Specific Nonpeptide Photoprobes as Tools for the Structural Study of the Angiotensin II AT_1 Receptor

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The aim of this work was to obtain photoactivatable nonpeptide antagonists of the angiotensin II AT_1 receptor. Based on structure−function relationships, two chemical structures as well as appropriate synthetic schemes were chosen as a frame for the design of radiolabeled azido probes. The feasibility of the strategy was first assessed by the synthesis of two tritiated ligands **21** and **22** possessing a high affinity for the AT_1 receptor and a low nonspecific binding to membrane or cell preparations. We then prepared two unlabeled azido derivatives **7** and **14** which retained a fairly high affinity for the AT_1 receptor. The latter compound proved to be suitable for receptor irreversible labeling and was prepared in its tritiated form **28**. This tritiated azido nonpeptide probe displayed a K_d value of 11.8 nM and a low nonspecific binding. It was suitable for specific and efficient covalent labeling of the recombinant AT_{1A} receptor stably expressed in CHO cells. The electrophoretic pattern of the specifically labeled entity was strictly identical to that of purified receptor photolabeled with a biotinylated peptidic photoactivatable probe. This new tool should be useful for the mapping of the nonpeptide receptor binding site. These potential applications are discussed in light of the current knowledge of molecular mechanisms of G-protein coupled receptor activation and inactivation.

Introduction

Besides their potential therapeutic applications, nonpeptide derivatives[1] constitute interesting tools for the structural analysis of G-protein coupled receptors. Ligand recognition and activation processes are closely interrelated. The existence of receptor active and inactive conformations has allowed one to provide mechanistic explanations for receptor constitutive activities, as well as inverse agonism properties found for many synthetic ligands.[2,3] As a consequence, the study of the mechanisms underlying blockade of receptor activation by nonpeptide antagonists, which in fact often behave as inverse agonists (when the appropriate experimental conditions are fulfilled), requires experiments which provide unambiguous data about inactive receptor conformations. The obvious limitations of mutagenesis studies, which do not allow one to discriminate between direct and indirect effects, are increased when the evaluation of mutant receptor properties is carried out through heterologous binding assays involving a tracer ligand and an unlabeled ligand which display optimal affinities for different receptor conformations.[4,5] This difficulty is overcome when radioactive nonpeptide ligands are available. A more difficult but straightforward manner of investigating receptor topography consists of its covalent labeling with appropriate synthetic ligands,[6−13] as previously carried out with peptidic angiotensin II-derived probes.[14−17] The goal of the present study was to design the first AT_1 specific photoactivatable nonpeptide derivative. The strategy used for the development of these compounds was based on previous structure−function relationships and the prevision of appropriate synthetic schemes.[18−22] We investigated a new series of molecules potentially suitable for the introduction of an azido function selected as the photoactivatable group and easy to be obtained in a tritiated form. This series was designed according to the nonpeptide AT_1 receptor antagonist SKF 108566[18,21,22] (Figure 1). SAR observed in this series is featured as follows:[22,23] (i) the N-1 phenylmethyl substituent bears an acidic functionality, (ii) the substituent in position 2 is an alkyl straight chain, (iii) the substituent in position 5 encompasses an aromatic moiety and an acidic function. Taking into account all these requirements, we focused on the general structure A as shown in Figure 1. This structure can accommodate an azido group in two distinct regions, either on the N-1 phenylmethyl substituent or on the phenyl linked to the C-5 imidazole nucleus substituent. Photoprobes corresponding to this latter possibility should allow one to check the validity of the previous proposal from Smith-Kline Beecham[21] that this portion of the molecule mimic the phenyl of Phe(8) of AII. Indeed efficient photolabeling of the AT_1 receptor was obtained using azido-Phe(8) angiotensin II-derived peptides[15,24] and recently applied to receptor mapping.[17,25]

The experimentation involved the following steps: (i) synthesis of two synthetic tritiated nonpeptide antagonists displaying a high affinity for the AT_1 receptor and a low nonspecific binding to membranes or intact cells

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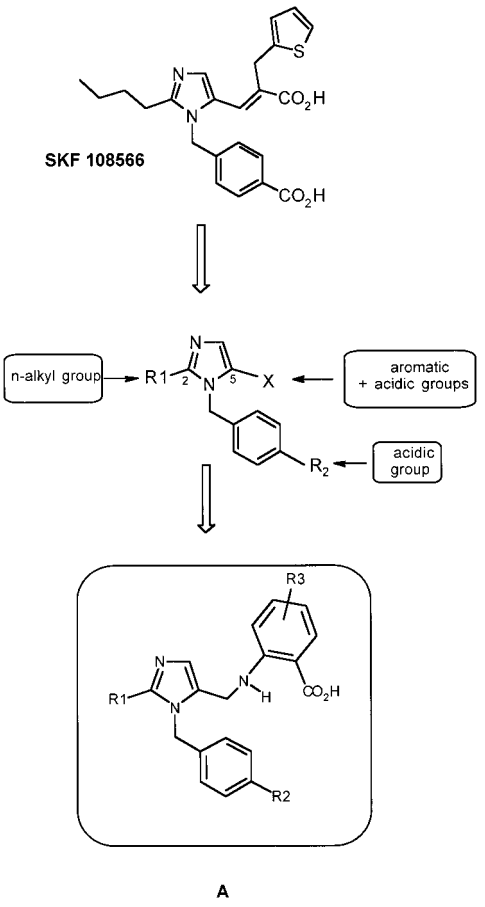


Figure 1. SAR of SKF 108566 family used for the design of general structure A.

expressing this receptor,[19] which is an essential prerequisite to decide the synthesis of photoactivatable derivatives (these radioligands constitute by themselves interesting tools for mapping studies); (ii) obtention of two nonradioactive photoactivatable derivatives through the introduction of an azido group into positions of the molecules potentially interacting with different portions of the receptor; (iii) evaluation of the ability of these compounds to irreversibly block AII binding sites upon irradiation; (iv) synthesis of the radioactive counterpart of the ligand considered as suitable on the basis of these experiments; (v) direct demonstration of the ability of the tritiated azido nonpeptide derivative to covalently label the AII receptor and characterization of the labeled entity.

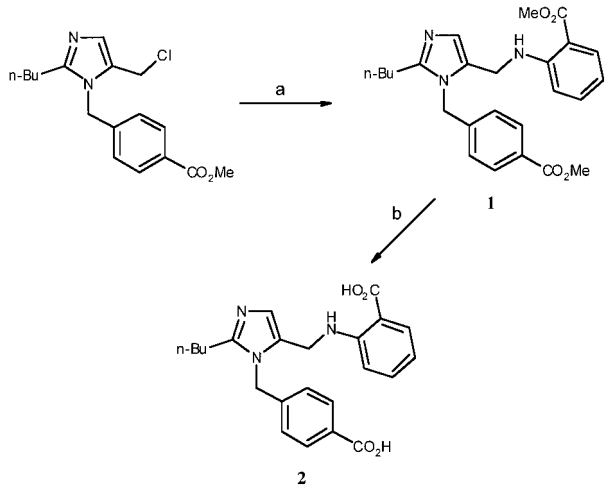
The advantage of this strategy was to avoid undue difficult synthesis of inefficient tritiated azido derivatives that would have involved several steps using labeled compounds.

Chemistry

The 1,2,5-trisubstituted imidazole **2** was prepared according to Scheme 1. The first step consisted of the nucleophilic displacement of the substituted 5-chloromethyl imidazole[21] by methyl 2-aminobenzoate in toluene containing 1 equiv of 2,6-dimethylpyridine to yield the expected ester intermediate **1**. Compound **2** was finally obtained by alcaline hydrolysis.

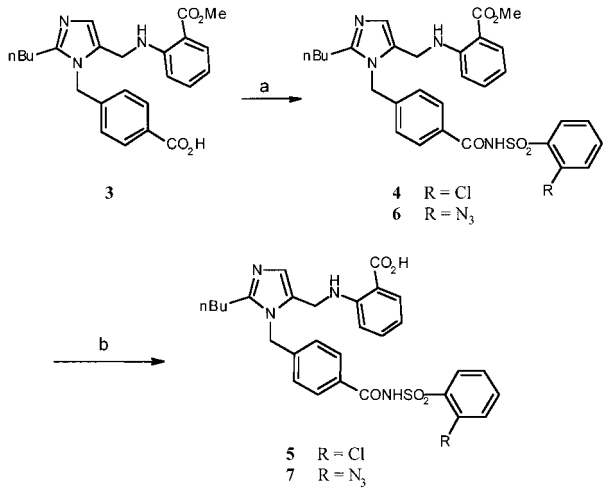
The chlorosulfonimide **5** was obtained according to Scheme 2. The required imidazole **3**[20] was coupled with 2-chloro-benzenesulfonamide to give the ester **4** which

Scheme 1[a]



[a] Reagents: (a) methyl 2-aminobenzoate, 2,6-dimethylpyridine, toluene, reflux; (b) 2 N NaOH, MeOH, reflux.

