SIDE REACTION DURING THE DEPROTECTION OF Cys(Acm)-CONTAINING PEPTIDES WITH IODINE. SYNTHESIS OF DISULFIDE FRAGMENTS FROM CATHEPSIN D STRUCTURE

Jan JEZEK, Vlasta VELKOVA, Vaclav KASICKA, Zdenek PRUSIK, Karel UBIK, Klaudie BARTOVA and Michael MARES

Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, 166 10 Prague 6, The Czech Republic

Received July 1, 1994
Accepted May 11, 1995

Peptides H-TPPQC(Acm)FTV-NH_2 (*I*) and H-VSVPC(Acm)QSASSAS-NH_2 (*III*) were prepared by the solid phase method. Their oxidation with iodine afforded hexadecapeptide *II*, tetracosapeptide *IV* and eicosapeptide *VI*. The disulfide peptides *II*, *IV* and *VI* are designed according to the sequence of the processing loop in human cathepsin D. The purity of the peptides was determined by analytical HPLC and capillary zone electrophoresis. In addition to the expected $[M + H]^+$ ion, FAB MS of HPLC-pure tetracosapeptide *IV* exhibited a molecular ion with the same relative molecular mass as the starting dodecapeptide *III*, in spite of clean HPLC separation of *III* and *IV*. Free-flow zone electrophoresis of *IV* separated peptide *V*, isomeric with *III*. Mass spectra, amino acid analysis and Edman sequencing revealed that the peptide *V* is a product of iodine-mediated S→O shift of Acm group in the serine-rich peptide *III*. Daughter-ion spectra of protonated molecules, recorded after collision-induced dissociation, have shown that the Acm moiety is bonded to Ser 9 or Ser 10.

Although many convenient protecting groups for the cysteine residue are available and several methods for their deprotection and oxidation to disulfide peptides[1,2] may be used, the synthesis of cysteine-containing peptides still presents some problems. To simplify the work-up procedure after hydrogen fluoride treatment, we made use of Acm as the protecting group[3]. Removal of the Acm group[4] and oxidation were performed simultaneously, by treatment with iodine. The synthesized peptides and their disulfide pairing were designed according to the sequence of the processing loop in human cathepsin D (ref.[5]). The present paper describes problems that emerged during preparation of relatively simple disulfide peptides.

RESULTS AND DISCUSSION

The peptides were synthesized by the solid phase method[1]. The final deprotection and detachment from the MeBHA resin was done with liquid hydrogen fluoride[7] in the

presence of 10% anisole. Octapeptide *I* was obtained in 72% yield and 96% purity (HPLC). The yield of dodecapeptide *III* was 94%, the HPLC purity of the crude product being 90%. Both the peptides were used without purification in oxidative dimerization experiments.

In order to obtain hexadecapeptide *II*, tetracosapeptide *IV* and eicosapeptide *VI*, we used iodine for Cys(Acm)* deprotection and simultaneous oxidation. We made some modifications of the described procedure[4]. To simplify the workup, the Acm-protected peptide was dissolved in 10% AcOH and treated with iodine in ethyl acetate. After 2 h, the excess iodine was extracted with ethyl acetate and the lyophilized product was purified by preparative HPLC. The fractions obtained were characterized by analytical HPLC, CZE, FAB MS and amino acid analysis.

Oxidation of octapeptide *I* yielded, after preparative HPLC purification, 48% of dimer *II*. Considerable amount of the starting monomer *I* was recovered. Mass spectrum (FAB) of the recovered monomer *I* (HPLC purity >98%, fraction 2) exhibited ions

