Synthesis of Human C-Type Natriuretic Peptide 22 Using Chlorotrityl Resin and Tetrafluoroboric Acid Deprotection[1)]

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Human C-type natriuretic peptide 22 (hCNP22), the third member of the natriuretic peptide family, was efficiently synthesized by Fmoc-based peptide chain construction on a 2-chlorotrityl (Clt) resin, followed by deprotection using tetrafluoroboric acid (HBF_4). The use of Clt resin was effective in suppressing racemization at the C-terminal cysteine residue caused by the base treatment for Fmoc-cleavage. The disulfide bond of hCNP22 was constructed using the silyl chloride-sulfoxide method to avoid oxidation at the Met residue. Using amino acid- and dipeptide-resin derivatives, the effects of bases, protecting groups and resin supports on the racemization at the C-terminal Cys residue were examined in detail.

Key words human C-type natriuretic peptide 22; tetrafluoroboric acid; 2-chlorotrityl resin; racemization; C-terminal cysteine; silyl chloride-sulfoxide method

The C-type natriuretic peptide (CNP), identified in porcine brain by Sudoh *et al.* in 1990, is the third member of the natriuretic peptide family.[2)] Amino acid sequences of rat and human CNP precursors have been deduced from nucleotide sequence analysis, indicating that the amino acid sequence of CNP22 of these three mammalian species is identical.[3,4)] CNP shows a pharmacological spectrum similar to those of atrial natriuretic peptide (ANP)[5)] and brain natriuretic peptide (BNP),[6)] including diuretic/natriuretic, hypotensive, and chick rectum relaxant effects, although the relative potencies are different.

Human CNP22 consists of 22 amino acid residues, including Met and C-terminal Cys residues, and contains one intramolecular disulfide bond (Fig. 1). The structure shows a high sequence homology to ANP and BNP within the 17 amino acid residue ring structure formed by an intramolecular disulfide linkage, although it completely lacks the C-terminal tail commonly found in ANPs and BNPs. We have synthesized a number of natriuretic peptide derivatives to determine the roles of individual amino acids of ANPs and BNPs in the biological activities.[7)] In this paper, we describe an efficient synthetic method for human CNP22[4)] which has provided a sufficient amount to allow investigation of the structure–activity relationships of CNPs.

In Fmoc-based solid phase peptide synthesis (SPPS), Atherton *et al.* briefly reported that the use of piperidine for Fmoc-cleavage during chain elongation had caused significant racemization at a C-terminal cysteine residue.[8)] This prompted us to reinvestigate the racemization at the C-terminal Cys residue in detail prior to the Fmoc-based synthesis of hCNP22 containing a C-terminal Cys residue.

Racemization of C-Terminal Cysteine First, in order to examine the effect of base, we exposed Boc–Cys(Acm)–OH linked to a 4-(benzyloxy)benzylalcohol resin (Wang resin)[9)] to four different kinds of bases, *i.e.*, 5% and 20% piperidine (PIP)/DMF, 5% diisopropylethylamine (DIEA)/DMF, and 5% Et_3N/DMF (Fig. 2a). For estimation of the racemization, resin-bound Cys (Acm) was cleaved by treatment with trifluoroacetic acid (TFA)–thioanisole (9:1) for 60 min at 25 °C. The D-isomer content of the product was examined using a Sumichiral OA-5000 column. With increasing concentration of piperidine for Fmoc cleavage, a higher degree of racemization was observed. In contrast, the use of Et_3N or DIEA did not affect the optical purity of Cys(Acm).

Next, we examined the effect of the *S*-protecting groups on the extent of the racemization (Fig. 2b). For this purpose, three kinds of resin-bound dipeptide derivatives were prepared; Val–Cys(R)–Wang resin (R = trimethylacetamidomethyl (Tacm),[10)] MeBzl, or *t*Bu). Each dipeptide-resin derivative was then treated with 20% PIP/DMF. The dipeptide was cleaved from the base-treated resin with TFA–thioanisole (9:1) as described above and the L-D-isomer content of the product was analyzed using reverse-phase (RP)-HPLC.[11)] More than 10% formation of the L-D-isomers was detected for all of the *S*-protecting groups, although there were some differences in the extent of racemization.

Then, the effect of the resin was examined using Wang, Merrifield, Pam,[12)] and 2-chlorotrityl (Clt) resins.[13)] Boc–Cys(Acm) bound to each resin was exposed to various bases, and the optical purity of the Cys(Acm) liberated from each resin with an acid (TFA or HF) was analyzed on the Sumichiral OA-5000 column. The products prepared from Pam, Wang, and Merrifield resins contained significant amounts of isomers, in that order. However, little racemization (<5%) was detected even after 24 h treatment with 20% PIP/DMF when Clt resin was used (Fig. 2c and 2d). These results suggest that the use of Clt resin will circumvent the racemization

