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173. Hormone-Receptor Interactions. Synthesis of a Biologically Active Cysteinyl-Angiotensin Derivative and its Use for the Preparation of Spin-labelled and Polymer-supported Molecules

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(7. VI. 74)

Summary. (N-t-Butoxycarbonyl-S-acetamido-methyl-L-cysteinyl)-[1-asparagine, 5-valine]-angiotensin II (**2**) was prepared from [1-asparagine, 5-valine]-angiotensin II (**1**). With respect to enhancement of rat blood pressure, it is a full agonist displaying about 20–25% of the potency of **1**. The sulfur atom of the cysteine group represents a specific nucleophile by which the hormone can easily be attached to other molecules in a well-defined manner. Thus, **2** has been added to N-substituted maleimides through its (deprotected) thiol group to produce the spin-labelled derivative **3**: {N-*t*-butoxy-carbonyl-S-[N-(1-oxy-2, 2, 5, 5-tetramethyl-pyrrolidin-3-yl-methyl)- imidosuccin-3-yl]-L-cysteinyl}-[1-asparagine, 5-valine]-angiotensin II, and the polymer-supported derivative **6**: S-(N-{5-[7-(sepharosyl-oxy-carbonimidoyl-amino)-4-azaheptylcarbamoyl]-pentyl}-imidosuccin-3-yl)-L-cysteinyl-[1-asparagine, 5-valine]-angiotensin II. The ESR. spectra of **3** in different solvents, and its principal NMR. characteristics, are reported. We are using the compound for studying its interaction with receptor molecules and for purification of target cells and membrane vesicles by affinity aggregation.

Introduction. – Labelling techniques and affinity chromatography are becoming increasingly important for the study of hormone-receptor interactions. Ideally, attachment of the marker group or of the polymeric support material to the hormone should leave the biological activity unimpaired in order to ensure correct reaction with the original set of receptor molecules. This condition can some-

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times be realized by specific covalent substitution at chemically and biologically well-defined points within a polypeptide hormone molecule [1].

Spin labelling – the introduction of paramagnetic substituents into diamagnetic molecules [2] – is a valuable tool for examining a variety of molecular properties and interactions in biological systems by electron spin resonance spectroscopy, ESR. (for reviews, *cf.* [3]). Special efforts have been directed towards studying the topography of protein active sites [4] and the supramolecular structure of natural [5] and artificial membranes (*e.g.* liposomes [6] and oriented lipid films [7]). It seems natural to extend the method for observing interactions between hormones and their discriminators (receptors) and other hormone-specific binding proteins.

Affinity chromatography requires the attachment of the 'ligand' – here a polypeptide hormone – to an insoluble, polymeric carrier [8]: ideally in a specific manner guaranteeing ligand exposure to the solvent and, also, if possible, the conservation of biological properties (*e.g.* hormone activity). The technique is proving its usefulness for the isolation of enzymes, antibodies, 'receptors' etc., although caution with regard to its limitations is necessary [9].

Our aim was to produce a biologically active angiotensin II derivative which could on the one hand easily adapt a spin label and on the other facilitate specific attachment to a polymeric support. We decided to use the sulfur atom of a cysteine group as a specific, nucleophilic handle which would be able to react with *e.g.* maleimide groups of spin labels [10] or of substituted polymeric supports as suggested by *Rudinger & Keller* (*cf.* [11]). Substitution at the N-terminus of the octapeptide **1** seems favorable, as such alterations either enhance biological activity [12] or attenuate it only slightly [13]; chemical changes at the C-terminus tend to abolish it [14].

Results and Discussion. – *Peptides.* The key compound described here is (N-*t*-butoxycarbonyl-S-acetamidomethyl-L-cysteinyl)-[1-asparagine, 5-valine]-angiotensin II (**2**). It was prepared from [Asn^1, Val^5]-angiotensin II (**1**) [15] by reaction with the N-hydroxysuccinimide ester of N-*t*-butoxycarbonyl-S-acetamidomethyl-L-cysteine [16] in a mixture of 2-propanol and water (dimethylformamide or trifluoroethanol were less suitable).

