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Preparation of tritiated oostatic peptides for study of radioactivity incorporation in flesh fly *Neobellieria bullata*

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Summary. A series of insect oostatic peptides containing 3,4-dehydroproline in the C-terminal part or inside of the peptide chain was synthesized and tritiated by addition of $^3H_2$ to double bond of 3,4-dehydroproline residue. ^{3}H-label was introduced also into tyrosine residue of oostatic tetra- and pentapeptides by isotopic exchange of benzyl β-hydrogens. In this way, three types of tritiated peptides were prepared, different in the radiolabeled amino acid position: [^{3}H] Tyr-Asp-Pro-Ala-OH, H-Tyr-Asp-[^{3}H] Pro-Ala-OH, [^{3}H] Tyr-Asp-Pro-Ala-Pro-OH, H-Tyr-Asp-[^{3}H] Pro-Ala-Pro-OH, H-Tyr-Asp-Pro-Ala-[^{3}H] Pro-OH, H-Tyr-Asp-Pro-Ala-Pro$_5$-[^{3}H] Pro-OH and H-Asp-[^{3}H] Pro-OH. These peptides made possible a highly sensitive comparative study on radioactivity incorporation into head and ovaries of the flesh fly *Neobellieria bullata*, which revealed this process to proceed differently. The reasons of the found differences are discussed.

Keywords: Oostatic peptide synthesis – 3,4-Dehydroproline – ^{3}H labeling – Incorporation in flesh fly

Abbreviations: The nomenclature and symbols of amino acids follow the Recommendations of IUPAC/IUB Joint Commission on Biochemical Nomenclature. Eur J Biochem (1984) 138: 9–37. Additional abbreviations include: AA, amino acid analysis; AcOH, acetic acid; Boc, *tert*-butoxycarbonyl; BrZ, 2-bromobenzyloxycarbonyl; DCC, *N,N′*-dicyclohexylcarbodiimide; DCM, dichloromethane; DIEA, *N,N*-diisopropylethylamine; DMA, *N,N*-dimethylacetamide; DMF, *N,N*-dimethylformamide; EDT, ethane-1, 2-dithiol; EtOAc, ethyl acetate; FAB MS, fast atom bombardment mass spectrometry; Fmoc, (fluoren-1-yl-methoxy)carbonyl; HOBt, 1-hydroxybenzotriazole; OBzl, benzyl ester; OMe, methyl ester; OSu, succinimide ester; PE, light petroleum; tBu, tert-butyl; TFA, trifluoroacetic acid; TFE, trifluoroethanol; TFMSA, trifluoromethane sulfonic acid; TIS, tri-isopropylsilane; TOTU, O-cyanoethoxycarbonylmethylidene amino-1,1,3,3-tetramethyl-uronium tetrafluoroborate; TPTU, O-(1,2-dihydro-2-oxo-1-pyridyl)-N,N,N′,N′-tetramethyluronium tetrafluoroborate.

Introduction

The study of insect pest management (Metcalf and Luckman, 1994) is an important task in food production. The most effective way involves application of insect growth regulators (IGRs) of different types (Büchel, 1983; Hodgson and Kuhr, 1990; Copping and Hewitt, 1998; Stenersen, 2004). Such compounds should have two basic features (Tykva, 1998): a high biological activity on the considered insect species and a low environmental impact on soils and ground waters. This impact depends not only on the applied IGR but also on its biodegradation by soil microorganisms that give rise to fragments representing additional environmental pollutants. Some of these soil biodegradation products are sometimes more detrimental for human health than the parent IGR.

In contrast to the majority of IGRs, we found that certain oostatic peptides (Hlaváček et al., 1997, 1998) and their metabolites in soil have no environmental impact (Tykva et al., 2004). Some of them have been found to function as the strong inhibitors of insect species Diptera, Orthoptera and Hemiptera reproduction (Tykva et al., 1999; Mařík et al., 2001; Němec et al., 2003). Thus, the morphological and histological observations of tetra- and pentapeptides H-Tyr-Asp-Pro-Ala-OH and H-Tyr-Asp-Pro-Ala-Pro-OH, respectively, have shown the devastating changes in ovarian development after their application to flesh fly *Neobellieria bullata*. Morphologically affected egg chambers have irregular appearance and their shape is often distorted. Histological evaluation has revealed very frequent changes throughout the ovary starting with growth of follicular epithelium toward to central part of the egg. The changes appear mostly in the development of the second batch of eggs (the second gonotrophic cycle); the changes in the first

gonotrophic cycle are manifested by decreased hatchability of eggs in the uterus.

To enhance our knowledge on the fate of oostatic peptides in the insect body, we focused our attention to their radiolabeled forms and used corresponding tritium labels (Slaninová et al., 2004). Such a labeling has been chosen since the peptides under study contain tyrosine and proline residues. While the tyrosine residue in the amino-terminus can be tritiated directly by isotopic exchange of benzyl β-hydrogens, the tritiated proline residues can be obtained by addition of $^3H_2$ to double bond of 3,4-dehydroproline residue (Klauschenz et al., 1981).

