An NMR fingerprint matching approach for the identification and structural re-evaluation of *Pseudomonas* lipopeptides.

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Classification

Biological Sciences: Microbiology

Keywords

NMR spectroscopy, Cyclic lipodepsipeptides, Pseudomonas, Stereochemistry, Dereplication

Author Contributions

M.H., R.D.M., A.M., and J.C.M. designed research; V.D.R., Y.V., L.G. and N.G. performed research; B.K. and N.G.. analyzed data; and V.D.R., Y.V., J.C.M. and N.G. wrote the paper.

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Abstract

Cyclic lipopeptides (CLiPs) are secondary metabolites secreted by a range of bacterial phyla. CLiPs display diverse structural variations in terms of the number of the amino acid residues, macrocycle size, amino acid identity and stereochemistry (e.g. D- vs. L-amino acids). Reports detailing the discovery of novel or already characterized CLiPs from new sources appear regularly in literature. However, in some cases, the lack of characterization detail threatens to cause considerable confusion, especially if configurational heterogeneity is present for one or more amino acids. The NMR fingerprint matching approach introduced in this work exploits the fact that the ^{1}H and ^{13}C NMR chemical shift fingerprint is sufficiently sensitive to differentiate the diastereomers of a particular CLiP even when they only differ in a single D/L configuration. This provides a means for a fast screening to determine whether an extracted CLiP has been reported before, by simply comparing the fingerprint of a novel CLiP with that of a reference CLiP. Even when the stereochemistry of a particular reference CLiP is unknown, the NMR fingerprint approach still allows to verify whether a CLiP from a novel source is identical to the reference. To facilitate this, we have made a publicly available 'knowledge base' at https://www.rhizoclip.be, where we present an overview of published NMR fingerprint data of characterized CLiPs, together with literature data on the originally determined structures.

Significance Statement

We present a full structural elucidation of MDN-0066, orfamide B and xantholysin A using an NMR fingerprint matching approach. In the process, we found that the structure of orfamide A should be revised, thereby illustrating the merits of our structure elucidation methodology and the need for such strategies by the community. Given the ubiquitous presence of CLiPs, reports on discovery of supposedly 'new' CLiPs keep on appearing. However, at the same time, a number of reports have recently been published, casting doubt on previous characterizations and discussing structural corrections and reassignment. The approach outlined in the current work, provides a straightforward and systematic route towards dereplication ("has the structure been found before?") as well as, even more importantly, structural assessment of CLiPs ("is the structure similar to that of a reference compound?").

Main Text

Introduction

Pseudomonads represent a diverse and ubiquitous bacterial genus that evolves in a wide range of ecological niches (1). To support their lifestyle and interactions with other organisms, they produce a variety of complex secondary metabolites including cyclic lipodepsipeptides or 'CLiPs' produced by non-ribosomal peptide synthetases (NRPSs) (2). Biological effects associated with these biosurfactant peptides include antimicrobial activity, control of bacterial motility and biofilm formation and phytotoxicity, among others (3, 4). They are also involved in the complex interplay between bacteria and plants at the level of the plant rhizosphere. This generates plant-growth promoting activity and protection against pathogens by direct antagonism or induced resistance (5, 6). Other reports have detailed anti-insecticidal activity (7, 8). As a result, this diverse array of functions of *Pseudomonas* CLiPs has sparked interest in their potential development in plant biocontrol applications and as biopesticides (9, 10). In turn, this also stimulates

screening efforts for their development as novel antimicrobials (3, 4). Indeed, the current dearth of novel compounds to fight multi-drug resistant bacteria or fungal infections combined with an increased focus on the development of narrow-spectrum or even species-specific antibiotics has renewed efforts towards mining bacteria for novel pharmaceutical leads (11). These have included CLiPs, (12, 13) not in the least motivated by the successful introduction of daptomycin and polymyxin B. In addition, several reports have highlighted anti-carcinogenic activities of *Pseudomonas* CLiPs, further illustrating their biomedical application potential (14-16). Therefore, both from a fundamental and application perspective, there is a pressing need to uncover the mode of action resulting in these biological functions, starting with the way these are related to the underlying chemical structures (4, 17, 18). Given that structure elucidation of CLiPs remains far from trivial, the development of reliable and straightforward approaches remains key. Moreover, such approaches should allow comparing different CLiPs to unequivocally establish their structural identity.

With well over 100 distinct chemical structures reported, *Pseudomonas* CLiPs invariably consist of an oligopeptide sequence of 8 to 25 residues which is partly cyclized into a depsipeptide through ester bond formation of the C-terminus with a preceding side-chain hydroxyl group (3, 4). The N-terminus of the oligopeptide sequence is capped by an acyl chain of varying length and constitution. The incorporation of non-proteinogenic amino acids, with a majority of residues displaying D-configuration through the action of epimerization domains in the non-ribosomal assembly line, generates additional structural diversity and further magnifies the structure elucidation challenge (19). The availability of rapid and affordable genome sequencing has stimulated the development of bioinformatic tools that allow to predict the CLiP structure produced by a particular NRPS by mapping its biosynthetic gene cluster (BGC) (20). The primary sequence is derived from analysis of the adenylation A-domains of the NRPS. Although separate epimerization domains are present in the siderophore synthetases of *Pseudomonas*, such activity is not found in their CLiP biosynthetic enzymes. Instead, condensation domains with intrinsic epimerization capacity (C/E) mimic the activity of a $^{D}C_{L}$ domain by converting the configuration of the C-terminal residue in the intermediate from L to D before extending the peptide (21). Putative attribution of D/L configuration can therefore be inferred from analysis of the condensation domains involved. For *Pseudomonas* CLiPs in particular, the dual condensation/epimerization C/E domains responsible for configurational inversion of the preceding L-amino acid in the growing peptide chain can be identified using such tools, however it turns out that the epimerization functionality can be inactive for reasons that remain unclear (22, 23). Consequently, as further demonstrated in this work, these predictions do not yet achieve the level of confidence necessary to eliminate the need for the more elaborate and labor-intensive chemical analysis methods. Furthermore, this genome-based approach does not provide information about the residues involved in the depsi bond, nor can the nature of the acyl chain be predicted. Thus, while analysis of the biosynthetic gene cluster coding for the NRPS represents a complementary asset during the chemical structure elucidation process, it cannot replace it.

