

128. Angiotensin II Analogues. Part II. Synthesis and Incorporation of the Sulfur-Containing Aromatic Amino Acids: L-(4'-SH)Phe, L-(4'-SO₂NH₂)Phe, L-(4'-SO₃)Phe and L-(4'-S-CH₃)Phe¹)²

by Emanuel Escher³), Michel Bernier and Paul Parent

Pharmacologie, Fac. de médecine, Université de Sherbrooke, Sherbrooke, Qué. Canada J1H 5NH

(25.III.83)

Summary

L-Phenylalanine has been treated with chlorosulfonic acid and the product was either hydrolyzed, ammonolyzed or reduced. The resulting sulfonic-acid and amino-sulfonyl 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'-SO₃H)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. Wunsch, 'Synthese von Peptiden', Vol. 15, part 1, of 'Houben-Weyl, Methoden der Organischen Chemie', E. Müller, ed. G. Thieme Stuttgart, G.F.R. 1974.

³) Author to whom correspondence should be addressed.

SAR studies. The aminosulfonyl derivative (4'-SO₂NH₂)Phe is a highly hydrophilic but uncharged amino acid, also useful for the above-mentioned purposes.

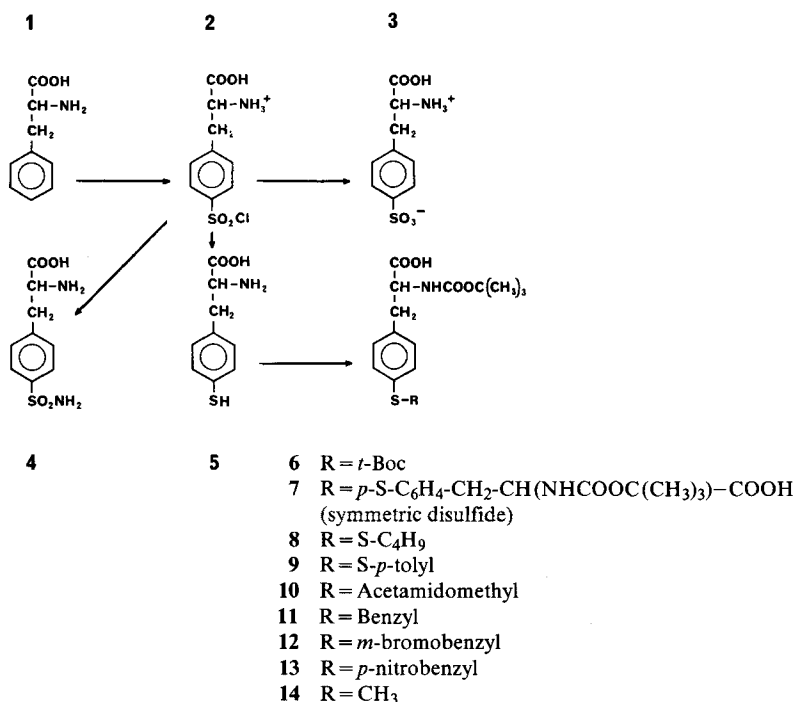
All these considerations seemed to be suitable for angiotensin II (Asp-Arg-Val-Tyr-Val-His-Pro-Phe, abbreviated as AT) since we have been studying the two aromatic positions of this peptide hormone since some time [5] [6]. It was observed that position 4 (Tyr) of AT affects the affinity of the hormone 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. NH₃, both **3** and the aminosulfonyl derivative **4** are obtained together. The free thiol **5** was more difficult to obtain, because several reduction attempts with NaBH₄ 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 **5** 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



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 ($\rightarrow$ **11** and **12**) or methyl iodide ($\rightarrow$ **14**) at neutral pH, followed by the isolation and purification after *N*^α-protection with bis(*t*-butyldicarbonate) (Boc₂O) [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 Ac_m-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].

This work has been financed by grants from the Medical Research Council of Canada and the Canadian Heart Foundation.

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 CHCl₃-solution. ¹H-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 0.25N 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 TLC., HF: anhydrous hydrogen fluoride, DEA: diisopropylethylamine, Ts: *p*-toluenesulfonyl, BrBzL: (*m*-bromobenzyl), Boc: *t*-butoxycarbonyl, Boc₂O: 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. NH₃ 3:1; B: BuOH/0.05M ammonium acetate 2:1, pH 7.0; C: BuOH/AcOH/H₂O 4:1:1; D: BuOH/AcOH/H₂O 5:2:3; E: BuOH/AcOH/H₂O/Pyridine 30:6:20:12; F: CHCl₃/AcOH/MeOH 95:5:3. RP-TLC. was performed on Whatman reversed-phase plates KC 18 in the solvent system 0.5M 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-mercaptopropionic acid in vacuum-sealed tubes. The amino-acid analyses were performed on a Beckman 119 CL analyzer.