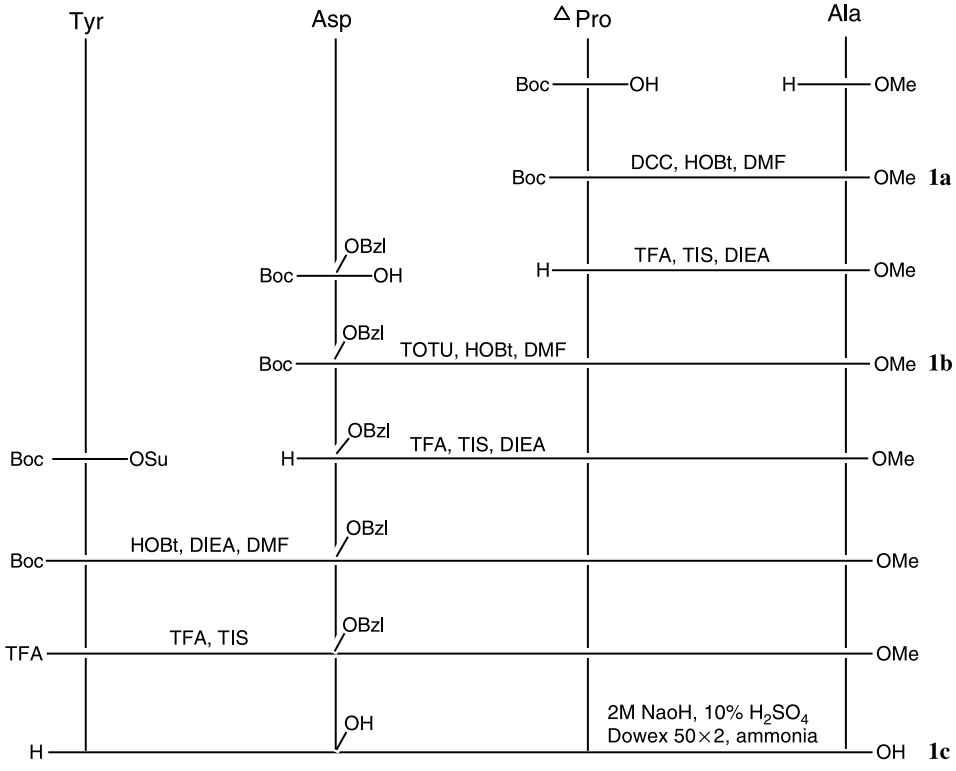
This non-coded amino acid has often been used as a substitute for proline residue in the structure-biological activity studies of biologically active peptides (Amoscato et al., 1984; Chan et al., 1986). However, it can be also utilized as the precursor of tritiated proline residue in peptides considered for a study of their metabolic pathways. The first step in such studies consists in a determination of the change in peptide radioactivity during the time for which the peptide is treated with a tissue or selected organ of given biological subject. In this study, an incorporation of radioactivity of tritiated peptides into the head and ovaries of our insect model flesh fly *Neobellieria bullata* (Diptera) was monitored and the results evaluated with respect to the sequence and level of oostatic activity of corresponding non-tritiated peptides.

Firstly, we synthesized the series of peptides containing 3,4-dehydroproline (Recommendations IUPAC/IUB, 1984) in their C-terminal part or in the middle part of their molecules to be used for tritiation and further studies on their oostatic potency.

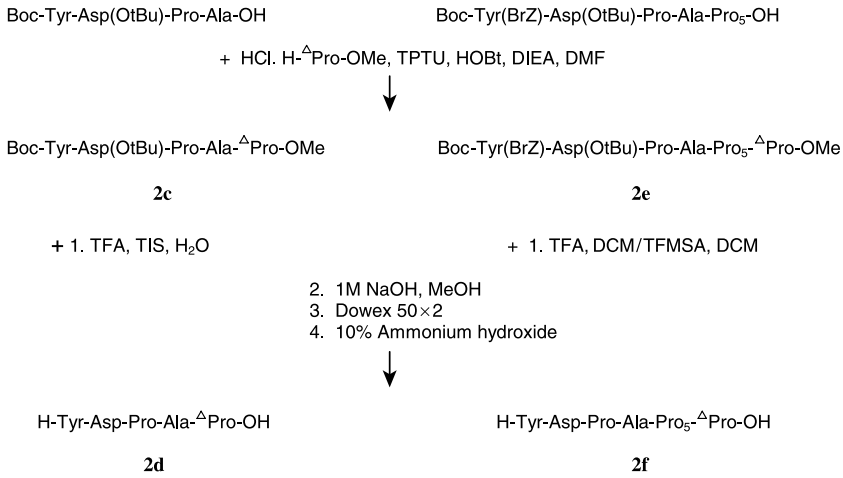
1a, Boc-$^{\Delta}$Pro-Ala-OMe; **1b**, Boc-Asp(OBzl)-$^{\Delta}$Pro-Ala-OMe; **1c**, H-Tyr-Asp-$^{\Delta}$Pro-Ala-OH; **1d**, H-Tyr-Asp-$^{\Delta}$Pro-Ala-Pro-OH; **2a**, Boc-Asp(OBzl)-$^{\Delta}$Pro-OMe; **2b**, H-Asp-$^{\Delta}$Pro-OH; **2c**, Boc-Tyr-Asp(OtBu)-Pro-Ala-$^{\Delta}$Pro-OMe; **2d**, H-Tyr-Asp-Pro-Ala-$^{\Delta}$Pro-OH; **2e**, Boc-Tyr(BrZ)-Asp(OtBu)-Pro-Ala-Pro_5-$^{\Delta}$Pro-OMe; **2f**, H-Tyr-Asp-Pro-Ala-Pro_5-$^{\Delta}$Pro-OH.

