

# Identification and differentiation of disulfide-bonded isomers of the $\mu$ -conotoxin PIIIA by trapped ion mobility spectrometry

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## Introduction

Cysteine-rich peptides, such as defensins and conotoxins, form intramolecular disulfide bonds which stabilize the peptide structure and contributes to the biological activity of the corresponding peptides. The correct connectivity of the disulfide bonds within the peptides is crucial for the bioactivity and is a key factor when such disulfide-rich peptides are applied in biopharmaceutical research. It is difficult to distinguish between different disulfide-bonded isomers using standard HPLC or MS methods, due to similar retention times and same masses. This study analyses different disulfide-bonded variants of the  $\mu$ -conotoxin PIIIA to demonstrate the capability of trapped ion mobility spectrometry (TIMS) to differentiate linkage isomers via altered collision cross section (CCS) values.

## Methods

The native 3-disulfide-bonded  $\mu$ -PIIIA isomers (**1**) (C4-C16, C5-C21, C11-C22) as well as 4 disulfide-bonded variants (**2-5**) were produced by solid-phase peptide synthesis using a targeted protecting group strategy and subsequent stepwise oxidation in solution. The cyclic  $\mu$ -PIIIA variants (**1-5**) were eluted from an ACQUITY UPLC CSH C18 1.7 $\mu$ m 2.1 x 150 mm column (Waters) with a 5 min gradient using an Elute UHPLC, and TIMS-MS spectra were acquired using a timsTOF Pro instrument (Bruker). The mobilograms were analyzed in DataAnalysis software (Bruker).

## Results

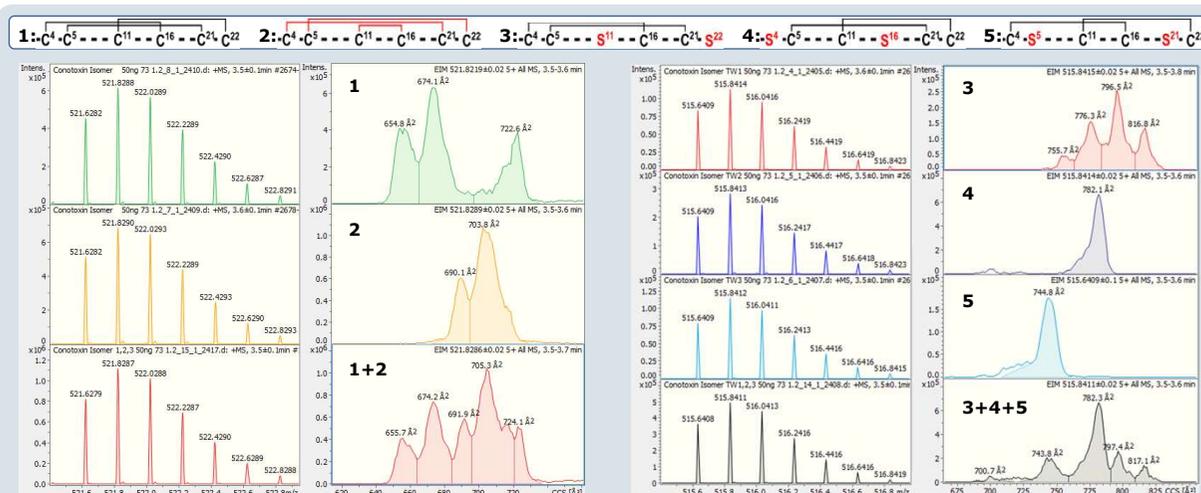
The native form of the  $\mu$ -conotoxin PIIIA is a 3-disulfide-bonded isomer (**1**) with the disulfide connectivity Cys4-Cys16, Cys5-Cys21 and Cys11-Cys22, which was compared to another 3-disulfide-bonded isomer (**2**) using TIMS-MS (**Fig. 1**). A differentiation of the two peptides (**1,2**) was observed by the mobility profile which was not possible by MS alone. The most preferred conformation of the naturally occurring isomer (**Fig. 1, 1**) shows a lower CCS

value (674.1 Å<sup>2</sup>) than the ions of the other isomer (**Fig. 1, 2**, CCS: 703.8 Å<sup>2</sup>), which goes in line with the more compact structure of the native isomer (**1**) compared to isomer **2**, as already described by Heimer et al. [1]. These isomers (**1,2**) were mixed in equimolar amounts and both isomers could still be observed and identified by mobility profile and CCS values, despite of their overlapping profiles (**Fig. 1, 1+2**).

Additionally three 2-disulfide-bonded variants (**3-5**), which varied in the amino acid sequence at two positions, where cysteines were replaced by serine residues, resulting in a reduction of the number of disulfide bonds in the PIIIA lead were analyzed [2]. Comparing the generally higher CCS values of these variants (**Fig. 2, 3-5**) with the native isomer (**Fig. 1, 1**) reflects the increased stability that the third disulfide bond provides.

In addition, **Fig. 2** shows that isomer **5** has the most compact structure of the disulfide deficient peptides (**3-5**), suggesting that the disulfide bond between Cys5-Cys21 has the lowest influence on the stability of the native PIIIA isomer **1**.

Again, **Fig. 2** shows that in a mixture experiment (**3+4+5**) the isomers could still be observed and identified by mobility profile and CCS values, and these could be quantified if fitting routines to a profile library were applied.



**Fig. 1** MS1 and TIMS mobility profiles for native  $\mu$ -conotoxin PIIIA isomer and a 3-disulfide-bonded isomer variant individually (**1** and **2**) and in equimolar mixture (**1+2**). The differences between the PIIIA variant and the native isomer are shown in red in the sequence.

**Fig. 2** MS1 and TIMS mobility profiles for three  $\mu$ -conotoxin PIIIA 2-disulfide-bonded isomer variants, individually (**3, 4** and **5**) and in equimolar mixture (**3+4+5**). (Top) The differences between the PIIIA variants and the native isomer (**Fig. 1, 1**) are shown in red in the sequence.

## References

- (1) A. A. Paul George et al., Mar Drugs 2019, 17, 390.
- (2) P. Heimer et al., Anal. Chem. 2018, 90, 3321.

## Conclusions

- TIMS-MS allowed differentiation of different isomeric disulfide-bonded  $\mu$ -conotoxin PIIIA variants
- CCS values correspond with known structural information from previous studies
- TIMS-MS analysis revealed an insight to the influence of particular disulfide bonds on peptide stability
- Individual isomers were identified and characterized in case of mixtures

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