Isolation and sequence analysis of peptides from the skin secretion of the Middle East tree frog *Hyla savignyi*

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**Abstract** Novel peptides were identified in the skin secretion of the tree frog *Hyla savignyi*. Skin secretions were collected by mild electrical stimulation. Peptides were separated by reversed-phase high-performance liquid chromatography. Mass spectra were acquired by electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS), and fragment ion spectra were obtained after collision-induced dissociation and electron capture dissociation. Peptides were analyzed by manual de novo sequencing and composition-based sequencing (CBS). Sequence analyses of three so far undescribed, structurally unrelated peptides are presented in this paper, having the sequences DDSEEEEVE-OH, P*EEVEEERJK-OH, and GJJDPJTGJVGGJJ-NH₂. The glutamate-rich sequences are assumed to be acidic spacer peptides of the prepropeptide. One of these peptides contains the modified amino acid hydroxyproline, as identified and localized by high-accuracy FTICR-MS. Combination of CBS and of experience-based manual sequence analysis as complementary and database-independent sequencing strategies resulted in peptide identification with high reliability.

**Keywords** Frog peptides · Composition-based sequencing · Peptide fragmentation · Sequence rearrangements · Proline effect

**Introduction**

Skin secretions of the dorsal glands of amphibians provide a cocktail of bioactive compounds which protect the animals from microorganisms and predators. The granular glands produce a secretion containing various compounds of biological activity. The main secretion components are peptides [1, 2] and alkaloids [3].

Amphibian peptides have a large variety of biological functions, such as antimicrobial [4–7], antiviral [8, 9], and anticancer [10]. Biological activity is induced by peptide–lipid interactions with target membranes, leading to membrane penetration and cell death. The mode of action, effected by cationic amphiphilic peptides, is described by diverse suggested mechanisms [11–14]. A sequence database of antimicrobial peptides from various biological sources was founded in 2003 [15] and updated recently [16].

The suitability of bioactive peptides in mammalian systems, however, is very limited so far. Pharmacokinetic and pharmacodynamic barriers, instability against proteases, systemic and local toxicity, or reduced activity based on salt and pH sensitivity make a medical application difficult [17, 18]. An additional problem is the high cost of chemical synthesis in the production of antimicrobial peptides.

A review on structure determination and bioactivity of frog peptides, mainly isolated from Australian species, was published recently [19, 20]. The frog species of our studies, *Hyla savignyi*, belongs to the Hylidae family and was collected in the southern part of Meshkin Shahr in the Ardabil province of Iran. We recently analyzed peptides of this species and classified them as tryptophyllin-like...
peptides [21]. Sequence analysis was presented and specific fragmentation features, such as formation of rearrangement products and the proline effect, were described. Peptides were found to show structural similarities to those found in the species Litoria rubella and Litoria electrica, called tryptophyllins L [22, 23]. Tryptophillin-like peptides were first isolated from Phyllomedusa rohdei [24].

Peptide characterization without employing database searching programs, called de novo sequencing [25–28], has become an important process in peptide analysis of non-standard species. The procedure often works reliably and fast if tryptic peptides having basic residues at the C-terminus are investigated by tandem mass spectrometry. If peptides are not digestion products or have non-basic amino acids at the C-terminal end, however, this procedure often fails [29]. Basic amino acids in the central part of a peptide sequence may cause highly complex fragmentation spectra [30]. Incomplete fragment ion information and posttranslational modifications can be an obstacle in finding the complete and correct sequence. Rearrangement products in collision-induced dissociation (CID) measurements, formed after cyclization reactions and subsequent ring opening at various peptide bonds, can lead to misinterpretations [31, 32]. CID experiments of proline-containing peptides can generate very specific MS/MS spectra, depending on the position of proline along a peptide chain [33–37], and may complicate peptide characterization as well. Novel frog peptides of the animal’s skin secretion are usually not listed in protein/peptide sequence databases. This requires an efficient database-independent sequencing strategy.

