potencies. Potencies are expressed as a percentage of glucagon activity in the same assay. Glucagon and [D-Phe⁴]glucagon were also tested for duration of activity (Figure 5) by exactly the same protocol, except that 0.5-mL blood samples were taken at 10, 20, 30, 45, 60, and 75 min after injection of test peptide, 20 min after the sodium pentobarbital dose. One milliliter of saline was injected intraperitoneally after taking the third 0.5-mL blood sample to replace fluid volume.

(33) Pugsley, L. I. *Endocrinology* **1946**, *39*, 161.

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Registry No. Glucagon, 16941-32-5; [Ala²]glucagon, 88200-39-9; [des-His¹,Arg¹²]glucagon, 88200-40-2; [D-*p*-Cl-Phe¹,D-Ala⁴,Arg¹²]glucagon, 88200-41-3; [Phe¹,Arg¹²]glucagon, 88200-42-4; [Arg¹²]glucagon, 78119-14-9; [D-Ala⁴,Arg¹²]glucagon, 88200-43-5; [3-Me-His¹,Arg¹²]glucagon, 88200-44-6; [Ac-His¹,Arg¹²]glucagon, 88200-45-7; [D-Phe⁴]glucagon, 88200-46-8.

Angiotensin II: Dependence of Hormone Affinity on the Electronegativity of a Single Side Chain[1]

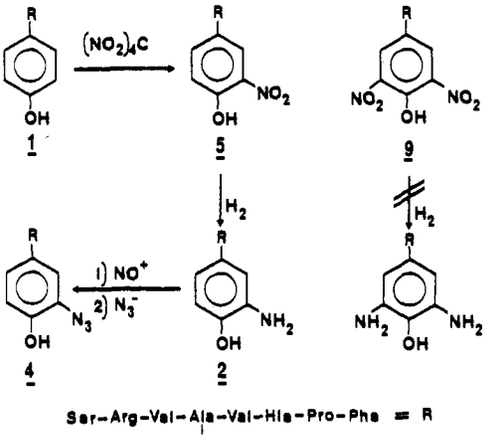
Gaëtan Guillemette, Michel Bernier, Paul Parent, Richard Leduc, and Emanuel Escher*

Department of Physiology and Pharmacology, Medical Faculty, University of Sherbrooke, Sherbrooke, Quebec J1H 5N4, Canada. *Received February 24, 1983*

Structure–activity studies on rabbit aorta of angiotensin II analogues have suggested a possible relationship between the electronegativity of the aromatic side chain in position 4 (Tyr) and the observed affinity. In order to test this hypothesis, several other analogues modified in position 4 have been prepared, and all available analogues were tested in three bioassays: in vitro on rabbit aorta strip, in vivo on the rat blood pressure, and the binding assay on beef adrenocortical membranes. In all three bioassays the postulated correlation has confirmed that angiotensin II affinity depends inversely on the electronegativity of the aromatic side chain in position 4.

Angiotensin II [Asp-Arg-Val-Tyr-Val-His-Pro-Phe (AT)] is one of the oldest and best known peptide hormones.[2] Its functions are mainly blood pressure control and are expressed at several sites, to mention only the most important: contraction of blood vessels[3] (increased vascular resistance), stimulation of aldosterone secretion from adrenals[4] (increased sodium uptake), actions on the central nervous system (CNS) to stimulate thirst perception, and centrally mediated blood pressure increase.[5] Many studies have shown the importance of the individual amino acid residues by structure–activity relationships (SAR), but most information was rather crude "yes or no" results. By studying position-4 modified analogues, we found several intriguing points that deserved further investigation:[6] The almost isosteric analogues [Sar¹,Tyr⁴]AT and [Sar¹,(4′-NO_2)Phe⁴]AT differ very much in their biological activity, the first being the "natural" substitution with 100% activity and the second being inactive. Preliminary ^1H and ^{13}C NMR studies[7] have been carried out on these two analogues, but no important conformational difference was visible, and no further data were available. Therefore, it was a still unanswered question if an intra- or intermolecular interaction was the reason for the striking biological difference. We therefore decided to investigate in more detail the possibility of an intermolecular interaction, i.e., the hormone-receptor interaction, involving this particular side chain. The possible interactions were lipophilicity, van der Waals interaction, hydrogen bridge, and electronegativity. The analogues available at this time gave a good correlation for electronegativity, but none was observed for the other parameters. New compounds were added to this correlation[8] and seemed to support this theory, at least on the in vitro bioassay rabbit aorta strip.

Scheme I



This example of electronegativity dependence is the first documented case on peptide hormones according to Pliska[9] and, therefore, had to be investigated in more detail. Two possible benefits of such a study would be (a) superagonists could be found by using amino acid analogues with exceptionally low electronegativity, and (b) if all bioassays indicate similar relationships, we should be able to postulate the identity of AT receptors from different target tissues. In this report we want to present the synthesis of several new, position-4 modified [Sar¹]AT analogues and the biological activity of all available analogues on the three mentioned bioassays and to discuss the results with regard

(1) Abbreviations are according to the IUPAC–IUB Commission for biochemical nomenclature; additional abbreviations are explained in the text. This work has been supported by grants from the Medical Research Council of Canada, the Canadian Heart Foundation, and the Canadian Kidney Foundation. E.E. is a scholar of the Canadian Heart Foundation.