The tetrapeptide **1c** and its protected precursors **1a** and **1b** were prepared using a solution procedure described in Scheme 1. The middle part of Scheme 1 (sequence Asp-Pro) depicts also preparation of dipeptide **2b** and its protected precursor **2a**, in which the proline methyl ester was used as the carboxyterminal component. The ester protection groups were removed by alkaline hydrolysis.

In the case of pentapeptide **2d** and decapeptide **2f**, the protected N-terminal tetra- and nonapeptides, respec-



Scheme 1. Synthesis of compounds **1a**–**1c**



Scheme 2. Synthesis of compounds **2c**–**2f**

tively, were prepared first on 2-chlorotrityl polystyrene resin. After detachment from the resin by a 20% AcOH in DCM, both the protected peptide acids were coupled with H-$^{\Delta}$Pro-OMe hydrochloride and corresponding protected penta- and decapeptides **2c** and **2e**, respectively, deprotected as described in Scheme 2.

The sequence of the pentapeptide **1d** was, however, assembled on 2-chlorotrityl-resin (Barlos et al., 1991) exclusively, followed by simultaneous detachment of this peptide from the resin and cleavage of the protecting groups by a TFA-anisole-TIS mixture.

The deprotected peptides **1c**, **1d**, **2b**, **2d** and **2f**, were purified by preparative HPLC. Characterization of peptides and their protected precursors was carried out by amino acid analysis, analytical HPLC and FAB mass spectrometry (Table 1).

The 3,4-dehydroproline containing peptides **1c**, **1d** and **2b**, **2d**, **2f** were tritiated in DMA using tritium gas in the presence of $PdO/BaSO_4$ catalyst. The ^3H label was also introduced into tyrosine residue of the most active oostatic tetra- and pentapeptides by isotopic exchange of benzyl β-hydrogens using tritium gas and $PdO/BaSO_4$ catalyst in aqueous 0.1 M ammonium carbonate (Evans, 1974). In such a way, various forms of tritiated oostatic peptides were prepared with respect to the tritium label position in the peptide sequence (Table 2). Accordingly, they were utilized in the study on radioactivity incorporation into head and ovaries of the flesh fly *Neobellieria bullata*.

Table 1. Analytical data of $^{\Delta}$Pro peptides and their protected precursors **1a**–**1d** and **2a**–**2f**

Compound	Formula[a] MW/(M + 1)$^+$	HPLC[b] RT (min)	Amino acid composition[c]				
			Tyr	Asp	Pro	Ala	$^{\Delta}$Pro
1a	$C_{14}H_{22}N_2O_5$ 298.4/299.1	16.81[d]	–	–	–	1.00	0.98
1b	$C_{25}H_{33}N_3O_8$ 503.6/504.5	24.25[d]	–	1.02	–	1.00	0.96
1c	$C_{21}H_{26}N_4O_8$ 462.5/463.3	7.53[d]	1.03	1.05	–	1.00	0.95
1d	$C_{26}H_{33}N_5O_9$ 559.6/560.1	8.58[d]	1.04	1.03	1.04	1.00	1.05
2a	$C_{22}H_{28}N_2O_7$ 432.5/333.2	22.08[d]	–	1.00	–	–	1.03
2b	$C_9H_{12}N_2O_5$ 228.2/229.1	7.27[e]	–	1.00	–	–	0.98
2c	$C_{36}H_{53}N_5O_{11}$ 729.9/730.1	19.49[d]	0.98	1.05	0.99	1.00	1.03
2d	$C_{26}H_{33}N_5O_9$ 559.6/560.4	9.48[d]	0.99	1.02	1.01	1.00	1.04
2e	$C_{68}H_{89}N_{10}O_{18}Br$ 1414.4/1413.6	25.23[f]	0.98	1.03	6.01	1.00	1.02
2f	$C_{51}H_{68}N_{10}O_{14}$ 1045.2/1045.5	18.49[f]	0.89	1.03	5.85	1.00	0.94

[a] Determined with FAB technique (VG Analytical, U.K.)
[b] Retention time in minutes, 25 × 0.4 cm column, 5 μm (LiChrospher WP-300, Merck Darmstadt, Germany), flow rate 60 ml/h, detection at 220 nm
[c] Amino acid analyses were performed on a Biochrom 20 instrument (Pharmacia, Sweden)
[d] Gradient 0–100% of ACN in 0.05% aqueous TFA, 40 min, flow 1 ml/min
[e] Isocratic HPLC in 0.05% aqueous TFA, flow 0.5 ml/min
[f] Gradient 0–100% of ACN in 0.05% aqueous TFA, 60 min, flow 1 ml/min

Table 2. Synthetic conditions and parameters of tritiated peptides

Tritiated peptide amount used (mg) yield (mg), activity (MBq)	$PdO/BaSO_4$ catalyst (mg) % of tritium	Solvent (ml)	HPLC[a] mobile phase in 0.05% TFA	Specific activity (TBq/mmol)	Radiochemical purity (%)
H-Asp[^{3}H]Pro-OH 0.9 0.27, 2020	2.5 100	[b] 0.2	[c]	1.82	>97
H-Tyr-Asp-[^{3}H]Pro-Ala-OH 0.9 0.52, 2020	7.1 100	[b] 0.3	7.3% ACN	1.80	>95
H-Tyr-Asp-[^{3}H]Pro-Ala-Pro-OH 0.4 0.17, 440	4.1 50	[b] 0.2	9% ACN	1.44	>98
H-Tyr-Asp-Pro-Ala-[^{3}H]Pro-OH 1.2 0.67, 2610	6.2 100	[b] 0.3	9% ACN	2.17	>97
H-Tyr-Asp-Pro-Ala-Pro_5-[^{3}H]Pro-OH 1.3 0.6, 1090	8.5 85	[b] 0.35	12% ACN	1.73	>95
H-[3H]Tyr-Asp-Pro-Ala-OH 0.25 0.11, 289	3.4 50	[d] 0.3	7.25% ACN	1.22	>98
H-[3H]Tyr-Asp-Pro-Ala-Pro-OH 0.4 0.18, 460	8.7 50	[d] 0.3	7.25% ACN	1.42	>98

