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Angiotensin II Analogues. 13. Role of the Hydroxyl Group of Position 4 Tyrosine in Pressor Activity[1]

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In order to determine the features of the phenolic ring in position 4 of [Asn[1],Ile[5]]angiotensin II that contribute to pressor activity, analogues with selected aromatic substituents were synthesized by the solid-phase method. They showed pressor activities in the rat: [Asn[1],Phe(4-NH_2)[4]]AII, 24%; [Asn[1],Phe(4-NO_2)[4]]AII, 0.1%; [Asn[1],Tyr(3,5-Me_2)[4]]AII, 2.2%; [Asn[1],D-Tyr(3,5-Me_2)[4]]AII, 1.4%. These results indicate that the activity contributed by the aromatic character of the phenyl ring in the side chain of position 4 is enhanced by a group in the para position that may function as a proton donor in hydrogen-bond formation. Bulky substituents ortho to this hydrogen-bonding group decrease activity by steric interference with hydrogen-bond formation. Bulky groups than cannot act as hydrogen donors in the para position of the aromatic ring drastically decrease the activating effect of the aromatic ring on pressor activity.

The tyrosine side chain of a peptide hormone is capable of participating in many types of interactions with structural components of its receptor. The phenolic hydroxyl group can form hydrogen bonds by proton donation or acceptance, or the aromatic ring can interact with other aromatic, hydrophobic, or polarizable systems. In angiotension II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) physical measurements by direct titration,[2] UV,[3] ^{1}H NMR,[4] and ^{13}C NMR[5] all indicate a normal pK_a of about 10 for the tyrosine phenolic group. These measurements further indicate the absence of any intramolecular interaction of the tyrosine hydroxyl group in the conformation of angiotensin II in solution. However, replacement of the hydroxyl group of tyrosine by a fluorine atom results in the loss of pressor activity and the acquisition of antagonistic activity.[6] This finding suggests a specific role for the tyrosine hydroxyl group at the hormone receptor, acting either directly or through its electronic effects on the aromatic ring.

In this study, we have prepared analogues of [Asn[1],-Ile[5]]angiotensin II with unnatural aromatic amino acids replacing the tyrosine in position 4. The *p*-aminophenylalanine and *p*-nitrophenylalanine analogues were prepared because of their hydrogen-bond donor and acceptor properties and to include analogues with electron-donating and electron-withdrawing substituent effects on the aromatic ring. The 3,5-dimethyltyrosine analogue was prepared because it retains the structural features of tyrosine, modified by bulky electron-donating groups flanking the phenolic hydroxyl function.

The peptides were synthesized using the solid-phase method,[7] with the Boc-protected amino acids incorporated in a stepwise manner by dicyclohexylcarbodiimide. Boc-DL-3,5-dimethyltyrosine was incorporated into the peptide without protection of the phenolic group. The resulting diastereomeric peptides [Asn[1],Tyr(3,5-Me_2)[4]]AII and [Asn[1],D-Tyr(3,5-Me_2)[4]]AII were separated by a combination of column chromatography and countercurrent distribution. The individual diastereomers were characterized by L-amino acid oxidase digestion of the peptide hydrolysates. The aminophenylalanine analogue [Asn[1],Phe(4-NH_2)[4]]AII was prepared by hydrogenation of [Asn[1],Phe(4-NO_2)[4]]AII.

Biological Results. The compounds were tested for pressor activity in nephrectomized, pentolinium-treated male rats that were anesthetized with pentobarbital.[8,9] The peptides were dissolved in normal saline containing 0.1% polyvinylpyrrolidone in siliconized glass containers. The molarities of the peptide solutions, including the angiotensin standard, were based on peptide content found from amino acid analyses of the peptide hydrolysates. Pressor activities calculated on a molar basis for the compounds from the present study are listed in Table I.

Discussion

The replacement of the tyrosine group in position 4 of angiotensin II by aliphatic residues results in loss of pressor activitiy as shown by the alanine[10,11] (0.3%), 1-aminocyclopentanecarboxylic acid[12] (0.1%), and glutamic acid[13](0.1%) analogues. The phenyl ring in the absence of the phenolic hydroxyl group still contributes significantly to pressor activity, as shown by the phenylalanine analogue,[14] with 10% the activity of angiotensin II.

