pK_a Determinations. The pK_a determinations were made at 30 °C as described by Albert and Serjeant.[20] The monooxalate salts were titrated using standardized carbonate-free KOH and a Radiometer Copenhagen Autoburette ABU12 and TTA60 titration assembly. The pH was measured using a Radiometer PHM standard pH meter. During the titration the solution was blanketed with N_2. The pK values for the amino and phenol were resolved using the Fortran program described in ref 20. Values reported for psilocin are the mean of four determinations and those for bufotenin the mean of duplicate determinations.

Octanol/Water Partition Coefficients. Partition coefficients were determined by standard methods[21] using 0.1 M phosphate buffer and 1-octanol, pH 7.40. Concentrations in the aqueous phases were determined using ultraviolet spectroscopy, and solutions were kept blanketed with N_2 to prevent oxidation. Apparent partition coefficients (P), uncorrected for ionization, are reported in Table II. Log P values reported in the table are for the un-ionized species.

Conversion of Bufotenin Monooxalate to the Free Base. Bufotenin monooxalate monohydrate (Sigma Chemical Co), 50 mg, was dissolved in distilled water (25 mL). Sodium bicarbonate was added in excess, and the free base was extracted with several 10-mL portions of $CHCl_3$. The combined organic extract was washed with saturated NaCl solution and dried (Na_2SO_4). Filtration and evaporation of the solvent gave the free base as a thick oil.

Psilocin was obtained as the free base from the National Institute on Drug Abuse. For solutions in D_2O, 1 equiv of reagent grade oxalic acid was added to the psilocin free base.

Acknowledgment. This research was supported by Grants 6 R01 ES00929 and 1 R01 DA02189. The NMR spectra were run at PUBMRL, which is supported by NIH Grant RR01077.

(20) Albert, A.; Serjeant, E. P. "The Determination of Ionization Constants"; 2nd ed., Chapman and Hall: London, 1971.
(21) Leo, A.; Hansch, C.; Elkins, D. *Chem. Rev.* 1971, *71*, 525.
(22) Vane, J. R. *Br. J. Pharmacol.* 1959, *14*, 87.

Studies on Position 1 of Angiotensin II: Effects on Affinity and Duration of Action from Alkyl Amide Substitution[1]

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The synthesis and the biological activities of [asparagine]angiotensin II analogues with alkyl-substituted amide groups are reported. This study was performed in order to elucidate further the importance and the influence of the side chain in position 1 of angiotensin II. The two synthesized analogues [1-(N^4,N^4-dipropyl)asparagine]- and [1-(N^4,N^4-diisopropyl)asparagine]angiotensin II were compared to [1-asparagine]angiotensin II (hypertensin, Ciba) and to [1-(N^4,N^4-dimethyl)asparagine]angiotensin II in vitro and in vivo. All compounds had full intrinsic activity, but their potency decreased with increasing alkyl size of the substituted carboxamide group. Despite their reduced potency, the alkylated analogues showed enhanced duration of action on rabbit aorta strips. The relative potencies of the series hypertensin, dimethyl, dipropyl, and diisopropyl analogues on rabbit aorta strips were 100, 46, 16, and 9%, respectively. In the rat blood pressure assay they were 100, 30, 9, and 7%, respectively.

The side chain of the aspartic acid residue in position 1 of angiotension II can be replaced by a free carboxamide group or other amino acids, such as sarcosine, either without a significant loss or an increase of activity.[2] However, its modification to a dimethyl amide, as in [1-(N^4,N^4-dimethyl)asparagine]angiotensin II, reduces the relative potency of the analogue, without altering the intrinsic activity.[3] In order to study further the influence of the carboxamide side chain on receptor binding and stimulation, we have synthesized analogues which have bulky alkylated amide groups. The analogue [1-(N^4,N^4-diisopropyl)asparagine]angiotensin II was synthesized according to the general solid-phase procedure,[4] using chloromethylated polystyrene resin, esterified with Boc-Phe by the *Loffet* esterification.[5] The synthesis of N^2-(*tert*-butyloxycarbonyl)-N^4,N_4-dipropylasparagine and N^2-(*tert*-butoxycarbonyl)-N^4,N^4-diisopropylasparagine was effected by coupling N^2-(benzyloxycarbonyl)aspartic acid α-benzyl ester with dipropyl- or diisopropylamine, a method we have described earlier.[6] The benzyl ester was cleaved with catalytic hydrogenation, and the free amino group was acylated with S-(butyloxycarbonyl)-4,6-dimethyl-2-mercaptopyrimidine.[7] The octapeptide analogues were liberated from the resin and from the side-chain protecting groups in liquid HF.[8] The resulting analogues were purified by gel filtration and partition chromatography, and their purity was assessed by TLC (thin-layer chromatography).