Peptides investigated in this study were characterized by manual de novo sequencing and by composition-based sequencing (CBS) [38]. The latter method is a database-independent sequencing strategy and includes two steps. In the first step, the amino acid composition (AAC) is determined by the evaluation of accurate MS and MS/MS mass values without employing probability functions. The second step determines the peptide sequence for the given AAC by scoring the agreement between the observed and expected fragment ion signals of permuted sequence propositions. The feasibility of the CBS strategy requires sufficient mass accuracy and (as every sequencing strategy) sufficient fragment ion information. In our previous studies of the tryptophyllins H [21], CBS turned out to be a valuable method since CID spectra were complex and most high-performance liquid chromatography (HPLC) fractions did not provide satisfying results when performing Edman degradation, probably due to sensitivity problems.

Peptide sequences of the present study were determined by CBS and manual spectra evaluation based on CID and electron capture dissociation (ECD) spectra. Since the amount of sample was limited, we were not able to extend the investigation by improving our Edman methodology. Deciphering I/L ambiguity was thus beyond the scope of the work presented here. Sequences of characterized peptides were confirmed by CID measurements of analog synthetic peptides.

Experimental

Collection of skin secretions

Adult specimens of H. savignyi were collected in the southern part of Meshkin Shahr in the Ardabil province of Iran. Frogs were submerged in a beaker containing deionized water and exposed to gentle electrical stimulation in ambient atmosphere using copper electrodes (3 V, 2 Hz, 4-ms pulse duration). Electrodes were rubbed on the skin surface for 40 s and a milky secretion was excreted and absorbed by the deionized water. Afterwards, animals were manually and gently submerged for an additional 60 s in the beaker to complete the extraction of skin secretion. The resulting aqueous solution was lyophilized with a freeze drying device and stored at −20 °C. Animals were released in their natural habitat after treatment.

HPLC separation

One milligram of the lyophilized sample was dissolved in 500 μl water containing 5% acetonitrile. An insoluble residue was removed by centrifugation. For nano-HPLC-MS analysis, a binary nano-HPLC pump/autosampler system (Ultimate, Dionex/LCpackings, Idstein, Germany) was employed. Of a 1:10 dilution of the sample, 1 μl was pre-focused on a trap column (C18 PepMap, I.D. 300 μm, length 5 mm, Dionex) and separated on a fused-silica C18 PepMap100 capillary column (3 μm, 100 Å; I.D. 75 μm; length 150 mm, Dionex). A multistep gradient from 10% to 50% solvent B [80% acetonitrile (v/v), 20% water (v/v), and 0.1% formic acid (v/v)] over a period of 25 min, followed by a 15-min isotropic step, and another gradient from 50% to 70% solvent B for a further 20 min was applied. Water containing 2% acetonitrile (v/v) and 0.1% formic acid (v/v) was used as solvent A. The flow rate was 0.2 μl/min. The eluent was monitored by ultraviolet absorbance at 214 nm.

A Summit microHPLC system (Dionex) was used for fractionation. Ten microliters was loaded onto a Microbore column (Macherey-Nagel, C18, 5 μm, 300 Å, I.D. 1 mm, length 150 mm). The flow rate was 40 μl/min. Multistep gradient and solvents were used as in the nano-HPLC separation.

Mass spectrometry

Mass spectra were acquired on a “Finnigan LTQ FT Ultra” hybrid instrument (Thermo Scientific GmbH, Bremen,
Germany) consisting of a linear quadrupole ion trap and a Fourier transform ion cyclotron resonance (FTICR) mass spectrometer equipped with a 6-T magnet. A nanospray ionization source was used for analyses (capillary temperature 250 °C, capillary voltage 10 V, tube lens 100 V). Nanospray needles for off-line measurements were employed (spray voltage 1.4 kV, O.D. 1.2 mm, I.D. 5.0 μm, DNU-MS, Berlin, Germany). For on-line measurements, Silica Tips (Tip I.D. 10 μm, New Objective, Woburn, MA, USA) were used at a spray voltage of 1.9 kV. Mass resolving power was set to 100,000 at m/z=400, allowing us to record one spectrum per second and thus several spectra per chromatographic peak. Mass accuracy after external calibration was better than ±2.0 ppm during both online and off-line measurements. Instrument-specific parameter settings for collision-induced dissociation in MS/MS experiments were as follows: activation energy 30 (normalized; manufacturer-specific units); activation duration 30 ms; activation Q 0.25 (manufacturer-specific units); wide scan range, three microscans per spectrum. The data acquisition software Xcalibur 2.0 SR2 (Thermo Fisher Scientific) was employed.

