Synthesis of Rat Brain Natriuretic Peptide 45 Using Tetrafluoroboric Acid Deprotection and Silyl Chloride Disulfide Formation[1)]

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A newly isolated rat brain natriuretic peptide 45 (rBNP45) was synthesized using the 9-fluorenylmethyloxycarbonyl (Fmoc)-based solid-phase method. Tetrafluoroboric acid (HBF_4) deprotection was successfully applied for this synthesis, while the conventional trifluoroacetic acid (TFA)–thioanisole method gave unsatisfactory results. The disulfide bond of rBNP45 was constructed by using the silyl chloride method within 10 min, which was extremely advantageous to avoid the formation of Met(O)–rBNP45. The chick rectum relaxant activity of the synthetic rBNP45 was three times as potent as that of α-rat atrial natriuretic peptide (α-rANP).

Keywords peptide synthesis; silyl chloride; tetrafluoroboric acid; rBNP45; disulfide bond

In 1989, Kambayashi *et al.*[2)] and Aburaya *et al.*[3)] isolated the 5 kDa cardiac natriuretic peptide, which had been identified in the rat heart,[4)] and determined its amino acid sequence. The 5 kDa cardiac natriuretic peptide was designated as rat brain natriuretic peptide 45 (rBNP45), since the sequence of this peptide was identical to the amino acid sequence [51—95] of the rat BNP precursor deduced from the cDNA sequence.[5)] From the analysis of peptides in the perfusate from isolated rat hearts, rBNP45 was identified as the major storage and secretory form derived from the BNP precursor in the rat heart. rBNP45 is composed of 45 amino acids and has a ring structure with 17 amino acids looped by the disulfide linkage (Fig. 1), which is essential for the biological actions of a family of natriuretic peptides.

Following our synthetic and structure–activity relationship studies on a series of natriuretic peptides,[6,7)] we have synthesized rBNP45 in order to obtain a sufficient amount to examine its biological relationship with α-atrial natriuretic peptides (α-ANPs). From a synthetic viewpoint, rBNP45 contains 6 synthetically problematic Arg residues[8)] as well as a Met residue which is known to be easily oxidized.[9)] The molecule also contains Asp–Ser (positions 3—4) and Asp–Gly (positions 40—41) sequences, which are very susceptible to acid- or base-catalyzed succinimide formation.[10,11)] Thus, the synthesis of rBNP45 appears to be a suitable test to evaluate the methodology for the synthesis of relatively large and complex cystine-peptides. In this paper, we wish to present a detailed account of our synthesis of the 45-residue peptide corresponding to the entire amino acid sequence of rBNP45.

Construction of Protected rBNP45–Resin In order to construct the peptide chain, the Fmoc-based solid-phase method[12)] was employed. Fmoc amino acid derivatives bearing protecting groups based on *tert*-butyl alcohol were employed, together with Arg(Mtr)[13)] and Cys(Tacm) developed by us.[14)] Fmoc-Phe-OH linked to a 4-(benzyloxy)-benzylalcohol resin (Wang resin)[15)] was employed as the starting resin derivative. The combination of piperidine treatment and the DIPCDI plus HOBt procedure served to elongate the peptide chain manually according to the published procedure.[16)] We encountered no serious difficulties during the elongation of the entire sequence of rBNP45, although the double coupling procedure was employed when the resin became positive to the ninhydrin test[17)] after a single coupling. The amino acid composition of the protected rBNP45–resin thus assembled was in good agreement with that predicted by theory after acid hydrolysis with 12 N HCl–propionic acid (1 : 1).