Scheme 2[a]



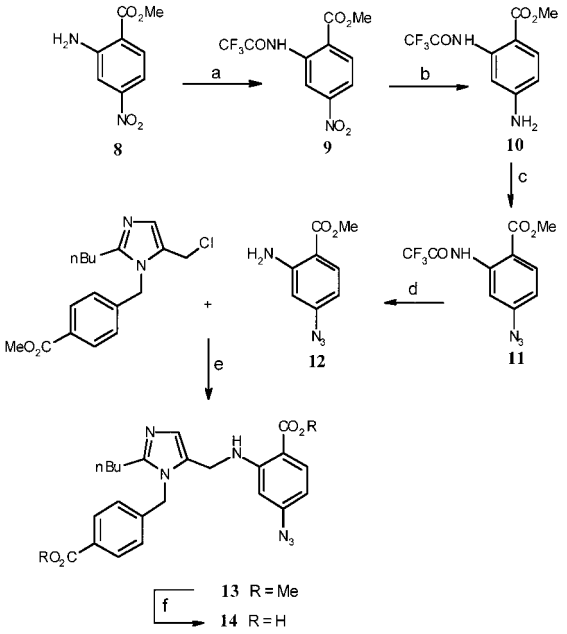
[a] Reagents: (a) 2-chloro-benzenesulfonamide or 2-azidobenzenesulfonamide, EDCI, DMAP, CH_2Cl_2; (b) 2 N NaOH, MeOH, H_2O, reflux.

was then hydrolyzed in alcaline medium to yield compound **5**. The azido derivative **7** was similarly obtained starting from 2-azido-benzenesulfonamide.[26]

The azido compound **14** was prepared in seven steps with an overall yield of ca. 15% (Scheme 3). Commercial 2-amino-4-nitro-benzoic acid was esterified to **8** which was protected as a trifluoroacetamide to afford **9** and catalytically reduced to **10**. Diazotization of **10** allowed its conversion into the corresponding azido derivative **11**, which was then smoothly deprotected with 7% aqueous K_2CO_3 to afford the key intermediate **12**. Nucleophilic displacement of the chlorine atom of methyl 4-[(2-butyl-5-chloromethyl-1*H*-imidazol-1-yl)-methyl]-benzoate[22] by **12** in the presence of 2,6-dimethylpyridine afforded the diester **13** which was converted to the final azido diacid **14** by alcaline hydrolysis.

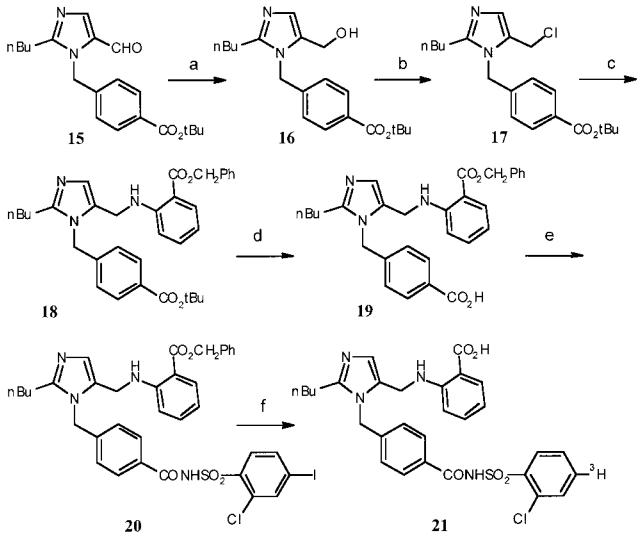
The tritiated compound **21** was obtained according to Scheme 4. Reduction of aldehyde **15** and chlorination of the resulting alcohol **16** gave **17**. Condensation of benzyl anthranilate with **17** led to the orthogonally protected diester **18** from which the desired monoacid monoester **19** was obtained by selective acidic hydrolysis. Acylation of known 2-chloro-4-iodo-benzene sulfona-

Scheme 3[a]



[a] Reagents: (a) $(CF_3CO)_2O$, toluene, reflux; (b) Pd/C 10%, DMF, 1 atm H_2; (c) $NaNO_2$, 2 N HCl, 0 °C, then NaN_3, 0 °C; (d) K_2CO_3, $MeOH/H_2O$; (e) 2,6-dimethylpyridine, toluene, reflux; (f) KOH, $MeOH/H_2O$, reflux.

Scheme 4[a]

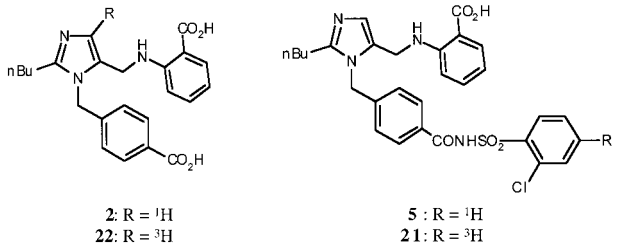


[a] Reagents: (a) $NaBH_4$, MeOH, 0 °C; (b) $SOCl_2$, CH_2Cl_2, 0 °C; (c) benzylanthranilate, 2,6-lutidine, toluene, reflux; (d) TFA, 0 °C; (e) 2-chloro-4-iodobenzenesulfonamide, DMAP, EDCI, CH_2Cl_2; (f) $[^3H]_2$, 1 atm, Pd/C 5%, MeOH.

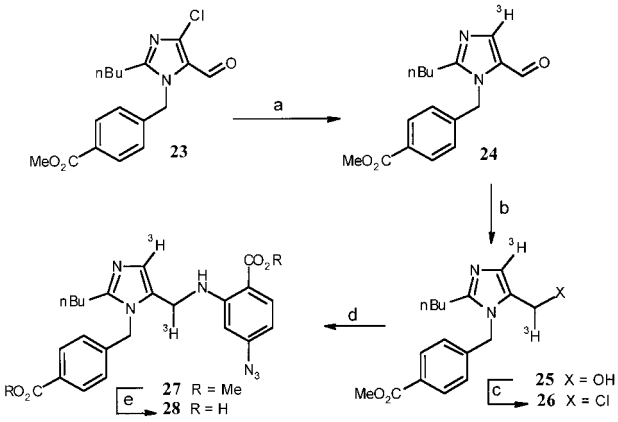
mide[27] with compound **19** in the presence of EDCI gave **20** which allowed us to obtain the desired monotritiated compound **21** in a single step. Compound **22** (Chart 1) was obtained in six steps according to the previously published procedure.[19]

The di-tritiated analogue of **14** (**28**) was prepared as indicated (Scheme 5). The chloroaldehyde **23**[22] was tritiated in position 4 of the imidazole ring by catalytic dehalogenation using Pd/C and tritium gas in 61% yield. The second tritium atom was introduced thanks to the reduction of the aldehyde **24** by $NaB[^3H]_4$ in **25** which was treated by $SOCl_2$ to afford the requisite synthon **26**. Displacement of the chlorine atom with **12** using

Chart 1



Scheme 5[a]



[a] Reagents: (a) $[^3H]_2$, Pd/C 10%, NEt_3, MeOAc; (b) $NaB[^3H]_4$, MeOH; (c) $SOCl_2$, $CHCl_3$; (d) **12**, 2,6-dimethylpyridine, toluene, reflux; (e) KOH, $MeOH/H_2O$, reflux.

the same procedure as described for the preparation of **13** gave the diester **27** in 45% yield, and final basic hydrolysis led to the di-tritiated target **28** in 81% yield.

Biology

Biological Properties of 2 and 5. 1. Antagonism of AII Action on Rabbit Aorta Ring. Compounds **2** and **5** (Chart 1) did not change the basal tension of the arteries but inhibited AII-induced contraction in a concentration-dependent manner, with IC_{50} values of 3.6 ± 0.4 nM and 0.8 ± 0.2 nM, respectively (mean of six experiments).

2. Binding Properties of the Tritiated Compounds 21 and 22 (Chart 1). Some binding properties of **22** have been previously described.[19] They emphasized a very low extent of nonspecific binding to rat liver membranes and membranes from CHO cells expressing the AT_1 receptor, as compared to the commercially available tritiated Losartan.[28] We found quite similar properties for **21** which displayed an improved affinity for the AT_1 receptor. The binding of **21** to rat liver membranes, which exclusively contain the AT_1 receptor subtype,[29,30] revealed a single class of high affinity binding sites characterized by K_d values of 2.55 ± 0.35 nM and 2.75 ± 0.30 nM (mean of three experiments) when nonspecific binding was evaluated using an excess of either Sar^1-AII or **5**, respectively. These quite similar values, systematically obtained within the same experiment, for both assay conditions (see typical experiment reported in Figure 2A) indicate the total absence of "pseudo-specific" binding which could be observed with tritiated Losartan.[28] They are consistent with K_i determined for **5** in competition binding assays using rat liver as receptor source and [^{125} I]-Sar^1-AII as tracer ligand (Table 1).

