Identification and characterization of the first class of potent bacterial acetyl-CoA carboxylase inhibitors with antibacterial activity

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Running title:
Novel bacterial acetyl-CoA carboxylase inhibitors
SUMMARY

The multisubunit acetyl-CoA carboxylase, which catalyzes the first committed step in fatty acid biosynthesis, is broadly conserved among bacteria. Its rate-limiting role in formation of fatty acids makes this enzyme an attractive target for the design of novel broad-spectrum antibacterials. However, no potent inhibitors have been discovered so far. This report describes the identification and characterization of highly potent bacterial acetyl-CoA carboxylase inhibitors with antibacterial activity for the first time. We demonstrate that pseudopeptide pyrrolidine dione antibiotics such as moiramide B inhibit the *E. coli* enzyme at nanomolar concentrations. Moiramide B targets the carboxyltransferase reaction of this enzyme with a competitive inhibition pattern versus malonyl-CoA ($K_i$ value = 5 nM). Inhibition at nanomolar concentrations of the pyrrolidine diones is also demonstrated using recombinantly expressed carboxyltransferases from other bacterial species (*S. aureus, S. pneumoniae* and *P. aeruginosa*). We isolated pyrrolidine dione-resistant strains of *E. coli, S. aureus* and *B. subtilis*, which contain mutations within the carboxyltransferase subunits AccA or AccD. We demonstrate that such mutations confer resistance to pyrrolidine diones. Inhibition values ($IC_{50}$) of >100 µM regarding an eukaryotic acetyl-CoA carboxylase from rat liver indicate high selectivity of pyrrolidine diones for the bacterial multisubunit enzyme. The natural product moiramide B and synthetic analogues show broad-spectrum antibacterial activity. The knowledge of the target and the availability of facile assays using carboxyltransferases from different pathogens will enable to evaluate the antibacterial potential of the pyrrolidine diones as a promising antibacterial compound class acting via a novel mode of action.
INTRODUCTION

The fatty acid synthesis (FAS) in living organisms comprises a repeated cycle of reactions involving the condensation, reduction, dehydration and subsequent reduction of carbon-carbon bonds. While higher eukaryotes carry out these reactions by a large multifunctional protein [type I pathway (1)], each reaction is catalyzed by discrete enzymes in bacteria, plant chloroplasts and *Plasmodium falciparum* [type II pathway (2)]. In bacteria, the pathway is essential for cell growth as shown for *E. coli* (3), since fatty acids are constituents of the membrane-building phospholipids (Fig. 1).

Due to the high structural conservation of many bacterial FAS enzymes among different species this pathway appears to be suited to be targeted by inhibitors with broad-spectrum antibacterial potency (4-6). Inhibitors of the enoyl-acyl carrier protein (enoyl-ACP) reductase FabI such as triclosan (7,8) or of the condensing enzyme isoforms FabF/FabB such as cerulenin (9,10) raised the hope that bacterial FAS represents a novel yet under-explored target area for the discovery of novel antibiotics. Nevertheless, critical aspects also accompany FAS as antibiotic target area. The fatty acid composition of bacteria varies from species to species, leading to variations in enzymatic properties of orthologs and to variable numbers of isoforms (11). For instance, the β-ketoacyl-ACP synthases III (FabH) of different species harbor various substrate specificities. None the less, some inhibitors targeting the enzymes of diverse species have been published recently (12-14). The enoyl-ACP reductase isoforms represent an example, in which enzymes even with the same substrate specificity might be difficult to be targeted efficiently by the same inhibitor. The enzyme FabK, which has been identified e. g. in *S. pneumoniae* and enterococci, acts analogous to FabI, but is insensitive to triclosan (15). Remarkably, FabI inhibitors with improved, but not yet equivalent FabK activity have been reported recently (16-18). In order to avoid difficulties mentioned before, another key enzyme of FAS has attracted attention, the bacterial acetyl-CoA carboxylase (ACC). It is a multisubunit enzyme that catalyzes the first committed step in FAS. It is broadly conserved among bacteria and no isoforms are known. Its rate-limiting role in FAS has been shown for *E. coli*,
where overexpression of this enzyme increased the rate of FAS (19). It was also demonstrated that ACC is a site of feedback inhibition by products of the FAS pathway, the acyl-ACPs (20). In addition, studies in *B. subtilis* indicated that the product of the ACC-catalyzed reaction malonyl-CoA plays major roles in regulating the expression of other FAS enzymes (21). Altogether, these findings suggest that the bacterial acetyl-CoA carboxylase could be an appropriate target for the design of novel broad-spectrum antibacterials.

The acetyl-CoA carboxylase reaction can be divided into two partial reactions (22) (Fig. 1). In a first step the biotin, attached to the biotin carboxyl carrier protein (BCCP = AccB), is carboxylated via ATP consumption by the biotin carboxylase AccC. Secondly, the carboxyl group is transferred to acetyl-CoA yielding malonyl-CoA by the carboxyltransferase, which is composed of the subunits AccA and D. Studies with the *E. coli* enzyme showed that the ACC complex contains each subunit as a dimer. After cell lysis the complex dissociates into two stable complexes, the biotin carboxylase and the carboxyltransferase sub-complexes (23). Only reaction intermediates and bisubstrate analogs without antibacterial activity have been published so far representing moderate inhibitors of the two partial reactions of ACC (24,25).

On the other hand, numerous natural compounds have been described as broad-spectrum antibacterials with unknown mode of action. Our efforts to decipher the targets of not yet mechanistically examined natural products led us to the identification of the first highly potent bacterial ACC inhibitors. We show that the pseudopeptide pyrrolidine dione antibiotics moiramide B (CPD1) and andrimid (CPD5; Fig. 2) (26-28) act by inhibiting the carboxyltransferases of bacterial ACCs.

