Bioorganic Chemistry **29,** 282–292 (2001)
doi:10.1006/bioo.2001.1210, available online at http://www.idealibrary.com on IDEAL®

Cyclic Analogs of Insect Oostatic Peptides: Synthesis, Biological Activity, and NMR Study

Jan Hlaváček,* Miloš Buděšínský,* Blanka Bennettová,† Jan Mařík,* and Richard Tykva*

**Institute of Organic Chemistry and Biochemistry, Academy of Sciences, 166 10 Prague 6, Czech Republic; and †Institute of Entomology, Academy of Sciences, 370 05 České Budějovice, Czech Republic*

Received January 17, 2001

Cyclic peptides **2a–2c**, derived from the sequence of the C-terminal shortened analogs of the oostatic decapeptide H-Tyr-Asp-Pro-Ala-Pro-Pro-Pro-Pro-Pro-Pro-OH **(1a)**, were synthesized and assayed on their effect in a reproduction of the flesh fly *Neobellieria bullata*. The cyclization of the N-terminal linear tetra- and pentapeptides **1b** and **1c** to the cyclotetra- and cyclopentapeptides **2b** and **2c** decreased the oostatic activity by one order of magnitude. The cyclodecapeptide **2a**, which emerged spontaneously during the pentapeptide cyclization, was quite inactive. Comparative ^{1}H and ^{13}C NMR study on a conformation of the cyclopeptides **2a–2c**, and their linear precursors **1b** and **1c** revealed that a space structure of the cyclic analogues **2b** and **2c** is too restricted to adopt a biological conformation necessary for receptor binding and therefore only minor oostatic activity is observed after their application. The lack of the oostatic activity in the case of the more flexible dimeric analogue **2a** is ascribed to the size of its molecule and its overall shape that is not compatible with a receptor binding.

Key Words: solid phase; solution synthesis; oostatic peptide; *Neobellieria bullata*; reproduction; cyclopeptide; ^{1}H and ^{13}C NMR study.

INTRODUCTION

Decapeptide H-Tyr-Asp-Pro-Ala-Pro-Pro-Pro-Pro-Pro-Pro-OH (**1a**), isolated from mosquito *Aedes aegypti* by Borovsky *et al. (1,2)*, was postulated to affect oogenesis through modulation of ovarian ecdysteroid synthesis, gut trypsin biosynthesis in the midgut of blood-sucking insects, 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 *(3,4)*.

In our previous studies, we investigated the effect of this decapeptide together with effect of its C-terminal truncated analogues *(5,6)* on a reproduction (development of eggs in ovaria and hatchability of larvae) of our insect model *Sarcophaga* (now *Neobellieria*) *bullata*. We found that the N-terminal tetra- and pentapeptide sequences

0045-2068/01 $35.00

1b and **1c** *(6)* exhibited an accelerated effect and a more pronounced oostatic activity than the original decapeptide.

H - Tyr - Asp - Pro - Ala - X - OH

$X = (Pro)_6$ [1a]

$X = 0$ [1b]

$X = Pro$ [1c]

In this paper we want to report on the synthesis and the insect development inhibitory activity of the cyclotetra- and cyclopentapeptides **2b** and **2c** derived from their linear precursors **1b** and **1c** and also on those of the cyclodecapeptide **2a**, that was detected and separated after cyclopentapeptide synthesis as the only by-product in a ratio 1:1.

cyclo(Tyr-Asp-Pro-Ala-Pro-Tyr-Asp-Pro-Ala-Pro) [2a]

cyclo(Tyr-Asp-Pro-Ala) [2b]

cyclo(Tyr-Asp-Pro-Ala-Pro) [2c]

Design of the cyclic peptides was also based on some preliminary calculations using MOPAC 7.01 program (PM 3 method) which suggested a pseudocyclic conformation in the linear tetrapeptide **1b** constrained by the proximity of the Ala^4 carboxyl terminus and either Tyr^1 and (or) Asp^2 amino groups (Fig. 1) or Tyr^1 hydroxyl group (Fig. 2).

A discussion on relationships between structure of the cyclic peptides **2a–2c** and their oostatic activity was carried out using results of morphological and histological assay and of detailed ^1H and ^{13}C NMR study, including linear precursors **1b** and **1c**.

