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Hydrogen Peroxide for Disulfide Bridge Formation in Methionine-containing Peptides[1]

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Abstract: Two methionine-containing peptides, endothelin 1 and the 1–16 fragment of the receptor of the plasminogen activator 1 for human urokinase, were synthesized and cyclized by hydrogen peroxide. Endothelin 1 was obtained by using regioselective and random schemes of disulfide bond formation. The conditions of cyclization that provided the target products in high purity were found. The general potential of disulfide bond formation by means of hydrogen peroxide was demonstrated for methionine-containing peptides. The method resulted in target products containing insignificant quantities of the corresponding Met-sulfoxide derivatives. Copyright © 2000 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: disulfide bridge formation; hydrogen peroxide; methionine

INTRODUCTION

Various methods for disulfide bond formation are known in modern peptide chemistry. However, most of them are of limited use in the synthesis of Met- and Trp-containing peptides due to the high sensitivity of these amino acid residues to oxidants [1,2]. Hydrogen peroxide has been used for a long time [3,4], but the potentialities of this reagent have been insufficiently investigated up to now. We methodically studied applications of hydrogen peroxide for disulfide bond formation and demonstrate its value during the synthesis of several natural Trp-containing peptides [5–7]. Eichler and Houghten also showed the usefulness of hydrogen peroxide in the synthesis of [$Cys^{2,6}$]-β-endorphin 1–6 [8] but noted the formation of the corresponding Met(O)-derivative in 47% yield during their synthesis. Thus, disulfide bond formation in methionine-containing peptides by means of hydrogen peroxide is still a problem. It is known that, in neutral or alkaline media, methionine is less sensitive to hydrogen peroxide than in acidic media [9]. This led us to suppose that the undesirable oxidation of methionine during the formation of SS-bonds may be minimized at pH >8. We carried out several experiments with Met-enkephalin to investigate the Met sensitivity to the hydrogen peroxide action at different pH values and found that the conversion into the corresponding Met(O) derivative was significantly slowed at basic pH values (Table 1).

We used the results of the model experiments above in the synthesis of two Met-containing peptides: endothelin 1 and the fragment corresponding to the 1–16 sequence of uPAR1 (Figure 1). For the oxidation reaction, we found conditions that provided target products with a high degree of purity.

Abbreviations: DIC, *N,N′*-diisopropylcarbodiimide; EDT, 1,2-ethanedithiol; ET-1, endothelin 1 (human, porcine, dog, or rat); ESI-MS, electrospray ionization mass spectrometry; For, formyl; HOBt, 1-hydroxybenzotriazole; NMP, *N*-methylpyrrolidone; uPAR1, the receptor of the plasminogen activator 1 for human urokinase.

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Table 1 The Oxidation of Met-enkephalin with 1.5 Equivalents of Hydrogen Peroxide for 5 min at the Peptide Concentration of 1 mg/ml

pH	Content of Met(O)-derivative % (HPLC)
3.0–4.0	44.2
4.0–5.0	14.0
7.5–8.0	7.5
8.5–9.0	2.8

Since endothelin 1 contained two disulfide bonds, it was synthesized by using both the regioselective and random schemes of the SS-bridge formation.

For the regioselective scheme, a linear precursor of endothelin 1 was prepared by the solid phase method using the Boc/benzyl protecting group scheme. Orthogonal protective groups were used for the thiol functions of Cys residues: acetamidomethyl (Acm; resistant to HF) for Cys^{1} and Cys^{15} and 4-methylbenzyl (Meb; cleavable by HF) for Cys^{3} and Cys^{11}. Previously, this combination of protective groups has been applied for the syntheses of peptides with two SS-bridges, including endothelin 1 [10]. 2,4-Dinitrophenyl (Dnp) and For protective groups were used for the imidazole ring of His and the indole ring of Trp, respectively. Couplings were achieved by the DIC–HOBt method. The resulting peptide was cleaved from the support by HF in the presence of *p*-cresol at 0°C (see Reference [10]). All the protective groups of side-chains except Acm and For were also split off during this treatment to give a crude product containing 31% of the target linear precursor of endothelin 1 (**I**) (Figure 2A).

The crude product was dissolved in 8 M urea solution to suppress any aggregation of **I** and oxidized by one to two equivalents of hydrogen peroxide at pH 8–8.5. After the oxidation, which was monitored by HPLC and Ellman's determination of sulfhydryl groups, was completed, nitrogen or helium was passed through the reaction mixture to remove the excess hydrogen peroxide, whose absence was then examined. Thereupon, the pH was adjusted to 4–5 with acetic acid. We found that nitrogen or helium blowing through is enough to prevent the formation of the Met(O)-derivative. Note that the acidification without preliminary blowing through with an inert gas resulted in almost quantitative formation of the corresponding Met(O)-derivative within a few seconds. The monocyclic intermediate **II** contained practically no Met(O)-impurity detectable by HPLC (Figure 3) or ESI-MS (Figure 4).

