"Biochemistry of the SH Group", Academic Press, New York, NY, 1972, p 344; (c) A. Meister, "Biochemistry of the Amino Acids", Vol. II, 2nd ed., Academic Press, New York, NY, 1965, pp 799–810.
(17) (a) A. I. Cederbaum and E. Rubin, *Biochem. Pharmacol.*, **25**, 2179 (1976); (b) E. G. DeMaster and H. T. Nagasawa, *Life Sci.*, **22**, 91 (1978).
(18) L. F. Chasseaud, *Biochem. Pharmacol.*, **23**, 113 (1974).
(19) S. Ratner and H. T. Clarke, *J. Am. Chem. Soc.*, **59**, 200 (1937).
(20) C. S. Alexander, H. T. Nagasawa, E. G. DeMaster, and D. J. W. Goon, "Recent Advances on Cardiac Structure and Metabolism", Vol. 12, T. Kobayashi, Y. Ito, and G. Rona, Eds., University Park Press, Baltimore, MD, 1978, p 347.
(21) D. J. W. Goon and H. T. Nagasawa, *Res. Commun. Chem. Pathol. Pharmacol.*, **16**, 746 (1977).

Biologically Active Derivatives of Angiotensin for Labeling Cellular Receptors

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The synthesis and potencies of several amino-terminal derivatives of angiotensin II to be used for the labeling of its receptors on vascular smooth muscle cells are reported here. The relative affinities on rabbit aortic strips of the compounds prepared, based on a potency of 100% for [Asp^1,Ile^5]angiotensin II (**4**), are N^α-(4-formylbenzoyl)-[Asn^1,Val^5]angiotensin II (**5**) 8%, N^α-(*N*-fluoresceinthiocarbamyl)-[Asp^1,Ile^5]angiotensin II (**6**) 1%, N^α-(*N*-rhodaminethiocarbamyl)-[Asp^1,Ile^5]angiotensin II (**7**) 4%, [2-methoxy-2,4-diphenyl-3(2*H*)-furanone]-[Asp^1,Ile^5]-angiotensin II (**8**) 3%, and N^α-[(2-nitro-5-azidobenzoyl)norleucyl]-[Asp^1,Ile^5]angiotensin II (**9**) 0.5%. Free angiotensin II was not detectable in peptides **5**–**9**. With the exception of **6** and **7** all peptides were of high purity by paper electrophoresis and thin-layer chromotography. Therefore the biological activities found for both **6** and **7** have the limitations that they represent the activity of a mixture. The peptides were not tested for antagonist activity.

Two classes of derivatives of angiotensin have been prepared for the study of its vascular receptor sites: fluorescent derivatives for light microscopic visualization of receptors (**6**–**8**) and reactive derivatives for the covalent labeling of receptors (**5** and **9**).

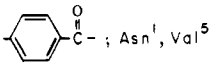
Fluorescent derivatives of small peptide hormones have rarely been prepared and characterized. However, N^α-(*N*-fluoresceinthiocarbamyl)tetragastrin[1] and fluorescamine labeled [Asp^1,Ile^5]angiotensin II[2] have been prepared by others and have been found to have high biological activity. We describe here the fluorescent derivatives of angiotensin with fluorescein isothiocyanate (**6**), tetramethylrhodamine isothiocyanate (**7**), and 2-methoxy-2,4-diphenyl-3(2*H*)-furanone (MDPF, **8**). These three compounds were prepared in order to have a range of fluorescence emission wavelengths for biological studies with vascular smooth muscle cells. Emission maxima for proteins derivatized with these fluorophores have been previously reported: fluorescein isothiocyanate–protein conjugates,[3] λ_{max} >520 nm, tetramethylrhodamine–protein conjugates,[3] λ_{max} >595 nm, and MDPF–γ-globulin conjugates,[4] λ_{max} 480 nm.

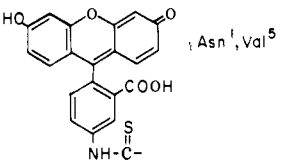
Several examples of chemically reactive peptide hormones are known. Chlorambucil-angiotensin II and chlorambucil-[des-Asp^1,Val^5]angiotensin II have been shown to be irreversible, noncompetitive inhibitors of angiotensin II on guinea pig ileum and rat uterus,[5] whereas chlorambucil-[des-Asp^1,Val^8]angiotensin I had no irreversible effects on guinea pig ileum, rat uterus, rabbit aorta, or rat blood pressure.[6] Chlorambucil-bradykinin had some irreversible effects on guinea pig ileum in vitro and on pulmonary kininases in vivo,[7] while (bromoacetyl)bradykinins had no irreversible effects on rat uterus in vitro or on rat blood pressure.[8] (Bromoacetyl)oxytocin was found to be an irreversible inhibitor of neurohypophyseal hormone stimulated adenylate cyclase in toad bladder, but only at 0.1 mM, where oxytocin itself was also an inhibitor.[9] Maleoyl derivatives of oxytocin had no irreversible effects on toad bladder or rat uterus in vitro.[10] Using a different approach, photolyzable aryl azide derivatives of peptide hormones have been described which are capable of covalent bond formation to any amino acid residues present in the hormone receptor site. Photolyzable insulin derivatives with high biological potency have been reported but were not tested for irreversible biological effects.[11] A photolyzable aryl azide derivative of a cholecystokinin fragment has been shown to be an irreversible agonist in stimulating discharge of exportable proteins from guinea pig exocrine pancreas in vitro.[12] Finally, a similar pho-

