# Angiotensin Analogues Palmitoylated in Positions 1 and 4

Lenka Maletínská, Witold Neugebauer, Jacqueline Pérodin, Marie Lefebvre, and Emanuel Escher*

*Département de Pharmacologie, Faculté de Médecine, Université de Sherbrooke, Sherbrooke, Québec J1H 5N4, Canada*

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Lipidated angiotensin II (Ang) agonists and antagonists were synthesized and evaluated for their biological activities for eventual use as antimyoproliferative agents. Solid phase peptide synthesis was used for the assembly of the peptides with the Fmoc protection scheme. *N*-Acetyl-Ser[1] Ang was palmitoylated on the serine hydroxyl function. The nonpalmitoylated analogue retained one-third of Ang's affinity toward the $AT_1$ receptor on bovine adrenal cortex membranes, and the palmitoylated analogue was essentially inactive. Upon enzymatic lipolysis or mild saponification of the palmitoylated peptide, biological activity was restored. An analogous compound of Ang, *N*-acetyl-Ser[1],*β*-D-naphthylalanine[8] ([NAcSer[1],D-Nal[8]]Ang), was a pure antagonist on rabbit aorta but with lower affinity. Its O-palmitoylated form was inactive as well but was easily converted to the nonlipidated active form by lipolysis or saponification. Direct palmitoylation of [sarcosine[1]]Ang with palmitoyl chloride was obtained on the free phenolic hydroxyl of Tyr[4] on solid phase on an otherwise fully protected peptide. This lipopeptide was fully active, was comparable to [Sar[1]]Ang, and exhibited strongly prolonged activity. Lipolysis and saponification under mild conditions yielded standard [Sar[1]]Ang. The corresponding [Sar[1],D-Nal[8]]Ang was a potent and very long-lasting antagonist ($pA_2 = 8.1$), and its analogous palmitoyl phenyl ester in position 4 was active in its palmitoylated form (antagonist) and, again, returned to the nonlipidated form upon saponification or lipolysis. [Sar[1],Tyr[4](*O*-octadecyl)]Ang, an analogue to Tyr-palmitoylated [Sar[1]]Ang with an octadecyl phenyl ether in position 4, was also prepared. Surprisingly, the ether compound was inactive. Premature hydrolysis of the palmitoyl phenyl ester peptide was excluded by HPLC analysis, and the activity of the ester peptide is attributed to a putative hydrogen bond that may be critical for biological activity. The discovery of potent biologically active lipidated antagonists of Ang gives access to potential antimyoproliferative agents with numerous application possibilities.

## Introduction

Drug delivery and bioavailability problems hinder a lot of otherwise interesting peptides to be introduced into standard clinical use. This problem has been aptly demonstrated by the vasoactive peptide angiotensin II (Ang, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) where literally hundreds of analogues, particularly those with an antagonistic character, have been prepared up to now. Unfortunately, all those analogues were characterized by a total lack of oral bioavailability. Furthermore, all analogues with antagonistic properties in *in vitro* assays had partial agonistic effects *in vivo* and had a biological half-life of only a few minutes.[1,2] Structural modifications that could reduce such disadvantages and lead to partial peptide and true non-peptide structures have been under intense investigation in the past decades.

Practically all peptide Ang antagonists are based on modifications at the C-terminal position 8 of the peptide molecule.[3] It was shown more than 30 years ago that the introduction of aliphatic amino acids to position 8 led to well-known peptide Ang antagonists (e.g., Saralasin, [Sar[1],Ala[8]]Ang[4]). Increasing the aromatic ring size of phenylalanine generally decreased progressively agonist efficacy toward pure antagonism in *in vitro* assays, whereas changes from the L- to the D-configuration directly conferred antagonistic properties to Ang analogues. Increasing the ring size and the ensuing increase in hydrophobicity also augmented the duration of action, producing insurmountable antagonists. [Sar[1],D-Trp[8]]Ang, [Sar[1],*β*-D-naphthylalanine[8]]Ang and [Sar[1],1-pyrenylalanine[8]]Ang are typical examples and have been shown to be quite potent antagonists.[3] Progressive introduction of halogen on the aromatic ring resulted in a similar pattern, in which the pentasubstituted series ([Sar[1],$Cl_5$Phe[8]]Ang and [Sar[1],$Br_5$Phe[8]]Ang) were particularly interesting, producing long lasting effects with *in vitro* actions of over 3 h.[5] [Sar[1],$Br_5$Phe[8]]Ang reduced endogenous Ang dependent blood pressure *in vivo*, when infused into the "low-sodium" dog model, with a continuous effect for several hours after the infusion period.[6]

The potency and the duration of action of agonists as well as antagonists are markedly enhanced when Asp is replaced with sarcosine (Sar).[7] It was proposed that the effect of the Sar residue was due to the combination of blocking aminopeptidase action ($N^{\alpha}$-methylation) and increasing the affinity of the analogues for its receptor,[8] though the exact mechanism of action is still speculative. Therefore, most of the interesting antagonists were synthesized with Sar in position 1 and showed disproportionately higher potencies when compared to antagonists containing Asp in position 1.[1] However, persistent partial agonistic effects and absence of oral bioavailability still preclude even limited clinical use.

Antagonism of analogues with changes in position 4 has also been observed.[9] Replacement of Tyr[4] by Phe gave a partial agonist with low affinity.[7,10] On the other hand, [Sar[1],Phe(*S*-ACM)[4]]Ang was described to be a

* Author to whom correspondence should be sent. Tel: (819) 564-5346. Fax: (819) 564-5400. E-mail: e.escher@courrier.usherb.ca.

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quite potent Ang antagonist,[11] and also [Sar$^1$,Tyr(*O*-Me)$^4$]Ang (Sarmesin) was shown to have lower but pure antagonistic potencies.[12] Due to the lowered potency observed with the position 4-modified Ang antagonists compared to the position 8 Ang antagonists, position 4 has been less explored.

The discovery of non-peptide antagonists of Ang renewed the therapeutic interest toward the angiotensin system, the Ang receptors in particular, and produced the first clinically accepted Ang receptor antagonist, Losartan, for the treatment of hypertension.[13] Using non-peptide analogues, two pharmacologically distinct receptors for Ang were demonstrated in mammals, and both receptors were then cloned.[14−17] The first, $AT_1$, mediates all known physiological actions of Ang, and the second, termed $AT_2$, is much less defined in its physiological role. Recent results indicate that the latter may act as a physiological antagonist toward $AT_1$.[18,19] Ang has been shown to be a potent mitogenic factor through its $AT_1$ receptor and through specific kinase cascades previously thought to be associated with growth factors only.[20−25] It is now evident that Ang contributes to various pathological situations of tissue hypertrophy and hyperplasia such as cardiac and arterial hypertrophy as well as post-angioplastic restenosis. The latter is a myoproliferative event occurring in the injured blood vessel after vascular remodeling procedures on stenosed coronary arteries. These vascular remodeling interventions have, in spite of a very favorable short-term success, an important stenosis recurrence rate (30−50%) within 6 months after the initial intervention.[26] In animal models of post-angioplastic restenosis and of neointima formation, many trophic factors, prominently Ang, have been found to contribute to vascular hypertrophy and hyperplasia.[25,27,28] Initial research on neointima inhibition with angiotensin-converting enzyme (ACE) inhibitors and then with Losartan proved interesting but with limited efficacy on certain animal models only.[29−32]

On the other hand, a peptide antagonist showed maximal efficacy in the prevention of neointima formation in the rat carotid assay, a model of post-angioplastic restenosis.[33] In this assay non-peptide antagonists, even at very high doses, exhibited reduced efficacy compared to the peptide antagonist [Sar$^1$,Br$_5$Phe$^8$]Ang. Infusion of this latter peptide at infusion rates far below those used for non-peptide antagonists practically inhibited all neointima formation and restenosis in the rat model.[34] In spite of all the disadvantages of peptide antagonists, our results obtained with the long-acting Ang antagonist gave new interest to search for antagonists of Ang with prolonged action for the prevention of post-angioplastic restenosis.

