Photoaffinity Labeling of the Angiotensin II Receptor. 1. Synthesis and Biological Activities of the Labeling Peptides

Emanuel H. F. Escher,* Thi Mai Dung Nguyen,[1] Hélène Robert, Serge A. St-Pierre, and Domenico C. Regoli

Département de Pharmacologie, Faculté de Médecine, Université de Sherbrooke, Sherbrooke, Québec, J1H 5N4, Canada.
Received December 16, 1977

The synthesis and biological activities of analogues of the peptide hormone angiotensin II (AT) for use in photoaffinity labeling and receptor isolation are described. In the modified sequence of AT, Sar-Arg-Val-Tyr-Val-His-Pro-Phe, the aromatic residues Tyr and Phe have been either singly or simultaneously replaced by L-4′-nitrophenylalanine, L-4′-amino-3′,5′-diiodophenylalanine, L-4′-aminophenylalanine, L-4′-diazoniumphenylalanine, and L-4′-azidophenylalanine. The peptides were assembled by solid-phase synthesis and the functional groups in position 4 and/or 8 chemically modified. Radioactivity was introduced by catalytic tritiation of the iodinated peptides to form the photolabeling precursors containing L-4′-amino-3′,5′-diiodophenylalanine. On rabbit aorta the AT analogues substituted in position 4 showed poor affinities (0–15%), in position 8 high relative affinities (16–118%), and in position 4 and 8 additive effects of simultaneous substitutions. It is also shown that the new Boc derivative of L-4′-amino-3′,5′-diiodophenylalanine can be used in peptide synthesis without side-chain protection.

The receptors of peptide hormones have been closely investigated for several years.[2a] In spite of intense efforts the understanding of events at the molecular level of the membrane receptor remains still very limited, in particular, how the receptor takes the message from the hormone, translates the signal, passes it through the membrane, and triggers a code in the cell interior. However, pharmacological studies together with peptide synthesis and physicochemical technique have identified many requirements for hormone response.[2a] The best known system so far, the insulin receptor, was intensively studied and shown to consist of intrinsic membrane proteins, which can be solubilized with retention of binding activity.[2b] However, sufficiently large quantities of pure receptor protein for chemical characterization have not yet been obtained. Unfortunately, not all peptide hormone receptors maintain their binding capacity upon solubilization and purification. This holds also for angiotensin II (AT), another quite well understood peptide hormone.[3] Receptor solubilization with retention of hormone binding capacity failed when using preparations from adrenals.[4] However, a preparation from rabbit aorta[5] showed comparable affinity upon solubilization, but, again, no pure protein was obtained.

Pharmacological studies are greatly facilitated if specific irreversible inhibitors are available.[6] A covalently linked specific label would permit the isolation of the receptor protein from the membrane. Long-acting or irreversible inhibitors of angiotensin II have not been prepared so far, even though several potent competitive inhibitors[7] are known. Attempts have been made to develop irreversible blockers.[8] To achieve this goal we intend to apply the photoaffinity-labeling technique previously utilized for smaller peptides and model receptors[9,10] to AT. Other peptide hormones have recently been studied using similar approaches.[11-13] However, previous studies with the chymotrypsin model[9,14] suggested that photoaffinity labeling must be performed cautiously to avoid various side reactions that can give rise to unspecific and misleading labeling.[15] The objective of this work is to find analogues that will allow for specific covalent labeling of the peptide hormone receptor site. This should enable us to (1) obtain an irreversible blocking of AT receptor for pharmacological studies and (2) isolate the AT receptor from the plasma membrane with radioactive photolabeling AT analogues.

We therefore undertook the synthesis of a series of AT analogues where the aromatic amino acid residues in the octapeptide sequence Sar-Arg-Val-Tyr-Val-His-Pro-Phe were replaced by photolabile derivatives of phenylalanine. The latter derivatives were preferred over photolabile peptide modifying agents previously used in studies of ACTH[11] and gastrin,[12] because they give rise to closer resemblance to the natural ligand. L-4′-Azidophenylalanine[16] (Pap), a well-studied photolabel,[9,17] and L-4′-nitrophenylalanine (Nip) were chosen as photolabeling derivatives. The use of the latter was shown successful in earlier studies,[9] even though its photochemistry is poorly understood.[18] Finally, L-4′-diazoniumphenylalanine (Dip) has also been considered as a potential photolabel because of the property of diazonium compounds to form radicals upon irradiation.[19]

The isolation of receptors requires radioactivity to be located on the labeling amino acids. This is done in efforts to prevent the loss of radioactivity from the photolabeled receptor by proteolytic action of nearby enzymes[20] after photoreaction and during isolation. Furthermore, the 4′-azidophenylalanine derivative was shown to be unstable in liquid hydrogen fluoride[18] and hydrogenation. The problem was overcome by introducing the labeling amino acid in the form of a precursor suitable for tritiation and azide formation on the final peptide[21] and not by direct incorporation into the peptide;[21,22] L-4′-amino-3′,5′-diiodophenylalanine (Adip) was the choice after its chemical stability in peptide synthesis was tested. Its conversion into L-4′-azido-3′,5′-ditritiophenylalanine has already been reported.[23]

This paper describes the synthesis and biological activities (rabbit aorta) of analogues of AT for their use as photoaffinity labels of the AT receptors.