L-4'-Sulphophenylalanine ((4'-SO₃H)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/H₂O, yield, 2.2 g of large, rhomboid crystals of **3**. - ¹H-NMR. (1N LiOD): 7.46 (*m*, AA'BB', 4 H); 3.43 (*m*, 1 H); 2.94 (*m*, 2 H).

L-*N*^a-*t*-Butoxycarbonyl-4'-sulfophenylalanine-diisopropylethylammonium salt (Boc-(4'-SO₃⁻)Phe · DEA, Boc-3 · DEA). Compound **3** (2.2 g, 9 mmol) was dissolved in 70 ml of H₂O 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 Boc₂O (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 2*N* H₂SO₄ and solid NaHSO₄. 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 1*N* NaOH. The solution was added to an ion-exchange column (3 × 20 cm) of BioRad AG 1X8, rinsed with water and eluted with 2*N* aq. DEA. After evaporation and lyophilization, 2.74 g of Boc-3 · DEA were recovered (64%). – ¹H-NMR. (D₂O, after addition of 2 equiv. of LiOD in D₂O 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'-SO₂NH₂)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 2*N* aq. NH₃ 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 0.2*N* AcOH yielded 1.47 g of pure **4** after lyophilization.

L-4'-(Aminosulfonyl)-*N*^a-*t*-butoxycarbonylphenylalanine (*N*^a-Boc-(4'-SO₂NH₂)Phe, Boc-**4**). Compound **4** (2.89 g, 11.7 mmol) was dissolved in 20 ml of 1*N* NaOH and diluted with 55 ml of H₂O and 50 ml of *t*-BuOH. At r.t. and under magnetic stirring 3.05 g of solid Boc₂O (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	[α] _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 ₃ H)Phe	3	0.12 (A), 0.026 (B), 0.12 (C)	– 11.8°	–	45%
<i>N</i> -Boc-(4'-SO ₃ ⁻)Phe	Boc- 3	0.48 (E)	+ 15.20°	–	64%*
H-(4'-SO ₂ NH ₂)Phe	4	0.26 (A), 0.13 (B), 0.26 (C)	– 6.25°	–	20%
<i>N</i> ^a -Boc-(4'-SO ₂ NH 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%
<i>N</i> -Boc-(4'-SH)Phe	<i>N</i> -Boc- 5	0.40 (F)	–	–	–
<i>N</i> -Boc-(4'-S-Boc)Phe	6	0.43 (F)	+ 8.95°	112.5–114.0°	22%
(H-4'-Phe) ₂ S ₂		0.40 (D), 0.38 (E)	–	–	–
(<i>N</i> -Boc-4'-Phe) ₂ S ₂	7	0.26 (F)	+ 47.25°	162.0–163.5°	20%
H-(4'-S-S-butyl)Phe		0.64 (D)	–	–	–
<i>N</i> -Boc-(4'-S-S-butyl)Phe	8	0.50 (F)	+ 10.00°	oil	6%
H-(4'-S-S-tolyl)Phe		0.62 (D)	–	–	6%
<i>N</i> -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%
<i>N</i> ^a -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%
<i>N</i> -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%
<i>N</i> -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)	–	–	–
<i>N</i> -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	H	N	S	Br
				Calc./Found	Calc./Found	Calc./Found	Calc./Found	Calc./Found
(4'-SO ₃ H)Phe	3	C ₉ H ₁₁ NO ₃ S · ½ H ₂ O	254.23	42.52/42.79	4.76/4.58	5.50/5.41	12.61/12.51	
N ^a -Boc-(4'-SO ₂ NH ₂)Phe	Boc-4	C ₁₄ H ₂₀ N ₂ O ₆ S · H ₂ O	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 ₆ S	397.50	57.41/57.40	6.85/6.64	3.52/3.72	8.07/8.18	
(N ^a -Boc-4'-Phe) ₂ S ₂	7	C ₂₈ H ₃₆ N ₂ O ₈ S 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 ₄ S 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- <i>p</i> -tolyl)Phe ^{a)}	9	C ₂₁ H ₂₅ NO ₄ S 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 ₂ O ₅ S	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 ₄ S	387.50	65.09/65.20	6.50/6.56	3.62/3.59	8.27/8.57	
N ^a -Boc-(4'-S- <i>m</i> -BrBzl)Phe	12	C ₂₁ H ₂₄ BrNO ₄ S	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 ₄ S	311.06	57.86/57.60	6.80/6.97	4.50/4.33	10.30/10.54	

^{a)} Compound disproportionates spontaneously.

