ANALYSIS FOR TRACE AMOUNTS OF ANALYTES BY ELECTROSPRAY MASS SPECTROMETRY*

Magdalena Wierzbicka†, Bartosz Setner, Zbigniew Szewczuk

Chemistry and Stereochemistry of Peptides and Proteins Group
Faculty of Chemistry, University of Wrocław
F. Joliot-Curie 14, 50-383 Wrocław, Poland

(Received June 29, 2016)

Electrospray mass spectrometry (ESI-MS) is the based analytical technique, used in proteomics research for biomarkers determination. However, some compounds do not undergo effective ionization process and, therefore, their trace amounts can be analysed only after proper derivatization. Properly designed ionization enhancers may reduce detection limit and allow for the analysis of minimal quantities of analytes by ESI-MS. The quantitative analysis is carried out using the isotope dilution. Recently, we developed synthetic methods of peptides derivatization by quaternary ammonium salts (QAS) and worked out their analysis at the attomole level. Unfortunately, such derivatives undergo the Hoffman elimination during peptide sequencing in tandem mass spectrometry (MS/MS). To overcome this neutral loss, we developed the synthetic methods of peptides derivatized by cyclic quaternary ammonium salts. In this work, we present synthesis of the azoniaspiro conjugate and its isotopologue deuterated on the α-carbon of the 5-azoniaspiro[4.4]nonyl group (ASN) and its analysis by ESI-FT-ICR-MS.

DOI:10.5506/APhysPolBSupp.9.345

1. Introduction

Mass spectrometry is nowadays one of the most sensitive analytical method. For this reason, it is commonly used in the field of medical research, biologically active compounds or environmental analysis. It is known that many biomarkers or biomodulators are often present at very low concentrations in real life samples and, therefore, have not been discovered by current

* Presented at the NICA Days 2015 Conference associated with WPCF 2015: XI Workshop on Particle Correlations and Femtoscopy, Warszawa, Poland, November 3–7, 2015.

† Corresponding author: magda61w@gmail.com

analytical methods. Furthermore, the ionization efficiency of some compounds is not sufficient in standard ESI-MS conditions, which is the main problem during the analysis of trace amounts of biologically active compounds, *e.g.* proteins and peptides. To overcome this limitation, ionization tags could be introduced into the peptide chain [1]. The ionization enhancers are known to reduce a detection limit. Recently, we developed the method of synthesis of peptide conjugates containing various N,N,N-trialkylglycine moieties [2]. They can reduce the detection limit to the attomole level during nano-LC-ESI-MRM experiment [3]. For qualitative and quantitative analysis, the derivatives were isotopically labelled by deuterons. It is known that the hydrogen atoms bound to heteroatoms in peptides and proteins easily undergo hydrogen–deuterium exchange (HDX). The α-carbon hydrogens of amino acid residues are usually not exchangeable but recently we discovered the HDX of N,N,N-trialkylglycine residues catalysed by a base [4]. Although these ionizations tags are useful in combinatorial chemistry, they cannot be applied in *de novo* peptide sequencing until they undergo Hoffman elimination [5]. To overcome this unwanted reaction, we developed the synthetic methods of peptides derivatized by cyclic quaternary ammonium salts [6]. The aim of our work was to examine new ionization tag: 5-azoniaspiro[4.4]nonyl group, which is stable during an MS/MS experiment and can be utilized for peptides sequencing. We also examined the application of the labelled tag for the isotopic dilution quantitative analysis. For this purpose, we examined the hydrogen–deuterium exchange at the α-carbon of the proline residue derivatized by azoniaspiro[4.4]nonyl group as well as its stability and susceptibility to back-exchange.

2. Experimental

We synthesized a model peptide H-MQIFVKT-OH on the solid support by a standard Fmoc procedure. It is a fragment of ubiquitin — ubiguitus protein responsible for marking the proteins for biodegradation. Then, we coupled this peptide with the Fmoc-L-Pro-OH residue (Fig. 1). After Fmoc protecting group cleavage, we performed the nucleophilic substitution with 1,4-dibromobutane in the presence of N,N-diisopropylethylamine to form the azoniaspiro[4.4]nonylcarboxyl group (Fig. 2). Finally, a peptide conjugate was cleaved from the solid support with TFA, lyophilised, and purified using a preparative reversed phase HPLC: Varian ProStar, column: TSKgel ODS-120T (21.5 mm $\times$ 30 cm, 10 μm) equipped with the UV detector (210 nm and 280 nm). The product structure was confirmed by the ESI-MS and ESI-MS/MS analysis. Mass spectrometry experiments were done on a Fourier transform ion cyclotron resonance (FT-ICR) Apex-Qe Ultra 7T Instrument (Bruker Daltonics, Brema, Germany).