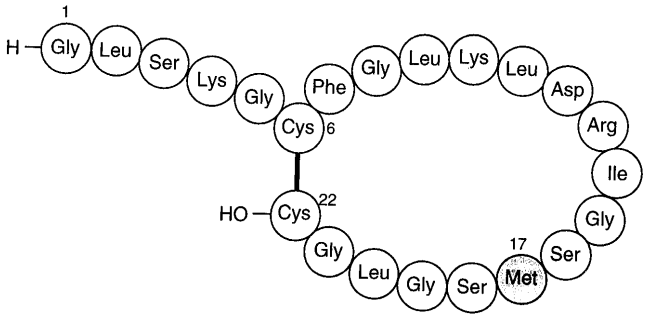

Fig. 1. Structure of Human C-Type Natriuretic Peptide 22

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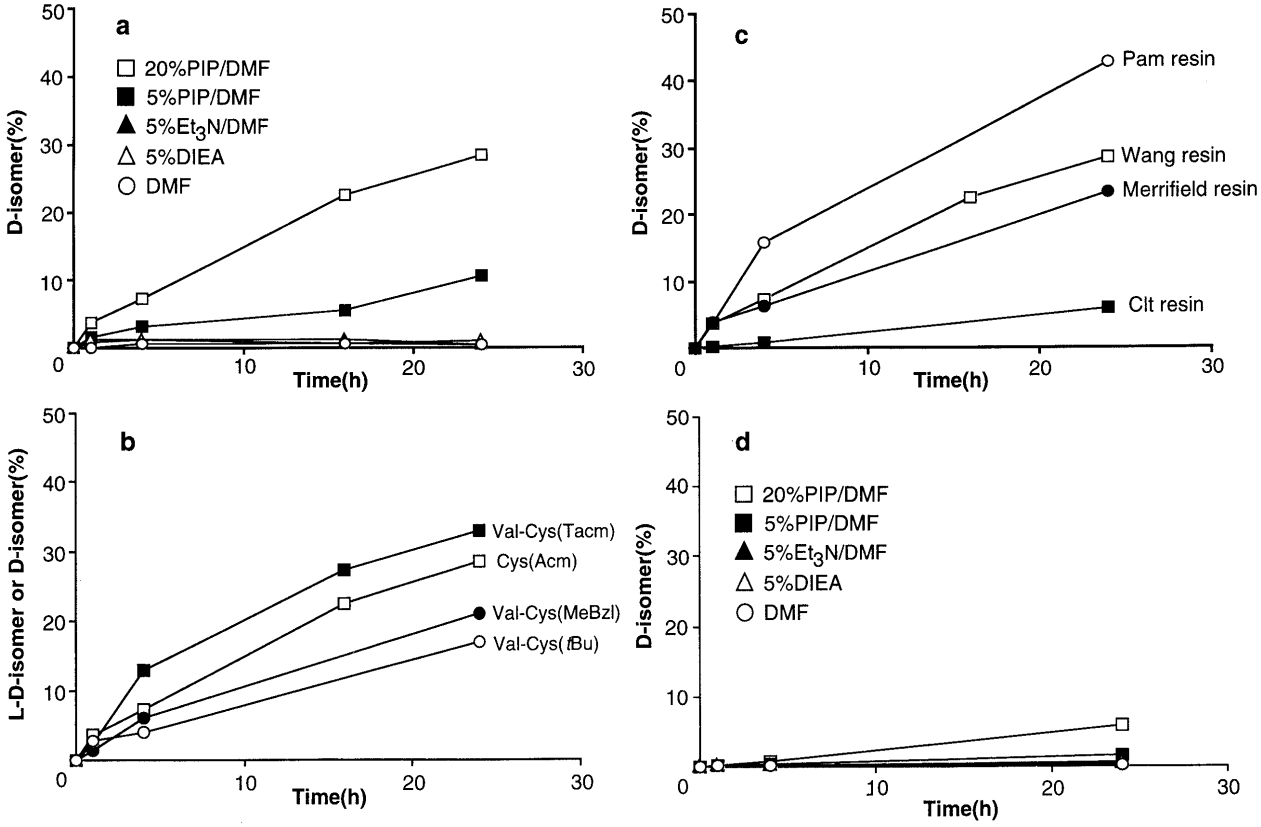

Fig. 2. Time Course of D-Isomer or L-D-Isomer Formation in Samples of Cys Derivative Attached to Resin under Various Basic Conditions

a, Cys(Acm) on Wang resin; b, *S*-protecting groups on Wang resin (20% PIP/DMF); c, Cys(Acm) on various resins (20% PIP/DMF); d, Cys(Acm) on Clt resin.

of C-terminal Cys in practical Fmoc-based SPPS. We consider that the bulky chlorotrityl group probably helps to suppress the racemization of C-terminal Cys residue due to its high steric hindrance.

