Hz, 3 H), 1.23 (s, 9 H), 1.12-1.68 (m, 5 H), 2.75-2.87 (m, 4 H), 4.59 (s, 2 H); ^{13}C NMR ($CDCl_3$) δ 21.96, 26.35, 30.30; 34.64, 50.24, 79.98, 92.11; MS, *m/e* (relative intensity) 302 (M + 1, 24), 200 (45), 186 (14), 128 (27), 112 (100), 73 (14). Anal. ($C_{11}H_{23}NO_2$) C, H, N.

1,6-Diaza-3,4,8,9,12,13-hexaoxabicyclo[4.4.4]tetradecane (HMTD) (9).[41] Hexamethylenetetramine (50 mmol, 7.0 g) was dissolved in 30% aqueous hydrogen peroxide (22.5 g) with stirring at 4 °C. Powdered citric acid hydrate (11.5 g) was added slowly over a period of 10 min, followed by continued stirring for 3 h at 4 °C. The reaction mixture was warmed to room temperature and **9** was obtained as fine white crystals (5.87 g, 57%) after washing with water (250 mL) and MeOH (50 mL) and air-drying: mp 154 °C exploded; ^1H NMR ($CDCl_3$) δ 4.80 (s). Anal. ($C_6H_{12}N_2O_6$) C, H, N.

Biological Methods. The in vitro assays were conducted by using a modification of the semiautomated microdilution technique of Desjardins et al.[56] and Milhous et al.[57] Two *P. falciparum* malaria parasite clones, designated as Indochina (W-2) and Sierra Leone (D-6), were utilized in susceptibility testing. Test compounds were dissolved in DMSO and serially diluted with culture media. The uptake of tritiated hypoxanthine was used as an index of inhibition of parasite growth. The compounds described herein were tested against a drug-sensitive strain of *P. berghei* (strain KBG 173) in mice according to a method previously described.[58]

Kinetic Experiments. The rates of hydrolysis of **4-8** were determined by ^1H NMR. Amine peroxides **4-8** were dissolved (45 mM) in CD_3CN-deuteriated buffer, 2:1, with 1,1,2,2-tetrachloroethane as an internal standard. The acetate (pH 5.2) and phosphate (pH 7.4) buffers (50 mM) were chosen to correspond to the average pH of the parasitic digestive vacuole and the extracellular fluid, respectively. The rates of hydrolysis were obtained by determining peak integration ratios for the methylene protons in **4-8** and the protons in the internal standard and comparison to standard curves.

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Registry No. 4, 116437-26-4; 5, 116437-27-5; 6, 64254-25-7; 7, 116437-28-6; 8, 116466-10-5; 9, 283-66-9; *tert*-butyl hydroperoxide, 75-91-2; 4-phenylpiperidine, 771-99-3; 1-phenylpiperazine, 92-54-6; *N*-ethylaniline, 103-69-5; *N*-methylcyclohexylamine, 100-60-7; 4-methylpiperidine, 626-58-4.

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Conformationally Restricted Analogues of Atriopeptin(103-125)amide

Kerry L. Spear,* Emily J. Reinhard, Ellen G. McMahon, Gillian M. Olins, Maria A. Palomo, and Dennis R. Patton

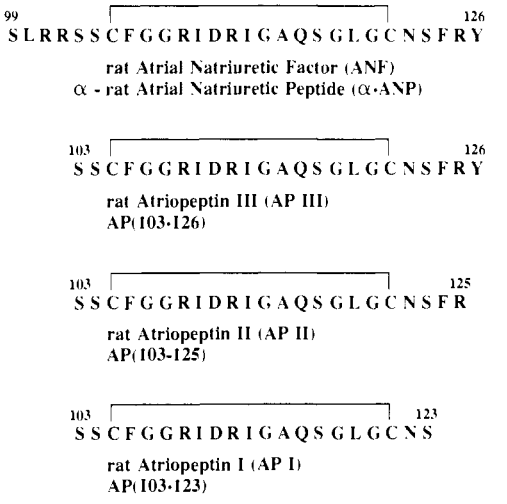
Cardiovascular Diseases Research Department, Searle Research and Development Division, G. D. Searle and Company, c/o Monsanto Company, 700 Chesterfield Village Parkway, Chesterfield, Missouri 63198. *Received February 19, 1988*

Conformationally restricted analogues of atriopeptin(103-125)amide were prepared by synthesizing novel bicyclic peptides in which a second disulfide bridge linking residues 108 and 117 was introduced. These syntheses were shown to proceed with no significant scrambling of the disulfide bonds and demonstrated that structurally defined bicyclic analogues of atrial peptides could be easily prepared. The conformationally restrained analogues described here were found to be biologically active with potencies (EC_{50}s) ranging from 0.05 to 3 μM. In addition, these bicyclic peptides (and many of the monocyclic precursors) were found to bind selectively to a class of specific tissue binding sites that have not been shown to be associated with any known second messenger system (NVR binding sites). Since affinity for the receptor class linked to vasorelaxation was negatively affected by the conformational restrictions described here, binding of atrial peptides to this class of receptors appears to have more specific conformational requirements than does binding to the NVR sites.