The acetamidomethyl group of **2** can easily be removed with Hg^{2+} ions. The resulting sulfhydryl group reacts smoothly with 3-maleimidomethyl-2,2,5,5-tetramethyl-1-pyrrolidinyl-oxide (here designated as 'free label', **FL**) to produce {N-*t*-butoxycarbonyl-S-[N-(1-oxy-2,2,5,5-tetramethyl-pyrrolidin-3-yl-methyl)-imidosuccin-3-yl]-L-cysteinyl}-L-asparaginyl-L-arginyl-L-valyl-L-tyrosyl-L-valyl-L-histidyl-L-prolyl-L-phenylalanine (**3**).

Elimination of both the *t*-butoxycarbonyl and the acetamidomethyl groups from **2** afforded L-cysteinyl-[Asp^1, Val^5]-angiotensin II, which was not isolated but allowed to react directly with the N^1-(5-maleimido-caproyl) derivative **5** of 7-(sepharosyl-oxycarbonimidoyl-amino)-4-azaheptylamine (**4**). Substitution was approximately 0.25 μmol of cysteinyl-angiotensin per ml of the moist gel.

Biological activity. Examination of the hypertensive activity in one individual rat revealed that **2** is a full agonist (same maximal response as for **1**), but possesses only about 20–25% of the potency of **1** (Fig. 1). This behavior is similar to that of

other angiotensin II derivatives modified at the N-terminal amino group [13]. In contrast to other analogues which in addition are modified at the C-terminus – such as [1-sarcosine,8-isoleucine]- or [1-sarcosine-8-alanine]-angiotensin II –, **2** displays no inhibitory properties. Its effect is of normal duration, although a prolongation could have been expected because of a possible influence of the fully protected cysteine residue on enzymatic degradation; ([1-β-aspartyl, 5-valine]-angiotensin II has a prolonged action, which was paralleled by its reduced sensitivity towards aminopeptidases [17]).

The results indicate that introduction of an N- and S-substituted cysteine residue does not seriously impair the biological activity of angiotensin II if situated at the N-terminus of the hormone. For our purposes, therefore, the use of the sulfur atom

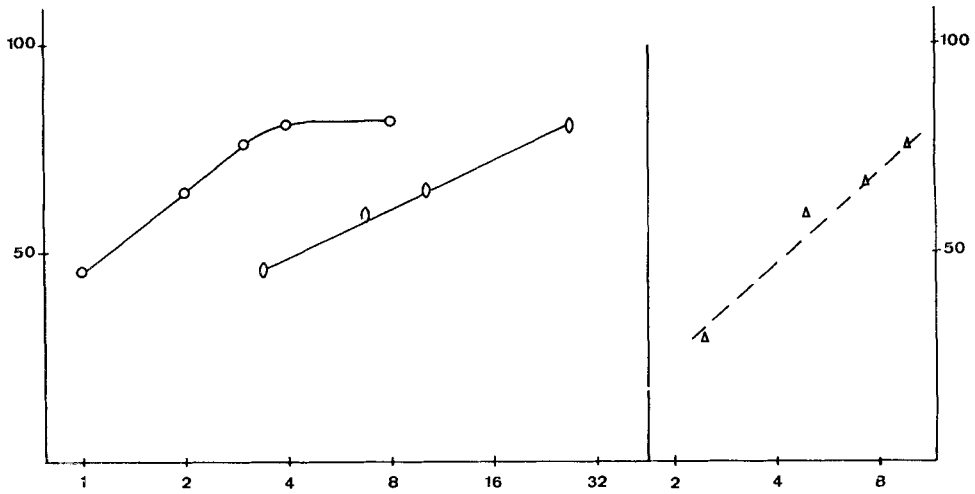


Fig. 1. *Log dose-response curves.* Abscissa with log dose in μg of **1** (○) and **2** (◊), and in international mU of vasopressin (△). Ordinate with blood pressure rise in mm Hg.

as a point of subsequent substitution is not *a priori* excluded, although the biological activity of specific derivatives, such as **3** or **6**, remains to be determined.

