Discovery of actinoplanic acid pathway in *Streptomyces rapamycinicus* reveals a genetically conserved synergism with rapamycin

Peter Mrak\textsuperscript{a,}\textsuperscript{d,*}, Philipp Krastel\textsuperscript{b}, Petra Pivk Lukančič\textsuperscript{a}, Jianshi Tao\textsuperscript{c}, Dominik Pistorius\textsuperscript{b}, Charles M Moore\textsuperscript{b,\textsuperscript{*}}

From the \textsuperscript{a}Novartis Technical Operations, MS&T Antiinfectives, SI-1234 Mengeš, Slovenia; \textsuperscript{b}Novartis Institutes for BioMedical Research, Novartis Campus, 4056 Basel, Switzerland; \textsuperscript{c}Genomics Institute of the Novartis Research Foundation, San Diego, CA 92121, USA; \textsuperscript{d}University of Ljubljana, Slovenia

Running title: *Actinoplanic acid biosynthesis and synergy with rapamycin*

*To whom correspondence should be addressed: Peter Mrak: Novartis Technical Operations, MS&T Antiinfectives, Kolodvorska 27, SI-1234 Mengeš; peter.mrak@novartis.com; Tel. (+386) 1 721 7583

*To whom correspondence should be addressed: Charles M Moore: Novartis Institutes for BioMedical Research, Novartis Campus, 4056 Basel, Switzerland; charles-1.moore@novartis.com; Tel. (+41) 79 727 7876

Keywords: actinoplanic acid, rapamycin, synergy, biosynthesis, actinobacteria, secondary metabolism, natural product, polyketide synthase, nonribosomal peptide synthetase, tricarballylic moiety

**ABSTRACT**

Actinobacteria possess a great wealth of pathways for production of bioactive compounds. Following advances in genome mining, dozens of natural product (NP) gene clusters are routinely found in each actinobacterial genome; however, the modus operandi of this large arsenal is poorly understood. During investigations of the secondary metabolome of *Streptomyces rapamycinicus*, the producer of rapamycin, we observed accumulation of two compounds never before reported from this organism. Structural elucidation revealed actinoplanic acid A and its desmethyl analogue. Actinoplanic acids (APL) are potent inhibitors of Ras farnesyltransferase and therefore represent bioactive compounds of medicinal interest. Supported with the unique structure of these polyketides and using genome mining, we identified a gene cluster responsible for their biosynthesis in *S. rapamycinicus*. Based on experimental evidence and genetic organization of the cluster, we propose a stepwise biosynthesis of APL, the first bacterial example of a pathway incorporating the rare tricarballylic moiety into a NP. Although phylogenetically distant, the pathway shares some of the biosynthetic principles with the mycotoxins fumonisins. Namely, the core polyketide is acylated with the tricarballylate by an atypical nonribosomal peptide synthetase-catalyzed ester formation. Finally, motivated by the conserved co-localization of the rapamycin and APL pathway clusters, in *S. rapamycinicus* and all other rapamycin-producing actinobacteria, we confirmed a strong synergism of these compounds in antifungal assays. Mining for such evolutionarily conserved co-harboring of pathways would likely reveal further examples of NP sets, attacking multiple targets on the same foe. These could then serve as a guidance for development of new combination therapies.

Actinoplanic acids A and B are actinobacterial secondary metabolites first isolated from *Streptomyces sp*. MA7099 and *Actinoplanes sp*. MA7066 for their farnesyltransferase inhibitory activity (1). Actinoplanic acid A is a polyketide with a 30-membered carbon backbone, assembled from malonate, methyl-malonate and ethyl-malonate units. It contains a unique tricarballylic acid-bridged structure, cyclizing a part of the polyketide into a double ester lactone (2, Fig. 1B). In actinoplanic acid B, this structure remains acyclic due to the absence of the hydroxyl group on carbon 36 (3). Stereo-chemical characterization of these compounds has remained elusive to date due to the complex nature of the NMR spectra and the inability to obtain crystalline material that would permit X-ray studies (3). Despite their unique structural features, little attention has been devoted to actinoplanic acids after initial discovery and
characterization of their bioactive properties (1). In these reports, actinoplanic acids A and B were found to be highly potent and selective competitive inhibitors of Ras farnesyl-protein transferase (FTI). Limited structure-activity relationship observations suggest that the tricarballylic moieties and more specifically its free carboxylic groups are important for the biological activity of actinoplanic acids (3). Ras GTP-ase, one of the most prevalent oncogenes, is an important target in development of anti-cancer drugs (4) and although FTI have not yielded approved therapies thus far, significant efforts are being invested into harnessing their potential (5). In addition, FTI inhibitors have been successfully used for the treatment of progeria and are being considered for the treatment of Alzheimer’s disease and certain protozoan infections (6).

In contrast to the actinoplanic acids, which remained unexplored in their biosynthetic origin, the only other known natural products featuring a tricarballylate moiety, the fungal toxins fumonisins (7, Fig. 1B), have received significantly more attention. Encoded by 15 genes covering approximately 30 kb (8), the fumonisin biosynthetic pathway is initiated with the formation of a linear polyketide, which is assembled by an iterative type I polyketide synthase (PKS) (9). The nascent polyketide is released by a unique mechanism, involving a condensation reaction with alanine or glycine to form a full-length, free, linear intermediate (10), which is then modified further by a series of post-PKS enzymes. Perhaps the most interesting among these is the nonribosomal peptide synthetase (NRPS)-catalyzed acylation of the intermediate, resulting in an ester with two tricarballylic acids (11). The origin of the tricarballylate has also been investigated, revealing an interesting reduction ofaconitic acid while thioester-tricarballylate has also been investigated, revealing an ester-forming NRPS and the accompanied reduction of the tricarboxylic acid (TCA) intermediate.

The actinomycete *Streptomyces rapamycinicus*, initially classified as *S. hygroscopicus*, is best known for its production the immnosuppressant rapamycin (13). Following the discovery of rapamycin’s inhibition of TOR complex signaling, this macrolide has proven an invaluable asset for cell biology research, as well as clinical use (13). Due to its medical and industrial importance, significant efforts have been invested into understanding the biosynthesis of rapamycin and improving the yield from *S. rapamycinicus* (13). In addition to rapamycin, this organism has been reported to produce other secondary metabolites including elaiophylin and nigericin (14). Recently the genome sequence of *S. rapamycinicus* ATCC 29253 was published, proving to be one of the largest actinobacterial genomes, rich in NP biosynthetic gene clusters (15).

In this study we report accumulation of actinoplanic acid A (2) and its novel desmethyl analogue, which we have designated the actinoplanic acid C, in the fermentation broth of *S. rapamycinicus* ATCC 29253. These compounds are unrelated to any of the secondary metabolites previously reported from this microorganism. Through genome mining, using PKS-domain architecture prediction tools, we identified the putative actinoplanic acid (APL) biosynthetic gene cluster. We further describe the APL biosynthetic pathway based on in silico analysis and experimental evidence, which revealed the key biochemical steps. CRISPR genome editing tools (16) were developed in-house for this organism and applied to the dissection of the pathway. Most intriguing are the post-PKS steps, especially the mechanism for acylation of the nascent polyketide with tricarballylate moieties, which resembles the fumonisin biochemistry in the involvement of an ester-forming NRPS and the accompanied reduction of the tricarboxylic acid (TCA) intermediate.

Furthermore, we have identified the APL cluster in other bacterial genomes, without exception in the presence of the rapamycin pathway. Taking into account the biochemical similarities and genetic differences, both between the bacterial representatives and in comparison to fumonisins, we discuss the evolutionary implications. Among these, the most notable example is our discovery of a significant synergistic effect in antifungal activity of rapamycin and actinoplanic acid A, unveiling nature’s strategy to attack multiple targets on the same foe. Our findings invite important questions in the quest for new natural compounds of medicinal value. Namely, how frequently have similar genetically conserved synergies evolved in secondary metabolism and how can we identify potential synergistic compounds at the primary DNA sequence level?

**Results and discussion**

**Identification of actinoplanic acids in *S. rapamycinicus***

LC-MS analysis of *S. rapamycinicus* ATCC 29253 cultures revealed a compound with *m/z* [M+NH₄]⁺ 966.5 in the polar section of the chromatogram (Fig. 1A). High-resolution mass spectrometry (HRMS) suggested a molecular formula C₁₅₁₆₀₁₆, corresponding to actinoplanic acid A, 1. We also identified a second compound (1:5 ratio compared to 1) with *m/z* [M+NH₄]⁺ 952.5 and a proposed molecular formula of C₂₈₂₁₂₁₆. This corresponds to a novel actinoplanic acid desmethyl analogue which we designated actinoplanic acid C, 2. Both compounds were isolated to yield white amorphous solids. 1D and 2D NMR was applied to confirm the chemical structures (Fig. 1B, Fig. S1, S2). In addition, previously undescribed open-chain variants of both
compounds were found with \( m/z \) \([M+NH_4]^+ \) 970.5 and \( m/z \) \([M+NH_4]^+ \) 984.5 (4 and 3, respectively) in the same 1:5 ratio (Fig. 1A). The HRMS data agrees with the proposed molecular formulas \( C_{50}H_80O_{17} \) and \( C_{51}H_82O_{17} \), respectively. The levels of these compounds were two orders of magnitude lower in comparison to 1 and 2. Nevertheless, a suitable amount of 3 could be isolated to allow structure confirmation by NMR (Fig. S3). Only traces of actinoplactic acid B (\( 5, m/z \) \([M+NH_4]^+ \) 954.5) and its novel desmethyl analogue, actinoplactic acid D (\( 6, m/z \) \([M+NH_4]^+ \) 968.5) were found at the limit of detection. A similar distribution of actinoplactic acid species, in somewhat higher overall levels, was observed from a rapamycin producer \( S. \) rapamycinicus R073 isolated from ATCC 29253 (Fig. 1A).

**Sequence analysis of the \( S. \) rapamycinicus APL gene cluster.**

The genome sequence of \( S. \) rapamycinicus ATCC 29253 (15) was analyzed with antiSMASH 3.0 (17), revealing a gene cluster with PKS domain logic and predicted enzymatic features which correspond well to the structural properties of actinoplactic acids. Similarity searches in the non-redundant databases revealed putative APL clusters in two other genomes, namely in *Streptomyces iranensis* HM35 (18) and *Actinoplanes* sp. N902-109 (19) and were used comparatively to roughly frame the cluster (Fig. 2, Table S1). In order to improve the gene calling and overall quality of the data, we have re-sequenced the \( S. \) rapamycinicus ATCC 29253 using PacBio RS II technology (GeneBank QYCY00000000).