The amino analogue [Asn[1],Phe(4-NH_2)[4]]AII prepared in this study is 24% as active as angiotensin II in the rat pressor assay (Table I). A recent report by Escher[15] of [Sar[1],Phe(4-NH_2)[4],Val[5]]AII showed this analogue to possess 15% of the activity of [Sar[1],Val[5]]AII in vitro

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(rabbit aorta). Thus, an aromatic amine is less effective than a phenol, but more effective than an unsubstituted phenyl ring in eliciting biological activity. This is consistent with the fact that both aromatic hydroxyl and amine groups could act as proton donors in hydrogen-bond formation with a structural element of the hormone receptor and that the hydroxyl group would form a stronger hydrogen bond than the amino group.[16]

Masking of the phenolic hydroxyl group as the *O*-methyl ether[13,17] reduces the pressor activity significantly (0.2–1% that of angiotensin II) relative to either the tyrosine or phenylalanine substitution. This loss in activity relative to tyrosine would be expected if the para substituent must act as a proton donor in enhancing the binding of the aromatic ring to the receptor. The bulk of the *O*-methyl group could also interfere with receptor association compared with the stronger effect produced by the unsubstituted phenyl ring.

The nitro analogue [Asn^1,Phe(4-NO_2)^4]AII prepared in this study is essentially inactive (0.1%) in the rat pressor assay (Table I). These data are complemented by the report of Escher[15] that [Sar^1,Phe(4-NO_2)^4,Val^5]AII is inactive in vitro (rabbit aorta) and does not act as an antagonist. This is consistent with a requirement for a small proton-donating group in the para position, since the nitro group, like the inactive methoxy group, can function as a proton acceptor but not a proton donor. Both nitro and methoxy groups would place bulk in a sterically sensitive location for receptor binding.

Escher[15] has reported that the 4-position phenylalanine analogues of [Sar^1,Val^5]AII with azido ($-N_3$) and diazonium ($-N_2^+$) groups in the para position, which were prepared as potential photoaffinity probes of receptors, showed low activities (0.26 and 0.66%, respectively) in vitro (rabbit aorta). Both analogues possess bulky groups that could not act as proton donors.

The 3,5-dimethyltyrosine analogue [Asn^1,Tyr(3,5-Me)^4]AII prepared in this study showed a low pressor activity, 2.2% that of angiotensin II (Table I). The diastereomeric peptide containing the D-3,5-dimethyltyrosine residue [Asn^1,D-Tyr(3,5-Me_2)^4]AII was less active (1.4%), which is consistent with the very low pressor activity reported[18] for [Asn^1,D-Tyr^4,Val^5]AII. The low activity of [Asn^1,Tyr(3,5-Me_2)^4]AII could be attributed to steric interference by the bulky *o*-methyl groups with hydrogen-bond formation between the phenolic hydroxyl group and receptor.

Iodine substitution ortho to the phenolic hydroxyl group led to reduced but still significant pressor activity.[19] The monoiodotyrosine analogue [Asn^1,Tyr(3-I)^4,Val^5]AII showed 33% the rat pressor activity of angiotensin II, while the 3,5-diiodotyrosine analogue [Asn^1,Tyr(3,5-I_2)^4,Val^5]AII was 13% as active as angiotensin II in the rat pressor assay. Although bulky iodine atoms, like methyl goups, inhibit hydrogen bonding by a steric effect, ortho halogen atoms enhance polarization and ionization of the phenolic hydroxyl group, which has a counteracting beneficial effect on intermolecular hydrogen-bond formation.[20] The less sterically hindered monoiodo analogue is more active than the diiodo analogue, which is consistent with the concept that the phenolic hydroxyl group acts as a proton donor in hydrogen-bond association with a sterically sensitive region of the receptor. The related 3,5-diiodo-4-aminophenylalanine analogue [Sar^1,Phe(3,5-I_2-4-NH_2)^4,Val^5]AII was reported[15] to show 2.7% the activity of [Sar^1,Val^5]AII in the rabbit aorta. This reduction in activity by diiodination of the *p*-aminophenylalanine analogue could also be caused by bulky iodine atoms hindering hydrogen-bond association with the receptor.

