Natural Products Hot Paper

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Discovery of Clostrubin, an Exceptional Polyphenolic Polyketide Antibiotic from a Strictly Anaerobic Bacterium**

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Abstract: *Genome mining of the strictly anaerobic bacterium Clostridium beijerinckii, an industrial producer of solvents, revealed the presence of several cryptic gene clusters for secondary metabolite biosynthesis. To unearth its metabolic potential, a C. beijerinckii strain was cultured under various conditions, which led to the discovery of a deep purple pigment. This novel metabolite, named clostrubin (**1**), was isolated and its structure was fully elucidated. The pentacyclic polyphenol features a benzo[a]tetraphene ring topology that is unprecedented for natural products. Stable-isotope labeling experiments showed that **1** is an aromatic polyketide that folds in a noncanonical manner to form the unusual perifused ring system. In addition to being the first reported polyketide from an anaerobic bacterium, **1** is a potent antibiotic with pronounced activity against various pathogenic bacteria, such as MRSA, VRE, and mycobacteria, with minimum inhibitory concentrations (MIC) of 0.12–0.97 μM.*

Bacterial infectious diseases are a persistent global concern and causing millions of deaths annually. According to the WHO, the rapid emergence of resistant pathogens and the lack of new therapeutics portend a return to the pre-antibiotic era.[1] Increased infections caused by the hospital-acquired pathogens methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterocci (VRE), combined with the emergence of multidrug-resistant tuberculosis (MDR-TB), demand the urgent development of new antibiotic agents.[2,3] Natural products, in particular microbial secondary metabolites, are the most important source of pharmacologically active compounds.[4] Traditionally, actinomycetes have been regarded as the mainstay of natural-product discovery in bacteria.[5] However, a problem inherent in screening these well-investigated organisms is the high rediscovery rate of known compounds and scaffolds.[6] In addition to myxobacteria[7], neglected bacterial species, the secondary metabolic potential of which has been poorly studied, have thus been considered as alternative sources of new bioactive compounds.[8] Indeed, the analyses of sequenced bacterial genomes for gene clusters for secondary metabolite biosynthesis have shown that unidentified leads may be unearthed from obscure bacterial lineages.[9] Even bacteria that were once completely discounted as a source of natural products, such as the anaerobes, contain the genetic material for the production of a wide array of secondary metabolites.[10] This holds true for the clostridia, a heterogeneous group of Gram-positive spore-forming obligate anaerobes that includes well-known pathogens that produce severe neurotoxins and numerous non-pathogenic strains that are used on an industrial scale for cellulose degradation and solvent production.[11] Through the isolation of the rare polythioamide antibiotic closthioamide from *Clostridium cellulolyticum*, we have shown that these organisms can indeed produce unusual biologically active compounds with novel structural features.[12] Herein, we report the discovery of an unprecedented type of antibiotic from *Clostridium beijerinckii* and demonstrate its high potency against nosocomial pathogens such as MRSA, and VRE, and mycobacteria. We also report this new compound as the first polyketide from an anaerobic organism and demonstrate that the unusual polyphenolic ring system derives from a noncanonical chain-folding pattern.

In the course of investigating different *Clostridium* species for their potential to produce secondary metabolites, we noted that the genome of *C. beijerinckii* (NCIMB 8052, JGI project ID 3634512) harbors orphan secondary metabolite biosynthesis genes.[10a] Surprisingly, *C. beijerinckii* has been extensively used for the biotechnological production of the solvents acetone, butanol, and ethanol,[13] yet no secondary metabolite has been reported from this organism. Given the presence of secondary metabolite biosynthesis genes in the type strain *C. beijerinckii* (NCIMB 8052), we reasoned that other *C. beijerinckii* strains may also possess these genes, and because the industrially used strain was not readily available, we investigated a related *C. beijerinckii* strain from the HKI strain collection. Initial fermentation of *C. beijerinckii* (HKI0724) in liquid media showed the cultures to be deep red to purple in color, most likely owing to the formation of an unknown pigment. Interestingly, similarly colored *C. beijerinckii* strains have not been reported in the literature, and in general, pigments are not a common feature of *Clostridium* species. As such, we sought to identify the type and composition of this pigment.

