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Mass spectrometric study of peptides secreted by the skin glands of the brown frog *Rana arvalis* from the Moscow region

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A high-performance liquid chromatography nano-electrospray ionization Fourier transform mass spectrometry (HPLC/nanoESI-FTMS) approach involving recording of collision-activated dissociation (CAD) and electron-capture dissociation (ECD) spectra of an intact sample and two its modifications after performic oxidation and reduction followed by carboxamidomethylation helps to establish peptide profiles in the crude secretion of frog species at mid-throughput level, including *de novo* sequencing. The proposed derivatization procedures allow increasing of the general sequence coverage in the backbone, providing complementary information and, what is more important, reveal the amino acid sequence in the cystine ring ('rana box'). Thus purely mass spectrometric efficient sequencing becomes possible for longer than usual proteolytic peptides.

Seventeen peptides belonging to four known families were identified in the secretion of the European brown frog *Rana arvalis* inhabiting the Moscow region in Russia. Ranatuerins, considered previously a unique feature of the North American species, as well as a new melittin-related peptide, are worth special mention. The developed approach was previously successfully used for the identification of peptides in the skin secretion of the Caucasian green frog *Rana ridibunda*.

Several recent papers published by our group deal with mass spectrometric study of the primary structure of peptides generated by the dorsal glands of ranid frogs inhabiting Russia.[1–3] Antibacterial peptides secreted by amphibians in response to external impulses possess double scientific interest. Due to their wide spectrum of biological activity they are considered to be a powerful source of future pharmaceuticals,[4] while they may also serve as taxonomic markers allowing distinction of the related species.[5] In addition, the establishment of the composition of the peptide fraction of the amphibian skin secretion allows phylogenetic connections to be found between various species and families in the order of tailless amphibia *Anura rafinesque*, required to understand pathways of evolutionary development of hylid and ranid frogs.[6,7]

It is known that the skin secretion of amphibians is a complex mixture of biologically active compounds including biogenic amines, alkaloids and peptides.[8] The latter group of compounds in the case of ranid frogs usually includes the following components: (1) neuropeptides of bombesins,[9] ranatensins,[9] tachykinins[10] and bradykinins[11]; (2) long bactericidal disulphide-containing peptides belonging to 13 various families: brevinins-1 and -2;[12] esculentins-1 and -2;[13,14] ranatuerins-1 and -2;[15] ranalexins;[16,17] palustrins-1, -2 and -3;[18] tigerinins;[19] japonicins-1 and -2;[20] and, finally, (3) temporins – the shortest species among the known antimicrobial peptides.[21]

Direct sequencing of the intact disulphide-containing antimicrobial peptides by means of mass spectrometry (MS) is rarely successful, although the use of negative ion MS allowed the primary structure of the authentic brevinin-1E, isolated from the skin secretion of the green frog *Rana ridibunda*, to be established.[22] Unfortunately, in the majority of the known examples this approach does not bring the desired results. Thus, preliminary destruction of the S–S bond is required for complete C-terminal sequencing. There are two general methods of S–S bond modification in peptides: (1) reduction followed by derivatization of the reduced cystine residues and (2) oxidation. A recent review[23] summarizes the known agents for reducing disulphide bonds as well as derivatizing agents. The reported variants of oxidation are also listed.

The present work aims at establishing the primary structure of the peptides constituting the secretion of the skin glands of the common brown frog *Rana arvalis* from the Moscow region in Russia by means of mass spectrometry. *Rana arvalis* inhabits a vast territory from south Sweden and Finland to France, south-east Europe and even Siberia.[24]

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There are no published data on the skin peptides of this species.

The present study involved consecutive nanospray electrospray ionization Fourier transform mass spectrometry (nanoESI-FTMS) analysis of the crude secretion of *Rana arvalis* and two samples of the same crude secretion after modification of the S–S bonds. Reduction with dithiothreitol followed by carboxamidomethylation as well as oxidation with performic acid were used. Since each of these two procedures has some advantages and shortcomings their joint application provides complementary structural information, increasing the reliability of the mass spectrometric sequencing of the disulphide-containing peptides.[25] To further increase the reliability electron capture dissociation (ECD) and collision-activated dissociation (CAD) spectra were recorded in each case. The developed approach was previously successfully used for the identification of peptides in the skin secretion of the Caucasian green frog *Rana ridibunda*.[2]

EXPERIMENTAL

Skin secretion

Ten male and female specimens of the Moor frog *R. arvalis* were caught near Zvenigorog in the Moscow region (Russia). The animals were maintained in captivity under conditions close to natural ones.