The second disulfide bond was formed by iodine treatment of **II** in methanol [10] (Figure 2A). The bicyclic Trp(For)-derivative of endothelin 1 (**III**) was obtained with 96% purity after desalting by HPLC. The N^{indole}-formyl protective group was removed by the treatment with 0.1 N NaOH followed by HPLC desalting. ET-1 with 97% purity resulted. Its yield was 7% from the starting amino acid connected to resin.

The next stage consisted of the random cyclization of the tetra-SH-derivative of ET-1, which required a simultaneous formation of two disulfide bonds (Figure 2B). The tetra-Acm-derivative of ET-1 was synthesized by using the Fmoc-scheme and the DIC–HOBt method of coupling. The peptide was

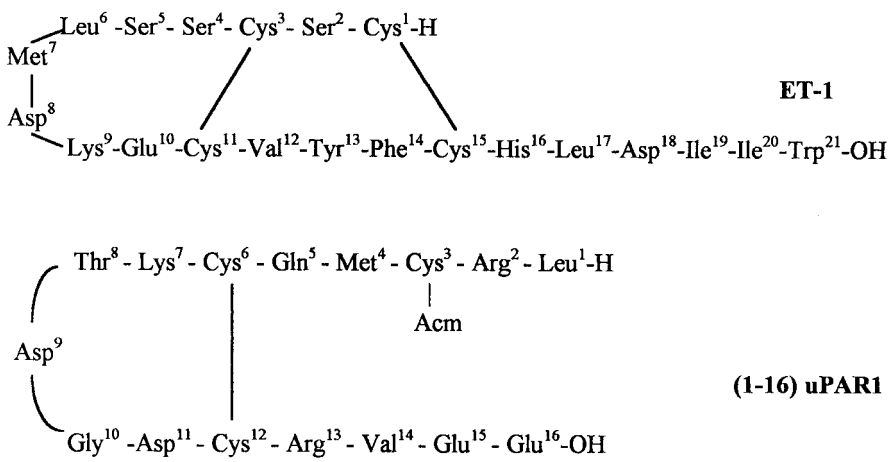

Figure 1 The structures of human, porcine, dog and rat endothelin 1 and the fragment corresponding to a 1–16 sequence of the receptor of plasminogen activator 1 for human urokinase.

