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Synthesis of Octanoyl[8-leucyl]angiotensin II, a Lipophilic Angiotensin Antagonist

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Octanoyl[8-leucyl]angiotensin II (oct-LAT) was synthesized with the aim of obtaining a longer acting angiotensin (AT) inhibitor. The new compound, with a partition coefficient $K = 7.00$ in *n*-BuOH–HOAc–H_2O, was compared with [Leu^8]angiotensin II (LAT, $K = 0.18$) as an AT antagonist in two isolated smooth muscle preparations and in the rat blood pressure assay. The two compounds were equally potent in the rat uterus, but LAT was more effective in the guinea pig ileum and the in vivo assay. LAT's effect was longer lasting in the smooth muscles, but the duration of in vivo inhibition was the same for the two compounds. It is concluded that partitioning between external medium and biophase is not a limiting factor for antagonistic potency and permanence of effects of 8-substituted AT derivatives.

The important finding that [Ala^8]angiotensin II is a competitive inhibitor of angiotensin II[1] (AT) has led to the description of numerous antagonistic AT analogues with aliphatic side chains in position 8.[2] These antagonists have proved to be useful tools in the study of the role of the renin–angiotensin system in human hypertension[3] and have potential uses as diagnostic and therapeutic agents. However, the compounds hitherto described have the disadvantage of a short in vivo half-life, and attempts to prolong their action by the introduction of enzyme-resistant peptide linkages have not been successful.[4]

An alternative way for obtaining longer acting angiotensin antagonists would be to increase the permanence of these compounds in the biophase[5] by increasing their lipophilicity. Lipophilic derivatives would also be of use for depot administration. In order to test these possibilities we have synthesized a derivative of [Leu^8]-AT[6] (LAT) in which the N-terminal amino group was acylated by *n*-octanoic acid (oct-LAT). The amino group was chosen for anchoring the lipophilic moiety because its acylation does not affect the pressor action of AT[7] and protects the peptide against attack by aminopeptidases. The results of the study of oct-LAT as an antagonist of the myotropic and pressor activities of AT are presented in this paper.

Experimental Section

Materials. The chloromethylated copolymer of styrene and divinylbenzene (2% cross-linking, 0.9 mequiv/g) was from Bio-Rad (lot 6554) and the *tert*-butyloxycarbonyl (Boc) amino acid derivatives were from Bachem. Bradykinin, [Sar^1, Ala^8]-AT, and LAT[8] were synthetic products of this laboratory.

Peptide Synthesis. Oct-LAT was prepared by the solid-phase method.[9] Boc-Leu was attached to the polymer support,[10] and chain elongation was performed with the aid of an automatic peptide synthesizer.[11] CH_2Cl_2 was used as solvent for all reagents and *tert*-butyloxycarbonylamino acids with the exception of CF_3COOH, used as a 30% (v/v) solution in $CHCl_3$, and of Boc-Arg(Tos), which was dissolved in CH_2Cl_2–DMF (2:1). The amino acids with reactive side chains were used in the form of the following derivatives: Boc-His(Tos), Boc-Tyr(Bzl), Boc-Arg(Tos), and Boc-Asp(Bzl). All the coupling reactions, done with 2.5 equiv of *tert*-butyloxycarbonylamino acid and of DCI, were monitored with the ninhydrin reaction[12] and were completed in 6 h or less. The Boc groups were removed by treatment with 30% (v/v) CF_3COOH in $CHCl_3$ for 30 min. After the last cycle (coupling of *n*-octanoic acid), the peptide-resin was cleaved by treatment with anhydrous HF containing 5% (v/v) anisole for 45 min at 0 °C. After removal of HF and anisole, by vacuum distillation and washing with EtOAc, the peptide was extracted with glacial HOAc and lyophilized.

Table I. Comparison of Some Physical Properties of LAT and Oct-LAT

Compd	Partition coeff[a]	Electrophoretic migration[b] pH 2.8	pH 4.9	pH 9.9	R_f[c] A	B	C
LAT	0.18	0.75	0.44	0.43	0.24	0.57	0.44
Oct-LAT	7.00	0.48	0.21	0.18	0.55	0.70	0.71

[a] Obtained from countercurrent distribution in *n*-BuOH–HOAc–H_2O (4:1:5). [b] Relative to histidine (pH 2.8), arginine (pH 4.9), and picric acid (pH 9.9). [c] Solvent systems described in the Experimental Section.