H-TPPQC(Acm)FTV-NH_2
I

H-TPPQCFTV-NH_2
|
H-TPPQCFTV-NH_2
II

H-VSVPC(Acm)QSASSAS-NH_2
III

H-VSVPCQSASSAS-NH_2
|
H-VSVPCQSASSAS-NH_2
IV

$^{9\ 10}$
H-VSVPCQSASSAS-NH_2
V
Acm group on 9 or 10 Ser

H-TPPQCFTV-NH_2
|
H-VSVPCQSASSAS-NH_2
VI

* The symbols and abbreviations obey the published recommendations[6]. In addition, the following abbreviations are used: AA amino acid; Acm acetamidomethyl; AcOH acetic acid; AcOEt ethyl acetate, BGE background electrolyte; BOP benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; CZE capillary zone electrophoresis; DCC *N*,*N*′-dicyclohexylcarbodiimide; DCM dichloromethane; DIEA diisopropylethylamine; DMF dimethylformamide; HOBt 1-hydroxybenzotriazole; MCA FAB MS multichannel fast atom bombardment mass spectrometry; MeBHA methylbenzhydrylamine; RP HPLC reversed-phase high-performance liquid chromatography; R_T retention time; TFA trifluoroacetic acid.

$[M + H]^+$ 962.7 and 891.6 in the ratio 8 : 1. These values were in accord with those calculated for octapeptide *I* with and without the Acm group (962.5 and 891.4, respectively). The FAB MS fragmentation of Acm groups in Cys(Acm)-containing peptides corresponds to that reported in the literature[8]. The FAB MS of dimer *II* (HPLC purity >98%, fraction 3) displayed two $[M + H]^+$ ions, 1 779.7 and 891.7 in the ratio 1 : 5; these values correspond to the calculated mass for dimer *II* (1 779.9) and octapeptide *I* without the Acm group (891.4). Amino acid analyses of both the monomer *I* and dimer *II* support the given structure.

Oxidation of dodecapeptide *III* and subsequent HPLC purification yielded 45% of dimer *IV*. A part of the monomer *III* was recovered. Mass spectrum (MCA FAB) of the isolated monomer *III* (HPLC purity >98%, fraction 3) displayed two $[M + H]^+$ ions, 1 192.6 and 1 121.6, in the ratio 8 : 1. These values correspond to protonated dodecapeptide *III* with and without Acm group (calculated 1 192.6 and 1 121.5, respectively). The MCA FAB MS of the dimer *IV* (HPLC purity >98%) exhibited an ion $[M + H]^+$ at *m/z* 2 241.5 and two protonated molecules at *m/z* 1 192.5 and 1 121.5 in the ratio 1 : 1.3 : 4. The calculated value for protonated dimer *IV* is 2 241.5. The presence of protonated species of *m/z* 1 192.5 is surprising because the dimer *IV* and monomer *III*, both HPLC pure, have different HPLC retention times. To corroborate the purity of RP HPLC separated peptides we used capillary zone electrophoresis (CZE). The CZE analyses showed high purity (99%) of octapeptide *I* and only small amount (4%) of *I* was found in hexadecapeptide *II* (CZE purity 96%). Dodecapeptide *III* (CZE purity 84%) contained two unidentified impurities. Tetracosapeptide *IV* (CZE purity 78%) contained, beside one unidentified admixture (3%), roughly 19% of a faster moving compound *V* which was not identical with the starting dodecapeptide *III*. On the basis of HPLC, CZE, FAB MS and amino acid analysis data we suggest that the side product *V* is a peptide in which the Acm group has shifted to one of the five serine residues in the molecule. This would explain why in the MCA FAB spectra of the tetracosapeptide *IV* we found also a molecular ion of the same mass as the monomer *III*. A contamination of the HPLC-pure tetracosapeptide *IV* with the starting monomer *III* can be excluded because the HPLC retention times of both compounds are different.

To determine the position of the Acm group in the peptide *V*, we utilized a collision-induced dissociation of protonated molecules in connection with daughter-ion scan at constant B/E. Our results indicate that the Acm moiety is bonded to Ser 9 or Ser 10. The presence of ions at *m/z* 907 (Y''_{10} ions according to the Roepstorff and Fohlman nomenclature[9]), at *m/z* 701 (B_7 ions) and at *m/z* 1 088 (B_{11} ions) excludes bonding of Acm to Ser 2, Ser 7 and Ser 12, respectively.

