

## ENZYMATICALLY CATALYZED SYNTHESIS OF OXYTOCIN FRAGMENTS 1—6 AND 7—9\*

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Papain,  $\alpha$ -chymotrypsin, thermolysin and elastase were utilized in the synthesis of peptide bonds of the protected oxytocin nonapeptide, except the S-benzylcysteine-proline bond. Amino groups were protected with benzyloxycarbonyl or tert-butyloxycarbonyl groups, carboxy groups as ethyl ester, phenylhydrazides or amides. The cysteine sulfhydryl group was blocked with the benzyl group whereas the tyrosine hydroxyl was unprotected. Most of the fragments were synthesized in satisfactory yields using an equimolar ratio of both reaction components and minimal (experimentally determined) amount of the given enzyme.

Until recently, the synthesis of the peptide bond by means of proteolytic enzymes was studied<sup>1-4</sup> mainly concerning the selection of optimal reaction conditions, reaction mechanism or applicability of the individual enzymes to preparation of peptides from the chosen amino acid derivatives\*\*. Some of the results have been utilized in enzymatic resyntheses of human insulin (see *e.g.* ref.<sup>7</sup>). The use of proteinases in the synthesis of biologically active peptides has been described for fragment condensations of [Asn<sup>1</sup>, Val<sup>5</sup>] angiotensin II (ref.<sup>8</sup>) and analogs of enkephalin<sup>9</sup>. Recently, preparative-scale partially or fully enzymatic syntheses have been described of enkephalins<sup>10,11</sup>, dynorfin<sup>12</sup>, caerulein<sup>13</sup>, CCK-8 (ref.<sup>14</sup>), four tetrapeptide MSH fragments<sup>15</sup>, eledoisin<sup>16</sup>, substance P (ref.<sup>17</sup>) sequences and some fragments of mouse epidermal growth factor<sup>18</sup>. The thermolysin-catalyzed synthesis of aspartyl-phenylalanine methyl ester<sup>19</sup> is also attractive.

Interestingly enough, the biologically important neurohypophyseal hormones, oxytocin and vasopressin, have not been so far used as model compounds for enzymatically catalyzed syntheses. The only exception appears to be the synthesis of the oxytocin fragments, N-terminal tripeptide and tert-butyloxycarbonylasparaginyl-S-benzylcysteine phenylhydrazide<sup>20</sup>. In the present communication we describe enzymatically catalyzed synthesis of the protected N-terminal hexapeptide *Ia* and the

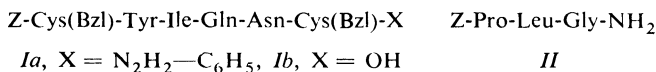
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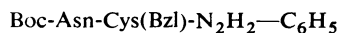
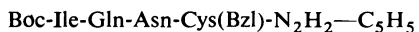
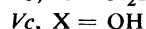
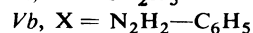
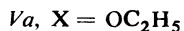
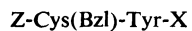
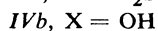
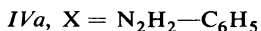
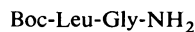
\*\* The amino acids used in this work are of the L-configuration. The nomenclature and symbols of the amino acids and peptides obey the published recommendations<sup>5,6</sup>.

C-terminal tripeptide *II*. At the present state of knowledge, these two fragments (after removal of the benzyloxycarbonyl group from *II* and conversion of *Ia* into *Ib* with aqueous ferric chloride<sup>21</sup>) cannot be coupled enzymatically and this condensation can be realized only by the already described classical organic synthesis<sup>22</sup>.