Results and Discussion

The structure and biological activities of the AT analogues are presented in Table I.

The peptides **1–5** were prepared by solid-phase synthesis,[27] cleaved from the resin by liquid hydrogen fluoride,[28] and purified by gel-filtration, ion-exchange, and partition chromatography.[29] Peptides **1–5** were derivatized according to Scheme I. Hydrogenation gave peptides **6**, **8**, and **10**, subsequent diazotization provided peptides **12–14**, and azide addition produced analogues **7**, **9**, and **11**. Tritiation of **1** and **2** gave **15** and **16** with specific activities of 44 and 73 Ci/mmol, respectively.

The final peptides except **12–14** were analyzed by amino acid analysis, UV or IR spectroscopy, and elemental analysis (only **2**). The L-4′-azidophenylalanine analogues **7**, **9**, and **11** were rehydrogenated to products which were identical with their L-4′-aminophenylalanine (Amp) precursors. The purity of the peptides was tested in three different TLC systems and by electrophoresis.

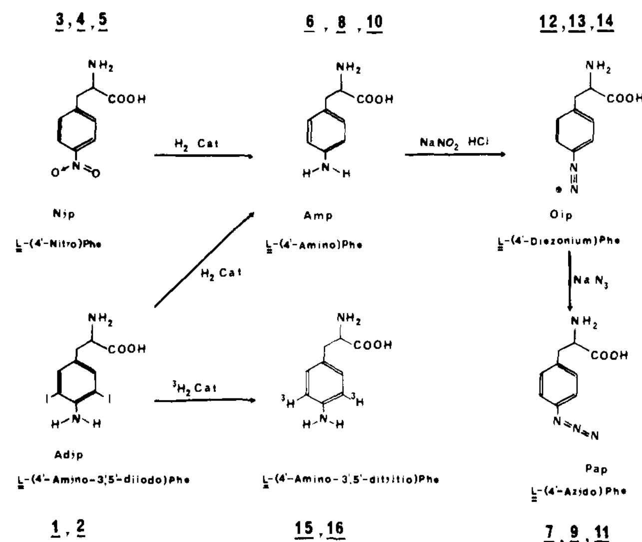
0022-2623/78/1821-0860$01.00/0 © 1978 American Chemical Society

Table I. Structure and Biological Activity of [1-Sarcosine]angiotensin II Analogues

Sar-Arg-Val·AA⁴-Val-His-Pro-AA⁸						
$AA^{4\,a}$	$AA^{8\,a}$	compd no.	$\alpha^{E\,b}$	$pD_2{}^c$	$pA_2{}^d$	rel affinity[e]
Tyr	Phe	0[g]	1	9.23		100
Adip	Phe	1	1	7.68		2.7
Tyr	Adip	2	0		9.30	118
Nip	Phe	3	0		0.00	0.00
Tyr	Nip	4	0.46	9.07	9.43	69
Nip	Nip	5	0.5	6.02	6.12	0.06
Amp	Phe	6	1	8.41		15
Pap	Phe	7	0.75	6.65		0.26
Tyr	Amp	8	1	8.80		37
Tyr	Pap	9	1	8.72		31
Amp	Amp	10	1	8.17		8.7
Pap	Pap	11	0.8	6.12		0.07
Dip	Phe	12	1	7.05		0.66
Tyr	Dip	13	1	8.33		13
Dip	Dip	14	1	7.36		1.3
$(3',5'\text{-}{}^3H_2)$Amp	Phe	15				f
Tyr	$(3',5'\text{-}{}^3H_2)$Amp	16				f

[a] Adip = L-(4-amino-3',5'-diiodo)Phe, Nip = L-(4'-nitro)Phe, Amp = L-(4'-amino)Phe,[25] Pap = L-(4'-azido)Phe, Dip = L-(4'-diazonium)Phe. Biological activities were determined with the rabbit aorta test.[26] [b] α^E = intrinsic activity. [c] pD_2 = log of the concentration of peptide producing 50% of maximal response. [d] pA_2 = log of the concentration of agonist as partial agonist that reduces the effect of a double dose of agonist to that of a single dose. [e] Relative affinity in percent of standard affinity of 0 (=100%). [f] Not determined. [g] See ref 24.

