Topographic Probes of Angiotensin and Receptor: Potent Angiotensin II Agonist Containing Diphenylalanine and Long-Acting Antagonists Containing Biphenylalanine and 2-Indan Amino Acid in Position 8†

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A series of phenylalanine-mimicking amino acids with increasing conformational restraint were prepared and incorporated into angiotensin II, in order to develop topographic probes of angiotensin useful for probing receptor boundaries by molecular graphics analysis and for conformational analysis of the ligand by NMR. In binding studies, all analogues displayed high affinity for rat uterus (K_i of 0.74–6.08 nM) and brain (0.46–1.82 nM) receptors. In smooth muscle (rat uterus) contraction assay, the diphenylalanine-containing [Sar^1,Dip^8]AII and [Sar^1,D-Dip^8]AII were potent agonists with respectively 284% and 48% activity of [Asn^1]AII. In contrast, the biphenylalanine-containing [Sar^1,Bip^8]AII, [Sar^1,D-Bip^8]AII, and the 2-indan amino acid containing [Sar^1,2-Ind^8]AII were potent inhibitors, approximately 9, 2, and 1.4 times more effective than a standard antagonist, [Sar^1,Leu^8]AII. Their respective pA_{10} values in rat uterus assay were 8.87, 8.70, and 8.82. By comparison, the pA_{10} value for [Sar^1,Leu^8]AII was 8.35. In rats, a single dose of 10 μg of [Sar^1,2-Ind^8]AII or [Sar^1,Bip^8]AII produced prolonged blockade of the pressor response toward angiotensin II for over 90 min. The very different pharmacological profiles of these rigid aromatic analogues suggest that the angiotensin receptor activation site consists of a relatively wide and elongated pocket with a narrow opening.

The discovery that the endogenous opioid, enkephalin, binds to morphine receptor[1-3] and induces analgesia and addiction strongly suggests that pharmacophores essential to the highly flexible and linear enkephalin peptide can assume a biologically active conformation resembling that of the rigid alkaloid morphine. Other than providing a rationale for the pharmacological actions of exogenous drugs, this finding further indicates the feasibility of developing nonpeptidic therapeutic agents from peptide structures, i.e., morphine from enkephalin. This is an especially exciting possibility in view of the many genetically engineered peptides created by the rapidly advancing recombinant biotechnology that may find therapeutic applications in the near future.[4,5]

Because of the inherent flexibility of the peptide chain, multiple conformations of the ligand and multiple receptor subtypes are often observed. This conformational multiplicity, in turn, suggests that a peptide spends only a portion of its time in the biologically active conformation. If such a conformation can be imposed, a far more effective ligand could result. In addition, non-peptide structure retaining this three-dimensional conformation should mimic the actions of the ligand, much like the morphine interaction with enkephalin receptors. Complementary to this need of defining the transition-state conformation of the ligand for developing non-peptide drug from peptide structure, an understanding of the topographic boundaries of the different receptor types can provide insight into their conformational complementarity with the ligand and advance the design of receptor-selective ligands. For both purposes, it is necessary to develop rigid ligands with well-defined topography that do not undergo conformational transition during ligand–receptor interaction.[6]

In an initial step to develop such a probe, we use the well-studied angiotensin (AII, Asp-Arg-Val-Tyr-Val-His-Pro-Phe) as the experimental model. Extensive SAR (structure–activity relationship) studies showed that while its carboxylate terminus[7] and the N$^\tau$-H structure[8] of the 6-His are important for receptor recognition, replacement of the 8-Phe by aliphatic amino acids led to potent antagonists[9-13] and modification of the 4-Tyr produced a weak inhibitor.[14] The potential interaction of these closely clustered essential pharmacophores is implicated by CD studies in which the 4-Tyr chromophore was shown affected by the 8-substituents,[15] yet NMR evidence indicated aromatic stacking of 8-Phe with 6-His but not with 4-Tyr.[16]

† The abbreviations used to denote amino acids and peptides are those recommended by the IUPAC Commission on Nomenclature (*Biochemistry* **1975**, *14*, 449; **1967**, *6*, 362). Other abbreviations are as follows: AII, angiotensin II (Asp-Arg-Val-Tyr-Val-His-Pro-Phe); Bip, biphenylalanine; Dip, diphenylalanine; 2-Ind, 2-aminoindan-2-carboxylic acid; Pgl, phenylglycine; Boc_2O, di-*tert*-butyl dicarbonate; Me_2SO, dimethyl sulfoxide; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole monohydrate; DFP, diisopropyl fluorophosphate; DTT, dithiothreitol; EGTA, ethyleneglycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; PMSF, (phenylmethyl)sulfonyl fluoride.

