Novel Synthesis of Cyclic Amide-Linked Analogues of Angiotensins II and III[†,‡]

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Cyclic amide-linked angiotension II (ANGII) analogues have been synthesized by novel strategies, in an attempt to test the ring clustering and the charge relay bioactive conformation recently suggested. These analogues were synthesized by connecting side chain amino and carboxyl groups at positions 1 and 8, 2 and 8, 3 and 8, and 3 and 5, N-terminal amino and C-terminal carboxyl groups at positions 1 and 8, 2 and 8, and 4 and 8, and side chain amino to C-terminal carboxyl group at positions 1 and 8. All these analogues were biologically inactive, except for cyclic [Sar[1],Asp[3],Lys[5]]ANGII (analogue **10**) which had high contractile activity in the rat uterus assay (30% of ANGII) and [Lys[1],Tyr(Me)[4],Glu[8]]ANGII (analogue **7**) which had weak antagonist activity ($PA_2 \approx 6$). Precyclic linear peptides synthesized using 2-chlorotrityl chloride resin and N^{α}-Fmoc-amino acids with suitable side chain protection were obtained in high yield and purity and were readily cyclized with benzotriazol-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate as coupling reagent. Molecular modeling suggests that the ring structure of the potent analogue can be accommodated in the charge relay conformation proposed for ANGII.

Introduction

The linear octapeptide angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe; ANGII), a potent pressor agent, has a vital role in the regulation of blood pressure. This hormone has been the subject of numerous studies since its discovery 50 years ago.[1] These investigations have included theoretical approaches,[2–6] physicochemical studies,[7–12] and spectroscopic examinations[13–23] in a variety of solvents of different dielectric constants. In addition, a large number of sterically restricted ANGII analogues have been synthesized in order to help establish the roles of the ANGII residues and the relationship between biological activity and conformation. This work has identified residues 2, 4, 6, and 8 for activity and 3, 5, and 7 for proper backbone orientation and has led to the proposal of several structural models for ANGII, including conformations which contain an α-helix, a β-turn, a γ-turn, an ion–dipole interaction, and a charge relay system.[7,9,13,17,23,24]

As a result of a high degree of flexibility, a peptide hormone in water readily interconverts between conformations so that an equilibrium exists in several different structural forms. However, as the hormone approaches the receptor, it presumably adopts a predominant conformation with much reduced flexibility. The issue therefore is to determine which of the conformations observed in solution best approximates the receptor-bound conformation. One way of probing the biologically active conformation is by using solvents of intermediate or low polarity, which allow a more ordered peptide structure. Current hypotheses emphasize the role of lipid-induced peptide folding in peptide hormone–receptor interactions.[25–27] Therefore the use of solvents of lower dielectric constant than H_2O ($\epsilon \sim 80$), such as DMSO ($\epsilon \sim 45$), can be justified.[28,29]

Another way of probing the receptor conformation of ANGII is via conformational restriction. Structural restrictions in the molecule afford analogues in which one or more conformational alternatives have been precluded. Several attempts to conformationally restrict ANGII via cyclization have been reported. Jorgensen and Patton were the first to our knowledge, to report synthesis of a cyclic ANGII analogue.[30] De Coen et al. published cyclization of [Asn[1],Val[5]]ANGII by forming an amide-linkage between the N- and C-termini to afford an inactive product.[30] Miranda and Juliano reported the synthesis of several analogues having a cystine moiety at various locations.[31] Most recently, Spear et al. reported the preparation of disulfide cyclic analogues of ANGII having high activity.[32] Cyclic disulfide analogues of [Sar[1],Ile[8]]ANGII with antagonist activity have been also recently reported.[33] Other cyclic ANGII analogues have been previously reported.[34,35] Our group has also previously reported the synthesis of [1,8-cysteine]ANGII.[36]

Our own interest in the renin–angiotensin system and the conformational model of ANGII, as well as our experience in the synthesis of acid-labile resins[37,38] and partially protected peptides,[39,40] has prompted us to undertake a systematic synthesis of cyclic amide analogues of ANGII. Cyclization was achieved by forming an amide-linkage between the $-NH_2$ and -COOH groups of Lys and Glu/Asp residues located at various positions

[†] Parts of this work are incorporated in the Ph.D. Dissertations of John Hondrelis and George Agelis.

[‡] Abbreviations used are in accordance with the rules of IUPAC–IUB Commission on Biochemical Nomenclature. *Eur. J. Biochem.* **1984**, *138*, 9–37; *J. Biol. Chem.* **1989**, *264*, 663–673. Other abbreviations: Ⓟ, poly(styrene); AcOH, acetic acid; EtOAc, ethyl acetate; DCM, dichloromethane; Et_2O, diethyl ether; TFE, 2,2,2-trifluoroethanol; DMF, *N,N*-dimethylformamide; *i*-PrOH, 2-propanol; TFA, trifluoroacetic acid; DIPEA, *N,N*-diisopropylethylamine; NMM, *N*-methylmorpholine; MeOH, methanol; BOC, (*tert*-butyloxy)carbonyl; *t*-Bu, *tert*-butyl; Bzl, benzyl; Trt, triphenylmethyl; Fmoc, (9-fluorenylmethoxy)carbonyl; Mtr, 4-methoxy-2,3,6-trimethylbenzenesulfonyl; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; Z, (benzyloxy)carbonyl; DCC, *N,N'*-dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; DMSO, dimethyl sulfoxide; Acp, aminocaproic acid; Des, amino acid omitted.

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Table 1. Protocol of the Solid-Phase Peptide Synthesis using N^{α}-Fmoc-amino Acids and 2-Chlorotrityl Chloride Resin[a]

	procedure	reagents/solvents	volume[a] (mL)	time (min)
1.	washing (3×)	dimethylformamide	10	2
2.	Fmoc deprotection	20% piperidine in dimethylformamide	10	30
3.	washing (3×)	dimethylformamide	10	2
4.	washing (3×)	2-propanol	10	2
5.	washing (3×)	dimethylformamide	10	2
6.	coupling	2.5 equiv of Fmoc-amino acid, 3.75 equiv of HOBt, and 2.75 equiv of DCC in 8 mL of dimethylformamide	10	150
7.	washing (3×)	dimethylformamide	10	2
8.	washing	2-propanol	10	2
9.	washing (3×)	dimethylformamide	10	2
10.	washing (3×)	2-propanol	10	2

[a] Quantities are relative to 1 g of resin.

of the ANGII precyclic analogue. Cyclization was also possible by connecting NH_2/NH and COOH termini groups. The synthesis of suitably protected linear precyclic analogues was possible by Fmoc methodology and use of the acid-sensitive 2-chlorotrityl chloride resin. Novel strategies were followed for obtaining the several cyclic products.

Chemistry

All peptides described here were synthesized by solid-phase peptide synthesis methodology as described by Barlos et al.[37-40] using N^{α}-Fmoc-amino acids and the 2-chlorotrityl chloride resin. Attachment of the first amino acid to the resin from the α-carboxyl or the side chain amino or carboxyl group was achieved by a simple, fast, and racemization-free reaction using diisopropylethylamine (DIPEA) in dichloromethane (DCM) solution at room temperature. Suitable protecting groups compatible with the desired synthesis were used for all trifunctional amino acids. Every step of the solid-phase peptide synthesis can be easily monitored by TLC after treatment of an aliquot of the peptide–resin with 2–3 drops of DCM–AcOH–TFE (7:1:2) using the appropriate TLC solvent systems (see the Experimental Section). In this paper, we present the synthesis of ANGII amide cyclic analogues containing bridges of different sizes. These analogues have been synthesized through novel strategies in an attempt to test the charge relay system conformation which has been recently suggested.[24,28,29]

Cyclization of the peptides, in solution, by connecting an amino with a carboxyl group was attempted by both DCC/HOBt and BOP coupling agents. The cyclization reaction using DCC (2 equiv) and HOBt (4 equiv) in DMF proceeded sluggishly and required prolonged time (5–7 days), and the yield was very poor (~15%). The cyclic product was also contaminated with other side products as shown by TLC and HPLC. However, the BOP reagent[44] (benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate) is ideally suited for solid-phase peptide synthesis as the yield and the rate of coupling are higher, when compared to other coupling agents.[45] The effective use of the BOP reagent in side chain to side chain solid-phase peptide cyclization[46] prompted us to evaluate the use of this coupling reagent in a variety of ANGII cyclization reactions: head to tail (analogues **1–3**), side chain to side chain (analogues **4–10**), and side chain to C-terminal carboxyl group (analogue **11**). Purification of the final product was achieved by HPLC, and identity was confirmed by amino acid analysis and FAB MS.

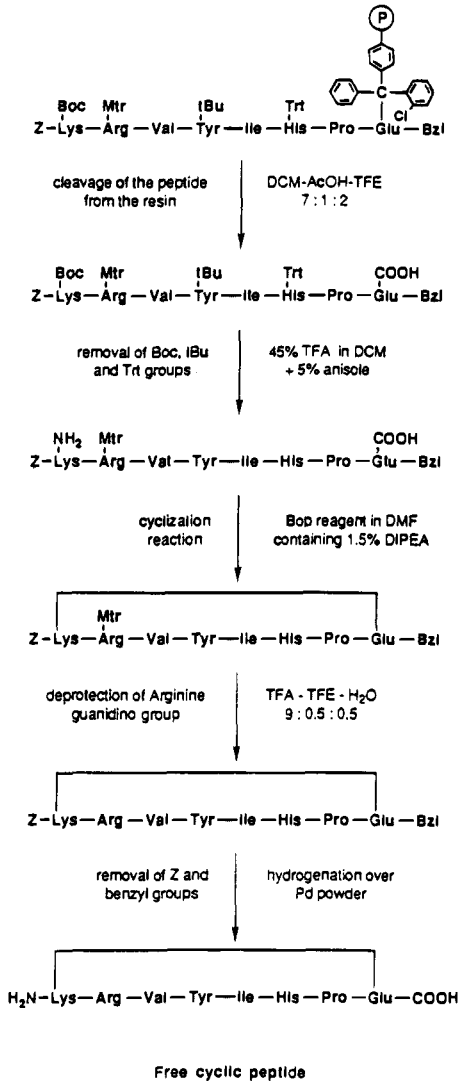
When linear angiotensin precyclic analogues were side chain to side chain cyclized, the α-carboxyl group of the C-terminal amino acid was protected as the benzyl ester (analogues **4–10**). Thus, a free C-terminal carboxyl group, important for biological activity, was available after the final hydrogenolysis step. The BOC group was used for the protection of α-amino groups in cases of head to tail cyclization (analogues **1–3**), while the (benzyloxy)carbonyl (Z) group was used in cases of side chain to side chain and side chain to C-terminal carboxyl group cyclizations (analogues **4–11**). According to our experimental data, the Z group is a better protecting group than the [(9-fluorenylmethyl)oxy]carbonyl (Fmoc) group for the cyclization reaction, mostly for steric reasons.