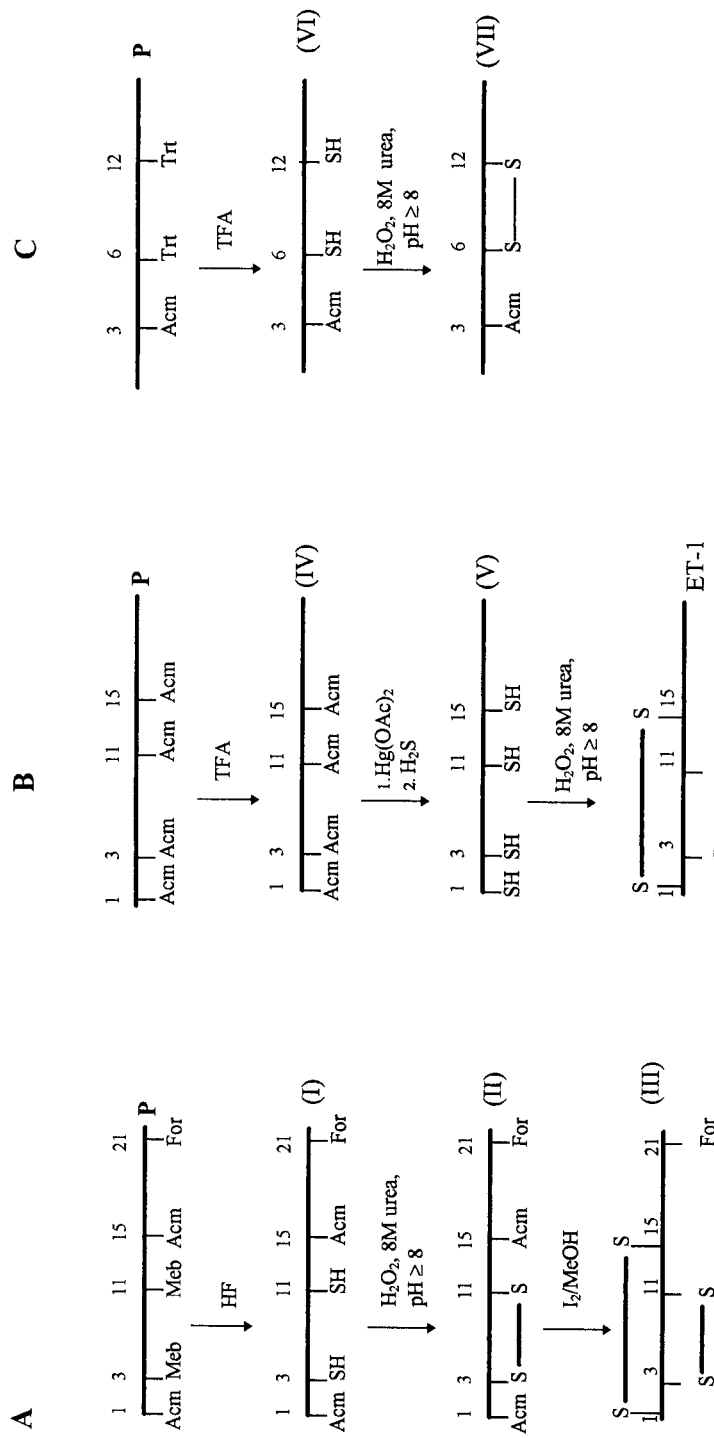

Figure 2 Synthetic schemes for (A) regioselective and (B) random closure of SS-bonds in endothelin 1 and (C) for 1–16 fragment of the receptor of plasminogen activator 1 for human urokinase.

