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Ion Mobility Mass Spectrometry as an Efficient Tool for Identification of Streptorubin B in *Streptomyces coelicolor* M145

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Abstract

Ion mobility spectrometry was utilized to corroborate the identity of streptorubin B (**2**) as the natural product produced by *Streptomyces coelicolor*. Natural product **2** was initially assigned as butylcycloheptylprodigiosin (**3**), and only relatively recently was this assignment clarified. We present additional evidence of this assignment by comparing collisional cross sections (Ω) of synthetic standards of **2**, **3**, and metacycloprodigiosin (**4**) to the cyclic prodiginine produced by *S. coelicolor*. Calculated theoretical Ω values demonstrate that cyclic prodiginines could be identified without standards. This work highlights ion mobility as an efficient tool for the dereplication of natural products.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jnatprod.9b00828>.

Drift chromatograms, ion mobility calibration information, ¹H NMR spectrum of **2**, and optimized 3D structures of prodiginines (PDF)

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Prodiginines were first discovered in 1819 when pharmacist Bartolomeo Bizio sought to identify the cause of the reddening of a farmer's polenta. He discovered that a microorganism was the cause of the reddening instead of a diabolical occurrence, which had previously been postulated.¹ Several prodiginines that have been found in various bacterial species are depicted in Figure 1. *Streptomyces coelicolor* M145 primarily produces two prodiginines: undecylprodigiosin (**1**) and streptorubin B (**2**).^{2–4} However, the identity of the latter species remained contentious for decades after its discovery from *Streptomyces* sp. Y-42 in 1975 by Gerber, who initially assigned the structure as butylcycloheptylprodigiosin (**3**).⁵ Although Gerber did reassign the structure to **2** in later biosynthetic studies, this went largely unnoticed, and in 1985, Floss discovered a pink compound from *S. coelicolor* A3(2) and assigned it as **3** based on Gerber's original report.^{2,6} In 1991, Weyland and co-workers reported a strain of actinomycete that produced a compound whose structure corresponded to **2**, not **3**.⁷ Synthetic work from the Fürstner and Reeves laboratories in 2005 and 2007, respectively, provided an authentic sample of **3** whose ¹H NMR spectrum was reported to match that of the authentic pigment isolated by Floss.^{8,9} On this basis, it was concluded that **3** was a natural product. Subsequently, in 2008, while conducting biosynthetic studies in *S. coelicolor* A3(2), Challis and co-workers isolated the cyclic prodiginine, and through extensive NMR studies showed that it corresponded to the structure of **2**.⁴ Thomson and co-workers later completed an enantioselective synthesis of **2**.¹⁰ Both groups concluded that **2** was not only the molecule produced by *S. coelicolor* A3(2) but that it also existed as slowly converting atropisomers.^{6,10,11} Additionally, in 2011, Thomson and co-workers undertook the synthesis of **3**, and by mass spectrometry fragmentation analysis, they demonstrated that the structure of **3** was inconsistent with the natural product from *S. coelicolor*. Their analysis of the fragmentation patterns was more consistent with **2**.¹² Clearly, substantial effort has been devoted to the correct identification of this natural product, largely through traditional isolation, synthesis, and NMR characterization, which is incredibly resource-intensive and challenging to accomplish. Therefore, we sought to employ ion mobility mass spectrometry to provide a case study for use with natural products, as it is a rapid and powerful technique but currently underutilized.

As the need for rapid methods of detection and identification of metabolites becomes more apparent, ion mobility spectrometry (IMS) provides a compelling and simple addition to the toolkit for small molecule analysis. This method provides an orthogonal physical measurement to traditional methodologies, the collisional cross section (Ω), a measure of the mobility of an ion in a gas-filled cell as it is colliding with neutral buffer gas molecules in a

weak and uniform electric field, which results in a size-based separation of analyte molecules as a consequence of their ionized 3D conformation in the gas phase.^{13,14} This measurement yields additional structural information because the size and shape of molecules can be gleaned from Ω values, which enables the differentiation and rationalization of structures for isomeric species that may not be elucidated by mass spectrometry fragmentation or complex NMR studies. Ion mobility has a significant advantage in that it is easily amended to LC-MS methodology as LC-IM-MS. This is due to the time scale of analysis of IMS being in the milliseconds, whereas LC and TOF-MS occur in the second and microsecond time scale, respectively.¹⁵ Recently, the use of LC-IM-MS was demonstrated to provide significant improvement over standard LC-MS analysis, owing to its ability to enable the identification of coeluting compounds and isobaric species.¹⁶

