# N-Terminal Tagging Strategy for *De Novo* Sequencing of Short Peptides by ESI-MS/MS and MALDI-MS/MS

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The major portion of skin secretory peptidome of the European Tree frog *Hyla arborea* consists of short peptides from tryptophyllin family. It is known that *b*-ions of these peptides undergo head-to-tail cyclization, forming a ring that can open, resulting in several linear forms. As a result, the spectrum contains multiple ion series, thus complicating *de novo* sequencing. This was observed in the Q-TOF spectrum of one of the tryptophyllins isolated from *Hyla arborea*; the sequence FLPFFP-$NH_2$ was established by Edman degradation and counter-synthesis. Though no rearrangements were observed in FTICR-MS and MALDI-TOF/TOF spectra, both of them were not suitable for mass-spectrometry sequencing due to the low sequence coverage. To obtain full amino acid sequence by mass spectrometry, three chemical modifications to N-terminal amino moiety were applied. They include acetylation and sulfobenzoylation of N-amino group and its transformation to 2,4,6-trimethylpyridinium by interaction with 2,4,6-trimethylpyrillium tetrafluoroborate. All three reagents block scrambling and provide spectra better than the intact peptide. Unfortunately, all of them also readily react with lysine side chain. Hence, all investigated procedures can be used to improve sequencing of short peptides, while acetylation is the recommended one. It shows excellent results, and it is plain and simple to perform. This is the procedure of choice for MS-sequencing of short peptides by manual or automatic algorithms. (J Am Soc Mass Spectrom 2010, 21, 104–111) © 2010 American Society for Mass Spectrometry

Anuran skin is a well-known source of bioactive peptides [1–3] responsible for high immunity of these amphibians [4]. The peptide activity range is extremely broad: from antimicrobial, antitumor, fungicide activities to neuropeptides and analgesic peptides [2].

During last few years, we have been carrying out mass spectrometric studies of the primary structures of the skin peptides isolated from amphibians inhabiting Russia and the former USSR [5–9]. The skin peptidome of the European tree frog *Hyla arborea*, caught in the vicinity of Tbilisi, became the object of the present study. It is known that the majority of hylid peptides belong to the tryptophyllin family. Tryptophyllins are pretty short peptides normally lacking any basic amino acids [10–15]. They always contain tryptophan in their sequence: this fact is reflected in the peptide family name. Hylid peptides without tryptophan but resembling structural analogies with the known tryptophyllins have been suggested to share the same family name [16].

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Physiologic activity of tryptophyllins is still unclear [17]. Some members of this family influence smooth muscles of arteries and intestine (ex. tryptophyllin PdT-1 from *Pachymedusa dacnicolor* [16]), but others do not. An increase in biosynthesis of liver enzymes and weight gain under the influence of some tryptophyllins was also reported for the experiments with rats [18]. This class of peptides does not possess antibiotic or neuronal NO-synthase inhibitory activity [19, 20].

Upon studying the peptide profile of *Hyla arborea* species (presented earlier [21, 22]), we faced the problem involving ambiguous fragmentation of 5–6 member peptides. The ion peaks presenting in MS/MS spectra reveal multiple amino acid sequences of several isomeric peptides. The above mentioned fragmentation pattern is called "indirect" (unlike "direct" one giving rise mostly to standard *b*- and *y*-ion series) [23].

Short peptides less than seven amino acids long are known to produce "indirect" ions as a result of gas-phase cyclization of their *b*-ions inside the mass spectrometer. There are several mechanisms for this process described earlier [23–26], while cyclization with consequential cycle reopening being a key step. Attention was focused so far on $b_4$ and $b_5$ fragment ions as well as on related $a_4$ and $a_5$ fragment ions [27–29]. According to


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Harrison et al. [23], $MH^+$ ion has possibilities either to form regular linear oxazolone *b*-ion or cyclic "head-to-tail" $b_n^+$ ion. The presence of cyclic ions was confirmed by characteristic IR spectra [30], $MS^3$, and ion mobility experiments [31, 32]. An energy gap between cyclic and linear *b*-ions was estimated to be 15.4 kJ/mol [30]. Though formation of linear product ions is thermodynamically more favorable, cyclic ones also occur in significant amount due to modest energy differences. Cyclic intermediate could reopen, giving rise to a number of linear oxazolone ions and, consequently, one can find in the resulting spectra fragment ions produced by any of them. Therefore, the primary structure of such short peptides may remain unattainable either by manual or by automatic *de novo* sequencing algorithms.

There is much less available information about formation of cyclic *b* ions longer than $b_5$. Nevertheless, the formation of $b_6$ and $b_7$ cyclic ions has been reliably proven in [33], while the Spanish team recorded cyclic ions $b_8$ and $b_9$ [24].

The key aspect in gas-phase rearrangement of peptide ions is duration of interactions inside the collision cell [33]. The longer activation times result in greater amount of rearrangement ions. According to recent data [24, 33], collision time less than 1 ms is necessary to suppress indirect fragmentation pathways. As time frames for rearrangement are not realized in sector instruments and MALDI experiments, one can hardly observe such ions using these methods. On the contrary, quadrupole and ion trap instruments favor cyclization [24, 34]; application of heavy inert gases Xe and Ar as additives to He in collision cell may enhance fragmentation and allow one to obtain more structural information [35]; Vachet et al. [34] observed a substantial increase of cyclization when 5% Xe in He was infused into collision cell.

Cyclization can be avoided in two ways: by changing the activation method to one with shorter collision time or by N-terminal $NH_2$-moiety modification to prevent head-to-tail interaction. The main goal of the present contribution deals with the study of the influence of different modifications of N-terminal amino group in natural peptide $Phe_3LeuPro_2$-$NH_2$ isolated from the skin secretion of the European tree frog *Hyla arborea* on the fragmentation pattern observed in ESI and MALDI experiments.

