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Cystobactamids: Myxobacterial Topoisomerase Inhibitors Exhibiting Potent Antibacterial Activity**

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Abstract: *The development of new antibiotics faces a severe crisis inter alia owing to a lack of innovative chemical scaffolds with activities against Gram-negative and multiresistant pathogens. Herein, we report highly potent novel antibacterial compounds, the myxobacteria-derived cystobactamids* ***1–3****, which were isolated from Cystobacter sp. and show minimum inhibitory concentrations in the low* $\mu g\,mL^{-1}$ *range. We describe the isolation and structure elucidation of three congeners as well as the identification and annotation of their biosynthetic gene cluster. By studying the self-resistance mechanism in the natural producer organism, the molecular targets were identified as bacterial type IIa topoisomerases. As quinolones are largely exhausted as a template for new type II topoisomerase inhibitors, the cystobactamids offer exciting alternatives to generate novel antibiotics using medicinal chemistry and biosynthetic engineering.*

The discovery of antibiotics and their application to cure a wide range of infectious diseases revolutionized modern medicine during the last century. However, an increase in antimicrobial resistance is seen as a severe public health threat, exacerbated by the fact that the development of new antibiotics in the pharmaceutical industry has largely been downsized or even terminated because it is difficult and not profitable.[1] Most target-driven approaches relying on chemical libraries did not yield novel antibiotics at the scale required to combat resistance, especially among ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp.) pathogens.[2] These bacteria are the cause of a vast majority of nosocomial infections with limited treatment options because of their intrinsic or acquired multidrug resistance (MDR) mechanisms.[3] Traditionally, anti-infectives were mostly derived from microbial natural products, leading to a golden era of antibiotics in the 60s and 70s of the last century. Since then, very few novel compound classes have been identified and developed for application. Natural products are thought to be evolutionarily optimized to overcome the highly problematic issue of cell wall penetration in bacteria. However, the natural product field is complicated by its high attrition rate that is due to the rediscovery of known chemical scaffolds. Therefore, new screening methods[4] and the analysis of untapped or underexplored resources are seen as promising approaches to discover novel antibacterial compounds.[5]

We have focused our attention on gliding bacteria, including the myxobacteria, as a source for novel anti-infective natural products.[6] For example, we recently identified the disciformycins as novel antimicrobials with a unique mode of action and minimum inhibitory concentrations (MIC; $<1\ \mu g\,mL^{-1}$) against resistant *Staphylococcus aureus* isolates.[7]

In our search for new compounds exhibiting activity against Gram-negative bacteria, a novel class of natural products from *Cystobacter* sp. came to our attention, whose isolation and structure elucidation is described herein. Furthermore, we elucidate the nonribosomal biosynthetic origin of this novel compound class, which we named cystobactamids, and decipher their molecular targets.

Crude extracts prepared from small-scale cultivations of *Cystobacter* sp. Cbv34 were found to efficiently inhibit the growth of several Gram-positive and Gram-negative bacteria. Following an LC-HRMS-assisted bioactivity-guided screening approach, we were able to identify cystobactamids 919-1 (**1**), 919-2 (**2**), and 507 (**3**) as active components (Figure 1). To isolate and characterize those, Cbv34 was grown on 10 L scale yielding approximately 1 mg of each derivative (for details on the isolation procedures, see the Supporting Information).

Elucidation of the planar structures was performed using various spectroscopic/spectrometric techniques, including high-resolution ESI-MS and 1D and 2D NMR (HSQC,

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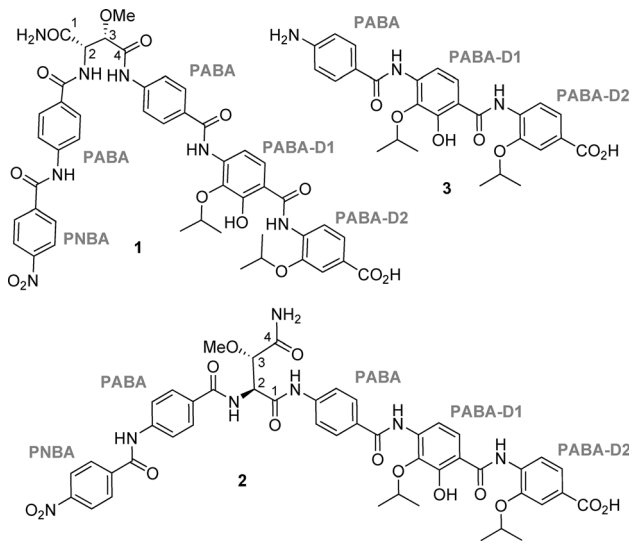

