(17) N. C. Chaturvedi, W. K. Park, R. R. Smeby, and F. M. Bumpus, *J. Med. Chem.*, **13**, 177 (1970).
(18) R. Schwyzer, *Ergeb. Physiol. Biol. Chem. Exp.*, **53**, 1 (1963).
(19) S. Y. Lin, H. Ellis, B. Weisblum, and T. L. Goodfriend, *Biochem. Pharmacol.*, **19**, 651 (1970).
(20) S. W. Dietrich, E. C. Jorgensen, P. A. Kollman, and S. Rothenberg, *J. Am. Chem. Soc.*, **98**, 8310 (1976).
(21) E. C. Jorgensen, G. C. Windridge, W. Patton, and T. C. Lee, *J. Med. Chem.*, **12**, 733 (1969).
(22) E. C. Jorgensen, G. C. Windridge, and T. C. Lee, *J. Med. Chem.*, **13**, 352 (1970).
(23) E. C. Jorgensen and R. A. Wiley, *J. Pharm. Sci.*, **52**, 122 (1963).
(24) E. Schnabel, *Justus Liebigs Ann. Chem.*, **702**, 188 (1967).

Photoaffinity Labeling of the Angiotensin II Receptor. 3. Receptor Inactivation with Photolabile Hormone Analogues

Emanuel H. F. Escher* and Gaëtan Guillemette[1]

Département de Pharmacologie, Faculté de Médecine, Université de Sherbrooke, Sherbrooke, Québec, J1H 5N4, Canada.
Received February 13, 1979

It has been shown that the receptor of angiotensin II (AT) in rabbit aorta strips, rat aorta, and rat stomach can be blocked specifically and irreversibly by several photolabile analogues of Sar-Arg-Val-Tyr-Val-His-Pro-Phe ([Sar[1]]AT) with irradiation. The effectiveness of a photolabel with light of wavelength 365 nm depends on the labeling amino acid (L-4′-nitrophenylalanine, L-4′-diazoniumphenylalanine, or L-4′-azidophenylalanine) and on its position in the peptide (replacing Tyr[4] and/or Phe[8]). The (4′-azido)Phe-containing analogues are all good to fair photoinactivators. Their decreasing order of effectiveness is as follows: [Sar[1],(4′-azido)Phe[8]]AT, [Sar[1],(4′-azido)Phe[4,8]]AT, and [Sar[1],(4′-azido)Phe[4]]AT. The (4′-nitro)Phe analogues show the opposite relation: the good ligand [Sar[1],(4′-nitro)Phe[8]]AT is almost ineffective, but the nonligand [Sar[1],(4-nitro)Phe[4]]AT exhibits good, specific photoinactivation. This can be explained by the existence of a different photolysis pathway for (4′-nitro)Phe: this analogue probably undergoes a multiphoton decay with a long-lived first excited state. A peptide in this state may differ in its pharmacological properties from the ground state and become a ligand.

The isolation and purification of peptide hormone receptors are the goals of continuing research efforts. Up to now, all the successful isolations[2-4] were of receptors which retained hormone-binding ability after solubilization of the cell membrane. Unfortunately, several peptide hormone receptors lose their binding ability upon total solubilization and have, thus, resisted isolation. For example, the homogenization, solubilization, and subsequent isolation of the angiotensin II (AT) receptor has often been attempted. Preparations from adrenals have always lost AT affinity upon addition of detergent.[5] A preparation from rabbit aorta retained some affinity in the presence of low detergent concentration, but no isolation was achieved.[6] Affinity labeling studies[7] were similarly unsuccessful.

In a previous paper,[8] we described the aim of this research and the synthesis and biological activities of several AT analogues designed for photoaffinity labeling of the AT receptor. In a later paper,[9] these biological activities were discussed in more detail and new conclusions were drawn about structure–activity relationships of AT. We now report the influence of photolabile peptide analogues on several AT-sensitive tissues in the presence of ultraviolet light. In a preliminary report, we have already presented the first example of irreversible and specific inactivation of the AT response on rabbit aorta strip with one of our peptides.[10]

The investigated peptides (see Table I) contained either L-(4′-nitro)Phe, L-(4′-azido)Phe, or L-(4′-diazonium)Phe in positions 4 and/or 8 of Sar-Arg-Val-Tyr-Val-His-Pro-Phe ([Sar[1]]AT). The azido compounds are well-known photoaffinity labels,[11,12] but the nitro compounds have also proven useful.[11] The diazonium compounds are potential photolabels known to be photosensitive and to yield radicals upon photolysis.[13] In the absence of photolysing radiation, the peptides exhibit great differences in biological activity, which are summarized in Table I.

