doi:10.2533/chimia.2023.250 Chimia 77 (2023) 250–253

Pyoverdine Analysis – From High-resolution MS/MS Fragmentation to Ion Mobility Measurements

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[§]SCS-DSM Award for best poster in Analytical Sciences

Abstract: Microorganisms produce iron chelators called siderophores that are a rich source for drug discovery or plant protective agents. Pyoverdines are a class of siderophores from fluorescent *Pseudomonas* members and consist of different peptide chains specific to each bacterial species. The structural elucidation and characterization of pyoverdines require comprehensive analytical methods as bacterial extracts are complex mixtures. Here, we present a high-throughput UHPLC-MS/MS pipeline and the application of ion mobility spectrometry to facilitate research in the field of medicine and agriculture.

Keywords: Mass Spectrometry · *Pseudomonas* · Siderophore · Structural Elucidation

Karoline Rehm obtained her Bachelor's and Master's degree in Chemistry at the University of Zurich. Under the supervision of Prof. Dr. Laurent Bigler, she conducted her Master's thesis on the quantification of progestogen metabolites in animal plasma. She continued her studies in analytical chemistry during her PhD in the research group of Prof. Bigler. Her main research focus is the application of high-resolution mass spectrometry in the field of small molecules, ranging from structural elucidation to quantification and untargeted analysis.

1. Introduction

Siderophores are a class of secondary metabolites secreted by microorganisms that have a high binding affinity to iron.[1] They have great potential in the field of medicine, industry and agriculture.[2] For example, their ability to protect plants from pathogen infections or bacterial communities from pathogen invasion was recently demonstrated.[3,4] They have also gained interest as drugs in the fight against cancer in recent years.[5] To date, several hundreds of chemically distinct siderophores have been characterized.[1] Most species produce their own unique metabolite that is recognized by their cognate uptake receptor in order to gain a competitive advantage.[6]

Pyoverdines are a subclass of siderophores and are solely found among fluorescent *Pseudomonas* members. In Fig. 1, their basic structure is illustrated by taking the pyoverdine PAO1 as an example. They are chromopeptides consisting of 6–14 amino acids that heavily vary depending on the bacterial strain. Multiple pyoverdines with different side chains or with linear and cyclized entities may also be encountered in a single bacterial extract. While succinic acid and succinic amide are the most common side chains, other possibilities such as glutamic acid or succinic imide have also been reported. Only the chromophore core is a conserved element. For this reason, over 60 pyoverdine types have already been described in the literature.[7]

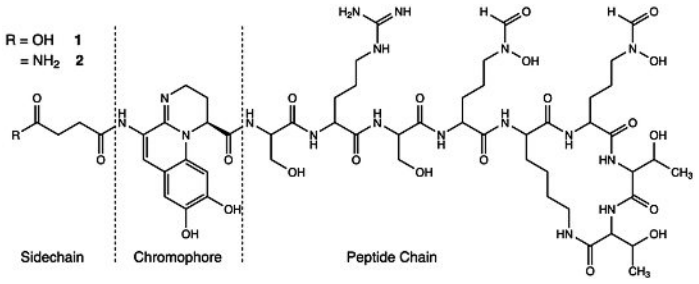

Fig. 1. Example of a pyoverdine structure: PAO1 consisting of a side chain (succinic acid (**1**) or succinic amide (**2**)), the characteristic pyoverdine chromophore and a structurally diverse peptide chain. The figure was taken from ref. [11].

In recent studies, it was shown that pyoverdines are a key factor in promoting or impeding invasions in bacterial communities.[3,4] However, the chemical structures of the respective pyoverdines remained unknown. Identifying the pyoverdines secreted by a bacterium is quite challenging, as bacterial extracts are always complex mixtures and many possible variations in the pyoverdine framework may be present. Even though *de novo* structural elucidation is frequently conducted by the interpretation of MS/MS fragmentation spectra for pyoverdines, no universally applicable method has been developed up to now.[7,8]

Alternatively, unknown pyoverdines can also be characterized by matching a measured internal characteristic against a library database of known pyoverdines. For example, determining the exact mass of a pyoverdine together with its isoelectrofocusing (IEF) pattern or iron uptake behavior provides sufficient information to unambiguously identify a pyoverdine.[9,10] However, this requires the performance of at least two experiments, which is time and cost-intensive.

In our research, we aimed to solve both problems: Firstly, we developed a universal high-throughput UHPLC-MS/MS pipeline, that is comprehensive and easy to replicate, to elucidate the pep-

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tide chain of pyoverdines and their derivatives in bacterial liquid cultures.[11] Secondly, we propose the measurement of collision cross sections (CCS) values by ion mobility spectrometry (IMS) as an alternative identification marker replacing the need for iron uptake or IEF experiments.[12]

2. Pyoverdine Structural Elucidation Pipeline

The first step in the structural elucidation pipeline of pyoverdines is the sample preparation as presented in Fig. 2.

