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Synthesis of Angiotensin II Antagonists by Incorporating α-Methylalanine or O-Methylthreonine Residues in Angiotensin II Analogues[1,2]

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[1-Sarcosine,5-O-methylthreonine,8-isoleucine]- (I), [1-sarcosine,5-O-methylthreonine,8-O-methylthreonine]- (II), [1-sarcosine,8-α-methylalanine]- (III), [1-α-methylalanine,8-α-methylalanine]- (IV), and [1-sarcosine,2-sarcosine,-8-threonine]angiotensin II (V) were synthesized by Merrifield's solid-phase procedure to evaluate these as antagonists of the pressor and myotropic activities of angiotensin II. The in vitro studies were carried out on rabbit aortic strips. Analogues I–V possessed negligible myotropic activity up to a dose level of 10^{-6} M/L. The pA_2 values obtained were 9.50, 9.50, 8.70, 7.22, and 8.78, respectively. In general, the duration of action of these antagonists (rabbit aortic strip) was 2–3 h. V showed maximum duration (3 h) while complete reversal of antagonistic activity of IV occurred after 1 h. The in vivo studies were carried out in ganglion-blocked vagotomized rats. Analogues I–V possess respectively 1.01, 0.46, 0.52, 0.24, and 0.00% pressor activity of angiotensin II (single injection procedure). Unless stated the infusion studies were carried out at 250 ng/kg/min. The analogues caused an initial rise in blood pressure (at 30-min infusion period) of 18.50, 14.50 (125 ng/kg/min), 16.60, 14.33, and 11.66 mmHg; dose ratios obtained were ~50, 32.73 (125 ng/kg/min), 19.76, 3.40, and 1.93, respectively. During infusion of I and II, the effect of anesthesia subsided making it difficult to complete the experiment. The results indicate that (a) replacement of positions 5 and/or 8 with O-methylthreonine significantly increased the potency of angiotensin II antagonists; the antagonistic potency was also enhanced when alanine in position 8 was replaced with α-methylalanine; (b) replacement of position 1 with α-methylalanine significantly reduced the antagonistic properties of III; and (c) replacement of positions 1 and 2 in [Sar[1],Thr[8]]angiotensin II with sarcosylsarcosine linkage did not affect its in vitro antagonistic activity but this change considerably reduced its in vivo antagonistic properties.

Angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) is known for its multiplicity of biological actions that are either vascular, related to endocrine, or are connected to the central and peripheral nervous system. Previous work from our laboratories indicated that the antagonists of angiotensin II, such as [Sar[1],Ala[8]]- or [Sar[1],Ile[8]]angiotensin II, are not tissue or organ specific for angiotensin II and demonstrate variable agonist–antagonist ratio when tested on different systems of the same species and possess short in vivo half-life (for detailed reviews, see ref 7–9). Our endeavor therefore has been to produce tissue specific antagonists with minimum or no agonist properties but with increased antagonistic potencies and binding affinity to the receptor. Single injection and infusion studies in rats with several analogues indicated that the antagonistic properties of [Sar[1],Thr[8]]angiotensin II were comparable to that of [Sar[1],Ile[8]]angiotensin II with the additional advantage that the initial pressor activity of [Sar[1],-Thr[8]]angiotensin II was 50% less than that of [Sar[1],-Ile[8]]angiotensin II.[10-14] A comparative antagonistic activity of [Sar[1],Thr[8]]angiotensin II in rabbit aortic strips, cat adrenal medulla, and cat adrenal cortex indicated that this analogue was a very potent antagonist of the angiotensin II induced responses in all the three tissues.[11,15] [Sar[1],-Thr[8]]angiotensin II also showed agonist properties in various tissues even though the activity was less than other compounds of this series. For example, as compared to angiotensin II, the myotropic (rabbit aortic strips) and the secretory activities in isolated cat adrenal medulla and cat adrenal cortex were 0.5, 0.1, and 1.0%, respectively.[9,11,15] There are two approaches available to minimize or eliminate these effects: (a) to find those structural features that should be responsible for these agonistic effects and to eliminate these or (b) to increase the antagonistic potency in these peptides so as the effective dosage could be lowered. The present investigation is an attempt to explore these possibilities.

Previous structure–activity relationships in our laboratory indicated that replacement of threonine with O-methylthreonine considerably enhanced the antagonistic properties of [Sar[1],Thr[8]] angiotensin II.[11] However, the initial pressor activity elicited by [Sar[1],Thr(Me)[8]]angiotensin II was higher than that of [Sar[1],Thr[8]]angiotensin II. Since O-methylthreonine is isosteric with isoleucine we thought it of interest to synthesize [Sar[1],Thr(Me)[5],Ile[8]]- and [Sar[1],Thr(Me)[5],Thr(Me)[8]]angiotensin II to study the effect of O-methylthreonine in position 5 of these antagonists.