The amino-terminal hexapeptide was built from the dipeptide fragments *III*, *IVb*, and *Vc*. The dipeptide *III* was synthesized using papain, similarly as described in the literature<sup>20</sup>. The synthesis of the dipeptide *IVa* was successful only with elastase, starting from tert-butyloxycarbonylisoleucine methyl ester; however, neither an excess of the amino component nor a greater amount of the enzyme gave satisfactory yields. Glutamine is obviously an unsuitable substrate for the P<sub>1</sub>' position of common proteinases. The location of isoleucine in position P<sub>1</sub> also restricts the applicability of these enzymes. However, formation of the peptide bond between isoleucine and glutamine residues placed at the corresponding ends of two peptide fragments might give better results. The synthesis of *IVa* totally failed with papain; since commercial papain contains glutamine cyclotransferase<sup>23</sup>, this result is not surprising. The dipeptide *IVb* was prepared from the compound *IVa* using ferric chloride. The amino-terminal dipeptides *Va* and *Vb* were synthesized in the presence of papain, without protecting the tyrosine hydroxyl. With tyrosine ethyl ester as the amino component in a mixture of an acetate buffer and dimethylformamide we observed cleavage of the ester bond in the dipeptide. This cleavage was suppressed by working with ethanol instead of dimethylformamide. The dipeptide *Vc* was obtained either by alkaline hydrolysis of the compound *Va* or from the compound *Vb* by treatment with ferric chloride. No sulfoxide of the S-benzylcysteine residue was detected in the latter reaction. The tetrapeptide *VI* was prepared from the dipeptide *IVb* and compound *III* (after removal of the tert-butyloxycarbonyl group with trifluoroacetic acid) in the presence of papain. The tert-butyloxy carbonyl protecting group was again removed by treatment with trifluoroacetic acid and the tetrapeptide trifluoroacetate was condensed with the dipeptide *Vc* in good yield using thermolysin. Attempted condensation of the tetrapeptide with the dipeptide *Va* or *Vc* in the presence of chymotrypsin was unsuccessful.

Synthesis of the tripeptide *II* was based on condensation of benzyloxycarbonylproline with leucyl-glycine amide trifluoroacetate in the presence of thermolysin. Of the available enzymes only thermolysin catalyzes synthesis of the peptide bond from proline carboxyl group. Only two similar examples have been described so far<sup>24,25</sup>. The dipeptide *VII* was synthesized with chymotrypsin from tert-butyloxycarbonylleucine methyl ester. With four-fold molar excess of glycine amide hydrobromide an almost quantitative yield (according to HPLC analysis) of the product was achieved after two hours using the kinetic approach.



*III**VI**VII*

Enzymatic syntheses of peptides described in the literature use large amounts of the enzyme and often a substantial excess of one of the reaction components in order to achieve maximal preparative yields. In the present study we tried to find such experimental conditions which would lead to satisfactory yields with minimal amount of the enzyme and equimolar amount of both the reaction components. This goal has been achieved in the synthesis of the N-terminal hexapeptide using the thermodynamic approach. An excess of the amino component was necessary in the synthesis of the dipeptide *VII* in which the kinetic approach was used.