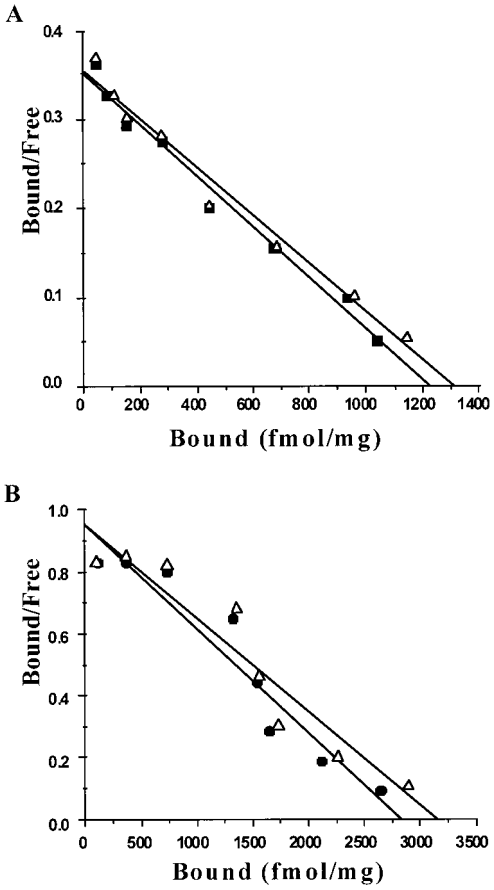


Figure 2. Binding properties of the tritiated nonpeptide compound **21** to the AT_1 receptor from membrane preparations. The binding of the tritiated nonpeptide compound **21** to membrane preparations from rat liver or CHO cells stably transfected with the AT_{1A} receptor cDNA was carried out as described in the Experimental Section. The nonspecific binding was evaluated using an excess of either Sar^1-AII or unlabeled nonpeptide derivative **5**. (A) Rat liver membranes: the binding parameters, determined through Scatchard analysis of the represented typical experiment (representative of three separate experiments), were $K_d = 2.8$ nM, $B_{max} = 1.2$ pmol/mg (■) and $K_d = 3.0$ nM, $B_{max} = 1.3$ pmol/mg (△) when nonspecific binding was determined using an excess of either Sar^1-AII or **5**, respectively. The nonspecific binding represented only 6% and 17% of total binding for radioligand concentrations 2 nM and 16 nM, respectively. (B) Membranes from transfected CHO cells: the binding parameters, determined through Scatchard analysis of the represented typical experiment (representative of four separate experiments), were $K_d = 1.7$ nM, $B_{max} = 2.8$ pmol/mg (●) and $K_d = 1.8$ nM, $B_{max} = 3.1$ pmol/mg (△) when nonspecific binding was determined using an excess of either Sar^1-AII or **5**, respectively. The nonspecific binding represented only 6% and 16% of total binding for radioligand concentrations of 1.9 nM and 18 nM, respectively. The B_{max} values were similar to those obtained for the binding of [^{125}I]Sar^1-AII ($K_d = 0.6$ nM, $B_{max} = 2.6$ pmol/mg).

We established similar properties for the binding of **21** to membranes from CHO cells stably expressing the AT_{1A} receptor: the mean K_d values for four separate experiments were 2.85 ± 0.7 nM and 2.0 ± 0.3 nM when nonspecific binding was evaluated with excess Sar^1-AII or **5**, respectively, with close values for both conditions within the same experiment (typical experiment represented in Figure 2B).

The compared bindings of **21** and **22** to intact CHO cells are represented in Figure 3. They confirm the better receptor affinity of **21** as compared to **22**, with respective K_d values of 1.3 ± 0.2 nM and 3.5 ± 0.5 nM (mean of four experiments). These results make the two nonpeptide radioligands convenient tools for mapping studies based on the evaluation of binding properties of mutated receptors.

3. Selectivity for the AT_1 Receptor. To assess their selectivity for the AT_1 receptor subtype, we checked the ability of **2** and **5** to inhibit [^{125}I]-Sar^1-AII binding to the AT_2 receptor. The experiments were performed on ewe lamb uterus membranes which contain both receptor subtypes and which have been previously treated with dithiothreitol to selectively abolish AII binding to the AT_1 receptor.[31] When tested at concentrations up to 10^{-5} M, **2** and **5** did not significantly reduce the binding of [^{125}I]-Sar^1-AII to the ewe lamb AT_2 receptor (data not shown).

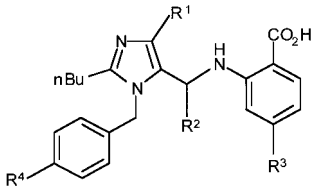
Biological Properties of the Nonpeptidic Azido Derivatives 14 and 7. The properties of the two unlabeled azido compounds **14** and **7** were compared to those of the parent derivatives **2** and **5**, the binding properties of which fulfilled essential requirements for the overall strategy, as assessed by the characteristics of their tritiated counterparts **22** and **21**, respectively.

1. Antagonist Properties. The introduction of an azido substituent into the structures of **2** and **5** does not alter significantly their antagonist properties (Table 1). Inhibition of AII-induced contraction of rabbit aorta rings by azido compounds **14** and **7** were characterized by IC_{50} values of 6.3 nM (confidence interval: 3.5−11.5 nM) and 3.5 nM (confidence interval: 1.1−11.2 nM), respectively. These values are close to those found for the parent compounds **2** and **5** (3.6 nM and 0.8 nM, respectively; see ref 19 and Table 1). Moreover, all these compounds were able to block AII-stimulated inositol phosphate production in CHO cells expressing the AT_{1A} receptor (data not shown).

2. Inhibition of [^{125}I]Sar^1-AII Binding to the Rat Liver AT_1 Receptor. The affinities of the two azido compounds **14** and **7** were evaluated for their ability to compete for the binding of [^{125}I]Sar^1-AII to purified rat liver membranes which contain only the AT_1 receptor subtype,[29] predominantly the AT_{1A} isoform.[30] These experiments were carried out in the absence of BSA, which can trap nonpeptide antagonists,[32] and in the presence of 0.1 mg/mL bacitracin which ensures satisfactory preservation of the tracer peptide against proteolysis.[19] Under these conditions, the K_i values for **14** and **7** were 22 nM and 1.5 nM, respectively (Table 1); they did not markedly differ from those obtained for the parent compounds **2** and **5** (12.1 nM and 2.5 nM, respectively). The direct binding properties of the tritiated azido compound **28**, which will be detailed in a next paragraph, confirm the competition binding assays. Taken together these data constitute a validation of the selected synthetic strategies.

3. Irreversible Binding of Azido Probes 7 and 14 to the AT_{1A} Receptor. CHO cells stably expressing the AT_{1A} receptor at high densities (1.2 pmol/10^6 cells) were chosen as a system for future analysis of covalent nonpeptide antagonist−receptor complexes. Preliminary evaluation of the properties of our azido compounds were carried out as followed: intact cell binding sites were saturated at 4 °C with each of the two probes and the irreversible labeling upon UV irradiation was as-

Table 1. Structure and Pharmacological Properties of Parent Nonpeptide Ligands, Their Radioactive and Photoactivatable Derived Probes



compd	R^1	R^2	R^3	R^4	IC_{50}[a] (nM)	K_i[b] (nM)	K_d[c] (nM)
2	H	H	H	CO_2H	3.6 ± 0.4	12.1 ± 2.9	
14	H	H	N_3	CO_2H	6.3± 1.3	22.0 ± 3.4	
5	H	H	H	$CONHSO_2(C_6H_4)$-2-Cl	0.8 ± 0.2	2.5 ± 0.8	
7	H	H	H	$CONHSO_2(C_6H_4)$-2-N_3	3.5 ± 0.6	1.5 ± 0.4	
22	[^{3}H]	H	H	CO_2H			3.5 ± 0.5
28	[^{3}H]	[^{3}H]	N_3	CO_2H			11.8 ± 1.1
21	H	H	H	$CONHSO_2(C_6H_4)$-2-Cl,4-[^{3}H]			1.3 ± 0.2

[a] Inhibition of AII-induced contraction in rabbit aorta strips. [b] Inhibition of [^{125}I]Sar^1-AII binding to purified rat liver membranes. [c] Direct binding of tritiated compounds to the recombinant $AT1_A$ receptor stably expressed in CHO cells.

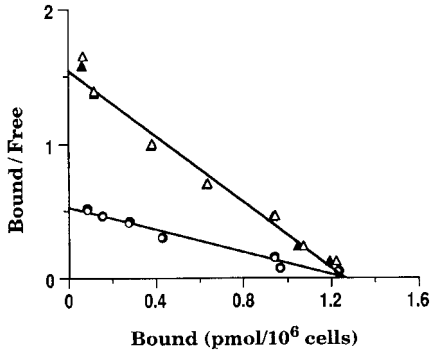


Figure 3. Binding properties of the tritiated nonpeptide derivatives **22** and **21** to intact CHO cells expressing the recombinant receptor. The binding of the two nonpeptide radioligands was carried out on CHO cells expressing the AT_{1A} receptor as described in the Experimental Section: ●, ○; binding of **22**, nonspecific binding measured in the presence of an excess of Sar^1-AII or unlabeled nonpeptide ligand **2**, respectively; ▲, △: binding of **21**, nonspecific binding measured in the presence of an excess of Sar^1-AII or unlabeled nonpeptide ligand **5**, respectively. The nonspecific binding never exceeded 5% of total **22** binding (concentration range 0.42−46 nM) and 4% of total **21** binding (concentration range 0.18−19 nM). The binding parameters deduced from Scatchard analysis of this typical experiment (representative of three separate experiments) are $K_d = 3.9$ nM, $B_{max} = 1.25$ pmol/10^6 cells for **22**, $K_d = 1.3$ nM, $B_{max} = 1.2$ pmol/10^6 cells for **21**.

sessed by measuring [^{125}I]Sar^1-AII binding, after dissociation of noncovalently bound ligand. The UV irradiation by itself (5 min, 254 nm) only moderately altered the AII binding sites (Table 2). Cells photolyzed in the presence of the azido compound **14** displayed a greatly decreased binding capacity for [^{125}I]Sar^1-AII, in favor of receptor covalent labeling with a predictive fairly good yield. The results obtained for control nonphotolyzed cells are indicative of a good dissociation of the ligand under the experimental conditions used. Similar results were obtained with the peptide azido derivative [Sar^1, Val^5, Phe $(4N_3)^8$]AII, a classical photoaffinity probe,[15] with the expected noncomplete dissociation of this high affinity peptide ligand in the absence of UV irradiation. On the contrary, the analysis of experiments carried out on cells saturated with the azido compound **7** did not allow one to conclude that receptor photolabeling, if any, had occurred at a high yield. Therefore, despite its lower affinity, we decided to use **14** for photolabeling experiments and prepared its tritiated form **28**.

