162. N-Carbobenzoxy-L-pyroglutamyl-L-histidyl Peptides

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Zusammenfassung. N-Benzyloxycarbonyl-L-pyroglutamyl-L-histidyl-peptide reagieren mit Methanol unter Bildung von N-Benzyloxycarbonyl-L-(0^5-methyl)glutamyl-L-histidyl-peptiden (Hauptprodukte) und L-Pyroglutamyl-L-histidyl-peptiden (Nebenprodukte), wenn die basische Histidin-Seitenkette nicht protoniert ist. Solche basenkatalysierte Methanolysen wurden an einem Di- und an einem Tripeptid-Amid beobachtet. Die entsprechende Äthanolyse verläuft wesentlich langsamer.

The thyrotropin-releasing hormone (TRH) isolated from ovine, bovine, and porcine *hypothalami* was shown to be L-pyroglutamyl-L-histidyl-L-prolinamide (**4**) (for recent reviews see [1] [2])[1]. During its structure elucidation **4** was prepared by synthesis [3] [4]. Later synthetic work (see *e.g.* [5]) was undertaken to make TRH available in sufficient quantities for biological studies.

It appears that in all the reported syntheses chromatographic purifications were required to render the TRH (**4**) sufficiently pure for biological and chemical studies. TRH is an amorphous solid which cannot be purified by crystallization. Therefore an attempt was made to develop a TRH synthesis employing a protected tripeptide which could be rigorously purified, if possible by crystallization, and would allow liberation of **4** quantitatively thus eliminating the purification of the TRH made in this manner. The Z-L-pyroglutamyl-L-histidyl-L-prolinamide (**3**)[2] was shown to satisfy the above requirements[3].

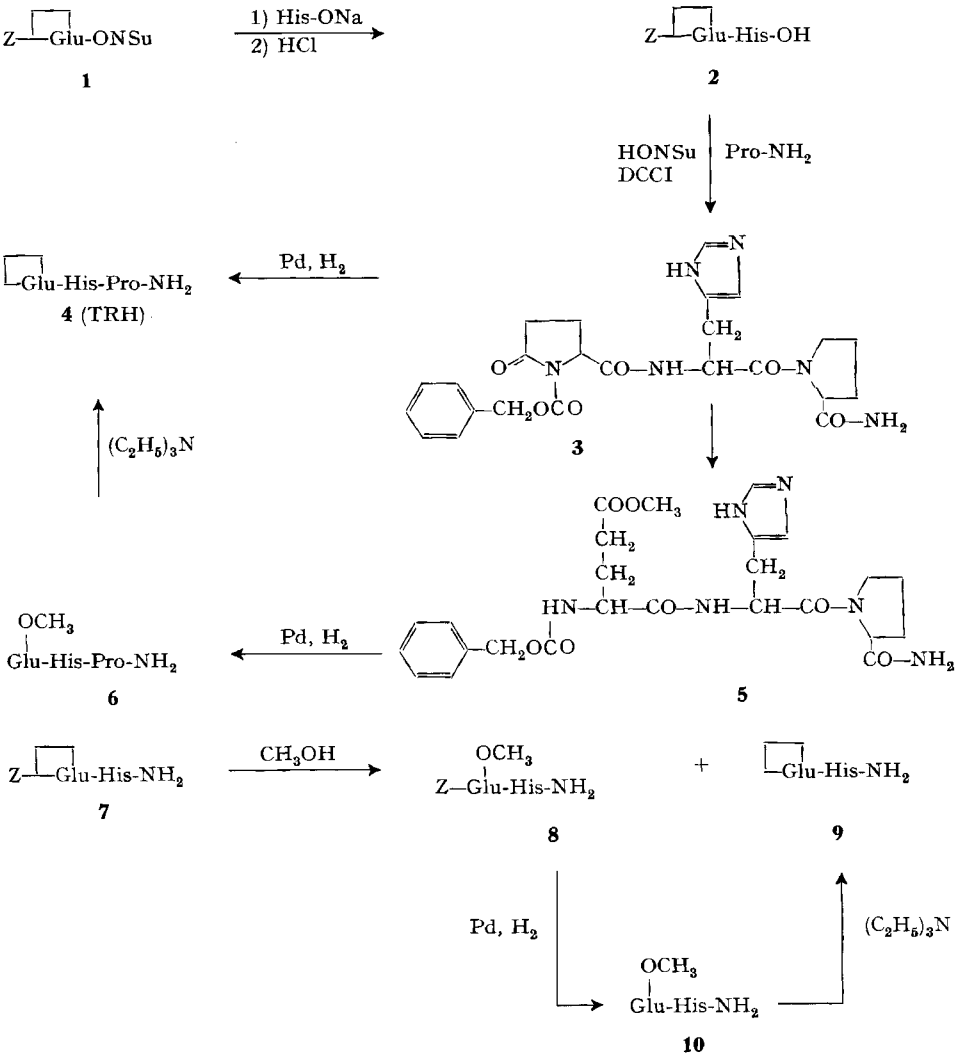
The conventional synthetic approach to prepare **3** starting with L-prolinamide would have to proceed through the Boc-L-histidyl-L-prolinamide. *Flouret* [5] showed this intermediate to be an oil which was purified by chromatography. For this reason the present synthesis was approached from the N-terminus. The known Z-L-pyroglutamic acid [6] was esterified with N-hydroxy-succinimide (HONSu) in the presence of dicyclohexylcarbodiimide (DCCI) to give the active ester **1**. The latter (**1**), upon reacting with the sodium salt of L-histidine and subsequent acidification of the reaction mixture, gave rise to the crystalline Z-L-pyroglutamyl-L-histidine (**2**). Coupling of **2** with prolinamide in the presence of DCCI and HONSu afforded the crystalline tripeptide **3**. Hydrogenolysis of **3** led to the desired pure TRH (**4**) in almost quantitative yield. No further purification of the TRH (**4**), prepared in this manner, was necessary. In contrast to the hydrogenolysis of **3**, the reduction of Z-L-pyroglutamyl-