Studies on model compounds containing α-methylamino acids indicated that these compounds are resistant to chemical hydrolysis[16] and to enzymatic attack by both endopeptidases and exopeptidases.[17,18] For this reason we have synthesized [Sar[1],Ala(α-Me)[8]]- and [Ala(α-Me)[1],-Ala(α-Me)[8]]angiotensin II to study the effect of α-methylation in positions 1 and/or 8. A similar study has been recently reported by Turk et al.[19]

Since substitution of sarcosine in the 1 position of angiotensin II and its analogues has been shown to enhance the biological activity of these peptides by increased binding affinity for the receptor site as well as by decreased rate of degradation,[20] we have synthesized [Sar[1],Sar[2],-Thr[8]]angiotensin II to study the effect of sarcosylsarcosine linkage at the N terminus of [Sar[1],Thr[8]]angiotensin II.

The biological activities of these new analogues as compared to [Sar[1],Ala[8]]-, [Sar[1],Ile[8]]-, [Sar[1],Thr[8]]-, and [Sar[1],Thr(Me)[8]]angiotensin II are listed in Tables I and II.

All the analogues reported in this paper were synthesized by the solid-phase procedure of Merrifield.[21] The protocol has been modified to ensure completion of deblocking and coupling steps and to minimize truncated or failure sequences (cf. Experimental Section). Determination of the pressor activity[22] of the analogues as compared to angiotensin II (expressed as percent) and of the comparative antagonist activity[13] (expressed as dose ratio) was carried out on vagotomized ganglion-blocked rats, as were the infusion studies to determine the initial pressor activity (expressed as mmHg). Inhibition of contractile activity of angiotensin II was studied on isolated spirally cut rabbit aortic strips[23] and is expressed as the pA_2 value.[24]

Duration of response was obtained by testing a standard dose of angiotensin II (10^{-9} M/L) and then incubating with 10^{-6} M/L analogue for 5 min. The analogue was washed out of the bath and the angiotensin II response was tested at 5, 30, 60, 120, and 180 min.

Results

Rabbit Aortic Strips. When tested on rabbit aortic

Table I. Comparative Agonist and Antagonist Effects of Analogues of Angiotensin II

Compd	Angiotensin II analogue	Agonist activity		Antagonist activity[c]		
		Pressor[a]	Myotropic[b]	Log k_2	n	pA_2
I	[Sar^1,Thr(Me)^5,Ile^8]-	1.01	0.10	8.32 ± 0.13	0.88 ± 0.14	9.50 ± 0.14 (5)
II	[Sar^1,Thr(Me)^5,Thr(Me)^8]-	0.46	0.00	7.14 ± 0.20	0.76 ± 0.22	9.50 ± 0.22 (5)
III	[Sar^1,Ala(α-Me)^8]-	0.52	0.00	9.21 ± 1.19	1.06 ± 0.14	8.70 ± 0.11 (5)
IV	[Ala(α-Me)^1,Ala(α-Me)^8]-	0.24	0.00	5.14 ± 0.60	0.71 ± 0.09	7.22 ± 0.21 (5)
V	[Sar^1,Sar^2,Thr^8]-	0.00	0.00	3.40 ± 0.30	0.39 ± 0.04	8.78 ± 0.40 (6)
VI	[Sar^1,Ala^8]-[d]		0.50			8.61 ± 0.03
VII	[Sar^1,Ile^8]-[e]	1.00	1.00			9.17 ± 0.04
VIII	[Sar^1,Thr^8]-[f]	0.60	0.50	8.10 ± 0.05	0.93 ± 0.13	8.79 ± 0.14
IX	[Sar^1,Thr(Me)^8]-[g]	0.48	0.50	8.39 ± 0.62	0.95 ± 0.06	8.76 ± 0.08

[a] Bolus injection in ganglion-blocked vagotomized rats. Pressor activity relative to [Asp^1,Ile^5]angiotensin II = 100. [b] Rabbit aortic strips, up to a maximum dose level of 10^{-6} M/L. [c] Rabbit aortic strips. The pA_2 value has been defined[24] as the negative logarithm of the molar concentration of a competitive antagonist that reduces the effect of a double concentration of agonist to that of a single one, or, in other words, pA_2 = log k_2/n wherein k_2 and n are constants. n = slope of the line obtained from a plot of [log dose ratio − 1] vs. [−log antagonist concentration], and k_2 = intersect of the line of the y axis and represents the affinity constant. [d] For details of the data see ref 34. [e] For details of the data see ref 27. [f] For details of the data see ref 10. [g] For details of the data see ref 11.

strips (Table I), [Sar^1,Thr(Me)^5,Ile^8]angiotensin II (I) showed 0.1% myotropic activity of angiotensin II while compounds II–V did not show any myotropic activity up to a concentration of 10^{-6} M/L.

