BIOORGANIC CHEMISTRY **26,** 131–140 (1998)
ARTICLE NO. BH981092

The C-Terminus Shortened Analogs of the Insect Peptide Oostatic Hormone with Accelerated Activity

Jan Hlaváček,* Richard Tykva,* Blanka Bennettová,† and Tomislav Barth*

**Institute of Organic Chemistry and Biochemistry, Academy of Sciences, 166 10 Prague, Czech Republic; and †Institute of Entomology, Academy of Sciences, 370 05 České Budějovice, Czech Republic*

Received February 11, 1998

Shortened analogs **1a–1f** of the oostatic hormone **1g** with a decreased number or an omission of the C-terminal Pro residues were synthesized for investigation of their effect on oogenesis in the flesh fly *Sarcophaga bullata Parker* (Diptera). Peptides **1b–1g** were prepared by solid-phase synthesis on polystyrene/divinylbenzene polymer with a 2-chlorotrityl linker using Fmoc/tBu strategy in dimethylformamide (DMF). Tetrapeptide **1a,** devoid of all the carboxy-terminal Pro residues, was prepared by stepwise procedure in DMF solution from HCl-H-Ala-OMe using Boc-Pro-OH, Z-Asp(OtBu)-OSu, and Boc-Tyr-OSu. In general, the peptides assayed affected processes of egg development in 20–80% of ovarioles causing changes in the follicular epithelium, proliferation of its nuclei, and cell division toward the inner part of the egg chamber. This process leads to the occurrence of a multilayered follicular epithelium which later fills up almost the whole egg chamber and then degenerates. Such ovarioles are never able to produce eggs. The shortening of the peptide sequence accelerated the observed effects on egg development. The application of the ^{125}I-labeled peptides made possible to express quantitatively the time dependence of the radioactivity distribution in the insect body, including ovaries through which it is transferred to the next F1 generation. The radioactivity was measured in larvaes and, consequently, in newborn flies. For different peptide sequences applied, different radioactivity distributions in the insect body were found using the same radiolabeling.

INTRODUCTION

Oostatic or antigonadotropic insect hormones have been found in a number of insect species (*1*). Some time ago, Borovsky *et al.* isolated another member of this class of reproductive and developmental hormones from mosquito and described it (*2*) to be an unusual decapeptide **1g** containing six C-terminal Pro (*3*) residues. This molecule was postulated to affect oogenesis through modulation of ovarian ecdysteroid synthesis, gut trypsin synthesis, or egg development neurosecretory hormone release in the Diptera and to influence a juvenile hormone action on ovarian yolk uptake in some Hemiptera. The peptide was designed to function as a signal that terminates vitellogenesis.

In our preliminary communication (*4*) we described the solution synthesis and oostatic properties of some peptides with the sequence of the oostatic hormone **1g** and its shorter analogs **1d–1f,** differing in a number of the C-terminal Pro residues.

0045-2068/98 $25.00

A comparative study of the hormone **1g** and one of the shorter analogs (**1e**) revealed certain effects on processes of egg development. They were manifested by disturbances of yolk deposition, changes of follicular epithelium, and formation of nonviable eggs. The presence of Tyr in the peptides prepared allowed us to carry out the radiotracer distribution studies using ^{125}I-labeled molecules of **1d** and **1g** and we quantitatively described a presence of the labeled peptide material in various parts of the insect body (ovaries, head, intestine).

H-Tyr-Asp-Pro-Ala-Pro$_n$-OH
1a $n = 0$, **1b** $n = 1$, **1c** $n = 2$, **1d** $n = 3$, **1e** $n = 4$, **1f** $n = 5$, **1g** $n = 6$
2a Boc-Pro-Ala-OMe
2b Z-Asp(OtBu)-Pro-Ala-OMe
2c Boc-Tyr-Asp(OtBu)-Pro-Ala-OMe
2d Boc-Tyr-Asp(OtBu)-Pro-Ala-OH

The results obtained in our first study have inspired us with an idea to perform similar studies on analogs with a peptide sequence even more shortened from the C-terminus. Therefore, we synthesized peptides **1a–1c** to compare their oostatic properties with peptides described earlier, including the native decapeptide **1g.**

Tetrapeptide **1a,** devoid of all C-terminal Pro residues, was prepared by stepwise procedure in solution. The synthesis was started from HCl · H-Ala-OMe which was coupled to Boc[1]-Pro-OH in DMF using DCC and HOBt in the presence of DIEA. The protected dipeptide **2a** was treated with TFA and the resulting TFA.H-Pro-Ala-OMe acylated by Z-Asp(OtBu)-OSu in DMF to give the protected tripeptide **2b.** After removal of the Z-protecting group by hydrogenolysis on Pd black in MeOH, the Boc-Tyr-OSu was used to complete the sequence of the protected tetrapeptide **2c** which was saponified to acid **2d** which was finally deprotected by the mixture TFA–anisole (9 : 1).