Sequence analysis

Calculations of accurate mass values of peptide ions and peptide fragment ions were performed using the computer program Peptide Composer 1.0 (copyright Bernhard Spengler). Peptide composition analysis, CBS, and manual de novo sequencing are supported by this program. Calculations were performed with a mass tolerance setting of ±2.0 ppm for precursor and fragment ions. Based on possible compositions of fragment ions and precursor ion, AACs of the peptides were determined. The composition analysis algorithm of CBS performs a combinatorial analysis and does not contain experience-based evaluation steps. Because of the identical mass values of leucine and isoleucine, all calculations were performed with treating both amino acids as being equal (L/I=J). The number of all amino acids (including phosphorylation) was set to unknown. Starting the AAC calculations, peptides were treated as having one of three possible structures: free acid, acid amide, or cyclic structure. A decision on the structural property was established as soon as no possible AACs remained for two of the three C-terminal structures during the composition analysis. After precursor ion calculation and generation of an AAC list, composition analysis of fragment ions (within the candidate AACs of the precursor) was carried out starting with the CID spectra signal of the lowest mass value. The AAC list of the precursor ion was then checked against the AAC list of the fragment ion. This was executed in several iteration steps after each fragment composition analysis until all fragment ion mass values were evaluated.

Lists of sequence propositions were generated from determined AACs by exhaustive permutation. After in silico fragmentation of sequence propositions, theoretical and observed fragment ions were compared and matching ions were registered by the CBS procedure. Each hit was weighed with a fragment type-specific factor. In addition, we carried out a manual evaluation of the CID and ECD spectra.

Syntheses of peptides

Peptides were synthesized by GL Biochem (Shanghai) Ltd. (Shanghai, China) and were delivered with a purity of >80%.

Results and discussion

Figure 1 shows the HPLC separation of the skin secretion. HPLC fractions were labeled A to L, and the corresponding identified peptides are listed in Table 1. Fractions C to K contained peptides that were characterized and classified as tryptophyllins H in a previous study [21]. They share sequence similarities with peptides found earlier in the skin secretion of the frogs L. rubella and L. electrica, called tryptophyllins L [22, 23]. Fractions between 24 and 30 min were investigated by MS/MS analysis. No peptides were found in these fractions. Sequence analysis of peptides found in fractions A, B, and L are discussed in this paper, and the results are listed in Table 1. Peptides were named savignins by us, according to the taxonomic name H. savignyi. CBS was carried out for the peptides of fractions A and L (savignin 1+3). The fraction B peptide (savignin 2) could be easily sequenced by manual evaluation of the ECD spectrum. All peptide sequences were confirmed by CID measurements of analog synthetic peptides. First bioassays, implemented on antibacterial activity against Micrococcus luteus and Escherichia coli, gave no significant results and are not described in this paper.

According to the accepted nomenclature [39, 40], b-type fragment ions include the N-terminus of the peptide. In our studies, we also observed two-step (internal) b-type fragments that are labeled as “b_{x-y}”, with x and y being the start and end positions of the terminating amino acids of the fragment ion within the peptide chain [41]. The same nomenclature is applied to a- and z-type ions.

Savignin 1

Composition analysis of the fraction A peptide (savignin 1) resulted in 1,276 possible AAC candidates belonging to a C-terminal free acid, 997 AAC candidates of acid amide, and 1,591 AAC candidates of cyclic structure (Table 2).