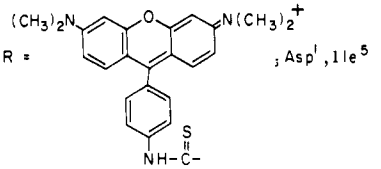
R-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe

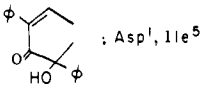
1, R = H; Asn^1, Val^5

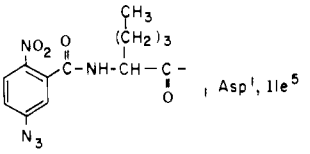
4, R = H; Asp^1, Ile^5

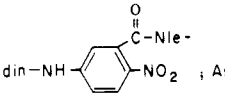
5, R =  ; Asn^1, Val^5

6, R =  ; Asn^1, Val^5

7, R =  ; Asp^1, Ile^5

8, R =  ; Asp^1, Ile^5

9, R =  ; Asp^1, Ile^5

10, R = avidin-NH-  ; Asp^1, Ile^5

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tolyzable derivative of epidermal growth factor has been reported to label a single membrane protein in target cells.[13]

In this study, chemically reactive derivatives of angiotensin (5 and 9) were prepared in order to test the possibility of designing new irreversible inhibitors of angiotensin and to provide specific irreversible tags for angiotensin receptors for morphological and biochemical identification. (2-Nitro-5-azidobenzoyl)norleucyl-[Asp[1],Ile[5]]angiotensin II (9) is convertible by photolysis to a reactive arylnitrene which is capable of insertion into carbon–hydrogen bonds.[14,15] The presence of norleucine allows quantitation of the attachment of 9 to a protein by amino acid analysis for norleucine. (4-Formylbenzoyl)-[Asn[1],Val[5]]angiotensin II (5) can form imines with amino groups which, in turn, can be reduced to stable secondary amines with sodium borohydride. The 4-formylbenzoyl group has been used to link a hemepeptide to antibodies by this method.[16]

Finally, avidin-angiotensin II 10 was prepared by photolyzing 9 and avidin together in relatively concentrated solution. Avidin-angiotensin can, in principle, be indirectly visualized in the electron microscope by employing biotin–ferritin[17] and thus can be used to label angiotensin receptors.

The preparation, potency on rabbit aortic strip, and some other properties of compounds 1–10 are described here.

Results

Angiotensin Peptides 1–4. [Asn[1],Val[5]]angiotensin II (1) was prepared previously by solid-phase peptide synthesis in 42%[18] yield. [Asp[1],Ile[5]]angiotensin II and its C-terminal hexapeptide 2 and heptapeptide 3 were prepared similarly except that successive double couplings of *tert*-butyloxycarbonylamino acids were employed and tyrosine was protected by either the 2,6-dichlorobenzyl[19,20] or *o*-bromocarbobenzoxy[21] group instead of benzyl.[18] The use of the two latter tyrosine-protecting groups reduced the yield of the side-product 3-benzyltyrosine peptides from about 20% (with benzyl protection) to less than 10% (with 2,6-dichlorobenzyl or *o*-bromocarbobenzoxy protection) of the total recovered peptide.

Difficulty was experienced in purifying [Asp[1],Ile[5]]angiotensin II (4) compared to [Asn[1],Val[5]]angiotensin II (1). A single chromatography on Bio-Rex 70 (H^+ form) was sufficient to produce pure 1[18] but yielded 4 contaminated by an impurity with partition coefficient in countercurrent distribution (CCD) system G of K_G 0.46 (this material, although never completely characterized, will be designated as **4a**). After this CCD in system G, 4 (K_G 0.37) and 4a (K_G 0.46) both showed additional components on high-voltage electrophoresis: $R_{1.9}$ (4) 0.70, $R_{1.9}$ (impurity in 4 and 4a, designated **4b**) 0.50, $R_{6.5}$ (4) 0.40, $R_{6.5}$ (**4b**) 0.20. CCD in system H produced pure 4 (K_H 0.26), **4a** (K_H 1.2), and an additional component, **4b**, at K_H 0.60 ($R_{1.9}$ 0.50), all with amino acid analyses identical with that of 4. Pure 4 could only be obtained after CCD in G, followed by H. Pure 4, after standing several hours in 20%, 50% (v/v), or 100% acetic acid yielded a mixture of 4 and the impurity: $R_{1.9}$ 0.50, $R_{6.5}$ 0.20. This acid-generated impurity appeared to be identical with the material, **4b**, with K_H 0.60. However, the identity of the acid-generated impurity and **4b** (K_H 0.60) has not been established nor are their individual structures known. This impurity ($R_{1.9}$ 0.50, $R_{6.5}$ 0.20) could also be generated from [Asp[1],Ile[5]]angiotensin samples received from Schwarz-Mann, Dr. R. J. Freer (University of Virginia Medical College), and Dr. A. C. N. Paiva (Escola Paulista de Medicina, Brazil) but not from compound 1, 2, or 3 by standing in acetic acid. The impurity **4b** was shown not to be [β-Asp[1],Ile[5]]angiotensin (generated according to Riniker and Schwyzer[22] by heating 4 at neutral pH) by electrophoresis at pH 3 and by TLC.