Peptides **1b** and **1c** and the larger peptides described previously (**1d–1g,** Ref. 4) were prepared by solid-phase approach using a polymer with a 2-chlorotritylchloride linker. We have utilized Fmoc/tBu protection of amino acids which were coupled in DMF by means of DCC/HOBt. The Fmoc protecting group has been removed by piperidine in DMF.

In the last step of the syntheses the peptides were deprotected and split off the resin by trifluoroacetic acid in the presence of anisole.

The peptides synthesized were purified by preparative HPLC and characterized by means of amino acid analysis, mass spectrometry, and analytical HPLC.

To illustrate the fate of the shortened analogs after their application to insect, basic morphological, histological, and radiotracer studies were carried out using tetrapeptide **1a** and pentapeptide **1b,** respectively, including their ^{125}I labeling. The results were compared with those obtained for the larger peptides.

[1] Abbreviations used: Boc, *tert*-butyloxycarbonyl; DCC, dicyclohexylcarbodiimide; DIEA, diisopropylethylamine; DMF, dimethylformamide; HOBt, hydroxybenzotriazole; OSu, succinimide ester; tBu, *tert*-butyl; TDM, 4,4′-tetramethyldiamino-diphenylmethane; TFA, trifluoroacetic acid.

MATERIAL AND METHODS

Materials

Z-Asp(OtBu)-OSu was purchased from Bachem (Switzerland), whereas all other amino acid derivatives were prepared in our laboratory following general protocols (*5*) and were checked for their purity by TLC, HPLC, elemental analysis, and mass spectrometry. 2-Chlorotritylchloride resin (1.3 mmol/g) was purchased from Calbiochem–Novabiochem AG (Switzerland).

General Methods

TLC of the protected amino acids and peptides prepared was performed on precoated Silufol$_{254}$ plates (Kavalier, Czech Republic) in the following 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); and 1-butanol–pyridine–acetic acid–water (15 : 10 : 3 : 6) (S4). The compounds were visualized directly under an ultraviolet light or by ninhydrin and chlorine–TDM detection (*6*). Melting points were measured on a Kofler block and were not corrected. Solvents were evaporated *in vacuo* on a rotary evaporator (bath temperature 30°C); DMF was evaporated at 30°C and 150 Pa. Analytical electrophoresis was carried out in a moist chamber on Whatman 3MM paper (20 V/cm) in 6% acetic acid (pH 2.4) and in a pyridine–acetate buffer (pH 5.7) for 60 min. Optical rotations were obtained on a Perkin–Elmer 141 MCA polarimeter at 20°C. The samples for amino acid analysis were hydrolyzed by 6 M HCl at 110°C for 20 h with 3% phenol. The amino acid analyses were performed on a Durum D-500 amino acid analyzer (Durum Instruments, USA). The molecular weights of the peptides were determined using mass spectrometry with FAB technique (VG Analytical, England). For HPLC, a Spectra Physics instrument with an SP 8800 pump, an SP 8450 UV detector, and an SP 4290 integrator was used. Peptides **1a–1g** were purified by preparative HPLC on a 35 × 2.5-cm RP-18 column, 10-μm (Vydac, The Separations Group, Hesperia, USA). Analytical HPLC was carried out on a 250 × 4-mm RP-18 column, 5-μm (LiChrospher WP-300, Merck Darmstadt, Germany). The samples for the elemental analysis performed at the analytical department of the above institute were dried over P_2O_5 at room temperature at 150 Pa.