After precursor ion calculation, the list of possible peptide amino acid compositions was narrowed down by...
fragment composition analyses (Table 3). Most fragment
ion signals gave no hits for acid amide structure, so that this
structure could be excluded in an early stage and is not
considered in the table. Starting with the signal of the
lowest mass value in the CID spectrum (Fig. 2a), calcula-
tion of fragment ion 247.12898 u gave a y-type ion of
possible compositions (D1J1) and (E1V1) when checked
against the precursor AAC list. Based on this result of
fragment composition analysis, the precursor AAC list was
updated and gave 172 possibilities of cyclic and 113
possibilities of free acid structures. The procedure was carried
out in several iteration steps, and the precursor AAC list was
updated after each fragment ion calculation (as shown in
Table 3). After the composition analysis procedure, the
peptide was found to have one out of two possible AACs
of free acid (D2E3J1S1 or D2E3S1V1) and one cyclic structure
(D2E3J1M2S1). Fragment ions of high signal intensity at
834.26340 u and 933.33114 u gave no hit for a cyclic peptide
structure. Furthermore, compositions of y-type fragment ions
that were found within the peptide composition list of cyclic
structure could only be interpreted as (isobaric) b+H2O ions
since cyclic peptides have no C-terminal end. The
corresponding b ions, however, were not found in the MS/
MS spectrum. Thus, the cyclic structure could be discarded.
CBS analysis of the two remaining AAC candidates with
C-terminal free acid gave DDSEEEVE as the overall
highest sequence score. The precursor mass value (Table 2)
therefore had a deviation of +0.94 ppm compared to the
calculated mass value (540.69621 u). The CBS results (top 4
of each sequence analysis) are presented in Table 4.

CBS analysis of the ECD spectrum (Fig. 2b) confirmed
the sequence found after an evaluation of CID mass data.
The corresponding fragment ion mass list is shown in
Table 5. Mass accuracy was improved by internal fine
calibration as described earlier [38].

The ECD spectrum is unexpectedly dominated by a
series of b and y ions (besides the expected c ions). With
the exception of z5 ion, ions of z-type occurred as two-step
fragments. The signal at 687.25841 u had a considerable
intensity, but was not matched in the CBS procedure. Based

Table 1 Savignin peptides from the skin secretion of H. savignyi

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>m/z [M+H]+ (monoisotopic)</th>
<th>Deviation (ppm)</th>
<th>HPLC fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Experimental</td>
<td>Theoretical</td>
<td></td>
</tr>
<tr>
<td>Savignin 1</td>
<td>DDSEEEVE–OH</td>
<td>540.6967</td>
<td>540.6962</td>
<td>+0.94</td>
</tr>
<tr>
<td>Savignin 2</td>
<td>P<em>EEVEERJK–OH</em></td>
<td>637.3166</td>
<td>637.3172</td>
<td>−1.83</td>
</tr>
<tr>
<td>Savignin 3</td>
<td>GJJDPITGJGVGGJ–NH2*</td>
<td>668.9128</td>
<td>668.9136</td>
<td>−1.15</td>
</tr>
</tbody>
</table>

P* hydroxyproline

*T J = L/I

Fig. 1 Online nano-LC-MS
separation (plot of total ion
count) of the skin secretion of
H. savignyi. Fractions C to K
contain tryptophyllin-like
peptides (tryptophyllins H) that
were described recently [21]
on the high mass accuracy, this fragment was identified as internal $z^i$ type ion with a loss of water ($z_{i(3–8)}^i$–H$_2$O), and no further fragment candidate was found. When the sequence ladder is pursued, it is obvious that (due to the identical amino acid composition) the signal at 600.22652 u may consist of both $z_5^i$–H$_2$O and $z_{i(4–8)}^i$–H$_2$O. Accordingly, it is assumed that the signal at 471.18378 u has a high ratio of $z_{i(5–8)}^i$–H$_2$O compared to $z_{4}^i$–H$_2$O.