The crude peptide was submitted to 200 transfers of countercurrent distribution in *n*-BuOH–HOAc–H_2O (4:1:5), followed by chromatography on a 10 × 1.8 cm carboxymethylcellulose column with a linear gradient between 0.01 M NH_4OAc (pH 5.0) and 0.5 M NH_4OAc (pH 8.6). The peptide-containing eluate was lyophilized until constant weight to remove NH_4OAc. Amino acid analyses were made on a Beckman 120C analyzer, after hydrolysis with 2 mL of 6 N HCl containing 0.1 mL of 10% (v/v) mercaptoethanol and 0.04 mL of 5% (v/v) phenol in nitrogen, for 72 h at 110 °C, yielding the following molar ratio: Asp, 1.01; Arg, 1.00; Val, 1.02; Tyr, 0.94; Ile, 0.98; His, 1.04; Pro, 1.01; Leu, 1.01. The peptide content was 88%, as expected from the diacetate form of the pure peptide. Only one spot was detected with Sakaguchi, Pauly, and hypochlorite reagents after TLC on silica gel (Eastman "Chromagram" plates, 0.1 mm) with the following solvent systems: (A) *n*-BuOH–HOAc–H_2O (5:1:1); (B) *n*-BuOH–EtOAc–HOAc–H_2O (1:1:1:1); (C) *n*-BuOH–pyridine–HOAc–H_2O (15:10:3:12). The R_f values are shown in Table I. Only one component, with the expected mobility, was detected after paper electrophoresis at 1000 V for 60 min in the following buffer systems: 1 M HOAc (pH 2.8), 0.1 M pyridine acetate (pH 4.9), and 0.2 M sodium carbonate–bicarbonate (pH 9.9). The relative mobilities, expressed as the ratio of the peptide's migration to that of a simultaneously run amino acid standard, are shown in Table I.

Bioassays. The preparations of the guinea pig isolated ileum,[13] rat isolated uterus,[14] and the rat blood pressure assay[15] were described in detail elsewhere. To avoid the interference of tachyphylaxis with our observations, in the case of the guinea pig ileum, the AT administrations were spaced at 15- to 20-min intervals to allow recovery from the tachyphylactic state.[16] In the rat uterus, the intervals between administrations could be

Table II. Antagonistic Activities of LAT and Oct-LAT

Compd	Guinea pig ileum			Rat uterus			Rat blood pressure	
	pA_2	pA_h	Recovery $t_{1/2}$, min	pA_2	pA_h	Recovery $t_{1/2}$, min	Dose ratio slope,[a] (kg min)/μg	Recovery $t_{1/2}$, min
LAT	8.92 ± 0.05	7.3 ± 0.1	3.8 ± 0.4	8.33 ± 0.05	6.2 ± 0.2	28.1 ± 0.7	10.2 ± 1.1	12 ± 2
Oct-LAT	7.64 ± 0.06	6.2 ± 0.2	1.7 ± 0.6	8.42 ± 0.08	6.0 ± 0.2	6.6 ± 0.8	2.3 ± 0.5	12 ± 1

[a] Taken from the plot of Figure 2.

5 min because no tachyphylaxis is observed in the conditions of the assay (pH 8.0, $[Ca^{2+}] = 5.4 \times 10^{-4}$ M).[17]

To determine the in vitro inhibition of the myotropic action, dose–response curves for AT and for bradykinin (control) were first obtained in organs that had been previously equilibrated at the assay conditions for 30 min. The organ was then equilibrated for 15 min with a medium containing the smallest concentration of inhibitor to be studied, and a new dose–response curve for AT was obtained in the presence of that medium. The process was repeated with higher inhibitor concentrations and at the highest concentration a dose–response curve for bradykinin was also obtained. This protocol allowed the study of two to three inhibitor concentrations in the same organ in 5–8 h. To ensure that tachyphylaxis did not interfere in the measurement of the inhibitor's effect on the maximum response to AT, freshly prepared isolated ilei were equilibrated for 30 min, and then their response to a maximal dose of histamine (10^{-5} M) was recorded. After 15 min, a maximal dose of AT (10^{-7} M) was given, followed by a 30-min recovery period and 30-min equilibration with the desired inhibitor concentration. The responses to one maximal dose each of histamine (10^{-5} M) and angiotensin (10^{-7} to 4×10^{-6} M) were then recorded.