The APL genes of \( S. \) rapamycinicus show a typical type I PKS cluster organization (Fig. 2). The core of the cluster is composed of three large, modular PKS ORFs (\( aplA, aplB \) and \( aplC \)), followed immediately downstream by two ORFs (\( aplD \) and \( aplE \)), collectively encoding components of a single-module NRPS. Next are a short-chain reductase (\( aplF \)) and a putative \( \alpha/\beta \) fold hydrolase/acylase (\( aplG \)). Additionally, downstream of \( aplG \), a putative SARP transcriptional regulator was identified which is not conserved in the other two organisms.

Upstream of the PKS ORFs is a freestanding ACP (\( aplI \)), followed by a putative 2-methylcitrate dehydratase (\( apl2 \)) and a CYP450 (\( apl3 \)). This organization is conserved in \( S. \) rapamycinicus and \( S. \) iranensis, but not in the *Actinoplanes* sp., which is devoid of the dehydratase gene. In all three clusters an \( \alpha/\beta \)-fold hydrolase/acylase is found next to the CYP450. In the \( S. \) rapamycinicus and \( S. \) iranensis clusters, a putative SAM-dependent methyltransferase is encoded next, while in *Actinoplanes* sp. this gene appears to be absent.

\( aplA, aplB \) and \( aplC \) encode the thiotemplate for actinoplactic acid core polyketide.

Analysis of the PKS domain architecture by antiSMASH revealed a 15-module PKS encoded within \( aplA, aplB \) and \( aplC \). The PKS domain composition and extender prediction fits well to the structural properties of the core polyketide (Fig. 3A). As is often the case with modular type 1 PKS, there are several predicted dehydratase (DH) domains within the PKS, which need to be inactive in order to yield the actinoplactic acid structure. Specifically, this is observed with modules 2, 7 and 9. Similarly, the ketoreductase (KR) domain in module 14 is apparently inactive. Beyond module 15, remnants of an additional module can be observed, indicated by the presence of a keto-synthase (KS) and acyltransferase (AT) domain, but in the absence of an acyl carrier protein (ACP) as well as any of the reductive dehydratase (DH) domains within the PKS, which need to be inactive in order to yield the actinoplactic acid structure.

We have introduced a frameshift deletion into the 5’ end of \( S. \) rapamycinicus \( aplA \) gene, and analyzed the mutant cultures by LC-MS. Complete absence of actinoplactic acids was observed (Fig. 3C), while the accumulation of other secondary metabolites remained largely unaffected (Fig. S4A). This confirms that the described PKS system is responsible for the biosynthesis of actinoplactic acids.

**Actinoplactic acid PKS module 14 is promiscuous, incorporating either ethylmanonyl-CoA or methylmalonyl-CoA.**

Isolation of the novel actinoplactic acid C, having a methyl substituent at carbon 4 instead of an ethyl moiety, lead us to assume that there is a level of promiscuity associated with the AT domain of the module 14. Promiscuity of AT domains is frequently observed with various type I PKS products (21). In these cases, the rate of incorporation of a certain extender-CoA moiety largely depends on relative availability of the competing substrates from the cellular pool. Moreover, by feeding of synthetic extender-SNAC analogues in the absence cluster-encoded biosynthesis of suitable extenders, this promiscuity can be fully exploited to dictate exclusive production of polyketide products with the desired substituents (22).

In order to reduce ethylmalonyl-CoA levels in the cellular pool, and measure the consequent relative changes in the amounts of 1 vs. 2 in the cultures, we targeted the crotonyl-CoA reductase (CCR) genes of \( S. \)
**rapamycinicus.** Several highly conserved homologues were found in the genome. Two instances (M271_06415 and M271_13255) were comparatively highly expressed according to qPCR analysis. Sequence analysis of the genomic context revealed that M271_06415 is located in the elaiophylin gene cluster, a biosynthesis pathway known to utilize ethylmalonyl-CoA extender units (14, 23). The second CCR (M271_13255) is positioned next to a putative ethylmalonyl-CoA mutase, which likely makes it a part of the generally conserved ethylmalonyl-CoA (EMC) pathway (24). An M271_06415 in-frame deletion mutant was constructed and cultures analyzed by LC-MS. The levels of extender-CoA in the CCR mutant were not directly measured, however lower relative levels of elaiophylin, and of the recently described rapamycin analogues, the homorapamycins (25), indicated a reduction in ethylmalonyl-CoA cellular pool. Indeed, the ratio of 1 vs. 2 changed from 5:1 in the w.t. strain to 2:1 in the M271_06415 mutant while the sum of both remained at similar levels. When a double M271_06415, M271_13255 mutant was investigated, the change in the ratio was even more pronounced, inverting from 5:1 to 1:5 (Fig. 3D, Fig. S5).

These results imply that the outcome of the APL pathway is dependent on the cellular extender-CoA pool composition. Moreover, with the absence of cluster-encoded CCR, an inter-pathway substrate supply is indicated, not only for actinoplastic acid but also for the rapamycin pathway. The latter is being reflected in reduced production of the homorapamycins, which was imposed by the CCR mutations. Despite this lack of self-sufficiency in ethylmalonyl-CoA supply, actinoplastic acid A is most likely the true end-product of the pathway, since the accumulation of desmethyl congeners in *S. rapamycinicus* seems to be an exception among the known producers of actinoplastic acids (1).

*Cluster-encoded NRPS initiates the first of the post-PKS modifications, the acylation with tricarballylic acid.*

The *aplD* and *aplE* genes are found in the APL cluster immediately downstream of the PKS core genes and are predicted to contain components of a single module NRPS; *aplD* encoding an adenylyating (A) domain and peptidyl carrier protein (PCP), whereas the *aplE* encodes a free-standing condensation (C) domain (Table S1). The coding domains are overlapping, and appear to be organized into a bi-cistronic operon. An in-frame deletion of *aplD* in *S. rapamycinicus* resulted in loss of actinoplastic acid production according to LC-MS analysis. Instead, the bare PKS products 7 and 8 with *m/z* [M+H]+ 635.5 and 621.5 (respectively) were found accumulating in the cultures together with their respective methyl esters, 9 and 10 (Fig. 4A). These intermediates, which again differ in presence of either methyl or ethyl group on carbon 4, were found in the same 5:1 ratio that can be observed between 1 and 2 in the w.t. strain. Identical results were obtained with an *aplE* in-frame deletion mutant (Fig. S6). 9 and 10 readily hydrolyzed to yield 7 and 8 under alkaline conditions (Fig. S7). We were also able to obtain 7 and 8 by alkaline hydrolysis of respective actinoplastic acids 5, 6, 11 and 12. Structures of 7, 8, 9 and 10 were then confirmed by comparative HRMS/MS fragmentation of these different sources (Fig. S7).

The methyl esterification of the PKS backbone is unexpected since no trace of it can be found on any other actinoplastic acid species. These results may imply a pathway non-relevant methylation event, with the acting methyltransferase being selective toward non-acylated PKS intermediates. Significant differences in charge and polarity brought by the acylation could very well support such selectivity. A putative class I SAM-dependent methyltransferase is encoded at the border of the *S. rapamycinicus* APL cluster (*apl5*) and members of this superfamily have been known to catalyze formation of methyl esters (26, 27). Since *apl5* is not conserved in the clusters of all three hosts, we presumed that this gene is unlikely to be of critical importance for the pathway. Therefore, experimental data to support its role, or the role of the observed methylation are not available at this time.

*In trans* complementation of the mutations in *aplD* and *aplE* by chromosomally integrated copy of *aplDE* under control of the strong *ermE* promoter reversed the mutant phenotype back to normal actinoplastic acid production with concomitant near-disappearance of methyl esters (26, 27). Since *apl5* is not conserved in the clusters of all three hosts, we presumed that this gene is unlikely to be of critical importance for the pathway. Therefore, experimental data to support its role, or the role of the observed methylation are not available at this time.

Typically, NRPS and hybrid PKS-NRPS complexes function as chain elongation assembly lines. The growing chain is tethered with the terminal carboxylic group to the ACP/PCP of the module last in operation. This keeps the carboxylic group of the growing chain activated for condensation with the extender, which is waiting, also thioester bound, on the next module in line (Fig. 4C). Thus, the direction of the chain elongation is defined (29). In contrast, the NRPS of the APL pathway has a function in post-PKS ornamenation, acting on positions distal to the terminal carboxylic group of the core polyketide (Fig. 4C). This atypical activity does not require the nascent polypeptide to remain phosphopantetheinyl-bound during the acylation. Significant accumulation of the full-length, free
intermediate in the aplD and aplE mutants indicates that the polyketide backbone can be efficiently released from the PKS without being acylated. Small amounts of 7, 8, 9 and 10 can be also be detected in the w.t. cultures (Fig. 4A). Considering all the above, it is highly likely that AplD/AplE NRPS is acting on the released polyketide intermediate. Based on the domain prediction, the APL PKS seems to lack a release TE domain; instead, one of the conserved hydrolases encoded within the cluster (aplG, apl4) may be involved in this release.

We have attempted to confirm acylation of the free core polyketides 7 and 8 by feeding them to cultures of S. rapamycinicus ΔaplA, harbouring an additionally integrated copy of aplDE. Judging from the complementation experiments described above (Fig. S6), this construct should provide efficient expression of the NRPS. Despite several attempts, which were extended to \textit{in vitro} experiments employing cell-free extracts of the above cultures in presence of various TCA substrates and ATP, we have failed to obtain convincing results. The likely reason is the poor solubility of intermediates 7 and 8 in aqueous environment at mild conditions, which was confirmed by complete absence of 7, 8, 9 and 10 in samples of \textit{in vitro} experiments as well as in intact or disrupted cultures of \textit{S. rapamycinicus AplID}, unless an organic solvent was used for extraction (Fig. S9).

