SYNTHESIS OF PRESSINOIC ACID BY ENZYMATICALLY CATALYZED FORMATION OF PEPTIDE BONDS*

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Three fully enzymatic syntheses of the 1–6 vasopressin hexapeptide were investigated using papain, α-chymotrypsin and thermolysin. Best results were obtained with thermolysin in the 2 + 4 fragment condensation. The α-chymotrypsin-catalyzed 3 + 3 condensation is less advantageous and the 4 + 2 condensation with papain gave only low yield. Using the mentioned enzymes, further fragments of vasopressin molecule were prepared. Amino groups were protected with benzyloxycarbonyl or tert-butyloxycarbonyl groups, carboxyl groups as phenylhydrazides or methyl esters, and the cysteine sulfhydryl group as the benzyl derivate. The tyrosine hydroxyl was not protected.

Neurohypophyseal hormones oxytocin and vasopressin have been utilized as models for enzymatically catalyzed synthesis of peptide bonds in only a few cases. The preparation of fragments 1–3 and 5–6 (ref.[1]) and our synthesis[2] of fragments 1–6 and 7–9 represent the only hitherto described enzymatic syntheses of protected oxytocin fragments. Our present work is an extension of enzymatic synthesis to the total synthesis of the protected N-terminal vasopressin hexapeptide *IIa*. Removal of the phenylhydrazide protecting group in *IIa* by treatment with aqueous ferric chloride[3] afforded the hexapeptide *IIb* as an intermediate in the synthesis of vasopressin and its analogues. Reduction of the hexapeptide *IIb* with sodium in liquid ammonia and subsequent cyclization with air yielded pressinoic acid** *I* (refs[6,7]).

The hexapeptide *IIa* was obtained from the dipeptides *III* and *V*, synthesized already previously using papain[2]. The dipeptide *IVa* was successfully synthesized only with α-chymotrypsin, starting from tert-butyloxycarbonylphenylalanine methyl ester[8] and glutamine phenylhydrazide trifluoroacetate. However, the enzyme was ineffective when the acylating component[9] had free carbonyl group. Experiments with papain, starting from the methyl ester[10] or from the unesterified acylating component[11], also failed because of rapid concurrent cyclization reaction, catalyzed

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** Amino acids used in this study are of the L-configuration. The nomenclature and symbols of the amino acids and peptides obey the published recommendations[4,5].

with glutaminecyclotransferase present in the commercial papain[12]. Negative results were obtained with carboxypeptidase-Y[13] and thermolysin[14], too. In the enzymatic synthesis of the N-terminal oxytocin hexapeptide this step proved to be critical and the analogous dipeptide Boc-Ile-Gln-N_2H_2—C_6H_5 was prepared only in a low yield using elastase[2]. The dipeptide *IVb* was prepared by oxidative cleavage of compound *IVa* with ferric chloride. On an analytical scale, we studied also the carboxypeptidase-Y-catalyzed condensation of tert-butyloxycarbonylphenylalanine methyl ester and tert-butyloxycarbonylisoleucine methyl ester with a large excess of glutamine: the positive results corresponded to the published data[13].

Also the preparation of the tetrapeptide *VI* by papain-catalyzed 2 + 2 fragment condensation of the dipeptide *IVb* with asparaginyl-S-benzylcysteine phenylhydrazide trifluoroacetate was successful, in accord with the literature[2]. On the other hand, the attempted condensation of tert-butyloxycarbonylphenylalanine methyl ester with glutaminyl-asparaginyl-S-benzylcysteine phenylhydrazide in the presence of α-chymotrypsin completely failed.

Papain-induced synthesis of the dipeptides *VII* and *VIII* afforded good preparative yields only with compound *VIII*. Because of difficulties with removal of the protecting groups, this intermediate was not suitable for further synthesis of the peptide chain. Replacement of the benzyloxycarbonyl protecting group in glutamine by tert-butyloxycarbonyl group in the condensation with asparagine phenylhydrazide trifluoroacetate shifted very unfavourably the equilibrium in the direction of peptide bond hydrolysis.

