128. Angiotensin II Analogues. Part II. Synthesis and Incorporation of the Sulfur-Containing Aromatic Amino Acids: L-(4'-SH)Phe, L-(4'-SO_2NH_2)Phe, L-(4'-SO_3^-)Phe and L-(4'-S-CH_3)Phe[1)2)]

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Summary

L-Phenylalanine has been treated with chlorosulfonic acid and the product was either hydrolyzed, ammonolyzed or reduced. The resulting sulfonic-acid and aminosulfonyl derivatives have been employed for peptide synthesis with Boc-protection of the N^{α}-position only. The reduction product L-(4'-SH)Phe has been protected by formation of asymmetric disulfides or with various thiol protecting groups (benzyl-, methyl- and acetamidomethyl groups, the latter being the most suitable for peptide synthesis).

With these protected amino acids several analogues of angiotensin II have been synthesized by the solid-phase method. These analogues have been used for structure-activity relationship studies on three different bioassays.

Introduction. – Sulfur-containing aromatic amino acids have been prepared almost a generation ago to search for possible chemotherapeutic applications [1] [2]. In the last years, however, only a preliminary report has been published, where sulfonated phenylalanine was used as an analogue of the sulfate ester of tyrosine in a gastrointestinal peptide, but no details were given [3]. However, phenylalanine derivatives substituted in the *p*-position with sulfur could be very versatile analogues for a wide variety of applications: (4'-SH)Phe is an analogue of tyrosine for structure-activity relationship (SAR) studies, it can be used to form cyclic analogues and for very specific attachment of a peptide to a support through the maleimid reaction [4] or by Hg-complexation, *e.g.* for antibody production or for affinity purification procedures. The sulfonic-acid derivative (4'-SO_3H)Phe can be seen as a very hydrophilic analogue or as a stable anion, which could be very useful for

1) Part of the M. Sc. thesis of *P. Parent* and *M. Bernier*.

2) Abbreviations follow the recommendation of the IUPAC-IUB Commission for biochemical nomenclature, see *E. Wünsch*, 'Synthese von Peptiden', Vol. 15, part 1, of 'Houben-Weyl, Methoden der Organischen Chemie', E. Müller, ed. G. Thieme Stuttgart, G.F.R. 1974.

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SAR studies. The aminosulfonyl derivative (4′-SO_2NH_2)Phe is a highly hydrophilic but uncharged amino acid, also useful for the above-mentioned purposes.

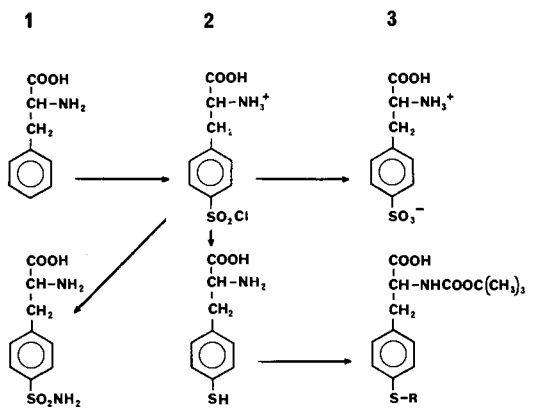
All these considerations seemed to be suitable for angiotensin II (Asp-Arg-Val-Tyr-Val-His-Pro-Phe, abbreviated as AT) since we have been studying the two aromatic positions of this peptide hormone since some time [5] [6]. It was observed that position 4 (Tyr) of AT affects the affinity of the hormone to its receptor through the electronegativity of the substituted aromatic ring [5]. This electronegativity dependance does not play a role for position 8 (Phe), where rather hydrophobicity and steric parameters are important [6]. In a foregoing contribution [7], we prepared and tested several analogues with highly hydrophobic substituents in position 8 of AT. In this paper we report on the synthesis of such hydrophilic, S-containing aromatic amino acids, their protection schemes for peptide synthesis and their application as tyrosine analogues (position 4) and of phenylalanine analogues (position 8) in AT.

Syntheses. – The introduction of sulfur into phenylalanine was carried out by direct chlorosulfonation of phenylalanine, a procedure which does not affect the chiral center of the amino acid. This pathway was preferred over the cumbersome strategy already reported [1], where a *Sandmeyer*-like reaction of 4′-aminophenylalanine with xanthate leads *via* several steps to (non-identified) 4′-mercaptophenylalanine (**5**). Our initial product, the instable chlorosulfonyl derivative **2** is the intermediate for all subsequent syntheses (see the *Scheme*). In a first pathway, the hydrolysis of **2** in aqueous medium affords 4′-sulfophenylalanine (**3**) a very well crystallized product. If the hydrolysis is carried out in presence of aq. NH_3, both **3** and the aminosulfonyl derivative **4** are obtained together. The free thiol **5** was more difficult to obtain, because several reduction attempts with $NaBH_4$ did not lead to the desired product. Reduction with Fe or Zn in HCl were not successful but Sn with HCl led to **5**. However, the lability against air oxidation was high and the symmetrical disulfide, almost insoluble in the usual solvents, was rapidly formed.

For peptide synthesis the 4′-sulfo derivative **3** and the 4′-aminosulfonyl derivative **4** had only to be protected at N^{α}, whereas for **3** some coupling problems were encountered because of the anionic-hydrophilic nature of this compound. In the case of **5** possible thiol protecting groups had to be investigated in detail, because the stability of the aromatic thiols is not identical to that of the aliphatic thiols, like cystein [8]. *t*-Butoxycarbonyl protection (Boc) was easily feasible to form **6**, but the N^{α}-Boc-group was cleaved under the same conditions as for the 4′-S-Boc group and therefore not attractive for peptide synthesis.