[1] It has been suggested that human TRH also has this structure [1].

[2] The abbreviations Z for the benzyloxycarbonyl group and Boc for the *t*-butoxycarbonyl group will be used.

[3] *M. Fujino, S. Kobayashi, T. Fukuda, M. Obayashi & S. Shinagawa*, Takeda Chemical Industries, Ltd., Osaka, Japan, informed us in a private communication of a similar approach in their laboratories.

N^{1m}-benzyl-L-histidyl-L-prolinamide gave, after chromatography of the reduction product, pure TRH (**4**) in 68% yield [7].



Prior to the finding that **3** could be obtained by direct crystallization from the crude coupling mixture the latter was purified by column chromatography on silica gel. Elution of the column with methanol/chloroform gave, in addition to the expected product **3**, two additional compounds formed during the purification process. The first substance which was less polar than **3** was formulated as Z-L-(0^5-methyl)-glutamyl-L-histidyl-L-prolinamide (**5**) on the basis of spectroscopic and analytical data. The second more polar compound was shown to be TRH (**4**). The above observation could be explained by the reaction of pure **3** with methanol at room temperature to

give **4** and **5** in 12% and 85% yields, respectively. Chemical proof for the structural assignment of **5** was obtained when it was shown that its unstable hydrogenolysis product **6** reacted in the presence of base [4] to form TRH (**4**).

That the above discussed methanolysis was not peculiar to **3** was demonstrated when it was found that Z-L-pyroglutamyl-L-histidinamide (**7**), prepared by condensation of **1** with L-histidinamide, was converted on standing in a methanol solution to Z-L-(0^5-methyl)glutamyl-L-histidinamide (**8**) and L-pyroglutamyl-L-histidinamide (**9**). Hydrogenolysis of **8** afforded the unstable amino-ester **10** which could be cyclized to **9** on treatment with base [4].