Conclusions. The tyrosine residue in position 4 of angiotensin II contributes to the hormonal response through two structural features, the aromatic ring and the phenolic hydroxyl group. The receptor association occurs as a structurally and sterically specific association of the aromatic ring with a hydrophobic region of the receptor, complemented by proton donation by the phenolic hydroxyl group to a proton acceptor group of the receptor. A closely coupled steric relationship exists between the aromatic ring-binding and hydrogen-bonding structures of the receptor. A bulky group that is in the para position of the aromatic ring and that is functionally incapable of hydrogen donation interferes with receptor binding and activation. Bulky substituents ortho to the phenolic hydroxyl group reduce hydrogen-bond formation, although iodine atoms reduce activity less than do methyl groups because of their beneficial electronic effects on the phenolic group.

Experimental Section

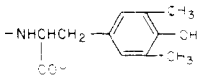
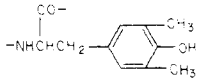
Melting points (Thomas-Hoover Uni-melt) are uncorrected. Microanalyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley. Where analysis is indicated only by symbols of the elements, analytical results obtained for these elements were within ±0.4% of the theoretical values. Precoated silica gel 60 F254 on glass plates (E. Merck) were used for TLC with the following solvent systems: (I) *sec*-butyl alcohol–3% NH_4OH (100:44), (II) butanol–acetic acid–H_2O (4:1:5, upper phase). Electrophoresis was carried out on Whatman no. 1 paper (0.16-mm thick) at 5000 V using AcOH–HCOOH buffer, pH 1.85, in a Savant apparatus. E_H indicates electrophoretic mobility relative to histine = 1.00. Peptides were hydrolyzed for 48 h under N_2 in constant-boiling HCl containing D-alanine as an internal standard. Hydrolyses were carried out in the presence of phenol to protect tyrosine from degradation. Amino acid analyses (Spinco Model 116 analyzer) were obtained using the standard 4-h methodology. Peptide content was calculated in terms of free peptide.

Asn-Arg-Val-Phe(4-NO_2)-Ile-His-Pro-Phe (1) The octapeptide-polymer, Z-Asn-Arg(Tos)-Val-Tyr(Bzl)-Ile-His(Tos)-Pro-Phe-polymer, was synthesized by the stepwise solid-phase method[7] as described previously.[1] Treatment of 3.2 mmol of octapeptide-polymer with liquid HF in the presence of anisole gave 547 mg of deprotected peptide 1. The peptide was purified by countercurrent distribution for 600 transfers between the upper and lower phases of the mixture of butyl alcohol–*tert*-butyl alcohol–H_2O (5:2:5). The fractions of $K = 0.15$ were combined (145 mg) and purified further through the picrate salt[21] to give 126 mg of peptide: TLC R_f (I) 0.18, R_f (II) 0.15, E_H 0.65. An acid hydrolysate had the following amino acid ratios: Asp, 1.02; Arg, 1.01; Val, 1.00; Phe(4-NO_2), 1.01; Ile, 0.99; His, 0.99; Pro, 1.00; Phe, 1.01; peptide content 89%. A 48-h acid hydrolysate incubated with *C. adamanteus* L-amino acid oxidase[22] for 48 h showed the following amino acid ratios: Asp, 0.77; Arg, 0.03; Val, 0.01; Phe(4-NO_2), 0.06; Ile, 0.02; His, 0.06; Pro, 1.00; Phe, 0.03.