Scheme I



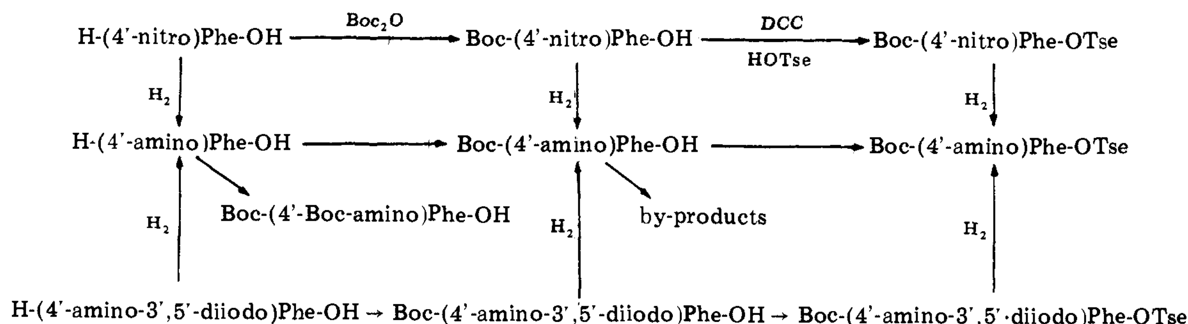
It was predicted that L-(4'-amino-3',5'-diiodo)Phe could be used in standard solid-phase synthesis without side-chain protection. The two bulky iodine atoms should provide enough steric hindrance to prevent acylation of the 4'-amino group. This was demonstrated by synthesizing protected (4'-amino)-, (4'-amino-3',5'-diiodo)-, and (4'-nitro)Phe derivatives and comparing their hydrogenation products (Scheme II). Upon using an excess of di-*tert*-butyl dicarbonate[30] (Boc_2O), (4'-nitro)- and (4'-amino-3',5'-diiodo)Phe formed only mono-Boc compounds, whereas (4'-amino)Phe yielded a second product which lacked a free amino group. After esterification with 2-(*p*-toluenesulfonyl)ethanol[31] (HOTse) and dicyclohexylcarbodiimide (DCC), Boc-(4'-nitro)- and Boc-(4'-amino-3',5'-diiodo)Phe gave only one product, while Boc-(4'-amino)Phe gave several. Hydrogenation of crude Boc-(4'-nitro)Phe and Boc-(4'-amino-3',5'-diiodo)Phe gave only pure Boc-(4'-amino)Phe. After the same treatment, the respective crude 2-(*p*-toluenesulfonyl)ethyl ester gave only Boc-(4'-amino)Phe-OTse.

The ultimate proof was obtained from the synthesis of peptides **1**–**4** (see Table I). Upon hydrogenation, both **1** and **3** gave peptide **6**; similarly, **2** and **4** gave **8**. The identity of the hydrogenation products was demonstrated by thin-layer chromatography (see the Experimental Section), electrophoresis, biological activity,[26] and amino acid analysis.

4'-Amino-3',5'-diiodophenylalanine was stable in liquid HF for several hours at room temperature but was readily hydrogenated to 4'-aminophenylalanine or tritiated to 3',5'-tritio-4'-aminophenylalanine.[23]

All analogues of AT exhibit moderate to strong specific affinity to the AT receptor of rabbit aortas, except **3** which does not bind at all. The agonists and **2**, an inhibitor, are competitive ligands in the absence of UV radiation. The analogues having a photosensitive group in position 4 of AT exhibit very weak affinities (**7** and **12**) or even none (**3**); the same can be stated about the doubly substituted

Scheme II



analogues **5**, **11**, and **14**. Substitution of position 8 gives analogues with high relative affinities (16–118%) although the intrinsic activity varies from 0% (**2**) to over 50% (**4**) to 100% (**8**, **9**, **13**).

Conclusions

4′-Amino-3′,5′-diiodophenylalanine was easily incorporated in peptides and quickly modified in situ to 4′-azidophenylalanine or 4′-amino-3′,5′-ditritiophenylalanine with specific activity of about 50 Ci/mmol. This makes 4′-amino-3′,5′-diiodophenylalanine a quite universal photoaffinity-labeling precursor whenever peptides are involved. We therefore intend to use the same approach for bradykinin, substance P, and other peptides in the near future.

The AT analogues substituted with photolabeling derivatives in position 8 are well suited for further pharmacological and biochemical studies with the influence of UV light. This work is presently under investigation in our laboratory.