Combined application of mass spectrometric (MS) and nuclear magnetic resonance spectroscopic (NMR) methods allows, in principle, to establish the composition of the oligopeptide sequence and the nature of the acyl chain as well as the cyclisation site but does not provide access to stereochemistry. For this, chemical derivatization methods combined with chromatographic separation, such as Marfey's analysis, are typically employed to derive the number and configuration of individual amino acids (24-26). However, the total hydrolysis of the lipopeptide into its constituent amino acids required in the process also causes the loss of positional information. This introduces positional ambiguity when a particular amino acid with multiple occurrences in the sequence is configurationally heterogenous i.e., both the D- and L-configuration occur (25, 27). As a result, the elucidation of stereochemistry remains incomplete, such as is the case for the *Pseudomonas* CLiPs MDN-0066 (16), PPZPM-1a (28) and lokisin (29), among others. While workarounds exist, they are tedious and not error-proof (23, 25). In other cases, configurational analysis is omitted and configurational attribution is claimed by relying on either the conservation of the known pattern of D/L-configurations along a sequence (30, 31), or solely on genomic prediction (32, 33). However, the latter ignores the problem of inactive epimerization domains (*vide supra*) while the former disregards the natural occurrence of diastereoisomeric lipopeptides having identical constitution but differing in configuration at one (or more) position(s). This is illustrated by, for example, the *Pseudomonas* CLiP pairs viscosin/WLIP or viscosinamide/pseudodesmin A, syringostatin/syringomycin and fengycin/plipastatin.

Thus, new approaches remain in high demand to move the structure elucidation beyond these configurational dead-ends.

Simultaneously, the increased screening of *Pseudomonas* strains for CLiP production also calls for the introduction of dereplication approaches that confidently distinguish a known versus a novel CLiP structure early on, this to avoid the costly re-elucidation of previously described compounds. In line with this, multiple reports show a growing tendency to characterize known CLiPs from novel sources solely through comparison of their high-resolution mass to those of previously characterized ones (30, 31, 34-39). However, these approaches overlook the possibility of isobaric lipopeptides which occur more commonly than is perhaps anticipated. For instance, several members of the Viscosin group of CLiPs, such as viscosin and massetolide F have a distinct molecular topology yet the same molecular formula, a situation that also occurs for milkisin and tensin, both members of the Amphisin group. Even lipopeptides from distinct CLiP groups can be isobaric, such as strikingly demonstrated by gacamide/cocoyamide and putisolvin II (40-42). Indeed, these share the same molecular formula of $C_{66}H_{115}N_{13}O_{19}$ yet differ in the total number of amino acids (12 vs. 11), the number of amino acids involved in the respective macrocycles (5 vs. 4) and the constitution of the acyl chain (3R-OH C10:0 vs. C6:0).

Previously, we noted that the combined ^{1}H and ^{13}C NMR chemical shift fingerprint obtained from ^{1}H-{^{13}C} HSQC spectra is sufficiently sensitive to differentiate diastereomers of a particular CLiP even when they differ in only a single D/L configuration (43). More so, it is even sensitive to the stereochemistry of the 3-hydroxy moiety of the fatty acid tail as well (44). By integrating NMR spectral fingerprinting and total synthesis into the existing bioinformatic and chemical analyses workflows we here show, for three representative cases, that configurational analysis dead-ends can be removed. This involves matching the ^{1}H-{^{13}C} HSQC spectral fingerprint of newly isolated *Pseudomonas* CLiPs with that of CLiPs with known configuration obtained from total synthesis as convincingly illustrated on the CLiP produced by *Pseudomonas azadiae* SWRI103^{T}. Next, the general applicability of our approach is shown by settling the stereochemistry of orfamide B from *Pseudomonas aestus* CMR5c and xantholysin A from *Pseudomonas mosselii* BW11M1, representative members from two other distinct *Pseudomonas* CLiP groups. In the process, we revise the stereochemistry of several orfamide homologues, including orfamide A from *P. protegens* Pf-5, the original prototype CLiP defining the Orfamide group (45). In the results section, we will first apply the characterization techniques that are typically used when a CLiP is extracted from a newly isolated *Pseudomonas* species, leading to a stereochemical dead-end for each of our CLiPs. By chemically synthesizing the possible sequences, and matching their NMR spectra with that of the natural compound, we are able to unambiguously determine the stereochemistry of each of the CLiPs. More importantly, using the NMR spectra of the natural compound as a reference (its stereochemistry is now determined), we are able to solve the stereochemical dead-end for several other reported CLiPs from other bacterial sources. Thus, the results presented here establish how simple matching of the NMR spectral fingerprint of newly isolated CLiPs with reference data available from a spectral database holds great promise for dereplication purposes. For example, the stereochemical dead-ends of MDN-0066 (16) and the xantholysin-homologue produced by *Pseudomonas* sp. 250J (46) were resolved by only making use of published NMR chemical shifts, enabling a NMR matching with our fully-characterized novel compounds, without the need for additional syntheses. Finally, even when the stereochemistry of an existing sequence is unknown, similarity in NMR fingerprint can rapidly and clearly indicate whether a newly isolated compound is identical to a previously extracted one.

Results

1. A new isolated Bananamide group member from *P. azadiae* SWRI103

1.1. Isolation, bioinformatic and initial chemical structure characterization

P. azadiae SWRI103 was originally collected from the rhizosphere of wheat in Iran, as part of a campaign to assess the plant growth stimulating potential of fluorescent pseudomonads (47). Initial PCR screening targeting initiation and termination domains indicated the presence of a lipopeptide-specific NRPS system (48). More recently, whole genome sequencing revealed a biosynthetic gene cluster (BGC) (49) with similarities to that of several Bananamide (8:6) producers, such as *Pseudomonas bananamidigenes* BW11P2 (50), *Pseudomonas botevensis* COW3 (51) and *Pseudomonas prosekii* LMG26867^{T} (52). Here,

the (8:6) refers to the (*ℓ:m*) notation we introduce to provide a simple but effective classification of CLiPs belonging to the same group from a chemical structural perspective, as explained in Materials and Methods. As the stereochemistry of all these bananamides was unknown, we used strain SWRI103 to develop our expanded workflow for the unambiguous structure elucidation of CLiPs.

Firstly, analysis of the retrieved BGC predicted a Leu – Asp – Thr – Leu – Leu – Ser – Leu – Ile octapeptide sequence from the associated NRPS. The total peptide sequence length and amphipathic profile matches that of previously reported and characterized bananamides (50, 51) but differs in amino acid composition. Therefore, it may represent a novel member of the Bananamide group (8:6). Next, bioinformatic analysis of the condensation/epimerization (C/E) domains was applied for the configurational analysis of the amino acids. The initial C_{start} domain is responsible for the incorporation of a fatty acid (FA) moiety at the N-terminus of the peptide, the exact nature of which cannot be predicted. This domain is followed by six dual activity C/E domains, responsible for the condensation of the newly recruited L-amino acid to the growing peptide chain along with by L to D epimerization of the preceding residue in the sequence (53, 54). This is followed by one $^{L}C_{L}$-type domain which lacks the epimerization functionality thus retaining the L-configuration of the preceding residue while incorporating an L-amino acid as final residue before cyclisation by a tandem transesterification (TE) domain. Assuming that all dual C/E domains have functional epimerization activity, the combined A- and C-domain analysis predicts a FA – D-Leu – D-Asp – D-aThr – D-Leu – D-Leu – D-Ser – L-Leu – L-Ile sequence as the most likely lipopeptide biosynthesized by *P. azadiae* SWRI103 (Table 1).