Table 2. Irreversible Binding of Photoactivatable Nonpeptidic or Peptidic Probes

ligand	[^{125}I]-Sar^1-AII specific binding[a] (% of control)	
	without UV irradiation	with UV irradiation
none	100 ± 8	84 ± 2
14	91 ± 1	64 ± 3
7	50 ± 1	55 ± 1
[Sar^1, Val^5, Phe$(4N_3)^8$]AII	75 ± 4	50 ± 1

[a] CHO cells expressing the AT_{1A} receptor were incubated in the absence or presence of the various photoactivatable compounds, then irradiated under conditions indicated in the Experimental Section. The remaining binding sites were titrated by incubation of the cells in the presence of [^{125}I]-Sar^1-AII. The figures represent the mean of triplicate assays carried out in a typical experiment. Similar results were obtained in three separate experiments.

Binding Properties of the Tritiated Azido Nonpeptidic Probe 28. Photolabeling of the AT_{1A} Receptor. 1. Binding Properties. The binding properties of **28** have been evaluated on CHO cells overexpressing the AT_{1A} receptor. This overexpression suitable for future mapping approaches was fortuitously obtained by replacement of Cys^{121} (located in TM III) by an alanine (2.4−5.4 pmol/10^6 cells for the C121A receptor, as compared to 1.2 pmol/10^6 cells for the wild type receptor); it is reminiscent of the overexpression obtained upon replacement of Cys^{116} located in TM III of the β_2-adrenergic receptor.[33] This mutant receptor was pharmacologically identical to the wild type receptor (J. Marie, unpublished results). Compound **28** bound to the C121A receptor in a saturable and reversible manner (Figure 4) and with a high affinity ($K_d = 11.8 \pm 1.1$ nM, three experiments). The binding parameters were independent of the ligand (Sar^1-AII or unlabeled nonpeptide ligand **14**) added in excess to evaluate nonspecific binding which never exceeded 3% of total binding. The binding capacity (Bmax = 2.4−5.4 pmol/10^6 cells) was identical to that found for [^{125}I]Sar^1-AII in the same experiments.

2. Receptor Photolabeling. The photoaffinity labeling of the AT_{1A} receptor with **28** was carried out as follows: after equilibrium binding of the tritiated nonpeptide photoactivatable probe, washed cells were irradiated for 5 min at 254 nm (this photolysis time was

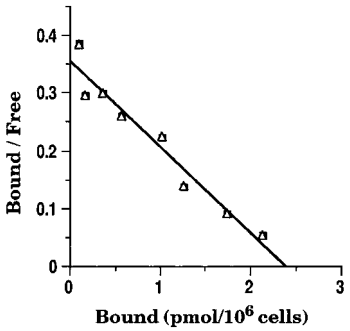


Figure 4. Binding properties of the radioactive photoactivatable probe **28**. The binding properties of **28** have been studied on CHO cells overexpressing the C121A AT_{1A} mutant receptor: binding of **28**, nonspecific binding measured in the presence of an excess of either Sar^1-AII (■) or unlabeled **14** (△). The nonspecific binding never exceeded 3% of total binding (concentration range 0.6−70 nM). The binding parameters deduced from Scatchard analysis of the presented experiments are $K_d = 11.1$ nM, $B_{max} = 2.4$ pmol/10^6 cells. The mean values ± SD obtained for three separate experiments were $K_d = 11.8$ ± 1.1 nM, B_{max} ranging from 2.4 to 5.4 pmol for 10^6 cells.

found to give an optimal photolabeling yield in pilot experiments). Photolyzed cells were then efficiently solubilized (yield = 95−97%, five experiments) with Triton × 100. The omission of binding medium during irradiation together with the absence of washing between photolysis and solubilization steps allowed rigorous evaluation of initially occupied binding sites and subsequent photolabeling yield. As for the receptor covalently labeled with peptidic probes, the receptor photolabeled with the nonpeptide derivative was efficiently adsorbed to hydroxylapatite gels. This chromatography step allowed elimination of dissociated noncovalently bound ligand. Covalent labeling yields were estimated from covalent complexes eluted with high ionic strength; they varied from 8% to 12% over five experiments and were systematically identical to those obtained for peptide−receptor covalent complexes obtained in the same experiments. Besides a partial purification, the hydroxylapatite step provided a way of concentrating the tritiated complexes before SDS−PAGE analysis.[16] Autoradiographic analysis of electrophoretic gels revealed nonpeptide labeling of a 78 kDa apparent molecular weight entity (Figure 5a, lane B). The autoradiographic pattern was similar to that obtained for purified receptor previously photolabeled with the peptidic probe biotin-NH-$(CH_2)_2$-S-S-$(CH_2)_2$-CO-[Ala^1, Phe$(4N_3)^8$]AII[16,17] (Figure 5A, lane A), as assessed by the perfect superimposition of tritium and iodine radioactivities in sliced gels (Figure 5B). This labeling was specific, as it was suppressed when initial incubation was carried out in the presence of an excess of either unlabeled Sar^1-AII (Figure 5A, lane C) or azido nonpeptide derivative (Figure 5B, lane D). No labeling was observed when UV irradiation was omitted (Figure 5A, lane E).

Discussion

Extensive mutagenesis experiments have been used to study the interaction of nonpeptide antagonists with peptide hormone receptors belonging to the GPCR family,[34−38] including the AT_1 receptor.[39−41] They led to the agreement that the binding sites for peptidic and

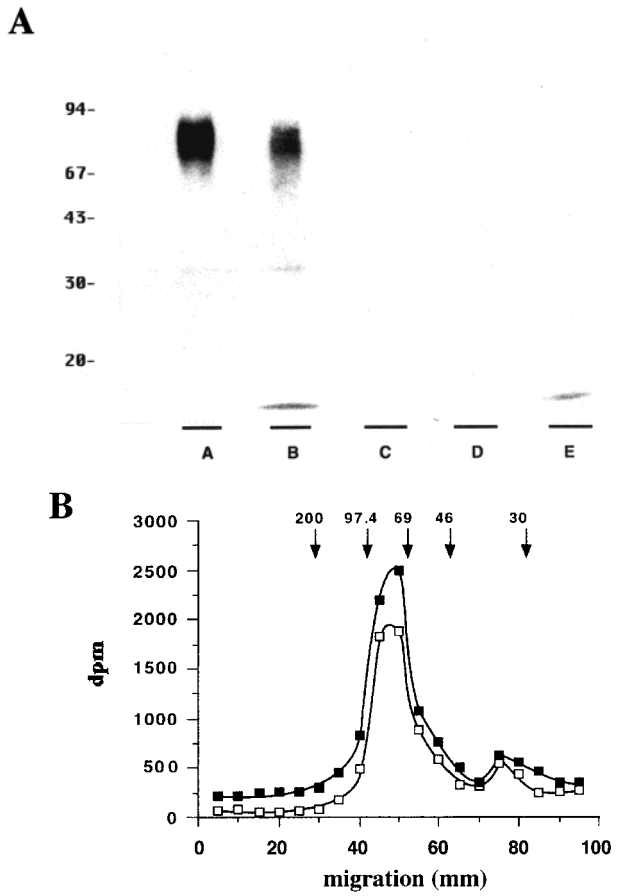


Figure 5. Photoaffinity labeling of the AT_{1A} receptor by the nonpeptide photoactivatable probe **28**. Intact CHO cells overexpressing the mutant C121A AT_{1A} receptor were photolabeled, and cells were solubilized using Triton × 100 as detergent. The samples containing covalent probe−receptor complexes were partially purified and concentrated through hydroxylapatite chromatography before electrophoresis (12.5% acrylamide gels) and autoradiography. (A) SDS−PAGE and autoradiographic analysis of photolabeled receptors. Lane A: control purified receptor previously photolabeled with a peptide biotinylated probe[16] under identical experimental conditions (3 fmol). Lane B: receptor photolabeled with the nonpeptide tritiated probe (5 pmol). Lanes C and D: nonspecific photolabeling obtained in the presence of excess Sar^1-AII or **14**, respectively, during initial receptor saturation. Lane E: control nonphotolyzed cells. Similar results were obtained in four separate experiments. (B) Compared electrophoretic patterns of receptor photolabeled by peptidic and nonpeptidic probes. Samples of receptor photolabeled by **28** and purified receptor previously photolabeled by a radioiodinated peptide probe[16] were analyzed in the same electrophoresis run. The wet unfixed gel was cut into 5 mm slices for tritium (□) or iodine (■) determinations. Molecular weights of precolored markers are indicated on the abscissa in kilodaltons.

nonpeptidic compounds are distinct. However it is not always possible to draw clear-cut conclusions from the pharmacological properties of mutated or chimeric receptors.[42] Receptor covalent labeling using affinity or photoaffinity probes,[6−17] although it is often a difficult task, can provide valuable information about ligand−receptor chemical interactions as previously shown for the AII−AT_1 receptor system.[15−17] Reported data relative to the biochemical mapping of peptide hormone receptors have been obtained using peptidic probes, while a limited number of studies have been undertaken using nonpeptide photoactivatable probes.[43,44]

The purpose of the present work was to design a nonpeptide derivative suitable for the covalent labeling of the AT_1 angiotensin II receptor. The first step of the strategy consisted of the synthesis of two tritiated nonpeptide antagonists, **22**[19] and **21**, giving negligible nonspecific binding. On the basis of the previous development of appropriate synthetic schemes,[18−22] we have prepared two photoactivatable compounds bearing an azido group in two positions of the molecule potentially interacting with different portions of the receptor. Irreversible binding could be evidenced for one of them, **14**, thus justifying the synthesis of the corresponding tritiated compound. The tritiated azido probe **28** displayed a satisfactory affinity for the AT_{1A} recombinant receptor expressed in CHO cells (K_d = 11.8 nM). We demonstrated its ability to specifically and efficiently photolabel this receptor. The apparent molecular weight of the labeled entity (78 kDa) and the yield of covalent labeling (about 10%) were identical to those found for the receptor photolabeled with a peptidic probe bearing an azido group on the C-terminal phenylalanine.[15−16]

If required, another probe expectedly displaying a higher affinity might be synthesized through the introduction of an azido group into the anthranilate moiety of compound **5**.

