SYNTHESIS OF THE AMINO-TERMINAL DECAPEPTIDE OF HUMAN CALCITONIN, IN WHICH THE DISULFIDE BOND IS REPLACED BY A THIOETHER GROUP*

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A fully protected amino-terminal decapeptide *Ib* with the sequence of human calcitonin, in which the S—S bridge was replaced by the S—CH_2 group (a so-called 7-carba-analogue), was synthetized mainly by the stepwise method. The cyclization of the linear decapeptide was performed by means of active ester.

The way in which the biological activities were influenced by substituting the individual sulfur atoms by methylene groups was studied in the case of oxytocin[1-6], vasopressin[7-11], vasotocin[9], insulin[12], somatostatin[13,14] and calcitonin from eel[15] and salmon[16,17]. Recently, an analogue of human calcitonin was described[18] that had the disulfide bond replaced by an ethylene group (a so-called dicarba analogue). In the case of neurohypophyseal hormones, this type of modification was found to decrease biological activities considerably, whereas the substitution of the disulfide bond by a thioether group (the so-called mono-carba-analogue) led to an increase of activities. The activity of the dicarba-analogue of eel calcitonin was decreased by only 20% as compared with the parent hormone, and the corresponding analogue of human calcitonin even had higher activity than its natural prototype.

We were therefore interested in investigating how the biological activities of human calcitonin are influenced by the replacement of only one sulfur atom, resulting in a mono-carba-analogue.

This paper describes the synthesis** of the amino-terminal decapeptide (*Ib*) of human calcitonin, in which the disulfide bridge is replaced by a —S—CH_2— group (the so-called 7-monocarba analogue). We chose the classical method for peptide synthesis in solution and the main part of the peptide chain was prepared by the stepwise

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** The amino acids used in this work were of L-configuration. The nomenclature and symbols of amino acids and peptides were based on the published suggestions[19]. For the nomenclature and symbols of cystathionine peptides, see ref.[20].

method. The fragments were connected by means of azide synthesis. The protecting groups we used (*o*-nitrobenzenesulphenyl and triphenylmethyl) were easily removed by acidolytic cleavage; the side chains (the N^{α}-amino group of cystathionine, the hydroxyl groups of serine and threonine) were also protected by groups which were easily split off acidolytically (tert-butyloxycarbonyl and O-tert-butyl).

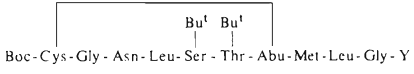
As the key intermediary product, a suitably substituted cystathionine derivative[21] *II* was used, which was subjected to azide condensation with pentapeptide *VIb*, giving heptapeptide *VIIa*. The pentapeptide hydrazide *VIb* was prepared from the corresponding pentapeptide ester *VIa*, which was synthetized by the stepwise method from dipeptide *IIIa* or *IIIb*.

The protected dipeptide *IIIa* was prepared by means of dicyclohexylcarbodiimide from the dicyclohexylammonium salt of N-*o*-nitrobenzenesulphenyl-O-tert-butylserine[22] and O-tert-butylthreonine methyl ester hydrochloride obtained from N-benzyloxycarbonyl-O-tert-butylthreonine methyl ester[23]. After the *o*-nitrobenzenesulphenyl protecting group had been split off by 1 equivalent of hydrogen chloride in methanol[24], the product was condensed with the dicyclohexylammonium salt of *o*-nitrobenzenesulphenylleucine[25] by means of the carbodiimide method, resulting in the formation of tripeptide *IV*. Similarly, the protected tripeptide *IV* was prepared *via* the intermediary product *IIIb*, which was prepared like dipeptide *IIIa* but using the dicyclohexylammonium salt of N-benzyloxycarbonyl-O-tert-butylserine[26]. Dipeptide *IIIb* was obtained in the form of an oil-like product; it was then subjected to hydrogenolytic cleavage of the benzyloxycarbonyl group and transformed into dipeptide hydrochloride from which the above-mentioned tripeptide *IV* was prepared by carbodiimide synthesis with the dicyclohexylammonium salt of *o*-nitrobenzenesulphenylleucine[25]. Further elongation of the chain was performed always after the removal of the *o*-nitrobenzenesulphenyl protecting group by means of 1 equivalent of hydrogen chloride in methanol[24], using *o*-nitrobenzenesulphenylasparagine 2,4,5-trichlorophenyl ester for obtaining the tetrapeptide *V* and triphenylmethylglycine *p*-nitrophenyl ester for obtaining pentapeptide *VIa*.

