New Approaches to the Synthesis of Cystine Peptides Using *N*-Iodosuccinimide in the Construction of Disulfide Bridges†

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Received July 16, 1992 (Revised Manuscript Received March 8, 1993)

N-Halosuccinimides have been found to convert cysteine, *S*-(acetamidomethyl)-, *S*-(*p*-methoxybenzyl)-, or *S*-(*p*-methylbenzyl)cysteine into cystine. *N*-Iodosuccinimide, the mildest reagent among *N*-halosuccinimides, is applied successfully in the synthesis of (Arg^8)-vasopressin and oxytocin by a fully automated process of solid-phase peptide synthesis or by cyclizing the released thiol peptides in DMF–CH_2Cl_2. These simple and synthetically useful methods have led to the fully automated solid-phase synthesis of (Cys^5,Cys^{12}) human growth hormone releasing factor (1–29) NH_2 and apamin based on the combined processes of stepwise addition of Boc-amino acids and controlled stepwise formation of the disulfide bonds. These new approaches may be useful in the synthesis of larger and more complex cystine peptides.

Disulfide bridges are important structures for the biological activities of peptide and protein molecules. The chemistry of disulfide bond formation is complicated in peptide synthesis, especially in multi-cystine peptides such as apamin,[1-3] insulin-like growth factor I (IGF-I),[4,5] and IGF-II.[6] Usually, the last step in the synthesis of cystine peptides is the time-consuming and nonspecific oxidation of the assembled thiol peptides in dilute solution in order to minimize unwanted side products resulting from intermolecular and intramolecular cyclizations. A few examples of direct solid-phase synthesis of cystine peptides on a resin support have been reported.[7-13] These approaches take advantage of the favored intramolecular cyclization due to pseudodilution phenomena.[14]

The disulfide bond can be formed by the coupling of two free thiol radicals[15] or two sulfenyl halides.[16-20] An alternative potential method of disulfide formation was to use *N*-halosuccinimides, the reactions of which proceeded via radical[21-23] or electrophilic[24-27] halogenation mechanisms. In order to find a simple method for the synthesis of cystine peptides in nonaqueous media, the formation of disulfide bonds by *N*-halosuccinimides was investigated. The reactions of L-cysteine hydrochloride hydrate (1 mmol) with *N*-halosuccinimides (0.55 mmol) gave good yields (68.3–84.1%) of cystine. Treatments of Boc-Cys(Acm)-OH (1 mmol) (Acm, acetamidomethyl) with *N*-halosuccinimides (1.1 mmol) followed by removal of the Boc protecting group using 45% trifluoroacetic acid (TFA) in CH_2Cl_2 afforded reasonable yields of cystine (40–41.7% yields for the two-step reaction). Treatment of Boc-Cys(4-MeOBzl)-OH (1 mmol) (4-MeOBzl, *p*-methylbenzyl) with *N*-iodosuccinimide (NIS, 1.1 mmol) followed by removal of the Boc protecting group also gave a reasonable yield of cystine (51.3% overall yield). Similar treatment of Boc-Cys(4-MeBzl)-OH (4-MeBzl, *p*-methylbenzyl) with NIS gave a less satisfactory result (5.7%, 80% pure). In the crude products of these two reactions, *N*-(*p*-methoxybenzyl)- or *N*-(*p*-methylbenzyl)succinamic acid usually coprecipitated with cystine, making purification of cystine difficult, especially in the latter case. In the protocol for these conversions of Boc-cysteine derivatives into cystine, TFA was used to remove the Boc protecting group, also resulting in difficult isolation of free cystine due to the high solubility of the TFA salt of cystine in water and in most organic solvents such as CH_2Cl_2, EtOAc, or MeOH. The preferred method for the neutralization of excess TFA in the crude products of

† Presented in part at the Fifth National Meeting, American Association of Pharmaceutical Scientists, Nov 4, 1990, Las Vegas, NV.

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cystine was to use 5 N NaOH instead of triethylamine (TEA), which usually resulted in cystine containing a small amount of the TEA salt of cystine in most cases. Based upon the encouraging results described above, a novel and promising approach was developed using *N*-halosuccinimides to effect the formation of disulfide bonds of cystine peptides employing previously well-established procedures of solid-phase peptide synthesis.[28] In order to examine the usefulness of *N*-halosuccinimides in the synthesis of cystine peptides, NIS, the mildest reagent among the *N*-halosuccinimides, was used to prepare cystine peptides by a fully automated process of solid-phase peptide synthesis or by cyclizing the released thiol peptides in DMF–CH_2Cl_2.