Deprotection and Cleavage The protected rBNP45–resin thus obtained was then treated with 1 M HBF_4–thioanisole[18)] at 4 °C for 90 min to cleave the peptide chain from the resin and at the same time to remove all side-chain protecting groups employed except for the Tacm

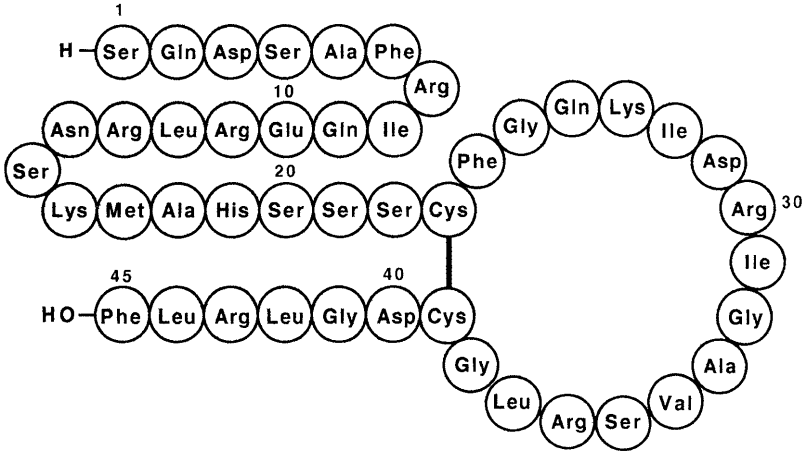

Fig. 1. Structure of rBNP45

group from the Cys. The deprotected peptide was partially purified by gel-filtration on Sephadex G-25 to give [Cys(Tacm)^{23,39}]–rBNP45, showing a sharp main peak on HPLC. However, the isolation yield was moderate (47%) due to incomplete cleavage of the long-chain peptide from the resin, probably because of the insufficient acidity of the deprotecting reagent. Thus, in order to find efficient deprotecting conditions for the synthesis of rBNP45, we selected 2 M HBF_4, instead of 1 M HBF_4, as a deprotecting reagent and compared the cleavage yield obtained by using this deprotecting reagent with those obtained by using the standard TFA–thioanisole method[19] and stronger acid deprotecting reagents (1 M TFMSA[20] and HF[21]).

As model experiments, the protected rBNP45–resin was treated with each deprotecting reagent mentioned above under the reaction conditions shown in Table I. After gel-filtration on Sephadex G-25, the yield and the purity of the deprotected peptides were compared. Nearly quantitative yields were obtained using all the cleavage reagents examined except for 1 M HBF_4 (Table I). However, a single main peak was not obtained when the protected peptide–resin was treated with the standard TFA–thioanisole method (Fig. 2, i). In addition, the product obtained by the strong acid deprotection (HF or 1 M TFMSA) contained higher impurity peaks than those contained in the product obtained by the 2 M HBF_4 deprotection (Fig. 2, ii—v).

TABLE I. Cleavage Conditions and Yields from Wang Resin

Reagent	Temperature (°C)	Time (h)	di-Tacm rBNP45 Yield (%)
TFA	25	24	84
1 M HBF_4	4	1.5	47
2 M HBF_4	4	1.5	92
1 M TFMSA	4	1.5	99
HF	4	1.5	99

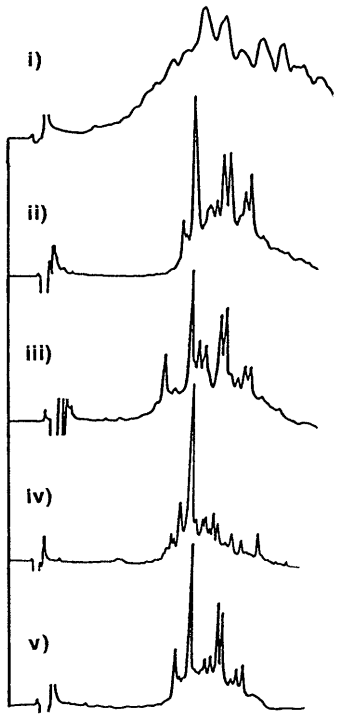

Fig. 2. HPLC Profiles of the Crude di-Tacm rBNP45 after Deprotection and Cleavage with Various Reagents

i) TFA, ii) 1 M TFMSA, iii) HF, iv) 1 M HBF_4, v) 2 M HBF_4; HPLC on YMC AM302 (4.6 × 150 mm), gradient elution with CH_3CN (10—60%, 30 min) in 0.1% TFA, flow rate 0.7 ml/min.