The elucidation of the mechanism of GPCR activation must take into account the existence of inactive and active receptor conformations and their preferential recognition and (or) induction by various ligands according to their pharmacological behavior as agonists, inverse agonists, or pure antagonists.[2,3] Recent data suggest that transmembrane helix movements, especially movements of helices III and VI,[45−48] are associated with the transition from an inactive to an active receptor conformations, thus resulting in marked structural changes. As a consequence, binding site studies can often be confusing when they are based on the evaluation of mutant receptor properties through heterologous competition binding experiments,[4,5] a situation which can be circumvented when radioactive nonpeptide ligands, such as those described in the present paper, are available. Moreover, hypotheses about the superimposition of peptide agonists and nonpeptide antagonists can appear still more speculative.[1,21,22] Therefore the need for the obtention of direct biochemical evidences about receptor−ligand interaction is reinforced. In this respect the original tritiated nonpeptide photoactivatable probe reported in the present work constitutes a valuable tool for mapping studies. The absence of nonspecific binding of both radioligands **21** and **22**, extended to receptors transiently expressed in COS-7 cells, allowed accurate evaluation of mutant receptors having lost much affinity for nonpeptidic derivatives (i.e. AT_1 N111A and S105A mutants[41,49]), as well as verification of the expression of mutant having completely lost their AII binding properties (S105A mutant[41]). It is noticeable that the new nonpeptide derivatives behaved as inverse agonists when tested in experimental situations where the AT_1 receptor basal activity was enhanced.[49] As a consequence, biochemical mapping using the nonpeptidic azido probe should help to define the inactive receptor conformation. As nonpeptidic ligands display a reduced conformational flexibility as compared to AII-derived peptides, they can be used as frameworks in order to refine the positioning of transmembrane helices.

A previous work has demonstrated the possibility to covalently label the AT_1 receptor with agonist AII-derived probes possessing an azido phenylalanine at their C-terminus;[15] it was further applied to receptor purification using azido biotinylated compounds.[16] We recently reassessed the preliminary characterization of small photolabeled tryptic fragments[17] to unambiguously demonstrate that the C-terminal phenylalanine of AII interacts with a receptor zone comprising transmembrane domain VI and part of the third extracellular domain.[25]

The photolabeling experiments carried out with peptidic agonists and nonpeptide antagonists (or inverse agonists) should thus be helpful for the building of reliable models of ligand−receptor interactions which integrate the molecular mechanisms underlying transitions from inactive to active receptor conformations and their differential recognition or induction[50,51] by the various classes of pharmacological agents.

Experimental Section

Chemistry. Melting points were determined either on a hot-stage Kofler or on a Büchi melting point apparatus and are uncorrected. IR spectra were measured on a Perkin-Elmer 782 spectrophotometer. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker AC 300 spectrometer using tetramethylsilane as internal reference. Structural assignments for all new compounds were consistent with their spectra. Elemental analyses were performed on a Perkin-Elmer 240 C apparatus. Molecular formula followed by the symbols C, H, N indicate that elemental analyses were found to be within ±0.4% of the theoretical values for C, H, and N. Sar^1-AII was purchased from Bachem (Bubendorf, Switzerland). It was radioiodinated as previously described[16] [Sar^1, Val^5, Phe(4N$_3$)^8]-AII was kindly supplied by E. Escher (Sherbrooke, Canada).

Methyl 2-[[[2-Butyl-1-[[4-(methoxycarbonyl)phenyl]-methyl]-1*H*-imidazol-5-yl]methyl]amino]benzoate (1). Methyl 4-[(2-butyl-5-chloromethyl-1*H*-imidazol-1-yl)methyl]-benzoate hydrochloride[22] (8 g, 22.3 mmol) was suspended in 80 mL of anhydrous toluene. Next, 10.15 g (67.1 mmol) of methyl 2-aminobenzoate and 4.79 g (44.7 mmol) of 2,6-dimethyl pyridine were added. The reaction mixture was refluxed for 8 h and then poured into cold water. The product was extracted and purified by chromatography with toluene−2-PrOH (9:1) to afford 9 g (92%) of **1** as an orange oil: ^{1}H NMR ($CDCl_3$) δ 7.91 (d, J = 8.3 Hz, 2H), 7.82 (dd, J = 1.57 Hz, J = 7.98 Hz, 1H), 7.67 (t, J = 4.80 Hz, 1H), 7.28 (m, 1H), 7.04 (s, 1H), 6.92 (d, J = 8.3 Hz, 2H), 6.63 (m, 2H), 5.18 (s, 2H), 4.20 (d, J = 4.80 Hz, 2H), 3.90 (s, 3H), 3.77 (s, 3H), 2.56 (t, J = 8 Hz, 2H), 1.70 (m, 2H), 1.30 (m, 2H), 0.87 (t, J = 7.3 Hz, 3H).

2-[[[2-Butyl-1[(4-carboxyphenyl)methyl]-1*H*-imidazol-5-yl]methyl]amino]benzoic Acid (2). Compound **1** (5.1 g, 11.7 mmol) was dissolved in 50 mL of methanol. Next, 17.6 mL (35.2 mmol) of 2 N NaOH solution was added, and the mixture was stirred under reflux for 4 h. Methanol was evaporated, and the residue was dissolved in cold water. The diacid was precipitated by addition of 1 N HCl to pH 4. The solid was filtered off, washed with water, and dried over P_2O_5. The crude product was recrystallized from methanol to afford 3.5 g (73.5%) of **2** as a pale yellow solid: mp 234 °C; ^{1}H NMR (DMSO-d_6) δ 13.0 (bs, 2H), 8.0 (bs, 1H), 7.75 (d, J = 8.26 Hz, 2H), 7.70 (d, J = 7.95 Hz, 1H), 7.30 (m, 1H), 7.0 (d, J = 8.13 Hz, 2H), 6.93 (s, 1H), 6.75 (d, J = 8.42 Hz, 1H), 6.55 (m, 1H), 5.32 (s, 2H), 4.31 (s, 2H), 2.50 (t, 2H), 1.45 (m, 2H), 1.21 (m, 2H), 0.77 (t, J = 7.35 Hz, 3H).

Methyl 2-[[[2-Butyl-1-[(4-carboxyphenyl)-methyl]-1*H*-imidazol-5-yl]methyl]amino]benzoate (3). Sodium hydroxide (0.68 g, 20 mmol) and 10 mL of water were added to a solution of 8.3 g (19.1 mmol) of **1** in 80 mL of methanol. The

mixture was heated at 50 °C for 3.5 h and concentrated, and the residue was diluted with 150 mL of water. The aqueous layer was washed with EtOAc and acidified to pH 5 with 1 N HCl. The product was extracted with EtOAc. The organic layers were washed with water, dried ($MgSO_4$), and concentrated. The crude product was purified by chromatography on silica with CH_2Cl_2−MeOH (95:5) to give 4.8 g of a solid which was recrystallized from EtOAc, yielding 4.41 g (55%) of **3** as a white solid: mp 160 °C; ^{1}H NMR ($CDCl_3$) δ 7.92 (d, J = 6.64 Hz, 2H), 7.80 (d, J = 8.61 Hz, 1H), 7.68 (bt, 1H), 7.30 (t, J = 9 Hz, 1H), 7.19 (s, 1H), 6.92 (d, J = 8.3 Hz, 2H), 6.63 (m, 2H), 5.3 (bs, 1H), 5.22 (s, 2H), 4.20 (d, J = 4.9 Hz, 2H), 3.79 (s, 3H), 2.88 (t, J = 7.6 Hz, 2H), 1.88 (m, 2H), 1.33 (m, 2H), 0.88 (t, J = 7.3 Hz, 3H).

Methyl 2-[[[2-Butyl-1-[(4-((((2-azidophenyl)sulfonyl)amino)carbonyl)phenyl)methyl]-1*H*-imidazol-5-yl]methyl]amino]benzoate (6). To a suspension of 2.22 g (5.3 mmol) of **3** in 55 mL of CH_2Cl_2 were added 1.15 g (5.8 mmol) of 2-azidobenzenesulfonamide, 0.71 g (5.8 mmol) of DMAP, and 1.11 g (5.8 mmol) of EDCI. The mixture protected from light was stirred during 3 days at room temperature and concentrated in vacuo to afford a residue which was purified by flash chromatography on silica gel using toluene-2-PrOH (6:4) as eluent to afford **6** (2.23 g, 70%) as a yellow solid which was kept away from light: mp 162 °C, ^{1}H NMR (DMSO-d_6) δ 7.89 (dd, J = 7.7 Hz and J = 1.5 Hz, 1H), 7.77 (m, 3H), 7.70 (t, J = 5.7 Hz, 1H), 7.44 (dt, J = 7.1 Hz and J = 1.5 Hz, 1H), 7.33 (dt, J = 7.1 Hz and J = 1.5 Hz, 1H), 7.21 (m, 3H), 6.82 (m, 4H), 6.58 (t, J = 7.8 Hz, 1H), 5.23 (s, 2H), 4.32 (d, J = 4.6 Hz, 2H), 3.66 (s, 3H), 2.47 (t, J = 7.8 Hz, 2H), 1.47 (m, 2H), 1.23 (m, 2H), 0.78 (t, J = 7.3 Hz, 3H).