Examples of ester-forming NRPS systems are scarce (30), and the APL pathway therefore adds to a small, yet growing list of secondary metabolite enzymes possessing this activity. Incidentally, the first ester-forming NRPS described, Fum10/Fum14, catalyzes the tricarballylate esterification in the fumonisin pathway (11, 31). Fum14, which encodes a C domain and PCP, has been shown to act on a non-tethered PKS intermediate \textit{in vitro} (11). Recently, another representative of these freestanding, ester-forming C proteins was examined in detail (32). The SgcC5, involved in the biosynthesis of streptomycetal C-1027 enediyne chromophore (33), was also confirmed to act by acylation of a free substrate. Based on phylogenetic relations, the authors propose a new family of ester-forming C enzymes, which constitute a distinctive clade with SgcC5 and possess a signature HHXXXDX_3Y motif in the active site (32). Reusing input data of this study, we have positioned the Fum14 and the AplE homologues into the phylogenetic tree of NRPS C domains. Interestingly, the tricarballylic acid-transferring enzymes do not follow the proposed conserved active site rule or share phylogenetic relations. Instead, these proteins form divergent clades; one containing the Fum14 orthologues and the other the three known AplE orthologues (Fig. 4C, 4D, S8).

\textbf{Origin of the tricarballylic acid.}

In fumonisin biosynthesis, the starting block for the synthesis of the tricarballylate side chains originates from the TCA cycle. A specific mitochondrial exporter, Fum11, is supplying cis-aconitate to the fumonisin pathway (34). The substrate for the NRPS A domain (11), is thereby sequestered from the highly active, reversible mitochondrial aconitase by action of this transporter. An analogous mechanism was proposed for supply of cis-aconitate to the itaconate biosynthesis in \textit{Aspergillus terreus} (35). In bacteria, subcellular compartmentalization is absent, therefore the starting block for the tricarballylate moiety in the APL pathway could be directly supplied from the TCA cycle. Interestingly, just upstream of the actinoplanic acid PKS ORFs in \textit{S. rapamycinicus} genome, we have found a putative methylcitrate dehydratase-encoding gene (MmgE/PrpD superfamily), \textit{apl2}. In addition to methylcitrate, certain bacterial methylcitrate dehydratases can also catalyze dehydration of citrate and isocitrate at somewhat lower rates. In contrast to aconitases they are unable to re-hydrate the cis-aconitate and related nonsaturated compounds (36). Contrary to our suspicions, an in-frame deletion of \textit{apl2} in \textit{S. rapamycinicus} proved non-critical for the APL pathway under various conditions tested (Fig. S10), and was not investigated further.

By drawing parallels to fumonisin biosynthesis, and presuming that cis-aconitate originating from TCA cycle is the starting block for the acylation, it is clear that a saturation step is needed at some point to yield the tricarballylic groups of the actinoplanic acids. Immediately adjacent to the \textit{aplD/aplE} pair, we have located a putative NAD(P)H-dependent short-chain reductase gene, the \textit{aplF}. Similarity search and conserved domain analysis predicted that AplF is most closely related to enoyl-ACP reductases (ER) and 3-oxoacyl-ACP reductases (KR) involved in fatty acid and polyketide biosynthesis. Interestingly, the gene is in opposite orientation to the \textit{aplD/aplE} and therefore transcribed independently (Fig 2).

An in-frame deletion of \textit{aplF} resulted in loss of actinoplanic acid production. Instead, a group of new compounds 11 and 12 were found accumulating, with \textit{m/z} [M+NH_4]^+ 964.5 and 950.5. Detailed investigation with HRMS confirmed molecular formulas \textit{C}_{53}H_{78}O_{16} and \textit{C}_{50}H_{76}O_{16}, revealing that the new compounds are tetradehydro- analogues of 5 and 6 (respectively). More specifically, these compounds have aconitic instead of tricarballylic acid incorporated at both of the expected positions. In total, eight chromatographically separated isomers were found for each, 11 and 12 (Fig. 5A). This abundance of isomers is presumably due to the ability of
the aconitic acid moiety to act as a conjugated system, allowing spontaneous double bond translocation (12). The possible isomers therefore include the geometric and regio isomers for each of the aconit side chains; that is the trans/cis and the 2'-ene/3'-ene translocation. Alkaline hydrolysis of 11 and 12 isomers gave single peaks of 7 and 8, respectively (Fig. S7). This, together with the HRMS data, confirms the structures of 11 and 12, as well as the assumption that the isomerization is occurring on the aconityl moieties. In trans complementation of the mutation with the aplF expressed under control of permE* partially reversed the phenotype as indicated by the presence of actinoplanic acids 1 and 2 in the fermentation cultures. (Fig. S11).

The above results resemble the phenotype obtained when fum7 of the fumonisin biosynthetic pathway is blocked (12) and confirm the reductive function of ApIF. These data are insufficient however, to determine at which point in the post-PKS sequence the reduction of aconitoyl moieties takes place, i.e. on the free aconitic acid, the aconityl-PCP or on 11 and 12. Based on similarity, ApIF can most accurately be described as an enoyl-ACP reductase, thereby requiring a thioester-bound substrate such as acyl-ACP (PCP) or acyl-CoA for activity (37). This excludes the aconityl esters 11 and 12 or the free aconitic acid as likely substrates for the ApIF. It is evident from the results that the ApID/AplE NRPS is able to catalyze the acylation of the actinoplanic polyketide with aconityl moiety in the absence of ApIF activity. Thus, the aconitate must be associated with PCP of the ApID at some point to allow this outcome. Complete loss of acylation observed with the inactivation of ApID (Fig. 4A) confirms the criticality of the A domain and PCP of this protein, thereby offering a clue on the activation mechanism for the free TCA starting block as well as providing a plausible substrate for the ApIF.

Therefore, we propose that the most likely order of events in the actinoplanic acid biosynthesis is as follows: the aconitate is loaded to the PCP of ApID via adenylation, reduced by action of ApIF to form tricarballyl-PCP, which is then used for acylation of the PKS product by ApIE, resulting in 5 and 6 (Fig. 3B). In this scenario, 11 and 12 are only shunt products unique to the apIF mutant and not intermediates in the pathway. A similar conclusion was made for the tetradehydro analogues of fumonisines (12).

Although the mechanism of post-PKS acylation in biosynthesis of fumonisins and actinoplanic acids seems strikingly similar, significant evolutionary distances between the involved pathways suggest a convergent evolution of this biochemistry. In addition to distances observed between the condensation domains of both ester-forming NRPS, this is also reflected in the fact that Fum7 is a member of the iron-containing alcohol dehydrogenases (12), while ApIF is a short-chain reductase/dehydrogenase. Given the rarity of natural products containing tricarballylic acid and the peculiarity of the enzymology, the above is a noteworthy observation.

**CYP450 monoxygenase Apl3 makes the first step toward the lactonized actinoplanic acids.**

A putative P450 hydroxylase gene, apl3, was found upstream of the core PKS genes. The genetic organization suggests an independently transcribed gene, lacking the often adjacentally encoded ferredoxin. In-frame deletion of apl3 resulted in complete absence of actinoplanic acids 1, 2, 3 and 4, with concomitant appearance of actinoplanic acids B and D (5 and 6), indicated by separate peaks with m/z [M+NH₄⁺] 968.5 and 954.5 (respectively) in the usual 5:1 ratio (Fig. 5B). HRMS confirmed the identity of 5 and 6 while their alkaline hydrolysis yielded 7 and 8 (Fig. S7). Complementation of the mutation with chromosomally integrated apl3 under the control of permE* resulted in partial reversion of the phenotype (Fig. S12). The formation of the hydroxyl group of carbon 36, and subsequently, the ability to form lactonized species of actinoplanic acids is therefore dependent of the function of this gene.

The order of events in the pathway is indicated by the absence of core polyketide intermediates hydroxylated at position 36 in the cultures of aplID deletion mutant, suggesting that the hydroxylation is carried out only after the acylation of the PKS backbone. In other words, the hydroxyl group must be introduced only after the formation of 5 and 6. Given the structural similarity of these compounds with 11 and 12, which are accumulating in the aplF mutant, it is surprising that the latter are not hydroxylated further by the action of Apl3. It is unlikely that the apIF mutation would have a polar effect on ap3 expression since the genes are located at the opposite sides of the gene cluster (Fig. 2). Indeed, reappearance of actinoplanic acids 1 and 2 upon in trans complementation of the apIF mutation (Fig. S11), confirms that Apl3 is active in this mutant. Considering all the above, it is possible that the structural differences imposed by the aconityl side chains, albeit relatively distal to the hydroxylation position, are the cause for the inability of 11 and 12 to serve as a substrate for Apl3.

Hydroxylated, open-chain derivatives 3 and 4, which were found in the culture broth of w.t. *S. rapamycinicus* in significantly higher amount compared to 5 and 6 (Fig. 5B), indicate that lactonization rather than hydroxylation is the limiting step in the pathway. In
contrast, *Actinoplanes* sp. MA 7066 was reported to produce a mixture of actinoplanic acids A and B (1 and 5) while the *Streptomyces* sp. MA7099 produced exclusively the actinoplanic acid B under the conditions used in the study by Singh *et al.* (3). It is not clear at this time how the closing of the double-lactone ring occurs; however, it is plausible that one of the conserved, cluster-encoded α/β fold hydrolases/acylases (AplG or Apl4) has a role in this reaction.

**APL gene cluster is co-localized with the rapamycin cluster.**

In the genomic context, the APL cluster is located adjacently to the rapamycin gene cluster in *S. rapamycinicus* (Fig. 2). Although regions condensed with secondary metabolite gene clusters outside of the core of the genome are not unusual in actinomycetes, the immediate proximity of the two clusters may have a more intimate evolutionary, and therefore also a functional background (38). Making genome-wide searches across all major publically available bacterial genome databases (NCBI, Broad institute, Joint Genome Institute), we were only able to find relevant similarity hits for the APL pathway in the genomes of the three known rapamycin producers (13). Vice versa, despite the increasing number of sequenced actinobacterial genomes, the *S. rapamycinicus* ATCC 29253, *S. iranensis* HM 35 and *Actinoplanes* sp. N902-109 remain the only repositories of the rapamycin pathway sequenced to date. In this respect, it would be interesting to examine the genomes of the original actinoplanic acid producers, *Streptomyces* sp. MA7099 and *Actinoplanes* sp. MA7066 (1), however these have not been sequenced yet. As in *S. rapamycinicus*, the APL and rapamycin gene clusters are also found in immediate proximity in the *Actinoplanes* sp. genome, but not in *S. iranensis* where the two clusters seem to be located at the opposite arms of the chromosome. In light of all the above, we wondered if perhaps the conserved co-localization of the pathways was more than a mere coincidence. Based both on the gene topology and similarity results, we have made synteny analysis between genome loci harboring the rapamycin and APL clusters of the three organisms. While both clusters are conserved at least in their essential functionality, we have found significant difference in genetic organization of the regions surrounding the PKS genes (Fig 2). Clearly, multiple recombination events had to occur in each of the hosts from the presumably common, ancient genetic origin, to come to this situation. This leads one to consider a possible evolutionary co-dependence of the products of both pathways.

