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Disciformycins A and B: 12-Membered Macrolide Glycoside Antibiotics from the Myxobacterium *Pyxidicoccus fallax* Active against Multiresistant Staphylococci**

*Frank Surup, Konrad Viehrig, Kathrin I. Mohr, Jennifer Herrmann, Rolf Jansen, and Rolf Müller**

Abstract: *Two macrolide glycosides with a unique scaffold were isolated from cultures of the myxobacterium Pyxidicoccus fallax. Their structures, including absolute configurations, were elucidated by a combination of NMR, MS, degradation, and molecular modeling techniques. Analysis of the proposed biosynthetic gene cluster led to insights into the biosynthesis of the polyketide and confirmed the structure assignment. The more active compound, disciformycin B, potently inhibits methicillin- and vancomycin-resistant Staphylococcus aureus.*

Staphylococcus aureus causes severe hospital- and community-acquired infections and consequently the steadily rising occurrence of multiresistant and methicillin-resistant *S. aureus* (MRSA) poses a serious public health threat.[1] Consequently, development of novel antibiotics active against such pathogens is urgently needed.[2]

Myxobacteria are a prolific source of novel bioactive secondary metabolites, for example the anticancer drug epothilone,[3] the antibacterial sorangicin,[4] and the antifungal soraphen.[5] In order to access the tremendous genetic potential of yet unexplored metabolites,[6] a selection of our myxobacterial strain collection comprising 8550 species is currently being re-assessed for the production of novel antibiotics.[7,8] In this rescreening, *Pyxidicoccus fallax* strain AndGT8 stood out with a bioactivity against Gram-positive indicator bacteria. Because none of the UV-MS-characterized compounds in the bioactive fractions obtained by RP-HPLC was included in our internal database "Myxobase"[9] or in the "*Dictionary of Natural Products*",[10] this strain was chosen for comprehensive examination.

Bioactivity-guided isolation of an initial cultivation on a 10 L scale led to the isolation of 1.0 mg of disciformycin A (**1**); *N. flava* was used as the indicator organism. HRESI-MS provided the elemental composition $C_{27}H_{40}O_{10}$ implying eight double-bond equivalents. Interpretation of the HMQC spectrum (CD_3OD) enabled the assignment of all protons to their respective carbon atoms, leaving only three exchangeable protons.

$^1H,^1H$-COSY and $^1H,^1H$-TOCSY correlations showed six ^1H spin systems (Figure S1, Table S1, Supporting Information). These partial structures were subsequently linked by series of $^1H,^{13}C$ HMBC correlations: the correlation of methyl group H_3-15 to C-1, of methylene H_2-4 and methine H-6 to C-5, of methine H-7 and methyl H_3-16 to C-8, and of H-11 and H_3-17 to C-12 established the carbon skeleton of the aglycon. The low-field shift of H-11 ($\delta_H = 5.61$ ppm) indicated an ester linkage at this position which was verified by a HMBC correlation between H-11 and C-1, establishing the lactone ring closure of the aglycon. The configuration of the methyl-substituted double bonds was derived from ROESY correlations. A strong ROESY correlation between H_3-15 and H-3 indicated the *Z* configuration of the $\Delta^{2,3}$ double bond, while the ROESY correlation between H_3-16 and H_a-10 supported an *E* configuration of the $\Delta^{8,9}$ bond. Finally, a ROESY correlation between H-13 and H_3-17 indicated the $\Delta^{12,13}$ *Z* configuration in the side chain of the aglycon. A $^1H,^{13}C$ HMBC correlation between H-6 and C-1′ proved the ester linkage of 3-methylbutyric acid to C-6, simultaneously explaining the low-field shift of H-6 ($\delta_H = 5.05$ ppm).