Three strategies were followed for the synthesis of the cyclic analogues described herein.

I. The cyclic peptides **1–8** and **11** were synthesized as outlined in Scheme 1. The linear precursor peptides were synthesized stepwise by Fmoc solid-phase peptide synthesis. Cleavage of the finished peptide from the 2-chlorotrityl chloride resin using a mixture of DCM–AcOH–TFE (7:1:2) gave a fully protected peptide which was purified by flash column chromatography. Treatment of the protected peptide with trifluoroacetic acid in dichloromethane containing anisole as scavenger resulted in a partially protected peptide with the desired amino and carboxyl groups available for cyclization. In the cases of cyclic analogues **1–3**, cyclization was attempted between the C-terminal carboxyl group and the N-terminal amino group using BOP reagent in a large volume of DMF containing excess of DIPEA. In the cases of cyclic compounds **4–8**, cyclization was carried out by the same method, between the ε-amino group of lysine and the γ-carboxyl group of glutamic acid. Finally in the case of cyclic peptide **11**, cyclization was performed as above between the C-terminal carboxyl group of phenylalanine and the side chain amino group of lysine. Acidolysis of the Mtr or Pmc side chain protecting group of arginine with TFA in the presence of scavenger followed by hydrogenolysis over Pd to free the α-amino terminus (analogues **4–8** and **11**) from the carbobenzoxy group and the C-terminus from the benzyl group (analogues **4–8**) afforded the final free cyclic product. Purification was achieved by HPLC.

II. The synthesis of cyclic analogue **9** was carried out as shown in Scheme 2. The linear dipeptide BOC-Acp-Lys(N^{α}-Fmoc)-OBzl was synthesized in solution in good overall yield by the mixed anhydride method, employing N^{α}-Fmoc protection for lysine, (*tert*-butyloxy)carbonyl protection for the amino group of aminocaproic acid (Acp), and esterification of the α-carboxyl group of lysine with the benzyl group. The dipeptide was then attached

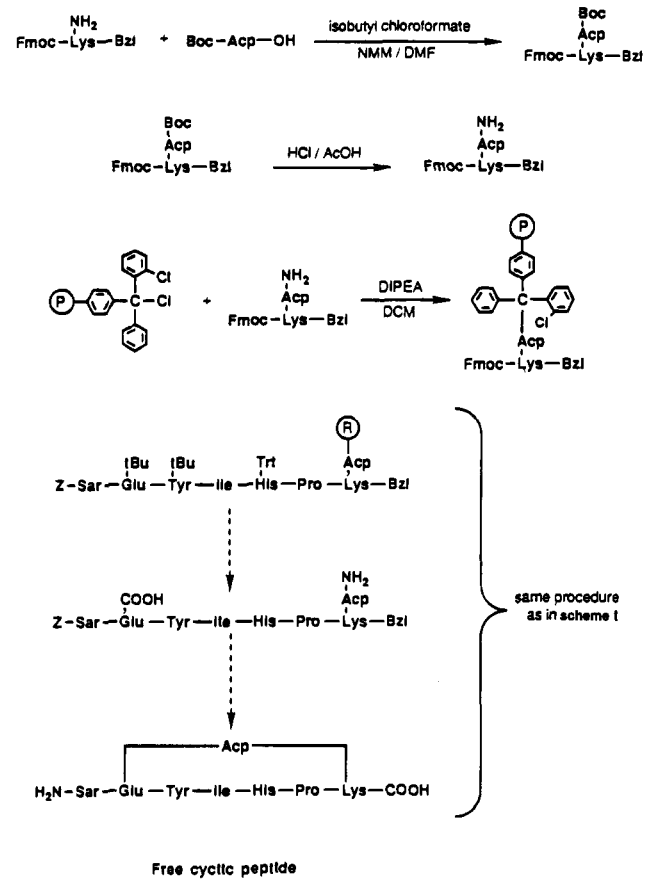
Scheme 1. General Synthetic Procedure for Cyclic Analogues **1–3** (Head to Tail Cyclization), **4–8** (Side Chain to Side Chain Cyclization), and **11** (Side Chain to C-Terminal Tail Cyclization)



to the resin via the amino group of Acp and the octapeptide synthesized stepwise by solid-phase peptide synthesis. After the cleavage of the finished peptide from the resin, the same procedure as above was followed to obtain the final cyclic product.

III. A slightly different strategy was followed for the synthesis of cyclic analogue **10** (Scheme 3). The octapeptide precursor was synthesized stepwise by Fmoc solid-phase peptide synthesis. The completed linear peptide was cleaved from the resin using the DCM–AcOH–TFE (7:1:2) cleavage solution and purified by flash column chromatography. The C-terminal carboxyl group of the peptide was esterified with the benzyl group using benzyl bromide and cesium carbonate or triethylamine in DMF at room temperature. The fully protected peptide was purified by flash column chromatography. Treatment of the pure peptide with trifluoroacetic acid in dichloromethane, containing anisole as scavenger, resulted in a partially protected peptide with the ϵ-amino group of lysine and the β-carboxyl group of aspartic acid to be free. Application of the same procedure as in Schemes **1** and **2** resulted in the final free cyclic (3^{β}-5^{ϵ})-[Sar^1,Asp^3,Lys^5]ANGII analogue which was found biologically active in the rat uterus.

Scheme 2. Synthetic Procedure for Cyclic Analogue **9**

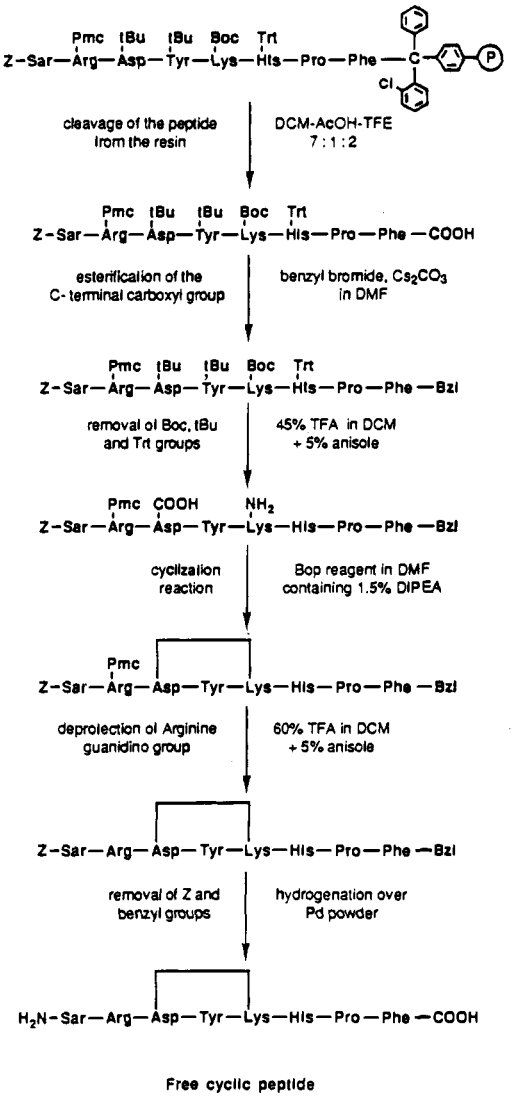


Results and Discussion

Tables 2 and 3 show the chemical data and biological activities, respectively, of the various angiotensin II cyclic analogues. Schemes **1–3** describe the routes which were followed for the synthesis of the cyclic analogues. Scheme **1** shows the synthesis of cyclic analogues in which the residue at position 8 is involved in cyclization. However only analogues **4–8** provide a free terminal carboxylate. All of these analogues have been found to be inactive (Table 3). Scheme 2 shows the synthesis of the cyclic nonapeptide (3^{γ}-Acp-8^{ϵ})-[Des^1,Sar^2,Glu^3,Lys^8]ANGII (analogue **9**). Acp was used to connect the side chains at positions 3 (Glu) and 8 (Lys) in order to increase the ring size of the [Des^1,-Sar^2,Glu^3,Lys^8]ANGII analogue and subsequently increase the number of ring conformers (one of which might be active). However this analogue was also found to be inactive. Scheme 3 shows the synthesis of the cyclic analogue [Sar^1,Asp^3,Lys^5]ANGII which is a potent agonist (Table 3). Cyclization by connecting residues 3 and 5 allowed appropriate conformational constraints which, according to molecular modeling, should not disturb the ring clustering important for the formation of the charge relay system which activates the receptor. Application of molecular orbital calculations using the AM1 Hamiltonian (MOPAC 5.0) with full geometry optimization based on line minimization algorithms[56] indicated that the proposed[28,29,50] "charge relay" conformation represents a valid candidate for the receptor-bound conformation of angiotensin II and related analogues.[53,55]

As shown in Table 3, cyclic analogues of ANGII have been synthesized by connecting:

Scheme 3. Synthetic Procedure for Cyclic Analogue **10**



(a) the C-terminal carboxyl group of Phe^8 with a N-terminal group at positions 1, 2, and 4,

(b) the C-terminal γ-carboxyl group of Glu or the ϵ-amino group of Lys with side chain amino or carboxyl groups at positions 1 and 2, thus retaining the C-terminal carboxylate, and

(c) the Phe C-terminal carboxylate to the ϵ-amino group of Lys.