Secretion from the skin glands was provoked by mild electrical stimulation. The procedure details have been described previously.[26] The skin of the animal was moistened with deionized water and then treated with a bipolar platinum electrode connected to a laboratory electrostimulator (ESL-1). The pulse parameters were as follows: voltage, 10 V; pulse duration, 5 ms; pulse frequency, 50 Hz. The skin secretion was washed out with a small amount (up to 25 mL) of deionized water into a container with an equal volume of methanol.

The mixture was then centrifuged for 15 min at 3000 rpm, the supernatant was filtered through a Millex-FH membrane (PTFE 0.45 μm; Millipore, Billerica, MA, USA) and concentrated at 35°C on a rotary evaporator to a volume of 1 mL. To avoid possible seasonal changes in the peptide profile, 'milking' was always performed in the same month of the year.

HPLC separation of the skin secretion

High-performance liquid chromatography (HPLC) fractionation was carried out using a C_{18} reversed-phase analytical column (5 μm, 110 Å, 150 × 4 mm; BioChimMac, Moscow, Russia), equilibrated with 10% acetonitrile/aqueous 0.1% trifluoroacetic acid (TFA) (Acros, Geel, Belgium). The single injection volume was less than 20 μL to improve the separation. Peptide separation was performed with an HPLC system from ThermoSeparation Products (Piscataway, NJ, USA), equipped with two P2000 gradient pumps (ThermoSystem, San Jose, CA, USA). Peptides were purified using a linear gradient from 10 to 70% (60 min) acetonitrile containing 0.1% TFA. The flow rate was 1.0 mL/min. The absorption was measured using a model UV3000 UV detector at 214 nm (Spectra Systems, San Jose, CA, USA). The dry samples were stored at −26°C.

Mass spectrometric sequencing of the peptides

All experiments were performed on a 7-Tesla hybrid mass spectrometer with linear ion trap, quadrupole and ion cyclotron resonance (ICR) cell (LTQ FT; Thermo Fisher Scientific, Bremen, Germany) coupled with an 1100 nanoflow system (Agilent, Santa Clara, CA, USA). The mass spectrometer toggles between a survey MS scan (resolving power 100 000) and consecutive ECD and CAD MS/MS scans (resolving power 25000) of the two most abundant peptides detected eluting at that moment from the nano-LC column. The experimental details are described in Kocher *et al*.[27]

Disulphide bond reduction and alkylation

The HPLC-purified and lyophilized crude secretion was redispersed in ammonium buffer (100 mM NH_4HCO_3, pH 8.0) with 10 mM iodoacetamide and incubated in the dark for 1 h at 37°C. Prior to treatment with iodoacetamide, dithiothreitol was added at a concentration of 4 mM while nitrogen was flushed into the sample to provide an inert atmosphere throughout the reaction.[28]

Disulphide bond oxidation

Performic oxidation of the peptides was performed as described previously.[29] Formic acid (Sigma, St. Louis, MO, USA) was mixed with 30% hydrogen peroxide (Sigma) (19:1 v/v) and incubated for 1 h at room temperature, forming performic acid. The peptides were dissolved in formic acid and incubated for 1 h with a triple volume excess of the performic acid at 0°C. Finally, the sample was diluted fivefold with Milli-Q water, and the resulting solution was lyophilized.

Edman degradation

Automated Edman sequencing was performed using a standard procedure on a model 470 peptide sequencer (Applied Biosystems, Foster City, CA, USA), equipped with a 900A data-processing module.[30] Best results were achieved when peptides were adsorbed from 90% acetonitrile in water on an Immobilon membrane treated with ethanolic bioprene solution.

Peptides synthesis

Synthetic samples of brevinin-1AVa (molecular weight (MW) 1809 Da) and the C-terminal fragment of ranatuerin-2AVa (MW 1902 Da) (85% purity) were prepared by GenScript Corporation (Piscataway, NJ, USA) using L-isomers of amino acids.