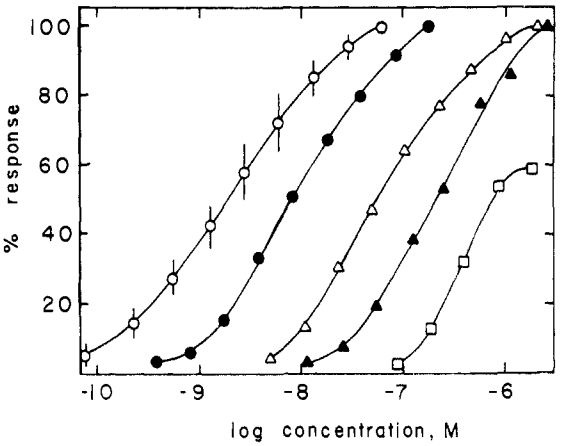

Figure 1. Dose–response curves in rabbit aortic strip for 4 (○), 1 (●), 8 (△), 6 (▲), and 9 (□). The dose–response curve for 4 shows the standard deviations for the average of five experiments.

Fluorescent Angiotensins 6–8. The pyrrolinone-angiotensin 8 was easily prepared to give a homogeneous product in good yield (66% yield by weight of material containing 66% peptide by amino acid analysis = 44% yield). In contrast, fluorescein and rhodamine peptides 6 and 7 were not obtainable in homogeneous form and were recovered in low overall yields, 12 and 9%, respectively. The structures shown for 6 and 7 are the structures assumed for isomer I of the fluorescein (6) and rhodamine (7) isothiocyanates. The structure shown for 8 is assumed to be the product of the reaction of 2-methoxy-2,4-diphenyl-3(2*H*)-furanone with primary amines.[4]

Reactive Angiotensins 5 and 9. The aldehyde derivative 5 of angiotensin was easily prepared in homogeneous form, as was 9, the photolyzable aryl azide derivative.

Avidin-angiotensin 10. Avidin-angiotensin 10 was obtained in 90% yield, based upon the starting amount of the protein. Avidin-angiotensin 10 retained all of avidin's biotin binding capacity and was prepared with 2–5 mol of angiotensin/mol of avidin. The structure shown for 10 is the assumed product of the insertion of the nitrene generated from the aryl azide into a carbon–hydrogen bond in avidin (see ref 14).

Biological Results. The results of the assays of 1–10 on the rabbit aortic strip are shown in Table I. Figure 1 shows dose–response curves obtained for 1, 4, 6, 8, and 9. Relative affinity gives the relative ED_{50} of the peptide compared to that of 4, taking ED_{50} of 4 to be 100%. The ED_{50} of 4 (the dose required to elicit half-maximum contraction) was 2 nM (lit.[23] 1.4 nM). The dose of 10 was measured as the concentration of macromolecule-bound angiotensin, rather than as the concentration of the macromolecule itself.

Several of the peptides were assayed in a preparation of dispersed single smooth muscle cells from rabbit aorta.[24] Peptides 1 and 4–6 caused contraction of the cells. Peptides 2, 3, and 7–10 were not tested in this preparation. Fluorescence of peptide 6 on these cells was impossible to distinguish from autofluorescence of the cells which occurs at similar wavelengths. The results of labeling studies with 5–9 will be reported in future publications.

Table I. Relative Potencies of Angiotensin Peptides in Rabbit Aortic Strip

no.	peptide, molecular weight	rel affinity[a]
1	[Asn^1,Val^5]angiotensin II, 1031	23 (18)
2	[des-Asp^1,des-Arg^2,Ile^5]-angiotensin II, 775	0.03 (0.1)
3	[des-Asp^1,Ile^5]angiotensin II, 931	2 (5.6)
4	[Asp^1,Ile^5]angiotensin II, 1046	100 (100)
5	(4-(formylbenzoyl)-[Asn^1,Val^5]angiotensin II, 1163	8
6	N^{α}-(*N*-fluoresceinthiocarbamyl)-[Asn^1,Val^5]angiotensin II, 1421	1
7	N^{α}-(*N*-rhodaminethiocarbamyl)-[Asp^1,Ile^5]angiotensin II, 1447	4
8	MDPF-[Asp^1,Ile^5]angiotensin II, 1281	3
9	(2-nitro-5-azidobenzoyl)norleucyl-[Asp^1,Ile^5]angiotensin II, 1350	0.5
10	avidin-[Asp^1,Ile^5]angiotensin II	0.1

[a] The relative affinities were estimated from the ratio of the ED_{50} of **4** to that of each of the other peptides. Figures in parentheses are literature values.[23]

Discussion

Angiotensin Peptides 1–4. The difficulties encountered in preparing [Asp^1,Ile^5]angiotensin II (**4**) have not yet been resolved. Apparently, a peptide can be generated during purification of **4** in acid solution (and presumably also during deprotection and HF cleavage of **4**) with K_H 0.60, $R_{1.9}$ 0.50, $R_{6.5}$ 0.20, $R_{f(A)}$ 0.90, and $R_{f(C)}$ 0.50, which we have called **4b**. This impurity can be removed by CCD at neutral pH in system H. Although this peptide has not been further characterized, its generation must involve the N-terminal aspartic acid residue, since neither **2** nor **3** exhibits the generation of an impurity in acidic solution. This impurity is not [β-Asp^1,Ile^5]angiotensin II and has not been previously reported to our knowledge. The biological activities of **1–4** are in satisfactory agreement with the literature values reported in Table I.

