solution of iodomethane (2.4 g, 0.017 mol) and 2-methoxyethanol (10 mL). After the solution was stirred for 1 h with exclusion of light, additional iodomethane (0.5 g) in 2-methoxyethanol (5 mL) was added, and the solution was stirred at ambient temperature overnight. The solution was concentrated under reduced pressure, and the residual oil was triturated with ether to afford a solid. Recrystallization from methanol–ether gave **4j** as colorless crystals. Properties of **4j**, and **4n** prepared in a similar manner from **4m**, are included in Table I.

(±)-4,5-Dihydro-2-(4-methyl-1-piperazinyl)-4-phenyl-3*H*-1,3-benzodiazepine (4k). A mixture of **4j** (4.8 g, 0.012 mol) and 1-methylpiperazine (35 mL) was refluxed with stirring under a nitrogen atomosphere until the evolution of methyl mercaptan ceased. The 1-methylpiperazine hydriodide that crystallized was removed by filtration. The filtrate was diluted with chloroform and washed with 5% NaOH solution. The dried (Na_2SO_4) organic phase was filtered, the filtrate was concentrated, and the crude product was crystallized twice from cyclohexane to afford **4k** as a colorless solid. Properties of **4k** are included in Table I.

(±)-4,5-Dihydro-2-(4-morpholinyl)-4-phenyl-3*H*-1,3-benzodiazepine (4l). This compound was prepared from **4j** (3.0 g, 0.076 mol) and morpholine (30 mL) to afford **4l** in a manner analogous to that described for **4k**. Properties of **4l** are included in Table I.

Pharmacological Method. Spontaneously Hypertensive Rat Assay. All compounds were evaluated for antihypertensive activity in spontaneously hypertensive rats (Okamoto-Aoki strain) at an oral screening dose of 50 mg/kg. Systolic blood pressures were determined by tail-cuff plethysmography predose on day 1 (zero time) and 2 h postdose on day 3. Details of the method are described by Buggy et al.[5] The test compounds were suspended in distilled water with Tween 80 and administered orally at 50 mg/kg unless otherwise indicated. Animals were dosed every day. Four animals per drug were used in the preliminary screen.

Acknowledgment. The authors express their appreciation to Marc N. Agnew and Anastasia Rizwaniuk for spectral data and to Susan Raite and Joan Galbraith for performing pharmacological assays. We also gratefully acknowledge June D. Baird-Strupczewski and Ann Van Dine for library research and Grace M. Naumovitz for assistance in preparation of the manuscript.

Registry No. (±)-1, 80806-39-9; (±)-**2a**, 88057-41-4; (±)-**2b**, 80805-99-8; (±)-**2c**, 88057-42-5; (±)-**2d**, 88057-43-6; (±)-**2e**, 88057-44-7; (±)-**2f**, 80806-00-4; (±)-**4a**, 88057-56-1; (±)-**4a**·HCl, 80806-15-1; (±)-**4b**, 88057-57-2; (±)-**4b**·HCl, 80806-23-1; (±)-**4c**, 88057-58-3; (±)-**4c**·HCl, 88057-45-8; (±)-**4d**, 88057-59-4; (±)-**4d**·HCl, 88057-46-9; (±)-4e, 88057-60-7; (±)-**4e**·HCl, 88057-47-0; (±)-**4f**, 88057-61-8; (±)-**4f**·HCl, 88057-48-1; (±)-**4g**, 88057-62-9; (±)-**4g**·HCl, 88057-49-2; (±)-**4h**, 80806-26-4; (±)-**4i**, 88057-50-5; (±)-**4j**·HI, 88057-51-6; (±)-**4k**, 88057-52-7; (±)-4l, 88057-53-8; (±)-**4m**, 88057-54-9; (±)-**4n**·HI, 88057-55-0; (±)-**4j**, 88057-63-0; CS_2, 75-15-0; 1-methylpiperazine, 109-01-3; morpholine, 110-91-8.

(5) Buggy, J.; Fink, G. D.; John, A. R.; Brody, M. J. *Circ. Res.* **1977**, *Suppl. 1*, 4015, I-110.

