

Total Synthesis and Structure Correction of the Cyclic Lipodepsipeptide Orfamide A

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Abstract: A total synthesis of the cyclic lipodepsipeptide natural product orfamide A was achieved. By developing a synthesis format using an amino acid ester building block and SPPS protocol adaptation, a focused library of target compounds was obtained, in high yield and purity. Spectral and LC-HRMS data of all library members with the isolated natural product identified the ⁵Leu residue to be D- and the 3'-OH

group to be R-configured. The structural correction of orfamide A by chemical synthesis and analysis was confirmed by biological activity comparison in *Chlamydomonas reinhardtii*, which indicated compound configuration to be important for bioactivity. Acute toxicity was also found against *Trypanosoma brucei*, the parasite causing African sleeping sickness.

Introduction

Pseudomonads are ubiquitous, Gram-negative gammaproteobacteria that produce a range of biologically active natural products.^[1,2] Among them, cyclic lipodepsipeptides (CLPs) act as biosurfactants, as antimicrobials, or as biocontrol agents.^[3] Typical CLPs biosynthesized by pseudomonads such as viscosin (1), orfamide A (2,3), or anikasin (4) contain 8–25 amino acids of which 4–10 residues form a macrocycle (Figure 1).^[2] Moreover, all feature a lipidated N-terminus. Orfamide A from *Pseudomonas protegens* Pf-5 or CHA0^[4,5] has been characterized as an insecticidal,^[6,7] an antifungal,^[8] and as an algicidal agent.^[9] Intriguingly, orfamide A triggers an increase in cytosolic Ca²⁺ in the green alga *Chlamydomonas reinhardtii*, causing its deflagellation, and thus leads to its immobilization (IC₅₀ = 4.1 μM).^[9,10] Orfamide A immobilizes several flagellate algae from the class

of Chlorophyceae but not select algae from other classes.^[9] A total synthesis of orfamide A has not been reported to date, except for a patent claim.^[11] We report here on the total synthesis and the resulting revision of the chemical structure of orfamide A (2→3), based on an improved, generally reliable synthetic route to CLP natural products.

Results and Discussion

Orfamide A features ten amino acids and a β-hydroxytetradecanoic acid (β-HTDA). The 3'-hydroxy group had been allocated S-configuration (→2),^[4] in contrast to many β-hydroxy acids found in other CLPs from *Pseudomonas*,^[2] which are R-configured. In order to clarify this issue, we synthesized both enantiomers of β-HTDA in high e.e. by using Noyori's hydrogenation chemistry^[12,13,14] (see Supporting Information for details). Then, a method was adapted that had originally been reported for the synthesis of pseudodesmin A.^[12] By applying solid-phase peptide synthesis (SPPS) with side-chain anchored serine, on-resin esterification, and subsequent cyclization, the orfamide stereoisomers 2 and 5 (Table 1) were initially obtained in approx. 2% yield (see Supporting Information, Scheme S3).

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Table 1. Orfamide A isomers prepared in this study. The configuration that was finally confirmed is highlighted.

Compound	A.A. residue 1	A.A. residue 5	3'-OH	Yield ^[a]
2	L-Leu	L-Leu	S	2%, 36% ^[b]
5	L-Leu	L-Leu	R	2%, 38% ^[b]
6	D-Leu	L-Leu	S	18% ^[b]
7	D-Leu	L-Leu	R	6%, 40% ^[b]
8	L-Leu	D-Leu	S	36% ^[b]
3	L-Leu	D-Leu	R	7%, 35% ^[b]
9	D-Leu	D-Leu	S	44% ^[b]
10	D-Leu	D-Leu	R	12%, 44% ^[b]

[a] Yield calculated from resin loading; [b] yield obtained by using optimized procedures (Scheme 2).