The tripeptides *IXa* and *IXb* were synthesized by condensation of the dipeptide *IIIb* with phenylalanine methyl ester hydrochloride or phenylalanine phenylhydrazide trifluoroacetate in the presence of thermolysin, the latter tripeptide being prepared in higher yield. The tripeptide *IXb* was also obtained from compound *IIIa* with α-chymotrypsin, but in lower yield than with thermolysin. Reaction of the dipeptide *IIIb* with phenylalanine phenylhydrazide (2 + 1) using papain resulted in a transpeptidation reaction which led to undesired benzyloxycarbonyl-S-benzylcysteinyl--phenylalanine phenylhydrazide. Also attempted condensation of benzyloxycarbonyl--S-benzylcysteine with tyrosyl-phenylalanine phenylhydrazide trifluoroacetate (1 + 2) in the presence of papain was unsuccessful affording, contrary to a report[1], only a mixture containing small amount of the desired tripeptide *IXb*. The tripeptide *IXc* was prepared either by alkaline hydrolysis of *IXa* or by oxidative cleavage of *IXb*.

Synthesis of the tripeptide *X* consisted in condensation of benzyloxycarbonylglutamine with asparaginyl-S-benzylcysteine phenylhydrazide trifluoroacetate in the presence of papain and gave good preparative yield.

The tetrapeptide *XIa* was synthesized by thermolysin-catalyzed 2 + 2 fragment condensation from the dipeptide *IIIb* and phenylalanyl-glutamine phenylhydrazide trifluoroacetate. Experiments with α-chymotrypsin, starting from the dipeptide *IIIa* (2 + 2) or from the tripeptide *IXa* (3 + 1), were unsuccessful: in both cases only

hydrolysis of the ester groups took place. The tetrapeptide *XIb* was prepared from *XIa* by oxidative removal of the phenylhydrazide protecting group.

Cys-Tyr-Phe-Gln-Asn-Cys
I

Z-Cys(Bzl)-Tyr-Phe-Gln-Asn-Cys(Bzl)—X
IIa, X = N_2H_2—C_6H_5
IIb, X = OH

Z-Cys(Bzl)-Tyr—X
IIIa, X = OEt
IIIb, X = OH

Boc-Phe-Gln—X
IVa, X = N_2H_2—C_6H_5
IVb, X = OH

Boc-Asn-Cys(Bzl)-N_2H_2—C_6H_5
V

Boc-Phe-Gln-Asn-Cys(Bzl)-N_2H_2—C_6H_5
VI

Boc-Tyr-Phe-N_2H_2—C_6H_5
VII

Z-Gln-Asn-N_2H_2—C_6H_5
VIII

Z-Cys(Bzl)-Tyr-Phe—X
IXa, X = OMe
IXb, X = N_2H_2—C_6H_5
IXc, X = OH

Z-Gln-Asn-Cys(Bzl)-N_2H_2—C_6H_5
X

Z-Cys(Bzl)-Tyr-Phe-Gln—X
XIa, X = N_2H_2—C_6H_5
XIb, X = OH

For the final synthesis of the hexapeptide *IIa*, thermolysin-catalyzed 2 + 4 condensation proved to be the method of choice. The desired product was obtained in good preparative yield from the dipeptide *IIIb* and tetrapeptide *VI* after removal of the tert-butyloxycarbonyl protecting group. Under the same reaction conditions, the use of subtilisin[11] resulted only in cleavage of the tetrapeptide. The 3 + 3 condensation, catalyzed by α-chymotrypsin, was successful only with considerable amounts of the enzyme; it was carried out with the tripeptide *IXc* and glutaminyl-asparaginyl-S-benzylcysteine phenylhydrazide, prepared by removal of the benzyloxycarbonyl protecting group from the tripeptide *X*. With *IXa* the experiment completely failed. In a blank experiment, compound *IXc* was completely resistant to α-chymotrypsin at pH 7, whereas at pH 10 the enzyme hydrolyzed the ester group of *IXa* without affecting the peptide bonds. The worst results were obtained in the 4 + 2 fragment condensation, catalyzed with papain: reaction of the tetrapeptide *XIb* with asparaginyl-S-benzylcysteine phenylhydrazide trifluoroacetate afforded the product in low yield and an unidentified side-product was formed. Blank experi-

ment showed no peptide bond cleavage in the tetrapeptide *XIb* with papain but confirmed formation of the same unidentified product as in the synthesis.