NMR. spectra. The NMR. spectra of **1**, **2** and **3** agreed with the assumed chemical constitutions. The table lists some of the salient features. With the introduction of the Boc-Cys(Acm) residue, the resonances b, c, and e – attributed to this group – appear with correct relative intensities. Spin-label attachment causes the Acm resonances, one of which is c, to disappear. It also broadens all lines, as would be expected for the influence of the unpaired electron of the N-oxide radical on relaxation-times (decrease of T2).

ESR. spectra. In 0.1 M sodium acetate solution, the resonance lines of the labelled peptide **3** appear at almost the same fields as those of the free N-oxide-label, **FL**, (Fig. 2), but with slightly lower amplitudes, indicating a small difference between the mobilities of the respective N-oxide groups. The rotational correlation times, τ(o), were calculated according to [10] and found to be 7.9×10^{-10} s for **FL** and 3.5×10^{-9} s for **3**. A certain broadening of the lines and a slight reduction of the value of

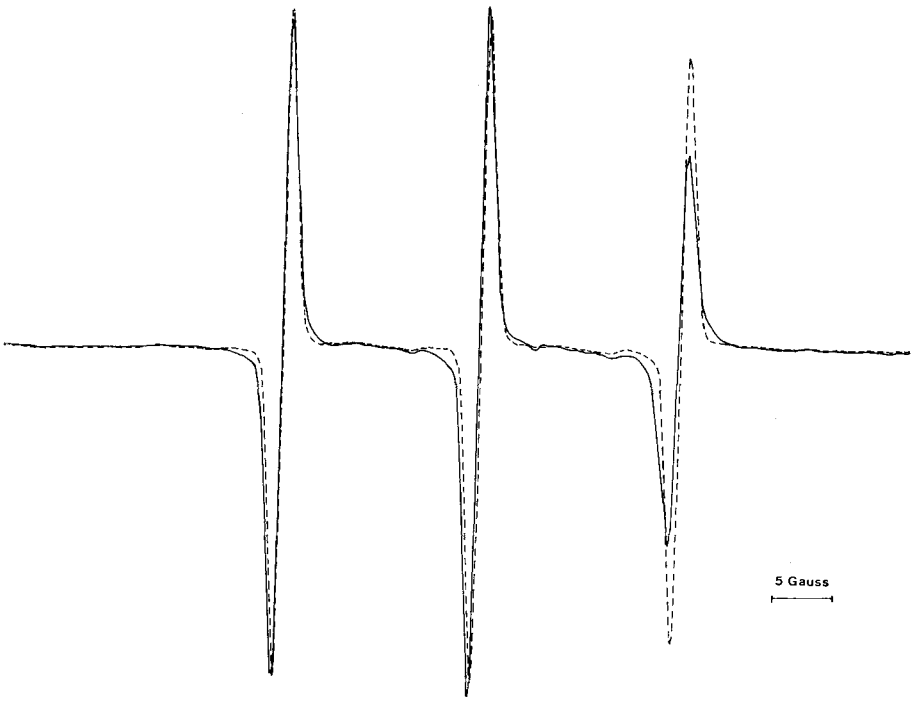


Fig. 2. *ESR. spectra of* 10^{-4} M **3** (——) *and* **FL** (– – –) *in 0.1* M *sodium acetate solution at 20°*

