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SOLID-PHASE APPROACHES TO REGIOSPECIFIC DOUBLE DISULFIDE FORMATION. APPLICATION TO A FRAGMENT OF BOVINE PITUITARY PEPTIDE

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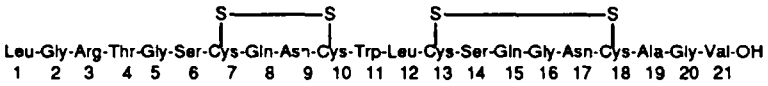
ABSTRACT: A 21-residue, two disulfide-containing peptide has been synthesized on solid phase. Three alternative protection schemes, based on Boc/benzyl chemistry and combinations of the 4-methylbenzyl with either acetamidomethyl, 9-fluorenylmethyl or 3-nitro-2-pyridylsulfenyl groups for pairs of cysteine residues have been examined. The most successful route involved formation of the first disulfide on the resin *via* 9-fluorenylmethylcysteine deprotection-oxidation.

INTRODUCTION

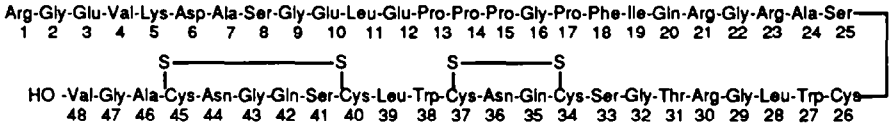
Synthesis of multiple disulfide-containing peptides remains one of the undiminished challenges of peptide chemistry. When the goal is the native form of a given peptide, spontaneous oxidation can usually be expected to lead to the desired product[1], although yields are often low. In all other cases, however, the thermodynamically favored isomer may not coincide with the synthetic target and regiospecific disulfide formation methods become a must. Recent accounts of directed double-disulfide peptide syntheses include apamin[2], conotoxins[3], endothelins[4], and enterotoxins[5]. In these syntheses a fully protected peptide sequence bearing two orthogonal (*e.g.*, HF-stable Acm[6] and HF-labile Meb) cysteine protecting groups was prepared and partially deprotected to give the dihydro peptide, which was then air-oxidized to the first disulfide. Subsequent deprotection-oxidation of the two other thiol functions gave rise to the second disulfide. In all these instances, assembly of the peptide sequence was done either in solution or in the solid phase but disulfide bond formation was invariably carried out in solution.

The possibilities of disulfide bond formation on the polymer phase have only been moderately explored so far[7,8]. Furthermore, it has been assumed for some time that "assymetrical disulfides are particularly unstable in markedly acidic solutions"[9] and therefore synthetic schemes in which disulfide bridges are exposed to final HF acidolysis have not been favored. However, successful reports of disulfide formation in the polymer phase with subsequent recovery of the HF-cleaved peptide in unaltered, correctly oxidized form have recently appeared[10,11,12], highlighting the potential advantages of this approach.

As part of our ongoing research in directed disulfide formation schemes[13,14,15], this paper describes the synthesis of peptide **1**, a 21-residue sequence corresponding to the C-terminal section of posterior pituitary peptide, **2**[16]. Although structurally similar to other peptide hormones such as lysine vasopressin, oxytocin, or corticotropin releasing factor, no significant biological activity has so far been reported for **2**[17] . However, its distribution of cysteine and hydrophobic residues is quite homologous to a multigenic family of proteins from the African swine fever virus[18]. in which we are interested[19]. We have consequently set out to prepare **1** as a preliminary step in the study of potential functional similarities with the gene products of the mentioned multigenic family.