EXPERIMENTAL

Melting points were determined on a Kofler block and are uncorrected. Solvents were removed from the reaction mixtures on a rotatory evaporator at 30°C. Analytical samples were dried over phosphorus pentoxide at room temperature and 150 Pa. Thin-layer chromatography on silica gel was carried out on Silufol plates (Kavalier) in the following solvent systems: 2-butanol–98% formic acid–water (75 : 13·5 : 11·5) (S1), 2-butanol–25% aqueous ammonia–water (87 : 7·5 : 7·5) (S2), 1-butanol–acetic acid–water (4 : 1 : 1) (S3), 1-butanol–pyridine–acetic acid–water (15 : 10 : 3 : 6) (S4). Electrophoresis was performed in moist chamber on a Whatman 3MM paper at 20 V/cm for 1 h in 1 mol l^{-1} acetic acid (pH 2·4) or in a pyridine–acetate buffer (pH 5·7). Spots were detected with ninhydrin or by the chlorination method. Samples for amino acid analysis were hydrolyzed by heating with 6 mol l^{-1} HCl to 105°C for 20 h. The analyses were carried out on AAA 339 (Mikrotechna) or Durrum D-500 analyzers. Optical rotations were measured on a Perkin–Elmer 141 MCA polarimeter. High performance liquid chromatography (HPLC) was carried out on a Spectra Physics SP 8700 instrument equipped with an SP 8400 UV-detector and SP 4100 integrator. Analyses were done on a 15 × 0·32 cm glass column packed with Separon SIX C-18, flow rate 30 ml/h, detection at 222 nm; mobile phase methanol, containing 0·05% aqueous trifluoroacetic acid (k' values of all the prepared products were determined with mobile phase containing 75% methanol). For semipreparative purposes we used a 25 × 0·8 cm column, packed with the same stationary phase, flow rate 180 ml/h (repeated injections for larger quantities of material). "The usual work-up procedure" means that the precipitate was washed successively with 1 mol l^{-1} HCl (compounds with the Boc protecting group were washed with an HSO_4^- buffer pH 2), water, 0·5 mol l^{-1} $NaHCO_3$, again water, and dried. Phenylhydrazides of tert-butyloxycarbonylamino acids were prepared using papain according to ref.[15]. Trifluoroacetates of amino acid and peptide derivatives were obtained by treatment of the corresponding tert-butyloxycarbonyl derivatives with trifluoroacetic acid and their homogeneity was checked by paper electrophoresis and HPLC. The benzyloxycarbonyl group was removed with hydrogen bromide in glacial acetic acid and the free peptide was obtained using an ion-exchanging resin. All the enzymatic syntheses were based on precipitation of the products from the reaction medium consisting of an aqueous buffer and a water-miscible organic solvent. Most of the syntheses were performed with a 10% excess of the amino component. Syntheses with papain were carried out at pH 4·8—5·0 in an acetate buffer, with α-chymotrypsin at pH 10 in a carbonate–bicarbonate buffer or at pH 7 in a Tris–maleate buffer, with thermolysin at pH 7 in a Tris–maleate buffer. The course of the enzymatic reactions was monitored by HPLC to determine the reaction end and to follow side-reactions. The papain used was a Sigma product, α-chymotrypsin and thermolysin were purchased from Serva, subtilisin from Novo and carboxypeptidase-Y from Biochimreaktiv (U.S.S.R).