The expected pharmacological effect of photoaffinity labeling the AT receptor was either permanent stimulation or permanent block of the AT response, giving further information about the receptors' kinetics and phenomena such as tachyphylaxis. If the photolabeling process does not significantly alter the receptor conformation, the following possible consequences can be considered: a permanently activated response would suggest an occupation mechanism where the receptor is locked in a "on" position. This has been observed with a photoaffinity labeling experiment on the gastrin receptor.[14] Conversely, a permanent block could, under certain conditions, support the rate model.[15] In this theory, transition between unoccupied and occupied receptor is the response releasing principle.

From earlier results with chymotrypsin[11] and other experiments,[16] it was known that photoactivated labels which are not in contact with the "receptor" can rearrange and react subsequently by nucleophile attack on any part of the proteins in the cell membrane. The work with chymotrypsin showed that this undesirable side reaction can be eliminated by the addition of the scavenger L-(4′-amino)Phe to the photolysis solution. It was also shown that the chymotrypsin "receptor" (tosyl-hole) can be protected from photolabeling by a specific reversible inhibitor and that this protection was strictly competitive. Digestion of photolabeled chymotrypsin showed that the incorporation of the previously competitive photolabel was highly specific for the chymotrypsin "receptor".[17] Another possible side reaction which has been established[10] is photosensitized or photooxidized inactivation due to irradiation of preformed photoproducts. Experiments with prephotolyzed peptides showed that all our analogues

0022-2623/79/1822-1047$01.00/0

Table I. Photoinactivation of the Angiotensin II Response of Rabbit Aorta Strips[a]

		biol act. in the dark[b]			photolabeling expts				
no.	peptide	αE	pD_2	rel aff, %	concn, μM	Na response	compet. protect.[b]	1st treatment	2nd treatment
0	[Sar^1]AT	1.0	9.23	100	0.2	100 ± 10	100 ± 8	92 ± 10	85 ± 8
1	[Sar^1,(4'·NO_2)Phe^4]AT	0	<5.0[c]	0.00	10	105 ± 5	102 ± 10	43 ± 19	19 ± 8
2	[Sar^1,(4'·NO_2)Phe^8]AT	0.46	9.07	69	0.4	95 ± 10		89 ± 12	76 ± 5
3	[Sar^1,(4'-NO_2)Phe^{4,8}]AT	0.5	6.02	0.06	10	99 ± 2		83 ± 8	60 ± 11
4	[Sar^1,(4'-N_3)Phe^4]AT	0.75	6.65	0.26	5	98 ± 5	64 ± 11	54 ± 19	31 ± 13
5	[Sar^1,(4'·N_3)Phe^8]AT	1	8.72	31	0.4	100 ± 4	103 ± 5	46 ± 21	19 ± 12
6	[Sar^1,(4'-N_3)Phe^{4,8}]AT	0.8	6.12	0.07	10	100 ± 6	92 ± 6	56 ± 21	32 ± 18
7	[Sar^1,(4'-N_2^+)Phe^4]AT	1	7.05	0.66	10			98 ± 5[d]	
8	[Sar^1,(4'-N_2^+)Phe^8]AT	1	8.33	13	5	78 ± 12	72 ± 10	72 ± 14	51 ± 20
9	[Sar^1,(4'-N_2^+)Phe^{4,8}]AT	1	7.36	1.3	10			96 ± 4[d]	

[a] Abbreviations used are: αE, intrinsic activity; PD_2, negative log of the concentration of an agonist which causes half maximal concentration; pA_2, negative log of the concentration of an antagonist which reduces the response of a double dose of agonist to that of a single dose. NA, noradrenaline (0.3 μM). Each point represents the mean ± SE of at least seven experiments. [b] Protected with 5 μM [Leu^8]AT. [c] Also pA_2 < 5.0; no inhibitory properties were detectable. [d] Only preliminary tested (two experiments each).

formed products with agonistic properties upon photolysis. It was therefore important to carry out parallel experiments with flash photolysis, which permits simultaneous activation of all photolabels in solution within <1 ms (and with no subsequent irradiation), and irradiation experiments with prephotolyzed peptides and with the labeling amino acids.

