Natural Product Biosynthesis

DOI: 10.1002/anie.201310641

Heterologous Reconstitution of Ikarugamycin Biosynthesis in *E. coli***

*Janine Antosch, Françoise Schaefers, and Tobias A. M. Gulder**

Abstract: *Polycyclic tetramate macrolactams (PTMs) are a family of biomedically promising natural products with challenging molecular frameworks. Despite these interesting properties, so far only relatively little is known about the biosynthetic origin of PTMs, in particular concerning the mechanism by which their ring systems are formed. Herein we present the first insights into these processes by using the biosynthesis of ikarugamycin as an example. This has been facilitated by the first heterologous expression of a PTM biosynthetic gene cluster in Escherichia coli. With this approach it will not only become possible to mechanistically investigate already known PTM biosynthetic pathways in more detail in the future, but also to interrogate cryptic PTM biosynthetic pathways chemically and biochemically.*

Polycyclic tetramate macrolactams (PTMs) are structurally and biosynthetically intriguing examples of complex bacterial natural products. They all have a tetramic acid moiety in common that is incorporated into a macrolactam ring system. Directly fused to the macrolactam is a set of two or three five- or six-membered carbacycles that give rise to the impressive structural diversity of PTMs. Representative examples are HSAF (**1**)[1] and the frontalamides (**2**),[2] both equipped with a 5-5-6-polycyclic ring system, ikarugamycin (**3**)[3] and the clifednamides (**4**),[4] bearing a 5-6-5-cyclization pattern, as well as cylindramide (**5**)[5] and alteramide A (**6**),[6] characterized by two five-membered rings (Figure 1).

A broad variety of biological activities has been reported, depending on the individual structures of the PTMs, which range from antibacterial and antifungal to cytotoxic properties.[1–6] Despite this promising biomedical potential of the PTMs and their challenging molecular frameworks, surprisingly little is known about their biosynthetic origin. Even though putative PTM biosynthetic pathways are widespread in phylogenetically diverse bacteria,[2] the only PTM chemical structures that have been linked to biosynthetic pathways are

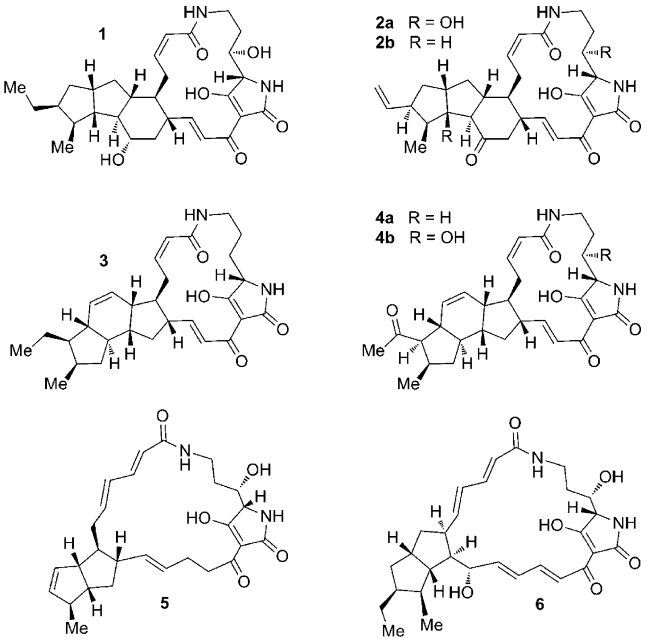


Figure 1. The structural diversity of PTM natural products exemplified by HSAF (**1**), the frontalamides (**2**), ikarugamycin (**3**), the clifednamides (**4**), cylindramide (**5**) and alteramid A (**6**).

those of **1**[7] and **2**,[2] two PTM family members with identical carbon skeletons. The respective gene clusters consequently are likewise highly similar, being composed of a hybrid polyketide synthase/nonribosomal peptide synthetase (PKS/NRPS)[8] equipped with a series of red/ox-tailoring enzymes (Figure 2). Most intriguingly, the PKS only consists of a single module, thus strongly suggesting an iterative mechanism (*i*PKS). Although this is a common situation in fungal PKSs,[8d] it is very unusual in bacterial polyketide pathways.[9] The only PTM biosynthetic steps that have so far been investigated in more detail revealed the origin of the hydroxy function attached to the ornithine building blocks of **1** and **2**, which gets introduced by a post-PKS/NRPS-hydroxylation,[2,10] and the formation of the tetramic acid moiety catalyzed by the NRPS-derived thioesterase domain,[7b] an enzyme that also exhibits unusual protease and peptide ligase activity.[11] However, many key steps in PTM biosynthesis still remain elusive, in particular the mechanism of the biocatalytic formation of the polycyclic ring system.