Experimental Section

Elemental analyses were performed by Galbraith Laboratories Inc., Knoxville, Tenn. NMR spectra were obtained with a Varian T-60 spectrometer, using tetramethylsilane (Me_4Si) as an internal reference. UV–visible spectra were recorded on a Beckman 25 spectrophotometer and IR spectra of KBr pellets on a Perkin-Elmer 457 instrument, and optical rotations were measured with a Zeiss Model O.L.D. 5 polarimeter. Amino acid analysis were performed on a Technicon TSM analyzer equipped with an Autolab integrator. Melting points were measured on a Thomas-Hoover melting point apparatus. *tert*-Butyloxycarbonylamino acids, peptide reagents, and chloromethylated resin (copolystyrene–1% divinylbenzene, 0.75 mmol of Cl/g of resin) were obtained from Bachem Fine Chemicals Inc., if not otherwise stated, and were used without further purification. Thin-layer chromatography (TLC) was performed on Merck precoated silica gel plates (type G60-F254) in the following solvent systems: BAW, 1-butanol–acetic acid–water, 10:2:3; BAWP, 1-butanol–acetic acid–water–pyridine, 15:3:12:10; BIWCl, 1-butanol–2-propanol–water–monochloroacetic acid, 65:15:20:3; CMA, chloroform–methanol–acetic acid, 95:5:3. The spots were visualized with UV fluorescence, ninhydrin, Pauly reagent, or a modified Reindel–Hoppe procedure.[32] Electrophoresis was performed on Merck precoated cellulose plates at pH 2.1 and the R_f values are correlated to picric acid (R_f 1.0). Amino acid analyses have been performed on peptides 1–11. Val has been chosen as internal standard of 2.00. Sar and His are poorly resolved; the integral is multiplied with the mean of their correction factors. Arg has been partially decomposed to Orn; both added, they gave 0.98 ± 0.12 for all samples. Peptide samples were hydrolyzed during 24 h at 110 °C in 6 N HCl + 0.2% phenol in vacuum sealed tubes. Biological activities were measured on rabbit aorta strips and described previously.[26] All synthesis, purifications, manipulations, and biological and instrumental analysis were performed under special lighting conditions with exclusion of long-wavelength UV light.

4′-Amino-3′,5′-diiodophenylalanine (H-Adip-OH) (MW 432.00). 4′-Aminophenylalanine (5 g, 27.77 mmol, MW 180.21) was dissolved in glacial AcOH (100 mL), iodine monochloride (ICl, Alfa Inorganics, 17.99 g, 111.0 mmol) was added, and the reaction was stirred at room temperature for 2 h. The solvent and excess of ICl were evaporated and the residue was extracted several times with CH_3OH, until a white powder was obtained. Recrystallization from neutralized 2 M NH_3 yielded 7.03 g of Adip (MW 432.00, 58%): mp 185 °C dec; TLC R_f^{BAW} 0.40; NMR (TFA-*d*) 7.8 (2 H, s, aromatic), 5.0 (1 H, α-H), 3.3 ppm (2 H, β-H). Anal. ($C_9H_{10}N_2O_2I_2$) C, H, N, I.

N$^{\alpha}$**-*tert*-Butyloxycarbonyl-4′-amino-3′,5′-diiodophenylalanine (Boc-Adip-OH) (MW 532.12).** 4′-Amino-3′,5′-diiodophenylalanine (5 g, 11.6 mmol) was reacted with Boc_2O (5.1 g, 23.2 mmol):[34] yield 5.74 g (93%); mp 168–171 °C; TLC R_f^{CMA} 0.42; $[\alpha]^{25}_D$ +60.03° (*c* 1, ethanol); NMR 1.4 (9 H, Boc), 7.5 ppm (2 H, aromatic protons). Anal. ($C_{14}H_{18}N_2O_4I_2$) C, H, N, I.

N$^{\alpha}$**-*tert*-Butyloxycarbonyl-4′-aminophenylalanine (Boc-Amp-OH) (MW 280.33).** (1) *tert*-Butyloxycarbonyl-4′-amino-3′,5′-diiodophenylalanine (10 mg) crude from the above reaction was hydrogenated in CH_3OH (2 mL) with palladium on charcoal (10%) (Pd/C) (2 mg) for 6 h. (2) *tert*-Butyloxycarbonyl-4′-nitrophenylalanine (150 mg, TLC R_f^{CMA} 0.66) was hydrogenated the same way with Pd/C (10 mg). (3) 4′-Aminophenylalanine (110 mg, 0.58 mmol) was reacted with Boc_2O (190 mg, 1.75 mmol) for 24 h; TLC (CMA) showed for the products of reactions 1 and 2 only one spot at R_f 0.14. Reaction 3 was shown to contain two products by the same TLC system, one main spot at R_f 0.14 and a faint one at R_f 0.40, which became more intense when the TLC was repeated after 4 days of reaction. All spots at R_f 0.14 were ninhydrin positive (pink), but the spot at R_f 0.40 became ninhydrin positive only after trifluoroacetic acid (TFA) treatment.

N$^{\alpha}$**-*tert*-Butyloxycarbonyl-4′-nitrophenylalanine 2-(*p*-Toluenesulfonyl)ethyl Ester (Boc-Nip-OTse) (MW 492.51).** N^{α}-*tert*-Butyloxycarbonyl-4′-nitrophenylalanine (1 g, 33.22 mmol) was dissolved in pyridine (25 mL); 2-(*p*-toluenesulfonyl)ethanol (HOTse, 0.71 g, 3.52 mmol, MW 200.26) and dicyclohexylcarbodiimide (DCC) (0.80 g, 3.86 mmol, MW 206.33) were added. After 12 h, dicyclohexylurea was filtered off and the filtrate evaporated to dryness. The product was dissolved in EtOAc and washed with $NaHSO_4$–Na_2SO_4 buffer. The organic layer was extracted with saturated $NaHCO_3$ and dried with anhydrous Na_2SO_4. The solution was evaporated and the product crystallized from EtOAc–petroleum ether: TLC R_f^{CMA} 0.80, ninhydrin positive only after TFA treatment; yield 870 mg (55.5% or 1.79 mmol); mp 90–94 °C; NMR 1.4 (9 H, Boc), 2.4 (3 H, CH_3-OTse), 7.2–8.2 ppm (8 H, aromatic).