Except for [Ala(α-Me)^1,Ala(α-Me)^8]angiotensin II, that showed moderate antagonistic activity, all other analogues were found to be potent antagonists of the myotropic response to angiotensin II. Both [Sar^1,Thr(Me)^5,Ile^8]- and [Sar^1,Thr(Me)^5,Thr(Me)^8]angiotensin II gave a pA_2 value of 9.50 which is higher than any other analogue tested so far. The log dose–response curves with all the analogues were paralleled and shifted to the right with increasing doses, thus suggesting competitive inhibition. However, at higher dose levels (>10^{-6} M/L) the log dose–response curves were not paralleled to the control and the maximum response was reduced, thus indicating noncompetitive antagonism.

In general, the duration of action of these antagonists in rabbit aortic strips was 2–3 h. [Sar^1,Sar^2,Thr^8]angiotensin II showed maximum duration (3 h) while complete reversal of antagonistic activity following washing of [Ala(α-Me)^1,Ala(α-Me)^8]angiotensin II from the strip occurred after 1 h.

Rat Pressor Assay. When administered as a single injection all of the analogues, except V, showed low pressor activity (0.46–1.01% of angiotensin II) (Table I). Similarly, an initial rise in blood pressure (12–19 mmHg) was observed (Table II) when these analogues were infused in ganglion-blocked vagotomized rats for 30 min.

In Vivo Antagonistic Activity in Rats (Table II). Substitution of isoleucine in position 5 with *O*-methylthreonine significantly increased the in vivo antagonistic activity of [Sar^1,Ile^8]- or [Sar^1,Thr(Me)^8]angiotensin II. In fact, [Sar^1,Thr(Me)^5,Thr(Me)^8]angiotensin II has been found to be the most potent antagonist of the pressor action of angiotensin II under the present experimental conditions. As compared to the control the dose–response curve obtained with these compounds was flatter, indicating noncompetitive antagonism. Antagonistic potency of [Sar^1,Ala^8]angiotensin II was also increased by replacing alanine in position 8 with α-methylalanine. However, simultaneous replacement of positions 1 (sarcosine) and 8 (alanine) with α-methylalanine significantly reduced the in vivo antagonistic activity of [Sar^1,Ala^8]angiotensin II.

Discussion

The present results indicate that replacement of isoleucine with its isostere, *O*-methylthreonine, in position 5 of [Sar^1,Ile^8]- and [Sar^1,Thr(Me)^8]angiotensin II significantly increased the in vitro (rabbit aortic strips) and in vivo (rat) antagonistic potencies of these peptides. The myotropic activity was either drastically reduced (0.1%) as in [Sar^1,Thr(Me)^5,Ile^8]angiotensin II (I) or was totally abolished as in [Sar^1,Thr(Me)^5,Thr(Me)^8]angiotensin II. Pressor activity in rats (single injection of both these compounds) was unaffected, while comparative infusion studies indicated that, as compared to VII and IX, the initial rise in blood pressure obtained with I and II was higher by 4 and 6 mmHg, respectively (Table II).

Since the *O*-methyl group can act only as a proton acceptor, it is likely that increase in antagonistic potencies due to the presence of the ether side chain may be due to increased binding affinity for the receptor. This is evident from the fact that both I and II had 3–7 times higher antagonistic activity (rabbit aortic strips) than the corresponding 5-isoleucine analogues (VII and IX). In the in vivo studies (rats) both I and II showed significantly enhanced dose ratios at 125 ng/kg/min. However, at 250 ng/kg/min, the effect of anesthesia subsided, making it difficult to complete the entire experiment.

Replacement of alanine with α-methylalanine in position 8 of [Sar^1,Ala^8]angiotensin II (VI) had little effect on the antagonistic potency on rabbit aortic strips. However, the dose ratio was increased from 7.9 for VI to 19.8 for [Sar^1,Ala(α-Me)^8]angiotensin II (III). These results are similar to those reported recently for [Sar^1,Val(αMe)^8]-angiotensin II.[19] But in spite of this enhanced activity, the in vivo antagonistic potency of III was still lower than that of [Sar^1,Ile^8]- (VII) or [Sar^1,Thr^8]angiotensin II (VIII) and there was no significant increase in the duration of action of III (rabbit aortic strips) when compared to VII or VIII.