The use of the symmetrical disulfide **7** was possible [9] but a synthesis attempt with this intermediate failed due to very poor yields. The asymmetric disulfides **8** and **9** were obtained with the thiosulfinic-ester method [10], but these compounds were not useful due to their spontaneous disproportionation to **7** upon standing. Acetamidomethyl (Acm)-protection by the standard method for cystein [11] led to a good intermediate **10** and the Acm-group was not cleaved even if the product was treated in liq. HF following the standard procedure (1 h at 0°) but it was readily removed under conditions normally used for Cys-Acm-cleavage [11] [12]. As a third group, benzyl protection was evaluated: since the alkylthioethers are

Scheme



6 R = *t*-Boc
7 R = *p*-S-C_6H_4-CH_2-CH(NHCOOC$(CH_3)_3$)–COOH (symmetric disulfide)
8 R = S-C_4H_9
9 R = S-*p*-tolyl
10 R = Acetamidomethyl
11 R = Benzyl
12 R = *m*-bromobenzyl
13 R = *p*-nitrobenzyl
14 R = CH_3

somewhat more stable than arylthioethers, we attempted to introduce benzyl-, 3′-bromobenzyl- and 4′-nitrobenzyl-groups leading to the products **11**, **12** and **13** and not the methylbenzyl- or methoxybenzyl-groups frequently used for Cys. As a last analogue which could function as a substitute of methionine or *O*-methyltyrosine, 4′-(methylthio) derivative **14**, was prepared.

These alkylations were achieved by the reaction of the freshly formed **5** with the corresponding benzyl bromide (→ **11** and **12**) or methyl iodide (→ **14**) at neutral pH, followed by the isolation and purification after N^{α}-protection with bis(*t*-butyldicarbonate) (Boc_2O) [13]. However, the reaction of *p*-nitrobenzyl bromide with **5** did not lead to **13** for unknown reasons. This protection could have been very attractive because of the high acid stability of the *p*-nitrobenzyl group which would be cleavable under milder acidic conditions after reduction to the corresponding aminobenzyl group [14].

The peptide synthesis was carried out mainly by the solid-phase method following the procedures already described [15]. The classical chloromethylated polystyrene was used as solid support and the Boc-group for α-amino-protection. Side-chain protecting groups were cleaved simultaneously with the attachment to the solid support by liq. HF and the peptides were purified by gel filtration, partition chromatography, and reversed-phase chromatography. The purity of peptides was assessed in two different TLC. systems, reversed-phase-TLC. and by analytical

HPLC. The only problem encountered during the synthesis was the N^{α}-Boc-protection of **3**. This amino acid had to be coupled as its DEA-salt in DMF with DCC as condensing agent.

Biological activities were measured *in vitro* on strips of rabbit aorta, *in vivo* on the blood pressure of anesthetized rats and on the binding potency towards purified bovine adrenocortical membranes (see *Table 2*). The most interesting compounds were the sulfonate **16** and the Acm-protected **20** modified in position 8. The first was a complete non-agonist, up to millimolar concentrations in all three bioassays, the second was a potent antagonist on rabbit aorta but with quickly reversible action, contrary to that observed with lipophilic substituents in position 8 [7].

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Experimental Part

General. Melting points (m.p.) were determined in open capillaries and are uncorrected. Optical rotations $[\alpha]_D$ were measured in a *Zeiss OLD*-Polarimeter in 0.5% EtOH-solutions. UV. spectra were recorded on a *Beckman 25* spectrophotometer. IR. spectra were carried out on a *Perkin-Elmer 457* instrument with nujol suspension on NaCl- or in $CHCl_3$-solution. ^{1}H-NMR. spectra were recorded on a *Varian T60* spectrometer, chemical shifts are indicated in ppm against tetramethylsilane, *s* = singulet, *d* = dublett, *t* = triplet, *qa* = quadruplet, *m* = multiplet. Elementary analyses have been carried out by *Galbraith Laboratories* (Knoxville, Tennessee, U.S.A.). Analytical HPLC. was carried out on a *Waters M45* instrument with a gradient of 0 to 45% acetonitrile in 0.25 N ammonium acetate pH 5.0 and 2.5% 2-propanol on a *μ-Bondapak C-18* column (4×300 mm), all peptides produced single, symmetrical peaks.

Abbreviations: AT: angiotensin II, DCC: dicyclohexyl-carbodiimide, *i.v.*: *in vacuo*, TLC.: thin layer chromatography, RP.-TLC.: reversed-phase TCL., HF: anh. hydrogen fluoride, DEA: diisopropylethylamine, Ts: *p*-toluenesulfonyl, BrBzL: (*m*-bromobenzyl), Boc: *t*-butoxycarbonyl, Boc_2O: di-*t*-butyldicarbonate, DMF: dimethylformamide, TFA: trifluoroacetic acid, r.t.: room temperature.

L-*t*-Butoxycarbonylamino acids, peptide reagents, chloromethylated resin (copolystyrene/1% divinylbenzene, 0.75 mmol of Cl/g of resin) were obtained from *Bachem Feinchemikalien A.G.*, Switzerland, if not otherwise stated, and were used without further purification. DCC was purified by dissolving the commercial product *(Aldrich Chemicals)* in ether; the insoluble material was removed by filtration and the ether was evaporated *i.v.* All reagents used for solid-phase synthesis were of analytical-reagent quality and solvents were redistilled before use. TLC. was performed on *Merck* precoated silica-gel plates (type *G60-F254*) in the solvent systems A-F (in *v/v*); A: 2-propanol/conc. aq. NH_3 3:1; B: BuOH/0.05 M ammonium acetate 2:1, pH 7.0; C: BuOH/AcOH/H_2O 4:1:1; D: BuOH/AcOH/H_2O 5:2:3; E: BuOH/AcOH/H_2O/Pyridine 30:6:20:12; F: $CHCl_3$/AcOH/MeOH 95:5:3. RP-TLC. was performed on *Whatman* reversed-phase plates *KC 18* in the solvent system 0.5 M ammonium acetate with 40% acetonitrile and 7% 2-propanol. The spots were visualized with UV. fluorescence, ninhydrin for amino acids or *Pauly* reagent for AT-peptides. Peptide samples for amino-acid analyses were hydrolyzed during 24 h at 110° in TFA/conc. HCl 1:1 with 1% of 3-mercapto-propionic acid in vacuum-sealed tubes. The amino-acid analyses were performed on a *Beckman 119 CL* analyzer.

