1-DEAMINO-1(15)-CARBA AND -DICARBA ANALOGUES OF ENDOTHELIN-1

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Sulfanyl-methylene and ethylene bridges were inserted into the molecule of endothelin-1 (ET-1) as other possible isosteric replacements of its outer disulfide linkage. The [1-deamino-1-carba]ET-1, [1-deamino-15-carba]ET-1 and [1-deamino-1,15-dicarba]ET-1 were synthesized either by a fragment condensation of protected cyclic pentadecapeptides with carboxy-terminal hexapeptides of the endothelin-1 sequence or by step-wise coupling on polymer support of the entire henicosapeptide sequences from carboxy-terminus. The analogues were devoid of uterotonic activity in comparison with the parent ET-1.

Key words: Peptides; Solid phase synthesis; Step-wise procedure; Fragment condensation; Endothelin; Disulfide isosters; Uterotonic activity.

Endothelins are a group of extremely potent vasoactive cyclic peptides which consist of 21 amino acids and contain two disulfide bonds. They exhibit a wide spectrum of functions in pathophysiological states of various organs in the body[1].

Their analogues with modified amino acid residues, disulfide bonds, amino terminal rings and others were synthesized and investigated to understand endothelin physiology[2]. The cyclic arrangement of endothelins stabilized by two disulfide bonds was found to be important for maintaining a high receptor binding activity. For example a reduction of the outer disulfide bond between the Cys residues in positions 1 and 15 interfered with the vasoconstrictive and binding activity in corresponding analogues[3] while the endothelin-1 (ET-1) analogue with amide bond replacing the outer disulfide linkage exhibited antagonistic activity[4].

We found interesting to introduce sulfanyl-methylene or ethylene bridges into the molecule of ET-1 as another possible isosteric replacement of this disulfide linkage and focussed our effort on the synthesis of

1-deamino-1-carba (**3a**), 1-deamino-15-carba (**3b**) and 1-deamino-1,15-dicarba (**3c**) analogues of ET-1. Firstly, we have replaced the outer disulfide linkage which is reported to be more important than the inner one in interaction with a corresponding receptor[5,6]. A monocyclic analogue of 1-deamino-ET-1 was reported to lack vasoconstrictor activity; however, it has been also described, that 1-deamino replacement of the amino-terminus in some peptide hormones does not interfere with their biological activity[7,8]. Therefore we have also utilized that replacement in this study to facilitate the preparation of these analogues. While dicarba substitution of the outer disulfide bond has been already used in the synthesis of corresponding [Ala^{3,11}]Et-1 analogue[6], the synthesis of monocarba analogues was partially described in our preliminary communications[9,10].

Now, we want to report in detail on a modified synthesis of both the 1-carba and 15-carba analogues of 1-deamino-ET-1 and also on the first synthesis of the corresponding dicarba analogue with 2-aminosuberic (2-aminooctanediotic) acid imitating the outer disulfide bond of the ET-1. The method of "building blocks" applied to creation of isosteric bonds in which one or both sulfur atoms of corresponding disulfide linkage are replaced by methylene group has been developed and found to be useful in a differentiation between steric and functional roles of the disulfide bond in neurohypophyseal hormone[11] and calcitonin[12] analogues.

To compare alternative synthetic routes, the synthesis of 1-deamino-1-carba or 1-deamino-15-carba analogues of the ET-1 (**3a** and **3b**) was carried out either by segment condensation of the protected cyclic carba pentadecapeptides **1a** and **1b** or **2a** and **2b** with the carboxy-terminal hexapeptides **4a** and **4b** (strategy *A*), or by step-wise assembling the entire henicosapeptide sequences from carboxyl terminus on a polymer support (strategy *B*). The step-wise strategy *B* was also used in the synthesis of 1-deamino-1,15-dicarba analogue of the ET-1 (**3c**).

Met-Leu-(*t*Bu)Ser-(*t*Bu)Ser-(Trt)Cys-(*t*Bu)Ser-OC-CH_2-CH_2-X-Y-CH_2
|
Asp(O*t*Bu)-Lys(Boc)-Glu(O*t*Bu)-Cys(Trt)-Val-Tyr(*t*Bu)-Phe-NH-CH-R

1a, X=CH_2, Y=S, R=COOH; **1c**, X=CH_2, Y=S, R=His(Boc)-Leu-Asp(O*t*Bu)-Ile-Ile-Trp-O-WR
1b, X=S, Y=CH_2, R=COOH; **1d**, X=S, Y=CH_2, R=His(Boc)-Leu-Asp(O*t*Bu)-Ile-Ile-Trp-O-WR

The linear forms of the cyclic protected 1-deamino-1-carba- and 1-deamino-15-carbapentadecapeptides **1a** and **1b** (strategy *A*) were prepared on a weak acid labile 2-chlorotrityl chloride resin[13,14], using Fmoc/*t*Bu protection of the corresponding amino acids with an exception of the Cys(Trt)

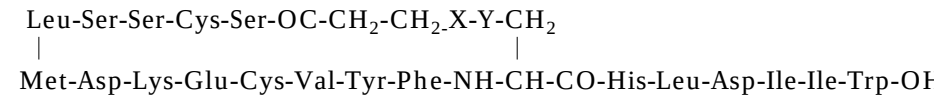
residue, starting with Fmoc-Phe-OH. Fmoc-Cys(CH_2-CH_2-CH_2-COOH)-OAll (**5c**) and Fmoc-Hcy(CH_2CH_2COOH)-O-All (**5f**) were coupled in the last step of the syntheses *via* their side-chain carboxyl groups. In a cleavage of the protected peptides from the resin by AcOH a mixture of solvents containing TFE has been used. The TFE, known to interfere with hydrogen bonds in peptide chains, alleviated a low solubility of the protected pentadecapeptides. To close the 1-carba and 15-carba bridges in the corresponding pentadecapeptides, the TPTU reagent in the same solvent mixture was successfully used as a substitute for the BOP used in our previous syntheses. As a consequence, increased reaction rates with nearly 90% yields of the cyclization reactions were achieved. After the allyl protecting group was removed by [$Pd(Ph_3P)_4$] in the presence of morpholine the protected cyclopeptides **1a** and **1b** were separately coupled to the side-chain protected hexapeptide bound to the Wang resin **4a** and the resin bound protected peptides **1c** and **1d** were obtained. After a simultaneous removal of the side-chain protecting groups and the 1-deamino-1-carba- and 1-deamino-15-carbahenicosapeptides from the resin with TFA, the inner disulfide linkage between Cys residues in the positions 3 and 11 was closed by air oxidation at pH 8.5 in an ammonium–trifluoroacetate buffer (procedure *A*) giving analogues **3a** and **3b**.