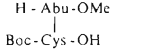
Heptapeptide *VIIa* was transformed into an active ester by means of bis-(*p*-nitrophenyl) sulfite[27]. After the removal of the triphenylmethyl amino-protecting group by 80% acetic acid, cyclization was performed in pyridine solution. The monomeric cyclopeptide *VIII* was isolated by gel filtration on Sephadex LH-20 in methanol and by chromatography on a silica gel column in 90% aqueous 2-butanol. The cyclic heptapeptide ester *VIII* could not be used for further synthesis because during its hydrazinolysis to the corresponding hydrazide or during attempts at alkaline hydrolysis products were formed that had a considerably lower content of glycine and, in some cases, of asparagine, as determined by amino-acid analysis.

For this reason, the linear heptapeptide *VIIa* was transformed into the corresponding hydrazide *VIIb* and subjected to azide copulation with tripeptide *IXa* (after removing the tert-butyloxycarbonyl group by trifluoroacetic acid), thus producing

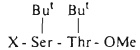
the protected decapeptide *X*. For the cyclization of linear decapeptide *X*, it was more suitable to transform it into the active ester by means of bis-(2,4,5-trichlorophenyl) sulfite[11] than by using bis-(*p*-nitrophenyl) sulfite. Afterwards, the same procedure as in the case of heptapeptide *VIIa* was employed. After purification by gel filtration on Sephadex LH-20 in dimethylformamide, the cyclic decapeptide ester *Ia* was transformed by alkaline hydrolysis into the final product *Ib*.



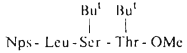
Ia, Y = OEt
Ib, Y = OH



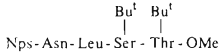
II



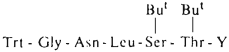
IIIa, X = Nps
IIIb, X = Z



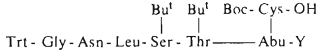
IV



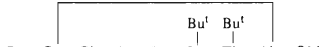
V



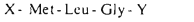
VIa, Y = OMe
VIb, Y = N_2H_3



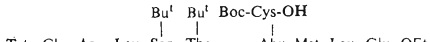
VIIa, Y = OMe
VIIb, Y = N_2H_3



VIII



IXa, X = Boc, Y = OEt
IXb, X = H, Y = OH



X

EXPERIMENTAL

Melting points (uncorrected) were taken on a Kofler block. Samples for elemental analysis were dried for 24 h at room temperature and 150 Pa. Thin-layer chromatography was performed on silica gel sheets (Silufol, Kavalier) in the following solvent systems: 2-butanol–98% formic acid–water (75 : 13·5 : 11·5) (S1), 2-butanol–25% aqueous ammonia–water (85 : 7·5 : 7·5) (S2), 2% ethanol in benzene (S3), n-heptane–pyridine–tert-butanol (5 : 1 : 1) (S4), 1-butanol–acetic acid–water (4 : 1 : 1) (S5), 1-butanol–pyridine–acetic acid–water (15 : 10 : 3 : 6) (S6), 2-butanol–water (9 : 1) (S7).

Paper electrophoresis was performed in a moist chamber on paper Whatman 3 MM in 1M acetic acid (pH 2·4) and pyridine–acetate buffer (pH 5·7) at 20 V/cm for 60 min. Spots were detected by means of the ninhydrin reaction or by the chlorination method. Samples for amino-acid analyses were hydrolysed for 20 h at 105°C in 6M-HCl (in ampoules sealed at 150 Pa). The analyses were carried out on an automatic analyzer (Development Workshops, Czechoslovak Academy of Sciences, Prague, type 6020). Reaction mixtures were evaporated on a rotatory evaporator at a bath temperature of 30–40°C under reduced pressure (water pump). The dimethylformamide-containing mixtures were evaporated at 150 Pa. In the course of chromatography, peptide material was continuously detected by a differential refractometer or UV spectrophotometer. In some cases the optical activity at various wavelengths was measured by the Jasco-UV-ORD-5 apparatus.