L-*4'-Sulfophenylalanine* ((4'-SO_3H)Phe, **3**). Chlorosulfonic acid (5 g, 43 mmol) was chilled to $-15°$; 3.3 g (20 mmol) of L-phenylalanine *(Sigma)* were added under stirring with a glass rod. The mixture was left overnight in an ice bath, ice was added, the precipitate filtered off and recrystallized from 2-propanol/H_2O, yield, 2.2 g of large, rhomboid crystals of **3**. – ^{1}H-NMR. (1 N LiOD): 7.46 (*m*, *AA'BB'*, 4 H); 3.43 (*m*, 1 H); 2.94 (*m*, 2 H).

L-*N^{α}-t-Butoxycarbonyl-4'-sulfophenylalanine-diisopropylethylammonium salt* (Boc-(4'-SO_3^-)Phe · DEA, Boc-**3** · DEA). Compound **3** (2.2 g, 9 mmol) was dissolved in 70 ml of H_2O and 60 ml of *t*-BuOH. The pH of the solution was adjusted to 9 with DEA *(Aldrich)* and further 9 mmol of DEA were added together with 2.36 g (10.8 mmol) of Boc_2O *(Aldrich)*. The reaction was left overnight under stirring and tested for completion by TLC. The resulting mixture was acidified carefully to pH 2 at 0° with 2N H_2SO_4 and solid $NaHSO_4$. The mixture was extracted twice with EtOAc, the org. phase rejected, the aq. phase diluted to 200 ml with thawing ice and the pH was adjusted to 9 with 1N NaOH. The solution was added to an ion-exchange column (3×20 cm) of *BioRad AG 1X8*, rinsed with water and eluted with 2N aq. DEA. After evaporation and lyophilization, 2.74 g of Boc-**3** · DEA were recovered (64%). – ^{1}H-NMR. (D_2O, after addition of 2 equiv. of LiOD in D_2O and lyophilization): 7.30 (*m*, *AA'BB'*, 4 H); 3.50 (*m*, 1 H); 2.98 (*m*, 2 H); 1.30 (*s*, 9 H).

L-*4'-(Aminosulfonyl)phenylalanine* ((4'-SO_2NH_2)Phe, **4**). Chlorosulfonic acid (20 ml, 300 mmol) was reacted with 5 g (30 mmol) of L-phenylalanine as described for **3**, leading to a homogeneous mixture. The temperature was raised to 0° for 2 h and finally the mixture was poured onto 300 g of ice. Further dilution to 500 ml afforded a clear solution which was immediately brought into contact with 100 g of regenerated ion-exchange resin *AG 50W-X4* (strong acid), and washed rapidly with ice-cold water until the eluent had a pH of 5. Incubation of the resin with 400 ml of 2N aq. NH_3 followed by filtration and lyophilization of the filtrate gave a mixture containing **3** and **4** according to TLC. Gel filtration over *Sephadex G10* (3×60 cm) with 0.2N AcOH yielded 1.47 g of pure **4** after lyophilization.

L-*4'-(Aminosulfonyl)-N^{α}-t-butoxycarbonylphenylalanine* (N^{α}-Boc-(4'-SO_2NH_2)Phe, Boc-**4**). Compound **4** (2.89 g, 11.7 mmol) was dissolved in 20 ml of 1N NaOH and diluted with 55 ml of H_2O and 50 ml of *t*-BuOH. At r.t. and under magnetic stirring 3.05 g of solid Boc_2O (14.0 mmol) were added and the mixture was stirred for 24 h. Ice was added to the mixture and the pH was adjusted to 2 with