Medication for the prevention of post-angioplastic restenosis may be done by the usual means of oral administration during the weeks and months following angioplasty, with the usual considerations of bioavailability, toxicity, target concentration, and therapeutic half-life. On the other hand, angioplasty and other vascular remodeling techniques are limited invasive procedures that permit, during the actual process, either to deliver a limited amount of medication to the dilated site or to leave in place an intraluminal prosthesis (stent). It appears that local medication could therefore be applied in depot form to the site of treatment during the remodeling process without additional intervention, if this medication can be made available in an appropriate depot form. The present contribution therefore puts forth the necessary analogue characteristics for eventual use as antimyoproliferative drugs. Much work has been put into trying to confer increased duration of action to peptide analogues. Unfortunately, most strategies to block proteolysis through peptide bond isosteres (e.g., retroinverso, deoxo, ketomethyl, introduction of D-isomers, C- or N-alkylation, and cyclizations) yielded analogues with very low biological activities in the case of Ang.[35−37] Therefore, we turned to "hydrophobic extraction" as a potential means for increased duration of action.

In our first study on lipidated analogues, position 1 of Ang was chosen as a target for potential changes.[38] Peptides containing Ser(*O*-Pal) or Cys(*S*-Pal) in position 1 (i.e., with a free amino group) were prone to the well-known O → N or S → N acyl shift producing N-acylated peptides that are inactive and which may not be reactivated. Problems with these compounds were avoided by using deamino derivatives of the amino acids mentioned above, i.e., lactic acid and 3-mercaptopropionic acid, respectively. The low potency observed with these analogues in their nonlipidated form led us to search for other modification strategies. Lipidation of the peptides was expected to decrease the susceptibility of the peptides to proteolytic degradation by extracting these peptides from the proteolytic activity in the aqueous compartment and to release on a day-to-week time scale the active peptides after lipolytic activity from the lipid compartment into the initial aqueous compartment. Slow lipolysis was a further goal, and our initial study showed that *O*-palmitoyl peptides had a 2-fold longer half-life than *O*-decanoyl peptides under the same enzymatic lipolysis conditions.[38] Hence, the structural modifications of peptides with cleavable lipid groups offer means for increased proteolytic stability and prolonged half-life and tissue residence time due to partitioning behavior. Peptides with prodrug characteristics could be deposited and stored in the affected tissues and would be continuously activated through lipolytic cleavage.

The present study therefore explores other modification schemes that permit the synthesis of stable peptide lipid esters that, upon lipolysis, produce Ang analogues of sufficient potency to be of interest for eventual use as antagonists against post-angioplastic restenosis.

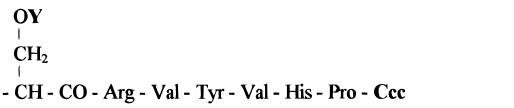
## Chemistry

Ang analogues were modified in positions 1, 4, and 8. First, we carried out several position 1 modifications, and then the attachment of a fatty acid was performed in order to obtain the lipopeptides. After structure−activity studies of the agonists, changes in position 8 were made and the corresponding antagonists were obtained. The same scheme was applied for palmitoylation of Tyr in position 4. The structures of the Ang analogues are shown in Table 1, and the physicochemical properties of all synthesized analogues are shown in Table 2.

Both Boc/TFA/HF and Fmoc/piperidine/TFA strategies were used for the synthesis of these compounds. Peptides **1**−**4** and **13** were synthesized by the Boc strategy as described previously.[38] Compound **9** was

**Table 1.** Structure of Ang Analogues[a]

A. General Structures of Position 1 Lipidated Peptides




| compd no. | structure | X | Y | Ccc |
|---|---|---|---|---|
| **1** | [Ser$^1$]Ang | $NH_2$ | H | Phe |
| **2** | [Lac$^1$]Ang | H | H | Phe |
| **3** | [Lac(*O*-Pal)$^1$]Ang | H | Pal | Phe |
| **4** | [Lac$^1$,D-Phe$^8$]Ang | H | H | D-Phe |
| **5** | [Ac-Ser$^1$]Ang | NHAc | H | Phe |
| **6** | [Ac-Ser(*O*-Pal)$^1$]Ang | NHAc | Pal | Phe |
| **7** | [Ac-Ser$^1$,D-Nal$^8$]Ang | NHAc | H | D-Nal |
| **8** | [Ac-Ser(*O*-Pal)$^1$,D-Nal$^8$]Ang | NHAc | Pal | D-Nal |

B. General Structures of Position 4 Lipidated peptides

**Aaa - Arg - Val - Tyr (O-Z) - Val - His - Pro - Ccc**

| compd no. | structure | Aaa | Z | Ccc |
|---|---|---|---|---|
| h-Ang[b] | | Asp | H | Phe |
| **9** | [Sar$^1$]Ang | Sar | H | Phe |
| **10** | [Sar$^1$,Tyr(*O*-Pal)$^4$]Ang | Sar | Pal | Phe |
| **11** | [Sar$^1$,D-Nal$^8$]Ang | Sar | H | D-Nal |
| **12** | [Sar$^1$,Tyr(*O*-Pal)$^4$,D-Nal$^8$]Ang | Sar | Pal | D-Nal |
| **13** | [Sar$^1$,Tyr(*O*-$C_{18}H_{37}$)$^4$]Ang | Sar | *n*-$C_{18}H_{37}$ | Phe |

[a] Lac = lactyl, Pal = palmitoyl, D-Nal = D-$\beta$-naphthylalanine, Ac = acetyl. [b] Human angiotensin II, [Asp$^1$,Ile$^5$]Ang.