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Table I. Physicochemical Characterizations of Unusual Amino Acid Derivatives Prepared for This Study

synthetic amino acid derivatives	yield, %	mp, °C	FABMS,[a] M + 1	TLC, R_f[b] CMA	TLC, R_f[b] sBuOH·NH_3	NMR
Boc-DL-Dip	38	135–138	342	0.84	0.48	($CDCl_3$) δ 1.53 (s, 9 H, $(CH_3)_3CO$), 4.47 (d, 1 H, $C_\alpha H$), 5.0 and 5.13 (bs, 2 H, $C_\beta H$ and N*H*), 7.3 (s, 10 H, diphenyl $C_{12}H_{10}$), 9.6 (s, 1 H, COO*H*)
DL-Dip-OBzl·HCl	46	191–194	332	0.71	0.77	($CDCl_3$–Me_2SO-d_6) δ 4.67 (bs, 2 H, β and α*H*), 4.90 (d, 2 H, benzylic CH_2), 6.83–7.23 (bs, 10 H, diphenyl $C_{12}H_{10}$), 7.40 (d, 5 H, benzylic C_6H_5), 8.3 (s, 3 H, NH_3^+)
2-Ind-OBzl·HCl[c]	61	197–199	268	0.56	0.74	($CDCl_3$–Me_2SO-d_6) δ 3.64 (s, 4 H, indane β,β′ CH_2), 5.23 (s, 2 H, benzylic CH_2), 7.2 (s, 4 H, indane C_6H_4), 7.3 (s, 5 H, benzylic C_6H_5), 9.2 (bs, 3 H, NH_3^+)
Boc-DL-Bip[d]	30	145–147	342	0.83	0.39	($CDCl_3$) δ 1.57 (s, 9 H, $(CH_3)_3CO$), 3.2 (d, 2 H, $C_\beta H_2$), 4.67 and 5.08 (bs, 2 H, C*H* and N*H*), 7.23–7.65 (m, 9 H, biphenyl $C_{12}H_9$), 10.07 (s, 1 H, COO*H*)
DL-Bip-OBzl·HCl	12[e]	200–203	332	0.60	0.72	($CDCl_3$–Me_2SO-d_6) δ 3.33 (d, 2 H, $C_\beta H_2$), 4.17–4.4 (m, 1 H, $C_\alpha H$), 7.27–7.57 (m, 14 H, $C_{12}H_9$ and C_6H_5)

[a] Fast atom bombardment mass spectrometry gave the molecular ion for the desalted form of amino acid benzyl ester. [b] R_f value in the solvent system of 85:15:10 chloroform–methanol–acetic acid (CMA) or 100:44 of *sec*-butyl alcohol –3% NH_4OH (sBuOH·NH_3). [c] Boc-2-Ind was reported in a previous study.[11] [d] Incorporation of Boc-D-Bip into LHRH was indicated,[20] although detailed information is not available. [e] Yield of the crude product was 51%, which was further purified on a silica gel column eluted with 9:1 $CHCl_3$–CH_3OH.

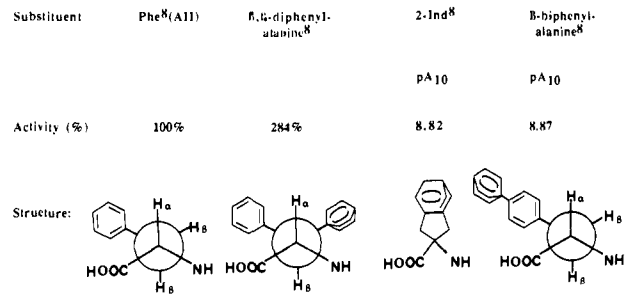

Figure 1. Projection of the position 8 substituents. In rat uterus contraction assay, [Sar[1],Dip[8]]AII was 284% as active as [Asn[1]]AII. Both [Sar[1],2-Ind[8]]AII and [Sar[1],Bip[8]]AII were potent antagonists with a calculated pA_{10} of 8.82 and 8.87, respectively.

Because of the crucial role of the 8-Phe residue in both recognition and activation of the receptor, in addition to its effect on angiotensin II conformation, we prepared a series of phenylalanine-mimicking analogues with increasing bulk and conformational restraint. These analogues contain a second phenyl ring in the β or para position of 8-Phe or have its Phe ring locked to the peptide backbone (Figure 1). Because of the well-defined size, shape, and orientation of the planar phenyl ring, these topographic probes are useful for molecular graphics analyses of receptor boundaries normally occupied by Phe in the ligand. In addition, the improved capacity to exert paramagnetic shielding or deshielding of neighboring pharmacophores in NMR by the expanded aromatic region in these probes facilitates conformational analyses of the ligands by NMR. Incorporation of these novel amino acids into angiotensin gave [Sar[1]]AII containing diphenylalanine (Dip), biphenylalanine (Bip), or 2-aminoindan-2-carboxylic acid (2-Ind) in position 8. These analogues were examined for their ability to bind to peripheral (rat uterus) and CNS (rat brain) receptors, their pharmacological profile in a myotropic (rat uterus) assay, and their therapeutic potential in AII-mediated hypertension.

Results and Discussion

Chemical synthesis of the unnatural amino acids diphenylalanine[17] (DL-Dip) and biphenylalanine[18] (DL-Bip) through acetamidomalonate condensation with the appropriate aralkyl halide was according to a reported procedure. 2-Aminoindan-2-carboxylic acid (2-Ind) was prepared through Strecker synthesis with 2-indanone as described.[19]

Enzymatic resolution of Ac-DL-Bip to L-Bip by acylase from the *Aspergillus* genus has been reported,[18] and Boc-D-Bip has been incorporated into luliberin (LHRH).[20] In preliminary studies, both Ac-DL-Dip and Ac-DL-Bip were relatively resistant to hog kidney acylase and carboxypeptidase resolution. Therefore, the racemic amino acids were used for subsequent experiments.

