Natural Products

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Closthioamide: An Unprecedented Polythioamide Antibiotic from the Strictly Anaerobic Bacterium *Clostridium cellulolyticum***

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Bacteria belonging to the genus *Clostridium* are among the most prominent microorganisms that lead an obligate anaerobic lifestyle. Clostridia occur in gastrointestinal tracts and are ubiquitously distributed in soil and sediments, rapidly decaying organic matter.[1] Owing to their potent catabolic properties, interest in these organisms has grown rapidly over the past few years. Not only are clostridia routinely employed to degrade anthropogenic cellulosic waste products, they have also been increasingly exploited to meet the need for renewable chemicals and biofuels.[2] Although various pathogenic species produce the most powerful neurotoxins known to mankind, the tetanus and botulinum toxins, respectively,[3] no secondary metabolites have yet been isolated from these or any other strictly anaerobic bacteria. However, bioinformatics analysis ("mining") of the recently sequenced genomes of *Clostridium* spp., for example, *Clostridium kluyveri*[4] and *Clostridium cellulolyticum*, indicated that these bacteria harbor genes for the biosynthesis of secondary metabolites. Since the encoded cryptic natural products have been overlooked so far, it appears the biosynthesis genes remain dormant under standard laboratory conditions and are only triggered in the presence of particular stimuli. Herein we report the discovery of the first secondary metabolite, a hitherto fully unprecedented type of polythioamide, from a strictly anaerobic bacterium, *Clostridium cellulolyticum*.

C. cellulolyticum is an anaerobic nonruminal Gram-positive bacterium that was isolated from decayed grass compost and is an important industrial strain as a result of its ability to degrade crystalline cellulose.[5] Yet, no secondary metabolite has been reported from this well-known model organism. To investigate secondary-metabolite production, we cultivated a strain of *C. cellulolyticum*, DSM 5812 (= ATCC 35319), in 1 L fermenters with pH control by using complex media. However, under these standard growth conditions as reported for this strain, no secondary-metabolite formation was observed. Therefore, we sought to induce natural product biosynthesis by applying external triggers. Several supplements, such as nutrients and various chemicals, were administered, and stress conditions were used (see the Supporting Information). All cultures were extracted and analyzed by reversed-phase (RP) HPLC. Unfortunately, no secondary metabolites were detected in any of the extracts.

We then sought to exploit yet unknown environmental cues that occur in the natural habitat. Thus, as the bacterium had been isolated from decayed grass compost, we added an aqueous soil extract to the fermenter prior to inoculation. Most surprisingly, under these conditions, the RP-HPLC profile showed new peaks with a maximum absorbance at 270 nm (Figure 1). This effect could be reproduced with various soil samples (fen, compost) from different sources, geographical locations, and layers. Careful analysis of the soil extracts demonstrated unequivocally that the new compounds were not components of the supplement (see the Supporting Information).

The major metabolite, compound **1**, was isolated from 5 L of culture broth and purified by sequential open-column chromatography and RP-HPLC, which yielded 1.07 mg of a pale-yellow compound. According to HRMS measurements, **1** had the molecular formula $C_{29}H_{38}N_6O_2S_6$ and thus appeared to be remarkably rich in sulfur atoms. Furthermore, NMR spectroscopic data showed that the molecule was symmetrical: the ^{13}C NMR and DEPT spectra indicated the presence of six methylene, two methine, and five quaternary carbon atoms. In the ^1H NMR spectrum, signals corresponding to two olefinic and six methylene hydrogen atoms were observed. The HMBC correlation between 2-H ($\delta = 3.62$ ppm) and C2′ ($\delta = 44.1$ ppm) confirmed the symmetrical structure of **1**. Extensive ^1H-^1H COSY, single-bond ^1H-^{13}C HSQC, and ^1H-^{13}C long-range HMBC analyses revealed one *p*-hydroxybenzoyl, one diaminopropane, and two β-alanine units. However, several lines of evidence excluded the possibility of a canonical amide linkage of these building blocks. First, an amide carbonyl carbon shift would be expected at around 170 ppm. Instead, the signals of the quaternary carbon atoms C9 ($\delta =$ 199.1 ppm), C6 ($\delta = 203.3$ ppm), and C3 ($\delta = 202.9$ ppm)