To understand the formation of internal $z$ ions, we examined the C-terminal amino acid glutamic acid. Pyroglutamic acid is a common modification in peptides containing glutamic acid. This amino acid is known to

Table 2 Number of possible AACs of savignin 1 (with a mass tolerance setting of ±2.0 ppm) before fragment ion calculation

<table>
<thead>
<tr>
<th>Precursor [M+2H]^{2+} (monoisotopic)</th>
<th>$\Delta$ (ppm)</th>
<th>Number of possible AACs (outset)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Free acid</td>
</tr>
<tr>
<td>540.69672</td>
<td>2.00</td>
<td>1,276</td>
</tr>
</tbody>
</table>

Table 3 Calculation of fragment ion mass values of savignin 1 (generated by CID) with a mass tolerance setting of ±2.0 ppm

<table>
<thead>
<tr>
<th>Fragment mass (u)</th>
<th>$\delta$ (ppm)</th>
<th>Fragment ion type</th>
<th>Possible fragment AACs</th>
<th>Number of possible peptide AACs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cyclic</td>
</tr>
<tr>
<td>247.12898</td>
<td>0.53</td>
<td>y</td>
<td>D$_2$J$_1$ V$_1$</td>
<td>172</td>
</tr>
<tr>
<td>358.16073</td>
<td>−0.42</td>
<td>b</td>
<td>D$_2$E$_1$J$_1$ V$_1$</td>
<td>82</td>
</tr>
<tr>
<td>376.17135</td>
<td>−0.24</td>
<td>y</td>
<td>D$_2$E$_1$J$_1$ V$_1$</td>
<td>82</td>
</tr>
<tr>
<td>447.13498</td>
<td>−1.79</td>
<td>a</td>
<td>D$_2$E$_1$ S$_1$ T$_1$</td>
<td>11</td>
</tr>
<tr>
<td>505.21371</td>
<td>−0.63</td>
<td>y</td>
<td>D$_2$E$_1$J$_1$ V$_1$</td>
<td></td>
</tr>
<tr>
<td>576.17724</td>
<td>−1.98</td>
<td>a</td>
<td>D$_2$E$_1$ S$_1$ T$_1$</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>634.25594</td>
<td>−1.09</td>
<td>y</td>
<td>D$_2$E$_1$J$_1$ V$_1$</td>
<td></td>
</tr>
<tr>
<td>705.22039</td>
<td>−0.82</td>
<td>a</td>
<td>D$_2$E$_1$ S$_1$ T$_1$</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>763.29942</td>
<td>0.26</td>
<td>b</td>
<td>D$_2$E$_1$ M$_2$ V$_1$</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>y</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>834.26340</td>
<td>−0.19</td>
<td>a</td>
<td>D$_2$E$_4$ S$_1$</td>
<td></td>
</tr>
<tr>
<td>850.33130</td>
<td>0.06</td>
<td>a</td>
<td>D$_2$E$_1$J$_1$ M$_3$</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b</td>
<td>D$_2$E$_1$M$_2$ S$_1$</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>y</td>
<td>D$_2$E$_1$S$_1$ V$_1$</td>
<td>2</td>
</tr>
<tr>
<td>933.33114</td>
<td>−0.90</td>
<td>b</td>
<td>D$_2$E$_1$J$_1$ S$_1$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
</tr>
</tbody>
</table>

Each composition is given in alphabetical order with the corresponding ion type

* $a$ Checked against precursor AAC list that was updated in each iteration step
* $b$ Checked against fragment AAC list after each fragment calculation
* $c$ The corresponding fragment ion compositions were not found within the peptide composition list of the respective structure
form a lactam after cyclization and elimination of water. Since this amino acid is found at the C-terminus of savignin 1, we propose an analogous mechanism (Scheme 1) derived from the mentioned cyclization reaction. After the nucleophilic attack of the amide nitrogen to the side chain carboxyl group, pyroglutamic acid is split off as a neutral compound and an internal z ion remains.