Since the structures of vasopressin and oxytocin are well defined as comparatively rigid conformations, they are useful models for methodological studies of disulfide bond formation using NIS. With this knowledge different reaction conditions were carried out by treating the mixture of resin and released dithiol vasopressin with NIS. The reaction using 0.11 mmol of NIS (0.22 equiv to 0.5 mmol of starting resin) for 17 h gave (Arg^8)-vasopressin in 34% overall yield. The yield was 30.6% for the reaction using 0.3 mmol of NIS (0.6 equiv) for 1 h. However, the reaction using 0.5 mmol of NIS (1.0 equiv) for 2 h gave (Arg^8)-vasopressin in a yield of 44.3% (70% pure). The discrepancy among the yields of these reactions could possibly result from purification processes and suggested that the transformation of a dithiol peptide into a cystine peptide only required a catalytic amount of NIS to proceed readily under mild conditions through the coupling of two free thiol radicals.[15] However, the cyclization efficiency of NIS was considered as good as that of established methods such as potassium ferricyanide[29] or air oxidation, which also gave a 30% yield of (Arg^8)-vasopressin. In addition, the tyrosine residue(s) remained intact during cyclization with NIS.

To perform the direct formation of the disulfide bond on protected peptide-resin via a fully automated process in a peptide synthesizer, the assembled Cys(4-MeOBzl)-vasopressin-resin was treated with 0.55 mmol of NIS (1.1 equiv) for 2 h to give (Arg^8)-vasopressin in 41% overall yield. In another experiment, treatment of the assembled Cys(4-MeBzl)-vasopressin-resin with 0.55 mmol of NIS (1.1 equiv) for 2 h gave a 20% overall yield of (Arg^8)-vasopressin. These results were similar to those obtained from the NIS conversions of Boc-Cys(4-MeOBzl)-OH and Boc-Cys(4-MeBzl)-OH into cystine. The higher yield in the direct conversion of the Cys(4-MeOBzl)-OH to cystine compared to that of the analogous reaction of Cys(4-MeBzl)-OH could be attributed to the stronger electron-donating character of the *p*-methoxy substituent compared to that of the *p*-methyl group. The electronic substituted effect on the aromatic ring in turn affected the stability of the leaving benzyl group during cyclization. This study of the leaving group could possibly reveal the electronic effects of the substituents on the efficiency of the cyclization reaction in other *S*-benzyl cysteine systems. However, the same treatment of the assembled Cys(4-MeO-Bzl)-oxytocin-resin with 0.55 mmol of NIS (1.1 equiv) resulted in a 7% crude yield of oxytocin (1% after further purification). Molecular iodine was observed as a side product in the NIS reactions. It was known that iodine could oxidatively convert Cys(Acm)-OH to cystine[30] but the transformation did not work for Cys(4-MeBzl)-OH and Cys(4-MeOBzl)-OH. Apparently, the mechanism for NIS conversion of Cys(4-MeBzl)-OH or Cys(4-MeOBzl)-OH to cystine was different from the iodine conversion of Cys(Acm)-OH to cystine.[30] In addition, *N*-(*p*-methoxybenzyl)- and *N*-(*p*-methylbenzyl)succinamic acid or their corresponding triethylamine salts of *N*-benzylsuccinamic acids were observed as byproducts in the previous NIS conversions of Boc-Cys(4-MeOBzl)-OH and Boc-Cys(4-MeBzl)-OH into cystine. This evidence suggested that the mechanism of these conversions was likely to involve the formation of the disulfide bond via the coupling of sulfur radicals mediated by succinimidyl and/or iodine radicals.[31-34]

Contrary to previous cyclization reactions examined, the postulated mechanisms for the conversion of Cys(Acm)-OH to cystine by *N*-halosuccinimides possibly involved electrophilic attack of *N*-halosuccinimides[24-27] at the sulfur atom of Cys(Acm) to yield the intermediate sulfenyl iodides (RSI),[16-20] which were then coupled to form cystine. To perform direct cyclization on Cys(Acm)-peptide-resin, a series of reactions was carried out by treatments of the assembled Cys(Acm)-oxytocin-resin with 1.1–3.3 mmol of NIS (2.2–6.6 equiv) for 2–17 h. Unfortunately, all efforts to isolate the desired oxytocin from the reaction using 1.1 mmol of NIS (2.2 equiv) for 2–17 h failed. The isolated product appeared to be Cys(Acm)-oxytocin. The reaction using 2.2 mmol of NIS (4.4 equiv) for 2 h gave a small amount of oxytocin (>3% yield). It was found that the reaction using 3.3 mmol of NIS (6.6 equiv) for 2 h afforded oxytocin in >7% crude yield (1.5% after further purification). In order to examine the electrophilic effects of *N*-halosuccinimides on the efficiency of the cyclization reactions, a smaller amount of NCS (1.1 equiv) was used to cyclize the Cys(Acm)-oxytocin-resin. A similar yield of oxytocin was obtained. These results indicated that a chlorine atom, a less bulky halogen, attacked the *S*-Acm group of the protected oxytocin-resin more readily to form the *S*-halo intermediate. However, treatment of Cys(Acm)-vasopressin-resin with 2.2 mmol of NIS (4.4 equiv) afforded (Arg^8)-vasopressin in 10% overall yield. It is known that the conformations of oxytocin and vasopressin are almost identical. Both have the rigid structure of antiparallel "pleated sheets", composed of six amino acids and cyclized by a disulfide bond. However, the linear "tail" portion of oxytocin is also rigidly held in a folded conformation different from the conformationally free "tail" portion of vasopressin.[35] In accordance with previous reports that iodine oxidation of Cys(Acm)-oxytocin-resin gave only a 6% yield of oxytocin,[8,13] the low yields of these cyclization reactions using *N*-halosuccinimides apparently resulted from conformational restriction and steric hindrance. Therefore it could be postulated that due to their amino acid sequences, the protected thiol vasopressin-resin and especially the protected thiol oxytocin-resin induced intramolecular aggregations in which the *S*-Acm group of the protected thiol