(2) Regoli, D., Park, W. K.; Rioux, F. *Pharmacol. Rev.* **1974**, *26*, 69.
(3) McCubbin, J. W.; Soares de Monra, R.; Page, I. H.; Olsmted, H. *Science* **1965**, *149*, 1394.
(4) Braun-Mendenez, E.; Fasciolo, J. C.; Leloir, L. F.; Munoz, J. M. *Rev. Soc. Argent. Biol.* **1940**, *16*, 398.
(5) Phillips, M. I. *Neuroendocrinology* **1978**, *25*, 354.
(6) Escher, E.; Nguyen, T. M. D.; Regoli, D. *Can. J. Physiol. Pharmacol.* **1978**, *56*, 956.
(7) Deslauriers, R., NRC Ottawa, personal communciation.
(8) (a) Escher, E.; Guillemette, G.; Leukart, O.; Regoli, D. *Eur. J. Pharmacol.* **1980**, *66*, 267. (b) Escher, E.; Bernier, M. In "Peptides", Proceedings of the 16th European Peptide Symposium; Brunfeldt, K., Ed.; Scriptor: Copenhagen, 1981; p 457.
(9) Pliska, V. In "Perspectives in Peptide Chemistry"; Eberle, A., et al., Eds.; S. Karger: Basel, 1982, p 221.

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Table I. Biological Activities of Position-4 Modified [Sar¹]-angiotensin II Analogues

peptide	no.	biological activities,[a] % in vitro rabbit aorta	in vivo rat BP	binding adreno-cortical membranes	parameters[b] σ	π	MR
[Sar¹]AT	1	100	100	100	−0.37	1.29	27.18
[Sar¹,(3′-NH_2)Tyr⁴]AT	2	120	184	132	−0.53	+0.06	31.57
[Sar¹,(3′-Cl)Tyr⁴]AT	3	103	82	87	−0.44/0.00	+2.0/−1.2	32.18/c
[Sar¹,(3′-N_3)Tyr⁴]AT	4	63	63	61	−0.10	1.75	36.35/c
[Sar¹,(3′-NO_2)Tyr⁴]AT	5	38	16	75	−0.10/+0.34	1.01/−2.19	33.51
[Sar¹,(3′,5′-Cl_2)Tyr⁴]AT	6	22	19	28	−0.07/+0.37	2.71/−0.49	37.18/c
[Sar¹,(4′-NH_2)Phe⁴]AT	7	15	14	28	−0.66	0.73	29.75
[Sar¹,Phe⁴]-AT	8	9.8	8.1	18	0.00	1.96	25.36
[Sar¹,[3′,5′-$(NO_2)_2$]Tyr⁴]AT	9	7.8	0.5	1.5	+0.61	−2.47	39.84/c
[Sar¹,(4′-SAcm)Phe⁴]AT	10	3.5	*b*	28	~0.2[d]	+1.6[d]	64.09[d]
[Sar¹,(4′-SO_2NH_2)Phe⁴]AT	11	3.2	0.9	3.8	+0.57	+0.14	36.61
[Sar¹,(4′-NH_2-3′,5′-I_2)Phe⁴]AT	12	2.7	5.3	16	+0.04	2.97	55.57
[Sar¹,(4′-SH)Phe⁴]AT	13	0.9	*b*	0.5	+0.15	2.35	35.55
[Sar¹,(4′-N_3)Phe⁴]AT	14	0.3	*b*	2.6	+0.15	2.42	34.53
[Sar¹,(4′-NO_2)Phe⁴]AT	15	0.0	0.2	1.5	+0.78	1.68	31.69
[Sar¹Car⁴]AT	16	0.0	*b*	0.5	+1.5 to +2.0[d]	3.00[d]	45[d]
[Sar¹,(4′-SO_3^-)Phe⁴]AT	17	0.0	0.0	0.0	+0.09	−2.8	c

[a] Expressed as a percentage of the activity of 1. All compounds were pure agonists or inactive and devoid of any inhibitory character in vitro and in vivo. On rabbit aorta, at least eight independent determinations have been carried out for each compound and the standard error was always less than 21% of the indicated relative affinity (RA) value. The standard 1 was determined with a half maximal dose of 7.2×10^{-10} M as 100%. Rat blood pressure has been determined for each substance with at least five experiments, and the standard error is always less than 27% of the indicated relative blood pressure potency (RBP) value. The standard 1 produced a 20-mm pressure increase at a dose of 3×10^{-10} mol/kg 100%. Membrane binding has been carried out on at least nine independent experiments with each compound, and the standard error was always less than 36% of the indicated RA value. The reference 1 had a IC_{50} (see Experimental Section) of 9.8×10^{-9} M (= 100%). [b] Aromatic substituent parameters are compiled from Hansch et al.:[16] σ is the Hammett factor for electronegativity, π the lipophilicity, and MR the molecular refractory index. [c] Values not available. [d] Approximate values. The values for 3, 5, and 6 are for the protonated and the deprotonated forms; for 9, only values for the deprotonated form are given.