Antimicrobial activity tests

Synthetic samples of brevinin-1AVa and the C-terminal fragment of ranatuerin-2AVa were tested for their antimicrobial activity at the Department of Microbiology of the Adelaide Institute of Medicine and Veterinary Science (Adelaide, Australia). The method involved establishing inhibition zones produced by the testing peptides on a thin plate covered by agar in the presence of various microorganisms. The process is discussed in detail in Jorgensen

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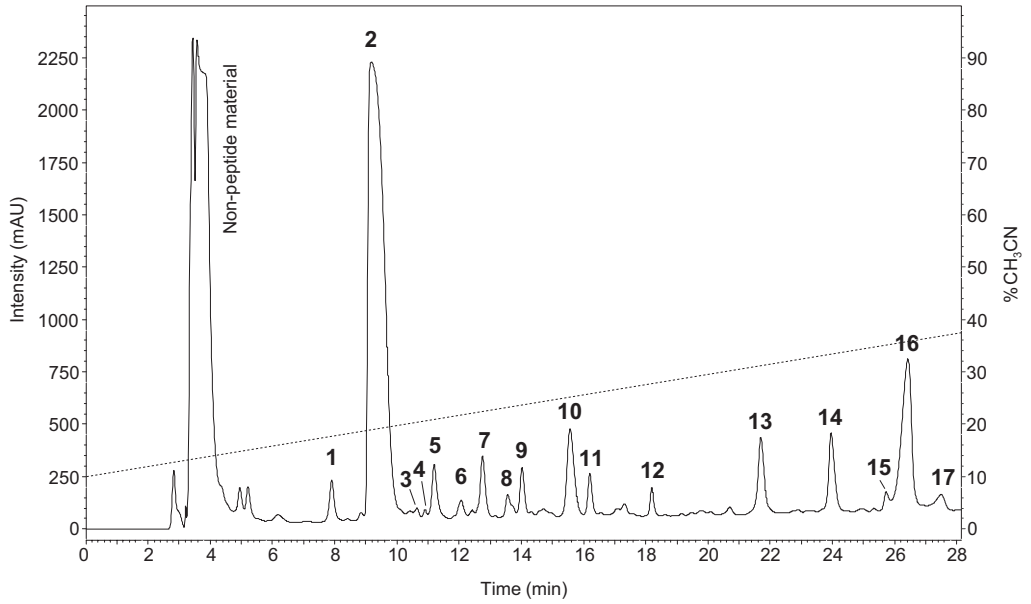

Figure 1. HPLC chromatogram of intact skin secretion of *R. arvalis*.

et al.[31] The peptides were tested towards Gram-positive bacteria: *Bacillus cereus*, *Enterococcus faecalis*, *Leuconostic lactis*, *Listeria innocua*, *Micrococcus luteus*, *Staphylococcus aureus*, *Staphylococcus epidermis*, *Staphylococcus uberis* and Gram-negative species: *Enterobacter clorae*, *Escherichia coli*, *Pasteurella multocida* and *Pseudomonas aeruginisa*.

RESULTS AND DISCUSSION

Figure 1 represents a general view of the HPLC chromatogram of the authentic skin secretion of *R. arvalis*. The sequences of 17 peptides identified in the secretion of this frog are summarized in Table 1. Four disulphide-containing peptides (**1–4**), one melittin-related peptide **5**, neuropeptide bradykinin **6** with its nine intermediates (**7–15**) as well as intermediates of two bradykinin modifications (**16–17**) are listed.

Disulphide-containing peptides in the secretion are presented by brevinin-1 and ranatuerin-2 families. In the opinion of some researchers the array of disulphide-containing peptides in the skin secretion of ranid frogs may characterize certain species and thus these compounds may be treated as a group of taxonomic markers.[5] Conlon *et al*.,[5] summarizing the data on frog peptides available in 2004, concluded that ranatuerins-2 may be treated as identity markers for the North American species, never having been reported in the European species. Nevertheless, two peptides from the ranatuerin-2 group were discovered by us in the secretion of the European frog *R. arvalis* (see Table 1). The unique characteristic feature of these peptides involves formation of a six-membered C-terminal disulphide ring, via the thiol groups of the side chains of cysteins.

Figure 2 represents mass spectra of a peptide with nominal molecular mass of 2824 Da in the intact crude *R. arvalis*

Table 1. Peptides isolated from the skin secretion of the brown frog *R. arvalis* from the Moscow region

№	MW	Name	Sequence	Peak
1	1809	Brevinin-1AVa	FLPLLAASFACTVTKKC-OH	13
2	1872	Brevinin-1AVb	FVPLLVSKLVCVVTKKC-OH	14
3	2824	Ranatuerin-2AVa	GLLDVVKGAAKNLLASALDKLKCKVTGC-OH	16
4	2911	Ranatuerin-2AVb	GLMoDMVKGAAKNLFASALDTLKCKITGC-OH	15
5	2361	Melittin-related peptide	FVGAALKVLANVLPPVISWIKQ-NH_2	17
6	1059	Bradykinin	RPPGFSPFR-OH	2
7	806	[des-Arg^1,Pro^2]Bradykinin	PGFSPFR-OH	3
8	903	[des-Arg^1]Bradykinin	PPGFSPFR-OH	4
9	1187	AR-11	AGRPPGFSPFR-OH	5
10	1243	RA-11	RPPGFSPFRIA-OH	7
11	1285	RI-11	RPPGFSPFRII-OH	1
12	1340	RP-12	RPPGFSPFRIAP-OH	5
13	1498	RS-14	RPPGFSPFRIAPAS-OH	6
14	1712	RL-16	RPPGFSPFRIAPASTL-OH	9
15	2355	RD-21	RPPGFSPFRIAPASTLKR(DE)D-OH	10
16	1728	[Hyp^3]RL-16	RPHypGFSPFRIAPASTL-OH	8
17	1225	[Glu^5]RA-11	RPPGESPFRIA-OH	5

