ChemPubSoc Europe

DOI: 10.1002/chem.201503548

CHEMISTRY A European Journal Full Paper

Biosynthesis

Evaluation of the Synthetic Potential of an AHBA Knockout Mutant of the Rifamycin Producer *Amycolatopsis mediterranei*

Ilona Bułyszko, Gerald Dräger, Anja Klenge, and Andreas Kirschning*[a]

Abstract: Supplementing an AHBA(−) mutant strain of *Amycolatopsis mediterranei*, the rifamycin producer, with a series of benzoic acid derivatives yielded new tetraketides containing different phenyl groups. These mutasynthetic studies revealed unique reductive properties of *A. mediterranei* towards nitro- and azidoarenes, leading to the corresponding anilines. In selected cases, the yields of mutaproducts (fermentation products isolated after feeding bacteria with chemically prepared analogs of natural building blocks) obtained are in a range (up to 118 mg L^{-1}) that renders them useful as chiral building blocks for further synthetic endeavors. The configuration of the stereogenic centers at C6 and C7 was determined to be 6*R*,7*S* for one representative tetraketide. Importantly, processing beyond the tetraketide stage is not always blocked when the formation of the bicyclic naphthalene precursor cannot occur. This was proven by formation of a bromo undecaketide, an observation that has implications regarding the evolutionary development of rifamycin biosynthesis.

Introduction

The ansamycins comprise an important and growing class of microbial macrolactam polyketides characterized by a macrocyclic structure in which an aliphatic *ansa* chain forms a bridge between two non-adjacent positions of a cyclic π-system.[1] Whereas geldanamycin **3** and ansamitocin P3 **4** are benzenic ansamycin antibiotics, rifamycins **1a–c** belong to the group of naphthalenic ansamycins (Figure 1). Rifampicin **2** and rifaximin are semisynthetic rifamycin derivatives that display antibacterial activity against both Gram-positive (e.g. *Mycobacterium tuberculosis* and *Mycobacterium leprae*) and Gram-negative bacteria, and are in clinical use, for example, for the treatment of tuberculosis, leprosy, and AIDS-associated mycobacterial infections.[2]

Ansamycin antibiotics are biosynthesized by type 1 polyketide synthases (PKSs) from 3-amino-5-hydroxybenzoic acid (AHBA) **5** as a starting building block.[3] This is selectively activated by the acyl transferase (AT_L) from which it is transferred onto the first acyl carrier protein (ACP_L) by transesterification.[4–6]

The biosynthesis of rifamycin B **1c** also requires AHBA which is uploaded onto the loading module by the AT_L (Scheme 1). After complete PKS processing, an amide synthase performs the macrolactamization to presumably yield proansamycin X **9**. A series of tailoring transformations proceed via rifamycins W **1a**, S **1b** and SV **10** that complete the biosynthesis. Naphthalenic ansamycins like rifamycin B involve a unique biosynthetic sequence (**6**→**8**) leading to the dihydronaphthoquinone moiety, which is proposed to take place en route from module 3 to 4 (Scheme 1a).[1]

First, oxidation of the benzene ring in **6** is expected to yield hydroquinone **11** and this is further oxidized to the quinone **12a** (Scheme 1b). Deprotonation of the 1,3-diketo moiety initiates an intramolecular Michael addition to yield dihydronaphthoquinone **13**, which is further processed to oligoketide **8** in module 4.[5] Support for the timing of the cyclization was derived from the inactivation of the rifF gene because formation of linear polyketides of different chain lengths was observed.[7,8] Whereas the penta- to decaketides carry the naphthoquinone ring, the tetraketide still contains the benzene ring. The enzymes involved in this sequence are not part of the rif-PKS and only limited information has so far been collected on their possible nature. Floss and coworkers studied the rif-Orf19 gene which encodes a 3-(3-hydroxyphenyl) propionate hydroxylase-like protein.[6] Similar genes are involved in the biosynthesis of geldanamycin **3**, for example. When rif-Orf19 was inactivated, the biosynthesis of rifamycin B was blocked and only the formation of tetraketides was observed. It was suggested that this enzyme acts as a separate protein that interacts with the rif-PKS and introduces a hydroxy group into the ACP-bound tetraketide. It remains unclear whether the next steps towards hydronaphthoquinone **13** are enzyme-catalyzed or proceed spontaneously.

It has been shown by our group and others[9–11] that ansamycin antibiotics are ideally suited for preparing new derivatives

[a] *I. Bułyszko, Dr. G. Dräger, A. Klenge, Prof. Dr. A. Kirschning*
Institute of Organic Chemistry and Center of Biomolecular Drug Research (BMWZ), Leibniz Universität Hannover
Schneiderberg 1B, 30167 Hannover (Germany)
E-mail: andreas.kirschning@oci.uni-hannover.de

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.201503548: Detailed descriptions of experimental procedures including the syntheses of mutasynthons and Mosher ester analysis, as well as analytical characterizations and copies of NMR spectra of new tetraketides.

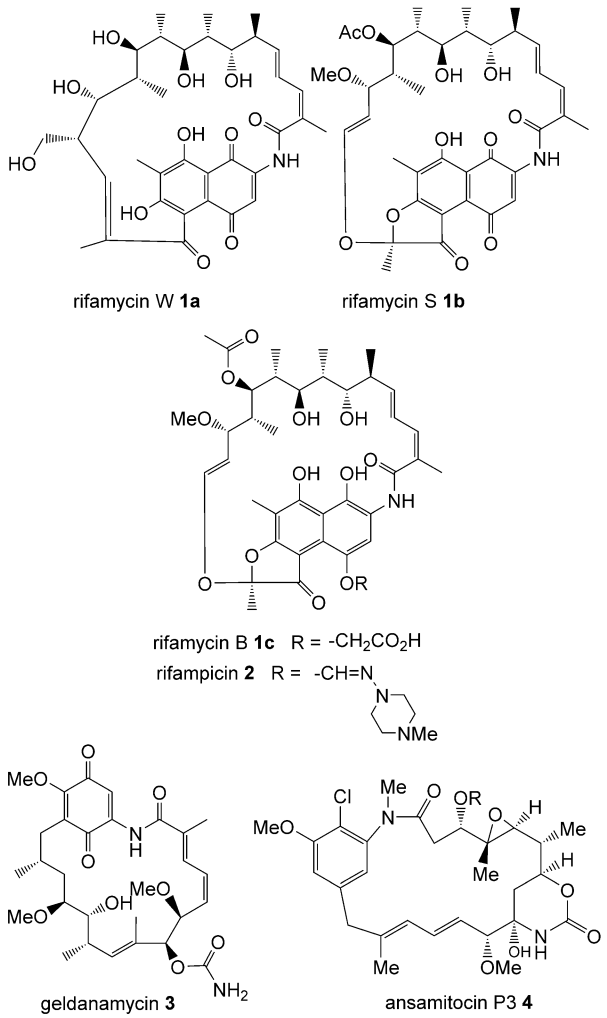


Figure 1. Structures of rifamycins W, S, and B (**1a**–**c**), rifampicin (**2**), geldanamycin (**3**), and ansamitocin P3 (**4**).

by mutational biosynthesis or, in short, mutasynthesis. Mutasynthesis requires the generation of mutant strains of a producer organism that are blocked in the formation of a biosynthetic building block of the end product.[12] Administration of chemically prepared analogs of these building blocks (mutasynthons) to the blocked mutant results in new metabolites. AHBA **5**, the universal starting building block for all ansamycin antibiotics, is a secondary metabolite and therefore blocking of its biosynthesis does not have an effect on the survival of the producer organism. For example, supplementing the AHBA(−) mutant strains of *Streptomyces hygroscopicus*, the producer of geldanamycin[9,10] and *Actinosynnema pretiosum*, which biosynthesizes the ansamtocins,[11] provided libraries of new ansamycin antibiotics modified in the aromatic moiety. It was reported that the rifamycin-producing polyketide synthase in *Amycolatopsis mediterranei* has several interesting features. The activities of AT_L and ACP_L were reconstituted in vitro and kinetic parameters for covalent arylation of the loading module were measured directly for different benzoates, as well as in competition experiments, to determine the relative rates of incorporation. Similarly to the loading modules of the geldanamycin (gdn) and ansamitocin synthetases (asm)[11,12] the rifamycin loading module showed substrate tolerance, preferentially for 3,5-disubstituted benzoic acids.[13] Indeed, there is 64% sequence identity between the acyl transferases (AT_L) in the rif- and asm-PKSs and 65% sequence identity between those in the rif- and gdn-PKSs.[14]

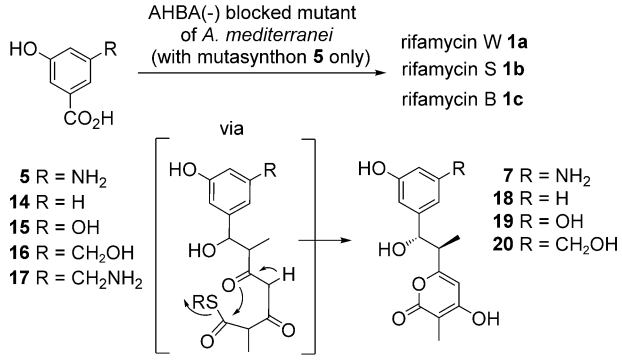
Another unusual feature of the rifamycin synthetase was first noted by Ghisalba et al.[15] and later confirmed by Floss and Khosla.[5,16] In selected UV-mutant strains, as well as specifically blocked mutant strains of *A. mediterranei*, the formation and release of the natural tetraketide in the form of 4-hydroxy-2*H*-pyran-2-one **7** was observed. In combination with mutasynthetic experiments using an AHBA(−) strain of *A. mediterranei* the formation and isolation of derivatives of **7** were also reported.[5] These data all suggest that failure of ring closure to give the dihydronaphthoquinone leads to rejection of the nascent ketide by module 4 of the PKS and release of the respective tetraketide.

Results and Discussions

Mutasynthetic experiments

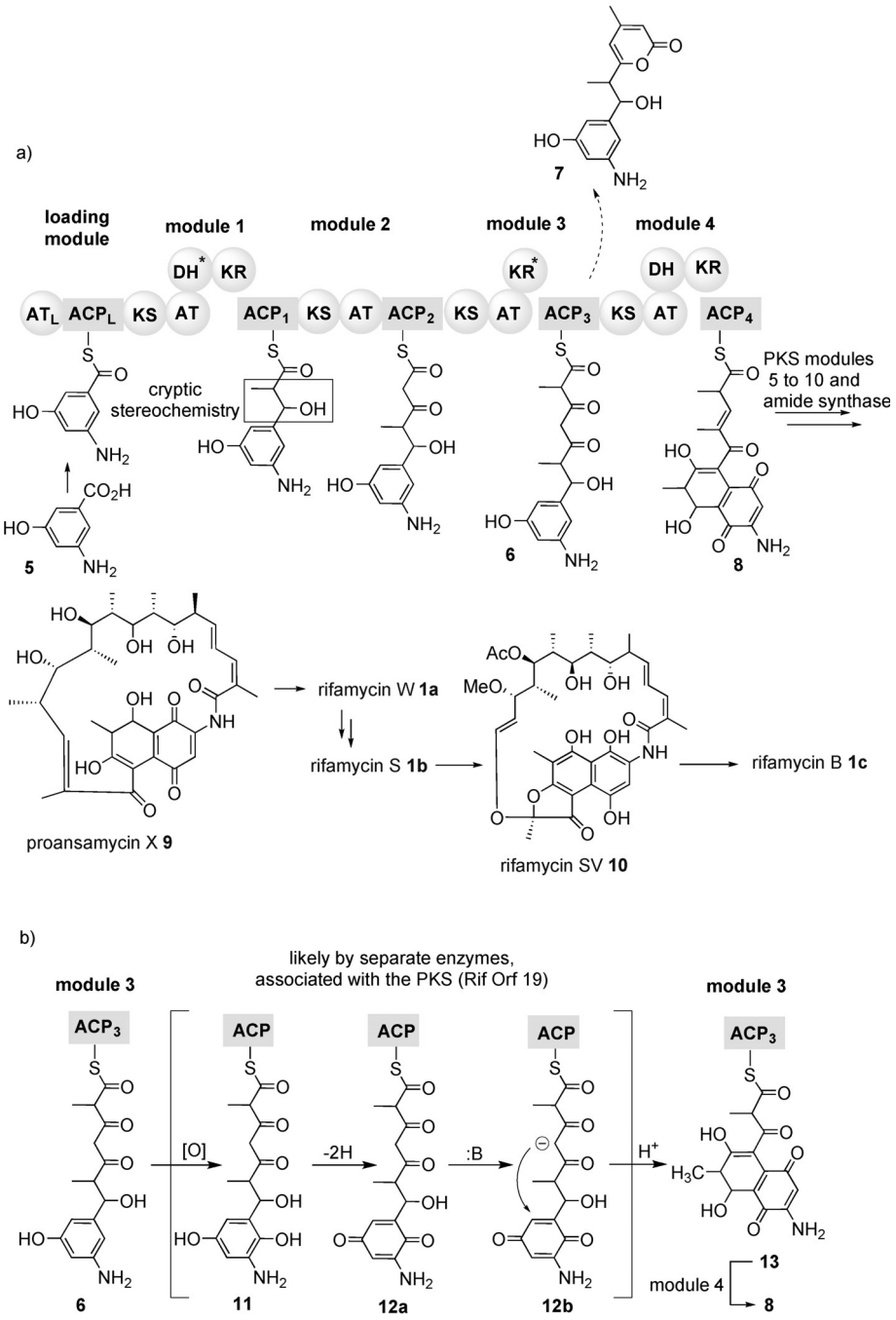
In continuation of our earlier work on AHBA blocked mutants of the geldanamycin **3** and ansamitocin P3 **4** producers, we extended our mutasynthetic studies to the reported AHBA(−) mutant strain of *A. mediterranei*. It was our intention to further evaluate the scope and limitations of such mutant strains as synthetic tools for creating libraries of advanced intermediates. We planned to include electron-deficient aromatic building