Figure 1. Chemical structures of cystobactamids 919-1 (**1**), 919-2 (**2**), and 507 (**3**).

HMBC, COSY, ROESY) experiments (see the Supporting Information). Cystobactamid 919-1 (**1**) and cystobactamid 919-2 (**2**) consist of five aromatic moieties, namely *para*-nitrobenzoic acid (PNBA), two *para*-aminobenzoic acid units (PABA), and two PABA derivatives with different oxidation patterns (PABA-D1 and PABA-D2), which are linked through an iso-β-methoxyasparagine unit (**1**) or a β-methoxyasparagine unit (**2**) between the two PABA units. Cystobactamid 507 (**3**) has the tripeptidic sequence PABA–PABA-D1–PABA-D2 and is thus a fragment of **1** and **2**.

Structurally, the cystobactamids show various special features. The PABA chain scaffold is unique for natural products, and to the best of our knowledge, the first example of this compound class. Furthermore, the structures of cystobactamids 919-1 (**1**) and 919-2 (**2**) comprise a rare nitro group, most likely originating from the oxidation of the amine group in one PABA moiety. Moreover, the isopropoxylation of the 3-hydroxy-4-aminobenzoic acid moieties (PABA-D1 and PABA-D2) seems to be unprecedented in natural products. Notably, the β-methoxyasparagine moiety is either included through a 2,4-linkage (**1**) or a 1,2-linkage (**2**).

The relative configuration of the β-methoxyasparagine unit was assigned as *anti* by analysis of the homonuclear vicinal coupling constants ($^3J_{H,H} \approx 7$–8 Hz). The interpretation of ROESY-NMR data for derivatives **1** and **2** revealed a *threo* configuration. The absolute configuration was assigned as "*SS*" using optical rotation measurements and a comparison to literature values of β-oxyasparagine derivatives. Details of the structure elucidation and the analysis of the stereochemical configuration are described in the Supporting Information.

When evaluating the antimicrobial activities of the purified compounds (Table 1 and Table S1 in the Supporting Information), we found that **1**–**3** exhibited a comparable activity spectrum to those of the crude extracts from Cbv34 that were initially screened. This confirmed that the novel compound family was at least in part responsible for the strong antimicrobial activity of the extracts. All of the

Table 1: Minimum inhibitory concentrations (MICs in $\mu g\,mL^{-1}$) of **1**–**3** and Cp.

	1	**2**	**3**	Cp
Acinetobacter baumannii	> 59	7.4	32.5–65.0	0.2–0.4
Escherichia coli	14.7–29.4	0.9	16.3	0.013
Enterococcus faecalis	3.7–7.4	0.1	4.1	0.8
Staphylococcus aureus	32.5	0.1	8.1	0.05–0.1
Streptococcus pneumoniae	14.7	0.1	4.1	0.8–1.6

derivatives inhibited the growth of *E. coli* at concentrations of as low as $1\,\mu g\,mL^{-1}$. Especially **2** strongly inhibited the growth of various pathogens, including *A. baumannii*, one of the Gram-negative bacteria of the ESKAPE panel. Hexapeptide **2** was also effective in inhibiting the growth of various Gram-positive bacteria, such as *E. faecalis* and *S. pneumonia*, at concentrations of approximately $0.1\,\mu g\,mL^{-1}$, which is comparable to or even exceeds the activity of ciprofloxacin (Cp), a second-generation fluoroquinolone antibiotic currently used in the clinic.[8] Importantly, none of the tested derivatives exhibited pronounced antifungal or cytotoxic activities at concentrations of up to approximately 50–$100\,\mu g\,mL^{-1}$ (Tables S1 and S3).

