168. Lipid Masking and Reactivation of Angiotensin Analogues

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Studies on post-angioplastic restenosis have shown the implication of angiotensin II (Ang) as a myoproliferative mediator. The antiproliferative efficacy of non-peptide Ang antagonists on the rat carotid model is of 50%, whereas a continuously infused peptide antagonist at low doses totally blocks neointimal growth. To explore the feasibility of depot forms of Ang that may be introduced during angioplasty and thus prevent restenosis, lipid-masked Ang analogues of the following general structure were prepared: [Xxx^0,Yyy^1]Ang with Xxx = decanoyl or palmitoyl and Yyy = Ser, Cys, Asp, β-lactoyl, 3-mercaptopropanoyl or succinyl. All fatty acylated peptides [Xxx^0,Yyy^1]Ang were practically inactive, and *O*- or *S*-esterified Ser and Cys peptides underwent intramolecular transacylation giving inactive N^{α}-acylated peptides. O-Acylated [β-mercaptopropanoyl^1]Ang were easily hydrolyzed into their biologically active [Yyy^1]Ang forms, either by mild saponification or by lipase activity.

Introduction. – Transluminal angioplasty is an increasingly important alternative to surgery in the treatment of obstructive arteriosclerotic diseases. Despite a significant increase in primary success, post-angioplastic restenosis reduces up to 50% the long-term success of this therapy. Post-angioplastic restenosis is a myoproliferative response that has been significantly reduced in the rat model by administration of angiotensin II (Ang) antagonists. To this date, our results show that non-peptide antagonists, selective for the AT_1 receptor (*e.g.* Losartan, L-158,809), have only a partial efficacy. On the other hand, our non-selective peptide antagonist [Sar^1,Phe(Br$_5$)^8]Ang is capable of blocking neointimal proliferation altogether, even at several hundred-fold lower doses compared to non-peptide antagonists [1] [2]. Unfortunately, peptides have major disadvantages in therapeutic use, such as no oral bioavailability, an extremely short half-life, hypertensive features such as partial agonistic properties in *in vivo* assays, and non-selectivity towards the two recognized Ang receptor subtypes AT_1 and AT_2.

Amino-acid residues in position 1 of Ang analogues have been shown to contribute primarily to the duration of action of Ang [3]. Therefore, this position was chosen as a target for our structure-activity studies. Few reports indicate that lipidation of peptides can be exploited in using lipopeptides as drugs, *i.e.*, to decrease the susceptibility of peptides to proteolytic degradation [4]. Development of such new drugs may lead to vascular remodeling therapies without the complication of neointimal proliferation. Lipopeptides with pro-drug characteristics, due to their cleavable lipidic group, may be stored in target tissues and continuously activated through lipolytic cleavage after transluminal application during angioplasty.

The past several years have seen a steady increase in studies concerned with co- or post-translational modifications of membrane-associated proteins by specific lipids. This

process, termed lipidation, involves protein palmitoylation, myristoylation, and prenylation. Lipid-modified proteins are classified on the basis of the identity of the attached lipid. Palmitoylated proteins generally contain a fatty acyl group attached by a labile thioester bond to a cystein residue. The relative lability of *S*-acylation allows the process to be reversible, showing the inherent instability of the thioester bond. Myristoylation results in a stably modified *N*-acylated protein, whereas for prenylated proteins, the lipids are attached to Cys residues by a stable thioether bond. Multiple physiological roles for attached lipids are found in protein-protein communications (G-proteins, tyrosin kinases, see reviews in [5] and [6]). Protein palmitoylation is associated with regulatory processes such as lateral diffusion, signaling, and receptor endocytosis [7]. Besides the thioester linkage, *O*-ester linkage has also been demonstrated, *e.g.*, in toxins isolated from spider venoms [8] [9]. Generally, however, it has been shown that protein lipidation induces membrane association.

Fatty-acid acylation observed in regulatory processes subsequently led to the synthesis as well as the use of lipopeptides. Synthetic lipopeptides have been proven useful for immunological purposes [10–12], and recently [13], peptides containing novel dialkyl-chain amphiphiles have also been synthesized as surfactants.

In this study, the interesting features of lipopeptides, *i.e.*, their hydrophobicity and the labile ester or thioester bond, were explored on the angiotensin peptide structure in order to find new molecular tools against post-angioplastic restenosis. The anticipated transluminal application of such depot-drugs during the angioplasty procedure or by means of drug-containing stents does not request for the oral bioavailability of any compound. Therefore, modified peptide molecules are of interest as drug since such topical, single applications need much smaller amounts of substance than systemic or even oral medication.

Henceforth, masked Ang analogues were synthesized using the Boc strategy, and peptides of the following general structure were prepared: **Xxx-Yyy**-Arg-Val-Tyr-Val-His-Pro-Phe-OH with Xxx = decanoyl (Dec), palmitoyl (= hexadecynoyl; Pal) or hexadecyl (Cet) and Yyy = Ser, Cys, Asp, β-lactoyl (= 3-hydroxypropanoyl; Lac), 3-mercaptopropanoyl (Mpr), or succinyl (= butanedioyl; Suc)[1]).

In these Ang analogues, the fatty-acid (or fatty alcohol) residue Xxx^0 was attached to the residue Yyy^1 of the octapeptide through an ester or a thioester bond (*e.g.* decanoylation/palmitoylation of N^{α}-protected Ser^1 or Cys^1). These lipopeptides were prepared in order to see if lipidation would temporarily inactivate these peptides and, following hydrolysis or lipolysis, convert them to their biologically active form. The synthesis of peptides containing Ser or Cys in position 1 (*i.e.* Yyy^1 with a free NH_2 group) imposes a problematic outcome in the form of intramolecular transacylation, the well known $O \rightarrow N$ or $S \rightarrow N$ acyl shift. Problems of instability of these compounds were avoided by using deamino derivatives Yyy^1 of the amino acids Ser, Cys, and Asp, *i.e.*, β-lactic (Lac) 3-mercaptopropanoic (Mpr), and succinic (Suc) acid, respectively.