The methanolyses of **3** to yield **4** and **5** and of **7** to give **8** and **9** are contrasted by the stability of Z-L-pyroglutamyl-L-histidine (**2**) in methanol containing solutions. The stability of **2** may be explained by internal salt formation between the carboxylic acid group and the imidazole ring system, thereby effectively lowering the basicity of the histidine side chain. Internal neutralization of the basic side chain in the amides **3** and **7** is not possible. Therefore it was postulated that the methanolysis of **3** and **7** was base catalyzed by the imidazole ring system present in both compounds. Indeed **3** was stable in methanol containing a small amount of acetic acid for a period of several days. The sensitivity of Z-L-pyroglutamyl peptides to external bases was demonstrated by the work of *Gibian & Klieger* [6].

The above findings led us to avoid any contact of the TRH-precursor **3** with methanol. It was found that the analogous alcoholysis with ethanol was sufficiently slow that **3** could be readily recrystallized from ethanol. The hydrogenolysis of **3** to form **4** was carried out in aqueous tetrahydrofuran to avoid alcoholysis.

The authors are indebted to Mr. *W.H. Washburn* and his staff for IR. spectra, to Dr. *R.S. Egan* and Mrs. *Ruth Stanaszek* for NMR. spectra, to Mrs. *Julie Hood* for microanalyses, and to Messrs. *D.A. Dunnigan & J.B. Holland* for catalytic hydrogenations. We wish to thank Drs. *W. Cole & J. Tadanier* for stimulating discussions during the course of this investigation.

Experimental Part

General Remarks. The m.p. were determined on a *Fisher-Johns* melting point apparatus. Optical rotations were measured with a *Hilger & Watts* polarimeter using solutions of N,N-dimethylformamide (DMF) or acetic acid (AcOH), and the IR. spectra were obtained with a *Perkin-Elmer* Model 421 grating spectrophotometer and KBr pellets. The NMR. spectra were determined at 100 MHz with a *Varian* HA-100 spectrometer employing deuterioacetic acid (AcOH-d_4) and trifluoroacetic acid (TFA) as solvents. Chemical shifts were reported in ppm δ from internal tetramethylsilane (δ = O); δ-values for multiplets refer to the center of the observed peaks. Thin layer chromatography (tlc.) was done on silica gel G plates which were developed with chloroform/methanol 2:1 and 1:2; the spots were detected with the chlorine/*o*-toluidine system.

Z-L-Pyroglutamic Acid N-Hydroxysuccinimide Ester (**1**). To a stirred and ice-acetone cooled solution of 15.79 g (60 mmol) of Z-L-pyroglutamic acid [6] and 7.59 g (66 mmol) of HONSu in 60 ml of 1,2-dimethoxyethane were added 13.61 g (66 mmol) of DCCI. The reaction mixture was stirred in the cold for 2 h and then at room temperature overnight. The precipitated dicyclohexylurea was removed by filtration and washed with two small portions of 1,2-dimethoxyethane. The combined filtrates were concentrated under reduced pressure to a viscous oil which was crystallized from about 100 ml of 2-propanol. The crystals were collected on a filter, washed with 2-propanol and ether, and dried to give 16.43 g (76% yield) of **1**, m.p. 131–132°; $[\alpha]_D^{25} = -23°$ (c = 1.80, DMF); $\tilde{\nu}_{max}$($CHCl_3$) 1820, 1796, 1744 cm^{-1}; NMR. (AcOH-d_4): 7.39 (*m*, Z-Ar), 5.28 (AB-*q*, Z-CH_2), 5.11 (*m*, α-CH pGlu), 2.88 (*s*, CH_2CH_2 Su), 2.2–3.0 ppm (*m*, β, γ-CH_2 pGlu).

$C_{17}H_{16}N_2O_7$ (360.314) Calc. C 56.67 H 4.47 N 7.77% Found C 56.57 H 4.52 N 7.73%