Considering their chemical structures, it seemed likely that the cystobactamids are products of a nonribosomal peptide synthetase (NRPS).[9] Intriguingly, the major building block of these compounds is PABA, a moiety rarely incorporated in secondary metabolites.[10] However, it is a starter unit in the polyketide-synthase-driven biosynthesis of candicidins, but to the best of our knowledge, it has not been found in NRPS-derived peptides. Interestingly, the respective biosynthetic cluster also contains two genes required for PABA synthesis, an aminodeoxychorismate (ADC) synthase and an ADC lyase.[10] To identify the cystobactamid biosynthetic gene locus we successfully screened the genome of Cbv34 for an NRPS locus that also comprises ADC lyase and anthranilate synthase homologues. The gene cluster contains three large NRPS genes as well as various genes that encode tailoring enzymes, transporters, and putative resistance genes (Tables S4 and S5). A detailed analysis of the modular structure of the NRPS together with feeding experiments (Figures S1 and S2) allowed us to propose a model for cystobactamid biosynthesis as depicted in Figure 2 and described in the Supporting Information. Intriguingly, the biosynthesis exhibits a number of mostly unusual features, including a loading process of an extender unit (isoasparagine or asparagine) in trans, the isopropoxylation by SAM-dependent methylation of a phenolic hydroxy group followed by two further methylations, which are catalyzed by a radical SAM-dependent methyl transferase, as well as the oxidation of an amine to a nitro group. The genetic locus also encodes a putative self-resistance protein, CysO, which belongs to the pentapeptide repeat protein family. Some of these proteins are known resistance factors against topoisomerase poisons, including fluoroquinolones (Qnr and MfpA), microcin B17 (McbG), and albicidin (AlbG),[11] suggesting that the cystobactamids act as inhibitors of bacterial type IIa topoisomerases.

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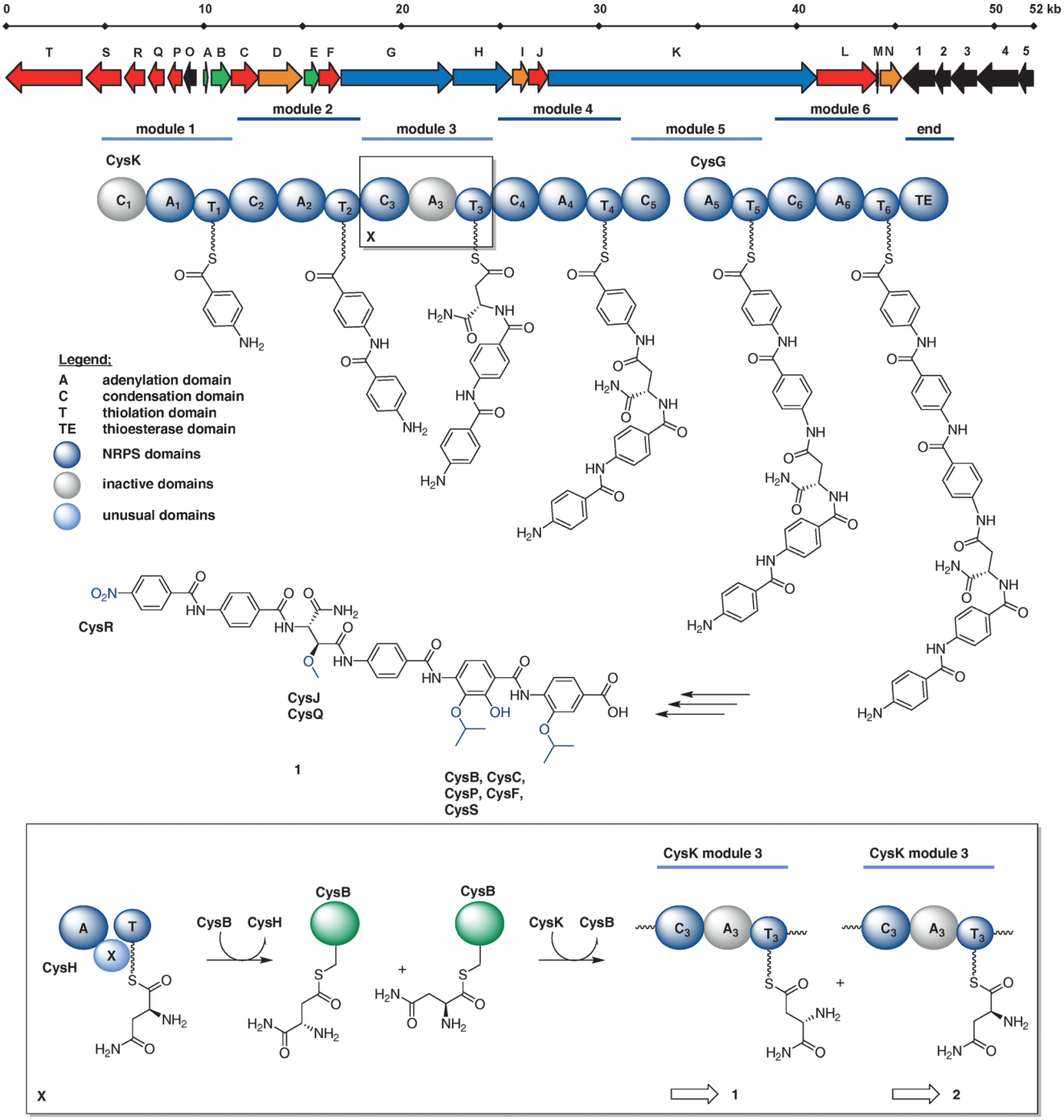