Tert-butyloxycarbonylphenylalanyl-glutamine Phenylhydrazide (*IVa*)

A solution of tert-butyloxycarbonylphenylalanine methyl ester (430 mg) and glutamine phenylhydrazide trifluoroacetate (650 mg) in a mixture of methanol (4 ml) and 0·2 mol l^{-1} carbonate–bicarbonate buffer, pH 10·5 (16 ml), was adjusted to pH 10. α-Chymotrypsin (10 mg) was added and the mixture was stirred for 3 h at room temperature. The precipitate was collected on filter and worked up as usual, affording 395 mg (52%) of the product, m.p. 200—202°C. Crystallization

from methanol–ether gave 324 mg (43%) of *IVa*, m.p. 202–203°C, R_F 0·74 (S1), 0·69 (S2), 0·72 (S3), 0·75 (S4); $k' = 0·78$. $[\alpha]_D$ −11·4° (*c* 0·3, methanol). Amino acid analysis: Glu 1·00, Phe 1·01. For $C_{25}H_{33}N_5O_5.0·5\ H_2O$ (492·6) calculated: 60·95% C, 6·96% H, 14·21% N; found: 61·25% C, 6·76% H, 14·39% N.

Tert-butyloxycarbonylphenylalanyl-glutamine (*IVb*)

A solution of ferric chloride (4·5 g) in water (15 ml) was added to a suspension of phenylhydrazide *IVa* (475 mg) in dioxane (20 ml) and the mixture was stirred for 30 min at 35°C. After addition of 2 mol l^{-1} NaOH (30 ml), the precipitate was separated by centrifugation. The supernatant was stripped of dioxane, acidified with 1 mol l^{-1} HCl, the product was taken up in ethyl acetate, dried over sodium sulfate and the solvent was evaporated. The residue was crystallized from ethyl acetate–light petroleum, affording 290 mg (75%) of *IVb*, m.p. 112–114°C. R_F 0·71 (S1), 0·15 (S2), 0·68 (S3), 0·58 (S4); $k' = 0·42$; $[\alpha]_D$ −3·2° (*c* 0·3, methanol). For $C_{19}H_{27}N_3O_6$ (393·4) calculated: 58·00% C, 6·92% H, 10·68% N; found: 57·78% C, 6·96% H, 10·31% N.

Tert-butyloxycarbonylphenylalanyl-glutaminyl-asparaginyl-S-benzylcysteine Phenylhydrazide (*VI*)

Ethylenediaminetetraacetic acid (3 mg) and cysteine hydrochloride (5 mg) were added to a solution of *IVb* (200 mg) and asparaginyl-S-benzylcysteine phenylhydrazide trifluoroacetate (320 mg) in a mixture of ethanol (1·5 ml) and 0·2 mol l^{-1} acetate buffer, pH 5·8 (3·5 ml). The resulting solution had pH 4·8. After addition of papain (20 mg) the mixture was incubated for 20 h at 38°C. The precipitate was filtered and processed as usual, affording 345 mg (87%) of *VI*, m.p. 228–229°C, which on crystallization from methanol–ether melted at 229–230°C (315 mg; 80%). R_F 0·68 (S1), 0·53 (S2), 0·68 (S3), 0·76 (S4), $k' = 1·63$; $[\alpha]_D$ −35·9° (*c* 0·2, dimethylformamide). Amino acid analysis: Asp 1·02, Glu 1·00, Phe 1·00, Cys(Bzl) 0·99. For $C_{39}H_{50}N_8O_8S.0·5\ H_2O$ (799·9) calculated: 58·56% C, 6·43% H, 14·01% N; found: 58·43% C, 6·21% H, 13·87% N.

Tert-butyloxycarbonyltyrosyl-phenylalanine Phenylhydrazide (*VII*)

Ethylenediaminetetraacetic acid (6 mg) and cysteine hydrochloride (20 mg) were added to a solution of tert-butyloxycarbonyltyrosine (565 mg) and phenylalanine phenylhydrazide trifluoroacetate (815 mg) in a mixture of ethanol (4 ml) and 0·2 mol l^{-1} acetate buffer, pH 4·8 (16 ml). After adjusting to pH 4·8, papain (85 mg) was added and the mixture was incubated at 38°C for 25 h. The product was extracted many times into ethyl acetate and the organic solution was washed successively with an HSO_4^- buffer pH 2, water, 0·5 mol l^{-1} $NaHCO_3$ and water, dried over sodium sulfate and taken down. Crystallization of the residue from ethyl acetate–light petroleum gave 370 mg (36%) of *VII*, m.p. 203–205°C. R_F 0·84 (S1), 0·80 (S2), 0·82 (S3), 0·81 (S4); $k' = 1·08$. An analytical sample was recrystallized from ethyl acetate, m.p. 207–208°C; $[\alpha]_D$ −31·9° (*c* 0·3, dimethylformamide). Amino acid analysis: Tyr 0·93, Phe 1·07. For $C_{29}H_{34}$. $(N_4O_5$ (518·6) calculated: 67·16% C, 6·61% H, 10·80% N; found: 66·88% C, 6·55% H, 10·56% N.