Z-L-Pyroglutamyl-L-Histidine (**2**). A solution of 13.51 g (37.5 mmol) of the active ester **1** in 25 ml of dioxane was added to a stirred solution of L-histidine sodium salt, prepared from 6.42 g (41.25 mmol) of L-histidine and 5.12 g (41.25 mmol) of sodium carbonate pentahydrate in 50 ml of water. The reaction mixture was stirred at room temperature for 4 h and concentrated to a volume of 30 ml under reduced pressure at a bath temperature below 40°. The solution was neutralized with 86.5 ml of 0.957 N hydrochloric acid and concentrated to about 20 ml. The resulting precipitate was collected on a filter, washed with two 10-ml portions of ice-water and recrystallized from 30% aqueous methanol, to yield, after drying, 9.40 g (62% yield) of white needles. This substance (**2**) did not melt but started to decompose at 149°; $[\alpha]_D^{25} = -1°$ ($c = 1.08$, DMF); $\tilde{\nu}_{max}$ 1796, 1674, 1630, 1590 cm^{-1}; NMR. (AcOH-d_4): 8.62 (*d*, 4-His Ar), 7.34 (*m*, Z-Ar), 7.28 (*d*, 2-His Ar), 5.26 (*s*, Z-CH_2), 4.85 (*m*, α-CH His and pGlu), 3.25 (*m*, β-CH_2 His), 2.2–2.8 ppm (*m*, β,γ-CH_2 pGlu).

$C_{19}H_{20}N_4O_6 \cdot 1^1/_2\ H_2O$	Calc.	C 53.39	H 5.42 N 13.11%
(427.406)	Found	,, 53.95	,, 5.53 ,, 13.24%

Z-L-Pyroglutamyl-L-Histidyl-L-Prolinamide (**3**). A solution of 1.005 g (2.48 mmol) of **2** and 0.342 g (3 mmol) of L-prolinamide, obtained from L-prolinamide hydrochloride by treatment with Rexyn® 201 (OH), in 7 ml of DMF, was cooled in an ice-bath; 0.578 g (2.8 mmol) of DCCI and 0.5 ml of DMF were added, stirring was started and continued overnight as the reaction mixture attained room temperature. The precipitated dicyclohexylurea was collected on a filter and washed with three 1-ml portions of DMF. The combined filtrates were added dropwise, with stirring, to 200 ml of ether. The supernatant was decanted and the residue dissolved in 10 ml. of ethanol which was added to 190 ml of ether. The white powder obtained was collected and crystallized from ethanol to yield 0.459 g of the desired peptide **3**, m.p. 184–186°. The combined ethanolic mother liquors (from the ether precipitation and recrystallization) were evaporated to dryness to leave a residue of 1.070 g which was purified by chromatography on 40 g of silica gel. Elution of the column with two 100-ml portions of ethanol/chloroform 2:3 removed the nonpolar impurities. The desired peptide was then eluted with three 100-ml portions of ethanol/chloroform 3:2. After evaporation of the solvent and crystallization of the residue from ethanol an additional 0.328 g of **3** was obtained, m.p. 180–183°; the total yield was 63%.

An analytical sample had the following physical constants: m.p. 186–187°; $[\alpha]_D^{25} = -43°$ ($c = 1.02$, DMF); $\tilde{\nu}_{max}$ 1783, 1680, 1622 cm^{-1}; NMR. (AcOH-d_4): 8.69 (*d*, 4-His Ar), 7.35 (*m*, Z-Ar and 2-His Ar), 5.26 (*s*, Z-CH_2), 5.15 (*m*, α-CH His), 4.82 and 4.56 (*m*, α-CH pGlu, Pro), 3.70 (*m*, δ-CH_2 Pro), 3.21 (*m*, β-CH_2 His), 1.8–2.8 ppm (*m*, β,γ-CH_2 pGlu, Pro).

$C_{24}H_{28}N_6O_6 \cdot {}^1/_2\ H_2O$	Calc.	C 57.02	H 5.78 N 16.63%
(505.520)	Found	,, 56.82	,, 5.74 ,, 16.75%

L-Pyroglutamyl-L-Histidyl-L-Prolinamide (**4**). A solution of 1.000 g (1.98 mmol) of **3**-hemihydrate in 50 ml of tetrahydrofuran and 50 ml of water was hydrogenolyzed over 0.200 g of palladium black. The catalyst was collected on a filter and washed with several portions of tetrahydrofuran/water 1:1. The filtrate was concentrated under reduced pressure at a bath temperature below 35°, to about 20 ml. The concentrated aqueous solution was filtered through a Millipore® filter and the filter was rinsed with two 10-ml portions of water. The resulting solution was lyophilized to leave 0.704 g (98% yield) of pure TRH (**4**): $[\alpha]_D^{25} = -47°$ ($c = 1.00$, AcOH); the Rf-values in several tlc.-systems as well as the IR. and NMR. spectra were identical to those of a reference sample of TRH (**4**) [5].