Thus, for the preparative experiments, we selected 2 M HBF_4–thioanisole in TFA as a suitable cleavage and deprotecting reagent. [Cys(Tacm)^{23,39}]–rBNP45 can be efficiently cleaved from the protected rBNP45–resin by treatment with the 2 M HBF_4 system at 4 °C within a relatively short time (90 min). The crude di-Tacm peptide thus obtained was then successively purified by gel-filtration on Sephadex G-25 and fast protein liquid chromatography (FPLC) on a YMC-ODS-AQ120(S50) column. The purified peptide exhibited a single main peak on analytical HPLC. The product possessed amino acids in ratios consistent with those predicted from the sequence of rBNP45 after acid hydrolysis or leucine aminopeptidase (LAP) digestion. These results indicate that the purified di-Tacm peptide contains no aspartimide peptide.

Disulfide Bond Formation and Characterization The FPLC-purified [Cys(Tacm)^{23,39}]–rBNP45 was treated with $AgBF_4$[22] (60 eq) in the presence of anisole (10 eq) for 60 min at 4 °C to remove the two Tacm groups. The Ag–peptide salt obtained was then treated with DTT (80 eq) at 25 °C for 24 h. The reduced peptide, after gel-filtration on Sephadex G-25, was subjected to air-oxidation at pH 7.5 to form the disulfide bond. The progress of the reaction was monitored by HPLC (Fig. 3). After 2 days' reaction, the crude air-oxidized peptide was isolated by lyophilization of the reaction mixture. The product was then purified by FPLC to give a homogeneous peptide. However, the isolation yield was fairly low due to the formation of Met(O)–rBNP45 during the air-oxidation procedure. The complete separation of this Met(O)-form was rather difficult, although most of the Met(O)–peptide could be reduced back to the desired rBNP45 by treatment with $SiCl_4$–anisole without affecting the disulfide bond.[23]

Fig. 3. HPLC of the Reaction Mixture after the Air-Oxidation

i) Crude sample after air-oxidation (S–S form). ii) Reduced rBNP45 after $AgBF_4$ treatment (SH form). HPLC on YMC AM302 (4.6 × 150 mm), gradient elution with CH_3CN (25—40%, 30 min) in 0.1% TFA, flow rate 0.7 ml/min.

The appearance of this Met(O)-form has also been observed during the isolation of rBNP45 from the rat atrium, or even in the extract of the perfusate from isolated rat heart.[2] These observations indicate that the Met residue in rBNP45 is very susceptible to oxidation and the long reaction/manipulation time for disulfide bond formation is the main reason for the accumulation of the Met(O)-form.

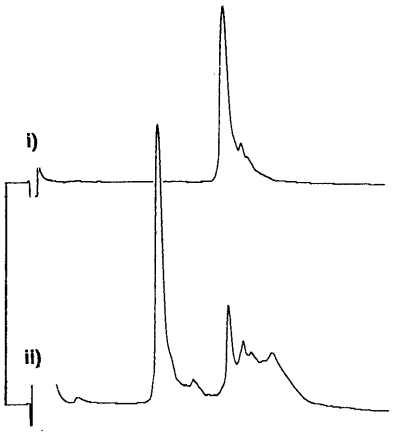

Fig. 4. HPLC of the Reaction Mixture after the Silyl Chloride Treatment

i) di-Tacm rBNP45. ii) Crude sample after a 10 min treatment with CH_3SiCl_3–PhS(O)Ph. HPLC on YMC AM302 (4.6 × 150 mm), gradient elution with CH_3CN (25—40%, 30 min) in 0.1% TFA, flow rate 0.7 ml/min.