Benzyloxycarbonylglutaminyl-asparagine Phenylhydrazide (*VIII*)

Ethylenediaminetetraacetic acid (3 mg) and cysteine hydrochloride (10 mg) were added to a solution of benzyloxycarbonylglutamine (281 mg) and asparagine phenylhydrazide trifluoroacetate (681 mg) in a mixture of dimethylformamide (1 ml) and 0·2 mol l^{-1} acetate buffer pH 4·8 (9 ml), and the solution was adjusted to pH 4·8. After addition of papain (21 mg), the mixture was incubated for 24 h at 38°C. The precipitate was filtered and processed as usual, affording 350 mg .72%) of the product, m.p. 253–254°C. An analytical sample was obtained by recrystallization

from methanol; m.p. 253°C, $[\alpha]_D$ −24·3 (*c* 0·3, dimethyl sulfoxide). Amino acid analysis: Asp 0·97, Glu 1·00. For $C_{23}H_{28}N_6O_6.0{\cdot}5\,H_2O$ (493·5) calculated: 55·97% C, 5·92% H, 17·03% N; found: 55·54% C, 5·80% H, 16·79% N.

Benzyloxycarbonyl-S-benzylcysteinyl-tyrosyl-phenylalanine Methyl Ester (*IXa*)

Calcium chloride (0·5 mg) was added to a solution of *IIIb* (51 mg) and phenylalanine methyl ester hydrochloride (25 mg) in a mixture of dimethylformamide (0·5 ml) and 0·2 mol l^{-1} Tris–maleate buffer, pH 7 (2 ml). After adjusting to pH 7·0 and addition of thermolysin (2 mg), the mixture was incubated at 38°C for 24 h, acidified with 1 mol l^{-1} HCl and the product was taken up in ethyl acetate. The organic solution was washed successively with 1 mol l^{-1} HCl, water, 0·5 mol l^{-1} $NaHCO_3$ and again water, dried over sodium sulfate and taken down. The residue on triturating with ether gave 40 mg (60%) of *IXa*, m.p. 162°C; $[\alpha]_D$ −36·50 (*c* 0·3, methanol). Amino acid analysis: Tyr 0·99, Phe 1·03, Cys(Bzl) 0·98. Reported[16] m.p. 154°C; $[\alpha]_D$ −33·7° (*c* 1·5, methanol).

Benzyloxycarbonyl-S-benzylcysteinyl-tyrosyl-phenylalanine Phenylhydrazide (*IXb*)

A) With thermolysin: Calcium chloride (0·5 mg) was added to a solution of *IIIb* (51 mg) and phenylalanine phenylhydrazide trifluoroacetate (47 mg) in a mixture of dimethylformamide (0·5 ml) and Tris–maleate buffer, pH 7 (2 ml). After adjusting to pH 7·0 and addition of thermolysin (2 mg), the mixture was incubated at 38°C for 6 h and the precipitate was filtered and worked up in the usual manner. The product, m.p. 243–245°C (70 mg; 94%), was crystallized from methanol to give 63 mg (84%) of *IXb*, m.p. 244–245°C. R_F 0·88 (S1), 0·77 (S2), 0·88 (S3), 0·82 (S4); $k' = 3{\cdot}50$. $[\alpha]_D$ −41·2° (*c* 0·3, dimethylformamide). Amino acid analysis: Tyr 0·95, Phe 1·11, Cys(Bzl) 0·94. For $C_{42}H_{43}N_5O_6S.0{\cdot}5\,H_2O$ (754·9) calculated: 66·82% C, 5·87%H, 9·27% N; found: 66·45% C, 5·73% H, 9·36% N.