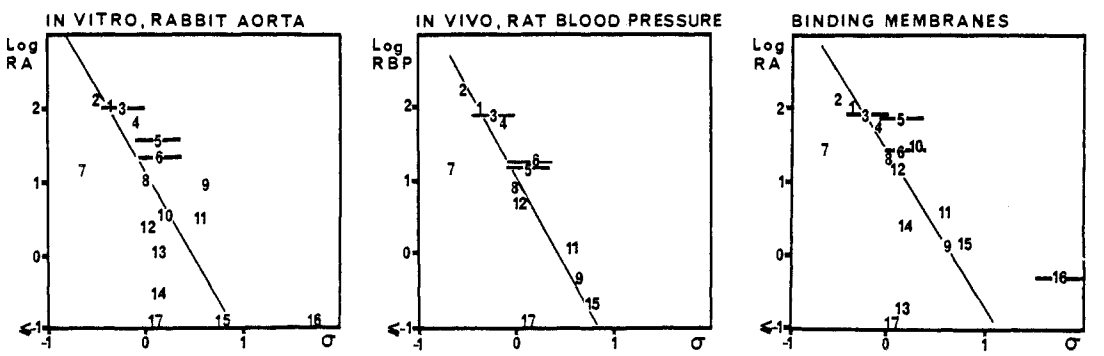

Figure 1. Correlation of electronegativity vs. relative affinity of position-4 modified [Sar¹]-angiotensin II analogues on three bioassays. σ is the Hammett factor (expression for electronegativity) of the aromatic side chain substituent of position 4 according to Hansch.[16] The horizontal bars indicate the values of protonated and deprotonated forms if both are reasonably present at physiological pH; otherwise, only the predominant form is considered (e.g., 1 is indicated in its protonated form and 9 in the deprotonated form). The oblique line is a calculated linear regression of all values with exclusion of 17. Compounds with affinities equal or lower than 0.1% are indicated on this line. Correlation coefficients are as follows: for rabbit aorta, 0.7527 (n = 16); for rat blood pressure, 0.8644 (n = 12); for the binding assay, 0.7352 (n = 16); significance in all three is therefore better than 0.001 (highly significant). Numerical values (log RA and σ) are from Table I.

to the formulated questions.

Syntheses. Peptides 1, 7, 12, and 14–16 were already available from the former studies,[6,8] peptides 10, 11, 13, and 17 are published elsewhere,[10] and peptides 2, 5, and 9 had been included in a preliminary communication,[8] but no experimental details have yet been published. The syntheses, purifications, and analyses of peptides 2–6, 8, and 9 are presented in this report.

Several analogues have been prepared by modification of the already available analogue 1: it has been submitted to nitration on Tyr,[4] which yielded the analogues 5 and 9; reduction of the nitrotyrosine peptide 5 produced 2, and subsequent diazotation and addition of azide gave 4 (see Scheme I). The reduction of 9, the dinitrotyrosine peptide, produced diaminotyrosine, but this compound is almost immediately oxidized and inaccessible for studies. Already the aminotyrosine peptide 2 decomposes very rapidly in pharmacological concentrations and has to be produced immediately before use. Peptides 3, 6, and 8 were synthesized by the classical solid-phase method using the Boc strategy and mainly symmetrical anhydrides for couplings.[13]

(10) Escher, E.; Bernier, M.; Parent, P. *Helv. Chim. Acta* **1983**, *66*, 1355.
(11) Capponi, A. M.; Catt, K. J. *J. Biol. Chem.* **1980**, *255*, 12081.
(12) Guillemette, G.; Escher, E. *Biochemistry* **1983**, *22*, 5591.
(13) Leukart, O.; Escher, E.; Regoli, D.; Schwyzer, R. *Helv. Chim. Acta* **1979**, *62*, 546.

Results and Discussion

All compounds were tested on three different biological systems: in vitro on rabbit aorta strip, in vivo on the blood pressure of the anesthetized rat, and on competitive binding to beef adrenocortical membranes. The results are presented in Table I, and the correlations with the electronegativity σ are given in Figure 1. It is seen immediately that the postulated correlation is valid and that in the three systems the tested compounds align well, with very few exceptions, in the same potency order. Compound **17**, the SO_3^- analogue, is always dropping out of the correlation. Also, the sulfhydryl compound **13** is not a chemically stable moiety, especially at the low concentrations used in the tests with oxygen saturation and the schedule used to prepare it free of interfering reductant (mercaptoethanol interfers in both the binding assay and the rabbit aorta). The sulfonic acid analogue **17** was found to be totally inactive in all tests, even at concentrations up to 10^{-4} M. This is probably due to the strong charge of the sulfonic acid anion, which must be hydrated in the conditions of the bioassays and, therefore, would interfere with the hormone–receptor alignment.

The overall correlation is quite evident; however, upon closer examination we can see a deviation of all values of tyrosine derivatives (i.e., analogues that contain the phenolic OH: **1–6** and **9**) to the right side of the regression curve, especially on rabbit aorta, which indicates a comparatively higher affinity. The analogue **7**, very closely resembling tyrosine but having an amino group instead of an hydroxyl, is on the left side, having relatively low affinity. Since aromatic amines are not protoned at physiological pH, they are mainly present in the sp^2-hybridized state, and therefore the amine nitrogen cannot accept a further hydrogen for protonation or hydrogen bridging. On the other hand, all tyrosine analogues maintain the capacity for hydrogen bridging as hydrogen acceptors at physiological pH either in the ionized (e.g., **9**) or the unionized form (e.g., **1**).

If the regression is repeated separately for the analogues capable of acting as hydrogen acceptors and for those unable to do so on the rabbit aorta assay, two new curves emerge that have narrow scattering (see Figure 2, left). It is therefore reasonable to assume that for position 4 of AT an additional parameter may play a role for affinity: its capacity to act as an hydrogen-bridge acceptor in the 4′-position.