## Methods

### Skin Secretions

Four European tree frogs *Hyla arborea* were caught near Tbilisi, Georgia. Secretion from the skin glands was provoked by mild electric stimulation. The procedure details were described earlier [36]. Briefly, the method involved the following: moistened with deionized water, the skin of the animal was treated during 40 s with a bipolar platinum electrode connected to the 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 with a small amount (up to 25 mL) of deionized water and was immediately diluted with an equal volume of methanol. The mixture was then centrifuged for 15 min at 3000 rpm, filtered through Millex HV (Millipore, Billerica, MA, USA) membrane (0.45 $\mu$L) and concentrated at 35 °C on a rotary evaporator to the volume of ~1 mL.

### HPLC Separation

HPLC fractioning was carried out using a reverse-phase analytical column $C_{18}$ (5 $\mu$m, 80 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 $\mu$L to improve the separation. Peptide separation was performed with HPLC system ThermoSeparation Products, equipped with binary gradient pump ThermoSystem P2000 (Piscataway, NJ, USA). Peptides were separated using a linear gradient from 10% to 70% (60 min) acetonitrile containing 0.1% aqueous trifluoroacetic acid. The flow rate was 0.8 mL/min. The absorption was measured at 214 nm (UV detector Spectra System UV3000). Each fraction was submitted to MALDI-MS analysis (see below). The fraction containing the peptide $Phe_3LeuPro_2$-$NH_2$ ($MH^+$ 766.43 Da) was collected and lyophilized.

### Mass Spectrometric Sequencing of the Peptides

The ESI spectra of intact tryptophyllin $Phe_3LeuPro_2$-$NH_2$ was recorded with QTOF instrument (Waters, Manchester, UK). All the following electrospray experiments were performed with a 7-Tesla hybrid linear ion trap ICR instrument (LTQ-FT Thermo Electron, Bremen, Germany) modified with a nano-ESI ion source (Proxeon Biosystems, Odense, Denmark). The experimental details are described in [37].

Collected fractions of crude skin secretion were analyzed with MALDI-TOF/TOF mass spectrometer (Ultraflex II, Bruker Daltonik, Bremen, Germany). The spectra were acquired in LIFT mode using *m/z* range 50–2000 Da. External calibration was made using a standard peptide mixture (PepMix-2; Bruker Daltonik, Bremen, Germany).

The samples were deposited on the plate being mixed with saturated solution of 2,5-dihydroxybenzoic acid (DHB; Acros) in acetonitrile/water/TFA (50:50:0.1 vol. %).

### Edman Degradation

Automated Edman sequencing was performed using a standard procedure on an Applied Biosystems 492 peptide sequenator equipped with 900 A data processing module [38]. The best results were achieved when peptides were adsorbed from 90% aqueous acetonitrile

on an Immobilon membrane treated with ethanolic bioprene solution.

### *Acetylation Protocol*

Acetylation was done by a slightly modified well-known procedure [39]. Briefly, it involves the following steps. Acetylation reagent was prepared by mixing 20 μL of acetic anhydride (Acros) with 60 μL of methanol (Merck, Darmstadt, Germany) (1:3 vol). A small amount of peptide was dissolved in 20 μL of 50 mM $NH_4HCO_3$ buffer solution followed by addition of 50 μL of acetylation reagent. The mixture was incubated at room temperature for 1 h and then lyophilized to dryness.

### *Sulfobenzoylation Protocol*

A 0.1 M solution of 2-sulfobenzoic acid cyclic anhydride (Acros) in dry THF (Sigma, St. Louis, MO, USA) was prepared fresh before the reaction. A small amount of peptide dissolved in 20 μL of 50 mM aqueous triethylamine (Sigma) was mixed with 2 μL of cyclic anhydride solution and vortexed for 30 s. After 2 min of incubation, an aliquot was taken, mixed with matrix solution, and analyzed by MALDI [40].

### *Synthesis of 2,4,6-Trimethylpyrillium Tetrafluoroborate*

Synthesis was performed as described earlier [41]. The yield of a pale yellow product was dried on filter paper overnight and recrystallized with ethanol-methanol 1:1, affording 3.84 g (44%) of colorless powder. The structure was confirmed by elemental analysis, $^1H$, and $^{13}C$ NMR.

### *N-Terminal Modification with 2,4,6-Trimethylpyrillium*

Peptide was dissolved in 8 μL of potassium carbonate buffer (pH 9), and 20-fold excess (200 nmol) of freshly prepared 2,4,6,-trimethylpyrillium tetrafluoroborate (4 μL of 0.05M solution) was added. Reaction mixture pH was adjusted to 6.5–7 with $HBF_4$. The reaction proceeds for 48 h at room temperature was then subjected to HPLC purification, followed by MS analysis.

### *Peptide Synthesis*

Synthetic sample of tryptophyllin with the following amino acid sequence FLPFFP-$NH_2$ was prepared by GenScript Corporation (Piscataway, NJ, USA) by standard Fmoc-method using L-isomers of amino acids.

## Results and Discussion

### *Intact Tryptophyllin $Phe_3LeuPro_2$-$NH_2$*

Tandem mass-spectrum of tryptophyllin $Phe_3LeuPro_2$-$NH_2$ is presented in Figure 1. The mentioned sequence was confirmed by Edman degradation. By referring the most abundant ions as *b*- and *y*-series PFFLFP-$NH_2$ primary structure should be proposed. However, quite a number of intensive peaks remain unattained in this case. As the spectrum was acquired with Q-TOF instrument, time frames are suitable for gas-phase cyclization.

Gas-phase head-to-tail cyclization of $b_6^+$ -ion of this peptide followed by reopening results in six various linear $b_6$-ions with scrambled amino acid sequence (Scheme **1**). The C-terminal amino acid in each case possesses oxazolone structure. Fragment ions of *b*- and *a*-series originating from each linear precursor and their *m/z* values are listed in Table 1. It is worth mentioning that only a few proposed ions have not been detected in the mass-spectrum (indicated with dashes in Table 1). It should also be emphasized that the most abundant ions (marked with bold font in Table 1) originated from different linear precursors, additionally complicating sequencing.