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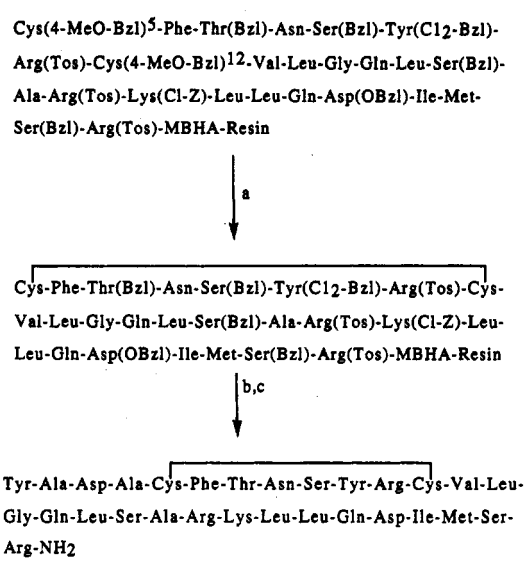
Scheme I

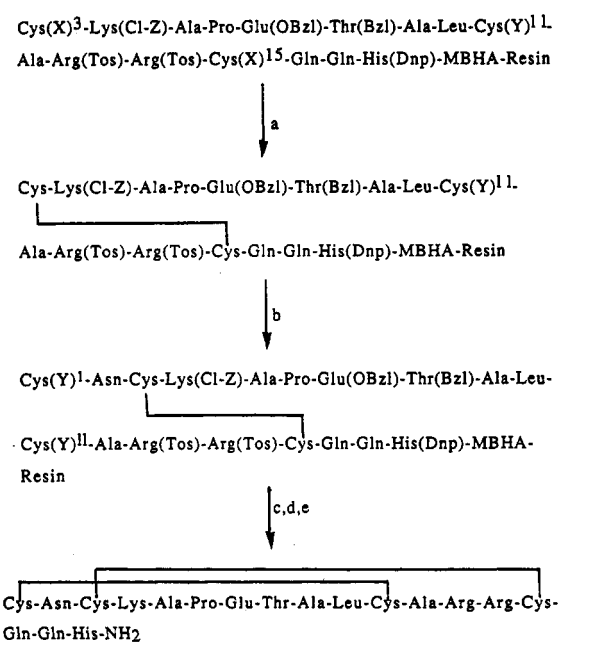


[a] Key: (a) NIS; (b) stepwise addition with Boc-Ala-OH, Boc-Asp(OcHex)-OH, Boc-Ala-OH, and Boc-Tyr(Cl_2Bzl)-OH; (c) HF–anisole–EMS (10:1:1), 0 °C, 1 h.

peptide-resin were less accessible to the attack of the halogen atom resulting in low yields of the cystine peptides. To avoid the difficulties encountered in direct synthesis of sequence-dependent cystine peptides on a resin support, it is recommended that these cystine peptides be prepared by treatment of a thiol peptide or a Cys(Acm)-peptide using NIS in nonaqueous media.

