Structure of the heme biosynthetic

_Pseudomonas aeruginosa_ Porphobilinogen Synthase in

Complex with the Antibiotic Alaremycin

Running title: Molecular function of alaremycin

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### ABBREVIATIONS

1. **ALA** 5-aminolevulinic acid  
2. **PBGS** porphobilinogen synthase  
3. **PBG** porphobilinogen  
4. **MIC** minimal inhibitory concentration  
5. **IPTG** isopropyl-1-thio-ß-D-galactopyranoside  
6. **IC\textsubscript{50}** inhibitor concentration reducing enzyme activity to 50%
The recently discovered antibacterial compound alaremycin produced by *Streptomyces sp.* A012304 structurally closely resembles 5-aminolevulinic acid, the substrate of porphobilinogen synthase. During the initial steps of heme biosynthesis two molecules of 5-aminolevulinic acid are asymmetrically condensed to porphobilinogen. Alaremycin was found to efficiently inhibit the growth of both Gram negative and positive bacteria. Using the newly created heme-permeable *E. coli* strain CSA1, we are able to uncouple heme biosynthesis from bacterial growth and demonstrate that alaremycin targets the heme biosynthetic pathway. Further studies focused on alaremycin activity against the opportunistic pathogenic bacterium *Pseudomonas aeruginosa*. The minimal inhibitory concentration (MIC) of alaremycin was determined to be 12 mM. Alaremycin was identified as a direct inhibitor of recombinant purified *P. aeruginosa* porphobilinogen synthase with a *K*<sub>i</sub> of 1.33 mM. To understand the molecular basis of alaremycin antibiotic activity at the atomic level, the *P. aeruginosa* porphobilinogen synthase was co-crystallized with the alaremycin. The crystal structure at 1.75 Å resolution reveals the antibiotic to efficiently block the active site of PBGS. The antibiotic binds as a reduced derivative of 5-acetamido-4-oxo-5-hexenoic acid. The corresponding methyl group is, however, not coordinated by any amino acid residues of the active site, excluding its functional relevance for alaremycin inhibition. Alaremycin is covalently bound by the catalytically important active site lysine residue 260 and tightly coordinated by several active site amino acids. Our data provide a solid structural basis to further improve alaremycin activity for rational drug design. Potential approaches are discussed.
INTRODUCTION

Modified tetrapyrroles are complex macromolecules and the most abundant pigments found in nature. Tetrapyrroles such as hemes and chlorophyll are essential prosthetic groups involved in numerous electron transport chains for energy recovery in essentially all forms of life. The biosynthetic pathway of tetrapyrroles is correspondingly highly conserved (16, 30), making heme biosynthesis an attractive target for antibacterial drug discovery and application (24). In fact, the tetrapyrrole biosynthetic pathway serves both as source for the production of antibiotics such as asukamycin (25) and as target for antibiotics as in the case of gabaculin (1, 19). Recently, the antibiotic alaremycin was isolated from the culture broth of the actinomycete Streptomyces sp. A012304. Its structure, determined to be 5-acetamido-4-oxo-5-hexenoic acid (2) (Figure 1B), is related to 5-aminolevulinic acid (ALA), the first common precursor molecule of all tetrapyrroles. The enzyme porphobilinogen synthase (PBGS, EC 4.2.1.24) asymmetrically condenses two such molecules of ALA to generate porphobilinogen (26, 27) (Figure 1A). The antimicrobial activity of alaremycin against E. coli is enhanced by the presence of ALA, implying that its antimicrobial activity derives from the inhibition of PBGS (2). During tetrapyrrole biosynthesis, four molecules of porphobilinogen are fused to generate the first tetrapyrrole uroporphyrinogen III. Thereafter the biosynthetic pathways of heme and chlorophyll separate from that of vitamin B\textsubscript{12}, siroheme and factor F\textsubscript{430} due to distinct modifications of the initial tetrapyrrole skeleton (30).