N-*o*-Nitrobenzenesulphenyl-O-tert-butylseryl-O-tert-butylthreonine Methyl Ester (*IIIa*)

N-Benzyloxycarbonyl-O-tert-butylthreonine methyl ester[23] (14 g) was dissolved in methanol (100 ml) and acetic acid (20 ml) and hydrogenated in the presence of Pd-black (from 4 g of $PdCl_2$). The catalyst was removed by filtration and the filtrate was evaporated to dryness. The residue was dissolved in a small amount of ether, 2·03M-HCl in ether (22·6 ml) was added and the solution was diluted with light petroleum. The precipitated oily product was repeatedly triturated with light petroleum, then recrystallized from ether and light petroleum, filtered off, washed with light petroleum and dried. The yield was 8·6 g (88%) of product, R_F 0·50 (S1), 0·48 (S2).

O-Tert-butylthreonine methyl ester hydrochloride (1·4 g) and the dicyclohexylammonium salt of N-*o*-nitrobenzenesulphenyl-O-tert-butylserine[22] (3·2 g) were dissolved in dichloromethane (20 ml), the solution was cooled to −20°C and dicyclohexylcarbodiimide (1·4 g) was added. The mixture was stirred for 1 h at −15°C and after 12 h of standing at 0°, the precipitated dicyclohexylurea was removed by filtration and the filtrate was evaporated to dryness. The residue was dissolved in ethyl acetate and the solution was sequentially extracted with 0·1M-H_2SO_4, water, 0·5M-$NaHCO_3$ and water, dried with sodium sulfate and evaporated. The residue was crystallized from methanol and water. The yield was 2·4 g (79%) of product, m.p. 74–76°C. $[\alpha]_D^{25}$ +6·0° (*c* 0·5, dimethylformamide). R_F 0·30 (S3), 0·58 (S4), electrophoresis after removing the *o*-nitrobenzenesulphenyl protecting group by HCl in methanol: $E_{5,7}^{His}$ 0·83, $E_{2,4}^{His}$ 0·66. For $C_{22}H_{35}N_3O_7S$ (485·6) calculated: 54·41% C, 7·27% H, 8·65% N; found: 54·72% C, 7·21% H, 8·82% N.

o-Nitrobenzenesulphenylleucyl-O-tert-butylseryl-O-tert-butylthreonine Methyl Ester (*IV*)

A. To the solution of protected dipeptide *IIIa* (9·9 g) in methanol (50 ml), 0·364M-HCl in methanol (56·5 ml) was added and the solution was stirred for 25 min at room temperature. Methanol was evaporated, the remnant was evacuated, dissolved in dichloromethane (100 ml), the dicyclohexylammonium salt of *o*-nitrobenzenesulphenylleucine[25] (9·7 g) was added to the solution

and after cooling to −20°C dicyclohexylcarbodiimide (4·7 g) was added. The reaction mixture was stirred for 1 h at −10°C and then left at 0°C for 12 h; precipitated dicyclohexylurea was removed by filtration and the filtrate was evaporated to dryness. The remnant was dissolved in ethyl acetate and the solution was sequentially extracted with 0·2M-H_2SO_4, water, 0·5M-$NaHCO_3$, water, dried with sodium sulfate and evaporated. The residue was dissolved in a small amount of methanol, ether and light petroleum were then added to the solution and the mixture was cooled to 0°C. After filtration and washing with light petroleum, the yield was 9·6 g (79%) of product, m.p. 147–149°C. The sample for analysis was recrystallized in the same way; the m.p. did not change; $E^{Gly}_{2.4}$ 0·83, $E^{His}_{5.7}$ 0·67 (after removing the *o*-nitrobenzenesulphenyl protecting group by HCl in methanol); R_F 0·66 (S1), 0·70 (S2), 0·62 (S5); $[\alpha]_D^{25}$ +8·3° (*c* 0·5, dimethylformamide). For $C_{28}H_{46}N_4O_8S$ (598·7) calculated: 56·17% C, 7·75% H, 9·36% N; found: 55·97% C, 7·78% H, 9·93% N.

B. The solution of the dicyclohexylammonium salt of N-benzyloxycarbonyl-O-tert-butylserine[26] (13·1 g) and O-tert-butyl-threonine methyl ester hydrochloride (6·1 g – preparation described under compound *IIIa*) in dichloromethane (110 ml) was cooled to −20°C and dicyclohexylcarbodiimide (6·6 g) was added. The reaction mixture was stirred for 12 h at 0°C, the precipitated dicyclohexylurea was removed by filtration and the filtrate was evaporated. The remnant was dissolved in ethyl acetate and the solution was sequentially extracted with 0·2M-H_2SO_4, water, 0·5M-$NaHCO_3$, water, dried with sodium sulfate and evaporated. $E^{Gly}_{2.4}$ 1·26, $E^{His}_{5.7}$ 0·81 (after removing the protecting groups by 35% HBr in acetic acid).