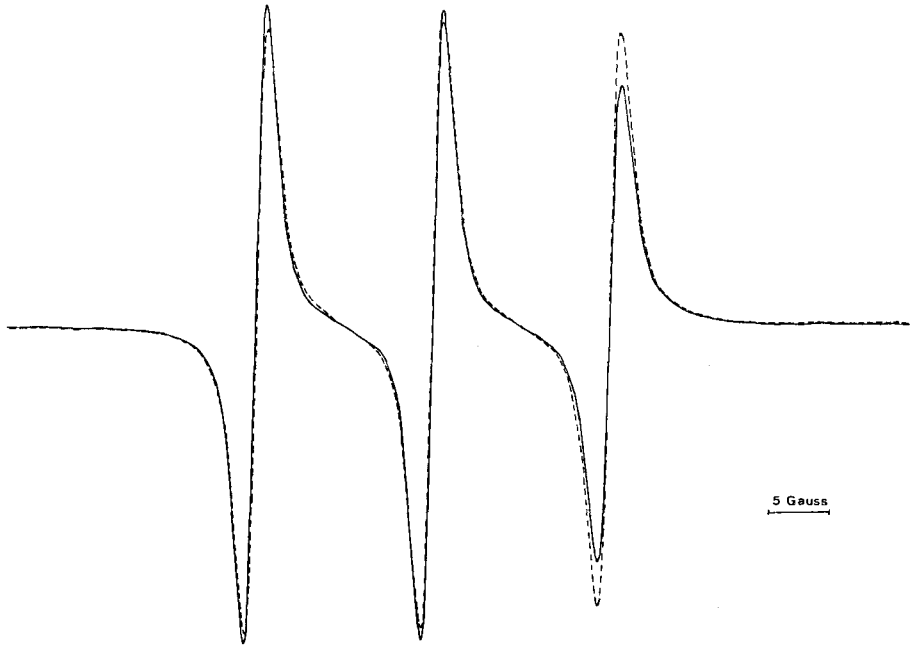


Fig. 3. *ESR. spectra of* 10^{-3} M **3** (——) *and* **FL** (– – –) *in pyridine at 20°*

the hyperfine coupling (distance between the lines) is observed for **3** as compared with **FL**. In pyridine and methanol solutions (Fig. 3 and 4) the lines become very broad, probably as a result of the very low ionic strength.