Table 1. *Data of the compounds* **1**, **3**–**12** *and* **14**

Compound	No.	TLC.-Rf	$[\alpha]_D$	M.p.	Yield[a])
H-Phe	**1**	0.47 (D), 0.50 (E)	−34.8°	–	–
Boc-Phe	Boc-**1**	0.44 (F)	+25.2°	86.0–88.0°	–
H-(4'-SO_3H)Phe	**3**	0.12 (A), 0.026 (B), 0.12 (C)	−11.8°	–	45%
N-Boc-(4'-SO_3^-)Phe	Boc-**3**	0.48 (E)	+15.20°	–	64%*
H-(4'-SO_2NH_2)Phe	**4**	0.26 (A), 0.13 (B), 0.26 (C)	−6.25°	–	20%
N^{α}-Boc-(4'-SO_2NH_2)Phe	Boc-**4**	0.22 (F)	−8.79°	84.0–85.0°	59%*
H-(4'-SH)Phe	**5**	0.46 (D), 0.55 (E)	–	–	20–28%
N-Boc-(4'-SH)Phe	*N*-Boc-**5**	0.40 (F)	–	–	–
N-Boc-(4'-S-Boc)Phe	**6**	0.43 (F)	+8.95°	112.5–114.0°	22%
(H-4'-Phe)$_2S_2$		0.40 (D), 0.38 (E)	–	–	–
(*N*-Boc-4'-Phe)$_2S_2$	**7**	0.26 (F)	+47.25°	162.0–163.5°	20%
H-(4'-S-S-butyl)Phe		0.64 (D)	–	–	–
N-Boc-(4'-S-S-butyl)Phe	**8**	0.50 (F)	+10.00°	oil	6%
H-(4'-S-S-tolyl)Phe		0.62 (D)	–	–	6%
N-Boc-(4'-S-S-tolyl)Phe	**9**	0.56 (F)	+32.47°	97.0–98.5°	5%
H-(4'-S-Acm)Phe		0.38 (D), 0.46 (E)	–	–	12%
N^{α}-Boc-(4'-S-Acm)Phe	**10**	0.15 (F)	+18.58°	152.5–154.0°	11%
H-(4'-S-Bzl)Phe		0.59 (D), 0.61 (E)	−35.58°	–	28%
N-Boc-(4'-S-Bzl)Phe	**11**	0.49 (F)	+10.98°	133.0–134.0°	24%
H-(4'-S-BrBzl)Phe		0.59 (D), 0.61 (E)	−31.79°	–	28%
N-Boc-(4'-S-BrBzl)Phe	**12**	0.49 (F)	+10.31°	145.0–146.5	24%
H-(4'-S-CH_3)Phe		0.54 (D)	–	–	–
N-Boc-(4'-S-CH_3)Phe	**14**	0.47 (F)	+6.73°	119.5–121.0°	0.5%
H-Cys		0.31 (D)	+7.2°	–	–

[a]) Yields are based on the initial quantity of **1** (Phe) with the exception of *.

Table 2. *Elemental analyses*

Compound	No.	Formula	M.W.	C Calc./Found	H Calc./Found	N Calc./Found	S Calc./Found	Br Calc./Found
(4′-SO_3H)Phe	**3**	$C_9H_{11}NO_5S \cdot \frac{1}{2} H_2O$	254.23	42.52/42.79	4.76/4.58	5.50/5.41	12.61/12.51	
N^a-Boc-(4′-SO_2NH_2)Phe	Boc-**4**	$C_{14}H_{20}N_2O_6S \cdot H_2O$	362.40	46.62/46.98	6.12/6.00	7.73/7.70	8.63/8.32	
N-Boc-(4′-S-Boc)Phe	**6**	$C_{19}H_{27}NO_6S$	397.50	57.41/57.40	6.85/6.64	3.52/3.72	8.07/8.18	
(N^a-Boc-4′-Phe)$_2S_2$	**7**	$C_{28}H_{36}N_2O_8S_2$	592.74	56.74/57.02	6.12/6.42	4.73/4.43	10.87/10.98	
N^a-Boc-(4′-S-S-butyl)Phe[a])	**8**	$C_{18}H_{27}NO_4S_2$	385.54	56.08/58.26	7.06/7.38	3.63/3.54	16.63/13.27	
N^a-Boc-(4′-S-S-*p*-tolyl)Phe[a])	**9**	$C_{21}H_{25}NO_4S_2$	419.57	60.12/59.45	6.01/6.10	3.34/3.27	15.28/15.28	
N^a-Boc-(4′-S-Acm)Phe	**10**	$C_{17}H_{24}N_2O_5S$	368.46	55.42/55.42	6.57/6.60	7.60/7.40	8.70/8.78	
N^a-Boc-(4′-S-Bzl)Phe	**11**	$C_{21}H_{25}NO_4S$	387.50	65.09/65.20	6.50/6.56	3.62/3.59	8.27/8.57	
N^a-Boc-(4′-S-*m*-BrBzl)Phe	**12**	$C_{21}H_{24}BrNO_4S$	466.40	54.08/54.25	5.19/5.62	3.00/3.30	6.87/6.94	17.13/17.43
N^a-Boc-(4′-S-CH_3)Phe	**14**	$C_{15}H_{21}NO_4S$	311.06	57.86/57.60	6.80/6.97	4.50/4.33	10.30/10.54	

[a]) Compound disproportionates spontaneously.

2N H_2SO_4. The white precipitate was extracted twice with EtOAc, the org. phases were pooled, dried over anh. Na_2SO_4 and evaporated. The crude product was further purified by chromatography on a pre-packed lobar silica-gel column (*Merck*, size *C*) with $CHCl_3$/MeOH 40:1, 2.38 g of pure Boc-**4**. – ^{1}H-NMR. (D_6-DMSO): 7.42 (*m*, *AA'BB'*, 4 H); 7.2–6.7 (*m*, 3 H); 1.26 (*s*, 9 H).

L-*4'-Mercaptophenylalanine* ((4'-SH)Phe, **5**). L-Phenylalanine (**1**) (5.0 g, 30 mmol) was stirred mechanically at −15°. Under exclusion of moisture, 35 g of chlorosulfonic acid (300 mmol), precooled to −15°, were added cautiously. Stirring was continued for 4 h with the ice/MeOH bath removed. The mixture was poured onto 300 g of crushed ice, filtered and the residue transferred to a two-neck flask equipped with a refluxing condenser, containing 25 g of ice and 30 ml of conc. HCl; 17.8 g (150 mmol) of Sn-powder were slowly added and the mixture was kept on reflux for 2 h. After all Sn had disappeared, the solution was diluted with 300 ml of H_2O and H_2S was bubbled through the solution at 40°. The precipitated Sn-sulfide was filtered off and some additional H_2S was given as a test for residual Sn in solution. After all Sn had been precipitated, the solution was reduced *i.v.* to 100 ml and used for the ensuing protection procedures without further purifications. TLC. positive on *Ellman* reagent for thiols [16].