The residue of N-benzyloxycarbonyl-O-tert-butylseryl-O-tert-butylthreonine methyl ester (*IIIb*) was dissolved in methanol (200 ml) and water (4 ml) and was hydrogenated in the presence of Pd-black (from 2 g of $PdCl_2$). In the course of hydrogenation, the pH value of the reaction mixture was maintained at 5·0 by the addition of 1M-HCl (approximately 26 ml; pH-stat). The catalyst was removed, the filtrate was taken to dryness and azeotropically dried. The remnant was dissolved in dichloromethane (150 ml), the dicyclohexylammonium salt of *o*-nitrobenzenesulphenylleucine[25] (12·7 g) was added, the mixture was cooled to −20°C and dicyclohexylcarbodiimide (6·2 g) was then added. The reaction mixture was stirred for 1 h at −10°C and after 12 h at 0°C treated as sub *A*). The yield was 11·8 g (73% on the dicyclohexylammonium salt of benzyloxycarbonyl-O-tert-butylserine) of product with m.p. 143–146°C, undepressed on admixture with the sample prepared sub *A*.

o-Nitrobenzenesulphenylasparaginyl-leucyl-O-tert-butylseryl-O-tert-butylthreonine Methyl Ester (*V*)

To the solution of protected tripeptide *IV* (13·5 g) in methanol (55 ml), 0·364M-HCl in methanol was added (63·2 ml), the mixture was left for 20 min at room temperature, methanol was then evaporated and the remnant evacuated. The remnant was dissolved in dimethylformamide (30 ml) and dichloromethane (80 ml); N-ethylpiperidine (3·2 ml) and *o*-nitrobenzenesulphenyl-asparagine 2,4,5-trichlorophenyl ester (11·0 g) were then added. After 24 h of stirring at room temperature, further portions of N-ethylpiperidine (1·6 ml) and active ester (4·0 g) were added and after 24 h the mixture was taken to dryness. The remnant was dissolved in ethyl acetate and the solution was sequentially extracted with 0·2M-H_2SO_4, water, 0·5M-$NaHCO_3$, water, dried with sodium sulfate and evaporated. The residue was crystallized from nitromethane and ether. The yield was 10·0 g (65%) of product, m.p. 180–182°C. $[\alpha]_D^{25}$ 10·2° (*c* 0·5, dimethylformamide); R_F 0·25 (S4); $E^{Gly}_{2.4}$ 0·81, $E^{His}_{5.7}$ 0·61 (after removing the *o*-nitrobenzenesulphenyl group by HCl in methanol). For $C_{32}H_{52}N_6O_{10}S$. 0·5 H_2O (721·8) calculated: 53·24% C, 7·40% H, 11·65% N; found: 53·24% C, 7·05% H, 11·73% N.

Triphenylmethylglycine *p*-Nitrophenyl Ester

To the solution of triphenylmethylglycine[28] (25·4 g) in pyridine (80 ml), bis-(*p*-nitrophenyl) sulfite[27] (28·5 g) was added. The reaction was performed under nitrogen. After 3 h of stirring at room temperature, the reaction mixture was evaporated, the residue was dissolved in ethyl acetate and sequentially extracted with a saturated solution of Na_2SO_4, dried with sodium sulfate and evaporated. The residue was crystallized from ethanol. The yield was 26·4 g (74%) of product, m.p. 155–157°C. Data published earlier give m.p. 150–155°C (ref.[29]), 152–155°C (ref.[30]) and 153–154°C (ref.[25]).

Triphenylmethylglycyl-asparaginyl-leucyl-O-tert-butylseryl-O-tert-butylthreonine Methyl Ester (*VIa*)