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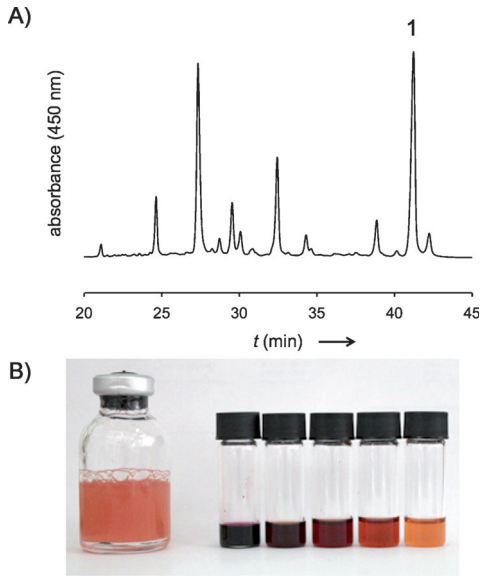

Figure 1. A) HPLC–UV/Vis profiles of ethyl acetate extracts of *C. beijerinckii* (HKI0724; wild-type); B) Liquid culture of *C. beijerinckii* (HKI0724) showing the pink color and gradient-diluted solutions of **1**.

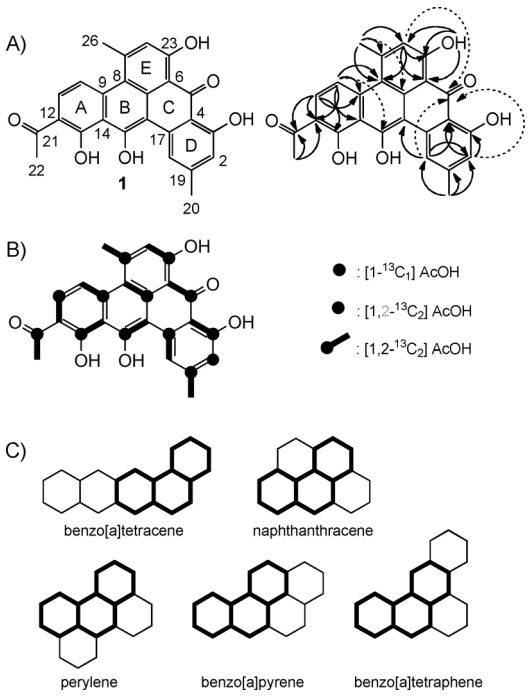

Figure 2. A) The structure of clostrubin A (**1**) and ^{2}J, ^{3}J (arrows) and ^{4}J (dashed arrows) HMBC correlations. B) Enriched carbon atoms observed in the ^{13}C NMR spectra following labeled-acetate feeding experiments. C) Ring topologies of pentacyclic polyphenols found in nature; the benzo[a]tetraphene scaffold is new.

HPLC analysis of ethyl acetate extracts of bacterial cultures revealed the presence of several peaks with UV/Vis maxima at 480 nm, consistent with a complex of compounds that appear visually pink or purple (Figure 1). Given the similar UV/Vis spectra, it appeared that these compounds were congeners. Extraction and isolation of these compounds proved to be unexpectedly arduous, with multiple rounds of ethyl acetate extraction failing to completely remove the purple color from the cells, and extraction with other solvents yielded similarly unsatisfactory results. The aggressive adhesive ability of the pigment was further observed during open-column processing with resins such as amberlite XAD16, silica gel, and octadecylsilyl (ODS). To minimize compound loss during purification, large-scale (20 L) fermentation cultures were extracted by pH-dependent partitioning of the aqueous and solvent phases, and washing with diverse organic solvents after precipitation on a glass filter. Finally, by using preparative HPLC, we succeeded in isolating 5 mg of the major component of the complex (**1**, at a retention time of 42 min) in sufficient purity for structural analysis.

