Antibiotics

Oak-Associated Negativicute Equipped with Ancestral Aromatic Polyketide Synthase Produces Antimycobacterial Dendrubins

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Abstract: Anaerobic bacteria have only recently been recognized as a source of antibiotics; yet, the metabolic potential of Negativicutes (Gram-negative staining Firmicutes) such as the oak-associated *Dendrosporobacter quercicolus* has remained unknown. Genome mining of *D. quercicolus* and phylogenetic analyses revealed a gene cluster for a type II polyketide synthase (PKS) complex that belongs to the most ancestral enzyme systems of this type. Metabolic profiling, NMR analyses, and stable-isotope labeling led to the discovery of a new family of anthraquinone-type polyphenols, the dendrubins, which are diversified by acylation, methylation, and dimerization. Dendrubin A and B were identified as strong antibiotics against a range of clinically relevant, human-pathogenic mycobacteria.

Microbes represent a major source of biologically active natural products that may be developed into therapeutics such as antibiotics. The increasing dereplication of known compounds from typical producers, however, has necessitated the exploration of yet under-investigated microbial sources.[1] Genome analyses have suggested that anaerobic bacteria, which have been known for their ability to produce solvents and protein toxins, have the potential to synthesize a range of secondary metablites.[2] Examples of antimicrobial secondary metabolites discovered from anaerobic bacteria include closthioamide,[3] clostrubin,[4] barnesin,[5] clostrindolin,[6] clostrocyloin,[6] and wexrubicin.[7]

All of these anaerobe-derived antimicrobials were isolated from the class Clostridia, and one example involves Epsilonbacteria. Yet, other classes of anaerobes have not yet been explored for their potential to produce secondary metabolites. A particularly intriguing and little explored class of bacteria in the phylum Firmicutes are the Negativicutes, which are characterized as Gram-positive bacteria with an unusual cell wall (Gram-negative staining) that inhabit a wide variety of niches, including aquatic environments, saprolites, and intestines.[8] A remarkable member of the Negativicutes is *Dendrosporobacter quercicolus*, which was isolated from discolored heart wood of oak trees.[9] Yet, nothing has been known about the metabolic potential of this rare plant-associated anaerobe. Here we report that *D. quercicolus* harbors genes for an ancestral type II PKS and produces a range of polyphenols, of which some show potent and highly selective activity against a number of clinically relevant mycobacteria.

To evaluate the biosynthetic potential of *D. quercicolus*, we sequenced its genome (JABTVI000000000) and searched for gene clusters coding for secondary metabolite biosynthetic pathways (Independently, the DOE Joint Genome Institute submitted a genome sequence as GCA 900104455.1). In the *D. quercicolus* genome we noted a gene locus (*den*) tentatively coding for a type II polyketide synthase (PKS). Specifically, the *den* gene cluster comprises genes (*denABC*) encoding for the minimal PKS, ketosynthase (KS_α), chain-length factor (CLF, also referred to as KS_β), and an acyl carrier protein (ACP), which are the minimal requirement for aromatic polyketide biosynthesis in bacteria.[10] Furthermore, we identified genes for two cyclases (*denD*, *denF*), and an oxidoreductase (*denE*), in addition to several regulator and transporter genes (Figure 1 A, Table S1). Typically, type II PKSs are found in actinomycetes, Gram-positive, and aerobic bacteria.[10] The *den* type II PKS (DenA-F) is rather unrelated to these systems. A database search (BlastP) using the DenB sequence revealed three orthologous gene clusters (Tables S1 and S2). The gene cluster with highest similarity to the *den* locus is harbored in the genome of the Negativicute *Methyromusa anaerophila* (Table S2). Moreover, the deduced *den* type II PKS is remarkable as it has a specific architecture (KS-CLF-ACP-TcmN-TcmN-OxyN) that points to a partic-