Initial attempts to prepare amino acid benzyl ester through anhydrous HCl-catalyzed or toluenesulfonic acid catalyzed esterification[21] with benzyl alcohol presented problems. The incomplete esterification even after prolonged reaction (over 2 weeks) made it difficult to separate the highly lipophilic, unreacted amino acid from its benzyl ester. Instead, we observed that N-terminal protection of DL-Dip and DL-Bip using di-*tert*-butyl dicarbonate[22] (Boc_2O) in dimethyl sulfoxide–*tert*-butyl alcohol with tetramethylammonium hydroxide proceeded satisfactorily at 60 °C overnight. Subsequent reaction of the tetramethylammonium salt of Boc-DL-Dip, Boc-2-Ind, or Boc-DL-Bip with benzyl chloride in Me_2SO at 60 °C gave the appropriate benzyl ester derivatives, which were treated with 4 N HCl in dioxane to give the corresponding DL-Dip-OBzl·HCl, 2-Ind-OBzl·HCl, and DL-Bip-OBzl·HCl. A list of their yield, melting point, molecular weight as determined by fast atom bombardment mass spectrometry and proton magnetic resonance patterns is shown in Table I.

Stepwise synthesis of Boc-Sar-Arg(NO_2)-Val-Tyr-(Bzl)-Val-His(Bzl)-Pro-resin by the solid-phase method[23] followed by $Pd(OAc)_2$-catalyzed hydrogenolysis of the peptide-resin[24] gave Boc-Sar-Arg-Val-Tyr-Val-His-Pro, which was purified to homogeneity by SP-Sephadex ion-exchange chromatography as described.[25] Solution cou-

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Table II. Synthetic and Analytical Data of Angiotensin Analogues Prepared for This Study

compound	yield,[a] %	R_f value[b] BPAW	R_f value[b] BAW	k value[c] in CCD	amino acid analysis 1	2	3	4	5	6	7	8	FABMS (M + 1)	peptide content, %
[Sar^1,DL-Dip^8]AII	35	0.26	0.29		1.03	0.98	1.01	1.07	1.01	*d*	0.89	*d*	1065	73
[Sar^1,Dip^8]AII	6	0.25	0.29	1.30	1.02	1.15	0.89	0.96	0.89	*d*	1.08	*d*	1065	94
[Sar^1,D-Dip^8]AII	7	0.26	0.29	1.64	1.09	1.15	0.87	0.98	0.87	*d*	1.01	*d*	1065	57
[Sar^1,2-Ind^8]AII	18	0.26	0.29		1.07	1.01	1.03	1.11	1.03	0.89	0.94	0.94	1006	66
[Sar^1,DL-Bip^8]AII	51	0.28	0.29		1.05	0.95	1.01	1.10	1.01	*e*	0.88	*e*	1065	63
[Sar^1,Bip^8]AII	5	0.28	0.29	1.61	0.81	1.22	0.91	1.01	0.91	*e*	1.15	*e*	1065	70
[Sar^1,D-Bip^8]AII	10	0.28	0.29	1.47	0.94	1.16	0.94	1.00	0.94	*e*	1.03	*e*	1065	92

[a] Maximal yield for the diastereomer mixture is 100%, and that for either the L or D congener is 50%. [b] R_f value in the upper phase of the solvent system of 8:1:2:9 of 1-butanol–pyridine–acetic acid–water (BPAW) or 4:1:5 of 1-butanol–acetic acid–water (BAW). [c] Partition coefficient for countercurrent distribution in 8:1:2:9 1-butanol–pyridine–acetic acid–water for 300 transfers for the Dip-containing analogues and for 1090 transfers for the Bip-containing analogues. [d] Dip was coeluted with His in amino acid analysis. [e] Bip was coeluted with His in amino acid analysis.