Methanolysis of Z-L-Pyroglutamyl-L-Histidyl-L-Prolinamide (**3**). A solution of 2.002 g (3.96 mmol) of **3**-hemihydrate in 200 ml of methanol was allowed to stand at room temperature for 9 days. The starting material was quantitatively converted to yield two new compounds (tlc.). The mixture obtained after evaporation of the solvent, 2.101 g, was separated by chromatography on 60 g of silica gel.

The residues of *Z-L-(O⁵-methyl)glutamyl-L-histidyl-L-prolinamide* (**5**) (1.782 g, 85% yield) obtained after the evaporation of the chloroform/methanol 4:1 eluates were shown to be pure by tlc., but could not be induced to crystallize. A dry sample had $[\alpha]_D^{25} = -22°$ ($c = 1.06$, DMF); $\tilde{\nu}_{max}$ 1720, 1675, 1640 cm^{-1}; NMR. (AcOH-d_4): 8.72 (*d*, 4-His Ar), 7.40 (*d*, 2-His Ar), 7.34

(*m*, Z-Ar), 5.17 (*m*, α-CH His), 5.13 (*s*, Z-CH_2), 4.60 and 4.32 (*m*, α-CH Glu Pro), 3.75 (*m*, δ-CH_2 Pro), 3.64 (*s*, OCH_3), 3.28 (*m*, β-CH_2 His), 1.8–2.7 ppm (*m*, β,γ-CH_2 Glu and Pro).

$C_{25}H_{32}N_6O_7$ (528.554) Calc. C 56.81 H 6.10 N 15.90% Found C 56.72 H 6.14 N 15.95%

Evaporation of the chloroform/methanol 1:2 eluates left a residue of 0.165 g (12% yield) of *L-pyroglutamyl-L-histidyl-L-prolinamide* (**4**) which was shown to be pure by tlc. The substance was dissolved in 20 ml of water; the solution was filtered through a Millipore® filter and lyophilized. A residue of 0.142 g of **4** was obtained, $[\alpha]_D^{25} = -48°$ ($c = 1.02$, AcOH); the Rf-values in several tlc.-systems, the IR. and NMR. spectra were identical to those of reference sample of TRH (**4**).

L-(O⁵-Methyl)glutamyl-L-histidyl-L-prolinamide (**6**) *dihydrochloride.* A solution of 0.448 g (0.85 mmol) of **5** in 50 ml of methanol containing 1.65 ml (1.7 mmol) of 1.03N hydrochloric acid was hydrogenolyzed over 0.450 g of palladium black for 90 min. The catalyst was collected on a filter and washed with several small portions of methanol. The solution was evaporated under reduced pressure to leave a residue of 0.393 g which was dissolved in 20 ml of methanol. The methanolic solution was added to 500 ml of ether with stirring to give a white precipitate which was collected on a filter, washed with several small portions of ether and dried. The residue amounted to 0.341 g (86% yield) of **6** dihydrochloride which decomposed between 195 and 200° and showed $[\alpha]_D^{26} = -29°$ ($c = 1.10$, H_2O); $\tilde{\nu}_{max}$ 1730, 1675, 1640 cm^{-1}, NMR. (TFA): 8.64 (*m*, 4-His Ar), 7.64 (*m*, 2-His Ar), 5.45 (*m*, α-CH His), 4.75 (*m*, α-CH Glu, Pro), 3.95 (*m*, δ-CH_2 Pro), 3.85 (*s*, OCH_3), 3.57 (*m*, β-CH_2 His), 2.83 (*m*, γ-CH_2 Glu), 1.5–2.7 ppm (*m*, β-CH_2 Glu β- and γ-CH_2 Pro).