Protected tetrapeptide *V* (10·8 g) was dissolved in methanol (60 ml), 0·332M-HCl in methanol (46·0 ml) was then added, and after 20 min at room temperature, methanol was evaporated. The remnant was dissolved in dichloromethane (100 ml); N-ethylpiperidine (2·15 ml) and triphenylmethylglycine *p*-nitrophenyl ester (7·0 g) were then added to the solution. After 24 h of stirring at room temperature, further portions of N-ethylpiperidine (1·0 ml) and active ester (3·0 g) were added to the reaction mixture; after 24 h the mixture was evaporated. The residue was dissolved in ethyl acetate and thoroughly extracted with 0·5M-$NaHCO_3$, water, dried with sodium sulfate and evaporated. The residue was crystallized under a mixture of ether and light petroleum, the product filtered off and washed on the filter with ether. The yield was 12·8 g (98%) of product, m.p. 188–190°C. $[\alpha]_D^{25}$ +14·3° (*c* 0·5, dimethylformamide); R_F 0·25 (S4); $E_{2.4}^{Gly}$ 0·72, $E_{5.7}^{His}$ 0·49 (after removing the triphenylmethyl group by 80% acetic acid). For $C_{47}H_{66}N_6O_9.0{\cdot}5H_2O$ (868·0) calculated: 65·03% C, 7·78% H, 9·68% N; found; 65·17% C, 7·57% H, 9·97% N. Amino-acid analysis: Asp 1·01, Gly 1·00, Leu 1·02, Ser 0·97, Thr 0·99.

Triphenylmethylglycyl-asparaginyl-leucyl-O-tert-butylseryl-O-tert-butylthreonine Hydrazide (*VIb*)

To the solution of protected pentapeptide ester *VIa* (12·8 g) in methanol, hydrazine hydrate (12·5 ml) was added. After 4 days at room temperature, the reaction mixture was diluted with water, stored for 6 h at 5°C and the precipitate was then filtered off and washed with water. After crystallization from methanol and water, 12·0 g of product were obtained, m.p. 176–178°C. $[\alpha]_D^{25}$ +8·0 (*c* 0·5, dimethylformamide), −9·2° (*c* 0·5, methanol). For $C_{46}H_{66}N_8O_8.0{\cdot}5H_2O$ (868·0) calculated: 63·65% C, 7·78% H, 12·91% N, 3·58% N_2H_3; found: 63·60% C, 7·57% H, 13·20% N, 3·81% N_2H_3.

$N^{\alpha'}$-(Triphenylmethylglycyl-asparaginyl-leucyl-O-tert-butylseryl-O-tert-butylthreonyl)-N^{α}-tert-butyloxycarbonylcystathionine α'-Methyl Ester (*VIIa*)

Protected pentapeptide hydrazide *VIb* (11·0 g) was dissolved in dimethylformamide (150 ml), the mixture was cooled to −50°C, and 5·7M-HCl in tetrahydrofuran (14·0 ml) and n-butyl nitrite (1·7 ml) were then added. The solution was warmed to −20°C in the course of 5 min, the temperature was maintained at this level for 10 min and then decreased to −40°C in 5 min. N^{α}-tert-butyloxycarbonylcystathionine α'-methyl ester[21] *II* (4·3 g) dissolved in dimethylformamide (100 ml) and N-ethylpiperidine (2·5 ml) was added to the solution. The pH value of the mixture was adjusted to 8·5–9·0 (moist pH paper) with N-ethylpiperidine. The reaction mixture was kept for 1 h at −20 to −30°C, for 16 h at −20°C, for further 24 h at 0°C and then evaporated to dryness. The product was triturated with water, cooled, filtered off and washed with water.

After crystallization from methanol and ether, 11·5 g (80%) of product was obtained, m.p. 156–159°C. $[\alpha]_D^{25}$ −12·8° (*c* 0·22, methanol); $E_{2,4}^{Gly}$ 0·43 (after removing the triphenylmethyl group by 80% acetic acid). For $C_{59}H_{86}N_8O_{14}S$ (1 163·4) calculated: 60·85% C, 7·46% H, 9·64% N; found: 60·70% C, 7·54% H, 9·65% N. Amino-acid analysis: Asp 1·00, Cyth 0·99, Gly 1·00, Leu 1·01, Ser 0·93, Thr 1·00.

N$^{\alpha'}$-(Triphenylmethylglycyl-asparaginyl-leucyl-O-tert-butylseryl-O-tert-butylthreonyl)-N$^{\alpha}$-tert-butyloxycarbonylcystathionine α′-Hydrazide (*VIIb*)

To the solution of protected peptide *VIIa* (3·8 g) in methanol (40 ml), hydrazine hydrate (7·2 ml) was added and after 3 days at room temperature the mixture was diluted with ether, stored for several hours at 0°C and the precipitated product was then filtered off, washed with ether, dried and reprecipitated from methanol and ether. The yield was 3·58 g (94%) of product, m.p. 142 to 145°C. $[\alpha]_D^{25}$ +1·8° (*c* 0·5, dimethylformamide), +4·0° (*c* 0·2, methanol). The sample for analysis was reprecipitated from methanol and water, m.p. 145–147°C. For $C_{58}H_{86}N_{10}O_{13}S$ (1 163·4) calculated: 59·90% C, 7·45% H, 12·04% N; found: 59·99% C, 7·56% H, 12·63% N.