In order to overcome this difficulty, we adopted the silyl chloride–sulfoxide method[24] instead of air-oxidation for the construction of the disulfide bridge of rBNP45. The silyl chloride–sulfoxide method developed by us can cleave various thiol-protecting groups of cysteine within 10—30 min to form a disulfide bond directly. The reaction is much faster than conventional air-oxidation; this would be extremely advantageous to avoid the formation of Met(O)–peptide during the reaction. The purified di-Tacm rBNP45 obtained above was treated with CH_3SiCl_3–PhS(O)Ph in TFA at 25 °C for 10 min to form the disulfide bond (Fig. 4). The reaction was stopped by the addition of NH_4F and the product was isolated by extraction with 4 N AcOH. The crude product gave a single main peak on HPLC, showing that no oxidation had occurred at the Met residue of rBNP45, as expected. After purification by FPLC, the homogeneous peptide was obtained in 9% yield (calculated from the starting C-terminal amino acid), approximately twice as high as that obtained by the synthesis using the air-oxidation method.

The purified rBNP45 exhibited a single sharp peak on analytical HPLC and a single spot on TLC. The product was proved to be a monomer by fast atom bombardment–mass spectrometry (FAB-MS). Its purity was further confirmed by amino acid analysis after 6 N HCl hydrolysis and LAP digestion. Both hydrolysates gave amino acid ratios in good agreement with the theoretical values. The chick rectum relaxant activity of the synthetic rBNP45 was

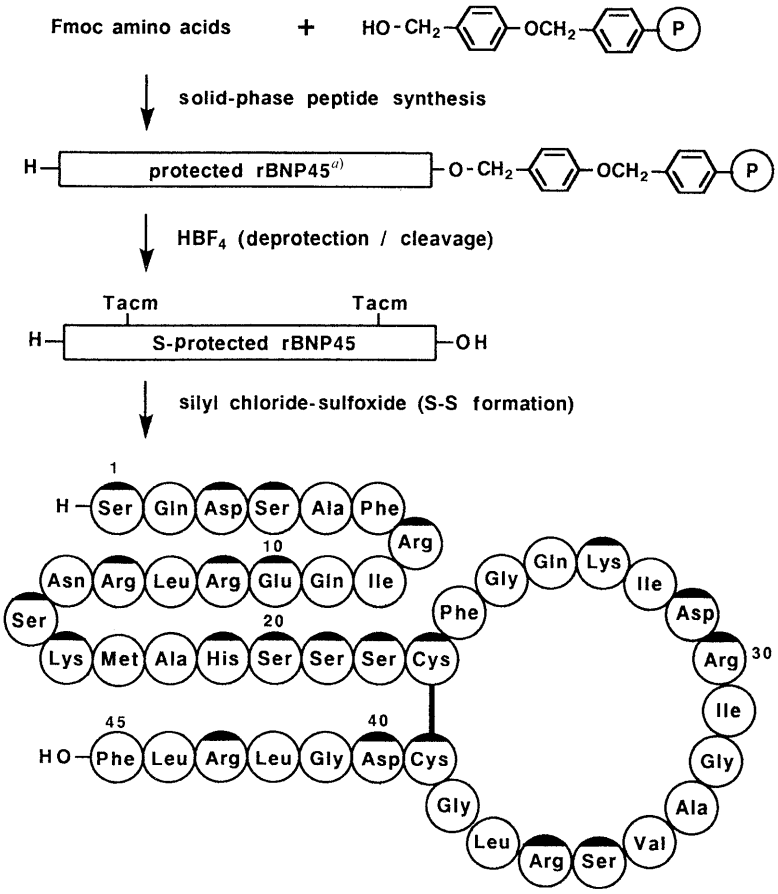

Chart 1. Synthetic Scheme for rBNP45

a) Protected amino acids; Asp(OBu^t), Glu(OBu^t), Ser(Bu^t), Thr(Bu^t), Tyr(Bu^t), Lys(Boc), His(Bum), Arg(Mtr), Cys(Tacm); positions of these amino acids are shown by partly filled circles.