Figure 2. Model of cystobactamid biosynthesis. Two PABA units are activated by the adenylation domains A_1 and A_2 of CysK. A_3 of CysK is thought to be inactive, and it is assumed that T_3 of CysK is primed in trans by CysH/CysB. CysH activates an asparagine unit, which is either used directly (**2**) or isomerized to isoasparagine by the unusual ammonia/amine-ligase-like domain of CysH (**1**) prior to transfer to the T domain of CysK module 3. The transfer of the Asn or iso-Asn building block to T_3 of CysK may be realized either directly or aided by CysB, which serves as a shuttle protein. After elongation of the module 2 bis-PABA intermediate on CysK by module 3, biosynthesis is continued on module 4, a split module 5 encoded on CysK and CysG, as well as on module 6, which results in the incorporation of three additional PABA building blocks. CysG releases the linear hexapeptide, which is further modified by various tailoring enzymes. Gene cluster color code: NRPS genes (blue), regulatory and "helper" protein genes (green), tailoring genes (red), PABA biosynthesis genes (orange), resistance and efflux genes (black). For details, see the Supporting Information.

Indeed, subsequent biochemical assays showed that the cystobactamids were able to inhibit the activity of the bacterial topoisomerases, such as gyrase and topoisomerase IV from *E. coli* (Tables 2 and S6; Figures 3, S3, S4, and S7). Compound **2** showed IC_{50} values for *E. coli* gyrase in the nanomolar range, thus being equally potent as Cp. For the

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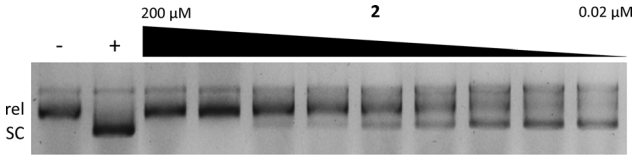
Table 2: Half-inhibitory concentrations (IC_{50} values in μM) of **1–3** and Cp in gyrase supercoiling assays and topoisomerase IV relaxation assays.

	1	2	3	Cp
E. coli gyrase	21.3 ± 6.0	0.26 ± 0.06	20.2 ± 2.2	0.40 ± 0.05
E. coli topo IV	89.5 ± 0.8	38.0 ± 0.7	86.0 ± 2.8	nd[a]

[a] nd: not determined.