To exclude wrong labeling mechanisms and to establish specificity for the AT receptor, the following precautions were used: (a) addition of (4'-amino)Phe (scavenger) before photolysis; (b) activity control of other hormones or agonists on the treated tissue, e.g., bradykinin, serotonin, noradrenaline, potassium, and histamine; (c) competitive protection of the receptor with the reversible AT-inhibitor [Leu^8]AT;[18] (d) parallel photolabeling experiments with monochromatic flash photolysis instead of classical mercury black light sources (365 nm); (e) tissue irradiation in the presence of prephotolyzed labeling peptides and in the presence of the labeling amino acids.

All photolabile peptides were assayed for photoinactivation on rabbit aorta strips,[19] the test which has been used before to establish their biological activity.[9] The most successful peptides were further tested on rat stomach strips and rat aorta in order to generalize any positive effect found with rabbit aorta.

Results and Discussion

The results of the inactivation experiments are summarized in Table I. Rabbit aorta strips have been incubated and after equilibration their sensitivity to AT and the other myotropic agonists has been tested. After relaxation, the analogues **0** to **9** were added in receptor saturating quantities, together with the scavenger (4'-amino)Phe. On maximal contraction, the tissues were exposed to UV light for 10 min or flashed. The tissues relaxed immediately to their basal tension due to photorelaxation, which itself is completely reversible. An exception was peptide **1**, which was a nonligand; therefore, an arbitrary dose was given and the noncontracted tissue was irradiated. After the removal of the light source, the tissues regained some of their initial contraction and were washed. Subsequent doses of AT to tissues treated with **0** and UV light gave the full response. The residual activity of the tissues treated with the photolabels **1** to **9** varied from strong inactivation to almost complete retention of activity (see Table I). None of the tissues showed loss of sensitivity to the other agonists, except those treated with **8**. Subsequent AT doses to inactivated tissues did not enhance the residual activity. The diazonium peptides **7** and **9** had low affinities of 0.66 and 1.3%, respectively. These (reversible) activities in the dark did not come from the peptides in question (which should be complete nonligand according to the electronegativity dependance) but rather from the hydrolysis products formed in the Krebs solution.[9] These impurities are inevitable and will form tyrosine-containing products which are the natural agonist or similar to it. It was therefore not surprising that both **7** and **9** did not show any photoinactivation. As predicted and described in a preliminary communication,[10] the most promising peptide **5** gave very good photoinactivations, ranging from almost total inactivation to about 60% residual activity in a single experiment (distinct differences of the inactivation rate were observed between different batches of rabbits). The other compounds showed weaker effects, with the exception of the nitropeptides **1** and **2**. Peptide **2** was not able to inactivate at all even if the treatment was repeated, although it was a very good ligand. However, peptide **1**, which did not show any agonistic or antagonistic properties in the dark, almost equaled the results obtained with peptide **5**. Peptide **6** showed a weaker inactivation and seemed to be more or less specific, but the high quantities used (10 μM) made it rather unattractive. Peptide **3** was a rather weak inactivator but did not harm other receptors. Peptide **4**, which was the most promising position 4 label, was not competitively binding-site directed because it caused considerable inactivation of the AT response even if the receptor was protected with the competitive antagonist [Leu^8]AT. Reference irradiation of the tissues in the presence of the labeling amino acids and of the prephotolyzed analogues did not give any significant inactivation.

We tried to broaden the results obtained using other bioassays such as rat aorta strips and rat stomach strips (Figure 1). In the rat aorta test, the results very closely resembled those obtained with the rabbit aorta. The rat stomach strip test was somewhat more difficult because the nonphotolyzed **5** in the control caused a very long-lasting tachyphylaxis, which was not reversible within the tissue life time. In this case, peptide **1** proved to be very helpful: as a nonligand the control tissues did not develop any tachyphylaxis and the inactivation observed was only due to the photolabeling process. As already mentioned, the experiments have been repeated with flash photolysis irradiation on rabbit aorta strips (Figure 1). With peptide **5**, the results were similar to the results obtained with normal mercury vapor lamps. However, peptide **1** did not