PBGS from a range of organisms have been investigated biochemically and structurally providing in-depth insights into PBGS catalysis. (4, 7-10). The two ALA substrate molecules occupy two distinct binding sites in PBGS referred to as A- and P-site to indicate that they contribute either acetic acid or propanoic acid side chains to porphobilinogen. Each ALA is covalently bound through a stable Schiff base with the lysine residues 205 and 260, respectively (12). Although PBGS from different organisms differs with respect to the metal ion requirements and localization, the amino acid sequence is highly conserved between bacteria, archaea and eukaryotes. Whereas human PBGS is...
Zn$^{2+}$-dependent (17) catalysis by PBGS from the opportunistic human pathogen *Pseudomonas aeruginosa* is metal-independent but retains a structural Mg$^{2+}$-site near the active site (9, 13).

Many organisms including bacteria produce dedicated antimicrobial compounds to ward off infections or competing microbes. *Streptomyces sp.* 012304 hence produces the compound alaremycin, thereby preventing growth in neighboring microorganisms. Strategies to counteract the effect of alaremycin on *Streptomyces sp.* 012304 itself have so far not been described. We have investigated the molecular basis for antimicrobial activity of alaremycin, by analyzing its activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Bacillus megaterium*, *Streptomyces coelicolor* and *Streptomyces avermitilis*. As PBGS is the molecular target of alaremycin, we studied the impact of the antibiotic on recombinant Mg$^{2+}$-stimulated *P. aeruginosa* and Zn$^{2+}$-dependent *Methanosarcina barkeri* PBGS. Alaremycin was furthermore co-crystallized with the PBGS from *P. aeruginosa* and its structure solved at 1.75 Å to describe its mode of PBGS inactivation at the atomic level.
EXPERIMENTAL

MATERIAL AND METHODS

Materials. ALA was purchased from Merck (Darmstadt, Germany), porphobilinogen from Porphyrin Products (Logan, USA), other chemicals from Sigma-Aldrich (Hamburg, Germany). Oligonucleotides were purchased from Metabion (Planegg-Martinsried, Germany), Protino® Ni-IDA Resin from Machery-Nagel (Düren, Germany) and ethylmethane sulfonate from ABCR (Karlsruhe, Germany).

Bacterial strains and growth conditions. To determine the antibacterial effect of alaremycin, *P. aeruginosa* PA01 was grown in AB minimal media, *Bacillus subtilis* JH642, *S. coelicolor* (DMS 40233) and *S. avermitilis* (DSM 46492) in minimal media SMM, *E. coli* CSA1 in M9 minimal media containing 20 µM hemin (20). *B. megaterium* (DSM 319) was grown in MOPSO minimal media. To quantify PBGS activity in cell free extracts, *S. avermitilis* was cultured in 2 % oat meal, pH 7.2 at 30 °C, 200 rpm for 4 days. *S. coelicolor* was grown in 0.4 % glucose, 0.4 % yeast extract, 1 % malt extract, pH 7.2 at 30°C, 200 rpm for 4 days. *Streptomyces* sp. A012304 was grown in seed medium (4 % glucose, 1 % dry bouillon, 0.3 % soy bean meal and 0.3 % CaCO$_3$, pH 7.0) at 30 °C, 200 rpm for 2 days. All other bacterial strains were grown in LB-medium at 37°C for 20 h.

Isolation of alaremycin. For alaremycin production, *Streptomyces* sp. A012304 was cultivated in production medium containing 6 % dextrin, 2 % yeast extract, 0.3 % NaCl, 0.3 % CaCO$_3$, 0.1 % dry bouillon and 0.1 % K$_2$HPO$_4$, pH 7.0, at 30 °C, 200 rpm for 4 days. Alaremcyin was isolated as described (2). It was shown to be at least 98% pure as analyzed by NMR spectroscopy (2).