[a] 25 × 0.4 cm Column, 5 µm, LiChrosphere WP 300 RP-18 (Merck, Darmstadt, Germany), flow 0.6 ml/min, isocratic mobile phase only, detection at 215 nm, and by radioactivity measurement
[b] N,N-Dimethylacetamide
[c] Only 0.05% TFA was used
[d] 0.1 M solution of $(NH_4)_2CO_3$

Materials and methods

General methodology

Protected amino acids were purchased from Bachem (Bubendorf, Switzerland) or were prepared in our laboratory following general protocols (Wünsch, 1974) 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 (Läufelfingen, Switzerland) and TOTU and TPTU reagents from Senn Chemicals International (Gentilly, France). Operations with tritium gas were carried out using an original apparatus (Hanuš and Vereš, 1971). DMA was carefully purified and all aqueous peptide solutions were prepared from twice distilled water (Černý and Hanuš, 1981). Catalyst $PdO/BaSO_4$ for tritiation was prepared according to literature (Kuhn and Haas, 1955). During preparation of 3,4-dehydroproline containing peptides the solvents were evaporated in vacuum on a rotary evaporator (bath temperature 30 °C); DMF was evaporated at 30 °C and 150 Pa. Progress in solid phase synthesis was monitored by a ninhydrine (Kaiser et al., 1970) and bromophenol blue (Krchňák et al., 1988) tests. 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, U.K.). 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 preparative HPLC on a 25 × 2.2 cm column, 10 µm Vydac RP-18 (The Separations Group, Hesperia, CA, U.S.A.), flow rate 7 ml/min, detection at 220 nm using a 0–100% gradient of MeOH in 0.05% aqueous TFA, 120 min, unless otherwise stated. The analytical HPLC was carried out on a 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 a 0–100% gradient of ACN in 0.05 aqueous TFA, 40 or 60 min.

Examples are presented to demonstrate application of tritiated oostatic peptides in analysis of the time course of a total radioactivity within ovaries and head after injection to *Neobellieria bullata*.

Synthesis of 3,4-dehydroproline containing peptides and their protected derivatives

Boc-$^{\Delta}$Pro-Ala-OMe, **1a**

A solution of Boc-$^{\Delta}$Pro-OH (0.21 g; 1 mmol), HOBt (0.15 g; 1.1 mmol) and DCC (0.23 g; 1.1 mmol) in DMF (2 ml) was stirred at −10 °C for 30 min and then filtered to a cooled solution of HCl. H-Ala-OMe (0.28 g; 2 mmol) in DMF (1 ml) and pH 7.5 adjusted by DIEA. The reaction mixture was stirred at 0 °C for 2 h followed by stirring at room temperature, overnight. The mixture was cooled in refrigerator and the precipitated dicyclohexylurea separated by filtration and DMF evaporated. The residue was dissolved in EtOAc (20 ml) 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 solution was evaporated and product purified on a column of silica Merck 60 in a 20% EtOAc-PE solvent mixture to give 0.28 g of product **1a**.

Boc-Asp(OBzl)-$^{\Delta}$Pro-Ala-OMe, **1b**

Compound **1a** (0.13 g; 0.44 mmol) was treated with a TFA (4 ml) – H_2O (0.13 ml) – TIS (0.05 ml) mixture at room temperature for 1 h. The reaction mixture was evaporated to dryness and corresponding trifluoroacetate (0.12 g) was dissolved in DMF (1 ml) and added to a stirred mixture of Boc-Asp(OBzl)-OH (0.19 g; 0.6 mmol), HOBt (0.11 g; 0.8 mmol) and TOTU (0.26 g; 0.8 mmol) in DMF (1 ml) adjusted to pH 7.5 with DIEA (moisted paper). After 16 h of stirring at room temperature, the mixture was evaporated to dryness and residue was worked up as described in compound **1a**, to yield 0.22 g of product **1b**.