Synthesis of Somatostatin In order to examine the usefulness of the Clt resin in practical synthesis, we synthesized somatostatin as a model peptide using Fmoc–Cys(Acm)–Clt resin (Fig. 3). The attachment of the C-terminal Cys(Acm) residue to the Clt resin was carried out according to the published procedure without racemization.[13c] The peptide backbone was constructed by the standard Fmoc-based SPPS using diisopropylcarbodiimide (DIPCDI) condensation and piperidine deprotection.[14] The fully protected peptide resin was treated with 1 M HBF_4–thioanisole/TFA[15] in the presence of *m*-cresol and EDT to cleave the peptide from the resin and, at the same time, to remove all the protecting groups except for two *S*-Acm groups. The *S*-protected product was purified by fast protein liquid chromatography (FPLC) and then treated with silver tetrafluoroborate ($AgBF_4$)–anisole/TFA[16] to remove the remaining two *S*-Acm groups. To construct the intramolecular disulfide bridge, the reduced peptide was subjected to air-oxidation at pH 7.8 under a high dilution condition. The progress of the reaction was monitored by RP-HPLC (Fig. 4a). The crude air-oxidized compound had a sharp main peak and was purified by preparative FPLC to give a homogeneous peptide in 26% yield (calculated from the starting Fmoc–Cys(Acm)–Clt resin). For comparison, SPPS of somatostatin using the same procedure as above, but starting from the conventional Wang resin was conducted. Alternatively, [D-Cys[14]]-somatostatin was prepared starting from Fmoc–D-Cys(Acm)–Wang resin to confirm that the side product after air-oxidation was [D-Cys[14]]-somatostatin. As shown in Fig. 4b, the crude product after air-oxidation contained 10% [D-Cys[14]]-somatostatin as a side product, and the isolation yield of the desired product was 17% (calculated from the starting Fmoc–Cys(Acm)–Wang resin). The results clearly show that Clt resin is more effective than the conventional Wang resin for suppression of racemization at the C-terminal Cys residue during Fmoc-based SPPS.

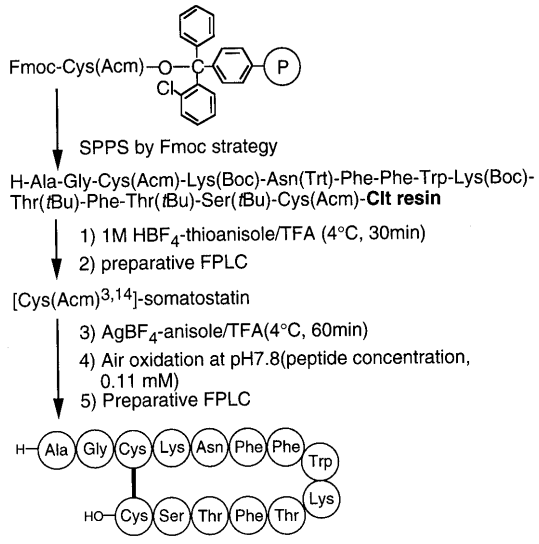

Fig. 3. Synthetic Scheme for Somatostatin

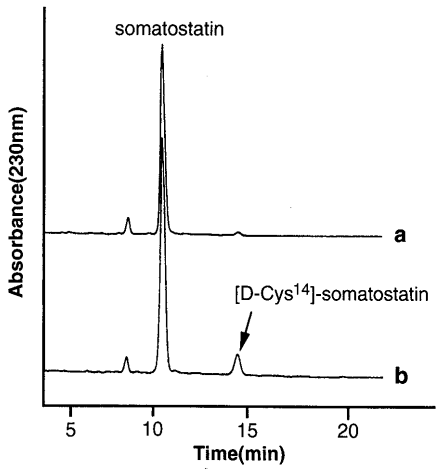

Fig. 4. HPLC Elution Patterns of Crude Somatostatin after Air-Oxidation

a, Clt resin; b, Wang resin. HPLC conditions: column, YMC AM302 (4.6 × 150 mm); eluent, 27% CH_3CN/0.1% TFA; flow rate, 1.0 ml/min.

Synthesis of hCNP22 We then applied the above SPPS method using Clt resin and HBF_4 deprotection procedure to the efficient synthesis of hCNP22. For the construction of a disulfide bond in hCNP22, we employed a silyl chloridesulfoxide method[17] instead of the air-oxidation method adopted above for somatostatin synthesis. The silyl chloride method can cleave various *S*-protecting groups of cysteine within 10—30 min to form a disulfide bond without affecting Met residue. We have already demonstrated in the previous synthesis of human BNP32 that this superior property of the silyl chloride method is especially suitable for efficient synthesis of a Met-containing cystine peptide.[17a,b]

Our synthetic scheme for hCNP22 is shown in Fig. 5. The fully protected hCNP22 was assembled on the Clt resin using standard Fmoc-based SPPS as described for the somatostatin synthesis. The protected hCNP22 resin was treated with 1 M HBF_4–thioanisole/TFA in the presence of *m*-cresol and EDT in an ice-bath for 30 min to remove all the protecting groups except for the two *S*-Acm groups and to cleave the peptide from the resin. The di-Acm form of hCNP22 thus obtained was purified by FPLC on ODS column. The purified di-Acm peptide was then treated with CH_3SiCl_3 in the presence of PhS(O)Ph at 25 °C for 15 min to form the disulfide bond (Fig. 6a). The reaction was stopped by the addition of NH_4F and the product was isolated by extraction with 4 N AcOH. After gel-filtration on a Sephadex G-10 column, the crude product showed a single main peak on HPLC, indicating that no oxidation had occurred at the Met residue of hCNP22, as expected. The product was purified by FPLC, and the homogeneous peptide was obtained in 28% yield (calculated from Fmoc–Cys(Acm)–Clt resin). The purified hCNP22 exhibited a single sharp peak on analytical HPLC and was proved to be a monomer by fast atom bombardment-mass spectrometry (FAB-MS). The purity of the purified hCNP22 was further confirmed by amino acid analysis after 6 N HCl hydrolysis and leucine-aminopeptidase (LAP) digestion.