Met-Leu-(Bzl)Ser-(Bzl)Ser-(MeBzl)Cys-(Bzl)Ser-OC-CH_2-CH_2-X-Y-CH_2
|
Asp(OcHx)-Lys(ClZ)-Glu(OcHx)-Cys(MeBzl)-Val-Tyr(Bzl)-Phe-NH-CH-R

2a, X=CH_2, Y=S, R=COOH; **2c**, X=CH_2, Y=S, R=His(Bom)-Leu-Asp(OcHx)-Ile-Ile-Trp(For)-O-MR
2b, X=S, Y=CH_2, R=COOH; **2d**, X=S, Y=CH_2, R=His(Bom)-Leu-Asp(OcHx)-Ile-Ile-Trp(For)-O-MR
2e, X=CH_2,Y=CH_2, R= His(Bom)-Leu-Asp(OcHx)-Ile-Ile-Trp(For)-O-MR

The linear forms of the protected 1-deamino-1-carba- and 1-deamino-15-carbapentadecapeptides **2a** and **2b** (strategy *A*) were alternatively prepared on the Merrifield chloromethyl resin using N^{α}-Fmoc and the side-chain protection: Asp(OcHx), Glu(OcHx), Tyr(Bzl), Lys(ClZ), Cys(MeBzl), Ser(Bzl) with the exception of aminoterminal Boc-Ser(Bzl)-OH. The Fmoc-Cys($CH_2CH_2CH_2COOtBu$)-OH for 1-carba or Fmoc-Hcy($CH_2CH_2COOtBu$)-OH for 15-carba linkages were coupled to the resin as Cs salts in the first step. After the cleavage of the N^{α}-Boc protection group of Ser residue in position 2 and *t*Bu group in the side chain of the bridge units (simulated position 1 in the natural endothelin) with TFA, the outer sulfanyl–methylene bond, isosteric with disulfide bridge, was again closed using TPTU in solvent mixture containing TFE. The protected cyclic peptides **2a** and **2b** were split off

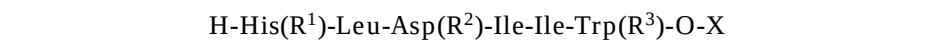
the resin with 0.1 M NaOH in MeOH-dioxane[15] and after acidification their carboxylic termini were separately coupled to the hexapeptide bound to Merrifield resin **4b** yielding the protected peptides on resin **2c**, **2d**. After the peptides were deprotected and cleaved from the resin simultaneously using TFMSA (ref.[16]), they were directly subjected to the modified air oxidation procedure different from that used for intermediates obtained from the peptide resins **1c** and **1d**. To alleviate the low solubility of these peptides, we chose air oxidation at high concentration of DMSO in aqueous solution[17] (procedure *B*) to close the inner disulfide bond. The degree of oxidation was monitored by HPLC.



3a, X=S, Y=CH_2; **3b**, X=CH_2, Y=S; **3c**, X=CH_2, Y=CH_2

The step-wise syntheses (strategy *B*) of the protected precursors **2c**–**2e** of [1-deamino-1-carba]- (**3a**), [1-deamino-15-carba]- (**3b**) and [1-deamino-1,15-dicarba]endothelin-1 (**3c**) from their carboxylic termini were also started with the carboxy-terminal hexapeptide built on the Merrifield resin **4b**. The α-amino and side chain protection, coupling reagents, conditions, and the removal of protecting groups were essential the same as those used in the preparation of the protected peptides **2c** and **2d** by the segment condensation. In addition, the Fmoc-Asu(O*t*Bu)-OH (**5g**) for 1,15-dicarba linkage was utilized. The closures of the 1-carba, 15-carba and 1,15-dicarba linkages were performed with the whole henicosapeptide sequences bound to Merrifield resin.

The peptides were simultaneously deprotected and detached from the resin and their inner disulfide groups closed according to procedure *B* used for the peptides obtained from peptide resins **2c** and **2d**, which were prepared by the segment condensation (strategy *A*). The HPLC elution times for the analogs **3a** and **3b** with 1-carba and 15-carba bridges, respectively, were identical. The retention time of the dicarba analogue **3c** was increased. The scarcely soluble analogues **3a**–**3c** were converted to their sodium salts which could be then easily handled during their HPLC purification and the following bioassay. Uterotonic activity of compounds **3a**–**3c** was assayed and compared with that of the native ET-1.



4a, R^1 = Boc, R^2 = O*t*Bu, R^3 = H, X = Wang resin; **4b**, R^1 = Bom, R^2 = OcHx, R^3 = For, X = Merrifield resin

The protected hexapeptide segments bound to the Wang and Merrifield resins **4a** and **4b** were prepared according to the synthetic protocols for the syntheses of the pentadecapeptides **1a**, **1b** and **2a**, **2b** using the corresponding protected amino acids and resins.

Fmoc-AA(R^1)-O-R^2

5a, AA = Cys, R^1 = $CH_2CH_2CH_2COOtBu$, R^2 = H
5b, AA = Cys, R^1 = $CH_2CH_2CH_2COOtBu$, R^2 = All
5c, AA = Cys, R^1 = $CH_2CH_2CH_2COOH$, R^2 = All
5d, AA = Hcy, R^1 = $CH_2CH_2COOtBu$, R^2 = H
5e, AA = Hcy, R^1 = $CH_2CH_2COOtBu$, R^2 = All
5f, AA = Hcy, R^1 = CH_2CH_2COOH, R^2 = All
5g, AA = Asu, R^1 = O*t*Bu, R^2 = H

EXPERIMENTAL

Chloromethylated Merrifield (0.6 mmol Cl/g), Boc-Trp(For)-Merrifield (0.46 mmol/g), 2-chlorotritylchloride (1.3 mmol/g) and 4-(benzyloxy)benzyl alcohol Wang (0.74 mmol/g) resins were purchased from Calbiochem–Novabiochem AG (Läufelfingen, Switzerland), TPTU, TBTU and HBTU from Senn Chemicals International (Gentilly, France) and protected amino acids were purchased from Bachem (Bubendorf, Switzerland) or were prepared in our laboratory following general protocols[18] and published papers[19–27]. In the Fmoc- and Boc-amino acid preparations, the prescribed pH of the reaction mixtures was maintained using a pH meter with an automatic titrator (Radiometer, Copenhagen, Denmark). They were checked for purity by thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), elemental analysis and mass spectroscopy. The TLC of the protected amino acids prepared was performed on precoated Silica gel 60 F_{254} plates (Merck, Darmstadt, Germany) in the following systems: butan-2-ol–98% formic acid–water (75 : 13.5 : 11.5) (S_1); butan-2-ol–25% aqueous ammonia–water (85 : 7.5 : 7.5) (S_2); butan-1-ol–acetic acid–water (4 : 1 : 1) (S_3); butan-1-ol–pyridinel–acetic acid–water (15 : 10 : 3 : 6) (S_4). Column chromatography was carried out with Silica gel 60 (Merck, Darmstadt, Germany). The compounds were visualized directly under ultraviolet light or by the ninhydrin and chlorine-TDM detection[28]. Melting points were measured on a Kofler block and were not corrected. Optical rotations were measured on a Perkin–Elmer 141 MCA polarimeter at 22 °C and $[\alpha]_D$ values are given in 10^{-1} deg cm^2 g^{-1}. Solvents were evaporated *in vacuo* on a rotary evaporator (bath temperature 30 °C); DMF was evaporated at 30 °C and 150 Pa. An average level of the first amino acid substitution on the corresponding resin was determined from a weight increase, spectroscopically by the measurement of the dibenzofulvene–piperidine complex absorption after the Fmoc group cleavage by 5% piperidine in DCM–DMF (1 : 1) for 10 min and 20% piperidine in DMF for 15 min, and by the quantitative amino acid analysis of the amino acid loaded on the resin. In the Boc-amino acid substitution assessment, only the weight increase and amino acid analysis were used. Progress in peptide synthesis was followed by the Kaiser[29] and Bromophenol Blue[30] tests. The samples for amino acid analysis were hydrolyzed with 6 M HCl containing 3% of phenol at 110 °C for 20 h. The amino acid analyses were

performed on a Biochrom 20 instrument (Pharmacia, Sweden). ^{1}H and ^{13}C NMR spectra were measured on FT NMR spectrometer Varian 500 (^{1}H at 500 MHz, ^{13}C at 125.7 MHz) in DMSO-d_6 with tetramethylsilane as an internal reference. Molecular weights of the peptides were determined using mass spectroscopy with FAB technique (Micromass, Manchester, England). For HPLC, a Spectra Physics instrument with an SP 8800 pump, an SP 4290 integrator and Thermo Separation Products Spectra 100 UV detector was used. Peptides **3a**–**3c** were purified by semipreparative HPLC on 250 × 10 mm columns: 10 μm Vydac RP-18 (The Separations Group, Hesperia CA, U.S.A.) and 10 μm LiChroCard, Purosphere RP-18 (Merck, Darmstadt, Germany), flow rate 3 ml/min, detection at 200 nm using gradient 50-100% ACN in 0.05% aqueous TFA within 60 min. The analytical HPLC was carried out on the 250 × 4 mm column: 5 μm LiChrospher WP-300 RP-18 (Merck, Darmstadt, Germany), flow rate 1 ml/min, detection at 200 nm, 5–100% gradient of ACN in 0.05% aqueous TFA within 60 min.