Determination of the minimal inhibitory concentration (MIC). MICs were determined using microdilution techniques: two-fold dilutions (1 – 100 mg/L) of alaremycin in double distilled water.
were placed in microtiter plates and \(10^8\) cells/mL exponentially growing *B. subtilis*, *B. megaterium*, *P. aeruginosa*, *S. coelicolor*, *S. avermitilis*, *E. coli* DH10b and *E. coli* strain CSA1 were added. *E. coli* strain CSA1 was incubated in the presence and absence of 50 µg/mL hemin. For all strains at least 4 independent growth curves over 24 h with different alaremycin concentrations were recorded. The MIC was determined as the lowest alaremycin concentration that inhibited growth for 5 h.

**Expression vectors for recombinant *P. aeruginosa* and *Methanosarcina barkeri* PBGS genes.**

*M. barkeri* hemB was amplified from chromosomal DNA by PCR using the primers 5′-CCGGAATTCCGGATGTTTCCAGATGTCAGGTTAAG-3′ and 5′-CCGCTCGAGCGGTTACTTCAACATGCGGGCAGC-3′ containing *Eco*RI and *Xho*I sites, respectively. The resulting 975-bp PCR product was digested with *Eco*RI and *Xho*I and inserted into pET32a (Novagen, Madison WI, USA) to create pET32aMbhemB. The vector pGEXhemB containing *P. aeruginosa* PBGS was kindly provided by Dr. N. Frankenberg-Dinkel (University Bochum, Germany) (10).

**Protein production and purification.** Recombinant *P. aeruginosa* PBGS was produced and purified as described (10). Protein integrity was analyzed by mass spectrometry and Western blot analysis. Recombinant *M. barkeri* PBGS was produced using *E. coli* strain BL21(DE3)RIL (Stratagene, Heidelberg, Germany) in LB-medium at 37°C and 180 rpm. At OD\(_{578} = 0.7\), protein production was induced by 150 µM isopropyl-1-thio-β-D-galactopyranoside (IPTG). Cells were cultivated overnight at 17 °C and 150 rpm, harvested, washed with buffer A (50 mM Tris HCl, pH 8.5, 300 mM NaCl, 10 mM ZnCl\(_2\)) and resuspended in a minimal volume of buffer A. After cell disruption by sonication (Bandelin HD 2070) and centrifugation, (100,000 g for 45 min), the soluble fraction was applied to Protino® Ni-IDA agarose (Machery-Nagel, Düren, Germany), washed and PBGS eluted with 300 mM imidazole in buffer A. Further purification steps involved anion exchange chromatography on a DEAE Sepharose column (PBGS elution with 200 mM NaCl in...
buffer A) and gel permeation chromatography on a 30 mL Superdex 200 HR 10/30 (GE Healthcare) (0.5 mL/min) in buffer A. The overall yield was ~9 mg of *M. barkeri* PBGS per litre of culture.

**Enzyme activity assay.** PBGS activity was quantified by a modified Ehrlich’s test based on the reaction between the product PBG with 4-(dimethylamino)-benzaldehyde (10, 15). The Michaelis Menten constant \( (K_M) \), the maximal velocity \( v_{\text{max}} \) and the catalytic constant \( k_{\text{cat}} \) were determined by measuring the constant rate of PBG formation for 0 - 10 mM ALA and iteratively optimized Lineweaver Burke plots using SigmaPlot 8.0 Enzyme Kinetics v1.1. The catalytic efficiency \( k_{\text{cat}} \) was obtained by dividing \( v_{\text{max}} \) by the enzyme concentration. PBGS activity in cell free extracts was determined by adjusting protein concentrations to an absorbance \( A_{280} \) of 5 with buffer K1. As a control, cell free extracts were inactivated by heating to 95 °C for 10 minutes resulting in negligible background activity. Reactions without additional ALA were used to determine the concentration of cellular PBG in cell free extracts.