N$^{\alpha}$**-*tert*-Butyloxycarbonyl-4′-amino-3′,5′-diiodophenylalanine 2-(*p*-Toluenesulfonyl)ethyl Ester (Boc-Adip-OTse) (MW 714.38).** N^{α}-*tert*-Butyloxycarbonyl-4′-amino-3′,5′-diiodophenylalanine (250 mg, 0.48 mmol) was reacted with HOTse and DCC, as described above: yield 225 mg (64.29%, 0.30 mmol); mp 82–85 °C; TLC R_f^{CMA} 0.89, ninhydrin positive after TFA treatment; NMR 1.4 (9 H), 2.4 (3 H), 7.2–8.0 ppm (6 H).

N$^{\alpha}$**-*tert*-Butyloxycarbonyl-4′-aminophenylalanine 2-(*p*-Toluenesulfonyl)ethyl Ester (MW 462.59).** (1) Crude *tert*-butyloxycarbonyl-4′-nitrophenylalanine 2-(*p*-toluenesulfonyl)ethyl ester (100 mg) from above was hydrogenated as described above. The product showed one spot by TLC, R_f^{CMA} 0.46, ninhydrin positive. (2) Crude N^{α}-*tert*-butyloxycarbonyl-4′-amino-3′,5′-diiodophenylalanine 2-(*p*-toluenesulfonyl)ethyl ester (75 mg) was hydrogenated as above: one spot at R_f^{CMA} 0.46. (3) N^{α}-*tert*-Butyloxycarbonyl-4′-aminophenylalanine (500 mg, 1.86 mmol) was esterified with HOTse (410 mg, 2.05 mmol) and DCC (460 mg, 2.23 mmol): TLC spots at R_f^{CMA} 0.20, 0.24, 0.34, and 0.46; no further identification.

Peptide synthesis was carried out by either using a Beckman Peptide Synthesizer Model 990B (method A) or a Burrell shaker with glass reaction vessels[33] (method B) according to a slightly modified schedule.[34] Coupling steps were monitored for completion by means of the ninhydrin test.[35] Single couplings were performed, using a threefold excess of *tert*-butyloxycarbonylamino acid. If the reaction was still incomplete after two couplings, the peptides were acetylated.[36] The first *tert*-butyloxycarbonylamino acid was attached to the resin using the cesium salt procedure.[37] The degree of substitution was evaluated by the picric acid method[38] and by elemental analysis. Simultaneous cleavage of the peptide from the resin and of the side-chain protecting groups was performed in liquid HF–anisole (5:1) for 30 min at −20 °C, followed by 60 min at 0 °C, using a Kel-F/Teflon apparatus from Protein Research Foundation, Japan. The peptides were extracted from the resin with 50% acetic acid and lyophilized.

[1-Sarcosine,4-(4′-amino-3′,5′-diiodophenylalanine)]-angiotensin II (1). Method A. This peptide was prepared from *tert*-butyloxycarbonylphenylalanyl-resin (2.5 g, 0.23 mmol/g), using the following protected L-amino acids: Boc-Pro-OH, Boc-His(Tos)-OH, Boc-Val-OH, Boc-Arg(Tos)-OH, Boc-Adip-OH, and Boc-Sar-OH.

[1-Sarcosine,8-(4′-amino-3′,5′-diiodophenylalanine)]-angiotensin II (2). Method A. The synthesis of this peptide was carried out with N^{α}-*tert*-butyloxycarbonyl-4′-amino-3′,5′-diiodophenylalanyl-resin (4 g, 0.23 mmol/g, determined by iodine