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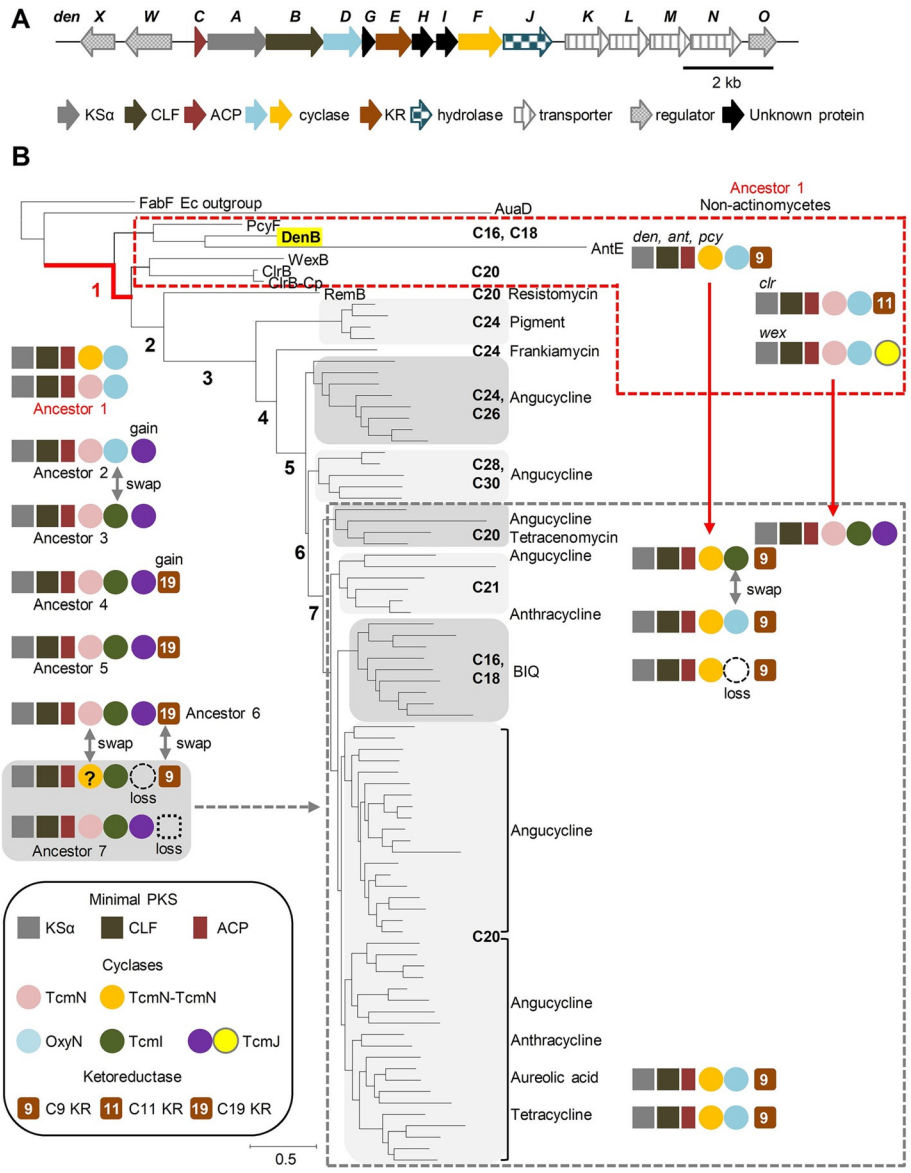


Figure 1. Gene cluster encoding a type II PKS in *Dendrosporobacter quercicolus* and its phylogenetic placement. A) Organization of the *den* gene locus. B) Phylogenetic tree of deduced *den* chain length factor (CLF) and related CLFs. Proposed evolution of type II PKS, annotated in analogy to the report of Hillenmeyer et al. The ancestor numbers guide the proposed key ancestoral type II PKS gene clusters. The red dotted area (ancestor 1) indicates the most anciently diverged type II PKS gene clusters and the gray dotted area are the more recently diverged gene clusters. The scale bar indicates amino acid substitutions per site. Gray coloration was used to emphasize the clade borders. Clades correspond with polyketide chain length formed in the KSα-CLF cavity and the polyphenol scaffolds. BIQ; benzoisochromanequinones.

ular evolutionary placement. On the basis of bioinformatics analyses it has been proposed that such type II PKS systems from non-actinomycete are ancestral.[12]

To corroborate the evolutionary relationship between the type II PKS encoded in the genome of *D. quercicolus* and other bacterial type II PKSs, we performed a phylogenetic analysis of representative PKS components, KSα and CLF using the maximum likelihood (ML) algorithm (Figure 1 B, Figures S1–S4). Our phylogenetic analysis revealed that a C20 clade of ClrB, ClrB Cp (clostrubin),[4] and WexB (wexrubicin)[7] from clostridia fall into an ancestral clade upstream of RemB (resistomycin)[13] from actinomycetes (Figure 1 B, Figure S1). A C16/C18 clade composed of DenB (dendrubin) from the Negativicute, AntE (AQ-256) from γ-Proteobacteria,[11c] and PcyF (pyxidicycline) from δ-Proteobacteria,[11d] which are all encoded in non-actinomycete genomes, are positioned further upstream of clostridial CLFs. Surprisingly, the gene clusters from Gram-negative bacteria and the *den* gene cluster code for KS-CLF-ACP-TcmN-TcmN-OxyN, in which the first cyclase TcmN (monomeric TcmN) is duplicated as TcmN-TcmN (dimeric TcmN) (Figures 1 B, S3–S4).