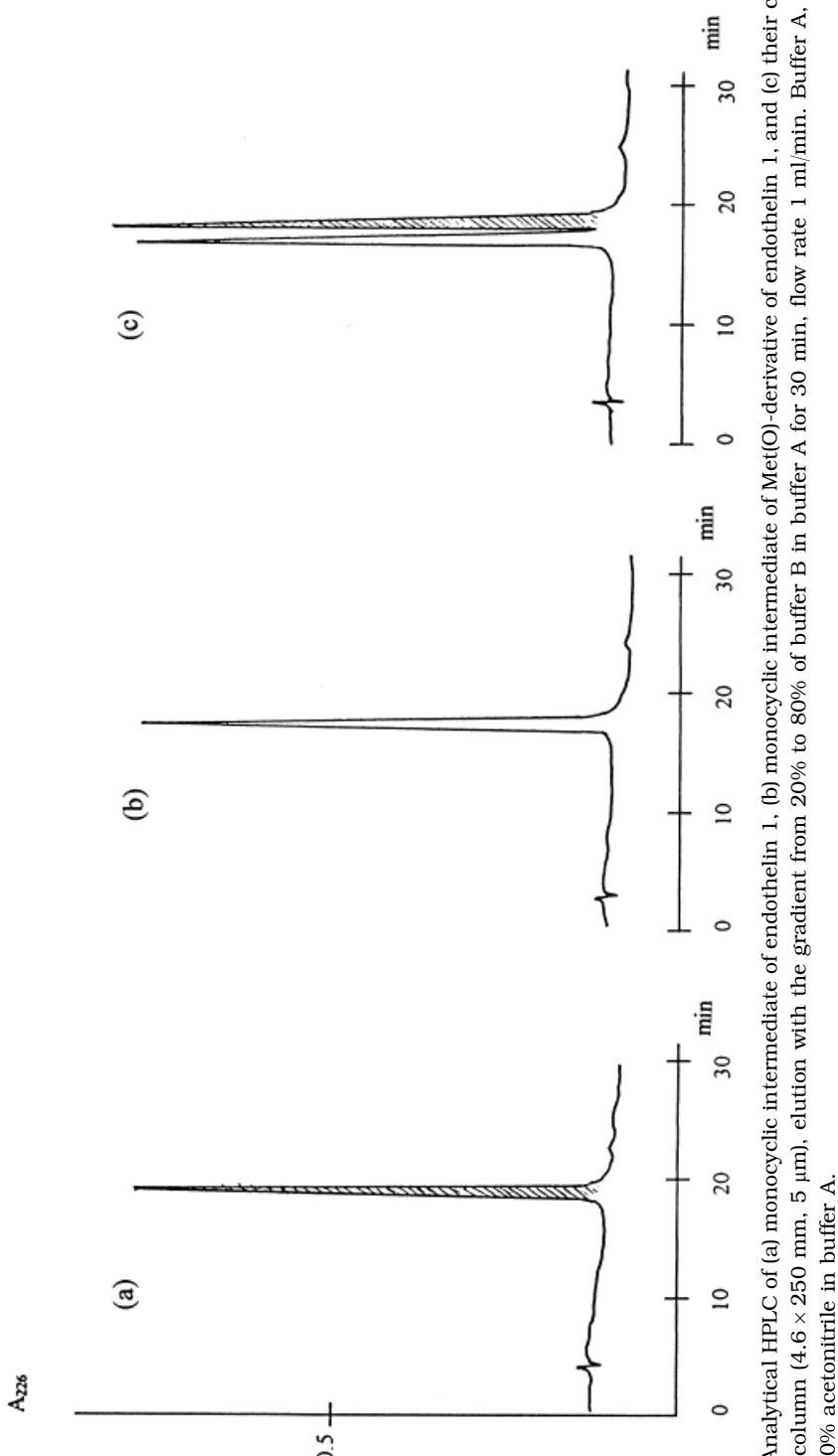

Figure 3 Analytical HPLC of (a) monocyclic intermediate of endothelin 1, (b) monocyclic intermediate of Met(O)-derivative of endothelin 1, and (c) their coinjection. Vydac C_{18} column (4.6 × 250 mm, 5 μm), elution with the gradient from 20% to 80% of buffer B in buffer A for 30 min, flow rate 1 ml/min. Buffer A, 0.1% TFA; buffer B, 80% acetonitrile in buffer A.

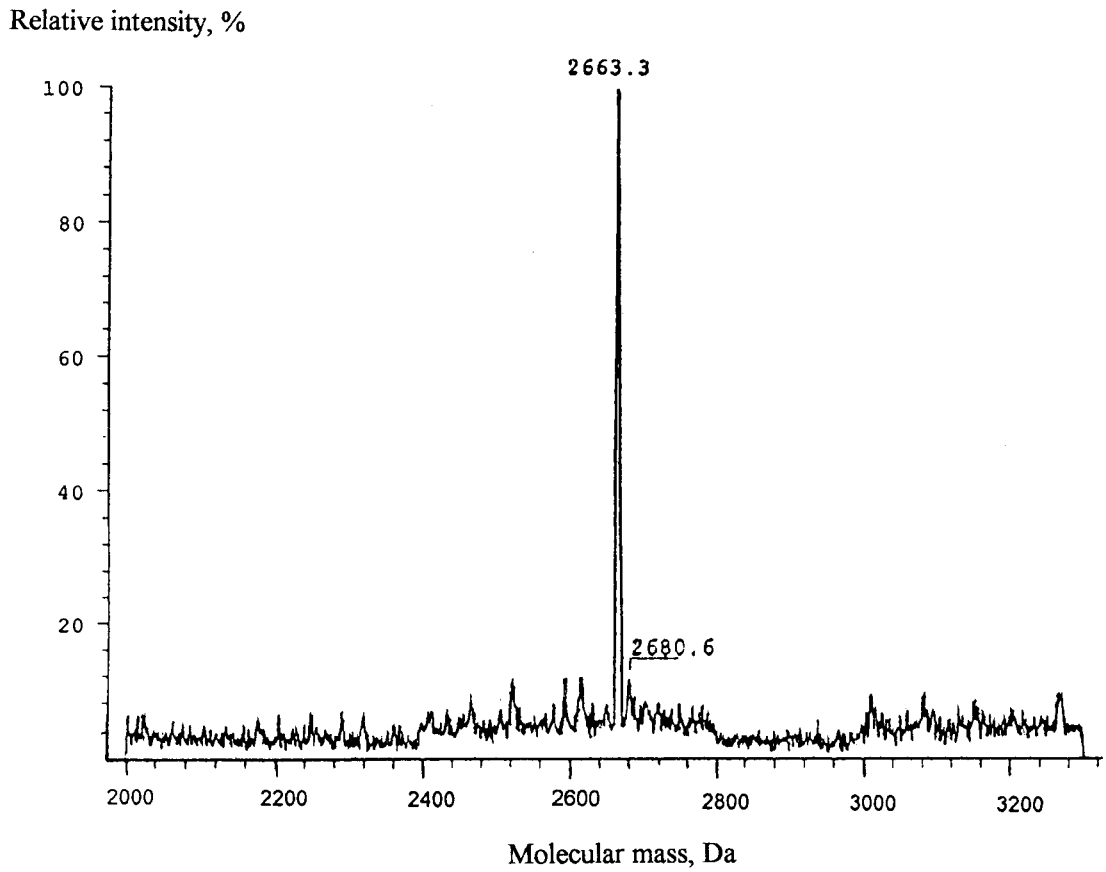

Figure 4 Mass spectrum of monocyclic intermediate of endothelin 1.