From HRMS measurements (*m*/*z* 414.1101), we deduced that compound **1** has a molecular formula of $C_{25}H_{18}O_6$. ^{1}H and ^{13}C NMR spectra of **1** suggested that it is a polyphenolic compound since it shows limited proton signals and multiple aromatic carbon signals. Detailed analysis of the ^{1}H NMR spectrum indicated the presence of three methyl, five aromatic, and two phenolic protons. ^{13}C NMR and DEPT135 spectra revealed three methyl, five aromatic methine, and 17 quaternary carbon atoms, including four phenolic and two carbonyl carbon atoms. Extensive HMBC and HSQC analyses showed three ring systems; rings D and E, which are di- and trisubstituted cresols, respectively, and ring A, which is a disubstituted 1-(2-hydroxyphenyl)ethan-1-one (Figure 2). Ring D is connected to ring E by three ^{4}J HMBC correlations from three singlet methine protons; H24 (δ 6.80), H2 (δ 6.60), and H18 (δ 9.30); to keto carbonyl carbon C5 (δ 184.5). Two other ^{4}J HMBC correlations from doublet methine proton H10 (δ 7.60) to phenolic carbon C15 (δ 178.0) and methyl proton H26 (δ 2.90) to quaternary carbon C7 (δ 133.6) suggested the presence of ring C. Finally, five aromatic rings were determined by two ^{3}J HMBC correlations from H18 to C16 (δ 104.3) and H10 to C8 (δ 114.1). Only one quaternary carbon (C7) was not assigned because of a lack of HMBC correlations. To overcome this challenge and to rigorously determine the structure, we performed stable-isotope labeling. Cultures were grown anaerobically in minimal media and supplemented with either 1-^{13}C or 1,2-^{13}C labeled acetic acid, and the isotopically enriched metabolite was purified and analyzed by ^{13}C NMR spectroscopy. This process and ^{13}C chemical-shift prediction (Table S1 in the Supporting Information) confirmed the presence of a number of quaternary carbon atoms, the correlations of which could now be reliably assigned. Thus, we inferred the unusual pentacyclic structure of **1**, which was termed clostrubin after its origin and visual appearance.

The isotope labeling pattern revealed that the red pigment is a polyphenol of polyketide origin. Notably, **1** represents the first polyketide to be identified from a strictly anaerobic organism. Particularly striking is the benzo[a]tetraphene skeleton of **1**, which demarks a novel ring topology for a natural product. Previously described architectures of pentacyclic polyphenols comprise pentangular benzo[a]tetracene systems[14] (such as benastatin,[15] fredericamycin,[16] and griseorhodin[17]) and the rare discoid naphthanthrene deriv-

ative resistomycin.[18] Furthermore, perylene systems are known that result from the fusion of two naphthylene moieties, and recently, the natural benzo[a]pyrene benzopyranomycin was isolated from a *Streptomyces* species.[19] In light of the hundreds of known aromatic polyketides from aerobic bacteria, it is remarkable that the architecture of this anaerobe metabolite is unprecedented.

The structure of **1** is also intriguing from a biosynthetic perspective because the chain folding appears to be exceptional. Fortuitously, our stable-isotope labeling experiments grant first insight into the polyketide assembly. Although other scenarios would be conceivable, the orientation of the intact acetate units and the position of the isolated C2 acetate-derived carbon atom strongly suggest that **1** originates from a decaketide chain, which folds into an angucyclic precursor. However, the folding of the clostrubin polyketide chain seems to deviate from the cyclization patterns that have been observed in aerobic microorganisms. In typical angucyclic decaketides, the first cyclization is a C7/12 fusion, whereas a C9/14 connection yields the A ring of **1**. Furthermore, in all known polyphenol pathways, the polyketide chain folds upwards in relation to the D ring.[20] In stark contrast, the clostrubin polyketide chain folds downwards to give rise to this rare skeleton (Figure 3 A). The downstream biosynthetic pathway would involve several cyclodehydration steps and decarboxylation, which leads to the loss of one C1 carbon. The labeling experiments also imply that the polycyclic core is subject to acetylation at the A ring, and that the E ring could be formed by condensation with an activated acetoacetyl building block (Figure 3 B). These tailoring steps are in full accord with the ring-carbon reactivity of the proposed precursor.

Apart from its unusual structure, it is surprising to discover an aromatic polyketide like **1** in an anaerobic bacterium. Bacterial polyphenols are typically produced by type II polyketide synthases (PKS). The corresponding PKS genes are very frequently found in the genomes of actinomycetes,[21] with only two characterized examples of non-actinomycete type II PKS.[22] Surprisingly, a large survey of over 200 sequenced anaerobe genomes revealed that type II PKS gene clusters are absent in all of the genomes analyzed.[10a] Likewise, no type II PKS gene cluster could be detected in the sequenced industrial *C. beijerinckii* strain, and it appears that the isolate used in this study has a distinctive genetic trait. Full genome sequencing and elucidation of the PKS gene locus will be the subject of future investigations.