Simultaneous replacement of positions 1 and 8 in VI with α-methylalanine as in [Ala(α-Me)^1,Ala(α-Me)^8]angiotensin II (IV) reduced the antagonistic potency on rabbit aortic strips and, rather drastically, in rats. These results suggest that steric hindrance imposed due to α-methylation in position 1 reduced the binding properties. This is evident from the fact that the antagonistic activity of this analogue was reversed in 1 h. A change in the conformation of the peptide and its mode of degradation are additional factors that may be responsible for the considerable decrease in the in vivo antagonistic potency of this peptide.

Replacement of positions 1 and 2 with sarcosylsarcosine in [Sar^1,Thr^8]angiotensin II (VIII) did not affect the in vitro antagonistic potency. Both analogues, [Sar^1,Thr^8]- (VIII) and [Sar^1,Sar^2,Thr^8]angiotensin II (V), gave pA_2 values of 8.8. As compared to other analogues of this series

Table II. Comparative Agonist and Antagonist Effects of Analogues of Angiotensin II. Infusion into Ganglion-Blocked Vagotomized Rats

Compd	Angiotensin II analogue	Dose, ng/kg/min	Rise in blood pressure during analogue infusion, mmHg ± SEM[a]			Angiotensin II $ED_{20} \times 10^{-9}$ ± SEM		Dose ratio[b]	p^c
			3 min	10 min	30 min	Before infusion of the analogue	During infusion of the analogue		
I	[Sar^1,Thr(Me)^5,Ile^8]-[e]	125	11.0 ± 1.21	16.82 ± 1.42	19.8 ± 1.74	1.78 ± 0.13	41.16 ± 1.84	24.15 ± 2.82 (6)[d]	<0.001
		250	19.0 ± 1.59	18.83 ± 2.07	18.50 ± 3.68	1.72 ± 0.09	~90	~50 (6)	
II	[Sar^1,Thr(Me)^5,Thr(Me)^8]-[e]	125	8.33 ± 1.35	13.00 ± 2.06	14.50 ± 1.17	1.37 ± 0.15	42.66 ± 2.57	32.73 ± 3.47 (6)	<0.001
III	[Sar^1,Ala(α-Me)^8]-	250	11.0 ± 0.84	13.16 ± 1.68	16.6 ± 2.06	1.82 ± 0.10	35.64 ± 2.90	19.76 ± 1.59 (7)	<0.001
IV	[Ala(α-Me)^1,Ala(α-Me)^8]-	250	5.16 ± 0.74	8.50 ± 1.08	14.33 ± 2.09	1.50 ± 0.23	5.75 ± 2.02	3.40 ± 0.67 (7)	<0.05
V	[Sar^1,Sar^2,Thr^8]-	250	3.16 ± 0.40	7.0 ± 0.85	11.66 ± 1.20	1.52 ± 0.15	3.07 ± 0.75	1.93 ± 0.34 (6)	<0.05
VI	[Sar^1,Ala^8][f]	250	15.11 ± 1.48	16.88 ± 1.87	17.44 ± 1.66	1.89 ± 0.19	14.16 ± 1.88	7.92 ± 1.21	
VII	[Sar^1,Ile^8]-[g]	250	17.81 ± 0.99	18.27 ± 1.72	14.81 ± 2.16	2.19 ± 0.15	59.18 ± 15.03	27.79 ± 6.25	
VIII	[Sar^1,Thr^8]-[h]	250	9.46 ± 0.79	9.66 ± 2.00	9.20 ± 4.26	1.71 ± 0.26	44.55 ± 8.20	26.91 ± 3.30	
IX	[Sar^1,Thr(Me)^8][i]	125	5.25 ± 1.26		8.75 ± 2.67	1.45 ± 0.13	17.13 ± 5.48	11.65 ± 3.01	
		250	13.82 ± 0.98	15.88 ± 0.85	15.06 ± 0.10	1.55 ± 0.10	95.26 ± 23.83	62.52 ± 14.93	

[a] Unless stated, the level of significance between pressor response to analogue and saline infusion was $p < 0.001$. [b] The ED_{20} (Y) of angiotensin II was obtained from the dose-response curve. (The ED_{20} was the concentration of angiotensin II producing 20 mmHg rise in blood pressure.) This was followed by infusion of the analogue. At 30-min period, the ED_{20} value (Z) of angiotensin II was again determined while the analogue was still being infused. Dose ratio = Z/Y. [c] Level of significance between ED_{20} values. [d] The number in parentheses is the number of rats used. [e] During infusion at 250 ng/kg/min the effect of anesthesia on the animals subsided, thereby making it difficult to complete the entire experiment. The slope of the dose-response curve to angiotensin II was flattened compared to the control dose-response curve. [f] For details of the data see ref 13. [g] For details of the data see ref 13 and 27. [h] For details of the data see ref 10–12 and 14. [i] For details of the data see ref 11.