In order to avoid tachyphylaxis to submaximal doses of AT,[16] the study of the kinetics of the guinea pig ileum's recovery from inhibition was made with a protocol in which AT doses were administered with a fixed frequency throughout the experiment. The organ was initially treated, at regular time intervals and 2-min contact, with 2×10^{-9} M AT until a stable response was obtained. An equipotent antagonistic dose of either LAT (2×10^{-8} M) or oct-LAT (2×10^{-7} M) was then administered 3 min before one of the regular treatments, and the response to AT in its presence was recorded. After the 2-min contact time, the organ was washed with fresh medium and the response to the next AT treatment was recorded to evaluate the recovery from inhibition in the period since the time of washout. By varying the time interval between AT treatments in each series of observations, recovery at different times after washout could be measured. For each recovery time, 7–11 independent measurements were made. In the rat uterus preparations, from five to eight independent measurements for each recovery time were also made, but in this case the AT (2×10^{-9} M) treatments were always spaced at 5-min intervals. During the time between washout and the test dose of AT, the uterus continued to contract regularly by the administration of 6×10^{-4} M $BaCl_2$ every 5 min. The LAT and oct-LAT concentrations were the same as those used in the guinea pig ileum.

The recovery from inhibition in both organs closely followed first-order kinetics, as attested by correlation coefficients ranging from −0.996 to −1.000 in linear least-square plots of log (100 − percent recovery) vs. time. Recovery $t_{1/2}$ values were obtained by interpolation in these plots.

For the in vivo assays, in the rat blood pressure preparation, it was not feasible to obtain full dose–response curves. Control responses, in the range 10–40 mmHg, were first recorded by the administration of three to five different doses of AT through one of the femoral veins. The inhibitor, dissolved in 0.15 M NaCl, was then infused through the other femoral vein, at a constant rate of 0.06 mL/min and a new dose–response curve to AT, in the range 10–40 mmHg, was obtained during the infusion period. The pressor response to test doses of epinephrine was used as controls for the specificity of the AT inhibition. After the infusion was discontinued, the recovery from inhibition was followed by the responses to test doses of AT given at regular intervals. The ratio of the AT doses that produced the same response in the presence and in the absence of a given treatment (dose ratio) was used as an estimate of the degree of inhibition.

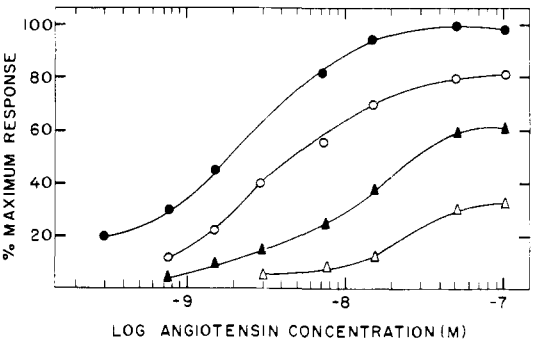

Figure 1. Angiotensin II log dose–response curves obtained in the rat isolated uterus in the absence (●) and in the presence of increasing concentrations of oct-LAT: (○) 5×10^{-9} M; (▲) 5×10^{-8} M; (△) 10^{-7} M.

The values presented in this paper are averages ± standard deviations.

Results

The comparison between the partition coefficients of oct-LAT and LAT (Table I) shows that the introduction of the *n*-octanoyl moiety greatly decreased the polarity of the molecule. Oct-LAT, however, was still soluble enough in water to allow the preparation of the dilute aqueous solutions used for the biological assays.

In Vitro Assays. The agonistic activities of LAT and oct-LAT, expressed as percentages of AT's activities, were, respectively, 0.016 ± 0.001 and 0.025 ± 0.008 in the ileum, 0.15 ± 0.02 and 0.21 ± 0.02 in the uterus. Oct-LAT was also found to be a potent specific angiotensin antagonist in the two muscle preparations. In either preparation, a considerable deviation from the typical competitive inhibitor behavior was observed with both LAT and oct-LAT, as shown by the example of Figure 1. The inhibition produced not only a displacement of the dose–response curves to the right but also a marked concentration-dependent decrease in the maximum response to AT. The dose–response curves to bradykinin, in the same organs, were not altered in the presence of the highest inhibitor concentrations employed. The AT inhibition was reversible, and the recovery of the organs' response after removal of the antagonist from the medium is indicated by the $t_{1/2}$ values shown in Table II. In spite of the relatively large errors associated with these values it is clear that, contrary to what was expected, the permanence of inhibition after antagonist washout was longer in the case of LAT than in that of oct-LAT.