**EXPERIMENTAL PROCEDURES**

*Media, materials and strains* - Various media for cell growth were used: DO (29), LB (Difco), M9 (Difco) and Brain Heart Infusion medium (Oxoid) as well as Isosensitest Broth (Oxoid). Columbia
agar plates with 5% sheep blood (Becton Dickinson), bovine serum (Oxoid) and Aerocult C filter-paper bags (Merck, Darmstadt, Germany) were necessary for *S. pneumoniae* cultivation. Radioactively labeled metabolites were purchased from Amersham. Other purchased reagents included calf intestinal alkaline phosphatase, T4 DNA ligase, DNA polymerase, protein and DNA molecular weight markers (Invitrogen), various substrates and ingredients for enzymatic assays (Sigma) as well as antibiotics for growth selection (Sigma). The compounds CPD1 to 5 were synthesized according to previously published procedures (30).

Cloning was generally performed in *E. coli* XL1Blue (Stratagene). The strain *E. coli* BLR(DE3) carrying the plasmid pMSD8 (19) was used for overproduction of the complete ACC complex. The plasmid pS47 was a derivative of the expression vector pQE30 (Qiagen) carrying a fragment of accC from *E. coli* MG1655 (codons 2 to 451 including the termination codon). Recombination experiments in *B. subtilis* were performed with strain 168 (31) using the plasmids pDG1731xyl (32) and pS42 (33). Other bacterial strains used in this study were *E. coli* MG1655 (PC 4007, Phabagen collection, Utrecht University, Netherlands), *E. coli* strain „Neumann“ (DSM no. 10650, DSMZ, Braunschweig, Germany), *E. coli* HN818 carrying a deletion of *acrA* encoding an efflux system component (34), *S. aureus* isolate 133, *S. pneumoniae* isolate G9A, *P. aeruginosa* strain PAO1 (NCCB 2452, Netherlands Culture Collection) and *P. aeruginosa* strain PAO200 carrying a deletion of *mexAB-oprM* encoding an antibiotic efflux system (35).

**Cloning of carboxyltransferases** - The subunits *accA* and *accD* of different organisms were cloned as one operon such as previously described for *E. coli* (36). The primers used for amplification of the genes *accA* and *accD* are listed in Table 1. The obtained PCR products of each species were fused via a second crossover PCR using the 5'-terminal *accA* and 3'-terminal *accD* primers (called ACCA1A and ACCD2B). The resulting plasmids pGF1, pS39, pS38 and pS35 contained *accAD* from *E. coli, S. aureus, S. pneumoniae* and *P. aeruginosa*, respectively. The plasmids enabled to
express the carboxyltransferases with a 6-amino acid His-tag fused to the amino terminus of the alpha subunit AccA.

The operon accDA amplified from the pyrrolidine dione resistant B. subtilis strain was ectopically expressed behind a xylose-inducible promoter P_{xylA} in B. subtilis 168, carrying a deletion of the accDA wild-type locus. The resulting strain was B. subtilis MH110. The applied primers and cloning strategy has been previously described for the ectopic expression of the wild-type allele (32,33).

*Metabolite incorporation assays* - Cells of E. coli MG1655, exponentially growing to OD₅₃₅ of 0.1 in DO medium (1.25 ml) supplemented with 2 µg/ml PMBN, were labeled with 7.5 kBq each of L-[U-¹⁴C]leucine (10.96 GBq/mmol), [U-¹⁴C]uridine (18.56 GBq/mmol), [2-¹⁴C]thymidine (2.22 Gbq/mmol) and N-acetyl-D[1-¹⁴C]glucosamine (1.85 GBq/mmol), in order to monitor effects on translation, transcription, replication and cell wall biosynthesis, respectively. After 5 min the test compound was added (defined as time point 0) to the cultures at various concentrations. Samples (0.1 ml) were taken in regular time intervals and precipitated with 6% perchloric acid in a multi-screen filter plate (Millipore, 0.45 µm). After washing the precipitates with 0.1 ml ethanol, plates were dried and radioactivity was determined in a MicroBeta TriLux β-counter (Wallac).

Phospholipid synthesis was determined by metabolic labeling of 0.1 ml exponentially growing E. coli MG1655 cells at OD₅₃₅ of 0.1 with 370 kBq [³H]acetate (189 GBq/mmol) in M9 medium supplemented with 0.4% glycerol and 2 µg/ml PMBN. At regular time intervals after addition of the test compound (time point 0) the cells were labeled for 10 min. Each labeling reaction was stopped by the addition of 0.6 ml CHCl₃/methanol (1:2). After phase separation with 0.2 ml CHCl₃ and 0.2 ml H₂O the organic phase was recovered by centrifugation and washed three times each with 0.6 ml of 2 M KCl and 0.1 M sodium acetate. Radioactivity in aliquots of the organic phase was determined by liquid scintillation counting.
The incorporation of $[^{14}\text{C}]$malonyl-CoA into fatty acids was measured in cell extracts of *E. coli* MG1655. The cell extract generation and subsequent fatty acid synthesis assay, including the saponification and extraction of fatty acids into hexane, was performed as described previously (37).

*Enzyme purification* - The *E. coli* strains containing the plasmids pGF1, pS35, pS38, pS39 and pS47 were handled for protein overexpression as described previously (36). The proteins were purified in a single step and under native conditions using nickel-nitrilotriacetic acid (Ni-NTA) columns according to manufacturer’s instructions (Qiagen, QIAexpressionist manual). Changes in comparison to these instructions concerned the lysis and elution buffers, which were published previously (36). Proteins were quantified using bovine serum albumin as standard (38). The purified proteins, which were >95% pure as estimated by SDS-PAGE, were stored at 4°C.