The ^{13}C NMR data of **1** were characteristic for an α-arabinofuranose configuration (observed $\delta_C = 110.6$, 83.8, 79.1, 86.3, 63.0 ppm; methyl furanoside $\delta_C = 109.3$, 81.9, 77.5, 84.9, 62.4 ppm).[11] The absolute D-(−) configuration of the arabinose moiety was determined by GC-MS comparison of the (−)-2-butyl glycoside derivative to authentic standards.[12]

The relative configuration of the aglycon of **1** could be derived from vicinal coupling constants and ROESY correlations (Figure S2). The large coupling constant of 9.5 Hz between H-6 and H-7 and the absence of a ROESY correlation indicated their *trans* configuration. Strong NOEs

[*] Dr. F. Surup, Dr. K. I. Mohr, Dr. R. Jansen, Prof. Dr. R. Müller
Helmholtz Center for Infection Research (HZI)
Department Microbial Drugs
Inhoffenstrasse 7, 38124 Braunschweig (Germany)
K. Viehrig, Dr. J. Herrmann, Prof. Dr. R. Müller
Helmholtz Institute for Pharmaceutical Research Saarland (HIPS)
Helmholtz Center for Infection Research and Pharmaceutical Biotechnology, Saarland University Campus
Building C2.3, 66123 Saarbrücken (Germany)
E-mail: rom@helmholtz-hzi.de
Dr. F. Surup, K. Viehrig, Dr. K. I. Mohr, Dr. J. Herrmann, Dr. R. Jansen, Prof. Dr. R. Müller
German Center for Infection Research (DZIF)
Location: Braunschweig (Germany)

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were observed for H-6 and H_b-10 with methyl H_3-16, but not with H-11. On the other side H-7 showed a strong NOE with H-9, which itself correlated to H-11 and H_a-10, indicating a cisoidal relation between H-7 and H-11.

The absolute configuration of the aglycon was derived from the previously determined absolute configuration of the D-(−)-arabinosyl residue: A strong ROESY correlation between H-1″ and H-7 confirmed the typical conformation of the glycoside in solution. Consequently, a weak but unambiguous correlation between H-4″ and methyl H_3-16 was used to assign the configuration of the chiral center C-7 (Figure 1). Thus the absolute configuration of the disciformycin A aglycon (**1**) was assigned as 6*S*,7*R*,11*R*.

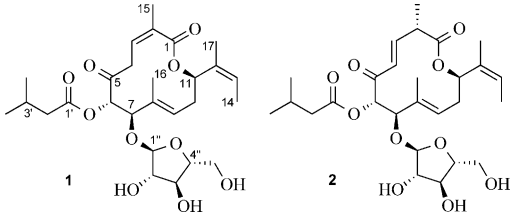

Figure 1. Structures of disciformycin A (1) and B (2).

After the strain and culture conditions had been optimized, a 70 L fermentation of strain AndGT8 provided 25.4 mg of **1** as well as 7.6 mg of isomer **2** with identical molecular formula $C_{27}H_{40}O_{10}$. 1D and 2D NMR data showed that the only difference between **1** and **2** was the shifting of the $\Delta^{2,3}$ double bond to position $\Delta^{3,4}$ with *E* configuration, which was indicated by the large coupling constant ($J_{3,4}$ = 15.3 Hz). Coupling constants and ROESY correlations of the C-6 to C-11 part remained largely unchanged compared to **1** (Table S2). Therefore, a 6*S*,7*R*,11*R* configuration can be concluded for **2** as well.

To determine the configuration at C-2 a structure model of compound **2** was calculated by PM3 with HyperChem (Figure 2). Due to the rigid structure elements, that is, the double bond, the α,β-unsaturated ketone, and the ester, the core part of **2** is locked in a twisted configuration, with protons H-2, H-4, and H-6 and methyl group H_3-16 located above the main plane. Strong ROESY correlations between H-4 and H-2, and between H-3 and the methyl group H_3-15 indicate a 2*S* configuration.

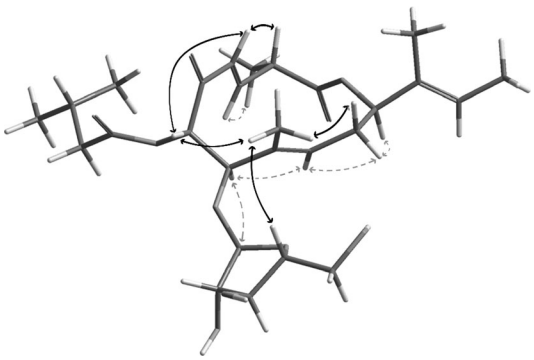

Figure 2. Model of **2** calculated by PM3 with HyperChem. Selected ROESY correlations are marked with arrows.