H-Tyr-Asp-$^{\Delta}$Pro-Ala-OH, **1c**

Protected tripeptide **1b** (0.24 g; 0.45 mmol) was treated with a TFA (7 ml)-water (0.2 ml)-TIS (0.1 ml) mixture at room temperature. After 1 h, the solution was evaporated to dryness and TFA.Asp(OBzl)-$^{\Delta}$Pro-Ala-OMe (HPLC peak retention time 14.51 min) obtained was reacted with Boc-Tyr-OSu (0.24 g; 0.6 mmol) and HOBt (0.08 g; 0.6 mmol) in DMF (4 ml). The reaction mixture was stirred at pH 7.5, adjusted by DIEA, at room temperature for 20 h. After DMF was evaporated, the residue was worked up similarly to compound **1a** to yield 0.26 g (0.40 mmol) of protected peptide Boc-Tyr-Asp(OBzl)-$^{\Delta}$Pro-Ala-OMe. AA: Ala 1.00, $^{\Delta}$Pro 0.94, Asp 1.03, Tyr 1.02. Analytical HPLC peak retention time 21.05 min. Protected tetrapeptide was treated with a TFA (6.2 ml)-water (0.18 ml)-TIS (0.1 ml) mixture at room temperature. After 1 h, the solution was evaporated to dryness and the corresponding di-ester of tetrapeptide trifluoroacetate in a water-acetone 1:1 mixture (6 ml) was saponified by 2 M NaOH (0.44 ml) at room temperature for 2 h. The alkaline solution was acidified to pH 2 on addition of 10% H_2SO_4 and acetone was evaporated. An acidic water solution was applied on the top of the Dowex 50 × 2 column (20 ml), sulfate ions were eluted by H_2O and peptide **1c** by 10% NH_4OH. After the ammonia was evaporated, the water solution was freeze dried and tetrapeptide **1c** (0.16 g) purified by preparative HPLC on Vydac 25 × 2.2 cm column, gradient 0–50% of MeOH in 0.05% aqueous TFA, 120 min, flow 5 ml/min, 280 nm, to yield 0.11 g of pure peptide. The main peak was collected at 24.07 min.

H-Tyr-Asp-$^{\Delta}$Pro-Ala-Pro-OH, **1d**

Fmoc-Pro-OH was loaded to 2-chlorotrityl chloride resin (1 g) with a substitution of 0.43 mmol/g of the resin (Hlaváček et al., 2001). After Fmoc deprotection by a 20% piperidine/DMF (10 ml)-20% TFE/DCM (10 ml) mixture, 20 min and 20% piperidine/DMF (20 ml), 30 min, the H-Pro-resin was successively acylated with Fmoc-derivatives of Ala, $^{\Delta}$Pro, Asp(OtBu) and Tyr(tBu) using a mixture of DIC-HOBt reagents. After the last Fmoc deprotection by above mixture, a detachment of peptide from the resin and the side-chain deprotection were performed simultaneously by treatment with a 50% TFA-5% anisole-5% TIS mixture in DCM. The solution was evaporated to dryness and pentapeptide **1d** was then purified by preparative HPLC using the conditions described in the purification of peptide **1c**, to yield 0.12 g of pure peptide.

Boc-Asp(OBzl)-$^{\Delta}$Pro-OMe, **2a**

The HCl · H-$^{\Delta}$Pro-OMe (0.082 g; 0.5 mmol) was added to solution of Boc-Asp(OBzl)-OH (0.24 g; 0.75 mmol), HOBt (0.14 g; 1 mmol) and TOTU (0.33 g; 1 mmol) in DMF (1.5 ml). The reaction mixture was adjusted to pH 7.5 with DIEA (moisted paper) and was stirred at room temperature for 16 h. After evaporation of DMF the residue was worked up as described in the preparation of compound **1a** to give 0.21 g of protected dipeptide **2a**, which was purified on precipitation from an EtOAc-PE mixture to yield 0.15 g of pure compound.

H-Asp-$^{\Delta}$Pro-OH, **2b**

The acidic and alkaline hydrolysis described in preparation of peptide **1c** was used to deprotect compound **2a** (0.14 g; 0.32 mmol) and to obtain dipeptide **2b**, which was purified by isocratic preparative HPLC on VYDAC 25 × 2.2 cm column, mobile phase 0.05% aqueous TFA, flow 3 ml/min to yield 60 mg of pure compound. The main peak was eluted at 15.78 min.

Boc-Tyr-Asp(OtBu)-Pro-Ala-$^{\Delta}$Pro-OMe, **2c**

Boc-Tyr-Asp(OtBu)-Pro-Ala-OH (Hlaváček et al., 1998) (0.15 g; 0.24 mmol) was dissolved in DMF (1 ml) and to this solution, adjusted to pH 7.5 with DIEA (moisted paper), the HOBt (0.08 g; 0.6 mmol), TPTU (0.18 g; 0.6 mmol) and HCl · H-$^{\Delta}$Pro-OMe (0.075 g; 0.46 mmol) were added under stirring at 0 °C. The reaction mixture was stirred at room temperature for 16 h, then evaporated to dryness and worked up similarly to preparation of compound **1a** to yield 0.14 g of protected pentapeptide methyl ester **2c**, which was purified on precipitation from an EtOAc-PE mixture.

H-Tyr-Asp-Pro-Ala-$^{\Delta}$Pro-OH, **2d**

Compound **2c** (0.07 g; 0.125 mmol) was deprotected using acidic and alkaline hydrolysis, and purified by ion exchange chromatography as already described in the preparation of peptide **1c**. Product was purified by preparative HPLC on the Vydac column 25 × 2.2 cm, gradient 0–50% of MeOH in 0.05% aqueous TFA, 60 min, flow 7 ml/min, 280 nm, to obtain 32 mg of pure peptide **2d**.

Boc-Tyr(BrZ)-Asp(OtBu)-Pro-Ala-Pro_5-$^{\Delta}$Pro-OMe, **2e**

According to the procedure described (Hlaváček et al., 1998), however, using a different tyrosine protection and detachment from the resin (20% AcOH in DCM), the Boc-Tyr(BrZ)-Asp(OtBu)-Pro-Ala-Pro_5-OH was prepared (0.71 g; 0.54 mmol). Protected nonapeptide acid was added to a mixture of HCl · H-$^{\Delta}$Pro-OMe (0.18 g; 1.1 mmol), HOBt (0.115 g; 0.85 mmol) and TPTU (0.26 g; 0.85 mmol) in DMF (1.5 ml) at 0 °C. Reaction mixture, adjusted to pH 7.5 with DIEA (moisted paper), was stirred at 0 °C for 2 h and overnight at room temperature. The DMF was evaporated to dryness and the residue was worked up as described in preparation of compound **1a** to yield 0.73 g of protected decapeptide.