N$^{\alpha}$-Tert-Butyloxycarbonyl-α-hemicystathionyl-glycyl-asparaginyl-leucyl-O-tert-butylseryl-O-tert-butylthreonyl-α′-hemicystathionine α′-Methyl Ester (*VIII*)

The preparation of the active ester and cyclization were performed under nitrogen. To the solution of protected peptide *VIIa* (6·0 g) in a mixture of pyridine (100 ml) and dimethylformamide (100 ml), bis-(*p*-nitrophenyl) sulfite[27] (10 g) was added. The reaction mixture was stirred for 8 h at room temperature, diluted with pyridine (50 ml), a further portion (10 g) of reagent was added, stirred for 12 h, a further portion (5 g) of reagent was added, and after 5 h the mixture was evaporated to dryness, the residue was triturated with ether, filtered off, washed with ether, water and dried in a desiccator. This yielded 5·2 g of chromatographically (benzene) pure product.

The active ester (5·2 g) was dissolved in 80% acetic acid (240 ml) and left for 1 h at room temperature. The mixture was then diluted with water (1 200 ml) and freeze-dried. The lyophilisate was triturated with ether (150 ml) and 2M-HCl in ether (4·3 ml); after 10 min at room temperature the product was filtered off, washed with ether and dried in a desiccator (NaOH). The product was dissolved in dimethylformamide (120 ml) and the solution was added in the course of 12 h to a mixture of pyridine (1 200 ml) and N-ethylpiperidine (1·1 ml) while stirring and heating to 50°C. After 12 h the reaction mixture was evaporated to dryness, the residue was dissolved in methyl acetate and the solution was sequentially extracted with 0·2M-H_2SO_4, 0·5M-$NaHCO_3$, water, dried with sodium sulfate and evaporated. The yield was 3·3 g (87%) of product. The product was subjected to gel filtration on Sephadex LH-20 in methanol (the column measured 2 × 120 cm, 1·7 g of product was dissolved in 4 ml of methanol, the flow rate was 65 ml per h, peptide material was detected by means of a UV spectrophotometer at 220 nm and simultaneously by ORD at 240 nm. Fractions from 230 to 310 ml of the eluted volume were isolated; after evaporation the yield was 1·85 g (49%). The product was then purified by chromatography on a silica gel column in 90% aqueous 2-butanol (silica gel 60–120 μ; 2·5 × 140 cm column; 900 mg of product was applied in 5 ml of 90% aqueous 2-butanol; flow rate 33 ml per h; detection by UV spectroscopy at 220 nm). Fractions from 430–550 ml of the eluted volume were isolated after evaporation, 1·15 g (30%) of chromatographically pure product was obtained. The sample for analysis was reprecipitated from methanol and water; m.p. 134–136°C, $[\alpha]_D^{25}$ −34·0° (*c* 0·2, methanol); R_F 0·75 (S1), 0·76 (S2), 0·75 (S6), 0·62 (S7); $E_{2,4}^{Gly}$ 0·76, $E_{5,7}^{His}$ 0·42 (after removing the protecting groups by trifluoroacetic acid). For $C_{40}H_{70}N_8O_{13}S$ (903·1) calculated: 53·15% C, 7·81% H, 12·40% N; found: 52·99% C, 7·71% H, 12·12% N. Amino-acid analysis: Asp 1·03,

Cyth 0·93, Gly 1·02, Leu 1·00, Ser 0·94, Thr 1·00. The number of amino groups in the molecule was determined by partial substitution and by means of electrophoretic analysis[31,32].