**Table 2.** Physicochemical Properties of Ang Analogues[a]

| | MW | | | HPLC | | |
|---|---|---|---|---|---|---|
| compd no. | calcd | found ($ES^+$/MS) | TLC $R_f$ | $t_R$ (min) | % $CH_3CN$ gradient | yield (%) |
| h-Ang | 1046.3 | | 0.33 | 6.4 | 26 | |
| **1** | 1003.2 | 1004.0 | 0.33 | 6.2 | 25 | 43 |
| **2** | 989.2 | 989.5 | 0.41 | 7.2 | 29 | 25 |
| **3** | 1227.5 | 1227.04 | 0.59 | 16.2 | 65 | 3 |
| **4** | 989.2 | 988.67 | 0.41 | 7.2 | 29 | 30 |
| **5** | 1045.1 | 1045.68 | 0.45 | 7.2 | 29 | 15 |
| **6** | 1283.3 | 1283.90 | 0.61 | 16.0 | 64 | 5 |
| **7** | 1096.2 | 1096.7 | 0.59 | 8.8 | 35 | 11 |
| **8** | 1334.5 | 1334.9 | 0.65 | 15.8 | 63 | 6 |
| **9** | 987.2 | 987.92 | 0.33 | 6.0 | 24 | 35 |
| **10** | 1226.5 | 1225.80 | 0.54 | 15.2 | 61 | 4 |
| **11** | 1038.3 | 1037.64 | 0.54 | 8.0 | 32 | 10 |
| **12** | 1276.6 | 1276.37 | 0.58 | 15.3 | 61 | 7 |
| **13** | 1239.2 | 1240.33 | 0.56 | 17.2 | 69 | 6 |

[a] TLC was performed in a solvent system of butanol/AcOH/water, 5:2:3. HPLC was carried out on a $C_{18}$ reversed phase column; elution with the gradient 0−95% $CH_3CN$ in 0.05% TFA was used. Calculation of yield is based on the resin capacity.

used as a standard to confirm the correct sequence of the common C-terminal heptapeptide and was synthesized by Boc and Fmoc strategies.

For the synthesis of most peptides and related non-lipopeptides, the Fmoc strategy was used. Previous synthesis using the Boc scheme resulted in small yields probably due to the HF treatment that cleaves the less stable ester bond of the lipopeptides.

On-resin palmitoylation was used for the synthesis of all lipopeptides (compounds **3**, **4**, **6**, **8**, **10**, and **12**) using palmitoyl chloride in the presence of DIEA. Palmitoylation was carried out as the very last step of the synthesis. Acidic conditions during TFA cleavage and purification on reversed phase $C_{18}$ columns kept the susceptible lipopeptides relatively stable, and hydrolysis was minimized.

The use of a deamino derivative of Ser, i.e., lactyl, for lipopeptide synthesis solved the problem of O → N acyl shift. Unfortunately, [Lac$^1$]Ang is not a very potent agonist (5−8% of Ang), and therefore we searched for another possibility in order to avoid the problem of transpalmitoylation. Previous acetylation of the $NH_2$ group of Ser showed to be sufficient since no transacylation occurred in this case. Mild saponification cleaved the palmitoyl group completely, demonstrating the presence of only O-palmitoylation. For the palmitoylation of Ac-Ser in position 1-containing peptides (compounds **6** and **8**), a special protection scheme was used. Fmoc-Ser(*O*-Trt) was coupled to the heptapeptide-resin, and after Fmoc deprotection and N-acetylation, the trityl group was specifically cleaved by 1% TFA for 5 min, leaving the other protecting groups intact[39] before palmitoylation. This protection scheme requested side chain protection of His which was different from the widely used Trt. Both Boc and Dnp protection were appropriate, and Boc protection was preferred since the subsequent cleavage was easier to perform.

Synthesis of compounds **10** and **12** was provided with a unprotected side chain of Tyr. It has already been demonstrated that Fmoc-Tyr can be successfully incorporated without side chain protection.[40,41] Palmitoylation was performed as a last step after coupling of Sar to position 1. In spite of the reputed instability of the phenyl ester bond, peptides **10** and **12** were obtained in reasonable yields.

For synthesis of the antagonists (compounds **7**, **8**, **11**, and **12**)—with the exception of compound **4**—D-$\beta$-naphthylalanine (D-Nal) in position 8 was used. Fmoc-D-Nal was attached to Wang resin using the mild method reported by Sieber[42] in order to avoid racemization during (dimethylamino)pyridine activation.

## Results and Discussion

**Structure−Activity Relationship.** The basic pharmacological properties of Ang analogues were examined using two biological assays. $AT_1$ receptor affinities were determined by binding studies on bovine adrenocortical membranes. Functional properties were provided by rabbit aorta strip contractions *in vitro*. Table 3 summarizes p$D_2$ or p$A_2$, p$K_D$, and relative affinity (RA) values obtained for all compounds. Commercial human angiotensin was used as standard. To evaluate the potencies of each group of compounds, biological assays of all peptides with Phe in position 8 were done first, and all these analogues exhibited agonistic effects.

As described earlier, if Asp in position 1 is replaced by Ser **(1)**, binding affinities and activities on aorta are decreased.[38] Replacement of Ser by the deamino derivative Lac **(2)** led to a more important decrease in its binding affinity as well as in its biological activity compared to Ang. The corresponding antagonist [Lac$^1$,D-Phe$^8$]Ang (**4**) showed a purely competitive antagonism (Figure 2a), unfortunately with very low potencies (p$A_2$ = 5.31).

Contrary to the deamino−amino modification in **2**, acetylation of Ser in position 1 did not significantly lower biological potencies, and it has activities comparable to [Ser$^1$]Ang (**1**; Table 3). As expected, [Ac-Ser(*O*-Pal)$^1$]Ang (**6**) was almost inactive and became active after mild saponification or lipolysis (see below). Syn-

**Table 3.** Biological Properties of Ang Analogues[a]

| compd no. | rabbit aorta contraction | | | binding to adrenocort | |
|---|---|---|---|---|---|
| | $pD_2$[b] | $pA_2$[c] | RA[d] | $pK_D$[e] | RA[d] |
| h-Ang | 8.33 ± 0.09 | | 100 | 8.24 ± 0.06 | 100 |
| **1** | 7.68 ± 0.15 | | 22.40 | 8.12 ± 0.11 | 75.76 |
| **2** | 7.23 ± 0.28 | | 7.95 | 6.98 ± 0.26 | 5.53 |
| **3** | 5.46 ± 0.28 | | 0.13 | 5.56 ± 0.05 | 0.21 |
| **4** | | 5.31 ± 0.19 | | 5.19 ± 0.05 | 0.09 |
| **5** | 7.54 ± 0.24 | | 16.23 | 7.75 ± 0.08 | 32.36 |
| **6** | <5.5 | | | 6.31 ± 0.01 | 1.17 |
| **7** | | 6.99 ± 0.18 | | 6.49 ± 0.07 | 1.78 |
| **8** | | <5.5 | | 5.65 ± 0.10 | 0.21 |
| **9** | 8.57 ± 0.23 | | 173.98 | 8.85 ± 0.15 | 407.38 |
| **10** | 7.61 ± 0.36 | (7.44 ± 0.09) | | 8.45 ± 0.06 | 162.18 |
| **11** | | 8.10 ± 0.10 | | 7.78 ± 0.08 | 34.67 |
| **12** | | 6.99 ± 0.17 | | 6.65 ± 0.10 | 2.57 |
| **13** | <5.0 | | <0.05 | 5.81 ± 0.18 | 0.37 |