To avoid any speculations and false identification, the peptide of interest has been individually separated by HPLC and subjected to Edman degradation, which revealed its structure to be FLPFFP-$NH_2$. This peptide was synthesized and analyzed by ESI and MALDI-MS/

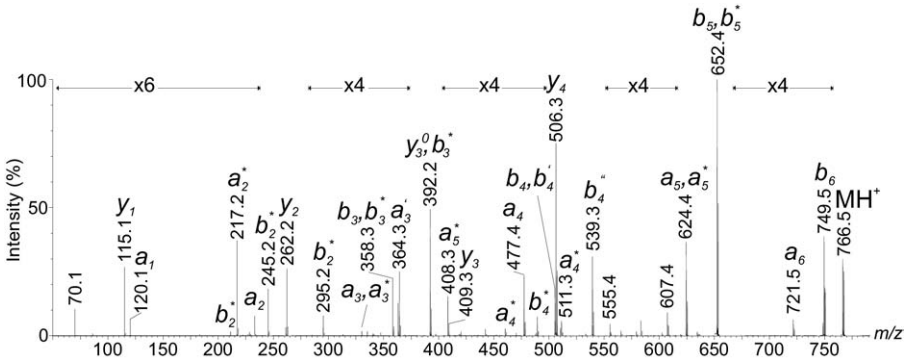
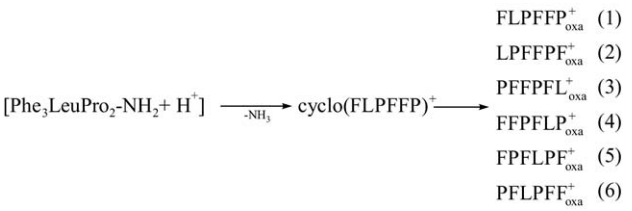



**Figure 1.** ESI-QTOF tandem mass-spectrum of a tryptophyllin isolated from skin secretion of *Hyla arborea.* Ions corresponding to "indirect" fragmentation are marked with asterisks and quotation marks; $y_3^0$ is $y_3$-ion after ammonia loss.




**Scheme 1.** Gas-phase cyclization with cycle reopening.

MS. Both spectra of synthetic and natural peptides were completely identical. This may confirm our assumption about $b_6$-ion cyclization.

It was interesting to compare spectra of the same peptide recorded with Q-TOF and FTICR instruments. The latter is presented in Figure 2a. Due to the large time frame of the FTICR instrument, we might expect that the resulting spectra would consist of scrambled ions only [34]. FTICR spectrum in general (Figure 2a) is much poorer compared with Q-TOF spectrum (Figure 1). Registered fragments cover only 3 of 5 possible backbone cleavages, thus making sequence concealed. Two major fragment ions (*m/z* 506 and 652) are $y_4$ and $b_5$, respectively. The latter may be attributed either to initial (1) or rearranged (4) sequences (Scheme **1**). The remaining six fragment ions of low abundance (excluding $b_2$ ion of *m/z* 261) may be formed from various rearranged structures.

Only a few cleavages can be observed in MALDI-TOF/TOF mass-spectrum of this peptide (Figure 2b). Two most abundant peaks in the spectrum correspond to the cleavages at the N-terminal side of $Pro_3$ and $Pro_6$. Though no rearrangement ions are observed in this spectrum, sequence information is unclear due to poor fragmentation and insufficient resolution of the instrument used. The positions of the first two residues from the N-terminus remain unknown.

To get a reliable sequence of this peptide by means of mass spectrometry, an approach involving preliminary chemical derivatization was selected. A number of chemical modifications of N-terminal amino moiety were used to prevent formation of cyclic $b_6$-ion.

## *Acetylation of $Phe_3LeuPro_2$-$NH_2$*

N-terminal acetylation is known to suppress *b*-ion cyclization [32–34]. The recorded spectrum of N-acetylated peptide proves this fact.

ESI spectrum recorded on FTICR MS instrument (Figure 3a) is much simpler in comparison with that of authentic peptide (Figure 1) and permits establishing complete sequence, including interposition of the first two amino acids. Ion peaks of direct fragmentation are present exclusively. The relative abundance of ion of *m/z* 392 is much lower than that in Q-TOF spectrum. This *m/z* value identifies either $ProPhe_2^+$ ion formed from three different scrambled linear oxazolone $b_6$-ions (Structures 3, 4, 5 in Table 1) or $y_3^0$-ion arisen from direct fragmentation pattern. Since cyclization is suppressed, three out of four possible ways to form the ion of *m/z* 392 are "inactive," and peak intensity drops. As $y^0$-ions are structurally similar to *b*-ions, we can observe CO loss from $y_3{}^0$(*m/z* 364 on Figure 3a).

MALDI-$MS^2$ spectrum of acetylated peptide FLPFFP-$NH_2$ (Figure 3b) demonstrates enhanced fragmentation as well. The sequence coverage is higher in comparison with the intact peptide: five *y*- and five *b*-ion peaks are present. Acetylation procedure has at least three major advantages making it handy: (1) it is simple to perform, (2) the reaction mixture can be easily purified by lyophilization, and (3) it suppresses cyclization and enhances fragmentation. Although the peptide studied in the present work does not contain lysine, it is known that acetic anhydride can react with lysine side chains [39]. There are publications dealing with selective derivatization of N-terminal and lysine amino groups [39, 42].