The two lines are, however, not significantly (more than 0.05) different, mainly due to the larger scattering of the non-hydroxyl analogues. However, the excellent correlation for the hydroxyl values (0.9697) is quite suggestive of such an additional interaction. The relative low correlation of the other values probably stems from other parameters that are not included in this simple approach and for themselves are not predominant (see Figure 2, middle and right). A complete Free–Wilson–Hansch analysis would probably improve this and is currently under investigation.

Analysis of the lipophilicity (π) and molecular refractory index (MR) against biological activity (see Figure 2, middle and right) produce very wide scattering and nonsignificant correlations (more than 0.1 significance). These factors may therefore play only a secondary role, if any.

If the same analysis is carried out for position 8 of AT, for positions 5 and 8 of bradykinin, and for positions 7 and 8 of substance P, positions which naturally contain phe-

Table II

no.	peptide	M_r[a]	R_f in the following solvent systems: BAW	BAWP	RPTLC	yield, %	amino acid anal.[b]: Sar	Arg	Val	His	Pro	Phe	Xxx
1[c]	Sar-Arg-Val-Tyr-Val-His-Pro-Phe	1108.28	0.40	0.36	0.30								
5	[Sar^1,(3′-NO_2)Tyr^4]AT	1153.28	0.40	0.39	0.29	82	*	0.97	2.01	0.98	1.01	1.03	NH_2-Tyr*
9	[Sar^1,[3′,5′-$(NO_2)_2$]Tyr^4]AT	1198.28	0.47	0.56	0.44	40	*	0.94	2.17	0.93	1.07	0.89	
2	[Sar^1,(3-NH_2)Tyr^4]AT	1123.29	0.18	0.33	0.27	97	*	0.97	1.89	0.94	1.04	1.16	NH_2-Tyr*
4	[Sar^1,(3′-N_3)Tyr^4]AT	1149.29	0.41	0.40	0.34	59	*	0.95	1.92	1.09	1.10	1.06	NH_2-Tyr*
3	[Sar^1,(3′-Cl)Tyr^4]AT	1142.72	0.41	0.39	0.31	7.9	*	1.07	1.85	1.02	1.02	1.00	Cl-Tyr, 1.08
6	[Sar^1,(3′,5′-Cl_2)Tyr^4]AT	1177.16	0.42	0.44	0.29	30	*	1.07	2.02	1.04	0.92	0.96	Cl_2-Tyr, 1.05
8	[Sar^1,Phe^4]AT	1092.28	0.41	0.39	0.36	27	*	1.08	1.87	0.94	1.05	2.10	

[a] Calculated as the diacetate. [b] An asterisk indicates that an amino acid is present in the analysis but not quantified. [c] [Sar^1]AT.

(14) Lemaire, S.; Yamashiro, D.; Behrens, R.; Li, C. H. *J. Am. Chem. Soc.* **1977**, *99*, 1577.
(15) Laczko, E.; Escher, E. *Helv. Chim. Acta* **1981**, *64*, 621.

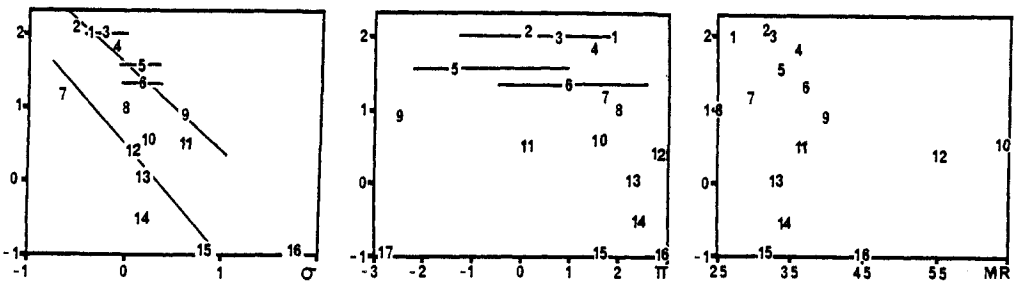

Figure 2. Comparison of physicochemical parameters on position 4 of [Sar[1]]angiotensin II on rabbit aorta. Left: Correlation between log relative affinity vs. electronegativity (σ). The upper oblique line represents the linear regression calculated for the Tyr derivatives 1–6 and 9 with an excellent correlation coefficient of 0.9697 (significance better than 0.001). The lower line represents the linear regression calculated of the non-hydroxyl derivatives with a correlation coefficient of 0.7861, $n = 9$ (significance better than 0.01). Exclusion of 11, which has hydrogen bond acceptor functions, improves the significance to 0.001. The difference between the two regressions is greater than 0.05 (significant). Middle: Correlation between log relative affinity vs. hydrophobicity (π); correlation coefficient, 0.3715; not significant at 0.1. Right: Correlation between log relative affinity vs. molecular refractory index (MR). No values were available for ionized forms on this plot: correlation coefficient, 0.2906; not significant at 0.1.

nylalanine, no significant correlation is possible for electronegativity as for position 4 of AT. Other parameters must be responsible in these positions.[6,16-18]

The observed electronegativity phenomenon on position 4 of AT is the reflection of some interactions of this side chain which increase or decrease the total binding energy of the hormone–receptor complex. Such an increase or decrease could be induced by a hormone-internal interaction (intramolecular) or by a direct hormone–receptor interaction (intermolecular). In our view, the most probable explanation is a hormone–receptor interaction, because electronegativity of aromatics mainly affects the aromatic–aromatic interaction, like charge-transfer complexes or base stacking in DNA. Phenylalanine in position 8 of AT is the only other aromatic residue in the peptide and does not exhibit a similar behavior. We therefore propose that the aromatic nucleus of tyrosine in position 4 of AT interacts directly with an aromatic system of the receptor molecule, probably of a rather electronegative nature [Tyr binds better than (4′-NO_2)Phe].