Table II. Synthetic, Physicochemical, and Analytical Data of [1-Sarcosine]angiotensin II Analogues

derivatives	MW, acetate free	peptide yield		UV		IR, cm^{-1}	TLC, R_f				amino acid analyses					
		mg	%	λ_{max}	ϵ		BAW	BAWP	BIWCl	Electroph	Pro	Val	Tyr	Phe	His + Sar	Arg
[Sar^1]-AT$_{II}$ (0)[a]	987.18			260	907		0.40	0.53	0.15	0.186						
1	1237.97	40	4	280	1360		0.40	0.57	0.15	0.186	1.08	2.0		0.88	2.01	0.80
2	1253.97	127	11	280/260	1547/1413		0.40	0.57	0.16	0.186	1.17	2.00	0.87		2.0	0.97
3	1016.16	54	4.4	280	5600		0.40	0.55	0.15	0.186	1.06	2.00		0.89	1.88	0.82
4	1032.16	188	11.7	280	4000		0.40	0.51	0.13	0.186	1.15	2.00	0.81		1.89	0.83
5	1061.16	211	17.1	280	7867		0.40	0.55	0.15	0.186	1.18	2.00			2.14	0.68
6	986.18	3	60	285	213		0.18	0.39	0.075	0.209	1.22	2.00		1.18	1.84	0.87
7	1012.17	3.5	50			2100 ($-N_3$)	0.43	0.53	0.16	0.186	1.04	2.00		1.12	1.81	0.69
8	1002.18	36	72	260	853		0.19	0.41	0.077	0.209	1.19	2.00	0.94		1.95	0.88
9	1028.17	2.3	77			2100 ($-N_3$)	0.48	0.51	0.14	0.186	0.97	2.00	0.85		2.12	0.82
10	1001.20	26	50	285	200		0.04	0.22	0.06	0.279	0.94	2.00			2.19	0.81
11	1053.18	2.5	83			2100 ($-N_3$)	0.58	0.57	0.17	0.186	1.12	2.00			1.87	0.92
15	990.18	0.8	19				0.18	0.39	0.075							
16	1006.18	1.2	29				0.19	0.41	0.077							

[a] See ref 24.

analysis) using the same *tert*-butyloxycarbonylamino acid derivatives as above plus Boc-Tyr(Bzl)-OH.

[1-Sarcosine,4-(4′-nitrophenylalanine)]angiotensin II (3). Method B: From 3.0 g of *tert*-butyloxycarbonylphenylalanyl-resin and *tert*-butyloxycarbonyl-4′-nitrophenylalanine; same procedure as for compound **1**.

[1-Sarcosine,8-(4′-nitrophenylalanine)]angiotensin II (**4**) **and [1-Sarcosine,4-(4′-nitrophenylalanine),8-(4′-nitrophenylalanine)]angiotensin II** (**5**). **Method B.** From 4.9 g of *tert*-butyloxycarbonyl-4′-nitrophenylalanyl-resin (0.75 mmol/g, determined by nitrogen analysis), Boc-Val-His(Tos)-Pro-4′-nitrophenylalanine was assembled on the resin. The peptide resin was divided into two equal portions. Peptide **4** was prepared from the first portion using Boc-Tyr(2-Br-Z)-OH and peptide **5** from the second portion.

Peptide Purification. The crude peptides **1**–**5** were dissolved in 0.2 M acetic acid and the solution was applied to a 2.5 × 100 cm column of Sephadex G-15. The peptide fraction was lyophilized and further purified by ion-exchange chromatography on Biorex 70 resin (Bio-Rad), using an acetic acid gradient (0–50%). If necessary, a last step of purification by partition chromatography[29] on Sephadex G-25 M was performed using a 1-butanol–acetic acid–water (4:1:5) system. After a final purification by gel filtration on G-15, the peptides were homogeneous in the three TLC systems, BAW, BAWP, and BIWCl, and by electrophoresis.

The peptides cited hereafter are all moderately to strongly photosensitive, except the 4′-aminophenylalanine-containing peptides. Therefore, their optical rotation has not been measured.

[1-Sarcosine,4-(4′-aminophenylalanine)]angiotensin II (6), [1-Sarcosine,8-(4′-aminophenylalanine)]angiotensin II (8), and [1-Sarcosine,4-(4′-aminophenylalanine),8-(4′-aminophenylalanine)]angiotensin II (10). The peptides **1**–**5** (3–5 mg) were dissolved in 0.2 M acetic acid (1–5 mL), Pd/C (1–5 mg) was added, and the hydrogenation was carried out at normal pressure and room temperature for 3 h. The catalyst was filtered off and the reaction mixture immediately applied to a G-15 column equilibrated with 0.2 M acetic acid. The products **6**, **8**, and **10** were collected and lyophilized.

[1-Sarcosine,4-(4′-azidophenylalanine)]angiotensin II (7), [1-Sarcosine,8-(4′-azidophenylalanine)]angiotensin II (9), and [1-Sarcosine,4-(4′-azidophenylalanine),8-(4′-azidophenylalanine)]angiotensin II (**11**). To a solution of the above peptides **6**, **8**, or **10** (3 mg each) in 2 mL of 0.1 M HCl at 0 °C, 90 μL of a 0.1 M solution of $NaNO_2$ was added under stirring. After 10 min, diazotization was controlled with iodine–starch paper. If negative, more of the $NaNO_2$ solution was added until a positive reaction was obtained. After 5 min, a 0.1 M solution of sulfamic acid (100 μL) was added, followed by a 0.1 M solution of NaN_3 (60 μL). After 5 min, the reaction mixture was neutralized with $NaHCO_3$ and this solution applied to a column of Sephadex G-15. The collected peptides were further purified by partition chromatography and lyophilized. The intermediate diazonium peptides **12**–**14** were not characterized (see Table II).