A Cyclic Angiotensin Antagonist: [1,8-Cysteine]angiotensin II

John M. Matsoukas, Martin N. Scanlon, and Graham J. Moore*

Department of Medical Biochemistry, University of Calgary, Calgary, Alberta, T2N 1N4, Canada. *Received July 7, 1983*

[Cys(*S*-acetamidomethyl)[1,8]]angiotensin II has been synthesized by the solid-phase method and purified by carboxymethylcellulose chromatography and reversed-phase HPLC. Treatment of this peptide with iodine gave the cyclic octapeptide [Cys[1,8]]angiotensin II, which was isolated by Sephadex G-25 chromatography and reversed-phase HPLC. Comparison of the circular dichroism spectra of the open-chain and cyclic peptides, respectively, demonstrated the existence of a disulfide bond with right-handed chirality in the cyclic peptide. In the isolated rat uterus assay, the open-chain peptide was found to be a potent antagonist of angiotensin II, having approximately 10% of the activity of [Sar[1], Ile[8]]angiotensin II, and the cyclic peptide had about 10% of the antagonist potency of its open-chain synthetic precursor.

The design of potent in vivo antagonists of biologically active peptides is desirable for clarification of the physiological functions of hormones and for therapeutic application in certain disease states. Conformational restriction of peptides via cyclization has proved to be a very valuable tool in the design of peptide hormone analogues that have resistance to metabolic degradation.[1-3] Several magnetic resonance studies have suggested a near-cyclic conformation for angiotensin II (ANG II, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe).[4-6] Cyclization of ANG II with water-soluble carbodiimide has resulted in an inactive product,[7] possibly because the C-terminal α-carboxyl group was derivatized in the cyclization step. We have synthesized a cyclic analogue of ANG II in which the C-terminal carboxyl group remains free, by substituting Cys for the N-terminal and C-terminal amino acids of the molecule. These substitutions would be anticipated to produce an angiotensin antagonist, since the presence of a straight or branched side chain at the C terminus of ANG II is known to abolish intrinsic activity without severely influencing receptor binding affinity. In general, substitution of the N-terminal amino acid of ANG II appears to influence only the rate of metabolic degradation of the molecule and has a minor influence on receptor binding.[8]

Results and Discussion

The synthesis of the cyclic peptide [Cys[1,8]]ANG II generally followed expectations, although the yields of intermediate and final products, both from the synthesis

(1) Veber, D. F. "Peptides: Synthesis, Structure, Function" (Proceedings of the American Peptide Symposium, 7th, University of Wisconsin, Madison, WI, June 14–19, 1981), Rich, B. H.; Gross, E., Eds.; Pierce Chemical Co.: Rockford, IL, 1981; p 685.
(2) Sawyer, W. H.; Pang, P. K. T.; Seto, J.; McEnroe, M. *Science* **1981** *212*, 49.
(3) Schiller, P.; Eggimann, B.; DiMiao J.; Lemieux, C.; Nguyen, T. M. D. *Biochem. Biophys. Res. Commun.* **1981**, *101*, 337.
(4) Printz, M. P.; Nemethy, G.; Bleich, H. *Nature (London)* **1972**, *237*, 135.
(5) Fermandjian, S.; Piriou, F.; Sakarellos, C.; Lintner, K.; Khosla, M. C.; Smeby, R. R.; Bumpus, F. M. *Biopolymers* **1981**, *20*, 1971.
(6) Lenkinski, R. B.; Stephens, R. L. *Inorg. J. Biochem.* **1981**, *15*, 95.
(7) DeCoen, J. L.; Ralston, E.; Durieux, J. P. Loffet, A. "Peptides: Chemistry, Structure, and Biology (Proceedings of the American Peptide Symposium, 4th, New York, June 1–6, 1975); Walter, R.; Meienhofer, J., Eds.; Ann Arbor Science: Ann Arbor, MI, 1975; p 553.
(8) Hollenberg, N. K. *Annu. Rev. Pharmacol. Toxicol.* **1979**, 559.