Strategy A

Protected Cyclopentadecapeptides **1a** and **1b**

Fmoc-Phe-OH (0.21 g, 0.78 mmol) was loaded to the 2-chlorotritylchloride resin (1 g) with an average level of the substitution 0.42 mmol/g of the resin. After the Fmoc protecting group was removed, the H-Phe-resin was then gradually acylated with 3 equivalents (1.3 mmol) of Fmoc-amino acids with corresponding side-chain protection [Tyr(*t*Bu), Val, Cys(Trt), Asp(O*t*Bu), Lys(Boc), Glu(O*t*Bu), Met, Leu, Ser(*t*Bu) twice, Cys(Trt) and Ser(*t*Bu)] using TBTU (0.49 g, 1.5 mmol) and DIEA (0.52 ml, 3 mmol) in DMF (20 ml). The Fmoc groups were removed by treatment with 20% piperidine in DMF (2 × 20 ml), 10 and 30 min. After the last Fmoc deprotection, the peptide resin was washed with MeOH, dried in desiccator and divided into two parts. Both parts were swollen in DMF (10 ml) and acylated either with Fmoc-Cys($CH_2CH_2CH_2COOH$)-O-All (**5c**; 0.32 g, 0.7 mmol; for the compound **1a**) or with Fmoc-Hcy(CH_2CH_2COOH)-O-All (**5f**; 0.32 g, 0.7 mmol; for the compound **1b**) *via* their side-chain carboxylic groups as described above. After the Fmoc deprotection and final washings with DMF (5 × 10 ml), both peptides were split off the resin by treating with an AcOH (5 ml)–TFE (5 ml)–DCM (30 ml)–DMF (2 ml) mixture for 30 min at room temperature. The solvents were evaporated, the solid residues dissolved in a DCM (30 ml)–TFE (3 ml)–DMF (30 ml) mixture and stirred with TPTU (0.45 g, 1.5 mmol), HOBt (0.2 g, 1.5 mmol) and DIEA (0.6 ml, 3 mmol) at room temperature for about 3 days until the Kaiser test indicating the presence of free α-amino group was negative. Both reaction mixtures were evaporated to dryness and ethyl acetate was added to the residues which precipitated. After filtration and washing with ethyl acetate, the protected cyclic pentadecapeptide allyl esters were dried in a desiccator to dryness, 0.4 g (0.15 mmol) of each was dissolved under argon in DCM (12 ml)–TFE (2 ml)–DMF (12 ml) and [$Pd(Ph_3P)_4$] (0.23 g, 0.2 mmol) with PPh_3 (0.06 g, 0.2 mmol) and morpholine (0.018 ml, 0.2 mmol) were added to the solutions. The reaction mixtures were stirred under argon for 2 h at room temperature, solvents were evaporated, the crude cyclic pentadecapeptide acids **1a** and **1b** were washed with water, ethyl acetate, diethyl ether and purified with a DMF–diethyl ether mixture. After drying in desiccator, the yields were 0.23 g (0.09 mmol; 43%) and 0.21 g (0.08 mmol; 38%) of the protected cyclopeptides **1a** and **1b**, respectively, which were checked for homogeneity by HPLC, FAB MS and amino acid analysis (Table I).

TABLE I
Analytical data of protected cyclic pentadecapeptides **1a**, **1b**, **2a**, **2b** and analogues **3a–3c**

Compound	Formula[b] MW/(M^+ + 1)	HPLC[c]	Amino acid composition[a]							
			Trp	Ile	Asp	Leu	His	Phe	Tyr	Cys(C_3)[d]
			Val	Cys	Glu	Lys	Met	Ser	Asu	Hcy(C_2)[e]
1a	$C_{138}H_{191}N_{15}O_{26}S_4$	42.75	–	–	1.01	0.97	–	1.06	0.91	1.01
	2 604.4/2 603.3		1.00	1.81	1.05	1.02	0.92	2.60	–	–
1b	[f]	42.54	–	–	1.05	0.99	–	1.04	0.94	–
			1.00	1.84	1.02	1.03	0.87	2.57	–	0.97
2a	$C_{133}H_{168}ClN_{15}O_{26}S_4$	40.23	–	–	1.07	1.05	–	1.02	0.96	0.97
	2 556.6/2 555.1		1.00	1.86	1.01	1.07	0.91	2.68	–	–
2b	[f]	40.05	–	–	1.02	1.02	–	1.02	0.92	–
			1.00	1.81	0.99	1.05	0.86	2.54	–	1.02
3a[g]	$C_{110}H_{160}N_{24}O_{32}S_4$	22.15	0.74	1.76	2.01	1.82	1.03	1.03	0.98	1.04
	2 458.9/2 458		1.00	1.83	1.05	1.04	0.89	2.62	–	–
3a[h]	[f]		0.76	1.76	1.98	1.85	0.99	1.03	1.01	1.02
			1.00	1.81	1.04	1.06	0.93	2.63	–	–
3b[g]	[f]	22.12	0.76	1.72	1.97	1.85	1.01	1.02	0.99	–
			1.00	1.85	1.01	1.03	0.92	2.65	–	0.95
3b[h]	[f]		0.78	1.75	1.99	1.88	0.99	1.01	1.01	–
			1.00	1.83	1.02	1.01	0.95	2.68	–	0.96
3c	$C_{111}H_{162}N_{24}O_{32}S_3$	22.98	0.81	1.73	1.98	1.84	1.04	1.05	1.01	–
	2 440.9/2 440		1.00	1.91	1.02	1.01	0.89	2.57	0.93	–

[a] Amino acid analyses were performed on Biochrom 20 instrument (Pharmacia, Sweden). [b] Determined with FAB technique (VG Analytical, England). [c] Retention time in minutes, 25 × 0.4 cm RP-18 column, 5 μm (Lichrospher WP-300, Merck Darmstadt, Germany), flow rate 60 ml/h, detection at 222 nm, gradient 50–100% of acetonitrile in 0.05% aqueous TFA, 60 min, Spectra Physics SP 8800 HPLC pump, an SP 4290 integrator and Thermo Separation Products Spectra 100 UV detector. [d] H-Cys($CH_2CH_2CH_2COOH$)-OH. [e] H-Hcy(CH_2CH_2COOH)-OH. [f] The same molecular weight was confirmed by mass spectroscopy. [g] Prepared by strategy *A*. [h] Prepared by strategy *B*.