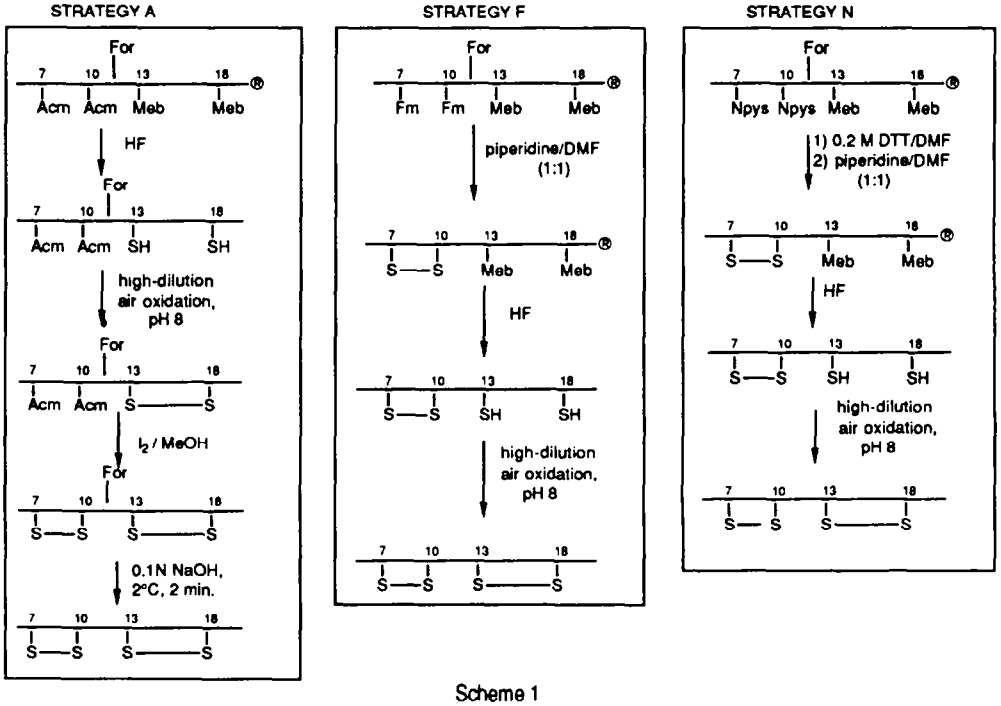
Peptide 1



Peptide 2

RESULTS AND DISCUSSION

In designing strategies for the solid phase synthesis of 1, particular attention has been given to the protection of the four cysteine and one tryptophan residues.Three alternative schemes (Scheme 1, where ® symbolizes the polymer support), all of them based on Boc/benzyl-type chemistry, have been tested. Common to all these strategies is: i) the use of the HF-labile Meb protecting group for Cys^{13} and Cys^{18} and ii) the protection of the indole ring of Trp^{11} with the formyl (For) group. The other two cysteine residues (7 and 10) have been protected with either Acm (strategy A), or Npys (strategy N), or Fm (strategy F). All three schemes have been devised to allow stepwise regiospecific disulfide formation (either on-the-resin or in solution) based on the orthogonality of the Cys protecting groups used in each case.



Scheme 1

Strategy A

This strategy is quite similar to the one successfully employed by the group of Sakakibara in their recent synthesis of endothelins A, B and C[5]. In our case, the fully protected peptide resin was submitted to HF acidolysis to give a partially protected [$Cys^{7,10}$ (Acm), Trp^{11}(For)] peptide which, after DTT reduction to regenerate some prematurely oxidized-polymerized material, and preparative MPLC purification, was air-oxidized under high dilution conditions to give the $Cys^{13,18}$ disulfide. Formation of the second disulfide ($Cys^{7,10}$) took place upon iodine-promoted deprotection of the Acm group[20]. The fully oxidized, indole-protected peptide was isolated from the reaction mixture[21] and then briefly treated with 0.1 M NaOH at 2°C to remove the For group. The completely deprotected material (>80% by analytical HPLC) was further purified by preparative MPLC to give an homogeneous product in an overall synthesis-cleavage-purification yield of 6%. Figure 1 illustrates different stages along the deprotection, disulfide formation and purification of peptide **1** by this strategy. Further characterization was done by amino acid analysis, plasma desorption (Cf^{252}) time-of-flight mass spectroscopy[22], and HPLC comparison with a sample of the correctly folded peptide (prepared by strategy F, see below). The latter method allowed to conclude that the pattern of cysteine connectivities was the desired one.