Finally, since aromatic polyketides often have antibiotic and/or antitumoral activities, we performed antibacterial and cytotoxicity assays with **1**. In a standardized antibiotic assay,[23] **1** proved to be superior to ciprofloxacin at inhibiting the growth of several Gram-positive bacteria, including the hospital pathogens MRSA and VRE (Table 1). We also found that **1** is active against mycobacteria, with potent antibiotic activity and MIC values between 0.12 and 0.48 μM (Table 1). Furthermore, in standardized cell-culture assays (see the Supporting Information),[24] we found that **1** shows only moderate antiproliferative and cytotoxic activities. Altogether, the bioactivity profile is highly promising for a potential therapeutic, and the strongly adhesive properties may qualify the antibiotic for coating of medical devices such as catheters.

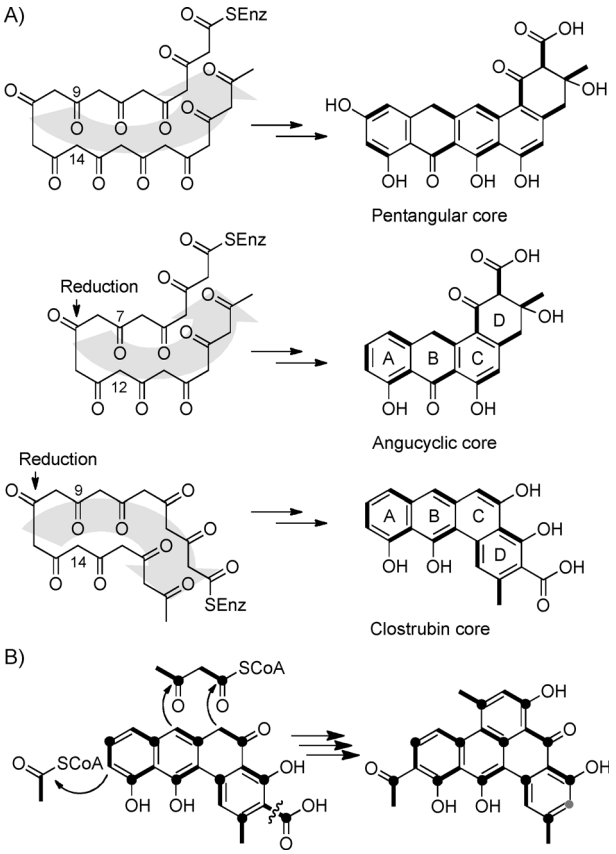

Figure 3. A) Typical angucyclic folding observed in aerobic bacteria versus noncanonical polyketide cyclization in the anaerobe, as deduced from isotope labeling. B) A model for the biosynthesis of clostrubin A (**1**).

Table 1: Antibacterial activity data for **1** and ciprofloxacin (cip).

Cpd.	MIC [μM]							
	Ec	Bs	MRSA	VRE	Ms	Ma	Mv	Mf
1	>60.0	0.075	0.12	0.97	0.48	0.12	0.12	0.12
Cip	0.010	0.080	37	2.3	4.71	0.30	1.20	0.60

Ec: *Escherichia coli*, Bs: *Bacillus subtilis*, MRSA: methicillin-resistant *Staphylococcus aureus*, VRE: vancomycin-resistant *Enterococcus faecalis*, Ms: *M. smegmatis*, Ma: *M. aurum*, Mv: *M. vaccae*, Mf: *M. fortuitum*.

In summary, we have succeeded in the challenging isolation of clostrubin, the first polyketide metabolite from a strictly anaerobic bacterium. Surprisingly, this novel compound was discovered in a species that has been extensively used in industry. Clostrubin features a highly unusual ring topology, which is unprecedented for natural products. Labeling experiments revealed that the polyphenolic compound emerges from a noncanonical polyketide folding, which departs from the conserved folding patterns of aerobic microorganisms. Furthermore, clostrubin exhibits remarkable antibiotic activity, notably including potent antimycobacterial activity, which holds promise for the further development of this compound into an antibacterial therapeutic. Whilst the

genetic basis for the production of this compound is not yet identified, the structural data suggest a unique biosynthetic route not previously observed in nature. These data, coupled with the identification of polythioamide-bearing secondary metabolites in another *Clostridium* species,[12] should now cement the place of anaerobes as a viable source of novel natural product scaffolds.

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