Table 1. Mutasynthetic supplementation of AHBA(−) mutant strain of *A. mediterranei* with different *m*-hydroxybenzoic acids **5** and **14**–**17**.



	1a [mg L^{-1}]	1b [mg L^{-1}]	1c [mg L^{-1}]	Pyranone (mg L^{-1})
5	12.8[a] 2.4[a]	1.1[b] –	20[b]	**7**[c]
14	–	–	–	**18** (118)[b]
15	–	–	–	**19** (4.2)[b]
16	–	–	–	chemically labile
17	–	–	–	not formed

[a] fermentation was carried out on solid agar medium (see the Experimental Section). [b] Fermentation was carried out in liquid medium (see the Experimental Section). [c] UPLC-MS detection: *m/z* calcd for $C_{15}H_{18}NO_5$: 292.1185 $[M+H]^+$; found: 292.1182.



Scheme 1. Simplified overview of the biosynthesis of rifamycin B **3** and proposed biosynthetic steps from phenol **6** to dihydronaphthoquinone **13** [asterisks signify that the dehydratase (DH) and ketoreductase (KR) are nonfunctional].

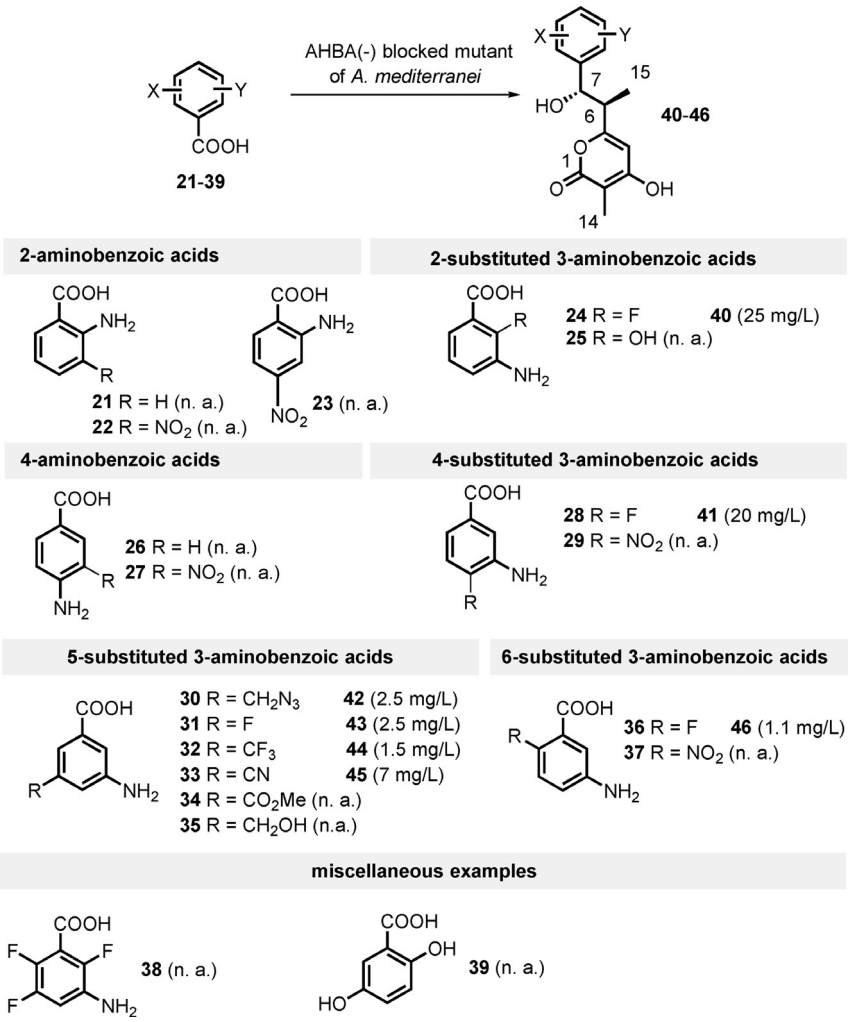
blocks that might be processed to modified tetraketides, able to form new dihydronaphthalenes by intramolecular cyclization of the β-ketothioester similarly to the proposed transformation of quinone **13** into dihydronaphthoquinone **8**. We sought to achieve this either by choice of appropriate phenols that are oxidized to hydroquinones (like **6**→**11**) or by choice of electron-withdrawing ester, nitrile, or nitro groups.

The AHBA(−) mutant strain of *A. mediterranei* was generated by a knockout of rifK activity responsible for the aromatization of the AHBA precursor, amino-dehydroshikimic acid.[17] Cultivation of this mutant strain was first carried out in the presence of AHBA **5** in liquid culture and, as expected, the production of rifamycin **1c** was restored (Table 1). Additionally, rifamycin W **1a** and rifamycin S **1b** were also isolated, whereas traces of the released tetraketide **7** were only detectable by UPLC-MS. Next, 3-hydroxybenzoic acids **14** and **15** were probed and these were also processed, in this case yielding the known 2*H*-pyran-2-ones **18** and **19**, respectively, with the former in preparatively excellent yield.[6] However, we did not observe hydroxylation and hence hydroquinone or naphthoquinone formation. As proposed, liberation from module 3 probably takes place by a spontaneous intramolecular release mechanism. As

oxidation and hence cyclization do not take place, further processing seems to be kinetically hampered and the intermediate remains attached to ACP_3 until pyranone formation takes place.

Additionally, we employed benzyl alcohol **16** and benzylamine **17** as mutasynthons, based on our recent observation that the geldanamycin producer *S. hygroscopicus* is able to accept benzyl alcohol **16** and process it to give ring-enlarged macrolactones.[18] In the present case, after supplementing the AHBA(−) strain of *A. mediterranei* with **16**, the corresponding chemically labile pyranone **20** was formed. The corresponding benzylamine **17** was not processed at all under the fermentation conditions employed.

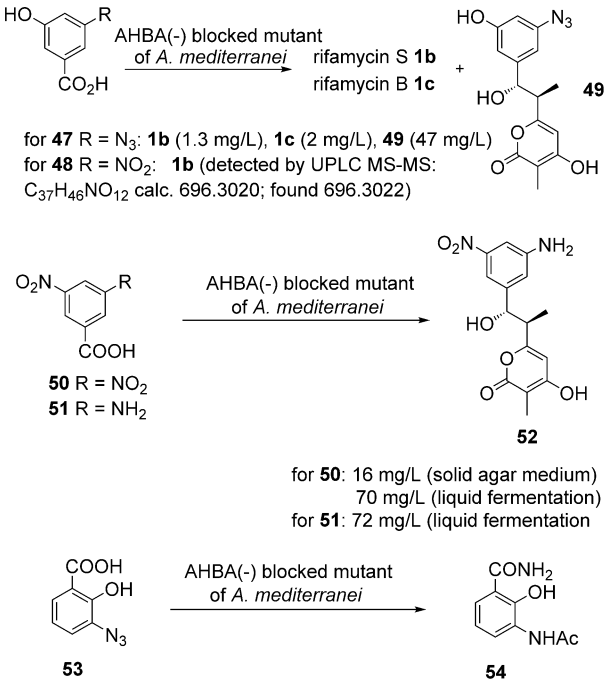
We extended our studies on the promiscuity of the rif-PKS loading module by using a library of differently substituted and functionalized benzoic acids **21**–**39** (Scheme 2). Anthranilic acid **21**, as well as two nitrosubstituted anthranilic acid derivatives **22** and **23**, were not accepted, in agreement with the kinetic data that was previously collected for the loading module.[13] 4-Amino benzoates **26** and **27** were also not consumed after being fed to the AHBA(−) mutant strain of *A. mediterranei*. Consequently, we prepared a library of differently functionalized and substituted 3-aminobenzoic acids. 2-Fluoro-substituted mutasynthon **24** was processed to give tetraketide **40** in surprisingly good yield but a hydroxy group at position 2 in the benzoic acid **25** inhibited processing by *A. mediterranei*. Likewise, 4-substituted benzoic acid **29** gave the same negative result, whereas the fluoro-substituted **28** was well accepted by the mutant strain. With the exception of **21** and **50** (see Scheme 3) these observations are in line with previously collected kinetic data.[13] However, substitution at position 5 in 3-aminobenzoic acids, as in **30**–**33**, makes them well-suited mutasynthons, from which 2*H*-pyran-2-ones **42**–**45** can be obtained in preparative amounts. One important finding of these feeding experiments is that electron-deficient mutasynthons **32** and **33**, which contain a trifluoromethyl and a nitrile group, respectively, are processed but do not undergo cyclization to the corresponding dihydronaphthalene but are also processed to the stage of the tetraketide before they are released from the rif-PKS. In addition, it is noteworthy that fluorine-containing mutasynthons such as **24**, **28**, and **36** that contain a substitution pattern not favored to be accepted by AT_L (functionali-



Scheme 2. Mutasynthetic supplementation with aminobenzoic acids **21**–**39**. X, Y = different substituents; n. a. = not accepted but detoxification products, including benzamides and acetylated amides, were detected (these are listed in the Supporting Information).

zation in the 2-,4-, or 6-position), are transformed into the corresponding 2*H*-pyran-2-ones **40**, **41**, and **46**, respectively. Very likely, fluorine is sufficiently small to accommodate the arene ring in the active site of AT_L–ACP_L of the loading module, in a similar manner to the unsubstituted aminobenzoic acid **14** (Table 1).

During our investigation of mutasynthetic options with ansamycin antibiotics, we reported on a unique and unprecedented reductive property of the geldanamycin producer *S. hygroscopicus* towards aryl azides. Selected examples were transformed into the corresponding anilines.[19] We have now found that *A. mediterranei* is the second microbial source able to reduce the bioorthogonal functional azido group (Scheme 3).