Hillenmeyer et al. suggested that monomeric TcmN and dimeric TcmN (TcmN-TcmN) diverged before type II PKSs proliferated in actinomycetes.[12] Consequently, the *ant* and *den* (*pcy*) gene clusters would represent ancestors of those for short-chain polyphenols, benzoisochromanequinones (BIQ), anthracyclines, tetracyclines and angucyclines in actinomycetes (Fig-

ures 1B and S5). This evolutionary model is also supported by our phylogenetic analysis of KSα (Figure S2). Taken together, these data allowed us to update the proposed type II PKS evolution route (Figures 1B and S5) and suggested that the products of this ancestral type II PKS were worth investigating.

HPLC analysis of red-pigmented *D. quercicolus* cultures (Figure 2A) revealed the presence of several phenolic compounds with absorption at 440 nm (Figure 2B). The crude ethyl acetate extract of a 5-L fermentation broth of *D. quercicolus* was subjected to open-column chromatography followed by preparative HPLC to afford six aromatic polyketides, compounds **1** (6.4 mg), **2** (14.3 mg), **3** (0.6 mg), **4** (3.7 mg), **5** (5.0 mg) and **6** (0.8 mg). Owing to their origin and color, the new compounds were named dendrubins A–F, respectively. Their structures were fully elucidated by a combination of mass spectrometry (MS) and 1D- and 2D-nuclear magnetic resonance (NMR) analyses (Figures S6–S49).

The HRMS of dendrubin A (**1**) shows a pseudo-molecular ion with *m/z* 337.0712 $[M-H]^-$, from which a sum formula of $C_{19}H_{14}O_6$ and thus 13 ring and double-bond equivalents were deduced (Figure S39). The ^1H NMR spectrum revealed the presence of three aromatic protons, three methyl groups and two phenolic hydrogens (Figure S7). All directly connected ^{13}C signals were assigned by an HSQC experiment (Figure S10). HMBC correlations from H-19 to C-18 (δ 198.0) and H-17 to C-16 (δ 202.6) assigned two of the methyl groups as parts of acetyl moieties (Figures S6 and S11, Table S5). An aromatic partial structure was established by a COSY correlation from H-7 to H-8, and HMBC correlations from H-7 and H-8 to C-9/C-11 and C-6/ C-10, respectively (Figures S6, S9, and S11). An HMBC correlation from H-8 to C-18 revealed that the first acetyl group is positioned in *ortho* position to C-8. Based on its ^{13}C chemical shift (δ 162.2), C-10 is connected to an oxygen atom, which could be verified by HMBC correlations from a phenolic singlet proton (δ 13.00) at C-10 to C-9, C-10, and C-11 (Figure S6). Thus, we identified the first partial structure as 5,6-substituted 2-acetylphenol. The second phenolic ring was inferred from HMBC correlations of H-3 to C-13 and C-15, and the phenolic singlet hydrogen (δ 12.23) at C-14 (δ 159.5) to C-13, C-14, and C-15. Furthermore, HMBC correlations of H-1 to C-2, C-3, and C-15 as well as H-17 to C-15 indicated that the methyl group and the second acetyl group are connected to this ring system. Accordingly, the second partial structure was identified as 5,6-substituted 2-acetyl-3-methyl-phenol. The connection of the two aromatic partial structures was guided by HMBC correlations from H-7 and H-3 to the carbonyl C-5 (δ 181.0). Connecting carbonyl C-12 (δ 192.5) to C-7 and C-13 revealed the two missing ring and double-bond equivalents, and established the 1,8-dihydroxyanthrone scaffold of **1** (Figure 2C).