A group of cyclic peptides[1] has been isolated from mammalian atria that mediate a variety of in vitro and in vivo responses, including vasorelaxation, diuresis, and natriuresis. This family of compounds varies in length from 21 to 28 amino acids and shares a common cyclic core of 17 residues (see Scheme I). Several different names have been applied to this hormone, including atriopeptin (AP), atrial natriuretic factor (ANF), and atrial natriuretic peptide (ANP).

Because of their unique physiological activity, both the physical and biological properties of AP have been intensively studied. Unfortunately, despite the availability of a variety of spectral information, the solution-phase conformational properties of these peptides remain poorly defined. For example, nuclear magnetic resonance (NMR)

Scheme I. Amino Acid Sequences and Proposed Names of Atrial Peptides



(1) For recent reviews, see: Needleman, P.; Adams, S. P.; Cole, B. R.; Currie, M. G.; Geller, D. M.; Michener, M. L.; Saper, C. B.; Schwartz, D.; Standaert, D. G. *Hypertension* **1985**, 7, 469. Ballerman, B. J.; Brenner, B. M. *J. Clin. Invest.* **1985**, 76, 2041. *Atrial Hormones and Other Natriuretic Factors*; Mulrow, P. J., Schrier, R., Eds.; American Physiological Society: Bethesda, MD, 1987. *Endocrinology and Metabolism Clinics of North America*; Rosenblatt, M., Jacobs, J. W., Eds.; W. B. Saunders Co.: Philadelphia, PA, 1987; Vol. 16.

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spectroscopy[2] suggests that AP exists as a random coil in solution. Although purification and partial characterization[3] of an AP receptor has been achieved, no spectral information about the peptide/receptor complex is yet available. Interestingly, Epand and co-workers[4,5] did recently report that, when associated with synthetic lipid vesicles, atriopeptin(103–126) (1) adopted a highly ordered structure that was determined to be predominately β-sheet by circular dichroism, differential scanning calorimetry, and infrared spectroscopy. No useful information about the conformation of AP was obtained if phospholipids were not present in the aqueous medium. In any event, even if available, it is unclear whether a conformation determined in solution will provide any insight about the receptor-bound conformation.

In the absence of definitive information about the secondary structure of AP, we designed and synthesized a series of analogues in which conformational alternatives were limited. It was anticipated that an improved understanding of the conformation of the receptor-bound hormone would permit a more rational design of novel therapeutic agents. There is increasing evidence[6] that potency or selectivity of some peptides can be potentiated by appropriately restricting conformational mobility. Presumably, analogues that exhibit such properties have been constrained to a conformation that closely resembles that required for productive peptide/receptor interactions. One approach to conformational restriction of peptides that has afforded promising results in the past is the preparation of cyclic analogues of normally acyclic peptides. This approach has been successfully applied to a variety of small, linear peptides such as melanotropin[7] or enkephalin.[7,8] While AP is already somewhat conformationally constrained as a cyclic peptide, model building shows that the molecule is still quite flexible. Accordingly, we prepared a series of analogues that could be further restricted by incorporation of a second disulfide bond within the cyclic core.

Prior work, here[9] and elsewhere,[10] clearly showed that acyclic atrial peptides could be readily oxidized to a single product that was identical both chemically and biologically to the natural product. This facile cyclization with recovery of full activity suggested that folding of the acyclic peptide in solution was such that the cysteine residues in positions 105 and 121 were proximal to each other and encouraged us to believe that it would be possible to control the intramolecular oxidation of a pair of cysteines in the presence of a preexistent cystine 105/121 linkage. However, a stepwise oxidation of first one cysteine pair to afford a monocyclic peptide followed by oxidation of the second pair of thiols to afford the corresponding bicyclic