Table 1: Sequence, configurational analysis, and assignment of a novel bananamide from *P. azadiae* SWRI103. Underlined amino acids are part of the macrocycle.

Bananamide from SWRI103	AA1	AA2	AA3	AA4	AA5	AA6	AA7	AA8
Bioinformatic analysis workflow								
A domain	Leu	Asp	Thr	Leu	Leu	Ser	Leu	Ile
C domain	C_{start}	C/E	C/E	C/E	C/E	C/E	C/E	$^{L}C_{L}$
Prediction	D	D	D	D	D	D	L	L
Chemical analysis workflow								
NMR analysis	Leu	Glu	<u>Thr</u>	<u>Leu</u>	<u>Leu</u>	<u>Ser</u>	<u>Leu</u>	<u>Ile</u>
Marfey's analysis	L/D	D	D	L/D	L/D	D	L/D	L
Combined analysis and synthesized (8:6) Lx library								
	Leu	Glu	<u>Thr</u>	<u>Leu</u>	<u>Leu</u>	<u>Ser</u>	<u>Leu</u>	<u>Ile</u>
(8:6) L1 (1)*	**L**	D	D-a	D	D	D	L	L
(8:6) L4 (2)*	D	D	D-a	**L**	D	D	L	L
(8:6) L5 (3)*	D	D	D-a	D	**L**	D	L	L

* The nomenclature of the synthetic compounds is based on the (*ℓ:m*) notation of the Bananamide group (8:7), followed by the position of the elusive L-Leu in the sequence (position 1, 4 or 5).

Secondly, and independently from the bioinformatic analysis workflow, we engaged into the chemical analysis workflow of the putative novel bananamide. Following incubation of *P. azadiae* SWRI-103 in M9 minimal salt medium, a single CLiP-containing fraction could be extracted and purified. From high resolution MS analysis, a molecular mass of 1053.67 Da could be established i.e., within the expected range for an (8:6) CLiP. Next, NMR was used to elucidate the planar structure. That is, combined COSY/TOCSY analysis showed the presence of a single glutamic acid, serine, threonine, and isoleucine and four leucine residues in the peptide sequence while NOESY and ^{1}H-{^{13}C} HMBC experiments independently allowed to place these in the same order as predicted from the A-domain analysis, validating the latter (Table 1). Note however that although in position 2 an aspartic acid residue was predicted from the bioinformatic analysis, the presence of a glutamic acid was observed here using NMR spectroscopy. This is not surprising, as it was previously shown that Asp/Glu selectivity from genomic predictions is not always clear cut (50). Additionally, the ^{1}H-{^{13}C} HMBC spectrum showed a clear $^{3}J_{CH}$ correlation between Ile8 C' and Thr3 H^{β}, thereby unambiguously establishing that the ester bond occurs between the hydroxyl

side chain of threonine and the isoleucine carboxyl end, thereby revealing the six-residue macrocycle expected for a member of the Bananamide group. Taking into account that $C_{52}H_{92}N_8O_{14}$ was derived as molecular formula from the HR-MS data, a 3-hydroxydecanoic moiety was inferred and confirmed from the NMR data while its linkage to the N-terminus resulted from characteristic $^3J_{CH}$ contacts with Leu1 in the ^{1}H-{^{13}C} HMBC spectrum. As neither MS nor NMR analysis allows establishing the configuration of the individual amino acid residues, we proceeded to Marfey's method for the analysis of amino acid configuration. Following total hydrolysis of the CLiP, Marfey's analysis allowed to unambiguously determine the configuration of all uniquely occurring residues. For the four leucines however, a 2:2 D:L ratio was found, indicating configurational heterogeneity and, as a result, the exact distribution of D and L-Leu residues within the oligopeptide sequence remained hidden. Therefore, at the end of the chemical analysis workflow, a total of six distinct sequences which all satisfy the 2:2 ratio but differ in the distribution of D- and L-leucines should therefore be considered. As a result, the definitive elucidation of the stereochemistry and therefore the chemical structure thus reached a dead-end.

When information regarding a CLiP sequence is available from both bioinformatic and chemical analysis workflows, the configurational ambiguity is sometimes resolved by proposing that one of the sequences issued from chemical analysis matches the bioinformatic prediction. However, such proposals should always be treated with caution since the epimerization functionality of C/E domains can be inactive in a currently unpredictable manner. Moreover, as aptly illustrated in all cases reported here, the predicted configuration from C-domain analysis may not even match those originating from the chemical analysis workflow. Here, for instance, the bioinformatic analysis proposes a 3:1 D:L ratio for the four leucines rather than the 2:2 found experimentally. Nevertheless, the bioinformatic analysis can still provide guidance to narrow down the number of possible diastereomeric sequences by noting that the ^LC_L domains, which lack the epimerization functionality, will maintain the L-configuration of the amino acid introduced at the preceding position (*vide supra*). Since such ^LC_L domain is present in the final NRPS module of *P. azadiae* SWRI103, the penultimate Leu7 residue will invariably retain its L-configuration upon recruitment. This narrows the number of possible sequences down to three: (8:6)-L1 (**1**), (8:6)-L4 (**2**) and (8:6)-L5 (**3**) (Table 1) depending on which leucine position features the remaining L-configuration, thereby showing the benefit of combined bioinformatic and chemical analysis. In all cases, the 3-hydroxy fatty acid tail is assumed to be (R)-configured, as is generally observed for *Pseudomonas* CLiPs.

1.2. Extending the chemical analysis workflow by a synthesis add-on

As recently reviewed by Götze and Stallforth, several approaches exist that allow to tackle the issue of configurational ambiguity (23). In the absence of suitable crystals for X-ray diffraction-based structure elucidation, the most common one resorts to mild acid catalyzed hydrolysis conditions or enzymatic degradation of linearized lipopeptides to yield oligopeptide fragments. These then need to be isolated, characterized by NMR or MS, and subjected to Marfey's method to identify the correct position of the corresponding D- or L-amino acids, introducing an extensive workload without guarantee that the matter will be settled. An alternative solution using MS makes clever use of deuterated amino acids and the fact that D-amino acids are, with very few exceptions, generated through epimerization from L-amino acids during biosynthesis (22, 55). Moreover, in all cases, a separate analysis should be performed to elucidate the stereochemistry of the 3-hydroxy fatty acid moiety (56). Here, we rather build on our previously developed total chemical synthesis route for Viscosin (9:7) group CLiPs (44, 57) to synthesize the three remaining (8:6)-Lx (x=1, 4, 5) sequences. The total chemical synthesis strategy mostly relies on solid phase peptide synthesis, affording considerable automation and rapid access to multiple homologous sequences through parallel synthesis (44, 57, 58). With one residue less in the macrocycle and a D-Glu2 rather than D-Gln2 as main differences, the synthesis could proceed with minimal change to the original strategy. A more extensive discussion of the applied synthesis route and its key features can be found in the Supplementary Materials section.