H-Tyr-Asp-Pro-Ala-Pro_5-$^{\Delta}$Pro-OH, **2f**

The compound **2e** (0.07 g; 0.05 mmol) was successively treated with a thioanisol-EDT 2:1 (0.3 ml) mixture and with TFA (2 ml) at room temperature, each for 10 min, and subsequently with TFMSA (0.1 ml) added drop-wise at −10 °C. The reaction mixture was stirred at room temperature for 1 h and than evaporated to dryness. After dissolving in an acetone-water 2:1 mixture (6 ml), the remaining methyl ester was treated with 4 M NaOH (0.2 ml) at room temperature for 1 h. Acetone was evaporated, the alkaline solution acidified with 10% H_2SO_4 and applied on the top of a Dowex 50 × 2 column (15 ml). After elution of sulfate ions by water, the peptide **2f** was eluted by 10% NH_4OH. The ammonia was evaporated and the remaining solution was freeze dried to give 0.42 g of the free decapeptide that was purified by preparative HPLC using standard procedure to yield 0.27 g of pure decapeptide **2f**.

Preparation of ^{3}H-labeled oostatic peptides

Method A: addition of tritium to double bond of 3,4-dehydroproline residue

The peptide was dissolved in N,N-dimethylacetamide, PdO/$BaSO_4$ catalyst (10% Pd) was added and this mixture was stirred with tritium gas

(80 kPa) at room temperature for 2 h. After the reaction mixture was freeze-dried, the residue was dissolved in water (0.5 ml), the catalyst was removed by centrifugation and washed with water (0.5 ml). The combined water solutions were freeze-dried and product was purified by radio-HPLC on a 25×0.4 cm column, 5 μm, LiChrosphere WP 300 RP-18 (Merck), flow 0.6 ml/min, detection at 215 nm, and by radioactivity measurement, using either a gradient of ACN in 0.05% aqueous TFA or using an isocratic mode of 0.05% aqueous TFA. The labeled peptide purity was checked by analytical HPLC using the same column and radioanalytical detector, flow 1 ml/min and a gradient 0–100% ACN in 0.05% aqueous TFA, 40 or 60 min.

Method B: isotopic exchange of benzyl hydrogen in tyrosine residue by tritium

The peptide H-Tyr-Asp-Pro-Ala-OH (4P) or H-Tyr-Asp-Pro-Ala-Pro (5P) was dissolved in aqueous 0.1 M $(NH_4)_2CO_3$ and the labeling as well as working up of the reaction mixture including radio-HPLC purification were performed using the same conditions described in the method A.

UV spectrophotometry at 275 nm using Helios β instrument (Unicam, USA) was utilized for determination of the mass of products. The parameters of peptide tritiation following the method A and B, and HPLC purification of tritiated peptides are shown in Table 2.

Measurement of radioactivity after peptide application

The insect species used, including its development, was described previously (Slaninová et al., 2004). 37 kBq of a labelled peptide (Table 2) in 5 μL of Ringer solution was injected into the upper part thorax of ethanol-anesthesized 3–4 days old females *Neobellieria bullata*. Each experimental group consisted of 10 flies. Their ovaries or head, resp., were dissected in appropriate time intervals. Each separated sample (a head or a pair of ovaries) was placed into a scintillation vial and covered by 1 ml of a Tissue Solubilizer (NCS-II, Amersham) and 10 ml of a liquid scintillator EcoLite (ICN Biochemicals Inc.) was added after 2 days. Before measurements the chitinans remaining from heads were not removed. The radioactivity was measured by a liquid scintillation spectrometer (Beckman LS-6500) with a standard deviation lower than ±1%. In each experimental group the highest and the lowest measured value was eliminated and the arithmetic means of the remaining eight values were used in expression of the radioactivity dependence on time after injection. The standard deviations in all measured sets were lower than ±35%.

Results and discussion

In order to measure the radioactivity incorporated into the target organ of *Neobellieria bullata* after a treatment with tritiated oostatic peptides and to compare it with the head radioactivity, we firstly synthesized their precursors containing 3,4-dehydroproline (Table 1). This amino acid was placed either in the C-terminal or in the central part of peptide molecules. Besides the tetra and pentapeptides **1c**, **1d** and **2d** corresponding to the most potent oostatic peptides we also prepared analog **2b** of the less potent deleted oostatic dipeptide together with analog **2f** corresponding to the natural decapeptide (Borovsky, 2003) with the lowest oostatic activity from the series (Slaninová et al., 2004). The physicochemical studies using CD and VCD spectra revealed a tendency of this decapeptide to adopt a helical structure, from which the more active peptide sequences are released gradually – as the proline residues are successively cleaved by enzymes from its carboxy-terminus (Maloň et al., 2003).

