NMR (CD_3SOCD_3) δ 12.87 (broad s, COOH), 7.87 (d, J = 7.5 Hz, 2H), 7.75 (d, J = 8.4 Hz, 1H), 7.70 (d, J = 7.5 Hz, 2H), 7.10−7.47 (m, 12H), 5.48 (s, 1H), 4.18-4.30 (m, 3H), 4.03−4.10 (m, 1H), 2.81 (dd, J = 4.5 and 13.5 Hz, 1H), 2.64 (dd, J = 9.9 and 13.5 Hz, 1H). Anal. Calcd. for $C_{31}H_{25}NO_5S$ (523.61): C, 71.11; H, 4.81; N, 2.68; S, 6.12. Found: C, 70.96; H, 4.76; N, 2.65; S, 6.00.

***N*$^\alpha$-(9-Fluorenylmethyloxycarbonyl)-*S*-(9*H*-xanthen-9-yl)cysteine Pentafluorophenyl Ester [Fmoc-Cys(Xan)-OPfp].** *N*,*N*′-Dicyclohexylcarbodiimide (DCC) (0.77 g, 3.7 mmol) was added in small portions to a suspension of Fmoc-Cys(Xan)-OH[10b] (1.86 g, 3.6 mmol) and 2,3,4,5,6 pentafluorophenol (0.79 g, 4.3 mmol) in freshly distilled CH_2Cl_2 (70 mL) over 10 min. A clear solution formed immediately, followed by precipitation about 2 min later. The reaction mixture was stirred under N_2 at 25 °C overnight, filtered to remove the insoluble urea, concentrated to ∼10 mL under reduced pressure, and then stored at 4 °C for 2 h. A new filtration removed further urea, and the filtrate was diluted with hexane (20 mL) and stored at 4 °C for 4 h. The resultant TLC-pure white precipitate was collected by filtration, washed with CH_2Cl_2−hexane (1:2, 3 × 5 mL), and dried *in vacuo*: yield 2.32 g (94%); mp 151−153 °C; R_f [hexane−EtOAc (3:1)] 0.61; ^{1}H NMR ($CDCl_3$) δ 7.77 (d, J = 7.5 Hz, 2H), 7.58 (d, J = 7.5, 2H), 7.22−7.45 (m, 8H), 7.11 (dd, J = 3.3 and 7.8 Hz, 4H), 5.38 (s, 1H), 5.14 (d, J = 8.1 Hz, 1H), 4.62 (dd, J = 6.0 and 13.5 Hz, 1H), 4.41 (d, J = 6.9 Hz, 2H), 4.21 (t, J = 6.9 Hz, 1H), 2.88 (d, J = 6.3 Hz, 2H). Anal. Calcd for $C_{37}H_{24}F_5NO_5S$ (689.66): C, 64.44; H, 3.51; F, 13.77; N, 2.03; S, 4.65. Found: C, 64.57; H, 3.71; F, 13.01; N, 2.26; S, 4.30.

***N*$^\alpha$-(9-Fluorenylmethyloxycarbonyl)-*S*-(2,4,6-trimethoxybenzyl)cysteine Pentafluorophenyl Ester [Fmoc-Cys(Tmob)-OPfp].** DCC (0.88 g, 4.3 mmol) was added in five portions over 10 min to a solution of Fmoc-Cys(Tmob)-OH[14b] (2.13 g, 4.1 mmol) and 2,3,4,5,6-pentafluorophenol (0.90 g, 4.8 mmol) in freshly distilled CH_2Cl_2 (35 mL). The reaction mixture was stirred under N_2 at 25 °C overnight, and further steps followed very closely the above procedure for the corresponding Cys(Xan) derivative: yield 2.53 g (90%); mp 108−110 °C; R_f [hexane−EtOAc (3:1)] 0.40; ^{1}H NMR ($CDCl_3$) δ 7.74 (d, J = 7.5 Hz, 2H), 7.61 (d, J = 7.5 Hz, 2H), 7.26−7.41 (m, 6H), 6.13 (s, 2H), 5.94 (d, J = 7.8 Hz, 1H), 4.97 (m, 1H), 4.49 (d, J = 6.9 Hz, 2H), 4.26 (t, J = 6.9 Hz, 1H), 3.79 (s, 9H), 3.10 (dd, J = 4.5 and 14.4 Hz, 1H), 2.95 (dd, J = 7.8 and 14.4 Hz, 1H). Anal. Calcd for $C_{34}H_{28}F_5NO_7S$ (689.66): C, 59.21; H, 4.09; F, 13.77; N, 2.03; S, 4.65. Found: C, 59.00; H, 4.16; F, 13.50; N, 2.20; S, 4.81.