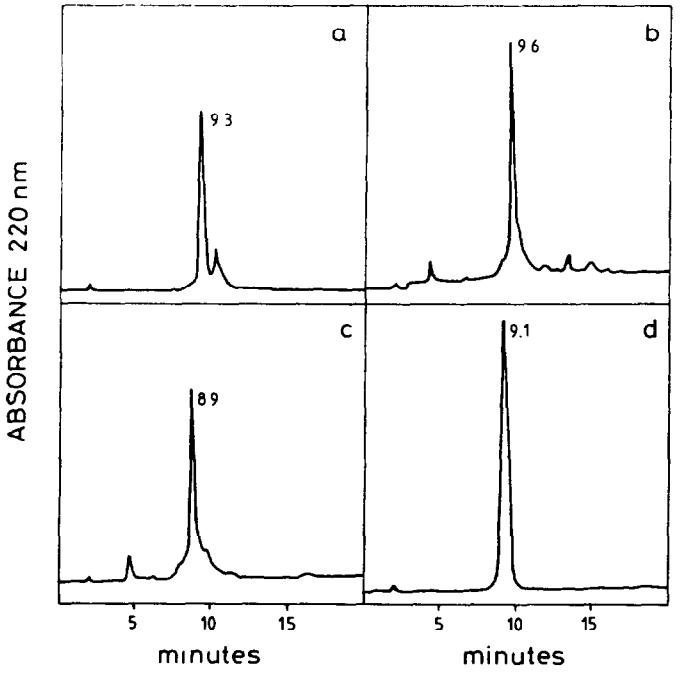


Figure 1. HPLC analysis of the disulfide formation, formyltryptophan deprotection and purification of peptide **1** by strategy A. Panel a: crude, partially protected [$Cys^{7,10}$(Acm),Trp^{11}(For)] peptide **1** after HF cleavage and DTT reduction; b: previous product after air-oxidation (formation of $Cys^{13,18}$ disulfide); c: crude from iodine-promoted Acm deprotection-oxidation (Trp^{11}-protected); d: deformylated final product **1**, after MPLC purification. HPLC conditions : Vydac C_{18} (5 µm, 25 x 0.46 cm), flow 1.5 mL/min,10-80%B in 20 min. A: H_2O (0.045% TFA), B: CH_3CN (0.035% TFA).

Strategy F

This strategy has been largely influenced by chemistry developed in our laboratory, which has shown the adequacy of Fm for orthogonal protection of cysteine in Boc/benzyl-based synthetic strategies[23]. After assembly of the protected peptide resin, a treatment with 1:1 DMF-piperidine for 3 h allowed formation of the $Cys^{7,10}$ disulfide with simultaneous deprotection of formyltryptophan. The completeness of the oxidation reaction was assessed by qualitative Ellman determination of thiol groups on the resin[24]. HF acidolysis of this partially protected peptide resin gave a crude with an HPLC profile (Fig. 2a) showing a major component and the significant absence of absorption at 300 nm, confirming the complete removal of the Fm and For groups. Without further purification, this crude was air-oxidized to give the second disulfide. The product exhibited a major component by analytical HPLC (Fig. 2b) which was purified to homogeneity by MPLC. The global yield was in this case 9%.

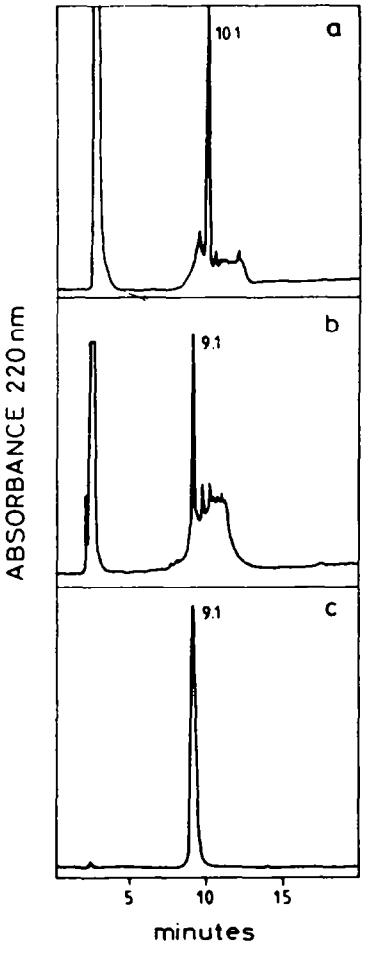
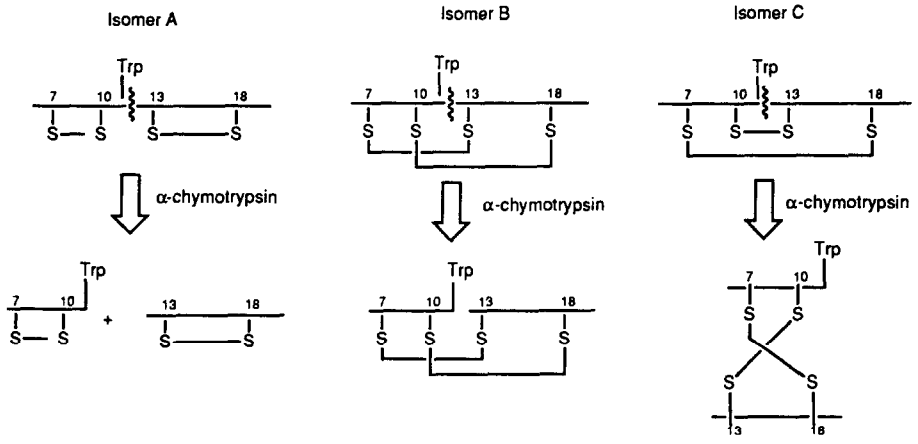


Figure 2. HPLC analysis of the disulfide formation, formyltryptophan deprotection and purification of peptide 1 by strategy F. Panel a: crude, HF-cleaved product after piperidine-promoted, on-the-resin disulfide ($Cys^{7,10}$) formation and For deprotection; b: previous product after air-oxidation to give the second disulfide; c:same, after final MPLC purification. HPLC conditions same as Fig. 1.