The compounds were tested on rabbit aorta strips (in vitro) and in the rat blood-pressure test (in vivo), and their biological activities were compared to those of hypertensin and to the aforementioned dimethyl analogue. The results of both biological assays were very similar and are present in Table I.

(1) All optically active amino acids are to the L configuration. Abbreviations used follow the recommendations of the IUPAC–IUB as found in *Biochemistry*, 14, 449 (1975) and *Biochem. J.*, 126, 773 (1972).
(2) D. Regoli, W. K. Park, and F. Rioux, *Pharmacol. Rev.*, 26, 69 (1974).
(3) P. Cordopatis, J. Brison, J. Dirkx, and D. Theodoropoulos, *Experientia*, 35, 849 (1979).
(4) R. B. Merrifield *J. Am. Chem. Soc.*, 85, 2149 (1963); R. B. Merrifield, *Adv. Enzymol.*, 32, 221 (1969).
(5) A. Loffet, *Int. J. Protein Res.*, 3, 297 (1971).
(6) T. Caplaneris, P. Cordopatis, J. Matsoukas, and D. Theodoropoulos, *Tetrahedron*, 34, 696 (1978).
(7) J. Nagasawa, K. Kuroiwa, K. Narita, and U. Isowa, *Bull. Chem. Soc. Jpn.*, 46, 1269 (1973).
(8) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Odaka, and H. Sugihara, *Bull. Chem. Soc. Jpn.*, 40, (1967).

0022-2623/81/1824-0209$01.00/0

Table I. Comparison of the Biological Activities of Hypertensin II and Its 1-Position Analogues[a]

compd	in vitro, rabbit aorta								in vivo, rat blood pressure	
	pD_2	F	%	α^E	$t_{1/2}$	$F_{1/2}$	$t_{3/4}$	$F_{3/4}$	%	F
[1-Asn]AII (hypertensin)	7.73 ± 0.13[b]	1	100	1.0	5.0	1	8.7	1	100	1
[1-(N^4,N^4-Me_2)Asn]AII	7.39 ± 0.14	2.2	46	1.0	6.7	1.3	20	2.3	30	3.3
[1-(N^4,N^4-Pr_2)Asn]AII	6.93 ± 0.20	6.3	16	1.0	18.7	3.7	44	5.1	9	11.1
[1-(N^4,N^4-*i*-Pr_2)Asn]AII	6.69 ± 0.15	11.0	9	1.0	14.3	2.9	36.7	4.2	7	14.3

[a] pD_2 is the negative log of the half-maximal dose; α^E is the intrinsic activity; $t_{1/2}$ is the time needed to relax the maximally contracted tissue to 50% of this contraction after the first bath change; $t_{3/4}$ indicates the relaxation time on 25% contraction; F is the factor between the hypertensin value and the corresponding analogue. All analogues had dose–response curves parallel to hypertensin. The peptides are calculated as acetate trihydrate as found in the elementary analysis. [b] Lit.[2] 8.11.

It has already been stated earlier[2] that the carboxyl group of angiotensin or the amide group of [1-Asn]angiotensin II is not essential, but beneficial for biological activity.

These results indicate for hypertensin that the Asn[1] amide protons do not play an important role in binding and no role in response release: the bulkier the substituent of the amide group, the weaker is the affinity of the analogue.

It is recognized that the residue in position 1 contributes primarily to the duration of action of angiotensin II through two possible mechanisms: (a) it makes the molecule more or less resistant to aminopeptidases and (b) it increases the binding affinity of the peptides for the receptor.[2,9] In an effort to quantitate the duration of action of a given agonist, the relaxation of the treated tissue was evaluated: At maximal contraction the unbound agonist was removed with several washings and the tissues relaxed more or less quickly to the tension before contraction. This relaxation behavior was used to determine the relative duration of action (see Table I). The observed differences in the relaxation velocity on rabbit aorta strips in comparison to hypertensin are probably due to the augmented lipophilicity in position 1, because metabolism is negligible in the in vitro assay and the apparent affinities (pD_2) are lower. Recent studies[10,11] show that enhanced lipophilicty can lead to increased duration of action even at lower apparent affinity, for reasons which are not yet clear. It is conceivable that the peptide is retained on or in the vicinity of the membrane receptors due to its more lipophilic character which slows down the dissociation velocity.