We alternatively synthesized hCNP22 starting from Fmoc–Cys(Acm)–Wang resin. In this alternative synthesis, the peptide chain construction and the following deprotection were conducted in the same way as above. The disulfide bond was constructed by conventional air-oxidation instead of the silyl chloride–sulfoxide method. To determine the quantity of [D-Cys^{22}]-hCNP22 in the crude peptide after air-oxidation, [D-Cys^{22}]-hCNP22, as a reference compound, was prepared starting from Fmoc–D-Cys(Acm)–Wang resin. The crude product contained large amounts of [Met^{17}(O)]- and [D-Cys^{22}]-forms as shown in Fig. 6 and the isolation yield of the desired peptide was only 7%.

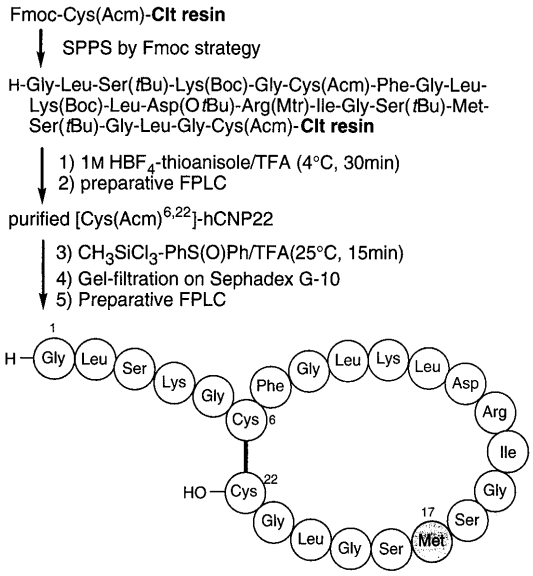

Fig. 5. Synthetic Scheme for hCNP22

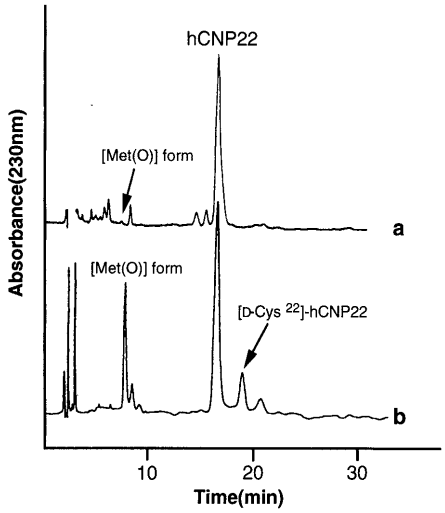

Fig. 6. HPLC Elution Patterns of Crude hCNP22

a, Clt resin (after CH_3SiCl_3–PhS(O)Ph/TFA); b, Wang resin (after air oxidation). HPLC conditions: column, YMC AM 302 (4.6 × 150 mm); eluent, 24% CH_3CN/0.1% TFA; flow rate, 1.0 ml/min.

Conclusion

We have synthesized hCNP22 containing a C-terminal Cys and a Met by combination of Fmoc-based SPPS using the Clt-resin with the HBF_4 deprotection method. In the model experiment, the Clt resin was found to be the most effective to suppress racemization of the C-terminal Cys residue, due to the steric hindrance of the 2-chlorotrityl

group. Using Clt resin, somatostatin and hCNP22 both containing C-terminal Cys residues were obtained in excellent yield without racemization. For the synthesis of hCNP22, the silyl chloride method was found to be suitable to construct the disulfide bond without oxidation at the Met residue. These results indicate that the synthetic method adopted for the present synthesis (Fig. 5) is suitable for the syntheses of complex cystine-peptides, especially those having a C-terminal cysteine residue.

Experimental

Fmoc amino acid derivatives, H–Cys(Acm)–OH·HCl, H–D-Cys(Acm)–OH·HCl, 4-(benzyloxy)benzylalcohol resin (Wang resin), 2-chlorotrityl (Clt) resin, and Boc–Cys(Acm)–Pam resin were purchased from Nova Biochem (Switzerland). Fmoc–D-Cys(Acm)–OH, Fmoc–D-Cys(*t*Bu)–OH, and Boc–Cys(Acm)–Merrifield resin were purchased from Watanabe Chemical Ind. (Hiroshima). Leucine-aminopeptidase (LAP, lot No. 61H-7045) was purchased from Sigma. Analytical HPLC and amino acid analysis were conducted with a Hitachi L-6200 and a Hitachi L-8500, respectively. FAB-MS was obtained on a JEOL JMS-SX102A spectrometer equipped with the JMA-DA7000 data system. Optical rotation was determined with a Horiba SEPA-200 polarimeter. Preparative FPLC and HPLC were conducted with a Pharmacia FPLC system and a Shimadzu LC-4A, respectively.