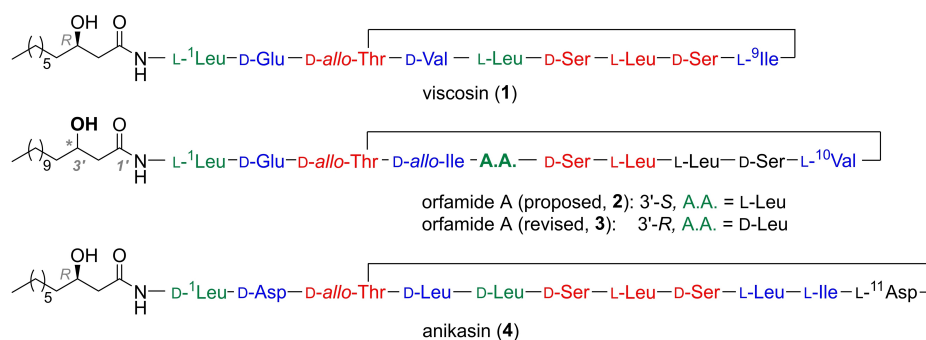


Figure 1. Cyclic lipodepsipeptides (CLPs) viscosin,^[28] orfamide A,^[4] and anikasin^[29] from *Pseudomonas* spp. Amino acid (A.A.) residues above are color-coded to indicate preserved (red), similar (blue), or stereoisomeric residues (green).

Compounds **2** and **5** were compared with an authentic sample by using LC/HRMS analysis (Figure 2) and ¹H NMR spectroscopy. Both synthetic CLPs were surprisingly distinct from the natural product, indicating the originally proposed

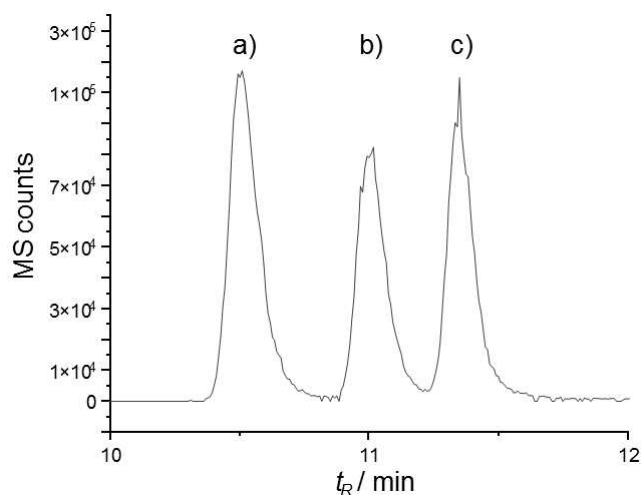


Figure 2. Extracted ion chromatogram (MS range: 1295.8 ± 0.5) of a mixed sample containing synthesized compounds **2** and **5** and isolated orfamide A (NP). a) **2**, b) **5**, c) NP. See Supporting Information for experimental details.

structure **2** to deserve a more detailed reinvestigation beyond side chain hydroxylation stereochemistry.

NMR- and MS data indicated the compound in question to be an isomer of the proposed structure, likely a stereoisomer. The NRPS (non-ribosomal peptide synthetase) biosynthesis genes of orfamide A in *P. protegens* Pf-5 were hence analyzed using antiSMASH version 6.0.^[15] The NRPS genes *orfA*, *orfB*, and *orfC*, which code for the biosynthetic machinery of the orfamides, were further examined using NRPSpredictor2 to determine the amino acid specificity of the adenylation (A) domains. In addition, a phylogenetic algorithm was employed to assign the condensation (C) domain functionalities (Figure 3).^[16] The A-domain analysis was in good agreement with the structure proposed earlier.^[4] All but two A-domain specificities showed a 100% match with the code introduced by Stachelhaus *et al.*^[17] and were in accordance with the originally assigned structure of the orfamides. The only exceptions were the A domains 2 and 10, which were predicted to incorporate Asp (80% match) and a hydrophobic amino acid (not further specified), respectively. Such ambiguous or deviating classifications are regularly encountered in bioinformatics analyses. Importantly, the predicted character of the two amino acids (acidic and hydrophobic) matched the expected structure. However, analysis of the C domains revealed stereochemical inconsistencies for the residues ¹Leu and ⁵Leu. These amino acids had originally been assigned with L-configuration, but