V showed the longest duration of action (~3 h) on rabbit aortic strips. Since pA_2 values of [Sar^1,Sar^2,Thr^8]- and [Sar^1,Thr^8]angiotensin II are the same, this suggests that the guanidino side chain in position 2 is not necessary for binding to the receptor. On the other hand, the low in vivo antagonistic activity of [Sar^1,Sar^2,Thr^8]angiotensin II suggests other inactivating mechanisms such as nonspecific binding that may prevent this analogue to reach the receptor site. Longer lasting effects suggest that its low in vivo activity could not be due to an increased degradative action. These results, however, again support the importance of the guanidino side chain in position 2 for the in vivo antagonistic potency.

In conclusion, replacement of positions 5 and/or 8 with *O*-methylthreonine in angiotensin analogues significantly enhanced the potency of angiotensin II antagonists. Potency was also enhanced when alanine in position 8 was replaced with α-methylalanine. On the contrary, replacement of position 1 with α-methylalanine reduced the antagonistic activity. Although none of these peptides were devoid of initial pressor activity, agonistic effects could be minimized by administering low dosage of these highly potent antagonists.

At present there are no guidelines to increase the in vivo half-life of these antagonists. Sarcosylsarcosine linkage at the N terminus increased the in vitro duration of action but reduced the in vivo potency. However, we have observed that by using lipophilic vehicles (e.g., cottonseed or linseed oil) for subcutaneous injections, the antagonist is slowly released into plasma over a period of 6–8 h.[25]

Experimental Section

TLC of angiotensin II analogues was conducted on cellulose supported on glass plates (Brinkmann Celplate-12). The solvent systems (upper phase) used were (a) *n*-BuOH–AcOH–H_2O (BAW), 4:1:5 or 4:1:1; (b) *n*-BuOH–AcOH–H_2O–pyridine (=Prd) (BAWP), 30:6:24:20; (c) *n*-BuOH–AcOEt–AcOH–H_2O (BEAW), 1:1:1:1; (d) *n*-BuOH–Prd–H_2O (BPW), 10:2:5 or 65:35:35 or 65:35:65; (e) *n*-PrOH–H_2O (PW), 2:1. Ionophoresis was carried out on filter paper strips on S & S 2043A filter paper strips in a Beckman electrophoresis cell (Durrum type) Model R, Series D, at 400 V, using HCO_2H–AcOH buffer prepared by diluting 60 mL of HCO_2H and 240 mL of AcOH to 2 L with distilled H_2O (pH 1.95) and Beckman barbiturate buffer B-2 (pH 8.6). Histidine was run simultaneously as a reference compound and *E*(His) indicates the electrophoretic mobility relative to histidine = 1.00. Location of compounds on chromatograms was carried out with ninhydrin reagent and/or diazotized sulfanilic acid. For locating *tert*-butyloxycarbonylamino acid derivatives, the plates were first stored in a closed tank saturated with HCl vapors, followed by spraying with ninhydrin. Single symmetrical spots were observed for purified compounds.

Free peptides were hydrolyzed in sealed tubes under N_2 in the presence of phenol in 6 N HCl at 110 °C for 24 h or in a 1:1 mixture of 12 N HCl–propionic acid;[26] peptide polymer esters were invariably hydrolyzed in the latter solvent system. Amino acid analyses were performed on a Jeolco-5AH amino acid analyzer. Optical rotations were determined in a Perkin-Elmer Polarimeter Model 141, equipped with a digital readout. Elemental analyses were performed by Microtech Laboratories, Skokie, Ill. Where analyses are indicated only by symbols of the elements or functions, analytical results obtained for these elements or functions were within ±0.4% of the theoretical values.

Boc-Thr(Me) and other *tert*-butyloxycarbonylamino acids were purchased from Bachem, Inc.