Methyl 4-[2-Butyl-5-[[2-[(2-chlorophenyl)sulfonylaminocarbonyl]phenyl]aminomethyl]-1*H*-imidazol-1-ylmethyl]benzoate (4). Compound **4** was synthesized from **3** and 2-chloro-benzenesulfonamide by the same procedure as decribed above for the synthesis of **6**: mp 125 °C; yield 78%; ^{1}H NMR ($CDCl_3$) δ 8.17 (d, 1H), 7.87 (d, 2H), 7.78 (d,1H), 7.65 (s, 1H), 7.20 (m, 5H), 6.70 (d, 2H), 6.52 (m, 2H), 5.12 (s, 2H), 4.07 (s, 2H), 3.67 (s, 3H), 2.50 (m, 2H), 1.40 (m, 2H), 1.21 (m, 2H), 0.72 (t, 3H).

2-[[[2-Butyl-1-[(4-((((2-azidophenyl)sulfonyl)amino)carbonyl)phenyl)methyl]-1*H*-imidazol-5-yl]methyl]amino]benzoic Acid (7). To a solution of 0.83 g (1.4 mmol) of **6** in 20 mL of methanol was added 0.42 g (10.5 mmol) of NaOH in 2 mL of water. The mixture was heated under reflux during 4 h. Evaporation of the methanol afforded a residue which was taken up in water. The pH of the resulting solution was adjusted to 5 with an aqueous 1 N HCl solution from which the desired product **7** precipitated as a white solid which was dried in vacuo in the presence of P_2O_5 (0.64 g, 79%) and which was kept away from light: mp 180 °C dec; ^{1}H NMR (DMSO-d_6) δ 8.10 (bs, 1H), 7.86 (m, 4H), 7.52 (dt, J = 7.8 Hz and J = 1.5 Hz, 1H), 7.28 (m, 4H), 7.06 (d, J = 8.3 Hz, 2H), 6.69 (d, J = 8.4 Hz, 1H), 6.60 (t, J = 7.7 Hz, 1H), 5.46 (s, 2H), 4.42 (s, 2H), 2.72 (t, J = 7.5 Hz, 2H), 1.43 (m, 2H), 1.21 (m, 2H), 0.78 (t, J = 7.2 Hz, 3H). Anal. ($C_{29}H_{29}N_7O_5$, 0.57 H_2O) C, H, N.

2-[[[2-Butyl-1-[(4-((((2-chlorophenyl)sulfonyl)amino)carbonyl)phenyl)methyl]-1*H*-imidazol-5-yl]methyl]amino]benzoic Acid (5). This product was synthesized from **4** by the same procedure as decribed above for the synthesis of **7**: mp 198 °C; yield 88%; ^{1}H NMR (DMSO-d_6) δ 13.20 (bs, 1H), 8.16 (m, 1H), 8.0 (d, 1H), 7.86 (d, 2H), 7.80 (d, 1H), 7.46 (m, 4H), 7.34 (t, 1H), 7.04 (d, 2H), 6.65 (m, 2H), 5.49 (s, 2H), 4.46 (s, 2H), 2.80 (t, 2H), 1.42 (m, 2H), 1.21 (m, 2H), 0.76 (t, 3H). Anal. ($C_{29}H_{29}ClN_4O_5S$) C, H, N.

Methyl (4-Nitro-2-trifluoromethylcarbonylamino)benzoate (9). To a solution of **8** (7 g, 36 mmol) in anhydrous toluene was added 30 mL (214 mmol) of trifluoroacetic anhydride. The mixture was heated under reflux for 24 h, then washed with water until neutrality, dried ($MgSO_4$), and evaporated. Recrystallization from diisopropyloxide gave **9** as ochreous needles (70%): mp 138−140 °C; ^{1}H NMR (DMSO-d_6) δ 11.91 (s, 1H), 8.70 (d, J = 2.2 Hz, 1H), 8.23 (dd, J = 8.7 and 2.2 Hz, 1H), 8.16 (d, J = 8.6 Hz, 1H), 3.89 (s, 3H). Anal. ($C_{10}H_7F_3N_2O_5$) C, H, N.

Methyl (4-Amino-2-trifluoromethylcarbonylamino)benzoate (10). A mixture of **9** (7 g, 24 mmol) in 70 mL of anhydrous DMF and 0.72 g of 10% palladium on charcoal was stirred under l atm H_2 pressure for 12 h. The catalyst was filtered off, and the DMF solution was diluted with water, extracted with toluene, and dried ($MgSO_4$). Evaporation gave **10** as an ochreous solid (80%): mp 161−162 °C; ^{1}H NMR (DMSO-d_6) δ 12.44 (s, 1H), 7.73 (d, J = 8.8 Hz, 1H), 7.62 (d, J = 2.2 Hz, 1H), 6.48 (s, 2H), 6.43 (dd, J = 8.8 and 2.2 Hz, 1H), 3.80 (s, 3H). Anal. ($C_{10}H_9F_3N_2O_3$) C, H, N.

Methyl (4-Azido-2-trifluoromethylcarbonylamino)benzoate (11). To a cold suspension (−3 °C) of **10** (4.8 g, 18 mmol) in 30 mL of aqueous 2 N HCl was added dropwise a solution of 1.85 g (27 mmol) of $NaNO_2$ in 12 mL of water. The mixture was stirred for 5 h vigorously at − 3 °C. Then a solution of 1.75 g (27 mmol) of NaN_3 in 11 mL of water was added dropwise. After the mixture was stirred for 2.5 h at −3 °C, the precipitate formed was filtered, washed with water, and dried in a desiccator (P_2O_5) to give **11** as a white-cream solid (95%) which was protected from light and kept at 4 °C: mp 83 °C; ^{1}H NMR (DMSO-d_6) δ 11.99 (s, 1H), 8.03 (d, J = 8.6 Hz, 1H), 7.84 (d, J = 2.2 Hz, 1H), 7.17 (dd, J = 8.6 and 2.3 Hz, 1H), 3.87 (s, 3H). Anal. ($C_{10}H_7F_3N_4O_3$) C, H, N.

Methyl 2-Amino-4-azidobenzoate (12). Compound **11** (1.03, 3.6 mmol) was dissolved in 50 mL of a solution of methanol−water (5:2) containing 7% of K_2CO_3. The mixture protected from light was stirred for 18 h at room temperature, diluted with water, and extracted with EtOAc. The organic layers were washed with water, dried, and concentrated to a residue which was washed with Mill Q water, taken up in ether, dried, and evaporated to yield **12** as a yellow solid (89%) which was kept away from light: mp 69 °C; ^{1}H NMR (DMSO-d_6) δ 7.72 (d, J = 8.6 Hz, 1H), 6.82 (1, 2H), 6.50 (d, J = 2.3 Hz, 1H), 6.27 (dd, J = 8.7 and 2.3 Hz, 1H), 3.77 (s, 3H). Anal. ($C_8H_8N_4O_2$) C, H, N.

Methyl 2-[[[2-Butyl-1-[[4-(methoxycarbonyl)phenyl]methyl]-1*H*-imidazol-5-yl]methyl]amino]-4-azidobenzoate (13). Methyl 4-[(2-butyl-5-chloromethyl-1*H*-imidazol-1-yl)methyl] benzoate, hydrochloride[22] (1.69 g, 4.7 mmol) was suspended in 50 mL of anhydrous toluene. Next, 2.72 g (14 mmol) of methyl 2-amino-4-azido-benzoate **12** and 1.1 mL (9.4 mmol) of 2,6-dimethylpyridine were added. The reaction mixture was heated at 120 °C for 5.5 h and then poured into a saturated $NaHCO_3$ solution. The product was extracted with EtOAc, dried, evaporated, and purified by flash chromatography with toluene-2-PrOH (9:1). The solid obtained was dissolved into freshly distillated acetone. Evaporation of this solution at 60 °C under vacuum gave **13** as a beige solid (45%) which was kept away from light: mp 130−132 °C; ^{1}H NMR ($CDCl_3$) δ 7.91 (d, J = 8.2 Hz, 2H), 7.84 (t, J = 5.0 Hz, 1H), 7.80 (d, J = 8.6 Hz, 2H), 7.05 (s, 1H), 6.92 (d, J = 8.1 Hz, 2H), 6.29 (dd, J = 8.6 Hz, J = 2.0 Hz, 1H), 6.21 (d, J = 2.0 Hz, 1H), 5.17 (s, 2H), 4.16 (d, J = 5.1 Hz, 2H), 3.91 (s, 3H), 3.76 (s, 3H), 2.57 (t, J = 7.5 Hz, 2H), 1.70 (m, 2H), 1.36 (m, 2H), 0.88 (t, J = 7.3 Hz, 3H). Anal. ($C_{25}H_{28}N_6O_4$) C, H, N.