In this study, only the N-terminal position of the classical basic Ang agonist molecule was studied to simplify pharmacological evaluation. An eventually successful scheme should then be transformed to the appropriate antagonist structures.

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[1]) Abbreviations follow the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature [14].

Results and Discussion. – *Synthesis.* The structures of the synthesized Ang analogues **1**–**17** modified in position 1 are shown in *Table 1*. The C-terminal octapeptides were built on *Merrifield* resin using solid-phase peptide synthesis with the (*tert*-butoxy)carbonyl (Boc)/CF_3COOH strategy and HF cleavage of the peptide-resin ester. Crude peptides were purified by gel filtration, followed by reversed-phase (C_{18}) chromatography. Correct peptide structure was confirmed by electrospray (ES) MS and purity by HPLC and TLC. *Table 1* shows the physicochemical properties of all synthesized analogues. The correct sequence of the common C-terminal heptapeptide was confirmed by a control synthesis of [Sar^1]Ang as standard. All octapeptides were obtained in good yield without synthetic complications. For the synthesis of [Mpr^1]Ang (**12**) and [Cys(*S*-Acm)^1]Ang (**9**), Mpr or Boc-Cys with the mercapto group protected by means of the acetamidomethyl (Acm) group was used. Acm protection, resisting to CF_3COOH and HF cleavage, allowed us to obtain stable [Mpr(*S*-Acm)^1]Ang (**11**) and [Cys(*S*-Acm)^1]Ang (**9**). The Acm group was then removed with mercuric acetate under acidic conditions followed by H_2S treatment to give free [Mpr^1]Ang (**12**) and [Cys^1]Ang, the latter being less stable.

For synthesis of the lipopeptides, *i.e.*, coupling of the acid (or alcohol) Xxx^0 to position 1 of Ang (*Table 1*), different strategies were used. First, the synthesis of [O-Pal^0,Ser^1]Ang (**16**) and [*S*-Pal^0,Cys^1]Ang (**17**) was attempted. It is well known that Ser- and Thr-containing peptides can generate two acylation products of the same molecular weight by either $O \rightarrow N$ or $N \rightarrow O$ acyl shift. Even under slightly basic conditions, easy $O \rightarrow N$ acyl shift is expected [15]. In the case of Ser in position 1, the peptide chain was terminated by a *N*-acyl group instead of the desired *O*-palmitoylation. The base-catalyzed intramolecular $S \rightarrow N$ acyl shift in Cys residues occurs even faster than the $O \rightarrow N$ acyl shift observed in Ser [16]. For the synthesis of compounds **16** and **17**, N^{α}-Boc-protected Ser and Cys were used. First, both amino acids were coupled to the heptapeptide-resin with the SH group of Cys temporarily protected by trityl (Trt; cleavable with 2-mercaptoethanol [17]) and *O*-unprotected Ser. After coupling of palmitoyl chloride to the OH or the SH group of Ser or Cys, respectively, the resins were subjected to HF cleavage and the peptides extracted with 50% AcOH/H_2O to give crude *O*-palmitoylated [Ser^1]Ang and crude *S*-palmitoylated [Cys^1]Ang. To have standards for evaluation of the eventual transacylation, we also synthesized [*N*-Pal^0,Ser^1]Ang (**5**) and [*N*-Pal^0,Cys^1]Ang (**10**) by coupling of Boc-Ser(*O*-Bzl) and Boc-Cys(*S*-MeBzl) to the heptapeptide-resin, followed by CF_3COOH deprotection of the N^{α}-Boc group, reaction with palmitoyl chloride, cleavage, and extraction. Both products **5** and **10** were chemically stable against hydrolysis as described below. The crude *O*- and *S*-palmitoylated peptides were then analyzed by reversed-phase (C_{18}) HPLC and their retention times t_R (see *Table 1*) compared with those of **5** and **10**. In the case of the crude *O*-palmitoylated peptide, two peaks were detected in ratio of 65:35, corresponding to [*O*-Pal^0,Ser^1]Ang (**16**) and [*N*-Pal^0,Ser^1]Ang (**5**). As for the crude *S*-palmitoylated peptide, HPLC analysis revealed the presence of 80% of [*N*-Pal^0,Cys^1]Ang (**10**) and 20% of [*S*-Pal^0,Cys^1]Ang (**17**). After HF treatment, peptides were kept in acidic conditions in which $O \rightarrow N$ or $S \rightarrow N$ acyl shift should not occur. HPLC Analysis of the crude peptides after lyophilization showed the same results as after HF cleavage. The peptides were purified by reversed-phase HPLC (C_{18}) under acidic conditions (MeCN gradient with 0.05% CF_3COOH, pH 2) as usual. Mass spectra confirmed their respective molecular weights: 1242 for **16** and 1258 for **17** (*Table 1*). Acidic conditions during purification kept the compounds relatively stable; similar re-