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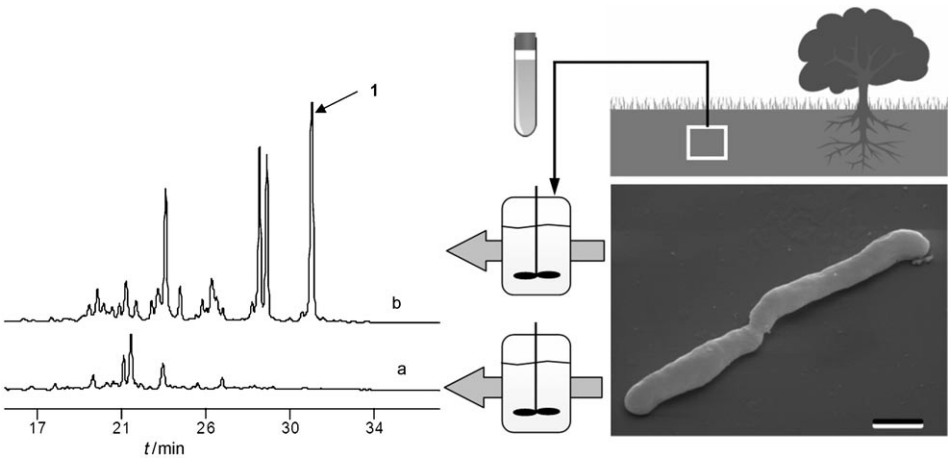
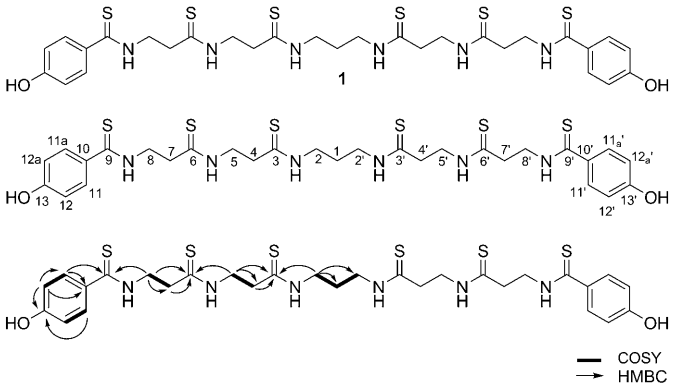


Figure 1. Left: HPLC profiles of extracts from *C. cellulolyticum* cultures in a) a complex medium and b) complex medium supplemented with aqueous soil extract; UV detection at 270 nm. Bottom right: scanning electron micrograph of *C. cellulolyticum* cells; scale bar: 1 μm.

appeared much further downfield. Second, the HRMS data indicated the presence of six sulfur atoms in the molecule; the chemical shifts clearly indicated that the sulfur atoms were incorporated into thioamide moieties (Scheme 1). The pres-



Scheme 1. Structure of closthioamide (**1**) and 2D NMR correlations.

ence of thioamide moieties was supported by the observation of intense, characteristic $\nu_{C=S}$ bands[6] at 838 cm^{-1} in the infrared spectrum of **1**. In conclusion, the physicochemical data unequivocally revealed that **1** is a symmetrical polythioamide composed of a central diaminopropyl, four β-alanyl, and two terminal *p*-hydroxybenzoyl units.

Compound **1**, which was named closthioamide, is unique for various reasons. First, the symmetric (thio)amide structure is quite unusual. Related phenolic polyamine conjugates, some of which have a symmetrical structure (e.g. N^1,N^{12}-bis-(dihydrocaffeoyl)spermine, kukoamine A), are primarily known from the plant kingdom;[7] a rare exception is the phenolic polyamine petrobactin, which occurs in bacteria.[8] Second, the six thioamide groups of **1** make the compound unique. Thioamide moieties are frequently employed in synthetic medicinal chemistry[9,10] as isosters for amide bonds, as they lend greater rigidity and stability towards proteases.[11–13] In stark contrast, thioamides are extremely scarce among naturally occurring organic compounds. Only four of an estimated 170000 known natural products feature thioamide groups. Cycasthioamide and (4-methoxyphenyl)-*N*-methyl-2-oxothioacetamide were isolated from a cycad, *Cycas revoluta*,[14] and a tunicate, *Polycarpa aurata*,[15] respectively. The latter compound, however, could be an artifact by-product from the major component polycarpine. More recently, the unusual chalcophore methanobactin was reported as a metabolite of the methane-oxidizing bacterium *Methylosinus trichosporium*,[16,17] and thioviridamide was identified as a novel apoptosis inducer from *Streptomyces olivoviridis*.[18] Whereas a natural thioamide is rare, an entirely polythioamidated metabolite is totally unprecedented.