Next, the exact stereochemistry of the natural compound is revealed by matching their NMR spectral fingerprint to those of the synthetic compounds. The NMR matching approach relies on previous observations where, using the ^{1}H-{^{13}C} HSQC experiment, we established that the inversion in the configuration of a single Cα atom introduces prominent changes in the ^{1}H and ^{13}C chemical shifts of the corresponding (C–H)α group, even when the three-dimensional structure of the CLiP was retained (43).

The same trend was found for the stereochemistry of the 3-hydroxy fatty acid moiety. Thus, the (C–H)α fingerprint region of the HSQC spectra of the natural compound and its synthetic (8:6)-Lx analogue are expected to be identical while the two other sequences will display clearly distinct HSQC spectra in general and (C–H)α fingerprint regions in particular as they feature two D:L inversions compared to the natural compound (Table 1).

By individually overlaying the ^{1}H-{^{13}C} HSQC spectra of each synthesized (8:6)-Lx variant with that of the natural compound from *P. azadiae* SWRI103, a straightforward visual assessment concerning similarities and differences in the ^{1}H and ^{13}C chemical shifts can be made (Figure 1). Being the most sensitive reporters of backbone stereochemistry, we focus here on the cross-peaks in the (C-H)α fingerprint regions (Figure 1). Accordingly, the (8:6)-L4 (**2**) variant shows an excellent match with the (C–H)α fingerprint of the natural compound since all (C-H)α cross peak pairs belonging to the same residue in the sequence show excellent overlap (Figure 1A). In contrast, a considerable mismatch exists with (8:6)-L1 (**1**) as visualized by 5 clearly non-overlapping cross-peaks (Figure 2B). This mismatch becomes even more pronounced for (8:6)-L5 (**3**), where essentially none of the cross-peaks overlap (Figure 1C). Based on this, we can establish the stereochemistry of the *P. azadiae* SWRI103 bananamide as identical to that of the (8:6)-L4 (**2**) sequence (i.e. 3R-OH C10:0 – D-Leu – D-Glu – D-aThr – L-Leu – D-Leu – D-Ser – L-Leu – L-Ile), revealing that the C/E domain in the fifth module of the NRPS system is non-functional for epimerization.

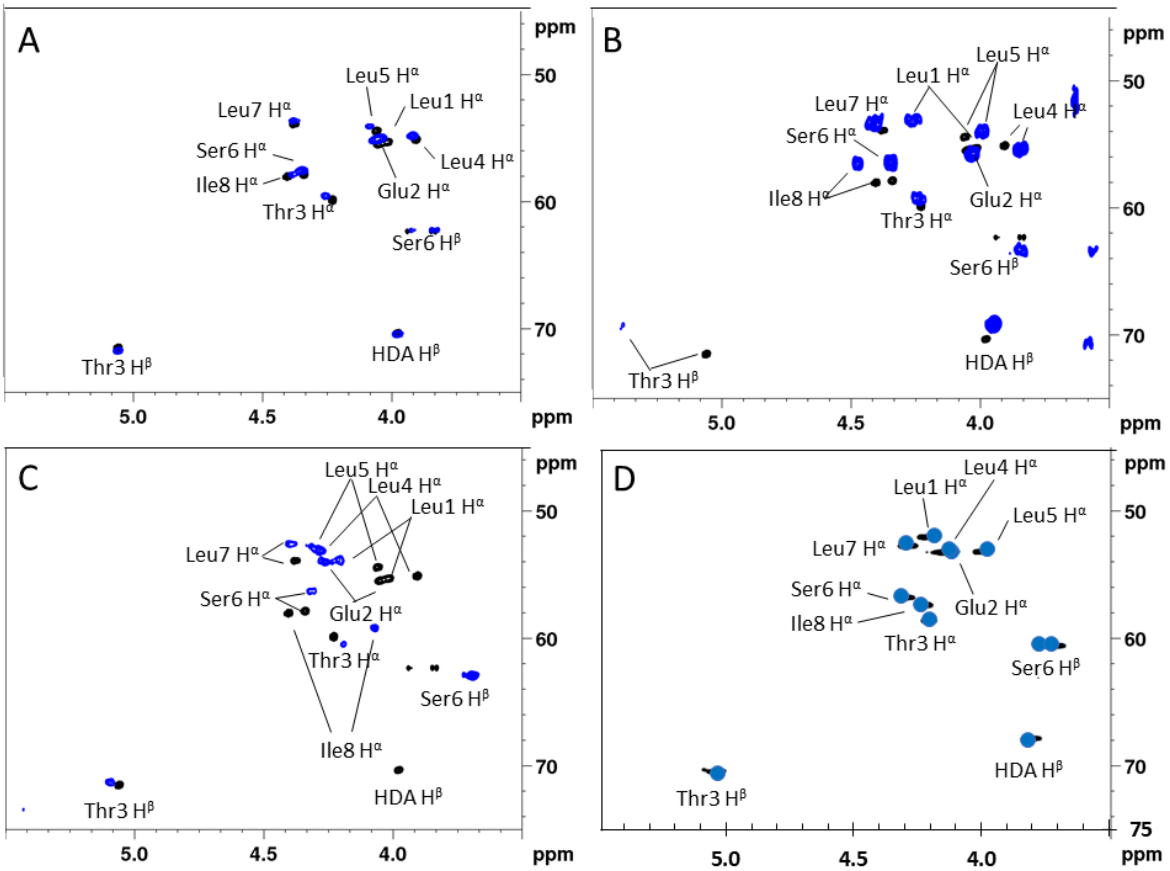

Figure 1: Comparison of the ^{1}H-{^{13}C} HSQC (CH)α fingerprints of the various synthetic (8:6)-Lx variants (blue) with that of the natural bananamide compound produced by *P. azadiae* SWRI103 (black) recorded in acetonitrile-d3 at 700 MHz, 303K. A) matching (8:6)- L4 variant B) (8:6)- L1 variant and C) (8:6)- L5 variant. D) shows the spectrum of the natural compound but now recorded in DMSO-d6 at 298K (black)

overlayed with a representation of the spectrum of MDN-0066, generated from the chemical shift data in the original report (16).