cleaved from the support by reagent K [11], which resulted in a crude product containing 58% of tetra-Acm-ET-1 (**IV**). We must mention an exceptionally low solubility and high aggregation capacity of **IV** in aqueous buffer within a wide range of pH, a fact that complicated our work. However, a mixture of 8 M urea, acetic acid (30–50%) and *n*-butanol (5–10%) was successfully used for the dissolution of **IV**, which was purified by preparative HPLC on Vydac C_{18} or Diasorb C_{16} columns, with the second column giving better results. After purification, the homogeneity of **IV** was 93–97% and the yield was 31% from the starting amino acid connected to resin. Tetra-Acm-ET-1 had the correct amino acid composition and molecular mass. We used mercuric acetate for the deprotection of sulfhydryl groups in this compound. The mercuric ions were removed by treatment with excess 2-mercaptoethanol or hydrogen sulfide. 2-Mercaptoethanol turned out to be ineffective for the destruction of the ET-1 mercaptide. The main product of this reaction was the compound containing two atoms of mercury per one molecule of peptide according to ESI-MS. Hydrogen sulfide successfully destroyed this compound and was therefore used in our subsequent experiments.

Random disulfide bond formation was carried out in 8 M urea at pH 8.5 by using two equivalents of hydrogen peroxide as described above. We used HPLC and Ellman's determination of sulfhydryl groups for monitoring the conversion of the SH- to SS-containing compounds. The oxidation reaction was completed after 30–40 min and resulted in mixtures containing 75–82% of the natural isomer (Figure 5). The target product was isolated by preparative HPLC. Its yield was 26% from the linear derivative **IV** or 8% from the starting amino acid attached to resin. The isolated product contained no admixture of the corresponding Met(O)-derivative according to HPLC and ESI-MS.

The solid phase synthesis of the linear precursor of the 1–16 fragment of uPAR1 was performed using the Fmoc-scheme by analogy with the synthesis of tetra-Acm-ET-1. The peptide was cleaved

from the support by reagent K. The crude product contained 64% of **VI**. It was cyclized by using one to two equivalents of hydrogen peroxide at pH 8.5. In this case, the nitrogen or helium blowing through was not sufficiently effective since the reaction mixture contained approximately 10% of corresponding Met(O)-derivative after acidification. The Met(O)-derivative was isolated by HPLC and identified by ESI-MS. Better results were obtained when the reaction mixture was evaporated to a half of its volume before the acidification. It is interesting that an attempt to form the disulfide bond in **VI** by air oxidation was unsuccessful. The reaction mixture contained no more than 5% of disulfide **VII** after 24 h intensive stirring at pH 8. On the other hand, the addition of one to two equivalents of hydrogen peroxide resulted in complete cyclization within 15 min.

Thus, we demonstrated a general possibility of disulfide bond formation by hydrogen peroxide for methionine-containing peptides. Our method allows the preparation of target products containing insignificant quantities of the corresponding Met(O)-sulfoxide derivatives. The oxidant must be removed from the reaction mixture before acidification. Such simple operations, such as inert gas blowing through or partial evaporation of the reaction mixture in a vacuum, may by used for this purpose.

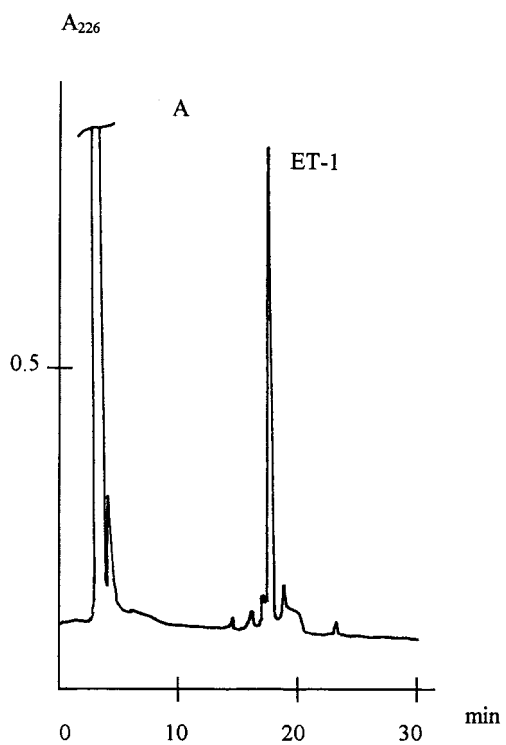

Figure 5 Analytical HPLC of crude endothelin 1 obtained by random cyclization with hydrogen peroxide. Ultrasphere ODS column (4.6 × 250 mm, 5 μm), elution with the gradient from 20% to 80% of buffer B in buffer A for 30 min, flow rate 1 ml/min. Buffer A, 0.1% TFA; buffer B, 80% acetonitrile in buffer A.