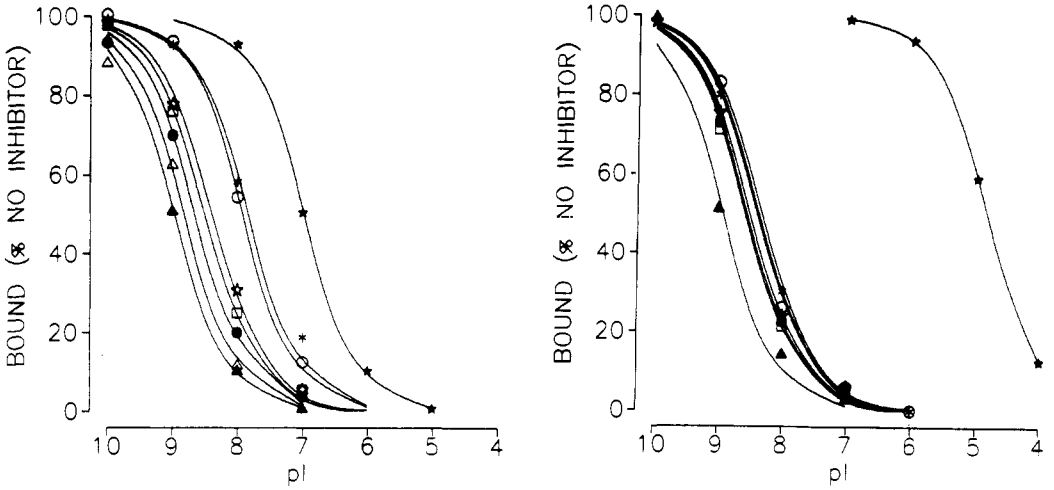

Figure 2. Competition for ^{125}I-[Sar^1,Ile^{5,8}]AII binding sites by angiotensin II and analogues, in rat uterus (left) and brain (right). Symbols are ★, [Sar^1,Pro-NH$_2$^{7},desPhe^8]AII; *, [Asn^1]AII; ○, [Sar^1,D-Dip^8]AII; ☆, [Ile^5]AII; □, [Sar^1,Bip^8]AII; ●, [Sar^1,Dip^8]AII; △, [Sar^1,2-Ind^8]AII; ▲, [Sar^1,D-Bip^8]AII. p*I* is the negative log of the concentration of the competing analogue. Specific binding of the tracer (220 pM) to uterus (approximately 10 mg initial wet weight) was 4700–5200 cpm. Nonspecific binding ranged from 400 to 800 cpm. Thus, specific binding represented about 88% of total binding. Specific binding in the brain (approximately 9 mg initial wet weight) was 5300–6400 cpm. Nonspecific binding ranged from 2100 to 3100 cpm. Thus, specific binding represented 69% of total binding.

pling of this [Boc-Sar^1,des-Phe^8]AII heptapeptide with DL-Dip-OBzl·HCl, 2-Ind-OBzl·HCl, or DL-Bip-OBzl·HCl by dicyclohexylcarbodiimide/hydroxybenzotriazole[26] in dimethylformamide at 60 °C for 2–3 days gave the appropriate benzyl ester derivatives.

Purification of [Boc-Sar^1,2-Ind-OBzl^8]AII by ion-exchange chromatography (CM-Sephadex) followed by catalytic hydrogenation and acidolysis deprotection of the product gave [Sar^1,2-Ind^8]AII, which was eluted from SP-Sephadex with 0.2 N NH_4OAc.

In contrast, elution of the highly lipophilic [Boc-Sar^1,DL-Dip-OBzl^8]AII and [Boc-Sar^1,DL-Bip-OBzl^8]AII from SP-Sephadex column required unusually high ionic strength (1 N NH_4OAc in 20% AcOH). Subsequent N- and C-terminal deprotection gave [Sar^1,DL-Dip^8]AII and [Sar^1,DL-Bip^8]AII, which still required high ionic strength (0.4 N NH_4OAc) for elution from the SP-Sephadex column. Each diastereomeric mixture was clearly separated into two fractions by countercurrent distribution. Tentative stereochemical assignments of the resultant diastereomers were based on observations that the L diastereomer was biologically more active than its D counterpart in smooth muscle contraction assays either as an agonist[9] or as an antagonist to angiotensin.[9,25] This assignment remains to be confirmed by a complementary method. The synthetic and analytical data for these analogues are listed in Table II.

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Table III. Binding of Angiotensin Analogues to Rat Uterus and Brain Receptors

	K_i,[a] nM uterus	brain
[Ile^5]AII	1.90 ± 0.38	1.37 ± 0.56
[Asn^1]AII	5.48 ± 4.42	1.64 ± 0.98
[Sar^1,Pro-NH$_2$^{7},des-Phe^8]AII[b]	52.8 ± 14.0	9310[c]
[Sar^1,Dip^8]AII	1.23 ± 0.25	0.85 ± 0.88
[Sar^1,D-Dip^8]AII	6.08 ± 2.61	1.64 ± 0.87
[Sar^1,2-Ind^8]AII	1.52 ± 0.63	1.82 ± 1.09
[Sar^1,Bip^8]AII	1.74 ± 0.10	1.00 ± 0.61
[Sar^1,D-Bip^8]AII	0.74 ± 0.12	0.46 ± 0.25

[a] K_i value was determined from IC_{50}, which is the concentration of AII or analogues producing 50% displacement of the tracer [Sar^1,Ile^{5,8}]AII. K_D for ^{125}I-[Sar^1,Ile^{5,8}]AII binding to uterus and brain homogenates was 347 ± 67 and 252 ± 131 pM, respectively. Results are the mean ± SD in three experiments, and each experiment was performed in duplicates. [b] The heptapeptide amide had a M + 1 of 841 in fast atom bombardment mass spectrometry (FABMS) analysis, and a hydrolysate of the peptide gave the appropriate amino acids in the correct ratio. [c] $N = 1$, with two other experiments indicated IC_{50} values $\gg 10^{-6}$ M.

Biological studies were conducted to determine whether the increased bulk of the ligands would lead to their steric repulsion by the receptor and whether receptors from different tissues display differential binding to these closely related probes as a result of topographic differences and regioselectivity of receptor subtypes. Binding studies using rat uteri or brain homogenate and ^{125}I-[Sar^1,Ile^{5,8}]AII as the radioligand[27] were performed at different concen-