B) With α-chymotrypsin: A solution of *IIIa* (67 mg) and phenylalanine phenylhydrazide trifluoroacetate (185 mg) in a mixture of dimethylformamide (2·25 ml) and 0·2 mol l^{-1} carbonate–bicarbonate buffer pH 10·5 (2·75 ml) was adjusted to pH 10. After addition of α-chymotrypsin (5 mg), the mixture was stirred for 1 h at room temperature, diluted with 1 mol l^{-1} HCl, the precipitate collected on filter and processed as usual. The obtained crude *IXb* (62 mg), containing some starting dipeptide *IIIa*, was crystallized from methanol to afford 50 mg (54%) of homogeneous product, m.p. 243–244°C, which had the same R_F and k' values as the compound prepared according to procedure *A*. Amino acid analysis: Tyr 0·97, Phe 1·11, Cys(Bzl) 0·92.

Benzyloxycarbonyl-S-benzylcysteinyl-tyrosyl-phenylalanine (*IXc*)

A) By alkaline hydrolysis: Tripeptide *IXa* (44 mg) was dissolved in dioxane (0·2 ml) and hydrolyzed with 1 mol l^{-1} NaOH (0·1 ml) for 3 h at room temperature. The mixture was diluted with 1 mol l^{-1} HCl and the product was taken up in ethyl acetate. The extract was washed with water, dried over sodium sulfate and the solvent was evaporated. Crystallization of the residue from ether gave 32 mg (74%) of *IXc*, m.p. 177–178°C; R_F 0·86 (S1), 0·35 (S2), 0·82 (S3), 0·65 (S4), $k' = 2{\cdot}18$, $[\alpha]_D$ −21·7° (*c* 0·2, methanol). For $C_{36}H_{37}N_3O_7S.1\,H_2O$ (673·8) calculated: 64·17% C, 5·83% H, 6·24% N; found: 64·30% C, 5·62% H, 6·41% N.

B) By oxidative cleavage: A solution of tripeptide *IXb* (40 mg) in dioxane (2 ml) was oxidized with ferric chloride (0·3 g) in water (1 ml) at 35°C for 30 min. After addition of 2 mol l^{-1} NaOH the precipitate was separated by centrifugation and dioxane was evaporated from the supernatant. After acidification with 1 mol l^{-1} HCl, the title compound (22 mg; 62%) was isolated as described under *A* and had the same melting point, R_F and k'.

Benzyloxycarbonylglutaminyl-asparaginyl-S-benzylcysteine Phenylhydrazide (*X*)

Ethylenediaminetetraacetic acid (3 mg) and cysteine hydrochloride (10 mg) were added to a solution of benzyloxycarbonylglutamine (281 mg) and asparaginyl-S-benzylcysteine phenylhydrazide trifluoroacetate (560 mg) in a mixture of ethanol (2 ml) and 0·2 mol l^{-1} acetate buffer, pH 4·8 (8 ml). After adjusting to pH 4·8 and addition of papain (42 mg), the mixture was incubated for 24 h at 38°C. The precipitate was collected on filter and processed in the usual manner, affording 493 mg (73%) of *X*, m.p. 244—246°C, which was crystallized from dioxane without change in the melting point (464 mg; 68%); R_F 0·69 (S1), 0·61 (S2), 0·70 (S3), 0·75 (S4), $k' = 0·72$, $[\alpha]_D$ −31° (*c* 0·3, dimethylformamide). Amino acid analysis: Asp 1·00, Glu 1·01, Cys(Bzl) 0·98. For $C_{33}H_{39}$. .$N_7O_7S.H_2O$ (695·8) calculated: 56·96% C, 5·94% H, 14·09% N; found: 56·92% C, 5·45% H, 14·19% N.