The observed change in log dose–response slopes and the decrease of maximum responses of the guinea pig ileum and the rat uterus (Figure 1) appear to be a more common property of 8-substituted AT antagonists. We have observed a similar behavior with [Sar[1],Ala[8]]-AT, in contrast with the parallel displacement of the AT log dose–response curves seen with this compound in the case of the rabbit isolated aorta.[18]

In view of the change in slope of the log dose–response curves, pA_2 values[19] (Table II) were calculated from dose

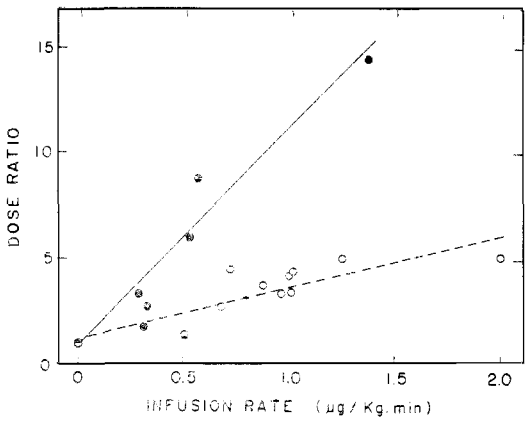

Figure 2. Angiotensin dose ratios for obtaining the same pressor response in the presence and in the absence of LAT (●) and oct-LAT (○), as a function of antagonist infusion rate.

ratios taken from the low portion of the curves, where lack of parallelism was less pronounced. The quantitation of the inhibitory effect was also made by determination of pA_h,[20] defined as the negative logarithm of the inhibitor concentration that reduces by one-half the maximum response to the agonist. The pA_h values estimated from our data are also shown in Table II. Although these values are significantly lower than the corresponding pA_2's, both parameters indicate that oct-LAT's antagonistic efficiency in the guinea pig ileum was one order of magnitude less than that of LAT, while both antagonists were equally potent in the rat uterus.

In Vivo Assays. Oct-LAT showed a small agonistic activity in the rat blood pressure (0.10 ± 0.02% of AT's activity), which was similar to that of LAT (0.09 ± 0.02). Intravenous infusion of either compound, at the rates employed in this study, produced a small transient pressor response followed by marked inhibition of responses to AT. The antagonistic potencies may be assessed from plots of the dose ratio as a function of perfusion rate. Figure 2 shows that a linear dependence was found with both LAT (r = 0.97 ± 0.02) and oct-LAT (r = 0.8 ± 0.1). The ratio of the two slopes (Table II) indicates that LAT was 4.4 times more potent than oct-LAT as an AT antagonist in the rat blood pressure. As for the recovery from inhibition after interruption of treatment, no difference was found between the $t_{1/2}$ values for LAT and oct-LAT (Table II).

Discussion

Smooth Muscles. Oct-LAT was found to be a potent inhibitor of AT's myotropic action, although less efficient than LAT in the guinea pig ileum. A striking property of both antagonists was the lowering of the maximum responses of the ileum and of the uterus to AT, a characteristic usually associated with noncompetitive inhibition. The 8-substituted AT analogues are generally believed to be competitive inhibitors of that peptide,[2] but some conflicting results have been reported. Regoli et al.[21] observed that LAT decreased the slope of log dose–response curves and depressed the maximum response to AT in the rat isolated stomach strip. This was not confirmed in a later report from the same laboratory,[22] but typical noncompetitive behavior was reported for [Sar^1, Ala^8]-LAT in the same muscle, and we have found the same in the guinea pig ileum and the rat uterus. Similar behavior may have not been detected in other 8-substituted AT antagonists because the dose effect curves reported in the literature were often obtained only with relatively low agonist concentrations, to avoid interference from tachyphylaxis. The depressions of maximum responses described in this paper were measured with all precautions to exclude tachyphylaxis, and they suggest that this may be a more general property of these antagonists, at least in the two smooth muscles studied here and in the rat stomach.[21,23] This brings doubt on the value of previously reported[8] pA_2 values, since this parameter only has meaning in fully competitive situations. For this reason, we have also evaluated the antagonistic potencies of LAT and oct-LAT using pA_h.[20] This parameter is equivalent to pD_2', proposed for noncompetitive inhibition,[24] and would be a measure of the negative logarithm of the dissociation constant of the antagonist–receptor complex if the response were directly proportional to agonist–receptor concentration. The pA_h values given in Table II are one or two orders of magnitude lower than the respective pA_2's but confirm the relative antagonistic potencies of LAT and oct-LAT found using the latter parameter. However, we do not attach greater theoretical significance to these pA_h values, because it is difficult to accept the concept of reversible noncompetitive AT inhibition by the 8-substituted analogues. Besides the fact that no instance of truly specific noncompetitive antagonism has been demonstrated,[5] the close structural relationships between AT, LAT, and oct-LAT point to an interaction with the same receptor sites. We believe that the depression of the maximum responses to AT, shown in Figures 1 and 2, may be due to either (a) a slow dissociation of the antagonists from the receptors or (b) triggering by the antagonists of a change in the receptor, whose return to a normal responsive state would be slow, even after the antagonist is dissociated from its binding sites. Either of these occurrences would be compatible with the observation that the $t_{1/2}$ values for recovery from LAT and oct-LAT antagonism are in the order of minutes (Table II) while that to AT's agonistic action is in the order of seconds.[25]