Asn-Arg-Val-Phe(4-NH_2)-Ile-His-Pro-Phe (2). Compound 1 (69 mg) was hydrogenated with 37 mg of Pd/C under 1 atm of H_2 for 12 h to give 56 mg of 2. The peptide was purified by countercurrent distribution for 700 transfers between the upper and lower phases of the mixture of butanol–acetic acid–H_2O (4:1:5). The fractions of $K = 0.034$ were combined (29 mg) and purified further through the picrate salt[21] to give 16 mg: TLC R_f (I) 0.16, R_f (II) 0.04, E_H 0.78. An acid hydrolysate had the following amino acid ratios: Asp, 0.96; Arg, 1.05; Val, 0.98; Phe(4-NH_2), 1.05; Ile, 0.96; His, 1.05; Pro, 0.96; Phe, 1.00; peptide content 79%. A 48-h acid hydrolysate incubated with L-amino acid oxidase[22] for 60 h showed the following amino acid ratios: Asp, 0.03; Arg, 0.04; Val, 0.01; Phe(4-NH_2), 0.07; Ile, 0.02; His, 0.12; Pro, 1.00; Phe, 0.03.

Z-Asn-Arg(NO_2)-Val-DL-Tyr(3,5-Me_2)-Ile-His(Bzl)-Pro-Phe-polymer (3). Ile-His(Bzl)-Pro-Phe-polymer was prepared by the stepwise solid-phase method.[1,7] Boc-DL-Tyr(3,5-Me_2) was synthesized by reaction of DL-3,5-dimethyltyrosine[23] with *tert*-

Table I. Relative Pressor Activities of [Asn^1,Ile^5]Angiotensin II Analogues in the Rat

angiotensin II analogues	struct of position 4 substit	pressor act.
[Asn^1,Val^5]AII (angiotensin II)	-NHCHCH$_2$-C$_6$H$_4$-OH CO-	100
[Asn^1,Phe(4-NH$_2$)^4,Ile^5]AII	-NHCHCH$_2$-C$_6$H$_4$-NH$_2$ CO-	24
[Asn^1,Phe(4-NO$_2$)^4,Ile^5]AII	-NHCHCH$_2$-C$_6$H$_4$-NO$_2$ CO-	0.1
[Asn^1,Tyr(3,5-Me$_2$)^4,Ile^5]AII		2.2
[Asn^1,D-Tyr(3,5-Me$_2$)^4,Ile^5]AII		1.4

butyloxycarbonyl azide according to the Schnabel procedure,[24] mp 179–180 °C. Anal. ($C_{16}H_{23}NO_5$) C, H, N. This racemic amino acid (371 mg, 1.2 mmol) was incorporated into the tetrapeptide-polymer (0.3 mmol) by dicyclohexylcarbodiimide (247 mg, 1.2 mmol). Boc-Val, Boc-Arg(NO_2), and Z-Asn-ONp were condensed with the pentapeptide-polymer in a stepwise manner to give **3**.

Asn-Arg-Val-Tyr(3,5-Me$_2$)-Ile-His-Pro-Phe (4) and Asn-Arg-Val-D-Tyr(3,5-Me$_2$)-Ile-His-Pro-Phe (5). Treatment of **3** with HBr/TFA cleaved the partially protected and diastereomeric peptide (310 mg) from the polymer. A portion of the peptide (250 mg) was chromatographed on a 3 × 45 cm column of Sephadex LH 20 and eluted with 40% isopropyl alcohol in water. The eluate was analyzed by TLC and separated into two fractions. Fraction I (183–186 mL) was combined to give 30 mg: TLC R_f (I) 0.29, R_f (II) 0.26. An acid hydrolysate had the following amino acid ratios: Asp, 1.16; Arg, 0.80; Val, 1.04; Tyr(3,5-Me$_2$), 1.00; Ile, 0.98; His(Bzl), 0.84; Pro, 1.00; Phe, 1.02; peptide content 60%. A 48-h acid hydrolysate incubated with L-amino acid oxidase[22] for 48 h showed the following amino acid ratios: Asp, 0.90; Arg, 0.04; Val, 0; Tyr(3,5-Me$_2$), 0.66; Ile, 0.01; His(Bzl), 0.15; Pro, 1.00; Phe, 0.07. Tyr(3,5-Me) was eluted from the long column 28 min after Phe using the standard 4-h methodology and had a color value 81% that of Phe. His(Bzl) emerged from the short column at 57 min with a color value 90% of Arg (49 min), when eluted with pH 5.26 (0.35 N) sodium citrate buffer for 20 min followed by pH 7.00 (0.38 N) sodium citrate buffer for 60 min.