In particular, cyclization of N- and C-termini of the superagonist [Sar^1]ANGII resulted in cyclic [Sar^1,Phe^8]-ANGII (analogue **1**) which was found to be inactive as agonist or antagonist. This is an expected result since the C-terminal carboxylate is considered to be an important element of the charge relay system believed to be present in angiotensin II.[28,50] Replacement of Sar in [Sar^1]ANGII with the longer chain Acp and cyclization of the N- and C-termini resulted in cyclic [Acp^1,-Phe^8]ANGII (analogue **2**) which was also found to be inactive. Cyclization of the pentapeptide fragment [Des^{1,2,3}]ANGII which retains the active site of ANGII resulted in cyclic [Des^{1,2,3},Phe^8]ANGII (analogue **3**) which also was found to be inactive as agonist or antagonist.

We pursued the synthesis of some Phe^8 C-terminal-cyclized compounds to test the cyclization procedure under the applied conditions in a number of linear angiotensin II precursors of different lengths. Furthermore we were interested in comparing the conformation of those analogues with the conformation of ANGII and active analogues characterized by ring cluster essential for activity,[28,29] in order to refine the proposed model.

Since the C-terminal carboxylate is essential for activity, we attempted to prepare cyclic analogues of ANGII retaining the C-terminal carboxyl. Thus, replacement of residues 1 and 8 of ANGII with Lys and Glu, respectively, provided the necessary NH_2 and COOH side chain functional groups to form the desired amide linkage. This synthesis was possible by attachment of Fmoc-Glu(COOH)-OBzl to the resin through the γ-carboxyl group and then stepwise Fmoc synthesis and finally coupling of Z-Lys(BOC)-COOH to the N-terminus of the heptapeptide–resin. Subsequent splitting of the protected peptide from the resin with the mild cleavage solution DCM–AcOH–TFE (7:1:2), partial deprotection with TFA–CH_2Cl_2 (1:1), and cyclization between Glu γ-COOH and Lys ϵ-NH_2 groups provided a partially protected cyclic analogue, [Z-Lys^1,Glu–OBzl^8]ANGII. Hydrogenolysis afforded the free cyclic analogue [Lys^1,-Glu^8]ANGII (analogue **6**), which was found to be inactive. Lack of agonist activity may relate to the known importance of the aromatic side chain at the C-terminus. Furthermore, 1,8 cyclization may result in a disruption of the Tyr/His proximity, which is believed to be important for the formation of the tyrosinate species which triggers activity.

Methylation of the tyrosine hydroxyl of the cyclic octapeptide [Lys^1,Glu^8]ANGII and the cyclic heptapeptide [Des^1,Lys^2,Glu^8]ANGII resulted in cyclic analogues [Lys^1,Tyr(Me)^4,Glu^8]ANGII and [Des^1,Lys^2,Tyr(Me)^4,-Glu^8]ANGII which also were found to lack agonist activity, although the former retained measurable antagonist activity (analogues **7** and **8** in Table 3). The synthesis of Tyr(Me) cyclic analogues was prompted by our findings that methylation of the Tyr hydroxyl in [Sar^1]ANGII results in a potent competitive ANGII antagonist.[47–49]

In another series of cyclic analogues, Lys was the C-terminal residue while Glu was the N-terminal. This was possible by attachment of Fmoc-Lys(NH_2)-OBzl to the resin through the ϵ-amino group of Lys and then stepwise Fmoc synthesis and final coupling of Z-Glu(*t*-Bu)COOH to the N-terminus of the peptide–resin chain. Subsequent splitting of the protected peptide from the resin with 35% TFA in DCM, partial deprotection with TFA, and cyclization between Lys ϵ-NH_2 and Glu γ-COOH groups provided a partially protected analogue, [Des^1,Z-Glu^2,Lys-OBzl^8]ANGII. Hydrogenolysis afforded the free cyclic analogue [Des^1,Glu^2,Lys^8]ANGII (analogue **4**), which was found to be inactive. Lack of activity may be due to the absence of an aromatic ring at position 8 and the disruption of the Tyr/His proximity, imposed by the 1.7 cyclization.

Only a few syntheses of ANGII amide cyclic analogues have been reported.[30,34,35] Synthesis of these analogues has proven to be a difficult task due to (a) steric effects, (b) polymerization, and (c) use of inappropriate resins and protective groups during the solid-phase peptide synthesis of the linear precursor. We believe that the choices of the right protection for each residue and especially of the right resin for the synthesis of the linear precursor are the most important aspects of the

Table 2. Chemical Data on ANGII Amide-Linked Cyclic Analogues[a]

analogue	TLC, R_f		amino acid analysis									
	BPAW	CMAW	Asp	Arg	Val	Tyr	Ile	His	Pro	Phe	Glu	Lys
(1) cyclo-(1−8)-[Sar^1]ANGII	0.57	0.42	–	1.00	0.90	0.95	0.84	0.93	1.04	1.20	–	–
(2) cyclo-(1−8)-[Acp^1]ANGII	0.55	0.43	–	1.08	0.89	0.77	0.89	0.86	1.00	0.88	–	–
(3) cyclo-(4−8)-[Des^{1,2,3}]ANGII	0.55	0.41	–	–	–	0.87	0.84	0.74	1.00	0.88	–	–
(4) cyclo-(2$^\gamma$-8$^\epsilon$)-[Des^1,Glu^2,Lys^8]ANGII	0.59	0.39	–	–	0.93	0.94	0.84	0.87	1.00	–	0.97	1.09
(5) cyclo-(2$^\epsilon$-8$^\gamma$)-[Des^1,Lys^2,Glu^8]ANGII	0.58	0.39	–	–	0.85	0.87	0.79	0.79	1.00	–	0.92	0.93
(6) cyclo-(1$^\epsilon$-8$^\gamma$)-[Lys^1,Glu^8]ANGII	0.59	0.46	–	1.00	1.08	0.81	0.90	0.83	0.94	–	0.91	0.97
(7) cyclo-(1$^\epsilon$-8$^\gamma$)-[Lys^1,Tyr(Me)^4,Glu^8]ANGII	0.58	0.39	–	1.00	0.89	0.72	0.90	0.82	1.00	–	0.95	0.94
(8) cyclo-(1$^\epsilon$-8$^\gamma$)-[Des^1,Lys^2,Tyr(Me)^4,Glu^8]ANGII	0.56	0.43	–	–	0.90	0.83	0.95	0.71	1.00	–	0.93	0.79
(9) cyclo-(3$^\gamma$-Acp-8$^\epsilon$)-[Des^1,Ser^2,Glu^3,Lys^8]ANGII	0.55	0.48	–	–	–	0.91	0.87	0.89	1.00	–	0.93	0.94
(10) cyclo-(3$^\beta$-5$^\epsilon$)-[Sar^1,Asp^3,Lys^5]ANGII	0.56	0.50	1.02	1.00	–	0.85	–	0.82	0.92	1.30	–	0.93
(11) cyclo-(1$^\epsilon$-8)-[Lys^1]ANGII	0.65	0.43	–	1.04	0.80	0.71	0.90	0.78	1.00	0.94	–	1.05

[a] Conversion of Tyr(Me) to Tyr during acid hydrolysis is not always quantitative. N-Methylated amino acids give very low color yields by amino acid analysis and could not be reliably estimated.

Table 3. Rat Uterus Biological Activities of ANGII Amide-Linked Cyclic Analogues[a]

analogue	agonist activity (% of ANGII)	antagonist activity (pA_2)
A. N-Terminal Amino Group to C-Terminal Carboxyl Group		
(1) [Sar^1,Phe^8]ANGII	<0.1 (3)	<5.0 (3)
(2) [Acp^1,Phe^8]ANGII	<0.1 (4)	<5.0 (3)
(3) [Des^{1,2,3},Tyr^4,Phe^8]ANGII	<0.1 (2)	<5.0 (2)
B. Side Chain to Side Chain		
(4) [Des^1,Glu^2,Lys^8]ANGII	<0.1 (3)	<5.0 (2)
(5) [Des^1,Lys^2,Glu^8]ANGII	<0.1 (2)	<5.0 (2)
(6) [Lys^1,Glu^8]ANGII	<0.1 (3)	<5.0 (3)
(7) [Lys^1,Tyr(Me)^4,Glu^8]ANGII	<0.1 (3)	6.0 (3)
(8) [Des^1,Lys^2,Tyr(Me)^4,Glu^8]ANGII	<0.1 (4)	<5.0 (3)
(9) [Des^1,Sar^2,Glu^3,Lys(Acp)^8]ANGII	<0.1 (3)	<5.0 (2)
(10) [Sar^1,Asp^3,Lys^5]ANGII	30 (5)	
C. Side Chain to C-Terminal Carboxyl Group		
(11) [Lys^1,Phe^8]ANGII	<0.1 (4)	<5.0 (5)

[a] Values are given as mean ± SEM (number of experiments) with human ANGII as standard. Abbreviations are standard except: Acp, 6-aminocaproic acid; Des, amino acid omitted.

procedure. We have found that the acid-sensitive 2-chlorotrityl chloride resin is an excellent one for pursuing cyclic products. The merits of this resin have been recently reported by Barlos et al.[37−40]

This resin allows splitting of the peptide from the resin by mild conditions, DCM−AcOH−TFE (7:1:2), affording a clean product in high yield and all protecting groups attached. In a few cases of splitting, part of the His residue was deprotected from the trityl group and this peptidic side product (15−20%) was removed by flash column chromatography. Selective deprotection of the clean linear precursor by TFA−DCM (1:1) at the residues to be interconnected allows the ready cyclization using the BOP coupling agent. If the cyclic product is still partially protected by carbobenzoxy (at the N-terminus) or benzyl (at the C-terminal residue), hydrogenolysis affords the final free cyclic product.