Determination of disulfide connectivities

In contrast to other synthetic targets[4], the fact that no significant bioassay for either **1** or **2** is yet available prevented us from using such criterium in the structural assignation of products obtained by the different strategies. In the original work of Preddie[17], assignment of Cys parings was considerably facilitated by the Trp residue in position 38 of **2** (corresponding to residue 11 of **1**), which defined a major cleavage point with chymotrypsin. We have used a similar reasoning for unequivocal assignation of the disulfides in the synthetic products. Thus, of all possible forms of disulfide pairing in **1**, that corresponding to the native structure (Isomer A, Scheme 2) is unique in that cleavage at residue 11 will lead to fragments of smaller size whereas other disulfide arrangements will tend to give a single major product with the amino acid composition of the full sequence.



Scheme 2

Chymotryptic digestion of the purified final product from strategy F produced a mixture of three peptides (Fig. 6) corresponding to fragments 2-11, 12-21 and 2-21 of the sequence of **1** released by cleavage at residues Leu^{1} and Trp^{11}.These findings confirmed that the product of synthetic strategy F indeed possessed the desired disulfide arrangement.

Strategy N

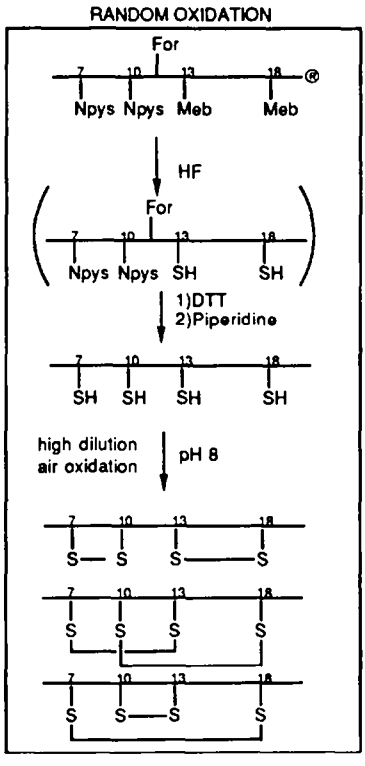
Several procedures to form the $Cys^{7,10}$ disulfide on the peptide resin by selective deprotection of the Npys group were attempted: i) piperidine-DMF (1:1, v/v), 4 hr; ii) 0.1 M DTT in DMF, 1h followed by piperidine-DMF (1:1, v/v), 3 hr; iii) 0.1 M DTT in DMF, 30 min, followed by 0.01 M potassium ferricyanide in DMF-H_2O(9:1, v/v), 3 h.; and iv) 0.1 M DTT in DMF, 30 min, followed by 0.1 M N-methylmorpholine acetate in DMF (pH *ca.* 8), 3 h. HPLC analysis of the HF-cleaved crude products corresponding to each one of these protocols were rather complex, evidencing extensive intermolecular

disulfide formation. No attempts have been made to characterize the components of these mixtures.

Several interpretations of these results are possible. One possibility is that protocol i) could produce slow, partial deprotection of the Npys groups, which in turn would favor both intra- and intermolecular disulfide formation by reaction of the newly released thiolate anions with the highly reactive, Npys-activated cysteine residues. This hypothesis is somewhat antagonical, however, to the fact that fast and complete Npys deprotection is known to be achieved under the non-basic conditions of the first part of protocol ii, yet similar results are obtained. An alternative explanation would be to assume that, under these given conditions, effective site isolations (*i.e.*, ideal pseudo-dilution state) is not attainable. Again, the fact that satisfactory levels of intramolecular disulfide formation were found in strategy F for a peptide resin of similar substitution under relatively comparable conditions seems to contradict this view. Finally, a third possibility could be that random intra- and intermolecular disulfide formation, catalyzed by 3-nitro-2-thiopyridone incompletely washed off the peptide resin during the Npys deprotection step, takes place either during HF cleavage or the ensuing workup steps.

Spontaneous air-oxidation of the tetrahydro peptide.