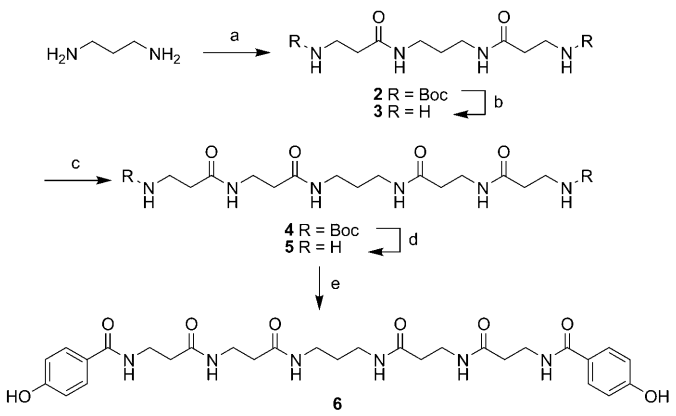
Surprisingly, this unusual compound also proved to be a novel type of antibiotic. In a standardized antimicrobial assay,[19] we found that **1** was highly active against a pathogenic, methicillin-resistant *Staphylococcus aureus* (MRSA) strain with a minimum inhibitory concentration (MIC) of 0.4 $\mu g\,mL^{-1}$ (0.58 μM). Compound **1** is even active against vancomycin-resistant enterococci (VRE) with the same low MIC value, and is thus significantly more potent against these bacteria than ciprofloxacin, the standard drug used against VRE, with a remarkable strain selectivity (Table 1). Furthermore, in a standardized cytotoxicity assay,[20] **1** showed moderate antiproliferative and cytotoxic effects (see the Supporting Information).

To investigate the role of the unusual thioamide moieties in antibiotic activity, we synthesized the corresponding hexaoxa analogue, closamide (**6**), by the fusion of diaminopropane, β-alanine, and hydroxybenzoate building blocks on the basis of a peptide-synthesis protocol (Scheme 2). Comparison of the biological-activity profiles of **1** and the synthetic analogue **6** gave a remarkable result, which highlights the importance of the sulfur-containing thioamide

Table 1: Antibiotic activity of closthioamide (**1**) versus ciprofloxacin (Cip) and closamide (**6**).[a]

Compound	MIC [μM]			
	Ec	MRSA	VRE	Mv
1	9.00	0.58	0.58	72.03
Cip	0.15	37.8	4.71	0.60
6	>167	>167	>167	>167

Ec: *Escherichia coli*; MRSA: methicillin-resistant *Staphylococcus aureus*; VRE: vancomycin-resistant *Enterococcus faecalis*; Mv: *Mycobacterium vaccae*.



Scheme 2. Synthesis of closamide (**6**), a hexaoxa analogue of **1**: a) Boc-β-Ala-OH, EDC, Et_3N, HOBt, DMF; b) TFA; c) β-Ala-Boc, PyBOP, Et_3N, HOBt, DMF; d) TFA; e) *p*-hydroxybenzoic acid, PyBOP, Et_3N, HOBt, DMF. Boc = *tert*-butoxycarbonyl, DMF = *N*,*N*-dimethylformamide, EDC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, HOBt = 1-hydroxy-1*H*-benzotriazole, PyBOP = benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate, TFA = trifluoroacetic acid.

functionalities: the hexamide **6** was fully inactive against the test strains.

Closthioamide is the first reported secondary metabolite from a bacterium of the genus *Clostridium*, and to the best of our knowledge the first from any of the large group of strictly anaerobic microorganisms. Why has this potent antibiotic been overlooked in a well-studied industrial microorganism? It is conceivable that anaerobes have a well-regulated metabolism to avoid wasting their expensively produced energy for the production of molecules that are not essential for their survival. Thus, the requisite biosynthesis genes would remain silent in the absence of environmental cues or particular triggers. In the last decade, various strategies have been employed to trigger silent biosynthetic pathways to yield "cryptic natural products". Chemical or physical stimuli have been used, as well as cocultivation and genomic approaches, such as genome mining, epigenetic remodeling, and engineered pathway activation.[21–23] In this respect, it is particularly intriguing that the biosynthesis of an antibacterial *Clostridium* metabolite was induced by the addition of compost or soil extract to mimic the natural environment. In future studies we aim to elucidate the cues for activation of the cryptic pathway as well as the biochemical basis of polythioamide formation.

In conclusion, by systematically altering the culture conditions, we discovered that aqueous soil extracts dramatically alter the metabolic profile of *Clostridium cellulolyticum*. The isolation and structure elucidation of the major metabolite led to the discovery of the symmetrical polythioamide closthioamide (**1**), which is unprecedented among natural products. Furthermore, we found that closthioamide is a novel type of antibiotic that is highly active against multiresistant staphylococci. The importance of the thioamide moieties for antibiotic activity was corroborated through the synthesis of the hexaoxa analogue closamide (**6**), which was fully inactive against the test strains. To the best of our knowledge, no secondary metabolite from a strictly anaerobic bacterium has been reported previously. This finding provides great encouragement to delve deeper into the underexplored metabolome of anaerobes.

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