In this study, traveling wave IMS (TWIMS) was used as it is commercially available in the Waters Synapt high definition mass spectrometers.^{17,18} TWIMS works by “pushing” ions through a drift tube using a wave that is generated by repeating DC pulses along the gas-filled tube instead of a constant electric field, which generally results in shorter analysis time. TWIMS is effective for analysis and separation of metabolites.^{19–21} IMS analysis has a clear place in aiding in the identification of metabolites as it is quick and amenable to current workflows and orthogonal to most other techniques, which is demonstrated here by the confirmation in which multiple closely related structures were readily resolved to provide further experimental evidence that *S. coelicolor* produces **2** and not the initially identified structure (**3**).

Collisional Cross Sections of Synthetic Standards.

To establish the difference in collisional cross section between **2** and **3**, three wave velocities were examined, 500, 600, and 700 m s⁻¹ (enables evaluation of the standard deviation of the Ω measurements). However, measured Ω values are not directly proportional to measured drift times, as in conventional drift-tube IMS (DT-IMS).²⁸ Thus, calibration is required for determination of the measured Ω with comparable accuracy to DT-IMS.²⁹ Fortunately, this is not an issue in our analysis as we are comparing measured Ω values of standards to match the measured Ω value of a biological compound, and exact measured Ω values are not needed. We also included metacycloprodigiosin (**4**) in our analysis to further demonstrate the utility of this method to distinguish closely related isomers.

Comparison of the standards, prepared according to published procedures,^{10,12,30,31} indicated that **2**, **3**, and **4** had at least a 1 Å² difference in their collisional cross section values, which has been previously reported to be sufficient to differentiate between structural isomers (Figures S1–S3).³² As shown in Figure 2, there is a difference in peak position for each compound. When the drift times at each wave velocity were converted to Ω , the averaged values of 205.41 ± 0.36 , 208.20 ± 0.28 , and 209.73 ± 0.13 Å² were obtained for **4**, **2**, and **3**, respectively. Whereas **4** was not a debated structure for the cyclic prodiginine in *S. coelicolor*, it is a related compound that is produced by *Streptomyces longispororuber*, which has an orthologous gene to *redG*, *mcpG*, which encodes for the protein that performs the oxidative carbocyclization reaction to transform **1** into **4** rather than **2**.³³ The difference between the primary compounds of interest, **2** and **3**, was found to be significant ($p =$

9.01×10^{-11}). Additionally, the measured Ω values correspond to the expected shape/size of each compound (Figure 1). Molecule **3** has a measured Ω value slightly larger than that of **2** as the *n*-butyl group is present on a carbon further from the core of the molecule, whereas **4** has a shorter alkyl moiety and correspondingly smaller measured Ω value in comparison to that for the other two species. Our data indicate that we can readily measure the difference between synthetic standards of these compounds and rationalize their relative mobility based upon their structures.

The use of ion mobility measurements on small molecules is a growing field that enables interrogation of the physical properties of metabolites in a rapid manner. We were able to effectively measure the difference in the measured Ω value of synthetic cyclic prodiginines, which due to their similar structures can be distinguished from one another by mass spectrometry and NMR but require complex data interpretation that has previously led to mischaracterization. In contrast, ion mobility provides rapid and easily interpreted results that can be rationalized by the 3D structures of the molecules. As an additional characterization technique, ion mobility can be a powerful tool for the dereplication and identification of natural products.

Collisional Cross Section of *Streptomyces coelicolor* M145 Prodiginine and Ion Mobility Coelution Studies.