## EXPERIMENTAL

Melting points were determined on a Kofler block and are uncorrected. The reaction mixtures were evaporated on a rotatory evaporator at bath temperature 30°C. Analytical samples were dried over phosphorus pentoxide at room temperature and 150 Pa. Thin-layer chromatography (TLC) was performed on silica gel-coated sheets (Silufol, Kavalier) in the 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), 1-butanol-acetic acid-water (4 : 1 : 1) (S3), 1-butanol-pyridine-acetic acid-water (15 : 10 : 3 : 6) (S4). Electrophoresis was carried out in moist chamber on a paper Whatman 3MM at 20 V/cm for 1 h in 1 mol l<sup>-1</sup> acetic acid (pH 2.4) or in a pyridine-acetate buffer (pH 5.7). Compounds were detected with ninhydrin or by the chlorination method. Samples for amino acid analysis were hydrolyzed with 6 mol l<sup>-1</sup> HCl at 105°C for 20 h. The analyses were performed on an AAA 339 (Microtechna) or on a Durrum D-500 analyzers. Optical rotations were measured on a Perkin-Elmer 141 MCA polarimeter. High performance liquid chromatography (HPLC) was performed on a Spectra Physics SP 8700 instrument, equipped with an SP 8400 UV detector and an SP 4100 integrator, on a 15 × 0.4 cm column packed with Separon SIX C-18; flow rate 42 ml h<sup>-1</sup>, detection at 222 nm, mobile phase methanol with 0.05% aqueous trifluoroacetic acid (the amount of methanol in % (vol) is given as subscript at the *k'* values). The papain used was an Enzymase (Belgium) product, α-chymotrypsin and thermolysin were purchased from Serva and pancreatic elastase from Merck. The enzymatic reactions were monitored by HPLC in order to find the end and/or to follow side-reactions. Trifluoroacetates of the amino acid and peptide derivatives were prepared from the corresponding tert-butyloxycarbonyl compounds by treatment with trifluoroacetic acid and their homogeneity was checked by paper electrophoresis and HPLC.

### Benzyloxycarbonyl-S-benzylcysteinyl-tyrosine Ethyl Ester (*Va*)

Ethylenediaminetetraacetic acid (6 mg) and cysteine hydrochloride (10 mg) were added to a solution of benzyloxycarbonyl-S-benzylcysteine (691 mg) and tyrosine ethyl ester hydrochloride

(492 mg) in a mixture of ethanol (9 ml) and 0.2 mol l<sup>-1</sup> acetate buffer, pH 4.8 (11 ml). After adjusting to pH 4.8, papain (8.5 mg) was added and the mixture was incubated at 38°C for 24 h. The product was taken up in ethyl acetate and the organic extract was washed successively with 1 mol l<sup>-1</sup> HCl, water, 0.5 mol l<sup>-1</sup> NaHCO<sub>3</sub> and water. After drying over sodium sulfate and evaporation, the residue was crystallized from ethyl acetate–light petroleum, affording 440 mg (41%) of the product of m.p. 104°C. The mother liquors furnished further product (100 mg; 9%) of the same m.p..  $R_F$  0.89 (S1), 0.78 (S2), 0.85 (S3), 0.83 (S4);  $k'_{7.5} = 2.11$ .  $[\alpha]_D^{24}$  (c 0.6, chloroform). Amino acid analysis: Tyr 1.00, Cys(Bzl) 1.12. For C<sub>29</sub>H<sub>32</sub>N<sub>2</sub>O<sub>6</sub>S. 0.5 H<sub>2</sub>O (545.7) calculated 63.84% C, 6.09% H, 5.13% N; found: 64.19% C, 5.85% H, 4.95% N. Reported<sup>26</sup> m.p. 103–104°C,  $[\alpha]_D^{26}$  (c 3.58, chloroform).

#### Benzoyloxycarbonyl-S-benzylcysteinyl-tyrosine Phenylhydrazide (Vb)

Ethylenediaminetetraacetic acid (3 mg) and cysteine hydrochloride (10 mg) were added to a solution of benzoyloxycarbonyl-S-benzylcysteine (346 mg) and tyrosine phenylhydrazide trifluoroacetate (390 mg) in a mixture of dimethylformamide (4.5 ml) and 0.2 mol l<sup>-1</sup> acetate buffer, pH 4.8 (5.5 ml). The mixture was adjusted to pH 4.8 and after addition of papain (21 mg) incubated at 38°C for 24 h. The precipitate was collected on a filter, washed with water, 1 mol l<sup>-1</sup> HCl, water, 0.5 mol l<sup>-1</sup> NaHCO<sub>3</sub>, again with water and dried, affording 310 mg (52%) of the product, m.p. 198–199°C.  $R_F$  0.90 (S1), 0.79 (S2), 0.84 (S3), 0.82 (S4);  $k'_{7.5} = 1.84$ . An analytical sample was crystallized from methanol, m.p. 198°C,  $[\alpha]_D -26.6^\circ$  (c 0.25, dimethylformamide). Amino acid analysis: Tyr 1.00, Cys(Bzl) 0.88. For C<sub>33</sub>H<sub>34</sub>N<sub>4</sub>O<sub>5</sub>S.0.5H<sub>2</sub>O (607.7) calculated: 65.22% C, 5.80% H, 9.22% N; found: 65.24% C, 5.45% H, 9.04% N.