Scheme 3. Studies on the reductive properties of *A. mediterranei.*

When feeding 3-azido-5-hydroxybenzoic acid **47** to the AHBA(−) mutant strain, rifamycin B (**1 c**) was isolated along with rifamycin S (**1 b**) and 2*H*-pyran-2-one **49** as the main product. The rifamycins only form after reduction of the aryl azide and formation of the pyranone **49** strongly indicates that reduction of the azido group takes place prior to PKS processing, which would be in line with our studies on the geldanamycin producer *S. hygroscopicus*.[19,20]

Secondly, 3-hydroxy-5-nitrobenzoic acid **48** was also converted into rifamycin S **1 b** in trace amounts, which is the first example of a nitroarene being reduced by one of the ansamycin producers in our hands. *A. mediterranei* exerted a more impressive reductive power towards nitro groups when it was supplemented with 3,5-dinitrobenzoic acid **50**. Aniline **52** was isolated in a similarly excellent yield as when 3-amino-5-nitrobenzoic acid **51** was fed to the AHBA(−) mutant strain. The formation of tetraketides **49** and **52** confirms that electron-withdrawing nitro groups are not sufficient to induce intramolecular Michael addition to create the dihydronaphthalene unit (see also mutasynthons **32** and **33**). One may speculate why reduction only occurs for one nitro group. Clearly, the resulting aminonitroarene is a new substrate with different binding properties for the cryptic enzyme and likely no longer a suitable substrate.

At this point, we had only encountered reduction by *S. hygroscopicus* of aryl azides that have the same 1,3,5-trisubstitution pattern found in AHBA **5**. When 2,3-disubstituted benzoic acid **53** was fed to the AHBA(−) mutant strain of *A. mediterranei*, formation of the tetraketide was not observed, which can be explained by the fact that 2,3-disubstituted benzoates are not accepted by the loading module (see also **25**, Scheme 2). However, we isolated the reduction product **54**, which was also detected after feeding 3-amino-2-hydroxybenzoic acid **25**, an experiment that was carried out for comparison. In earlier studies on the reductive power of the geldanamycin producer *S. hygroscopicus*, we provided evidence that the reduction of the azido group occurs prior to loading onto the PKS starter module and the transformation **53**→**54** provides some support for the present case.

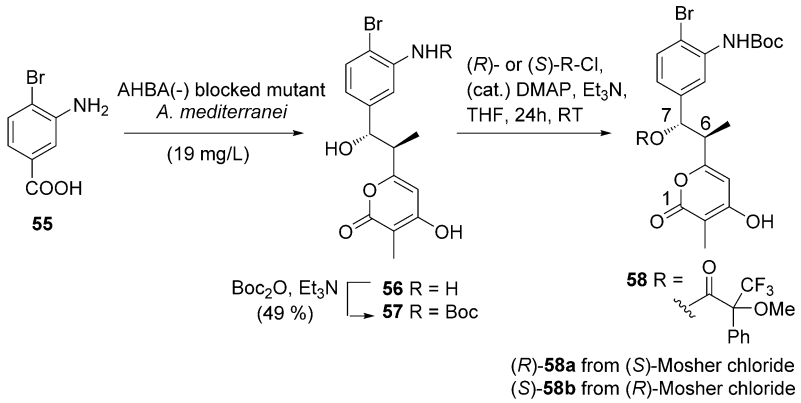
Structure elucidation of the stereochemistry at C6 and C7 in tetraketides

The two stereogenic centers generated in module 1 are also present in tetraketide **7** but disappear during downstream processing. We chose tetraketide **56** for determining the absolute configuration of both stereogenic centers at C6 and C7, respectively. Tetraketide **56** was obtained in good yield after feeding of 3-amino-4-bromobenzoic acid **55** to the AHBA(−) strain. This result is remarkable in view of the fact that 4-substituted 3-aminobenzoic acids, with the exception of benzoic acid **28** bearing a small fluorine substituent, are commonly poor substrates for PKS processing (Scheme 2).

After protection of the aniline group as Boc-carbamate, the secondary alcohol in **57** was treated with both enantiomeric Mosher chlorides to yield diastereomeric esters **58 a** and **58 b** (Scheme 4). Analysis of the ^{1}H NMR data established the stereogenic center at C7 to be *S* (see the Supporting Information). The configuration at C6 was determined to be *R* by using the SPARTAN '08 software (Wavefunction, Inc., Irvine, CA, USA). The analysis was based on the relationship between the H–H coupling of the vicinal protons at C6 and C7 ($J = 8.7$ Hz), as observed in the ^{1}H NMR spectrum, and the torsion angle (179.4°). Both stereochemical assignments are in accordance with the results reported for a different approach by Hartung et al.[21]

Besides the tetraketide **56**, the undecaketide **59** was also detected in the crude fermentation material by UPLC-HRMS (Figure 2). Attempts to isolate it by HPLC were not successful, likely due to chemical lability of the enedione structure. For analytical purposes we therefore prepared the methyl ester **60** from the crude fermentation product but chemical lability of both undecaketides hampered isolation in amounts sufficient for full characterization.

It is generally accepted that formation of the naphthoquinone moiety takes place during the chain extension of the



Scheme 4. Feeding of 3-amino-4-bromobenzoic acid **55** to the AHBA(−) blocked mutant of *A. mediterranei* and stereochemical assignment of C6 and C7 after Mosher esterification. DMAP = 4-dimethylaminopyridine.

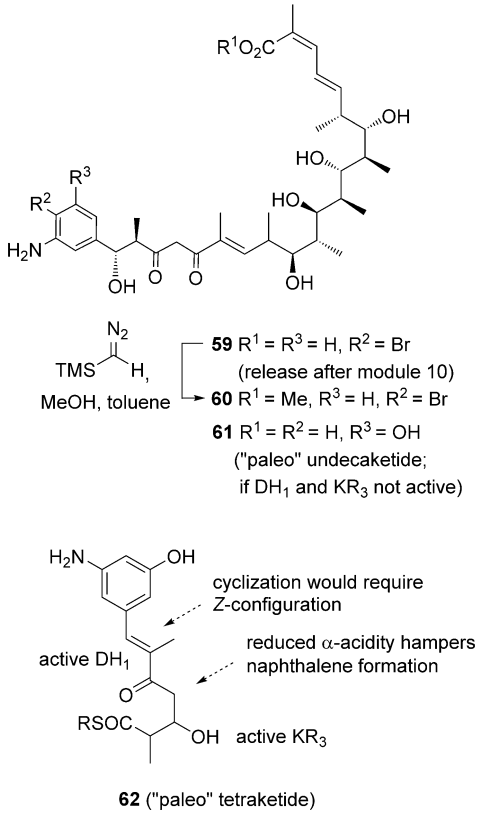


Figure 2. Structures of bromo undecaketide **59**, methyl ester **60**, "paleo" undecaketide **61**, and "paleo" tetraketide **62**.

polyketide, specifically between the tetraketide and the pentaketide stage.[6] Failure of ring closure leads to release of the tetraketide. The formation of bromo undecaketide **59** is remarkable, as it demonstrates that the rif-PKS is also able to process a tetraketide that is not transformed into the dihydronaphthoquinone moiety (Figure 2).

As a consequence, natural undecaketide **61** may be regarded as a potential evolutionary predecessor of an unknown rif-PKS product. As a result of an evolutionary optimization process, new enzymes that were not part of the PKS became part of rifamycin biosynthesis. These are responsible for dihydronaphthoquinone formation (**6**→**8**). Such evolutionary changes are also evident in the rif-PKS architecture, which contains an inactive DH (module 1) and KR (module 3; Scheme 1). Their evolutionary inactivation might have been crucial for naphthalene formation, because the hypothetical "paleo"-tetraketide **62** lacks structural and reactivity features to facilitate cyclization.

All of these alterations may have led to survival advantages for the producer organism. In fact, the importance of such evolutionary analysis of PKSs was recently discussed by Kalesse and co-workers, who reported on the synthesis and biological evaluation of "paleo"-soraphens that remarkably showed different biological activity than natural soraphen, thereby addressing other biological targets.[22,23]

Conclusions

In conclusion, we extended the synthetic potential of the AHBA(−) blocked mutant of *A. mediterranei*, the producer of rifamycin B **1 c**, using the concept of mutasynthesis. As reported for the loading domains of geldanamycin and ansamitocin polyketide synthases, the rifamycin AHBA loading domain (AT_L–ACP_L) is also highly promiscuous for unnatural benzoic acids; 3-aminobenzoic acids with a third substituent located at position 5 are preferentially processed. The fact that we did not find any oxidation products derived from any of the mutasynthons fed reveals the high substrate specificity of the enzymes involved in naphthalene formation (**6**→**8**). Several of these tetraketides are produced in such amounts that they can be employed as highly functionalized chiral building blocks.

In addition, we found that *A. mediterranei* shows remarkable reductive properties towards aryl azides and nitroarenes. This is the second example of a microorganism with reductive properties towards aryl azides. Notably, in both cases it is associated with aminohydroxybenzoic acids and ansamycin biosynthesis. As observed for *S. hygroscopicus*, the reduction is very likely not associated with the PKS but occurs prior to loading onto the ansamycin PKS. One may speculate about the nature of this cryptic enzyme; all of our attempts to date to pinpoint

it to enzymes associated with the AHBA biosynthesis, which is a common biosynthetic feature in *A. mediterranei* and *S. hygroscopicus*, have failed.[24]

Finally, the formation of undecaketide **59** reveals that the rif-PKS is principally able to further process intermediate polyketides that cannot be cyclized to the naphthalene moiety. Along with the silent DH_1 and KR_3 in PKS modules 1 and 3, this observation may be interpreted to indicate that naphthalene formation became part of rifamycin biosynthesis late in the evolution of this secondary metabolite.

Experimental Section

General information

^{1}H NMR spectra were recorded with a Bruker Avance-400 (400 MHz) with DPC console and Bruker DRX-500 (500 MHz) with DRX-console at room temperature. Multiplicities are described with the following abbreviations: s singlet, d doublet, t triplet, q quartet, m multiplet, b broad.

^{13}C NMR spectra were recorded at 100 MHz with a Bruker Avance-400 and at 125 MHz with a Bruker DRX-500. Chemical shifts of ^{1}H and ^{13}C NMR spectra are reported in δ (ppm) relative to TMS as the internal standard. Multiplicities are described with the following abbreviations: s singlet (due to quaternary carbon), d doublet (methine), t triplet (methylene), q quartet (methyl), m multiplet. All coupling constants *J* are expressed in Hz. Supporting ^{1}H–^{1}H correlation (COSY) and ^{1}H–^{13}C correlation (HSQC, HMBC) experiments were performed for interpretation of mutaproduct spectra.

Mass spectra were recorded with a type QTOF premier (MICROMASS) spectrometer (ESI mode) in combination with a Waters Acquity S2 UPLC system equipped with a Waters Acquity UPLC BEH C18 1.7 μm (SN 01473711315545) column [solvent A = water + 0.1% v/v formic acid; solvent B = MeOH + 0.1% v/v formic acid; flow rate = 0.4 mL min^{-1}; gradient (*t* [min]/solvent B [%]): 0/5; 2.5/95; 6.5/95; 6.6/5; 8/5]. Ion mass signals (*m/z*) are reported as values in atomic mass units. Retention times (t_R) are given in the experimental part.

Rifamycin derivatives were isolated by using preparative high-performance liquid chromatography on a VARIAN PROSTAR system [pump Prepstar Model 218, variable wavelength detector Prostar (λ = 220–400 nm, preferred monitoring at λ = 248 nm)] with the column TRENTEC Reprosil-Pur 120 C18 AQ (5 μm, 250 mm × 25 mm, with guard column 40 mm × 8 mm) with parallel mass spectrometric detection (MICROMASS type ZMD ESI-Quad spectrometer). Semi-preparative high-performance liquid chromatography was performed by using a MERCK HITACHI Model 7000 system with the column NUCLEODUR C18 ISIS (5 μm, 250 mm × 8 mm). Separation conditions and retention times (t_R) are reported in the experimental part. Flash column chromatography was performed on MACHEREY–NAGEL silica gel (grain size 40–63 μm).

Commercially available reagents and solvents were used as received or purified by conventional methods prior to use, as reported previously.[25] For thin-layer chromatography, precoated silica gel 60 F254 plates (MERCK, Darmstadt) were used and the spots were visualized with UV light at 254 nm or alternatively by staining with ninhydrin or permanganate solutions.