The classical pharmacological definition of a receptor postulates receptor identity, if the order of potency of a series of agonists is the same on two bioassays. Furthermore, the use of specific and competitive antagonists is suggested. In biochemical terms one can speak of identity only if two proteins have the same amino acid sequence. Between these two definitions are a broad range of other possible definitions; we do not intend to propose a new one, but we feel that such distinct interactions (electronegativity, hydrogen bridge) indicate a high degree of similarity among the binding sites of the AT receptors in our bioassays. Photoaffinity labeling studies of AT receptors in several tissues have produced similar molecular weights for the hormone binding subunit (dog adrenocortex, dog uterus,[11] and bovine adrenocortex[12]). We therefore propose that the AT receptors tested in our study are very similar, if not identical, in their structure (beef adrenocortex, rat blood pressure, and rabbit aorta).

Conclusions

With 17 analogues of angiotensin II that were modified in the 4-position (Tyr), we have shown that the hormone affinity depends on the electronegativity of the aromatic side chain (see Figure 1). High electronegativity produces low affinity; low electronegativity gives good hormone affinities. If the substituent on the aromatic side chain in position 4 can act as a hydrogen-bridge acceptor, affinity is also enhanced; therefore, the compound containing (3′-NH_2)Tyr (2) is the best agonist, having an affinity in all bioassays superior to 1, the Sar[1] analogue of natural AT. This is the first case where a dependence of the biological activity on the electronegativity at an amino acid side chain could be demonstrated in peptide hormones. On other aromatic side chains, either of AT, bradykinin, or substance P as examples, no such relationship was observed.[6,16-18] Because the electronegativity interaction is most probably with some aromatic residues on the AT receptor, we can assume that these aromatic residues of the receptor are localized at the same place in the different receptor-bearing tissues tested so far. We therefore propose that the AT receptors on bovine adrenal cortex are identical, or at least very similar, with the AT receptors of rabbit aorta and those responsible for the blood pressure response in the in vivo test.

If we were capable of designing aromatic amino acids that are even more electropositive and still accepting a hydrogen bridge, we could probably synthesize AT analogues even more potent than peptide 2.

Experimental Section

General Procedures. N^{α}-Butyloxycarbonyl-protected amino acids and peptide reagents were obtained either from Bachem AG, Switzerland, or Chemalog Chemical Dynamics Corp. or prepared in our laboratory. Dicyclohexylcarbodiimide (DCC) was purified by dissolving the commerical product (Aldrich Chemical Co.) in dry diethyl ether; the insoluble material, dicyclohexyl urea, was removed by filtration, and the ether was evaporated in vacuo. All solvents and reagents used for solid-phase synthesis were of analytical quality and were redistilled before use. Solid-phase peptide synthesis and HF cleavage were performed as already reported.[13] Crude peptides were filtered in a first purification over Sephadex G-15 (3 × 85 cm) with 0.2 M acetic acid. The peptide fractions were detected by recording the absorbance of the eluent at 280 nm and by spotting the individual fractions on TLC. TLC were performed on Merck precoated silica gel plates (Type G60-F254) in the solvent system BAW (1-butanol–acetic acid–water, 10:2:3) or BAWP (1-butanol–acetic acid–water–pyridine, 15:3:10:6). Reversed-phase TLC was carried out on Whatman KC 18 plates in the solvent system 30% acetonitrile 2.5%–2-propanol in 0.25 M ammonium acetate at pH 5.0. Peptides were visualized by UV fluorescence and by Pauly reagent (4-diazoniophenyl sulfonate in 2 N Na_2CO_3); amino acids were visualized by ninhydrin. Pure peptide fractions were pooled and lyophilized twice. Peptide purity was tested on the three TLC systems mentioned above and on isocratic HPLC on reversed-phase support 5 μ C-18 with the eluent 17.5% acetonitrile, 2.5% 2-propanol in 0.25 M ammonium acetate at pH 5.0. All products

(16) Hansch, C.; Leo, A.; Unger, S. H.; Kim, K. H.; Nikaitani, D.; Lien, E. J. *J. Med. Chem.* **1973**, *16*, 1207.
(17) Escher, E.; Couture, R.; Champagne, G.; Mizrahi, J.; Regoli, D. *J. Med. Chem.* **1982**, *25*, 470.
(18) Escher, E.; Laczko, E.; Guillemette, G.; Regoli, D. *J. Med. Chem.* **1981**, *24*, 1409.

produced single spots and single symmetrical peaks, respectively. IR spectra were recorded on a Perkin-Elmer 457 instrument in KBr pellets. UV spectra were recorded on a Beckman spectrophotometer with peptides dissolved in 0.5 N acetic acid. Peptide samples for amino acid analyses were hydrolyzed at 110 °C during 24 h in 6 N HCl plus 0.2% ethanethiol in vacuum-sealed tubes. The amino acid analyses were carried out on a Beckman 119 amino acid analyzer.