## *Sulfobenzoylation of $Phe_3LeuPro_2$-$NH_2$*

Another way to prevent cyclization of $b_6$-ion involves N-terminal modification using cyclic anhydride of 2-sulfobenzoic acid. It is especially efficient for MALDI-MS/MS experiments where the presence of additional proton is very desirable [43–45]. Along with scrambling suppression, this modification also introduces a strong acidic group at N-terminus. The latter can protonate amide bonds in peptide backbone and improve fragmentation of the molecular ion. MALDI spectrum of modified peptide is presented in Figure 4. We did not succeed in registering ESI spectrum of this peptide due to low stability of its molecular ion.

Figure 4 clearly indicates that sulfobenzoylation results in increasing of *y*-series ion intensities as expected [40, 46]. Head-to-tail cyclization is suppressed. Complementary *y*- and *b*-ion series (see scheme under the spectrum in Figure 4) cover full sequence, including

**Table 1.** The *m/z* values of the corresponding *a*- and *b*-ions depending on the sequence of the reopened $b_6^+$ cyclic ion

| | FLPFFPoxa (**1**) | LPFFPFoxa (**2**) | PFFPFLoxa (**3**) | FFPFLPoxa (**4**) | FPFLPFoxa (**5**) | PFLPFFoxa (**6**) |
|---|---|---|---|---|---|---|
| $b_2/a_2$ | 261/233 | 211/183 | 245/**217** | 295/267 | 245/**217** | 245/**217** |
| $b_3/a_3$ | 358/330 | 358/330 | **392**/364 | **392**/364 | **392**/364 | 358/330 |
| $b_4/a_4$ | 505/477 | 505/477 | 489/461 | 539/511 | 505/477 | –/– |
| $b_5/a_5$ | **652/624** | 602/– | –/608 | **652/624** | 602/– | 602/– |

The most abundant ions are in bold font.

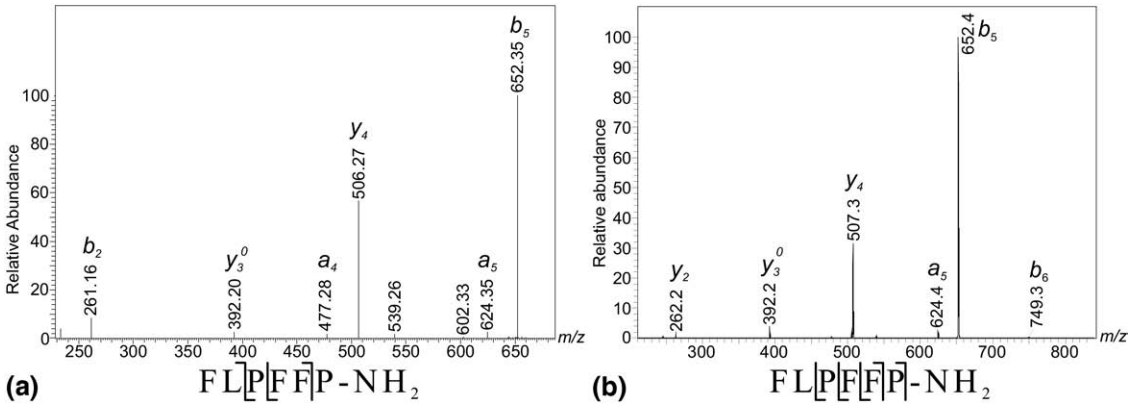



**Figure 2.** ESI-FTICR (**a**) and MALDI-TOF/TOF (**b**) spectrum of tryptophyllin FLPFFP-$NH_2$.

interposition of two first residues unidentified from intact peptide spectrum. It should be noted that only $y_3^0$ rather than $y_3$ is registered. Ions $y_{1-3}$ abundances are too low. The advantage of this method is simple and fast sample preparation, while the main shortcoming is liability of the tag in the gas phase. Thus, the peak corresponding to the tag loss (*m/z* 750) is the most intense in the mass spectrum (Figure 4). Another disadvantage for any peptides deals with possible side reaction with lysine. All mentioned restrictions make sulfobenzoylation inappropriate for *de novo* sequencing.

## *N-Terminal Fix Charge Introduction*

It is worth mentioning that both derivatization methods described above do not provide a sound fragmentation between the two first N-terminal amino acids, although there are publications reporting N-terminal modifications inducing cleavages after the first amino acid [42, 47]. Thus, the third tested approach engaged fixed charge introduction. The main feature of the fixed charge at N-terminus is lowering fragmentation energy of a peptide and enhancing the intensity of *b*-series ions. To achieve this modification, N-terminal amino group may be transformed to pyridinium moiety by reaction with 2,4,6-trimethylpyridinium [48]. Nevertheless this approach does not work for FLPFFP-$NH_2$ peptide, and we had to modify the procedure. A new modification procedure is based on reaction of N-terminus and 2,4,6-trimethylpyrillium tetrafluoroborate, while pH of the reaction media plays the decisive role in this modification. It should be maintained in the range between 6.5 and 7. At lower pH, amino group is protonated, at higher pH, pyrillium base is hydrolyzed rapidly.

Since sample purity is crucial for MS analysis, MS-compatible ammonium carbonate buffer is desirable. However it cannot be used interacting with modification reagent. Potassium and sodium carbonate buffers were tested and the former proved to be better. Because pyrillium salts are stable in acidic media, we prepared 0.05 M solution of 2,4,6-trimethylpyrillim tetrafluoroborate with addition of small amount of 40% $HBF_4$ (1000:1 vol/vol). Addition of double volume of potassium carbonate buffer to the reagent provides the desired pH value (6.5–7). It was proven experimentally that 48 h is sufficient for nearly quantitative reaction processing. The proposed modification is not selective for N-terminal amino groups readily involving ε-amino groups of lysine. HPLC was used for purification of the reaction