Segment Synthesis of the Protected 1-Deamino-1-carba- (**1c**) and 1-Deamino-15-carba- (**1d**) henicosapeptides on Resin

The protected cyclic peptides **1a** and **1b** (0.13 g, 0.05 mmol of each) were separately agitated with the side-chain protected hexapeptide **4a** linked to Wang resin (0.2 g, 0.4 mmol/g for each of the cyclic peptides) in the mixture of solvents DCM (15 ml)–DMF (7.5)–TFE (3 ml) with HBTU (0.11 g, 0.3 mmol) in the presence of DIEA (0.1 ml) at 40 °C for 3 days in ultrasonic bath. The peptide resins **1c** and **1d** were separated by filtration, washed with the same solvent mixture (3 × 15 ml), with DCM (3 × 15 ml), MeOH (3 × 15 ml) and dried in desiccator.

Analogues **3a** and **3b** by Oxidative Procedure *A*

Peptide-resins **1c** and **1d** were treated with 50% TFA in DCM (3 × 10 ml) in the presence of EDT (0.5 ml) and anisole (1 ml). The resins were filtered off, washed with 50% AcOH (3 × 10 ml), the filtrates evaporated to one third of their volumes and diluted with 100 ml of water. The solutions were washed with ethyl acetate and diethyl ether to remove the EDT and anisole and were freeze-dried (both about 0.11 g). Both products were oxidized by air in an AcOH–TFA (1 : 1; 10 ml) solution at pH 8.5, which was adjusted and maintained by 50% ammonium hydroxide. The peptide solutions (about 400 ml of each) were stirred at room temperature for 24 h, then acidified with AcOH to pH 4.5, evaporated to one third of their volumes and finally freeze-dried to obtain the crude analogues **3a** and **3b** which were purified by preparative HPLC (Table I).

Protected Cyclopentadecapeptides **2a** and **2b**

The Cs salts of the Fmoc-Cys($CH_2CH_2CH_2COO$*t*Bu)-OH (**5a**; 0.45 g, 0.65 mmol) or Fmoc-Hcy(CH_2CH_2COO*t*Bu)-OH (**5d**; 0.45 g, 0.65 mmol) were separately loaded as the first amino acid residues to chloromethylated Merrifield resin (0.5 g) in DMF (15 ml) at 50 °C for 30 h in the presence of 18-crown-6 (0.24 g, 0.65 mmol). The average substitution on the resin was 0.25 mmol/g. Fmoc group in this and all the following steps was removed by treatment with 20% piperidine in DMF (2 × 10 ml), 15 min. The amino acid-resin was washed with DMF (5 × 10 ml) and then gradually acylated with 3 equivalents (0.37 mmol) of Fmoc-amino acids with corresponding side-chain protection [Phe, Tyr(BrZ), Val, Cys(MeBzl), Asp(OcHx), Lys(ClZ), Glu(OcHx), Met, Leu, twice Ser(Bzl), Cys(MeBzl)] using TBTU (0.12 g, 0.4 mmol) and DIEA (0.13 ml, 0.8 mmol) in DMF (10 ml). The last coupling was carried out using Boc-Ser(Bzl)-OH. After cleavage of the Boc and *t*Bu ester groups with 50% TFA/DCM (2 × 10 ml), 1 h, a closure of the 15-carba bridge was carried out on the resin using TPTU (0.23 g, 0.74 mmol), HOBt (0.11g, 0.74 mmol) and DIEA (0.24 ml, 1.48 mmol) in DMF (10 ml) at 50 °C for 3 days in an ultrasonic bath. Each of the full-sequence-peptide resins was treated at room temperature with a mixture of MeOH (5 ml)–dioxane (13 ml)–4 M NaOH (0.5 ml) in ultrasonic bath for 3 min at room temperature and then AcOH (0.5 ml) was added. The resins were filtered off and washed with a DCM–DMF–TFE mixture (3 : 3 : 1; 3 × 10 ml). This cleavage was repeated twice and finally all filtrates were collected and solutions evaporated to dryness. The residues were triturated with ethyl acetate, separated by filtration and purified from a DMF–diethyl ether mixture. The yields were 0.13 g (0.05 mmol; 40%) and 0.12 g (0.047 mmol; 38%) of the protected cyclopeptides **2a** and **2b**, respectively, which were checked for homogeneity by HPLC, FAB MS and amino acid analyses (Table I).

Segment Synthesis of the Protected 1-Deamino-1-carba- (**2c**) and 1-Deamino-15-carba- (**2d**) henicosapeptides on Resin

Solutions of protected cyclic pentadecapeptides **2a** or **2b** (0.08 g, 0.03 mmol), HBTU (0.06 g, 0.15 mmol), HOBt (0.04 g, 0.3 mmol) and DIEA (0.1 ml, 0.6 mmol) in a DMF (3 ml)–DCM (3 ml)–TFE (1 ml) mixture were stirred at room temperature for 30 min and then added separately to side-chain protected hexapeptide **4b** (0.11 g, 0.46 mmol/g) linked to Merrifield resin. The condensation was allowed to proceed in ultrasonic bath at 50 °C for 3 days. The peptide-resins were separated by filtration, washed with a DCM–DMF–TFE mixture (3 : 1; 3 × 10 ml), MeOH (3 × 10 ml) and dried in desiccator.

Strategy B

Step-Wise Synthesis of the Protected 1-Deamino-1-carba- (**2c**), 1-Deamino-15-carba- (**2d**) and 1-Deamino-1,15-dicarba- (**2e**) henicosapeptides on Resin

Each of the Fmoc-Cys($CH_2CH_2CH_2COOtBu$)-OH (**5a**; 0.24 g, 0.5 mmol), Fmoc-Hcy($CH_2CH_2COOtBu$)-OH (**5d**; 0.24 g, 0.5 mmol) and Fmoc-Asu(O*t*Bu)-OH (**5 g**; 0.23 g, 0.5 mmol) in DMF solution (20 ml) was activated with HBTU (0.6 g, 1.5 mmol), HOBt (0.2 g, 1.5 mmol) in the presence of DIEA (0.51 ml, 3 mmol) and coupled to the hexapeptide (0.36 g, 0.46 mmol/g) on the Merrifield resin **4b** with yielding the corresponding carboxy-terminal resin bound heptapeptides. The Fmoc protecting group in this and all the following steps was removed by treatment with 20% piperidine in DMF (2 × 10 ml), 30 min. However, after this coupling (which was repeated twice with the five fold excess of **5a**, **5d** and **5g**), the amino acid substitution was found to be 0.15 mmol/g only. Therefore each of the hepta-peptide chains bound to resin was capped by treatment with an acetic acid anhydride–DIEA mixture (2 : 1; 20 ml) for 2 h, washed with DCM (3 × 20 ml), DMF (5 × 20 ml) and gradually acylated with 3 equivalents (0.16 mmol) of side-chain protected Fmoc-amino acids [Phe, Tyr(BrZ), Val, Cys(MeBzl), Asp(OcHx), Lys(ClZ), Glu(OcHx), Met, Leu, twice Ser(Bzl), Cys(MeBzl)] using TBTU (0.05 g, 0.16 mmol) and DIEA (0.08 ml, 0.48 mmol) in DMF (10 ml). The last coupling was carried out using Boc-Ser(Bzl)-OH. After cleavage of the Boc and *t*Bu ester groups with 50% TFA in DCM (2 × 10 ml), 1 h, the peptide-resins were treated with TPTU (0.08 g, 0.25 mmol), HOBt (0.06 g, 0.49 mmol) and DIEA (0.12 ml, 0.73 mmol) in DMF (15 ml) at 50 °C for 3 days in ultrasonic bath. The cyclized peptides bound to the resin **2c**, **2d** and **2e** were separated from the reaction mixture by filtration and were washed with 3 × 20 ml portions of DMF, isopropyl alcohol, MeOH and dried in desiccator.