The remaining eluate from the Sephadex LH 20 column was pooled to give 129 mg of peptide which was rechromatographed on the same column in the same manner. Fraction II (141–144 mL) was isolated to give 8 mg: TLC R_f (I), 0.22, R_f (II) 0.21. An acid hydrolysate had the following amino acid ratios: Asp, 1.24; Arg, 0.88; Val, 1.09; Tyr(3,5-Me$_2$), 1.24; Ile, 0.94; Pro, 1.00; Phe, 0.98; peptide content 73%. A 48-h acid hydrolysate incubated with L-amino acid oxidase[22] for 48 h showed the following amino acid ratios: Asp, 1.00; Arg, 0.04; Val, 0.02; Tyr(3,5-Me$_2$), 0.23; Ile, 0.01; His(Bzl), 0.08; Pro, 1.00; Phe, 0.04.

The remaining eluate from the second Sephadex LH 20 column was combined (93 mg) and was purified by countercurrent distribution for 200 transfers between the upper and lower phases of the solution of butyl alcohol–tert-butyl alcohol–H_2O (5:2:5). Fractions of K = 9.5 were combined to give 11 mg. When analyzed by TLC and electrophoresis, this material was identical with fraction I (30 mg) from the Sephadex LH 20 chromatography. Fractions of K = 2.3 were combined to give 34 mg, which was identical with material (8 mg) isolated as fraction II on Sephadex LH 20 chromatography. After appropriate fractions were combined, a portion (40 mg) of the peptide having K = 9.5 was hydrogenated under 2 atm of H_2 for 48 h in the presence of 45 mg of 10% Pd/C catalyst to give 32 mg of **5**: TLC R_f (I) 0.29, R_f (II) 0.26. An acid hydrolysate had the following amino acid ratios: Asp, 0.97; Arg, 1.02; Val, 0.99; Tyr(3,5-Me$_2$), 1.05; Ile, 0.99; His, 0.99; Pro, 1.01; Phe, 0.99; peptide content 56%. A 48-h acid hydrolysate incubated with L-amino acid oxidase[22] for 48 h showed the following amino acid ratios: Asp, 0.49; Arg, 0.04; Val, 0.02; Tyr(3,5-Me$_2$), 0.91; Ile, 0.02; His, 0.10; Pro, 1.00; Phe, 0.05. A portion (40 mg) of the combined peptide fractions having K = 2.3 was hydrogenated to give 30 mg of **4**: TLC R_f (I) 0.22, R_f (II) 0.21. An acid hydrolysate had the following amino acid ratios: Asp, 1.02; Arg, 1.01; Val, 1.00; Tyr(3,5-Me$_2$), 1.06; Ile, 0.98; His, 1.03; Pro, 0.99; Phe, 0.99; peptide content 56%. A 48-h acid hydrolysate incubated with L-amino acid oxidase[22] for 48 h showed the following amino acid ratios: Asp, 0.54; Arg, 0.05; Val, 0.02; Tyr(3,5-Me$_2$), 0.12; Ile, 0.01; His, 0.08; Pro, 1.00; Phe, 0.04.

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References and Notes

(1) (a) The abbreviations used to denote amino acids and peptides are those recommended by the IUPAC–IUB Commission on Biochemical Nomenclature [*J. Biol. Chem.*, **247**, 977 (1972), and *Biochemistry*, **6**, 362 (1967)]. Additional abbreviations are: Phe(4-F), *p*-fluorophenylalanine; Phe(4-NH_2), *p*-aminophenylalanine; Phe(4-NO_2), *p*-nitrophenylalanine; Tyr(Me), tyrosine methyl ether; Tyr(3,5-Me_2), 3,5-dimethyltyrosine; Tyr(3-I), 3-iodotyrosine; Tyr(3,5-I_2), 3,5-diiodotyrosine; AII, angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe). (b) For paper 12 in this series, see K. H. Hsieh, E. C. Jorgensen, and T. C. Lee, *J. Med. Chem.*, **22**, preceding paper in this issue (1979).
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Photoaffinity Labeling of the Angiotensin II Receptor. 3. Receptor Inactivation with Photolabile Hormone Analogues

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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

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