[a] Values are expressed as mean ± SEM. Activities were measured within the concentration range of $5 \times 10^{-11}$–$10^{-5}$ M for each peptide (at least three evaluations for each value were carried out). [b] $pD_2$ is the negative log of the dose of agonist that produces half-maximal contraction. [c] $pA_2$ is the negative log of the dose of antagonist that reduces response of a double dose of Ang to that of a single dose. [d] RA is the relative activity (affinity) of the analogue compared to the affinity of Ang (Ang = 100%). [e] $pK_D$ is the negative log of the dissociation constant $K_D$ calculated from the individual $IC_{50}$ values with the Cheng–Prussof equation.[60]

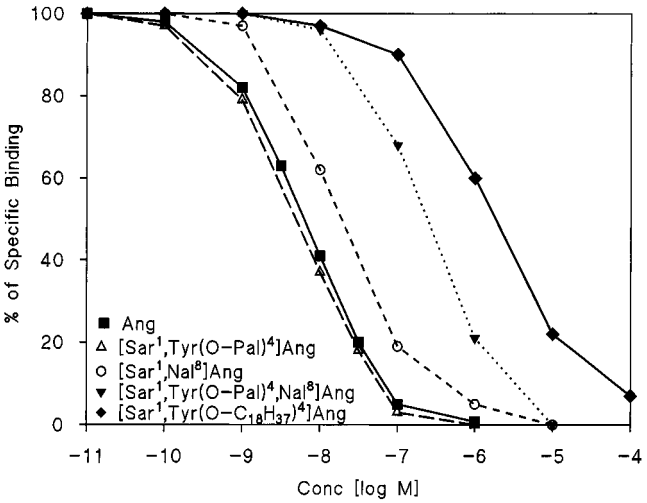



**Figure 1.** Displacement of [$^{125}$I]Ang analogues on adrenocortical membranes. The values are expressed as a percent of the value obtained with 30 pM [$^{125}$I]Ang alone. (The shown results are a representative example of at least three identical experiments carried out in duplicate.)

thesis of compound **7** with D-Nal in position 8 led to antagonistic features, but with a disappointingly low affinity.

Our search for possible sites of palmitoylation brought us back to antagonists with Sar in position 1. First, [Sar$^1$]Ang was used for palmitoylation in position 4 on the resin. Changes in position 4 were investigated in the past, but lower potencies and partial agonistic features were observed.[9] In our case, compound **13** containing a lipid ether bond was examined first in order to assess the activity on a stable compound. An Ang analogue with Tyr(*O*-$C_{18}H_{37}$) in position 8 has already been assayed[3] and was found inactive. This amino acid was introduced into position 4, and the resulting compound **13** had very low binding affinity to the $AT_1$ receptor (see Figure 1) and was inactive on the aorta assay.

On the other hand, [Sar$^1$,Tyr(*O*-Pal)$^4$]Ang **(10)**, which differs from **13** just by the change from $CH_2$ to C=O on the α carbon of the aliphatic chain (phenyl ester *versus* phenyl ether), showed high affinity to adrenocortical membranes (Figure 1) which was comparable to that of Ang. The compound was completely stable in a buffered solution (pH 7.5) at room temperature for up to 10 h and no hydrolysis occurred during the binding test (controlled by HPLC analysis). The difference of affinities could be explained by a possible hydrogen bonding of the para-substituent in position 4 of Ang. It has been proposed[10] that substituents on the aromatic side chain in position 4 can act as a hydrogen-bridge acceptor. Therefore, [Sar$^1$,Tyr(*O*-Pal)$^4$]Ang as well as [Sar$^1$]Ang dispose of such an ability (free carboxyl group or hydroxyl group, respectively) in contrast to analogue **13**. This hypothesis can also explain the lower potencies of Sarmesin and [Sar$^1$,Tyr(*O*-Et$^4$)]Ang which also contain a phenyl ether bond.

On rabbit aorta tests, [Sar$^1$,Tyr(*O*-Pal)$^4$]Ang (**10**) behaved like a full agonist, however with a $pD_2$ of about 1 log unit lower than expected from the binding experiment (Table 3). A special phenomenon was observed when searching for long-lasting effects of this analogue. Subsequent cumulative dose–response curves of Ang were done 30 min and 3 h after a single dose of **10** (washing every 20 min). A suppression of the Ang curves—a shift of the slope toward the right as well as a decrease of the maximum—was observed. This feature was strong even 40 min after the addition of peptide **10**, and it still remained at about 50% even after 3 h of washing whereas, normally, Ang sensitivity is fully recovered after 30 min. The palmitoyl on Tyr$^4$ is probably associated with a lipophilic part of the receptor and is only slowly released during washing. The long-lasting presence of this compound near or in the receptor seems to cause a prolonged desensitization of the receptor.

For the corresponding antagonists, the bulky and hydrophobic naphthylalanine was used in its D-form because it is known that [Sar$^1$,D-Nal$^8$]Ang is an antagonist without partial agonistic features in the rabbit aorta assay and with prolonged duration of action.[3] The antagonists [Sar$^1$,D-Nal$^8$]Ang (**11**) and [Sar$^1$,Tyr(*O*-Pal)$^4$,D-Nal$^8$]Ang (**12**) showed strongly prolonged action on aorta (in the case of **11**, around 1.5 h to reach one-half of the original response of Ang; in the case of **12**, more than 3 h of repeated washing). Both **11** and **12** were pure antagonists on the aorta assay of which Figure 2b shows a typical experiment. Opposite to reversible competitive antagonism (as in Figure 2a), characterized by a parallel shift of the concentration–response curves to the right while still reaching the same maximal contraction, is the case of compounds **7**, **11**, and **12** which are so-called "slowly reversible" or "insurmountable" antagonists. This is characterized not only by a shift of the concentration–response curves but also by a decrease of the maximal response, especially at higher doses of the antagonist.[43] This feature was already observed with high concentrations of Saralasin, with small concentrations of [Sar$^1$,Br$_5$Phe$^8$]Ang[5,44] and with some non-peptide antagonists of Ang.[45,46] In this case, $pA_2$ values cannot be calculated by a Schild plot; however, it is possible to estimate the $pA_2$ from experiments with lower concentrations of antagonist, though these values may be overestimated. As seen in Table 3, estimated $pA_2$ values were however in good correla-