Table IV. Pharmacological Profiles of the Topographic Probes on Rat Uterine Preparations

	agonistic[a] activity (%)	inhibitory activity[b] (fold of shift in AII DRC): dose of inhibitors, ng/mL			calcd pA_{10}
		2	4	6	
[Asn^1]AII	100				
[Sar^1,Leu^8]AII		1.7 ± 0.1 (5)	3.9 ± 0.4 (6)	22.2 ± 2.3 (5)	8.35
[Sar^1,Dip^8]AII	284 ± 13 (3)				
[Sar^1,D-Dip^8]AII	48 ± 2 (3)				
[Sar^1,2-Ind^8]AII	negligible	8.7 ± 0.5 (3)	16.2 ± 3.7 (3)	31.8 ± 11.2 (3)	8.82
[Sar^1,Bip^8]AII	negligible	9.2 ± 1.5 (5)	71.7 ± 13.3 (4)	208.2 ± 26 (4)	8.87
[Sar^1,D-Bip^8]AII	negligible	7.2 ± 1.3 (3)	24.6 ± 5.6 (4)	44.7 ± 5.1 (4)	8.70

[a] Agonistic activity of an analogue was expressed as the ratio of the dose of [Asn^1]AII producing a 50% maximal response (ED_{50}) to the dose of the analogue producing the same response. Results are the mean ±SEM, with the number of experiments indicated in parentheses. [b] Inhibitory potency of an analogue was calculated as the ratio of the dose of [Asn^1]AII required to produce 50% of the control maximal response in the presence and absence of the inhibitor.

trations of each analogue. The IC_{50} value was determined by Hill analysis (Figure 2), and the K_i value was determined from the IC_{50} value as described by Cheng and Prusoff.[28] In this system, [Sar^1,Dip^8]AII, [Sar^1,2-Ind^8]AII, and [Sar^1,Bip^8]AII bound to both uterus and brain receptors with high affinity (K_i of 1.23, 1.52, and 1.74 nM vs 1.90 for [Ile^5]AII and 5.48 for [Asn^1]AII for uterine homogenates), as did the D congeners (Table III). Because the receptor binding assay assesses the extent of complementarity of ligand with receptor but does not measure intrinsic activity, it does not distinguish agonists from antagonists. In addition, the binding affinity of the ligand does not always parallel its ability to promote or inhibit receptor activation, for which the nature of the activation pharmacophores of the ligand and their spatial orientation appear to be a major determinant. These considerations may account for the higher potency of the [Sar^1,D-Bip^8]AII over its L congener in the binding assay but not the biological assays. The binding studies suggest that the increased size of the probes does not exceed the critical limit that can be accommodated by the receptor active sites. Interestingly, differences between uterus and brain receptors appear to exist, in that deletion of 8-Phe in the [Sar^1,Pro-NH_2^7,des-Phe^8]AII heptapeptide amide resulted in a 10–30-fold decrease in affinity for uterus but over 5000-fold reduction in brain binding in comparison to [Asn^1]AII and [Ile^5]AII. On the other hand, binding to the uterus was relatively sensitive to N-terminal variation, i.e., K_i of 1.90 vs 5.48 nM for the equipotent [Ile^5]AII and [Asn^1]AII, while binding to the brain was not.

When the analogues were assayed for smooth muscle contraction response, very different pharmacological profiles were observed (Table IV). On isolated rat uterine preparations, [Sar^1,Dip^8]AII was a superactive agonist with 284% activity, while [Sar^1,Bip^8]AII was an unusually potent inhibitor, about 9 times more effective than [Sar^1,Leu^8]AII.

These results can be interpreted in terms of angiotensin–receptor interaction. Because the normal motions of 8-phenylalanine would include the rotation of the C_α–C_β bond (Figure 1) as well as rotation of the phenyl ring along its 1′–4′ axis, a β,β-diphenylalanine can occupy the same receptor pocket for a freely rotating phenylalanine. Thus, the highly agonistic nature of [Sar^1,Dip^8]AII suggests that the receptor pocket is wide enough to simultaneously accommodate two bulky phenyl groups rotating along their 1′–4′ axes. Since [Sar^1,Ile^5]AII was reported to have 140% activity of [Ile^5]AII in rat uterus assay,[28] 1-Sar substitution is expected to increase activity by 1.4-fold. Therefore, the 284% myotropic activity for [Sar^1,Dip^8]AII would suggest that 8-Dip substitution produced a 2-fold increase in activity. This result is consistent with a diphenylalanine ligand having both phenyl rings rotating freely around the C_β atom, thereby activating the receptor twice as frequently as the single phenyl group in angiotensin.

However, receptor activation appears mediated by selective orientation of the phenyl ring rather than by a general aromatic interaction. In a freely rotating phenylalanine, the orientation of the phenyl pharmacophore relative to the carboxylate recognition site can be coplanar, skew, or perpendicular. In rat uterus assay, the 2-indane amino acid analogue, [Sar^1,2-Ind^8]AII, in which the nearly planar, bicyclic indan ring is perpendicular to the peptide backbone (Figure 1), was a potent antagonist with negligible myotropic activity. Thus, the steric arrangement of 8-Phe in the biologically active conformation of AII can be further limited to having the phenyl ring being coplanar with or skew to the peptide backbone.