To shed more light on the biosynthetic logic leading to PTMs we set out to identify a gene cluster that encodes a PTM with a cyclization pattern deviating from that of **1** and **2**. As a consequence of its potential as a biochemical tool to investigate cellular processes associated with endocytosis,[12] we selected ikarugamycin (**3**) as the target molecule. Fiedler, Zeeck et al. showed that this metabolite is produced by

[*] M.Sc. J. Antosch, Dipl.-Chem. F. Schaefers, Dr. T. A. M. Gulder
Kekulé-Institut für Organische Chemie und Biochemie
Universität Bonn
Gerhard-Domagk-Strasse 1 (Germany)
M.Sc. J. Antosch, Dipl.-Chem. F. Schaefers, Dr. T. A. M. Gulder
Department Chemie, Technische Universität München
Lichtenbergstrasse 4, 85748 Garching (Germany)
E-mail: tobias.gulder@ch.tum.de
Homepage: http://www.biosystemchemie.ch.tum.de.

[**] J.A. thanks the Fonds der Chemischen Industrie (FCI) for her PhD scholarship. This project is generously supported by the North Rhine-Westphalian Academy of Scienes, Humanities and the Arts ("Junges Kolleg") and the DFG (GU1233/2-1). We thank Prof. Dr. H.-P. Fiedler for providing *Streptomyces* sp. Tü 6239.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201310641.

Streptomyces sp. Tü 6239.[13] Degenerate primers selectively targeting putative PTM thioesterase domains were designed to allow for a PCR-directed identification of the PTM biosynthetic gene cluster in this strain (see the Supporting Information). Based on the sequence data of the thus-obtained amplicon, specific primers were developed as probes to screen for the ikarugamycin biosynthetic gene cluster in a pCC1FOS genomic library of *Streptomyces* sp. Tü 6239 (1056 clones). This resulted in the identification of four clones bearing the desired sequence, one of which was identified to be a promising candidate to harbor the complete PTM gene cluster by end sequencing of the isolated fosmid Ika-Fos8. This fosmid was fully sequenced (GATC) and the resulting data bioinformatically analyzed, thereby revealing the organization of the putative ikarugamycin (**3**) pathway (*ika*, Figure 2). As expected, the *ika* locus likewise harbored the unusual PKS/NRPS system. In addition, only two oxidoreductases were identified, thus reducing the number

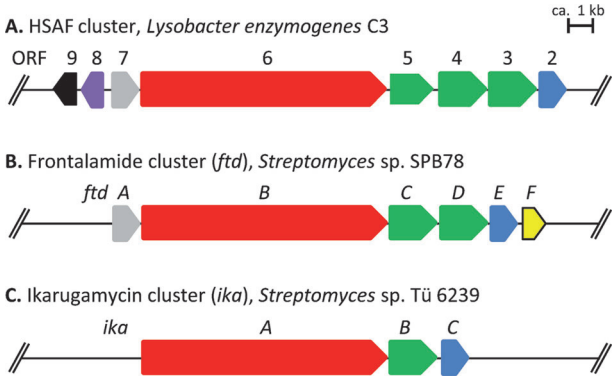


Figure 2. Biosynthetic gene clusters of HSAF (**1**, A), the frontalamides (**2**, B), and ikarugamycin (**3**, C). Red = iPKS/NRPS (domains shown in Scheme 1), green = FAD-dependent oxidoreductase, blue = alcohol dehydrogenase, yellow = cytochrome P450, gray = sterol desaturase, violet = ferredoxin reductase, black = arginase.

of potential post-PKS/NRPS modifications compared to the two known PTM gene clusters. A number of genes that have not been reported in the two characterized PTM biosynthetic pathways were found up- and downstream of these PTM core genes (see Table S4 in the Supporting Information).