Conformation. We have recently suggested a conformational model for ANGII characterized by clustering of aromatic rings and a charge relay system involving the triad Tyr hydroxyl−His imidazole−Phe carboxylate.[24,28,29] This model was based on structure−activity studies, NMR studies (1D-NOE, 2D-ROESY), and nanosecond-resolved tyrosinate fluorescence lifetime studies.[24,28,29,48,50,51] Activities and NMR data for a series of recently synthesized linear ANGII analogues, in which position 7 was occupied by secondary cyclic amino acids of variable size (Aze, Pro, Pip), supported the proposed model.[52] Distance geometries based on NOE data and molecular dynamics for [Sar^1]ANGII have furthermore supported this conformation.[53] For this reason, we have been interested in the synthesis of cyclic ANGII analogues which would enable us to test this model.

Cyclization of peptides is believed to restrict the number of possible conformations that a peptide can assume and has proved to be a very valuable tool in the design of inhibitors with resistance to metabolic degradation in other systems. However, this approach requires at least two structural changes to be made within a single analogue, and it is important that the two residues to be connected should be the least important for biological activity. For ANGII, structure−activity studies have shown that the functional groups of residues 2, 4, 6, and 8 appear to be important for biological activity.[53,55] Replacement of these residues with other functional residues (Cys, Asp, Lys), therefore, should result in substantial loss of agonist activity, even if the backbone conformation closely approximates that of the natural product. However, uncharged nonaromatic amino acids, at position 8, produce type I antagonists with high receptor affinity, and the lack of activity of cyclic analogues with cross-linked Glu or Lys at position 8 must be due to disruption of the bioactive conformation induced by cross-linking (molecular modeling suggests disruption of Tyr/His functional groups in particular). On the other hand, residues 3, 5, and 7 seem to play an important role for the proper backbone conformation, and the steric influence of these side chains appears to function to properly orient the functional groups at the active center of the hormone for effective receptor−ligand interaction.[53,55] If true, then conformational restrictions imposed by replacement of two of these amino acids, with residues providing functional side chains for intramolecular linkage (Lys and Glu/Asp or vice versa), could still afford active analogues. This is particularly applicable to Val^3 and Ile^5 whose side chains are expected to exist on the other side of the molecule from the functionally important aromatic side chains.[28] Indeed, cyclic [Sar^1,Asp^3,Lys^5]-ANGII was found to be a potent agonist (30% of ANGII). In this regard, ring size could be a critical factor for maximum activity. On the other hand, cyclic analogues

cyclized at positions which bear side chains important for activity (residues 2, 4, and 8) have been found inactive. These data emphasize the role of residues 4, 6, and 8 which, at solvents of low or intermediate polarity such as DMSO (representing the receptor lipophilic environment),[25-27] are closely spaced allowing the formation of tyrosinate species in the proposed charge relay system mechanism for receptor activation.

Conclusion. Use of the novel 2-chlorotrityl resin and application of the Fmoc methodology allowed an efficient stepwise synthesis of linear ANGII peptide fragments with suitable side chain protection at the residues to be amide bond linked during the cyclization step. A TFA-free mild cleavage solution was used to remove the fully protected peptide from the resin. Selective deprotection of the residues to be cross-linked allowed for efficient cyclization with BOP reagent.

Cyclization of ANGII across residues 3 (Asp) and 5 (Lys) has resulted in a potent cyclic analogue revealing structural requirements necessary for activity. Apparently the side chains of residues 3 and 5 are not important for activity and exist on the other side of the molecule from the functionally important aromatic side chains, and this structure can be accommodated in the charge relay conformation proposed for ANGII. The absence of bioactivity for the remaining cyclic analogues synthesized in this study could derive from a number of factors, including (1) inappropriate conformation, (2) neutralization of functional groups (e.g., the carboxylate groups in analogues **1–3** and **11** and the Phe[8] ring in analogues **4–9**), and (3) imposition of a bridging group which prevents appropriate access of ligand to receptor. On the basis of molecular modeling considerations, we believe that (3) largely explains why analogues **4–9** do not demonstrate significant antagonist activity. The fundamental conclusion arising from this work is that cross-bridging is only possible between positions 3 and 5 and not between positions 1 and 8, 2 and 8, 3 and 8, and 1 and 5.

This research is aiming at refining the charge relay system conformational model for ANGII which has been recently proposed on the basis of structure–activity relationships, nuclear magnetic resonance, and fluorescence lifetime studies. Additional structure–activity studies to further explore the effects of cyclization between 3 and 5 residues in terms of ring size and location of the side chains to be amide bond linked will permit a more detailed analysis of the structural requirements for maximum receptor–ligand interaction. In addition we anticipate that detailed analysis of constrained cyclic ANGII analogues using modern two-dimensional NMR techniques will allow for a better understanding of the ANGII conformation.

Experimental Section

FAB MS. The identity of cyclic products was established by FAB MS. FAB spectra were run on a AEI M29 mass spectrometer modified as described elsewhere.[54] FAB gun was run at 1 mA discharge current at 8 kV. The FAB matrix used was a mixture of dithiothreitol/dithioerythritol (6:1) (Cleland Matrix).

TLC. The purity of all products (cyclic and linear) was established also by thin layer chromatography (TLC). TLC was carried out with precoated silica gel on glass (Merck Kieselgel 60 F254) TLC plates. The following solvent systems were used, (A) $CHCl_3$–AcOH–MeOH (9:0.3:1), (B) $CHCl_3$–AcOH–MeOH (8:1:2), (C) *n*-BuOH–pyr–AcOH–H_2O (30:20:6:24), (D) *n*-BuOH–AcOH–H_2O (4:1:1), (E) $CHCl_3$–AcOH–MeOH (8:0.5:2), (F) $CHCl_3$–AcOH–MeOH (9.5:0.05:2.8), (G) $CHCl_3$–MeOH (6:1), (H) $CHCl_3$–MeOH (9:1), (I) toluene–AcOH–MeOH (7:1.5:1.5), (J) toluene–AcOH–MeOH (8:2:0.5), (K) hexane–EtOAc (7:3), (L) $CHCl_3$–MeOH (9.5:0.5), (M) *n*-BuOH–pyr–AcOH–H_2O (15:10:3:6) (BPAW), and (N) $CHCl_3$–MeOH–AcOH–H_2O (15:10:2:3) (CMAW).

Amino Acid Analysis. Amino acid analysis was performed on a Beckman 6300 high-performance analyzer. Compositional analysis data were collected from 6 N HCl hydrosylates (110 °C, 18 h) with ninhydrin-based analysis.

Preparative Reversed-Phase HPLC. A Varian HPLC system equipped with a Vista 401 microprocessor controller was used for purification of peptides. Separations were achieved on a Bio-Rad Hi-Pore 318 reversed-phase preparative column (25.0 × 2.15 cm^2) at 25 °C using a stepped linear gradient of acetonitrile in 0.1% CF_3COOH at a flow rate of 7.5 mL/min. A nitrogen pressurized rheodyne injector with a 2.0 mL sample loop was used for automated repetitive injections of peptides (5 × 5 mg). One-fifth of the total sample was injected during each run by lowering the flow rate from 7.5 to 4.0 mL/min for a 0.1 min "inject" period. One cycle consisted of the following events: 0 → 10 min, 7.5 mL/min, 90% H_2O/10% of 1% aqueous CF_3COOH; 10 → 11 min, 4.0 mL/min; 11 → 11.1 min, "inject"; 11.1 → 13 min, 7.5 mL/min, 70% H_2O/20% CH_3CN/10% of 1% CF_3COOH; 13 → 30 min, 45% H_2O/45% CH_3CN/10% of 1% CF_3 COOH; 30 → 42 min, 90% CH_3CN/10% of 1% CF_3COOH; 42 → 50 min, 100% H_2O.

A Gilson Model 201 fraction collector was used to collect fractions at 0.1 min intervals. The collector was programmed to collect for a 5 min period centered around the elution time, 23–27 min, of the major product. The Vista 401 microprocessor controller restarted the fraction collector at the beginning of each HPLC run so that the material eluting with the same retention time was repeatedly deposited in the same tuber. The peptide was detected simultaneously from the absorbances at 254 nm (Varian UV-1) and 230 nm (Kratos SF 769Z). Fractions containing the required peptide were pooled, and CH_3CN was removed on a rotary evaporator at 40 °C. The fractions were lyophilized and stored at −20 °C.

Rat Uterus Bioassay. Uterine horns from diethylstilbestrol-primed virgin Sprague–Dawley rats (150–250 g) were defatted and cut in half. Each tissue was suspended under 1 g of tension in a 3 mL tissue bath containing 150 mM NaCl, 5.6 mM KCl, 0.18 mM $NaHCO_3$, and 1.4 mM glucose at pH 7.0 gassed with oxygen. Contractions were monitored with Gould Metripak 763341–4202 isotonic transducers coupled to Gould 13–4615–50 transducer amplifiers housed in a Gould 2600s recorder. Agonist activity was determined by matching the response with an equivalent response to ANGII (human). Antagonist potencies were determined as the minimum effective concentration, i.e., the minimum concentration of antagonist required to completely block the response to an ED_{75} dose of ANGII.