In the synthesis, the solution, solid phase and a combination of both the methods were utilized, depending on the length of peptide chain and on the position of 3,4-dehydroproline in a given peptide sequence. Thus, the shorter peptides **1c** and **2b** were prepared exclusively in solution. We experienced that the imino group of 3,4-dehydroproline exhibits lower nucleophile reactivity than that of proline itself. Therefore, the acylation of this imino acid was mediated by TOTU reagent that has been already successfully used in the solid phase synthesis of difficult sequence of the human prion peptide HuPrP 106–126 (Šebestík et al., 2004). Using this reagent, satisfactory yields of couplings in corresponding derivatives were obtained. A combination of solid and solution synthetic approach was utilized in the preparation of penta- and decapeptide **2d** and **2f**, respectively. We have found it advantageous to synthesize the protected N-terminal tetra- and nonapeptide, respectively, on 2-chlorotrityl-polymer (Barlos et al., 1991) first and then, after detachment of the protected peptide acids from the resin, to couple these segments with methylester of 3,4-dehydroproline using the racemization suppressing TPTU as the reagent of choice. In such a way, this imino acid was introduced into the C-terminus of protected peptides **2c** and **2e** with satisfactory yields. Due to the utilization of coupling reagent mixture of the above reagent with HOBt as a racemization-suppressing additive, no racemization was observed in the peptide bond formation. This result can be also ascribed to usage of exact equivalent amount of DIEA for adjusting the pH 7.5 of the reaction mixture. The peptide **1d** was synthesized exclusively on polymer support. The 2-chlorotrityl linker on polystyrene resin is particularly suited to synthesis of proline peptides. The extreme steric hindrance of the trityl group means that formation of dioxopiperazine at the dipeptide step, with the consequent loss of peptide from the resin, is totally suppressed. Also in the synthesis of peptide **1d**, 2-chlorotrityl-resin was used successfully. Achieving the high yield (over 99%) in coupling reaction – a necessary assumption of successful solid phase synthesis – was attained by using a four-fold excess of protected amino acids and the reagents, as well, used for the coupling.

The difference in the length of peptides in our series required a careful selection of conditions for their HPLC purification. The longer and lipophylic peptides **1c**, **1d**, **2d** and **2f** were purified using a gradient of ACN in 0.05%

aqueous TFA, while the dipeptide **2b** was purified using isocratic system of 0.05% aqueous TFA, only.

The peptides **1c**, **1d**, **2b**, **2d** and **2f** were radiolabeled by addition of tritium to double bond of 3,4-dehydroproline residue, in the presence of barium catalyst. The catalytic exchange of benzyl hydrogens in the tyrosine residue for tritium, introduced ^{3}H label also into the amino-terminus of tetra- and pentapeptides, containing proline residues only.

A method used for preparation of ^{3}H labeled peptides corresponded to substrate for tritiation. The addition of tritium to double bond was carried out in N,N-dimethylacetamide because this solvent is efficient enough to dissolve highly hydrophilic peptides and also stable enough to avoid side-products formation that could decrease the efficiency of catalyst used. The exchange of tritium for benzyl hydrogens in tyrosine residue was carried out in ammonium carbonate to ensure a correct pH of reaction mixture necessary for high yield exchange.

Dependencies of the measured total radioactivity on time after injection of tritiated **2f**, **2d** and **2b** were in all cases comparable for ovaries and also for head. As is shown for **2f** and **2b** in Fig. 1, the total radioactivity was decreased from 1 to 144 h in head while it was stable in ovaries where approximately 1% of the radioactivity of the applied peptide was accumulated. It can be supposed that after rapid metabolic cleavage, predominantly C-terminal radioproline (Tykva et al., 2005a) was accumulated in ovaries while successively eliminated from head. On these processes partly also some shorter labelled sequences after metabolic degradation of an applied radiopeptide **2f** or **2b** could partly take part as well as the non-degraded applied labeled peptides themselves. Anyway, the total radioactivity of the target organ was found stable during the whole investigated period of 1 h to 6 days after a labelled peptide injection. The end of this time interval was chosen as a start of the second gonotrophic cycle which was previously found to be decisive for oostatic effects and their histological observations (Slaninová et al., 2004).

For further analysis in ovaries, shorter time intervals after peptide injection were used for radiopeptides of **2d** and **1d** (5P) as well as **1c** (4P) (Fig. 2) having the highest oostatic effect (Slaninová et al., 2004). The comparable courses of total radioactivities for all radiopeptides tested have been approximately stable already from the beginning of the measurements. The level of total radioactivity corresponds to longer time intervals (Fig. 1) and does not depend on the position of proline labelling. For comparison, 4P or 5P labelled in Tyr^{1} were injected. For both peptides, the total radioactivity in ovaries at a several times higher level was detected, starting 1 h after injection (Fig. 2).