Solid Phase Peptide Synthesis The assembly of H–Val–Cys(R)–Wang resins (R = Tacm, MeBzl, or *t*Bu) was manually performed starting from Fmoc–Cys(R)–Wang resins. Each dipeptide-resin derivative was constructed according to the following protocol: (1) 20% PIP/DMF (1 × 1 min, 1 × 20 min); (2) DMF (6 × 1 min); (3) Fmoc-amino acid coupling (90 min); (4) DMF (6 × 1 min). The condensation reaction of Fmoc–Val–OH (3 eq) was performed by use of DIPCDI (3 eq) in the presence of HOBt (3 eq). H–Val–Cys(R)–Wang resins were obtained by removal of the N-terminal Fmoc group with 20% PIP/DMF.

For the syntheses of somatostatin and hCNP22, the peptide chain assembly was performed on a continuous-flow Milligen 9050 peptide synthesizer, starting from Fmoc–Cys(Acm)–Wang resin, Fmoc–D-Cys(Acm)–Wang resin, or Fmoc–Cys(Acm)–Clt resin. The flow rate of the unit pump was set at 5.0 ml/min and the following synthetic schedule was used: Fmoc-group deprotection with 20% PIP/DMF (7 min), DMF washing (12 min), Fmoc-amino acid coupling (30 min), and DMF washing (12 min). Fmoc-amino acids (4 eq) and HOBt (4 eq) were dissolved to a final concentration of 0.3 M with a solution of DIPCDI (0.3 M) in DMF.

Racemization of C-Terminal Cysteine Wang Resin: Boc–Cys(Acm)–Wang resin (50 mg, 26 μmol) was exposed to the following bases (5% PIP/DMF, 20% PIP/DMF, 5% DIEA/DMF, or 5% Et_3N/DMF) (1 ml). Aliquots (*ca.* 10 mg) of this resin were sampled at different times (1 h, 4 h, 16 h, and 24 h) and each resin obtained was treated with TFA–thioanisole (9 : 1, 1 ml) at 25 °C for 60 min. Dry ether (4 ml) and 4N AcOH (2 ml) were added to the reaction mixture. The aqueous phase was washed with dry ether three times, and the lower phase was lyophilized. The powder thus obtained was dissolved in H_2O (200 μl), and a definite volume (3 μl) was applied to a Sumichiral OA-5000 column. The eluate was monitored by UV absorption measurement at 254 nm. L-Cys(Acm): 10.36 min, D-Cys(Acm): 13.96 min [columm, Sumichiral OA-5000 (4.6 × 150 mm); eluent, aqueous 1% copper(II) sulfate solution; flow rate, 1.0 ml/min]. D-Enantiomer content of Cys(Acm) in each sample was determined by comparison of the areas of L-form and D-form on HPLC. These results are shown in Fig. 2a.

S-Protecting Groups: H–Val–Cys(R)–Wang resin (R = Tacm, MeBzl, *t*Bu) (50mg), prepared according to the manual schedule described above, was exposed to the following bases (5% PIP/DMF, 20% PIP/DMF, 5% DIEA/DMF, 5% Et_3N/DMF) (1 ml). Aliquots (*ca.* 10 mg) of these resins were sampled at different time intervals and each resin obtained was treated with TFA–thioanisole (9 : 1, 1 ml) at 25 °C for 60 min. Dry ether (4 ml) and 4N AcOH (2 ml) were added to the reaction mixture. The aqueous phase was washed with dry ether four times, and the lower phase was lyophilized. The powder thus obtained was dissolved in H_2O (300 μl), and a definite volume (5 μl) was applied to an RP-HPLC column. The eluate was monitored by UV absorption measurement at 230 nm. H–Val–D-Cys(R)–Wang resin (R = Tacm, MeBzl, *t*Bu) was also assembled essentially as described above. Three kinds of L-D-forms of dipeptide derivatives was obtained by treatment with TFA-thioanisole (9 : 1) at 25 °C for 60 min. H–Val–Cys(Tacm)–OH: 14.08 min, H–Val–D-Cys(Tacm)–OH: 27.04 min [YMC AM302 (4.6 × 150 mm); 9% CH_3CN in 0.1% TFA, 0.9 ml/min]. H–Val–Cys(MeBzl)–OH: 19.03 min, H–Val– D-Cys(MeBzl)–OH: 21.51 min [YMC AM302 (4.6 × 150 mm); CH_3CN (10—60%, 30 min) in 0.1% TFA; 0.9 ml/min]. H–Val–Cys(*t*Bu)–OH: 12.26 min, H–Val–D-Cys(*t*Bu)–OH: 16.42 min [YMC AM302 (4.6 × 150 mm); CH_3CN (10—60%, 30 min) in 0.1% TFA; 0.9 ml/min]. The isomer content in each sample was determined by comparison of the areas of L-L-form and L-D-form on RP-HPLC. These results are shown in Fig. 2b.