Scheme II. Generalized Synthetic Route to Bicyclic Peptides

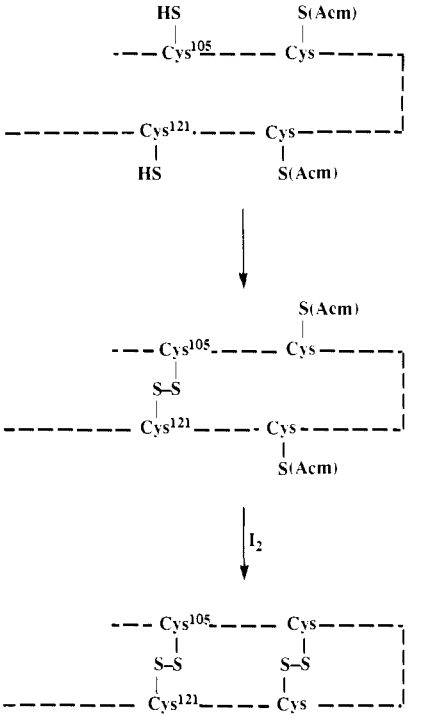

Table I. Relative Vasorelaxant Activities and Relative Binding Affinities of AP Analogues with AP(103–126) as the Standard

analogue of AP(103–125)NH_2[a]	vasorelaxation: relative potency[b] ± SE	binding: relative potency NVR[c]	VR[d]
1, AP(103–126)	1.0[e]	1.0[f,g]	
2, AP(103–125)NH_2	1.70 ± 0.46	3.2[g]	
3, Cys(Acm)^{108}	1.03 ± 0.20	4.3[g]	
4, Cys(Acm)^{117}	0.21 ± 0.07	3.7[g]	
5, Cys^{105,117}Cys(Acm)^{108,121}	0.042 ± 0.011	3.1	0.014
6a, Cys^{105,117}Cys^{108,121}	0.0007 ± 0.0001	2.0	0.0017
6b, Cys^{105,117}Cys^{108,121}	0.0007 ± 0	2.0	0.0017
7, Cys(Acm)^{105,117}Cys^{108,121}	0.0022 ± 0.0001	1.6	0.0096
8, Cys(Acm)^{105,108}Cys^{117,121}	0.023 ± 0.0005	2.8	0.024
9a, Cys^{105,108}Cys^{117,121}	0.028 ± 0.005	not tested	
9b, Cys^{105,108}Cys^{117,121}	0.013 ± 0.003	2.2	0.0053
10, Cys^{105,108}Cys(Acm)^{117,121}	0.0043 ± 0.0009	1.2	0.0088
11, Cys^{105,121}Cys(Acm)^{108,117}	0.43 ± 0.06	0.48[g]	
12a, Cys^{105,121}Cys^{108,117}	0.014 ± 0.002	1.4	0.020
12b, Cys^{105,121}Cys^{108,117}	0.030 ± 0.004	1.8	0.015
13, Cys(Acm)^{105,121}Cys^{108,117}	0.0085 ± 0.0006	3.5	0.032
14, Cys^{105,121}D-Cys(Acm)^{108,117}	0.044 ± 0.006	0.91[g]	
15, Cys^{105,121}D-Cys^{108,117}	0.0073 ± 0.0006	5.0	0.016
16, Cys^{105,121}Hcy(Acm)^{108}Cys-(Acm)^{117}	0.24 ± 0.02	0.15[g]	
17, Cys^{105,121}Hcy^{108}Cys^{117}	0.049 ± 0.004	2.3	0.016
18, Cys^{105,121}Hcy(Acm)^{108,117}	0.068 ± 0.009	0.84[g]	
19, Cys^{105,121}Hcy^{108,117}	0.032 ± 0.003	2.8	0.071

[a] All structures are analogues of AP(103–125)NH_2 unless otherwise specified. Amino acid analyses and FABMS agree with the theoretical values unless otherwise specified. [b] $n = 2$ unless otherwise specified. [c] Nonvasorelaxant binding site. [d] Vasorelaxant binding site. [e] $EC_{50} = 2.3 \pm 1.6$ nM ($n = 8$). [f] Apparent $K_i = 0.25 \pm 0.16$ nM ($n = 6$). [g] Displacement curve could not distinguish between binding sites.

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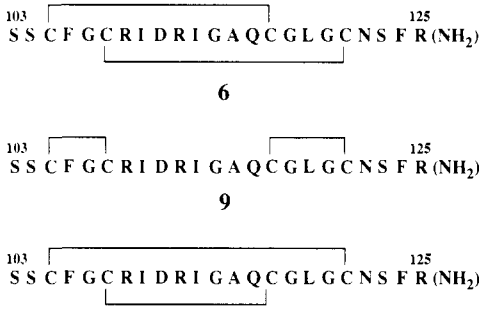
Scheme III. Amino Acid Sequence and Possible Disulfide Linkages of [Cys^{105,108,117,121}]AP(103–125)amide



isomer could not be presumed a priori to proceed with no disulfide interconversion. Accordingly, the stability of these disulfide bridges under the appropriate reaction conditions was also examined. We report here the synthesis of several structurally defined bicyclic AP analogues and biological characterization of the vasorelaxant and receptor-binding properties associated with these novel peptide hormones.