Oxidative cross-coupling of octapeptide *I* and dodecapeptide *III* and subsequent purification by HPLC afforded eicosapeptide *VI* in the yield of 34%. Whereas its purity (determined by HPLC) was 98%, the purity found by CZE was only 53%, the main contamination (40%) being octapeptide *I*. Consequently, MCA FAB MS of the HPLC-

pure eicosapeptide *VI* exhibited also a peak at *m/z* 963.4 corresponding to protonated molecule of *I*, in addition to the expected $[M + H]^+$ ion at 2 011.1 and fragments at *m/z* 1 122.5 and 891.5.

Capillary zone electrophoresis has proved to be very valuable technique for determination of purity of peptides isolated by HPLC. Due a separation principle different from RP HPLC and due to high separation power it is capable of separating eicosapeptide *VI* from octapeptide *I* and tetracosapeptide *IV* from peptide *V* which are not separable by RP HPLC. Tetracosapeptide *IV* and eicosapeptide *VI* were subjected to free-flow zone electrophoresis (FFZE) and both were obtained sufficiently pure (CZE purity 92–94%).

When our experiments were in progress, Lamthanh[10] observed an S→O shift of Acm group from cysteine to the OH group of serine and/or threonine during thallic trifluoroacetate or mercuric acetate mediated *S*-Acm deprotection in Ser- and Thr-rich peptides. Now we are describing an analogous side reaction for iodine-mediated *S*-Acm deprotection and oxidation. We tried to localize exactly the Ser residue to which the Acm group was shifted by the Edman degradation of peptide *V*. However, under these conditions serine afforded didehydroalanine and the only conclusion obtained is that the Acm group is not bonded to the Cys residue.

Oxidative cross-coupling of *I* and *III* should afford, in addition to the expected products *II*, *IV* and *VI*, also peptides with $[M + H]^+$ ions higher by 71.1 units, corresponding to peptides with one Acm group because the peptide *V* generated from *III* by S→O Acm shift and the analogous peptide derived from *I* will also take part in the oxidative coupling. The MCA FAB spectra of crude eicosapeptide *VI* indeed contained also peaks of protonated molecules at *m/z* 2 312.4, 2 082.3 and 1 852.7, corresponding to compounds *IV* + Acm, *VI* + Acm and *II* + Acm (calculated values 2 312.6, 2 082.4 and 1 852.2, respectively). We have not attempted to isolate these side products.

EXPERIMENTAL

Analytical RP HPLC was performed with a Spectra-Physics 8700 apparatus (Darmstadt, Germany) equipped with a column (250 × 4 mm) packed with Separon SGX-RPS, particle size 10 μm (Tessek, Prague). The sample (1 mg) was dissolved in water (100 μl) and 8 μl of this solution was injected. The chromatography was performed in a gradient of methanol–water (2%/min, both phases contained 0.1% TFA) starting with 20% of methanol. Flow rate 1.0 ml/min, detection at 220 nm. Preparative RP HPLC was carried out using a Knauer apparatus (Bad Homburg, Germany) equipped with a 250 × 10 mm Separon SGX-RPS column, particle size 10 μm (Tessek, Prague). The sample (50 mg of crude peptide) was dissolved in water and chromatographed in a gradient of methanol–water (1%/min, both phases contained 0.1% TFA), starting with 100% water (tetracosapeptide *IV*), 20% of methanol (hexadecapeptide *II*), or 10% of methanol (eicosapeptide *VI*). Flow rate 1.7 ml/min, detection at 220 nm.