*Biotin carboxylase assay* - The activity of biotin carboxylase was measured by following the production of ADP using the coupling enzymes pyruvate kinase and lactate dehydrogenase as described previously (39). The reaction was measured at room temperature in 384 microtitre plates using a mix (60 µl) containing 100 mM Tris-Cl, pH 8.0, 0.1 mM DTT, 0.1% bovine serum albumin, 8 mM MgCl$_2$, 20 mM biotin, 3 mM ATP, 8 mM KHCO$_3$, 0.5 mM phosphoenolpyruvate, 0.2 mM NADH, 5 U lactate dehydrogenase and 3 U pyruvate kinase. The reaction was started with various amounts of biotin carboxylase and the oxidation of NADH was followed at 340 nm using Spectrafluor Plus (Tecan).

*Carboxyltransferase assay* - The carboxyltransferase activity was measured in the reverse direction using a spectrophotometric assay, in which the production of acetyl-CoA was coupled to the citrate synthase/malate dehydrogenase reaction cascade leading to NAD reduction (36,40). The reaction was measured at room temperature in 384 microtitre plates using a mixture (60 µl) with 100 mM
Tris-Cl, pH 8.0, 10 mM L-malate, 0.5 mM NAD, 0.06% bovine serum albumin, 6.8 U/ml (0.1 mg/ml) citrate synthase, 3.6 U/ml (0.07 mg/ml) malic dehydrogenase, biocytin, malonyl-CoA and carboxyltransferase. The reduction of NAD was measured at 340 nm using Spectrafluor Plus (Tecan). The standard assay concentrations of the carboxyltransferase substrates biocytin and malonyl-CoA were 10 mM and 0.1 mM, respectively. In case of the E. coli and S. aureus enzyme, initial velocities were obtained by varying the substrate concentrations in order to determine maximum velocities and Michaelis constants. The data were calculated by non-linear regression of initial velocity values and substrate concentrations fitted to Michaelis-Menten equations (36) using the software GraphPad Prism (vers. 3; GraphPad software Inc., San Diego, CA).

E. coli ACC assay - Extracts of E. coli BLR(DE3) cells carrying the plasmid pMSD8 were prepared as described previously (19). ACC activity was assayed by the acetyl-CoA-dependent incorporation of $^{14}$CO$_2$ from $[14$C]bicarbonate into the acid-stable product malonyl-CoA (19,41). Changes compared to the published assay concerned the total reaction volume of 25 µl, the concentration of 5 mM NaH$^{14}$CO$_3$ (0.3 µCi) and the amount of cell-free extract protein (0.02 mg).

Rat liver ACC isolation and assay - The isolation of rat liver ACC was performed similar to published protocols (42,43) with several modifications. Rat livers were freeze-clamped in liquid nitrogen and homogenized in 2 vol. of ice-cold homogenization buffer [150 mM NaCl, 1mM EDTA, 1 mM DDT, 10 % Glycerol, 0.5% protease inhibitor (Sigma) and 100 mM Tris-Cl, pH 7.4]. After centrifugation at 16.000 g, the supernatant fluid was incubated with immobilized monomeric avidin (Pierce, Rockford, IL) for 60 min at 4°C. Subsequently, the immobilized avidin was packed into a column and washed with 2-3 vol. of homogenization buffer. ACC was eluted with homogenization buffer containing 2 mM biotin.

Inhibition of rat liver ACC was determined in 20 mM MgCl$_2$, 20 mM citrate, 5 mM NaHCO$_3$, 1 mM ATP, 0.1% bovine serum albumin and 50 mM Tris-Cl, pH 7.4. Following a pre-incubation of
ACC with test compounds for 10 min, the reaction was started with 0.2 mM acetyl-CoA. After 90 min, the reaction was stopped with perchloric acid, neutralized with KHCO$_3$ and malonyl-CoA synthesis was evaluated by HPLC similar to Hosokawa et al. (44). CoA-esters were separated by reverse phase HPLC (C18 Nucleosil 120-3, Macherey & Nagel, Dueren, Germany) in 0.2 M sodium phosphate (pH 5.0) with a gradient of 0 to 12% acetonitrile and detected by UV absorption at 254 nm. The peak area was determined for malonyl-CoA using an external standard and inhibition by test compounds was quantified by percent reduction in malonyl-CoA.

**Enzyme inhibition studies** - The initial velocity of the enzyme activities was determined in the presence of various concentrations of a test compound. IC$_{50}$ values were obtained by fitting the data to a sigmoid dose response equation using the software GraphPad Prism. The data for competitive and noncompetitive inhibition by CPD1 in case of the carboxyltransferase of *E. coli* were fitted to equations \[1; v = V_m \times A / (K_m(1+I/K_i) + A)\] and \[2; v = V_m \times A / (K_m(1+I/K_i) + A(1+I/K_{ii}))\], respectively.

The reversibility of CPD1 binding to the carboxyltransferase of *E. coli* was examined by dilution experiments. The enzyme without inhibitor as well as together with 15 and 30 nM CPD1, respectively, was diluted several times. The initial reaction velocity was immediately measured, whereas the activities of the inhibitor-free enzyme dilutions additionally were measured in the presence of 15 nM and 30 nM CPD1, respectively.

**Antibiotic susceptibility tests** - Microdilution MICs were determined against different bacterial strains in 96-well microtitre plates with growth medium containing serial dilutions (2-fold) of antibiotics. A starting inoculum of 1.0 x 10$^5$ c.f.u. ml$^{-1}$ derived from overnight cultures was used. *S. pneumoniae* cells were directly taken from colonies, grown overnight on columbia agar plates with 5% sheep blood (Becton Dickinson). The strains were aerobically incubated in Isosensitest medium at 37°C except *S. pneumoniae*, which was incubated at 37 °C in Brain Heart Infusion broth.
supplemented with 10% bovine serum using an anaerobic jar with AerocultC (Merck, Darmstadt, Germany). The MIC was the lowest concentration of drug that yielded no visible growth after incubation for 18-24 h at 37 °C. Endpoints were determined by measuring the optical density at 600 nm with the microtiter plate reader EL312e (Bio-Tec Instruments).