Table 1. *Structure and Physicochemical Properties of Ang Analogues*

Xxx-Yyy-Arg-Val-Tyr-Val-His-Pro-Phe

0 1 2 3 4 5 6 7 8

	Structure[a])		Formula
	Xxx	Yyy	
Ang	H	Asp	$C_{50}H_{71}N_{13}O_{12}$
1	*N*-Pal	Asp	$C_{65}H_{99}N_{13}O_{13}$
2	H	Suc	$C_{49}H_{68}N_{12}O_{13}$
3	*O*-Cetyl	Suc	$C_{65}H_{100}N_{12}O_{12}$
4	H	Ser	$C_{48}H_{69}N_{12}O_{11}$
5	*N*-pal	Ser	$C_{64}H_{99}N_{12}O_{11}$
6	H	Lac	$C_{48}H_{68}N_{12}O_{11}$
7	*O*-Dec	Lac	$C_{58}H_{86}N_{12}O_{12}$
8	*O*-Pal	Lac	$C_{64}H_{98}N_{12}O_{12}$
9	*S*-Acm	Cys	$C_{51}H_{75}N_{14}O_{11}S$
10	*N*-Pal	Cys	$C_{64}H_{99}N_{13}O_{11}S$
11	*S*-Acm	Mpr	$C_{51}H_{73}N_{13}O_{11}S$
12	H	Mpr	$C_{48}H_{68}N_{12}O_{10}S$
13	*S*-Dec	Mpr	$C_{58}H_{69}N_{12}O_{11}S$
14	*S*-Pal	Mpr	$C_{64}H_{98}N_{12}O_{11}S$
15	H	Pal	$C_{65}H_{99}N_{13}O_{13}$
16	*O*-Pal	Ser	$C_{64}H_{99}N_{12}O_{11}$
17	*S*-Pal	Cys	$C_{64}H_{99}O_{13}H_{11}S$

Mol. wt.		R_f (TLC)[c])	HPLC[d])		Yield [%][e])
calc.	found[b])		t_R [min]	% MeCN (gradient)	
1046	–	0.33	6.4	26	–
1270.5	1270	0.57	14.6	58	8.8
1017.2	1016.7	0.44	7.0	28	39.5
1241.5	1242.0	0.58	16.2	65	2.0
1003.2	1004.0	0.33	6.2	25	42.9
1242.5	1242.1	0.58	14.6	58	20.9
989.2	989.5	0.41	7.2	29	25.0
1143.4	1143.4	0.58	12.4	50	6.3
1227.5	1227.0	0.59	16.2	65	2.8
1090.3	1090.8	0.35	6.4	26	36.2
1258.6	1258.1	0.58	15.0	60	11.4
1076.2	1075.5	0.40	7.8	31	22.8
1005.2	1004.8	0.39	8.0	32	60.0[f])[g])
1159.5	1158.8	0.58	13.2	53	9.8
1243.5	1242.9	0.58	16.2	65	6.8
1155.4	1155.1	0.58	15.6	62	23.0
1242.5	1241.9	0.58	15.2	61	[g])
1258.6	1257.9	0.59	15.6	62	[g])

[a]) Lac = β-lactoyl, Mpr = 3-mercaptopropanoyl, Pal = palmitoyl, Dec = decanoyl, Cetyl = hexadecyl, Acm = acetamidomethyl. [b]) Determined by ES-MS (positive mode). [c]) TLC solvent system BuOH/AcOH/H_2O 5:2:3. [d]) HPLC on C_{18} reversed-phase column, elution with gradient 0–95% MeCN in 0.05% aq. CF_3COOH solution. [e]) Calculation of yield is based on the resin capacity. [f]) Yield of [Mpr^1]Ang was calculated with respect to [Acm-Mpr^1]Ang. [g]) Unstable product.

sults were found by *Branton et al.* [18]. During Fmoc solid-phase synthesis of a tetrapeptide containing Thr(*O*-Pal) in position 3, $O \rightarrow N$ acyl shift occurred when Fmoc-Thr(*O*-Pal) was used for coupling. The authors proved that piperidin treatment during Fmoc synthesis caused transpalmitoylation. Both compounds **16** and **17** were unstable under basic conditions (1M 2-aminoethano, 1M NaOH, or 0.1M KOH, resp., 1 h of incubation) as expected. HPLC Analysis and mass spectra confirmed O or $S \rightarrow N$ transacylation with partial depalmitoylation. To evaluate the stability of compounds **16** and **17** under conditions used for biological assays, incubations of the peptides with *Tris* · HCl buffer, pH 7.4, and *Krebs* buffer were performed. HPLC Analysis after 24 and 48 h of incubation showed degradation of the peptides, with $O \rightarrow N$ and $S \rightarrow N$ shift of the palmitoyl group. Because of their instability, biological assays were not carried out on **16** and **17**.

To avoid the problem of intramolecular transpalmitoylation of palmitoylated Ser[1] and Cys[1], deamino derivatives of Ser and Cys (or Asp) (*i.e.*, Yyy[1] = Lac, Mpr, and Suc, resp.) were used for the following lipopeptide syntheses. A successful method for the synthesis of *O*-acylated [Lac[1]]Ang, *i.e.*, compounds **7** and **8**, was the reaction of the acid chlorides of the fatty acids, *i.e.*, decanoyl or palmitoyl chloride, respectively, directly with the heptapeptide-resin in presence of $(i\text{-Pr})_2$EtN. After HF treatment and extraction with 50% AcOH/H_2O, a mixture of [Lac[1]]Ang and compounds **7** or **8** in a ratio of *ca.* 1:1 was obtained (HPLC and TLC analyses; for data, see *Table 1*). Similarly, 1-bromohexadecane (= cetyl bromide) was used for coupling of the fatty alcohol moiety cetyl to the carboxyl group of succinyl in [Suc[1]]Ang (→ **3**). Synthesis of *S*-acylated [Mpr[1]]Ang **13** and **14** was carried out both in solution and in solid phase. For synthesis in solution, [Mpr(*S*-Acm)[1]]Ang (**11**) was used. After removal of the Acm group (→ **12**), the peptide