Enzymatic Analyses. Peptide **2**, **4**, **5**, or **9** (1.5 mg) was dissolved in 500 μL of 50 mM sodium phosphate buffer at pH 8.0. Bovine trypsin (Sigma Chemical Co., 0.17 mg) was added, and the mixture was incubated at 37 °C for 8 h. The enzyme was denatured by heating to 100 °C for 5 min, and the mixture was added to a solution of 500 μL of 50 mM sodium phosphate buffer, pH 8.2, 10 mM in magnesium chloride and containing 20 μg of porcine leucine amino peptidase (Sigma Chemical Co.) at 37 °C for 24 h. The proteins were precipitated by adding 100 mg of trichloroacetic acid, and then filtered and desalted on strong acid ion exchange resin AG 50-W-X4. The amino acids were eluted with 2 N aqueous ammonia, lyophylized, and identified on TLC. With this technique the modified amino acids were compared to synthetic amino acids: system A, 1-butanol–acetic acid–water (4:1:1); system B, 1-butanol–ammonium acetate (50 mM, pH 7.0, 2:1).

Sar-Arg-Val-Phe-Val-His-Pro-Phe ([Sar^1,Phe^4]AT, 8). Boc-Val-His(Tos)-Pro-Phe-resin ester (2 g),[12] initially substituted with 0.42 mmol of Boc-Phe per gram of resin, was coupled, as indicated above, in sixfold excess with the following protected amino acids: Boc-Phe, Boc-Val, Boc-Arg(Tos), and Cbz-Sar. After synthesis, cleavage, and the first gel filtration, the peptide fractions were pooled and neutralized to pH 6 with ammonium acetate. This solution was loaded directly onto a reversed-phase column (1.5 × 30 cm), Michel–Miller containing nucleosil 30 μ, C-18 (Macherey-Nagel, Germany), preequilibrated with 0.1 M ammonium acetate, pH 7.0, and washed after loading with 200 mL of the same buffer. The peptide was eluted with a linear gradient of 10% acetonitrile to 35% in 0.25 M ammonium acetate, pH 5.0, containing 7% 2-propanol, at a mean pressure of 8 atm and 10 mL min^{-1}; 250 mg of pure **8** was obtained.

Sar-Arg-Val-(3′-Cl)Tyr-Val-His-Pro-Phe ([Sar^1,(3′-Cl)-Tyr^4]AT, 3). L-N^{α}-Boc-*O*-(3″-BrBzl)(3′-Cl)Tyr was prepared from L-(3′-Cl)Tyr (our laboratory) by the method of Lemaire et al.[14] To 1 g of Boc-Val-His(Tos)-Pro-Phe resin ester (same as above) were coupled 815 mg of N^{α}-Boc-*O*-(3″-BrBzl)(3′-Cl)Tyr, Boc-Val, Boc-Arg(Tos), and Cbz-Sar. After HF cleavage, 260 mg of raw product was purified with two successive partition chromatographies after gel filtration. The first partition chromatography was carried out on Sephadex G-25 (fine) in a glass column (3 × 95 cm), saturated with the lower phase of the system 1-butanol–acetic acid–water, 4: 1: 5, and eluted with the upper (organic) phase. The second partition chromatography was carried out on a similar column but with the system 1-butanol–acetic acid–water–pyridine, 50:1:110:30. The yield was 20.5 mg of pure **3**.

Sar-Arg-Val-(3′,5′-Cl$_2$)Tyr-Val-His-Pro-Phe ([Sar^1,(3′,5′-Cl$_2$)Tyr^4]AT, 6). To 1.5 g of the same resin, Boc-Val-His-(Tos)-Pro-Phe-resin ester, were coupled the following protected amino acids: 1.3 g of N^{α}-Boc-*O*-(3″-BrBzl)(3′,5′-Cl$_2$)Tyr, Boc-Val, Boc-Arg(Tos), Cbz-Sar. The halogenated analogue of Tyr was prepared from L-(3′,5′-Cl$_2$)Tyr (our laboratory) according to Lemaire et al.[14] After HF cleavage, 500 mg of raw material was purified identically with **3**, and 226 mg of pure **6** resulted.

Sar-Arg-Val-(3′-NO$_2$)Tyr-Val-His-Pro-Phe ([Sar^1,(3′-NO$_2$)Tyr^4]AT, 5). Compound **1** (from our laboratory, 30 mg) was dissolved in 5 mL of a mixture of ethanol–0.01 M ammonium acetate, pH 7.0, 1:1, and stirred at room temperature. Tetranitromethane (Sigma Chemical Co., 240 mg) in 5 mL ethanol was added slowly over 30 min and stirred for another 90 min. Then glacial acetic acid was added until an apparent pH of about 4 was obtained. This mixture was directly applied to a gel filtration column (Sephadex G-15, 1.7 × 106 cm) and eluted with 0.2 M acetic acid. Under these conditions, already pure **5** (25.6 mg) was obtained (yellow powder): UV λ_{max} 359 nm (log ε 2.69) 278 (3.51). Enzymatic analysis: R_f (A) 0.34, R_f (B) 0.25 yellow, identical with (3′-NO$_2$)Tyr.