Electrophoretic homogeneity of the peptides after HPLC purification was tested by CZE in 0.5 M acetic acid, pH 2.5, as BGE. The analyses were performed on a home-made apparatus[11] equipped with an untreated fused silica capillary (0.05 mm i.d., 0.15 mm o.d., effective length 200 mm, total length 310 mm) and a UV detector (206 nm). Peptides (0.75–0.9 mg/ml) were dissolved in the BGE

and the solution was introduced into the capillary manually, forming a hydrostatic pressure (50 mm height difference of capillary tips) for 5–15 s. The applied voltage was 10 kV (anode at the injection end of the capillary) and the current was 10.5–10.8 μA at 23 °C. The purity was calculated from the relative peak height, i.e. the peak height of the main component was related to the sum of heights of all peaks present in the electrophoreogram[12].

Continuous FFZE was performed in a home-made apparatus[11] equipped with a glass chamber cooled on both sides (500 × 500 × 0.5 mm), in 0.5 M acetic acid as BGE. Flow-through period of BGE and sample 31 min, flow rate 1.6 ml/h, sample concentration 30 mg/ml. The experiments were performed at constant voltage 3 000 V, current 112–116 mA, glass plate coolant temperature –2.5 to –3 °C. The preparative fractions were evaluated by off-line UV absorption measurements at 225 nm and (after lyophilization) by CZE analysis.

The Edman degradation was carried out on a Model 470A Gas Phase Protein Sequencer (Applied Biosystems, U.S.A.) according to a modified standard program 03CPTH supplied by the manufacturer. The content of PTH's in the individual cycles was assayed by HPLC using a system consisting of Beckman 114 Solvent Delivery Module, Beckman 421 Controller with 420 Pneumatic Interface Card, Beckman 340 Organizer and Altex 210 injection valve. The effluents were monitored at 269 nm by a Shimadzu SPD-2A spectrophotometric detector combined with a Shimadzu Chromatopac C-R3A integrator.

Positive-ion FAB spectra were recorded on a ZAB-EQ mass spectrometer (VG Analytical, Manchester, U.K.), with an IonTech gun and glycerol matrix. Collision-induced dissociation spectra were generated using helium as a target gas in the first field-free region at 50% transmission of parent ions. The daughter-ion linked scans at constant B/E were recorded on the $[M + H]^+$ ions.

Samples for amino acid analysis were hydrolyzed in 6 M HCl at 110 °C for 20 h and analyzed on a D-500 Durrum Amino Acid Analyzer (Durrum Corporation, Palo Alto, CA, U.S.A.). The analyses were done either classically or with performic acid oxidation (i.e. Cys was converted to cysteic acid). For estimation of Cys(Acm), Boc-Cys(Acm) was treated under the same conditions as the corresponding peptides and used as a standard.

Analytical samples were dried over P_2O_5 at 6.5 Pa and 25 °C for 8 h.

Peptide Synthesis

All the Boc-amino acids were from Bachem (Bubendorf, Switzerland). Peptide synthesis was performed manually using the solid phase methodology[1] with Boc/Bzl strategy. Protecting groups used: Bzl for Ser and Thr, Acm for Cys. The solid support consisted of *p*-methylbenzhydrylamine . HCl-type resin (Peptides International, Louisville, KY, U.S.A.), 0.92 mmol NH_2/g. The washing protocol was: 1) DCM, 1 × 30 s; 2) 55% TFA in DCM, 5 + 25 min; 3) DCM, 4 × 1 min; 4) 10% DIEA in DCM, 2 × 2 min; 5) DCM, 2 × 1 min; 6) DMF, 1 × 1 min; 7) 3-fold molar excess of Boc-AA, HOBt and DCC, preactivation in minimum of DCM–DMF 9 : 1 (for Boc-Gln DCM–DMF 1 : 1 was used) for 20 min at room temperature. The precipitated dicyclohexylurea was filtered off, washed with small amount of DCM, the filtrate was concentrated in vacuo, dissolved in DMF to give a 0.2 mol/l solution and added to the resin. After 60 min Kaiser test[13] was done. When positive, the solvents were filtered off, the resin was neutralized, washed twice with DMF and the coupling was repeated with 3-fold molar excess of BOP reagent and Boc-AA and 6-fold excess of DIEA in DMF; 8) DMF, 1 × 1 min; 9) DCM, 3 × 1 min.