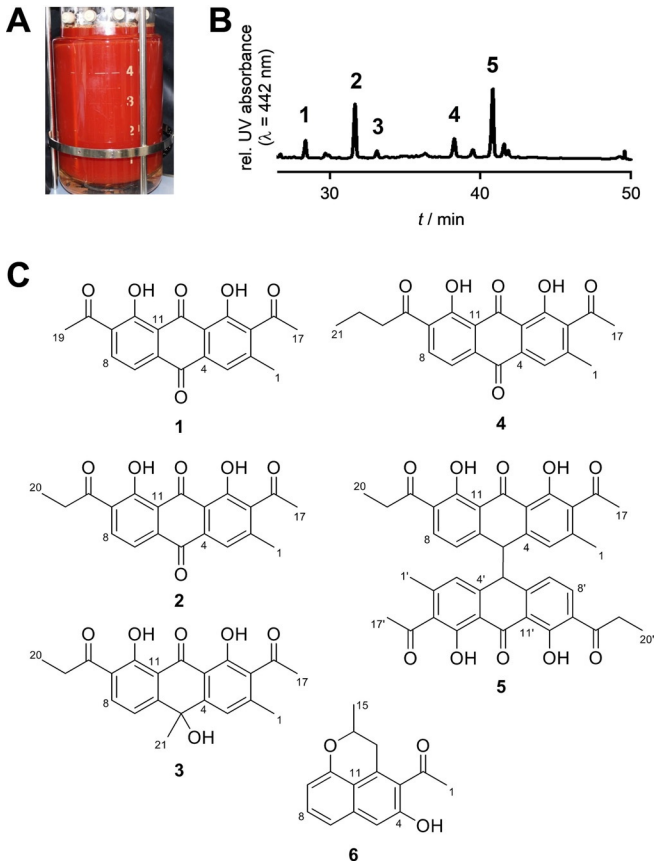


Figure 2. Metabolic profile and structures of polyphenols isolated from *D. quercicolus*. **A**) Photograph of fermenter showing red pigmentation of *D. quercicolus* culture. **B**) HPLC-profile of culture extract. **C**) Structures of dendrubins A–F (**1**–**6**).

Dendrubin B (**2**) has a sum formula of $C_{20}H_{16}O_6$ according to HRMS (*m/z* 351.0868 $[M-H]^-$) and thus formally differs from **1** by an additional methylene unit (Figure S41). In fact, comparison of NMR spectra indicated that **2** is a congener of **1** that bears a propionyl moiety in lieu of one acetyl group (Figures S6–S15). Three HMBC correlations of H-20, H19, and H-8 to C-18 elucidated the exact position (Figures S6 and S15). The comparison of NMR spectra of **4** with those of **1** and **2** revealed that dendrubin D (**4**) ($C_{21}H_{18}O_6$ *m/z* 365.1034 $[M-H]^-$) is a higher homologue of **1** and **2** that features a butyryl side chain (Figures S6, S21–S25, S45, Table S6).

NMR data of dendrubin C (**3**) suggested that this compound has the same overall scaffold as **2**, yet has an additional methyl group (H-21), which is supported by the deduced sum formula $C_{21}H_{20}O_6$ of **3** (Figures S16–S20, S43). Analysis of its ^{13}C NMR spectrum further revealed the presence of a quaternary carbon instead of an anthrone carbonyl at C-5 (Figure S17, Table S7). The chemical shift of C-5 (δ 70.5) as well as HMBC correlations of H-7, H-3, and H-21 to C-5 established the 4-hydroxy-4-methylcyclohexa-2,5-dien-1-one partial structure of **3** (Figures S6 and S20). Unfortunately, the low amount hampered the elucidation of the absolute configuration of **3**.

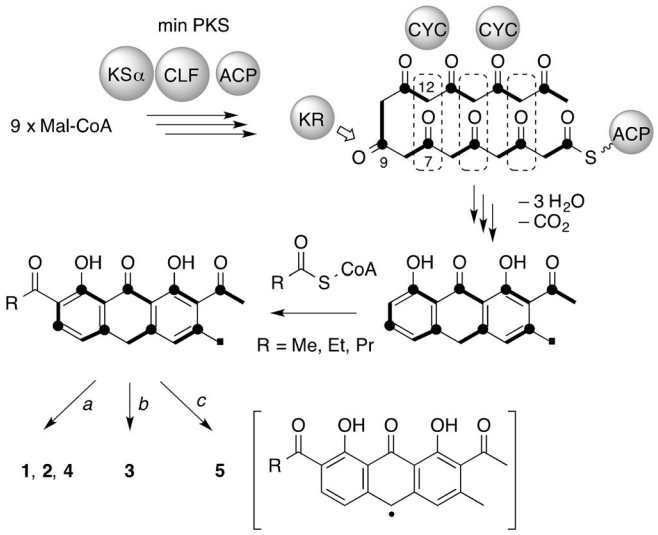
Dendrubin E (**5**) possesses a sum formula of $C_{40}H_{34}O_{10}$ based on HRMS analysis (*m/z* 673.2078 $[M-H]^-$) (Figure S47). Analysis of ^1H and ^{13}C data showed that the overall scaffold of **5** is similar to compound **2** but contains two methine carbons C-5 (δ 56.0) and C5′ (δ 56.2) instead of anthrone carbonyl groups at C-5 (Figures S26 and S27). HMBC correlations from H-5 to C-4′/ C-6′ and from H-5′ to C-4 and C-6 revealed that **5** is a dimer of **2** which is linked at position C-5 and C-5′ (Figures 2C, S6, S30, and Table S9).