The regioselective and random methods of disulfide bond formation provided comparable yields of the target product in the synthesis of ET-1. We want to stress that the use of both hydrogen peroxide and air oxygen provides a preferable formation of natural ET-1 during the oxidative cyclization. Thus, the ratios of the natural and two isomeric disulfides were 10:1:0 and 3:1:1 in the case of the hydrogen peroxide and the air oxygen oxidations, respectively [10]. However, the hydrogen peroxide cyclization proceeded more rapidly (< 40 min) than the air oxygen cyclization, which took several hours [10].

EXPERIMENTAL

L-Amino acid derivatives from Bachem (Switzerland) and phenol from Merck (Germany) were used. Mercuric acetate, anisole, *p*-cresol, DIC, EDT, 5,5′-dithiobis-2-nitrobenzoic acid (Ellman's reagent), HOBt, hydrogen peroxide (30%), and thiophenol were from Fluka (Switzerland). Before oxidation, hydrogen peroxide was diluted to the desired concentration and titrated by 1 N $KMnO_4$. For peptide synthesis, we used dichloromethane, methanol, NMP, piperidine, and TFA from Applied Biosystems (USA). A Gilson chromatograph (France) equipped with an Ultrasphere ODS (4.6 × 250 mm, 5 μm, Beckman, USA) or a Vydac C_{18} (4.6 × 250 mm, 5 μm, Sigma, USA) column was used for analytical HPLC. Solvent A was 0.1% TFA, and solvent B was 80:20 acetonitrile:solvent A mixture. Gradients of solvent A in solvent B from 20% to 80% for 30 min (gradient **1**), from 30% to 70% for 40 min (gradient **2**), and from 0% to 60% for 30 min (gradient **3**) were used. The flow rate was 1 ml/min; peptides detection was at 226 nm. Preparative HPLC was carried out on a Beckman chromatograph (USA) with a Diasorb C_{16} (25 × 250 mm, 10 μm) or a Vydac C_{18} (16 × 250 mm, 10 μm) column. Acetonitrile from Technopharm (Russia) was used for HPLC. Amino acid analyses were performed on an automatic Biotronik 5001 analyzer (Germany); prior to the analysis, peptides were hydrolyzed with 6 N HCl containing 2% thioglycolic acid at 110°C for 48 h for ET-1 or for 24 h for 1–16 fragment of uPAR1. Mass spectra were recorded on a quadrupole Finnigan

MAT TSQ 700 mass spectrometer (Germany) with an electrospray (API) ionic source (Finnigan MAT).

Solid phase peptide synthesis was carried out on an Applied Biosystems 431 automatic synthesizer. ET-1 was synthesized on the copolymer of styrene with 1% divinylbenzene as a solid support with the 4-hydroxymethylphenylacetamidomethyl anchoring group for the peptide attachment (PAM-resin). We started from Boc-Trp(For)-resin (0.14 g, 0.10 mmol) containing 0.70 mmol/g of Boc-Trp(For). Boc-amino acids were coupled stepwise to the resin according to the standard protocol. The synthetic cycle included the following procedures: deprotection of the α-amino group with 50% solution of TFA in dichloromethane for 20 min, neutralization with 5% *N*,*N*-diisopropylethylamine in NMP for 7.5 min, 20 min activation of the amino acid derivative being coupled by DIC (one equivalent) in the presence of HOBt (one equivalent) in NMP, coupling of the corresponding activated amino acid derivative (tenfold excess) in NMP for 70 min, and all the necessary intermediate washings of peptidyl resin. After the synthesis was completed, peptidyl resin was washed with dichloromethane and dried *in vacuo*. The resulting peptidyl resin (0.47 g) was treated with a DMF–thiophenol mixture for 4 h at 20°C under stirring for the removal of Dnp. After filtration, washings with DMF (3×10 ml), water (1×5 ml), methanol (3×10 ml), and dichloromethane (3×10 ml) it was dried *in vacuo*, whereupon it was treated with 1:1 TFA:dichloromethane mixture for 1 h at 20°C under stirring. After filtration and washings with dichloromethane (2×10 ml), methanol (2×10 ml), and dichloromethane (5×10 ml) the resin was dried over P_2O_5. Treatment with HF (5 ml) in the presence of *p*-cresol (1 g) at 0°C for 1 h, evaporation of HF, and trituration with ether afforded a mixture of the crude product and resin. The peptide was taken up in 20 ml TFA and precipitated with dry ether. The crude product was washed with ether (5×10 ml) and ethyl acetate (3×5 ml); and dried over NaOH to give 0.20 g of material. It contained 31% of **I** on the basis of a HPLC analysis.