Resins: Boc–Cys(Acm)–Clt resin was prepared according to Barlos's procedure without racemization.[14c] Boc–Cys(Acm)–Merrifield resin (0.47 mmol/g, 50 mg), Boc–Cys(Acm)–Pam resin (0.50 mmol/g, 50 mg), and Boc–Cys(Acm)–Clt resin (0.46 mmol/g, 50 mg) were exposed to the four bases (5% PIP/DMF, 20% PIP/DMF, 5% DIEA/DMF, 5% Et_3N/DMF) (1 ml). Aliquots (*ca.* 10 mg) of these resins were sampled at different time intervals and each resin obtained was treated with TFA-thioanisole (9 : 1, 1 ml) at 25 °C for 60 min or HF–*m*-cresol (9 : 1, 1 ml) at 4 °C for 60 min. The D-enantiomer content of Cys(Acm) in each sample was determined as described above in the case of Wang resin. These results are shown in Fig. 2c and 2d.

Preparation of Somatostatin Using Clt Resin [Cys(Acm)^{3,14}]-Somatostatin: A fully protected somatostatin resin [H–Ala–Gly–Cys(Acm)–Lys(Boc)–Asn(Trt)–Phe–Phe–Trp–Lys(Boc)–Thr(*t*Bu)–Phe–Thr(*t*Bu)–Ser(*t*Bu)–Cys(Acm)–Clt resin] was assembled using a Milligen 9050 peptide synthesizer, starting from Fmoc–Cys(Acm)–Clt resin (395 mg; 0.20 mmol), according to the continuous flow synthetic procedure.[14] The protected somatostatin resin (200 mg) was treated with 1 M HBF_4–thioanisole in TFA (7 ml) in the presence of *m*-cresol (570 μl) and EDT (450 μl) in an ice-bath for 30 min. The crude product was extracted with 6 M guanidine·HCl (16 ml). The solution was applied to a Sephadex G-15 column (30 × 600 mm), which was eluted with 4N AcOH. The solvent of the fractions corresponding to the main peak was removed by lyophilization to give a powder (66 mg). The product was further purified by FPLC to afford a white powder: yield 47.5 mg (50%, calculated from Fmoc–Cys(Acm)–Clt resin), HPLC on a YMC AM302 (4.6 × 150 mm) [retention time; 19.16 min, on gradient elution with CH_3CN (10—60%, 30 min) in 0.1% TFA, 0.9 ml/min]. Amino acid analysis after 6N HCl hydrolysis and LAP digestion (numbers in parentheses): Asp × 1, 1.04 (N.D.); Thr × 2, 1.94 (2.24); Ser × 1, 0.55 (1.06); Gly × 1, 1.22 (1.04); Ala × 1, 1.04 (1.00); Phe × 3, 3.00 (3.00); Lys × 2, 2.06 (2.06); Trp × 1, 0.13 (1.02); Cys(Acm) × 2, N.D. (N.D.).

Somatostatin: The above di-Acm peptide (20 mg) was treated with $AgBF_4$ (220 mg, 100 eq) in the presence of anisole (121 μl) in TFA (3 ml) in an ice-bath for 60 min. Dry ether (30 ml) was added to the reaction mixture. The resulting powder was collected by centrifugation and dissolved in 4N AcOH (15 ml), then DTT (345 mg, 200 eq) was added. The solution was stirred at 25 °C overnight and the supernatant was applied to a column of Sephadex G-10 (20 × 300 mm) using 4N AcOH as an eluant. The fractions corresponding to the front main peak were collected and diluted with H_2O (100 ml). The pH of this solution was adjusted to 7.8 with 5% NH_4OH and the solution was kept standing at 25 °C. The progress of air oxidation was monitored by HPLC (Fig. 4a). After 14 h, the entire solution was lyophilized to give a powder. The crude peptide was purified by FPLC on a YMC ODS-AQ120 (15 × 500 mm) column, which was eluted with a linear gradient of 60% CH_3CN/0.1% TFA (15—100%, 400 min) in 0.1% aqueous TFA at flow rate of 4.0 ml/min. The eluate corresponding to the main peak was collected and lyophilized to yield a white fluffy powder: yield 9.4 mg (52%, calculated from di-Acm somatostatin; overall yield 26%, calculated from Fmoc–Cys(Acm)–Clt resin), $[\alpha]_D^{28}$ −35.3° (*c* = 0.22, 1N AcOH). HPLC on a YMC AM302 (4.6 × 150 mm) [retention time; 10.52 min, 27% CH_3CN in 0.1% TFA, 1.0 ml/min]. Amino acid analysis after 6N HCl hydrolysis and LAP digestion (numbers in parentheses): Asp × 1, 1.06 (N.D.); Thr × 2, 1.99 (2.30); Ser × 1, 1.09 (1.17); Gly × 1, 1.09 (1.13); Ala × 1, 1.02 (1.08); Cys × 1, 0.72 (1.01); Phe × 3, 3.00 (3.00); Lys × 2, 1.99 (2.08); Trp × 1, 0.35 (1.03). FAB-MS, *m/z* 1637.730 for $[M+H]^+$ (Calcd 1637.722 for $C_{76}H_{105}N_{18}O_{19}S_2$).