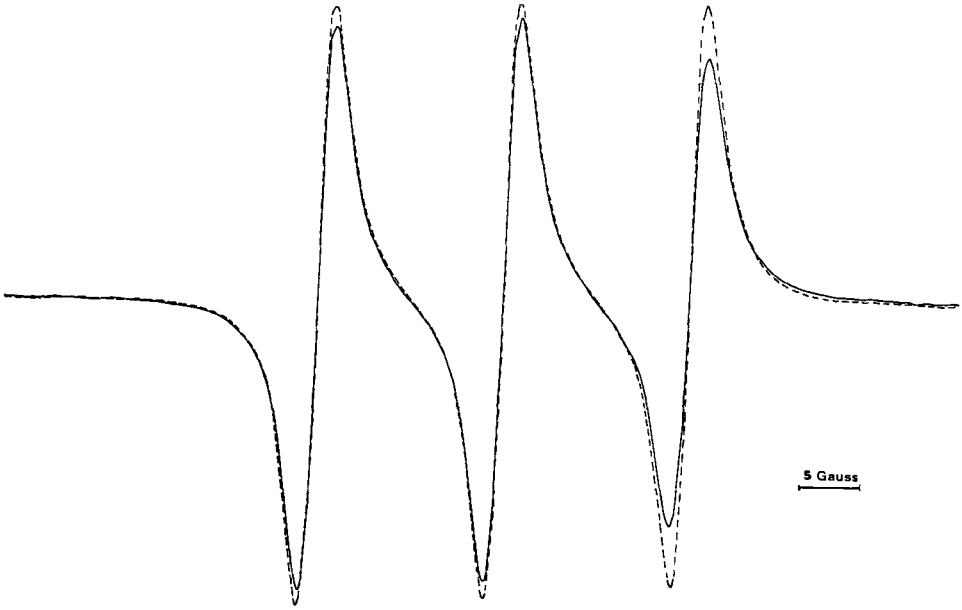
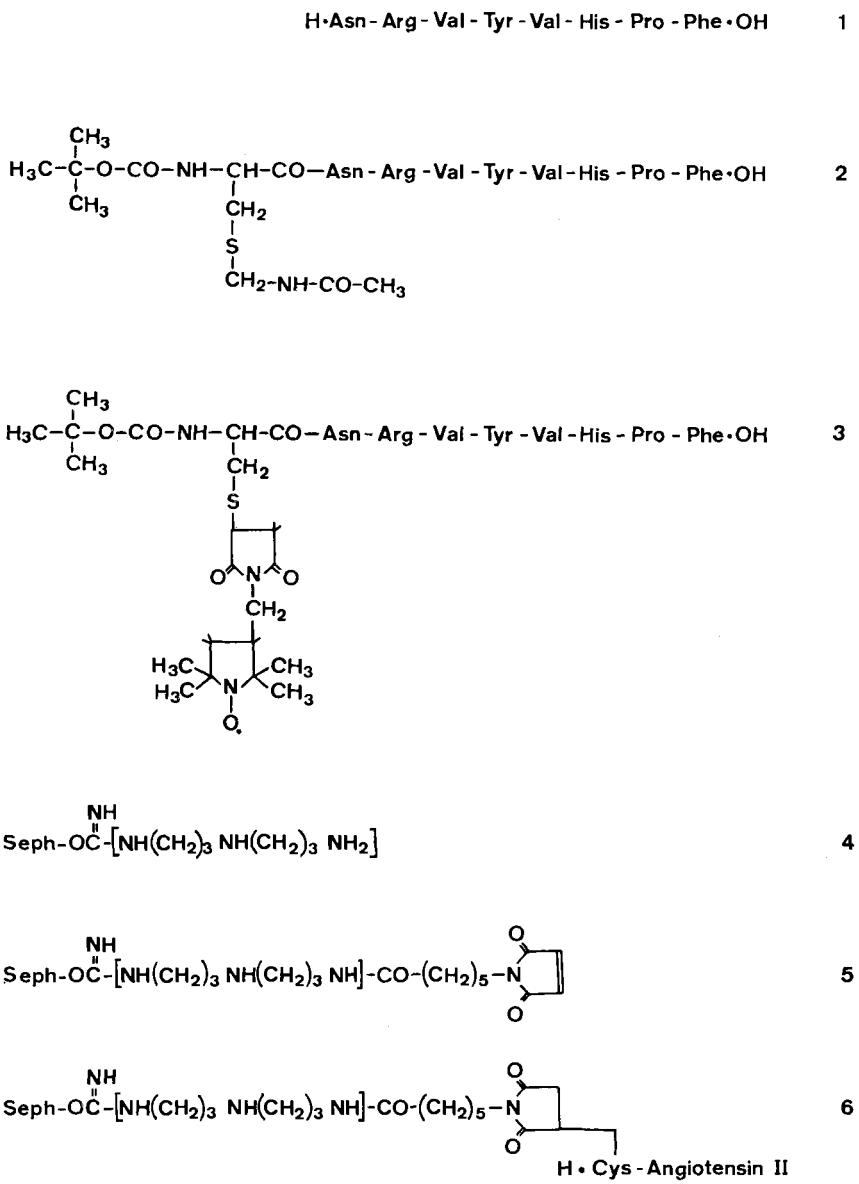


Fig. 4. *ESR. spectra of* 10^{-3} M **3** (——) *and* **FL** (– – –) *in methanol at 20°*