1.3. Matching our reference compound with literature data

The effort to elucidate the stereochemistry of the *P. azadiae* SWRI103 (8:6) bananamide also allows to settle that of MDN-0066, an (8:6) CLiP produced by *Pseudomonas granadensis* F-278,770^{T}, which showed distinct bioactivity in a renal carcinoma cell model (16). Using a chemical analysis methodology similar to the one described above, Cautain *et al* elucidated the peptide sequence by relying on MS analysis and NMR spectral assignment. However, the detailed stereochemical elucidation of MDN-0066 was left incomplete as Marfey's analysis revealed configurational heterogeneity given the presence of 2 D-Leu and 2x L-Leu, a result similar to that of the *P. azadiae* SWRI103 bananamide. Using the tabulated ^{1}H and ^{13}C NMR chemical shifts reported for MDN-0066 in DMSO-d6, the spectral fingerprint matching could be performed against the data of the SWRI103 bananamide recorded under identical conditions. The result, shown in Figure 1D, shows a quasi-perfect match with (8:6)-L4 (**2**). This proves that MDN-0066 is identical to bananamide SWRI103, thereby disambiguating the stereochemistry of MDN-0066.

In order to assess the general applicability of our approach to *Pseudomonas* CLiPs, we then turned to two additional case studies, being the stereochemical elucidation of orfamide B and xantholysin A.

2. Configuration elucidation of (10:8) orfamide B from *P. aestus* CMR5c

Orfamides are important for bacterial motility, cause lysis of oomycete zoospores, and play a role in biocontrol activity against fungal pathogens and insects (5, 45, 59-61). The name-sake of the Orfamide (10:8) group, orfamide A, was extracted from *Pseudomonas protegens* Pf-5 and fully characterized by mass spectrometry, NMR spectroscopy and chiral gas chromatography. (45) Orfamides B and C were extracted as minors, and their planar structures were characterized as well. Since its original discovery, orfamide B is often found as the major compound produced by newly isolated bacterial strains. (33) Additionally, the orfamide group has expanded with additional orfamide-homologues. (28, 33, 62, 63) However, in many cases, the stereochemistry of the orfamide homologues was defined based on sequence similarity with the original orfamide A as extracted from *P. protegens* Pf-5, a situation we wish to rectify now, starting with the stereochemical determination of orfamide B as produced by *Pseudomonas aestus* CMR5c.

2.1. Isolation, bioinformatic and initial chemical structure characterization of orfamide B

P. aestus CMR5c was originally isolated from the rhizosphere of red cocoyam in Cameroon in a screen for biocontrol agents against the cocoyam root rot disease caused by *Pythium myriotylum* (10). We already reported the initial genome mining and bioinformatic analysis of the *P. aestus* CMR5c BGC which revealed the presence of three genes with ~80% similarity to the *ofaA*, *ofaB* and *ofaC* NRPS genes from *P. protegens* Pf-5 (33). MS and NMR analysis further confirmed the predicted primary sequence and evidenced the incorporation of a 3-hydroxy-tetradecanoic acid moiety at the N-terminus. Thus, the major CLiP produced by *P. aestus* CMR5c has a planar structure identical to orfamide B. Based on this similarity in primary sequence and 80% genomic similarity between the respective BGCs, orfamide B from *P. aestus* CMR5c was proposed to also possess only L-leucines (33), as was previously also found for the original orfamide A (45).

C-domain analysis showed that the initial C_{start} type domain involved in acylating the first amino acid is followed by six dual activity C/E domains (modules 2–7), two non-epimerizing $^{L}C_{L}$ type domains (modules 8 and 9) and a final C/E domain in the last module (Table 2). Taking into account the distribution of $^{L}C_{L}$ and C/E domains, the bioinformatic analysis predicts the stereochemistry as shown in Table 2, with D-Leu residues occurring at positions 1 and 5 (Table 2) contradicting the all-L-Leu configuration proposed earlier. Marfey's analysis confirmed the configuration predictions for singly occurring amino acids and the 1:1 D:L ratio for both valines. For the leucines however, a 1:3 D:L ratio was found, invalidating the 2:2 ratio predicted from bioinformatic analysis and their all-L configuration proposed by Ma et al (33).

Table 2: Sequence, configurational analysis, and assignment of orfamide B from *P. aestus* CMR5c

CMR5c	AA[1]	AA[2]	AA[3]	AA[4]	AA[5]	AA[6]	AA[7]	AA[8]	AA[9]	AA[10]
Bioinformatic analysis workflow										
A domain	Leu	Glu	aThr	Val/Ile	Leu	Ser	Leu	Leu	Ser	Val
C domain	Cstart	C/E	C/E	C/E	C/E	C/E	C/E	$^{L}C_{L}$	$^{L}C_{L}$	C/E
Prediction	D	D	D	D	D	D	L	L	D	L
Proposal Ma et al. 2016 (33)	L	D	D	D	L	D	L	L	D	L
Chemical analysis workflow										
NMR analysis	Leu	Glu	Thr	Val	Leu	Ser	Leu	Leu	Ser	Val
Marfey's analysis	L/D	D	D-a	L/D	L/D	D	L/D	L/D	D	L/D
Synthesized Lx(10:8) library										
	Leu	Glu	Thr	Val	Leu	Ser	Leu	Leu	Ser	Val
(10:8)-L1 (4)	L	D	D-a	D	**D**	D	L	L	D	L
(10:8)-L5 (5)	**D**	D	D-a	D	L	D	L	L	D	L
(10:8) L1L5 (6)	L	D	D-a	D	L	D	L	L	D	L

* The nomenclature of the synthetic compounds is based on the (***ℓ:m***) notation of the Bananamide group (8:7), followed by the position of the elusive L-Leu in the sequence (position 4, 5 or 4&5).

Considering the experimental evidence from the chemical analysis workflow, a total of 8 sequences should be considered since the configurational D:L heterogeneity of the Leu and Val residues are combinatorically independent. Conveniently, the bioinformatic analysis allows to trim this down to two sequences only, reducing the synthetic effort required. First, the ambiguity regarding the valines can be settled by noting that, as the final residue, Val10 is not subjected to any epimerization activity. This pins the valine configurations down as D-Val4 end L-Val10. Next, the presence of $^{L}C_{L}$ domains in module 8 and 9 allows to unequivocally attribute the L-configuration to Leu7 and L-Leu8. The remaining D and L configured leucines are to be distributed over positions 1 and 5. To finalize configuration assignment, the (10:8)-L1 (**4)** and (10:8)-L5 (**5**) sequences were synthesized (Table 2) using the same strategy as discussed above for the bananamide analogues. This included the incorporation of a 3R-hydroxydecanoic moiety (3-OH C10:0) at the N-terminus rather than a 3R-hydroxytetradecanoic one (3-OH C14:0) mainly because of synthetic availability of the precursor and the fact that previous investigation of C_{10}, C_{12} and C_{14} pseudodesmin analogues evidenced that lengthening of the acyl chain did not affect the ^{1}H and ^{13}C chemical shifts of the peptide moiety in any way (58).