Preparation of Somatostatin Using Wang Resin [Cys(Acm)^{3,14}]-Somatostatin: A fully protected somatostatin Wang resin was constructed in essentially the same manner as above, starting from Fmoc–Cys(Acm)–Wang resin (395 mg; 0.20 mmol). The protected somatostatin resin (200 mg) was treated with 1 M HBF_4–thioanisole in TFA (5 ml) in

the presence of *m*-cresol (380 μl) and EDT (300 μl) in an ice-bath for 90 min. The crude product was purified by FPLC to afford a white powder: yield 42.2 mg (46%, calculated from Fmoc–Cys(Acm)–Wang resin). HPLC on a YMC AM302 (4.6 × 150 mm) [retention time; 19.14 min, on gradient elution with CH_3CN (10—60%, 30 min) in 0.1% TFA, 0.9 ml/min].

Somatostatin: The di-Acm peptide (20 mg) in TFA (3 ml) was treated with $AgBF_4$ (220 mg, 100 eq) in the presence of anisole (121 μl), followed by DTT (345mg). After air oxidation at pH 7.8 for 14 h (Fig. 4b), the major and the minor product were eluted at retention times of 10.54 min and 14.76 min, respectively. The major product was purified by FPLC to give a white fluffy powder: yield 6.7 mg (38%, calculated from di-Acm somatostatin; overall yield 17%, calculated from Fmoc–Cys(Acm)–Wang resin), $[\alpha]_D^{28}$ −32.7° (c=0.33, 1 N AcOH). HPLC on a YMC AM302 (4.6 × 150 mm) [retention time; 10.54 min, 27% CH_3CN in 0.1% TFA, 1.0 ml/min]. The minor peak shown in Fig. 4b was co-eluted with the following [D-Cys^{14}]-somatostatin, prepared alternatively.

[D-Cys^{14}]-Somatostatin: [D-Cys^{14}]-Somatostatin was prepared by essentially the same method as described above for the synthesis of somatostatin, starting from Fmoc-D-Cys(Acm)–Wang resin (417 mg; 0.20 mmol). Finally, purified [D-Cys^{14}]-somatostatin was obtained in 10% yield (calculated from Fmoc-D-Cys(Acm)–Wang resin). $[\alpha]_D^{28}$ −15.6° (c=0.21, 1 N AcOH), HPLC on a YMC AM302 (4.6 × 150 mm) [retention time; 14.75 min, 27% CH_3CN in 0.1% TFA, 1.0 ml/min].

Preparation of hCNP22 Using Clt Resin [Cys(Acm)^{6,22}]-hCNP22: A fully protected hCNP22 Clt resin [H–Gly–Leu–Ser(*t*Bu)–Lys(Boc)–Gly–Cys(Acm)–Phe–Gly—Leu–Lys(Boc)–Leu–Asp(O*t*Bu)–Arg(Mtr)–Ile–Gly–Ser(*t*Bu)–Met–Ser(*t*Bu)–Gly–Leu–Gly–Cys(Acm)–Clt resin] was constructed using a Milligen 9050 peptide synthesizer, starting from Fmoc–Cys(Acm)–Clt resin (642 mg; 0.10 mmol). The protected peptide resin (400 mg) was treated with 1 M HBF_4-thioanisole in TFA (5 ml) in the presence of *m*-cresol (327 μl) and EDT (26 μl) in an ice-bath for 30 min. The crude product was extracted with 4 N AcOH (15 ml). The solution was applied to a Sephadex G-25 (M) column (27 × 900 mm), which was eluted with 1 N AcOH. The solvent of the fractions corresponding to the main peak was removed by lyophilization to give a powder (68 mg). The di-Acm-hCNP22 was further purified by FPLC to afford a white powder: yield 36 mg (49%, calculated from Fmoc–Cys(Acm)–Clt resin), HPLC on a YMC AM302 (4.6 × 150 mm) [retention time; 18.55 min, on gradient elution with CH_3CN (10—60%, 30 min) in 0.1% TFA, 0.9 ml/min]. Amino acid analysis after 6 N HCl hydrolysis and LAP digestion (numbers in parentheses): Asp × 1, 1.10 (0.86); Ser × 3, 2.75 (2.69); Gly × 6, 6.09 (5.84); Met × 1, 0.99 (1.02); Ile × 1, 0.99 (1.00); Leu × 4, 4.00 (4.00); Phe × 1, 1.00 (1.09); Lys × 2, 1.97 (2.02); Arg × 1, 0.96 (0.94); Cys(Acm) × 2, N.D. (N.D.).