Isolation of resistant mutants - LB medium (3 ml) containing serial dilutions (2-fold) of the antibiotic was inoculated with overnight cultures of *B. subtilis* 168, *S. aureus* 133 and *E. coli* HN818, respectively (starting OD<sub>600</sub> = 0.01). After overnight incubation at 37 °C, the culture with the highest antibiotic concentration, at which the cells reached the same optical density at 600 nm as the antibiotic-free culture, was used for inoculation of a novel series of antibiotic dilutions in LB medium. This procedure was repeated six times. The resistant cells then were plated on antibiotic-free LB agar plates and incubated overnight at 37°C for isolation of single colonies. The obtained clones were tested for antibiotic susceptibility, in order to confirm their antibiotic resistance.

RESULTS

Metabolite incorporation and other technologies - We measured the effects of CPD1 on macromolecular syntheses in *E. coli*. The compound selectively inhibited incorporation of radioactively labeled acetate into chloroform/methanol extractable phospholipids. In contrast, the incorporation of labeled thymidine, uridine, leucine and N-acetyl-glucosamine into DNA, RNA, proteins and murein, respectively, was not inhibited (Fig. 3). These data suggested phospholipid synthesis as the possible target area of CPD1. In order to narrow down the target candidates, we measured incorporation of malonyl-CoA into hexane extractable fatty acids in the presence of the natural product. No effects up to 10 μM could be observed, while the FAS inhibitor cerulenin inhibited the incorporation by 83% (Fig. 4). We concluded that CPD1 probably inhibits the conversion of acetyl-CoA to malonyl-CoA, which is catalyzed by the key enzyme acetyl-CoA carboxylase.
We also applied other technologies coming to the same conclusions. Fourier-Transform Infrared (FT-IR) technology-based experiments with *B. subtilis* classified CPD1 as a FAS inhibitor (data not shown). In addition, transcriptome analyses with *B. subtilis* suggested the malonyl-CoA production as the point of inhibition (33).

**Enzymatic studies** - In order to show inhibition of ACC, we recombinantly expressed the carboxyltransferase subunits AccA and D and the biotin-carboxylase subunit AccC and tested their enzymatic activity in the presence of CPD1. While the biotin-carboxylase reaction was measured in physiological direction, the carboxyltransferase assay was designed to measure the decarboxylation of malonyl-CoA, i.e. the reverse reaction. Indeed, the carboxyltransferase was inhibited by CPD1 (Fig. 5), whereas the enzymatic activity of AccC was not influenced. Our kinetic studies of AccAD revealed apparent Michaelis constants of 100 ± 30 µM for malonyl-CoA and 10 ± 5 mM for biocytin. The specific activity of the enzyme preparation was 9 µmol/min/mg. We demonstrated that the inhibitor was competitive to malonyl-CoA with a Kᵢ value of approx. 5 nM and non-competitive to the biotin-derivative biocytin (Fig. 6). The reversibility of the binding mode of CPD1 was indicated by enzyme-inhibitor dilution experiments (Fig 7).

In a next step we expressed and tested carboxyltransferase components of other bacteria being important human pathogens. Kinetic analyses with the enzyme of *S. aureus* revealed apparent Michaelis constants of 150 ± 50 µM for malonyl-CoA and 70 ± 30 mM for biocytin roughly resembling the *E. coli* values. However, the specific activity of the enzyme preparation was one magnitude lower (0.4 µmol/min/mg) than in case of the *E. coli* enzyme. Inhibition experiments with the *E. coli* and *S. aureus* enzyme as well as with the not kinetically characterized enzyme preparations of *P. aeruginosa* and *S. pneumoniae* showed that CPD1 is a potent inhibitor of all these enzymes. While the Gram-negative enzymes exhibited IC₅₀ values of approx. 6 nM, the Gram-positive enzymes displayed IC₅₀ values of approx. 96 nM (*S. aureus*) and 450 nM (*S. pneumoniae*), respectively. A few derivatives of CPD1 were also tested for inhibition of the *E. coli*...
and *S. aureus* enzyme (Table 2). CPD2 and CPD3 gave IC$_{50}$ values similar or slightly better than CPD1, while CPD4 and CPD5 were less active. The antibacterial activity correlated with target activity in case of *E. coli*. However, the whole cell activity against *S. aureus* did not reflect variations in IC$_{50}$ values (Table 2).

We also tested the effect of pyrrolidine diones on the complete physiological ACC reaction in extracts of *E.coli* overexpressing all four ACC subunits (Fig. 5). The incorporation of [¹⁴C]-hydrogencarbonate into malonyl-CoA was measured. The obtained IC$_{50}$ values displayed the same ranking of potency for CPDs 1-4 as obtained with the partial reverse AccAD assay, despite some deviation of absolute values (Table 2).

Finally, we tested two compounds (CPD1 and CPD 3) for inhibition of the eukaryotic ACC isolated from rat liver and discovered no IC$_{50}$ up to 100 µM. These data indicated the selectivity of pyrrolidine diones for the prokaryotic version of ACC.

**Antibacterial susceptibility studies** - Compounds tested in enzyme assays were also tested for their antibacterial activity against *E. coli* and *P. aeruginosa* including corresponding pump deletion strains, against the Gram-positive pathogens *S. aureus* and *S. pneumoniae* as well as the Gram-positive model organism *B. subtilis* (Table 2). All species showed sensitivity to our pyrrolidine diones. However, in case of *P. aeruginosa* this sensitivity could only be observed in an efflux pump deletion mutant. Also a pump deletion strain of *E. coli* exhibited improved sensitivity.