Peptide Synthesis. All trifunctional amino acids were suitably protected. The ω-carboxyl groups of aspartic and glutamic acids and the hydroxyl group of tyrosine were protected with the *tert*-butyl (*t*-Bu) group. The ε-amino group of lysine was protected with the (*tert*-butyloxy)carbonyl (BOC) group. The histidine imidazole ring was protected with the triphenylmethyl (Trt) group[43] and the guanidino function of arginine with the 4-methoxy-2,3,6-trimethylbenzenesulfonyl (Mtr) group[41] or with the more acid-labile 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) group.[42]

Solid-phase peptide synthesis was carried out on 2-chlorotriphenylmethyl (trityl) resin (1.4–1.6 mmol of Cl^-/g) using a manually handled reaction vessel (2 × 12 cm^2) equipped with a porous G filter (size 2) and a tap at the bottom connected with a water aspirator. A vibrator was used for shaking the reaction vessel throughout the several steps. The protocol used for the peptide synthesis is shown in Table 1.[39] One coupling step with 2.5 equiv of N^{α}-Fmoc-protected amino acid and coupling agent were usually employed for each amino acid; the coupling was mediated by *N,N'*-dicyclohexylcarbodiimide (DCC) in the presence of 1-hydroxybenzotriazole (HOBt) for

150 min. Completion of couplings was verified by the ninhydrin test. A second coupling under the same conditions was employed in cases of incomplete coupling. Removal of the Fmoc protective groups was carried out by incubation in 20% piperidine in DMF. Splitting of the peptides from the 2-chlorotrityl chloride resin, with all protecting groups attached, was effected by treating the peptide–resin with the splitting solution: dichloromethane–acetic acid–2,2,2-trifluoroethanol (DCM–AcOH–TFE, 7:1:2) for 1 h at room temperature. Cleavage of the linear precursor of cyclic analogue **4** (Table 3), attached to the resin through the Lys ϵ-amino group, required stronger acidic conditions: 35% TFA in DCM.

The final protected linear peptide after the cleavage was easily purified by flash column chromatography using silica gel and a suitable (TLC) solvent system. In all cases, treatment of the received protected linear peptide with trifluoroacetic acid in dichloromethane in the presence of scavengers resulted in a partially protected peptide. In the case of side chain to side chain cyclization (analogues **4–10**), treatment with the above mixture resulted in a partially protected peptide with the ϵ-amino group of lysine and the ω-carboxyl groups of aspartic and glutamic acids free. In the case of side chain to C-terminal carboxyl group cyclization (analogue **11**), this treatment resulted in a partially protected peptide with the ϵ-amino group of lysine free, while the same treatment in the case of head to tail cyclization (analogues **1–3**) resulted in the deprotection of the α-amino group.

The cyclization reaction was carried out using BOP reagent (3 equiv) in DMF, containing excess of DIPEA, and afforded relatively pure cyclic products and good yields (65–70%) in overnight excperiments (12–19 h). Cyclization reactions were followed by TLC using the systems: *n*-BuOH–AcOH–H_2O (4:1:1) and $CHCl_3$–MeOH (6:1).

Before cyclization, we neutralized the acetate or TFA peptide salt with triethylamine in DMF and precipitated the neutralized lipophilic peptide with H_2O. Alternatively we lyophilized the acetate peptide from 1% HCl solution. HCl, as a stronger acid, readily replaced TFA and CH_3COOH.

Deprotection of the guanidino group of arginine (1) for Mtr, by treatment with 90% trifluoroacetic acid in dichloromethane containing 5% H_2O and 5% TFE as scavengers, and (2) for Pmc, by treatment with 60% trifluoroacetic acid in dichloromethane containing 5% anisole as scavenger, and hydrogenation over Pd, to free the α-amino and the C-carboxyl terminus from the (benzyloxy)carbonyl and benzyl groups correspondingly gave the final cyclic peptide.

2-Chlorotrityl chloride resin, Fmoc-His(Trt)-OH, Fmoc-Tyr(*t*-Bu)-OH, and Fmoc-blocked amino acids were purchased from C.B.L. (Chemical and Biopharmaceutical Laboratories) of Patras or synthesized by methods described elsewhere. Fmoc-Arg(Mtr)-OH, Fmoc-Arg(Pmc)-OH, Z-Lys(BOC)-OH, Z-Glu(*t*-Bu)-OH, Fmoc-Lys(BOC)-OH, and Fmoc-Glu(*t*-Bu)-OH were purchased from Bachem Inc. or Nova-Biochem. BOP reagent was purchased from Richelieu Biotechnologies Inc. BOC-Sar-OH and BOC-Acp-OH were purchased from Chemalog.

Sequence of Peptide 10. Sequence of active peptide **10** was carried out on an Applied Biosystems 470A protein sequencer, identifying wanted product. Sequencing was done through four cycles, and the following were identified at each step: step 1, Sar; step 2, Arg; step 3, blank; step 4, Tyr. The yields of Sar, Arg, and Tyr were similar.

Synthetic Procedure: 1. **Synthesis of Cyclo-(1–8)-[Sar^1]ANGII (Analogue 1). I. Preparation of N^α-Fmoc-Phe-2-chlorotrityl Chloride Resin.** 2-Chlorotrityl chloride resin (1 g, 1.4–1.6 mequiv of Cl^-/g of resin) in dry dichloromethane (10 mL) was stirred in a round bottom flask. Diisopropylethylamine (DIPEA) (0.545 mL, 3.2 mmol) and N^α-Fmoc-Phe-OH (387 mg, 1 mmol) were added, and the solution was stirred for 20 min at room temperature. A mixture of MeOH (3 mL) and DIPEA (0.5 mL) was then added and the mixture stirred for another 10 min at room temperature. The Fmoc-Phe-resin was filtered, subsequently washed with DMF (3 × 10 mL), DCM (2 × 10 mL), *i*-PrOH (2 × 10 mL), and Et_2O (2 × 10 mL), and dried in vacuo for 24 h at room temperature. The loading of the amino acid/g of substituted resin was 0.70 mmol/g (calculated by quantitative Kaiser test).

II. Preparation of Sar-Arg(Mtr)-Val-Tyr-Ile-His-Pro-Phe-OH. N^α-Fmoc-Phe-2-chlorotrityl chloride resin (150 mg, 0.70 mmol/g) was used for the synthesis of the titled linear peptide precursor following the protocol shown in Table 1. The finished peptide–resin was dried in vacuo (270 mg) and treated with the splitting mixture DCM/AcOH/TFE (3 mL, 7:1:2) for 1 h at room temperature to remove the peptide from the resin. The mixture was filtered off and the resin washed with the splitting mixture and DCM several times. The solvent was removed on a rotary evaporator, and the obtained oily product was treated for 45 min at room temperature with 45% trifluoroacetic acid in DCM (2 mL) containing 5% anisole as scavenger. The mixture then was evaporated to ~0.5 mL and the peptide precipitated from dry diethyl ether as a white amorphous solid. Flash column chromatography (silica gel, 230–400 mesh, 15 g) using $CHCl_3$–AcOH–MeOH (150 mL, 8:0.5:2) as elution solvent provided 109 mg (78%) of **1–II** as a light yellow oil, homogeneous on TLC. R_f = 0.41 in D.

III. Preparation of Sar-Arg(Mtr)-Val-Tyr-Ile-His-Pro-Phe. To a solution of the linear octapeptide **1-II** (60 mg, 0.045 mmol) in DMF (10 mL) containing DIPEA (1.5% v/v, 0.15 mL) was added BOP reagent (119 mg, 6 equiv). The reaction mixture was stirred for 19 h at room temperature, and the solvent was removed under reduced pressure affording a yellow oily residue. The reaction was followed by TLC. R_f = 0.71 in D.

IV. Preparation of Sar-Arg-Val-Tyr-Ile-His-Pro-Phe. The oily residue **1-III** was treated with 90% TFA in DCM (3 mL) containing 5% TFE and 5% H_2O as scavengers for 2 h at room temperature. The resulting solution was concentrated to a small volume (~0.5 mL), and the final free cyclic product was precipitated from diethyl ether as a white amorphous solid. Yield: 36 mg (82%); after HPLC, 12 mg (27%). R_f = 0.13 in D. FAB MS $(M + H)^+$: 985.

2. Synthesis of Cyclo-(1–8)-[Acp^1]ANGII (Analogue 2). I. Preparation of Acp-Arg(Mtr)-Val-Tyr-Ile-His-Pro-Phe-OH. N^α-Fmoc-Phe-2-chlorotrityl chloride resin (100 mg, 0.70 mmol/g) was used for the synthesis of the titled linear peptide following the protocol shown in Table 1. Cleavage of the finished octapeptide from the resin within 1 h at room temperature using DCM–AcOH–TFE (2 mL, 7:1:2) and subsequent removal of the solvent under reduced pressure afforded a light yellow oily residue. The oily product was treated for 45 min at room temperature with 45% TFA in DCM (2 mL), containing 5% anisole as scavenger. The mixture was evaporated to ~0.5 mL, and the product was precipitated from diethyl ether as a white amorphous solid. Flash column chromatography on silica gel (230–400 mesh, 10 g) using $CHCl_3$–ACOH–MeOH (100 mL, 8:0.5:2) as elution solvent provided an oily material, 72 mg (75%), homogeneous on TLC. R_f = 0.25 in D.

II. Preparation of Acp-Arg(Mtr)-Val-Tyr-Ile-His-Pro-Phe. The octapeptide derivative **2-I** (50 mg, 0.04 mmol) was reacted at room temperature in DMF (8 mL) with DIPEA (1.5% v/v, 0.12 mL) and BOP reagent (106 mg, 6 equiv). After 19 h, the reaction mixture was concentrated in vacuo to afford an oily residue which was homogeneous on TLC. R_f = 0.67 in D.

III. Preparation of Acp-Arg-Val-Tyr-Ile-His-Pro-Phe. The oily residue **2-II** was treated with TFA–H_2O–TFE (2 mL, 9:0.5:0.5) for 2 h at room temperature. The mixture was concentrated to a small volume, and the final cyclic product precipitated as a white amorphous solid upon addition of diethyl ether. Yield: 35 mg (85%); after HPLC, 11 mg (26%). R_f = 0.10 in D.