$C_{17}H_{26}N_6O_5 \cdot 2\,HCl$ (467.356) Calc. C 43.69 H 6.04 N 17.98%
Found ,, 43.48 ,, 6.10 ,, 17.92%

L-(O⁵-Methyl)glutamyl-L-histidyl-L-prolinamide (**6**) *acetate.* A solution of 0.791 g (1.5 mmol) of **5** in 99 ml of methanol and 1 ml of acetic acid was hydrogenolyzed over 0.80 g of palladium black. The catalyst was collected on a filter and washed with several small portions of methanol. The combined methanolic solution was evaporated and the residue was dissolved in a total of 20 ml of water. The solution was filtered through a Millipore® filter and lyophilized to leave a residue of 0.704 g of substance. The NMR. spectrum revealed that the compound was an acetate salt, and the tlc. showed that the substance contained a small amount of TRH (**4**)[4]).

L-Pyroglutamyl-L-histidyl-L-prolinamide (**4**). A solution of 0.291 g of the above prepared **6** acetate salt in 15 ml of dioxane and 15 ml of water containing 0.141 g of triethylamine (pH ~8.5) was allowed to react at room temperature overnight. Evaporation of the solvent under reduced pressure left a residue of 0.409 g which was purified by chromatography on 20 g of silica gel. The residues, obtained by evaporation of the chloroform/methanol 1:2 eluates containing the desired substance **4** in pure form (tlc.), were dissolved in a total of 15 ml of water, filtered through a Millipore® filter, and lyophilized to leave a residue of 0.238 g of **4** acetate salt. The salt character of the substance was evident from both the NMR. (δ 2.27 ppm) and the IR. (shoulders at about 1600 cm^{-1}) spectra; $[\alpha]_D^{25} = -44°$ ($c = 1.06$, AcOH) (*cf.* [4]). Treatment of 0.221 g of the above salt with 1 g of Rexyn® 201 (OH) in methanolic solution and extraction of the resin with several small portions of methanol led, after evaporation of the solvent, to the isolation of a residue (0.228 g) which was dissolved in a total of 12 ml of water, filtered through a Millipore® filter, and lyophilized to leave 0.195 g of pure **4**, $[\alpha]_D^{26} = -47°$ ($c = 1.01$, AcOH). The identity of this substance was confirmed by the NMR. and IR. spectra.

Z-L-Pyroglutamyl-L-histidinamide (**7**). A solution of 1.246 g of L-histidinamide, liberated from 1.703 g (7.5 mmol) of L-histidinamide dihydrochloride in 7 ml of water and 20 ml of methanol by treatment with 21 g of Rexyn® 201 (OH) in the usual manner, and 2.620 g (7.27 mmol) of the active ester **1** in 15 ml of DMF was stirred at room temperature overnight. The reaction mixture was added dropwise to 550 ml of ether with stirring; the flask was rinsed with two 5-ml portions of DMF which were likewise added to the ether suspension. The precipitate was collected on a filter and washed with several small portions of ether. The solid was dissolved in 70 ml of dioxane and the solution was added to 800 ml of ether with stirring. The precipitate was collected on a filter, washed with several small portions of ether and dried. The dry solid (2.483 g) was dissolved in 50 ml of ethanol and the solution was added dropwise to 700 ml of ether with stirring. The

[4]) Omitting the acetic acid addition in the hydrogenolysis step led to the formation of a considerable amount of TRH (**4**).

precipitate was collected on a filter, washed with ether, and dried to give 2.286 g of substance **7** which still contained a small amount of HONSu (δ = 2.71 ppm). Attempted purification of the above substance by chromatography on 90 g of silica gel, led to the isolation of 1.757 g of **7** still containing a small amount of HONSu according to the NMR.-spectrum. The substance could not be induced to crystallize, $[\alpha]_D^{26} = -10°$ (c = 0.98, DMF); $\tilde{\nu}_{max}$ 1788, 1673 cm^{-1}; NMR. (AcOH-d_4): 8.71 (*d*, 4-His Ar), 7.35 (*m*, 2-His Ar and Z-Ar), 5.24 (*s*, Z-CH_2), 4.87 (*m*, α-CH pGlu and His), 3.27 (*m*, β-CH_2 His) 2.0–2.8 ppm (*m*, β, γ-CH_2 pGlu).