Table 2. *Biological Properties of Ang Analogues*[a])

	Rabbit aorta contraction			Binding to adrenocortical membranes		
	pD_2[b])	rel. activity[c])	*n*	pK_D[d])	rel. activity[c])	*n*
Ang	8.45 ± 0.16	100	7	8.24 ± 0.06	100	9
1	< 5.0	< 0.03	3	4.76 ± 0.09	0.03	3
2	7.01 ± 0.33	3.63	3	6.65 ± 0.10	2.57	3
3	< 5.0	< 0.03	3	5.70 ± 0.14	0.29	3
4	7.68 ± 0.15	16.99	3	8.12 ± 0.11	75.76	3
5	< 5.0	< 0.03	3	5.38 ± 0.06	0.14	3
6	7.23 ± 0.28	5.96	4	6.98 ± 0.26	5.53	3
7	5.72 ± 0.33	0.19	3	5.65 ± 0.10	0.26	3
8	5.46 ± 0.28	0.10	3	5.56 ± 0.05	0.21	3
9	7.08 ± 0.38	3.81	3	7.39 ± 0.15	13.79	3
10	< 5.0	< 0.03	3	5.75 ± 0.07	0.32	3
11	6.84 ± 0.45	2.46	3	7.04 ± 0.03	6.35	3
12	6.96 ± 0.37	3.14	3	6.68 ± 0.06	2.73	3
13	5.85 ± 0.35	0.25	3	5.98 ± 0.26	0.55	3
14	5.08 ± 0.11	0.04	3	5.55 ± 0.15	0.20	3
15	< 5.0	< 0.03	3	4.76 ± 0.09	0.02	3

[a]) Values are expressed in mean ± s.e.m., *n* = number of experiments. Activities were measured in the concentration range of $5 \cdot 10^{-11}$–10^{-5} M of each peptide. [b]) pD_2 is the negative log of the concentration of agonist that produces half-maximal contraction. [c]) Relative activity (affinity) of analogue to Ang = 100%. [d]) pK_D is the negative log of the concentration of analogue which displaces half of the specifically bound ^{125}I-Ang.

was reacted with acyl chlorides in presence of $(i\text{-Pr})_2EtN$ (slightly basic pH was necessary). The reaction was completed within 1–4 h, as exhibited by HPLC analysis. Mass spectra of the purified material showed each time a mixture of mono- and di-decanoylated or -palmitoylated peptides. Indeed, because of the unprotected status of the peptide, palmitoylation was not restricted to Mpr *S*-esterification but probably also to *O*-esterification of Tyr[4]. Finally, in spite of the instability of the thioester bond, solid-phase synthesis was the most successful. A strategy similar to that leading to the lipopeptides **7** and **8** containing Lac in position 1 was used. The resulting yield of [*S*-Acyl[0],Mpr[1]]Ang peptides **13** or **14** was of 20–50% with respect to [Mpr[1]]Ang (**12**) (HPLC and TLC analyses; for data, see *Table 1*).

Table 1 shows the yields and mass spectra of all synthesized compounds. Small yields were probably caused by incomplete acylation during coupling. Synthesis of longer and more complex peptides probably limits the use of on-resin approaches due to the greater potential of incomplete palmitoylation [19]. Mass spectra of all compounds obtained were in agreement with calculated values.

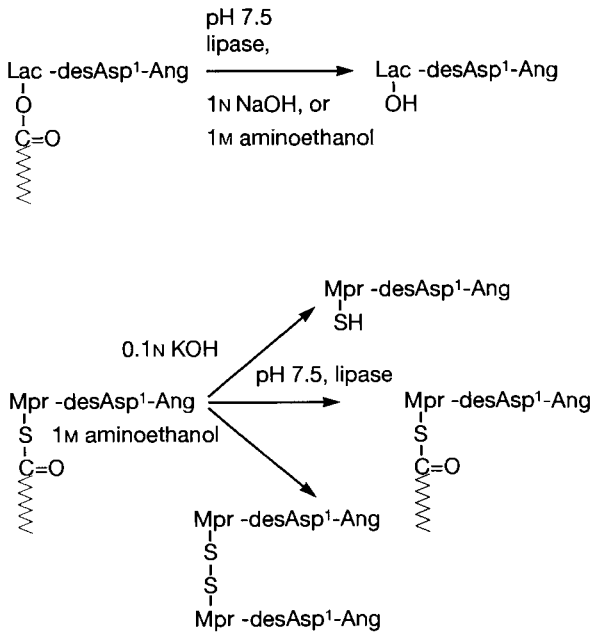
Structure-Activity Relationship. Two biological assays were used to assess the basic pharmacological properties of the Ang analogues. AT_1 Receptor affinities were determined by binding studies on bovine adrenocortical membranes. The ability of the analogues to contract rabbit aorta strips *in vitro* was also measured. *Table 2* summarizes pD_2 and pK_D values obtained for all compounds; human angiotensin was used as standard. All the analogues exhibited agonistic with, however, different potencies.

All lipopeptides were generally much less active than their corresponding [Yyy[1]]Ang peptides or totally inactive. On the other hand, incorporation of hydrophobic moieties into the Ang peptide resulted in a significant increase of the duration of action [20–22]. This behavior was also observed on the *S*- or *O*-esterified lipopeptides, although their potencies were quite low when compared to Ang on the rabbit aorta assay (data not shown).