The successful preparation of (Arg^8)-vasopressin and oxytocin via a fully automated process suggests that stepwise addition of Boc-amino acids on the monocyclic peptide-resin can offer a larger and more complex cystine peptide after release from the resin. Accordingly, this approach was applied to prepare (Cys^5,Cys^{12}) human growth hormone releasing factor (1–29) NH_2, an analog of hGHRF-(1–29)-NH_2. Therefore, the sequence of (Cys^5,Cys^{12})hGHRF-(5–29)-NH_2 was assembled on a MBHA (*p*-methylbenzhydrylamine) resin, while Cys-5 and Cys-12 were protected by 4-MeOBzl groups (Scheme I). Cyclization of (Cys^5,Cys^{12})hGHRF-(5–29)-resin was accomplished by treatment with 0.5 mmol of NIS (1.0 equiv) for 2 h in the peptide synthesizer. Subsequent elongation of the monocyclic (Cys^5,Cys^{12})hGHRF-(5–29)-resin with Boc-Ala-OH, Boc-Asp(OcHex)-OH, Boc-Ala-OH, and Boc-Tyr(Cl_2Bzl)-OH completed the sequence to generate (Cys^5,Cys^{12})hGHRF-(1–29)-resin. After cleavage, deblocking, and purification of the peptide, (Cys^5,Cys^{12})-hGHRF-(1–29)-NH_2 was obtained in 15.6% (93% pure) overall yield. Apparently, the S-protected cysteine in this peptide was more conformationally accessible to attack by NIS and methionine was not sensitive to NIS during cyclization. The result also indicated that the direct conversion of Cys(4-MeOBzl)-OH to cystine on the peptide-resin favored intramolecular cyclization[14] and provided an efficient synthesis for cystine peptides. Instead, the time-consuming ferricyanide oxidation[29] of assembled dithiol (Cys^5,Cys^{12})hGHRF-(1–29)-NH_2 only afforded less than 3% overall yield of methionine sulfoxide form, which was also obtained by bubbling (Cys^5,Cys^{12})hGHRF-(1–29)-NH_2 with air in aqueous solution. Apparently, this peptide can not be prepared by the conventional air oxidation of dithiol hGHRF-(1–29)-NH_2 in dilute solution due to the oxygen-sensitive methionine residue.[11]

Scheme II



[a] Key: X = Acm, Y = 4-MeOBzl. (a) NIS; (b) stepwise addition with Boc-Asn-OH and Boc-Cys(4-MeOBzl)-OH; (c) NIS; (d) 10% thiophenol in DMF, 2 h; (e) HF–anisole–EMS (10:1:1), 0 °C, 1 h. X = 4-MeOBzl, Y = Acm. (a) NIS; (b) stepwise addition with Boc-Asn-OH and Boc-Cys(Acm)-OH; (c) NIS; (d) 10% thiophenol in DMF, 2 h; (e) HF–anisole–EMS (10:1:1), O °C, 1 h.

Apamin, a bi-cystine peptide with 18 amino acid residues, is a neurotoxic component of bee venom. Previously, preparation of apamin had been extensively studied using different S-protecting groups, such as *tert*-butylsulfenyl,[1] 4-MeOBzl,[2] Acm,[2] and 4-MeBzl[3] for temporary protections of the four cysteine residues during solid-phase synthesis. The formation of disulfide bridges by random oxidation was always the last step of the operation for the synthesis of this peptide. However, stepwise formation of disulfide bonds had been used in the synthesis of multi-cystine peptides such as conotoxin GI[9] and insulin.[36] The successful approach to the preparation of (Cys^5,Cys^{12})hGHRF-(1–29)-NH_2 opens up the possibility for the fully automated solid-phase synthesis of apamin via the combined processes of stepwise addition of Boc-amino acids and controlled stepwise formation of disulfide bonds, which can be constructed from different pairs of S-protected cysteine residues. Thus Cys-3 and Cys-15 were protected by Acm groups during synthesis of apamin, while Cys-1 and Cys-11 were protected by 4-MeOBzl groups (Scheme II). The assembled apamin-(3–18)-resin was cyclized to the corresponding Cys-3–Cys-15 monocyclic form by treatment with 2.2 mmol of NIS (2.2 equiv to starting resin) for 2 h in a peptide synthesizer. The Cys-3–Cys-15 monocyclic form of the Apamin (3–18)-resin was further elongated with Boc-Asn-OH and Boc-Cys(4-MeOBzl)-OH to give the Cys-3–Cys-15 monocyclic form of the apamin resin. The formation of the second disulfide bond between Cys-1 and Cys-11 on the monocyclic apamin-resin was carried out by 1.1 mmol of NIS (1.1 equiv) for 1 h. After cleavage, deblocking, and purification of the peptide, apamin was obtained in 11% overall yield based on the starting resin. In the comparative experiments (Scheme II), Cys-3 and Cys-15 were protected by

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Table I. Conversion of Cysteine Derivatives into Cystine by *N*-Halosuccinimides

cysteine deriv	route	reagent	amt of reagent (equiv)	reactn time (h)	isol yield (%)
cysteine·HCl·H_2O	1[a]	NIS	0.55	1	69
	1[a]	NCS	0.55	1	84.1
	1[a]	NBS	0.55	1	68.2
Boc-Cys(Acm)-OH	2[b]	NIS	1.1	2	40
	2[b]	NCS	1.1	2	41.7
	2[b]	NBS	1.1	2	40
Boc-Cys-(4-MeOBzl)-OH	3[a]	NIS	1.1	1	53.1
Boc-Cys-(4-MeBzl)-OH	3[a]	NIS	1.1	1	5.7 (80% pure)

[a] Solvent, DMF–CH_2Cl_2. [b] Solvent, CH_2Cl_2.