$C_{19}H_{21}N_5O_5$ (399.398) Calc. C 57.13 H 5.30 N 17.54% Found C 56.56 H 5.41 N 17.60%

Methanolysis of Z-L-pyroglutamyl-L-histidinamide (**7**). A solution of 1.080 g (2.71 mmol) of **7** in 100 ml of methanol was allowed to stand at room temperature for 5 days. Evaporation of the reaction mixture led to the recovery of a foamy residue which was chromatographed on 80 g of silica gel. Elution with chloroform/methanol 4:1 gave, after evaporation of the solvent, 0.765 g of the less polar substance. Recrystallization of this foam from methanol afforded 0.553 g of analytically pure *Z-L-(O⁵-methyl)glutamyl-L-histinamide* (**8**) which had a m.p. of 204–205°; $[\alpha]_D^{25} = -6°$ (c = 1.02, DMF); $\tilde{\nu}_{max}$ 1729, 1697, 1674, 1643, 1535 cm^{-1}; NMR. (TFA): 8.38 (*m*, 4-His Ar), 7.38 (*m*, Z-Ar and 2-His Ar), 5.23 (*s*, Z-CH_2), 5.07 (*m*, α-CH His), 4.52 (*m*, α-CH Glu), 3.82 (*s*, OCH_3), 3.40 (*m*, β-CH_2 His), 2.67 (*t*, γ-CH_2 Glu), 2.25 (ppm (*m*, β-CH_2 Glu).

$C_{20}H_{25}N_5O_6$ (431.440) Calc. C 55.67 H 5.84 N 16.23% Found C 55.53 H 5.94 N 16.41%

The eluates of the above chromatogram with methanol/chloroform 1:2 left a residue of 0.082 g after evaporation of the solvent. The substance was recrystallized from ethanol to afford 0.029 g of pure *L-pyroglutamyl-L-histidinamide* (**9**): m.p. 232–234°; $[\alpha]_D^{26} = -28°$ (c = 0.99, AcOH); $\tilde{\nu}_{max}$ 1673, 1642, 1540 cm^{-1}; NMR. (TFA): 8.62 (*m*, 4-His Ar), 7.45 (*m*, 2-His Ar), 5.11 (*m*, α-CH His), 4.75 (*m*, α-CH pGlu), 3.48 (*m*, β-CH_2 His), 2.0–3.0 ppm (*m*, β, γ-CH_2 pGlu).

$C_{11}H_{15}N_5O_3$ (265.270) Calc. C 49.80 H 5.70 N 26.40% Found C 49.67 H 5.93 N 26.65%

L-Pyroglutamyl-L-histidinamide (**9**). A solution of 0.432 g (1 mmol) of **8** in 100 ml of methanol was hydrogenolyzed over 0.43 g of palladium black. The catalyst was collected on a filter and washed with several small portions of methanol. Evaporation of the methanolic solution left a residue of 0.317 g of crude *L-(O⁵-methyl)glutamyl-L-histidinamide* (**10**), which was dissolved in 15 ml of water and 15 ml of dioxane and treated with 0.149 g of triethylamine (pH ~8.5) at room temperature overnight. Evaporation of the reaction mixture left a residue of 0.318 g which was purified by chromatography on 30 g of silica gel. Evaporation of the chloroform/methanol 1:2 eluates afforded 0.164 g of residue which was recrystallized from ethanol to yield 0.106 g of **9**: m.p. 231–233°; $[\alpha]_D^{26} = -28°$ (c = 1.07, AcOH); $\tilde{\nu}_{max}$ 1672, 1642, 1541 cm^{-1}; NMR. (TFA): 8.63 (*m*, 4-His Ar), 7.46 (*m*, 2-His Ar), 5.12 (*m*, α-CH His), 4.74 (*m*, α-CH pGlu), 3.47 (*m*, β-CH_2 His), 2.0–3.0 ppm (*m*, β,γ-CH_2 pGlu).

$C_{11}H_{15}N_5O_3$ (265.270) Calc. C 49.80 H 5.70 N 26.40% Found C 49.91 H 5.71 N 26.15%

This substance was fully identical with the above **9** obtained from methanolysis as well as with a reference sample of **9** prepared by a different procedure[5]).

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