**Determination of inhibition constants.** Two \( \mu \text{g/mL} \) *P. aeruginosa* PBGS and 25 \( \mu \text{g/mL} \) *M. barkeri* PBGS were diluted to a final absorbance of 5 at \( \lambda_{280} \) with kinetic buffer K1 (100 mM BTP, pH 8.5). 40 mM ALA and alaremycin solutions were prepared in buffer K1. For *P. aeruginosa* PBGS 10 mM \( \text{MgCl}_2 \) was added, for *M. barkeri* PBGS 10 mM \( \text{ZnCl}_2 \). Protein, buffer and alaremycin (0 - 10 mM) were mixed and incubated at 37 °C for 10 minutes. Longer incubation times did not affect enzyme inhibition. Five mM substrate were added to start the reaction. The PBGS-catalyzed reaction was stopped at time points between 1 and 60 min by adding equivalent volumes of stop reagent (50 % trichloroacetic acid, 100 mM \( \text{HgCl}_2 \)) to the reaction mixture. After centrifugation (3 min at 5000 g), the supernatant was treated with an equivalent amount of Ehrlich’s reagent (0.4 g 4-dimethylamino benzaldehyde in 10 mL acetic acid and 10 mL \( \text{HClO}_4 \)). After 15 min incubation at room temperature, the product was quantified by absorbance at 555 nm \( (\varepsilon = 60,200 \text{ M}^{-1} \text{ cm}^{-1}) \). \( \text{IC}_{50} \) for alaremycin was
determined for both *P. aeruginosa* and *M. barkeri* PGBS. As inhibition was time independent, kinetic inhibition constants $k_i$ were determined by Michaelis-Menten kinetics.

**Crystallization and structure determination.** 20 mg/mL recombinant *P. aeruginosa* PBGS was mixed with 5 mM alaremycin (both in 100 mM Tris-HCl, pH 7.5 and 10 mM MgCl$_2$). The purity of the alaremycin was at least 98% as determined by NMR spectroscopy (2). Crystallization conditions were identified using Crystal Screen and Crystal Screen 2 (Hampton Research, Aliso Viejo, CA) in 96-well sitting-drop racks (Douglas Instruments) with 100 µL reservoir solution and drops of 3 µL of reservoir and 3 µL protein solution. *P. aeruginosa* PBGS crystals (space group I422, $a = b = 84$ Å, $c = 159$ Å) grew in 12 h at 17°C in 100 mM Hepes pH 7.5, 200 mM MgCl$_2$, 30 % PEG400. Crystals were cryo-protected by 20% (v/v) PEG 400 and flash-cooled in liquid N$_2$.

Data were collected at 100 K at beamline BL1 (BESSYII, Berlin, Germany) and indexed, integrated and scaled using HKL2000 (23). Data statistics are listed in table 3. The structure of *P. aeruginosa* PBGS in complex with alaremycin was solved by molecular replacement using Phaser (5) (6) and the A-chain of a related crystal (PDB code 1B4K). The structure was refined by restrained refinement with TLS-protocols (22). For manual adjustments and quality control the graphics program Coot was used (7). Molecular depictions were prepared using PyMOL (www.pymol.org).

Atomic coordinates have been deposited with the Protein Data Bank, accession number 3B1C.
RESULTS AND DISCUSSION

Rationale of the experimental approach

Alaremycin was recently shown to inhibit the growth of an *E. coli* laboratory strain (2). To determine the general applicability of alaremycin as an antibiotic, a range of bacterial species was exposed to the compound. Gram positive representatives include *B. megaterium* and *B. subtilis*, Gram negative bacteria *P. aeruginosa* and *E. coli* DH10b. Alaremycin-producing and non-producing *Streptomyces* strains were analyzed for their intrinsic immunity towards the antibiotic. To identify the cellular target of alaremycin activity, a heme permeable *E. coli* strain CSA1 was created to test alaremycin independently of intrinsic heme biosynthesis. Alaremycin inhibition of PBGS was determined in cell-free extracts for the species listed above and for purified enzyme from *P. aeruginosa* and *M. barkeri*. PBGS from *P. aeruginosa* was also used for biochemical characterization as well as for co-crystallization with alaremycin and crystal structure determination.