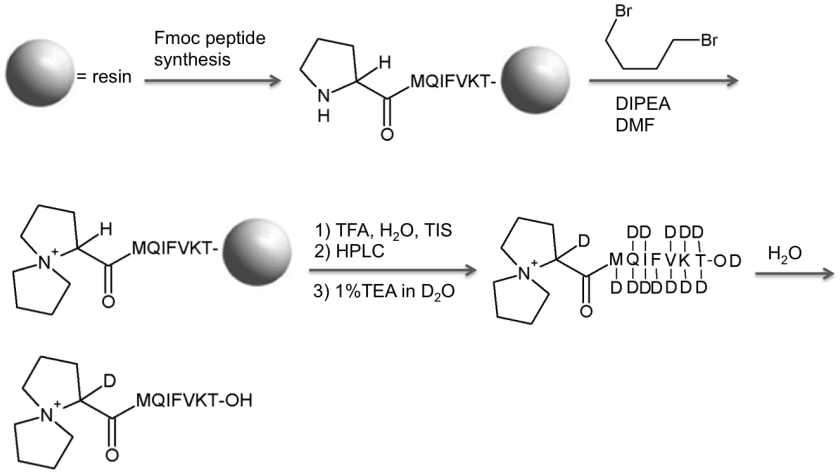

Fig. 1. The scheme of synthesis of ASN^+-CO-MQIFVKT-OH, DIPEA = N,N-Diisopropylethylamine, DMF = N,N-Dimethylformamide, TFA = Trifluoroacetic acid, TIS = Triisopropylsilane, TEA = Triethylamine, D_2O = deuterated water.

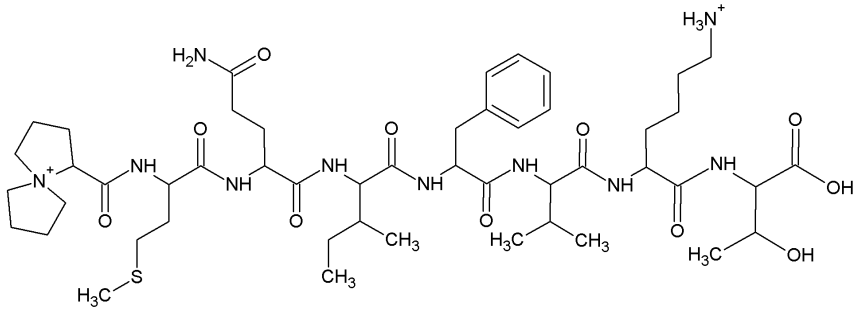

Fig. 2. Synthesized analog of the ubiquitin fragment.

To examine the hydrogen–deuterium exchange of the synthesized conjugate, we dissolved the ASN^+-CO-MQIFVKT-OH in 1% TEA/D_2O and incubated for 2, 5, 10, 20 and 30 minutes at room temperature. After lyophilization, we redissolved the samples in H_2O. Finally, we performed the MS/MS analysis of deuterated and non-deuterated analogues.

3. Results and discussion

The peptide conjugate was synthesized with a high yield. The N-terminal proline residue was converted into the azoniaspiro[4.4]nonylcarbonyl group by nucleophilic substitution with 1,4-dibromobutane. The structure of ASN^+-CO-MQIFVKT-OH was confirmed by ESI-MS and ESI-MS/MS analysis.

There are two clear signals in the ESI-MS spectrum (Fig. 3): the most abundant signal (509.23) results from the protonated compound $[MH]^{2+}$ and the signal at m/z 1017.58, corresponding to M^+. Its isotopic pattern relates to the simulated isotopic pattern of $C_{49}H_{81}N_{10}O_{11}S$ (Fig. 4). Application of the 5-azoniaspiro[4.4]nonylcarbonyl ionization tag increases 100 times the ionization efficiency.

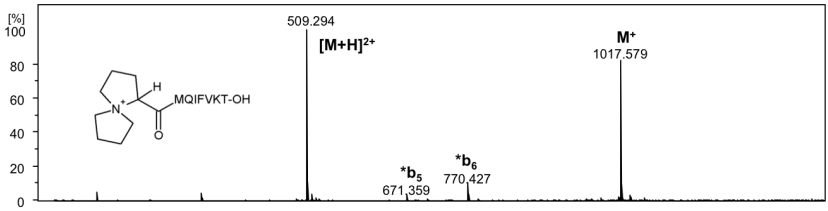

Fig. 3. ESI-MS spectrum of synthesized ASN^+-CO-MQIFVKT-OH.