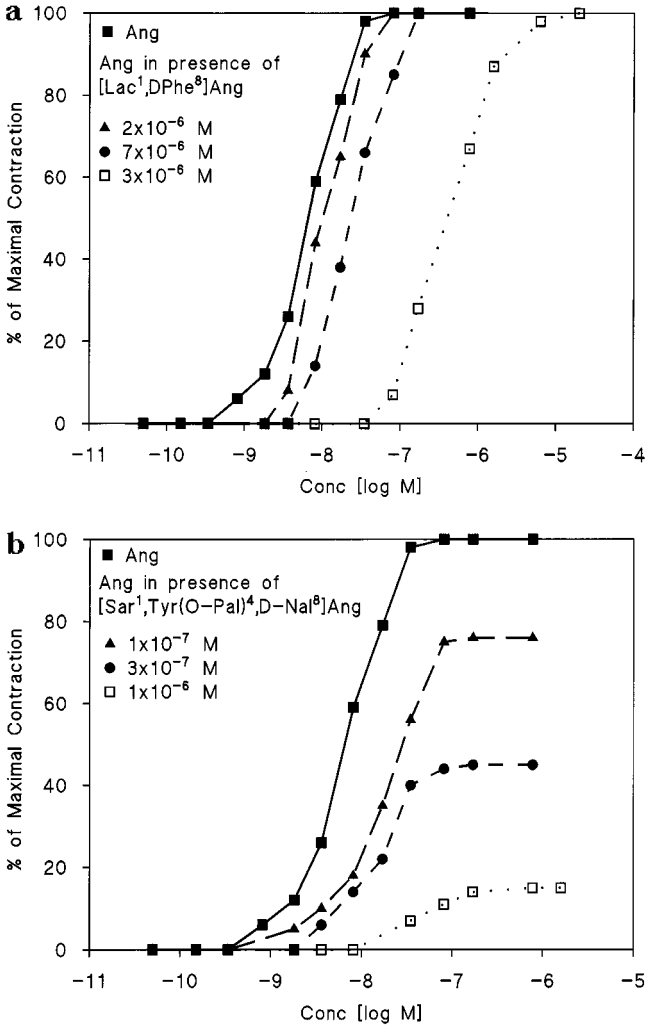



**Figure 2.** Effect of increasing concentrations of (a) [Lac[1],D-Phe[8]]Ang and (b) [Sar[1],Tyr(*O*-Pal)[4],D-Nal[8]]Ang on the contractile responses to Ang on rabbit aorta strips. Values are expressed in percent maximal contraction obtained by $10^{-7}$ M Ang (representation is a typical example of at least three identical experiments).

tion with the corresponding p$K_D$ values. In conclusion, [Sar[1],D-Nal[8]]Ang (**11**) is a quite potent long-lasting antagonist. [Sar[1],Tyr(*O*-Pal)[4],D-Nal[8]]Ang can be converted into compound **11** by hydrolysis or lipolysis, and it enhances the prolonged action of [Sar[1],D-Nal[8]]Ang. Therefore, this analogue is a prime candidate antagonist for later use in our model of post-angioplastic restenosis.

**Saponification and Lipolysis of Lipopeptides.** Peptides containing ester or phenyl ester bonds can be easily hydrolyzed and transformed to their nonlipidated form. All *O*-acylated peptides were hydrolyzed under basic conditions in order to confirm the ester nature of the fatty acid−peptide bond. The palmitoyl group was removed from the lactyl ester **3** after 1 h of hydrolysis in 1 M NaOH. [Ac-Ser(*O*-Pal)[1]]Ang (**6**), containing the same type of bond, was already completely hydrolyzed after 1 h in 0.1 M KOH, probably because of intramolecular activation. The susceptibility of the phenyl ester was also high since 0.1 M KOH treatment for 1 h cleaved all palmitoyl phenyl esters (**10** and **12**) whereas the phenyl ether peptide **13** was completely stable under these conditions. To figure out the stability of the lipopeptides for biological assays, incubation of these compounds in a Tris/HCl-buffered solution (pH 7.5) at room temperature was carried out followed by HPLC

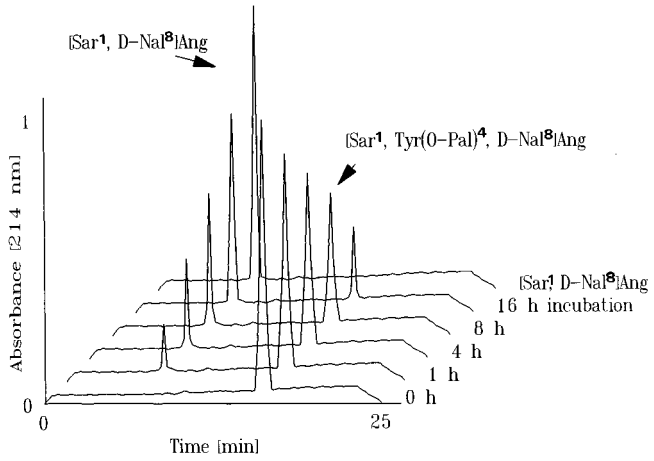



**Figure 3.** Reversed phase HPLC of [Sar[1],Tyr(*O*-Pal)[4],D-Nal[8]]-Ang during lipolysis. A gradient of 0−95% acetonitrile in the presence of 0.05% TFA was used for elution with a flow rate of 1 mL/min. Peptides were incubated with lipase from *C. cylindracea* (300 units/sample) in a Tris/HCl buffer, pH 7.5, at 37 °C.

analyses. Compounds **6** and **10** were hydrolyzed up to 50% after 2 days of incubation. The corresponding antagonists **8** and **12** were more stable since they were hydrolyzed up to about 50% only after more than 3 days.

Selective enzymatic cleavage of the ester or phenyl ester bond was provided using lipase from *Candida cylindracea* (LCC) as described previously.[38] There are several advantages in using LCC such as water solubility, possibility of incubation in organic cosolvents (DMSO), commercial availability, and the previously described use in enzymatic peptide synthesis.[47,48] Optimum pH (7.5) and temperature (37 °C) were used, and lipolysis was followed by HPLC analyses (Figure 3). Hydrolysis of the peptides during incubation was monitored using a control sample (peptide incubated without the enzyme). Time courses of lipolysis of compounds **3**, **6**, **10**, and **12** are shown in Figure 4a. The peak area of depalmitoylated peptide was calculated versus the log-scale of time. All peptides were treated with 300 units of LCC/peptide sample. Linear regression curves show different speeds of lipolysis for each peptide. While the half-life of [Lac(*O*-Pal)[1]]Ang (**3**) is about 35 h, the half-life of compounds **10** and **6** is only about 2 h. On the other hand, antagonist **12**, containing the bulky D-Nal, is a lesser substrate for lipase since it is depalmitoylated more slowly (half-life of about 8 h). When higher concentrations of LCC were used, the speed of lipolysis was increased. Figure 4b shows the time course of lipolysis of **12** with three different concentrations of LCC. A 10-fold increase of the concentration of LCC, i.e., to 3000 units/sample, changed the half-life from 8 h to 15 min. In the case of other lipopeptides (**6**, **8**, and **10**), the buffer-induced solvent hydrolysis was approximately as fast as in the case of **12**, i.e., with a half-life of about 16 h. Not only did the lipopeptide **3** have a long half-life toward lipolysis but the hydrolysis at 37 °C and pH 7.5 was also extremely long with a half-life of hydrolysis of about 128 h.