Hydrolysis and Lipolysis of Peptides. The most important reason for the synthesis of *O*- and *S*-acylated peptides, *i.e.*, peptides containing ester or thioester bonds, was to demonstrate that *S*- or *O*-acylated peptides can easily be hydrolyzed and, thus, transformed to their biologically active form, *i.e.* [Yyy[1]]Ang peptides. *N*-Acylated Ang analogues on the other hand (fatty acid attached through an amide bond) are not easily cleavable (hydrolysis, lipolysis). The *Scheme* shows the hydrolytic treatment of the synthesized lipopeptides by either a chemical or an enzymatic procedure. All acylated peptides were hydrolyzed under basic conditions in order to find out whether it is possible to cleave the fatty acid group. The lability of the thioester bond in [Mpr[1]]Ang compounds **13** and **14** allowed the use of relatively mild conditions, 0.1M KOH, for full saponification. The decanoyl or the palmitoyl group was split from the [Lac[1]]Ang peptides **7** and **8** after 1 h of hydrolysis in 1M NaOH; however, after 5 h of 0.1M NaOH treatment, hydrolysis was not yet completed. The resulting hydrolysis products were identical to either [Mpr[1]]Ang (**12**) or [Lac[1]]Ang (**6**) (according to their t_R in the HPLC or their MS, see *Table 1*); thus, they also produced the same effects on aorta strips. As assumed, *N*-palmitoylated peptides **1**, **5**, and **10** remained without changes after hydrolysis.

For comparison, 2-aminoethanol as a nucleophile was used to hydrolyze the lipopeptides. Treatment of *O*-acylated peptides produced the same results as treatments with NaOH. In the case of *S*-acylated compounds, *e.g.* **13** or **14**, the MS of the products

Scheme. *Lipopeptide Degradation*



showed disulfide-bridge formation, *i.e.*, presence of a dimer *S*,*S*′-bis-[Mpr¹]Ang (molecular weight 2008) (see *Scheme*).

Selective enzymatic cleavage of the ester bond in *O*-acylated peptides **7** or **8** was shown using lipase from *Candida cylindracea* (LCC). LCC (EC 3.1.1.3) is a stereospecific triacylglycerol hydrolase suitable for the preparative resolution of racemic acids and alcohols [23]. Lipases are usually active at the interface of oil-H_2O microemulsions, but LCC is H_2O-soluble, and incubation can be done with organic co-solvents [24] such as DMSO. LCC has also other advantages: it is commercially available, it has been successfully used for deprotection of the OH group in the synthesis of pyranoses [25] and, a crucial point in the present application, it is essentially free from proteases. Optimum pH for lipase activity is between 7 and 9, and optimal temperature is 37°. In our case, a simple system was used. Briefly, lipopeptides were dissolved as usual and incubated in 50 mM *Tris* · HCl buffer (pH 7.5, 37°, 2% DMSO), in which the lipase was soluble. The background during HPLC analyses remained without changes. Eventual degradation of the peptides during incubation was monitored using a control sample (peptide incubated without the enzyme). The reaction was selective for the *O*-ester bond. No cleavage of the *S*-ester bond was observed in peptides **13** and **14** or the *N*-palmitoylated peptides **5** and **10**. As seen by saponification, the fatty acid group of the *O*-esters **7** and **8** was cleaved and the biologically active [Lac¹]Ang (**6**) obtained (aorta contraction). *Fig. 1* shows the HPLC analyses of [Pal⁰,Lac¹]Ang (**8**) during lipolytic cleavage. Time courses of

lipolysis of compounds **7** and **8** are shown in *Fig. 2*; the peak area of [Lac^1]Ang (**6**) was calculated *vs.* the log-scale of time. Curves are linear and show a slightly faster cleavage of the palmitoyl group from peptide **8** when compared to that of the cleavage of the decanoyl group from **7**. To prevent bacterial growth (2 weeks of incubation at 37°), all solutions were filtered through 0.2 μm membranes before the experiments. However, partial degradation of control samples after 256 h of incubation occurred (*ca.* 20%), which is probably due to the hydrolytic instability of these compounds. Different concentrations of LCC were tested. Up to 30 units/sample, the effect of LCC was not detectable (no changes in the peptides were found until 48 h of incubation). Cleavage with 300 units and 3000 units of LCC per sample were measured, but no major difference in the rate of degradation was observed. As shown in the literature [26], long incubation periods (128, 264 h) were necessary for complete cleavage by LCC. It is, therefore, evident that lipo- or glycopeptides are possible but not optimal substrates for lipases. This fact can be an advantage in our case since we are searching for depot, long-lasting analogues of Ang, releasing slowly the active non-lipidic form. Approximative half-life of [Pal^0,Lac^1]Ang (**8**) and [Dec^0,Lac^1]Ang (**7**) were of 35 and 55 h, respectively, under our conditions (see *Fig. 2*).

Conclusions. – Drug delivery and bioavailability problems hinder a lot of otherwise interesting peptide analogues to be introduced into clinical use. Structural modifications and protection of peptides with cleavable lipid groups offer means for increased proteolytic stability and prolonged half-life and tissue residence time, especially if no oral application is anticipated as in the present case. Peptides with a pro-drug characteristic could be deposited and stored in the affected tissues during the actual vascular remodeling procedure either by transmural injection or by a medicated prosthesis (stent) and could be continuously activated through lipolytic cleavage.