Fluorescent Angiotensins 6–8. The lack of homogeneity of **6** and **7** may be due to the inhomogeneity of the starting fluorescent isothiocyanates (see Experimental Section) which are notoriously impure,[25] to the known lactone–acid equilibrium in these fluorescent dyes,[26] or to reaction of the isothiocyanates with other nucleophiles in angiotensin besides the α-amino group. Lack of homogeneity of **6** and **7** may complicate quantitative interpretation of receptor-labeling studies and prevents the assessment of the individual biological activities of the component peptides of the mixture. However, the absence of contaminating angiotensin II in both **6** and **7** suggests that the fluorescent peptides are biologically active and, therefore, could be used to label angiotensin receptors. Peptide **8** was easily obtained in homogeneous form. However, its fluorescence emission maximum is similar to that of fluorescein and thus may be difficult to distinguish from the autofluorescence of the smooth muscle cells (green). Peptide **7**, with emission λ_{max} >595 nm, may be more easily visualized against the autofluorescence.

Reactive Angiotensins 5 and 9. The aldehyde-antiotensin **5** has not yet been tested as an irreversible inhibitor of angiotensin. The attachment of **9** to avidin by photolysis suggests that **9** should be capable of irreversibly labeling angiotensin receptors.

Avidin-Angiotensin 10. The macromolecular angiotensin **10** has very low relative affinity in the aortic strip (0.1%) and there is no direct chemical evidence for the nature of the covalent bond linking avidin and angiotensin in **10**. Therefore, it is not certain at this time whether the 0.1% affinity found for **10** is due to covalently bound angiotensin, to contaminating free angiotensin, or to inability of the angiotensin linked to the bulky protein to penetrate whole aortic strips.

Experimental Section

Melting points were determined with a hot-stage microscope and are corrected. Elemental analyses were performed by Baron Consulting Co., Milford, CT. Amino acid analyses were performed on a Durrum 500 amino acid analyzer after hydrolysis in 6 N hydrochloric acid containing 0.2% phenol for 24 or 48 h. Values reported below are molar ratios relative to phenylalanine. Solid-phase peptide synthesis and high-voltage paper electrophoresis (HVE) were performed as previously described.[18] Relative electrophoretic migration distances are given based on *R* of histidine = 1.0 at pH 1.9 and 6.5, denoted by $R_{1.9}$ and $R_{6.5}$, respectively. Thin-layer chromatography (TLC) was on analytical glass or plastic-backed plates (E. Merck) in (A) *n*-BuOH–HOAc–H_2O (4:1:5), upper phase on cellulose, (B) *n*-BuOH–HOAc–H_2O (4:1:5), upper phase on silica gel, (C) EtOAc–pyridine–HOAc–H_2O (5:5:1:3), (D) *n*-BuOH–HOAc–H_2O–pyridine (30:6:24:30), (E) EtOAc, and (F) CH_3CN (C–F on silica gel). In system B preparative (2 mm) layers gave different R_f values than the analytical (0.25 mm) layers. Peptides were located with ninhydrin or Pauly reagents,[18] except that **7** was too intensely colored for detection by either reagent. Countercurrent distribution was determined in (G) *n*-BuOH–HOAc–H_2O (4:1:5) or (H) *n*-BuOH–0.4 M NH_4OAc (1:1), with partition coefficients denoted by K_G and K_H. Tryptic digestion was at 37 °C for 20 min with Worthington TPCK-trypsin 0.2% in 1% NH_4HCO_3 at a peptide concentration of 1%.

Weight yields of peptides isolated by lyophilization were calculated as (mg found)/(mg theoretical). The fraction of peptide in solid products was calculated as percent peptide = (mg of peptide present determined by amino acid analysis)/(mg of solid by weight). Molecular weights of peptides were assumed to be those of the pure peptides, without the presence of water of hydration or acetate or other salts. Products which contain a low percentage of peptide weight compared to product weight (for example, **6** is 27% peptide) presumably contain water of hydration and acetate or other salts but contain no unidentified peptide material. Solvent–mixture ratios are volume per volume.

Biological Assay. Peptides were assayed on rabbit aortic strips as described by Furchgott and Bhadrakom[27] except that 1–2-cm strips under 5-g tension were used. A Grass Model 70 polygraph with FT 0.03 strain guage was employed to record isometric contraction. Some of the peptides were assayed in a preparation of dispersed single smooth muscle cells.[24] Responses in this latter preparation are assayed by observing contraction of the living cells under phase or Nomarski interference contrast optics. Quantitative dose–responses are not obtainable at this time due to the qualitative observations of contraction and variability of the sensitivity of preparations. In the strip assay, dose–response curves were determined at least once for each peptide in each of two separate strips. A dose–response to **4** was always obtained either before or after each peptide was tested. Relative affinities were estimated from the ratio of the ED_{50} of **4** to that of the unknown peptide.