Heme biosynthesis is the target of alaremycin activity

*E. coli* laboratory strains have generally lost their ability to import heme from the surrounding medium (29). We have created an *E. coli* strain capable of heme uptake by treating the *hemA*^-^ *E. coli* strain EV61 (28), which normally requires ALA for growth, with ethylmethane sulfonate to induce chemical mutagenesis (20, 21). Colonies were screened for their ability to grow in ALA-free, heme-containing media. Our isolated heme-permeable *hemA*^-^ strain was designated *E. coli* CSA1. If heme biosynthesis is the target of alaremycin activity heme addition to *E. coli* CSA1 should overcome the growth inhibitory phenotype. Alaremycin treatment [≤ 40 mg/mL] of *E. coli* CSA1 in the presence and absence of ALA completely inhibited bacterial growth. Further addition of heme rendered *E. coli* CSA1 alaremycin insensitive. This implicates heme biosynthesis as the target of alaremycin.
Alaremycin inhibits the growth of Gram negative and positive bacteria

Minimal inhibitory concentrations of alaremycin (MIC) for several Gram positive and negative bacteria ranged between 4 mM and over 20 mM (Table 1). For example for *E. coli* the MIC for alaremycin was 4 mM. Eight and 14 mM alaremycin inhibited growth of *S. coelicolor* and *S. avermitilis* in complex media, whereas the producer strain *Streptomyces sp.* A012304 was not inhibited by alaremycin concentrations of up to 20 mM.

Quantifying PBGS inhibition by alaremycin in cell-free extracts

As alaremycin is structurally related to ALA (the substrate of PBGS), we analyzed the effect of 0, 5 and 10 mM alaremycin on PBGS-activity in cell-free extracts of *S. coelicolor*, *S. avermitilis*, *S. sp.* A012304, *E. coli*, *B. megaterium*, *B. subtilis*, *P. aeruginosa*, *E. coli* DH10b and *E. coli* CSA1. Surprisingly, the trend of alaremycin inhibition of PBGS in cell-free extracts is distinct from that of the corresponding organisms (Table 1). Thus 5 mM alaremycin essentially abolishes PBGS activity of *B. megaterium* cell-free extract, whereas *S. coelicolor* (22%), *P. aeruginosa* (25%) and *B. subtilis* (30%) cell-free extracts retain residual PBGS activity even at 10 mM alaremycin. Differences in alaremycin inhibition between intact organism (MIC) and cell free extracts (PBGS) activity observed for *P. aeruginosa* are presumably due to variable permeability and up-take capacities of individual organisms. Interestingly, *Streptomyces sp.* A012304 cell-free extracts retain 78% PBGS activity in the presence of 10 mM alaremycin, implying PBGS from this species to possess significant immunity against this antibiotic.

Determination of the inhibitory effect on recombinant PBGS

To unambiguously identify the molecular target of the alaremycin, we tested its effect on recombinant, purified PBGS from *P. aeruginosa* (metal-independent) and *M. barkeri* (Zn²⁺-dependent). The kinetic parameters (Table 2) of *P. aeruginosa* PBGS are essentially as published (11). The specific activity of purified native *M. barkeri* PBGS is 0.39 µmol min⁻¹ mg⁻¹ (3), while the
KM = 0.07 µM is quite low. To quantify the potency of the inhibitor, IC50 values were determined as described (2) and listed in table 2. No time dependence of inhibition was observed. The k_i for P. aeruginosa PBGS is 1.33 mM, that of M. barkeri is 1.51 mM.