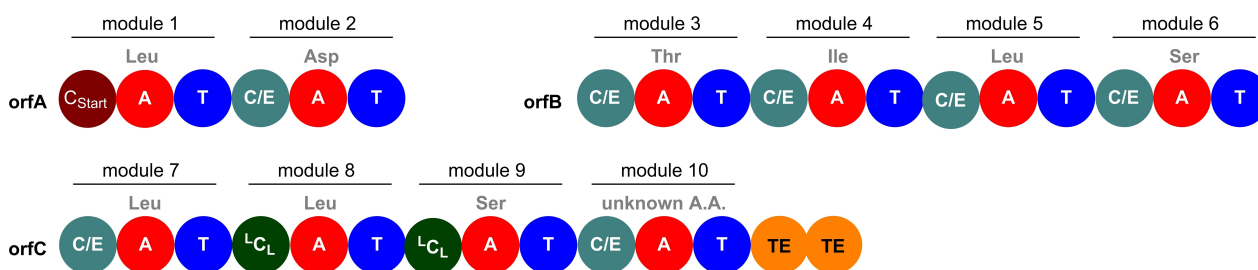
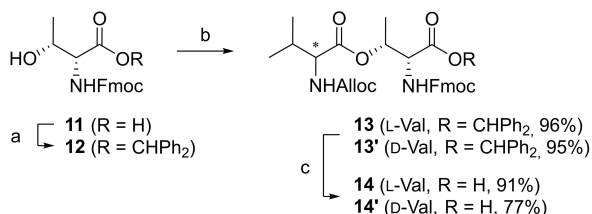


Figure 3. Module structure of the three NRPS genes that code for the biosynthesis of the orfamides in *P. protegens* Pf-5 (Gene Bank accession number CP000076), as predicted by antiSMASH. Expected A-domain specificity (NRPSpredictor2) and C-domain subtype (NaPDoS) are indicated (see Supporting Information for methods). The residue loaded by module 10 is predicted to be hydrophobic. Abbreviations: orf = Orfamide synthase; C/E = Condensation-epimerization domain; C_{Start} = Starter condensation domain; ¹C_L = Condensation domain for linking two L-A.A.s; TE = Thioesterase domain; A = Adenylation domain; T = Thiolation domain.