For further corroboration of the observed structure we sequenced and screened the AndGT8 genome sequence for a suitable gene cluster using antiSMASH.[13] A candidate gene cluster was found in the partial genome sequence encoding a linear type I PKS that consists of six separate PKS modules with a domain organization in line with the observed reduction stages of the polyketide. The core biosynthetic gene cluster is shown in Figure 3A, with identified open reading frames (ORFs) and their assumed function listed in Figure 3C. ORFs *difB* to *difG* encode the PKS genes, *difA* encodes the tailoring cytochrome P450. The functions of *difH* and *difI* are yet unclear. The PKS domain organization, as obtained from sequence analysis with the NRPS/PKS predictor[14] is shown in Figure 3B, together with a biosynthesis proposal.

Assembly of the polyketide starts at DifG, which resembles a fused starter and extender module, as observed previously in the stigmatellin synthase.[15] The polyketide is then extended at modules DifB–DifF and released by macrocyclization. DifF contains a domain of unknown function between the DH and the KR domain. This position is usually occupied by an ER domain in fully reductive modules. A Rossman fold for NADP(H) binding is found in the sequence by BLAST analysis, but other typical sequence motifs of ER domains are absent, as well as other hints for its potential function.

The substrate specificity of the seven AT domains was predicted by multiple alignments of their respective amino acid sequences and comparison of key residues to published data.[16] The predicted substrate of the AT domains from modules DifB, DifD, DifE, and DifG1 are malonyl-CoA, while AT domains from modules DifC, DifF, and DifG2 are predicted to be specific for methylmalonyl-CoA, as observed in the disciformycin molecule. A detailed alignment is presented in the Supporting Information.

To further validate our biosynthesis proposal, the *dif* KR domain sequences were screened for described motifs, which allows the prediction of the configuration of the hydroxy groups and double bonds in the molecule.[17,18] Based on conserved sequence motifs, most KR domains can be categorized as either A or B type, which reduces their ketoacyl substrate to either *S*- or *R*-configured secondary alcohols, respectively. In the case of elimination after keto reduction, the configuration of the cryptic KR-derived alcohol can be predicted with this method. This approach has been used previously to determine the stereochemistry of molecules in which the absolute configuration could not be determined by experimental methods.[19,20]

In the case of disciformycin, the *R*-configured hydroxy group at carbon atom C-7 results from keto reduction by the KR of DifD, which shows B-type sequence features. The KR of DifB, which reduces the keto group at C-11, cannot be categorized as A or B type. For the *Z*-$\Delta^{12,13}$ double bond and the *E*-$\Delta^{8,9}$ double bond, the KR domain sequences correspond to a A-type KR for DifG and a B-type KR for DifC, respectively.