Fermentation of *Amycolatopsis mediterranei* (mutant strain HGF003)

General information on fermentations

To conduct mutasynthetic experiments, a blocked mutant of *Amycolatopsis mediterranei* (strain HGF003) was used. It is a knockout of rifK, which is one of the genes indispensable in the biosynthesis of AHBA. Consequently, the bacterial strain is unable to produce rifamycin unless AHBA is added to the culture medium. All work with bacteria was performed by using sterile devices and media. The cultivation of *A. mediterranei* strain HGF003 was carried out on agar plates at 28 °C in an Heraeus or Thermo Scientific Heratherm IGS100 incubator, whereas the cultivation in liquid medium was performed by using New Brunswick Scientific Innova 4900 gyratory multi-shaker or New Brunswick Scientific Innova 44/44R stackable shaker at 200 rpm at 28 °C. The cultivation media were prepared by using distilled water and sterilized by autoclaving: YMG agar: 10 g L^{-1} malt extract (Sigma Aldrich, Roth), 4 g L^{-1} yeast extract (Bacto, Duchefa Biochemie), 4 g L^{-1} D-(+)-glucose·H_2O (Roth), 22 g L^{-1} agar (Bacto, Roth); vegetative medium: 5 g L^{-1} beef extract powder (Sigma Aldrich), 5 g L^{-1} peptone (Roth), 5 g L^{-1} yeast extract (Bacto, Duchefa Biochemie), 2.5 g L^{-1} caseine hydrolysate (enzymatic, Roth), 1.5 g L^{-1} NaCl (Honeywell), 22 g L^{-1} glucose·H_2O; production medium: 25 g L^{-1} peanut meal, 9.6 g L^{-1} $(NH_4)_2SO_4$, 9.5 g L^{-1} $CaCO_3$, 126.5 g L^{-1} glucose·H_2O, 1 mL L^{-1} trace elements solution (1 g L^{-1} $MgSO_4$·H_2O, 1 g L^{-1} $CuSO_4$·5 H_2O, 1 g L^{-1} $FeSO_4$·7 H_2O, 1 g L^{-1} $MnSO_4$·H_2O, 1 g L^{-1} $Co(NO_3)_2$·6 H_2O).

Cultivation parameters

Amycolatopsis mediterranei (strain HGF003) was stored as spore suspensions in 40% v/v glycerol/water solution at −80 °C and used for the inoculation of agar plates. Test fermentations were carried out on agar plates at 28 °C for 14 days (after 7 days 10 mg of substrate dissolved in 1.5 mL of DMSO/water mixture per plate were added). For cultivation in liquid medium, a few colonies were transferred directly from the agar plate after 7 days of growing to the 500 mL bottom-baffled flasks or flasks with steel springs containing vegetative medium (50 mL per flask). Precultures were shaken for 3 days at 28 °C before inoculation of the main cultures (500 mL bottom-baffled flask or flask with steel springs charged with 50 mL of production medium, 0.0625 mmol of substrate dissolved in 4 mL of DMSO/water mixture, preferably 1:1). Shaking was continued at 28 °C for 4 days for total cultivation time of 13 days.

Scale-up fermentations were carried out in vegetative (50 mL per flask) and production media (1 L, 1.25 mmol L^{-1} of substrate in 4 mL of DMSO/water mixture, 4 mL of inoculum from vegetative medium per flask) as described above.

The substrate solution was added onto an agar plate or into the production medium respectively by sterile filtration after pH adjustment to 7 with 1 M NaOH. When cultivation was completed, the culture broth was extracted with ethyl acetate, the combined extracts were dried over $MgSO_4$ and the solvent was removed under reduced pressure.

Simultaneously, the microbial strain was fed with the natural starter building block 3-amino-5-hydroxybenzoic acid **5** to monitor the productivity of the strain (positive control). The inoculated medium with no mutasynthon added constituted the zero control.

For detection of the products from the fermentation cultures, samples from the culture broth (200 μL) were dissolved in methanol

(200 μL), centrifuged (3 min, 4 °C) and the clear supernatant was screened by UPLC-ESI-MS.

Mutasynthetic experiments

Rifamycin W 1a: Rifamycin W **1a** was obtained as a yellow solid after supplementing a culture of the blocked mutant of *A. mediterranei* (strain HGF003) with 3-amino-5-hydroxybenzoic acid **5** (fed 50 mg, 0.33 mmol per 125 mL; 10 mg per plate) carried out on agar plates (isolated amount = 1.6 mg; 12.8 mg L^{-1}). 1st HPLC: preparative HPLC (C18): solvent A = water + 0.1 % v/v formic acid, solvent B = MeOH + 0.1 % v/v formic acid; flow rate = 15.0 mL min^{-1}; gradient (*t* [min]/solvent B [%]): 0/20; 5/20; 50/55; 90/100; 100/100; t_R = 67.0 min. 2nd HPLC: semi-preparative HPLC (C18-ISIS): solvent A = water, solvent B = MeOH; flow rate = 2.5 mL min^{-1}; gradient (*t* [min]/solvent B [%]): 0/20; 5/20; 70/55; 90/100; 100/100; t_R = 74.0 min. The analytical data are in accordance with those reported previously:[6,26] ^{1}H NMR (500 MHz, CD_3OD, CHD_2OD = 4.87 ppm): δ = 8.40 (bs, 1H, 2-NH), 7.57 (s, 1H, 3-H), 6.50 (dd, *J* = 16.0, 11.0 Hz, 1H, 18-H), 6.41 (d, *J* = 10.0 Hz, 1H, 29-H), 6.25 (d, *J* = 11.0 Hz, 1H, 17-H), 6.08 (dd, *J* = 16.0, 6.7 Hz, 1H, 19-H), 4.61 (bs, 1H, 6-OH), 4.39 (bs, 1H, 8-OH), 4.04 (dd, *J* = 10.0, 1.0 Hz, 1H, 21-H), 4.01 (dd, *J* = 10.0, 1.0 Hz, 1H, 23-H), 3.58 (dd, *J* = 10.9, 7.9 Hz, 1H, 34a-H), 3.48 (dd, *J* = 10.0, 2.0 Hz, 1H, 25-H), 3.44 (dd, *J* = 10.8, 6.5 Hz, 1H, 34b-H), 3.21–3.13 (m, 1H, 28-H), 2.63 (dd, *J* = 16.6, 7.3 Hz, 1H, 27-H), 2.38–2.32 (m, 1H, 20-H), 2.16 (s, 3H, 14-H), 2.09 (bs, 3H, 13-H), 2.08 (bs, 3H, 30-H), 1.88–1.85 (m, 1H, 22-H), 1.82–1.79 (m, 1H, 24-H), 1.44–1.38 (m, 1H, 26-H), 1.06 (d, *J* = 7.1 Hz, 3H, 33-H or 34-H), 0.92 (d, *J* = 7.0 Hz, 3H, 31-H), 0.73 (d, *J* = 6.8 Hz, 3H, 33-H or 34-H), 0.43 ppm (d, *J* = 7.0 Hz, 3H, 32-H); ^{13}C NMR (125 MHz, CD_3OD, CD_3OD = 49.0 ppm): δ = 201.2 (s, C11), 187.1 (s, C4), 183.4 (s, C1), 172.2 (s, C15), 164.7 (s, C8), 164.1 (s, C6), 142.6 (d, C19), 141.5 (d, C29), 140.9 (s, C12), 140.8 (s, C2), 135.2 (d, C17), 132.1 (s, C16), 129.9 (s, C10), 126.3 (d, C18), 122.2 (s, C5), 119.0 (s, C7), 118.0 (d, C3), 107.5 (s, C9), 79.0 (d, C27), 74.8 (d, C23), 71.0 (d, C21), 69.1 (d, C25), 64.5 (t, C34a), 49.6 (d, C28), 44.1 (d, C26), 39.1 (d, C20), 37.9 (d, C24), 34.3 (d, C22), 20.2 (q, C30), 18.0 (q, C13), 12.7 (q, C34), 11.7 (q, C32), 11.2 (q, C31), 8.9 (q, C33), 8.5 ppm (q, C14); UPLC-MS (MeOH) t_R = 2.9 min; HRMS-ESI: *m/z* calcd for $C_{35}H_{46}NO_{11}$: 656.3071 $[M+H]^+$; found: 656.3068.

Rifamycin S 1b: Rifamycin S **1b** was obtained as a yellow solid after supplementing a culture of the blocked mutant of *A. mediterranei* (strain HGF003) with 3-hydroxy-5-nitrobenzoic acid **48** (fed 229 mg, 1.25 mmol L^{-1}; HRMS), 3-azido-5-hydroxybenzoic acid **47** (fed 168 mg, 0.94 mmol per 750 mL; isolated amount = 1.0 mg; 1.3 mg L^{-1}) and 3-amino-5-hydroxybenzoic acid **5** (fed 191 mg, 1.25 mmol L^{-1}; isolated amount = 1.1 mg L^{-1}). Additionally, fermentation with AHBA **5** on agar plates (fed 50 mg, 0.33 mmol per 125 mL; 10 g/plate) yielded 0.3 mg of rifamycin S **1b** (2.4 mg L^{-1}). 1st HPLC: preparative HPLC (C18): solvent A = water + 0.1 % v/v formic acid, solvent B = MeOH + 0.1 % v/v formic acid; flow rate = 15.0 mL min^{-1}; gradient (*t* [min]/solvent B [%]): 0/20; 5/20; 50/55; 90/100; 100/100; t_R = 77.0 min. 2nd HPLC: semi-preparative HPLC (C18-ISIS): solvent A = water, solvent B = MeOH; flow rate = 2.5 mL min^{-1}; gradient (*t* [min]/solvent B [%]): 0/20; 5/20; 70/55; 90/100; 100/100; t_R = 84.0 min. 3rd HPLC: semi-preparative HPLC (C18-ISIS): solvent A = water, solvent B = MeOH; flow rate = 2.5 mL min^{-1}; gradient (*t* [min]/solvent B [%]): 0/20; 5/20; 50/60; 100/100; t_R = 68.0 min. The analytical data are in accordance with those reported previously:[27] ^{1}H NMR (500 MHz, C_6D_6, C_6D_5H = 7.16 ppm): δ = 12.73 (s, 1H, 8-OH), 8.34 (s, 1H, 3-H), 8.29 (s, 1H, 2-NH), 6.34 (d, *J* = 12.6 Hz, 1H, 29-H), 6.28 (dd, *J* = 15.7, 8.3 Hz, 1H, 18-H), 5.86 (d, *J* = 8.3 Hz, 1H, 17-H), 5.77 (dd, *J* = 15.7, 8.3 Hz, 1H, 19-H), 5.27 (dd, *J* = 12.6, 8.6 Hz, 1H, 28-H), 4.63 (d, *J* = 1.5 Hz, 1H, 25-H), 3.91 (d, *J* = 4.2 Hz, 1H, 23-OH), 3.71 (s, 1H, 21-OH), 3.49 (d, *J* = 9.5 Hz, 1H, 21-H), 3.01 (dd, *J* = 9.0, 3.4 Hz, 1H, 27-H), 2.96 (ddd, *J* = 10.1, 4.3, 1.7 Hz, 1H, 23-H), 2.78 (s, 3H, 37-H), 2.37 s, 3H, 14-H), 2.28–2.24 (m, 1H, 20-H), 1.90–1.87 (m, 1H, 26-H), 1.79 (s, 3H, 30-H), 1.60 (s, 3H, 13-H), 1.59 (s, 3H, 36-H), 1.50 (m, 1H, 22-H), 1.47 (m, 1H, 24-H), 0.99 (d, *J* = 7.0 Hz, 3H, 32-H), 0.64 (d, *J* = 7.3 Hz, 3H, 31-H), 0.45 (d, *J* = 7.1 Hz, 3H, 33-H), 0.23 ppm (d, *J* = 7.1 Hz, 3H, 34-H); ^{13}C NMR (125 MHz, C_6D_6, C_6D_6 = 128.06 ppm): δ = 190.7 (s, C11), 185.4 (s, C1), 181.8 (s, C4), 173.5 (s, C35), 172.5 (s, C6), 169.2 (s, C15), 167.0 (s, C8), 146.3 (d, C29), 142.5 (d, C19), 139.8 (s, C2), 133.1 (d, C17), 131.5 (s, C16), 131.5 (s, C10), 124.7 (d, C18), 117.1 (d, C3), 115.2 (d, C28), 115.1 (s, C7), 111.5 (s, C5), 111.4 (s, C9), 109.1 (s, C12), 82.8 (d, C27), 78.0 (d, C23), 73.9 (d, C21), 73.9 (d, C25), 56.3 (q, C37), 40.4 (d, C20), 37.7 (d, C26), 37.4 (d, C24), 33.1 (d, C22), 22.8 (q, C13), 20.6 (q, C36), 20.3 (q, C30), 16.6 (q, C31), 12.4 (q, C34), 11.8 (q, C32), 8.9 (q, C33), 7.5 ppm (q, C14); UPLC-MS (MeOH); t_R = 3.0 min; HRMS-ESI: *m/z* calcd for $C_{37}H_{46}NO_{12}$: 696.3020 $[M+H]^+$; found: 696.3022.