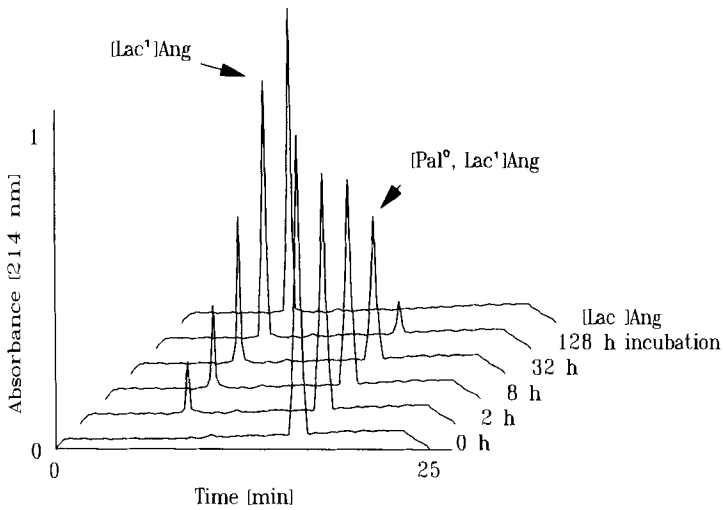


Fig. 1. *Reversed-phase HPLC of [Pal^0,Lac^1]Ang* (**8**) *during lipolysis.* Elution with a gradient of 0–95% MeCN in 0.05% aq. CF_3COOH solution flow rate 1 ml/min; peptides were incubated with lipase from *Candida cylindracea* (300 units/sample) in *Tris* · HCl buffer, pH 7.5 at 37°.

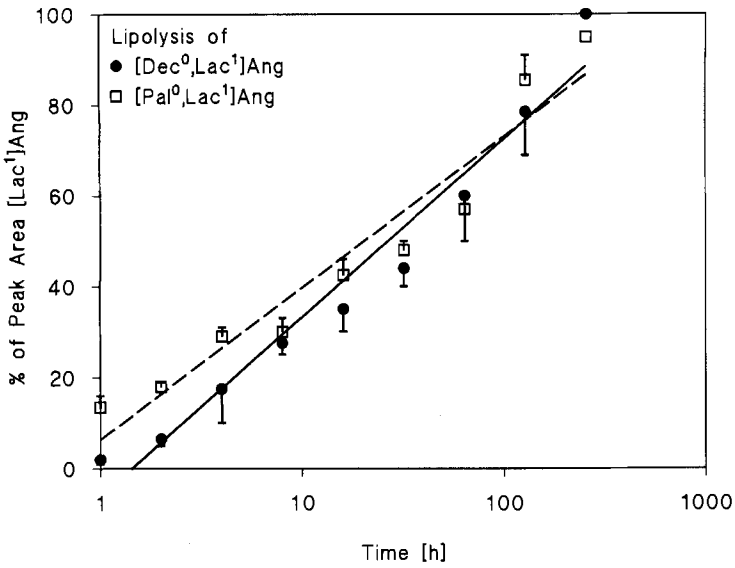


Fig. 2. *Time course of lipolysis of* O-*acylated [Lac^1]Ang* **7** *and* **8**: *HPLC peak area of [Lac^1]Ang* (**6**) vs. *log-scale of time*. For conditions, see *Fig. 1*. Values are expressed in mean ± s.e.m. (n = 3).

Therefore, we explored the possibility to produce Ang analogues that are masked by esters of fatty acids or fatty alcohol which can be transformed under lipolytic conditions into a pharmacologically active form. From these results, we can conclude that hydrolytic or lipolytic cleavage of lipopeptide ester bonds allows activation of these peptides once back to their [Yyy^1]Ang form. Synthesis of corresponding antagonists and their subsequent application to a post-angioplastic restenosis model (studies of myoproliferation inhibition) are presently under investigation.

Experimental Part

General. Protected standard amino acids were purchased from *Advanced Chemtech* (Louisville, USA) and *Novabiochem* (San Diego, USA). Mpr(*S*-Acm) was from *Peptides International* (Louisville, USA). ^{125}Iodine (^{125}I) was obtained from *Amersham* (Oakville, Canada) and *Iodo-Gen* from *Pierce* (Rockford, USA). Lipase from *Candida cylindracea* and human angiotensin II were purchased from *Sigma* (St. Louis, USA). All other reagents were from *Aldrich* (Milwaukee, USA) or *Sigma* (St. Louis, USA) and were of anal. grade. Solvents for solid-phase peptide synthesis were redistilled before use. TLC: silica gel *60-F$_{254}$* alumunium sheets (*Merck*, Darmstadt, Germany).

General Procedure for Solid-Phase Peptide Synthesis. The N^{α}-Boc strategy was used on a *Peptomat* automatic synthesizer according to an earlier published procedure [27]. The Boc-protected Phe was esterified by the Cs-salt method [28] with 1 mol-equiv. of 2% cross-linked *Merrifield* resin (*Sigma*, St. Louis, USA): A four-fold excess of each protected amino acid was used for the formation of symmetrical anhydrides with dicyclohexylcarbodiimide (DCC) [29]. The Boc protecting group was removed before the next coupling by 40% CF_3COOH in CH_2Cl_2 for 15 min. To liberate the free amino function of the maturing peptide, 5% $(i\text{-}Pr)_2EtN$ in CH_2Cl_2 was used. The completion of every coupling was checked with the ninhydrin test [30], and coupling was repeated if necessary.

After the completed synthesis, the resin was washed with CH_2Cl_2 and dried under vacuum. Then, simultaneous cleavage of the side-chain protecting group (except of Acm) and the resin-ester was performed in liquid HF at 0° 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 a scavenger. After removal of the reactants by flushing with N_2 and drying under high vacuum, the residue was washed twice with dry Et_2O. Crude peptide was extracted with aq. AcOH, 50% in the case of lipopeptides or 25% for all other peptides, and the extract was then lyophilized.