#### Benzoyloxycarbonyl-S-benzylcysteinyl-tyrosine (Vc)

A solution of ferric chloride (3 g) in water (10 ml) was added at 35°C to a rigorously stirred suspension of benzoyloxycarbonyl-S-benzylcysteinyl-tyrosine phenylhydrazide (300 mg) in dioxane (10 ml). The mixture was stirred at 35°C for 30 min and made alkaline with 2 mol l<sup>-1</sup> NaOH. The precipitated ferric hydroxide was removed by centrifuging and the supernatant was taken down and acidified with 3 mol l<sup>-1</sup> HCl. The product was taken up in ethyl acetate and the extract was washed with water, dried over sodium sulfate and taken down. The residue was crystallized from ethyl acetate, affording 190 mg (75%) of the product, m.p. 196°C.  $R_F$  0.89 (S1), 0.54 (S2), 0.88 (S3), 0.73 (S4);  $k'_{6.5} = 3.67$ .  $[\alpha]_D -16.8^\circ$  (c 0.5, methanol). Amino acid analysis: Tyr 1.00, Cys(Bzl) 0.95. For C<sub>27</sub>H<sub>28</sub>N<sub>2</sub>O<sub>6</sub>S (508.6) calculated: 63.73% C, 5.55% H, 5.51% N; found: 63.66% C, 5.59% H, 5.40% N. Reported<sup>26</sup> m.p. 199–201°C,  $[\alpha]_D -17^\circ$  (c 3.9, pyridine). Oxidative cleavage gave a product free of the corresponding sulfoxide (according to HPLC analysis<sup>27</sup>). The dipeptide Vc was prepared also according to the literature<sup>26</sup> by alkaline hydrolysis of the corresponding ester.

#### Tert-butyloxycarbonylasparaginyl-S-benzylcysteine Phenylhydrazide (III)

Ethylenediaminetetraacetic acid (3 mg) and cysteine hydrochloride (10 mg) were added to a solution of tert-butyloxycarbonylasparagine (235 mg) and S-benzylcysteine phenylhydrazide trifluoroacetate (416 mg) in a mixture of ethanol (1 ml) and 0.2 mol l<sup>-1</sup> acetate buffer, pH 4.8 (9 ml). After adjusting to pH 4.8 and addition of papain (42 mg), the mixture was incubated at 38°C for 24 h. The precipitate was filtered, washed with water, an HSO<sub>4</sub><sup>-</sup> buffer (pH 2), water, 0.5 mol l<sup>-1</sup> NaHCO<sub>3</sub>, again with water, and dried. This product (400 mg; 78%), m.p. 203–204°C was recrystallized from ethyl acetate; yield 375 mg (73%) of the title compound, m.p. 202–203°C,

$R_F$  0.83 (S1), 0.71 (S2), 0.81 (S3), 0.79 (S4);  $k'_{65} = 4.31$ .  $[\alpha]_D - 37.8^\circ$  ( $c$  0.3, dimethylformamide). Amino acid analysis: Asp 1.00, Cys(Bzl) 0.94. For  $C_{25}H_{33}N_5O_5S$  (515.6) calculated: 58.23% C, 6.45% H, 13.58% N; found: 57.83% C, 6.20% H, 13.80% N. Reported<sup>20</sup> m.p. 197–199°C,  $[\alpha]_D - 40.2^\circ$  ( $c$  2, dimethylformamide).