**Identification of resistance determinants** - We generated resistant mutants of *B. subtilis*, *S. aureus* and *E. coli*, which were less sensitive to CPD2. Since we were not able to isolate spontaneously resistant mutants among $10^9$ cells, we transferred overnight-growing cells six times at sub-lethal antibiotic concentrations (see EXPERIMENTAL PROCEDURES). The isolated clones exhibited MIC values from 64 µM to $>$128 µM versus CPD2, while the original strains harbored MIC values of 1 to 4 µM. Sequencing the respective *accDA* operons revealed that each resistant clone contained
a single amino acid exchange in one of the two subunits AccA and D (Fig. 8). In *S. aureus* and *B. subtilis* the mutations were located at homologous amino acid positions of AccA within a motif that is highly conserved among bacterial AccA molecules (G->A and G->S at pos. 196 in *S. aureus* and pos. 199 in *B. subtilis*, respectively). In *E. coli* the mutation was found in subunit AccD at a similar amino acid position (S->Y at pos. 207).

The mutated *accDA* operon as well as the corresponding wild-type allele of *B. subtilis* were each ectopically put into the chromosomal *thrC* locus of *B. subtilis* behind a xylose-dependent promoter, while the wild-type locus of *accDA* was replaced by a neomycin resistance cassette. The mutant carrying the mutated *accDA* version was 8 to 64-fold less sensitive than the strain carrying the wild-type version in dependence of the xylose concentration (Table 3). These data proved that the mutation in *accA* of *B. subtilis* conferred resistance to CPD2.

**DISCUSSION**

*Antibacterial activity of pyrrolidine diones* - Pyrrolidine dione antibiotics were isolated between 1987 and 1994 from three distinct bacterial species of the gamma-subdivision of proteobacteria: *Enterobacter* sp., *Vibrio* sp. and *Pseudomonas fluorescens* (26-28). Additional publications deal with the chemical synthesis of such compounds (30,45,46). Only one mechanistic study has been published. Singh et al. (47) reported that CPD5 appeared to have preferential effects on uridine uptake and RNA synthesis. The antibiotics were reported to show broad-spectrum antibacterial activities against Gram-positive bacteria such as staphylococci and bacilli as well as Gram-negatives such as *E. coli*. We could confirm broad-spectrum activity of pyrrolidine diones, with good MIC values for *B. subtilis* and *S. aureus* (0.5-2 µg/ml of CPD2) and moderate MIC values for *E. coli* and *S. pneumoniae* (each 16 µg/ml of CPD2). Pyrrolidine diones seem to be substrates for efflux systems such as AcrAB in *E. coli* and MexAB-OprM in *P. aeruginosa*, since respective efflux system deletion strains were significantly more sensitive (MICs of CPD2: 2-8 µg/ml). *P.
aeruginosa even obtained sensitivity only by elimination of such an efflux system. On the other hand, the moderate susceptibility of S. pneumoniae might rather be caused by medium effects (e.g. by serum albumin binding), since this was the only species tested in medium containing serum. Certainly, more species in different media need to be tested, in order to evaluate the broad-spectrum potency of this compound class. Nevertheless, the reported antibacterial activity provides a promising starting point for further optimization regarding broad-spectrum potency and improved membrane penetration in Gram-negative bacteria.

**Target elucidation** - In order to start such a chemical optimization program, one important requirement is the knowledge about the mode of action of such compounds. Therefore, the aim of this study was the elucidation of the molecular target of pyrrolidine diones. The metabolite incorporation assays with CPD1 did not confirm recently published effects of pyrrolidine diones on RNA synthesis (47). While replication, transcription, translation and cell wall synthesis were not influenced, a clear inhibitory effect was observed for acetate incorporation into phospholipids. The measurement of malonyl-CoA incorporation into fatty acids enabled to narrow down the target area of pyrrolidine diones to the early FAS pathway. Remarkably, our transcriptome analyses with B. subtilis represented a complementary approach to pinpoint this target area (33).

The enzyme responsible for production of the central FAS precursor malonyl-CoA is ACC. Therefore, we applied several assay systems, in order to measure inhibition of the partial reactions as well as the complete reaction of ACC. It was shown that the carboxyltransferase reaction is inhibited by pyrrolidine diones. We obtained similar IC\(_50\) ranking with the spectrophotometric carboxyltransferase assay, which measured the reverse reaction with recombinant enzymes and malonyl-CoA and biocytin as substrates, as with the radioactive ACC assay, which measured the complete reaction in physiological direction in E. coli extracts. Our IC\(_50\) determinations even indicated that target activity correlates with antibacterial activity against the E. coli pump deletion strain HN818. However, in case of the Gram-positive pathogen S. aureus good antibacterial activity
was retained despite a higher IC₅₀ value of CPD4. Such data indicate that other parameters such as physicochemical properties, which influence bacterial membrane penetration, certainly need to be considered. Indeed, correlation between target activity and whole cell activity can only confidently be deduced on the basis of numerous derivatives with diverse potencies. In comparison, the reverse carboxyltransferase assay is much more facile than the ACC extract assay and appears to deliver results with physiological relevance. Therefore the assay is suitable for screening and studying novel ACC inhibitors, harboring the potential to become antibacterials.

**Carboxyltransferases from various species** - We demonstrated that the carboxyltransferase overexpression strategy described for the recombinant *E. coli* enzyme AccAD was also applicable for orthologous enzymes from other species. Enzymes from important pathogens (*S. aureus*, *S. pneumoniae* and *P. aeruginosa*) were tested with the carboxyltransferase assay for inhibition by pyrrolidine diones. The IC₅₀ values obtained with the Gram-positive enzymes were increased by a factor of ten in comparison to the Gram-negative ones. Nevertheless, the nanomolar inhibition values demonstrate the potency of this compound class versus a broad-spectrum of bacterial ACC targets.