One indication of such co-dependence would be an intertwined regulation of expression of the two clusters in either synchronous or contingency mode. To study these connections, we have targeted the putative transcriptional regulators of both pathways. In-frame deletion of the SARP gene found at the flank of the APL cluster of *S. rapamycinicus*, the aplR, had no influence on the production of actinoplanic acids or rapamycin compared to the w.t. strain (Fig. S4C). On the other hand, deletion of the rapG and rapH genes, the regulators of the rapamycin pathway (39), resulted in complete loss of rapamycin production but had no influence on the levels of actinoplanic acids (Fig. S4B). Thus, the expression control of the APL pathway, as well as any regulatory relations with the rapamycin pathway remain unresolved.

Nevertheless, under the cultivating conditions used in this study, the production of rapamycin and actinoplanic acids seems to be temporally coordinated (Fig. S13). Therefore, we have examined a potential functional synergy of the two compounds. Since both rapamycin and actinoplanic acids have been shown to be active against eukaryotic targets, the TOR complexes (40) and Ras farnesyl-protein transferase (2), respectively, antifungal assays were chosen for the purpose at hand.

**Actinoplanic acid A and rapamycin show strong synergism in antifungal activity.**

We selected three diverse fungal species, *Aspergillus fumigatus*, *Candida albicans*, and *Rhizopus oryzae* for growth inhibition assays with rapamycin and actinoplanic acid A (1), the major end-product of the APL pathway. The concentrations of the two compounds in the assays were kept well within the biosynthetic potential of *S. rapamycinicus* ATCC 29253, which accumulates rapamycin and 1 at ~55 µM and ~120 µM, respectively.

Fungal cells were embedded in agar medium along with sub-inhibitory concentrations of 1. When the agar solidified, rapamycin solution was spotted on top. After incubation, the growth inhibition was observed in the form of clear areas in the well. As expected, a growth inhibition activity of rapamycin (40) was observed. This activity was significantly enhanced with the addition of sub-inhibitory amounts of 1 (Fig. 6A).

To ensure that the observed synergistic activity is not due to an artifact arising from the order of compound addition, the *R. oryzae* test was repeated. This time the cells were first embedded into the agar in presence of rapamycin, and 1 was then spotted on top. This experiment resulted in similar inhibition patterns. In addition, concentration levels of both compounds were adjusted to reflect maximal sub-inhibitory concentrations within the assay. While both 1 and 7...
rapamycin failed to show a clear inhibitory effect by their own, the wells with inclusion of as little as 0.32 nM of rapamycin in the agar resulted in clear inhibition zones providing that I was present (Fig. 6B). It should be noted, that a difference in potency of actinoplanic acid species was reported before (3). This may also hold true for the synergistic activity with rapamycin, but remains to be tested.

To gain further insight into the effects underlying the growth inhibition observed with the initial assays, R. oryzae was grown in chamber slides in potato dextrose broth (PDB), supplemented with various concentrations of rapamycin and I (Fig. 6C). In agreement with the results observed in the agar tests, I alone, even at the highest concentration tested (10 µM), had no apparent antifungal activity. Rapamycin on the other hand did show partial inhibition of conidia germination, but only at the highest concentration tested (25 nM). Strikingly, combining 1 nM rapamycin with 2 µM of I resulted in close to full inhibition of R. oryzae spore germination. Again, the results demonstrate a clear synergistic effect of these two compounds against fungal growth. The morphological state of the exposed cultures suggests a developmental arrest; the synergy could therefore lie in the enhancement of rapamycin’s mode of action. The latter is also supported by the apparent absence of antifungal activity of actinoplanic acid A alone, even at relatively high concentrations (Fig 6).

Antifungal activity of rapamycin is due to inhibition of cellular signaling through TOR Complex 1 (TORC1), thereby interfering with cell cycle progression, growth and development, and with general synthesis of cellular components. Specifically, rapamycin arrests fungal cell cycle at the G1 phase (40). While the role of the TORC1 has been investigated in relative detail, the understanding of its rapamycin-insensitive counterpart, the TORC2, is relatively poor (41). In an intertwined upstream and downstream signaling network, the two complexes coordinate processes such as protein synthesis, ribosome biogenesis, transcription, cell-cycle progression, cytoskeleton organization, stress response and autophagy (42). Despite the wide conservation of the basic functionality throughout eukaryotes, the composition of the TOR-centered signaling network may differ significantly between various organisms (43, 44). Still, most signaling pathways upstream of the TOR complexes involve one or more small, farnesylated GTPases of the Ras superfamily. Most notably, the small G proteins Rheb, Rab, Ras and others have been found involved with the TOR activation in various organisms (45, 46).

Unlike the zaragozic acids, which also feature FTI activity and a tricarboxylic structural moiety (47), actinoplanic acids were found inert toward geranylgeranyl-pyrophosphate synthase and squalene synthase (1). Notwithstanding the possibility of alternative mode of action, it is tempting to speculate on the plausible role of actinoplanic acids as potent and selective farnesyl-protein transferase inhibitors (FTI, 1), thereby escalating the effects of rapamycin. Indeed, mutations in TOR-activating GTPases RhbI and GtrI in C. albicans, have been shown to induce hypersensitivity to rapamycin (48, 49). In addition, synergistic anti-proliferative activity of an FTI and rapamycin has been found in several mammalian cancer cell lines (50, 51, 52). Again, this was postulated to be due to inhibition of the Rheb GTPase, which is normally directly activating the TORC1 (46). Subsequently, several combination therapies with a rapamycin-derived drug and an FTI have been subjected to clinical testing recently (53, Table S2).

Whichever target of actinoplanic acid may prove to be valid, the mechanism of the synergetic activity with rapamycin is worth of further investigation since the signaling network involving both TOR complexes is of principal interest to cell biologists and is a recognized target of clinical value.

Conclusions

Even well studied organisms such as S. rapamycinicus still hold surprises to be unveiled; in our case, the biosynthesis of actinoplanic acid and its novel analogues that lay undiscovered for over two decades. These findings led to the identification of the actinoplanic acid biosynthetic gene cluster and elucidation of the pathway sequence and biochemistry. In addition, we have provided insight into the origin of the structural diversity among the actinoplanic acid species. An important motivator for our efforts was the rare biochemistry involved with the tricarballylate group incorporated into the actinoplanic acids, the only known bacterial compounds to possess such a moiety. Our work provides an outline of the second example of this biochemistry in addition to the well-examined fungal fumonisins. The core of the pathway is comprised of a hybrid PKS-NRPS system, which is perhaps counter-intuitive at a first glance due to the absence of amide bonds in the actinoplanic acid structure. Interestingly, the divergent NRPS catalyzes the formation of esters between the tricarballylic moieties and the unmodified product of the PKS. It is surprising to see that despite the clear evolutionary distances and rarity of the chemistry involved, there is a close resemblance between the fumonisin and actinoplanic acid pathways, suggesting convergent evolution of the two systems.

Furthermore, the evolutionary connection reflected in the conserved cohabitation of biosynthetic clusters for
rapamycin and actinoplanic acids invited us to explore the synergism between two secondary metabolites produced by a single organism. It is increasingly evident that in their natural environment secondary metabolites produced by one organism often act in unison against multiple targets on the same foe, and so one of the paths toward network pharmacology (54) may lead from this unexpected source. Rapamycin, first isolated for its relatively weak anti-fungal properties, can now perhaps be better appreciated in the context of its partner, actinoplanic acid A as a potent “one-two punch” anti-fungal assault. This combination attack is of course not unique. One of the most widely used anti-infectives today for example, the amoxicillin-clavulanic acid combination antibiotic drug (55), has its ancient blueprint in nature. The genome of the producer of clavulanic acid, the actinomycete *Streptomyces clavuligerus*, encodes adjacent, co-regulated biosynthetic gene clusters for a β-lactam antibiotic, β-lactamase inhibitor pair: the cephapramycin C and clavulanic acid (56). This genetically imprinted functional synergy of the two secondary metabolites makes perfect sense, both in evolutionary and ecological aspect (38), as it increases the potency of the attack and therefore gives a competitive advantage to the host (Fig. 7A).

Despite the huge diversity in chemical space that the natural products represent, the repetitive use of similar search strategies has led to redundancy and a decline in the number of fundamentally novel therapies (57, 58). One plausible reason is that, restricted by the single-compound therapy paradigm, we have largely neglected the evolutionary events resulting in genetically conserved co-harboring of natural product pathways, their co-production and the teachings hidden therein. The number of known synergistic relationships (38, 59) is surprisingly small, considering the thousands of discovered actinobacterial NP (60) and the enormous arsenal of these compounds revealed by the rapid development of (meta)genomics. Nevertheless, increased interest in synergistic activity of natural products has already resulted in several successful attempts in systematic discovery of such NP pairs (59, 61, 62, 63). Many of these approaches are based on functional profiling of NP, however few exploit evolutionary origins or ecological roles in their strategy. This is surprising, since the sequence data of NP-producer genomes is one of the most widely accessible and rich resources. Unfortunately, the existing bioinformatic algorithms (64) process the secondary metabolite pathways in an isolated manner, leaving the evolutionary conservation hidden in plain sight.

Thus, it may be possible to extend the search for such synergies into a wider scope, with suitably adapted and systematic genome mining (Fig. 7B). Incidentally, this could prove to be the only practical way to address the opportunities presented by synergistic natural products when considering the enormous arsenal of these compounds revealed by the rapid development of (meta)genomics in the recent years.