Rifamycin B 1c: Rifamycin B **1c** was obtained as an orange solid after supplementing a culture of the blocked mutant of *A. mediterranei* (strain HGF003) with 3-hydroxy-5-nitrobenzoic acid **48** (fed 229 mg, 1.25 mmol L^{-1}; HRMS, LC-MS/MS), 3-azido-5-hydroxybenzoic acid **47** (fed 168 mg, 0.94 mmol per 750 mL; isolated amount = 1.5 mg; 2 mg L^{-1}) and 3-amino-5-hydroxybenzoic acid **5** (fed 191 mg, 1.25 mmol L^{-1}; isolated amount = 20 mg L^{-1}). 1st HPLC: preparative HPLC (C18): solvent A = water + 0.1 % v/v formic acid, solvent B = MeOH + 0.1 % v/v formic acid; flow rate = 15.0 mL min^{-1}; gradient (*t* [min]/solvent B [%]): 0/20; 5/20; 50/55; 90/100; 100/100; t_R = 82.0 min. 2nd HPLC: preparative HPLC (C18): solvent A = water, solvent B = MeOH; flow rate = 15.0 mL min^{-1}; gradient (*t* [min]/solvent B [%]): 0/40; 5/40; 20/50; 50/50; 100/100; t_R = 75.0 min. 3rd HPLC: preparative HPLC (C18): solvent A = water, solvent B = MeOH; flow rate = 15.0 mL min^{-1}; gradient (*t* [min]/solvent B [%]): 0/10; 5/10; 85/100; 100/100; t_R = 69.0 min. UPLC-MS (MeOH) t_R = 3.2 min; HRMS-ESI: *m/z* calcd for $C_{39}H_{50}NO_{14}$: 756.3231 $[M+H]^+$; found: 756.3228.

Tetraketide 18: Tetraketide **18** was obtained after supplementing a culture of the blocked mutant of *A. mediterranei* (strain HGF003) with 3-hydroxybenzoic acid **14** (fed 173 mg, 1.25 mmol L^{-1}). The fermentation was carried out in liquid medium providing tetraketide **18** as a colorless foam (isolated amount = 118.0 mg). HPLC: preparative HPLC (C18): solvent A = water + 0.1 % v/v formic acid, solvent B = MeOH + 0.1 % v/v formic acid; flow rate = 15.0 mL min^{-1}; gradient (*t* [min]/solvent B [%]): 0/20; 5/20; 50/55; 90/100; 100/100; t_R = 44.0 min. The analytical data are in accordance with those reported previously:[5] ^{1}H NMR (400 MHz, CD_3OD, CD_2HOD = 3.31 ppm): δ = 7.15 (t, *J* = 8.1 Hz, 1H, H-12), 6.82–6.76 (m, 2H, H-11 or H-13, H-9), 6.73–6.66 (m, 1H, H-11 or H-13), 6.06 (s, 1H, H-4), 4.67 (d, *J* = 8.8 Hz, 1H, H-7), 2.83 (dq, *J* = 14.1, 7.1 Hz, 1H, H-6), 1.88 (s, 3H, H-14), 0.98 ppm (d, *J* = 7.0 Hz, 3H, H-15); ^{1}H NMR (400 MHz, $[D_8]$THF, $[D_8]$THF = 1.73 ppm): δ = 7.06 (t, *J* = 7.8 Hz, 1H, H-12), 6.75–6.73 (m, 2H, H-11 or H-13, H-9), 6.62 (ddd, *J* = 8.0, 2.3, 1.0 Hz, 1H, H-11 or H-13), 5.85 (s, 1H, H-4), 4.64 (d, *J* = 8.4 Hz, 1H, H-7), 2.71 (dq, *J* = 15.0, 7.1 Hz, 1H, H-6), 1.83 (s, 3H, H-14), 0.93 ppm (d, *J* = 7.1 Hz, 3H, H-15); ^{13}C NMR (100 MHz, $[D_8]$THF, $[D_8]$THF = 25.5 ppm): δ = 166.0 (s, C3), 165.4 (s, C5), 165.2 (s, C1), 158.6 (s, C10), 146.0 (s, C8), 129.6 (d, C12), 118.6 (d, C13), 115.2 (d, C11), 114.6 (d, C9), 100.9 (s, C2),98.7 (d, C4), 76.2 (d, C7), 47.4 (d, C6), 15.3 (q, C15), 8.7 ppm (q, C14); UPLC-MS (MeOH) t_R = 2.4 min; HRMS-ESI: *m/z* calcd for $C_{15}H_{17}O_5$: 277.1076 $[M+H]^+$; found: 277.1075.

Tetraketide 19: Tetraketide **19** was obtained after supplementing a culture of the blocked mutant of *A. mediterranei* (strain HGF003)

with 3,5-dihydroxybenzoic acid **15** (fed 193 mg, 1.25 mmol L^{-1}). The fermentation was carried out in liquid medium providing tetraketide **19** as a colorless solid (isolated amount = 4.2 mg). The analytical data are in accordance with those reported previously:[5] 1st HPLC: preparative HPLC (C18): solvent A = water + 0.1 % v/v formic acid, solvent B = MeOH + 0.1 % v/v formic acid; flow rate = 15.0 mL min^{-1}; gradient (*t* [min]/solvent B [%]): 0/20; 5/20; 50/55; 90/100; 100/100; t_R = 41.0 min. 2nd HPLC: semi-preparative HPLC (C18-ISIS): solvent A = water, solvent B = MeOH; flow rate = 2.5 mL min^{-1}; gradient (*t* [min]/solvent B [%]): 0/10; 5/10; 80/45; 90/100; 100/100; t_R = 32.0 min. 3rd HPLC: semi-preparative HPLC (C18-ISIS): solvent A = water, solvent B = MeOH; flow rate = 2.5 mL min^{-1}; gradient (*t* [min]/solvent B [%]): 0/10; 5/10; 60/25; 90/90, 100/100; t_R = 37.0 min. ^{1}H NMR (400 MHz, CD_3OD): δ = 6.29 (d, *J* = 2.2 Hz, 2H, H-9, H-13), 6.19 (t, *J* = 2.2 Hz, 1H, H-11), 6.04 (s, 1H, H-4), 4.58 (d, *J* = 8.8 Hz, 1H, H-7), 2.78 (dq, *J* = 14.2, 7.1 Hz, 1H, H-6), 1.87 (s, 3H, H-14), 0.99 ppm (d, *J* = 7.1 Hz, 3H, H-15); ^{13}C NMR (100 MHz, CD_3OD): δ = 169.4 (s, C3), 169.0 (s, C5), 165.9 (s, C1), 159.6 (s, C10, C12), 146.1 (s, C8), 106.4 (d, C9, C13), 103.0 (d, C11), 102.7 (s, C4), 99.0 (d, C2), 77.0 (d, C7), 47.2 (d, C6), 15.6 (q, C15), 8.3 ppm (q, C14); UPLC-MS (MeOH) t_R = 2.2 min; HRMS-ESI: *m/z* calcd for $C_{15}H_{17}O_6$: 293.1025 $[M+H]^+$; found: 293.1028.

Tetraketide 20: Tetraketide **20** was obtained after supplementing a culture of the blocked mutant of *A. mediterranei* (strain HGF003) with 3-hydroxy-5-(hydroxymethyl)benzoic acid **16** (fed 210 mg, 1.25 mmol L^{-1}). The fermentation was carried out in liquid medium providing compound **20** as a colorless solid which gradually decomposed during attempts to purify it by HPLC. 1st HPLC: preparative HPLC (C18): solvent A = water + 0.1 % v/v formic acid, solvent B = MeOH + 0.1 % v/v formic acid; flow rate = 15.0 mL min^{-1}; gradient (*t* [min]/solvent B [%]): 0/20; 5/20; 50/55; 90/100; 100/100; t_R = 38.0 min. 2nd HPLC: semi-preparative HPLC (C18-ISIS): solvent A = water, solvent B = MeOH; flow rate = 2.5 mL min^{-1}; gradient (*t* [min]/solvent B [%]): 0/10; 5/10; 80/45; 90/100; 100/100; t_R = 30.0 min. 3rd HPLC: semi-preparative HPLC (C18-ISIS): solvent A = water, solvent B = MeOH; flow rate = 2.5 mL min^{-1}; gradient (*t* [min]/solvent B [%]): 0/10; 5/10; 60/25; 90/90, 100/100; t_R = 47.0 min. ^{1}H NMR (400 MHz, CD_3OD): δ = 6.81 (s, 1H, Ar), 6.74 (s, 1H, Ar), 6.68 (s, 1H, Ar), 6.04 (s, 1H, H-4), 4.67 (d, *J* = 8.8 Hz, 1H, H-7), 4.53 (s, 2H, $ArCH_2$), 2.82 (dq, *J* = 14.2, 7.1 Hz, 1H, H-6), 1.86 (s, 3H, H-14), 0.97 ppm (d, *J* = 7.0 Hz, 3H, H-15); UPLC-MS (MeOH) t_R = 2.0 min; HRMS-ESI: *m/z* calcd for $C_{16}H_{19}O_6$: 307.1182 $[M+H]^+$; found: 307.1187.

Tetraketide 40: Tetraketide **40** was obtained after supplementing a culture of the blocked mutant of *A. mediterranei* (strain HGF003) with 3-amino-2-fluorobenzoic acid **24** (fed 194 mg, 1.25 mmol L^{-1}). The fermentation was carried out in liquid medium providing compound **40** as a colorless solid (isolated amount = 25 mg). 1. HPLC: preparative HPLC (C18): solvent A = water + 0.1 % v/v formic acid, solvent B = MeOH + 0.1 % v/v formic acid; flow rate = 15.0 mL min^{-1}; gradient (*t* [min]/solvent B [%]): 0/20; 5/20; 50/55; 90/100; 100/100; t_R = 39.0 min. 2. HPLC: semi-preparative HPLC (C18-ISIS): solvent A = water, solvent B = MeOH; flow rate = 2.5 mL min^{-1}; gradient (*t* [min]/solvent B [%]): 0/5; 10/5; 20/10; 60/40; 90/100; 100/100; t_R = 46.0 min. 3. HPLC: preparative HPLC (C18): solvent A = water + 0.1 % v/v formic acid, solvent B = MeOH + 0.1 % v/v formic acid; flow rate = 15.0 mL min^{-1}; gradient (*t* [min]/solvent B [%]): 0/10; 10/10; 20/10; 60/40; 100/100; t_R = 68.0 min. ^{1}H NMR (400 MHz, CD_3OD): δ = 6.90 (t, *J* = 7.8 Hz, 1H, H-12), 6.78–6.71 (m, 2H, H-13, H-11), 6.06 (s, 1H, H-4), 5.09 (d, *J* = 8.9 Hz, 1H, H-7), 2.90 (dq, *J* = 14.4, 7.2 Hz, 1H, H-6), 1.86 (s, 3H, H-14), 1.01 ppm (d, *J* = 7.0 Hz, 3H, H-15); ^{13}C NMR (100 MHz, CD_3OD): δ = 169.2 (s, C3), 168.2 (s, C5), 165.7 (s, C1), 150.7 (s, C9), 136.7 (s, C10), 130.5 (s, C8), 125.3 (d, C12), 117.4 (d, C13), 117.2 (d, C11), 102.5 (d, C4), 99.2 (s, C2), 70.3 (d, C7), 46.8 (d, C6), 15.3 (q, C15), 8.3 ppm (q, C14); UPLC-MS (MeOH) t_R = 2.3 min; HRMS-ESI: *m/z* calcd for $C_{15}H_{17}FNO_4$: 294.1142 $[M+H]^+$; found: 294.1140.