L-N,S-*Bis(*t*-butoxycarbonyl)-4'-mercaptophenylalanine* (N-Boc-(4'-S-Boc)Phe, **6**). To the residue of the above reaction at r.t. were given under stirring 100 ml *t*-BuOH 2.4 g (11 mmol) of Boc_2O and 4N NaOH until a pH of 10 was obtained. The pH was adjusted several times with 2N Na_2CO_3 to 10 during the 8 h reaction time and the completion of the reaction was followed with TLC. (F). The mixture was evaporated to half of the initial volume, chilled with crushed ice, acidified with $NaHSO_4$ to pH 2 and extracted twice with EtOAc. The org. phases were pooled, dried over anh. Na_2SO_4 and evaporated *i.v.*. The residual yellow oil was loaded onto a prepacked silica-gel column (*Merck*, size C), washed with toluene/CH_2Cl_2 1:1, eluted with a gradient of CH_2Cl_2/MeOH, 0 to 2% and 2.6 g of a white powder were collected. – ^{1}H-NMR. ($CDCl_3$): 8.80 (*m*, 1 H); 7.17 (*AA'BB'*, 4 H); 5.00 (*m*, 1 H); 4.43 (*m*, 1 H); 3.08 (*m*, 2 H); 1.48 (*s*, 9 H); 1.38 (*s*, 9 H).

L,L-*Bis(*N-t*-butoxycarbonyl-4'-phenylalanyl)disulfide* ((N^a-Boc-4'-Phe)$_2S_2$, **7**). O_2 was bubbled through the residue of the mixture containing **5** (see above), and the reaction was followed on TLC. (E). After all free thiol **5** had disappeared (about 5 h), the pH was increased with 4N NaOH to 10, and 100 ml of *t*-BuOH were added together with 2.4 g (11 mmol) of Boc_2O. The reaction was controlled and worked up as mentioned above, and the crude **7**, a yellow oil, was dissolved in DMF and loaded onto a reversed-phase column (2.2×30 cm, containing 30 *μ C-18* modified spherical silica-gel, *Macherey-Nagel*) and eluted with a maximal pressure of 6 atm with a gradient of H_2O/MeOH 0 to 70%. The product-containing fractions were pooled, partially evaporated and extracted twice with EtOAc. The org. phases were pooled, dried over anh. Na_2SO_4 and evaporated. Recrystallization from EtOAc/petrol ether yielded 1.8 g of **7** as a white powder.

*(*L-N-t-*Butoxycarbonyl-4'-phenylalanyl) butyl disulfide* (N-Boc-(4'-S-S-butyl)Phe, **8**). Butyl 1-thiobutane-1-sulfinate (1.0 g, 5.0 mmol) [17], prepared according to [18], was added in 100 ml of EtOH to a batch of crude **5** and the mixture was stirred overnight at 40°. The volume was reduced to 70 ml *i.v.* and extracted twice with Et_2O. The org. phase was rejected and a precipitation was observed in the aq. phase which was completed at 4° overnight. Filtration and subsequent washings with cold EtOH and H_2O gave a product which was dissolved in 25 ml 1N NaOH, diluted with 100 ml of H_2O and 100 ml of *t*-BuOH. Introduction of Boc was carried out as usual with 500 mg (2.3 mmol) of Boc_2O. The crude **8**, a yellow oil, was purified as described for **7**, 700 mg of a clear yellow oil resulted (1.8 mmol from **1**). – ^{1}H-NMR. ($CDCl_3$): 1067 (*m*, 1 H); 7.23 (*m*, *AA'BB'*, 4 H); 5.13 (*m*, 1 H); 4.47 (*m*, 1 H); 3.08 (*m*, 2 H); 2.67 (*m*, 2 H); 1.53 (*m*, 4 H); 1.40 (*s*, 9 H); 0.90 (*m*, 3 H).

Immediately after purification no trace of **7** on TLC. (F) was visible but after 1 day at 4° already some **7** and a strongly reducing spot at Rf ≈ 1 were detected.

*(*L-N-t-*Butoxycarbonyl-4'-phenylalanyl)* p-*tolyl disulfide* (N-Boc-(4'-S-S-*p*-tolyl)Phe, **9**). *p*-Tolyl-(*p*-toluene)thiosulfinate [19] (1.3 g, 5.1 mmol), prepared in our laboratory according to [18], have been dissolved in 100 ml of dioxane, added to a batch of crude **5** and stirred at 40° overnight. The formed precipitate was filtered, washed with cold EtOH and ice-cold water. The product was converted to its Boc-derivative and isolated as described above. The product was purified by chromatography on a prepacked silica-gel column, size C, eluted with a gradient of $CHCl_3$/MeOH 0 to 2%. The collected fractions were pooled, evaporated and recrystallized from EtOAc/petrol ether, 600 mg of **9**. – ^{1}H-NMR. ($CDCl_3$): 9.38 (*m*, 1 H); 7.12 (*m*, 8 H); 5.00 (*m*, 1 H); 4.40 (*m*, 1 H); 3.02 (*m*, 2 H); 2.27 (*s*, 3 H); 1.33

(*s*, 9 H). This product was instable upon storage and produced considerable amounts of **7** upon standing.