Crystal structure of the P. aeruginosa PBGS-alaremycin complex

P. aeruginosa PBGS was co-crystallized with alaremycin to elucidate its mode of inhibition in detail. The structure was solved and refined to 1.75 Å resolution. The overall structure is unchanged from that of earlier reports (10, 13), despite a change in space group from P42_12 (one dimer per asymmetric unit) to I422 (one monomer per asymmetric unit). Interestingly, the antibiotic is observed to bind as 5-acetamido-4-oxo-5-hexanoic acid in the active site of PBGS, a reduced derivative of 5-acetamido-4-oxo-5-hexenoic acid. We correspondingly refer to this compound as alaremycin 2 (Figure 3C). Alaremycin 2, located in the active site (Figure 2) and clearly defined in the electron density, is coordinated by the P-site Lys260 via a Schiff base to its C4 atom. This resembles substrate ALA binding to the P-site and is comparable in its binding mode to the inhibitor 5-fluorolevulinic acid (Figure 3) (9, 14). Inhibitor binding is stabilized by two hydrogen bonds from the antibiotic carboxylate to Ser286 as described for 5-hydroxylevulinic acid, a substrate analogue, in the P-site of P. aeruginosa PBGS (12). Alaremycin 2 thus mimics the P-site substrate ALA rather than the product PBG. The Schiff base bond of a second, A-site substrate ALA with Lys205 is not mirrored by alaremycin 2 coordination. Instead, the amino group of A-site Lys205 hydrogen bonds the C7 keto group of alaremycin 2. Inhibitor binding is further stabilized by hydrogen bonds to water molecules that in turn hydrogen bond Gln233, Asp127, Ser175. A Mg^{2+} in the active site (12) is coordinated by Asp139, Asp131 and Asp176. The part of the antibiotic that blocks the A-site of the active site has a similar conformation as previously observed for inhibitors mimicking the intermediate of the condensation reaction (18). The methyl group attached to the non-planar carbon atom C5 of alaremycin 2 is only coordinated by weak van der Waals interactions (≥ 3.9Å) in the active site of PBGS. Implying that the modification to alaremycin may be of limited relevance for its...
antibiotic function. The conversion of alaremycin (characterized by NMR (2)) to alaremycin 2 observed in the active site of PBGS by x-ray crystallography) appears to occur during crystallization by an as yet unknown mechanism. However, the inhibitory features of the compound are not affected.

Alaremycin as lead compound for antibiotic development

An amino acid sequence alignment of PBGS from a range of bacteria demonstrates the high degree of conservation in the catalytic site. Correspondingly, amino acid residues in contact with alaremycin2 are conserved in many bacteria explaining the broad antibiotic activity of the compound. It also provides a structural basis for rational drug design to further improve its specificity and affinity. Clearly, the observed alaremycin 2 IC₅₀ value of 2.1 mM for the P. aeruginosa PBGS (Table 2) would need to be further improved prior to any clinical applications.

Previous investigations of the catalytic mechanism of P.aeruginosa PBGS using synthetic inhibitors provide a rational basis for this (12). These inhibitors, with a (carbon) backbone of 11 atoms, bear ether, amine, thioether, sulfoxide and sulfonyl groups at the central position 6. All were co-crystallized with P. aeruginosa PBGS. The “amine” and “thioether” derivatives revealed IC₅₀ values of around 0.3 mM. Their coordination by PBGS differs somewhat from that of alaremycin largely based on the fact that alaremycin is shorter than the other inhibitors. Thus, inter alia, it lacks the A-site carboxyl group. Extending alaremycin to a chain length of 11 atoms and restoring the terminal carboxyl group would be one way of increasing the overall binding affinity. As described, alaremycin is unable to utilize the second Schiff base coordination by Lys205, despite possessing a carbonyl group at position 7 positioned within 2.8 Å of the lysine amine group. The geometry is however such that a Schiff base bond would require the introduction of a carbonyl group at carbon 8 in the potentially optimized inhibitor could restore this interaction. Finally, the described synthetic amine and thioether derivatives partly serve as substrate for the enzyme, leading to C – C bond formation between C3 and C8 mirroring the ring closure via aldol addition of A-side C3 to P-side C8.
of the natural substrate and leading to a dead-end product. Larger and charged atoms in position 6, such as sulfoxide and sulfanyl prevent this reaction. In contrast, alaremycin2 with an amine group in this position could provide a good starting point for the synthesis of a novel inhibitory derivative, since the product of the natural PBGS catalysis also carries an amino group in this position. Overall, the crystal structure of the \textit{P. aeruginosa} PBGS/alaremycin complex provides a solid basis for the rational development of novel antibiotic compounds targeted to bacterial heme biosynthesis.
TABLES