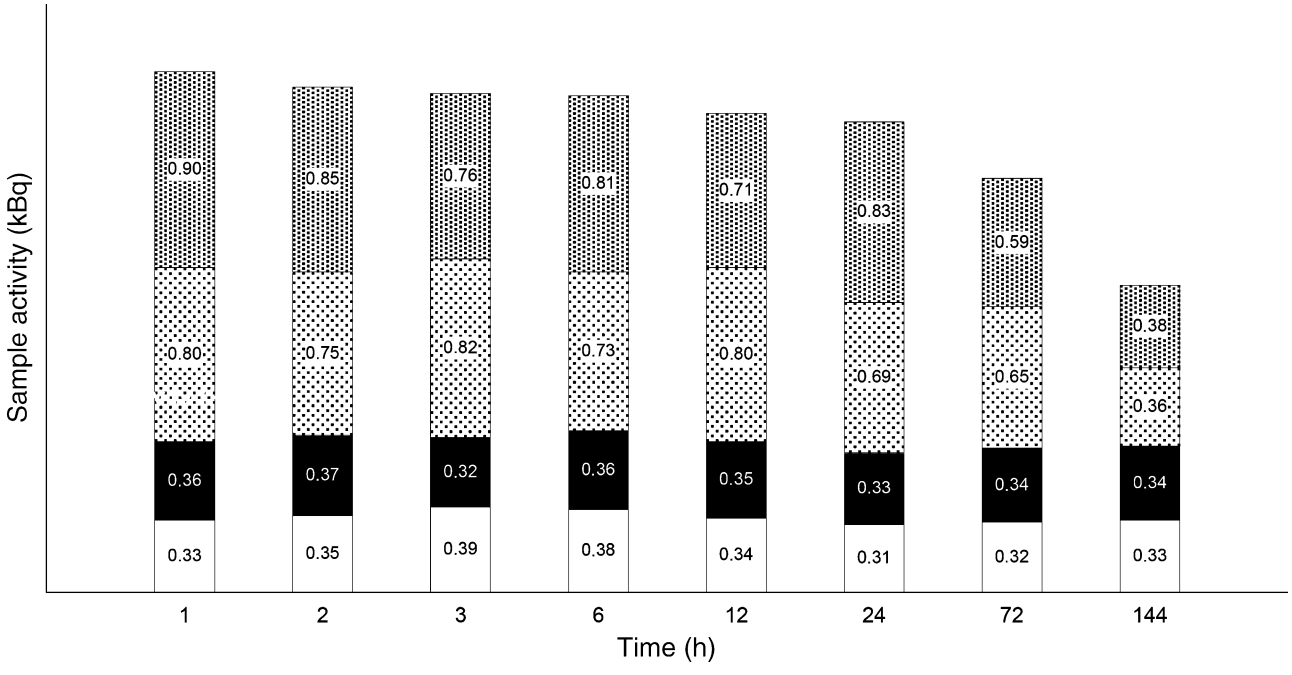


Fig. 1. Dependence of total radioactivity of a pair of ovaries or a head, respectively, isolated from *Neobellieria bullata* on time after injection of 37 kBq of a peptide tritiated in C-terminal proline: (S.D. $< \pm 35\%$): H-Asp-[^{3}H]Pro-OH – □ ovaries; ▦ head; H-Tyr-Asp-Pro-Ala-Pro_5-[^{3}H]Pro-OH – ■ ovaries; ⚄ head

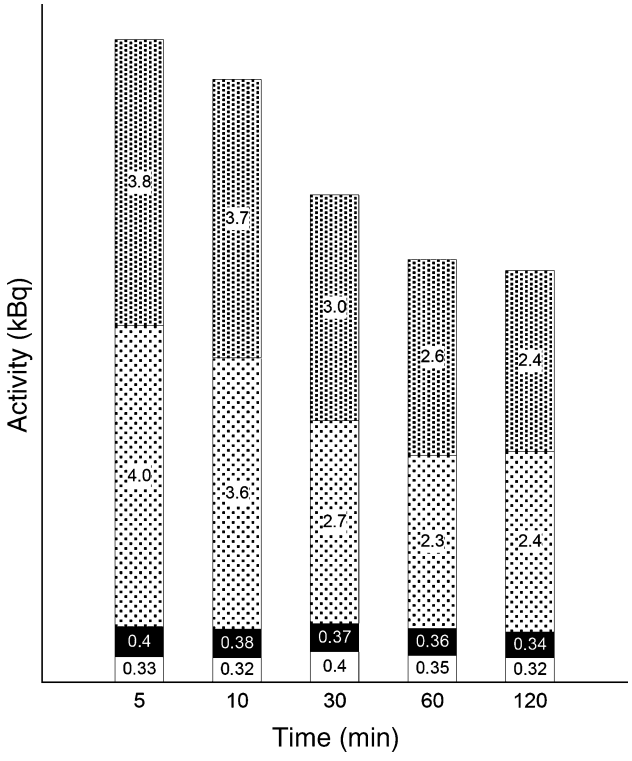


Fig. 2. Dependence of total radioactivity of ovaries isolated from *Neobellieria bullata* on time after injection of 37 kBq of a peptide tritiated in different positions of the peptide chain: (S.D. $< \pm 35\%$): ⚄ [^{3}H]Tyr^1-5P; □ [^{3}H]Pro^3-5P; ■ [^{3}H]Pro^5-5P; for comparison: ▦ [^{3}H]Tyr^1-4P

Between 5 min and 1 h it was continuously decreasing. It can be supposed that these differencies are caused by different metabolism of tyrosine and proline.

Since insects possess an open circulatory system, hemolymph can freely circulate around all body organs. It can be expected that peptides injected into the body cavity are transported into all parts of the body quite easily. Therefore, the radioactivity consisting of an applied peptide and its radioactive decay products was detected also in head. Its relatively high values, especially, shortly after a peptide application as well as the radioactivity decreasing depending on time could be explained by the hemolymph transportation. The brain is mainly composed of lipidic material and there is a very little demand for protein turnover.

On the contrary, the ovary presents an unique composition of tissues equipped with metabolic system concentrated on accumulation of yolk building components for rapidly growing egg. Both the follicular and nutritive cells actively transport vitellogenic substances from the hemolymph into the oocyte and thus the radioactivity intake of ovaries mirrors the actual stages of vitellogenesis.

The obtained results formed an interesting basis for metabolic studies of an insect oostatic peptide beeing under way (Tykva et al., 2005b).

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