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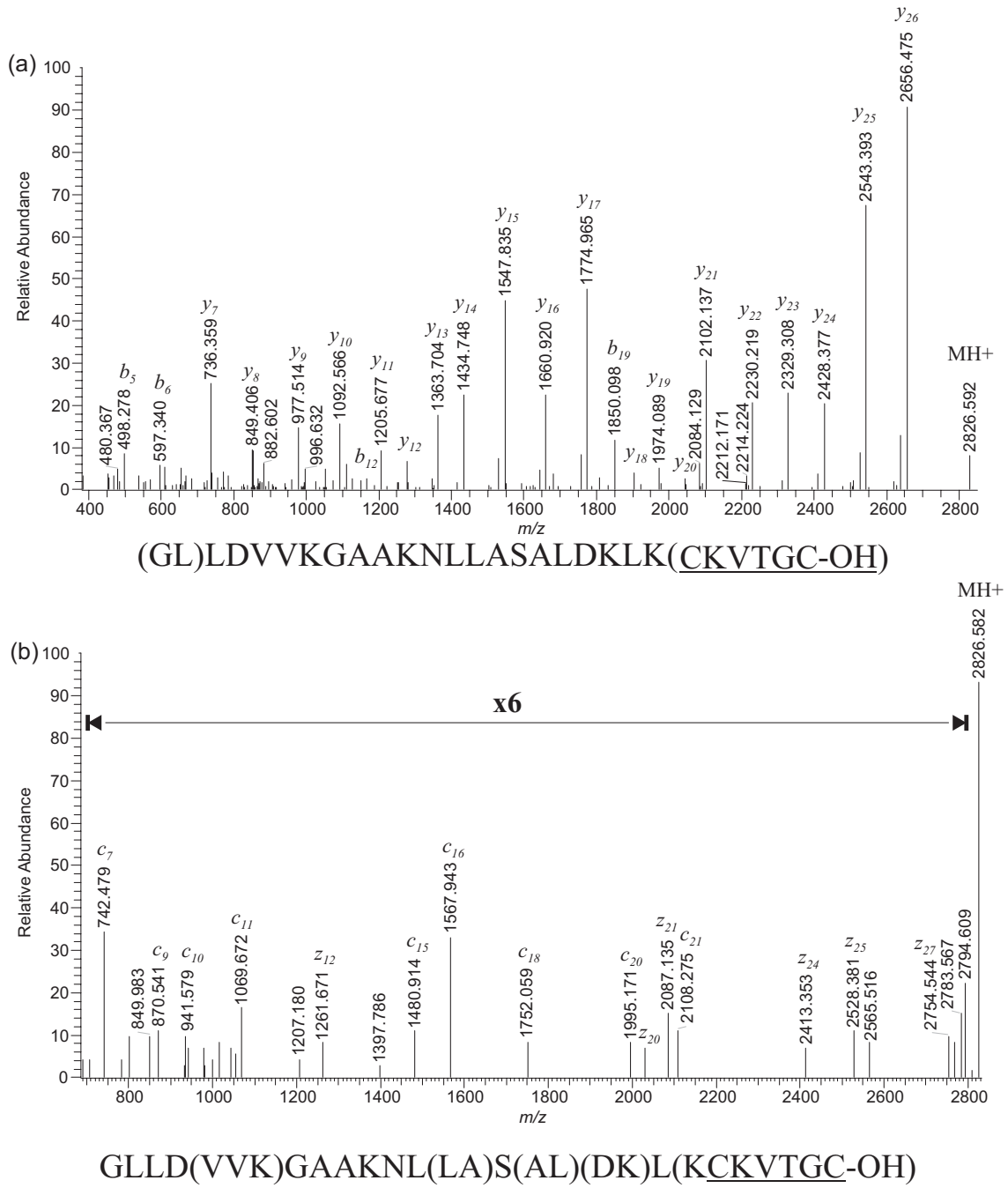

Figure 2. Mass spectra of the peptide with nominal molecular mass of 2824 Da: (a) CAD spectrum and (b) ECD spectrum. If no cleavages are observed between some amino acids those groups are presented in parentheses. Series of *y* and *b* ions and *z* and *c* ions were used to assign fragment identities.

sample. The spectra were recorded in CAD (Fig. 2(a)) and ECD (Fig. 2(b)) modes.