A final aspect of interest in this study was to investigate the result of spontaneous, high-dilution air oxidation of the fully deprotected, reduced peptide. The composition of the resulting mixture would reflect the relative stabilities of the three possible disulfide arrangements. Cleavage of the peptide resin prepared according to strategy N, followed by Npys removal with DTT and Trp(For) deprotection with piperidine under the same reductive conditions, gave a tetrahydro derivative (Scheme 3 and Fig. 3) which, upon air oxidation, yielded a mixture of three disulfide products in a 70:18:12 ratio (Fig. 3c). The major component was separated by MPLC (global yield 5%) and shown to coelute with the products of strategies A and F above. Its chymotryptic map was also comparable to that obtained for strategy F. Thus, in this case the product of directed disulfide formation by strategies A and F coincided with the thermodinamically favored isomer.



Scheme 3

Conclusion

The usefulness of stepwise, regiospecific methods for the preparation of double-disulfide structures such as **1** has been established. Although in this case both A and F synthetic strategies happen to lead to the same product obtained by atmosferic oxidation of the fully reduced tetrahydro peptide derivative, their general validity for any multiple disulfide-containing peptide must be fully appreciated. In particular, the results of strategy F complement previous work which had shown the potential of Fm cysteine protection for the preparation of single disulfide peptides[23] The convenience of this approach becomes even more evident in the present case, where it allows a more advantageous use of solid phase chemistry resulting in fewer purification steps and improved yields.

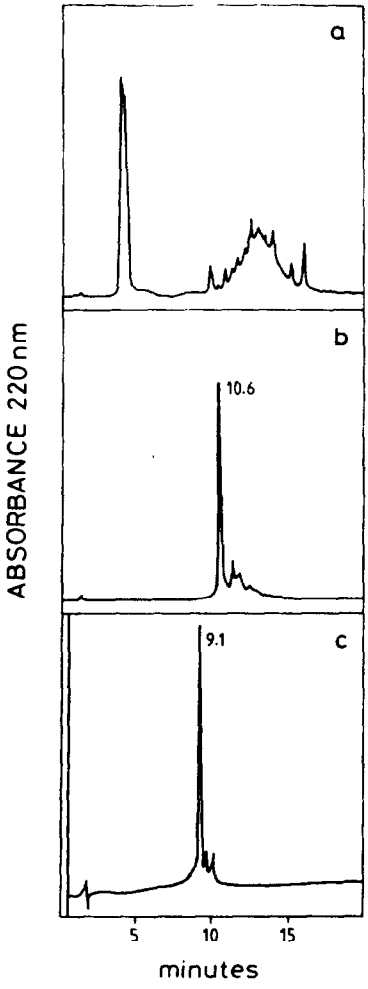


Figure 3. HPLC analysis of the spontaneous disulfide formation, formyltryptophan deprotection and purification of the tetrahydro derivative of peptide **1**. Panel a: crude, HF-cleaved product; b: previous product after DTT reduction and piperidine-promoted For deprotection (tetrahydro peptide); c: mixture obtained after air-oxidation of tetrahydro peptide. HPLC conditions: same as Fig. 1.

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MATERIALS AND METHODS

Chemicals

Aminomethyl resin (0.65 mmol/g) and protected amino acids were purchased from Novabiochem AG (Läufelfingen, Switzerland) except Boc-Cys(Npys)-OH, which was synthesized in our laboratory according to Bernatowicz et al[25]. Boc-Val-OCH_2-C_6H_4-CH_2-COOH was prepared according to Mitchell et al[26]. DTT, N-methylmorpholine and piperidine were from Merck (analytical grade) and used without further purification. Bovine α-chymotrypsin, immobilized on carboxymethylcellulose, was from Sigma. Other peptide synthesis reagents and solvents (CH_2Cl_2, DCC, DIEA, DMF and TFA) were of analytical grade and purified as previously described[19].

Synthesis of (S)-9-fluorenylmethylcysteine.

5.4 g (34.4 mmol) of L-cysteine hydrochloride and 15.6 g (43 mmol) of 9-fluorenylmethyl tosylate dissolved in 150 mL of DMF and 18 mL of DIEA (105.8 mmol) were slowly added dropwise over a 2.5 h period. A gelatinous white precipitate formed overnight which was filtered and dried to give the title compound in quantitative yield.

Synthesis of N^{α}-tert-butyloxycarbonyl-(S)-9-fluorenylmethylcysteine.