Results

Chemistry. The APs discussed here are all numbered as fragments of the 126 amino acid prohormone. All peptides were prepared via solid-phase peptide synthesis methodology, and no difficulties were encountered either during synthesis or purification. In order to better correlate with other ongoing structure/activity relationship studies, analogues of **2**, the C-terminal carboxamide derivative of AP(103–125), were synthesized. This nonnatural variant is somewhat more potent than **1** (see Table I). The route of cyclization is outlined in Scheme II. During synthesis, thiol protecting groups were chosen to facilitate selective deprotection at a later time. Protection of one pair of cysteine thiols employed 4-methylbenzyl (MeB) groups and the second pair of cysteine residues was blocked with acetamidomethyl (Acm) groups. Since the MeB group is not stable to hydrogen fluoride (HF), deprotection is simultaneous with cleavage of the peptide from its resin support. In contrast, the Acm group is relatively stable to HF. As a result, after HF cleavage and purification of the resulting acyclic peptide, only two free sulfhydryl functional groups were available for further reaction. Cyclization afforded the expected monocyclic AP analogue. After further purification, the Acm protecting groups were removed with iodine in acetic acid according to the method of Kamber.[11] Cleavage of the protecting groups under these conditions was accompanied by concomitant oxidation to afford the bicyclic target molecule.

Since disulfide exchange is promoted by thiolate anions, it seems likely that oxidation in an acidic environment would minimize the possibility of scrambling of the disulfide bonds. In order to demonstrate this conclusively, all bicyclic analogues of [Cys^{105,108,117,121}]AP(103–125)NH$_2$ were synthesized. As summarized in Scheme III, only three different monomeric isomers of this analogue (or any peptide containing four cysteine residues and two disulfide bonds) are possible. Moreover, each of these bicyclic isomers can be synthesized via two different routes, depending on the order in which the cysteine residues are oxidized. As expected, the order of cyclization does not appear to affect the structure of the product. The required monocyclic analogues (**5**, **7**, **8**, **10**, **11**, **13**) were readily synthesized, purified, and characterized. Separate Acm-deprotection/oxidation of these analogues was similarly facile, affording only a single product in each case. The presence of AP oligomer was not detected. Fast atom bombardment mass spectroscopy (FABMS) confirmed that the Acm protecting groups were cleaved and that the molecular ion peaks were the same. Ellman's colorimetric[12] test showed that no free sulfhydryl groups were present. Amino acid analysis and peptide sequence analysis of all six products were identical, suggesting that they differed only in the linkage of the two disulfides. These isomers could be divided into two groups by comparing the HPLC retention times (see Figure 1 and Table II). The products formed upon treatment of **5** or **7** with iodine could not be separated by HPLC, but had a clearly different retention time from that of the other four isomers. Similarly, after

Table II. HPLC Retention Times of the Bicyclic Isomers of [Cys^{105,108,117,121}]AP(103–125)NH$_2$

bicyclization reaction	HPLC retention time,[a] min
5 → **6a**	12.6 ± 0.1
7 → **6b**	12.6 ± 0.1
8 → **9a**	13.7 ± 1
10 → **9b**	13.7 ± 1
11 → **12a**	14.0 ± 1
13 → **12b**	14.0 ± 1

[a] The peptides were separated on the gradient shown in Figure 1. Absorbance of the column effluent was measured at 220 nm.