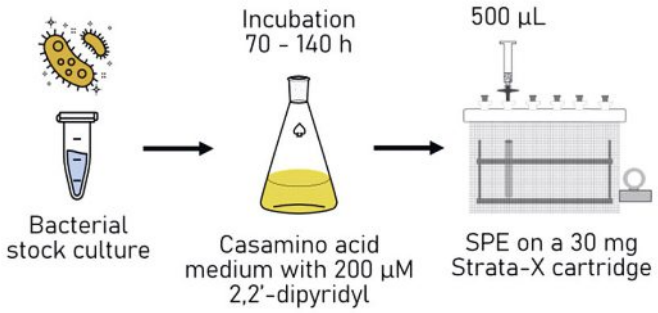

Fig. 2. Basic steps for the sample preparation of bacterial liquid cultures for LC-MS analysis.

Briefly, a bacterial stock culture was incubated in casamino acid medium under iron-deficient conditions in order to stimulate siderophore production. Afterwards, a small aliquot of 500 µL liquid bacterial culture was purified by a small-scale solid phase extraction (SPE) by washing the loaded cartridge with water and eluting the pyoverdines with 30% aq. MeOH. The resulted sample did not require any further reconstitution and could be directly submitted to liquid chromatography. Multiple samples can be purified simultaneously in this fashion. The presence of pyoverdines in the extracts can also be checked under UV-light as their chromophore core emits fluorescence (Fig. 3).

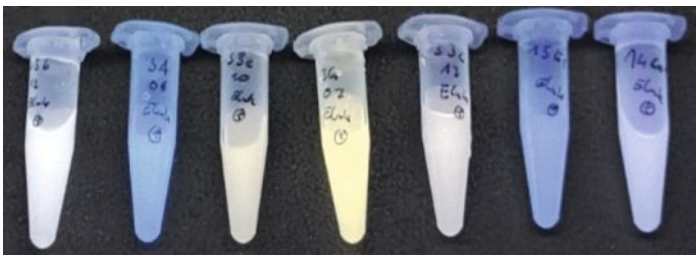

Fig. 3. Typical fluorescent glow of SPE purified pyoverdine extracts under UV light.

What follows is the analysis of the extract by UHPLC-HR-MS/MS, which is illustrated in Fig. 4. Within 15 min, the polar components of the bacterial extracts were separated with a high peak capacity on a HSS C18 column, using water and MeCN with 0.1% formic acid as an eluent.

A Q Exactive hybrid quadrupole-Orbitrap mass spectrometer was coupled to the UHPLC system and an all-ion-fragmentation (AIF) scan was recorded besides a full MS scan. In this way, characteristic fragments for pyoverdine (204 *m/z*) and for its biological precursor ferribactin (136 *m/z*) were generated. Localizing these fragments allowed for the determination of the intact pyoverdine mass.

In a last step, the $[M+H]^{2+}$ ions of the pyoverdine candidates were isolated and fragmented at multiple collision energies, generating high-quality MS/MS spectra for all types of pyoverdines. While cyclized pyoverdines tended to be more stable and required higher fragmentation energies, linear pyoverdines started to fragment already at lower energies. The generated MS/MS spectra contained mostly B and Y″ ions that could be used to assemble the peptide sequence. Since this MS/MS interpretation is time-consuming, a pyoverdine fragmentation predictor programmed in Excel was developed. In this way, measured and theoretical masses can be quickly compared.