Compounds **3a**, **3b** and **3c** by Oxidative Procedure *B*

Each of the protected peptide resins **2c**, **2d** and **2e** was stirred with an EDT (0.2 ml)–*m*-cresol (0.8 ml)–DMS (3 ml)–TFA (5 ml)–TFMSA (1 ml) mixture at –5 to 0 °C for 30 min and then it was filtered, washed with cold diethyl ether and dried to remove residual ether. The peptide-resins were further treated with a thioanisole–EDT 2 : 1 mixture (1.5 ml) for 10 min at room temperature and after cooling in an ice bath, TFA (10 ml) was added under stirring. After 10 min, TFMSA (1 ml) was added dropwise and the reaction was stirred for another 1.5 h at room temperature. The peptides were precipitated under argon stream with diethyl ether and together with the resin filtered off the solvent containing EDT, *m*-cresol and thioanisole. After washing with diethyl ether (3 × 50 ml), the peptides were dissolved with

TFA (5 ml each), filtered into cold diethyl ether (200 ml) to precipitate and the solids were dried in desiccator. Each of the henisocapeptides containing the free sulfanyl groups in the Cys^3, Cys^{11} residues was oxidized in aqueous 80% DMSO solution (500 ml) at pH maintained at 7–8 by aqueous ammonia for 4 h. After acidification of the mixtures to pH 5, the solutions were freeze-dried and the crude analogues **3a**–**3c** subjected to HPLC purification in the gradient of ACN (50–100%) in 0.05% TFA within 60 min. A LiChroCard column with the Purosphere RP-18 stationary phase recommended for hydrophobic peptides was used. The purified analogues **3a** and **3b** exhibited the HPLC, FAB MS and amino acid analyses data identical to those obtained for the same analogues prepared from peptides **1c** and **1d** bound to the Wang resin (Table I).

H-His(Boc)-Leu-Asp(O*t*Bu)-Ile-Ile-Trp-O-WR (**4a**)

Fmoc-Trp-OH (0.52 g, 1.2 mmol) was coupled to 4-benzyloxyalcohol resin (WR; 0.5 g) using a Sieber method[31] with substitution of 0.4 mmol/g, then deprotected with 20% piperidine in DMF and gradually acylated with 3 equivalents (0.6 mmol) of side chain protected Fmoc-amino acids [Ile twice, Asp(O*t*Bu), Leu and His(Boc)] using TBTU (0.22 g, 0.7 mmol) and DIEA (0.25 ml, 1.4 mmol) in DMF (20 ml). After the final Fmoc deprotection and washing with DMF, propan-2-ol and MeOH, the partially protected hexapeptide resin **4a** was dried in desiccator (0.62 g) and divided into two parts which were used for the segment condensation with the cyclic pentadecapeptides **1a** and **1b**.

H-His(Bom)-Leu-Asp(OcHx)-Ile-Ile-Trp(For)-O-MR (**4b**)

After the Boc deprotection with 50% TFA in DCM and neutralization with 5% DIEA in DCM, the H-Trp(For)-O-MR (2.0 g, 0.46 mmol/g) was gradually acylated with 3 equivalents (2.76 mmol) of side-chain protected Boc-amino acids [Ile twice, Asp(OcHx), Leu and His(Bom)] using DIC (0.43 ml, 2.76 mmol) and HOBt (0.37 g, 2.76 mmol) in DMF (30 ml). After the final Boc deprotection, neutralization and washing with DCM, propan-2-ol and MeOH, the side-chain protected hexapeptide-resin **4b** was dried in desiccator (2.6 g) and divided into five parts which were used either for the segment condensation with the cyclic pentadecapeptides **2a** and **2b** or for the step-wise synthesis of the henicosapeptides **2c**–**2e**.

Fmoc-Cys($CH_2CH_2CH_2COO$*t*Bu)-OH (**5a**)

A solution of H-Cys($CH_2CH_2CH_2COO$*t*Bu)-OH (6.6 g, 25 mmol) in water (500 ml) was stirred at pH 9.3 (adjusted and maintained with 10% Na_2CO_3) with Fmoc-OSu (8.5 g, 25.2 mmol) in acetone (150 ml) for 3 h. Acetone was evaporated, the alkaline solution was washed with diethyl ether (4 × 100 ml) and acidified with aqueous HCl to pH 3. The separated oil was extracted with ethyl acetate (3 × 100 ml), the organic solution was washed with saturated $KHSO_4$ and Na_2SO_4 and evaporated to dryness. The residue was purified on a column of Silica gel 60 in the gradient of solvents EtOAc–PE yielding 8.7 g (17.9 mmol; 72%) of pure product. M.p. 89–90 °C; $[\alpha]_D$ –27.9 (*c* 0.59, DMF), [ref.[22] m.p. 142–143 °C for dicyclohexylammonium salt; $[\alpha]_D$ +2.8 (*c* 0.36, EtOH)]. The HPLC retention time was 19.6 min (60–100% MeOH in 0.05% TFA, 30 min). TLC: R_F 0.78 (S_1), 0.30 (S_2), 0.84 (S_3), 0.49 (S_4), 0.33 (40% EtOAc in PE). ^{1}H NMR: 12.88 b, 1 H (carboxyl); 7.75 d, 1 H (carbamate); 7.33 dt, 7.42 bt, 7.73 bd, 7.89 bd, 8 H (fluorene, arom.); 4.28 dd, 2 H and 4.31 dd, 2 H (2 × CH_2O); 4.23 bt, 1 H (fluorene, alif.); 4.12 ddd, 1 H (α-CH); 2.74 dd and 2.90 dd, 2 H (β-CH_2); 2.52 m, 2 H (δ-CH_2); 2.27 t, 2 H

(ζ-CH_2); 1.72 t, 2 H (ε-CH_2); 1.38 s, 9 H ($C(CH_3)_3$). ^{13}C NMR: 172.49, 1 C (COOH); 171.96, 1 C (CO, ester); 156.19, 1 C (CO, carbamate); 143.98, 140.91, 127.82, 127.24, 125.49, 120.30, 12 C (fluorene, arom.); 79.79, 1 C ($C(CH_3)_3$); 65.91, 1 C (CH_2O); 54.24, 1 C (α-CH_2); 46.80, 1 C (fluorene, alif.); 33.78, 1 C (ζ-CH_2); 32.71, 1 C (δ-CH_2); 30.82, 1 C (β-CH_2); 27.91, 3 C ($C(CH_3)_3$); 24.70, 1 C (ε-CH_2). For $C_{26}H_{31}NO_6S$ (485.6) calculated: 64.31% C, 6.43% H, 2.88% N, 6.60% S; found: 64.25% C, 6.44% H, 3.16% N, 6.39% S. FAB MS, *m*/*z*: 486.2 (M + 1).