**Experimental procedures**

**Strains and media**

*S. rapamycinicus* ATCC 29253 (*S. hygroscopicus* subsp. *hygroscopicus* NRRL 5491) (65) and *S. rapamycinicus* R073, a rapamycin producer isolated from ATCC 29253, were used in this study. R073 and ATCC 29253 share 99.1% nucleotide and 98.7% CDS AA sequence identity over the rapamycin and APL pathway region. All manipulation on solid media was done with the TAA4 agar plates (Table S3), incubated at 28 °C for 9 days. Where appropriate, apramycin (60 mg/L) and theophylline (4 mM) were supplemented to the medium. Spores obtained from ~1 cm² of the confluent, 9 days old TAA4 agar plate culture were used as an inoculum for the shake flask fermentation. Cultures were incubated on an orbital shaker in 100 mL flasks containing 15 mL of RAP-1P medium (Table S4) at 28 °C and 260 rpm (2.5 cm throw) for 7 days. At this time, the cultures were sampled for analysis of secondary metabolites. At least three independently isolated strains were used for the phenotype characterization of genetic modifications in all cases. The sampling was performed by weighing 3 g of the culture broth diluted with 6 mL of acetonitrile:water (2:1) mixture. The samples were immediately centrifuged for 10 min at 15000 x g and 4°C. The cleared supernatants were decanted into a fresh vial and stored at -75 °C until the analysis.

**Genome sequencing, in silico analysis and design**

Genomic DNA was extracted from early stationary phase cultures of *S. rapamycinicus* ATCC 29253 using the Nicodinovic method (66). Genome sequencing was performed on the PacBio Sequel platform with sequencing library preparation performed using the SMRTbell template prep kit and SequelTM Binding Kit 2.0. Sequence reads were de novo assembled using HGAP2 software (Pacific Biosciences, Menlo Park, CA USA). Gene calling was performed using Prodigal software (67) and natural products pathways were annotated using antiSMASH 3.0 (16). Analysis of the biosynthetic genes was performed using BLAST algorithms (68) and CLUSTAL Omega (69). Genetic design of constructs and sgRNAs were performed using Geneious software (Biomatters LTD., Auckland, NZ).
MUSCLE (70) was also used for multiple sequence alignment. Phylogenetic trees were built using FastTree 2 (71), incorporated in the Geneious. The newly sequenced genome of *S. rapamycinicus* ATCC 29253 is deposited under GeneBank accession number QYCY00000000.

**Molecular methods**

CRISPR genome editing was carried out with an in-house-developed, single-plasmid-based approach, using pREP_P1_w.t.cas9 (Fig. S14). The plasmid backbone is derived from the unstable replicon, originating from pJTU412 (72) and in addition contains transfer functions *(traJ, oriT)* and an apramycin resistance marker *(aac(3)-IV)* from pSET152 (73). The *S. pyogenes* cas9 (GeneBank: NC_002737.2, Locus tag: SPy_1046) was codon optimized for expression in *Streptomyces* and put under control of the *ermE* promoter, which was fused to a theophylline riboswitch (74). Single guide RNA design (17) was expressed from a gene with a synthetic P21 promoter (75), and the native *S. pyogenes* tracrRNA terminator (76). Guide RNAs were designed for target homing using the integrated function of Geneious R8 (Geneious 8.1.9, Biomatters, 2016) by crosschecking for off-target potential within the genome of *S. rapamycinicus* as well as the genome of the transient host, *E. coli* ET12567. The editing template was delivered on the same plasmid and typically included 500 bp to 1000 bp long homology flanks to support double strand break repair by homologous recombination. Both the editing template and the sgRNA gene were obtained synthetically from Genewiz, USA. The plasmid was delivered to the host by conjugal transfer (77), and the plasmid was selected for with 60 mg/L apramycin. Exoconjugants where then sub-plated to agar plates containing theophylline (4 mM) to induce expression of the Cas9. After incubation, colonies were transferred onto plates without antibiotic pressure and tested for the loss of apramycin resistance. Details for construction of gene inactivation CRISPR constructs are available in Table S5. Details on PCR confirmation of genotypes are listed in Fig. S15 and Table S6. Complementation experiments were performed by standard methods using the integrative pSET152 plasmid (73), with genes of interest under control of the strong *ermE* promoter *(permE)*. Complementation vectors were assembled by taking fragments from the following genomic positions, according to the deposited *S. rapamycinicus* genome, GeneBank accession number CP006567.1: *aplDE*, 3155 bp (878447 - 881601); *apl3*, 1891 bp (9921254 - 9923144); *aplF*, 930 bp (10010246 - 10011175).

**Purification and structure elucidation of the actinoplanic acids**

After 7 days of fermentation, the pH of the broth was lowered to 2.5 using hydrochloric acid. 200 g/L of Na₂SO₄ was added in order to facilitate extraction of fermentation products with ethyl-acetate. Whole-broth extraction with 2 vol. of ethyl-acetate was repeated 4 times. Ethyl-acetate phases were pooled, dried over MgSO₄ and filtered. Evaporation of the solvents yielded a crude product in the form of yellow-brown oil. For purposes of structure confirmation, compounds were further purified using preparative reverse phase (C-18) HPLC. This purification yielded in 23.8 mg Actinoplanic acid A (1) and 8.2 mg Actinoplanic acid C (2). From a 100 L fermenter, additionally 43.2 mg of 3 could be isolated. The structure of 1, 2 and 3 was determined using 1D and 2D-NMR spectroscopy and comparison of the NMR data with the published data of Actinoplanic acid A (2). NMR spectra were recorded on a Bruker Avance 600MHz instrument (Bruker, USA) with samples dissolved in d₆-DMSO. Alkaline hydrolysis of 9 and 10 was performed by dissolving the extract of the *aplD* deletion strain in DMSO. 100 μL of 5M NaOH was added to 600 μL of the DMSO solution to give a pH between 13.5 and 14. The mixture was incubated for 3 h at RT, and then neutralized with HCl. For hydrolysis of 1, 2, 3, 4, 5, 6, 11 and 12, the fermentation broth of appropriate strains was extracted with 2 vol. of methanol. 200 μL of Ca(OH)₂ suspension (300 g/L in methanol) was added to 600 μL of clarified supernatant. The mixture was incubated for 3 h at 50 °C and then clarified by centrifugation.

**UPLC-MS and HRMS analytics**

Routine analysis of compounds in *S. rapamycinicus* cultures was performed by UPLC-MS. Clarified sample supernatant (5 μL) was injected onto an Agilent Poroshell 120 EC – C18 column (150 x 2.1 mm, 2.7 μm) with a flow rate of 0.9 mL/min, column temperature 60 °C. Mobile phase A (pH 4.8): 10 mM ammonium formiate; 0.1% formic acid; 2% acetonitrile (v/v) and mobile phase B: 80% acetonitrile;15% methanol (v/v), were used in the gradient profile. The method was initiated at 50% mobile phase B for the first 5 min and then progressed linearly to 73% in the next 45 min, with re-equilibration to starting conditions for 3 min. MS detection was performed with an LCQ iontrap mass spectrometer (ThermoFischer, USA) equipped with an ESI source after 1:1 split of the flow. Positive ionization (source voltage 5 kV, capillary temperature 250 °C, sheath gas 35 AU, auxiliary gas 5 AU) and full-scan monitoring with m/z range 300-1000 allowed detection of the compounds, primarily as proton [M+H]⁺ and ammonium...
adducts [M+NH₄]⁺. UV diode array multi-wavelength detection was performed in parallel. High-resolution mass spectrometry (HRMS) was performed on a Q-Exactive quadrupole/orbitrap accurate mass spectrometer (ThermoFischer, USA), using the same chromatographic and ionization conditions described above. The instrument was calibrated with Pierce™ LTQ Velos ESI Positive Ion Calibration Solution (ThermoFischer, USA) prior to the measurements. The rapamycin peak was used as internal standard to check the accuracy of the instrument and was within ± 0.3 ppm in all cases.

Antifungal assays

Aspergillus fumigatus ATCC MYA-3627, Candida albicans ATCC 24433, and Rhizopus oryzae ATCC MYA-4621 were used in the antifungal assays. Conidia of A. fumigatus and R. oryzae (both harvested from 5 days old Potato Dextrose Agar plate) were used as inoculum for the assays. In the case of C. albicans, a liquid culture with ~5x10⁶ cells/mL was used. Fungal cells (~1x10⁵) were embedded into 1 mL of Difco Potato Dextrose Agar (PDA, Becton, Dickinson and Company, USA) together with a DMSO solution of rapamycin and/or actinoplanic acid A. After solidifying, 2 µL of DMSO solution of the remaining compound was spotted on top of the agar. The plates were incubated at 35 °C for 24 h. Liquid culture experiments with R. oryzae were performed in chamber slides filled with 0.1 mL of Difco Potato Dextrose Broth (PDB, Becton, Dickinson and Company, USA) supplemented with the appropriate concentration of rapamycin and/or actinoplanic acid solution. The chambers were inoculated with ~1x10⁵ R. oryzae conidia and incubated for 16 h at 35 °C. Microphotographs were produced with an Olympus IX81 (Olympus, USA) inverted microscope, using phase contrast light source and the 20X objective lens.

Acknowledgments

The authors thank Tjaša Drčar and Eva Knapič for support with culture cultivation; Matej Ošlaj and Marko Trebušak and Nina Pirher for assistance with the LC-MS analytics; Monika Kozlevčar and Bernarda Skok for technical assistance with genome editing; and Jerome Cluzeau for advising methods for hydrolysis of actinoplanic acids. We also thank Yue Fu for support with the antifungal assays as well as to Lukas Oberer, Thomas Lochmann and Trixie Wagner for their support in NMR analytics. We thank Edward Oakley, Ulrike Naumann and Robert Brucoleri for support with sequencing and assembly. We thank Frank Peterson for useful comments on this work. A special gratitude goes to Gregor Kopitar for his enthusiastic and long-standing support in every endeavor we have undertaken.

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Authors contributions

P.M and C.M.M conceived and designed the study; P.M. and C.M.M analyzed and interpreted bioinformatic data; P.M. and P.P.L designed and prepared the mutant strains; P.M and P.K analyzed and interpreted the phenotypes and compound structures; D.P and J.T. designed, performed and analyzed the antifungal assays; P.M. and C.M.M. wrote the manuscript; P.M., P.K., P.P.L, J.T., D.P. and C.M. M. critically reviewed the study.