Benzyloxycarbonyl-S-benzylcysteinyl-tyrosyl-phenylalanyl-glutamine Phenylhydrazide (*XIa*)

Calcium chloride (5 mg) was added to a solution of *IIIb* (102 mg) and phenylalanyl-glutamine phenylhydrazide trifluoroacetate (110 mg) in a mixture of dimethylformamide (3 ml) and 0·2 mol . . l^{-1} Tris–maleate buffer, pH 7·3 (7 ml). Thermolysin (5 mg) was added to the solution (pH 7·0) which was then incubated for 20 h at 38°C. The precipitate was filtered and worked up in the usual manner. The product (138 mg; 79%), m.p. 228—231°C, was crystallized from methanol affording 124 mg (71%) of *XIa*, m.p. 231—233°C, R_F 0·82 (S1), 0·69 (S2), 0·78 (S3), 0·80 (S4); $k' = 2·04$, $[\alpha]_D$ −27·6° (*c* 0·3, dimethylformamide). Amino acid analysis: Glu 1·0, Tyr 0·9, Phe 1·0, Cys(Bzl) 0·9. For $C_{47}H_{51}N_7O_8S.1\ H_2O$ (892·1) calculated: 63·27% C, 5·99% H, 10·99% N; found: 63·36% C, 5·87% H, 11·23% N.

Benzyloxycarbonyl-S-benzylcysteinyl-tyrosyl-phenylalanyl-glutamine (*XIb*)

Tetrapeptide *XIa* (100 mg) in dimethylformamide (5 ml) was oxidized with ferric chloride (600 mg) in water (2 ml) at 35°C for 30 min. The mixture was diluted with dimethylformamide (5 ml), treated with 2 mol l^{-1} NaOH (3 ml) and the precipitate removed by centrifugation. The supernatant was neutralized with 1 mol l^{-1} HCl and the solvent was evaporated at 30°C *in vacuo*. The residue was triturated with ether, washed with 1 mol l^{-1} HCl and water, and dried. The crude product (63 mg; 70%), m.p. 195—198°C, was crystallized from methanol–ether to give 57 mg (64%) of *XIb*, m.p. 198—200°C, R_F 0·81 (S1), 0·20 (S2), 0·78 (S3), 0·60 (S4), $k' = 1·09$; $[\alpha]_D$ −34·8° (*c* 0·3, dimethylformamide). For $C_{41}H_{45}N_5O_9S.0·5\ H_2O$ (792·9) calculated: 62·10% C, 5·85% H, 8·83% N; found: 62·23% C, 5·85% H, 8·82% N.

Benzyloxycarbonyl-S-benzylcysteinyl-tyrosyl-phenylalanyl-glutaminyl-asparaginyl-S-benzylcysteine Phenylhydrazide (*IIa*)

A) *With thermolysin*: Calcium chloride (5 mg) and thermolysin (5 mg) were added (at pH 7·0) to a solution of *IIIb* (102 mg) and phenylalanyl-glutaminyl-asparaginyl-S-benzylcysteine phenylhydrazide trifluoroacetate (180 mg) in a mixture of dimethylformamide (3·5 ml) and 0·2 mol l^{-1} Tris–maleate buffer, pH 7 (6·5 ml). The mixture was incubated for 20 h at 38°C, the precipitate was filtered and processed as usual. The material (202 mg; 86%), m.p. 248—250°C, was crystallized from methanol to afford 178 mg (75%) of *IIa*, m.p. 260—261°C; $k' = 3·71$, $[\alpha]_D$ −35·3° (*c* 0·2, dimethylformamide). Amino acid analysis: Asp 1·07, Glu 1·10, Tyr 0·92, Phe 1·06, Cys(Bzl) 1·71. For $C_{61}H_{68}N_{10}O_{11}S_2.3\ H_2O$ (1235) calculated: 59·32% C, 6·04% H, 11·34% N; found: 59·38% C, 5·58% H, 11·61% N.