11.5 g (34.4 mmol) of (S)-9-fluorenylmethylcysteine were dissolved in 35 mL of 2:1 (v:v) *tert*-butanol-H_2O and 14.0 g of Boc_2O in 40 mL of *tert*-butanol were added. The pH was adjusted to 9-9.5 with 10% Na_2CO_3 and stirring was continued until no further reaction was observed by TLC ($CHCl_3$ / MeOH / HOAc, 85:10:5). After 6 h the mixture was acidified with HCl 1N at pH 2 and filtered. The aqueous solution was extracted with EtOAc, the organic layer was dried with $MgSO_4$ and the solvent was removed. The resulting oil was dissolved in EtOH and the product precipitated by the addition of water. After drying *in vacuo* 10.2 g (25.5 mmol) of Boc-Cys(Fm)-OH (74%) were obtained, mp 74-75° C, TLC, R_f=0.32 ($CHCl_3$ / MeOH / HOAc, 85:10:5), ^1H NMR (200 MHz, DMSO-d_6) d= 7.91-7.12 (m, 8H, Ar, Fm), 4.12 (m, 1H, CH, Fm), 3.32 (broad s, 1H, CH_2, Cys), 3.07 (d, 2H, CH_2, Fm), 2.82 (m, 2H, CH_2, Cys), 1.3 (s, 9H, CH_3, Boc).

Synthesis of Trp(For)-Leu-Cys(4-MeBzl)-Ser(Bzl)-Gln-Asn-Cys(Meb)-Ala-Gly-Val-OCH_2-Pam-Phe-resin..

The synthesis of the undecapeptide resin common to all three strategies was carried out in the manual mode starting with 2.5 g of aminomethyl resin. Boc-Phe (2.5 equiv.) was anchored to the polymer as internal standard with DCC (2.5 equiv., 50 min). After deprotection of the Boc group with 40% (v/v) TFA/CH_2Cl_2 and neutralization, the C-terminal Val residue was incorporated as Boc-Val-OCH_2-C_6H_4-CH_2-COOH (1.5 equiv.) in the presence of 1.5 equiv. of DCC (20h). For the remaining residues of the sequence, the following coupling protocol was used (15-20 mL solvent/ g resin) : (1) 40% TFA-CH_2Cl_2 (1 x 1 min; 1 x 20 min); (2) CH_2Cl_2 (5 x 1 min); (3) 5% DIEA-CH_2Cl_2 (3 x 2 min); (4) CH_2Cl_2 (4 x 1 min); (5a) Boc-amino acid, 2.5 equiv. in 5 mL CH_2Cl_2 (2 min), then (5b) 2.5 mL of 1 M DCC in CH_2Cl_2 (50 min); (6) CH_2Cl_2 (2 x 1 min); (7) DMF (2 x 1 min); (8) CH_2Cl_2 (2 x 1 min). Couplings were monitored by the qualitative ninhydrin test[27] and repeated if necessary (steps 3 to 8 above). Exceptions to this protocol were Boc-Arg(Tos), Boc-Asn and Boc-Gln which were coupled in DMF-CH_2Cl_2. The weight of the peptide resin at the end of these cycles was 5.0 g, corresponding to 78% from the theoretical value. Amino acid analysis of the peptide resin hydrolysate (12 N HCl -

propionic acid (1:1), 3 h, 160°C) gave Phe 1.0 (1, internal standard), Val 1.0 (1), Gly 2.15 (2), Ala 0.89 (1), Asp 0.90 (1), Glu 0.84 (1), Leu 0.87 (1), Ser 0.68 (1) [Cys and Trp not determined].

Protected peptide resins A, F and N

The above peptide resin was split into three one-gram batches upon which the syntheses according to strategies A, F and N were continued, following identical protocols. Amino acid analyses of resin hydrolysates were as follows: Resin A : Asp 1.78 (2); Thr 0.80 (1); Ser 1.54 (2); Glu 1.67 (2); Gly 3.71 (4); Ala 0.98 (1); 1.00 Val (1); Leu 1.72 (2); Arg 0.76 (1). Resin F: Asp 1.81 (2); Thr 0.79 (1); Ser 1.52 (2); Glu 1.68 (2); Gly 3.67 (4); Ala 1.01 (1); Val 1.02 (1); Leu 1.66 (2); Arg 0.77 (1). Resin N: Asp 1.75 (2); Thr 0.71 (1); Ser 1.42 (2); Glu 1.65 (2); Gly 3.62 (4); Ala 0.98 (1); Val 1.01(1); Leu 1.60 (2); Arg 0.80 (1).

Strategy A

Cleavage, partial deprotection of peptide resin A and stepwise disulfide formation.