(2-Nitro-5-azidobenzoyl)norleucine *N*-Hydroxysuccinimide Ester. The *N*-hydroxysuccinimide ester of 2-nitro-5-azidobenzoic acid was prepared as previously described.[15] To 1.5 g (5 mmol) of this ester in 20 mL of dioxane was added 0.96 g (7.5 mmol) of DL-norleucine (Sigma Chemical Co.) in 30 mL of 0.5 M $NaHCO_3$. After standing overnight the reaction mixture was diluted with 100 mL of 0.5 M $NaHCO_3$, extracted with EtOAc, acidified to pH 2 with HCl, and extracted again with EtOAc. The acidic extract was dried over $MgSO_4$, partially evaporated, and crystallized upon addition of heptane. Recrystallization from EtOAc gave 0.7 g (43%) of (2-nitro-5-azidobenzoyl)norleucine, mp 155–156 °C. Anal. ($C_{13}H_{15}N_5O_5$) C, H, N. To 0.64 g (2 mmol) of (2-nitro-5-azidobenzoyl)norleucine and 0.23 g (2 mmol) of *N*-hydroxysuccinimide (Sigma) in 6 mL of dioxane at 4 °C was added 0.41 g (2 mmol) of dicyclohexylcarbodiimide (Aldrich). After standing overnight at 4 °C the dicyclohexylurea was removed by filtration, and the filtrate was evaporated to an oil which crystallized from a small volume of EtOAc to yield 0.47 g (56%) of (2-nitro-5-azidobenzoyl)norleucine *N*-hydroxysuccinimide ester, mp 141–143 °C. Anal. ($C_{17}H_{18}N_6O_7$) C, H, N.

Solid-Phase Peptide Synthesis. [Asp[1],Ile[5]]angiotensin II (4) and its C-terminal heptapeptide **3** and hexapeptide **2** were prepared by solid-phase synthesis as described for 1.[18] N^{α}-*tert*-Butyloxycarbonylamino acids (Protein Research Foundation, Beckman, or Vega-Fox) were coupled in methylene chloride for two successive 30-min reactions with a 2- to 2.5-fold excess of *tert*-butyloxycarboxylamino acid and dicyclohexylcarbodiimide. In one synthesis, tyrosine was coupled as Boc-Tyr(2,6-Cl_2Bzl). In a second, compound **4** was prepared using Boc-Tyr(*o*-BrZ). The other trifunctional amino acids were coupled as Boc-His(Tos), Boc-Arg(Tos), and Boc-Asp(Bzl). Deprotection and cleavage from the resin were as previously described.[18] Purification of the crude peptides was as described below.

Val-Tyr-Ile-His-Pro-Phe (2). Initial purification was by ion-exchange chromatography on a 2.5 × 180 cm column of Bio-Rex 70 (H^+ form, Bio-Rad), eluting with a gradient of 10–50% HOAc.[18] Only a minor fraction (3% of the total peptide recovered) eluting near the end of the gradient appeared tyrosine deficient and contained 3-benzyltyrosine.[18] Additional purification of the major fraction from the Bio-Rex 70 chromatography was by countercurrent distribution (300 transfers in system G), K_G 0.57 (peptide **2**). The final yield (based on analysis of the starting phenylalanine resin) was 25% by weight of lyophilized powder: $R_{f(A)}$ 0.80, $R_{f(C)}$ 0.70, $R_{f(D)}$ 0.60, $R_{1.9}$ 0.70, $R_{6.5}$ 0.50; amino acid analysis Pro 1.07, Val 0.96, Ile 0.96, Tyr 0.97, Phe 1.00, His 0.93. This material was 89% peptide, determined by amino acid analysis.

Arg-Val-Tyr-Ile-His-Pro-Phe (3). Initial purification was by countercurrent distribution (100 transfers in system G), K_G 1.1 (peptide **3**). For this peptide, the fraction (K_G 1.9) appearing tyrosine deficient on amino acid analysis represented approximately 7% of the recovered peptide. For additional purification of the major fraction, the distribution was repeated in the same system. The final yield of **3** was 15% by weight: $R_{f(A)}$ 0.80, $R_{f(C)}$ 0.70, $R_{f(D)}$ 0.60 and 0.80 (trace impurity, ≤1%), $R_{1.9}$ 0.80, $R_{6.5}$ 0.80; amino acid analysis Pro 1.03, Val 1.04, Ile 0.98, Tyr 0.99, Phe 1.00, His 1.02, Arg 1.02. This material was 72% peptide, determined by amino acid analysis.

Asp-Arg-Val-Tyr-Ile-His-Pro-Phe (4). Initial purification was by ion-exchange chromatography as described for **2**. Again, only a small fraction of the recovered peptide (2%) appeared tyrosine deficient on amino acid analysis. Countercurrent distribution of the major fraction from Bio-Rex 70 after 550 transfers in system G yielded two major components: **4** with K_G 0.37, $R_{f(A)}$ 0.60, and **4a** with K_G 0.46, $R_{f(A)}$ 0.70. An impurity [**4b**, $R_{1.9}$ 0.50, $R_{6.5}$ 0.20, $R_{f(A)}$ 0.90, $R_{f(C)}$ 0.50] was still present in **4** and could be removed by CCD in system H where **4** has K_H 0.26 and **4b** has K_H 0.60. The final yield of **4** was 18%. Amino acid analysis: Asp 1.02, Pro 1.03, Val 1.04, Ile 0.94, Tyr 1.02, Phe 1.00, His 0.92, Arg 0.95; 75% peptide determined by amino acid analysis, $R_{f(A)}$ 0.60, $R_{f(B)}$ 0.15, $R_{f(C)}$ 0.60, $R_{1.9}$ 0.70, $R_{6.5}$ 0.40. In the second synthesis of **4** using Boc-Tyr(*o*-BrZ) rather than Boc-Tyr(2,6-Cl_2Bzl), initial purification was again by ion-exchange chromatography as for **2**. About 10% of the recovered peptide appeared tyrosine deficient upon amino acid analysis. Additional purification of the major fraction was by countercurrent distribution in system H, followed by a second distribution of the major fraction in system G, and a final distribution in system H to give pure **4** in 8% yield by weight, homogeneous, as above, on thin-layer chromatography and electrophoresis. Amino acid analysis: Asp 0.98, Pro 0.99, Val 0.95, Ile 0.96, Tyr 1.00, Phe 1.00, His 0.96, Arg 1.00; 75% peptide, determined by amino acid analysis.