Tetraketide **41**: Tetraketide **41** was obtained after supplementing a culture of the blocked mutant of *A. mediterranei* (strain HGF003) with 3-amino-4-fluorobenzoic acid **28** (fed 194 mg, 1.25 mmol L^{-1}). The fermentation was carried out in liquid medium providing compound **41** as a colorless solid (isolated amount = 20 mg). 1. HPLC: preparative HPLC (C18): solvent A = water + 0.1 % v/v formic acid, solvent B = MeOH + 0.1 % v/v formic acid; flow rate = 15.0 mL min^{-1}; gradient (*t* [min]/solvent B [%]): 0/20; 5/20; 50/55; 90/100; 100/100; t_R = 46.0 min. 2. HPLC: semi-preparative HPLC (C18-ISIS): solvent A = water, solvent B = MeOH; flow rate = 2.5 mL min^{-1}; gradient (*t* [min]/solvent B [%]): 0/5; 10/5; 20/10; 60/40; 90/100; 100/100; t_R = 50.0 min. ^{1}H NMR (400 MHz, CD_3OD): δ = 6.90 (dd, *J* = 11.2, 8.3 Hz, 1H, H-12), 6.83 (dd, *J* = 8.7, 2.1 Hz, 1H, H-9), 6.60 (ddd, *J* = 8.3, 4.4, 2.1 Hz, 1H, H-13), 6.04 (s, 1H, H-4), 4.62 (d, *J* = 8.8 Hz, 1H, H-7), 2.79 (dq, *J* = 14.1, 7.1 Hz, 1H, H-6), 1.86 (s, 3H, H-14), 0.95 ppm (d, *J* = 7.0 Hz, 3H, H-15); ^{13}C NMR (100 MHz, CD_3OD): δ = 169.2 (s, C3), 168.1 (s, C5), 166.0 (s, C1), 152.7 (s, C11), 140.1 (s, C8), 136.7 (s, C10), 117.4 (d, C13), 116.7 (d, C9), 115.6 (d, C12), 102.3 (d, C4), 99.2 (s, C2), 76.6 (d, C7), 47.3 (d, C6), 15.6 (q, C15), 8.3 ppm (q, C14); UPLC-MS (MeOH) t_R = 2.4 min; HRMS-ESI: *m/z* calcd for $C_{15}H_{17}FNO_4$: 294.1142 $[M+H]^+$; found: 294.1144.

Tetraketide **42**: Tetraketide **42** was obtained after supplementing a culture of the blocked mutant of *A. mediterranei* (strain HGF003) with 3-amino-5-(azidomethyl)benzoic acid **30** (fed 240 mg, 1.25 mmol L^{-1}). The fermentation was carried out in liquid medium providing compound **42** as a colorless solid (isolated amount = 2.5 mg). 1. HPLC: preparative HPLC (C18): solvent A = water + 0.1 % v/v formic acid, solvent B = MeOH + 0.1 % v/v formic acid; flow rate = 15.0 mL min^{-1}; gradient (*t* [min]/solvent B [%]): 0/20; 5/20; 50/55; 90/100; 100/100; t_R = 36.0 min. 2. HPLC: semi-preparative HPLC (C18-ISIS): solvent A = water, solvent B = MeOH; flow rate = 2.5 mL min^{-1}; gradient (*t* [min]/solvent B [%]): 0/10; 5/10; 50/35; 90/80; 100/100; t_R = 49.0 min. ^{1}H NMR (500 MHz, CD_3OD): δ = 6.69 (t, *J* = 1.6 Hz, 1H, H-13), 6.65–6.60 (m, 2H, H-9, H-11), 6.04 (s, 1H, H-4), 4.64 (d, *J* = 8.8 Hz, H-7), 4.21 (s, 2H, $ArCH_2$), 2.81 (dq, *J* = 14.1, 7.1 Hz, H-6), 1.86 (s, 3H, H-14), 0.97 ppm (d, *J* = 7.1 Hz, H-15); ^{13}C NMR (125 MHz, CD_3OD): δ = 169.4 (s, C3), 168.9 (s, C5), 165.9 (s, C1), 149.6 (s, C10), 145.4 (s, C8), 137.9 (s, C12), 117.5 (d, C13), 115.7 (d, C11), 114.8 (d, C9), 102.7 (d, C4), 99.1 (s, C2), 77.0 (d, C7), 55.7 (t, $ArCH_2$), 47.3 (d, C6), 15.6 (q, C15), 8.3 ppm (q, C14); UPLCMS (MeOH) t_R = 2.54 min; HRMS-ESI: *m/z* calcd for $C_{16}H_{19}N_4O_4$: 331.1406 $[M+H]^+$; found: 331.1406.

Tetraketide 43: Tetraketide **43** was obtained after supplementing a culture of the blocked mutant of *A. mediterranei* (strain HGF003) with 3-amino-5-fluorobenzoic acid hydrochloride **31**·HCl (fed 239 mg, 1.25 mmol L^{-1}). The fermentation was carried out in liquid medium providing tetraketide **43** as a colorless solid (isolated amount = 2.5 mg). 1st HPLC: preparative HPLC (C18): solvent A = water + 0.1 % v/v formic acid, solvent B = MeOH + 0.1 % v/v formic acid; flow rate = 15.0 mL min^{-1}; gradient (*t* [min]/solvent B [%]): 0/20; 5/20; 50/55; 90/100; 100/100; t_R = 39.0 min. 2nd HPLC: semi-preparative HPLC (C18-ISIS): solvent A = water, solvent B = MeOH; flow rate = 2.5 mL min^{-1}; gradient (*t* [min]/solvent B [%]): 0/10; 5/10; 80/45; 90/100; 100/100; t_R = 62.0 min. 3rd HPLC: semi-preparative HPLC (C18-ISIS): solvent A = water, solvent B = MeOH; flow rate = 2.5 mL min^{-1}; gradient (*t* [min]/solvent B [%]): 0/10; 10/10; 20/30; 60/40; 90/90, 100/100; t_R = 43.0 min. ^{1}H NMR (500 MHz, CD_3OD): δ = 6.45 (t, *J* = 2.0 Hz, 1H, H-9), 6.35–6.32 (dt, *J* = 11.0, 2.0 Hz 1H, H-13), 6.31–6.29 (dt, *J* = 11.0, 2.0 Hz, 1H, H-11), 6.04 (s,

1H, H-4), 4.59 (d, J=8.7 Hz, H-7), 2.77 (dq, J=14.0, 7.1 Hz, H-6), 1.85 (s, 3H, H-14), 0.98 ppm (d, J=7.1 Hz, H-15); ^{13}C NMR (125 MHz, CD_3OD): δ=169.3 (s, C3), 168.8 (s, C5), 165.7 (s, C1), 165.3 (s, C12), 146.9 (s, C10), 146.8 (s, C8), 110.5 (d, C9), 103.0 (d, C13), 102.7 (s, C2), 101.8 (d, C11), 99.1 (d, C4), 76.7 (d, C7), 47.1 (d, C6), 15.6 (q, C15), 8.3 ppm (q, C14); UPLC-MS (MeOH) t_R=2.3 min; HRMS-ESI: m/z calcd for $C_{15}H_{17}FNO_4$: 294.1142 $[M+H]^+$; found: 294.1140.

Tetraketide 44: Tetraketide **44** was obtained after supplementing a culture of the blocked mutant of *A. mediterranei* (strain HGF003) with 3-amino-5-(trifluoromethyl)benzoic acid **32** (fed 256 mg, 1.25 mmol L^{-1}). The fermentation was carried out in liquid medium providing tetraketide **44** as a colorless solid (isolated amount= 1.0 mg). 1st HPLC: preparative HPLC (C18): solvent A=water+ 0.1% v/v formic acid, solvent B=MeOH+0.1% v/v formic acid; flow rate=15.0 mL min^{-1}; gradient (t [min]/solvent B [%]): 0/20; 5/ 20; 50/55; 90/100; 100/100; t_R=57.0 min. 2nd HPLC: preparative HPLC (C18): solvent A=water, solvent B=MeOH; flow rate= 15.0 mL min^{-1}; gradient (t [min]/solvent B [%]): 0/10; 5/10; 50/35; 90/100; 100/100; t_R=74.0 min. 3rd HPLC: semi-preparative HPLC (C18-ISIS): solvent A=water, solvent B=MeOH; flow rate= 2.5 mL min^{-1}; gradient (t [min]/solvent B [%]): 0/20; 5/20; 50/55; 90/100; 100/100; t_R=68.0 min. 4th HPLC: semi-preparative HPLC (C18-ISIS): solvent A- water, solvent B- MeOH; flow rate= 2.5 mL min^{-1}; gradient (t [min]/solvent B [%]):0/10; 5/10; 50/35; 90/ 100; 100/100; t_R=69.0 min. ^{1}H NMR (500 MHz, CD_3OD): δ=6.92– 6.88 (m, 1H, H-11), 6.86 (m, 2H, H-13, H-9), 6.01 (s, 1H, H-4), 4.72 (d, J=8.5 Hz, 1H, H-7), 2.81 (dq, J=14.0, 6.9 Hz, 1H, H-6), 1.87 (s, 3H, H-14), 1.00 ppm (d, J=7.1 Hz, 3H, H-15); ^{13}C NMR (125 MHz, CD_3OD): δ=179.4 (s, C3), 169.3 (s, C5), 165.5 (s, C1), 150.2 (s, C10), 146.0 (s, C8), 132.4 (s, C12), 125.8 (s, CF_3), 117.6 (d, C9), 112.8 (d, C13), 111.3 (d, C11), 102.7 (d, C4), 99.2 (s, C2), 76.6 (d, C7), 47.1 (d, C6), 15.3 (q, C15), 8.3 ppm (q, C14); UPLC-MS (MeOH) t_R=2.6 min; HRMS-ESI: m/z calcd for $C_{16}H_{17}F_3NO_4$: 344.1110 $[M+H]^+$; found: 344.1114.

Tetraketide 45: Tetraketide **45** was obtained after supplementing a culture of the blocked mutant of *A. mediterranei* (strain HGF003) with 3-amino-5-cyanobenzoic acid **33** (fed 102 mg, 0.63 mmol per 500 mL). The fermentation was carried out in liquid medium providing tetraketide **45** (isolated amount=3.5 mg, 7.0 mg L^{-1}) as a colorless solid. 1st HPLC: preparative HPLC (C18): solvent A= water+0.1% v/v formic acid, solvent B=MeOH+0.1% v/v formic acid; flow rate=15.0 mL min^{-1}; gradient (t [min]/solvent B [%]): 0/ 10; 5/10; 90/100; 100/100; t_R=41.0 min. 2nd HPLC: preparative HPLC (C18): solvent A=water, solvent B=MeOH; flow rate= 15.0 mL min^{-1}; gradient (t [min]/solvent B [%]): 0/20; 5/20; 70/50; 90/100; 100/100; t_R=51.0 min. 3rd HPLC: semi-preparative HPLC (C18-ISIS): solvent A=water, solvent B=MeOH; flow rate= 2.5 mL min^{-1}; gradient (t [min]/solvent B [%]): 0/20; 5/20; 70/50; 90/100; 100/100; t_R=21.0 min. 4th HPLC: semi-preparative HPLC (C18-ISIS): solvent A=water, solvent B=MeOH; flow rate= 2.5 mL min^{-1}; gradient (t [min]/solvent B [%]): 0/5; 5/5; 50/5; 90/ 100; 100/100; t_R=65.0 min. ^{1}H NMR (500 MHz, CD_3OD): δ=6.90– 6.86 (m, 2H, H-11, H-9), 6.84 (dd, J=2.1. 1.5 Hz, 1H, H-3), 6.04 (s, 1H, H-4), 4.65 (d, J=8.5 Hz, 1H, H-7), 2.81 (dq, J=14.2, 7.1 Hz, 1H, H-6), 1.86 (s, 3H, H-14), 0.99 ppm (d, J=7.1 Hz, 3H, H-15); ^{13}C NMR (125 MHz, CD_3OD): δ=169.3 (s, C3), 168.3 (s, C5), 165.6 (s, C1), 150.7 (s, C10), 146.7 (s, C8), 120.5 (s, CN), 119.8 (d, C11), 118.8 (d, C9), 117.4 (d, C13), 113.7 (s, C12), 102.7 (d, C4), 99.5 (s, C2), 76.4 (d, C7), 47.2 (d, C6), 15.6 (q, C15), 8.5 ppm (q, C14); UPLC-MS (MeOH) t_R=2.1 min; HRMS-ESI: m/z calcd for $C_{16}H_{17}N_2O_4$ 301.1188 $[M+H]^+$; found: 301.1140.