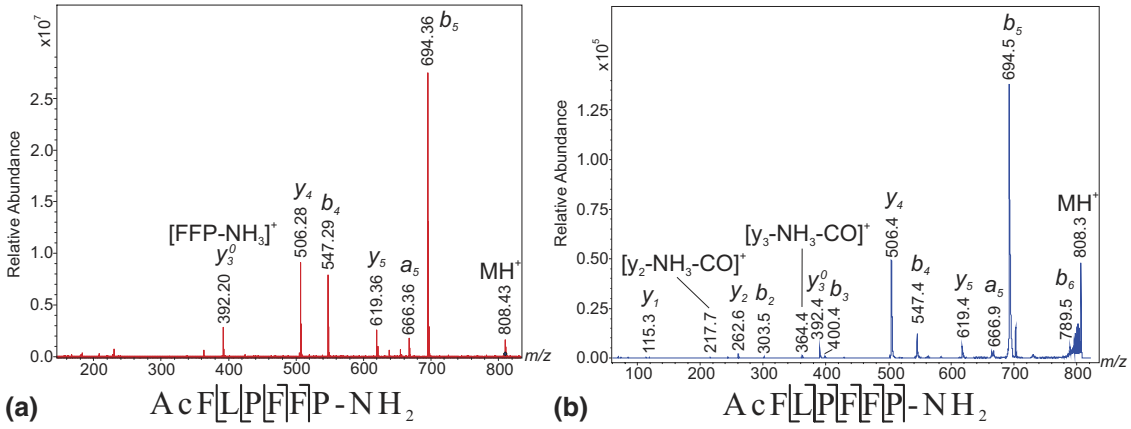



**Figure 3.** Mass-spectra of acetylated FLPFFP-$NH_2$ ESI-FTICR (**a**) and MALDI-TOF/TOF (**b**).

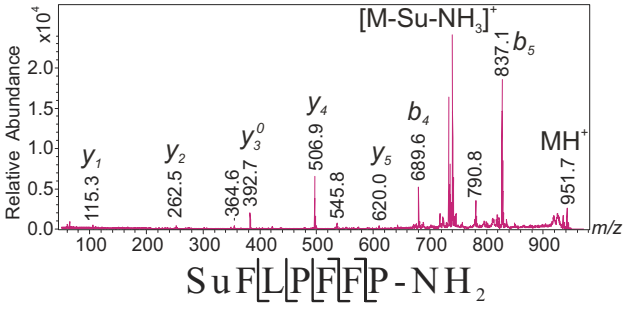



**Figure 4.** MALDI-TOF/TOF mass spectrum of the N-terminally sulfobenzoylated FLPFFP-$NH_2$.

media. The procedure is smooth and takes place under common conditions (room temperature, air).

The MALDI-TOF/TOF spectrum of FLPFFP-$NH_2$ modified with trimethylpyrillium is represented in Figure 5b. Additional charge at N-terminus improves fragmentation in MALDI. One can find all the ions of *b*-series including $b_1$, which have not been detected earlier. Since MALDI produces only singly protonated ions, we expected the charge to be localized in pyridinium moiety of the modified peptide. This fact makes *b*-ions more favorable; no proton transfer is needed for its formation. Moreover, there is no competition for proton between *b*- and *y*-ions. The highest peak in Figure 5b corresponds to $a_2$-ion. Non-common bicyclic (Figure 5c) structure can be proposed for this ion to rationalize its formation and stability. Almost complete *y*-series can also be observed in MALDI-MS/MS spectrum. Together with *b*-ions, it provides full sequence information in the same manner as acetylation approach does.

Fragmentation in ESI spectrum (Figure 5a) is poorer than that in MALDI and does not provide full sequence information. Since doubly charged peptide ion $[M + Na]^{2+}$ was used as a precursor, fragment ions $[y_4 + Na]^+$ and $[y_5 + Na]^+$ can be identified in mass-spectrum. Complementary pair $b_1$-$y_5$ allows reliable identification of the first residue. Together, with high-resolution data and data obtained for intact peptide full sequence can be elucidated.

Therefore, 2,4,6-trimethylpyrillium tetrafluoroborate can be used for fixed charge introduction at N-terminus to assist *de novo* sequencing of natural peptides, both in MALDI and ESI mass spectrometry.

## Conclusions

While elucidating the primary structure of tryptophyllin FLPFFP-$NH_2$ isolated from skin secretion of the European tree frog *Hyla arborea* with Q-TOF instrument, a notable amino acid scrambling was observed. Almost every possible rearrangement ion was found in the corresponding spectrum. This can be rationalized by assumption of gas-phase formation of cyclic (head-to-tail) form of $b_6$-ion that can give rise to a number of isomeric oxazolone $b_6$-ions. Therefore, a number of chemical modifications on N-terminal amino moiety, acetylation, sulfobenzoylation, and transformation to 2,4,6-trimethylpyridinium were tested to enhance mass-spectrometric sequencing possibilities and prevent amino acid scrambling. First two modifications were carried out by the methods reported earlier, while introduction of 2,4,6-trimethylpyridinium moiety was successfully conducted only by modified procedure using 2,4,6-trimethylpyrillium tetrafluoroborate in potassium carbonate buffer with pH adjusted to 6.5–7 during 48 h at room temperature. Unfortunately, all reagents can interact with ε-amino group of lysine easier than with N-terminal amino group. All three procedures successfully block formation of cyclic ion, thus preventing amino acid scrambling. Any chemical modification results in better fragmentation compared with authentic peptide, both in MALDI and ESI. Full amino acid sequence can be established using any of the modified peptides by MALDI and only with acetylated one using ESI. Tryptophillin with 2,4,6-trimethylpyridinium substituent gives reliable *b*-series ions in MALDI. However, because of the use of potassium carbonate buffer, an extensive purification is needed