Tetra-Acm-ET-1 was obtained by the solid phase synthesis according to the Fmoc scheme starting from 0.33 g (0.25 mmol) of Fmoc-Trp-resin (Bachem, Switzerland) containing 0.76 mmol/g of Fmoc-Trp. A copolymer of styrene with 1% divinylbenzene as a solid support and 4-hydroxymethylphenoxymethyl anchoring group as a peptide resin spacer (the Wang resin) were used. The *tert*-butyl protecting groups were applied for the protection of side-chain functional groups of Ser, Thr, Tyr, Asp, and Glu. The Boc and Trt protective groups were used for ε-amino group of Lys and N^{im} of His, respectively. The fragment of uPAR1 was synthesized starting from 0.41 g (0.25 mmol) Fmoc-Glu(OBu^t)-resin (Bachem, Switzerland) containing 0.61 mmol/g Fmoc-Glu(OBu^t). The *tert*-butyl groups were used for the protection of functional groups in side chains of Asp, Glu, Thr, and Lys. The Pmc and Trt protective groups were used for Arg and carboxamide function of Gln, respectively. Sulfhydryl groups of Cys^6 and Cys^{12} were also blocked with Trt, whereas Acm was used for the Cys^3 sulfhydryl group. Fmoc-amino acids were attached to the resin according to the standard protocol for stepwise coupling. The synthetic cycle was as for the Boc-amino acids except that 20% piperidine in NMP was used for deblocking the *N*-termini. The activated amino acid derivatives were added in fourfold excess. After the last Fmoc removal the resin was washed with dichloromethane and after drying was stirred with a 8.25:0.5:0.5:0.5:0.25 TFA:phenol:water:thioanisole:EDT mixture (10 ml) for 1.5 h at 20°C. The resin was filtered off and washed with TFA (3×1 ml), and the filtrate was precipitated with dry ether. The precipitate was filtered, washed with ether and ethyl acetate, and dried. The material (0.48 g) contained 58% of tetra-Acm-ET-1 (**IV**) according to HPLC. The linear precursor of the uPAR1 fragment **VI** being prepared in analogy to the synthesis of **IV** gave 0.55 g of material containing 64% of the desired peptide.

H-Cys(Acm)-Ser-Cys-Ser-Ser-Leu-Met-Asp-Lys-Glu-Cys-Val-Tyr-Phe-Cys(Acm)-His-Leu-Asp-Ile-Ile-Trp(For)-OH (II)

Crude **I** (0.10 g) was dissolved in urea (8 M; 100 ml). The solution was adjusted to pH 8.0–8.5 with 25% ammonia, and 1% hydrogen peroxide (0.04 ml) was added, then the reaction mixture was kept for 10–15 min, with monitoring the SS bridge formation by Ellman's test and HPLC (gradient **1**). Nitrogen or helium was then passed through the reaction mixture, and the absence of hydrogen peroxide was examined by iodometric test. The reaction mixture was acidified with acetic acid (1–2 ml) and chromatographed on a Vydac C_{18} column (16×250 mm). Elution with a gradient from 20 to 60% of solvent B in solvent A for 80 min at the flow rate of 5 ml/min resulted in fractions containing the target product. These were combined and evaporated. The residue was dissolved in water and lyophilized to

give (**II**). Yield 44.5 mg, 17% from the starting amino acid attached to the resin; t_R 18.54 min (gradient **1**, Vydac C_{18}); molecular mass by ESI-MS: 2663.3, calculated 2663.9.

H-Cys-Ser-Cys-Ser-Ser-Leu-Met-Asp-Lys-Glu-Cys-Val-Tyr-Phe-Cys-His-Leu-Asp-Ile-Ile-Trp-OH (ET-1)

A solution of iodine (0.06 g, 0.25 mmol) in methanol (2 ml) was added to a solution of **II** (44.5 mg, 0.0167 mmol) in a 8:2 methanol:water mixture containing 5 N HCl (0.09 ml). The reaction mixture was kept for 40 min at room temperature whereupon the oxidation was stopped by adding a 5% solution of ascorbic acid in a citrate buffer (pH 5). The reaction mixture was evaporated to a final volume of 10 ml and chromatographed on a Vydac C_{18} column (16×250 mm) as described for **II**. The yield of ET-1 was 16 mg, 39% from **II** and 7% from the starting amino acid, attached to the resin; t_R 19.03 min (gradient **1**, Vydac C_{18}); molecular mass by ESI-MS: 2491.0, calculated 2491.9; amino acid analysis: Asx 2.02 (2), Ser 1.96 (3), Glx 1.10 (1), Val 1.05 (1), Met 1.03 (1), Ile 1.60 (2), Leu 2.06 (2), Tyr 0.99 (1), Phe 0.97 (1), His 1.00 (1), Trp 0.48 (1), and Lys 1.00 (1).