During purification, peptides were analyzed by TLC ($BuOH/AcOH/H_2O$ 5:2:3; visualization first by UV fluorescence and then with *Pauly* reagent [31]) and anal. HPLC (*Waters 600E* instrument with *Waters Bondapak* C_{18} reversed-phase column (3.9 × 300 mm); linear gradient 0–95% of 0.05% CF_3COOH/MeCN in 0.05% aq. CF_3COOH soln., flow rate 1 ml/min; 214 nm UV detection). Purification of non-lipopeptides was carried out on *Sephadex G-15* (column 30 × 350 mm, 0.2M AcOH). The peptide-containing fractions, detected by UV absorption (280 nm) during elution and by TLC, were lyophilized. The lyophilizate was dissolved in H_2O or 25% $AcOH/H_2O$ and then loaded onto a reversed-phase *Michel-Miller* column filled with 30 μm *Nucleosil-*C_{18} material (*Macherey-Nagel*, Darmstadt, Germany). According to the amount of the applied peptide, different column dimensions were used (10 × 150 mm or 20 × 300 mm). Loaded samples were eluted with a linear gradient of 0.05% CF_3COOH/MeCN in 0.05% aq. CF_3COOH soln., delivered at $8–10 \cdot 10^5$ Pa with a *FMI Lab* pump, model *Q650* (*Fluid Metering Inc.*, Oyster Bay, USA), 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 homogeneous in TLC. The structure of all peptides was confirmed by electrospray-ionization (ES) mass spectrometry (pos. mode; *VG Quattro*, Manchester, U.K.).

Ang (1–7)-Resin. At the beginning of the synthesis, the C-terminal heptapeptide of the Ang sequence, *i.e.*, Arg-Val-Tyr-Val-His-Pro-Phe-OH, was built on resin (5 g, substitution range 0.35–0.60 mmol/g), and this peptide-resin was used for the following synthesis of all peptides. Amino acids Boc-Arg(N^{α}-Tos), Boc-Val, Boc-Tyr(*O*-BrBzl), Boc-His(*im*Tos), Boc-Pro, and Boc-Phe were used. The correct sequence of amino acids of the heptapeptide was verified by the synthesis of [Sar^1]Ang as a standard compound.

[N-Pal^0,Asp^1]Ang (**1**). Boc-Asp (*O*-cHex) was coupled to the heptapeptide-resin (0.25 mmol), and the Boc group was removed. Palmitoyl chloride (4-fold excess), dissolved in CH_2Cl_2 with addition of $(i\text{-}Pr)_2EtN$ (4-fold excess), was used for coupling of Pal to position 0. Then the peptide-resin was subjected to HF cleavage and **1** purified as usual.

[Suc^1]Ang (**2**). A 6-fold excess of succinic acid in CH_2Cl_2 and DMF was used for the coupling with 1 equiv. of DCC and 1-hydroxy-1*H*-benzotriazole (HOBt). Then, the resin (0.5 mmol) was divided into two equal parts: the first part was cleaved and the resulting **2** purified according to the *General Procedure*, the second part was used for the synthesis of **3**.

[O-Cetyl^0,Suc^1]Ang (**3**). A soln. of 1-bromohexadecane in CH_2Cl_2 and $(i\text{-}Pr)_2EtN$ was added in 8-fold excess to the peptide-resin containing peptide **2** overnight. After HF cleavage, the crude **3** was purified as described for lipopeptides.

[Ser^1]Ang (**4**). Boc-Ser (*O*-Bzl) (4-fold excess) was coupled to the heptapeptide-resin (0.5 mmol) and then the resin split into two equal parts. After HF cleavage and purification, pure **4** was obtained from the first part. The second part was used for the synthesis of **5**.

[N-Pal^0,Ser^1]Ang (**5**). The N^{α}-Boc protecting group was removed from the peptide-resin containing **4**. Palmitoyl chloride and $(i\text{-}Pr)_2EtN$ (6-fold excess) in CH_2Cl_2 were added, and completion of the reaction was obtained after 2 h. Then, **5** was split from the resin and purified.

[Lac^1]Ang (**6**). β-Lactic acid (6-fold excess) in DMF was coupled to the heptapeptide-resin (0.75 mmol) by means of DCC, HOBt and $(i\text{-}Pr)_2EtN$ (6-fold excess, 6 h of reaction). Then, the resin was divided into three equal portions. One portion was subjected to HF cleavage; after purification, **6** was obtained. The second and third part were used for the synthesis of **7** and **8**.

[O-Dec^0,Lac^1]Ang (**7**). The second part of the peptide-resin containing **6** was reacted with an 8-fold excess of decanoyl chloride and $(i\text{-}Pr)_2EtN$ in CH_2Cl_2 overnight. After HF cleavage, crude **7** was purified.

[O-Pal^0,Lac^1]Ang (**8**). Peptide **8** was prepared by the same way as **7** using palmitoyl chloride instead of decanoyl chloride.

[Cys(S-Acm)^1]Ang (**9**). Boc-Cys (*S*-Acm) in 4-fold excess was coupled to the heptapeptide-resin (0.25 mmol). Then, the peptide-resin was subjected to HF cleavage and **9** purified.

[N-Pal^0,Cys^1]Ang (**10**). Boc-Cys(*S*-MeBzl) (4-fold excess) was coupled to the heptapeptide-resin (0.25 mmol). The peptide-resin with [Cys^1]Ang was treated with CF_3COOH for N^{α}-Boc deprotection, and a 6-fold excess

of palmitoyl chloride and (i-Pr)$_2$EtN in CH_2Cl_2 were added and left for coupling overnight. After HF cleavage, **10** was purified.