Synthesis and Purification of Angiotensin II Analogues. C-Terminal *tert*-butyloxycarbonylamino acid was attached to chloromethylated polystyrene, 2% cross-linked with divinylbenzene (Lab Systems, Inc.) and chain elongation was performed on a manual, nitrogen-stirred apparatus.[27] Side-chain functional protecting groups employed were Arg(NO_2), Tyr(2,6-Cl_2Bzl), and His(Bzl); α-amino groups were blocked with the *tert*-butyloxycarbonyl group. 1-Hydroxybenzotriazole was used as an additive

to avoid racemization of the histidine residue during coupling with Boc-His(Bzl).[28] This was carried out by using a preformed active ester of *tert*-butyloxycarbonyl-N^{im}-benzylhistidine with a 1 molar excess of this reagent.[10] Coupling of each amino acid residue was carried out by utilizing the cycle of reactions and washes given below. The esterified polymer was (1) washed with glacial CH_3CO_2H (three times for 3 min); (2) washed with CH_2Cl_2 (three times for 3 min); (3) washed with CF_3CO_2H–CH_2Cl_2 (4:6) (once for 1 min); (4) Boc group removed by treatment with CF_3CO_2-H–CH_2Cl_2 (4:6) for 15 min; (5) steps 1–4 were repeated; (6) washed with $CHCl_3$ (five times for 3 min); (7) the amino group liberated by treatment with NEt_3–$CHCl_3$ (1:9) (three times for 5 min); (8) washed with $CHCl_3$ (five times for 3 min); (9) washed with CH_2Cl_2 (three times for 3 min); (10) *tert*-butyloxycarbonylamino acid (1.5 M) in CH_2Cl_2 was added and mixed for 10 min; (11) DCC (1.5 M) in CH_2Cl_2 was added and mixed for 2 h; (12) washed with 1:2 MeOH–$CHCl_3$ (three times for 3 min); (13) washed with $CHCl_3$ (three times for 3 min); (14) washed with $HCONMe_2$ (three times for 3 min); (15) steps 10–14 were repeated except that coupling of *tert*-butyloxycarbonylamino acid was carried out for 6 h using 1:1 CH_2Cl_2–$HCONMe_2$ as the solvent; (16) residual amino groups were acetylated (except the N-terminal amino acid) by using 3:1:20 Ac_2O–NEt_3–$HCONMe_2$ (two times for 30 min);[21] (17) washed with $HCONMe_2$ (three times for 3 min); (18) washed with $CHCl_3$ (three times for 3 min).

The above coupling procedure was applicable to all amino acid derivatives except the following that are insoluble in CH_2Cl_2: (a) Boc-His(Bzl) (threefold excess) was dissolved by repeated extraction with small portions of $HCONMe_2$ on a sintered funnel; any insoluble material was filtered and the filtrate treated with 1-hydroxybenzotriazole and DCC to obtain the active ester.[10] The latter was added to the peptide polymer (steps 10 and 11) and the mixture stirred for 6 h; (b) Boc-Arg(NO_2) (threefold excess) was dissolved in a minimum volume of $HCONMe_2$, the solution was mixed with $^1/_3$ vol of CH_2Cl_2, and the coupling carried out by adding a solution of DCC (threefold excess) in 3:1 $HCONMe_2$–CH_2Cl_2.

In general, the coupling with sterically hindered amino acids, e.g., Boc-Ala(α-Me), was carried out either by activation with Woodward's reagent K[29,30] or through preformed active ester with a 1 molar excess of 1-hydroxybenzotriazole.[10] At the end of the synthesis, the peptide was cleaved from the polymer and partially deblocked with HBr–CF_3CO_2H at room temperature.[31] Complete deblocking of the peptide was carried out by hydrogenolysis in a mixture of 5:1:1 MeOH–AcOH–H_2O under 2 atm of H_2 for 36–48 h in a Parr hydrogenation apparatus.[32] Amino acid analysis of the crude hydrogenolyzed products was carried out routinely to detect the presence of His(Bzl) or Arg(NO_2). We have already reported that tyrosine is not converted into β-cyclohexylalanine under the above conditions of hydrogenolysis.[11]

Purification of the desired peptides was carried out by ion-exchange chromatography on a column of AG-1 X2 (200–400 mesh in acetate form using NH_4OAc buffer of pH 8.6) followed by partition chromatography on Sephadex G-25 using *n*-BuOH–Prd–H_2O (10:2:5) or *n*-BuOH–AcOH–H_2O (4:1:5) as the solvents.[33] Fractions in the column chromatography were cut with emphasis on purity rather than on yield and no attempt was made to rechromatograph the minor fractions for identification purposes. The homogeneity of the compounds was determined by (a) thin-layer chromatography in solvent systems of different pH, (b) electrophoresis at pH 1.95 and 8.6, and (c) amino acid analysis.