If the permanence of the inhibitory effect were controlled by the rate of diffusion of the antagonist from the hydrophobic biophase into the aqueous medium, recovery from oct-LAT inhibition should be slower than in the case of LAT. One would also expect oct-LAT to be a more potent inhibitor, if partitioning between external medium and biophase were a limiting factor for this activity. However, oct-LAT presented approximately the same antagonistic activity as [Suc^1]-LAT, which differs from LAT by the lack of the amino group, and has a partition coefficient of 0.79 in the n-BuOH–HOAc–H_2O system.[8] Thus, the dominant factor in determining the relative antagonistic potencies of LAT and oct-LAT in the smooth muscle preparations appears to be the blocking of the N-terminal amino group. This finding parallels the observation of a lack of correlation between partition coefficients and agonistic effectiveness of AT analogues.[25]

Blood Pressure. In the rat blood pressure assay, the antagonistic potency of LAT was 4.4 times greater than that of oct-LAT. This was probably not due to the blocking of the amino group in the latter compound, since this group is not as important for the pressor as it is for the myotropic activities of AT derivatives.[8] The antagonistic action of LAT was not prolonged by the addition of the n-octanoyl moiety (Table II), indicating that diffusion between biophase and external medium is not a limiting factor also for the inhibition of pressor activity. In this case, however, the $t_{1/2}$ values for recovery were the same for the two antagonists, indicating that the amino group is not as important for the duration of action as it is in the isolated smooth muscles. For this reason, although the addition of N-terminal nonpolar residues is not a promising means for obtaining prolonged in vivo activity

of 8-substituted AT inhibitors, these derivatives may be useful for depot administration.

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Cyanoguanidine-Thiourea Equivalence in the Development of the Histamine H_2-Receptor Antagonist, Cimetidine

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In the histamine H_2-receptor antagonist metiamide (**2a**) isosteric replacement of thione sulfur (=S) by carbonyl oxygen (=O) or imino nitrogen (=NH) affords the urea **2c** and guanidine **2d** which are antagonists of decreased potency. The guanidine is very basic and at physiological pH is completely protonated. However, introduction of strongly electronegative substituents into the guanidine group reduces basicity and gives potent H_2-receptor antagonists, viz. the cyanoguanidine **2b** (cimetidine, "Tagamet") and nitroguanidine **2e**. A correspondence between the activity of thioureas and cyanoguanidines is demonstrated for a series of structures 1–4. The close correspondence between cyanoguanidine and thiourea in many physicochemical properties and the pharmacological equivalence of these groups in H_2-receptor antagonists leads to the description of cyanoguanidine and thiourea as bioisosteres. Acid hydrolysis of the cyanoguanidine **2b** yields the carbamoylguanidine **2f** at ambient temperatures and the guanidine **2d** at elevated temperatures. Cimetidine is slightly more active than metiamide in vivo as an inhibitor of histamine-stimulated gastric acid secretion and has clinical use in the treatment of peptic ulcer and associated gastrointestinal disorders.

The discovery of the selective antagonist burimamide (**1a**, Table I) permitted the characterization of histamine H_2-receptors and furnished a class of drug with a completely novel pharmacological action.[1] Chemical modification of burimamide led to the orally active antagonist metiamide[2,3] (**2a**) which proved sufficiently active to allow the exploration of the therapeutic potential of this new type of drug. Clinical studies established that metiamide is a highly effective inhibitor of gastric acid secretion and that it gave marked symptomatic relief to patients with peptic ulcer;[4] healing of recalcitrant multiple ulcers following treatment with metiamide was also reported.[5,6] To explore further the structural requirements for H_2-receptor antagonism, we have investigated the effect of replacing the thiourea group of metiamide. A factor that has emphasized the importance of this investigation has been the finding of kidney damage and agranulocytosis in high-dosage chronic toxicity tests with metiamide[7,8] and the possibility that these effects are attributable to the presence of a thiourea group in the drug molecule. As part of this study we have investigated the isosteric replacement of the thiourea sulfur atom of metiamide.

Carbonyl oxygen (=O) and imino nitrogen (=NH) are well precedented[9] as isosteres of thione sulfur (=S) (e.g., in the barbiturates) and, initially, these isosteric replacements were considered for metiamide. The urea **2c**