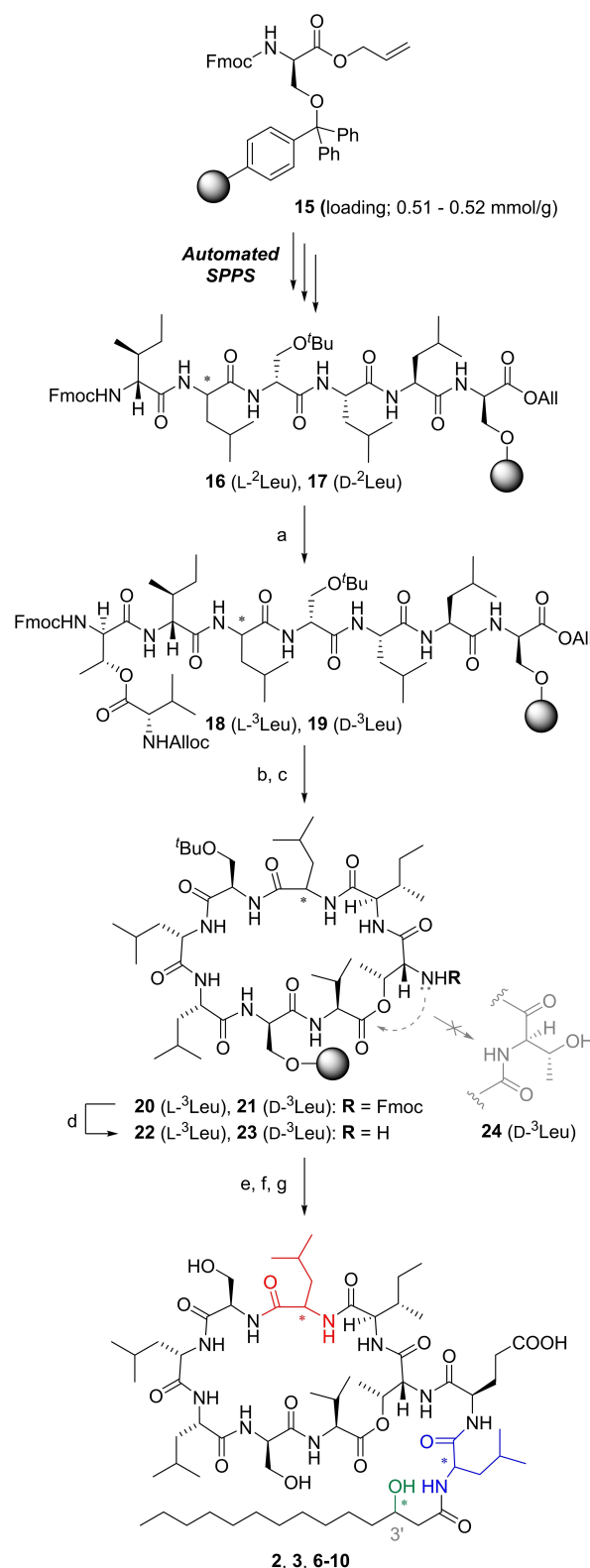
condensation-epimerization (C/E) domains subsequent to modules 1 and 5 should produce *D*-configured residues. Nonetheless, non-functional epimerization domains have been observed in *Pseudomonas*,^[2,18] precluding an assignment from NRPS gene sequencing data *a priori* in these organisms today. Therefore, all possible stereoisomers at the ambiguous positions were selected as candidates for comparison by synthesis (3, 6–10, Table 1).

In our early synthesis experiments (Supporting Information, Scheme S3), we had found the on-resin esterification procedure to be slow and prone to epimerization, in line with other reports.^[19,20] Synthesis reorganization only led to modest improvements (Supporting Information, Schemes S2 and S3). To establish a more reliable method, we envisioned using a building block with a preformed ester bond.^[20,21] Such building blocks were previously employed for the synthesis of A54145B^[22] and ophiotine.^[23] Notably, *O*→*N* acyl migrations from β -oxygen atoms to proximal α -amino groups are facile under basic conditions and have indeed been applied to dedicated peptide bond syntheses.^[20,21,24] To study if regular Fmoc protection could be kept for SPPS and the migration still be prohibited, Fmoc-*D*-allo-Thr (11) was protected with a diphenylmethyl (Dpm) group^[25,26] and esterified with Alloc-L-Val to yield ester 13 in excellent stereoisomeric purity (>97%, Scheme 1 and Supporting Information). TFA-mediated cleavage of the Dpm group produced the ester building block 14 in 81% overall yield for direct use. Isomer 14' was prepared for comparison (d.e. determination).

Automated SPPS using Fmoc/^tBu strategy and side-chain anchored serine on trityl resin at moderate loading (0.5 mmol/g) cleanly provided the resin-bound peptides 16 and 17 (Scheme 2). Coupling with dimer 14 gave the linear octadepsipeptides 18 and 19. During the coupling of ester 14, elimination of Alloc-L-Valine to give a dehydrothreonine residue was observed as a side reaction,^[20] which was suppressed after suitable optimization (see Supporting Information for details). Simultaneous allyl deprotection and on-resin macrolactamization efficiently led to the cyclic octadepsipeptides 20 and 21. Fmoc deprotection tests with linear peptide 19 and cyclic peptide 21 indicated the *O*→*N* acyl migration rate of cyclic peptide 21 (23→24) to be significantly lower than that of linear peptide 19, probably owing to a more rigid structure (see Supporting Information for details). After optimization, the *O*→*N* acyl migration could be suppressed for the cyclic peptides 20