3. Synthesis of Cyclo-(4–8)-[Des^{1,2,3}]ANGII (Analogue 3). I. Preparation of H_2N-Tyr-Ile-His-Pro-Phe-OH. N^α-Fmoc-Phe-2-chlorotrityl chloride resin (100 mg, 0.70 mmol/g) was used for the synthesis of the above linear pentapeptide by solid-phase peptide synthesis. Cleavage of the finished peptide from the resin with DCM–AcOH–TFE (2 mL, 7:1:2)

for 1 h at room temperature afforded a light yellow oily product. Treatment of the oily residue with 45% TFA in DCM (2 mL), containing anisole as scavenger, resulted in a free linear peptapeptide. The crude product was purified by flash column chromatography (silica gel, 230–400 mesh, 10 g) using toluene/AcOH/MeOH (100 mL, 7:1.5:1.5) as elution solvent to afford 42 mg (76%) of **3-I** as a white solid, homogeneous on TLC. R_f = 0.32 in D.

II. Preparation of Tyr-Ile-His-Pro-Phe. The cyclization reaction was carried out in a solution of the pentapeptide derivative **3-I** (25 mg, 0.031 mmol) in DMF (7 mL) containing DIPEA (1.5% v/v, 0.10 mL) and BOP reagent (82 mg, 6 equiv). After 19 h at room temperature, the reaction mixture was concentrated under reduced pressure and the cyclic peptide precipitated from MeOH/diethyl ether as a white amorphous solid. Yield: 19 mg (95%); after HPLC, 6 mg (30%). R_f = 0.71 in D.

4. Synthesis of Cyclo-(2$^{\gamma}$-8$^{\epsilon}$)-[Des^1,Glu^2,Lys^8]ANGII (Analogue 4). I. Preparation of N^{α}-Fmoc-Lys(BOC)-OBzl. N^{α}-Fmoc-Lys(BOC)-OH (468 mg, 1 mmol) was dissolved in DMF (3 mL). Cesium carbonate (977 mg, 3 mmol) and benzyl bromide (0.180 mL, 1.5 mmol) were then added, and the mixture was stirred at room temperature for 1 h. The reaction mixture was then treated with H_2O (50 mL) and extracted with diethyl ether (2 × 50 mL). The ether layer was washed with H_2O (2 × 50 mL), 5% Na_2CO_3 (1 × 50 mL), and H_2O (2 × 50 mL) and dried over anhydrous sodium sulfate, and the solvent was removed in vacuo. The crude product was purified by flash column chromatography (silica gel, 230–400 mesh, 15 g) using hexane–EtOAc (200 mL, 7:3) as elution solvent. The product-containing fractions were collected, the solvent was evaporated, and the solid residue was recrystallized from EtOAc–Petroleum ether affording 0.370 g (66%). Mp 103–4 °C. R_f = 0.20 in K, 0.63 in L.

II. Preparation of N^{α}-Fmoc-Lys®-OBzl. Treatment of N^{α}-Fmoc-Lys(BOC)-OBzl (0.370 g) with HCl in Et_2O 5.12 M (3 mL) at room temperature for 1 h, concentration of the solvent, and trituration with diethyl ether afforded 0.3 g (94%) of N^{α}-Fmoc-Lys-OBzl. A suspension of 2-chlorotrityl chloride resin 0.5 g (1.4–1.6 mequiv of Cl^-/g of resin), DIPEA (0.32 mL, 3.2 equiv), and N^{α}-Fmoc-Lys-OBzl (0.5 mmol, 250 mg) in dry dichloromethane (3 mL) was stirred in a round bottom flask for 20 min at room temperature. A mixture of MeOH (1.5 mL) and DIPEA (0.25 mL) was then added and the mixture stirred for another 10 min at room temperature. The reaction mixture was then filtered off, and the remaining solid was washed with DMF, DCM, *i*-PrOH, and Et_2O for several times and dried in vacuo for 24 h at room temperature. The substitution of the resin was 0.41 mmol of amino acid/g of substituted resin (0.41 mmol/g).

III. Preparation of Z-Glu(COOH)-Val-Tyr-Ile-His-Pro-Lys(NH_2)-OBzl. The synthesis of the titled linear heptapeptide was carried out by solid-phase peptide synthesis, using 0.5 g of the amino acid–resin **4-II** following the protocol shown in Table 1. The completed peptide–resin was dried in vacuo (644 mg) and treated with 35% TFA in dichloromethane (4 mL) containing 5% anisole as scavenger. After 45 min at room temperature, the mixture was evaporated to 0.5 mL. The product was precipitated from diethyl ether as a white amorphous solid (224 mg). Purification of the peptide by flash column chromatography (silica gel, 230–400 mesh, 20 g) using $CHCl_3$–AcOH–MeOH (200 mL, 8:0.5:2) as elution solvent afforded 175 mg (70%) of **4-III** as a white solid, homogeneous on TLC. R_f = 0.36 in D, 0.66 in C. FAB MS (M^+): 1109.

IV. Preparation of Z-Glu-Val-Tyr-Ile-His-Pro-Lys-OBzl. To a solution of the linear heptapeptide **4-III** (175 mg, 0.143 mmol) in DMF (30 mL) containing DIPEA (1.5% v/v, 0.45 mL) was added BOP reagent (190 mg, 3 equiv). The reaction mixture was stirred for 19 h at room temperature, and then the solvent was removed under reduced pressure. The cyclic product was precipitated from chilled water as a white solid, 125 mg (80%), homogeneous on TLC. R_f = 0.60 in D, 0.18 in H, and 0.37 in G. FAB MS (M^+): 1091.

V. Preparation of H_2N-Glu-Val-Tyr-Ile-His-Pro-lys-COOH. The protected cyclic heptapeptide **4-IV** (115 mg) was hydrogenated over Pd for 12 h in MeOH solution containing 5% water. The reaction mixture was filtered off, evaporated to dryness, and lyophilized to afford 60 mg (66%) of a white powder, homogeneous on TLC. Yield after HPLC: 28 mg (31%). R_f = 0.15 in D. FAB MS ($M + H)^+$: 868.

5. Synthesis of Cyclo-(2$^{\epsilon}$-8$^{\gamma}$)-[Des^1,Lys^2,Glu^8]ANGII (Analogue 5). I. Preparation of N^{α}-Fmoc-Glu(*t*-Bu)-OBzl. To a solution of N^{α}-Fmoc-Glu(*t*-Bu)-OH·H_2O (2 g, 4.51 mmol) in DMF (10 mL) were added cesium carbonate (4.4 g, 13.53 mmol) and benzyl bromide (0.72 mL, 6 mmol), and the mixture was reacted at room temperature for 3 h. The reaction mixture was then treated with water (2 × 50 mL) and extracted with diethyl ether (2 × 50 mL). The organic layer was washed with water (2 × 50 mL), 5% sodium carbonate (1 × 50 mL), and water (2 × 50 mL) and dried over anhydrous sodium sulfate, and the solvent was removed under reduced pressure. Flash column chromatography (silica gel, 230–400 mesh, 20 g) using hexane–EtOAc (300 mL, 7:3) as elution solvent afforded a solid residue which was recrystallized from ethyl acetate–petroleum ether to afford 1.210 g (52%). Mp 100–1 °C. R_f = 0.35 in K (homogeneous spot).

II. Preparation of N^{α}-Fmoc-Glu(®)-OBzl. Treatment of N^{α}-Fmoc-Glu(*t*-Bu)-OBzl (1.210 g) with 35% TFA in DCM (5 mL) for 45 min at room temperature, subsequent concentration of the reaction mixture, and trituration with diethyl ether afforded 0.9 g (84%) of N^{α}-Fmoc-Glu-OBzl, homogeneous on TLC. R_f = 0.40 in G. A suspension of 2-chlorotrityl chloride resin (2 g, 1.4–1.6 mequiv of Cl^-/g of resin), DIPEA (1.09 mL, 3.2 equiv), and N^{α}-Fmoc-Glu-OBzl (0.9 g, 1.96 mmol) in dry dichloromethane (20 mL) was stirred in a round bottom flask for 20 min at room temperature. A mixture of MeOH (3 mL) and DIPEA (0.5 mL) was then added and the mixture stirred for another 10 min at room temperature. The reaction mixture was then filtered off, and the remaining solid was washed with DMF, DCM, *i*-PrOH, and Et_2O several times and dried in vacuo for 24 h at room temperature. The substitution of the Fmoc-amino acid–resin was 0.6 mmol/g (calculated by quantitative Kaiser test).

III. Prepration of Z-Lys(BOC)-Val-Tyr(*t*-Bu)-Ile-His(Trt)-Pro-Glu(COOH)-OBzl. The amino acid–resin **5-II** (0.5 g) was used for the stepwise solid-phase peptide synthesis of the linear heptapeptide following the protocol shown in Table 1. The finished peptide–resin was dried in vacuo (657 mg) and treated with the cleavage mixture DCM–AcOH–TFE (8 mL, 7:1:2) for 1 h at room temperature. The mixture was filtered off and the resin washed with DCM several times. The solvent was removed under reduced pressure, and the product precipitated from diethyl ether as a white solid. Yield: 260 mg (57%). R_f = 0.53 in G (homogeneous spot).

IV. Preparation of Z-Lys(NH_2)-Val-Tyr-Ile-His-Pro-Glu(COOH)-OBzl. The heptapeptide derivative **5-III** (260 mg) was treated with 45% TFA containing 5% anisole as scavenger. After 45 min at room temperature, the mixture was evaporated to ~0.5 mL and the peptide precipitated from diethyl ether as a white amorphous solid. The crude peptide was purified by flash column chromatography (silica gel, 230–400 mesh, 20 g) using $CHCl_3$–AcOH–MeOH (200 mL, 8:0.5:2) as elution solvent. Yield: 172 mg (81%). R_f = 0.31 in D.