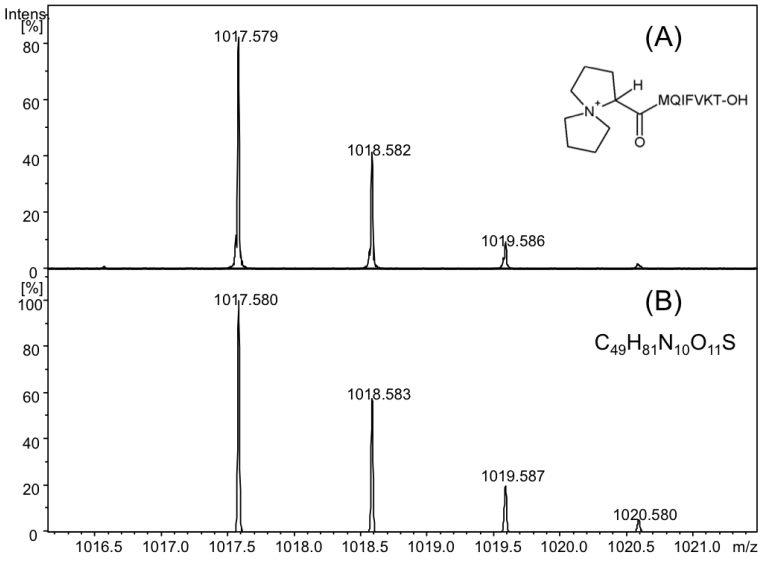

Fig. 4. Isotopic envelope: experimental (A) and simulated (B): the mass accuracy is high (1.67 ppm) and the resolving power is about 70 000 FWHM.

Hydrogen–deuterium exchange takes place in the solution of 1% TEA in D_2O, and the mass spectrum shows that the peptide m/z value was shifted from 1017.58 to 1031.66, which indicates that 14 protons undergo a hydrogen–deuterium exchange. After dissolving in H_2O, the m/z value of the major peak decreased to m/z 1018.58. The labile deuterium atoms undergo back-exchange in water solution at neutral pH. But the hydrogen on α-carbon of the azoniaspiro[4.4]nonylcarbonyl does not undergo a back-

exchange in water solution. The evolution of the HDX spectra with time (Fig. 5) shows that the hydrogen–deuterium exchange is complete within 20 minutes at room temperature.

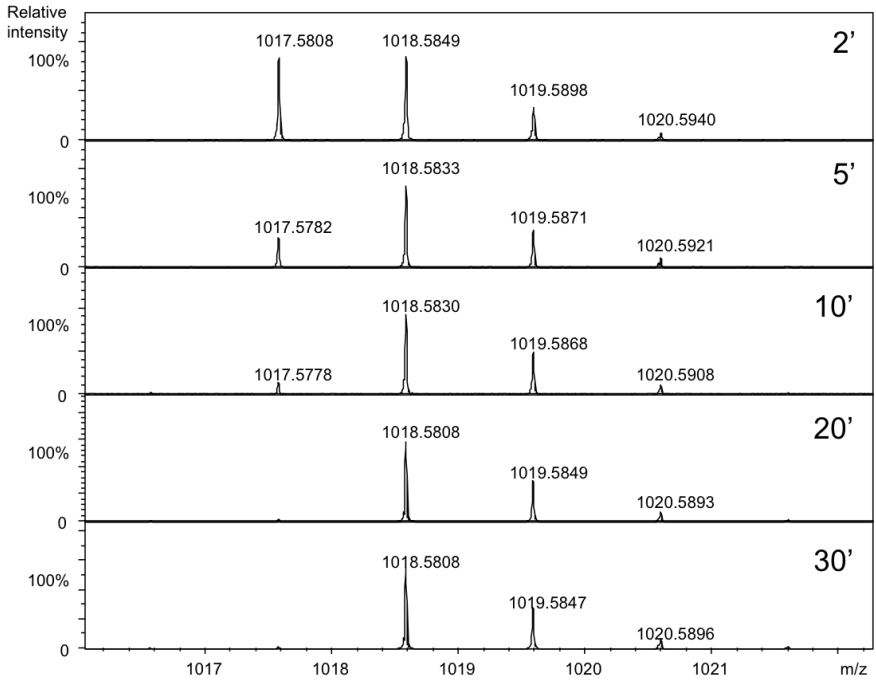

Fig. 5. Mass spectra of ASN^{+}-CO-MQIFVKT-OH recorded after 2, 5, 10, 20 and 30 minutes of incubation in 1% TEA in D_2O, followed by lyophilisation and redissolving in H_2O.