The practical use of a probe, like spin-labelled angiotensin **3**, is with a view to determining the thermodynamic parameters of its specific association with receptor molecules and also certain topographic features of the specifically labelled site. Evaluation of changes in band-width, which reflect correlation- and relaxation-time alterations, have been used to determine association constants in biological systems involving labelled drugs such as sulfonamides, barbiturates, choline esters, and local anesthetics [18], as well as haptenes [19], coenzymes [20], and serine enzyme inhibitors [21]. The method has its limitations because (a) rotational correlation-times greater than about 1 nanosecond can be estimated only with difficulty and (b) the concentrations must be rather high ($\sim 10^{-4}$ M). This last condition is hard to fulfill with receptors *in situ, e.g.* on isolated adrenal cells, where an estimated maximal hormone-receptor complex concentration of $\sim 10^{-8}$ to 10^{-9} M can be attained with a cell concentration of about 50 mg dry cell weight per ml. Perhaps isolated receptors would offer a greater possibility; lacking solubilized receptor molecules, no answer to this question is possible at the moment. Neither limitation applies to fluorescence polarization methods (*cf.* [1]); however these require more elaborate instrumentation and are more prone to interference by other fluorescing molecules (paramagnetic interference is much less probable).

It remains to be stated that *Weinkam & Jorgensen* [22] have prepared free-radical containing peptides of angiotensin II with replacement of histidine by its analogue



'nitronylnitroxyalanine' in order to study the conformation of the carboxyl-terminal region of the hormone.

Aggregation of isolated adrenal cortex cells by the insolubilized angiotensin **6**. Earlier work claims the aggregation or binding of intact fat cells by sepharose (Seph) beads containing covalently – but unspecifically – bound insulin [23]. The published microscopic pictures show single cells adhering to the beads. They are practically identical with our own pictures: a careful inspection of cell-to-bead aggregates in different

focal planes shows that the number of such contacts is very much higher for the angiotensin-loaded beads **6** than for control beads **4**. However, we do not believe that these results can be entirely convincing until a separation of angiotensin target cells from other cells contained in the suspensions has been achieved.

This work was supported by the *Schweizerischer Nationalfonds zur Förderung der wissenschaftlichen Forschung* (Gesuch No. 3.374.70). We thank Mr. *Werner Schlegel*, dipl. Natw. ETH Zürich, and Miss *Ursula Walty* who helped with cell aggregation experiments.

Experimental Part

General. M.p. were determined in open capillary tubes and are uncorrected. Optical rotations were measured with a *Perkin Elmer* 141 Polarimeter. Amino acid analyses were carried out in the laboratory of Prof. Dr. *H. Zuber* (this institute) with a *Beckman* Amino Acid Analyzer Model 120 B. ^{1}H-NMR. spectra were measured by Mr. *J.-P. Meraldi* of the laboratory of Prof. Dr. *K. Wüthrich* (this institute) with a *Brucker* 270-MHz NMR.-Spectrometer on the premises of *Spectrospin* AG., Zürich. The EPR. spectra were obtained with a *Varian* E-4 EPR.-Spectrometer.

Main educts for the synthetic work were: [1-asparagine, 5-valine]-angiotensin II (**1**) [15]; 3-maleimidomethyl-2, 2, 5, 5-tetramethyl-1-pyrrolidinyl oxide, **FL**, of *Synvar Chem. Co.*, Palo Alto, California, USA; 7-(sepharosyl-oxycarbonimidoyl-amino)-4-azaheptylamine (**4**) beads ('Affinose', Seph[AP2]amine, **4**) of *Affitron Corp.*, Rosmead, California, USA, containing 5 μmol of amino groups per ml of moist gel; and 5-maleimidylhexanoic acid N-hydroxysuccinimide ester (Diss. *O. Keller*), prepared by *O. Keller* and Prof. Dr. *J. Rudinger* (this institute). Isolated cells from cow adrenal cortex were prepared, without using enzymes to separate the cells, by Mr. *Werner Schlegel* and Miss *Ursula Walty* (this institute, preparation to be reported elsewhere).