Syntheses

tert-Butyloxycarbonyl-prolyl-alanine methyl ester (**2a**). A solution of Boc-Pro-OH (13.8 g; 70 mmol), HOBt (10.3 g; 77 mmol), and DCC (14.4 g; 70 mmol) in DMF (40 ml) was stirred at −10°C for 30 min and then filtered to a cooled solution of HCl.H-Ala-OMe (10 g, 72 mmol) in DMF (10 ml) and pH 7.5 adjusted by DIEA. The reaction mixture was stirred for 2 h at 0°C followed by stirring overnight at room temperature. The mixture was then cooled in refrigerator and the precipitated dicyclohexylurea separated by filtration and DMF evaporated. The residue was dissolved in ethyl acetate (EtOAc) and the solution washed three times with 1 M $NaHCO_3$, H_2O, 20% citric acid, and a saturated solution of Na_2SO_4. After drying

by anhydrous Na_2SO_4 the EtOAc solution was evaporated and the resulting product crystallized from EtOAc–light petroleum to give 13.4 g of the protected dipeptide **2a** with m.p. 77–80°C. $[\alpha]_D$ −57.7° (c 0.5, DMF), −93.3° (c 0.4, MeOH). For $C_{14}H_{24}N_2O_5$ (300.4) calculated: 55.99% C, 8.05% H, 9.33% N; found: 55.78% C, 8.01% H, 9.21% N. Mass spectrum (FAB), *m/z* 301.2 (M^+ +1). Amino acid composition: Ala (1), Pro (1.02).

N-Benzyloxycarbonyl-O-tert-butylaspartyl-prolyl-alanine methyl ester (**2b**). The Boc-Pro-Ala-OMe (**2a,** 6.5 mmol, 1.95 g) was treated with TFA (10 ml) for 30 min and the resulting TFA · H-Pro-Ala-OMe ($E^{2,4}_{Gly}$ 1.44; $E^{2,4}_{His}$ 0.95; $E^{5,7}_{His}$ 0.99) was acylated by Z-Asp(OtBu)-OSu (7 mmol, 2.8 g) in DMF (15 ml) in the presence of HOBt (6.5 mmol, 0.87 g) at room temperature and pH 7 was maintained by DIEA. The reaction mixture was worked up after 2 days similarly to preparation of **2a** to give 3.9 g of the protected tripeptide **2b.** The pure product with m.p. 103–105°C was obtained on crystallization from EtOAc–light petroleum. $[\alpha]_D$ −67.2° (c 0.3, DMF). For $C_{25}H_{35}N_3O_8$ (505.6) calculated: 59.39% C, 6.98% H, 8.31% N; found: 59.32% C, 7.01% H, 8.45% N. Mass spectrum (FAB), *m/z*: 506.2 (M^+ +1). Amino acid composition: Asp (1.02), Ala (1.0), Pro (0.95).

N-tert-Butyloxycarbonyltyrosyl-O-tert-butylaspartyl-prolyl-alanine methyl ester (**2c**). The Z-Asp(OtBu)-Pro-Ala-OMe (**2b,** 2.02 g, 4 mmol) in MeOH (100 ml) was hydrogenated in the presence of Pd black for 3 h, and the resulting H-Asp(OtBu)-Pro-Ala-OMe ($E^{2,4}_{Gly}$ 0.99; $E^{2,4}_{His}$ 0.67; $E^{5,7}_{His}$ 0.76) was acylated by Boc-Tyr-OSu (4 mmol, 1.5 g) in DMF (15 ml) in the presence of HOBt (4 mmol, 0.54 g) for 2 days. The reaction mixture was worked up similarly to the synthesis of **2a** with a yield of the amorphous **2c** (2.4 g). TLC: 0.73(S1), 0.77(S2). For $C_{31}H_{46}N_4O_{10}$ (634.7) calculated: 58.66% C, 7.30% H, 8.83% N; found: 58.44% C, 7.41% H, 8.97% N. Mass spectrum (FAB), *m/z* 635.3 (M^+ +1). Amino acid composition: Tyr (0.97), Asp (1.02), Ala (1.0), Pro (1.01).

N-tert-Butyloxycarbonyltyrosyl-O-tert-butylaspartyl-prolyl-alanine (**2d**). The solution of the Boc-Tyr-Asp(OtBu)-Pro-Ala-Pro-OMe (**2c,** 1.27 g; 2 mmol) in acetone (30 ml)–water (3 ml) was stirred with 2 M NaOH (1 ml; 2 mmol) at room temperature for 3 h. Water was added (10 ml), acetone evaporated, and the alkaline solution washed with EtOAc and acidified by 20% citric acid to pH 3.5. The oily product was taken to EtOAc and the EtOAc solution washed by water, dried by anhydrous Na_2SO_4, and after filtration evaporated to dryness. The residue was solidified from ether–light petroleum to give the protected tetrapeptide acid **2d** (0.98 g) with TLC: 0.57 (S2), 0.62 (S4). $[\alpha]_D$ −112° (c 0.4; DMF). For $C_{30}H_{44}N_4O_{10}$ (620.7) calculated: 58.05% C, 7.15% H, 9.03% N; found: 57.81% C, 7.04% H, 9.29% N. Mass spectrum (FAB), *m/z* 621.3 (M^+ +1). Amino acid composition corresponded to that of the methyl ester **2c.**