It is an accepted concept that linear peptides of the size of angiotensin II are characterized by a certain flexibility which corresponds to an equilibrium of conformational families. Indeed, seven angiotensin II conformations have been proposed during the last few years.[12,13] An interpretation of the loss of activity with [1-(N^4,N^4-dialkylated)Asn]angiotensin II analogues could be explained on the basis of the proposed conformation by Fermandjian et al.[13] According to it, the N-terminal sequence Asp-Arg-Val-Tyr forms a β turn, which could be stabilized by hydrogen bonds between the Asp carbonyl group and the Tyr NH and/or the Val NH. In accord with this hypothesis, alkyl groups on the N^4 of the asparaginyl carboxamide could sterically interfere with the formation of hydrogen bonding or repulse side chains of essential residues. This would produce changes in the preferred backbone conformation of the hormone[13] and deserves further investigation. Another explanation would be a direct steric interference of the modification with the receptor which might impair ideal receptor binding of the analogue and thus reduce its potency.

Experimental Section

Melting points were determined in open capillaries and are uncorrected. Optical rotations ($[\alpha_D]$) were measured with a Carl Zeiss precision polarimeter (±0.005). TLCs were performed on precoated silica gel plates, Type G, F-254 (E. Merck or Riedel de Haen) with 10–50 μg samples. The plates were developed for 10–15 cm in the following solvent systems (all by volume): (A) 1-butanol–acetic acid–water (4:1:5, upper phase); (B) 1-butanol–acetic acid–water (4:1:1); (C) 1-butanol–acetic acid–pyridine–water (30:6:20:24); (D) chloroform–methanol–acetic acid (95:5:3). The spots were detected by UV and visualized by ninhydrin and Reindel Hoppe. Elemental analyses were performed by the Microanalytical Laboratory of the National Hellenic Research Foundation, and the data (C, H, N) fall within ±0.4% of the theoretical values. Peptide samples were hydrolyzed in sealed tubes with 6 N HCl at 110 °C for 16–22 h, evaporated, and analyzed on a Durrum D-500 amino acid analyzer.[14,15] The amino acid derivatives were supplied by Protein Research Foundation (Japan), while the other reagents and solvents were from Fluka (Switzerland) and were analytical grade. All products were checked for purity before use.

N^2-(*tert*-Butyloxycarbonyl)-N^4,N^4-diisopropylasparagine. Coupling of N^2-(benzyloxycarbonyl)aspartic acid α-benzyl ester (0.72 g, 2 mmol) with diisopropylamine was effected in the usual manner by the mixed anhydride procedure in THF solution.[6] The oily product, N^2-(benzyloxycarbonyl)-N^4,N^4-diisopropylasparagine benzyl ester (0.65 g, 74% yield), was hydrogenated over PdO in 2-propanol–water (14:1, 20 mL). After 4 h the catalyst was filtered off, the filtrate was evaporated under reduced pressure, and the residue, N^4,N^4-diisopropylasparagine, was triturated with acetone–ether (1:9): yield 0.25 g (80%); mp 203–206 °C; $[\alpha]^{22}_D$ +4.45° (*c* 1, 1 N HCl); TLC R_f (A) 0.35, R_f (B) 0.26. Anal. ($C_{10}H_{20}N_2O_3$) C, H, N.

A portion of N^4,N^4-diisopropylasparagine (0.17 g, 0.8 mmol) was dissolved in dioxane–water (2:7). Triethylamine (0.13 mL, 0.9 mmol) and *S*-(butyloxycarbonyl)-4,6-dimethyl-2-mercaptopyrimidine[7] (0.24 g, 0.96 mmol) were added and stirred for 24 h at room temperature. The reaction mixture was diluted with water (20 mL) and was extracted twice with ethyl acetate. The aqueous layer was cooled and acidified with 5 N HCl (pH 1.5), and the