2.2. Extending the chemical analysis by a synthesis add-on

Figure 2 shows the overlay of the (C–H) α fingerprint region of each synthesized (10:8)-Lx sequence with that of natural orfamide B from *P. aestus* CMR5c. A straightforward visual assessment clearly indicates that the (10:8)-L1 (**4**) variant displays a quasi-perfect fingerprint match while none of the (C–H)α cross peaks of (10:8)-L5 (**5**) overlap with those of the CMR5c orfamide. The latter probably indicates a major conformational effect caused by the L to D inversion at Leu5. To remove any doubts and independently exclude the all-L-Leu configuration originally proposed from sequence similarity with orfamide B from *P. protegens* Pf-5, we also committed to synthesize the corresponding (10:8)-L1L5 (**6**) analogue which again showed significant mismatch with the natural CMR5c orfamide. (See Supplementary data) All data together establish that the stereochemistry of orfamide B from *P. aestus* CMR5c corresponds to that of the (10:8)-L5 (**5**) sequence (3R-OH C14:0 – L-Leu – D-Glu – D-aThr – D-Val – D-Leu – D-Ser – L-Leu – L-Leu – D-Ser – L-Val), indicating the C/E domain in the second module is non-functional for epimerization.

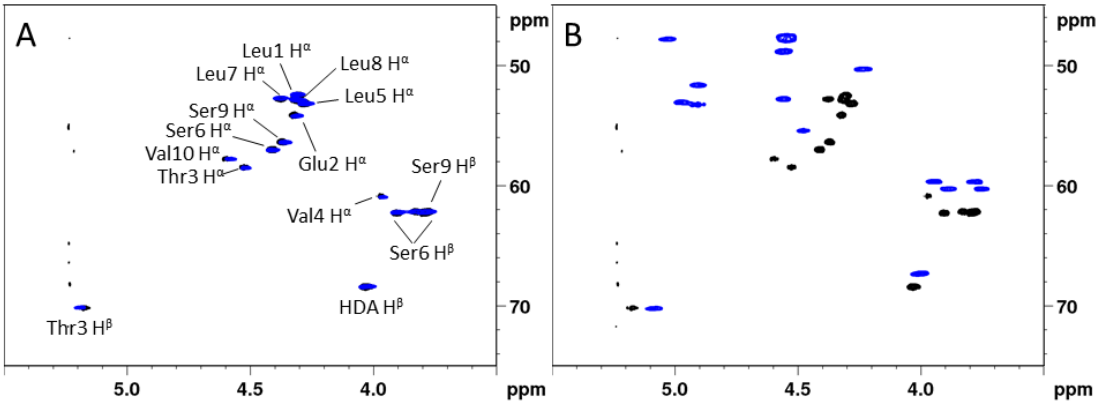

Figure 2: Comparison of the ^{1}H-{^{13}C} HSQC (CH)α fingerprint of synthetic (10:8)-Lx variants (blue) with that of the natural compound extracted from *P. aestus* CMR5c, all recorded in DMF-d7 at 500 MHz and 298K,. A) overlay of the synthetic (10:8)-L1 variant (blue) and B) the synthetic (10:8)-L5 variant (blue).

2.3. Matching our reference compound with literature data

The results presented above appear to indicate that orfamide B from *P. aestus* CMR5c is a D-Leu5 diastereoisomer of orfamide B from *P. protegens* Pf-5 (45), since the latter is reported to possess a L-Leu5. So far, examples of CLiPs from the same (***ℓ:m***) group that feature a configurational difference are few, possibly due to the lack of extensive configurational assignment among *Pseudomonas* CLiPs. The best-known example occurs within the Viscosin (9:7) group where CLiPs can be divided in a L-subgroup (14 sequences) and D-subgroup (5 sequences) depending on the configuration of the leucine, also at position 5 (64). Given that orfamides have been reported from multiple bacterial sources since their initial discovery, the question raised as to whether such division is also present for the Orfamide (10:8) group, which would indicate configurational variation to be a more general feature present in the NRPS of *Pseudomonas* CLiPs. Conveniently, the major orfamide produced by other reported bacterial sources feature planar structures identical to either orfamide A from *P. protegens* Pf-5 or orfamide B from *P. aestus* CMR5c. To be able to screen directly against a reference spectrum of orfamide A under standardized conditions, *P. protegens* Pf-5 was cultured to obtain orfamide A, together with a series of minor compounds including orfamide B and 4 previously unreported ones which we named orfamides J – M (See supplementary information).

As mentioned before, there are numerous other orfamide producers described, and their stereochemical identification was mainly based on homology with the original orfamides. Using our NMR matching approach, we are able to unambiguously determine their structural similarity to our reference compounds, without the need for additional synthesis, as long as NMR data is available. For the orfamide B producer *Pseudomonas sessilinigenes* CMR12a (65), the ^{1}H-{^{13}C} HSQC spectrum revealed spectral fingerprints identical to the reference orfamide B from *P. aestus* CMR5c. This indicated that these molecules have identical stereochemistry and feature a D-Leu5. Genome exploration revealed an orfamide-type BGC in *Pseudomonas* sp. PH1b (66). Characterization of orfamides from this isolate presented an interesting case as it produces a major and a minor orfamide with sequence identical to orfamide A and orfamide B, respectively, thus only differing by a Ile4/Val4 substitution resulting from A-domain substrate flexibility in module 4 of the NRPS. The (C–H) α spectral fingerprint of its major orfamide matched with that of orfamide A produced by *P. protegens* Pf-5 indicating an L-configuration for Leu5. Surprisingly, however, the spectrum of the minor orfamide of *Pseudomonas* sp. PH1b matched with that of orfamide B from *P. aestus* CMR5c, therefore indicating a D-Leu5 configuration. This result was highly unexpected as it implied that in *Pseudomonas* sp. PH1b, the same NRPS assembly line would yield different configurations for Leu5. More specifically, it would require the occurrence of epimerization by the C/E domain of module 6 to correlate with the sequence composition of the growing peptide chain. Since the all-L Leu configuration of the original

orfamide A from *P. protegens* Pf-5 was derived from extensive and high-quality chemical analysis but not confirmed through total synthesis, it appeared more likely that an error was made in establishing the configuration at this position. To settle this matter, we reinvestigated the stereochemistry of the original orfamide A, as extracted from *P. protegens* Pf-5. In contradiction with the report by Gross *et al*, where chiral GC revealed a 0:4 D:L ratio for the leucines, our Marfey's analysis yielded a 1:3 D:L ratio, definitively settling the case in favor of a D-Leu5. As a result, configurational variability within one NRPS assembly line can be invalidated, and a revision is required for the configuration of Pf-5 based orfamides as shown in Table 2. Gratifyingly, this outcome was recently also arrived at using a completely independent approach involving total synthesis of orfamide A by Bando et al. (REF), whereby the original stereochemistry led to loss of green algal deflagellation activity, while the corrected one was as active as the natural compound (67). Finally, we could establish that the orfamide produced by *Pseudomonas* sp. F6 (7) is identical to the revised orfamide A, by only making use of the tabulated chemical shift values of the published compound, and matching this to our reference spectrum of orfamide A,.