4-MeOBzl groups, while Cys-1 and Cys-11 were protected by Acm groups. Similar methodology was applied to give apamin in 8.3–10.5% overall yield. Since both Cys(Acm) and Cys(4-MeOBzl) residues on peptide-resin would be converted to cystine by NIS through electrophilic and radical mechanisms, respectively, the possibility of formation of intramolecular disulfide bridges between Cys-1 and Cys-3, between Cys-3 and Cys-11, or between Cys-11 and Cys-15 could not be excluded. However, these side reactions could possibly be minimized as shown by the good yields of apamin due to stepwise formation of disulfide bridges formed by different amount of NIS through different mechanisms. In addition, histidine was not sensitive to NIS during the cyclization reaction. The results also indicated that the monocyclic form of the protected peptide-resin did not necessarily induce intramolecular aggregation to hinder the formation of the second disulfide bond and that the fully automated solid-phase procedure using the combined processes of stepwise addition of Boc-amino acids and stepwise formation of disulfide bonds provided a practical approach for synthesis of multi-cystine peptides. Furthermore, all results shown suggest that in addition to the convenience of a nonaqueous workup, NIS is a mild and useful reagent in the synthesis of cystine peptides.

In conclusion, the studies of direct conversion of cysteine, Cys(Acm)-OH, Cys(4-MeOBzl)-OH, or Cys(4-MeBzl)-OH to cystine using *N*-halosuccinimides have led to the successful utilization of NIS in the synthesis of (Arg^8)-vasopressin and oxytocin under mild conditions by a fully automated process of solid-phase peptide synthesis or by cyclizing the released thiol peptides in DMF–CH_2Cl_2. These simple and synthetically useful methods provided new approaches for the fully automated solid-phase synthesis of (Cys^5,Cys^{12})hGHRF-(1–29)-NH_2 and apamin based on the combined processes of stepwise addition of Boc-amino acids and controlled stepwise formation of disulfide bonds using NIS. The scope, limitations, and applications of these approaches to the synthesis of larger and more complex cystine peptides are under investigation.

Experimental Section

Boc-amino acid derivatives were purchased from Peninsula (Belmont, CA) and Bachem (Torrance, CA). TFA and TEA were distilled prior to use. All other solvents and chemicals were of analytical grade.

Solid-Phase Synthesis of Peptides. Peptides were assembled by stepwise solid-phase procedure[28] on a MBHA-resin (usually, substitution = 0.574) using a Beckmann System 990 synthesizer. Side-chain protecting groups of Boc-amino acids were Tos (*p*-toluenesulfonyl) for arginine, 4-MeOBzl, 4-MeBzl, or Acm for cysteine, Cl_2Bzl (2,4-dichlorobenzyl) for tyrosine, ClZ (2-chlorobenzyloxycarbonyl) for lysine, Dnp (2,4-dinitrophenyl) for histidine, Bzl (benzyl) for glutamic acid, serine, and threonine, and Bzl or cHex (cyclohexyl ester) for aspartic acid. General procedures for peptide synthesis involved the coupling of DCC-Boc-amino acid-resin (2:2:1 mmolar equiv) for 2 h and regular deprotection of the Boc group with 45% TFA in CH_2Cl_2 containing 0.1% indole for 20 min. The DCC/HOBt (1-hydroxybenzotriazole) method was used to couple Boc-Gln-OH and Boc-Asn-OH only. The peptides were cleaved from the resin with removal of the side-chain protecting groups by anhydrous HF–anisole–EMS (ethyl methyl sulfide) (10:1:1) at 0 °C for 1 h and then washed alternatively with ether and CH_2Cl_2 followed by extraction with 2 N AcOH and lyophilization. The peptide-resin was treated with 10% thiophenol in DMF for 2 h to remove the Dnp group before the procedures of cleavage and deblocking.

General Procedures for the Formation of Disulfide Bond of Cystine Peptides Using NIS. Two general strategies were utilized to construct disulfide bridges of cystine peptides. In method A, the mixture of resin and released dithiol peptide was treated with NIS in 40 mL of DMF–CH_2Cl_2 under nitrogen at room temperature. The reaction mixture was filtered and washed with MeOH to give crude cystine peptide. In method B, direct cyclization on S-protected peptide-resin was carried out in a solution of NIS in 25 mL of DMF–CH_2Cl_2 for 2 h in the vessel of the synthesizer. In this automated process, the reaction mixture was drained and washed with two cycles of MeOH and CH_2Cl_2.

Identification of Cystine and Cystine Peptides. All cystine and cystine peptides were detected by TLC (silica gel 60 F_{254}; solvent systems: *n*-butanol–pyridine–AcOH–H_2O, 4:4:1:1) staining with ninhydrin and/or Pauly reagent. ^1H NMR spectra were recorded at 500 MHz. The characterizations of cystine peptides were determined by HPLC employing a C_{18}-bonded reversed phase silica column (Vydac194C_{18}Al) using a linear acetonitrile gradient (0–80%) as eluent at a flow rate of 1 mL/min. The purified products were compared with authentic or reference compounds and characterized by amino acid analysis after hydrolysis in 6 M hydrochloric acid (110 °C, 20 h) in tubes sealed under vacuum. Amino acid analyses were carried out using Amino Quant of Hewlett Packard. Low-resolution fast atom bombardment (FAB) mass spectra (MS *m/z* (relative intensity, ion)) for apamin were obtained in the Mass Spectrometry Facility, The Research Institute of Scripps Clinic, La Jolla, CA.