The kinetic studies with the *E. coli* and *S. aureus* enzyme revealed, that the Michaelis constants were similar for malonyl-CoA (approx. 100 µM and 150 µM, respectively) and only slightly different for biocytin (approx. 10 and 70 mM, respectively) with the *S. aureus* biocytin value still harboring a considerable error of ± 42%. Our kinetic data with the *E. coli* enzyme were in agreement with the ones published by Blanchard and Waldrop (K_iₐ for malonyl-CoA = 100 µM and K_m for biocytin = 6.3 mM) (36).

**Inhibition pattern of pyrrolidine diones** – Recently, a bisubstrate analog, that consists of covalently linked biotin and coenzyme A, was published as a weak inhibitor of the *E. coli* carboxyltransferase. It exhibited competitive inhibition versus malonyl-CoA and non-competitive inhibition versus
biocytin. Such an inhibition pattern shows an ordered binding of substrates to the carboxyltransferase, in which malonyl-CoA binds first followed by biocytin. Our inhibition experiments with CPD1 using the *E. coli* enzyme revealed the same inhibition pattern, but with a $K_i$ value reduced by three orders of magnitude ($K_i$ for CPD1 = 0.005 µM; $K_i$ for the bisubstrate analog = 23 µM) (24). Enzyme/inhibitor dilution experiments indicated that CPD1 binds to AccAD reversibly. Experiments with radioactively labeled substrate would confirm our results. Little is known about the catalytic mechanism of the carboxyltransferase in contrast to the other partial ACC reaction catalyzed by the biotin carboxylase. For instance, no three-dimensional structure of the carboxyltransferase sub-complex has been published so far. Attempts to obtain crystals that diffract to high resolution are still ongoing (22). As soon as structural data are available, docking studies or co-crystallization with these highly affine inhibitors will open up new avenues to study their binding mode and the catalytic mechanism of the carboxyltransferase.

*Selectivity of pyrrolidine diones for bacterial ACC* - We also performed first inhibition studies with pyrrolidine diones on the eukaryotic ACC isolated from rat liver. IC$_{50}$ values above 100 µM indicated that the pyrrolidine diones are selective for the bacterial multisubunit ACC. In mammals including humans two isoforms of ACC exist. Each enzyme represents a single polypeptide chain with multiple domains. Further tests with the separate isoforms ACC1 and ACC2 (48) are required to ensure the high preference of pyrrolidine diones for the prokaryotic enzyme. Such a selectivity is an important prerequisite for clinical use of pyrrolidine diones, in order to avoid target-based toxicity effects in humans.

*Resistance mutations* - We tried to obtain mutants of *B. subtilis, S. aureus* and *E. coli* spontaneously resistant to the pyrrolidine dione CPD2. We were not able to easily identify such strains by plating experiments, indicating the low resistance rate of this compound (see RESULTS). Therefore we isolated resistant strains by several transfers of growing cultures at sub-lethal antibiotic
concentrations as in resistance induction experiments. We cannot rule out that several mutation events could have been taken place during such a procedure. Nevertheless, we could find mutations within the accDA operon. By introducing the mutated accDA operon from B. subtilis into an originally sensitive wild-type strain, we observed an 8 to 64-fold decreased sensitivity versus CPD2 leading to MIC values of 16 µg/ml (compared to 0.25 – 2 µg/ml of the wild-type strain depending on the expression level of accDA). This way, an additional proof was delivered that ACC inhibition is the cause for bacterial growth inhibition by pyrrolidine diones.

Remarkably, the mutations of the isolated resistant strains of S. aureus and B. subtilis are located at the same position within a conserved motif of AccA. The mutation identified in the resistant E. coli strain was not located in the alpha-subunit but in the beta-subunit AccD at a position similar to the one of AccA, since both subunits can be considered to be related from the primary sequence point of view. Although the overall sequence identity is below 20%, multiple alignments reveal several amino acid positions significantly conserved across both AccA and AccD sequences derived from genomic sequences of numerous different species (data not shown). All three mutations identified were found close to a conserved glycine position (see Fig. 8). Seemingly, important sites for the interaction with the inhibitor near a structurally or functionally conserved amino acid position have been mutated in all three species.

Conclusions - This report describes the elucidation of the mode of action of natural product-derived pyrrolidine diones. These compounds represent the first bacterial ACC inhibitors with antibacterial activity. For a long time, it has been predicted that bacterial ACC might be a suitable target for antibacterial drug discovery. The data presented here prove this hypothesis. The knowledge of the target and the availability of facile assays using carboxyltransferases from different pathogens now allow to evaluate the antibacterial potential of pyrrolidine dione derivatives. This study might serve as a starting point for further improvement of potency, leading to a therapeutically useful new class of antibacterials with a novel mode of action.
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TABLE 1. Oligonucleotides used in this study