In addition, we isolated a compound (dendrubin F, **6**) that appeared to be a biosynthetic shunt product. Extensive 2D

NMR data analyses of **6** suggested that **6** has a benzo[de]chromene backbone (Figures S6, S31–S35, and Table S10). Specifically, the structure was deduced from observed spin systems of aromatic protons H-7 to H-9 and methylene proton H-13 to methyl proton H15 in COSY spectrum, and the HMBC correlations from H-5 to C-3/C-7/C-11, H-7 to C-9/C-11, H-8 to C-6/C-10, and H-13 to C-3/C-11/C-12 (Figure S6). Two correlations from methyl proton H-1 to C-2/C-3 in HMBC spectrum connected an acetyl group to main backbone (Figure S6).

Although anthraquinone natural products are widely distributed in nature,[14] the structures of the dendrubins are new. Notably, the presence of two acyl side chains is a rare feature in anthraquinone natural products, and no structural analogues of the dendrubins with this acylation pattern have been reported.

To gain insight into the biosynthesis of the dendrubins and to correlate their production with the *den* gene cluster, we sought to generate a *D. quercicolus* null mutant that is deficient in an essential type II PKS gene. However, despite many attempts employing various genetic manipulation methods, including single-crossover and gene editing, it has not been possible to generate a knockout mutant. Apparently, *D. quercicolus* is not genetically tractable by established methods. Likewise, all attempts to heterologously express the *den* PKS genes and to obtain soluble proteins have been futile. As an alternative, we conducted a stable-isotope-labeling study with 1–^{13}C- and 1,2–$^{13}C_2$-enriched sodium acetate. The incorporation pattern (Scheme 1, Figures S36, S37, and Table S6) confirmed that dendrubins are aromatic polyketides. On the basis of the ^{13}C labeling experiments and the diverse structures of the isolated dendrubins we propose a biosynthetic Scheme (Scheme 1). The *den* PKS would form a nonaketide, which would undergo three cyclodehydration reactions to form the anthrone core. The labeling pattern indicates that the carboxy terminus of the nonaketide is truncated by decarboxylation as recently proposed for intermediates in pyxidicycline biosynthesis.[11d] Structural diversity may be introduced at the anthrone stage; oxygenation would lead to the corresponding anthraquinone,[15] methylation and hydroxylation would lead to the hydroxymethyl derivative **3** (dendrubin C, and radical dimerization would yield to the bis-anthrone **5** (dendrubin E). We have observed similar reaction channels, which depend on enzymatic methylation and oxidative power, in the biosynthesis of benastatins.[16] However the *den* gene cluster does not contain any methyltransferase and oxidase/oxygenase genes (Table S1). Thus, these genes are likely located elsewhere in the genome. The ^{13}C acetate labeling experiments (Scheme 1, Figures S35, S36, and Table S6) demonstrated that the second acyl side chain (acetyl, propyl, and butyl) of the dendrubins is not derived from type II PKS. Thus, these side chains may be introduced by an enzymatic Friedel–Crafts or Fries-like acylation catalyzed by an acyltransferase as in the biosynthesis of diacetylphloroglucinol (DHPG).[17] However, the *D. quercicolus* genome does not harbor any orthologues of the *Pseudomonas* acyltransferase gene. Moreover, there seems to be a degree of flexibility in this acylation step since propionyl and butyryl units may be installed in lieu of the acetyl group.



Scheme 1. Model of dendrubin biosynthesis on the basis of deduced gene functions and stable isotope labeling experiments. *a*, anthrone oxidation to anthraquinone; *b*, methylation/hydroxylation; *c*, dimerization of anthrone radicals.