0022-2623/84/1827-0404$01.50/0

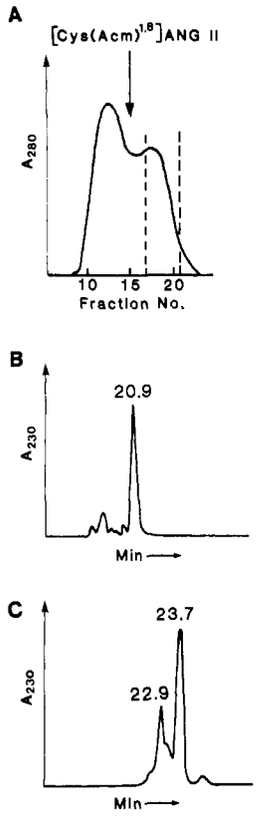

Figure 1. Purification of angiotensin analogues by gel filtration and HPLC. (A) Separation of the products obtained after deprotection and cyclization of [Cys(Acm)^{1,8}]ANG II with iodine by Sephadex G-25 chromatography eluting with 50% acetic acid. The dashed lines indicate the fractions containing monomer which were pooled and lyophilized. The elution position of [Cys(Acm)^{1,8}]ANG II is shown for reference purposes and demonstrates, as has been shown previously,[17] that the elution volume is dependent on molecular diameter rather than on molecular weight in this chromatography system. (B) Purification by reversed-phase HPLC (see Experimental Section) of the product obtained after carboxymethylcellulose chromatography of [Cys(Acm)^{1,8}]ANG II. The peak eluting at 20.9 min was collected. (C) Purification by reversed-phase HPLC (see Experimental Section) of the monomer fraction of [Cys^{1,8}]ANG II obtained by Sephadex G-25 chromatography. The peak eluting at 23.7 min was collected.

and purification steps, were substantially lower than have been encountered in previous syntheses and purifications that have followed a similar basic strategy.[9] However, the products obtained were of reasonably high purity and could be purified to homogeneity by reversed-phase HPLC.

Circular dichroism (CD) spectra of these peptides have been used to verify the structure of the cyclic octapeptide. A disulfide moiety gives CD bands near 290 and 240 nm.[10] The ellipticity bands near 290 and 240 nm are negative and positive, respectively, when the molecule is in a left-hand screw sense. The opposite situation is observed when the disulfide bridge is in the right-hand helical chirality, i.e., positive band near 290 nm and negative band near 240 nm. In oxytocin analogues, a positive band at 250–260 nm and a negative band at 230–240 nm have been taken to indicate a right-handed chirality for the disulfide bridge in oxytocin.[11] In our linear analogue [Cys(Acm)^{1,8}]ANG II, there

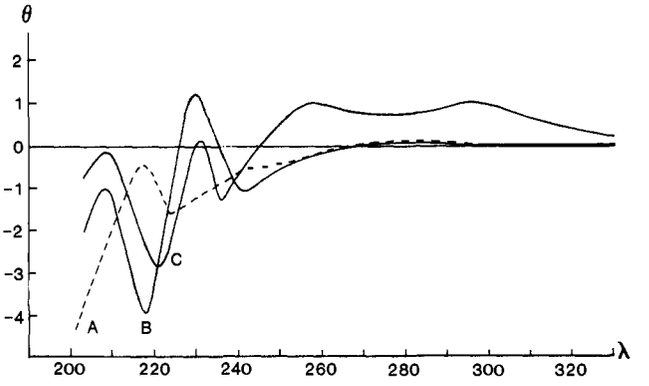

Figure 2. CD spectra of ANG II (A), [Cys(Acm^{1,8})]ANG II (B), and [Cys^{1,8}]ANG (C) in water (see Experimental Section for details). The CD spectrum for ANG II was taken from DeCoen et al.[7]

Table I. Antagonist Properties of Angiotensin Analogues

peptide	antagonist act.: MEC,[a] M (no. of experiments)
[Sar^1, Ile^8]ANG II	$2.1 \pm 0.4 \times 10^{-8}$ (4)
[Cys(Acm)^{1,8}]ANG II	$2.4 \pm 0.5 \times 10^{-7}$ (4)
[Cys^{1,8}]ANG II	$3.3 \pm 1.2 \times 10^{-6}$ (4)
[Cys^{1,8}]ANG II dimer[b]	$9.6 \pm 3.1 \times 10^{-6}$ (3)