Savignin 2

The CID spectrum of savignin 2 (Electronic supplementary material (ESM) Fig. S1) showed a series of internal fragment ions of types ‘b’ and ‘y’. The ECD (Fig. 3) spectrum instead expressed a lower complexity and was used for sequence determination. The sequence could be
easily read from the spectrum and no CBS analysis was carried out. The z' and c ions gave complementary and complete sequence information. Signals of c9 and z9 could be resolved by FTICR-MS as shown in the spectrum. The doubly charged precursor mass value (637.31658 u) had a deviation of −1.83 ppm compared to the theoretical value (637.31720 u). The mass value of hydroxyproline (113.04768 u) is rather close to that of leucine/isoleucine (113.08407 u). Accurate mass data allowed us to identify hydroxyproline at the N-terminal end and gave the sequence P*EEVEEERJK-OH. Mass data of the ECD measurement are given in Table 6.

Savignin 3

Sequence analysis of the fraction L peptide (savignin 3) turned out to be rather difficult. CBS analysis was carried out as described above. The results of composition analysis (precursor ion evaluation before fragment ion evaluation) are shown in Table 7 considering free acid, acid amide, and cyclic structure.

After precursor ion evaluation, the list of amino acid composition candidates was reduced as much as possible by an evaluation of all CID fragments detected by FT-MS (Fig. 4). MS3 analysis (MS/MS of 399.22360 u) gave an additional fragment ion at 171.11270 u (not shown). Composition analysis gave a list of 43 fragment ions considered in the evaluation procedure (ESM Table S1). Peptide composition analysis resulted in 86 AAC candidates of acid amide, eight AAC candidates of free acid, and ten AAC candidates of cyclic peptides after fragment ion evaluation. The final amino acid composition was determined by increment analysis as H(D1G4J6P1T1V1)NH2.
Amino acids were identified by an evaluation of accurate increment mass values obtained in the MS/MS spectrum (see labeled sequence ladder in Fig. 5). The C-terminal amide structure was found based on a series of two-step fragment ions \(b_{x-14}\) derived from y-type ions after loss of ammonia. The precursor mass value of Table 7 had a deviation of \(-1.15 \text{ ppm}\) compared to the calculated value (668.91357 u). All fragment ions had a deviation within \(\pm 2.0 \text{ ppm}\).

Knowing the amino acid composition of the sample peptide, the number of sequences to be tested is 
\[ P = k! \]
where \(k\) is the number of permutations and \(k\) the peptide length. Therefore, the number of permutations (possible sequences) of \(H(D_iG_iJ_iP_iT_iV_i)NH_2\) is 
\[ P = 14! = 87,178,291,200. \]
Since the determined composition contains two multiplets of amino acids, the number of permutations reduces to 
\[ P = \frac{14!}{4!5!} = 5045040. \]

The number is reduced further by grouping of several amino acids. The intense b ion at 399.22360 u has the composition \((D_iG_i)\). The corresponding intense y ion at 938.60370 u includes the remaining amino acids of the peptide and is of composition \((G_iJ_iP_iT_iV_i)\). Within the latter AAC, the compositions \((G_jJ_iV_j)\) and \((J_iP_i)\) were grouped additionally. The input string for permutational sequence analysis was then \{[DGJ][GGJ][GJ][JP]T\} [38]. The number of sequences to be tested was thus 
\[ P = \frac{14!}{2!3!4!} = 86400. \]

CBS analysis gave sequence results with rather close score values (top 4 shown in Table 8). The highest and second highest scored sequence was tested in more detail by manual spectra analysis as discussed in the following. The number of matched fragment ions conforms to the number of assigned ion signals in the CID spectrum (+ fragment ion at 171.11270 u as described above) less the number of fragments that are rearrangement products and do not directly originate from the peptide sequence (fragment ion signals at 994, 1,109 and 1,220 u). Fragment ions \(b_3\) and \(y_9\) were not detected in the MS/MS spectrum. These ion signals would enable us to distinguish partial sequences \"JP\" and \"PJ\" at positions 5 and 6. The ECD spectrum (not shown) did not give additional sequence information. Responsible for different score values of the first two scored sequences was the b type fragment ion at 400.27925 u and the y type fragment ion at 938.60370 u.