Extension of the aromatic region results in the biphenylalanine analogue in which the two rings form a rigid and elongated cylinder (Figure 1). Since the rigid biphenyl structure retains the aromatic ring of phenylalanine and its full motional freedom, if the receptor pocket were deep enough to accommodate the full length of the second phenyl group and permit the proper alignment of the internal phenyl group relative to the carboxylate, a fully active agonist would be expected. Instead, this analogue had negligible agonistic activity, but in fact was seven times more effective than [Sar^1,2-Ind^8]AII as an angiotensin antagonist (Table IV). Although the low K_i values (1.74 and 1.00 nM) of this analogue suggest its tight binding with the receptor, its antagonistic nature nevertheless indicates an imperfect fit, probably as a consequence of the elongated biphenyl structure approaching the limit of the receptor boundary. In which case the length of the receptor pocket can be deduced from the longitudinal distance of the biphenyl structure.

In contrast to the potent agonistic or antagonistic nature of [Sar^1,Dip^8]AII or [Sar^1,Bip^8]AII, shortening of the distance between the aromatic ring to the peptide backbone by one carbon to phenylglycine gave the weak antagonist [Sar^1,Pgl^8]AII, comparable to [Sar^1,Ala^8]AII in inhibitory potency and residual agonistic activity.[29] This finding suggests that the receptor contains a sterically hindered region normally reserved for angiotensin carboxylate binding and that this region cannot accommodate a bulky and rigid phenyl group in the α position, thus accounting for the considerably reduced inhibitory activity of [Sar^1,Pgl^8]AII.

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Taken together, our results suggest that the angiotensin receptor active site in rat uterus consists of a relatively wide and elongated pocket with a narrow opening near the C_α region of position 8 in the peptide backbone. Beyond the C_α region, this narrow opening may continue for some distance. Thus, [Sar[1],D-Dip[8]]AII, which requires a "wide" space within the narrow passage was five times weaker in affinity to the uterus receptor than its L congener. In contrast, [Sar[1],D-Bip[8]]AII was twice as effective as its L congener in receptor binding (Table III), suggesting that accommodation of the "elongated" D-Bip structure by the narrow passage is not a problem. In smooth muscle contraction assay, [Sar[1],D-Dip[8]]AII had considerable agonistic activity (48%), whereas [Sar[1],D-Bip[8]]AII displayed significant antagonistic effect (Table IV).

For conformational analysis of angiotensin II, the expanded aromatic region of these topographic probes is valuable in that it can provide improved NMR monitoring of a broad area of the AII molecule unaccessible to the 8-Phe ring, and the added size of this region further reduces its mobility and conformational flexibility. Thus, the availability of the closely related superactive agonist [Sar[1],Dip[8]]AII, antagonist [Sar[1],Bip[8]]AII, and their less active D congeners offers an excellent opportunity to investigate possible conformational differences between AII agonists and antagonists by molecular graphics and NMR analyses. For the latter purpose, the fixed orientation of [Sar[1],2-Ind[8]]AII is especially useful for identifying the relative location of essential pharmacophores in the vicinity of the indan ring. This information can, in turn, help elucidate the three-dimensional arrangement of these pharmacophores, which is central to the long-term design of a nonpeptidic drug for angiotensin receptor blockade.

To evaluate the relative therapeutic usefulness of [Sar[1],2-Ind[8]]AII, [Sar[1],Bip[8]]AII, and [Sar[1],D-Bip[8]]AII, in AII-initiated hypertension, we compared the doses required to achieve the same level of AII inhibition by these analogues and by the standard inhibitor, [Sar[1],Leu[8]]AII. Rats (n = 3–5 for each analogue) received bolus injections of the inhibitors, followed by [Asn[1]]AII (80 ng) at 15-min intervals. This permitted concurrent evaluation of the effectiveness and duration of inhibition of the antagonists, through comparison of the time periods required for the pressor response of AII to return to 50% of its pretreatment levels. At the respective dose ratio of [antagonist]/[AII] of 12.5, 37.5, and 125, bolus injections of 1, 3, and 10 μg of [Sar[1],2-Ind[8]]AII gave the 50% recovery times of 25 ± 2, 63 ± 17, and 96 ± 22 min. Although [Sar[1],Bip[8]]AII was about seven times more potent than [Sar[1],2-Ind[8]]AII in vitro (Table IV), 1, 3, and 10 μg of [Sar[1],Bip[8]]AII gave the less-than-expected but still prolonged 50% recovery times of 62 ± 18, 73 ± 18, and 97 ± 17 min. Interestingly, [Sar[1],D-Bip[8]]AII was less effective than [Sar[1],2-Ind[8]]AII in vivo and had the 50% recovery times of 14 ± 2, 41 ± 6, and 73 ± 18 min. In comparison, 10, 30, and 60 μg of [Sar[1],Leu[8]]AII (dose ratio of 125, 375, and 750) gave the 50% recovery times of 34 ± 6, 72 ± 11, and 83 ± 3 min. Thus, to achieve an antihypertensive duration of 1 h, [Sar[1],Bip[8]]AII, [Sar[1],2-Ind[8]], and [Sar[1],D-Bip[8]]AII were, respectively, 23, 8, and 4 times more effective than [Sar[1],Leu[8]]AII (Figure 3).

