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Synthesis of Angiotensin II Antagonists Containing Sarcosine in Position 7

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Received October 10, 1978

Analogues of the type [1-sarcosine,7-sarcosine,8-X]angiotensin II, where X = isoleucine, leucine, alanine, methionine, *O*-methylthreonine, or DL-alloisoleucine, were synthesized by the solid-phase method and purified by partition chromatography, cation-exchange chromatography, and high-pressure liquid chromatography. In the isolated rat uterus, these analogues had activities of <0.1, <0.1, <0.1, <0.1, <0.1, and 0.7%, respectively, of the myotropic activity of angiotensin II and inhibited the contractile response to angiotensin II with pA_2 values of 8.1, 7.2, 6.7, 7.7, 7.4, and 8.4, respectively. In the vagotomized ganglion blocked rat, the analogues had 0.7, 0.21, 0.06, 0.72, 0.13, and 12.5%, respectively, of the pressor activity of angiotensin II.

Studies of structure–activity relationships of angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) analogues have shown that replacement of the C-terminal phenylalanine residue with a nonaromatic lipophilic residue (e.g., alanine,[1] isoleucine[2]) invokes antagonist properties to the molecule. Similarly, the substitution of a residue with a lipophilic side chain terminating in a hydrophilic functional group (e.g., methionine,[3] threonine,[4] *O*-methylthreonine[3]) or an *N*-methyl-substituted amino acid (e.g., *N*-methylisoleucine,[3] *N*-methylphenylalanine[5]) at the C terminus produces inhibitors of the myotropic and pressor responses to angiotensin II. We thought it of interest to study the penultimate amino acid at the C terminus of the molecule as a possible functional site which could be modified to produce useful analogues of angiotensin II. Earlier work has shown that replacement of the proline residue in position 7 of angiotensin II with glycine[6] or alanine[6] produces analogues with very weak activities, whereas substitution of *N*-methylalanine in position 7 results in retention of reasonable activity.[7] We have substituted sarcosine (*N*-methylglycine) in place of the proline residue normally occupying position 7 of angiotensin II. Sarcosine, like proline, can take up either the cis or the trans conformation at the peptide bond and can also produce a bend in the peptide backbone.

Results and Discussion

The results in Table I indicate that replacement of the proline residue in angiotensin II with sarcosine does not severely alter the properties of the molecule with respect to receptor interactions. [Sar[1],Sar[7]]angiotensin II is a potent agonist having about one-fifth of the myotropic and pressor activities of angiotensin II and, in keeping with this, [Sar[1],Sar[7],Ile[8]]antiotensin II is a potent antagonist in the isolated rat uterus. Similarly, all of the analogues studied of the type [Sar[1],Sar[7],X[8]]angiotensin II, where X is a nonaromatic hydrophobic amino acid, demonstrate antagonist properties, although none of these analogues is a more potent antagonist than [Sar[1],Ile[8]]angiotensin II in the rat uterus assay.

With respect to intrinsic activity, as determined on the rat uterus in vitro and the in vivo rat pressor assay, these analogues appear to follow the same general trend as many other angiotensin II antagonists; i.e., they exhibit a very low myotropic activity and a low, but significant, pressor activity. There is also a general trend with the analogues reported here for the pressor activity to parallel the in vitro antagonist potency, suggesting a dependence on binding affinity for the receptors in both situations. The only exception is [Sar[1],Sar[7],DL-aIle[8]]angiotensin II, which has an unusually high agonist activity both in vivo and in vitro.

It has been predicted on the basis of conformational calculations at the receptor that the C-terminal portion of the angiotensin II molecule plays a minor role, if any, in actual binding to the receptors.[9] The results presented here could be in agreement with this possibility, since the replacement of proline by sarcosine has minimal effects on the properties of angiotensin II analogues. It is concluded that a bend produced by the presence of a secondary amino acid at position 7 of angiotensin II analogues is of critical importance in maintaining the molecule in a conformation which is recognized by angiotensin II receptors. Moreover, the decreased agonist and antagonist activities of analogues which contain the less constrained sarcosine residue in place of proline may result from a decrease in the angle of a turn[16] in the C-terminal part of the molecule, resulting in slightly less effective binding to angiotensin II receptors.