Boc-Ala(α-Me). To a solution of α-methylalanine (1 g) in 1 N NaOH (20 mL) was added EtOH until a slight turbidity appeared. Boc-N_3 (5 mL) was added and the mixture stirred magnetically while the pH was maintained between 9 and 10. After 48 h, water (200 mL) was added and the mixture extracted with Et_2O to remove unreacted Boc-N_3. The aqueous layer was concentrated to approximately 25 mL, cooled to −5 °C, and treated with citric acid to pH 4. The solution was now saturated with NaCl and extracted with AcOEt. The extract was washed (H_2O and saturated NaCl solution) and dried (Na_2SO_4) and the solvent removed in vacuo to give 500 mg of Boc-Ala(α-Me). TLC (silica gel) showed that the product was homogeneous: R_f 0.47 (EtOH), R_f 0.56 ($CHCl_3$–MeOH, 1:3), R_f 0.74 ($CHCl_3$–MeOH–AcOH, 85:10:5). Anal. ($C_9H_{17}NO_4$) N.

[Sar^1,Sar^2,Thr^8]angiotensin II (V): TLC (cellulose) R_f 0.46 (BAW, 4:1:5), R_f 0.75 (BEAW), R_f 0.24 (BPW, 10:2:5), R_f 0.43 (BPW, 65:35:35), R_f 0.63 (BAWP), R_f 0.76 (PW); *E*(His) 0.78 (pH 1.95), *E*(His) 0.88 (pH 8.6); amino acid ratio in the acid hydrolysate Sar 2.00, Val 1.16, Tyr 1.16, Ile 1.00, His 1.00, Pro 1.16, Thr 1.0; $[\alpha]^{25}_D$ −93.04° (*c* 0.5, 5 N AcOH).

[Sar^1,Ala(α-Me)^8]angiotensin II (III): TLC (cellulose) R_f 0.46 (BAW, 4:1:5), R_f 0.50 (BAW, 4:1:1), R_f 0.26 (BPW, 65:35:35), R_f 0.22 (BPW, 65:35:65), R_f 0.62 (BAWP); *E*(His) 0.90 (pH 1.95), *E*(His) 0.91 (pH 5.9, Prd–AcOH–H_2O 10:90:900); amino acid ratio in the acid hydrolysate Sar 1.00, Arg 1.00, Val 1.15, Tyr 1.10, Ile 1.11, His 0.90, Pro 1.00, Ala(α-Me) 1.00. The retention time of α-methylalanine (long column for acidic and neutral amino acids) was approximately 82 min, which is the same for alanine, and it had a color constant of $^1/_{20}$nd that of alanine. In order to obtain a reasonable peak size excessive amounts of the peptide were hydrolyzed: $[\alpha]^{25}_D$ −73.16° (*c* 0.5, 5 N AcOH).

[Ala(α-Me)^1,Ala(α-Me)^8]angiotensin II (IV). Boc-Ala(α-Me) (1.14 g) was activated by treatment with NEt_3 (0.79 mL) and Woodward's reagent K (1.42 g) in $HCONMe_2$ and the solution added to the heptapeptide polymer. The coupling step was repeated twice to ensure completion. The octapeptide was cleaved from the polymer, hydrogenated, and purified in the usual way: TLC (cellulose) R_f 0.47 (BAW, 4:1:5), R_f 0.26 (BPW, 10:2:5), R_f 0.41 (BPW, 65:35:35), R_f 0.43 (BPW, 65:35:65), R_f 0.64 (BAWP), R_f 0.83 (PW, 2:1); *E*(His) 0.89 (pH 1.95), *E*(His) 1.04 (pH 8.6); amino acid ratio in the acid hydrolysate Ala(α-Me) 2.0, Arg 1.0, Val 1.1, Tyr 1.0, Ile 1.0, His 1.1, Pro 1.0; $[\alpha]^{25}_D$ −71.1° (*c* 0.5, 5 N AcOH).

[Sar^1,Thr(Me)^5,Ile^8]angiotensin II (I): TLC (cellulose) R_f 0.35 (BAW, 4:1:5), R_f 0.08 (BPW, 10:2:5), R_f 0.25 (BPW, 65:35:35), R_f 0.25 (BPW, 65:35:65); *E*(His) 0.89 (pH 1.95), *E*(His) 0.89 (pH 5.9, Prd–AcOH–H_2O 10:90:900); amino acid ratio in the acid hydrolysate Sar 0.9, Arg 1.0, Val 1.1, Tyr 1.0, Thr 0.9, His 0.9, Pro 1.0, Ile 1.2; $[\alpha]^{25}_D$ −66.52° (*c* 0.5, 5 N AcOH).