Tert-Butyloxycarbonylmethionyl-leucyl-glycine Ethyl Ester (*IXa*)

A solution of HBr in acetic acid (35%, 10 ml) was added to the ethyl ester of benzyloxycarbonyl-leucyl-glycine[33–35] (7·0 g). After 20 min at room temperature, the reaction mixture was diluted with ether (200 ml) and the precipitated dipeptide hydrobromide was filtered off, dried in a desiccator and dissolved in dimethylformamide (40 ml). N-Ethylpiperidine (pH 6, moist pH paper), dicyclohexylammonium salt of tert-butyloxycarbonylmethionine[36] (8·6 g) and 1-hydroxybenzotriazole[37] (2·7 g) were added to the solution. The mixture was cooled to −20°C, its pH value was adjusted to 7·5 to 8·0 (moist pH paper) and dicyclohexylcarbodiimide (4·6 g) was added. The solution was stirred for 1 h at −10°C and for 12 h at 0°C. The precipitated dicyclohexylurea was removed by filtration, the filtrates were evaporated and the remnant was dissolved in ether. The solution thus obtained was sequentially extracted with 0·2M-H_2SO_4, water, 0·5M-$NaHCO_3$, water, dried with sodium sulfate, evaporated and crystallized from ether and light petroleum. The yield was 6·0 g (68%) of product, m.p. 118–120°C. The sample for analysis was recrystallized in the same way without change in the m.p. $[\alpha]_D^{25}$ −46·7° (*c* 0·52, ethanol). $E_{2.4}^{Gly}$ 0·92, $E_{5.7}^{His}$ 0·48 (after removing the protecting group by trifluoroacetic acid). For $C_{20}H_{37}N_3O_6S$ (447·6) calculated: 53·66% C, 8·33% H, 9·39% N; found: 54·05% C, 8·45% H, 9·40% N.

Methionyl-leucyl-glycine (*IXb*)

To the protected tripeptide *IXa* (2·4 g), trifluoroacetic acid (8 ml) was added and after 1 h at room temperature, the reaction mixture was diluted with toluene (10 ml) and evaporated to dryness. The residue was dissolved in ethanol (40 ml), 1M-NaOH (20 ml) was added and after 1 h at room temperature ethanol was evaporated and the aqueous solution was applied onto a column of Dowex 50 (H^+-cycle, 100 ml). The column was washed through with water and the product was eluted by 10% aqueous pyridine. The eluate was evaporated to dryness and crystallized from water and ethanol. The yield was 0·9 g (53%) of product, m.p. 215–217°C; $E_{2.4}^{Gly}$ 0·94, $E_{5.7}^{His}$ 0·00; $[\alpha]_D^{25}$ −38·8° (*c* 0·2, pyridine), −2·4° (*c* 0·5, 1M-HCl). For $C_{13}H_{25}N_3O_4S$. 0·5 H_2O (328·4) calculated: 47·65% C, 7·98% H, 12·81% N; found: 47·96% C, 7·61% H, 12·97% N.

$N^{\alpha'}$-(Triphenylmethylglycyl-asparaginyl-leucyl-O-tert-butylseryl-O-tert-butylthreonyl)-N^{α}-tert-butyloxycarbonylcystathionyl-α′-methionyl-leucyl-glycine Ethyl Ester (*X*)

To the protected tripeptide *IXa* (1·4 g, 3·1 mM), trifluoroacetic acid (5 ml) was added and after 1 h at room temperature, the reaction mixture was diluted with toluene (5 ml) and evaporated to dryness. The remnant was dissolved in dimethylformamide (10 ml) and N-ethylpiperidine (2 ml) was added to the solution.

To the solution of protected peptide hydrazide *VIIb* (1·8 g, 1·55 mM) in dimethylformamide (15 ml), cooled to −40°C, 8·0M-HCl in tetrahydrofuran (1·1 ml) and n-butyl nitrite (0·25 ml) were added. The solution was warmed to −20°C in the course of 5 min, kept at this temperature for 10 min and then cooled to −40°C in 5 min. To the reaction mixture the previously prepared solution of methionyl-leucyl-glycine ethyl ester was added and the pH was adjusted to 8·5 (moist pH paper). The reaction mixture was kept for 1 h at −20 to −30°C, overnight at −20°C and for 72 h at 0°C; it was then evaporated. The product was triturated with water, stored for several hours at 0°C, filtered off and washed with water (2·05 g, 90%). The crude product was recrystallized from methanol, filtered off and washed with methanol and ether. The yield was 1·00 g (45%) of product, m.p. 228–233°C. $[\alpha]_D^{25}$ −7·9° (*c* 0·2, dimethylformamide); $E_{2.4}^{Gly}$ 0·57 (after

removing all protecting groups by HBr in acetic acid). For $C_{73}H_{111}N_{11}O_{17}S_2.2\,H_2O$ (1 514·9) calculated: 57·80% C, 7·65% H, 10·18% N; found: 57·52% C, 7·47% H, 10·50% N. Amino-acid analysis: Asp 1·04, Gly 1·99, Cyth 1·03, Leu 2·00, Met 1·00, Ser 0·95, Thr 1·03.