Experimental Section

tert-Butyloxycarbonyl-blocked amino acids were purchased from Bachem Inc. and examined for purity by TLC before use. A sample of Boc-aIle, specified by the manufacturer to contain only the L isomer, was found to contain equal amounts of the L and D isomers (determined by amino acid analysis after deprotection and treatment with either L- or D-amino acid acid oxidase). [Sar[1],Ile[8]]angiotensin II was a product of Peninsula Labs. 1-Hydroxybenzotriazole (Aldrich) was dehydrated and purified by refluxing with benzene in a Dean and Stark reflux apparatus, followed by drying in vacuo over P_2O_5.

TLC of the analogues was carried out on silica gel on glass plates (Brinkmann Instruments, 60F-254) in the following solvent systems: *n*-butanol–pyridine–acetic acid–water (BPAW; 15:10:3:6, v/v) and chloroform–methanol–acetic acid–water (CMAW; 15:10:3:6, v/v). Thin-layer electrophoresis was conducted on cellulose on plastic sheets (Brinkmann, Polygram Cell 400 UV) in pyridine–acetate buffer, pH 6.5, at 500 V for 1 h. Detection of peptides on chromatograms was sequentially by UV fluoresence quenching, ninhydrin spray reagent, and chlorination followed by starch–KI spray reagent.

Boc-amino acid-resins were hydrolyzed in sealed tubes in 12 N HCl–propionic acid (1:1, v/v) at 165 °C for 15 min. Peptides

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Table I. Agonist and Antagonist Properties of Angiotensin II Analogues[a]

peptide	rat uterus		vagotomized ganglion blocked rat: pressor act., %[b]
	intrinsic act., %[b]	antagonist act., pA_2[c]	
[Sar^1,Sar^7]AII[d]	22 ± 3 (6)		17 ± 3 (6)
[Sar^1,Sar^7,Ile^8]AII[d]	<0.1	8.1 ± 0.1 (9)	0.7 ± 0.2 (5)
[Sar^1,Sar^7,Leu^8]AII	<0.1	7.4 ± 0.2 (5)	0.21 ± 0.02 (4)
[Sar^1,Sar^7,Ala^8]AII	<0.1	7.0 ± 0.2 (5)	0.06 ± 0.01 (5)
[Sar^1,Sar^7,Met^8]AII	<0.1	7.7 ± 0.2 (7)	0.82 ± 0.08 (5)
[Sar^1,Sar^7,Thr(Me)^8]AII	<0.1	7.4 ± 0.2 (7)	0.13 ± 0.02 (4)
[Sar^1,Sar^7,DL-aIle^8]AII	0.7 ± 0.2 (5)	8.4 ± 0.2 (5)	12.5 ± 1.0 (4)
[Sar^1,Ile^8]AII	<0.1	8.6 ± 0.2 (6)	1.1 ± 0.2 (4)

[a] Values are given as mean ± SEM (no. of experiments). [b] Relative to [Ile^5]angiotensin II (MRC Research Standard A, 70/302). [c] The pA_2 is the negative logarithim of the concentration of antagonist required to halve the response to an ED_{50} dose of angiotensin II. [d] See ref 8.

were hydrolyzed in 6 N HCl in the presence of 1% 2-mercaptoethanol and 1% phenol for 18 h at 110 °C in vacuo. Amino acid analyses were obtained with a Beckman 121 M amino acid analyzer. Sarcosine, which had a very low color yield (0.3% of that of glycine), could only be estimated by applying a 1000-fold greater quantity of the hydrolyzate to the amino acid analyzer.