(9) R. R. Smeby and S. Fermandjian in "Chemistry and Biochemistry of Amino Acids, Peptides and Proteins", B. Weinstein, Ed., Marcel Dekker, New York and Basel, 1978, p 117.
(10) V. Pliska and P. Marbach, *Eur. J. Pharmacol.*, **49**, 213 (1978).
(11) E. Escher, G. Guillemette, O. Leukart, and D. Regoli, *Eur. J. Pharmacol.*, **66**, in press (1980).
(12) R. R. Smeby, K. Arakawa, F. M. Bumpus, and M. M. Marsh, *Biochim. Biophys. Acta*, **58**, 550 (1962); T. B. Paiva, A. C. M. Paiva, and H. A. Scheraga, *Biochemistry*, **2**, 1327 (1963); M. P. Printz, H. P. Williams, and L. C. Craig, *Proc. Natl. Acad. Sci. U.S.A.*, **69**, 378 (1972); R. J. Weikam and E. C. Jorgensen, *J. Am. Chem. Soc.*, **93**, 7038 (1971).
(13) S. Fermandjian, P. Fromageot, A. M. Tistechenko, J. P. Leichnoam, and M. Lutz, *Eur. J. Biochem.*, **28**, 174 (1972); S. Fermandjian, J. L. Morgat, P. Formageot, C. Legreussus, and P. Meyer, *FEBS Lett.*, **16**, 192 (1971); S. Fermandjian, J. L. Morgat, and P. Formageot, *Eur. J. Biochem.*, **24**, 252 (1971).
(14) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).
(15) S. Moore, in "Chemistry and Biology of Peptides", J. Meinhofer, Ed., Ann Arbor, Mich., 1973, p 629.

resulting oil was extracted with ethyl acetate. The organic layer was washed with 5% HCl and concentrated NaCl solution and dried with anhydrous Na_2SO_4. After evaporation of the solvent, an oily product was obtained: yield 0.24 g (95%); TLC R_f (B) 0.9, R_f (D) 0.84; NMR ($CDCl_3$, 60 MHz Varian T60) showed incorporation of additional 9 H (singlet) at 1.4 ppm. To 10 mg of this oily product was added 1 mL of 4 N HCl in AcOEt, and the solution stirred at room temperature for 20 min. After evaporation, the hydrochloride gave identical $[\alpha]_D$ and TLC values as the orginal N^4,N^4-diisopropylasparagine.

N^2-(*tert*-Butyloxycarbonyl)-N^4,N^4-dipropylasparagine. In a similar manner to that described for the diisopropyl analogue, N^4,N^4-dipropylasparagine was prepared: mp 187–189 °C; $[\alpha]^{20}_D$ +7.5° (*c* 1, 1 N HCl); TLC R_f (B) 0.32, R_f (C) 0.59. Anal. ($C_{10}H_{20}N_2O_3$) C, H, N. A portion of it was acylated with *S*-(butyloxycarbonyl)-4,6-dimethyl-2-mercaptopyrimidine as previously described to give the desired product in oily form: TLC R_f (B) 0.9, R_f (D) 0.84. The product was identified in the same manner as above.

[1-(N^4,N^4-Dipropyl)asparagine]angiotensin II. The peptide was synthesized by the solid-phase method on a Beckmann Model 990 peptide synthesizer. Chloromethylated polystyrene resin cross-linked with 1% divinylbenzene containing 9.75 mequiv of Cl/g was obtained from Bio-Rad Labs, Inc. (Bio-Beads X-X1, 200–400 mesh). Attachment of the Boc-Phe to the resin was done by the Loffet[5] esterification procedure, and substitution of 0.56 mmol of Boc-Phe/g of resin was obtained. Couplings were preformed with 2.5 equiv of *tert*-butyloxycarbonyl-protected amino acids and DCC in CH_2Cl_2 for 120–240 min. The coupling was judged complete (>99% yield) by the Kaiser ninhydrin test.[16] The following side-chain-protected amino acid derivatives were used: Boc-His(Tos), Boc-Tyr(2-BrZ), Boc Arg(NO_2). Removal of the Boc group in each deprotection cycle was accomplished with 25% CF_3COOH in CH_2Cl_2 containing 2% anisole (twice for 15 min). Neutralization was effected with 10% triethylamine in CH_2Cl_2. Between individual operations, extensive washings was performed with CH_2Cl_2 or DMF, followed by absolute methanol.