[a] Minimum effective concentration plus or minus the standard error of the mean, of peptide required to abolish the response to an ED75 dose of ANG II (10^{-8} M) in the isolated rat uterus assay. [b] The first peak eluted from the Sephadex G-25 column (see Experimental Section) without further purification.

were no CD bands in the range 250–300 nm, as would be expected for an open-chain peptide lacking the inherent optical activity imposed by a disulfide bridge. In contrast, strong optical activity was observed in the cyclic analogue, [Cys^{1,8}]ANG II, as demonstrated by positive bands near 295 and 255 nm and a negative band at 230–240 nm. The CD spectrum therefore suggests a right-handed helical chirality for the disulfide bridge in the cyclic analogue and confirms the cyclic structure of this peptide.

The results in Table I demonstrate that replacement of the N- and C-terminal residues of ANG II with Cys(Acm) produces a molecule with antagonist properties, having about 10% of the antagonist activity of the most potent known antagonist in this tissue, i.e., [Sar^1,Ile^8]ANG II. When the acetamidomethyl groups were removed from [Cys(Acm)^{1,8}]ANG II and when the peptide was cyclized by formation of a disulfide bond, the antagonist activity of the resulting molecule decreased to about 10% of the activity of its open-chain synthetic precursor. The progressively decreasing antagonist activities observed on going from [Sar^1,Ile^8]ANG II to [Cys(Acm)^{1,8}]ANG II to [Cys^{1,8}]ANG II could be interpreted as manifestations of significant changes in peptide conformation. On the other hand, the decreasing antagonist potency observed for this series of peptides may reflect differences in the length of the side chain of the C-terminal amino acid, since it is known that an optimum length for the side chain in position 8 is important for antagonist activity.[8] Thus, the antagonist potencies of these peptides could be attributed to the length of the side chain occupying position 8, i.e., optimum length for [Sar^1,Ile^8]ANG II, too long for [Cys(Acm)^{1,8}]ANG II, and essentially infinite length for [Cys^{1,8}]ANG II. Certainly the CD spectra indicated that, apart from formation of the disulfide bond, no major

(9) Moore, G. J.; Oudeman, E. M.; Ko, D.; Nystrom, D. *J. Med. Chem.* **1979**, *22*, 1147.
(10) Linderburg, J.; Michl, J. *J. Am. Chem. Soc.* **1970**, *92* 2619.
(11) Urry, D. W.; Quadrifolgio, F.; Walter, R.; Schwartz, I. L. *Proc. Natl. Acad. Sci. U.S.A.* **1968**, *60*, 697.
(12) Arunlakshna, O.; Schild, H. O. *Br. J. Pharmacol.* **1959**, *14*, 48.

change in conformation occurred on going from [Cys(Acm)^{1,8}]ANG II to [Cys^{1,8}]ANG II, supporting a primary role for the side chain in position 8 in directing the antagonist activity of these compounds. Interestingly, the [Cys^{1,8}]ANG II dimer also appeared to retain significant antagonist activity (Table I).

These studies support the hypothesis based on nuclear magnetic resonance spectroscopy that ANG II adopts a near-cyclic conformation in solution and may also indicate that this conformation is essentially maintained when the hormone is bound to its receptors. These findings also demonstrate that it is possible to synthesize cyclic analogues of ANG II that are biologically active. Further refinement of the cyclic peptide reported here may produce potent antagonists with increased resistance to metabolic degradation and perhaps therapeutically useful analogues of ANG II for the treatment of cardiovascular disease.