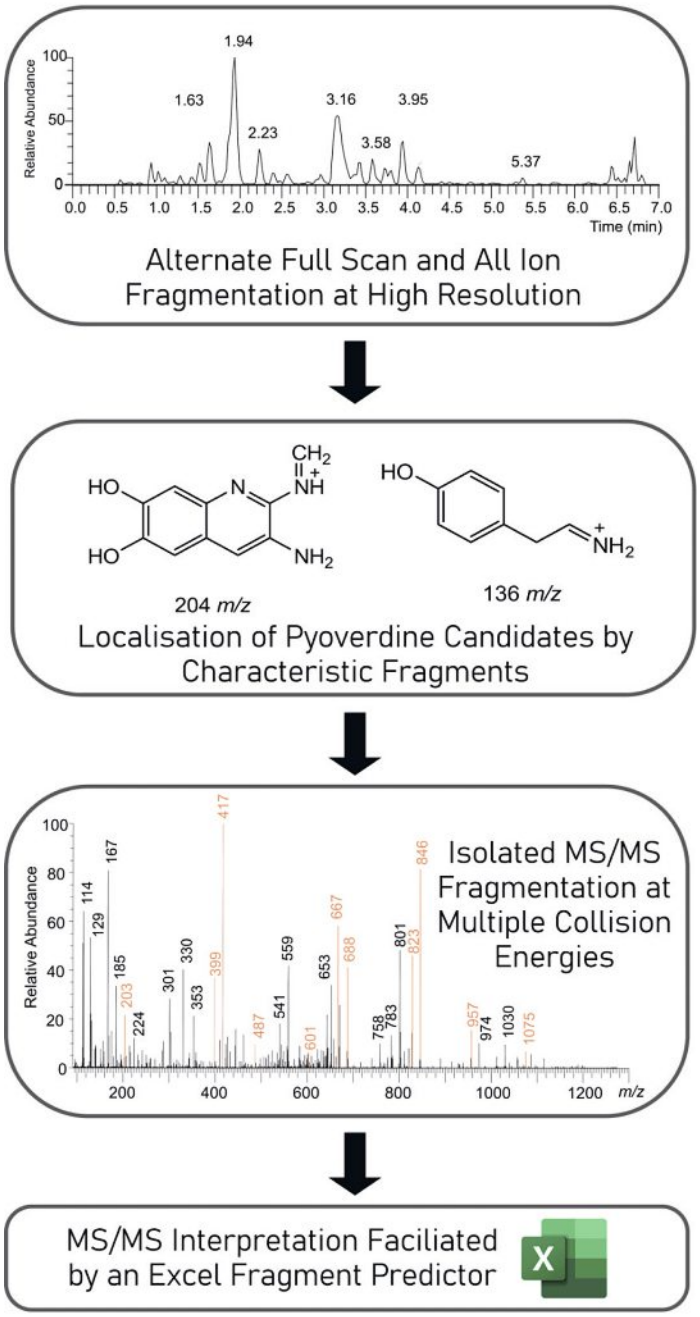

Fig. 4. Workflow for the structural elucidation of unknown pyoverdines.

This workflow was optimized and validated by using four well-known pyoverdines differing in their structure. Afterwards, it was successfully applied to elucidate the unknown pyoverdines found in the bacterial extracts of 13 fluorescent *Pseudomonas* species sampled from the Irchel park (Zurich). Thereby, four new pyoverdine peptide chains were discovered.[11]

3. Collision Cross Sections: An Alternative Characterization Marker

Trapped ion mobility spectrometry (TIMS) is a new technique for characterizing compounds by measuring their collision cross sections (CCS).[13] CCS values are commonly expressed in Å^2, an area that relates to the size of a molecule. By combining TIMS with UHPLC and MS, a three-dimensional separation is achieved: Polarity, shape, and mass. This offers the additional advantages that isomers and complex mixtures can be readily separated. While already popular in the fields of lipidomics, proteomics and positional post-translation modification,[14] no ion mobility data on pyoverdine and its iron bond complex was published up to date to the best of our knowledge.

In our work,[12] we determined the CCS values of 17 pyoverdines and their iron-complexes using a timsTOF Pro hybrid quadrupole-time-of-flight (QTOF) mass spectrometer. In their apo (iron-free) form, pyoverdines showed one dominant ion mobility signal. An exemplary ion mobilogramm of the pyoverdine S3a05 is shown in Fig. 5a.

Furthermore, we found a positive correlation between the pyoverdine mass and its corresponding CCS value (see Fig. 6).

These CCS values were very characteristic for each compound and remained stable during repeated measurements. Since this intrinsic CCS can be determined simultaneously in an UHPLC-MS measurement, ion mobility has a high potential to replace older differentiation methods such as IEF or iron uptake experiments.

For additional annotation confidence, broadband collision-induced dissociation (bbCID) can be included in the measurement run to confirm the pyoverdine nature of the analyte based on its characteristic 204 *m/z* fragment.