Fmoc-Cys($CH_2CH_2CH_2COO$*t*Bu)-O-All (**5b**)

A mixture of Fmoc-Cys($CH_2CH_2CH_2COO$*t*Bu)-OH (**5a**; 9.6 g, 20 mmol) and $NaHCO_3$ (1.8 g, 21 mmol) in water (32.4 ml), Aliquat 336 (9.6 ml) and allyl bromide (2.5 g, 21 mmol) in DCM (31.9 ml) was stirred at room temperature for 30 h. Allyl bromide (2.5 g, 21 mmol) and 0.5 M $NaHCO_3$ (5.3 ml) were added and the mixture was stirred for another 24 h. The product was extracted from aqueous layer with DCM (3 × 100 ml), the DCM solution was dried with Na_2SO_4, evaporated to dryness and the diester was purified on a Silica gel 60 column in a EtOAc–PE gradient yielding pure compound **5b** (5.2 g, 49.5%). The HPLC retention time was 25.18 min (60–100% MeOH in 0.05% TFA, 30 min). TLC: R_F 0.85 (40% EtOAc in PE) and 0.69 (30% EtOAc in PE). For $C_{29}H_{35}NO_6S$ (525.7) calculated: 66.26% C, 6.71% H, 2.66% N, 6.10% S; found: 66.61% C, 6.48% H, 2.39% N, 6.51% S. FAB MS, *m*/*z*: 526.4 (M + 1).

Fmoc-Cys($CH_2CH_2CH_2COOH$)-O-All (**5c**)

The diester **5b** (5.3 g, 10 mmol) was treated with TFA (6 ml) in the presence of diethyl sulfide (1.1 ml, 10 mmol) at room temperature for 2 h. TFA was evaporated and the residue evaporated three times with toluene. The oily product (4 g) was purified on a Silica gel 60 column using 50% EtOAc in PE yielding 3.2 g (6.8 mmol; 68%) of the pure acid **5c**, m.p. 94–96 °C; $[\alpha]_D$ –28.4 (*c* 0.47, DMF). The HPLC retention time was 16.43 min (60–100% MeOH in 0.05% TFA, 30 min). TLC: R_F 0.16 (30% EtOAc in PE). ^{1}H NMR: 12.29 b, 1 H (carboxyl); 7.75 d, 1 H (NH, carbamate); 7.89 bd, 7.73 bd, 7.42 bt, 7.33 dt, 8 H (fluorene, arom.); 5.89 m, 1 H (β-allyl ester); 5.29 dq and 5.20 dq, 2 H (2 × γ-allyl ester); 4.58 m, 2 H (α-allyl ester); 4.31 dd and 4.28 dd, 2 H (CH_2O); 4.23 bt, 1 H (fluorene, alif.); 4.12 m, 1 H (α-CH); 2.52 m, 2 H (δ-CH_2); 2.27 t, 2 H (ζ-CH_2); 2.91 dd and 2.75 dd, 2 H (β-CH_2); 1.72 t, 2 H (ε-CH_2). ^{13}C NMR: 173.15, 1 C (COOH); 171.46, 1 C (CO, ester); 156.32, 1 C (CO, carbamate); 143.98, 140.93, 127.83, 127.25, 125.38, 120.32, 12 C (fluorene, arom.); 117.92, 1 C (γ-allyl ester); 132.56, 1 C (β-allyl ester); 65.83, 1 C (CH_2O); 65.18, 1 C (α-allyl ester); 54.14, 1 C (α-CH); 46.81, 1 C (fluorene, alif.); 33.58, 1 C (ζ-CH_2); 30.82, 1 C (β-CH_2); 32.71, 1 C (δ-CH_2); 24.72, 1 C (ε-CH_2). For $C_{25}H_{27}NO_6S$ (469.6) calculated: 63.95% C, 5.80% H, 2.98% N, 6.83% S; found: 63.22% C, 5.67% H, 2.89% N, 6.23% S. FAB MS, *m*/*z*: 470.1 (M + 1).

Fmoc-Hcy(CH_2CH_2COO*t*Bu)-OH (**5d**)

The same reaction and purification conditions as described for compound **5a** were used in the reaction of H-Hcy(CH_2CH_2COO*t*Bu)-OH (5.3 g, 20 mmol) with Fmoc-OSu (7.1 g, 21 mmol). The yield of pure **5d** was 6.7 g (69%). The data of TLC, HPLC, elemental and FAB MS analyses were close to those obtained for the Cys derivative **5a**. M.p. 86–89 °C; $[\alpha]_D$ –21.3 (*c* 0.52, DMF), [ref.[23] m.p. 87–89 °C; $[\alpha]_D$ –17.8 (*c* 1.8, DMF)]. ^{1}H NMR: 12.87 b, 1 H (carboxyl); 7.85 d, 1 H (NH, carbamate); 7.89 bd, 7.71 bd, 7.42 bt, 7.33 dt, 8 H (fluorene, arom.); 4.32 m, 2 H (CH_2O); 4.23 bt, 1 H (fluorene, alif.); 4.22 m, 1 H (α-CH); 2.67 t, 2 H (ε-CH_2); 2.57 m, 2 H

(γ-CH_2); 2.51 m, 2 H (ζ-CH_2); 1.92 m, 2 H (β-CH_2); 1.38 s, 9 H ($C(CH_3)_3$). ^{13}C NMR: 173.19, 1 C (COOH); 171.45, 1 C (CO, ester); 156.34, 1 C (CO, carbamate); 143.98, 140.93, 127.83, 127.25, 125.38, 120.32, 12 C (fluorene, arom.); 79.98, 1 C (**C**$(CH_3)_3$); 65.98, 1 C (CH_2O); 54.64, 1 C (α-CH); 46.80, 1 C (fluorene, alif.); 32.73, 1 C (ζ-CH_2); 31.02, 1 C (β-CH_2); 27.89, 3 C ($C(\mathbf{C}H_3)_3$); 27.65, 1 C (γ-CH_2); 24.04, 1 C (ε-CH_2).

Fmoc-Hcy(CH_2CH_2COO*t*Bu)-OAll (**5e**)

Using the reaction and purification conditions described for the synthesis of the Cys derivative **5b**, Fmoc-Hcy(CH_2CH_2COO*t*Bu)-OH (**5d**; 3.8 g, 7.9 mmol), $NaHCO_3$ (1.34 g) in water (15.8 ml) and Aliquat 336 (3.6 ml) were treated with allyl bromide (1.96 g, 16.2 mmol) in DCM (12 ml) to afford 2.1 g of pure **5e** (50%) with analytical HPLC, TLC, elemental and FAB MS analysis data close to those obtained for the Cys derivative **5b**.

Fmoc-Hcy(CH_2CH_2COOH)-OAll (**5f**)

The same reaction and purification conditions described in preparation of Cys derivative **5c** were used in treatment of diester **5e** (2.65 g, 5.0 mmol) with TFA (3 ml) in the presence of diethyl sulfide (0.55 ml). Pure product **5f** was obtained in a yield of 1.6 g (68%), m.p. 93–95 °C; $[\alpha]_D$ –22.3 (*c* 0.42, DMF) and exhibited analytical HPLC, TLC, elemental and FAB MS analysis data close to those obtained for the Cys derivative **5c**. ^{1}H NMR: 12.29 b, 1 H (carboxyl); 7.85 d, 1 H (NH, carbamate); 7.89 bd, 7.71 bd, 7.42 bt, 7.33 dt, 8 H (fluorene, arom.); 5.89 m, 1 H ($OCH_2C\mathbf{H}=CH_2$); 5.29 dq and 5.20 dq, 2 H (2 × $OCH_2CH=C\mathbf{H}_2$); 4.58 m, 2 H ($OC\mathbf{H}_2CH=CH_2$); 4.32 m, 2 H (CH_2O); 4.23 bt, 1 H (fluorene, alif.); 4.22 m, 1 H (α-CH); 2.67 t, 2 H (ε-CH_2); 2.57 m, 2 H (γ-CH_2); 2.51 m, 2 H (ζ-CH_2); 1.92 m, 2 H (β-CH_2). ^{13}C NMR: 173.19, 1 C (COOH); 171.96, 1 C (CO, ester); 156.34, 1 C (CO, carbamate); 143.98, 140.93, 127.83, 127.25, 125.38, 120.32, 12 C (fluorene, arom.); 117.91, 1 C ($OCH_2CH=\mathbf{C}H_2$); 132.54, 1 C ($OCH_2\mathbf{C}H=CH_2$); 65.83, 1 C (CH_2O); 65.08, 1 C ($O\mathbf{C}H_2CH=CH_2$); 53.04, 1 C (α-CH); 46.82, 1 C (fluorene, alif.); 34.66, 1 C (ζ-CH_2); 31.00, 1 C (β-CH_2); 27.64, 1 C (γ-CH_2); 26.30, 1 C (ε-CH_2).