0.52 g of Leu-Gly-Arg(Tos)-Thr(Bzl)-Gly-Ser(Bzl)-Cys(Acm)-Gln-Asn-Cys(Acm)-Trp(For)-Leu-Cys(Meb)-Ser(Bzl)-Gln-Asn-Cys(Meb)-Ala-Gly-Val-OCH_2-Pam-Phe-resin was treated with 4.5 mL HF and 0.5 mL of *p*-cresol at 0°C for 1 h. After HF evaporation, the residue was washed with anhydrous ether and extracted with 10% HOAc and lyophilized. The resulting powder was treated with 7 mL of 10 mM DTT in water for 3 h at 30°C and then adsorbed onto Diaion HP-20 (20 g). The column was exhaustively washed with water until the eluent gave no reaction with Ellman's reagent[28]. At this point the peptide was eluted with 8:1 (v/v) CH_3CN - H_2O (0.1% TFA) and further purified by MPLC on Vydac C_{18} (15-20 μm, 25 x1.2 cm) using a 15-80% linear CH_3CN gradient. Fractions containing the purified major product were combined and added slowly dropwise over 500 mL of 0.05 M TrisHCl, pH 8, with vigorous stirring in a flat crystallization dish. When the addition was complete, the pH was adjusted to 8 with 0.1 M Tris.HCl, pH 8, and the oxidation reaction was allowed to proceed for 36 h at room temperature.

The reaction mixture was next loaded onto Diaion HP-20 and, after exhaustive column washing with H_20 the adsorbed peptide was eluted as above with 8:1 (v/v) CH_3CN / H_2O (0.1%TFA) and lyophilized. The residue was dissolved with 3:1 (v/v) H_2O /MeOH in the presence of 15 equiv. of hydrochloric acid and the solution was vigorously stirred in one portion into 16.5 mL of 0.1 M iodine in MeOH solution The oxidation reaction was stopped after 40 min by addition of saturated solution of ascorbic acid in 0.05 M sodium citrate buffer, pH 5, until total decoloration. After adsorption - elution on Diaion HP-20 as above and lyophilization, the salt-free peptide was treated with 0.1 N NaOH at 2°C in a UV-cell for 2 min. Fast UV scans of the solution showed immediate disappearance of the 300 nm absorption due to the formyl group. The reaction was quenched by addition of 10% HOAc, the peptide material was once again isolated by means of the Diaion HP-20 column and purified by MPLC on Vydac C_{18} (15-20 μm, 25 x 1.2 cm) using a 10-70% linear CH_3CN gradient. Fractions homogeneous by analytical HPLC were combined to give 3 mg of peptide **1**, characterized by amino acid analysis [Asp 1.89 (2), Glu 1.87 (2), Ser 1.64 (2), Thr 0.86 (1), Gly 3.90 (4), Ala 1.00 (1), Val 0.89 (1), Leu 1.90 (2), Arg 0.87 (1)] and ^{252}Cf time-of-flight mass spectrometry [molecular weight: calculated, 2153.2 , found,2154.0 (M+H)].

Strategy F

On-the-resin formation of $Cys^{7,10}$ disulfide

0.59 g of Leu-Gly-Arg(Tos)-Thr(Bzl)-Gly-Ser(Bzl)-Cys(Fm)-Gln-Asn-Cys(Fm)-Trp(For)-Leu-Cys(Meb)-Ser(Bzl)-Gln-Asn-Cys(Meb)-Ala-Gly-Val-O-CH_2-Pam-Phe-resin was swollen in CH_2Cl_2 and treated with 1:1 (v/v) piperidine-DMF for 3 h. After washings with DMF, CH_2Cl_2 and MeOH, the resin was dried and an aliquot submitted to an Ellman test which showed the absence of thiols.

Cleavage and total deprotection of peptide F and second disulfide bond formation

The above resin was treated with 4.5 mL of HF and 0.5 mL of *p*-cresol at 0°C for 1 h. After HF evaporation, the residue was washed with anhydrous ether and extracted with 10% HOAc, lyophilized and desalted on Sephadex G-15 eluted with 0.1 N HOAc. The peptide fractions were collected and added dropwise over 500 mL of 0.05 M Tris.HCl, pH 8. The pH was adjusted to 8 and the mixture was stirred vigorously for 36 h. The oxidation reaction was quenched with 10% HOAc, concentrated, desalted on Sephadex G-15 (0.1 N HOAc) and lyophilized. MPLC purification on Vydac C_{18} (15-20 μm, 25 x 1.2 cm), using a 15-80% linear acetonitrile gradient gave 11 mg of **1**, which was characterized by amino acid analysis [Asp 1.99 (2), Glu 2.10 (2), Ser 1.65 (2), Thr 0.84 (1), Gly 3.80 (4), Ala 1.00 (1),Val 0.93 (1), Leu 1.89 (2), Arg 0.90 (1)] and ^{252}Cf time-of-flight mass spectrometry [molecular weight: calculated, 2153.2 , found, 2176.3 (M+Na), 2154.6 (M+H)].