[1-Sarcosine,4-(4′-amino-3′,5′-ditritiophenylalanine)]-angiotensin II (**15**) **and [1-Sarcosine,8-(4′-amino-3′,5′-ditritiophenylalanine)]angiotensin II** (**16**). The peptides **1** and **2** (5 mg each) were dissolved in 0.5 μL of dimethylacetamide, 2 mg of Pd/C was added, and tritiation was performed during 30 min at a pressure of 600 Torr of $^3H_2$. After exchange of labile ^3H the peptide solution was filtered through a column of Sephadex G-15. The tritiated peptides showed the same R_f values and UV spectra as the cold one. The specific radioactivity in Ci/mmol was determined by β counting and the Lowry test, peptide **15** showing 44 Ci/mmol and peptide **16** showing 73 Ci/mmol.

Acknowledgment. We gratefully acknowledge Dr. Jean-Louis Morgat from the Service de Biochimie, Centre d'Etudes Nucléaires de Saclay (France), for his help and the opportunity to work in his laboratory for the tritiation. Thanks are due to Dr. David Gibson and Mr. Claude Lazure from the Department of Biochemistry, Faculté de Médecine, Université de Sherbrooke, for the amino acid analyses and to Mrs. J. Pépin and T. Carrier for typing the manuscript. The Boc_2O was a kind gift from O. Keller,

Ph.D., Fluka AG Buchs, Switzerland. This work was supported by a grant from the Medical Research Council of Canada, No. DG 154.

References and Notes

(1) (1) Part of the M.Sc. thesis of T. M. D. Nguyen, April 1978.
(2) (a) B. I. Posner, *Can. J. Physiol. Pharmacol.*, **53**, 689 (1975); (b) P. Cuatrecasas, *J. Biol. Chem.*, **247**, 1980 (1972).
(3) D. Regoli, W. K. Park, and F. Rioux, *Pharmacol. Rev.*, **26**, 69 (1974).
(4) Personal communication from W. Schlegel, J. C. Bonafous, and R. Schwyzer, unpublished results.
(5) M. A. Devynck, M. G. Pernollet, P. Meyer, S. Fermandjian, P. Fromageot, and F. M. Bumpus, *Nature (London)*, **249**, 67 (1974).
(6) H. P. Rang, *Q. Rev. Biophys.*, **7**, 283 (1975).
(7) M. C. Koshla, R. A. Leese, W. L. Maloy, A. T. Ferreira, R. R. Smeby, and F. M. Bumpus, *J. Med. Chem.*, **15**, 792 (1972); D. Regoli, F. Rioux, and W. K. Park, *Rev. Can. Biol.*, **31**, 73 (1972).
(8) T. B. Paiva, A. C. M. Paiva, R. I. Freer, and J. M. Stewart, *J. Med. Chem.*, **15**, 6 (1972).
(9) E. Escher and R. Schwyzer, *FEBS Lett.*, **46** (1), 347 (1974).
(10) E. Escher, Thesis, ETH No. 5363, Zürich, 1974.
(11) J. Ramachandran and E. Canova-Davis in "Peptides", Proceedings of the 5th American Peptide Symposium, M. Goodman and J. Meienhofer, Ed., Wiley, New York, N.Y., 1977, p 553.
(12) R. E. Galardy, L. C. Craig, J. D. Jamieson, and M. P. Printz, *J. Biol. Chem.*, **249**, 3510 (1974).
(13) M. I. Titov and A. A. Az'muko, *Bioorg. Khim.*, **3**, 842 (1977).
(14) W. Fischli, O. Leukart, and R. Schwyzer, *Helv. Chim. Acta*, **60**, 959 (1977).
(15) A. E. Ruoho, H. R. Kiefer, P. E. Roeder, and S. J. Singer, *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 2567 (1973).
(16) R. Schwyzer and M. Caviezel, *Helv. Chim. Acta*, **54**, 1395 (1971).
(17) N. Sonenberg, M. Wilchek, and A. Zamir, *Biochem. Biophys. Res. Commun.*, **72**, 1534 (1976).
(18) E. Escher, *Helv. Chim. Acta*, **60**, 339 (1977).
(19) J. de Jonge, *Recl. Trav. Chim. Pays-Bas*, **72**, 846 (1952).
(20) F. Rioux, W. K. Park, and D. Regoli, *Can. J. Physiol. Pharmacol.*, **53**, 383 (1975).
(21) E. Escher, R. Jost, H. Zuber, and R. Schwyzer, *Isr. J. Chem.*, **12**, 129 (1974).
(22) T. H. Wieland, A. von Dungen, and C. H. Birr, *Justus Liebigs Ann. Chem.*, **752**, 109 (1971).
(23) W. Fischli, M. Caviezel, A. Eberle, E. Escher, and R. Schwyzer, *Helv. Chim. Acta*, **59**, 878 (1976).
(24) W. K. Park, [Sar[1],Val[5]]-AT_{II}, unpublished synthesis; biological activity and R_f values were identical with [Sar[1],-Ile[5]]-AT_{II} from D. Regoli, W. K. Park, and F. Rioux, *Can. J. Physiol. Pharmacol.*, **51**, 114 (1973).
(25) F. Bergel and J. A. Stock, *J. Chem. Soc.*, 2409 (1954).
(26) J. St-Louis, D. Regoli, J. Barabé, and W. K. Park, *Can. J. Physiol. Pharmacol.*, **55**, 1056 (1977).
(27) B. W. Ericson and R. B. Merrifield in "The Proteins", Vol. II, 3rd ed, H. Neurath, R. L. Hil, and C. L. Boeder, Ed., Academic Press, New York, N.Y., 1976, pp 251–527.
(28) S. Sakakibara in "Chemistry and Biochemistry of Amino Acids, Peptides and Proteins", Vol. I, B. Weinstein, Ed., Marcel Dekker, New York, N.Y., 1971, p 51.
(29) D. Yamashiro and C. H. Li, *Int. J. Pept. Protein Res.*, 4, 181 (1972).
(30) L. Moroder, A. Hallet, E. Wünsch, O. Keller, and G. Wersin, *Z. Physiol. Chem.*, **357**, 1651 (1976).
(31) A. W. Miller and C. J. M. Stirling, *J. Chem. Soc.*, 2612 (1968).
(32) E. Von Arx, M. Faupel, and M. Brugger, *J. Chromatogr.*, **120**, 224 (1976).
(33) W. K. Park and D. Regoli, *Can. J. Biochem.*, **50**, 755 (1972).
(34) B. F. Gisin and R. B. Merrifield, *J. Am. Chem. Soc.*, **94**, 3102 (1972).
(35) E. Kaiser, R. L. Colescott, C. D. Bossinger, and P. I. Cook, *Anal. Biochem.*, **34**, 595 (1970).
(36) E. Bayer, H. Eckstein, K. Hägele, W. A. König, W. Brüning, H. Hagenmaier, and W. Parr, *J. Am. Chem. Soc.*, **92**, 1735 (1970).
(37) B. F. Gisin, *Helv. Chim. Acta*, **56**, 1476 (1973).
(38) B. F. Gisin, *Anal. Chim. Acta*, **58**, 248 (1972).