The information provided by the *b*-*y* and *c*-*z* ion series allows us to obtain the complete sequence, excluding the disulphide C-terminal ring. The spectrum of the quadruply charged molecule is richer than that of triply charged one (data not shown). Unfortunately, higher charge states are not generated in the experiment as they are unstable for a peptide with a molecular mass below 3 kDa.[32]

To define the C-terminal sequence of the peptide the original crude sample was oxidized with performic acid. This procedure results in the transformation of the thiol moieties of the cysteins into sulphonic acid groups (-SO_3H – groups, denoted as C_{Ox} below).[33] Figure 3 represents CAD and ECD spectra of a peptide with molecular mass 2824 Da after its oxidation.

The information obtained in the two experiments is sufficient to establish the complete sequence of this peptide, including the C-terminus. The only difficulty involves the relative positions of two residues (Ala17 and Leu18). Thus, four spectra of this peptide consisting of 28 amino acid residues (Figs. 2 and 3) provide its complete sequence:

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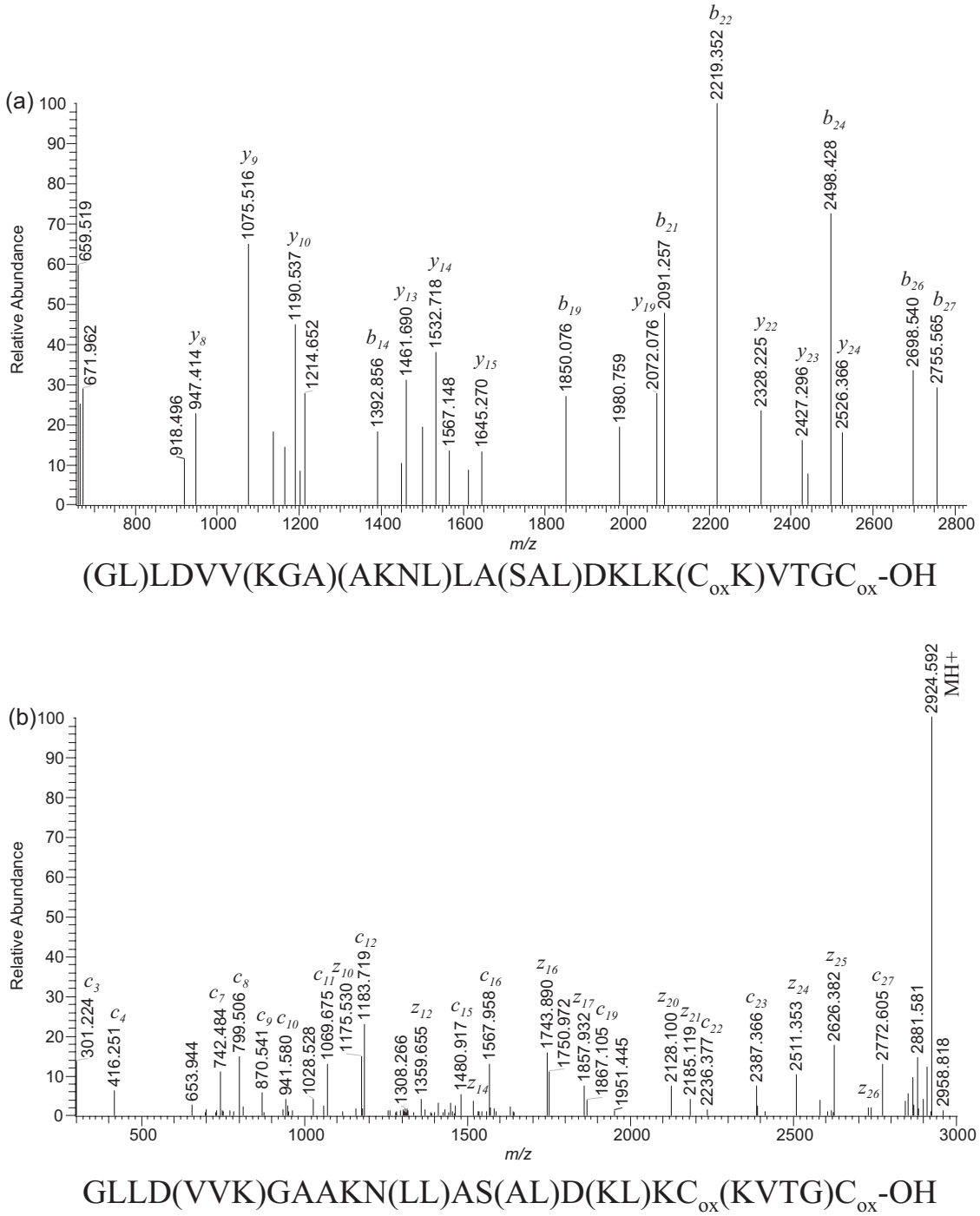