Recent literature has unequivocally shown that the prodiginine found from *S. coelicolor* is compound **2**.^{10,11} Our data confirm this as a measured Ω value of $208.20 \pm 0.28 \text{ \AA}^2$ was obtained from the pink metabolite found in the culture media, which is identical to that of the synthetic standard of **2** (Figure S4). Ion mobility coelution studies were also performed to bolster our confirmation that **2** was produced by *S. coelicolor*. Each synthetic standard was added to the *S. coelicolor* prodiginine solution, and wave velocities of 500, 600, and 700 m s^{-1} were again used (Figures S5–S7). However, it is known that as wave velocity increases, the resolving power also increases at the cost of reduced Ω accuracy.^{28,34} Although it was not possible to fully resolve each species from one another, at 700 m s^{-1} , the peak shapes in the samples mixed with **3** and **4** are clearly distorted compared to the isolated samples (Figure 3A,B). Additionally, overlaying the isolated samples onto the mixed samples illustrates that the peak shapes are the result of the mixture. As expected, the sample mixed with **2** appeared identical to that of the standard of **2** (Figure 3C). Thus, measured Ω values and ion mobility coelution studies provide definitive evidence that **2** is produced by *S. coelicolor*, as previously reported.^{10,11}

Our results show that metabolites in a complex matrix, such as a minimally purified bacterial media solution, can be orthogonally investigated using ion mobility. This enabled us to differentiate constitutionally isomeric prodiginines and provided additional experimental confirmation that **2** is the true structure that is produced by *S. coelicolor* M145. This strategy has great potential for the efficient identification of natural products from biological media, prior to any extensive purification efforts. Ion mobility requires both less time and material to analyze a biologically produced compound in comparison to NMR, assuming that a synthetic standard is available. Even when a synthetic standard is not available, the use of

molecular modeling has become a powerful tool to validate a structural hypothesis about a compound through comparison of calculated and measured Ω values.^{14,34} The straightforward differentiation of prodiginines by ion mobility in this work exemplifies the power of this technique in the identification of small molecules and will be applicable to prodiginines produced by other organisms, as well as other classes of natural products.

Theoretical Collisional Cross Sections of Cyclic Prodiginines.

In most cases during natural product discovery, related structural standards of compounds are not available. However, Ω values can be calculated once putative structures have been determined and can be used as comparators to experimental measurements. We sought to apply this method to the cyclic prodiginines and evaluate this strategy for other natural product structure identification cases. The A-position pyrrole (Figure 1) was chosen for protonation as it was previously shown to be the most favorable by density functional theory calculations and provided the possibility for proton stabilization by hydrogen bonding with the methoxy group on the B-position pyrrole, which was also found to be true in this work as the A pyrrole protonated structures resulted in the lowest energies.³⁵ We found that the calculated Ω values for **2**, **3**, and **4** with the A pyrrole protonation site matched well to the measured Ω values with a less than 2% difference for each, which is within the typical error produced by MOBCAL.³⁶ However, the calculated Ω values for **2** and **3** appear to be more accurate with percent differences of <1%, whereas **4** is >1% (Table 1). This suggests that the calculated Ω values could be utilized to confidently assign a structure to measured Ω values even if standards of the cyclic prodiginines had not been available.

Interestingly, a shoulder appears in the drift chromatograms before both the major peak in the *S. coelicolor* prodiginine solution and the standard of **2** (Figures 3C and S8), which has a measured Ω value of $196.94 \pm 0.47 \text{ \AA}^2$. We initially hypothesized that the peak was due to the minor *syn*-atropisomer of **2** that has been reported in previous isolation and synthetic studies (Figure 4). The synthetic standard of **2** that we analyzed was found to contain both atropisomers using NMR methods (Figures S9 and S10).^{10,11} Theoretically, the calculated Ω values could be used to test whether the shoulder peak was due to the atropisomers, as standards of the minor atropisomers alone are unavailable. However, the percent difference (>3%) in the calculated Ω value refuted this as this is larger than what would be acceptable for a clear confirmation that the shoulder was *syn*-**2**. However, the value was lower than the *anti*-atropisomer as expected (Table 1). To further explore the origin of the shoulder peak from **2**, we calculated the theoretical Ω values of the cyclic prodiginines with the B pyrrole protonated. This revealed that *anti*-**2** with a B-protonated pyrrole had a calculated Ω value that matched the measured shoulder peak Ω value with less than a 2% difference (Table 1). This unique situation highlights the power of utilizing theoretical Ω values to understand gas-phase conformations of natural products, which due to the complex structural nature of these compounds, and multiple possible protonation sites can be especially difficult to interpret.