1. Cystine. Route 1. A mixture of cysteine hydrochloride hydrate (176 mg, 1 mmol) and NIS (130 mg, 0.55 mmol) in DMF–CH_2Cl_2 (15 mL) was stirred under nitrogen gas at room temperature for 1 h. The reaction mixture was evaporated under vacuum. MeOH was added to the residue and then the mixture was titrated with 5 N NaOH to pH 4–5. The precipitate was filtered and washed with MeOH to give 83.2 mg (69%) of cystine, which was identical to an authentic sample: ^1H NMR (D_2O) 3.99 (q, 2 H, CH), 3.25 (dd, 2 H, SCH_2), 3.04 (q, 2 H, SCH_2). Similar treatments with NCS and NBS gave 101.8 mg (84.1%) and 82.6 mg (68.2%) of cystine, respectively.

Route 2. A mixture of Boc-Cys(Acm)-OH (292 mg, 1 mmol) and NIS (260 mg, 1.1 mmol) in CH_2Cl_2 (2.8 mL) was stirred at room temperature for 2 h. TFA (2.3 mL) was added to the reaction mixture and the mixture was stirred for another 20 min. The reaction mixture was evaporated under vacuum. MeOH was added to the residue and then the mixture was titrated with 5 N NaOH to pH 4–5. The precipitate was filtered and washed with MeOH to give 48.1 mg of cystine (40% overall yield). Similar treatments with NCS and NBS gave 50.4 mg (41.7%) and 48 mg (40%) of cystine, respectively.

Route 3. A mixture of Boc-Cys(4-MeOBzl)-OH (260 mg, 1 mmol) and NIS (260 mg, 1.1 mmol) in DMF–CH_2Cl_2 (2.8 mL) was stirred at room temperature for 1 h. TFA (2.3 mL) was added to the reaction mixture, and the mixture was stirred for another 20 min. The reaction mixture was evaporated under vacuum. MeOH was added to the residue and then the mixture was titrated with 5 N NaOH to pH 4–5. The precipitate was filtered and washed with MeOH to give crude product which was

Table II. Conversion of S-Protected Peptidyl Resins into Cystine Peptides by NIS

cystine peptide	route	S-protected peptidyl resin	molar ratio (NIS/resin)	reactn time (h)	isol yield based on starting resin (%)
(Arg^8)-vasopressin	1[a]	4-MeOBzl	0.22	17	34
	1[a]	4-MeOBzl	0.6	1	30.6
	1[a]	4-MeOBzl	1	2	43 (70% pure)
	2[b]	4-MeOBzl	1.1	2	41
	3[b]	4-MeBzl	1.1	2	20
	4[b]	Acm	4.4	2	10
oxytocin	1[b]	4-MeOBzl	1.1	2	1[c]
	2[b]	Acm	6.6	2	1.5[b]
(Cys^5,Cys^{12})hGHRF-(1–29)-NH_2	1[b]	4-MeOBzl	1	2	15.6 (93% pure)
apamin	1[b]	$Cys^{3,15}$-Acm and $Cys^{1,11}$-4-MeOBzl	2.2	2	11 (95% pure)
			1.1	1	
	2[b]	$Cys^{3,15}$-4-MeOBzl and $Cys^{1,11}$-Acm	1.1	1	10.5 (86% pure)
			2.2	2	
	2[b]	$Cys^{3,15}$-4-MeOBzl and $Cys^{1,11}$-Acm	0.6	2	8.3 (83% pure)
			2.2	2	

[a] Cyclizing the released thiol peptide in DMF–CH_2Cl_2. [b] Fully automated process of solid-phase peptide synthesis. [c] >7% crude yield. [d] >7% crude yield.

further heated with AcOH (1 mL) at 75 °C for 30 min and then evaporated under vacuum. MeOH was added to the residue, and the precipitate was filtered and washed with MeOH to give 62 mg of cystine (53.1% overall yield). Usually, the crude product of cystine was coprecipitated with *N*-(*p*-methoxybenzyl)succinamic acid: ^1H NMR (D_2O) 6.8 (d, aromatic H), 7.2 (d, aromatic H), 3.71, (s, 3 H, CH_3O), 3.68 (s, 2 H, $-CH_2N$), 2.64 (s, 4 H, $-CH_2-CH_2-$). Similar treatment of Boc-Cys(4-MeBzl)-OH with NIS gave 6.9 mg (5.7%, >80% pure) of cystine containing *N*-(*p*-methylbenzyl)succinamic acid: ^1H NMR (D_2O) 7.1 (m, aromatic H), 3.6 (s, 2 H, $-CH_2N$), 2.64 (s, 4 H, $-CH_2CH_2-$), 2.2 (s, 3 H, CH_3). No further purification was attempted.