Figure 3. Mass spectra of the peptide with nominal molecular mass 2824 Da after performic oxidation: (a) CAD spectrum and (b) ECD spectrum. C_{ox} is used to indicate triply oxidized cysteine. If no cleavages are observed between some amino acids those groups are presented in parentheses. Series of *y* and *b* ions and *z* and *c* ions are used to assign fragment identities.

GLLDVVKGAAKNLLASALDKLKCKVTGC-OH. According to the accepted nomenclature,[5] and taking into account the structural similarity approach[34] (number of amino acid residues in the chain and six-membered C-terminal cycle), we classified it as a new ranatuerin-2, called ranatuerin-2AVa.

The residues in positions 7, 11, 20, 22, 24 of the ranatuerin-2AVa backbone are considered to be Lys, based on the exact mass measurements. The amino acid residues in the second and third positions were assigned as Leu on the basis of the MS^3 experiments, carried out on the doubly charged b_{10} ion. Figure 4 shows the ECD product ion spectrum of the doubly charged b_{10} ion.

ECD spectra allow us to distinguish between the isomeric pairs Leu/Ile, based on the losses of isopropyl or ethyl radicals from the respective *z* ions.[35] Figure 4 demonstrates

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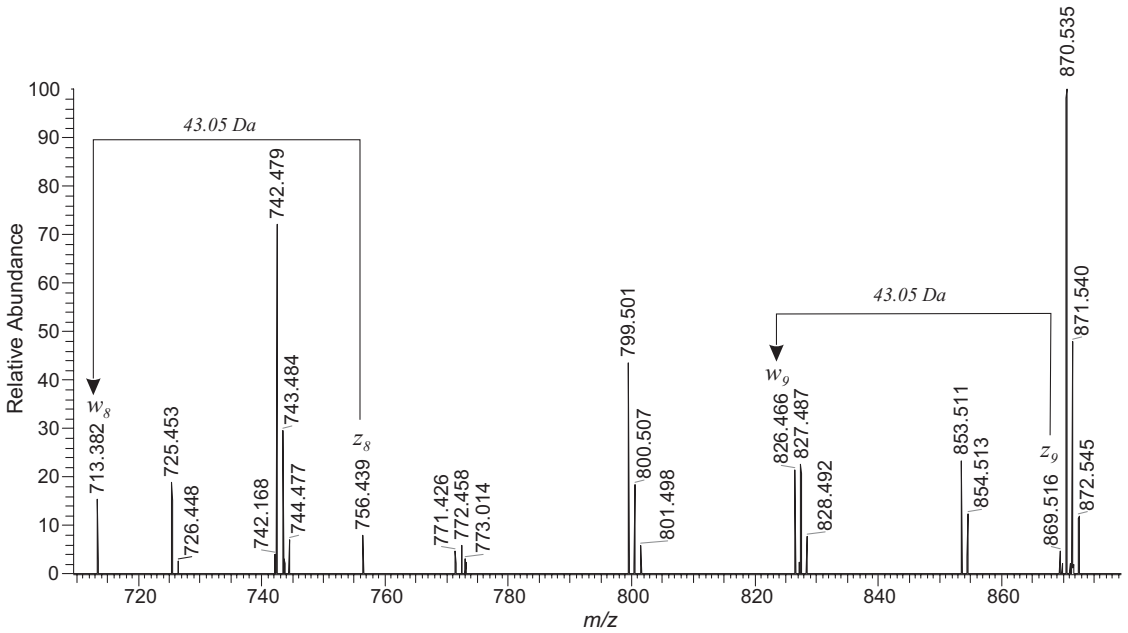

Figure 4. Part of the ECD spectrum of the doubly charged b_{10} ion of the peptide with nominal molecular mass 2824 Da (without extraction).