References

1. Silverman, K. C., Cascales, C., Genilloud, O., Sigmund, J. M., Gartner, S. E., Koch, G. E., Gagliardi, M. M., Heimbuch, B. K., Nallin-Omstead, M., Sanchez, M., Diez, M. T., Matrin, I., Garrity, G. M., Hirsch, C. F., Gibbs, J. B., Singh, S. B., and Lingham, R. B. (1995) Actinoplanic acids A and B as novel inhibitors of farnesyl-protein transferase. Appl. Microbiol. Biotechnol. 43(4), 610-616. doi:10.1007/BF001647
2. Singh, S. B., Liesch, J. M., Lingham, R. B., Goetz, M. A., and Gibbs, J. B. (1994) Actinoplanic Acid A: A Macrocyclic Polycarboxylic Acid Which Is a Potent Inhibitor of Ras Farnesyl-Protein Transferase. J. Am. Chem. Soc., 116(25), 11606-11607. doi:10.1021/ja00104a066
3. Singh, S. B., Liesch, J. M., Lingham, B. R., Sileverman, K. C., Sigmund, J. M., and Goetz, M. A. (1995) Structure, Chemistry, and Biology of Actinoplanic Acids: Potent Inhibitors of Ras Farnesyl-Protein Transferase. J. Org. Chem., 60, 7896-7901. doi:10.1021/jo00129a033
4. Saxena, N., Lahiri, S. S., Hambarde, S., and Tripathi, R. P. (2008) RAS: target for cancer therapy. Cancer Invest, 26(9), 948-955. doi:10.1080/0735790802087275
5. Wang, J., Yao, X., and Huang, J. (2017) New tricks for human farnesyltransferase inhibitor:. Med. Chem. Commun.(8), 841-54. doi: 10.1039/c7md00030h
6. Moorthy, N. S., Sousa, S. F., Ramos, M. J., and Fernandes, P. A. (2013) Farnesyltransferase inhibitors: a comprehensive review based on quantitative structural analysis. *Curr. Med. Chem.*, 20(38), 4888-4923. doi:10.2174/0929867313206660262

7. Deepa, N., and Sreenivasa, M. Y. (2017) Fumonisins: A Review on its Global Occurrence, Epidemiology, Toxicity and Detection. *J. Vet. Med. Res.*, 4(6), 1093.

8. Proctor, R. H., Desjardins, A. E., Plattner, R. D., and Hohn, T. M. (1999) A polyketide synthase gene required for biosynthesis of fumonisin mycotoxins in Gibberella fujikuroi mating population A. *Fungal Genet. Biol.*, 27(1), 100-112. doi:10.1006/fgbi.1999.1141

9. Proctor, R. H., Brown, D. W., Plattner, R. D., and Desjardins, A. E. (2003) Co-expression of 15 contiguous genes delineates a fumonisin biosynthetic gene cluster in Gibberella moniliformis. *Fungal Genet. Biol.*, 38(2), 237-249. doi:10.1016/S1087-1845(02)00525-X

10. Gerber, R., Lou, L., and Du, L. (2009) A PLP-Dependent Polyketide Chain Releasing Mechanism in the Biosynthesis of Mycotoxin Fumonisins. *J. Am. Chem. Soc.*, 131(9), 3148-3149. doi:10.1021/ja8091054

11. Zaleta-Rivera, K., Xu, C., Butchko, R. A., Proctor, R. H., Hidalgo-Lara, M. E., Raza, A., Dessault, P. H., and Du, L. (2006) A bidomain nonribosomal peptide synthetase encoded by FUM14 catalyzes the formation of tricarballylic esters in the biosynthesis of fumonisins. *Biochemistry*, 45(8), 2561-2569. doi:10.1021/bi052085s

12. Li, Y., Lou, L., Cerny, R. L., Butchko, R. A., Proctor, R. H., Shen, Y., and Du, L. (2013) Tricarballylic ester formation during biosynthesis of fumonisin mycotoxins in *Fusarium verticillioides*. *Mycology*, 4(4), 179-186. doi:10.1080/21501203.2013.874540

13. Yoo, J. H., Kim, H., Park, S. R., and Yoon, Y. J. (2017) An overview of rapamycin: from discovery to future perspectives. *J. Ind. Microbiol. Biotechnol.*, 44, 537-553. doi:10.1007/s10295-016-1834-7

14. Fang, A., Wong, G. K., and Demain, A. L. (2000) Enhancement of the antifungal activity of rapamycin by the coproduced elaiophylin and nigericin. *J. Antimicrob. Chemother.*, 45(5), 158-162. doi:10.1094/ ДибиотиСнот.53.158

15. Baranasic, D., Gacesa, R., Stravecic, A., Zucko, J., Blažič, M., Horvat, M., Gjuračić, K., Fuis, Š., Hranueli, D., and Petković, H. (2013) Draft Genome Sequence of *Streptomyces rapamycinicus* Strain NRRL 5491, the Producer of the Immunosuppressant Rapamycin. *Genome Announc.*, 1(4), e00581-13. doi:10.1128/genomeA.00581-13

16. Ran, F. A., Hsu, P. D., Wright, P., Agarwala, V., Scott, D. A., and Zhang, F. (2013) Genome engineering using the CRISPR-Cas9 system. *Nature Protocols*, 8, 2281-2308. doi:10.1038/nprot.2013.143

17. Weber, T., Blin, K., Deddela, S., Krug, D., Kim, H. U., Brucoleri, R., Lee, S. Y., Fischbach, M. A., Mueller, R., Wohlleben, W., Breitling, R., Takano, E., and Medema, M. H. (2015) antiSMASH 3.0—a comprehensive resource for the genome mining of biosynthetic gene clusters. *Nucleic Acids Research*, 43(W1), W237-W243. doi:10.1093/nar/gkv437

18. Horn, F., Schroeckh, V., Netzker, T., Guthke, R., Brakhage, A. A., and Linde, J. (2014) Draft Genome Sequence of *Streptomyces iranensis*. *Genome Announc.*, 2(4), e00616-14. doi:10.1128/genomeA.00616-14

19. Huang, H., Ren, S. X., Yang, S., and Hu, H. F. (2015) Comparative analysis of rapamycin biosynthesis clusters between *Actinoplanes sp.* N902-109 and *Streptomyces hygroscopicus* ATCC29253. *Chin. J. Nat. Med.*, 13(2), 60012-60017. doi:10.1016/S1875-5364(15)60012-7

20. Bisang, C., Long, P. F., Cortes, J., Westcott, J., Crosby, J., Mantharu, A. L., Cox, R. J., Simpson, T. J., Staunton, J., and Leadley, P. F. (1999) A chain initiation factor common to both modular and aromatic polyketide syntheses. *Nature*, 401(6752), 502-505. doi:10.1038/46829

21. Ladner, C. C., and Williams, G. J. (2016) Harnessing natural product assembly lines: structure, promiscuity, and engineering. *J. Ind. Microbiol. Biotechnol.*, 43(6), 371-387. doi:10.1007/s10295-015-1704-8

22. Kosec, G., Goranovič, D., Mrak, P., Fuis, S., Kusčer, E., Horvat, J., Kopitar, G., and Petković, H. (2012) Novel chemobiosynthetic approach for exclusive production of FK506. *Metab. Eng.*, 14(1), 39-46. doi:10.1016/j.ymben.2011.11.003

23. Haydock, S. F., Mironenko, T., Ghoorahoo, H. I., and Leadley, P. F. (2004) The putative elaiophylin biosynthetic gene cluster in *Streptomyces sp.* DSM4137 is adjacent to genes encoding adenosylcobalamin-dependent methylmalonyl CoA mutase and to genes for synthesis of cobalamin. *J. Biotechnol.*, 113(1-3), 55-68. doi:10.1016/j.jbiotec.2004.03.022

12
24. Blažič, M., Kosec, G., Baebler, Š., Gruden, K., and Petković, H. (2015) Roles of the crotonyl-CoA carboxylase/reductase homologues in acetate assimilation and biosynthesis of immunosuppressant FK506 in Streptomyces tsukubaensis. Microb. Cell. Fact., 14(164). doi:10.1186/s12934-015-0352-z

25. Kong, F., Zhu, T., Yu, K., Pagano, T. G., Desai, P., Radebaugh, G., and Fawzi, M. (2011) Isolation and Structure of Homotemsirolimuses A, B, and C. J. Nat. Prod., 74(4), 547-553. doi:10.1021/np1003388

26. Petromikolou, N., and Nair, S. K. (2015) Biochemical Studies of Mycobacterial Fatty Acid Methyltransferase: A Catalyst for the Enzymatic Production of Biodiesel. Chem. Biol., 22(11), 1480-1490. doi:10.1016/j.chembiol.2015.09.011

27. Seo, H. S., Song, J. T., Cheong, J. J., Lee, Y. H., Lee, Y. W., Hwang, I., Lee, J.S., and Choi, Y. D. (2001) Jasmonic acid carboxyl methyltransferase: a key enzyme for jasmonate-regulated plant responses. Proc. Natl. Acad. Sci. U.S.A., 98(8), 4788-4793.

28. Struck, A. W., Thompson, M. L., Wong, L. S., and Micklefield, J. (2012) S-adenosyl-methionine-dependent methyltransferases: highly versatile enzymes in biocatalysis, biosynthesis and other biotechnological applications. Chembiochem, 13(18), 2642-2655. doi:10.1002/cbic.201200556

29. Conduro, H. L., and Bruner, S. D. (2012) Structure and noncanonical chemistry of nonribosomal peptide biosynthetic machinery. Nat. Prod. Rep., 29(10), 1099-1110. doi:10.1039/c2np20023f

30. Bloudoff, K., and Schmenig, T. M. (2017) Structural and functional aspects of the nonribosomal peptide synthetase condensation domain superfamily: discovery, dissection and diversity. Biochim. Biophys. Acta., 1865(11 Pt B), 1587-1604. doi:10.1016/j.bbapap.2017.05.010

31. Du, L., and Lou, L. (2010) PKS and NRPS release mechanisms. Nat. Prod. Rep., 27(2), 255-278. doi:10.1039/b912037h.

32. Chang, C., Lohman, J. R., Huang, T., Michalska, K., Bigelow, L., Rudolf, J. D., Jedrzejczak, R., Yan, X., Ma, M., Babnigg, G., Joachimiak, A., Phillips, G. N., and Shen, B. (2018) Structural Insights into the Free-standing Condensation Enzyme SgcC5 Catalyzing Ester Bond Formation in the Biosynthesis of the Enediyne Antitumor Antibiotic C-1027. Biochemistry, 57(23), 3278-88. doi:10.1021/acs.biochem.8b00174