The shoulder peak being the result of a protonation site rather than atropisomerism raised the question as to why the other cyclic prodiginines do not also exhibit a shoulder peak. The calculated Ω Values suggest that for both **3** and **4** the Ω differences between their A and B

pyrrole protonation confirmations are only 3.45 and 6.52 Å², respectively (Table 1). This is much lower than the 11.26 Å² seen in the **2** and *S. coelicolor* prodiginine samples. Therefore, it is possible that the resulting peaks may exist, but lie under the main peak in the **3** and **4** samples. To confirm this, it would require an ion mobility instrument with higher resolving power. This discrepancy is especially interesting in the case of **2** and **3** as the only difference between these compounds is the attachment point of the 10-membered ring. The optimized structure of B pyrrole protonated **2** appears to adopt a more condensed structure as the C pyrrole and B pyrrole both have their nitrogen atoms facing the four-membered alkyl chain, possibly interacting through van der Waals forces (Figures S11 and S12). This is in contrast to the A pyrrole protonated structures of **2** and **3**, in which the B and C pyrrole nitrogen atoms face opposite directions from one another, as would be expected to reduce repulsion (Figures S11–S13). However, this is not the case for **4** as the B and C pyrrole nitrogen atoms face the same direction in structures from both protonation sites (Figure S14). The aromaticity of the pyrroles may also have a role in dictating the preferred gas-phase confirmations when protonated at different sites, resulting in a smaller calculated Ω when the B pyrrole is protonated. Clearly, using theoretical Ω values and optimized structures can enable a better understanding of the possibly large effects of small structural differences between natural products in their gas-phase conformations, which will be increasingly important as ion mobility mass spectrometry is integrated into natural product discovery and dereplication workflows.

Comparing theoretical and measured Ω values of natural products provides insight into the complex and rich structures of these compounds. We highlighted this by showing that calculated Ω values could be used to identify a suspected natural product by assessing similarity to the measured Ω value. Furthermore, conformation differences can be elucidated using theoretically optimized structures and calculated Ω values, which could likely be used as a further differentiator from other compounds. It is clear that ion mobility mass spectrometry, in combination with theoretical modeling, has the potential to be an impressive natural product identification technique. Improvements in instrumentation (i.e., greater resolving power) and decreasing computational times will result in the ability to investigate the many possible conformations that a natural product may take. The continued development and use of these techniques in the natural product community is certain to expedite dereplication and discovery campaigns.

EXPERIMENTAL SECTION

Bacteria Strain, Culturing, and Media Composition.

Wild-type *Streptomyces coelicolor* M145 (ATCC BAA-471) was used in this work. *S. coelicolor* M145 spore stock (100 μ L) was added to 200 mL of R5 media in a 500 mL baffled flask. The culture was grown for 7 days at 27°C with shaking at 200 rpm. The R5 media consisted of 103 g of sucrose, 0.25 g of K₂SO₄, 10.12 g of MgCl₂·6H₂O, 10 g of glucose, 0.1 g of Difco casamino acids, 2 mL of trace element solution, 5 g of Difco yeast extract, 5.73 g of TES buffer, 1 mL of KH₂PO₄ (0.5%), 0.4 mL of CaCl₂·2H₂O (5M), 1.5 mL of L-proline (20%), and 0.7 mL of NaOH (1M) in 1000 mL total volume. Trace element solution consisted of 40 mg of ZnCl₂, 200 mg of FeCl₂·6H₂O, 10 mg of CuCl₂·2H₂O, 10 mg of

MnCl₂·4H₂O, 10 mg of Na₂B₄O₇·10H₂O, and 10 mg of (NH₄)₆Mo₇O₂₄·4H₂O in 1000 mL. Media were autoclaved for 20 min at 115°C. The culture medium was collected and centrifuged at 4000 rpm for 10 min to remove cells. Translucent dark blue *S. coelicolor* medium supernatant was stored at –20°C until analysis.

Ion Mobility Measurements.