w_8 and w_9 (m/z 713.38 and 826.46) ions, formed due to losses of 43.05 Da from the z_8 and z_9 ions (m/z 756.44 and 869.52), and thus confirming the presence of two Leu residues in the second and third positions of the ranatuerin-2AVa sequence. The remaining four Leu residues (positions 13, 14, 18 and 21) are defined by the Edman degradation of the ranatuerin-2AVa fragment, formed by the proteolytic rupture of the peptide bond at the N-terminus of Lys11 (MW 1901 Da, peak 11 in the chromatogram in Fig. 1). The standard procedure for obtaining skin secretions of *R. arvalis* by electrostimulation of the dorsal glands[26] always leads to the formation of two ranatuerin pieces in the peptide mixture: chromatographic peaks №11 (MW 1901 Da, KNLLASALDKLKCKVTGC-OH) and №12 (MW 1923 Da, KNLFASALDTLKCKITGC-OH). To prevent this process taking place, we slightly changed the procedure – washing the secretion with deionized water from the skin into the container already filled with methanol. Thus, the time available for the endoproteolytic reaction of splitting the peptides at the Ala–Lys bond was minimized.

Ranatuerin-2AVb (MW 2911 Da) was also identified on the basis of mass spectra and other available information. The assignment of Leu residues in positions 2, 13, 18 and 21 was made by analogy with the related ranatuerin-2AVa. The identified C-terminal sequence completely coincided with that characteristic of ranatuerins-2: CKITGC.[34] Thus, the amino acid residue in the cystine ring was assumed to be Ile. A peculiarity of this peptide is the presence of two methionines in its sequence. One of them in position 3 was always detected in the oxidized form – $Met_{ox}3$. We did not succeed in detecting the non-oxidized form of ranatuerin-2AVb in the authentic crude serum of *R. arvalis*. However, the secretion always contains two peptides formed by proteolytic cleavage at the N-terminus of Lys in ranatuerin-2AVb: GLMDMVKGAA-OH (MW 991 Da) and KNLFASALDTLKCKITGC-OH (MW 1923 Da). The first one contains both methionines in the non-oxidized form. Therefore, oxidation of the authentic peptide takes place during sample preparation.

In addition to the two ranatuerins, two new disulphide-containing 17-residue peptides with molecular masses 1809 and 1872 Da were detected in the secretion. Their sequences were also established on the basis of the ECD and CAD spectra of the authentic and modified samples. Two types of modification were used: (1) performic oxidation of the S–S bond and (2) reduction of the S–S bond followed by carboxamidomethylation. Distinguishing between Leu/Ile pairs was achieved by ECD spectra. The w_{13}, w_{14} and w_{16} ions in the ECD spectrum of the doubly protonated peptide with MW 1809 Da, as well as the w_9, w_{13}, w_{14} ions in the ECD spectrum of the doubly protonated peptide with MW 1872 Da, were formed as a result of $C_3H_7^{\cdot}$ (43.055 Da) losses from the corresponding z ions, proving the presence of Leu in the mentioned positions. The sequences of these two peptides established by means of mass spectrometry were confirmed by Edman degradation. They were assigned to the brevinin-1 family and named brevinin-1AVa and brevinin-1AVb, respectively. Structurally (the number of the amino acid residues in the chain and the size of the disulphide cycle) both new brevinins are closely related to the 17-residue brevinin-1Ta detected previously in the secretion of the European brown frog *R. temporaria*: FITLLLRKFICSITKKC.[8] However, the sequences (CTVTKKC or CVVTKKC) of their disulphide moieties (excluding the second residue) coincide with these of ranalexines, skin peptides of the North American frogs: CAVTKKC.[34]

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Biotesting of brevinin-1AVa and the C-terminal fragment, consisting of 18 residues, of ranatuerin-1AVa towards 15 species of Gram-positive and Gram-negative bacteria proved their activity for *Leuconostic lactis:* their IC_{50} values are 32.4 and 72.9 μM, respectively.

Bradykinin (**6**) and bradykinin-related peptides (**7**–**17**) are also present in the skin secretion of *R. arvalis*. Bradykinin itself appeared to be the major component of the peptide mixture (Fig. 1). This compound is a powerful neuropeptide present in the skin secretion of various types of ranid frogs. It guarantees the functioning of the defense properties of amphibian skin.[34] The secretion also contains [des-Arg^1, Pro^2]bradykinin (**7**) and [des-Arg^9]bradykinin (**8**). It is worth mentioning that the latter is also present in the plasma of human blood, being generated from bradykinin by carboxypeptidase-C.[36]