MATERIAL AND METHODS

TPTU[1] was purchased from Senn Chemicals International (Gentilly, France) and protected amino acids were purchased from Bachem (Bubendorf, Switzerland) or were prepared in our laboratory following general protocols *(7)* and were checked

[1] Abbreviations used: ACN, acetonitrile; AcOH, acetic acid; COSY, 2D-NMR correlation spectroscopy; DIC, *N,N*-diisopropylcarbodiimide; DIEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; Et_2O, diethyl ether; Fmoc, fluorenylmethyloxycarbonyl; HMQC, heteronuclear multiple quantum coherence; HOBt, 1-hydroxybenzotriazole; MeOH, methanol; Me_2SO, dimethyl sulfoxide; FAB MS, fast atom bombardment mass spectroscopy; NOE, Nuclear Overhauser Effect; Pic, picric acid; ROESY, 2D-NOE in rotating frame; tBu, tert-butyl; TFA, trifluoroacetic acid; TOCSY, total correlation 2D-NMR spectroscopy; TPTU, 2-(2-pyridon-1-*yl*)-1,1,3,3-tetramethyl uronium tetrafluoroborate. The nomenclature and symbols of amino acids follow published recommendations of the IUPAC/IUB Joint Commission on Biochemical Nomenclature (Nomenclature and Symbolism for Amino Acids and Peptides (1984) *Eur. J. Biochem.* **138,** 9–37).

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

Progress in peptide synthesis was monitored by the Kaiser *(8)* and bromophenol blue *(9)* tests. Analytical electrophoresis $E_{5.7}^{Pic}$ was carried out in a moist chamber on Whatman 3MM paper (20 V/cm) in a pyridine-acetate buffer (pH 5.7) for 60 min. The compounds were visualized by the ninhydrin or chlorine-KI-*o*-tolidine detection, *(10)*. Optical rotations were measured on a Perkin–Elmer 141 MCA polarimeter at 22°C and $[\alpha]_D$ values are given in 10^{-1} deg cm^2 g^{-1}. Solvents were evaporated *in vacuo* on a rotary evaporator (bath temperature 30°C); DMF was evaporated at 30°C and 150 Pa. The samples for amino acid analysis were hydrolyzed with 6 M HCl containing 3% of phenol at 110°C for 20 h. The amino acid analyses were performed on Biochrom 20 instrument (Pharmacia, Sweden). Molecular weights of the peptides were determined using mass spectroscopy with FAB technique (Micromass, Manchester, England). For HPLC a Spectra Physics instrument with an SP 8800 pump, an SP 4290 integrator and Thermo Separation Products Spectra 100 UV detector were used. The compounds were purified by semipreparative HPLC on the 25 × 1 cm column, 10 μm Vydac RP-18 (The Separations Group, Hesperia CA), flow rate 3 ml/min, detection at 220 nm using gradient 0–100% ACN in 0.05% aqueous TFA within 60 min. The analytical HPLC was carried out on the 25 × 0.4 cm column, 5 μm LiChrospher WP-300 RP-18 (Merck, Darmstadt, Germany), flow rate 1 ml/min, detection at 220 nm, using 0–100% gradient of ACN in 0.05 aqueous TFA within 60 min.

Syntheses

Solid-Phase Synthesis of Linear Precursors of Cyclopeptides **2b** *and* **2c**. Fmoc-Ala-OH (0.85 g; 2.7 mmol) or Fmoc-Pro-OH (0.91 g; 2.7 mmol) was loaded on 2-chlorotritylchloride resin (1 g) with the substitution 0.43 mmol/g of the resin *(11)*. The loading of the Fmoc-amino acids on the resin was calculated as the average of the values determined by (a) weight increase, (b) measurement of the dibenzofulvene–piperidine complex absorption after cleavage by 5% piperidine in DCM—DMF 1:1 for 10 min and 20% piperidine in DMF for 15 min, and (c) AAA. After the Fmoc deprotection, the H-Ala-resin was sequentially acylated with three equivalents of Fmoc-Pro-OH (0.44 g), Fmoc-Asp(OtBu)-OH (0.54 g) and Fmoc-Tyr(tBu)-OH (0.60 g) activated with HOBt (0.19 g; 1.4 mmol) - DIC (0.16 g; 1.3 mmol) mixture in DMF (30 ml). The H-Pro-resin was similarly acylated with Fmoc-Ala-OH (0.41 g), Fmoc-Pro-OH (0.44 g), Fmoc-Asp(OtBu)-OH (0.54 g), and Fmoc-Tyr(tBu)-OH (0.60 g). The Fmoc groups were removed by 20% piperidine in DMF (2x 20 ml), 10 and 30 min. After the last Fmoc deprotection, peptide-resins were washed with MeOH, dried in desiccator and stirred with an AcOH (20 ml)–CH_2Cl_2 (60 ml) mixture for 2x 90 min at room temperature to detach the partially protected peptides off the resin which was filtered off. The solutions were concentrated by evaporation of CH_2Cl_2, diluted with water and were freeze dried yielding the side-chain protected derivative of the **1b**, H-Tyr(tBu)-Asp(OtBu)-Pro-Ala-OH [0.23 g; 0.39 mmol; HPLC purity