#### Tert-butyloxycarbonylisoleucyl-glutamine Phenylhydrazide (IVa)

*A) Enzymatically:* Calcium chloride (0.5 mg) was added to a solution of tert-butyloxycarbonylisoleucine methyl ester (10 mg) and glutamine phenylhydrazide trifluoroacetate (70 mg) in a mixture of dimethylformamide (50  $\mu$ l) and 0.2 mol l<sup>-1</sup> carbonate-bicarbonate buffer, pH 10.5 (430  $\mu$ l). The solution was adjusted to pH 10 with 2 mol l<sup>-1</sup> NaOH and after addition of elastase (0.5 mg) incubated at 38°C for 24 h. The precipitate was filtered, washed with water, an HSO<sub>4</sub><sup>-</sup> buffer pH 2, water, 0.5 mol l<sup>-1</sup> NaHCO<sub>3</sub>, again with water and dried, affording 1.4 mg (8%) of the product, m.p. 212°C.  $R_F$  0.71 (S1), 0.66 (S2), 0.70 (S3), 0.74 (S4);  $k'_{65} = 2.44$ .  $[\alpha]_D - 15.2^\circ$  ( $c$  0.2, dimethylformamide). Amino acid analysis: Glu 1.00, Ile 0.88.

*B) Chemically:* A solution of glutamine phenylhydrazide trifluoroacetate (710 mg) in dimethylformamide (8 ml) was made alkaline with N-ethylpiperidine. After addition of tert-butyloxycarbonylisoleucine pentafluorophenyl ester (1.06 g) the mixture was stirred for 20 h at room temperature. Dimethylformamide was evaporated, the residue was triturated with ether, washed with ether and water and processed further as described above. Yield 527 mg (59%) of product melting at 214–216°C, chromatographically identical with that prepared by procedure A;  $[\alpha]_D - 14.7^\circ$  ( $c$  0.3, dimethylformamide). For  $C_{22}H_{25}N_5O_5$  (449.6) calculated: 58.77% C, 7.85% H, 15.58% N; found: 58.39% C, 7.76% H, 15.12% N.

#### Tert-butyloxycarbonylisoleucyl-glutamine (IVb)

A solution of ferric chloride (1.8 g) in water (6 ml) was added to a suspension of tert-butyloxycarbonylisoleucyl-glutamine phenylhydrazide (180 mg) in dioxane (10 ml) and the mixture was stirred at 35°C for 30 min. After addition of 2 mol l<sup>-1</sup> NaOH (11 ml), the ferric hydroxide was removed by centrifuging. Dioxane was evaporated, the aqueous residue was acidified with cold 1 mol l<sup>-1</sup> HCl and the product was taken up in ethyl acetate. The organic solution was dried and evaporated and the residue was crystallized from ethyl acetate–light petroleum to give 110 mg (76%) of the product m.p. 108–109°C.  $R_F$  0.74 (S1), 0.13 (S2), 0.69 (S3), 0.66 (S4);  $k'_{65} = 1.02$ ;  $[\alpha]_D - 23.2^\circ$  ( $c$  0.3; methanol). Amino acid analysis: Glu 1.00, Ile 0.95. For  $C_{16}H_{29}N_3O_6$  (359.4) calculated: 53.47% C, 8.13% H, 11.69% N; found: 53.68% C, 8.05% H, 11.39% N.