hCNP22: The di-Acm hCNP22 (15.0 mg) dissolved in TFA (20 ml) was treated with CH_3SiCl_3 (189 μl, 250 eq) in the presence of PhS(O)Ph (26 mg, 20 eq) and anisole (70 μl, 100 eq) at 25 °C for 15 min. NH_4F (118 mg) was added to the reaction mixture. Dry ether (100 ml) was added, and the precipitate was dissolved in 1 N AcOH (10 ml). This solution was applied to a Sephadex G-10 column (27 × 300 mm), which was eluted with 4 N AcOH. After lyophilization, the crude product was purified by FPLC on a YMC ODS-AQ120 (15 × 500 mm) column, which was eluted with a linear gradient of 60% CH_3CN/0.1% TFA (15—100%, 400 min) in 0.1% aqueous TFA at a flow rate of 4.0 ml/min. The eluate corresponding to the main peak was collected and lyophilized to yield a white fluffy powder: yield 7.9 mg (57%, calculated from di-Acm hCNP22; overall yield 28%, calculated from Fmoc–Cys(Acm)–Clt resin), $[\alpha]_D^{28}$ −49.7° (c=0.20, 1 N AcOH). HPLC on a YMC AM302 (4.6 × 150 mm) [retention time; 16.56 min, 24% CH_3CN in 0.1% TFA, 1.0 ml/min]. Amino acid analysis after 6 N HCl hydrolysis and LAP digestion (numbers in parentheses): Asp × 1, 1.00 (1.05); Ser × 3, 2.84 (2.86); Gly × 6, 6.00 (6.00); Cys × 1, 0.96 (0.82); Met × 1, 1.18 (0.99); Ile × 1, 1.03 (0.91); Leu × 4, 4.00 (4.00); Phe × 1, 1.02 (1.01); Lys × 2, 1.96 (1.94); Arg × 1, 0.96 (0.99). FAB-MS, m/z 2197.098 for $[M+H]^+$ (Calcd 2197.082 for $C_{93}H_{158}N_{27}O_{28}S_3$).

Preparation of hCNP22 Using Wang Resin [Cys(Acm)^{6,22}]-hCNP22: A fully protected hCNP22 Wang resin was assembled on the Fmoc–Cys(Acm)–Wang resin (300 mg; 0.16 mmol) according to the schedule described above. The protected peptide resin (150 mg) was treated with 1 M HBF_4–thioanisole in TFA (7 ml) in the presence of *m*-cresol (760 μl) and EDT (30 μl) in an ice-bath for 90 min. The crude product was purified by FPLC to afford a white powder: yield 30 mg (48%, calculated from Fmoc–Cys(Acm)–Clt resin). HPLC on a YMC AM302 (4.6 × 150 mm) [retention time; 18.46 min, on gradient elution with CH_3CN (10—60%, 30 min) in 0.1% TFA, 0.9 ml/min].

hCNP22: The above di-Acm peptide (22 mg) in TFA (4 ml) was treated with $AgBF_4$ (110 mg, 100 eq) in the presence of anisole (100 μl), followed by DTT (112 mg). After air oxidation at pH 7.8 for 14 h (Fig. 6b), the purity of crude hCNP22 was confirmed by analytical HPLC. The major and the minor products were eluted at retention times of 16.44 min and 19.84 min, respectively. The major product was purified by FPLC to give a white powder: yield 5.6 mg (15%, calculated from di-Acm hCNP22; overall yield 7%, calculated from Fmoc–Cys(Acm)–Wang resin), $[\alpha]_D^{28}$ −52.6° (c=0.18, 1 N AcOH). HPLC on a YMC AM302 (4.6 × 150 mm) [retention time; 16.45 min, 24% CH_3CN in 0.1% TFA, 1.0 ml/min]. Further, the minor product obtained after FPLC was purified by preparative HPLC to afford a white powder: yield 0.7 mg (2%, calculated from di-Acm hCNP22; overall yield 1%, calculated from Fmoc–Cys(Acm)–Wang resin). HPLC on a YMC AM302 (4.6 × 150 mm) [retention time; 19.54 min, 24% CH_3CN in 0.1% TFA, 1.0 ml/min]. The minor peak shown in Fig. 6b was co-eluted with the following [D-Cys^{22}]-hCNP22, prepared alternatively. H_2O_2 treatment of the purified product gave its oxidized form [retention time; 7.92 min, on HPLC described above].

[D-Cys^{22}]-hCNP22: [D-Cys^{22}]-hCNP22 was prepared by essentially the same method as described above for the synthesis of hCNP22, starting from Fmoc-D-Cys(Acm)–Wang resin (383 mg; 0.19 mmol). Finally, the purified [D-Cys^{22}]-hCNP22 was obtained in 9% yield (calculated from Fmoc-D-Cys(Acm)–Wang resin). $[\alpha]_D^{28}$ −29.3° (c=0.15, 1 N AcOH), HPLC on a YMC AM302 (4.6 × 150 mm) [retention time; 19.50 min, 24% CH_3CN in 0.1% TFA, 1.0 ml/min].

References and Notes

1) A preliminary report of the racemization-free synthesis has been published: Fujiwara Y., Akaji K., Kiso Y., *Chem. Pharm. Bull.*, **42**, 724 (1994). The following abbreviations are used: Fmoc = 9-fluorenylmethyloxycarbonyl, Boc = *tert*-butyloxycarbonyl, Acm = acetamidomethyl, *t*Bu = *tert*-butyl, Mtr = 4-methoxy-2,3,6-trimethylbenzenesulfonyl, Trt = trityl, HOBt = 1-hydroxybenzotriazole, DMF = *N*,*N*-dimethylformamide, EDT = ethane-1,2-dithiol, DTT = dithiothreitol, PhS(O)Ph = diphenylsulfoxide.
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11) The D-isomer content of H–Cys(Acm)–OH cleaved from Boc–Cys(Acm)–Wang resin could be directly determined on the Sumichiral OA-5000 column as described in the text. However, the isomer from Boc–Cys(R)–OH(R = Tacm, MeBzl, *t*Bu) could not be separated on the Sumichiral column probably due to the high lipophilicities of these Cys derivatives.
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