N^{α}-(4-Formylbenzoyl)-[Asn[1],Val[5]]angiotensin II (5). To 10.3 mg (10 μmol) of [Asn[1],Val[5]]angiotensin II in 0.4 mL of dimethylformamide–pyridine (1:1) was added 2.5 mg (10 μmol) of the *N*-hydroxysuccinimide ester of 4-formylbenzoic acid.[16] After standing for 2 days at room temperature, the reaction mixture was chromatographed on a 0.9 × 50 cm column of Bio-Rex 70 as described for **2**. Peptide **5** was eluted by an additional volume (100 mL) of 50% HOAc after the 10–50% HOAc gradient (100 mL each). Evaporation of the 50% HOAc, followed by lyophilization from glacial HOAc, gave 2.66 mg (23% yield by weight) of N^{α}-(4-formylbenzoyl)-[Asn[1],Val[5]]angiotensin II (**5**): $R_{1.9}$ 0.40, $R_{6.5}$ 0.50, $R_{f(B)}$ 0.10, $R_{f(C)}$ 0.60; Pauly reagent positive, ninhydrin negative; 45% peptide by amino acid analysis: Asp 0.95, Pro 0.86, Val 2.16, Tyr 1.06, Phe 1.00, His 0.97, Arg 0.98; free of angiotensin II by TLC and HVE. An 11.2 mM solution of **5** in water had an absorbance of 0.024 at 295 nm, indicating 1.00 mol of 4-formylbenzoic acid (ϵ_{295} 2150, shoulder, in water) per mole of peptide.

N^{α}-(*N*-Fluoresceinthiocarbamyl)-[Asn[1],Val[5]]angiotensin II (6). To 19.8 mg (19.2 μmol) of [Asn[1],Val[5]]angiotensin II in 0.3 mL of pyridine–water (1:1) was added 8.71 mg (22 μmol) of fluorescein isothiocyanate (BBL Bioquest isomer I). The fluorescein isothiocyanate itself was not homogeneous by TLC: $R_{f(E)}$ 0.80, 0.70, 0.60; $R_{f(F)}$ 0.90, 0.80, 1.0. The reaction was allowed to proceed for 20 h in the dark, and all subsequent chromatography and manipulations were performed in the dark. Chromatography on Bio-Rex 70 as for **5** yielded a major fluorescent fraction (two overlapping bands) and a minor, later eluting fluorescent band, all of which contained angiotensin by amino acid analysis. TLC of the major fraction in system B showed five fluorescent bands. The major band was isolated by preparative TLC in system B ($R_{f(B)}$ 0.30 2-mm layer). Repeated rechromatography of this material in system B always produced a major band migrating with $R_{f(B)}$ 0.50 on analytical (0.25 mm) layers and minor bands (5–10%) at $R_{f(B)}$ 0.30 and 0.80 (analytical 0.25-mm layer). Rechromatography of $R_{f(B)}$ 0.30 (2-mm layer) material on Bio-Rex 70 again produced a major fluorescent fraction (two overlapping bands) and a minor band eluting later. This decomposition or isomerization may be related to the known lactonization of fluorescein in solution.[26] The final yield of **6** was 12 mg (43% by weight), which was 27% peptide by amino acid analysis: Asp 0.43, Pro 1.00, Val 1.97, Tyr 0.95, Phe 1.00, His 0.95, Arg 0.90; $R_{1.9}$ 0.60, $R_{6.5}$ 0.0; ninhydrin negative (or not detectable against the intense fluorescein color), Pauly positive; $R_{f(B)}$ 0.50 (major), 0.30 (minor), 0.80 (minor); $R_{f(C)}$ 0.70 (major), 0.80 (minor), 0.95 (minor); $R_{f(D)}$ 0.60 (major), 0.75 (minor), 0.80 (minor); free of angiotensin II by HVE and TLC. Tryptic digestion yielded a new fluorescent component, hexapeptide, and a minor amount of Asn-Arg by HVE.