L-S-*(Acetamidomethyl)*-N$^{\alpha}$-t-*butoxycarbonyl-4'-mercaptophenylalanine* (N$^{\alpha}$-Boc-(4'-S-Acm)Phe, **10**). The pH of crude **5** was adjusted to 3 with 4N NaOH, the solution cooled to 0° and N_2 was bubbled through. After 1 h, 830 mg of *N*-(hydroxymethyl)acetamide [11], (9.3 mmol), from our laboratory) were added together with 5.4 ml of conc. HCl. The reaction was followed from time to time by TLC. and the mixture was stirred for 3 days at r.t. until completion. The pH was increased to 10 and the product was acylated with 2.2 g of Boc_2O, isolated and purified on silica-gel as usual, using a gradient of $CHCl_3$ 0 to 2.5% MeOH, and a second purification on a reversed-phase column with a gradient of water with 0 to 50% MeOH. The product, 1.23 g, recrystallized from EtOAc/petrol ether, was obtained as a white powder. – ^{1}H-NMR. ($CDCl_3$); 9.72 (*s*, 1 H); 7.19–6.92 (*m*+*AA'BB'*, 4 H + 1 H); 5.27 (*m*, 1 H); 4.85–4.33 (*m*, 3 H); 3.08 (*m*, 2 H); 1.91 (*s*, 3 H); 1.43 (*s*, 9 H).

L-S-*Benzyl*-N-t-*butoxycarbonyl-4'-mercaptophenylalanine* (Boc-(4'-S-Bzl)Phe, **11**). To a batch of crude **5**, neutralized with 4N NaOH to pH 7 and diluted with 300 ml of EtOH, was added a solution of benzyl bromide (1.6 g, 9.2 mmol) in 25 ml of EtOH together with 3.4 ml of Et_3N (25 mmol). The mixture was stirred at r.t. for 15 h and the reaction was followed by TLC.(E). The solution was evaporated to dryness, redissolved in boiling water at pH 6, filtered hot and the clear solution kept at 4° overnight. The formed precipitate was filtered, redissolved in 25 ml of 1N NaOH, diluted with 250 ml of H_2O and 100 ml of *t*-BuOH and acylated with Boc_2O (2.2 g, 10 mmol) as described above. The crude, oily **10** was purified on a reversed-phase column as described for **7** with a gradient of H_2O/MeOH 20 to 90%. Purified **10** was recrystallized from Et_2O/petrol ether and yielded 3.3 g of **10** as white powder. – ^{1}H-NMR. ($CDCl_3$): 11.10 (*s*, 1 H); 7.17 (*s*, 5 H); 7.08 (*m*, *AA'BB'*, 4 H); 5.00 (*m*, 1 H); 4.47 (*m*, 1 H); 4.02 (*s*, 2 H); 3.03 (*m*, 2 H); 1.38 (*s*, 9 H).

L-S-*(m-Bromobenzyl)*-N-*(t-butoxycarbonyl)-4'-mercaptophenylalanine* (Boc-(4'-S-BrBzl)Phe, **12**). Crude **5** was treated with *m*-bromobenzyl bromide (2.3 g, 9.2 mmol) as described for **11**; 3.9 g of pure **12** were collected after purification. – ^{1}H-NMR. ($CDCl_3$): 11.42 (*s*, 1 H); 7.13 (*m*, 8 H); 5.20 (*m*, 1 H); 4.50 (*m*, 1 H); 3.96 (*s*, 2 H); 3.07 (*m*, 2 H); 1.38 (*s*, 9 H).

Attempted synthesis of L-N$^{\alpha}$-*(t-Butoxycarbonyl)*-S-*(p-nitrobenzyl)-4'-mercaptophenylalanine* (**13**). Crude **5** was treated with *p*-nitrobenzylbromide (2.0 g, 9.2 mmol) as described for **11**. Before acylation with Boc_2O no major product could be detected around Rf 0.60 (D,E) and after acylation only a few mg of heterogeneous product had been collected. This behaviour was not changed in several repetitions with slight modifications of the reaction conditions.

L-N-*(t-Butoxycarbonyl)*-S-*methyl-4'-mercaptophenylalanine* (Boc-(4'-SCH_3)Phe, **14**). Crude **5** has been treated with CH_3I (2.13 g, 15 mmol) as described for **11**, and only 47 mg of **14** were obtained. – ^{1}H-NMR. ($CDCl_3$): 11.45 (*s*, 1 H); 7.10 (*m*, 4 H); 5.07 (*m*, 1 H); 4.43 (*m*, 1 H); 3.06 (*m*, 2 H); 2.40 (*s*, 3 H); 1.37 (*s*, 9 H).

HF-cleavage of **10**, **11**, and **12**. Each compound (100 mg) was treated with 5 ml of liq. HF with 100 µl diethyl sulfide and 1 ml of anisole at 0° for 30 min. After removal of the reactants by flushing with N_2 and followed by high vacuum, the residue was dissolved in 5 ml of 0.2N AcOH and controlled on TLC. (D and E). Compound **10** was converted to (4'-S-Acm)Phe without formation of **5**. Compound **11** was completely converted to **5**, no trace of (4'-S-Bzl)Phe was detectable. Compound **12**, however, contained important amounts of (4'-S-BrBzl)Phe on TLC.; density scanning of TLC. indicated about 15% still bromobenzylated **5**.

Sar-Arg-Val-(4'-sulfo)Phe-Val-His-Pro-Phe ([Sar^1-(4'-SO_3H)Phe^4]-AT, **16**). Of the Boc-Phe resin ester (0.36 mmol/g) 10 g were used to start the syntheses. The Boc-Val-His-Pro-Phe-tetrapeptide resin ester was prepared with symmetrical anhydrides following described procedures [15] using the following protected amino acids: Boc-Val, N$^{\alpha}$-Boc-N^{im}-Tos-His, and Boc-Pro. The tetrapeptide resin ester was split in 10 equal portions and the synthesis of **16** was continued with one portion and the following amino acids: Boc-Sar, N$^{\alpha}$-Boc-N^{g}-Tos-Arg-, Boc-Val and Boc-**3** · DEA.