Determination of disulfide connectivity by enzymatic hydrolysis

0.5 mg of pure peptide **1** were dissolved in 500 μL of H_2O and treated with 50 μL of a suspension of α-chymotrypsin linked to carboxymethylcellulose (9 mg/mL) for 150 min at 37°C. The reaction mixture was centrifuged and chymotryptic peptides in the supernatant were analyzed by HPLC on a Vydac C_{18} column (5 μm, 25 x 0.46 cm) using a linear 10-80% acetonitrile gradient in water (0.05% TFA) over 20 minutes. The eluent was monitored at 220 and 280 nm. Each peak was collected, hydrolyzed with 6 N hydrochloric acid for 24 h at 110°C and submitted to amino acid analysis.

Strategy N

*Attempts to prepare **1** by on-the-resin $Cys^{7,10}$ disulfide formation*

The procedure for protocol ii of the Results section was as follows. 80 mg of Leu-Gly-Arg(Tos)-Thr(Bzl)-Gly-Ser(Bzl)-Cys(Npys)-Gln-Asn-Cys(Npys)-Trp(For)-Leu-Cys(Meb)-Ser(Bzl)-Gln-Asn-Cys(Meb)-Ala-Gly-Val-OCH_2-Pam-Phe-resin was placed in a propylene syringe fitted with a polyethylene fritted disk, swollen in CH_2Cl_2 and treated repeatedly with 0.2 M DTT in DMF for 1 h. After washings with DMF, DCM and MeOH the resin was re-swollen in DCM and treated 1:1 (v/v) piperidine-DMF for 3 h, then washed and dried. As discussed in the Results section, HF cleavage of the resulting resin gave crudes of considerable complexity by HPLC. This route was therefore not pursued.

Preparation of fully reduced and deprotected peptide

117 mg of Leu-Gly-Arg(Tos)-Thr(Bzl)-Gly-Ser(Bzl)-Cys(Npys)-Gln-Asn-Cys(Npys)-Trp(For)-Leu-Cys(Meb)-Ser(Bzl)-Gln-Asn-Cys(Meb)-Ala-Gly-Val-OCH_2-Pam-Phe-resin was treated with 4.5 mL of anhydrous HF in the presence of 0.5 mL of *p*-cresol at 0°C for 1 h. After HF evaporation the resin was washed with ether and extracted with 10% HOAc. 11.3 μmol of the crude peptide was treated with 5 mL of 0.2 M DTT in 0.1 N HOAc (100 x excess) for 8 h at 24°C, then piperidine was added until pH 9.5 and for 5 min. The reaction was quenched by addition of glacial HOAc until pH 4.5. The reaction crude was then loaded to a Sephadex G-15 column eluted with 0.1 N HOAc. The front peptide fractions were pooled, lyophilized and purified by MPLC on Vydac C_{18} (15-20 μm, 25 x 1.2 cm) using a 10-70% linear gradient of acetonitrile in water (0.05% TFA).

Random oxidation of the tetrahydro derivative. Identification of the major product

Purified reduced peptide fractions from the MPLC purification were slowly added dropwise over 500 mL of 0.1 M Tris HCl, pH 8, in a flat crystallizing dish with vigorous stirring. The oxidation reaction was allowed to proceed for 36 h, then quenched with 10% HOAc and the resulting solution was concentrated by rotary-evaporation, placed in a polyethylene container and lyophilized. The oxidized material was finally desalted by molecular filtration on Sephadex G-10 eluted with 0.1 M HOAc. Semi-preparative HPLC of the product allowed the separation of the three expected oxidation products. Fractions corresponding to the major component were lyophilized, dissolved in 250 μL of phosphate buffer, pH 8, and submitted to chymotryptic hydrolysis as described above.

REFERENCES AND NOTES

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[6]Common abbreviations are: Acm, acetamidomethyl; Boc_2O, di-*tert*-butyl dicarbonate; DIEA, N,N-diisopropylethylamine; DMF, N,N-dimethylformamide; DCC, dicyclohexylcarbodiimide; DTT, 1,4-dithiothreitol; For, formyl; Fm, 9-fluorenylmethyl; Meb, 4-methylbenzyl; Npys, 3-nitro-2-pyridylsulfenyl.

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