33. Lin, S., Van Lanen, S. G., and Shen, B. (2009) A free-standing condensation enzyme catalyzing ester bond formation in C-1027 biosynthesis. Proc. Natl. Acad. Sci. U.S.A., 106(11), 4183–8. doi:10.1073/pnas.0808880106

34. Butchko, R. A., Plattner, R. D., and Proctor, R. H. (2006) Deletion analysis of FUM genes involved in tricarballylic ester formation during fumonisin biosynthesis. Butchko, R. A., Plattner, R. D., and Proctor, R. H. (2006) Deletion analysis of FUM genes involved in tricarballylic ester formation during fumonisin biosynthesis. J. Agric. Food. Chem., 54(25), 9398-9404. doi:10.1021/jf0617869

35. Jaklitsch, W. M., Kubicek, C. P., and Scrutton, M. C. (1991) The subcellular organization of itaconate biosynthesis in Aspergillus terreus. Microbiology, 137, 533-539. doi:10.1099/00221287-137-3-533

36. Blank, L., Green, J., and Guest, J. R. (2002) AcnC of Escherichia coli is a 2-methylcitrate dehydratase (PrpD) that can use citrate and isocitrate as substrates. Microbiology, 148(Pt 1), 133-146. doi:10.1099/00221287-148-1-133

37. Massengue-Tiasse, R. P., and Cronan, J. E. (2009) Diversity in Enoyl-Acyl Carrier Protein Reductases. Cell. Mol. Life Sci., 66(9), 1507. doi:10.1007/s00018-009-8704-7

38. Challis, G. L., and Hopwood, D. A. (2003) Synergy and contingency as driving forces for the evolution of multiple secondary metabolite production by Streptomyces species. Proc. Natl. Acad. Sci. U.S.A., 100(Suppl 2), 14555-14561. doi:10.1073/pnas.1934677100

39. Kuščer, E., Coates, N., Challis, I., Gregory, M., Wilkinson, B., Sheridan, R., and Petković, H. (2007) Roles of rapH and rapG in positive regulation of rapamycin biosynthesis in Streptomyces hygroscopicus. J Bacteriol., 189(13), 4756-63. doi:10.1128/JB.00129-07

40. Heitman, J., Movva, N. R., and Hall, M. N. (1991) Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. Science, 253(5022), 905-9. doi:10.1126/science.1715094

41. Gaubitz, C., Prouteu, M., Kusmider, B., and Loewith, R. (2016) TORC2 Structure and Function. Trends Biochem. Sci., 41(6), 532-545. doi:https://doi.org/10.1016/j.tibs.2016.04.001

42. Gonzales, S., and Rallis, C. (2017) The TOR Signaling Pathway in Spatial and Temporal Control of Cell Size and Growth. Front.Cell Dev. Biol., 5, 61. doi:https://doi.org/10.3389/fcell.2017.00061

43. van Dam, T. J., Zwartkruis, F. J., Bos, J. L., and Snell, B. (2011) Evolution of the TOR Pathway. J. Mol. Evol., 73(3-4), 209–220. doi:10.1007/s00239-011-9469-9

44. Tatebe, H., and Shiozaki, K. (2017) Evolutionary Conservation of the Components in the TOR Signaling Pathways. Biomolecules, 1(7), E77. doi:10.3390/biom7040077.
Khanna, A., Lofti, P., Chavan, A. J., Montano, N. M., Bolourani, P., Weeks, G., Shen, Z., Briggs, S. P., Pots, H., Van Haastert, P. J. M., Kortholt, A., and Charest, P. G. (2016) The small GTPases Ras and Rap1 bind to and control TORC2 activity. Sci. Rep., 6, 25823. doi:10.1038/srep25823.

Duran, R. V., and Hall, M. N. (2012) Regulation of TOR by small GTPases. EMBO Rep., 13(2), 121-8. doi:10.1038/embor.2011.257.

Bergstrom, J. D., Dufresne, C., Bills, G. F., Nallom Omstead, M., and Byrne, K. (1995) Discovery, biosynthesis, and mechanism of action of the zaragozic acids: potent inhibitors of squalene synthase. Annu. Rev. Microbiol., 49, 607-639. doi:10.1146/annurev.mi.49.100195.003135.

Flanagan, P. R., Liu, N. N., Fitzpatrick, D. J., Hokamp, K., Köhler, J. R., and Moran, G. P. (2017) The Candida albicans TOR-Activating GTPases Gtr1 and Rhb1 Coregulate Starvation Responses and Biofilm Formation. mSphere, 2(6), e00477-17. doi:6). pii: e00477-17.

Tsao, C. C., Chen, Y. T., and Lan, C. Y. (2009) A small G protein Rhb1 and a GTPase-activating protein Tsc2 involved in nitrogen starvation-induced morphogenesis and cell wall integrity of Candida albicans. Fungal Genet. Biol., 46(2), 126-136. doi:10.1016/j.fgb.2008.11.008.

Melchinger, W., Zierock, L., Wehrle, B., and Marks, R. (2013) Inhibitors Of Farnesyltransferase and Everolimus Act Synergistically In Growth Inhibition Of Human T-NHL Cells By Involvement Of AMPK. Blood, 122(1), 3076.

Cheong, J., Eom, J. I., Lee, H. W., Park, I., Kim, Y., Kim, J. I., and Min, Y. (2007) mTOR Inhibitor Rapamycin Interacts Synergistically with Farnesyltransferase Inhibitor FTI-277 To Induce Growth Inhibition in Human Leukemia Cells. Blood, 110(11), 1821.

Niessner, H., Beck, D., Sinnberg, T., Lasithiotakis, K., Maczey, E., Gogel, J., Venturelli, S., Berger, A., Mauthe, M., Toulany, M., Flaherty, K., Schaller, M., Schadendorf, D., Proikas-Cezanne, T., Schitteck, B., Garbe, C., Kulms, D., and Meier, F. (2011) The farnesyl transferase inhibitor lonafarnib inhibits mTOR signaling and enforces sorafenib-induced apoptosis in melanoma cells. J. Invest. Dermatol., 131(2), 468-479. doi:10.1038/jid.2010.297.

Clinicaltrials.gov. (n.d.). (NIH. U.S. National Library of Medicine) Retrieved 6 24, 2018, from https://clinicaltrials.gov/

Hopkins, A. L. (2008) Network pharmacology: the next paradigm in drug discovery. Nat. Chem. Biol., 4, 682-690. doi:10.1038/nchembio.118.

WHO. (2017). 20th WHO Essential Medicines List (EML).

Paradkar, A. (2013) Clavulanic acid production by Streptomyces clavuligerus: biogenesis, regulation and strain improvement. J. Antibiot. (Tokyo), 66, 411-420. doi:10.1038/ja.2013.26.

Patridge, E., Gareiss, P., Kinch, M. S., and Hoyer, D. (2011) Synergistic drug combinations for tuberculosis therapy identified by a novel high-throughput screen. Proc. Natl. Acad. Sci. U.S.A., 108(14), 5601-5606. doi:10.1073/pnas.1014680114.

Wambaugh, M. A., Shakya, V. P., Lewis, A. J., Mulvey, M. A., and Brown, J. C. (2017) High-throughput identification and rational design of synergistic small-molecule pairs for combating and bypassing antibiotic resistance. PLoS Biol., 15(6), e2001644. doi:10.1371/journal.pbio.2001644.

Katz, L., and Baltz, R. H. (2016) Natural product discovery: past, present, and future. J. Ind. Microbiol. Biotechnol., 43(2-3), 155-176. doi:10.1007/s10295-015-1723-5.

Arp, J., Götz, S., Mukherji, R., Mattern, D. J., García-Altares, M., Klapper, M., Brock, D. A., Brakhage, A. A., Strassmann, J. E., Bardl, Q. D. C. B., Willing, K., Peschel, G., and Stallforth, P. (2018) Synergistic activity of cosecreted natural products from amoeba-associated bacteria. Proc. Natl. Acad. Sci. U.S.A., 115(12), 3758-3763. doi:10.1073/pnas.1721790115.

Ramón-García, S., Ng, C., Anderson, H., Chao, J. D., Zheng, X., Pfeifer, T., Av-Gay, Y., Roberge, M., and Thompson, C. J. (2011) Synergistic drug combinations for tuberculosis therapy identified by a novel high-throughput screen. Antimicrob. Agents Chemother., 55(6), 3861-3869. doi:10.1128/AAC.00474-11.