Dihydrooxytocin (H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2). The detailed experimental design, HPLC documentation (Figure 1), and essential conclusions are described in the text. In brief, Fmoc-Cys(Xan)-Tyr(*t*Bu)-Ile-Gln-Asn-Cys-(Xan)-Pro-Leu-Gly-PAL-PEG-PS (300 mg, 0.15 mmol/g initial loading) was assembled by Fmoc chemistry, with 1-h couplings in DMF of all residues [except that Asn and Gln were incorporated as their corresponding Pfp esters (with HOBt, 1:1 ≡ 6 equiv), and Cys as specified] mediated by BOP/HOBt/NMM (4:4:8 equiv with respect to peptide-resin; 5-min preactivation). Cleavage/deprotection with reagent R was carried out for 1 h at 25 °C. The filtrate from the cleavage reaction was evaporated partially under a stream of N_2, diluted with CH_2Cl_2 (1 mL for 20 mg peptide-resin), and then precipitated with anhydrous Et_2O (10 mL). The procedure of adding CH_2Cl_2 followed by Et_2O precipitation was repeated a total of three times, in order to remove all low molecular weight organic compounds. The residue was dissolved in the mixture of

HOAc−H_2O (1:4) (1 mg/mL) and applied to HPLC analysis (Figure 1). Diastereomeric dihydrooxytocins were separated by semipreparative HPLC (Waters Delta Prep 3000, using a linear gradient of 0.1% TFA in CH_3CN and 0.1% aqueous TFA from 1:19 to 2:3 over 50 min, flow rate 5.0 mL/min, detection at 220 nm) and analyzed by mass spectrometry: FABMS calcd monoisotopic mass of dihydrooxytocin 1008.44, found positive and negative of isolated dihydrooxytocin $[M + H]^+$ 1009.6, $[M-H]^-$ 1007.7, Found positive and negative of racemized dihydrooxytocin $[M + H]^+$ 1009.6, $[M-H]^-$ 1007.6.

Synthesis and Analysis of Standard H-Gly-L-Cys-Phe-NH_2 and H-Gly-D-Cys-Phe-NH_2. Starting with Fmoc-PAL-PEG-PS (1.0 g, 0.15 mmol/g), peptide chain assembly was carried out using 1-h DIPCDI/HOBt (4:4 equiv with respect to peptide−resin, 0.18 M, 5-min preactivation)-mediated couplings in DMF (3.3 mL). In separate syntheses, Fmoc-Cys-(Xan)-OH and Fmoc-D-Cys(Xan)-OH were incorporated; otherwise, Fmoc-Phe-OH and Boc-Gly-OH were used. Fmoc removal was carried out with piperidine−DMF (1:4, 2 + 8 min), followed by washes with DMF (5 × 2 min). Final release of peptides was achieved with reagent R at 25 °C for 1 h. Crude peptides were purified on semipreparative HPLC (Waters Delta Prep 3000, using a linear gradient of 0.1% TFA in CH_3CN and 0.1% aqueous TFA from 0:10 to 3:2 over 50 min, flow rate 5.0 mL/min, detection at 220 nm). Purified H-Gly-L-Cys-Phe-NH_2 and H-Gly-D-Cys-Phe-NH_2 were characterized by HPLC (Figure 2) and FABMS analyses (matrix: glycerol−TFA): calcd monoisotopic mass of $C_{14}H_{20}N_4O_3S$ 324.4, found positive and negative of H-Gly-L-Cys-Phe-NH_2 $[M + H]^+$ m/z 325.2, $[M - H]^-$ m/z 323.2, found positive and negative of H-Gly-D-Cys-Phe-NH_2 $[M + H]^+$ m/z 325.2, $[M - H]^-$ m/z 323.1.

Examination of Cysteine Racemization during Stepwise Solid-Phase Peptide Synthesis of H-Gly-Cys-Phe-NH_2 (Tables 1 and 2). Each experiment started with Fmoc-PAL-PEG-PS (50 mg, 0.15 mmol/g = 1 equiv resin) and followed the overall experimental outline of the above description of standards. Fmoc-Cys derivatives with the indicated side-chain protection were incorporated by 1-h couplings mediated by the coupling reagents, additives, and bases indicated in Tables 1−3 [no. of equiv in parentheses; concentration of Cys derivative always 0.18 M unless indicated otherwise]; solvent and preactivation time (expressed in min) as specified. After completion of the chain assembly, the tripeptide H-Gly-Cys-Phe-NH_2 was released from the support by treatment with Reagent R at 25 °C for 1 h, precipitated by cold diethyl ether, dissolved in deionized H_2O, lyophilized, and analyzed by HPLC (see legend of Figure 2 for details). However, for those experiments using Cys(Acm), the following steps were carried out after completion of chain assembly, but before cleavage: peptide-resin was treated with $Hg(OAc)_2$ solution (0.06 M) in DMF in the dark at 25 °C for 3 h and then washed with β-mercaptoethanol−DMF (1:19, v/v, 3 × 2 min).

Examination of Cysteine Racemization during Stepwise Solid-Phase Peptide Synthesis of H-Gly-Cys-Phe-NH_2 with Preformed Symmetrical Anhydrides (Table 3, entry 1). The general outline described above was followed. Symmetrical anhydrides[6b,21] were prepared *in situ* by combining AA/DIPCDI (8:4 = 0.16 M) in CH_2Cl_2−DMF (10:1) for N^{α}-Fmoc-, *S*-Tmob-, *S*-Trt-, and *S*-Acm-protected cysteine derivatives and by combining AA/DIPCDI (8:4 = 0.12 M) in CH_2Cl_2−DMF (2:1) for Fmoc-Cys(Xan)-OH. After 5 min, these reaction mixtures were diluted with CH_2Cl_2 to provide a final solvent milieu of CH_2Cl_2−DMF (1:1) and a concentration of 0.09 M activated species for coupling.

Examination of Cysteine Racemization during Stepwise Solid-Phase Peptide Synthesis of H-Gly-Cys-Phe-NH_2 with Pentafluorophenyl Esters (Table 3, entries 2−5). The general outline described above was followed; concentrations of the active species were 0.18 M. Additives, when used in Pfp ester couplings, were introduced to the peptide-resin ahead of other reagents.

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