3. Settling the elusive stereochemistry of xantholysin A

An additional example illustrating the strength of our combined synthesis and spectral matching approach concerns xantholysin A. First reported as the main CLiP produced by *Pseudomonas mosselii* BW11M1, together with minor congeners B to D, it is the prototype that defines the Xantholysin (14:8) group (68). Since no configurational analysis was performed at the time, NMR and MS only allowed to derive its planar structure. Subsequent reports proposed the production of xantholysins by other *Pseudomonas* strains, uncovering a diverse portfolio of biological functions in the process. Pascual *et al* (15) reported the production of xantholysins A to D by *Pseudomonas soli* F-279,208^{T} solely based on HR-MS of the isolates and characterized their cytotoxic activity against the RCC4 kidney carcinoma cell line. Lim *et al* (8) reported HR-MS and ^{1}H NMR identical to those of xantholysin A and B for two lipopeptides from *Pseudomonas* sp. DJ15 and demonstrated their insecticidal activity against the green peach aphid, a major peach tree pest also serving as plant virus vector. In the context of exploring the tolerance of cocoyam plants against *Pythium myriotylum* in the field, Oni *et al.* (40) showed that *Pseudomonas* sp. COR51 produces a lipopeptide with identical planar structure to that of xantholysin A. A large-scale bacterial screening for antibiotic activity proposed the identification of xantholysins A-D in *Pseudomonas* sp. 250J, based on HR-MS data and high similarity in the NRPS genomic make-up to that of *P. mosselii* BW11M1 (69). Later on, combination of Marfey's analysis with mapping of the C/E and $^{L}C_{L}$ domains in its NRPS led to propose a stereochemistry for xantholysin A, although the distribution of the remaining D-Leu and L-Leu over positions 9 and 11 remained tentative (46). Shimura *et al* (70) reported the total synthesis of MA026, a xantholysin-like CLiP from *Pseudomonas* sp. RtlB026 with potent anti-hepatitis C activity. Discrepancies in physicochemical properties of synthetic and natural MA026 led Uchiyama *et al.* (71) to revise its structure by exchanging residues at position 10 and 11, as previously suggested by Li *et al* (68), thus leading to a planar structure identical to xantholysin A. With more than 90% sequence similarity between their respective BGC, it was proposed that the stereochemistry of xantholysin A would be identical to that of MA026. However, they also noted that the stereochemical assignment for xantholysin A from *Pseudomonas* sp. 250J did not match with MA026, the latter containing L-Gln6 and D-Gln13, while the reverse configuration was proposed for the former.

To resolve this on-going issue, we first considered applying our combined approach to the original xantholysin A from *P. mosselii* BW11M1. However, the presence of configurational heterogeneity for the leucine (D:L 2:1) and glutamine/glutamic acid (Glx) residues (D:L 4:1) independently act to generate 15 possible sequences without further prioritization possibility. Comparison of the (C–H)α fingerprint of MA026 (with known stereochemistry) to that of original xantholysin A should in principle allow to either quickly establish configurational identity or eliminate one sequence, however, NMR data for MA026 was listed without explicit assignment. To circumvent this, we adapted our synthesis scheme to produce MA026 and subsequently established that its (C–H)α fingerprint is indeed identical with that of the original xantholysin A (Figure 3), thus avoiding the need for further synthesis. Used as reference compound, simple comparison of its (C–H)α fingerprint with that of putative xantholysin A lipopeptides allowed to also extend the assigned stereochemistry to the major xantholysin extracted from *Pseudomonas.* sp. COR51 (40) and *Pseudomonas* sp. 250J (46), for which the NMR data was available or provided for comparison by the

original authors, respectively. (See supporting information) In conclusion, the structure of xantholysin A was determined to be 3R-OH C10:0 – L-Leu – D-Glu – D-Gln – D-Val – D-Leu – L-Gln – D-Ser – D-Val – D-Leu – D-Gln – L-Leu – L-Leu – D-Gln – L-Ile. This reveals that two modules (2 in XtlA and 7 in XltB) lack the epimerization capability expected for the respective C/E-classified domains.

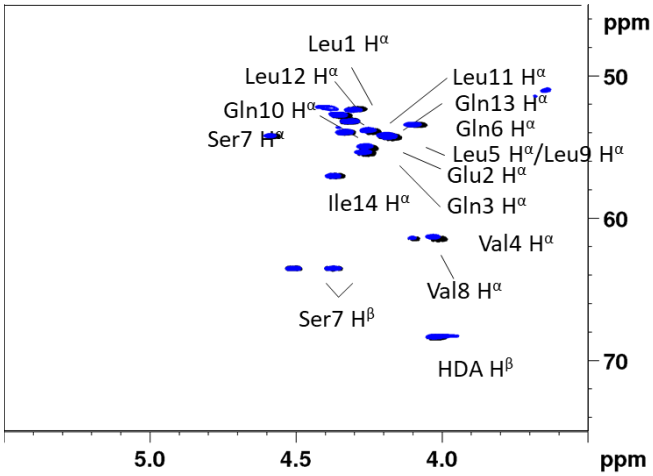

Figure 3: Comparison of the ^{1}H-{^{13}C} HSQC (C-H)α fingerprints of natural xantholysin A (14:8) produced by *P. mosselii* BW11M1 (black) with that of MA026 obtained through synthesis (blue). DMF-d7, 328K, 700MHz.