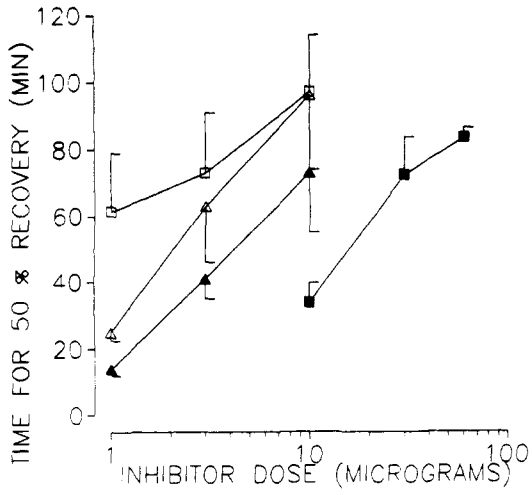

Figure 3. Time course for [Asn[1]]AII (80 ng) induced pressor response in rats to return to 50% of pretreatment level after a bolus injection of the inhibitors. Bars represent standard errors of means determined for each analogue in 3–6 rats. Symbols are □, [Sar[1],Bip[8]]AII; △, [Sar[1],2-Ind[8]]AII; ▲, [Sar[1],D-Bip[8]]AII; ■, [Sar[1],Leu[8]]AII.

Significance

The phenylalanine residue in many peptides, including angiotensin and the pressure-regulatory atriopeptin[30] and bradykinin,[31] is essential to receptor recognition–activation. An important issue is how Phe exerts its effect on ligand conformation and by what approach superactive agonists and antagonists can be developed. The exciting findings of this study show that by modifying the topography of Phe in a systematic manner, extremely effective analogues resulted. These analogues are useful for probing receptor boundaries. In binding studies, the brain and uterus receptors differ in their absolute requirement for the peptide backbone of 8-Phe. It is conceivable that other topographic difference in receptors exists that may be utilized to develop angiotensin analogues selectively active in the CNS. Such analogues are extremely desirable for the unambiguous determination of the physiological functions of the brain renin–angiotensin system in the absence of peripheral interference. As to the biologically active conformation of angiotensin II, the increase in agonistic activity by [Sar[1],Dip[8]]AII over [Asn[1]]AII is impressive but does not approach that expected of a transition-state conformation. In this regard, the potent antagonism displayed by [Sar[1],2-Ind[8]]AII, in which the rigid indan ring is perpendicular to the C-terminal binding site, further suggests that a coplanar or skew orientation of these pharmacophores may be present in the transition-state conformation of angiotensin II.

Two limitations have been associated with the development of therapeutically useful angiotensin and other peptide antagonists: the presence of residual agonist activity[32] and their rapid inactivation by proteolytic enzymes.[5] In this study, we showed that an unexpected benefit of the topographic probes is that conformationally rigid amino acids can impart proteolytic resistance to the resultant peptides. For example, both Dip and Bip derivatives were resistant to acylase and carboxypeptidase proteolysis, and a single injection of 10 μg of [Sar[1],2-Ind[8]]AII or [Sar[1],Bip[8]]AII was effective for 1–2 h. This metabolic stability is probably due to the steric hindrance inherent in conformational rigidity. In addition, the inability of the rigid analogues to adopt the proper molecular orientation and alignment required for receptor activation should be advantageous for developing pure antagonists.

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In in vivo studies, [Sar^1,2-Ind^8]AII had less than 0.2% of the pressor effect of [Asn^1]AII, while [Sar^1,Bip^8]AII, [Sar^1,D-Bip^8]AII, and [Sar^1,Leu^8]AII had respectively, 0.4%, 2.4%, and 1.1% agonistic activity. Thus, in comparison with the more flexible analogues containing an aliphatic amino acid in position 8, the conformational probes [Sar^1,2-Ind^8]AII and [Sar^1,Bip^8]AII also had less residual agonistic effect, approximately $^1/_6$ and $^1/_3$ of that for [Sar^1,Leu^8]AII.

Experimental Section

All chemicals were of reagent grade. N^α-(*tert*-Butyloxycarbonyl)-L-amino acids were supplied by Bachem, Inc., Torrance, CA. Hog kidney acylase and carboxypeptidase Y were obtained from Sigma, St. Louis, MO. Sephadex G-25 and SP-Sephadex C-25 were obtained from Pharmacia, Piscataway, NJ. Microgranular carboxymethylcellulose (CM 52) was from Whatman, Inc., NJ. Melting points (Thomas-Hoover Uni-melt) are uncorrected. Proton magnetic resonance spectra in deuterated chloroform and/or dimethyl sulfoxide containing tetramethylsilane were obtained on a Varian T-60 (60 MHz). Homogeneity of amino acid derivatives and of the synthetic peptides was assessed by thin-layer chromatography (TLC) on Merck precoated silica gel glass plates (type G60-F254) in different solvent systems. The products were identified by a combination of UV, Ninhydrin, Chlorox–KI, and Pauly sprays. Peptide samples were hydrolyzed for 24 h at 110 °C in 6 N HCl + 0.2% phenol containing norleucine or D-alanine as an internal standard in sealed tubes. Amino acid analyses were performed on a Beckman Model 119 analyzer equipped with a Beckman system AA computing integrator using the 4-h methodology. Peptide content was calculated in terms of free peptide.

2-Ind was eluted after tyrosine.[11] Because the unusual amino acid, diphenylalanine or biphenylalanine, was coeluted with histidine, fast atom bombardment mass spectrometry was used to determine the molecular weight of the intact peptides, thus providing direct evidence of the presence of these amino acids in the appropriate analogues.