#### Tert-Butyloxycarbonylisoleucyl-glutaminy-l-asparaginy-l-S-benzylcysteine Phenylhydrazide (VI)

Ethylenediaminetetraacetic acid (1 mg) and cysteine hydrochloride (3 mg) were added to a solution of tert-butyloxycarbonylisoleucyl-glutamine (108 mg) and asparaginy-l-S-benzylcysteine phenylhydrazide trifluoroacetate (190 mg) in a mixture of ethanol (0.9 ml) and 0.2 mol l<sup>-1</sup> acetate buffer, pH 5.8 (2.1 ml). Papain (12.5 mg) was added to this solution at pH 4.9 and the mixture was incubated for 20 h at 38°C. The precipitate was filtered, washed with water, an HSO<sub>4</sub><sup>-</sup> buffer (pH 2), water, 0.5 mol l<sup>-1</sup> NaHCO<sub>3</sub>, water and dried, affording 198 mg (87%) of the product, m.p. 236–237°C.  $R_F$  0.65 (S1), 0.54 (S2), 0.69 (S3), 0.78 (S4);  $k'_{65} = 7.64$ . An analytical sample was crystallized from methanol–ether; m.p. 238°C;  $[\alpha]_D - 40.1^\circ$  ( $c$  0.3, dimethylformamide). Amino acid analysis: Asp 1.00, Glu 0.94, Ile 0.92, Cys(Bzl) 1.03. For  $C_{36}H_{52}N_8O_8S \cdot 0.5 H_2O$  (756.9) calculated: 56.45% C, 6.97% H, 14.63% N; found: 56.09% C, 6.73% H, 14.32% N.

Benzyloxycarbonyl-S-benzylcysteinyl-tyrosyl-isoleucyl-glutaminy-l-asparaginy-l-S-benzylcysteine Phenylhydrazide (*Ia*)

Calcium chloride (1 mg) was added to a solution of benzyloxycarbonyl-S-benzylcysteinyl-tyrosine (51 mg) and isoleucyl-glutaminy-l-asparaginy-l-S-benzylcysteine phenylhydrazide trifluoroacetate ( $E_{\text{Gly}}^{2,4} = 0.67$ ,  $E_{\text{His}}^{5,7} = 0.41$ ;  $k'_{65} = 1.51$ ; 78 mg) in a mixture of dimethylformamide (1.5 ml) and  $0.2 \text{ mol l}^{-1}$  Tris-maleate buffer, pH 7, (3.5 ml). The solution was adjusted to pH 7 and after addition of thermolysin (2.5 mg) incubated at  $38^\circ\text{C}$  for 24 h. The precipitated solid was filtered, washed with water,  $1 \text{ mol l}^{-1}$  HCl, water,  $0.5 \text{ mol l}^{-1}$   $\text{NaHCO}_3$ , water and dried, affording 100 mg (86.9%) of crude product, m.p.  $261\text{--}264^\circ\text{C}$  which was further extracted with methanol and ether. Yield 83 mg (72%) of the title compound, melting at  $262^\circ\text{C}$ ;  $[\alpha]_{\text{D}} -37.9^\circ$  ( $c$  0.3, dimethylformamide). Amino acid analysis: Asp 1.14, Glu 1.09, Ile 1.00, Tyr 0.97, Cys(Bzl) 1.90. For  $\text{C}_{58}\text{H}_{70}\text{N}_{10}\text{O}_{11}\text{S}_2 \cdot 2 \text{H}_2\text{O}$  (1183) calculated: 58.87% C, 6.30% H, 11.84% N; found: 58.58% C, 5.98% H, 11.84% N.

Benzyloxycarbonyl-S-benzylcysteinyl-tyrosyl-isoleucyl-glutaminy-l-asparaginy-l-S-benzylcysteine (*Ib*)