N$^{\alpha}$-Tert-butyloxycarbonyl-α-hemicystathionyl-glycyl-asparaginyl-leucyl-O-tert-butylseryl-
-O-tert-butylthreonyl-α′-hemicystathionyl-methionyl-leucyl-glycine Ethyl Ester (*Ia*)

The preparation of the active ester and cyclization were performed under nitrogen. To the solution of protected peptide *X* (1·0 g) in a mixture of pyridine (30 ml) and dimethylformamide (30 ml), bis-(2,4,5-trichlorophenyl) sulfite (2·0 g) was added. The reaction mixture was stirred for 8 h at room temperature and diluted with pyridine (20 ml). Then, a further portion (2·0 g) of the reagent was added, the mixture was stirred for 12 h, 1·0 g more of reagent was added, after 5 h the reaction mixture was evaporated to dryness, the residue was triturated with ether, water and dried. The product obtained was chromatographically pure (benzene).

The active ester was dissolved in 80% acetic acid (40 ml), after 1 h the reaction mixture was diluted with water and freeze-dried. To the remnant (950 mg) ether (50 ml) and 2M-HCl in ether (0·5 ml) were added, and after 10 min at room temperature, the product was filtered off, washed with ether and dried in a desiccator (NaOH). The product was dissolved in dimethylformamide (100 ml) and this solution was added in the course of 12 h to a mixture of pyridine (1 300 ml) and N-ethylpiperidine (0·15 ml) while stirring and heating to 50°C. After 12 h, the reaction mixture was evaporated to dryness, triturated with ether, filtered off, washed with ether and water. After drying, 720 mg of crude product were obtained. The product was purified by gel filtration on Sephadex LH-20 in dimethylformamide (1 × 200 cm column; 60—90 mg portions were applied in 1·0 ml of dimethylformamide; flow rate 12 ml per h; peptide material was detected by means of a differential refractometer and/or ORD at 310 nm). Fractions from approximately 77·0 to 84·0 ml of the eluted volume were isolated. (A second peak appeared at approximately 67·0 ml of the eluted volume.) After evaporation and trituration with ether, 368 mg (44%) of product were obtained, $[\alpha]_D^{22}$ −12·7° (*c* 0·2, dimethylformamide); $E_{2,4}^{Gly}$ 0·62, $E_{5,7}^{His}$ 0·31 (after removing the protecting groups by trifluoroacetic acid). For $C_{54}H_{95}N_{11}O_{16}S_2.2\,H_2O$ (1 263·6) calculated: 51·33% C, 7·98% H, 12·19% N; found: 51·23% C, 7·62% H, 12·15% N. Amino-acid analysis: Asp 0·98, Thr 1·02, Ser 1·02, Gly 1·93, Met 0·98, Cyth 1·07, Leu 2·00.

N$^{\alpha}$-Tert-butyloxycarbonyl-α-hemicystathionyl-glycyl-asparaginyl-leucyl-O-tert-butylseryl-
-O-tert-butylthreonyl-α′-hemicystathionyl-methionyl-leucyl-glycine (*Ib*)

The protected decapeptide ester *Ia* (320 mg) was dissolved in dimethylformamide (1·5 ml) and methanol (10 ml); 1M-NaOH (2·0 ml) was then added to the solution. After 1 h at room temperature, the pH value of the solution was adjusted to 4·0—5·0 by 20% citric acid and the solution was diluted with water to 100 ml. The mixture was left at 0°C for 1 h, the product was then isolated by centrifugation, washed with water and dried. The yield was 180 mg (57%) of product with m.p. above 270°C (decomposition). The test for sulfoxides was negative[38]. $[\alpha]_D^{25}$ −9·0° (*c* 0·2, dimethylformamide), $E_{2,4}^{Gly}$ 0·62, $E_{5,7}^{His}$ 0·00 (after removing the protecting groups by trifluoroacetic acid); R_F 0·61 (S6), 0·00 (S4). For $C_{52}H_{90}N_{11}O_{15}S_2$ (1 189·5) calculated: 52·50% C, 7·62% H, 12·95% N; found: 52·50% C, 7·49% H, 12·62% N. Amino-acid analysis: Asp 0·99, Thr 0·96, Ser 1·00, Gly 2·00, Met 1·01, Cyth 1·01, Leu 2·00.

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