2N H₂SO₄. The white precipitate was extracted twice with EtOAc, the org. phases were pooled, dried over anh. Na₂SO₄ and evaporated. The crude product was further purified by chromatography on a pre-packed lobar silica-gel column (Merck, size C) with CHCl₃/MeOH 40:1, 2.38 g of pure Boc-4. – ¹H-NMR. (D₆-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 H₂O and H₂S was bubbled through the solution at 40°. The precipitated Sn-sulfide was filtered off and some additional H₂S 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 Boc₂O and 4N NaOH until a pH of 10 was obtained. The pH was adjusted several times with 2N Na₂CO₃ 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 NaHSO₄ to pH 2 and extracted twice with EtOAc. The org. phases were pooled, dried over anh. Na₂SO₄ and evaporated *i.v.*. The residual yellow oil was loaded onto a prepacked silica-gel column (Merck, size C), washed with toluene/CH₂Cl₂ 1:1, eluted with a gradient of CH₂Cl₂/MeOH, 0 to 2% and 2.6 g of a white powder were collected. – ¹H-NMR. (CDCl₃): 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)₂S₂, 7). O₂ 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 Boc₂O. 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 H₂O/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. Na₂SO₄ 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-thio-butane-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 Et₂O. 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 H₂O gave a product which was dissolved in 25 ml 1N NaOH, diluted with 100 ml of H₂O and 100 ml of *t*-BuOH. Introduction of Boc was carried out as usual with 500 mg (2.3 mmol) of Boc₂O. 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). – ¹H-NMR. (CDCl₃): 10.67 (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 R_f ≈ 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 CHCl₃/MeOH 0 to 2%. The collected fractions were pooled, evaporated and recrystallized from EtOAc/petrol ether, 600 mg of 9. – ¹H-NMR. (CDCl₃): 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^α-t-butoxycarbonyl-4'-mercaptophenylalanine (N^α-Boc-(4'-S-Acm)Phe, **10).** The pH of crude **5** was adjusted to 3 with 4N NaOH, the solution cooled to 0° and N₂ 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 Boc₂O, isolated and purified on silica-gel as usual, using a gradient of CHCl₃ 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. – ¹H-NMR. (CDCl₃): 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 Et₃N (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 H₂O and 100 ml of *t*-BuOH and acylated with Boc₂O (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 H₂O/MeOH 20 to 90%. Purified **10** was recrystallized from Et₂O/petrol ether and yielded 3.3 g of **10** as white powder. – ¹H-NMR. (CDCl₃): 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. – ¹H-NMR. (CDCl₃): 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^α-(*t*-Butoxycarbonyl)-S-(*p*-nitrobenzyl)-4'-mercaptophenylalanine (13**).** Crude **5** was treated with *p*-nitrobenzyl bromide (2.0 g, 9.2 mmol) as described for **11**. Before acylation with Boc₂O no major product could be detected around R_f 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'-SCH₃)Phe, **14).** Crude **5** has been treated with CH₃I (2.13 g, 15 mmol) as described for **11**, and only 47 mg of **14** were obtained. – ¹H-NMR. (CDCl₃): 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 N₂ 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 ((Sar¹-(4'-SO₃H)Phe⁴)-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^α-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^α-Boc-N^B-Tos-Arg-, Boc-Val and Boc-3·DEA.

Until the coupling of Boc-3, the couplings were carried out in CH₂Cl₂/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 1/2 equiv. of DCC. The next couplings up to Boc-Sar were carried out in DMF/CH₂Cl₂ 10:1 without further problems, except Boc-Val which made one repetition necessary.

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/H₂O 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 ([Sar¹-(4'-SO₂NH₂)Phe⁴]-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 ([Sar¹-(4'-S-Acm)Phe⁴]-AT, **18**). One portion of the tetrapeptide resin Boc-Val-His-Pro-Phe was used together with Cbz-Sar, N^α-Boc-N^ε-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 ([Sar¹-(4'-S-Acm)Phe⁸]-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 ([Sar¹-(4'-SH)Phe⁴]-AT, **19**) and *Sar-Arg-Val-Tyr-Val-His-Pro-(4'-mercapto)Phe* ([Sar¹-(4'-SH)Phe⁸]-AT, **21**) and *bis(Sar-Arg-Val-Tyr-Val-His-Pro-(4'-)-Phe) disulfide* ([Sar¹-(4'-)-Phe⁸]-AT)₂S₂, **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, 18 μmol) were added and stirred for 1 h. H₂S 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 N₂ (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)Phe⁸]-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 Liebig's 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. Denkwalter & 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).