E. coli gyrase DNA supercoiling

Figure 3. Titration of *E. coli* gyrase supercoiling assays with cystobactamid 919-2 (**2**). Controls: (−) without enzyme; (+) untreated. Details of the assay are given in the Supporting Information. rel and SC denote relaxed and supercoiled *E. coli* DNA, respectively.

same enzyme, **1** and **3** show at least 80-fold higher IC_{50} values (two-digit micromolar range). However, the presence of the first two PABA units and, more importantly, the constitution of the central amino acid (L-iso-Asn vs. L-Asn) and thus the overall shape of the molecule boost the activity from the micromolar to the nanomolar range. Intriguingly, **1–3** showed only moderate activities in *E. coli* topoisomerase IV relaxation assays (Table 2), thus indicating that the main target of the cystobactamids in *E. coli* is DNA gyrase.

Type IIa topoisomerase inhibitors can be generally divided into two classes: topoisomerase poisons and competitive ATP-binding pocket inhibitors, such as the aminocoumarin natural product novobiocin.[12] Topoisomerase poisons such as the quinolones promote the formation of double-stranded DNA breaks by stabilizing the formed covalent GyrA tyrosyl–DNA adducts, which can be visualized in vitro using a plasmid linearization assay.[13] The formation of such linearized plasmids is clearly visible for Cp and **2** (Figure S5). Furthermore, the inhibitory activity of **2** could not be diminished by the addition of high concentrations of ATP (Figure S6), which suggests that cystobactamids do not bind to the ATP-binding pocket located on the GyrB subunit of gyrase. Taken together, this indicates that the cystobactamids are indeed type II topoisomerase poisons and can thus be regarded as "natural quinolones", which are one of the most successful classes of antibiotics. The results also suggest that the primary binding site of the cystobactamids on gyrase is located at the GyrA–DNA interphase.

This hypothesis prompted us to analyze potential cross-resistance between **1–3** and Cp. Resistance to quinolones is reported to be mediated by mutations in *gyrA* and *parC*, which leads to alterations in the drug binding sites. In GyrA, the quinolone-resistance-determining region (QRDR) is located between amino acids 67 and 106, whereas S83 and D87 are most often involved.[14] We compared the MIC values of **1–3** with Cp using a quinolone-susceptible *E. coli* strain and isogenic mutants in the *gyrA* gene (Table S2). Minimum inhibitory concentrations for Cp were found in accordance with literature values;[15] the MIC values increased approximately by a factor of 30–60 for single GyrA mutants. A combination of both mutations (S83L, D87G) resulted in clinical resistance ($MIC \geq 1\ mg\,L^{-1}$). The activity of **2**, the most potent derivative described herein, was reduced with the S83 and D87 mutants to a smaller extent (2–7-fold), and a combination of both GyrA mutations had a larger impact. These findings suggest an overlapping, but not identical binding site of cystobactamids and quinolones in GyrA, as the impact of the quinolone resistance mutations on cystobactamid activity is less pronounced.

In summary, we have identified a novel class of antibacterial compounds that exhibit promising activity towards a validated drug target. Cystobactamids were highly active against various Gram-positive pathogens. Most importantly, **2** also efficiently inhibited the growth of *E. coli* and *A. baumannii*, confirming that the compounds can penetrate the outer membrane of Gram-negative bacteria. Numerous other cystobactamid derivatives were detected in crude extracts from *Cystobacter* sp. in minute amounts only, which makes separation very challenging. Thus we currently cannot rule out the possibility that the moderate activity of **3** results from minor contamination with highly potent full-length cystobactamids. Current research aims at the production optimization and characterization of new natural derivatives, molecular definition of the target site as well as total synthesis and biosynthetic engineering of the currently most active hexapeptide **2** and its medicinal chemistry to define the structure–activity relationships of the cystobactamids. Furthermore, we focus on the development of second-generation cystobactamids with improved Gram-negative coverage especially within the ESKAPE panel. Given their novel structural scaffold and the limited cross-resistance, we hope that the cystobactamids will serve as a starting point to develop a novel class of antibiotics exhibiting broad-spectrum antibacterial activity.

Experimental Section

All gyrase and topoisomerase IV assays described herein have been performed using commercial kits from Inspiralis, Norwich, UK.

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