The prolonged half-life of D-Nal[8]-containing peptides, when compared to Phe[8]-containing peptides, can be an advantage for future *in vivo* use. Analogue **12**, containing non-DNA-coded amino acids in both the N- and C-terminal positions, is even more protected by palmi-

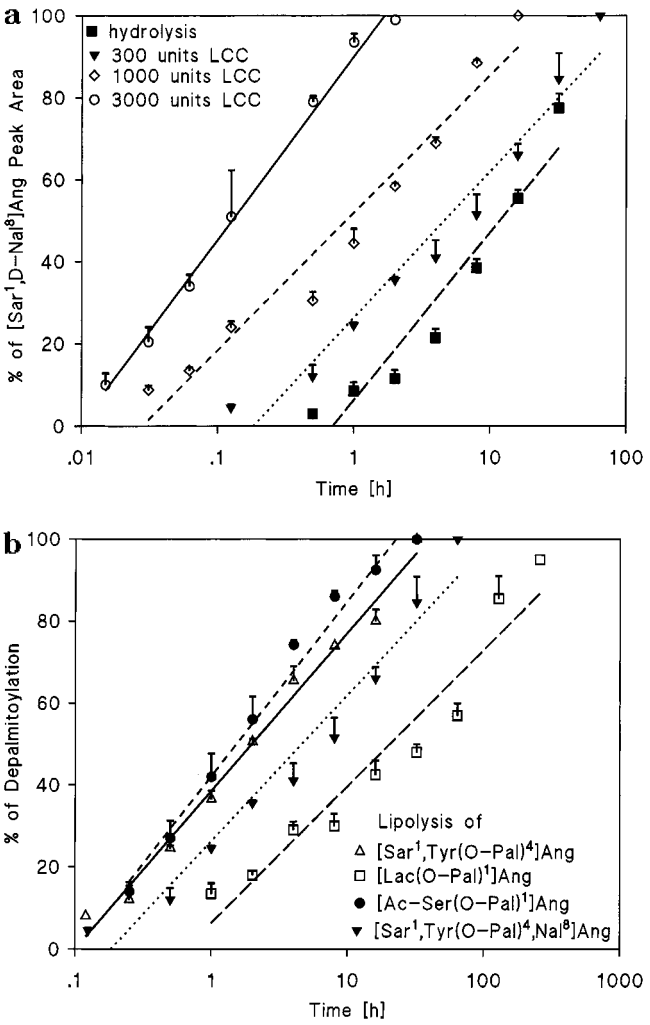



**Figure 4.** (a) Time course of lipolysis of O-palmitoylated lipopeptides. Peptides were incubated with lipase from *C. cylindracea* (300 units/sample) in Tris/HCl buffer, pH 7.5, at 37 °C and then analyzed by HPLC. Depalmitoylation, i.e., peak area of depalmitoylated peptide, was calculated versus log-scale of time. Values are expressed as mean ± SEM ($n = 3$). (b) Time course of hydrolysis and lipolysis of [Sar$^1$,Tyr(*O*-Pal)$^4$,D-Nal$^8$]Ang. The peptide was incubated with different concentrations of lipase from *C. cylindracea* (LCC, values in units per sample) or without LCC (hydrolysis) in a Tris/HCl buffer, pH 7.5, at 37 °C and then analyzed by HPLC. Peak area of [Sar$^1$,D-Nal$^8$]Ang was calculated versus log-scale of time. Values represent the mean ± SEM ($n = 3$).

toylation since lipolysis of the phenyl ester bond is significantly slower.

## Conclusions

Lipid masking of Ang analogues in positions 1 and 4 was explored in order to obtain bioactive depot analogues. O → N acyl shift of the free amino group-containing lipopeptides was prevented by the use of deamino derivatives or by acetylation of the corresponding amino acid, although this modification led to partial loss of biological potency. The modification in position 4, i.e., palmitoylation of Tyr, was successful and gave a surprisingly potent analogue, [Sar$^1$,Tyr(*O*-Pal)$^4$]Ang. The phenyl ester but not the ether bond of the lipid chain attached to Tyr$^4$ preserves the biological potencies of the analogue.

Corresponding antagonists [Sar$^1$,D-Nal$^8$]Ang and [Sar$^1$,-Tyr(*O*-Pal)$^4$,D-Nal$^8$]Ang are quite active analogues with prolonged action and slowly reversible antagonistic features. The speed of lipolysis is higher in the case of agonists but slower in the case of antagonists. The antagonist [Sar$^1$,Tyr(*O*-Pal)$^4$,D-Nal$^8$]Ang is a very promising agent in the model of post-angioplastic restenosis.

## Experimental Section

**General.** Protected standard amino acids were purchased from Advanced Chemtech (Louisville, KY) and Novabiochem (San Diego, CA). Fmoc-His(Boc) and Fmoc-His(Dnp) were from Bachem California (Torrance, CA). Fmoc-D-$\beta$-Nal and Fmoc-Ser(*O*-Trt) were from Bachem Biosci (Bubendorf, Switzerland). $^{125}$Iodine was obtained from Amersham (Oakville, Canada) and Iodo-Gen from Pierce (Rockford, IL). Lipase from *C. cylindracea* and human angiotensin II were purchased from Sigma (St. Louis, MO). All other reagents were from Aldrich (Milwaukee, WI) or Sigma (St. Louis, MO) and were of analytical grade. Solvents for solid phase peptide synthesis were redistilled before use. TLC was performed on silica gel 60-$F_{254}$ aluminum sheets (Merck, Darmstadt, Germany).

Melting points (mp) were determined in open capillaries and are uncorrected. IR spectra were carried out on a Perkin-Elmer 457 instrument in $CHCl_3$ solution (absorption in $cm^{-1}$, s = strong, m = medium, w = weak). $^1$H-NMR spectra were recorded on a Varian T60 spectrometer; chemical shifts are indicated in ppm against tetramethylsilane (s = singlet, d = doublet, t = triplet, m = multiplet).

**Synthesis of Boc-Tyr(*O*-$C_{18}H_{37}$).** This compound was synthesized by the Williamson synthesis. Sodium (0.04 mol) was dissolved in 100 mL of absolute ethanol, and 0.02 mol of Boc-Tyr dissolved in 20 mL of ethanol was added. The solution was vigorously stirred while 0.02 mol of $C_{18}H_{37}Br$ in 20 mL of ethanol was added. The mixture was stirred at 60 °C under reflux for 2 h and then at room temperature overnight. The reacting mixture was heated for 6 h at 60 °C, and alcohol was evaporated under reduced pressure. The precipitate was suspended in ice−water, stirred, and acidified to pH 3. The white precipitate was filtered and the water solution extracted with ethyl acetate (unreacted Boc-Tyr). The white precipitate was dissolved in warm 2-propanol and precipitated with water. The crude precipitate was dried at room temperature and suspended in diethyl ether. The insoluble part was filtered, and the white solid was obtained (yield of 7 g, 73%, mp 87 °C). TLC was performed in $CHCl_3$/AcOH/methanol at a ratio of 95:5:3, respectively, and visualized by ninhydrin ($R_f = 0.63$). IR: 1180 and 1220 $\nu_{C-O}$(s), 2880 and 1950 $\nu_{C-H}$(s), no absorption observed between 3200 and 3400 $\nu$ (i.e., no free hydroxyl group). $^1$H-NMR ($CDCl_3$): 0.7 (t, -$CH_3$), 1.1 (m, (-$CH_2$-)$_{16}$), 1.2 (s, ($CH_3$)$_3$), 1.55 (t, -$CH_2$-), 3.7 (t, arom -$CH_2$-), 3.9 (t, chiral -CH-), 4.25 (s, NH), 6.6 (d, arom -H), 6.9 (d, arom -H).