[Sar^1,Thr(Me)^5,Thr(Me)^8]angiotensin II (II): TLC (cellulose) R_f 0.28 (BAW, 4:1:5), R_f 0.18 (BPW, 65:35:35), R_f 0.47 (BAWP); *E*(His) 0.86 (pH 1.95), *E*(His) 0.95 (pH 5.8, Prd–AcOH–H_2O 10:90:900); amino acid ratio in the acid hydrolysate Sar 1.0, Arg 1.11, Val 1.0, Tyr 1.0, Thr 2.0, His 1.0, Pro 1.0; $[\alpha]^{25}_D$ −60.2° (*c* 0.5, 5 N AcOH).

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(1) Presented in part by Khosla et al. in (a) Symposium on Cardiovascular Agents, Centennial Meeting of the American Chemical Society (171st National Meeting), New York, N.Y., April 4–9, 1976;[3] (b) XIVth European Peptide Symposium, Wepion, Belgium, April 11–17, 1976;[4] and (c) Symposium on Recent Advances in Peptide Hormones, Eighth Central Regional Meeting of the American Chemical Society, Akron, Ohio, May 19–21, 1976.[5]

(2) Abbreviated designation of amino acid derivatives and peptides is according to the recommendations of the IUPAC–IUB Commission (IUPAC Information Bulletin No. 26). Symbols for structural variants of naturally occurring amino acids were adopted from the list compiled by M. C. Khosla and W. E. Cohn.[6]

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2-Deaminoactinomycin D, Synthesis and Interaction with Deoxyribonucleic Acid

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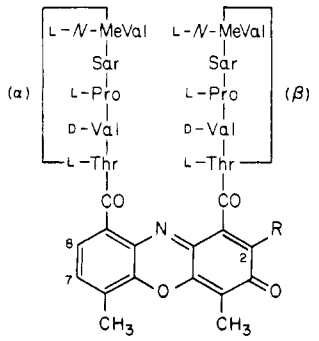
2-Deaminoactinomycin D has been synthesized and characterized. It binds to DNA by intercalation according to NMR, CD, thermal denaturation, and unwinding studies on the drug–DNA complex. Loss of the 2-amino group does not seriously affect binding parameters relative to actinomycin D; affinity for calf thymus DNA may even be increased, according to ΔT_m measurements. The unwinding of circular DNA caused by this compound is at least as large as that effected by actinomycin D and ethidium bromide. Nevertheless, 2-deaminoactinomycin D is less effective than actinomycin D in inhibiting nucleic acid syntheses in L1210 cell culture and in in vivo antitumor activity against P388 leukemia.

Actinomycin D (1), an antiobiotic with antitumor activity limited to a few specific tumors,[1-3] has been extensively studied, not only to effect modifications to improve its therapeutic index but also to clarify its mode of action as an effective drug. It has been established that actinomycin inhibits DNA-dependent RNA synthesis[4-8] and also, to a lesser degree, DNA synthesis, apparently by forming an intercalation complex with DNA.[9-11]



1, R = NH_2
2, R = OH
3, R = Cl
4, R = H

Derivatives of 1 in which the 2-amino group on the chromophore has been replaced by OH (2), Cl (3), or mono- or disubstituted amines (NRH or NR_2) have been nearly or completely inactive biologically.[12-16] It has been assumed therefore that the 2-amino group is required for DNA binding and biological activity.[17] However, the 2-deamino analogue 4 has not been prepared and studied. [The material referred to in early literature[12,16] as "de(s)aminoactinomycin" is actually described as 2-deamino-2-hydroxyactinomycin. The product reported by Moore et al.[13] might be 2-deamino-2-chloroactinomycin.] In this paper we are reporting the synthesis of 4 and certain characteristics of its complex with DNA.

Conversion of 1 to 3, via intermediate 2, was carried out according to reported procedures.[12,13,16] The product 3 showed an absorption spectrum almost identical with that of "chloroactinomycin C_3" reported by Brockmann[12] but different from the spectrum reported by Moore et al.[13] The latter group also indicated that the melting point of their product was 30 °C lower than ours. Movement on thin-layer chromatographic plates was also somewhat different from our results.

Although 2-deamino-2-chloroactinomycins C_2 and C_3 have been treated with a variety of amines to give *N*-alkyl analogues,[14,15] we found that displacement of the chlorine in 3 by catalytic hydrogenation proceeded very slowly. The course of the reaction was followed by monitoring the