Solid-Phase Peptide Synthesis. The C-terminal Boc-protected amino acid was coupled to the chloromethylated 1% cross-linked polymer (Bio-Rad, 0.89 mequiv of Cl/g) via the cesium salt.[10] The use of limiting amounts of the Boc-protected amino acid cesium salt enabled substitution in the range 0.25–0.55 mmol of Boc-protected amino acid/g of resin (amino acid analysis). Unreacted groups on the resin were subsequently acetylated with 5 equiv each of acetic anhydride and triethylamine in $HCONMe_2$ for 1 h.

The peptide chain was elongated using a Beckman 990 peptide synthesizer. The reagents used and the general methodology were essentially as described previously.[11] Peptides were usually synthesized on a 0.5 mmol scale. The tosyl, benzyl, and nitro groups were used to protect histidine, tyrosine, and arginine, respectively. One cycle of the synthesis utilized the following series of reactions and washing steps: (1) $CHCl_3$ (3 × 2 min); (2) CF_3CO_2H–$CHCl_3$, 1:3 (2 and 25 min); (3) $CHCl_3$ (4 × 2 min); (4) Et_3N–$CHCl_3$, 1:9 (2 and 15 min); (5) $CHCl_3$ (2 × 2 min); (6) CH_2Cl_2 (2 × 2 min); (7) 2.5 equiv of the Boc-protected amino acid in 12 mL of CH_2Cl_2 or $HCONMe_2$–CH_2Cl_2 (1:4) and stir for 10 min; (8) add 2.5 equiv of DCC in 4 mL of CH_2Cl_2 and stir for 3 h; (9) CH_2Cl_2 (2 × 2 min); (10) ETOH (3 × 2 min); (11) $CHCl_3$ (2 × 2 min). Except for the coupling step, reagent and washing volumes were 20 mL. Exceptions to the general procedure were: (1) dipeptide-resins were neutralized with Et_3N–$CHCl_3$, 1:9 (2 × 2 min), and DCC was added to the reaction vessel prior to the addition of the next Boc-protected amino acid; (2) 2.5 equiv of 1-hydroxybenzotriazole was added for the coupling of Boc-Sar (position 7) and Boc-His(Tos), and the reactions were allowed to proceed for 6 h. Completeness of coupling reactions was monitored by the ninhydrin method;[12] occasionally, a coupling reaction was found to be incomplete and a repeat coupling step was carried out under the same conditions.

The completed peptide was removed from the resin and simultaneously deprotected by reaction with anhydrous HF (20 mL) in a Teflon apparatus (Protein Research Foundation, Japan) at 0 °C for 1 h in the presence of anisole (2 mL). HF was removed in vacuo, the peptide was dissolved in CF_3CO_2H (60 mL), and the resin was removed by filtration. The solvent was removed on a rotary evaporator, and the peptide was obtained as a white amorphous solid by tituration with ether. Yields of the crude peptides were generally in excess of 90%. Purification of peptides was carried out by partition chromatography and cation-exchange chromatography as described below. Yields of the peptides were generally of the order of 50% (based on starting Boc-amino acid-resin). Peptides which were not homogenous after column chromatography were subjected to high-pressure liquid chromatography as a final purification step.

Partition Chromatography. Peptides (150–250 mg) were purified by partition chromatography in *n*-butanol–acetic acid–water (4:1:5) as described previously.[8] A column (90 × 2.5 cm) of Sephadex G-25 was equilibrated with the lower layer of the two-phase system. The peptide, dissolved in 2 mL of the upper layer, was run through the column, eluting with the upper phase at a flow rate of 30–40 mL/h. The absorbance of the effluent was measured at 280 nm, and fractions (6 mL) containing the major peak of peptide material[8] were pooled, lyophilized, and relyophilized from 2% acetic acid.

Cation-Exchange Chromatography. A column (80 × 1.6 cm) of carboxymethylcellulose (Whatman CM23) was equilibrated sequentially with 0.05 N NaOH, 0.2 N HCl, and 0.01 M ammonium acetate, pH 5.0. The peptide was applied to the column in the starting buffer (10 mL) and eluted with a linear gradient to 0.5 M ammonium acetate, pH 8.0, at a flow rate of 40–50 mL/h. The absorbance of the effluent was measured at 280 nm, and fractions (10 mL) containing the required peptide were pooled, lyophilized, and relyophilized twice from 5% acetic acid. In cases where an incomplete separation was obtained, the peptide was rerun through the column using a linear gradient of ammonium acetate from 0.01 M, pH 5.0, to 0.25 M, pH 8.0.