81.6% and that of the **1c**, H-Tyr(tBu)-Asp(OtBu)-Pro-Ala-Pro-OH [0.26 g; 0.38 mmol; HPLC purity 86% (Table 1).

*Cyclo(Tyr-Asp-Pro-Ala) (***2b**). H-Tyr(tBu)-Asp(OtBu)-Pro-Ala-OH (0.15 g; 0.26 mmol) in DMF (125 ml) was added dropwise to solution of HOBt (0.1 g; 0.75 mmol) and TPTU (0.2 g; 0.67 mmol) in DMF (250 ml) at room temperature during 6 h at pH 7 adjusted with DIEA. The DMF was evaporated to dryness, the residue was dissolved in EtOAc and the solution was washed with 1 N $NaHCO_3$, 20% citric acid, brine (2x 20 ml of each) and was dried over Na_2SO_4. The EtOAc was evaporated to give 0.14 g of the side-chain protected derivative of cyclopeptide **2b** (HPLC peak retention time was 30.57 min). This product was treated with a TFA (4.5 ml)–anisole (0.5 ml) mixture at room temperature for 1 h, the TFA was evaporated, the residue triturated with Et_2O, and dissolved in 20% AcOH. The solution was freeze dried yielding the cyclopeptide **2b** (90 mg), which was purified by preparative HPLC to afford 70 mg of pure peptide (Table 1); the ^1H and ^{13}C NMR—see Tables 2 and 3.

*Cyclo(Tyr-Asp-Pro-Ala-Pro) (***2c***) and cyclo(Tyr-Asp-Pro-Ala-Pro)*$_2$ *(***2a***).* The reaction and purification conditions described for compound **2b** were used in the cyclization of H-Tyr(tBu)-Asp(OtBu)-Pro-Ala-Pro-OH (0.11 g; 0.17 mmol) in DMF (80 ml) with HOBt (0.08 g; 0.6 mmol) and TPTU (0.11 g; 0.37 mmol) in DMF (150 ml). The side-chain protected cyclopentapeptide (HPLC peak retention time was 31.45 min) and its cyclodecapeptide dimer (HPLC peak retention time was 44.90 min) were detected in the reaction mixture in the ratio 1:1. A treatment of this mixture (0.1 g) with TFA (4.5 ml)-anisole (0.5 ml) and the HPLC purification described for the compound **2b** yielded pure cyclopeptides **2c** (40 mg) and **2a** (30 mg) (see Table 1); the ^1H and ^{13}C NMR—see Tables 2 and 3.

Biological Activity Assay

Peptides were separately injected in Ringer solution (1 μg/1 μl, 5 μl per female) into the upper part of the thorax of Et_2O-anestetized, 24-h old females of *Neobellieria bullata* (Diptera). Treated female flies were placed into cages with untreated males and dissected at regular time intervals (on days 1, 2, 8, and 15 after application). The ovaries were checked for signs of resorption and hatchability of eggs in the uterus during the first gonotrophic cycle. The ovaries undergoing the second gonotrophic cycle were examined as well. Histological preparations made of suspect ovaries of both gonotrophic cycles were prepared by standard procedure. They were stained with Mallory's or Mayer's hematoxyline.