**General Procedure for Solid Phase Peptide Synthesis. (a) Boc Synthesis.** The $N^{\alpha}$-Boc strategy was used on a Peptomat automatic synthesizer according to an earlier published procedure.[21] The Boc-protected Phe was esterified by the Cs-salt method[50] with 1 mol equiv of 2% cross-linked Merrifield resin (Sigma, St. Louis, MO). A 4-fold excess of each protected amino acid was used for the formation of symmetrical anhydrides with DCC.[51] The Boc protecting group was removed before the next coupling by 40% TFA in DCM for 15 min; 5% DIEA in DCM was used for the neutralization of the free amino function of the growing peptide. The completion of every coupling was tested with the ninhydrin test,[52] and coupling was repeated if necessary. After completed synthesis, the resin was washed with DCM and dried under vacuum. Then, simultaneous cleavage of the side chain-protecting group and the resin ester was performed in liquid HF at 0 °C for 1 h. Peptide-resin (0.5−2.0 g) was placed in a Kel-F reactor of an all-Teflon-fluorocarbon cleavage instrument (Peptomat cleaver) with 10% anisole as scavenger. After removal of the reactants by flushing with $N_2$ and followed by high vacuum, the residue was washed twice with dry diethyl ether. The crude peptide was extracted with aqueous acetic acid, 50% (in the case of lipopeptides) or 25% (all other peptides), and was then lyophilized.

**(b) Fmoc Synthesis.** The first amino acid (Fmoc-protected) was attached to 4-alkoxybenzyl alcohol resin (Wang resin) according to the method of Sieber[42] using 2,6-dichlorobenzoyl chloride as acylating agent. Substitution was measured by UV determination of the 9-fluorenylmethylpiperidine after cleavage of the Fmoc group with piperidine.[52] Peptides were synthesized using *N*-methylpyrrolidinone as solvent in a mechanically agitated reactor. Protected amino acids were coupled in 2-fold excess in the presence of 2-fold excess of TBTU as activating reagent and 4-fold excess of DIEA. The Fmoc protecting group was removed by treatment with 50% piperidine in DMF for 15 min. The completion of the coupling was monitored by ninhydrin test as in the case of Boc synthesis. After completed synthesis, the resin was washed with DCM and dried under vacuum. The peptide was cleaved from the resin, and amino acid side chains were deprotected by treatment with reagent K (composition: 82.5% TFA, 5% thioanisole, 5% phenol, 5% $H_2O$, 2.5% 1,2-ethanedithiol),[53] volume 10 mL/g of resin for 3 h. Then, filtration with diethyl ether followed by centrifugation of the precipitated peptide was done. The crude peptide was dissolved in aqueous acetic acid and lyophilized.

**(c) Purification.** During purification, the peptides were analyzed by TLC and HPLC. TLC was performed in butanol/AcOH/$H_2O$, 5:2:3. Postmigration visualization was carried out first with UV fluorescence and then with Pauly reagent.[54] Analytical HPLC was carried out on a Waters 600E instrument with a Waters Bondapak $C_{18}$ reversed phase column (3.9 × 300 mm) with a flow rate of 1 mL/min and 214 nm UV detection. A gradient of 0−95% acetonitrile in 0.05% aqueous TFA was used.

Purification of non-lipopeptides was carried out on a Sephadex G-15 column (30 × 350 mm) eluted with 0.2 M AcOH. The peptide-containing fractions, detected by UV absorption (280 nm) during elution and by TLC, were pooled and lyophilized. The lyophilisate was dissolved (in water or 25% aqueous AcOH) and then loaded onto a reversed phase Michel-Miller column filled with 30 $\mu$m Nucleosil-$C_{18}$ material (Macherey-Nagel, Darmstadt, Germany). According to the amount of applied peptide, different column dimensions were used: 10 × 150 or 20 × 300 mm. Loaded samples were eluted with a gradient of $CH_3CN$ in 0.05% TFA, delivered at 8−10 × $10^5$ Pa with a FMI Lab pump, model Q650 (Fluid Metering Inc., Oyster Bay), through a 280 nm UV recorder. Lipopeptides were directly purified by reversed phase chromatography. After HPLC control pure peptide fractions were pooled and lyophilized. Peptides were considered pure if the HPLC peptide peak integral was at least 95% of total combined peak integrals and if they were homogenous in TLC. The structure of all peptides was confirmed by electrospray ionization mass spectrometry ($ES^+$/MS; VG Quattro, Manchester, U.K.).

**Ang (2−8) Resins.** Three types of C-terminal heptapeptides of the Ang sequence were used: Arg-Val-Tyr-Val-His-Pro-Phe-OH using Boc strategy, the same heptapeptide using Fmoc strategy, and Arg-Val-Tyr-Val-His-Pro-D-Nal-OH using Fmoc strategy. In both cases of peptides with Phe in position 8, Fmoc-Phe or Boc-Phe-resin with a substitution range of 0.35−0.60 mmol/g was used. In the case of Fmoc-D-Nal-resin, the substitution was lower (about 0.25 mmol/g). This peptide-resin was used for the following synthesis of all peptides. For the Boc strategy, the amino acids Boc-Arg($N^{\gamma}$-Tos), Boc-Val, Boc-Tyr(*O*-BrBzl), Boc-His($^{im}$Tos), and Boc-Pro were used. For the Fmoc strategy, the amino acids Fmoc-Arg(Pmc), Fmoc-Val, Fmoc-Tyr(tBu), Fmoc-His(Trt), and Fmoc-Pro were used (excluded were a few exceptions described below). The correct sequence of amino acids of the heptapeptide was verified by synthesis of [Sar$^1$]Ang as a standard compound. Peptides **1**−**4** have been reported earlier.

**[Ac-Ser$^1$]Ang (5) (Fmoc Synthesis).** Fmoc-Ser(*O*-Trt) was coupled to the heptapeptide-resin (0.5 mmol) containing His in position 6 with Boc or Dnp side chain protection. *N*-Acetylation was carried out by means of acetic anhydride (8-fold excess) in the presence of DIEA in DCM for 15 min. Then, the resin was dried and divided into two equal parts. Dnp protection of His was removed from one part by means of thiophenol,[56] and then, the peptide-resin was cleaved with reagent K and peptide **5** was purified.

**[Ac-Ser(*O*-Pal)$^1$]Ang (6) (Fmoc Synthesis).** The second part of the resin with [Ac-Ser$^1$]Ang was used. *O*-Trt side chain protection group of Ser was removed by mild treatment with 2% TFA for 5 min, and 6-fold excess of palmitoyl chloride and DIEA in DCM were added and left for coupling overnight. After cleavage, peptide **6** was purified.

**[Ac-Ser$^1$,D-Nal$^8$]Ang (7) (Fmoc Synthesis).** Fmoc-Ser(*O*-Trt) was coupled to the heptapeptide-resin (0.5 mmol) containing the Arg(Pmc)-Val-Tyr(tBu)-Val-His(Boc or Dnp)-Pro-D-Nal sequence. Acetylation was done as described for **5**; the resin was separated into two parts. Peptide **7** was cleaved from one part of the resin and purified.

**[Ac-Ser(*O*-Pal)$^1$,D-Nal$^8$]Ang (8) (Fmoc Synthesis).** After selective deprotection of Trt from the second part of the resin containing peptide **7**, palmitoylation was carried out overnight. The peptide was cleaved from the resin and purified.