Sar-Val-[3′,5′-(NO$_2$)$_2$]Tyr-Val-His-Pro-Phe ([Sar^1,[3′,5′-(NO$_2$)$_2$]Tyr^4]AT, 9). Compound **1** (15 mg) was treated as described for **5** but with 600 mg of tetranitromethane and in 1 M ammonium acetate, adjusted to pH 10 with ammonia. This reaction produced a mixture of **5** and **9**, and purification on G-15 yielded 4.8 mg of dark yellow **9** and 3.8 mg of **5**: UV λ_{max} 355 nm (log ε 2.78). Enzymatic analysis: R_f (A) 0.35, R_f (B) 0.14, yellow, identical with [3′,5′-(NO$_2$)$_2$]Tyr.

Sar-Arg-Val-(3′-NH$_2$)Tyr-Val-His-Pro-Phe ([Sar^1,(3′-NH$_2$)Tyr^4]AT, 2). Compound **5** (20 mg, 20 μmol) was hydrogenated with 2 mg of palladium on active carbon in 0.2 M acetic acid according to an earlier publication,[15] which permits hydrogenation at about 8 atm of H_2; the hydrogenation was complete after 1 h. β-Mercaptoethanol (50 μL) was added to protect the product from oxidation and to deplace the product from the adsorbing catalyst, and the mixture was filtered and lyophilized twice. This produced 19 mg of apparently pure **2**. For the biological tests, a different method was used to produce **2**. Because of its sensitivity to oxidation, it had to be prepared immediately before use when applied in low concentrations: 10 μL of a 10^{-3} M solution of **5** was added to 10 μL of a 0.1 M solution of sodium dithionite, which discolored the yellow solution of **5** immediately. This mixture was immediately diluted with ice-cold 0.9% saline to the appropriate concentration and applied to the bioassay. Control tests have shown no interference of sodium dithionite at the concentrations used with any bioassay (**2**, 10^{-7} to 10^{-9} M; sodium dithionite, 10^{-5} to 10^{-7} M). Peptide **2** prepared in this way was identical on TLC with the above material: UV λ_{max} 277 nm (log ε 3.03). Enzymatic analysis: R_f (A) 0.13, R_f (B) 0.11, identical with (3′-NH$_2$)Tyr.

Sar-Arg-Val-(3′-N$_3$)Tyr-Val-His-Pro-Phe ([Sar^1,(3′-N$_3$)-Tyr^4]AT, 4). Compound **2** (10 mg, 10 mol) was dissolved in 1 mL of 0.1 N HCl at 0 °C under stirring. A 1 N solution of $NaNO_2$ (30 μL) was added and stirred for 10 min. An iodine–starch paper test was positive, and 35 μL of a 1 N sulfamic acid solution was added, which produced a negative repetition of the test. After the addition of 20 μL of a 1 N NaN_3 solution, the reaction mixture was neutralized with solid ammonium acetate, and the volume was expanded to 5 mL with water. This mixture was lyophilized in the dark and purified by partition chromatography on a Sephadex G-25 fine column (1 × 75 cm) with the system 1-butanol–acetic acid–water, 4:1:5, as described for **3** and producing 6.0 mg of **4**: IR 2110 (N$_3$) cm^{-1}. Enzymatic analysis: R_f (A) 0.49, R_f (B) 0.33, identical with synthetic (3′-N$_3$)Tyr.

Sar-Arg-Val-[3′,5′-(NH$_2$)$_2$]Tyr-Val-His-Pro-Phe ([Sar^1,-[3′,5′-(NH$_2$)$_2$]Tyr^4]AT). Compound **9** (1 mg) was hydrogenated as under **2**, but no product was recovered. On TLC only a faint brown streak was observed from R_f 0.0 to 0.7. Other reductions with sodium dithionite or sodium borohydride gave the same results.

Biological Assays. Preparation of Purified Adrenal Vesicular Membranes and Binding Experiments. Bovine adrenal glands were obtained from a nearby slaughter house immediately after slaughtering and kept in ice-cold saline until dissected free of adipose tissue. The membrane preparation was carried out as already described by some of us.[12] Purified adrenal microsomal membranes (approximately 50–100 μg of protein) were incubated in 100 μL of assay buffer in the presence of a radioactive analogue of AT, [Sar^1,(3′,5′-$^3H_2$-4′-NH$_2$)Phe^4]AT ([^{3}H]AT, 5 × 10^{-9} M). Varying concentrations of AT or 4th-position substituted analogues were added between 10^{-9} and 10^{-4} M to generate binding inhibition curves. All bindings are corrected for nonspecific binding in the presence of excess unlabeled AT (10^{-4} M). Binding studies are performed in triplicate at 20 °C for 15 min. Separation of bound and free hormones are achieved by Millipore filtration by using HAWP (0.45 μm) nitrocellulose filters (Millipore Corp., Bedford, MA). Filters are dissolved in Aquasol and counted on a Beckman LS 6800 liquid scintillation counter. Receptor binding is expressed as a ratio of specific counts bound in the presence of varying ligand concentrations to specific counts bound in the absence of added ligand (B/BO). The IC_{50} of each analogue is the concentration of the peptide that displaces 50% [^{3}H]AT from the receptor. The relative affinity indicated in Table I is the relationship between the IC_{50} of **1** and the IC_{50} of the tested compound.