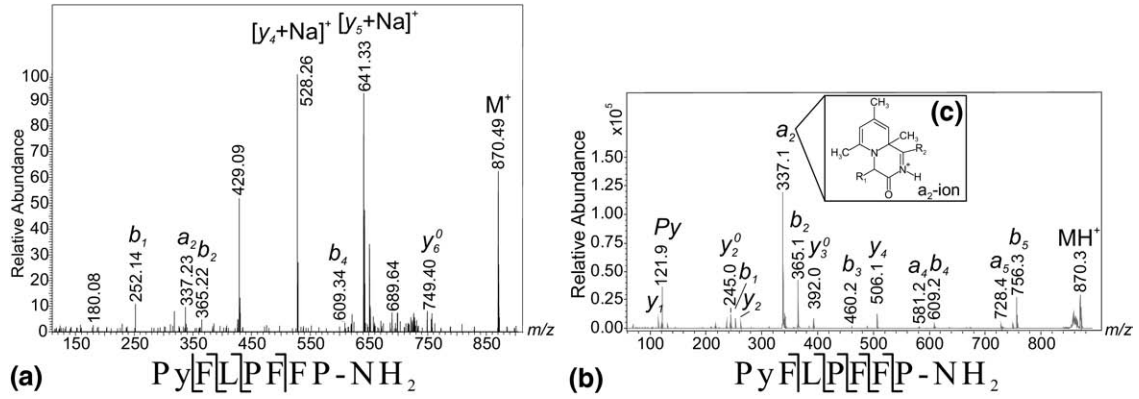



**Figure 5.** Mass spectra of FLPFFP-$NH_2$ peptide modified with 2,4,6-trimethylpyrillium tetrafluoroborate. ESI-FTICR (**a**) and MALDI-TOF/TOF (**b**).

before MS-analysis. N-terminally sulfobenzoylated peptide gives mostly *y*-series ions that include all possible cleavages. Unfortunately, the stability of this tag under experimental condition is low, and it readily dissociates from the peptide. Both MALDI and ESI spectra of acetylated peptide give two series of complementary ions and observed cleavages cover the full amino acid sequence. Together with the fact that acetylation is the simplest and "clean" (easy to purify) procedure among tested ones, it might be recommended for application during *de novo* sequencing of short peptides.