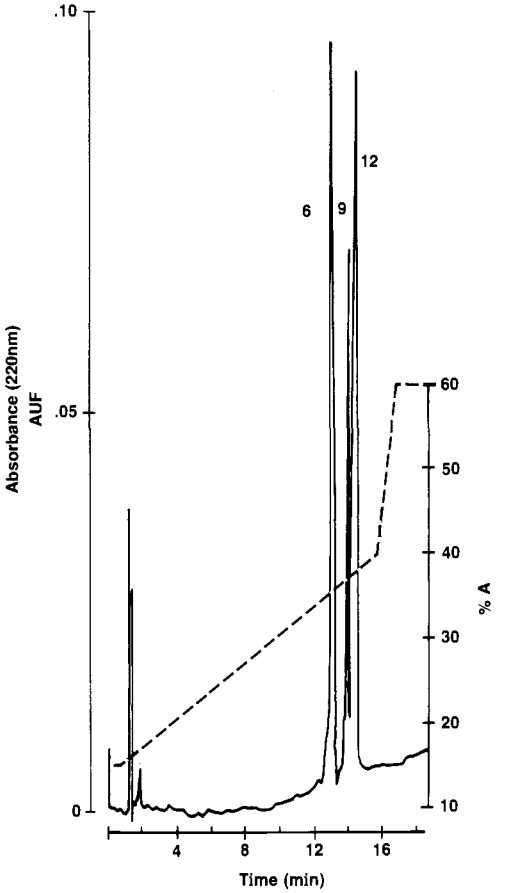


Figure 1. HPLC profile of analogues **6a**, **6b**, **9a**, **9b**, **12a**, and **12b** using a Vydac C$_{18}$ analytical column (4.6 mm i.d. × 15 cm; 5 μm; 300 A). Conditions: 15% A to 40% A over 16 min. A: acetonitrile (+ 0.05% TFA); water (+ 0.05% TFA). Flow rate: 1.5 mL/min.

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(12) Deakin, H.; Ord, M. G.; Stocken, L. A. *Biochem. J.* **1963**, *89*, 296.

cyclization, the peptides derived from **8** and **10** had identical HPLC retention times which were different from the others. Finally, **11** and **13** afforded products having indistinguishable HPLC retention times. Although the inability to separate two compounds is not proof that they are identical, these three pairs of isomers correspond precisely to those pairs of peptides that were predicted to afford the same product upon bicyclization. Moreover, it is clear that those bicyclic isomers that were predicted to be different did indeed have different HPLC retention times. The findings are consistent with the absence of disulfide exchange under these conditions. We conclude that bicyclic analogues of AP can be prepared by selective deprotection and oxidation of blocked cysteine residues.

Biology. The receptor-binding properties of the AP analogues discussed here were evaluated in a test involving competitive displacement of ^{125}I-AP(103–126) from a rabbit lung membrane preparation. Specific, high-affinity atriopeptin receptors have been identified in particulate fractions prepared from rabbit lung.[13] Competitive binding studies and affinity cross-linking experiments using a linear AP analogue revealed the presence of two populations of AP binding sites, a small proportion (approximately 27%) of which are linked to guanylate cyclase activity.[14] Binding of AP analogues to the cyclase-linked receptors correlates with smooth muscle relaxant activity.[15] For convenience, this receptor subclass is referred to as the vasorelaxant (VR) receptor. The other population of high-affinity binding sites (approximately 73%) is not associated with guanylate cyclase or vasorelaxant activity and so are termed nonvasorelaxant (NVR) binding sites. Those analogues that appear to be nonselective have apparent K_i values for the two binding sites that are about equal. Multiple classes of AP binding sites have been reported[16–20] for other tissues, but the NVR sites do not seem to be associated with any known second messenger system.

The vasorelaxant activity of the peptides was measured with norepinephrine (NE) contracted rabbit aortic rings and compared to the activity recorded for **1** in a second aortic ring cut from the same preparation.

Discussion

From model building, it appears that conformational restriction of AP by incorporation of a second disulfide linkage across residues 108 and 117 could stabilize an antiparallel β-structure in which the β-turn occurs at residues 111–114 (Asp-Arg-Ile-Gly). Epand[4] has suggested that a β-structure that he observed for **1** in the presence of phospholipid membranes is directly relevant to the conformation that this peptide must assume in order to be recognized by the receptor. It is also interesting that imposition of this particular secondary structure results in a β-sheet having considerable amphiphilicity. Although no evidence exists that indicates that AP assumes an amphiphilic conformation when associated with its receptor(s), it has been proposed[21,22] that specific peptide/receptor interactions can be facilitated in some cases by the peptide assuming an amphiphilic secondary structure. We felt that these factors made replacement of residues 108 and 117 with cysteine moieties an attractive conformational restriction of the AP molecule.

The synthesis of **3** confirmed that replacement of glycine at position 108 with an Acm-protected cysteine resulted in only a modest drop in vasorelaxant activity while maintaining full binding potency. Although **4** (replacement of Ser^{117} with Cys(Acm)) was still highly potent in the muscle relaxation assay, it was 1 order of magnitude less active than **2**. Unexpectedly, this analogue proved to be modestly selective for the NVR binding site. Binding potency to this site marginally increased while a drop in affinity to the VR receptor was observed. Nevertheless, this preliminary data confirmed that the choice of conformational restriction by modifications of the 108 and 117 positions was acceptable.