B) *With α-chymotrypsin*: α-Chymotrypsin (5 mg) was added to a solution of *IXc* (13·2 mg) and glutaminyl-asparaginyl-S-benzylcysteine phenylhydrazide (12 mg) in a mixture of dimethylformamide (0·3 ml) and 0·2 mol l^{-1} Tris–maleate buffer, pH 7·0 (0·7 ml), the mixture was incubated for 20 h at 38°C and worked up as described under *A*. The product (20 mg; 85%), m.p. 248—250°C, was crystallized from methanol to give 15 mg (64%) of *IIa*, m.p. 258—260°C, $k' =$ = 3·71; $[\alpha]_D$ −34·4° (*c* 0·2, dimethylformamide). Amino acid analysis: Asp 1·14, Glu 1·13, Tyr 0·92, Phe 0·95, Cys(Bzl) 1·71.

C) *With papain*: Ethylenediaminetetraacetic acid (0·3 mg) and cysteine hydrochloride (1 mg) were added to a solution of *XIb* (16 mg) and asparaginyl-S-benzylcysteine phenylhydrazide trifluoroacetate (13 mg) in a mixture of dimethylformamide (0·3 ml) and 0·2 mol l^{-1} acetate buffer, pH 4·8 (0·7 ml). After adjusting to pH 4·8, papain (2·1 mg) was added and the mixture was incubated for 20 h at 38°C. The work-up procedure was the same as described under *A* and afforded 15 mg (64%) of material, m.p. 248—251°C. Crystallization from methanol yielded 8 mg (34%) of *IIa*, m.p. 257—259°C, $k' =$ 3·71. Amino acid analysis: Asp 0·97, Glu 1·08, Tyr 0·96, Phe 0·97, Cys(Bzl) 2·04.

Benzyloxycarbonyl-S-benzylcysteinyl-tyrosyl-phenylalanyl-glutaminyl-asparaginyl-S-benzylcysteine (*IIb*)

A solution of ferric chloride (900 mg) in water (3 ml) was added to a solution of *IIa* (120 mg) in dimethylformamide (10 ml) and stirred for 30 min at 35°C. After dilution with dimethylformamide (10 ml), 2 mol l^{-1} NaOH (5·5 ml) was added and the precipitate was removed by centrifugation. The supernatant was taken down at 30°C and the residue triturated with ether, washed with 1 mol l^{-1} HCl, water and dried, affording 75 mg (68%) of product, m.p. 219—221°C, which on crystallization from methanol gave 65 mg (59%) of *IIb*, m.p. 220—221°C, R_F 0·78 (S1), 0·21 (S2), 0·79 (S3), 0·66 (S4); $k' = 2{\cdot}16$, $[\alpha]_D$ −20·9° (*c* 0·25, dimethylformamide). Amino acid analysis: Asp 1·06, Glu 1·11, Tyr 0·91, Phe 1·05, Cys(Bzl) 1·73. For $C_{55}H_{62}N_8O_{12}S_2.2\,H_2O$ (1127) calculated: 58·61% C, 5·90% H, 9·94% N; found: 58·78% C, 5·61% H, 10·24% N.

Pressinoic Acid (*I*)

Hexapeptide *IIb* (50 mg) was reduced with sodium in liquid ammonia (50 ml) for 20 sec similarly as described in ref.[7]. After addition of a small amount of ammonium chloride, ammonia was evaporated and the residue dissolved in 0·05% trifluoroacetic acid (100 ml). The mixture was adjusted to pH 7 with 3% ammonia, oxidized with air for 6 h, acidified to pH 3·5 and freeze-dried. The residue was dissolved in 0·05% trifluoroacetic acid (10 ml) and the product was isolated by HPLC (gradient elution); yield 19 mg (53%) of the lyophylisate; R_F 0·18 (S1), 0·04 (S2), 0·06 (S3), 0·36 (S4), $k' = 0{\cdot}31$; $E^{2,4}_{Gly}$ 0·69, $E^{5,7}_{Gly}$ 0·0. $[\alpha]_D$ −9·2 (*c* 0·2; 1 mol l^{-1} acetic acid). Amino acid analysis: Asp 1·04, Glu 1·08, 1/2 Cys 1·81, Tyr 0·97, Phe 1·01. For $C_{33}H_{42}N_8O_{10}S_2.CF_3COOH.3\,H_2O$ (943) calculated: 44·58% C, 5·24% H, 11·88% N; found: 44·37% C, 4·60% H, 11·49% N.

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