H-Cys(Acm)-Ser-Cys(Acm)-Ser-Ser-Leu-Met-Asp-Lys-Glu-Cys(Acm)-Val-Tyr-Phe-Cys(Acm)-His-Leu-Asp-Ile-Ile-Trp-OH (IV)

The solid phase synthesis resulted in 480 mg of crude product. This was dissolved in 70-mg portions in a 4.5:4.5:1.0 mixture of 8 M urea:30% acetic acid:*n*-butanol and chromatographed on a Diasorb C_{16} column (25×250 mm) by elution with a 0.5%/min gradient of solvent B in solvent A starting from 20% of solvent B at the flow rate of 12 ml/min. Fractions containing the target product were combined and evaporated. The residue was dissolved in water and lyophilized. The yield was 220 mg, 32% from the starting amino acid attached to the resin; t_R 18.9 min (gradient **1**, Diasorb C_{16}); molecular mass by ESI-MS: 2779.9, calculated 2780.0; amino acid analysis: Asx 1.92 (2), Ser 2.40 (3), Glx 1.10 (1), Val 0.98 (1), Met 0.89 (1), Ile 1.53 (2), Leu 1.93 (2), Tyr 1.03 (1), Phe 1.03 (1), His 0.90 (1), Trp 0.42 (1), and Lys 1.00 (1).

H-Cys-Ser-Cys-Ser-Ser-Leu-Met-Asp-Lys-Glu-Cys-Val-Tyr-Phe-Cys-His-Leu-Asp-Ile-Ile-Trp-OH ET-1, random cyclization

Mercuric acetate (18.2 mg, 0.057 mmol) was added to a solution of **IV** (20 mg, 0.0072 mmol) in a 1:1 mixture of 50% acetic acid (3 ml) and 8 M urea (3 ml). The reaction mixture was stirred for 1.5 h at 20°C, and hydrogen sulfide was then passed through the solution for 20 min. The precipitated mercuric sulfide was filtered off and washed with 5% acetic acid. The filtrate was diluted with 8 M urea (20 ml), blown through with helium, adjusted to pH 8.5 with 25% ammonia, and stirred with 1% hydrogen peroxide (0.05 ml, 0.0144 mmol) for 30–40 min. The SS bridge formation was monitored by the Ellman test and HPLC. Helium was then blown through the reaction mixture for 20 min, with the absence of hydrogen peroxide being tested by iodometric test. The reaction mixture was acidified to pH 4–5 with acetic acid (1 ml) and the main reaction product was isolated by preparative HPLC as described above for **IV**. The yield of ET-1 was 4.6 mg, 26% from **IV**; t_R 18.1 min (gradient **1**, Diasorb C_{16}) and 19.1 min (gradient **2**, Diasorb C_{16}); molecular mass by ESI-MS: 2491.2, calculated 2492; amino acid analysis: Asx 2.00 (2), Ser 2.42 (3), Glx 1.12 (1), Val 0.99 (1), Met 0.92 (1), Ile 1.83 (2), Leu 1.90 (2), Tyr 0.98 (1), Phe 1.10 (1), His 1.03 (1), Trp 0.52 (1), and Lys 1.00 (1).

H-Leu-Arg-Cys(Acm)-Met-Gln-Cys-Lys-Thr-Asp-Gly-Asp-Cys-Arg-Val-Glu-Glu-OH (VII)

The solution of crude **VI** (0.25 g) in water (250 ml) was adjusted to pH 8.5 with 25% ammonia (0.25 ml) and treated with 1% hydrogen peroxide (0.4 ml). The reaction mixture was stirred for 25 min, evaporated *in vacuo* at 35°C to a final volume of 125 ml, acidified with acetic acid (1 ml) to pH 5, and chromatographed on a Diasorb C_{16} column (25×250 mm) eluted with a 0.5%/min gradient of solvent B in solvent A starting from 0% of solvent B at the flow rate of 12 ml/min. Fractions containing the target product were combined and evaporated. The residue was dissolved in water and lyophilized. The yield was 0.04 g, 18% from the starting amino acid attached to the resin; t_R 16.4 min (gradient **3**, Diasorb C_{16}); molecular mass by ESI-MS: 1955.1, calculated 1955.3; amino acid analysis: Asp 2.06 (2), Thr 0.91 (1), Glx 3.22 (3), Gly 1.05 (1), Val 1.06 (1), Met 0.97 (1), Leu 0.93 (1), Lys 0.93 (1), and Arg 1.87 (2) (Cys was not determined).

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