Quite a number of peptide intermediates having several additional amino acid residues at the C- or N-termini of bradykinin were also detected. These are: AR-11 – N-extension; RA-11, RI-11, RP-12, RS-14, RL-16, and RD-21 – C-extension. These compounds are considered to form as a result of interaction of preprobradykinin with various endoproteases. The detected substitution of one residue at the terminal extension may be rationalized by expression of more than one gene coding bradykinins and by the fact that preprobradykinins contain several copies of the target peptide.[36,37]

R. arvalis secretion contains also intermediates of two modifications of bradykinin (**16**, **17**). The first one is [Hyp^3]RL-16 with Pro3 substituted for hydroxyproline. The intermediate of the modified bradykinin [Glu^5]RA-11 has a substitution of phenylalanine for Glu. The biological activity of hydroxyprolylbradykinin is comparable with that of bradykinin itself, while data on the activity of [Glu^5]-bradykinin are absent so far.[34] Non-modified [Hyp^3]bradykinin and [Glu^5]bradykinin, formed due to proteolysis of **16** and **17** at the C-terminus of Arg, were not detected. This may be a result of the rapid deactivation of endoproteases by methanol during secretion collection.

Since bradykinin is the major component of the skin secretion of the brown frog *R. arvalis*, it is not surprising that an acidic spacer of preprobradykinin DEDEDEYAGEA-KAEDVKR-OH was also discovered. It is known that peptide predecessors are kept in the glands of amphibians in a deactivated form. Stimulation of the dorsal glands results in secretion of the inactive propeptides consisting of an acidic spacer and the peptide itself. Elimination of the spacer requires interaction with endoproteases which are secreted by frogs together with the peptides. The liberated peptide then becomes active.[6,7,38]

It is worth specially mentioning an interesting peptide **5** (Table 1) identified in the secretion of *Rana arvalis*. Melittin is the basic active component of the venoms of honeybees *Apis mellifera* and *Apis florae*.[39] Melittin-related peptides (MRPs) have been reported as components of the secretions of brown frogs: European *R.temporaria* [21,40] and Japanese *R. tagoi* [41] and *R. sakuraii*.[42] MRPs as well as authentic melittin are active towards Gram-positive and Gram-negative microorganisms as well as fungi. It should be stressed that their cytolytic activity is lower than that of melittin itself.[38,39] The MRP from the brown frog *R. arvalis* is a C-terminal α-amidated peptide, containing 22 amino acid residues: FVGAALKVLANVLPP-VISWIKQ-NH_2. Its sequence is 50% analogous to melittin (48% and 52% – to the MRP from brown Japanese frogs *R. tagoi* and *R. sakuraii* and 77% to MRP from European *R. temporaria*). Leu9, Leu13 and Ile20 are established on the basis of the ECD spectrum of the triply protonated MRP with MW 2361 Da. Leu6 and Ile17 are assigned using an analogous principle and taking into account the sequences of melittin and other known MRPs. Reaction of the original skin secretion sample with performic acid resulted in parallel oxidation of tryptophan residues. The masses of the tryptophan-containing product ions were 15.995 *m/z* units higher. An earlier publication[43] states that hydroxytryptophan (HTRP) formation takes place. Similarly to the bradykinin case an acidic spacer of the predecessor of MRP was detected in the secretion of *R. arvalis*. Its sequence – EEENGGEVKEEE – completely coincides with that of the acidic spacer of the prepropeptides of MRPs of the brown Japanese frogs *R. tagoi* and *R. sakuraii*.[44]

CONCLUSIONS

A HPLC/nanoESI-FTMS approach involving recording of CAD and ECD spectra of an intact sample of crude secretion and two samples of the same crude secretion after its performic oxidation and reduction followed by carboxamidomethylation allows us to successfully establish peptide profiles of the skin secretion of frog species at the mid-thoughput level, including *de novo* sequencing of rather long peptides containing the 'rana box' feature.

Seventeen peptides belonging to four known families were identified in the secretion of the European brown frog *R. arvalis* inhabiting the Moscow region in Russia, with the neuropeptide bradykinin being the major constituent.

Seventy-seven precent of the sequence of the newly discovered melittin-related peptide FVGAALKVLANVLPP-VISWIKQ-NH_2 coincides with that of the MRP identified in the skin secretion of the related species of the European brown frog *R. temporaria*.

The secretion from the Moscow species of *R. arvalis* contains ranatuerins-2 closely related to those found in the secretion of various North American species.

The IC_{50} values of brevinin-1AVa and the 17-residue C-terminal fragment of ranatuerin-1AVa towards Gram-positive bacteria *Leuconostic lactis* are 32.4 and 72.9 μM, respectively.

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