Table 1: Minimal inhibitory concentrations (MICs)

Minimal inhibitory concentration (MIC) and PBGS synthase activity in cell-free extracts for alaremycin inhibition were determined as described above. PBGS activity in cell-free extracts of each strain without alaremycin was set to 100%. PBGS activity after alaremycin additions are relative values. The standard error is estimated at 10 and 15%.

<table>
<thead>
<tr>
<th>Species</th>
<th>MIC (mM)</th>
<th>PBGS activity (mg/L)</th>
<th>5 mM alaremycin</th>
<th>10 mM alaremycin</th>
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<tbody>
<tr>
<td>Escherichia coli DH10b</td>
<td>4</td>
<td>740</td>
<td>30%</td>
<td>&lt; 1%</td>
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<tr>
<td>Pseudomonas aeruginosa</td>
<td>12</td>
<td>2220</td>
<td>92%</td>
<td>25%</td>
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<td>Bacillus subtilis</td>
<td>14</td>
<td>2590</td>
<td>61%</td>
<td>30%</td>
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<tr>
<td>Bacillus megaterium</td>
<td>4</td>
<td>740</td>
<td>3%</td>
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<td>8</td>
<td>1480</td>
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<td>22%</td>
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<tr>
<td>Streptomyces avermitilis</td>
<td>8</td>
<td>1480</td>
<td>68%</td>
<td>&lt; 1%</td>
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<tr>
<td>Escherichia coli CSA1</td>
<td>10</td>
<td>1850</td>
<td>36%</td>
<td>&lt; 1%</td>
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<tr>
<td>Streptomyces sp. A012304</td>
<td>&gt; 20</td>
<td>&gt; 3700</td>
<td>90%</td>
<td>79%</td>
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</table>
Table 2: Kinetic parameters of several PBGS and IC\textsubscript{50} values for alaremycin

The Michaelis Menten constant $K_M$, the $k_{cat}$ and the $k_{cat}/K_M$ values for the various PBGS were determined from substrate velocity plots by measuring the constant velocity formation of porphobilinogen from 5-ALA over a substrate range from 1 - 10 mM. Values were determined by iterative, curve-fitting Lineveaver Burk plots (SigmaPlot 8.0 Enzyme Kinetics v1.1). For inhibition studies 0 - 40 mM alaremycin were added to the enzyme activity test. The standard error of the shown results was between 5 and 10%.

<table>
<thead>
<tr>
<th></th>
<th>$P. aeruginosa$</th>
<th>$M. barkeri$</th>
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<tr>
<td>General catalytic</td>
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<tr>
<td>properties</td>
<td>$K_M$ (µM)</td>
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<tr>
<td></td>
<td>$k_{cat}$ (s\textsuperscript{-1})</td>
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<td>$k_{cat}/K_M$ (µM\textsuperscript{-1}s\textsuperscript{-1})</td>
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<td>Inhibition by</td>
<td></td>
<td></td>
</tr>
<tr>
<td>alaremycin</td>
<td>$IC_{50}$ (mM)</td>
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<tr>
<td></td>
<td>$k_i$ (mM)</td>
<td>1.33</td>
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Values were determined by iterative, curve-fitting Lineveaver Burk plots (SigmaPlot 8.0 Enzyme Kinetics v1.1). For inhibition studies 0 - 40 mM alaremycin were added to the enzyme activity test. The standard error of the shown results was between 5 and 10%.
### Table 3 Data collection and refinement statistics