2-[[[2-Butyl-1-[(4-carboxyphenyl)methyl]-1*H*-imidazol-5-yl]methyl]amino]-4-azidobenzoic Acid (14). A suspension of 0.17 g (0.4 mmol) of **13** in 2 mL of distilled methanol in 0.16 mL of water and 90 mg (1.6 mmol) of KOH was heated under reflux for 1 h. Evaporation of the methanol afforded a residue which was taken up in water. The pH of the resulting solution was adjusted to 5 with an aqueous 1 N HCl solution from which the desired product **14** precipitated as a white solid which was dried in vacuo in the presence of P_2O_5 (0.13 g, 81%): mp 215 °C dec; ^{1}H NMR (DMSO-d_6) δ 8.08 (s, 1H), 7.81 (d, J = 8.1 Hz, 2H), 7.75 (d, J = 8.5 Hz, 1H), 6.98 (d, J = 8.1 Hz, 2H), 6.87 (s, 1H), 6.38 (d, J = 1.9 Hz, 2H), 6.32 (dd, J = 8.5 Hz and J = 2.0 Hz, 1H), 5.31 (s, 2H), 4.34 (s, 2H), 2.47 (t, J = 7.8 Hz, 2H), 1.49 (m, 2H), 1.23 (m, 2H), 0.77 (t, J = 7.2 Hz, 3H). Anal. ($C_{23}H_{24}N_6O_4$, 0.74 H_2O) C, H, N.

(1,1-Dimethylethyl) 4-[[2-Butyl-5-(hydroxymethyl)-1*H*-imidazol-1-yl]methyl]benzoate (16). To a solution of 4.6 g

(13.4 mmol) of **15**[20] in 60 mL of methanol cooled to 0 °C was added 0.61 g (16 mmol) of sodium borohydride. After 0.25 h, the reaction mixture was concentrated, and the residue was dissolved in water and extracted with EtOAc. The organic layer was washed with brine, dried ($MgSO_4$), and concentrated to afford **16** as a crude solid which was directly used in the next step: mp 163 °C; ^{1}H NMR ($CDCl_3$) δ 7.9 (d, J = 8.3 Hz, 2H), 7.0 (d, J = 8.36 Hz, 2H), 6.93 (s, 1H), 5.26 (s, 2H), 4.46 (s, 2H), 2.53 (t, J = 7.8 Hz, 2H), 2.05 (bs, 1H), 1.63 (m, 2H), 1.57 (s, 9H), 1.32 (m, 2H), 0.87 (t, J = 7.3 Hz, 3H).

(1,1-Dimethylethyl) 4-[[2-Butyl-5-(chloromethyl)-1*H*-imidazol-1-yl]methyl]benzoate (17). To a solution of 4.5 g (13 mmol) of **16** in 100 mL of CH_2Cl_2 cooled to 0 °C were added 5.3 mL (72.7 mmol) of thionyl chloride dropwise. The solution was allowed to warm to room temperature during 5 min. The mixture was concentrated, and the residue was taken up in 100 mL of toluene and concentrated again to afford 5.1 g (97%) of crude **17** as a beige solid: mp 191 °C dec; ^{1}H NMR ($CDCl_3$) δ 16.83 (s, 1H), 8.05 (d, J = 8.34 Hz, 2H), 7.54 (s, 1H), 7.09 (d, J = 8.305 Hz, 2H), 5.45 (s, 2H), 4.42 (s, 2H), 3.08 (t, J = 7.8 Hz, 2H), 1.80 (m, 2H), 1.59 (s, 9H), 1.41 (m, 2H), 0.90 (t, J = 7.3 Hz, 3H). This compound was used in the next step without further purification.

(1,1-Dimethylethyl) 4-[[2-Butyl-5-[[2-(phenylmethoxycarbonyl)phenyl]aminomethyl]-1*H*-imidazol-1-yl]methyl]benzoate (18). A mixture of 1 g (2.5 mmol) of **17**, 1.83 g (2.5 mmol) of benzyl anthranilate, and 0.53 g (2.5 mmol) of 2,6-lutidine into 35 mL of toluene was stirred under reflux for 1 h and at room temperature for 14 h. The mixture was partitioned between water and EtOAc. The organic layers were washed, dried ($MgSO_4$), and concentrated, and the crude residue was purified by flash chromatography with methylcyclohexane−acetone (7:3) to afford 0.7 g (50%) of **18** as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 7.90 (m, 3H), 7.70 (bt, 1H), 7.39−7.25 (m, 6H), 7.03 (s, 1H), 6.90 (d, J = 8.34 Hz, 2H), 6.60 (m, 2H), 5.23 (s, 2H), 5.16 (s, 2H), 4.16 (d, J = 4.87 Hz, 2H), 2.58 (t, J = 7.58 Hz, 2H), 1.70 (m, 2H), 1.58 (s, 9H), 1.39 (m, 2H), 0.88 (t, J = 7.2 Hz, 3H).

4-[[2-Butyl-5-[[2-(phenylmethoxycarbonyl)phenyl]-amino-methyl]-1*H*-imidazol-1-yl]methyl]benzoic Acid (19). To 2.6 g (4.7 mmol) of **18** were added 10 mL of TFA at 0 °C, and the obtained solution was stirred at 0 °C for 3 h. The reaction mixture was evaporated to dryness, 50 mL of water was added, the pH of the mixture was adjusted to 6 with NaOH, and the organic layers were washed with water, dried ($MgSO_4$), and concentrated. The crude product was purified by flash chromatography with CH_2Cl_2−MeOH (9:1) to afford **19** (2.2 g, 94%) as a yellowish solid: mp 90 °C; ^{1}H NMR ($CDCl_3$) δ 13.0 (bs, 1H), 8.03 (d, J = 8.26 Hz, 2H), 7.95 (d, J = 9.5 Hz, 1H), 7.88 (bs, 1H), 7.43−7.28 (m, 7H), 7.0 (d, J = 8.25 Hz, 2H), 6.65 (dt, J = 7.4 Hz, 1H), 6.55 (d, J = 8.48 Hz, 1H), 5.30 (s, 2H), 5.24 (s, 2H), 4.22 (d, J = 3.2 Hz, 2H), 2.86 (t, J = 7.6 Hz, 2H), 1.70 (m, 2H), 1.33 (m, 2H), 0.85 (t, J = 7.25 Hz, 3H).

Benzyl 2-[[[2-Butyl-1-[(4-((((2-chloro-4-iodophenyl)sulfonyl)amino)carbonyl)phenyl)methyl]-1*H*-imidazol-5-yl]-methyl]amino]benzoate (20). To a suspension of 3.85 g (7.7 mmol) of **19** in 100 mL of CH_2Cl_2 were added 2.7 g (8.5 mmol) of 2-chloro-4-iodo-benzene sulfonamide, 1.04 g (8.5 mmol) of DMAP, and 1.62 g (8.5 mmol) of 1-(3-dimethyl-amino propyl)-3-ethylcarbodiimide hydrochloride. The mixture was stirred at room temperature for 4 days. The organic layer was washed with water, dried ($MgSO_4$), and concentrated to afford a crude residue. The desired product was purified by flash chromatography with toluene−2-PrOH (1:1) to afford 4.26 g (69%) of **20** as an orange solid, mp 155 °C: ^{1}H NMR (DMSO-d_6) δ 7.77 (m, 7H), 7.38 (m, 6H), 6.82 (m, 4H), 6.59 (t, J = 7.4 Hz, 1H), 5.22 (s, 2H), 5.20 (s, 2H), 4.31 (d, J = 5.2 Hz, 2H), 2.48 (t, J = 7.8 Hz, 2H), 1.48 (m, 2H), 1.24 (m, 2H), 0.78 (t, J = 7.3 Hz, 3H).

2-[[[2-Butyl-1-[(4-((((2-chloro-4-*t*-phenyl)sulfonyl)amino)carbonyl)phenyl)methyl]-1*H*-imidazol-5-yl]methyl]-amino]benzoic Acid (21). A mixture of 0.05 g (0.063 mmol) of **20** and 0.0378 g (0.38 mmol) of triethylamine in 5 mL of anhydrous DMF with 0.01 g of 10% palladium on carbon was stirred under 1 atm $[^3H]_2$ pressure for 6 h at room temperature. The catalyst was then filtered off, and the solvent evaporated. The residue is solubilized in MeOH (5 mL) and purified on preparative TLC (silica gel 60F 254) with $CHCl_3$−MeOH (7:3). After elution the desired compound is obtained after desorption by MeOH. The HPLC analysis (Lichrospher RP C8; eluent: KH_2PO_4 5 mM−MeOH (53:47)) revealed that the tritiated compound is identical to the unlabeled compound **5**.

Methyl 4-[[2-Butyl-5-formyl-4-*t*-imidazol-1-yl]methyl]-benzoate (24). A mixture of 0.2 g (0.6 mmol) of methyl 4-[[2-butyl-4-chloro-5-formyl-imidazol-1-yl]methyl]benzoate **23**[22] in 10 mL of methyl acetate, 0.1 mL (0.72 mmol) of triethylamine, and 20 mg of 10% palladium on charcoal was stirred under l atm of $[^3H]_2$ for 2 h. The catalyst was filtered off, the solvent was evaporated, and the resulting residue was partitioned between EtOAc and water. The organic layer was then washed with water, dried over $MgSO_4$, and concentrated. The crude product was purified by preparative TLC on silica gel using toluene-2-PrOH (9:1) as eluent to afford 0.11 g (61%) of **24** which coeluted with an authentic sample of the corresponding nontritiated compound.

Methyl 4-[[2-Butyl-5-hydroxymethyl-*t*-4-*t*-imidazol-1-yl]methyl]benzoate (25). To a solution of 0.11 g (0.37 mmol) of **24** in 5 mL of MeOH cooled to 0 °C were added 18.7 mg (0.44 mmol) of $NaB[^3H]_4$. The mixture was stirred for 20 min and the methanol was then evaporated to afford a residue which was taken up in water. The pH of the resulting solution was adjusted to 6 with an aqueous 1 N HCl solution from which the desired product **25** was extracted with EtOAc. The organic layer was washed with water, dried over $MgSO_4$, and concentrated to afford a crude product which was purified by preparative TLC on silica gel using CH_2Cl_2/MeOH (95/5) as eluent to afford 0.10 g (89%) of **25** which coeluted with an authentic sample of the corresponding nontritiated compound.