three times as potent as that of rANP, approximately the same potency as pBNP.[7]

Conclusion

We have synthesized rBNP45, containing synthetically problematic Arg, Asp, and Met residues, by the combination of HBF_4 deprotection and the silyl chloride method for the disulfide bond formation. In this synthesis, we showed that HBF_4 has suitable acidity as a cleavage and deprotecting reagent to be applied in Fmoc-based solid-phase peptide synthesis. In contrast, the conventional TFA–thioanisole method gave unsatisfactory results, requiring a long reaction time and giving the cleaved peptide in low purity, in the synthesis of the 45-residue peptide. Strong acid deprotecting reagents caused relatively large extents of modification at the peptide chain. The disulfide bond of rBNP45 was constructed by means of a 10 min treatment with silyl chloride–sulfoxide, whereas 2 d were necessary when the conventional air-oxidation method was employed for this purpose. No oxidation at the Met residue of rBNP45 was observed by using this silyl chloride disulfide formation. Thus, the synthetic method adopted for the present synthesis (Chart 1) appears to be not only efficient for the synthesis of rBNP45 but also generally applicable for the syntheses of large, complex cystine-peptides.

Experimental

Fmoc amino acid derivatives, 4-(benzyloxy)benzylalcohol resin (Wang resin), and Fmoc–Phe–OH linked to Wang resin were purchased from Nova Biochem (Switzerland). LAP (lot No. 117F-8085) was purchased from Sigma. TLC was effected with silica gel (Kieselgel $60F_{254}$, Merck) on precoated aluminum sheets using *n*-BuOH : AcOH : pyridine : H_2O = 4 : 1 : 1 : 2 as a solvent system. Analytical HPLC and amino acid analysis were conducted with a Hitachi 655A and a Hitachi L8500, respectively. The FAB mass spectrum was obtained on a VG Analytical 2AB-2SEQ spectrometer equipped with the 11-250J data system.

Solid-Phase Synthesis Protected rBNP45 resin was prepared by the solid-phase method starting from Fmoc–Phe–OH linked to a Wang resin (208 mg, 0.1 mmol) according to the published procedure.[16] The following Fmoc amino acids and derivatives were used to construct the peptide chain on the resin: Gly, Ala, Val, Met, Ile, Leu, Phe, Asp(OBu^t), Glu(OBu^t), Ser(Bu^t), Lys(Boc), His(Bum),[25] Arg(Mtr),[13] and Cys(Tacm).[14] The condensation reactions (2 h each) of the derivatives (2.5 eq each) were conducted by the use of DIPCDI (2.5 eq) in the presence of HOBt (2.5 eq). The reaction was repeated when the resin became positive to the Kaiser test. After assembly of the respective amino acids, the N-terminal Fmoc group was removed by treatment with 20% piperidine/DMF and the resulting protected peptide resin was hydrolyzed in 12 N HCl–propionic acid (1 : 1). Amino acid ratios in the hydrolysate (numbers in parentheses are theoretical): Asp 4.63 (4), Ser 5.28 (7), Glu 3.54 (4), Gly 4.44 (4), Ala 2.88 (3), Val 1.07 (1), Met 0.92 (1), Ile 2.79 (3), Leu 4.40 (4), Phe 3.00 (3), Lys 1.82 (2), His 0.88 (1), Arg 5.60 (6).