Discussion

Because of their broad range of antimicrobial activities, interest in structure-activity relations (SAR) of natural compounds such as *Pseudomonas* CLiPs, is increasing (33, 58). In this respect, detailed knowledge of their structure, including stereochemical make-up, is of vital importance to draw meaningful associations. In contrast, we observed an increasing number of reports where lipopeptides from newly isolated bacterial sources are incompletely characterized, using only mass spectrometry or genomic predictions. Consequently, the antimicrobial activities of the newly extracted compounds cannot easily be compared to already existing ones. Additionally, since new *Pseudomonas* CLiPs and CLiP groups continue to be found, it seems likely that many reports will dwell on rediscoveries of existing CLiPs as they continue to be extracted from novel bacterial strains. Indeed, due to the ubiquitous nature of the CLiP-producing bacteria, once a new structure is described, possibly, it will also be found in other environments. Because of this, complete characterization (including the labor-intensive stereochemical validation) – while essential – may not provide a 'return-on-investment'. In this context, the NMR fingerprint approach illustrated in this work will be able to support researchers on two levels. The first concerns dereplication i.e., has this particular lipopeptide been isolated before? By matching NMR spectra of a CLiP from a newly isolated bacterial source with those of an existing (reference) CLiP – irrespective of the stereochemical characterization of the latter – one can determine whether they are identical or not. This is exemplified by the fact that, for each lipopeptide in this study, we were able to compare their (C–H) α NMR fingerprints with that of several identical CLiPs from other bacterial sources, by using only their ^{1}H and ^{13}C chemical shifts found in literature. In this way, we could elucidate their stereochemical make-up as well. Secondly, provided the stereochemistry of the matching reference has been established, the stereochemistry of the new sequence is also established, including that of the fatty acid tail. It is important to note that in both cases, total synthesis is thus no prerequisite for dereplication. In other words, the (C–H) α NMR fingerprint matching only requires the comparison of NMR spectra to assess structural similarity to the reference. In this way, our approach provides a fast screening method, potentially eliminating additional labor-intensive analyses, especially when it concerns CLiPs with a configurational heterogeneity of the amino acid content. Only when a new CLiP structure is found and/or no reference compound is available, additional analysis is desired/required. In such cases, we have shown that genomic and chemical methods, in combination with solid-phase peptide synthesis can provide unambiguous characterization of CLiP structures.

Using (C–H) α NMR fingerprint matching, we established that the CLiP extracted from the newly isolated producer *P. azadiae* SWRI103 belongs to the (8:6) Bananamide group. More specifically, by utilizing our NMR spectral matching methodology, it was found identical to MDN-0066 of the *P. granadensis* type-strain, thereby settling the stereochemistry of this compound as well (16). Using the same approach, we determined that a structure revision is required for several, if not all, Orfamide(10:8) group members. While initially only investigating the stereochemical make-up of orfamide B as extracted from *P. aestus* CMR5c, we observed that this compound possesses a D-Leu5, instead of an L-Leu5 as previously published (33, 45). To exclude the possibility that the CMR5c orfamide was a stereoisomer of the originally reported orfamide B, we synthesized the latter sequence as well. Comparing the ^{1}H-{^{13}C} HSQC spectra of other orfamides, produced by *P. protegens* Pf-5*, P. sessilinigenes* CMR12a, *Pseudomonas* sp. F6 and *Pseudomonas*. sp. PH1b, all revealed the presence of a D-Leu5. Moreover, it was determined that all orfamide members possess a 3R-hydroxyl fatty acid tail, in contrast to earlier literature reports asserting the presence of a 3S-hydroxyl fatty acid tail (45, 56). Finally, we determined the stereochemistry of xantholysin A. Notably, with 15 possible sequences resulting from the combination of information obtained from both genomic as well as chemical analysis protocols, a large synthetic effort would have been required for the elucidation of the lipopeptides' stereochemistry. Instead, we utilized available literature data to prioritize possible sequences, resulting in only a single preferred sequence to be synthesized, thereby solving the stereochemistry of xantholysin A (as produced by various bacterial strains; using their reported literature data) and MA026.

Dereplication should permit the better mining of NRPSs by providing reference data for mining software suites, thereby improving genomic prediction of CLiP structures. As previously observed for *Pseudomonas* lipopeptides from the Viscosin, Poaeamide, Tolaasin, Factin, Peptin, and Mycin families (72, 73) and further evidenced in this report for CLiPs from three additional groups (Bananamide, Orfamide, Xantholysin), the bioinformatic prediction of epimerization functionality in the non-ribosomal biosynthesis

of CLiPs remains inaccurate. In each of these groups, at least one module appears catalytically inactive for epimerization. Only in the Amphisin (11:9) and Gacamide/Cocoyamide (11:5) groups, the predictions of dual activity condensation domains match with the elucidated CLiP stereochemistry. It can be anticipated that ensuring availability of unambiguous stereochemical information and scrutinizing corresponding C/E domain sequences for motifs linked to epimerization (in)activity (21), ideally complemented with insight from currently unavailable 3D structural C/E domain data, should allow an improved genomic prediction of the affected amino acid configurations.

Finally, incomplete (or erroneous) structural characterization currently generates 'scientific noise' in the field of lipopeptide research, hampering a concerted research action for the generation of structure-activity relationships (17). The (C–H)α NMR fingerprint matching approach will prevent this by offering a rapid, unambiguous characterization of CLiP structures. By standardizing the relevant experimental conditions as much as possible and by making reference data publicly available, it will allow for a rapid elucidation of the complete structure of newly extracted lipopeptides, including stereochemical make-up of both the oligopeptide and the fatty acid moieties. While limited to *Pseudomonas* CLiPs, our results support the (C–H) α NMR fingerprint matching as a tool for dereplication beyond this genus, such as those produced by other bacterial phyla, including *Bacillus*, *Burkholderia* and *Streptomyces* spp. While awaiting the development of a (dedicated) NMR data sharing platform – most likely by integration of the reference spectra into existing (lipopeptide)databases, such as Norine (74, 75) – we have made a publicly available 'knowledge base' at https://www.rhizoclip.be, where we present an overview of downloadable (C–H)α NMR fingerprint data of characterized CLiPs, together with literature data on the originally determined structures. The latter includes a description of the CLiPs' original description, molecular mass, three dimensional structures (if available), and a summary of published antimicrobial activities. Moreover, a detailed protocol will be made available for researchers that wish to record NMR data of their newly extracted lipopeptides to compare them to the publicly available reference data. Finally, we invite all researchers to submit NMR data of new CLiPs, such that they can be included in the knowledge base.

Materials and Methods

Detailed materials and experimental methods can be found in the SI appendix together with additional tables and figures.

Data Availability

All study data are included in the article and *SI Appendix*. NMR data for (C – H) spectral fingerprint matching can be found at https://www.rhizoclip.be

Acknowledgments

AM and JM are recipient of a concerted research action grant (MEMCLiP) from Ghent University supporting this research (GOA-028-19). JM and MH are recipients of the Excellence of Science grant RhizoCLiP (EOS ID 30650620) supporting this research. The NMR equipment used in this work was funded through the FFEU-ZWAP initiative and grants from the Hercules Foundation (AUGE09/06 and AUGE15/12) awarded to a consortium headed by J. Martins. The NMR equipment is part of the NMR Centre of Expertise at UGent. We would like to thank Pauline De Coster and Ilse Delaere for practical assistance with bacterial cultures, HPLC purifications and NMR characterization. Thanks are extended to Kok-Gan Chan (University of Malaya, Malaysia) for use of the *Pseudomonas* sp. PH1B strain and Carlos Molina Santiago for the NMR spectral data of xantholysin A as extracted from *Pseudomonas* sp. 250J.

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Figures and Tables

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