The bicyclic analogues **6** and **9**, as well as their monocyclic precursors (**5**, **7**, **8**, **10**) all exhibited reduced vasorelaxant potencies. In light of the fact that these six structures were prepared in order to test the synthetic method rather than to probe a specific conformational restriction, these results are not surprising. On the other hand, the radioreceptor assay (RRA) data are unexpected. In each case, affinity to the VR binding sites is significantly lower than that of **1**. However, binding to the NVR sites is very high, affording relative potencies about equal to that of **1**. It is also interesting that the pairs of bicyclic analogues that differ only by the method of synthesis afford similar values in both biological assays. This provides additional evidence that these pairs of peptides are conformationally equivalent as well as structurally identical.

As previously described, the target analogue **12** was prepared via two different routes. The monocyclic precursor **11** is structurally similar to the natural product and has excellent vasorelaxant activity and a similarly high, nonselective binding potency; bicyclization to **12a** is accompanied by 1 order of magnitude drop in vasorelaxation. Interestingly, this conformationally restricted peptide is also found to be selective for the NVR binding sites. A similar binding selectivity is observed for **13**, in which less conformational restriction is imparted by shifting the disulfide linkage from the 105/121 positions to residues 108/117.

Although bicyclization of **11** affords a loss in vasorelaxant activity, the product **12** is still quite active, having an EC_{50} in the 10^{-7} M range. This suggests that, while restriction in this manner is not ideal, it does allow the molecule to assume a conformation that permits effective interactions with the receptor linked to vasorelaxation. Despite the inability to relate the NVR class of binding sites to biological activity, the RRA clearly shows that this conformationally restricted analogue has the same affinity for these sites as does **1**.

Model building, in which the previously described β-sheet structure is enforced on **12**, allows two observations to be made. First, the disulfide linkage between residues 108 and 117 is sterically bulky and extends into the hydrophobic face of the β-sheet. Such bulk might interfere with effective receptor/ligand interactions. While the disulfide group is necessary to conformationally restrict the molecule, its precise location relative to the putative

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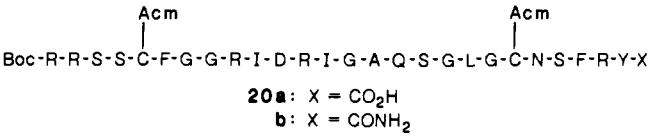
β-sheet structure can be varied somewhat. Examination of the model shows that replacement of residues 108 and 117 with D-cysteine moieties would shift this disulfide bridge from the hydrophobic face to the hydrophilic face. In fact, the monocyclic peptide **14** is nonselective and exhibits a vasorelaxant activity that is about 1 order of magnitude less than that of **11**. Bicyclization results in an additional 6-fold loss of vasorelaxant activity together with a high degree of selectivity, once again to the NVR binding sites.

Further examination of the model shows that the disulfide bridge resulting from the cystine moiety at positions 108/117 tends to pinch the β-sheet, distorting hydrogen bonding between the two strands and causing the fully extended model to bend slightly, in much the same fashion as an open book. This distortion can be relieved somewhat by replacing one or both of these residues with homocysteine, effectively inserting one or two additional methylene units into the bridge. In fact, the monocyclic peptides **16** and **18** are less potent in the rabbit aorta vasorelaxation assay than the corresponding cysteine analogue **11**, and the RRA shows both to be nonselective. Once again, bicyclization results in an additional loss of vasorelaxant activity and the appearance of selectivity favoring the NVR sites. While each of these analogues is about equipotent to **12**, the lower potency of the monocyclic precursors results in a smaller additional drop in vasorelaxant activity (5-fold and 2-fold, respectively).

Although a second disulfide bridge between residues 108 and 117 can stabilize a β-sheet conformation, none of the analogues described here exhibit enhanced vasorelaxation. Seemingly, this would indicate that such a β-sheet does not allow appropriate interactions with the VR subclass of binding sites. However, these receptors appear to have very specific requirements for effective interactions with the ligand. Although the bicyclic peptides described here have lower potency in the vasorelaxation assay and reduced affinity for the VR receptors, they are still potent analogues (EC_{50}s of 0.05–0.3 μM). Accordingly, we feel that these peptides represent examples of AP analogues that can assume a conformation that closely approximates that of the natural product when it is productively associated with the VR receptor. Since these bicyclic analogues are structurally perturbed as well as conformationally restricted, it is not possible to conclude which factor is predominately responsible for the observed decrease in activity. While the data presented here do not prove the validity of a β-sheet model for interaction of AP with the VR receptor, it does suggest that effective interactions with the VR receptors can still be achieved by incorporating a second disulfide bridge between residues 108 and 117. Varying the location of this second disulfide could provide additional insights into the validity of this model. These studies are being pursued.