NMR Spectra Measurement

NMR spectra were measured on Varian UNITY-500 spectrometer (^1H at 500 MHz, ^{13}C at 125.7 MHz) in Me_2SO-d_6. Complete assignment of all proton signals was derived from COSY, TOCSY, and ROESY experiments. The amino acid residues were identified according to their characteristic spin-systems, scalar-coupling connectivity patterns derived from COSY and TOCSY spectra, and NOE contacts (aromatic protons). Sequential assignment of the amino acid residues was carried out from the analysis of ROESY spectrum (mainly NH(i + 1)-Hα (i) NOE contacts). Proton chemical shifts and coupling constants are given in Table 2. "Attached proton test" ^{13}C NMR spectra allowed distinguishing C, CH, CH_2, and CH_3 carbon signals. Their

structural assignment (except C = O signals) was done using ^{1}H, ^{13}C-correlated HMQC spectra—for data see Table 3.

RESULTS AND DISCUSSION

The synthesis of cyclopeptides **2a–2c** was carried out by a cyclization of their linear, *t*Bu side-chain protected, tetra- and pentapeptide precursors followed by the side-chain deprotection. The linear precursors were prepared on a solid support with 2-chlorotrityl linker *(11,12)*, which is particularly suited for the synthesis of the peptides containing C-terminal proline using corresponding N^{α}-Fmoc-amino acids. The extreme steric hindrance of the trityl group effectively suppresses a formation of dioxopiperazine at the dipeptide step and a premature cleavage of the peptide from the resin. Prior to each synthetic step mediated by DIC and HOBt as coupling reagents in DMF, the N^{α}-Fmoc protecting group was removed by 20% piperidine in DMF. After the last Fmoc-deprotection, the peptides were cleaved off the resin quantitatively, with an AcOH-CH_2Cl_2 mixture 1:3. The cyclization was carried out in high DMF dilution with TPTU-HOBt coupling reagents in the presence of DIEA. At this step, the dimerization of the linear pentapeptide occurred yielding a mixture of the side-chain protected cyclopenta- and cyclodecapeptides. Finally, the *t*Bu protecting groups

TABLE 1

Analytical Data on Protected Linear Peptides and Cyclopeptides **2a–2c**

Compound HPLC[a]	Formula[b] M.W./(M^+ + 1)	$[\alpha]_D$[c]	AAA[d] Tyr	Asp	Pro	Ala	$E_{5.7}$[e] Pic
H-Tyr(tBu)Asp(OtBu) ProAla-OH 24.97[f]	$C_{29}H_{44}N_4O_8$ 576.7/577.3	ND	1.00	1.08	0.92	1.00	
H-Tyr(tBu)Asp(OtBu) ProAlaPro-OH 26.23[f]	$C_{34}H_{51}N_5O_9$ 673.8/674.4	ND	0.98	1.07	1.85	1.00	
c(TyrAspProAla) **2b** 13.72[f]	$C_{21}H_{26}N_4O_7$ 446.4/447.1	− 86.5° (c 0.06)	0.92	0.99	1.03	1.00	0.26
c(TyrAspProAlaPro) **2c** 15.81	$C_{26}H_{33}N_5O_8$ 543.6/544.2	−112.8° (c 0.12)	0.92	0.99	2.03	1.00	0.21
c(TyrAspProAlaPro)$_2$ **2a** 22.34	$C_{52}H_{66}N_{10}O_{16}$ 1087.2/1087.7	−138.8° (c 0.07)	0.94	0.97	1.98	1.00	0.19

[a] Retention time in minutes; 250×4-mm column RP-18, 5 μm (Lichrospher WP-300, Merck, Darmstadt, Germany), flow rate 1 ml/min, detection at 220 nm. Spectra Physics 8800 pump with an integrator SP 4290 and a UV/VIS detector TSPS 100.

[b] Determined with FAB MS technique (Micromas, Manchester, England).

[c] Measured in 1 M AcOH solution on a Perkin–Elmer 141 MCA polarimeter at 22°C. ND, not determined.

[d] Amino acid analyses were performed on a Biochrom 20 (Pharmacia, Sweden).

[e] Analytical electrophoresis was carried out in a moist chamber on a Whatman No. 3MM paper (20 V/cm) in a pyridine-acetate buffer (pH 5.7) for 60 min.

[f] 0–100% gradient of ACN in 0.05% aqueous TFA within 60 min.