High Pressure Liquid Chromatography. As a final purification step, peptides were subjected to reverse-phase high-pressure liquid chromatography (Spectra-Physics SP 8000) on a column (25 × 0.46 cm) of LiChrosorb RP-10A (Brownlee Labs) with a linear gradient of 20–55% acetonitrile in 0.01 M ammonium acetate, pH 4.1, at 40 °C and a flow rate of 4 mL/min. The major peak detected by the absorbance of the effluent at 254 nm was collected, lyophilized, and relyophilized from 5% acetic acid.

Bioassays. The isolated rat uterus assay was carried out on uteri from diethylstilbestrol-primed female Sprague-Dawley rats (150–250 g) as described by Freer and Stewart.[13] The tissue was challenged every 12 min in all experiments. For the measurement of the antagonist potencies of analogues, the antagonist was given 2 min before angiotensin II. Antagonist potencies were determined as pA_2 values[14] (the negative logarithmn of the concentration of antagonist required to halve the response of an ED_{50} dose of angiotensin II).

In vivo pressor assays were carried out on the anesthetized vagotomized ganglion blocked rat preparation described by Pickens et al.,[15] except that the ganglion blocker was mecamylamine hydrochloride (0.3 mg/100 g subcutaneous in 10% polyvinylpyrrolidone). Pressor potencies of analogues were determined by bolus injection using the conventional 2+2 assay system against [Asp^1,Ile^5]angiotensin II (MRC Research Standard A, 70/302).

[Sar^1,Sar^7,Ile^8]angiotensin II: TLC R_f (BPAW) 0.44, R_f (CMAW) 0.30. Amino acid analysis: Val, 1.00; Ile, 2.00; Tyr, 0.97; His, 1.04; Arg, 1.00; Sar, ~2.

[Sar^1,Sar^7,Leu^5]angiotensin II: TLC R_f (BPAW) 0.52, R_f (CMAW) 0.60. Amino acid analysis: Val, 0.96; Ile, 1.00; Leu, 1.01; Tyr, 0.87; His, 0.89; Arg, 1.01; Sar, ~2.

[Sar^1,Sar^7,Ala^8]angiotensin II: TLC R_f (BPAW) 0.36, R_f (CMAW) 0.26. Amino acid analysis: Ala, 0.97; Val, 0.99; Ile, 1.00; Tyr, 0.94; His, 0.92; Arg, 0.89; Sar, ~2.

[Sar^1,Sar^7,Met^8]angiotensin II: TLC R_f (BPAW) 0.40, R_f (CMAW) 0.42. Amino acid analysis: Val, 1.11; Met, 0.90; Ile, 1.00; Tyr, 0.96; His, 0.91; Arg, 0.92; Sar, ~2.

[Sar^1,Sar^7,Thr(Me)^8]angiotensin II: TLC R_f (BPAW) 0.24, R_f (CMAW) 0.25. Amino acid analysis: Thr(Me), 0.96; Val, 0.91; Ile, 1.00; Tyr, 0.95; His, 1.14; Arg, 1.04; Sar, ~2.

[Sar^1,Sar^7,DL-aIle^8]angiotensin II: TLC R_f (BPAW) 0.40, R_f (CMAW) 0.31. Amino acid analysis: Val, 1.02; aIle, 1.07; Ile,

1.00; Tyr, 0.94; His, 1.12; Arg, 1.08; Sar, ~2.

Acknowledgment. This investigation was supported by the Alberta Heart Foundation and the Canadian Heart Foundation.