Pivaloyl Esters of N,N-Dialkylated Dopamine Congeners. Central Dopamine–Receptor Stimulating Activity

Håkan Wikström,* Per Lindberg, Per Martinson, Stephan Hjorth, Arvid Carlsson,

Department of Pharmacology, University of Göteborg, Fack, S-400 33 Göteborg, Sweden

Uli Hacksell, Uno Svensson, and J. Lars G. Nilsson

Department of Organic Pharmaceutical Chemistry, Biomedical Center, University of Uppsala, S-751 23 Uppsala, Sweden.
Received April 4, 1978

In order to test for dopamine–receptor stimulating activity a new, sensitive biochemical screening method was designed. For behavioral studies and for determination of the duration of action on the compounds, motor activity measurements were used. *O,O'*-Dipivaloyl-*N,N*-dipropyldopamine (4) was the only derivative of a series of dipivaloyl-*N,N*-dialkyldopamines studied that showed any significant activity. However, the monopivaloyl ester 2-(3-pivaloyloxyphenyl)-*N,N*-dipropylethylamine (8) seemed to be more potent. The same relationship was found for the corresponding phenols, *N,N*-dipropyldopamine (3) and 2-(3-hydroxyphenyl)-*N,N*-dipropylethylamine (7), although both were more active than their pivaloyl esters.

Substances which stimulate dopamine receptors in the central nervous system have attracted increasing clinical interest.[1] Such compounds are known to induce hyperactivity and stereotyped behavior in animals. Numerous papers describing syntheses and pharmacology of such dopamine receptor agonists have appeared in the literature over the last decade.[2-13]

Dopamine itself does not pass the blood–brain barrier. Several simple dopamine derivatives, however, have shown central dopaminergic activity after peripheral administration.[4,13] Thus some *N,N*-dialkyldopamine derivatives, e.g., *N*-methyl-*N*-propyl-, *N*-butyl-*N*-methyl-, and *N*-butyl-*N*-propyldopamine, have been shown to induce turning in nigral-lesioned rats when given intraperitoneally.[4]

Apomorphine is a direct stimulant of dopamine receptors in the brain.[14] Its clinical use, however, is limited because of short duration and low bioavailability when administered orally. Borgman et al.[15-17] have studied diester derivatives of apomorphine, e.g., *O,O'*-dipivaloylapomorphine, and reported a prolonged duration of action. This compound was independently synthesized and

0022-2623/78/1821-0864$01.00/0 © 1978 American Chemical Society