Experimental Section

tert-Butyloxycarbonyl-blocked amino acids were purchased from Peninsula Laboratories. Boc-Cys(Acm) was coupled to chloromethylated 1% divinylbenzene–styrene copolymer (Bio-Rad) by the cesium salt method,[13] and the substitution was determined by amino acid analysis [0.27 mmol of Cys(Acm)/g of resin]. Solid-phase peptide synthesis was carried out on a 0.25-mmol scale with a Beckman 990 peptide synthesizer essentially by procedures described previously[9] to give Boc-Cys(Acm)-Arg-(Tos)-Val-Tyr(Br-Z)-Ile-His(Tos)-Pro-Cys(Acm)-resin. Two coupling steps with 2.5 equiv of Boc-protected amino acid and coupling reagent were employed for each amino acid; the first coupling was mediated by dicyclohexylcarbodiimide (DCC) in the presence of hydroxybenzotriazole for 4 h and the second was mediated by EEDQ for 8 h. Completion of couplings was verified by the ninhydrin test.[14] The completed peptide was removed from the resin and simultaneously deprotected by reaction with anhydrous HF (20 mL) in the presence of anisole (1 mL), ethyl methyl sulfide (0.1 mL), and indole (0.1 g) for 30 min at 0 °C: yield 290 mg (45%). After purification by carboxymethylcellulose chromatography and reversed-phase HPLC (see below), the product was obtained in 8% yield (22 mg).

[Cys(Acm)^{1,8}]ANG II (20 mg) was dissolved in 75% aqueous methanol (160 mL), and iodine (20 mg) in methanol (6 mL) was added dropwise during 1 h to the stirred solution.[15,16] After a further 5 h, one drop of acetic acid was added, and the solution was taken to dryness on a rotary evaporator. The product was dissolved in 50% acetic acid (1 mL) and chromatographed on a column (60 × 1.6 cm) of Sephadex G-25, eluting with 50% acetic acid at a flow rate of 5 mL/h. Two peaks of similar size were detected from the absorbance at 280 nm (first peak, elution time 6 h; second peak, elution time 8.5 h). A subsequently determined elution time for [Cys(Acm)^{1,8}]ANG II of 7.5 h suggested that, based on molecular diameters,[17] the second peak was the required cyclic monomer and the first peak was dimeric material resulting from intermolecular disulfide bond formation. Fractions (30 min) corresponding to monomer and dimer, respectively, were cut for purity rather than yield and were pooled, diluted with water, and lyophilized: yield of monomer, 4 mg (20%); yield of dimer, 6 mg (30%). The monomer was purified to homogeneity by reversed-phase HPLC (see below): yield 3 mg.

Carboxymethylcellulose Chromatography. The crude product obtained after reaction with HF was dissolved in 7% acetic acid (10 mL), clarified by centrifugation, and chromatographed on a column (70 × 1.6 cm) of Whatman CM23 carboxymethylcellulose using a linear gradient of ammonium acetate from 0.01 M, pH 5.0, to 0.5 M, pH 8.0. Fractions of the major peak detected from the absorbance at 280 nm were collected (cut for purity rather than yield), lyophilized, and relyophilized from 1% acetic acid.

Semipreparative Reversed-Phase HPLC. Final purification of peptides was accomplished with a Varian 5000 liquid chromatograph equipped with a Vista 401 microprocessor controller. Separations were achieved on a C_{18} Varian MCH-10ncap column (30 × 0.8 cm) at 40 °C using a stepped linear gradient of acetonitrile in 0.1% trifluoroacetic acid (TFA) at a flow rate of 2.5 mL/min. Automated repetitive injections of peptides (20 × 0.5 mg) were made from a nitrogen-pressurized Rheodyne injector with a 2.0-mL sample loop. The loop was loaded with 10 mg of peptide in 2 mL of 0.25% acetic acid. One-twentieth of the total sample was injected during each run by lowering the flow rate to 0.2 mL/min for a 30-s "inject" period. One cycle consisted of the following events: 0–10 min, flow 2.5 mL/min, 90% H_2O/10% of 1% TFA; 10–10.5 min, flow 0.2 mL/min, "inject"; 10.5–11 min, flow 2.5 mL/min, "load"; 11–13 min, → 70% H_2O/20% CH_3CN/10% of 1% TFA; 13–30 min, → 45% H_2O/45% CH_3CN/10% of 1% TFA; 30–35 min, → 90% CH_3CN/10% of 1% TFA; 35–42 min, 90% CH_3CN/10% of 1% TFA; 42–47 min, → 100% H_2O; 47–50 min, 100% H_2O.