| Primer name   | Sequence (5'->3' direction)                                      |
|---------------|-----------------------------------------------------------------|
| ACCA1A_ECO    | CCGGGGAATTCCATATGAGTCTGAATTTCTTGTATTTTGAA                      |
| ACCA1B_ECO    | CTCATCTCGAGTTTCTTACGCGTAAACGTTAGCTCATCAGGCG                   |
| ACCD2A_ECO    | CGTAAGGAGAACTCGAGATGAGCTGGATTGAAACGAAATTAAAAGC                 |
| ACCD2B_ECO    | CCGGGGATCCCTCACGGCCTCAGGCCTAGTGCTTCTGTACGTCGGTAC              |
| ACCA1A_SA     | CCGGGAAATTCCATATGAGTCTGAATTTCTTGTATTTTGAA                      |
| ACCA1B_SA     | AACATTCTAGATTCTCCTATTCTATATATAAGAAACGATATTTCTG                |
| ACCD2A_SA     | AATAAGGAGAAATCTAGAATGTATTAAAGATTTTTTAAATCGAAC                 |
| ACCD2B_SA     | CCGGCTCGAGTTATTTAGTCACCTTTGTATGG                              |
| ACCA1A_SP     | CCGGGAAATTCCATATGAGTCTGAATTTCTTGTATTTTGAA                      |
| ACCA1B_SP     | GCCATTTCTAGATTCTCCTTTATATATATCAAATCGGATAG                    |
| ACCD2A_SP     | ACTAAGGAGAAATCTAGAATGTATTAAAGATTTTTTAAATCGAAC                 |
| ACCD2B_SP     | CCGGCTCGAGTTATTTAGTCACCTTTGTATGG                              |
| ACCA1A_PAE    | CCGGGAAATTCCATATGAGTCTGAATTTCTTGTATTTTGAA                      |
| ACCA1B_PAE    | CTCAATTCTAGATTCTCCTTTACGCGCGGCGCCGAGTCCTG                    |
| ACCD2A_PAE    | CGTAAGGAGAAATCTAGAATGAGCAACTGGGTCTGGTAGAC                     |
| ACCD2B_PAE    | CCGGCGGATCCCTCATCGGGATACGGGGGCTC                              |

The restriction sites for cloning of the fusion-PCR products are underlined (see text).
TABLE 2. IC₅₀ values and MIC data of pyrrolidine dione derivatives.

| Compound | IC₅₀ AccAD [nM]ᵃ | IC₅₀ ACC [nM]ᵇ | MIC E. coli [µg/ml] | MIC E. coli HN818 [µg/ml] | MIC E. coli Neumann [µg/ml] | MIC S. aureus 133 [µg/ml] | MIC S. aureus 133 G9A [µg/ml] | MIC B. subtilis 168 [µg/ml] | MIC P. aeruginosa PAO1 [µg/ml] | MIC P. aeruginosa PAO200 [µg/ml] |
|----------|------------------|----------------|-------------------|--------------------------|-----------------------------|-----------------------------|--------------------------------|-------------------------------|-------------------------------|--------------------------------|
| CPD1     | 6                | 15             | 4                 | 32                       | 96                          | 8                           | 32                             | 1                             | >64                           | 16                             |
| CPD2     | 4                | 11             | 1                 | 16                       | 91                          | 2                           | 16                             | 0.5                           | >64                           | 8                              |
| CPD3     | 1                | 8              | 1                 | 8                        | 50                          | 8                           | 16                             | 16                            | >64                           | 16                             |
| CPD4     | 37               | 288            | >64               | >64                      | >64                         | 540                         | 2                              | 16                            | n. d.                         | >64                            |
| CPD5     | 13               | n. d.          | 32                | >64                      | 305                         | 8                           | 8                              | n. d.                         | >64                           | n. d.                          |

ᵃ AccAD = recombinant carboxyltransferase from E. coli and S. aureus, respectively;
ᵇ ACC: acetyl-CoA carboxylase measured in E. coli cell extracts
TABLE 3. Antibacterial activity of CPD2 versus *B. subtilis* carrying a mutated *accDA* operon<sup>a</sup>

| Compound   | MIC [ µg/ml] | *B. subtilis* MH108 | *B. subtilis* MH110 |
|------------|--------------|---------------------|---------------------|
|            | 0.0025% xylose | 0.25% xylose | 0.0025% xylose | 0.25% xylose |
| CPD2       | 0.25         | 2               | 16                 | 16          |
| Ciprofloxacin | 0.04        | 0.08            | 0.08               | 0.08        |
| Tetracyclin | 8            | 8               | 8                  | 8           |

<sup>a</sup> MIC values in µg/ml of CPD2 determined with recombinant *B. subtilis* strains conditionally expressing a wild-type *accDA* operon [strain MH108, which is also called ACCDA(-) (33)] and a mutated *accDA* allele (MH110) in dependence of the inducing agent xylose. The strain MH108 exhibits differential sensitivity towards CPD2 depending on the expression level of *accDA* (0.25 - 2 µg/ml), while strain MH110 shows non-differential and significantly reduced sensitivity to that compound (16 µg/ml). Control antibiotics such as ciprofloxacin and tetracyclin give (nearly) the same MIC values each with both strains and xylose concentrations.
FIGURE LEGENDS

Fig. 1. Bacterial fatty acid synthesis pathway (FAS type II). Acetyl-CoA is converted to malonyl-CoA by the acetyl-CoA carboxylase complex (AccABCD). Malonyl-CoA is transferred to the acyl-carrier protein (ACP) by FabD and initially condensed with acetyl-CoA or in some species with branched chain acyl-CoA primers (= R-CoA) by the β-ketoacyl-ACP synthase III (FabH). The resulting β-ketoacyl-ACP enters the elongation cycle leading to long-chain acyl-ACP by reduction (FabG), dehydration (FabZ), reduction (FabI, FabL, FabK) and elongation (FabB, FabF) consuming additional malonyl-ACP molecules. The enoyl-ACP reductases FabI and FabK are different isoforms catalyzing the same reaction (see text). The enzyme FabL, which is not discussed in the text, is another isoform in some species, which exhibits similarities to FabI.

Fig. 2. Structures of pyrrolidine diones (CPD1 = moiramide B; CPD5 = andrimid)

Fig. 3. Metabolite incorporation in *E. coli* MG1655 in the presence of CPD1 in comparison to controls without the antibiotic. The fourfold MIC of CPD1 in minimal media containing 2 µg/ml PMBN (= 32 µg/ml) was applied (see EXPERIMENTAL PROCEDURES).