In contrast, the NVR binding sites appear to tolerate a wide range of conformational restrictions in the AP ligand. Other reports have shown that a cyclic 15 amino acid ring delted peptide,[23] a 21 amino acid truncated, linear analogue,[24] and an eight amino acid linear fragment of AP[25] all bind selectively to the NVR binding sites with very high affinity. Accordingly, it seems clear that a β-sheet is not required for effective interactions of atrial peptides with this subclass of binding sites.

The effects of reducing conformational restrictions by breaking the disulfide bridge between residues 105 and 121 were not directly examined in this laboratory. However, several reports[26–28] indicate that such acyclic AP analogues are less potent than the corresponding cyclic peptides. For example, Napier and co-workers[27] have shown that **20a** and **20b** both have reduced potencies (by about 2 orders of



20a: X = CO_2H
b: X = $CONH_2$

magnitude) and excellent correlation between the functional assay (rabbit aorta tissue) and the RRA (both rabbit aorta and rabbit kidney membranes). The ability of these analogues to relax vascular tissue is similar to that of **12**. At the same time, no clear selectivity to the NVR binding sites is observed. It seems likely that a favorable conformational stabilization is imparted by the cystine of the natural product. The additional constraints of a second disulfide bridge between residues 108 and 117 have a negative impact on biological activity. However, any disruption of the conformational stabilization imparted by the cystine at residues 105 and 121 appears to be limited to the VR subclass of receptors since these bicyclic analogues all maintain full affinity to the NVR binding sites.

In conclusion, conformational restriction of AP by incorporating a second disulfide bridge across residues 108 and 117 does not improve the vasorelaxant potency of **2**. However, these unusual bicyclic peptides are still very potent, having EC_{50}s approaching 100 nM in the rabbit aorta vasorelaxant assay. Even more interesting is the appearance of binding selectivity in many of these analogues. Since binding to the NVR site is invariably favored here, it seems clear that the VR receptors are considerably more sensitive to the conformational restrictions enforced on these molecules than are the NVR binding sites. The requirements for effective interactions between these bicyclic AP analogues and the VR subclass of receptors appear to be very specific, and while the conformational restrictions described here are not ideal, these ligands still exhibit excellent binding affinity to the VR sites (app K_is ≅ 25 nM). We conclude that the analogues described here can still assume a conformation that permits effective interactions with both the NVR and the VR binding sites.

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Experimental Section

Compositional analysis data were collected from 6 M HCl hydrolysates (vapor phase, 110 °C, 24 h) by ninhydrin-based analysis performed on a Beckman 6300 high-performance analyzer. Automated Edman degradation chemistry was used to determine the peptide sequence analysis data. An Applied Biosystems, Inc. (ABI) Model 470A gas-phase sequencer equipped with an on-line ABI Model 120 HPLC was employed for the degradations. Positive-ion FABMS was carried out on a VG-ZAB SE double focusing mass spectrometer. The FAB beam consisted of xenon neutrals at an acceleration of 9 kV (1 mA). The mass spectrometer was scanned at a rate of 10 s/decade over a mass range of 100–3000 daltons at a resolution of 1000. The FAB matrix used was a mixture of glycerol/thioglycerol/HCl/dimethyl sulfoxide. HPLC was carried out on a Waters system consisting of two Model 510 pumps connected to a Model 680 automated gradient controller. Additional solvent mixing was accomplished by an Axxiom Model 400 mixer. Column effluent was monitored at 220 nm by a Waters Model 481 spectrophotometer and peak area was measured with a Waters Model 740 integrator. All eluents for both high-pressure and medium-pressure liquid chromatography were HPLC grade and 0.05% trifluoroacetic acid (TFA; spectrograde) was added.

Peptide Synthesis. Solid-phase peptide syntheses were carried out on *p*-methylbenzhydrylamine hydrochloride resin (~0.45 mequiv/g) with an ABI Model 430A peptide synthesizer. All protected amino acids, reagents, and solvents were purchased from ABI and used directly with no further purification. The peptides were removed from resin and deprotected with anhydrous hydrogen fluoride (HF; 10 mL/g of resin-bound peptide) containing anisole (1 mL/g) and 2-mercaptopyridine (0.1 g/g) at 0° C for 1.5 h on a Peptide Institute Type I HF-Reaction Apparatus. After evaporation of the HF, the residue was triturated successively with ether (5 × 50 mL) and ethyl acetate (4 × 50 mL). The residue was then washed with aqueous acetic acid (50 mL of 30% AcOH followed by 2 × 50 mL of 5% AcOH). The aqueous fractions were combined and lyophilized. The crude peptide was dissolved in 0.1 M tris(hydroxymethyl)aminomethane (Tris) buffer (pH 8; 25 mL/g). An equal weight of dithiothreitol (DTT) was added and the resulting solution was stirred at room temperature for 1 h before being filtered and loaded directly onto a glass column (350 mm × 40 mm i.d.) packed with C_{18} silica gel (Vydac; 15–20 μm, 300 A) and equilibrated with 5% acetonitrile in water. Eluent flow (5% acetonitrile; ~6 mL/min) was initiated, and isocratic conditions were maintained until DTT and Tris eluted. At this time, a gradient was initiated by adding 50% acetonitrile (1000 mL) dropwise to the solvent reservoir at such a rate the reservoir level was maintained at 1000 mL. Fractions (~8 mL) were collected and like fractions were combined and lyophilized to afford a solid that was generally >80% pure.