V. Preparation of Z-Lys-Val-Tyr-Ile-His-Pro-Glu-OBzl. A solution of the heptapeptide derivative **5-IV** (172 mg, 0.14 mmol) in DMF (32 mL) with DIPEA (1.5% v/v, 0.48 mL) and BOP reagent (186 mg, 3 equiv) was stirred for 21 h at room temperature. The reaction mixture was then concentrated under reduced pressure to afford an oily product which was triturated with water. The precipitated solid product was dried in vacuo. Yield: 125 mg (82%), homogeneous on TLC. R_f = 0.50 in D, 0.12 in G. FAB MS ($M + H^+$): 1092.

VI. Preparation of H_2N-Lys-Val-Tyr-Ile-His-Pro-Glu-COOH. The cyclic protected peptide **5-V** (115 mg) was dissolved in a mixture of MeOH–DMF–H_2O (15:7:0.5) and hydrogenated over Pd for 7 h. The reaction mixture was filtered off and then evaporated to dryness. The remaining

oily product was dissolved in 12% AcOH and lyophilized. Yield: 59 mg (65%); after HPLC, 23 mg (25%). R_f = 0.12 in D. FAB MS (M^+): 867.

6. Synthesis of Cyclo-(1$^{\epsilon}$-8$^{\gamma}$)-[Lys^1,Glu^8]ANGII (Analogue 6). I. Preparation of Z-Lys(BOC)-Arg(Mtr)-Val-Tyr(*t*-Bu)-Ile-His(Trt)-Pro-Glu(COOH)-OBzl. N^{α}-Fmoc-Glu(resin)-OBzl (**5-II**) (0.5 g, 0.6 mmol/g) was used for the stepwise solid-phase peptide synthesis of the titled linear octapeptide following the protocol shown in Table 1. The completed peptide was cleaved from the resin using DCM–AcOH–TFE (8 mL, 7:1:2) and precipitated from diethyl ether. The peptide was purified by flash column chromatography (silica gel, 230–400 mesh, 20 g) using $CHCl_3$–MeOH (250 mL, 7:2) as elution solvent to afford 344 mg (61%) of **6-I** as a white solid, homogenous on TLC. R_f = 0.50 in G.

II. Prepration of H$_2$N-Lys-Arg-Val-Tyr-Ile-His-Pro-Glu-COOH. The octapeptide derivative **6-I** (340 mg) was treated with 45% TFA in DCM containing 5% anisole as scavenger. After 45 min at room temperature, the reaction mixture was evaporated to ~0.5 mL and the peptide was precipitated from diethyl ether as a white amorphous solid. The cyclization reaction was carried out using BOP reagent (342 mg, 6 equiv) in a solution of the octapeptide (207 mg, 0.13 mmol) in DMF (32 mL) containing DIPEA (1.5% v/v, 48 mL). After 19 h at room temperature, the reaction mixture was concentrated under reduced pressure to afford an oily and viscous product (189 mg). R_f = 0.81 in D, 0.13 in G. A portion (120 g) of the resulting oily product was dissolved in MeOH (20 mL) and hydrogenated over Pd for 14 h. The reaction mixture was filtered off, and the solvent was removed on a rotary evaporator. A mixture of TFA–TFE–H_2O (4 mL, 9:0.5:0.5) was added to remove the Mtr group from arginine. The mixture was stirred for 2 h at room temperature and then evaporated to ~0.5 mL. The free cyclic product precipitated from diethyl ether as a yellowish solid. Yield: 41 mg (50%); after HPLC, 16 mg (19.5%). R_f = 0.18 in D.

7. Synthesis of Cyclo-(1$^{\epsilon}$-8$^{\gamma}$)-[Lys^1,Tyr(Me)^4,Glu^8]ANGII (Analogue 7). I. Preparation of Z-Lys(NH$_2$)-Arg(Mtr)-Val-Tyr(OMe)-Ile-His-Pro-Glu(COOH)-OBzl. Stepwise solid-phase peptide synthesis of the titled linear octapeptide was carried out using N^{α}-Fmoc-Glu(resin)-OBzl (**5-II**) (0.5 g, 0.6 mmol/g) following the protocol shown in Table 1. The finished peptide–resin was dried in vacuo (815 mg), and treatment with the cleavage mixture DCM–AcOH–TFE (7:1:2) afforded 390 mg of crude material. Purification of this material by flash column chromatography (silica gel, 230–400 mesh, 25 g) using $CHCl_3$–MeOH (300 mL, 6:1) as elution solvent afforded 331 mg (60%) of **7-I** as a white solid material, homogeneous on TLC. R_f = 0.58 in G. Treatment of this peptide with 45% TFA in DCM (3 mL) containing 5% anisole as scavenger resulted in a yellow oily product. The peptide precipitated upon addition of diethyl ether. Yield: 266 mg (92%), homogeneous on TLC. R_f = 0.23 in G.

II. Preparation of Z-Lys-Arg-Val-Tyr(Me)-Ile-His-Pro-Glu-OBzl. To a solution of the linear octapeptide derivative **7-I** (266 mg, 0.17 mmol) in DMF (40 mL) containing DIPEA (1.5% v/v, 0.6 mL) was added BOP reagent (225 mg, 3 equiv). The reaction mixture was stirred for 19 h at room temperature and then evaporated to dryness to afford a yellow oily product. R_f = 0.80 in D, 0.74 in G. The oily product was treated with 90% TFA in DCM (3 mL), for 2 h at room temperature, to remove the Mtr group from the guanidino group of arginine. The reaction mixture was concentrated under reduced pressure, and the cyclic peptide precipitated from diethyl ether as a yellow solid material. Yield: 193 mg (90%). R_f = 0.29 in D.

III. Preparation of H$_2$N-Lys-Arg-Val-Tyr(Me)-Ile-His-Pro-Glu-COOH. A portion (120 mg) of the protected peptide **7-II** was dissolved in DMF (20 mL) and then hydrogenated over Pd for 14 h. The reaction mixture was filtered off, and the solvent was removed under reduced pressure. Trituration with diethyl ether gave the product as a white solid. Yield: 70 mg (71%); after HPLC, 20 mg (20%). R_f = 0.10 in D. FAB MS (M^+): 1037.

8. Synthesis of Cyclo-(2$^{\epsilon}$-8$^{\gamma}$)-[Des^1,Lys^2,Tyr(Me)^4,Glu^8]-ANGII (Analogue 8). I. Preparation of Z-Lys(BOC)-Val-Tyr(Me)-Ile-His(Trt)-Pro-Glu(COOH)-OBzl. N^{α}-Fmoc-Glu-(resin)-OBzl (**5-II**) (0.5 g, 0.6 mmol/g of resin) was used for the stepwise solid-phase peptide synthesis of the linear heptapeptide following the protocol shown in Table 1. The finished peptide–resin was dried in vacuo (630 mg) and treated with DCM–AcOH–TFE (4 mL, 7:1:2) for 1 h at room temperature to remove the peptide from the resin. The mixture was filtered off, the resin was washed with DCM several times, and the solvent was removed under reduced pressure. Diethyl ether was then added, and the precipitated product was collected by filtration as a white amorphous solid. Flash column chromatography (silica gel, 230–400 mesh, 25 g), using toluene–AcOH–MeOH (300 mL, 8:2:0.5) as elution solvent, furnished a solid product which upon trituration with diethyl ether afforded 273 mg (62%), homogeneous on TLC. R_f = 0.45 in J.

II. Preparation of Z-Lys(NH$_2$)-Val-Tyr(Me)-Ile-His-Pro-Glu(COOH)-OBzl. The protected linear octapeptide derivative **8-I** (273 mg) was treated with 45% TFA in DCM (3 mL) containing 5% anisole as scavenger. After 1 h at room temperature, the reaction mixture was evaporated to ~0.5 mL, diethyl ether was then added, and the product was collected by filtration. Yield: 198 mg (86%). R_f = 0.43 in D.

III. Preparation of Z-Lys-Val-Tyr(Me)-Ile-His-Pro-Glu-OBzl. To a solution of the linear heptapeptide **8-II** (198 mg, 0.16 mmol) in DMF (34 mL) containing DIPEA (1.5% v/v, 0.51 mL) was added BOP reagent (212 mg, 3 equiv). The reaction mixture was stirred for 18 h at room temperature, and the solvent then was removed under reduced pressure. The cyclic product was precipitated from chilled water to afford 167 mg (95%) of **8-III** as a solid material. R_f = 0.91 in D, 0.65 in G.

IV. Preparation of H$_2$N-Lys-Val-Tyr(Me)-Ile-His-Pro-Glu-COOH. A portion (152 mg) of the cyclic compound **8-III** was hydrogenated over palladium powder in DMF (15 mL) for 14 h and worked up as for **7-III**. The product was lyophilized twice providing 35 mg (29%) of **8-IV**. Yield after HPLC: 13 mg (11%). R_f = 0.14 in D. FAB MS (M^+): 881.

9. Synthesis of Cyclo-(3$^{\gamma}$-Acp-8$^{\epsilon}$)-[Des^1,Sar^2,Glu^3,Lys^8]-ANGII (Analogue 9). I. Preparation of Fmoc-Lys(Acp-BOC)-OBzl. To a chilled solution of *N*-*t*-BOC-Acp-OH (374 mg, 1.617 mmol) in dry DMF (5 mL) were added *N*-methylmorpholine (NMM) (0.18 mL, 1.617 mmol) and isobutylchlorocarbonate (0.21 mL, 1.617 mmol). The mixture was kept for 3 min at −15 °C, and then it was mixed with N^{α}-Fmoc-Lys-OBzl·HCl (0.5 g, 1.01 mmol) (synthesized by the same procedure as product **4-I**) and NMM (0.11 mL, 1.01 mmol) in DMF (5 mL) precooled at −15 °C. After 3.5 h at −15 °C and 15 min at room temperature, the solvent was evaporated in vacuo and the remaining oily residue was taken up with ethyl acetate (70 mL), washed with 5% $NaHCO_3$ (50 mL), brine solution (2 × 50 mL), 5% citric acid (50 mL), and water (50 mL), and dried over Na_2SO_4. The solvent was evaporated under vacuum, and the oily residue was crystallized in the refrigerator. Recrystallization from ethyl acetate–petroleum ether afforded 0.505 g (74%) of a white solid material, homogeneous on TLC. Mp 84–5 °C. R_f = 0.66 in H.