At the end of the synthesis, the Boc group was cleaved off, the resin was washed with DCM, DMF, ethanol and ether (four times each) and the peptide resin was dried. The detachment[7] of the peptide from the resin and the side chain deprotection were carried out in liquid HF–anisole 9 : 1, 10 ml/g of peptide resin, 1 h at 0 °C. The HF was blown off by stream of nitrogen and the crude mixture was

extracted three times with ether to remove the scavenger. The peptide, at the 2 mmol scale, was extracted with 20% AcOH (5 × 40 ml) and the extracts were diluted with water (200 ml) and lyophilized. The yields are not corrected for solvation.

H-Thr-Pro-Pro-Gln-Cys(Acm)-Phe-Thr-Val-NH_2 (*I*)

The synthesis was carried out on a MeBHA resin (2.17 g, 2 mmol). No coupling had to be repeated. Yield 4.95 g of peptide–resin adduct. Deprotection with HF afforded 1.39 g (72%) of crude peptide *I*, HPLC purity 96%, R_T 26.30 min. This compound was used for dimerization experiments without further purification. The following data relate to peptide *I*, recovered after preparative HPLC purification of crude hexadecapeptide *II*. Analytical HPLC showed purity >98%, CZE purity was 99%. For peptide without Acm ($C_{40}H_{62}N_{10}O_{11}S$) calculated relative molecular mass 891.1, monoisotopic mass 890.4. For peptide *I* ($C_{43}H_{67}N_{11}O_{12}S$) calculated relative molecular mass 962.1, monoisotopic mass 961.5. FAB MS (HPLC fraction 2), *m/z*: 962.7 $[M + H]^+$ and 891.6 $[M + H - Acm]^+$ in the ratio 1 : 8; calculated for *I* 962.5, for *I* without Acm 891.4.

Amino acid analysis (without oxidation): Thr 1.97, Pro 1.87, Glu 0.90, Cys(Acm) 0.98, Phe 0.98, Val 1.00. With oxidation: Thr 1.94, Pro 1.88, Glu 0.93, $CysSO_3H$ 0.94, Phe 0.98, Val 1.00. For $C_{43}H_{67}N_{11}O_{12}S$. CF_3COOH . 3 H_2O (1 130.2) calculated: 47.82% C, 6.60% H, 13.63% N, 2.84% S, 5.04% F; found: 47.97% C, 6.48% H, 13.84% N, 2.61% S, 4.76% F.

Hexadecapeptide *II*

A solution of iodine (305 mg, 1.2 mmol) in AcOEt (100 ml) was added to a vigorously stirred solution of crude octapeptide *I* (385 mg, 0.4 mmol) in 10% AcOH (200 ml) and stirring was continued for 2 h. The organic phase was discarded, the aqueous one was washed with AcOEt (2 × 100 ml) at 5 °C and slightly concentrated in vacuo to remove traces of AcOEt. Lyophilization yielded 364 mg of the product.

Analytical HPLC showed two dominant peaks with R_T 26.30 min and 34.30 min in the ratio 1 : 5.25, corresponding to the starting octapeptide amide *I* and hexadecapeptide *II*, respectively. Preparative HPLC afforded 53 mg of the starting peptide *I* (fraction 2) and 172 mg (48%) of hexadecapeptide *II* (fraction 3). HPLC purity of both *I* and *II* was >98%, CZE purity was 99% for *I* and 96% for *II*. For hexadecapeptide *II* ($C_{80}H_{122}N_{20}O_{22}S_2$) calculated relative molecular mass 1 780.1 and monoisotopic mass 1 778.9. FAB MS (HPLC fraction 3), *m/z*: 1 779.7 $[M + H]^+$ and a fragment 891.7 in the ratio 1 : 5; calculated for hexadecapeptide *II* 1 779.9 and for octapeptide amide without Acm 891.4.