Preliminary Experiment for Deprotection of the Protected Peptide Resin The protected rBNP45–resin (50 mg) was treated with each of the following deprotecting reagents: 1 M solution of HBF_4 or TFMSA in TFA (1.8 ml) in the presence of 1 M thioanisole and two additional scavengers, *m*-cresol (54 μl, 100 eq) and EDT (131 μl, 300 eq); TFA (1.71 ml)–thioanisole (0.09 ml); HF (4 ml) in the presence of *m*-cresol (54 μl, 100 eq) and EDT (4.5 μl, 10 eq). The same protected resin (100 mg) was also treated with 2 M HBF_4–thioanisole in TFA (1.9 ml) in the presence of *m*-cresol (109 μl, 100 eq) and EDT (262 μl, 300 eq). After being stirred with the above HBF_4 or TFMSA reagent under the reaction conditions shown in Table I, AcOEt (10—50 ml) and 6 M guanidine–HCl (3—10 ml) were added to the reaction mixture. The aqueous phase of the mixture was filtered and the filtrate was applied to a column of Sephadex G-25 (3.2 × 45 cm), which was eluted with 4 N AcOH. The eluate was monitored by ultraviolet (UV) absorption measurement at 254 nm. The fractions corresponding to each front main peak (6 ml each) were combined and the solvent was removed by lyophilization to give a powder. After the HF or TFA treatment, the excess reagent was removed by evaporation. Dry ether (10 ml) was added to the residue and the resulting precipitate, collected by centrifugation, was dissolved in 4 N AcOH (2 ml). The solution was filtered to remove the resin and the filtrate was gel-filtered as mentioned above. Yields calculated from the starting C-terminal amino acid are listed in Table I. The HPLC profile of each product is shown in Fig. 2.

[Cys(Tacm)^{23,39}]–rBNP45 The protected rBNP45–resin (200 mg) was treated with 2 M HBF_4–thioanisole/TFA (7 ml) in the presence of *m*-cresol (218 μl, 100 eq) and EDT (524 μl, 300 eq) at 4 °C for 90 min. The product was isolated and gel-filtered as stated above to give di-Tacm rBNP45 as a white powder. The gel-filtered sample was dissolved in 4 N AcOH (5 ml). The solution was applied to an FPLC column (YMC ODS-AQ300, 2 × 50 cm) eluted with a linear gradient of 60% CH_3CN/0.1% aqueous TFA (0—100%, 400 min) in 0.1% aqueous TFA at the flow rate of 3.0 ml/min. The eluate was monitored by measuring the UV absorption at 230 and 254 nm. The desired fractions were lyophilized to give a white fluffy powder: 21.7 mg (20%, calculated from the starting C-terminal amino acid), *Rf* 0.19, HPLC on a YMC AM302 (4.6 × 150 mm) [retention time; 17.90 min, on gradient elution with CH_3CN (10—60%, 30 min) in 0.1% aqueous TFA, 0.7 ml/min]. Amino acid ratios after 6 N HCl hydrolysis and LAP digestion (numbers in parentheses); Asp × 4, 4.26 (2.70); Ser × 7, 5.36 (6.80); Glu × 4, 3.69 (0.99); Gly × 4, 4.38 (4.10); Ala × 3, 2.95 (2.91); Val × 1, 1.06 (1.04); Met × 1, 0.90 (1.08); Ile × 3, 2.87 (3.04); Leu × 4, 4.23 (4.33); Phe × 3, 3.00 (3.00); Lys × 2, 1.94 (1.98); His × 1, 0.96 (1.02); Arg × 6, 5.47 (5.70); Cys(Tacm), ND (2.06).