Scheme 1. Synthesis of dipeptide building blocks 14 and 14'. *Reagents and conditions:* a) Benzophenone hydrazone, PIDA, *cat.* I₂, CH₂Cl₂, 0 °C, 93%^[25,26] b) Alloc-L-Val or Alloc-D-Val, EDCl, DMAP, CH₂Cl₂, 0 °C to r.t.; c) TFA, TIS, CH₂Cl₂, 0 °C to r.t.



Scheme 2. Total synthesis of orfamide A and its stereoisomers (see Table 1). *Reagents and Conditions:* a) piperidine/DMF (20 vol%); 14, HATU, HOAt, 2,4,6-collidine, DMF, r.t.; b) Pd(PPh₃)₄, PhSiH₃, CH₂Cl₂, r.t.; c) HATU, HOAt, 2,4,6-collidine, DMF, r.t.; d) DBU/piperidine/DMF (2 vol%, 2 vol%), 2 × 30 s; e) Fmoc-A.A., HBTU, HOBT, DIEA, DMF, r.t.; piperidine/DMF (20 vol%); f) (*R*)- or (*S*)-3-TBSOxy tetradecanoic acid, HBTU, HOBT, DIEA, DMF, r.t.; g) 0.1 N HCl/HFIP, TIS (1 vol%), r.t.^[27,30]

and **21** by applying rapid Fmoc deprotection by brief (30s, 2x) exposure to 2% DBU/2% piperidine/DMF solution and rapid washing with DMF to minimize the peptide exposure period to basic conditions, immediately followed by subsequent amino acid couplings. Finally, acylation with β -HTDA completed the syntheses of the target compounds **2**, **3**, and **5–10** in very appreciable yields (Table 1), likely the result of significantly facilitated compound purification.

All synthesized candidates were subsequently characterized by using RP-HPLC. Candidates with retention times on RP-HPLC similar to authentic material were further compared by using LC/HRMS (Figure 4). Only the characteristics of compound **3** (L¹-Leu, D⁵-Leu, 3'-R-HTDA) agreed with the isolated natural product. Unambiguous verification of the structure of orfamide A was then achieved by ¹H NMR through comparison of synthetic **3**, the natural product, and their mixture, all showing identical ¹H NMR spectra in acidic buffer (see Supporting Information, Figures S5–S10).

To compare the biological response of synthetic CLPs **2** and **3** to isolated orfamide A in *C. reinhardtii*, we used the transgenic AEQ34 cell line that expresses apo-aequorin for measuring cytosolic Ca²⁺ levels (for details see Supporting Information).^[9,10] CLP **3** showed identical characteristics to the natural product, with a rapidly occurring initial Ca²⁺ release signal and a secondary Ca²⁺-release that persisted over several minutes. These data additionally confirmed that compound **3** has the correct structure of orfamide A. CLP **2**, which differs from CLP **3** only in the configuration of two residues, showed a distinct Ca²⁺ signature, where the initial signal was strongly reduced and the secondary release was absent (Figure 5). Beyond biological identity, these data indicate that its stereochemistry profoundly affects the biological properties of orfamide A. This