Z-Asu-OBzl

Z-Asu-OH (refs[24,26]) (6.0 g, 18.6 mmol) was stirred with TFE (2.6 ml, 18.6 mmol) and benzyl bromide (3.5 g, 2.4 ml, 20.4 mmol) in DMF (4 ml) at room temperature for 20 h. The reaction mixture was poured into a water with ice (40 ml) and the organic layer was taken up with EtOAc. This solution was washed with 5% $NaHCO_3$ and water, dried with Na_2SO_4 and evaporated to dryness yielding an oily product (6.4 g, 15.5 mmol). The purity of 88% was assessed by HPLC in the 0–100% gradient of ACN in 0.05% TFA, 60 min, 220 nm, elution time 35 min (content of the benzyl ester in this product was 5.6 g (13.6 mmol)).

H-Asu(O*t*Bu)-OH

Using general procedure[18], Z-Asu-OBzl (4.1 g, 10 mmol) was converted to the *tert*-butyl ester oily product (4.6 g) containing 92% of Z-Asu(O*t*Bu)-OBzl (by HPLC as described above), elution time 44.8 min. The diester was saponified in acetone (40 ml) solution with 1 M NaOH (10 ml) at 0–5 °C for 5 h. After acidification with 1 M HCl, the separated oil was extracted with EtOAc and this solution was washed with water, dried with Na_2SO_4 and evaporated to

dryness. The oily residue (3.3 g), free of the benzyl ester as assessed by HPLC using the 0–100% gradient of ACN in 0.05% TFA, 60 min, elution time 37.6 min, was hydrogenated in propan-2-ol (100 ml) solution on Pd black (prepared by general procedure from 9 ml of 10% $PdCl_2$) for 2 h at room temperature. The catalyst was filtered off and propan-2-ol eas evaporated yielding 2.1 g (86%) of H-Asu(O*t*Bu)-OH (HPLC elution time 20.1 min in the gradient described above).

Fmoc-Asu(O*t*Bu)-OH (**5g**)

The same reaction and purification conditions as described with compound **5a** were used in the reaction of H-Asu(O*t*Bu)-OH (3.2 g, 13 mmol) with Fmoc-OSu (4.7 g, 13.9 mmol). The crude compound was purified on a Silica gel 60 column in the gradient of solvents EtOAc–PE yielding 4.3 g (71%) of pure derivative **5g**, m.p. 89–91 °C; $[\alpha]_D$ –16.9 (*c* 0.51, DMF). HPLC retention time was 41.6 min (0–100% ACN in 0.05% TFA, 60 min). TLC: R_F 0.78 (S_1), 0.32 (S_2), 0.85 (S_3), 0.54 (S_4), 0.39 (40% EtOAc in PE). ^{1}H NMR: 12.88 b, 1 H (carboxyl); 7.64 d, 1 H (NH, carbamate); 7.33 dt, 7.42 bt, 7.73 bd, 7.89 bd, 8 H (fluorene, arom.); 4.26 dd and 4.29 dd, 2 H (2 × CH_2O); 4.22 bt, 1 H (fluorene, alif.); 3.92 ddd, 1 H (α-CH); 2.17 bt, 2 H (ζ-CH_2); 1.67 m and 1.59 m, 2 H (β-CH_2); 1.47 m, 2 H (ε-CH_2); 1.38 s, 9 H ($C(CH_3)_3$); 1.26 m, 2 H (δ-CH_2). ^{13}C NMR: 174.17, 1 C (COOH); 172.44, 1 C (CO, ester); 156.34, 1 C (CO, carbamate); 144.05, 140.90, 127.81, 127.24, 125.48, 120.30, 12 C (fluorene, arom.); 79.55, 1 C (**C**$(CH_3)_3$); 65.74, 1 C (CH_2O); 53.88, 1 C (α-CH); 46.85, 1 C (fluorene, alif.); 34.85, 1 C (ζ-CH_2); 30.75, 1 C (β-CH_2); 28.07, 1 C (δ-CH_2); 27.94, 3 C (C(**C**$H_3)_3$); 24.58, 1 C (γ-CH_2); 25.38, 1 C (ε-CH_2). For $C_{27}H_{33}NO_6$ (467.6) calculated: 69.36% C, 7.11% H, 3.00% N; found: 68.96% C, 7.18% H, 3.13% N. FAB MS, *m*/*z*: 467.2 (M + 1).

Biological Assay

Uterotonic Activity Assessment

Compound **3a**, **3b** or **3c** (0.3 mg) was dissolved in 1 M NaOH (0.5 ml) and 1 M HCl was added dropwise until pH 6 of the solution was reached. Peptides were tested for uterotonic activity *in vitro* in the previously described rat uterotonic test for oxytocin according to Holton[32] in the Munsick[33] solution either in the absence of Mg^{2+} or in the presence of 1 mM Mg^{2+}. Synthetic endothelin was used as a standard for determination of both the agonistic and antagonistic activities. Female rats were estrogenized 24–48 h before the experiment. Cumulative dose response curves were constructed using data from experiments in which doses were added successively in double concentrations to the bath with an uterus strip in 1 min intervals without changing the fluid until a maximal response was obtained. The activity was determined by comparing the threshold doses of the standard and the respective analog (IU/mg or EC_{50}). In the case of antagonistic activity, the dose of the antagonists was applied to the bath with the uterus strip, 1 min prior to the standard dose of endothelin. The antagonistic activity was expressed as EC_{50} or pA_2, *i.e.* the concentration of the analogue, which reduced the effect of the double dose of agonist, in our case endothelin, to the effect of dose *x*, and the negative decadic logarithm of the EC_{50}, respectively. Each analog was tested on uteri from 3–5 different Wistar rats.

RESULTS AND DISCUSSION

In general, a combination of the solid phase and solution approaches has been chosen in the synthesis of ET-1 analogues **3a**–**3c** using strategies *A* and *B*.

The strategy *A* has followed a convergent approach consisting in segment condensation of the formerly prepared protected cyclic pentadecapeptide acids **1a**, **1b** and **2a**, **2b** with corresponding side-chain protected hexapeptides bound to resins **4a** and **4b** using the same coupling reagents and conditions.

However, the preparation of **1a**, **1b** was different from that of **2a**, **2b** (and consequently, the preparation of **4a** different from that of **4b**) as regards the peptide chain assembly and the side-chain protection. Compounds **1a**, **1b** and **4a** were built up on the mild-acid-labile resins with *t*Bu and Trt side-chain protection and the Fmoc-Cys-OAll (**5c**) or Fmoc-Hcy-OAll (**5f**) containing 1-carba(methylene-sulfanyl) or 15-carba(sulfanyl-methylene) linkages were inserted into corresponding peptide chains in the last solid-phase step. The following cyclization and the carboxy group deprotection were carried out in solution.