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## References

1. Simmaco, M.; Mignogna, G.; Barra, D. Antimicrobial Peptides from Amphibian Skin: What Do They Tell Us? *Biopolymers* **1998,** *47*(6), 435–450.
2. Pukala, T. L.; Bertozzi, T.; Donnellan, S. C.; Bowie, J. H.; Surinya-Johnson, K. H.; Liu, Y.; Jackway, R. J.; Doyle, J. R.; Llewellyn, L. E.; Tyler, M. J. Host-Defense Peptide Profiles of the Skin Secretions of Interspecific Hybrid Tree Frogs and Their Parents, Female *Litoria splendida* and male *Litoria caerulea*. *FEBS J.* **2006,** *273*(15), 3511–3519.
3. Tomas, G. Biosynthesis of Defensins and Other Antimicrobial Peptides. In *Ciba Foundation Symposium 186—Antimicrobial Peptides*, Marsh, J.; Goode, J. A., Eds.; John Wiley & Sons: Chichester, UK, 1994; pp 62–76.
4. Erspamer, V. Bioactive Secretions of the Amphibian Integument. In *Amphibian Biology* Vol. I, Heatwole, H.; Barthalmus, G. T.; Heatwole, A. Y., Eds.; Surrey Beatty and Sons: Chipping Norton, 1994; pp. 178–350.
5. Artemenko, K. A.; Samgina, T. Y.; Lebedev, A. T.; Doyle, J. R.; Llewellyn, L. E.; Bilusich, D.; Bowie, J. H. Host-Defense Peptides from the Skin Secretion of the European Marsh Frog *Rana ridibunda*. *Mass-Spektrometria* **2007,** *4*(2), 79–88.
6. Samgina, T. Y.; Artemenko, K. A.; Gorshkov, V. A.; Lebedev, A. T. Bioactive Peptides from *Ranid* Frog Skin Secretions: Modern Approach to Mass Spectrometric *de novo* Sequencing. *Russ. Chem. Bull.* **2008,** *57*(5), 1061–1072.
7. Samgina, T. Y.; Artemenko, K. A.; Gorshkov, V. A.; Ogourtsov, S. V.; Zubarev, R. A.; Lebedev, A. T. *De Novo* Sequencing of Peptides Secreted by the Skin Glands of the Caucasian Green Frog *Rana ridibunda*. *Rapid Commun. Mass Spectrom.* **2008,** *22*(22), 3517–3525.
8. Samgina, T. Y.; Artemenko, K. A.; Gorshkov, V. A.; Ogourtsov, S. V.; Zubarev, R. A.; Lebedev, A. T. Mass Spectrometric Study of Peptides Secreted by the Skin Glands of the Brown Frog *Rana arvalis* from the Moscow Region. *Rapid Commun. Mass Spectrom.* **2009,** *23*(9), 1241–1248.
9. Samgina, T. Y.; Artemenko, K. A.; Gorshkov, V. A.; Poljakov, N. B.; Lebedev, A. T. Oxidation Versus Carboxyamidomethylation of S–S Bond in *Ranid* frog peptides: Pro and Contra for *De Novo* MALDI-MS Sequencing. *J. Am. Soc. Mass Spectrom.* **2008,** *19*(4), 479–487.
10. Erspamer, V.; Melchiorri, P.; Falconieri Erspamer, G.; Montecucchi, P. C.; de Castiglione, R. Phyllomedusa Skin: A Huge Factory and Store-House of a Variety of Active Peptides. *Peptides* **1985,** *6*(Suppl. 3), 7–12.
11. Gozzini, L.; Montecucchi, P.; Erspamer, V.; Melchiorri, P. Tryptophyllins from Extracts of *Phyllomedusa rhodei* Skin: New Tetra-, Penta- and Heptapeptides. Further Studies. *Int. J. Pept. Prot. Res.* **1985,** *25*(3), 323–329.
12. Perseo, G.; De Castiglione, R. Syntheses of Tetra- and Pentapeptides from Skin Extracts of *Phyllomedusa rhodei* (tryptophyllins). *Int J Pept Protein Res.* 1984, *24*(2), 155–160.
13. Steinborner, S. T.; Wabnitz, P. A.; Waugh, R. J.; Bowie, J. H.; Gao, C. W.; Tyler, M. J.; Wallace, J. C. The Structures of New Peptides From the Australian Red Tree Frog *Litoria rubella*. The Skin Peptide Profile as a Probe for the Study of Evolutionary Trends of Amphibians. *Aust. J. Chem.* **1996,** *49*(9), 955–963.
14. Steinborner, S. T.; Gao, C. W.; Raftery, M. J.; Waugh, R. J.; Blumenthal, T.; Bowie, J. H.; Wallace, J. C.; Tyler, M. J. The Structures of Four Tryptophyllin and Three Rubellidin Peptides from the Australian Red Tree Frog *Litoria rubella*. *Aust. J. Chem.* **1994,** *47*(11), 2099–2108.
15. Wabnitz, P. A.; Bowie, J. H.; Wallace, J. C.; Tyler, M. J. Peptides from the Skin Glands of the Australian Buzzing Tree Frog *Litoria electrica*. Comparison with the Skin Peptides of the Red Tree Frog *Litoria rubella*. *Aust. J. Chem.* **1999,** *52*(7), 639–646.
16. Chen, T.; Orr, D. F.; O'Rourke, M.; McLynn, C.; Bjourson, A. J.; McClean, S.; Hirst, D.; Rao, P.; Shaw, C. *Pachymedusa dacnicolor* Tryptophyllin-1: Structural Characterization, Pharmacological Activity, and Cloning of Precursor cDNA. *Regul. Pept.* **2004,** *117*(1), 25–32.
17. Conlon, J. M.; Jouenne, T.; Cosette, P.; Cosquer, D.; Vaudry, H.; Taylor, C. K.; Abel, P. W. Bradykinin-Related Peptides and Tryptophyllins in the Skin Secretions of the Most Primitive Extant Frog, *Ascaphus truei*. *Gen. Comp. Endocrinol.* **2005,** *143*(2), 193–199.
18. Renda, T.; D'Este, L.; Buffa, R.; Usellini, L.; Capella, C.; Vaccaro, R.; Melchiorri, P. Tryptophyllin-Like Immunoreactivity in Rat Adenohypophysis. *Peptides* **1985,** *6* (Suppl. 3), 197–202.
19. Doyle, J.; Llewellyn, L. E.; Brinkworth, C. S.; Bowie, J. H.; Wegener, K. L.; Rozek, T.; Wabnitz, P. A.; Wallace, J. C.; Tyler, M. J. Amphibian Peptides that Inhibit Neuronal Nitric Oxide Synthase. Isolation of Lesuerin from the Skin Secretion of the Australian Stony Creek Frog *Litoria lesueuri*. *Eur J Biochem.* **2002,** *269*(1), 100–109.
20. Rollins-Smith, L. A.; Conlon, J. M. Antimicrobial Peptide Defenses Against Chytridiomycosis, an Emerging Infectious Disease of Amphibian Populations. *Dev. Comp. Immunol.* **2005,** *29*(7), 589–598.
21. Samgina, T. Y.; Kovalev, S. V.; Vorontsov, Y. A.; Gorshkov, V. A.; Lebedev, A. T. Bioactive Peptides in the Skin Secretion of Ranid and Hilyd Frogs: Complex Approach for the Mass Spectrometric *De Novo* Sequencing (ThP 186). *Proceedings of the 57th ASMS Conference on Mass Spectrometry and Allied Topics*; Philadelphia, PA, May, 2009.
22. Lebedev, A. T.; Kovalev, S. V.; Gorshkov, V. A.; Samgina, T. Y. Sequencing of the Skin Peptides Secreted by Caucasian Common Tree Frog *Hyla arborea* (PF7). *Proceedings of the 22nd Biennial Mass Spectrometry Conference in Australia and New Zealand*; Sydney, Australia, January, 2009.
23. Harrison, A. G.; Young, A. B.; Bleiholder, C.; Suhai, S.; Paizs, B. Scrambling of Sequence Information in Collision-Induced Dissociation of Peptides. *J. Am. Chem. Soc.* **2006,** *128*(32), 10364–10365.
24. Yague, J.; Paradela, A.; Ramos, M.; Ogueta, S.; Marina, A.; Barahona, F.; Lopez de Castro, J. A.; Vazquez, J. Peptide Rearrangement During Quadrupole Ion Trap Fragmentation: Added Complexity to MS/MS Spectra. *Anal. Chem.* **2003,** *75*(6), 1524–1535.
25. Tang, X. J.; Boyd, R. K. Rearrangements of Doubly Charged Acylium Ions from Lysyl and Ornithyl Peptides. *Rapid Commun. Mass Spectrom.* **1994,** *8*(9), 678–686.
26. Tang, X. J.; Thibault, P.; Boyd, R. K. Fragmentation Reactions of Multiply-Protonated Peptides and Implications for Sequencing by Tandem Mass Spectrometry with Low-Energy Collision-Induced Dissociation. *Anal. Chem.* **1993,** *65*(20), 2824–2834.
27. Bleiholder, C.; Osburn, S.; Williams, T. D.; Suhai, S.; Van Stipdonk, M.; Harrison, A. G.; Paizs, B. Sequence-Scrambling Fragmentation Pathways of Protonated Peptides. *J. Am. Chem. Soc.* **2008,** *130*(52), 17774–17789.
28. Bythell, B.; Erlekam, U.; Van Stipdonk, M. J.; Paizs, B. Infrared Spectroscopy of Fragment Ions of Protonated Peptides. *Proceedings of the 57th ASMS Conference on Mass Spectrometry and Allied Topics*; Philadelphia, PA, May, 2009.
29. Gaskell, S. J. Award Lecture: Award for Distinguished Contribution in Mass Spectrometry. *Proceedings of the 57th ASMS Conference on Mass Spectrometry and Allied Topics*; Philadelphia, PA, May, 2009.
30. Polfer, N. C.; Oomens, J.; Suhai, S.; Paizs, B. Infrared Spectroscopy and Theoretical Studies on Gas-Phase Protonated Leu-Enkephalin and Its Fragments: Direct Experimental Evidence for the Mobile Proton. *J. Am. Chem. Soc.* **2007,** *129*(18), 5887–5897.
31. Riba-Garcia, I.; Giles, K.; Bateman, R. H.; Gaskell, S. J. Evidence for Structural Variants of *a*- and *b*-Type Peptide Fragment Ions Using Combined Ion Mobility/Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* **2008,** *19*(4), 609–613.
32. Polfer, N. C.; Bohrer, B. C.; Plasencia, M. D.; Paizs, B.; Clemmer, D. E. On the Dynamics of Fragment Isomerization in Collision-Induced Dissociation of Peptides. *J. Phys. Chem. A* **2008,** *112*(6), 1286–1293.
33. Jia, C.; Qi, W.; He, Z. Cyclization Reaction of Peptide Fragment Ions During Multistage Collisionally Activated Decomposition: An Inducement to Lose Internal Amino-Acid Residues. *J. Am. Soc. Mass Spectrom.* **2007,** *18*(4), 663–678.
34. Vachet, R. W.; Bishop, B. M.; Erickson, B. W.; Glish, G. L. Novel Peptide Dissociation: Gas-Phase Intramolecular Rearrangement of Internal Amino Acid Residues. *J. Am. Chem. Soc.* **1997,** *119*(24), 5481–5488.
35. Bordoli, R. S.; Bateman, R. H. The Effect of Collision Energy, Target Gas, and Target Gas Purity on the High Energy Collision Induced Product Ion Spectrum of Renin Substrate. *Int. J. Mass Spectrom. Ion Processes* **1992,** *122,* 243–254.
36. Tyler, M. J.; Stone, D. J.; Bowie, J. H. A Novel Method for the Release and Collection of Dermal, Glandular Secretions from the Skin of Frogs. *J. Pharmacol. Toxicol. Methods* **1992,** *28*(4), 199–200.
37. Kocher, T.; Savitski, M. M.; Nielsen, M. L.; Zubarev, R. A. PhosT-Shunter: A Fast and Reliable Tool to Detect Phosphorylated Peptides in Liquid Chromatography Fourier Transform Tandem Mass Spectrometry Data Sets. *J. Proteome Res.* **2006,** *5*(3), 659–668.
38. Hunkapiller, M. W.; Hewick, R. M.; Dreyer, W. J.; Hood, L. E. High-Sensitivity Sequencing with a Gas-Phase Sequenator. *Methods Enzymol.* **1983,** *91,* 399–413.
39. Riordan, J. F.; Vallee, B. L.; Hirs, C. H. W.; Serge, N. T. [41] Acetylation. In *Methods in Enzymology* Vol. XXV; Academic Press: Oxford, UK, 1972; pp 494–499.