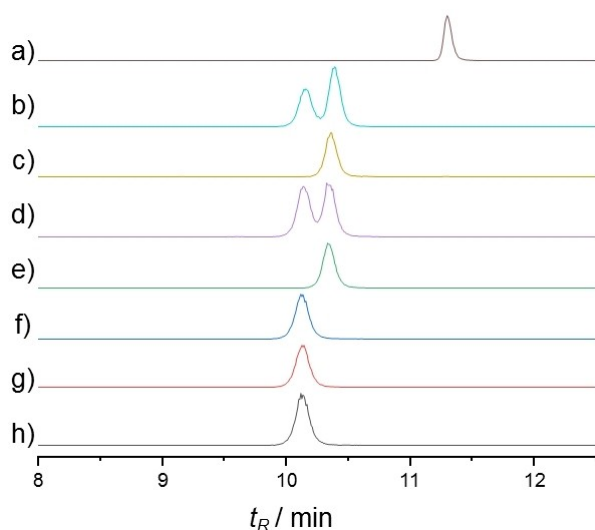


Figure 4. Extracted ion chromatogram ($m/z = 1295.8 \pm 0.5$ Da) of the natural product (NP) and selected orfamide A derivatives (see Table 1): a) **10**; b) mixture of **9** and NP; c) **9**; d) mixture of **7** and NP; e) **7**; f) **3**; g) mixture of **3** and NP; h) NP. Peak heights normalized for comparison, for individual full traces see Supporting Information (Figure S12).

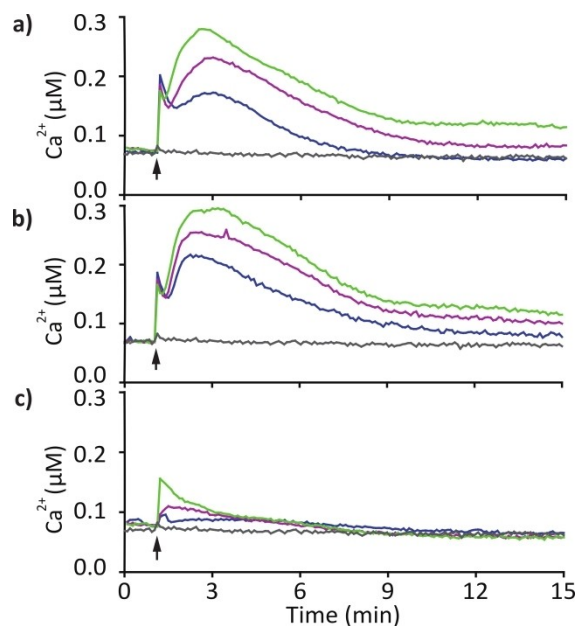


Figure 5. Time course of cytosolic Ca²⁺ concentrations in *C. reinhardtii* upon compound treatment. Compounds were dissolved as a stock solution in MeOH and further diluted in TAP medium. Each line represents the mean of three independent biological replicates, and each biological replicate includes three technical replicates. For details see Supporting Information. Color code: MeOH control (gray), 2 μM compound (blue), 5 μM (purple), 10 μM (green). a) Synthetic orfamide A (**3**), b) isolated orfamide A (NP), c) compound **2**.

is corroborated by other biological activities and the activity of further orfamide A variants.^[31]

Since orfamide A causes immobilization in several flagellated chlorophyte algae,^[9] we also studied the medically relevant *Trypanosoma brucei*, a flagellated parasitic microorganism that causes African sleeping sickness. While orfamide A (**3**) did not cause deflagellation of *T. brucei*, it nonetheless inhibited its growth ($IC_{50} = 6 \mu\text{M}$, see Supporting Information for details), as it did in *C. reinhardtii*.^[9] It was furthermore found to be acutely toxic to trypanosoma cells immediately upon exposure.

Conclusion

In summary, we have completed the first total synthesis of orfamide A and determined its correct structure (**3**) by independently applying synthetic chemistry, bioinformatics analysis, and bioassays. These results emphasize once again the importance of total synthesis in natural products chemistry, especially when stereochemistry is concerned.^[32] We have established a novel, robust, versatile, and high yielding synthetic route to CLPs by using a preformed ester bond in building block **14** and by suppressing O→N acyl migration during Fmoc deprotection. This methodology was implemented on solid support and is compatible with automated SPPS. Thereby, orfamide A analogs and other related CLPs will now be easily accessible for detailed biological or structural studies.

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Conflict of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: algae · natural products · peptides · structure-activity relationship · total synthesis

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