On the contrary, in preparation of **2a** and **2b** the peptide chains were built up on the strong acid or base-labile resin with a benzyl-type side-chain protection. The 1-carba or 15-carba linkages were inserted into peptide chains in the first coupling step when the corresponding compounds Fmoc-Cys($CH_2CH_2CH_2COO$*t*Bu)-OH (**5a**) or Fmoc-Hcy(CH_2CH_2COO*t*Bu)-OH (**5d**) Cs salts were coupled to the Merrifield resin. The final cyclization was performed on the resin and the C-terminal carboxy group deprotection was, in fact, identical with the cleavage of the protected cyclized peptides **2a**, **2b** from the resin.

The strategy *B* consisted in the step-wise construction of the whole peptide sequence from the C-terminal Trp residue bound to the Merrifield resin. The Fmoc-Cys($CH_2CH_2CH_2COO$*t*Bu)-OH (**5a**) or Fmoc-Hcy(CH_2CH_2COO*t*Bu)-OH (**5d**), and also Fmoc-Asu(O*t*Bu)-OH (**5g**) bearing the ethylene (1,15-dicarba) linkage, were coupled to the growing peptide chain in the seventh step. This synthetic procedure was similar to that utilized for peptides **2a**, **2b** and **4b** due to the same reagents and the same amino acid side-chain protection used. The only difference is the closure of carba bridges, performed in the last step of the syntheses on the polymer, just before the deprotection and detachment of the henicosapeptides from the resin.

The strategy *B* seems the most straightforward and the simplest one due to the overall shorter synthetic route. However, we observed a large decrease in substitution of growing peptide on the Merrifield resin after cou-

pling of the carba bridge containing compounds **5a**, **5d** and **5g**, respectively, in the seventh step. The decreased yields of the coupling were also observed when the compounds **5a** and **5d** as Cs salts were linked to the same type of the resin at the beginning of the syntheses of protected pentadecapeptides **2a** and **2b** when following the strategy *A*.

This disadvantage could be excluded by coupling of the 1-carba or 15-carba linkage containing compounds **5c** or **5f** *via* their side-chain carboxylic groups in the synthesis of protected cyclic pentadecapeptides **1a** and **1b** (strategy *A*). The reaction route was generally more complicated; however, it afforded better yields of solid-phase couplings and possibility to purify individual intermediates in solution after the pentadecapeptide sequences of the **1a** and **1b** were detached from the resin. A slight excess of hexapeptide bound resin **4a** and a larger excess of reagents was necessary for its satisfactory condensation with the protected acids **1a** and **1b**. In comparison with the strategy *B* and the strategy *A* where the cyclic protected pentadecapeptides **2a**, **2b** and hexapeptide bound resin **4b** were elaborated, the employment of the AcOH-labile 2-chlorotrityl resin (**1a** and **1b**) and the TFA-labile Wang resin (**4a**) allowed the usage of milder conditions for side-chain deprotection and detachment of the whole henicosapeptide sequences from the resin (**1c**, **1d**) without any damage to the peptide molecules.

For the oxidative closure of the inner disulfide bridge between Cys^3 and Cys^{11} residues, basically two approaches were utilized. The first one (procedure *A*) consisted in the oxidation by air oxygen in a highly diluted ammonium acetate–trifluoroacetate solution at pH 8, the second one (procedure *B*) used DMSO as a solvent and an oxidation reagent, as well. Even the second approach was found to be more efficient due to a shorter time of the oxidation, we have encountered well known problems with evaporation of DMSO and with a measurement of exact pH values in aqueous 80% DMSO.

Compounds **3a**–**3c** were subjected to a bioassay of a smooth muscle contraction and the results were compared with that obtained in application of ET-1. None of the compounds used, at any concentration, produced contraction of the rat uterus "*in vitro*". Nor was antagonistic activity detected for the three compounds tested. The results of the bioassay have confirmed a suggestion that α-amino group of ET-1 is a structural element essential for binding of the peptide to a corresponding receptor and for a transduction of biological activity. The deletion of this group in 1-deamino analogues **3a**–**3c** has caused the drop in the ET vasoconstrictory activity described already by Japanese authors[6] for [$Ala^{3,11}$,Asu^{15}]ET-1.

On the other hand, the substitution of the outer disulfide bridge by the sulfanyl-methylene (carba-1, carba-15) or ethylene (1,15-dicarba) bridges could be hardly responsible for such dramatic extinction of biological activity. The cyclic arrangement, suggested to be important for achieving a high receptor binding activity of ET-1, was also preserved in these carba analogues. It was even reported that the two ET-1 analogues, in which Cys residues of either one of the disulfide bonds have been replaced by Ala thus giving rise to a monocyclic analogue, still possess about 2 and 10% of the activity of ET-1 (refs[5,34]). These analogues readily bind to the rat cerebral membrane and are full agonists when tested with the rat aorta[35,36]. Cleavage of the Cys^3-Cys^{11} disulfide bond, and protection as a Cys(Acm) or, alternatively, reduction of both disulfides with subsequent carboxamidomethylation give rise to a weak partial agonist (with respect to ET-1) judging from the constriction test with porcine aorta[37]. Hence the decrease in biological activity seems to be rather due to the character of the protecting groups than to the reduction of disulfide bonds and the disulfide bond cannot be a necessary prerequisite for the activation and recognition of the receptor.

Therefore, the synthesis and bioassay of the ET-1 analogues with the cystathionine building block containing the carba linkage and bearing also the α-amino group, are necessary for finding the true effect of the carba substitution on the ET-1 conformation and for recognition of its receptors.

SYMBOLS

ACN, acetonitrile; AcOH, acetic acid; OAll, allyl ester; Asu, 2-aminosuberic acid; Boc, *tert*-butyloxycarbonyl; BOP, [(benzotriazol-1-yl)oxy]tris(dimethylamino)phosphonium hexafluorophosphate; BrZ, [(2-bromobenzyl)oxy]carbonyl; Bom, benzyloxymethyl; Bz, Benzyl; cHx, cyclohexyl; ClZ, [(2-chlorobenzyl)oxy]carbonyl; DCM, dichloromethane; DIC, *N*,*N*-diisopropylcarbodiimide; DIEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DMS, dimethyl sulfide; DMSO, dimethyl sulfoxide; EC_{50}, effective concentration; EDT, ethane-1,2-dithiol; ET, endothelin; EtOAc, ethyl acetate; Fmoc, (fluoren-1-yl-methoxy)carbonyl; For, formyl; HBTU, *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; Hcy, homocysteine; HOBt, 1-hydroxybenzotriazole; MeBzl, 4-methylbenzyl; MR, Merrifield resin; OSu, succinimide ester; PE, light petroleum; *t*Bu, *tert*-butyl; TBTU, *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TDM, 4,4′-bis(dimethylamino)diphenylmethane; TFE, 2,2,2-trifluoroethan-1-ol; TFMSA, trifluoromethanesulfonic acid; TFA, trifluoroacetic acid; TPTU, *O*-[2-(1*H*)-oxopyridin-1-yl]-1,1,3,3-tetramethyluronium tetrafluoroborate; Trt, triphenylmethyl; WR, Wang resin. The nomenclature and symbols of amino acids follow published recommendations of the IUPAC/IUB Joint Commission on Biochemical Nomenclature (*Eur. J. Biochem.* **1984**, *138*, 9).

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