II. Preparation of Fmoc-Lys(Acp-®)-OBzl. A portion of the dipeptide **9-I** (200 mg) was treated with 1.21 N HCl in acetic acid (2.5 mL) for 1.5 h at room temperature. The solvent was removed on a rotary evaporator, and the product was then precipitated from diethyl ether as a white amorphous solid (123 mg). 2-Chlorotrityl chloride resin (200 mg) was swelled in dichooromethane (2 mL), and to this suspension was added a freshly prepared solution of Fmoc-Lys(Acp)-OBzl (123 mg, 0.2 mmol) and DIPEA (0.11 mL, 0.64 mmol) in dichloromethane (3 mL). The mixture was stirred for 20 min at room temperature, and then methanol (1.5 mL) and DIPEA (0.25 mL) were added. The reaction mixture was stirred for another 10 min at room temperature, and then it was filtered, washed with DMF (3 × 10 mL), DCM (2 × 10 mL), *i*-PrOH (3 × 10

mL), DMF (2 × 10 mL), *i*-PrOH (2 × 10 mL), MeOH (2 × 10 mL), and Et_2O (2 × 10 mL), and dried in vacuo for 24 h. The substitution of the resin was 0.63 mmol of dipeptide/g of substituted resin.

III. Preparation of Z-Sar-Glu(COOH)-Tyr-Ile-His-Pro-Lys(Acp-NH_2)-OBzl. The N^{α}-Fmoc-dipeptide–resin **9-II** (100 mg, 0.63 mmol/g) was used for the stepwise solid-phase peptide synthesis of the linear precyclic peptide following the protocol shown in Table 1. The completed peptide–resin was dried in vacuo (160 mg) and treated with the cleavage mixture DCM–AcOH–TFE (3 mL, 7:1:2) for 1.5 h at room temperature. The resin was separated by filtration and washed with the splitting mixture and dichloromethane several times. The solvent was removed under vacuum, and then 45% TFA in dichloromethane (2 mL) was added. After 30 min at room temperature, the mixture was concentrated to ~0.5 mL and the peptide was precipitated from diethyl ether as a white amorphous solid. Yield: 61 mg (74%). R_f = 0.24 in D.

IV. Preparation of Z-Sar-Glu-Tyr-Ile-His-Pro-Lys-(Acp)-OBzl. To a solution of the linear octapeptide **9-III** (40 mg, 0.031 mmol) in DMF (7 mL) containing DIPEA (1.5% v/v, 0.11 mL) was added BOP reagent (82 mg, 6 equiv). The reaction mixture was stirred for 19 h at room temperature, and then the solvent was removed under reduced pressure to afford the cyclic peptide as an oily product. Yield: 25 mg (69%). R_f = 0.67 in D.

V. Preparation of H_2N-Sar-Glu-Tyr-Ile-His-Pro-Lys-(Acp)-COOH. The protected cyclic octapeptide **9-IV** (25 mg) was hydrogenated over Pd powder in DMF (10 mL) for 6 h, to remove the carbobenzoxy and benzyl groups. The reaction mixture was filtered off and evaporated to dryness to afford a sticky material upon addition of MeOH–Et_2O. Yield: 10 mg (50%); after HPLC, 6 mg (30%). R_f = 0.10 in D.

10. Synthesis of Cyclo-(3$^{\beta}$-5$^{\epsilon}$)-[Sar^1,Asp^3,Lys^5]ANGII (Analogue 10). I. Preparation of Z-Sar-Arg(Pmc)-Asp(*t*-Bu)-Tyr(*t*-Bu)-Lys(BOC)-His(Trt)-Pro-Phe-COOH. N^{α}-Fmoc-Phe-2-chlorotrityl chloride resin (0.2 g, 0.45 mmol/g), synthesized by the same procedure as product **1-I**, was used for the stepwise solid-phase peptide synthesis of the linear precyclic peptide following the protocol shown in Table 1. The completed peptide–resin was suspended in the cleavage mixture DCM–AcOH–TFE (7 mL, 7:1:2) and stirred at room temperature for 1 h. The mixture was filtered, and the resin was washed with the splitting mixture and dichloromethane several times. The solvent was removed on a rotary evaporator, and the product was precipitated from diethyl ether as a white amorphous solid. Yield: 140 mg (84%), homogeneous on TLC. R_f = 0.23 in I.

II. Preparation of Z-Sar-Arg(Pmc)-Asp(*t*-Bu)-Tyr(*t*-Bu)-Lys(BOC)-His(Trt)-Pro-Phe-OBzl. To a solution of the linear peptide **10-I** (140 mg, 0.075 mmol) in DMF (2 mL) were added cesium carbonate (73 mg, 0.225 mmol) and benzyl bromide (13 mL, 0.112 mmol), and the mixture was stirred for 3 h at room temperature. The reaction mixture was worked up as for products **4-I** and **5-I**, and the product was purified by flash column chromatography (silica gel, 230–400 mesh, 10 g), using toluene–AcOH–MeOH (100 mL, 7:1.5:1.5) as elution solvent. The pure peptide was precipitated from diethyl ether to afford 110 mg (75%), homogeneous on TLC. R_f = 0.71 in A, 0.40 in I.

III. Preparation of Z-Sar-Arg(Pmc)-Asp(COOH)-Tyr-Lys(NH_2)-His-Pro-Phe-OBzl. The fully protected peptide **10-II** (110 mg) was treated with 45% TFA in dichloromethane (2 mL) for 1 h at room temperature. The reaction mixture was then evaporated to ~0.5 mL volume, and the product was precipitated from water. Yield: 70 mg (74%). R_f = 0.24 in D. Before cyclization, we neutralized the TFA peptide salt with triethylamine in DMF and precipitated the neutralized peptide with H_2O.

IV. Preparation of Z-Sar-Arg(Pmc)-Asp-Tyr-Lys-His-Pro-Phe-OBzl. To a solution of the linear neutralized octapeptide **10-III** (70 mg, 0.044 mmol) in DMF (10 mL) containing DIPEA (1.5% v/v, 0.15 mL) was added BOP reagent (117 g, 6 equiv). The mixture was stirred for 19 h at room temperature, and then the solvent was removed under reduced pressure. The cyclic product precipitated from diethyl ether. Yield: 45 mg (69%). R_f = 0.63 in D.

V. Preparation of H_2N-Sar-Arg-Asp-Tyr-Lys-His-Pro-Phe-COOH. The protected cyclic peptide **10-V** (45 mg) was treated with 60% TFA in dichloromethane (2 mL) containing 5% anisole as scavenger. After 2 h at room temperature, the mixture was concentrated to ~0.5 mL volume and diethyl ether was then added. The resulting white amorphous solid was dissolved in MeOH (5 mL), containing 1% H_2O, and hydrogenated over Pd for 16 h. The reaction mixture was filtered, evaporated to dryness, and lyophilized. Yield: 18 mg (58%); after HPLC, 10 mg (32%). R_f = 0.10 in D. FAB MS (M^+): 1015.

11. Synthesis of Cyclo-(1$^{\epsilon}$-8)-[Lys^1]ANGII (Analogue 11). I. Preparation of Z-Lys(NH_2)-Arg(Mtr)-Val-Tyr-Ile-His-Pro-Phe-COOH. N^{α}-Fmoc-Phe-2-chlorotrityl chloride resin (**1-I**) (300 mg, 0.70 mmol/g) was used for the synthesis of the linear precursor peptide following the protocol shown in Table 1. The finished peptide–resin was dried in vacuo (470 mg) and treated with DCM–AcOH–TFE (3 mL, 7:1:2) for 1 h at room temperature, to remove the peptide from the resin. The mixture was filtered off, the resin was washed with DCM several times, and the solvent was removed on a rotary evaporator. The remaining oily residue was treated with 45% TFA in DCM (3 mL) containing 5% anisole as scavenger. After 1 h at room temperature, the mixture was evaporated to ~0.5 mL and the peptide precipitated from diethyl ether. Purification of this peptide by flash column chromatography (silica gel, 230–400 mesh, 20 g), using $CHCl_3$–MeOH (250 mL, 6:1) as elution solvent, afforded 227 mg (71%), homogeneous on TLC. R_f = 0.64 in D.

II. Preparation of Z-Lys-Arg(Mtr)-Val-Tyr-Ile-His-Pro-Phe. A solution of the octapeptide derivative **11-I** (207 mg, 0.14 mmol) in DMF (36 mL), DIPEA (1.5% v/v, 0.54 mL, and BOP reagent (371 mg, 6 equiv) was stirred for 19 h at room temperature. The mixture was evaporated to a small volume (0.5 mL), and the product was precipitated upon addition of water. Yield: 180 mg (92%). R_f = 0.70 in D.

III. Preparation of H_2N-Lys-Arg-Val-Tyr-Ile-His-Pro-Phe. The protected cyclic peptide **11-II** (180 mg) was dissolved in DMF (20 mL) and hydrogenated over Pd powder for 9 h, to remove the carbobenzoxy group of lysine. The reaction mixture was evaporated to dryness, and the obtained yellow oil was treated with TFA–H_2O–TFE (3 mL, 9:0.5:0.5) for 2 h to remove the Mtr group from arginine. The solvent was removed under reduced pressure, and the free cyclic product was precipitated upon addition of diethyl ether as a white amorphous solid. Yield: 112 mg (83%); after HPLC, 52 mg (38%). R_f = 0.10 in D. FAB MS ($M + H^+$): 1042.

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