Mott, B. T., Eastman, R. T., Guha, R., Sherlach, K. S., Sirwardana, A., Shinn, P., McKnight, M., Michael, S., Lacerda-Queiroz, N., Patel, P. R., Khine, P., Sun, H., Kasbekar, M., Aghdam, N., Fontaine, S. D., Liu, D., Mierzwa, T., Mathews-Griner L. A., et al. (2015). High-throughput matrix screening identifies synergistic and antagonistic antimalarial drug combinations. Sci. Rep., 5, 13891. doi:10.1038/srep13891.
64. Ziemert, N., Alanjary, M., and Weber, T. (2016) The evolution of genome mining in microbes – a review. Nat. Prod. Rep., 33, 988-1005. doi:10.1039/C6NP00025H
65. Kumar, Y., and Goodfellow, M. (2008) Five new members of the Streptomyces violaceusniger 16S rRNA gene clade: Streptomyces castelarensis sp. nov., comb. nov., Streptomyces himastatinicus sp. nov., Streptomyces mordarksii sp. nov., Streptomyces rapamycinicicus sp. nov. and Streptomyces ruanii sp. nov. Int. J. Syst. Evol. Microbiol., 58(Pt 6), 1369-1378. doi:10.1099/ijs.0.65408-0
66. Nikodinovic, J., Barrow, K. D., and Chuck, J. A. (2003) High yield preparation of genomic DNA from Streptomyces. Biotechniques., 35(5), 932-4, 936.
67. Hyatt, D., Chen, G. L., Land, M. L., Larimer, F. W., and Hauser, L. J. (2010) Prodigal: prokaryotic gene recognition and translation initiation site identification. BMC Bioinformatics, 11(119). doi:10.1186/1471-2105-11-119
68. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) Basic local alignment search tool. J. Mol. Biol., 215(3), 403-10. doi:10.1016/S0022-2836(05)80360-2
69. Sievers, F., Wilm, A., Gibson, T. J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Soding, M., Thompson, J. D., and Higgins, D. G. (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol. Syst. Biol., 7(539). doi:10.1038/msb.2011.75
70. Edgar, R. C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res., 32(5), 1792-7. doi:10.1093/nar/gkh340
71. Price, M. N., Dehal, P. S., and Arkin, A. P. (2010) FastTree 2 -- Approximately Maximum-Likelihood Trees for Large Alignments. PLoS ONE, 5(3), e9490. doi:10.1371/journal.pone.0009490
72. Sun, Y., He, X., Liang, J., Zhou, X., and Deng, Z. (2009) Analysis of functions in plasmid pHZ1358 influencing its genetic and structural stability in Streptomyces lividans 1326. Appl. Microbiol. Biotechnol., 82(2), 303-310. doi:10.1007/s00253-008-1793-7
73. Bierman, M., Logan, R., O’Brien, K., Seno, E. T., Rao, R. N., and Schoner, B. E. (1992) Plasmid cloning vectors for the conjugal transfer of DNA from Escherichia coli to Streptomyces spp. Gene, 116(1), 43-49. doi:10.1016/0378-1119(92)90627-2
74. Rudolph, M. M., Vockenhuber, M., and Suess, B. (2013) Synthetic riboswitches for the conditional control of gene expression in Streptomyces coelicolor. Microbiology, 159, 1416-1422. doi:10.1099/mic.0.067322-0
75. Siegl, T., Tokovenko, B., Myronovskiy, M., and Luzhetskyy, A. (2013) Design, construction and characterisation of a synthetic promoter library for fine-tuned gene expression in actinomycetes. Metab. Eng., 19, 98-106. doi:10.1016/j.ymeng.2013.07.006
76. Deltcheva, E., Chylinski, K., Sharma, C. M., Gonzales, K., Chao, Y., Pirzada, Z. A., Eckert, M. R., Vogel, J., and Charpentier, E. (2011) CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. Nature, 471, 602-607. doi:10.1038/nature09886
77. Kieser, T., Bibb, M., Buttner, M., Chater, K., and Hopwood, D. A. (2000) Practical Streptomyces Genetics (1 ed.). Colney, Norwich NR4 7UH, England: John Innes Foundation.
Figure 1. LC-MS chromatogram of *S. rapamycinicus* ATCC 29253 and *S. rapamycinicus* R073 culture extracts. (A) Full scan LC-MS data in the m/z range 300-1000 Da with normalized intensities on the vertical axis. Extracted MS chromatograms show the major actinoplanic acid species (1, 2, 3 and 4). Mass spectra of actinoplanic acid A (1) and actinoplanic acid C (2) are shown. Rapamycin-related compounds populate the right part of the chromatogram. 

HRMS: 1, C₅₁H₈₀O₁₆; measured [M+NH₄]⁺ 966.57913; calculated [M+NH₄]⁺ 966.579014; Δ 0.12 ppm. 2, C₅₀H₇₈O₁₆; measured [M+NH₄]⁺ 952.56320; calculated [M+NH₄]⁺ 952.563364; Δ -0.17 ppm. 3, C₅₁H₈₂O₁₇; measured [M+NH₄]⁺ 984.58960; calculated [M+NH₄]⁺ 984.589579; Δ 0.02 ppm. 4, C₅₀H₈₀O₁₇; measured [M+NH₄]⁺ 970.57431; calculated [M+NH₄]⁺ 970.573929; Δ 0.39 ppm. (B) Structures of actinoplanic acids found in *S. rapamycinicus* are depicted on the far right. For comparison, the structure of fungal toxins fumonisins B and C is also shown. The tricarballylic moieties are colored red.
Figure 2. Genetic organization and synteny of rapamycin and actinoplanic acid gene clusters in genomes of the known rapamycin producers. Genomic loci encoding the rapamycin and actinoplanic acid clusters of *Streptomyces iranensis* HM35, *Streptomyces rapamycinicus* ATCC 29253 and *Actinoplanes* sp. N902-109 were compared. The scale of the large PKS genes is compressed two fold. The *S. rapamycinicus* APL gene cluster is labeled with gene designations. CDS locus tags, as found in the deposited genome annotations, are listed in Table S1.
Figure 3. The proposed biosynthesis of actinoplanic acids in *S. rapamycinicus*. (A) PKS assembly. Extender unit utilization and polyketide condensation sequence as derived from the bioinformatic prediction and structural properties of actinoplanic acid. Each module is numbered and depicted with the encoded catalytic domains. The domains, predicted to be inactive, are crossed-out. (B) Post-PKS sequence. The proposed NRPS-catalyzed acylation, with tricarballylate side chains resulting in the double-lactone structure of 1 and 2. (C) Phenotype of *S. rapamycinicus ΔaplA* mutant. Extracted LC-MS chromatograms* show the major actinoplanic acid species (1, 2, 3 and 4) in *S. rapamicinicus* R073 (w.t.) and *S. rapamycinicus* R073 ΔaplA. (D) Phenotypes of *S. rapamycinicus ΔCCR* mutants. Extracted chromatograms* show the major actinoplanic acid species (1, 2, 3 and 4) in *S. rapamicinicus* R073 (w.t.), *S. rapamycinicus* R073 ΔM271_06415 and *S. rapamycinicus* R073 ΔM271_06415 ΔM271_13255. *Normal intensity values are shown on the vertical axis.
Figure 4. (A) Phenotype of the S. rapamicinicus aplD deletion mutant. Extracted LC-MS chromatograms show the major actinoplanic acid species (1 and 2) and the core PKS intermediates in S. rapamicinicus R073 (w.t.) and S. rapamycinicus R073 ΔaplD. Normalized intensities are shown on the vertical axis. Mass spectra of 7 and 8 are shown.

(B) The proposed structures of 7, 8, 9 and 10 are based on comparative HRMS/MS data with analogues derived synthetically from authentic actinoplanic acid samples (Fig. S7). HRMS: 7, C39H70O6; measured [M+H]+ 635.52426; calculated [M+H]+ 635.525065; Δ -0.64 ppm. 8, C38H68O6; measured [M+H]+ 621.50867; calculated [M+H]+ 621.509415; Δ -0.55 ppm. 9, C40H72O6; measured 649.54022; calculated 649.540715; Δ -0.15 ppm. 10, C39H70O6; measured 635.52484; calculated 935.525065; Δ 0.27 ppm. (C) Approximately-maximum-likelihood, bootstrapped phylogenetic tree of various subtypes of condensation proteins/domains* with schematic mode of action for assembly-line C domains (blue) compared to ester-forming C domains (yellow and green) acting on non-tethered substrates. (D) Active site motif conservation*; multiple alignments were made from: consensus – the complete dataset from C; Fum14 - five analogues from Fusarium and Aspergillus; AplE- the three known AplE homologues; and SgcC5 – dataset for ester-forming clade surrounding the SgcC5. *See Fig. S8 and Dataset S1 for details.
Figure 5. Phenotype of the *S. rapamicinicus* aplF deletion mutant. (A) Extracted LC-MS chromatograms* show the major actinoplanic acid species (1 and 2) and the isomers of the nonsaturated analogues of actinoplanic acids B and D (11 and 12) in *S. rapamicinicus* R073 and *S. rapamycinicus* R073 ΔaplF. Mass spectra are from the largest extracted peak for each 11 and 12 and were found representative for all other peaks. HRMS: 11, C₅₁H₇₈O₁₆; measured [M+NH₄]⁺ 964.56298; calculated [M+NH₄]⁺ 964.563364; Δ: -0.39 ppm. 12, C₅₀H₇₆O₁₆; measured [M+NH₄]⁺ 950.54733; calculated [M+NH₄]⁺ 950.547714; Δ: -0.40 ppm. (B) Deletion of apl3 results in a shift to production of actinoplanic acids B and D (5, 6). Extracted chromatograms* show the major actinoplanic acid species (1, 2, 3, 4, 5 and 6) in *S. rapamicinicus* R073 (w.t.) and *S. rapamycinicus* R073 Δapl3. Mass spectra of 5 and 6 are given on the right. HRMS: 5, C₅₁H₇₀O₁₆; measured [M+NH₄]⁺ 968.59425; calculated [M+NH₄]⁺ 968.594664; Δ: -0.43 ppm. 6, C₅₀H₇₈O₁₆; measured [M+NH₄]⁺ 954.57867; calculated [M+NH₄]⁺ 954.579014; Δ: -0.36 ppm. *Vertical axis show normalized intensities.
Figure 6. Synergistic antifungal activity of rapamycin and actinoplanic acid A. (A) Agar assays with *Aspergillus fumigatus*, *Rhizopus oryzae*, and *Candida albicans*. Actinoplanic acid A was embedded into the PDA along with fungal cells. Rapamycin was then spotted on top. Plates were photographed after 24 h of growth. (B) A repeated assay where first rapamycin was embedded into the PDA along with *R. oryzae* conidia, and actinoplanic acid was spotted on top of the agar. (C) Synergistic antifungal activity of rapamycin and actinoplanic acid A in PDB liquid culture. Microphotographs of *R. oryzae* conidia grown in chamber slides. The photographs were taken after overnight incubation at 35 °C. Various concentrations of rapamycin and actinoplanic acid A were supplemented to the medium.
Figure 7. (A) Schematic representation of independent vs. synergistic natural product (NP) attacks. Competitive advantage for carriers of synergistic NP promotes evolutionary conservation of NP gene cluster co-harboring. (B) A possible workflow for systematic genome mining for synergistic NP, based on pathway co-harboring. NP gene cluster prediction is computed by antiSMASH for each genome. NP gene cluster inventories are built wherein unique IDs are assigned to groups of highly similar gene clusters. A matrix of genome-NP gene cluster inventory enables the identification of co-harbored clusters and their ranking based on conservation of pathway co-harboring. NP discovery efforts can then be focused to genomes harboring high-ranking pathway sets.
Discovery of actinoplanic acid pathway in *Streptomyces rapamycinicus* reveals a genetically conserved synergism with rapamycin.
Peter Mrak, Philipp Krastel, Petra Pivk Lukancic, Jianshi Tao, Dominik Pistorius and Charles M Moore

*J. Biol. Chem. published online October 16, 2018*

Access the most updated version of this article at doi: [10.1074/jbc.RA118.005314](https://doi.org/10.1074/jbc.RA118.005314)

Alerts:
- When this article is cited
- When a correction for this article is posted

[Click here](https://www.jbc.org) to choose from all of JBC's e-mail alerts