40. Keough, T.; Youngquist, R. S.; Lacey, M. P. A Method for High-Sensitivity Peptide Sequencing Using Postsource Decay Matrix-Sssisted Laser Desorption Ionization Mass Spectrometry. *Proc. Natl. Acad. Sci. U.S.A.* **1999,** *96*(13), 7131–7136.
41. Balaban, A. T.; Boulton, A. J. In *Organic Syntheses, Collective Volumes*, Vol. V, Baumgarten, H. E., Ed.; Wiley-VCH: 1973; p. 1112.
42. Munchbach, M.; Quadroni, M.; Miotto, G.; James, P. Quantitation and Facilitated *de novo* Sequencing of Proteins by Isotopic N-Terminal Labeling of Peptides with a Fragmentation-Directing Moiety. *Anal. Chem.* **2000,** *72*(17), 4047–4057.
43. Barlet, O.; Yang, C. Y.; Gaskell, S. J. Influence of Cysteine to Cysteic Acid Oxidation on the Collision-Activated Decomposition of Protonated Peptides—Evidence for Intraionic Interactions. *J. Am. Soc. Mass Spectrom.* **1992,** *3*(4), 337–344.
44. Dai, J.; Zhang, Y.; Wang, J.; Li, X.; Lu, Z.; Cai, Y.; Qian, X. Identification of Degradation Products Formed During Performic Oxidation of Peptides and Proteins by High-Performance Liquid Chromatography with Matrix-Assisted Laser Desorption/Ionization and Tandem Mass Spectrometry. *Rapid Commun. Mass Spectrom.* **2005,** *19*(9), 1130–1138.
45. Keough, T.; Youngquist, R. S.; Lacey, M. P. Sulfonic Acid Derivatives for Peptide Sequencing by MALDI MS. *Anal Chem.* **2003,** *75*(7), 156A–165A.
46. Samyn, B.; Debyser, G.; Sergeant, K.; Devreese, B.; Van Beeumen, J. A Case Study of *de novo* Sequence Analysis of N-Sulfonated Peptides by MALDI TOF/TOF Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* **2004,** *15*(12), 1838–1852.
47. Chacon, A.; Masterson, D. S.; Yin, H.; Liebler, D. C.; Porter, N. A. N-Terminal Amino Acid Side-Chain Cleavage of Chemically Modified Peptides in the Gas Phase: A Mass Spectrometry Technique for N-Terminus Identification. *Bioorg. Med. Chem.* **2006,** *14*(18), 6213–6222.
48. Li, X.; Cournoyer, J. J.; Lin, C.; O'Connor, P. B. The Effect of Fixed Charge Modifications on Electron Capture Dissociation. *Proceedings of the 55th ASMS Conference on Mass Spectrometry and Allied Topics*; Indianapolis, IN, June, 2007.