2. (Arg^8)-vasopressin, Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2. Route 1. (Arg^8)-vasopressin was synthesized from Cys(4-MeOBzl)-vasopressin-resin by method A using 70 mg of NIS (0.3 mmol) for 1 h. The crude product was purified on a carboxymethylcellulose CM-32 cation exchange column using 0.01 M NH_4OAc, pH 4.5–0.2 M NH_4OAc, pH 6.5 as eluent and a Sephadex G-25 partition column using *n*-butanol–ethanol–pyridine–0.2 N AcOH (4:1:1:7) as eluent. The overall yield of (Arg^8)-vasopressin was 30.6% (166 mg) based on 0.5 mmol of starting resin. Amino acid analysis: Asn 1.13 (1), Gln 1.14 (1), Gly 1.12 (1), Arg 1.04 (1), Tyr 0.92 (1), Phe 1.00 (1), Pro 1.02 (1).

Route 2. (Arg^8)-vasopressin was prepared from Cys(4-MeOBzl)-vasopressin-resin by method B using 130 mg of NIS (0.55 mmol). The crude product was purified on a carboxymethylcellulose CM-32 cation exchange column using 0.01 M NH_4OAc, pH 4.5–0.2 M NH_4Ac, pH 6.5 as eluent and a Sephadex G-25 partition column using *n*-butanol–ethanol–pyridine–0.2 N AcOH (4:1:1:7) as eluent. The overall yield of (Arg^8)-vasopressin was 41% (224 mg) based on 0.5 mmol of resin.

Route 3. Similarly, (Arg^8)-vasopressin was prepared from Cys(4-MeBzl)-vasopressin-resin by method B using 130 mg of NIS (0.55 mmol). The crude product was purified on a Sephadex G-25 partition column using *n*-butanol–AcOH–H_2O (4:1:5) as eluent to give (Arg^8)-vasopressin in 20% (105.5 mg) overall yield based on 0.5 mmol of resin.

Route 4. Similarly, (Arg^8)-vasopressin was synthesized from Cys(Acm)-vasopressin-resin by method B using 540 mg of NIS (2.2 mmol). After purification on a Sephadex G-50 column using 30% AcOH as eluent and a carboxymethylcellulose CM-32 cation exchange column using 0.01 M NH_4OAc, pH 4.5–0.2 M NH_4OAc, pH 6.5 as eluent, the overall yield of (Arg^8)-vasopressin was 10% (54.5 mg) based on 0.5 mmol of resin.

3. Oxytocin, Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2. Route 1. Oxytocin was prepared from Cys(4-MeOBzl)-oxytocin-resin by method B using 130 mg of NIS (0.55 mmol). The crude peptide was purified on a Sephadex G-50 column using 30% AcOH as eluent and a carboxymethylcellulose CM-32 cation exchange column using 0.01 M NH_4OAc, pH 4.5–0.1 M NH_4OAc, pH 6.5 as eluent to give >7% crude yield of oxytocin. The impure compound was further purified on the same CM-32 column and two Sephadex G-25 partition columns subsequently using *n*-butanol–AcOH–H_2O (4:1:5) and *n*-butanol–ethanol–pyridine–0.2 N AcOH (4:1:1:7) as eluent. The overall yield of oxytocin was 1% (4 mg) based on 0.5 mmol of resin. Amino acid analysis: Asn 1.00 (1), Gln 1.08 (1), Gly 1.17 (1), Tyr 0.79 (1), Ile 0.99 (1), Leu 1.09 (1), Pro 0.91 (1).

Route 2. Similarly, oxytocin was prepared from Cys(Acm)-oxytocin-resin by method B using 780 mg of of NIS (3.3 mmol). The crude product was purified on a Sephadex G-50 column using 30% AcOH as eluent to give >7% crude yield of oxytocin. The impure compound was further purified on a carboxymethylcellulose CM-32 cation exchange column using 0.01 M NH_4OAc, pH 4.5–0.1 M NH_4OAc, pH 6.5 as eluent. The overall yield of oxytocin was 1.5% (7.5 mg) based on 0.5 mmol of resin.