Fig. 4. Incorporation of [14C]malonyl-CoA into fatty acids using cell extracts of *E. coli* MG1655 in the presence of various concentrations of CPD1 and cerulenin, respectively.

Fig. 5. Inhibition of *E. coli* ACC by CPD1. The enzymatic activities of A) the recombinant carboxyltransferase (measured in reverse direction) and B) of the complete ACC (measured in physiological direction in cell extracts) are plotted against increasing concentrations of CPD1. The obtained IC50 values of A) 6 nM and B) 19 nM are marked on the x-axes.
Fig. 6. Inhibition of *E. coli* carboxyltransferase by increasing concentrations of CPD1 with respect to biocytin (A) and malonyl-CoA (B), and *K*<sub>i</sub> determination of CPD1 (C). A) and B): The reciprocals of the initial reaction velocities and substrate concentrations are plotted. The lines represent the best fit of data to equation 2 (A) or 1 (B) (see EXPERIMENTAL PROCEDURES). When the concentration of biocytin was varied, the malonyl-CoA was held constant at 100 µM. In case of malonyl-CoA being the variable substrate the biocytin concentration was fixed at 10 mM. C) The slope values of the lines from graph B are plotted versus the inhibitor concentrations giving a line obtained by linear regression. The intercept point of this line with the x-axis gives an approximate *K*<sub>i</sub> value of 5 nM for CPD1.

Fig. 7. Carboxyltransferase/inhibitor dilution experiments. The initial velocities of *E. coli* carboxyltransferase dilutions, which were differently treated with the inhibitor CPD1, are plotted in percent of the non-inhibited enzyme activity (control) versus the dilution factor. The maximal enzyme concentration used for dilution was 2.4 µg/ml. The 1<sup>st</sup> column (black) represents the enzyme activity without CPD1, which is set as 100% initial velocity after each dilution step. The 2<sup>nd</sup> and 3<sup>rd</sup> columns (white and gray) show the diluted enzyme activities, which were measured at fixed CPD1 concentrations of 15 nM and 30 nM, respectively. The 4<sup>th</sup> and 5<sup>th</sup> columns (dotted) represent the activities of carboxyltransferase, obtained by dilution of the enzyme together with 15 nM and 30 nM CPD1, respectively. No additional inhibitor was added during enzyme kinetics. Eight-fold dilution of the carboxyltransferase (from 2.4 to 0.3 µg/ml) together with the inhibitor gives activities in the range of the inhibitor-free enzyme dilution, indicating the reversibility of CPD1 binding to the enzyme.

Fig. 8. Multiple amino acid sequence alignments of the carboxyltransferase subunits AccA and D around the glycine position 206 of *E. coli*-AccA and 205 of *E. coli*-AccD (marked by a star), which is highly conserved across AccA and D subunits of diverse species. The exchanged amino acids
identified in the CPD2-resistant strains *E. coli* RMH100, *S. aureus* RMH102 and *B. subtilis* RMH103 are written in bold and framed. The amino acid stretches (motifs) highly conserved across the species in AccA and AccD, respectively, are marked by gray background color. The MIC values of CPD2 with respect to the resistant strains and their wild-type counterparts were 1 µg/ml (*E. coli* HN818), 32 µg/ml (*E. coli* RMH100), 2 µg/ml (*S. aureus* 133), >64 µg/ml (*S. aureus* RMH102), 0.5 µg/ml (*B. subtilis* 168) and >64 µg/ml (*B. subtilis* RMH103).
Fig. 1
Fig. 2

CPD1  \( R = \begin{array}{c}
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Fig. 3

Fig. 4
Fig. 5
Fig. 6

[Graph showing enzyme activity across different dilutions]

**Enzyme dilution**

Fig. 7

**AccA**

- *E. coli*  
  \[...QLTVFVICTVIGEGCGGALIAIGVGDKVNI... 219\]
- *P. aeruginosa*  
  \[...PLKPIIATVIGEGCGGALIAIGVCDQILM... 216\]
- *S. aureus* 133  
  \[...SLKVPVIAVTIGEGCGGALIGIGIANKVL... 212\]
- *S. aureus* RMH102  
  \[...SLKVPVIAVTIGEGCGGALIGIGIANKVL... 212\]
- *S. pneumoniae*  
  \[...DLKVPVIAIITIGEGCGGALAVADRVWM... 162\]
- *B. subtilis* 168  
  \[...GLRVFVICTVIGEGCGGALGVGNHLHM... 215\]
- *B. subtilis* RMH103  
  \[...GLRVFVICTVIGEGCGGALGVGNHLHM... 215\]

**AccD**

- *E. coli* HN818  
  \[...EERGLFISVLTDPTTGGVSASFAHLDLNI... 218\]
- *E. coli* RMH100  
  \[...EERGLFISVLTDPTTGGVSASFAHLDLNI... 218\]
- *P. aeruginosa*  
  \[...EEGIPFVSVLTDPTTGGVSASFAHLDLNI... 222\]
- *S. aureus* 133  
  \[...DASLGYISYLVHTTGGVSASFAHLDGIDL... 221\]
- *S. pneumoniae*  
  \[...NAAGFLYLTILTTGVTASAHEGDDIL... 236\]
- *B. subtilis* 168  
  \[...EEQLIIISVNHPTTTGGVSASFAHLDYNF... 192\]

Fig. 8
Identification and characterization of the first class of potent bacterial acetyl-CoA carboxylase inhibitors with antibacterial activity
C Freiberg, N. A. Brunner, G. Schiffer, T. Lampe, J. Pohlmann, M. Brands, M. Raabe, D. Häbich and K. Ziegelbauer

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