Tyrosyl-aspartyl-prolyl-alanine (**1a**). The protected tetrapeptide **2d** (0.19 g; 0.3 mmol) was dissolved in a mixture of TFA (5 ml)–anisole (0.5 ml) at room temperature and after 1 h the solution was evaporated and the residue dissolved in 10% AcOH (200 ml), washed three times with ethyl acetate, and freeze dried. The peptide was purified by preparative HPLC on a 30 × 2.5-cm column filled with Vydac stationary phase with a gradient of 5–20% ACN in 0.05% TFA in 30 min

TABLE 1
Analytical Data on Peptides **1a–1c**

Compound[a] HPLC[e]	Formula[b] MW/(M^+ + 1)	AAA[c] Tyr	Asp	Ala	Pro	$E^{2.4,d}$ E_{gly}	E_{his}	$E^{5.7}$ E_{Pic}
HTyrAspProAlaOH 8.59 (**1a**)	$C_{21}H_{28}N_4O_8$ 464.8/465.3	1.03	1.01	1.0	0.98	0.75	0.51	0.29
HTyrAspProAlaProOH 11.80 (**1b**)	$C_{26}H_{35}N_5O_9$ 561.2/562.2	1.02	1.02	1.0	2.1	0.72	0.48	0.27
HTyrAspProAlaPro$_2$OH 14.17 (**1c**)	$C_{31}H_{42}N_6O_{10}$ 658.7/659.2	1.04	1.03	1.0	2.9	0.68	0.44	0.24

[a] Analytical data of peptides **1d–1g,** see (4).
[b] Determined with FAB technique (VG Analytical, England).
[c] Amino acid analyses were performed on a Durum D-500 AA analyzer (Durum Instruments, Corp., Palo Alto, CA).
[d] Analytical electrophoresis was carried out in a moist chamber on Whatman 3MM paper (20 V/cm) in 6% acetic acid (pH 2.4) and in a pyridine–acetate buffer (pH 5.7) for 60 min.
[e] Retention time in minutes, 25 × 0.4-cm RP-18 column, 5-μm (LiChrospher WP-300, Merck Darmstadt, Germany), flow rate 60 ml/h, detection at 222 nm, gradient 5–50% of acetonitrile in 0.05% aqueous TFA, 25 min; Spectra Physics 8800 HPLC pump with SP 8450 UV/VIS detector and SP 4290 integrator.

and characterized by mass spectrometry, amino acid analysis, electrophoresis, and analytical HPLC (Table 1).

Solid-Phase Synthesis of Peptide **1b**

The solution of Fmoc-Pro-OH (0.92 g; 2.73 mmol) in dry DCM (30 ml) containing 2 ml of dry DMF (just enough to make the amino acid dissolve in DCM) was added to the 2-chlorotritylchloride resin (3 g) together with about one-third of the total amount (1.2 ml, 7 mmol) of DIEA. After 5 min of stirring the remainder of the DIEA in DCM (1 : 1) was added. Vigorous stirring was prolonged for an additional 60 min. At the end of this time an excess of HPLC-grade MeOH (3 ml) was added and stirring continued for 10 min to end cap the remaining trityl groups on the resin. The resin was then washed three times with 30-ml portions of DCM, DMF, methanol, and diethylether and dried over KOH in a dessicator (3.6 g).

The Fmoc-Pro-2-chlorotrityl resin (0.6 g) was swollen in DMF/DCM (1 : 1) and deprotected by 5% piperidine in DMF for 10 min (30 ml) followed by 20% piperidine in DMF (30 ml) for 15 min. The free amino acid resin was then gradually acylated with three equivalents of Fmoc-Ala-OH (0.29 g), Fmoc-Pro-OH (0.31 g), Fmoc-Asp(tBu)-OH (0.38 g), and Fmoc-Tyr(tBu)-OH (0.43 g) activated by HOBt/DCC mixture (0.19 g, 1.4 mmol/0.21 g/1.02 mmol) in DMF (30 ml). At each step cleavage of the Fmoc group has been done by 20% piperidine in DMF (2× 30 ml, 30 and