A solution of ferric chloride (450 mg) in water (1.5 ml) was added to a stirred solution of benzyloxycarbonyl-S-benzylcysteinyl-tyrosyl-isoleucyl-glutaminy-l-asparaginy-l-S-benzylcysteine phenylhydrazide (60 mg) in dimethylformamide (4 ml). After stirring for 30 min at  $35^\circ\text{C}$  the mixture was diluted with dimethylformamide (4 ml) and  $2 \text{ mol l}^{-1}$  NaOH (2.5 ml) was added. The precipitated ferric hydroxide was separated by centrifuging and the supernatant was evaporated at  $30^\circ\text{C}$ . The residue was triturated with ether, washed with water,  $1 \text{ mol l}^{-1}$  HCl and again water, dried and washed with ether. The obtained crude product (39 mg) was washed with boiling methanol and then with cold methanol and ether, affording 28 mg (51%) of *Ib*, m.p.  $225\text{--}226^\circ\text{C}$ ;  $[\alpha]_{\text{D}} -33.2^\circ$  ( $c$  0.3; dimethylformamide). Amino acid analysis: Asp 1.09, Glu 1.07, Ile 1.00, Tyr 0.96, Cys(Bzl) 1.87. Reported<sup>22</sup> m.p.  $226\text{--}227^\circ\text{C}$ ;  $[\alpha]_{\text{D}} -32.4^\circ$  ( $c$  0.3; dimethylformamide).

Tert-butyloxycarbonylleucyl-glycine Amide (*VII*)

$\alpha$ -Chymotrypsin (33 mg) was added at pH 10 to solution of tert-butyloxycarbonylleucine methyl ester (750 mg) and glycine amide hydrobromide (1.86 g) in a mixture of dimethylformamide (3 ml) and  $0.2 \text{ mol l}^{-1}$  carbonate-bicarbonate buffer (pH 10; 27 ml). After stirring for 1 h at  $25^\circ\text{C}$ , the reaction was quenched by rapid cooling and cautious acidification with  $1 \text{ mol l}^{-1}$  HCl to pH 3.5. The product was taken up in ethyl acetate, the extract was washed with an  $\text{HSO}_4^-$  buffer (pH 2), water, and evaporated. The residue was dissolved in 50% aqueous methanol, containing 10% dimethylformamide, and the solution was filtered through a column of Amberlite IR-4B (10 ml) in the same solvent mixture. The filtrate was evaporated and the residue triturated and then washed with light petroleum to give 425 mg (49%) of the product, m.p.  $58\text{--}60^\circ\text{C}$ .  $R_{\text{F}}$  0.66 (S1), 0.56 (S2), 0.63 (S3), 0.69 (S4);  $k'_{65} = 1.28$ ;  $[\alpha]_{\text{D}} -10.4^\circ$  ( $c$  0.3; methanol). Amino acid analysis: Gly 1.00, Leu 1.01. For  $\text{C}_{13}\text{H}_{25}\text{N}_3\text{O}_4$  (287.4) calculated: 54.32% C, 8.77% H, 14.62% N; found: 53.80% C, 8.64% H, 14.11% N.

Benzyloxycarbonylprolyl-leucyl-glycine Amide (*II*)

Calcium chloride (0.5 mg) was added to a solution of benzyloxycarbonylproline (100 mg) and leucyl-glycine amide trifluoroacetate (65 mg) in a mixture of dimethylformamide (0.2 ml) and  $0.2 \text{ mol l}^{-1}$  Tris-maleate buffer, pH 7 (1.8 ml). After adjusting to pH 7 with  $4 \text{ mol l}^{-1}$  NaOH

and addition of thermolysin (4 mg), the mixture was incubated at 38°C for 2 h. The product was extracted several times with ethyl acetate and the combined extracts were taken down. The residue was dissolved in 30% methanol and filtered successively through a column of Dowex-50 (2 ml) and Amberlite IR-4B (2 ml). The filtrate was taken down and the residue was triturated with ether, affording 37 mg (43% based on trifluoroacetate of the dipeptide amide) of the product, m.p. 162–163°C;  $[\alpha]_D -68.15^\circ$  (c 0.3; ethanol). The product was chromatographically (TLC, HPLC) identical with the compound prepared by the classical procedure. Reported m.p. 165 to 165.5°C,  $[\alpha]_D -70^\circ$  (c 0.2, ethanol) (ref.<sup>28</sup>), m.p. 162–163°C (ref.<sup>29</sup>) and 163–163.5°C (ref.<sup>30</sup>).

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