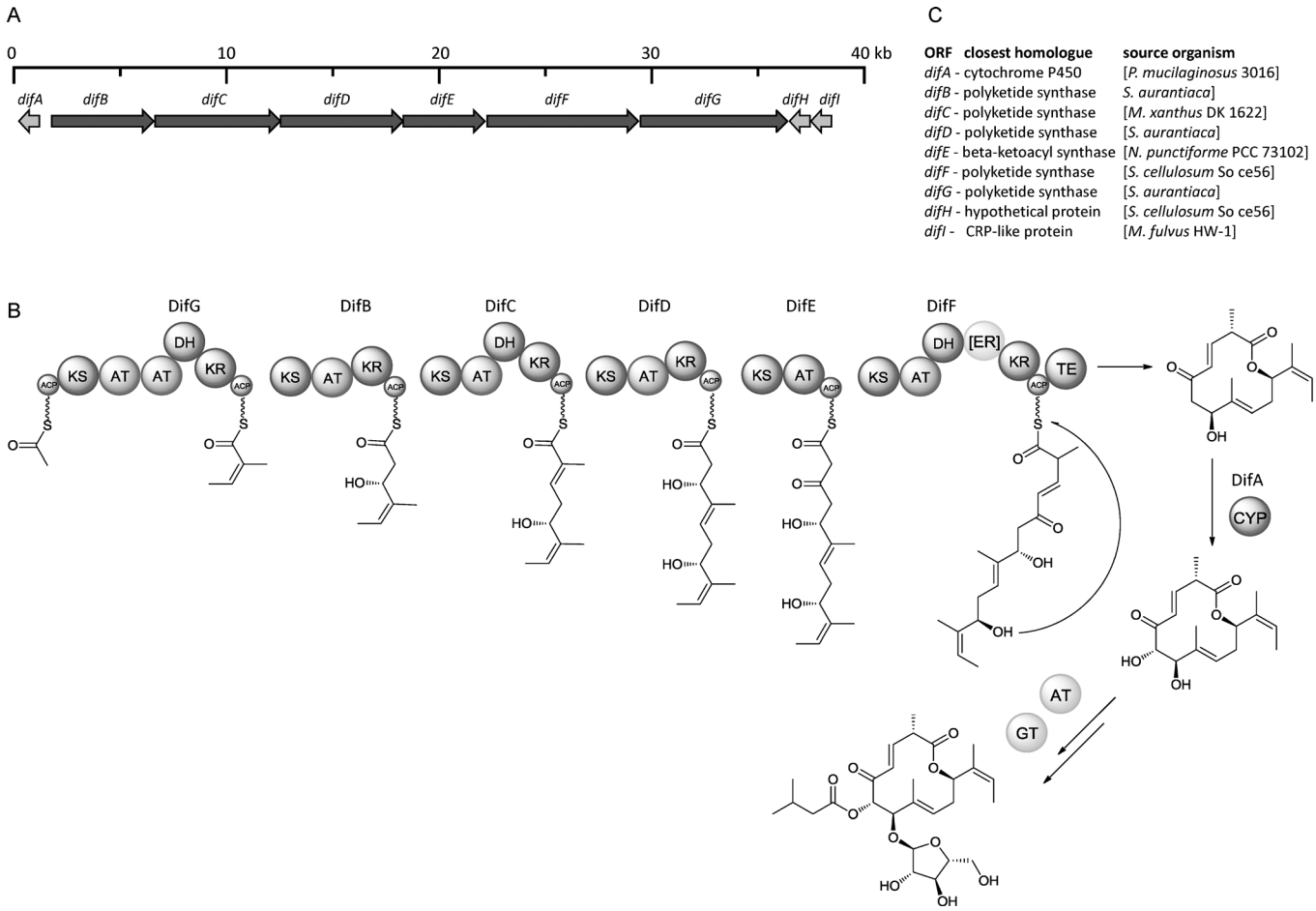

Figure 3. A) Disciformycin biosynthetic gene cluster. PKS genes are displayed in dark gray, other ORFs are shown in light gray. B) Proposed disciformycin B assembly. The hydroxy group at C6 is introduced by the cytochrome P450 enzyme DifA. The tailoring glycosyltransferase and acyltransferase are yet unknown and must be located elsewhere in the genome. ACP = acyl carrier protein domain; AT = acyl transferase (domain); CYP = cytochrome P450; DH = dehydrogenase domain; ER = enoyl reductase domain; GT = glycosyl transferase; KS = ketosynthetase domain; KR = ketoreductase domain; TE = thioesterase domain. C) Annotated ORFs in the *dif* cluster and closest homologues found by BLAST search.