### Table 6 Fragment ions after ECD of savignin 2: experimental mass values are those determined after internal fine calibration

<table>
<thead>
<tr>
<th>Fragment mass (u)</th>
<th>(d_{\text{exp/calc}}) (ppm)</th>
<th>Fragment ion</th>
</tr>
</thead>
<tbody>
<tr>
<td>400.27847</td>
<td>-1.94</td>
<td>(z_3)</td>
</tr>
<tr>
<td>529.32139</td>
<td>-0.85</td>
<td>(z_4)</td>
</tr>
<tr>
<td>658.36456</td>
<td>0.19</td>
<td>(z_5)</td>
</tr>
<tr>
<td>728.39389</td>
<td>-0.50</td>
<td>(w_6)</td>
</tr>
<tr>
<td>787.40726</td>
<td>-0.04</td>
<td>(z_6)</td>
</tr>
<tr>
<td>803.42571</td>
<td>-0.04</td>
<td>(y_6)</td>
</tr>
<tr>
<td>886.47563</td>
<td>0.22</td>
<td>(z_7)</td>
</tr>
<tr>
<td>902.49473</td>
<td>0.65</td>
<td>(w_7)</td>
</tr>
<tr>
<td>956.50516</td>
<td>-0.11</td>
<td>(w_8)</td>
</tr>
<tr>
<td>1015.51830</td>
<td>0.27</td>
<td>(z_8)</td>
</tr>
<tr>
<td>1031.46442</td>
<td>0.44</td>
<td>(c_8)</td>
</tr>
<tr>
<td>1085.54772</td>
<td>-0.11</td>
<td>(w_9)</td>
</tr>
<tr>
<td>1144.54804</td>
<td>0.49</td>
<td>(c_9)</td>
</tr>
<tr>
<td>1144.56156</td>
<td>0.84</td>
<td>(z_9)</td>
</tr>
</tbody>
</table>
Three novel, structurally unrelated peptides were reliably characterized by database-independent sequencing strate-
**Fig. 4** CID spectrum of savignin 3 (a mass range 270–700 u; b mass range 700–1,000 u; c mass range 1,000–1,250 u). Fragments labeled in red are discussed in the text. Fragments at 994, 1,109, and 1,220 u are rearrangement products and are not matched by CBS.
Savignin 1 was easily sequence-analyzed by CBS using CID and ECD mass data as having the sequence DDSEEEVE-OH. The ECD spectrum unexpectedly showed a high number of b and y ions. Internal ions of z type were observed as well, and a mechanism of ion formation was proposed by us. Sequence determination of savignin 2 was carried out based on ECD data, and the sequence could unequivocally be determined from the spectrum as P*EEVEERJK-OH. Hydroxyproline was identified and localized by accurate FT-MS analysis. Characterization of savignin 3 turned out to be rather difficult. In-depth sequence analysis by CBS and accurate mass increment evaluation led to the proposed sequence GJJDPJTGJVGJGJ−NH$_2$. This sequence could finally be verified by comparing the CID spectra of the natural peptide and the synthesized homologue.

Sequence analysis of savignin 3 showed that the occurrence of rearrangement products and multiple assignable fragment ions of identical mass and composition are fundamental obstacles in de novo sequencing in general. A non-direct sequence ion led to a misinterpretation of the N-terminal sequence part of savignin 3. Another rearrange-
ment product, however, was helpful in identifying the sequential order of proline and leucine/isoleucine in the central part of the peptide chain.

The feasibility of the CBS method depends (as every sequencing strategy) on the availability of sufficient sequence information and on a limited complexity of MS/MS spectra. The software provides sequence results that are exclusively based on the mass calculation of fragment ions and precursor ion without employing database information. The process is experience-independent, only leaving the necessity to interpret the occurrence of rearrangement products and of the proline effect manually. However, the software performs a very comprehensive and exhaustive sequence analysis, including ion type characterization and composition analyses without “gaps” where all fragment ions are taken into account.

Savignin 1 and 2 are assumed to be non-active spacer or signal peptides [42, 43], being proteolytically cleaved off from a prepropeptide upon stimulation. Extensive series of bioactivity tests of savignin 3 on various pathogens will be implemented in the future.

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