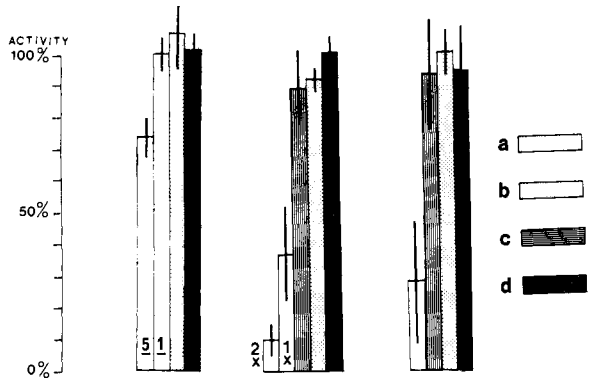

Figure 1. Photoinactivation with flash photolysis and other tissues: (a) irradiation in the presence of photolabel; (b) competitive protection with [Leu^8]AT (5 μM); (c) control irradiation in the presence of **0** (0.5 μM); (d) residual response to noradrenalin, 0.3 μM (left and middle), or to serotonin, 2.6 μM (right). Left: rabbit aorta flashed with **5** (0.05 μM) and **1** (0.5 μM). Middle: rat aorta treated once (1×) and twice (2×) with **5** (0.1 μM) and mercury vapor lamp irradiation. Right: rat stomach treated with **1** (1 μM) and mercury vapor lamp irradiation. Peptide **5** gave high tachyphylaxis in the reference (see text).

show any photoinactivation.

In order to understand these findings, the photochemistry of (4′-nitro)Phe and (4′-azido)Phe has been reinvestigated, both with classical mercury vapor lamps and with monochromatic flash photolysis. Both amino acids undergo photolysis with the continuous mercury source of 365 nm at about the same velocity.[21] If the experiments are repeated with flash photolysis at the same wavelength, (4′-azido)Phe readily undergoes decomposition, whereas (4′-nitro)Phe is completely stable.

A possible explanation of this photochemical finding, together with an additional theory, might help to clarify the photolabeling results of **1** and **2**. We have recently postulated[9] that the affinity of AT analogues depends on the electronegativity of the aromatic nucleus in position 4 of AT. It is shown that the logarithm of the relative affinity of an AT analogue depends inversely on the σ_p (Hammett factor) of the substitution of the aromatic nucleus of position 4 in AT. The photolysis experiments of (4′-nitro)Phe strongly suggest that the decomposition of this amino acid involves a multiphoton process: upon capture of the first photon, (4′-nitro)Phe goes to a first excited state (probably singlet), which relaxes immediately into a long-lived (triplet) state. If it then captures a further photon, it will undergo dissociative decay, giving reactive intermediates, probably radicals;[21] if not, it will relax into the initial ground state. In the long-lived triplet state, σ_p could be lowered to a less electronegative value, the peptide would thus become a ligand and bind to the receptor as long as it is in this excited state. There it might capture the second photon and act as a photoaffinity label. In the same way, the good ligand but poor photoaffinity label **2** can be explained. This compound is a good ligand in the ground state and would have about the same affinity in the excited state, because only stereochemical influences seem to be important in this position 9. In continuous irradiation the population of excited molecules is rather low. There will be competition for the receptor between a large population of nonexcited and a small population of excited ligands. Therefore, the latter will be replaced competitively before they can capture the required second photon. In the case of peptide **1** there is no such competition, because the ground-state molecule is a nonligand and labeling is therefore possible. All good photolabels gave a permanent block of the AT response; no permanent stimulation was observed. If the labeling process is at least as specific and localized as it has been shown on chymotrypsin,[19] there should be a very small difference between the noncovalent and the covalent receptor–hormone complexes. This assumption is reasonable because of the much higher affinity of AT compared with chymotrypsin ligands. For the same reason, labels in position 4 of AT should not directly interfere with the receptor area specific for residue 8 and vice versa. Phe^8 is dominant for response release[22,23] and should be still functional after photolabeling with **1**. On the basis of a receptor mechanism similar to the occupation model, this should result in permanent contraction. This is not observed. We therefore favor the rate model[15] for the vascular receptor of AT.

Conclusion. The receptor of AT in smooth-muscle strips has been irreversibly and specifically blocked by the action of several photosensitive AT analogues in the presence of UV light. The best labeling peptides were [Sar^1]AT analogues with either (4′-azido)Phe in position 8 or (4′-nitro)Phe in position 4. Although the photochemistry of the latter is more complicated, this proved advantageous with receptors sensitive to tachyphylaxis. The photolysis of (4′-azido)Phe is a single-photon process, whereas the (4′-nitro)Phe probably involves a multiphoton mechanism. Furthermore, the labeling results suggest a receptor mechanism similar to the postulated rate model.[15]

Experiments on other tissues and receptor isolation with radioactive analogues are currently under investigation.