Tetraketide 46: Tetraketide **46** was obtained after supplementing a culture of the blocked mutant of *A. mediterranei* (strain HGF003) with 3-amino-6-fluorobenzoic acid **36** (fed 194 mg, 1.25 mmol L^{-1}). The fermentation was carried out in liquid medium providing tetraketide **46** as a colorless solid (isolated amount=1.1 mg). 1st HPLC: preparative HPLC (C18): solvent A=water+0.1% v/v formic acid, solvent B=MeOH+0.1% v/v formic acid; flow rate= 15.0 mL min^{-1}; gradient (t [min]/solvent B [%]): 0/20; 5/20; 50/55; 90/100; 100/100; t_R=26.0 min. 2nd HPLC: semi-preparative HPLC (C18-ISIS): solvent A=water, solvent B=MeOH; flow rate= 2.5 mL min^{-1}; gradient (t [min]/solvent B [%]): 0/10; 5/10; 80/45; 90/100; 100/100; t_R=50.0 min. ^{1}H NMR (500 MHz, CD_3OD): δ=8.25 (s, residual signal, OH), 7.64 (dd, J=6.3, 2.6 Hz, H-12), 7.61–7.57 (m, 1H, H-9), 7.09–7.02 (m, 1H, H-11), 5.95 (s, 1H, H-4), 5.15 (d, J= 8.4 Hz, 1H, H-7), 2.89 (dq, J=14.5, 7.0 Hz, 1H, H-6), 1.85 (s, 3H, H-14) 1.06 ppm (d, J=7.0 Hz, H-15); ^{13}C NMR (125 MHz, CD_3OD): δ= 164.2 (s, C3), 163.7 (C5) 161.5 (s, C1), 159.4 (s, C13), 135.4 (s, C10), 131.7 (s, C8), 122.3 (d, C11), 121.0 (d, C9), 117.0 (s, C2), 116.4 (d, C12), 98.0 (d, C4), 69.8 (d, C7), 46.8 (d, C6), 15.1 (q, C15), 8.6 ppm (q, C14); UPLC-MS (MeOH) t_R=2.2 min; HRMS-ESI: m/z calcd for $C_{15}H_{17}FNO_4$: 294.1142 $[M+H]^+$; found: 294.1130.

Tetraketide 49: Tetraketide **49** was obtained after supplementing a culture of the blocked mutant of *A. mediterranei* (strain HGF003) with 3-azido-5-hydroxybenzoic acid **47** (fed 168 mg, 0.94 mmol per 750 mL). The fermentation was carried out in liquid medium providing tetraketide **49** as brown crystals (isolated amount=35 mg; 47 mg L^{-1}). 1st HPLC: preparative HPLC (C18): solvent A=water+ 0.1% v/v formic acid, solvent B=MeOH+0.1% v/v formic acid; flow rate=15.0 mL min^{-1}; gradient (t [min]/solvent B [%]): 0/20; 5/ 20; 50/55; 90/100; 100/100; t_R=53.0 min. 2nd HPLC: preparative HPLC (C18-ISIS): solvent A=water, solvent B=MeOH; flow rate= 2.5 mL min^{-1}; gradient (t [min]/solvent B [%]): 0/10; 5.0/10; 50/55; 90/100; 100/100; t_R=55.0 min. ^{1}H NMR (400 MHz, CD_3OD): δ=8.06 (s, residual signal, OH), 6.53 (s, 1H, 9-H), 6.46 (s, 1H, 13-H), 6.33 (t, J=2.1 Hz, 1H, 11-H), 6.01 (s, 1H, 4-H), 4.62 (d, J=8.4 Hz, 1H, 7-H), 3.89 (s, residual signal, OH), 2.76 (dq, J=8.3, 7.1 Hz, 1H, 6-H), 1.81 (s, 3H, 14-H), 0.95 ppm (d, J=7.1 Hz, 3H, 15-H); ^{13}C NMR (100 MHz, CD_3OD): δ=169.1 (s, C3), 167.8 (s, C5), 165.6 (s, C1), 159.9 (s, C12), 147.1 (s, C10), 142.41 (s, C8), 112.0 (d, C9), 109.3 (d, C11), 106.3 (d, C13), 102.3 (s, C2), 99.3 (d, C4), 76.5 (d, C7), 47.1 (d, C6), 15.3 (q, C15), 8.3 ppm (q, C14); UPLC-MS (MeOH) t_R=2.3 min; HRMS-ESI: m/z calcd for $C_{15}H_{16}N_3O_5$ 318.1090 $[M+H]^+$; found: 318.1080.

Tetraketide 52: Tetraketide **52** was obtained after supplementing a culture of the blocked mutant of *A. mediterranei* (strain HGF003) with 3,5-dinitrobenzoic acid **50** (fed 27 mg, 0.12 mmol per 100 mL) and 3-amino-5-nitrobenzoic acid **51** (fed 57 mg, 0.31 mmol per 250 mL). The fermentations were carried out in liquid medium providing compound **52** as a yellow solid (isolated 7.0 mg; 70 mg L^{-1} and 18.0 mg; 72 mg L^{-1}, respectively). Fermentation with 3,5-dinitrobenzoic acid **50** (fed 50 mg, 0. 24 mmol per 125 mL; 10 mg per plate) carried out on agar plates also provided the title compound **52** (isolated amount=2.0 mg, 16 mg L^{-1}). 1st HPLC: semi-preparative HPLC (C18-ISIS): solvent A=water+0.1% v/v formic acid, solvent B=MeOH+0.1% v/v formic acid; flow rate=15.0 mL min^{-1}; gradient (t [min]/solvent B [%]): 0/20; 5.0/20; 50/55; 90/100; 100/ 100; t_R=18.0 min. 2nd HPLC: semi-preparative HPLC (C18-ISIS): solvent A=water, solvent B=MeOH; flow rate=2.5 mL min^{-1}; gradient (t [min]/solvent B [%]): 0/15; 5.0/15; 80/45; 90/100; 100/100; t_R=33.0 min. 3rd HPLC: semi-preparative HPLC (C18-ISIS): solvent A=water, solvent B=MeOH; flow rate=2.5 mL min^{-1}; gradient (t [min]/solvent B [%]): 0/5; 5.0/5; 15.0/5; 90/100; 100/100; t_R= 68.0 min. ^{1}H NMR (400 MHz, CD_3OD): δ=7.51–7.43 (m, 1H, H-13), 7.40 (t, J=2.2 Hz, 1H, H-11), 7.00–6.94 (m, 1H, H-9), 6.05 (s, 1H, H-

4), 4.76 (d, $J=8.3$ Hz, 1H, H-7), 4.59 (broad residual signal, 1H, OH), 2.86 (dq, $J=8.3$, 7.1 Hz, 1H, H-6), 1.87 (s, 3H, H-14), 1.03 ppm (d, $J=7.1$ Hz, 3H, H-15); ^{13}C NMR (100 MHz, CD_3OD): $\delta=171.4$ (s, C3), 169.8 (s, C5), 164.7 (s, C1), 150.9 (s, C12), 150.7 (s, C10), 146.6 (s, C8), 119.8 (d, C11), 110.6 (d, C13), 109.3 (d, C4), 108.5 (d, C9), 98.5 (s, C2), 76.2 (d, C7), 47.0 (d, C6), 15.2 (q, C15), 8.5 ppm (q, C14); UPLC-MS (MeOH) $t_R=2.5$ min; HRMS-ESI: *m/z* calcd for $C_{15}H_{17}N_2O_6$ 321.1087 $[M+H]^+$; found: 354.1065.

Tetraketide 56: Tetraketide **56** was obtained after supplementing a culture of the blocked mutant of *A. mediterranei* (strain HGF003) with 3-amino-4-bromobenzoic acid **55** (fed 214 mg, 0.99 mmol per 800 mL). The fermentation was carried out in liquid medium providing tetraketide **56** as a colorless solid (isolated amount = 15.0 mg, 19.0 mg L^{-1}). The yield is better than the one obtained after fermentation on agar plates (fed 300 mg, 0. 94 mmol per 750 mL; 10 mg per plate; isolated amount = 7.0 mg/750 mL, 9.0 mg L^{-1}). 1st HPLC: preparative HPLC (C18): solvent A = water + 0.1% v/v formic acid, solvent B = MeOH + 0.1% v/v formic acid; flow rate = 15.0 mL min^{-1}; gradient (*t* [min]/solvent B [%]): 0/20; 5/20; 50/55; 90/100; 100/100; $t_R=50.0$ min. 2nd HPLC: preparative HPLC (C18): solvent A = water, solvent B = MeOH; flow rate = 15.0 mL min^{-1}; gradient (*t* [min]/solvent B [%]): 0/10; 5/10; 50/55; 90/100; 100/100; $t_R=56.0$ min. 3rd HPLC: semi-preparative HPLC (C18-ISIS): solvent A = water, solvent B = MeOH; flow rate = 2.5 mL min^{-1}; gradient (*t* [min]/solvent B [%]): 0/10; 5/10; 80/45; 90/100; 100/100; $t_R=64.0$ min. ^{1}H NMR (400 MHz, CD_3OD): $\delta=7.33$ (d, $J=8.2$ Hz, 1H, H-12), 6.82 (d, $J=2.0$ Hz, 1H, H-9), 6.54 (dd, $J=8.2$, 2.0 Hz, 1H, H-13), 6.04 (s, 1H, H-4), 4.63 (d, $J=8.7$ Hz, 1H, H-7), 2.81 (dq, $J=14.1$, 7.0 Hz, 1H, H-6), 1.87 (s, 3H, H-14), 0.99 ppm (d, $J=7.1$ Hz, 3H, H-15); ^{13}C NMR (100 MHz, CD_3OD): $\delta=169.2$ (s, C1), 168.2 (s, C3), 165.8 (s, C-5), 146.5 (s, C10), 144.3 (s, C8), 133.3 (d, C12), 118.1 (d, C13), 115.4 (d, C9), 108.9 (s, C11), 102.4 (d, C4), 99.2 (s, C2), 76.6 (d, C7), 47.2 (d, C6), 15.5 (q, C15), 8.3 ppm (q, c-14); UPLC-MS (MeOH) $t_R=2.6$ min; HRMS-ESI: *m/z* calcd for $C_{15}H_{17}BrNO_4$ 354.0341 $[M+H]^+$; found: 354.0341.

The undecaketide **59** was also present in the crude fermentation material as judged by UPLC-HRMS analysis: **59**: *m/z* calcd for $C_{35}H_{53}BrNO_9$: 710.2904 $[M+H]^+$; found: 710.2906; *m/z* calcd for $C_{35}H_{51}BrNO_9$: 708.2753 $[M-H]^-$; found: 708.2751; $t_R=3.34$ min. For methyl ester formation, see the Supporting Information.