N^{α}-(*N*-Rhodaminethiocarbamyl)-[Asp[1],Ile[5]]angiotensin II (7). To 23 mg (22 μmol) of [Asp[1],Ile[5]]angiotensin II in 0.3 mL of pyridine–H_2O (1:1) at 0 °C in the dark was added 10.5 mg (22 μmol) of tetramethylrhodamine isothiocyanate hydrochloride (BBL Bioquest, isomer R characterized by $R_{f(E)}$ 0.90, 0.75, 0.15, 0.0; $R_{f(F)}$ 0.95, 0.85, 0.80, 0.0) in 100 μL of pyridine. After standing overnight at 40 °C in the dark, the reaction mixture was evaporated to dryness and distributed for 60 transfers in CCD system H. The center portion of the major fluorescent band with K_H 1.0 was partially evaporated to remove all of the *n*-BuOH and then lyophilized to give 4.67 mg (16% by weight) of material which was 55% peptide as determined by amino acid analysis: Asp 0.41, Pro 1.15, Val 1.04, Ile 1.08, Tyr 1.01, Phe 1.00, His 0.93, Arg 1.02; $R_{1.9}$ 0.40; $R_{f(B)}$ 0.15 (major), 0.20 (minor); $R_{f(C)}$ 0.40 (major), 0.20 (minor), 0.70 (minor); $R_{f(D)}$ 0.60 (major), 0.40 (minor), 0.70 (minor); free of angiotensin II by HVE and TLC. Tryptic digestion yielded a new fluorescent component, hexapeptide, and a minor amount of Asp-Arg by HVE.

MDPF-[Asp[1],Ile[5]]angiotensin II (8). To 20.8 mg (20 μmol) of [Asp[1],Ile[5]]angiotensin II in 1.0 mL of dimethylformamide–pyridine–water (5:5:1) was added 12.5 mg (46 μmol) of 2-methoxy-2,4-diphenyl-3(2*H*)-furanone (the gift of Dr. M. Weigele, Hoffman-La Roche Inc., Nutley, NJ), and the reaction mixture was allowed to stand overnight at 43 °C. Chromatography on Bio-Rex 70 (H^+ form), as for compound **5**, yielded a fluorescent band which, upon concentration and lyophilization from a small volume of glacial acetic acid, yielded 17 mg (66% yield by weight) of **8**, 66% peptide by amino acid analysis: Asp 0.25, Pro 1.00, Val 1.06, Ile 0.94, Tyr 0.98, Phe 1.00, His 0.97, Arg 0.98; $R_{1.9}$ 0.45, $R_{f(B)}$ 0.50, $R_{f(C)}$ 0.75, $R_{f(D)}$ 0.75; ninhydrin negative, Pauly positive; free of angiotensin II by electrophoresis and TLC. A 0.113 mM solution of **8** in 1% NH_4HCO_3 had an absorbance of 0.760 at 385 nm, indicating 1.04 mol of fluorophore per mole of peptide (ϵ_{385} 6500 for the MDPF fluorophore[4]).

N^{α}-[(2-Nitro-5-azidobenzoyl)norleucyl]-[Asp[1],Ile[5]]angiotensin II (9). To 16.3 mg (15.5 μmol) of [Asp[1],Ile[5]]angiotensin II in 0.4 mL of dimethylformamide–pyridine–water (5:5:1) was added 24 mg (57 μmol) of (2-nitro-5-azidobenzoyl)-DL-norleucine *N*-hydroxysuccinimide ester, and the reaction mixture was allowed to stand overnight at 43 °C in the dark. Chromatography, as for compound **5**, yielded a band containing

both the 2-nitro-5-azidobenzoyl chromophore and peptide, which upon evaporation and lyophilization from a small volume of glacial acetic acid gave 13 mg (64% yield by weight) of **9**, which was 63% peptide by amino acid analysis: Asp 1.03, Pro 1.03, Val 0.88, Ile 0.98, Nle 0.83, Tyr 0.97, Phe 1.00, His 0.99, Arg 0.99; $R_{1,9}$ 0.40, $R_{f(B)}$ 0.60, $R_{f(C)}$ 0.75, $R_{f(D)}$ 0.75; ninhydrin negative, Pauly positive; free of angiotensin II by HVE and TLC.

Avidin-[Asp^1,Ile^5]angiotensin II (10). N^{α}-[(2-Nitro-5-azidobenzoyl)norleucyl]-[Asp^1,Ile^5]angiotensin II (**9**) (6.5 mg, 3000 nmol, of peptide) and 5.8 mg of avidin (Sigma, 40% protein by weight by amino acid analysis, 30 nmol) were dissolved in 0.4 mL of 0.1 M boric acid adjusted to pH 9 with NaOH. This mixture was photolyzed employing a Hanovia 450-W high-pressure mercury lamp (Ace Glass) within a Corning No. 3220 glass filter in a borosilicate immersion well[15] for 1.5 h and then chromatographed on a 1.2 × 60 cm column of Sephadex G-50 (fine) in 1% NH_4HCO_3. The protein peak in the void volume was analyzed for angiotensin content by ultraviolet spectroscopy of the photolyzed 2-nitro-5-azidobenzoyl chromophore and by amino acid analysis. By ultraviolet absorbance at 317 nm, 3 mol of angiotensin per mole of avidin was found: ϵ_{317} (compound **9**) 13000, ϵ_{317} (photolyzed compound **9**) 6600, ϵ_{280} (avidin) 110000. By amino acid analysis for norleucine, an average of 2 mol of angiotensin per mole of avidin was found, and this ratio was assumed to be the more accurate determination. The recovery of avidin was 90% by amino acid analysis. Avidin-angiotensin II retained 100% of the biotin binding ability of native avidin, assayed by the method of Green.[28] Gel filtration and analysis of this preparation after several months of storage at 4 °C again showed 2 mol of angiotensin per mole of avidin.