To prove the function of the *ika* cluster we chose its heterologous reconstitution in *E. coli*. As a production host we selected *E. coli* BAP1,[14] as it harbors a chromosomal copy of the promiscuous phosphopantetheinyl transferase Sfp from the surfactin biosynthetic pathway that catalyzes posttranslational activation of PKS and NRPS carrier proteins.[15] Based on pHIS8,[16] vectors were constructed by homologous recombination[17] that put the expression of the desired genes under the control of the inducible T7 promotor (see the Supporting Information). Three plasmids were generated, harboring all the genes from *ikaA* to Orf + 7 (pJA_Orf + 7), the three genes *ikaABC* (pJA_*ikaABC*), or only *ikaAB* (pJA_*ikaAB*) (see Table S4 in the Supporting Information). In the expression experiments carried out with these constructs, the formation of **3** was not only evident with pJA_Orf + 7, but also—even with significantly improved production titers—with pJA_*ikaABC* that is reduced to three genes (Figure 3). In experiments with pJA_*ikaAB*, by contrast, **3** was not detectable. The thus-generated proof that the three proteins IkaABC are sufficient and essential for the production of **3** reveals a remarkable contrast between the

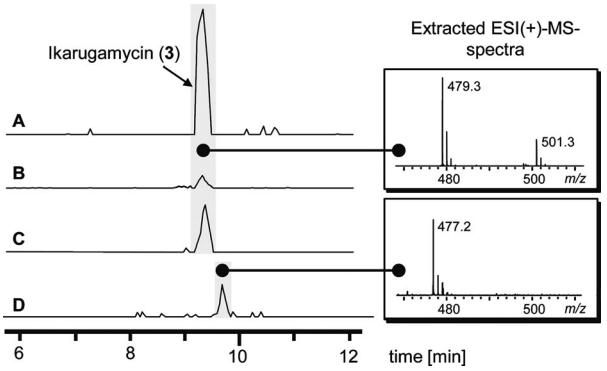
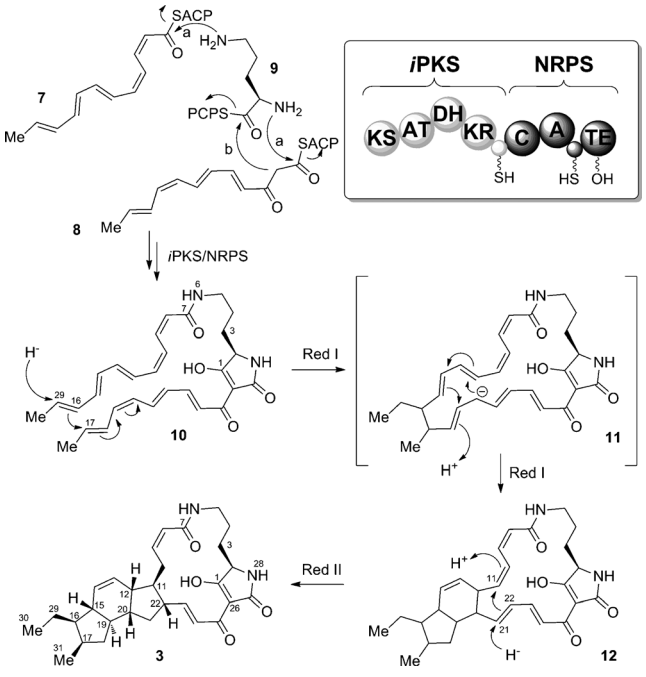


Figure 3. Analysis of the heterologous expression experiments with *E. coli* BAP1 by HPLC-ESI(+)-MS. Extracted ion chromatograms (EICs) of the masses for *m/z* of **3** and **12**. A = standard solution of **3** (0.1 mg mL^{-1}); extracts of *E. coli* BAP1 pJA_Orf + 7 (B), pJA_*ikaABC* (C), or pJA_*ikaAB* (D, intensity × 10).

simplicity of the *ika* locus and the structural complexity of **3**. Our experiments furthermore constitute the first experimental confirmation of the iterative mechanism of the PKS module in PTM biosynthesis.