Preparation of rBNP45 Using a Silyl Chloride–Sulfoxide Method [Cys(Tacm)^{23,39}]–rBNP45 (15 mg, 2.94 μM) obtained above in TFA (5 ml) was treated with CH_3SiCl_3 (86 μl, 250 eq) in the presence of PhS(O)Ph (6 mg, 10 eq) and anisole (32 μl, 100 eq) at 25 °C. The mixture was stirred for 10 min, then NH_4F (32 mg, 300 eq) was added. Dry ether (120 ml) and 4 N AcOH (120 ml) were then added. The solvent of the aqueous phase was removed by lyophilization. The product was purified by gel-filtration on Sephadex G-25 (4 N AcOH) followed by FPLC as described above to give a sample having a sharp single peak on analytical HPLC: yield 6.5 mg (45%, calculated from di-Tacm rBNP45; overall yield 9%, calculated from the starting C-terminal amino acid). *Rf* 0.17 (single spot), HPLC on a YMC AM302 (4.6 × 150 mm) [retention time; 12.20 min, on gradient elution with CH_3CN (25—40%, 30 min) in 0.1% aqueous TFA, 0.7 ml/min]. Amino acid ratios after 6 N HCl hydrolysis and LAP digestion (numbers in parentheses); Asp × 4, 3.97 (2.70); Ser × 7, 5.06 (7.00); Glu × 4, 3.95 (0.99); Gly × 4, 4.03 (4.01); Ala × 3, 3.33 (3.08); Val × 1, 1.03 (1.17); Met × 1, 0.96 (1.08); Ile × 3, 2.99 (3.04); Leu × 4, 4.06 (4.33); Phe × 3, 3.00 (3.00); Lys × 2, 1.99 (1.98); His × 1, 1.00 (1.02); Arg × 6, 5.81 (5.70); Cys, 0.88 (0.80). FAB-MS, *m/z* 5041.2 for $[M+H]^+$ (Calcd 5041.8 for $C_{213}H_{349}N_{71}O_{65}S_3$).

Preparation of rBNP45 Using an Air-Oxidation Method FPLC purified [Cys(Tacm)^{23,39}]–rBNP45 (21.7 mg, 5.63 μM) in TFA (2.0 ml) was treated with $AgBF_4$ (65.8 mg, 60 eq) in the presence of anisole (6.1 μl, 10 eq) at ice-bath temperature for 60 min, then dry ether was added. The resulting powder was dissolved in 4 N AcOH (4 ml) and DTT (70 mg, 80 eq) was added. This solution was stirred at 25 °C for 14 h and the supernatant was applied to a column of Sephadex G-25 eluted with 4 N AcOH. The fractions corresponding to the main peak were combined and diluted with H_2O (total volume 300 ml). The pH of this solution was adjusted to 7.5 with 5% NH_4OH. The solution was kept standing at 4 °C and the progress of air-oxidation was monitored by HPLC. After 2 d, the entire solution was lyophilized to give a powder. The crude product was dissolved in 4 N AcOH (5 ml) and the solution was applied to an FPLC column (YMC ODS-AQ300, 2 × 50 cm) eluted with a linear gradient of 60% CH_3CN/0.1% aqueous TFA (40—60%, 300 min) in 0.1% aqueous TFA at the flow rate of 3.0 ml/min. The solvent of desired fractions was removed by lyophilization to give rBNP45: yield 5.3 mg (26%, calculated from di-Tacm rBNP45; overall yield 5%, calculated from the starting C-terminal amino acid). HPLC on a YMC AM302 (4.6 × 150 mm) [retention time; 11.94 min, on gradient elution with CH_3CN (25—40%, 30 min) in 0.1% aqueous TFA, 0.7 ml/min]. H_2O_2 treatment of the product gave its oxidized form [retention time; 11.36 min, on HPLC described above]. The oxidized form could be converted back to the rBNP45 by $SiCl_4$–anisole treatment.

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References and Notes

1) Amino acids and peptide derivatives used here are of the L-configuration. The following abbreviations are used: Boc = *tert*-butyloxycarbonyl, Bu^t = *tert*-butyl, Bum = *tert*-butyloxymethyl, DIPCDI = diisopropylcarbodiimide, DMF = *N*,*N*-dimethylformamide, DTT = dithiothreitol, EDT = ethane-1,2-dithiol, Fmoc = 9-fluorenylmethyloxycarbonyl, HOBt = 1-hydroxybenzotriazole, Mtr = 4-methoxy-2,3,6-trimethylbenzenesulfonyl, PhS(O)Ph = diphenylsulfoxide, Tacm = trimethylacetamidomethyl, TFA = trifluoroacetic acid, TFMSA = trifluoromethanesulfonic acid.
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