Cyclization. The partially purified, acyclic peptide was dissolved in 0.1 M ammonium bicarbonate (1.5 mg/mL) and stirred open to the air. The course of the reaction was monitored via HPLC. After cyclization was complete (several h to several days), the solution was acidified (30% AcOH) and lyophilized. The resulting solid was purified via HPLC on a C_{18} silica gel column (Vydac; 22 mm i.d. × 250 mm, 15–20 μm, 300 A) eluting with a linear gradient of 15–35% acetonitrile over 25 min at a flow rate of 9 mL/min.

Bicyclization was accomplished by dissolving the purified monocyclic peptide in a solution of 0.02 M iodine in 80% AcOH (1 mg/mL). The course of the reaction (<15 min to 2 h) was monitored by HPLC. Iodine was removed by diluting the reaction mixture with water to about 4 times the original volume and extracting with ether until no color remained in the aqueous phase. Final purification was accomplished via HPLC as described above. Yields were unoptimized. Greater emphasis was placed on peptide purity, which resulted in decreased yields. Moreover, only a sufficient quantity of peptide was purified to complete the necessary analyses/assays. All peptides were purified to greater than 97% purity. Amino acid analyses were in agreement with the expected results.

Biological Assays. Receptor-Binding Assay. Atrial peptide analogues were studied in a competitive binding assay with rabbit lung membranes as described previously.[13] The binding assay consisted of 0.25 mL of a solution containing 50 mM Tris (pH 7.5), 0.1% bovine serum albumin, 100 μg of membrane protein, and ^{125}I-AP(103–126) ((2–3) × 10^4 cpm; specific activity of 500–1000 Ci/mmol) in the absence or presence of different concentrations of unlabeled peptide analogue. The reaction was initiated by the addition of membranes and the mixture was incubated at 25 °C for 30 min. The incubation was terminated with 4 mL ice-cold 50 mM Tris (pH 7.5) and the mixture was filtered to separate membrane-bound labeled peptide from free ligand with a PHD Cell Harvester (Cambridge Technology, Inc.). The assay tube and filter were washed with ice-cold buffer. Filters were assayed for radioactivity in a Micromedic gamma counter. Nonspecific binding was defined as binding in the presence of a 10^{-6} M unlabeled 1. Specific binding was calculated as total binding minus nonspecific binding. Binding data were analyzed by a nonlinear least-squares fitting program.

Rabbit Aorta Vasorelaxant Activity Assay. Male New Zealand White rabbits (2–2.5 kg) were sacrificed with an overdose of pentobarbital. The thoracic aorta was removed, cleaned of adherent fat and connective tissue, and then cut transversely into 3-mm ring segments. The endothelium was removed from the rings by gently sliding a rolled-up piece of filter paper into the vessel lumen. The rings were then mounted in a water-jacketed tissue bath, maintained at 37 °C, between a moveable and fixed stainless steel wire, with the moveable end attached to an FT03 Grass transducer coupled to a Model 7D Grass Polygraph for recording isometric force responses. The bath was filled with 20 mL of oxygenated (95% oxygen/5% carbon dioxide) Krebs solution of the following composition (mM): 130 NaCl, 15 $NaHCO_3$, 15 KCl, 1.2 NaH_2PO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$, and 11.4 glucose. The preparations were equilibrated for 1 h before approximately 1 g of passive tension was placed on the rings. NE was then added to the bath to achieve a final concentration of 50 nM and when the isometric tension response had reached a plateau level, 1 or the test peptide was added to the bath cumulatively (every 10 min) until relaxation back to the baseline was achieved. A plot of log [peptide] vs % relaxation was then used to calculate the EC_{50} value for each test peptide. These values were then compared to the EC_{50} response for the standard (1) which was obtained from concentration–response data measured in an identical ring segment from the same rabbit. Each test peptide was evaluated in aortas from two rabbits.

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