Amino acid analysis (without oxidation): Thr 3.92, Pro 3.68, Glu 1.83, Cys 2.12, Phe 1.97, Val 2.00. With oxidation: Thr 4.16, Pro 3.94, Glu 1.97, $CysSO_3H$ 2.00, Phe 2.06, Val 2.00. For $C_{80}H_{122}N_{20}O_{22}S_2$. CF_3COOH . CH_3COOH . 4 H_2O (2 026.2) calculated: 49.79% C, 6.72% H, 13.83% N, 3.17% S, 2.81% F; found: 49.84% C, 6.79% H, 13.62% N, 3.01% S, 2.56% F.

H-Val-Ser-Val-Pro-Cys(Acm)-Gln-Ser-Ala-Ser-Ser-Ala-Ser-NH_2 (*III*)

The synthesis was carried out with an MeBHA resin (2.17 g, 2 mmol). Only the coupling of Cys^5, Val^3 and Ser^2 had to be repeated. Yield 5.6 g of peptide on resin. Deprotection with HF afforded 2.25 g (94%) of crude peptide, HPLC purity 90%, R_T 15.98 min. This product was used without further purification for the oxidation experiments.

The following data relate to peptide *III*, recovered after preparative HPLC purification of the crude tetracosapeptide *IV*. Analytical HPLC showed R_T 15.98 min and purity >98%. For peptide without Acm ($C_{44}H_{76}N_{14}O_{18}S$) calculated: relative molecular mass 1 121.2, monoisotopic mass 1 120.5. For peptide *III* ($C_{47}H_{81}N_{15}O_{19}S$) calculated: relative molecular mass 1 192.3, monoisotopic mass 1 191.6. MCA

FAB (HPLC fraction 3), *m/z*: 1 192.6 $[M + H]^+$ and 1 121.6 in the ratio 8 : 1; calculated: for *III* 1 192.6, for *III* without Acm 1 121.5.

Amino acid analysis (without oxidation): Val 2.07, Ser 4.6, Pro 0.97, Cys(Acm) 0.85, Glu 0.93, Ala 2.0. With oxidation: Val 1.96, Ser 4.5, Pro 0.93, $CysSO_3H$ 0.97, Glu 0.94, Ala 2.0. For $C_{47}H_{81}N_{15}O_{19}S$. CF_3COOH . 2 H_2O (1 342.4) calculated: 43.84% C, 6.46% H, 15.65% N, 2.39% S, 4.25% F; found: 43.90% C, 6.64% H, 15.35% N, 2.21% S, 3.97% F.

Tetracosapeptide *IV*

Iodine (305 mg, 1.2 mmol) in AcOEt (100 ml) was added to a vigorously stirred solution of crude dodecapeptide *III* (476 mg, 0.4 mmol) in 10% AcOH (200 ml) and stirring was continued for 2 h. The organic phase was discarded and the aqueous one washed with AcOEt (2 × 100 ml) at 5 °C and slightly concentrated in vacuo to remove traces of AcOEt. Lyophilization yielded 440 mg of the product.