Rabbit Aorta Strips. New Zealand rabbits of either sex, weighing 1.5–2.0 kg, were killed by stunning and exsanguination. The thoracic aorta were excised and immediately immersed in

cold, oxygen saturated Kreb's solution. The aortas were freed from adrentia and helically cut into a 5-mm large band. Two-centimeter strips of this band were suspended in 5-mL baths containing Kreb's solution at 37 °C; they were continually aerated with a mixture at 95% O_2/5% CO_2. A tension of 2 g was applied at the beginning and was adjusted several times during the 60–90-min equilibration period. The bath fluid was changed at intervals of 10–15 min. Concentrations indicated were always final concentrations in the tissue bath. Biological activities of the tissue strips in response to the applied peptides were recorded with force-displacement transducers (Grass FT 0.3) on a Grass polygraph Model 7 (Grass Co. Quincy, MA). The biological activities are expressed by the relative affinity (RA) compared to 1.

In Vivo Rat Blood Pressure. Male and female Wistar rats, weighing between 300 and 500 g, were used and purchased from Charles River Canada Inc., St-Constant, Quebec. Before the experiment, the animals were anesthetized with urethane (1.4 g/kg intraperitoneally), tracheotomized, and self-ventilated. The left jugular vein and the right carotid artery were carefully dissected. A catheter was implanted into each vessel leading to the body for drug injections (jugular vein) and direct blood pressure recording (carotid artery). The arterial blood pressure was monitored with arterial pressure transducers (Statham P 23Db) attached to the side arm of the carotid cannula.

The drugs were injected in volumes of 0.1 mL, followed by 0.2 mL of physiological saline (NaCl, 0.9%, w/v) in order to rinse the cannula.

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Synthesis of a Novel Class of Heteroaromatic Amino Acids and Their Use in the Preparation of Analogues of Luteinizing Hormone-Releasing Hormone[1]

John J. Nestor, Jr.,*[†] Bonnie L. Horner,[†] Teresa L. Ho,[†] Gordon H. Jones,[†] Georgia I. McRae,[‡] and Brian H. Vickery[‡]

Institutes of Bio-Organic Chemistry and Biological Sciences, Syntex Research, Palo Alto, California 94304.
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A novel class of heterocyclic aromatic amino acids based on the 3-(2-benzimidazolyl)alanine system has been generated by chiral synthesis from D- or L-aspartic acid. The use of variously substituted *o*-phenylenediamines for condensation with the β-carboxyl function of α-benzyl *N*-(benzyloxycarbonyl)-D-aspartate has led to a series of amino acids of graded hydrophobicity with a steric bulk similar to that of tryptophan. In a similar fashion, we have prepared 3-(2-benzothiazolyl)-D-alanine from *o*-aminothiophenol and 3-(2-benzoxazolyl)-D-alanine from *o*-aminophenol. Incorporation of these amino acids into the 6-position of luteinizing hormone-releasing hormone (LH-RH) led to a series of very potent agonist analogues (up to 160 times LH-RH potency), active in doses ranging from 0.1 to 0.5 μg by twice daily injection in a rat estrus cyclicity suppression assay designed to show the paradoxical antifertility effects of these compounds.

The preparation of unnatural amino acids with specially designed physical and chemical properties (e.g., basicity, lipophilicity, steric requirements, etc.) for incorporation into polypeptides is of increasing importance in peptide chemistry.[2-6] Such an approach can be useful in the investigation of specific sites in polypeptide hormones with binding and effector functions. Of particular importance for binding and effector functions are the indole[7] and imidazole[8] containing side chains of Trp and His.

The demonstration that the C-terminal carboxylic acid of peptides could be converted into benzimidazole functions[9] led us to use a similar route to convert the β-carboxylic acid function of Asp into a novel series of heteroaromatic amino acids with steric requirements similar to Trp (Scheme I). The use of N, O, and S as members of the heteroaromatic ring will allow the study of the effects of a range of basicities and lipophilicities with little change in steric requirements. In addition, the facile use of substituents on the aromatic portion of the side chain allows the study of a range of lipophilic and electronic effects.

An area of particular interest to us[6] has been the regulation of fertility using hydrophobic analogues of luteinizing hormone-releasing hormone (<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, LH-RH). Chronic administration of pharmacological doses of long lived LH-RH

Table I. Basicity of Side-Chain Functional Groups

compd	pK_a	method[a]
Dcb	3.4	A
Bia	4.7	A
Dmb	5.4	A
benzimidazole	5.5 (5.5)[b]	A
Tba	7.5	B

[a] The pK_a's were determined either spectrophotometrically in a series of graded aqueous buffers (method A) or by titration (method B) in aqueous medium (D. Nagami and T.-Y. Yang, unpublished results). For reference, the pK_a of His is 6.1.[36] The pK_a values were not determined for Nia (insoluble) or for the very weakly basic Boa and Bta. [b] Reference 18.

(1) Contribution no. 166 from the Institute of Bio-Organic Chemistry, Syntex Research. The unnatural amino acids have been given the following abbreviation: Bia, 3-(2-benzimidazolyl)-alanine (3a); Nia, 3-(2-naphthimidazolyl)alanine (3b); Dmb, 3-(5,6-dimethylbenzimidazol-2-yl)alanine (3c); Dcb, 3-(5,6-dichlorobenzimidazol-2-yl)alanine (3d); Bta, 3-(2-benzothiazolyl)alanine (3e); Boa, 3-(2-benzoxazolyl)alanine (3f); Tba, 3-(4,5,6,7-tetrahydrobenzimidazol-2-yl)alanine (6). The abbreviations for natural amino acids and protecting groups follow the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature (*J. Biol. Chem.* 1971, *247*, 977).

[†] Institute of Bio-Organic Chemistry.
[‡] Institute of Biological Sciences.

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