The biological assays were carried out by Mrs. *J. Vašak* in the laboratory of Dr. *V. Plíška* (this institute). The influence on the blood pressure of physiological saline solutions of the peptides, injected into the *vena jugularis*, was tested with a male rat weighing 260 g; the animal had not been nephrectomized, but its blood pressure had been lowered and stabilized by pretreatment with phenoxybenzamine (= N-(2-chloroethyl)-N-(1-methyl-2-phenoxyethyl)-benzylamine). Preliminary tests for inhibition were carried out by injecting alternately the test and reference peptides at intervals of 30 min to avoid tachyphylaxis.

*{N-t-Butoxycarbonyl-S-acetamidomethyl-*L*-cysteinyl}-[1-asparagine, 5-valine]-angiotensin II* (**2**). [1-Asparagine, 5-valine]-angiotensin II (**1**) (130 mg, 0.1 mmol) was dissolved in 10 ml of 2-propanol water 1:1 (v/v). The solution was adjusted to pH 9.5 with triethylamine, whereupon N-*t*-butoxycarbonyl-S-acetamidomethyl-L-cysteine-hydroxysuccinimide ester [16] (200 mg, 0.51 mmol) was added. After keeping for 15 h at 20° under nitrogen, the 2-propanol was evaporated *in vacuo*. The aqueous residue was slightly diluted with water, well extracted with ethyl acetate, and lyophilized: residue 130 mg (70% yield) of crude **2**. This product was purified by countercurrent distribution in the system *n*-butanol/5% acetic acid to remove the educt **1**. After lyophilization, 67 mg (35%) of pure **2** were obtained. Thinlayer chromatography (TLC.) on silica gel in 2-propanol/conc. NH_3/H_2O 7:1:2 (v/v) revealed a single spot (ninhydrin, *Pauly, Reindel-Hoppe*), Rf = 0.52. $[\alpha]_D^{25} = -52.8°$ ($c = 0.25$, methanol); m.p. 200–220° (dec.). – Amino acid analysis (molar ratios): Cys (as cysteinic acid) 0.97, Asp 1.04, Arg 1.02, Val 1.89, Tyr 0.99, His 0.93, Pro 0.99, Phe 1.00 (reference). – (If dimethylformamide or trifluoroethanol were substituted for 2-propanol/H_2O as solvents, the yields were considerably lower. Attempts to use N-acetyl-DL-homocysteine-thiolactone for introducing a thiol function were unsuccessful.)

*{N-t-Butoxycarbonyl-S-[N-(1-oxy-2, 2, 5, 5-tetramethyl-pyrrolidin-3-yl-methyl)-imidosuccin-3-yl]-*L*-cysteinyl}-[1-asparagine, 5-valine]-angiotensin II* (**3**). A solution of **2** (10 mg, 0.01 mmol) in 10 ml of water was adjusted to pH 4 with acetic and treated with 3.5 mg (0.011 mmol) $Hg(OAc)_2$ to split off the S-acetamidomethyl group [16]. After stirring for 3 h under nitrogen at room temp., dihydrogen sulfide was bubbled through the solution, and the precipitated HgS removed by filtration. The excess dihydrogen sulfide was removed with nitrogen and the solution was treated with 3-maleimidomethyl-2, 2, 5, 5-tetramethyl-1-pyrrolidinyl oxide (2.75 mg, 0.011 mmol, dissolved in a small amount of methanol) after having been adjusted to pH 6 with ammonia. After keeping the solution for 15 h in the dark and under nitrogen, the methanol was evaporated *in vacuo*, and

the aqueous residue was diluted with water and extracted with ethyl acetate. The aqueous phase was lyophilized after addition of a slight amount of *t*-butyl alcohol. The white residue weighed 10 mg (about 80% yield) and, according to TLC., was pure (ninhydrin, *Pauly, Reindel-Hoppe*), Rf = 0.60 (2-propanol/conc. NH_3/H_2O 7:1:2 (v/v). $[\alpha]_D^{25} = -57.0°$ ($c = 0.13$, methanol); m.p. 200–220° (dec.). – Amino acid analysis (cysteine, after oxidation, appears, as 2, sometimes 3 peaks: 1 at the position of cysteinic acid, and 1 or 2 just preceding aspartic acid; the total area was measured): Cys 1.07, Asp 1.05, Arg 0.92, Val 2.03, Tyr 1.01, His 0.76, Pro 1.08, Phe 1.00 (reference).