General Procedure for the Synthesis of Boc-amino Acid. Except for Boc-2-Ind,[11] which was prepared by reacting Boc_2O with 2-Ind in aqueous NaOH–*t*BuOH at room temperature,[22] reaction of the more lipophilic amino acids under similar conditions was very slow and incomplete. Instead, DL-Dip·HCl or DL-Bip·HCl was suspended in methanol and treated with a 2.5-fold excess of tetramethylammonium hydroxide in methanol. This solution was evaporated to dryness, and the residue was suspended in 1:4 Me_2SO–*t*BuOH (2–3 mL/mmol amino acid). To this solution was added Boc_2O (2-fold excess), and the mixture was stirred at 60 °C overnight.

The reaction mixture was diluted with petroleum ether and washed with 2 N HCl, and the product was extracted into 2 N NaOH. Acidification of the NaOH solution with 4 N HCl to pH 1, followed by extraction with Et_2O–EtOAc, gave the corresponding Boc-amino acid. This was recrystallized from EtOAc–petroleum ether. When necessary, silica gel chromatography with $CHCl_3$–CH_3OH elution was performed to further purify the product.

Preparation of Amino Acid Benzyl Ester Hydrochloride Salt. Boc-amino acid from the previous procedure or Boc-2-Ind was treated with excess tetramethylammonium hydroxide in methanol (1.5-fold excess) and evaporated to dryness. The residue was dissolved in Me_2SO (2–3 mL/mmol amino acid), to which benzyl chloride (2-fold excess) was added, and the mixture was stirred at 60 °C until the reaction was complete (4 h to 4 days).

The mixture was diluted with petroleum ether, washed with 2 N NaOH and 2 N HCl, and dried (Na_2SO_4). The solvent was removed, and the residue was treated with 4 N HCl in dioxane at room temperature for 2 h. The corresponding amino acid benzyl ester hydrochloride salt was precipitated from the dioxane solution by the addition of Et_2O, petroleum ether, or their mixture. When necessary, silica gel chromatography eluted with $CHCl_3$–CHOH was performed to further purify the product.

Peptide synthesis was carried out by using Merrifield's stepwise solid-phase method[23] as described previously.[25] The first Boc-amino acid was attached to the chloromethylated polystyrene–1% divinylbenzene resin by using the cesium procedure. Deprotection of the amino group was accomplished with 25% trifluoroacetic acid in methylene chloride. Neutralization was carried out twice by using 5% triethylamine in methylene chloride. Coupling was performed by using a 3-fold excess of Boc-amino acid and dicyclohexycarbodiimide (DCC), and 1-hydroxybenzotriazole was added during coupling of Boc-His(Bzl). Coupling steps were monitored for completion by the Kaiser test[33] and coupling was repeated when indicated. Incomplete sequence was terminated with *N*-acetylimidazole following the coupling step.

Solution coupling of Boc-Sar-Arg-Val-Tyr-Val-His-Pro with the appropriate amino acid benzyl ester was mediated by DCC/HOBt. The octapeptide product was chromatographed, deprotected with catalytic hydrogenation (10% Pd/C, 30 psi H_2 for 2 days) followed by acidolysis (25% CF_3COOH/CH_2Cl_2, 30 min), and purified to homogeneity by ion-exchange chromatography. The diastereomeric pair was well separated by countercurrent distribution, and each diastereomer was further purified by gel-filtration chromatography on Sephadex G-25.

Binding study was performed on Sprague-Dawley rat uteri or brain homogenates in sodium phosphate (50 mM) buffered saline (pH 7.1–7.2) containing $MgCl_2$ (10 mM), EGTA (1 or 5 mM), DTT (1 or 5 mM), and PMSF (0.1 mM) or DFP (1 mM), as described.[27] The membrane fraction was obtained as the 40000*g* precipitate of tissue homogenate suspended for three times in 10 volumes of buffer. To 0.1 mL of the tracer solution and 0.05 mL of the competing ligand solution in phosphate buffered saline containing 0.1 mg/ML of albumin was added 0.1 mL of the membrane suspension. The mixture was incubated at 22 °C for 1 h. Bound and free ^{125}I-[Sar^1,Ile^{5,8}]AII were separated by filtration on glass fiber filter (GF/B, Whatman Inc., Clifton, NJ), and specific binding was determined as the difference between radioligand bound in the presence and absence of unlabeled AII or analogues.

Bioassay of angiotensin II analogues was performed on isolated rat (Sprague-Dawley, 230–260 g) uterine strips perfused with deJalon's solution according to a reported procedure,[34] with n = 3–6 rats for each analogue. Agonistic activity of an analogue was expressed as the ratio of [the dose of AII required to produce a half-maximal response]/[the dose of analogue required to produce half-maximal response]. Inhibitory potency of an analogue was calculated as the ratio of [ED_{50} of AII in the presence of the inhibitor]/[ED_{50} of AII in the absence of such an inhibitor] and was expressed as the magnitude of the displacement of the AII dose–response curve to the right by a select dose of the inhibitor.

Antihypertensive assay of the angiotensin II antagonists was performed on male Sprague-Dawley rats (260–370 g), anesthetized with urethane (0.12 g/100 g body weight) and cannulated (carotid artery and jugular veins). Blood pressure was recorded on a Grass Model 7D physiograph, and the pressure response to 80 ng of [Asn^1]AII was measured at 15-min intervals after a bolus injection of the inhibitors.

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