All drift time measurements were performed on a Waters Synapt G2. Drift times were collected at wave velocities of 500, 600, and 700 m s^{–1} and 40 V wave height. Drift cell nitrogen pressure was kept at 3.3 mbar with a flow of 100 mL min^{–1}. Drift times were converted to Ω using a standard poly-DL-alanine calibration technique (Figure S15).^{22,23} Synthetic standards of **2**, **3**, and **4** were dissolved in 50:50 MeCN/H₂O at approximately 10 μ g mL^{–1}. Freezing of the supernatant material for storage caused pink metabolites to crash out of the solution. This pink material was collected by centrifugation of 1 mL of the supernatant/precipitant mixture (15 000 rpm, 5 min, room temperature) and the pellet dissolved in 1 mL of 50:50 MeCN/H₂O, 0.1% formic acid, resulting in a solution of *S. coelicolor* prodiginines. Approximately 10 μ g of **2**, **3**, or **4** was added to the dissolved pink metabolite solutions obtained from the *S. coelicolor* supernatant for the ion mobility coelution studies. Chromatographic separations were performed on a Waters Acquity UPLC system with a Zorbax C18 1.8 μ m particle column (2.1 $\times$ 50 mm). A 10 min separation at a flow rate of 0.4 mL min^{–1} consisted of a gradient analysis of 0–2 min of 3% B, 2–8 min increasing from 3 to 100% B and 8–10 min of 100% B. A = 100% H₂O, 0.1% formic acid, B = 100% MeCN, 0.1% formic acid. Injection volumes of 5 μ L were used. Driftscope v2.7 was used for data processing.

Theoretical Collisional Cross Sections.

Structures of *anti*-**2**, *syn*-**2**, **3**, and **4** were generated using Chem3D 16.0 with the top pyrrole of each prodiginine protonated. Confirmations were generated using the MMF94 algorithm allowing 1000 steps of minimization and generation of 50 confirmations. The three lowest energy confirmations were chosen for further optimization. Structures were optimized using Gaussian 09 with the B3LYP/3–21 basis set and were then further optimized, and energies were calculated with the B3LYP/6–31G basis set.²⁴ Theoretical Ω values were then obtained from MOBCAL using the nitrogen drift gas-optimized code and increasing the imp parameter to 1250 to account for fewer configurations compared to multiple molecular dynamics simulations.^{25–27} This procedure was repeated for each prodiginine with the A and B pyrroles protonated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Prodiginine structures. Prodigiosin is the prototypical prodiginine. Undecylprodigiosin (**1**) and streptorubin B (**2**) are produced by *S. coelicolor*. Both metacycloprodigiosin (**4**) and butylcycloheptylprodigiosin (**3**) are possible constitutional isomers of **2**. Pyrrole rings are labeled A, B, and C for reference.

Figure 2. Overlay of drift time chromatograms of synthetic standards **2**, **3**, and **4**. Each peak was obtained at 600 m s^{-1} wave velocity and normalized to the highest abundance value.

Figure 3.

Ion mobility coelution drift time chromatograms overlaid with isolated drift time chromatograms of (A) **4** and *S. coelicolor* prodiginine, (B) **3** and *S. coelicolor* prodiginine, and (C) **2** and *S. coelicolor* prodiginine. Each peak was obtained at 700 m s^{-1} and normalized to the highest abundance value.

Figure 4.

Atropisomers of **2**. The 7' position can exist as either the *R* or *S* configuration. The depicted structures are in the *S* configuration.

Table 1.

Calculated Collisional Cross Section (CCS) and Relative Energies for Cyclic Prodiginines

name/pyrrole protonated	relative energy (kcal/mol) ^a	CCS (Å ²) ^a	percent difference from measured
<i>anti</i> -2, A	0	206.51 ± 1.14	−0.81%
<i>anti</i> -2, B	7.32	194.39 ± 0.60	−6.63%, −1.29% ^b
<i>syn</i> -2, A	1.26	203.27 ± 0.99	−2.37%, 3.21% ^b
<i>syn</i> -2, B	14.79	191.96 ± 0.22	−7.80%, −2.53% ^b
3, A	0	208.91 ± 1.18	−0.39%
3, B	4.82	205.46 ± 1.61	−2.04%
4, A	0	201.67 ± 1.39	−1.85%
4, B	8.13	195.09 ± 1.59	−5.03%

^aRelative energy and CCS are average values.^bPercent difference from shoulder peak in **2**.