Methyl 4-[(2-Butyl-5-chloromethyl-*t*-4-*t*-1*H*-imidazol-1-yl)-methyl]benzoate, Hydrochloride (26). To a solution of 0.1 g (0.33 mmol) of **25** in 5 mL of chloroform cooled to 0 °C was slowly added 0.11 g (0.196 mmol) of thionyl chloride. The resulting mixture was stirred for 1.5 h at 0 °C and then concentrated. The residue was taken up in a few milliliters of toluene and concentrated. This operation was reproduced three times in order to eliminate all traces of thionyl chloride and afforded the desired **26** which was directly used in the next step to prepare **27**.

Methyl 2-[[[2-Butyl-1-[[4-(methoxycarbonyl)phenyl]-methyl-4-*t*-]-1*H*-imidazol-5-yl]methyl-*t*]amino]-4-azido-benzoate (27). This product was prepared following the same procedure described for the preparation of **13** starting from **26**. Its purification has been carried out by preparative TLC on silica gel using toluene/EtOAc (3/2) as eluent to afford **27** which coeluted with **13**.

2-[[[2-Butyl-1-[(4-carboxyphenyl)methyl]-4-*t*-1*H*-imidazol-5-yl]methyl-*t*]amino]-4-azidobenzoic Acid (28). This product was prepared following the same procedure described for the preparation of **14** starting from **27** instead of **13**. Its purification has been carried out by preparative TLC on silica gel using EtOAc/HCO_2H/H_2O (60/5/35) as eluent to afford **28** which coeluted with **14**. The chemical purity of **28** has been measured to >99.2% by HPLC using the following conditions: Lichrospher RP C8, 5 mm, 1 mL/min, eluent: KH_2PO_4 5 mM, pH 3/H_3PO_4/acetonitrile (60/40), detection UV 247 nm and beta counter. The product has been obtained with a specific activity of 22.6 Ci/mmol.

Biology. Biological Assay. The ability of the compounds to inhibit AII-induced contractile response was examined in the isolated rabbit aorta as previously described.[19,52] A segment of thoracic aorta was isolated, and the endothelium was mechanically rubbed off. Rings of 4 mm in length were prepared and suspended on triangular stainless steel wires in 20 mL jacketed organ baths maintained at 37 °C. One hook was suspended from a Gould-Statham UC_2 or UTC_1 transducer, and the other was fixed to a plastic support leg. Changes in isometric tension were continuously recorded. Rings were left unstretched for 30 min and were then stretched under a

passive tension of 2 g. Vessel segments were maintained in a Krebs-Henseleit solution bubbled with O_2/CO_2 (95%/5%) throughout the experiments. Three to five different concentrations of the antagonist were tested against the contraction to AII added at a concentration of 3 nM which produces the maximal response. Only one concentration of antagonist was tested in any one ring of artery. Subsequently, the concentration of antagonist reducing the response to AII by 50% (IC_{50}) was determined.

CHO Cells. CHO cells expressing the AT_{1A} receptor were kindly supplied by K. E. Bernstein (Atlanta, GA) and E. Clauser (Paris, France).

Membrane Preparations. Rat liver membranes were purified as described previously[16] and were stored frozen in liquid nitrogen before use. Crude membranes from CHO cells expressing the AT_{1A} receptor were prepared at 4 °C as follows. Cells were lysed in ice-cold 10 mM Tris·HCl, pH 7.4, and then homogenized with a Dounce homogenizer. The supernatant of a first 300*g* centrifugation was centrifuged at 48000*g* for 20 min. The pellet was resuspended in 10 mM Tris·HCl, pH 7.4, and aliquots of the membrane suspension were frozen in liquid nitrogen. Protein measurements in membrane preparations were carried out according to the method of Lowry et al.[52]

Binding Assays. Liver Membranes. Membranes (50−100 mg/assay, volume 180 mL) were incubated for 30 min at 30 °C in binding medium (50 mM phosphate, pH 7.4, 5 mM $MgCl_2$, 0.1 mg/mL bacitracin, 0.6% Me_2SO) with various amounts of the tested radioligands. Nonspecific binding was determined by addition to the assays of an excess (10^{-5} M final concentration) of either unlabeled Sar^1-AII or unlabeled nonpeptide antagonist). The assays were carried out in triplicate, in polypropylene tubes to minimize the adsorption of nonpeptide ligands. Bound radioactivity was estimated by filtration through GF/C filters (presoaked in a 1% BSA solution). Competition binding assays using [^{125}I]Sar^1-AII as tracer ligand assays were carried out with 10 μg membrane protein/assay in a 90 μL volume. The K_i values of the nonpeptide antagonists in competition experiments were calculated according to Ekins equation.[53]

CHO Cell Membranes. The binding medium was similar to that used for rat liver membranes (no BSA, 0.1 mg/mL bacitracin). The membrane amounts were 10 μg/assay (90 μL volume) and 125 μg/assay (450 μL volume) for the binding of [^{125}I]Sar^1-AII and **21**, respectively.

Intact CHO Cells. CHO cells expressing the rat AT_{1A} receptor (wild type or C121A mutant) were grown in F12 medium supplemented with 10% fetal calf serum (heat-inactivated), 100 U/mL penicillin, 100 μg/mL streptomycin, and 400 μg/mL Geneticin. Cells were plated in 12-well tissue culture clusters and grown to confluence (about 5×10^5 cells/well). After removal of the culture medium, the cells were washed twice with binding buffer (PBS, pH 7.4, 5 mM $MgCl_2$, 0.1 mg/mL bacitracin, 0.6% Me_2SO), then incubated for 4 h at 4 °C under gentle agitation, with 300 μL of binding buffer containing various radioligand concentrations. The reaction was stopped by removal of medium followed by two rapid washings with ice-cold binding buffer.

The cells were collected after addition of 400 μL of 0.1 N NaOH to each well, and the associated radioactivities estimated by either gamma or liquid scintillation counting (after neutralization by addition of 100 μL of 0.5 N AcOH).

Irreversible Binding of Unlabeled Azido Derivatives to the AT_{1A} Receptor Expressed in CHO Cells. CHO cells expressing the AT_{1A} receptor were grown in 24-well tissue culture clusters. They were incubated for 4 h at 4 °C in the dark, in the presence of the photoactivatable nonpeptide ligands **14** or **7** (50 nM and 10 nM, respectively) or photoactivatable peptide ligand (Sar^1, Val^5, Phe (4 N_3)^8)-AII (10 nM) in PBS, pH 7.4, 5 mM $MgCl_2$, 0.1 mg/mL bacitracin. After two rapid washings with ice-cold binding medium, cells were irradiated for 5 min at 0 °C at 254 nm (five TUV6 Philips lamps). Then the cells were submitted to two 30 min incubations at 30 °C in binding medium to allow dissociation of noncovalently bound ligands. Remaining AII binding sites were titrated by cell incubation in the presence of [^{125}I]Sar^1-AII (5 nM, 2 h at 4 °C).

Photoaffinity Labeling, Solubilization, and Analysis of Nonpeptide Antagonist−Receptor Covalent Complexes. CHO cells expressing the C121A mutant AT_{1A} receptor were grown to confluence in 23 cm × 23 cm square dishes as described in the preceding paragraph. Cells were washed twice with binding medium (PBS, pH 7.4, 5 mM $MgCl_2$, 0.1 mg/mL bacitracin, 0.6% Me_2SO) then incubated for 4 h at 4 °C in the dark, under gentle agitation, in 30 mL of the same medium containing the photoactivatable probe **28** (40−60 nM), in the presence or absence of an excess of either unlabeled Sar^1-AII or **14** (10^{-5} M).

The incubation medium was discarded, and each dish was rinsed twice with 30 mL of cold binding medium without bacitracin. The cells were irradiated at 0 °C for 5 min at 254 nm (five TUV6 Philips lamps), without any added medium. They were then solubilized with 10 mM phosphate, pH 6.0, 5 mM EDTA, 1 mM PMSF, 0.5 mM NEM, 1.5% Triton × 100 (33 mL/dish) for 1 h at room temperature. The detergent-treated cells were centrifuged for 1 h at 200000*g*.

After dissociation of noncovalently bound ligand (1 h, 30 °C) and EDTA complexation with the appropriate amount of $MgCl_2$ (10 mM final concentration), the solubilized samples were incubated under gentle agitation with hydroxylapatite-Biogel P.30 for 90 min at room temperature (3.2 mL gel/dish previously equilibrated in 10 mM phosphate, pH 6.0, 1 mM PMSF, 0.5 mM NEM, and 0.5% Triton × 100). The gel was then packed into a column and rinsed thoroughly. Receptor was eluted with 0.3 M phosphate, pH 6.0, 0.2% SDS, 5 mM EDTA, 1 mM PMSF, and 0.5 mM NEM, and then concentrated and dialyzed using Amicon Centricon P30 microconcentrators before SDS−PAGE analysis (four cycles, dilution with 0.1% SDS between two consecutive cycles).

Electrophoresis and Autoradiography. SDS−PAGE of covalent nonpeptide antagonist−receptor complexes was carried out in reducing conditions on 12.5% acrylamide gels. The gels were treated with EN^{3}HANCE from Dupont-NEN before autoradiography using Kodak XAR-5 films.

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