[Mpr(S-Acm)^1]Ang (**11**). Mpr(*S*-Acm), dissolved in a DMF/CH_2Cl_2 1:1, was coupled to the heptapeptide-resin (0.5 mmol) by means of DCC and HOBt (4-fold excess). After 6 h of coupling, the resin was split into two parts. The first part was cleaved with HF and the second used for the synthesis of **12**. Peptide **11** was purified as described above.

[Mpr^1]Ang (**12**). The Acm group was removed from **11** by means of mercury acetate [32]: to a soln. of **11** (30 mg) in H_2O (10 ml) of pH 4.0 (adjusted with AcOH), mercuric acetate (1 equiv.) was added while stirring and the pH readjusted to 4.0. The soln. was stirred at r.t. for 2 h until HPLC showed a completed reaction. The mixture was then diluted with H_2O, and H_2S was bubbled into the mixture to precipitate the Hg salt. The mixture was then filtered and the filtrate lyophilized to give **12**.

[S-Dec^0,Mpr^1]Ang (**13**). a) *Solid-Phase Synthesis.* In an ice bath, 3-mercaptopropanoic acid (0.05 mol) was stirred with benzene. Triphenylmethyl chloride (1 equiv.) and Et_3N (1.1 equiv.) were then slowly added. The reaction was followed by TLC and the end of the reaction (absence of free SH groups) confirmed by *Ellmann's* reagent [33]. After evaporation, the product Mpr(*S*-Trt) was extracted with hexan/Et_2O. Mpr(*S*-Trt), being soluble in hexan, was used for the coupling to position 1 of the heptapeptide-resin (0.25 mmol). Then the Trt group was removed by reaction with 80% CF_3COOH (5 min). After washing of the peptide-resin with MeOH and CH_2Cl_2, an 8-fold excess of decanoyl chloride and (i-Pr)$_2$EtN in CH_2Cl_2 were added and left overnight. The resulting peptide-resin was subjected to HF cleavage and the crude **13** purified as usual.

b) *Synthesis in Solution.* To a soln. of purified **12** (10 mg) in DMF, decanoyl chloride (2 equiv.) was added and the 'pH' adjusted to 7–8 with (i-Pr)$_2$EtN. The mixture was stirred at r.t. for 2–6 h until HPLC analysis showed a completed reaction. Then, DMF and (i-Pr)$_2$EtN were evaporated, the peptide was repurified by HPLC (C_{18} column, 30–90% of 0.05% CF_3COOH/MeCN linear gradient in 0.05% aq. CF_3COOH soln.), and the fractions containing pure **13** were lyophilized.

[S-Pal^0,Mpr^1]Ang (**14**). Peptide **14** was synthesized both on solid phase and in soln., as described for **13** except that palmitoyl chloride instead of decanoyl chloride was used.

[Pal^1]Ang (**15**). Palmitoyl chloride (6-fold excess) was used for coupling of Pal to position 1 of the heptapeptide-resin (0.25 mmol). After HF cleavage, the crude **15** was purified as described in the *General Procedure.*

[O-Pal^0,Ser^1]Ang (**16**). Boc-Ser was dissolved in CH_2Cl_2 and, in 4-fold excess, coupled to the heptapeptide-resin (0.25 mmol). Then, palmitoyl chloride and (i-Pr)$_2$EtN (6-fold excess) in CH_2Cl_2 were used for coupling overnight. Peptide **16** was cleaved from the resin, then extracted with 50% AcOH/H_2O, and purified as described above.

[S-Pal^0,Cys^1]Ang(**17**). Boc-Cys(*S*-Trt) in 4-fold excess was coupled to position 1 of the peptide-resin (0.25 mmol). Deprotection of trityl was carried out in presence of 2-mercaptoethanol overnight [17]. After washing with CH_2Cl_2, palmitoyl chloride and (i-Pr)$_2$EtN (6-fold excess) in CH_2Cl_2 were used for coupling overnight. The resulting peptide-resin was subjected to HF cleavage and the crude **17** purified.

Saponification of Lipopeptides. Peptides were hydrolyzed with 0.1M or 1M NaOH in H_2O/dioxan (*O*- and *N*-decanoylated and -palmitoylated peptides, [*O*-Cetyl^0,Suc^1]Ang (**3**)) or in 0.1M KOH in MeOH (*S*-decanoylated and -palmitoylated peptides) for 1 h. Analysis was done by HPLC followed by an evaluation of their biological activity on rabbit aorta. Lipopeptides were also hydrolized with 1M – 10^{-3} M 2-aminoethanol for 1–8 h and then analyzed by HPLC and ES^+/MS (pos. mode).

Lipolysis of Peptides. Lipolysis of the lipopeptides was performed by incubating peptides ($5 \cdot 10^{-3}$ final concentration) and lipase from *Candida cylindracea* (final concentration 300 and 3000 units, *i.e.* 0.17 and 1.7 mg/ml) at 37° in buffer (50 mM *Tris* · HCl, pH 7.5, 50 µl final volume). Before use, all solns. were sterilized by filters with a pore size of 0.2 µm. The incubation was stopped by denaturation of the enzyme at 100° for 5 min, after which samples were centrifuged (1000 g, 5 min) to separate the 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 treated in the same fashion, though without enzyme, and analyzed by HPLC. The peak area was calculated by means of the chromatography computer program Baseline 810 (*Waters*, CA, USA).

Bioassays. Bovine adrenocortical membranes were prepared according to the procedure reported by *Glossman et al.* [34], ^{125}I-Ang was obtained by the method of iodation using *Iodo-Gen* [35], and the assay was performed as described earlier [36].

Rabbit Aorta Strips Contraction. This assay was performed according to the previously described method [36].

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