N-(5-maleimido-caproyl)-Seph[AP2]amine (**5**). 1.0 ml Seph[AP2]amine (**4**) and 15.5 mg (50 μmol) of 5-maleimido-hexanoic acid hydroxysuccinimide ester [11] in 3 ml of dimethylformamide/water 1:1 (v/v) were mixed by slow rotation, taking care not to damage the beads mechanically, and allowed to react at 20° for 15 h, when there was no longer any reaction with 2,4-dinitrofluorobenzene.

S-(N-{5-[7-(sepharosyl-oxycarbonimidoyl-amino)-4-azaheptyl-carbamoyl]-pentyl}-imidosuccin-3-yl)-L-cysteinyl-[1-asparagine, 5-valine]-angiotensin II (**6**). 15.0 mg of **2** (10 μmol) were dissolved in 1.0 ml of 90% trifluoroacetic acid and kept for 40 min at 20°. About 0.5–0.7 ml of the solvent were evaporated *in vacuo*, and the residue treated with excess diethyl ether. The white precipitate of S-acetamidomethyl-L-cysteinyl-[1-asparagine, 5-valine]-angiotensin II trifluoroacetate (ninhydrin-positive) was used without further purification. It was dissolved in 5 ml of water, acidified to pH 4 with acetic acid, and treated with 5.25 mg of $Hg(OAc)_2$ in an atmosphere of nitrogen. After keeping for 2 h at 20°, the metal ions were precipitated with dihydrogen sulfide and removed by filtration. The filtrate was lyophilized and the colourless residue dissolved in 0.5 ml of water, titrated to pH 6.0 with ammonia, and treated with 1.0 ml of the solution of **5**, prepared as above. After careful agitation for 15 h at 20°, the substituted sepharose was filtered and washed with water. A sample was hydrolysed and used for amino acid determination. Because of the sepharose, only approximate figures were obtained; they indicated a content of about 0.25 μmol of the angiotensin **6** per ml of the moist gel.

Table. *Chemical shifts δ in ppm from tetramethylsilane for typical resonances of* **1**, **2** *and* **3** ($3 \cdot 10^{-3}$ M solutions in $(CD_3)_2SO$ at 270 MHz)

			1	**2**	**3**
a)	Val	CH_3	0.74	0.74	0.74
b)	Boc	CH_3		1.41	1.39
c)	Acm	CH_3		1.93	
d)	Phe	C_6H_5	7.20	7.23	7.20
e)	Cys	NH		7.90	7.90
f)	AcOH	CH_3	1.91	1.88	1.82
g)	DMSO	CH_3	2.51	2.54	2.51

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174. Reaktionen von Glutaminsäuredimethylester im Massenspektrometer

21. Mitteilung über das massenspektrometrische Verhalten von Stickstoffverbindungen[1][2]

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(18. VI. 74)

Summary: The mass spectral fragmentation of dimethyl glutamate (**1**) and its deuterated derivatives **1a**, **1b** and **1c** has been investigated. By loss of a methoxycarbonyl group from the molecular ion an ion of *m/e* 116 is generated. The latter splits off methanol (*m**), the resulting fragment of *m/e* 84 giving raise to the base peak of the spectrum. Only part of the hydrogen

[1] 20. Mitt., s. [1].
[2] *E. Lerch*, Teil der geplanten Dissertation, Universität Zürich.