Analytical HPLC showed two dominant peaks with R_T 15.98 and 22.02 min in the ratio 1 : 2.3 (at 220 nm) corresponding to the starting dodecapeptide amide *III* and tetracosapeptide *IV*, respectively. Preparative HPLC afforded 53 mg of the starting peptide *III* and 203 mg (45%) of tetracosapeptide *IV*, both of HPLC purity >98%. The CZE purity of *III* and *IV* was 84% and 78%, respectively. For tetracosapeptide *IV* ($C_{88}H_{150}N_{28}O_{36}S_2$) calculated relative molecular mass: 2 240.5, monoisotopic mass: 2 239.0. MCA FAB (*m/z*): 2 241.5 $[M + H]^+$ and fragments 1 192.7 and 1 121.7 in the ratio 1 : 1.3 : 4. Calculated for tetracosapeptide *IV* 2 241.5. The measurement was repeated in the FAB MS mode (*m/z*): 1 192.8 $[M + H]^+$ and fragment 1 121.8 in the ratio 1 : 4; calculated: for *III* (or isomeric compound *V*) 1 192.6, for dodecapeptide amide without Acm 1 121.5.

Amino acid analysis (without oxidation) for *IV*: Val 4.18, Ser 9.0, Pro 2.02, Cys 2.14, Glu 1.92, Ala 4.0. With oxidation: Val 4.14, Ser 8.62, Pro 1.88, $CysSO_3H$ 2.01, Glu 1.97, Ala 4.0.

The tetracosapeptide *IV* (330 mg) was purified by continuous FFZE under the above-described conditions. Yield 167 mg (51%) of *IV*, CZE purity 94%. MCA FAB, *m/z*: 2 241.6 $[M + H]^+$ and fragment 1 121.9 in the ratio 1 : 4. Calculated: for tetracosapeptide *IV* 2 241.5, for dodecapeptide amide without Acm 1 121.5. For $C_{88}H_{150}N_{28}O_{36}S_2$. 2 CH_3COOH . 2 H_2O (2 396.6) calculated: 46.11% C, 6.81% H, 16.36% N, 2.68% S; found: 46.02% C, 6.98% H, 16.16% N, 2.39% S.

The purity of peptide *V* isolated after continuous FFZE was 67.5% according to CZE and 77% according to HPLC. The crude tetracosapeptide *IV* (330 mg) afforded 26 mg (8%) of peptide *V*. FAB MS, *m/z*: 1 193.1 $[M + H]^+$ and fragment 1 122.1 $[M + H - Acm]^+$ in the ratio 1 : 10; calculated: for *V* 1 192.6, for *V* without Acm 1 121.5.

Amino acid analysis (without oxidation): Val 2.06, Ser 4.6, Pro 0.97, Cys(Acm) 0.95, Glu 0.92, Ala 2.0. With oxidation: Val 1.96, Ser 4.65, Pro 0.93, $CysSO_3H$ 0.98, Glu 0.93, Ala 2.0. For $C_{47}H_{81}N_{15}O_{19}S$. CH_3COOH . 2 H_2O (1 288.4) calculated: 45.68% C, 6.96% H, 16.31% N, 2.49% S; found: 45.90% C, 6.74% H, 16.35% N, 2.21% S.

Eicosapeptide *VI*

A solution of iodine (152 mg, 0.6 mmol) in AcOEt (100 ml) was added to a vigorously stirred solution of crude octapeptide amide *I* (192 mg, 0.2 mmol) and dodecapeptide amide *III* (238 mg, 0.2 mmol) in 10% AcOH (200 ml) and stirring was continued for 2 h. The organic phase was discharged and the aqueous one washed with AcOEt (2 × 100 ml). Lyophilization afforded 412 mg of product.

Analytical HPLC showed peaks with R_T 15.98 min (corresponding to dodecapeptide *III*), 22.02 min (tetracosapeptide *IV*), 26.30 min (eicosapeptide *VI*; octapeptide *I* had the same HPLC R_T) and 34.30 min (hexadecapeptide *II*) in the ratio 1 : 0.9 : 4.6 : 1.5. Preparative HPLC afforded 44 mg of peptide *III* (fraction 3), 52 mg of tetracosapeptide *IV* (fraction 4), 137 mg (34%) of eicosapeptide *VI* (fraction 5;

this fraction contained also octapeptide *I*) and 44 mg of hexadecapeptide *II* (fraction 6). Purity (HPLC) of fractions 3, 4, 5 and 6 was >98%. Injection of a mixture of *I* and *VI* afforded a single symmetrical peak of R_T 26.30 min. CZE purity of eicosapeptide *VI* was 53%.