A second preparation of compound **10** by this method but with three successive additions of compound **9**, each followed by 1.5 h of photolysis, yielded avidin-[Asp^1,Ile^5]angiotensin II with 5 mol of angiotensin per mole of avidin, determined by amino acid analysis.

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References and Notes

(1) C. R. Sachatello, J. Sedwick, C. L. Moriarity, O. Grahl-Nielsen, and G. L. Tritsch, *Endocrinology*, **88**, 1300 (1971).
(2) G. Forget, S. Heisler, W. K. Park, P. Sirois, D. Gagnon, and D. Regoli, *J. Pharm. Pharmacol.*, **27**, 491 (1975).
(3) R. C. Nairn, "Fluorescent Protein Tracing", E. S. Livingston, Ltd., London, 1964, p 44.
(4) M. Weigele, S. DeBernardo, W. Leimgruber, R. Cleeland, and E. Grunberg, *Biochem. Biophys. Res. Commun.*, **54**, 899 (1973).
(5) T. B. Paiva and A. C. M. Paiva, *J. Med. Chem.*, **15**, 6 (1972).
(6) A. C. M. Paiva, V. L. A. Nouailetas, M. E. Miyamoto, G. B. Mendes, and T. B. Paiva, *J. Med. Chem.*, **16**, 6 (1973).
(7) R. J. Freer and J. M. Stewart, *J. Med. Chem.*, **15**, 1 (1972).
(8) J. Turk, P. Needleman, and G. R. Marshall, *J. Med. Chem.*, **18**, 1135 (1975).
(9) R. Walter, I. L. Schwartz, O. Hechter, T. Dousa, and P. L. Hoffman, *Endocrinology*, **91**, 39 (1972).
(10) D. H. Rich, P. D. Gesellchen, A. Tong, A. Cheung, and C. K. Buckner, *J. Med. Chem.*, **18**, 1004 (1975).
(11) D. Levey, *Biochim. Biophys. Acta*, **322**, 329 (1973).
(12) R. E. Galardy and J. D. Jamieson, *Mol. Pharmacol.*, **13**, 852 (1977).
(13) M. Das, T. Miyakawa, C. F. Fox, R. Pross, A. Aharanov, and H. R. Herschman, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 2790 (1977).
(14) J. R. Knowles, *Acc. Chem. Res.*, **5**, 155 (1972).
(15) R. E. Galardy, L. C. Craig, J. D. Jamieson, and M. P. Printz, *J. Biol. Chem.*, **249**, 3510 (1974).
(16) J. P. Kraehenbuhl, R. E. Galardy, and J. D. Jamieson, *J. Exp. Med.*, **139**, 208 (1974).
(17) H. Heitzman and F. M. Richards, *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 3537 (1974).
(18) R. E. Galardy, H. E. Bleich, P. Ziegler, and L. C. Craig, *Biochemistry*, **15**, 2303 (1976).
(19) B. W. Erickson and R. B. Merrifield, *J. Am. Chem. Soc.*, **95**, 3750 (1973).
(20) D. Yamashiro and C. H. Li, *J. Am. Chem. Soc.*, **95**, 1310 (1973).
(21) D. Yamashiro and C. H. Li, *J. Org. Chem.*, **38**, 591 (1973).
(22) B. Riniker and R. Schwyzer, *Helv. Chim. Acta*, **47**, 2357 (1964).
(23) D. Regoli, W. K. Park, and F. Rioux, *Pharmacol. Rev.*, **26**, 69 (1974).
(24) H. E. Ives, R. E. Galardy, and J. D. Jamieson, *J. Cell Biol.*, **70**, 328a (1976).
(25) R. M. McKinney, J. J. Spillane, and G. W. Pearce, *Anal. Biochem.*, **7**, 74 (1964).
(26) M. Rozwadowski, *Acta Physiol. Pol.*, **20**, 1005 (1961).
(27) R. F. Furchgott and S. Bhadrakom, *J. Pharmacol. Exp. Ther.*, **108**, 129 (1952).
(28) N. M. Green, *Methods Enzymol.*, **18A**, 418 (1970).

Trichloroacetamidines, a New Class of Positive Inotropic Agents

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A series of trichloroacetamidine derivatives, obtained by addition of amines to trichloroacetonitrile, was evaluated for positive inotropic activity on isolated cat heart papillary muscles. Increased contractility, not antagonized by β-adrenergic blockade with sotalol or reserpine pretreatment, was observed in this assay with a variety of N-substituted trichloroacetamidine derivatives. More extensive pharmacological studies with the 3-indolylmethyl analogue **2** showed that this amidine in dogs, 5 mg/kg iv, produced a positive inotropic effect more pronounced than that of ouabain, 50 μg/kg iv. Several of the trichloroacetamidines were found to be inhibitors of guinea pig kidney and calf heart Na–K-dependent ATPase and to have a specificity for these enzymes different from that of ouabain. Bacterial mutagenic activity was observed with three members, **2**, **3**, and **12**, of the series.

The use of myocardial stimulants, such as the cardiac glycosides and sympathomimetic amines, currently employed in the treatment of heart failure is limited by undesirable effects of these agents upon the heart or peripheral circulation. During the course of a program aimed at developing a positive inotropic agent with a

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