Experimental Section

Bioassays. Biological activities of the tissues in response to AT, its analogues, and other agonists were recorded with force-displacement transducers (Grass FT 03) on a Grass polygraph mod. 7 (Grass Co. Quincy, Mass.). The experiments were performed in the absence of ultraviolet radiation,[24] except when indicated. Albino rabbits (New Zealand) of either sex, weighing 1.5–2.0 kg, were killed by stunning and exsanguination. Wistar rats of either sex, weighing 300 g, were killed by stunning and decapitation. The respective organs were excised and prepared in the same way. Tissue strips were prepared according to the literature[19] and suspended in 5-mL organ 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 are always final concentrations in the tissue bath.

Irradiation Equipment. Four mercury vapor lamps (Westinghouse JC Par-38, 100 W), capped with Raymaster 5 in. black light filters (G.W. Gates, Long Island, N.Y.), were focussed onto a tissue bath. The light path was interrupted by a water-jacketed tubular shutter around the tissue bath; for tissue irradiation the shutter was lifted. Further filteration through the Pyrex glass and bath fluid ensured that only light of wavelengths in a narrow band around 365 nm reached the tissues. For the flash photolysis experiment, a thermostated tissue bath with built-in tubular black light filter (type 5040, G.W. Gates Co.) was surrounded with a helical flash lamp (type L-3071, length 74 cm, inner quartz tube diameter 6 mm, mercury doped xenon filling, pulse width $\simeq$100 μs, maximal lamp discharge 1000 J). A corresponding power supply of 15-kV charging capacity for single-flash operation was purchased from the same company (ILC Technology, San Carlos, Calif.). For standard operation, the lamp was fired with 12 kV.

Inactivation Experiments. In a typical experiment, the tissues were washed every 10–15 min with fresh Krebs solution. After 60 min, a first dose of 200 ng/mL (0.2 μM) of AT was given, followed by a second dose at 120 min. After relaxation, the agonists noradrenalin (3 μM), serotonin (26 μM), bradykinin (20 μM), histamine (70 μM), and potassium chloride (100 mM) were added sequentially in at least two inactivation experiments per analogue. In the other experiments, noradrenaline (aorta) or

serotonin (stomach) was always tested. At 180 min, the tissues were incubated with the photolysis solution containing the peptide to be tested (for concentrations used, see Table I) and (4'-amino)Phe (0.6 mM). After 5 min at maximal contraction, the tissues were exposed for 10 min to the light source (or flashed in the flash experiment) and washed after exposure. At 280 min, the residual activity of the tissue was tested with AT and the other agonists and/or the treatment was repeated. The residual activity of the doubly treated tissues was controlled at 390 min. After a first residual activity control, the tissue was tested for any change in residual activity with further doses of AT every 45 min until the reference tissues were almost exhausted. This happened normally between 9 and 12 h of incubation. For the competitivity experiments, the tissues were incubated with 5 μg/mL [Leu^8]AT (5 μM) for 15 min before the addition of the photolabel and washed only after photolysis. The photoinactivations (one and two treatments) are given as a percentage of the recovered activity compared to the nonirradiated, identically incubated references. These references had at least 92 (first treatment) or 87% (second treatment) of the initial activity and/or of the untreated control reference. If these reference activities were lower (fatigue or tachyphylaxis), the results were not counted. The reference activities for the protection experiments with [Leu^8]AT were 88% ± 7 due to the large inhibitor concentrations. The references of **5** on rat stomach developed high tachyphylaxis, which did not recover within 3 h. On the other hand, the aorta tests normally proceeded without this problem.