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Book Reviews

Sustained and Controlled Release Drug Delivery Systems. Volume 6. Drugs and the Pharmaceutical Sciences. Edited by Joseph R. Robinson. Marcel Dekker, New York. 1978. x + 713 pp. 16 × 23.5 cm. $59.75.

As stated by the editor, this volume was designed to "fulfill a perceived need to provide a comprehensive picture of the sustained release drug product area". To this end, the editor and the various authors have succeeded in bringing together a vast amount of literature and providing the reader with a comprehensive reference list.

A novice in this area will find that the first three chapters of the book bring them up to date in classical sustained release theory and approach. Those with some experience in sustained release drug development will find these chapters an excellent review. The section on biological drug properties, particularly as they relate to pharmacokinetic considerations in the design of sustained release drug delivery systems, is especially good. The presentation of the various physical strategies used in formulation is valuable, although theoretical aspects are somewhat watered down.

The discussion of the physical approach to sustained release drug development is continued in Chapter 4 with a particular emphasis on drug implants. Readers only peripherally involved with this topic may find the concepts and mathematics difficult. Although basically well written, the chapter is somewhat repetitious and also overlaps with some material presented in later chapters.

Chapter 5 deals with the evaluation of injury to the injection site and contains information essential to those involved in the formulation of parenteral drug products. The detail into which this chapter goes, however, might better have been left to a more comprehensive treatment of parenteral drug development.

The next chapter, which discusses the chemical approach to sustained released drug delivery, will be of particular interest to medicinal chemists. The chapter is excellent both with regard to material presented and literature surveyed.

Chapter 7 summarizes the philosophies of the biomedical engineers in developing what have come to be known as therapeutic systems (i.e., Ocusert®, Progestasert®, etc.). The material is interesting and is a necessary component of the book, but the manner of presentation provides little mechanistic insight. The two concluding chapters summarize, in a form not readily found elsewhere, the application of classical pharmacokinetics to sustained release drug disposition.

In summary, this book will be a valuable aid to anyone interested in understanding sustained and controlled release drug delivery systems. It is comprehensive, provides an outstanding literature survey, and is remarkably free of technical and typographical errors. The inclusion of separate author and drug indicies is also useful. Some repetition, particularly in the early chapters, may have made the book longer than required. This point becomes important primarily from the standpoint that had the book been shorter it might have been made more affordable.

The University of Kansas **Thomas F. Patton**

Advances in Experimental Medicine and Biology. Volume 98. Immunobiology of Proteins and Peptides. 1. Edited by M. Z. Atassi and A. B. Stavitsky. Plenum Press, New York. 1978. x + 513 pp. 25 × 18 cm. $45.00.

This book contain a series of articles which relate to (1) antigenic structure of proteins, (2) immunobiology of proteins and peptides, (3) immunobiology of protein conjugates, (4) immune responses to synthetic polymers and to proteins, and (5) structure of lymphocyte membranes. Current data from a series of independant investigators are included which relate the molecular structure of proteins and peptides to their activity in either the activation or inhibition of immune response mechanisms. Each series of papers is followed by a discussion paper which in every case extracts significant key points from the articles and suggests possible new directions for research. This book is especially useful for immunochemists involved in research and/or teaching.

Staff

Neurotransmitter Receptor Binding. Edited by H. Yamamura, S. Enna, and M. Kuhar. Raven Press, New York. 1978. ix + 195 pp. 16 × 24 cm. $17.00.

One of the more important recent developments in neurochemistry which has a direct and practical application to medicinal chemistry is the use of radioactively labeled agonists and antagonists to study neurotransmitter receptors. These receptor-binding techniques have vastly increased our knowledge of the biochemical characteristics of receptors and, in addition, provide a relatively simple and rapid means of screening for new drugs. A book outlining the basic theoretical and practical background to this subject is, thus, particularly welcome.

The volume begins with an excellent introductory overview by Snyder, who is one of the worlds leading exponents of this technique, and this is followed by a very clear presentation of the essential elements of the kinetics of small molecule–macromolecule interactions. The next two chapters by Burt and Bennett cover