Finally, we performed MS/MS analysis of deuterated (Fig. 6 (c)) and non-deuterated (Fig. 6 (b)) peptide conjugates. At the collision energy set to 12 eV, mostly *b and *a daughter ions were observed (Fig. 6 (a)). The azoniaspiro[4.4]nonylcarbonyl group does not undergo fragmentation in that conditions what facilitate interpretation of the spectrum. All of the *b and *a peaks on spectrum obtained for the deuterated peptide conjugate were shifted by 1.004 Da as compared to its non-deuterated counterpart what confirms that the isotopically labelled peptide contained one deuterium atom.

In conclusion, the azoniaspiro[4.4]nonyl system increases the ionization efficiency. The ionization tag can be easily introduced into peptides containing a proline residue at the *N*-terminus on the solid support. Our results show that the hydrogen–deuterium exchange of the derivatized proline residue is feasible and the obtained isotopologue is stable at neutral conditions, which make it potentially useful in isotopic dilution for quantitative

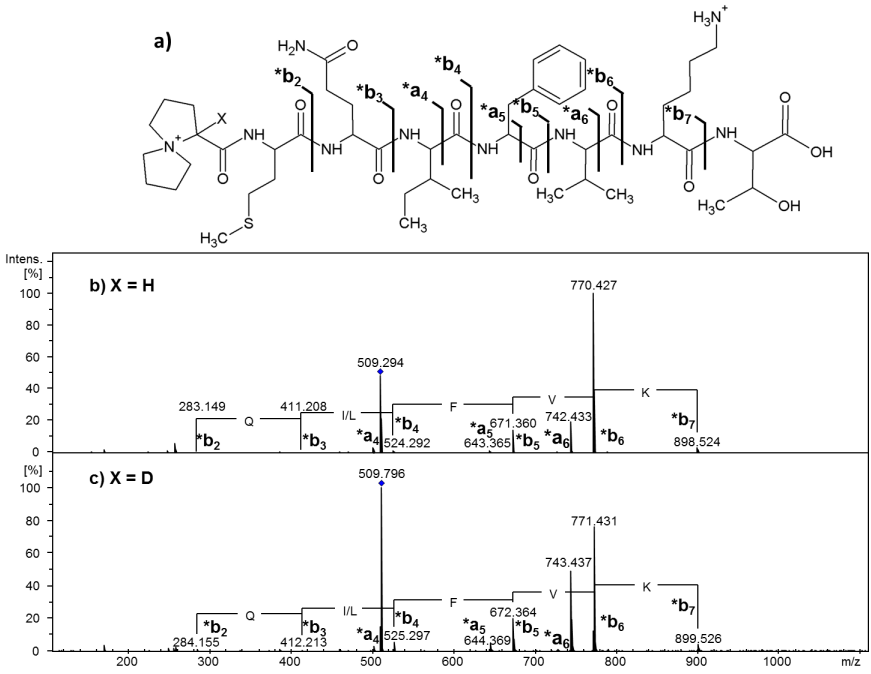

Fig. 6. (a) Fragmentation of ASN$^+$-CO-MQIFVKT-OH, (b) MS/MS spectrum of non-deuterated compound (parent ion at m/z 509.3), (c) MS/MS spectrum of deuterated compound (parent ion at m/z 509.8).

peptides analysis. Furthermore, a high stability of the ionization tag facilitates the peptides sequencing. Its possibility to increase the ionization efficiency may lead to development of new biomarkers based on proteins of low abundance.

This work was supported by a grant No. UMO 2013/09/B/ST4/00277 from the National Science Centre, Poland.

REFERENCES

[1] P. Stefanowicz, A. Kluczyk, Z. Szewczuk, *Amino Acids, Pept. Proteins* **40**, 36 (2016).
[2] M. Cydzik, M. Rudowska, P. Stefanowicz, Z. Szewczuk, *J. Pep. Sci.* **17**, 445 (2011).
[3] R. Bąchor *et al.*, *Int. J. Mass Spectrom.* **362**, 32 (2014).
[4] M. Rudowska *et al.*, *J. Am. Soc. Mass Spectrom.* **23**, 1024 (2012).
[5] R. Bąchor *et al.*, *Mol. Divers.* **16**, 613 (2012).
[6] B. Setner *et al.*, *J. Mass Spectrom.* **49**, 995 (2014).