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<tr>
<td>Resolution range (Å)</td>
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<td>Completeness (%)</td>
<td>100 (99.9)</td>
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<td>Redundancy</td>
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<td>$R_{merge}$</td>
<td>6.9 (50.4)</td>
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<td>$I/σ_I$</td>
<td>46.0 (5.3)</td>
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<td>Temperature factor from Wilson plot (Å$^2$)</td>
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<th>Refinement</th>
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<td>Reflections</td>
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<td>$R$-factor (%)</td>
<td>14.6 (21.5); 17.8 (25.4)</td>
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<td>Non-H protein atoms/monomer</td>
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<td>Water molecules/monomer</td>
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<td>Ions/monomer: Mg$^{2+}$</td>
<td>4</td>
</tr>
<tr>
<td>Average $B$-factor (Å$^2$)</td>
<td>16.1</td>
</tr>
<tr>
<td>Ramachandran plot: preferred; allowed; outliers (%/#)</td>
<td>97.0/292; 2.7/8; 0.3/1</td>
</tr>
<tr>
<td>R.m.s. deviation from ideality: bond lengths (Å); bond angles (deg.)</td>
<td>0.015; 1.49</td>
</tr>
<tr>
<td>Estimate overall coordinate error, based on maximum likelihood (Å)</td>
<td>0.06</td>
</tr>
<tr>
<td>PDB code</td>
<td>3B1C</td>
</tr>
</tbody>
</table>

---

1. Value for shell of highest resolution in parentheses.
2. $>1σ$.
3. $R_{merge} = 100(\sum_h \sum_j |I_{hj} - I_h|)/(\sum_h \sum_j I_{hj})$ for all observations $I_{h,j}$ contributing to $I_h$.
4. 5% of data were omitted from refinement.
5. Coot (7)
6. Refmac (21)


FIGURE LEGENDS

Figure 1.
Panel A: Alaremycin (5-acetamido-4-oxo-5-hexenoic acid). Panel B: Enzyme reaction of porphobilinogen synthase (PBGS). PBGS catalyzes the first common step in tetrapyrrole biosynthesis involving the asymmetric condensation of two 5-aminolevulinic acid molecules to form the monopyrrolic porphobilinogen. Panel C: Alaremycin 2 (5-acetamido-4-oxo-5-hexanoic acid).

Figure 2.
Stereo image of the catalytic domain of Pseudomonas aeruginosa PBGS in complex with alaremycin 2. Alaremycin 2 binds to Lys260 through a Schiff base while its keto-oxygen forms a hydrogen bond to Lys205 (A-site).

Figure 3.
Comparison of the binding modes of alaremycin 2 (A) and the two 5-fluorolevulinc acid (FLA) substrate analoge molecules (B) in the PBGS catalytic site. The two FLA substrate molecules and the alaremycin 2 inhibitor are indicated in orange, the conserved lysine residues of the P- and A- sites in violet, amino acid residues involved in substrate recognition in black, hydrogen bonding as dotted lines. The two F-ALA molecules are covalently bound to Lys260 (P-site) and Lys205 (A-site) by Schiff bases, linked by a Na\(^+\). The natural inhibitor alaremycin 2 is bound by a Schiff base bond to Lys260 (P-site) but additionally partly blocks the A-site.

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REFERENCES


A

\[
\text{Alaremycin}
\]

B

\[
\text{5-Aminolevulinic acid} \xrightarrow{\text{Porphobilinogen synthase (PBGS)}} \text{Porphobilinogen}
\]

C

\[
\text{Alaremycin 2}
\]