Until the coupling of Boc-**3**, the couplings were carried out in CH_2Cl_2/DMF 10:1. A first attempt to couple Boc-**3** with the same solvent system failed, but repetition in DMF produced a satisfactory coupling (negative ninhydrin test) after 1 h initial coupling with 1 equiv. of DCC followed by a second h and an additional ½ equiv. of DCC. The next couplings up to Boc-Sar were carried out in DMF/CH_2Cl_2 10:1 without further problems, except Boc-Val which made one repetition necessary.

Table 3. *Physicochemical and biological properties of synthetic angiotensin II analogues*

Name	No.	TLC.-Rf			Amino-acid analysis								Yield[a]	Biological activity			
		D	E	RP-TLC.										Rabbit aorta a^{Eb})	R.A.[c])	Blood pressure R.B.P.[d])	Binding R.A.[e])
[Sar[1]]-AT	**15**	0.375	0.398	0.271	Sar	Arg	2Val	Tyr	His	Pro	Phe	–	–	1.0	100%	100%	100%
[Sar[1]-(4′-SO_3^-)Phe[4]]-AT	**16**	0.317	0.296	0.534	[f])	0.97	2.05	–	0.84	1.09	1.05	1.00 of **3**	50%	–	0%	0%	0%
[Sar[1]-(4′-SO_2NH_2)Phe[4]]-AT	**17**	0.356	0.394	0.391	[f])	1.11	2.00	–	1.00	1.12	1.05	0.74 of **3**	50%	1.0	3.2%	0.9%	3.8%
[Sar[1]-(4′-S-Acm)Phe[4]]-AT	**18**	0.356	0.398	0.323	[f])	1.01	2.00	–	1.04	1.05	1.07	–	32%	1.0	3.5%	–	28%
[Sar[1]-(4′-SH)Phe[4]]-AT	**19**	0.397	0.434	0.142	–	–	–	–	–	–	–	–	–	1.0	0.9%	–	0.5%
[Sar[1]-(4′-S-Acm)Phe[8]]-AT	**20**	0.327	0.380	0.417	[f])	0.97	2.00	0.95	1.00	1.01	–	–	26%	0.0	9%[g])	–	23.0%
[Sar[1]-(4′-SH)Phe[8]]-AT	**21**	0.382	0.361	0.290	–	–	–	–	–	–	–	–	–	0.54	5.7%	–	8.2%
([Sar[1]-(4′-S)Phe[8]]-AT)$_2$	**22**	0.376	0.380	0.201	[f])	1.03	1.87	0.62	1.05	1.04	–	–	50%	0.20	4.6%	–	–
AT-(Asp-Arg-Val-Tyr-Ile-His-Pro-Phe)	**23**	0.476	0.388	–	Asp	Arg	Val	Tyr	Ile	His	Pro	–	–	1.0	90%	–	32.0%
[(4′-S-Acm)Phe[8]]-AT	**24**	0.386	0.393	0.491	1.16	0.90	0.82	1.00	0.92	0.99	1.04	–	38%	0.2	8.5%	–	–

[a]) The yield is calculated from the initial substitution of the peptide resin and the obtained pure peptide. The yield of **22** is calculated from the amount of **20** used.

[b]) a^E is the intrinsic activity of a given peptide, a full agonist having $a^E = 1$ and a pure antagonist $a^E = 0$.

[c]) R.A. is the relative affinity of an analogue compound to **15** which produces half maximal response to 0.74×10^{-9} M.

[d]) R.B.P. is the relative blood pressure potency compared to **15**.

[e]) In the binding assay, R.A. is the relative affinity of an analogue compared to **15** which displaces half of the specifically bound radioactive [Sar[1]]-AT [7] at a concentration of 9.8×10^{-9} M.

[f]) Amino acid present but not quantified; sarcosine gives a very weak peak close to histidine.

[g]) Peptide **20** is a pure antagonist on rabbit aorta with an ID_{50} of 8.1×10^{-9} M.

After HF-cleavage, the crude peptide was subjected to gel filtration on *Sephadex G 15* (2.5 × 100 cm), eluted with 0.2N AcOH followed by a partition chromatography on *Sephadex G25* (2.5 × 80 cm) saturated with the lower phase of the system BuOH/AcOH/H_2O 4:1:5 and eluted with the upper phase. The peptide fraction was recovered from the last fractions of the elution, affording 184 mg of **16**.

Sar-Arg-Val-(4'-aminosulfonyl)Phe-Val-His-Pro-Phe ([Sar^1-(4'-SO_2NH_2)Phe^4]-AT, **17**). One part of Boc-Val-His-Pro-Phe-resin mentioned above was used at the beginning of this synthesis. The same amino acids and Boc-**4** instead of Boc-**3** · DEA were used. After HF-cleavage, gel filtration and partition chromatography 188 mg of **17** were obtained.

Sar-Arg-Val-(4'-S-acetamidomethyl)mercapto-Phe-Val-His-Pro-Phe ([Sar^1-(4'-S-Acm)Phe^4]-AT, **18**). One portion of the tetrapeptide resin Boc-Val-His-Pro-Phe was used together with Cbz-Sar, N^{α}-Boc-N^{g}-Tos-Arg, Boc-Val and **10**. After completion of the synthesis, HF-cleavage and a first *Sephadex G15* gel filtration (see above), the crude peptide has been loaded onto a reversed-phase column (see the synthesis of **7**) and eluted with 7% 2-propanol in 0.25n ammonium acetate at pH 5 with a gradient of 15 to 35% acetonitrile. Lyophilization of the homogeneous fractions yielded 145 mg of pure **18**.