A highly efficient biosynthetic pathway to **3** can be devised on the basis of these results. The *i*PKS module of IkaA consecutively provides two ACP-bound unsaturated hexaketides **7** and **8** that get attached to the nitrogen functions of PCP-bound ornithine (**9**) by action of the NRPS condensation domain (Scheme 1, arrows a).[18] The thioesterase subsequently catalyzes the formation of the tetramic acid moiety (arrow b),[7b] which concomitantly leads to cleavage of the precursor molecule **10** from the synthetase. Intermediate **10** is further processed by hydride-induced cyclization/protonation catalyzed by the two oxidoreductases IkaBC. Reductase I (Red I) triggers the formation of a bond between C16 and C17. The theoretical intermediate **11** further cyclizes to give **12**, which already bears two carbacycles. The last cyclization event between C22 and C11 is then catalyzed by reductase II (Red II), again by reductive cyclization.[19] This concludes the biosynthetic sequence to **3**, exclusively utilizing the three enzymes IkaABC. The proposed biosynthetic pathway, however, also raises the question of which of the two oxidoreductases is responsible for which ring-closing reaction. The analysis of the heterologous expression experiment with pJA_*ikaAB* shows that, instead of **3**, a metabolite with a slightly higher retention time accumulates. This metabolite has the molecular mass of the postulated intermediate **12** (Figure 3D, for better visibility the relative intensity of chromatogram B was increased by a factor of 10 compared to A–C). Although structural elucidation of this metabolite by NMR was not possible because of the low production level, this observation strongly indicates that the



Scheme 1. Potential biosynthetic pathway leading to **3**. Reactions catalyzed by (a) C or (b) TE. KS = ketosynthase, AT = acyltransferase, DH = dehydratase, KR = ketoreductase, C = condensation domain, A = adenylation domain, TE = thioesterase domain, the unlabeled, thiol-containing domains in the *i*PKS and NRPS are carrier proteins.

initial cyclization cascade is induced by IkaB (= Red I), while IkaC (= Red II) catalyzes the formation of the last carbacycle. If IkaC is missing, as in pJA_*ikaAB*, the final cyclization does not occur and the biosynthesis halts at intermediate **12**. This assumption is supported by bioinformatic analysis of putative PTM biosynthetic gene clusters in sequenced bacterial genomes (not shown), thus showing that all such clusters carry a homologue of *ikaB*, while not all harbor a copy of an alcohol dehydrogenase, here coded by *ikaC*. This is consistent with the observation that all the PTMs isolated so far show a cyclization between C16 and C17 and thus require a copy of Red I, whereas some representatives, such as **5** and **6**, only bear two small carbacycles and therefore should not contain a functional copy of Red II.

In conclusion, we developed PCR probes for the selective amplification of PTM thioesterase domains that were utilized for the identification of the ikarugamycin biosynthetic gene cluster *ika*. This system was transferred into an inducible expression vector by homologous recombination, transformed into *E. coli*, and used for the heterologous production of **3**. The findings obtained suggest a very compact biosynthetic pathway to **3**, thus revealing a large discrepancy between the simplicity of the *ika* locus, consisting of only three genes, and the complexity of its encoded natural product **3**. Our investigations constitute one of the few examples of the successful heterologous expression of a bacterial PKS system in *E. coli* and the first for a bacterial *i*PKS. The introduced simple system for PTM production can now be used to investigate cryptic *i*PKS/NRPS biosynthetic pathways found in other bacteria. It, therefore, constitutes a valuable approach for the directed discovery of new PTMs and thus of potent biologically active natural products.

Received: December 8, 2013
Published online: February 12, 2014

Keywords: biosynthesis · heterologous expression · ikarugamycin · natural products · PTMs

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[18] The configurations of the double bonds in **7** and **8** after their assembly by the *i*PKS and potential changes in their geometry catalyzed by the downstream reductases can currently not be predicted.
[19] Alternatively, **10** might also directly get protonated at C18 after initial cyclization, followed by a (non-enzymatic) Diels–Alder cycloaddition of diene at C12 to C15 with the in that case *trans*-configured double bond at C19/C20; the reductive cyclization steps could furthermore also occur after an initial Diels–Alder cycloaddition.