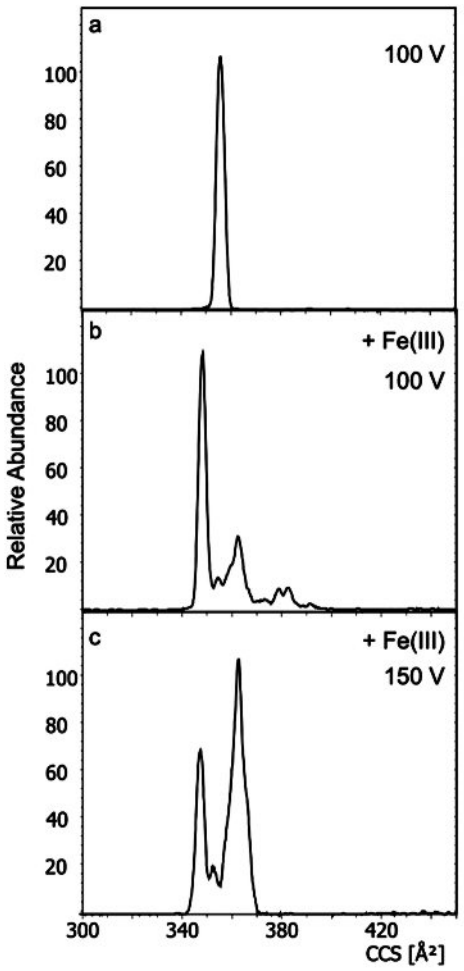

Fig. 5. Ion mobility patters of pyoverdine S3a05: a) apo-pyoverdine measured at Δ6 = 100 V, b) ferripyoverdine measured at Δ6 = 100 V, c) ferripyoverdine measured at Δ6 = 150 V.

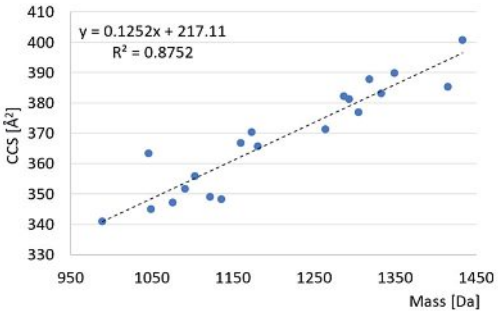

Fig. 6. Graphic illustration of CCS values from pyoverdines of different masses. The figure was adapted from ref. [11].

The determination of the CCS values of apo-pyoverdines is not possible in analytical laboratories that are not free of iron contaminations.[15] For this reason, ferripyoverdines were also investigated by adding $FeCl_3$ to the apo-pyoverdine extracts. For ferripyoverdines, an unpredictable change in ion mobility pattern was observed in comparison to the corresponding apo-pyoverdines. For example, the splitting of the ion mobility signal of S3a05 after the addition of iron is illustrated in Fig. 5b.

Potential explanations for this splitting of ion mobility patterns could be coordination isomerism, helical handedness or partial molecule unfolding. The latter was further investigated by increasing the TIMS Δ6 voltage and thereby stimulating collision-induced unfolding (CIU). This parameter adjusts the velocity of ions as they move from the accumulation tunnel to the separation tunnel in the mass spectrometer. At higher voltages, the ions will collide with the drift gas with larger collision energies, resulting in the energetic activation of the complex.[16]

It was found that some ferripyoverdines remained stable and their ion mobility patterns did not change upon activation. In contrast, other ferripyoverdine ion mobility patterns, such as the one of S3a05 (Fig 5c), changed drastically at higher Δ6 voltages. This emphasizes the need to describe all instrument parameters to obtain comparable results across platforms. If this is taken into account, CCS measurements of ferripyoverdines are just as suitable as those of apo-pyoverdines as an alternative identification marker. Thus, our method can also be used in iron-contaminated laboratories.

4. Conclusion and Outlook

Siderophores such as pyoverdines gained a large interest due to their properties to combat pathogens and their potential applications in medicine and agriculture. Their structural characterization, however, has been challenging due to the lack of analytical methods. In our research, we developed versatile approaches for the analysis of pyoverdines. To date, the gold standard for pyoverdine identification remains the interpretation of their MS/MS spectra. For this reason, we developed a high-throughput structural elucidation pipeline that was validated using 17 different pyoverdine extracts. Even though bacterial extracts are complex mixtures, AIF and/or bbCID can be applied in order to identify pyoverdines or their biological precursor, ferribactin. High-quality MS/MS spectra for the structural interpretation were generated by the application of multiple collision energies.

We also investigated the CCS values of apo- and ferripyoverdines by TIMS. These CCS values were highly characteristic and proved to be a suitable alternative identification marker, rendering other analytical methods obsolete. Thereby, instrument settings should be reported in detail to allow data comparability.

In the future, our analytical approach can also serve as a basis for investigating other siderophore classes such as citrate derivatives. In this way, we make the world of microbial secondary metabolites more accessible and facilitate research on the applications of pyoverdines in the field of agriculture and medicine.

Acknowledgements

We thank the Swiss Chemical Society and DSM Nutritional Products for their support and the best poster award in analytical sciences. The research was funded in part by the Swiss National Science Foundation (SNSF, grant no. 31003A_182499 to RK).

Received: January 6, 2023

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