4. (Cys^5,Cys^{12})hGHRF-(1–29)-NH_2, Tyr-Ala-Asp-Ala-Cys-Phe-Thr-Asn-Ser-Tyr-Arg-Cys-Val-Leu-Gly-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Met-Ser-Arg-NH_2. The assembled (Cys^5,Cys^{12})hGHRF-(5–29)-resin was cyclized by method B using 120 mg of NIS (0.5 mmol) for 2 h. The monocyclic form of (Cys^5,Cys^{12})hGHRF-(5–29)-resin was subsequently elongated with Boc-Ala-OH, Boc-Asp(OcHex)-OH, Boc-Ala-OH, and Boc-Tyr(Cl_2Bzl)-OH to generate (Cys^5,Cys^{12})hGHRF-(1–29)-resin (see Scheme I). After the procedures of cleavage and deprotection, the crude peptide was purified on a Sephadex G-50 column using 30% AcOH as eluent to give (Cys^5,Cys^{12})hGHRF-(1–29) NH_2 in 15.6% (260 mg, 93% pure) overall yield based on 0.5 mmol of resin. Amino acid analysis: Asx 3.0 (3), Glx 2.4 (2), Ser 3.0 (3), Leu 4.3 (4), Gly 1.7 (1), Ala 2.6 (3), Arg 2.9 (3), Thr 1.1 (1), Tyr 1.6 (2), Val 1.1 (1), Ile 1.1 (1), Met 1.3 (1), Phe 1.0 (1), Lys 1.2 (1).

5. Apamin, Cys-Asn-Cys-Lys-Ala-Pro-Glu-Thr-Ala-Leu-Cys-Ala-Arg-Arg-Cys-Gln-Gln-His-NH_2. Route 1. The sequence of apamin was assembled on MBHA-resin (substitution = 0.39) according to Scheme II. Cys-3 and Cys-15 were protected by Acm groups, while Cys-1 and Cys-11 were protected by 4-MeOBzl groups. The first disulfide bond between Cys-3 and Cys-15 on the asembled apamin-(3–18)-resin was constructed by method B using 520 mg of NIS (2.2 mmol) for 2 h. The Cys-3–Cys-15 monocyclic form of apamin-(3–18)-resin was subsequently elongated with Boc-Asn-OH and Boc-Cys(4-MeOBzl)-OH to generate the Cys-3–Cys-15 monocyclic form of apamin-resin. The formation of second disulfide bond between Cys-1 and Cys-11 on the monocyclic apamin-resin was carried out by method B using 260 mg of NIS (1.1 mmol) for 1 h to generate apamin-resin. After cleavage from the resin with removal of the side-chain protecting groups, the crude product was purified on a Sephadex G-50 column using 30% AcOH as eluent and a carboxymethylcellulose CM-32 cation exchange column using 0.01 M NH_4OAc, pH 4.5–0.3 M NH_4OAc, pH 6.5 as eluent. The overall yield of apamin was 11% (223 mg, 95% pure) based on 1.0 mmol) of resin. Amino acid analysis: Asx 0.9 (1), Glx 3.3 (3),

His 1.0 (1), Ala 2.9 (3), Arg 2.0 (2), Leu 0.9 (1), Lys 1.0 (1), Pro 0.9 (1), Thr 0.9 (1); MS *m/z* 2027 (100, calcd MW = 2027.3).

Route 2. The sequence of apamin was assembled on MBHA-resin (substitution = 0.574). Cys-3 and Cys-15 were protected by 4-MeOBzl groups, while Cys-1 and Cys-11 were protected by Acm groups. The assembled apamin-(3–18)-resin was cyclized to the Cys-3–Cys-15 monocyclic form by method B using 130 mg of NIS (0.55 mmol) for 1 h. The monocyclic form of apamin-(3–18)-resin was subsequently elongated with Boc-Asn-OH and Boc-Cys(Acm)-OH to generate the Cys-3–Cys-15 monocyclic apamin-resin. The formation of second disulfide bond between Cys-1 and Cys-11 on the monocyclic apamin-resin was accomplished by method B using 260 mg of NIS (1.1 mmol) for 2 h to generate apamin-resin. After the usual procedures of cleavage and deprotection, the crude product was purified on a Sephadex G-50 column using 30% AcOH as eluent, and a carboxymethylcellulose CM-32 cation exchange column using 0.01 M NH_4OAc, pH 4.5–0.3 M NH_4OAc, pH 6.5 as eluent. The overall yield of apamin was 10.5% (107 mg, 86% pure) based on 0.5 mmol of resin. In another experiment, the assembled apamin-(3–15)-resin was cyclized to the Cys-3–Cys-15 monocyclic form by method B using 70 mg of NIS (0.3 mmol) for 2 h. The formation of second disulfide bond between Cys-1 and Cys-11 on the monocyclic apamin-resin was carried out by method B using 260 mg of NIS (1.1 mmol) for 2 h. The crude product was purified on a Sephadex G-50 using 30% AcOH as eluent, a carboxymethylcellulose C-32 cation exchange column using 0.01 M NH_4OAc, pH 4.5–0.3 M NH_4OAc, pH 6.5 as eluent and a Sephadex G-25 partition column subsequently using *n*-butanol–AcOH–H_2O (4:1:5) and then 30% AcOH as eluents. The overall yield was 8.3% (84 mg, 83% pure) based on 0.5 mmol of starting resin.

Acknowledgment. I wish to thank Dr. Nicholas Ling and Dr. Stuart G. Levy for their helpful discussions. This work was supported by the National Institutes of Health Grants DK18811 and HD 09690.