Cleavage of the octapeptide from the resin with concomitant removal of the protecting groups was effected with HF containing 10% of anisole (by volume) for 1 h at 0 °C. After removal of HF and drying under vacuum, the resin was washed several times with dry diethyl ether and extracted with CH_3COOH (2 M). The extract was lyophylized and gave 600 mg (95.2%) of crude peptide. A portion (250 mg) was partially purified by gel filtration on a Sephadex G-15 column (2.6 × 100 cm) with 5% (v/v) acetic acid. Fractions of 3 mL were collected at a flow rate of 10 mL/h and peptide material was detected by monitoring the absorbancy at 280 nm. On the basis of the elution profile and amino acid analysis, fractions 53–68 were collected and lypohilized to give 110 mg (44%). TLC showed one major spot, R_f 0.13, and two contaminants with R_f 0.12 and 0.15 in system A. A portion (60 mg) of the above material was further purified by partition chromatography on a Sephadex G-15 column (1.5 × 150 cm) equilibrated with both phases of the solvent system 1-BuOH–AcOH–H_2O (4:1:5). The column was eluted with the upper phase at 7 mL/h and fractions of 4 mL were collected. the peptide containing fractions 89–95 were pooled and diluted with water. The organic phase was evaporated under vacuum and the residue was lyophilized, yielding 50 mg of product. TLC in system A gave a major spot with R_f 0.13 and a minor spot with R_f 0.15. This product was subjected to a final partition chromatography on a Sephadex G-25 column (1.5 × 100 cm) and eluted with the same solvent system at 8 mL/h into 4-mL fractions: yield 34 mg, 24% based on the phenylalanine initially attached to the polymer. This final product gave single spots on TLC: R_f (A) 0.13, R_f (B) 0.26, R_f (C) 0.45, revealed by standard methods and with diazolized sulfanilic acid; mp 199–202 °C dec, softening at 183 °C; $[\alpha]^{25}_D$ −55.3° (*c* 0.5, 1 N AcOH). Amino acid analysis gave the following ratios: Asp, 1.03; Arg, 1.01; Val, 1.05; Tyr, 1.03; Ile, 0.99; His, 0.95; Pro, 0.92; Phe, 1.04. Anal. ($C_{50}H_{84}N_{14}O_{11}\cdot C_2H_4O_2\cdot 3H_2O$) C, H, N (peptide acetate trihydrate).

[1-(N^4,N^4-Diisopropyl)asparagine]angiotensin II. This analogue was synthesized, purified, and detected according to the procedure described above for the [1-(N^4,N^4-dipropyl)-asparagine]angiotensin II. The yield of crude product was 305 mg (93%). After the purifications, the final product (30 mg, 20.5% overall yield) gave single spots on TLC: R_f (A) 0.12; R_f (B) 0.25, R_f (C) 0.44; mp 203–206 °C dec, softening at 197 °C; $[\alpha]^{25}_D$ −46.7° (*c* 0.5, 1 N AcOH). Amino acid analysis gave the following molar ratios: Asp, 0.98, Arg, 1.01; Val, 1.06; Tyr, 1.04; Ile, 0.99; His, 0.90; Pro, 0.96; Phe, 1.07. Anal. ($C_{50}H_{84}N_{14}O_{11}\cdot C_2H_4O_2\cdot 3H_2O$) C, H, N (peptide acetate trihydrate).

Biological Assays. Rabbit Aorta Strips. 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 Mass.) New Zealand rabbits of either sex, weighing 1.5–2.0 kg, were killed by stunning and exsanguination. The thoracic aortas were excised and immediately immersed in oxygen-saturated cold Krebs solution. The aortas, freed from adventitia, were helically cut into a 5-mm large band. Two-centimeter strips of this band were suspended in 5-mL baths, containing Krebs solution at 37 °C; they were continuously aerated with a mixture of 95% O_2 and 5% CO_2. A tension of 2.0 g was applied at the beginning and was adjusted several times during the 60–90 min of the equilibration period. The bath fluid was changed at intervals of 10–15 min. Concentrations indicated were always final concentrations in the tissue bath.

Rat Blood Pressure. In vivo experiments were performed on male Wistar rats, weighing about 250 g. The animals were anesthetized with urethane (1.4 g/kg, sc). The right jugular vein and the left carotid artery were cannulated. peptide solutions were injected through the vein, and the blood pressure was recorded in the artery with a Statham pressure transducer (Hato Rey, Puerto Rico), connected to a Grass polygraph.

Angiotensin II and the tested analogues were dissolved in distilled water as 1 mg/mL. This solution was stored at −20 °C for a maximum of 14 days. It was diluted with 0.9% saline to the desired concentrations immediately before use. The purity of these products was checked from time to time on TLC.

Acknowledgment. Thanks are due to Dr. J. Brison, of the University of Mons (Belgium), for his contribution in the early stages of this work. D. T. thanks the National Hellenic Research Foundation, and G.G. and E.E. thank the Medical Research Council of Canada and the Canadian Heart Foundation for partial support of this work.

(16) E. Kaiser, R. L. Colescoot, C. D. Bossinger, and P. I. Cook, *Anal. Biochem.*, **34**, 595 (1970).