3-Acetamido-2-hydroxybenzamide 54: 3-Acetamido-2-hydroxybenzamide **54** was obtained after supplementing a culture of the blocked mutant of *A. mediterranei* HGF003 with 3-azido-2-hydroxybenzoic acid **53** (fed 224 mg, 1.25 mmol L^{-1}). The fermentation was carried out in liquid medium providing compound **54** as a colorless solid (isolated amount = 11.1 mg). 1st HPLC: preparative HPLC (C18): solvent A = water + 0.1% v/v formic acid, solvent B = MeOH + 0.1% v/v formic acid; flow rate = 15.0 mL min^{-1}; gradient (*t* [min]/solvent B [%]): 0/20; 5/20; 50/55; 90/100; 100/100; $t_R=$ 23.0 min. 2nd HPLC: preparative HPLC (C18): solvent A = water, solvent B = MeOH; flow rate = 15.0 mL min^{-1}; gradient (*t* [min]/solvent B [%]): 0/10, 20/15, 60/25, 90/45, 100/100; $t_R=41.0$ min. 3rd HPLC: semi-preparative HPLC (C18-ISIS): solvent A = water, solvent B = MeOH; flow rate = 2.5 mL min^{-1}; gradient (*t* [min]/solvent B [%]): 0/10, 20/15, 60/15, 90/45, 100/100; $t_R=7.0$ min. 4th HPLC: semi-preparative HPLC (C18-ISIS): solvent A = water, solvent B = MeOH; flow rate = 2.5 mL min^{-1}; gradient (*t* [min]/solvent B [%]): 0/5, 20/10, 60/10, 90/45, 100/100; $t_R=7.0$ min. ^{1}H NMR (400 MHz, $[D_6]$DMSO, $[D_6]$DMSO = 2.5 ppm): $\delta=13.94$ (s, 1H, OH), 9.21 (s, 1H, NHAc), 8.67 (s, 1H, $CONH_2$), 8.07 (dd, $J=7.8$, 1.0 Hz, 1H, H-6), 7.93 (s, 1H, $CONH_2$), 7.56 (dd, $J=8.0$, 1.2 Hz, 1H, H-4), 6.75 ppm (t, $J=$ 8.0 Hz, 1H, H-5); ^{13}C NMR (100 MHz, CD_3OD): $\delta=174.5$ (s, $CONH_2$), 171.9 (s, $NHCOCH_3$), 154.2 (s, C2), 128.6 (s, C3), 127.0 (d, C4), 124.1 (d, C6), 118.7 (d, C5), 115.3 (s, C1), 23.8 ppm (q, $NHCOCH_3$); UPLC-MS (MeOH) $t_R=1.9$ min; HRMS-ESI: *m/z* calcd for $C_9H_{11}N_2O_3$ 195.0770 $[M+H]^+$; found: 195.0768; *m/z* calcd for $C_9H_9N_2O_3$ 193.0619 $[M-H]^-$; found: 193.0612.

Acknowledgements

This work has been carried out as an integral part of the BIOFABRICATION FOR NIFE Initiative, which is financially supported by the ministry of Lower Saxony and the Volkswagen Stiftung. We are thankful to Heinz G. Floss for providing mutant strain HGF003 and for helpful discussions when preparing this manuscript. We thank Davina Guhr and Christian Bartens for expert technical assistance.

Keywords: antibiotics · azides · mutasynthesis · natural products · polyketide synthase

[1] H. G. Floss, T.-W. Yu, *Chem. Rev.* **2005**, *105*, 621 – 632.
[2] a) W. Wehrli, M. Staehelin, *Bacteriol. Rev.* **1971**, *35*, 290 – 309; b) P. A. Aristoff, G. A. Garcia, P. D. Kirchhoff, H. D. H. Showalter, *Tuberculosis* **2010**, *90*, 94 – 118; c) A. Nigam, K. H. Almabruk, A. Saxena, J. Yang, U. Mukherjee, H. Kaur, P. Kohli, R. Kumari, P. Singh, L. N. Zakharov, Y. Singh, T. Mahmud, R. Lal, *J. Biol. Chem.* **2014**, *289*, 21142 – 21152; d) M. R. K. Ali, S. R. Panikkanvalappil, M. A. El-Sayed, *J. Am. Chem. Soc.* **2014**, *136*, 4464 – 4467.
[3] a) C. T. Walsh, S. W. Haynes, B. D. Ames, *Nat. Prod. Rep.* **2012**, *29*, 37 – 59; b) H. G. Floss, T.-W. Yu, K. Arakawa, *J. Antibiot.* **2011**, *64*, 35 – 44.
[4] H. G. Floss, T.-W. Yu, *Curr. Opin. Chem. Biol.* **1999**, *3*, 592 – 597.
[5] D. Hunziker, T.-W. Yu, C. R. Hutchinson, H. G. Floss, C. Koshla, *J. Am. Chem. Soc.* **1998**, *120*, 1092 – 1093.
[6] J. Xu, E. Wan, C.-J. Kim, H. G. Floss, T. Mahmud, *Microbiology* **2005**, *151*, 2515 – 2528.
[7] A. Stratmann, C. Toupet, W. Schilling, R. Traber, L. Oberer, T. Schupp, *Microbiology* **1999**, *145*, 3365 – 3375.
[8] T.-W. Yu, Y. Shen, Y. Doi-Katayama, L. Tang, C. Park, B. S. Moore, C. R. Hutchinson, H. G. Floss, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 9051 – 9056.
[9] a) S. Eichner, H. G. Floss, F. Sasse, A. Kirschning, *ChemBioChem* **2009**, *10*, 1801 – 1805; b) J. Hermane, I. Bułyszko, S. Eichner, F. Sasse, W. Collisi, A. Poso, E. Schax, J.-G. Walter, T. Scheper, K. Kock, C. Herrmann, P. Aliuos, G. Reuter, C. Zeilinger, A. Kirschning, *ChemBioChem* **2015**, *16*, 302 – 311.
[10] a) H. G. Menzella, T.-T. Tran, J. R. Carney, J. Lau-Wee, J. Galazzo, C. D. Reeves, C. Carreras, S. Mukadam, S. Eng, Z. Zhong, P. B. M. W. M. Timmermans, S. Murli, G. W. Ashley, *J. Med. Chem.* **2009**, *52*, 1518 – 1521; b) W. Kim, J. S. Lee, D. Lee, X. F. Cai, J. C. Shin, K. Lee, C.-H. Lee, S. Ryu, S.-G. Paik, J. J. Lee, Y.-S. Hong, *ChemBioChem* **2007**, *8*, 1491 – 1494.
[11] a) K. Harmrolfs, L. Mancuso, B. Thomaszewski, F. Sasse, A. Kirschning, *Beilstein J. Org. Chem.* **2014**, *10*, 535 – 543; b) S. Eichner, T. Knobloch, H. G. Floss, J. Fohrer, K. Harmrolfs, J. Hermane, A. Schulz, F. Sasse, P. Spiteller, F. Taft, A. Kirschning, *Angew. Chem. Int. Ed.* **2012**, *51*, 752 – 757; *Angew. Chem.* **2012**, *124*, 776 – 781; c) T. Knobloch, G. Dräger, W. Collisi, F. Sasse, A. Kirschning, *Beilstein J. Org. Chem.* **2012**, *8*, 861 – 869; d) T. Knobloch, H. G. Floss, K. Harmrolfs, T. Knobloch, F. Sasse, F. Taft, B. Thomaszewski, A. Kirschning, *ChemBioChem* **2011**, *12*, 540 – 547; e) K. Harmrolfs, M. Brünjes, G. Dräger, H. G. Floss, F. Sasse, F. Taft, A. Kirschning, *ChemBioChem* **2010**, *11*, 2517 – 2520; f) F. Taft, M. Brünjes, T. Knobloch, H. G. Floss, A. Kirschning, *J. Am. Chem. Soc.* **2009**, *131*, 3812 – 3813; g) T. Taft, M. Brünjes, H. G. Floss, N. Czempinski, S. Grond, F. Sasse, A. Kirschning, *ChemBioChem* **2008**, *9*, 1057 – 1060; h) T. Kubota, M. Brünjes, T. Frenzel, J. Xu, A. Kirschning, H. G. Floss, *ChemBioChem* **2006**, *7*, 1221 – 1225.
[12] a) A. Kirschning, F. Hahn, *Angew. Chem. Int. Ed.* **2012**, *51*, 4012 – 4022; *Angew. Chem.* **2012**, *124*, 4086 – 4096; b) J. Kennedy, *Nat. Prod. Rep.* **2008**, *25*, 25 – 34; c) A. Kirschning, F. Taft, T. Knobloch, *Org. Biomol. Chem.* **2007**, *5*, 3245 – 3259; d) S. Weist, R. F. Süssmuth, *Appl. Microbiol. Biotechnol.* **2005**, *68*, 141 – 150.
[13] S. J. Admiraal, C. T. Walsh, C. Khosla, *Biochemistry* **2001**, *40*, 6116 – 6123.

[14] Analyzed by blastp:BLOSUM62 matrix; The rif loading domain is rather unique, as Walsh and Khosla demonstrated in ref. [13]. The rif loading domain functions like a NRPS module, activating AHBA as the adenylate and then charging it onto ACP_L.
[15] O. Ghisalba, H. Fuhrer, W. J. Richter, S. Moss, *J. Antibiot.* **1981**, *34*, 58 – 63.
[16] P. R. August, L. Tang, Y. J. Yoon, S. Ning, R. Müller, T.-W. Yu, M. Taylor, D. Hoffmann, C.-G. Kim, X. Zhang, C. R. Hutchinson, H. G. Floss, *Chem. Biol.* **1998**, *5*, 69 – 79.
[17] C.-G. Kim, A. Kirschning, P. Bergon, P. Zhou, E. Su, B. Sauerbrei, S. Ning, Y. Ahn, M. Breuer, E. Leistner, H. G. Floss, *J. Am. Chem. Soc.* **1996**, *118*, 7486 – 7491.
[18] S. Eichner, T. Eichner, H. G. Floss, J. Fohrer, E. Hofer, S. Florenz, C. Zeilinger, A. Kirschning, *J. Am. Chem. Soc.* **2012**, *134*, 1673 – 1679.
[19] L. Mancuso, G. Jürjens, J. Hermane, K. Harmrolfs, S. Eichner, J. Fohrer, W. Collisi, F. Sasse, A. Kirschning, *Org. Lett.* **2013**, *15*, 4442 – 4445.
[20] It should be noted that enzyme-bound **49** may alternatively be reduced in competition with its release, and the reduced product further reacts to form rifamycins.
[21] For the natural hydroxymethyl derivative, Hartung et al. reported the same absolute configurations by feeding all four possible stereoisomers of the natural diketide activated as SNAC (two enatiomeric syn-AHBA diketides and two enatiomeric anti-AHBA diketides) ester and analyzed which stereoisomer was further processed: I. V. Hartung, M. A. Rude, N. A. Schnarr, D. Hunziker, C. Khosla, *J. Am. Chem. Soc.* **2005**, *127*, 11202 – 11203. It is very likely that all new tetraketides reported herein adopt the *erythro* configuration found by us and Hartung et al.
[22] H.-H. Lu, A. Raja, R. Franke, D. Landsberg, F. Sasse, M. Kalesse, *Angew. Chem. Int. Ed.* **2013**, *52*, 13549 – 13552; *Angew. Chem.* **2013**, *125*, 13791 – 13795.
[23] R. Perez-Jimenez, A. Inglés-Prieto, Z.-M. Zhao, I. Sanchez-Romero, J. Alegre-Cebollada, P. Kosuri, S. Garcia-Manyes, T. J. Kappock, M. Tanokura, A. Holmgren, J. M. Sanchez-Ruiz, E. A. Gaucher, J. M. Fernandez, *Nat. Struct. Mol. Biol.* **2011**, *18*, 592 – 596.
[24] a) C. Oberhauser, Masters thesis, Leibniz Universität Hannover, **2015**; b) G. Jürjens, Ph.D. thesis, Leibniz Universität Hannover, **2014**.
[25] J. Leonard, B. Lygo, G. Procter, *Praxis in der organischen Chemie*, VCH, Weinheim, **1996**.
[26] O. Ghisalba, P. Traxler, J. Nüesch, *J. Antibiot.* **1978**, *31*, 1124 – 1131.
[27] a) E. Rubio, I. Merino, A. Garcia, M. Cabal, C. Ribas, M. Bayod-Jasanada, *Magn. Reson. Chem.* **2005**, *43*, 269 – 282; b) L. Santos, M. A. Medeiros, A. Santos, M. C. Costa, R. Tavares, M. J. M. Cutro, *J. Mol. Struct.* **2001**, *563 – 564*, 61 – 78.

Received: September 5, 2015
Published online on November 12, 2015