Finally, all new compounds were subjected to a panel of whole-cell bioassays to evaluate their antimicrobial and antiproliferative/cytotoxic potencies. In agar diffusion experiments with *Bacillus subtilis*, *Staphylococcus aureus*, *Mycobacterium vaccae*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Sporobolomyces salmonicolor*, *Candida albicans* and *Penicillium notatum*, revealed main compounds **1** and **2** as selective antimycobacterial agents. In HeLa cell assays both compounds showed no cytotoxic effects up to the maximum test concentration of 50 μg mL^{-1}. Furthermore, the antiproliferative activity of **1** and **2** was only moderate against the leukemia cell line K-562, while compound **2** showed a GI_{50} of 3.6 μg mL^{-1} against HUVEC endothelial cells (Table S11). Owing to their low cytotoxicity the antimycobacterial activities of **1** and **2** were evaluated in more detail to obtain information on the scope and species-specific minimal inhibitory concentrations (MIC) (Figure 3 and Table S12). Whereas both compounds were inactive against *Mycobacterium chimaera* and *Mycobacterium abscessus*, *M. vaccae* is strongly inhibited with a MIC of 1.56 μg mL^{-1}. Compounds **1** and **2** inhibit *Mycobacterium fortuitum* with a MIC of 12.5 and 6.25 μg mL^{-1}, respectively. Lower potency was observed against *Mycobacterium smegmatis* and *Mycobacterium aurum*. Furthermore, **1** and **2** showed activity against *Mycobacterium marinum* with a MIC of 5.25 and 0.78 μg mL^{-1}, *Mycobacterium tuberculosis* H37Rv with a MIC of 3.13 and 0.78 μg mL^{-1} (compared to MIC of rifampicin: 0.12 μg mL^{-1}) and *Mycobacterium kansasii* with a MIC of 12.5 and 1.56 μg mL^{-1} respectively (Figure 3 and Table S12).

These findings are important as the reveal dendrubins as strong antibiotics against clinically relevant mycobacteria. Notably, *M. fortuitum* and *M. kansasii* are the causative agents of non-tuberculous mycobacterioses,[18] *M. vaccae* can cause sporadic cutaneous and pulmonary infections,[19] *M. marinum* is able to infect skin and soft tissue after injuries that are exposed to an aquatic environment or marine animals,[20] and *M.*

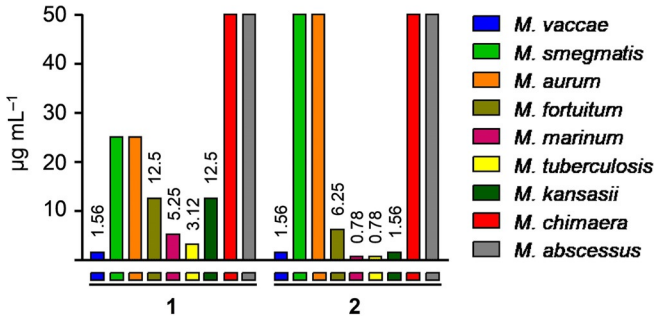


Figure 3. Antimycobacterial activities of compounds **1** and **2**.

tuberculosis is the causative agent of tuberculosis, a disease affecting currently one-third of the world population.[21] With the rise of multi-drug-resistant mycobacteria strains, the discovery of novel antimycobacterial agents is a important task in antimicrobial drug discovery.[22]

In summary, we report the discovery of an ancestral type II PKS through genome mining of oak-associated bacteria that belong to the little explored class of Negativicutes. The *den* PKS represents a rare example of an aromatic PKS from Negativicutes. Moreover, we identified dendrubins as a new family of antimycobacterial polyphenols produced by the anaerobe and propose their biosynthetic pathway based on bioinformatics and stable-isotope labeling experiments. The dendrubins are an important addition to the small number of known natural products from anaerobes and represent the first identified secondary metabolites from a Negativicute. Considering that anaerobic microbes were the first living beings on earth, these findings are intriguing from an evolutionary point of view, as dendrubins may represent ancestral natural products. More importantly, we identified dendrubins as potent antibiotics against a range of clinically relevant mycobacteria. Thus, this study not only consolidates anaerobes as promising source of novel natural products but also sets the basis for the development of urgently needed antimycobacterial agents.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: anaerobes · antibiotics · genome mining · mycobacteria · polyphenols

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