Sar-Arg-Val-Tyr-Val-His-Pro-(4'-S-acetamidomethyl)mercapto-Phe ([Sar^1-(4'-S-Acm)Phe^8]-AT, **20**). **10**-Resin ester (1.5 g), produced with the C_s-salt method [19] and with a substitution of 0.35 meq./g (measured according to [20]) were used for this synthesis. *N*-Boc-*O*-BrBzl-Tyr and the usual amino acids were condensed to this resin. After completion of the synthesis, treatment by HF and the usual gel filtration, the crude product was purified on a reversed-phase column eluted with a gradient of 7% 2-propanol, 0.25N ammonium acetate pH 5 and 10 to 30% acetonitrile, 150 mg of pure **20** were collected after lyophilization.

Sar-Arg-Val-(4'-mercapto)Phe-Val-His-Pro-Phe ([Sar^1-(4'-SH)Phe^4]-AT, **19**) *and Sar-Arg-Val-Tyr-Val-His-Pro-(4'-mercapto)Phe* ([Sar^1-(4'-SH)Phe^8]-AT, **21**) *and bis(Sar-Arg-Val-Tyr-Val-His-Pro-(4'-)-Phe) disulfide* (([Sar^1-(4'-)Phe^8]-AT)$_2S_2$, **22**). Peptide **18** or **20** (19,1 mg, 18 µmol) were dissolved in 2 ml of 2N ammonium acetate at pH 4.4. To this solution mercuric acetate (5.7 mg, 18µmol) were added and stirred for 1 h. H_2S was bubbled through this solution during 40 min, followed by the addition of 100 µl of mercaptoethanol. The precipitated mercury sulfide was filtered off and the solution evaporated under N_2 (TLC.-*Ellmann* positive). This product was either directly used for pharmacological tests or lyophilized twice, redissolved in ammonium-acetate buffer at pH 4.4 and oxidized by air overnight to form **22**. This product was purified by gel filtration over *Sephadex G15* (8 mm × 30 cm), eluted with 0.2N AcOH and 9.5 mg of pure **22** were collected (TLC.-*Ellmann* negative).

Asp-Arg-Val-Tyr-Ile-His-Pro-4'-(S-acetamidomethyl)mercapto-Phe ([4'-S-Acm)Phe^8]-AT, **24**). The same **10**-resin ester (1.0 g) as used for **20**, and the usual amino acids, N-Boc-OBzl-Asp and Boc-Ile were used. After completion, cleavage and the first gel filtration, the product was subjected to a reversed-phase chromatography in a gradient of 7% 2-propanol, 0.25N ammonium acetate at pH 5 with 5 to 28% acetonitrile, affording after lyophilization 150 mg of **24**.

REFERENCES

[1] *F. Bergel & J.A. Stock,* J. Chem. Soc. *1959*, 90; *D.F. Elliott, A.T. Fuller & C.R. Harrington,* J. Chem. Soc. *1949*, 1374.
[2] *J.C. Nevenzel, W.E. Shelberg & C. Niemann,* J. Am. Chem. Soc. *71*, 3024 (1949).
[3] *A. Agosti, I. Orlandini & R. de Castiglione,* Experientia *27*, 412 (1971).
[4] *N.H. Alexander,* Anal. Chem. *30*, 1292 (1958).
[5] *E. Escher, T.M.D. Nguyen, H. Robert, S. St-Pierre & D. Regoli,* J. Med. Chem. *21*, 860 (1978) and also *E. Escher, T.M.D. Nguyen & D. Regoli,* Can. J. Physiol. Pharmacol. *56*, 956 (1978).
[6] *E. Escher, G. Guillemette, O. Leukart & D. Regoli*, Eur. J. Pharmacol. *66*, 267 (1980).
[7] *R. Leduc, M. Bernier & E. Escher,* Helv. Chim. Acta *66*, 960 (1983).
[8] *S. Patai,* 'The Chemistry of the Thio Group', Vol. I, J. Wiley Inc. N.Y. 1974, p. 395.
[9] *W. Lunkenheimer & H. Zahn,* Justus Liebigs Ann. Chem. *740*, 1 (1970).
[10] *U. Weber & P. Hartter,* Z. Physiol. Chem. *351*, 1384 (1970).

[11] *D. F. Veber, J. D. Milkowski, R. G. Denkewalter & R. Hirschmann,* Tetrahedron Lett. *1968*, 3057.
[12] *D. A. Onties, J. C. Roberts, J. F. Hennessy, H. J. Burford & C. W. Cooper,* Endocrinology *92*, 1780 (1973).
[13] *L. Moroder, A. Hallet, E. Wünsch, O. Keller & G. Wersin,* Z. Physiol. Chem. *357*, 1651 (1976).
[14] *K. Noda, S. Terada & N. Izumiya,* Bull. Chem. Soc. Jpn. *43*, 1883 (1970) and also *F. H. Carpenter & D. T. Gish,* J. Am. Chem. Soc. *74*, 3818 (1952).
[15] *O. Leukart, E. Escher, D. Regoli & R. Schwyzer,* Helv. Chim. Acta *62*, 546 (1979).
[16] *C. B. Glaser, H. Maeda & J. Meienhofer,* J. Chromatogr. *50*, 151 (1970).
[17] *L. V. D. Small, J. H. Bailey & C. J. Cavallito,* J. Am. Chem. Soc. *69*, 1710 (1947).
[18] *J. L. Kice & J. P. Cleveland,* J. Am. Chem. Soc. *95*, 104 (1973).
[19] *H. Bredereck, A. Wagner, H. Beck & R. J. Klein,* Chem. Ber. *93*, 2736 (1960).
[20] *B. F. Gisin,* Anal. Chim. Acta *58*, 248 (1972).