The DifF KR domain is predicted to be B type; therefore an *R*-hydroxy intermediate is expected to be the substrate for the DH domain of DifF, which is then processed to either a *Z*-$\Delta^{2,3}$ double bond in **1** or an *E*-$\Delta^{3,4}$ double bond in **2**. Therefore, a double-bond shift has occurred in **2**. Such double-bond shifts in PKS products have been reported previously, e.g. in rhizoxin,[21] ansamitocin,[22] corallopyronin,[23] and bacillaene,[28] and have been shown to take place during biosynthesis while the substrate is tethered to the PKS. Since the *dif* cluster contains no separate "shifting module" as is the case for the corallopyronin cluster, we expect the double-bond migration to be introduced by the DH domain of DifF, as observed in ansamitocin biosynthesis. To our knowledge, no sequence motifs hve been described yet by which a DH domain could be predicted to perform such double-bond shifts along with the dehydration step, and future work is required to clarify this question in disciformycin biosynthesis.

The OH group at C-6, to which the valerate is attached in the final compound, must result from hydroxylation by a tailoring enzyme during or after assembly, most likely a P450 enzyme encoded by *difA*. Suitable genes for the acylation and glucosylation could not be found in the vicinity of the PKS gene cluster, but numerous such functionalities are encoded in the genome. Currently, further studies are underway to identify the missing functionalities. However, despite serious efforts the producing strain currently cannot be genetically manipulated.[24,25]

The bioactivity assessment of **1** and **2** revealed strong antimicrobial activity (Tables 1 and S3) against Gram-positive bacteria, for example, *Bacillus subtilis* DSM-10. Disciformycins were found to be especially active against staphylococci, such as *S. carnosus* DSM-20501 and *S. aureus* Newman.[26] In addition, both tested strains of methicillin-resistant *S. aureus* (MRSA) were inhibited: *S. aureus* DSM-11822 and *S. aureus* N315.[27] Moreover, these MRSA strains show reduced susceptibility to other antibiotic classes, such as macrolides and quinolones, indicating a novel target to be addressed by disciformycins. The MIC values were in the range of the reserve antibiotic vancomycin (Table S3). Importantly, no cross-resistance was observed to vancomycin, which is currently in use for human therapy as the antibiotic of last resort; the disciformycins displayed pronounced activity against the methicillin- and vancomycin-resistant *S. aureus* (MRSA/VRSA) Mu50 (ATCC 700699). Importantly, at

Table 1: Minimum inhibitory concentrations (MICs in $\mu g\,mL^{-1}$) for selected bacteria and half-inhibitory concentrations (IC_{50} in μM) for cell lines measured for disciformycin A (**1**), disciformycin B (**2**), and vancomycin (VAN).

	1	**2**	**VAN**
Bacillus subtilis DSM-10	4.2	0.8	0.25
Nocardioides simplex DSM-20130	33.3	16.6	0.42
Paenibacillus polymyxa DSM-36	16.6	16.6	–
Staphylococcus carnosus DSM-20501	7.8	2.4	0.25
Staphylococcus aureus Newman	8.0	1.2	0.5
Staphylococcus aureus DSM-11822 (MRSA)	4.0	0.6	1.0
Staphylococcus aureus N315 (MRSA)	8.0	1.2	1.0
Staphylococcus aureus Mu50 (MRSA/VRSA)	2.0	0.6	16.0
Colon carcinoma cells HCT-116	>10	>10	–
Murine fibroblast-like cells L929	>10	>10	–
Chinese hamster ovary cells CHO-K1	>10	>10	–

concentrations of up to 10 μM no cytotoxicity was found against human HCT-116 colon carcinoma cells, murine fibroblast-like L929 cells, and Chinese hamster ovary CHO-K1 cells.

In conclusion, we present the structure elucidation of two new antibiotics from *Pyxidicoccus fallax* AndGT8 which were discovered by rescreening our myxobacterial strain collection. Identification and analysis of the fundamental biosynthetic gene cluster properly matches the stereochemistry determined by structure elucidation for the more active metabolite **2**, which is most likely the primary product of the biosynthesis. Disciformycins A (**1**) and B (**2**) might be suitable starting points for antibiotic development; ongoing work in our laboratory aims to identify the presumably novel bacterial target of these antibiotics. In any case, the compounds provide a novel scaffold for future total synthesis approaches, which will provide insight into their structure–activity relationship.

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