The eicosapeptide *VI* (389 mg) was purified by continuous FFZE, yield 195 mg (50%), CZE purity 92%. For eicosapeptide *VI* ($C_{84}H_{136}N_{24}O_{29}S_2$) calculated: relative molecular mass 2 010.3, monoisotopic mass 2 008.9. MCA FAB, *m/z*: 2 011.2 $[M + H]^+$ and fragments 1 122.4 and 891.5 in the ratio 2 : 1 : 13; calculated for eicosapeptide *VI* 2 011.3.

Amino acid analysis (without oxidation): Val 3.09, Ser 4.56, Pro 3.01, Cys 2.12, Glu 2.15, Ala 2.0, Thr 2.03, Phe 1.05. With oxidation: Val 3.09, Ser 4.58, Pro 3.13, $CysSO_3H$ 2.00, Glu 2.11, Ala 2.00, Thr 2.01, Phe 1.06. For $C_{84}H_{136}N_{24}O_{29}S_2$. 2 CH_3COOH . 3 H_2O (2 184.4) calculated: 48.39% C, 6.92% H, 15.39% N, 2.94% S; found: 48.45% C, 6.84% H, 15.29% N, 2.66% S.

The authors express their thanks to Dr M. Pavlík for Edman degradation, to Dr V. Pechanec for elemental analyses and to Mr J. Zbrozek for amino acid analyses. Part of this work was supported by the Grant Agency of the Czech Republic (Grant No. 203/93/0718; Z. P.).

REFERENCES

1. Barany G., Merrifild R. B. in: *The Peptides. Analysis, Synthesis, Biology* (E. Gross and J. Meienhofer, Eds), Vol. 2, Part A, p. 1. Academic Press, New York 1980.
2. Bodanszky M.: *Peptide Chemistry. A Practical Textbook*, p. 88. Springer, Berlin 1988.
3. Veber D. F., Milkowski J. D., Varga S. L., Denkewalter R. G., Hirschmann R.: J. Am. Chem. Soc. *94*, 5456 (1972).
4. Kamber B., Hartmann A., Eisler K., Riniker B., Rink H., Sieber P., Rittel W.: Helv. Chim. Acta *63*, 899 (1980).
5. Faust P. L., Kornfeld S., Chirgwin J. M.: Proc. Natl. Acad. Sci. U.S.A. *82*, 4910 (1985).
6. *Nomenclature and Symbolism for Amino Acids and Peptides. Recommendations 1983*. Eur. J. Biochem. *138*, 9 (1984).
7. Tam J. P., Merrifield R. B. in: *The Peptides. Analysis, Synthesis, Biology* (S. Udenfriend and J. Meienhofer, Eds), Vol. 9, Part C, p. 185. Academic Press, New York 1987.
8. Grandas A., Pedroso E., Figueras A., Rivera J., Giralt E.: Biomed. Environ. Mass. Spectrom. *15*, 681 (1988).
9. Roepsterff P., Fohlman J.: Biomed. Environ. Mass Spectrom. *11*, 601 (1984).
10. Lamthanh H., Roumestand Ch., Deprun C., Menez A.: Int. J. Pept. Protein Res. *41*, 85 (1993).
11. Prusik Z., Kasicka V., Mudra P., Stepanek J., Smekal O., Hlavacek J.: Electrophoresis *11*, 932 (1990).
12. Kasicka V., Prusik Z., Smekal O., Hlavacek J., Barth T., Weber G., Wagner H.: J. Chromatogr. *656*, 99 (1994).
13. Kaiser E., Colescott R. L., Bossinger C. D., Cook P. I.: Anal. Biochem. *34*, 595 (1970).