**[Sar$^1$]Ang (9) (Boc and Fmoc Synthesis).** Peptide **9** was synthesized by both Boc and Fmoc syntheses as a standard compound. In both cases, Boc-Sar (4-fold excess) was coupled to position 1 of Ang heptapeptide-resin (Merrifield or Wang, respectively). Once the reaction was complete, the peptide-resin was subjected to HF or TFA cleavage and purified and its identity was confirmed by comparison to reference material.

**[Sar$^1$,Tyr(*O*-Pal)$^4$]Ang (10) (Fmoc Synthesis).** The C-terminal heptapeptide of Ang was synthesized as usual but using instead Fmoc-Tyr with an unprotected side chain. At last, Boc-Sar was coupled, and a 6-fold excess of palmitoyl chloride and DIEA in DCM was left to react overnight. Peptide **10** was cleaved from the resin and purified. Elemental analysis of $C_{64}H_{99}O_{11}N_{13}\cdot 4^1/_2CF_3COOH$ expected C = 49.71, H = 6.00, and N = 10.47; determined values C = 50.00, H = 6.18, and N = 10.20 (Guelph Chem. Lab., Guelph, Ontario).

**[Sar$^1$,D-Nal$^8$]Ang (11) (Fmoc Synthesis).** The peptide-resin with D-Nal in position 8 (i.e., the first amino acid attached on the resin) was synthesized, and Boc-Sar was coupled to position 1. The peptide was split from the resin by means of reagent K and purified by gel filtration followed by reversed phase chromatography.

**[Sar$^1$,Tyr(*O*-Pal)$^4$,D-Nal$^8$]Ang (12) (Fmoc Synthesis).** The peptide was synthesized as described for **10** but with D-Nal instead of Phe in position 8.

**[Sar$^1$,Tyr(*O*-$C_{18}H_{37}$)$^4$]Ang (13) (Boc Synthesis).** The Boc-Sar-Arg(Tos)-Val-Tyr(*O*-$C_{18}H_{37}$)-Val-His(Tos)-Pro-Phe octapeptide was built on the resin. Tyr(*O*-$C_{18}H_{37}$) was coupled to position 4 in 4-fold excess by means of DCC and HOBt. After synthesis, the Boc group was cleaved, and final HF treatment followed before purification of peptide **13**.

**Saponification of Lipopeptides.** Peptides were hydrolyzed with 1 M NaOH or 0.1 M KOH in methanol for 1 h. Analysis was done by HPLC followed by the test on rabbit aorta. Lipopeptides were also incubated with 50 mM Tris/HCl buffer, pH 7.5, at room temperature and analyzed by HPLC.

**Lipolysis of Peptides.** Lipolysis of the lipopeptides was performed by incubating the peptides (5 × $10^{-3}$ final concentration) and the lipase from *C. cylindracea* (final concentration 300, 1000, and 3000 units, i.e., 0.17, 0.6, and 1.7 mg/mL) at 37 °C in a buffer (50 mM Tris/HCl, pH 7.5, 50 $\mu$L final volume). Before use, all solutions were sterilized by filters with a pore size of 0.2 $\mu$m. The incubation was stopped by denaturation of the enzyme at 100 °C for 5 min, after which the samples were centrifuged (1000*g*, 5 min) in order to separate precipitated enzyme. The time course of the lipolysis was determined by analyzing the peptide content in the supernatant of samples by reversed phase HPLC. The peak area of each sample was compared to a reference sample which was also analyzed by HPLC treated in the same fashion, though without enzyme. The peak area was calculated by means of a chromatography computer program named Baseline 810 (Waters, CA).

**Binding on Bovine Adrenocortical Membranes.** Bovine adrenocortical membranes were prepared according to the procedure reported by Glossman et al.[56] [$^{125}$I]Ang was obtained by the method of iodination using Iodo-Gen.[57] Samples (0.5 mL containing about 100 mg of protein) were incubated in 25

mM Tris/HCl buffer (100 mM NaCl, 5 mM $MgCl_2$, 0.2% BSA, pH 7.4). The appropriate concentrations of peptide were added followed by the addition of [$^{125}$I]Ang to a final concentration of 30 pM. After an incubation period of 45 min at 22 °C, ice-cold buffer (25 mM Tris/HCl, 100 mM NaCl, 5 mM $MgCl_2$, pH 7.4) was added followed immediately by filtration through Gelman Science A/E glass fiber filters. The radioactivity associated with the membranes was measured by an Automatic gamma counter (Wizard 1470, Wallac, Finland). The total binding was measured in the absence of unlabeled Ang, and the nonspecific binding was established in the presence of 1 $\mu$M Ang. Nonspecific binding was always less than 10% of total binding.

**Rabbit Aorta Strip Contraction.** The test was performed according to a previously described method.[58] New Zealand rabbits were killed by stunning and exsanguination; the thoracic aorta was excised, defatted, and then cut into 5 mm rings. Four pieces of rings were placed into baths (37 °C) containing an oxygenated Krebs solution (118 mM NaCl, 4.7 mM KCl, 1.18 mM $MgSO_4$, 1.18 mM $KH_2PO_4$, 1.1 mM glucose, 25 mM $NaHCO_3$, 25 mM $CaCl_2$, pH 7.4). A tension of 2 g was applied and adjusted during a 90 min equilibration period. Isometric contractions were measured by using force displacement transducers (Grass FTO.3) and were recorded using a Grass polygraph (Grass Co., Quincy, MA). Cumulative dose−response curves for Ang, as well as for each peptide with an agonistic activity, were expressed as the percentage of the maximal stimulation obtained by Ang. In the case of antagonist, aorta rings were incubated for 10 min with different single concentrations of the analogue. Then, concentration−response curves of Ang in the absence or presence of antagonist were made. $pA_2$ values were calculated using Schild plot.[59] In the case of long-acting analogues, $pA_2$ values were estimated from lower doses. The long-lasting effect of the antagonists was monitored by cumulative curves of Ang 30 min, 1.5 h, and 3 h after the injection of the antagonist. Aorta rings were washed with buffer every 20 min.

**Blood Pressure Measurements.** Animals were anesthetized by an intramuscular injection of a ketamine−xylazine solution (87 and 13 mg/kg, respectively). Systolic and diastolic blood pressure of unconscious male Sprague−Dawley rats (300−325 g) were measured directly with a catheter (intramedic polyethylene tube, PE50) in the right carotid artery using a Grass polygraph with 23 × 1 Statham pressure transducers.

Bolus injections of antagonists and the challenging dose of Ang (volume of 0.1 mL) were administered intravenously in the left jugular vein. After the bolus injection of antagonists, the challenging dose of Ang (0.3 ng/kg) was administered 2.5 min after the antagonist followed by subsequent injections every 15 min.

**Abbreviations.** Abbreviations follow the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (*Eur. J. Biochem.* **1984**, *138*, 9−37). Additional abbreviations: Ac, acetyl; AcOH, acetic acid; Ang, angiotensin II; D-Nal, D-$\beta$-naphthylalanine; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; DIEA, diisopropylethylamine; DMF, dimethylformamide; HOBt, hydroxybenzotriazole; Lac, lactyl; LCC, lipase from *Candida cylindracea*; Pal, palmitoyl; Pyr, pyrenylalanine; TBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoracetic acid.

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