Fractions were collected at 0.1-min intervals with a Gilson Model 201 fraction collecter programmed to collect for a 5-min period centered around the elution time of the major product. The fraction collector was restarted by the Vista 401 at the beginning of each HPLC run so that material eluting at the same elution time was repeatedly deposited in the same tubes. Elution of the peptide was detected simultaneously from the absorbances at 254 (Varian UV-1) and 230 nm (Kratos SF769Z). Fractions containing the required peptide were pooled; after removal of CH_3CN on a rotary evaporater at 40 °C, the fractions were lyophilized and stored at −20 °C. Amino acid analyses were carried out in 6 N HCL (containing 1% cresol to prevent loss of Tyr) at 110 °C for 18 h in vacuo.

CD Spectra. CD spectra were taken with a Jasco 50-U spectropolarimeter and were accumulated eight times. Samples were dissolved at a concentration of 0.67 mg/mL in water, pH 6.0, at 20 °C. Ellipticity (θ) was determined as 10^4 deg cm^2 dmol^{-1}.

Bioassay. The isolated rat uterus assay was carried out on uteri from diethylstilbestrol (DES) primed female Sprague–Dawley rats (150–250 g) as described by Freer and Stewart.[16] Contractions were measured with Grass force-displacement transducers connected to a Beckman dynograph. The tissue was challenged every 12 min in all experiments, and the antagonist was given 2 min before ANG II. Antagonist potencies were determined as the minimum effective concentration, i.e., the minimum concentration of antagonist required to completely block the response to an ED75 dose of ANG II. This procedure was adopted because a Schild plot determined for the standard antagonist, [Sar^1,Ile^8]ANG II, in this bioassay suggested noncompetitive antagonism for this peptide, thus invalidating the use of pA_2 values for comparing antagonist potencies.[12]

[Cys(Acm)^{1,8}]ANG II. Amino acid analysis: Val, 1.00; Pro, 1.01; Cys, 0.95; Ile, 0.92; Tyr, 1.05; His, 1.21; Arg, 1.06. Mass ion (FAB-MS): 1131; HPLC elution time 20.9 min; CD maxima 209 nm (θ −1.0), 230 (+1.2), 280 (+0.2); CD minima 218 nm (θ 3.9), 240 (−1.2).

[Cys^{1,8}]ANG II. Amino acid analysis: Val, 1.00; Pro, 0.98; Cys, 1.50; Ile, 0.84; Tyr, 0.95; His, 1.15; Arg, 1.13. Mass ion (FAB-MS) 987; HPLC elution time 23.7 min; CD maxima 209 nm (θ −0.2), 231 (+0.2), 257 (+1.0), 294 (+1.0); CD minima 221 nm (θ −2.8), 236 (−1.3), 275 (+0.7).

Acknowledgment. The authors thank Dr. A. Hogg, University of Alberta, for FAB-MS analyses, Dr. D. McKay for amino acid analyses, and Keith Jorgenson for assistance with CD measurements. We are grateful to Dr. R. Roche for helpful discussion in the interpretation of CD spectra. This work was supported by the Alberta Heart Foundation and the Alberta Heritage Foundation for Medical Research.

Registry No. Boc-Cys(Acm), 19746-37-3; [Cys(Acm)^{1,8}]ANG II, 87937-68-6; [Cys^{1,8}]ANG II, 87937-69-7; [Cys^{1,8}]ANG II dimer, 87937-71-1; ANG II, 4474-91-3.

(13) Gisin, B. F. *Helv. Chim. Acta* **1973**, *56*, 1476.
(14) Kaiser, E.; Colescott, R. L.; Bossinger, C. B.; Cook, P. I. *Anal. Biochem.* **1978**, *34*, 595.
(15) Marbach, P.; Rudinger, J. *Helv. Chim. Acta* **1974**, *57*, 403.
(16) Freer, R. J.; Stewart, J. M. *J. Med. Chem.* **1973**, *16*, 733.
(17) Moore, G. J. *Biochem. J.* **1978**, *173*, 402.