Kinetics of Photolysis. A small test tube containing 1 μg (1 nmol) of peptides **1**–**6** in 2 mL of Krebs solution at 37 °C was inserted into the tissue bath. The samples were irradiated for 0.5, 1, 2, 5, 10, or 20 min, and the contents were lyophilized, resuspended in 10 μL of 50% acetic acid, and spotted onto silica gel plates (Merck, precoated silica gel plates G60-F254). The TLC plates were eluted in the following system: 1-butanol/acetic acid/water (10:2:3). The spots were visualized with UV light, Pauly reagent, or a modified Reindel–Hoppe procedure.[25] After 2 min of irradiation, traces of the initial peptides were still visible at R_f 0.40–0.48 but none after 10 min. In the flash photolysis experiments, peptide quantities of 0.2 μg (0.2 nmol) of **1** and **5** in 1 mL of Krebs solution were flashed with 12 kV as described above. TLC showed for **1** an R_f value of 0.40 (educt) only; for **5** multiple R_f values of 0.0 to 1.0 were found (R_f educt 0.45).

Acknowledgment. We thank Dr. D. Regoli and Dr. F. Rioux for helpful discussions, Mrs. T. M. D. Nguyen for her contribution in the early stages of this work, and Mrs. Diane Tousignant for typing this report. This work has been supported by grants from the Medical Research Council of Canada, from the Conseil de la Recherche en Santé du Québec, and from the Canadian Heart Foundation.

References and Notes

(1) Part of the M.Sc. Thesis of G. Guillemette, April 1979.
(2) S. Jacobs, Y. Schechter, K. Bissel, and P. Cuatrecasas, *Biochem. Biophys. Res. Commun.*, **77**, 981 (1977).
(3) A. Welton, P. Lad, A. Newby, H. Yamamura, S. Nicosia, and M. Rodbell, *J. Biol. Chem.*, **252**, 5947 (1977).
(4) C. McIntosh, J. Warnecke, M. Nieger, A. Barner, and J. Köberling, *FEBS Lett.*, **66**, 149 (1976).
(5) Personal communcations from W. Schlegel, J. C. Bonafous, and R. Schwyzer, unpublished results.
(6) M. A. Devynck, M. G. Pernollet, P. Meyer, S. Fermandjian, P. Fromageot, and M. F. Bumpus, *Nature (London)*, **249**, 67 (1974).
(7) T. B. Paiva, A. C. M. Paiva, R. I. Freer, and J. M. Stewart, *J. Med. Chem.*, **15**, 6 (1972).
(8) E. Escher, T. M. D. Nguyen, H. Robert, S. St-Pierre, and D. Regoli, *J. Med. Chem.*, **21**, 860 (1978).
(9) E. Escher, T. M. D. Nguyen, and D. Regoli, *Can. J. Physiol. Pharmacol.*, **56**, 956 (1978).
(10) E. Escher, T. M. D. Nguyen, G. Guillemette, and D. Regoli, *Nature (London)*, **275**, 145 (1978).
(11) E. Escher and R. Schwyzer, *FEBS Lett.*, **46**, 347 (1974).
(12) N. Sonenberg, M. Wilchek, and A. Zamir, *Biochem. Biophys. Res. Commun.*, **72**, 1534 (1976).
(13) S. de Jonge, *Recl. Trav. Chim. Pays-Bas*, **72**, 846 (1952).
(14) R. E. Galardy, L. C. Craig, J. D. Jamieson, and M. P. Printz, *J. Biol. Chem.*, **249**, 3510 (1974).
(15) W. D. M. Paton, *Proc. R. Soc. London, Ser. B*, **154**, 21 (1961).
(16) A. E. Ruoho, H. Kiefer, P. E. Roeder, and S. J. Singer, *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 2567 (1973).
(17) W. Fischli, Thesis, ETH 1 Zurich, Switzerland (in press).
(18) D. Regoli, W. K. Park, F. Rioux, and C. S. Chan, *Rev. Can. Biol.*, **30**, 319 (1971).
(19) R. F. Furchgott and S. Bhadrakom, *J. Pharmacol. Exp. Ther.*, **108**, 129 (1953).
(20) R. F. Furchgott, S. J. Ehrreich, and J. Greenblatt, *J. Gen. Physiol.*, 44, 501 (1961).
(21) E. Escher, *Helv. Chim. Acta*, **60**, 339 (1977).
(22) D. Regoli, W. K. Park, and F. Rioux, *Pharmacol. Rev.*, **26**, 69 (1974).
(23) F. M. Bumpus, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **36**, 2128 (1972).
(24) E. Escher and R. Schwyzer, *Helv. Chim. Acta*, **58**, 1465 (1975).
(25) E. Von Arx, M. Faulpel, and M. Brugger, *J. Chromatogr.*, **120**, 224 (1976).